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Semicarbazone-based inhibitors of cathepsin K, are they prodrugs for aldehyde inhibitors?

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Abstract—Starting from potent aldehyde inhibitors with poor drug properties, derivatization to semicarbazones led to the identification of a series of semicarbazone-based cathepsin K inhibitors with greater solubility and better pharmacokinetic profiles than their parent aldehydes. Furthermore, a representative semicarbazone inhibitor attenuated bone resorption in an ex vivo rat calvarial bone resorption model. However, based on enzyme inhibition comparisons at neutral pH, semicarbazone hydrolysis rates, and ^{13}C NMR experiments, these semicarbazones probably function as prodrugs of aldehydes.

As the average lifespan continues to increase in the developed world due to better nutrition and improved medicinal intervention, societies are being increasingly burdened by the diseases of aging. Therefore, increased effort is being directed toward addressing the maladies of the elderly. As part of this endeavor, the search for therapeutic interventions to reduce fractures attributed to osteoporosis has intensified. Supplementing research into anabolic agents that induce net bone formation are investigations aimed at reducing bone loss in order to maintain skeletal integrity. Bone loss results from the resorption of the mineral and matrix components of bone by acid and enzymes, respectively, secreted by specialized cells called osteoclasts.[1] The C1A family cysteine protease cathepsin K is the major bone resorbing protease in osteoclasts.[2] In complex with glycosaminoglycans, it rapidly hydrolyzes the major component of bone matrix, type I collagen. Inhibitors of cathepsin K should therefore decrease the rate of bone loss, and such inhibitors have in fact been shown to attenuate bone resorption in animal models of osteoporosis.[3] Furthermore, the rare autosomal recessive osteopetrotic trait known as pycnodysostosis, which results from deactivating mutations in cathepsin K, provides evidence that cathepsin K plays a similar role in humans.[4] With the mounting evidence of the potential utility of cathepsin K inhibitors in preventing excess bone resorption, much research has focused on the development of cathepsin K inhibitors as drugs for the treatment of osteoporosis.[5]

As part of a broader effort to develop novel cathepsin K inhibitors, researchers from these laboratories recently reported the discovery of the aldehyde cathepsin K inhibitors **1a** (IC_{50} = 51 nM), **1b** (IC_{50} = 1.8 nM), **1c**

Keywords: Cathepsin K inhibitors; Cysteine protease inhibitors; Semicarbazones; Aldehydes; Prodrugs.

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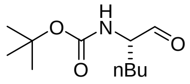
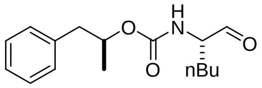
(IC_{50} = 0.50 nM), and **1d** (IC_{50} = 0.35 nM).[6,7] Although these aldehyde-based inhibitors provided valuable structure/activity relationship data for optimizing S^1, S^2, and S^3 subsite binding, they did not occupy the $S^{1'}$ or $S^{2'}$ subsites in the enzyme. Furthermore, they exhibited poor solubility and metabolic stability, precluding further evaluation in animal models of osteoporosis. Semicarbazones have previously been disclosed as cysteine protease inhibitors.[8] Believing that semicarbazones should enhance solubility and allow exploration of the $S^{1'}$ and $S^{2'}$ subsites of cathepsin K, investigations of the ability of semicarbazones derived from aldehyde **1a** to inhibit cathepsin K were undertaken.



1a



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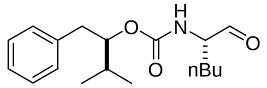
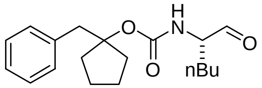


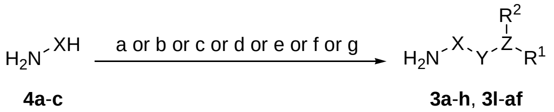
1c



1d

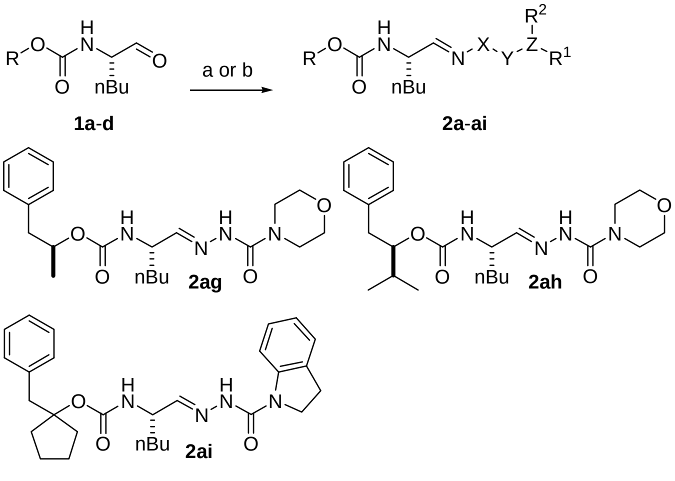
The imine analogs **2a**–**ai** were prepared by one of two methods as depicted in Scheme 1. In the first method, the known aldehydes **1a**–**d** were coupled under acidic catalysis either to semicarbazides **3a**–**h**, **3l**–**n**, and **3u**–**ab**, hydrazides **3o**, **3p**, and **3s**, *iso*-propyl carbazate **3q**, *iso*-propyl thiocarbazate **3r**, *iso*-propyl thiosemicarbazide **3t**, 2-thiazolidinylidene hydrazine **3ac**, aminoguanidine **3ad**, *N*,*N*-dimethyl sulfamic acid hydrazide **3ae**, or morpholinocarbonyl hydroxylamine **3af** to provide analogs **2a**–**h** and **2l**–**2ai**. Alternatively, aldehyde **1a** was treated with hydrazine to give the hydrazone, which was combined with commercially available isocyanates to afford semicarbazones **2i**–**k**.

The requisite coupling partners were either purchased or were prepared as depicted in Scheme 2. The semicarbazides **3a** and **3f**, hydrazides **3o** and **3p**, 2-thiazolidinylidene hydrazine **3ac**, and aminoguanidine **3ad** were commercially available. Semicarbazides **3b**–**e**, **3g**–**h**, and **3m** were derived from the reaction of commercially available isocyanates with excess hydrazine, whereas semicarbazides **3l**, **3n**, and **3u**–**ab** were prepared from the reaction of the in situ generated carbamoyl chlorides, derived from the commercially available amines and phosgene, with excess hydrazine. Similarly, the carbazate **3q** was synthesized by reacting *iso*-propylchloroformate with excess hydrazine. Likewise, 2-propanethiol was treated with phosgene and the resulting thiochloroformate was added to excess hydrazine to afford the thiocarbazate **3r**. Also, addition of isovaleryl chloride to excess hydrazine provided the hydrazide **3s**. Correspondingly, the thiosemicarbazide **3t** was synthesized by adding *iso*-propyl isothiocyanate to excess hydrazine. The sulfamic acid hydrazide **3ae** was produced by addition of the dimethyl sulfamoyl chloride, derived from sulfuryl chloride and dimethylamine, to excess hydrazine. Finally, morpholinocarbonyl hydroxylamine **3af** was prepared from hydroxylamine hydrochloride. First, protection of the amine as the *tert*-butyl carbamate, followed by reaction with 4-morpholinecarbonyl chloride, and subsequent cleavage of the amine masking group afforded the morpholinocarbonyl hydroxylamine **3af**.



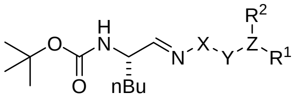
Scheme 2. Reagents and conditions: (a) $R^1N{=}C{=}O$, PhMe, 43–97%; (b) R^1R^2NH or R^1SH, $COCl_2$, dioxane, 0 °C to rt; N_2H_4, dioxane, 24–98%; (c) R^1COCl, PhMe, 45%; (d) R^1OCOCl, PhMe, 86%; (e) $R^1N{=}C{=}S$, PhMe, 92%; (f) R^1R^2NH, Et_3N, $CHCl_3$, SO_2Cl_2, dioxane, 0 °C to rt; N_2H_4, THF, 91%; (g) $(t\text{-}BuOCO)_2O$, $NaHCO_3$, CH_2Cl_2, H_2O, 91%; 4-morpholinecarbonyl chloride, $i\text{-}Pr_2NEt$, dioxane, 23%; 4 N HCl, dioxane, 99%.

As shown in Table 1, the parent semicarbazone **2a** (IC_{50} = 350 nM) was only a ~7-fold less active inhibitor of cathepsin K than the starting aldehyde **1a** (IC_{50} = 51 nM) at pH 5.5. Since imines are less electrophilic than aldehydes and semicarbazones should pay a higher desolvation cost in binding to the enzyme, this modest decrease in potency was encouraging. It was



Scheme 1. Reagents: (a) **3**, PPTS, THF, 27–81%; (b) N_2H_4, THF, 27%; $R^1N{=}C{=}O$, PhMe, 18–77%.

Table 1. Inhibition of human cathepsin K



Compound	X	Y	Z	R^1	R^2	IC$_{50}$ nM[a]
2a	NH	C=O	N	H	H	350
2b	NH	C=O	N	Me	H	280
2c	NH	C=O	N	Et	H	490
2d	NH	C=O	N	*i*-Pr	H	170
2e	NH	C=O	N	*t*-Bu	H	160
2f	NH	C=O	N	Ph	H	400
2g	NH	C=O	N	$PhCH_2$	H	410
2h	NH	C=O	N	$Ph(CH_2)_2$	H	260
2i	NH	C=O	N	CO_2Me	H	180
2j	NH	C=O	N	CH_2CO_2Et	H	520
2k	NH	C=O	N	$(CH_2)_2CO_2Et$	H	470
2l	NH	C=O	N	$Ph(CH_2)_2$	Me	45
2m	NMe	C=O	N	$Ph(CH_2)_2$	H	11,000
2n	NMe	C=O	N	$Ph(CH_2)_2$	Me	520
2o	NH	C=O	Ph	—	—	220
2p	NH	C=O	CF_3	—	—	24
2q	NH	C=O	O	*i*-Pr	H	130
2r	NH	C=O	S	*i*-Pr	H	830
2s	NH	C=O	CH_2	*i*-Pr	H	200
2t	NH	C=S	N	*i*-Pr	H	170
2u	NH	C=O	N	Me	Me	72
2v	NH	C=O	N	Et	Et	40
2w	NH	C=O	1-pyrrolidine	—	—	36
2x	NH	C=O	1-piperidine	—	—	58
2y	NH	C=O	4-morpholine	—	—	35
2z	NH	C=O	2,3-dihydroindole	—	—	48
2aa	NH	C=O	1,2,3,4-tetrahydroquinoline	—	—	59
2ab	NH	C=O	1,2,3,4-tetrahydroisoquinoline	—	—	93
2ac	NH	—	4,5-dihydrothiazole	—	—	470
2ad	NH	C=NH	N	H	H	33,000
2ae	NH	SO_2	N	Me	Me	72
2af	O	C=O	4-morpholine	—	—	1100

[a] Inhibition of recombinant human cathepsin K activity in a fluorescence assay using 10 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, and 120 mM NaCl, pH 5.5. The IC$_{50}$ values are means of two or three inhibition assays, individual data points in each experiment were within a 3-fold range of each other.

believed that inhibitory activity could be recovered by generating additional inhibitor interactions with the S$^{1'}$ and S$^{2'}$ subsites of cathepsin K. Probing for hydrophobic interactions in the S$^{2'}$ subsite of the enzyme to improve potency, alkyl groups were connected to the terminal nitrogen of semicarbazone **2a**. However, the mono-substituted alkyl semicarbazones **2b–e** (IC$_{50}$ = 160–490 nM) all exhibited cathepsin K inhibitory activity similar to that of the parent **2a**. Hoping to enhance potency by picking up potential π–π stacking interactions with aryl groups in S′ subsites of the protein, phenyl rings were appended to the prototype semicarbazone by various tether lengths, but aryl analogs **2f–h** (IC$_{50}$ = 260–410 nM) were not significantly different from the other semicarbazone analogs **2a–e** tested. An alternative strategy to improve inhibitory activity by attaching hydrogen bond acceptors to the archetype semicarbazone **2a** was also pursued. Analogs **2i–k** (IC$_{50}$ = 180–520 nM) were prepared, but, unfortunately, once again no improvement in potency was achieved.

To explore the necessity of the terminal hydrogen bond donating nitrogen of the semicarbazone, the oxygen analog **2q** (IC$_{50}$ = 130 nM) of semicarbazone **2d** (IC$_{50}$ = 170 nM) was prepared. Since these analogs were equipotent and paid similar desolvation costs, it is unlikely that the NH of the semicarbazone donates a hydrogen bond to the enzyme. The inhibitory activity of the carbon analog **2s** (IC$_{50}$ = 200 nM) was also not significantly different from those of the nitrogen or oxygen compounds. The phenyl hydrazone **2o** (IC$_{50}$ = 220 nM) also displayed similar activity. Since the enthalpic penalty to desolvate a methylene group is substantially less than a heteroatom, the similar activity of these analogs makes it difficult to draw any conclusions regarding the hydrogen bond accepting requirements of these heteroatoms for inhibitor potency. The thiocarbazate **2r** (IC$_{50}$ = 830 nM) was slightly less active than the hydrazide **2s**, but the nature of the group adjacent to the carbonyl does not seem to have a significant influence on inhibitory activity.

In contrast to monosubstitution of the terminal semicarbazone nitrogen, dialkylation improved potency. Thus, the *N*-methyl *N*-phenethyl analog **2l** (IC_{50} = 45 nM) was equipotent to the starting aldehyde **1a** (IC_{50} = 51 nM) and 5-fold more potent than its monosubstituted analog **2h** (IC_{50} = 260 nM). The dimethyl analog **2u** (IC_{50} = 72 nM) and the diethyl analog **2v** (IC_{50} = 40 nM) were also equipotent to the precursor aldehyde. Furthermore, the cyclic amine substituted analogs **2w**–**ab** also exhibited similar inhibitory activity. Thus, the pyrrolidine **2w** (IC_{50} = 36 nM), the piperidine **2x** (IC_{50} = 58 nM), the morpholine **2y** (IC_{50} = 35 nM), the indoline **2z** (IC_{50} = 48 nM), the tetrahydroquinoline **2aa** (IC_{50} = 59 nM), and the tetrahydroisoquinoline **2ab** (IC_{50} = 93 nM) were all equipotent cathepsin K inhibitors to the aldehyde **1a**.

An attempt was made to explore the $S^{1'}$ subsite, by attaching an alkyl group to the nitrogen adjacent to the imine nitrogen. The *N*-methyl analog **2m** (IC_{50} = 11,000 nM) was greater than 40-fold less active than the corresponding unsubstituted semicarbazone **2h** (IC_{50} = 260 nM). Replacement of the $P^{1'}$ nitrogen of semicarbazone **2y** (IC_{50} = 35 nM) with an oxygen, as in oxime derivative **2af** (IC_{50} = 1100 nM), also led to a decrease in potency. Consistent with the structure/activity relationship showing that complete alkylation of the terminal semicarbazone nitrogen enhanced inhibitory activity, disubstitution of the $S^{1'}$ probe **2m** gave the fully alkylated semicarbazone **2n** (IC_{50} = 520 nM) which recovered over an order of magnitude of potency relative to its antecedent **2m**.

Table 2. Cathepsin B, H, and L inhibition and selectivity

Compound	Cat K IC_{50} (nM)	Cat B IC_{50} (nM)[a]	Cat H IC_{50} (nM)[b]	Cat L IC_{50} (nM)[c]
1a	51	32,000	>13,000	8900
1b	1.8	460	40	740
1c	0.50	27	18	12
1d	0.35	810	>13,000	2000
2y	35	32,000	>13,000	11,000
2z	48	35,000	>13,000	14,000
2ag	0.94	270	230	540
2ah	0.33	18	190	12
2ai	1.1	1800	>13,000	3400

[a] Inhibition of recombinant human cathepsin B activity in a fluorescence assay using 10 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, and 120 mM NaCl, pH 5.5. The IC_{50} values are means of two or three inhibition assays, individual data points in each experiment were within a 2-fold range of each other.

[b] Inhibition of recombinant human cathepsin H activity in a fluorescence assay using 50 μM L-Arg-β-naphthalamide as substrate in 100 mM NaOAc, 10 mM DTT, and 120 mM NaCl, pH 5.5.

[c] Inhibition of recombinant human cathepsin L activity in a fluorescence assay using 5 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, and 120 mM NaCl, pH 5.5.

The significance of the semicarbazone carbonyl to cathepsin K inhibitory activity was also investigated. The replacement of the carbonyl oxygen of semicarbazone **2d** (IC_{50} = 170 nM) with sulfur to give the thiosemicarbazone **2t** (IC_{50} = 170 nM) afforded no change in potency. Cyclization of the thiosemicarbazone to give the 2-thiazolidinylidine hydrazine **2ac** (IC_{50} = 470 nM) also did not significantly alter potency. Furthermore, replacement of the carbonyl of semicarbazone **2u** (IC_{50} = 72 nM) with SO_2 to give the sulfamic acid hydrazone **2ae** (IC_{50} = 72 nM) did not affect inhibitory activity either. In contrast, the substantially more basic guanidine derivative **2ad** (IC_{50} = 33,000 nM) was ~100-fold less active than the parent semicarbazone **2a** (IC_{50} = 350 nM).

The semicarbazones **2ag**–**i** of the more potent aldehyde inhibitors **1b**–**d** were also synthesized. As shown in Table 2, the potency of the semicarbazones **2ag**–**i** versus cathepsin K was comparable to that of their corresponding aldehydes **1b**–**d**. Furthermore, their selectivity ratios relative to cathepsins B, H, and L did not differ significantly from the respective aldehydes.

Most of these analogs showed reasonable stability in plasma. Moreover, as shown in Table 3, these semicarbazones exhibited good to excellent permeability in an in vitro Madin–Darby canine kidney (MDCK) permeation assay (P_{APP} = 62–770 nm/s).[9] Furthermore, they were more soluble in fasted state-simulated intestinal fluid (FS-SIF = 0.033–0.38 mg/mL) at pH 6.8 than the aldehyde inhibitor **1a** (FS-SIF <0.010 mg/mL).[10,11] Unfortunately, during solubility studies in simulated gastric fluid it was discovered that these semicarbazones were rapidly hydrolyzed at pH 1.0. A study of the rate of hydrolysis versus time at various pHs disclosed that the rate constant for hydrolysis increased over 3 orders of magnitude from pH = 6.0 to pH 1.0. In addition, these semicarbazones were also rapidly metabolized in rat S9 liver incubations ($t_{1/2}$ < 1 h).[12]

Table 3. Serum pharmacokinetics of combination analogs

Compound	*c*log*P*	MDCK P_{APP} (nm/s)	Sol. FS-SIF[a] (mg/mL)	$t_{1/2}$[b] (min)	C_l[c] (mL/min/kg)	V_{SS}[d] mL/kg	*F*[e] (%)
2w	3.8	200	0.071	18	18	430	0
2y	3.2	62	0.120	22	43	570	3.9
2z	5.1	150	0.033	10	40	540	0
2ae	2.7	770	0.380	39	25	390	26
2ag	4.4	140	0.060	9	16	220	0

[a] FS-SIF is the equilibrium solubility in fasted state-simulated intestinal fluid at pH 6.8. The values are means of two measurements.

[b] $t_{1/2}$ is the iv terminal half-life dosed as a solution in male Han Wistar rats. All in vivo pharmacokinetic values are means of two experiments.

[c] C_l is the total clearance.

[d] V_{SS} is the steady state volume of distribution.

[e] *F* is the oral bioavailability when dosed as a solution.

Despite these discouraging stability issues, the iv and p.o. pharmacokinetics of several analogs were profiled in male Han Wistar rats. As shown in Table 3, the compounds exhibited short terminal elimination half-lifes ($t_{1/2}$ = 9-39 min), moderate steady state volumes of distribution (V_{SS} = 220–570 mL/kg), and moderate to high clearances (C_l = 16–43 mL/min/kg). Despite improved permeabilities and aqueous solubilities, oral bioavailabilities were low (F = 0–26%), suggesting that chemical instability is the key determinant to the pharmacokinetic profile of the semicarbazones. Interestingly, semicarbazone **2ag** was still able to attenuate deoxypyridinoline crosslink release in an ex vivo rat calvarial resorption assay (IC_{50} = ~1000 nM), despite its poor stability and modest inhibitory activity versus rat cathepsin K (rat cathepsin K IC_{50} = 130 nM).[13,14]

X-ray quality co-crystals of cathepsin K incubated with 13 different semicarbazones were obtained. Although a few structures showed some partial electron density for the semicarbazone portion of the inhibitor, the vast majority of these structures only contained the aldehyde. Apparently, the semicarbazone is hydrolyzed to the aldehyde under the crystallization conditions. Neither aldehyde nor semicarbazone co-crystals with cathepsin K have shown covalent adducts by mass spectrometry. Two representative structures of cathepsin K incubated with inhibitors **2ah** and **2ai** are shown in Figure 1. These structures shed additional light on the nature of the S^2 and S^3 binding interactions of the aldehyde inhibitors **1c** and **1d**. These reversible inhibitors form a covalent hemithioketal intermediate between the aldehyde group of the inhibitor and the active site ^{25}Cys of the enzyme. The hemithioketal hydroxyl is stabilized by two hydrogen bonds to the side chain of ^{19}Gln, and the backbone amide of ^{25}Cys, consistent with other aldehyde co-crystal structures from this group.[6,7] The carbamate NH and the carbamate carbonyl of the inhibitor are stabilized by two additional hydrogen bonds between ^{161}Asn and ^{66}Gly in the peptide backbone recognition site of the enzyme. Moreover, the S^1 wall composed of ^{23}Gly, ^{24}Ser, ^{64}Gly, and ^{65}Gly forms van der Waals interactions with one face of the *n*-butyl group of the norleucine-derived inhibitor. The terminal carbon of the *n*-butyl group is solvent exposed and oriented in different directions in the two structures. Furthermore, the P^2 *iso*-propyl substituent of **1c** forms significant lipophilic interactions with the deep S^2 pocket composed of ^{67}Tyr, ^{68}Met, ^{134}Ala, ^{163}Ala, and ^{209}Leu. The cyclopentyl group of inhibitor **1d** occupies an even greater area of the S^2 pocket. Finally, the P^3 phenyl moieties interact with the active site trough as well as pointing into the S^3 subsite formed by ^{60}Asn, ^{61}Asp, ^{65}Gly, ^{66}Gly, and ^{67}Tyr. The accommodation of the P^2 cyclopentyl group into the S^2 subsite causes the P^3 phenyl substituent of analog **1d** to rotate perpendicular to the P^3 phenyl of analog **1c**. The P^2 cyclopentyl fragment probably is better accommodated by the cathepsin K S^2 pocket than the cathepsins B, H, and L S^2 pockets, explaining its increased selectivity.

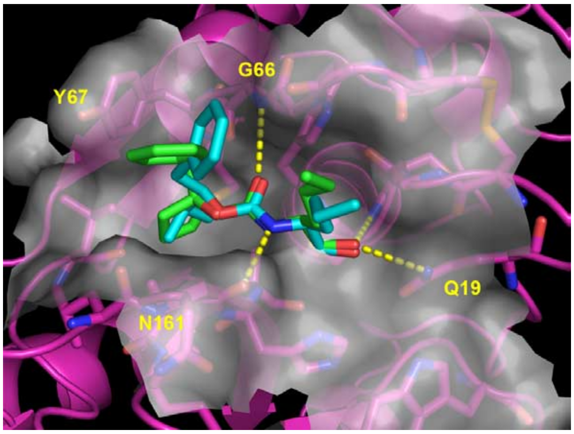


Figure 1. An overlay of the active sites of the X-ray co-crystal structures of compounds **1c** (from **2ah**) and **1d** (from **2ai**) complexed with cathepsin K. The cathepsin K carbons are colored magenta with inhibitor **1c** carbons colored cyan and inhibitor **1d** carbons green. The semi-transparent white surface represents the molecular surface, while hydrogen bonds are depicted as yellow dashed lines. The coordinates have been deposited in the Brookhaven Protein Data Bank Accession Nos. 2AUX & 2AUZ. This figure was generated using PYMOL version 0.97 (Delano Scientific, www.pymol.org).

It is possible that hydrolysis of the semicarbazones to the aldehydes is an artifact of the slow crystallization process. Another interpretation is that the active species is in fact the aldehyde and the semicarbazone merely acts as a pro-drug being hydrolyzed to the aldehyde under the pH 5.5 buffer conditions of the enzyme assay, as well as in the resorption lacunae. This hypothesis was supported by the fact that none of the semicarbazones were more potent than their corresponding aldehydes. Furthermore, the structure–activity relationships for the semicarbazones were relatively flat, with the exception that the sterically encumbered imine semicarbazones **2m** and **2n** were substantially less potent than their unmethylated congeners **2h** and **2l**, respectively.

To test this supposition, several semicarbazones were assayed at pH 5.5 and 7.0 in the presence of a large excess of their corresponding semicarbazide, added to try to slow hydrolysis. As shown in Table 4, inhibition of cathepsin K by semicarbazones at pH 5.5 was not significantly affected by running the assay with added semicarbazide at a concentration 15-fold greater than the

Table 4. Cathepsin K inhibition at pH

Compound	IC_{50} at pH 5.5[a] (nM) w/o **3**	IC_{50} at pH 5.5[b] (nM) w/15 × **3**	IC_{50} at pH 7.0[c] (nM) w/15 × **3**
2c	490	900	>13,000
2s	200	160	11,000
2v	40	47	1,800
2y	35	50	2,100
2ae	72	220	1,900

[a] Inhibition of recombinant human cathepsin K activity in a fluorescence assay using 10 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, and 120 mM NaCl, pH 5.5.

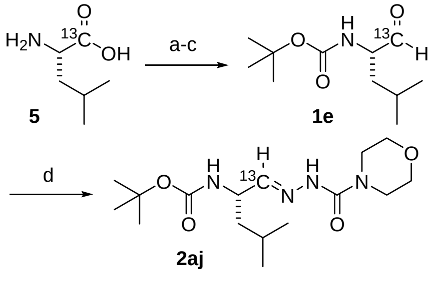
[b] The assay was performed in a similar manner except that the corresponding semicarbazide was added in a 15-fold excess of the inhibitor.

[c] The assay was performed in a similar manner except that the corresponding semicarbazide was added in a 15-fold excess of the inhibitor and the assay was performed at pH 7.0.

inhibitor concentration, suggesting that the added semicarbazide did not affect the rate of hydrolysis. In contrast, at pH 7.0, the semicarbazones were significantly poorer inhibitors of cathepsin K under similar assay conditions. Cathepsin K hydrolyzes Cbz-Phe-Arg-AMC at a slower rate at pH 7.0 than at pH 5.5, but it still rapidly cleaves the substrate.[15] These results can be explained by a model in which hydrolysis of the semicarbazones to generate the active inhibitor aldehydes is rapid at pH 5.5, and not influenced by the presence of excess semicarbazide, whereas the acid catalyzed hydrolysis is slowed at pH 7.0, decreasing the concentration of active aldehyde and therefore the apparent potency of the inhibitor. Thus, these data support the hypothesis that semicarbazones inhibit cathepsin K by acting as prodrugs of their aldehydes.

To further explore this hypothesis, a ^{13}C labeled inhibitor was prepared for a NMR experiment with cathepsin K. The carbonyl labeled Boc-Leu-H aldehyde **1e** and semicarbazone **2aj** were synthesized as shown in Scheme 3. The amine of commercially available ^{13}C labeled leucine was converted to its *tert*-butyl carbamate. Then, in situ generation of the mixed anhydride, from the acid, followed by sodium borohydride-mediated reduction provided the alcohol. The alcohol was subsequently oxidized to the aldehyde **1e**. Then, employing a similar method as detailed in Scheme 1, the aldehyde was converted into the semicarbazone **2aj**.

Both the aldehyde **1e** (IC_{50} = 31 nM) and the semicarbazone **2aj** (IC_{50} = 110 nM) were potent inhibitors of cathepsin K. The ^{13}C NMR spectrum of aldehyde **1e** in D_2O showed a small ^{13}C resonance (~5%) at 206 ppm for the carbonyl carbon and a large resonance (~95%) at 93 ppm for the hydrate, whereas the ^{13}C NMR spectrum of semicarbazone **2aj** in D_2O displayed a large ^{13}C resonance (~98%) at 150 ppm for the imine carbon and a small resonance (~2%) at 92 ppm for the hydrate. When added to cathepsin K at pH = 5.5, the imine carbon of the semicarbazone inhibitor **2aj** showed a resonance at 149 ppm. The spectrum also exhibited a small resonance at 204 ppm and a large resonance at 91 ppm representing the free aldehyde and hydrate, respectively, from hydrolysis of the semicarbazone catalyzed by the acidic buffer, as well as a new resonance at 80 ppm corresponding to the hemithioketal adduct with cathepsin K. Importantly, no new resonance for a thioaminal adduct of the semicarbazone with cathepsin K was detected. It was therefore concluded that, although it is possible that some of these semicarbazones inhibit cathepsin K directly, they most likely function as delivery vehicles for the aldehyde inhibitors.



Scheme 3. Reagents and conditions: (a) $(t\text{-BuOCO})_2O$, NaOH, dioxane, H_2O, 99%; (b) *i*-PrOCOCl, NEt_3, THF, −10 °C; $NaBH_4$, H_2O, 0 °C, 88%; (c) pyridine · SO_3, NEt_3, DMSO, CH_2Cl_2, −10 °C, 90%; (d) **3y**, PPTS, THF, 45%.

In summary, this report details the discovery and investigation of the mechanism of action of semicarbazone-based inhibitors of cathepsin K. Semicarbazone hydrolysis rates at acidic pH, cathepsin K inhibition assays at pH 7.0, and ^{13}C NMR experiments support a theory that these semicarbazones serve as prodrugs for the actual aldehyde inhibitors. Although these semicarbazones were more water soluble than the corresponding aldehydes, they offer only slightly enhanced pharmacokinetic profiles than the parent aldehydes, presumably due to their instability under acidic conditions. Nevertheless, based on surrogate markers, these semicarbazones were able to attenuate bone resorption in an ex vivo model of osteoporosis, most likely via in situ conversion to known aldehyde cathepsin K inhibitors in the acidic resorption lacunae of osteoclasts.

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