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Effect of the (+)-CC-1065–(*N*3-Adenine)DNA Adduct on in Vitro DNA Synthesis Mediated by *Escherichia coli* DNA Polymerase†

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Received September 16, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: (+)-CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis*. Previous studies have shown that the potent cytotoxic and antitumor activities of (+)-CC-1065 are due to the ability of this compound to covalently modify DNA. (+)-CC-1065 reacts with duplex DNA to form an *N*3-adenine DNA adduct which lies in the minor groove of the DNA helix overlapping with a 5-base-pair region. As a consequence of covalent modification with (+)-CC-1065, the DNA helix bends into the minor groove and also undergoes winding and stiffening [Lee, C.-S., Sun, D., Kizu, R., & Hurley, L. H. (1991) *Chem. Res. Toxicol. 4*, 203–213]. In the studies described here, in which we have constructed site-directed DNA adducts on single-stranded DNA templates, we have shown that (+)-CC-1065 and select synthetic analogues, which have different levels of cytotoxicity, all show strong blocks against progression of Klenow fragment, *E. coli* DNA polymerase, and T4 DNA polymerase. The inhibition of bypass of drug lesions by polymerase could be partially alleviated by increasing the concentration of dNTPs and, to a small extent, by increasing polymerase levels. Klenow fragment binds equally well to a DNA template adjacent to a drug modification site and to unmodified DNA. These results taken together lead us to suspect that it is primarily inhibition of base pairing around the drug modification site and not prevention of polymerase binding that leads to blockage of DNA synthesis. Unexpectedly, the exact termination site of the in vitro DNA synthesis by Klenow fragment is not dependent on the species of covalently bound drug molecule but on the sequence to the 5′ side of the drug-modified adenine. Misincorporation of dA for dG by Klenow fragment occurred at the secondary pausing site specifically for (+)-CC-1065 contained within the covalently modified sequence 5′-GATTA-3′. Although (+)-CC-1065 and its analogues evaluated in this study did not produce dramatically different effects on DNA polymerases when the drugs were bound to a single-stranded template, polymerization from a primer site containing a drug lesion in the duplex region did show a selective inhibitory effect with (+)-CC-1065 and (+)-AB′C′. When this observation is considered alongside results of experiments showing selective inhibition by these same compounds of T4 ligase and helicase II, the winding phenomena uniquely found with these compounds may be associated with the potent biological effect known as delayed lethality.

(+)-CC-1065 is a potent antitumor antibiotic produced by *Streptomyces zelensis*, which is active against several experimental tumors in vivo and is at least 50–1000 times more cytotoxic than other clinically used anticancer drugs (Hanka et al., 1978; Neil et al., 1981; Bhuyan et al., 1982; Li et al., 1982). There is overwhelming evidence that DNA is the principal biological target of (+)-CC-1065 and that the alkylating properties of this drug molecule are largely responsible for the potent cytotoxic effects produced via the formation of the (+)-CC-1065–DNA adduct (Li et al., 1982; Reynolds et al., 1986). Previous studies have revealed that (+)-CC-1065 forms a covalent adduct through *N*3 of adenine in a highly sequence-specific manner, in which the drug molecule lies within the minor groove covering 4- and 1-base-pair regions to the 5′ and 3′ sides, respectively, of the covalently modified adenine (Reynolds et al., 1985; Scahill et al., 1990; Hurley et al., 1990; Lin & Hurley, 1991).

Recently we have demonstrated that (+)-CC-1065 induces local bending (17–19° in toward the minor groove), winding, and helix-stiffening of DNA molecules as a consequence of *N*3-adenine covalent adduct formation (Lee et al., 1991; Sun & Hurley, 1992a). Previous work has shown that, in L1210 cells, (+)-CC-1065 inhibited DNA synthesis much more than it inhibited RNA or protein synthesis (Bhuyan et al., 1982).

† This research was supported by the Public Health Service (CA-49751), The Welch Foundation, and the Burroughs Wellcome Fund.
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Inhibition of DNA replication due to the strong irreversible binding of (+)-CC-1065 to double-stranded DNA has been proposed to be responsible for the cytotoxicity of (+)-CC-1065 (Li et al., 1982). The DNA replication process consists of three consecutive steps: recognition of replication origins, unwinding of duplexes, and synthesis of a new complementary DNA. In this in vitro study, complementary strand synthesis of a single-stranded template has been chosen as a model for the replication process. It is generally recognized that most DNA-modifying agents that react with DNA to produce bulky-type lesions result in a DNA template which acts as a block to DNA synthesis (Piette & Hearst, 1983; Singer & Grunberger, 1984; Pinto & Lippard, 1985; Brown & Romano, 1991). For this purpose, a DNA template containing a site-directed (+)-CC-1065–DNA adduct was constructed and used to monitor whether DNA polymerase bypasses the drug–DNA adducts or terminates at the drug lesion site. At the drug modification site, we have utilized two different sequences, 5′-GATTA*-3′ and 5′-AGTTA*-3′ (* indicates drug-modified adenine). These two sequences, 5′-AGTTA and 5′-GATTA, have markedly different reactivities toward (+)-CC-1065 (Theriault et al., 1988), the former reacting much faster than the latter. A comparison of (+)-CC-1065–DNA adducts on slightly different sequences allows us to investigate the sequence dependency of bypass or termination by polymerase at the drug-modification site. In these studies, we have also compared the four (+)-CC-1065 analogues shown in Figure 1. These analogues were synthesized by scientists at The Upjohn Company as part of their ongoing program to develop new chemotherapeutic agents based on the (+)-CC-1065 structure that are more efficacious and do not cause the delayed lethality exhibited by (+)-CC-1065 and (+)-AB′C′ (Warpehoski, 1986; Kelly et al., 1987; Warpehoski & Bradford, 1988; Warpehoski et al., 1988). By comparison of the effects of these analogues, we hope to relate the results from this present study to the differential biological activities of each drug molecule published in previous in vivo and in vitro studies (Warpehoski & Bradford, 1988; Warpehoski et al., 1988).

Although these drug molecules showed different levels of antitumor and cytotoxic activity in in vivo and in vitro studies (Warpehoski & Bradford, 1988), the in vitro results presented in this paper clearly show that all the drug–DNA adducts act as approximately equal strong blocks against the progression of DNA polymerase through the adduct site, except where polymerization initiates from a duplex region covalently modified with (+)-CC-1065 and (+)-AB′C′. In addition, the termination sites of the in vitro DNA synthesis on drug-modified template are not dependent on the species of drug molecules covalently attached to the template but on the sequence to the 5′ side of the drug-modified adenine.

Finally, we have evaluated the fidelity of in vitro DNA synthesis around the drug adduct site. (+)-CC-1065 was found to cause misincorporation of the nucleotide prior to the modified adenine when 5′-GATTA-3′ was selected as the drug-bonding site. This result can be used to rationalize the previous report that (+)-CC-1065 is a very potent mutagen (Harbach et al., 1986). A preliminary account of these results has appeared (Sun & Hurley, 1991). In a recently published study (Weiland & Dooley, 1991) it has been shown that when the modified bacteriophage T7 polymerase Sequenase is used in the presence of (+)-ABC″-modified plasmid DNA, the polymerase is inhibited at the nucleotide adjacent to the modified base.

Materials and Methods

Materials. (+)-CC-1065 and its synthetic analogues

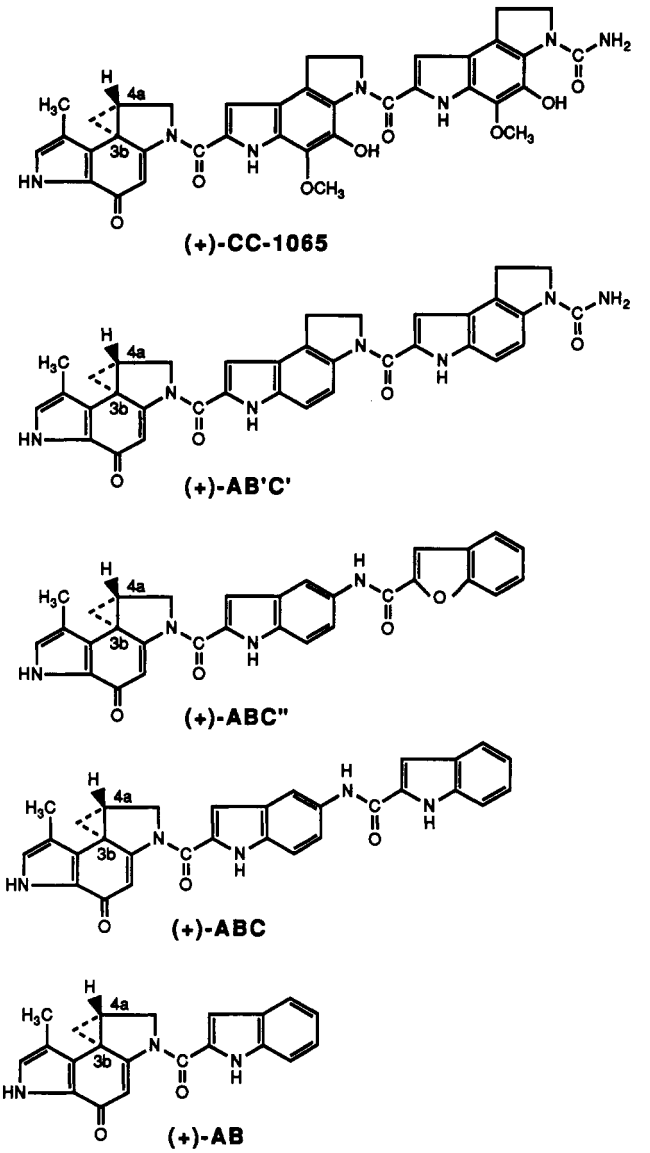

FIGURE 1: Structures of (+)-CC-1065 and its synthetic analogues used in this investigation.

(Figure 1) used in this study were provided by J. Patrick McGovren at The Upjohn Company, Kalamazoo, Michigan. Electrophoretic reagents [acrylamide, TEMED,[1] ammonium persulfate, and bis(acrylamide)] were purchased from Bio-Rad. Klenow fragment, *Escherichia coli* DNA polymerase I, and T4 DNA polymerase were from BRL. DNase I was from Sigma, restriction endonucleases used in this study were from New England Biolabs, and T4 polynucleotide kinase and reagents for dideoxy DNA sequencing were from U. S. Biochemicals. Other chemicals for chemical DNA sequencing were from Aldrich Chemical Co.; [γ^{32}P]ATP was from ICN; and X-ray film, intensifying screens, and developing chemicals were from Kodak.

Oligonucleotide Synthesis. The series of oligonucleotides used in this study (Table I) were synthesized on an automated DNA synthesizer (Applied Biosystems 381A) by the phosphoramidite method. The oligomers were then deprotected

[1] Abbreviations: bp, base pair; ss, single stranded; DDW, double-distilled water; Tris, tris(hydroxymethyl)aminomethane; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; OP-Cu-phenanthrene, bis(*O*-phenanthroline)copper(I); DNase I, bovine pancreatic deoxyribonuclease I; dNTP, deoxyribonucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid.

Table I: Sequences of Templates and Primers Used in This Study[a]

T-44MER	5′-TGGGCGGAGTTAGGGGCGGGTGGATCAGGCAGTCAGTTACGTAG-3′
16MER	3′-CGTCAGTCAATGCATC-5′
20MER	3′-ACCCGCCTCAATCCCCGCCC-5′
T-45MER	5′-ACCATGATTACGGATTCGAGATCAGCAGTCAGTTACGTAGATCAC-3′
16MER	3′-CGTCAGTCAATGCATC-5′
21MER	3′-CGTCAGTCAATGCATCTAGTG-5′
35MER	3′-GCCTAAGCTCTAGTCGTCAGTCAATGCATCTAGTG-5′
20MER	3′-TGGTACTAATGCCTAAGCTC-5′
T-51MER	5′-CTCGAATCCGTAACTATGGTCGGTGATTACGTAGAGATCAGCGCTAGCATG-3′
21MER	3′-CATCTCTAGTCGCGATCGTAC-5′
20MER	3′-ACCAGCCACTAATGCATCTC-5′

[a] Bold letters indicate drug modification sites.

separately with saturated ammonium hydroxide at 55 °C overnight, dried under vacuum, and redissolved in DDW.

Preparation of 5′-End-Labeled Primers. Approximately 10 μg of each primer was separately 5′-end-labeled with [γ^{32}P]ATP and T4 polynucleotide kinase mixed in 25 μL of solution containing 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM dithiothreitol for 1 h at 37 °C. Labeled oligonucleotides were electrophoresed on a 12% denaturing polyacrylamide gel and located by autoradiography. Gel fragments containing DNA were excised from the gel, minced with a blade, extracted with DDW, and ethanol-precipitated. DNA pellets were dried under vacuum and redissolved in DDW.

Preparation of Site-Directed Adducts on Single-Stranded DNA Templates. The oligomers (T-44, T-45, and T-51 in Table I) containing 5′-GATTA-3′ or 5′-AGTTA-3′ as the drug-bonding sequence (Table I) were separately annealed in a solution of 100 mM NaCl and 10 mM Tris-HCl (pH 7.5) to the complementary 20-mer single-stranded oligomer to form a partial duplex DNA. The drug-bonding site was positioned within the duplex region so that the reaction occurred exclusively within this region to form a covalent adduct with T-44, T-45, or T-51. The resulting partial duplexes were modified with drug molecules [(+)-CC-1065, (+)-AB′C′, (+)-ABC″, (+)-ABC, and (+)-AB], as previously described (Lee et al., 1991). Unbound drug molecules were removed by ethanol precipitation, and resulting DNA pellets were redissolved in sequencing dye solution [90% w/v) formamide in 0.1 M Tris-HCl (pH 7.8)]. After the DNA samples were heated for 1 min at 95 °C, they were subjected to denaturing 12% polyacrylamide gel electrophoresis (7 M urea) to separate drug-modified ss DNA from the complementary strand by the size difference between two species. After electrophoresis, the gel was transferred to plastic wrap and placed over a TLC plate containing fluorescent indicator, and the locations of DNA molecules were visualized under short-wavelength UV light. Gel fragments containing the desired DNA fragments were cut from the gel, minced with a blade, extracted with distilled water, and dried under vacuum. Dried DNA pellets were dissolved in distilled water, ethanol-precipitated, and redissolved in distilled water. Purified drug-modified ss DNAs were annealed to appropriate 5′-^{32}P-end-labeled primers in 2× polymerase buffer by incubating at 50 °C and slowly cooling to room temperature, and these were then used as a DNA template.

Preparation of Template DNA Modified with Drug Molecules at the Priming Site. The oligomer T-45-mer was annealed to the primer, 5′-end-labeled 21-mer, to form a partial duplex, and the resulting duplex was modified with (+)-CC-1065 or select analogues. Unbound drug molecules were removed by ethanol precipitation, and drug-modified templates

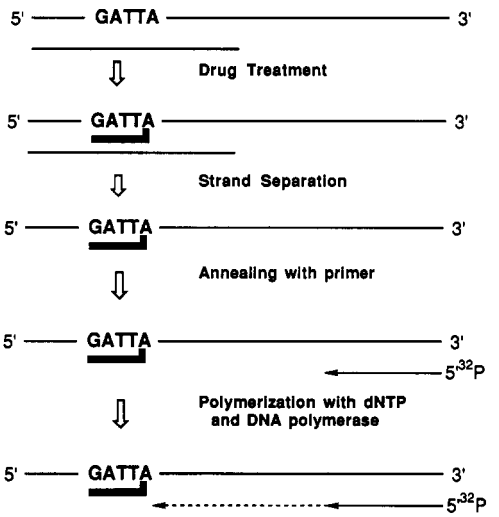

FIGURE 2: Schematic diagram showing the strategies used to construct site-directed (+)-CC-1065- and analogue-modified template for the DNA polymerase experiments. A partial duplex of DNA containing a unique drug-bonding sequence within the duplex region was prepared and modified with drug, and the strands were separated by denaturing polyacrylamide gel electrophoresis to purify a site-directed drug-modified single strand, as described in Materials and Methods. DNA polymerization extension was carried out from a primer annealed to the 3′ end of the drug-modified ss DNA template. Solid fold bars represent drug molecules covalently attached to DNA.

were further purified by electrophoresis on nondenaturing 8% polyacrylamide gels as described above.

Heat-Induced Strand Breakage Assay of Drug-Modified Single-Stranded DNA. (+)-CC-1065– and analogue–(*N*3-adenine)DNA adducts can be released from the DNA strand by thermal treatment to also induce DNA strand breakage (Reynolds et al., 1985). After 5′-end-labeling with [γ^{32}P]ATP, drug-modified ss DNAs were subjected to thermal treatment for 2 h at 95 °C to locate the drug-bonding site and determine the extent of reaction at the desired sequence on ss DNA. Heat-treated samples were dried, redissolved in formamide (80%)–NaOH (10 mM) dye solution, and then subjected to denaturing 12% polyacrylamide gel electrophoresis run in parallel with DNA sequencing reactions.

DNA Polymerase Reaction. Template DNA (about 50 nM) was incubated with 1 unit of Klenow fragment, *E. coli* DNA polymerase I, or T4 DNA polymerase in 10 μL of 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 10 mM $MgCl_2$ for 30 min in the presence of the indicated amount of dNTPs. Reactions were quenched by addition of the same volume of sequencing dye.

Results

Design and Characterization of Drug-Modified DNA Templates for DNA Polymerization Experiments. Template DNA molecules for polymerization experiments were designed to have the drug-bonding sequence (5′-GATTA-3′ or 5′-AGTTA-3′) on the 5′ side of the ss region with at least a 4-bp gap remaining between the 3′ terminus of the primer and the drug-modified adenine. This template was used to monitor the effect of drug–DNA adducts on the in vitro DNA synthesis mediated by DNA polymerase. The overall strategy for preparation of drug-modified templates is shown in Figure 2. The stability of covalently attached drug molecules on the DNA template during the preparation of the template was confirmed by chemical footprinting and a spectrophotometric assay (unpublished results). In the latter assay, ss DNA (T-45-mer in Table I) modified with (+)-CC-1065 exhibits a second absorption peak around 360 nm, which can be at-

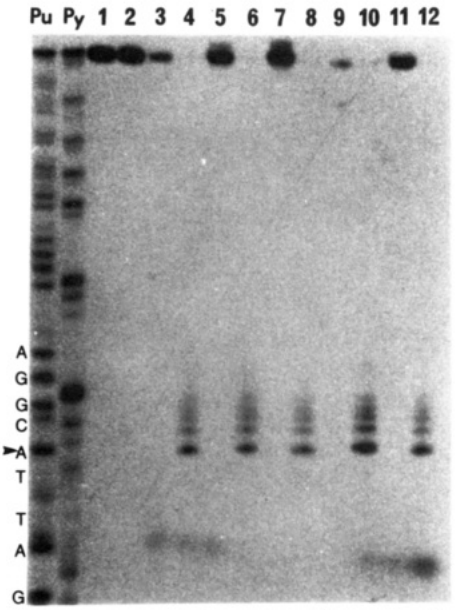

FIGURE 3: Autoradiogram of the results of the thermally induced strand breakage assay (Reynolds et al., 1985) of 5′-labeled site-directed drug-modified T-45-mer used to locate the drug-modified adenine and determine the extent of drug modification. Pu and Py are AG- and TC-specific chemical sequencing reactions, respectively. Lane 1 refers to control DNA and lanes 3, 5, 7, 9, and 11 refer to T-45-mer modified with (+)-CC-1065, (+)-AB′C′, (+)-ABC″, (+)-ABC, and (+)-AB, respectively. Lanes 2, 4, 6, 8, 10, 11, and 12 are the same as the preceding lanes, except that they were heat treated (95 °C for 2 h). The DNA sequence around the drug-bonding site and the drug-modified adenine (arrowhead) are shown at left. The multiplicity of the bands is explained in footnote 2.

tributed to drug molecules when compared to the unmodified T-45-mer (data not shown). In addition, 5′-^{32}P-end-labeled drug-modified ss DNAs were subjected to thermal treatment (Reynold et al., 1985) to induce DNA strand breakage in order to identify the drug-modified adenine and also to monitor the extent of drug modification at the desired site. As shown in Figure 3, thermal treatment induced almost quantitative strand cleavage of drug-modified ss DNA (lanes 4, 6, 8, 10, and 12) at the expected site on DNA backbone[2] (5′-GATTA*; asterisk indicates drug-modified adenine), while non-drug-modified ss DNA subjected to thermal treatment (lane 2) or drug-modified ss DNA not subjected to heating (lanes 3, 5, 7, 9, and 11) remained intact, indicating that all the templates were saturated with drug molecules at the desired site.

Determination of the Extent of the Polymerization Reaction by Klenow Fragment on Single-Stranded DNA Templates Modified by (+)-CC-1065 and Select Analogues. When the drug-modified ss DNA template was used, (+)-CC-1065 and its analogues were found to be strong blocks to progression of DNA polymerase. While in control experiments using the highest concentration of dNTPs the major product was fully elongated primers, in the presence of drug-modified templates only small amounts of full-length molecules were synthesized (Figure 4). The same two major termination sites were found for all five drug-modified templates, in which at the highest concentration of dNTP (lane 3) the termination step was one nucleotide further than at the lower dNTP concentrations. The

[2] The higher molecular weight bands appearing just above the band corresponding to the covalently modified adenine (arrow) are attributed to intermediates in the DNA backbone degradation (Reynolds et al., 1985). Subsequent thermal treatment under alkaline conditions results in conversion to the product migrating with the adenine sequencing reaction. The bands appearing at the bottom of the gel are an artifact created during electrophoresis and not a second species of DNA.

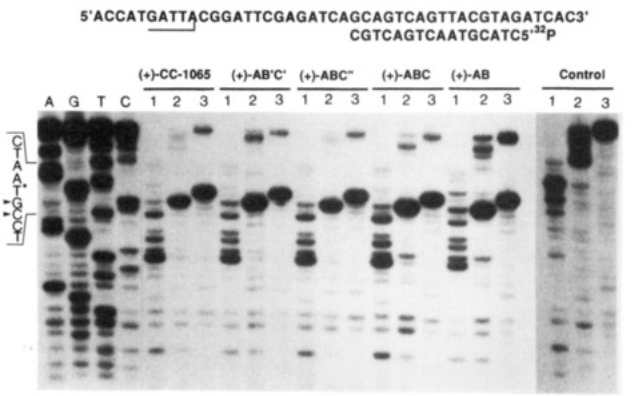

FIGURE 4: Effect of dNTP concentration on the termination site of in vitro DNA synthesis by Klenow fragment on the ss drug-modified template shown at the top of the figure. Lanes A, G, T, and C refer to the dideoxy sequencing reactions carried out with Klenow fragment on 5′-^{32}P-end-labeled 16-mer annealed to the T-45-mer. Lane headings refer to drug molecules used in template modification. Lanes 1, 2, and 3 are reactions containing 1, 10, and 100 μM dNTPs, respectively. 5′-^{32}P-End-labeled 16-mers (50 nM) annealed to either the T-45-mer or the T-45-mer containing drug–DNA adduct were incubated with 0.1 unit/μL (180 nM) Klenow fragment at 30 °C for 0.5 h as a function of dNTP concentration. The sequence within brackets is that of the primer-extended strand at the end of the sequence, and lower and upper arrowheads indicate the predominant termination sites of DNA synthesis at 10 and 100 μM dNTPs, respectively. The nucleotide indicated by an asterisk is the nucleotide opposite the drug-modified adenine.

termination sites were mapped exactly by using dideoxy sequencing of a control template. The primary termination step that was found at 10 μM dNTP concentration (lane 2) was mapped at the second nucleotide prior to the drug-modified adenine, while at the highest concentration of dNTP (100 μM, lane 3) this termination site disappeared and a new one appeared just one nucleotide prior to the drug-modified adenine. All the drug–DNA adducts caused the same pattern of termination in DNA synthesis mediated by the Klenow fragment, implying that each drug–DNA adduct changed the DNA structure in a manner such that the base-pairing step was impaired at the nucleotide prior to the modified adenine, as well as at the drug-modified adenine. The amount of bypass product was measured by densitometric scanning after autoradiography and expressed as a function of time at 10 and 100 μM dNTP concentrations (Figure 5, top and middle panels), and the effect of the amount of Klenow fragment on the formation of bypass product was also monitored (Figure 5, bottom panel). The extent of bypass product was reproducibly found to be dependent upon both dNTP concentration and, to a lesser extent, concentration of Klenow fragment. This observation suggests that the cause of the termination of DNA synthesis is predominantly the impairment of the base-pairing step at the adduct site, and this is a more significant factor than the lowering of the affinity of the Klenow fragment for the drug-modified DNA template. DNase I footprinting of the Klenow fragment on DNA in the presence and absence of drug also showed that DNA polymerase binds equally well to the drug-modified DNA template even after the polymerase reaches the drug-modified adenine (data not shown).

Determination of the Fidelity of DNA Polymerase in Incorporation of a Nucleotide One Base Prior to the (+)-CC-1065 Modification Site. The DNA polymerase incorporates the nucleotide 1 bp prior to the (+)-CC-1065 modification site only with low efficiency at 10 μM dNTP concentrations. Therefore, we evaluated whether this step might also be carried out with lower than usual fidelity. For this experiment, four parallel incubations were prepared containing, in each case,

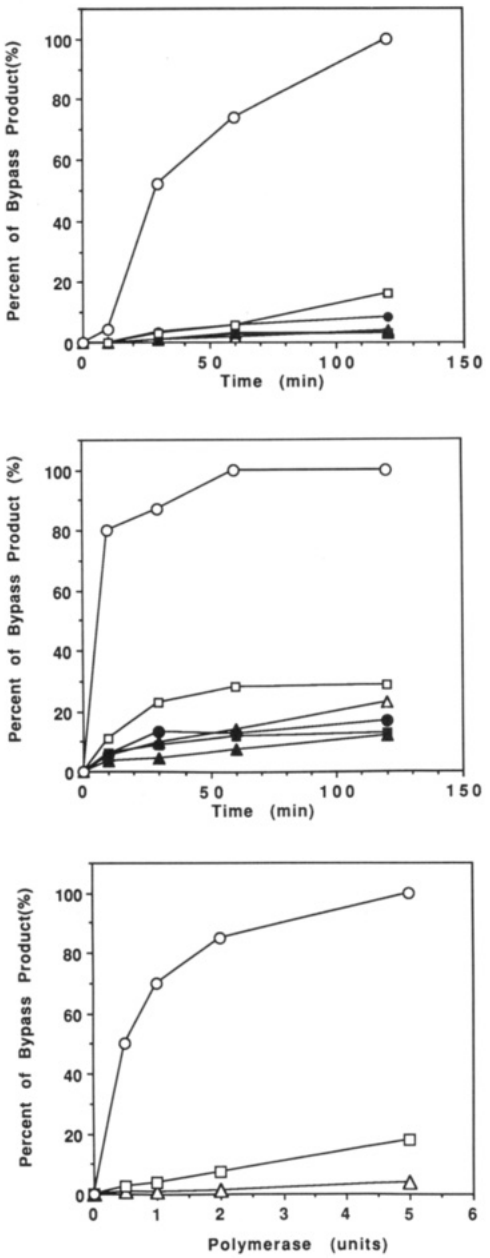

FIGURE 5: Effect of time of incubation (top and middle panels) and amount of Klenow fragment (bottom panel) on the extent of polymerase bypass product at a given concentration of dNTPs. In the top and middle panels are (5′-^{32}P-end-labeled 16-mer (50 nM) annealed to T-45-mer or drug-modified T-45-mer incubated with 10 and 100 μM dNTPs, respectively. In the bottom panel the same amount of template DNA was incubated with 10 μM dNTPs for 0.5 h with different amounts (in units) of Klenow fragment. Reactions were carried out with control template (○) and (+)-CC-1065- (△), (+)-AB′C′- (●), (+)-ABC″- (▲), (+)-ABC- (■), and (+)-AB-modified template (□).

10 μM dNTP, but individual tubes contained just one of the four dNTPs at 100 μM. As expected, at 100 μM dGTP, the primary pausing at the second nucleotide from the covalently modified adenine was removed, since dGTP is the complementary nucleotide for incorporation at the next site (Figure 6, lane 3). However, while supplements with 100 μM dTTP and dCTP did not cause elongation past the primary pausing site, a 100 μM dATP supplement in the presence of 10 μM of each of the other dNTPs allowed considerable passage to the secondary pausing site, which is presumably due to misincorporation of adenine opposite cytosine.

To further explore the possibility of misincorporation of bases at the site opposite this cytosine in the presence of

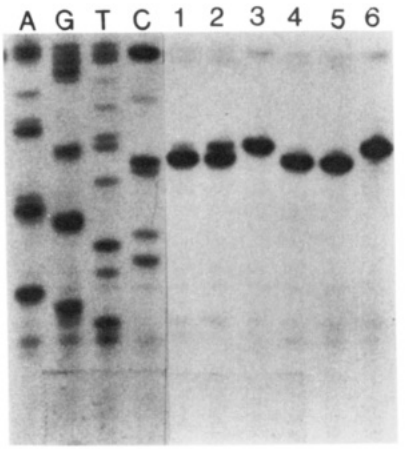

FIGURE 6: Effect of dNTP concentration bias on Klenow fragment termination sites in the presence of site-directed (+)-CC-1065-modified ss templates. 5′-^{32}P-End-labeled 16-mer (50 nM) annealed to T-45-mer modified with (+)-CC-1065 was incubated with 0.1 unit/μL Klenow fragment for 30 min in the presence of the indicated amount of dNTPs. A, G, T, and C refer to dideoxy sequencing reactions with non-drug-modified templates. Lanes 1–6 contain 10 μM dNTPs; 10 μM dNTPs and 100 μM dATP; 10 μM dNTPs and 100 μM dGTP; 10 μM dNTPs and 100 μM dTTP; 10 μM dNTPs and 100 μM dCTP; and 100 μM dNTPs in reaction mixtures, respectively.

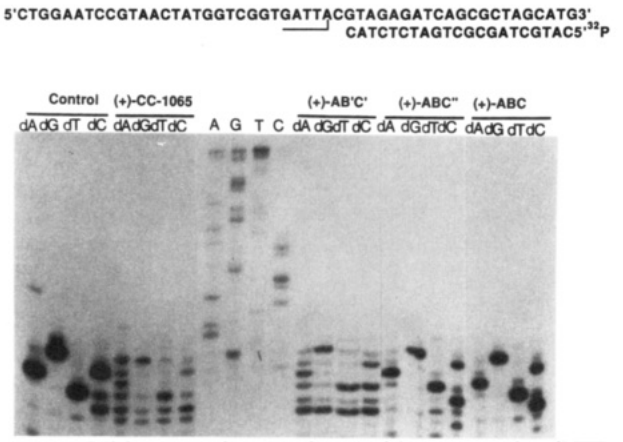

FIGURE 7: Misincorporation experiment without competing dNTPs for (+)-CC-1065-, (+)-AB′C′-, (+)-ABC″-, and (+)-ABC-modified 51-mer. To construct the template, which has a one-nucleotide gap between the 3′-end of the primer and the drug-modified adenine, a 5′-^{32}P-end-labeled 21-mer (45 nM) was annealed to T-52-mer or a site-directed drug-modified T-52-mer. Template DNA (45 nM) was incubated with 0.1 unit/μL Klenow fragment for 10 min in the presence of 100 μM dATP (lane heading dA), dGTP (lane heading dG), dCTP (lane heading dC), or dTTP (lane heading dT). Lanes A, G, T, and C refer to dideoxy sequencing reactions with non-drug-modified template.

(+)-AB′C′, (+)-ABC″, and (+)-ABC, as well as (+)-CC-1065, a second template (see Figure 7) was constructed in which the primer is complete up to the position at which guanine should be incorporated. Upon addition of DNA polymerase and, in separate experiments, each dNTP at 100 μM concentrations, only in the case of (+)-CC-1065 and dATP is misincorporation found to any significant extent (Figure 7). This experiment differs from the previous one in that no competing dNTPs were present in the inhibition mixture; nevertheless, it confirms the result shown in Figure 6.

Comparison of the DNA Polymerase Termination Sites Using Different Polymerases and Drug-Bonding Sequences. Since the bonding sequence 5′-AGTTA* shows such different reactivity with (+)-CC-1065 than 5′-GATTA* (Theriault et al., 1988), this sequence was also used in this investigation for comparison of termination sites using the Klenow fragment

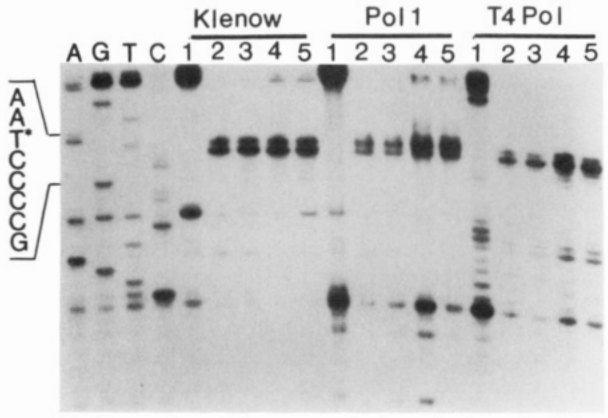

FIGURE 8: Comparison of the extent of polymerization using Klenow fragment, *E. coli* DNA polymerase, and T4 DNA polymerase using a 5′-AGTTA drug-bonding sequence. 5′-^{32}P-End-labeled 16-mer (50 nM) was annealed to the T-44-mer or a drug-modified T-44-mer. The polymerization reaction was carried out with 0.1 unit/μL Klenow fragment, *E. coli* DNA polymerase, or T4 DNA polymerase in the presence of 100 μM dNTPs for 30 min. Lanes 1–5 refer to polymerization reactions with control, (+)-CC-1065-, (+)-AB′C′-, (+)-ABC″-, and (+)-ABC-modified templates, respectively. The sequence around the drug-bonding site is given to the left of the gel.

(Figure 8). Unexpectedly, even with the lowest dNTP concentration used (10 μM), the major termination site occurred at *one* nucleotide prior to the drug-modified adenine, and the minor termination site also occurred at the drug-modified adenine, in contrast to the previous experiments with the bonding sequence, 5′-GATTA-3′, in which termination occurred *two* nucleotides prior to this adenine except at high concentration of dNTPs. Different kinds of DNA polymerase were also used in this study and found to show a similar pattern of termination sites. This pattern of further extension of primer was observed irrespective of the 3′-side flanking sequence of the drug-bonding site and also with the different polymerases (data not shown). These results suggest that two different bonding sequences (5′-**GA**TTA-3′ and 5′-**AG**TTA-3′) result in different types of association of the drug-modified template with DNA polymerase and, consequently, different polymerase termination sites.

Determination of the Effect of Drug Modification of the Priming Site on the Polymerization Process. (+)-CC-1065 and (+)-AB′C′ are unique in this class of compounds in that they are known to produce an unusual DNA winding effect, which affects the periodicity of DNA duplexes (Lee et al., 1991). Our recent studies on the effect of (+)-CC-1065 and related drug–DNA adducts on T4 DNA ligase (Sun & Hurley, 1992a) and helicase II (Sun & Hurley, 1992b) activity have also provided strong evidence that this DNA winding exerted by (+)-CC-1065 and (+)-AB′C′ on DNA interferes significantly with the DNA ligation or unwinding process. These observations led us to suspect that (+)-CC-1065 and (+)-AB′C′ modification in the vicinity of the priming site of the template could potentially affect overall DNA synthesis, since these drug molecules can affect the stability and the orientation of the 3′ terminus of the DNA primer. For this experiment, template DNA molecules modified with each of the drug molecules shown in Figure 1 in proximity to the priming site were constructed and used in the polymerase reactions to monitor any differences in efficiency of DNA synthesis between different drug molecules. As shown in Figure 9, the modification of template DNA with (+)-CC-1065 and (+)-AB′C′ severely blocked the elongation of primer DNA, while the modification with other analogues had little effect on this process. Since (+)-AB′C′ and (+)-CC-1065 are

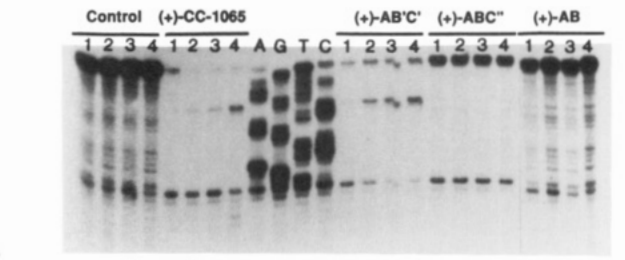

FIGURE 9: Effect of drug modification of the template strand in the vicinity of the primer on the polymerase extension reaction. Templates (50 nM) modified with the drug molecules indicated by the lane heading were incubated with 1 unit (0.1 unit/μL) of Klenow fragment for 30 min in the presence of 10 μM (lane 1), 20 μM (lane 2), 60 μM (lane 3), and 80 μM (lane 4) dNTPs.

unique in producing winding of DNA, in addition to the bending and stiffening of DNA that is common to all of the drugs used in this study, we propose that it is the change in helical periodicity on the priming site produced by drug modification that is mainly responsible for the inhibition of the polymerization process. It is also important to note that the DNA polymerase can still utilize the bent DNA template produced by drug modification on the priming site.

Discussion

Using a ss template containing a site-directed drug–(*N*3-adenine)DNA adduct, we have shown that (+)-CC-1065 and its analogues (Figure 1) block in vitro DNA synthesis mediated by Klenow fragment, *E. coli* DNA polymerase I, and T4 DNA polymerase, presumably due to covalent modification of adenine. This result is consistent with the previous reports that the cytotoxicity of (+)-CC-1065 is primarily mediated by the inhibition of DNA synthesis rather than inhibition of RNA or protein synthesis (Bhuyan et al., 1982), and the Sequenase results recently published (Weiland & Dooley, 1991). The ss templates used for the studies reported here were constructed from partial duplex DNA molecules that contain a site-directed drug–DNA adduct in the duplex region. The ss DNA containing the drug–DNA adduct was purified by strand separation on denaturing polyacrylamide gel electrophoresis. This was then annealed with a primer that is complementary to a sequence to the 3′ side of the drug–DNA adduct site. The stability of the covalent drug adduct during this treatment was determined by a spectrophotometric assay and an OP-Cu-phenanthrene footprinting assay of the drug–duplex adduct (unpublished results). In the latter case, the duplex for footprinting was constructed by annealing a complementary strand to a drug-modified ss DNA prepared in the usual way. The saturation of the drug-bonding site and the location of the adduct on the template were determined by a thermal cleavage strand breakage assay (Reynolds et al., 1985). Each DNA template that was constructed contained only one drug-modified adenine at full saturation at the desired site, which allowed us not only to quantitate the amount of polymerase bypass product through the drug–DNA adduct but also to determine the polymerase pausing site(s) caused by this adduct.

The results of this study show that (+)-CC-1065– and analogue–(*N*3-adenine)DNA adducts were not absolute blocks for the different kinds of DNA polymerase used in this study, since up to 25% full-length products were reproducibly observed in (+)-AB-modified and other analogue-modified templates. However, for the formation of bypass product, high

concentrations of dNTP and DNA polymerase were required, together with longer time incubation compared to control template, which do not compare to the in vivo situation. Moreover, it is important to recognize that this work was carried out in vitro with an incomplete replication system. These results also suggest that drug modification of the template prevents the base-pairing process, rather than lowering the affinity of the DNA polymerase for the template at the drug–DNA adduct site, since polymerase binds equally well to drug-modified and unmodified templates (unpublished results).

Since (+)-CC-1065 is one of the most potent mutagenic agents known (Bhuyan et al., 1982), we also tested the possibility of drug-induced base misincorporation near the adduct site during the polymerization process. The results showed that the (+)-CC-1065–DNA adduct was particularly prone to produce misincorporation of the nucleotide *prior to modified adenine* when it was covalently attached to the sequence 5′-GATTA*-3′ (* indicates the drug-modified adenine), while other (+)-CC-1065 analogues did not induce significant misincorporation. This appears to be an unusual case, since most mutagenic compounds cause the base mispairing *at the lesion site* (Palejwala et al., 1991). However, in the particular sequence examined, only dA was significantly misincorporated for dG at the nucleotide prior to the modified adenine. Overall, these results imply that the potent mutagenicity of (+)-CC-1065 (Harbach et al., 1986) may be caused by the misincorporation of nucleotides during DNA synthesis. What has not been addressed in this present study is the possibility of misincorporation of nucleotides opposite the covalent adduct site and in the drug overlap region. Experiments are in progress using an M13 viral DNA bearing a site-specific (+)-CC-1065-DNA adduct in a transfection system to investigate this possibility.

The DNA polymerase termination site could be modulated more significantly by increased dNTP concentration than by higher polymerase concentration. While the exact termination site was dependent upon the covalent bonding sequence, it was independent of drug structure within the (+)-CC-1065-evaluated series and type of DNA polymerase. Specifically, the primary (pausing) termination site for the polymerases was *one* nucleotide prior to the covalently modified adenine for the bonding sequence 5′-AGTTA*, but for 5′-GATTA* the primary pausing site was *two* nucleotides prior to the drug-modified adenine. The only difference between the two bonding sequences is the AG vs the GA step, 4 and 3 bps, respectively, to the 5′ side of the covalently modified adenine. Since this apparent minor difference causes a change in the pausing site for the DNA polymerase, this is suggestive of a more significant conformational difference in the two drug–DNA adducts. We have noted other differences between these two sequences in relative reactivity to (+)-CC-1065 (Theriault et al., 1988), recognition by UvrA (Sun and Hurley, unpublished results), and magnitude of drug-induced bending (Sun and Hurley, unpublished results). In addition, our recent two-dimensional ^{1}H- and ^{31}P-NMR study on a 12-mer containing the 5′-AGTTA sequence has revealed a transient kink associated with the 5′-AC and 5′-CT steps on the complementary strand opposite 5′-AGT (Lin et al., 1992). This observed difference in polymerase pausing site may be yet another indication of the unique structure of the highly reactive sequence 5′-AGTTA and, consequently, its unique secondary structure as the drug–DNA adduct (Lin & Hurley, 1992).

The main purpose of this investigation was to determine the effect of (+)-CC-1065-modified DNA template on polymerase extension past the adduct site. The results show that, irrespective of the (+)-CC-1065 analogue tested, the in vitro DNA synthesis on a ss template is inhibited to about the same extent. This was surprising, since (+)-CC-1065 and the analogues used here show different levels of cytotoxicity and antitumor activity, and only (+)-CC-1065 and (+)-AB′C′ show the delayed lethality (McGovren et al., 1984; Warpehoski et al., 1988). However, what is dramatically different is the effect (+)-CC-1065 and (+)-AB′C′ have on polymerization from a primer site containing a drug lesion in the duplex region relative to (+)-ABC, (+)-AB, and (+)-ABC″. While (+)-CC-1065 and (+)-AB′C′ selectively inhibit DNA synthesis from a primer in a duplex region containing the adduct (see template inset in Figure 9), the other drugs have little effect on the polymerase activity. (+)-CC-1065 and (+)-AB′C′ are unique not only in this selective effect on DNA polymerase but also in producing an unusual winding effect alongside the bending and stiffening of the helix common to other members of this drug series (Lee et al., 1991). This winding effect, which changes the periodicity of DNA by about the equivalent of 1 base pair per adduct site, is associated with the inside-edge substituents of the pyrrole rings of subunits B and C (see Figure 1) and has also been associated with a much stronger inhibition of T4 ligase (Sun & Hurley, 1992a) and helicase II catalyzed unwinding of DNA (Sun & Hurley, 1992b). Since these compounds are also unique in producing the delayed lethality in mice, the winding effect is a possible candidate.

In conclusion, covalent modification of DNA molecules with (+)-CC-1065 and its analogues was found to be a strong hindrance to in vitro DNA synthesis mediated by *E. coli* DNA polymerase, Klenow fragment, and T4 polymerase and to cause pretermination of polymerization around the adduct site, which then inhibits the DNA replication process.

ACKNOWLEDGMENTS

We thank Mr. David M. Bishop for editorial assistance.

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Flanking Sequence Effects within the Pyrimidine Triple-Helix Motif Characterized by Affinity Cleaving†

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Received September 3, 1991; Revised Manuscript Received December 30, 1991

ABSTRACT: Nearest neighbor interactions affect the stabilities of triple-helical complexes. Within a pyrimidine triple-helical motif, the relative stabilities of natural base triplets T·AT, C+GC, and G·TA, as well as triplets, D_3·TA and D_3·CG, containing the nonnatural deoxyribonucleoside 1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamido)phenylimidazole (D_3) were characterized by the affinity cleaving method in the context of different flanking triplets (T·AT, T·AT; T·AT, C+GC; C+GC, T·AT; G+GC, C+GC). The T·AT triplet was shown to be insensitive to substitutions in either the 3′ or 5′ directions, while the relative stabilities of triple helices containing C+GC triplets decreased as the number of adjacent C+GC triplets increased. Triple helices incorporating a G·TA interaction were most stable when this triplet was flanked by two T·AT triplets and were adversely affected when a C+GC triplet was placed in the adjacent 5′ direction. Similarly, complexes containing D_3·TA or D_3·CG triplets were most stable when the triplet was flanked by two T·AT triplets but were destabilized when the adjacent 3′ neighbor position was occupied with a C+GC triplet. This information regarding sequence composition effects in triple-helix formation establishes a set of guidelines for targeting sequences of double-helical DNA by the pyrimidine triple-helix motif.

Triple-stranded polynucleotide RNA complexes were identified shortly after the discovery of the DNA double helix (Felsenfeld et al., 1957). These structures consist of one purine and two pyrmidine strands, and it was postulated that this interaction occurs through the formation of a triple helix in which a pyrimidine strand binds in the major groove of double-helical DNA (or RNA) with specific hydrogen bonds formed to the Watson–Crick purine strand. To date, no high-resolution X-ray structure for a triple helix has been reported; however, chemical and spectroscopic studies support triple-helical structures derived from the formation of T·AT and C+GC base triplets (Felsenfeld et al., 1957; Lipsett, 1963, 1964; Michelson et al., 1967; Morgan & Wells, 1968; Lee et al., 1979; Moser & Dervan, 1987; Praseuth et al., 1988; Rajagopal & Feigon, 1989a,b; de los Santos et al., 1989; Sklenar & Feigon, 1990; Pilch et al., 1990; Radhakrishnan et al., 1991a) (Figure 1).

In exploring the possibility that the triple-helix structure could function as a recognition motif for single sites in large double-helical DNA, it was demonstrated that a pyrimidine oligonucleotide binds specific purine tracts within large duplex DNA (Moser & Dervan, 1987; Le Doan et al., 1987). The pyrimidine third strand is parallel to the purine Watson–Crick strand (Moser & Dervan, 1987). Since these initial findings, the pyrimidine triple-helix motif has been utilized to target single sites within megabase DNA (Strobel & Dervan, 1990, 1991); the recognition code has been broadened to include G·TA triplets (Griffin & Dervan, 1989); and a nonnatural

† We are grateful to the Office of Naval Research for generous support.
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0006-2960/92/0431-2829$03.00/0