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Novel semicarbazide-derived inhibitors of human dipeptidyl peptidase I (hDPPI)

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Abstract—Human dipeptidyl peptidase I (hDPPI, cathepsin C, EC 3.4.14.1) is a novel putative drug target for the treatment of inflammatory diseases. Using **1** as a starting point ($IC_{50} > 10$ μM), we have improved potency by more than 500-fold and successfully identified novel inhibitors of DPPI via screening of a one-bead-two-compounds library of semicarbazide derivatives. Selected compounds were shown to inhibit intracellular DPPI in RBL-2H3 cells. These compounds were further characterized for adverse effects on HepG2 cells (cytotoxicity and viability) and their metabolic stability in rat liver microsomes was estimated. One of the most potent inhibitors, **8** ($IC_{50} = 31 \pm 3$ nM; $K_i = 45 \pm 2$ nM, competitive inhibition), is selective for DPPI over other cysteine and serine proteases, has a half-life of 24 min in rat liver microsomes, shows approximately 50% inhibition of intracellular DPPI at 20 μM and is noncytotoxic.
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1. Introduction

Human dipeptidyl peptidase I (hDPPI, cathepsin C, EC 3.4.14.1), first discovered by Gutmann and Fruton in 1948,[1] is a lysosomal cysteine exopeptidase that belongs to the papain family.[2,3] The enzyme is constitutively expressed in many tissues with highest levels in lung, kidney, liver and spleen. The cloning and sequencing of cDNAs encoding rat, human and murine DPPI show that the enzyme is highly conserved.[2,4,5] DPPI is a 200 kDa tetramer consisting of four identical subunits, each composed of three different chains: a heavy chain, a light chain and an exclusion domain.[6a,b] The crystal structure of DPPI shows that the enzyme has four active site clefts with the active site residues Cys 234 and His 381 forming an ion pair near the scissile peptide bond. The S1 pocket is located near the surface, exposed to the solvent. The S2 pocket is deep and hydrophobic, with a chloride ion and two solvent molecules at the bottom, and Asp 1 at the entrance to anchor the substrate via an electrostatic interaction. The exclusion domain blocks extension beyond the S2 pocket, and thus prevents endopeptidase activity. DPPI is synthesized as an inactive precursor (zymogen), and is activated by a non-autocatalytic excision of an internal activation peptide within the N-terminal propeptide.[7] Once activated, DPPI catalyzes sequential removal of dipeptides from the N-termini of peptide and protein substrates with broad specificity.[3,8,9] However, DPPI only cleaves substrates with a free N-terminal amino group, and does not cleave substrates with P1 or P1′ Pro, P1 Ile and P2 ornithine, Lys or Arg.

DPPI is an important enzyme in lysosomal protein degradation and recent studies in DPPI knock-out mice show that DPPI functions as a key enzyme in the activation of granule serine proteases in cytotoxic T lymphocytes and natural killer cells (granzymes A and B),[3,10a] mast cells (chymase and tryptase),[10b,c] and neutrophils (cathepsin G, proteinase 3 and elastase).[11]

Keywords: Human dipeptidyl peptidase I; Inflammation; Protease inhibitors; Semicarbazides.
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Dominant-negative mutations within the human DPPI genes are linked to the Papillon-Lefèvre syndrome,[12] a disease characterized by early periodontitis, palmoplantar hyperkeratosis and a predisposition to bacterial infections. This indicates that DPPI plays an important role in the immune system. Recent data[13] show that disruption of DPPI genes is beneficial for the survival from sepsis and indicate that DPPI is a potential new target for the treatment of sepsis. Thus, in combination with predictive animal models, inhibitors of DPPI will be beneficial for further studies on the physiological role of DPPI.

Inhibitors of DPPI include dipeptide diazomethyl ketones (e.g., Gly-Phe-CH_2N_2, Fig. 1),[14a,b] a dipeptide nitrile,[15] dipeptide vinyl sulfones,[16] dipeptide acyloxymethyl- and fluoromethyl-ketones,[16] dipeptide *O*-acyl hydroxamic acids,[17] arginine-based peptides[18] and phosphinic tripeptides.[19] The naturally occurring cysteine protease inhibitor E-64 also inhibits DPPI at high concentrations.[20]

Herein, we report semicarbazide-derived inhibitors of DPPI, also known as azapeptides when flanked by amino acid residues.[21] Azapeptides have been reported as inhibitors of Hepatitis C Virus NS3 protease,[22,23] Human Rhinovirus 3C protease,[24] papain[25,26] and cathepsins B and K.[25] Zoladex®, an azapeptide analogue of the peptide hormone luliberin (LHRH), is used for treatment of prostate cancer. Azapeptides contain one or more amino acids in which the central α-carbon has been replaced by a nitrogen. Replacement of the P1 α-carbon with a nitrogen decreases the electrophilicity of the P1 carbonyl group and changes the geometry of the α-position from tetrahedral to nearly trigonal, resulting in loss of chirality and an orientation of the side chain somewhere in between the D- and L-configuration of the corresponding amino acid. Some azapeptides are metabolically more stable towards enzymatic degradation than their natural peptide counterparts.[27] Azapeptides form inactivating carbamoyl–enzyme complexes with cysteine and serine proteases, which undergo slow hydrolysis.[22,24] The rate and extent of acylation–deacylation depend on the reactivity of the leaving group.[22] Azapeptides with poor leaving groups fail to acylate the enzyme and hence display competitive inhibition, whereas azapeptides with reactive leaving groups form carbamoyl–enzyme complexes that are more stable than normal acyl–enzymes.[26]

2. Results and discussion

Using **1** (Fig. 1, $IC_{50,\,DPPI} > 10\ \mu M$) as a starting point, we initially screened a 64,000 member unbiased, one-bead-two-compounds combinatorial library of semicarbazides (**2**). The one-bead-two-compounds library[28–30] allows screening of a large number of compounds in a competitive fashion using biocompatible beads. Each bead contains an internally quenched fluorogenic substrate and a putative inhibitor. Upon incubation with a protease the substrate will be cleaved in the presence of a weak inhibitor, resulting in bright beads under UV-light due to separation of the quencher from the immobilized fluorophore. Vice versa, the darkest beads contain potent inhibitors, which prevent substrate cleavage by binding to the protease. Thus, dark beads can be selected and the identity of the inhibitors (hits) can be elucidated by MALDI-TOF MS. The hits from the screening are verified by resynthesis of the identified structure and subsequent IC_{50} determination.

Based on the results from the first one-bead-two-compounds library screening, **8** (Fig. 1, Table 1, $IC_{50} = 31 \pm 3$ nM) was synthesized. Subsequently, a second focused 8000 member library was screened, leading to the identification of **9** ($IC_{50} = 21 \pm 3$ nM), and the design of **10**–**14**. Overall, more than 500-fold increase in potency was achieved compared to **1**.

To reduce both molecular weight and the peptidic character of the compounds, we substituted the P1′–P2′ dipeptide moiety with various aromatic groups (Table 2, entries **19**–**46**) leading to the identification of **28** ($IC_{50} = 560 \pm 26$ nM) and the more potent analogue **35** ($IC_{50} = 123 \pm 16$ nM).

The selectivity of a range of inhibitors was determined by screening against a panel of relevant cysteine- and serine-proteases, as well as CYP450 enzymes. Selected compounds were also tested in a cellular DPPI assay using a RBL-2H3 rat mast cell line (Fig. 3), and their

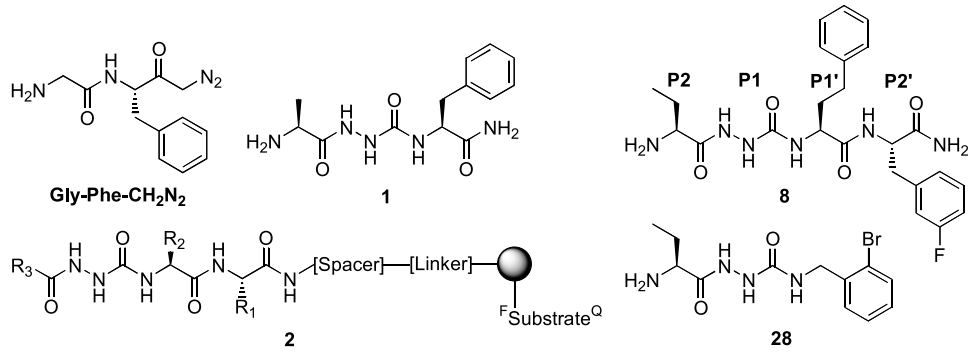
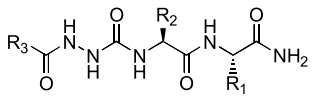


Figure 1. Structures of Gly-Phe-CH_2N_2 (or $GFCH_2N_2$), semicarbazides **1, 8**, **28** and the one-bead-two-compounds library **2**; F, fluorophore; Q, quencher.

Table 1. IC_{50} values for **3–18**



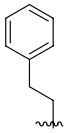
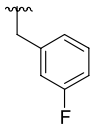
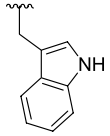
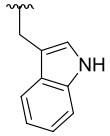
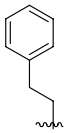
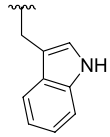
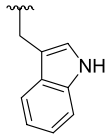
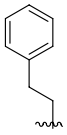
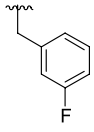
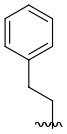
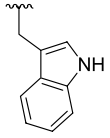
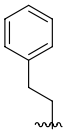
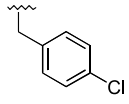
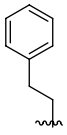
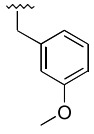
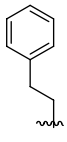
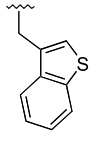
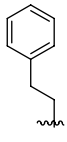
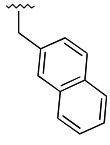
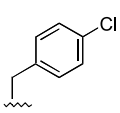
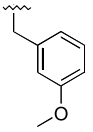
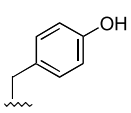
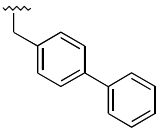
#	R_3	R_2	R_1	IC_{50} (DPPI) nM
3				622 ± 13
4				933 ± 251
5				378 ± 50
6				968 ± 185
7				10,967 ± 3519
8				31 ± 3
9				21 ± 3
10				45 ± 4
11				39 ± 3
12				22 ± 2
13				19 ± 6

Table 1 (*continued*)

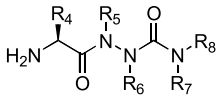
#	R_3	R_2	R_1	IC_{50} (DPPI) nM
14				445 ± 10
15				219 ± 10
16				108 ± 23
17				440 ± 64
18				496 ± 70

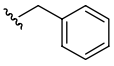
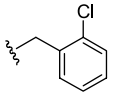
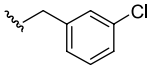
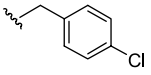
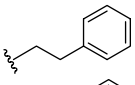
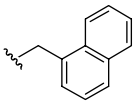
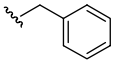
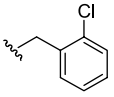
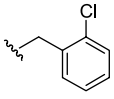
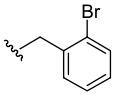
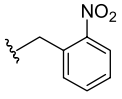
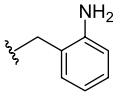
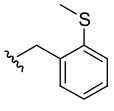
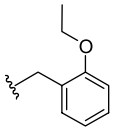
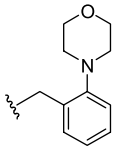
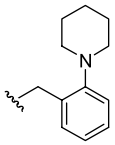
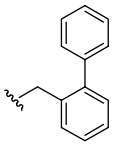
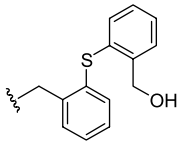
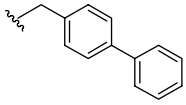
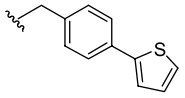
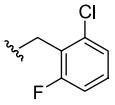
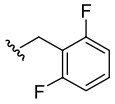
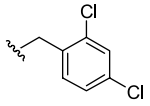
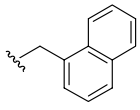
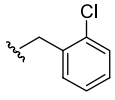
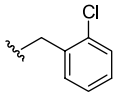
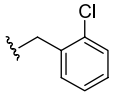
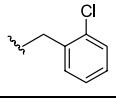
cytotoxicity and viability in HepG2 cells was determined. The metabolic stability of these compounds was evaluated in rat liver microsomes.

2.1. Chemistry

For solution-phase synthesis (Scheme 2, **19–44**) the semicarbazide bond was formed by acylation of the amine with the appropriate 1-acyl-1′-carbonylimidazolylhydrazide, formed in situ by reaction of the amino acid hydrazide with CDI. The hydrazides were formed by in situ activation of the appropriate amino acid with CDI, followed by reaction with hydrazine. The crude products were usually used directly in the next step. When using the solid phase approach (Scheme 1, **3–18**) Fmoc–$NHNH_2$ was used to introduce the semicarbazide bond via in situ activation with CDI, similar to what was previously reported.[25] The Fmoc group could subsequently be removed by standard treatment with piperidine in DMF, followed by acylation at the 1-position with an activated carboxylic acid derivative (e.g., TBTU activation). Substituents in the 2-position of the semicarbazide functionality can be introduced via substituted hydrazines (Scheme 3), as described previously.[31] Thus, benzylhydrazine was selectively Boc-protected on the 1-position with excess Boc_2O at −78 °C, followed by Fmoc-protection of the 2-position with Fmoc–Cl and DIPEA. Treatment with TFA gave 1-Fmoc-2-benzylhydrazine **I** in 46% yield after work-up. Reaction with phosgene in toluene afforded crude 2-(chlorocarbonyl)-1-Fmoc-2-benzylhydrazine, which was reacted directly with 2-chlorobenzylamine to give the 1-Fmoc-2-benzyl-

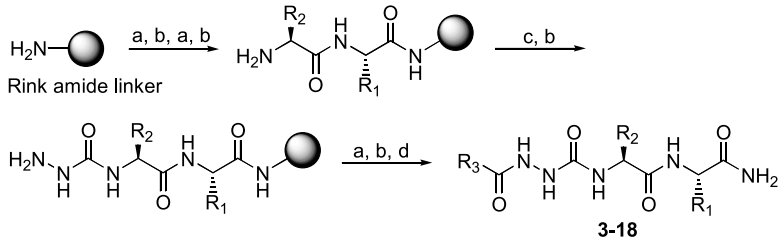
Table 2. IC_{50} values for **19-46**



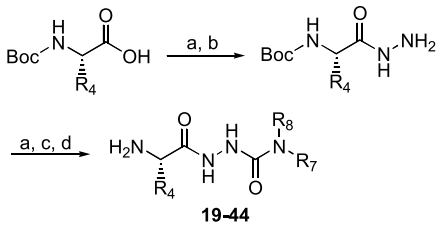
#	R_4	R_5	R_6	R_7	R_8	IC_{50} (DPPI) nM
19	Me	H	H	H		8066 ± 252
20	Me	H	H	H		1396 ± 13
21	Me	H	H	H		13,167 ± 1018
22	Me	H	H	H		6292 ± 419
23	Me	H	H	H		NI[a]
24	Me	H	H	H		1622 ± 101
25	Et	H	H	H		2045 ± 277
26	Et	H	H	H		759 ± 117
27	Et	H	H	Me		NI[a]
28	Et	H	H	H		560 ± 26
29	Et	H	H	H		562 ± 91
30	Et	H	H	H		5422 ± 117
31	Et	H	H	H		269 ± 26
32	Et	H	H	H		698 ± 53
33	Et	H	H	H		422 ± 25
34	Et	H	H	H		600 ± 37
35	Et	H	H	H		123 ± 16
36	Et	H	H	H		157 ± 23
37	Et	H	H	H		678 ± 30
38	Et	H	H	H		530 ± 56
39	Et	H	H	H		494 ± 29
40	Et	H	H	H		986 ± 36
41	Et	H	H	H		1284 ± 175
42	Et	H	H	H		684 ± 55
43	Bu	H	H	H		2312 ± 303
44	Bn	H	H	H		1227 ± 167
45	Et	H	Bn	H		NI[a]
46		H	H			NI[a]

[a] No inhibition at 10 μM.

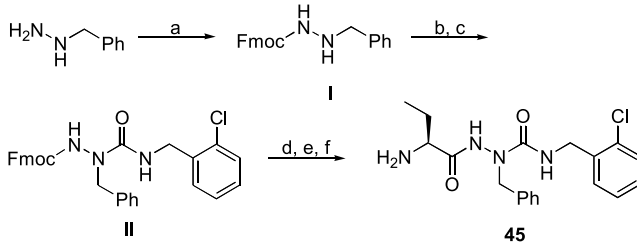
4-(*o*-chlorobenzyl)semicarbazide **II**. The Fmoc group was removed with piperidine and the final (2*S*)-2-aminobutanoyl substituent was introduced via the DIC-mediated anhydride of (2*S*)-*N*-Boc-aminobutyric acid to give **45** after deprotection and purification.



Scheme 1. Solid-phase synthesis of semicarbazides **3**–**18**. Reagents and conditions: (a) Fmoc–AA–OH, TBTU, NEM, DMF; or Fmoc–AA–OPfp, DhbtOH, DMF; (b) 20% piperidine in DMF; (c) Fmoc–$NHNH_2$, CDI, DMF; (d) TFA–TIPS 98:2.



Scheme 2. Synthesis of semicarbazides **19**–**44** in solution. Reagents and conditions: (a) CDI, THF; (b) NH_2NH_2, THF; (c) R_7R_8NH, THF; (d) TFA–H_2O 95:5.



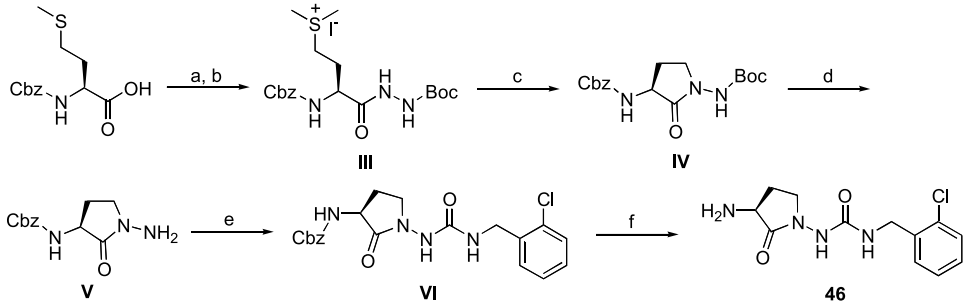
Scheme 3. Synthesis of **45**. Reagents and conditions: (a) (i) Boc_2O, DIPEA, $CHCl_3$, −78 °C; (ii) Fmoc–Cl, DIPEA; (iii) TFA–H_2O 95:5 (46%, three steps); (b) $COCl_2$, toluene; (c) 2-chlorobenzylamine, DIPEA, CH_3CN; (d) piperidine; (e) Boc-Abu-OH, DIC, THF; (f) TFA–H_2O 95:5 (9%, five steps).

The conformationally constrained analogue **46** was prepared via the pyrrolidinone **V** (Scheme 4) following a previous procedure,[32] although experimental details and analytical data were not reported. Thus, Cbz-L-methionine was converted to the sulfonium salt **III** by condensation with *tert*-butylcarbazate via activation with CDI, followed by reaction with methyl iodide. Cyclization was achieved using NaH, affording **IV** in 50% yield. Removal of the Boc group with HCl in dioxane gave **V** as the HCl salt, which was converted to the protected semicarbazide **VI** by activation with CDI followed by displacement with *o*-chlorobenzylamine. The Cbz group was removed with HBr in acetic acid to give **46**.

All target compounds were purified by HPLC to a minimum purity of 95% as determined by HPLC and characterized by ^1H NMR and ES-MS.

2.2. Structure–activity relationships

Starting with the weak inhibitor **1** we initially synthesized and screened a 64,000 member one-bead-two-compounds combinatorial library of semicarbazides, four amino acids in length in order to explore the P2′ binding site as well. The crystal structure of human DPPI[6] revealed that the S2′ pocket is located in the Gln 228-Cys 234 loop, however, very little is known about the P2′ SAR. Based on the results from the first round of library screening, we synthesized **3**–**7**, and from **4** (IC_{50} = 933 ± 251 nM) it became evident that addition of P2′ Trp increased the potency >10-fold compared to **1**, indicating that this position can indeed have a significant impact on binding. Further analysis revealed that for the P2 residue the preference is ethyl > methyl ≫ piperidinyl (**4**, **5** and **7**), consistent with previous findings that dipeptides with small aliphatic P2 groups are optimal substrates, and a free



Scheme 4. Synthesis of **46**. Reagents and conditions: (a) CDI, THF; then $BocNHNH_2$ (96%); (b) MeI (100%); (c) NaH, DMF, DCM (50%); (d) HCl in dioxane (49%); (e) CDI, THF, *o*-chlorobenzylamine (34%); (f) HBr in acetic acid (83%).

(primary) terminal amino group is crucial.[8] The latter was also confirmed by various mono- and di-*N*-alkylations of **1**, which resulted in complete loss of activity at 20 μM (**S1**–**S6**, Table S1; see Supplementary data). The former was confirmed by comparing **20** (IC_{50} = 1396 ± 13 nM), **26** (IC_{50} = 759 ± 117 nM), **43** (IC_{50} = 2312 ± 303 nM) and **44** (IC_{50} = 1227 ± 167 nM), although the benzyl- and methyl-analogues (**20** and **44**) were surprisingly equally potent. However, the *p*-bromobenzyl analogue **S42** (Table S2) was only weakly active at 10 μM, indicating that the bottom of the S2 pocket might have been reached. P2 isopropyl (**S39**) and 3-thiabutyl (**S41**) were also tolerated. The conformationally constrained analogue **46** was inactive at 10 μM, as was the *R*-isomer (data not shown).

Intrigued by the fact that **3** (IC_{50} = 622 ± 13 nM) with P2 cyclohexyl and **6** (IC_{50} = 968 ± 185 nM) with P2 pyrrolidine, both with P1′ homophenylalanine, were surprisingly potent considering their seemingly unfavourable P2 group, we synthesized **8** giving a significant boost in potency. We then aimed at increasing potency by screening a second, 8000 member library focussed around the general P2–P1′–P2′ motif 'small aliphatic–aromatic–aromatic'. This resulted in the identification of **9** with a minor increase in potency. Attempts to optimize the P2′ substituent further did not lead to a significant increase in potency (**10**–**14**). With respect to the P1′ residue hydrophobic, aromatic groups were generally well tolerated, consistent with the shallow and hydrophobic nature of the S1′ pocket,[6] although homophenylalanine was far superior to both *p*-chlorophenylalanine, Trp, 2-naphthylalanine and *p*-phenylphenylalanine, when comparing the screening hits **14** (IC_{50} = 445 ± 10 nM), **16** (IC_{50} = 108 ± 23 nM), **17** (IC_{50} = 440 ± 64 nM) and **18** (IC_{50} = 496 ± 70 nM) to **8** (IC_{50} = 31 ± 3 nM) and **11** (IC_{50} = 39 ± 3 nM). Discouraged by the complete lack of activity at 10 μM for **45** with P1 benzyl, a preferred substrate P1 side chain,[8] no attempts were made towards introducing further P1 diversity. It might be speculated that P1 substitution of the semicarbazides results in an unfavourable orientation of the side chain due to the resulting nearly trigonal geometry. It is known that D-amino acids are not tolerated at this position.[8] However, an electronic effect cannot be ruled out since *N*-substitution decreases the electrophilicity of the semicarbazide carbonyl, thus affecting the putative interaction with the active site cysteine.

In an attempt to reduce molecular weight and the number of rotatable bonds, a series of less peptidic semicarbazides were prepared (**19**–**44**). In this series P1′ benzyl (**19**; IC_{50} = 8066 ± 252 nM and **25**; IC_{50} = 2045 ± 277 nM) and 1-naphthylmethyl (**24**; IC_{50} = 1622 ± 101 nM and **42**; IC_{50} = 684 ± 55 nM) was initially identified as suitable groups. Benzyl was selected as a starting point for potency optimization due to the wide variety of commercially available benzylamines, thus facilitating rapid parallel synthesis. The preferred aromatic monosubstitution $o > p > m$ (**20**–**22**) was quickly identified. In the *ortho* position, many groups were tolerated, with phenyl (**35**; IC_{50} = 123 ± 16 nM) being the best in the series. In an attempt to rapidly further explore the SAR in the *ortho* position, we prepared a series of anilides (**S44**–**S57**, Table S3). However, judging from the resulting 15- to 1000-fold loss in potency compared to **35**, this was not a well tolerated modification. In contrast, the thioether **36** (IC_{50} = 157 ± 23 nM) retained its potency, indicating that rather bulky and flexible groups are tolerated, although it appears that planarity is potentially important (**33**; IC_{50} = 422 ± 25 nM and **34**; IC_{50} = 600 ± 37 nM). Although **35**, the most potent compound in this series, is at least 5-fold less potent than the best compounds from the first series (**3**–**18**), further optimization should be feasible.

2.3. Reversibility, selectivity, metabolic stability and cellular activity

The reversibility of inhibition of DPPI by **8** was investigated by comparing its kinetics with the irreversible inhibitor Gly-Phe-CH_2N_2 ($GFCH_2N_2$, Fig. 2).[33] During the first 600 s of the reaction with an initial substrate concentration of 150 μM ($1 \times K_m$), the velocity of the substrate cleavage in the presence of 150 nM **8** was measured as lower (2.4 RFU/s) than the measured velocity of the substrate cleavage in the control reaction (6.6 RFU/s). After addition of substrate to 1.5 mM ($10 \times K_m$, at 6000 s), the velocity of substrate cleavage in the presence of 150 nM **8** was equal to the velocity of substrate cleavage in the control reaction (approximately 11 RFU/s for both reactions, in the interval 6000–7000 s), indicating reversible inhibition. At the same high substrate concentration, 20 μM of the inhibitor $GFCH_2N_2$ reduced the velocity of substrate cleavage significantly (a reduction from 11 to 2 RFU/s), indicating an irreversible mode of inhibition for this

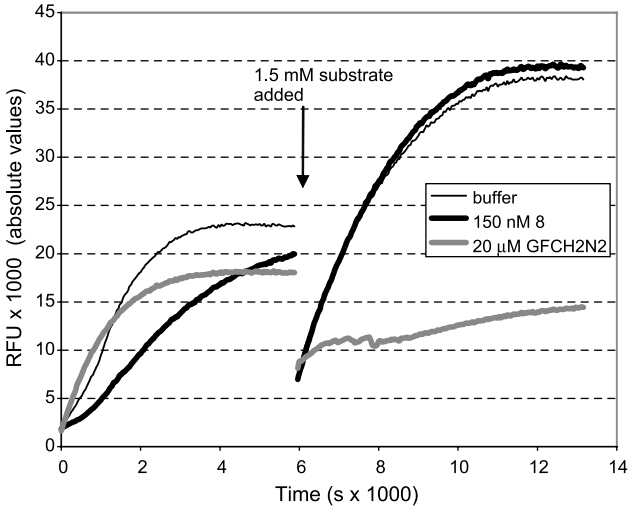


Figure 2. Reversibility studies using 150 nM **8** and 20 μM$GFCH_2N_2$ at 150 μM substrate concentration (left-hand side). Addition of saturating substrate (1.5 mM, $10 \times K_m$; right-hand side) restores enzymatic activity in the presence of **8**, indicating reversible inhibition, whereas $GFCH_2N_2$ inhibits the enzyme in an irreversible manner. The experiment was performed twice.

inhibitor.[34] The lack of inhibition with $GFCH_2N_2$ in the initial period (600 s) is presumably because this inhibitor requires a certain time period to irreversibly occupy all the substrate binding sites in the enzyme. In the period from 4000 to 6000 s, $GFCH_2N_2$ completely inhibits substrate cleavage despite the fact that there is still noncleaved substrate present in the solution (i.e., comparing the absolute fluorescence value of approximately 18,000 RFU to the maximal fluorescence value of approximately 23,000 RFU reached in the control reaction), further indicating an irreversible mode of inhibition for $GFCH_2N_2$. The sudden decrease in fluorescence signal at 6000 s is caused by substrate quenching due to the high amount of substrate added to the reactions ($10 \times K_m$). This quenching phenomenon is known as the inner filter effect.[35]

Selected compounds from each series were screened for their effect on other proteases (Table 3). No inhibition of the serine proteases cathepsin G, neutrophil elastase, tryptase and chymase were observed at 10 μM for any of the compounds. In addition, compounds **3**, **8**, **26**, **28**, **39** and **42**–**44** did not inhibit the serine protease dipeptidyl peptidase IV[36] (DPPIV) at 10 μM (data not shown). However, **8**–**13** all displayed moderate to weak inhibition of the cysteine proteases cathepsin B and H. No inhibition of the cysteine protease cathepsin L was observed. Compounds **28** and **42**–**44** all displayed $IC_{50} > 10$ μM against cathepsins B, H and L. Selected compounds were also screened against a panel of cytochrome P450 enzymes (Table 4). Only the peptidic series of semicarbazides **8**–**13** and **16** showed significant inhibition. For all seven compounds this effect was observed with respect to CYP3A4, the major cytochrome responsible for metabolism of nearly 50% of marketed drugs.[37] Whether CYP3A4 was inhibited by the tested compounds or whether these compounds were metabolized by CYP3A4, thereby causing apparent inhibition, was not investigated further. The metabolic stability of **8**, **9**, **16**, **28**, **38** and **39** was determined in rat liver microsomes. All compounds except **39** were significantly metabolized after 15 min with half-lives in the range of 17–24 min (Table 5). Finally, the effect of **3**, **8**, **26**, **28** and **42**–**44** was tested in a cellular DPPI assay using a RBL-2H3 rat mast cell line (Fig. 3). Several compounds displayed cellular efficacy toward DPPI at 20 μM with **8** and **42** being the most potent in the series, although both were less potent than the irreversible inhibitor $GFCH_2N_2$. In addition, **3**, **8**, **26**, **28** and **42**–**44** showed neither cytotoxicity (HepG2 cells; LDH release ≪50%

Table 3. Selectivity screening[a]

#	Cat B	Cat G	Cat H	Cat L	N. elastase	Tryptase	Chymase
8	4500 ± 275	>10,000	3200 ± 581	>10,000	>10,000	>10,000	>10,000
9	927 ± 47	>10,000	2182 ± 383	>10,000	>10,000	>10,000	>10,000
10	6499 ± 997	>10,000	≈10,000[b]	>10,000	>10,000	>10,000	>10,000
11	6992 ± 832	>10,000	3152 ± 428	>10,000	>10,000	>10,000	>10,000
12	2602 ± 186	>10,000	7341 ± 897	>10,000	>10,000	>10,000	>10,000
13	2200 ± 272	>10,000	1000–10,000[c]	>10,000	>10,000	>10,000	>10,000
28	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	ND
42	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	ND
43	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	ND
44	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	ND

[a] IC_{50} values or estimated IC_{50} values (from cut-off determinations) in nM.
[b] Cut-off at 10 μM showed approximately 50% inhibition (double determination).
[c] Based on cut-off at 1 μM and 10 μM (double determinations); ND—not determined.

Table 4. Cytochrome P450 inhibition[a]

#	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
3	>20,000	>20,000	>20,000	>20,000	>1500
8	>20,000	>20,000	>20,000	>20,000	593 ± 485
9	>20,000	>20,000	>20,000	>20,000	984 ± 121
10	>20,000	>20,000	>20,000	>20,000	100–500[b]
11	>20,000	>20,000	>20,000	>20,000	100–500[b]
12	>20,000	>20,000	>20,000	>20,000	100–500[b]
13	>20,000	>20,000	>20,000	>20,000	100–500[b]
16	>20,000	>20,000	>20,000	>20,000	898 ± 236
26	>20,000	>20,000	>20,000	>20,000	>20,000
28	>20,000	>20,000	>20,000	>20,000	17,335 ± 8886
35	>10,000	>10,000	>10,000	2274 ± 174	4749 ± 3700
42	>5000	>20,000	>5000	>20,000	>10,000
43	>20,000	>20,000	>20,000	>20,000	>15,000
44	>20,000	>20,000	>20,000	>20,000	>1500

[a] IC_{50} values or estimated IC_{50} values (from cut-off determinations) in nM.
[b] Cut-off at 100 and 500 nM concentrations, determined in doublets.

Table 5. Metabolic stability[a]

#	8	9	16	28	38	39
%[b]	69(24)	65(20)	58(17)	59(22)	55	85

[a] In rat liver microsomes. All values are the mean of two determinations.
[b] % remaining after 15 min; $t_{1/2}$ in minutes given in parentheses. The $t_{1/2}$ values for reference compounds imipramine, verapamil and terfenadine are 5, 24 and 20 min, respectively.

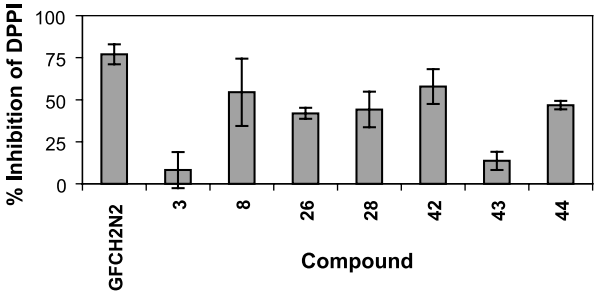


Figure 3. Inhibition of DPP-I in total cell lysate from a rat mast cell line (RBL-2H3) incubated in the presence of 20 μM **3**, **8**, **26**, **28** or **42**–**44** for 30 min. DMSO was used as control.

at 100 μM) nor reduced viability (HepG2 cells; viable cells ≫50% at 100 μM).

3. Conclusion

Starting with the weak semicarbazide-based inhibitor **1**, we have identified novel DPPI inhibitors based on a semicarbazide scaffold, using a one-bead-two-compounds library approach to aid our design. In P2, aminobutyric acid was preferred, and for the peptidic semicarbazides **3**–**18** homophenylalanine was preferred in P1′, whereas the best of the nonpeptidic analogues **19**–**46** had substituted benzylic groups in this position with the *ortho* position being favoured. In P2′ several aromatic groups were tolerated. Introduction of a benzyl group in P1 gave rise to loss of activity, as did the introduction of conformational constraint in P2. In a selectivity study with other cysteine and serine proteases, the inhibitors were selective for DPPI, although moderate to weak inhibition of cathepsins B and H was observed for the peptidic semicarbazides. The same compounds also showed significant inhibition of CYP3A4. The half-lives in rat liver microsomes were around 20 min for selected compounds and several inhibitors showed cellular efficacy towards DPPI. None of these compounds was cytotoxic or influenced cell viability. One of the best inhibitors was **8**, with an $IC_{50} = 31 \pm 3$ nM and $K_i = 45 \pm 2$ nM against DPPI. The compound showed competitive, reversible inhibition. Although the nonpeptidic semicarbazides were generally less potent, the discovery of **35** ($IC_{50} = 123 \pm 16$ nM) indicates that further optimization should be feasible. X-ray crystallography studies with these inhibitors are on-going and the results will be reported elsewhere. The results reported herein can be used to further optimize inhibitors of DPPI with respect to potency, selectivity and pharmacokinetics, which may serve to further validate DPPI as a potential drug target in inflammation.

4. Experimental

4.1. Abbreviations

AA, amino acid; Abz, 2-aminobenzoyl; AFC, 7-amino-trifluoromethyl coumarin; AMC, 7-amido-4-methyl-coumarin; Boc, *tert*-butyloxycarbonyl; CDI, 1,1′-carbonyldiimidazole; DCM, dichloromethane; DE, diethylether; DhbtOH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, 1,4-dithiothreitol; EtOAc, ethyl acetate; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; Im, 1-imidazolyl; NEM, *N*-ethylmorpholine; PE, petroleums ether (bp 60–80 °C); Pfp, pentafluorophenyl; PEGA, poly(ethylene glycol)–poly(acryl amide) copolymer; Rink amide linker, *p*-[(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid; TBTU, *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)-methylene]-*N*-methylmethan-aminium tetrafluoroborate *N*-oxide; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIPS, triisopropylsilane; TMSBr, trimethylsilyl bromide; One- and three-letter codes are used for the amino acids according to IUPAC.

4.2. Materials and methods

Experiments were conducted at room temperature (20 °C), unless otherwise noted. All solvents were HPLC grade. Anhydrous solvents were obtained by storing over 4 Å activated molecular sieves. Unless otherwise noted, starting materials were purchased from commercial suppliers and used without further purification. Yields were generally of purified product and were not optimized.

Solid phase reactions run at room temperature were performed in flat-bottom polyethylene syringes equipped with sintered Teflon filters (70 μm pores), Teflon tubing, Teflon valves for flow control and suction to drain the syringes from below, or on a VacMaster parallel synthesis rack. Fmoc deprotection was performed with 20% (v/v) piperidine in DMF (2 + 10 min). TBTU couplings were performed by dissolving the acid (3 equiv) in DMF with NEM (4 equiv), followed by addition of TBTU (2.88 equiv). The resulting solution was preactivated for 10 min before use. The reactions were

monitored using the Kaiser test.[38] Pfp esters (3 equiv) were coupled with DhbtOH (1 equiv) present. The disappearance of the bright yellow colour indicated complete capping of the resin-bound amino groups. Solid phase reactions were generally run in an amount of solvent that was sufficient to cover the resin. Resin loadings were determined by spectrophotometric analysis at 290 nm of the dibenzofulvene–piperidine adduct formed upon Fmoc deprotection, using a Perkin–Elmer Lambda 7 UV/vis spectrometer and a calibration curve from a known sample.

NMR spectra were acquired on a Bruker Advance DRX 250. Chemical shifts are reported in ppm, relative to internal solvent peaks (2.49 for DMSO-d_6, 7.25 for $CDCl_3$, 4.75 for D_2O, 3.35 for CD_3OD). Coupling constants J are reported in hertz. All key compounds were characterized by NMR and for several of the target semicarbazides the presence of rotamers was evident from isomeric resonances.

ES-MS spectra were obtained on a Micromass Quattro micro™ instrument in the positive mode. Analytical HPLC was performed on a Gilson system (UV/vis-155 detector at 215 and 254 nm, 402 syringe pump, 819 injection module, valvemate 35, 864 degasser, 233 XL on-line column switching module, and a Zorbax 300SB RP-18 column, 4.6 × 50 mm with a 322 pump), using two eluent systems. System 1: Eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) were used in a linear gradient (0% B → 100% B in 7 min) with a flow of 1 mL/min. System 2: Eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) were used in a linear gradient (0% B → 90% B in 10 min) with a flow of 0.5 mL/min. Purity (given in parentheses) is at 215 nm. Preparative HPLC was performed on the same Gilson system, using a Zorbax 300SB RP-18, 21.2 mm × 25 cm column, with a flow of 15 mL/min.

TLC plates used were Merck silica gel $60F_{254}$ on aluminium. Visualization was achieved with UV light when applicable, or developed by staining with the AMC reagent (21 g $(NH_4)_6Mo_7O_{24}$, 1 g $Ce(SO_4)_2$, 31 mL H_2SO_4, 500 mL water) or phosphormolybdic acid (2.5 g phosphormolybdic acid in 500 mL ethanol).

Elemental analysis was performed by Microanalytisches Laboratorium, University of Vienna (Austria). Evaluation of compound metabolic stability in rat liver microsomes was performed by Cerep (Seattle).

4.3. Synthesis of inhibitors 3–18

Compounds **3**–**18** were all prepared using TentaGel S-NH_2 (L = 0.41 mmol/g, 150 mg, 0.062 mmol) or $PEGA_{800}$ resin derivatized with the Rink amide linker. Coupling of individual amino acid derivatives were performed using TBTU or Pfp-ester chemistry, as described in Section 4.2. The semicarbazide bond was formed using Fmoc–NHNH–COIm, as described in **3**. Compounds **9**–**18** were prepared by Schafer-N (Copenhagen, Denmark).

4.3.1. 1-(1-Aminocyclohexane-1-carbonyl)-4-{2*S*-*N*-[2*S*-3-(*m*-fluorophenyl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semicarbazide (3). Fmoc–Rink–$PEGA_{800}$ resin (1.6 g, L = 0.25 mmol/g, 0.4 mmol) was Fmoc deprotected and washed with DMF (×5). Fmoc-3-fluorophenylalanine (487 mg, 1.2 mmol) was coupled using TBTU (370 mg, 1.15 mmol) and NEM (203 μL, 1.59 mmol) in dry DMF (12 mL). The resin was washed with DMF (×5), Fmoc deprotected and washed with DMF (×5). Fmoc–homophenylalanine (482 mg, 1.2 mmol) was likewise coupled using TBTU and NEM. Following a cycle of wash (DMF × 5), deprotection and wash (DMF × 5) the semicarbazide bond was formed by overnight reaction with Fmoc–NHNH–COIm, formed from Fmoc–$NHNH_2$ (302 mg, 1.2 mmol) and CDI (193 mg, 1.2 mmol) in dry DMF (3 mL, 2 h preactivation, then the resulting solution was added to the resin along with further 9 mL dry DMF). At this point a Kaiser test was found slightly positive and the coupling was repeated. The resin was washed with DMF (×5) and DCM (×3) and dried in vacuo. A portion of the resin (1294 mg, 318 μmol) was subjected to another cycle of wash (DMF × 5), deprotection and wash (DMF × 5), and then Boc-1-amino-1-cyclohexane carboxylic acid (232 mg, 954 μmol) was coupled using TBTU (294 mg, 916 μmol) and NEM (161 μL, 1.27 mmol) in DMF (10 mL). The resin was washed with DMF (×5) and DCM (×3) and treated with neat TFA–TIPS 98:2 for 1 h and twice for 30 min. The combined fractions were concentrated and the residue was purified by HPLC to give the title compound as a white solid. Yield: 100 mg (60%); HPLC (1): t_R = 4.21 min (>99%); HPLC (2): t_R = 7.00 min (>98%);^{1}H NMR (DMSO-d_6/D_2O 10:1, 250 MHz): δ 7.33–6.95 (m, 9H), 4.50–4.44 (m, 1H), 4.13–4.07 (m, 1H), 3.09–3.02 (dd, J = 5.0, 13.8, 1H), 2.88–2.79 (dd, J = 9.7, 13.8, 1H), 2.53–2.44 (2H, partially under DMSO signal), 2.07–1.99 (m, 2H), 1.87–1.14 (m, 10H); ES-MS: mass calcd for $C_{27}H_{36}FN_6O_4$ 527.3 (MH^+). Found m/z 527.2.

4.3.2. 1-(2*S*-2-Aminopropanoyl)-4-{2*S*-*N*-[2*S*-3-(indol-3-yl)propan-2-yl-amide]-3-phenylpropan-2-yl-amide}semicarbazide (4). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 13.1 mg (44%); HPLC (1): t_R = 3.56 min (>99%); HPLC (2): t_R = 6.36 min (>98%); ^{1}H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.69–7.66 (d, J = 7.6, 1H), 7.42–7.39 (d, J = 7.9, 1H), 7.31–7.02 (m, 8H), 4.56–4.41 (m, 2H), 3.91–3.75 (1H, partially under water signal), 3.24–3.16 (dd, J = 5.3, 14.6, 1H), 3.09–2.99 (m, 2H), 2.86–2.77 (dd, J = 8.1, 13.8, 1H), 1.44–1.41 (d, J = 7.0, 3H); ES-MS: mass calcd for $C_{24}H_{30}N_7O_4$ 480.2 (MH^+). Found m/z 480.1.

4.3.3. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(indol-3-yl)-propan-2-yl-amide]-3-phenylpropan-2-yl-amide}semicarbazide (5). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 13.3 mg (44%); HPLC (1): t_R = 3.64 min (>99%); HPLC (2): t_R = 6.41 min (>98%); ^{1}H NMR (D_2O, 250 MHz): δ 7.66–7.63 (d, J = 7.6, 1H), 7.52–7.49 (d, J = 8.0, 1H), 7.34–7.13 (m, 6H), 7.01–6.97 (m, 2H), 4.61–4.56 (m, 1H), 4.35–4.29 (t, J = 7.4, 1H), 4.02–3.96 (t, J = 6.6,

1H), 3.36–3.28 (dd, $J = 5.9$, 14.7, 1H), 3.19–3.10 (dd, $J = 8.4$, 14.7, 1H), 2.88–2.77 (m, 2H), 1.99–1.87 (m, 2H), 1.04–0.98 (t, $J = 7.6$, 3H); ES-MS: mass calcd for $C_{25}H_{32}N_7O_4$ 494.2 (MH^+). Found *m/z* 494.

4.3.4. 1-(2*S*-Pyrrolidin-2-carbonyl)-4-{2*S*-*N*-[2*S*-3-(indol-3-yl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semicarbazide (6). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 16.2 mg (51%); HPLC (1): $t_R = 3.90$ min (>99%); HPLC (2): $t_R = 6.79$ min (>97%); ^{1}H NMR (DMSO-d_6/D_2O 10:1, 250 MHz): δ 7.72–7.69 (d, $J = 7.6$, 1H), 7.44–7.04 (m, 9H), 4.62–4.57 (m, 1H), 4.32–4.21 (m, 2H), 3.38–3.03 (m, 4H), 2.63–2.57 (1H, partially under DMSO signal), 2.43–2.36 (m, 1H), 2.08–1.80 (m, 6H); ES-MS: mass calcd for $C_{27}H_{34}N_7O_4$ 520.3 (MH^+). Found *m/z* 520.1.

4.3.5. 1-(2*S*-Piperidin-2-carbonyl)-4-{2*S*-*N*-[2*S*-3-(indol-3-yl)propan-2-yl-amide]-3-phenylpropan-2-yl-amide}semicarbazide (7). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 13.7 mg (43%); HPLC (1): $t_R = 3.58$ min (>99%); HPLC (2): $t_R = 6.47$ min (>97%); ^{1}H NMR (DMSO-d_6/D_2O 10:1, 250 MHz): δ 8.16–8.12 (d, $J = 8.1$, 1H), 7.61–7.58 (d, $J = 7.6$, 1H), 7.34–7.31 (d, $J = 7.9$, 1H), 7.23–6.96 (m, 7H), 4.51–4.34 (m, 2H), 3.75–3.59 (1H, partially under water signal), 3.23–2.70 (m, 6H), 2.08–1.21 (m, 6H); ES-MS: mass calcd for $C_{27}H_{34}N_7O_4$ 520.3 (MH^+). Found *m/z* 520.1.

4.3.6. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(*m*-fluorophenyl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semicarbazide (8). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 21.6 mg (54%); HPLC (1): $t_R = 4.17$ min (>99%); HPLC (2): $t_R = 6.95$ min (>97%); ^{1}H NMR (D_2O, 250 MHz): δ 7.22–6.72 (m, 9H), 4.53–4.45 (m, 1H), 3.92–3.81 (m, 2H), 3.15–3.07 (dd, $J = 5.3$, 14.4, 1H), 2.85–2.75 (dd, $J = 10.4$, 14.4, 1H), 2.32–2.26 (t, $J = 7.8$, 2H), 1.88–1.59 (m, 4H), 0.92–0.81 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{24}H_{32}FN_6O_4$ 487.24 (MH^+). Found *m/z* 487.2. Anal. ($C_{24}H_{31}FN_6O_4 \cdot 1H_2O \cdot 1CF_3COOH$) C, H, N: Calcd 50.48, 5.54, 13.59; found, 50.59, 5.36, 13.42.

4.3.7. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(indol-3-yl)-propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semicarbazide (9). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 14 mg (28%); HPLC (1): $t_R = 4.03$ min (>98%); HPLC (2): $t_R = 7.11$ min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.74–7.55 (d, $J = 7.0$, 1H), 7.33–7.00 (m, 8H), 4.79–4.70 (m, 1H), 4.17–4.12 (dd, $J = 5.3$, 8.2, 1H), 3.88–3.83 (t, $J = 6.6$, 1H), 3.43–3.32 (m, 1H, partially hidden under methanol signal), 3.22–3.12 (dd, $J = 8.6$, 14.7, 1H), 2.58–2.49 (t, $J = 8.5$, 2H), 2.05–1.69 (m, 4H), 1.14–1.08 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{26}H_{34}N_7O_4$ 508.3 (MH^+). Found *m/z* 508.5.

4.3.8. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(*p*-chlorophenyl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semicarbazide (10). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 8 mg (15%); HPLC (1): $t_R = 4.35$ min (>99%); HPLC (2): $t_R = 7.37$ min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.47–7.24 (m, 9H), 4.86–4.79 (dd, $J = 5.3$, 9.5, 1H), 4.35–4.27 (dd, $J = 5.4$, 8.1, 1H), 4.04–3.96 (app t, $J = 6.5$, 1H), 3.45–3.36 (dd, $J = 5.3$, 14.0, 1H), 3.15–3.06 (dd, $J = 9.6$, 14.0, 1H), 2.78–2.66 (t, $J = 8.4$, 2H), 2.19–1.95 (m, 4H), 1.30–1.20 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{24}H_{32}ClN_6O_4$ 503.2 (MH^+). Found *m/z* 503.4.

4.3.9. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(*m*-methoxyphenyl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}-semicarbazide (11). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 23 mg (46%); HPLC (1): $t_R = 4.03$ min (>99%); HPLC (2): $t_R = 6.87$ min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.51–7.30 (m, 6H), 7.04–7.02 (m, 2H), 6.92–6.88 (m, 1H), 4.88–4.82 (dd, $J = 5.1$, 9.7, 1H), 4.32–4.27 (dd, $J = 5.3$, 7.9, 1H), 4.05–4.00 (app t, $J = 6.5$, 1H), 3.89 (s, 3H), 3.15–3.05 (dd, $J = 5.1$, 13.9, 1H), 2.77–2.63 (dd, $J = 9.7$, 13.9, 1H), 2.20–1.95 (m, 4H), 1.31–1.20 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{25}H_{35}N_6O_5$ 499.3 (MH^+). Found *m/z* 499.4.

4.3.10. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(benzo[*b*]-thiophen-3-yl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}-semicarbazide (12). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 8 mg (16%); HPLC (1): $t_R = 4.48$ min (>99%); HPLC (2): $t_R = 7.52$ min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.99–7.92 (d, $J = 7.9$, 1H), 7.86–7.83 (d, $J = 7.6$, 1H), 7.45–7.11 (m, 8H), 4.91–4.72 (m, partially under water signal, 1H), 4.15–4.09 (dd, $J = 5.3$, 8.1, 1H), 3.88–3.83 (app t, $J = 6.5$, 1H), 3.62–3.47 (dd, $J = 5.3$, 14.8, 1H), 3.35–3.19 (m, partially under methanol signal, 1H), 2.56–2.46 (t, $J = 8.3$, 2H), 2.06–1.71 (m, 4H), 1.14–1.03 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{26}H_{33}N_6O_4S$ 525.2 (MH^+). Found *m/z* 525.4.

4.3.11. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(2-naphthyl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semicarbazide (13). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 5 mg (10%); HPLC (1): $t_R = 4.50$ min (>98%); HPLC (2): $t_R = 7.61$ min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.85–7.74 (m, 4H), 7.46–7.37 (m, 3H), 7.23–6.90 (m, 5H), 4.91–4.77 (m, partially under water signal, 1H), 4.12–4.07 (dd, $J = 5.6$, 7.9, 1H), 3.87–3.82 (app t, $J = 6.6$, 1H), 3.49–3.41 (dd, $J = 5.1$, 13.8, 1H), 3.19–3.09 (dd, $J = 9.7$, 14.0, 1H), 2.46–2.40 (t, $J = 8.1$, 2H), 2.02–1.70 (m, 4H), 1.14–1.01 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{28}H_{35}N_6O_4$ 519.3 (MH^+). Found *m/z* 519.5.

4.3.12. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(*m*-methoxyphenyl)propan-2-yl-amide]-3-(*p*-chlorophenyl)propan-2-yl-amide}semicarbazide (14). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 12 mg (23%); HPLC (1): $t_R = 4.14$ min (>96%); HPLC (2): $t_R = 7.31$ min (>97%); ^{1}H NMR (CD_3OD, 250 MHz; one signal hidden under water peak): δ 7.41–7.18 (m, 5H), 6.98–6.89 (m, 4H), 4.78–4.70 (m, 1H), 4.62–4.44 (dd, $J = 5.6$, 7.8, 1H), 3.33–

2.91 (m, 4H), 2.17–1.88 (m, 2H), 1.29–1.18 (t, $J = 7.6$, 3H); ES-MS: mass calcd for $C_{24}H_{32}ClN_6O_5$ 519.2 (MH^+). Found *m/z* 519.4.

4.3.13. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(1,1′-biphenyl-4-yl)propan-2-yl-amide]-3-(*p*-hydroxyphenyl)propan-2-yl-amide}semicarbazide (15). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 27 mg (49%); HPLC (1): $t_R = 4.16$ min (>98%); HPLC (2): $t_R = 7.06$ min (>99%); ^1H NMR (CD_3OD, 250 MHz): δ 7.50–7.40 (m, 4H), 7.34–7.16 (m, 5H), 6.89–6.86 (d, $J = 8.5$, 2H), 6.60–6.54 (d, $J = 8.5$, 2H), 4.56–4.50 (dd, $J = 5.5$, 8.6, 1H), 4.28–4.23 (dd, $J = 5.9$, 7.7, 1H), 3.70–3.64 (app t, $J = 6.6$, 1H), 3.16–3.08 (dd, $J = 5.6$, 13.9, 1H), 2.92–2.78 (m, 2H), 2.70–2.61 (dd, $J = 7.8$, 14.0, 1H), 1.90–1.69 (m, 2H), 1.02–0.86 (t, $J = 7.4$, 3H); ES-MS: mass calcd for $C_{29}H_{35}N_6O_5$ 547.3 (MH^+). Found *m/z* 547.5.

4.3.14. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(*m*-fluorophenyl)propan-2-yl-amide]-3-(indol-3-yl)propan-2-yl-amide}-semicarbazide (16). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 13 mg (25%); HPLC (1): $t_R = 3.97$ min (>99%); HPLC (2): $t_R = 7.02$ min (>99%); ^1H NMR (CD_3OD, 250 MHz): δ 7.73–7.70 (d, $J = 7.3$, 1H), 7.49–7.03 (m, 8H), 4.75–4.68 (m, 1H), 4.63–4.58 (t, $J = 7.0$, 1H), 3.93–3.88 (m, 1H), 3.35–3.17 (m, 3H), 3.04–2.95 (dd, $J = 8.4$, 13.8, 1H), 2.11–1.92 (m, 2H), 1.23–1.15 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{25}H_{31}FN_7O_4$ 512.3 (MH^+). Found *m/z* 512.4.

4.3.15. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(*m*-fluorophenyl)propan-2-yl-amide]-3-(2-naphthyl)propan-2-yl-amide}-semicarbazide (17). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 8 mg (15%); HPLC (1): $t_R = 4.39$ min (>96%); HPLC (2): $t_R = 7.44$ min (>98%); ^1H NMR (CD_3OD, 250 MHz): δ 7.89–7.68 (m, 4H), 7.50–7.24 (m, 4H), 7.16–6.92 (m, 3H), 4.68–4.62 (dd, $J = 5.7$, 8.8, 1H), 4.58–4.53 (dd, $J = 5.4$, 8.1, 1H), 3.81–3.76 (app t, $J = 6.6$, 1H), 3.28–3.17 (m, 2H), 3.12–2.92 (m, 2H), 2.02–1.78 (m, 2H), 1.08–1.02 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{27}H_{32}FN_6O_4$ 523.2 (MH^+). Found *m/z* 523.4.

4.3.16. 1-(2*S*-2-Aminobutanoyl)-4-{2*S*-*N*-[2*S*-3-(*m*-fluorophenyl)propan-2-yl-amide]-3-(1,1′-biphenyl-4-yl)propan-2-yl-amide}semicarbazide (18). Following the procedure for **3** with the appropriate starting materials. White solid. Yield: 5 mg (9%); HPLC (1): $t_R = 4.68$ min (>99%); HPLC (2): $t_R = 7.89$ min (>98%); ^1H NMR (CD_3OD, 250 MHz): δ 7.65–6.92 (m, 13H), 4.68–4.63 (dd, $J = 5.7$, 8.7, 1H), 4.51–4.46 (dd, $J = 5.7$, 7.9, 1H), 3.82–3.77 (app t, $J = 6.6$, 1H), 3.25–2.88 (m, 4H), 1.99–1.87 (m, 2H), 1.15–1.02 (t, $J = 7.5$, 3H); ES-MS: mass calcd for $C_{29}H_{34}FN_6O_4$ 549.3 (MH^+). Found *m/z* 549.4.

4.4. Synthesis of inhibitors 19–44

The compounds were prepared starting from the Boc-protected amino acid hydrazide derivatives via activation with CDI, followed by displacement with the appropriate amine, as described below.

4.5. N^{α}-Boc-L-alaninehydrazide

CDI (4.71 g, 29.1 mmol) was added slowly to a solution of N^{α}-Boc-L-alanine (5.0 g, 26.4 mmol) in 50 mL dry THF. After addition the solution was stirred for 30 min. Then, hydrazine monohydrate (3.85 mL, 79.3 mmol) was added and the resulting solution was stirred overnight. The reaction mixture was concentrated and the white solid was dissolved in 1,4-dioxane and lyophilized overnight. The crude product was suspended in diethyl ether, cooled to −18 °C and filtered. The filtrate was dried in vacuo overnight yielding 5.6 g of crude product. The solid was a mixture of imidazole and the desired product (imidazole–product 2:1, determined by ^1H NMR) and was used without further purification. Yield (estimated): 3.3 g (62%). ^1H NMR (DMSO-d_6 exchanged with D_2O, 250 MHz): δ 3.96–3.85 (m, 1H), 1.39–1.31 (s, 6H), 1.18–1.10 (d, $J = 7.2$, 3H).

4.6. N^{α}-Boc-L-aminobutyric acid hydrazide

CDI (1.76 g, 10.8 mmol) was added slowly to a solution of N^{α}-Boc-L-aminobutyric acid (2.0 g, 9.84 mmol) in 20 mL dry THF. After addition the solution was stirred for 1 h. Then, hydrazine monohydrate (1.44 mL, 29.5 mmol) was added and the resulting solution was stirred overnight. The reaction mixture was concentrated and the white solid re-dissolved in 1,4-dioxane and lyophilized overnight yielding 3.85 g of crude product. The solid was a mixture of imidazole and desired product (imidazole–product 2:1, determined by ^1H NMR) and was used without further purification. Yield (estimated): 2.14 g (100%). ^1H NMR (DMSO-d_6 250 MHz): δ 9.08–8.89 (s, 1H), 6.75–6.10 (d, $J = 8.2$, 1H), 3.79–3.64 (q, $J = 7.91$, 14.4, 1H), 1.61–1.34 (m, 2H), 1.34–1.18 (s, 9H), 0.81–0.63 (t, $J = 7.54$, 3H).

4.7. General procedure

CDI (1.1 equiv) was added to a solution of either N^{α}-Boc-L-alaninehydrazide or N^{α}-Boc-L-aminobutyric acid hydrazide (1 equiv) in dry THF (1 mL/0.24 mmol hydrazide). The resulting solution was stirred for minimum 2 h and the appropriate amine (1.1 equiv) dissolved in dry THF (0.5 mL/0.27 mmol amine) was added. The reaction mixture was stirred overnight and then concentrated. The residue was treated with 95% TFA/H_2O (1 mL/0.24 mmol hydrazide) for 5 min and concentrated followed by HPLC purification.

4.7.1. 1-(2*S*-2-Aminopropionyl)-4-benzylsemicarbazide (19). Yield: 23 mg (36%); HPLC (1): $t_R = 2.57$ min (>99%); HPLC (2): $t_R = 4.97$ min (95%); ^1H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.40–7.23 (m, 5H), 4.32 (s, 2H), 4.15–4.05 (q, $J = 7.2$, 1H), 1.55–1.49 (d, $J = 7.2$, 3H); ES-MS: mass calcd for $C_{11}H_{17}N_4O_2$ 237.1 (MH^+). Found *m/z* 237.

4.7.2. 1-(2*S*-2-Aminopropionyl)-4-(*o*-chlorobenzyl)semicarbazide (20). Yield: 24 mg (33%); HPLC (1): $t_R = 3.02$ min (>99%); ^1H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.23–7.01 (m, 4H), 4.18 (s, 2H), 3.94–3.84 (q, $J = 7.2$, 1H), 1.35–1.27 (d, $J = 7.2$, 3H);

ES-MS: mass calcd for $C_{11}H_{16}ClN_4O_2$ 271.1 (MH^+). Found *m/z* 271.0.

4.7.3. 1-(2*S*-2-Aminopropionyl)-4-(*m*-chlorobenzyl)semicarbazide (21). Yield: 47 mg (70%); HPLC (1): t_R = 3.17 min (>98%); ^{1}H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.31–7.21 (m, 3H), 7.18–7.11 (m, 1H), 4.29 (s, 2H), 4.16–4.05 (m, J = 7.2, 1H), 1.61–1.53 (d, J = 7.2, 3H); ES-MS: mass calcd for $C_{11}H_{16}ClN_4O_2$ 271.1 (MH^+). Found *m/z* 271.1.

4.7.4. 1-(2*S*-2-Aminopropionyl)-4-(*p*-chlorobenzyl)semicarbazide (22). Yield: 52 mg (71%); HPLC (1): t_R = 3.30 min (>97%); ^{1}H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.37–7.30 (m, 2H), 7.27–7.19 (m, 2H), 4.29 (s, 2H), 4.16–4.05 (q, J = 7.2, 1H), 1.55–1.49 (d, J = 7.2, 3H); ES-MS: mass calcd for $C_{11}H_{16}ClN_4O_2$ 271.1 (MH^+). Found *m/z* 271.1.

4.7.5. 1-(2*S*-2-Aminopropionyl)-4-(2-phenylethyl)semicarbazide (23). Yield: 45 mg (66%); HPLC (1): t_R = 3.11 min (>99%); HPLC (2): t_R = 5.35 min (96%); ^{1}H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.37–7.21 (m, 5H), 4.10–4.02 (q, J = 7.2, 1H), 3.40–3.31 (td, J = 6.9, 2.5, 2H), 2.79–2.72 (t, J = 6.9, 2H), 1.50–1.45 (d, J = 7.2, 3H); ES-MS: mass calcd for $C_{12}H_{19}N_4O_2$ 251.1 (MH^+). Found *m/z* 251.0.

4.7.6. 1-(2*S*-2-Aminopropionyl)-4-(1-naphthylmethyl)semicarbazide (24). Yield: 42 mg (55%); HPLC (1): t_R = 3.50 min (>97%); HPLC (2): t_R = 6.34 min (>99%); ^{1}H NMR (D_2O, 250 MHz): δ (rotamers observed) 8.03–7.81 (m, 3H), 7.63–7.40 (m, 4H), 4.77 (s, 2H), 4.13–4.03 (q, J = 7.2, 1H), 1.52–1.45 (d, J = 7.2, 3H); ES-MS: mass calcd for $C_{15}H_{19}N_4O_2$ 287.1 (MH^+). Found *m/z* 287.0.

4.7.7. 1-(2*S*-2-Aminobutanoyl)-4-benzylsemicarbazide (25). Yield: 34.8 mg (60%); HPLC (1): t_R = 2.56 min (>99%); HPLC (2): t_R = 4.82 min (96%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.31–7.21 (m, 5H), 4.36 (s, 2H), 3.85–3.80 (t, J = 6.6, 1H), 1.99–1.86 (m, 2H), 1.09–1.03 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{12}H_{19}N_4O_2$ 251.1 (MH^+). Found *m/z* 251.3.

4.7.8. 1-(2*S*-2-Aminobutanoyl)-4-(*o*-chlorobenzyl)semicarbazide (26). Yield: 9.4 mg (13%); HPLC (1): t_R = 3.39 min (>99%); HPLC (2): t_R = 5.76 min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.41–7.28 (m, 2H), 7.26–7.13 (m, 2H), 4.41 (s, 2H), 3.80–3.71 (t, J = 6.6, 1H), 1.96–1.79 (m, 2H), 1.07–0.95 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{12}H_{18}ClN_4O_2$ 285.1 (MH^+). Found *m/z* 285.1.

4.7.9. 1-(2*S*-2-Aminobutanoyl)-4-methyl-4-(*o*-chlorobenzyl)semicarbazide (27). Yield: 52 mg (75%); HPLC (1): t_R = 3.28 min (>99%); HPLC (2): t_R = 6.15 min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.42–7.37 (m, 1H), 7.32–7.23 (m, 3H), 4.65 (s, 2H), 3.87–3.81 (t, J = 6.6, 1H), 2.96 (s, 3H), 2.00–1.87 (m, 2H), 1.11–1.05 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{13}H_{20}ClN_4O_2$ 299.1 (MH^+). Found *m/z* 299.3.

4.7.10. 1-(2*S*-2-Aminobutanoyl)-4-(*o*-bromobenzyl)semicarbazide (28). Yield: 49 mg (65%); HPLC (1): t_R = 3.12 min (>99%); HPLC (2): t_R = 5.99 min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.57–7.53 (m, 1H), 7.43–7.28 (m, 2H), 7.19–7.12 (m, 1H), 4.42 (s, 2H), 3.86–3.81 (t, J = 6.6, 1H), 2.03–1.86 (m, 2H), 1.09–1.03 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{12}H_{18}BrN_4O_2$ 329.1 (MH^+). Found *m/z* 329.1.

4.7.11. 1-(2*S*-2-Aminobutanoyl)-4-(*o*-nitrobenzyl)semicarbazide (29). Yield: 26 mg (37%); HPLC (1): t_R = 2.80 min (>96%); HPLC (2): t_R = 5.34 min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ 8.06–8.03 (m, 1H), 7.68–7.66 (m, 2H), 7.54–7.45 (m, 1H), 4.67 (s, 2H), 3.85–3.80 (t, J = 6.6, 1H), 1.99–1.86 (m, 2H), 1.09–1.03 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{12}H_{18}N_5O_4$ 296.1 (MH^+). Found *m/z* 296.3.

4.7.12. 1-(2*S*-2-Aminobutanoyl)-4-(*o*-aminobenzyl)semicarbazide (30). This compound was an intermediate in the synthesis of **S44**–**S57** (Table S3) and was unstable to storage. Yield: 15 mg (69%); ES-MS: mass calcd for $C_{12}H_{20}N_5O_2$ 266.2 (MH^+). Found *m/z* 266.4.

4.7.13. 1-(2*S*-2-Aminobutanoyl)-4-(*o*-methylthiobenzyl)-semicarbazide (31). Yield: 20 mg (29%); HPLC (1): t_R = 3.27 min (99>%); HPLC (2): t_R = 5.91 min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.34–7.22 (m, 3H), 7.17–7.10 (m, 1H), 4.42 (s, 2H), 3.84–3.79 (t, J = 6.5, 1H), 1.99–1.86 (m, 2H), 1.09–1.03 (t, J = 7.4, 3H); ES-MS: mass calcd for $C_{13}H_{21}N_4O_2S$ 297.1 (MH^+). Found *m/z* 297.2.

4.7.14. 1-(2*S*-2-Aminobutanoyl)-4-(*o*-ethoxybenzyl)semicarbazide (32). Yield: 28 mg (41%); HPLC (1): t_R = 3.27 min (>99%); HPLC (2): t_R = 6.10 min (>98%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.27–7.17 (m, 2H), 6.93–6.83 (m, 2H), 4.36 (s, 2H), 4.12–4.03 (q, J = 6.95, 2H), 3.84–3.79 (t, J = 6.5, 1H), 2.01–1.80 (m, 2H), 1.46–1.40 (t, J = 7.0, 3H), 1.09–1.03 (t, J = 7.6, 3H); ES-MS: mass calcd for $C_{14}H_{23}N_4O_3$ 295.2 (MH^+). Found *m/z* 295.2.

4.7.15. 1-(2*S*-2-Aminobutanoyl)-4-[*o*-(*N*-morpholinyl)benzyl]-semicarbazide (33). Yield: 32 mg (41%); HPLC (1): t_R = 2.55 min (>99%); HPLC (2): t_R = 4.75 min (97%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.52–7.30 (m, 4H), 4.49 (s, 2H), 4.03–3.99 (m, 4H), 3.87–3.82 (t, J = 6.6, 1H), 3.39–3.30 (m, 4H), 2.03–1.86 (m, 2H), 1.10–1.04 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{16}H_{26}N_5O_3$ 336.2 (MH^+). Found *m/z* 336.3.

4.7.16. 1-(2*S*-2-Aminobutanoyl)-4-[*o*-(*N*-piperidinyl)benzyl]semicarbazide (34). Yield: 7 mg (9%); HPLC (1): t_R = 2.71 min (>96%); HPLC (2): t_R = 4.75 min (95%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.77–7.52 (m, 4H), 4.43 (s, 2H), 3.87–3.82 (t, J = 6.5, 1H), 3.70–3.57 (m, 4H), 2.21–2.02 (m, 4H), 1.99–1.71 (m, 4H), 1.11–1.05 (t, J = 7.4, 3H); ES-MS: mass calcd for $C_{17}H_{28}N_5O_2$ 334.2 (MH^+). Found *m/z* 334.2.

4.7.17. 1-(2*S*-2-Aminobutanoyl)-4-[(1,1′-biphenyl-2-yl)-methyl]semicarbazide (35). Yield: 63 mg (83%); HPLC: t_R = 3.97 min (>97%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.50–7.19 (m, 9H), 4.28 (s, 2H), 3.82–3.77 (t, J = 6.6, 1H), 2.01–1.85 (m, 2H), 1.09–1.03 (t, J = 7.4, 3H); ES-MS: mass calcd for $C_{18}H_{23}N_4O_2$ 327.2 (MH^+). Found m/z 327.4.

4.7.18. 1-(2*S*-2-Aminobutanoyl)-4-[*o*-(*o*-hydroxymethyl-phenylthio)benzyl]semicarbazide (36). Yield: 25 mg (28%); HPLC (1): t_R = 3.66 min (>98%); HPLC (2): t_R = 6.63 min (>98%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.57–7.07 (m, 8H), 4.72 (s, 2H), 4.46 (s, 2H), 3.85–3.79 (t, J = 6.6, 1H), 1.99–1.86 (m, 2H), 1.09–1.03 (t, J = 7.4, 3H); ES-MS: mass calcd for $C_{19}H_{25}N_4O_3S$ 389.2 (MH^+). Found m/z 389.1.

4.7.19. 1-(2*S*-2-Aminobutanoyl)-4-[(1,1′-biphenyl-4-yl)-methyl]semicarbazide (37). Yield: 34 mg (45%); HPLC (1): t_R = 3.86 min (>99%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.60–7.55 (m, 4H), 7.45–7.29 (m, 5H), 4.41 (s, 2H), 3.86–3.81 (t, J = 6.5, 1H), 2.00–1.87 (m, 2H), 1.10–1.04 (t, J = 7.4, 3H); ES-MS: mass calcd for $C_{18}H_{23}N_4O_2$ 327.2 (MH^+). Found m/z 327.2.

4.7.20. 1-(2*S*-2-Aminobutanoyl)-4-[*p*-(thiophen-2-yl)benzyl]semicarbazide (38). Yield: 29 mg (37%); HPLC (1): t_R = 3.92 min (>99%); HPLC (2): t_R = 6.79 min (98%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.60–7.54 (m, 2H), 7.35–7.31 (m, 4H), 7.08–7.05 (m, 1H), 4.38 (s, 2H), 3.85–3.80 (t, J = 6.6, 1H), 2.00–1.86 (m, 2H), 1.10–1.04 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{16}H_{21}N_4O_2S$ 333.1 (MH^+). Found m/z 333.2.

4.7.21. 1-(2*S*-2-Aminobutanoyl)-4-(2-chloro-6-fluorobenzyl)semicarbazide (39). Yield: 14 mg (20%); HPLC (1): t_R = 3.03 min (>99%); HPLC (2): t_R = 5.69 min (98%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.37–7.24 (m, 2H), 7.13–7.06 (m, 1H), 4.56 (s, 2H), 3.81–3.76 (t, J = 6.5, 1H), 1.98–1.84 (m, 2H), 1.09–1.03 (t, J = 7.6, 3H); ES-MS: mass calcd for $C_{12}H_{17}ClFN_4O_2$ 303.1 (MH^+). Found m/z 303.0.

4.7.22. 1-(2*S*-2-Aminobutanoyl)-4-(2,6-difluorobenzyl)-semicarbazide (40). Yield: 13 mg (19%); HPLC (1): t_R = 2.74 min (>99%); ^{1}H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.44–7.32 (m, 1H), 7.08–6.97 (m, 2H), 4.45 (s, 2H), 4.04–3.99 (t, J = 6.6, 1H), 2.02–1.90 (m, 2H), 1.06–1.00 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{12}H_{17}F_2N_4O_2$ 287.1 (MH^+). Found m/z 287.2.

4.7.23. 1-(2*S*-2-Aminobutanoyl)-4-(2,4-dichlorobenzyl)-semicarbazide (41). Yield: 26 mg (36%); HPLC (1): t_R = 3.50 min (>99%); HPLC (2): t_R = 6.51 min (95%); ^{1}H NMR (D_2O, 250 MHz): δ (rotamers observed) 7.51–7.50 (m, 1H), 7.34–7.32 (m, 2H), 4.41 (s, 2H), 4.07–4.01 (t, J = 6.6, 1H), 2.04–1.92 (m, 2H), 1.07–1.01 (t, J = 7.6, 3H); ES-MS: mass calcd for $C_{12}H_{17}Cl_2N_4O_2$ 319.1 (MH^+). Found m/z 319.1.

4.7.24. 1-(2*S*-2-Aminobutanoyl)-4-(1-naphthylmethyl)semicarbazide (42). Yield: 32 mg (46%); HPLC (1): t_R = 3.53 min (>99%); HPLC (2): t_R = 6.49 min (>95%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 8.10–8.06 (m, 1H), 7.90–7.78 (m, 2H), 7.54–7.39 (m, 4H), 4.84 (partially hidden under H_2O, 2H), 3.85–3.79 (t, J = 6.6, 1H), 1.99–1.86 (m, 2H), 1.09–1.02 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{16}H_{21}N_4O_2$ 301.2 (MH^+). Found m/z 301.3.

4.7.25. 1-(2*S*-2-Aminohexanoyl)-4-(*o*-chlorobenzyl)semicarbazide (43). CDI (45 mg, 0.28 mmol) was added slowly to a solution of N^{α}-Boc-L-aminocaproic acid (58 mg, 0.25 mmol) in 1.0 mL dry THF. After addition the solution was stirred for 2 h. Then, hydrazine monohydrate (13.4 μL, 0.28 mmol) was added and the resulting solution was stirred overnight. CDI (45 mg, 0.28 mmol) was added and the solution was stirred for 6 h. *o*-Chlorobenzylamine (27 μL, 0.23 mmol) was added and the reaction mixture was stirred overnight, and concentrated. The residue was treated with 1 mL 95% TFA/H_2O for 2 h, and concentrated followed by HPLC purification. Yield: 30 mg (38%); HPLC (1): t_R = 3.46 min (>99%); HPLC (2): t_R = 6.44 min (97%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamers observed) 7.44–7.33 (m, 2H), 7.31–7.21 (m, 2H), 4.46 (s, 2H), 3.88–3.82 (t, J = 6.9, 1H), 1.96–1.79 (m, 2H), 1.47–1.36 (m, 4H), 0.99–0.92 (t, J = 7.22, 3H); ES-MS: mass calcd for $C_{14}H_{22}ClN_4O_2$ 313.1 (MH^+). Found m/z 313.1.

4.7.26. 1-(2*S*-2-Amino-3-phenyl-propionyl)-4-(*o*-chlorobenzyl)semicarbazide (44). CDI (45 mg, 0.28 mmol) was added slowly to a solution of N^{α}-Boc-L-phenylalanine (67 mg, 0.25 mmol) in 1.0 mL dry THF. After addition the solution was stirred for 2 h. Then, hydrazine monohydrate (13.4 μL, 0.28 mmol) was added and the resulting solution was stirred overnight. CDI (45 mg, 0.28 mmol) was added and the solution was stirred for 6 h. 2-Chlorobenzylamine (27 μL, 0.23 mmol) was added and the reaction mixture was stirred overnight, and concentrated. The residue was treated with 1 mL 95% TFA/H_2O for 2 h, and concentrated followed by HPLC purification. Yield: 15.6 mg (18%); HPLC (1): t_R = 3.71 min (>97%); HPLC (2): t_R = 6.85 min (95%); ^{1}H NMR (CD_3OD, 250 MHz): δ (rotamerism observed) 7.38–7.14 (m, 9H), 4.41 (s, 2H), 3.80–3.61 (m, 3H); ES-MS: mass calcd for $C_{17}H_{20}ClN_4O_2$ 347.1 (MH^+). Found m/z 347.1.

4.7.27. 1-(2*S*-2-Aminobutanoyl)-2-benzyl-4-(*o*-chlorobenzyl)semicarbazide (45). (a) Preparation of 1-Fmoc-2-benzylhydrazine (**I**, following a procedure for 1-Fmoc-2-methylhydrazine[31]): Benzylhydrazine dihydrochloride (2 g, 10.25 mmol) and DIPEA (3.5 mL, 20.5 mmol) were dissolved in dry $CHCl_3$ (10 mL). This solution was added in a single portion to a stirring solution of Boc_2O in dry $CHCl_3$ (10 mL) at −78 °C. The reaction was allowed to come to rt and then stirred for 2 h. Fmoc–Cl (2.7 g, 10.25 mmol) was then added in a single portion and DIPEA (1.8 mL, 10.25 mmol) was added dropwise. The resulting solution was stirred at rt for 6 h and then TFA–H_2O 95:5 (15 mL) was added carefully. After 2 h the bright yellow solution was taken to dryness. The residue was redissolved in EtOAc (100 mL), washed carefully with satd $NaHCO_3$ (×3) and brine (×1) and dried

($MgSO_4$). Evaporation gave a yellowish solid, which was purified on silica, using PE–EtOAc 2:1. The resulting off-white solid (3 g) was dissolved in hot EtOAc (20 mL), then cooled slowly to rt and finally kept at 5 °C overnight. The precipitate was washed with cold EtOAc and dried to give a white solid. Yield 1.2 g (34%). The filtrate was concentrated to dryness and then heated briefly in DE (20 mL). Upon cooling, wash with DE and drying a further 0.4 g white solid could be isolated having the same purity as the first crop. Combined yield: 1.6 g (46%). HPLC (1): t_R = 5.39 (>98%); ^{1}H NMR (DMSO-d_6 250 MHz): δ 8.73 (br s, 1H), 7.90–7.87 (d, J = 7.4, 2H), 7.71–7.68 (d, J = 7.3, 2H), 7.45–7.21 (m, 9 H), 4.95 (br s, 1H), 4.35–4.33 (d, J = 6.5, 2H), 4.25–4.19 (t, J = 6.5, 1H), 3.91 (s, 2H); ^{13}C NMR (DMSO-d_6 250 MHz): δ 156.8, 143.7, 140.7, 138.6, 128.4, 128.0, 127.6, 127.0, 126.8, 125.2, 120.0, 65. 4, 54.2, 46.7; ES-MS: mass calcd for $C_{22}H_{21}N_2O_2$ 345.15 (MH^+). Found *m*/*z* 345.21.

(b) Preparation of 1-(2*S*-2-Aminobutanoyl)-2-benzyl-4-(*o*-chlorobenzyl)semicarbazide (**45**): 1-Fmoc-2-benzylhydrazine (**I**, 100 mg, 0.29 mmol) was dissolved in dry THF (1 mL). Phosgene (1.9 M solution in toluene, 300 μL, 0.58 mmol) was added at 0 °C and the reaction mixture was then cooled to rt and stirred for 16 h. Evaporation to dryness gave crude 2-(chlorocarbonyl)-1-Fmoc-2-benzylhydrazine as a clear oil. ES-MS: mass calcd for $C_{23}H_{19}ClN_2O_3Na$ 429.11 (MNa^+). Found *m*/*z* 429.1. The crude product was dissolved in dry CH_3CN (1 mL) and DIPEA (50 μL, 0.29 mmol) and 2-chlorobenzylamine (39 μL, 0.32 mmol) was added. After 10 min the reaction mixture had turned nearly solid and a further 1.5 mL of CH_3CN was added. After 3 h LC–MS showed complete conversion to **II** (*m*/*z* 512.1; MH^+ for $C_{30}H_{27}ClN_3O_3$). Piperidine (86 μL, 0.87 mmol) was added and the resulting bluish-green solution was stirred at rt overnight. The now brownish reaction mixture was split into two equal portions. To one portion was added $(Boc\text{-}Abu)_2O$ (formed from Boc-Abu-OH (177 mg, 0.87 mmol) and DIC (68 μL, 0.44 mmol) in 1 mL dry THF; reaction time 20 min). After 3 days the mixture was treated with TFA–H_2O 95:5 (5 mL) for 1 h and evaporated to dryness. The dark residue was purified by HPLC to give the title compound as a white solid. Yield: 4.6 mg (9%); HPLC (1): t_R = 4.03 min (>99%); HPLC (2): t_R = 6.92 min (>95%); ^{1}H NMR (CD_3OD, 250 MHz): δ 7.58–7.37 (m, 9H), 5.15–5.00 (m, 1H; partially hidden under water signal), 4.74–4.60 (m, 3H), 3.93–3.88 (app t, 1H), 2.03–1.79 (m, 2H), 1.50–1.46 (t, J = 7.5, 3H); ES-MS: mass calcd for $C_{19}H_{23}ClN_4O_2$ 375.2 (MH^+). Found *m*/*z* 375.2.

4.7.28. 1,1-(2*S*-1,4-*cyclo*-Aminobutanoyl)-4-(*o*-chlorobenzyl)semicarbazide (46). (a) Preparation of Cbz-L-Met-NHNHBoc: Cbz-L-methionine (5 g, 17.65 mmol) was dissolved in dry THF (20 mL) and cooled in an ice bath. CDI (3.15 g, 19.42 mmol) was added in one portion, resulting in vigorous evolution of gas. After 20 min $BocNHNH_2$ (2.57 g, 19.42 mmol) was added and the resulting solution was stirred for a period of 20 h, at which time it was poured into water (150 mL) and extracted with EtOAc (×3). The combined solvents were washed once with satd aq $NaHCO_3$, 10% aq citric acid and brine. Drying over $MgSO_4$ followed by evaporation gave an oil, which was dried in vacuo to give a white solid. Yield: 6.75 g (96%); HPLC (1): t_R = 5.00 (>99%); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 9.00 (br, 1H), 7.29 (m, 5H), 7.27 (br, 1H), 6.15–6.11 (d, J = 8.4, 1H), 5.12–4.98 (m, 2H), 4.45–4.40 (m, 1H), 2.57–2.52 (t, J = 7.1, 2H), 2.13–1.89 (m, 5H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$, 250 MHz): δ 171.7, 156.7, 155.7, 136.5, 128.9, 128.5, 128.4, 81.9, 67.5, 52.8, 32.3, 30.3, 28.5, 15.6; ES-MS: mass calcd for $C_{18}H_{28}N_3O_5S$ 398.2 (MH^+). Found *m*/*z* 398.1.

(b) Preparation of Cbz-L-Met-NHNHBoc methyl sulfonium iodide (**III**): Cbz-L-Met-NHNHBoc (6.65 g, 16.73 mmol) was stirred in methyl iodide (25 mL) at rt for a period of 72 h. DCM (50 mL) was added and the yellow solution was concentrated to give a yellow oil, which was dried in vacuo to give the sulfonium salt as a yellow solid. Yield: 9.1 g (100%); HPLC (1): t_R = 3.86 (>99%); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 9.33 (br, 1H), 7.27–7.19 (m, 5H), 6.93 (br, 1H), 6.38–6.36 (d, J = 7.1, 1H), 5.01 (s, 2H), 4.61–4.59 (m, 1H), 3.75–3.58 (m, 2H), 3.08 (s, 3H), 2.99 (s, 3H), 2.45–2.30 (m, 2H), 1.34 (s, 9H); ^{13}C NMR ($CDCl_3$, 250 MHz): δ 171.1, 156.9, 155.8, 136.5, 129.0, 128.6, 128.4, 82.0, 67.7, 52.3, 40.3, 28.7, 26.8, 26.4; ES-MS: mass calcd for $C_{19}H_{30}N_3O_5S$ 412.2 (M^+). Found *m*/*z* 412.1.

(c) Preparation of 3(*S*)-benzyloxycarbonylamino-1-*tert*-butoxycarbonylaminopyrrolidin-2-one (**IV**): Cbz-L-Met-NHNHBoc methyl sulfonium iodide (**III**, 9 g, 16.68 mmol) was dissolved in dry DCM–DMF 1:1 (338 mL). The resulting yellow solution was cooled in an ice bath under Ar and NaH (60% suspension in mineral oil; 1344 mg, 34 mmol) was added in one portion. The solution was stirred at 0 °C for 3 h, during which time the colour changed from yellow to various shades of red. The reaction was quenched by addition of EtOAc (115 mL) and water (27 mL), and then stirred overnight at room temperature. The volume was concentrated to ca. 100 mL, diluted with water (300 mL) and extracted with EtOAc (×3). The combined organics were washed with brine, dried over $MgSO_4$ and concentrated to give the crude product as a semisolid residue (6 g after drying). This was dissolved in hot EtOAc–DE 1:2 (60 mL), then cooled slowly to rt and finally to −18 °C. After 1 h a further 10 mL DE and 20 mL *n*-heptane were added portionwise to the cold solution. Upon storage at −18 °C overnight a white solid was isolated, washed with *n*-heptane (×3) and dried. Yield: 2.9 g (50%); HPLC (1): t_R = 4.68 (>86%)/4.97 (>12%) (isobaric peaks); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.28–7.19 (m, 5H), 6.81 (s, 1H), 5.51–5.49 (d, J = 7.1, 1H), 5.03 (s, 2H), 4.30–4.20 (m, 1H), 3.58–3.37 (m, 2H), 2.58–2.47 (m, 1H), 2.02–1.70 (m, 1H), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$, 250 MHz): δ 172.1, 156.8, 154.7, 136.7, 128.9, 128.5, 128.4, 82.2, 67.3, 51.2, 46.3, 28.5, 26.4; ES-MS: mass calcd for $C_{17}H_{24}N_3O_5$ 350.2 (MH^+). Found *m*/*z* 350.1.

(d) Preparation of 3(*S*)-benzyloxycarbonylamino-1-aminopyrrolidin-2-one (**V**): 3(*S*)-Benzyloxycarbonyl-

amino-1-*tert*-butoxycarbonylaminopyrrolidin-2-one (**IV**, 100 mg, 0.286 mmol) was dissolved in HCl in dioxane (4 M, 2 mL) and left at rt for 18 h. The solution was evaporated to dryness and the crude residue was purified by preparative HPLC to give a white solid. Yield: 35 mg (49%); HPLC (1): t_R = 3.61 (>99%); ES-MS: mass calcd for $C_{12}H_{16}N_3O_3$ 250.1 (MH^+). Found *m*/*z* 250.1.

(e) Preparation of 1,1-(2*S*-1,4-*cyclo*-benzyloxycarbonyl-aminobutanoyl)-4-(*o*-chlorobenzyl)semicarbazide (**VI**): 3(*S*)-Benzyloxycarbonylamino-1-aminopyrrolidin-2-one (**V**, 35 mg, 0.14 mmol) and NEM (35 μL, 0.28 mmol) were dissolved in dry THF (1.5 mL) and CDI (25 mg, 0.154 mmol) was added. After a period of 21 h at rt *o*-chlorobenzylamine (19 μL, 0.154 mmol) was added. After 4.5 h the crude product was purified by preparative HPLC to give a white solid. Yield: 20 mg (34%); HPLC (1): t_R = 4.84 (>99%); ES-MS: mass calcd for $C_{20}H_{22}N_4O_4$ 417.1 (MH^+). Found *m*/*z* 417.0.

(f) Preparation of 1,1-(2*S*-1,4-*cyclo*-aminobutanoyl)-4-(*o*-chlorobenzyl)semicarbazide (**46**): 1,1-(2*S*-1,4-*cyclo*-benzyloxycarbonylaminobutanoyl)-4-(*o*-chlorobenzyl)-semicarbazide (**VI**, 19 mg, 46 μmol) was dissolved in glacial acetic acid (300 μL). HBr in acetic acid (5.7 M, 20 μL, 114 μmol) was added. After 22 h a further 20 μL HBr in acetic acid was added and yet another 20 μL after a further 3.5 h. Three hours later the crude product was purified by preparative HPLC to give a white solid. Yield: 10.7 mg (83%); HPLC (1): t_R = 3.34 (>99%); HPLC (2): t_R = 5.69 min (>99%); ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.56–7.34 (m, 4H), 4.59 (s, 2H), 4.29–4.21 (t, J = 9.1, 1H), 3.85–3.66 (m, 2H), 2.78–2.66 (m, 1H), 2.34–2.21 (m, 1H); ES-MS: mass calcd for $C_{12}H_{16}ClN_4O_2$ 283.1 (MH^+). Found *m*/*z* 283.1.

4.8. Enzymatic assays

All enzyme assays were performed at 37 °C in duplicate in 96-well plates. The reaction was monitored for 10 min. In the software (SOFTmax Pro) used for data collection from the fluorometer (Molecular Devices: Gemini EM) or the spectrophotometer (Molecular Devices: SPECTRAmax 190), it was ensured that the measured control slopes were linear ($R^2 > 0.99$). For K_m determinations, data were exported to GraphPad Prism and fitted to the Michaelis–Menten equation. For IC_{50} determinations, data were exported to GraphPad Prism and nonlinear regression were performed using the option Sigmoidal dose–response (variable slope). Data for K_i determinations were analyzed using the SigmaPlot Enzyme Kinetics Module (SPSS Science).

4.9. Recombinant human dipeptidyl peptidase I (DPPI) assay

Human DPPI was expressed and purified as described previously.[7] The enzyme was activated by incubation with an equal volume of 200 mM cysteamine at 37 °C for 5 min. Inhibitor and 150 μM substrate (Gly-Tyr(3′NO_2)-Gly-Pro-Pro-Lys(ε-Abz)-Gly, Schafer-N, Denmark) were incubated for 5 min at 37 °C in assay buffer (100 mM sodium phosphate pH 6.0, 150 mM KCl, 1.5 mM EDTA and 5 mM cysteamine). The reaction was initiated by the addition of 1 mU enzyme. Excitation: 320 nm, emission: 420 nm.

K_i Determination was performed using four different substrate concentrations (100, 150, 225 and 300 μM) in the absence or presence of **8** at five different concentrations: 10, 25, 50, 100 and 150 nM.

Reversibility studies were performed using 150 nM of **8** and compared with the reaction in absence of inhibitor, or in the presence of 20 μM of the irreversible inhibitor $GFCH_2N_2$. After 6000 s, substrate was added to a concentration of 1.5 mM ($10 \times K_m$).[33] The reaction was followed for additional 7000 s.

4.10. Human liver cathepsin B assay

The human liver cathepsin B (enzyme system products) assay was performed in assay buffer (0.1 M MES buffer pH 6.1, 1 mM EDTA, 5 mM DTT). Inhibitor and 1 ng enzyme were incubated for 5 min at 37 °C. The reaction was initiated by addition of the following substrate: Boc-Leu-Arg-Arg-AFC*2TFA (Enzyme System Products) to 600 μM. Excitation: 400 nm, emission: 505 nm.

4.11. Human cathepsin G assay

Assay buffer (100 mM Tris/HCl pH 8.3, 500 mM NaCl, 20 mM $CaCl_2$, 125 μM DTNB), 20 ng human cathepsin G (Calbiochem) and inhibitor were incubated for 10 min at 37 °C. The reaction was started by the addition of substrate (Suc-Ala-Ala-Pro-Phe-SBzl, Bachem) to 200 μM. The absorbance was monitored at 410 nm.

4.12. Human liver cathepsin H assay

Human liver cathepsin H (enzyme system products) was pre-incubated in the following buffer: 50 mM sodium phosphate buffer pH 6.0, 2 mM EDTA, 0.012% TX-100, 5 mM DTT, for 5 min on ice. The final assay was performed with inhibitor, 40 ng enzyme and 15 μM substrate (Ala-Arg-Gly-AFC*2HBr, enzyme system products). Excitation: 400 nm, emission: 505 nm.

4.13. Human liver cathepsin L assay

Human liver cathepsin L (enzyme system products) was activated for 5 min on ice in assay buffer (20 mM sodium acetate buffer pH 5.5, 4 mM EDTA, 0.012% TX-100, 5 mM DTT). The final assay was performed with inhibitor, 80 nU enzyme and 10 μM substrate (Z-Phe-ARG-AFC*TFA, enzyme system products). Excitation: 400 nm, emission: 505 nm.

4.14. *Aspergillus* DPPIV assay

Aspergillus DPPIV (0.04 mU, enzyme system products) was added to a 30 °C pre-heated assay buffer (50 mM Tris buffer pH 7.4, 0.1% Triton X-100) containing inhibitor. Substrate (Gly-Pro-AFC*TFA, enzyme system products) was added to 100 μM. The reaction was monitored at 30 °C. Excitation: 400 nm, emission: 505 nm.

4.15. Human neutrophil elastase assay

Human neutrophil elastase (Calbiochem) activity was measured in assay buffer (50 mM sodium acetate buffer pH 5.5, 200 mM NaCl). The assay was performed with inhibitor, 10 ng enzyme and 400 μM substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC, Calbiochem). Excitation: 380 nm, emission: 460 nm.

4.16. Tryptase assay

Recombinant human skin β-tryptase (Promega) was diluted in assay buffer (100 mM HEPES pH 7.5, 10% glycerol, 0.1 mg/mL heparin). Inhibitor and 0.4 ng enzyme were incubated for 10 min at 37 °C. Substrate (Tyr(3′NO_2)-Arg-Lys-Tyr-Arg-Met-Ala-Ser-Ala-Lys(ε-Abz), Schafer-N, Denmark) was added to 7.5 μM. Excitation: 320 nm, emission: 420 nm.

4.17. Cytochrome P450 assays

Vivid®CYP assay kits for CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 were purchased from invitrogen. Assays were performed according to the manufacturer's instructions.

4.18. Cellular assays

4.18.1. Cell culture. The RBL-2H3 (rat basophilic leukaemia cell line) and HepG2 (human epithelial hepacellular carcinoma) cells were obtained from the American Type Culture Collection (ATCC). RBL-2H3 cells were grown in RPMI Medium (GIBCO) and HepG2 cells were grown in minimum essential medium (MEM) α medium. Media for cell culture were supplemented with 10% heat-inactivated foetal bovine serum (FBS; GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO). Cells were cultured at 37 °C in humidified air atmosphere with 5% CO_2.

4.18.2. Cytotoxicity and viability assays. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) was set up by seeding 4×10^4HepG2 cells pr. well into a 96-well plate (Corning). After attachment of the cells, the compounds were added for a test period of 48 h. Cytotoxicity and cell viability were measured by detection of lactate dehydrogenase release, as described by the manufacturer's instruction. Measurement of fluorescence as a consequence of lactate dehydrogenase enzymic activity was performed at excitation/emission wavelengths 560/590 nm.

4.18.3. Cellular DPPI assay. All cell-based experiments employing inhibitors of DPPI were performed by addition of the compound for indicated time and concentrations to sub-confluent cell cultures growing on culture dishes. All experiments were set up in triplicates. Following the treatment, cells were washed twice in ice cold PBS and scraped from the culture dish. After another wash in PBS, cells were lysed in ice cold H_2O containing 0.3% Triton X-100. An equal volume of buffer containing 40 mM MOPS, 2 M NaCl, 2 mM EDTA and 20% of glycerol was added. The cell lysate was centrifugated at 3000 rpm for 10 min. The resulting supernatant was transferred to eppendorf tubes. The Coomassie Plus Protein Assay (Pierece) was used to determine protein levels of the samples.

4.18.4. Measurement of cellular DPPI activity. The activity of DPPI in cell lysates was performed in 100 mM sodium phosphate, 150 mM KCl, 1.5 mM EDTA (pH = 6) using 50 μM of Gly-Tyr(3′NO_2)-Gly-Pro-Pro-Lys(ε-Abz)-Gly (Schafer-N, Denmark) as a substrate. For each measurement, cell lysate corresponding to 50 μg of protein was used. All samples were prepared in duplicates.

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Supplementary data

Tables S1 (**S1**–**S6**), S2 (**S7**–**S43**) and S3 (**S44**–**S57**) and experimental details for **1**, **S1**–**S6** and **S44**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.04.048.

References and notes

1. Gutmann, H. R.; Fruton, J. S. *J. Biol. Chem.* **1948**, *174*, 851–858.
2. Ishidoh, K.; Muno, D.; Sato, N.; Kominami, E. *J. Biol. Chem.* **1991**, *266*, 16312–16317.
3. McGuire, M. J.; Lipsky, P. E.; Thiele, D. L. *Arch. Biochem. Biophys.* **1992**, *295*, 280–288.
4. Rao, N. V.; Rao, G. V.; Hoidal, J. R. *J. Biol. Chem.* **1997**, *272*, 10260–10265.
5. Pham, C. T. N.; Armstrong, R.; Zimonjic, D. B.; Popescu, N. C.; Payan, D. G.; Ley, T. J. *J. Biol. Chem.* **1997**, *272*, 10695–10703.
6. (a) Turk, D.; Janjic, V.; Stern, I.; Podobnik, M.; Lamba, D.; Dahl, S. W.; Lauritzen, C.; Pedersen, J.; Turk, V.; Turk, B. *EMBO J.* **2001**, *20*, 6570–6582; (b) Olsen, J. G.; Kadziola, A.; Lauritzen, C.; Pedersen, J.; Larsen, S.; Dahl, S. W. *FEBS Lett.* **2001**, *506*, 201–206.
7. Dahl, S. W.; Halkier, T.; Lauritzen, C.; Dolenc, I.; Pedersen, J.; Turk, V.; Turk, B. *Biochemistry* **2001**, *40*, 1671–1678.
8. Tran, T. V.; Ellis, K. A.; Kam, C.-M.; Hudig, D.; Powers, J. C. *Arch. Biochem. Biophys.* **2002**, *403*, 160–170.
9. McDonald, J. N.; Zeitman, B. B.; Reilly, T. J.; Ellis, S. *J. Biol. Chem.* **1969**, *244*, 2693–2709.
10. (a) Pham, C. T. N.; Ley, T. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8627–8632; (b) Sheth, P. D.; Pedersen, J.; Walls, A. F.; McEuen, A. R. *Biochem. Pharmacol.* **2003**, *66*, 2251–2262; (c) Wolters, P. J.; Pham, C. T. N.; Muilenberg, D. J.; Ley, T. J.; Caughey, G. H. *J. Biol. Chem.* **2001**, *276*, 18551–18556.
11. Adkinson, A. M.; Raptis, S. Z.; Kelley, D. G.; Pham, C. T. N. *J. Clin. Invest.* **2002**, *109*, 363–371.
12. Hart, P. S.; Zhang, Y.; Firatli, E.; Uygur, C.; Lotfazar, M.; Michalec, M. D.; Marks, J. J.; Lu, X.; Coates, B. J.; Seow, W. K.; Marshall, R.; Williams, D.; Reed, J. B.;

Wright, J. T.; Hart, T. C. *J. Med. Genet.* **2000**, *37*, 927–932.
13. Mallen, St.; Clair, J.; Pham, C. T. N.; Villalta, A.; Caughey, G. H.; Wolters, P. J. *J. Clin. Invest.* **2004**, *113*, 628–634.
14. (a) Thiele, D. L.; Lipsky, P. E.; McGuire, M. J. U.S. Patent 5,602,102, 1997; (b) Green, G. D. J.; Shaw, E. *J. Biol. Chem.* **1981**, *256*, 1923–1928.
15. Thompson, S. A.; Andrews, P. R.; Hanzlik, R. P. *J. Med. Chem.* **1986**, *29*, 104–111.
16. Kam, C.-M.; Götz, M. G.; Koot, G.; McGuire, M.; Thiele, D.; Hudig, D.; Powers, J. C. *Arch. Biochem. Biophys.* **2004**, *427*, 123–134.
17. Demuth, H.-U.; Heiser, U.; Niestroj, A. WO 03/022871 A2, 2003.
18. Horn, M.; Pavlik, M.; Doleckova, L.; Baudys, M.; Mares, M. *Eur. J. Biochem.* **2000**, *267*, 3330–3336.
19. Mucha, A.; Pawelczak, M.; Hurek, J.; Kafarski, P. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3113–3116.
20. Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. *Biochem. J.* **1982**, *201*, 189–198.
21. Gante, J. *Synthesis* **1989**, *38*, 405–413.
22. Zhang, R.; Durkin, J. P.; Windsor, W. T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1005–1008.
23. Bailey, M. D.; Halmos, T. H.; Goudreau, N.; Lescop, E.; Llinás-Brunet, M. *J. Med. Chem.* **2004**, *47*, 3788–3799.
24. Venkatraman, S.; Kong, J.-s.; Nimkar, S.; Wang, Q. M.; Aubé, J.; Hanzlik, R. P. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 570–580.
25. Wieczerzak, E.; Drabik, P.; Lankiewicz, L.; Oldziej, S.; Grzonka, Z.; Abrahamson, M.; Grubb, A.; Brömme, D. *J. Med. Chem.* **2002**, *45*, 4202–4211.
26. Xing, R.; Hanzlik, R. P. *J. Med. Chem.* **1998**, *41*, 1344–1351.
27. Dutta, A. S.; Giles, M. *J. Chem. Soc., Perkin Trans. 1* **1976**, 244–248.
28. Tornøe, C. W.; Sanderson, S. J.; Mottram, J. C.; Coombs, G. H.; Meldal, M. *J. Comb. Chem.* **2004**, *6*, 312–324.
29. Buchardt, J.; Schiødt, C. B.; Krog-Jensen, C.; Delaissé, J.-M.; Foged, N. T.; Meldal, M. *J. Comb. Chem.* **2000**, *2*, 624–638.
30. Graven, A.; Hilaire, P. M. St.; Sanderson, S. J.; Mottram, J. C.; Coombs, G. H.; Meldal, M. *J. Comb. Chem.* **2001**, *3*, 441–452.
31. Gibson, C.; Goodman, S. L.; Hahn, D.; Hõlzemann, G.; Kessler, H. *J. Org. Chem.* **1999**, *64*, 7388–7394.
32. Duffy, K. J.; Ridgers, L. H.; DesJarlais, R. L.; Tomaszek, T. A.; Bossard, M. J.; Thompson, S. K.; Keenan, R. M.; Veber, D. F. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1907–1910.
33. McGuire, M. J.; Lipsky, P. E.; Thiele, D. L. *J. Biol. Chem.* **1993**, *268*, 2458–2467.
34. Thornberry, N. A.; Peterson, E. P.; Zhao, J. J.; Howard, A. D.; Griffin, P. R.; Chapman, K. T. *Biochemistry* **1994**, *33*, 3934–3940.
35. Liu, Y.; Kati, W.; Chen, C.-M.; Tripathi, R.; Molla, A.; Kohlbrenner, W. *Anal. Biochem.* **1999**, *267*, 331–335.
36. Weber, A. E. *J. Med. Chem.* **2004**, *47*, 4135–4141.
37. Rendic, S. *Drug Metab. Rev.* **2002**, *34*, 83–448.
38. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 596–598.