Different inhibition characteristics of intracellular transglutaminase activity by cystamine and cysteamine

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Abstract

The treatment of cystamine, a transglutaminase (TGase) inhibitor, has beneficial effects in several diseases including CAG-expansion disorders and cataract. We compared the inhibition characteristics of cystamine with those of cysteamine, a reduced form of cystamine expected to be present inside cells. Cystamine is a more potent inhibitor for TGase than cysteamine with different kinetics pattern in a non-reducing condition. By contrast, under reducing conditions, the inhibitory effect of cystamine was comparable with that of cysteamine. However, cystamine inhibited intracellular TGase activity more strongly than cysteamine despite of cytoplasmic reducing environment, suggesting that cystamine itself inhibits *in situ* TGase activity by forming mixed disulfides.

Keywords: cystamine; cysteamine; inhibitor; polyamine; transglutaminase

Introduction

Transglutaminases (TGases) are a family of enzymes that catalyze the transamidation reaction between the γ-carboxyamide of a peptide-bound glutamine residue and the ε-amino group of a peptide-bound lysine residue or the primary amine group of a polyamine *via* forming a thioester acylenzyme intermediate at the active site cysteine (Lorand *et al.*, 1984). Thus, TGase activity produces cross-linked proteins or amine conjugates.

At least 3 types of TGases (TGase 1, 2 and 3) are expressed in human brain (Cooper *et al.*, 2002). Given the fact that the catalytic properties of TGases acting on selected glutamine residues generate protein aggregates, TGase activity has been implicated in a number of neurodegenerative disorders caused by an abnormal expansion of polyglutamine repeats, such as Dentatorubral-pallidoluysian atrophy, Spino-bulbar muscular atrophy, Spinocerebellar ataxia and Huntington's disease (HD) (Cooper *et al.*, 2002; Gentile *et al.*, 2004). Recent reports showed that the treatment with cystamine, a TGase inhibitor, resulted in prolonged survival and decreased abnormal movements in a murine model of HD (Dedeoglu *et al.*, 2002; Karpuj *et al.*, 2002), suggesting a possible role for TGases in the pathogenesis of neurodegeneration. Thus, the development of TGase inhibitors based on the inhibition mechanism of cystamine could lead to novel pharmacological strategies for the treatment of neurodegenerative disorders.

Cystamine (β,β'-diaminodiethyl disulfide) is a thiolamine joined by a disulfide bridge between two cysteamine (β-mercaptoethylamine), and has been used to inhibit TGase activity in both *in vitro* and *in vivo* assay systems. However, the inhibition mechanism of cystamine has not been clearly understood. Since cystamine may be converted to cysteamine in cytoplasmic reducing environment (Cooper *et al.*, 2002; Gentile *et al.*, 2004), it has been thought that cysteamine, not cystamine, is an intracellular form of TGase inhibitor. Nonetheless, it is not known as to whether the inhibitory effect of cysteamine on TGase activity is comparable with cystamine. In this study, we compared the inhibitory effects of cystamine with those of cysteamine on TGase activity and showed that cystamine is a more potent inhibitor of TGase than cysteamine in the intact cells.

Materials and Methods

Materials

5-(biotinamido)pentylamine was obtained from Pierce and other chemicals were purchased from Sigma (St. Louis, MO). [^{14}C]putrescine was obtained from Perkin-Elmer. All culture media were purchased from Invitrogen.

Cell culture

A549, MCF7, T47D, HepG2 and K562 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Saos2, HEK293, SH-SY-5Y, HeLa, PC3M, HaCaT and DU145 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HLE-B3 cells were grown in modified Eagle's medium containing 20% fetal bovine serum. Penicillin (100 U/ml), streptomycin sulfate (100 µg/ml) and glutamine (2 mM) were supplemented to all cell culture media. All cell lines were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Production of human TGase 2 protein

Recombinant human TGase 2 protein was produced using baculovirus expression system (Pharmingen, San Diego, CA) and a nickel-nitrilotriacetic acid column (Qiagen, Hilden, Germany) as previously described (Shin *et al.*, 2004), monitoring TGase activity by [^{14}C]putrescine incorporation assay (Jeon *et al.*, 2002). Purified TGase 2 was dialyzed against a buffer (30 mM Tris-Cl, pH 7.5, 15 mM NaCl) and stored at -20°C in a buffer (30 mM Tris-Cl, pH 7.5, 15 mM NaCl and 50% glycerol).

Microtiter plate assay for *in vitro* TGase activity

Each well of a 96-well microtiter plate was coated with N,N'-dimethylcasein at 10 µg/ml (100 µl/well) in a buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA) for 1 h at room temperature. The wells were overcoated with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature. The wells were then washed three times with PBS containing 0.1% Tween 20 (PBST). TGase 2 (10 ng of purified protein) was added to each well which contains 5-(biotinamido)pentylamine (BP; 0-20 µM) in the 100 µl of a buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10 mM CaCl$_2$ and 0.1% Triton X-100). In experiments under reducing conditions, either dithiothreitol or glutathione at 0-20 mM, respectively, was included in buffer R. In experiments with TGase inhibitors, five amine compounds (cystamine, cysteamine, putrescine, spermidine and spermine) at 0-5 mM were included in the reaction mixture. The plates were further incubated for 1 h at 37°C, followed by washing three times with PBST. The incorporation of BP into N,N'-dimethylcasein was measured with horseradish peroxidase-conjugated streptavidin in 5% BSA (1.25 mg/ml, 100 µl/well) for 45 min at 37°C. After washing the cells with PBST five times, the wells of plates were incubated with O-phenylenediamine dihydrochloride (0.4 mg/ml, 50 µl/well) in citrate-phosphate solution (0.1 M citric acid, 0.2 M Na$_2$HPO$_4$) for 5-15 min at room temperature before the addition of 1 M H$_2$SO$_4$ (100 µl/well) solution. The color development was quantitated by measuring the absorbance at 490 nm on microplate spectrophotometer (Molecular Devices).

in situ TGase assay

in situ TGase activity was measured as previously described (Jeon *et al.*, 2003a; b). Briefly, the cells (2 ×10^6) were labeled with BP at 1 mM or amine compounds (0 to 1.0 mM) in serum-free medium for 1 h prior to harvesting. After washing twice with PBS, the cells were suspended in PBS containing protease inhibitors and sonicated (2 s pulse/2 s pause × 5, 20% amplitude). The homogenate was centrifuged for 10 min at 20,000 *g* at 4°C. The cell extract (0.2 mg/ml, 50 µl/well) was coated in the wells of a 96-well microtiter plate for 12 h at 4°C. The wells were then overcoated with 5% BSA in PBS for 1 h at room temperature. After washing three times with PBST, BP incorporated into the cellular proteins was assessed as performed in microtiter plate assays.

Statistical analysis

The mean values were compared using analysis of variance (one-way ANOVA) plus Student's *t*-test. Statistical significance was set at a *P*-value $<$ 0.05. GraphPad Softwares were used for statistical analysis and curve fitting.

Results

Inhibition of *in vitro* TGase activity by cystamine or cysteamine

To compare the difference of inhibition characteristics between cystamine and cysteamine in a reducing condition, we first examined the effects of reducing agents on *in vitro* TGase activity. Dithiothreitol (DTT) or reduced-glutathione (GSH) increases TGase activity at higher concentration (Figure 1A). The maximum increase of the TGase activity was 3.8-fold with DTT at a concentration of 10 mM, whereas the TGase activity was 2.7-fold increase with GSH at 5 mM. Interestingly, in contrast to DTT, the TGase activity with GSH higher than 5 mM was remarkably diminished.
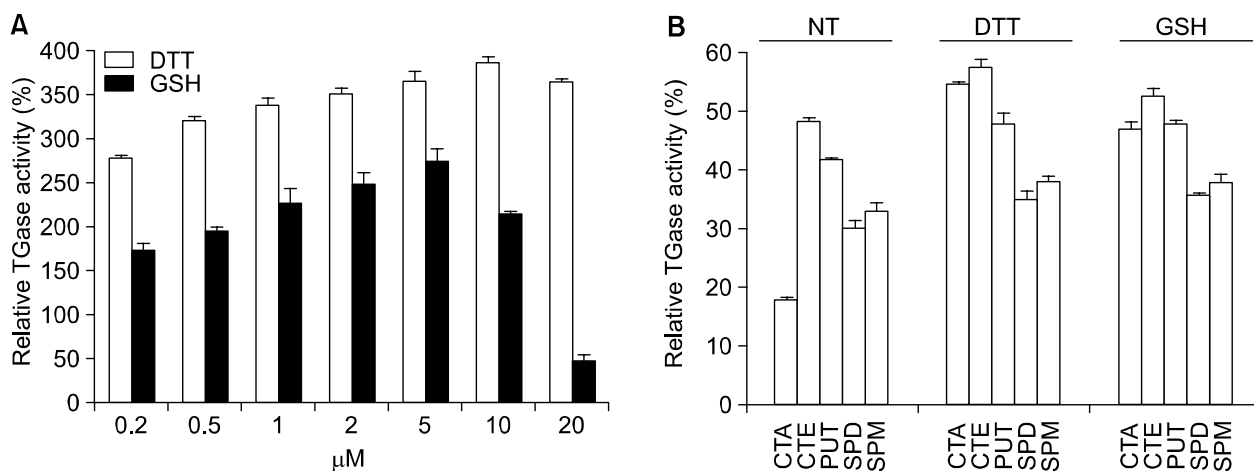
A

B

Figure 1. Inhibitory effects of cystamine and cysteamine on *in vitro* TGase activity. Recombinant TGase 2 (10 ng) was incubated with BP (5 μM) in the absence or presence of either DTT or GSH at various concentrations. The incorporation of BP to N,N'-dimethylcasein was measured with horseradish peroxidase-conjugated streptavidin and quantitated in terms of the absorbance at 490 nm. Relative TGase activity is calculated based on the negative control, without DTT and GSH, as 100%. (A) Effects of DTT or GSH on *in vitro* TGase activity. (B) Inhibition of *in vitro* TGase activity by amine compounds. Each amine compound (200 μM; except cystamine at 100 μM in the reaction with reducing conditions) was added to the reaction mixture. NT denotes the experimental condition without DTT and GSH. The figures represent the mean values ± SEM. (*n* = 3 independent experiments performed with the samples in triplicate). CTA, cystamine; CTE, cysteamine; PUT, putrescine; SPD, spermidine; SPM, spermine.

Table 1. Inhibitory effects of cystamine or cysteamine on *in vitro* TGase activity in the presence of DTT or GSH.

Inhibitors	IC_{50} (μM)		
	NT	DTT (DTT/NT)	GSH (GSH/NT)
Cystamine	22 ± 5	212 ± 24 (9.6)	124 ± 19 (5.6)
Cysteamine	178 ± 17	232 ± 31 (1.3)	218 ± 27 (1.2)

Recombinant TGase 2 (10 ng) reacted with BP (5 μM) in the absence (NT) or in the presence of either DTT or GSH at 2 mM, respectively. Cystamine or cysteamine at varying concentrations (0-5,000 μM) was added to the reaction mixture. The table represents the mean values ± SEM (*n* = 3). IC_{50} denotes the inhibitory concentration resulting in a 50% inhibition of TGase activity.

We next compared the effects of cystamine and cysteamine on *in vitro* TGase activity in the presence or absence of reducing agents (Table 1). Cystamine and cysteamine inhibited *in vitro* TGase activity with IC_{50} values of 22 and 178 μM, respectively. In the presence of reducing agents, the inhibitory capacity of cystamine for TGase activity decreased by 5.6-fold (GSH) or 9.6-fold (DTT). On the other hand, the inhibition by cysteamine decreased only slightly (1.2- to 1.3-fold). These results indicate that cystamine is a more potent inhibitor for *in vitro* TGase activity than cysteamine in a non-reducing condition.
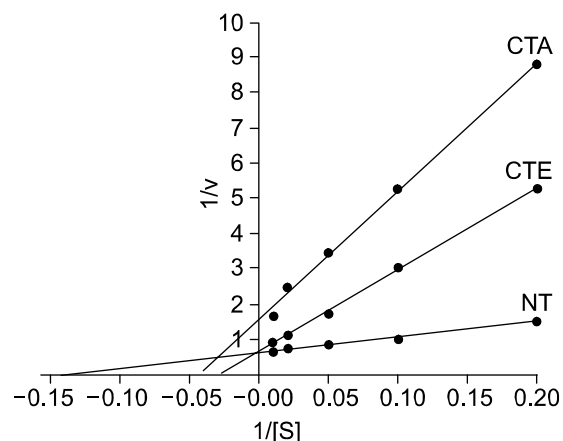

Figure 2. Kinetic analysis of TGase inhibition by cystamine or cysteamine under a non-reducing condition. Recombinant TGase 2 was incubated with varying concentrations of BP in the absence and presence of 500 μM cystamine or cysteamine, respectively. The BP incorporated into N,N'-dimethylcasein was measured with horseradish peroxidase-conjugated streptavidin by absorbance at 490 nm. Representative data from a typical experiment are presented (*n* = 3 independent experiments performed with the samples in duplicate).

Comparison of inhibitory effects of cystamine with that of polyamines

To determine whether the presence of disulfide bond of cystamine is associated with inhibitory capacity of cystamine, we compared the effects of naturally oc-curring polyamines including putrescine, spermidine and spermine, which have two primary amine groups

without disulfide bond, on TGase with those of cysta-mine and cysteamine. Among five compounds exa-mined under non-reducing condition, cystamine is the most, and cysteamine the least effective inhibitor of TGase activity with following order: cystamine > spermidine ≈ spermine > putrescine > cysteamine (Figure 1B). Putrescine, spermidine, spermine and cy-steamine exhibited a similar inhibitory activity for TGase regardless of the presence of reducing agents. By contrast, only cystamine decreased its inhibitory capacity significantly under the reducing conditions.

A Lineweaver-Burk plot showed that Km, Vmax and slope were all affected by cystamine. The graph pattern seemed to be intermediate between those for competitive and non-competitive inhibitions. However, competitive inhibition pattern was noted only with cy-steamine (Figure 2). By contrast, in the presence of reducing agents, both cystamine and cysteamine showed competitive inhibition kinetics (Cooper *et al.*, 2002). Taken together, these results indicate that a disulfide bond of cystamine may react with the active site cysteine of TGase, which is required for the efficient inhibition of *in vitro* TGase activity.
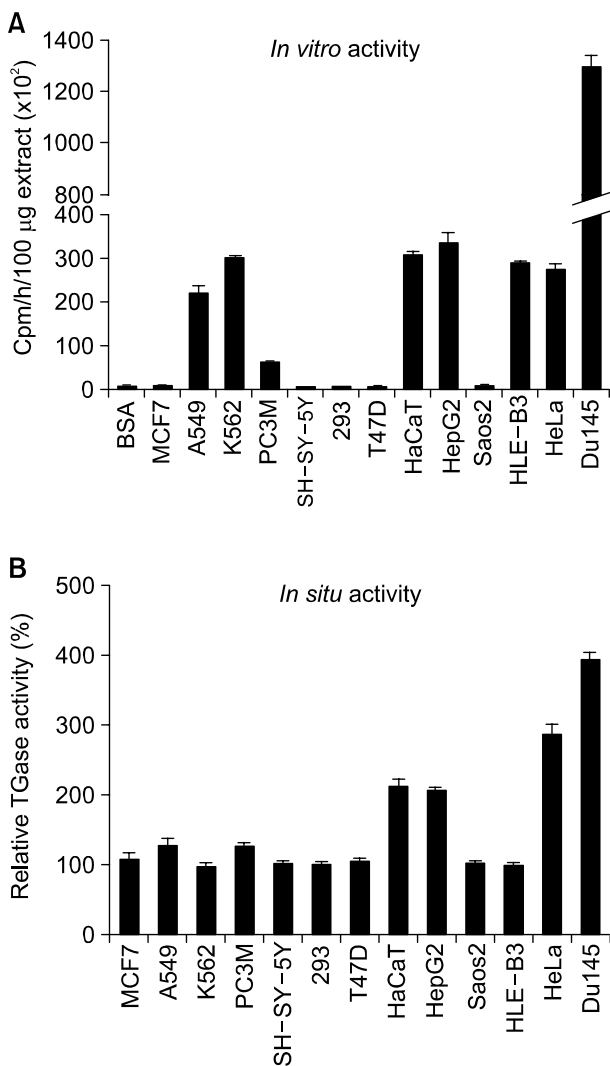

Figure 3. *in vitro* and *in situ* TGase activity of cultured cell lines. (A) *in vitro* TGase activity for each cell line was expressed in (cpm/h) per 100 μg extract. BSA was adopted as a negative control for samples. The figures represent the mean values ± SEM of three determinations. (B) The *in situ* TGase 2 activity of each cell line is expressed relative to that of control cells cultured in the absence of BP (100%). The figures represent the mean values ± SD of four determinations.
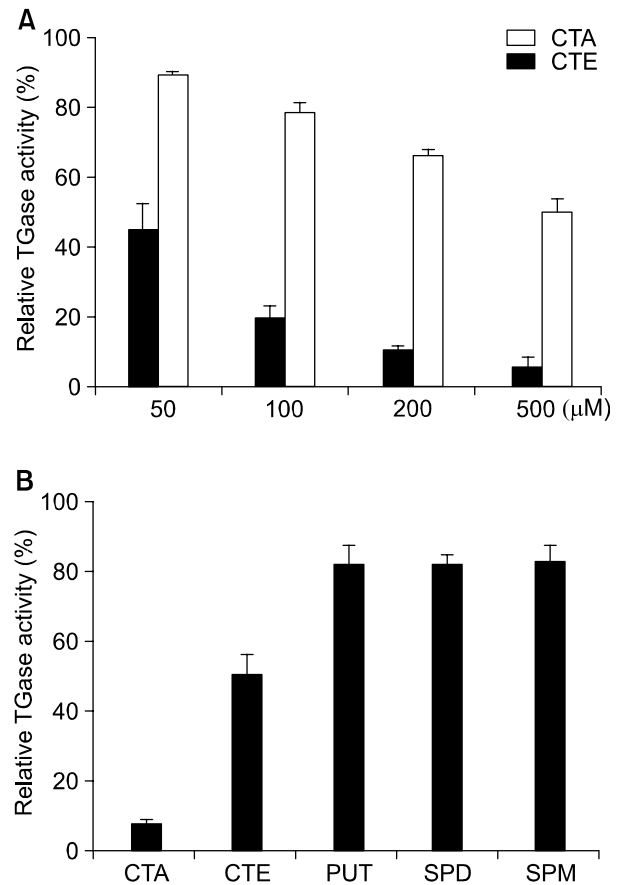

Figure 4. Inhibition of *in situ* TGase activity of DU145 cells by cystamine and cysteamine. (A) The inhibition of cellular *in situ* TGase activity with a various amount of cystamine or cysteamine. DU145 cells were labeled with BP in the presence of cystamine or cysteamine, respectively. (B) The inhibition of cellular *in situ* TGase activity with cystamine, cysteamine, putrescine, spermidine or sper-mine at 500 μM, respectively. TGase activity is expressed relative to that of the cells cultured without cystamine or cysteamine (100%). The figures represent the mean values ± SD (*n* = 3-4 independent experiments performed with the samples in triplicate). The mean values were compared using analysis of variance (one-way ANOVA) plus Student's *t*-test.

Inhibition of *in situ* TGase activity by cystamine or cysteamine

Cystamine may be converted to cysteamine in the cytoplasmic reducing environment (Cooper *et al.*, 2002; Gentile *et al.*, 2004). Thus, we questioned whether the inhibitory effect of cysteamine on *in situ* TGase activity is different from that of cystamine in cultured cell system. To address this question, a number of cell lines were screened in terms of *in vitro* and *in situ* TGase activities to select a suitable cell line. While A549, K562, PC3M, HepG2, HaCaT, HLE-B3, HeLa and DU145 cells showed *in vitro* TGase activities (Figure 3A), HepG2, HaCaT, HeLa and DU145 cells exhibited remarkable intrinsic *in situ* TGase activities (Figure 3B). Among the cell lines examined, DU145 cells exhibited strong positive TGase activities both *in vitro* and *in situ*, and was selected for investigating *in situ* TGase activities.

Cystamine and cysteamine showed a concentration-dependent inhibition of *in situ* TGase activity of DU145 cells. However, it is apparent that cystamine is a more effective inhibitor than cysteamine (Figure 4A). When the inhibitory effects of five amine compounds on the *in situ* TGase activity were compared with each other, cystamine was found to be the most effective inhibitor (Figure 4B). These results indicate that *in situ* TGase activity may be inhibited by cystamine per se in cell culture experiments, and that cystamine may be not completely converted to cysteamine in the intracellular environment.

Discussion

Cystamine has been used as a TGase inhibitor in experiments *in vitro* as well as *in vivo*. It is proposed that cystamine inhibits TGase activity by forming mixed disulfide bonds that may block the formation of a thioester acylenzyme intermediate or by competing with transamidation reaction between the ε-amino group of a peptide-bound lysine residues and the γ-glutamyl residue of proteins bound to TGase (Lorand *et al.*, 1979; Cooper *et al.*, 2002). We found that the inhibition potency of cystamine is significantly decreased in the presence of reducing agents such as DTT or GSH, indicating that the disulfide bond of cystamine may be an important structural component in inhibiting TGase activity. Indeed, these data suggest that the formation of mixed disulfide bonds at active site cysteine residue of TGase mainly contribute to the inhibitory mechanism of cystamine. However, our kinetic studies suggest that the primary amine group of cystamine may also contribute to the competitive inhibition. These findings may be important for developing pharmacologic agents that can inhibit TGase activity *in vivo*.

The cytoplasmic environment is considered to be under a reducing condition (Cooper *et al.*, 2002; Gentile *et al.*, 2004). However, our results demonstrated that cystamine inhibited *in situ* TGase activity far more effectively than cysteamine. In addition, the current data indicate that the inhibitory capacity of cystamine was less affected by GSH than that by DTT, suggesting that cellular GSH redox regulation is not capable of effectively converting cystamine to cysteamine. However, since pharamacokinetic profiles, such as uptake rate and half-life, of thiolamines and polyamines were not examined in this study, we did not discriminate whether the differential effects between cystamine and cysteamine are caused by reducing capacity or pharmacokinetic parameters. The development of convenient assay system for detecting intracellular thiolamines is required to clarify these points.

In eukaryotic cells, three naturally occurring polyamines, putrescine, spermidine and spermine, are derived from L-arginine and L-methionine *via* a series of enzymatic reactions (Wallace *et al.*, 2003). These polyamines are positively charged aliphatic cations. Since most polyamines in cells are sequestered by the association with negatively charged molecules, such as nucleic acids or phospholipids (Igarashi and Kashiwagi, 2000), only a small portion of free polyamines would be available as amine donors for TGase. Thus, this hypothesis may explain the poor capacity of natural polyamines for the inhibition of TGase activity *in situ*. Cysteamine is a small compound that contains one amine group and sulfhydryl group, and therefore, less prone to interact with other negatively charged molecules within cells. Thus, the availability of free amine group in the molecules may be a critical factor to determine its inhibitory capacity.

Despite positive therapeutic effects of cystamine in a murine model of HD (Dedeoglu *et al.*, 2002; Karpuj *et al.*, 2002), the mechanism by which cystamine treatment is beneficial to the mice is not understood. In addition to TGase inhibition, cystamine is able to replenish glutathione and to inhibit caspase activity (Lesort *et al.*, 2003). Moreover, in contrast to cysteamine, it is known that cystamine does not cross the blood brain barrier (Cooper *et al.*, 2002). Thus, it is probable that cystamine may act in symptomatic improvement of HD model mice by mechanism other than TGase inhibition, and that the metabolic effects of cysteamine could contribute to the therapeutic benefits in a murine model of HD.

Cysteamine is a metabolite of pantetheine or coenzyme A and serves as a precursor for hypotaurine and taurine that is a neurotransmitter, exerting neuroprotective actions in neural tissue from ischemia, oxidative stress and the presence of free radicals (Vecsei *et al.*, 1990; Saransaari *et al.*, 2000). In

addition, since cross-linking reaction between proteins is competitively inhibited by cysteamine (Lorand *et al.*, 1984; Cooper *et al.*, 2002), this inhibition leads to the accumulation of amine-conjugated proteins within the cells. Thus, the functional alteration of target proteins by thiolamine incorporation needs further characterization.

In summary, the inhibitory effects of cystamine and cysteamine on TGase activity were analyzed. The inhibitory capacity of cystamine *in vitro* was largely affected by the extent of the reduced form of the molecule. Nonetheless, cystamine inhibits *in situ* TGase activity decidedly stronger than cysteamine. These results may provide some insights for future investigation, regarding cystamine or its derivatives, to develop a treatment for TGase-related disorders.

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