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Design and synthesis of arylaminoethyl amides as noncovalent inhibitors of cathepsin S. Part 1

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Abstract—A series of N_{α}-acyl-α-amino acid-(arylaminoethyl)amides were found to be potent and noncovalent cathepsin S inhibitors. Compound **20** possessed high cathepsin S affinity (K_i = 3.3 nM) and showed excellent selectivity over cathepsin K, L, F, and V. Molecular modeling, design, synthesis, and in vitro activity are described.

Cathepsin S is a lysosomal cysteine protease of the papain family. It is primarily expressed in antigen presenting cells (APC) and has been demonstrated to play a major role in antigen presentation through the targeted degradation of the invariant chain (li) that is associated with the major histocompatibility class II complex (MHC II).[1,2] The invariant chain blocks the MHC II binding groove and requires proteolytic removal prior to productive antigen loading on the MHC II complex.[3–5] Cathepsin S deficient mice displayed profound impairment of invariant chain degradation in APC. More importantly, the mice were viable and showed a minimally altered phenotype.[3,4] Compared with the wild type, cathepsin S deficient mice showed marked resistance to the development of collagen-induced arthritis and autoimmune myasthenia gravis.[4,6] These facts suggest that cathepsin S is an attractive therapeutic target for the regulation of immune hyperresponsiveness,[1] such as rheumatoid arthritis, multiple sclerosis,[7] asthma, and allergy.[5]

Unlike most other cathepsins, cathepsin S remains active at neutral pH.[8] Secreted cathepsin S has been shown to degrade all of the major components of the extracellular matrix and has been implicated in the pathogenic response that ultimately leads to atherosclerosis, emphysema, and chronic obstructive pulmonary disease (COPD).[1,3,9] Abundant cathepsin S is present in human atherosclerotic lesions. When fed a high cholesterol diet, the severity of atherosclerosis developed in cathepsin S and LDL receptor (LDLr) double knockout mice was significantly diminished relative to the LDLr deficient mice alone.[10] In keeping with its prominent collagenolytic and elastolytic activities in lung, cathepsin S has been proposed to play an important role in tissue remodeling in emphysema.[11] Blocking cathepsin S activity inhibited IFN-γ-induced alveolar epithelial cell apoptosis.[12] Furthermore, cathepsin S is believed to be involved in tumor invasion, as immunohistochemical analysis found elevated levels of cathepsin S in astrocytoma cells, and LHVS, an irreversible cathepsin S inhibitor, reduced the cell invasion rate in vitro.[13] Cathepsin S deficient mice displayed defective microvessel development, implying an essential role of cathepsin S for extracellular matrix degradation in angiogenesis.[14] More recently, cathepsin S inhibitors have been claimed for their therapeutic use in treating pain of various etiologies.[15]

High throughput screening of our compound collections against recombinant human cathepsin S was performed by measuring the rate of cleavage of a synthetic tetrapeptide (P4-P1) coumarin substrate.[16] Compound **1** (Table 1) was selected for further profiling. Unlike the majority of cysteine protease inhibitors reported in the literature that are peptide-based analogues bearing a

Keywords: Cathepsin S; Cathepsin; Cysteine protease inhibitor; Peptidomimetics; Noncovalent inhibitors.
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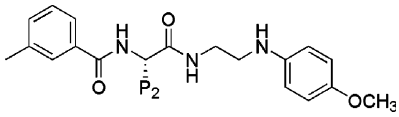
Table 1. Inhibition of cathepsin S, K, and L—variation of P_2[a]



Compounds	P_2	Cathepsin S (K_i, nM)	Cathepsin K (K_i, nM)	Cathepsin L (K_i, nM)
1	*i*-Butyl	62	43	7.2
5a	*t*-Butylmethyl	46	246	216
5b	Cyclohexyl	23	66	22
5c	Cyclohexylmethyl	9.7	205	4.6
5d	Cyclohexylethyl	38	>100,000	326
5e	Phenyl	285	>30,000	134
5f	$C_6H_5CH_2$	29	123	3.7
5g	4-Cl-$C_6H_4CH_2$	16	>100,000	1.4
5h	3-Cl-$C_6H_4CH_2$	33	>100,000	1.5
5i	2-Cl-$C_6H_4CH_2$	35	595	5.0
5j	4-CH_3-$C_6H_4CH_2$	27	>65,000	3.4
5k	4-CF_3-$C_6H_4CH_2$	19	>100,000	2.8
5l	4-CH_3O-$C_6H_4CH_2$	66	>100,000	9.3
5m	4-CH_3CONH-$C_6H_4CH_2$	123	>100,000	23
5n	3-Picolyl	3707	>100,000	673
5o	4-Picolyl	6769	>100,000	251
5p	Furan-2-ylmethyl	>20,000	>30,000	2991
5q	Thiazol-5-ylmethyl	>65,000	>100,000	4214
5r	2-Naphthyl-CH_2	16	>100,000	1.1
5s	1*H*-Indol-2-yl-CH_2	29	>100,000	7.2
5t	4-Biphenyl-CH_2	34	>100,000	1.2
5u	$C_6F_5CH_2$	>20,000	>100,000	25

[a] Values are means of three experiments.

reactive or 'quiescent' functional group (e.g., warhead) at the scissile amide bond,[17,18] compound **1** possesses no apparent warhead that could form a covalent interaction with the cysteine thiol at the active site, but is nonetheless a potent inhibitor of cathepsin S, K, and L with K_i values of 62, 43, and 7 nM, respectively.[19] Lineweaver–Burk analysis of **1** demonstrated that it is purely a competitive inhibitor. Reversible binding was established by an experiment in which inhibitor **1** was incubated with cathepsin S at high concentration to ensure loss of enzyme activity. Then upon dilution with assay buffer containing substrate, enzymatic activity was restored. It is believed that the noncovalent inhibitors are most likely to offer the highest selectivity and the lowest likelihood for adverse side effects that can result from undesired nonspecific or irreversible binding to other proteins.[17] Selective cathepsin S inhibitors are needed to clearly delineate the centrality of this target for the specific inhibition of antigen presentation and elastolytic activities.[20] Hence, we set out to explore the structure–activity relationship of this chemotype with the aim of identifying potent and selective cathepsin S inhibitors.[21]

Though the high sequence homology among cathepsin S, L, and K presented considerable challenges to designing selective cathepsin S inhibitors, it has been recognized that the side chain of Phe211 near the bottom of the S2 pocket of cathepsin S can undergo a conformational switch that is unique to cathepsin S. Namely, the Phe211 side chain can assume two stable conformations to accommodate either a small (conformation *a*) or a bulky P2 side chain (conformation *b*).[22] The structural plasticity of the S2 pocket in cathepsin S was elected as key focal point in the initial rational exploration. Figure 1 shows the S2 pocket of cathepsin S docked with the prototype inhibitor **1**.

A solid-phase synthesis of compounds **5a**–**u** is depicted in Scheme 1. PAL aldehyde (4-formyl-3,5-dimethoxy-

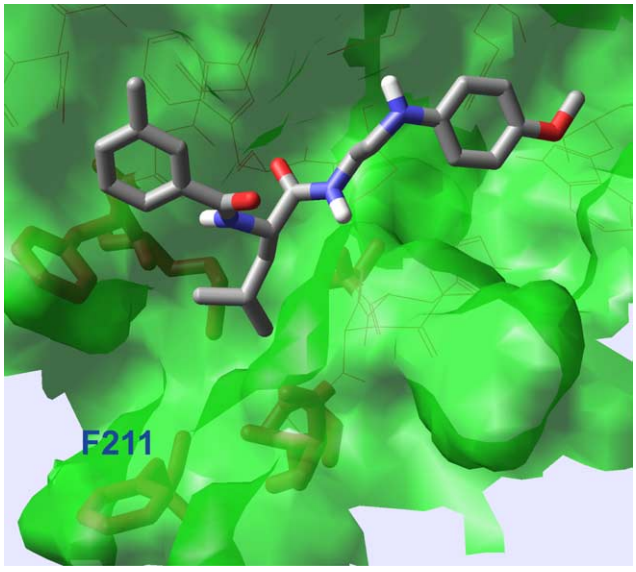
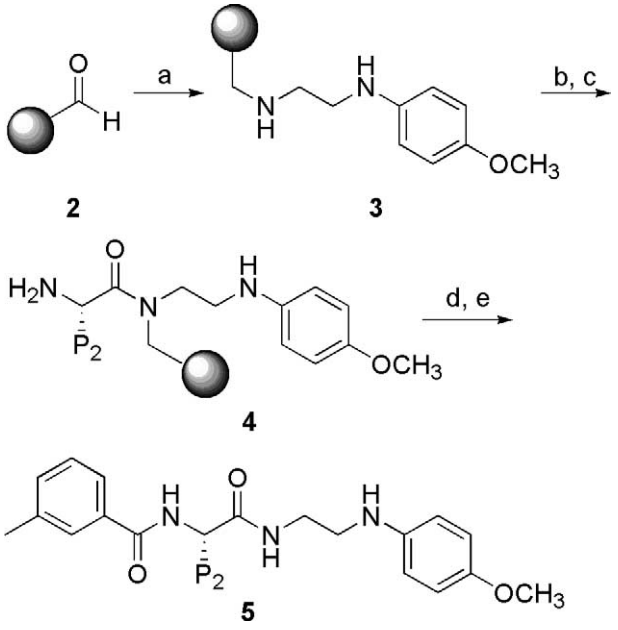


Figure 1. The active site of cathepsin S in conformation *b* docked with compound **1**.



Scheme 1. Reagents: (a) N^1-(4-Methoxy-phenyl)-ethane-1,2-diamine, AcOH; $NaHB(OAc)_3$; (b) (L)-$FmocHNCHP_2CO_2H$, HOBt, DIC; (c) 20% piperidine in DMF; (d) *m*-toluic acid, HOBt, DIC; (e) TFA/CH_2Cl_2/H_2O: 45:45:10, v/v. Overall yields after LCMS purification ranged 20–60%.

phenoxymethyl) resin **2** is reductively aminated with N^1-(4-methoxy-phenyl)-ethane-1,2-diamine to obtain the resin bound intermediate **3**, which is then acylated with a Fmoc-protected amino acid. The Fmoc group is removed to provide **4**. After coupling with *m*-toluic acid under DIC mediated amide formation conditions, cleavage from resin using TFA affords the desired product **5** on 10–20 mg scale in 20–60% overall yield.[23]

As expected, bulkier aliphatic P2 enhanced cathepsin S potency (Table 1, **5a**, **b**).[24] The cyclohexylmethyl P2 (**5c**) yielded 6-fold improvement in affinity for cathepsin S compared to **1**. The higher potency of **5c** was attributed to its enhanced hydrophobic interactions with the S2 pocket.[25] Affinity to cathepsin K was completely abolished by cyclohexylethyl and phenyl P2 (**5d**, **e**). Phenylalanine side chains were well accepted by the S2 pocket of cathepsin S (**5f**–**i**). The significant loss of binding affinity with cathepsin K by *p*- and *m*-substitutions (**5g**, **h** vs. **5f**, **i**) indicated that the steric restrictions imposed by the Leu205 and the Tyr67 side chains prevented these inhibitors from binding to cathepsin K (Fig. 2).[22] A variety of small substitutions at the *p*-position provided moderately potent cathepsin S inhibitors (**5j**–**m**) that were completely selective over cathepsin K. The lower potency of **5l** and **5m** is perhaps due to the desolvation energy required for these polar groups to bind in the highly hydrophobic S2 pocket. Accordingly, several polar bioisosteres of the phenylalanine side chain (**5n**–**q**) were found to be detrimental to the binding of cathepsin S. Compounds with fused biaryl P2, such as **5r** and **5s**, exhibited cathepsin S affinity and selectivity against cathepsin K similar to those of compounds **5f**–**h** and **5j**–**m**. The cathepsin S affinity of **5t** highlighted the great flexibility of the S2 pocket. Upon interaction

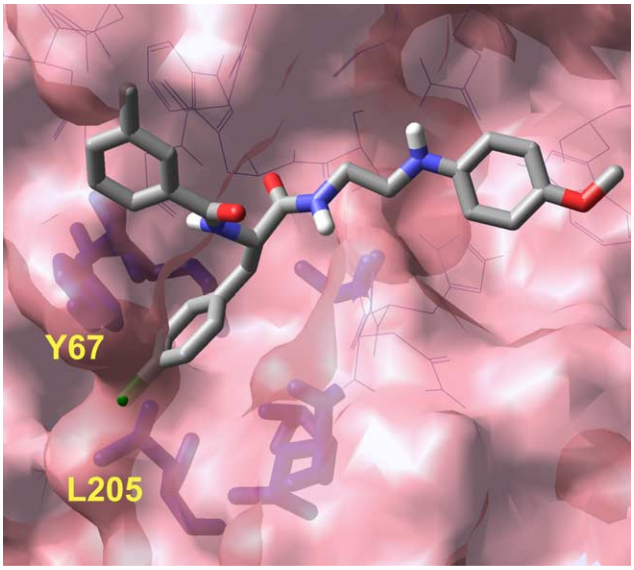


Figure 2. The active site of cathepsin K docked with compound **5g**.

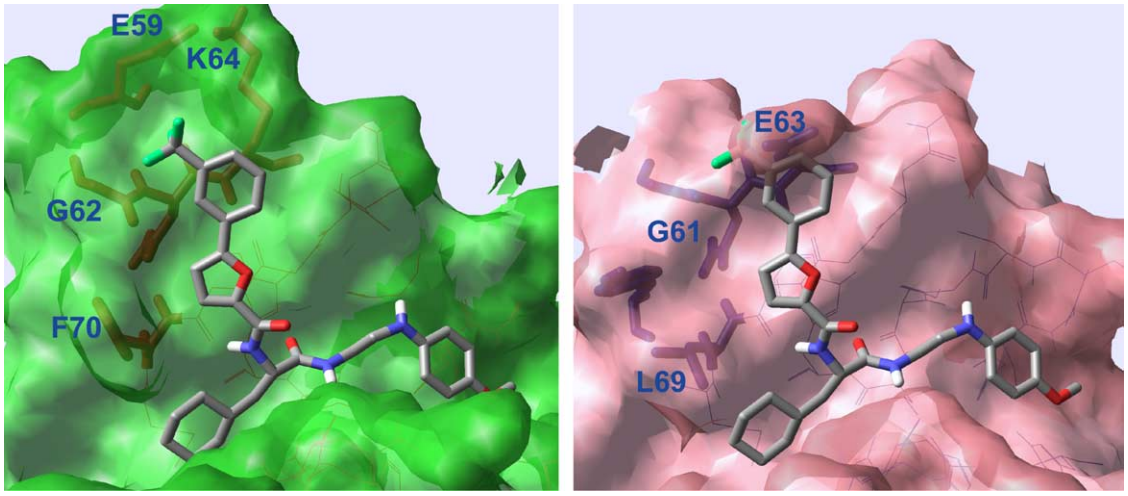
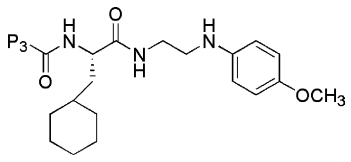
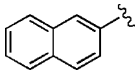
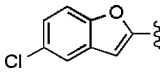
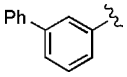
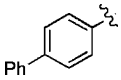
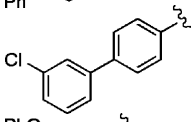
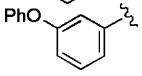
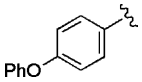
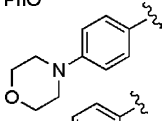
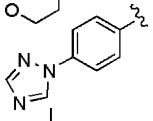
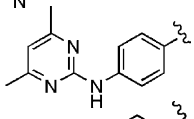
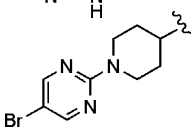
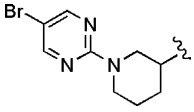
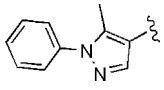
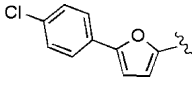
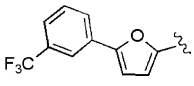


Figure 3. The active sites of cathepsin S (left) and cathepsin L (right) docked with compound **20**.

Table 2. Inhibition of cathepsin S, K, and L—variation of P_3[a]



Compounds	P_3	Cathepsin S (K_i, nM)	Cathepsin K (K_i, nM)	Cathepsin L (K_i, nM)
6		5.0	55	13
7		9.4	58	68
8		2.9	>20,000	14
9		9.1	>30,000	>37,000
10		7.4	>100,000	>79,000
11		8.0	>100,000	106
12		17	>30,000	>37,000
13		4.0	341	148
14		26	718	303
15		2.0	438	176
16		107	>100,000	7950
17		49	>100,000	1296
18		34	1581	316
19		7.1	>10,000	>6440
20		3.3	>65,000	>17,400

[a] Values are means of three experiments.

with the 4-biphenylmethyl P2, sufficient space was created to accommodate the bulkier biphenyl group through the conformational adjustment of Phe211 at the distal position of the S2 pocket.[22] Unfortunately, none of the P2 modifications described above imparted the desired selectivity against cathepsin L. The potency in-

crease for **5f–k** over **1** against cathepsin L can be rationalized by the substrate specificity data. Incorporating aromatic amino acids at the P2 position of the peptide substrate generally provides good recognition for the S2 pocket of cathepsin L.[26] It was intriguing that the perfluoro phenylalanine derivative **5u** was found to be a selective cathepsin L inhibitor.

The active sites of cathepsin S and L were compared subsequently to identify chemical opportunities for building selectivity over cathepsin L into compound **5**. Cathepsin S is known to have a poorly defined S3 pocket.[22] Receptor optimization calculation revealed that a unique Lys64 residue residing in the cathepsin S S3 pocket can re-orient its side chain to accommodate an extended P3 moiety of the inhibitors (Fig. 3).[27] In contrast, the analogous S3 region of cathepsin L is occluded by Glu63 (vide infra). On the basis of this computational analysis, we envisioned that selectivity between cathepsin S and L could be achieved by judicious extension of the P3 moiety to invoke steric clash with the Glu63 side chains of cathepsin L.

The initial P3 optimization maintained a cyclohexylalanine in the P2 position because of the favorable balance between cathepsin S potency and selectivity over cathepsin K and L as in compound **5c**. In addition, incorporation of a cyclohexylalanine residue at the P2 position of the peptide substrates dramatically increased the cleavage efficiency for cathepsin S, while either decreasing or maintaining affinity for cathepsin L and K.[26]

Although the selectivity over cathepsin K with compounds **6** and **7** was reduced relative to compound **5c**, they did exhibit improved selectivity over cathepsin L (Table 2). 3-Biphenyl P3 (**8**) exhibited poor activity toward cathepsin K, while its selectivity over cathepsin L had only marginally improved. However, dramatic increases (>3000-fold) in the selectivity over both cathepsin K and L were observed for the first time with compounds **9** and **10** by incorporating a 4-biphenyl group in P3, albeit their affinity to cathepsin S was slightly diminished as compared to **8**. 3- and 4-Phenoxyphenyl analogues (**11** and **12**) showed potency and selectivity similar to those of **8** and **9**, respectively. Unfortunately, compounds **9–12** exhibited poor solubility in aqueous buffer (<1 mg/L) which precluded any further profiling. Hence, in an attempt to improve the solubility, we investigated various hydrophilic moieties in the P3 position. Replacement of the pendant phenyl group in **9** with a heterocycle had little effect on cathepsin S activity, but usually resulted in decreased selectivity against cathepsin K and L (**13** and **14**). The analogue **15** with 4-(pyrimidin-2-ylamino)-phenyl P3 was very potent but of only modest selectivity. Analogues **16** and **17** in which the proximal phenyl ring was replaced with a piperidine ring exhibited poor affinity to both cathepsin K and L. However, their potencies toward cathepsin S were considerably diminished. Heteroaryls were also introduced at the proximal phenyl position of compound **9**. Compound **18** displayed modest potency and selectivity, upon which further optimization led to the 5-phenyl-furan-2-yl analogues **19** and **20**. Both of these inhibitors bound tightly to cathepsin S, and more importantly, they exhibited greater than 1000-fold selectivity over cathepsin K and L.

To understand the mechanism underlying the excellent selectivity of compounds **19** and **20** between cathepsin S and L, a 3-dimensional structure of cathepsin S-**20** complex, obtained by a modeling study, was examined (Fig. 3). The furan moiety of **20** efficiently fills the S3 pocket of cathepsin S shaped by Gly62 and Phe70. One edge of the furan ring packs against the aromatic ring of Phe70, with which it appears to be involved in an edge-face π–π interaction with a centroid separation and dihedral angle of ~5 Å and 75°, respectively.[28] Lys64 side chain swings away to accommodate the distal 5-phenyl ring in the created space. The positively charged ammonium group of Lys64 is positioned at a distance of 2.8 Å from the negatively charged carboxylate oxygen of Glu59, which could lead to a favorable electrostatic interaction. On the other hand, modeling suggests that compound **20** is not able to bind to cathepsin L as in the cathepsin S-**20** complex, because a steric clash would occur between the distal 5-phenyl group with Glu63 of cathepsin L and the furan group of compound **20** cannot make close contacts with Leu69 and Gly61 in the S3 pocket of cathepsin L.

In summary, SAR studies on a lead compound **1** identified from screening led to the discovery of a series of potent and selective cathepsin S inhibitors. The judicious combination of moderately sized aliphatic P2 and extended P3 moieties proved to be crucial for conferring the selectivity of these inhibitors. Compounds **19** and **20** are among the most potent and selective cathepsin S inhibitors reported to date lacking an electrophilic cysteinyl trap.[21,29] The discovery of potent, selective, and noncovalent cathepsin S inhibitors provides a valuable tool to further probe the biological and pathological functions of this enzyme.[30] The therapeutic applications of selective cathepsin S inhibitors will be reported in due course.

Acknowledgments

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27. Receptor optimization was performed, to reflect the induced fit upon ligand binding, using mixed Monte Carlo Multiple Minimum/Large Scale LowMode conformational search strategy available in MacroModel (version 7.0; Schrodinger, Inc.). Flexible ligand docking was performed with Glide (version 2.0; Schrodinger, Inc.). The van der Waals radii of nonpolar protein atoms were scaled by a factor of 0.9 and those of the ligand were scaled by factor 0.8. The enclosing box for ligand docking was centered on active site residues and was 34 Å in size along each dimension. Maximum number of poses selected for refinement was 5000, and maximum number of refined poses selected final minimization and scoring was set at 400. The crystal structures of cathepsin S and K used in the docking experiments were obtained in-house (Clark, K. L. unpublished results). The crystal structure of cathepsin L was from PDB (Accession No. 1ICF). The amino acid residue numbering scheme used in discussions is for each individual cathepsin.
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