



# A potential new prodrug for the treatment of cystinosis: Design, synthesis and in-vitro evaluation

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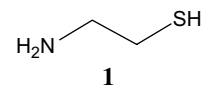
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**Abstract**—Nephropathic cystinosis is a rare autosomal recessive disease characterised by raised lysosomal levels of cystine in the cells of most organs. The disorder is treated by regular administration of the aminothiol, cysteamine, an odiferous and unpleasant tasting compound that along with its metabolites is excreted in breath and sweat, leading to poor patient compliance. In an attempt to improve patient compliance a series of novel prodrugs has been designed and evaluated as a potential new treatment for nephropathic cystinosis. The first of the prodrugs tested, **3a**, was found to decrease the levels of intracellular cystine in cystinotic fibroblasts. This is the first report of a potential new therapeutic treatment for nephropathic cystinosis since the advent of cysteamine bitartrate.

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Nephropathic cystinosis is a rare autosomal recessive disease. It is characterised by raised lysosomal levels of cystine in the cells of most organs. If untreated, the disease, results in death from renal failure by the second decade of life. The condition is characterised by poor growth, renal Fanconi syndrome, renal glomerular failure and impairment of other tissues and organs (e.g. thyroid, pancreas, CNS). If treatment is started just after birth this can attenuate the rate of renal failure, however, glomerular damage present at the time of diagnosis (usually about 12 months of age) is irreversible and may result in the need for renal transplant.<sup>1–4</sup>

Cystinosis is caused by a defect in the lysosomal transport mechanism for cystine and results from mutations in the CTNS gene found on chromosome 17p13, which codes for cystinosin, a lysosomal membrane transport protein. A number of mutations have been reported, the most common being a 57Kb deletion present in about 50% of cystinotic patients of Western European ancestry.<sup>5</sup> Treating patients with cystinosis involves administration of glucose and electrolytes to reverse the effects of Fanconi syndrome as well as corneal and renal transplantation. Furthermore, the disorder is treated



**Figure 1.** Structure of 2-aminoethanethiol, cysteamine.

ted by administration of the aminothiol, cysteamine (**1**) (as the bitartrate salt, Cystagon<sup>®</sup>), **Figure 1**, which acts to lower intracellular levels of cystine by forming a cysteamine–cysteine mixed disulfide. The mixed disulfide is spatially similar in structure to the amino acid lysine and can egress the lysosome using the undamaged excretion pathway for lysine.<sup>6</sup> Cysteamine, however, possesses an offensive taste and smell and irritates the gastrointestinal tract leading to nausea and vomiting following administration. In addition, cysteamine and its metabolites are excreted in breath and sweat. As a result of these problems patient compliance can be poor.<sup>7</sup>

Given the well established beneficial effects of **1** we envisaged developing a prodrug that was pharmacologically inactive, thereby masking the offensive taste and smell of the thiol, but metabolically activated in-vivo, releasing the active compound thus allowing for effective oral administration. Our research was directed towards the design, synthesis and biological evaluation of novel cysteamine derivatives, intended to preserve the advantageous cystine depletive effects, while reducing the ad-

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verse consequences of administration that occur as a direct result of treatment with **1**. It was hoped that by decreasing the solubility of the prodrug, the metabolism of cysteamine in the plasma would also be decreased leading to lower concentrations of the metabolites responsible for halitosis (dimethylsulfide and methanethiol).<sup>8</sup> Furthermore, such a prodrug strategy focuses on increasing the cellular/lysosomal concentration of cysteamine whilst decreasing circulatory levels, either through increased cellular uptake or diminished plasma reduction/methylation processes.

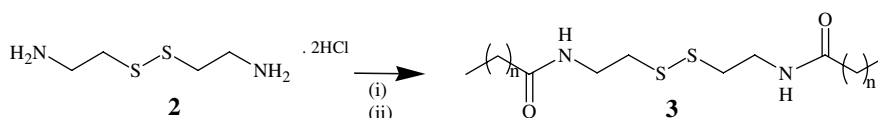
A series of novel compounds based on the disulfide counterpart of **1**, cystamine dihydrochloride (**2**), has been designed and synthesised. The taste of cysteamine may be disguised by synthesis of salts or derivatives which have low water solubility. These derivatives often take the form of embonates, palmitates and stearates (previously used successfully to disguise the bitter taste of the antibiotics chloramphenicol and erythromycin).<sup>9</sup> Based on this rationale, a library of lipophilic prodrugs was synthesised (**3a–3m**). The synthesis was achieved through the N-acylation of cystamine by a method analogous to peptide coupling techniques (Scheme 1). All compounds were prepared in high yield and characterised by <sup>13</sup>C, <sup>1</sup>H NMR, mass spectroscopy and IR spectroscopy.

This study is focused on compound **3a**,<sup>10</sup> the first compound to be evaluated for its in-vitro capacity as a prodrug of cysteamine (decanoic acid [2-(2-decanoylaminoethyl)disulfanyl]ethyl]amine), selected due to its

favourable solubility in ethanol. Toxicity studies on this compound were carried out on both human umbilical vein endothelial cells (HUVEC) and cystinotic fibroblasts. The proliferation assay was carried out on the HUVE cells with various concentrations of **3a** in 1% EtOH solution, and observed over a 48 h period. The cystinotic fibroblasts were subjected to 50 μM **3a** and the proliferation assay followed over a 6 day period, Table 1. It was determined from this study that at 72 h there was no significant difference in cell growth of the cystinotic fibroblasts ( $p < 0.05$ ) from the addition of 50 μM **3a** using the Mann–Whitney test. Both toxicity studies were carried out utilising an alamar blue proliferation assay. This confirms that the compound has negligible toxicity at the concentrations and time frame utilised in this study.

A range of methods have been reported in the literature for the quantitative detection of thiols.<sup>11</sup> Using a combination of these methods a reverse phase HPLC assay for the in-vitro determination of cysteine has been established. This reverse phase HPLC assay employs a thiol specific UV tagging agent (**4**)<sup>12</sup> to quantify cysteine levels in the lysosome using a DAD detector. Compound **4** (1-methyl-2-chloroquinolinium), Figure 2, has been synthesised and characterised in our laboratory.<sup>13</sup> The tagged cysteine elutes the column with a retention time of 12.5 min (Figs. 3a and b).

Cystinotic fibroblasts<sup>14</sup> were seeded in a 75 cm<sup>3</sup> vented flask and allowed to reach a confluency approximating 80% before being spiked with 50 μM either **1**, or **3a** in



**Scheme 1.** Reagents and conditions: (i) DMF, Et<sub>3</sub>N (2 equiv); (ii) relevant fatty acid (4 equiv), DIPEA (6 equiv), HOBt (3.92 equiv), PyBOP (3.94 equiv), rt 2 h, H<sub>2</sub>O wash (4 × 50 ml). Recrystallised if required. 3(a)  $n = 8$ ; 3(b)  $n = 10$ ; 3(c)  $n = 12$ ; 3(d)  $n = 13$ ; 3(e)  $n = 14$ ; 3(f)  $n = 15$ ; 3(g)  $n = 16$ ; 3(h)  $n = 17$ ; 3(i)  $n = 16$  [elaidioate *trans*(CH<sub>2</sub>)<sub>8</sub> = (CH<sub>2</sub>)<sub>8</sub>]; 3(j)  $n = 16$  [oleiate *cis*(CH<sub>2</sub>)<sub>8</sub> = (CH<sub>2</sub>)<sub>8</sub>]; 3(k)  $n = 16$  [linoleate (CH<sub>2</sub>)<sub>4</sub>CH = CH<sub>2</sub>CH = CH(CH<sub>2</sub>)<sub>8</sub>]; 3(l)  $n = 16$  [linolenate *cis, cis, cis*(CH<sub>2</sub>)<sub>4</sub>(CH = CH<sub>2</sub>)<sub>3</sub>(CH<sub>2</sub>)<sub>6</sub>]; 3(m)  $n = 20$  [eruciate (CH<sub>2</sub>)<sub>9</sub>CH = CH(CH<sub>2</sub>)<sub>9</sub>].

**Table 1.** Toxicity study on the effect of **3a** in 1% EtOH on HUVE cells and cystinotic fibroblasts

Time (h)	Control <sup>a,b,c</sup>	<b>3a</b> <sup>c,d</sup>	Control <sup>a,b,e</sup>	<b>3a</b> <sup>a,e,f</sup>	<b>3a</b> <sup>a,e,g</sup>	<b>3a</b> <sup>a,e,h</sup>	<b>3a</b> <sup>a,e,i</sup>	<b>3a</b> <sup>a,d,e</sup>
18	0.09(±0.03)	0.08(±0.01)	0.50(±0.06)	0.51(±0.17)	0.53(±0.09)	0.54(±0.07)	0.49(±0.14)	0.52(±0.13)
24	0.06(±0.02)	0.07(±0.01)	0.49(±0.06)	0.49(±0.17)	0.52(±0.10)	0.52(±0.07)	0.47(±0.15)	0.51(±0.12)
48	0.17(±0.02)	0.16(±0.01)	0.45(±0.06)	0.47(±0.17)	0.50(±0.12)	0.51(±0.07)	0.45(±0.14)	0.47(±0.12)
72	0.33(±0.01)	0.25(±0.01)	—	—	—	—	—	—
144	0.45(±0.02)	0.44(±0.01)	—	—	—	—	—	—

No significant difference in the growth of cells was evident at the time scales and concentrations utilised in this study, as determined using the Mann–Whitney test.

<sup>a</sup> Values are means of six experiments, standard deviation is given in parentheses. Absorbance recorded at 595 nm. Mean net absorbance relative to time zero.

<sup>b</sup> Control consisted of 0 μM **3a**, 1% EtOH.

<sup>c</sup> Proliferation assay carried out in cystinotic fibroblasts.

<sup>d</sup> 50 μM **3a** in 1% EtOH.

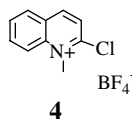
<sup>e</sup> Proliferation assay carried out in HUVE cells.

<sup>f</sup> 2 μM **3a**, 1% EtOH.

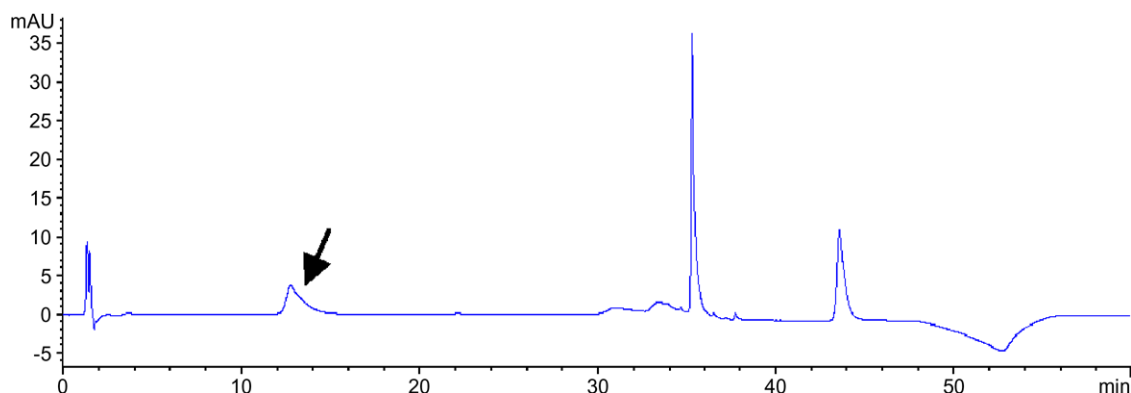
<sup>g</sup> 10 μM **3a**, 1% EtOH.

<sup>h</sup> 20 μM **3a**, 1% EtOH.

<sup>i</sup> 40 μM **3a**, 1% EtOH.



**Figure 2.** UV tagging agent, 1-methyl-2-chloroquinolinium tetrafluoroborate.



**Figure 3a.** Sample prepared containing 20  $\mu\text{M}$  cysteine.

15  $\text{cm}^3$  Eagles minimum essential media supplemented with 15% FBS, 200 U/ml penicillin, 200  $\mu\text{g}/\text{ml}$  streptomycin and 2 mM glutamine containing 1% ethanol. This was then incubated at 37  $^\circ\text{C}$  and 5%  $\text{CO}_2$  for 72 h. The cells were harvested, frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until the cysteine concentration was determined per quantity of protein. The procedure was based

on a method described by de Graaf-Hess et al.,<sup>11a</sup> with some modifications<sup>15</sup> for determining cysteine in fibroblasts. The protein determination was carried out using the Bradford method<sup>16</sup> and recording absorbance at 595 nm. Quantification was done using the UV active tagging agent **4** with a gradient RP-HPLC assay<sup>17</sup> separating the tagged thiols.

**Table 2.** Relative cystine depletion after incubation with compounds **1** and **3a** for 72 h

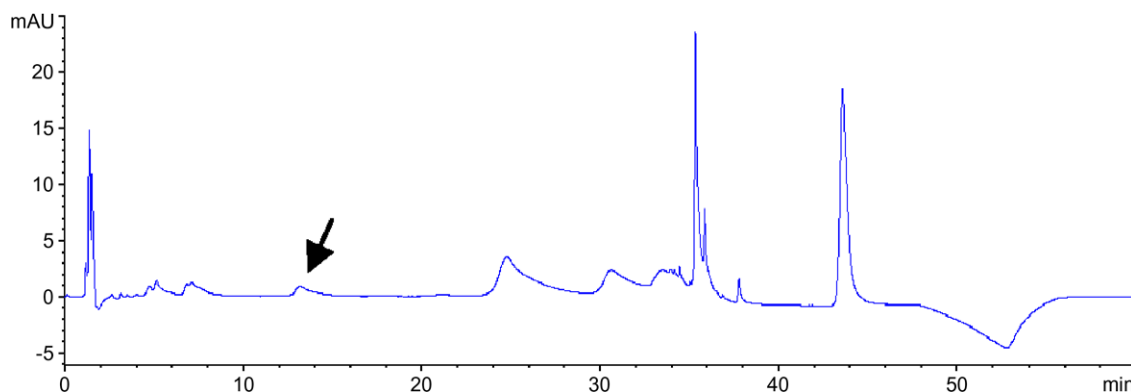
Experiment No.	<b>1</b> <sup>a</sup>	<b>3a</b> <sup>a</sup>
1	0.69	0.63
2	0.54	0.80
3	0.91	0.91
4	0.80	0.77
5	0.66	0.63
6	0.88	0.85
7	0.81	0.85

<sup>a</sup> Values given are relative to control cells (1% EtOH) incubated alongside 50  $\mu\text{M}$  compounds **1** and **3a**. Control normalised for each experiment.

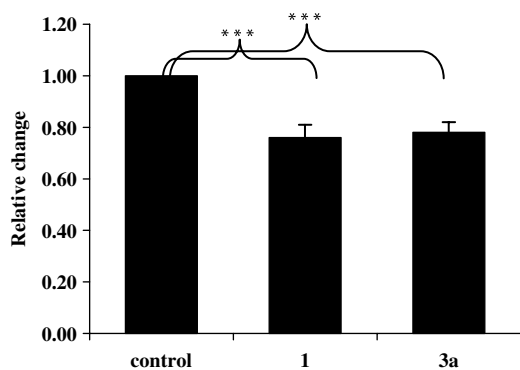
The results obtained for compound **3a** when compared with the control and **1** are displayed in Figure 4 and Table 2. It can be concluded that 50  $\mu\text{M}$  **3a** significantly depletes the levels of cystine in cystinotic fibroblasts relative to the control and is comparable to 50  $\mu\text{M}$  of cysteamine, **1**, after 72 h incubation.

Importantly, this is consistent with the requirement for biological activation of the prodrug in-vitro to generate the active form following absorption of the prodrug into cells.<sup>18,19</sup>

In conclusion, we have succeeded in the design and synthesis of a novel prodrug with the potential to rival the present treatment for nephropathic cystinosis.



**Figure 3b.** Actual flask containing cystinotic fibroblasts with 1% EtOH (control). Sample illustrated contains 2.72  $\mu\text{M}$  cysteine.



**Figure 4.** Relative cystine depletion in nmol cystine/mg protein for compounds **3a** and **1** compared to control. The level of significance was determined using a modified one tailed Students *t*-test, where  $t = (\text{mean} - 1)/\text{SEM}$  at  $n - 1$  d.f., where  $n = 7$  ( $***p < 0.001$ ).

Compound **3a** has been shown to deplete levels of lysosomal cystine with negligible toxicity to cultured cells. It is anticipated that compounds of this type could lead to an oral treatment with improved pharmacodynamic and pharmacokinetic parameters, leading to better compliance among cystinotic patients and an improved quality of life. Future work will expand these current data to include time dependence studies as well as the investigation of further compounds already prepared. These studies are currently underway in our laboratories.

### Acknowledgments

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- Diisopropylethylamine (1.54 ml, 8.8 mmol) was added to a stirring solution of cystamine dihydrochloride (1.0 g, 4.4 mmol) in anhydrous DMF (20 cm<sup>3</sup>). The reaction was stirred at room temperature for 15 min. HOBt (2.63 g, 17.2 mmol), PyBOP (9.02 g, 17.3 mmol), diisopropylethylamine (4.60 ml, 26.4 mmol) and decanoic acid (3.04 g, 17.6 mmol) were added to the solution and the reaction stirred for 1.5 h. A white crystalline precipitate formed from the solution with the addition of excess water (100 cm<sup>3</sup>). The solid was filtered, re precipitated from EtOH (70 °C) and dried in the vacuum oven at 65 °C. <sup>1</sup>H NMR (C<sub>5</sub>D<sub>5</sub>N at 55 °C 400 MHz) δ 8.4 (2H, br s), δ 3.8 (4H, q), δ 3.1 (4H, t), δ 2.4 (4H, t), δ 1.8 (4H, qu), δ 1.45 (4H, m), δ 1.25 (20H, m), δ 0.9 (6H, t); LCMS *m/z*: ES (+), (M+Na) 483; ES (-), (M+Cl) 495.
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- <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub> 400 MHz) δ 7.9(1H, d), δ 7.7(1H, d), δ 7.6(1H, t), δ 7.5(1H, d), δ 7.2(1H, t), δ 6.6 (1H, d), δ 3.6(3H, s); LC-MS *m/z*: M, 178.
- Cystinotic fibroblasts (GM00008) were purchased from Coriell Repositories and cultured in Eagle's minimum essential media supplemented with 15% FBS, 200 U/ml penicillin, 200 µg/ml streptomycin and 2 mM glutamine.
- The corresponding procedure is as follows: after the cells were recovered from storage at -80 °C, they were resuspended in 200 µl 1 mM *N*-ethyl malimide prepared in phosphate buffer (pH 7.2). They were sonicated 3 times for 10 s with 20 s cooling intervals on ice. Solution was centrifuged at 800g for 10 min at 4 °C in Biofuge primo R Heraeus centrifuge. Cell supernatant (120 µl) was then added to 15 µl 4 mM homocysteine with 20 µl 1.67 mM dithioerythritol in 2 mM EDTA, 30 µl octanol and 40 µl 4 M NaBH<sub>4</sub> in 7:3 0.1 M NaOH/DMSO. Thirty microlitres of 1.8 M HCl was added dropwise, the solution was vortex mixed and after 1.5 min, 100 µl 1.6 M *N*-ethyl morpholine, 375 µl water and 20 µl 0.1 M **4** were added, the solution was vortex mixed and after 3 min 50 µl glacial acetic acid was added dropwise. One hundred microlitre solution was injected onto an Agilent C-18 eclipse column for HPLC analysis.
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- The RP-HPLC analysis was carried out on an Agilent 1200 series LC/MS using an Agilent Eclipse XDB-C18, 5 µm, 4.6 × 150 mm column. The mobile phase consisted of water (solvent A), acetonitrile (solvent B) and 0.05% trichloroacetic acid buffered to pH 3.15 with LiOH (solvent C), with the gradient as follows: 0 min, 41% A, 9% B; 30 min, 41% A, 9% B; 35 min, 0% A, 50% B; 45 min, 0% A, 50% B; 50 min, 41% A, 9% B; 60 min 41% A, 9% B.
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