Efficient, Specific Interstrand Cross-Linking of Double-Stranded DNA by a Chlorambucil-Modified, Triplex-Forming Oligonucleotide

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Selective and irreversible gene inactivation should be attainable by sequence-targeted interstrand cross-linkage of the genomic DNA. Oligodeoxynucleotides (ODNs) capable of forming sequence-specific triple helices with double-stranded DNA targets and which bear alkylating groups for covalent modification of the target strands offer a means of testing this hypothesis. Covalent modification of *one* strand of the targeted duplex has been demonstrated for polypyrimidine ODNs conjugated to a single reactive group.[1] These ODNs irreversibly react with the homopurine-containing strand within the triplex to block transcription[2] and possibly replication.

ODNs which react with only one strand of the duplex target would be expected to exert transitory effects due to DNA repair. Triple-helix-forming ODNs which covalently react with both strands of a DNA target might mutationally inactivate the targeted gene through an error-prone repair pathway, thus permanently inhibiting gene expression. ODNs bearing the pendant bifunctional photoactive cross-linker psoralen can meet this specification[3] but will be limited to topical applications in therapeutics due to the requirement for activation by ultraviolet light. We and others have found that electrophilic moieties like α-haloacetamides[1c,4] react too readily with biological amines and thiols.[5] Nitrogen mustards offer the advantage of low reactivity until the formation of a highly reactive aziridinium ion and have been used in ODN-directed duplex[6] and triplex[1a,b] monofunctional cross-linking. We report here that triple-helix-forming ODNs modified at the 3′- and 5′-ends with the clinically used nitrogen mustard chlorambucil are able to rapidly alkylate both strands of a DNA target with excellent efficiency and specificity.

The ODNs used in this study are shown in Figure 1. The double-stranded target formed by **I** + **II** is a 40-mer, with **II** containing a 23-base-long A-rich homopurine tract. Polypyrimidine 20-mers **III**–**VI** form the triplex by binding, with Hoogsteen H-bonding, to the purines of **II** within the **I**–**II** duplex. Chlorambucil (ClAmb) was coupled to **IV**–**VI** through 3′ and/or

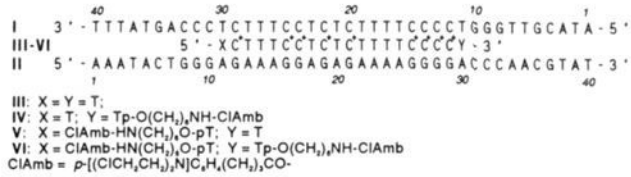

Figure 1. ODNs used in this study. The double-stranded target formed by ODNs **I** and **II** contains a homopyrimidine/homopurine run which differs by only two base pairs from the target used by Maher II et al.[15]

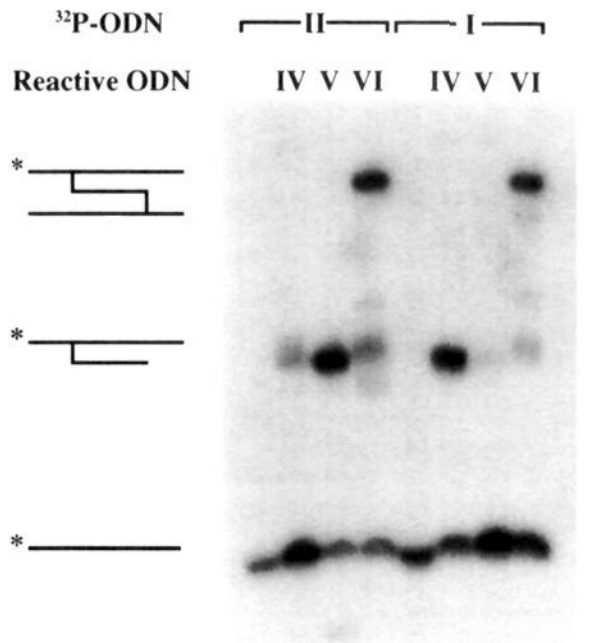

Figure 2. Analysis of reaction mixtures containing 5′-^{32}P-labeled duplex **I** + **II** and reactive ODNs **IV**–**VI**. Preformed duplex (2×10^{-8} M labeled strand and 4×10^{-8} M unlabeled strand) was incubated for 6 h at 37 °C with triple-helix-forming ODNs **IV**–**VI** (1×10^{-7} M) in 140 mM KCl, 10 mM $MgCl_2$, 1 mM spermine, and 20 mM HEPES, pH 6.0. Aliquots were analyzed on an 8% denaturing polyacrylamide gel. The first lane in each series is a control reaction mixture which lacked any triplex-forming ODN. The structures of the products are schematically represented to the left of the gel.

5′-aminohexyl phosphate groups[7] added to the end(s) of the ODN at the time of synthesis.[8] All of the triple-helix-forming ODNs contained 5-methylcytosine (C^+) residues to stabilize triplex formation.[9]

Alkylation of the targeted duplex strands by reactive ODNs **IV**–**VI** was monitored by denaturing polyacrylamide gel electrophoresis (Figure 2). Two classes of reaction products were resolved, a mono-cross-linked product linking the reactive ODN with one target strand, and a bis-cross-linked product linking it with both target strands. These results show that, for this target,

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(2) Young, S. L.; Krawczyk, S. H.; Matteucci, M. D.; Toole, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10023.

(3) (a) Takasugi, M.; Guendouz, A.; Chassignol, M.; Decout, J. L.; Lhomme, J.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5602. (b) Duval-Valentin, G.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 504. (c) Giovannangeli, C.; Thuong, N. T.; Helene, C. *Nucleic Acids Res.* **1992**, *20*, 4275. (d) Giovannangeli, C.; Rougee, M.; Garestier, T.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8631. (e) Grigoriev, M.; Praseuth, D.; Guieysse, A. L.; Robin, P., Thuong, N. T.; Helene, C.; Harel-Bellan, A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3501.

(4) Meyer, R. B.; Tabone, J. C.; Hurst, G. D.; Smith, T. M.; Gamper, H. *J. Am. Chem. Soc.* **1989**, *111*, 8517.

(5) For instance, the reaction of a bromoacetamidopropyl-derivatized ODN with 1 mM cysteine is 90% complete after 1 h at 37 °C.

(6) (a) Knorre, D. G.; Vlassov, V. V. *Genetica* **1991**, *85*, 53. (b) Knorre, D. G.; Zarytova, V. F. In *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS*; Wickstrom, E., Ed.; Wiley-Liss: New York, 1991; p 195.

(7) The reagent for the 5′-aminohexyl modification is commercially available from Glen Research, Sterling, VA. The method for the 3′-aminohexyl modifications is as described: Petrie, C. R.; Reed, M. W.; Adams, A. D.; Meyer, R. B., Jr. *Bioconjugate Chem.* **1992**, *3*, 85–87.

(8) The coupling reaction employed the 2,3,5,6-tetrafluorophenyl ester of chlorambucil. Modified ODNs were purified by reverse-phase chromatography, concentrated by extraction with 1-butanol, and precipitated in acetone containing 2% $LiClO_4$. All manipulations were performed in ice to minimize reaction of the bis-*N*,*N*-(2-chloroethyl)amine residue. The modified ODNs were stored in aqueous solution at −70 °C. All of the chlorambucil-modified oligomers showed a single band on reverse-phase HPLC. Reaction of these modified ODNs with 1 M tetraethylenepentamine in water for 6 h at 37 °C, followed by electrophoretic analysis, indicated that >85% of the ODN possessed the expected alkylating ability.

(9) (a) Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059. (b) Collier, D. A.; Thuong, N. T.; Helene, C. *J. Am. Chem. Soc.* **1991**, *113*, 1457.

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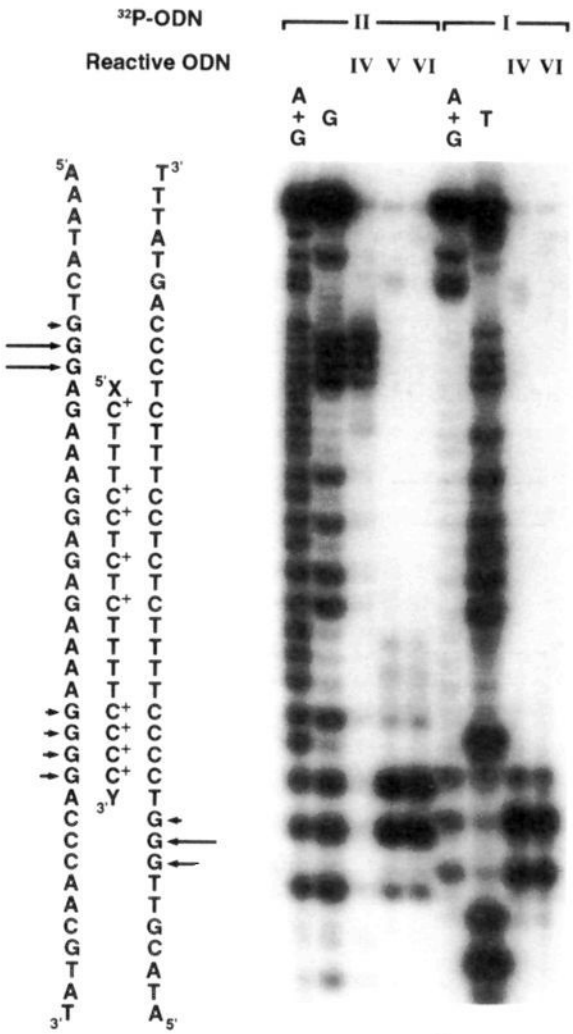

Figure 3. Determination of the sites of alkylation in 5′-^{32}P-labeled duplex strands **I** and **II** by reactive ODNs **IV**–**VI**. The major cross-linked products generated by each reactive ODN were isolated by denaturing PAGE (see Figure 2), and the products were eluted from the appropriate sections of the gel which had been cut out. They were treated for 15 min at 95 °C in 1 M pyrrolidine[16] to cleave the DNA backbone at the sites of alkylation, evaporated twice in water, and run on an 8% denaturing polyacrylamide gel. The positions of alkylation are indicated in the triple strand to the left of the gel. For reactive ODN **VI**, only the interstrand bis-cross-linked products were analyzed; hence, no cleavage at G26–G29 in target strand **II** is observed with this ODN. Because of the low level (<5%) of modification of target strand **I** by reactive ODN **V**, no attempt was made to analyze these reaction products.

the 5′-chlorambucil group preferentially alkylated target strand **II**, while the 3′-chlorambucil group preferentially alkylated target strand **I**. When both chlorambucil residues were present on the same ODN, efficient alkylation of both strands occurred.[10] These results were obtained using a molar ratio of reactive ODN to double-stranded target of 5:1 in a pH 6.0 buffer containing physiologically relevant concentrations of K^+, Mg^{2+}, and spermine.[11] Under these conditions in 6 h at 37 °C,[12] **IV** mono-cross-linked to **I** with 62% efficiency, **V** mono-cross-linked to **II** with 77% efficiency, and **VI** bis-cross-linked to **I** and **II** with 44% efficiency. When the molar ratio was increased to 100:1, the efficiency of bis-cross-linkage with **VI** increased to 80%.

The double-stranded target used here was synthesized with guanines at positions 8, 9, and 10 on both strands **I** and **II**. These bases bracketed both ends of the reactive ODN within the triple-stranded complex and provided, as suggested by molecular modeling studies, sites for alkylation by the 3′- and 5′-chlorambucil groups. To determine exactly which guanines were alkylated, we isolated the mono- and bis-cross-linked complexes from the gel shown in Figure 2 and treated them with pyrrolidine at 95 °C to cleave the DNA backbone at the sites of reaction. The sequencing gel in Figure 3 shows that the 5′-chlorambucil group alkylated residues G9 and G10 on target strand **II** while the 3′-chlorambucil group alkylated residues G-8 and G-9 on target strand **I**. A minor reaction product between **IV** and **II** was also analyzed and the site of alkylation shown to be the guanines at positions 25–28 involved in triple-strand formation. The accessibility of these guanines to the 3′-chlorambucil group suggests that the 3′-terminus of the triple strand (as defined by the polarity of the homopurine-containing duplex strand) may be relatively unstable due to the abundance of C^+:G:C triplets.[3d,13]

In summary, we have demonstrated efficient chemical cross-linkage of a polypyrimidine ODN to *both* the Watson and Crick strands within a triple-stranded complex. Alkylation occurred at the desired guanines flanking the triplex region, as predicted by molecular modeling. By attaching the same groups to ODNs which are able to form stable triplexes at physiological pH, it should be possible to directly introduce sequence-specific cross-links into the DNA of living cells and thereby cause permanent gene inactivation through error-prone repair. This could find application in the treatment of chronic viral diseases characterized by integrated viral genomes. Other duplex sequences flanking the triplex region could react with the chlorambucil group, since the N7 positions of both guanine and adenine can be alkylated by this nitrogen mustard. In the case of flanking 5′-GXC-3′ sequences, a single chlorambucil group might react with the purines to generate the bis-cross-link.[14]

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(10) No cross-linkage was observed when the **I** + **II** duplex or the **I** + **II** + **III** triplex was incubated in the presence of a randomized 20-mer containing a terminal chlorambucil group.

(11) The pH 6.0 buffer used in this study was required for protonation of the 5-methylcytosine residues in ODNs **III**–**VI**. Gel retardation analysis indicated that ODN **III** does not form a stable triplex with the **I** + **II** duplex at pH 7.2 (data not shown). The levels of monovalent and multivalent cations employed in this study approximate those found in the intracellular environment, see: Alberts, B. *Molecular Biology of the Cell*; Garland: New York, 1989; p 304.

(12) The time course of reaction between **V** and **II** within a triple helix was followed at 37 °C by periodically removing reaction aliquots, which were then stored at −20 °C until all samples could be analyzed electrophoretically at the same time. After 45 min of incubation, alkylation was 50% complete using conditions described in the text (data not shown).

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(15) Maher, L. J., II; Wold, B.; Dervan, P. B. *Science* **1989**, *245*, 725.

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