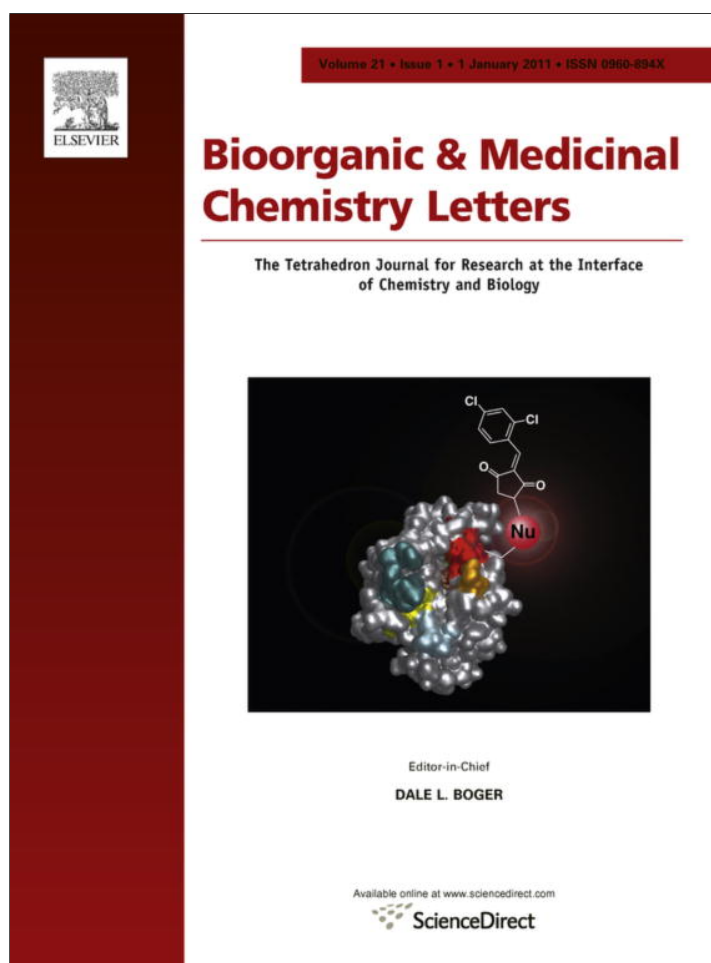


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## PEGylated derivatives of cystamine as enhanced treatments for nephropathic cystinosis

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### ABSTRACT

The genetic disease, nephropathic cystinosis is characterized by lysosomal accumulation of the amino acid cystine. Crystallization of cystine in affected organs, if untreated, results in mortality of the affected individuals by their middle to late teens. The only approved treatment for cystinosis is administration of cysteamine. However, cysteamine is associated with an offending odor and taste and this, coupled to a rapid first pass metabolism and a 6 h dosing regimen, suggest a clear need to improve the therapy. A number of PEGylated derivatives of cystamine, the disulfide counterpart of cysteamine, have been synthesized and evaluated in cultured cystinotic fibroblasts for toxicity and efficacy. All of the tested compounds were non-cytotoxic and displayed a remarkable depletion of intralysosomal cystine.

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Nephropathic cystinosis is a rare autosomal recessive disease 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. Symptoms include poor growth, renal Fanconi syndrome, renal glomerular failure and impairment of other tissues and organs (e.g., thyroid, pancreas and 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</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. This gene codes for cystinosin, a lysosomal membrane transport protein. A number of mutations have been reported, the most common being a 57 Kb deletion present in about 50% of cystinotic patients of Western European ancestry.<sup>2</sup> Treatment of cystinosis involves administration of electrolytes to reverse the effects of Fanconi syndrome as well as corneal and renal transplantation. Furthermore, the disorder is treated by administration of the aminothioli, cysteamine, **1** (Fig. 1) (as the bitartrate salt, Cystagon<sup>®</sup>), which acts to lower intracellular levels of cystine by forming a cysteamine–cysteine mixed disulfide.<sup>3</sup> Treatment with cysteamine, although successful, may be associated with poor patient compliance due to the unpalatable taste and smell of cysteamine, gastrointestinal side-effects such as nausea and vomiting, coupled with a requirement for 6 h dosing to overcome extensive

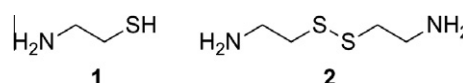


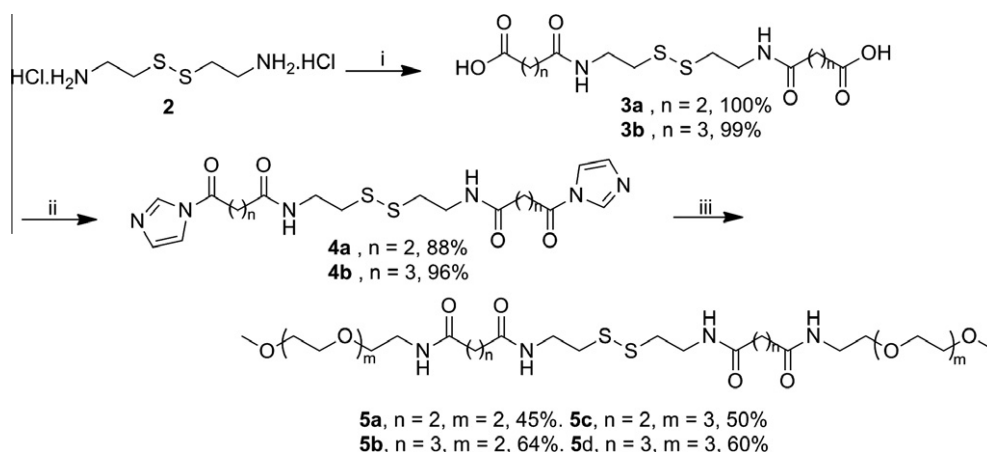
Figure 1. Chemical structures of cysteamine **1** and cystamine **2**.

first pass metabolism of the active. In an attempt to overcome these unpleasant side-effects, recent work has concentrated on the synthesis and evaluation of novel pro-drugs of cysteamine and cystamine.<sup>4,5</sup> As part of this ongoing study a number of polyethylene glycol derivatives of cystamine, **2** (Fig. 1), the disulfide counterpart of cysteamine, have been synthesized, purified and fully characterized. These derivatives were designed to be less volatile and more palatable than the current treatment. We report here the synthesis of a small library of PEGylated derivatives and in vitro evaluation of their cytotoxicity and cystine depleting efficacy.

As part of our work to modify the short aminothioli used for the treatment of cystinosis,<sup>4,5</sup> it was decided to employ PEGylation as a means of addressing the shortcomings of current therapy.<sup>6,7</sup> PEGylation has seen widespread use in the modification of the physicochemical properties of proteins<sup>8</sup> and more recently of small drug molecules.<sup>9,10</sup> A small library of PEGylated derivatives of cystamine was synthesized. The synthesis was achieved from the versatile cystamine derivatives **3a–b**<sup>11,12</sup> (Scheme 1). The activation of the carboxylate terminals of these derivatives was achieved by means of treatment with an excess of carbonyldiimidazole (CDI).<sup>11</sup> The resultant compounds **4a–b** were then coupled with amino polyethylenglycol to yield the attempted pro-drugs **5a–c** with overall yields of 40–61%.

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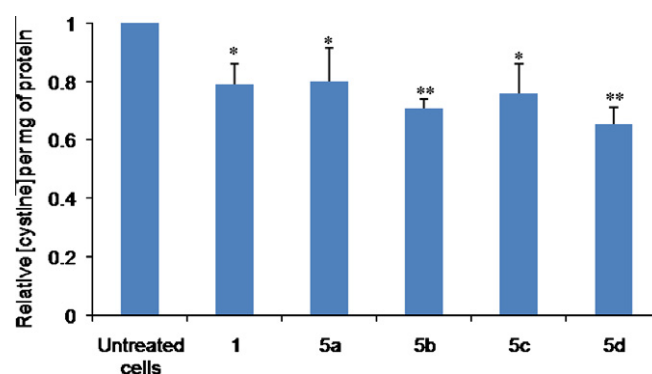
**Scheme 1.** Chemical synthesis of prod-drugs **5a–d**. Reagents and conditions: (i) relevant acid anhydride (3 equiv), NaOH, H<sub>2</sub>O, pH 10, rt; (ii) CDI (3 equiv), DMF, rt, 2 h; (iii) PEG–NH<sub>2</sub> (2.8 equiv), DMF, 85 °C, 12 h.

The cytotoxicity of pro-drugs **5a–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.<sup>13</sup> The cystinotic fibroblasts were subjected to 50 μM of the current treatment, **1**, and the compounds **5a–c**, and cell growth measured over a 72 h period. The results, displayed in Figure 2, show that there is no significant difference in cell growth of the cystinotic fibroblasts through 72 h which confirms that **1** and **5a–c** have negligible toxicity at the concentrations and time scales utilised in this study. This result is not unexpected since cysteamine, cystamine succinic acid, glutaric acid and polyethylene glycol are established safe pharmaceutical agents.

Intralysosomal cystine was measured using the commercially available Thiol and Sulfide Quantification Kit<sup>®</sup> (Molecular Probes). Lysates of cystinotic fibroblasts were treated for 24 h with 50 μM of **1** or compounds **5a–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 results obtained for compounds **5a–d** when compared with the control and **1** are displayed in Figure 3. The data are presented as μM cysteine per mg of protein relative to control as determined by the Bradford method.<sup>14</sup> It can be concluded that 50 μM **5a–d** significantly deplete the levels of cystine in cystinotic fibroblasts relative to the control and that this depletion is comparable to 50 μM of cysteamine, **1**, after 24 h incubation.

In conclusion, a library of PEGylated derivatives of cystamine has been established. These compounds are non-cytotoxic up to 72 h of incubation and display a statistically significant reduction in levels of intralysosomal cystine when evaluated in cultured



**Figure 3.** Relative cystine depletion in cystinotic fibroblasts measured after 24 h of incubation with compounds **1** and **5a–d**. The data shown are the mean of four independent experiments ±S.E., each measurement was carried out in triplicate. 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  $n = 4$  (\* $p < 0.05$ , \*\* $p < 0.005$ ).

cystinotic fibroblasts. These pro-drugs should lead to an oral treatment with improved pharmacodynamic and pharmacokinetic parameters, leading to better compliance among cystinotic patients and an improved quality of life.

#### Acknowledgments

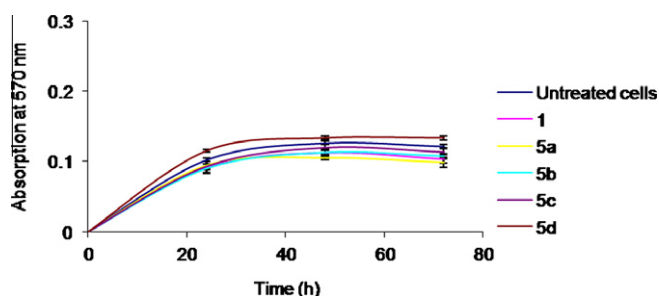
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#### Supplementary data

Supplementary data (synthetic procedures for **5a–d**, Alamar blue and thiol assays protocols) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.085.

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**Figure 2.** Alamar blue absorbance at 24, 48 and 72 h intervals for compounds **1** and **5a–d**. The data shown are a mean of eight independent experiments ±S.E., each measurement was carried out in triplicate.

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