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Research paper

Synthesis of diacylated γ-glutamyl-cysteamine prodrugs, and *in vitro* evaluation of their cytotoxicity and intracellular delivery of cysteamine

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A R T I C L E I N F O

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A B S T R A C T

To overcome the major disadvantages of cysteamine, the only registered treatment for the rare genetic disease cystinosis, nine prodrugs of γ-glutamyl-cysteamine (**4**) were synthesized for evaluation. Esterification of the thiol conferred oxidative stability, while sufficient lipophilicity for oral bioavailability was achieved by acylation of the α-carboxyl group of γ-glutamyl-cysteamine (**4**). Low cytotoxicity was observed in cultured HaCaT keratinocytes using the MTT assay, with EC_{50} values higher than or similar to that of cysteamine. Successful uptake of the esterified prodrugs and the subsequent release of cysteamine into cultured human proximal tubule epithelial cells were demonstrated using CMQT derivatisation and HPLC with UV detection. These prodrugs show potential as novel delivery vehicles of cysteamine to improve the treatment of the genetic disorder nephropathic cystinosis.

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1. Introduction

Nephropathic cystinosis is an autosomal recessive disorder, characterized by the accumulation of cystine crystals within the lysosomes of all cells, as a result of deficiency of the cystine transporter protein. The initial symptoms of cystinosis result from the failure of renal tubules to reabsorb small molecules, causing Fanconi syndrome and the associated polyuria, glucosuria, phosphaturia, and proteinuria [1]. While kidney involvement is one of the classical features of cystinosis, it has been recognized more recently as a multi-systemic disease, due to cystine accumulation in, and damage to, non-renal organs and tissues [1,2]. Untreated, this disease progresses, usually resulting in damage to all organs and death by the age of 10 [3]. Treatment of cystinosis is typically by administration of the aminothiol, cysteamine (as the bitartrate salt, Cystagon®, or as its sustained release formulation, Procysbi®), which reduces lysosomal cystine levels and therefore delays disease progression. However, there are several problems with its administration, which can lead to non-compliance in a large proportion of those affected [4]. Even as the bitartrate salt, cysteamine has an intensely unpleasant taste and smell, resulting in nausea and vomiting [1], and frequently causes disturbance of the gastrointestinal (GI) mucosa; gastric or duodenal ulceration is a common side effect [5]. Extensive first pass metabolism of cysteamine after oral administration leads to urinary excretion of its conjugates, an estimated bioavailability of 10–30% [2], and significant amounts of dimethyl sulfide and methanethiol exhaled in the breath and through the pores of the skin as body odour [6]. To maintain a therapeutic plasma concentration, 60–90 mg/kg/day of cysteamine is required in four divided doses [7], reaching 1 g (as either 50 or 150 mg tablets) per dose for many patients. The importance of a six hourly intake of Cystagon®, despite disruption to sleep, was demonstrated by a significant increase in polymorphonuclear cystine levels after 9 hourly dosing, when compared to a 6 h dosing interval [8]. The sustained release form Procysbi® allows dosing at 12 h intervals, alleviating the issue of sleep disturbance, but, as it still releases cysteamine in the GI tract, the problems of first pass

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metabolism, resulting in low bioavailability, the release of strong smelling metabolites, and the gastric disturbance issues are not significantly addressed [9]. Additionally, a cysteamine concentration effect on cell viability and cell proliferation was observed in several cell lines, including cultured human fibroblasts, and excessively high doses of cysteamine have been linked to the appearance of bruise-like lesions on a number of cystinotic patients [10].

Cysteamine is also of interest for its potential in treating other diseases, such as Huntington's disease [11,12], cystic fibrosis [13–15], chronic kidney disease [16], and non-alcoholic fatty liver disease (NAFLD), including non-alcoholic steatohepatitis (NASH) [17]. A current clinical trial is evaluating the potential of Procysbi® as a disease-modifying treatment for Huntington's disease. If the scope of cysteamine as a treatment increases to include these other diseases, similar compliance problems due to its side effects are likely and alternative delivery approaches therefore have wider potential application than to cystinosis alone.

Cysteamine exerts its therapeutic effect due to its ability to undergo a thiol exchange with cystine, resulting in the formation of cysteine and a cysteine-cysteamine mixed disulfide [18] (Scheme 1), which can efflux the lysosome *via* the transport protein PQLC2. Evidence for the involvement of this transporter in the cysteamine-driven depletion of cystine was obtained by its genetic inactivation or silencing [19,20]. Molecular modelling has shown the structural and electronic similarities of the L-cysteine-cysteamine mixed disulfide to L-lysine, facilitating its use of this transporter [21].

The thiol and amino groups of cysteamine are therefore vital for its role in the treatment of cystinosis, but the unpleasant taste and smell contribute to low levels of patient compliance. Our initial work demonstrated the feasibility of amino acid-cysteamine conjugates as prodrugs, the depletion of cystine providing evidence of their delivery of cysteamine to cystinotic cells, with a delayed response consistent with the requirement for hydrolysis of the amino acid-cysteamine amide bond to release the active agent cysteamine [21]. A major drawback to these simple amino acid-cysteamine derivatives was their lack of clinical relevance; in particular, the thiol group confers nucleophilic and reducing properties, with disulfide bond forming potential, while α-amino acid prodrugs are known to be readily hydrolysed in the blood [22], which would release cysteamine and fail to prevent its metabolism. However, the initial studies also showed good depletion of accumulated cystine in cystinotic cells by γ-glutamyl-cysteamine, providing evidence of hydrolysis of the γ-glutamyl amide bond and release of cysteamine, while the α-glutamyl-cysteamine analogue performed poorly in comparison [21]. Initial viability experiments using γ-glutamyl-cysteamine in cells with a high expression of γ-glutamyl transpeptidase (GGT), an external cell surface enzyme that recognises and hydrolyses γ-glutamyl-derivatives, indicated low cytotoxicity [21], providing encouragement for the further development of γ-glutamyl-cysteamine into a clinical candidate.

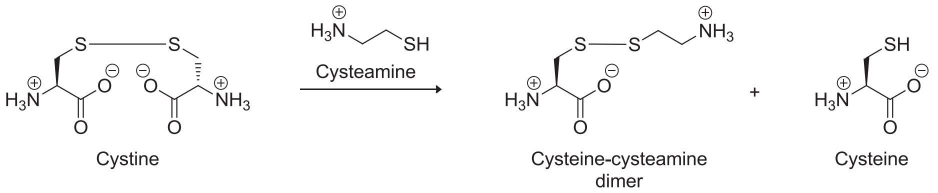
1.1. *Prodrug design and rationale*

Two further groups that are capable of bioactivation were required to convert γ-glutamyl-cysteamine into a candidate for clinical use: acylation of the thiol group to confer oxidative stability and an α-carboxyl ester to address the likely limitation to oral bioavailability caused by the zwitterionic nature of γ-glutamyl-cysteamine **4** (Fig. 1). These modifications resulted in *S*-thioester and α-carboxyl ester γ-glutamyl-cysteamine derivatives **1a-c**, **2a-c**, and **3a-c** (Fig. 1). The combined effects of varied thioester and ester groups enable a limited investigation of suitable prodrug groups with desirable pharmacokinetic and pharmacodynamic properties for the effective treatment of cystinosis by oral administration. Esterification of carboxylic acid, alcohol and thiol groups on pharmaceutical products is a successful strategy to overcome chemical instability and limited oral bioavailability, with convincing evidence from their extensive clinical application [23–25]. Ester prodrugs are hydrolysed rapidly after uptake from the GI tract by blood and liver esterases, human carboxylesterases 1 and 2 (hCE1 and hCE2), with half-lives typically 10–30 min [24].

There are three distinct enzyme cleavage steps to release cysteamine from the prodrugs (Scheme 2), presenting two main ways to deliver the active agent cysteamine into cells after absorption from the GI tract. Firstly, satisfactory lipophilicity of the prodrugs permits rapid uptake into cells directly from the blood. Once inside the cells, the prodrugs are hydrolysed to release cysteamine, by (thio)esterase activity and hydrolysis of the γ-glutamyl bond, probably by γ-glutamyl cyclotransferase. Alternatively, after uptake from the GI tract into the hepatic portal vein, the prodrugs undergo rapid esterase hydrolysis of the α-carboxyl ester (R_1) and thioester (R_2) groups in the blood and/or liver to release the key intermediate **4**, targeted to γ-glutamyl transpeptidase (GGT) on the surface of cells. Interaction of γ-glutamyl-cysteamine **4** with membrane-bound GGT results in the hydrolysis of γ-glutamyl-cysteamine **4** and local release of cysteamine on the surface of the cell, allowing its rapid internalisation. Our previous studies provide evidence for the success of this approach: incubation of cystinotic cells in medium containing γ-glutamyl-cysteamine **4** resulted in significant depletion of cystine [21].

Although a low level of serum GGT is normal in humans and is increased in various disease states, such as alcoholic liver disease, the normal activity of serum GGT is around 30 times lower than that of membrane-bound GGT [26] and is likely to make only a small contribution to the release of cysteamine in the circulation. The greater resistance of γ-glutamyl amide derivatives than their α-amide isomers to proteolytic degradation by serum proteases, due to low levels of serum γ-glutamyl hydrolysis [27], minimizes premature proteolysis of the γ-glutamyl-cysteamine bond in serum.

GGT is known to recognize a variety of small molecules linked to glutamate *via* an amide bond at the γ-carboxyl group [28,29]. It is expressed on the surface of many cells across various tissues, including the proximal convoluted tubule cells of the kidney [30], the intestinal epithelium [31], and the luminal surface of the blood



Scheme 1. Intralysosomal thiol exchange of cysteamine with cystine forms a cysteine-cysteamine dimer and cysteine, both of which can efflux the lysosome.

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