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Design of small molecule ketoamide-based inhibitors of cathepsin K

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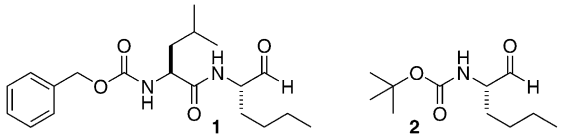
Abstract—A novel series of ketoamide-based inhibitors of cathepsin K has been identified. Modifications to P^2 and P^3 elements were crucial to enhancing inhibitory activity. Although not optimized, a selected inhibitor was effective in attenuating type I collagen hydrolysis in a surrogate assay of bone resorption.

Osteoporosis is a skeletal disorder characterized by enhanced bone resorption relative to bone formation resulting in decreased bone mass and increased susceptibility to fracture.[1] Specialized cells, called osteoclasts, secrete acid and proteolytic enzymes to remove the mineral and matrix components of bone. Cathepsin K is the major proteolytic enzyme implicated in degradation of bone matrix.[2] It is a cysteine protease of the papain superfamily highly expressed in osteoclasts. As part of a complex with glycosaminoglycans, cathepsin K efficiently degrades type I collagen, the major component of bone matrix.[3] Small molecule inhibitors of cathepsin K are efficacious in attenuating bone resorption in animal models of osteoporosis.[4]

As part of a larger program to develop new osteoporosis therapies, the discovery of small molecule cathepsin K inhibitors was pursued. A directed screen of peptide aldehydes identified calpeptin **1** as a potent cathepsin K inhibitor ($IC_{50} = 0.11$ nM).[5] A truncated analogue, Boc-Nle-H **2**, retained reasonable potency ($IC_{50} = 51$ nM). Since aldehydes can be metabolically unstable, replacements for this electrophilic group were explored.[6] Considering the chronic nature of osteoporosis therapies, reversible inhibitors were required. In addition to aldehydes, several other electrophiles including ketones, α-dicarbonyl derivatives, α-ketoheterocycles, and nitriles have been employed in reversible cysteine protease inhibitors.[7] Desiring to explore both S and S′ enzyme subsites, ketoamides were selected as the aldehyde replacement. Besides being more electrophilic than ketones and nitriles,[8] α-ketoamide-based inhibitors have also demonstrated oral bioavailability.[9]



1 **2**

Three general routes were utilized to synthesize the α-ketoamides. The first method employed a modified cyanohydrin procedure based on the precedent of Harbeson and colleagues.[10] As depicted in Scheme 1, the aldehyde **2** was converted to a diastereomeric mixture of cyanohydrins **3**. Subsequent acid catalyzed hydrolysis of the nitrile with concomitant loss of the carbamate protecting group followed by reprotection of the primary amine provided the known α-hydroxyacids **4**.[11,12] Carbodiimide-based amide bond formation with various $P^{1'}$ amines[13] and subsequent oxidation afforded the ketoamides **5a–k**. By early incorporation of the P^2 substituent, the cyanohydrin protocol allows the rapid generation of $P^{1'}$ analogues. It also is amenable to the synthesis of P^2 analogues. Furthermore, it employs

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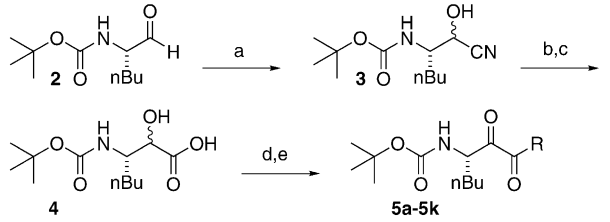
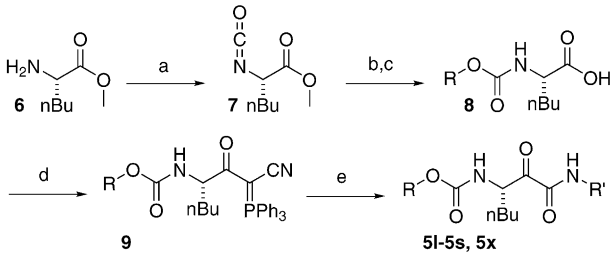


Scheme 1. (a) Acetone cyanohydrin, KCN, nBu_4NI, PhMe, H_2O; (b) concd HCl, 100 °C; (c) 1N NaOH, Boc_2O, THF; (d) amine, EDC, HOBt, iPr_2NEt, DMF, 40–77% over 4 reactions; (e) TEMPO, 5% NaOCl, KBr, $NaHCO_3$, CH_2Cl_2, 42–89%.



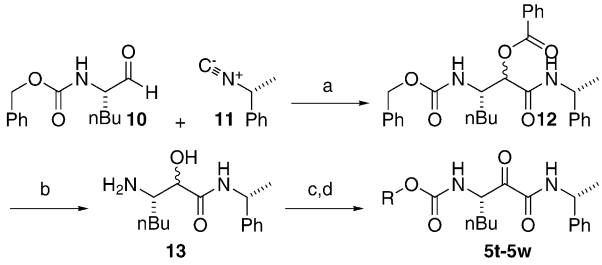
Scheme 2. (a) Pyridine, CH_2Cl_2, 0 °C; 1.93 M $COCl_2$ in PhMe, 0 °C; 1N HCl, 0 °C; distilled @ 2 Torr, 94%; (b) alcohol, PhMe, 85 °C, sealed tube, 89–99%; (c) LiOH·H_2O, THF, H_2O; 1N HCl; (d) Ph_3P=CCN, DMAP, EDC, CH_2Cl_2, 46–79% over two reactions; (e) O_3, CH_2Cl_2, −78 °C; N_2; amine, −78 °C to rt; $AgNO_3$, THF, H_2O, 19–45%.

inexpensive reagents and is readily amenable to multigram scale.

The second process applied the acyl cyanophosphorane oxidative cleavage and amine coupling procedure of Wasserman to generate the ketoamides.[14] As displayed in Scheme 2, the amino acid ester **6** was transformed into the isocyanate **7**. Subsequent coupling to a P^2–P^3 alcohol[15] followed by hydrolysis of the esters provided acids **8**. Coupling the acids **8** with cyanomethyltriphenylphosphonium ylide yielded the phosphoranes **9**. In situ generation of acyl nitriles via ozonolysis of the phosphorus–carbon double bond, followed by displacement of cyanide with a $P^{1'}$ amine gave the desired ketoamides **5l–s** and **5x**. This procedure provided the best method to quickly vary the $P^{1'}$ substituent, although chemical yields of the final reaction decreased with increasing scale.

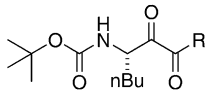
The Passerini reaction was exploited as a third technique to produce ketoamides.[16] As shown in Scheme 3, the aldehyde **10**[17] was reacted with the isonitrile **11**[18–20] and benzoic acid to give the α-acyloxyamides **12**. Hydrolysis of both the ester and carbamate afforded the β-amino-α-hydroxyamides **13**. Various P^2–P^3 alcohols[15] were converted to chloroformates and coupled to the amines **13** followed by oxidation to afford the ketoamides **5t–w**. The Passerini method allowed the swift synthesis of compounds with different P^2 groups, however volatile isonitriles can be toxic and smelly.

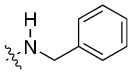
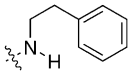
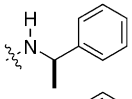
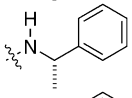
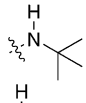
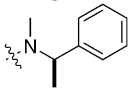
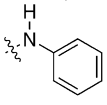
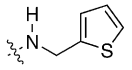
As shown in Table 1, replacement of the aldehyde warhead of inhibitor **2**, by an electrophilic ketoamide group as in analogue **5a** resulted in a 100-fold loss in inhibitory activity. Attempts were made to regain activity by



Scheme 3. (a) PhCOOH, CH_2Cl_2, 60%; (b) NaOH, dioxane, H_2O, 100 °C, 95%; (c) alcohol, 1.93 M $COCl_2$ in PhMe, pyridine, CH_2Cl_2, −20 °C to rt; **13**, iPr_2NEt, dioxane, 22–91%; (d) Dess–Martin periodinane, CH_2Cl_2, 55–78%.

Table 1. Inhibition of human cathepsin K by $P^{1'}$ analogues



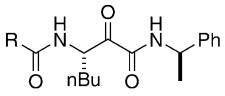
Compd	R	IC_{50} μM[a]	Compd	R	IC_{50} μM[a]
5a		5.9	**5g**		2.9
5b		21	**5h**		5
5c		18	**5i**		1.2
5d		9.1	**5j**		4.2
5e		> 100	**5k**		> 100
5f		6.3	**5l**		3.4

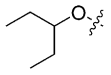
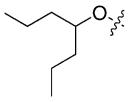
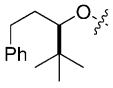
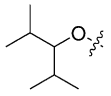
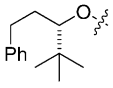
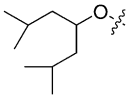
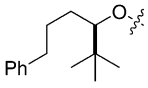
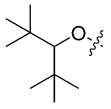
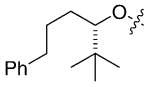
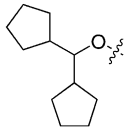
[a] Inhibition of recombinant human cathepsin K activity in a fluorescence assay using 10 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, 120 mM NaCl, pH = 5.5. The IC_{50} values are the mean of two or three inhibition assays, individual data points in each experiment were within a 3-fold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

probing the $S^{1'}$ subsite. Adding simple lipophilic groups like the methyl, ethyl, or isopropyl moieties in analogues **5b–d** did not improve potency. Furthermore, the *t*-butyl amide **5e** demonstrated that additional steric bulk adjacent to the amide was detrimental to inhibitory potency.

Since the cathepsins contain a conserved tryptophan (^{184}Trp in cathepsin K) in their active site, amines linked to phenyl substituents were also employed in attempts to form π–π stacking interactions with its conserved indole.[21] Unfortunately, the phenyl, benzyl, and phenethyl analogues **5f–h** offered no significant potency advantage over the primary amide **5a**. Attempting to increase the population of the active site rotamer of analogue **5g**, a methyl group was inserted into the

Table 2. Inhibition of human cathepsin K by P^2 analogues



Compd	R	IC_{50} nM[a]	Compd	R	IC_{50} nM[a]
5m		34	**5s**		76
5n		20	**5t**		32
5o		1.1	**5u**		2.5
5p		912[b]	**5v**		69
5q		0.79	**5w**		0.71
5r		1400[c]	**5x**		6.3

[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, 120 mM NaCl, pH = 5.5. The IC_{50} values are the mean of two or three inhibition assays, individual data points in each experiment were within a 3-fold range of each other.
[b] This analogue contains ~0.1% of **5o**.
[c] This analogue contains ~0.1% of **5q**.

benzylic carbon to give **5i** and **5j**. Although **5i**, derived from the *R*-enantiomer, was apparently more active than **5j**, derived from the *S*-enantiomer, no significant gains in potency were produced. Furthermore, disubstitution of the amide nitrogen was not tolerated. The *N*-methyl analogue **5k** lost all activity. However, replacement of the phenyl ring, as in thiophene **5l**, was tolerated and could offer a potential site to alter drug properties of this class of inhibitor. While this preliminary exploration of the $S^{1'}$ subsite provided no significant boost in enzyme inhibitory activity, the α-methyl benzyl amide moiety of analogue **5i** was adopted as a suitable $P^{1'}$ substituent for further modifications in the ketoamide inhibitor series.

Since the primary determinant of enzyme specificity in the cathepsins is the S^2 pocket, it was perceived that P^2 region modifications might prove more fruitful. Calpeptin **1** contains an isobutyl group as its P^2 substituent. Since the *tert*-butyl moiety of **5i** does not reach as deeply into the S^2 pocket, it was extended by an additional carbon, as in **5m** and **5n**, to better mimic this feature of calpeptin. As shown in Table 2, these modifications resulted in 35-fold and 60-fold increases in potency over their precursor **5i**.

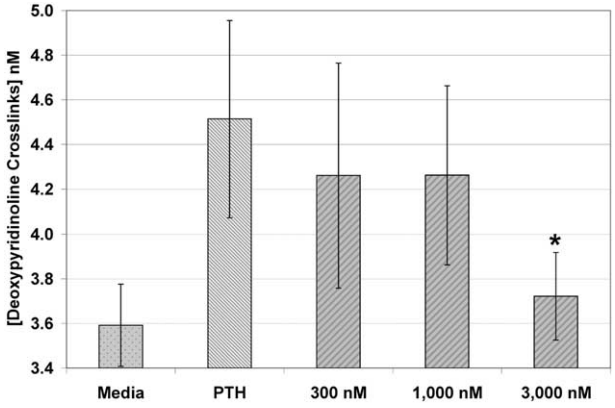


Figure 1. Rat calvarial resorption assay with **5u**. *2 sided *t*-test, $p = 0.03$.

The appendage of a P^3 substituent to analogue **5m** should further increase inhibitory activity. Taking a clue from the benzyl carbamate of calpeptin **1**, a phenyl ring was tethered to the neopentyl carbon of **5m** by carbon linkers of various lengths, giving analogues **5o**–**r**. The two-atom tether **5o** was 30-fold more potent than its precursor. Since analogue **5n** has equivalent activity to **5m**, most of this improvement in activity of **5o** versus **5m** probably arises from binding interactions in the S^3 subsite rather than from P^2 rotamer stabilization by the chiral center. The more conformationally flexible three-atom linker **5q** retained equivalent activity to **5o** despite its potentially greater entropic cost on binding. In contrast, their corresponding diastereomers **5p** and **5r** are substantially less active.[22]

The insertion of a P^3 substituent into these inhibitors had conferred a substantial boost in potency, but added another stereogenic center to the inhibitors resulting in a more complex synthesis and the need for the chiral separation of enantiomeric precursor alcohols via HPLC. To address these concerns a series of achiral P^2 substituents were incorporated into the inhibitors **5s**–**x**. The di-*iso*-propyl, di-*tert*-butyl, and dicyclopentyl analogues **5u**, **5w**, and **5x** were significantly more active than the neopentyl analogue **5m**. In addition to occupying the S^2 pocket, these P^2 residues may pick up some binding interactions in the active site trough that accommodates the substrate backbone.

Because the active site of rat cathepsin K differs from the human enzyme (^{61}Asp→Tyr in S^3 and ^{133}Ala→Ser in S^2),[23] many human cathepsin K inhibitors are less potent against the rat ortholog making it difficult to assess their efficacy in rat osteoporosis models.[24] Wishing to evaluate the potential developability of this potent class of cathepsin K inhibitors, analogue **5u**, which is only 10-fold less active versus the rat enzyme (rat $IC_{50} = 20$ nM), was selected for further evaluation. In vitro incubations with rat S9 liver homogenates rapidly metabolized **5u**.[25] In spite of this ex vivo prediction of rapid clearance, **5u** exhibited a low clearance and long terminal half-life in rats ($t_{1/2} = 290$ min, $C_l = 8.7$ mL/min/kg, $V_{ss} = 1900$ mL/kg). However, this analogue was poorly soluble in fasted state simulated intestinal fluid (0.012 mg/mL)[26,27] and exhibited moderate

membrane permeability in the Madin–Darby canine kidney cell monolayer assay ($P_{APP} = 57 \pm 4$ nm/s).[28] Not surprising given the low solubility, **5u** had poor oral bioavailability (F = 3%) in rats.

This analogues poor oral bioavailability precluded a chronic pharmacodynamic study in the OVX rat. However **5u** was profiled in the ex vivo rat calvaria resorption assay, a model often predictive of anti-resorptive activity.[29,30] As shown in Figure 1, treatment with parathyroid hormone enhanced bone resorption as measured by deoxypyridinoline (DPD) crosslinks release. Analogue **5u** dose-dependently attenuated this release of DPD crosslinks from type I collagen in the rat calvaria resorption assay. This efficacy reached statistical significance at the 3000 nM dose (2 sided *t*-test, $p = 0.03$). Thus, this ketoamide cathepsin K inhibitor acts as an antiresorptive agent by attenuating type I collagen hydrolysis in bone.

In summary, this report highlights the discovery of a potent series of cathepsin K inhibitors. Starting from the directed screening hit **2**, the aldehyde warhead was replaced with a more drug-like ketoamide electrophile. The resulting drop in potency was recovered and enhanced via modifications to the $P^{1'}$, P^2 and P^3 substituents. In addition, a representative inhibitor in this ketoamide series was efficacious in attenuating bone resorption in a surrogate assay of osteoporosis. Subsequent reports will detail efforts to improve the oral bioavailability of these cathepsin K ketoamide inhibitors.

Acknowledgements

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