Peroxynitrite-Induced Reactions of Synthetic Oligo 2′-Deoxynucleotides and DNA Containing Guanine: Formation and Stability of a 5-Guanidino-4-nitroimidazole Lesion†

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Received February 20, 2002; Revised Manuscript Received April 9, 2002

ABSTRACT: Peroxynitrite is a strong oxidizing agent that is formed in the reaction of nitric oxide and superoxide anion. It is capable of oxidizing and nitrating a variety of biological targets including DNA, and these modifications may be responsible for a number of pathological conditions and diseases. A recent study showed that peroxynitrite reacts with 2′,3′,5′-tri-*O*-acetylguanosine to yield a novel compound, tri-*O*-acetyl-1-(*β*-D-*erythro*-pentafuranosyl)-5-guanidino-4-nitroimidazole, and, unlike other peroxynitrite-mediated guanine oxidation products, it is a stable and significant component formed even at low peroxynitrite concentrations. In this work, we studied the in vitro formation of the guanine-derived product, 5-guanidino-4-nitroimidazole, in synthetic oligonucleotides and DNA treated with peroxynitrite. When calf thymus DNA or oligonucleotides were reacted with peroxynitrite at ambient temperature, the modified base 5-guanidino-4-nitroimidazole was generated along with several other products. The oligonucleotides containing the 5-guanidino-4-nitroimidazole modification were purified by reverse-phase and anion-exchange HPLC and characterized by matrix-assisted laser desorption mass spectrometry. 5-Guanidino-4-nitroimidazole formation in peroxynitrite-treated DNA was characterized after enzymatic digestion of the reacted DNA to the nucleoside level. HPLC purification and electrospray ionization mass spectrometry (with selected reaction monitoring) enabled the analysis of this modified nucleoside with high sensitivity. The yield of 5-guanidino-4-nitroimidazole formed in single-stranded DNA was approximately 10-fold higher than that found in duplex DNA. With calf thymus DNA, 5-guanidino-4-nitroimidazole was dose-dependently formed at low peroxynitrite concentrations. In stability tests, a synthetic oligonucleotide containing the 5-guanidino-4-nitroimidazole modification was only partially cleaved by hot piperidine and was a weak substrate for Fpg glycosylase repair enzyme; in addition, this site was not cleaved by endonuclease III. These results suggest that nuclear DNA containing 5-guanidino-4-nitroimidazole may not be quickly repaired by DNA repair enzyme systems. Finally, primer extension experiments revealed that this lesion is a potential DNA replication blocker when polymerization is catalyzed by polymerase α and polymerase I (Klenow fragment, lack of exonuclease activity) but not with human polymerase *β*. Replication fidelity experiments further showed that 5-guanidino-4-nitroimidazole may cause G→T and G→C transversions in calf thymus polymerase α and *E. coli* polymerase I.

Although it remains a daunting challenge to trap or assay peroxynitrite ($ONOO^-$)[1] because of its high reactivity, there is much evidence supporting the notion that peroxynitrite, a powerful oxidant, is formed in vivo (*1*−*6*). The formation of peroxynitrite is thought to occur through a diffusion-limited radical combination of nitric oxide with superoxide anion at an average rate constant of 1.9×10^{10} M^{-1} s^{-1} (*4*, *7*, *8*), which is about 3 times faster than the rate at which superoxide dismutase can scavenge superoxide (*9*). In chronic pathophysiologic states, inflammatory cells such as macrophages overproduce both nitric oxide and superoxide (*10*−*12*). Recently, Giulivi et al. (*13*, *14*) others (*15*−*17*) reported mitochondrial production of NO by nitric oxide synthase located at the inner membrane. Ischiropoulos et al. (*18*) suggested that the kinetic rate of peroxynitrite production by activated macrophages is about 100 pmol $(10^6$ cells$)^{-1}$ min^{-1}. A kinetic model, based on the reaction of nitric oxide with superoxide anion and the half-life of peroxynitrite,

† This work was supported by Grants 5-P01-CA26731-23 (to S.R.T.) and 5-RO1-GM48802-08 (to R.A.J.) from the National Institutes of Health. The tandem mass spectrometer was purchased through a grant (DE-FG02-957E0056) from the U.S. Department of Energy.

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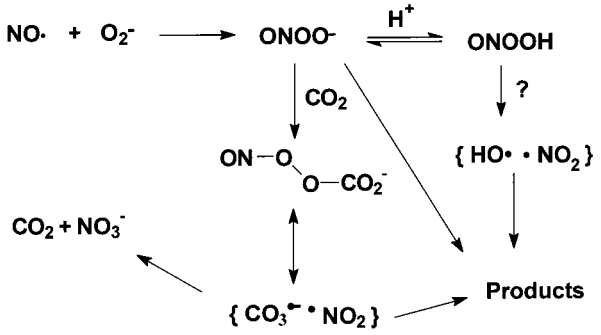
10.1021/bi020148q CCC: $22.00
Published on Web 05/16/2002

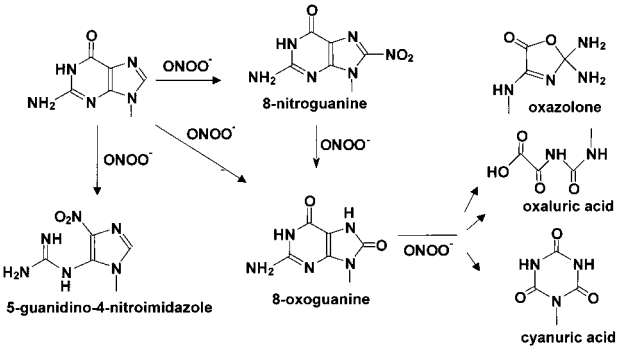
Scheme 1: Peroxynitrite Formation and Reaction with CO_2



indicated that the local concentration of peroxynitrite adjacent to activated macrophages could be around 60 nM (*19*). Under physiological conditions, the peroxynitrite anion is quickly protonated to become peroxynitrous acid, which can undergo homolysis to give the caged radical pair $^{\bullet}OH$ and $^{\bullet}NO_2$, with a half-life of about 1 s at 37 °C (*6*). On the other hand, CO_2 can also play a significant role in peroxynitrite chemistry and biochemistry. For example, CO_2 reacts with peroxynitrite to give the adduct nitrosoperoxycarbonate ($ONOOCO_2^-$), a very reactive species that can rapidly decompose to give rise to $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ via homolytic cleavage of the weak O−O bond (Scheme 1) (*20*−*22*). The radicals formed from these transformations are believed to be the ultimate oxidants that can react with various biological targets. In addition to macrophages, several other cell types, including neutrophils (*23*) and cultured endothelial cells (*24*), also produce peroxynitrite. It was shown that peroxynitrite-related anions, radicals, or neutral peroxynitrous acid may traverse cell membranes by diffusion or through anion channels (*25*, *26*). Since these reactive species can readily cross cell walls, all cellular compartments, including the nucleus and its DNA content, may be exposed to them.

The biological effects of oxidative damage to biomolecules have received a great deal of attention because of their importance in understanding cytotoxic and promutagenic properties of xenobiotics and endogenous toxins, particularly with regard to the aging process, neurodegenerative diseases, and cancer study. Peroxynitrite has specifically been correlated with many disease states (*27*), and various studies have shown that it can oxidize and nitrate a variety of biological substrates, including small organic molecules such as anti-oxidants (*28*), and larger biomolecules including lipids (*29*), proteins (*30*, *31*), and DNA (*32*). The nitration product of the tyrosine residue in proteins, i.e., 3-nitrotyrosine, has been specifically used as an indicator for peroxynitrite production in vivo (*33*−*36*), although some recent results

Scheme 2: Pathway of Products Formed via the Reaction of Peroxynitrite with Deoxyguanosine



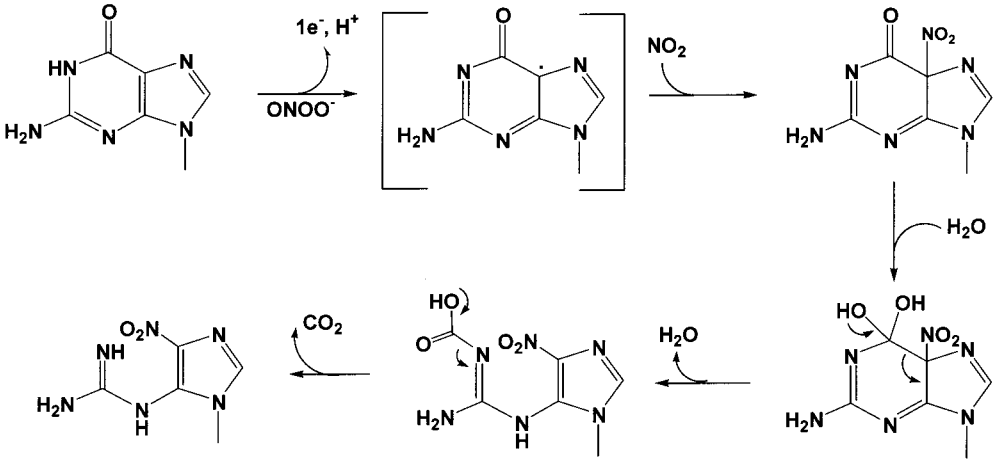
indicated that this molecule may not be generated exclusively via peroxynitrite chemistry (*37*, *38*). In DNA, both the deoxyribose (*39*) and the nucleobase (*40*, *41*) constituents can undergo oxidation. Of the bases, guanine is oxidized preferentially by peroxynitrite because it has the lowest oxidation potential of the four nucleobases (*41*). Some of the major initial products from the reaction of deoxyguanosine with peroxynitrite include 8-oxo-7,8-dihydro-2′-deoxyguanosine (*41*) and 8-nitro-2′-deoxyguanosine (*42*). Because these intermediates have even lower redox potentials compared to their guanine precursor, they are readily oxidized to give secondary oxidation products when excess peroxynitrite is present (*39*, *41*). For example, 8-oxo-7,8-dihydro-2′-deoxyguanosine is at least 1000 times more reactive toward $ONOO^-$ than the parent 2′-deoxyguanosine (*20*, *43*, *44*). Tretyakova et al. (*45*) recently demonstrated that 8-oxoG-containing oligonucleotides upon reaction with $ONOO^-$ underwent site-specific oxidative degradation to yield the corresponding oxazolone, oxaluric acid, and cyanuric acid derivatives in vitro (Scheme 2).

Most recently, a novel product, 1-(β-D-*erythro*-pentafuranosyl)-5-guanidino-4-nitroimidazole, was identified and characterized from the reaction of peroxynitrite with 2′,3′,5′-tri-*O*-acetylguanosine in the presence of CO_2 (*46*). The mechanism for the formation of 5-guanidino-4-nitroimidazole (Scheme 3) involves a radical cation intermediate by way of an initial one-electron oxidation of the guanine base. Unlike 8-nitroguanine and 8-oxoguanine that can undergo further oxidation, 5-guanidino-4-nitroimidazole is a stable and significant reaction product generated directly from guanosine and peroxynitrite even at low peroxynitrite concentrations. Whereas the oxazolone and oxaluric acid derivatives, as well as the cyanuric acid derivatives, are guanine oxidation products of other in vivo oxidants such as singlet oxygen (*47*) or metal complex ions (*48*), 5-guanidino-4-nitroimidazole is formed only through peroxynitrite-related chemistry. Thus, this lesion may be important in understanding peroxynitrite-induced mutagenesis and may serve as a specific biomarker of peroxynitrite-induced DNA damage.

Previously, tri-*O*-acetylated guanosine was used as a model compound in studies of 5-guanidino-4-nitroimidazole formation (*46*). In this paper, we report the in vitro formation of 5-guanidino-4-nitroimidazole from the reaction of peroxynitrite with synthetic oligonucleotides and calf thymus DNA. In addition, the stability of this lesion in oligonucleotides was determined, and the biological consequences were

[1] Abbreviations: 5-guanidino-4-nitroimidazole (or nitroimidazole), 1-(β-D-*erythro*-pentafuranosyl)-5-guanidino-4-nitroimidazole or 1-(2′-deoxy-β-D-*erythro*-pentafuranosyl)-5-guanidino-4-nitroimidazole; $ONOO^-$, peroxynitrite; dG, 2′-deoxyguanine; 8-oxoguanosine, 1-(2′-deoxy-β-D-*erythro*-pentafuranosyl)-8-oxo-7,8-dihydroguanine; cyanuric acid, 1-(2′-deoxy-β-D-*erythro*-pentafuranosyl)cyanuric acid; oxazolone, 2,2-diamino-4-[(2′-deoxy-β-D-*erythro*-pentafuranosyl)amino]-5(2*H*)-oxazolone; oxaluric acid, 3-(2′-deoxy-β-D-*erythro*-pentafuranosyl)oxaluric acid; Fpg enzyme, formamidopyrimidine-DNA glycosylase; endonuclease III (endo III), thymine glycol-DNA glycosylase; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; ESI, electrospray ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SRM, selective reaction monitoring; PAGE, polyacrylamide gel electrophoresis.

Scheme 3: Proposed Mechanism for the Formation of 5-Guanidino-4-nitroimidazole from the Reaction of Peroxynitrite with Deoxyguanosine



examined in vitro. A sensitive LC-MS/MS method was developed for the detection of the 5-guanidino-4-nitroimidazole nucleoside lesion in DNA. Using this method, we found that the amount of 5-guanidino-4-nitroimidazole formed in single-stranded DNA was about 10-fold higher than that in duplex DNA. Furthermore, a linear dose response was found for the formation of 5-guanidino-4-nitroimidazole in peroxynitrite-treated calf thymus DNA at low concentrations of peroxynitrite.

MATERIALS AND METHODS

All synthetic DNA oligonucleotides (Figure 1) were obtained from Synthetic Genetics (San Diego, CA). NAP-10 DNA desalting columns were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). T4 polynucleotide kinase, T4 DNA ligase, and Klenow fragment of DNA polymerase I lacking the 3′ to 5′ exonuclease activity were purchased from New England Biolabs (Boston, MA). DNA polymerases α and β were purchased from ChimeRx (Milwaukee, WI). *E. coli* endonuclease III (endo III) and formamidopyrimidine-DNA glycosylase (Fpg) were from Trevigen (Gaithersburg, MD). Calf thymus DNA, nuclease P1, calf spleen phosphodiesterase, snake venom phosphodiesterase (type I), and alkaline phosphatase were obtained from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]-ATP was from New England Nuclear-Dupont (Boston, MA). Fifteen percent denaturing polyacrylamide gels were prepared with Sequagel stock from National Diagnostics (Atlanta, GA). Gel electrophoresis was performed in a 1× TBE buffer at room temperature using a GIBCO BRL or BioRad instrument at 800−1500 V. All other chemicals, reagents, and solvents (Omnisolve grade) were from Sigma-Aldrich, EM Science, VWR Scientific, and other chemical distributors.

UV/Vis measurements were conducted using an HP8452 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA). HPLC was performed with an Agilent 1100 system, which consisted of a binary pump, an autosampler, and a diode array UV/Vis detector. LC-MS/MS analyses were conducted using an Agilent 1100 microflow pump LC system interfaced with a TSQ 7000 triple stage quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA).

Preparation of 2-^{13}C-1,7-NH$_2$-^{15}N$_3$-2′-Deoxyguanosine and the Corresponding Labeled Nitroimidazole Standards. The isotopically labeled deoxyguanosine was synthesized at Rutgers University by procedures reported earlier (*49*, *50*). The corresponding 5-guanidino-4-nitroimidazole compound was prepared by the reaction of the labeled diacetylated-dG with peroxynitrite. The desired product was purified by HPLC as described previously (*46*).

DNA Oligonucleotide Preparation, Separation, and ^{32}P-Labeling. The raw oligonucleotides, obtained from commercial sources, were desalted by passing through NAP-10 columns and were either used directly or further purified by 15% PAGE or HPLC (see below), if necessary. Gel purification was performed using a 15% gel, and the appropriate bands were cut under UV detection, extracted with water, desalted, and lyophilized. When radioactive labeling was needed, the 5′-end of oligonucleotides was labeled with [γ-^{32}P]-ATP using a T4 polynucleotide kinase. The labeled oligonucleotides were purified on a 15% gel and filmed, and the appropriate bands were excised, crushed, soaked with sterile water, and desalted with NAP-10 columns for later use.

Reaction of Peroxynitrite with DNA Oligonucleotides. Peroxynitrite was prepared by ozonolysis of sodium azide in 0.1 M NaOH solution and was stored at −80 °C until it was used as described previously (*51*, *52*). The concentrations of peroxynitrite were measured by UV absorption in a 0.1 M NaOH solution (ϵ = 1670 M^{-1} cm^{-1}, λ = 302 nm). Purified DNA oligonucleotides were dissolved in 150 mM potassium phosphate/25 mM sodium bicarbonate buffer at pH 7.2 in an Eppendorf tube. The appropriate volume of peroxynitrite was placed on the underside of the lid. The tube was carefully closed, and the contents were mixed and immediately vortexed for 3 min. For the hairpin duplex, the oligonucleotide was annealed by heating to 80 °C for 5 min and then slowly cooling to room temperature before the reaction with peroxynitrite. After the reaction, the samples were purified by HPLC.

HPLC Separation of Oligonucleotide Reaction Products. Two types of columns were used for the separation and purification of DNA oligonucleotides: a Supelco (Supelcosil LC-18-DB) column (25 cm × 2.1 mm, 5 μm) and a TSKgel DEA-NPR (Tosoh, Tokyo, Japan) anion exchange column. For the C-18 column, the mobile phase was 150 mM aqueous ammonium acetate (A) and 100% acetonitrile (B) with a

gradient of 7−12% B over 50 min at a flow rate of 250 μL/min. Anion exchange chromatography was performed with 1 M NaCl in 20 mM Tris-HCl (A) and 20 mM 1,3-diaminopropane adjusted to a pH of 10.5 with HCl (B) with a gradient of 0−100% B over 30 min at a flow rate of 1 mL/min. The detection wavelengths were set at 254 and 380 nm.

Ligation of Oligonucleotides. To examine the damage to oligonucleotides by $ONOO^-$, a modified 12-mer (D_8) was first ligated with a 14-mer (D_2) to form the 26-mer (D_9). A 20-mer (D_3) complementary to both the 12-mer and the 14-mer was used as the template for the ligation. The ligation was performed as follows: the 14-mer [300 pmol in 50 μL of 1× T4 ligase buffer (New England Biolab)] was phosphorylated at the 5′-termini with T4 polynucleotide kinase (1 unit) for 1 h at 37 °C. The kinase was then deactivated by heat (90 °C for 20 min), and equimolar amounts of the 12-mer and 20-mer were added to the solution at 90 °C. The mixture was allowed to cool slowly to room temperature in 1 h, and then T4 ligase (400 units) was added with additional ligase buffer (5 μL) to make the final concentration of buffer equal to 1× according to NEB. The incubation was held at 16 °C for 48 h. The ligated 26-mer was then purified by gel electrophoresis.

MALDI-TOF Mass Spectrometry. A PerSeptive Biosystems (Framingham, MA) Voyager Elite DE MALDI-TOF mass spectrometer was operated in the negative linear mode at an accelerating voltage of 25 000 V. The pulse delay time was set at 220 ns, and the grid voltage was 95% of the accelerating voltage. A 337 nm UV nitrogen laser was used for the power, and the average shots required to obtain molecular weights of the oligonucleotides were between 50 and 200. The samples were mixed and calibrated with two oligonucleotides of known sequence as internal standards to ensure the accuracy of the molecular weight. The matrix was a mixture of anthranilic acid, nicotinic acid, and ammonium citrate (molar ratio of 2:1:0.003) as previously reported (*53*). In general, the desalted oligonucleotide samples (about 1 pmol of each) were dissolved in 2−4 μL of matrix, spotted on the MALDI plate, and air-dried before analysis.

Enzymatic sequencing of the 26-mer was conducted using a 1 μL sample containing 30 pmol of the 26-mer with both snake venom phosphodiesterase (VPDE) and bovine spleen phosphodiesterase (SPDE). For the VPDE digest, 1 μL of VPDE (0.001 unit) was added to 1 μL of the oligomer solution in 6 μL of 100 mM ammonium citrate (pH 9.2 with NH_4OH) and 6 μL of H_2O. In the SPDE digestion, 1 μL of enzyme (0.001 unit) was added to 1 μL of oligomer solution in 12 μL of H_2O. The digestion solutions were heated at 37 °C. A 1 μL aliquot of the digest solution was removed at timed intervals, mixed with 2 μL of matrix solution, and then spotted on the plate. After air-drying, the sample plate was placed in the MALDI instrument for mass spectrometric analysis.

Reaction of Peroxynitrite with Calf Thymus DNA. Calf thymus DNA (300 μg) was dissolved in 600 μL of a pH 7.2 buffer containing potassium phosphate (150 mM) and sodium bicarbonate (25 mM). The guanine content was calculated to be ∼465 μM in this solution. The solution was divided into six aliquots, and each aliquot was further diluted to a volume of 400 μL with potassium phosphate/sodium bicarbonate buffer (116 μM guanine). Varying amounts of the $ONOO^-$ stock solution (concentration measured as described above) were added to the buffered DNA solution followed by rapid vortexing for 1 min. The samples were left at room temperature for 1 h and then desalted by passing through NAP-10 columns. The recovery of the DNA in the samples was over 90% as judged by UV−Vis spectroscopy. A known amount of the labeled nitroimidazole nucleoside standard was then added to each of the samples. Control calf thymus DNA treated with a deactivated solution of $ONOO^-$ was assayed under the same conditions to determine any background presence in the analysis.

Enzymatic Digestion of Oligonucleotides and Calf Thymus DNA and HPLC Purification of the 5-Guanidino-4-nitroimidazole Product. $ONOO^-$-reacted oligonucleotides were digested as follows: 2 nmol of the oligonucleotide in 40 μL of 100 mM Tris-HCl buffer (pH 8.8) and 15 mM $MgCl_2$ was incubated with 300 milliunits of snake venom phosphodiesterase and 20 units of alkaline phosphatase at 37 °C for 1 h.

Calf thymus DNA (50 μg) was digested by incubation with 4 milliunits of calf spleen phosphodiesterase and 5 units of nuclease P1 in 100 μL of buffer (200 mM succinic acid and 100 mM $CaCl_2$, pH 6) for 2 h at 37 °C. Then, 3 milliunits of snake venom phosphodiesterase and 5 units of alkaline phosphatase in 10 μL of the alkaline phosphatase buffer (500 mM Tris and 1 mM EDTA, pH 8.5) were added, and the mixture was incubated for an additional 2 h at 37 °C. After enzymatic hydrolysis, the incubation mixture was centrifuged, and the supernatant was collected. Water (100 μL) was added to the precipitate, and after mixing and centrifugation, the aqueous layer was combined with the collected supernatant fraction. CH_2Cl_2 (100 μL) was then added to the aqueous fraction to precipitate the enzymes. Following centrifugation, the aqueous layer was transferred to HPLC sample vials. Purification of the 5-guanidino-4-nitroimidazole product in the samples was conducted by HPLC using a 250 × 2.1 mm, 5 μm Supelcosil LC-18-DB column (Supelco, Bellafonte, PA). The mobile phase consisted of 150 mM aqueous ammonium acetate (A) and 100% acetonitrile (B) with a gradient of 3−20% B over 30 min at a flow rate of 250 μL/min.

LC-MS/MS Analysis of 5-Guanidino-4-nitroimidazole. The HPLC fraction containing both 5-guanidino-4-nitroimidazole and its stable isotope-labeled analogue was collected and concentrated under vacuum. The dried samples were dissolved in water, and an aliquot was analyzed by on-line HPLC-ESI-MS/MS using an Agilent 1100 series capillary liquid chromatography system interfaced with a Finnigan TSQ 7000 quadrupole mass spectrometer equipped with an electrospray ionization source.

Chromatographic separation was achieved using a Microbore C-18 reverse-phase Zorbax column (0.5 × 150 mm, 5 μm, Agilent Technologies). The solvent system consisted of 95% water and 5% acetonitrile with the pH adjusted to 4.7 with 1 M acetic acid. Isocratic conditions were used with a flow rate of 20 μL/min. The mass spectrometer was operated in the positive ion mode, and the samples were analyzed using an SRM procedure, which monitors collision-induced dissociation of a precursor ion to its product ion. The SRM analyses were performed by monitoring the transition of *m*/*z* 287 → *m*/*z* 171 for the 5-guanidino-4-nitroimidazole product and *m*/*z* 291 → *m*/*z* 174 for the stable isotope-labeled internal

standard. Instrumental parameters were optimized before use by adjusting the capillary and tube lens voltages to ensure maximum sensitivity. Electrospray ionization was achieved at a spray voltage of 4.5 kV. The heated capillary was set at 165−170 °C, and nitrogen sheath gas was held at 70 psi. During SRM scanning, a collision offset energy of −18 eV was used, and the gas (Ar) cell pressure was set at 1.45 mTorr. A calibration plot was constructed from the SRM analysis of the unlabeled 5-guanidino-4-nitroimidazole nucleoside versus its labeled analogue over the range of 0−25 pmol/sample. Quantification of the analyte in the sample was performed using the peak areas as determined by the integration routine in the data system.

Stability Tests of Oligonucleotides Containing Modified Bases and Repair Experiments with Fpg and Endonuclease III. Piperidine treatments were performed by heating the oligonucleotides (1 pmol ^{32}P-labeled) in 100 μL of 1 M piperidine at 90 °C for 30 min. Piperidine was removed by coevaporation with water (3 × 100 μL) under vacuum. The samples were then dissolved in loading buffer and analyzed by PAGE.

A 1 pmol ^{32}P-labeled lesion containing 26 nucleotides was annealed with its complementary 26-mer strand (2 pmol) by heating at 90 °C for 2 min and then slowly cooling the solution to room temperature in 2 h. The enzymatic reactions were performed in a 20 μL solution of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM KCl at 37 °C for 3 h with either 200 ng of Fpg or 400 ng of endo III. The resulting reaction mixtures were denatured, dried in vacuo, and then subjected to gel electrophoresis analysis.

Primer Extension Experiments. Three DNA polymerases were used in these experiments: calf thymus polymerase α, human polymerase β, and *E. coli* polymerase I. Reactions catalyzed by polymerase I were carried out in 10 μL of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.05 mg/mL BSA, and 1 mM DTT. Primer extension reactions using polymerases α and β were performed in 10 μL of 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM KCl, 0.2 mg/mL BSA, and 1 mM DTT. The buffered solutions containing the oligonucleotide template (a 26-mer, 100 nM) and the 5′-end-labeled primer (30 nM) were heated to 60 °C for 5 min and then cooled to 4 °C in 2 h. DNA polymerization reactions were conducted with either a 10 μM solution of a single dNTP or a mixture of all four dNTPs. The solutions were maintained at 37 °C for 1 h. The reactions were stopped by heating the mixtures to 90 °C for 10 min in order to deactivate the polymerases. The duplexes were denatured and subjected to PAGE analysis.

RESULTS

5-Guanidino-4-nitroimidazole Formation from the Reaction of Peroxynitrite with a *Single-Stranded 12-mer Oligonucleotide.* All oligonucleotides used in this study are shown in Figure 1. Initial experiments were performed to determine $ONOO^-$ damage to a single-stranded oligonucleotide containing only one guanine base in the strand. D_1 is a 12-mer oligonucleotide that has an identical sequence to the *supF* gene (*54*) from position 132 to position 143. Previous point mutation assays showed that the one guanine site on this 12-mer is a major mutation "hotspot" in the *supF* gene (*54*).

D_1: 5′-TCTAAATCTGCC-3′
D_2: 5′-GTCATCGACTTCGA-3′
D_3: 3′-TTAGACGGCAGTAGCTGAAG-5′
D_4: 5′-TCTAAATCTGCCGTCATCGACTTCGA-3′
D_5: 5′-TAGAT-L_9-ATCTA-3′ (L_9 = -$(OCH_2CH_2)_3O$-)
D_6: 3′-AGATTTAGACGGCAGTAGCTGAAGCT-5′
D_7: 3′-GGCAGTAGCTGAAGCT-5′
D_8: 5′-TCTAAATCTG^{NI}CC-3′
D_9: 5′-TCTAAATCTG^{NI}CCGTCATCGACTTCGA-3′
D_{10}: 5′-TCTAAATCTG^{OXO}CCGTCATCGACTTCGA-3′

FIGURE 1: Sequences of the synthetic oligonucleotides that were used in this study. L_9, a chemical linker to form a hairpin type of duplex structure; G^{NI}, 5-guanidino-4-nitroimidazole lesion that arises from the oxidation of guanine by peroxynitrite; Goxo, 8-oxoguanine lesion.

HPLC-purified D_1 was treated with a 50-fold excess of $ONOO^-$ in carbonated phosphate buffer at pH 7.2 at room temperature. After the reaction, the mixture was directly analyzed by HPLC, and the oligonucleotides were purified and characterized by C-18 column chromatography. Figure 2A shows the HPLC chromatograms of the reaction mixture. In general, a majority of the oligomer remains unchanged as indicated by its elution at 36 min. The presence of many small peaks eluting before the major peak at 36 min is presumably oligonucleotides containing different base modifications arising from the reaction of peroxynitrite. Unlike the reaction of 8-oxoguanine with peroxynitrite, in which the starting materials were almost entirely consumed when a 20-fold excess of peroxynitrite was used (*45*), the yields of the oxidation products derived from the direct oxidation of guanine as well as other bases are quite low. This is consistent with results obtained from the $ONOO^-$ oxidation of guanosine (*46*) versus 8-oxoguanine (*52*). As a means of searching for possible 5-guanidino-4-nitroimidazole-containing products from the reaction mixture, a full UV scan was obtained for each of the small peaks. A peak at 34.7 min was detected with a UV absorption maximum at 380 nm, which is assumed to contain the nitroimidazole moiety based on the preceding investigation in which triacetylated guanosine was reacted with $ONOO^-$ (*46*). In that study, 1-(2′,3′,5′-tri-*O*-acetyl-β-D-*erythro*-pentafuranosyl)-5-guanidino-4-nitroimidazole had UV maxima at 230 and 380 nm; the latter absorption maximum is unusual and characteristic of this compound; no other major oxidative lesions have a maximum absorption at this wavelength. The oligonucleotide-containing peak was therefore collected from multiple HPLC injections, and the fractions were combined and repurified by anion-exchange column chromatography. A chromatogram of the purified peak eluting at 34.7 min is illustrated in Figure 2B. A mass spectrum of this oligonucleotide obtained by MALDI-TOF showed a $[M-H]^-$ ion at *m*/*z* 3798.5, which is 19 Da higher than that of the parent compound (Figure 3). This is in agreement with a 5-guanidino-4-nitroimidazole modification at the original guanine position (Figure 1, D_8).

To obtain additional supporting evidence that the modification is indeed at the guanine site, we performed a sequencing experiment with this modified oligonucleotide using exonuclease digestion. A combination of exonuclease digestion and soft ionization mass spectrometry, such as

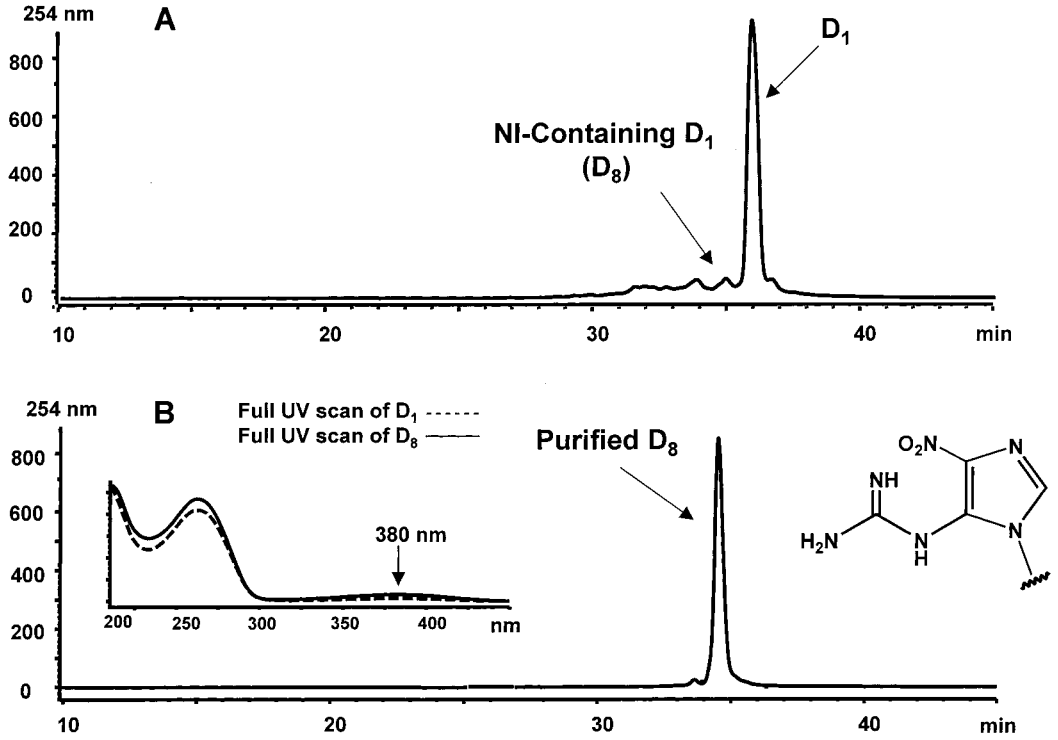


FIGURE 2: HPLC chromatograms of (A) peroxynitrite-reacted D_1 and (B) purified 5-guanidino-4-nitroimidazole-containing oligo D_8 from the reaction of D_1 with peroxynitrite. Two UV−Vis channels at 254 and 380 nm were used to detect the 5-guanidino-4-nitroimidazole-modified oligo. The HPLC conditions are described under Materials and Methods.

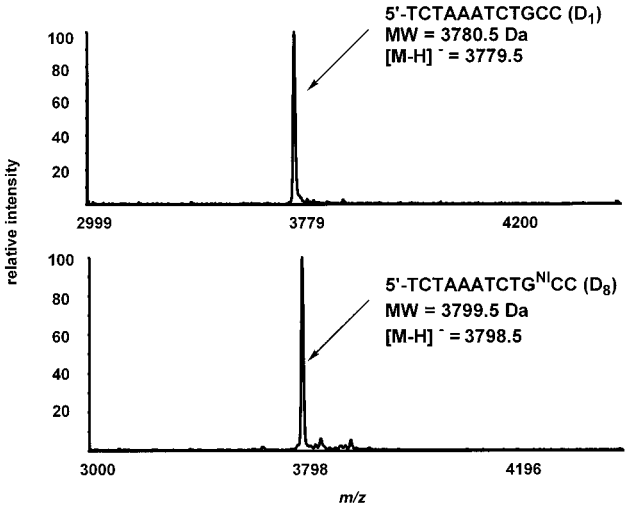


FIGURE 3: MALDI-TOF negative ion spectra of (top) D_1 and (bottom) purified D_9. A mass difference of 19 Da is observed.

MALDI-TOF or ESI-MS, has been used to sequence DNA oligonucleotides (*53*, *55*). Two enzymes were chosen for the stepwise digestions: 5′-exonuclease snake vemon phosphodiesterase (VPDE) for digestion of the oligonucleotide starting from 3′-end, and 3′-exonuclease calf spleen phosphodiesterase (SPDE) which sequentially cleaves the deoxynucleoside 3′-monophosphates from the oligonucleotide's 5′-terminus. To efficiently perform this sequencing technique, we extended the modified D_1 to form a 26-mer. We chose to ligate the 12-mer (D_8) with the 14-mer 5′-GTCATC-GACTTCGA-3′ (D_2) that is derived from the *SupF* gene (see Figure 1). A 20-mer oligonucleotide, D_3, which is complementary to both D_1 and D_2, was chosen as the template for the ligation. D_2 was first 5′-phosphorylated with T4 polynucleotide kinase, and the ligation was then carried out by using T4 DNA ligase and at a standard of 16 °C. The product was purified by gel electrophoresis, and the resulting 26-mer (D_9) was analyzed by MALDI mass spectrometry. A $[M-H]^-$ ion present at *m*/*z* 7899.2 is in agreement with its theoretical value (Figure 4A).

Exonuclease digestions were subsequently performed with the D_9 oligonucleotide. The digestions were quenched at different times, and the samples were mixed with a matrix of anthranilic acid, nicotinic acid, and ammonium citrate, and directly analyzed by MALDI-TOF mass spectrometry (Figure 4). At early time points, the oligomers were missing several nucleotides and ladders were observed. The mass unit between adjacent peaks indicates at which point a nucleotide is lost (Figure 4C,F). Surprisingly, unlike the case with other types of lesions, neither VPDE nor SPDE was able to overpass the 5-guanidino-4-nitroimidazole lesion site and continue the hydrolysis. Even at lower concentrations of the oligonucleotide or with prolonged incubation times, the hydrolysis stopped at this specific site. These results provide evidence that the lesion is on the guanine base (Figure 4D,G).

5-Guanidino-4-nitroimidazole Formation in Single- and Double-Stranded Oligonucleotides. One of our objectives was to measure the amount of 5-guanidino-4-nitroimidazole formed in single-stranded versus double-stranded oligonucleotides. In recent years, liquid chromatography combined with electrospray ionization mass spectrometry has provided high-sensitivity detection and selectivity in DNA adduct studies (*56*−*58*). Therefore, we performed enzymatic hydrolysis of D_8, which is a purified product from D_1, and the crude desalted mixtures from the reaction of D_1 and peroxynitrite, to the corresponding nucleoside level using a combination of enzymes for the digestion (phosphodiesterases and alkaline phosphatase). Labeled 2-^{13}C-1,7-NH_2-$^{15}N_3$-2′-deoxyguanosine (*49*) was used to synthesize the

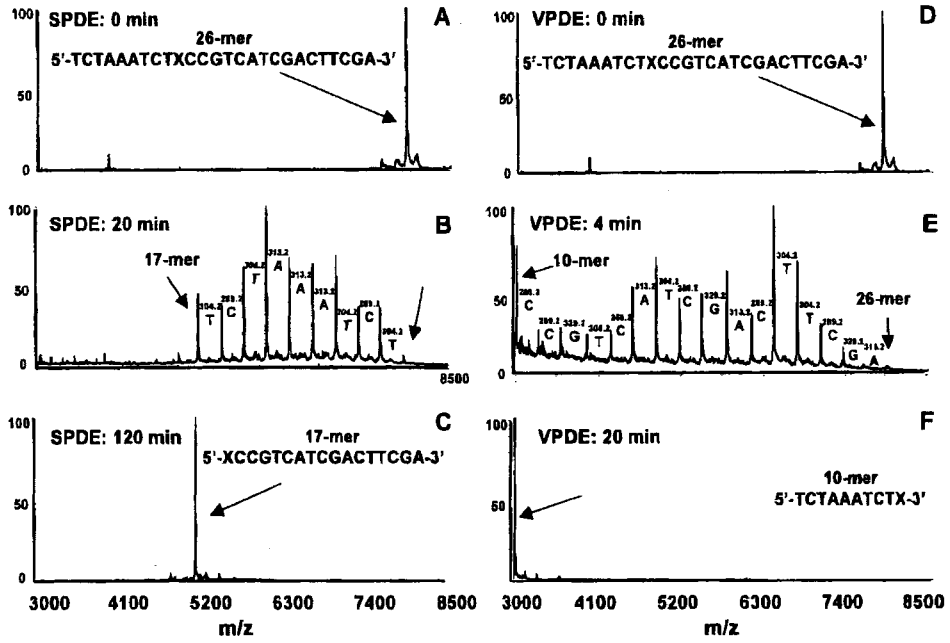


FIGURE 4: MALDI-TOF negative-ion spectra of the exonuclease-digested 26-mer D_9. (A) The starting 26-mer D_9. (B, C) Time-dependent results from the digestion with 3′-exonuclease bovine spleen phosphodiesterase. (D−F) Time-dependent results from the digestion with 5′-exonuclease snake venom phosphodiestease. The identification of the nucleotide lost from the oligonucleotides (both 3′-end and 5′-end) was determined by the mass unit difference between two adjacent peaks. For example, dG = 329.2, dA = 313.2, dC = 289.2, and dT = 304.2. The errors were within 0.5 Da.

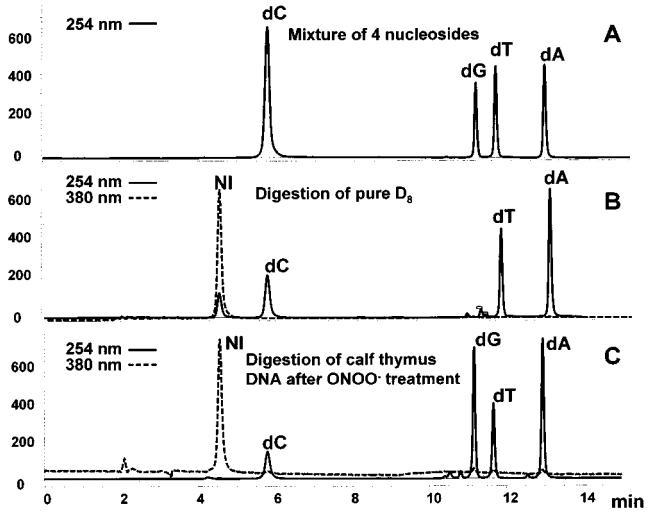


FIGURE 5: HPLC chromatograms of (A) mixture of the four normal DNA nucleosides, (B) D_8 following complete enzymatic digestion, and (C) peroxynitrite-treated calf thymus DNA after enzymatic digestion; the sample contains the stable isotope-labeled 5-guanidino-4-nitroimidazole nucleoside as internal standard. Absorption at 380 nm indicates the peak containing the 5-guanidino-4-nitroimidazole product.

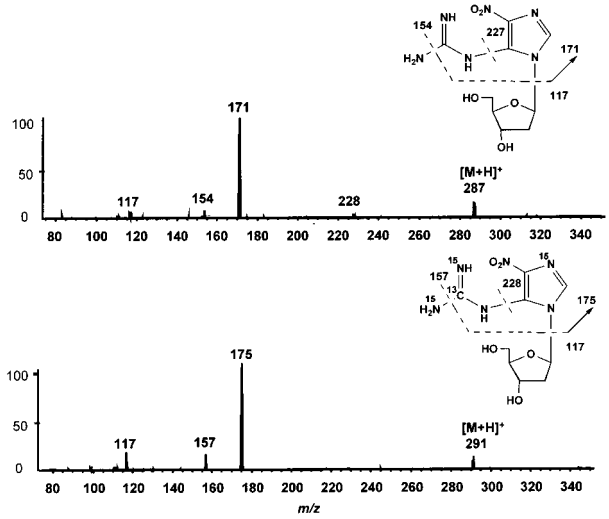


FIGURE 6: Product ion scans of (top) the unlabeled 5-guanidino-4-nitroimidazole nucleoside and (bottom) its stable isotope-labeled analogue. The ESI mass spectrometric conditions are described under Materials and Methods.

labeled 5-guanidino-4-nitroimidazole as an internal standard for the LC-MS/MS analysis. For these studies, a known amount of the labeled internal standard was mixed with the digestion solution from the D_1 hydrolysis. The resulting nucleoside fraction was purified by HPLC using a C-18 column with a flow rate of 250 μL/min. As shown in Figure 5B, the 5-guanidino-4-nitroimidazole-modified nucleoside elutes at an earlier retention time than the four normal nucleosides. The HPLC fractions containing 5-guanidino-4-nitroimidazole were collected and dried under vacuum. The samples were next dissolved in water and analyzed by LC-MS/MS.

To increase the sensitivity of the mass spectrometer, we used small-diameter (0.5 mm) columns for the LC separation with a flow rate of 20 μL/min. Figure 6 shows the product ion mass spectra of the unlabeled and stable isotope-labeled 5-guanidino-4-nitroimidazole-modified nucleosides. The labeled 5-guanidino-4-nitroimidazole derivative has a molecular mass that is 4 Da greater than that of its unlabeled counterpart; as expected, these compounds gave protonated parent ions $[M+H]^+$ at *m*/*z* 287 and *m*/*z* 291, respectively. Identical fragmentation was observed for both compounds, and predominant collision-induced dissociation ions were present at MH^+-116. This fragmentation corresponds to loss of the deoxyribose moiety from the molecules and is common for deoxyribose-containing DNA adducts (*59*, *60*). Figure 7

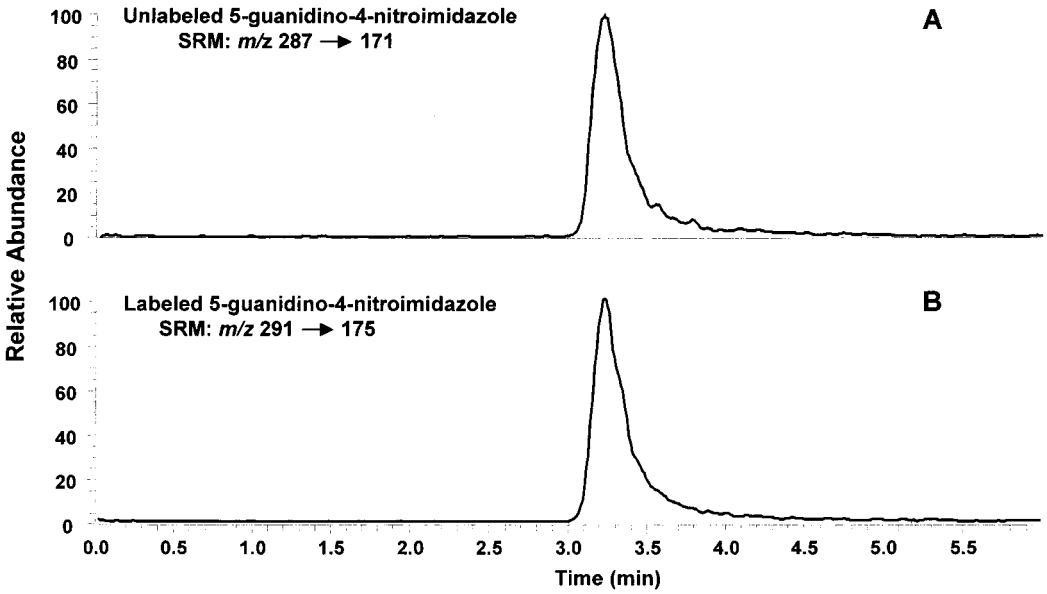


FIGURE 7: LC-ESI-MS/MS SRM analysis of (A) unlabeled 5-guanidino-4-nitroimidazole nucleoside and (B) its stable isotope-labeled analogue. The LC-MS/MS conditions are described under Materials and Methods.

shows an analysis by SRM mode of a standard mixture of the unlabeled and labeled 5-guanidino-4-nitroimidazole compounds. A calibration plot constructed from the SRM analysis of unlabeled 5-guanidino-4-nitroimidazole versus its labeled analogue was linear over the range of 0−25 pmol/sample ($r = 0.99$, slope = 0.98), with an intercept close to zero. Using the unlabeled standard, the lower limit of detection was 35 fmol (10 pg) of 5-guanidino-4-nitroimidazole injected on the column (signal/noise ∼ 3).

The yield of 5-guanidino-4-nitroimidazole from the D_1 oligonucleotide after treatment with a 50-fold excess of peroxynitrite was 2.3 ± 0.2%. For comparison, the amount of 5-guanidino-4-nitroimidazole formed from double-stranded DNA under similar peroxynitrite conditions was also determined. To ensure efficient duplex formation at room temperature with a requirement of only one GC base pair in the duplex, we prepared two 5-mer complementary DNA strands connected by a linker to form a hairpin-like structure (D_5 of Figure 1). D_5 was annealed to form a duplex in carbonate−phosphate buffer and then was treated with 50-fold excess of peroxynitrite at room temperature. The reaction mixture was desalted by the Nap-10 desalting column procedure (described above) and, following enzymatic digestion, was purified by HPLC and analyzed under conditions analogous to those of D_1. The yield of 5-guanidino-4-nitroimidazole from the reaction of $ONOO^-$ with D_5 was 0.3 ± 0.05%, which is about 10-fold less than that obtained for D_1. These results indicate that oxidation of guanine by peroxynitrite is more difficult when DNA is paired in the duplex form. This is not unexpected given our previous observations of different peroxynitrite reactivity with single- and double-stranded DNA (*41*). Among the many possible explanations are (1) steric hindrance, (2) charge repulsion effects in the interaction of negatively charged peroxynitrite with the sugar−phosphate backbone, and (3) possible alterations in the electronic properties of guanine in the different structural environments of single- and double-stranded DNA.

5-Guanidino-4-nitroimidazole Formation in Calf Thymus DNA. We next turned to duplex DNA and determined the amount of 5-guanidino-4-nitroimidazole formed in reactions of peroxynitrite with polymeric duplex DNA. It was of interest to examine 5-guanidino-4-nitroimidazole formation in DNA treated with varying concentrations of peroxynitrite and determine whether a dose-dependent response is present. Based on the former study, the reaction of peroxynitrite with deoxyguanosine demonstrated 5-guanidino-4-nitroimidazole formation at very low concentrations of peroxynitrite (*46*).

It can be roughly assumed that calf thymus DNA has an equal amount of each of the normal four nucleobases, and, therefore, the guanine content is about 25%. Calf thymus DNA was then reacted with 0.5-, 1-, 2-, 3.5-, 5-, 10-, and 20-fold amounts of peroxynitrite (compared to the DNA guanine content). The DNA was desalted, and, after spiking with a known amount of the labeled 5-guanidino-4-nitroimidazole internal standard, the samples were digested to the nucleoside level using a cocktail of hydrolytic enzymes. The nucleosides were separated and purified by HPLC as described above, and the 5-guanidino-4-nitroimidazole-containing fractions were analyzed by LC-MS/MS using SRM. Figure 8 depicts the amount of 5-guanidino-4-nitroimidazole formed in the reaction of DNA with varying concentrations of $ONOO^-$. As can be seen, the formation of this compound is proportional to the concentration of peroxynitrite used in the reactions. A linear response was obtained when the concentration of $ONOO^-$ was below 600 μM, corresponding to about 5-fold excess in concentration compared to the guanine content in the DNA.

Stability of 5-Guanidino-4-nitroimidazole-Containing Oligonucleotides. We were also interested in determining the stability of nitroimidazole lesions in DNA. Three 5′-end ^{32}P-labeled 26-mer DNA oligonucleotides, D_4, D_{10}, and D_9, were therefore treated with 1 M piperidine at 90 °C for 1 h and then cooled to room temperature. In another set of experiments, D_4, D_{10}, and D_9 were annealed with their complementary strand (D_6), and the resulting duplex strands were mixed with two DNA repair enzymes: Fpg and endonuclease III (endo III) in their required buffer solutions. After incubation at 37 °C for 3 h, the oligonucleotides were loaded onto denaturing polyacrylamide gel slabs and analyzed by electrophoresis. Figure 9 illustrates the cleavage reaction of

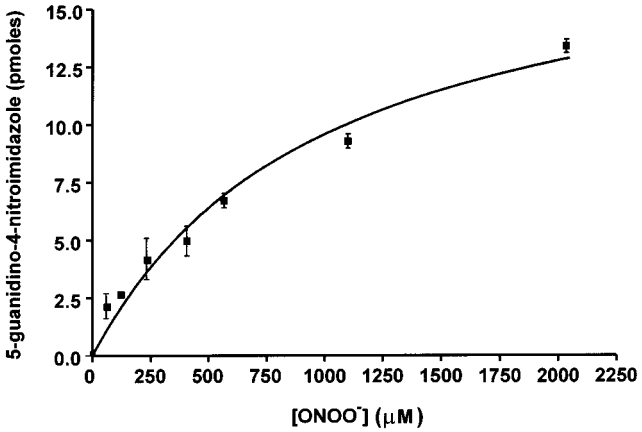


FIGURE 8: Formation of 5-guanidino-4-nitroimidazole in calf thymus DNA treated with varying concentrations of peroxynitrite. The levels were determined by SRM analysis of the modified nucleoside. The data points are the mean values obtained from two sets of experiments.

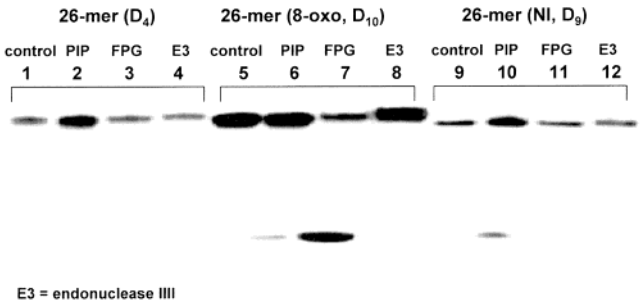


FIGURE 9: Stability tests of D_4, D_9, and D_{10}. Lanes 1−4: the unmodified D_4 oligonucleotide (stable to various treatment procedures). Lanes 5−8: 8-oxoguanine lesion showing partial cleavage by hot piperidine treatment (lane 6), and significant cleavage by the Fpg repair enzyme (lane 7), but no cleavage by endo III. Lanes 9−12: 5-guanidine-4-nitroimidazole lesion showing partial cleavage by hot piperidine (lane 10), and slight cleavage by Fpg (lane 11). It is not a substrate for endo III.

the oligonucleotides by hot piperidine, as well as that by the DNA repair enzymes. It can be seen that the normal oligonucleotide D_4 remained intact after base treatment (lane 2) and treatment with DNA repair enzymes (lanes 3 and 4). D_{10}, which contains the 8-oxoguanine lesion, was slightly cleaved by hot piperidine (lane 6) and significantly cleaved by Fpg (lane 7). These results are in agreement with previous reports [see, for example, (*61*)]. The 26-mer oligonucleotide D_9, in which the normal G site is replaced by 5-guanidino-4-nitroimidazole, behaved somewhat like 8-oxoguanine in that hot piperidine treatment (lane 10) and Fpg enzyme (lane 11) only partially cleaved the oligonucleotide. The sole difference is that Fpg cleavage of D_9 is much less significant in comparison to its reaction with D_{10}. Thymine glycol-DNA glycosylase (endo III) did not cleave either the 8-oxoguanine or the 5-guanidino-4-nitroimidazole residues, whereas this enzyme is capable of cleaving 5-hydroxydeoxyuracil (data not shown).

Base Incorporation Opposite the 5-Guanidino-4-nitroimidazole Lesion. To determine the mutagenic potential of the 5-guanidino-4-nitroimidazole lesion, we decided to undertake primer extension experiments to determine base incorporation opposite the 5-guanidino-4-nitroimidazole moiety. The 26-mer D_9 oligonucleotide was employed as the template, and a 16-mer D_7 complementary to D_9 from its 3′-end to the base next to the lesion site was used. The 5′ terminus of D_9 was labeled with ^{32}P. The primer was extended using three different DNA polymerases, human polymerase β (pol β), calf thymus polymerase α (pol α), and bacterial polymerase I (Klenow fragment with lack of exonuclease activity), in the presence of either a single dNTP or a mixture of all four dNTPs. Figure 10 shows the gel electrophoresis results from the extended primers after the polymeration reactions were quenched.

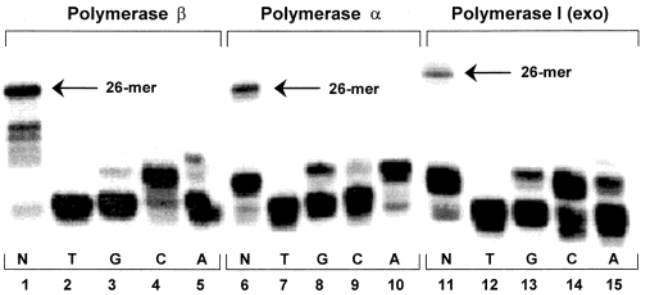


FIGURE 10: Primer experiments with three different polymerases: pol α (lanes 6−10), pol β (lanes 1−5), and pol I (Klenow fragment without exonuclease activity) (lanes 11−15). The primer (D_9) and ^{32}P -labeled primer (D_7) were annealed to form a duplex: D_7(3′-GGCAGTAGCTGAAGCT-^{32}P-5′)/D_9 (5′-TCTAAATCTG^{NI}CCGT-CATCGACTTCGA-3′). The experiments were carried out in the presence of dNTP (all four nucleoside triphosphates; lanes 1, 6, 11), dTTP (lanes 2, 7, 12), dGTP (lanes 3, 8, 13), dCTP (lanes 4, 9, 14), and dATP (lanes 5, 10, 15).

Interestingly, the 5-guanidino-4-nitroimidazole lesion in D_9 exhibited a significant replication block with pol α (lane 6) and pol I (lane 11) but not with pol β (lane 1) when all four nucleoside triphosphates were introduced in the mixture. Relatively full extension of the 26-mer was observed when pol β was used in the experiment. When only one triphosphate was added in the mixture, misincorporations occurred with three bases, except for T, at the site opposite 5-guanidino-4-nitroimidazole. Human pol β was able to incorporate for the most part the "right" C base opposite to NI (lane 4), while pol α mistakenly inserted chiefly A (lane 10) and G (lane 8). For pol I, which has a lack of proofreading activity, the trend is C > A > G (lanes 12−15).

DISCUSSION

Previous work from this laboratory identified the novel product 1-(β-D-*erythro*-pentafuranosyl)-5-guanidino-4-nitroimidazole from the reaction of peroxynitrite with 2′,3′,5′-tri-*O*-acetylguanosine (*46*). In this paper, we have shown that 5-guanidino-4-nitroimidazole is formed from the oxidation of guanine when oligonucleotides and DNA are treated with peroxynitrite. Unlike many other oxidation products, which are unstable intermediates or secondary reaction products, this compound is formed as a direct oxidation product of guanine. It was detected at low peroxynitrite concentrations in DNA and found to be extremely stable. Therefore, it is quite likely that this lesion may accumulate in DNA at the site of generation, and—unless repaired—may contribute to significant mutagenic effects.

Unlike the 8-oxoguanine-containing oligonucleotide, in which 100% conversion into three major oxidative products was reported (*45*), the reaction of peroxynitrite with a 12-mer oligonucleotide (containing only one guanine moiety) is more complex, and the yield of 5-guanidino-4-nitro-

imidazole is relatively low. Measurement of the molecular weight of the modified 5-guanidino-4-nitroimidazole-containing oligonucleotides by MALDI mass spectrometry combined with the characteristic UV absorption analysis at 380 nm facilitated the identification of this compound in complex mixtures of reaction products. By monitoring the partial digestion by mass spectrometry using two DNA exonucleases, we sequenced the ligated 26-mer oligonucleotide (D_9). The results clearly indicate that the guanine base was the site of oxidation in the oligonucleotide.

Several groups have elucidated the limitations of the traditional ^{32}P-postlabeling method in DNA adduct detection and have introduced alternative LC-MS/MS assays (*58*, *59*). These newer methods were found to approach comparable sensitivity and in addition have an advantage in that structural specificity is also obtained. We applied this modern technology to develop a method for the detection of 5-guanidino-4-nitroimidazole. In our experiments, synthetic oligonucleotides and calf thymus DNA following treatment with peroxynitrite were enzymatically hydrolyzed to the nucleoside level. After HPLC purification of the 5-guanidino-4-nitroimidazole fraction, the samples were analyzed by LC/ESI-MS/MS. Microflow HPLC employing a SRM scanning protocol provided the high sensitivity and selectivity needed in this research.

We found that 5-guanidino-4-nitroimidazole formation is proportional to the starting peroxynitrite concentration at low peroxynitrite concentrations. In addition, the amount of 5-guanidino-4-nitroimidazole formed in duplex DNA is about 10-fold less than that formed in single-stranded DNA. As discussed earlier, the reduced reaction of peroxynitrite with guanine in duplex DNA could be the result of steric hindrance, charge repulsion, or altered base electronics.

Many of the modifications that result from oxidation of a nucleic acid are labile toward hot base treatment. It is also known that several of these lesions are repaired by DNA repair enzymes. For example, 8-oxoguanine is a substrate for the *E. coli* enzyme formamidopyrimidine-DNA glycosylase (Fpg) (*61*). Fpg excises 8-oxoguanine from duplex DNA when it is paired with cytosine, leaving a gap flanked by 5′- and 3′-phosphate termini that are subsequently processed to afford a normal GC base pair. It was also demonstrated that among the secondary oxidation products of 8-oxoguanine, oxaluric acid is efficiently excised by Fpg, whereas oxazolone is partially removed (*44*, *62*). The cyanuric acid derivative, on the other hand, is not a substrate of Fpg (*62*). Our results show that the 5-guanidino-4-nitroimidazole lesion is only slightly cleaved by hot piperidine. Furthermore, the repair of this site by Fpg appears to be noneffective; therefore, because of its stability in DNA, this lesion may accumulate during the time course necessary for the expression of possible genetic toxicity within cells. In addition to the 8-oxoguanine adduct, 5-guanidino-4-nitroimidazole is not a substrate for endo III, which primarily recognizes only modified pyrimidine bases.

Nonrepairable lesions in DNA are important because they are most likely to be mutagenic and/or lethal to cells. A majority of DNA lesions, if not efficiently repaired, can exhibit their mutagenic or carcinogenic effects by two mechanisms: (i) by blocking DNA synthesis, which is lethal; or (ii) by causing miscoding, which is premutagenic (*63*). For example, many DNA products produced by ionizing radiation are lethal modifications (*64*), whereas other lesions such as 8-oxoguanine are bypassed during DNA replication and often produce miscoding reactions (*63*). Our in vitro experiments revealed that 5-guanidino-4-nitroimidazole causes a replication block in two of the three repair enzymes examined. Moreover, it induces both G→T and G→C transversions in polymerase α and polymerase I. Juedes and Wogan (*54*) found that peroxynitrite treatment of DNA containing the *supF* gene, followed by transfection, induced mainly G→T and G→C transversions when replicated in bacterial or mammalian cells. The specific lesions for these mutations are still unknown; however, it is understood that 8-oxoguanine, while subject to secondary oxidation, can cause primarily G→T transversions when replicated. More recently, using a genomic construction methodology, Henderson et al. (*65*) showed that all three secondary oxidation products arising from 8-oxoguanine were easily bypassed in vivo to cause exclusively G→T transversions at frequencies at least 10-fold higher than that found for 8-oxoguanine. Considering these results, our current findings indicate that formation of 5-guanidino-4-nitroimidazole may be one of the sources of the G→C transversions reported earlier (*54*).

In conclusion, our work provides evidence that 5-guanidino-4-nitroimidazole is formed in single-stranded and duplex DNA treated with peroxynitrite. The stability of 5-guanidino-4-nitroimidazole suggests that it may be an important lesion in peroxynitrite-mediated DNA damage.

ACKNOWLEDGMENT

We acknowledge Dr. Jacquin C. Niles for his pioneering work in identifying this lesion at the nucleoside level and his constructive suggestions and comments during this research. We are also grateful to Dr. Paul Henderson of Professor John Essigmann's group at MIT for providing help and lending laboratory space for experiments with ^{32}P labeling.

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BI020148Q