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Association of Chromatin with Anticancer Antibiotics, Mithramycin and Chromomycin A_3

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Abstract—Mithramycin and chromomycin A_3 are two anticancer antibiotics, which inhibit protein biosynthesis via transcription inhibition. They bind reversibly to DNA with (G.C) base specificity. At and above physiological pH in the absence of DNA, they form two types of complexes with Mg^{2+}, complex I (1:1 in terms of antibiotic: Mg^{2+}) and complex II (2:1 in terms of antibiotic: Mg^{2+}). These are the DNA binding ligands. In vivo, the antibiotics interact with chromatin, a protein–DNA complex. In order to understand the mode of action of these antibiotics at molecular level, we have carried out spectroscopic, gel electrophoretic and *UV* melting studies of complex I of these antibiotics with rat liver chromatin, nucleosome core particle and DNA stripped of all chromosomal proteins. Analysis of the results has led us to propose that the antibiotic: Mg^{2+} complex binds to both nucleosomal and linker DNA in native chromatin. Histone proteins reduce the binding potential and accessibility of the complexes to the minor groove of (G.C) rich regions of chromosomal DNA. The antibiotic: Mg^{2+} complex stabilizes DNA duplex and histone- DNA contacts in chromatin fiber. It also leads to the aggregation of chromatin fibers. From a comparison of the association of the antibiotic: Mg^{2+} complexes with different levels of chromatin structure and their effects upon the structure, we suggest that the sugar moieties of the antibiotics play a role in the binding process. Significance of these results to understand the molecular basis of the transcription inhibition potential of the antibiotics in eukaryotes is discussed.

Introduction

Aureolic acid group of antitumor antibiotics, mithramycin (MTR) and chromomycin A_3 (CHR), from *Streptomyces plicatus* and *Streptomyces griseus*, respectively, are clinically employed for testicular carcinoma and Paget's disease.[1] With a gross structural similarity, MTR and CHR have difference in the nature of sugar rings connected to aglycone ring via *O*-glycosidic bond (Fig. 1, panels a and b). Antitumor properties of MTR and CHR in experimental tumors have been ascribed due to their inhibitory roles in replication and transcription process during macro molecular biosyntheses.[2] They inhibit the expression of some proto-oncogenes like c-myc, having an important role in the regulation of cell proliferation and differentiation.[3] Recently, a potential use of these antibiotics has been proposed as neurological therapeutics for the treatment of diseases associated with aberrant activation of apoptosis.[4] The prime cellular target of these antibiotics is DNA. Bivalent cation such as Mg^{2+} is an essential requirement for their association with DNA at and above physiological pH.[5] DNA binding properties of these antibiotics have been very well studied in our and other laboratories.[5–11] We have shown that in absence of DNA these antibiotics interact with Mg^{2+} and form two different types of complexes, complex I (1:1 in terms of antibiotic: Mg^{2+}) and complex II (2:1 in terms of antibiotic: Mg^{2+}).[5–8] These complexes are DNA binding ligands at and above physiological pH and bind to DNA via minor groove.[5–9] It was established in our laboratory from spectroscopic and thermodynamic studies that the modes of binding of the two ligands with natural DNA, polynucleotides and oligomeric duplexes are different.[5–9] We also illustrated the role of DNA minor groove size and the accessibility of the 2-amino group in the minor groove of guanosine in drug-DNA interaction using designed nucleotide sequences.[8,9] Detailed NMR studies from other laboratories have helped to understand how the bulky complex II is accommodated at the cost of a considerable widening of the minor groove in B-DNA type structure.[10,11]

These studies have focused upon molecular details of interaction with DNA. Very few studies, on the other

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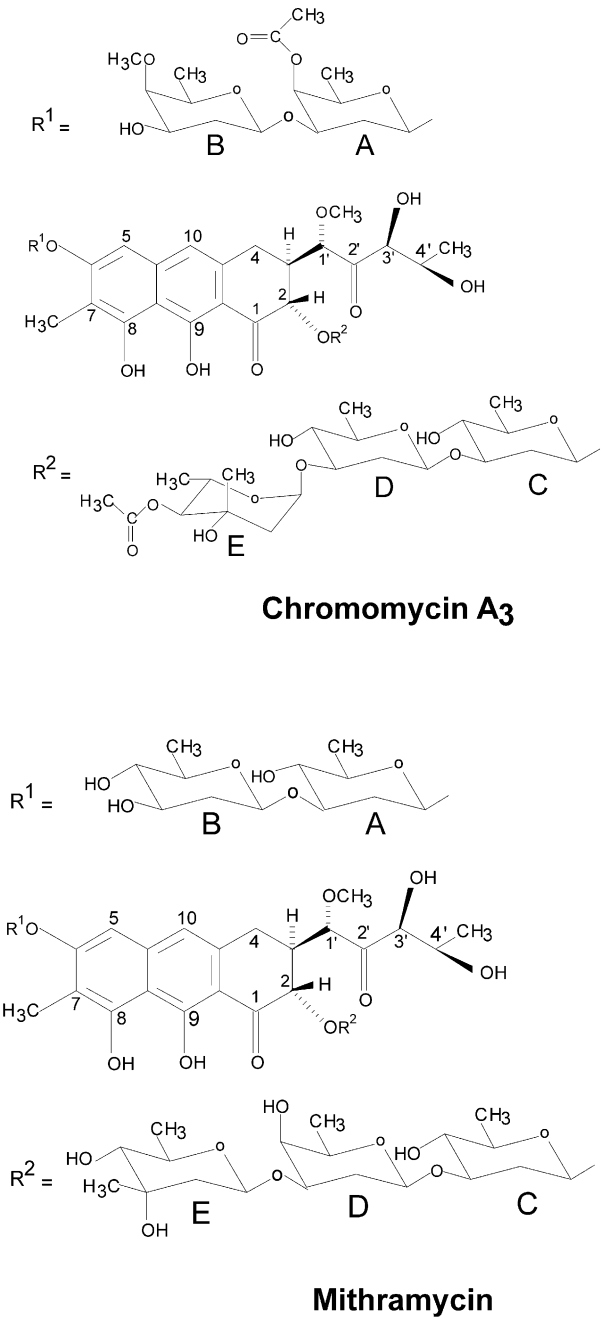

Figure 1. Structures of the antibiotics: (a) Chromomycin A_3 and (b) Mithramycin.

hand, have attempted to understand the structural basis of the association of antibiotic: Mg^{2+} complexes with chromatin, the constituent of the chromosome. Packaging of DNA in an eukaryotic nucleus involves several distinct hierarchical events. The first level of compaction occurs when DNA is wrapped around an octamer of histones to form a repeating subunit called the nucleosome.[12] Histone octamer-DNA complexes are spaced at nearly 200 base pair intervals to form nucleosomal arrays, which interact with linker histones to form a highly folded transcriptionally suppressive '30 nm diameter' chromatin fiber.[13] Minor grooves of DNA in chromatin are not freely accessible to the ligands because of their occupancy with N-terminal tail domains and arginine side chains of histone proteins in the nucleosome core region of chromatin.[14] The only report, which showed the binding of chromomycin with chromatin, isolated from mouse and rat liver, had the following lacunae.[15] Chromomycin concentration used was greater than 50 μM, at which it aggregates. Mg^{2+} concentration used was in micromolar range (45–328 μM). Under this condition a mixed population of complexes, I and II, is formed. Knowledge about chromatin structure and its role in transcription was also preliminary. Recently, we have reported a spectroscopic study of the interaction of complexes, I and II, of mithramycin with rat and chicken liver chromatin and nucleosome core particle.[16] The results from this report. show that presence of histones adversely influences the association of both complexes with DNA and they interact differentially with same chromatin. In the present paper, we have reported a detailed investigation of the association of complex I of two antibiotics with chromatin/nucleosome core particle and naked DNA from rat liver. It describes spectroscopic, thermal melting and gel electrophoretic studies on the interaction of complex I of both antibiotics (CHR and MTR) with native chromatin, nucleosomal core particle and naked DNA. The present study has two objectives as follows: (i) to probe, characterize and compare the binding sites of complex I for both CHR and MTR on the chromatin and to examine the effect of histones upon the binding process and (ii) to detect the effects of the association upon the chromatin structure. The results have thrown light upon the potential deleterious effects of the binding of complex I (for both MTR and CHR) upon chromatin to function as a transcription template. A relative study of the two antibiotics has provided important information about the role of sugar moieties. Concentrations of the antibiotics (CHR/MTR) and Mg^{2+} were so chosen from the spectroscopic titration of antibiotic and Mg^{2+} that they led to the formation of complex I only.[5,6]

We employed spectroscopic studies like absorbance, fluorescence and CD to characterize the association, because they permit us to work at micromolar concentration of the drug (10–50 μM). At this concentration range, there is no alteration in the state of aggregation in the ligand as detected by the optical spectroscopic tools. This concentration of the ligand lies within the LD 50 dose of the antibiotic.[2] The reason for confining our studies to complex I is as follows. Spectroscopic studies require optically clear solutions. Complex I of both the antibiotics is formed at a lower concentration of Mg^{2+} (200–300 μM) where chromatin does not aggregate to precipitate. In the case of chromomycin, formation of complex II requires minimum Mg^{2+} concentration of 3.5 mM. Chromatin undergoes extensive condensation and aggregation to phase out at this concentration of Mg^{2+}. So, the spectroscopic studies with complex II are not reliable. There are reported fluctuations in Mg^{2+} concentration in the carcinogenic tissues.[17] At times it reaches micro molar level, when complex I will be the active species.

Results

Characterization of chromatin and nucleosome core

Chromatin and nucleosome core particles were isolated and purified as described in Materials and Methods. To check the purity of the sample, histones from both chromatin and nucleosome core particle were isolated and run on SDS PAGE (not shown). We observed the presence of all core histones (H2A, H2B, H3 and H4) and linker histone H1 in chromatin. Nucleosome was tested similarly to contain only core histones. DNA was also isolated from chromatin and nucleosome core particle as described above and run on agarose gel. Length of the chromosomal DNA, as estimated from the comparison with standard DNA marker, showed the chromatin preparation containing 6 to 20 nucleosomes. We observed that nucleosomal core DNA was 147 base pairs long. These experiments were repeated after treatment of the chromatin and nucleosomal core particle at saturating concentrations of antibiotic: Mg^{2+} complex and incubated for 30 min. We did not notice any change in the band pattern of SDS PAGE of histones and agarose gel electrophoresis of DNA. However, longer incubation of the core particle with higher concentration antibiotic: Mg^{2+} complex for more than 4 h leads to DNA release as mentioned earlier.[24] Therefore, all experiments in the case of core particle was completed within 1 h and at about ten-fold lower concentration of the antibiotic. Each sample after the experiments was also checked for any DNA release.

Binding of complex I of chromomycin and mithramycin with chromatin, nucleosome core particle and naked DNA

Changes in the absorption (Fig. 2) and fluorescence (Fig. 3) spectra of complex I of both antibiotics in the presence of chromatin, nucleosome core particle and naked DNA originate from an association between them. Changes induced in the spectrum of the ligands depend upon the nature of the polymer to which the ligand binds. Figure 2 (panels a and c) show the absorption spectra of complex I for both antibiotics upon addition of different concentrations of native chromatin. The set of spectrum for the ligand alone and in the presence of native chromatin contains one isosbestic point suggesting the formation of a single type of complex between them. Figure 2 (panel b) shows the changes in absorption spectrum of MTR: Mg^{2+} complex in the presence of the saturating concentrations of different polymers. While the principal features of these spectral changes are red shift and broadening of the band associated with hyperchromocity, the nature of the spectra with different polymers are non- overlapping. These changes are the characteristic of the association of the complexes with DNA.[5,6] Similar changes mark the association of complex I of CHR with the above polymers (panel d of Fig. 2). Non-overlapping nature of the set of spectra shown in the panels b and d of Figure 2 implies that the electronic environment of the chromophore in the DNA bound complex I are not similar for the three polymers.

Figure 3 shows the increase in fluorescence intensity of ligands upon their interaction with native chromatin. Blue shift of the peak accompanies the increase in fluorescence. Similar changes in fluorescence properties were observed for both ligands during their association with the other polymers. Comparison of the spectra shown in different panels of the figure shows that the extent of blue shift and increase in fluorescence intensity of the ligands depend upon the nature of the polymer and

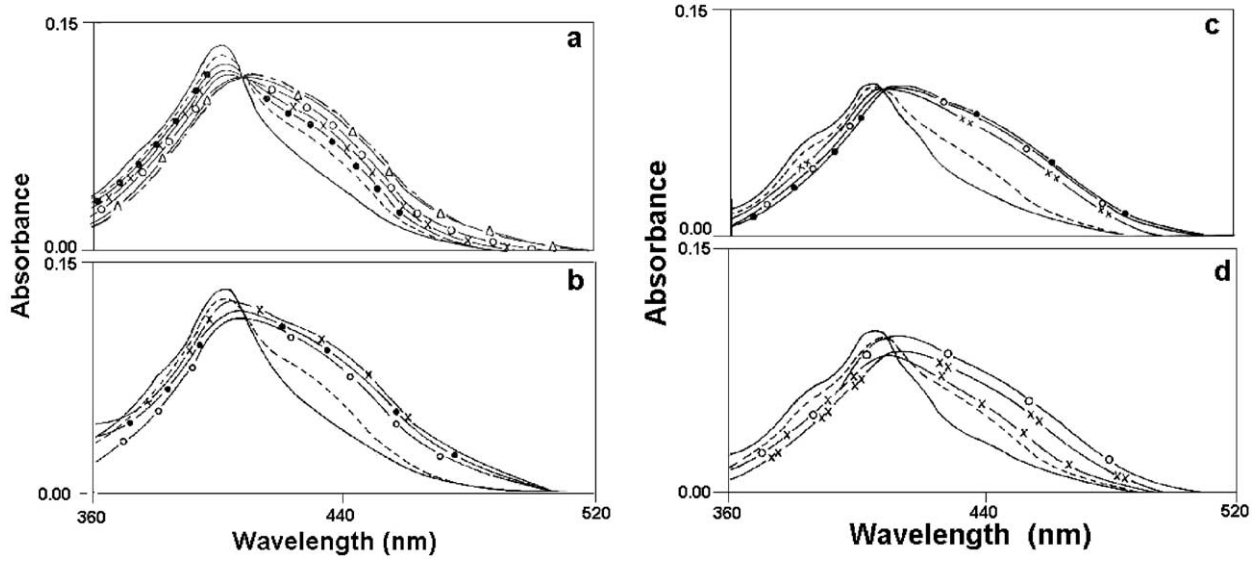

Figure 2. Absorption spectra (360–520 nm) of Mithramycin under different conditions in 10 mM Tris–HCl, pH 8.0 at 25 °C. (a) MTR alone ([MTR] = 13.5 μM, —); complex I ([MTR] = 13.5 μM, $[Mg^{2+}]$ = 230 μM, - - -); complex I in presence of native chromatin (110 μM, — ● —; 220 μM, — X —; 340 μM, — ○ —; 450 μM, — △ — and 500 μM — ——); (b) Complex I in presence of saturating concentrations of naked DNA (200 μM, — X —); native chromatin (450 μM, — ● —); and nucleosome core particle (600 μM, — ○ —); (c) CHR alone ([CHR] = 13.5 μM, —); complex I ([CHR] = 13.5 μM, $[Mg^{2+}]$ = 300 μM, - - -) and complex I in presence of native chromatin (150 μM, — ● —; 300 μM, — ○ —, 450 μM, — XX —); (d) CHR alone ([CHR] = 13.5 μM, —); complex I ([CHR] = 13.5 μM, $[Mg^{2+}]$ = 300 μM, - - -); complex I in presence of saturating concentrations of native chromatin (450 μM, — ○ —), naked DNA (150 μM, — XX —) and nucleosome core particle (470 μM, — X —).

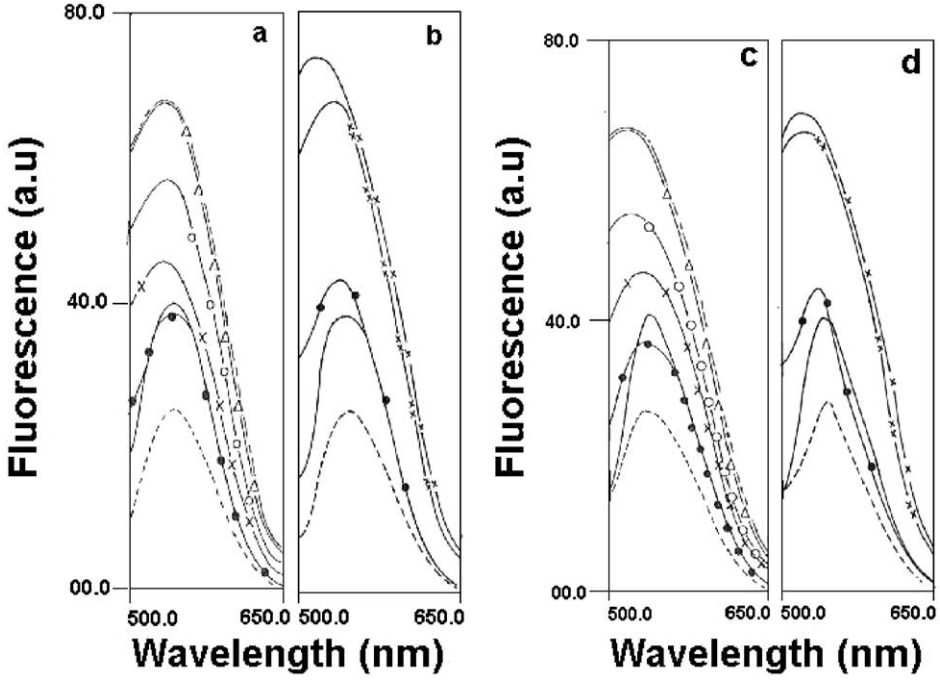

Figure 3. Fluorescence emission spectra (500–650 nm, $\lambda_{ex} = 470$ nm) of antibiotics under different conditions in 10 mM Tris–HCl, pH 8.0 at 20 °C: (a) MTR alone ([MTR] = 13.5 μM, —); complex I ([MTR] = 13.5 μM, [Mg^{2+}] = 230 μM, - - -); complex I in presence of native chromatin (110 μM — ● —; 240 μM, — X —; 340 μM, — ○ —; 450 μM, — Δ — and 550 μM, — –—); (b) Complex I of mithramycin in presence of saturating concentrations of naked DNA (200 μM, — X —); native chromatin (450 μM, — XX —) and nucleosome core particle (600 μM, — ● —); (c) CHR alone ([CHR] = 13.5 μM, —); complex I ([CHR] = 13.5 μM, [Mg^{2+}] = 300 μM, - -); complex I in presence of native chromatin (110 μM — ● —; 240 μM, — X —; 340 μM, — ○ —; 450 μM, — Δ —– and 550 μM — –—); (d) Complex I of chromomycin in presence of saturating concentrations of naked DNA (300 μM, — X —), native chromatin (450 μM, — XX —) and nucleosome core particle (500 μM, — ●—).

the antibiotics as well. Extent of increase in fluorescence of the ligand in presence of the polymers follows the order: naked DNA > native chromatin > core particle. The extent of blue shift follows the same trend. The extent of blue shift for a particular system is comparatively more in case of chromomycin than mithramycin.

We compared the CD spectra of the two ligands in presence of increasing concentrations of different polymers. Red shift of the peak to the longer wavelength region of antibiotic: Mg^{2+} complex of both antibiotics upon addition of chromatin, nucleosome core particle and naked DNA is a common feature. However, there are notable differences in the features of the spectra as given below. Figure 4 (panel a) shows the representative CD spectra of MTR: Mg^{2+} complex in presence of different concentrations of native chromatin. They are characterized by an isosdichroic point, thereby indicating the formation of a single type of complex. Isodichroic points were also observed with other systems. CD spectra of both complexes (panels b and c) are different in presence of chromatin/core particle/dehistonized DNA. The difference in CD spectral features of a particular ligand in chromatin/core particle/dehistonized DNA may be ascribed to a difference in the chiro-optical environments of the chromophore in the

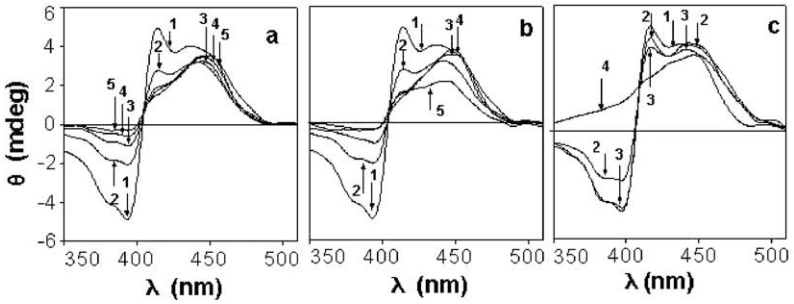

Figure 4. CD spectra (340–510 nm) of complex I of mithramycin and chromomycin under different conditions in 10 mM Tris–HCl buffer, pH 8.0 at 25 °C: (a) MTR (15 μM) alone (1), complex I [MTR (15 μM) plus Mg^{2+} (230 μM)] (2), complex I in presence of 110 μM (3), 240 μM (4) and 450 μM (5) of native chromatin (b) MTR (15 μM) alone (1), complex I [MTR (15 μM) plus Mg^{2+} (230 μM)] (2), complex I in presence of saturating concentrations of 450 μM native chromatin (3), 220 μM naked DNA (4) and 600 μM nucleosome core particle (5), (c) complex I of CHR [CHR (15 μM) plus Mg^{2+} (300 μM)] (1), complex I in presence of saturating concentrations of 450 μM native chromatin (2), 500 μM nucleosome core particle (3) and 220 μM naked DNA (4).

ligand as a result from an altered accessibility of the ligand to DNA in different polymers.

Figure 4 (panels b and c) shows the CD spectra of antibiotic(s): Mg^{2+} complex in presence of saturating concentrations of different polymers. Changes in the profile and band intensity of visible CD spectra of the ligand(s) occur as a result of association with DNA in chromatin and nucleosome core particle. That is why spectra of the complex in presence of dehistonized DNA have been recorded. Comparison of the spectra shown in the two panels shows that the effect of association with a particular polymer upon the spectrum of antibiotic(s): Mg^{2+} complex is dissimilar for CHR and MTR. In contrast to MTR, parental spectral features of the complex of CHR are retained after association with nucleosome core and chromatin. In case of dehistonized DNA, where parental spectral features of both antibiotic(s): Mg^{2+} complexes are lost during the association and the spectra for both antibiotics are comparable.

Estimation of binding parameters, dissociation constant and binding stoichiometry (or binding site size)

We estimated the binding parameters, dissociation constant and binding stoichiometry, for the ligand–polymer interaction from fluorescence titration of the ligands with varying concentrations of polymers. Representative curves for CHR: Mg^{2+} complex are shown in Figure 5. Nature of the curves shows a non-cooperative binding characteristic of a single type of complex, as supported from the presence of a single cross-over point in the absorption and CD spectra. Dissociation constant (K_d) was estimated by means of nonlinear curve fit analysis. However, the method gives an average value of the dissociation constant if there exists a microheterogenity in the binding sites. Binding stoichiometry (or site size) was estimated as explained under Materials and Methods.

Table 1 summarizes the binding parameters for the interaction of both ligands with different polymers. At 20 °C both ligands bind to chromatin and core particle with comparable affinity. There is a marked increase in the binding affinity for both ligands with dehistonized DNA. This shows the negative role of histones upon the binding process. In general, the binding site size (number of DNA base/molecule of bound ligand) follows the decreasing order: nucleosome core particle (where most of the DNA is wrapped around histone octamer and linker DNA and histone H1 are absent) > native chromatin > dehistonized DNA (where histone proteins are stripped off).

Evaluation of thermodynamic parameters

van't Hoff thermodynamic parameters for the interactions of both ligands with different polymers were determined by using eq 3 and 4. Figure 6 shows the representative plots for the interaction of antibiotic: Mg^{2+} complex of both antibiotics with chromatin. They are linear, suggesting an absence of dependence of ΔH values upon temperature within the range measured. Table 2 lists the thermodynamic parameters for the interaction of both complexes with native chromatin, nucleosome core particle and dehistonized DNA. From the table it is clear that association of both complexes with different polymers is enthalpy driven process. We have observed a small but consistent reduction in the negative enthalpy value from native chromatin to dehistonized DNA. Such trend has also been reported from the studies on the interaction of ethidium bromide and netropsin with chicken erythrocyte chromatin.[25,26] The reduction in negative enthalpy is compensated by a reduction in the negative entropy value, thereby leading to comparable free energy value. While the heat content of association of a antibiotic: Mg^{2+} complex with different polymers are not much different, entropy of binding varies significantly.

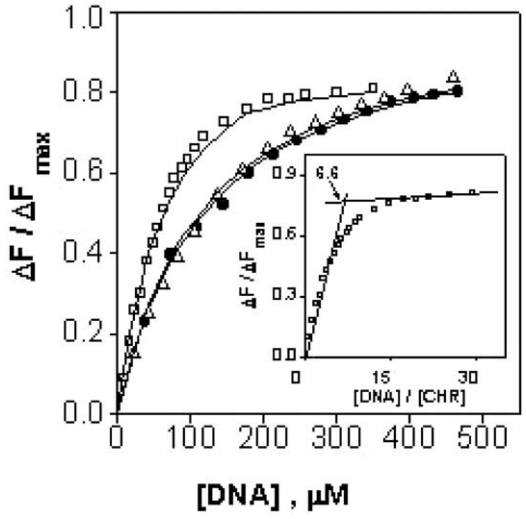

Figure 5. Curve fitting analyses to evaluate dissociation constant for the interaction of complex I of chromomycin with naked DNA (□), native chromatin (Δ) and nucleosome core particle (●) in 10 mM Tris–HCl buffer, pH 8.0 at 20 °C. Inset in the figure illustrates the method to determine binding site size as discussed in Materials and Methods.

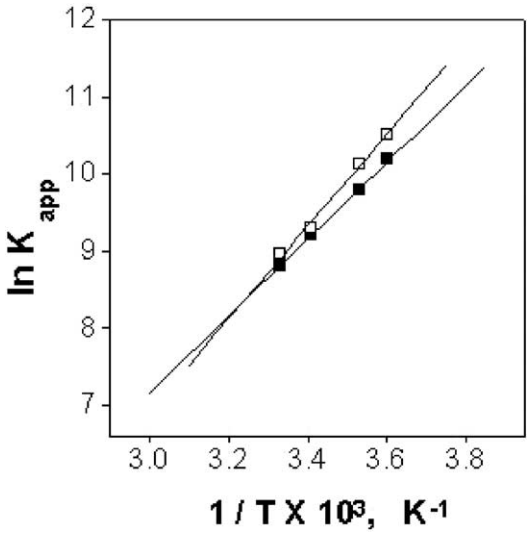

Figure 6. van't Hoff plots for the interaction of complex I of chromomycin (■) and mithramycin (□) with native chromatin in 10 mM Tris–HCl buffer, pH 8.0.

Table 1. Binding parameters for the interaction of complex I (chromomycin A_3 and mithramycin) with rat liver native chromatin, nucleosome core particle and naked DNA in 10 mM Tris HCl buffer pH 8.0 at 20 °C

System	K_d[a] (μM)	*n* (number of DNA bases/ molecule of bound ligand)
CHR: Mg^{2+} complex		
Native chromatin	110	13±2.0
Nucleosome core particle	116	18.0±2.0
Naked DNA	54	6.0±0.6
MTR: Mg^{2+} complex		
Native chromatin	107	14±2.0
Nucleosome core particle	154	18.0±2.0
Naked DNA	33	5.2±0.5

[a]The standard deviation from three sets of experiments is 15%.

Table 2. Thermodynamic parameters for the interaction of complex I of (chromomycin A_3 and mithramycin) with chromatin, nucleosome core particle and naked DNA in 10 mM Tris HCl pH 8.0 at 20 °C

System	ΔG (*K*cal/mol)	ΔH (*K*cal/mol)	ΔS(e.u)
CHR: Mg^{2+} complex	−5.4	−9.9	−15.4
Native chromatin	−5.3	−7.7	−8.1
Nucleosome core particle	−5.7	−5.2	1.7
Naked DNA			
MTR: Mg^{2+} complex			
Native chromatin	−5.4	−12.0	−22.6
Nucleosome core particle	−5.1	−9.8	−16.0
Naked DNA	−5.9	−7.5	−5.5

The standard deviation from two sets of experiments is 15%.

The dissociation constant and site size (DNA base/ molecule of bound ligand) for both complexes is smaller in naked DNA stripped of histone proteins compared to native chromatin. It implies that histone proteins obstruct access of both ligands to the potential (G.C) sites in chromosomal DNA. A consistent increase in the binding site size characterizes the interaction of both complexes with core particle. Such increase in the binding site size in the core particle relative to the native chromatin means presence of potential binding site(s) in the linker DNA. Increase in the binding site size due to a reduction of potential binding sites in core particle correlates well with the consistent decrease in negative binding enthalpy from the native chromatin to core particle. These results support our suggestion that the complexes interact with both nucleosomal DNA wrapped around the histone core and linker DNA in the chromatin fiber.

UV melting studies

Differential plots of change in the absorbance with temperature as a function of temperature indicated three thermal transitions in chromatin: T1, T2 and T3 numbered from lower to higher temperature (Table 3). Figure 7 (panels a and b) shows the representative derivative melting profiles of native chromatin in presence and absence of antibiotic: Mg^{2+} complex for both antibiotics. Triphasic melting profiles of chromatin is in agreement with the earlier literature.[26–31] Both table and Figure 7 (panels a and b) demonstrate an increase in the temperatures, T1, T2 and T3, when the ligands

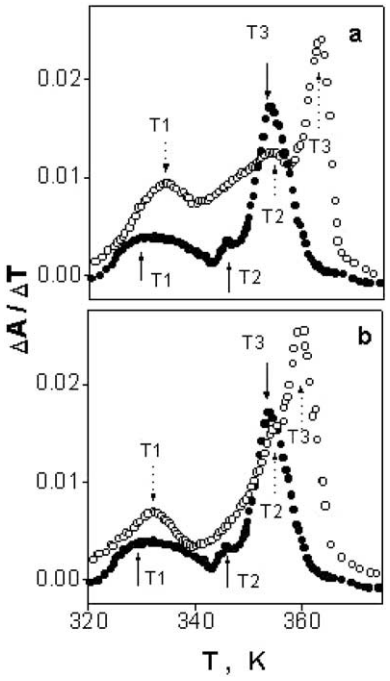

Figure 7. Derivative melting profiles of native chromatin in presence and absence of complex I of CHR (Fig. 7a) and complex I of MTR (Fig. 7b) in 5 mM Hepes buffer, pH 8.0: Native chromatin (250 μM) in presence of 230 μM Mg^{2+} (●); native chromatin (250 μM) in presence of antibiotic : Mg^{2+} complex I ([antibiotic] = 25 μM, [Mg^{2+}] = 230 μM), (○).

are bound. This suggests antibiotic: Mg^{2+} complex induces stabilization of the chromatin structure. Transition T1 (Table 3) in the derivative melting profile is due to denaturation of DNA unprotected by histones that is, linker DNA in chromatin.[30] Transition, T2 is dependent on the integrity of both DNA and histones and corresponds to the nucleosomal collapse during melting process, in which a disruption of protein-nucleic acid interactions takes place.[29,31] Transition T3 corresponds to the unstacking of bases in nucleosomal DNA.[31] We observed the stability of all three transition temperatures of chromatin by both antibiotic: Mg^{2+} complex. We observed that transition T2 is always stabilized more by CHR: Mg^{2+} complex compared to MTR: Mg^{2+} complex. In contrast, transitions, T1 and T3, are stabilized more by MTR: Mg^{2+} complex compared to

Table 3. Melting temperatures of chromatin in 5 mM Hepes buffer pH 8.0 and 230 μM Mg^{2+} in presence and absence of complex I of (chromomycin A_3 and mithramycin)

System	Complex I	Melting Temp (T_m, °C)			Relative change in (T_m, °C)		
		T1	T2	T3	ΔT1	ΔT2	ΔT3
CHR: Mg^{2+} complex							
Native chromatin	(−)	57.0	72.7	80.7			
Native chromatin	(+)	58.0	82.0	87.0	1.0	9.3	6.3
MTR: Mg^{2+} complex							
Native chromatin	(−)	57	72.7	80.7			
Native chromatin	(+)	61.1	81.1	90.4	4.1	8.4	9.7

ΔT values are reproducible within ±1 °C.

CHR: Mg^{2+} complex. Melting studies suggest that antibiotic: Mg^{2+} complex of both antibiotics binds to both nucleosomal and linker DNA in the chromatin fiber, since all three transition temperatures of chromatin fiber increase in its presence. It may be mentioned here that we did not attempt DSC for examination of the effect of ligand(s) upon the melting transitions. This is because independent experiments by means of direct calorimetric methods show a large and anomalous change in the heat of dilution in the case of MTR: Mg^{2+} complex.

Scattering and agarose gel electrophoresis to detect the ultra structural changes in chromatin fibers in the presence of antibiotic: Mg^{2+} complexes

Figure 8 shows the scattering of chromatin samples in presence and absence of antibiotic: Mg^{2+} complex. From the figure it is clear that both ligands induce aggregation of chromatin fibers. The dependence of the extent of scattering upon the input concentration of the antibiotic(s) implies that it results from the association of the ligand(s) with chromatin. At comparable concentration, chromomycin induces the aggregation of chromatin fibers to an enhanced degree compared to mithramycin. Further increase in the concentration of antibiotic: Mg^{2+} complex results in the precipitation of chromatin fibers. We isolated the histones from both soluble and precipitated fractions; we did not find any change in the band pattern.

Aggregation of chromatin fibers was further checked by agarose gel electrophoresis experiments. Glutaraldehyde fixation of chromatin samples (see Materials and Methods) was used to avoid the reversion of ultra structural changes, if any, during electrophoresis.[23] Treatment of chromatin with 230 μM Mg^{2+} as a control system does not show any significant change in electrophoretic mobility of chromatin (Fig. 9, lane 2 in panels a and b).

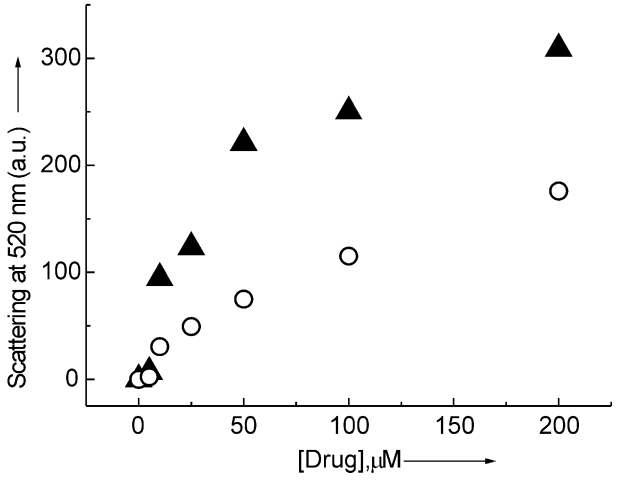

Figure 8. Scattering of chromatin in presence and absence of antibiotic: Mg2+ complex. 300 μM chromatin in presence of different concentrations of chromomycin (▲); 300 μM chromatin in presence of different concentrations of mithramycin (○). Values of scattering on the y-axis are corrected for the control value of scattering in case of chromatin (300 μM) containing Mg^{+} (230 μM) only.

Effect of the treatment of chromatin with two different input concentrations of complex I of chromomycin and mithramycin are shown in the lanes 3 and 4 of both figures. Lane 5 in both panels illustrates another control of chromatin pretreated with sodium chloride, that is known to induce compaction in chromatin structure resulting in enhanced electrophoretic mobility.[23] It is interesting to note that association with antibiotic: Mg^{2+} complex leads to a retardation in the mobility. Concentration dependent nature of the retardation implies that it originates from the association of the ligand(s) with chromatin fiber and not as a sequel to any artifact. Antibiotic: Mg^{2+} complex induced band retardation can then be ascribed to an aggregation of chromatin in the presence of antibiotic: Mg^{2+} complex. At comparable concentration of the antibiotic, the aggregation potential is relatively higher for chromomycin than mithramycin (compare lane 3 in panels a and b) as observed in the scattering experiment. Summing up, results from scattering and gel electrophoretic studies are consistent with each other and indicate in favor of drug induced aggregation of the chromatin.

We have carried out micrococcal nuclease digestion of chromatin in presence and absence of antibiotic: Mg^{2+} complexes to check the accessibility of the chromatin for the nuclease. Figure 10 shows 1.5 percent agarose gel electrophoreses of chromatin, which has been digested with micrococcal nuclease in absence and presence of antibiotic: Mg^{2+} complexes. Digestion of chromatin with micrococcal nuclease for 10 min in absence of antibiotic: Mg^{2+} complex degrades the chromatin predominantly to mono nucleosomal level (lane 2, Fig. 10). On the other hand, pretreatment with antibiotic: Mg^{2+}

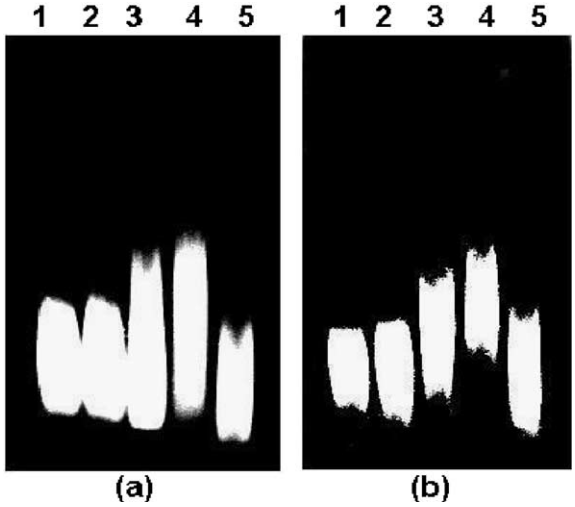

Figure 9. Agarose gel electrophoresis of native chromatin in presence and absence of MTR: Mg $^{2+}$ complex I (Fig. 9a) and CHR: Mg^{2+} complex I (Fig. 9b) under different conditions: Chromatin (200 μM) in 5 mM Tris–HCl buffer, pH 8.0 (lane 1), Chromatin (200 μM) in 5 mM Tris–HCl buffer, pH 8.0 and 230 μM Mg^{2+} (lane 2), Chromatin (200 μM) in 5 mM Tris–HCl buffer, pH 8.0 and MTR: Mg^{2+} complex I ([MTR] = 13.3 μM, [Mg^{2+}] = 230 μM) or CHR: Mg^{2+} complex ([CHR] = 13.3 μM, [Mg^{2+}] = 300 μM) (lane 3), Chromatin (200 μM) in 5 mM Tris–HCl buffer, pH 8.0 and MTR: Mg^{2+} complex ([MTR] = 28 μM, [Mg^{2+}] = 230 μM) or CHR: Mg^{2+}: complex ([CHR] = 28 μM, [Mg^{2+}] = 300 μM) (lane 4), Chromatin (200 μM) in 5 mM Tris HCl pH 8.0 and 60 mM NaCl (lane 5).

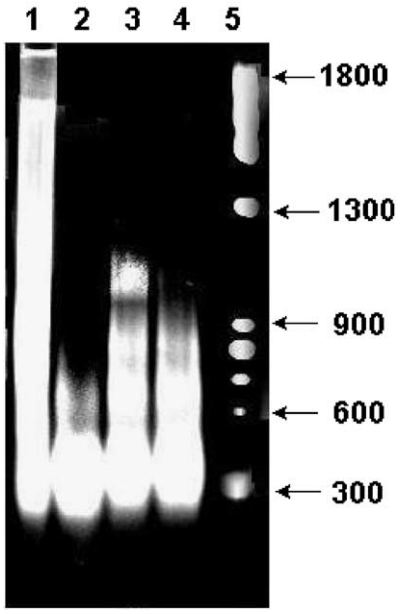

Figure 10. 1.5 percent agarose gel electrophoresis of chromatin subjected to micrococcal nuclease digestion for 10 min in presence and absence of antibiotic: Mg^{2+} complex I: undigested native chromatin (450 μM) in presence of $[Mg^{2+}]=230$ μM (lane 1), native chromatin (450 μM) in presence of $[Mg^{2+}]=230$ μM after digestion with micrococcal nuclease (lane 2), Chromatin (450 μM) bound to CHR: Mg^{2+} complex I ($[CHR]=40$ μM, $[Mg^{2+}]=230$ μM) after digestion with micrococcal nuclease (lane 3). Chromatin (450 μM) in presence of MTR: Mg^{2+} complex I ($[MTR]=40$ μM, $[Mg^{2+}]=230$ μM) after digestion with micrococcal nuclease (lane 4). DNA marker (lane 5). Numbers on the right indicate length of marker DNA in terms of bp.

complex(es) leads to the presence of higher oligomer bands of chromatin. Such protection against digestion by the nuclease is more pronounced in the case of chromomycin (lane 3), where we notice the presence of band corresponding to higher oligomer. These results establish that binding of antibiotic: Mg^{2+} complex in the linker region of chromatin imparts protection against digestion by micrococcal nuclease and the extent of protection varies with the nature of the antibiotic. CHR: Mg^{2+} complex imparts better protection.

Discussion

Since the first report of association of CHR with chromatin, there has been a radical progress in the knowledge about chromatin structure and its various levels of organization.[15] Studies of mithramycin with nucleosomes reconstituted from naked DNA fragments have suggested that this drug binds to GC rich regions in which minor groves face away from protein core.[32] Here we have presented an investigation of the association of the two aureolic acid group of antibiotics with their prime cellular target chromatin. The results obtained from the study lead to the following conclusions.

The absorption (Fig. 2a) and CD spectral studies (Fig. 4) have shown that there is a single type of complex formed between each and the three polymers, chromatin, core particle and naked DNA. Nature of the binding curve (Fig. 5) further corroborates this conclusion. None of the two ligands interacts with any histone isolated from native chromatin. Independent experiments using fluorescence property of the antibiotic have demonstrated the absence of such interaction (not shown). We did not notice any change in the composition of chromatin after treatment with the ligands. Neither did we detect any DNA release from the core particle. However, histone proteins reduce the binding potential and accessibility of the complexes to the minor groove of (G.C) rich regions of chromosomal DNA. This is apparent from a comparison of the dissociation constant, binding site size (Table 1) and associated thermodynamic parameters (Table 2) for the interaction of the two ligands with chromatin and naked DNA.

Similar comparison for the interaction of the two ligands with nucleosome core particle and chromatin leads to the conclusion that the ligands bind to core particle and linker DNA as well. Ligand-induced alteration in the melting profile of the chromatin also supports the suggestion that the antibiotic: Mg^{2+} complexes bind to both nucleosomal and linker DNA. The increase in the transition temperatures ($\Delta T1$ and $\Delta T3$ in Table 3) occurs as a sequel to antibiotic: Mg^{2+} complex(es) induced stabilization of both linker and nucleosomal DNA in chromatin. Inhibition to the nuclease digestion of the chromatin in presence of the ligands further supports the proposition that they bind to the linker region.

As reported earlier, DNA is the principal target for these antibiotics at the chromosomal level.[16] Thermal melting studies showing increase in T2 (Table 3) implies stabilization of histone-DNA contacts in presence of antibiotic: Mg^{2+} complex(es). Scattering assay supplemented by gel electrophoretic studies have clearly indicated that the association with antibiotic: Mg^{2+} complex(es) leads to an aggregation of the chromatin fiber.

From the above discussion of the results it follows that the association of the antibiotic: Mg^{2+} complex(es) with the chromatin has the following deleterious effects upon the ability of chromatin to act as transcription template. First, stablization of the nucleosomal, linker DNA and histone-DNA contacts would make the RNA polymerase induced opening of the duplex at the promoter site energetically costly during eukaryotic transcription. During nucleosome tracking, a key step in eukaryotic transcription, histone-DNA contacts in the nucleosome region of chromatin need to be ruptured. Increase in T2 (Table 3) upon addition of ligand(s) implies a stabilization of the histone-DNA contacts. So, these ligands could potentially block transcription not only by stabilizing the duplex but by inhibiting the nucleosome tracking as well. Second, results from micrococcal nuclease digestion studies imply these sequence selective DNA binding drugs could inhibit transcription factors from binding to their target sites in the linker DNA.[26] Third, de condensation of chromatin structure by chromatin remodeling apparatus is an important step in the gene activation. Induction of aggregation by these transcription inhibitors would disfavor such de condensation.

Present results are not sufficient to propose whether all or any one of the three deleterious effects causes their transcription inhibitory property in vivo.

Another important feature of the results follows from an analysis of the binding of the two ligands with the same polymer and its relative effects upon the chromatin structure. The results have clearly established that the binding potentials and associated thermodynamic parameters of the two ligands are different. In addition, stabilization of the chromatin structure occurs to different degree (compare $\Delta T1$, $\Delta T2$ and $\Delta T3$ values for both ligands in Table 3). Inhibition of the nuclease digestion is also dependent upon the nature of the ligands. Scattering and gel mobility assay have shown that the chromatin aggregation occurs to different degree in the presence of the two ligands. The trend is consistent with what has been reported earlier in the case of linear DNA from our laboratory.[9] The substituents in the sugar moieties (Fig. 1) are not same in the two antibiotics. We propose that these substituents are either involved in binding or they lead to a difference in the conformations of the two ligand(s), which recognize the chromatin and exert their effect upon the structure to different degrees. In a recent study using oligonucleotide of defined sequence we have shown that sugar residues play an important role in the binding process with DNA.[33] Our present results and these observations offer a plausible explanation in terms of the structure of the antibiotics for the unequal cytological effects of the two antibiotics. As a general trend, we notice that chromomycin: Mg^{2+} complex has relatively more deleterious effects upon the chromatin structure. A priori one might expect that this will affect relatively to a greater degree the function of chromatin to act as the transcription template. It might be a potential factor for its greater cytotoxic property.[1] Further studies are going on in our laboratory to establish this aspect.

Materials and Methods

Mithramycin, chromomycin, Tris, Magnesium chloride solution (4.9M), Calcium chloride solution(1M), proteinase K, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid, disodium salt (EDTA), triton-X-100 and micrococcal nuclease were from Sigma Chemical Company, USA. All buffers were prepared in quartz distilled deionized water from Milli-Q source, Millipore Corporation, USA.

Preparation of chromatin and nucleosome core particle

Male albino Sprague–Dowley rats weighing 150 ± 10 g, about 2–3 months old, were used throughout this work. Nuclei were isolated from the homogenized liver by the standard method,[18] given briefly as follows. Liver was homogenized in 10 mM Tris–HCl buffer of pH 7.4, containing 0.3 M sucrose, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5 percent triton X-100 and 40 mM sodium bisulfite. Homogenate was filtered through four layers of cheese cloth, followed by centrifugation at 800 *g* for 10 min. Pellet was suspended in TM buffer (50 mM Tris HCl buffer pH 7.4 plus 10 mM $MgCl_2$) containing 2.2M sucrose. Suspension was slightly homogenized and centrifuged for 1 h at 105,000 *g*. The pellet thus obtained was resuspended in TM buffer, containing 1M sucrose and centrifuged at 10,000 *g* for 10 min. The pellet obtained at this step contained nuclei. Outer membrane of the nuclei was removed by two successive washings with TM buffer containing 0.34 M sucrose and 1 percent Triton X-100.

Purified nuclei were digested with micrococcal nuclease to prepare the nucleosome core particle with DNA length of 147 base pairs.[19,20] 100 μL of nuclear pellet ($A_{260} = 228$) was digested with 25 units of micrococcal nuclease for 0.5 min. for the preparation of chromatin. For the preparation of nucleosome core particle 50 units of enzyme were used and the digestion time was increased up to 5 min. Reaction was terminated by 30 mM EDTA. Digested nuclei were incubated in 10 mM $NaHSO_3$, pH 7.5, 1 mM EDTA. for 30 min and centrifuged at 10,000 *g* for 10 min. Supernatant contained chromatin or mononucleosomes along with some longer chromatin fragments. Final purification of the nucleosome core particle was achieved by centrifugation in 5% to 20% (W/V) sucrose gradients. Purity of the nucleosome core particle was checked from identification of the histones in SDS–PAGE and acid urea gel after comparison with commercially obtained samples of histones.[21] Nucleosomal DNA was checked from agarose gel with 100 base pair DNA ladder as a standard marker. Concentration of nucleosome core particles was estimated in terms of DNA base. DNA was extracted from the nucleosome/chromatin by repeated phenol/chloroform extraction for quantitative estimation of base.[22]

Absorbance and fluorescence measurements

Absorption and fluorescence spectra were recorded with a Hitachi U-2000 spectrophotometer and a Shimadzu RF-540 spectrofluorometer, respectively. Concentrations of mithramycin and chromomycin were estimated from molar absorption coefficient of 10,000 (at 405 nm) and 8800 (at 400 nm) $M^{-1}cm^{-1}$, respectively[7,8] Fluorescence excitation wavelength was 470 nm in order to avoid photodegradation of the antibiotic.[7,8] During fluorescence, absorbance of the samples did not exceed 0.05. Therefore, we did not correct the emission intensity for inner filter effect. In the course of spectrophotometric titration, small aliquot of the polymer was added to the antibiotic–Mg^{2+} complexes and the equilibrium spectrum corresponding to each addition was noted at the time when there was no further change in the spectrum with time. During the titration, total dilution due to addition of the polymers was restricted to 5% of the initial volume and correction due to dilution was incorporated in the calculation of binding parameters.

CD spectroscopic study

CD Spectra of both complexes in presence of different polymers were recorded at room temperature in a cuvette

of 1-cm path length using a Jasco J-720 spectropolarimeter. All spectra are average of four runs. They were smoothed within the permissible limits by inbuilt software of the instrument.

Analysis of binding data

Results from fluoremetric titrations were analyzed by the following methods. Apparent dissociation constant ($K_d = 1/K_{app}$) was determined using non-linear curve fitting analysis [eq 1 and 2] based on the following equilibrium:

$$L + P \rightleftharpoons LP$$

L represents ligand and P represents polymer (chromatin/core particle/dehistonized DNA.

All experimental points for binding isotherms were fitted by least-square analysis.

$$K_d[C_o - (\Delta F/\Delta F_{max}).\ C_o].[C_p - (\Delta F/\Delta F_{max}).C_o]/ [(\Delta F/\Delta F_{max}).C_o] \quad (1)$$

$$C_o.(\Delta F/\Delta F_{max})^2 - (C_o + C_p + K_d).(\Delta F/\Delta F_{max}) + C_p = 0 \quad (2)$$

ΔF is the change in fluorescence emission intensity at 540 nm ($\lambda_{ex} = 470$ nm) for each point of titration curve, ΔF_{max} is the same parameter when the ligand is totally bound to polymer (chromatin/nucleosome core particle/dehistonized DNA), C_p is the concentration of the polymer (Chromatin/nucleosome core particle/dehistonized DNA) and C_o is the initial concentration of the antibiotic and K_d is the apparent dissociation constant. Double reciprocal plot was used for determination of ΔF_{max} using eq 3.

$$1/\Delta F = 1/\Delta F_{max} + K_d/[\Delta F_{max}(C_p - C_o)] \quad (3)$$

Details of the method are given in an earlier report.[16]

As described earlier,[8,16] binding site size was estimated from an intersection of two straight lines obtained from the least-square fit plot of normalized increase in fluorescence against the ratio of input concentrations (in terms of DNA base) of chromatin/core particle/dehistonized DNA and drug. It is expressed as number of DNA bases bound per ligand molecule.

Evaluation of thermodynamic parameters

Thermodynamic parameters, ΔH (van't Hoff enthalpy), ΔS (entropy) and ΔG (free energy) were determined using the following equations.

$$\ln\ K_{app} = -\Delta H/RT + \Delta S/R \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

Where R at T are the universal gas constant and absolute temperature, respectively. K_{app} ($1/K_d$) was determined at four different temperatures. ΔH and ΔS were determined from the slop and intercept of a plot of ln Kapp against 1/T. ΔG was determined from equation 5 after the incorporation of ΔH and δS values obtained from eq 4.

Thermal denaturation studies

Ultraviolet melting studies of the samples were carried out by using Hitachi U-3300 spectrophotometer, which is equipped with a programmable thermoelectric temperature controller. All samples used for this study were in 5 mM Hepes buffer pH 8.0 and pre incubated with the required concentration of Mg^{2+} for 20 min. Concentrations of chromatin and antibiotic were so chosen to ensure the saturation of chromatin by the ligand. After addition of antibiotic: Mg^{2+} complex, the samples were further incubated for 30 min to attain equilibrium. During the melting experiments the samples were heated at a rate of 1 °C per min, until the temperature reached 25 °C. Further heating was carried out slowly, at the rate of 0.5 °C per min while continuously monitoring the absorbance at 260 nm. Using appropriate control, we checked that the DNA binding potential of the antibiotic: Mg^{2+} complex remains unaltered after cooling from high temperature.

Scattering assay for ligand induced aggregation of chromatin

Antibiotic: Mg^{2+} complex induced chromatin aggregation was studied by light scattering experiment. 300 μL of chromatin (300 μM in terms of DNA base) in 10 mM Tris HCl pH 8.0 and 230 μM Mg^{2+} were incubated overnight with different concentrations MTR: Mg^{2+} complex at 4 °C. The control samples contain same concentration of Mg^{2+} as in MTR: Mg^{2+} with no drug. The experiment with CHR: Mg^{2+} was repeated in the same way as in MTR, except the concentration of Mg^{2+} was 300 μM. Aggregation of both antibiotic: Mg^{2+} complex treated and control samples was checked by monitoring the scattering at 520 nm.

Gel electrophoresis

0.7 percent w/v agarose gel electrophoresis was used for further examination of ultra structural changes of chromatin by antibiotic: Mg^{2+} complex of both antibiotics. In case of mithramycin, 200 μL of chromatin (300 μM in terms of DNA base) in 10 mM Tris HCl pH 8.0 and 230 μM Mg^{2+} were incubated with MTR:Mg^{2+} complex (24 μM or 48 μM) for 4 h. Control samples contain same concentration of Mg^{2+} as MTR: Mg^{2+} complex with no drug. For CHR: Mg^{2+} complex, the experiment was repeated in the same way except the concentration of Mg^{2+} was 300 μM. Both control and drug treated samples were fixed with 0.1 percent v/v glutaraldehyde overnight at 4 °C. An aliquot of the mixture was run in 0.7 percent agarose gel for 1 h at 45 volts. A chromatin sample in the same buffer containing 100 mM NaCl to induce chromatin condensation[23] was fixed in the same

way and run as a control. The condensed chromatin sample shows enhance electrophoretic mobility in accordance with the reported literature.[23]

Micrococcal nuclease digestion of chromatin

100 μL of chromatin in 10 mM Tris HCl, pH 8.0, (450 μM in terms of DNA base) were incubated with drug (45 μM): Mg^{2+} complex of both antibiotics for 1 h. Control samples contained same concentration of Mg^{2+} as in antibiotic: Mg^{2+} complex with no drug. Before digestion, both control and antibiotic: Mg^{2+} complex treated samples were incubated with 0.05 mM $CaCl_2$ at 37 °C for 2 min. Concentration of $CaCl_2$ was carefully chosen so that it is enough as necessary cofactor for the digestion but it is not too high for displacement of Mg^{2+} from antibiotic: Mg^{2+} complex. Both samples were digested with 2 μL (5 units/μL) of micrococcal nuclease for 10 min at 37 °C. Reaction was terminated by the addition of EDTA to a final concentration of 10 mM. Samples were immediately run in 1.5 percent w/v agarose gel at 70 volts for 3 h to check the difference in digestion pattern.

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References and Notes

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