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Synthesis and in vitro evaluation of novel pro-drugs for the treatment of nephropathic cystinosis

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ABSTRACT

As part of our continuing work to obtain new pro-drugs for the treatment of nephropathic cystinosis, a number of glutaric and succinic acid derivatives of cystamine have been designed, synthesised and biologically evaluated in vitro. These compounds have been designed as odourless and tasteless pro-drugs which will release multiple molecules of cysteamine upon administration. All of the synthesised compounds evaluated in this study were non-cytotoxic and displayed a greater ability than cysteamine to deplete the levels of cystine in cultured fibroblasts.

1. Introduction

Nephropathic cystinosis is a rare, autosomal, recessive disease characterised by raised intracellular levels of the amino acid, cystine. Crystals of cystine are present in lysosomes, bone marrow aspirates, leucocytes, cornea, and conjunctiva. Symptoms of the disease include poor growth, renal Fanconi syndrome (impairment in proximal tubule function), renal glomerular failure and accumulation of cystine crystals in almost all cells, leading to tissue damage. Treatment begun just after birth can attenuate the rate of renal failure; however glomerular damage present at the time of diagnosis (approximately 12 months) is irreversible and may result in the need for renal transplant.[1]

The disease is caused by a defect in the lysosomal transport mechanism for cystine and results from mutations in the CTNS gene, which codes for cystinosin, a membrane transport protein.[2] Treatment of cystinosis includes administration of electrolytes to reverse the effects of Fanconi syndrome, phosphate, vitamin D carnitine and human growth hormone in addition to corneal and renal transplant. Administration of the aminothiol, cysteamine ($H_2NCH_2CH_2SH$, marketed as the bitartrate salt, Cystagon™), acts to lower intracellular levels of cysteine by forming a cysteamine–cysteine mixed disulphide which can egress the lysosome using the lysine transporter excretion pathway which remains intact in cystinosis.[3]

There are major problems, however, with administration of Cystagon. The molecule possesses an offensive taste and smell and irritates the gastrointestinal (GI) tract leading to nausea and vomiting following administration. In addition, cysteamine and its metabolites are excreted in breath and sweat, which leads to halitosis and body odour. Furthermore, some patients exhibit more serious side-effects, such as neutropenia. As a result of these problems, patient compliance can be poor.[4] In addition, because of a combination of high first-pass metabolism and a short half-life, Cystagon only removes cystine crystals for a period of 6 h after the medication has been taken. This means that it must be given every 6 h, every day for life.[4,5]

In the last few years, efforts in our laboratory have been concentrated on the design of pro-drugs of cysteamine that produce the same, or even better, cystine depleting ability than the current treatment but are devoid of the bad taste and smell of the parent drug.[6–8] The aim of the current project is to extend previous work by synthesising novel pro-drugs that in addition to be odourless and tasteless, release multiple molecules of cysteamine (up to six per pro-drug). Furthermore, in an attempt to increase the uptake of the drug into intestinal mucosal cells[9]; we have incorporated the amino acid, phenylalanine, at the amino terminus of pro-drugs **1**-**c** and **1**-**d**.

2. Chemistry

Compounds **1a**–**d** were obtained from the disulphide derivative of cysteamine, cystamine dihydrochloride **2** (Scheme 1). The latter was transformed to *N*,*N*′-disuccinoyl[10] and *N*,*N*′-diglutaroyl[11] derivatives **3a**–**b**, with almost quantitative yields, by reaction with the corresponding dicarboxylic anhydrides in basic aqueous solution. The activation of terminal carbonyl groups of the

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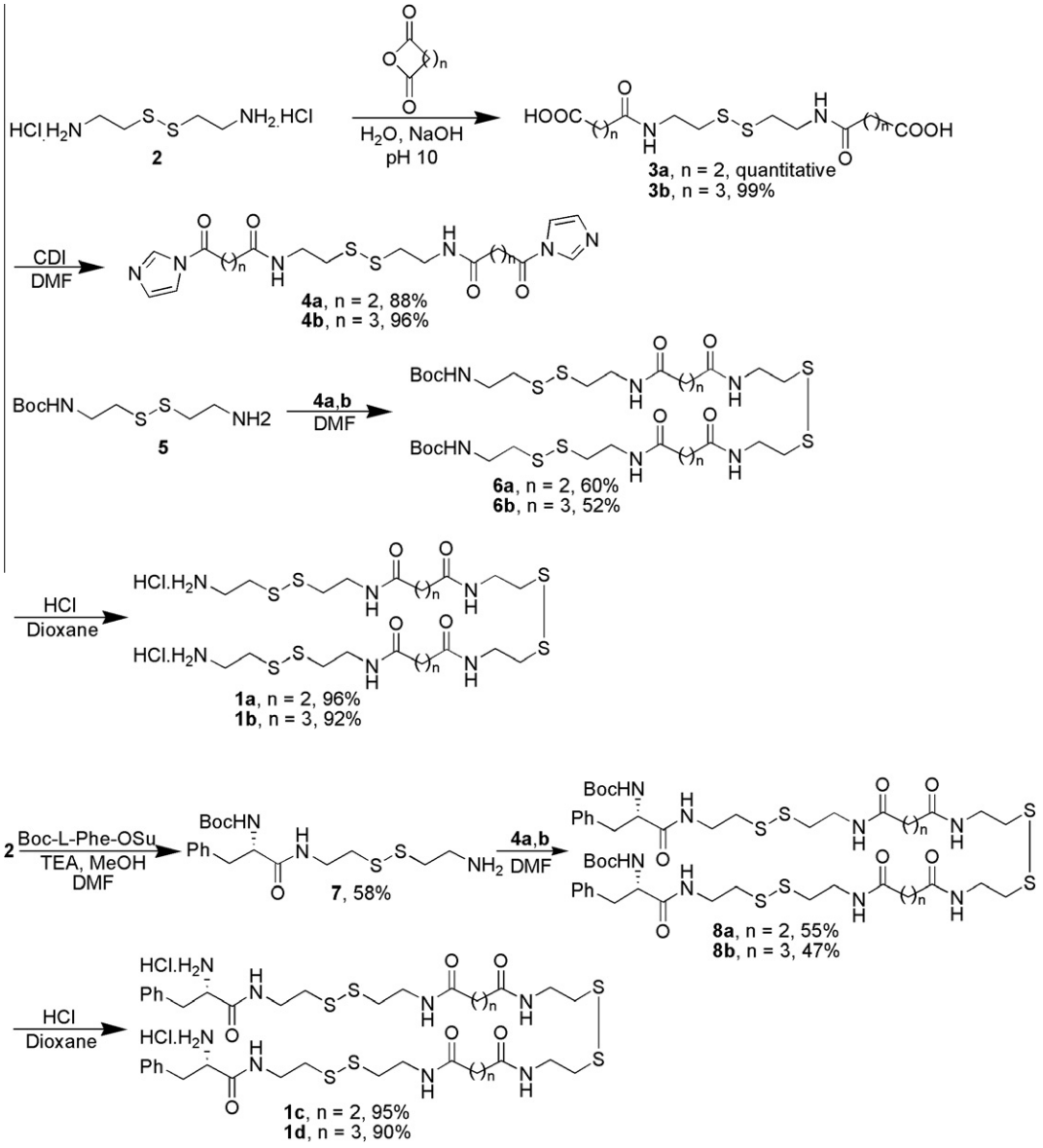

Scheme 1. Chemical synthesis of pro-drugs **1a**–**d**.

diamides **3a**–**b** as bisazolides was achieved by using carbonyldiimidazole (CDI).[7,10] The imidazolide derivatives **4a**–**b** were then coupled with 2 equiv of mono-Boc-cystamine **5**[12] to yield the multicystamines derivatives **6a**–**b**. In the same way, the compounds **8a**–**b** were obtained in moderate yields by reacting the imidazolyl derivatives **4a**–**b** with the mono-phenylalanyl-cystamine **7**.[13] The latter was prepared by coupling the commercially available *N*-Boc-L-phenylalanine-*N*-hydroxysuccinimide ester with cystamine dihydrochloride **2** in triethylamine–methanol–DMF mixed solvent. The Boc protecting group of the compounds **6a**–**b** and **8a**–**b** was removed by treatment with 4 M hydrogen chloride solution in dioxane[14] to result in the pro-drugs **1a**–**d** as white odourless crystalline powders with excellent yields.

3. Biological evaluation

3.1. Toxicity profile

The cytotoxicity of pro-drugs **1a**–**d** was determined to confirm that any change in cystine burden observed was not a consequence of cell death or an increase in cell proliferation. The test was carried out on human cystinotic fibroblasts using the Alamar blue cell proliferation assay.[15] The cystinotic fibroblasts were subjected to 50 μM of the current treatment, cysteamine, and the compounds **1a**–**d**, and cell growth was measured over a 72 h period. The results are shown in Figure 1.

3.2. Measurement of intralysosomal cystine

Intralysosomal cystine was measured using the commercially available Thiol and Sulfide Quantification Kit® (Molecular Probes). Lysates of cystinotic fibroblasts were treated for 24 h with 50 μM of cysteamine or compounds **1a**–**d**. Intralysosomal cystine was isolated from the lysates, converted to cysteine and the concentration was then measured on a multiwell plate reader at 410 nm by comparison to known standards of cysteine. The effects of compounds **1a**–**d** on the levels of intralysosomal cystine relative to control are shown in Figure 2. The data are presented as μM cysteine per mg of protein as determined by the Bradford method.[16]

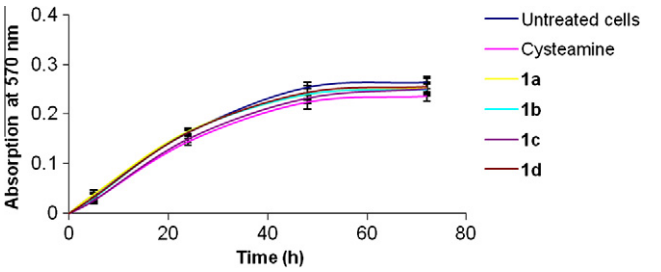

Figure 1. Net change of Alamar blue absorbance at 6, 24, 48 and 72 h intervals for compounds **1** and **1a–d**. The data shown are a mean of 6 independent experiments ± SE, each measurement was carried out in triplicate.

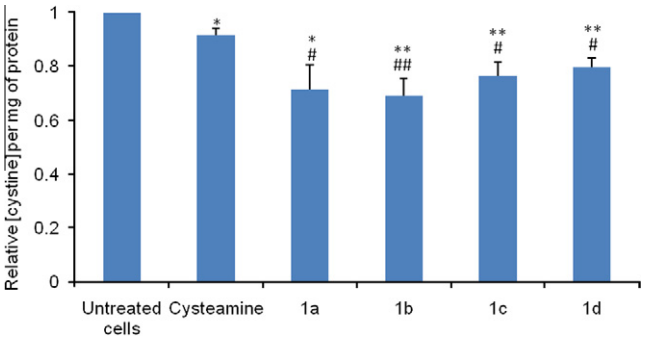

Figure 2. Relative cystine depletion in cystinotic fibroblasts measured after 24 h of incubation with compounds **1** and **1a–d**. The data shown is the mean of 4 independent experiments ± SE. Each measurement was carried out in triplicate. The level of significance was determined using a one-tailed the Student's *t*-test, and is represented as follows; comparison to untreated cells $^{*}p$ <0.05, $^{**}p$ <0.01 and in comparison to cysteamine treated cells $^{\#}p$ <0.05, $^{\#\#}p$ <0.01.

4. Discussion

It was determined from the Alamar blue study that treatment of the cystinotic fibroblasts with 50 μM of the compounds **1a–d** or cysteamine has no significant difference in cell growth of the fibroblasts through 72 h. Figure 1 shows that cysteamine and **1a–d** have negligible toxicity at the concentrations and time frame utilised in this study.

Furthermore, the results displayed in Figure 2 shows that the synthesised pro-drugs **1a–d** significantly reduce the lysosomal cystine levels relative to control, and, more importantly, the pro-drugs deplete cystine significantly better than the current treatment, cysteamine. The observed increase in the efficacy of **1a–d** is attributed to intracellular hydrolysis of the amide linkages and subsequent release of cysteamine (up to six cysteamine molecule per pro-drug if the hydrolysis goes to completion). However, the measured depletion of intracellular cystine is not stoichiometric (i.e., pro-drugs **1a–d** are not six times more effective than cysteamine). This suggests that hydrolysis within the cells may not proceed to completion. Notwithstanding this outcome, we propose that the increased efficiency of the multi-cysteamine pro-drugs approach has been demonstrated.

5. Conclusion

The design, synthesis and biological evaluation of a series of multi-cysteamine pro-drugs have been achieved. These pro-drugs have been shown to deplete the cysteine levels in cystinotic cells with no toxicity and with a greater efficacy than the established treatment. It is anticipated that this type of pro-drug should allow less frequent administration than the current treatment and may be better tolerated by cystinotic patients. It is hoped that in this way, patient compliance and quality of life amongst cystinotic patients will be improved.

6. Experimental section

6.1. Chemistry

6.1.1. General

All chemicals were purchased from Sigma–Aldrich (UK). ^{1}H NMR and ^{13}C NMR were run at 400 and 100 MHz, respectively, on Bruker 400 Ultrashield Plus machine. Coupling constants (J) are quoted in Hz and chemical shifts (δ) are given in parts per million (ppm) using the residue solvent peaks as reference relative to TMS. High resolution mass spectra were recorded on Thermofisher LTQ Orbitrap XL instrument at National Mass Spectrometry Service Centre, Swansea, UK and the assistance of EPSRC is acknowledged.

6.1.2. Synthesis

6.1.2.1. 5-Imidazol-1-yl-5-oxo-pentanoic acid {2-[2-(5-imidazol-1-yl-5-oxo-pentanoylamino)-ethyldisulfanyl]-ethyl}-amide 4b. 1 g (2.6 mmol) of *N,N′*-diglutaroylcystamine was dissolved in 10 ml of anhydrous DMF and 1.05 g (6.5 mmol) of CDI was added. Bubbles of CO_2 indicated the start of the reaction, and the reaction product rapidly precipitated. The reaction was allowed to continue for 3 h under reduced pressure. After dilution with 100 ml of anhydrous ethyl acetate, the product was collected by filtration, rinsed twice with 100 ml of anhydrous ethyl acetate and carefully dried in the vacuum oven at 50 °C.

Yield: 96%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.86 (quint, J = 7.6 Hz, 4H), 2.18 (t, J = 7.6 Hz, 4H), 2.73 (t, J = 7.6 Hz, 4H), 3.01 (t, J = 7.2 Hz, 4H), 3.31 (q, J = 7.2 Hz, 4H), 7.05 (s, 2H), 7.68 (s, 2H), 8.09 (t, J = 7.2 Hz, 2H), 8.39 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 19.74, 33.80, 33.96, 37.23, 37.88, 116.72, 130.22, 136.96, 170.22, 171.63.

6.1.2.2. General procedures for the synthesis of compounds 6a-b and 8a-b. To a solution of the mono-substituted cystamine compounds **5** or **7** (4.5 mmol) in 5 ml of dry DMF, was added the corresponding activated cystamine derivatives **4a-b** (1.5 mmol). The reaction mixture was stirred overnight at room temperature. The solvent was then evaporated and 50 ml of distilled water were added to the residue. The white precipitate was filtered and washed by 3 × 50 ml of aqueous 0.1 M HCl, aqueous 0.1 M NaOH, then by 3 × 50 ml of AcOEt. The product was then dried in the vacuum oven at 70 °C.

6.1.2.2.1. [2-(2-{4-[2-(2-{4-[2-(2-tert-Butoxycarbonylamino-ethyldisulfanyl)-ethylcarbamoyl]-propionylamino}-ethyldisulfanyl)-ethylcarbamoyl]-propionyllamino}-ethyldisulfanyl)-ethyl]-carbamic acid tert-butyl ester ***6a****.* Yield: 60%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.37 (s, 18H), 2.30 (s, 8H), 2.74 (m, 12H), 3.19 (m, 4H), 3.30 (m, 8H), 6.99 (t, J = 4.8 Hz, 2H), 8.04 (t, J = 5.2 Hz, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 28.23 (6C), 30.66 (4C), 37.20 (4C), 37.24 (2C), 37.20 (4C), 37.98 (2C), 77.80 (2C), 155.52 (2C), 171.45 (4C). HR-MS calculated for $C_{30}H_{57}N_6O_8S_6$ $(M+H)^+$: 821.2557, found: 821.2545.

6.1.2.2.2. [2-(2-{4-[2-(2-{4-[2-(2-tert-Butoxycarbonylamino-ethyldisulfanyl)-ethylcarbamoyl]-butyrylamino}-ethyldisulfanyl)-ethylcarbamoyl]-butyrylamino}-ethyldisulfanyl)-ethyl]-carbamic acid tert-butyl ester ***6b****.* Yield: 52%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.36 (s, 18H), 1.68 (quint, J = 7.6 Hz, 4H), 2.04 (t, J = 7.6 Hz, 8H), 2.74 (m, 12H), 3.17 (m, 12H), 6.98 (t, J = 7.2 Hz, 2H), 7.99 (t, J = 6.0 Hz, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 21.36 (2C), 28.18 (6C), 34.65 (4C), 37.18 (4C), 37.24 (2C), 37.48 (4C),

37.83 (2C), 77.76 (2C), 155.47 (2C), 171.84 (4C). HR-MS calculated for $C_{32}H_{61}N_6O_8S_6$ $(M+H)^+$: 849.2870, found: 849.2863.

6.1.2.2.3. (1-{2-[2-(3-{2-[2-(3-{2-[2-(2-(S)-tert-Butoxycarbonylamino-3-phenyl-propionylamino)-ethyldisulfanyl]-ethylcarbamoyl}-propionylamino)-ethyldisulfanyl]-ethylcarbamoyl}-propionylamino)-ethyldisulfanyl]-ethylcarbamoyl}-2-(S)-phenyl-ethyl)-carbamic acid tert-butyl ester ***8a***. Yield: 55%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.28 (s,18H), 2.31 (s, 8H), 2.73 (m, 12H), 2.92 (m, 4H), 3.34 (m, 12H), 4.10 (m, 2H), 6.90 (d, J = 8.4 Hz, 2H), 7.23 (m, 10H), 8.04 (t, J = 2.8 Hz, 4H), 8.11 (t, J = 4.8 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 28.15 (6C), 30.68 (4C), 37.01 (2C), 37.19 (4C), 37.67 (2C), 37.95 (2C), 37.99 (4C), 55.77 (2C), 77.98 (2C), 126.18 (2C), 128.10 (4C), 129.19 (4C), 138.15 (2C), 155.19 (2C), 171.47 (4C), 171.73 (2C). HR-MS calculated for $C_{48}H_{75}N_8O_{10}S_6$ $(M+H)^+$: 1115.3925, found: 1115.3905.

6.1.2.2.4. (1-{2-[2-(4-{2-[2-(4-{2-[2-(2-(S)-tert-Butoxycarbonylamino-3-phenyl-propionylamino)-ethyldisulfanyl]-ethylcarbamoyl}-butyrylamino)-ethyldisulfanyl]-ethylcarbamoyl}-butyrylamino)-ethyldisulfanyl]-ethylcarbamoyl}-2-(S)-phenyl-ethyl)-carbamic acid tert-butyl ester ***8b***. Yield: 47%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.35 (s,18H), 1.77 (quint, J = 7.2 HZ, 4H), 2.12 (t, J = 7.2 Hz, 8H), 2.81 (m, 12H), 2.98 (m, 4H), 3.41 (m, 12H), 4.18 (m, 2H), 6.98 (d, J = 8.8 Hz, 2H), 7.31 (m, 10H), 8.07 (t, J = 5.0 Hz, 4H), 8.18 (t, J = 5.0 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 21.36 (2C), 28.09 (6C), 34.65 (4C), 36.93 (2C), 37.15 (4C), 37.66 (2C), 37.83 (4C), 37.89 (2C), 55.72 (2C), 77.91 (2C), 126.12 (2C), 127.96 (4C), 129.13 (4C), 138.10 (2C), 155.14 (2C), 171.68 (2C), 171.84 (4C). HR-MS calculated for $C_{50}H_{79}N_8O_{10}S_6$ $(M+H)^+$: 1143.4238, found: 1143.4218.

6.1.2.3. General procedures for the synthesis of the pro-drugs 1a–d. To a solution of the compounds **6a-b** or **8a-b** (0.04 mmol) was added 5 ml of 4 M hydrogen chloride solution in dioxane, at 0 °C under a nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 30 min and then for further 30 min at room temperature. The white precipitate was filtered and washed by 3 × 50 ml of AcOEt and 3 × 50 ml of CH_2Cl_2. The product was then dried in the vacuum oven at 70 °C.

6.1.2.3.1. Pro-drug ***1a***. Yield: 96%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.31(s, 8H), 2.76 (m, 8H), 2.94 (m, 4H), 3.07 (m, 4H), 3.31 (m, 8H), 8.11 (m, 10H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 30.61 (4C), 34.00 (2C), 36.98 (2C), 37.14 (2C), 37.79 (2C), 37.87 (2C), 37.94 (2C), 171.48 (2C), 171.54 (2C). HR-MS calculated for $C_{20}H_{41}N_6O_4S_6$ $(M+H)^+$: 621.1508, found: 621.1503.

6.1.2.3.2. Pro-drug ***1b***. Yield: 92%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.70 (quint, J = 7.2, 4H), 2.07 (t, J = 7.2, 8H), 2.78 (m, 8H), 2.95 (m, 4H), 3.08 (m, 4H), 3.32 (m, 8H), 8.09 (m, 10H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 21.42 (2C), 34.04 (2C), 34.09 (4C), 37.09 (2C), 37.22 (2C), 37.77 (2C), 37.82 (2C), 37.89 (2C), 171.90 (2C), 171.96 (2C). HR-MS calculated for $C_{22}H_{45}N_6O_4S_6$ $(M+H)^+$: 649.1821, found: 649.1819.

6.1.2.3.3. Pro-drug ***1c***. Yield: 95%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.31 (s, 8H), 2.63 (m, 4H), 2.74 (t, J = 6.4 Hz, 8H), 3.05 (m, 4H), 3.29 (m, 8H), 3.43 (m, 4H), 4.00 (m, 2H), 7.26 (m, 10 H), 8.14 (m, 4H), 8.36 (m, 6H), 8.80 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 30.70 (4C), 36.70 (2C), 36.95 (2C), 37.05 (2C), 37.21 (2C), 37.81 (2C), 38.00 (2C), 38.11 (2C), 53.47 (2C), 127.13 (2C), 128.49 (4C), 129.54 (4C), 134.97 (2C), 167.95 (2C), 171.47 (2C), 171.53 (2C). HR-MS calculated for $C_{38}H_{59}N_8O_6S_6$ $(M+H)^+$: 915.2876, found: 915.2878.

6.1.2.3.4. Pro-drug ***1d***. Yield: 90%, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.70 (quint, J = 7.6 Hz, H-7, 4H), 2.06 (t, J = 7.6 Hz, 8H), 2.64 (m, 4H), 2.74 (t, J = 6.8 Hz, 8H), 3.02 (m, 4H), 3.30 (m, 8H), 3.43 (m, 4H), 3.96 (m, 2H), 7.30 (m, 10 H), 8.04 (m, 4H), 8.20 (m, 6H), 8.64 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 21.46 (2C), 35.81 (4C), 36.69 (2C), 36.96 (2C), 37.08 (2C), 37.23 (2C), 37.82 (2C), 37.90 (2C), 37.99 (2C), 53.48 (2C), 127.14 (2C), 128.50 (4C), 129.54 (4C), 134.69 (2C), 167.96 (2C), 171.93 (2C), 171.96 (2C). HR-MS calculated for $C_{40}H_{63}N_8O_6S_6$ $(M+H)^+$: 943.3189, found: 943.3185.

6.2. Biology

6.2.1. General

Human cystinotic fibroblasts (GM00008) were purchased from Coriell Cell Repositories (NJ, USA) and cultured in Eagle's minimum essential media supplemented with 15% FBS, 200 U/ml penicillin, 200 μg/ml streptomycin and 2 mM glutamine at 37 °C in 5% CO_2. Alamar blue reagent was purchased from (Serotech, UK). Thiol and sulphide quantification kit (Molecular Probes T6060) was purchased from FisherSicentific (UK). Bradford reagent was purchased from Sigma (UK).

6.2.2. Alamar blue assay

Cystinotic fibroblasts cultured in 96 well plates were incubated for 0–72 h in the presence of 50 μM either cysteamine, or **1a–d** in media supplemented with 10% Alamar blue. Cell growth was measured over a 72 h period on a multiwell plate reader Biotek FL6000 and is presented as the net change in absorbance at 570 nm relative to the reading at time 0 h.

6.2.3. Thiol assay

Cystinotic fibroblasts were seeded in a 25 cm^3 vented flask and allowed to reach approximately 80% confluence before the addition of the test compounds; 50 μM either cysteamine, or **1a–d** in 4 cm^3 Eagles minimum essential media supplemented with 15% FBS, 200 U/ml penicillin, 200 μg/ml streptomycin and 2 mM glutamine. This was then incubated at 37 °C and 5% CO_2 for 24 h. The cells were harvested, frozen in liquid nitrogen and stored at −80 °C until the cysteine concentration was determined per quantity of protein. The cells were recovered from storage at −80 °C and suspended in 100 μl 1 mM *N*-ethylmalimide prepared in phosphate buffer (pH 7.6) followed by sonication for 10 s which was repeated three times with 20 s cooling intervals on ice. The solution was centrifuged at 800 g for 10 min at 40 °C (Biofuge primo R Heraeus centrifuge). Cell supernatant (40 μl) was then added; 4 μl of 4 M $NaBH_4$ in 7:3 0.1 M NaOH/DMSO. After 5 min incubation at room temperature; 800 μl of sodium acetate buffer (pH 4.7) was added. A 5 μl volume of the diluted solution was then added to 100 μl of 0.6 mg/ml solution of Papain-$SSCH_3$ in 96 wells plate and incubated for 1 h at room temperature. A 100 μl volume of 4.9 mM L-BAPNA solution in sodium acetate buffer (pH 4.0) was then added to each well of the 96 well plate, gently mixed and incubated for further 1 h at room temperature. The absorption at 410 nm was measured and the cysteine levels were calculated by comparison to known cysteine standards.

The protein concentration in every sample was determined according to Bradford method[14] at the same time of the thiol assay. Briefly, 200 μl of Bradford reagent were added to 5 μl of the previously obtained cell supernatant in each well of a 96 well plate and incubated for 5 min at room temperature and the absorption at 595 nm was measured. The protein concentrations were calculated using a range of concentrations of bovine serum albumin as a standard. The cysteine levels are presented following normalisation to μM cysteine per mg of protein.

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References and notes

1. Gahl, W. A.; Thoene, J. G.; Schneider, J. A. Cystinosis: A Disorder of Lysosomal Membrane Transport. In *The Metabolic and Molecular Basis of Inherited Disease*; Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Vogelstein, B., Eds.; McGraw-Hill: NY, 2001. eighth ed., pp 5085–5108.
2. Town, M.; Jean, G.; Cherqui, S.; Attard, M.; Forestier, L.; Whitmore, S. A.; Callen, D.; Gribouval, O.; Broyer, M.; Bates, G. P.; Van't Hoff, W.; Antignac, C. *Nat. Genet.* **1998**, *18*, 319.
3. Pisoni, R.; Thoene, J.; Christtensen, H. *J. Biol. Chem.* **1995**, *260*, 4791.
4. Cardwell, W. A.; Cairns, D.; Anderson, R. J. *J. Pharm. Pharmacol.* **1997**, *49*, 99.
5. Gahl, W.; Theone, J. G.; Schneider, J. A. *New Engl. J. Med.* **2002**, *347*, 111.
6. McCaughan, B.; Kay, G.; Knott, R. M.; Cairns, D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1716.
7. Omran, Z.; Kay, G.; Di-Salvo, A.; Knott, R. M.; Cairns, D. *Bioorg. Med. Chem. Lett.* **2010**, *21*, 45.
8. Omran, Z.; Kay, G.; Hector, E. E.; Knott, R. M.; Cairns, D. *Bioorg. Med. Chem. Lett.* **2010**, *21*, 2502.
9. Hu, M.; Borchardt, R. T. *Pharm. Res.* **1990**, *7*, 1313.
10. Nicolas, F. L.; Gagnieu, C. H. *Biomaterials* **1997**, *18*, 807.
11. Shu, X. PCT Int. Appl. (2008), CODEN: PIXXD2 WO 2008061427.
12. Girgenti, E.; Ricoux, R.; Mahy, J. P. *Tetrahedron* **2004**, *60*, 10049.
13. Zhang, B.; Cech, T. R. *Chem. Biol.* **1998**, *5*, 539.
14. Han, G.; Tamaki, M.; Hruby, V. J. *J. Pept. Res.* **2001**, *4*, 338.
15. Ahmed, S. A.; Gogal, R. M., Jr.; Walsh, J. E. *J. Immunol. Methods* **1994**, *170*, 211.
16. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.