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Identification of α-dicarbonyl scavengers for cellular protection against carbonyl stress

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Abstract

Tissue deterioration and aging have long been associated with the accumulation of chemically induced protein and DNA damage. Reactive oxygen species (ROS) and reactive carbonyl species (RCS), especially α-dicarbonyl compounds, are key mediators of damage caused by oxidative stress, glycation, and UV-irradiation. The toxic effects of ROS are counteracted *in vivo* by antioxidants and antioxidant enzymes, and the deleterious effects of one RCS, methylglyoxal, are counteracted by a ubiquitous glyoxalase system. Carbonyl stress as a result of toxic effects of various mono-dicarbonyls (e.g. 4-hydroxynonenal) and α-dicarbonyls (e.g. glyoxal and deoxyosones) cannot be directly antagonized by antioxidants, and only a small number of biological carbonyl scavengers like glutathione (GSH) have been identified to date. We have developed a new screening method for the identification of carbonyl scavengers using a rapid glycation system that proceeds independent of oxygen and therefore, excludes identification of inhibitory compounds acting as antioxidants. Using this screening assay adapted to 96-well microtiter plates, we have identified the cysteine derivative 3,3-dimethyl-D-cysteine as a potent inhibitor of non-oxidative advanced glycation. Comparative kinetic analyses demonstrated the superior α-oxoaldehyde-scavenging activity of D-penicillamine over that of aminoguanidine. D-Penicillamine traps α-oxoaldehydes by forming a 2-acylthiazolidine derivative as shown by structure elucidation of reaction products between D-penicillamine and methylglyoxal or phenylglyoxal. We demonstrated that upon co-incubation, D-penicillamine protects human skin keratinocytes and fibroblasts (CF3 cells) against glyoxal- and methylglyoxal-induced carbonyl toxicity. Our research qualifies α-amino-β-mercapto-β,β-dimethyl-ethane as a promising pharmacophore for the development of related α-dicarbonyl scavengers as therapeutic agents to protect cells against carbonyl stress. © 2002 Published by Elsevier Science Inc.

Keywords: Glycation; α-Dicarbonyl compounds; Carbonyl scavenger; HaCat keratinocytes; CF3 fibroblasts; D-Penicillamine

1. Introduction

Tissue deterioration and aging have been widely associated with the accumulation of damage from chemical processes induced by oxidative stress, glycation, and UV-irradiation [1,2]. All of these are potent inducers of ROS and RCS, which are key intermediates of cumulative protein damage during general aging and several pathological conditions, e.g. chronic inflammatory diseases [3], psoriasis [4], and diabetes [5,6]. RCS, as reactive intermediates of cellular carbonyl stress, originate from a multitude of mechanistically related pathways, like glycation [7], sugar autoxidation [8], lipid peroxidation [9], and UV-photodamage [10] (Fig. 1). Glycation, the spontaneous amino-carbonyl reaction between reducing sugars and long-lived proteins, is a major source of RCS production, leading to cellular carbonyl stress. Reactive α-dicarbonyl intermediates, such as glyoxal, methylglyoxal, and 3-deoxyosones, are generated by both oxidative (glycoxidative) and non-oxidative reaction pathways of glycation [7]. The complex reaction sequence is initiated by the reversible formation of a Schiff base, which undergoes an Amadori rearrangement to form a relatively stable ketoamine

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Abbreviations: ADP-ribose, adenosine 5′-diphosphoribose; AGE, advanced glycation end product; AGE–BSA, BSA modified with advanced glycation end products; CML, N^{ε}-carboxymethyllysine; DTPA, diethylenetriamine-pentaacetic acid; GOLD, glyoxal–lysine dimer; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; MOLD, methylglyoxal–lysine dimer; NAC, N^{α}-acetyl-L-cysteine; RCS, reactive carbonyl species; ROS, reactive oxygen species.

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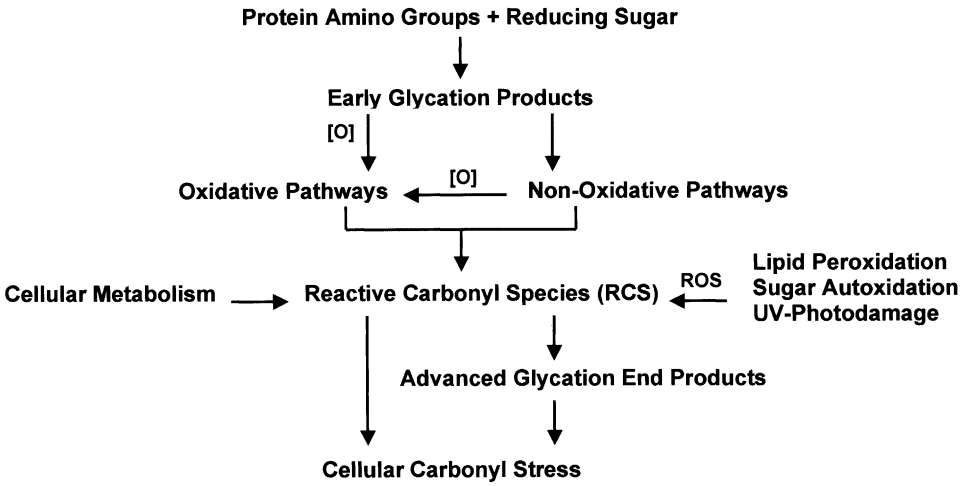

Fig. 1. Cellular carbonyl stress as a result of glycation, lipid peroxidation, sugar autoxidation, and metabolism. Oxygen-dependent and -independent pathways lead to the formation of various reactive carbonyl species, including α-dicarbonyls, as key intermediates for the accumulation of protein damage.

product during early glycation. A series of further reactions involving sugar fragmentation and the formation of α-dicarbonyl compounds as key reactive intermediates yields stable protein-bound advanced glycation end products (AGEs) [7,11,12]. Interestingly, RCS and AGEs can exert their detrimental cellular effects by increasing ROS production [13], thereby forming a vicious cycle of ROS and RCS production. AGE formation is accompanied by the accumulation of AGE-specific fluorescence (λ_{ex} = 370 nm, λ_{em} = 440 nm) and protein cross-linking, which are measures of overall protein damage in tissue [5]. The arginine-derived imidazolium AGE products [14], the glyoxal–lysine dimer (GOLD) and the methylglyoxal–lysine dimer (MOLD) [6], have been identified in aged human lens crystallin and skin collagen, implicating α-dicarbonyl stress in the aging of tissue. Additionally, RCS, like glyoxal, the direct precursor of AGE N^{ε}-carboxymethyllysine (CML), are generated by free radical damage to polyunsaturated fatty acids in cellular membranes [9]. UV-irradiation is another source of tissue carbonyl stress, as evidenced by the accumulation of CML in sun-exposed lesions of actinic elastosis [10]. Therefore, AGE products like CML and GOLD may be regarded as biomarkers of tissue carbonyl stress.

Methylglyoxal is an important glycation intermediate [7] that is also generated as a biological metabolite by non-enzymatic and enzymatic degradation of glycolytic triose phosphate intermediates and from threonine catabolism [15]. Increased levels of methylglyoxal are found in the blood from diabetic patients [16] and in the lens of streptozotocin-induced diabetic rats. A recent study on the formation of AGEs in endothelial cells cultured under hyperglycemic conditions indicated that methylglyoxal was the major precursor of AGEs [17]. Various methylglyoxal-derived AGEs have been identified in human tissues, such as fluorescent 5-methylimidazolone derivatives in atherosclerotic lesions of aorta [18], or MOLD and N^{ε}-carboxyethyl-L-lysine in aged skin collagen [6]. Recently, the cytotoxic effects of the glycation intermediates methylglyoxal and 3-deoxyglucosone on neuronal cells, such as PC12 cells [19] and cultured cortical neurons [20], have attracted considerable attention because of their suspected participation in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease [21] and amyotrophic lateral sclerosis [22].

As another result of oxidative and carbonyl stress, protein damage by carbonylation has been associated with aging and a number of diseases such as the premature aging diseases, progeria and Werner's syndrome [2]. The amount of carbonyl groups in human skin fibroblast proteins strongly correlates with the age of the donor [23]. Recently, elevated levels of histone H1 carbonylation *in vivo* as an indicator of nuclear oxidative and glycoxidative stress have been reported by our group [24].

A very limited number of inhibitors of cellular carbonyl stress have been identified to date, their therapeutic potential being realized only recently. Some inhibitors of glycation interfere with the reaction by trapping intermediate α-dicarbonyls, whereas other inhibitory substances act merely as antioxidants and transition metal chelators, thereby inhibiting advanced glycoxidation but not glycation [25]. Systemic administration of the hydrazine derivative and carbonyl reagent aminoguanidine, a member of the first class of glycation inhibitors, effectively suppresses secondary complications in diabetic rodents with experimental diabetes and inhibits skin collagen cross-linking [26,27]. Recently, a nucleophilic bidentate, phenylacylthiazolium bromide, has been shown to protect *Escherichia coli* against methylglyoxal toxicity [28]. Other nucleophilic compounds acting as carbonyl traps like tenilsetam [29], pyridoxamine [30], and the glyoxal-trapping biguanidine metformin [31] are being evaluated for prevention of secondary diabetic complications.

In vitro screening for potential α-dicarbonyl scavengers is complicated by the nature of most of the currently employed glycoxidative reaction systems, which measure

the suppression of oxygen-dependent AGE formation as assessed by AGE fluorescence or immunological quantification of specific AGEs like CML [25,29,31]. Consequently, in these glycoxidation systems AGE formation is inhibited effectively by compounds with antioxidant and metal-chelating activity. Recently, oxygen-independent advanced glycation by pentoses with formation of AGE fluorescence and protein cross-linking has been demonstrated and linked mechanistically to non-oxidative formation of deoxypentosones as reactive α-dicarbonyl intermediates [32]. Based upon our identification of an accelerated glycation reaction between the phosphate-substituted pentose adenosine 5′-diphosphoribose (ADP-ribose) and histone H1, which produces AGEs without involvement of oxygen [24], we developed an assay for the screening of glycation inhibitors acting as carbonyl scavengers. Among other test substances, nucleophilic thiol compounds were selected to screen for a promising pharmacophore with carbonyl scavenging activity. In our study, 3,3-dimethyl-D-cysteine (D-penicillamine) was identified as an effective inhibitor of non-oxidative advanced glycation, most probably acting as an α-dicarbonyl scavenger. The chemistry of the trapping reaction of the α-oxoaldehydes methylglyoxal and phenylglyoxal by D-penicillamine was investigated in detail. A cell culture model of protection against exposure to methylglyoxal and glyoxal was established to evaluate the potential usefulness of D-penicillamine and derivatives for protection of human skin against carbonyl stress.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from the Sigma. Calf tissue (thymus) frozen in liquid nitrogen immediately after collection was from Pel-Freez Biologicals.

2.2. Preparation of AGE–BSA

BSA modified with advanced glycation end products (AGE–BSA) was prepared as described by Takata *et al.* [33]. Briefly, 1.6 g of BSA and 3.0 g of D-glucose were dissolved in 10 mL of 0.5 M sodium phosphate buffer, pH 7.4, containing 0.05% NaN_3. The solution was filter sterilized through a 0.45 μm filter and incubated in the dark for 90 days at 37°. Following dialysis against water, the sample was lyophilized.

2.3. Isolation of histone H1 from calf thymus

All operations were carried out at 4°. Chromatin was isolated from fresh calf thymus by extraction with 0.14 M NaCl, 0.05 M $Na_2S_2O_5$, pH 5, as described earlier [24]. After repeated extraction with 5% $HClO_4$ and centrifugation (1500 *g*, 4°, 30 min), histone H1 was precipitated from the supernatant by the addition of trichloroacetic acid (TCA; 20% final concentration, v/v). The histone H1 precipitate was collected by centrifugation (12,000 *g*, 4°, 30 min) and resuspended in deionized water. After extensive dialysis (MW cut-off: 12,000–14,000) against water for 48 hr, the sample was lyophilized, and the protein was stored at 4°. SDS-PAGE (12%) was used to analyze the purity of the preparation.

2.4. Screening inhibitors of non-oxidative advanced glycation

For the development of a glycation inhibitor screen, the reaction mixture was prepared in a 1 mL reaction volume as described previously [24]. For screening purposes, the reaction was performed on a 96-well microtiter plate where each well contained 1.5 mg/mL of histone H1, 1 mM ADP-ribose in 50 mM KH_2PO_4 buffer, pH 7.4, 37° (containing 0.015% NaN_3 to inhibit microbial growth). The test compound was added from a concentrated solution to a final concentration range between 1 and 10 mM in a total reaction volume of 300 μL to duplicate wells. The microtiter plate was covered with a watertight plastic sheet to avoid loss of volume during the incubation period. The detected reaction parameter representing the accumulation of protein damage is AGE fluorescence ($\lambda_{ex} = 370$ nm; $\lambda_{em} = 440$ nm), which is suppressed by the presence of a compound with inhibitory activity. The plate was read on a conventional fluorescence microtiter plate reader with a filter setting approaching the above conditions (e.g. $\lambda_{ex} = 355$ nm; $\lambda_{em} = 405$ nm), which is in the range of the broad excitation/emission maxima of AGE compounds.

The following screening scheme, diagrammed in Fig. 2, was used: AGE fluorescence was determined at

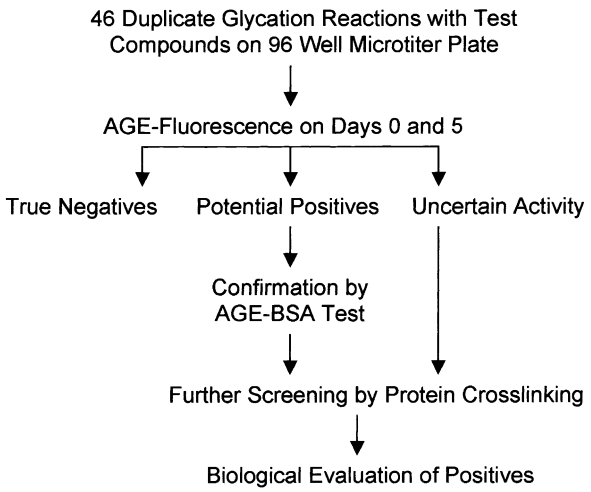

Fig. 2. Screening scheme for the rapid identification of inhibitors of non-oxidative advanced glycation on a 96-well microtiter plate.

the beginning and after 5 days of incubation. Test compounds that are inherently fluorescent (designated false negatives) were identified by the initial fluorescence measurement. Since these are compounds of uncertain activity, they were diverted directly to the second stage of the screen. The known glycation inhibitor aminoguanidine was used as a positive control for suppression of the increase in AGE fluorescence. After 5 days of incubation and plate reading, fluorescence quenchers (designated false positives) were excluded by measuring the quenching activity of the test compound by the addition of an AGE-modified protein having known fluorescence activity to one microtiter plate well containing test compound in the complete reaction mixture. For this, we used AGE–BSA (1 mg/mL), prepared as described above, as an AGE-type fluorescence standard. We refer to this as the AGE–BSA test for exclusion of false positive compounds that function by fluorescence quenching. If fluorescence quenching occurred, the test compound was excluded from further screening. Potential positive compounds were analyzed further by measuring inhibition of protein cross-linking by 12% SDS-PAGE analysis of a 3 μL aliquot taken from the reaction well on the plate. The protein was visualized by silver staining of the gel. Untreated histone H1 and the positive control containing aminoguanidine were loaded onto the gel together with the samples of potential positive test compounds. A compound that passed the first and the second stage of the screen was considered a glycation inhibitor and was evaluated further for biological activity as described below.

2.5. Fluorescence reading

Fluorescence measurements of 1 mL glycation reaction mixtures were performed using a Hitachi F-2000 fluorescence spectrophotometer at excitation/emission wavelengths of $\lambda_{ex} = 370$ nm/$\lambda_{em} = 440$ nm. Before fluorescence measurement, the protein sample was dialyzed extensively against water (MW cut-off 10,000), lyophilized, and reconstituted in reaction buffer. For inhibitor screening, the 96-well microtiter plate was read on a Fluoroskan II plate reader (Titertek, ICN) at excitation/emission wavelengths of $\lambda_{ex} = 355$ nm/$\lambda_{em} = 405$ nm (bandwidth 35 nm).

2.6. Preparation and structure elucidation of the D-penicillamine-methylglyoxal reaction product as 2-acetyl-5,5-dimethyl-thiazolidine-4-carboxylic acid

To a solution of D-penicillamine (350 mg, 2.3 mmol) in 50 mL of aqueous 0.20 M phosphate buffer (pH 7.4) was added methylglyoxal (40% in H_2O/620 μL, 3.45 mmol). The reaction mixture was stirred at 37° for 24 hr. The solvent was concentrated to half volume at reduced pressure, and the residue was desalted on Amberchrome CG 71 ms resin (1.5 cm × 45 cm) (TosoHaas). The column was developed with water. The UV absorbing peaks were pooled, and the water was evaporated at reduced pressure. The crude product was purified by anion exchange chromatography on a 1.5 cm × 45 cm column of QAE Sephadex 25 (Sigma), developed by application of a linear gradient formed between 200 mL of distilled water and 200 mL of 0.2 M NH_4HCO_3. Fractions were collected, and absorbance at 254 nm was measured. Fractions constituting a single major peak eluting about midway in the gradient were pooled and concentrated.

The ^{1}H NMR spectrum exhibited the following signals [δ_H (D_2O) in ppm]: 1.03 (3H, s, CH_3), 1.05 (3H, s, CH_3), 1.90 (3H, s, CO-CH_3), 3.96 and 3.98 (diastereomeric, 1H, s, CH-COOH). Mass spectrometric analysis by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) revealed an $[M + Na]^+$ of 226 Da.

2.7. Preparation and structure elucidation of the D-penicillamine-phenylglyoxal reaction product as 2-benzoyl-5,5-dimethyl-thiazolidine-4-carboxylic acid

Phenylglyoxal (final concentration 10 mM) and D-penicillamine (final concentration 20 mM) were reacted in 50 mM KH_2PO_4 buffer, pH 7.4, at room temperature. The progress of the reaction was monitored by HPLC analysis of reaction aliquots at 254 nm. After 40 min of reaction time, more than 90% conversion of the phenylglyoxal peak into a single product peak of higher retention time was observed. The reaction product was obtained by preparative HPLC, lyophilized, and analyzed by ^{1}H NMR spectroscopy.

The ^{1}H NMR spectrum of the compound exhibited the following signals: [δ_H (D_2O) in ppm]: 1.38 (3H, s, CH_3), 1.45 (3H, s, CH_3), 4.12 (1H, s, CH-COOH), 7.42–7.82 (5H, m, ArH).

2.8. Reaction kinetics of α-dicarbonyl trapping

The reactions of phenylglyoxal with test compounds were carried out in 10 mM phosphate buffer, pH 7.4, at 37° and were followed by HPLC analysis. The reaction kinetics were studied at a phenylglyoxal concentration of 50 μM and at 250 and 500 μM carbonyl scavenger concentrations (D-penicillamine, aminoguanidine). Over the course of the reaction, aliquots were analyzed by HPLC. In the case of D-penicillamine, which required shorter sampling periods, reaction aliquots were taken every 20 s, and kept on dry ice until analysis. The initial reaction rates of phenylglyoxal with the test compounds were monitored by following the disappearance of phenylglyoxal over time. The reaction between phenylglyoxal and the test compounds is a second-order reaction with a rate equation of $-dc/dt = k_{2nd}$ [phenylglyoxal] [dicarbonyl scavenger]. The reaction was conducted in the presence of excess test compounds (ratio of 1:5 and 1:10 phenylglyoxal to test compound), to

convert it to pseudo first-order reaction kinetics as demonstrated by the apparent dependency of the reaction rate constant (k_{1st}) on the concentration of the test compound (data not shown). A plot of log area under the curve (AUC) for phenylglyoxal vs. time resulted in a slope equal to $k_{1st}/2.303$. The measured first-order rate constant (k_{1st}) was then used to calculate the second-order rate constant (k_{2nd}) by applying the following equation: $k_{2nd} = k_{1st}$ [α-dicarbonyl scavenger]. The calculated second-order rate constants determined at two reactant ratios (5:1 and 10:1) were in good agreement.

2.9. Cell culture

A continuous cell line of human epidermal keratinocytes (HaCat cells) and human dermal fibroblasts (CF3 cells) were routinely cultured in 75 cm^2 flasks, split biweekly in DMEM containing 10% fetal bovine serum, and kept in a humidified atmosphere containing 5% CO_2 at 37°. Human keratinocytes were split using 5% trypsin, and for the human fibroblasts 1% trypsin was employed. All experiments were carried out on 6-well dishes (Falcon), where keratinocytes were seeded at 2×10^4 cells per well and fibroblasts at 4×10^4 cells per well. Cells were left overnight to attach to the plate, and the appropriate carbonyl scavenger was then added 15 min prior to the addition of the α-dicarbonyl stress compounds glyoxal or methylglyoxal. Following a 72 hr exposure to the α-dicarbonyl stress, cells were counted using a Coulter counter. The protective effects of the α-dicarbonyl scavengers were assessed by comparing the growth of untreated cells with cells exposed to α-dicarbonyl stress ± test compound.

2.10. HPLC systems

For preparative HPLC of the D-penicillamine-phenylglyoxal reaction product, a C4-Rainin-Microsorb column (4.6 mm × 250 mm, 300 Å, 5 μm particle size) from Varian was used with a gradient elution. Running solution A was 0.1% trifluoroacetic acid (TFA) and running solution B was 50% acetonitrile, 50% water (0.1% TFA). The gradient was 100% A to 100% B over 30 min, 100% B for 15 min, back to 100% A over 5 min, and A for 5 min. The flow rate was 1 mL/min, and the elution was monitored at 254 nm. For kinetic studies of phenylglyoxal trapping, the same column as above was operated with a mobile phase of 20% acetonitrile and 80% water (0.1% TFA) and a flow rate of 1 mL/min. The eluate was monitored by UV detection at 254 nm.

2.11. Mass spectrometry

Mass spectrometry was performed using a Kratos Kompact Seq MALDI-TOF-MS. Spectra were recorded in positive ion mode in linear configuration using α-cyano-4-hydroxycinnamic acid as the matrix.

2.12. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded in D_2O on a Varian Gemini-200 (200 MHz) spectrometer.

3. Results

3.1. Development of a screening assay for inhibitors of non-oxidative advanced glycation

Based on our discovery of a rapid and potent nuclear glycation reaction between the pentose ADP-ribose and the lysine-rich histone H1 that proceeds *in vitro* via non-oxidative pathways, we have developed a rapid, simple, and sensitive assay for inhibitors of non-oxidative advanced protein glycation. After 5 days of incubation, the reaction between H1 and ADP-ribose, performed in 1 mL of reaction volume, produced an AGE fluorescence signal about 20 times greater than background fluorescence represented by histone H1 incubated in the absence of sugar (Fig. 3). The sugar incubated in the absence of protein did not produce AGE fluorescence. A strong fluorescence signal also was observed when the reaction was performed in a 300 μL volume on a 96-well microtiter plate (Table 1). The reaction in the presence of the transition metal ion chelator diethylenetriamine-pentaacetic acid (DTPA) (5 mM) and under argon to ensure antioxidative reaction conditions produced the same fluorescence increase as in the presence of oxygen (Table 1). However, this increase was fully suppressed by the presence of the standard glycation inhibitor and α-dicarbonyl scavenger aminoguanidine (1–10 mM) (Table 1 and Fig. 3). Therefore,

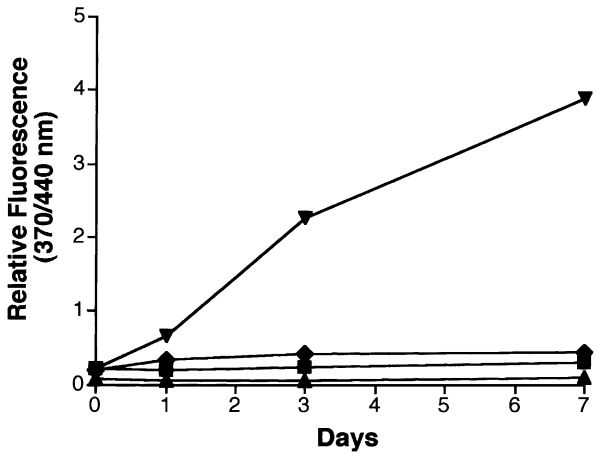

Fig. 3. Formation of AGE fluorescence on histone H1 at pH 7.4. Reaction mixtures in phosphate buffer, pH 7.4, were analyzed for the generation of AGE-type fluorescence. H1 and ADP-ribose, after dialysis, lyophilization, and reconstitution in phosphate buffer, pH 7.4 (▼); H1 alone (■); ADP-ribose alone (▲); and H1, ADP-ribose, and 5 mM aminoguanidine, after dialysis, lyophilization, and reconstitution in phosphate buffer, pH 7.4 (◆). A representative experiment out of six separate experiments is shown.

Table 1
Screening of inhibitors of non-oxidative advanced glycation: AGE fluorescence on 96-well microtiter plate

Sample	AGE fluorescence[a] (day 0)	AGE fluorescence[a] (day 5)	AGE–BSA test[a,b]
Histone H1 blank	1.1 (0.0)	1.5 (0.2)	
Complete reaction			
-Under argon (+5 mM DTPA)	1.1 (0.1)	23 (1.1)	32
-Under air	1.1 (0.1)	21 (0.9)	30
+Compound			
Aminoguanidine (mM)			
1	1.2 (0.1)	4.3 (0.2)	
5	1.2 (0.1)	2.0 (0.0)	
10	1.3 (0.1)	1.8 (0.0)	10
Rutin (μM)			
200	1.0 (0.0)	3.0 (0.1)	5.7
NADH (mM)			
5	52 (0.3)	31 (0.0)	
L-Cys-Gly (mM)			
1	1.3 (0.3)	17 (0.0)	
5	1.2 (0.2)	26 (0.4)	
10	1.2 (0.1)	40 (0.0)	
GSH (mM)			
1	1.3 (0.0)	19 (0.3)	
5	1.1 (0.3)	17 (0.3)	
10	1.1 (0.0)	14 (0.5)	
l-Cys (mM)			
1	1.2 (0.1)	14 (0.3)	
5	1.1 (0.1)	11 (0.2)	
10	1.2 (0.1)	9.0 (0.2)	
l-Cys-OMe (mM)			
1	1.2 (0.1)	15 (0.1)	
5	1.3 (0.1)	12 (0.8)	
10	1.2 (0.1)	9.0 (0.2)	
NAC (mM)			
1	1.2 (0.2)	11 (0.6)	
5	1.0 (0.1)	10 (0.4)	
10	1.1 (0.1)	9.4 (0.6)	
d,L-Homocysteine (mM)			
1	1.3 (0.0)	16 (1.1)	
5	1.2 (0.2)	22 (0.1)	
10	1.1 (0.1)	27 (1.7)	
Cysteamine (mM)			
1	1.0 (0.1)	10 (0.6)	
5	1.1 (0.1)	9.0 (0.7)	
10	1.2 (0.2)	6.0 (0.3)	
d,L-Penicillamine (mM)			
1	1.1 (0.2)	13 (0.0)	
5	1.1 (0.1)	2.7 (0.0)	
10	1.1 (0.1)	1.7 (0.0)	15
d-Penicillamine (mM)			
1	1.2 (0.0)	11 (0.6)	
5	1.1 (0.1)	2.9 (0.3)	
10	1.1 (0.0)	1.4 (0.1)	12
2-Thiobarbituric acid (mM)			
1	1.1 (0.1)	12 (0.4)	
5	1.2 (0.1)	5.5 (0.4)	
10	1.3 (0.1)	2.3 (0.0)	12
l-Ergothioneine (mM)			
1	1.0 (0.1)	13 (0.0)	
5	1.2 (0.0)	9.3 (0.2)	
10	1.2 (0.1)	7.4 (0.1)	
Thiourea (mM)			
1	1.1 (0.1)	18 (0.1)	
5	1.1 (0.1)	15 (0.0)	
10	1.1 (0.2)	11 (0.3)	

[a] Fluorescence in relative units (±range, $n = 2$).
[b] AGE–BSA test was performed as indicated in Section 2.

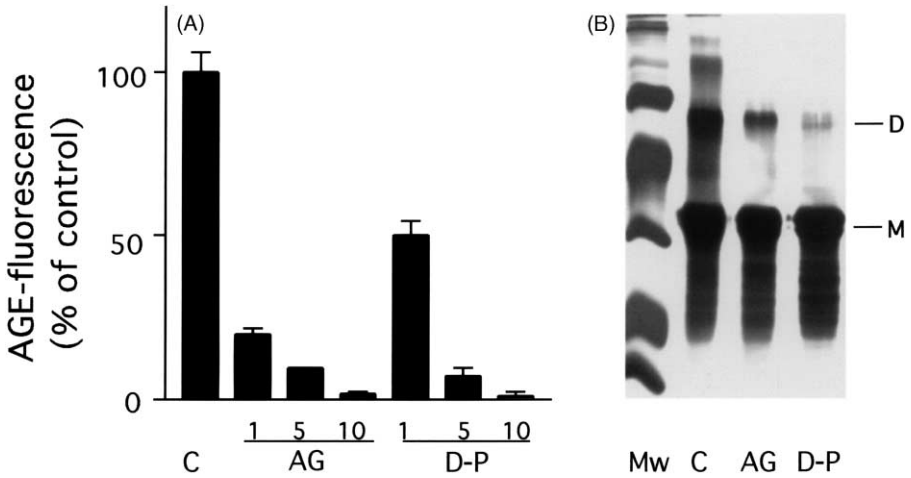

Fig. 4. Aminoguanidine and D-penicillamine as inhibitors of non-oxidative advanced glycation of histone H1 by ADP-ribose. Panel A: after 5 days of incubation on a 96-well microtiter plate, reaction mixtures in phosphate buffer, pH 7.4, were analyzed for the generation of AGE-type fluorescence: (C) control reaction between H1 and ADP-ribose; (AG) reaction in the presence of aminoguanidine (1–10 mM); and (D-P) reaction in the presence of D-penicillamine (1–10 mM). Values represent the means ± SD of three measurements. Panel B: analysis of protein cross-linking was performed with reaction aliquots by 12% SDS-PAGE with subsequent silver staining. Inhibitor concentration was 5 mM: (MW) molecular weight standard (kDa, from low to high): 14.3, 18.4, 29, 43, 68, 97.4, and 200. M and D refer to the migration position of histone H1 monomer and dimer, respectively.

aminoguanidine was selected as a positive control in the screening assay. Test substances, which substantially suppressed the increase of AGE-specific fluorescence on the microtiter plate were designated ''potential positives''. As indicated for the positive control aminoguanidine, the AGE–BSA test was performed on ''potential positives'' to exclude false positive activity due to quenching of AGE-type fluorescence (Table 1; see ''Section 2'' for a description of the AGE–BSA test). The second phase of the screen assay tested for suppression of protein cross-linking as a consequence of AGE formation by potential positives. A reaction aliquot of the day 5 glycation incubation was assessed by 12% SDS-PAGE analysis (Fig. 4). Consistent with our previous report [24], we observed that histone cross-linking by ADP-ribose occurred equally under anaerobic conditions with metal chelation (5 mM DTPA) as well as under air (data not shown). Therefore, we conclude that histone cross-linking is a consequence of non-oxidative advanced glycation. The presence of aminoguanidine (5 mM) clearly inhibited glycation-induced cross-linking of histone H1 (Fig. 4B). After establishing the feasibility of screening large numbers of samples on microtiter plates using the above-described histone H1/ADP-ribose glycation system, a primary screen of 15 different compounds in various concentrations (1–10 mM) was performed, as shown in Table 1. Fluorescence measurements on day 0 detected NADH as a strongly fluorescent substance (false negative), but examined in the secondary screen, it showed no inhibitory activity on protein cross-linking (data not shown). The flavonoid rutin was identified as a false positive because it quenched the fluorescence of AGE–BSA when added to the test well at the end of the incubation (Table 1, AGE–BSA test), and did not inhibit histone cross-linking (data not shown).

3.2. Identification of D-penicillamine as a potent inhibitor of non-oxidative advanced glycation in vitro

Nucleophilic compounds are promising candidates as inhibitors of advanced glycation due to their potential to scavenge electrophilic α-dicarbonyl intermediates [28]. Several thiol compounds tested showed clear inhibitory activity in our screen. However, even at the highest concentration tested (10 mM), L-cysteine-*O*-methylester (L-Cys-OMe), L-cysteine (L-Cys), and N^{α}-acetyl-L-cysteine (NAC) achieved only 50% inhibition in the screen. Somewhat better inhibition was observed with the thiol cysteamine and the mercaptoimidazole derivative L-ergothioneine. In contrast, D,L-homocysteine and the dipeptide NH_2-L-Cys-Gly-COOH increased AGE fluorescence above control most probably by undergoing glycation reactions themselves. The enhanced production of fluorescent AGEs of unknown reactivity by these substances excluded them from further screening. Apart from 2-thiobarbituric acid, which was not examined further at this point, D-penicillamine and its racemic mixture were clearly the only thiol compounds tested that showed strong suppression of AGE fluorescence (5–10 mM, Fig. 4A) comparable to that of aminoguanidine. False positive activity due to fluorescence quenching was excluded by the AGE–BSA test (Table 1). Therefore, the potential positive test compound D-penicillamine was subsequently assessed for inhibition of glycation-induced cross-linking of histone H1 (Fig. 4B). Inhibition of glycation-induced cross-linking of histone H1 by D-penicillamine was clearly more potent than by the standard inhibitor aminoguanidine (5 mM each). As a result of screening numerous compounds, 1-amino-2-mercapto-2,2-dimethylethane was identified as a putative pharmacophore for

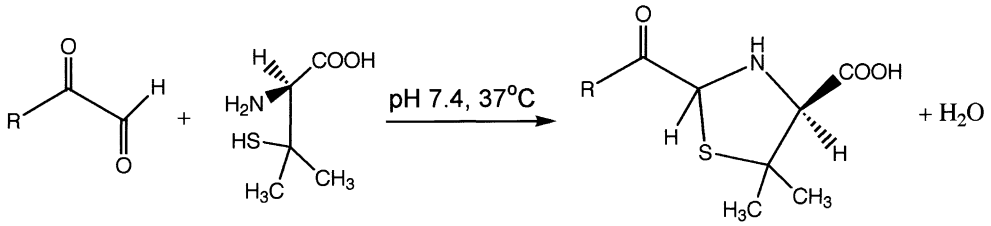

Fig. 5. Trapping of α-oxoaldehydes by D-penicillamine via 2-acylthiazolidine formation. Reaction of D-penicillamine with phenylglyoxal (R = C_6H_5) or methylglyoxal (R = CH_3) under conditions of physiological temperature and pH rapidly converts the α-dicarbonyl species into a stable diastereomeric thiazolidine derivative.

full inhibitory activity on non-oxidative glycation of histone H1 by ADP-ribose.

3.3. Irreversible trapping of α-dicarbonyl compounds by D-penicillamine

The strong inhibitory activity of D-penicillamine on the development of AGE fluorescence and protein cross-linking suggested a scavenging activity towards RCS as key intermediates of non-oxidative advanced glycation [7,32,34]. Therefore, chemical reactivity towards selected α-dicarbonyl compounds under conditions of physiological pH and temperature was investigated. Methylglyoxal and phenylglyoxal readily reacted with D-penicillamine under these conditions. The homogeneous reaction product between methylglyoxal and D-penicillamine was isolated and subjected to analysis by NMR and mass spectrometry. Mass spectrometric analysis by MALDI-TOF-MS revealed a molecular mass $[M + Na]^+$ of 226 Da compatible with the calculated mass for $C_8H_{13}NO_3S$ of 203.26 Da. The aldehyde–thiazolidine adduct formed presumably after nucleophilic ring closure of the initially formed Schiff base (Fig. 5). Consistently, all ^1H NMR signals could be assigned to 2-acetyl-5,5-dimethyl-thiazolidine-4-carboxylic acid as the structure of the reaction product, with the exception of the missing signal of the very acidic proton in position 2 of the thiazolidine ring, which probably exchanges with D_2O. No aldehyde proton was detected excluding formation of the isomeric thiazolidine at C-2 of methylglyoxal. Moreover, ^{13}C NMR analysis of this compound clearly indicated two carbonyl groups at $\delta =$ 199 ppm ($CH_3C{=}O$) and $\delta = 209$ ppm (COOH). ^{13}C NMR analysis of the homogeneous phenylglyoxal–D-penicillamine adduct isolated by preparative HPLC revealed analogous signals of the expected thiazolidine derivative with five proton signals in the aromatic region. Again, the signal of the acidic proton in position 2 of the thiazolidine ring was not detected due to the expected exchange with D_2O, and no aldehyde proton was observed. Therefore, the structure of the phenylglyoxal–D-penicillamine adduct was tentatively assigned as being 2-benzoyl-5,5-dimethyl-thiazolidine-4-carboxylic acid. In conclusion, α-dicarbonyl trapping by 2-acyl-5,5-dialkyl-thiazolidine formation as a mechanism of inhibition of non-oxidative advanced glycation may explain the efficacy of inhibition of D-penicillamine relative to other cysteine derivatives. The 5,5-dialkyl substitution may sterically favor closure of the thiazolidine ring and prevent the reverse reaction by hydrolysis.

3.4. Comparative reaction kinetics of α-dicarbonyl trapping by aminoguanidine and D-penicillamine

The strong inhibitory effect of D-penicillamine on advanced non-oxidative glycation and the fast formation of thiazolidine derivatives with α-oxoaldehydes were taken as evidence that D-penicillamine acts as a potent α-dicarbonyl scavenger *in vitro*. To further define the strong reactivity of D-penicillamine as compared to the known α-dicarbonyl scavenger aminoguanidine, comparative reaction kinetics of α-dicarbonyl trapping were performed. Using phenylglyoxal as an UV-active α-oxoaldehyde compound, the kinetics of α-dicarbonyl scavenging by D-penicillamine and aminoguanidine were determined (Fig. 6A). Among the test compounds, D-penicillamine clearly was the most rapid α-dicarbonyl trapping agent, derivatizing phenylglyoxal more than 60 times faster than aminoguanidine. Trapping by L-cysteine occurred with a rate comparable to aminoguanidine [k_{2nd} (L-cysteine) $= 0.63 \pm 0.04$/M s]. To determine the influence of the α-oxo-substitution on the progress of the aldehyde-trapping reaction, an additional analysis was undertaken comparing the reactivity of D-penicillamine towards an α-oxoaldehyde compound with the analogous aldehyde. For this goal, the second-order rate constant for the reaction of D-penicillamine with phenylacetaldehyde was determined (Fig. 6B). D-Penicillamine trapped phenylacetaldehyde approximately 14 times less efficiently ($k_{2nd} = 1.83$) than phenylglyoxal. Thus, we conclude that D-penicillamine traps α-oxocarbonyl compounds more effectively than the analogous aldehydes.

3.5. D-Penicillamine protection of cultured skin cells from glyoxal- and methylglyoxal-induced toxicity

The cellular toxicity of α-dicarbonyl compounds like glyoxal and methylglyoxal has been well established in various neuronal cells [20,22] and macrophage-derived cell lines [35]. We were interested in assessing α-dicarbonyl toxicity and protection by α-dicarbonyl scavengers

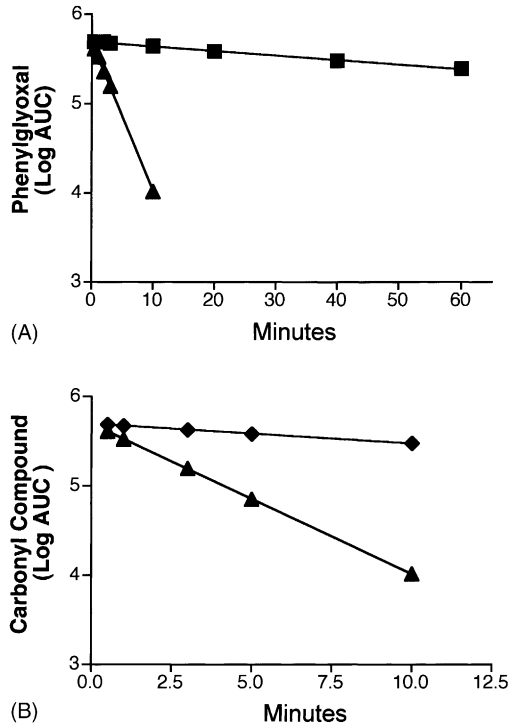

Fig. 6. Comparative reaction kinetics of α-oxoaldehyde scavenging by D-penicillamine and aminoguanidine. Panel A: scavenging of phenylglyoxal as a model α-oxoaldehyde with D-penicillamine (▲) as compared to aminoguanidine (■). The second-order rate constants (k_{2nd}) were calculated from the observed pseudo first-order rate constants that were obtained from the slope of the curves indicating the time course of the disappearance of phenylglyoxal as measured by the AUC during the reaction with the trapping reagent: k_{2nd} (D-penicillamine) = 24.8 ± 1.3/M s); k_{2nd} (aminoguanidine) = 0.4 ± 0.01/M s. Panel B: comparative reaction kinetics of mono-oxoaldehyde (phenylacetaldehyde, ◆) vs. α-oxoaldehyde compound (phenylglyoxal, ▲) scavenging by D-penicillamine: k_{2nd} (phenylacetaldehyde) = 1.83 ± 0.05/M s; k_{2nd} (phenylglyoxal) = 24.8 ± 1.3/M s. Values represent the means ± SD (error bars are within the symbols) of four measurements.

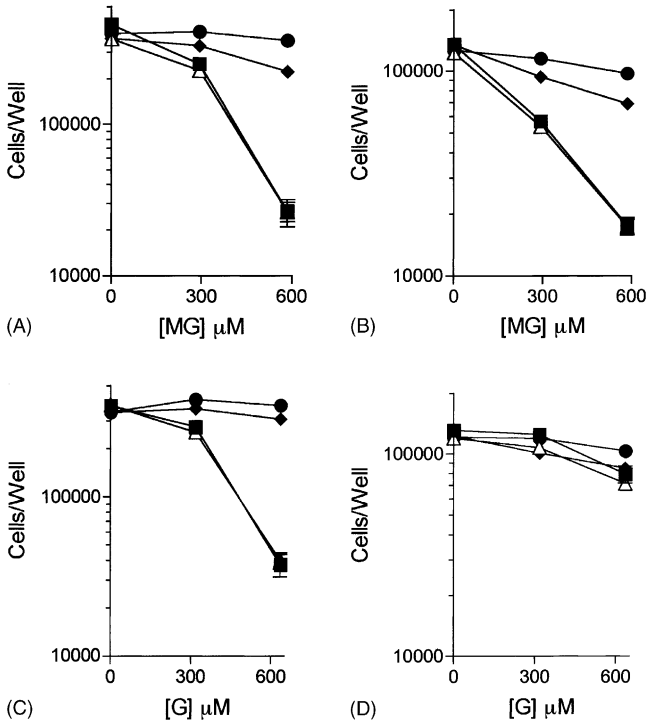

Fig. 7. Protection of human epidermal HaCat keratinocytes and human dermal CF3 fibroblasts from α-dicarbonyl stress by aminoguanidine and D-penicillamine. Protection of HaCat keratinocytes (panels A and C) and CF3 fibroblasts (panels B and D) against methylglyoxal (MG)-induced (panels A and B) and glyoxal (G)-induced (panels C and D) carbonyl stress: (■) α-dicarbonyl alone; (△) l-alanine (1 mM); (◆) aminoguanidine (1 mM); and (●) D-penicillamine (1 mM). Values represent the means ± SD of three samples.

using two types of human skin cell culture models (Fig. 7). First, human epidermal HaCat keratinocytes (Fig. 7, A and C) and human dermal CF3 fibroblasts (Fig. 7, B and D) were exposed to increasing concentrations of methylglyoxal (panels A and B) or glyoxal (panels C and D) in the absence or presence of aminoguanidine or D-penicillamine as α-dicarbonyl scavengers (1 mM each). The effectiveness of the test compounds as α-dicarbonyl scavengers *in vivo* was assessed by their protective effects on cell growth. L-Alanine was chosen as a negative control, as this compound is not expected to scavenge RCS. No significant cellular toxicity was observed with the test compounds alone during a 72 hr exposure to either keratinocytes or fibroblasts. Increasing concentrations of methylglyoxal resulted in a concentration-dependent growth inhibition. Concentrations of 600 μM were completely growth inhibitory in both cell lines. Glyoxal treatment had effects on keratinocytes similar to those of methylglyoxal, whereas fibroblasts were less sensitive towards glyoxal. As predicted, co-incubation of α-dicarbonyls with L-alanine showed no protective effects. Although aminoguanidine partly rescued both keratinocytes and fibroblasts from methylglyoxal-induced growth inhibition, D-penicillamine had superior protective effects and almost totally blocked the toxic effects of methylglyoxal. Aminoguanidine and D-penicillamine equally

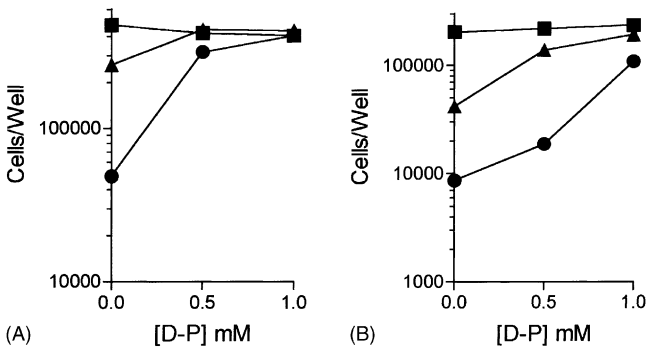

Fig. 8. Protection of HaCat keratinocytes and CF3 fibroblasts from methylglyoxal-induced α-dicarbonyl stress by D-penicillamine. Protection of HaCat keratinocytes (panel A) and CF3 fibroblasts (panel B) by increasing concentrations of D-penicillamine (D-P) against methylglyoxal-induced carbonyl stress: (■) no methylglyoxal; (▲) methylglyoxal (300 μM); and (●) methylglyoxal (600 μM). Values represent the means ± SD (error bars are within the symbols) of three samples.

protected keratinocytes against growth inhibition by glyoxal. The protective effect is most likely due to direct chemical scavenging of the toxic RCS, because pre-incubation of cells with aminoguanidine or D-penicillamine for 24 hr followed by exposure to the α-dicarbonyl in the absence of either compound did not show any protective effect (data not shown). Next, a concentration-range effect of the cytoprotection against methylglyoxal by D-penicillamine was investigated (Fig. 8). Increasing concentrations of D-penicillamine resulted in increased protection in both cell types. However, as opposed to keratinocytes (Fig. 8A), fibroblasts (Fig. 8B) were more sensitive to methylglyoxal toxicity. Further, D-penicillamine (1 mM) effected only 47% survival in fibroblasts treated with 600 μM methylglyoxal, whereas in keratinocytes we observed 100% cell survival under the same conditions.

4. Discussion

Carbonyl stress is an important mechanism of tissue deterioration in several pathological conditions like diabetes [5], atherosclerosis [34], uremia [36], dialysis-related amyloidosis [37], and general aging [2]. Therefore, compounds acting as RCS scavengers may reduce detrimental effects of processes such as oxidative stress and glycation, which are mediated, in part, by production of RCS, especially α-dicarbonyls [7,32,34]. A microtiter plate assay was designed combining a rapid glycation system with a simple way to assess the progress or inhibition of the reaction in the presence of a potential inhibitor by measuring the generation of AGE-specific fluorescence as the primary screen. Accumulation of AGE fluorescence and protein cross-linking of histone H1 have been established as valid markers of protein damage due to non-oxidative advanced glycation in this system [24]. Screening for compounds acting as antioxidants and inhibitors of glycoxidation is excluded because this unique glycation system has been shown to proceed independent of the presence of oxygen and transition metal ions. Non-oxidative advanced glycation by ADP-ribose is consistent with the recent finding that oxygen is not required for the browning and cross-linking of RNase A by pentoses [32]. Different reagents including the metal ion chelator DTPA under argon, the hydroxyl radical scavenger mannitol, and the presence of catalase for the removal of traces of hydrogen peroxide did not interfere with the formation of AGE fluorescence and histone cross-linking by ADP-ribose [24]. Only the α-dicarbonyl scavenger aminoguanidine blocked AGE fluorescence and cross-linking in this ADP-ribose-dependent glycation system. To observe inhibition of the strong glycation activity of ADP-ribose at the millimolar test concentration chosen to create a rapid glycation reaction, millimolar concentrations of inhibitory test compounds were required. Although aminoguanidine as a hydrazine derivative shows an unfavorable toxicity profile *in vivo*, it was chosen as a reference inhibitor of glycation by α-dicarbonyl scavenging [26,38]. Preliminary experiments indicated that nucleophilic monoamines (such as carnosine) and polyamines (such as spermine) did not suppress formation of AGE fluorescence or histone cross-linking in our screening system (data not shown), most probably undergoing preferential glycation themselves. In this study, we focused our screening efforts on thiol compounds as another class of nucleophilic agents expected to interfere with glycation. L-Cysteine has been reported to protect against acetaldehyde toxicity *in vivo* by its carbonyl scavenging activity [39], but its cellular toxicity [40] and rapid catabolism limit its usefulness *in vivo*. NAC acts as an antioxidant and GSH precursor *in vivo* [41], but proved to be less effective than L-cysteine as an inhibitor of non-oxidative advanced glycation *in vitro*. The decarboxylated cysteine derivative cysteamine showed improved inhibitory activity, whereas D,L-homocysteine was not effective. These data indicated the requirement of an α-amino-β-mercaptoethane structure for the highest degree of inhibitory activity. Additionally, differential susceptibility towards autoxidative degradation and inactivation of thiol compounds may contribute to the observed differences in inhibitory activity, and compounds unstable under the conditions of the assay will be screened out as negatives with little potential for therapeutical development. Among the test compounds, penicillamine, either racemic or the therapeutically approved D-enantiomer, was the most potent compound, inhibiting both AGE fluorescence and histone cross-linking. The inhibitory effects of D-penicillamine on oxygen-dependent glycation damage by D-glucose [27] and D-fructose *in vitro* [42] have been ascribed to its role as a metal chelator and antioxidant. Consistently, D-penicillamine inhibited glycoxidative damage of tail tendon collagen after 28 days of implantation into the peritoneal cavity of streptozotocin-diabetic rats [25]. As opposed to action as a reducing agent or metal chelator, the inhibitory effects of D-penicillamine on non-oxidative advanced glycation as observed in the reaction between ADP-ribose and histone H1 may be linked mechanistically to its very strong reactivity towards α-dicarbonyls as the key reactive intermediates of both glycation and lipid peroxidation reactions. In a model reaction under conditions of physiological pH and temperature using methylglyoxal or phenylglyoxal as RCS, D-penicillamine readily reacted with the aldehyde group forming thiazolidine derivatives, thereby inactivating these α-dicarbonyl structures. Much work has been dedicated to elucidating the complex kinetics and mechanism of the reaction of NAC [43], aminoguanidine [38], and metformin [31] with several α-dicarbonyl compounds under physiological conditions. In a simplified approach, the comparative kinetics of the phenylglyoxal scavenging activity of aminoguanidine and D-penicillamine revealed a more efficacious scavenging activity of the latter under conditions of physiological

pH *in vitro*. Interestingly, D-penicillamine trapped phenylglyoxal much faster than the corresponding aldehyde, phenylacetaldehyde, a finding that may be explained by the expected higher electrophilicity of the α-dicarbonyl compound. The α-dicarbonyl adduct is stable in water, whereas the monocarbonyl-derived thiazolidine adducts form reversibly with subsequent release of the aldehyde [44]. Consistently, *N*,*S*-isopropylidene-D-penicillamine, the acetone adduct of D-penicillamine, was almost as equally effective as D-penicillamine when tested in our *in vitro* screen (data not shown). In conclusion, the α-amino-β-mercapto-β,β-dimethylethane pharmacophore was identified as a structural requirement for rapid α-dicarbonyl scavenging by 2-acyl-5,5-dimethyl-thiazolidine formation. D-Penicillamine is a drug approved for systemic administration in copper storage disease (Wilson's disease), cystinuria, heavy metal ion intoxication, and as a second-order medication for rheumatoid arthritis [45]. As a very potent Cu-ion chelator, it acts as an antioxidant presumably by formation of several stable Cu(I) or Cu(II)/penicillamine complexes that are inactive towards the Fenton reaction [46] and exhibit superoxide dismutase (SOD) activity [47]. D-Penicillamine has been shown to interfere with collagen biosynthesis by reversible thiazolidine formation with lysine and hydroxylysine aldehyde groups, thereby inhibiting collagen maturation by cross-linking [44]. Although severe systemic toxicity upon long-term administration has been described in patients taking high doses of D-penicillamine [45], the preferential and irreversible reactivity towards electrophilic α-dicarbonyls and the expected micromolar tissue concentrations of the target α-dicarbonyls [16] may qualify this drug or derivatives as protective agents against carbonyl stress.

Skin deterioration and aging have been widely associated with the accumulation of damage by chemical processes involving ROS and RCS, the most important being oxidative stress, solar light, and glycation [48]. Consistently, increased levels of oxidized proteins, glycated proteins, and proteins modified by the lipid peroxidation product 4-hydroxy-2-nonenal were observed in lysates of epidermal cells from donors with increasing age [49]. Moreover, the CML levels of human skin elastin in sun-exposed areas are significantly higher than those in areas not exposed to the sun [10]. This is explained by increased UV-induced oxidative stress leading to formation of the RCS glyoxal, the direct precursor of CML, from lipid peroxidation and/or glycation. Moreover, oxidative keratin damage in the stratum corneum was demonstrated leading to an epidermal keratin oxidation gradient in normal human skin [48]. Therefore, protection of epidermal and dermal types of skin cells by D-penicillamine and aminoguanidine against α-dicarbonyl toxicity as demonstrated in our study may be of therapeutic potential. D-Penicillamine and aminoguanidine were well tolerated and clearly protected both types of skin cells against the toxic action of methylglyoxal. D-Penicillamine was more protective than aminoguanidine and protected skin cells in a concentration-dependent manner. Fibroblasts were generally more sensitive towards methylglyoxal toxicity than keratinocytes. As suggested by experiments in which methylglyoxal and D-penicillamine or aminoguanidine were co-incubated prior to exposure to cells, methylglyoxal was most probably detoxified by direct chemical trapping. This mechanism is consistent with an earlier report describing prevention of 3-deoxyglucosone neurotoxicity by co-incubation with aminoguanidine [20]. More complex mechanisms, however, such as elevation of intracellular GSH for the enzymatic detoxification of methylglyoxal by the glyoxalase system, may be partly involved in the action of D-penicillamine [50].

α-Dicarbonyl compounds as key reactive intermediates of cellular carbonyl stress may be important targets for therapeutic intervention in pathological conditions involving enhanced carbonyl stress. The screening method presented here will allow further optimization of α-amino-β-mercapto-β,β-dimethyl-ethane as a lead pharmacophore for α-dicarbonyl scavengers acting by formation of stable 2-acyl-5,5-dialkyl-thiazolidine adducts as a new class of therapeutic agents. Furthermore, our approach should be useful for the identification of new chemical entities as potential carbonyl trapping agents for cellular protection in disease states that involve carbonyl stress.

Acknowledgments

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