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

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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.

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.[1–4]

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.[5] 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 by administration of the aminothiol, cysteamine (**1**) (as the bitartrate salt, Cystagon®), 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.[6] 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.[7]

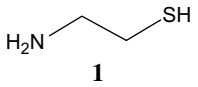

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Figure 1. Structure of 2-aminoethanethiol, cysteamine.

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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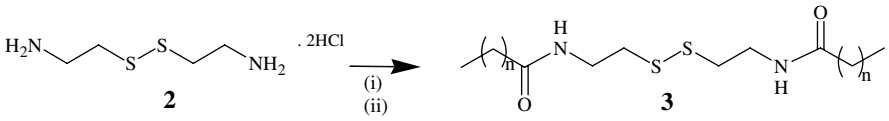
verse consequences of administration that occur as a direct result of treatment with **1**. It was hoped that by decreasing the solubility of the prodrug, the metabolism of cysteamine in the plasma would also be decreased leading to lower concentrations of the metabolites responsible for halitosis (dimethylsulfide and methanethiol).[8] 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).[9] 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 ^{13}C, ^{1}H NMR, mass spectroscopy and IR spectroscopy.

This study is focused on compound **3a**,[10] the first compound to be evaluated for its in-vitro capacity as a prodrug of cysteamine (decanoic acid [2-(2-decanoylaminoethyldisulfanyl)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.[11] 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**)[12] 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.[13] The tagged cysteine elutes the column with a retention time of 12.5 min (Figs. 3a and b).

Cystinotic fibroblasts[14] were seeded in a 75 cm^3 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_3N (2 equiv); (ii) relevant fatty acid (4 equiv), DIPEA (6 equiv), HOBt (3.92 equiv), PyBOP (3.94 equiv), rt 2 h, H_2O wash (4 × 50 ml). Recystallised 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*$(CH_2)_8 = (CH_2)_8$]; 3(j) $n = 16$ [oleiate *cis*$(CH_2)_8 = (CH_2)_8$]; 3(k) $n = 16$ [linoleate $(CH_2)_4CH = CH_2CH = CH(CH_2)8$]; 3(l) $n = 16$ [linolenate *cis*, *cis*, *cis*$(CH_2)_4(CH = CH_2)_3(CH_2)_6$]; 3(m) $n = 20$ [eruciate $(CH_2)_9CH = CH(CH_2)_9$].

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

Time (h)	Control[a,b,c]	**3a**[c,d]	Control[a,b,e]	**3a**[a,e,f]	**3a**[a,e,g]	**3a**[a,e,h]	**3a**[a,e,i]	**3a**[a,d,e]
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(±80.17)	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.

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

[b] Control consisted of 0 μM **3a**, 1% EtOH.

[c] Proliferation assay carried out in cystinotic fibroblasts.

[d] 50 μM **3a** in 1% EtOH.

[e] Proliferation assay carried out in HUVE cells.

[f] 2 μM **3a**, 1% EtOH.

[g] 10 μM **3a**, 1% EtOH.

[h] 20 μM **3a**, 1% EtOH.

[i] 40 μM **3a**, 1% EtOH.