Reversibility of the Covalent Reaction of CC-1065 and Analogues with DNA

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ABSTRACT: Covalent DNA adducts of the antitumor antibiotic CC-1065 and its analogues undergo a retrohomologous Michael reaction in aqueous/organic solvent mixtures to regenerate the initial cyclopropylpyrroloindole (CPI) structure and, presumably, intact DNA. This reaction, which at higher temperatures competes with depurination of the *N*3-alkylated adenine, also occurs to a significant extent at 37 °C in neutral aqueous solution. Tritium-labeled adozelesin, covalently bonded to a 3-kilobase DNA restriction fragment which was exhaustively extracted to remove unbonded drug, was efficiently transferred to a 1-kilobase fragment upon coincubation for 20 h at 37 °C in aqueous buffer. Covalent adducts of adozelesin, but not CC-1065, on calf thymus DNA were cytotoxic to L1210 cells after incubation for 3 days at 37 °C, indicating that reversal of DNA alkylation can mediate potent cellular effects for simplified CC-1065 analogues.

It is generally presumed that most chemical agents which alkylate nucleophilic base atoms in DNA[1] achieve an essentially irreversible modification of the macromolecule (Basu & Essigmann, 1988; Dabrowiak, 1983). The antitumor antibiotic CC-1065 (**1a**, Figure 1) (Hanka et al., 1978; Martin et al., 1980), whose strong interaction with DNA could not be reversed by a variety of experimental manipulations (Li et al., 1982; Swenson et al., 1982), was shown to alkylate the N3 atom of adenine in certain sequences of double-helical DNA (Hurley et al., 1984; Reynolds et al., 1985). The stability of these covalent molecular lesions has been considered to be an important factor in the biochemical actions and extraordinary potency of CC-1065 and some of its analogues (Li et al., 1982; Warpehoski & Hurley, 1988; Hurley et al., 1987; Weiland & Dooley, 1991).

Direct experimental characterization of DNA–CC-1065 covalent adducts has included NMR studies (Scahill et al., 1990; Lin & Hurley, 1990; Powers & Gorenstein, 1990; Lin et al., 1991) as well as circular dichroism (CD) combined with extraction procedures (Krueger et al., 1985, 1986; Krueger & Prairie, 1987; Theriault et al., 1988). Noncovalent complexes of CC-1065 and its analogues with certain polynucleotides and oligonucleotides show CD spectra distinct from that of covalent adducts. The former can be dissociated completely by extraction with organic solvents such as phenol. Covalent adducts are impervious to such extraction procedures, and their molar CD is not reduced upon dilution (Krueger et al., 1986). Indirect detection of covalent adducts on a DNA restriction fragment by a thermal (90–100 °C) strand break assay identified the same sites detected by (methidiumpropyl-EDTA)–Fe and DNAse I footprinting experiments carried out at 25 °C (Hurley et al., 1988). Together these observations have supported the assumption that the covalent reaction of CC-1065 or its analogues with DNA was irreversible under ordinary conditions.

We have been intrigued by the unusual resistance to thermal depurination exhibited by DNA alkylated at adenine N3 by CC-1065 and related agents (Reynolds et al., 1985). In the course of an investigation of the butanol-soluble products of the thermal depurination reaction of DNA covalently bonded to a CC-1065 analogue, it was unexpectedly observed that a small amount of the intact CPI was recovered, which could not be attributed to unreacted starting material. At temperatures at which depurination was relatively slow, this CPI regeneration in aqueous/organic solvent mixtures became the major reaction pathway of the covalent adducts. This paper describes our initial study of the dependence of this "reverse alkylation", formally a retrohomologous Michael reaction, on reaction conditions and on CPI drug structure and an assessment of the relevance of this process to the biological action of CPI's.

Materials and Methods

Preparation of Calf Thymus DNA–CPI Adducts. CC-1065 was isolated (Martin et al., 1981) and **1b** and **1c** were synthesized (Kelly et al., 1987; Warpehoski et al., 1988) at the Upjohn Company. Organic solvents were reagent grade and were presaturated with the aqueous buffer. Preparation and characterization of the DNA–CPI adducts was carried out essentially as described below for **1b**. To 0.25 mmol bp of calf thymus DNA (Sigma) in 150 mL of 0.01 M SPB, pH 7.0, was added 7.2 mg (0.014 mmol) of adozelesin (**1b**) in 3 mL of DMA. The mixture was stirred at 20 °C for 4 h and then at 30 °C for 18 h (for **1c**, 1 week at room temperature yielded 60% conversion to the adduct). Potassium acetate (4.5 g) was added, and the DNA was precipitated with ethanol. The slightly yellow DNA filtrand was dissolved in 120 mL of 0.02 M SPB, pH 7.4, filtered, and extracted with 1:1 phenol–chloroform, with ethyl acetate to remove all traces of phenol, and finally with 1-butanol. UV analysis of the DNA adduct solution indicated 80% recovery of DNA and a significant adduct chromophore absorbance. One milliliter of this solution (0.0016 mmol bp) was diluted with water and vigorously stirred with butanol for 15 min at 92 °C. No drug chromo-

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[1] Abbreviations: bp, base pair; nt, nucleotide; cDNA, complementary DNA; DNA, deoxyribonucleic acid; kb, kilobase; CPI, cyclopropylpyrroloindole; DMA, dimethylacetamide; DMF, dimethylformamide; SSPE buffer, 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4; SPB, sodium phosphate buffer; TAE, 40 mM Tris–acetate and 1 mM EDTA, pH 7.5; TE, 10 mM Tris-HCl and 1 mM EDTA, pH 7.4; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; tlc, thin-layer chromatography; RP, reversed phase; CD, circular dichroism; UV, ultraviolet; NMR, nuclear magnetic resonance; FAB, fast atom bombardment.

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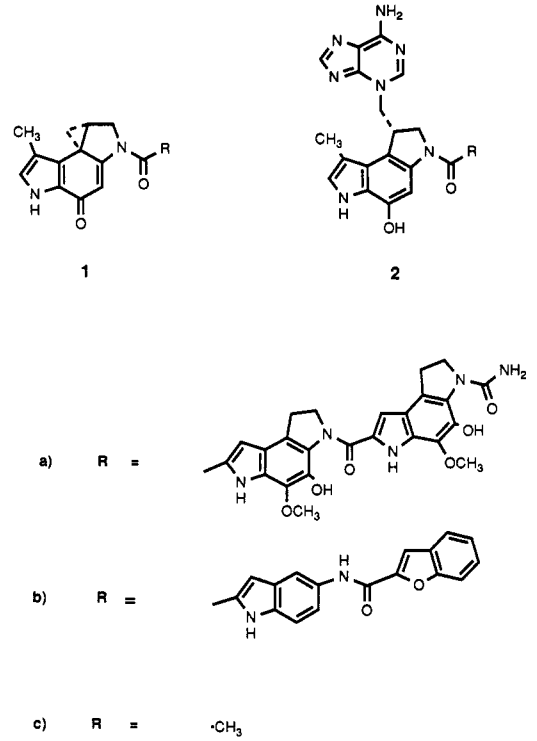

FIGURE 1: Structures of CC-1065 and analogues described in this work and of the corresponding (*N*3-adenine) conjugates.

phore remained in the aqueous DNA solution. The butanol phase contained the *N*3-adenine–adozelesin conjugate, **2b**, >90% pure by RP tlc. The structure of **2b** was previously confirmed by ^{1}H NMR (Scahill, unpublished work). Applying an extinction coefficient at 296 nm of 45 000, obtained from the acid solvolysis product of **1b** in butanol, to the UV spectrum indicated recovery of 0.0001 mmol of **2b**. The concentration of adduct in the DNA solution was thus estimated to be 0.1 mM. Complete depurination of the DNA adducts of **1a** and **1c** likewise gave **2a** (Hurley et al., 1984) and **2c** (verified by FAB mass spectrometry), respectively. Extinction coefficients of 50 000 (363 nm) for **2a** and 35 000 (252 nm) for **2c**, obtained from the acid solvolysis products of **1a** and **1c**, allowed estimation of the concentration of covalent drug adducts on the DNA.

Reversal of the Alkylation Reaction of Covalent DNA–CPI Adducts. A solution of the DNA–CPI adduct (0.3–2 mM bp) in buffer was vigorously stirred with butanol at the indicated temperature. Aliquots of the butanol phase were removed at intervals, dried over Na_2SO_4, and examined by UV spectroscopy. Aliquots of the aqueous phase were also monitored by UV spectroscopy for the drug adduct chromophore and gave the expected mass balance within experimental error. Variations in pH, buffer and salt concentration, temperature, DNA concentration, base pair to drug ratio, and organic solvent were examined as described in the Results section. The organic phase was evaporated to dryness under a stream of nitrogen, redissolved in butanol or DMF, and analyzed by tlc densitometry. The UV spectra of the bands confirmed the assignments based on retention. For **1a** and **2a**, and for **1b** and **2b**, separation was achieved on an analytical RP support (Whatman LKC18D) developed in 65–68% DMF in 0.01 M SPB, pH 7.0. For **1c** and **2c**, development in 60% methanol – 40% buffer achieved only a slight separation; however, silica gel tlc developed in 1:1 acetone–cyclohexane readily distinguished **1c** ($R_f \sim 0.5$) from **2c**, which remained at the origin. A preparative-scale reversal of DNA–**1b** adducts is described below.

To the calf thymus DNA–**1b** adduct solution (115 mL, 0.18 mmol bp, 0.012 mmol drug adducts) was added 4.1 g of Na_2HPO_4 to obtain a final solution of 0.25 M SPB, pH 8.4. Butanol (200 mL) was introduced and the mixture was stirred vigorously, under a nitrogen atmosphere, in a 55 °C bath. After 15 h, UV analysis of an aliquot of the aqueous phase indicated that about 80% of the drug chromophore was gone. The butanol phase was washed with water to remove buffer salts and then removed as an azeotrope with water under reduced pressure. Ethyl acetate extraction recovered the product, whose proton NMR spectrum (300 MHz, DMF-d_6), UV and CD spectra in butanol, FAB mass spectrum, and RP tlc retention were identical to those of authentic **1b**. The yield determined by UV absorbance, using an extinction coefficient of 31 000 at 365 nm (Warpehoski et al., 1988), was 80% of the estimated initial adducts. The product was >90% pure by tlc, with the main contaminant having the retention properties of **2b**.

Tritium-Labeled Adozelesin Transfer Experiments. ^{3}H-**1b** was synthesized with an initial specific activity of 9.20 Ci/mmol. At the time of these experiments the radiopurity of the compound was shown to be in excess of 88% and only a 2.95% decay correction was needed, yielding a compound with a specific activity of 8.93 Ci/mmol. The plasmid, pTP2, contains a 1.1-kb cDNA insert ligated into the *Sma*I site of pGEM-7Zf(+) vector (Promega), resulting in a plasmid of 4.1 kb. The plasmid was grown in *Escherichia coli* strain JM109 and purified via cesium chloride gradient centrifugation (Maniatis et al., 1982). The purified plasmid was digested with *Eco*RI and *Bam*HI, generating the 1.1- and 3-kb fragments, which were preparatively separated on a 0.8% low melting point agarose gel (BRL) in TAE buffer. The fragments were located by ethidium bromide staining and individually purified using Elutip-D columns (Schleicher & Schuell).

The 3-kb fragment was incubated with ^{3}H-adozelesin at a drug to nucleotide ratio of approximately 1:635 nt in 1× SSPE buffer. The reaction was incubated for 16 h at 25 °C. Noncovalently bound drug was removed from the reaction mixture by successive extraction using water-saturated phenol, phenol–chloroform (1:1), and chloroform. Extractions were repeated until no further counts could be removed from the aqueous layer. The aqueous phase was transferred to a clean 1.5-mL microfuge tube, a $^1/_{10}$ volume of 3 M sodium acetate, pH 5.4, was added, and the DNA was precipitated with 2.5 volumes of ethanol at −20 °C for 16 h. The DNA was pelleted, rinsed in 70% ethanol, and resuspended in TE buffer to a concentration of approximately 0.5 μg/μL.

Aliquots of the drug-reacted 3-kb fragment were subjected to reverse-phase HPLC with radiometric detection. Samples were first diluted with DMF to contain 40% DMF and injected onto an HPLC system fitted with a Vydac protein and peptide C_{18} column equilibrated with a mobile phase of 70% acetonitrile, 25% water, 5% tetrahydrofuran, and 0.05 M ammonium phosphate, pH 7.0. Samples were also assayed for labile tritium by lyophilization of a known aliquot of the radiolabeled sample while the volatiles were collected with a dry ice trap. Aliquots of the recovered water were assayed for tritium by liquid scintillation counting.

The reversal/transfer experiments were set up as 15-μL reactions containing 3 μg of the 1-kb fragment and 3 μg of drug-labeled 3-kb DNA in 1× SSPE, pH 7.4, and incubated at temperatures ranging from 0 to 55 °C for 0–72 h. The fragments from each reaction were resolved by 0.8% agarose gel electrophoresis in TAE buffer and located by ethidium

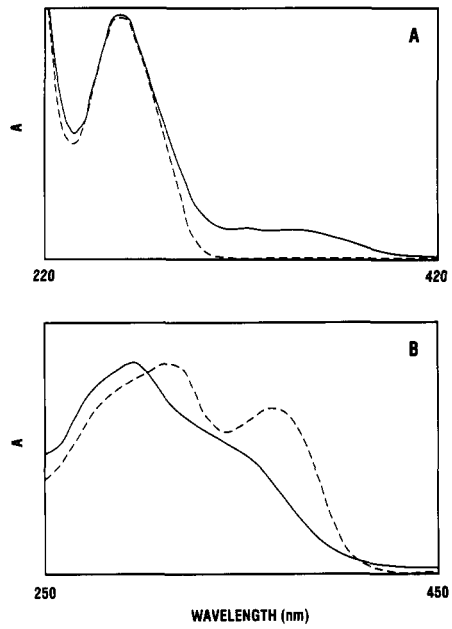

FIGURE 2: (A) Ultraviolet–visible spectra of calf thymus DNA (---) and calf thymus DNA–**1b** adducts (—) in 0.02 M SPB, pH 7.4. (B) Product of DNA–**1b** adduct reversal (**1b**), in butanol after 15 h at 55 °C (---), and **2b** obtained by depurination of the DNA–**1b** adducts, in butanol (—).

bromide staining. Each band was excised from the gel and transferred to an Ultrafree-MC 0.45-μm filter unit (Millipore). Each filter unit was placed into a 1.5-mL microfuge tube and centrifuged at approximately 10000 rpm for 10 min. This step was repeated three times with the addition of 100 μL of TE buffer after each centrifugation. The filter units were removed and the aqueous solution (400 μL) was extracted with an equal volume of water-saturated phenol–chloroform (1:1) followed by one extraction with chloroform. The DNA was then precipitated and resuspended as described above.

The DNA concentration in each sample was determined by UV absorption. Purity was assessed by A_{260}/A_{280} ratios. A 200-μL aliquot of each sample was mixed with 10 mL of Aquasol-2 LSC cocktail (Du Pont) and analyzed by liquid scintillation counting on a Beckman KS5000TA counter with a wide open ^{3}H window. Samples were counted for three 5-min cycles. Counting efficiency was determined from triplicate counts of an external ^{3}H standard and background corrections were made from two separate samples also counted for three 5-min cycles.

Reversal/transfer reactions were conducted and electrophoresed as described above. Individual lanes containing both fragments were excised from the gel. These excised slabs were then sectioned into 2-mm slices using a tube gel slicer (Bio-Rad). Each gel slice was dissolved in 10 mL of Aquasol-2 LSC cocktail and counted. A gel profile was then produced by plotting the total cpm in each section vs the distance migrated from the gel origin.

Cytotoxicity. Calf thymus DNA adducts of **1a** and **1b** were evaluated in the 3-day L1210 cytotoxicity assay (Li et al., 1987). Solutions of **2a** and **2b** in butanol, obtained from depurination of the DNA–drug adducts at 92 °C, were also assayed, either directly or after the butanol was evaporated under a stream of nitrogen and the residue was mixed with calf thymus DNA (1.2 mM bp, 0.01 M SPB, pH 7.4) for 1–2 h, prior to reextraction with butanol. In this way **2b** was readily recovered; however, **2a** was not efficiently reextracted into butanol at ambient temperature, and the sample was recovered by separating the phases after brief heating to 55 °C.

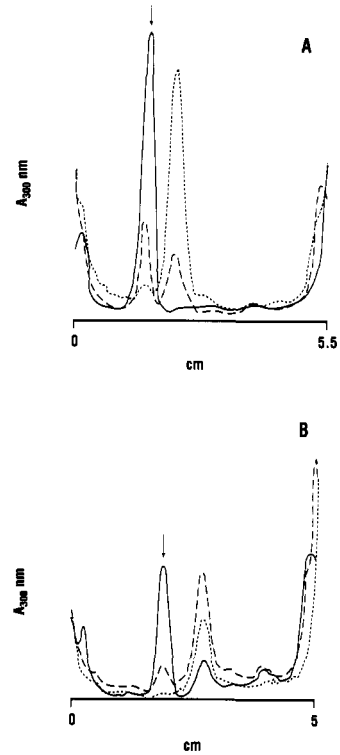

FIGURE 3: Densitometric scans, at 300 nm, of RP tlc chromatograms. (A) **2b** obtained by depurination of the DNA–**1b** adducts (···), **1b** recovered in nearly quantitative yield from the DNA–drug adducts in a mixture of butanol and 0.25 M SPB, pH 8.4, 55 °C, 20 h (—), and **1b** and **2b** recovered, in ca. 20% total yield, from DNA–**1b** adducts in a mixture of butanol and 0.02 M SPB, pH 7.4, 37 °C, 5 days (---). The arrow indicates the retention of **1b**. (B) **2a** obtained by depurination of the DNA–**1a** adducts (···), **1a** (major product) and **2a** recovered in 35–40% yield from the DNA–**1a** adducts, 0.25 M SPB, pH 8.4, 80 °C, 1 h (—), and **1a** and **2a** (major product) recovered in 35–40% yield from DNA–**1a** adducts, 0.02 M SPB, pH 7.4, 80 °C, 1 h (---). The arrow indicates the retention of **1a**.

RESULTS

Regeneration of Adozelesin from the DNA–Drug Adduct in Organic/Aqueous Solvent Mixtures. The UV (Figure 2A) and CD (not shown) spectra of the adozelesin- (**1b**-) modified DNA clearly showed the presence of covalently bonded drug. Complete depurination of the **1b**–DNA adducts at 92 °C in a mixture of butanol and 0.003 M SPB, pH 7.4, gave a nearly quantitative yield of the *N*3-adenine conjugate, **2b**, in the butanol phase (Figure 2B), supporting *N*3-adenine alkylation as the predominant reaction of **1b** with DNA, as expected. In contrast, incubation of the **1b**-modified DNA for 15 h at 55 °C, in a vigorously stirred mixture of butanol and 0.25 M SPB, pH 8.4, resulted in extraction of 80% of the **1b** chromophore into the butanol phase as intact **1b** (Figure 2B)! The yield of the recovered CPI was consistent with the adduct quantitation determined through the depurination reaction (Materials and Methods) and suggests that virtually all of the covalent adducts in this sequence-heterogeneous DNA are capable of undergoing reversal under these conditions.

Effect of Reaction Variables on the Recovery of Adozelesin from Its DNA Adducts. At 92 °C, in dilute buffer at pH 7.4, release of **2b** from DNA–**1b** adducts into the butanol phase was rapid and efficient. Only a small percentage of a minor product which comigrated with **1b** was detected (Table I and Figure 3A). At lower temperatures, the depurination reaction not only slowed dramatically but also was accompanied by a previously unrecognized reaction, formally the reverse of the

Table I: Recovery of Adozelesin (**1b**) from Its DNA Adducts[a]

SPB (M)	pH	temp (°C)	time (h)	% **1b**	% **2b**
0.003	7.4	92	0.25	<5	>90
0.02	7.4	37	116	10	10
0.25	8.4	37	5	15	
			20	40	
			116	85	
0.25	8.4	55	1	30	
			2	50	
			23	90	<5
0.25	7.9	55	1	20	
			2	30	
			20	85	<5
0.25	7.4	55	24	50	10–15
0.07[b]	8.0	55	1	10	
			2	20	
			31	75	5
0.07[c]	8.0	55	2	10	
			4	20	
			24	40	5

[a] Procedure as described in Materials and Methods. Butanol was the cosolvent unless noted otherwise. Yields of **1b** (UV) and **2b** (UV and tlc) estimated to the nearest 5%. [b] 0.08 M NaCl added. [c] Ethyl acetate as cosolvent. Experiments with or without 0.08 M NaCl gave identical results.

DNA alkylation, leading to regeneration of free **1b**. Table I lists the amounts of recovered **1b** as a function of time, temperature, and solvents. After 5 days at 37 °C, 80% of the covalently bonded **1b** remained on DNA, but the other 20% was found in the butanol phase as a roughly equal mixture of **1b** and **2b** (Table I and Figure 3A). The alkylation reversal pathway was strongly accelerated by raising the pH of the buffer and, independently, by increasing the buffer concentration. At pH 8.4 in 0.25 M SPB, **1b** was cleanly (Figure 3A) and nearly quantitatively recovered from its DNA adduct in about 1 week at 37 °C and within 1 day at 55 °C. Variations in the concentration of modified DNA, the base pair to drug ratio, and the addition of NaCl up to 0.35 M had no effect upon the rate of accumulation of **1b** in the butanol phase. Under otherwise comparable conditions, however, the substitution of ethyl acetate for butanol led to a significantly slower recovery of **1b**. Control experiments showed that alkylation of calf thymus DNA by **1b** in the two-phase butanol/buffer system at the concentrations relevant to the reversal experiments was extremely slow, even at pH 7.4 and 0.02 M SPB, indicating that rapid realkylation from the butanol-solubilized drug was not affecting the observed recovery of **1b**. In addition, the conjugate **2b** was stable in mixtures of butanol and 0.25 M SPB, pH 8.4, even when heated to 92 °C for 15 min, by UV and tlc analysis.

Tritium Transfer Experiments. Two assays were performed on aliquots of the ^{3}H-adozelesin-modified DNA sample to ensure that the sample contained no radioactivity attributable to noncovalently bound ^{3}H-**1b** or to labile radiolabel. HPLC analysis of the 3-kb fragment containing the tritiated adduct revealed no detectable noncovalently bound drug (not shown). We estimate the minimum detectable amount of radioactivity to be less than 1% of the label incorporated onto the restriction fragment. In addition, less than 0.5% labile tritium was detected in these samples (not shown).

A radioactivity profile of the gel lane corresponding to a reversal/transfer experiment carried out at 37 °C for 72 h is shown in Figure 4. Electrophoresis of the fragments produced a clean separation of the radiolabel, verifying that the tritium associated with the 1-kb fragment was not due to comigrating degradation products from the 3-kb fragment.

Coincubations of the labeled 3-kb fragment with the unlabeled 1-kb fragment showed a clear time and temperature

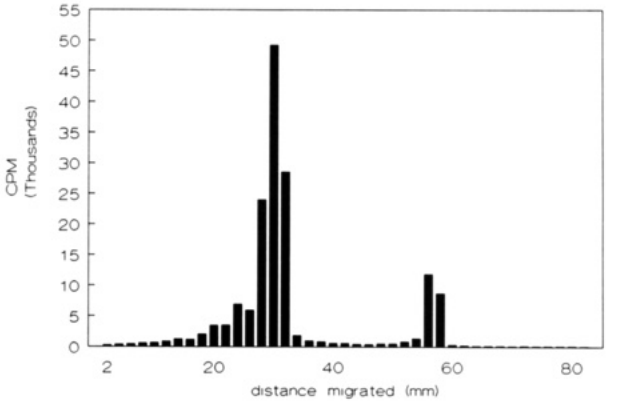

FIGURE 4: Histogram illustrating the distribution of radioactivity in a gel lane after electrophoresis of a reversal/transfer of ^{3}H-**1b** carried out at 37 °C for 72 h. The 3-kb fragment migrated to ca. 30 mm and the 1-kb fragment migrated to ca. 58 mm.

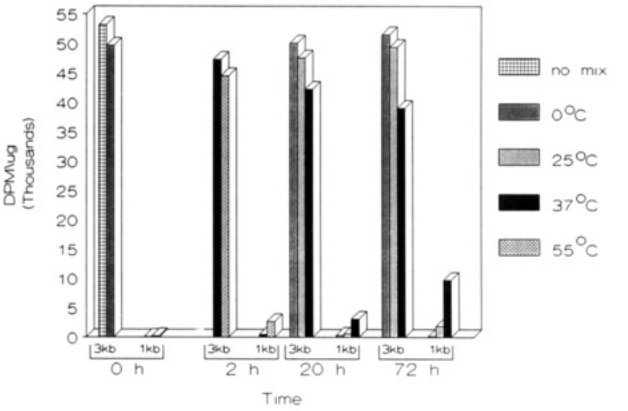

FIGURE 5: Histogram illustrating the transfer of ^{3}H-**1b** from a 3-kb DNA fragment to a 1-kb fragment during coincubation at the indicated times and temperatures.

dependence of the amount of labeled drug transferred to the 1-kb fragment (Figure 5). At 0 °C, negligible transfer was detected, even after 72 h. But at 37 °C, the detected transfer rose to nearly 20% of the total disintegrations per minute per microgram in 3 days. Even at 25 °C, a slow but measurable transfer of label to the 1-kb DNA occurred over several days. Since the newly alkylated 1-kb DNA fragment was present in equal base pair concentration to the 3-kb fragment, it might reasonably be inferred that a comparable amount of regenerated drug could also have realkylated other sites on the 3-kb fragment. Thus the actual percent reversed and dissociated from the original adduct site may be closer to twice the values measured in the experiment. The total disintegrations per minute per microgram remained fairly constant, indicating that little irreversible loss of drug (e.g., by depurination) occurred under these conditions.

Effect of Drug Structure on DNA–CPI Adduct Reversal. Figure 1 shows the structures of CC-1065 (**1a**) and the CPI analogues **1b** and **1c** which were reacted with calf thymus DNA to obtain the covalent adducts. DNA–CPI adduct reversal was examined in 0.25 M SPB, pH 8.4. Within the experimental accuracy, very little difference was detected in the rate or efficiency of the CPI formation and extraction into the butanol phase for DNA adducts of **1b** and **1c** at 37 °C (40% of **1b** and 50% of **1c** in 1 day) or 55 °C (30% of **1b** and 40% of **1c** in 1 h). However, **1a** showed a much slower overall rate of recovery from its DNA adducts. At 55 °C, in mixtures of butanol and 0.25 M SPB, pH 8.4, only about 20% of the drug chromophore was extracted into butanol after 20 h. This was not surprising, since at ambient temperature butanol is

Table II: Cytotoxicity of DNA Adducts

sample equiv	I/C_{50} (nM) drug equiv	base pair
1b	0.003	
DNA adduct of **1b**	0.04[a]	0.7
2b	0.25	
	0.9[b]	
1a	0.04	
DNA adduct of **1a**	>2.5	>80
2a	4[b]	
calf thymus DNA		>600

[a] Duplicate results of two separate adduct preparations. Range of results was ±20%. [b] Butanol solution of the conjugate was treated with calf thymus DNA as described in Materials and Methods.

not efficient at extracting even noncovalently bound **1a** from DNA. However, at 80 °C, **1a** could be efficiently recovered from its covalent DNA adduct into butanol (Figure 3B). About a third of the adducts were converted in 1 h at this temperature, in 0.25 M SPB, pH 8.4, with a 4:1 ratio of **1a** to **2a**. In 0.02 M SPB, pH 7.4, a similar net rat of conversion occurred, but in this medium the major product was **2a**, and **1a** was present in only minor amounts. Figure 3B also shows the chromatographic retention of **2a**, produced by heating the DNA adduct of **1a** for 15 min at 92 °C in a mixture of butanol and 0.004 M SPB, pH 7.4.

Cytotoxicity. The DNA–**1b** adducts showed surprisingly potent cytotoxicity, only about an order of magnitude less than the high potency of **1b** itself (Table II). This was consistent with the reversal of a significant percentage of the covalent adducts, releasing a biologically active form of the drug under the assay conditions. The depurination product, **2b**, showed substantially less potency, which was reduced still further by exposure of the product to calf thymus DNA, presumably removing traces of **1b** formed during the depurination reaction. However, the DNA–**1a** adducts showed no cytotoxicity at equivalent doses 60 times that of **1a**.

Discussion

A kinetic model for DNA–CPI interaction which recognizes the insolubility of the free drug in aqueous solution (Krueger et al., 1985), the reversibility of both noncovalent complexation and alkylation, and the known and inferred potential fates of the adduct once it is formed is presented in eqs 1–5.

$$\text{drug(agg) or drug(org)} \overset{K_{sol}}{\rightleftharpoons} \text{drug(aq)} \quad (1)$$

$$\text{drug(aq)} + \text{DNA} \overset{K_{bind}}{\rightleftharpoons} \text{complex} \quad (2)$$

$$\text{complex} \overset{K_{cov}}{\rightleftharpoons} \text{adduct} \quad (3)$$

$$\text{adduct} \xrightarrow{k_{dep}} \text{apurinic DNA} + N3\text{-adenine–drug conjugate} \quad (4)$$

$$\text{adduct} \rightarrow \text{subsequent biological effect on cellular processes involving DNA} \quad (5)$$

Part of the practical difficulty in recognizing the reversibility of the CPI covalent reaction is related to the extreme water insolubility of these drugs. The process of adduct → complex → drug(aq) + DNA is thus highly unfavorable. In a mixed organic/aqueous solvent system, the partitioning of the drug into the organic phase effectively favors dissociation of the complex (eq 2). This, in turn, disturbs the covalent equilibrium (eq 3) resulting in net alkylation reversal. At the ambient temperatures at which organic solvent extractions have usually been carried out, the rate of the reversal reaction is so slow that its existence was not detected, and only equilibria involving the much weaker noncovalent association were manipulated. On the other hand, aqueous butanol mixtures at 90–100 °C revealed only the organic-soluble product of alkylated adenine depurination (eq 4), which preceded DNA strand cleavage (Hurley et al., 1984). These manipulations did not reveal the adduct's potential for reversal of the alkylation.

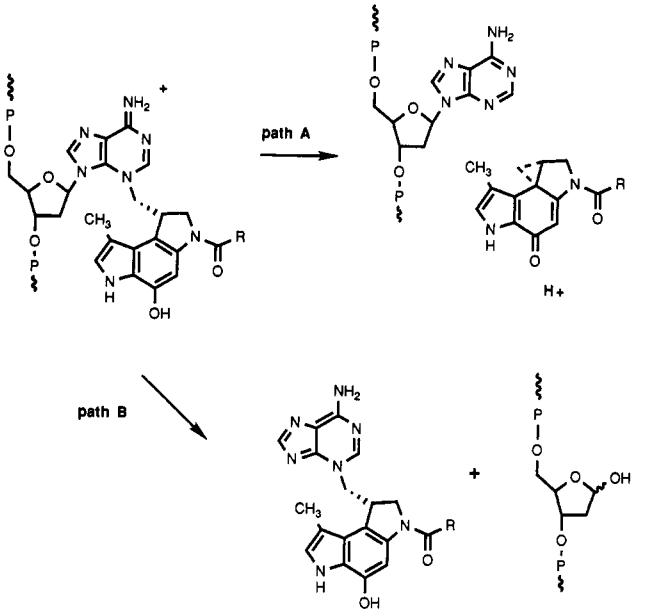

FIGURE 6: Thermal decomposition pathways observed for DNA–CPI adducts: path A, reversal of alkylation; path B, depurination.

Figure 6 illustrates the two principal thermal decomposition pathways observed for the DNA–CPI adduct. At temperatures above 90 °C, path B predominates. This may in part reflect a higher activation energy for path B. It may also indicate that the depurination, like that of 3-methyladenine adducts (Lawley & Brookes, 1963), can occur from either double- or single-stranded DNA. Path A, on the other hand, appears to be the microscopic reverse of the forward alkylation reaction. Like the alkylation reaction (Li et al., 1982), path A may require double-helical structure in the DNA. Thus, at temperatures which melt DNA, this pathway may be inhibited. The effects of DNA structure and sequence on the reversal and depurination pathways are currently under study in our laboratories.

In purely aqueous solution the adduct might reverse (path A, Figure 6) but then bond to the same site without dissociating, or it might migrate along the minor groove to an adjacent site. Such events would not be detectable in our radiolabel transfer experiments. If, however, free drug(aq) formed, it might readily react with either the original 3-kb DNA fragment or with the experimentally distinguishable 1-kb fragment. In the event, an unambiguous time- and temperature-dependent transfer of radiolabel from the 3-kb DNA to the smaller DNA fragment was observed (Figure 5). Although the rates of transfer in aqueous buffer cannot be rigorously compared to rates of CPI recovery in mixed solvent systems, the two methods indicate roughly comparable levels of reversal under similar conditions. About 10% of **1b** was recovered in the butanol phase from the DNA–drug adduct after 5 days at 37 °C in a mixture of butanol and neutral, dilute buffer (Table I). The transfer experiments, in purely aqueous solutions in which the intermediate noncovalent complex would be much more stable, indicated nearly 20% transfer in 3 days under otherwise comparable conditions

(Figure 5). That the rate of transfer is at least as rapid as the rate of extraction suggests that the adduct reversal reaction (eq 3), and not complex dissociation (eq 2), is rate determining for the ^{3}H-**1b** transfer in aqueous solution.

The slow release of CC-1065 from its covalent adducts in mixed solvent systems suggests that even in this milieu the dissociation of the noncovalent complex (eq 2) is rate determining, consistent with its exceptional stability (Krueger et al., 1985; Swenson et al., 1982). For the natural product, then, strong binding forces, attributable in large measure to the central and right-hand pyrroloindole subunits (Boger et al., 1990b), serve to counter the reversibility of the alkylation reaction and prevent accumulation of free CC-1065 in the organic phase, except at relatively high temperatures (Figure 3B). The lack of observable cytotoxicity of the DNA–CC-1065 adducts also indicates that they are effectively "irreversible" under the cell growth conditions.

In a general sense, this effective "irreversibility" of CC-1065, due to its extremely tight noncovalent binding, is reminiscent of proposals made by Coleman and Boger (Coleman & Boger, 1989; Boger et al., 1990a). On the basis of their computational studies, these investigators described the CPI alkylation of DNA as a "thermal neutral and potentially reversible covalent alkylation". In the case of CC-1065, this "thermodynamically poor alkylation" would be stabilized by strong noncovalent binding. Their language implied that the noncovalent binding forces stabilized the product (adduct) relative to the alkylation transition state, making alkylation reversal unfavorable.

While this hypothesis is consistent with our observations for CC-1065, it is also possible, and indeed seems likely, that the second step in the reversal reaction, namely, dissociation of the noncovalent complex to generate free drug, may be rate determining for CC-1065 under relevant experimental conditions and may principally account for the observed "irreversibility."

For the simplified analogues **1b** and **1c**, noncovalent complex dissociation in butanol/aqueous mixtures is extremely rapid and efficient, and hence the observed rates of reversal should reflect the relative free energy differences between adducts and alkylation transition states. That the release of **1c** was only slightly faster than the release of **1b** (50% vs 40%, respectively, in 1 day at 37 °C) indicates that this free energy difference is quite comparable for the two drugs. In other words, the additional binding stabilization available to **1b** (as compared to **1c**) provides only a small net stabilization of the product DNA adduct with respect to reversal.

In contrast to the comparable stability of the DNA adducts of **1b** and **1c**, the rate of alkylation of DNA is considerably slower for **1c** than for **1b**, although given adequate time DNA alkylation by **1c** is quite efficient (for **1b**, >85% adduct formation occurs in 1 day; for **1c**, 60% conversion to adduct occurs in 1 week).[2] Together these observations support our previous suggestion that the higher **1c** concentrations required to produce detectable alkylation in the strand break assay (and to kill cells in vitro) reflect the slower rate of DNA alkylation by **1c** relative to compounds like **1b** (Hurley et al., 1988). They do not support the proposal that these large reactivity and potency differences result from differences in net stabilization of covalent adducts (Boger et al., 1990a).

While the computational studies appear to have led to an underestimation of the thermodynamic stability of CPI adducts of agents such as **1c**, they presciently countered the prevailing assumption which overestimated that stability.[3]

The observation of covalent adduct reversibility not only impacts current mechanistic models of the CPI–DNA interaction but also must be considered in designing and evaluating in vivo and in vitro studies with these agents. The radiolabel transfer experiments, in particular, give the first quantitative assessment of the effective lifetime of DNA–adozelesin adducts under biochemically relevant conditions. They raise the possibility that an adozelesin adduct might effect its cytotoxic action catalytically (if eq 5 is an equilibrium process) or that the kinetic product of DNA alkylation may not necessarily reveal that ultimate target sequence. Together with recent reports presenting evidence for reversibility of covalent DNA adducts of mitomycin C (Borowy-Borowski et al., 1990) and cisplatinum compounds (Gaucheron et al., 1991), these results argue that a reevaluation of commonly held assumptions about the chemical stability of "irreversible" covalent bonds between DNA and drugs may be timely.

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[2] Recent kinetic measurements have shown that the rate constant for DNA alkylation is orders of magnitude slower for **1c** than for **1b** (Warpehoski and Harper, unpublished work).

[3] Despite the computational studies, all authors in this area have continued to describe the CPI alkylation of DNA by CC-1065 and the related duocarmycins as "irreversible" (Boger et al., 1990c; Hurley et al., 1990). The continued use of the term "irreversible" in light of the present work is no longer justified without carefully describing the conditions under which the given studies are carried out.

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Distortions Induced in DNA by *cis*-Platinum Interstrand Adducts†

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ABSTRACT: A 22 base pair double-stranded oligonucleotide containing a unique interstrand adduct resulting from chelation of the two guanine residues within the central sequence d(TGCT/AGCA) by a *cis*-platinum residue has been studied by means of gel electrophoresis, chemical probes, and molecular mechanics. The anomalously slow electrophoretic mobility of the multimers of the platinated and ligated oligomers suggests that the platinated oligonucleotide is bent. The two cytosine residues (complementary to the platinated guanines) are hyperreactive to hydroxylamine, indicating a large exposure of the two bases to the solvent. The adduct does not induce a local denaturation within the flanking sequences since the adenine residues are not reactive with diethyl pyrocarbonate. This is confirmed by the nonreactivity of the complementary T residues with osmium tetraoxide. These results and the molecular mechanics modeling suggest that the interstrand adduct bends the double helix by approximately 55° toward the major groove, that the double helix conserves its average twist angle, and that the distortion induced by the adduct is localized at the platinated sequence d(GC/CG).

cis-Diamminedichloroplatinum(II) (cis-DDP)[1] is widely used in the treatment of human tumors. Numerous results suggest that the cytotoxic action of *cis*-DDP is related to its ability to react with cellular DNA. Lesions produced in DNA have been characterized as bifunctional adducts including mainly intrastrand and interstrand cross-links [for general reviews, see Eastman (1987), Reedijk (1987), Johnson et al. (1989), and Lepre and Lippard (1990)]. The major adducts are DNA intrastrand cross-links. Although the interstrand cross-links represent a minor portion of the total lesions, they have often been implicated with cytotoxicity [for a general review, see Roberts et al. (1988)].

Structural knowledge of the lesions induced in DNA by the binding of *cis*-DDP is a necessary step to understand the antitumor activity of *cis*-DDP or at least to understand the processing of DNA lesions involved in mutagenesis and DNA repair. Several structural studies have been already devoted to the intrastrand adducts [for general reviews, see Eastman

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); OsO_4, osmium tetraoxide; HA, hydroxylamine; DEPC, diethyl pyrocarbonate.

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