Characterization of an adduct between CC-1065 and a defined oligodeoxynucleotide duplex

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ABSTRACT

CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis*. The drug binds covalently through N-3 of adenine and lies within the minor groove of DNA. Previous studies indicated that CC-1065 reacted with adenine in DNA to yield a thermally labile product that could be used to reveal its sequence specificity. These studies also provided insight into a DNA sequence (5'-CGGAGTTAGGGGCG-3') which should bind one molecule of CC-1065 in an unambiguous manner. This sequence, which contains the CC-1065 adenine binding site within the sequence 5'-TTA-3' was chemically synthesized together with the complementary strand. CC-1065 reacted with the oligoduplex to give an adduct that maintained the B-DNA form and had a final CD spectrum similar to those of the CC-1065 complexes formed with calf thymus DNA. The above 14mer was 5' end-labelled with ³²P, annealed with its complementary strand, reacted with CC-1065 and heated. Drug-mediated strand breakage was evaluated on a sequencing gel. A single break occurred in the labelled strands to give a fragment that migrated as an 8.5mer; subsequent piperidine treatment produced a fragment that migrated as a 7mer, which is the size expected from the known binding of CC-1065 at adenine in 5'-TTA-3' sequences.

INTRODUCTION:

CC-1065, an antitumor antibiotic produced by *Streptomyces zelensis*, is 100 times more potent than Adriamycin in the human tumor-cloning assay (1). The drug exerts its cytotoxic effect by binding covalently to DNA through N-3 of adenine (see Figure 1) and is proposed to lie within the minor groove (2,3). CC-1065 exhibits a marked sequence specificity (3), causes helix stabilization (4), and is a potent inhibitor of DNA synthesis (4). The drug has been shown to cause DNA strand scission and apurinic site formation *in vitro* upon heating (2), and causes prolonged depletion of NAD levels *in vivo*, most likely due to conversion of NAD to poly(ADP)ribose (5). CC-1065 is also mutagenic in the *Salmonella* forward mutation assay, increases SCE and chromosomal aberrations in V79 cells (6), and is associated with a delayed hepatotoxicity (7).

Figure 1. Reaction of CC-1065 with DNA to form the N-3 adenine alkyl adduct (3).

Sequence specificity studies (3) on the early promoter region of SV40 DNA have shown that CC-1065 binds to identical sites within the 21 base pair repeats. We have synthesized a 14 base pair oligodeoxynucleotide duplex with the sequence 5'-CGGAGTTAGGGGCG GCCTCAATCCCCGC-5', which contains the single CC-1065 binding site and examined the properties of the CC-1065 modified duplex.

In this paper, we describe the synthesis of the above oligomer, CD analysis of the duplex, and its CC-1065-adduct. Additionally, we report on the identification of a single unambiguous binding site of CC-1065 on the duplex, as well as partial chemical and enzymatic characterization of the drug-induced thermal strand break.

MATERIALS AND METHODS

Materials

CC-1065 was provided by The Upjohn Company, Kalamazoo, MI and (γ -³²P)ATP was enzymatically synthesized (8) employing enzymes purchased from Boehringer and ³²P-phosphate from ICN. Sequencing reagents were purchased from BioRad. Polynucleotide kinase was prepared from T-4 infected *E. coli* as described (9).

Oligomer Synthesis

The tetradecanucleotides 5'-CGGAGTTAGGGGCG-3' and its complement were synthesized by the solid-phase phosphite triester method (10) using a BioSearch SAM 1 synthesizer. The solid-phase polymer support was functionalized silica gel (11) with 5'-dimethoxytrityl-N-isobutyryl

deoxyguanosine 3'-linked at 42 μ mol/g (Vydak silica, 300 A pore size). All yields were calculated based on the acid-mediated (1.5% trichloroacetic acid (TCA)/methylene chloride) detritylation and measuring the absorbance at 504 nm.

The phosphite triester method specifically involved the use of 3'-N,N-dimethyl-aminophosphoramidites of the appropriately protected nucleosides: 5'-dimethoxytrityl with N-benzoyl deoxyadenosine, N-benzoyl deoxycytidine, N-isobutyryl deoxyguanosine and thymidine prepared according to standard procedures (10) and their purity checked by ^{31}P - and ^1H -NMR. The synthesis program employed an 8.5 minute coupling time on 50 mg of polymer support, 40-fold excess of nucleoside phosphoramidite, 400-fold excess of benzotriazole as the coupling activator and dry acetonitrile as solvent. A flow rate of 1.0 ml/min was employed during coupling and 3.5 ml/min during the other steps (washing, oxidation, capping, detritylation, (approx. 28 min. cycle time). A capping procedure with acetic anhydride/dimethylaminopyridine/tetrahydrofuran was employed. The oligonucleotides were ultimately deprotected with 1.5% TCA/ CH_2Cl_2 followed by thiophenol/triethylamine/dioxane and finally treated with ammonium hydroxide at room temperature (15 hours) and 50°C (5 hours) (11) to remove the N-protecting groups and cleave the chain from the silica. The yield of the G-rich tetradecanucleotide was 24% and the C-rich tetradecanucleotide was 27%.

The resulting material (70-80 O.D. units) was purified by preparative acrylamide gel electrophoresis (29:1 bis, 7 M urea, 20% cross-linked, 3 mm thick) and desalted on Sephadex G-25 using H_2O as elution buffer ($A_{260}:A_{280}=1.8$). Size and purity was checked by 5'-end labelling of the oligonucleotide with (γ - ^{32}P)ATP and running an analytical acrylamide gel (29:1 bis, 7 M urea, 24% cross-linked, 1.5 mm thick). Both sequences migrated as one spot. The C-rich tetradecanucleotide migrated as a 14mer while the G-rich tetradecanucleotide migrated as a 15mer. This is not unexpected in view of the high G content (57%).

CD Analyses

CD spectra were obtained on a JASCO 500C instrument at room temperature. The instrument was calibrated with D-10-camphor-sulfonic acid (12). Oligomer annealing for CD studies was carried out by incubating equimolar amounts of complementary tetradecanucleotides in 10 mM Tris-HCl (pH 7.8), 0.075 M NaCl at 65°C for 25 minutes followed by slow cool down to room temperature over a 4-hour period.

³²P Labelling of Oligomer for Sequencing Analysis

Single stranded synthetic oligomer (2 µg) was incubated in 50 mM Tris, pH 9.5, 10 mM MgCl₂, 5 mM DTT, 4% glycerol and 0.5 mCi (γ - ³²P)ATP in the presence of 10 units of polynucleotide kinase at 37°C for 10 minutes. The ³²P-labelled oligomer was separated from unreacted (γ - ³²P)ATP by Sephadex G-25 column chromatography as described previously (13).

Oligomer Annealing for Sequencing Analysis

³²P-labelled oligomer was incubated with an equimolar amount of unlabelled complementary strand in 20 µl of 0.1 M NaCl, 1.0 mM EDTA, and 10 mM Tris-HCl, pH 7.8 for 25 minutes at 65°C, after which the water bath was allowed to cool slowly to room temperature. Sodium acetate (200 µl of 0.3 M), 20 µl of a 1.0 mg/ml solution of t-RNA, and 1.0 ml ethanol was added, the solution was mixed well, cooled in liquid nitrogen, and centrifuged at 12,000 x g for 12 minutes. The supernatant was removed with a drawn Pasteur pipette, 1.0 ml ethanol was added to the pellet, centrifuged at 12,000 x g for 8 minutes, and then the supernatant was removed again. The sample was dried under vacuum at room temperature.

CC-1065 Binding to Oligomer for Sequencing Analysis

³²P single end-labelled oligomer duplex (2.6 µg) was incubated with 0.84 nmol CC-1065 in 20 µl deionized water at 4°C for 24 hr. This solution was ethanol precipitated, washed and dried as described before.

Thermal Strand Breakage

Drug bound duplex was dissolved in 100 µl of 10 mM NaCl and 1.5 mM sodium citrate, sealed in an Eppendorf snap-cap vial with conformable tape, and heated to 90°C for 30 minutes. Samples were ethanol precipitated, washed, and dried as before.

Piperidine Treatment

Samples were dissolved in freshly prepared 1 M piperidine, sealed in Eppendorf snap-cap vials with tape, and heated to 90°C for 30 minutes. The solution was cooled, transferred to a new vial, and lyophilized to dryness. Samples were resuspended and lyophilized 5 times in 20 µl of deionized water.

Bovine Alkaline Phosphatase (BAP), and Polynucleotide Kinase Treatment

Previously thermally treated samples were resuspended in 200 µl of 0.1M Tris (pH 8.0) and incubated in the presence of 12 U BAP at 37°C for 30 minutes. The solution was heated to 70°C for 1 hour, and extracted twice with 200 µl phenol saturated with 50 mM Tris, pH 8.0. The samples were then extracted 3 x 250 µl with diethyl ether and ethanol precipitated,

Figure 2. CD spectra of 14mer duplex (—) in 0.01 M Tris HCl buffer, pH 7.8, 0.075 M NaCl and calf thymus DNA (---) in 0.01 M phosphate buffer, pH 7.2 (A). CC-1065 CD spectra induced by the 14mer duplex (—) and calf thymus DNA (---). The molar ellipticity is with respect to CC-1065 at 0.37×10^{-5} M (B). CC-1065 CD spectra induced by the oligoduplex at various times after the addition of CC-1065: (—●—) 10 min, (—○—) 70 min, (—■—) 24 hrs, (—▲—) 7 days, (—□—) 72 days (C).

rinsed and dried. The sample was then ^{32}P end-labelled by the method described to label single strand oligomers (vida supra).

Oligomer Sequencing Reactions

Purine and pyrimidine-specific sequencing reactions were performed as described in (13).

Sequencing Gels

Samples were suspended in 80% deionized formamide, 10 mM NaOH, 1 mM EDTA and 1% xylene cyanol, applied to a 24 x 32 x 0.3 cm 24% acrylamide sequencing gel (29:1 bis, 7 M urea) (13) and electrophoresed at 1600 V. Gels were run in 50 mM Tris-borate, 1 mM EDTA, pH 8.3 buffer and stopped when a bromophenol blue marker dye adjacent to sample lanes reached 12 cm. Gels were wrapped in Saran Wrap and matted with X-ray film for visualization.

RESULTS

CD Studies

The oligomer chosen for this study was predicted to bind one molecule of CC-1065 in an unambiguous manner based upon previous studies (3) which were carried out to determine the sequence specificity of CC-1065 within the early promoter region of SV40 DNA. The CD spectrum of the oligodeoxynucleotide duplex molecule is similar to that of calf thymus DNA (ct-DNA) (Figure 2a). The CD induced in the CC-1065 electronic transition near 380 nm by the oligodeoxynucleotide duplex is similar to that induced by ct-DNA (Fig. 2b)(14).

DNA Sequence Analysis of the 14mer and its CC-1065-Adduct

The oligodeoxynucleotide duplex and its CC-1065 adduct were analyzed in separate experiments by gel electrophoresis after ^{32}P end-labelling each of the complementary strands. Maxam-Gilbert sequencing of the two strands (Figure 3, lanes 1, 2, and 13, 14) revealed the expected sequences. The two ^{32}P end-labelled duplexes were reacted with CC-1065 to produce saturation binding. Analysis of the ^{32}P -end labelled oligodeoxynucleotide sequence containing the CC-1065 covalent binding sequence (5'-TTA-3') is shown in Figure 3, lanes 6-9. The unheated CC-1065 duplex adduct (lane 6) shows a trace of a band at the 8.5mer position, corresponding to the location at which thermally induced breaks normally occur. Thermal treatment (lane 7) produced the expected break corresponding to the 8.5mer position, i.e., about 1.5 residues removed from the expected point of covalent attachment on adenosine. Exposure of the thermally treated CC-1065 duplex adduct to piperidine produced a band at a 7mer position corresponding to cleavage of the AP site (the N-3 adenine adduct position) (lane 9). The oligodeoxynucleotide strand predicted not to react covalently with CC-1065 (complementary strand) did not show any fragmentation when heated to 90° (lane 10).

In an experiment designed to probe the identity of the frayed end at the thermally induced break point, unlabelled CC-1065 duplex adduct was heated, followed by sequential treatment with BAP and polynucleotide kinase in the presence of (γ - ^{32}P)ATP. The electrophoresis pattern (lane 8) was identical to that produced by thermal treatment of the ^{32}P -labelled CC-1065 duplex adduct. In a control experiment using unlabelled 14mer, subsequent ^{32}P -labelling by polynucleotide kinase was found to be dependent upon prior treatment with BAP. This confirms that the BAP was capable of removing 5' phosphates.

Figure 3. Autoradiogram of the 5' ^{32}P end-labelled complementary strands of the duplex and its CC-1065-duplex adduct. Lanes 1 through 9 are the ^{32}P end-labelled 5'-CGGAGTTAGGCG-3' strand and lanes 10-14 are the complementary strand. Lanes 1 and 14 and 2 and 13 are the Maxam-Gilbert TC and AG lanes, respectively. Lanes 3 and 12 are unmodified duplex. Lane 4 is the unmodified duplex thermally treated (90° for 30 mins). Lane 5 is identical to lane 4 followed by piperidine treatment. Lanes 6 and 11 are CC-1065-duplex adducts. Lanes 7 and 10 are identical to 6 and 11 but thermally treated (90° for 30 mins). Lane 8 is identical to lane 7 but also incubated with BAP and polynucleotide kinase ($\gamma\text{-}^{32}\text{P}$)ATP. Lane 9 is identical to lane 7 followed by piperidine treatment.

DISCUSSION

The results of this investigation show that, as predicted, CC-1065 binds unambiguously to the adenine residue within the 5'-TTA-3' segment of the synthetic oligodeoxynucleotide duplex. CD analysis of the 14mer duplex and its CC-1065 adduct reveals that they both assume the B-form (Fig. 2a and b). The B-form of DNA is necessary for CC-1065 binding. Furthermore, Fig. 2b shows that the CC-1065 CD induced by the oligoduplex conforms in shape and position to that observed for ct-DNA. Whereas the bound species

of CC-1065 responsible for this CD is irreversibly bound, some poly- and oligonucleotides initially bind CC-1065 reversibly to yield an induced CD at longer wavelengths (Krueger, W., unpublished results). However, Fig. 2c shows that CC-1065 binds irreversibly to the oligoduplex even at incubation times of less than 24 hours.

In using restriction enzyme fragments of ct-DNA modified with CC-1065, our previous studies (3) have provided a number of important observations. Thermal treatment of the CC-1065-DNA adduct produces a strand break at about one residue removed from the adenine on the 3' side. Upon piperidine treatment of the previously thermally cleaved DNA fragment, the break is moved back about one residue to the 5' side, i.e., to the aforementioned adenine residue. The CC-1065 DNA sequence specificity extends on the 5' side from the point of thermal cleavage, i.e., 5'-PuNTTA-3'. These observations can be rationalized if heating of the CC-1065-DNA adduct independently produces both a single strand break and an AP site. Piperidine treatment then induces further cleavage from the thermally induced strand break to the 5' side of the AP site. However, the site and mechanism for thermal cleavage of the deoxyribosephosphate backbone by CC-1065 was not clear. The present studies using the 14mer in combination with various chemical and enzymatic treatments provide some insight into the possible mechanism and site for this thermally induced strand breakage.

It is reasonable to assume that the DNA strand break produced as a result of thermal treatment occurs before depurination, otherwise the CC-1065 would be liberated before it could catalyze strand breakage. Several mechanisms can be proposed for the generation of CC-1065 thermally induced DNA strand breaks. A general base catalysis originating from a suitable group on CC-1065 could result in β -elimination on a deoxyribose with concomitant DNA strand breakage. Alternatively, formation of a phosphotriester might produce the strand breakage upon thermal treatment. Both a β -elimination mechanism and phosphotriester hydrolysis will generate 3' or 5' phosphates. However, irrespective of which of these mechanisms might be operative BAP and polynucleotide kinase treatment of the thermal cleavage product should give rise to species with different electrophoretic mobilities. The results show that following BAP and polynucleotide kinase treatment (Figure 3, lane 8) the electrophoretic pattern is identical to that without BAP and polynucleotide kinase (Figure 3, lane 7). Therefore, we can rule out β -elimination or phosphotriester as mechanisms for the thermally induced strand breakage of DNA.

Figure 4. Stereo drawings of the proposed CC-1065-DNA adduct.

The results of the previous enzymatic experiment lead us to suspect that a "frayed end", possibly terminating at a partially degraded deoxyribose moiety, might be present. This is consistent with the piperidine treatment which will, through reaction of the AP-site followed by sequential β -eliminations, remove such a "frayed end". The antitumor antibiotic bleomycin produces a strand breakage of this type via a one electron oxidation process (15). We therefore examined stereo drawings of the CC-1065-DNA adduct (Figure 4) to determine whether a suitable CC-1065 functional group might be in proximity to the deoxyribose moiety on the 3' side of the adenine to which CC-1065 is covalently bound. The phenolic oxygen and indolic nitrogen of the alkylating subunit of CC-1065 are both in close proximity to the 4' and 5' positions of the deoxyribose on the 3' side of the adenine. It is therefore conceivable that a one electron oxidation arises from one of these groups generating a chain of events eventually leading to cleavage of the deoxyribose. Experiments are in progress to test this hypothesis. The mobility of the fragment containing a "frayed end" is in accord with a phosphorylated AP site with its charge "masked" by a terminal alkyl group.

The results described in this paper demonstrate that the synthesized 14mer binds CC-1065 in an unambiguous manner as predicted from the previous DNA sequence specificity experiments (3). The use of the 14mer has a

number of distinct advantages over using a restriction enzyme fragment for studying a defined CC-1065-DNA adduct. Future experiments using various smaller sub-fragments of the 14mer will be directed towards obtaining more direct evidence for the structure of the CC-1065-DNA adduct.

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