Folate pro-drug of cystamine as an enhanced treatment for nephropathic cystinosis

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Nephropathic cystinosis is a rare autosomal recessive disease characterised by raised intracellular levels of the amino acid, cystine. If untreated, the disease, progressively deteriorates towards end stage renal disease (ESRD) at the end of the first decade. The disease is caused by a defect in the lysosomal transport mechanism for cystine. The treatment of choice is the aminothiol cysteamine which acts as a lysine mimic. However, cysteamine possesses an offensive taste and smell and irritates the gastrointestinal tract leading to nausea and vomiting following administration. Furthermore, the rapid metabolism of cysteamine requires oral administration every 6 h for life, in consequence, the patient compliance is poor. As part of our continuing work to obtain new pro-drugs for the treatment of this genetic disease, we have synthesised a folate derivative of cystamine, the disulfide derivative of cysteamine. This new pro-drug was non cytotoxic, showed greater ability to deplete intralysosomal cystine than the current treatment, and, in fact has been the most effective reducer of intralysosomal cystine discovered in our laboratories to date.

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Nephropathic cystinosis is a rare, autosomal, recessive disease with an estimated incidence of 1 case per 100,000 to 200,000 live births. It is characterised by raised intracellular levels of cystine (the dimer of the essential amino acid, cysteine). The disease symptoms are renal tubular Fanconi syndrome, poor growth, hypophosphatemic rickets, impaired glomerular function, and accumulation of cystine crystals in almost all cells, leading to tissue destruction. The typical untreated child has short stature, rickets, and photophobia. The responsible gene, CTNS, encodes cystinosin, a 367 amino acid integral membrane protein that transports cystine out of the lysosome.

Treatment of cystinosis involves symptomatic and specific therapy. The symptomatic treatment consists in the administration of fluids and electrolytes to reverse the effects of Fanconi syndrome, phosphate and vitamin D to treat rickets. The specific therapy for cystinosis is cysteamine (H2NCH2CH2SH), which acts to lower intracellular levels of cystine by forming a cysteamine–cysteine mixed disulphide which can egress the lysosome using the lysine transporter excretion pathway which remains intact in cystinosis.

Cysteamine has 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. Finally, because of a combination of high first-pass metabolism and a short half-life, cysteamine only removes cystine crystals for a period of 6 h after the medication has been taken. This means that the drug must be given every 6 h, every day for life.

In an attempt to overcome these unpleasant side-effects, recent work in our laboratories has concentrated on the development of novel pro-drugs of cysteamine and cystamine (the disulphide counterpart of cysteamine) that have a similar, or enhanced efficacy than cysteamine, in lowering the intracellular levels of cystine, while displaying fewer side effects and a longer half-life.

As part of this project, we synthesised a folate derivative of cystamine 1, whereby two molecules of cystamine were covalently attached to the carboxylate termini of the folic acid by formation of amide bonds. Folic acid pro-drugs are reported to increase the renal uptake of the parent drugs. It was envisaged that this would be advantageous for cystinotic patients as the kidneys are one of the first organs affected in nephropathic cystinosis.

The pro-drug 1 was obtained by peptidic coupling of folic acid 2 with two molecules of mono-Boc-cystamine. The deprotection...
of the product so obtained, under acidic conditions\textsuperscript{5,10} yielded the attempted water-soluble pro-drug \textsuperscript{1} with an overall yield of 92%, Scheme 1.

The cytotoxicity of pro-drug \textsuperscript{1} was determined to confirm that any change in cystine burden observed on treatment was not a consequence of cell death or an increase in cell proliferation. The test was carried out on human cystinotic fibroblasts, the standard cell line for determination of cystine levels, using the Alamar blue cell proliferation assay.\textsuperscript{11} The cystinotic fibroblasts were subjected to 50 \( \mu \text{M} \) of the current treatment, cysteamine, and the compound \textsuperscript{1}, and cell growth measured over a 72 h period. The results, displayed in Figure 1, show that there is no significant difference in cell growth of the cystinotic fibroblasts through 72 h which confirms that cysteamine and our pro-drug \textsuperscript{1} have negligible toxicity at the concentrations and time scales utilised in this Letter.

Intralysosomal cystine was measured using the commercially available Thiol and Sulfide Quantification Kit\textsuperscript{6} (Molecular Probes). Cystinotic fibroblasts were treated for 24 h with 50 \( \mu \text{M} \) of cysteamine or compound \textsuperscript{1}. 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 compound \textsuperscript{1} when compared with the control and cysteamine are displayed in Figure 2. The data are presented as micromolar cysteine per mg of protein relative to control as determined by the Bradford method.\textsuperscript{12} It can be concluded that 50 \( \mu \text{M} \) \textsuperscript{1} significantly depletes the levels of cystine in cystinotic fibroblasts relative to the control and that this depletion is 3–4-fold greater than that exerted by 50 \( \mu \text{M} \) of the current treatment, cysteamine, after 24 h incubation: a result which means this pro-drug is the most potent compound reported from our laboratories to date.

In conclusion, a new folate pro-drug of cystamine has been developed. This new pro-drug is non-cytotoxic up to 72 h of incubation and is 3–4 times more active in depletion of intralysosomal cystine than the current treatment when evaluated in cultured cystinotic fibroblasts: a result which means \textsuperscript{1} is the most potent prodrug to emerge from our prodrug programme.

Further work is currently underway in our laboratories to address the tissue specific nature of \textsuperscript{1} and to investigate in vivo pharmacokinetic parameters using a murine model of cystinosis. If these studies are successful, we aim to produce an orally active treatment for cystinosis with a less frequent administration regimen. This in turn may lead to improved compliance and a significant improvement in the quality of life of patients with nephropathic cystinosis.

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**Supplementary data**

Supplementary data (synthetic procedure for compound \textsuperscript{1}, Alamar blue and thiol assays protocols) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.048.

**References and notes**