In Vitro Evaluation of Novel Cysteamine Prodrugs Targeted to γ-Glutamyl Transpeptidase

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AIMS

The overall aim of this work is to create novel prodrugs for cysteamine to increase tolerability, efficiency and patient compliance by:

- Diminishing the side effects associated with standard cysteamine treatment
- Improving the absorption and bioavailability of cysteamine and thus decreasing the quantity and frequency of dosing
- Adequately controlling cellular cystine levels to diminish the serious complications associated with disease progression

This will be achieved by:

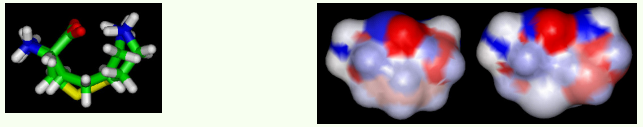
- Preserving the basic structure of cysteamine, which is essential to its mode of action
- Targeting the prodrugs to γ-glutamyl transpeptidase (GGT), an important enzyme in the glutathione cycle, with high expression on the surface of particular cells (*e.g.* kidney, liver and pancreatic cells).

BACKGROUND

Cystinosis, a rare autosomal recessive disorder, is characterised by the accumulation of cystine in the lysosomes of most tissues. The condition has been linked to mutations in the CTNS gene found on chromosome 17p13, which encodes for cystinosin,[1] a lysosomal membrane transport protein responsible for the passage of cystine from the lysosome back into the cytosol where cysteine can be recycled for protein synthesis and the glutathione cycle . As a result, the cystine content of leukocytes and cultured fibroblasts from cystinotic patients is 50 to 100 times the normal levels.[2] Symptoms include renal Fanconi syndrome, which can lead to end stage renal disease without adequate treatment.[3]

Currently, the standard treatment for cystinosis is regular doses of **cysteamine** (60-90 mg/kg every 6h), administered as the bitartrate salt, Cystagon®. Consistent adherence with this therapy can be very effective, achieving leukocyte cystine depletion of up to 95%.[4] Long term cysteamine treatment, from a young age, is beneficial in terms of survival, growth and preservation of renal function, as well as limiting other clinical manifestations (i.e. Diabetes Mellitus and pulmonary complications).[5,6]

Cysteamine reduces lysosomal cystine levels, through a thiol disulphide exchange to form cysteine and a cysteamine-cysteine mixed disulphide, which are both able to cross the lysosomal membrane, as the mixed disulphide is structurally analogous to the amino acid lysine (see Figure 1), enabling exploitation of the lysosomal lysine transporter.[7,8] The structure of cysteamine is essential to its success, hence we have concentrated on a prodrug approach to enhance delivery of cysteamine to cystinotic cells, while reducing its unpleasant taste, smell and side effects[7] and increasing its bioavailability and efficiency, which should result in lower doses being required.



A B

Figure 1: A Overlay of L-Lysine with L-Cysteine-cysteamine dimer showing similarity in shape, size and relative position of functional groups
B Comparison of surface charge distribution of L-Lysine (left) with L-cysteine-cysteamine dimer (right)

RATIONALE FOR TARGETED CYSTEAMINE PRODRUGS

The unpleasant taste and smell of cysteamine are due to the amino and thiol groups of this small molecule, both required for its efficient function in treating cystinosis. By derivatisation of both groups using prodrug moieties, their properties are changed and the taste / smell can be reduced to negligible. Amino acid prodrugs are known to have excellent solubility properties for oral administration, but there were concerns that amino acid derivatives would be targets for proteolytic enzymes in the gastrointestinal tract, which would result in breakdown of the prodrug before it had been absorbed. However, successful examples of amino acid-derivatised drugs already exist in the clinic; the use of γ-glutamyl derivatives, which are more resistant to proteolytic degradation due to the unusual amide linkage through the γ-carboxyl group, may further address these concerns. In fact, γ-glutamyl derivatives are expected to survive enzymic attack until hydrolysis by enzymes specific for γ-glutamyl derivatives, such as γ-glutamyl transpeptidase (GGT), an essential enzyme of the glutathione cycle.

GGT is found on the surface of most cells and is found in high concentrations on the surface of kidney, liver, skin and pancreatic cells, thus it provides a suitable target for γ-glutamyl-cysteamine prodrugs. Interaction of the prodrugs with GGT is expected to result in the internalisation of the prodrug and intracellular release of glutamate and cysteamine.

Recent evidence has highlighted a link between glutathione and cystinosis; the presence of higher levels of oxidised glutathione in cystinotic cells compared to normal cells suggested increased oxidative stress in these cells.[9] We have therefore chosen to concentrate on cysteamine prodrugs rather than cystamine derivatives, as the latter require intracellular reduction to release cysteamine, which may be inefficient in oxidatively stressed cells and could result in further depletion of valuable cellular reducing agents, possibly leading to further oxidative insult and even apoptosis, which has been linked to the phenotypic presentation of cystinosis.[10]

Prodrugs were synthesised where both the amino and thiol functionalities were derivatised. They have been targeted to GGT to overcome problems with taste, GI irritation and first pass metabolism; whilst also increasing bioavailability, increasing efficacy and reducing dosing. The γ-glutamyl moiety will direct the derivatised cysteamine to cells with an active glutathione cycle, therefore maximising treatment efficiency.

EXPERIMENTAL

A number of γ-glutamyl-cysteamine derivatives (prodrugs **1** - **9**) have been synthesised by standard peptide chemistry and fully characterised spectroscopically; their general structures are shown below (Figure 2).

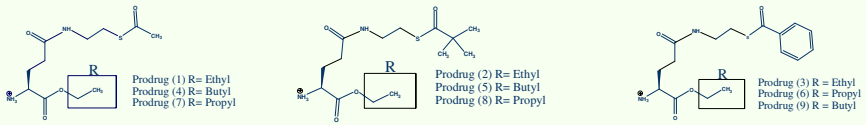


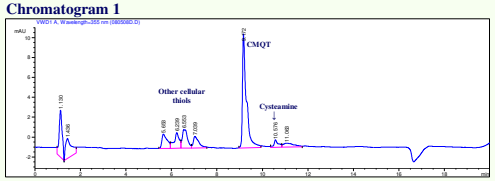
Figure 2: Prodrugs (1-9) structures – esterification of the free thiol and carboxyl groups of γ-glutamyl-cysteamine enhances absorption across lipophilic membranes, such as the GI tract.

Prodrugs **1** – **9** were incubated with Balkan Green Monkey renal (BGM) cells and human proximal tubular epithelial (HK2) cells and their ability to be internalised and release cysteamine was evaluated *via* HPLC.

A reverse phase, gradient HPLC method was adapted from current literature, employing 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) as a UV tagging agent, as demonstrated recently by McCaughan *et al.*.[11] This reagent reacts specifically with thiols and acts as a derivatising agent for the UV detection of thiol peaks, allowing the use of HPLC with standard UV detection.[12]

RESULTS & DISCUSSION

Chromatogram 1



Chromatogram 2

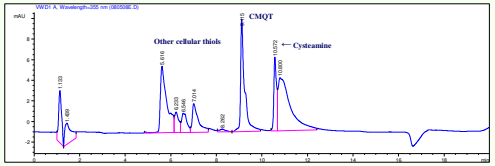


Figure 3: Thiol peaks from derivatised cell extracts, following incubation of HK2 cells with prodrug 1 (1mM) (chromatogram 2); and incubation in maturation medium only (chromatogram 1).

Peak Areas Chromatogram 1: CMQT = 136.227; Cysteamine-CMQT = 9.97 (RT determined through sample spiking with known concentration of cysteamine)
Chromatogram 2: CMQT = 130.716; Cysteamine = 59.760 (RT determined as above)

As shown above, there is an increase in the concentration of cysteamine in the cell extracts derived from prodrug treated HK2 cells compared to controls. This was found to be the case for most of the prodrugs (**1** – **7**) tested in HK2 cells (see Table 1 below). The results suggest that the cells are successfully able to internalise and breakdown the prodrugs to release cysteamine. The concentrations of cysteamine released were found to be comparable to those observed in cysteamine treated cells. Transient increases in glutathione and cysteine have also been observed, especially upon longer incubations with the prodrugs or with cysteamine; the reasons for these observations need to be further explored, although are consistent with earlier observations.[13,14,15] Peaks representing glutathione-CMQT and cysteine-CMQT were identified by spiking samples with known concentrations of these thiols.

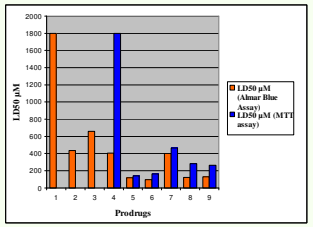
Prodrug	Peak Area observed for cysteamine	LD50 µM (Alamar Blue Assay)	LD50 µM (MTT Assay)	Toxicity Level
Control	50.031	N/A	N/A	N/A
Cysteamine	125.535	Not done	Not done	Low
γ-(*S*-acetyl cysteamine)glutamate-α-ethyl ester (1)	196.929	1800	Not done	Very Low
γ –(*S*-pivaloylcysteamine)glutamate-α-ethyl ester (2)	205.385	438	Not done	Moderate
γ –(*S*-benzoyl cysteamine)glutamate-α-ethyl ester (3)	217.583	658	Not done	Low
γ –(*S*-acetyl cysteamine)glutamate-α-propyl ester (4)	264.471	408	1800	Moderate / Low
γ-(*S*-pivaloylcysteamine)glutamate-α-propyl ester (5)	257.132	117	139.5	High
γ –(*S*-benzoyl cysteamine)glutamate-α-propyl ester (6)	104.744	93	163.1	High
γ –(*S*-acetyl cysteamine)glutamate-α-butyl ester (7)	78.673	399	467	Moderate
γ –(*S*-pivaloyl cysteamine)glutamate-α-butyl ester (8)	Not detected	122	280	High
γ –(*S*-benzoyl cysteamine)glutamate-α-butyl ester (9)	Not detected	129	265.4	High

Table 1: Peak areas observed for cysteamine, following 24H incubation of HK2 cells with cysteamine (1mM) and the various prodrugs (1mM); also includes toxicity data.

Cytotoxicity of Prodrugs

Prodrug cytotoxicity was measured using various methods, including Alamar Blue and MTT assays, as well as microscopic inspection of the cells. The most toxic of the compounds tested were two of the *S*-pivaloyl cysteamine derivatives (**5** and **8**) and two of the *S*-benzoyl cysteamine derivatives (**6** and **9**).

The drugs exhibiting the lowest cytotoxicity were the *S*-acetyl cysteamine derivatives (prodrugs **1**, **4** and **7**) and prodrug **3** (γ–(*S*-benzoyl cysteamine)glutamate-α-ethyl ester). These compounds (**1**, **4**, **7** and **3**) will now be taken forward and tested on cystinotic fibroblasts for their ability to deplete cystine compared to cysteamine.



Graph 1: Comparative representation of LD50 values obtained from Alamar Blue and MTT assays.

Future Work

- Evaluate the efficacy of the prodrugs in depletion of cystine from human cystinotic fibroblasts.
- Investigate the mechanism and efficiency of the intracellular breakdown of the prodrug to release cysteamine.
- Examine the stability of the most favourable prodrugs to gastrointestinal conditions and probe the pharmacokinetics.

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Acknowledgements

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