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Design and synthesis of dipeptidyl nitriles as potent, selective, and reversible inhibitors of cathepsin C

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ABSTRACT

A series of dipeptide nitriles with a thienyl alanine in P2 were identified as potent and selective cathepsin C inhibitors. Incorporation of a substituted cyclopropyl moiety in P1 effectively protects these derivatives against hydrolase activity in whole blood.

Cathepsin C (Cat C), also known as dipeptidyl peptidase I (hDPPI, EC 3.4.14.1), is a lysosomal cysteine protease from the papain family which sequentially removes dipeptides from the N-termini of substrates.[1] It is constitutively expressed in many tissues with highest levels in lung, kidney, liver, and spleen. This enzyme is highly conserved between rat, murine, and human homologs and is associated with the degradation of lysosomal proteins. It also has an important role in the activation of many proinflammatory granule serine proteases such as cathepsin G (CG), proteinase-3 (Pr-3), neutrophil elastase (NE), chymase, and β-tryptase from mast cells, and granzyme A and B from cytotoxic T-lymphocytes and natural killer cells.[2] Consequently, inhibition of Cat C represents an attractive therapeutic strategy for inflammatory diseases with a high neutrophil burden such as chronic obstructive pulmonary disease and cystic fibrosis.[3]

The X-ray crystal structure of Cat C has been determined and was shown to be tetrameric with four identical subunits, each composed of a heavy chain, a light chain, and an exclusion domain.[4] Recently the complex with the inhibitor Gly-Phe-CHN_2 was also reported.[5] Substrate specificity is dictated by the exclusion domain which is cleaved during activation of the proenzyme but remains non-covalently associated to the light and heavy chains from the papain-like domain. The presence of this exclusion domain is the key determinant in preventing the endopeptidase activity of Cat C. It effectively blocks substrate access beyond the S2 pocket in the active site, while the carboxy group of the Asp-1 side chain provides an essential binding interaction for the free amino group required for all substrates. Thus, Cat C cleaves a broad range of protein and peptide substrates with a free N-terminal amino group but will not cleave substrates with Pro in P1 or P1′, Ile in P1, and Lys, Arg or ornithine in P2.[6]

Many known inhibitors of Cat C form an irreversible covalent bond with the enzyme active-site Cys-234. These are mostly dipeptides bearing reactive moieties such as diazomethyl ketones,[7] vinyl sulfones,[8] acyloxymethyl and fluoromethyl ketones,[8a] and *O*-acyl hydroxamic acids.[9] The naturally occurring epoxysuccinyl derivative E64 also inhibits Cat C, albeit only at high concentrations.[10] Published reversible inhibitors of Cat C include dipeptide nitriles,[11] dipeptide semicarbazides,[12] arginine-based peptides,[13] and a series of non-competitive phosphinic tripeptides.[14]

We have recently reported the design and identification of potent and selective nitrile inhibitors of cathepsin S[15] and cathepsin K, which form a reversible thioimidate complex with the active-site cysteine.[16] Herein, we report on a series of dipeptidyl nitriles as inhibitors of Cat C.

Initial efforts in identifying a suitable lead focused on the preparation of non-covalent inhibitors with an α-amino amide motif mimicking endogenous substrates. Screening of a library of over 100 compounds prepared from available amines and amino acids provided the initial lead compound **1** (Fig. 1).

Incorporation of a nitrile 'warhead' into structure **1** was accomplished as described in Scheme 1. In general, the Boc-protected amino acid was converted to the α-amino nitrile via the corresponding primary amide. Conversion to the free amine was best accomplished with methanesulfonic acid in cold THF to avoid

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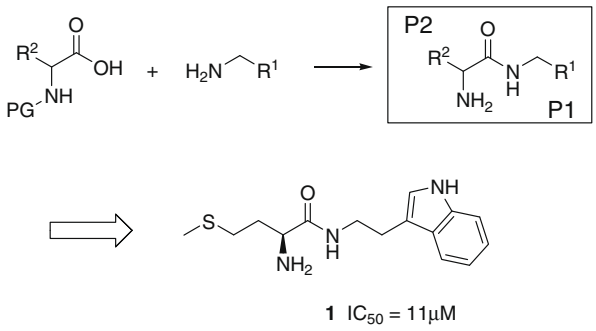
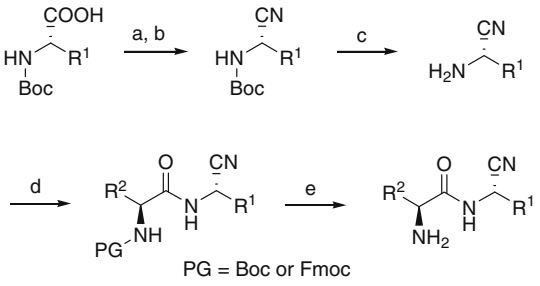


Figure 1. Preparation of α-amino amides and structure of lead **1**.



Scheme 1. Reagents and conditions: (a) EtCOCl, NMM, THF, 0 °C, then aq NH_3 or HATU, NH_4OH, DMF, rt; (b) TFAA, Et_3N, THF, 0 °C; (c) MsOH, THF, 0 °C or TFA, CH_2Cl_2; (d) HATU, 2,6-lutidine, DMF, BocNHCH(R^1)COOH or FmocNHCH(R^1)COOH; (e) MsOH, THF, 0 °C or piperidine, DMF.

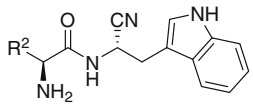
any decomposition. Coupling with another protected amino acid followed by final deprotection afforded the desired dipeptide nitriles. All final dipeptides were obtained as pure diastereomers (>98%) as determined by LC–MS and ^{1}H NMR.

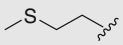
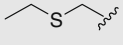
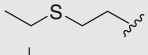
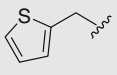
Addition of the nitrile moiety onto the lead compound **1** resulted in a 55-fold increase in intrinsic potency against Cat C (Table 1, entry **2**). SAR of the R2 group allowed us to identify 2-thienylmethyl as the optimal moiety in P2 (entry **6**). Replacement of the thiophene for a phenyl usually led to a ~30-fold loss of potency. The importance of the absolute configuration at both chiral centers was demonstrated by evaluation of the ***SR***, the ***RS***, and the ***RR*** isomers of compound **6** which suffers a 52-, 68-, and 3150-fold loss of potency, respectively (data not shown).

Optimization of the R1 substituent was then performed while maintaining the preferred R2 thiophene moiety. With no R1 substituent as in the alanine derived nitrile **7** (Table 2), the loss of potency is only threefold when compared to **6**. As described by others,[6,11b] we found that hydrophobic aromatic residues increased potency (entry **8** vs **9** and **10** vs **11**). Homologation of the Phe side chain in P1 resulted in a 12-fold loss of potency (entry **12**) whereas other types of non-polar benzyl surrogates gave compounds of similar or improved potency (entry **13**–**15**).

With potent inhibitors in hand, we began to evaluate their pharmacokinetic profiles with the ultimate goal of demonstrating that a Cat C inhibitor can effectively block the activation of downstream inflammatory serine proteases in vivo. However, when dosed intravenously in rats, all of these derivatives (e.g., **9**, **11**, **13**–**15**) suffered from very short half-lives (<30 min, clearance >100 ml/min/kg). Careful examination of the plasma samples did not indicate the presence of any of the oxidative metabolites. The only fragments identified were the P2 amino acid and the P1 amino nitrile resulting from amide hydrolysis and the same results were obtained from incubations in hepatocytes. To confirm that the action of hydrolases also present in blood was responsible for compound degradation, we incubated a number of inhibitors with rat whole blood at room temperature for 30 min (Table 3). Compounds such as **6** and **11** with a benzyl-type group in P1 are rapidly hydrolyzed to the corresponding amino acids and amino nitriles. Increasing the steric bulk in P1 significantly improves hydrolytic stability

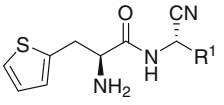
Table 1
SAR of P2 amino nitriles: IC_{50} values for **2**–**6**

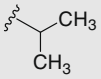
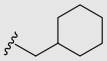
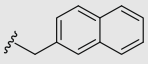
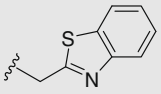
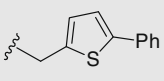


Entry	R^2	IC_{50} (Cat C)[a] (nM)[b]
2		200 ± 64
3		630 ± 211
4		1580 ± 486
5		330 ± 123
6		5.8 ± 3.8

[a] Purified human recombinant enzyme.
[b] Mean value of at least three experiments with standard deviation.

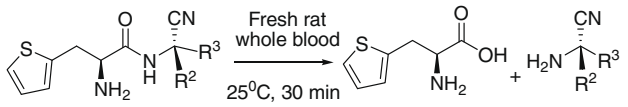
Table 2
SAR of hydrophobic P1 amino nitriles: IC_{50} values for **7**–**15**

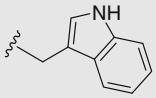
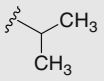


Entry	R^1	IC_{50} (Cat C)[a] (nM)[b]
7	H	17 ± 4.7
8		215 ± 78
9		3.8 ± 1.4
10		5.1 ± 2.8
11		0.9 ± 0.3
12		11 ± 4
13		1.0 ± 0.4
14		0.7 ± ± 0.2
15		0.3 ± 0.1

[a] Purified human recombinant enzyme.
[b] Mean value of at least three experiments with standard deviation.

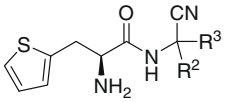
Table 3
Stability of **6**–**8**, **11**, **16**–**17** in rat whole blood

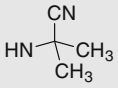
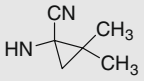
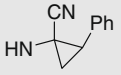
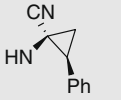
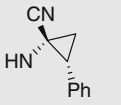


Entry	R^2	R^3	IC_{50} (Cat C)[a] (nM)[b]	Parent remaining (%)
6	H		5.8 ± 3.8	0
7	H	H	17 ± 4.7	6
8	H		215 ± 78	11
11	H		0.9 ± 0.3	0
16	H		>3000	83
17	$-CH_2CH_2-$		12.0 ± 4.4	92

[a] Purified human recombinant enzyme.
[b] Mean value of at least three experiments with standard deviation.

Table 4
SAR of dialkyl-P1 amino nitriles: IC_{50} values for **17**–**23**



Entry			IC_{50} (Cat C)[a] (nM)[b]
17			12 ± 4.4
18			1419 ± 320
19			1832 ± 436
20			63 ± 12
21		*trans* *racemic.*	1230 ± 332
22		*cis* *S,S*	17 ± 6.7
23		*cis* *R,R*	14 ± 3.6

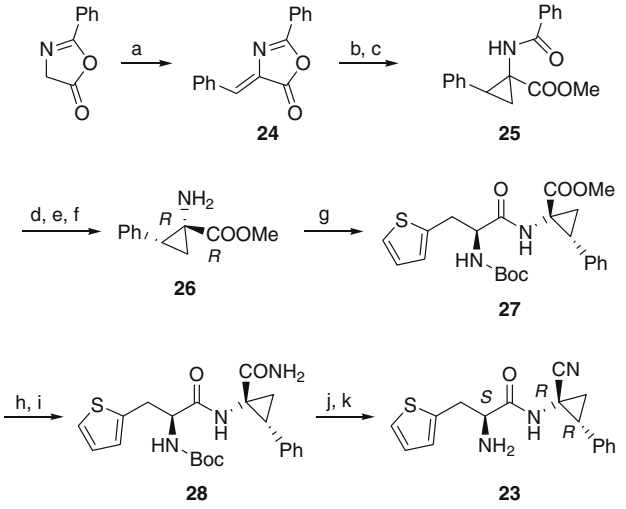
[a] Purified human recombinant enzyme.
[b] Mean value of at least three experiments with standard deviation.

but at the expense of reduced potency against Cat C (e.g., **7**, **8**, and **16**). One modification retaining potency and increased stability was identified in compound **17** which incorporates a 1,1-cyclopropyl amino nitrile moiety in P1, as previously described for inhibitors of cathepsin K.[17] Clearly, steric congestion in the proximity of the amide bond significantly reduces susceptibility toward enzymatic hydrolysis. All modifications of the α-amino amide motif such as introduction of a methyl in the α- or β-positions, incorporation of an α,β-cyclopropyl or N-methylation led to orders of magnitude in potency loss (data not shown).

To further explore P1 modifications, a number of 1,1-dialkylated dipeptide nitriles were synthesized to evaluate the tolerance of the S1 pocket. The tight fit of these inhibitors in the active site is highlighted by comparing **17** and **18** (Table 4). Opening the cyclopropyl group into a *gem*-dimethyl motif resulted in a 100-fold loss of potency and ring expansion to a cyclobutane was similarly detrimental (entry **19**). The α-position of the nitrile is consequently very sensitive to steric congestion. Branching from the β-position on the other hand is tolerated as evidenced by the dimethyl cyclopropyl derivative **20** which was only fivefold less potent than the parent unsubstituted cyclopropyl **17**. Further elaboration of this P1 residue led to phenyl cyclopropyl analogs **21**–**23**. Whereas the *trans* isomer **21** was two orders of magnitude less potent than **17**, both *cis* diastereomers **22** and **23** were equipotent against Cat C. Compound **23** was selected for further evaluation in vitro and in vivo.

The synthesis of **23** is outlined in Scheme 2. Knoevenagel condensation of benzaldehyde with 2-phenyl-5-oxazolone in acetic anhydride and acetic acid afforded the geometrically pure *Z* benzylidene oxazolone **24**. Cyclopropanation was accomplished using catalytic palladium(II) acetate and excess diazomethane in ether and subsequent methanolysis gave the trisubstituted cyclopropane **25** as the pure *cis* isomer in 30% yield. The benzamide could be selectively removed by treatment with Meerwein's salt in DCM followed by acid hydrolysis. Resolution of the racemic amino ester by chiral HPLC (ChiralPak-AD) afforded the enantiomerically pure *R,R*-cyclopropyl amino ester **26** in 35% yield. The absolute configuration of **26** and of its enantiomer was determined by preparing the corresponding *N*-Boc derivatives and comparing the optical rotation with the literature value.[18] Coupling of **26** with Boc-protected 2-thienyl alanine using agents such as HATU a base gave the protected dipeptide ester **27** in 95% yield. Saponification of the ester and amide formation using ethyl chloroformate and ammonia provided the primary amide **28** in almost quantitative yield. Dehydration of the amide into the nitrile moiety was accomplished with TFAA and a base and final deprotection of the amino group afforded the desired compound **23** in 42% yield.

When submitted to our standard stability assay in rat whole blood (30 min, 25 °C), compound **23** was found to be stable with no traces of amide hydrolysis and >98% of the intact drug remaining. In addition, this compound was determined to be non-cytotoxic to U937 cells at a concentration of 30 μM for up to 72 h. However, the pharmacokinetic profile of **23** in rats was only marginally improved with a relatively high clearance of 35 ml/min/kg and a half life of 1.4 h, but was superior to that of **17** ($T_{1/2}$ = 0.5 h, clearance = 113 ml/min/kg). Nonetheless, the potency and selectivity profile of this compound prompted us to perform



Scheme 2. Reagents and conditions: (a) benzaldehyde, AcOH, Ac_2O, 80 °C; (b) CH_2N_2, $Pd(OAc)_2$, CH_2Cl_2; (c) MeOH, DMAP (30%, 3 steps); (d) $(EtO)_3BF_4$, CH_2Cl_2; (e) HCl, Et_2O; (f) HPLC ChiralPak-AD (35%, 3 steps); (g) HATU, 2,6-lutidine, Boc(2-thienyl) alanine, DMF (95%); (h) LiOH aq, THF, MeOH; (i) EtOCOCl, Et_3N, THF; then NH_3 (97%); (j) TFAA, Et_3N, THF, 0 °C; (k) CH_3SO_3H, THF, 0 °C, (42%, 2 steps).

a number of key experiments (Table 5). Dosing **23** with a constant infusion paradigm, we were able to demonstrate that inhibition of Cat C blocks the activation of downstream serine proteases (such as NE, CG, and Pr-3) in vitro and in vivo but requires a sustained and high fractional inhibition.[19]

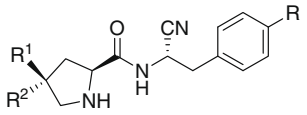
Modification of the phenyl and thiophene moieties in compound **23** such as halogenated analogs resulted in compounds with increased stability to incubations with hepatocytes (data not shown) but had little effect on the pharmacokinetic profile. Our suspicion was that the primary α-amino amide motif of this class of compounds is the key recognition element for another elimination pathway or pathways. So we then turned our attention to dipeptides containing a proline derivative in P2. As reported,[11b] the Pro-Phe-CN (Table 6, entry **29**) is not a very potent Cat C inhibitor with an IC_{50} around 5 μM. Based on predictive molecular modeling, we elected to extend further into the S2 pocket with lipophilic substitution at the 4-position of the proline ring. A methyl group *cis* to the carboxamide improved the potency by twofold whereas a methoxy led to a fivefold increase in potency (entries **30** and **31**). The chloro analog **32** was found to be almost 10-fold more potent than the parent **29**. To our surprise, the smaller fluorine atom led to another threefold increase in potency (entry **33**). Introduction of a methyl thioether (cf. **35**) resulted in the most potent proline modification. Clearly, the sulfur atom in this space relative to the α-amino amide moiety enjoys a favorable interaction with the enzyme. Following literature precedents,[11b] further modification of the P1 residue into a biphenyl led to a significant increase in potency giving inhibitors with IC_{50}'s below 10 nM (entries **38** and **39**). Not surprisingly however, the pharma-

Table 5
Selectivity profile of **23**

Enzyme[a]	IC_{50} (nM)
Cathepsin C	14
Cathepsin B	>50,000
Cathepsin L	5400
Cathepsin S	5900
Cathepsin H	>50,000
Cathepsin G	>50,000
Neutrophil elastase	>50,000
Proteinase-3	>50,000

[a] Isolated human enzymes.

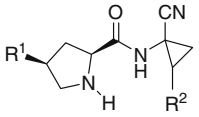
Table 6
SAR of P2 proline derived amino nitriles: IC_{50} values for **29**–**39**

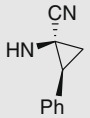
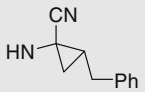
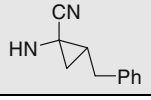


Entry	R	R^1	R^2	IC_{50} (Cat C)[a] (nM)[b]
29	H	H	H	4806 ± 741
30	H	Me	H	2502 ± 210
31	H	MeO	H	1081 ± 13
32	H	Cl	H	495 ± 29
33	H	F	H	133 ± 16
34	F	H	H	>10,000
35	H	MeS	H	29 ± 5
36	MeS	H	H	2545 ± 105
37	H	EtS	H	271 ± 12
38	H	F	Ph	8.2 ± 0.6
39	H	MeS	Ph	1.7 ± 0.1

[a] Purified human recombinant enzyme.
[b] Mean value of at least three experiments with standard deviation.

Table 7
SAR of P2 Pro P1 cyclopropyl derived dipeptides: IC_{50} values for **40**–**44**



Entry	R^1			IC_{50} (Cat C)[a] (nM)[b]
40	F			880 ± 107
41	MeS			336 ± 18
42	F			2193 ± 246
43	F		*cis* *isomer A* *isomer B*	523 ± 128 250 ± 30
44	MeS		*cis* *isomer A* *isomer B*	192 ± 13 67 ± 6

[a] Purified human recombinant enzyme.
[b] Mean value of at least three experiments with standard deviation.

cokinetic profile of these last two inhibitors was not suitable for further evaluation as a result of facile amide hydrolysis in vivo.

The combination of P2 Pro with an unsubstituted cyclopropyl nitrile in P1 (Table 7, entries **40** and **41**) gave inhibitors significantly less potent than the thienyl alanine analog **17** (Table 4). Incorporation of the phenyl cyclopropyl group did not result in the expected increase in potency (entry **42**). However, some intrinsic potency was gained by homologation of the phenyl into a benzyl substituted cyclopropane (entries **43** and **44**). Attempts to further improve the potency of these P2 Pro dipeptide nitriles met with limited success. In addition, these derivatives (e.g., **43**, isomer B) showed no improvement of their pharmacokinetic profiles.

In conclusion, we have designed a series of dipeptide nitriles as potent and selective inhibitors of Cat C. The presence of a sulfur atom in P2 appears to be involved in a favorable binding interaction in the active site. Incorporation of a cyclopropyl group in P1 significantly improves the stability of the dipeptides towards the action of hydrolases present in blood. Identification of **23** as a potent and selective inhibitor of Cat C allowed us to demonstrate in vivo the possibility to modulate the processing of other serine proteases through the inhibition of Cat C.[19] Additional optimization will be required in order to identify a candidate suitable for development.

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