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Synthesis and evaluation of arylaminoethyl amides as noncovalent inhibitors of cathepsin S. Part 3: Heterocyclic P3

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Abstract—A series of N_α-2-benzoxazolyl-α-amino acid-(arylaminoethyl)amides were identified as potent, selective, and noncovalent inhibitors of cathepsin S. Structure–activity relationships including strategies for modulating the selectivities among cathepsins S, K, and L, and in vivo pharmacokinetics are discussed. A X-ray structure of compound **3** bound to the active site of cathepsin S is also reported.

Cathepsin S is a lysosomal cysteine protease and a member of the papain family, and it is expressed primarily in antigen presenting cell. It has been shown to play a key 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). The primary role of the invariant chain is to block the MHC II binding groove, and proteolytic removal is required prior to productive antigen loading on the MHC II complex.[1] Cathepsin S knockout mice display significant impairment of invariant chain degradation in antigen presenting cells and consequently exhibit a clear resistance to the development of collagen-induced arthritis and autoimmune myasthenia gravis in comparison to the wild type mice.[2] These data point to cathepsin S as an attractive therapeutic target for the modulation and regulation of immune hyperresponsiveness, such as autoimmune diseases, asthma, multiple sclerosis, and rheumatoid arthritis.[3]

Compound **1** (Fig. 1) was originally reported by Altmann et al. as a noncovalent inhibitor of the highly homologous cysteine protease cathepsin K.[4] As a relatively nonselective inhibitor of this class of proteases, **1** was also pulled out of a high-throughput screen of our compound collection for inhibitors of cathepsin S. As we have reported previously,[5] early hit-to-lead optimization on this scaffold produced compound **2** as a potent

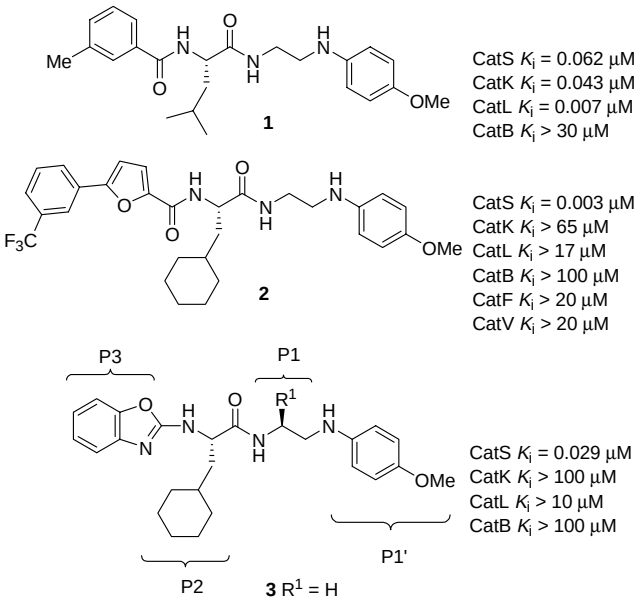

Figure 1.

Keywords: Cathepsin S; Cathepsin; Cysteine protease inhibitor; Noncovalent inhibitor; Peptidomimetics.
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(K_i = 3 nM) inhibitor of cathepsin S with greater than 5000-fold selectivity over cathepsins K, L, B, F, and V. Lineweaver–Burk analysis confirmed that compounds from this series are purely competitive inhibitors.[5,6] In addition, the nature of inhibition of arylaminoethyl amides was shown to be fully reversible as enzymatic activity is restored following dilution and dialysis of the enzyme–inhibitor complex.[4–7]

Compound **2**, however, suffered a short half-life and rapid clearance when administered intravenously in rats. Lead optimization efforts were initiated with the intent to decrease the peptidic nature of compound **2** and improve the pharmacokinetic (PK) and physicochemical properties of this series. Structure analysis suggested that the benzamide carbonyl in compounds **1** and **2** does not play a direct role in binding to cathepsin S. Thus, in order to reduce the peptidic character of **2** we envisioned that the benzamide P3 could be replaced with an NH-aryl/heteroaryl moiety in which the analogous hydrogen bond is formed with Gly69 as in **2**, and the NH-linked aryl/heteroaryl moiety would fill the shallow S3 pocket formed by Gly62 and Phe70 (Fig. 2).[5,8]

As shown in Table 1, our initial attempts at replacing the biaryl amide P3 subunit resulted in compound **3**, in which the biaryl amide was successfully replaced with a benzoxazole. The affinity toward cathepsin S was diminished about 10-fold versus compound **2**, nevertheless this modification eliminated an amide and decreased the molecular weight while simultaneously preserving the selectivity over cathepsins K and L. Furthermore, this modification of the P3 subunit had a dramatic effect on the in vivo pharmacokinetic parameters of this series. Upon intravenous administration (3 mg/kg) the half-life of **3** was nearly 5 h with a low clearance and moderate volume of distribution (Table 4). However, compound **3** had effectively no plasma exposure following a 10 mg/kg oral dose. The poor oral bioavailability is presumably due to rapid first pass metabolism since **3** exhibits poor in vitro metabolic stability, with <5% of parent compound remaining after incubation for 30 min in rat liver microsomes. Lead optimization of this series was then aimed primarily at improving the oral bioavailability and in vivo PK by exploring the SAR of the P3 heterocycle, the P1 side chain, and the aniline moiety at the prime side.

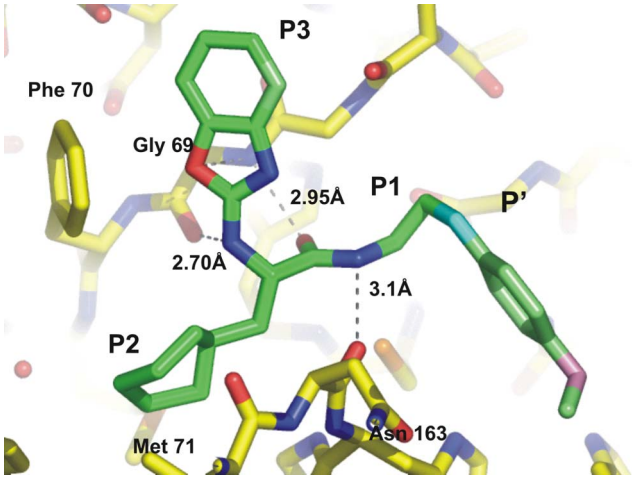


Figure 2. Crystal structure of cathepsin S with compound **3** at 1.8 Å resolution (RCSB PDB ID: 2F1G). Protein atoms are colored with yellow carbons, red oxygens, and blue nitrogens. Compound atoms are colored with cyan carbons, red oxygens, and blue nitrogens, while those atoms ill defined with no visible electron density (P′ aniline moiety) are labeled with the corresponding lighter shades. Potential hydrogen bonds are represented by dotted black lines. Figure generated using PyMOL.

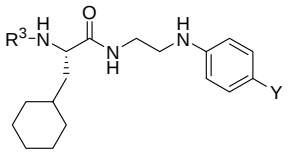
The synthesis of analogs lacking P1 substitution on the diamine side chain is depicted in Scheme 1. Substituted 2-chlorobenzoxazoles were prepared from the appropriate *ortho*-aminophenol.[9] N-Arylation of cyclohexylalanine methyl ester with the appropriate 2-chlorobenzoxazole **5** followed by saponification led to the *N*-aryl amino acid **6**. Condensation of **6** with either *N*-(4-methoxyphenyl)- or *N*-(4-fluorophenyl)-ethylene diamine[4–6] afforded compounds **3** and **7a**–**g**, respectively, in 25–50% overall yield.

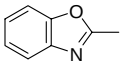
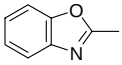
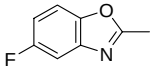
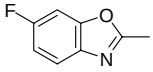
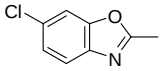
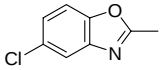
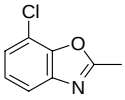
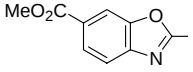
Substitution on the benzoxazole ring is fairly well tolerated as shown in Table 1. Replacement of the undesirable *para*-methoxyaniline group[10] with 4-fluoroaniline decreased the potency by about threefold (**3**–**7a**). The addition of a halogen to the 5-position of the benzoxazole resulted in a twofold loss in activity (**7b** and **e**), whereas substitutions at the 6-position of the benzoxazole were roughly equipotent (**7d**) or were slightly improved in their potency toward cathepsin S (**7c** and **g**), while still remaining selective over cathepsins K and L. The most significant improvement in potency was found with the 7-chlorobenzoxazole **7f**, which gave a nearly fivefold boost in affinity to cathepsin S over **7a**. Although highly selective over cathepsin K, the selectivity of **7f** over cathepsin L was reduced to about 30-fold.

The 6- and 7-chlorobenzoxazoles were chosen as the preferred P3 subunits for further exploration into the nature of the P1 side-chain and aniline moieties. The synthesis of diamines with a P1 side chain is shown in Scheme 2. The appropriate Cbz-protected amino acid **8** was first reduced to the alcohol and then oxidized to the aldehyde to give **9**. Reductive amination with the desired aniline or indoline afforded the *N*-aryl diamine **10**. Hydrogenolysis of the Cbz-group, followed by amide coupling to the *N*-aryl-cyclohexylalanine **6**, afforded both the 7-chlorobenzoxazole series **12a**–**g** and the 6-chlorobenzoxazole series **13a**–**i** in 20–40% overall yield from amino acid **8**.

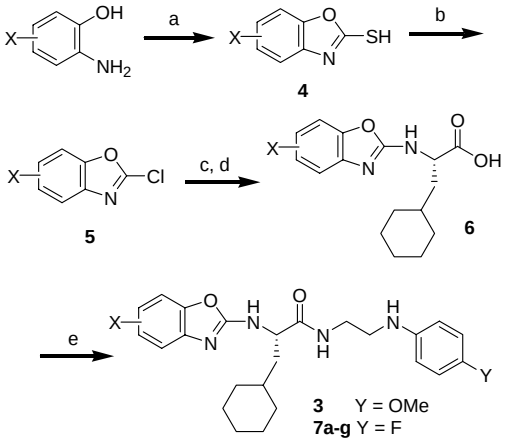
We have shown previously that incorporating an alkyl group onto the P1 side chain boosts the potency of compounds from this series over the unsubstituted ethylenediamine analogs.[11] Therefore, the diversification of the *N*-aryl moiety, which can have dramatic influence over the cathepsin S affinity selectivity over the related enzymes, was initiated with a (*S*)-methyl group in the P1. The 4-methoxyaniline **12a** (Table 2) is the most potent analog in the series (K_i = 0.001 μM), but it also showed a proportional increase in the affinity toward cathepsins K and L, leaving only a narrow window of selectivity over cathepsin L. The potency of the 7-chlorobenzoxaz-

Table 1. Inhibition of cathepsins S, K, and L: influence of substitution on P3 benzoxazole[a]



Compound	R^3	Y	CatS K_i (μM)	CatK K_i (μM)	CatL K_i (μM)
3		OMe	0.029	>30.0	>10.0
7a		F	0.094	>30.0	>10.0
7b		F	0.197	>30.0	8.37
7c		F	0.043	>30.0	9.04
7d		F	0.093	>20.0	7.46
7e		F	0.173	>20.0	11.8
7f		F	0.019	4.56	0.541
7g		F	0.043	>10.0	1.73

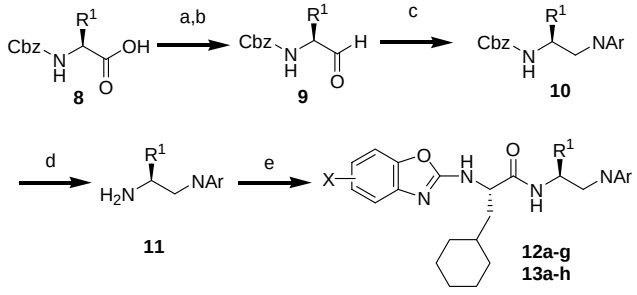
[a] Details of the assay conditions can be found in Supplementary material.



Scheme 1. Reagents and conditions: (a) KSCSOEt, EtOH, reflux; (b) PCl_5, $POCl_3$, 100 °C; (c) 3-cyclohexyl-L-alanine methyl ester hydrochloride, DIEA, DMF, 0 °C to rt, 70–85%; (d) LiOH, H_2O, dioxane, 95–99%; (e) N^1-(4-fluorophenyl)-ethylenediamine, DIEA, HATU, CH_2Cl_2, 75–85%.

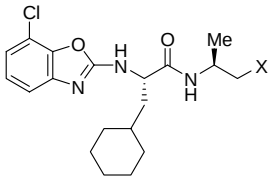
ole P3 subunit remained attractive, however, and we therefore sought to improve the selectivity profile by optimization of the aniline moiety at the prime side.

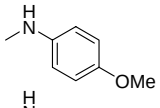
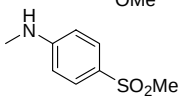
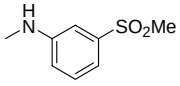
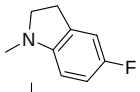
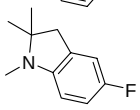
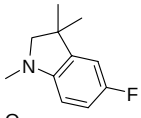
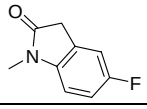
Electron-withdrawing groups on the aniline ring are also tolerated. Methylsulfones **12b** and **c** improved the solubility but led to an order of magnitude decrease in cathepsin S activity and contributed very little to any improvements in selectivity over cathepsins K and L. Replacement of the aniline with 5-fluoroindoline (**12d**) showed a significant improvement in the selectivity over both cathepsins K and L despite the somewhat diminished cathepsin S activity over the methoxyaniline. More importantly, the introduction of the indoline moiety gave a dramatic improvement in the pharmacokinetics of this series, as compound **12d** had a 24% oral



Scheme 2. Reagents and conditions: (a) i—*iso*-BuOCOCl, Et_3N, THF; ii—$NaBH_4$, H_2O, 60–85%; (b) Dess–Martin periodinane, CH_2Cl_2; (c) aniline or indoline, $NaB(CN)H_3$, AcOH, MeOH, 60–75% combined yield steps (b) and (c); (d) H_2, Pd/C, MeOH, 90–95%; (e) **6**, HATU, DIEA, CH_2Cl_2, 75–85%.

Table 2. Inhibition of cathepsins S, K, and L: variation of aniline moiety

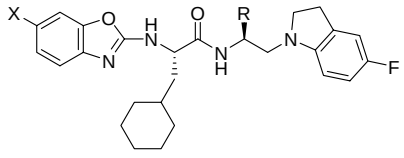


Compound	X	CatS K_i (μM)	CatK K_i (μM)	CatL K_i (μM)
12a		0.001	0.286	0.024
12b		0.011	1.05	0.108
12c		0.026	2.13	0.199
12d		0.015	14.7	0.849
12e		0.015	>100	4.67
12f		0.003	3.71	0.123
12g		12.3	>100	>30

bioavailability in rats. However, **12d** also had a rather high clearance (33.1 mL/min/kg) (Table 4).

With the 5-fluoroindoline in place, we sought to explore the effects of substitution on the P1 side chain, and determine what effects, if any, such substitution had on selectivity and PK of this series. In the case of unsubstituted benzoxazoles in P3 (**13a**, Table 3), the potency drops off considerably. However, the addition of a methyl group at P1 (**13b**) leads to a tenfold improvement in the K_i over **13a**. Yet another fivefold increase in activity is gained by further extending the side chain into P1 with a benzyloxy group as in **13c**, while simultaneously preserving a 300-fold selectivity over cathepsin L. Addition of a chlorine atom to the 6-position of the benzoxazole ring (**13d**) resulted in a tenfold reduction in activity

Table 3. Inhibition of cathepsins S, K, and L—exploration of P1 side chain



Compound	X	R^1	CatS K_i (μM)	CatK K_i (μM)	CatL K_i (μM)
13a	H	–H	0.222	>30	>10
13b	H	$-CH_3$	0.026	>10	2.83
13c	H	$-CH_2OCH_2Ph$	0.005	16.7	1.49
13d	Cl	$-CH_3$	0.151	>30	9.67
13e	Cl	$-CH_2CH_2SO_2Me$	0.021	4.87	0.369
13f	H	$-CH_2CO_2H$	0.012	>20	>10
13g	Cl	$-CH_2CO_2H$	0.027	>100	>70
13h	Cl	$-CH_2CO_2Et$	0.071	>100	>50
13i	Cl	$-CH_2CO_2$-*t*-Bu	0.730	>100	>100

versus the 7-chlorobenzoxazole **12d** for both cathepsins S and L. Incorporation of a more polar P1 moiety, such as the sulfone in compound **13e** which bears a side chain derived from methionine, returns the cathepsin S activity, but not without the emergence of moderate cathepsin L activity. On the other hand, increasing the polarity further, as in the aspartate derived side chains **13f–h**, afforded potent cathepsin S inhibitors while completely dialing out cathepsins K and L activity.[11,12] The positively charged guanidinium group in the S1 pocket emanating from Arg141 represented a distinct feature of cathepsin S that may have implications for the specificity of **13f–h** (Fig. 2). The analogous S1 region is occupied by Asn158 in cathepsin K and Asp162 in cathepsin L.[13]

The carboxylic acid derivative **13g** is not orally bioavailable, and it has a relatively short half-life and moderate clearance when administered intravenously. When its ethyl ester analog **13h** is administered orally at 10 mg/kg, it is not detected in the plasma, however its carboxylic acid metabolite **13g** is detected with a peak plasma concentration of over 1 μM, providing a 22% bioavailability of **13g** as its ethyl ester prodrug (Table 4). Interestingly, increasing the steric bulk of the side chain to the *tert*-butyl ester, as in compound **13i**, led to a significant drop in activity. This is in contrast to what was observed with **13c**, where the relatively large benzyloxymethyl side chain drove the cathepsin S K_i down to 5 nM, suggesting a preference for aromatics over bulky aliphatics in the S1 pocket.[14]

Metabolic ID studies on indolines **12d** and **13a–h** showed that one of the primary modes of metabolism is a hydroxylation/dehydration on the aminoethylindoline moiety, suggesting a possible explanation for the relatively high in vivo clearance and short half-lives (Table 4). Attempts to block this oxidative metabolism were made by introducing substituents onto the 2- and 3-positions on the indoline ring. Geminal dimethyl substitution at the 2-position of the indoline ring (**12e**, Table 2) led to a major boost in selectivity over cathepsin L to greater than 300-fold, while completely preserving cathepsin S activity (K_i = 0.015 μM). Although the half-life ($t_{1/2}$ = 4 h) improved significantly over **12d**, the AUC and C_{max} were not improved and as such did not warrant further iv profiling (Table 4). Moving this *gem*-dimethyl group over to the 3-position on the indoline ring (**12f**) resulted in a 5-fold gain in cathepsin S affinity (K_i = 0.003 μM). The selectivity of **12f** remained a robust 1200-fold over cathepsin K, but narrowed somewhat over cathepsin L (41×). More importantly, the *gem*-dimethyl substitution at the indoline 3-position considerably improved the PK, as **12f** has a long in vivo half-life in rats and low clearance. Compound **12f** is also orally bioavailable (F = 15%), achieving a C_{max} of over 1.2 μM with a half-life of over 5 h following oral administration. Interestingly, the 2-indolone derivative **12g** is virtually inactive toward all of the cathepsins assayed, suggesting that the indoline nitrogen lone pair plays an essential role in the binding to the enzyme.[15]

The X-ray co-crystal structure of compound **3** bound to the active site of cathepsin S is shown in Figure 2. Recombinant human cathepsin S was crystallized in the presence of 2-mercaptopyridine, which was added prior to crystallization to prevent oxidation of the catalytic cysteine (Cys25) and to aid in the crystallization itself. The cathepsin S crystals were soaked with compound **3** for 24 h in the presence of 4 mM DTT (also present 4 h prior to addition of **3**) to remove the 2-mercaptopyridine covalently bound to Cys25. The presence of the DTT reduced the occupancy of the 2-mercaptopyridine in the S1-prime site but did not eliminate it completely. As a result, the methoxyaniline group was completely disordered such that virtually no electron density was visible, and therefore was modeled into the S1-prime site. The lack of electron density for the methoxyaniline could have been due either to the residual 2-mercaptopyridine in the S1-prime site or to the intrinsic disorder within this sight. The two carbons of the ethylenediamine chain are completely visible however, and are situated 4.8 and 3.5 Å from the sulfur atom of Cys25. As expected, the cyclohexyl P2 group fills the enzyme S2 pocket, and the benzoxazole sits squarely in the S3 pocket, which is formed by Lys64, Gly62, Gly69, and Phe70 through induced fit. The hydrogen bonds formed between benzoxazole **3** and the enzyme are represented by the dotted lines in Figure 2. The N–H groups of both the cyclohexylalanine and the ethylenediamine amide each donates a putative hydrogen bond to the protein backbone carbonyls of Gly69 and Asn163, respectively, while the cyclohexylalanine carbonyl accepts a hydrogen bond from N–H of Gly69.

In summary, our early lead optimization from compound **2** led to a novel series of potent cathepsin S inhibitors lacking an electrophilic, covalent warhead.[16] By

Table 4. Pharmacokinetics of selected analogs[a]

Compound	Single iv dose (3 mg/kg)			Single po dose (10 mg/kg)			
	CL (mL/min/kg)	V_{ss} (L/kg)	$t_{1/2}$ (h)	AUC (min μg/mL)	C_{max} (nM)	$t_{1/2}$ (h)	F (%)
3	11.4	1.63	4.8	0.9	12	5.3	<1
12d	33.1	2.57	2.3	65	703	1.1	24
13g/h	23.2	0.74	1.0	95	1100	1.0	22[b]
12e	—	—	—	54	533	4.1	—
12f	10.1	0.90	8.3	144	1261	5.1	15

[a] Pharmacokinetic parameters of single iv dose (3 mg/kg) and po dose (10 mg/kg) in male Wistar rats. All in vivo pharmacokinetic values are means of three experiments.

[b] PK parameters shown are for IV administration of carboxylic acid **13g** and oral administration of ethyl ester prodrug **13h**, and the bioavailability of **13g** from prodrug **13h** is calculated using iv AUC of **13g**.

replacing the P3 amide of compound **2** with a heterocycle, the in vivo PK of this series was dramatically improved. Furthermore, optimization of the P1 aniline moiety to a more drug-like indoline group[10] has led to potent, orally bioavailable cathepsin S inhibitors, such as **12f**, with much improved pharmacokinetics in rats over the early lead compounds **2** and **3**.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.12.095.

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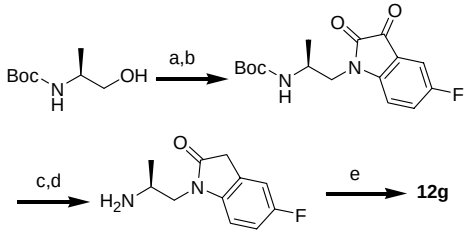

Reagents and conditions: (a) CBr_4 (1.0 equiv), PPh_3 (1.0 equiv), DCM (52%); (b) 5-fluoroisatin, NaH (1.2 equiv), DMF, (36%); (c) H_2NNH_2 (50%) in EtOH, reflux (89%); (d) TFA:DCM:H_2O (50:45:5) (99%); (e) **6**, HATU, DIEA, DCM (77%).
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