INHIBITION BY DIHYDRORIFAMPICIN OF RNA POLYMERASES I AND II ISOLATED FROM NUCLEI OF ROUS SARCOMA CELLS AND HUMAN TUMOR CELLS HEp-2

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Received August 24th, 1978

Dihydrorifampicin, a rifampicin derivative hydrogenated at the 18—19 carbon atoms of the aliphatic ansa chain of the rifampicin molecule, inhibits the enzymatic activity of RNA polymerases I and II, isolated from the nuclei of avian tumor cells (Rous sarcoma) and from the human tumor cell line HEp-2. The RNA polymerases from these tumors have been separated and partially purified by chromatography on DEAE Sephadex A-25 and characterized by the sensitivity to α-amanitin. The [^{3}H]UMP-labeled ribonucleic acids synthesized in the isolated nuclei of Rous sarcoma cells in the presence and absence of DHR were analyzed by sedimentation analysis in sucrose density gradients. It was found that the synthesis of rRNAs and mRNAs is very significantly inhibited by dihydrorifampicin, whereas the synthesis of tRNAs is much less inhibited at the same DHR concentration (100 μg/ml). The observed cytostatic effect of DHR on the growth of human tumor cells HEp-2 and embryonic cells in culture is apparently mediated by the selective inhibition of RNA polymerases I and II in human and avian cells. The relationship between the molecular structure of DHR and its affinity to RNA polymerases of eukaryotic cells is discussed.

Dihydrorifampicin (DHR), a new rifampicin derivative hydrogenated at the 18–19 carbon atoms of the aliphatic ansa chain of the rifampicin molecule, was synthesized by catalytic hydrogenation of rifampicin[1]. DHR has been shown to inhibit RNA synthesis (but not DNA synthesis) in eukaryotic cells and to exhibit a reversible cytostatic effect on the growth of human tumor cells HEp-2 and chick embryo fibroblasts in culture[2], at concentrations of 40 μg–100 μg DHR per ml.

The finding that dihydrorifampicin inhibits transcription in eukaryotic cells was surprising, because the parent substance, rifampicin, is known to be a potent inhibitor of bacterial RNA polymerases, but not mammalian RNA polymerases[3–6]. Several derivatives of rifamycin SV,

* *Abbreviations*: DHR, dihydrorifampicin; DMSO, dimethyl sulfoxide; RSV, Rous sarcoma virus; hnRNA, heterogeneous nuclear ribonucleic acid; TGMED buffer, solution containing 0·05M Tris-HCl, pH 7·9, 25% glycerol, 5 mM-$MgCl_2$, 0·1 mM-EDTA, and 0·5 mM dithiothreitol; PBS, isotonic NaCl solution containing 0·01M phosphate buffer, pH 7·4. Enzymes: DNA-dependent RNA polymerase (nucleoside triphosphate RNA nucleotidyl transferases, EC 2.7.7.6); pyruvate kinase (EC 2.7.1.40); hyaluronidase (EC 3·2·1·35).

especially AF/013 (O-n-octyloxime of 3-formyl rifamycin SV), are known as inhibitors of mammalian RNA polymerases[4,5,7–11], and mammalian DNA polymerases[12].

Multiple forms of DNA-dependent RNA polymerases have been found in eukaryotic cells[13,14,17,18]. Detailed characterization of eukaryotic RNA polymerases class I, II and III, including sensitivity of these enzymes to the inhibitory effect of α-amanitin[15,16] and their possible role in regulation of gene activity in eukaryotic cells, have been reviewed extensively[17,18].

In order to prove our suggestion that the molecular mechanism of the inhibitory activity of dihydrorifampicin on the growth and RNA synthesis of eukaryotic cells[2] involves the direct interaction of DHR with eukaryotic RNA polymerases, we have studied the inhibitory effect of DHR on the enzymatic activities of RNA polymerases I and II isolated and partially purified from the nuclei of avian tumor cells (Rous sarcoma) and from human tumor cells HEp-2. The results of these studies are presented in this communication.

EXPERIMENTAL

Chemicals: Dihydrorifampicin was synthesized by catalytic hydrogenation of rifampicin, purified by chromatography and characterized by nuclear magnetic resonance spectroscopy in the Isotope Laboratory, Czechoslovak Academy of Sciences, Prague, by Hanuš and coworkers[1] and kindly provided for biochemical studies in our laboratory. Ribonucleoside 5′-triphosphates (ATP, CTP, GTP, UTP), α-amanitin, phosphoenol pyruvate, pyruvate kinase, and dithiothreitol were obtained from Calbiochem, Lucern (Switzerland), the [5-^{3}H]uridine 5′-triphosphate (spec. activity 11 Ci/mmol) from The Radiochemical Center, Amersham (England) and DEAE Sephadex A-25 preparation from Pharmacia, Uppsala (Sweden). The other chemicals were commercial preparations of analytical purity. Lyophilized hyaluronidase preparation (Sevac, Prague) was used in experiments with Rous sarcoma tissue.

Cells: The human tumor cell line HEp-2 was kindly provided by Academician V. M. Zhdanov and Prof. F. I. Ershov, the D. I. Ivanovsky Institute of Virology, Academy of Medical Sciences of the U.S.S.R., Moscow. These tumor cells, originally isolated from a carcinoma of the larynx by Moore and coworkers[19] have been shown to produce a type D oncornavirus, immunologically similar to Mason–Pfizer monkey virus[20,21].

Isolation of Nuclei from Rous Sarcoma Tissue and Solubilization of RNA Polymerases

Rous sarcomas were induced in 6-week-old Brown Leghorn chickens by Rous sarcoma virus (strain Schmidt–Ruppin)[22]. Tumor tissue (70 g wet weight) was isolated from infected chicks 13 days after infection, minced with scissors and suspended in 200 ml of ice-cold 0·25M sucrose solution containing 2 mM-$MgCl_2$, 3 mM-$CaCl_2$, 10 mM Tris-HCl, pH 7·9, and 0·5 mM dithiothreitol. After addition of 1 mg of hyaluronidase to the suspension, the tumor tissue was homogenized at +3°C in a glass homogenizer with a motor-driven teflon pestle. The homogenate was centrifuged at low speed (750 rev./min) at 4°C for 10 min to pellet the unbroken cells, and the supernatant was centrifuged at 850*g* for 15 min at 4°C. The pelleted nuclei were resuspended in an ice-cold 0·34M buffered sucrose solution containing 0·05M-Tris-HCl, pH 7·5, 0·025M-KCl and 0·005M-$MgCl_2$, and further purified as described by Roeder and Rutter[14].

The suspension of nuclei was mixed gently with 2 volumes of 2·3M sucrose in the above mentioned buffer, underlaid with 2 ml of 2·3M sucrose in 25 ml centrifuge tubes (MSE-SW rotor) and centrifuged at 60000*g* and 4°C for 60 min. The pellet of purified nuclei was resuspended

in 10 ml of 0·05M Tris-HCl buffer, pH 7·9, containing 1M sucrose, 0·005M-$MgCl_2$ and 0·01M dithiothreitol. To the suspension of nuclei, 4M ammonium sulfate solution (adjusted to pH 7·9) was added to the final concentration of 0·3M and the nuclei were solubilized by sonication in an MSE Ultrasonic Disintegrator (100 Watt model) in an ice-cold tube at 5 . 10 s intervals during 2 min. The nuclear lysate was centrifuged at 70000*g* for 30 min at 4°C in an MSE 65 high-speed ultracentrifuge. The supernatant was brought to near-saturation with ammonium sulfate by the addition of 0·42 g of solid ammonium sulfate per ml of solution. The solution was cooled in an ice bath for 30 min, and the precipitate was collected by centrifugation for 1 h at 105000*g* at 4°C. The pellet of proteins was dissolved in 8 ml of a buffer containing 0·05M-Tris-HCl, pH 7·9, 25% glycerol, 5 mM-$MgCl_2$, 0·1 mM-EDTA, 0·5 mM dithiothreitol (TGMED buffer). The solution was dialyzed for 5 h at +2°C against the same buffer containing 0·05M ammonium sulfate The dialyzed solution was centrifuged at 110 000*g* and 4°C for 60 min. sealed in ampoules and stored in liquid nitrogen (Extract I).

Isolation of RNA Polymerases I and II from the Extract of Rous Sarcoma Cell Nuclei by Chromatography on DEAE Sephadex A-25

DEAE Sephadex A-25 was recycled and prepared in sulfate form according to the procedure described by Saunders and coworkers[23], and equilibrated in a column (2 × 16 cm) with TGMED buffer containing 0·05M ammonium sulfate. Soluble proteins extracted from nuclei of Rous sarcoma cells (extract I) were diluted with buffer TGMED containing 0·05M ammonium sulfate (592 mg proteins in 26 ml of TGMED buffer) and loaded on the DEAE Sephadex A-25 column. The chromatography on the DEAE Sephadex A-25 was performed in a refrigerator at +3°C.

The column was first washed with 40 ml TGMED buffer containing 0·05M-$(NH_4)_2SO_4$ and 2 ml fractions were collected at a flow rate of 2 ml per 3·5 min. Then the DEAE Sephadex column was eluted with a linear gradient of 0·1M-0·4M ammonium sulfate in TGMED buffer (using 70 ml of 0·1M-$(NH_4)_2SO_4$ and 70 ml 0·4M-$(NH_4)_2SO_4$ in TGMED buffer and a gradient mixer GM-1, Pharmacia, Uppsala). Bovine serum albumin (0·1 ml of 1% sterile solution) was then added each to 2 ml fraction, and the fractions were stored at —70°C. Aliquots (50 μl) from each fraction were used the next day for the determination of RNA polymerase activity.

RNA Polymerase Assay

The assay was performed essentially as described by Roeder and Rutter[13]. The components present in the reaction mixture were as follows: 30 μg denatured (100°C) calf thymus DNA, 2·5 μg pyruvate kinase (Calbiochem), 10 μmol Tris-HCl (pH 7·9), 0·2 μmol $MnCl_2$' 0·5 μmol phosphoenol pyruvate, 1·0 μmol KCl, 0·75 μmol NaF, 0·2 μmol dithiothreitol, 0·08 μmol each of ATP, GTP, CTP, 1 μCi [^{3}H]UTP (spec. activity 11 Ci/mmol), 5—10 μmol ammonium sulfate (as given for each experiment) and 0·05 ml of enzyme solution in a final volume of 0·25 ml. After incubation at 37°C for 10 min, the reaction mixture was cooled in ice and stopped by adding 3 ml of 10% trichloroacetic acid containing 0·04M sodium pyrophosphate. Bovine serum albumin (0·1 mg in 0·1 ml) was added to each sample and after standing for 2 h in ice, the precipitate was collected on membrane filters (Synpor, Czechoslovakia, pore diameter 0·23 μ) and washed eight times with 5% trichloroacetic acid. The filters were then dried and counted in a toluene solution of PPO and POPOP[24] in the Nuclear Chicago liquid scintillation spectrometer. The RNA polymerase activity was calculated in picomoles [^{3}H]UMP incorporated into RNA per 10 min.

Isolation of RNA Polymerases I and II from HEp-2 Cells and Purification by Chromatography on DEAE Sephadex A-25

Human tumor cells were grown in Eagle's minimal essential medium containing 10% inactivated calf serum and antibiotics in Falcon plastic dishes incubated at 37°C in a CO_2 thermostat as described earlier[2]. The medium was changed every two days, and the cells were harvested from 80 Falcon plastic dishes (60 mm diam.), 8 days after plating. After aspirating the medium from the cultures with a Pasteur pipette, the dishes were cooled in ice, and the cells were washed with an ice-cold sterile PBS solution[25] (isotonic NaCl solution containing 0·01M phosphate buffer pH 7·4). The cells were scraped with a sterile plastic spatula from the bottom of the dish, suspended in 0·5 ml of ice-cold PBS solution, and the pooled suspensions were centrifuged at low speed (750 rev. per min) for 10 min at +4°C. The supernatant was discarded and the pelleted HEp-2 cells (5 g wet weight, approximately 1 . 10^9 cells) were suspended in 10 ml TGMED buffer at 0°C, frozen at —70°C in sealed ampoules and stored in liquid nitrogen to the next day.

Extraction and chromatographic separation of RNA polymerases from HEp-2 cells was performed using the procedure descripeb by Hossenlopp, Wells and Chambon[26]. The suspension of frozen HEp-2 cells was thawed at +2°C, diluted with TGMED buffer to 30 ml, and 6·75 ml of saturated ammonium sulfate solution were added. The suspension was cooled in an ice-bath and gently mixed for 10 min. The cells were then homogenized by short sonication (6 . 10 s) in an MSE Ultrasonic Disintegrator in a tube cooled with ice and NaCl mixture. After sonication the viscosity of the solution decreased significantly. Powdered ammonium sulfate was then added to the homogenate (0·165 g per 1 ml) to 45% saturation. After 60 min cooling in an ice bath, the precipitated proteins were pelleted by centrifugation at 100000*g* and 4°C for 90 min in an MSE 65 ultracentrifuge. The pellet of proteins was suspended in 16 ml of ice-cold TGMED buffer and homogenized in a glass homogenizer at 0°C. To this solution 0·8 ml of 1% protamine in dist. H_2O was added, and after standing for 30 min in ice bath, the precipitate was removed by centrifugation at 90000*g* and 4°C for 30 min. The supernatant was stored in sealed ampoules for 3 days in liquid nitrogen (preparation PS).

DEAE Sephadex A-25 in sulfate form was prepared as described by Saunders and coworkers[23], and equilibrated in a column (2 × 16 cm) with TGMED buffer containing 0·05M ammonium sulfate (in a refrigerator at +3°C). Proteins extracted from HEp-2 cells (preparation PS) were diluted (at 0°C) with TGMED buffer to a final concentration of 1 mg protein per ml and 0·04M ammonium sulfate. Forty-five milliliters of this solution were loaded on the DEAE Sephadex A-25 column. All further operations were performed at +3°C.

The column was then washed with 20 ml of TGMED buffer containing 0·04M ammonium sulfate, 30 ml of 0·1 of 0·1M ammonium sulfate in TGMED, and then the proteins were eluted with a linear gradient of 0·1M—0·4M ammonium sulfate in TGMED buffer (total volume 120 ml). Two-milliliter fractions were collected at a flow rate of 2 ml/4 min. After addition of 0·1 ml of 1% bovine serum albumin solution to each fraction, the fractions were immediately frozen and stored at —70°C. The RNA polymerase activity in the eluted fractions was determined the next day using 50 µl aliquots from each fraction for enzyme assay. The selected fractions were then stored in liquid nitrogen[27].

Transcription in the Isolated Nuclei of Rous Sarcoma Cells and Analysis of the Ribonucleic Acids Synthesized in the Presence and Absence of Dihydrorifampicin

Isolation and partial purification of nuclei from Rous sarcoma tissue was performed by the method described by Marzluff and coworkers[28] and Udvardy and Seifart[29], with some modifications.

Forty grams of Rous sarcoma tissue, freshly isolated from Brown Leghorn chickens 13 days after infection with Rous virus[24], were homogenized in an ice-cold Potter–Elvehjem homogenizer

with a teflon pestle in 40 ml of solution containing 0·3M sucrose, 2 mM-Mg-acetate, 3 mM-$CaCl_2$, 10 mM Tris-HCl pH 7·9, 0·5 mM dithiothreitol, and 1 mg of hyaluronidase. The homogenate was centrifuged at 750 rev./min. and 4°C for 10 min. The supernatant containing nuclei and cytoplasmic components was mixed with 1 volume of sterile 2M sucrose solution containing 5 mM Mg-acetate, 10 mM Tris-HCl (pH 7·9) and 0·5 mM dithiothreitol. The mixture was layered over a 2 ml pad of the 2M sucrose buffer and centrifuged at 60000*g* in the Spinco SW 50·1 rotor at 4°C for 45 min.

The nuclear pellet was gently resuspended with a Pasteur pipette in 25% glycerol containing 5 mM Mg-acetate, 50 mM Tris-HCl (pH 7·9), 5 mM dithiothreitol and 0·1 mM-EDTA (to 4·5 mg nuclear DNA per ml). The isolated nuclei were used immediately for RNA biosynthesis experiments in the presence and absence of dihydrorifampicin.

The nuclear suspension (200 μl containing 1·8 mg of DNA) was mixed at 0°C with 800 μl of the incubation mixture containing 250 mM ammonium sulfate, 0·1M-KCl, 0·05 Tris-HCl (pH 7·9), 5 mM Mg-acetate, 2 mM-$MnCl_2$, 1 mM dithiothreitol, 4 mM ATP, GTP, CTP, 8 μCi [^{3}H]UTP (spec. activity 11 Ci/mmol), 12% glycerol, and 50 μl of DMSO or 50 μl of the solution of 200 μg DHR in DMSO. The mixture was incubated at 37°C for 30 min. The RNA synthesis was terminated by cooling the tube in an ice-bath, and 4 ml of ice-cold 0·25M sucrose were added. The nuclei were pelleted immediately by centrifugation at 850 g and 3°C for 10 min, the nuclear pellet was resuspended in 0·8 ml of 0·01M acetate buffer pH 5·2 and mixed immediately with 1 ml of distilled phenol saturated with 0·01M acetate buffer pH 5·2 and containing 0·1% 8-oxyquinoline. The mixture was heated 4 min at 65°C and shaken vigorously for 15 min. The aqueous phase was separated by centrifugation and extracted twice with 1 ml phenol, and twice with 1 ml ethylether. The ether was removed from the aqueous solution with a stream of nitrogen and the [^{3}H]UMP-labeled ribonucleic acids in the aqueous phase were analysed by ultracentrifugation in a linear sucrose density gradient.

The samples of [^{3}H]UMP-labeled ribonucleic acids (0·5 ml) were layered on a 4·5 ml volume of linear sucrose gradient (20%—5%) in a buffer containing 0·01M Tris-HCl (pH 7·5), 0·1M-NaCl, and 0·01M-DETA (gradient prepared in 5 ml nitrocellulose tubes), and centrifuged in an SW 39 rotor (Beckmann Spinco L50 ultracentrifuge) at 70000*g* and 4°C for 18 h. Standards of 28S rRNA, 18S rRNA labeled with [^{3}H]uridine (RNAs extracted with phenol from chick embryo fibroblasts labeled in cultur with [5-^{3}H]uridine for 4 h) were centrifuged simultaneously in the third cuvette of the SW-39 rotor. After centrifugation, 6-drop fractions (0·18 ml) were collected from the bottom of the tube, each fraction was precipitated with 3 ml of 10% trichloroacetic acid containing 0·04M pyrophosphate, filtered on the membrane filters (Synpor, pore diameter 0·23 μ) and washed eight times with 5% trichloroacetic acid (ice-cold). The dried filtérs were counted in scintillation solution as described earlier[24].

Other methods: Methods described by Lowry and coworkers[30] and Dische[31] were used for the determination of proteins and DNA respectively.

RESULTS

Inhibitory Effect of Dihydrorifampicin on Enzymatic Activity of RNA Polymerases Isolated from Nuclei of Rous Sarcoma Cells

Dihydrorifampicin inhibits selectively RNA synthesis but not DNA synthesis in avian and mammalian cells growing in tissue cultures[2]. In order to obtain direct experimental evidence that DHR inhibits the polymerization reaction catalyzed by RNA

polymerases from eukaryotic cells, we have studied the effect of DHR on RNA polymerases isolated from cell nuclei of an avian tumor, the Rous sarcoma.

The nuclei were isolated from freshly prepared Rous sarcoma tissue and extract I was prepared as described in Methods. Table I shows that dihydrorifampicin (40 μg or 80 μg per assay) inhibits the enzymatic activity of nuclear RNA polymerases I, II and III contained in the preparation by 55% and 86% respectively.

In a second experiment, the kinetics of enzymatic RNA synthesis *in vitro* catalyzed by RNA polymerases present in extract I from nuclei of Rous sarcoma cells was studied. As shown in Fig. 1, the rate of enzymatic RNA synthesis was linear during the first 10 min and then decreased slightly (Fig. 1, curve *1*). In the presence of dihydrorifampicin added simultaneously with the enzyme to the assay mixture, the rate of RNA synthesis was significantly lowered (Fig. 1, curves 2 and 3).

When dihydrorifampicin (50 μg per assay) was added to the enzymatic assay 5 min after initiation of RNA synthesis, the RNA synthesis was stopped very rapidly (Fig. 1, curve *4*), and the inhibition of enzymatic activity was very similar to the experiment, where DHR (50 μg per assay) was added before initiation of RNA synthesis (Fig. 1, curve 2). This experiment indicates that RNA polymerases from nuclei of Rous sarcoma cells are sensitive to the inhibitory effect of DHR even after formation of the complex between enzyme and DNA template. The inhibitory effect of DHR on RNA biosynthesis may therefore involve not only inhibition of initiation but also inhibition of elongation of RNA chains.

TABLE I

Inhibition by Dihydrorifampicin of RNA Polymerases Isolated from Nuclei of Rous Sarcoma Cells

Extract I prepared from nuclei of Rous sarcoma cells (see Methods) and containing RNA polymerases I, II and III was used in this experiment. The composition of the assay mixture is described in Methods. Dihydrorifampicin (DHR) was added in 10 μl of solution in DMSO to the assay mixture at 0°C before initiation of enzymatic reaction. RNA synthesis was started by addition of the enzyme (170 μg of protein per assay).

DHR μg/assay	[^{3}H]UMP incorporated in 10 min cpm	Inhibition of RNA polymerase activity %
0	770	0
40	348	55
80	109	86

Separation and Partial Purification of RNA Polymerases I and II Isolated from Nuclei of Rous Sarcoma Cells by Chromatography on DEAE Sephadex A-25

RNA polymerases present in extract I from the nuclei of Rous sarcoma cells were separated and partially purified by chromatography on a DEAE Sephadex A-25 column as described in Methods. As shown in Fig. 2, at least 5 distinct fractions with RNA polymerase activity were eluted from the column.

The isolated RNA polymerases I_A, I_B, II_A, II_B, and III (Fig. 2) were characterized by their sensitivity to α-amanitin. As shown in Table II, only RNA polymerases

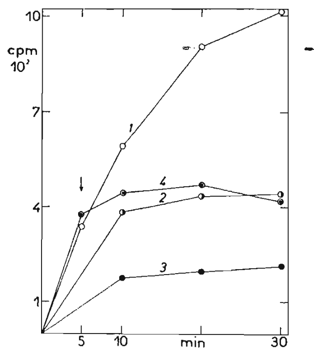

FIG. 1

Kinetics of RNA synthesis *in vitro* by RNA Polymerases Isolated from Nuclei of Rous Sarcoma Cells and Inhibition of the Enzymatic Activity by Dihydrorifampicin

Source of enzyme: Extract I, 170 µg protein per assay. *1* RNA synthesis without inhibitor; *2*, *3* RNA synthesis in the presence of 50 µg resp. 100 µg DHR per assay; *4* RNA synthesis when DHR (50 µg per assay) was added 5 minutes after initiation of the enzymatic reaction (indicated by arrow). The components of the reaction mixture and conditions of the RNA polymerase assay as described in Methods. Results are expressed in c.p.m. of [^{3}H]-UMP incorporated into RNA.

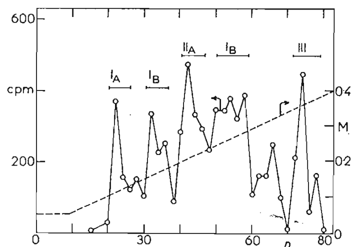

FIG. 2

DEAE Sephadex Chromatography of RNA Polymerases Extracted from Nuclei of Rous Sarcoma Cells

Extract I from nuclei of Rous sarcoma cells (592 mg protein in 6 ml of extract I) was chromatographed on a column of DEAE Sephadex A-25 (16 × 2 cm). Flow rate 2 ml/3·5 min (one fraction). Aliquots of each fraction were assayed as described in Methods. Broken line: ammonium sulfate concentration (M), full line: enzyme activity (in c.p.m. of [^{3}H]-UPM, incorporated into RNA). *n* fraction number.

II_A and II_B are highly sensitive to α-amanitin and are inhibited by 92% and 74% respectively in the presence of 1 μg of α-amanitin per assay. Under the same conditions RNA polymerases I_A and I_B are fully resistant to α-amanitin, thus demonstrating this characteristic property of RNA polymerase form I. RNA polymerase III was only very slightly inhibited in the presence of 1 μg α-amanitin per assay.

Chromatographic properties of the isolated RNA polymerases as well as the characteristic sensitivities of these enzymes to α-amanitin, allow us to characterize the enzymes I_A and I_B as RNA polymerases form I (A) and the enzymes II_A and II_B as RNA polymerases form II (B) (ref.[17,18]).

Dose-Dependent Inhibition of RNA *Polymerases* I *and* II *from Nuclei of Rous Sarcoma Cells by Dihydrorifampicin*

RNA polymerases I_B and II_A isolated from nuclei of Rous sarcoma cells and partially purified by chromatography on DEAE Sephadex A-25 column (Fig. 2) were assayed in the presence of increasing concentrations of dihydrorifampicin in order to test the sensitivity of these enzymes to this inhibitor. The results are summarized in Table III. Both enzymes are inhibited by dihydrorifampicin to a similar extent. Significant inhibition of RNA polymerases I_B and II_A was observed at a concentration as low as 20 μg DHR per assay (30% – 39% decrease of enzymatic activity). Practically absolute inhibition of both enzymes was achieved at a concentration of 160 μg DHR per assay (Table III).

TABLE II

Characterization of RNA Polymerases Isolated from Nuclei of Rous Sarcoma Cells and Separated by Chromatography on DEAE Sephadex A-25 by their Sensitivity to α-Amanitin

RNA polymerase activity was determined by standard assay (see Methods) in the presence of α-amanitin (1 μg per assay). 50 μl aliquots of pooled enzyme fractions were added to the assay mixture and incubated at 37°C for 10 min. Each sample was assayed in duplicate.

RNA polymerase preparation	RNA polymerase activity, pmol [^{3}H]UMP incorp./10 min		Inhibition %
	without α-amanitin	in presence of α-amanitin	
I_A	16·1	15·7	0
I_B	16·3	15·9	0
II_A	34·2	2·8	92
II_B	23·8	6·2	74
III	23·0	20·6	11

Partial Purification of RNA *Polymerases* I *and* II *from* HEp-2 *Cells by Chromatography on* DEAE *Sephadex* A-25 *Column and Inibition of These Enzymes by Dihydrorifampicin*

The method described by Hossenlopp, Wells and Chambon[26] for isolation and chromatographic separation of RNA polymerases from HeLa cells was used for isolation and partial purification of RNA polymerases I and II from human tumor cells HEp-2 as described in Methods. The proteins extracted from 5 g freshly isolated HEp-2 cells (approximately $1 . 10^9$ cells) and fractionated with ammonium sulfate and protamine treatment (preparation PS) were separated by chromatography on DEAE Sephadex A-25 column (2 × 16 cm) as described in Methods.

RNA polymerases I_A, I_B, II and III (Fig. 3) eluted from the DEAE Sephadex A-25 column by linear 0·1M–0·4M ammonium sulfate gradient in TGMED buffer, were characterized by the sensitivity to α-amanitin, RNA polymerases I_A and I_B being entirely resistant to 1 μg of α-amanitin per assay. The same concentration of α-amanitin inhibited enzymatic activity of RNA polymerase II (fraction 52, Fig. 3) by more than 96%.

TABLE III

Inhibition by Dihydrorifampicin of Enzymatic Activity of RNA Polymerases I and II Isolated from Nuclei of Rous Sarcoma Cells and Purified Partially by Chromatography on DEAE Sephadex A-25

RNA polymerase preparations I_B and II_A (Table II) were assayed in this experiment. Components of the enzymatic assay mixture are described in Methods. Dihydrorifampicin (dissolved in DMSO) was added in the indicated amounts to the assay mixture at 0°C and RNA synthesis was started by addition of 50 μl of enzyme solution and incubation at 37°C. Enzymatic activity is expressed in picomoles of [^{3}H]UMP incorporated into RNA per 10 min at 37°C.

Dihydro-rifampicin	RNA polymerase I_B activity	Inhibition of RNA polymerase I_B by DHR	RNA polymerase II_A activity	Inhibition of RNA polymerase II_A by DHR
μg/assay	pmol [^{3}H]UMP incorp./10 min	%	pmol [^{3}H]UMP incorp./10 min	%
0	14·7	0	30·2	0
10	14·2	3	25·7	15
20	10·1	31	18·5	39
40	7·5	48·6	7·3	76
80	4·1	72·3	4·7	84
160	0·5	96	1·9	94

The inhibitory effect of dihydrorifampicin on the enzymatic activities of RNA polymerases I_A and II (fractions No 22 and 52 from DEAE Sephadex A-25 chromatography, Fig. 3) was studied. DHR dissolved in DMSO was added simultaneously with the enzyme to the assay mixture before initiation of RNA synthesis (at 0°C)

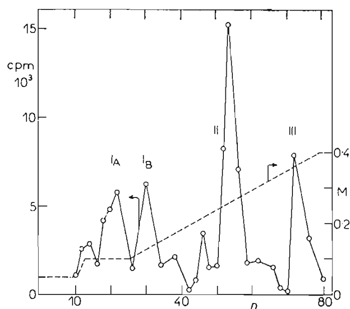

FIG. 3

DEAE Sephadex A-25 Chromatography of RNA Polymerase from Human Tumor Cells HEp-2

The RNA polymerases present in 1×10^9 cells were solubilized as described in Methods PS fraction, 45 mg protein in 45 ml, was subjected to chromatography on a 16 × × 2 cm column of DEAE Sephadex A-25 equilibrated with buffer TGMED containing 0·04M ammonium sulfate. After loading the protein, the column was washed as described in Methods and developed (at +3°C) using an 0·1M—0·4M linear ammonium sulfate gradient in TGMED buffer. Flow rate 2 ml/4 min (one fraction). To each fraction 1 mg bovine serum albumin was added and 50 µl aliquots of each fraction were assayed as described in Methods. Broken line ammonium sulfate concentration (M). Results are expressed in c.p.m. of [^{3}H]-UMP incorporated into RNA. *n* fraction number.

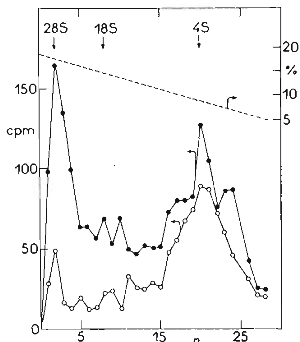

FIG. 4

Sedimentation Analysis in Linear Sucrose Gradients of [^{3}H]-UMP-labeled RNAs Synthesized in Isolated Nuclei of Rous Sarcoma Cells, and Inhibitory Effect of DHR on the Synthesis of High Molecular Weight RNA Speceis

Isolated nuclei from Rous sarcoma cells were labeled with [^{3}H]-UTP for 30 min as described in Methods, in the presence or absence of DHR. The [^{3}H]-UMP-labeled RNAs were extracted with phenol and analyzed in linear 5%—20% sucrose gradient at 70000*g* and 4°C for 18 h. Full circles: [^{3}H]RNA synthesized in the absence of DHR; open circles: [^{3}H]RNA synthesized in the presence of DHR (200 µg per of assay mixture). The positions of standards (28S rRNA, 18S rRNA, and 4S tRNA), centrifuged simultaneously, are indicated by arrows. Broken line sucrose concentration (%), *n* fraction number. Results are expressed in c.p.m. of [^{3}H]-UMP incorporated into RNA.

Table IV shows the dose-dependent inhibitory effect of DHR on RNA polymerases I_A and II from human tumour cells HEp-2. Both enzymes are inhibited by DHR, the degree of inhibition being apparently dependent on the ratio of the enzyme concentration to the concentration of the inhibitor. The human RNA polymerases I and II appear to be similarly sensitive to the inhibitory effect of DHR as corresponding RNA polymerases I and II from avian tumor cells.

The reversible cytostatic effect of dihydrorifampicin on the growth of avian and human cells in tissue culture[2] is therefore mediated by inhibition of RNA polymerases I and II in the DHR-treated cells, *i.e.* by inhibition of the synthesis of the high molecular weight precursors of ribosomal RNAs and messenger RNAs. This conclusion was also supported by the following experiment.

Selective Inhibition by Dihydrorifampicin of the Biosynthesis of High Molecular Weight RNA *Species in Isolated Nuclei from Rous Sarcoma Cells*

The cell nuclei freshly prepared from Rous sarcoma tissue and purified by the method described by Marzluff and coworkers[28]. were immediately incubated as described in Methods, using 1·8 mg nuclear DNA per assay. The same amount of nuclear suspension was incubated simultaneously in the presence of dihydrorifampicin

TABLE IV

Inhibitory Effect of Dihydrorifampicin on Enzymatic Activity of RNA Polymerases I and II Isolated from Human Tumor Cells HEp-2 and Purified Partially by Chromatography on DEAE Sephadex A-25 Column

RNA polymerases I_A and II (fractions number 22 and 52 from DEAE Sephadex A-25 chromatography, Fig. 3) were used in this experiment. Same RNA polymerase assay conditions as described in Methods and Table III.

Dihydro-rifampicin	RNA polymerase I_A activity	Inhibition of RNA polymerase I_A activity by DHR	RNA polymerase II activity	Inhibition of RNA polymerase II activity by DHR
μg/assay	pmol [^{3}H]UMP incorp./10 min	%	pmol [^{3}H]UMP incorp./10 min	%
0	35·2	0	79·2	0
20	—	—	73·1	6·7
40	12·5	66	43·2	45
80	3·7	90	16·8	80
160	0	100	5·7	93

(200 μg DHR per ml of assay mixture). After incubation, the [^{23}H]UMP-labeled RNAs were extracted with phenol and analyzed by ultracentrifugation in a linear sucrose density gradient as described in Methods.

Fig. 4 shows that the synthesis of the high molecular weight RNA species in isolated nuclei (very probably 45S ribosomal RNA precursor and hn RNA) is very significantly inhibited by dihydrorifampicin. On the other hand, the synthesis of 4S tRNAs was much less inhibited. The results indicate that the enzymatic activities of RNA polymerases I and II (synthesizing rRNAs and mRNAs respectively) are mainly inhibited by dihydrorifampicin in the isolated nuclei, whereas RNA polymerase III (participating in the tRNA biosynthesis) seems to be less sensitive to the inhibitory effect of DHR. Further experiments are required to analyze in more detail the synthesis of which RNA species (45S pre-rRNA, 28S and 18S rRNA, poly(A)-containing mRNAs, 5S rRNA and tRNAs) is inhibited by DHR in solated nuclei or in intact cells, and the extent of inhibition.

In agreement with this experiment, sedimentation analysis of [^{23}H]uridine-labeled RNA, labeled for 5 g in HEp-2 cells in the presence and absence of dihydrorifampicin (100 μg DHR/ml), showed that DHR inhibits mainly the biosynthesis of high molecular weight RNA species (rRNAs, mRNAs) and the synthesis of tRNAs is much less inhibited by DHR also in these human tumor cells (data not shown).

DISCUSSION

Experimental results presented in this communication clearly demonstrate the significant inhibitory effect of dihydrorifampicin on the enzymatic activities of RNA polymerases I and II isolated from human and avian tumor cells. The parent compound, rifampicin, which is a very potent inhibitor of prokaryotic RNA polymerases[3,32,33], does not show any inhibitory effect on eukaryotic RNA polymerases[4,5]. The surprising new fact that a relatively small structural change in the rifampicin molecule (the hydrogenation of 18–19 double bond in the ansa chain) increased very significantly the affinity of the resulting 18,19-dihydrorifampicin to eukaryotic RNA polymerases, is important and opens a new approach to the synthesis of new rifamycin SV derivatives hydrogenated at 18–19 carbon atoms of the ansa chain with probably increased affinity to mammalian RNA polymerases. On the other hand, hydrogenation of the double bonds of the ansa-bridge decreases the inhibitory activity of rifamycins against the bacterial RNA polymerases[34,35]. These facts probably reflect the structural differences between eukaryotic and prokaryotic RNA polymerases.

It is of interest that 16,17,18,19-tetrahydrorifampicin[1] does not inhibit the growth of HEp-2 cells in culture at a concentration of 50 μg/ml, and at a concentration of 100 μg/ml it exhibits only a partial and weaker cytostatic effect than DHR (ref.[2]).

Inhibition of eukaryotic RNA polymerases I and II by dihydrorifampicin is therefore the essential molecular mechanism of the reversible cytostatic effect of DHR on the growth of avian and human tumor cells in tissue cultures[2]. Inhibition by DHR of the biosynthesis of ribosomal RNAs and messener ribonucleic acids (*e.g.* histone mRNA with a short life[36]) may result in blocking the cell cycle and arresting the cell mitosis. Dihydrorifampicin seems to be a more specific inhibitor of RNA synthesis in eukaryotic cells and much less toxic in comparison with other rifamycin SV derivatives[11,12]. Our preliminary data[37] indicate that the cytostatic effect of dihydrorifampicin on cells growing in culture may be significantly increased in the presence of Amphotericin B (Fungizone. Squibb), due to the increased permeability of cell membranes treated with Amphotericin B (ref.[38]).

In our preliminary experiments[39] we observed a reversible formation of a complex between [18,19-^{3}H]-dihydrorifampicin[1] and RNA polymerases I and II isolated from nuclei of Rous sarcoma cells, which had the same sedimentation characteristics in glycerol density gradients as the free enzymes. We suggest therefore that dihydrorifampicin exerts an inhibitory action on eukaryotic RNA polymerases by reversible binding to the enzyme molecules. A similar mechanism of the inhibitory effect of another rifamycin SV derivative, the AF/013, on mammalian RNA polymerase II was suggested[5].

Our data also indicate that dihydrorifampicin inhibits with an equal efficiency the free RNA polymerase molecules as well as enzymes already engaged in RNA synthesis (Fig. 1). It seems therefore that DHR may inhibit not only initiation of RNA synthesis, but also elongation of the RNA chains. Definite solution of these problems requires further experimental studies to be done.

The observed relative resistance of tRNA biosynthesis to the inhibitory action of DHR in nuclei and cells treated with dihydrorifampicin involves probably the lower sensitivity of RNA polymerase III to DHR. This question deserves further study.

The inhibitory action of DHR on RNA polymerase II from avian and mammalian cells enables not only to regulate transcription of the cellular genes coding for cellular mRNAs, but also opens the possibility to inhibit the replication cycle of oncogenic RNA viruses, because RNA polymerase II very probably transcribes the integrated proviral DNA in tumor cells transformed by oncornaviruses[40,41]. In agreement with this assumption, we have found that dihydrorifampicin inhibits significantly the replication of Rous sarcoma virus in tissue culture of chick embryo fibroblasts infected with Rous sarcoma virus[42].

The inhibition of mitochondrial RNA synthesis by rifampicin has been reported by several authors[43–45]. The possibility that DHR also may inhibit the mitochondrial RNA polymerase cannot be excluded. However, considering that DHR is practically not toxic for mammalian cells[2], we can assume that dihydrorifampicin may also be much less harmful to the mitochondria than, for excample, the rifamycin derivative AF/013 (ref.[46]).

The regulation of RNA biosynthesis plays an important role in the control of tumor cell proliferation. The overlooked aspect of the mechanism of action of most antineoplastic drugs, involving the inhibition of RNA biosynthesis, should be therefore considered in a rational approach to cancer chemotherapy, as has been recently emphasized[47,48]. Specific inhibitory action on RNA biosynthesis and very low toxicity of dihydrorifampicin for human cells[2] opens up the possibility of practical use of this semisynthetic antibiotic in antineoplastic chemotherapy, especially in combination with other cytostatic drugs. We have recently observed a synergistic antileukemic effect of the combination of dihydrorifampicin, Amphotericin B and 6-azauridine in the treatment of Rauscher murine leukemia[37].

The authors wish to thank Academician V. M. Zhdanov and Professor F. I. Ershov, D. I. Ivanovsky Institute of Virology, Academy of Medical Sciences of the U.S.S.R., Moscow, for the kind gift of the human tumour cell line HEp-2, *and Dr J. Hanuš, Mr J. Kozel and Dr J. Beneš, Isotope Laboratory, Biological Institutes, Czechoslovak Academy of Sciences, Prague, for the preparation of dihydrorifampicin. The authors thank Academician J. Říman, Director of the Institute of Molecular Genetics, Czechoslovak Academy of Sciences, for his interest and kind support of this research work.*

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