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Synthesis and biological characterisation of sirtuin inhibitors based on the tenovins

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ABSTRACT

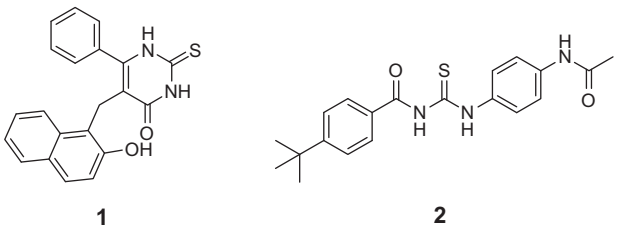
The tenovins are small molecule inhibitors of the NAD^+-dependent family of protein deacetylases known as the sirtuins. There remains considerable interest in inhibitors of this enzyme family due to possible applications in both cancer and neurodegenerative disease therapy. Through the synthesis of novel tenovin analogues, further insights into the structural requirements for activity against the sirtuins in vitro are provided. In addition, the activity of one of the analogues in cells led to an improved understanding of the function of SirT1 in cells.

1. Introduction

The human genome contains seven sirtuins (SirT1-7).[1] The major function of these enzymes is as NAD^+-dependent deacetylases of histone and non-histone substrates although some members of this protein family possess ADP ribosylase activity.[2] Recent studies have implicated the sirtuins in a range of important cellular processes and disease states. SirT1 has been implicated in the regulation of the transcriptional function of p53[3] and SirT2 has been linked with Parkinson's and Huntington's disease.[4] Initial interest in small molecule modulators of sirtuin function led to the identification of activators of potential relevance to the manipulation of cellular lifespan under conditions of calorie restriction.[5] More recently, there has been considerable interest in the development of sirtuin inhibitors and excellent review articles have been published that summarise the current situation.[6] Sirtuin inhibitors are interesting not only due to their potential use in therapeutic applications but also as chemical tools to help dissect the function of the various sirtuins.

Our contribution to date has focused on the two sirtuin inhibitor classes that possess activity in preclinical cancer models.[7a,8a] Modifications to the unselective inhibitor cambinol **1** (Fig. 1), originally discovered by Bedalov et al.,[7] have led to relatively active compounds that show either selective inhibition of SirT1 over SirT2 or vice versa.

In addition, we have reported the discovery of the tenovins.[8] A high-throughput screen designed to detect compounds that activate the tumour suppressor p53, led to the prioritisation of tenovin-1 (**2**) (Fig. 1) for target identification studies as **2** impaired the growth of BL2-derived tumour xenografts. However, the planned target identification studies proved difficult with **2** due to its limited water solubility. Here we describe the synthesis of a series of more water soluble analogues of **2** that, in general, retained the desired biological activity. One of these new



1 2

Figure 1. Structure of cambinol (**1**) and tenovin-1 (**2**).

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analogues, tenovin-6, was subsequently used to identify the sirtuins as an important target of this class of compounds.[8a] In addition, we report here the parallel synthesis of a range of tenovin-6 analogues enabling the determination of structure–activity relationships. Insights into the potential importance of an intramolecular hydrogen bond for the activity of this class of compounds were gained. Preliminary tenovin-6 metabolism studies are also reported. Studies with a potential metabolite of tenovin-6 led to an improved understanding of the function of SirT1 in cells.

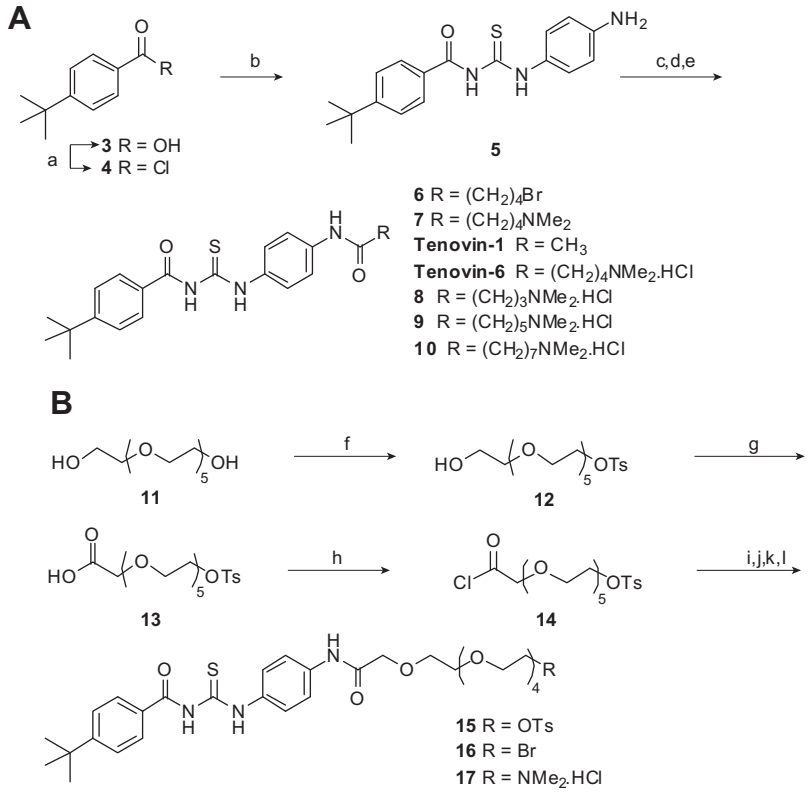
2. Results and discussion

2.1. Synthesis of more water soluble tenovin-1 analogues

Using a series of commercially available analogues, our previous studies showed that the *N*-acetyl group present in tenovin-1 could be modified without loss of activity (see tenovins-2, -4 and -5 in Ref. 8a) whereas replacement of the *tert*-butyl group in these specific cases led to a reduction in activity. It was therefore decided to prepare analogues of **2** containing an *N*-acyl group modified with a carbon linker of varying lengths and terminating in a tertiary amine (Scheme 1). Initially a 5-carbon (5C) linker was used in combination with a dimethylamino-group (tenovin-6). The synthesis of this analogue involved conversion of 4-*tert*-butyl benzoic acid (**3**) to the corresponding acid chloride **4** followed by reaction with sodium thiocyanate to generate an *N*-acyl-isothiocyanate which was reacted with 1,4-phenylenediamine to give the *N*-acyl thiourea **5**. Subsequent N-acylation of **5** with 5-bromovaleryl chloride, substitution of the bromine by dimethylamine and formation of the corresponding HCl salt gave the desired analogue, tenovin-6.

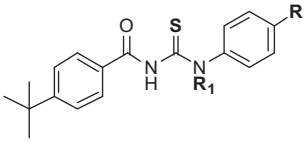
Tenovin-6 was tested in a cell-based p53 induction assay[8a] to assess the effect of the structural changes on activity in cells. MCF-7 cells were treated for 6 hours with tenovin-6 at concentrations ranging from 2 to 10 μM after which total p53 levels were analysed by Western blot. Tenovin-6 treatment not only led to an increase in p53 levels (Table 1 and Fig. S1[9]) but to a greater extent than tenovin-1 did (Table 1).[8a] As expected, it was shown that tenovin-6 was significantly more water soluble than tenovin-1 (**2**) (sixfold, Table 1). Subsequent studies focused on the outcome of varying the carbon linker chain length. An analogous synthetic route was used to prepare the 4C (**8**), 6C (**9**) and 8C (**10**) analogues (Scheme 1). In addition the all carbon linker was replaced by a polyethylene-glycol linker to give **17**. Synthesis of **17** required initial preparation of the PEG-containing linker for coupling to **5** (Scheme 1B). Conversion of hexaethylene glycol (**11**) to the mono-tosylate **12** was followed by oxidation of the remaining alcohol in **12** to give the carboxylic acid **13**.[10] Formation of the corresponding acid chloride **14** enabled coupling with **5** to give **15** in 46% yield. The tosylate group in **15** was then substituted by dimethylamine via bromide **16**. Finally, the hydrochloride salt was prepared giving **17**.

Biological analysis showed that extension of the linker was tolerated with **9** and **10** demonstrating a similar activity to tenovin-6 in the p53 assay (Table 1 and Fig. S1[9]). Shortening the linker or incorporation of the PEG-linker led to less active analogues with **17** being inactive in cells (Table 1). Following these studies, it was decided to carry out target identification work using tenovin-6 and this ultimately led to the realisation that tenovin-6 was an inhibitor of the two sirtuins SirT1 and SirT2.[8a] It was therefore decided to test the activity of the other tenovin analogues



Scheme 1. Reagents and conditions: (A) (a) $SOCl_2$, DCM, rt, 16 h, 96% (0.5 g scale), 98% (50 g scale); (b) NaSCN, acetone, rt, 8 h, then 1,4-phenylene-diamine, 16 h, 58% (4 g scale), 74% (50 g scale); (c) acid chloride, NEt_3, DCM, rt, 4–8 h; (d) 40% aq $HNMe_2$, DCM/H_2O, rt, 24 h; (e) 2 M HCl in diethyl ether, yield over three steps for tenovin-6 78% (6 g scale), 79% (35 g scale); for tenovin-7 55%; for tenovin-8 59%; for tenovin-9 62%; (B) (f) Ts-Cl, AgO, KI, DCM, rt, 1 h, 87% (see Ref. 10); (g) CrO_3/H_2SO_4, acetone, 0 °C–rt, 16 h, 65% (see Ref. 10); (h) $SOCl_2$, DCM, rt, 16 h; (i) **5**, NEt_3, DCM, rt, 16 h, 46% over two steps; (j) LiBr, acetone, reflux, 24 h, 81%; (k) 40% aq $HNMe_2$, DCM/H_2O, rt, 24 h, 91%; (l) 2 M HCl in diethyl ether, DCM, 73%.

Table 1
Biological data for selected tenovin-6 analogues



Compound	R	R_1	p53 levels[a,e]			SirT1 Inhib.[b]		SirT2 Inhib.[b]		Solubility[f] (μg/mL)
			2 μM	5 μM	10 μM	% Inhib 60 μM	IC_{50} (μM)	% Inhib 60 μM	IC_{50} (μM)	
Tenovin-1	$NHC(O)CH_3$	H	+[c]	++[c]	+++[c]	nd[d]	nd[d]	nd[d]	nd[d]	0.12
Tenovin-6	$NHCO(CH_2)_4NMe_2$	H	++	+++++	+++++	73.9 ± 1.3	37.5 ± 0.8[a]	94.9 ± 0.4	10.4 ± 0.1[a]	0.74
8	$NHCO(CH_2)_3NMe_2$	H	–	++	++	85.2 ± 3.0	35.8 ± 9.0	85.9 ± −0.6	12.8 ± 4.0	0.57
9	$NHCO(CH_2)_5NMe_2$	H	+	+++++	++++	96.2 ± 3.6	16.1 ± 2.9	77.8 ± 5.9	21.3 ± 4.2	>0.97
10	$NHCO(CH_2)_7NMe_2$	H	+	++++	+++++	70.9 ± 16.7	24.7 ± 9.8	59.9 ± 11.8	41.7 ± 17.1	>1.01
17	$NHCO(CH_2OCH_2)_5CH_2NMe_2$	H	–	–	–	29.6 ± 0.01	>60	19.0 ± 0.01	>90	>1.34
26	$NHCO(CH_2)_4NMe_2$	Me	–	–	–	6.6 ± 1.5	nd	46.7 ± 4.2	nd	nd

[a] p53 levels were detected in MCF-7 cells after 6 h treatment with the indicated compounds.
[b] Enzyme inhibition as determined using the commercially available assay kit (Bioml/Enzo Life Sciences). Results were obtained in triplicate. Values are expressed as means ± SD.
[c] Value differs from previously reported due to change in peptide substrate concentration (from 7 μM (Ref. 8a) to 25 μM).
[d] Not determined due to lack of water solubility.
[e] The following symbols were used to represent: +, small increase; ++, moderate increase; +++, medium increase; ++++, significant increase; +++++, large increase; –, no increase in p53 levels; nd, not determined.
[f] Solubility was determined using a by UV spectrophotometric method. The extinction co-efficient for each compound was calculated and used to determine the solubility (see Section 4.4).

against purified human recombinant SirT1 and SIRT2. Whilst relatively little difference in the activity against both enzymes in vitro was observed on varying the linker length (cf. tenovin-6 and analogues **8–10**), a clear difference came with **17** which was shown to no longer inhibit SirT1 or SirT2 (Table 1). This observation is consistent with the lack of p53 activation observed on treatment of MCF-7 cells with **17**.

2.2. Large scale synthesis of tenovin-6

As tenovin-6 retained the same biological activity as tenovin-1 and was of sufficiently high water solubility to enable the target identification studies to be carried out,[8a] it was decided that a large quantity of tenovin-6 should be prepared for use in preclinical efficacy models of human tumours. Increasing the scale of the synthesis of **5** proved particularly effective as on scales of over 15 g, **5** crashed out of the crude reaction mixture giving, after filtration, a solid of sufficient purity for use in the next step. Acylation of **5** with bromovaleryl chloride initially proved more challenging on a larger scale with the generation of an unknown by-product. However, optimised conditions were found that enabled the isolation of a pure sample of the required bromide **6** (Scheme 1) following filtration of the crude reaction mixture and washing the filter cake with dichloromethane. On scale up, the displacement reaction to give **7** proceeded in an analogous manner to the small scale reaction and did not require further optimisation. Formation of the HCl salt of **7** afforded a high yield of pure tenovin-6 on up to a 35 g scale.

2.3. Tenovins adopt a preferred conformation in solution that contains an intramolecular hydrogen bond

^1H NMR analysis of tenovins-1, 6 and analogues **8–10** showed the presence of an unexpectedly low field signal at higher than 12.5 ppm (see Figs. S2, S3 and Table S1). This signal was assigned to the N14-hydrogen atom (Fig. 2A) and its chemical shift was rationalised based on a proposal that in the preferred conformation of the tenovins the N14H forms an intramolecular hydrogen bond with the oxygen atom of the C11-carbonyl. Small molecule X-ray crystallographic analysis of a crystal of tenovin-1 also provided

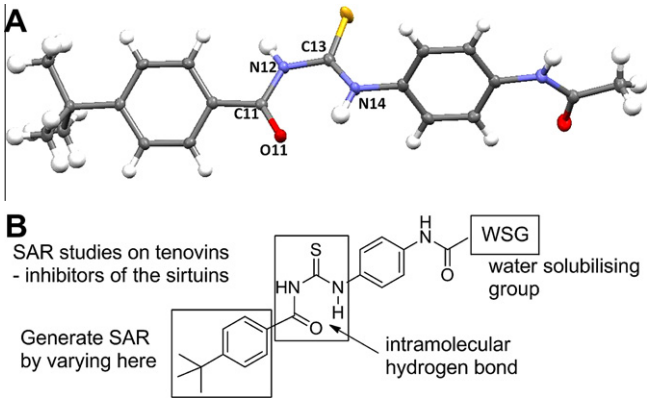
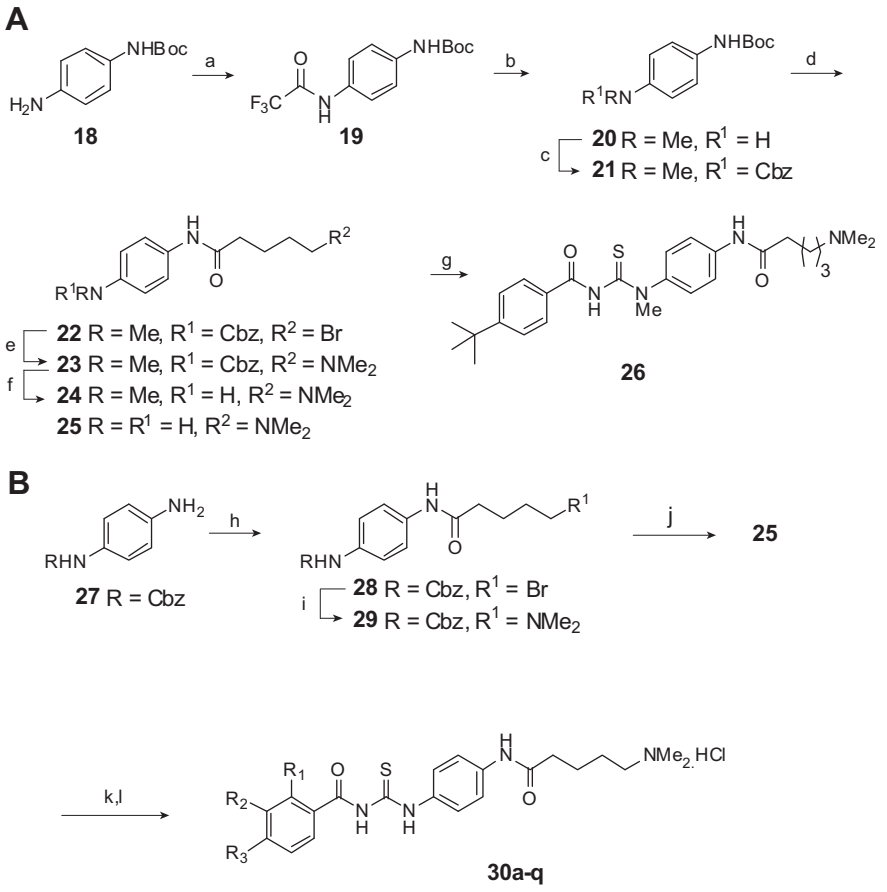


Figure 2. (A) Representation of the small molecule crystal structure of tenovin-1; (B) schematic of tenovin-6 highlighting important regions of the molecule.

evidence for the existence of this hydrogen bond in the solid state (Fig. 2A).[11] Interestingly, a previously reported co-crystal structure of glycogen phosphorylase (GP) with an *N*-acyl-*N*'-aryl substituted urea (structurally related to the *N*-acyl *N*'-aryl thiourea present in the tenovins) showed the GP-inhibitor bound to the protein in a conformation that contained an intramolecular hydrogen bond.[12]

In order to explore whether the ability to form the intramolecular hydrogen bond in the tenovins correlated with biological activity against the sirtuins, it was decided to prepare an analogue of tenovin-6 in which the N14H was replaced by a N14Me group (**26**, Scheme 2A). It was reasoned that **26** could not form the intramolecular hydrogen bond and would adopt an alternative preferred conformation in solution to tenovin-6. A modified version of our initial approach to the tenovins was developed to prepare analogue **26**. In accordance with literature precedent, *N*-Boc protected 1,4-phenylenediamine **18** was N-trifluoroacetylated to give **19** (Scheme 2A).[13] N-Methylation of **19** with concomitant removal of the trifluoroacetate group to give **20** was followed by reprotection of the N-methylated nitrogen with a Cbz-group leading to **21**. Removal of the *N*-Boc-protection and coupling of the resulting



Scheme 2. Reagents and conditions: (A) (a) NEt_3, trifluoroacetic anhydride, DCM, 0 °C–rt, 15 min, 98%; (b) ^tBuOK, THF, −78 °C, then MeI, 50 °C, ^tBuOK, 1.5 h, 59%; (c) benzyl chloroformate, DIPEA, DCM, 0 °C–rt, 16 h, 94%; (d) trifluoroacetic acid, DCM, rt, then 5-bromovaleryl chloride, NEt_3, DCM, rt, 2 h, 32%; (e) 40% aq $HNMe_2$, DCM/H_2O, rt, 24 h, 86%; (f) 48% w/v HBr in acetic acid, rt, 1 h, 96%; (g) 4-*t*-butylbenzoyl chloride, acetone, NaSCN, rt, 16 h, then **24**, rt, 16 h, 30%; (B) (h) 5-bromopentanoyl chloride, DIPEA, DCM, rt, 4 h, 71%; (i) 40% aq $HNMe_2$, DCM/H_2O, rt, 16 h, 68%; (j) 48% w/v HBr in acetic acid, rt, 1 h or 10% Pd/C, H-Cube, MeOH, quantitative; (k) acid chloride, NaSCN, acetone, rt, 30 min then **25**, acetone, rt, 16 h, 8–74%; (l) 2 M HCl in diethyl ether, acetone, 54–91%.

aniline to 5-bromovaleryl chloride gave the required intermediate **22**. Subsequent incorporation of the water solubilising tertiary amine led to **26** via **23** after hydrogenolysis to remove the Cbz-group (to give **24**), coupling with the *N*-acyl-isothiocyanate generated from **4** (Scheme 1A) and salt formation. Interestingly, **26** was found to be a very poor inhibitor of SirT1 and a relatively weak inhibitor of SirT2 (Table 1) and led to no increase in p53 levels in cells (Fig. S4[9]). This suggests that the tenovins may bind to SirT1 in a conformation that retains the intramolecular hydrogen bond although this can only be proved by solving a co-crystal structure of a tenovin analogue with the SirT1 enzyme.[14] Comparison of the intramolecularly hydrogen bonded conformer of the tenovins with the structure of cambinol (**1**) suggests that the two compounds may occupy the same or overlapping binding sites although the positions in which substituents are incorporated in the fixed six-membered ring in **1** differ from those in the tenovins. At this stage it was decided to prepare analogues of tenovin-6 that differed in structure in the *tert*-butyl-phenyl ring (Fig. 2B). Whilst modifications in this position had previously led to a reduction in activity (see Ref. 8a), these studies were limited and further studies were required in order to try and increase potency and selectivity.

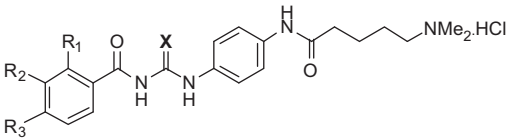
2.4. Parallel synthesis of tenovin-6 analogues and preliminary SARs for SirT1 and SirT2

Whilst practical for the large scale synthesis of tenovin-6, the synthetic route described in Scheme 1A was modified to enable rapid preparation of analogues of tenovin-6 that differed in the substituents in the *tert*-butyl-substituted aromatic ring. This approach was inspired by our route to **26** and involved the synthesis of the key intermediate aniline **25** (Scheme 2A and B). Compound **25** was initially prepared by HBr-mediated deprotection of the *N*-Cbz protected aniline **29** (prepared as shown in Scheme 2B from **27** via **28**). However, this route to **25** proved unreliable with samples of **25** decomposing possibly due to difficulties in removing excess HBr. Deprotection by hydrogenation using a palladium on carbon catalyst proved more robust, with samples of **25** being stable over extended periods of time after using this deprotection procedure. Standard parallel synthesis protocols were then used to prepare a range of tenovin-6 analogues using **25** with purification of the resulting free bases taking place on short silica plugs prior to hydrochloride salt formation (Scheme 2B and Table 2). The choice of substituents enabled exploration of the importance of the size and electronic properties of the 4-substituent as well as the effect of incorporating substituents at the 2- and 3-positions. Analogues containing two substituents were also prepared.

2.5. Biological activity of novel analogues in vitro and in cells

Assessment of the activity of the novel tenovin-6 analogues as inhibitors of SirT1 and SirT2 in vitro was then carried out. Table 2 shows the percentage inhibition at a final concentration of inhibitor of 60 μM for all analogues and selected IC_{50} data. Replacement of the 4-*tert*-butyl substituent in tenovin-6 with a 4-*n*-propyl, a

Table 2
Biological data for selected tenovin-6 analogues



	X	R_1	R_2	R_3	SirT1 inhibition		SirT2 inhibition		p53[b]			Ac-tubulin[c]		
					% Inhib 60 μM[a]	IC_{50}[a] (μM)	% Inhib 60 μM[a]	IC_{50}[a] (μM)	β-Gal 5 μM	β-Gal 10 μM	β-Gal 20 μM	10 μM	20 μM	30 μM
Tnv-6	S	H	H	*t*-Bu	73.9 (1.3)	37.5 (1.0)	94.9 (0.4)	10.4 (0.1)	+ (11)	+ (10.5)	+ (3.5)	+ (3.0)	– (0.86)[d]	Toxic[d]
30a	S	H	H	*n*-Pr	50.9 (3.4)	60.2 (4.7)	88.9 (3.0)	7.85 (1.3)	– (1.0)	+ (1.5)	+ (2.4)	– (1.0)	– (1.1)	+ (1.4)
30b	S	H	H	*iso*-Pr	52.9 (2.8)	42.7 (3.1)	78.4 (4.4)	5.71 (0.7)	+ (8.7)	+ (11.1)	+ (12)	+ (1.3)	+ (1.4)	+ (1.3)
30c	S	H	H	*n*-Bu	56.3 (2.8)	46.3 (1.4)	90.1 (3.7)	8.6 (1.1)	– (0.91)	– (0.93)	Tox (0.28)	+ (1.5)	Tox (0.66)	Toxic
30d	S	H	H	*n*-Pen	73.2 (1.9)	36.7 (1.7)	85.4 (7.4)	5.6 (0.9)	– (1.1)	– (1.1)	+ (1.2)	– (1.1)	– (1.2)	+ (1.4)
30e	S	H	H	H	3.5 (0.5)	>90	43.7 (4.2)	>90	– (1.1)	– (0.97)	– (0.92)	– (0.78)	– (0.90)	– (0.95)
30f	S	H	H	Me	47.3 (2.3)	>90	40.1 (3.5)	>90	– (1.2)	– (1.1)	– (1.1)	– (1.1)	– (0.99)	– (1.0)
30g	S	H	H	OMe	48.5 (0.9)	>90	51.1 (4.5)	50.6 (1.4)	– (1.0)	– (1.1)	– (1.12)	– (0.99)	– (1.0)	– (1.0)
30h	S	H	H	Cl	53.4 (1.2)	>90	61.5 (2.1)	>90	– (1.1)	– (1.2)	– (1.01)	– (1.0)	– (1.1)	– (1.2)
30i	S	H	H	Br	13.5 (1.0)	>90	47.7 (0.4)	55.0 (5.4)	– (1.0)	– (1.1)	– (1.01)	– (0.97)	+ (1.4)	+ (1.3)
30j	S	H	Me	H	5.2 (1.1)	>90	20.9 (2.7)	>90	– (0.94)	– (0.89)	– (0.89)	– (1.0)	– (1.2)	– (1.2)
30k	S	H	OMe	H	25.3 (6.9)	nd	30.5 (0.1)	nd	– (1.1)	– (1.1)	– (1.2)	– (1.0)	– (1.1)	– (1.1)
30l	S	H	Cl	H	29.2 (0.3)	nd	57.9 (0.1)	nd	– (1.1)	– (1.1)	– (1.0)	– (0.95)	– (1.2)	– (1.0)
30m	S	H	Br	H	70.5 (1.4)	39.9 (8.0)	81.1 (2.8)	21.2 (7.0)	– (1.1)	– (1.2)	+ (1.2)	– (1.0)	– (0.98)	– (0.97)
30n	S	H	Br	*t*-Bu	93.5 (0.4)	18.3 (0.3)	91.1 (0.7)	4.6 (0.6)	– (1.1)	– (1.1)	+ (1.4)	– (1.0)	– (1.1)	+ (1.3)
30o	S	H	Cl	Cl	8.1 (0.1)	70.9 (4.0)	68.5 (4.5)	31.9 (6.0)	– (1.04)	– (1.1)	– (0.97)	– (1.0)	– (1.9)	+ (1.2)
30p	S	Me	H	H	9.9 (0.9)	>90	48.5 (3.5)	42.5 (2.4)	– (0.99)	– (0.98)	– (0.84)	– (1.0)	– (1.1)	– (1.2)
30q	S	Cl	H	H	29.3 (0.9)	>90	54.3 (1.6)	>90	– (1.2)	– (1.1)	– (1.2)	– (1.0)	– (1.0)	– (1.0)
33	O	H	H	*t*-Bu	93.6 (4.5)	21.7 (1.3)	35.3 (14)	88.4 (5.2)	+ (6.9)	+ (7.3)	+ (6.5)	+ (1.4)	+ (1.8)	+ (2.1)
39	S	H	H	*t*-Bu	72.5 (2.1)	17.9 (3.1)	84.9 (1.6)	12.4 (1.3)	nd	+ (3.9)	+ (5.5)	– (1.2)	+ (1.6)	+ (1.9)

[a] Enzyme inhibition was determined using the commercially available assay kits (Biomol/Enzo Life Sciences). Results were obtained in triplicate. Values are expressed as means (SD).

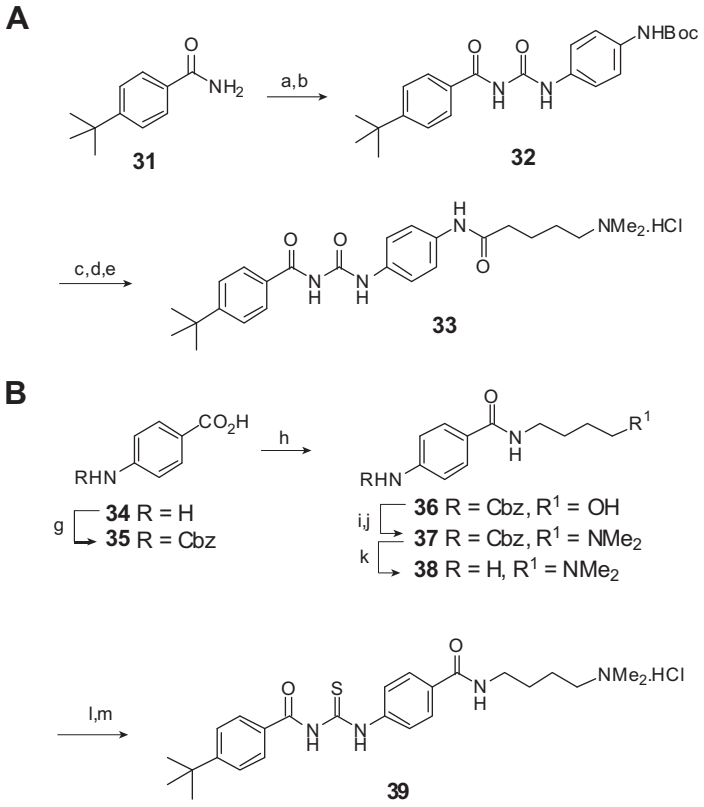
[b] p53 activation using a reporter gene assay was determined in ARN8 cells in triplicate. + Denotes active and – denotes no observed activity. Values in parentheses correspond to the ratio between compound treated cells and DMSO control cells to 2 significant figures. For further details see Section 4.

[c] Levels of K40 acetylated tubulin were determined in H1299 cells using western blotting in triplicate. + Denotes active and – denotes no observed activity. Values in parentheses correspond to the ratio between the intensity of the bands in the compound treated and DMSO control treated cells to 2 significant figures.

[d] Tenovin-6 was toxic to H1299 cells at higher concentrations, see Ref. 8a. For more information on the interpretation of cell data and examples of Ac-tubulin blots see Supplementary data (Figs. S8–S10).

4-*iso*-propyl, a 4-*n*-butyl or a 4-*n*-pentyl substituent led to only minor changes in activity against SirT2 indicating that this enzyme is relatively tolerant of the nature of the alkyl substituent at this position (cf. Table 2, analogues **30a–d**). Inhibition of SirT1 varied more with the structure of the 4 analogues. Interestingly, whilst the analogues with shorter alkyl chains (**30a** and **30b**) showed the initially expected activity in cells, analogues **30c** and **30d** (with longer *n*-alkyl chains) proved either toxic or inactive in cells. Differences in the cell permeability of these two analogues compared to tenovin-6 and analogues **30a** and **30b** may explain this observation. It should be noted that previous studies by us[7b,c,8a] and others[3e] have looked to correlate inhibition of SirT1 in vitro with p53 activation in cells and in vitro SirT2 inhibition with an increase in K40-acetylated tubulin levels in cells. Results presented in Section 2.6 below suggest that this approach may be an oversimplification.

A decrease in activity against both SirT1 and SirT2 was observed with a range of alternative 4-substituents including small substituents such as 4-H and 4-methyl (**30e** and **30f**), electron-donating (4-Me, 4-OMe, **30f** and **30g**) and electron-withdrawing (4-Cl and 4-Br, **30h** and **30i**) substituents. Some degree of selectivity for inhibition of SirT2 over SirT1 was observed when a 4-methoxy- or a 4-bromo-substituent was present (analogues **30g** and **30i**). Replacement of the 4-*tert*-butyl group in tenovin-6 with a 3-methyl, 3-methoxy or 3-chloro group led to a loss in activity (compounds **30j–l**) against both enzymes although incorporation of a 3-bromo-substituent both in the absence (**30m**) and the presence of the 4-*tert*-butyl group (**30n**) led to an improvement in activity with **30n** having IC_{50}s of 18.3 ± 0.3 μM and 4.6 ± 0.6 μM against SirT1 and SirT2 respectively. Unfortunately, the maintenance of in vitro activity (compared to tenovin-6) associated with the incorporation of a 3-bromo-substituent was not mirrored by the activity in cells. Incorporation of a 3,4-dichloro-substitution pattern had a detrimental effect on activity for both enzymes compared to tenovin-6 (compound **30o**). Further studies also suggested that incorporation of an electron-donating substituent at the 2-position of this aromatic ring may result in tuning of activity towards SirT2 with the 2-Me analogue showing better activity against SirT2 than SirT1 (**30p**). In addition to the in vitro characterisation of these analogues, **30a–q** were all tested in cells. An alternative method of assessing p53 activation in cells was used for these analogues compared to compounds **8–10**, **17** and **26** (see Table 2, Section 4 and Ref. 8a for details). This change was made as the p53-dependent transcription assay was judged to be more robust, quantitative (see values in Table 2) and cost effective for the analysis of an increased number of analogues. As observed for the sirtuin inhibitor cambinol (**1**),[7b] analogues that did not inhibit SirT1 activity in vitro failed to activate p53-dependent transcription in cells. However, analogues **30c**, **30d**, **30m** and **30n** did not activate p53-dependent transcription in cells despite inhibiting SirT1 in vitro. Unexpectedly poor physicochemical properties such as cell-permeability or cellular toxicity (see **30c**) may explain these results. The robust SirT1 inhibitors **30a** and **30b** did activate p53-dependent transcription. Unlike the in vitro SirT1 inhibition, which appears to be very sensitive to changes in compound structure, several of the novel analogues retained activity against SirT2 in vitro. In general, a reasonable correlation between SirT2 inhibition in vitro and increases in K40-acetylated tubulin levels in cells was observed, with 7 of the 10 in vitro SirT2 inhibitors leading to increased levels of K40-acetylated tubulin. All of the analogues that were found to increase acetylated tubulin levels in cells, inhibited SirT2 in vitro.



Scheme 3. Reagents and conditions: (A) (a) $(COCl)_2$, 1,2-DCE, reflux, 24 h; (b) **14**, MeCN, reflux, 3 h, 17%; (c) TFA, rt, 40 min quantitative; (d) 5-bromovaleryl chloride, NEt_3, DCM rt, 16 h (48%); (e) 40% aq $HNMe_2$, H_2O, DCM, rt, 20 h then 2 M HCl in diethyl ether, acetone 36%; (B) (g) benzyl chloroformate, satd aq $NaHCO_3$, THF, rt, 16 h, 85%; (h) methyl chloroformate, NEt_3, DCM, 0 °C–rt, 2 h then 4-aminobutanol, rt, 6 h, 72%; (i) CBr_4, PPh_3, DCM, 0 °C–rt, 4 h, 54%; (j) 40% aq $HNMe_2$, DCM/H_2O, rt, 16 h, 67%; (k) 10% Pd/C, H-Cube, MeOH, quantitative; (l) 4-*t*-butylbenzoyl chloride, NaSCN, acetone, rt, 30 min then **38**, acetone, rt, 16 h, 52%; (m) 2 M HCl in diethyl ether, acetone, 86%.

2.6. Other issues relating to the tenovin structure

Prior to carrying out mouse xenograft experiments,[8a] studies focused on possible methods by which the tenovins may be metabolised in vivo. This work was initially carried out in order to determine the half-life of tenovin-6 and hence to provide information on potential dosing regimes in xenograft experiments.[8a] Incubation of tenovin-6 with H1299 cells for 16 h, followed by cell lysis and extraction with methanol led to the identification of one major degradation product (Fig. S5[9] retention time (rt) (tenovin-6) = 5.61 min; rt (degradation product) = 4.46 min). Mass spectrometric analysis showed that degradation was accompanied by a loss of 16 Da (data not shown), which was predicted to arise from oxidative desulfurisation[15] of the thiourea functional group to the corresponding urea. Evidence to support this came following the synthesis of urea compound **33** (Scheme 3A). This was achieved in six steps from 4-*tert*-butylbenzamide (**31**) via the *N*-acyl-urea **32**. Comparison of the retention time and fragmentation pattern of synthetic **33** with those of the degradation product corroborates the assignment (Figs. S6 and S7[9]). Synthetic **33** inhibited SirT1 in vitro and also caused an increase in p53-dependent transcription in the cellular assay (Table 2).

It therefore seems likely that once in the cell both tenovin-6 and **33** can inhibit SirT1 function. Analogue **33** was also found to increase levels of K40-acetylated tubulin in cells (Table 2). Intrigued by this unexpected result, further experiments were carried out to investigate whether the ability of **33** to inhibit SirT1 could be responsible for this observation. A cytoplasmic extract from H1299 cells treated with trichostatin A was prepared. This extract contains relatively high levels of K40-acetylated tubulin (Fig. 3A). The extract was then treated with purified recombinant SirT1 in the presence/absence of NAD^+. As shown in Figure 3A a decrease in the levels of K40-acetylated tubulin was observed in the presence of SirT1 at two different concentrations and this decrease was NAD^+-dependent. In addition, overexpression of SirT1 in H1299 cells also led to a reduction in the levels of K40-acetylated tubulin in cells (Fig. 3B). These results are consistent with the unexpected increase in tubulin levels on treatment of H1299 cells with the apparently SirT1 selective inhibitor **33**.

1,4-Phenylenediamine is a known carcinogen.[16] As 1,4-phenylenediamine is also a potential metabolite of tenovin-6 concerns were raised about the possibility that this could explain the observed p53 activation. The fact that not all tenovin analogues prepared to date activate p53 despite containing the diamine unit argues against this. However, it was decided to prepare and assess the biological activity of **39**, an analogue in which one of the amide bonds is reversed and which cannot be metabolised to 1,4-phenylenediamine. **39** was prepared from 4-amino-benzoic acid (**31**) (Scheme 3B) and was shown to have comparable activity in the in vitro SirT1 and SirT2 assay to tenovin-6 (Table 2).

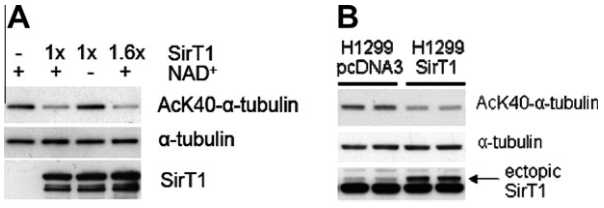


Figure 3. SirT1-mediated deacetylation of K40-acetylated α-tubulin. (A) A cytoplasmic extract prepared from H1299 cells containing acetylated tubulin was treated with purified recombinant His-tagged SirT1 in the presence/absence of NAD^+. Different concentrations of SirT1 were used; (B) comparison of K40-acetylated tubulin levels in vector transfected H1299 cells (H1299pcDNA3) or overexpressing SirT1 cells (H1299SirT1). Arrow indicates expression of ectopic SirT1 isoform a.

Interestingly, **39** activated p53 in cells, showing that the potential metabolism to 1,4-phenylenediamine is not essential for p53 activation by tenovin-6. However, **39** was not as potent as tenovin-6 in this assay. Subtle differences in the cell permeability of the two isomeric tenovins may explain this observation.

3. Conclusions

The development of sirtuin inhibitors continues to be of considerable interest and importance. Significant advances in our understanding of the detailed biology of this class of deacetylases are being made and potent and selective chemical tools can help with this. In addition, it has become clear that modulation of sirtuin function can be linked to a range of potential therapeutic applications and an increased range of chemical starting points for drug discovery applications is desirable. Here, the synthesis of a series of novel tenovin analogues is reported including a demonstration that the chemistry is relevant to large scale work and applicable to a medium-throughput format. During the course of the work, ^1H NMR and X-ray crystallographic analysis of the tenovins showed that this inhibitor class adopts a preferred conformation in solution and in the solid state that contains an intramolecular hydrogen bond. Evidence suggesting that the ability to form this hydrogen bond is linked to the desired biological activity is provided for SirT1 inhibition. The situation for SirT2 inhibition is less clear at present. This observation may aid future developments in sirtuin inhibitor development with fixed ring analogues of the tenovins being of potential interest. The results of our recent studies in this area will be reported in the near future. Finally, prior to xenograft experiments in mice,[8a] it was necessary to consider potential pathways by which the tenovins may degrade in cells. The oxidative desulfurisation of the thiourea functionality was identified as a potential issue and the synthesis of **33**, the oxo-version of tenovin-6, verified this. In addition, further experiments with analogue **33** provide new evidence that K40-acetylated tubulin is a substrate of SirT1 in cells. This conclusion is consistent with a previous report on tubulin deacetylation by sirtuins although no comment was made about SirT1's ability to deacetylate tubulin in the earlier report.[17] Synthesis of a reverse amide analogue of tenovin-6 also confirmed that the observed activation of p53 could not be explained by formation of 1,4-phenylenediamime.

4. Experimental section

4.1. Chemistry

Unless otherwise stated, starting materials and reagents were obtained from commercial suppliers and were used without further purification. ^1H and ^{13}C NMR spectra were measured on a Brüker Advance 300/400 instrument. Chemical shifts are calibrated with reference to the residual proton and carbon resonances of the solvent ($CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$ ppm; DMSO-d_6: $\delta_H = 2.50$, $\delta_C = 39.5$ ppm; CD_3OD: $\delta_H = 3.31$, $\delta_C = 49.0$ ppm). Low and high-resolution mass spectral analyses were recorded using chemical ionisation operating in positive or negative ion mode.

4.1.1. Preparation of tenovin-6 and analogues 8–10

4.1.1.1. 1-(4-Amino-phenyl)-3-(4-*tert*-butyl-benzoyl)-thiourea (5). To a solution of **4** (4.0 g, 20.4 mmol) in acetone (30 mL) was added sodium thiocyanate (1.7 g, 20.8 mmol). The resulting pale yellow suspension was stirred at rt for 8 h, then added to a solution of 1,4-phenylenediamine (4.4 g, 40.8 mmol) in acetone (30 mL) at 0 °C. The brown suspension was warmed to rt and stirred for 16 h. The solvent was concentrated in vacuo and the remaining residue

resuspended in CH_2Cl_2 and the insoluble solids filtered. The filtrate was concentrated in vacuo to give a brown solid which was purified by column chromatography on silica using petroleum ether/ethyl acetate (1:1) as the eluent and subsequent recrystallisation from ethyl acetate to give **5** (4.4 g, 13.5 mmol, 58%) as a pale yellow solid. Mp 185–186 °C; ν_{max} cm^{-1} (KBr) 3398, 3350, 3200, 2960, 1684, 1665, 1609, 1542, 1328, 1274, 1126, 846, 772, 720, 621; ^1H NMR (300 MHz, $CDCl_3$) δ 12.37 (1H, br s, NH), 9.02 (1H, br s, NH), 7.81 (2H, d, J = 10.8 Hz, ArH), 7.68 (2H, d, J = 8.6 Hz, ArH), 7.49 (2H, d, J = 8.6 Hz, ArH), 6.71 (2H, d, J = 11.8 Hz, ArH), 3.75 (2H, br s, NH_2), 1.36 (9H, s, $(CH_3)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.5, 166.8, 157.7, 145.4, 128.8, 128.7, 127.4, 126.2, 125.8, 115.1, 35.0, 31.1; LRMS (m/z): $[M+H]^+$ 329.56; HRMS (m/z): $[M]^+$ calcd for $C_{18}H_{22}N_3OS$, 328.1484; found, 328.1486.

4.1.1.2. 5-Bromo-pentanoic acid {4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-amide (6). Thiourea **5** (30.0 g, 91.5 mmol) was stirred in CH_2Cl_2 (600 mL) under N_2 atmosphere. 5-Bromovaleryl chloride (18.3 g, 91.5 mmol) was added, followed by triethylamine (12.6 mL, 91.5 mmol). The beige suspension was stirred at rt for 6 h. The suspension was filtered to afford **6** (36.0 g, 75.0 mmol, 82%) as a light yellow powder. An analogous protocol was used to prepare **6** (1.0 g, 2.0 mmol, 65%) from **5** (1.0 g, 3.1 mmol) except following reaction the solution was washed sequentially with 1 M aqueous HCl (1 × 15 mL), 1 M NaOH (1 × 15 mL) and brine (1 × 15 mL). The organic layer was dried over $MgSO_4$ and the solvent removed in vacuo to afford an off-white gum. The residue was purified by column chromatography using hexanes/ethyl acetate (1:1) as the eluent to afford the product. Mp 158–159 °C; ν_{max} cm^{-1} (KBr) 3358, 3210, 2964, 2947, 1695, 1657, 1604, 1558, 1347, 1269, 1174, 1113, 952, 842, 641; ^1H NMR (300 MHz, $CDCl_3$) δ 12.60 (1H, br s, NH), 9.05 (1H, br s, NH), 7.82 (2H, m, ArH), 7.68 (2H, d, J = 8.9 Hz), 7.57 (4H, m, ArH), 7.22 (1H, br s, NH), 3.45 (1H, t, J = 6.3 Hz, CH_2), 2.42 (1H, t, J = 6.9 Hz, CH_2), 1.93 (4H, m,$(CH_2)_2$), 1.36 (9H, s, $(CH_3)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.4, 170.9, 166.9, 157.9, 133.6, 128.6, 127.4, 126.3, 124.9, 119.9, 37.4, 33.6, 32.5, 31.1, 27.7, 24.6. LRMS (m/z): $[M]^+$ 512.06, $[M+Na]^+$; HRMS (m/z): $[M]^+$ calcd for $C_{23}H_{28}N_3O_2NaS^{79}Br$, 512.0983; found, 512.0995.

4.1.1.3. 5-Dimethylamino-pentanoic acid {4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (tenovin-6). To a stirred solution of **6** (35.0 g, 71.6 mmol) in DCM (600 mL) was added dimethylamine (40% aqueous solution) (240 mL) and water (600 mL) and the resulting biphasic mixture stirred rapidly at rt for 16 h. The mixture was diluted with CH_2Cl_2 (200 mL) and the resulting organic layer was washed with water (300 mL), 1 M aqueous sodium hydroxide (300 mL), saturated brine (2 × 300 mL), dried over $MgSO_4$ and concentrated in vacuo to give **7** as an off-white solid. Purification of **7** was accomplished by conversion to the corresponding HCl salt. Compound **7** was dissolved in a minimal amount of acetone and 2 M hydrochloric acid in ether solution was added dropwise until a yellow precipitate appeared. The solid was filtered to give **Tenovin-6** as a yellow solid (24.1 g, 49.4 mmol, 69%). An analogous protocol was used to prepare **Tenovin-6** (686 mg, 1.4 mmol, 67%) from **6** (1.0 g, 2.0 mmol). Mp 206–208 °C; ν_{max} cm^{-1} (KBr) 3236, 3040, 2952, 2663, 2503, 1674, 1610, 1577, 1348, 1270, 1159, 1113, 966, 834, 703; ^1H NMR (300 MHz, DMSO-d_6) δ 12.61 (1H, br s, NH), 11.43 (1H, br s, NH), 10.13 (1H, br s, NH), 9.77 (1H, br s, NH), 7.95 (2H, d, J = 8.4 Hz, ArH), 7.59 (6H, m, ArH), 3.06 (2H, m, CH_2), 2.75 (6H, s, $N(CH_3)_2$), 2.40 (2H, t, J = 6.1 Hz, CH_2), 1.66 (4H, m, $(CH_2)_2$), 1.33 (9H, s, $(CH_3)_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.4, 171.2, 168.5, 156.8, 137.9, 133.3, 129.7, 129.1, 125.8, 125.3, 119.5, 56.7, 42.5, 35.9, 35.3, 31.3, 23.8, 22.4; LRMS (m/z): $[M]^+$ 455.24, $[(M-HCl)+H]^+$; HRMS (m/z): $[M]^+$ calcd for $C_{25}H_{34}N_4O_2S$, 455.2474; found, 455.2477.

4.1.1.4. *N*-{4-[3-(4-*tert*-Butyl-benzoyl)-thioureido]-phenyl}-4-dimethylamino-butyramide hydrochloride (8). Thiourea **5** (200 mg, 0.61 mmol) was stirred in CH_2Cl_2 (10 mL) under N_2 atmosphere. 4-Bromobutyryl chloride (169 mg, 0.92 mmol) was added, followed by triethylamine (85 μL, 0.61 mmol). The orange solution was stirred at rt for 1.5 h and diluted with CH_2Cl_2 (10 mL). The organic solution was washed with 1 M aqueous hydrochloric acid (1 × 10 mL), 10% aqueous sodium hydroxide (1 × 50 mL), and saturated brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The resulting pale yellow foam was purified by flash chromatography on silica using hexanes/ethyl acetate (1:1) to give 4-bromo-*N*-{4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-butyramide (138 mg, 0.29 mmol, 48%) as a yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ 12.59 (1H, br s, NH), 9.08 (1H, br s, NH), 7.82 (2H, d, J = 8.7 Hz, ArH), 7.60 (6H, m, ArH), 3.52 (2H, t, J = 6.2 Hz, CH_2), 2.57 (2H, t, J = 7.0 Hz, CH_2), 2.26 (2H, m, CH_2), 1.35 (9H, s, $(CH_3)_3$). Treatment of the prepared bromo compound (138 mg, 0.29 mmol) with dimethylamine (3 mL) in H_2O (7.5 mL) gave a crude mixture which was dissolved in a minimal amount of acetone and 2 M hydrochloric acid in ether solution was added dropwise until a precipitate appeared. The solid was filtered and the yellow solid which was recrystallised from isopropanol to give **8** (75 mg, 0.16 mmol, 55%) as a yellow solid. ν_{max} cm^{-1} (NaCl, thin layer) 3239, 3044, 2921, 2342, 1659, 1603, 1514, 1074, 793; ^1H NMR (300 MHz, DMSO-d_6) δ 12.61 (1H, br s, NH), 11.44 (1H, br s, NH), 10.17 (1H, br s, NH), 9.64 (1H, br s, NH^+), 7.95 (2H, d, J = 8.5 Hz, ArH), 7.62 (6H, m, ArH), 3.09 (2H, m, CH_2), 2.79 (6H, d, J = 4.8 Hz, $N(CH_3)_2$), 2.44 (2H, t, J = 7.3 Hz, CH_2), 1.95 (2H, m, CH_2) and 1.32 (9H, s, $(CH_3)_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.4, 171.2, 168.3, 156.4, 137.6, 133.1, 129.5, 128.9, 125.5, 125.1, 119.1, 56.7, 42.5, 35.9, 35.3, 31.3; LRMS (m/z): $[M+H]^+$ 441.28; HRMS (m/z): $[M]^+$ calcd for $C_{24}H_{33}N_4O_2S$, 441.2318; found, 441.2322.

4.1.1.5. 6-Dimethylamino-hexanoic acid {4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (9). Thiourea **5** (200 mg, 0.61 mmol) was stirred in CH_2Cl_2 (10 mL) under N_2 atmosphere. 6-Bromohexanoyl chloride (195.8 mg, 0.92 mmol) was added, followed by triethylamine (85 μL, 0.61 mmol). The beige suspension was stirred at rt for 1.5 h and diluted with CH_2Cl_2 (10 mL). The organic solution was washed with 1 M aqueous hydrochloric acid (1 × 10 mL), 10% aqueous sodium hydroxide (1 × 50 mL), and saturated brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The resulting brown foam was purified by flash chromatography on silica (hexanes/ethyl acetate 1:1) to give 6-bromo-hexanoic acid {4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-amide (166 mg, 0.33 mmol, 55%) as a yellow oil. ^1H NMR (300 MHz, $CDCl_3$) δ 12.58 (1H, br s, NH), 9.10 (1H, br s, NH), 7.57 (7H, m, ArH, NH), 3.40 (2H, t, J = 6.7 Hz, CH_2), 2.36 (2H, t, J = 7.4 Hz, CH_2), 1.89 (2H, m, CH_2), 1.73 (2H, m, CH_2), 1.51 (2H, m, CH_2), 1.35 (9H, s, $(CH_3)_3$). Treatment of prepared bromo compound (136 mg, 0.27 mmol) with dimethylamine (2.5 mL) in H_2O (6.25 mL) gave a crude mixture containing that was dissolved in a minimal amount of CH_2Cl_2 and a 2 M hydrochloric acid in ether solution was added dropwise until a cream precipitate appeared. The solid was filtered and recrystallised from isopropanol to give **9** (80 mg, 0.16 mmol, 59%) as a yellow solid. ν_{max} cm^{-1} (NaCl, thin layer) 3372, 3039, 2961, 2863, 2707, 1701, 1698, 1664, 1609, 1516, 1472, 1270, 1125, 852, 772; ^1H NMR (300 MHz, DMSO-d_6) δ 12.59 (1H, s, NH), 11.42 (1H, s, NH), 10.08 (1H, s, NH), 9.83 (1H, br s, NH^+), 7.94 (2H, d, J = 8.4 Hz, ArH), 7.59 (6H, m, ArH), 3.01 (2H, m, CH_2), 2.73 (6H, d, J = 4.8 Hz, $N(CH_3)_2$), 2.35 (2H, t, J = 7.1 Hz, CH_2), 1.64 (4H, m, $(CH_2)_2$) and 1.32 (9H, s, $(CH_3)_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.7, 171.2, 168.0, 156.2, 137.4, 132.7, 129.2, 128.6, 125.3, 124.8, 118.9, 57.9, 43.9, 36.2, 34.8, 30.8, 26.1, 25.6, 24.8; LRMS

(*m/z*): $[M+Na]^+$ 491.38; HRMS (*m/z*): $[M]^+$ calcd for $C_{26}H_{37}N_4O_2S$, 469.2637; found, 469.2640.

4.1.1.6. 8-Dimethylamino-octanoic acid {4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (10). 8-Bromo-octanoic acid (2 g, 8.90 mmol) was stirred in CH_2Cl_2 (15 mL) under N_2 atmosphere and thionyl chloride (784 μL, 10.68 mmol) added followed by DMF (few drops). The solution was stirred for 16 h and concentrated in vacuo to give 8-bromooctanoyl chloride (2.04 g, 8.50 mmol, 95%) as a yellow oil which was used without further purification. Thiourea **5** (600 mg, 1.84 mmol) was stirred in CH_2Cl_2 (15 mL) under N_2 atmosphere. 8-Bromooctanoyl chloride (532 mg, 2.02 mmol) was added, followed by triethylamine (256 μL, 1.84 mmol). The beige suspension was stirred at rt for 16 h and diluted with CH_2Cl_2 (20 mL). The organic solution was washed with 1 M aqueous hydrochloric acid (1 × 20 mL), 10% aqueous sodium hydroxide (1 × 20 mL), and saturated brine (1 × 20 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo. The resulting yellow foam was purified by flash chromatography on silica (petroleum ether/ethyl acetate 1:1) to give 8-bromo-octanoic acid {4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-amide (579 mg, 1.09 mmol, 59%) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 12.59 (1H, s, NH), 9.04 (1H, s, NH), 7.82 (2H, d, J = 8.6 Hz, ArH), 7.63 (2H, d, J = 8.9 Hz, ArH), 7.56 (6H, m, ArH), 7.18 (1H, s, NH), 3.41 (2H, t, J = 6.8 Hz, CH_2), 2.37 (2H, t, J = 7.4 Hz, CH_2), 1.86 (2H, m, CH_2), 1.74 (2H, m, CH_2), 1.44 (6H, m, $(CH_2)_3$), 1.36 (9H, s, $(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.5, 171.6, 167.0, 157.8, 136.8, 133.4, 128.6, 127.5, 126.2, 124.8, 120.1, 37.6, 34.0, 32.7, 31.1, 29.1, 28.6, 28.0, 25.5. Treatment of the prepared bromo compound (487 mg, 0.92 mmol) with dimethylamine (10 mL) in H_2O (25 mL) gave a crude mixture which was dissolved in a minimal amount of CH_2Cl_2 and a 2 M hydrochloric acid in ether solution was added dropwise until a cream precipitate appeared. The solid was filtered to give a yellow solid which was recrystallised from isopropanol to give **10** (301 mg, 0.57 mmol, 62%) as a yellow-brown solid. ^1H NMR (400 MHz, DMSO-d_6) δ 12.60 (1H, s, NH), 11.44 (1H, s, NH), 10.08 (1H, br s, NH^+), 9.95 (1H, s, NH), 7.97 (2H, dd, J = 8.5, 16.5 Hz, ArH), 7.62 (6H, m, ArH), 2.99 (2H, m, CH_2), 2.72 (6H, br s, $N(CH_3)_2$), 2.33 (2H, m, CH_2), 1.62 (4H, m, $(CH_2)_2$), 1.32 (6H, m, $CH_2)_3$) and 1.32 (9H, s, $(CH_3)_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.5, 171.3, 168.0, 156.2, 137.4, 132.7, 129.2, 128.6, 125.3, 124.7, 118.9, 58.5, 44.4, 36.2, 34.8, 30.8, 28.2, 26.1, 25.6, 25.0 24.9; LRMS (*m/z*): $[M+H]^+$ 497.38; HRMS (*m/z*): $[M]^+$ calcd for $C_{28}H_{41}N_4O_2S$, 497.2950; found, 497.2946.

4.1.2. Synthesis of PEG analogue 17

4.1.2.1. Benzenesulfonic acid 2-[2-(2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester (12). Hexaethyleneglycol (**11**) (2.82 g, 10 mmol) was dissolved in CH_2Cl_2 (100 mL) under a N_2 atmosphere, and silver oxide (3.48 g, 15 mmol) added followed by tosyl chloride (2.10 g, 11 mmol) and potassium iodide (332 mg, 2 mmol). The solution was stirred at rt for 1 h then concentrated and filtered through Celite eluting with ethyl acetate. The filtrate was concentrated in vacuo to give a pale yellow oil which was purified by column chromatography on silica (ethyl acetate) to give **12** (3.79 g, 8.70 mmol, 87%) as a pale yellow oil. ^1H NMR (300 MHz, DMSO-d_6) δ 7.79 (2H, d, J = 8.4 Hz, ArH), 7.33 (2H, d, J = 8.0 Hz, ArH), 4.15 (2H, m, CH_2), 3.64 (22H, m, $(CH_2)_{11}$), 2.44 (3H, s, CH_3) and 1.80 (1H, br s, OH).

4.1.2.2. [2-(2-{2-[2-(2-Toluenesulfonyloxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-acetic acid (13). A 1.5 M aqueous solution of sulfuric acid (10 mL) was prepared at 0 °C and stirred while chromium trioxide was added to give an orange solution. The known alcohol **12** (1 g, 2.3 mmol) was dissolved in acetone (15 mL) and the solution added dropwise at 0 °C. The mixture was allowed to warm to rt and stirred for 16 h. The resulting green solution was filtered through Celite and concentrated in vacuo. The resulting filtrate was diluted with H_2O and extracted with CH_2Cl_2 (3 × 50 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to give **13** (672 mg, 1.6 mmol, 65%) as a pale yellow oil which was used without further purification. ^1H NMR (300 MHz, acetone-d_6) δ 7.82 (2H, d, J = 8.0 Hz, ArH), 7.49 (2H, d, J = 8.0 Hz, ArH), 4.13 (4H, m, $(CH_2)_2$), 3.59 (18H, m, $(CH_2)_9$), 2.46 (3H, s, CH_3); ^{13}C NMR (100 MHz, acetone-d_6) δ 172.7, 144.9, 132.9, 129.9, 127.9, 77.4, 77.1, 76.8, 71.3, 71.2, 70.8, 70.7, 70.6, 70.6, 70.5, 70.4, 70.4, 70.3, 69.3, 69.1, 68.8, 68.6, 68.5, 21.6; LRMS (*m/z*): $[M+Na]^+$ 459.16.

4.1.2.3. Toluene-4-sulfonic acid 2-[2-(2-{2-[2-({4-[3-(4-*tert*-butyl-benzoyl)-thioureido]-phenylcarbamoyl}-methoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyl ester (15). Acid **13** (117 mg, 0.26 mmol) was stirred in oxalyl chloride containing a catalytic amount of DMF. The solution was heated at reflux for 1 h then cooled to rt and the solvent concentrated in vacuo to give the corresponding acid chloride **14** which was used without further purification. Crude acid chloride **14** (138 mg, 0.29 mmol, 1.3 equiv) was stirred in dry CH_2Cl_2 (10 mL) under N_2 atmosphere and thiourea **5** (74 mg, 0.23 mmol, 1 equiv) was added to give a bright yellow solution. Triethylamine (41 μL, 0.23 mmol, 1 equiv) was added and the mixture stirred at rt for 16 h. The solution was diluted with CH_2Cl_2 (1 × 10 mL) and washed with 1 M aqueous hydrochloric acid (1 × 10 mL), 1 M aqueous sodium hydroxide followed by saturated brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and the solvent concentrated in vacuo. The resulting yellow foam was purified by flash chromatography on silica (petroleum ether:ethyl acetate 1:1) to give **15** (79 mg, 0.10 mmol, 46%) as a yellow oil. ν_{max} cm^{-1} (NaCl, thin layer) 3259, 3059, 2958, 2708, 2635, 1918, 1697, 1668, 1604, 1516, 1473, 1268, 1122, 1034, 852, 734; ^1H NMR (300 MHz, $CDCl_3$) δ 12.61 (1H, s, NH), 9.15 (1H, s, NH), 8.99 (1H, s, NH), 7.79 (4H, m, ArH), 7.67 (4H, m, ArH), 7.53 (2H, d, J = 8.6 Hz, ArH), 7.31 (2H, d, J = 8.0 Hz, ArH), 4.10 (4H, m, $(CH_2)_2$), 3.63 (18H, m, $(CH_2)_9$), 2.41 (3H, s, CH_3), 1.34 (9H, s, $(CH_3)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.3, 168.4, 166.9, 157.8, 144.8, 136.1, 133.7, 129.8, 128.6, 127.9, 127.5, 126.2, 126.1, 124.6, 120.2, 71.2, 70.7, 70.6, 70.5, 70.4, 70.1, 69.3, 68.7, 35.3, 31.1, 21.7; LRMS (*m/z*): $[M+Na]^+$ 782.28; HRMS (*m/z*): $[M]^+$ calcd for $C_{37}H_{49}N_3O_{10}NaS_2$, 782.2757; found, 782.2756.

4.1.2.4. *N*-{4-[3-(4-*tert*-Butyl-benzoyl)-thioureido]-phenyl}-2-[2-(2-{2-[2-(2-dimethylamino-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-acetamide hydrochloride (17). Lithium bromide (44 mg, 0.5 mmol) was added to a stirred solution of tosylate **15** (76 mg, 0.1 mmol) in acetone (3 mL). The mixture was heated at 50 °C for 24 h. The solvent was removed in vacuo and the resulting residue resuspended in ethyl acetate and the solid filtered. The filtrate was concentrated in vacuo to give **16** (54 mg, 0.1 mmol, 81%) as a pale yellow oil which was used without further purification. ν_{max} cm^{-1} (NaCl, thin layer) 3396, 3054, 2963, 2921, 2873, 2599, 1694, 1668, 1610, 1514, 1475, 1266, 1115, 947, 736; ^1H NMR (300 MHz, $CDCl_3$) δ 12.61 (1H, s, NH), 9.16 (1H, br s, NH), 9.06 (1H, s, NH), 7.82 (2H, d, J = 8.6 Hz, ArH), 7.70 (4H, q, J = 9.0 Hz, ArH), 7.55 (2H, d, J = 8.6 Hz, ArH), 4.18 (2H, s, CH_2), 3.71 (16H, m, $(CH_2)_8$), 3.45 (2H, t, J = 6.3 Hz, CH_2), 1.36 (9H, s, $(CH_3)_3$); LRMS (*m/z*): $[M+Na]^+$ 692.02. Bromide **16** (30 mg, 0.05 mmol) was converted to the dimethylamine derivative by stirring in 40% aqueous dimethylamine (0.75 mL), H_2O (1.8 mL) and CH_2Cl_2 (2 mL) at rt for 24 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and the organic phase washed with H_2O (1 × 10 mL), 1 M aqueous sodium hydroxide (1 × 10 mL) and saturated brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and the solvent concentrated in vacuo to give

the dimethylamine derivative. The pale yellow oil was taken up in a minimal amount of CH_2Cl_2 and a solution of 2 M hydrochloric acid in diethyl ether was added dropwise until a precipitate appeared. The mixture was concentrated in vacuo to give **17** (20 mg, 0.04 mmol, 73%) as a yellow foam. ν_{max} cm^{-1} (NaCl, thin layer) 3308, 2992, 2921, 2873, 1725, 1674, 1609, 1517, 1464, 1272, 1123, 841, 738; ^1H NMR (300 MHz, DMSO-d_6) δ 12.62 (1H, s, NH), 11.45 (1H, s, NH), 9.77 (1H, s, NH), 9.77 (1H, s, NH), 9.55 (1H, s, NH), 7.95 (2H, d, J = 8.4 Hz, ArH), 7.63 (6H, m, ArH), 4.10 (4H, m, $(CH_2)_2$), 3.61 (18H, m, $(CH_2)_9$), 2.77 (6H, d, J = 4.0 Hz, $N(CH_3)_2$), 1.32 (9H, s, $(CH_3)_3$); ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.2, 168.2, 168.0, 156.3, 136.4, 133.3, 129.2, 128.6, 125.3, 124.7, 119.6, 70.3, 70.1, 69.8, 69.7, 69.6, 69.5, 69.3, 64.4, 63.9, 63.3, 55.6, 42.6, 34.8, 30.8; LRMS (*m/z*): $[(M-HCl)+H]^+$ 632.82,; HRMS (*m/z*): $[M]^+$ calcd for $C_{32}H_{49}N_4O_7S$, 633.3322; found, 633.3327.

4.1.3. Synthesis of analogue 26

4.1.3.1. [4-(2,2,2-Trifluoro-acetylamino)-phenyl]-carbamic acid *tert*-butyl ester (19). A dry flask was charged with aniline **18** (1.02 g, 4.90 mmol) and CH_2Cl_2 (40 mL) and cooled to −78 °C. Trifluoroacetic anhydride (977 μL, 6.90 mmol) was added dropwise, followed by triethylamine (1.9 mL, 13.80 mmol). The cream slurry was stirred for 30 min and diluted with ethyl acetate (50 mL), and washed with 1 M aqueous hydrochloric acid (1 × 30 mL) and saturated brine (1 × 30 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to give **19** (1.43 g, 4.90 mmol, quantitative) as a cream solid. ^1H NMR (300 MHz, $CDCl_3$) δ 7.81 (1H, br s, NH), 7.49 (2H, d, *J* = 9 Hz, ArH), 7.39 (2H, d, *J* = 9.0 Hz, ArH), 6.57 (1H, br s, NH) and 1.52 (9H, s, $(CH_3)_3$).

4.1.3.2. (4-Methylamino-phenyl)-carbamic acid *tert*-butyl ester (20). Trifluoroacetamide **19** (695 mg, 2.36 mmol) was stirred in dry THF (10 mL) under N_2 atmosphere. To this stirring solution was added potassium-*t*-butoxide (264 mg, 2.46 mmol) and methyl iodide (147 μL, 2.83 mmol) and the reaction mixture heated at 50 °C for 5 min. A further portion of potassium-*t*-butoxide (409 mg, 3.65 mmol) in THF (10 mL) was added dropwise slowly. The resulting yellow suspension was stirred at rt for 3 h. The solvent was concentrated in vacuo and the resulting residue resuspended in ethyl acetate. The undissolved solid was filtered and the filtrate concentrated in vacuo to give a crude pale yellow oil which was purified by column chromatography on silica (petroleum ether:ethyl acetate 2:1) to give **20** (235 mg, 1.09 mmol, 46%) as a brown oil. ^1H NMR (300 MHz, $CDCl_3$) δ 7.13 (2H, d, *J* = 8.1 Hz), 6.55 (2H, d, *J* = 8.7 Hz, ArH), 6.20 (1H, br s, NH), 2.79 (3H, s, NCH_3) and 1.47 (9H, s, $(CH_3)_3$); LRMS (*m/z*): $[M+Na]^+$ 245.11.

4.1.3.3. Benzyl (4-((*tert*-butoxycarbonyl)amino)phenyl)(methyl) carbamate (21). To a solution of **20** (1.4 g, 6.4 mmol) in CH_2Cl_2 (20 mL) at −78 °C, under N_2, was added benzylchloroformate (1.1 mL, 7.7 mmol) followed by diisopropylethylamine (1.1 mL, 6.4 mmol) dropwise. The solution was allowed to warm to room temperature over 2 h. The solution was washed with water (10 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$ and the solvent removed to afford the crude product. The resulting residue was purified by column chromatography using petroleum ether/ethyl acetate (2:1) to afford the product as a white solid (2.2 g, 6.2 mmol, 97%). ^1H NMR (300 MHz, $CDCl_3$) δ 7.35–7.21 (7H, m, ArH), 7.08 (2H, d, *J* = 7.8 Hz, ArH), 6.40 (1H, s, NH), 5.07 (2H, s, CH_2), 3.21 (6H, s, $N(CH_3)_2$), 1.45 (9H, s, $(CH_3)_3$).

4.1.3.4. 5-(Dimethylamino)-*N*-(4-(methylamino)phenyl)pentanamide (24). To a solution of the known compound **21** (5.7 g, 15.8 mmol) in CH_2Cl_2 (100 mL) was added TFA (15 mL) and the resulting solution stirred at rt for 2 h before the solvent was removed in vacuo. The resulting deprotected amine salt was stirred in dry CH_2Cl_2 (40 mL) under N_2 atmosphere and triethylamine (4.4 mL, 31.6 mmol) was added and stirred for 20 min before addition of 5-bromovaleryl chloride (3.2 mL, 23.7 mmol) followed by further triethylamine (6.6 mL, 47.4 mmol). The reaction mixture was stirred at rt for 16 h then diluted with CH_2Cl_2 (1 × 50 mL) and washed with 1 M aqueous hydrochloric acid (1 × 100 mL), 1 M aqueous sodium hydroxide (1 × 100 mL) and saturated brine (1 × 100 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to give a crude brown oil which was purified by column chromatography on silica (petroleum ether/ethyl acetate 1:1) to give **22** (2.14 g, 5.1 mmol, 32%) as a brown solid. ^1H NMR (300 MHz, $CDCl_3$) δ 7.50 (2H, d, J = 8.6 Hz, ArH), 7.39–7.29 (5H, m, ArH), 7.21 (2H, d, J = 8.6 Hz, ArH), 5.19 (2H, s, CH_2), 3.47 (2H, t, J = 6.5 Hz, CH_2), 3.32 (3H, s, NCH_3), 2.41 (2H, d, J = 6.9 Hz, CH_2), 2.02–1.88 (4H, m, $(CH_2)_2$). **22** (500 mg, 1.30 mmol) was stirred in CH_2Cl_2 (4 mL), 40% aqueous dimethylamine (4 mL) and H_2O (10 mL) at rt for 20 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and washed with H_2O (1 × 10 mL), 10% aqueous NaOH (1 × 10 mL), and saturated brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to give **23** as a yellow oil (428 mg, 4.4 mmol, 86%) which was used without further purification. ν_{max} cm^{-1} (NaCl, thin layer) 3312, 3183, 3048, 2945, 2864, 2782, 2307, 1896, 1699, 1604, 1537, 1516, 1498, 1455, 1389, 1351, 1266, 1156, 843, 734; ^1H NMR (300 MHz, $CDCl_3$) δ 8.43 (1H, s, NH), 7.40 (2H, d, J = 8.4 Hz, ArH), 7.29–7.15 (5H, m, ArH), 7.06 (2H, d, J = 8.4 Hz, ArH), 5.07 (2H, s, CH_2), 3.20 (3H, s, NCH_3), 2.31–2.18 (4H, m, $(CH_2)_2$), 2.13 (6H, s, $N(CH_3)_2$), 1.66 (2H, app. quintet, J = 7.7 Hz, CH_2), 1.45 (2H, app. quintet, J = 7.7 Hz, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 171.6, 155.6, 136.6, 136.4, 128.5, 127.9, 127.7, 126.3, 120.3, 67.3, 58.8, 45.3, 37.9, 37.1, 26.5, 23.4.

Compound **23** (550 mg, 1.45 mmol) was stirred in 48% w/v hydrobromic acid in acetic acid (6 mL) for 1 h. Diethylether was added until a cream precipitate appeared and the ethereal solution was decanted. The resulting white sticky solid was suspended in CH_2Cl_2 (100 mL) and 1 M aqueous sodium hydroxide (20 mL). The aqueous layer was extracted with CH_2Cl_2 (1 × 50 mL) and the combined organic layers were washed with saturated brine (1 × 50 mL), dried ($MgSO_4$) and concentrated in vacuo to give **24** as a yellow oil (153 mg, 0.61 mmol, 42%). ν_{max} cm^{-1} (NaCl, thin layer) 3295, 3134, 3051, 2942, 2865, 2782, 2309, 1896, 1652, 1656, 1603, 1520, 1467, 1308, 1266, 1178, 824, 736; ^1H NMR (300 MHz, $CDCl_3$) δ 7.65 (1H, s, NH), 7.23 (2H, d, *J* = 8.8 Hz, ArH), 6.49 (2H, d, *J* = 8.8 Hz, ArH), 2.74 (3H, s, NCH_3), 2.26 (4H, app. quartet, *J* = 7.3 Hz, $(CH_2)_2$), 2.16 (6H, s, $N(CH_3)_2$), 1.67 (2H, app. quintet, *J* = 7.6 Hz, CH_2), 1.49 (2H, app. quintet, *J* = 7.1 Hz, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.1, 147.3, 130.2, 121.9, 121.8, 112.6, 59.9, 49.6, 45.5, 37.3, 27.8, 24.2; LRMS (*m/z*): $[M+H]^+$ 250.11; HRMS calcd for $C_{14}H_{24}N_3O$, 250.1919; found, 250.1922.

4.1.3.5. 4-(*tert*-Butyl)-*N*-((4-(5-(dimethylamino)pentanamido) phenyl)(methyl)carbamothioyl)benzamide (26). To a stirred solution of **4** (65 μL, 0.36 mmol) in dry acetone (2 mL) was added sodium thiocyanate (33 mg, 0.40 mmol) and the resulting yellow suspension was stirred at rt for 18 h. A solution of amine **24** (90 mg, 0.36 mmol) in dry acetone (1 mL) was added and the resulting suspension stirred at rt for a further 7 h. The solvent was removed in vacuo and the residue re-suspended in CH_2Cl_2 and the undissolved salts were removed by filtration. The filtrate was concentrated to afford a yellow solid which was purified by column chromatography on silica (CH_2Cl_2/MeOH 10:0.1) to afford **26** as a bright yellow solid (50 mg, 0.10 mmol, 28%). Mp 242–243 °C; ν_{max} cm^{-1} (KBr) 3392, 2960, 2925, 2855, 1675, 1662, 1610, 1514, 1484, 1408, 1267, 1113, 855, 779; ^1H NMR (300 MHz, acetone-d_6) δ 9.50 (1H, s, NH), 7.56 (2H, d, *J* = 8.8 Hz, ArH), 7.14 (6H, app. s, ArH), 3.60 (3H, s, NCH_3), 3.08 (2H, app. t,

J = 6.9 Hz, CH_2), 2.76 (6H, s, $N(CH_3)_2$), 2.32 (2H, app. t, J = 6.9 Hz, CH_2), 1.78–1.58 (4H, m, $(CH_2)_2$), 1.08 (9H, s, $(CH_3)_3$); ^{13}C NMR (75 MHz, aceone-d_6) δ 184.6, 178.2, 172.0, 156.7, 140.5, 139.9, 133.6, 129.7, 126.8, 125.9, 120.6, 120.5, 57.8, 46.2, 42.9, 36.7, 35.5, 31.2, 24.6, 23.1; LRMS (m/z): $[M+H]^+$ 469.31; HRMS calcd for $C_{26}H_{37}N_4O_2S$, 469.2637; found, 469.2629.

4.1.4. Synthesis of analogues 30a–q

4.1.4.1. Benzyl 4-(5-bromopentanamido)phenylcarbamate (28). Aniline **27** (12.69 g, 52.0 mmol) was stirred in CH_2Cl_2 (250 mL) at 0 °C and 5-bromovaleryl chloride (8.9 mL, 52.0 mmol) was added followed by DIPEA (9.0 mL, 52.0 mmol). The resulting white suspension was allowed to warm to room temperature and stirred for 3 h. The white solid was collected by filtration and recrystallised from ethyl acetate to give **28** as a white solid (14.6 g, 36.9 mmol, 71%). Mp 184–186 °C; ν_{max} cm^{-1} (KBr) 2971, 1656, 1548, 1305, 1233, 1066, 812; ^1H NMR (400 MHz, $CDCl_3$) δ 7.38 (2H, d, J = 8.9 Hz, ArH), 7.36–7.25 (7H, m, ArH), 7.02 (1H, s, NH), 6.56 (1H, br s, NH), 5.12 (2H, s, CH_2), 3.38 (2H, t, J = 6.4 Hz, CH_2), 2.32 (2H, t, J = 6.8 Hz, CH_2), 1.91–1.80 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0, 153.4, 136.0, 131.9, 131.6, 128.7, 120.8, 119.4, 67.5, 36.9, 33.7, 24.9, 24.1; LRMS (m/z): $[M+Na]^+$ 427.20; HRMS calcd for $C_{19}H_{21}N_2O_3Na^{79}Br$, 427.0633; found, 427.0637.

4.1.4.2. Benzyl 4-(5-(dimethylamino)pentanamido)phenylcarbamate (29). Bromide **28** (5.9 g, 12.0 mmol) was stirred in CH_2Cl_2 (150 mL) with 40% aqueous dimethylamine (10 mL) and water (25 mL) for 24 h. The solution was then diluted with CH_2Cl_2 (50 mL) and the organic phase washed with water (100 mL), 1 M aqueous sodium hydroxide (100 mL) and brine (100 mL), dried ($MgSO_4$), filtered and concentrated in vacuo to give **29** as a white solid (3 g, 8.1 mmol, 68%). Mp 141–144 °C; ν_{max} cm^{-1} (KBr) 2894, 1656, 1546, 1307, 1231, 1065; ^1H NMR (400 MHz, $CDCl_3$) δ 7.89 (1H, br s, NH), 7.39 (2H, d, J = 8.4 Hz, ArH), 7.35–7.22 (7H, m, ArH), 6.65 (1H, br s, NH), 5.16 (2H, s, CH_2), 2.32–2.26 (4H, m, $(CH_2)_2$), 2.18 (6H, s, $N(CH_3)_2$), 1.70 (2H, app. quintet, J = 8.3 Hz, 7.3 Hz, CH_2), 1.50 (2H, app. quintet, J = 8.3 Hz, 7.3 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3, 154.0, 136.2, 135.8, 133.7, 128.6, 128.3, 120.7, 67.0, 59.9, 45.4, 37.1, 26.6, 23.5; LRMS (m/z): $[M+H]^+$ 370.30; HRMS calcd for $C_{21}H_{28}N_3O_3$, 370.2131; found, 370.2131.

4.1.4.3. 5-Dimethylamino-pentanoic acid (4-amino-phenyl)-amide (25). *Method 1*: Compound **29** (1.94 g, 5.0 mmol) was stirred in 48% w/v hydrobromic acid in acetic acid (11 mL) for 1 h. Diethylether was added until a cream precipitate appeared and the ethereal solution decanted. The resulting white sticky solid was suspended in CH_2Cl_2 (100 mL) and 1 M aqueous sodium hydroxide (20 mL). The aqueous layer was extracted with dichloromethane (1 × 50 mL) and the combined organic layers were washed with saturated brine (1 × 50 mL), dried ($MgSO_4$) and concentrated in vacuo to give **25** as a pale yellow oil (975 mg, 4.1 mmol, 82%).

Method 2: A 0.1 M solution of the protected amine **29** (1 g, 2.8 mmol) in methanol (28 mL) was passed through the H-Cube®, which was equipped with a 10% Pd/C Cat-Cart, at 0 bar under the Full H_2 mode. The flow through was collected and concentrated in vacuo to afford **25** as a colourless oil (617 mg, 2.8 mmol, quant.) which was used without further purification. ν_{max} cm^{-1} (NaCl, thin layer) 3299, 3131, 3050, 2945, 2864, 2781, 2305, 1876, 1653, 1605, 1542, 1516, 1264, 1174, 831, 736; ^1H NMR (300 MHz, $CDCl_3$) δ 7.68 (1H, br s, NH), 7.22 (2H, d, J = 8.7 Hz, ArH), 6.57 (2H, d, J = 8.7 Hz, ArH), 2.30 (4H, app. quintet, J = 7.1 Hz, $(CH_2)_2$), 2.23 (6H, s, $N(CH_3)_2$), 1.70 (2H, app. quintet, J = 7.2 Hz, CH_2), 1.53 (2H, app. quintet, J = 7.1 Hz, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.6, 143.1, 129.7, 121.9, 115.4, 58.8, 45.2, 37.0, 26.5, 23.5; LRMS (m/z): $[M+H]^+$ 222.34.

4.1.5. General procedure for the synthesis of analogues 30a–q

To a solution of the acid chloride (1 equiv) in dry acetone (10 mL per mmol) under N_2, was added sodium thiocyanate (1 equiv). The resulting suspension was stirred at room temperature for 30 min before cooling to 0 °C. A solution of amine **25** (1 equiv) in dry acetone (5 mL per mmol) was added and the resulting suspension allowed to warm to room temperature and stirred for 16 h. The reaction was filtered through Celite and the filtrate concentrated in vacuo to give the crude product. The product was purified by column chromatography (CH_2Cl_2/MeOH 10:1) and conversion to the corresponding HCl salt provided **30a–q**.

4.1.5.1. 5-Dimethylamino-pentanoic acid {4-[3-(4-propyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30a). Yield (95 mg, 0.20 mmol, 45%), light yellow solid. Mp 161–162 °C; ν_{max} cm^{-1} (KBr) 3391, 2960, 2871, 2717, 2084, 1668, 1608, 1530, 1408, 1338, 1265, 1149, 838, 738; ^1H NMR (300 MHz, DMSO-d_6): δ 12.58 (1H, s, NH), 11.40 (1H, s, NH), 10.20 (1H, s, NH), 10.10 (1H, br s, NH^+), 7.92 (2H, d, J = 8.2 Hz, ArH), 7.62 (4H, AA'BB', J = 9.0, 20.9 Hz, ArH), 7.36 (2H, d, J = 8.3 Hz, ArH), 3.05 (2H, m, CH_2), 2.73 (6H, d, J = 4.9 Hz, $N(CH_3)_2$), 2.64 (2H, m, CH_2), 2.39 (2H, t, J = 6.5 Hz, CH_2), 1.64 (6H, m, $(CH_2)_3$), 0.90 (3H, t, J = 7.3 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 178.9, 170.8, 168.0, 148.0, 137.4, 132.9, 129.5, 128.7, 128.4, 124.7, 120.7, 56.0, 41.9, 37.0, 35.5, 33.0, 23.7, 23.1, 22.0, 13.6; LRMS (m/z): $[(M-HCl)+H]^+$ 441.20; HRMS (m/z): $[M]^+$ calcd for $C_{24}H_{33}N_4O_2S$, 441.2324; found, 441.2318.

4.1.5.2. 5-Dimethylamino-pentanoic acid {4-[3-(4-isopropyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30b). Yield (25 mg, 0.05 mmol, 34%), light yellow oil. ν_{max} cm^{-1} (NaCl, thin layer) 3246, 2961, 2705, 1668, 1606, 1516, 1408, 1343, 1265, 1150, 838, 768; ^1H NMR (400 MHz, DMSO-d_6): δ 12.59 (s, 1H, NH), 10.21 (s, 1H, NH), 10.16 (br s, 1H, NH), 7.93 (d, J = 8.3 Hz, 2H, ArH), 7.62 (AA'BB', J = 9.0, 27.4 Hz, 4H, ArH), 7.42 (d, J = 8.3 Hz, 2H, ArH), 3.02 (m, 1H, CH), 2.71 (m, 6H, $N(CH_3)_2$), 1.66 (m, 4H, $(CH_2)_2$), 2.37 (m, 2H, CH_2), 1.23 (d, J = 6.9 Hz, 6H, $CHMe_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 180.7, 173.6, 169.6, 155.9, 138.1, 135.1, 121.1, 129.6, 128.0, 125.8, 121.9, 58.7, 43.5, 36.8, 36.7, 25.2, 24.1, 23.3; LRMS (m/z): $[(M-HCl)+H]^+$ 441.22; HRMS (m/z): $[M]^+$ calcd for $C_{24}H_{33}N_4O_2S$, 441.2324; found, 441.2314.

4.1.5.3. 5-Dimethylamino-pentanoic acid {4-[3-(4-butyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30c). Yield (45 mg, 0.09 mmol, 14%), yellow solid. Mp 170–171 °C; ν_{max} cm^{-1} (KBr) 3415, 3250, 3042, 2957, 2933, 2714, 2472, 1675, 1638, 1611, 1529, 1408, 1271, 1147, 842, 742; ^1H NMR (300 MHz, DMSO-d_6): δ 12.58 (1H, s, NH), 11.44 (1H, s, NH), 10.13 (1H, s, NH), 9.70 (1H, br s, NH), 7.91 (2H, d, J = 8.2 Hz, ArH), 7.62 (4H, AA'BB', J = 9, 20.2 Hz, ArH), 7.36 (2H, d, J = 8.3 Hz, ArH), 3.06 (2H, m, CH_2), 2.75 (6H, d, J = 4.9 Hz, $N(CH_3)_2$), 2.67 (2H, m, CH_2), 2.39 (2H, t, J = 6.6 Hz, CH_2), 1.60 (6H, m, $(CH_2)_3$), 1.31 (2H, q, J = 7.3 Hz, CH_2), 0.91 (3H, t, J = 7.3 Hz, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 180.6 (C), 173.6 (C), 169.7 (C), 150.5 (C), 138.2 (C), 135.3 (C), 131.0 (C), 129.8 (CH), 129.3 (CH), 124.8 (CH), 121.0 (CH), 58.6 (CH_2), 43.4 ($N(CH_3)_2$), 36.7 (CH_2), 36.6 (CH_2), 34.7 (CH_2), 25.2 (CH_2), 23.4 (CH_2), 23.2 (CH_2), 13.9 (CH_3) LRMS (m/z): $[(M-HCl)+H]^+$ 455.2; HRMS (m/z): $[M]^+$ calcd for $C_{25}H_{35}N_4O_2S$, 455.2481; found, 455.2484.

4.1.5.4. 5-Dimethylamino-pentanoic acid {4-[3-(4-pentyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30d). Yield (178 mg, 0.35 mmol, 55%), yellow solid. Mp 173–174 °C; ν_{max} cm^{-1} (KBr) 3417, 3258, 3012, 2952, 2929, 2713, 2473, 1672, 1609, 1544, 1513, 1409, 1272, 1143, 840, 734; ^1H NMR (300 MHz, DMSO-d_6): δ 12.58 (1H, s, NH), 11.44 (1H, s, NH),

10.18 (1H, s, NH), 9.98 (1H, br s, NH), 7.91 (2H, d, J = 8.3 Hz, ArH), 7.62 (4H, AA'BB', J = 9.0, 20.1 Hz, ArH), 7.36 (2H, d, J = 8.4 Hz, ArH), 3.02 (2H, m, CH_2), 2.73 (6H, m, $N(CH_3)_2$), 2.66 (2H, m, CH_2), 1.62 (6H, m, $(CH_2)_3$), 1.29 (4H, m, $(CH_2)_2$), 0.86 (3H, t, J = 6.8 Hz, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 180.6, 173.5, 169.7, 150.5, 138.2, 135.3, 131.0, 130.0, 129.4, 125.8, 121.1, 58.7, 43.5, 36.8, 36.7, 32.6, 32.1, 25.2, 23.5, 23.2, 14.4; LRMS (m/z): $[(M-HCl)+H]^+$ 469.25; HRMS (m/z): $[M]^+$ calcd for $C_{26}H_{37}N_4O_2S$, 469.2637; found, 469.2637.

4.1.5.5. 5-Dimethylamino-pentanoic acid [4-(3-benzoyl-thioureido)-phenyl]-amide hydrochloride (30e). Yield (22 mg, 0.05 mmol, 55%), brown solid. Mp 174–175 °C; ν_{max} cm^{-1} (KBr) 3357, 2956, 2925, 2854, 1737, 1668, 1604, 1516, 1464, 1409, 1377, 1261, 1154, 802, 706; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.54 (1H, s, NH), 11.55 (1H, s, NH), 10.16 (1H, s, NH), 9.92 (1H, br s, NH^+), 7.97 (2H, d, J = 7.7 Hz, ArH), 7.59 (7H, m, ArH), 3.06 (2H, m, CH_2), 2.74 (6H, d, J = 4.9 Hz, $N(CH_3)_2$), 2.39 (2H, t, J = 6.9 Hz, CH_2), 1.64 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 180.6, 174.3, 169.9, 138.1, 135.3, 134.4, 133.8, 129.9, 129.3, 126.0, 121.3, 58.8, 44.0, 36.7, 25.2, 23.1; LRMS (m/z): $[(M-HCl)+H]^+$ 398.96; HRMS (m/z): $[M]^+$ calcd for $C_{21}H_{27}N_4O_2S$, 399.1855; found, 399.1861.

4.1.5.6. 5-Dimethylamino-pentanoic acid {4-[3-(4-methyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30f). Yield (46 mg, 0.10 mmol, 16%), pale brown solid. Mp 202–204 °C; ν_{max} cm^{-1} (KBr) 3417, 3257, 3025, 2939, 2675, 2467, 1689, 1666, 1647, 1603, 1521, 1302, 1248, 1155, 839; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.70 (1H, s, NH), 11.44 (1H, s, NH), 10.19 (1H, br s, NH), 8.00 (2H, d, J = 7.8 Hz, ArH), 7.75 (2H, d, J = 7.7 Hz, ArH), 7.45 (2H, d, J = 8.5 Hz, ArH), 7.30 (2H, d, J = 7.8 Hz, ArH), 3.16 (2H, m, CH_2), 2.84 (6H, d, J = 4.6 Hz, $N(CH_3)_2$), 2.42 (2H, m, CH_2), 2.15 (3H, s, CH_3), 1.74 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 180.7, 173.5, 169.7, 145.7, 138.2, 135.3, 130.8, 130.5, 129.6, 126.0, 121.3, 58.7, 43.5, 36.7, 25.2, 23.2, 21.6; LRMS (m/z): $[(M-HCl)+H]^+$ 413.18; HRMS (m/z): $[M]^+$ calcd for $C_{22}H_{29}N_4O_2S$, 413.2011; found, 413.2005.

4.1.5.7. 5-Dimethylamino-pentanoic acid {4-[3-(4-methoxy-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30g). Yield (23 mg, 0.05 mmol, 22%), pale yellow solid. Mp 190–191 °C; ν_{max} cm^{-1} (KBr) 3410, 2956, 2923, 2851, 1725, 1659, 1647, 1605, 1540, 1516, 1464, 1256, 1158, 1025; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.70 (s, 1H, NH), 11.44 (s, 1H, NH), 10.19 (br s, 1H, NH), 8.10 (d, J = 8.7 Hz, 2H, ArH), 7.66 (d, J = 8.7 Hz, 2H, ArH), 7.16 (d, J = 8.7 Hz, 4H, ArH), 3.93 (s, 3H, OMe), 3.08 (m, 2H, CH_2), 2.79 (d, J = 4.7 Hz, 6H, $N(CH_3)_2$), 2.34 (t, J = 8.5 Hz, 2H, CH_2), 1.72 (m, 2H, CH_2), 1.59 (m, 2H, CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 180.9, 174.0, 169.2, 165.5, 138.3, 135.3, 131.5, 125.8, 125.4, 121.2, 115.2, 59.6, 56.1, 44.5, 37.2, 26.7, 24.0; LRMS (m/z): $[(M-HCl)+H]^+$ 429.21; HRMS (m/z): $[M]^+$ calcd for $C_{22}H_{29}N_4O_3S$, 429.1960; found, 429.1970.

4.1.5.8. 5-Dimethylamino-pentanoic acid {4-[3-(4-chloro-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30h). Yield (44 mg, 0.09 mmol, 15%), yellow sticky oil. ν_{max} cm^{-1} (NaCl, thin layer) 3396, 3053, 2925, 2716, 1668, 1605, 1561, 1516, 1485, 1408, 1149, 1013, 842; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.54 (1H, s, NH), 11.76 (1H, s, NH), 10.34 (2H, br s, 2 × NH), 8.08 (2H, d, J = 7.8 Hz, ArH), 7.71 (6H, m, ArH), 3.15 (2H, m, CH_2), 2.82 (6H, d, J = 4.7 Hz, $N(CH_3)_2$), 2.49 (2H, m, CH_2), 1.75 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.7, 170.8, 167.1, 137.9, 137.5, 132.8, 131.0, 130.6, 130.2, 128.6, 128.5, 124.8, 120.1, 119.0, 56.0, 41.8, 35.5, 23.2, 22.0; LRMS (m/z): $[(M-HCl)+H]^+$ 433.15; HRMS (m/z): $[M]^+$ calcd for $C_{21}H_{26}N_4O_2SCl$, 433.1465; found, 433.1452.

4.1.5.9. 5-Dimethylamino-pentanoic acid {4-[3-(4-bromo-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30i). Yield (36 mg, 0.07 mmol, 11%), brown oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.41 (1H, s, NH), 11.65 (1H, s, NH), 10.52 (1H, br s, NH), 10.30 (1H, s, NH), 7.91 (2H, m, ArH), 7.75 (2H, d, J = 8.4 Hz, ArH), 7.66 (2H, m, ArH), 7.58 (2H, m, ArH), 3.06 (2H, m, CH_2), 2.73 (2H, d, J = 4.7 Hz, $N(CH_3)_2$), 2.39 (2H, m, CH_2), 1.66 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.7, 170.8, 167.3, 137.5, 132.7, 131.4, 131.4, 131.3, 129.7, 124.7, 119.2, 56.0, 41.8, 35.5, 23.2, 22.0; LRMS (m/z): $[(M-HCl)+H]^+$ 479.11; HRMS (m/z): $[M]^+$ calcd for $C_{21}H_{26}N_4O_2SBr$, 477.0960; found, 477.0965.

4.1.5.10. 5-Dimethylamino-pentanoic acid {4-[3-(3-methyl-benzoyl)-thioureido]-phenyl}-amide (30j). Yield (41 g, 0.09 mmol, 43%), white-yellow solid. Mp 175–176 °C; ν_{max} cm^{-1} (KBr) 3250, 2921, 2677, 1665, 1651, 1604, 1521, 1406, 1314, 1271, 1184, 837; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.54 (1H, s, NH), 11.50 (1H, s, NH), 10.21 (1H, s, NH), 10.17 (1H, br s, NH), 7.83 (1H, s, ArH), 7.77 (1H, d, J = 7.3 Hz, ArH), 7.66 (1H, d, J = 9.1 Hz, ArH), 7.60 (2H, m, ArH), 7.46 (2H, m, ArH), 7.13 (2H, d, J = 7.7 Hz, ArH), 3.05 (2H, m, CH_2), 2.71 (6H, m, $N(CH_3)_2$), 2.40 (3H, s, CH_3), 2.39 (2H, m, CH_2), 1.65 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 180.6, 174.3, 170.0, 140.0, 138.2, 135.3, 135.1, 133.7, 129.8, 129.7, 126.5, 125.8, 121.1, 59.0, 44.0, 36.8, 25.3, 23.2, 21.4; LRMS (m/z): $[(M-HCl)+H]^+$ 413.19; HRMS (m/z): $[M]^+$ calcd for $C_{22}H_{29}N_4O_2S$, 413.2011; found, 413.2007.

4.1.5.11. 5-Dimethylamino-pentanoic acid {4-[3-(3-methoxy-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30k). Yield (32 mg, 0.07 mmol, 11%), orange oil. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.53 (1H, s, NH), 11.55 (1H, s, NH), 10.17 (1H, s, NH), 9.99 (1H, br s, NH), 7.60 (6H, m, ArH), 7.45 (1H, t, J = 7.9 Hz, ArH), 7.22 (1H, dd, J = 2, 7.9 Hz, ArH), 3.85 (3H, s, OMe), 3.03 (2H, m, CH_2), 2.73 (6H, m, $N(CH_3)_2$), 2.38 (2H, m, CH_2), 1.64 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.8, 173.9, 170.8, 167.9, 159.0, 137.4, 133.4, 129.6, 124.7, 120.9, 119.4, 119.0, 113.2, 56.0, 55.4, 41.9, 35.5, 23.2, 22.0; LRMS (m/z): $[(M-HCl)+H]^+$ 429.17; HRMS (m/z): $[M]^+$ calcd for $C_{22}H_{29}N_4O_3S$, 429.1960; found, 429.1959.

4.1.5.12. 5-Dimethylamino-pentanoic acid {4-[3-(3-chloro-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30l). Yield (28 mg, 0.06 mmol, 23%), orange sticky oil. ν_{max} cm^{-1} (KBr) 3410, 2920, 2671, 2347, 1669, 1647, 1604, 1539, 1514, 1472, 1407, 1335, 1267, 1141, 836, 750; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.39 (1H, s, NH), 11.71 (1H, s, NH), 10.12 (1H, s, NH), 9.65 (1H, br s, NH^+), 8.03 (1H, s, ArH), 7.73 (1H, d, J = 8.2 Hz, ArH), 7.61 (5H, m, ArH), 3.06 (2H, m, CH_2), 2.75 (6H, m, $N(CH_3)_2$), 2.38 (2H, m, CH_2), 1.64 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.6, 174.0, 170.8, 166.8, 137.5, 134.2, 133.0, 132.7, 130.4, 128.4, 127.4, 124.7, 119.0, 56.0, 41.9, 35.5, 23.1, 21.4; LRMS (m/z): $[(M-HCl)+H]^+$ 433.13.

4.1.5.13. 5-Dimethylamino-pentanoic acid {4-[3-(3-bromo-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30m). Yield (74 mg, 0.13 mmol, 21%), yellow solid. Mp 114–115 °C; ν_{max} cm^{-1} (KBr) 3411, 3237, 3046, 2960, 2695, 2058, 1674, 1606, 1521, 1408, 1340, 1265, 1152, 839, 736; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.55 (1H, s, NH), 12.38 (1H, s, NH), 10.14 (1H, s, NH), 9.81 (1H, br s, NH), 8.15 (1H, t, J = 1.8 Hz, ArH), 7.93 (1H, d, J = 7.9 Hz, ArH), 7.85 (1H, d, J = 7.9 Hz, ArH), 7.64 (2H, d, J = 8.9 Hz, ArH), 7.57 (2H, d, J = 8.9 Hz, ArH), 7.49 (1H, t, J – 7.9 Hz, ArH), 3.05 (2H, app t, J = 7.6 Hz, CH_2), 2.70 (6H, d, J = 4.9 Hz, $N(CH_3)_2$), 2.38 (2H, app. t, J = 6.6 Hz, CH_2), 1.71–1.56 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.1, 171.2, 167.1, 137.8, 136.0, 134.8, 133.2, 131.6, 131.0, 128.1, 125.2, 121.8, 119.2, 56.5, 42.3, 35.9, 23.6, 22.3; LRMS

(*m/z*) $[(M-HCl)+H]^+$ 477.03; HRMS (*m/z*): $[M]^+$ calcd for $C_{21}H_{26}N_4O_2BrS$, 477.0960; found, 477.0958.

4.1.5.14. 5-Dimethylamino-pentanoic acid {4-[3-(3-bromo-4-*tert*-butyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30n). Yield (52 mg, 0.09 mmol, 35%), yellow oil. ν_{max} cm^{-1} (NaCl, thin layer) 3242, 3044, 2963, 2925, 2872, 2694, 2058, 1671, 1604, 1517, 1480, 1408, 1362, 1144, 839, 731; ^1H NMR (400 MHz, DMSO-d_6): δ 12.59 (1H, s, NH), 12.44 (1H, s, NH), 10.14 (1H, s, NH), 9.87 (1H, br s, NH^+), 8.22 (1H, d, *J* = 2.0 Hz, ArH), 7.92 (1H, dd, *J* = 2 Hz, 8.4 Hz, ArH), 7.67–7.60 (3H, m, ArH), 7.57 (2H, d, *J* = 8.9 Hz, ArH), 3.09–3.01 (2H, m, CH_2), 2.73 (6H, d, *J* = 4.8 Hz, $N(CH_3)_2$), 2.38 (2H, app. t, *J* = 7.1 Hz, CH_2), 1.73–1.56 (4H, m, $(CH_2)_2$), 1.49 (9H, s, $(CH_3)_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 180.5, 173.8, 168.0, 154.5, 138.2, 136.8, 135.2, 133.0, 130.8, 128.6, 126.0, 123.7, 121.3, 58.7, 44.0, 36.7, 30.3, 25.2, 23.2; LRMS (*m/z*) $[(M-HCl)+H]^+$ 533.16; HRMS (*m/z*): $[M]^+$ calcd for $C_{25}H_{34}N_4O_2BrS$, 533.1586; found, 533.1579.

4.1.5.15. 5-Dimethylamino-pentanoic acid {4-[3-(3,4-dichloro-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30o). Yield (90 mg, 0.18 mmol, 52%), brown oil. ^1H NMR (300 MHz, DMSO-d_6): δ 12.29 (1H, s, NH), 12.11 (1H, s, NH), 10.26 (1H, s, NH), 9.93 (1H, br s, NH^+), 7.88 (2H, d, *J* = 1.93 Hz, ArH), 7.72 (5H, m, ArH), 3.16 (2H, m, CH_2), 2.84 (6H, br s, $N(CH_3)_2$), 2.48 (2H, m, CH_2), 1.74 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 Hz, CD_3OD) δ 180.0, 173.6, 168.4, 138.6, 138.3, 125.2, 134.3, 133.4, 131.6, 131.0, 128.7, 125.8, 121.2, 58.7, 43.5, 36.8, 25.2, 23.3; LRMS (*m/z*): $[(M-HCl)+H]^+$ 467.11

4.1.5.16. 5-Dimethylamino-pentanoic acid {4-[3-(2-methyl-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30p). Yield (28 mg, 0.06 mmol, 10%), white solid. Mp 201–202 °C; ν_{max} cm^{-1} (KBr) 3388, 3227, 3041, 2957, 1590, 1716, 1674, 1651, 1604, 1521, 1406, 1308, 1256, 1165, 835, 746; ^1H NMR (400 MHz, DMSO-d_6): δ 12.46 (1H, s, NH), 11.69 (1H, s, NH), 10.20 (1H, s, NH), 10.13 (1H, br s, NH), 7.63 (4H, AA'BB', *J* = 9.1, 21.5 Hz, ArH), 7.50 (1H, d, *J* = 7.4 Hz, ArH), 7.43 (1H, m, ArH), 7.31 (2H, d, *J* = 8.0 Hz, ArH), 3.06 (2H, m, CH_2), 2.73 (6H, d, *J* = 4.9 Hz, $N(CH_3)_2$), 2.42 (3H, s, CH_3), 2.39 (2H, m, CH_2), 1.65 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, CD_3OD) 180.6, 173.7, 172.3, 138.2, 138.0, 135.4, 135.3, 132.4, 132.2, 128.7, 127.0, 125.8, 121.1, 58.7, 43.5, 36.7, 25.2, 23.2; LRMS (*m/z*): $[(M-HCl)+H]^+$ 413.15; HRMS (*m/z*): $[M]^+$ calcd for $C_{22}H_{29}N_4O_2S$, 413.2011; found, 413.1998.

4.1.5.17. 5-Dimethylamino-pentanoic acid {4-[3-(2-chloro-benzoyl)-thioureido]-phenyl}-amide hydrochloride (30q). Yield (22 mg, 0.05 mmol, 8%), yellow solid. Mp 188–190 °C; ν_{max} cm^{-1} (KBr) 3311, 3042, 2956, 2926, 2855, 2703, 1667, 1604, 1516, 1467, 1408, 1344, 1291, 1155, 839, 752; ^1H NMR (300 MHz, DMSO-d_6): δ 12.27 (1H, s, NH), 12.00 (1H, s, NH), 10.10 (1H, s, NH), 9.45 (1H, br s, NH), 7.56 (8H, m, ArH), 3.07 (2H, m, CH_2), 2.76 (6H, d, *J* = 3.7 Hz, $N(CH_3)_2$), 2.39 (2H, t, *J* = 6.6 Hz, 2H CH_2), 1.64 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 180.2, 174.0, 168.0, 138.2, 136.7, 136.5, 135.8, 135.2, 133.4, 132.4, 130.3, 125.8, 121.9, 58.7, 43.4, 36.7, 25.2, 23.1; LRMS (*m/z*): $[(M-HCl)+H]^+$ 433.11; HRMS (*m/z*): $[M]^+$ calcd for $C_{21}H_{26}N_4O_2SCl$, 433.1465; found, 433.1469.

4.1.6. Synthesis of 33

4.1.6.1. 5-Dimethylamino-pentanoic acid {4-[3-(4-*tert*-butyl-benzoyl)-ureido]-phenyl}-amide hydrochloride (33). 4-*t*-Butyl benzamide (**31**) (250 mg, 1.41 mmol) was added to a dry flask containing molecular sieves under N_2. 1,2-Dichloroethane (5 mL) was added and the mixture stirred whilst a solution of 2 M oxalyl chloride in CH_2Cl_2 (1.19 mL, 2.30 mmol) was added. The clear yellow solution was heated at reflux for 24 h and cooled to rt. The reaction was then concentrated in vacuo. After redissolving the residue in dry acetonitrile (5 mL) aniline **18** was added. The resulting mixture was heated at reflux for 3 h and after cooling, the resulting beige precipitate was collected by filtration to give **32** (400 mg, 0.24 mmol, 17%) as a beige solid which was not purified further. Mp decomp. >310 °C; ν_{max} cm^{-1} (KBr) 3396, 3363, 3244, 2972, 2905, 2597, 1694, 1674, 1607, 1559, 1521, 1479, 1411, 1367, 1265, 1220, 1164, 1058, 828, 771; ^1H NMR (300 MHz, DMSO-d_6) δ 10.91 (1H, s, NH), 10.77 (1H, s, NH), 9.31 (1H, s, NH), 7.97 (2H, d, J = 8.6 Hz, ArH), 7.55 (2H, d, J = 8.6 Hz, ArH), 7.43 (4H, m, ArH), 1.47 (9H, s, $(CH_3)_3$), 1.31 (9H, s, $(CH_3)_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.4, 156.1, 152.8, 151.1, 135.5, 131.9, 129.5, 128.2, 125.4, 120.3, 118.6, 78.9, 34.8, 30.8, 28.1.

N-Acyl urea **32** (300 mg, 0.73 mmol) was stirred in trifluoroacetic acid (1.2 mL) for 40 min and concentrated in vacuo to give the corresponding aniline 1-(4-amino-phenyl)-3-(4-*tert*-butyl-benzoyl)-urea as the trifluoroacetate salt (383 mg, 0.73 mmol, quantitative) as a brown solid which was not purified further. ^1H NMR (300 MHz, DMSO-d_6) δ 11.02 (1H, s, NH), 10.96 (1H, s, NH), 7.98 (2H, d, *J* = 8.6 Hz, ArH), 7.70 (2H, d, *J* = 8.9 Hz, ArH), 7.55 (2H, d, *J* = 8.6 Hz, ArH), 7.32 (2H, d, *J* = 8.8 Hz, ArH), 1.30 (9H, s, $(CH_3)_3$). The deprotected aniline (368 mg, 0.70 mmol) was then stirred in dry CH_2Cl_2 (10 mL) and triethylamine (98 μL, 0.70 mmol) was added to give a clear brown solution. 5-Bromovaleryl chloride (140 μL, 1.05 mmol) was added followed by triethylamine (98 μL, 0.70 mmol). The reaction mixture was stirred at rt for 16 h and then diluted with CH_2Cl_2 (1 × 20 mL) and washed with 1 M aqueous hydrochloric acid (1 × 20 mL), 1 M aqueous sodium hydroxide (1 × 20 mL) and saturated brine (1 × 20 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo to give the crude product which was purified by column chromatography on silica (petroleum ether/ethyl acetate 1:1) to give *N*-((4-(5-bromopentanamido)phenyl)carbamoyl)-4-(*tert*-butyl)-benzamide (153 mg, 0.34 mmol, 48%) as a yellow solid. ^1H NMR (300 MHz, DMSO-d_6) δ 10.93 (1H, s, NH), 10.82 (1H, s, NH), 9.90 (1H, s, NH), 7.98 (2H, d, *J* = 8.5 Hz, ArH), 7.54 (6H, m, ArH), 3.57 (2H, t, *J* = 6.6 Hz, CH_2), 2.33 (2H, t, *J* = 7.2 Hz, CH_2), 1.80 (4H, m, $(CH_2)_2$), 1.31 (9H, s, $(CH_3)_3$). The bromide (123 mg, 0.27 mmol) was then stirred in CH_2Cl_2 (10 mL), 40% aqueous dimethylamine (3 mL) and H_2O (7.5 mL) at rt for 20 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and washed with H_2O (1 × 10 mL), 10% aqueous NaOH (1 × 10 mL), and saturated brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to give a cream solid which was dissolved in acetone and treated with 2 M hydrochloric acid in diethyl ether. A white precipitate was obtained and isolated by filtration to give **33** (44 mg, 0.10 mmol 36%) as a cream solid. Mp 247–249 °C; ν_{max} cm^{-1} (KBr) 3415, 3238, 2965, 2903, 2659, 2471, 1699, 1667, 1610, 1570, 1514, 1481, 1409, 1275, 1228, 1128, 836, 770; ^1H NMR (400 MHz, DMSO-d_6) δ 10.93 (1H, s, NH), 10.82 (1H, s, NH), 9.98 (1H, s, NH), 9.63 (1H, br s, NH), 7.98 (2H, d, *J* = 8.6 Hz, ArH), 7.53 (6H, m, ArH), 3.05 (2H, m, CH_2), 2.75 (6H, s, $N(CH_3)_2$), 2.36 (2H, t, *J* = 6.7 Hz, CH_2), 1.64 (4H, m, $CH_2)_2$), 1.31 (9H, s, $(CH_3)_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.5, 169.4, 157.1, 152.1, 136.2, 133.8, 130.5, 129.5, 126.5, 121.5, 120.5, 57.2, 43.2, 36.5, 35.8, 32.0, 24.4, 23.0; LRMS (*m/z*): $[M]^+$ 439.13, $[(M-HCl)+H]^+$; HRMS (*m/z*): $[M]^+$ calcd for $C_{25}H_{35}N_4O_3$, 439.2709; found, 439.2696.

4.1.7. Synthesis of 39

4.1.7.1. Benzyl (4-((4-hydroxybutyl)carbamoyl)phenyl)carbamate (36). To a solution of the known acid **35** (1 g, 3.69 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added triethylamine (1.03 mL, 7.38 mmol) and methyl chloroformate (0.57 mL, 7.38 mmol) dropwise. The resulting solution was allowed to warm to rt and stirred for 2 h before cooling to 0 °C and 4-aminobutanol (0.68 mL, 7.38 mmol) added dropwise and the mixture stirred at rt for 6 h.

The solvent was removed in vacuo and water added (20 mL). The resulting white precipitate was filtered to afford **36** as a white solid (0.9 g, 2.66 mmol, 72%). Mp 164–165 °C; ν_{max} cm^{-1} (KBr) 3567, 3381, 3294, 3030, 2952, 2866, 2741, 1792, 1699, 1667, 1628, 1611, 1587, 1524, 1411, 1312, 1245, 1061, 736, 696; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (1H, s, NH), 7.49 (2H, d, J = 8.8 Hz, ArH), 7.44–7.32 (7H, m, ArH), 6.85 (1H, s, NH), 5.21 (1H, s, NH), 2.58 (1H, s, OH), 2.44–2.37 (4H, m, $(CH_2)_2$), 1.79 (2H, app. quintet, J = 7.5 Hz, CH_2), 1.60 (2H, app. quintet, J = 7.2 Hz, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.3, 154.2, 143.1, 134.6, 130.4, 128.3, 128.1, 119.5, 66.1, 58.7, 38.3, 26.1, 22.9; LRMS (m/z): $[M]^-$ 341.29 $[M-H]^-$;

4.1.7.2. Benzyl (4-((4-(dimethylamino)butyl)carbamoyl)phenyl) carbamate (37). To a stirred solution of **31** (1.2 g, 3.5 mmol) in CH_2Cl_2 (40 mL) was added CBr_4 (1.28 g, 3.9 mmol) and the solution cooled to 0 °C before PPh_3 (1.02 g, 3.9 mmol) was added slowly. The resulting mixture was allowed to warm to rt and stirred for 4 h. The solvent was removed and the residue purified by column chromatography on silica (CH_2Cl_2:MeOH 10:0.5) to afford the bromide (764 mg, 1.89 mmol, 54%) which was used immediately in the next step. Mp 161–162 °C; ν_{max} cm^{-1} (KBr) 3327, 3091, 2937, 1701, 1668, 1632, 1611, 1588, 1521, 1413, 1315, 1245, 1070, 738; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (2H, d, J = 8.7 Hz, ArH), 7.49 (2H, d, J = 8.3 Hz, ArH), 7.46–7.37 (5H, m, ArH), 6.88 (1H, s, NH), 6.14 (1H, t, J = 5.2 Hz, NH), 5.24 (2H, s, CH_2), 3.55–3.47 (4H, m, $(CH_2)_2$), 1.99 (2H, app. quintet, J = 6.7 Hz, CH_2), 1.81 (2H, app. quintet, J = 7.4 Hz, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.0, 167.2, 153.3, 141.1, 136.1, 129.1, 128.9, 128.8, 128.5, 118.4, 67.7, 39.5, 33.8, 30.4, 28.8. To a solution of the bromide (300 mg, 0.74 mmol) in CH_2Cl_2 (3 mL) was added a 40% aqueous solution of dimethylamine (1.5 mL) and water (12 mL) and the resulting biphasic mixture stirred at rt for 18 h. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with H_2O (1 × 10 mL), 10% aqueous sodium hydroxide (1 × 10 mL), and saturated brine (1 × 10 mL). The organic phase was dried over $MgSO_4$ and concentrated in vacuo to give **37** as an off-white sticky oil (185 mg, 0.50 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (1H, t, J = 5.4 Hz, NH), 7.74 (2H, d, J = 8.6 Hz, ArH), 7.48 (2H, d, J = 8.6 Hz, ArH), 7.42–7.34 (5H, m, ArH), 5.20 (2H, s, CH_2), 3.46–3.39 (2H, m, CH_2), 2.33 (2H, t, J = 6.4 Hz, CH_2), 2.22 (6H, s, $N(CH_3)_2$), 1.72–1.57 (4H, m, $(CH_2)_2$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.4, 167.0, 153.2, 141.0, 136.2, 131.3, 129.1, 128.8, 128.6, 118.2, 67.6, 59.5, 45.5, 40.2, 27.7, 25.5; LRMS (m/z): $[M]^+$ 370.25 $[M+H]^+$; HRMS calcd for $C_{21}H_{28}N_3O_3$, 370.2131; found, 370.2131.

4.1.7.3. 4-Amino-*N*-(4-(dimethylamino)butyl)benzamide (38). A 0.1 M solution of the protected amine **32** (185 mg, 0.50 mmol) in methanol (5.2 mL) was passed through the H-Cube®, which was equipped with a 10% Pd/C Cat-Cart, at 0 bar under the Full H_2 mode. The flow through was collected and concentrated in vacuo to afford **38** as a colourless oil (118 mg, 0.50 mmol, quant.) which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (2H, d, J = 8.6 Hz, ArH), 7.21 (1H, br s, NH), 6.67 (2H, d, J = 8.6 Hz, ArH), 3.97 (2H, br s, NH_2), 3.45 (2H, app. quintet, J = 5.9 Hz, CH_2), 2.36 (2H, app. t, J = 6.7 Hz, CH_2), 2.25 (6H, s, NMe_2), 1.73–1.59 (4H, m, $(CH_2)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 143.1, 131.7, 120.6, 116.3, 59.8, 45.9, 38.0, 26.2, 23.7; LRMS (m/z): $[M+H]^+$ 222.45.

4.1.7.4. 4-(*tert*-Butyl)-*N*-((4-((4-(dimethylamino)butyl)carbamoyl)phenyl)-carbamothioyl)benzamiide hydrochloride (39). To a solution of **4** (77 μL, 0.42 mmol) in dry acetone (4 mL) under N_2, was added sodium thiocyanate (34 mg, 0.42 mmol) The resulting suspension was stirred at room temperature for 30 min. before being cooled to 0 °C. A solution of amine **38** (99 mg, 0.42 mmol) in dry acetone (3 mL) was added and the resulting suspension allowed to warm to rt and stirred for 16 h. The reaction was filtered through celite and the filtrate concentrated to give the crude product. The product was purified by column chromatography (CH_2Cl_2:MeOH 10:1) and conversion to the corresponding HCl salt provided **39** (177 mg, 0.36 mmol, 86%). Mp 184–186 °C; ν_{max} cm^{-1} (KBr) 3253, 2921, 2641, 2478, 1709, 1662, 1609, 1564, 1365, 1261, 1126, 1014, 960, 849, 765; ^{1}H NMR (400 MHz, d_6-DMSO) δ 12.80 (1H, s, NH), 11.54 (1H, s, NH), 10.30 (1H, br s, NH), 8.63 (1H, t, J = 5.8 Hz, NH), 7.96–7.89 (4H, m, ArH), 7.82 (2H, d, J = 8.6 Hz, ArH), 7.56 (2H, d, J = 8.6 Hz, ArH), 3.28 (2H, app. quartet, J = 6.8 Hz, CH_2), 3.08–3.02 (2H, m, CH_2), 2.71 (6H, d, J = 4.9 Hz, $N(CH_3)_2$), 1.74–1.65 (2H, m, CH_2), 1.55 (2H, app. quartet, J = 6.9 Hz, CH_2), 1.31 (9H, s, $(CH_3)_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.3, 165.6, 156.2, 151.0, 140.2, 129.4, 129.3, 128.6, 125.6, 125.4, 118.8, 56.2, 42.0, 34.8, 30.8, 26.1, 21.2; LRMS (m/z): $[(M-HCl)+H]^+$ 455.25; HRMS (m/z): $[M]^+$ calcd for $C_{25}H_{34}N_4O_2S$, 455.2474; found, 455.2474.

4.2. Inhibition of SirT1 and SirT2

Compounds were tested for inhibition of human recombinant SirT1 and SirT2 using the Fluor de Lys fluorescence-based assay kits provided by Biomol (Plymouth Meeting, USA). The assays were carried out as described in Lain et al.[8a], except the final concentration of FdL substrate used per reaction was 25 μM. Raw data was processed using Microsoft Excel. SigmaPlot (Systat Software Inc.) was used to fit the data to a 4-parameter equation which was used to calculate the concentration of inhibitor resulting in 50% inhibition (IC_{50} value). Compounds were tested on two separate occasions for % inhibition at 60 μM and the values were used to calculate a mean weighted to the standard error. For IC_{50} values, compounds were tested on three separate occasions and the IC_{50} values were used to calculate a mean weighted to a standard deviation. These values are given in Tables 1 and 2.

4.3. Cell culture and Western Blotting

Human MCF-7 and H1299 cancer cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, UK) and RPMI respectively. Both were supplemented with 10% foetal calf serum (FCS, Hyclone, UK) and gentamycin (complete medium). Cells were seeded at a concentration of 2×10^5 (MCF-7) and 6×10^4 (H1299) in 6 well collagen precoated plates (TPP, Helena Biosciences, UK) and incubated in a humidified atmosphere containing 5% CO2–95% air at 37 °C for 42 h (MCF-7) and 24 h (H1299). Different concentrations of target compounds in DMSO were added to MCF-7 cells which were incubated for a further 6 h. Different concentrations of target compounds in DMSO were also added to H1299 cells, with 40 nM trichostatin (TSA) also added and incubated for a further 16 h. After lysing the cells with 1 × LDS sample buffer (100 μL per well for MCF-7 and 200 μL for H1299) (Invitrogen, UK), the protein concentration was assessed with a BCA protein assay kit (Pierce, UK) and the concentration of protein equalised with 1 × LDS sample buffer. Proteins were separated with 4–12% bis-tris gels (Invitrogen, USA) and electrophoretically transferred to PVDF transfer membranes (Millipore, UK). Membranes were blocked with Marvel nonfat milk (45 min, 5% solution in PBS/0.1% tween) and immunoblotted using DO.1 (anti p53, in-house produced) for the MCF-7 membranes. PC-10 mouse monoclonal antibody (in-house produced) was used to detect PCNA as a loading control. Anti-K40 acetylated α-tubulin (SIGMA) and anti α-tubulin (SIGMA) were used for the H1299 membranes. All the primary antibodies were diluted in Marvel nonfat milk (5% solution in PBS/0.1% tween). The secondary antibody used against the primary antibodies was a HRP-tagged polyclonal rabbit antimouse IgG (DAKO, UK). After incubation with

primary (1 h) and secondary antibodies (45 min), bound antibody was visualised with enhanced chemiluminescence (ECL) Western blotting developer (Amersham, UK) in a darkroom. Use of the β-galactosidase assay has been reported previously by us using T22 cells. In this study, the only change to this protocol was the use of ARN8 cells instead of T22s.[7b,c]

4.4. Cell assay using cellular extract containing K40-acetylated α-tubulin

H1299 cells were treated with trichostatin A (100 nM) for 6 h. Cytoplasmic extracts were prepared following the Dignam method and combined with dd H_2O, 10 × complete EDTA-free protease inhibitor (Roche), 527 mM Tris pH 7.5, 5 mM NAD^+, 5 mM DTT, 750 mM NaCl, 0.5 mM trichostatin A and 2 mM PMSF and incubated on ice prior to the addition of recombinant SirT1 (0.23 and 0.36 μM final concentrations, Enzo Life Sciences). Reactions were carried out for 90 min at 37 °C. Levels of K40-acetylated α-tubulin, total α-tubulin and SirT1 were detected by Western Blot analysis. Antibodies against K40-acetylated tubulin and tubulin (as described above) and SirT1 (# 05-1243, Millipore) were used.

4.5. SirT1 overexpression

H1299 cells were transfected with pcDNA3 or pcDNA3-SirT1, which was prepared by cloning SirT1 from pCMV-SirT1 (Origene) into pcDNA3. K40-acetylated tubulin, tubulin and SirT1 levels were detected by western blotting as described.

4.6. Protocol for solubility analysis

Relative solubilities of compounds were determined using a UV spectroscopy based method. Generation of the extinction coefficient for each compound was achieved by dissolving 1 mg of compound in spectroscopic grade acetonitrile (100 mL) (some compounds required dissolving in a minimal amount of DMSO first). A series of 6 × 2 fold dilutions were then made, giving a total of 7 solutions. These solutions were analysed by UV spectroscopy and their absorbance at 273 nM was plotted against molar concentration. The extinction coefficient was calculated from the gradient of the line of best fit. The extinction coefficient was then used to calculate the actual concentration of a 100 μM aqueous solution of compound.

The actual concentration of the 100 μM aqueous solution was determined by diluting an aliquot (4.2 μL) of a 40 mM DMSO solution to 1 mL with water. An aliquot (60 μL) of this solution was added to water (40 μL) and any undissolved compound was pelleted by centrifugation. The supernatant (80 μL) was diluted to 4 mL with acetonitrile to generate a theoretical 2×10^{-6} M solution. This solution was then analysed by UV spectroscopy.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.001.

References and notes

1. Haigis, M. C.; Sinclair, D. A. *Annu. Rev. Pathol.* **2010**, *5*, 253.
2. (a) Sauve, A. A.; Wolberger, C.; Schramm, V. L.; Boeke, J. D. *Annu. Rev. Biochem.* **2006**, *75*, 435; (b) Fahie, K.; Ho, P.; Swatkoski, S.; Cotter, R. J.; Zhang, Y.; Wolberger, C. *FEBS J.* **2009**, *276*, 7159; (c) Hawse, W. F.; Wolberger, C. *J. Biol. Chem.* **2009**, *284*, 33654.
3. (a) Vaziri, H.; Dessain, S. K.; Ng Eaton, E.; Imai, S. I.; Frye, R. A.; Pandita, T. K.; Guarente, L.; Weinberg, R. A. *Cell* **2001**, *107*, 149; (b) Luo, J.; Nikolaev, A. Y.; Imai, S.; Chen, D.; Su, F.; Shiloh, A.; Guarente, L.; Gu, W. *Cell* **2001**, *107*, 137; (c) Langley, E.; Pearson, M.; Faretta, M.; Bauer, U. M.; Frye, R. A.; Minucci, S.; Pelicci, P. G.; Kouzarides, T. *EMBO J.* **2002**, *21*, 2383; (d) Yi, J.; Luo, J. *Biochim. Biophys. Acta, Proteins Proteomics* **2010**, *1804*, 1684; (e) Van Leeuwen, I.; Lain, S. *Adv. Cancer Res.* **2009**, *102*, 171.
4. (a) Outeiro, T. F.; Kontopoulos, E.; Altmann, S. M.; Kufareva, I.; Strathearn, K. E.; Amore, A. M.; Volk, C. B.; Maxwell, M. M.; Rochet, J.; McLean, P. J.; Young, A. B.; Abagyan, R.; Feany, M. B.; Hyman, B. T.; Kazantsev, A. G. *Science* **2007**, *317*, 516; (b) Garske, A. L.; Smith, B. C.; Denu, J. M. *ACS Chem. Biol.* **2007**, *2*, 529; (c) Luthi-Cartera, R.; Taylora, D. M.; Pallos, J.; Lambert, E.; Amore, A.; Parker, A.; Moffitt, H.; Smith, D. L.; Runne, H.; Gokce, O.; Kuhn, A.; Xiang, Z.; Maxwell, M. M.; Reeves, S. A.; Bates, G. P.; Neri, C.; Thompson, L. M.; Marsh, J. L.; Kazantsev, A. G. *PNAS* **2010**, *107*, 7927; (d) Harting, K.; Knoell, B. *Eur. J. Cell Biol.* **2010**, *89*, 262.
5. (a) Howitz, K. T.; Bitterman, K. J.; Cohen, H. Y.; Lamming, D. W.; Lavu, S.; Wood, J. G.; Zipkin, R. E.; Chung, P.; Kisielewski, A.; Zhang, L.; Scherer, B.; Sinclair, D. A. *Nature* **2003**, *425*, 191; For recent reviews see: (b) Dittenhafer-Reed, K. E.; Feldman, J. L.; Denu, J. M. *ChemBioChem* **2011**, *12*, 281; Camins, A.; Sureda, F. X.; Junyent, F.; Verdaguer, E.; Folch, J.; Pelegri, C.; Vilaplana, J.; Beas-Zarate, C.; Pallas, M. *Biochim. Biophys. Acta* **2010**, *1799*, 740; Sauve, A. A. *Curr. Pharm. Des.* **2009**, *15*, 45; Alcaín, F. J.; Villalba, J. M. *Expert Opin. Ther. Patents* **2009**, *19*, 403.
6. For recent examples of the many excellent reviews see: (a) Blum, C. A.; Ellis, J. L.; Loh, C.; Ng, P. Y.; Perni, R. B.; Stein, R. L. *J. Med. Chem.* **2011**, *54*, 417; (b) Schemies, J.; Uciechowska, U.; Sippl, W.; Jung, M. *Med. Res. Rev.* **2010**, 861; (c) Cen, Y. *Biochim. Biophys. Acta, Proteins Proteomics* **2010**, *1804*, 1635; (d) Alcaín, F. J.; Villalba, J. M. *Expert Opin. Ther. Patents* **2009**, *19*, 283; (e) Li, H.; Simon, J.; Bedalov, A. *Methods Princ. Med. Chem.* **2009**, *42*, 225; (f) Grubisha, O.; Smith, B. C.; Denu, J. M. *FEBS J.* **2005**, *272*, 4607; g Mai, A.; Massa, S.; Lavu, S.; Pezzi, R.; Simeoni, S.; Ragno, R.; Mariotti, F. R.; Chiani, F.; Camilloni, G.; Sinclair, D. A. *J. Med. Chem.* **2005**, *48*, 7789; Grozinger, C. M.; Schreiber, S. L. *Chem. Biol.* **2002**, *9*, 3.
7. (a) Heltweg, B.; Gatbonton, T.; Schuler, A. D.; Posakony, J.; Li, H.; Goehle, S.; Kollipara, R.; Depinho, R. A.; Gu, Y.; Simon, J. A.; Bedalov, A. *Cancer Res.* **2006**, *66*, 4368; (b) Medda, F.; Russell, R. J. M.; Higgins, M.; McCarthy, A. R.; Campbell, J.; Slawin, A. M. Z.; Lane, D. P.; Lain, S.; Westwood, N. J. *J. Med. Chem.* **2009**, *52*, 2673; (b) Medda, F.; Joseph, T. L.; Pirrie, L.; Higgins, M.; Campbell, J.; Lain, S.; Verma, C.; Westwood, N. *J. MedChemCommun.* **2011**, *2*, 611.
8. (a) Lain, S.; Hollick, J. J.; Campbell, J.; Staples, O. D.; Higgins, M.; Aoubala, M.; McCarthy, A. R.; Appleyard, V.; Murray, K. E.; Baker, L.; Thompson, A.; Mathers, J.; Holland, S. J.; Stark, M. J.; Pass, G.; Woods, J.; Lane, D. P.; Westwood, N. J. *Cancer Cell* **2008**, *13*, 377; b McCarthy, A. R.; Hollick, J. J.; Westwood, N. J. *Semin. Cancer Biol.* **2010**, *20*, 40.
9. See ESI for Supplementary figures and tables.
10. Loiseau, F. A.; Hii, K. K.; Hill, A. M. *J. Org. Chem.* **2004**, *69*, 639.
11. Crystallographic data (excluding structure factors) for **4iv** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 857728. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
12. Klabunde, T.; Wendt, K. U.; Kadereit, D.; Brachvogel, V.; Burger, H.; Herling, A. W.; Oikonomakos, N. G.; Kosmopoulou, M. N.; Schmoll, D.; Sarubbi, E.; Von Roedern, E.; Schönafinger, K.; Defossa, E. *J. Med. Chem.* **2005**, *48*, 6178.
13. Klotz, P.; Chatrenet, B.; Coppo, M.; Rousseau, B.; Goeldner, M.; Hirth, C. *J. Labelled Compd. Radiopharm.* **1991**, *29*, 149.
14. For a recent publication on the challenges of X-ray crystallographic analysis of SirT1 see: Holbourn, K. P.; Lloyd, M. D.; Thompson, A. S.; Threadgill, M. D.; Acharya, K. R. *Acta Crystallogr., Sect. F* **2011**, *67*, 461.
15. Katori, E.; Nagano, T.; Kunieda, T.; Hirobe, M. *Chem. Pharm. Bull.* **1981**, *29*, 3075.
16. Chung, K.; Murdock, C. A.; Stevens, S. E., Jr.; Li, Y.; Wei, C.; Huang, T.; Chou, M. W. *Toxicol. Lett.* **1995**, *81*, 23.
17. North, B. J.; Marshall, B. L.; Borra, M. T.; Denu, J. M.; Verdin, E. *Mol. Cell* **2003**, *11*, 437.