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In Vitro and in Vivo DNA Bonding by the CC-1065 Analogue U-73975

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Received February 21, 1991; Revised Manuscript Received May 2, 1991

ABSTRACT: CC-1065, a cyclopropylpyrroloindole (CPI), is a highly potent antitumor DNA-alkylating agent. We have devised a simple method to detect CPI bonding sites on double-stranded DNA (dsDNA). The technique utilizes a modified form of bacteriophage T7 polymerase, Sequenase, to synthesize a radiolabeled nascent strand from dsDNA that has been reacted in vitro with the CC-1065 analogue U-73975 (adozelesin). The reaction products were electrophoresed on sequencing gels containing 8 M urea and visualized by autoradiography. The transit of this DNA polymerase is inhibited at the sites where CPIs are bound to the template strand. Thus, the enzyme stalls or stops at the nucleotide immediately adjacent to the modified base, resulting in the accumulation of DNA strands at these sites and in diminished read-through beyond these sites in a set of CPI-treated DNA molecules. The precise positions of polymerase inhibition can be determined by comparison of CPI-treated and unreacted DNA reactions. This modified dideoxynucleotide sequencing technique has been used to establish the sequence selectivity of U-73975. Approximately 1 kilobase of dsDNA has been analyzed to derive a consensus canonical bonding sequence, 5′(T/A)-T/A-T-A*-(C/G)-(G), where A* is the site of U-73975 alkylation and parentheses denote deoxynucleotide preferences. Noncanonical sites were also found at poly(A) sites. This technique yielded a consensus sequence for U-73975 bonding that is similar to, but not identical with, the published consensus obtained for CC-1065 by a modified Maxam and Gilbert sequencing technique. We have also examined the bonding of [^{3}H]U-73975 to the DNA of viable cultured mammalian cells, using gel electrophoresis and autoradiographic techniques. [^{3}H]U-73975 does appear to be covalently bound to nuclear DNA in vivo (intracellular) and to a subset of human highly repetitive sequence elements. U-73975 is bound predominantly to nuclear DNA at sites other than telomeric repeat sequences, which contain the potential CPI bonding sequence $(TTAGGG)_n$.

The interaction of cyclopropylpyrroloindole (CPI)[1] compounds with DNA has been established (Reynolds et al., 1986). CC-1065 is an extremely potent cytotoxic CPI compound produced by *Streptomyces zelensis* (Reynolds et al., 1986). Structurally, this unique compound consists of three repeating pyrroloindole subunits, one of which contains a potentially reactive cyclopropane ring (Hurley et al., 1990). The chemistry of CC-1065 and its biological properties have been documented (Reynolds et al., 1986). The intracellular target for CC-1065 is double-stranded DNA (dsDNA), with little or no affinity for protein, RNA, or single-stranded DNA (Reynolds et al., 1986). This agent binds in the minor groove of duplex B-DNA, where the cyclopropyl moiety reacts with the N3 of adenine to form a covalent adduct (Reynolds et al., 1985, 1986). It is this irreversible covalent association with DNA that is believed to be responsible for its extraordinary potent antitumor activity (Hurley, 1990).

A modified Maxam and Gilbert sequencing technique has demonstrated CC-1065 alkylation of dsDNA to be highly sequence-specific (Reynolds et al., 1985). Initial analysis of CC-1065 binding/bonding sites revealed that 5′PuNTTA* and

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[1] Abbreviations: bp, base pair; cDNA, complementary DNA; CPI, cyclopropylpyrroloindole; DNA, deoxyribonucleic acid; dNTP, deoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; kb, kilobase (1000 bp); NaOAc, sodium acetate; NaOH, sodium hydroxide; PBS, phosphate-buffered saline; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; dsDNA, double-stranded deoxyribonucleic acid; DMA, *N,N*-dimethyacetamide; DMF, dimethylformamide.

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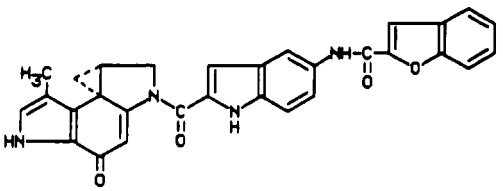

FIGURE 1: Structure of U-73975. The molecule consists of 3 subunits. The left-hand segment contains the potentially reactive cyclopropyl group. The cyclopropyl moiety forms covalent N3-adenine DNA adducts.

5′AAAAA*, where A* is the site of alkylation, are most reactive (Reynolds et al., 1985). Similar results were obtained by circular dichroism techniques for CC-1065 bound to synthetic DNA oligomers (Swenson et al., 1988). More recently, additional CC-1065 binding/bonding sites have been cataloged and have demonstrated that 5′A/T-A/T-A* is the essential component that conveys high reactivity (Hurley et al., 1990).

Studies using CC-1065 in vivo have demonstrated a drug-induced delayed death and hepatotoxicity at nontherapeutic doses (Hurley et al., 1990). Several CC-1065 analogues have been synthesized that maintain potent cytotoxicity without exhibiting the delayed death phenomenon. The highly potent CPI analogue U-73975 (adozelesin; Figure 1), currently in Phase I human clinical trials for cancer therapy, has recently been characterized with respect to its interaction with DNA. Unpublished results obtained at Upjohn Laboratories indicate that like CC-1065 U-73975 does not react appreciably with protein, RNA, or single-stranded DNA but does bond covalently with the N3 of adenine in duplex B-DNA. However, differences in reactivity to some oligonucleotide duplexes have suggested altered sequence-specific binding/bonding interactions for U-73975 (K.L.W., T.P.D., J. P. McGovren, G. L. Petzold, and W. C. Krueger, Upjohn Laboratories, unpublished results).

This report examines the interaction of U-73975 with DNA. Specific target consensus sequences for U-73975 binding/bonding have not yet been reported. Here we describe the development of a novel, rapid technique to determine sequence selectivity for bound CPI analogues. With use of this technique, the bonding of U-73975, as well as that of other DNA interactive agents, was investigated. Also, a consensus bonding sequence for U-73975 was derived.

The in vivo (intracellular) localization of CPI adducts on nuclear DNA was investigated. The DNA of eukaryotic organisms contains a substantial fraction of highly repetitive sequences, which are dispersed throughout the genome (Korenberg & Rykowski, 1988; Hutchison et al., 1989). These sequences are present at a high copy number, and some of the repeats characteristically have a 3′ A-rich region (Hutchison et al., 1989). In addition, a different conserved repetitive DNA sequence, $(TTAGGG)_n$, was found to be clustered at the telomeres of all human chromosomes (Moyzis et al., 1988). This repetitive sequence resembles the reported CC-1065 consensus and is potentially a very reactive binding/bonding sequence. Prior to this work, increased binding/bonding of CPI compounds to specific regions on chromosomes or nuclear DNA has not yet been demonstrated. The availability of tritiated U-73975 has allowed us to preliminarily address the bonding of U-73975 to telomeres, highly repetitive elements, and total nuclear dsDNA.

Materials and Methods

Compounds and Cell Lines. The CPI analogue U-73975 was provided by R. Kelly (Upjohn) as a 1 mg/mL solution in *N*,*N*-dimethylacetamide (DMA) and stored at −20 °C. [^{3}H]U-73975 was radiolabeled at Chemsyn Science Laboratories (Lenexa, KS) and supplied in DMF at a specific activity of 10 Ci/mmol, 1 mCi/mL. Dilutions of U-73975 were made in DMA immediately prior to use. Melphalan was purchased from Sigma Chemical Co. (St. Louis, MO).

Human HT-1080 fibrosarcoma cells (ATCC CCL121; Rasheed et al., 1974) were used to examine [^{3}H]U-73975 bonding in vivo.

In Vitro Bonding of U-73975 to Purified Plasmid DNA. Double-stranded, supercoiled, plasmid DNA was routinely isolated by alkaline lysis of bacterial host cells (Maniatis et al., 1982) and further purified either in cesium chloride gradients or over Elutip-d columns (Schleicher & Schuell). Five different plasmids were reacted with U-73975 and subjected to DNA sequencing (below). Vector plasmids pGEM3Z and pGEM7Zf+ were purchased from Promega Corp. (Madison, WI). Several cDNA inserts from λ gt11 libraries were subcloned into the pGEM vectors to facilitate sequencing. Two subclones contained human cDNAs; pWFD6, a 593-bp partial α_1-antitrypsin cDNA [EMBL/GenBank Accession No. X17122 (T.P.D. and K.L.W., unpublished results)], and a 442-bp involucrin cDNA, pGEM3.1-3 (S. Young, D. L. Hudson, K. L. Weiland, T. P. Dooley, M. Simon, and F. M. Watt, submitted for publication). This involucrin clone extends from position 1292 to 1733 of the human involucrin gene, exon 2 (Eckert et al., 1986). Portions of a 0.99-kb rat rhodanese cDNA, pRhoC (Weiland & Dooley, 1991), were also sequenced.

Sequencing Technique for Determining CPI Adduct Sites. Routinely, 2 μg of plasmid DNA was reacted with 200 pmol of U-73975 for 16 h at 22 °C in a 10-μL volume containing 0.1× SSPE (15 mM NaCl, 1 mM NaH_2PO_4, 0.1 mM EDTA, pH 7.4). The molar ratio of U-73975 (20 μM final concentration) to plasmid DNA ranged from approximately 160:1 to 260:1, depending on the size of the different plasmids. Incubation times, temperatures, and U-73975 concentrations were occasionally modified (refer to Results section).

The DNA was sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977). Both reacted and unreacted plasmid DNAs were denatured at room temperature for 5 min by the addition of NaOH to a final concentration of 0.2 N. The solution was adjusted to 0.3 M NaOAc, pH 7, and the DNA was precipitated with the addition of ethanol. Ethanol precipitation presumably removes unbound CPI. After centrifugation, the pellet was washed with 75% ethanol at room temperature and dried. Preliminary experiments using [^{3}H]U-73975-bound plasmid DNA have shown the CPI–DNA adduct to be stable under these conditions (data not shown). The denatured DNA was annealed for 30 min at 37 °C in sequencing buffer with 0.5 pmol of oligonucleotides complementary to vector sequences to serve as primers. The sequence reactions were performed with the Sequenase kit (U.S. Biochemicals, Cleveland, OH), which utilizes a modified form of bacteriophage T7 DNA polymerase, Sequenase, to synthesize a nascent strand incorporating [α-^{32}P]dATP. The reaction products were electrophoresed on 6% acrylamide–8M urea gels and visualized by autoradiography. The drug-treated DNA samples are subjected to the same dideoxynucleotide reactions as the control DNA. Therefore, polymerase inhibition sites are superimposed on the reference sequence ladder to facilitate precise identification of "termination sites" and to indicate the extent of reduced polymerase activity at primer-distal sites.

Exposure of Cultured Human HT-1080 Cells to [^{3}H]U-73975 and Extraction of Nuclear DNA. Routinely, subcon-

fluent cultures of human HT-1080 cells were treated with 20 nM [^{3}H]U-73975 (10 Ci/mmol) in Dulbecco's-modified Eagle's medium with 10% fetal calf serum for 17 h at 37 °C, 5% CO_2. The medium was removed, and the cells were rinsed with PBS. Lysis buffer (50 mM Tris-HCl, pH 8, 100 mM EDTA, 0.5% SDS) was added to disrupt the cells. The lysate was phenol–chloroform-extracted several times to eliminate cellular debris. The DNA was precipitated out of solution by the addition of sodium acetate to 0.3 M and 2 volumes of ethanol, and placed at −20 °C for 1 h. After centrifugation, the DNA pellet was washed with 75% ethanol at room temperature and dried. The pellet was resuspended in TE (10 mM Tris-HCl, pH 7.9, 1 mM EDTA) containing 10 μg/mL RNase A (Sigma Chemical Co.) and incubated at 37 °C for 30 min. The DNA was again precipitated as above.

Digestion of DNA To Investigate in Vivo Bonding of U-73975 to Telomeres. Untreated HT-1080 nuclear DNA was included as a control to confirm that the presence of the CPI bound to nuclear DNA does not interfere with the restriction of the DNA or the hybridization of probe. Each digest contained 10 μg of untreated or [^{3}H]U-73975-bound (specific activity = 5×10^3 cpm/μg; 65% counting efficiency) nuclear DNA. *Eco*RI (a six-base cutter) was used to resolve the DNA into a wide range of fragment sizes. A double digest using two enzymes, *Sau*3AI and *Alu*I (each four-base cutters), cleaves the DNA into extremely small molecular weight fragments. Because of the highly repetitive nature of telomeres, they lack restriction endonuclease cleavage sites and remain as high molecular weight fragments in these digests (greater than 3 kb).

Digested DNA was run on a 0.75% agarose gel and stained with ethidium bromide (EtBr) for visualization using ultraviolet light. Transfer of the untreated and electrophoresed DNA was carried out according to standard methodology (Southern, 1975) to a Nytran membrane (Schleicher & Schuell). After transfer, the DNA was permanently affixed to the membrane by placement at 80 °C in vacuo for 2 h. Prehybridization of the membrane was done for 1 h at 50 °C in 5× SSPE–0.3% SDS–2× Denhardt's solution. A telomere-specific oligonucleotide, $(TTAGGG)_3$ was synthesized and end-labeled with [γ-^{32}P]ATP, with use of T4 polynucleotide kinase, to a specific activity of 8×10^7 cpm/μg. This probe was added to the prehybridization solution at a concentration of 10^6 cpm/mL and hybridized for 16 h at 50 °C. The membrane was washed twice in 2× SSPE–0.2% SDS at 50 °C to remove unbound probe. The membrane was autoradiographed with XAR film (Kodak, Rochester, NY) for 2 weeks at −70 °C with an intensifier screen.

Autoradiography of [^{3}H]U-73975-bound nuclear DNA digests demonstrates the predominant CPI bonding patterns for comparison to the Southern blot, which was probed with the telomere-specific oligonucleotide. Digests of CPI-labeled nuclear DNA were run on a separate agarose gel. The radioactive signal was enhanced by soaking the gel in Enlightning (NEN, Boston, MA) for 20 min. The gel was dried under vacuum at 65 °C and subsequently autoradiographed with XAR film for 3 weeks at −70 °C.

Digestion of [^{3}H]U-73975-Bound Nuclear DNA To Resolve Repetitive Elements. Each restriction endonuclease digest contained 5 μg of [^{3}H]U-73975-bound nuclear DNA at a specific activity of 6.8×10^3 cpm/μg. The following enzymes were used to restrict the DNA at 37 °C for 16 hours in the appropriate buffers recommended by the supplier: *Eco*RI, *Bgl*II, *Kpn*I, *Bam*HI, *Sca*I, *Xba*I, *Nco*I, *Pst*I, *Hpa*I, and *Sph*I. The DNAs were electrophoresed on an 0.8% agarose gel and stained with EtBr for visualization of the DNA with ultraviolet light. The radiolabeled DNAs were enhanced by soaking the gel in Enlightning for 20 min. The gel was then dried at 65 °C under vacuum and autoradiographed for 4.5 weeks with XAR film at −70 °C.

Results and Discussion

The dideoxynucleotide sequence ladder of plasmid DNA reacted with U-73975 (refer to Materials and Methods) in Figure 2 shows several sites within the DNA where DNA polymerase (Sequenase) encountered DNA modifications (e.g., CPI adducts) resulting in premature blockage of chain elongation, which we presume are "termination sites". These sites do not occur in the unreacted control DNA ladder. It is presumed that these sites in CPI-treated DNA are caused by impedance or stoppage of this bacteriophage DNA polymerase read-through by U-73975 bound to the template strand. The sequence presented in Figure 2 represents the nascent strand or, in other words, the complement of the template strand (i.e., the nascent strand is similar to the polymerase-displaced strand). For reference, the drug-treated samples were subjected to the same dideoxy sequencing reactions as the control DNA, so that the inhibition sites are precisely identified and the extent of reduced downstream polymerase activity can be determined. Figure 3 diagrammatically conceptualizes how deoxynucleotide incorporation proceeds along a template until it encounters a CPI molecule. The presence of the CPI–N3-adenine adduct blocks the movement of this DNA polymerase. On these sequencing gels, polymerase stops or stalls at the position immediately adjacent to the modified adenine, as evidenced by the accumulation of ^{32}P-labeled DNA nascent strands at these juxtaposed sites. We presume that Sequenase cannot continue to read beyond CPI-bound sites; thus, the newly synthesized strand is terminated. Using this technique, one can not determine whether the final nucleotide of the arrested chain is correctly incorporated.

By comparison with unreacted sequence, the nucleotides flanking the termination sites can be determined. One kilobase (kb) of DNA sequence information was generated and determined to contain 55% G:C. Examination of these sequences yielded 27 unique U-73975 bonding sequences of a common motif, which are cataloged in Table I. The sequences presented are the complement of the experimental sequence data (as obtained directly from the sequencing gels), thus reflecting the actual template sequences read by the polymerase. The bonding sequences of this type are termed "canonical" by comparison with previously reported CC-1065 bonding sequences, where TTA* and ATA* are commonly bound (the asterisk indicates adduct site; Hurley et al., 1990). Alternatively, two sequences were "noncanonical" and represent poly(dA) tracts that were present infrequently in this 1 kb of DNA (Table I). Therefore, a minimum of four dA residues is sufficient for U-73975 bonding. Table II gives the percentage of frequency of occurrence for each nucleotide in each position of the canonical bonding sequences. The percentages were computed from 27 canonical bonding sequences for U-73975 found in 1 kb of sequence information. A consensus canonical bonding sequence for U-73975 was derived: 5′-(T/A)-T/A-T-A*-(C/G)-(G), where A* is the site of alkylation. The parentheses indicate a bias for those nucleotides; however, their occurrence is not absolute. The specificity of U-73975 for two types of binding/bonding sequences [canonical and poly(deoxyadenine)] is consistent with previous reports of CC-1065 having this property (Reynolds et al., 1985). The consensus bonding sequences obtained for these two CPI compounds by different techniques are similar, but

Table I: Bonding Sequences[a] of U-73975

5′	−4	−3	−2	−1	A	+1	+2	+3
				canonical				
	T	A	T	T	A	C	A	A
	C	T	A	T	A	G	T	G
	G	A	A	T	A	C	T	C
	C	T	A	T	A	G	G	T
	A	T	T	T	A	G	G	T
	C	T	T	T	A	T	G	C
	G	A	T	T	A	C	G	C
	G	C	T	T	A	T	C	G
	C	T	T	T	A	C	A	C
	C	A	T	T	A	G	G	C
	A	G	T	T	A	G	C	T
	A	A	T	T	A	A	T	G
	C	G	A	T	A	A	G*	C
	T	A	A	T	A	C	G	A
	C	T	A	T	A	G	G	G
	G	A	T	T	A	A	G*	T
	T	A	T	T	A	G	C	C
	G	G	A	T	A	A	C*	A
	G	T	T	T	A	T	T	G
	T	A	T	T	A	C	A*	A*
	C	T	A	T	A	G	G	A
	T	T	T	T	A	G	A*	A
	T	C	T	T	A	C	G	C
	G	T	T	T	A	C	G	A
	T	A	T	T	A	C	A*	A
	A	T	T	T	A	C	C*	C
	C	T	A	T	A	G	T	G
				noncanonical				
	T	A	A	A	A	C	G	A
	G	A	A	A	A	T	G	A

[a] *A* is the presumed site of U-73975 adduct formation. +1 corresponds to the site at which apparent dNTP misincorporation occurs adjacent to the adduct "A". The asterisks in +2 and +3 positions indicate additional nucleotides at which polymerase stalls.

Table II: Frequency of Nucleotides Flanking Canonical Adenine Adduct Sites of U-73975

5′	−4	−3	−2	−1	A	+1	+2	+3
G	26	11	0	0	0	37	44	22
A	15	37	33	0	100	15	18	30
T	26	44	67	100	0	11	18	15
C	33	7	0	0	0	37	18	33
A/T	41	82	100	100	100	26	37	44
G/C	59	18	0	0	0	74	63	56
consensus	N	(T/A)[a]	T/A	T	A	(C/G)	(G)	N

[a] Parentheses indicate a strong tendency for a particular base to occur at that position; however, its presence is not absolute.

not identical. CC-1065 was reported several years ago to exhibit selectivity for 5′PuNTTA*. Although the two bases 5′ proximal to the adenine are identical or similar, U-73975 has a T/A bias in the −3 position (relative to A*) and no preference for a purine at the −4 position, using our technique.

In order to verify the selectivity of U-73975 bonding to T/A-T-A sites, sequences having a potentially reactive TA dinucleotide elsewhere in the 1 kb of DNA that were *not* bound by U-73975 are cataloged in Table III. The −2 position of these sequences indicates an absolute C/G bias. The A/T requirement at the −2 position for U-73975 bonding may be described as a C/G "avoidance" (Hurley et al., 1990); thereby, the presence of a C/G at this position precludes bonding of U-73975. Perhaps the minor groove cleft is interrupted by the protruding 2-amino group of guanine, making covalent bond formation difficult. It may be inferred from these results that the −2 position T/A is a requirement for sequence specificity of U-73975 adduct formation. In addition, the +1 position frequently contains T in nonbound sites (67%). Perhaps a +1 T avoidance also occurs. Thus, a *nonbonding*

Table III: TA Dinucleotide Sequences within 1 kb of DNA That Are Not Bound by U-73975[a]

5′	−4	−3	−2	−1	A	+1	+2	+3
	G	T	C	T	A	G	A	G
	G	G	G	T	A	C	C	G
	A	G	C	T	A	T	G	A
	C	T	C	T	A	G	A	C
	T	T	G	T	A	T	G	A
	A	C	C	T	A	T	G	A
	C	A	C	T	A	A	G	G
	A	C	C	T	A	T	G	A
	A	A	G	T	A	T	T	C
	C	A	C	T	A	T	A	G
	A	G	C	T	A	T	T	T
	T	T	G	T	A	A	T	A
	C	G	C	T	A	T	T	A
	C	A	C	T	A	T	A	G
	C	C	C	T	A	T	T	A

[a] A is the potentially reactive adenine.

Table IV: Frequency of Nucleotides Flanking TA Dinucleotide Sequences Not Bound by U-73975

5′	−4	−3	−2	T	A	+1	+2	+3
G	13	27	27	0	0	13	33	33
A	33	27	0	0	100	13	27	47
T	13	27	0	100	0	67	33	7
C	40	20	73	0	0	7	7	13
A/T	47	53	0	100	100	80	60	53
G/C	53	47	100	0	0	20	40	47
consensus			C/G	T	A	(T/A)		

consensus that contains a TA dinucleotide (Table IV) was generated: C/G-T-A-(T/A).

Overlapping regions of U-73975-bound DNA (demonstrated to be double-stranded by restriction cleavage, data not shown) were sequenced to determine if CPI bound to the displaced strand affected polymerase read-through on the template. Separate sequencing reactions were done with plasmid pGEM7Zf+ with primers that flank the 120-bp polylinker region. The presence of U-73975 on the displaced strand had no effect on synthesis of the nascent strand (data not shown). Only sequences fitting the consensus rule on the template strand were observed as enzyme termination sites. In addition, if termination were caused by the displace strand, then one would predict that about half of all sites would appear as the reverse complement of our previously stated consensus rule, and yet all of the sites we discovered were due to template strand bound CPI. These results suggest that polymerase termination is not due merely to the presence of the CPI molecule within the minor groove but correlated with the covalent association of U-73975 with adenine of a specific target sequence *only* on the template strand.

Other aspects of U-73975-DNA interaction were addressed. Inhibition of strand synthesis by U-73975 was demonstrated in sequencing reactions using two different DNA polymerases, modified T7 DNA polymerase (Sequenase) and DNA polymerase I, large fragment (Klenow, data not shown). In addition, the effect of temperature on adduct formation was investigated. DNA was reacted with U-73975 as described previously; however, the temperature for adduct formation was varied. Polymerase read-through was arrested proximal to the priming site when incubated with CPI at 42 °C, when compared with the 37 and 4 °C incubation reactions (Figure 2A). This indicates that a greater extent of U-73975 bonding to DNA occurs at 42 °C. Adding a large molar excess of U-73975 can saturate the DNA to completely inhibit polymerase read-through or can be titrated to give minimal effects. We believe this titration result provides strong evidence that DNA polymerase is actually terminated and not merely stalled at

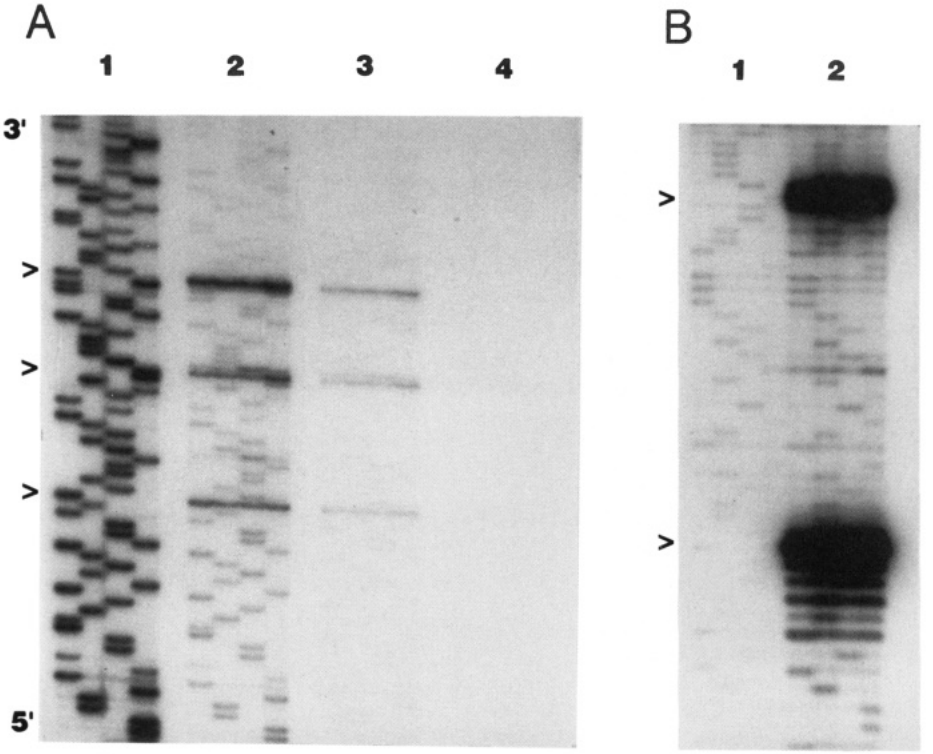

FIGURE 2: Sanger dideoxy sequencing reactions of pGEM7Zf+ plasmid DNA. The nascent strand is polymerized by Sequenase 5′ to 3′. The order of reaction lanes is GATC (left to right). Carets indicate termination sites. (A) Temperature effects on 20 μM U-73975 bonding: (1) control, untreated; (2) 4 °C incubation temperature; (3) 37 °C incubation temperature; (4) 42 °C incubation temperature. For reference the sequence presented in this figure starts with 5′-CCAACGCGTT. These three polymerase inhibition sites occur at 5′-GTATT, CTAAA, and GTAAT of the nascent strand, representing template-strand arrests at 5′-AATA*C, TTTA*G, and ATTA*C (where A* is the site of N3-adenine alkylation). (B) Preferred U-73975 bonding sites: (1) control, untreated; (2) 20 μM U-73975, room temperature incubation.

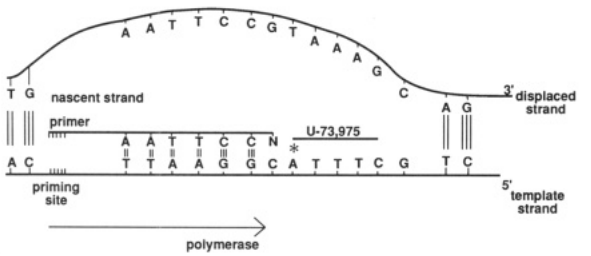

FIGURE 3: Model for DNA polymerase termination at U-73975 adduct sites. U-73975 is bound to a 5′TTA* site on the template strand. DNA polymerase transits from the priming site to the nucleotide adjacent to the adduct site (denoted by N). The N-site is the position at which ^{32}P-labeled DNA strands accumulated. The identity of the final nucleotide incorporated into the nascent strand cannot be determined by this technique.

CPI adduct sites in dsDNA. Comparison of termination sites within the same sequence ladder shows that some sites are preferentially bound by U-73975. Examples of preferred bonding sites are demonstrated in Figure 2B.

Negative supercoiled helicity of the DNA plays a surprisingly insignificant role in U-73975 bonding. Plasmid DNA, which is in a negative supercoiled state, was linearized with a restriction endonuclease at 37 °C prior to U-73975 treatment and yielded a result identical with that of unrelaxed plasmid DNA. Identical sites were occupied by this CPI at equal intensities (i.e., similar amounts bound) in both types of DNA (data not shown). This result suggests that negative supercoils have little, if any, effect on U-73975 sequence selectivity or affinity under these incubation conditions.

In Vivo Bonding of [^{3}H]U-73975 to Telomeres. The telomeres of each human chromosome possess clusters of tandemly repeated sequence $(TTAGGG)_n$ that are potential binding/bonding sites for U-73975 (Moyzis et al., 1988). An experiment was devised to determine if a majority of U-73975 bonding occurs *in vivo* at the telomeres. DNA was extracted from untreated HT-1080 cells and cells that were treated with a high concentration of [^{3}H]U-73975 as described in the Materials and Methods section. The DNA was restricted with *Eco*RI to resolve the DNA into a wide range of molecular weight fragments. A second digest was done with two enzymes, each having a four-base recognition sequence. This double four-cutter digest will cleave virtually all of the DNA into very small molecular weight fragments. Because of the repetitive nature of the telomeric sequences, they lack all of these restriction endonuclease cleavage sites and remain at relatively high molecular weights. Figure 4A shows the EtBr-Stained gel of the restriction digests. Lanes 1 and 2 contain untreated HT-1080 genomic DNA as a control to confirm that the presence of the CPI did not interfere with restriction of the DNA or hybridization of the probe. Lanes 3 and 4 are digests of DNA extracted from cells that were treated with [^{3}H]U-73975. Duplicates of these digests were run on a separate gel (Figure 4C) for autoradiography of the ^{3}H-labeled DNA (Figure 4D).

The electrophoresed DNA from the gel in Figure 4A was transferred to a nylon membrane and subsequently hybridized with a telomere-specific oligonucleotide probe, $(TTAGGG)_3$. The autoradiogram of the Southern hybridization blot in Figure 4B demonstrates that the telomeric sequences are greater than 3 kb. The autoradiogram of the [^{3}H]U-73975-bound DNA (Figure 4D) shows that the label is distributed throughout the DNA with no detectable concentration of [^{3}H]U-73975 in the range where telomeres are located (in the dual four-cutter lanes; e.g., Figure 4D, lane 2). This result suggests that U-73975 binds predominantly to nuclear DNA at sites other than telomeric repeats that contain numerous potential bonding sites. However, with circular dichroism

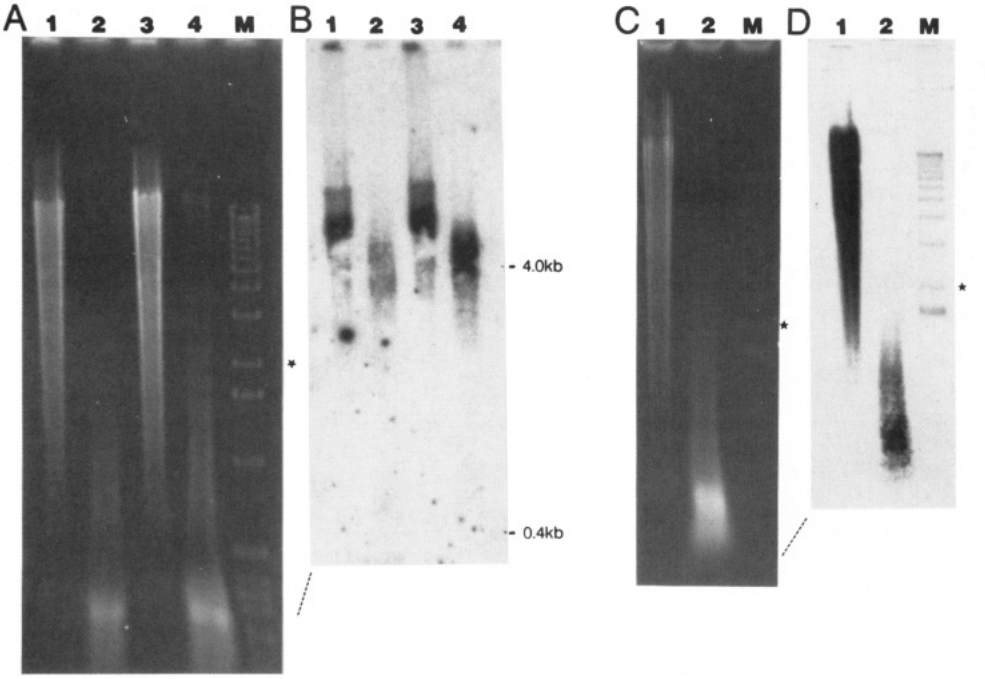

FIGURE 4: In vitro bonding of ^{3}H-U-73975 to telomeres. (A) EtBr-stained agarose gel containing digests of HT-1080 nuclear DNA. Lanes: (1) untreated DNA, *Eco*RI digested; (2) untreated DNA, *Sau*3AI/*Alu*I digested; (3) [^{3}H]U-73975-bound DNA, *Eco*RI digested; (4) [^{3}H]U-73975-bound DNA, *Sau*3AI/*Alu*I digested; (M) 1-kb ladder (Bethesda Research Laboratories, Bethesda, MD) molecular weight marker. (★ denotes 2-kb band). (B) Autoradiogram of DNA from gel in (A) hybridized with telomere-specific probe. Telomeres are larger than 3 kb. (C) EtBr-stained agarose gel of digested [^{3}H]U-73975-bound DNA. Lanes: (1) *Eco*RI; (2) *Sau*3AI/*Alu*I; (M) 1-kb marker. (★ denotes 2-kb band). (D) Autoradiogram of [^{3}H]U-73975-bound HT-1080 DNA digests from gel in (C). The radiolabeled DNAs were enhanced in Enlightning (NEN), and the gel was dried and autoradiographed. The label is randomly distributed throughout the genome with no apparent concentration of label found at the location of telomeres (>3 kb). (M) The 1-kb molecular weight marker was labeled with [^{32}P]dATP and Klenow. (★ denotes 2-kb band.)

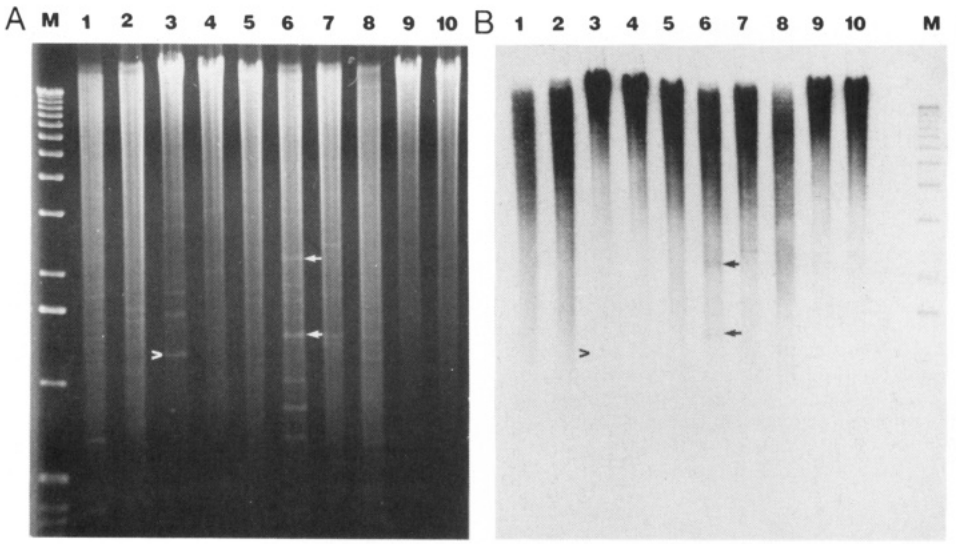

FIGURE 5: In vitro bonding of [^{3}H]U-73975 to repetitive DNA. (A) Restriction endonuclease digests of genomic DNA. Subconfluent HT-1080 cultures were treated with 20 nM [^{3}H]U-73975 (10 Ci/mmol) for 16 h. Nuclear DNA was isolated from these cells at a specific activity of 6.8×10^3 cpm/μg. Digests of 5 μg of DNA were incubated overnight at 37 °C. Lanes: (M) 1-kb ladder molecular weight marker; (1) *Eco*RI; (2) *Bgl*II; (3) *Kpn*I; (4) *Bam*HI; (5) *Sca*I; (6) *Xba*I; (7) *Nco*I; (8) *Pst*I; (9) *Hpa*I; (10) *Sph*I. (B) Autoradiogram of digested genomic DNA. The lanes contain the digests indicated above (A). The gel in panel A was soaked in Enlightning (NEN) for 20 min to enhance the radioactive signal and dried at 65 °C under vacuum. The gel was exposed to XAR film (Kodak) for 4.5 weeks. The arrows indicate [^{3}H]CPI-bound repetitive elements, whereas carets indicate repetitive sequence bands that contained significantly lower amounts of [^{3}H]CPI.

techniques, the ability of CC-1065 to react with a synthetic oligomer, 5′TTAGGGTTAGGG, in vitro has been examined (W. C. Krueger, personal communication). This sequence was found to be one of the best in vitro covalent bonding oligomers in a panel of potentially reactive oligomers. Likewise, we also observed U-73975 bonding in vitro to specific telomere-like sequences, TTAGG and GTTAG (Table I); the human telomeric sequence is very similar to our in vitro generated canonical consensus sequence (T/A)-T/A-T-A*-(C/G)-(G). Yet, we have demonstrated that almost all of [^{3}H]U-73975 was bound in vivo (intracellular) to sites other than telomeres. Perhaps either the telomeric repeats are not abundant enough for detection by [^{3}H]CPI autoradiography, or, alternatively, telomeric structures are not maintained within chromatin in a conformation that is permissive for CPI adduct formation.

Human Repetitive DNA. We wanted to further determine if CPIs exhibit intracellular sequence selectivity. To accomplish this we addressed whether different, highly repetitive

DNA elements varied in their ability to form U-73975 adducts in vivo [refer to Korenberg and Rykowski (1988) and citations therein regarding repetitive DNA]. Cultured HT-1080 cells were treated overnight with [^{3}H]U-73795 as previously described. The DNA was extracted from the cells and restricted with a battery of enzymes to generate a variety of electrophoretic patterns with distinguishable bands containing highly repetitive DNA elements. The fragments were resolved on agarose gels, dried, and autoradiographed. Figure 5 allows a comparison of the EtBr-stained gel and the corresponding autoradiogram. As indicated previously, the majority of [^{3}H]U-73975 is distributed randomly throughout the "smear" of restricted chromosomal DNA. However, the autoradiogram (Figure 5B) demonstrates an abundance of [^{3}H]U-73975 bound to some repeat elements (arrows) and not as extensively to others that have the same relative amount of DNA (carets) on comparison to the EtBr-stained gel (Figure 5A). These results suggest that there are regions of the genome that exhibit increased U-73975 bonding, relative to other repeat elements. The families of long and short interspersed nuclear elements have a characteristic A-rich 3′ end (Hutchison et al., 1989). Perhaps the elements that bond more [^{3}H]U-73975 seen here are related to those sequences and contain A-rich 3′ termini as well. Many of these repeat elements are currently being cloned and sequenced to determine their percentage of A/T richness and relatedness to other repetitive elements. Regardless of this information, we can conclude that U-73975 does bond covalently to nuclear DNA and exhibits some sequence selectivity with regard to highly repetitive elements.

Conclusions

In summary, with use of our modified dideoxynucleotide sequencing technology, a number of U-73975 bonding properties have been elucidated that, by extension, may have relevance to inhibition of DNA synthesis in vivo by this antineoplastic compound. Perhaps the transit of eukaryotic DNA or RNA polymerases will be terminated by CPI adducts on the template strand in a manner similar to that obtained for bacterial DNA polymerases. Temperature of adduct formation and CPI concentration can be varied to most effectively inhibit DNA polymerase read-through on dsDNA, with use of our new technique. Sequenase-based analysis of reacted DNA can be an expedient method for determining sequence selectivity and relative reactivity of these compounds, as well as other DNA alkylating agents. We are currently examining the interaction of other antineoplastic drugs to DNA using the polymerase assay. DNA adducts formed by the non-CPI alkylator melphalan (Pieper et al., 1989; Pieper & Erickson, 1990) were also detected by our Sequenase technique (K.L.W. and T.P.D., unpublished results).

In addition to studies of CPI-DNA alkylation in vitro, we have demonstrated that [^{3}H]U-73975 is bound to the DNA of treated, viable cells. Consistent with this result, fixation and autoradiography of cells treated with [^{3}H]U-73975 show that the majority of label is found in the nucleus of treated cells (K.L.W. and T.P.D., unpublished results). The majority of U-73975 is bound to nuclear DNA in vivo at sites other than telomeric sequences. This CPI appears to bond preferentially to a subset of human highly repetitive sequence elements. These studies further our understanding of the DNA sequence selectivity of U-73975 both in vitro and in the nuclear DNA of viable cells.

Acknowledgments

We are grateful to Drs. M. A. Mitchell and W. C. Krueger for helpful discussions, to R. C. Kelly for U-73975, and to N. T. Hatzenbuhler for oligonucleotide synthesis.

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