Site-specific DNA Cleavage by Antisense Oligonucleotides Covalently Linked to Phenazine Di-N-oxide*

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Site-specific degradation of DNA was achieved by the use of DNA oligonucleotides covalently tethered to phenazine 5,10-di-N-oxide. When annealed to a complementary DNA target strand, the antisense oligonucleotide effected alklylation of guanosine residues in proximity to the phenazine di-N-oxide prosthetic group. Admixture of dithiothreitol to the formed duplex resulted in reductive activation of the phenazine di-N-oxide moiety with concomitant generation of diffusible oxygen radicals; the latter effected strand scission of the target DNA oligonucleotide. Several parameters of DNA degradation were studied, including the effect on DNA degradation of chain length in the tether connecting the oligonucleotides and prosthetic group, the relative efficiencies of DNA cleavage when the prosthetic group was in the middle or at the end of the antisense oligonucleotide, and the effect of O2 on DNA degradation. Also studied was the actual chemistry of DNA oligonucleotide degradation and the ability of individual diastereomers of the modified oligonucleotides to mediate degradation of the target DNA.

In recent years, there has been growing interest in the design of molecules that can act as sequence-specific nucleic acid probes (see e.g. 1–3); one approach has involved the use of oligonucleotides able to bind to and destroy specific single-stranded RNA or DNA sequences (4–12). By structural alteration of these oligonucleotides, it has been possible to modify certain parameters of oligonucleotide-DNA (RNA) interaction, such as affinity or nuclease stability, without lessening the intrinsic specificity due to Watson-Crick base-pairing interactions (13–20). Although many technical challenges remain, antisense oligonucleotides can now reasonably be regarded as having significant potential as therapeutic agents (21–23). In fact, at an experimental level, antisense oligonucleotides have been used to interfere specifically with gene expression by human immunodeficiency virus (24–27), vesicular stomatitis virus (28), herpes simplex virus 1 (29), type A influenza (30) and encephalomyocarditis viruses (31) as well as trypanosomes (32).

At a technical level, the delivery of antisense oligonucleotides to the appropriate cellular targets in quantities sufficient to disrupt nucleic acid (e.g. mRNA) function constitutes an ongoing problem. One approach involves the attachment to the antisense oligonucleotide of a prosthetic group that can destroy the target cellular nucleic acid of interest in a site-specific fashion; this would allow the oligonucleotide to be used at lower concentration than if it simply acted via binding, especially if nucleic acid destruction were effected catalytically. Presently, there are many examples of oligonucleotides that can alkylate or oxidatively destroy their target RNA or DNA substrates (4–12, 33–37), but it is less clear whether any of these could function specifically within an intact cell.

Recently, we described the reductive activation of an aminoalkylphenazine di-N-oxide under conditions that could obtain within an intact cell (38). Presently, we describe the conjugation of this phenazine di-N-oxide derivative to several DNA oligonucleotides via N-alkylphosphoramide linkages and the use of the conjugates for the destruction of a complementary DNA oligonucleotide target molecule. We demonstrate that the oligonucleotide-linked phenazine di-N-oxide can both alkylate the DNA target and effect its oxidative destruction, and that the chemistry of oxidative destruction differs under aerobic and anaerobic conditions.

EXPERIMENTAL PROCEDURES

Materials
T4 polynucleotide kinase (30 units/ml; 1 unit is the amount of enzyme required to incorporate 1 nmol of 32P from [γ-32P]ATP into micrococcal nuclease-treated calf thymus DNA) was purchased from United States Biochemical Corp. [γ-32P]ATP was obtained from ICN Biomedicals, Inc. Superoxide dismutase and catalase were purchased from Sigma; where necessary, inactivation was achieved by heating solutions of the enzymes at 90 °C for 10 min. Nensorb columns were from Du Pont-New England Nuclear.

DNA synthesis was carried out on a Biosearch model 8600 DNA synthesizer essentially as described (39–44); chemicals were obtained from Milligen/Biosearch (dAdT CPG 500-A solid support, β-cyanoethylphosphoramidite activator solution, and β-cyanoethylphosphoramidites), Sigma (DMT' 2'-deoxyctydine H-phosphonate triethylammonium salt and pivaloyl chloride), Aldrich Chemical Co. (dry acetonitrile, acetic anhydride, N,N-methylimidazole, and dichloroacetic acid) and Mallinkrodt (resublimed iodine). Pyridine was distilled successively from p-toluenesulfonyl chloride and KOH, and stored over 4 Å molecular sieves. Tetrahydrofuran was freshly distilled from LiAlH4. Pivaloyl chloride was distilled prior to use, as was CCl4 (distilled from P2O5). DMF was dried by heating at reflux over CaH2 for 16 h, then distilled, and stored over 4 Å molecular sieves.

HPLC analysis was carried out on a Varian 2000 liquid chromatograph equipped with a gradient mixing system, Perkin-Elmer Cetus LC-235 diode array detector and a photometer output recorder. Reversed-phase HPLC analysis was carried out on a Ráinin Microsorb C8 (4.6 mm × 10 cm, 3 µm) or Alttech C18 (10 mm × 25 cm, 10 µm) column. Gradient elution was performed under ambient conditions using a linear gradient of acetonitrile in either 0.1 M H2O/NaAc buffer, pH 7.0, or 0.1 M Et3N·HOAc buffer, pH 7.0. UV and visible spectra

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1 The abbreviations used are: DMT, dimethoxytrityl; DTT, dithiothreitol; FAGE, polyacrylamide gel electrophoresis; PDNO, phenazine di-N-oxide; CPG, controlled pore glass beads; DMF, N,N-di-methylformamide; HPLC, high pressure liquid chromatography; nt, nucleotide.
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were obtained on a Perkin-Elmer Cetus Lambda Array 3840 spectrophotometer.

Methods

Synthesis of 2-(3'-Aminomethylaminophenazine Di-N-oxide)—A reaction mixture containing 500 mg (2.03 mmol) of 2-chlorophenazine 5,10-di-N-oxide (45, 46), 20 ml (240 mmol) of 1,3-diaminopropane, and 1.65 g (11.9 mmol) of K₂CO₃ in 10 ml of DMF was heated at 60 °C for 22 h, then cooled, and filtered. The filtrate was concentrated and purified by flash chromatography (47) on silica gel (elution was with 1:1 CH₂Cl₂-CH₂OH containing 1% NH₃OH), to afford 2-(3'-aminomethylaminophenazine di-N-oxide as a dark purple powder, mp 140–142 °C (dec); silica gel TLC (50:50:1 CH₂Cl₂-CH₂OH-NH₃OH) Rᵣ 0.17; 'H NMR (CDCl₃) δ 1.35–1.45 (m, 8), 1.75 (m, 2), 2.70 (t, 2, J = 7 Hz), 3.35 (q, 2, J = 6 Hz), 4.74 (br s, 1), 7.16 (dd, d, J = 10, 2 Hz), 7.42 (d, J = 2 Hz), 7.68 (t, J = 8 Hz), 7.78 (t, 1, J = 8 Hz), 8.50 (d, 1, J = 10 Hz), and 8.68 (d, 1, J = 8 Hz); mass spectrum (chemical ionization, isobutane) m/z 285 (M + H)⁺, 269 and 253.

The synthesis of 2-(6'-aminohexyl)aminophenazine 5,10-di-N-oxide was carried out analogously, starting from 2-chlorophenazine 5,10-di-N-oxide (247 mg, 1.0 mmol) and 1,6-diaminohexane. The product was obtained in 60% yield, mp 135–137 °C (dec); silica gel TLC (80:20:0.5 CH₂Cl₂-CH₂OH-NH₃OH) Rᵣ 0.57; 'H NMR (CDCl₃) δ 1.35–1.45 (m, 8), 1.75 (m, 2), 2.70 (t, 2, J = 7 Hz), 3.35 (q, 2, J = 6 Hz), 4.74 (br s, 1), 7.16 (dd, d, J = 10, 2 Hz), 7.42 (d, J = 2 Hz), 7.68 (t, J = 8 Hz), 7.78 (t, 1, J = 8 Hz), 8.50 (d, 1, J = 10 Hz), and 8.68 (d, 1, J = 8 Hz); mass spectrum (chemical ionization, isobutane) m/z 285 (M + H)⁺, 269 and 253.

The fully deblocked 21-nt oligomer was recovered from the initial Nensorb column, and the purity was determined using HPLC (28) (Scheme 1). The mixture was stirred at room temperature for 1 h, and the excess reagent was removed. The CPG support was washed with DMF and MeOH and then treated with 1 ml of 3:1 NH₄OH:ethanol. The tubes were sealed and maintained at 50 °C for 12 h; the column was removed from the DNA synthesizer and dried under argon. The CPG support was distributed into two 1.5-ml centrifuge tubes and a mixture of 2-(3'-aminopropanol)aminophenazine di-N-oxide (30 mg) and 2 ml of CCl₄ in 2 ml of DMF was added to the CPG support (Scheme 1). The mixture was stirred at room temperature for 1 h, and the excess reagent was removed. The CPG support was washed with DMF and MeOH and then treated with 1 ml of 3:1 NH₄OH:ethanol. The tubes were sealed and maintained at 50 °C for 12 h. The supernatant and washings were concentrated and applied to a Nensorb column that had been prewashed with 0.1 triethylammonium acetate buffer, pH 7.0, and then 20 ml of deionized water to remove failed sequences and deblocking of the CPG support. The assembly of the remaining 20 nucleosides was then carried out using H-phosphonate chemistry as a mixture of diastereomers, which was desalted by Nensorb chromatography (48); λₑₓ 258, 425 and 550 nm. The ratio of peak heights was consistent with that expected for a 15-nt product having the anticipated sequence and a single, covalently linked phena- zine di-N-oxide. Based on the extinction coefficient of 2-(3'-aminomethylaminophenazine di-N-oxide (ε₅₈₀ = 8.0 × 10⁵), the yield of oligonucleotide 1 was 129 mmol (12%).

For the separation of diastereomers of 1, the DMT-protected oligonucleotide was recovered from the initial Nensorb column, and the isomers were separated by HPLC (Fig. 2c). The purified DMT-protected diastereomers 1 were deblocked and desalted as indicated above.

Antisense oligonucleotide 2 was prepared in the same fashion as 1; the overall yield of the HPLC-purified oligonucleotide (Fig. 2b) was 8%. The 15 nt oligomer 1a was also prepared in the same fashion as 1, but using 2-6'-aminohexylaminophenazine di-N-oxide/CCI₄ for introduction of the phenazine di-N-oxide during the final coupling procedure. Purification was effected by C₁₈ reverse-phase HPLC (Alltech 10 urn column, 10 mm × 25 cm) using a gradient of CH₃CN (10% initially, then increasing 1%/min up to 40%) in 0.1 M NH₄OAc, pH 7.0, at a flow rate of 4.0 ml/min. The overall yield of purified 1a was 14%.

Synthesis of a 21-nt Oligomer Linked to Phenazine Di-N-oxide (3)—The synthesis of the fully protected oligonucleotide 5'-ATAGGCGATCCGGGA was carried out on a 1.05-μmol dA column (CPG support) using phosphoramidite chemistry (39, 40). DMT 2'-deoxyctydine was then introduced using H-phosphate chemistry (41–44). The overall and stepwise yields were 74 and 98%, respectively, based on released DMT cation. The column was removed from the DNA synthesizer and dried under argon. The CPG support was distributed into two 1.5-ml centrifuge tubes and a mixture of 2-(3'-aminomethylaminophenazine di-N-oxide (30 mg) and 2 ml of CCl₄ in 2 ml of DMF was added to the CPG support (Scheme 1). The mixture was stirred at room temperature for 1 h, and the excess reagent was removed. The CPG support was washed with DMF and MeOH and then treated with 1 ml of 3:1 NH₄OH:ethanol. The tubes were sealed and maintained at 50 °C for 12 h. The supernatant and washings were concentrated and applied to a Nensorb column that had been prewashed with 0.1 triethylammonium acetate buffer, pH 7.0, and then 20 ml of deionized water to remove failed sequences and deblocking of the CPG support. The CPG support was distributed into two 1.5-ml centrifuge tubes and a mixture of 2-(3'-aminomethylaminophenazine di-N-oxide (30 mg) and 1 ml of CCl₄ in 1 ml of anhydrous 1:1 DMF-CH₃CN was added to the support (Scheme 1). The mixture was stirred at room temperature for 2 h. The excess reagent was removed and the CPG support was washed with DMF and CH₃OH. The dried CPG support was used to pack a column, which was placed on the DNA synthesizer. The assembled yield of the sequence, 5'-AAACAT, was carried out using phosphoramidite chemistry (Scheme 1). The final 21-nt product (still containing the 5'-DMT protecting group) was treated with 2 ml of 3:1 NH₄OH:ethanol at 50 °C for 12 h; the supernatant and washings were concentrated and applied to a Nensorb column for removal of failed sequences and deblocking of the CPG support, as described above. The fully deblocked 21-nt product was purified further by C₁₈ reverse-phase HPLC (Alltech 10 urn column, 10 mm × 25 cm) using a linear gradient of acetonitrile (5% initially, then increasing 0.5%/min up to 20%) in 0.1 M NH₄OAc, pH 7.0, at a flow rate of 4.0 ml/min. The overall yield of purified 3 was 28 mmol (2%); λₑₓ 260, 427 and 550 nm.

Antisense oligonucleotide 3a was prepared in the same fashion as 3, but using 2-6'-aminohexylaminophenazine di-N-oxide/CCI₄ for introduction of the phenazine di-N-oxide moiety. Following purifi-
Preparation of 5'-32P-End-labeled DNA—A 5-μg sample of chemically synthesized 40-nt DNA having a sequence identical with nucleotide 2691–2730 of eX174 (+)-strand DNA (49) (Fig. 1) was phosphorylated in 100 μl (total volume) of 50 mM Tris-HCl, pH 7.6, containing 10 mM MgCl2, 5 mM DTT, 100 μM spermidine, 100 μM EDTA, 1.2 μM [γ-32P]ATP (7000 Ci/mmoll, and 250 units of T4 polynucleotide kinase. The reaction mixture was incubated at 37 °C for 30 min, and the DNA was recovered by precipitation with ethanol. The radiolabeled DNA was purified by PAGE on a 20% gel, then by Nensorb chromatography (48). The yield of radiolabeled DNA was 4.4 μg (88%), containing 5.5 × 10^6 cpm.

Cleavage of Radiolabeled DNA by Antisense Oligonucleotides Containing Phenazine Di-N-oxide—In a typical experiment a 20-μl solution (total volume) of 10 mM sodium cacodylate, pH 7.5, containing 500 nM 5'-32P-labeled DNA, 50 μM phenazine-containing oligonucleotide, 10 mM EDTA, and 50 mM NaCl was heated at 65 °C for 10 min and slowly cooled to room temperature (~1.5 h). The cleavage reaction was initiated by adding DTT to a final concentration of 10 mM; the reaction was maintained at 25 °C for 24 h. For reactions carried out anaerobically, both the DNA-containing solution and DTT solution were purged with argon prior to mixing; the combined solution was again purged with argon for 15 min. The reactions were terminated by the addition of 4 μg of sonicated calf thymus DNA, 3 M NaOAc (to a final concentration of 0.3 M), and three volumes of cold ethanol. The precipitated DNA was recovered by centrifugation, rinsed with EtOH and dried. For reactions involving alkali treatment, the residue was dissolved in 20 μl of 0.1 M NaOH and heated at 90 °C for 10 min. The cooled solution was adjusted to 0.3 M NaOAc, concentrated, and treated with three volumes of EtOH for recovery of DNA. Analysis of the DNA was accomplished by dissolving the individual samples in 80% formamide containing 1 mM EDTA, 50 mM Tris-borate buffer, pH 8.3, 0.1% xylene cyanol, and 0.1% bromphenol blue. The resulting solution was heated at 90 °C for 5 min, quick-chilled on ice, and applied to a 20% denaturing polyacrylamide, 8 M urea gel. Electrophoresis was carried out in 90 mM Tris-borate, pH 8.3, containing 2.5 mM EDTA (3 h, 2200 V). DNA sequencing lanes were produced by the method of Maxam and Gilbert (50).

**RESULTS**

Synthesis of Oligonucleotides Linked to Phenazine Di-N-oxide—The syntheses of the antisense oligonucleotides (Fig. 1) were performed on a solid support (Scheme 1). Each oligonucleotide contains an N-alkylphosphoramidate linkage, as well as the phosphodiester bonds normally present in DNA and RNA. The strategy employed for oligonucleotide synthesis involved the use of phosphoramidite chemistry (39, 40) to establish the unmodified phosphate ester bonds between individual nucleosides. H-phosphonate chemistry (41–44) was employed for the elaboration of the N-alkylphosphoramidate linkages; oxidation was carried out by treatment with CCl4 (51) in the presence of the 2-(aminomethyl)aminophenazine of interest. A similar strategy for obtaining functionalized N-alkylphosphoramidates has been described recently by Agrawal and Tang (52).

After separation of the “failed” sequences from the desired product on a Nensorb column (48) and removal of the 5'-dimethoxytrityl protecting group with 0.1% CF3COOH, the individual oligonucleotides were analyzed by C18 reverse-phase HPLC, eluting with a linear gradient of acetonitrile in 0.1 M (alkyl)ammonium acetate buffer. As shown in Fig. 2a for a 15-nt oligomer, the product consisted primarily of material that eluted as a single peak of the poorly resolved diastereomers of 1; the diastereomeric mixture was purified in each case by preparative C18 reverse-phase HPLC prior to utilization for degradation of target DNA. The separation of diastereomers of 1 could be accomplished prior to removal of the DMT protecting group (Fig. 2c); a quantity of each isomer of DMT-1 was prepared in this fashion and subsequently deblocked. When the Rp and Sp samples of 1 were used to degrade a 40-nt single-stranded DNA containing a sequence complementary to 1, both isomers were found to effect degradation at the same site (see below). Accordingly, for ease of operation most of the experiments were performed using purified diastereomeric mixtures of the modified oligonucleotides.

Cleavage of a 40-nt Single-stranded DNA by Complementary Oligonucleotides Linked to Phenazine Di-N-oxide—Cleavage of the target DNA sequence was carried out by combining the 32P-end-labeled DNA with an oligonucleotide, then heating, and slowly cooling the combined solution to facilitate annealing. Reactions were initiated by the addition of DTT in the
the phenazine-di-N-oxide moiety (38); the reactions were maintained at 25 °C for 24 h. As shown in Fig. 3, PAGE analysis of the treatment of the 40-nt DNA target with oligonucleotide 1 revealed cleavage at several positions from G23 to T26 (Fig. 3, lane 7; cf. Fig. 1). Oligonucleotide 2, which contains an additional three nucleotides at its 5' terminus with the phenazine di-N-oxide linked to the penultimate 5' phosphate, produced damage localized from T20-T22 (lane 6). Oligonucleotide 3, which is 21 nt in length but has the phenazine di-N-oxide attached in the same position as oligomer 1, produced much less damage to the target DNA under the same conditions (lane 5). Also clear from the figure is the fact that some of the DNA cleavage products did not comigrate with the analogous Maxam-Gilbert cleavage products, the latter of which are (oligo)nucleotide 3'- phosphates (cf. lanes 2 and 7). Treatment of the reaction mixtures containing 1-3 with alkali (0.1 M NaOH, 90 °C, 10 min) prior to PAGE analysis changed the observed cleavage patterns substantially. Oligonucleotide 1 produced bands corresponding to G18 and G21; oligomer 3 afforded damage at the same two sites but to a lesser extent. Interestingly, oligonucleotide 2 produced a lesion predominantly at G21. In all cases, the bands observed after alkali treatment all comigrated with the Maxam-Gilbert bands, suggesting that the species formed initially by treatment with oligomers 1-3 were converted to oligonucleotides 3'- phosphates by alkali treatment.

In order to assess the extent to which the length of the alkyl linkers between the oligonucleotides and phenazine di-N-oxide moiety influenced the site(s) of cleavage and the relative extents of cleavage by oligomers 1 and 3, we prepared two additional oligonucleotides (1a and 3a, Fig. 4) that differed from 1 and 3 only in the length of the linker. As shown in Fig. 5, oligonucleotide 1a (having six methylene groups between the phosphate of the oligonucleotide and phenazine di-N-oxide moiety) produced a cleavage pattern rather similar to that afforded by the structurally related oligomer 1. Oligonucleotide 3a, which in common with 3 is 21 nt in length and has the phenazine di-N-oxide moiety attached between C7 and A8 (cf. Figs. 1 and 4), also produced little DNA damage. When the reaction mixture containing 1a was treated with alkali prior to PAGE analysis, the gel contained two strong bands corresponding to DNA cleavage.
at G10 and G11 (not shown). Alkali treatment of the reaction mixture containing 3a produced no band above background. Thus, the length of the alkyl linker did not appear to control the positions of DNA cleavage. Further, the extent of cleavage actually appeared to diminish somewhat for the oligonucleotides containing the longer linkers, suggesting that linker length per se did not limit the site or efficiency of cleavage by oligonucleotides 1-3.

Effect of Catalase and Superoxide Dismutase on DNA Cleavage—Because the reductive activation of phenazine di-N-oxide under aerobic conditions is believed to result in the formation of O2, we studied the effect of superoxide dismutase and catalase on DNA cleavage by oligonucleotides containing an attached phenazine di-N-oxide group. As shown in Fig. 6, the presence of catalase at a concentration of 0.5 μg/μl had no effect on DNA cleavage by oligonucleotide 1. In contrast, the presence of 0.1 μg/μl superoxide dismutase increased the amount of DNA cleavage, and resulted in the appearance of DNA cleavage at new sites from C14 to T17. Admixture of both superoxide dismutase and catalase diminished the increase in DNA cleavage obtained with superoxide dismutase alone. The extent to which superoxide dismutase enhanced DNA cleavage by oligomer 1 was also found to increase as the concentration of the enzyme was increased to 1.0 μg/μl (Fig. 7). In a control experiment, it was shown that superoxide dismutase produced no DNA cleavage when used together with an unmodified oligonucleotide having the same sequence as oligomer 1.

These results were consistent with the interpretation that phenazine di-N-oxide does produce superoxide anion when activated reductively under aerobic conditions (see Scheme 2 and Ref. 38); superoxide dismutase would presumably act to facilitate the production of H2O2, the latter of which can produce highly reactive·OH (53). That catalase had no effect when added to the reaction mixture alone but diminished the increase in strand scission mediated by superoxide dismutase, argues strongly that O2, but not·OH, is produced by reductive activation of the phenazine di-N-oxide moiety in 1 under aerobic conditions.

Also evident in Figs. 6 and 7 are the presence of relatively strong DNA cleavage bands, the intensity of which was not changed significantly by the addition of catalase or superoxide dismutase. Further, as noted above, some of these bands clearly migrated differently than oligonucleotide 3'-phosphates or oligonucleotide 3'-phosphoroglycolates, which are known products of the destruction of DNA by diffusible oxygen radicals (54-56). These observations suggested strongly that some of the observed DNA cleavage (e.g. in Figs. 3 and 5) had resulted from a mechanism unrelated to the action of diffusible oxygen radicals.

Cleavage and Cross-linking of the DNA Substrate by Oligonucleotide 2—Although the relaxation of supercoiled covalently closed circular DNA by a phenazine di-N-oxide derivative was dependent on the presence of a reducing agent such
as DTT or NADPH (38), it seemed possible that maintenance of the phenazine di-N-oxide moiety in proximity to the DNA target by virtue of covalent attachment to a complementary oligonucleotide would facilitate reaction with the DNA substrate by processes not readily accessible to phenazine di-N-oxide itself.

In fact, when oligonucleotide 2 was annealed to the 40-nt DNA substrate and the formed duplex was maintained under ambient conditions without added reducing agent, a cross-linked product was formed (Fig. 8, lanes 10–13). This product was produced in the absence of light (cf. lanes 10 and 12) and also in the presence of DTT, albeit to a slightly lesser extent (lanes 10 versus 11 and 12 versus 13). When the reaction mixtures were heated at 90 °C for 5 min prior to PAGE analysis, a DNA cleavage band appeared between Tz2 and Tz3 (lanes 4–7). Again, the appearance of this band was not dependent on the presence of light. These results seemed consistent with the interpretation that oligonucleotide 2 alkylated the 40-nt single-stranded DNA target, producing the observed cross-linked product, and that subsequent heating effected strand scission of the alkylated DNA.

DNA Degradation by Rp-1 and Sp-1—The finding that oligonucleotides containing attached phenazine di-N-oxide moieties can alkylate complementary single-stranded DNA suggested that the orientation of the phenazine di-N-oxide moiety relative to the formed DNA duplex should influence the rate of alkylation (Fig. 9). In fact, the isomer of 1 that eluted more quickly from a C18 reverse-phase HPLC column as its DMT derivative was found to alkylate the 40-nt DNA substrate somewhat more effectively than its diastereomer (Fig. 10).

Cleavage of a Modified DNA Substrate by Oligonucleotide 2—In order to study the possible cleavage of the 40-nt DNA target by diffusible oxygen radicals resulting from reductive activation of the phenazine di-N-oxide moiety in the antisense oligomers, we sought to diminish the efficiency of the alkylation reaction. Since oligonucleotide 2 appeared to react

with the 40-nt DNA target primarily by alkylation of G21, we prepared a modified DNA containing deoxycytidine at position 21 (Fig. 1). Further, since reductive activation of phenazine di-N-oxide under anaerobic conditions appeared to afford 'OH rather than O2− (Scheme 2) (38), the reaction of 2 with the modified DNA was carried out anaerobically. As shown in Fig. 11, this procedure resulted in DTT-dependent DNA cleavage of moderate intensity at several sites in proximity to the position of the attached phenazine di-N-oxide moiety (i.e., G18 to Tz2, with the most intense cleavage at Cz1 and Tz2). Further, the observed products all comigrated with products of the Maxam-Gilbert sequencing procedure, suggesting that they were oligonucleotide 3'-phosphates.

**FIG. 9.** Implied orientation of the phenazine di-N-oxide moiety in the duplex formed by annealing of Rp-1 and Sp-1 to the 40-nt DNA target.

**FIG. 10.** Cleavage of the 40-nt DNA oligomer by the Rp and Sp-isomers of 1. Reaction mixtures containing oligonucleotide 1 as a mixture of diastereomers, or as single diastereomers, were incubated with the 40-nt DNA oligomer in the presence or absence of DTT. The reaction mixtures were maintained at 25 °C for 24 h; the recovered DNA samples were heated at 90 °C for 5 min prior to analysis on 20% polyacrylamide gels. Lane 1, G + A-lane; lane 2, G-lane; lanes 3 and 4, DNA alone; lanes 5 and 6, diastereomeric-1; lanes 7 and 8, isomer of 1 that eluted first from reverse-phase HPLC as its DMT derivative; lanes 9 and 10, slower eluting isomer of 1.

**DISCUSSION**

It has been shown previously (38) that an aminoalkyl derivative of phenazine di-N-oxide undergoes reductive activation in the presence of agents such as DTT or NADPH and that the activated species mediates DNA strand scission as judged by the relaxation of a supercoiled φX174 replicative
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The latter process is known to be slow (53), but the production of \( \cdot \text{OH} \) from \( \text{H}_2\text{O}_2 \) can be facilitated by the presence of certain metal ions (e.g. \( \text{Fe}^{2+} \)) (57); it may be noted, however, that EDTA had no effect on the facility of plasmid DNA relaxation by reductively activated phenazine di- or mono-N-oxides. Alternatively, peroxide could conceivably react directly with the radical anion derived from phenazine di-N-oxide (38, 53), i.e.

\[ \text{R}^* + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \cdot \text{OH} + \text{R} \]

Experimental support for Scheme 2 also derives from the observation that dimethyl sulfoxide, a known scavenger of \( \cdot \text{OH} \), inhibited phenazine di-N-oxide-mediated DNA relaxation (38). In the present study, it has also been shown that following reductive activation under aerobic conditions, an oligonucleotide (1) containing a phenazine di-N-oxide moiety was able to effect greater damage to a complementary target oligomer upon admixture of superoxide dismutase, and that catalase suppressed much of the enhanced damage produced by superoxide dismutase (Figs. 6 and 7). In the context of the foregoing discussion, it is logical to conclude that activation of oligonucleotide 1 (already bound to the target DNA oligomer as part of a duplex) resulted in the formation of superoxide in proximity to the DNA target. That the species which effects DNA damage is actually \( \cdot \text{OH} \) produced from \( \text{O}_2^* \) is suggested by the enhancement of DNA damage by superoxide dismutase (which produces \( \text{H}_2\text{O}_2 \) from \( \text{O}_2^* \)), by the diminution of superoxide dismutase-induced DNA cleavage by catalase, and by the observation that phenazine di-N-oxide-mediated DNA cleavage is suppressed by dimethyl sulfoxide.

In the aggregate, these data suggest that phenazine di-N-oxide can be activated reductively under conditions that can obtain within an intact cell, producing species \( \cdot \text{OH} \) capable of mediating DNA and RNA strand scission (4-7, 53, 55). The fact that oligonucleotides containing attached phenazine di-N-oxide produced DNA damage at several sites in proximity to the site at which this group was attached (e.g. Fig. 11) supports the intermediacy of oxygen free radicals in the observed cleavage of DNA (2, 4-7). It may be noted that in addition to its ability to be activated with a reductant (NADPH) present in abundance in intact cells, the reductive activation of phenazine di-N-oxide under aerobic conditions can potentially produce \( \text{O}_2^* \) catalytically, which should be of great value in the design of antisense oligonucleotides with improved properties.

In addition to the evidence for oxygen radical-mediated DNA degradation, our studies of oligonucleotides 1-3 clearly indicate the existence of another mechanism for DNA degradation. This pathway is evident in Fig. 8, which illustrates the cross-linking of oligonucleotide 2 to DNA in a reaction that was not dependent on the presence of a reducing agent. Also illustrated in Fig. 8 is the appearance of a band corresponding to cleavage of the DNA substrate when the reaction mixture containing cross-linked DNA-oligonucleotide 2 complex was heated prior to PAGE analysis. This observation suggests, but obviously does not prove, that the new bands may have been derived from the cross-linked DNA-oligonucleotide 2 complex. Because heating of DNA samples for purposes of denaturation is a routine part of the procedure employed for polyacrylamide gel analysis, we believe that the bands that appear at the same position in other gel analyses (e.g. Figs. 3 and 5) must also represent thermally induced cleavage at a site of DNA oligonucleotide cross-linking; in

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K. Nagai, unpublished results.
fact a number of the polyacrylamide gels obtained reflected the presence of limited amounts of the residual cross-linked products (not shown).

The nature of the chemistry that leads to cross-linking of the DNA target and the modified oligonucleotides seems likely to involve deoxyguanosine residues, based on the observation that alkali treatment of the thermally induced cleavage products gives bands that comigrate with bands produced in the G-lanes of Maxam-Gilbert sequencing reactions (Fig. 3). The G-specific reactions in the Maxam-Gilbert sequencing protocol involve alkylation of deoxyguanosine at N-7, followed by depurination to afford an apurinic acid, the latter of which can undergo strand scission. The chemistry of strand scission has been studied both for DNA and RNA (58); the initially formed product includes a 5′-oligonucleotide having the five-carbon fragment derived from (deoxy)ribose at the 3′ terminus. This intermediate, which is known to migrate more slowly on polyacrylamide gels than the same oligonucleotide containing a 3′-phosphate (59), can be converted to the oligomer having a 3′-phosphate by treatment under basic conditions (59, 60). Alkylation of guanosine at N-7 has been documented in a number of cases both for small molecules that bind to DNA (61), as well as for oligonucleotides containing electrophilic prosthetic groups (62-64).

The present results, reflected in Figs. 3, 5, 8, and 10, contain features strongly reminiscent of those obtained in other systems that proceed via alkylation of the N-7 position in deoxyguanosine. These include the appearance of a cross-linked product (Fig. 8), its putative thermal conversion to DNA cleavage products that do not comigrate with authentic oligonucleotide 3′-phosphates (Figs. 3 and 8), and conversion of the latter to faster-moving bands that comigrate with the bands in Maxam-Gilbert G-lanes (Fig. 3). On this basis we suggest (Scheme 3) that the modified oligonucleotides studied here degrade the 40-nt DNA primarily via alkylation of deoxyguanosine residues at N-7. As noted in the scheme, there are several potentially electrophilic sites on phenazine di-N-oxide; the site(s) actually employed for deoxyguanosine alkylation have not yet been characterized. Further, we cannot exclude the possibility that alkylation of deoxyguanosine occurs at a position other than N-7, producing a thermally unstable cross-linked intermediate.

Also of interest in the context of deoxyguanosine alkylation are the differences in G alkylation effected by oligonucleotides 1-3 (Fig. 3). As is clear from the figure, the 15-nt oligomer 1, having the phenazine di-N-oxide moiety between the first two nucleosides (Fig. 1), effected alkylation of G15 and G21, but primarily G21, while oligonucleotide 3 (having the phenazine di-N-oxide moiety attached at the same position as in 1, but within a presumptive double-stranded region in the formed DNA duplex) gave much less alkylation at G15 or G21. Computer-assisted molecular modeling studies using the MACROMODEL program (65) suggest that the phenazone di-N-oxide moiety in oligonucleotides 1 and 3 cannot react with N-7 of G15 via intercalation because the linker connecting the heterocycle and oligonucleotide is too short. The observations that the patterns of reactivity did not change when the linkers were extended (Fig. 5), and that oligonucleotide 1 actually preferentially alkylated G21, rather than G15, suggests strongly that alkylation occurred with greater facility within single-stranded regions of DNA. It is clear from modeling studies that alkylation of G21 by the phenazine di-N-oxide moiety in 1 would require “folding” of that (single-stranded) region of the DNA target that extends beyond the presumptive 15-nt duplex formed with oligonucleotide 1.

While the exact orientation of the phenazine di-N-oxide moiety in the formed duplex between 1 and the DNA target is not entirely clear, it is still not surprising that one of the two diastereomers alkylates the 40-nt DNA target with somewhat greater efficiency. No assignment of absolute stereochemistry can be made based on the data available at present, but it may be noted that in the model shown in Fig. 9, which assumes maintenance of the G18-C1 base pair between the target DNA and oligonucleotide 1, the Sp-isomer orients the phenazine di-N-oxide moiety in closer proximity to the DNA target.

Finally, it may be noted that while DNA degradation mediated by the modified oligonucleotides proceeded with much greater facility via deoxyguanosine alkylation than by free radical-mediated DNA strand scission, the specific heterocyclic di-N-oxide employed for these experiments has not been optimized for reductive generation of oxygen free radicals. In fact, while reductive activation of phenazone di-N-oxide can be achieved with NADPH in quantities sufficient to mediate DNA degradation, stoichiometric reduction of phenazone di-N-oxide cannot be achieved under reasonable conditions even with a large excess of DTT (as judged by the incomplete reductive deoxygenation of phenazone di-N-oxide when the substrate was reduced under anaerobic conditions (38)). Structural alteration of the heterocyclic di-N-oxide moiety may permit the design of an antisense oligonucleotide that destroys complementary nucleic acid targets primarily by the use of oxygen-free radicals.

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