Template-Directed Cross-Linking of Oligonucleotides: Site-Specific Covalent Modification of dG-N7 within Duplex DNA

Robert S. Coleman*,[1] and Edward A. Kesicki

Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208

Received July 24, 1995

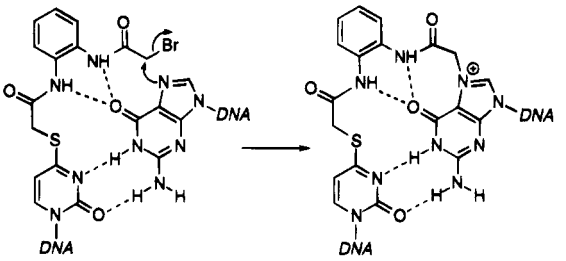
The interaction of small molecules with DNA is a fundamental mechanism in the chemotherapy of cancer and viral diseases. Although conventional small molecule drugs have proven effective as antitumor and antiviral agents, these compounds typically lack unique sequence-selective binding.[2] In contrast, oligonucleotides can exploit the recognition elements inherent in Watson–Crick or Hoogsteen base-pairing to target specific information contained within a DNA or mRNA molecule, thereby potentially achieving unique sequence selectivity.[3] We describe our preliminary studies on the use of oligonucleotide probes to direct chemically reactive functionality to targeted sequences of nucleic acids. In previous approaches to the problem of sequence selective covalent modification of oligonucleotides,[4] groups containing reactive functionality have been attached to short oligonucleotides complementary to discrete sequences of single- or double-stranded DNA.[5,6] Duplex (or triplex) formation delivers reactive functionality to the targeted sequence of the oligonucleotide. Herein, we describe methods to tether chemically reactive functionality *within* a targeted oligonucleotide sequence, and we report the high-yielding covalent modification of *specific* functional groups on the target oligomer. We have also examined the *sequence-dependence* of the covalent bond formation.

We have previously reported the incorporation of 4-thio-2′-deoxyuridine ($d^{S4}U$) into DNA by solid-phase synthesis, and we demonstrated the ability to chemoselectively modify the thiocarbonyl group in a post synthetic manner.[7,8] Now, in a realization of our goal to use antisense probes to form covalent cross-links within duplex DNA, we report the use of 4-thio-2′-deoxyuridine-containing oligomers as agents for specific dG–N7 adduct formation. We describe the incorporation of electrophilic groups into DNA within the major groove using the thiocarbonyl group of $d^{S4}U$; upon duplex formation these electrophiles are suitably positioned to react with the nucleophilic guanine–N7 to form covalent lesions.

Cross-linking studies were performed using a modified T7 RNA polymerase promoter sequence as the probe strand,[9] in which individual dC residues were replaced successively with $d^{S4}U$ bases. The target strands were designed so that deoxyguanosine residues[10] would be positioned complementary to the electrophilic base. Probes **2–4** were designed to examine the positional selectivity of cross-link formation by competition between the three contiguous dG residues (G_7, G_8, G_9) in the target strand.

						1a			**2a**	**3a**	**4a**		**5a**		($X = d^{S4}U$)		
						1b			**2b**	**3b**	**4b**		**5b**		($X = d^{RS4}U$)		
probe	5′–T_1	A	A	T	A	X_6	G	A	X_9	X_{10}	X_{11}	A	X_{13}	T	A	T	A_{17}–3′
target	3′–A_{17}	T	T	A	T	G_{12}	C	T	G_9	G_8	G_7	T_6	G_5	A	T	A	T_1–5′

For cross-linking probes, we designed an *o*-phenylenediamine tether to append an α-bromoacetamide in proximity to the complementary dG–N7. Design considerations included tether length and rigidity, electrophile reactivity, and complementarity of fit within the major groove.



Treatment of 17-mers **1a-5a** (1OD, 20 μL), prepared according to our previously described protocol,[8,11] in phosphate buffer (100 mM, pH 8, 50 μL) with *N,N′*-bis-(bromoacetyl)-*o*-phenylenediamine (**6**, 0.5 mg) in DMF (20 μL) at room temperature effected chemoselective S-alkylation of the $d^{S4}U$-residue[8] to afford the corresponding probe oligonucleotides **1b-5b** ($X = d^{RS}U$) after purification by spin-column gel filtration (Sephadex G-25, H_2O) and *n*-butanol precipitation. Complementary target oligomers were 5′-end labeled with [γ-^{32}P]-ATP and T4 kinase. The electrophilic strands **1b-5b** (30–100 × excess) were annealed with the target strand in phosphate buffer (pH 7.2, 10 mM, 15 μL) containing 130 mM NaCl for 2 h at 37 °C. Denaturing 20% polyacrylamide gel electrophoresis (PAGE) (0.3 mm, 7 M urea, 20% formamide, 50 mM Tris·borate, pH 8.3, 1 mM EDTA) and autoradiographic detection were used to analyze for cross-link formation. Oligomers containing covalent lesions were subjected to piperidine-induced depurination/strand cleavage[12] (1 M piperidine, 95 °C, 20 min), and the cleavage products were analyzed by denaturing PAGE by comparison with the Maxam–Gilbert G-specific lane.[13]

In Figure 1, gel electrophoresis data are presented for cross-linking between electrophiles appended at X_6, X_9,

(1) Recipient of a Dreyfus Foundation Distinguished New Faculty Award (1989–1994), an American Cancer Society Junior Faculty Research Award (1990–1993), the American Cyanamid Young Faculty Award (1993–1996), and an Alfred P. Sloan Foundation Research Fellowship (1995–1997).

(2) *Molecular Aspects of Anticancer Drug–DNA Interactions*; Neidle, S., Waring, M., Eds.; CRC Press: Boca Raton, FL, 1993. Dervan, P. B. *Science* **1986**, *232*, 464. Hurley, L. H.; Boyd, F. L. *Annu. Rep. Med. Chem.* **1987**, *22*, 259. Hurley, L. H. *J. Med. Chem.* **1989**, *32*, 2027.

(3) *Antisense Research and Applications*; Crooke, S. T., Lebleu, B., Eds.; CRC Press: Boca Raton, FL, 1993. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*; Cohen, J. S., Ed.; CRC Press: Boca Raton, FL; 1989. Kiely, J. S. *Annu. Rep. Med. Chem.* **1994**, *29*, 297. Matteucci, M. D.; Bischofberger, N. *Annu. Rep. Med. Chem.* **1991**, *26*, 287.

(4) Belikova, A. M.; Zarytova, V. F.; Grineva, N. I. *Tetrahedron Lett.* **1967**, 3557.

(5) Khalimskaya, L. M.; Levina, A. S.; Zarytova, V. F. *Biomed. Chem. Lett.* **1994**, *4*, 1083. Webb, T. R.; Matteucci, M. D. *Nucl. Acids Res.* **1986**, *14*, 7661. Tabone, J. C.; Stamm, M. R.; Gamper, H. B.; Meyer, R. B., Jr. *Biochemistry* **1994**, *33*, 375. Povsic, T. J.; Strobel, S. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 5934. Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 9428.

(6) For reviews, see: Knorre, D. G.; Vlassov, V. V. *Genetica* **1991**, *85*, 53. Uhlmann, E.; Peyman A. *Chem. Rev.* **1990**, *90*, 543. Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 165.

(7) Coleman, R. S.; Siedlecki, J. M. *J. Am. Chem. Soc.* **1992**, *114*, 9229.

(8) Coleman, R. S.; Kesicki, E. A. *J. Am. Chem. Soc.* **1994**, *116*, 11636.

(9) The normal T_{10}·dA_8 base pair in the promoter sequence was changed to $d^{S4}U$·dG.

(10) dG was chosen because of the nucleophilicity of the N7 position and due to the potential base-pair match with an S-alkyl $d^{S4}U$.

(11) Coleman, R. S.; Siedlecki, J. M. *Tetrahedron Lett.* **1991**, *32*, 3033.

(12) Mattes, W. B.; Hartley, J. A.; Kohn, K. W. *Biochim. Biophys. Acta* **1986**, *868*, 71.

(13) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560.

0022-3263/95/1960-6252$09.00/0

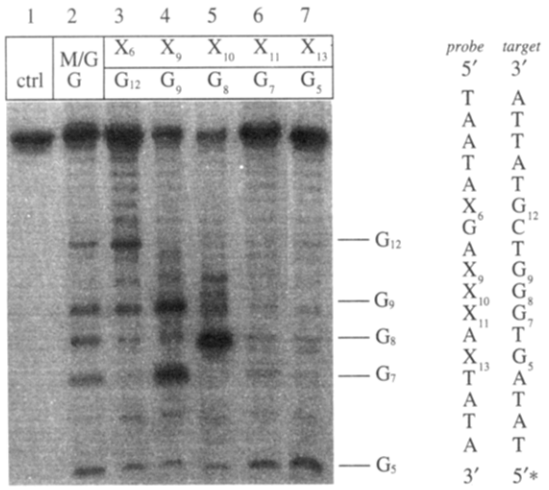
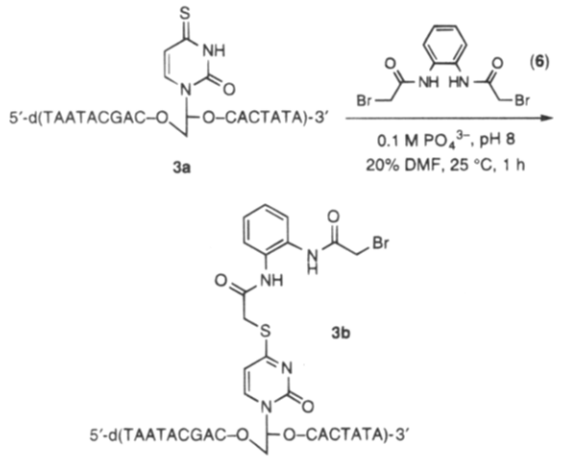


Figure 1. Denaturing PAGE autoradiogram of the reaction of **1b**-**5b** with ^{32}P 5′ end-labeled target after treatment with 1 M piperidine (95 °C, 20 min): lane 1, ^{32}P 5′-end-labeled target; lane 2, Maxam−Gilbert G-specific cleavage;[13] lanes 3−7, piperidine-treated probe-target duplexes.



X_{10}, X_{11}, or X_{13} and dG residues on the complementary strands. Data are shown for piperidine-treated oligomers and, hence, reveal higher mobility bands only at sites of dG−N7 alkylation. Efficient cross-link formation was observed by PAGE with probe **3b** (X_{10}), where alkylation occurred in high yield (74%) at the complementary dG_8 (lane 5). Probe **2b** (X_9) formed cross-links not only to the complementary dG_9 (37%), but also to dG_7 (36%), two bases removed to the 5′-side (lane 4). Conversely, probe **4b** (X_{11}) failed to cross-link to the complementary dG_7 and instead alkylated the accessible dG_5 (8%), two bases to the 5′-side, with low efficiency (lane 6). Also occurring with modest efficiency was cross-link formation between (**1b**) X_6 and dG_{12} (21%, lane 3) and between (**5b**) X_{13} and dG_5 (23%, lane 7). Product yields were estimated from scanned images of PAGE autoradiograms. In all cases, the covalently linked duplexes underwent depurination and strand cleavage upon treatment with piperidine, indicating that alkylation had occurred at N7 of dG.

We postulated that the absence of cross-linking with **4b** ($X_{11} \rightarrow dG_7$) was due to steric hindrance by the C5-methyl group of the proximal T_6 residue, which projects into the DNA duplex major groove. Computer models of the targeted sequence 5′-TG-3′ support this supposition, since in the standard B-DNA model[14] the C5-methyl of T is juxtaposed directly above the adjacent dG−N7 and would block the approach of electrophiles, thereby preventing cross-link formation. In the reversed target sequence 5′-GT-3′, the C5 methyl of T_6 is fairly remote from the dG_5−N7. This may explain why cross-linking was seen with probe **5b** ($X_{13} \rightarrow dG_5$), but not with **4b** ($X_{11} \rightarrow dG_7$). To confirm this hypothesis we prepared the oligonucleotide target wherein the T_6 was replaced with dU_6, thereby removing the C5-methyl impediment. In Figure 2, data are presented comparing the effect of T_6 (lane 1) versus dU_6 (lane 2) on cross-link formation between $X_{11} \rightarrow dG_7$. Removal of this steric block now enabled effective cross-link formation between $X_{11} \rightarrow dG_7$ (lane 2) and increased the amount of alkylation between $X_{11} \rightarrow dG_5$.

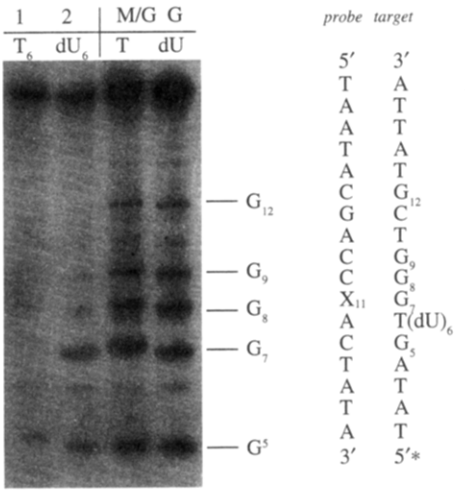


Figure 2. Denaturing PAGE autoradiogram of the reaction of **4b** with ^{32}P end-labeled target strands, after treatment with 1 M piperidine (95 °C, 20 min): lane 1, T_6-containing cleavage products; lane 2, dU_6-containing cleavage products.

The efficient cross-link formation that we have observed with *o*-phenylenediamine-linked α-bromoacetamides parallels that observed with the nitrogen mustard mechlorethamine,[15] where the most effective monoadduct and cross-link formation was seen at N7 of the central dG of the sequence 5′-GGG-3′. We observed less effective cross-linking to isolated dG residues, in accord with the expected decreased nucleophilicity of such bases. Our *o*-phenylenediamine tether was found to target dG residues in the complementary strand with high efficiency and was found to alkylate dG residues two bases in the 5′ direction with lower efficiency. This result appears to be the result of two different tether conformations, one that positions the electrophilic carbon near the complementary dG−N7 and the other that is capable of reaching two bases (but not one base) in the 5′ direction on the target strand. No example exists in the literature that suggests steric inhibition by a 5′-T residue of dG−N7 alkylation or cross-linking,[16] and our demonstration that removal of the C5-methyl group of T_6 enables alkylation of the adjacent dG residue illustrates the directionality of steric requirements in sequence selective cross-linking of DNA.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM-47991). We thank American Cyanamid, Bristol-Myers Squibb, and Pfizer for their generous support.

JO9513525

(14) Arnott, S.; Campbell-Smith, P.; Chandresekharan, P. In *CRC Handbook of Biochemistry*; CRC Press: Boca Raton, FL, 1976; Vol. 2.

(15) Hopkins, P. B.; Millard, J. T.; Woo, J.; Weidner, M. F.; Kirchner, J. J.; Sigurdsson, S. Th.; Raucher, S. *Tetrahedron* **1991**, *47*, 2475.

(16) Mattes, W. B.; Hartley, J. A.; Kohn, K. W. *Nucl. Acids. Res.* **1986**, *14*, 2971. Kohn, K. W.; Hartley, J. A.; Mattes, W. B. *Nucl. Acids Res.* **1987**, *15*, 10531.