Inhibition of Crystallin Ascorbylation by Nucleophilic Compounds in the hSVCT2 Mouse Model of Lenticular Aging

Xingjun Fan¹ and Vincent M. Monnier¹,²

PURPOSE. Senile cataracts are associated with oxidation, fragmentation, cross-linking, insolubilization, and yellow pigmentation of lens crystallins. This process is partially explained by advanced glycation end products (AGEs) from ascorbic acid (ASA), as the authors unequivocally demonstrated in an hSVCT2 transgenic mouse. The authors present the first pharmacologic intervention study against ascorbylation in these mice.

METHODS. Five groups of mice from 2 to 9 months of age (10 mice/group) were fed a diet containing 0.1% (wt/wt) amino-guanidine, pyridoxamine, penicillamine, and nucleophilic compounds NC-I and NC-II. AGEs were determined in crystallin digests using high-performance liquid chromatography, liquid chromatography–mass spectrometry, or gas chromatography–mass spectrometry. Lens protein extract was incubated in vitro with ASA or dehydroascorbic acid.

RESULTS. The ASA level increased approximately 10-fold in all groups and was unaffected by treatment. AGEs were increased several-fold in transgenic compared with control lenses. Body weight, food intake, lenticular glutathione, and glycated lysine level were unaltered. In vitro, all compounds inhibited AGE formation. In vivo, NC-I and NC-II significantly decreased protein fluorescence at λ exc/λ em 355/405 (P = 0.045, P = 0.017, respectively) and λ exc/λ em 370/440 (P = 0.029, P = 0.007, respectively). Other inhibitors had no effect. After 7 months, only NC-I and NC-II induced a 50% reduction in pentosidine (P = NS for NC-I; P = 0.035 for NC-II). NC-I also decreased carboxymethyllysine (P = 0.032) and carboxyethyllysine (P = NS). Fluorescent cross-link K2P was decreased by NC-I, NC-II, amidoguanidine, and pyridoxamine (P = NS).

CONCLUSIONS. Pharmacologically blocking protein ascorbylation with absorbable guanidino compounds is feasible and may represent a new strategy for the delay of age-related nuclear sclerosis of the lens. (Invest Ophthalmol Vis Sci. 2008;49:4945–4952) DOI:10.1167/iovs.08-1813

Advanced glycation end products (AGEs) are formed non-enzymatically by the reaction of reducing sugars and oxoaldehydes, with amino groups in proteins, lipids, and nucleic acids through a series of reactions (Maillard reaction), and their levels have been documented to increase with age in many tissues.¹⁻³ Reducing sugars are degraded before or during their interaction with proteins to form AGES.⁴⁻⁵ In fact, many AGES are formed from reactive degradation intermediates such as methylglyoxal and glyoxal. From this point of view, advanced glycation can be considered a set of posttranslational, nonenzymatic modifications of proteins by reactive carbonyl compounds.

With age, various postsynthetic modifications occur in human lens crystallins. These are particularly associated with the water-insoluble proteins and are characterized by the presence of yellow chromophores and non-tryptophan fluorescence,⁷,⁸ pigmentation,⁹ aggregation,¹⁰ insolubilization,¹¹ fragmentation, and cross-linking.¹² These modifications are greatly enhanced in senile and brunescent cataractous lenses.¹³ Various theories and mechanisms have been hypothesized to explain these changes, including oxygen free radicals and oxidation,¹⁴,¹⁵ glycation (Maillard reaction),¹⁶ and tryptophan oxidation products.¹⁷,¹⁸ In recent years, because of the presence of high concentrations of ascorbate in the human lens, considerable interest has focused on the potential role of ascorbate as a mediator of postsynthetic modifications of crystallins in the aging lens. It was found that many phenomena observed in aging and cataractous lenses could be duplicated by the reaction of lens crystallins with ascorbate.¹⁹,²⁰ This hypothesis has been unequivocally confirmed with our recently established mouse model of lens crystallin aging in which the human sodium-dependent vitamin C transporter 2 (hSVCT2) was overexpressed under the control of the mouse α-crystallin promoter fused to a chick lens δ-crystallin enhancer.²¹ With age, there was an increase in the ascorbic acid oxidation products and crystallin-bound AGES pentosidine, carboxymethyllysine (CML), vespertylsine A, and K2P. We also noticed that at 12 months, mouse lenses had acquired a yellow tint similar to that observed in old human lenses. This animal model compressed lens crystallin chemical aging from 70 years in the human to 12 months in the mouse, providing us the opportunity to test potential pharmacologic inhibitors of lens crystallin aging by the Maillard reaction in vivo.

In the present study, we treated 2-month-old transgenic mice with five different candidate inhibitors and analyzed lens crystallin-bound fluorescence and AGES markers after 7 months of treatment. Similar experiments were conducted in vitro.

METHODS

Animals

All animal experiments were conducted in accordance with procedures approved by the Case Western Reserve University Animal Care Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed under diurnal lighting conditions and allowed free access to food and water. hSVCT2 transgenic mice were generated as described previously.²² Genomic DNA from mouse tails was isolated and subjected to PCR screening using specifically designed primers. The genetic background of these transgenic mice is C57BL/6 after at least eight generations of cross-breeding with wild-type C57BL/6 mice.

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Treatment of hSVCT2 Mice with Candidate Ascorbylation Inhibitors

Pyridoxamine dihydrochloride (P9380), DL-penicillamine (P5125), aminoguanidine (A7259), and the guanidino compounds NC-I and NC-II were purchased from Sigma (St. Louis, MO). The structure is displayed in Figure 1. The mouse diet with 0.1% wt/wt inhibitors was produced by Bio-Serv using standard diet (Isopro 3000 Prolab 5P75; LabDiet, Richmond, IN). Doses of the inhibitors were chosen based on similar studies with nucleophilic AGE inhibitors. Transgenic and age-matched control mice (50/50 male/female), 10 mice per group, were maintained on a standard mouse diet or a special medical diet started at 2 months and continued until 9 months of age. Body weight and food intake were monitored monthly. Nine-month-old mice were killed, and eyes were removed and decapsulated to release the lenses, which were processed for AGE determination.

In Vitro Incubation of Lens Crystallins with Ascorbylation Inhibitors

To best mimic the in vivo formation of ascorbylation products, two models of incubation conditions were tested. Model A consisted of 5 mg calf lens protein homogenate (CLP) incubated with 1 mM dehydroascorbic acid (DHA), with or without 1.5 mM inhibitors, under anaerobic conditions in chelex-treated 5 mM sodium phosphate buffer (pH 7.0) at 37°C for 7 days. DHA and inhibitors were refreshed daily to take into account the short half-life of DHA. Model B consisted of a one-time addition of 25 mM ASA with or without 15 mM inhibitors in aerobic conditions, but with chelex-treated buffer for 7 days to slow down but not eliminate the oxidation of ASA. At the end of the incubations, the protein was dialyzed against phosphate-buffered saline (PBS) for 24 hours at 4°C, dialyzed twice against water for 48 hours at 4°C, and lyophilized. Half the incubated protein (approximately 2.5 mg) was used for enzymatic proteolysis, as previously described. The other half of the protein was used for hydrolysis with 6 N HCl. Enzymatic digest and hydrolysates were used for AGE determination.

Measurement of Ascorbic Acid and Glutathione

Lenses were homogenized in 200 μL of 10% trichloroacetic acid (TCA). The supernatant was used for derivatization with dimethyl-o-phenylene-diamine to determine ascorbic acid level and for derivatization with 1-fluoro-2,4-dinitrobenzene to determine glutathione (GSH), level as previously reported.

Enzymatic Digestion of Lens Proteins

The TCA precipitate of mouse lens protein was washed twice with 500 μL ethyl ether and was allowed to dry at room temperature for 10 minutes. The pellet was reconstituted in an Eppendorf tube with 500 μL of 5 mM argon-exchanged, chelex-treated phosphate buffer (pH 7.0). The protein pellet was enzymatically digested with a series of proteases, as previously described. Digestion efficiency varied from 64% to 75%. Corresponding enzyme blanks incubated without added protein were used as background control. After digestion, the samples were reconstituted with water for fluorescence measurement, dried, reconstituted with 0.01 M heptafluorobutyric acid in water, and subjected to HPLC assay for the determination of K2P, as described.

Protein concentrations of the samples were analyzed by means of a ninhydrin assay expressed as a leucine equivalent after enzymatic digestion and after 6 N HCl hydrolysis to evaluate digestion efficiency. Reported values may underestimate the true AGE content given that at best 80% digestion efficiency was obtained.

Fluorescence Spectroscopy

Fluorescence at λ ex=370/em=430 nm and 335/385 nm of the enzymatic lens protein digest was measured with a spectrophotometer (821-F; Jasco, Easton, MD). Data were expressed as fluorescence units per unit protein measured as leucine equivalent.

Determination of Advanced Glycation End Products

K2P, pentosidine, CML, CEL, and furosine were analyzed in this study. The acid-labile K2P was determined in lens protein digest, as reported...
Pentosidine, CML, CEL, and furosine were determined in lens protein acid hydrolysate, as previously reported.\textsuperscript{21}

### Statistical Analysis

All values were expressed as mean ± SD. The statistical significance of differences in mean values was assessed by repeated-measures ANOVA or Student’s t-test. \( P < 0.05\) was considered statistically significant.

### RESULTS

#### Parameters of Homeostasis

To better interpret our data, we first determined a number of parameters of homeostasis in the five treatment groups and compared the data with untreated wild-type and control mice. As shown in Fig. 2A, ascorbic acid was elevated in all hSVCT2 mice to levels similar to those of the human lens (2 mM) and were unaffected by treatment. Similarly, lenticular glucose concentrations as reflected by furosine (i.e., fructose-lysine) were not significantly different in any of the groups (Fig. 2B), suggesting that the tested drugs did not impair lenticular glucose levels. Finally, and most important, there was no change in lenticular glutathione concentration (Fig. 2C), which could be a confounding factor for the interpretation of the inhibition data.

#### Testing for Ascorbylation Inhibition In Vivo and In Vitro

The five candidate inhibitors aminoguanidine, pyridoxamine, penicillamine, and the guanidino compounds NC-I and NC-II were given to the mice at a concentration of 0.1% (wt/wt) in powdered laboratory chow. In addition to testing the effects of the inhibitors in vivo, these were also tested in vitro with ascorbic acid (model B) and dehydroascorbic acid (model A). The latter was applied with repetitive supplementation to account for its short half-life. We present the data in Figures 3 and 4 whereby the in vitro data obtained with ASA are displayed in the main figure and those obtained with DHA are shown in the inset.

#### Effect of Inhibitors on Protein-Bound Fluorescence at \(\lambda_{\text{ex}}335/\text{em}385\) nm and \(370/440\) nm

Although fluorescence was not specific, the similarity in the fluorescence spectra of various synthetic AGEs (including pentosidine and vesperlysine A) with those of human lens proteins supported the assumption that AGEs are the main fluorescent species that increase with age in the human lens.\textsuperscript{22} Usually, fluorescence is monitored for AGEs at \(\lambda_{\text{ex}}335/\text{em}385\) nm and \(\lambda_{\text{ex}}370/\text{em}440\) nm. Both types of protein-bound fluorescence were highly elevated in the lens protein digest from transgenic versus wild-type mice (Figs. 3A, 3C) and in the ASA-incubated calf lens crystallins (Figs. 3B, 3D). After 7 months of intervention, NC-I was able to significantly reduce the fluorescence at both \(\lambda_{\text{ex}}335/\text{em}385\) (\( P = 0.045\); Fig. 3A) and \(\lambda_{\text{ex}}370/\text{em}440\) (\( P = 0.029\); Fig. 3C). NC-II had a similar effect at both wavelengths (\(\lambda_{\text{ex}}335/\text{em}385, P = 0.017; \lambda_{\text{ex}}370/\text{em}440, P = 0.007\); Figs. 3A, 3C). Surprisingly, aminoguanidine, penicillamine, and pyridoxamine showed no fluorescence reduction at either...
In contrast to the in vivo data, in model B (ASA) all inhibitors suppressed both types of fluorescence in vitro, though to varying degrees (Figs. 3B, 3D). Moreover, aminoguanidine and penicillamine, which had no effect in vivo, suppressed fluorescence efficiently in vitro. Interestingly NC-I, NC-II, and pyridoxamine all inhibited fluorescence at both wavelengths to approximately the same extent, but only NC-I and NC-II had a significant effect in vivo. Model A (DHA) behaved similarly to ASA for fluorescence at 385 nm (Fig. 3B, inset), but the latter was curiously not suppressed by the guanidino compounds, including aminoguanidine.

The inhibition pattern of pentosidine formation in vivo (Fig. 3E) is similar to the data in Figure 3C and was significant for NC-II but not for NC-I. This is not entirely surprising because...
the excitation/emission wavelengths are similar for both parameters. Again the most potent inhibitors were NC-I and NC-II, with a positive trend for aminoguanidine. Paradoxically, NC-I and NC-II were relatively weak inhibitors in both in vitro models compared with the other inhibitors (Fig. 3F and inset). These discrepancies raised issues that will be addressed in the Discussion.

The glycoxidation/lipoxidation ascorbylation products CML and CEL were vigorously formed from ASA and DHA (Figs. 4B, 4D, and insets), as previously reported, but only NC-I had significant in vivo suppressive activity toward CML (Fig. 4A). A trend toward the suppression of carboxyethyl-lysine (CEL) was observed (Fig. 4C). An overall similar pattern of CML and CEL inhibition was observed in both models in vitro (Figs. 4B, 4D, 4F).
tendons31 and in cultured rabbit proximal tubular epithelial 
mation in diabetic animals. We also chose penicillamine, a thiol 
amine and aminoguanidine can dramatically inhibit AGE for-
werth,7,12 and us.12 The chemical pathways by which ASA is 
directly react with nucleophiles25or it can delactonize into 
of crystallins with DHA.
studied in vitro and in vivo28 –30. Studies revealed that pyridox-
amine and aminoguanidine, two AGE inhibitors extensively 
were found in the brains of patients with hyperarginine-
sequence, and AGEs found in old and cataractous human lens 
crystallins related to ascorbic acid and its oxidation products.21 
This was already postulated many years ago by Bensch,24 Ort-
them is highly elevated in the hSCT2 mouse.21 DHA can 
directly react with nucleophiles25or it can delactonize into 
2,3-diketogulonate, which spontaneously degrades into xy-
osone and erythrose26,27 (Fig 1B). Our in vitro modeling 
reactions now unequivocally confirmed that most compounds 
levels of ultraviolet (UV) light in the chromatogram. We 
reached because of the high standard errors in the control. No 
phore K2P in vivo (Fig 4E). Statistical significance was not 
pharmacologic agents that can delay the aging of lens crystallins and 
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abetic agents can delay the aging of lens crystallins and 
aging. Two inhibitors, NC-I and NC-II, were found to signifi-
carboxylic acid. Although the exact 
ning the human lens is crucial to the development of phar-
ning nucleophiles with a guanidino group in the structure that 
delay lens opacification and cataractogenesis. The trapping of 
coloration and fluorogen formation may have the potential to 
trapping of dicarbonyl compounds and can block AGE formation, 
provisionally named NC-I and NC-II. Guanidino compounds 
can also serve as free radical scavengers34; increased levels 
for hyperargininemia.35,36 However, their role in this condition and in healthy 
still unclear.56 
At the outset a major finding in this study was that all five 
hibitors showed inhibition of ascorbylation in vitro in both 
models, though to varying degrees. Aminoguanidine, pyridox-
amine, and penicillamine were the most potent inhibitors for 
specific AGIs (pentosidine, CML, and CEL). The most surpris-
ing finding, however, was that NC-I and NC-II, which had the 
which had the weakest in vitro activity, had the best in vivo activity (notably 
NC-I).
Of particular interest are the data with K2P. This molecule is a 
fluorescent and UV-active lysine–lysine cross-link and one of the major modifications in old and cataractous human 
lenses.37 In our previous study, there was a dramatic in-
crease in K2P between 9 and 12 months of age, suggesting 
the data might have reached significance if we had extended 
the study by another 3 months. The uniform trend toward its 
suppression by all nucleophiles except for penicillamine 
(Fig. 3C) suggests similar effects would have been observed 
in vitro. As previously mentioned, however, overlapping UV 
peaks precluded us from interpreting the in vitro data. 
Nevertheless, the in vivo suppressive effect of the drug 
appeared to be strong.

A more difficult issue to address was the discrepancy 
between the in vivo and in vitro effects of NC-I and NC-II 
compared with pyridoxamine, aminoguanidine, and penicil-
lamine. The metal chelation ability of the latter three com-
and in cultured rabbit proximal tubular epithelial cells,32 as well as AGE formation in bovine eyes incubated with 
increasing nucleophiles with a guanidino group in the structure that 
methylglyoxal improves the chaperone activity of the 
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lamine. The metal chelation ability of the latter three com-
pounds might have contributed to their strong in vitro 
anti-AGE property, as previously reported by Price et al.38 
Another possibility is that NC-I and NC-II have been shown 
elsewhere to penetrate the blood barrier, likely through an 
active transport. It may thus be possible that these agents 
achieved millimolar concentrations, thus providing strong 
dicarbonyl compound trapping activity. Nevertheless, the 
fact that aminoguanidine and pyridoxamine lowered K2P 
suggests that these nucleophiles are also able to reach the 
mas.35,36 However, their role in this condition and in healthy 
humans is still unclear.56 

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References


