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The Butter Flavorant, Diacetyl, Forms a Covalent Adduct with 2-Deoxyguanosine, Uncoils DNA, and Leads to Cell Death

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ABSTRACT: Diacetyl (DA), a natural butter flavorant, is a causative agent for the lung disease obliterative bronchiolitis. Mutagenic properties of 1,2-dicarbonyls have previously been empirically linked to their possible interaction with DNA nucleobases. This study for the first time identifies chemically the adduct of DA with 2-deoxyguanosine. Selective reactivity of DA with 5′-TTTGTTTTT-3′ over 5′-TTTTTTTTT-3′ indicated its propensity to modify specifically the guanosine residue. Treatment of plasmid DNA, pBR322, with DA induced changes in electrophoretic mobility that are typical of ternary structure disruption. Such DNA nucleobase interaction of DA translated into increased apoptosis in DA-treated SH-SY5Y cells in a dose-dependent manner (IC_{50} = 0.114 ± 0.0421 mM). The traditional carbonyl scavengers metformin, 2-thiobarbituric acid, and D-penicillamine protected cells from DA toxicity in proportion to their rates of reaction with DA, with D-penicillamine causing a maximal increase in the IC_{50} to 5.23 ± 0.0992 mM when co-incubated with DA.

KEYWORDS: *diacetyl, guanosine, DNA unwinding, apoptosis, carbonyl scavenger*

■ INTRODUCTION

Much of the notoriety gained by diacetyl (DA, 2,3-butanedione) is in light of its connection to an occupational health hazard, "popcorn lung," occurring in individuals working in popcorn-manufacturing facilities.[1] DA is not merely an additive in butter-based products; its presence is pervasive throughout the modern human diet. It occurs naturally in milk products such as butter and arises during fermentation in foods such as beer and wine. The known toxicity of DA is, however, limited to cases of exposure to DA well beyond that encountered in natural foods. There are several instances of bronchiolitis obliterans, an inflammatory lung disease of the small airways, or bronchioles, in popcorn factory workers that are linked conclusively to DA exposure.[2] There is a solitary case of a particularly avid popcorn consumer being diagnosed with bronchiolitis obliterans, the latter not being attributable empirically to anything but DA exposure.[3] Placement of regulatory hurdles for DA usage in consumer products has spurred utilization of entities such as the diacetyl trimer that release DA upon exposure to water and/or heat.[4] Although formally fulfilling regulatory requirements, these replacements that ultimately yield DA fumes are well recognized as not being a means to mitigate DA hazard.[5]

Acute toxicity studies of DA exposure indicate that the entire respiratory system is a target organ.[6] A direct chemical attack on lung epithelium is implicated, accompanied by a neutrophil-mediated inflammatory response that was reported to be devoid of lymphocytic involvement.[7] The latter finding is curious as DA has been shown to form an irreversible adduct with the guanidine function of the side chain of arginine, potentially setting the stage for epitope formation and initiation of an immunological response (Figure 1).[8,9] The DA−arginine interaction is also implicated in the DA-caused inactivation of vital protective cellular enzyme mechanisms such as glutathione reductase and glyoxalase-I.[10,11] DA is also known to react with cysteine, forming 5-membered heterocycles of unexplored toxicological profiles (Figure 1).[12] Indeed, a lymphocytic inflammatory component was detected in a murine study that simulated DA concentrations commonly encountered at various places in a popcorn-manufacturing plant. In the latter study, exposures to concentrations of 100 ppm or greater were associated with marked morbidity and toxic effects of DA on bronchioles being apparent at concentrations as low as 25 ppm.[13]

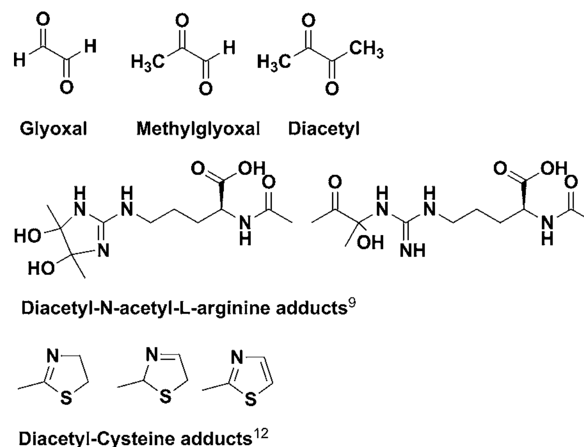

Figure 1. Structures of reactive dicarbonyls related structurally to diacetyl and covalent adducts of diacetyl with *N*-acetyl-L-arginine and L-cysteine.

Information about the chronic toxic effects of DA is surprisingly sparse. The interaction of structurally analogous dicarbonyls such as methylglyoxal and glyoxal (Figure 1) with guanosine has been studied in detail and the resulting adducts

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well-characterized.[14−16] Methylglyoxal is a documented mutagen at physiologically occurring concentrations, causing abnormal gene expression.[17] Covalent modification of guanosine with cyclic adduct formation is a common characteristic of compounds carrying the 1,2-dicarbonyl motif, many of those compounds being nutritionally relevant. We recently reported that dehydroascorbic acid, the oxidized form of vitamin C, forms a stable adduct with guanosine and alters protein expression.[18] In light of the ubiquity of DA and the growing interest in and alarm about its toxic effects, an investigation into its potential interactions with nucleobases and the subsequent effects on DNA structure is warranted. The present study focuses on several aspects of DA−DNA interaction: reactivity of DA with nucleobases alone and as part of a single-stranded oligomer, the effect of such a reaction on DNA tertiary structure, and the repercussions of such a phenomenon on cell survival.

■ MATERIALS AND METHODS

Ultraviolet absorption spectra were recorded on a BioTek Synergy HT plate reader (Biotek Instruments, Winooski, VT). NMR spectra were recorded on a Varian XL 600 MHz instrument. All ^{1}H and ^{13}C NMR experiments are reported in δ units (parts per million (ppm)). All coupling constants were reported in hertz. Melting points are uncorrected and obtained from a Mel-Temp II melting point apparatus. Mass analyses were performed on a 4800 MALDI TOF/TOF (Applied Biosystems Inc., Framingham, MA) at the Center for Mass Spectrometry and Proteomics, St. Paul, MN, and on an Agilent 1100 capillary flow HPLC equipped with an ESI source (Agilent Technologies, Palo Alto, CA) at the Masonic Cancer Center, University of Minnesota. A C18 column (Agilent Zorbax SB-C18, 150 mm, 5 μm particle size) in line with a Discovery Max (Thermoelectron, San Jose, CA) triple-quadrupole mass spectrometer was employed.

Drugs and Reagents. All chemicals and solvents required for chemical synthesis were purchased from Sigma-Aldrich (St. Louis, MO). DA, metformin, 2-thiobarbituric acid, D-penicillamine, ethidium bromide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Stock solutions of all compounds (10 mmol/L) were freshly prepared in PBS for every experiment. Stock solution of the MTT reagent was prepared in PBS at 5 mg/mL concentration and was stored at 4 °C for up to 2 weeks from preparation under protection from light. Oligomers (nonamers) used in this study were custom synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The cell culture media MEM, F12, and fetal bovine serum (FBS) and nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen (Carlsbad, CA). The plasmid DNA, pBR322, was purchased from New England Biolabs (Ipswich, MA).

Cell Culture. The human neuroblastoma cell line, SH-SY5Y, used in the present study was obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in MEM/F12 (1:1) medium supplemented with 10% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 1% nonessential amino acid (NEAA). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2/95% air.

Preparation of DA−2-Deoxyguanosine Adduct, 2. 2-Deoxyguanosine·H_2O (0.245 g, 0.85 mmol) was reacted with DA (0.473 g, 5.51 mmol) in anhydrous dimethylformamide (20 mL) under nitrogen atmosphere. The solution was warmed to 40 °C and stirred for 3 h. The reaction mixture was evaporated under reduced pressure to afford a dark brown syrup, **1** (0.305 g). Acetic anhydride (5 mL, 51.17 mmol) and pyridine (0.35 mL, 4.32 mmol) were added to the crude syrup, **1** (0.254 g) and refluxed overnight. The reaction mass was evaporated to give a dark brown oily residue. The crude product was purified by flash chromatography on SiO_2 using 5% methanol in ethyl acetate. Compound **2** was obtained as a white solid (0.204 g, 54.4% based on 2-deoxyguanosine).

Compound **2**: ^{1}H NMR (600 MHz, DMSO-d^6) δ 8.64 (bs, 1H, N^2-H), 8.01 (s, 1H, 8-H), 6.84 (s, 1H, −OH), 6.09 (s, 1H, −OH), 6.06 (t, 1H, H-1′, J = 7.05 Hz), 5.42 (m, 1H, H-4′), 4.38 (m, 1H, H-3′), 4.31 (m, 2H, H-5′), 3.03 (m, 2H, H-2′), 2.21 (s, 3H, $-OCOCH_3$), 2.15 (s, 3H, $-OCOCH_3$), 2.04 (s, 3H, $-CH_3$), 1.55 (s, 3H, $-CH_3$); ^{13}C NMR (150 MHz, DMSO-d^6) δ 170.62 (CO-3′), 170.47 (CO-5′), 156.01 (C-6), 154.90 (C-2), 151.22 (C-4), 135.58 (C-8), 118.67 (C-5), 93.66 (C−OH), 89.26 (C−OH), 83.25 (C-4′), 81.91 (C-1′), 74.82 (C-3′), 64.03 (C-5′), 35.98 (C-2″), 21.26 ($C-CH_3$-3′), 21.01 ($C-CH_3$-5′), 20.96 ($C-CH_3$), 20.73 ($C-CH_3$); ESI-HRMS m/z 418.1383 $(M - H_2O)^-$, $[C_{18}H_{23}N_5O_8 - H_2O]^-$ requires 418.1368; mp 230−235 °C (decomp).

Ultraviolet Absorption Spectroscopic Monitoring of DA−Deoxyguanosine Reaction. DA (250 μM) and 2-deoxyguanosine (50 μM) were incubated in 1 mL of 0.1 M potassium phosphate buffer (pH 7.1) at 37 °C for 12 h, and UV spectra were recorded from 220 to 300 nm on a BioTek Synergy HT plate reader at various time points.

Reaction of DA with Single-Stranded Oligonucleotides. The oligonucleotide (9mer) sequences selected were

5′-TTTGTTTTT-3′ (ssDNA-G), mol wt 2700

5′-TTTTTTTTT-3′ (ssDNA-no G), mol wt 2675

Stock solutions of DA and oligonucleotides were prepared in DMSO at a concentration of 10 μM. A 30 μL aliquot of DA stock was mixed with 10 μL of each of the oligonucleotides separately, and mixtures were allowed to incubate at 37 °C for 12 h. Each of the reaction mixtures was analyzed by MALDI using a 4800 MALDI TOF/TOF (Applied Biosystems Inc.). For each analysis, 1 μL of the reaction aliquot was mixed with an equal volume of a matrix containing 3-hydroxypicolinic acid and allowed to dry overnight before being analyzed by MALDI mass spectrometer. A 0.5 μL aliquot of each reaction mixture was also analyzed with an Agilent 1100 capillary HPLC in line with an Agilent 1100 ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA) operated in the ESI+ mode. The spectra were obtained by performing full scan MS within the m/z range of 200−2000. Chromatographic separation was achieved with an Agilent Zorbax 300 SB-C3 column (150 mm × 0.5 mm, 5 μm) eluted at a flow rate of 15 μL/min. The mobile phase consisted of 0.05% TFA in water (A) and 0.05% TFA in acetonitrile (B). The elution program started at 30% B for 5 min, followed by a linear increase to 80% B in 25 min. Under these conditions the adduct of 5′-TTTGTTTTT-3′ with DA eluted at 17 min and oligonucleotide 5′-TTTTTTTTT-3′ appeared at 18 min. Deconvolution of the resulting charge envelopes was performed using the Agilent ion trap deconvolution software.

Interaction of DA with pBR322 Plasmid DNA. Unwinding of supercoiled pBR322 DNA was studied by agarose gel electrophoresis. Solutions of pBR322 plasmid DNA (at concentration = 0.05 μg/μL) were incubated with DA at various concentrations (0−10 mM) in potassium phosphate buffer (0.1 M, pH 7.4) at 37 °C. The reaction mixtures were allowed to incubate for 72 h. At the end of incubation, the reaction mixtures were mixed with 6× loading buffer (0.25% bromophenol blue, 30% glycerol) and subjected to electrophoresis in 1% agarose gel. The gel was stained in TAE buffer containing ethidium bromide and visualized under UV light.

Cell Apoptosis Assay. SH-SY5Y cells were seeded in 4-well chamber slides (obtained from Fisher Scientific) at a density of 2×10^4 cells/well and incubated at 37 °C overnight. The cells were then treated in the absence or in the presence of DA (0, 50, 100, and 250 μM) for 48 h at 37 °C. The medium was then aspirated, and the cells were rinsed once with PBS. The cells were then incubated with a mixture of ethidium bromide and DAPI in PBS (1:1, each at a concentration of 100 μg/mL) for 15 min at room temperature, after which the cells were washed with PBS twice for 5 min each. At the end, PBS was removed completely; the wells were removed from the slide and were coverslipped with glycerol in PBS. The slides were imaged with Dage MTI Excel16 color digital camera coupled with a *CytoViva* VNIR Hyperspectral Imaging system using 10× objective (CytoViva, Inc., Auburn, AL).

Cytotoxicity Assay. The cytotoxicity of DA was measured by standard MTT assays in 96-well plates. SHSY5Y cells were seeded in a

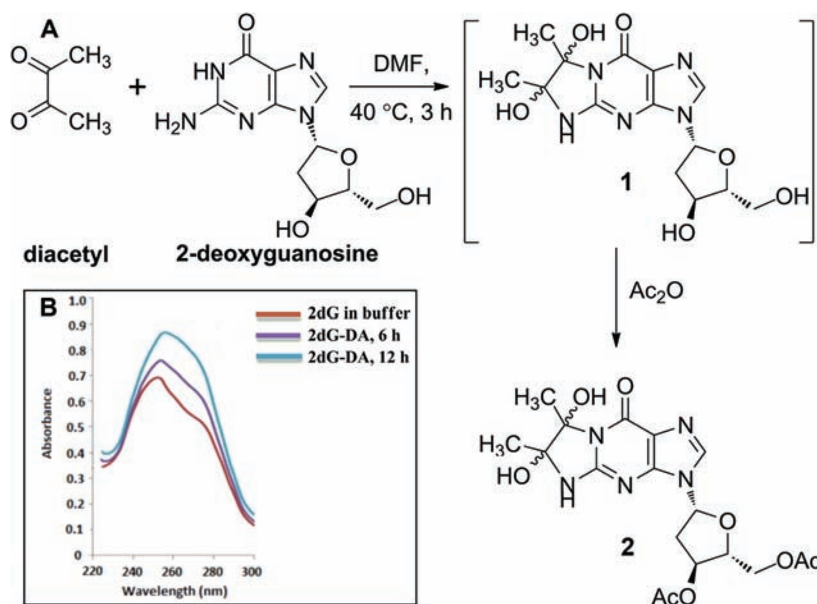

Figure 2. (A) Schematic depiction of the reaction between diacetyl and deoxyguanosine; (B) UV absorption changes of diacetyl reaction with 2-deoxyguanosine. Diacetyl was incubated with 2-deoxyguanosine (5:1) in 0.1 M potassium phosphate buffer (pH 7.1) at 37 °C, and the reaction was analyzed on BioTek Synergy HT plate reader in the wavelength range of 220−300 nm at various time intervals as described under Materials and Methods.

96-well plate at the density of 30000 cells/well. The next day, the cells were exposed to three different conditions: (1) DA or methylglyoxal alone; (2) pre-incubation of cells with carbonyl scavengers such as metformin, 2-thiobarbituric acid, or D-penicillamine at a concentration of 1 mM for 30 min, followed by the addition of DA at various concentrations; (3) incubation of carbonyl scavengers (metformin, 2-thiobarbituric acid, or D-penicillamine at 1 mM concentration) with various concentrations of DA for 30 min, followed by their addition to cells. The cells were allowed to incubate under the above conditions for 72 h at 37 °C. At the end of the incubation, the media containing treatment solutions were then replaced with fresh media, and 20 μL of MTT stock solution was added to each well. After an additional 3 h of incubation at 37 °C, the MTT reaction medium was discarded, and the purplish-blue MTT formazan crystals were dissolved by the addition of 100 μL of 0.1 N HCl in isopropanol. The optical density (OD), which is a reflection of the mitochondrial function of the viable cells, was read directly with a microplate reader (BioTek Synergy HT, BioTek Instruments, Inc.) at 580 nm and a reference wavelength of 680 nm. Concentration response graphs were generated for each drug using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). These graphs were analyzed using a curve fit for sigmoid dose−response, and IC_{50} values were derived. Results are expressed as mean IC_{50} with the standard error of the mean.

Statistical Analysis. All of the experiments were carried out in triplicate, and similar results were obtained. Data were analyzed by two-tailed unpaired *t* tests using Prism 5.0 software (GraphPad Software). $P < 0.05$ was considered to be statistically significant.

■ RESULTS AND DISCUSSION

Chemical Characterization of DA−Deoxyguanosine Adduct. There is great interest in the ability of 1,2-dicarbonyl compounds to react with nucleobases, especially guanosine. Structures of the reaction products of reactive dicarbonyls, such as glyoxal and methylglyoxal, have been unequivocally elucidated.[14] Our recent investigations have established that dehydroascorbic acid (DHA), another naturally occurring dicarbonyl, reacts with guanosine to form a cyclic adduct.[18] The presence of the 1,2-dicarbonyl motif in DA (Figure 1) makes it reasonable to speculate that its mutagenicity may arise from an interaction with a guanine nucleobase. Characterization of such an interaction was therefore regarded as a worthwhile pursuit. As seen in Figure 2A, the reaction of 2-deoxyguanosine with DA led to the formation of the intermediate **1**, which was detected by mass spectroscopy in the crude reaction mixture. For the purpose of isolation, we acetylated the reaction mixture containing **1** with acetic anhydride/pyridine. The adduct **2** was isolated in 54% yield based on the starting amount of 2-deoxyguanosine. NMR spectroscopy indicated that **2** is a tricyclic compound with a ring formed between N^1 and N^2 of the guanosine ring and the two carbonyls of DA to form a vicinal diol. The signals corresponding to exchangeable protons NH and OH were observed at δ 8.64, 6.84, 6.09. The hemiaminal carbons of the adduct **2** were assigned at δ 93.66 and 89.26.

The reaction between DA and 2-deoxyguanosine was also monitored by UV spectroscopy and examined for spectral changes due to adduct formation (Figure 2B). Significant changes in the spectra were observed after 6 h of incubation time. Wavelength shifts were observed between 220 and 300 nm. The shoulder occurring between wavelengths 270 and 280 nm diminished over the period of incubation and the absorbance at 257 nm increased during the course of incubation, reaching equilibrium at 12 h.

DA Reacts with Single-Stranded Oligonucleotides. On the basis of the reactivity of DA with 2-deoxyguanosine observed chemically and spectroscopically, we performed a reaction of DA with a single-stranded oligonucleotide, mimicking biological DNA (Figure 3). The two oligomers that were designed differed at one nucleobase site; one had a G in place of a T. The sequences selected were 5′-TTTGTTTTT-3′ (ssDNA-G) and 5′-TTTTTTTTT-3′ (ssDNA-no G). Comparative binding studies of DA with these oligomers (nonamer) were carried out using LC-MS and MALDI analyses. A 3-fold excess of DA was employed in the reaction with oligomer to ensure completion of the reaction. The selectivity of binding of DA (FW 86 Da) to the ssDNA-G oligomer (MW 2700 Da) was confirmed by the DA/ssDNA-G

Figure 3. (A, C) Incubation of oligo sequence 5′-TTTGTTTTT-3′ (ssDNA-G) with diacetyl. Adduct peaks were analyzed by LC/MS (A) and MALDI-TOF (C). Under the chromatographic separation conditions described under Materials and Methods, the adduct peak eluted at 17 min retention time (A; top). A mass corresponding to diacetyl with the oligomer was observed at 2787 Da in the full mass spectrum (A; bottom) and MALDI (C). (B, D) Incubation of oligo sequence 5′-TTTTTTTTT-3′ (ssDNA-no G) with diacetyl. Adduct peaks were analyzed by LC/MS (B) and MALDI-TOF (D). The reaction did not yield adduct peaks, and the parent oligomer was detected at 2675 Da. The oligomer eluted at the retention time of 18 min (B; top) under the chromatographic conditions described under Materials and Methods.

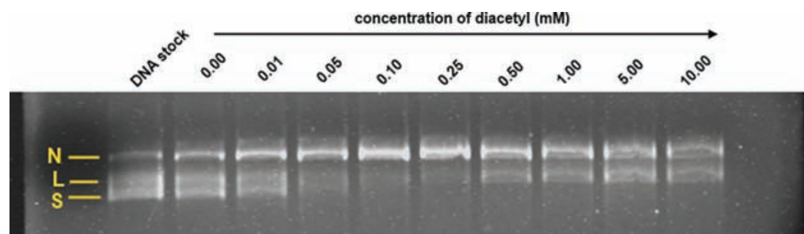

Figure 4. Effect of diacetyl on plasmid DNA unwinding. pBR322 plasmid DNA was incubated with various concentrations of diacetyl at 37 °C in potassium phosphate buffer (0.1 M, pH 7.4) for 72 h. Reaction mixtures were subjected to electrophoresis on 1% agarose gel as described under Materials and Methods. DNA unwinding was apparent at concentrations of diacetyl as low as 50 μM (N, nicked; L, linear; S, supercoiled).

adduct peak at 2787 Da (M + H) in both MALDI-TOF (Figure 3C) and LC-MS (Figure 3A). The LC-MS analysis of the reaction resulted in mixtures of two diastereomers of the adduct and were baseline resolved. The reactions were remarkably free of byproduct formation. The oligomer sequence ssDNA-no G (MW 2674 Da) incubated with DA (MW 86 Da), however, failed to exhibit such an adduct peak, and only the parent oligomer was detected by MALDI-TOF (Figure 3D) and LC-MS (Figure 3B) at 2675 Da (M + H). These results clearly demonstrate the reaction with guanosine as one of the pathways by which DA may cause DNA modification.

DA Induces Unwinding of Plasmid DNA. Following the chemical studies, we performed a biochemical assay to determine the effect of DA–DNA binding on ternary structure. Here, the effect of DA exposure (0.01−10 mmol/L concentrations) to pBR322 plasmid DNA was studied and is seen in Figure 4. In this particular experiment, the electrophoretic mobility of pBR322 DNA, an indicator of ordered

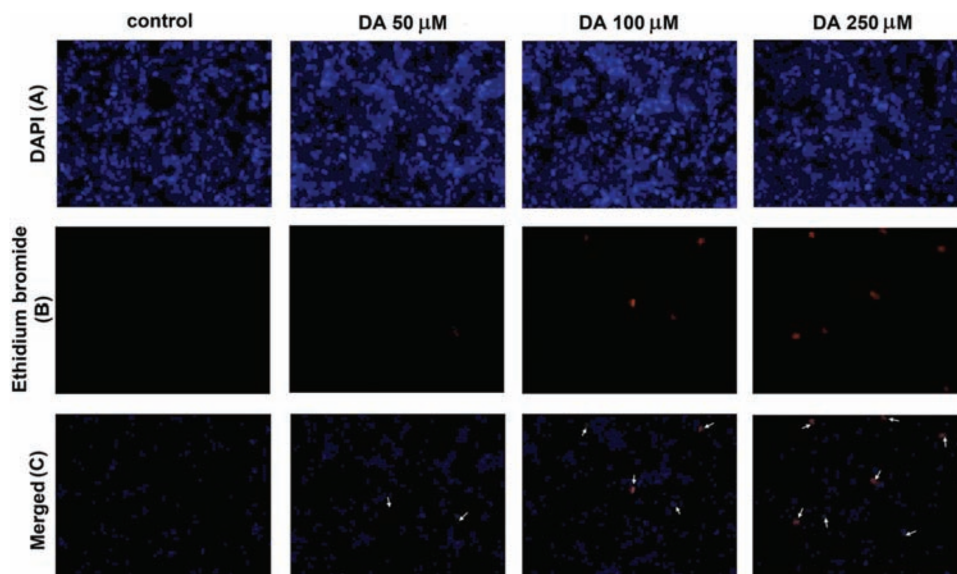

Figure 5. Characterization of diacetyl-induced cell death in SH-SY5Y cells. Cells were cultured with various concentrations of diacetyl (0, 50, 100, and 250 μM) for 48 h at 37 °C as described under Materials and Methods. Cells were stained with the nuclear stains DAPI (A) and ethidium bromide (B). (C) Merged image of DAPI- and ethidium bromide-stained cells. The white arrows indicate condensed/apoptotic nuclei. Number of apoptotic nuclei were proportional to the concentration of DA employed.

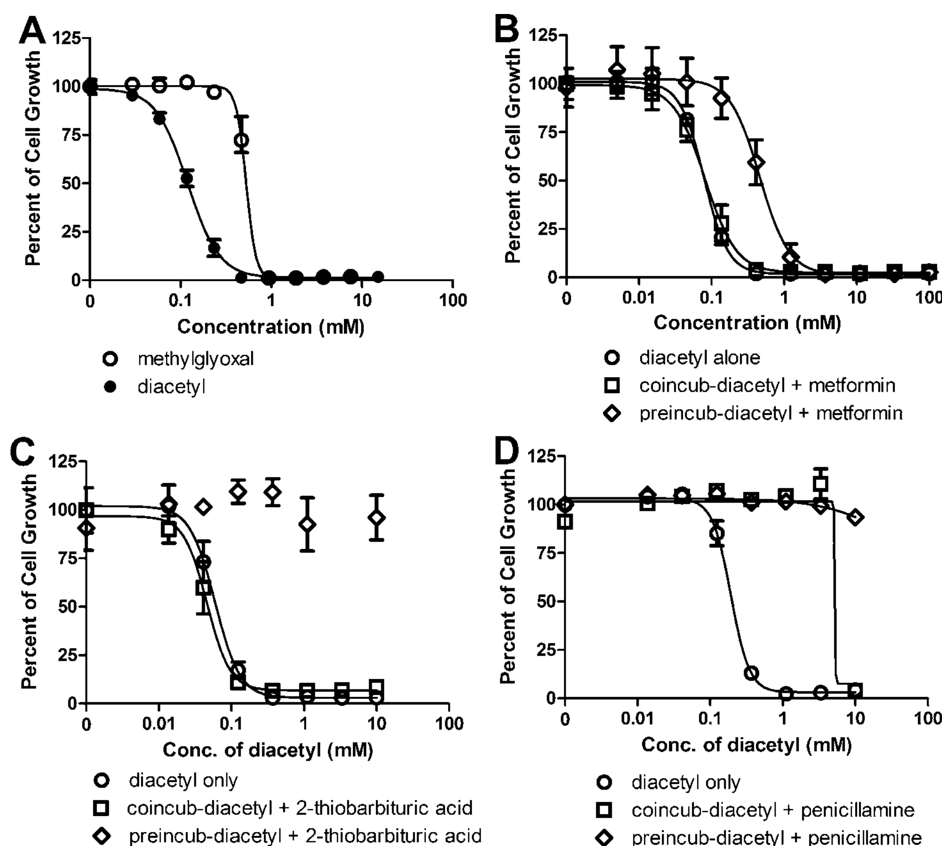

Figure 6. (A) Cytotoxicity of methylglyoxal and diacetyl in SH-SY5Y cells. Cells were seeded in a 96-well plate and incubated with or without methylglyoxal or diacetyl at various concentrations for 72 h at 37 °C. Cytotoxicity was determined by a standard MTT assay as described under Materials and Methods. (B−D) Protection against diacetyl cytotoxicity conferred by metformin, 2-thiobarbituric acid, and D-penicillamine, respectively. Co-incubation is defined as treatment of cells with metformin, 2-thiobarbituric acid, or D-penicillamine (each at 1 mM concentration) for 30 min, followed by addition of DA and continued incubation of cells with the scavenger and DA for 3 days. Pre-incubation is defined as the reaction of diacetyl with metformin, 2-thiobarbituric acid, or D-penicillamine for 30 min at 37 °C prior to being added to cells and incubation for an additional 72 h. Cytotoxicity was analyzed by a standard MTT assay as described under Materials and Methods. D-Penicillamine was found to be most effective in preventing diacetyl toxicity.

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structure, was reduced at DA concentrations as low as 50 μmol/L. Covalent modification of nucleobases by DA sequesters key H-bonding elements that are responsible for maintenance of DNA ternary structure and thus lead to decrease in order or "unwinding" as one of the outcomes. This study showed that exposure of DNA to DA results in disruption of DNA ternary structure and formation of lower ordered conformations, colloquially termed "uncoiling." Such conformational and chemical changes in DNA are expected to hamper normal transcription events, which in turn can disrupt expression of key proteins responsible for continued cell function and cell division. To examine such possibilities, the effect of DA exposure on the neuroblastoma cell line, SH-SY5Y, was next studied.

DNA Binding of DA Leads to Apoptosis in SH-SY5Y Cells. Cell morphology was highlighted through staining with DAPI and with ethidium bromide. Viable cells resist penetration of ethidium bromide but readily absorb DAPI, which binds to A—T-rich regions of DNA, causing nuclei to appear fluorescent blue. On the other hand, cells in various stages of apoptosis are permeable to ethidium bromide, which confers an orange coloration to chromatin, which is regarded as a positive indicator of apoptosis. DAPI/ethidium bromide double-staining (Figure 5) indicated scant apoptosis indicators in untreated cells after 48 h, whereas cells treated with 50, 100, and 250 μM concentrations of DA exhibited orange chromatin in condensed and fragmented nuclei (Figure 5). The number of cells exhibiting orange coloration of chromatin rose in a dose-dependent fashion with respect to DA. Apoptosis was evident at concentrations as low as 50 μM. DA has been reported to be mutagenic in nature. Recently, Mathews et al.[9] have reported an adduct of DA with *N*-α-acetylarginine, which indicates that DA may alter protein ternary structure and function. These interactions of DA with DNA and proteins form the basis of its mutagenicity and cytotoxicity.

Classical Carbonyl Scavengers Protect against DA Toxicity in Culture. The cytotoxic potential of diacetyl was determined in a standard MTT assay. Incubation of SH-SY5Y cells with diacetyl exhibited an IC_{50} of 0.119 μM, whereas its structurally similar analogue methylglyoxal showed an IC_{50} of 0.885 μM (Figure 6A). In work environment settings where continuous exposure to DA is expected, cytoprotective agents may be of prophylactic value. In this respect, three well-established carbonyl scavengers,[19] namely, D-penicillamine, metformin, and 2-thiobarbituric acid, were examined as potential cytoprotectives against DA toxicity (Figure 6). Such carbonyl scavengers have been extensively employed in the past for protection of DNA-damaging and toxic effects of dicarbonyls such as glyoxal and methylglyoxal.[20,21] To simulate a state of continuous dietary supplementation, the cells were pre-incubated with the carbonyl scavenger before exposure to DA. This condition is defined as "co-incubation" wherein cells are treated with metformin, 2-thiobarbituric acid, or D-penicillamine (each at 1 mM concentration) for 30 min, followed by the addition of DA and continued incubation of cells with the scavenger and DA for 3 days. Under these conditions, D-penicillamine raised the IC_{50} of DA from 0.114 ± 0.0421 to 5.23 ± 0.0992 mM ($p < 0.0001$; Figure 6D), a phenomenon that was not observed with metformin and 2-thiobarbituric acid (Figure 6, panels B and C, respectively). To examine the rate of inactivation of DA by the scavengers, they were premixed with DA prior to exposure of cells to the mixture (pre-incubation). Pre-incubation is defined as the reaction of diacetyl with metformin, 2-thiobarbituric acid, or D-penicillamine for 30 min at 37 °C prior to being added to cells and incubation for additional 72 h. Admixture of DA with each of the carbonyl scavengers followed by treatment of cells with the admixture resulted in significantly lower toxicity of DA: 10-fold increase of DA IC_{50} with 2-thiobarbituric acid, 5.9-fold with metformin, and practically complete protection with D-penicillamine (IC_{50}: DA only, 0.123 ± 0.0760 mM; DA + 2-thiobarbituric acid, 1.19 ± 0.294 mM; DA + metformin, 0.725 ± 0.0454 mM; and DA + D-penicillamine, >20 mM; $p < 0.0001$; Figure 6). The difference in reactivity was readily apparent, D-penicillamine being the most potent in protecting against DA cytotoxicity, followed by 2-thiobarbituric acid and metformin. The ability of carbonyl scavengers to rescue cells from prior exposure to DA was evaluated by exposure of cells to DA for 30 min followed by treatment with a 1 mM concentration of the carbonyl scavenger. In this case, only D-penicillamine reduced the toxicity of DA (10-fold increase in DA IC_{50}), whereas metformin and 2-thiobarbituric acid were ineffective (data not shown). In light of the extensive usage of DA as a flavorant, it was deemed worthwhile to examine the possibility of cell rescue after DA exposure with these carbonyl scavengers. D-Penicillamine was found to be the only viable rescue agent among the scavengers studied under various experimental conditions. D-Penicillamine is available as a commercial formulation (Cuprimine) and could be considered as a potential antidote against DA toxicity.

DA binds covalently to guanyl nucleotides under physiological conditions, affording a tangible product. This phenomenon could be responsible for the plasmid DNA unwinding and cellular apoptosis observed upon exposure to DA. To our knowledge, this is the first report that establishes affirmatively the mechanism through which DA causes DNA damage. The potential of D-penicillamine as a rescue agent against DA toxicity has been demonstrated. It is expected that these findings will bring to light the need for more thorough investigations into the toxicity of this ubiquitous but harmful flavoring agent.

■ SAFETY

As per the MSDS of diacetyl and the authors' own experiences, diacetyl should be handled strictly within the confines of a well-ventilated fume hood. Prolonged or repeated exposure to diacetyl should be avoided.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DA, diacetyl; SiO_2, silicon dioxide (silica gel); NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; MALDI, matrix-assisted laser desorption/ionization.

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