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The identification of potent, selective, and bioavailable cathepsin S inhibitors

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Abstract—Highly potent, selective, and bioavailable inhibitors of human, mouse, or rat cathepsin S are described. The key structural features combine a sulfonyl moiety attached to a large group in P2 and a small substituent in P3.

Cathepsin S (Cat S) is a cysteine protease that belongs to the papain superfamily.[1] It is most highly expressed in antigen presenting cells and can be induced by a number of mediators in different cell types. Cat S has substrate activity against many proteins, in particular the MHC class II invariant chain (Ii), as well as extracellular matrix proteins including elastin and collagen.[2] Cat S has been implicated in a wide range of diseases such as Alzheimer's disease, atherosclerosis, asthma, chronic obstructive pulmonary disease, cancer, osteoarthritis, neuropathic pain,[3] and a number of autoimmune disorders.[4] The identification of a potent, selective, and bioavailable Cat S inhibitor is therefore a valuable goal as it would allow the evaluation of the pharmacology due to Cat S inhibition and hence the therapeutic potential of such an inhibitor.

The crystal structure of human Cat S with and without inhibitors has been solved.[5] These data and our molecular modeling studies revealed that there are three significant differences between Cat S and Cat K in the S2 subsite. Using the numbering from PDB entry 1MS6 (Cat S) Gly137 and Gly165 are both Ala in Cat K. The resulting two extra methyl groups effectively act as 'gate keepers' to the S2 subsite in Cat K, whereas in Cat S the absence of these two methyl groups opens up the subsite both in terms of width and depth. The third difference is found at the end of the S2 subsite. In Cat K the subsite is terminated by a Leu group, however, in Cat S the residue is Phe211 which is directed up and out of the active site so that the end of the S2 subsite is not bound. As a result of these three differences it is expected that the S2 subsite in Cat S will accept significantly larger groups compared to the S2 subsite found in Cat K and this should provide a key to achieving selectivity between these two enzymes.

In a recent *Letter*,[6] we described L-873724, a Cat K inhibitor in the trifluoroethylamine series. Although this compound is a potent and selective inhibitor of Cat K, we thought that it would make an ideal starting point to identify a Cat S inhibitor seeing that it is reversible, non-lysosomotropic, and orally bioavailable (Fig. 1).

Our strategy was to replace the P2 leucine by cysteine, or serine, and use the heteroatom suitably located as a linker to quickly derivatize and probe the steric and

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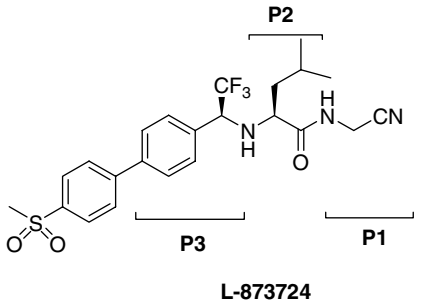
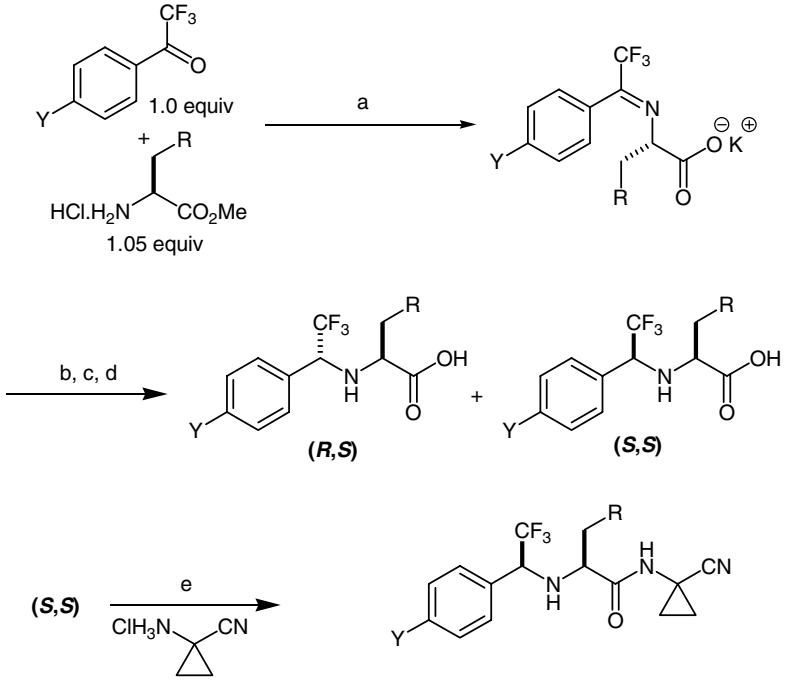


Figure 1. L-873724, a member of the trifluoroethylamine series of Cat K inhibitors.

electronic requirements in S2. To achieve this, we developed a stereoselective reductive amination of 2,2,2-trifluoroacetophenones and cysteine or serine derivatives (Scheme 1).[7]

In all cases, the reduction yielded a mixture of diastereoisomers obtained in a ratio of ~10:1 favoring the desired (*S*,*S*)-diastereoisomer. Following coupling (HATU) with aminocyclopropanecarbonitrile, the cysteinamides were oxidized to the sulfone (MMPP or Na_2WO_4/*n*-Bu_4HSO_4/H_2O_2). For the approach using serine, the hydroxyacid obtained from reductive amination was first transformed to the aminocyclopropanecarbonitrile derivative followed by conversion of the hydroxyl group to the bromide (CBr_4/PPh_3P). S_N2 displacement with mercaptans (K_2CO_3/DMF) provided the sulfides (oxidized as above). In all cases, simple trituration in MTBE (or diethyl ether) provided the (*S*,*S*)-diastereoisomer in a ratio of ~25:1.

We initiated our SAR study with the simple methylsulfonylcysteinamide derivative in P2 and 4-bromophenyl derivative in P3 (R = SO_2CH_3; Y = Br) as we envisaged using the bromide to eventually explore other P3 substitutions (see Table 1). This combination produced **2**, a relatively potent inhibitor of human Cat S with weak selectivity against Cat K (Cat K/S ~3-fold). The sulfide **3** and sulfanyl **4** were significantly less active against Cat S. This clearly established the preference of a sulfonyl moiety in P2.[8] We then tried to fill the S3 pocket by preparing the 4-fluorobiphenyl derivative **5** (obtained via Suzuki coupling reaction). Although this compound is still a potent inhibitor of Cat S, it is also potent against Cat K and Cat B. On the contrary, replacing bromine by fluorine provided **6**, showing a 6-fold increase in potency against Cat S and over 200-fold selectivity against Cat K, B, and L. The reduced size of the S3 pocket in Cat S has previously been observed from crystal structures of Cat S inhibitor complexes.[5] In addition, the preference for a large P3 substituent in S3 of Cat K has already been established in our previous *Letter*.[6] The optimal position of the fluorine at the *para* position is demonstrated with the preparation of the *meta*-fluoro derivative **8**, an inhibitor 3.5-fold less potent and significantly less selective against Cat K. Finally, replacing the halogen by hydrogen provided a potent inhibitor **9**, which also suffered from erosion of the Cat S/K selectivity (56-fold). The steric requirements in P2 were next evaluated. The isobutyl and cyclopropylmethyl derivatives **10**, **11**, and **12** (Y = F or H) provided subnanomolar inhibitors of Cat S. Moreover, these analogs are very selective against Cat K and B and at least 100-fold selective against Cat L. Molecular modeling performed with **10** clearly indicated that while Cat S accom-



Scheme 1. Reductive amination of fluoroacetophenones with *S*-alkyl-, *S*-benzyl-, and *S*-aryl-L-cysteine or serine (R = *S*-alkyl, *S*-benzyl, *S*-aryl, or R = –OH) methyl esters. Reagents and conditions: (a) $KOCH_3$ (2.0 equiv), CH_3OH, 0–25 °C, 16 h; (b) $Zn(BH_4)_2$, DME/CH_3CN (1:4), −40 °C, 2 h; (c) acetone, −40 to 25 °C; (d) 1 N HCl; (e) HATU, DIEA, DMF, 0–25 °C.

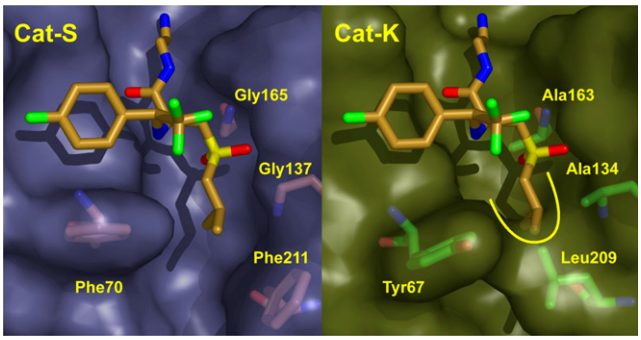


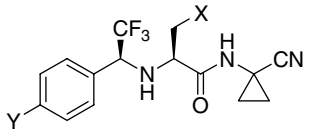
Figure 2. Average structure of compound **10** in Cat S (left) from molecular dynamics calculation (numbering based on X-ray structure of Cat S PDB entry 1MS6). The same structure superimposed on X-ray structure of Cat K (right; PDB entry 1BG0). Superposition is based on Cα's of the active site and shows penetration of the isobutyl moiety of **10** in the van der Waals volume of Cat K Leu209 (bottom right).

modates an isobutylsulfonylcysteinamide group in P2, unfavorable interactions with Leu209 in Cat K provide a rational for the selectivity observed with **10** (Fig. 2).

Selectivity against Cat K and Cat B could be further improved by replacing these P2 aliphatic substituents by benzyl groups. As exemplified with **13**–**17**, substitution around the phenyl ring was well tolerated in the Cat S active site but not in the Cat K active site. Thus, the 2,6-dichloro analog **16** was completely inactive against Cat K and B while being 350-fold selective against Cat L.

Substitution on the benzylic carbon was not tolerated: the *gem*-dimethyl derivative **18** was essentially inactive compared to **13** (~2000-fold). We also explored direct attachment of the aryl to the sulfone moiety. The 4-fluoro-, 4-bromo-, and 3-bromophenylsulfonyl derivatives **19**, **20**, and **21** although potent against Cat S suffered from significant erosion of the selectivity against Cat K. Replacement of the simple phenyl moiety by heteroaromatics was explored with the thiazolyl and benzothiazolyl derivatives **22** and **23**, both of which lost potency against Cat S. Further investigation of the substitution pattern for halophenyl derivatives finally led to the 3,4-dichlorophenyl derivatives **24** and **25** which

Table 1. In vitro activity[9] of representative cathepsin inhibitors[10]



Compound	X	Y	Human cathepsin IC_{50}[a] (nM)			
			Cat S	Cat K[b]	Cat B	Cat L
L-873724[c]			178	0.2	5239	264
1	2-Propyl	4-CH_3SO_2-phenyl	265	0.3	7740	456
2	SO_2CH_3	Br	11	31	282	427
3	SCH_3	Br	80	5.0	417	456
4	$SOCH_3$	Br	2173	639	8029	>10,000
5	SO_2CH_3	4-Fluorophenyl	10	5.3	170	>10,000
6	SO_2CH_3	F	1.8	405	1104	1013
7	$SOCH_3$	F	729	1117	9724	>10,000
8	SO_2CH_3	3-F	6.4	314	3854	911
9	SO_2CH_3	H	2.5	141	1980	1035
10	$SO_2CH_2CH(CH_3)_2$	F	0.4	3058	337	132
11	SO_2CH_2-cyclopropyl	H	0.6	555	287	68
12	SO_2CH_2-cyclopropyl	F	0.5	2003	1000	188
13	SO_2CH_2-4-fluorophenyl	H	0.5	>10,000	2777	86
14	SO_2CH_2-2,3-difluorophenyl	H	0.3	>10,000	1844	105
15	SO_2CH_2-2,3-difluorophenyl	F	0.3	>10,000	1319	121
16	SO_2CH_2-2,6-dichlorophenyl	F	0.7	>10,000	>10,000	249
17	SO_2CH_2-2-biphenyl	F	1.8	>10,000	4041	>10,000
18	$SO_2C(CH_3)_2$-4-fluorophenyl	F	627	>10,000	>10,000	>10,000
19	SO_2-4-fluorophenyl	F	0.4	305	7280	22
20	SO_2-4-bromophenyl	H	0.3	290	3453	15
21	SO_2-3-bromophenyl	H	0.2	269	6629	23
22	SO_2-2-thiazolyl	H	5	376	3612	181
23	SO_2-2-benzothiazolyl	H	1.3	825	2971	114
24	SO_2-3,4-dichlorophenyl	H	0.2	314	8507	24
25	SO_2-3,4-dichlorophenyl	F	0.2	691	4813	41
26 (LHVS[d])	—	—	1.2	6.2	4504	5.7

[a] IC_{50} values with a 15 min preincubation and representing an average of at least two independent titrations. Standard deviations for **2** were ±3, 4, 91, and 150 for Cat S, K, B, and L, respectively, and are typical of what is observed with other compounds.
[b] Humanized rabbit Cat K was used (see Ref. 10 for conditions).
[c] L-873724[6] has an unsubstituted cyanomethyl moiety in P-1 (–CH_2CN). All other compounds are cyanocyclopropyl derivatives; data for compound **1** are provided to show that this substitution in P1 does not significantly affect the in vitro profile of this series of compounds.
[d] LHVS is an irreversible cysteine protease inhibitor.[11]

offered an excellent profile of potency and selectivity. Both derivatives have IC_{50}s of 0.2 nM against Cat S, are more than 1000-fold selective against Cat K and B and are 100- to 200-fold selective against Cat L. Taken all together, these results establish that potent and selective inhibitors of Cat S require the combination of a critical sulfonyl moiety attached to a large group in P2 and a small substituent in P3, preferentially located in the *para* position (Table 1).

This SAR study allowed the identification of several potent and selective inhibitors of human Cat S. However, to support rodent disease models, we needed to evaluate these compounds for their inhibitory properties against mouse and rat enzymes. Data for representative compounds are listed in Table 2. With mouse enzymes, all compounds retained their potency at inhibiting Cat S (IC_{50}s $\leqslant$ 1.6 nM, except for **7** which is inactive across all species) and selectivity versus other cathepsins ($\geqslant$250-fold).

However, some potent inhibitors of human and mouse Cat S were generally weak inhibitors of rat Cat S. This is notably the case for **6** and **12** which are 700- and 1000-fold less active against the rat enzyme, respectively.[12] Our molecular modeling studies suggest that these variations are attributable to structural differences in the S2 active sites. In particular, two simultaneous side chain differences increase the lipophilic interactions between P2 and S2 in rat Cat S (compared to mouse or human). When performing molecular dynamic simulations on a homology model of the rat enzyme (obtained from a human Cat S crystal structure) we observed that the Phe212Tyr and Val163Met differences between human and rat in S2 confer an increased stability for the conformation of the rat Tyr212 in which the phenyl is pointing toward Thr73.[13] In this position, the phenyl ring hydroxyl group forms two H-bonds with Thr73, an interaction not possible in the mouse and human enzymes. As a result, in rat, S2 forms a cavity offering an advantageously favorable lipophilic interaction with an aromatic ring such as the benzyl derivative **15** or the phenyl derivative **20**. Ultimately, extensive SAR around the aromatic moiety allowed the identification of **24**, a potent inhibitor of rat Cat S, essentially inactive against rat Cat B and 3-fold selective against rat Cat L.

In summary, our SAR study identified several potent and selective inhibitors of either human, mouse, or rat Cat S. Based on its overall profile of activity and selectivity, **6** appears to be an optimal inhibitor of human or mouse Cat S, while **24** is preferred as a rat Cat S inhibitor.

The pharmacokinetic profiles of some inhibitors were evaluated in rats (see Table 3). Compounds **2** and **6**, bearing a simple methyl derivative in P2, had good bioavailability, high C_{max}, and relatively long $t_{1/2}$. However, analogs such as **10** and **12** suffered from either poor bioavailability, shorter $t_{1/2}$, and/or reduced C_{max}. To gain insight into the pharmacokinetic profile of these inhibitors, we examined their metabolic profiles in fresh rat hepatocytes after incubation for 2 h at 37 °C.[14] As opposed to **2** and **6** which were found to be stable under these conditions, **10** and **12** generated several metabolites, presumably via an oxidative pathway, resulting in de-alkylation and ultimately leading to the corresponding sulfinic and sulfonic acids. The benzyl derivative **15**, presumably also suffering from benzylic oxidation, had limited absorption and short $t_{1/2}$. This could be addressed with the 3,4-dichlorophenyl derivatives **24** and **25** which provided an acceptable pharmacokinetic and metabolic profile.

In addition, the exposures of compounds such as **6** in rodents were found to be non-linear which can seriously limit the evaluation of the pharmacology due to Cat S inhibition. For example, in C57BL/6 mice, dosing orally a 0.5% methocel/0.2% SDS suspension of **6** at 5 and 50 mg/kg resulted in AUCs of 43 and 111 μM * h (0–24 h), respectively. To address this issue, we identified a sulfoxide prodrug **7**, which is rapidly converted

Table 2. In vitro activity of representative cathepsin inhibitors against mouse and rat enzymes[a]

Compound	Cathepsins IC_{50}[b] (nM)						
	Cat S		Cat K[c]	Cat B		Cat L	
	Mouse	Rat	Mouse	Mouse	Rat	Mouse	Rat
L-873724	254	2441	43	540	925	254	90
2	1.6	1051	1030	69	66	2222	1278
6	0.6	426	1646	233	319	3037	3159
7	96	>10,000	5835	6702	>10,000	>10,000	>10,000
12	0.9	759	>10,000	253	272	332	738
15	0.8	21	>10,000	384	384	662	399
20	0.2	66	1324	1747	1809	39	72
21	<0.2	44	2553	4013	4702	90	132
24	<0.2	15	1464	3018	5694	59	48
26	0.5	1.7	388	1277	735	7.1	4.1

[a] Recombinant mouse Cat K and S were prepared in-house using standard procedures. Recombinant mouse pro-Cat B and L were obtained from R&D systems, Minneapolis, MN, USA 55413. Rat Cat S was isolated from spleen, whereas rat Cat B and L were purified from liver lysosomes. The conditions used to assess the potency of inhibitors were as described earlier (see Ref. 10).

[b] IC_{50} values with a 15 min preincubation and representing an average of at least two independent titrations. Standard deviations for **2** were ±0.9, 337, 559, 20, 10, 1024, and 635 for mouse or rat Cat S, K, B, and L, respectively, and are typical of what is observed with other compounds.

[c] The compounds were not tested against the rat Cat K enzyme.

Table 3. Pharmacokinetic profiles[a] of selected Cat S inhibitors

Compound	% F (10 mg kg)	C_{max} (μM)	$t_{1/2}$ (h)
2	40	3.3	11
10	5	0.14	1.6
12	40	2.8	3.8
15	3	0.046	1.2
6	48	6.3	5.2
24	9	0.088	1.7
24[b]	30	0.66	1.7
25[b]	35	0.25	1.2

[a] Conscious Sprague–Dawley rats ($n = 2$) were dosed orally with a suspension of individual test compound in 0.5% methocel/0.2% sodium dodecyl sulfate (SDS) at 10 mL/kg. Formulation in 60% aqueous PEG 400 was used for IV rats ($n = 2$) at 1 mL/kg.

[b] Dosed orally as a 1:1 Inwitor/Tween 80 solution.

in vivo to the parent sulfone **6**. In mice, the plasma AUC exposures of **6**, dosed as **7**, at 10, 50, and 150 mg/kg are 63, 274, and 750 μM * h (0–24 h). In addition, for chronic in vivo studies, mice can be most conveniently dosed using **7** in food. At doses of 0.01%, 0.05%, and 0.25 (w/w)% of **7** in regular rodent diet, the plasma AUC exposures of **6** are 7, 31, and 87 μM * h (0–24 h) after 5 days of dosing. Peak blood levels (6 am, lights on, mice start feeding less actively) were 1, 6, and 16 μM. Trough levels (6 pm) were 0.3, 2, and 3 μM. Therefore, the food formulation of **7** avoids the large peak to trough variation observed with BID dosing that is required for complete coverage, reduces the risk of off-target activity, and provides a significant simplification compared to multiple daily gavages.

The ability of **6** to inhibit Cat S was evaluated in two immune function-related assays. The first assay determines the ability of **6** to block the presentation of antigen by an engineered mouse B cell line to a responsive T cell hybridoma.[9] The second measures the accumulation of a 10 kDa fragment (p10) in mouse spleen which results from the inhibition of proteolytic digestion of the MHCII invariant chain peptide (Ii).[15]

In the mouse antigen presentation cell-based assay, **6** has an IC_{50} of 44 nM. The irreversible inhibitor LHVS, **26**, and the Cat K inhibitor L-873724 have IC_{50} values of 1.2 and 4814 nM, respectively, demonstrating that **6** is highly efficacious in this assay. For the in vivo p10 accumulation assay, C57BL/6 female mice were fed a food formulation containing 0.01 (w/w)% of **7** for 5 days (corresponding to approximately 3 mg/kg/day based on food consumption). Following the treatment period, analysis of p10 levels in spleen lysates showed ~24-fold accumulation vs vehicle. After returning a second cohort of mice to a regular diet for 3 days, analysis of the spleen lysates indicated that p10 levels had returned to levels comparable to that of vehicle treated animals. These results indicate that a high degree of reversible Cat S inhibition is achieved with **6**.[9]

In summary, highly potent and selective inhibitors of human, mouse, and rat Cat S were identified. The key structural features combine a critical sulfonyl moiety attached to a lipophilic substituent in P2 and a small substituent in P3. For the inhibition of human or mouse Cat S, **6** is preferred. However, interspecies variations were observed and potent rat Cat S inhibitors require an aromatic P2 moiety extending to an even more lipophilic cavity in S2, as is the case with **24**. In mice, **6** is highly potent in a cell-based assay of antigen presentation and causes a large increase in splenic MHC II invariant chain p10 levels, demonstrating high efficacy at inhibiting Cat S. The pharmacological outcomes of inhibiting Cat S in rodents will be the subject of subsequent reports.

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