Sequence-dependent cleavage of DNA by alkylation with antisense oligodeoxyribonucleotides containing a 2-(N-iodoacetylaminoethyl)thio-adenine

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ABSTRACT

Antisense oligodeoxyribonucleotides (15mers), containing a 2-(N-iodoacetylaminoethyl)thio-adenine, were synthesized and tested for their ability to cleave complementary DNAs (21mers). Cleavage of the target DNAs was done by alkylation followed by treatment with piperidine, and the positions of the alkylated sites were estimated by identification of the cleaved products. By using several combinations of the modified strands and their target DNAs, it was determined that alkylation occurred at adenine or guanine, depending on the torsion angle of the modified nucleoside.

INTRODUCTION

Sequence-dependent cross-linking and/or cleavage of single stranded DNA have been studied by using oligonucleotides carrying reactive groups that generate alkylating species (1–5) or oxygen radicals (6–14). The alkylation of nucleic acid bases has mostly targeted N-7 of guanine, which is in the major groove and is thought to be most reactive (15, 16). In the minor groove of DNA duplexes, cleavage reactions caused by the introduction of reactive groups at the termini of the third strand have been reported (11), however, no internal reactive groups that target adenines at the N-3 position in the minor groove have been investigated. As non-nucleotidyl compounds, the antibiotics CC-1065 (17, 18) and N-bromoacetyldistamycin (19) have been characterized by the alkylation of N-3 in the adenine ring.

In this paper we report the chemical synthesis of antisense oligodeoxyribonucleotides (15mers) containing a 2-(aminoethyl)thio-adenine, and its conversion to reactive strands by iodoacetylation. These alkylating oligonucleotides, which were designed to target the N-3 of adenine in the minor groove, were tested for their ability to alkylate complementary oligodeoxyribonucleotides (21mers).

The alkylation sites were analyzed by the identification of cleavage products and were found to be influenced by the torsion angle of the alkylating adenine ring.

EXPERIMENTAL

General methods

Thin layer chromatography (TLC) was done on Kiesel gel 60F₂₅₄ plates (Merck). For column chromatography, Wako gel C-200 and C-300 (Wako Pure Chemicals) and Preparative C-18 (Waters) were used.

¹H-NMR spectra were recorded on a JEOL FX-100 (100 MHz) spectrometer and reported in parts per million (ppm) from tetramethylsilane. ³¹P-NMR spectra were recorded on a JEOL FX-90Q (36.25 MHz) spectrometer and reported from trimethyl phosphate.

UV-spectra were recorded on a Shimadzu OPI-3 and a Beckman DU-65 spectrophotometer.

HPLC analysis and purification of oligonucleotides were done on a Gilson system using a YMC AM-324 column (10 mm I.D. × 300 mm L., Yamamura Chemical Laboratories) at a flow rate of 2.0 ml/min or a CHEMCOSORB 5-ODS-H column (4.6 mm × 250 mm L., Yamamura Chemical Laboratories) at a flow rate of 1.0 ml/min. For ion-exchange HPLC, a TSK gel DEAE-2SW column (4.6 mm I.D. × 250 mm L., Tosoh Corporation) was used at a flow rate of 1.0 ml/min on a Shimadzu LC-3A system.

Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems 380A) with 1 μmol nucleoside resin and deblocked as described (20). The modified 15mers were purified as described for 15mer-1.

Deoxyadenosine N-oxide (compound 1) (21–23)

2'-Deoxyadenosine (5.0 g, 19.9 mmol) and *m*-chloroperbenzoic acid (4.8 g, 39.8 mmol, 2 eq) were dissolved in 30% aqueous dioxane (500 ml), stirred at room temperature in the dark for 3 hr, and then 1% Pd-charcoal (1 g) was added to remove the peroxide from the mixture, which was monitored with KI-starch paper. After 1 hr, the solution was filtered and evaporated. The *m*-chlorobenzoic acid was removed with ether (40 ml × 4) and the residue was crystallized from EtOH-H₂O. Yield: 8.3 g (73%, as a monohydrate) mp: 234–238° (decomp) (lit. 228–229°, 23) UV_{λ_{max}}_{nm}: 230, 260 (H₂O).

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Yield: 0.80 g (89%) mp: 189–191° UVλ_{max}nm: 253, 310 (H₂O), 258, 315 (H⁺), 230, 258, 315 (OH[−]) MASS: m/z 526 (M⁺) ¹H-NMR (DMSO-d₆) ppm: 11.53 (s, 1H, *N*H*Bz*), 9.51

(t, br, 1H, NHCOCF_3), 8.56 (s, 1H, H-8), 7.53–8.16 (m, 5H, benzoyl), 6.53 (t, 1H, 1'H), 5.35 (d, 1H, 3'OH), 5.00 (t, 1H, 5'OH), 4.39 (br, 1H, 2'H), 3.50–3.90 (5H, 2', 3', 4', 5'H), 3.32 (t, 2H, SCH_2), 2.70 (q, 2H, CH_2NH).

Compound **7** (0.80 g, 1.52 mmol) was coevaporated with pyridine and dissolved in pyridine (5 ml). To this solution, dimethoxytrityl chloride (0.62 g, 1.8 mmol, 1.2 eq) was added and the mixture was stirred at room temperature. After 4 hr the reaction mixture was quenched with methanol (1.5 ml) and extracted with chloroform-water. The organic layer was dried with Na_2SO_4 , and evaporated and coevaporated with toluene. The residue was purified by column chromatography on silica gel, and eluted with 0–1% MeOH/ CHCl_3 . The product was concentrated and dissolved in chloroform (7 ml) and the solution was added dropwise with stirring into *n*-hexane (100 ml) to obtain a powder of product **8**.

Yield: 0.95 g (93%) mp: 118–122° $\text{UV}_{\lambda_{\text{max}}}$: 234, 310 (MeOH), 233, 258, 318 (H^+), 232, 315 (OH^-) $^1\text{H-NMR}$ (CDCl_3) ppm: 9.30 (s, 1H, NHCO), 8.78 (br, 1H, NH-6), 6.80–8.03 (m, 18H, aromatic and H-8), 6.4 (t, 1H, 1'H), 4.65 (q, 1H, 3'OH), 3.65–4.05 (m, 12H, $-\text{OCH}_3 + 2', 3', 4', 5'\text{H}$), 3.45 (m, 4H, $-\text{SCH}_2\text{CH}_2\text{NH-}$).

Compound **8** (0.30 g, 0.36 mmol) was coevaporated with pyridine and dissolved in 12 ml of CH_2Cl_2 . Under an N_2 gas atmosphere, *N,N*-diisopropylethylamine (0.25 ml, 1.44 mmol,

4 eq) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.12 ml, 1.2 eq) were added to the solution. After being stirred for 1 hr, the mixture was diluted with ethyl acetate (10 ml) and washed with *sat.* NaHCO_3 aq. (10 ml $\times$ 2) and *sat.* NaCl aq. (10 ml $\times$ 2). The organic layer was filtered with 1ps filter paper, concentrated, purified by column chromatography on silica gel, and eluted with 0–0.1% MeOH/ CHCl_3 containing 1% pyridine. The product was evaporated and dissolved in *dry* CH_2Cl_2 and dropped into *n*-pentane. It was centrifuged at 3000 rpm for 2 min and the supernatant was removed. The residue was dissolved in *dry* CH_2Cl_2 and evaporated to afford a foam of compound **9**.

Yield: 276 mg (75%) $^{31}\text{P-NMR}$ (CDCl_3) ppm: 146.53, 146.40.

Iodoacetylation of modified oligonucleotides

Oligonucleotide 15mer-1a (1.5 A_{260} units in 300 μl of pH 8.1 NaHCO_3 buffer) and a solution of *N*-[(iodoacetyl)oxy]-succinimide (**30**) (1000 eq) in DMF (200 μl) were mixed and shaken at room temperature for 2 hr. The oligomer 15mer-1b was then purified by gel filtration on a column (fine, 1.8 $\times$ 36 cm) of Sephadex G-25 in H_2O . To reveal the existence of the iodoacetyl group of the amino linker in the oligonucleotide, it was treated with thiophenol and analyzed by observing the change in the peak by reverse-phase HPLC.

Analysis of nucleoside composition

The oligonucleotide 15mer-1a (0.52 A_{260} unit) was dissolved in buffer (60 μl , 0.1M Tris HCl, 2mM MgCl_2 , pH8.2) and VPDase (5 μl , 1 mg/0.5 μl) was added. To this solution, a mixture of bacterial alkaline phosphatase (2 μl , 0.35 unit/ μl) and buffer (65 μl) was added (this mixture was first incubated at 100°C for 10 min to deactivate adenosine deaminase). The reaction mixture was incubated at 37°C for 20 hr and precipitated with EtOH. The retention time of the modified nucleoside was compared to standard nucleosides by reverse-phase HPLC, and identified. The nucleoside composition ratios were calculated from the areas of the peaks in the HPLC chart and the value of ϵ_{260} of each nucleoside. For the A^* calculation, 11.5×10^3 was used as the ϵ_{260} of A^* .

Alkylation and cleavage reaction of DNA

A target oligomer, 20 pmol of 5'- ^{32}P labeled 21mer-1 (10000 cpm per lane), and a 10-fold excess of 15mer-1b were added to 20 μl of pH 7.2 phosphate buffer (150 mM NaCl, 10 mM

Figure 2. Synthesis of 2-thio-2'-deoxyadenosine derivatives and preparation of a building block for DNA synthesis.

Figure 3. Purification of the modified oligonucleotide by column chromatography on C-18 silica gel with a linear gradient of acetonitrile (from 5–35%) in 50 mM TEAA (3a), by reverse-phase HPLC using a YMC A-324 column with a linear gradient of acetonitrile (from 9–15% in 20 min) in 0.1 M TEAA (3b), and by ion-exchange HPLC using a TSK gel DEAE-2SW column with a linear gradient of ammonium formate (from 0.4–0.9% in 25 min) in 20% aqueous acetonitrile (3c).

Figure 4. a. Analysis of the iodoacetylated oligonucleotide by reverse-phase HPLC using a YMC A-324 column with a linear gradient of acetonitrile (from 9–13% in 20 min) in 0.1 M TEAA. Peak a is 15mer-1a and peak b is the iodoacetylated oligonucleotide (15mer-1b). 4b. Analysis of the nucleoside composition of 15mer-1a by reverse-phase HPLC using a column of Chemcosorb 5-ODS-H with a linear gradient of acetonitrile (from 0–12.5% in 20 min) in 0.1 M TEAA.

sodium phosphate) and incubated at 47°C for 48 hr. The mixture was then treated with piperidine at 95°C for 30 min, evaporated *in vacuo*, and coevaporated with 20 μ l of H₂O twice. The sample was suspended in 5 μ l of loading solution (0.05% xylene cyanol, 0.05% bromophenolblue, 10 M urea), heat-denatured, and loaded on a 0.5-mm-thick, 40-cm-long, 7 M urea, 20% polyacrylamide gel. Electrophoresis was performed at 1800 V for 3.5 hr. Autoradiography of the gels was done at -80°C using Kodak X-Omat RP film. The amount of radioactivity in each band was measured in a liquid scintillation counter.

RESULTS AND DISCUSSION

Synthesis of the iodoacetyl derivative of oligonucleotides

For preparation of reactive oligodeoxyribonucleotides that alkylate a complementary strand at the N-3 of an adenine base, 2-(aminoethyl)thio-adenine was incorporated into 15mer oligonucleotides and the amino group was iodoacetylated. The sequence of an alkylating oligonucleotide (15mer-1) is shown in Figure 1a, together with the complementary 21mer. The iodoacetamide group is in the minor groove, as shown in Figure 1b, and is thought to attack the N-3 position of the neighboring adenine bases in the complementary strand. The key intermediate, 2-thio-2'-deoxyadenosine (compound 3), was synthesized via 2'-deoxyadenosine N-oxide (compound 1) and converted to the protected aminoethyl derivative (compound 4) (Figure 2). The modified nucleoside was phosphitylated after protection, as shown in Figure 2, to yield compound 9, which

was used as the condensing unit in the synthesis of 15mers by the phosphoramidite method (28, 29). The modified oligonucleotides were prepared on a DNA synthesizer under the usual conditions, except a longer coupling time (300 sec) was used for compound 9. The oligonucleotides were then deblocked. The ethylamino oligonucleotide (15mer-1a) was purified by reverse-phase chromatography before dedimethoxytritylation (Figure 3a) and was completely deprotected. The product was further purified by high performance liquid chromatography (HPLC), as shown in Figure 3b, and analyzed by ion-exchange HPLC (Figure 3c).

The alkylamino group of 15mer-1a was specifically iodoacetylated with N-[(iodoacetyl)oxy]-succinimide to convert it to 15mer-1b just before use. The product was analyzed by reverse-phase HPLC (Figure 4a) and characterized by reaction with thiophenol. The base-composition was confirmed by reverse-phase HPLC of 15mer-1a digested with venom phosphodiesterase and alkaline phosphatase (Figure 4b). The ratio of each nucleoside is calculated as follows; A: G: C: T: A* = 1: 6: 2: 5: 1 (calculated), = 1: 6: 1.9: 5: 0.9 (experimental). This result indicates that the oligomer has a modified nucleoside, but 15mer-1b does not give good results like 15mer-1a because of its reactive iodoacetyl group. Other oligonucleotides containing the iodoacetyl linker were also prepared by the same procedure. These modified oligonucleotides were very reactive and should

Figure 5. Each sample contains 20 pmol of target oligomer (21mer) and was incubated in 20 μ l of 10 mM phosphate buffer (pH 7.2) with 150 mM NaCl at 47°C, for 48 hr, except for the sample in lane 6. Lane 1: 21mer-1 + complementary 15mer (without A*, 10 eq), lane 2: 21mer-1 + 15mer-1a (10 eq), lane 3-6: 21mer-1 + 15mer-1b (10 eq), lane 8: 21mer-1 + 15mer-1b (30 eq), lane 9: 21mer (non-complementary) + 15mer-1b (10 eq). Samples in lanes 1, 2, 5, 6, 8, and 9 were treated with piperidine, lane 4 was heated without piperidine treatment, and lane 6 was incubated at 4°C. Lane 7: 21mer-1 Maxam-Gilbert G + A sequence ladders.

Figure 6. a. The sequences of newly synthesized oligonucleotides. b. Each reaction was done under the same conditions as 21mer-1 + 15mer-1b and the mixture was treated with piperidine. Lane 1, 4, 7, 10: Maxam-Gilbert G + A sequence ladders. Lane 2, 5, 8, 11: 21mer + 15mer-a (10 eq). Lane 3, 6, 9, 12: 21mer + 15mer-b (10 eq).

be useful for studies of interactions not only between nucleic acids but also between nucleic acids and proteins.

Alkylation and cleavage reactions with complementary DNA strands

The ethylamino oligonucleotide (15mer-1a) was annealed with a complementary oligodeoxyribonucleotide (21mer-1, Figure 1) in the presence of 100 mM sodium chloride in 10 mM sodium cacodylate (pH 7.0) and 1 mM EDTA. The T_m value for the duplex was 65°C and was almost the same as that of the parent duplex. The optimum temperature for alkylation of the target 21mer-1 was estimated to be 47°C from the T_m value. A 10-fold excess of the oligomer (15mer-1b) and the 5'-³²P-labeled 21mer-1 were incubated in 20 mM sodium phosphate (pH 7.2) at 47°C for 2 days, and the depurinated site was cleaved by treatment with piperidine at 95°C for 30 min. Figure 5 shows the results analyzed by polyacrylamide gel electrophoresis (PAGE). Two cleavage sites (G_{10} and A_{13}) were identified, as shown in lane 5. A large excess of the active strand yielded a similar extent of alkylation, as shown in lane 8. The cleavage was found to be sequence-dependent (lane 9). Cleavage at A_{13} was due to β -elimination of the 3'-phosphate from the site of depurination of the N-3 alkylated A_{13} (precisely, by the β -

elimination and the subsequent elimination of the partial sugar residue remaining at the 3'-end (19)). Cleavage at G_{10} of the 21mer indicated that the alkylating group attacked the N-7 of G_{10} which is in the major groove. This may result from the *syn*-conformation of the 2-substituted adenine ring of the 15mer. It has been reported that oligonucleotide reagents containing reactive 5-substituted uracils alkylate and cleave complementary strands at a guanine positioned 2-nts to the 5'-side of the modified base. The linker attached to the C-5 of uracil was a five- (5) or eight-atom arm (4) and the site of the alkylation by methyl-transfer was assigned to the N-7 of G (4). Since the above linker arms of adenine (*syn*-conformation) and uracil can have similar orientations in the major groove, this G-alkylation may occur at the N-7 position and at the second nucleotide (G_{10}). It is noted that the sample in lane 3, before piperidine treatment, showed higher molecular weight bands which appeared for the cross-linked compounds, and the cleavage product bands consisted of two sets of two bands which corresponded to the 5'-labeled products with two types of termini, respectively (19).

To examine the preferences of the reaction, several duplexes of the target oligonucleotide and the active strand were prepared (Figure 6). Three targets (21mer 2–4) contained T_{12} opposite the reactive adenine (as a *syn-anti*-conformer) and another strand (21mer-5) contained G_{12} , which was expected to have Hoogsteen-type hydrogen bonds with adenine in a *syn*-conformation (Figure 7, 31–34). Since 2-substituted adenosines tend to fold in a *syn*-conformation in a poly(2-alkyladenylic acid)-poly(5-bromouridylic acid) duplex (35, 36), the reactive adenine in 15mer-5b is assumed to have a perfect *syn*-conformation. The results of the cleavage reactions for these duplexes are summarized in Table 1. It was observed that reaction with adenine occurred at A_{13} (21mer-2). Deoxyadenosines beyond 13, or in the 5'-flanking positions were not attacked.

Deoxyguanosines located at the 5'-side (G_9 , G_{10} , and G_{11}) and the position opposite the reactive adenine were alkylated. These results are explained by the length and conformation of the adenine with the 2-iodoacetylaminoethyl group. The result from duplex-5 especially indicated that alkylation at A_{13} did not occur, whereas the G-reaction was observed at G_{10} and G_{12} . This would be due to the dominance of G-*anti*, A-*syn* pairing at position 12.

In conclusion, antisense oligonucleotides bearing 2-(N-iodoacetylaminoethyl)thio-adenosine were shown to crosslink and cleave complementary strands at sites of adenines and guanines. The alkylating reagent was expected to attack the N-3 of adenine from the minor groove, providing the substituted adenine was in the *anti*-conformation. Reactions with guanine suggested the participation of the *syn*-conformer and reactive groups in both the major and minor grooves of the duplex. The efficiency of alkylation was estimated by cleavage of the 5'-linked oligonucleotide after treatment with piperidine, and was found to be low. Free rotation of the reactive linker around the thio group may not favor targeting. More rigid tethers, such as amide linkages on the purine ring, may give higher selectivity of alkylation.

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Figure 7. G-A mismatched base pairs in the DNA

Table 1. Cleavage of 21mers. Duplex-5 includes a mismatched base pair. Cleavage was measured following piperidine treatment.

duplex	13	10	cleavage sites (cleavage yield%)
Duplex-1	3' -dCAA TAG CTG- 5'		A_{13} (4.5), G_{10} (3.6)
Duplex-2	3' -dCGA TGC CTG- 5'		A_{13} (2.6), G_{11} (1.9)
Duplex-3	3' -dCAG TGG CTG- 5'		-
Duplex-4	3' -dAAT TAG GTG- 5'		G_9 (1.2), G_{10} (1.6)
Duplex-5	3' -dCAA GAG CTG- 5'		G_{10} (4.0), G_{12} (4.2)

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