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Research Report

Cellular thiol pools are responsible for sequestration of cytotoxic reactive aldehydes: Central role of free cysteine and cysteamine

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ABSTRACT

Cellular thiol pools have been shown to be important in the regulation of the redox status of cells, providing a large antioxidant pool consisting of free thiols, thiols bound in the disulfide form and thiols bound to proteins. However, experimental studies with the thiol cysteamine and its disulfide cystamine have demonstrated dramatic cytoprotection in experimental models where antioxidants provide only minor protection. These data suggest that an alternate action of thiols is important in their cytoprotective actions. A common feature of the *in vitro* and *in vivo* models, where these thiol agents demonstrate cytoprotection, is the generation of cytotoxic aldehydes. We therefore studied the actions of cystamine, cysteamine and several reference thiol agents as cytoprotectants against cell death induced by increased "aldehyde load". We found that all the thiol agents examined provided dramatic protection against aldehyde-induced cell death in SN56 cholinergic neurons, under conditions in which acrolein induced 100% cell death. With regard to mechanism of action, the reference thiols cysteine, *N*-acetylcysteine, 2-mercaptoethanesulfonic acid, mercapto-propionyglycine, and cysteamine can directly sequester aldehydes. In addition, these thiols were all found to augment intracellular cysteine levels via disulfide interchange reactions. Cysteamine and cystamine also augmented basal intracellular cysteamine levels. Our data, for the first time, demonstrate the importance of intracellular thiols in sequestering toxic reactive aldehyde products of lipid peroxidation and polyamine metabolism. In addition it appears that pharmacological manipulation of intracellular thiol pools might offer a new approach in the design of neuroprotective drug candidates.

1. Introduction

The thiol agent cysteamine and its disulfide, cystamine, have been shown to be neuroprotective in a number of cell culture and animal models. *In vitro*, these include protection from glutamate toxicity in rat primary astroglial cultures (Ientile et al., 2003), from 3-nitropropionic acid toxicity in Huntington's disease knock-in murine striatal cells (Mao et al., 2006), and from toxicity in cellular models of polyglutamine aggregation (Fox et al., 2004). *In vivo*, neuroprotection has been demonstrated in the R6/2 murine model of Huntington's disease (Dedeoglu et al., 2002); in the YAC128 murine model

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of Huntington's disease (Pinto et al., 2005); against striatal lesions induced by parenteral injection of the mitochondrial toxin 3-nitropropionic acid (Fox et al., 2004) and in the MPTP murine model of Parkinson's disease (Tremblay et al., 2006). There is also a large historical database demonstrating cytoprotection with cysteamine and cystamine in animal models of chemically induced hepatotoxicity (Nagiel-Ostaszewski and Lau-Cam, 1990) and as radioprotectants (Stoklasova et al., 1980; Zheng et al., 1988).

These cytoprotective properties of cystamine and cysteamine are dramatic; however, the mechanism of action remains controversial. It is clear that cysteamine is most likely the active principal since cystamine is rapidly metabolized to cysteamine (Pinto et al., 2005). With this in mind, a number of molecular mechanisms have been studied. Inhibition of transglutaminases by cystamine led to its evaluation in murine models of Huntington's disease; however, while neuroprotection was demonstrated, studies of R6/2 mice expressing and not expressing transglutaminase concluded that transglutaminase inhibition is not the neuroprotective mechanism of cystamine (Bailey and Johnson, 2006). In contrast, dramatic and sustained increases in cellular cysteine and glutathione levels in human T4 lymphoblastoid cells (Jokay et al., 1998); in human neuroblastoma cells over-expressing transglutaminase (Lesort et al., 2003) and in PC12 cells transfected with huntingtin (Fox et al., 2004), suggest that augmentation of cellular thiol pools by cystamine and cysteamine may be important in their neuroprotective and cytoprotective actions. In this regard, *in vivo* studies have demonstrated that cystamine and cysteamine increase brain levels of cysteine (Fox et al., 2004; Pinto et al., 2005). Cystamine has also been shown to augment the non-protein thiol pool in bone marrow and small intestine (Stoklasova et al., 1980). While the intracellular thiol pool, including cysteine and

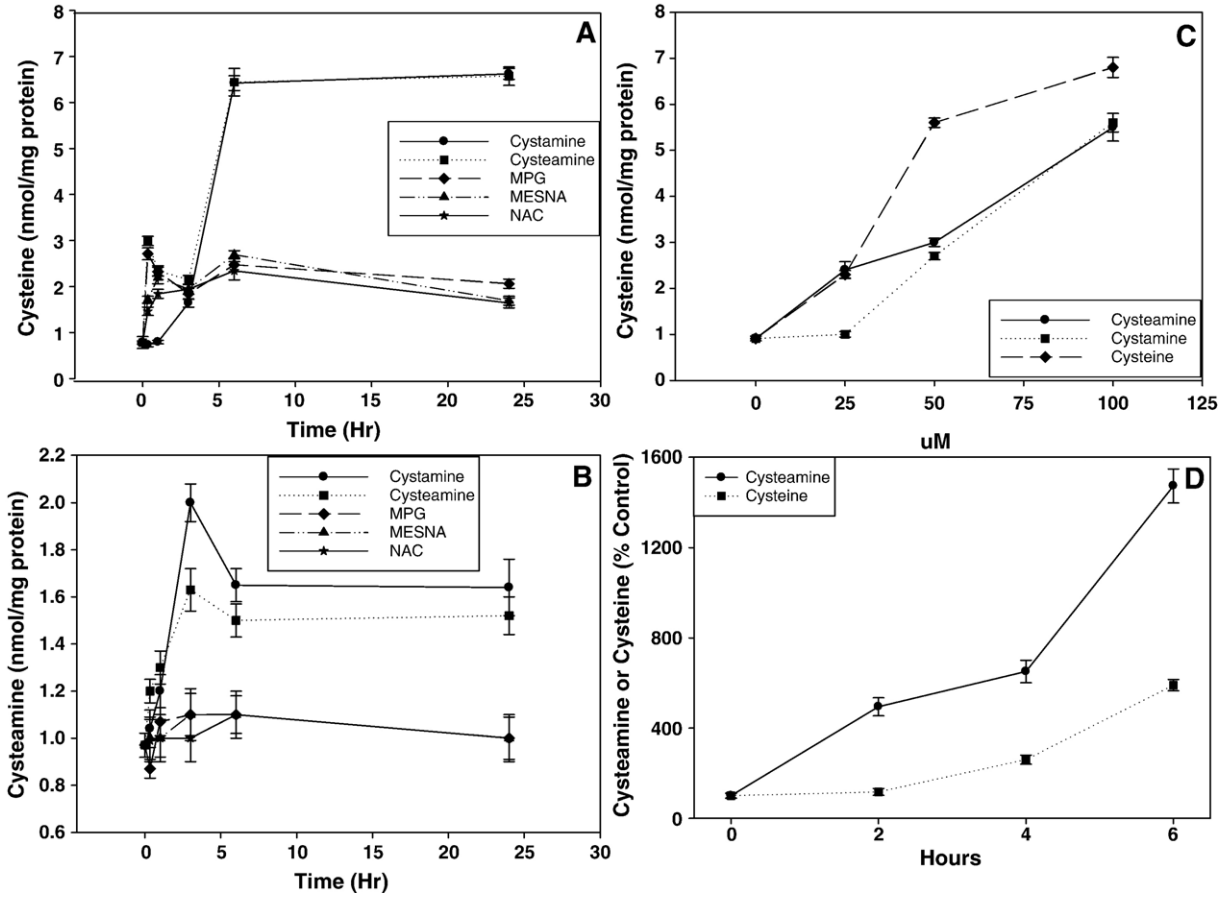
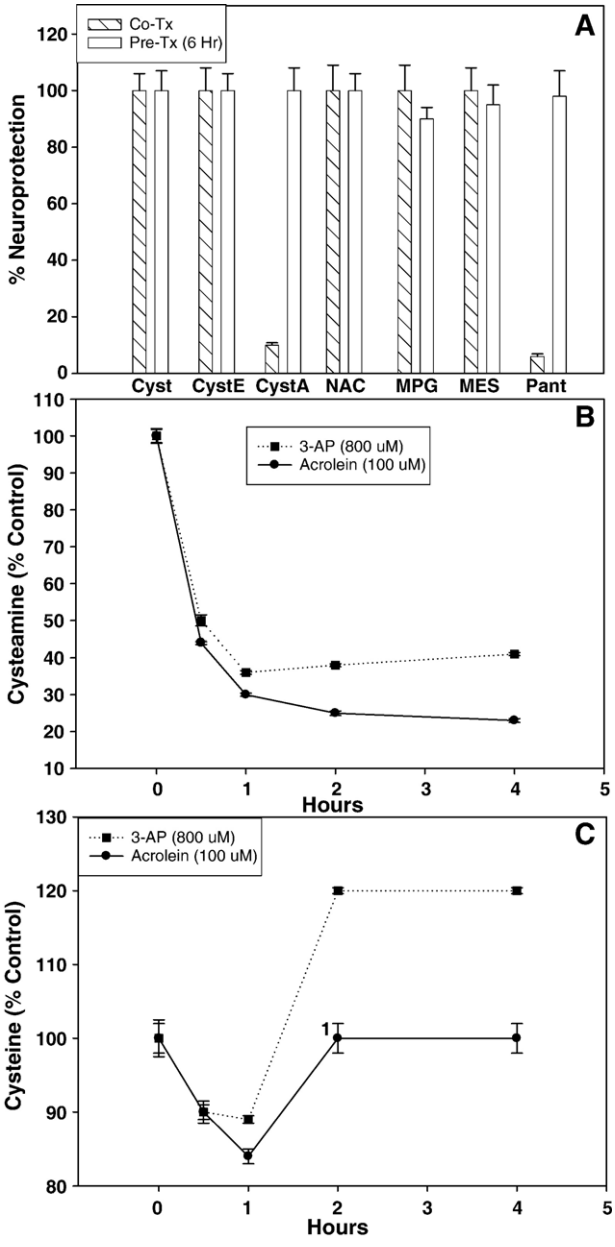


Fig. 1 – Time course for increases in cellular cysteine (A) and cysteamine (B) levels in SN56 cholinergic neurons incubated with 100 μM cystamine, cysteamine, mercaptopropionylglycine (MPG), 2-mercaptoethanesulfonic acid (MESNA), or *N*-acetylcysteine (NAC) for 20 min to 24 h. These increases in cysteine were concentration dependent after 6-h incubations with 25 to 100 μM cystamine, cysteamine or cysteine (C). The cysteamine precursor, pantethine (300 μM) also resulted in rapid increases in cellular cysteamine levels which in turn resulted in delayed increases in cellular cysteine levels (D). Data are presented as mean ± SEM (n=6 wells). *Statistics*: (A) All cysteine levels were significantly greater than controls (p<0.05) except with cystamine treatment at 0.5 and 1 h. (B) Cysteamine levels were significantly greater than controls (p<0.05) for cysteamine treatment starting at 0.5 h and starting at 1 h for cystamine treatment. (C) Cysteine levels were significantly greater than controls (p<0.05) at all points except for cystamine at 25 μM. (D) Cysteamine levels were significantly greater than controls (p<0.05) at all points while cysteine levels were significantly increased starting at 4 h.

cysteamine, is recognized as a key regulator of cellular redox status, the role of thiols in sequestering intracellular reactive aldehydes (Pocernich et al., 2001; Ivanova et al., 2002; Wood et al., 2006b,c) is less appreciated. In this regard, cytotoxic reactive aldehydes are known to be increased in a number of the experimental models listed above as well as in Huntington's disease and in Parkinson's disease. Specifically, reactive aldehydes are elevated in the caudate of Huntington's patients (Browne et al., 1999); in rats treated with 3-nitropropionic acid (Tadros et al., 2005); in experimental models of radiation toxicity (Vladimirov and Miasoedov, 1997) in preclinical models of ischemia–reperfusion injury (Ivanova et al., 1998); in preclinical models of chemically induced hepatotoxicity (Sener et al., 2005); and in Parkinson's disease (Ilic et al., 1999). We therefore decided to characterize the actions of cystamine and cysteamine on cellular cysteine and cysteamine levels more fully and determine if pharmacological augmentation of cysteine and cysteamine levels could effectively sequester large increases in "aldehyde load" that elicit neuronal cell death (Wood et al., 2006b,c).



2. Results

2.1. *Intracellular cysteine and cysteamine pools*

We have previously (Wood et al., 2006b,c) demonstrated neuroprotection against aldehyde-induced cell death with aldehyde-sequestering agents, including thiols. It is also important to note that antioxidants and free radical scavengers are ineffective against aldehyde-dependent cell death (Wood et al., 2006b). To further understand the role of endogenous thiol pools in protecting cells against increased "aldehyde load", we first evaluated the effects of augmenting the intracellular pool of cysteamine by loading SN56 cholinergic cells (Blusztajn et al., 1992) with cysteamine itself and with cystamine, the disulfide dimer of cysteamine. Both cystamine and cysteamine augmented intracellular cysteine (Fig. 1A) and cysteamine (Fig. 1B) levels. These actions were both time- (Fig. 1A and B) and concentration- (Fig. 1C) dependent, with cystamine, which is metabolized to cysteamine, not increasing cellular cysteine until 3 h, whereas cysteamine increased cellular cysteine within 30 min. In addition, the thiol agents mercaptopropionylglycine (MPG), *N*-acetylcysteine (NAC), and 2-mercaptoethanesulfonic acid (MESNA) increased intracellular cysteine (Fig. 1A) but not cysteamine (Fig. 1B) levels, albeit to a lesser extent (2-fold increases vs. 6-fold increases with cystamine and cysteamine). The cysteamine precursor pantethine (300 μM; Fig. 4) also elicited time-dependent increases in cellular cysteamine levels which in turn were followed by

Fig. 2 – Neuroprotection of SN56 cholinergic neurons from aldehyde-dependent cell death (A). Acrolein (100 μM) induced cell death was prevented by a 6-h pre-treatment (Pre-Tx) or by co-treatment (Co-Tx) with cysteine (Cyst, 300 μM), cysteamine (CystE, 100 μM), *N*-acetylcysteine (NAC, 300 μM), mercaptopropionylglycine (MPG, 300 μM) and 2-mercaptoethanesulfonic acid (MES, 300 μM). In the cases of cystamine (CystA; 100 μM) and pantethine (Pant, 100 μM), pre-treatments that augment intracellular thiols provided neuroprotection. However, co-treatments only provided partial protection. Data are presented as % neuroprotection ± SEM (n=6 wells) based on the WST-1 assay at 24 h post acrolein addition. The acrolein-treated cells exhibited no reduction of WST-1. Addition of the aminoaldehyde, 3-aminopropanal (3-AP, 800 μM) or the α,β-unsaturated aldehyde (100 μM), acrolein, rapidly reduced cellular cysteamine levels (B) to approximately 30% of control. Cellular cysteine levels also rapidly decreased by 10% to 15% but cellular levels were also rapidly restored or even rebounded in the case of 3-AP (C). Data are presented as mean ± SEM (n=6 wells). *Statistics*: (B) Cysteamine levels were significantly less than controls (p<0.05) at all time points. (C) Cysteine levels were significantly less than controls (p<0.05) at 0.5 and 1 h and significantly greater than controls with 3-AP treatment at 2 and 4 h.

increases in cellular cysteine levels with a time delay of about 2 h (Fig. 1D).

2.2. *Thiols and neuroprotection from reactive aldehydes*

We next reasoned that thiols possessing a free sulfhydral group should readily neutralize aldehydes without a pre-treatment period but that cystamine and pantethine which have to be metabolized to cysteamine would require a pre-treatment period in cellular assays of neuroprotection. This indeed was the case with both cystamine and pantethine pre-treatments but not co-treatments providing neuroprotection against acrolein, the α,β-unsaturated aldehyde product of lipid peroxidation and polyamine metabolism (Fig. 2A). In contrast cysteine, cysteamine, NAC, MPG and MESNA all provided dramatic neuroprotection both as co-treatments and pre-treatments (Fig. 2A), under conditions in which aldehyde treatments induced 100% cell death.

We also examined the effects of increased "aldehyde load" on cellular thiol levels and found that both acrolein and 3-aminopropanal, toxic aldehyde products of polyamine metabolism, rapidly decreased intracellular cysteamine levels to 25% to 40% of control, with decreased levels being sustained over the 4-h observation period (Fig. 2B). Acrolein and 3-aminopropanal also elicited rapid but less dramatic decreases of approximately 10% in cellular cysteine levels which rebounded to control levels or greater in the case of 3-AP treatment (Fig. 2C). These data demonstrate the important role of intracellular thiols in the removal of toxic aldehyde products resulting from lipid peroxidation, polyamine metabolism and intermediary metabolism. To validate sequestration of aldehydes by cysteamine, we loaded cells with 300 μM cysteamine/[$^2H_4$]cysteamine (1:1) for 6 h and then added 100 μM acrolein and incubated cells for a further 2 h. Selected ion monitoring (407 and 411; Section 4.3) of cellular extracts demonstrated the anticipated thiazolidine product (Esterbauer et al., 1976).

2.3. *Disulfide interchange reactions*

Next we investigated the role of disulfide interchange reactions (Gahl et al., 1985; Fig. 4) in the actions of thiols to increase intracellular cysteine levels. For these studies we utilized a balanced salt solution rather than complex media and demonstrated that cystine in the media is a major source of increased cellular cysteine levels after treatment with cystamine (Fig. 3A), cysteamine (Fig. 3B) and MPG (data not shown) since these agents did not increase cysteine levels in cystine-deficient balanced salt solution. These actions involve disulfide interchange reactions (Gahl et al., 1985) and may well include increased cystine uptake which has being shown to be stimulated by extracellular thiols (Issels et al., 1988; Jokay et al., 1998; Fox et al., 2004).

3. Discussion

Our data indicate that cellular cysteine and cysteamine pools play important roles in the sequestration of reactive aldehydes. Cysteamine is a product of pantethine metabolism (Fig. 4) and exists in the free form, protein-bound form and as oxidized forms which include the dimer cystamine, and mixed disulfides with cysteine, glutathione and homocysteine (Kataoka et al., 1993). Our data demonstrate that cysteamine, like cysteine, is effective in sequestering reactive aldehydes and preventing their cytotoxicity. These data suggest that further evaluation of the roles of intracellular cysteamine pools in the modulation of oxidative stress and reactive aldehyde metabolism is warranted. Increases in cellular cysteine levels after treatment with cysteamine, cystamine and other thiol agents presumably involve several mechanisms in concert. For example, increased cystine uptake as has been reported with cystamine, cysteamine and *N*-acetylcysteine treatment; with cystine subsequently being hydrolyzed, augmenting intracellular cysteine and glutathione levels (Issels et al., 1988; Jokay et al., 1998; Fox et al., 2004). In this regard, our data with SN56 cells support previous work that implicates disulfide exchange reactions in regulating

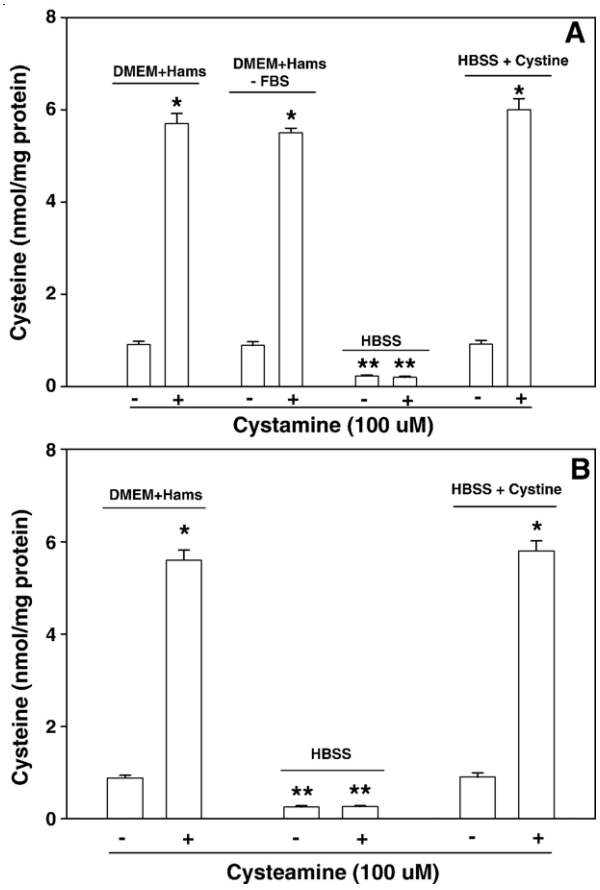


Fig. 3 – To determine if cystine was a major source of cellular cysteine levels with thiol treatment, incubations with cystamine (A) or cysteamine (B) were performed in Hanks' Balanced Salt Solution (HBSS)±200 μM cystine for 6 h. The robust cysteine increases were clearly found to be cystine-dependent. In addition, removing fetal bovine serum (FBS) from the DMEM/Ham's medium was not found to alter the actions of cystamine (A). Data are presented as mean±SEM (n=4–6 wells). *Statistics*: *Cysteine levels were significantly greater than controls (p<0.05). **Cysteine levels were significantly less than controls (p<0.05).

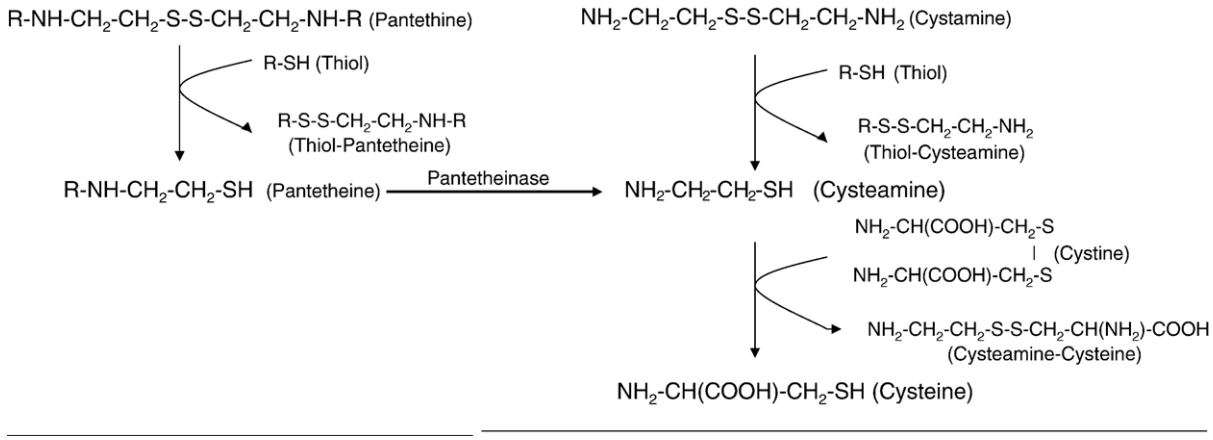


Fig. 4 – Scheme for the formation of cysteamine from disulfide interchange reactions of the endogenous thiol dimers cystamine, cystine and pantethine (R=–CO–CH_2–CH_2–NH–CO–CH(OH)–$C(CH_3)_2$–CH_2–OH). Cysteamine and other thiols augment cellular cysteine levels from cystine or other mixed cellular disulfides containing cysteine via disulfide interchange, as depicted.

intracellular free thiol levels (Gahl et al., 1985; Jokay et al., 1998; Pendyala et al., 2000).

The net result of cystamine or cysteamine treatment is a dramatic augmentation of intracellular thiol levels that will boost the antioxidant capacities of cells. In addition, our data are the first to demonstrate the importance of this intracellular thiol pool in the sequestration of reactive aldehydes. The importance of the aldehyde sequestering properties of augmented thiol pools is appreciated when one considers that thiols are cytoprotective in models of cell death where antioxidants are ineffective (Ivanova et al., 2002; Wood et al., 2006b,c). These data further suggest that pharmacological manipulation of intracellular thiol pools may represent a new approach in the design of neuroprotective agents.

4. Experimental procedures

4.1. *Cell culture*

The murine medial septal cholinergic neuronal cell line, SN56.B5.G4 (Blusztajn et al., 1992) was grown in DMEM/Ham's F12 (1:1) containing 10% FBS. For aldehyde toxicity experiments cells were grown in 48-well plates. For neurochemical studies cells were grown in 12-well plates.

4.2. *Cellular cysteine and cysteamine*

Cells were treated with cystamine or cysteamine and the concentration–response and time course for increases in cellular cysteine and cysteamine studied. In some experiments the medium was replaced by HBSS ± 200 μM cystine. Cellular cysteine was measured by GC–MS as previously described (Wood et al., 2006a). The same derivatization procedure resulted in the di-tBDMS derivatives of cysteamine and [$^2H_4$]cysteamine with the respective $[MH]^+$ ions of 306 and 310 monitored in ammonia PCI. Cysteine and cysteamine levels were expressed as nmol/mg of acid (0.1 N HCl; 25,000×*g*)-precipitable protein.

4.3. *Cysteamine–acrolein adduct formation*

To monitor for the predicted thiazolidine diadduct product (Esterbauer et al., 1976; M.Wt. = 193) of 2 mol of cysteamine and 1 mol of acrolein, product formation in PBS was first monitored. The reaction mix was Savant dried after 2 h and the diacyl derivative formed with trifluoroacetic anhydride was monitored by GC–MS. This derivative formed a strong $[M+ammonia]^+$ ion of 403 with cysteamine and 407 and 411 with cysteamine + [$^2H_4$]cysteamine (1:1) in ammonia PCI. To further validate this adduct, we loaded cells (75 cm^2 flask) with 300 μM cysteamine/[$^2H_4$]cysteamine (1:1) for 6 h and then added 100 μM acrolein and incubated the cells for a further 2 h. Cellular extracts yielded the anticipated thiazolidine reaction product.

4.4. *Aldehyde toxicity*

Aldehyde-induced cell death was examined with 100 μM acrolein, a concentration that elicited 100% cell death. Cysteine, cystamine, cysteamine and reference thiol agents were added as co-incubations or as 6-h pre-treatments. After 24 h, cell viability was assayed with the Roche WST-1 assay kit.

4.5. *Cellular thiols after aldehyde treatment*

Cells were incubated with 100 μM acrolein or 800 μM 3-aminopropanal for 0.5, 1, 2 or 4 h and intracellular cysteine and cysteamine levels were monitored by GC–MS as described above.

4.6. *Statistics*

All experimental groups consisted of 6 tissue culture wells and all experiments were repeated at least 2 times. Data were

analyzed with a one-way ANOVA followed by the Dunnett's t test.

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