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Cysteamine revisited: repair of arginine to cysteine mutations

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Abstract

Cysteamine is a small aminothiols endogenously derived from coenzyme A degradation. For some decades, synthetic cysteamine has been employed for the treatment of cystinosis, and new uses of the drug continue to emerge. In this review, we discuss the role of cysteamine in cellular and extracellular homeostasis and focus on the potential use of aminothiols to reconstitute the function of proteins harboring arginine (Arg) to cysteine (Cys) mutations, via repair of the Cys residue into a moiety that introduces an amino group, as seen in basic amino acid residues Lys and Arg.

Cysteamine has been utilized *in vitro* and *ex vivo* in four different genetic disorders, and thus provides “proof of principle” that aminothiols can modify Cys residues. Other aminothiols such as mercaptoethylguanidine (MEG) with closer structural resemblance to the guanidinium moiety of Arg are under examination for their predicted enhanced capacity to reconstitute loss of function. Although the use of aminothiols holds clinical potential, more studies are required to refine specificity and treatment design. The efficacy of aminothiols to target proteins may vary substantially depending on their specific extracellular and intracellular locations. Redox potential, pH and specific aminothiol abundance in each physiological compartment are expected to influence the reactivity and turnover of cysteamine and analogous drugs. Upcoming research will require the use of suitable cell and animal models featuring Arg to Cys mutations. Since Arg to Cys changes comprise in general about 8% of missense mutations, repair of this specific mutation may provide promising avenues for many genetic diseases.

Keywords

cysteamine; cysteine residue modification; aminothiols; repair Arg to Cys mutations

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1. Introduction

Rare diseases, as defined in Europe by affecting less than 1 in 2000 individuals (Orphanet), are individually rare, but as a group affect globally over 400 million individuals. At least 6000 different rare diseases are known, most of which are genetically determined, often chronic and life-threatening (Lynn et al 2017). For many of them, there is not yet any treatment and thus, new therapeutic approaches are urgently needed. Among the causes of rare inborn error of metabolism (IEM), the most common alterations seen are missense mutations (HGMD® professional release 2014.3) (Stenson et al 2014). Missense mutations may affect the protein structure preventing the proper folding and stability, and may lead to a functional decrease or even loss in enzyme activity. Different therapeutic strategies have been developed to correct protein folding and restore enzymatic function. Therapies based on pharmacological chaperones, molecular chaperones and proteostasis regulators have shown greater promise than other approaches (Gupta et al 2013, Muntau et al 2014, Yuste-Checa et al 2016).

This review focuses on the use of cysteamine and other aminothiols as mutation-tailored drugs. Aminothiols, such as glutathione, are essential for maintaining and compartmentalizing cellular redox potential and play a central function in cellular and extracellular homeostasis. Among aminothiols, cysteamine has long been used as a therapeutic option for patients with cystinosis. Excellent reviews by others cover the therapeutic applications of cysteamine in the field of cystinosis and neurodegenerative disorders (Besouw et al 2013, Gibrat and Cicchetti 2011). This review recapitulates advances on the use of cysteamine to repair Arg to Cys missense mutations in genetic disorders. The basic idea is that cysteamine can bind to protein-incorporated cysteine via a disulfide crosslink hereby creating a structure that “mimics” the original arginine residue (see Figure 1).

We have organized this review into three sections. First, we present an overview on the reactivity, cellular uptake and metabolism of aminothiols, with a focus on cysteamine. Then, a brief summary of the use of cysteamine in various diseases is provided, in particular its current application in cystinosis. Finally, we expand on the main topic reviewed in this article: the molecular targeting of Arg to Cys mutations by cysteamine and other aminothiols, along with the most relevant challenges to be considered. This final part also covers future perspectives for cysteamine as a potential treatment for genetic disorders.

1.1 Structure and Reactivity of Aminothiols

Aminothiols such as cysteine, homocysteine, taurine and glutathione play essential roles in redox homeostasis and signaling (Antelmann and Helmann 2011, Dalle-Donne et al 2007, Jones and Sies 2015, Winterbourn and Hampton 2008). Structurally, aminothiols are low molecular weight compounds, whose thiol moiety exists in equilibrium between protonated and deprotonated species (Singh and Whitesides 2010). The reactivity of these thiols toward thiol-containing targets depends largely on their thiol dissociation pKa and their redox potential (Table 1). The reactivity of free aminothiols with Cys residues of proteins is also influenced by the size of the aminothiol and the accessibility of the target Cys residue. The protein structure can strongly influence the pKa of the thiolate of cysteine residues. For

example, the pKa of Cys34 of human serum albumin is about 5.0 (Jr. 1995), hence approximately 3 units lower compared to free cysteine (Table 1). This implies that Cys34 exists in its thiolate form at physiological pH, increasing its reactivity with free low molecular weight thiols. The concentration of aminothiols differs greatly between different tissues (Turell et al 2013). Aminothiols undergo sulfide exchange reactions, a fast and reversible process mainly driven by the redox potential of the participating species (Mirzahosseini and Noszál 2016). In plasma, the predominant aminothiols are cysteine, cysteinylglycine, homocysteine, glutathione and γ -glutamylcysteine, and their corresponding protein mixed disulfides (Turell et al 2013). The most abundant thiol in plasma is Cys34 of albumin, with a concentration of $422 \pm 52 \mu\text{M}$ (Giustarini et al 2005) and it is mostly reduced (75%) (Turell et al 2013). In cells, thiols are found in millimolar concentrations and are mainly reduced. The total concentration of glutathione, the most abundant low-molecular weight aminothiol in cells, is between 2-17 mM and it is essentially all reduced (>90%). At the cellular level, it is of great interest to investigate the reactivity of aminothiols with the Cys residues of proteins. These reversible post-translational modifications can affect the function of proteins by altering their interactions with other cellular effectors (Roos et al 2013). The reactivity of Cys residues in proteins has been a matter of intense research, and excellent reviews are available on the topic (see for example (Go and Jones 2013, Jones and Go 2011, Marino and Gladyshev 2012, Rebrin et al 2004)). Work has emerged on the use of the endogenous aminothiols as cysteamine for the targeted reconstitution of function in proteins harboring Arg to Cys mutations (Inauen et al 2016).

The rationale of this approach is two-fold. First, cysteamine by binding to cysteine can prevent any ill effects that might be associated with the presence of the free thiol. Second, the structure of the cysteine-cysteamine molecule mimics the guanidinium function of Arg.

1.2 Biosynthesis and Fates of Cysteamine

Cysteamine is a decarboxylated derivative of cysteine. However, in mammals, cysteamine is not formed from cysteine directly by decarboxylation. Rather, cysteamine derives from coenzyme A degradation, a highly conserved pathway (Figure 2). Coenzyme A is synthesized from pantothenate (vitamin B5) and cysteine. The endogenous production of cysteamine occurs during the degradation of coenzyme A, in particular from pantetheine. The membrane associated protein pantetheinase, also called vanin, catalyzes the hydrolysis of pantetheine to produce cysteamine and pantothenic acid. Subsequently, cysteamine dioxygenase, mainly present in the liver, oxidizes the released cysteamine to hypotaurine. Afterward, cysteamine is removed via the taurine pathway in form of bile salts (Ripps and Shen 2012). If cysteamine is administered in high dose, an alternative catabolic route has been proposed. This pathway involves cysteamine conversion to *S*-methylcysteamine by a thiol-methyltransferase, which is consequently metabolized into methanethiol and acetamide by cytochrome P450. Next, another thiolmethyltransferase catalyzes the rapid conversion of methanethiol into dimethylsulfide (Besouw et al 2007, Gahl et al 1995). This dimethylsulfide has been proposed as the main cause of halitosis in cystinosis patients treated with cysteamine (Gahl et al 1995). In addition, it has been suggested that cysteamine can substitute for L-homocysteine as the thiol co-substrate in the cystathionine beta-synthase (CBS) catalytic reaction (Shen et al 2005), resulting in the formation of thialysine (S-(2-

aminoethyl)-L-cysteine). CBS is a pivotal enzyme in human sulfur and methyl group metabolism that catalyzes the condensation of L-homocysteine with L-serine to generate L-cystathionine (see also below). The alternative CBS reaction using cysteamine may occur in particularly in the brain where very little cysteamine dioxygenase is present and appreciable amounts of CBS are found. Thialysine has been detected in brain tissues following gavage feeding of cysteamine in rats (Pinto et al 2009). In addition, thialysine has been detected in urine of normal human adults, suggesting thialysine may even be a natural occurring metabolite (Yu et al 1997).

1.3 Cellular Uptake and Compartmentalization of Cysteamine

Oral and subcutaneous administration of cysteamine in rats leads to distribution of this aminothiols primarily in the kidney, the gastrointestinal tract (mainly the duodenum) and the liver (Khomenko et al 2012). The basal level of cysteamine in all organs is low (<100 pmol/mg protein) (Khomenko et al 2012). Gavage administration of cysteamine leads to marked accumulation of the aminothiol in the duodenum, and substantially lower levels are found in the stomach, jejunum, ileum or colon (Khomenko et al 2012). Regardless of the way of administration, cysteamine uptake reaches a maximum in the duodenum after 6 hours that is maintained for up to 12 hours, and efflux is observed only after 24 hours (Khomenko et al 2012). In general, cellular uptake of cysteamine occurs rapidly. In the human proximal tubular kidney cell line PT37, a maximum uptake occurs at approximately 2 hours when cysteamine is provided in the culture medium, and intracellular levels are maintained for about 6 hours (Frost et al 2016). An extracellular concentration of 20 mM cysteamine was shown not to promote additional uptake of the aminothiol, suggesting that the uptake system is saturable (Khomenko et al 2012). Studies performed with leukocytes isolated from patients with cystinosis treated with or without oral cysteamine demonstrated that lysosomal transport of cysteamine competes with cystine, suggesting a common transporter (Gahl et al 1983). Loading of lysosomes from leukocytes with cystine reaches a maximum at approximately 90 minutes, suggesting that import of cysteamine into the lysosomal compartment occurs too, within relatively short timeframes (Gahl et al 1983). Experiments performed with lysosomes isolated from human fibroblasts showed that maximum loading of [³H]-cysteamine occurs after 2 to 3 minutes (Pisoni et al 1995). Uptake of cysteamine is more favorable at alkaline pH compared to pH 5 (the pH inside lysosomes is 5.3) (Pisoni et al 1995, Smith et al 1987). This may be related to the pKa of the thiol group of cysteamine; the protonated form (HS-CH₂-CH₂-N⁺H₃) species is presumably less well transported into the lysosome compared to its thiolate form. Likewise, it was observed that an acidic pH limits the efflux of cysteamine from the lysosome, which increases the efficacy to metabolize accumulated cystine in cystinosis (Pisoni et al 1995). Thus, the ease of transport, compartmentalization, retention in the lysosome, and pH specificity of cysteamine makes this drug a promising candidate for the targeted modification of proteins in the lysosome. Apart from the above described characteristics, cystinosis renal epithelial cells exhibit an increased concentration of oxidized glutathione (GSSG), and supplementation with cysteamine (1mM) restored levels of reduced glutathione (GSH), thus repairing GSH/GSSG homeostasis (Wilmer et al 2011). Deficiencies of glutathione appear to be common among inherited diseases (Abeti et al 2016, Caterino et al 2015, Pastore et al 2014, Petrillo et al

2013). Therefore, it is possible that the benefits of cysteamine may extend beyond its proposed targeting of cystine in cystinosis.

2. Medical Use of Cysteamine

2.1 Introduction

The first report of cystamine (disulfide form of cysteamine) in a clinical trial concerns studies to test radiation sickness in patients receiving radiotherapy (Healy 1960). It had been previously suggested that cysteamine exerts a radio-protective effect by quenching of radicals as a possible mechanism of action (Bacq et al 1953). Cystamine was administered to adults at a dose of 100 to 400 mg daily (Healy 1960). Side effects of radiation include nausea and vomiting, and the treatment was rated as successful by reducing these symptoms in approximately 50% of patients. However, cysteamine did not prove to be more effective than the radio-protective effect of lactose administration (Healy 1960). Some years later in 1973, it was shown that high dose of cysteamine (750-900 mg/kg/day) caused ulcers in rats. The ulcerogenic properties of cysteamine motivated the development of an experimental model for production of perforating duodenal ulcers (Cahill et al 1986). This model mimics the pathological and functional abnormalities found in humans duodenal ulcers and became a useful tool to study the pathophysiology behind duodenal ulcer formation in humans (Cahill et al 1986). Additionally, cysteamine was tested as a possible treatment for different diseases showing limited positive results (Besouw et al 2013). In 1976, cysteamine was introduced as a possible therapy for cystinosis and since then it has been used for more than three decades for this propose (see also below) (Thoene et al 1976).

2.2 Cystinosis

Cysteamine was approved as a cystine-depleting agent for the treatment of cystinosis by the US Food and Drug Administration (FDA) in the USA (1994) and by the European Medicines Agency (EMA) in Europe (1997). Cystinosis is a lysosomal storage disorder, caused by mutations in the *CTNS* gene (OMIM 219800), encoding cystinosisin, the lysosomal cystine carrier protein. The pathologic landmark of the disease is the lysosomal accumulation of cystine, which crystallizes at low concentrations in the lysosomes in all cells of the body, triggering tissue damage. Cysteamine enters the lysosomal compartment through an unknown transporter and depletes lysosomal cystine via disulfide exchange reactions resulting in formation of cysteine and cysteine-cysteamine mixed disulfides. Cysteine can leave the lysosome through the cysteine transporter while cysteine-cysteamine mixed-disulfide escapes the lysosome via the lysine/cationic amino acid transporter PQLC2 (Jézégou et al 2012). In addition to reducing cystine, cysteamine is considered to play a crucial role in antioxidant defense and energy homeostasis in cystinosis. Cysteamine prevents lipid peroxidation and protein carbonylation thus protecting the activities of superoxide dismutase and glutathione peroxidase (Dubinsky and Gray 2006, Kessler et al 2008), normalizes the activities of creatine kinase and pyruvate kinase, and maintains the GSH pool (Rech et al 2008, Wilmer et al 2011). The recommended cysteamine bitartrate (Cystagon®) dose for cystinosis patients over 12 years of age and over 50 kg is a total of 2 g/day, divided in four doses daily. Cysteamine treatment improves overall prognosis by delaying the progression to end-stage renal disease and preventing the development of extra-

renal complications. Treatment of cystinosis should start as early as possible, ideally already at infant age, to allow prevention or at least delay of the complications of the disease (Elmonem et al 2016). A new twice-daily delayed-release enteric-coated formula of cysteamine bitartrate (Procysbi, Raptor Pharmaceuticals Inc.) has been approved for clinical use by the US FDA and EMA in 2013 (Veys et al 2016).

2.3 Other Potential Clinical Applications of Cysteamine

The use of cysteamine is currently being explored for treatment of various inherited and acquired conditions, including non-alcoholic fatty liver diseases, cancer, malaria, schizophrenia, Huntington disease, Parkinson disease (PD), and Alzheimer's disease (AD) (for reviews see Gibrat and Cicchetti 2011; Besouw et al 2013; De Stefano and Maiuri 2015). An interesting aspect addressed by Besouw et al., is the potential beneficial effect of cysteamine to treat neurological disorders as attention deficit of hyperactivity, Rett syndrome and neuropathic pain (Besouw et al 2013). Some preclinical and phase II/III clinical trials with cysteamine have been conducted over last few years. Because the various mechanisms of action of cysteamine identified (Figure 3) overlap with those driving the pathophysiology of neurological disorders, most of the cysteamine studies are focused in this area. A recent publication using an animal model of PD (induced by a toxin) shows that cysteamine can prevent and even reverse ongoing dopaminergic degeneration (Cisbani et al 2015).

3. Molecular Targeting of Arg to Cys Mutations

About 56% of disease-causing variants in humans involve missense mutations according to the last update of the Human Genome Mutation Database (January 2017) (Stenson et al 2014). Almost 15% of all missense mutations concern an Arg residue (Vitkup et al 2003). This is a consequence of the high mutability of the methylated CpG dinucleotides prone to natural deamidation of cytosine to thymine (Cooper et al 1988). Four out of the six different codons encoding Arg (CGU, CGC, CGA, CGG, AGA and AGG) contain CpG dinucleotides. Interestingly, more than half of the missense mutations of the Arg codon result in a substitution to Cys (Vitkup et al 2003). The overall consequence is that about 8% of all genetic diseases due to missense mutations are affected by Arg to Cys changes.

The loss of the guanidine moiety of the arginine in proteins undergoing Arg to Cys mutations can have drastic consequences on the protein structure and function (Beutler et al 2002; Cali et al 1991; Simon et al 2002; Willander et al 2014). Guanidine moiety interactions, such as H-bridges and Van der Waals force, may disappear. Charge changes and the potential cysteine thiol function properties are other interesting factors to be considered of Arg to Cys mutations, which may be potentially treatable by cysteamine or other amino thiols (see below). A list of natural Arg to Cys mutations involving the only four amino thiol-targeted proteins so far examined is provided in Table 2. If cysteamine forms a mixed disulfide with cysteine residues this 'new' amino acid that structurally mimics lysine, and enables the formation of H-bridges, van der Waals interactions and blocks the sulfhydryl function of cysteine (Figure 1). Both *in vitro* and *in vivo* studies suggest that cysteamine can restore the function of some Arg to Cys mutated proteins (Table 2, see below). Next, we focus on describing the earliest reports on cysteamine use to target human proteins back in

the 1980's, comprising studies with ApoE3 and Factor VIII proteins (Gahl et al 1985, Weisgraber et al 1981), as well as recent molecular targeting studies addressing argininosuccinate lyase (ASL) and CBS (Mendes et al 2015; Inauen et al 2016).

3.1 Apolipoprotein E3 and Cysteamine

Apolipoprotein E (ApoE) is a lipid-transport glycoprotein that plays multiple roles in the regulation of lipid and lipoprotein. ApoE is an integral component of chylomicrons, very low-density lipoproteins and high-density lipoproteins, and transports lipoproteins, fat-soluble vitamins, and cholesterol into the lymph system and then into the blood. Several variants have been identified in the human *APOE* gene. In particular, rs7412 (C/T) and rs429358 (C/T) are responsible for the three major alleles (ε2, ε3 and ε4). The major isoforms, ApoE2, ApoE3 and ApoE4, differ from each other by only one or two amino acid positions. ApoE3 has cysteine-112 and arginine-158, whereas ApoE4 and ApoE2 have arginine and cysteine, respectively, at both positions. Importantly, the difference in these positions dramatically changes the structure and function of ApoE. ApoE3 is the major variant in the normal human population. ApoE2 is defective in low density lipoprotein receptor (LDLR) binding and associated with type III hyperlipidemia and premature atherosclerosis (Mahley et al 1999). ApoE4 facilitates the formation of the amyloid plaques and neurofibrillary tangles and is associated with increased risk for both atherosclerosis and AD (Weisgraber et al 1994; Roses and Saunders 1994; Masliah et al 1996). Weisgraber et al., incubated purified ApoE2 protein and plasma of type III hyper-lipoproteinemic patients, homozygous for ApoE2, with a wide range of cysteamine doses (therapeutic levels of 50 μM) to determine if cysteamine could modify the cysteine residues of ApoE2 (Weisgraber 1990). Charge modifications were monitored by isoelectric focusing and showed a shift in the ApoE pattern from the variant ApoE2 (Cys 112 and 158) to the normal ApoE3 (Cys 112 and Arg 158) and ApoE4 (Arg 112 and 158). These *in vitro* studies indicated that cysteamine forms a mixed disulfide with the cysteine residue, which increased the net charge of the protein by 1 positive charge per cysteine residue. This charge shift in ApoE increased the binding of the modified apoE2 to LDLR by 2.7 to 17.6-fold (Gahl et al 1985). *In vivo*, similar results were obtained in plasma of cystinosis patients (homozygous for ApoE3) treated with cysteamine (Gahl et al 1985). It was shown that a small band was apparent in the ApoE4 position, indicating that cysteine-cysteamine disulfide formation mimics the original Arg residue in ApoE4. These results were the first evidence of the potential of cysteamine as a cysteine residue modifying reagent *in vivo*.

3.2 Factor VIII and Cysteamine

Human clotting Factor VIII (hFVIII, OMIM 300841) or anti-hemophilic factor is a plasma glycoprotein that plays a critical role in the intrinsic pathway of blood coagulation as a cofactor in the activation of Factor X. Its deficiency results in hemophilia A, a recessive rare X-linked coagulation disorder caused by mutations in the *F8* gene. hFVIII is synthesized in the liver and circulates in the bloodstream closely associated with its carrier protein, von Willebrand Factor (vWF) (Kaufman 1992). In response to injury, FVIII is activated by thrombin and separated from vWF. Thrombin cleaves FVIII after Arg residues 372 and 740 to yield heavy chain fragments and at Arg 1689 to yield the light chain fragment. Cleavage after Arg 1689 is required to dissociate the FVIII from vWF (Kaufman et al 1997). The

active FVIII acts as a cofactor for factor IXa that converts factor X to the activated form Xa and eventually leads to the formation of a blood clot. The substitution of arginine for cysteine at position 1689 has been identified in 5% of all patients with hemophilia A (Lazarchick and Hoyer 1978). This variant abolishes thrombin cleavage site and thereby blocks FVIII function. The possibility that FVIII may be susceptible to chemical modification by cysteamine was considered after the successful results shown for ApoE2 (Aly et al 1992, Gahl et al 1985). The incubation of plasma of FVIII hemophilic patients with cysteamine at micro- to millimolar levels increased FVIII activity 14fold and restored light chain cleavage by thrombin. Aly et al., suggested that Cys 1689 is converted by cysteamine treatment to a positively charged lysine analogue recovering the thrombin cleavage site (Aly et al 1992).

3.3 Argininosuccinate Lyase and Cysteamine

ASL is part of the urea cycle and an essential enzyme for ammonia detoxification and Arg synthesis. It catalyzes the hydrolytic cleavage of argininosuccinic acid (ASA) into arginine and fumarate. ASL deficiency (ASLD, OMIM 207900) is considered the second most common urea cycle disorder (UCD). Loss of ASL function leads to the accumulation of ASA, ammonia and citrulline in the blood along with accumulation of ASA in urine. ASLD-causing mutations are spread all over the *ASL* gene and have recently been reviewed (Balmer et al 2014). Remarkably, 12% of all known ASLD genotypes are Arg to Cys substitution (Balmer et al 2014). The high number of arginine to cysteine mutations in ASLD has triggered the interest of exploiting aminothiol therapy as a potential approach in ASLD patients. Cysteamine treatment has recently been studied in all known Arg to Cys mutations in ASLD (p.Arg94Cys; p.Arg95Cys; p.Arg168Cys; p.Arg379Cys and p.Arg385Cys) (Inauen et al 2016). Mutants were overexpressed in 293T cells and incubated with cysteamine from 0.05 to 0.1 mM. This range of cysteamine concentration (0.05 mM) yields a peak plasma concentration similarly found after 15 mg/kg oral cysteamine administration (Smolin et al 1988). The effect of cysteamine on ASL activity was examined in cell extracts after 48 h with cysteamine provided in the culture medium, and also after 1 h incubations with the aminothiol added directly to cell lysates. Cysteamine (0-1 mM) added to the culture medium for 48 h in transfected cells carrying Arg to Cys mutants and wild-type constructs showed no effect on ASL activity. Interestingly, the effect of direct incubation of cell lysates with cysteamine (0-0.1 mM at 37°C for 1h) was dependent on the location of the mutation in the recombinant ASL proteins. Increased ASL activities after cysteamine incubation (0.05 and 0.1 mM) were reported in cells lysates carrying p.Arg94Cys; p.Arg379Cys and p.Arg385Cys mutations (Inauen et al 2016). Notably, cell lysates carrying p.Arg385Cys incubated with cysteamine recovered similar activities to the untreated cell lysate carrying the p.Arg385Lys mutation, which is in line with the expected cysteamine-induced change of the cysteine residue to mimic a lysine analogue (Inauen et al 2016).

Wild-type ASL activity decreased when cell lysates were treated with cysteamine. A similar reduction in activity has been observed in FVIII wild-type plasma protein incubated at 37°C for 1 h with cysteamine (1-100 mM) (Aly et al 1992). Some reasons argued by the authors are: the accessibility of natural cysteine residues in the ASL protein by free cysteamine, the

possible impact of cysteamine in the wild-type protein by acting as a mild competitive inhibitor of ASA and the potential of cysteamine to modify cysteines in partner proteins and alter the 3D protein configuration, the protein process and the trafficking of ASL and other proteins.

Concerning the natural cysteine residues in the wild-type ASL protein, structural modeling and molecular dynamics studies reported two cysteine residues exposed to the surface (Cys264 and Cys426). Both of these cysteines could react with cysteamine, being Cys264 the preferred spot for docking. Analysis of the Cys264Lys mutant, which represents a structural analog of the cysteamine-cysteine-264 disulfide, showed that all the crucial bonding interactions in the ASL wild-type structure were preserved (Inauen et al 2016). *Ex vivo* and *in vitro*, modification of either of these cysteines was found not to impact the ASL tetramer structure or enzymatic function in the wild-type protein. The finding that low cysteamine concentrations can repair the function of Arg to Cys ASL mutants can be considered as a starting point for further studies in additional conditions.

3.4 Cystathionine Beta-Synthase and Cysteamine

CBS is a key regulatory enzyme that lies at the branch point of two metabolic pathways: methionine methylation cycle and transsulfuration pathway. It represents the only known catabolic route of L-homocysteine (Hcy) and L-methionine (Met) degradation. CBS binds two cofactors, pyridoxal 5'-phosphate and heme, to catalyze the condensation of L-serine and Hcy into L-cystathionine, the intermediate in the biosynthesis of cysteine (Kraus 1987, Kraus et al 1993). It is mostly expressed in liver and kidney and located mainly in the cytoplasm, although under specific conditions it can be re-localized to the nucleus (Kabil et al 2006, Markovic et al 2007). CBS is a multi-domain tetrameric protein allosterically activated by S-adenosylmethionine (SAM) a co-substrate involved in methyl group transfers. Mutations in human CBS result in homocystinuria (OMIM 236200) an autosomal recessive disorder biochemically characterized by severely elevated levels of Hcy and Met in plasma and urine. More than 192 causing-diseases mutations have been identified in the *CBS* gene. The State of Qatar has the highest rate of CBS deficiency in the world (approximately 1 in 1,800 births) (El-Said et al 2006, Zschocke et al 2009), due to a specific founder missense mutation, which results in an Arg to Cys replacement in the CBS, p.Arg336Cys. Untreated homozygous patients for this founder mutation are clinically severely affected with intellectual disability and other neuropsychiatric problems, thromboembolisms, ectopia lentis and orthopedic complications. Main treatment options are pharmacological doses of betaine and a strict life-long methionine-restricted diet. However, adherence to diet is poor particularly in adolescence and adulthood and there is a clear need for new therapies. Recently, Mendes et al., studied the potential repairing effect of cysteamine on the function of purified recombinant CBS p. Arg336Cys. Cysteamine (250 and 1000 μ M) showed a partial stabilization and an increase in the specific activity of the purified p.Arg336Cys protein (Mendes et al 2015). Analysis of thermal inactivation, proteolysis and differential scanning fluorometry profiles in the presence of cysteamine caused an increase in the specific activity of CBS, which was not a consequence of alterations in conformational stability. The findings point to an increase in the catalytic efficiency for both substrates Hcy and Ser, but the mechanism underlying this enhanced activity remains to be elucidated.

(Mendes et al 2015). Mendes et al., also explored the effect of another small aminothiols, mercaptoethylguanidine (MEG) (Mendes et al 2015). MEG is a commercially available aminothiol which contains a guanidine group in addition to a thiol (Figure 1). Thus, MEG treatment of Arg to Cys mutant proteins would be postulated to have a Cys-MEG disulfide residue that better resembles the structure of the wild-type Arg compared to the expected lysine analog formed by Cys-cysteamine linkage (Figure 1). A small concentration of MEG (25 μ M) was enough to stabilize and increase specific CBS activity *in vitro*. In this case, the effect of MEG was proportional to the incubation time; longer incubation times led to greater stabilization in the residual activity of the CBS variant. Furthermore, kinetics studies showed that MEG was able to improve the catalytic efficiency of the p.Arg336Cys variant for both substrates (serine and Hcy) (Mendes et al 2015). These results suggest that MEG is superior compared to cysteamine for the repair of CBS p.Arg336Cys. Mendes et al., also proposed that absence of a negative impact on the wild-type CBS activity was likely a consequence of the low accessibility of MEG to the other Cys residues in the CBS structure (Mendes et al 2015).

The results by Mendes et al., suggest that both aminothiols are able to partially restore the activity of CBS p.Arg336Cys variant (Mendes et al 2015). However, there is no structural evidence that the wild-type or mutant proteins are chemically modified by Cysteamine or MEG. Therefore, recovery of the specific CBS or ASL activity could be explained by two possible mechanisms. One involves the proposed repair via Cys-sulfur linkage using cysteamine or MEG as donating sulfur cross-linkers, and the other, could involve binding of these aminothiols to pockets of the protein not necessarily involving Cys residues. The latter mechanism could be pictured as a 'chaperone' function of the aminothiols, and could help to explain the moderate decrease in ASL and CBS activities in the native wild type proteins. It is plausible that the aminothiols could compete with native ligands for the active site, leading to mild inhibition of the native enzymes.

Even a partial recovery of CBS function may reduce the extremely elevated plasma Hcy levels observed in Qatari patients carrying the p.Arg336Cys mutation. In addition, several studies have shown that homocysteine may disturb protein folding in the ER, possibly inducing oxidative stress and leading to the up-regulation of some ER stress responsible genes and redox regulators genes (Ji and Kaplowitz 2003, Vijayan et al 2013, Werstuck et al 2001). The antioxidant properties of cysteamine by restoring the GSH pool (Wilmer et al 2011) along with the potency of MEG to scavenge reactive nitrogen species (Guyen et al 2008, Szabó et al 1997) suggest these two aminothiol compounds as good candidates to ameliorate the oxidative and endoplasmic reticulum stress in homocystinuria (Davì et al 2001, Gupta et al 2009, Martínez-Pizarro et al 2016, Vanzin et al 2011). Thus aminothiol therapy may have benefits via two different mechanisms in CBS deficient patients.

4. Caveats

Although the results above are promising, this potential therapy presents several challenges that have to be considered before clinical studies can be entered.

4.1 Chemical modification of Cys residues

A major premise of cysteine modification by MEG/Cysteamine, is the alteration of its chemical structure to a more Arg/Lys-like moiety that enables formation of H-bonds and salt bridges. The crystal structure of aspartate beta semialdehyde dehydrogenase (ASADH) with cysteamine bound covalently to Cys128 (PDB code 3Q1L) from *Streptococcus pneumonia* demonstrates that cysteamine indeed can form a disulfide with a cysteine residue. In this particular example, cysteamine acted as an inhibitor of the enzyme, by blocking the thiol group of Cys128 (Pavlovsky et al 2012). The incorporation of the aminothiol cysteamine to the Cys128 had no effect on the conformation of the protein, except for the formation of an expanded network of possible hydrogen bonds in its vicinity. Cysteamine moiety bound to active site residue Cys128 showed two spatial configurations, each of which facilitate H-bond formation with neighboring amino acid residues (Figure S1). This mimics the function of amino groups present in basic amino acid residues such as Lys and Arg.

4.2 Specificity towards selected Cys residues

The likelihood of repairing mutated Arg to Cys proteins activity with cysteamine will depend on its ability to react specifically with the variant Cys residue, as well as the impact of the formed cysteamine-cysteine residue on the function of the variant protein. This relative specificity is important because cysteamine may potentially react with any accessible wild-type cysteine residues in proteins hereby potentially causing damage. Notably, cysteine is a relatively rare amino acid; its probability for its occurrence in proteins is only 3.3% (Miseta and Csutora 2000). Another important aspect to consider is that cysteine is the least exposed residue in proteins (Poole 2015). This will influence their reactivity and accessibility by cysteamine. The attachment of a linker on cysteamine designed to target it to specific cellular compartments may improve its efficacy and specificity (Inauen et al 2016).

A potential side-effect of aminothiols is that they can inhibit the activity of native proteins. A 18% decrease in the enzymatic activity of wild-type CBS was documented after 8 h of incubation with cysteamine (Mendes et al 2015). A similar effect was also observed with ApoE3 (Weisgraber 1990) and factor VIII (Aly et al 1992). In addition, cysteamine failed to target CBS p.Arg336Cys in cultured lymphoblast of Qatari homocystinuria patients (Blom et al., unpublished results) and in cultured 293T cells containing Arg to Cys mutations in the expressed ASL protein (Inauen et al 2016). Oxidation of cysteamine in the culture medium or intracellularly, along with a limited efficacy of cysteamine in the cytosol possibly due to a fast uptake into the lysosomes are some of the hypotheses proposed to explain these findings (Besouw et al 2013, Hua Long and Halliwell 2001, Inauen et al 2016).

Side effects of cysteamine have been described in cystinosis patients including ulcers, skin, vascular, neurologic, and muscular problems, bone lesions and copper deficiency (Besouw et al 2013, Besouw et al 2011, Cahill et al 1986, De Stefano and Maiuri 2015), especially at high dose at beginning of the treatment. A recent study using the registry of Orphan Europe Record (license holder of Cystagon® in Europe) (Besouw et al 2011), reported “new” side effects concerning skin lesions and neurologic symptoms in 8 of 550 cystinosis patients followed up for 5 years (Besouw et al 2011). All patients showed recovery after dose

reduction. The reason why only a very small fraction of patients developed symptoms of cysteamine cytotoxicity, despite only three of these eight patients were treated with higher than recommended doses (<1.95 g/m²/d equivalent to 90 mg/kg/d), awaits further study. Besouw et al., suggested that differences in cysteamine uptake, transport or metabolism may increase cysteamine concentrations in some patients (Besouw et al 2013).

4.3 Dose-effect in target tissues and cellular compartments

At the organ and whole organism level, monitoring the delivery and distribution of aminothiols to specific tissues and compartments is challenging. Early studies testing the radio-protective effect of thiols have provided information about the high uptake of ³⁵S cysteamine in liver, spleen, intestinal mucosa, and kidney (Nelson and Ullberg 1960). Oral and subcutaneous administration of cysteamine in rats led to distribution of this aminothiol mainly in the kidney, the gastrointestinal tract, and the liver, as discussed before (Khomeenko et al 2012). Accurate determination of cysteamine concentration is a prerequisite to determine dose-effect of cysteamine in target tissues. In humans, cysteamine is detected at very low concentrations in plasma (<0.1 μ M) (Coloso et al 2006). After cysteamine ingestion, a peak concentration in plasma is reached after approximately one hour. Doses of cysteamine of 15-20 mg/kg lead to a peak plasma concentration of 30-50 μ M (Besouw et al 2007, Smolin et al 1988) whereas ingestion of cysteamine together with food decreases the absorption of the drug by approximately 30% (Dohil et al 2012).

4.4 Methodological constraints in the assessment of plasma and tissue levels of aminothiols

Abnormal content of aminothiols has been associated with several pathologies including liver damage, cancer, homocystinuria, AD and cardiovascular diseases (Basu et al 2015, Estrela et al 2006, Refsum et al 1998, Seshadri et al 2002). Therefore, the assessment of cellular and plasma levels of therapeutically administered cysteamine is of utmost importance for disease management. Thiol compounds have been detected by a variety of reagents and separation techniques such as HPLC, GC, CE and mass spectrometry between others as it has been published (Bayle et al 2004, Carlucci and Tabucchi 2009, Dohil et al 2012, Kataoka et al 1994, McMenamin et al 2009, Soriano et al 2012). It is uncertain whether cysteamine plasma levels reflect cysteamine tissue levels. Studies on animals treated with various doses of cysteamine permitted the assessment of the aminothiol concentration in various tissues, however, the published data differ by several orders of magnitude between groups (from 0.1 to 350 nmol/g of tissue) (Coloso et al 2006, Pinto et al 2009, Pitari et al 2000). Cysteamine levels in mouse liver, kidney and brain of 0.16, 0.35 and 0.11 mol/g of tissue, respectively, were reported after homogenates reduction with dithiothreitol (Coloso et al 2006). However, after reduction of perchloric acid-deproteinized homogenates with mercaptopropionic acid the levels of cysteamine reported in mouse kidney and liver were 15 and 24 nmol/g of tissue, respectively (Pitari et al 2000). Such inconsistency is possibly due to inadequate methodology to determine cysteamine levels in tissues. Cysteamine in mammalian tissues and serum can be found in the free-reduced, free-oxidized and protein-bound forms. Most of the cysteamine measured in the literature was probable in fact in mixed disulfide linkages with protein thiols. Assessment of free cysteamine available in target tissues after administration may be a crucial step to target Arg

to Cys mutant proteins. Although free cysteamine is devious to measure, 22 nmol of free cysteamine/mg protein has been reported in rat brain after intraperitoneal injection of 300 mg/kg (Ogony et al 2006). Novel and accurate methods to measure free and bound cysteamine must be developed.

5. Remarks and Conclusions

The potential use of cysteamine as a drug to target Arg to Cys mutations has successfully been employed, both *in vitro* and *ex vivo*, in various genetic disorders. While the use of aminothiols holds clinical promise, mechanistic studies are required to refine specificity and treatment design. For example, the efficacy of aminothiols in targeting proteins residing in plasma, such as ApoE3 and factor VIII, will be remarkably different from those targets located intracellularly. Each physiological compartment harbors specific redox potential and aminothiol abundance, all of which are expected to influence the reactivity and turnover of cysteamine and analogous drugs. MEG is another promising aminothiol analogue to repair loss of function due to Arg to Cys mutations, due to its enhanced structural resemblance to the analogous guanidinium moiety of Arg. However, studies with MEG are currently hampered by its high costs and the lack pre-clinical studies.

In vitro and *ex vivo* results have provided 'proof of principle' that aminothiols can modify Cys residues. Future research will demand the use of suitable animal models featuring Arg to Cys mutations. Ongoing studies examining the phenotype of CBS-p.Arg336Cys mutation in homocystinuria mice will pave the road for future studies on CBS function recovery upon different doses of cysteamine or MEG. If successful, this will set the stage for further studies in patients suffering from other genetic diseases caused by Arg to Cys mutations. In particular, cysteamine could be used to target lysosomal proteins harboring amino acid replacements of the type Arg to Cys, given its ease of transport, pH specificity and efficient retention in this organelle. Hence, lysosomal storage disorders such as Tay-Sachs disease and Pompe's disease may well be excellent model systems for future *in vivo* studies. Reconstitution of function of plasma proteins may also be feasible, given favorable oxidation potential and the easily reachable therapeutic concentrations of cysteamine in plasma after oral administration.

Taken together future research on aminothiols hold promise as a potential tool to target Arg to Cys mutations at large, and so in theory providing avenues to treat up to 8% of all missense mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Strategy of cysteine residues modification in proteins by aminothiols
Postulated compounds formed by L-cysteine (orange) and aminothiols compounds interactions: A) cysteamine and B) MEG (mercaptoethylguanidine). L-lysine (C) and L-arginine (D) structures. The guanidine moiety is highlighted in green. Figure adapted from Mendes et al 2015.

Figure 2. Biosynthesis and fates of cysteamine

Endogenous production of cysteamine occurs during degradation of coenzyme A, when pantothenic acid is formed. Vanin catalyzes the hydrolysis of pantetheine into pantothenic acid and cysteamine. Cysteamine is highlighted in a blue rectangle. Cysteamine as well as cysteine can be converted into hypotaurine, which is oxidized into taurine. Less than 3% of cysteamine administered has been postulated to be converted into *S*-methylcysteamine by a methyltransferase, and consequently metabolized into methanethiol and acetamide by cytochrome P450. Successively, another methyltransferase converts methanethiol into dimethylsulfide (Gahl et al 1985).

Figure 3. The mechanisms of actions of cysteamine in various diseases

Under different conditions cysteamine can exert a wide range of actions: as an antioxidant; changing gene expression; changing enzymatic activity and targeting Arg to Cys mutants (highlighted in an orange rectangle). NAFLD (non-alcoholic fatty liver disease); BDNF (brain-derived neurotrophic factor); Atg5 (autophagy related 5). Figure adapted from Besouw et al 2013.

Table 1
pKa and midpoint potentials of biological aminothiols

Aminothiol	pKa (SH)	Midpoint potentials (V) (RS/RSH); (RSSR/RSH)	Reference
Cysteine	8.6	-0.22	(Jocelyn 1967)
Homocysteine	10.0	-0.28	(Glushchenko and Jacobsen 2007; Baron and Sochor 2013)
Cysteamine	8.19	+0.921	(El-Hallag et al 2011, Serjeant and Dempsey 1979)
Glutathione	9.65	+0.92; -0.24	(Jocelyn 1967; Madej and Wardman 2007)

Table 2

Molecular targets of aminothiols

Molecular targets	Arg->Cys variants	Gene	Disease	Compartment	Recovery' of activity'	Reference
ApoE	E2 (p.Arg158Cys)	APOE	HLP	plasma	2.7 to 17.6-fold	Weisgraber et al 1984
Factor VIII	p.Arg1689Cys	F8	Hemophilia A	plasma	14-fold	Aly et al 1992
ASL	p.Arg94Cys	ASL	ASLD	cytoplasm	0%	Inauen et al 2016
	p.Arg95Cys	ASL	ASLD	cytoplasm	12.8%	Inauen et al 2016
	p.Arg168Cys	ASL	ASLD	cytoplasm	19.7%	Inauen et al 2016
	p.Arg379Cys	ASL	ASLD	cytoplasm	41.3%	Inauen et al 2016
	p.Arg385Cys	ASL	ASLD	cytoplasm	53.2%	Inauen et al 2016
CBS	p.Arg336Cys	CBS	CBSD	cytoplasm	133%	Mendes et al 2015

ApoE: Apolipoprotein E; HLP: type III hyperlipidemia; ASL: argininosuccinate lyase; CBS: cystathionine β-synthase