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DEGRADATION OF HELA S_3 CELL CHROMATIN BY AUROMOMYCIN AND ITS CHROMOPHORE

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The antitumor protein agent auromomycin was found to degrade chromatin structure primarily by inducing strand scissions in linker regions. The reaction was stimulated by dithiothreitol. The chromophore form of the drug caused similar effects on chromatin, but it appeared to function at a more rapid rate. There was no evidence that auromomycin could cause breakage in core regions of chromatin.

INTRODUCTION

The antitumor agent auromomycin (also referred to as macromomycin-I) consists of a 667 molecular weight chromophore hydrophobically bound to a 12,000 dalton protein (1-3). Both the holoantibiotic and isolated chromophore possess similar activities in that either form of the drug can inhibit cell growth or DNA synthesis and damage cellular or cell free DNA (4,5). The inhibition of DNA synthesis and drug cytotoxicity appears to be a result of drug induced damage to DNA (5). Using cell free systems, it was found that DNA damage was single stranded in nature, occurred preferentially at guanine sites, and was stimulated by dithiothreitol (6,7). The effects of auromomycin on DNA are similar to other DNA strand breakers such as neocarzinostatin and bleomycin (8).

Recent data on the effects of strand scission drugs on chromatin structure have demonstrated that both bleomycin and neocarzinostatin can break down chromatin (9,10). Damage by necarzinostatin appears to occur at both core and linker regions, but bleomycin cleaves only linker DNA (9,10). Our study of the effects of auromomycin on isolated HeLa S_3 chromatin conclude that this

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drug can also damage chromatin. A similar interaction with chromatin occurs with both the holoantibiotic and the chromophore form of the drug.

MATERIALS AND METHODS

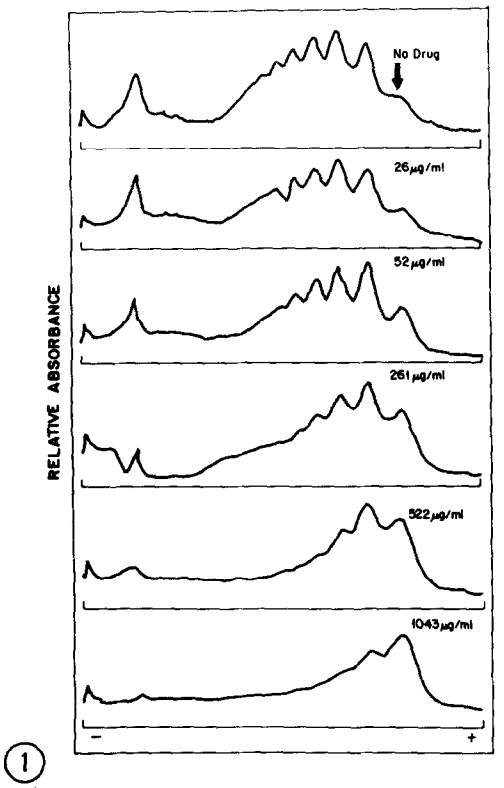
Maintenance of HeLa S_3 Culture and Chromatin Preparation. HeLa S_3 cells were grown in Spinner culture in Joklik's medium supplemented with 5% calf serum. Nuclei from these cells were isolated according to Fraser and Huberman (11). Chromatin was prepared by incubation of nuclei with micrococcal nuclease as described in (10). Growth inhibition studies were done using HeLa S_3 plate cultures grown in Minimum Essential Medium (Eagle) with 5% calf serum. Cell suspension (5ml) containing 200 cells was added to 60mm tissue culture dishes and treated with drug. After 7-9 days of growth the plates were fixed in methanol, stained with Giemsa, and scored for viable colonies.

Chemicals and Drugs. All tissue culture media and sera were obtained from Grand Island Biological. Ethidium Bromide came from Sigma. Neocarzinostatin (NSC #157365) and crude auromomycin (NSC #170105) were provided by the Developmental Therapeutics Program, National Cancer Institute. Auromomycin was purified as described in (3). The ability of drug to convert PM2-I (superhelical DNA) to PM2-II (open circular duplex DNA) was used as a standard measure of drug activity (12). The non-protein chromophore was extracted from lyopholized holoantibiotic as described previously (13). The concentration of chromophore was expressed in μg equivalent of native auromomycin per ml. All other chemicals were reagent grade.

Chromation Degradation and Gel Electrophoresis. Handling of auromomycin and all reactions were performed in semi or complete darkness. The standard assay for measuring chromatin degradation contained the appropriate drug, 10mM Tris (pH 7.8), 1.0μg chromatin and unless otherwise noted, 2mM dithiothreitol in a total volume of 25μl. After incubation at 37°C for various times, the reactions were stopped by heating samples for 15 minutes at 55°C in 0.1% sodium dodecyl sulfate. The deproteinized oligomeric DNA fragments were separated on native 1.5% agarose submersion gels. The running buffer was 50mM Tris pH 8.0/20mM NaAc/2mM EDTA. Samples containing 1μg DNA were loaded in 10% sucrose and 0.02% bromophenol blue as marker dye and electrophoresed at 30V for 20 hours. Identical reactions were run and the DNA fragments separated on high resolution 4% polyacrylamide (19:1 ratio of acrylamide to bisacrylamide) vertical slab gels (3mM thick). The running buffer was 36mM Tris-HCl/30mM NaH_2PO_4 (pH 7.8)/1mM EDTA/0.1% SDS. After electrophoresis at 30V for 14 hours, gels were soaked in 50% (V/V) methanol/water for a minimum of 3 hours (two changes) to remove SDS, then rinsed with water. After staining for 1 hour in 0.6μg/ml ethidium bromide, nucleosome bands were visualized under ultraviolet light and then photographed using a polaroid CU-5 camera. The negative was scanned with an Auto-Scanner FLUR-VIS (Helena Laboratories). The relative size of each oligomer peak was determined from the integrator output of the Auto-Scanner.

RESULTS

Incubation of auromomycin with isolated HeLa S_3 chromatin results in breakdown of the polynucleosome structure (Fig. 1). Detectable damage to chromatin is observed at a drug level of 26μg/ml as seen by a slight increase in the size of the mononucleosome band. At the highest drug level tested, 1043μg/ml, much of the higher molecular weight oligonucleosome structures have



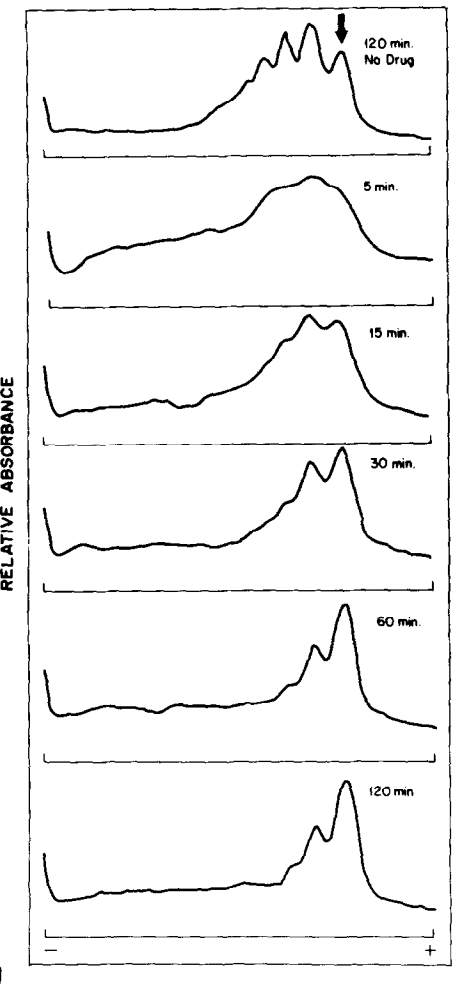


Fig. 1. Increased chromatin degradation with increasing auromomycin concentration. Chromatin was incubated with the indicated concentration of drug for 1 hour after which the DNA fragments generated were separated on agarose gels as described in Materials and Methods. The arrow on the control scan of this figure and subsequent ones indicates the position of the 165 base pair mononucleosome.

Fig. 2. Time course of chromatin degradation by auromomycin. Chromatin was incubated with 522μg/ml auromomycin for the times indicated on the graph and then analyzed by agarose gel electrophoresis.

been degraded, leaving predominantly dimer and monomer units. Material at the top of the gel represents residual high molecular chromatin that is not resolved by this gel system. The quantity varies with each chromatin prepara-

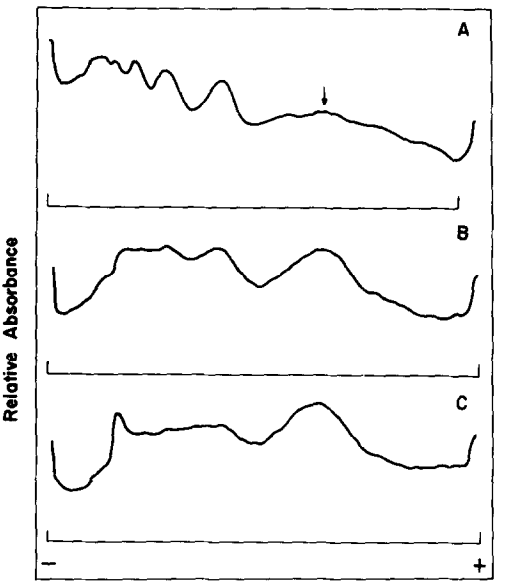


Fig. 3. Acrylamide gel analysis of chromatin degradation by auromomycin. Chromatin was incubated with zero (A), 261μg/ml drug (B), or 783μg/ml drug (C) for 1 hour.

tion, and its presence was found to not to interfere with the observed degradation patterns of the lower molecular weight chromatin.

A time course of auromomycin degradation of chromatin is shown in Fig. 2. During short incubations (5 min) there was a consistent smearing of nucleosomal bands which after longer treatment times showed a distinct progression of breakdown towards the mononucleosome form. If the increase in the mononucleosome band is followed, it is clear that degradation continues for up to one hour.

Since agarose gels have a limited ability to resolve any heterogeneity of nucleosome bands, auromomycin treated chromatin was also analyzed on acrylamide gels. The data in Fig. 3 represents breakdown of chromatin caused by low and moderate treatment with drug. The size of the mononucleosome bands at either drug level is 165 base pairs. Also the breadth of the monomer peak indicates a fair degree of heterogeneity when compared to chromatin that is further digested with micrococcal nuclease (data not shown).

Auromomycin is similar to another of the protein antitumor drugs, neocarzinostatin, in that it also contains a chromophore which expresses all of the drug's biological activities (14). Treatment of chromatin with isolated

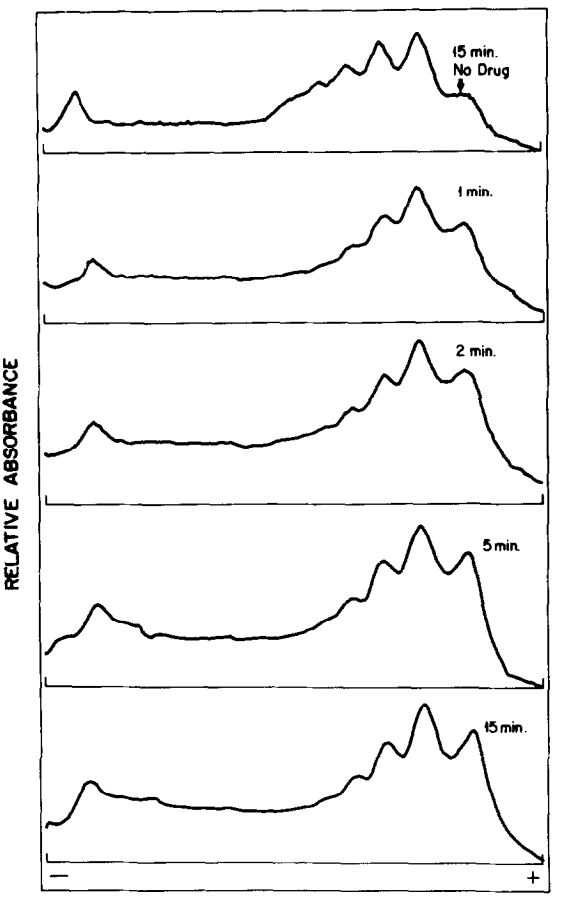


Fig. 4. Time course of chromatin degradation by chromophore of auromomycin. Chromophore extracted from the holoantibiotic was incubated with chromatin at a concentration of 783μg/ml for the times indicated on the graph and then analyzed by agarose gel electrophoresis.

chromophore resulted in degradation that gave a pattern similar to that seen with holoantibiotic (Fig. 4). A greater concentration of chromophore is needed to obtain chromatin breakdown comparable to that produced by the holoantibiotic. Comparisons of the cell free DNA strand scission activity of both drug forms showed that some chromophore activity was probably lost during its isolation. The time course indicates that chromophore cleaves chromatin very rapidly and that even with incubations as short as one minute there is an observable increase in the mononucleosome band. No further breakdown was seen beyond a 15 minute incubation (data not shown). Compared to holoantibiotic, the breakdown of chromatin is somewhat more rapid but also does not continue

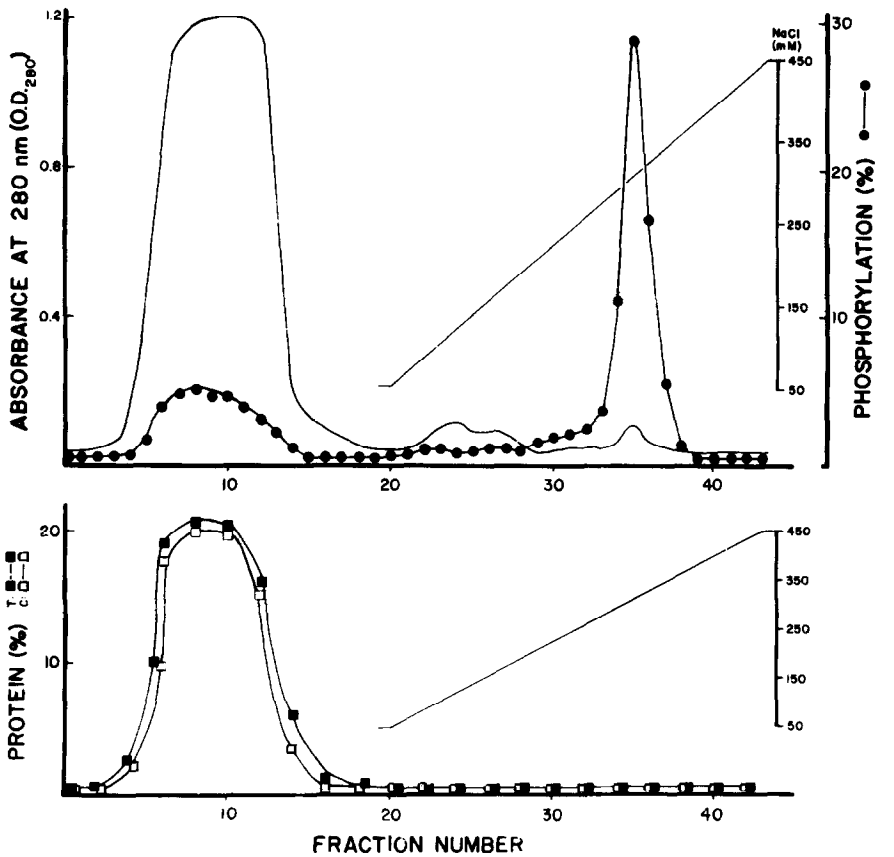


Figure 1. Elution of Ca^{2+}-calmodulin tubulin kinase activity (●-●), total protein (—), tubulin (■-■) and calmodulin (□-□) from cellulose phosphate column chromatography of brain cytosol. Brain cytosol from 10 rat brains was applied to the column and eluted with a NaCl gradient from 50 to 450 mM. Total protein was continuously monitored at 280 nM. Ca^{2+}-calmodulin tubulin kinase activity was assayed in each tube employing tubulin as the substrate (Methods). Calmodulin and tubulin levels were determined for each fraction by SDS-PAGE and quantitation of protein staining (Methods). Tubulin phosphorylation, calmodulin protein and tubulin protein levels are expressed as percent of total value for comparison.

(14.3%) and activity (43.6%) adhered to the calmodulin affinity column in the presence of Ca^{2+}. Washing the affinity column with 50 mM and 200 mM NaCl washes did not release significant bound tubulin kinase activity. However, elution with 1 mM EGTA and EDTA in 200 mM NaCl resulted in the quantitative recovery of the majority of bound tubulin kinase activity (Table 1). The relative specific activity of the calmodulin affinity tubulin kinase preparation was increased 26.7 times over the CP enzyme fraction. Some kinase activity passed through the column in the void, but this fraction had a low relative specific activity (Table 1) and was found to adhere to the affinity column upon

lower size nucleosomes culminating in greatly enhanced mononucleosome bands. From the data obtained in Fig. 3 it is clear that the 165 base pair mononucleosome generated by treatment with 261μg/ml of drug is not reduced to smaller sizes at higher drug concentrations (783μg/ml). This result is in contrast to micrococcal enzyme which produces similar size mononucleosomes (165 base pair), but when increased levels of enzyme are used, produces a 145 base pair monomer devoid of linker regions.

The damage to chromatin must be occurring preferentially at linker regions, as cutting in other additional regions would result in electrophoresis patterns with less defined nucleosome peaks. Earlier work with neocarzinostatin indicated that this drug could cleave both linker and core regions (10). Experiments with neocarzinostatin performed under conditions similar to those used for auromomycin does indicate that neocarzinostatin also has a preference for damaging linker regions, though there is also cutting in core areas (data not shown). Though we have not ruled out the possiblity that auromomycin can cleave core DNA, under the most severe conditions tested there is little indication of the appearance of subnucleosomal material or a broadening of the mononucleosomal band. This type of strong specificity for linker DNA is similar to that found with bleomycin (9).

An additional point of interest about the degradation of chromatin by drug is noted in a comparison of the breakdown of oligonucleosomes as determined by agarose (Fig. 1) and acrylamide gel electrophoresis (Fig. 3). The lack of resolution seen on agarose gels gives the appearance that all the degraded chromatin is eventually converted to mononucleosome bands. Examination of higher resolution acrylamide gels indicates that in addition to the increased mononucleosome band there is considerable smearing of the di, tri, and tetranucleosome. This is perhaps indicative of DNA strand breaks occurring throughout the linker region which would generate a distribution of oligomer peaks. Since acrylamide gels tend to spread out any size difference of the DNA, this filling in of some intranucleosomal spaces could result in the broad

banding patterns seen in Fig. 3. This data is quite similar to that obtained with neocarzinostatin (10).

It is generally accepted that there is a strong relationship between auromomycin induced damage to cellular DNA and cytotoxicity, but the exact mode of drug action is yet to be delineated. Our data on the cytotoxicity of purified auromomycin with HeLa S_3 cells, though it varies with each purification, generally gives ID_{50} levels of 10-20 pM. This compares to an ID_{50} of neocarzinostatin of 1-5mM. Yet, neocarzinostatin cleaves cell free DNA more strongly than auromomycin as measured by conversion of superhelical PM2 DNA to an open circular duplex form (neocarzinostatin is 2-3 fold more active than auromomycin). Possibly the very high cytotoxic effect of auromomycin is related to its ability to cleave cellular chromatin at particular sites. Currently we are examining how this drug damages nuclear chromatin.

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