ABSTRACT

CC-1065 is a potent antitumor antibiotic which is cytotoxic to P388 and L1210 leukemia cells in vitro and in vivo. CC-1065 covalently binds to calf thymus DNA preferentially to adenine-thymine regions at N3 of adenine. Here, we compare CC-1065 interaction with P388-derived chromatin, DNA, and histones as measured by electronic absorption and circular dichroism. Two CC-1065 analogues (U-71,184 and its enantiomer, U-71,185) which show different biological activities from CC-1065 were also studied.

The shape and temporal behavior of the induced circular dichroism curves generated by CC-1065 or its analogues bound to chromatin were similar to CC-1065 plus DNA. This suggested that CC-1065 and its analogues bind to the minor groove of chromatin DNA in a manner similar to calf thymus DNA. However, the binding of CC-1065 and its analogues to DNA induced a more intense circular dichroism band than binding to chromatin. The order of interaction for both chromatin and DNA was CC-1065 > U-71,184 > U-71,185. In contrast to the essentially irreversible binding to DNA after 24-h incubation, binding to chromatin was primarily a reversible interaction, the degree of reversibility being U-71,185 > U-71,184 = CC-1065. CC-1065 binds weakly and nonspecifically to histones.

INTRODUCTION

CC-1065, an antitumor antibiotic produced by Streptomyces zelensis (1–3), is one of the most cytotoxic antitumor agents known. It was active against P388 leukemia, L1210 leukemia, CDF81 mammary carcinoma, and colon 26 carcinoma in vivo (4). In comparison to Adriamycin, CC-1065 was 100-fold more potent against a broad panel of human tumors in a cloning assay (5). DNA synthesis was inhibited by CC-1065 much more than either RNA or protein synthesis (5, 6). Alkylation experiments, competitive binding experiments with netropsin, and studies using glycosylated T, DNA indicated that CC-1065 binds in the minor groove of DNA with preference for A-T-rich regions (7, 8). Thermal melting of DNA and differential circular dichroism studies indicated that CC-1065 dramatically stabilized the DNA helix upon binding, and about 7 to 11 base pairs per CC-1065 were required for saturation binding of DNA. CC-1065 and analogues bind to CT-DNA by at least 2 mechanisms, one which produces a reversibly bound (extractable) species and the other which produces an irreversibly bound (nonextractable) species, shown to be the adenine N3 adduct. The reversibly bound species converts to the adduct over a period of hours (9). CC-1065 did not intercalate since binding to supercoiled DNA produced no change in DNA mobility on agarose gels. CC-1065 did not bind to single-stranded DNA, RNA, or protein.

The development of CC-1065 toward clinical trials was stopped because later studies showed that CC-1065 caused delayed death at subtherapeutic doses (10). In order to obtain compounds that retained antitumor activity without appreciable toxicity, several CC-1065 analogues were prepared. Of these analogues, U-71,184 was chosen for detailed study, particularly because of its significant in vivo antitumor activity (11–14) without delayed toxicity. Like CC-1065, U-71,184 also bound to DNA and caused marked inhibition of DNA and RNA synthesis. However, doses required for inhibition of nucleic acid synthesis were much higher than the dose for cell lethality. This discrepancy suggested that inhibition of nucleic acid synthesis may not be causally related to lethality. Further studies showed that unbalanced growth and cell death may be related (15).

Most CC-1065 studies have primarily used extracted DNA or synthetic oligonucleotides for binding studies. However, nuclear DNA does not exist as loosely associated (naked) DNA but is packaged as chromatin (16). Chromatin consists of repeating nucleosome subunits composed of an interior core of histones around which are wrapped approximately 140 base pairs of the B-form of DNA. Each nucleosomal subunit is connected to the next with a stretch of DNA called the "linker" region. The organization of chromatin in eukaryotic nuclei plays an important role in the regulation of gene expression (17). Several investigators have used chromatin as a model to examine biochemical mechanisms of certain antitumor agents (18–21). In some cases, drug interaction with DNA was different from that with chromatin preparations. We report here the interaction of CC-1065 and two of its analogues (U-71,184 and its enantiomer, U-71,185) with DNA, chromatin, and histone. Parts of this paper were presented previously as an abstract (22).

MATERIALS AND METHODS

Drugs. CC-1065, a fermentation product (2), was supplied by Dr. David Martin at The Upjohn Company. CC-1065 analogues (U-71,184 and U-71,185) were synthesized by Dr. Martha Warpehoski at The Upjohn Company, Kalamazoo, MI (11, 12). All compounds were dissolved in dimethyl sulfoxide or dimethylacetamide at 1 mg/ml and were stored in glass vials at −20° C.

Cell Cultures. P388 mouse leukemia cells were obtained from the National Cancer Institute-Frederick Cancer Research Facility, Division of Cancer Treatment Tumor Repository (Frederick, MD). These cells were maintained in vitro in RPMI 1640 medium (K. C. Biologicals, Lenexa, KS) with 5% FCS (HyClone, Logan, UT) in a 5% CO2 humidified incubator. L1210 mouse leukemia cells were maintained in RPMI 1634 medium (Whittaker Bioproducts, Walkersville, MD) and supplemented with 5% FCS, 0.075% sodium bicarbonate, 100 μg/ml of penicillin, and 50 μg/ml of streptomycin. Both cell lines were grown exponentially and subcultured prior to reaching stationary phase. Cells were suspended in growth medium supplemented with 7% dimethyl sulfoxide and then stored in liquid nitrogen.

Chromatin Preparation. Unless otherwise indicated, the following steps were performed on ice. Nuclei were isolated by Triton X-100 treatment (23). Briefly, P388 cells (4 to 6 × 10^9/ml) were centrifuged and washed 3 times in phosphate-buffered saline. Pelleted cells were resuspended in 10 mM Tris (pH 7.4):10 mM NaCl:1 mM MgCl2 and allowed to swell on ice for 30 min. The cell suspension was homogenized with 10 strokes of a Potter-Elvehjem tissue grinder (Wheaton).
and then centrifuged, and the resulting pellet was resuspended in Solution 3 [0.32 M sucrose:1 mM MgCl₂·6H₂O·1.4% Triton X-100 (pH 7.2)] and homogenized. After centrifugation, the pellet was washed 3 times in Solution 3, washed twice, and resuspended in Solution 1 [0.32 M sucrose:2 mM MgCl₂·1 mM potassium phosphate (pH 6.8)]. The nuclear preparation was free of cytoplasmic contamination as judged by phase-contrast microscopy. The integrity of the nuclear membrane was checked after staining with 0.5% trypan blue by phase-contrast microscopy.

Chromatin was prepared from isolated nuclei as described previously (24). Briefly, isolated nuclei were washed in 10 mM Tris buffer (pH 8.0) and suspended in 25 mM EDTA:10 mM Tris (pH 8.0). The suspension was then homogenized with 5 strokes, centrifuged, and washed 3 times with 50 mM Tris buffer (pH 8.0). The resulting pellet was washed with 0.12 M NaCl:5 mM magnesium acetate:20 mM Tris (pH 8.0) and then resuspended in 0.01 M Tris (pH 7.2). The purity of chromatin preparation (25) was checked by spectrophotometric analysis (ratio of A₃₅₀ nm/A₂₆₀ nm, 0.96; A₂₆₀ nm/A₂₈₀ nm, 1.07; A₃₁₀ nm/A₂₆₀ nm, 0.56). The integrity of the chromatin preparation was indicated by CD measurements as described in the text (see "Results").

DNA Preparation. DNA was extracted from P388 cells as described (26) and treated with RNase and protease K. DNA was extracted with phenol/chloroform, ethanol precipitated, and resuspended in 10 mM Tris (pH 7.4):1 mM EDTA, and the concentration was determined at A₂₆₀ nm via baseline method (see below) by spectrophotometry. For some experiments, calf thymus DNA (CalBiochem, San Diego, CA) was used.

Preparation of Acid-soluble Nuclear Proteins. Nuclei were isolated from P388 cells as described previously (23). Acid-soluble nuclear proteins were prepared from nuclei pellets (27). Pelleted nuclei were extracted twice with 0.4 M H₂SO₄ for 2 h, and the supernatants were pooled. Histones and other acid-soluble nuclear proteins were precipitated in 20% trichloroacetic acid for 18 h. The final pellet was washed with acidified acetone (20 mM HCl) and then with acetone.

For certain experiments, calf thymus histones (Sigma Chemical Co., St. Louis, MO) were used with or without further purification as described. Histone protein concentration was estimated by A₂₆₀ nm, and each of four core histones and histone H₁ were identified by gel electrophoresis.

CD and Electronic Absorption Measurements. Electronic absorption spectra were obtained on Perkin-Elmer Lambda 5 or Lambda 7 instruments which were calibrated with dichromate solutions and holmium oxide. CD spectra were obtained on a Jasco 500-C CD instrument after calibration with D-10-camphor sulfonic acid (28). P388 chromatin, P388 DNA, and CT DNA were quantitated from their respective UV absorbance spectra by a baseline technique to avoid problems due to light-scattering effects. Here, a baseline was drawn from wing to wing of the A₃₅₀ nm band. The absorbance of the 260-nm band was defined as the distance between the curve and the baseline at A₃₅₀ nm. CT DNA had a molar absorptivity of 4900 by this method, and DNA from P388 chromatin and P388 DNA was assumed to have the same molar absorptivity.

A slight translucence and some light scattering (at <230 nm and >300 nm) were evident for chromatin fractions, but such light scattering was not seen for P388 DNA. These effects were probably due to greater DNA particle size in polycomplexes rather than the non-light-scattering mononucleosomes. Light scattering could cause damping and red shifts of CD bands. However, the induced CD effect is large relative to any possible scattering effects, especially for CC-1065 and U-71,184, and the qualitative nature of the results must be accepted if the binding properties of polycomplexes are to be studied. It is our experience from extraction experiments that light scattering in the 500-300-nm range (which presents a serious problem in the case of absorption measurements) does not cause a significant distortion of the CC-1065-induced CD band.

Induced CD measurements, expressed as molar ellipticity, were used as a measure of the degree of binding of drug to DNA, chromatin, or histones. Molar ellipticity = degrees of ellipticity concentration (g/ml) × path length (dm) × molecular weight 100

Unless stated otherwise, all CD interaction measurements were obtained after a 24-h interaction at 24°C in a 5.0-cm microcell and performed in 0.01 M Tris-HCl (pH 7.2). Drug concentration was 0.37 × 10⁻³ M, and molar ratio of nucleotide to drug (C₀/Cₚ) was 30. For histones, a molar ratio of protein to drug (C₀/Cₚ) of 30 was used.

Mixtures for spectral analysis were prepared by injecting about 0.02 ml of a solution of CC-1065 (or its analogues) in dimethyl formamide into 5.0 ml of aqueous buffer containing the biomolecule sample. The reversibly bound species of CC-1065, U-71,184, or U-71,185 were removed from chromatin or DNA with phenol extraction, followed by 3 butanol extractions (to remove the phenol) and then CHCl₃ to clear the solution for absorbance and CD measurements. In these experiments, the aqueous phase was repeatedly extracted with an equal volume of phenol until the CD and absorbance spectra of bound drug indicated that no more drug was being removed.

In Vitro Testing of Drug-Macromolecule Interaction. The ability of CC-1065 and its analogues to interact with chromatin and DNA chromatin components was further evaluated in a biological system (6). The drug was mixed with DNA, chromatin, or histone, and then the mixture was added to L1210 cells. The L1210 cells were then incubated for 3 days at 37°C, following which the cell number in the treated sample was compared to that in the control (drug alone without added macromolecule). If the drug was bound irreversibly to the macromolecule, then the degree of growth inhibition caused by the drug-macromolecule would be less than that caused by drug alone, and the decrease in inhibition would be a measure of drug binding to macromolecule. This method assumes that reversibly bound drug causes growth inhibition to the same extent as free drug. Different drug concentrations were prepared in sterile H₂O or growth medium, and 0.1 ml was added to a culture tube. Glass pipets and vials were used in diluting drugs such as CC-1065 binds to plastic. Different concentrations of macromolecules were prepared, and 0.1-ml aliquots were added to the culture tube. The culture tubes containing macromolecule and drug, or drug alone (positive control) were then incubated at 4°C for 4 h. A 4°C incubation temperature was used during premixing in order to avoid possible protease or nuclease degradation of the macromolecules.

RESULTS

Biological Activity of CC-1065 and Its Analogues. The structure and P388 cytotoxicity of CC-1065, U-71,184, and U-71,185 are shown in Table 1. U-71,184 is more cytotoxic than CC-1065 to P388 cells in vitro (Table 1) and has been shown to have greater antitumor effect in vivo (14). U-71,185, the mirror image isomer of U-71,184, is ½ as potent as U-71,184. Since U-71,185 may contain up to 1% of U-71,184, the inherent potency of U-71,185 cannot be distinguished from the contaminating active isomer.

CD Spectrum of P388 Chromatin and DNA. Isolated P388 chromatin and DNA were suspended in 10 μM Tris-HCl (pH 7.2) and analyzed by CD spectroscopy (Fig. 1) P388 chromatin showed a CD band at 272 nm (molar ellipticity = +6000), a shoulder near 245 nm, and a zero cross-over point of 258 nm. P388 DNA showed CD bands at 270 nm (+7300) and 245 nm (−7700) and a zero cross-over point of 256 nm. These CD data are in good agreement with those reported for DNA and chromatin by others (29, 30).

Comparison of Induced CD Spectra of CC-1065 and Its Analogues. In Fig. 2, induced CD curves for CC-1065 and its analogues with chromatin or DNA are compared to the molar ellipticity curves of drug alone at various incubation times. With the exception of U-71,185 plus chromatin, all drugs show intense induced CD bands (upon binding to the chromatin or DNA), suggesting an acquired helical asymmetry. The qualit-
Table 1 Properties of CC-1065 and its analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>P388 in vitro 90% growth inhibition (ng/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-1065</td>
<td>0.09</td>
</tr>
<tr>
<td>U71184</td>
<td>0.02</td>
</tr>
<tr>
<td>U71185</td>
<td>2.00</td>
</tr>
</tbody>
</table>

* After 3-day growth at 37°C.

Table 2 Induced molar ellipticity of CC-1065, U-71,184, and U-71,185

<table>
<thead>
<tr>
<th>Compound</th>
<th>P388 DNA</th>
<th>P388 chromatin</th>
<th>% of reduction of molar ellipticity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-1065</td>
<td>290,000</td>
<td>175,000</td>
<td>40</td>
</tr>
<tr>
<td>U-71,184</td>
<td>190,000</td>
<td>65,000</td>
<td>66</td>
</tr>
<tr>
<td>U-71,185</td>
<td>57,000</td>
<td>10,000</td>
<td>82</td>
</tr>
</tbody>
</table>

* Molar ellipticity was determined after 24-h incubation of drugs (0.37 × 10⁻⁶ M) with chromatin or DNA. CN/CT = 30.

Reversibility of Drug-Chromatin Interaction. The reduction of induced CD upon phenol extraction of the complex of CC-1065 (or its analogues) with DNA and chromatin is shown in Fig. 3. The unbound or reversibly bound CC-1065 separated in the phenol layer while the irreversibly bound CC-1065 partitioned in the buffer phase with the DNA or chromatin. In all cases, interaction of the drugs with P388 chromatin was mostly reversible. The percentage of reduction of induced CD after phenol extraction was 70% for CC-1065, 82% for U-71,184, and 90 to 100% for U-71,185. Thus, the degree of reversible binding to chromatin was U-71,185 > U-71,184 > CC-1065. In contrast, the interaction of naked DNA with the compound was mostly irreversible. CC-1065- or U-71,184-DNA complex showed between 0 and 5% reduction of induced CD upon phenol extraction. U-71,185 plus DNA showed a 21% reduction of induced CD upon phenol extraction. The degree of reversible binding to DNA was U-71,185 > U-71,184 = CC-1065.

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Fig. 2. Effect of period of incubation of drug plus DNA or chromatin on induced CD. The molar concentration ratio of nucleotide to drug (CN/CT) = 30. All experiments were performed in 0.01 M Tris-HCl (pH 7.2) at 24°C at a drug concentration of 0.37 × 10⁻⁶ M. The CD curves for drug only were obtained within the first hour of incubation, since drug settles out of solution with time (50% CD decrease after 24 h). In the presence of DNA or chromatin, the drug does not settle out.

Fig. 3. Circular dichroism spectra for P388 DNA and chromatin.

CC-1065 Interacts Weakly with Histones. Similar experiments as those described above were performed with isolated histones plus CC-1065 to determine the possible role of histones in binding drug to chromatin. There was no significant difference in absorbance spectrum between CC-1065 alone and CC-1065 plus histones (data not shown). We have observed that CC-1065 precipitates from a reaction mixture when there is no binding (9). In the case of histone plus CC-1065, precipitation of CC-1065 did not occur, suggesting that CC-1065 does bind to histones. However, this binding does not produce an induced CD (see below).

The CD curve of the histone-CC-1065 mixture is shown in Fig. 4 along with those of DNA and chromatin. The CD of the mixture cannot be distinguished from that of CC-1065. Thus, no significant induced CD is produced by the mechanism by which histones bind CC-1065. These results suggest that CC-1065 and, by extension, U-71,184 and U-71,185 bind very weakly and nonspecifically to P388 histones, and that the induced CD caused by chromatin results from DNA binding of the drug molecule.

Binding Studies in 2.0 M NaCl. In another experiment, we...
measured the effect of 2 m NaCl on the interaction of CC-1065 with chromatin. At this salt concentration, histones are dissociated from DNA (31, 32). If the histones have no direct effect on the binding process, then this experiment should produce results similar to those with naked DNA. Fig. 5 shows that this is the case: the induced CD curves produced by chromatin and naked DNA in 2.0 m NaCl are identical in position and intensity (within experimental error); i.e., the dissociated histones have no effect on the binding. This result also indicates that CC-1065 and, by extension, U-71,184 and U-71,185 bind to the DNA of chromatin and not to the dissociated histones. Furthermore, the similar wavelength positions of the two CD bands of Fig. 5 suggest that relative amounts of reversible and irreversible bound species are equivalent. Thus, the different binding properties of chromatin, as compared to naked DNA, cannot be accounted for as a result of histone-bound drug.

Cytotoxicity of the Drug after Binding to DNA or Chromatin. We next wanted to evaluate whether the difference between the DNA, CHROMATIN INTERACTION WITH CC-1065 AND ITS ANALOGUES

Fig. 4. CC-1065 interaction with histones. CC-1065 = 0.37 x 10^-5 M. Histone = 11.1 x 10^-5 M (residue). DNA or chromatin = 11.1 x 10^-3 M (nucleotides). Twenty-four h of incubation. Drug interaction with DNA and chromatin under conditions similar to that in Fig. 2 is shown for comparison.

Fig. 5. Induced CD for the interaction of CC-1065 plus chromatin or DNA in the presence of 2.0 m NaCl. CC-1065 (DNA) = 0.43 x 10^-5 M. CC-1065 (chromatin) = 0.37 x 10^-5 M. CH/Cr = 30. One-day incubation. Solid line, DNA curve; dashed line, chromatin curve.

Fig. 6. Comparison of growth inhibition by drug, drug-DNA, or drug-chromatin mixtures. DNA or chromatin at inumi final concentration was incubated with drug for 4 h at 4°C in growth medium, following which the complex was added to L1210. Drug alone was similarly incubated. Growth inhibition was compared to the effect of the drug alone. Cells were counted after 3 days of incubation at 37°C.

Fig. 2.

Fig. 3. CD spectra of drug-DNA and drug-chromatin mixtures with or without phenol extraction. Drug = 0.37 x 10^-5 M; CH/Cr = 30. Drug-DNA and drug-chromatin mixtures were incubated for 3 days, after which they were extracted with phenol (see "Materials and Methods") to remove unbound and reversibly bound drug. Solid lines, DNA curves; dashed lines, chromatin curves; circles, no phenol extraction; squares, after phenol extraction.
interaction of DNA and chromatin with drug would also be true in a biological system. Growth inhibition by the complex of drug and calf thymus DNA or chromatin is compared to that of drug alone in Fig. 6. These results clearly show that, when CC-1065 or U-71,184 was mixed with calf thymus DNA, the cytotoxicity of the drug decreased markedly. Thus, 0.04 ng/ml of CC-1065 alone caused 98% inhibition of growth compared to only 23% inhibition when mixed with calf thymus DNA. For both drugs, chromatin caused less reduction in growth inhibition as compared to DNA. Premixing of histones with the drugs did not cause any reduction in growth inhibition (not shown).

DISCUSSION

Most CC-1065 binding studies have used naked DNA or synthetic oligonucleotides as substrates. Our studies were intended to characterize the interaction of CC-1065 with a more complex structure, i.e., chromatin. The chromatin used in these studies, isolated without a nuclease digestion step, consisted of high-molecular-weight species rather than monomeric or oligomeric nucleosomes. Our CD spectra of DNA and chromatin from P388 cells correlated well with reports by others and are clearly different from nuclease-digested chromatin or nuclear preparations (34–35). The CD binding studies show that CC-1065 and analogues bind to P388 chromatin and that the induced CD spectra are similar to those obtained when CC-1065 binds to P388 DNA and many other synthetic polynucleotides (9). Since these drugs do not bind significantly to histones, and since all experiments to date indicate that CC-1065 and its analogues require the minor groove of B-form DNA as the binding site (4), we assume the binding site of chromatin to be the minor groove of the DNA molecule.

We showed that the intensity of the induced CD spectra and the rate of conversion of reversibly bound drug to irreversibly bound drug are less for chromatin than for naked DNA. Since histones themselves do not bind to CC-1065, this suggests that histones act indirectly to restrict the binding of CC-1065 to chromatin. This could occur through fewer binding sites being available on chromatin as compared to DNA or due to the less chiral environment of chromatin. We assume here that a good fit in the minor groove leads to a more chiral environment for the drug chromophore and, hence, a more intense induced CD. At the molecular level, the goodness of fit probably depends upon the conformation of the DNA molecule and whether or not the chromatin proteins are close enough to the binding site to sterically or electronically inhibit the binding. Our CD experiments do not distinguish between these two possibilities. Alternatively, since conformational transition states of chromatin are regulated by histones as well as nonhistone proteins (17), the latter nuclear proteins may modulate CC-1065 action. HMG-1, which binds to A-T-rich regions in dsDNA (36) and interacts with the minor groove of B-form DNA (37), may regulate CC-1065 binding.

Several other antitumor drugs have been shown to bind less to chromatin than DNA. For example, with Adriamycin (20) and nogalamycin (21), fewer binding sites are available in chromatin than in DNA. In the case of nogalamycin, drug-DNA interactions were inhibited by the presence of core histones, histone H1, or non-histone proteins. Some antitumor compounds can also alter the structural orientation of chromatin DNA. For example, netropsin and berenil interacted with subchromatin and induced the DNA to rotate about one-half a turn so that regions facing outside now faced inside and vice versa (38). Since both CC-1065 and netropsin share similar binding characteristics, CC-1065 might also be able to induce a similar rotational change in the chromatin DNA.

Qualitatively the comparative binding of CC-1065 and U-71,184 to DNA, chromatin, or histone as measured by the growth inhibition assay correlated with CD results. Both drugs show significantly reduced cytotoxicity when premixed with CT-DNA, which indicates that the drugs are bound tightly (irreversibly) to DNA and are, therefore, not available to inhibit growth. Also, the more reversible binding to chromatin results in less growth inhibition which agrees with the lower induced CD and reversibility seen on phenol extraction (Fig. 3). In general, the potency of CC-1065 analogues correlates with their ability to bind to DNA, although there does not appear to be a quantitative relationship. It would be unrealistic to expect a quantitative correlation between DNA or chromatin binding data obtained under one set of conditions and the action of the drug at the cellular level or in whole animals.

In conclusion, chromatin can serve as a suitable substrate for studying interaction with CC-1065 and its analogues. In one respect, drug interaction with chromatin is significantly different from that with DNA in that the former was reversible, whereas the latter was a nonreversible, covalent interaction. In trying to explain the molecular action of these DNA binding agents at the cellular level, it is necessary to study a more complex paradigm than naked DNA.

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