Evaluation of Carbohydrate-Cysteamine Thiazolidines as Pro-drugs for the Treatment of Cystinosis

Yasaman Ramazani,^a Elena N. Levchenko,^a Lambertus Van Den Heuvel,^b Ann Van Schepdael,^c Prasanta Paul,^c Ekaterina A. Ivanova,^a Anna Pastore,^d Trina M. Hartman,^e and Neil P. J. Price^{*e}

ABSTRACT

Cystinosis is a genetic disorder caused by malfunction of cystinosin and is characterized by accumulation of cystine. Cysteamine, the medication used in cystinosis, causes halitosis resulting in poor patient compliance. Halitosis is mainly caused by the formation of dimethylsulfide as the final product in the cysteamine metabolism pathway. We have synthesized carbohydrate-cysteamine thiazolidines, and hypothesized that the hydrolytic breakdown of cysteamine-thiazolidines can result in free cysteamine being released in target organs. To examine our hypothesis, we tested these analogs *in vitro* in patient-derived fibroblasts. Cystinotic fibroblasts were treated with different concentrations of arabinose-cysteamine, glucose-cysteamine and maltose-cysteamine. We demonstrated that the analogs break down into cysteamine extracellularly and might therefore not be fully taken up by the cells under the form of the pro-drug. Potential modifications of the analogs that enable their intracellular rather than extracellular breakdown, is necessary to pursue the potential of these analogs as pro-drugs.

Abbreviations:

L-Cys-T, lactose-cysteamine thiazolidine; R-Cys-T, ribose-cysteamine thiazolidine; A-Cys-T, arabinose-cysteamine thiazolidine; G-Cys-T, glucose-cysteamine thiazolidine; M-Cys-T, maltose-cysteamine thiazolidine

KEYWORDS: Cystinosis, Cysteamine, Halitosis, Carbohydrate-cysteamine thiazolidines, Pro-drug

1. Introduction

Cystinosis is a rare disorder characterized by a defective function of the lysosomal cystine transporter cystinosin, and is inherited in an autosomal recessive manner. Cystinosin is encoded by the CTNS gene on chromosome 17p3 and, currently, there are over 100 mutations identified in this gene that give rise to cystinosis [1,2]. The most frequent mutation, accounting for the majority of affected individuals in Northern Europe, is a deletion of 57,257 base pairs (first 10 exons) of the CTNS gene, referred to as 57kb deletion [1,3]. Cystinosis has a prevalence of 1 per 150.000 live births [4]. The general characteristic of the disease is the accumulation of cystine in cells of several organs, however, cystinosis can be classified to infantile, juvenile or ocular forms [5]. The infantile nephropathic form is the most frequent cause of inherited Fanconi syndrome, with generalized proximal tubular damage resulting in excessive excretion of amino acids, glucose, ions and other solutes in the urine [1,6]. Infantile nephropathic cystinosis manifests in polyuria, polydipsia and failure to thrive in the first year of life and can progress to end-stage renal disease around 10 years of age if left untreated [5]. As a result of cystine accumulation in cells throughout the body, other organs such as eyes, endocrine organs, muscles and central nervous system can also be affected [2]. Juvenile cystinosis occurs later and has a slower progress rate compared to nephropathic cystinosis. In ocular cystinosis, the cornea is attacked while other organs are unaffected [2,5]. The two latter account for less than 5% of all cystinosis incidence [2,6]. The mechanism by which cystine storage results in tissue damage is not clearly understood, however, studies have suggested a complex mechanism including enhanced rate of cell death, oxidative stress and altered vesicle trafficking and kinase signaling [7,8,9,10].

Cysteamine has been used for treatment of cystinosis since 1976 and is still the only available treatment [11]. Cysteamine enters the lysosome via an unknown transporter and through a disulfide exchange with cystine, converts cystine to two compounds: cysteine and cysteine-cysteamine. Cysteine exits the lysosome through a cysteine transporter, and the latter is removed from the lysosome through the PQLC2 transporter, a member of the PQ-loop protein family (Figure 1) [1,12,13].

Figure 1. Mechanism of action of cysteamine. Via a disulfide exchange with cystine, cysteamine produces cysteine-cysteamine that exits the lysosome through the PQLC2 transporter. Cysteine, another product of the reaction, exits the lysosome via a cysteine transporter. The defective cystinosin transporter is by-passed.

The oral administration of cysteamine in daily doses of 1.3 – 1.9 g/L reduces the progression of cystinosis toward end-stage renal disease and delays or prevents the extra-renal complications [14,15,16]. However, cysteamine has several side effects such as gastro-intestinal discomfort, halitosis and disagreeable sweat odor. Halitosis and disagreeable sweat odor are considered to be caused by cysteamine metabolites, methanethiol and to a larger extent dimethylsulfide [17,18]. These side effects and the frequency of administration can negatively influence the compliance of patients with cysteamine [19]. Therefore, there is a great demand for new cysteamine preparations that can reduce the side effects and increase patients' compliance. In this context, we report herein the synthesis of carbohydrate-cysteamine thiazolidines and their biological functional evaluation in patient-derived cystinotic fibroblasts.

Thiazolidines are sulfur analogs of oxazolidines, with a thioether ring containing an amine and dithiol group. They may be synthesized by a condensation reaction between a thiol and an aldehyde or ketone, a reaction that is generally considered to be reversible. Thiazolidines based on the reaction of cysteamine with sugars leads to ring opening of the monosaccharide during formation of the five-member sulfur/nitrogen-containing ring. This most probably occurs via a Schiff's base intermediate

between the carbohydrate and the cysteamine amino group, followed by a ring closure reaction of the adjacent thiol group (Figure 2). The hydrolytic reaction is probably the reverse of this, and for this reason the degradation of cysteamine thiazolidines is promoted by aqueous condition, and especially at high pH [20,21]. The hydrolysis of the thiazolidine generates the thiol and aldehyde or ketone from which it was synthesized, and therefore the facile breakdown of cysteamine thiazolidines leads to the formation of the sugar component, plus free cysteamine.

Figure 2. Reversible breakdown and synthesis of sugar cysteamine thiazolidines via a Schiff's base intermediate. R= mono- or disaccharide chains.

We hypothesize that if the breakdown of carbohydrate-cysteamine thiazolidines occurred in the target organs, the cysteamine thiazolidines might act as pro-drugs for the *in situ* formation of cysteamine, avoiding the adverse odor and taste of the free cysteamine while providing active drug for the potential treatment of cystinosis sufferers. Carbohydrate-cysteamine thiazolidines have been used as pro-drugs in the past as protective agents against γ -radiation-induced toxicity and mutagenesis [22,23,24]. In the present work we have synthesized several carbohydrate thiazolidines based on cysteamine and have evaluated their potential as pro-drugs for the treatment of cystinosis.

2. Results and discussion

2.1. Synthesis of Cysteamine-based Thiazolidines

Thiazolidines have been prepared in the past by refluxing equivalent amounts of aldehydes and aminothiols with a catalytic amount of concentrated HCl in dry benzene. Because of the known reverse hydrolysis reaction this required that water was continuously distilled from the reaction mixture by

employing a Dean-Stark trap [20,26]. Alternatively the reaction has been conducted in ethanol by stirring equimolar quantities of amine and aldehyde for four days [21], while others have favored the use of sodium methoxide in methanol for the formation of isopropylidene-protected thiazolidines [27]. The latter method, however, suffers from the need for a subsequent de-protection step. More conveniently, we have found that carbohydrate-cysteamine thiazolidines can be synthesized in near quantitative yields by heating reducing aldose sugars and cysteamine salts together at moderate temperatures in aqueous pyridine (Figure 3.A) or in methanolic pyridine. However, we also noted that a small amount of the dithiol, cystamine, was present, but generally at less than >10% (Figure 3.A).

Figure 3. A. NMR (^1H , ^{13}C , and HSQC) and B. GC/MS analyses and assignments of ribose-cysteamine thiazolidine (R-cys-T). A. The α and β assignments refer to the $1S$ and $1R$ stereoisomers, respectively, which for the R-cys-T are present in nearly equal amounts. Signals at 2.9/33 ppm and 3.4/38 ppm are due to the dithiol, cystamine. B. The GC peaks at 20.29 (β) and 20.60 (α), correspond to the peracetylated R-cys-T stereoisomers, with the α/β stereochemistry assigned arbitrarily.

The reaction of ribose or glucose and cysteamine in methanol compared to the reaction in methanolic pyridine confirmed that the pyridine was required for optimal yield (See supplementary figures S3 and S4). This was initially based on the observation that cysteamine thiazolidines could be prepared for the GC analysis of aldehydes in alcoholic beverages [28]. We subsequently looked at the reaction of various monosaccharides with cysteamine in pyridine, followed by acetylation with acetic anhydride as a method of derivatization of sugars for GC/MS analysis. From the GC/MS analysis, we

noted the formation of monosaccharide thiazolidines was quantitative under these conditions with no evidence of residual monosaccharide acetates or cysteamine acetate (Figure 3.B). However, we also noted that the solubility of some sugars, notably maltose and lactose, was quite poor in 100% pyridine. We therefore undertook a series of condensation reactions with lactose or maltose with an equimolar amount of cysteamine hydrochloride in different ratios of pyridine:water mixtures. Excellent yields of lactose and maltose thiazolidines were obtained in 70:30 v/v pyridine:water at 60 °C for 1 hour. The starting materials were fully soluble under these conditions and the reactions proceeded to completion. Noticeably the reactions in 25:75 pyridine:water and 100% water had considerable amounts of unreacted carbohydrate remaining.

Having optimized an expedient synthesis for mono- and disaccharide thiazolidines of cysteamine, we then scaled these up to the 5 g scale for ribose-cysteamine thiazolidine (R-Cys-T), arabinose-cysteamine thiazolidine (A-Cys-T), glucose-cysteamine thiazolidine (G-Cys), lactose-cysteamine thiazolidine (L-Cys-T), and maltose-cysteamine thiazolidine (M-Cys-T). In all cases the yields were excellent and provide crystalline water-soluble products with reduced thiol odor. The pentose based thiazolidines (R-Cys-T and A-Cys-T) had a notable ‘burnt popcorn-like’ odor that might be similar to that noted previously by Engel and Schieberle [29,30]. The products were characterized by NMR (¹H, ¹³C, COSY, HSQC, HMBC, and TOCSY), MALDI-TOF/MS and after peracetylation, by gas chromatography-mass spectrometry (GC/MS).

2.2. Biological function of carbohydrate-cysteamine thiazolidines

In order to evaluate the functionality of carbohydrate-cysteamine thiazolidines, we incubated patient-derived fibroblasts after 1 or 4 days of culture with 0.1 and 1 mM of each compound and measured the accumulated cystine.

2.2.1. Functional analysis of arabinose-cysteamine thiazolidine (A-cys-T)

Under basal cell culture conditions, cystinotic fibroblasts accumulated 11.4 versus 12.4 nmol cystine/mg protein on day 1 and 4 respectively. Treatment of cystinotic fibroblast with A-cys-T in concentrations of 0.1 and 1 mM resulted in significant reduction of cystine in cystinotic cells with the

Figure 4. Functional analysis of arabinose-cysteamine thiazolidine (A-cys-T). A-cys-T significantly depletes cystine from cystinotic fibroblasts in comparison with untreated cells. Data are represented

in percentage relative to untreated cystinotic fibroblasts as reference. Error bars represent standard error of the mean (SEM). * $p < 0.05$.

concentration of 1 mM being comparably efficient as cysteamine in depletion of cystine (Figure 4). A similar depleting pattern is visible in the series of cells kept in culture for 1 day and 4 days before incubation with drug analogs. The consistent pattern can be explained by the similar accumulated cystine levels in untreated cystinotic fibroblasts at day 4 compared to day 1. In other words, the culture duration did not significantly affect the cystine accumulation nor the efficacy of the analogs.

2.2.2. Functional analysis of glucose-cysteamine thiazolidine (G-cys-T) and maltose-cysteamine thiazolidine (M-cys-T)

Our experiments suggest that glucose and maltose analogs exert a similar cystine-depleting effect as displayed above for arabinose analogs (See supplementary figures S1 and S2).

2.3. Determination of the tendency towards hydrolysis of the analogs

We used CE to examine whether the analogs underwent intra- or extracellular hydrolysis. Analysis of the stability of the analogs in their solvent, PBS, as illustrated in Figure 5, suggests that the compounds are at least partly hydrolyzed to cysteamine prior to their addition to the cell culture medium.

Figure 5. Electropherograms of phosphate buffered saline (PBS, top panel), cysteamine (middle panel) and arabinose-cysteamine thiazolidine (bottom panel); the analog is hydrolyzed to cysteamine. Cysteamine is a product of disulfide bond between two cysteamines.

Cysteamine has been utilized for more than three decades for the treatment of cystinosis [1]. Several refinements have been performed in the preparation of cysteamine in order to ameliorate its side effects and increase the compliance of patients. Recently, an enteric-coated formulation of cysteamine, cysteamine bitartrate (Procysbi™), has been introduced. Procysbi™ is proved to have a safe profile and better pharmacokinetic properties. In addition, its twice daily administration compared to Cystagon (4 times per day), might enhance patients' compliance [1,19]. Halitosis, one of the significant obstacles to patients' compliance is mainly caused by cysteamine metabolites, dimethylsulfide and methanethiol [17]. Therefore, there is a great demand for new compounds in form of pro-drugs that guarantee the on-target release of cysteamine and thus minimizing the poor breath and sweat odors caused by exhalation of the

metabolites in breath and excretion of them in skin. To date, several pro-drugs have been synthesized and tested *in vitro* [31 – 35].

In line with the concept of developing pro-drugs for improving the adverse effect of cysteamine, we evaluated the efficiency of three sugar-conjugated cysteamine analogs in our *in vitro* cystinotic fibroblasts. The results have suggested that all three compounds significantly deplete cystine from the treated cells in comparison with the untreated ones. In order to find the most optimal concentration of these compounds in reducing cystine, we incubated the cells with two different concentrations of 0.1 and 1 mM of the analogs. The results suggest that 1 mM of the derivatives have the most efficient cystine-depleting properties.

Pesek and Frost observed that thiazolidines are readily hydrolyzed in strongly basic solution (>3 M aqueous NaOH), decomposing to the free aminothiol and the aldehyde [36]. However, no degradation was observed in acidic conditions (8 M HCl). They also noted that in deuterated water H-D exchange occurred at carbon-1 of the thiazolidine ring, implying that ring opening and closing occurs via an intermediate imine double bond. This would lead to ring enantiomers as we observed by the GC and NMR analysis. A mechanism of hydrolysis for N-H thiazolidines has been proposed to occur in two equilibrium steps; a prior ring opening to form a zwitterionic intermediate, followed by a base-dependent proton loss from nitrogen to give a Schiff base, which is subsequently hydrolyzes in the usual way [21]. The described prodrugs are designed to degenerate to free cysteamine at the site of action (that is, within the patient's stomach), but the rate of breakdown obviously also affects the shelf-life of the prodrugs. We have done some preliminary testing and found that the pentose thiazolidines (Rib and Ara) are more stable than the hexose analogs, and this has also been noted by others [23]. Also, that they are more stable when stored dry rather than in solution, and that this shelf-life is improved by the addition of anhydrous sodium bicarbonate.

To get an insight on the possible hydrolysis of the compounds, we used capillary electrophoresis and determined that, as anticipated, the thiazolidine analogs get hydrolyzed in their solvent. It's noteworthy, however, that our experiments were performed solely for qualitative purposes and that we cannot draw a definite conclusion as to what ratio of the compounds are hydrolyzed and what ratio remains intact. Chemical/structural modifications of the thiazolidine analogs, for instance, or the addition of stabilizers might significantly assist the stability of the intact analogs. Assessment of the precisely required preservation methods for cysteamine pro-drugs analogs until their delivery in intact form to the target cells is worthwhile.

3. Materials and methods

3.1. Materials and general methods

The cysteamine hydrochloride, pyridine, solvents, and the carbohydrates used were purchased from Sigma-Aldrich Corp., St. Louis, MO, and were of the highest purity available. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance instrument (Bruker BioSpin Corp., Billerica, MA). The spectra were recorded in D_2O , obtained from Cambridge Isotope Labs (Andover, MA). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) spectra were recorded on a Bruker Microflex instrument (Bruker Daltonics, Billerica, MA) operating in reflectron mode. An averaged 3000 shots were acquired at a frequency of 60 Hz and 75% laser power. The matrix was saturated with 2, 5-dihydrobenzoic acid, and the instrument was calibrated with defined series of maltose oligosaccharides. For chromatography mass spectrophotometry (GC/MS) analysis, the thiazolidine samples were treated with acetic anhydride and pyridine (1:1 v/v, 60 °C, 30 min), evaporated to dryness, and partitioned between water:ethyl acetate. The ethyl acetate phase was analyzed on an Agilent (Santa Clara, CA) 6890N gas chromatograph interfaced with an Agilent 5973N mass-selective detector configured in an electron impact (EI) mode. The column used was a Hewlett-Packard DB-5 MS (30 m by 0.25 mm), using helium as the carrier gas, and with the oven temperature ramped over a linear gradient from 150 to 300 °C at 10 °C/min. Positive-ion spectra were recorded over the range of m/z 43–550, and the analysis was done with Agilent Chemstation. MALDI-TOF/MS data were recorded on a Bruker-Daltonics Microflex LRF instrument (Bruker-Daltonics, Billerica, MA).

3.2. Gram-scale preparation of thiazolidines

The reactions were done with the carbohydrate (L-arabinose, D-ribose, D-glucose, lactose, or maltobiose) at a 27.8 mmol scale, and cysteamine hydrochloride (27.5 mmol). In a typical reaction, glucose (5.09 g) was added to a large tear-drop shaped rotory evaporator flask. Then cysteamine.HCl (3.01 g) was added, and water (30 mL) was added to the flask to dissolve the sugar and cysteamine. Pyridine (70 mL) was added to the tear-drop flask and swirled to mix, and the reaction flask was placed into a hot water bath at 70 °C for 2 h. The rotation was set low and without vacuum. Once the reaction was completed, the water bath was cooled to 50 °C and the vacuum was applied, slowly evaporating the water:pyridine solvent mixture and leaving a

golden-colored syrup. The vacuum rotation was continued for about 1h to remove any residual pyridine. Water (50 mL) was added to the syrup and swirled to dissolve and the flask was returned to the Roto-vap to re-evaporate. The syrup was dissolved in water (10 mL) and lyophilized to give a dry powder product. The material was sometimes purified further on a charcoal:celite column, or more simply by filtration through a charcoal:celite filter bed, washing with 5% v/v ethanol and eluting with 30% v/v ethanol. The products were characterized by NMR, MALDI-TOF/MS, and after peracetylation by gas chromatography/mass spectrometry.

3.3. Analytical characterization

3.3.1. **L-Arabinose-cysteamine thiazolidine.** ^1H NMR (500 MHz, in D_2O). δ 3.22 (t, H1' CH_2

of the thiazolidine ring), 3.60 (m, H2' CH_2 of the thiazolidine ring), 3.2-3.9 (H3 – H5 C-H sugar protons), 4.06 (d,d, H2 β , J = 6.2, 4.0 Hz), 4.26 (d,d, H2 α , J = 6.3, 2.6 Hz), 4.92 (d, H1 β *, J = 6.3 Hz), 4.95 (d, H1 α , J = 2.8 Hz).; ^{13}C NMR (125 MHz, in D_2O) δ 28.8 (C1' of the thiazolidine ring), 48.6 (C2' of the thiazolidine ring), 62.7 (C5), 65.9 (C1 β), 66.3 (C1 α), 69.7 (C2 β), 69.9 (C2 α), 70.4 (C3), 70.9 (C4). Mass spectrometry (MALDI-TOF/MS). m/z calcd for $\text{C}_7\text{H}_{15}\text{O}_4\text{NS}$, 209.072; found 210.063 $[\text{M}+\text{H}]^+$ and 232.078 $[\text{M}+\text{Na}]^+$; GC/MS (peracetylated). R_t = 18.5 min., m/z 299 $[\text{M}-120]$ (characteristic for pentose thiazolidine), 257 $[\text{M}-120\text{-ketene}]$, 214 (C3-C4 cleavage), 172 $[\text{M}-120\text{-ketene}]$, 130 (loss of thiazolidine), 88 $[\text{M}-120\text{-ketene}]$. *Note that the $\alpha\beta$ designations in the NMR assignments refer to the 1S or 1R stereoisomers of the thiazolidine ring.

3.3.2. **D-Ribose-cysteamine thiazolidine.** ^1H NMR (500 MHz, in D_2O). δ 3.20 (t, H1' CH_2 of

the thiazolidine ring), 3.55 (m, H2' CH_2 of the thiazolidine ring), 3.2-3.9 (H3 – H5 C-H sugar protons), 4.11 (d,d, H2 β , J = 6.2, 3.9 Hz), 4.23 (d,d, H2 α , J = 6.3, 2.8 Hz), 4.95 (d, H1 β , J = 6.2 Hz), 4.97 (d, H1 α , J = 2.8 Hz).; ^{13}C NMR (125 MHz, in D_2O) δ 28.7 (C1' of the thiazolidine ring), 49.2 (C2' of the thiazolidine ring), 63.5 (C5), 65.3 (C1 β), 67.0 (C1 α), 69.5 (C2 β), 70.3 (C2 α), 71.0 (C3), 72.2 (C4). Mass spectrometry (MALDI-TOF/MS). m/z calcd for $\text{C}_7\text{H}_{15}\text{O}_4\text{NS}$, 209.072; found 210.079 $[\text{M}+\text{H}]^+$ and 232.054 $[\text{M}+\text{Na}]^+$; GC/MS

(peracetylated), $R_t = 18.4$ min., m/z 299 [M-120] (characteristic for pentose thiazolidine), 257 [M-120-ketene], 214 (C3-C4 cleavage), 172 [214-ketene], 130 (loss of thiazolidine), 88 [130-ketene].

3.3.3. D-Glucose-cysteamine thiazolidine. ^1H NMR (500 MHz, in D_2O) δ 3.31 (t, H1' CH₂ of

the thiazolidine ring), 3.75 (m, H2' CH₂ of the thiazolidine ring), 3.2-3.9 (H3 - H6 C-H sugar protons), 4.02 (d,d, H2 β , $J = 6.2$, 3.9 Hz), 4.19 (d,d, H2 α , $J = 6.3$, 2.4 Hz), 4.91 (d, H1 β , $J = 6.4$ Hz), 4.97 (d, H1 α , $J = 4.0$ Hz).; ^{13}C NMR (125 MHz, in D_2O) δ 28.9 (C1' of the thiazolidine ring), 49.5 (C2' of the thiazolidine ring), 60 -75 (C3 - C6 sugar), 67.8 (C1 β), 68.2 (C1 α), 70.8 (C2 α), 72.0 (C2 β). Mass spectrometry (MALDI-TOF/MS) m/z calcd for $\text{C}_8\text{H}_{17}\text{O}_5\text{NS}$, 239.083; found 240.093 [M+H]⁺ and 262.044 [M+Na]⁺; GC/MS (peracetylated), $R_t = 22.0$ min., m/z 371 [M-120] (characteristic for hexose thiazolidine), 329 [M-120-ketene], 214 (C3-C4 cleavage), 172 [214-ketene], 130 (loss of thiazolidine), 88 [130-ketene].

3.3.4. D-Lactose-cysteamine thiazolidine. ^1H NMR (500 MHz, in D_2O) δ 3.31 (t, H1' CH₂ of

the thiazolidine ring), 3.75 (m, H2' CH₂ of the thiazolidine ring), 3.3-3.9 (C-H sugar protons), 4.00 (d,d, H2 β), 4.21 (d,d, H2 α), 4.59 (d, Gal H1'' β , 8 Hz), 4.92 (d, H1 β , $J = 6.5$ Hz), 4.85 (d, H1 α , $J = 3.9$ Hz). ^{13}C NMR (125 MHz, in D_2O) δ 28.9 (C1' of the thiazolidine ring), 49.5 (C2' of the thiazolidine ring), 60 -75 (C3 - C6 sugar), 66.9 (C1 β), 68.2 (C1 α), 70.8 (C2 α), 72.4 (C2 β), 94.3 (GalC1'' β). Mass spectrometry (MALDI-TOF/MS) m/z calcd for $\text{C}_{14}\text{H}_{27}\text{O}_{10}\text{NS}$, 401.135; found 402.241 [M+H]⁺ and 424.149 [M+Na]⁺; GC/MS (peracetylated), $R_t = 27.8$ min., m/z 432 [loss of Glc thiazolidine], 331 [M-loss of Gal], 130 (loss of thiazolidine), 88 [130-ketene].

3.3.5. D-Maltose-cysteamine thiazolidine. ^1H NMR (500 MHz, in D_2O) δ 3.31 (t, H1' CH₂ of

the thiazolidine ring), 3.75 (m, H2' CH₂ of the thiazolidine ring), 3.2-3.9 (C-H sugar protons), 4.02 (d,d, H2 β), 4.19 (d,d, H2 α), 4.91 (d, H1 β , $J = 6.4$ Hz), 4.97 (d, H1 α , $J = 4.0$ Hz), 5.17 (d, Glc H1'' α , $J = 4.0$ Hz). ^{13}C NMR (125 MHz, in D_2O) δ 29.1 (C1' of the thiazolidine ring), 49.5 (C2' of the

thiazolidine ring), 60 -75 (C3 – C6 sugar), 67.8 (C1 β), 68.2 (C1 α), 70.7 (C2 α), 72.1 (C2 β), 91.1 (Glc C1'' α). Mass spectrometry (MALDI-TOF/MS) m/z calcd for C₁₄H₂₇O₁₀NS, 401.135; found 402.324 [M+H]⁺ and 424.410 [M+Na]⁺; GC/MS (peracetylated), R_t = 27.45 min., m/z 432 [loss of Glc thiazolidine], 331 [M-loss of Glc], 130 (loss of thiazolidine), 88 [130-ketene].

3.4. Cell culture and incubation

Fibroblasts were cultured from skin biopsies of healthy controls and cystinotic patients as described previously [25]. Control fibroblasts and cystinotic fibroblasts (homozygous deletion of 57 kb) were kept in culture for either one or four days before adding cysteamine analogs. Fibroblasts were then sub-cultured in 6-well plate recipients in the density of 600,000 cells per well. In order to add cysteamine analogs, culture medium was replaced with freshly-prepared medium containing cysteamine analogs. In all experiments, cells were incubated with the analogs for 24 hours in triplicate. For comparison, cells were incubated with cysteamine (1 mM) for 24 hours.

3.5. Pellet preparation

After 24 hours, cells were washed once with 10% Dulbecco phosphate buffered saline (DPBS, Gibco). Each well was scraped with 200 μ l of PBS and centrifuged at 2500 rotation per minute (RPM) for 5 minutes. Pellets were dissolved in 5 mM *N*-Ethylmaleimide (NEM, Sigma-Aldrich) in 0.1 mM phosphate buffer (100 μ l). Each sample was subject to three times sonication of 30 seconds while kept on ice. 120g/L of 5-Sulfosalicylic acid dehydrate (SSA, Sigma-Aldrich) was added to cells in amount of 50 μ l prior to being centrifuged at 13000 RPM for 5 minutes. The acid soluble fraction was separated from the protein pellet and stored at -80 °C. Protein pellets were dissolved in 0.1 M sodium hydroxide (150 μ l) and stored at -80 °C. Acid soluble fractions and protein pellets were sent to the Laboratory of Metabolomics and Proteomics, Children's Hospital and Research Institute Bambino Gesù (Italy) for cystine measurement by high performance liquid chromatography (HPLC).

3.6. Capillary electrophoresis (CE) analysis of cysteamine analogs

3.6.1. Chemicals

The chemicals used were of analytical grade: ammonium hydroxide solution (25 % ammonia in water) (Riedel-de-Häen, Seelze, Germany), ammonium acetate (Fisher scientific,

Loughborough, UK), sodium hydroxide pellets (VWR, Leuven, Belgium), or of HPLC grade: methanol (Acros organics, Geel, Belgium). The water was purified (18 MΩ/cm) in a Milli-Q® system (Millipore, Milford, MA, USA). Cysteamine hydrochloride, 99.15 % was purchased from Acros organics. Due to stability reasons the solutions were prepared immediately before use and cysteamine.HCl as well as the cysteamine analogs were stored under refrigeration (4 °C).

3.6.2. Buffer and sample preparation

The buffers utilized were at most 3 days old. The amount corresponding to the indicated molarity was weighed and dissolved in water, and the pH was adjusted to 8.85 with dilute solutions of ammonium hydroxide solution. For the background electrolyte containing methanol, the reported pH corresponds to the aqueous solution before the addition of the alcohol. The pH was measured and adjusted with the aid of a pH-meter Metrohm 691 (Herisau, Switzerland). In order to ascertain the accuracy of these measurements, it was calibrated before each measurement with reference buffer solutions, as described in the Ph. Eur. The cysteamine standard was freshly dissolved in Milli-Q water at a concentration of 6 mg/mL. The samples of cysteamine analogs were diluted 6 times with water starting from the stock solutions of the corresponding compounds in PBS. For long term storage, all these solutions were kept at -80 °C.

3.6.3. Capillary electrophoresis

Capillary electrophoresis experiments were performed on a P/ACE™ MDQ equipment with diode array detector and the data acquisition was done by means of 32 Karat™ 4.0 software (both Beckman-Coulter, Fullerton, CA, USA). The capillaries used were purchased from Polymicro Technologies (Phoenix, AZ, USA). All of the capillaries used were uncoated fused silica, 75 µm ID, 40 cm long, with the detection window burnt at 10 cm from the opposite end. New capillaries were conditioned by rinsing with water for 5 min, then with NaOH (1 M) for 5 min and keeping them filled with NaOH (1 M) for 120 min. Finally they were rinsed with NaOH (0.1 M) followed by water, 5 min each. Daily, prior to analysis, the capillary was conditioned by the following washing sequence: water (5 min), 0.1 M NaOH (10 min), water (10 min), running electrolyte (10 min), and application of 15 kV (10 min). Between each run one single flush of 2 min was performed with running electrolyte. All the washing procedures were performed by applying a pressure of 138 kPa (20 psi). The capillary was thermostated at 25 °C during all of the experiments, including washing procedures. The inlet/outlet vials were replaced every 3 runs. A

voltage of 28 kV was applied. UV detection was performed at 195 nm. The composition of the background electrolyte was methanol-15 mM ammonium acetate (pH 8.85) (10:90, v/v).

3.7. Statistical analysis

Statistical significance for comparison of control and cystinotic fibroblasts was analyzed by IBM SPSS Statistics 23 software. Significant difference was considered at $p < 0.05$.

4. Conclusions

In summary, we present a novel and expedient synthesis of carbohydrate-based cysteamine thiazolidines on the gram-scale and their further functional testing on cystinotic fibroblasts. To this end, we demonstrated that the synthesis of the analogs, albeit novel, requires some modifications for *in situ* hydrolysis of the analogs in the cells. This research provides a premise for the potential synthesis of carbohydrate-cysteamine thiazolidines with consideration of possible methods to improve their properties to function as pro-drugs.

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