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INTERACTION OF COVALENTLY CLOSED CIRCULAR PM-2 DNA AND HEDAMYCIN

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Summary: The interaction of hedamycin with covalently closed circular PM-2 DNA was examined. Hedamycin produced strand breakage detectable in alkaline sucrose gradients. Under neutral conditions hedamycin inhibited ethidium bromide binding and induced conformational changes in PM-2 DNA.

Introduction: Hedamycin (HDM)[1] was isolated in the primary fermentation broth of Streptomyces griseoruber (1). Tests showed inhibition of the transplanted mouse sarcoma 180 tumor and that hedamycin was inhibitory to adenocarcinoma of the duodenum in hamsters and Walker 256 intramuscular tumor of rats (2).

Biochemical effects of the interaction of hedamycin with DNA included an increase in DNA melting temperature, inhibition of enzymatic digestion of native DNA by snake venom phosphodiesterase, and decreased bouyant density of DNA in cesium chloride (CsCl) gradients (3). The binding between hedamycin and DNA was probably non-covalent since the drug could be extracted with benzene from an aqueous solution of the drug-DNA complex (3). Hedamycin was a potent inhibitor of RNA polymerase (4). A 3-5 fold greater concentration of antibiotic was required to produce comparable effects on DNA synthesis. Hedamycin was able to block RNA polymerization even after its initiation (4).

[1]Abbreviations: Hedamycin (HDM); Covalently Closed Circular PM-2 DNA (CCC-PM-2 DNA); Ethylene Diamine Tetra-acetic Acid (EDTA); Cesium Chloride (CsCl).

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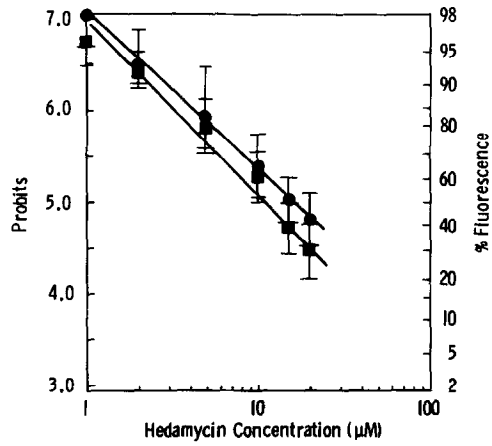

Figure 1. Probit analysis[2] of the fluorescence assay by HDM treatment. HDM was prepared at 1 mM stock solution of 50% dimethyl sulfoxide (DMSO). 25 μg of CCC PM-2 DNA was incubated with different concentrations of HDM for 30 minutes (-●-) and 60 minutes (- ■ -) and the fluorescence was recorded as described in "Materials and Methods". The final concentration of DMSO in the incubation mixture was from 0.1 to 1%. No effect on fluorescence due to DMSO was detected under these conditions.

Investigations presented here revealed hedamycin was a potent DNA binding and breakage agent. The drug caused both DNA degradation and inhibition of ethidium bromide binding to DNA when added to covalently closed circular (CCC) PM-2 DNA. The purpose of this investigation was to further characterize the properties of the interaction of HDM with DNA.

Materials and Methods: Covalently closed circular PM-2 DNA was isolated as previously described (6). The reaction of HDM with PM-2 DNA was performed, except as otherwise indicated, by adding drug to 25 μg PM-2 DNA in 0.5 ml of 50 mM Tris buffer plus 0.015 M NaCl at pH 9.5. The fluorescence assay for DNA breakage, and agarose gel electrophoretic assays were performed as previously described (6). Under the conditions employed, the order of anodal migration was: covalently closed circular DNA (form I), double-strand broken linear DNA (form III), and single strand broken open circular DNA (form II). Alkaline sucrose density gradient centrifugation was performed as previously described.

Results: A concentration dependent decrease in ethidium bromide fluorescence was observed following addition of HDM to PM-2 DNA in the fluorescence assay (fig. 1). Approximately 10 μM concentration

[2] The probit analysis tecnhique (15) is a mathematical transformation of the relative fluorescence decrease to allow more accurate estimation of the EC_{50} values.

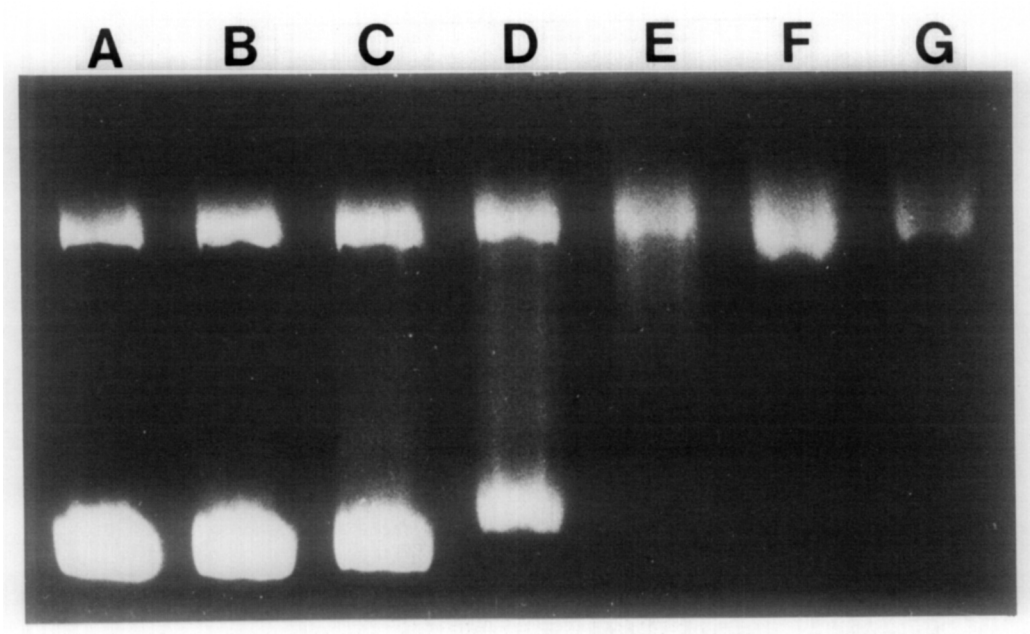

Figure 2. The effect of HDM on agarose gel electrophoretic pattern of PM-2 DNA. CCC PM-2 DNA was treated with HDM as described in figure 1 and electrophoretically separated on 1% agarose gel. The gel was stained in 0.5 μg/ml EB in electrophoresis buffer. From left to right: (A) control PM-2 DNA, (B) PM-2 DNA in the presence of 2.5% DMSO, (C) 2 μM, (D) 5 μM, (E) 10 μM, (F) 25 μM, (G) 50 μM HDM treatment of PM-2 DNA as indicated in "Materials and Methods".

of drug was required to produce a 50% decrease in fluorescence after 30 min. incubation. This decrease in fluorescence may have been caused by either DNA breakage, inhibition of ethidium bromide-DNA binding, or induction of alkali labile sites. Agarose gel electrophoresis of the products of HDM and DNA (fig. 2) incubation showed a concentration dependent decrease in the CCC PM-2 DNA (form I) observable following electrophoresis. However, the degradation products usually observed after incubation of CCC PM-2 DNA with nucleolytic agents (i.e., single strand broken relaxed circular and double strand broken linear duplex DNA) (6) were not detectable on agarose gels. Rather, reduced electrophoretic mobility and diffuse staining patterns were observed.

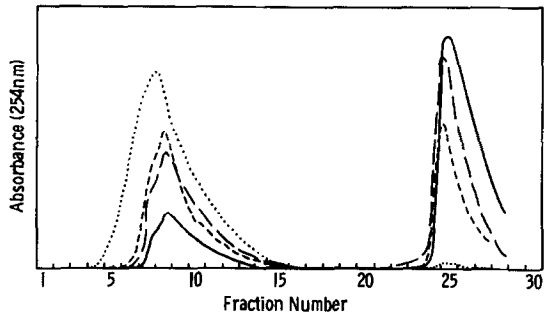

Figure 3. Alkaline sucrose gradient centrifugation analysis of HDM treated PM-2 DNA. After the PM-2 DNA was incubated with HDM for 1 hr, the incubation was centrifuged in 5-20% alkaline sucrose gradients. Sedimentation from left (top) to right (bottom). Control ——— ,5 μM HDM – – – –, 10 μM HDM -------, 50 μM ·····.

Alkaline sucrose density gradient centrifugation was employed to determine whether DNA breakage had occurred. Sedimentation of CCC PM-2 DNA on alkaline sucrose gradients following treatment with increasing amounts of HDM revealed a concentration dependent breakage of CCC PM-2 DNA (fig. 3). This breakage was manifested by a decrease in the amount of the 64S CCC PM-2 DNA and an increase in 18 and 20S single strand linear and circular forms (7), with increasing HDM concentrations. Thus, hedamycin produced CCC PM-2 DNA degradation detected by alkaline sucrose density gradient centrifugation.

The effect of HDM on ethidium bromide binding to CCC PM-2 DNA and the possibility that hedamycin preferentially inhibits ethidium bromide (EB) staining of form I PM-2 DNA were studied and shown in fig. 4. Approximately equal amounts of CCC PM-2 DNA (form I) and single strand broken circular PM-2 DNA (form II) were electrophoretically separated in cylindrical agarose gels. Following electrophoresis, the gels were incubated with hedamycin and then equilibriated with 0.5 μg/ml EB. The HDM treatment reduced ethidium bromide fluorescence in proportion to the HDM concentration. Furthermore, the fluorescence of ethidium bromide bound to form I or form II was inhibited similarly.

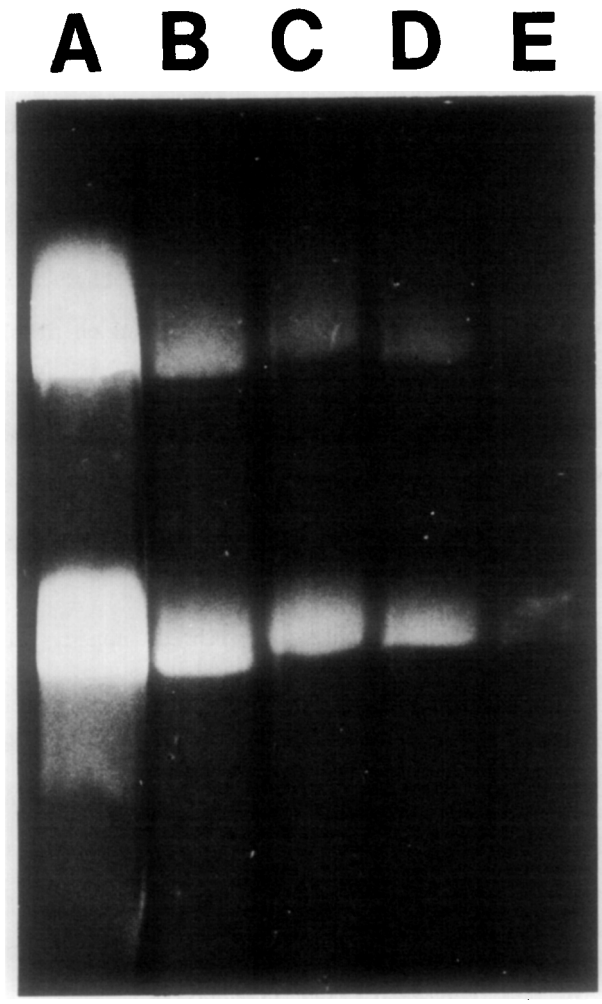

Figure 4. The inhibitory effect of HDM on PM-2 DNA staining by EB. Approximately equal amounts of form I and II DNA were separated in cylindrical agarose gels by electrophoresis. The tube gels were incubated in HDM solution for 3 hrs and then excessively stained with EB. (500 µg/ml, EB, 20 mM NaCl, 10 mM Tris buffer at pH 7.5) and equilibrated in 0.5 µg/ml EB staining solution. (A) control DNA without treatment with HDM, (B) 5 µM HDM treatment, (C) 10 µM HDM treatment, (D) 50 µM HDM treatment.

Discussion: An increasingly large number of antitumor drugs have now been identified that are capable, under appropriate conditions, of producing DNA breakage in vitro (6,8,9,10,11,12). HDM apparently may be added to this list based upon results of the alkaline sucrose sedimentation analysis of HDM treated CCC PM-2 DNA. However, the DNA

breakage observed under alkaline conditions may also have been caused by alkaline labile damage produced by HDM treatment. Alkali lability sites were observed in PM-2 DNA treated by other DNA breaking agents such as bleomycin and tallysomycin (11,6). The absence of normal DNA breakage products (increased relaxed circular and linear DNA) after electrophoresis of HDM treated PM-2 DNA, indicated that alkali lability may be an explanation for the DNA breakage observed on alkaline sucrose gradients. Furthermore, the diffuse ethidium bromide staining pattern observed after HDM treatment of PM-2 DNA, indicating possible conformational alterations in the superhelicity of PM-2 DNA, was similar to that identified with other agents that bind to DNA by intercalation (13,14).

Thus, the interaction of hedamycin with DNA appears to produce several effects: 1) inhibition of ethidium bromide binding to DNA; 2) DNA breakage and/or alkaline labile damage; and 3) possible conformational alterations in the superhelicity of CCC PM-2 DNA. That the apparent preferential loss of form I PM-2 DNA observed in fig. 2 is not due to preferential inhibition of staining of form I DNA is shown by fig. 4. Thus the preferential loss of form I DNA observed in fig. 2 must be due to preferential degradation and/or alteration in the mobility of form I DNA. HDM appears unique among the previously discovered DNA breaking antitumor agents in that the other DNA degradative agents have not been shown to inhibit ethidium bromide, whereas, HDM at low concentration caused rapid inhibition of EB binding to DNA (6,12).

HDM has been proposed to have multiple types of binding to DNA (3). Although the results of the present study did not differentiate different types of binding, the use of CCC PM-2 DNA may offer a sensitive and unique way to study the multiple types of interaction between HDM and DNA.

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