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Dipeptide nitrile inhibitors of cathepsin K

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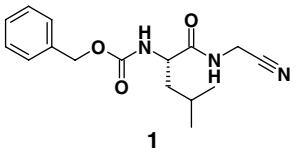
Abstract—A series of dipeptidyl nitriles as inhibitors of cathepsin K have been explored starting from lead structure **1** (Cbz–Leu–NH–CH_2–CN, IC_{50} = 39 nM). Attachment of non-natural amino acid side chains in P1 and modification of the P3 subunit led to inhibitors with higher potency and improved pharmacokinetic properties.

Bone remodeling is a complex and important process in the physiology of the skeleton. It is a tightly coupled process which is based on a precisely tuned interplay between bone resorbing osteoclasts and bone forming osteoblasts.[1,2] An imbalance in bone remodeling due to increased bone resorption will lead to reduced bone mass and disturbed microarchitecture causing bone fragility and fractures. Therapeutic inhibition of excessive bone resorption can be achieved in multiple ways. One possibility is to modulate the resorptive activity of the osteoclasts without affecting bone formation. Bone resorption by osteoclasts occurs in two distinct steps namely by first demineralization of the bone matrix via acid secretion and in a second phase by degradation of the organic bone matrix via proteolytic enzymes. Cathepsin K is the major osteoclastic protease[3] and has been proposed to play a central role in the degradation of type I collagen and other important components of the bone matrix.[4] Thus, specific inhibition of cathepsin K should substantially decrease bone resorption and therefore may offer an efficacious treatment for diseases characterized by excessive bone loss such as osteoporosis.

As part of our efforts to identify potent and reversible inhibitors of cathepsin K, we have explored a series of dipeptide nitriles starting from our lead structure **1** (Cbz–L-Leu–NH–CH_2–CN, IC_{50} cathepsin K = 39 nM).[5] Peptide nitriles as covalent, reversible inhibitors of papain were first described by Hanzlik and co-workers.[6] Three recent reports on peptidic nitrile inhibitors for cathepsins S[7], B,[8] and K[9] now prompt us to disclose our results in this area.



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Our initial studies aimed at exploring the effects of attaching an extended P1-substituent on potency and on the metabolic stability. These experiments were driven on the assumption that non-natural amino acid side chains in P1 would increase metabolic stability without negatively impacting the inhibitory potency. In a second round of optimization, we investigated the influence of the replacement of the P3 benzyloxycarbonyl group by different acyl moieties on potency, on the selectivity profile, and on physico-chemical properties. Finally, in vitro metabolism and pharmacokinetic properties of the most potent inhibitors were assessed to have an integral understanding of the drug-like properties of this series.

The synthesis of Cbz-protected dipeptide nitrile cathepsin K inhibitors is outlined in Schemes 1–3. Depending on the commercial availability of the amino acid precursors, different approaches were followed. Dipeptide nitriles **5a**, **5b**, and **5e** were prepared starting from the Boc-protected amino acids **2a**, **2b** and **2e** (Scheme 1). The mixed anhydride of the Boc-protected amino acids **2a**, **2b**, and **2e** with isobutyl-chloroformate was quenched with aqueous NH_3 to provide the amide

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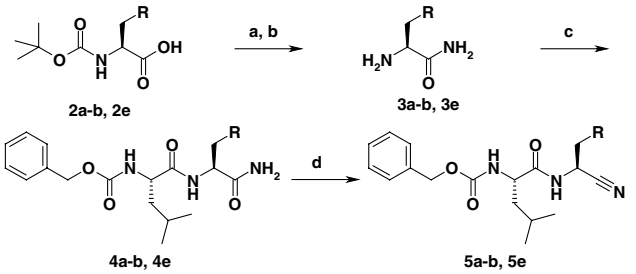
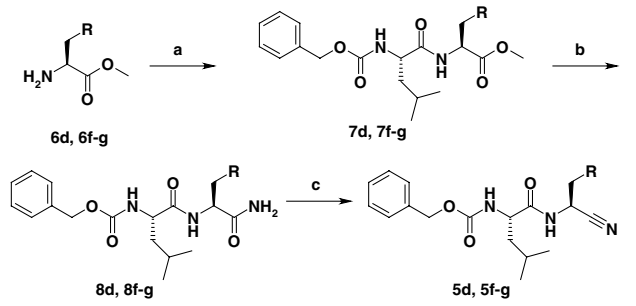
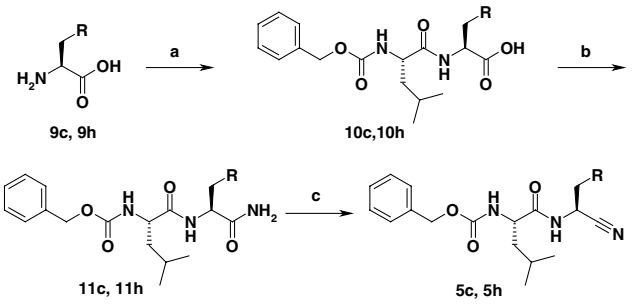


Scheme 1. Reagents and conditions: (a) 1—Isobutyl-chloroformate, *N*-methylmorpholine, THF, −15 °C, 15 min, 2—NH_3 (aq), 88–96%; (b) 4 N HCl/dioxane, rt, 6 h, 88–93%; (c) Cbz–Leu–OSu, DIEA, CH_2Cl_2, rt, 16 h, 65–88%; (d) $(CF_3CO)_2O$, Et_3N, THF, −15 °C, 2 h, 61–78%.



Scheme 2. Reagents and conditions: (a) Cbz–Leu–OSu, DIEA, CH_2Cl_2, rt, 16 h, 92–100%; (b) NH_3/MeOH, rt, 18 h, 61–84%; (c) $(CF_3CO)_2O$, Et_3N, THF, −15 °C, 2 h, 52–78%.



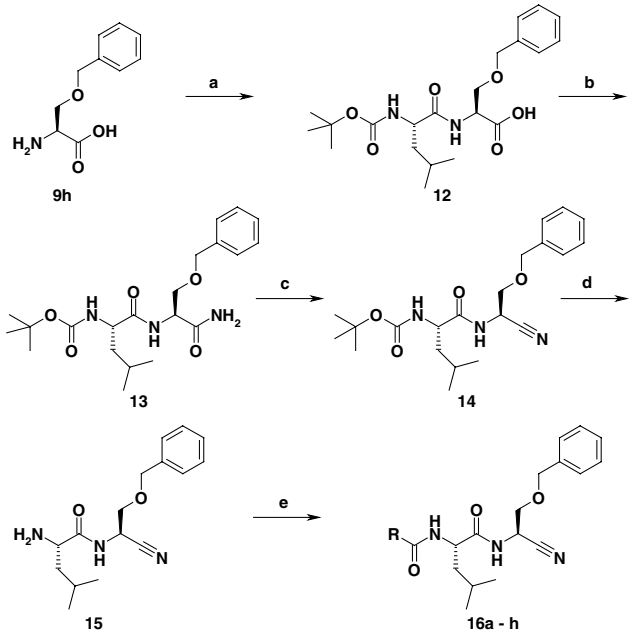
Scheme 3. Reagents and conditions: (a) 1—TMSiCl, DIEA, CH_2Cl_2, rt, 1 h, 2—Cbz–Leu–OSu, DIEA, rt, 6 h, 50–63%; (b) 1—Isobutyl-chloroformate, *N*-methylmorpholine, THF, −15 °C, 15 min, 2—NH_3 (aq), 62–96%; (c) $(CF_3CO)_2O$, Et_3N, THF, −15 °C, 2 h, 42–55%.

derivatives which upon removal of the Boc group (4 N HCl in dioxane), gave the amines **3a**, **3b**, and **3e**. Subsequent reaction with the *N*-hydroxysuccinimide ester of Cbz–Leu (Cbz–Leu–OSu) yielded the dipeptide amides **4a**, **4b**, and **4e**. Conversion to the target nitriles **5a**, **5b**, and **5e** was achieved by $(CF_3CO)_2O$-mediated dehydration[10] of **4a**, **4b**, and **4e**. Scheme 2 summarizes the synthesis of **5d** and **5f**–**g**. The dipeptides **7d**, **7f**–**g** were obtained by coupling of Cbz–Leu–OSu with amino acid esters **6d**, **6f**–**g**. Treatment of the methylester with ammonia afforded amides **8d**, **8f**–**g**, which were dehydrated with $(CF_3CO)_2O$ to produce the nitriles **5d**, **5f**–**g**.

The preparation of **5c** and **5h** is depicted in Scheme 3. The dipeptides **10c**, **10h** were synthesized according to a method originally described by Kricheldorf,[11] involving the in situ silylation of the C-terminal amino acids **9c**, **9h** and subsequent reaction with Cbz–Leu–OSu. The conversion to the target nitriles **5c**, **5h** was carried out utilizing the same strategy as depicted in Scheme 1.

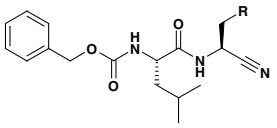
Preparation of compounds **16a**–**h** starts with the in situ silylation of *O*-benzyl-L-serine **9h**, followed by reaction with Boc–Leu–OSu to yield dipeptide **12**. The mixed anhydride of the Boc-protected dipeptide **12** with isobutyl-chloroformate was quenched with aqueous NH_3 to furnish the amide derivative **13**, which was converted to the nitrile compound **14** by $(CF_3CO)_2O$-mediated dehydration.[10] Removal of the Boc group was carried out with neat HCOOH and the resulting amine **15** was coupled with the appropriate carboxylic acid to yield the target dipeptide nitriles **16a**–**h** (Scheme 4).

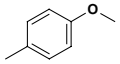
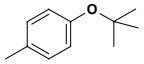
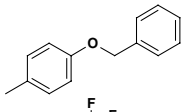
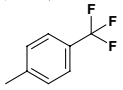
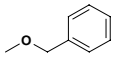
We first investigated the effects of attaching a P1-substituent to the α-position of the C-terminal Gly-nitrile moiety of our lead **1** on potency and selectivity for cathepsin K inhibition. In an attempt to exploit hydrophobic interactions within the lipophilic S1 subsite of cathepsin K, a series of compounds containing phenylalanine-derived P1 side chains were investigated. The biological activity of these analogs is summarized in Table 1. A 4-substituent on the phenyl ring proved favorable in terms of potency against cathepsin K and also had a positive effect on the specificity profile of the inhibitors. Compounds **5a** and **5f** incorporating a 4-methyl or a 4-trifluoromethyl-phenylalanine moiety were the most potent and selective inhibitors of cathepsin K within



Scheme 4. Reagents and conditions: (a) 1—TMSiCl, DIEA, THF, rt, 1 h, 2—Boc–Leu–OSu, DIEA, rt, 6 h, 96%; (b) 1—Isobutyl-chloroformate, *N*-methylmorpholine, THF, −15 °C, 15 min, 2—NH_3 (aq), 97%; (c) $(CF_3CO)_2O$, Et_3N, THF, −15 °C, 2 h, 70%; (d) HCOOH, rt, 18 h, 54%; (e) RCOOH, (benzotriazol-1-yloxy)-tris-(dimethylamin)-phosphonium-hexafluorophosphate (BOP), DIEA, CH_2CL_2, rt, 16 h, 41–87%.

Table 1. Inhibition of homologous cathepsins by compounds **5a–h**



Compound	R	IC_{50} (nM)		
		Cat K[a]	Cat L[b]	Cat S[c]
5a		48	9900	>10,000
5b		120	7700	2300
5c		63	1100	700
5d		398	1400	>10,000
5e		>1000	> 10,000	>10,000
5f		43	8000	>10,000
5g		240	2800	900
5h		9	1700	969

[a] Inhibition of recombinant human (rh) cathepsin K activity in a fluorescence assay using 48 μM Cbz–Phe-Arg–AMC as substrate in 100 mM NaH_2PO_4, 1 mM EDTA, 20 μM Tween 20, and 2 mM DTT, pH 7.

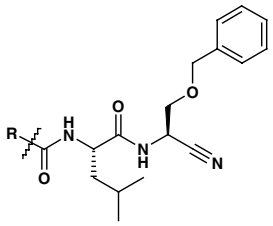
[b] Inhibition of rh cathepsin L activity using 3 μM Cbz–Phe-Arg–AMC as substrate in 100 mM NaOAc, 1 mM EDTA, 0.005% Brig 35, and 1 mM DTT, pH 5.5.

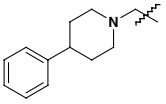
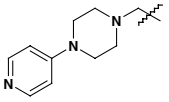
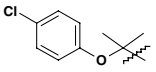
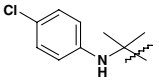
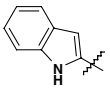
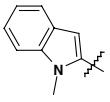
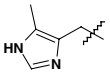
[c] Inhibition of rh cathepsin S activity using 11 μM Cbz–Leu-Leu-Arg–AMC as substrate in 100 mM NaOAc, 1 mM EDTA, 0.01% Triton X-100, 1 mM DTT, pH 5.5. Data represent means of two experiments performed in duplicate, individual data points in each experiment were within a 3-fold range of each other.

the phenylalanine series. However, increasing the steric bulk of the 4-substituent led to a gradual loss in potency for compounds **5a** → **5c** → **5d** → **5e**. A 3-substituent resulted in a less potent analog **5a** → **5b**. Compounds **5g** and **5h** are inhibitors incorporating O-protected L-serine derivatives as P1-subunit. Whereas the *O-tert*-butyl-ether derivative **5g** is not very active, compound **5h**, incorporating an *O*-benzyl serine P1 moiety, is the most potent inhibitor investigated within the Cbz-protected dipeptide nitrile series. In addition, **5h** exhibits good specificity for cathepsin K over the two highly homologous cathepsins L (K/L = 190) and S (K/S = 107).

Based on the most potent inhibitor **5h** we embarked on a second round of optimization that involved replacement of the benzyloxycarbonyl group by different acyl moieties. The S3 subsite of cathepsin K is rather hydrophilic in nature and therefore polar substituents should be tolerated as part of the P3 subunit, which might in fact allow to fine-tune the physico-chemical properties of our inhibitors. As illustrated by the data summarized in Table 2 this strategy afforded several highly potent cathepsin K inhibitors (**16c**, **16e**, and **16f**). Replacement of the benzyloxycarbonyl group by a 4-phenyl-piperidin-1-yl)-acetyl moiety led to a loss in potency (**5h** → **16a**). The introduction of a 4-pyridin-4-yl-piperazin-1-yl)-acetyl moiety resulted in a 9 nM inhibitor, but **16b** is significantly less specific for cathepsin K than

Table 2. Inhibition of homologous cathepsins by compounds **16a–h**



Compound	R	IC_{50} (nM)		
		Cat K[a]	Cat L[b]	Cat S[c]
16a		83	1200	140
16b		9	330	180
16c		<1	<30	52
16d		430	190	41
16e		<1	154	29
16f		<1	83	47
16g		24	110	130
16h		190	660	240

[a] Inhibition of recombinant human (rh) cathepsin K activity in a fluorescence assay using 48 μM Cbz–Phe-Arg–AMC as substrate in 100 mM NaH_2PO_4, 1 mM EDTA, 20 μM Tween 20, and 2 mM DTT, pH 7.

[b] Inhibition of rh cathepsin L activity using 3 μM Cbz–Phe-Arg–AMC as substrate in 100 mM NaOAc, 1 mM EDTA, 0.005% Brig 35, and 1 mM DTT, pH 5.5.

[c] Inhibition of rh cathepsin S activity using 11 μM Cbz-Leu-Leu-Arg-AMC as substrate in 100 mM NaOAc, 1 mM EDTA, 0.01% Triton X-100, and 1 mM DTT, pH 5.5. Data represent means of two experiments performed in duplicate, individual data points in each experiment were within a 3-fold range of each other.

compound **5h**. A 4-chlorophenoxy-2-methyl-propionyl P3 group afforded the sub-nanomolar inhibitor **16c**. However, this compound features a poor specificity profile. Surprisingly, compound **16d**, which differs from **16c** only by the replacement of the phenoxy- by a phenylamino moiety, shows a reversed selectivity profile in favor of cathepsin S. An unsubstituted and 1-methyl-indolyl moiety provide analogs **16e** and **16f**, both of which show also sub-nanomolar potency. These two compounds exhibit moderate specificity, in particular with regard to cathepsin S.[12]

The two imidazolyl-acetyl-based analogs **16g** and **16h** are essentially non-specific with a maximal specificity ratio of 5-fold in favor of cathepsin K.

One of the main challenges in the optimization of peptidic lead structures is to generate compounds with good pharmacokinetic properties, in order to allow for oral administration of a potential drug candidate. Compounds with promising in vitro potencies were thus evaluated in in vitro metabolism assays and their pharmacokinetic properties were determined in a cassette dosing experiment. The focus of these studies was to reveal potential weak points within the series to guide further modifications.

Metabolic stability of compounds **5h**, **16b**, **16c**, **16f**, and **16g** was assessed in vitro in rat or human liver microsomes as well as in rat plasma. Capillary HPLC/MS–MS was used to determine remaining parent compound and characterize potential metabolites.[13] Figure 1 shows the selected ion chromatogram of **5h** after 1 h incubation with rat liver microsomes.

The metabolism of all five compounds investigated followed a similar pattern which is exemplified in Figure 1 for compound **5h**. In all cases, O-debenzylation

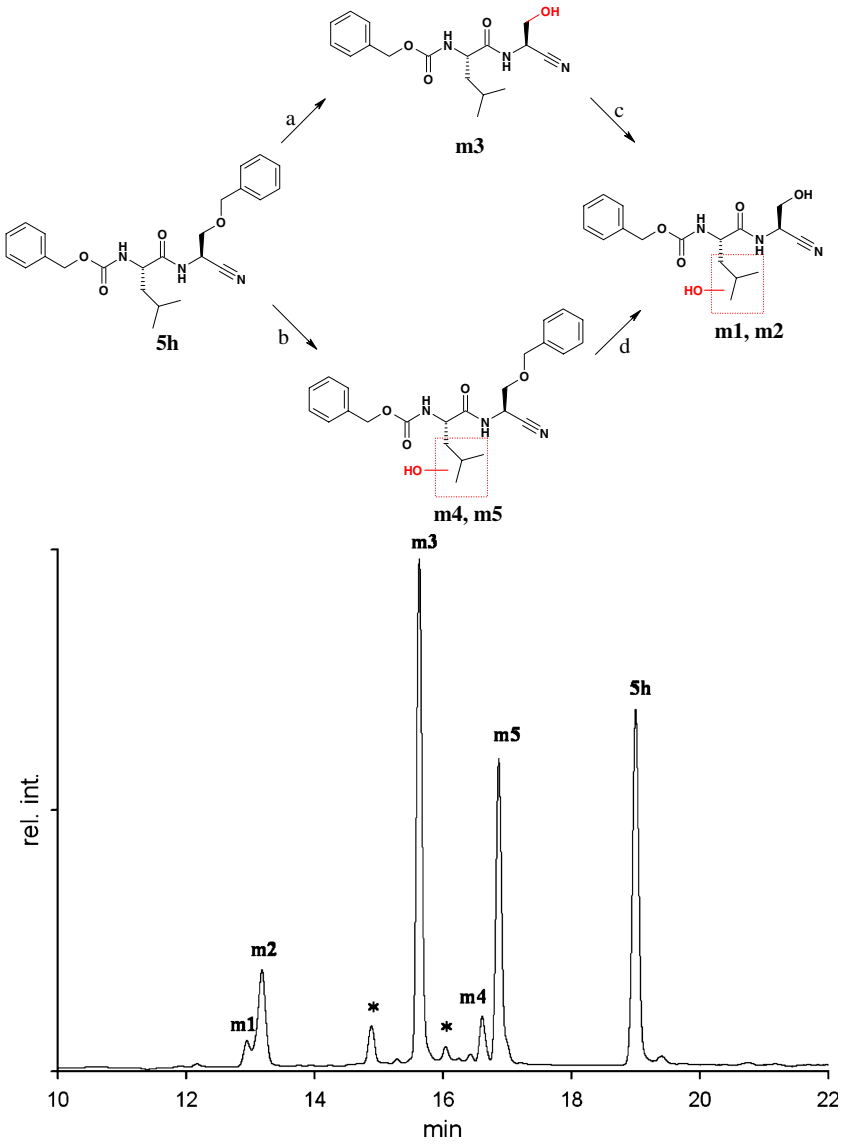


Figure 1. Proposed metabolic pathways of **5h** and selected ion chromatogram over $M+NH_4^+$ of **5h** (*m/z* 441), **m4/m5** (*m/z* 457), **m3** (*m/z* 351), and **m1/m2** (*m/z* 367), 1 h after incubation of **5h** in rat liver microsomes (* peaks are not related to **5h**).

of the serine side chain was observed (pathway a) and for **16b** this was the only transformation detected. For **5h**, **16c**, **16f**, and **16g** additional metabolites were formed through hydroxylation of the isobutyl side chain of leucine (pathway b; benzyl group still intact). A metabolite arising from both debenzylation and leucine hydroxylation was only observed for **5h** (pathways c or d). Metabolite patterns were similar between microsomes from rat and human.

In addition to liver metabolism, plasma stability plays an important role in drug discovery, as compounds with insufficient plasma stability tend to exhibit rapid clearance and short half-lives, which are associated with poor in vivo performance. We have thus investigated the stability of compounds **1**, **5h**, **16b**, **16c**, **16f**, and **16g** in freshly prepared rat plasma.[14] Lead compound **1** was degraded to a significant extent after 1 h (>50%).[15] In contrast, the optimized inhibitors proved to be completely stable, which validates our initial working hypothesis about the beneficial effects of extended P1-substituents on metabolic stability.

Pharmacokinetic profiles in rats were determined in a cassette dosing experiment with six different compounds, including **1**, which had been previously tested separately in a discrete dosing experiment, thus serving as a reference compound (no significant difference in F and other pharmacokinetic parameters between cassette and discrete dosing was observed). Pharmacokinetic data obtained in the cassette dosing experiment[16] are summarized in Table 3. The highly soluble compounds **16b** and **16g** are extremely rapidly cleared from blood after iv administration and exhibit very low oral bioavailability (3.5% and 4.6%, respectively). We assume that after po dosing, due to their high solubility in the acidic gastric environment, **16b** and **16g** may be partially degraded by proteolytic enzymes already in the stomach and upper gastrointestinal tract. Data on blood levels and the resulting very low C_{max} po for **16g** are shown in comparison to **5h** in Figure 2. High total clearance and low oral bioavailability are also observed for the less soluble compound **16c**, however, in contrast to **16b** and **16g**, this might occur via first-pass metabolism. A distinct reduction in total clearance resulted in a significant prolongation of terminal half-lives for inhibitors **5h** and **16f**. This, probably together with improved absorption and a reduced first-pass effect, also led to a substantial improvement in oral bioavailability for these two compounds (F = 26.4% and 30.1%, respectively), compared to our lead **1** (F = 5%).

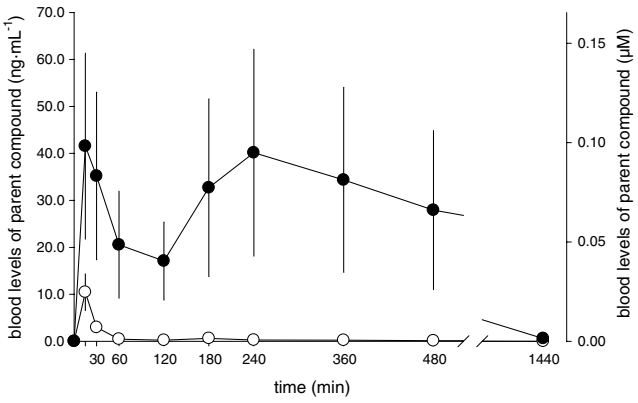


Figure 2. Blood levels of unchanged **5h** (●) and **16g** (○) after po administration (3.0 mg kg^{-1}) to conscious rats (n = 4). Dosing was in a cassette format together with four other compounds.

In summary, we have identified a series of potent dipeptide nitrile inhibitors of cathepsin K with considerable metabolic stability. Starting from lead structure **1** variations in the P1 and P3 substituents led to new analogs, which proved to be highly potent enzyme inhibitors. For several of these compounds, their in vitro metabolism was assessed in liver microsomes and in plasma, and the major metabolites were identified by HPLC/MS–MS. All optimized compounds proved to be completely stable in rat plasma. The pharmacokinetic properties of the best analogs were determined and for **16f** oral bioavailability was improved to 30% up from 5% for our initial lead structure **1**. The data presented in this study set the stage for additional medicinal chemistry efforts on dipeptide nitrile inhibitors of cathepsin K, and also other cathepsins, with even further enhanced properties.

Acknowledgments

We thank Werner Gertsch, Doris Huerzeler, Philippe Ramstein, Jean-Louis Runser, Gerard Schreiber, and Hans Zihlmann for excellent technical assistance.

Table 3. Rat pharmacokinetic data for compounds **1**, **5h**, **16b–c**, and **16f–g**

Compound	CL[a] (mL min^{-1} kg^{-1})	V_{ss}[b] (L kg^{-1})	$t_{1/2\ term}$[c] (h)	C_{max}[d] po (nM)	F[e] (%)
1	243 ± 27	3.5 ± 0.5	0.2 ± 0.03	5.4 ± 2.5	5.2 ± 2.9
5h	45 ± 12	4.6 ± 1.4	2.2 ± 0.4	44.3 ± 12.0	26.4 ± 12.7
16b	320 ± 78	18.0 ± 1.2	1.0 ± 0.1	2.5 ± 0.6	3.5 ± 0.9
16c	141 ± 26	13.6 ± 3.0	3.1 ± 0.5	3.5 ± 1.0	10.1 ± 5.2
16f	82 ± 37	7.7 ± 3.0	1.4 ± 0.4	51.1 ± 26.1	30.1 ± 9.5
16g	396 ± 94	4.3 ± 0.9	0.2 ± 0.1	11.3 ± 2.0	4.6 ± 1.6

[a] Total clearance.
[b] Volume of distribution at steady state.
[c] Terminal half life for elimination (non-compartmental estimate).
[d] Maximal blood concentration (dose-normalized to 1 mg kg^{-1}).
[e] Oral bioavailability.

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12. For two of the most potent compounds, **16e** and **16f**, IC_{50} values were also determined against cathepsin B and found to be >100 nM (selectivity ratio CatK/CatB $\gg$100).
13. Incubation in liver microsomes. Compounds were dissolved in DMSO (2 mM) and were added to rat or human liver microsomal suspensions (20 mg/mL protein) in phosphate buffer (pH 7.4) at 37 °C. The metabolic reaction was initiated by the addition of NADPH regenerating system. The incubation reactions were terminated at 1 h by addition of 500 μL chilled CH_3CN. The samples were stored at −80 °C for at least 1 h before centrifugation. After centrifugation, the supernatant was diluted 1:5 with H_2O/0.1% HCOOH and the samples were submitted for HPLC/MS–MS analysis.
14. Compounds were incubated at concentrations of 1 and 5 μM in freshly prepared rat plasma for 1 h at 37 °C. Incubation was stopped by addition of 500 μL chilled CH_3CN. After centrifugation, the supernatant was diluted 1:5 with H_2O/0.1% HCOOH and the samples were submitted for HPLC/MS–MS analysis.
15. No specific degradation products could be detected under our experimental conditions.
16. PK cassette-dosing in rats. The experiment was performed in conscious, fed, permanently cannulated rats kept under standard conditions. For iv bolus administration, all compounds were dissolved in *N*-methyl-2-pyrrolidone/PEG200 (30:70, v/v; 0.5 mL kg^{-1}) and dosed at 1 mg kg^{-1}. For oral administration compounds were dissolved/suspended and sonicated in a solution of tartaric acid (1%) with carboxymethyl cellulose (1%) in bidistilled water (2.5 mL kg^{-1}) and administered at 3 mg kg^{-1}. Blood samples were collected from the femoral artery for 24 h after iv and after oral dosing. Iv and po administration was carried out in the same animals with a 48 h washout period between administrations. After acetonitrile precipitation of plasma samples, parent drug concentrations in extracts were measured using a specific HPLC/MS method.