Paradoxical Impact of Antioxidants on Post-Amadori Glycoxidation

COUNTERINTUITIVE INCREASE IN THE YIELDS OF PENTOSIDINE AND N\textsuperscript{-}CARBOXYMETHYLLYSINE USING A NOVEL MULTIFUNCTIONAL PYRIDOXAMINE DERIVATIVE

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The inhibition of post-Amadori advanced glycation end product (AGE) formation by three different classes of AGE inhibitors, carbonyl group traps, chelators, and radical-trapping antioxidants, challenge the current paradigms that: 1) AGE inhibitors will not increase the formation of any AGE product, 2) transition metal ions are required for oxidative formation of AGE, and 3) screening AGE inhibitors only in systems containing transition metal ions represents a valid estimate of potential in vivo mechanisms. This work also introduces a novel multifunctional AGE inhibitor, 6-dimethylaminopyridoxidine (dmaPM), designed to function as a combined carbonyl trap, metal ion chelator, and radical-trapping antioxidant. Other AGE inhibitors including pyridoxamine, aminoguanidine, o-phenylenediamine, dipiroxylamine, and diethylenetriaminepentaacetic acid were also examined. The results for unreacted and interrupted ribose glycations show: 1) an unexpected increase in the yield of pentosidine in the presence of radical-trapping phenolic antioxidants such as Trolox and dmaPM, 2) significant formation of N\textsuperscript{-}carboxymethyllysine (CML) in the presence of strong chelators and phenolic antioxidants, which implies that there must be nonradical routes to CML, 3) prevention of intermolecular cross-links with radical-trapping inhibitors, and 4) that dmaPM shows excellent inhibition of AGE. Glucose glycations reveal the expected inhibition of pentosidine and CML with all compounds tested, but in a buffer free of trace metal ions the yield of CML in the presence of radical-trapping antioxidants was between the metal ion-free and metal ion-containing controls. Protein molecular weight analyses support the conclusion that Amadori decomposition pathways are constrained in the presence of metal ion chelators and radical traps.

Nonenzymatic protein glycation by reducing sugars, such as glucose or ribose, is a complicated cascade of condensations, rearrangements, fragmentations, and oxidative modifications that lead to a plethora of compounds collectively called advanced glycation end products (AGEs) (1). AGE products slowly build up and contribute to the age-dependent chemical modification of long-lived tissue proteins. Once formed, AGE products may prevent normal protein function and recognition or stimulate potentially detrimental interactions in signaling pathways (2, 3). Glycation reactions are believed to have a significant role in the progression of diabetic complications largely because of elevated levels of glucose. Indeed, the formation of AGE has been increasingly implicated in pathogenesis of diabetic complications, atherosclerosis, and Alzheimer’s disease (4–6). Inhibitors that act to reduce AGE formation may prove useful for limiting nonenzymatic protein modification associated in the pathology of these and other chronic age-related diseases.

The reaction of amino compounds, such as lysine, with reducing sugars is known as the Maillard reaction (7, 8). The “classical” or Hodge pathway begins with reversible formation of a Schiff base aldime adduct that undergoes rearrangement to a relatively irreversible ketoamine Amadori product (see supplemental material). This Amadori pathway has been considered the primary route to formation of AGE products in vivo (1). Post-Amadori pathways proceed through direct rearrangement and fragmentation of the Amadori compound and probably form a variety of reactive dicarbonyl compounds, especially α-ketoaldehydes (8, 9). Autoxidation of the reducing sugar or Schiff’s base adduct, the Wolff and Namiki pathways, respectively, can significantly complicate in vitro experiments designed to study AGE formation and inhibition (8). Interrupted glycation methods have been developed by Booth et al. (1) that allow Amadori-rich proteins to be isolated. This method allows more detailed studies to be carried out, which may provide insight into the specific mechanisms involved in post-Amadori formation of AGES.

Generally, glycation alters protein physicochemical properties by forming a variety of cross-links, browning (nonfluorescent AGE), and fluorescent AGE products. Much research has focused on investigating AGE inhibitors by assessing these nonspecific physicochemical properties, but these procedures have been questioned (10). Two specific AGE products commonly analyzed in protein glycation are N\textsuperscript{-}carboxymethyl lysine (CML) and pentosidine (10, 11). Quantitatively, CML is believed to be the major chemically characterized oxidatively formed glycation (glycoxidation) product (12). The highly fluo-

CML, N\textsuperscript{-}carboxymethyllysine; diPM, dipiroxylamine trihydrochloride; dmaPM, 6-dimethylaminopyridoxine dihydrochloride; dmaPN, 6-dimethylaminopyridoxine hydrochloride (6-dimethylaminopyridoxyl-HCl); DTPA, diethylenetriaminepentaacetic acid; MW, molecular weight; o-PD, ortho-phenylenediamine; PM, pyridoxamine dihydrochloride; PN, pyridoxine hydrochloride (pyridoxol); RI, refractive index; SEC, size exclusion chromatography; Tx, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid); HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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The abbreviations used are: AGE, advanced glycation end product; ABAP, 2,2’-azobis(2-amidinopropane) dihydrochloride; Ac-Pt, di-N-acetylpentosidine; AG, aminoguanidine; BSA, bovine serum albumin;
rescent lysine-arginine cross-linked product, pentosidine, is believed to be one of the major contributors to AGE related increases in blue (350–400 nm) fluorescence (13, 14).

Specific mechanisms for the late Maillard reaction conversion of the initial Amadori adducts to the irreversible formation of AGE involve complex sequential and parallel reactions that are not completely understood (8). AGE formation in vitro is typically accelerated by oxidative conditions (glycoxidation) using metal ions such as Fe$^{3+}$ or Cu$^{2+}$ (4, 15). It is generally accepted that transition metal ions may play a role during AGE formation in vivo and that they are required to catalyze electron transfer from ene-diol (or enaminol) intermediates, which leads to the generation of superoxide and α-dicarbonyls (16, 17). For example, oxidative conditions are required for the formation of the dicarbonyl intermediate glyoxal, and nonoxidative conditions can significantly decrease the amount of the lysine-glyoxal-derived adduct CML (18, 19). Pentosidine has also been considered to be an in vivo marker of glycoxidation that can reasonably be expected to arise from a 3-deoxyosone (hexose or pentose) precursor (20, 21). However, pentosidine formation during glycation of the pentose, arabinose, was comparable under both oxidative and nonoxidative conditions (18). This suggested a possible role for non–Metal-dependent intermolecular redox reactions during pentosidine formation. The mechanism by which pentosidine is formed from the unexpected ribose precursor $\text{N}^3\text{-2-hydroxy-4,5-dioxo-pentyl}-\text{L-lysine}$ has recently been elucidated (22). The generation of this novel deoxyosone requires carbonyl shifts along the entire carbohydrate backbone and opens new possibilities in considering Amadori degradation (23). Other research suggests that AGE intermediates may participate directly in redox chemistry to form free radicals without any requirement for transition metal ion catalysis. For example, enaminol intermediates have been proposed to provide “metal-free” formation of superoxide (24) (although alternative interpretations have been suggested (25, 26)). However, redox chemistry between two different AGE intermediates may also be involved in Maillard reaction pathways, especially under nonoxidative conditions (18, 26, 27).

Although a variety of AGE inhibitors have been identified, their mechanisms of action are not well understood (28, 29). The most common feature among the AGE inhibitors known to date is the presence of a nucleophilic functionality, such as an amine or hydrazone, which can intercept reactive carbonyl compounds and prevent progression to AGE products (6, 9). Other classes of inhibitors, such as metal ion chelators (diethylenetriaminepentaacetic acid (DTPA)) and radical-trapping antioxidants (α-tocopherol and ascorbate) exhibit their activity by limiting oxidative acceleration of glycation (8). The most reasonable point in the pathway to inhibit glycation from a pharmaceutical standpoint would be to block post-Amadori AGE formation. Inhibitors such as aminoguanidine (AG) were initially expected to block glycation at several points including direct reaction with reducing sugars, reactive dicarbonyls, Amadori products, and post-Amadori intermediates (8). However, by using interrupted glycation methods, Booth et al. (1) were able to demonstrate that AG provided little inhibition of post-Amadori AGE formation, whereas pyridoxamine (PM) was identified as an effective post-Amadori AGE inhibitor. Other AGE inhibitors have primarily been compared with AG in uninterrupted glycation experiments in the presence of transition metal ions, which makes it difficult to determine the points of effective inhibition because of high sugar levels and the complicating Wolff and Namiki pathways (30). In fact, it is difficult to discern in the literature whether some of the more promising AGE inhibitors owe their activities to carbonyl trapping or to metal ion chelation (30) (Structure 1).

Overall, the mechanism of action of AGE inhibitors is complex and dependent on the inhibitor. For example, PM has been reported to decrease the formation of fluorescent AGE products significantly and specifically to decrease the yield of CML. However, this inhibitor did not lower pentosidine cross-links (8). The only AGE inhibitor reported to specifically reduce the formation of both CML and pentosidine has been OPB-9195 (31). Both AG and PM are less active metal ion chelators than OPB-9195, and each of these compounds shows evidence for carbonyl trapping via formation of triazine, amide, or hydrazone adducts, respectively (30). However, the use of strong carbonyl traps, such as AG and OPB-9195, to retard AGE formation opens up the risk of pyridoxal deficiency in vivo by indiscriminately trapping necessary carbonyl species (10).

The present work describes a novel multifunctional AGE inhibitor designed to function as a carbonyl trap, metal ion chelator, and radical-trapping antioxidant and addresses the involvement of both reactive carbonyls and radical intermediates in the complex pathways of the Maillard reaction. Considering that the known carbonyl-trapping AGE inhibitor, PM, may be found bound to important post-Amadori intermediates (32, 33), we suspected that the phenol functionality might serve as a local protective agent against radical AGE intermediates. However, PM has been reported to have only weak hydrogen atom donating (radical-trapping) ability, making it only a marginally effective antioxidant (34, 35). Electron-donating para-substituents lower phenolic O–H bond dissociation enthalpies, which enhances radical trapping rates. Unfortunately, such substituents also lower the ionization potential of the phenol and can do so to such an extent that, for example, 4-dimethylaminophenol (which should be an extremely good radical trap) is of no practical value as an antioxidant because it reacts directly with dioxygen. Pratt et al. (36) have shown that strong electron-donating groups (such as dimethylaminopropyridoxine, damaPM) (59). A pyridoxine (PN) derivative (6-dimethylaminopyridoxine, damaPN) was also tested to study the importance of the nucleophilic amine group during glycation reactions. Both damaPM and damaPN were found to be much better radical-trapping antioxidants than PM or PN (59), as evaluated by
following the peroxyl radical-induced quenching of allopheochromycin fluorescence (37, 38). This new carbonyl group trapping and radical-trapping AGE inhibitor, dmaPM, and a number of other AGE inhibitors were tested in metal ion-containing (±M buffer) and metal ion-free (−M buffer) experiments.

We present unexpected results that provide mechanistic insight into inhibiting post-Amadori AGE formation by comparing three different classes of AGE inhibitors: carbonyl group traps, metal ion chelators, and radical-trapping antioxidants. The results during ribose glycations show: 1) a counterintuitive increase in the hML of predominately the presence of phenolic antioxidants, 2) significant formation of CML in the presence of strong metal ion chelators and radical-trapping antioxidants, which implies that there must be nonradical routes to CML, 3) prevention of intermolecular cross-links with radical-trapping inhibitors, and 4) that the novel carbonyl group and radical-trapping agent, dmaPM, shows multifunctional inhibition of AGE. Glucose glycations reveal the expected inhibition of pentosidine and CML with all compounds tested, but in a buffer free of trace metal ions the yield of CML in the presence of radical-trapping antioxidants was higher than in the metal ion-free control, although it was still lower than in the metal ion-containing control. Protein molecular weight analyses support the conclusion that Amadori decomposition pathways are limited by the presence of metal ion chelators and radical traps. Overall, our findings show a phenominal impact of radical-trapping antioxidants on post-Amadori glycoxidation and challenge the current paradigms that: 1) AGE inhibitors will not increase the formation of any AGE product, 2) transition metal ions are required for oxidative formation of AGE, and 3) screening AGE inhibitors only in systems containing transition metal ions represents a valid estimate of potential in vivo mechanisms.

MATERIALS AND METHODS

Reagents—Chemicals were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. All water used in the experiments was deionized and treated with a Millipore Milli-Q plus system (18 MΩ). Monobasic and dibasic sodium phosphate salts were SigmaUltra grade. Metal ion-containing buffer (+M buffer) was a 0.2 M phosphate buffer, pH 7.4, with no treatment to remove trace levels of transition metal ions. Metal ion-free buffer (−M buffer) was a 0.2 M phosphate buffer, pH 7.4, which was stirred with Chelex-100 resin (Bio-Rad) for 24 h and then removed from the buffer and the addition of the metal ion chelators DTPA (1 mM) and phytic acid (1 mM). Chelex-100-treated buffer (Chelex only) with no added chelating agents (DTPA or phytic acid) was also prepared and used for some dilutions or glycation experiments. All reaction buffers were filtered (0.45-μm Gelman GHP Acrodisc) before use. The buffers were tested for the presence of transition metal impurities by ascorbate autoxidation (266 nm) according to Buettner (39). Ascorbate loss (15 min at room temperature) was typically ~47% ± 9% for +M buffer and ~0.7 ± 0.2% for −M buffer. Ascorbate test loss for Chelex only buffer was ~2.8 ± 0.7% in 15 min. Dipirydoxaline (diPM) was synthesized according to the methods of Ikawa (40). The novel vitamin B6 derivatives dmaPM and dmaPN were synthesized according to the methods of Dyer et al. (15) with only minor modifications (see supplemental material). D-N-acetyl- pentosidine (Ac-Pt) was synthesized by the reaction of N-acetyl-lysine (6.2 mmol), ribose (6.2 mmol), and N-acetylglarginine (6.2 mmol) in 0.5 M phosphate buffer (62 ml), pH 9.0, for 48 h at 60 °C and purified by the methods of Dyer et al. (15) with only minor modifications (see supplemental material).

Uninterrupted Glycation—Bovine serum albumin (BSA) (10 mg/ml) essentially free of fatty acids was glycated in +M buffer or −M buffer at 37 °C, under air, with either (−)−glucose (50 mM), unless otherwise indicated. The inhibitors were dissolved in Chelex only buffer and added to either −M buffer or +M buffer experiments. The amount of Chelex only buffer present (10% v/v) was kept constant for all experiments. Toluene (1%, v/v) was added to prevent bacterial growth. Most experiments were performed in at least two side by side reactions; however, many experiments (typically, control and 5 mM inhibitor experiments) were performed on two or three separate occasions (see figure legends for the specific numbers of experiments). Aliquots were removed at various intervals (3–21 days) depending on the sugar and frozen at −75 °C until analyses.

Interrupted Ribose Glycation—BSA (10 mg/ml) was preglycated, under air, by incubation with ribose (0.5 mM) at 37 °C for 24 h in +M buffer, unless otherwise indicated. To remove the portions were dialyzed (molecular weight cut-off, 12 kDa) against either +M buffer or −M buffer at 4 °C (4 buffer changes over 24 h). The protein concentrations were determined by the method of Lowry as modified by Peterson (42). Interrupted glycation experiments of preglycated, diaxylated BSA (5 mg/ml) in +M buffer (±M buffer or −M buffer) were incubated at 37 °C under air, in the absence or presence of various inhibitors (5 mM) (the samples also contained toluene, 1% v/v). The aliquots were removed at 0, 1, 3.5, and 5 days and frozen at −75 °C until analyses.

Pentosidine Analysis by HPLC—The samples from the incubation mixtures (50 μl) were prepared according to the methods of Miyata et al. (10). The hydrolysates were evaporated to dryness under nitrogen and dissolved in 0.2 M phosphate buffer (filtered 0.2 μm), pH 7.4 (400 or 100 μl for ribose or glucose glycation samples, respectively). Pentosidine was quantified by fluorescence detection (excitation/emission = 336/385 nm) on a Discovery C18 HPLC column (5 μm particle size, 25 cm length × 4.6 mm inner diameter; Supelco) using PN (excitation/emission = 336/385 nm) as internal standards (fluorescence detector, 370-nm emission cut-off; excitation/emission time program, 336/385 nm 0–15 min and 25–60 min; 305/395 nm 15–25 min). A gradient program was used to separate pentosidine and internal standards (solvent A, 0.1% heptfluorobutyric acid in water; solvent B, 0.1% heptfluorobutyric acid in 1.1 acetonitrile/water: 100 μl, 15% A and 85% B; 35 μl, 65% A and 34% B; 40 μl, 65% A and 34% B; 50 μl, 0% A and 100% B; 58 μl, 0% A and 100% B; 59 μl, 100% A and 0% B; and 70 μl, 100% A and 0% B. The internal standard PN was added to confirm Ac-Pt stability and purity by monitoring a consistent known ratio of Ac-Pt/PN (e.g., Ac-Pt/PN = 1.1 ± 0.1) (standard mix typically, FN 108 μl and Ac-Pt 6.75 μl) (see Fig. 2).

CML Analysis by Gas Chromatography/Mass Spectrometry—CML samples (50 μl) were prepared according to the methods of Miyata et al. (10) with only minor modifications (see supplemental material). The hydrolysates were dried under nitrogen, and CML and tetra-deutero-CML were measured as Nd, trifluoroacetyl methyl esters (dissolved in dry methanol, 0.15 ml) by selected ion monitoring gas chromatography/ mass spectrometry (12, 41).

Size Exclusion Chromatography—Size exclusion chromatography (SEC) was performed on a Tosoh Biosep G3000SWx2 column (5 μm, 30 cm × 7.8 mm) (Supelco) with simultaneous sequential UV (280 and 340 nm), fluorescence (excitation/emission = 336/385 nm), and refractive index (RI) detection. The mobile phase was 25 mM phosphate buffer, pH 7.4 (±M buffer) or 0.2 M phosphate buffer, pH 7.4 (−M buffer) at 4 °C, with 5% methanol (v/v) delivered at 0.7 ml/min (back pressure was maintained below 80 bar). Incubation samples were injected (7 μl), and the percentage of protein cross-linked (aggregates/aggregates + monomer) was calculated from the average of the RI and 280 nm UV signals.

MALDI-TOF Analysis—The incubation samples (10 μl) were diluted (100 μl) and dialyzed in Dispo-Biodialyzers (molecular weight cut-off, 10 kDa) (Sigma) against deionized water at 4 °C (four changes over 24 h). The dialyzed samples were kept at ~20 °C until further analysis. The mass spectral analyses were carried out on a PerSeptive BioSystem Voyager DE-STR, MALDI-TOF mass spectrometer (Applied Biosystems Inc.). A nitrogen laser was used to desorb the molecules; the ionized proteins were then accelerated to 25 kV for mass analyses. For all of these experiments, the instrument was set at linear mode to increase the mass range and sensitivity. To ensure mass accuracy, an external calibration was performed prior to each sample spot, and a calibration check was performed after each sample to ensure that the calibration had not shifted (see supplemental material) (43, 44).

RESULTS

Unexpected Increase in Pentosidine Yields during Ribose Glycations—To study the complex and confusing effects of metal ion chelation on AGE inhibitor activity, we chose to conduct glycation experiments with the highly reactive pentose, ribose, in both metal ion-containing (+M buffer) and metal ion-free (−M buffer) buffers. The buffers were 0.2 M phosphate, pH 7.4 (made from high purity salts; SigmaUltra grade) either with no
treatment to remove trace metal ions (+M buffer) or Chelex-treated followed by addition of the chelators DTPA (1 mM) and phytic acid (1 mM) (−M buffer). Buffer quality was determined by monitoring ascorbate loss (39) (+M buffer, −47%; −M buffer, −0.7% ascorbate loss in 15 min, see “Materials and Methods”). Pentosidine was quantified (after acid hydrolysis) by fluorescence detection using the internal standards Ac-Pt and PN (Fig. 1A). A mixture of Ac-Pt and PN was added to neutralized hydrolysates just before analysis. The stability and purity of the Ac-Pt standard was confirmed in each sample by comparing the sample Ac-Pt/PN ratio to the standard mixture Ac-Pt/PN ratio.

On the basis of their structures, many of the AGE inhibitors tested are probably multifunctional but, nevertheless, they can still be classified as follows: 1) radical traps: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox or Tx), dmaPM, and dmaPN (these three compounds have been shown to have comparable abilities to retard allophycocyanin fluorescence quenching induced by peroxyl radicals (59), and allophycocyanin fluorescence quenching was retarded by o-PD at a level intermediate between PN and Tx); 2) carbonyl traps: dmaPM, PM, AG, and o-phenylenediamine (o-PD); and 3) chelators: dmaPM, PM, and diPM (other potential weaker chelators are AG, o-PD, dmaPN and PN). The three compounds classified as radical-trapping antioxidants, Tx, dmaPM, and dmaPN, gave an astonishing increase in the yields of pentosidine in both +M buffer (3–6-fold) and −M buffer (4.5–7-fold), as shown in Fig. 1B. o-PD also increased pentosidine yields in +M buffer and more noticeably in −M buffer, suggesting that it has radical trapping abilities (as indicated also by its retardation of allophycocyanin fluorescence quenching; see above). In the presence of metal ions, o-PD may act as a metal ion chelator; it can also autoxidize and may even be a pro-oxidant. The −M buffer and the metal ion chelators PM and diPM also increase pentosidine yields by a factor of up to 3 compared with the +M control experiment. The weaker chelators, PN and AG, also gave small increases in pentosidine (Fig. 1B). The phenolic radical-trapping antioxidant Tx produced a concentration-dependent pentosidine yield. The maximum increase in pentosidine (up to 10-fold) was observed at Tx concentrations between 0.3 and 1 mM (see supplemental material).

A few experiments were also conducted in +M buffer after a 24-h Chelex-100 only treatment (Chelex only buffer) and in a −M buffer with the added radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) (2 mM). The Chelex only buffer results were similar to −M buffer results when chelators or radical traps were present, PM, PN, diPM, and Tx, but were more similar to +M buffer results in the control and AG experiments (all inhibitors 5 mM) (see supplemental material). Control, PM, and dmaPM experiments with ABAP included as a source of water-soluble peroxyl radicals gave, surprisingly, results identical to those obtained with the same additive in the −M buffer (data not shown).

Some inhibition of pentosidine was observed at high inhibitor concentrations (Fig. 1C). High concentrations (25 mM) of Tx and AG both produced a significant inhibition of pentosidine in both the +M and −M buffers. PM (25 mM) only showed inhibition in the −M buffer (relative to its −M buffer control). Mixtures of Tx (1 mM), to amplify pentosidine formation, and a secondary inhibitor (PM, AG, or o-PD at 5 mM), to trap carbonyl intermediates, were tested to assess the action of traditional AGE inhibitors. The results (Fig. 1C) display a trend where there is greater inhibition of pentosidine, compared with 1 mM Tx alone, with the expected increasing strength of the carbonyl trap (PM < AG < o-PD). (Radical trapping by o-PD and its autoxidation are expected to be negligible in the presence of the strong antioxidant, Tx, and thus the o-PD will be more available to act as a carbonyl trap.) Interestingly, the maximum pentosidine increase (16-fold or 24.9 mmol of pentosidine/mol of Lys (BSA)) for all of the various glycation experiments was found when incubating 0.3 mM Tx and 0.3 mM AG in −M buffer for 14 days (data not shown).

The unexpected and counterruitive increase in pentosidine yields in uninterrupted ribose glycations with radical-trapping antioxidants led us to carry out a more thorough testing of glycation reactions (interrupted ribose and uninterrupted glu-
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Inhibition of Pentosidine and CML during Uninterrupted Glucose Glycations—The unexpected increased yield of pentosidine in ribose glycations led us to test glycations with the slower reacting hexose, glucose. Glycation conditions were similar to the ribose experiments, except that a higher concentration of glucose (250 mM) was used, and the reaction time was increased to 21 days. CML was analyzed by selected ion monitoring gas chromatography/mass spectrometry after reduction, precipitation, addition of tetra-deutero-CML, hydrolysis, and conversion to N,O-trifluoroacetyl methyl esters. As shown in Table I, nearly all of the AGE inhibitors tested, Tx, dmaPM, dmaPN, PM, PN, diPM, and -M buffer decreased the formation of both pentosidine and CML compared with +M control (AG decreased CML but not pentosidine). Chelators appear to provide the best inhibition of pentosidine and CML. Surprisingly, the radical-trapping antioxidants Tx, dmaPM, and dmaPN in -M buffer showed an increase in CML when compared with -M buffer control. Small increases in pentosidine were also noted with the radical-trapping antioxidants in -M buffer. The loss of these phenolic antioxidants was followed by HPLC analysis during the course of the reaction (at 7, 14, and 21 days). Tx was completely consumed between 7 and 14 days of reaction, but ~15% of the dmaPM inhibitor remained after 21 days (see supplemental material).

Incomplete CML Inhibition during Ribose Glycations—The effect of metal ion chelation, radical trapping, and carbonyl trapping on formation of the glycoxidation product CML was also investigated during ribose glycations. (N'-carboxymethyllysine formation was also estimated for some uninterrupted ribose experiments (see supplemental material).) In uninterrupted ribose glycations, metal ion chelators, -M buffer, dmaPM, PM, and diPM (45), show a ~2.5-fold decrease in CML compared with the +M buffer control (Fig. 3A). Similar results for CML inhibition were observed in the post-Amadori interrupted glycation model (Table II). Unexpectedly, a significant amount of CML was formed even in well chelated buffers (Fig. 3 and Table II). Even very high concentrations (up to 25 mM; Fig. 3B) of carbonyl trapping, dmaPM, PM, AG, and o-PD, or radical-trapping, Tx and dmaPM, inhibitors were not able to decrease CML below 30% of the +M buffer control. However, chelating agents do inhibit CML formation at low levels (e.g., 0.2 mM dmaPM and 0.05 mM diPM; Fig. 3B). Mixtures of Tx with added carbonyl traps, PM, AG, or o-PD, only show a change in CML formation, compared with Tx alone, when metal ion chelation was possible (1 mM Tx and 5 mM PM). Tx, and o-PD to a lesser degree, appear to increase CML in -M buffer compared with the -M buffer control. Uninterrupted ribose experiments were also conducted in -M buffer with added ABAP (2 mM); the results for control, PM, dmaPM, and dmaPN (5 mM inhibitor) experiments with ABAP present (data not shown) were unexpectedly identical to the -M buffer only CML values shown in Fig. 3A.

Prevention of Protein Cross-links during Ribose Glycations—Intermolecular protein cross-links were measured analytically by SEC with RI, UV, and fluorescence detection (Figs. 4 and 5). The RI response showed little or no dependence on glycation modifications. Protein browning (UV 340 nm) and fluorescence (excitation/emission = 336/385 nm) were recorded but not used to compare inhibitor differences because inhibitors such as PM and dmaPM would be expected to bind to glycation intermediates.

The HPLC conditions were Discovery C18 column, solvents A (water) and B (acetonitrile) both containing 0.1% heptfluoroisobutryic acid delivered at 1 ml/min as gradient: 0 min, 100% A; 30 min, 40% A; 92 min, 36% A; 35 min, 0% A; 47 min, 0% A; 48 min, 100% A; and 58 min, end. The inhibitors were quantified at 293 nm UV against calibration curves.
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**TABLE I**

Pentosidine and CML inhibition during uninterrupted glucose glycation in +M buffer or -M buffer

These results are after 21 days uninterrupted glucose glycation 10 mg/ml BSA and 250 mM glucose at 37 °C under air (n = 1 for all experiments).

| Experiment | [Inhibition] | Relative pentosidine* | Relative CML* |
|------------|-------------|-----------------------|---------------|
|            | +M buffer   | -M buffer             | +M buffer     | -M buffer     |
| Control    | 0           | 100                   | 20.1          | 100           | 5.5           |
| Tx         | 5           | 23.7                  | 31.4          | 40.5          | 36.1          |
| dmaPM      | 5           | 35.4                  | 62.7          | 14.5          | 19.5          |
| dmaPN      | 5           | 66.7                  | 63.1          | 47.0          | 14.8          |
| PM         | 5           | 35.4                  | 13.4          | 5.9           | 4.5           |
| PN         | 5           | 47.3                  | 18.0          | 72.8          | 5.4           |
| diPM       | 0.05        | 12.4                  | 17.7          | 6.7           | 4.8           |
| AG         | 5           | 120.9                 | 18.0          | 54.8          | 4.6           |

* Relative pentosidine, % of +M control = 0.069 mmol pentosidine/mol Lys (BSA).
* Relative CML, % of +M control = 44.2 mmol CML/mol Lys (BSA).

**TABLE II**

CML inhibition during interrupted ribose glycations in +M buffer or -M buffer

The results are after 5 days (post-dialysis), unless otherwise indicated, interrupted ribose glycation (preglycated, dialyzed BSA 5 mg/ml at 37 °C under air; preglycated for 24 h at 37 °C in +M buffer at BSA 10 mg/ml and ribose 0.5 M and then dialyzed against +M buffer or -M buffer as indicated) (n = 1 for all experiments).

| Experiment | [Inhibition] | Relative CML* |
|------------|-------------|---------------|
|            | +M buffer   | -M buffer     |
| Con (0 days)| 0           | 28.6          | 26.0         |
| Control    | 0           | 100           | 42.7         |
| Tx         | 5           | 79.6          | 48.3         |
| dmaPM      | 5           | 42.5          | 39.2         |
| PM         | 5           | 49.9          | 42.2         |
| AG         | 5           | 82.6          | 39.1         |

* Relative CML, % of +M control (5 days) = 77.0 mmol CML/mol Lys (BSA).

![Fig. 3. CML inhibition by metal ion chelators (−M buffer, dmaPM, PM, and diPM) during uninterrupted ribose glycation.](image)

A, CML after 7 days of uninterrupted ribose glycation (10 mg/ml BSA and 50 mM ribose at 37 °C under air) in the absence (Con) or presence of various inhibitors (5 mM as indicated) in +M buffer (open bars) or −M buffer (solid bars). CML reported as a relative percentage of the +M Control (+M Control = 69 ± 4.5 mmol of CML/mol of Lys (BSA)). B, other inhibitor concentrations (as indicated) and two inhibitor experiments (1 mM Tx and 5 mM second inhibitor as indicated). The other details are as indicated for A. (For all experiments n = 2 (duplicate analysis)).

Interrupted ribose glycation demonstrated more complex cross-linking profiles. As shown in Fig. 5, high amounts (>40%) of protein cross-links were formed during the preglycation and dialysis steps, control 0 days (post-dialysis). Cross-links were largely maintained in the interrupted control experiments, suggesting that initial cross-links could mature to become irreversible. Inhibitors generally reduced the extent of cross-linking at both 0 and 5 days post-dialysis, which implies that maturation of initial (reversible) cross-links can be prevented (Fig. 5). The radical-trapping antioxidants Tx and dmaPM were also the most effective inhibitors of intermolecular cross-linking during interrupted ribose glycation.

**Metal Ion Dependence on Protein MW Increase during Glycations**—The gradual decrease in SEC retention time for BSA monomer (Fig. 4A) qualitatively illustrates the protein MW increase during glycation, whereas MALDI-TOF analysis provides quantitative information about the average mass increase (Figs. 6 and 7). Formation of Amadori adducts increase the protein MW by +132 atomic mass units for ribose and +162 atomic mass units for glucose. Many AGE products require a decrease in the mass of the initial Amadori adduct. For example, CML and pentosidine products result in +58- and +99-atomic mass unit increases, respectively, rather than the +132-atomic mass unit increase from the ribose Amadori adduct.

Uninterrupted ribose and glucose glycations demonstrated metal ion-dependent MW increases. The −M buffer control ribose glycation (50 mM) at 7 days showed an average MW of...
~69,800 atomic mass units, which corresponds to at least ~25 Amadori adducts on the protein (Fig. 6A) (there are 60 lysine and 26 arginine amino acid residues in the BSA sequence; the structure of bovine serum albumin (accession number ABBOS, version GI 418694) was obtained on PubMed). The average MW for the +M buffer control ribose glycation was 69,000 atomic mass units corresponding to 20 Amadori adducts. Except for Tx, glycations in the presence of inhibitors, dmaPM, PM, and AG, displayed MW results similar to the control experiments (Fig. 6A). Compared with controls, Tx experiments showed a lower average MW in both the +M buffer (~13 Amadori adducts) and −M buffer (~20 Amadori adducts). Glycations at lower levels of ribose (1 and 5 mM) resulted in a MW increase corresponding to ~12 Amadori adducts in −M buffer (Fig. 6A). Glucose glycations in −M buffer showed MW increases corresponding to ~16 glucose Amadori adducts at 7 days and ~27 Amadori adducts at 21 days of incubation (Fig. 6B). In contrast, +M buffer uninterrupted glucose glycations indicate MW increases of ~7.5 and 12 Amadori adducts at 7 and 21 days, respectively. During these control glucose glycations, the pres-
The present work introduces a novel multifunctional AGE inhibitor, dmaPM, designed to function as a carbonyl trap, metal ion chelator, and radical-trapping antioxidant. The 6-dimethylamino substituent increases the radical-trapping ability of the phenolic group of pyrrodoxamine. We have used the highly water-soluble α-tocopherol analog Tx to study and isolate the effects of radical traps on the formation of AGE products. The PN derivative (dmaPN) was also tested to study the importance of the nucleophilic amine group during glycation reactions. Both dmaPM and dmaPN are comparable with Tx in their abilities to retard peroxyl radical induced quenching of allophecoxyanin fluorescence, PN and PM being much less efficient (50). This new AGE inhibitor, dmaPM, and a number of other AGE inhibitors were tested in metal ion-containing (+M buffer) and metal ion-free (−M buffer) experiments. As has already been mentioned, on the basis of their structures, many of the AGE inhibitors tested are probably multifunctional but can still be classified as: 1) radical traps: Tx, dmaPM, and dmaPN; 2) carbonyl traps: dmaPM, PM, AG, o-PD; and 3) chelators: dmaPM, PM, and diPM (AG, o-PD, dmaPN and PN will be weaker chelators).

In the presence of radical-trapping antioxidants, Tx, dmaPM, and dmaPN, we found an unexpected (3–7-fold, compared with +M buffer control) increase of the specific AGE product pentosidine during both uninterrupted (Fig. 1) and interrupted (Fig. 2) ribose glycations. However, although the radical-trapping antioxidants increased the yield of the lysine-arginine cross-link, pentosidine, they decreased intermolecular protein cross-linking dramatically (Fig. 4). Uninterrupted ribose glycations demonstrated an interesting [Tx] dependence with the maximum pentosidine yields occurring between 0.3 and 1 mM Tx. Metal ion chelators, such as −M buffer, PM, and diPM, were also found to increase pentosidine −2–3-fold, compared with +M buffer control (Figs. 1 and 2). When the carbonyl-trapping inhibitors, PM, AG, or o-PD, were co-incubated with 1 mM Tx (Fig. 1A), pentosidine decreased with increasing strength of the carbonyl trap (PM < AG < o-PD). Thus, effective carbonyl trapping of pentosidine precursors was more pronounced when the Amadori decomposition pathways were limited by the presence of both metal ion chelators and radical traps.

An unexpected increase in the yield of the important AGE product CML was observed in the presence of Tx in metal ion-free glycations with both ribose (Fig. 3) and glucose (Table 1). Metal ion chelators, −M buffer, PM, and diPM, in uninterrupted glucose glycations inhibited CML formation by 15–20-fold, compared with +M buffer control. The addition of Tx, dmaPM, or dmaPN to −M buffer glucose glycations resulted in a 3–7-fold increase in CML, compared with −M buffer control. However, these radical-trapping inhibitors did decrease glucose derived CML in both the +M and −M buffers when compared with the +M buffer control. Multifunctional dmaPM provided better inhibition of CML in +M buffer than Tx or dmaPN, probably because it chelates metal ions. As shown in Fig. 3, CML was also increased in the presence of Tx during ribose glycations in −M buffer, compared with +M buffer control. Metal ion chelators (and even high inhibitor concentrations) were not able to decrease ribose derived CML by more than −2.5–3-fold compared with +M buffer control. These results imply that there must be nonradical routes to CML, especially during ribose glycations.

**DISCUSSION**

*Effective AGE Inhibitors Can Increase the Formation of Specific AGE Products—*Radical-trapping antioxidants have long been known to inhibit the Maillard reaction (8, 47, 48). However, little work has been done on the effects of radical traps on AGE chemistry. Ascorbic acid and α-tocopherol have been examined to some degree (48, 49, 50), but both of these bio-antioxidants have significant disadvantages for understanding protein glycation chemistry. The lipophilic antioxidant α-tocopherol is only sparingly soluble in aqueous protein solutions and is unlikely to be associated with the highly polar protein or glycation residues. The ene-diol anion of ascorbate is so susceptible to autoxidation via metal ions or direct electron transfer to oxygen that it rapidly decomposes to short chain carbo-hydrate intermediates that are themselves potent glycating agents (51, 52).

The impact of antioxidants on post-Amadori glycoxidation was studied in the presence of metal ions (+M buffer) caused the average MW to increase only half as much as in the metal ion-free (−M buffer) experiments.

Different aspects of metal ion-dependent MW increases were observed in interrupted ribose glycations. Preglycation of BSA (10 mg/ml) with ribose (0.5 M) at 37 °C for 24 h resulted in a MW increase via formation of Amadori adducts and possibly some AGE products. After dialysis, the average MW decreased as the Amadori-rich protein was incubated, and more AGE products formed (Fig. 7). Surprisingly, the dialysis (4 °C, 24 h) as indicated in Fig. 7 (controls at 0 days), caused a large increase in the average MW after 5 days of post-dialysis incubation (approximately −1800 atomic mass units) was approximately the same for both +M buffer and −M buffer experiments when the preglycation was in +M buffer. To prevent premature decomposition of Amadori adducts, BSA was preglycated in −M buffer and dialyzed against −M buffer (striped bars for all experiments, n = 1).

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**Transition Metal Ions Are Not Necessarily Required for AGE Formation—**The pentosidine results during ribose glycations indicate that prevention or scavenging of AGE radical intermediates leads to an increase in the formation of pentosidine (Figs. 1 and 2), whereas carbonyl traps can decrease the formation of this specific AGE cross-link (Fig. 1C). These unex-
expected findings give strong support to the mechanism elucidated by Biemel et al. (22) that requires carbonyl shifts along the entire carbohydrate backbone to form the important pentosidine precursor, \( \text{N}^6-(2\text{-hydroxy-4,5-dioxo-pentyl})-\text{L-lysinate} \). The serial tautomerizations involved in this recently identified mechanism form ene-diol groups at several points on the carbohydrate backbone. The increased yield of pentosidine in the presence of transition metal ion chelators and radical-trapping antioxidants implies that at least some pentosidine precursors can decompose to form both pentosidine and other glycoxidation products and that the latter decomposition route can be inhibited by chelators and radical traps. We suggest that radical-trapping phenolic antioxidants reduce radical intermediates (Fig. 8) such as enaminol radicals and ene-diol radical anions back to their parent enaminols or ene-diol anions, respectively. Based on the dramatic increases observed in pentosidine formation, we also suggest that deprotonated ene-diols may autoxidize via electron transfer to oxygen, without any requirement for catalysis by transition metal ion. (In the absence of oxygen, electron transfer may occur between glycation intermediates (18, 27).)

In the presence of Tx the carbonyl-trapping agents, PM, AG, and o-PD, caused pentosidine yields to decrease relative to Tx alone (Fig. 1C). In this connection, there is evidence in the literature for the interception of pentosidine intermediates at various stages in the dideoxyosone pathway by o-PD and AG (21, 53). However, in both +M and −M buffer these carbonyl traps did not inhibit pentosidine formation (Fig. 1B). We propose that this difference arises because radical-trapping antioxidants significantly limit the participation of reactive carbonyl compounds in the decomposition of the Amadori adducts and thus more of the decomposition chemistry occurs via the dideoxyosone pathway. It also seems probable that Tx at high concentrations (e.g. 25 mM) can act as a nucleophile\(^3\) capable of stabilizing Amadori intermediates and thus preventing AGE product formation (Fig. 1C). We suspect that higher concentrations of o-PD would also inhibit the initial ribose-derived pentosidine yield increase in Fig. 1B based on recent experiments by Biemel et al. (53).

Glucose glyations showed pentosidine inhibition with all of the AGE inhibitors tested (Table I), but on mechanistic grounds, we may expect glucosepane yields to be increased in the presence of radical-trapping antioxidants and, possibly, metal ion chelators. In this connection, the glucose-derived lysine-arginine cross-link, glucosepane, has recently been identified as a dominant Maillard cross-link product in human serum albumin and lens proteins (23). The reaction proceeds via \( \text{N}^\text{6}-(2,3\text{-dihydroxy-5,6-dioxo-hexyl})-\text{L-lysinate} \) (which has recently been confirmed to form on a lysozyme in a site specific manner (53)) to produce glucosepane rather than pentosidine as the end product (22). Formation of pentosidine from a hexose such as glucose requires, at some point, oxidative cleavage to a five-carbon chain (possibly via arabinose formation (18, 54)). Radical-trapping antioxidants and metal ion chelators (to a lesser degree) would be expected to favor formation of glucosepane by a mechanism similar to that proposed for the increased yield of pentosidine (Fig. 8).

One possible explanation for the Tx-induced increase of CML yield in −M buffer is an increased formation of hydrogen peroxide. If deprotonated enaminols or ene-diols react directly with oxygen in the absence of metal ions, then more superoxide may be produced in a metal ion-free system containing radical-trapping antioxidants, and the dismutation of the superoxide would yield more hydrogen peroxide. Elgawish et al. (55) have proposed that 2,3-dicarbonyl intermediates may be oxidized by hydrogen peroxide to produce CML and acetamide cross-links via a Bayer-Villiger oxidation. To our knowledge, detailed comparisons of CML yields in metal ion-free systems with a wide range of antioxidants have not been made. Future work should evaluate the role of hydrogen peroxide in the radical-trapping antioxidant-induced increase in CML (and possibly, acetamide cross-links) in metal ion-free systems. We speculate that other

\(^3\) The chroman structure and methyl substituents on Tx donate electron density to the aromatic ring, which would be expected to increase both hydrogen atom donating ability and nucleophilic activity of the phenol.
nonradical (or internal redox) mechanisms to CML formation may also be involved (especially for the background level in ribose glycations).

Transition metal ion chelators, −M buffer and diPM, showed no effect on the formation of intermolecular protein cross-links during ribose glycation of BSA. Carbonyl-trapping inhibitors, PM, AG, and o-PD, significantly decreased intermolecular cross-links, whereas the radical-trapping inhibitors, Tx and dmaPM, almost completely inhibited the formation of cross-links (Fig. 4). Furthermore, cross-link analysis during interrupted ribose glycations indicated that during the early stages of the reaction the intermolecular cross-links are labile, although they eventually mature into stable cross-links (Fig. 5). Again, this process was not observed to be metal ion-dependent. The absence of a chelator effect also indicates that metal ion catalysis is probably irrelevant in the formation of many of the protein cross-links.

Protein MW analysis (determined by MALDI-TOF) supports the conclusion that Amadori decomposition pathways are limited by the presence of metal ion chelators and radical traps. The presence of metal ions (+M buffer) leads to a decrease in MW compared with reactions in metal ion-free (−M) buffer (Figs. 6 and 7). We presume that this comparative loss in MW is primarily due to transition metal ion catalyzed loss of low molecular weight AGE products from the glycated protein. (During uninterrupted ribose glycations (Fig. 6a), Tx shows a lower MW increase than control. We suggest that fewer AGE products may be contributing to the protein MW increase above a steady state level of Amadori adducts.) Rapid acceleration of Amadori product decomposition is supported by the metal ion-dependent loss of MW during +M buffer and −M buffer dialysis of interrupted ribose glycation samples (Fig. 7). In this case, a significant loss of MW occurred at 4 °C in just 24 h (compare +M and −M buffer controls at 0 days). MW loss continued at similar rates for +M buffer and −M buffer during incubation at 37 °C for 5 days. However, interrupted ribose experiments preglycated in −M buffer demonstrated a lower initial increase in MW and showed a slower rate of MW loss during incubation (Fig. 7, striped bars). We presume that in the interrupted experiments with +M buffer, the preglycation steps must be forming a different set of glycation adducts compared with −M buffer preglycation. It is expected that metal ion-free reactions would favor formation of Amadori adducts with less interference from the complicating Wolff and Namiki pathways. The MW differences observed for glucose experiments (Fig. 6b) suggest that Amadori adducts form readily from glucose even under conditions that should minimize glucose autoxidation to more active glycating species.

Non-Metal-dependent AGE Chemistry Should Be Considered When Trying to Identify AGE Inhibitors for Potential in Vivo Mechanisms—We believe that the results of this work provide a new perspective for understanding AGE formation and inhibition processes in vivo where the levels of free metal ions, such as Cu²⁺ and Fe³⁺, are very low. Powerful metal ion-chelating agents and radical-trapping antioxidants, such as carnosine and ascorbate (respectively), may be present at substantial concentrations in tissues where AGE products form (30). Penicillamine and CML yield increases in strongly chelated glycations containing radical-trapping antioxidants imply that metal catalyzed “glycoxidation” is unlikely to be the only mechanistic explanation for the presence of these specific AGE products in vivo (especially for CML formation) (6, 56, 57). To understand how carbonyl-trapping AGE inhibitors may function in vivo, the possibility must be considered that metal-catalyzed in vitro methods may be misleading because they push the decomposition chemistry into more complicated pathways than are actually relevant.

Because radical-trapping antioxidants significantly alter and minimize AGE formation, the new multifunctional AGE inhibitor dmaPM may provide a significant advantage over the typical carbonyl-trapping (and metal ion-chelating) AGE inhibitors. We suspect that the carbonyl-trapping ability of dmaPM may help to localize its radical-trapping activity to sites where glycation chemistry is occurring. This may help to further limit AGE product formation and prevent AGE-induced oxidative modification of protein amino acids (10, 58) by scavenging radical intermediates. The ability of dmaPM to inhibit advanced lipoxidation end products may be even more dramatic. PM has been found to inhibit keto-octadecadienoic acids during linoleic acid oxidation (34). Other researchers have identified oxidized phosphotidylcholine species, incorporating a terminal γ-hydroxy(=oxo)-α,β-unsaturated carboxyl, as high affinity ligands for the macrophage scavenger receptor, CD36 (46). Interception of reactive lipoxidation carbonyl intermediates by dmaPM may prevent protein modification as well as break radical chain reactions leading to more lipoxidation intermediates. We believe that the novel carbonyl- and radical-trapping agent, dmaPM, shows promise as a multifunctional inhibitor of both AGE and advanced lipoxidation end products.

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