Biochemical Effects of Mithramycin on Cultured Cells

Gretajo Northrop, Samuel G. Taylor, III, and Robert L. Northrop

Departments of Microbiology and Medicine, Presbyterian–St. Luke’s Hospital and University of Illinois College of Medicine and Graduate College at the Medical Center, Chicago, Illinois 60612

SUMMARY

The biochemical effects of mithramycin, an antibiotic employed clinically as an antitumor and antihypercalcemic agent, were studied in mouse embryonic, BHK-21, and Chang’s human conjunctiva cell cultures. Following treatment with mithramycin, RNA synthesis was decreased by 85% in mouse embryo and BHK-21 cell cultures and by 45% in human conjunctiva cell cultures when compared to controls. The inhibition of RNA synthesis in BHK-21 cells was dose-dependent and encompassed all cellular types of RNA. Although mithramycin inhibited protein and DNA syntheses in BHK-21 cells, these were not dose-related, which suggested that the primary site of action of this antibiotic was on RNA synthesis.

INTRODUCTION

Mithramycin, an antibiotic derived from cultures of Streptomyces (8), has been employed clinically in the treatment of certain neoplastic diseases (9) and is currently being evaluated for the treatment of hypercalcemia (1, 5, 10). Early studies on the mechanism of action of this antibiotic in cell-free systems revealed that mithramycin inhibited DNA-directed RNA synthesis (13). Subsequently, Yarbro et al. (15) reported inhibition of 32P incorporation into cellular RNA of mouse ascites tumor cells and mouse liver cells by mithramycin. Incorporation of 32P into DNA was unaffected by this inhibitor in their study.

Studies presented here were undertaken to elucidate further the effect of mithramycin not only on nucleic acid synthesis but also on protein synthesis in mammalian cells in culture.

MATERIALS AND METHODS

HuC4 cells (2) were grown at 37°C in 30-ml plastic flasks (Falcon Plastic Division of B-D Laboratories, Inc., Los Angeles, California) containing Eagle’s basal medium (3), penicillin (100 units/ml), and streptomycin (100 μg/ml), and supplemented with 20% horse serum. BHK-21 cells (12) were grown as monolayers at 37°C in Eagle’s basal medium containing both penicillin (100 units/ml) and streptomycin (100 μg/ml), to which 10% fetal calf serum was added. Primary cultures of ME cells were prepared from 10- to 12-day-old embryos. The minced embryonic tissues were suspended in 0.2% trypsin in CA++- and Mg++-free saline at 37°C. The cells obtained from this treatment were grown at 37°C in the same medium used for the BHK-21 cells plus 20 μg/ml amphotericin B. When the primary cultures became confluent monolayers, they were dispersed with 0.02% trypsin and were subcultured using the same conditions as described for the primary cultures. Only secondary cultures of ME cells were used in the experiments reported here.

RNA, DNA, and protein syntheses were assessed by labeling the cultures at 37°C with media containing 5 μc/ml of uridine-5-3H, 25–30 mc/mmole (Nuclear-Chicago, Des Plaines, Illinois), 0.5 μc/ml of thymidine-214C, 25–30 mc/mmole, (New England Nuclear Corporation, Boston, Massachusetts) for one hour, or 0.5 μc/ml of a mixture of amino acids-14C, 1 mc/mmole, (New England Nuclear Corporation, Boston, Massachusetts) for 30 minutes. Incorporation was terminated by replacing the radioactive media with medium containing 10% glycerol precooled to 4°C and then by freezing the cultures at -70°C. The cells were removed by the scraping action of the ice slush during subsequent thawing, and the cell suspensions from the 30-ml flasks were transferred to a centrifuge tube. Following centrifugation at 500 X g for 10 minutes, the supernatant was discarded, and the pellet was suspended in 1 ml of 0.01 M TMK (pH 7.4) containing 1.5 mM MgCl2 and 0.025 M KCl. The proteins and nucleic acids were precipitated from the suspension by the addition of 1 ml of cold 15% TCA and were maintained at 4°C for 10-12 hours to permit complete precipitation. Following centrifugation, the pellets were dispersed in 1 ml of 0.1 M PPO (pH 7.4) containing 1.5 mM MgCl2, 0.025 M KCl, 2.5-diphenyloxazole, POPOP, and 1,4-bis(5-phenyloxazolyl)benzene, and the absorbance was determined at 450 nm.

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4 HuC4, Chang’s human conjunctiva cells; ME, mouse embryo; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl) benzene; TMK, tris(hydroxymethyl)amino-methane-HCl buffer.
pellet was washed 3 times with cold 5% TCA and once each with cold absolute ethanol and cold ethyl ether. The washed precipitate was solubilized in 0.5 ml Hyamine 10X (Packard Instruments Co., Inc., Downers Grove, Illinois) and diluted with 15 ml of scintillation fluid (4 gm PPO and 0.3 gm POPOP in 1000 ml of toluene). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, Illinois).

Density gradient studies were performed on the isotopically labeled cellular components of BHK-21 cells obtained from two 250-ml plastic flasks. These cells were cultured and harvested as described above. Following centrifugation, the pellet of cells was suspended in 0.8 ml of TMK, incubated at 4°C for 20 minutes, and then homogenized in a Potter-Elvehjem Teflon-glass homogenizer. Sodium deoxycholate and BRIJ-58 (polyoxyethylene lauryl alcohol, Atlas Powder Company, Wilmington, Delaware) were added to a final concentration of 0.5% each. The sample was layered onto a previously prepared and chilled, linear, 10 to 60% sucrose-TMK gradient. The gradient was centrifuged at 40,000 rpm for 3 hours at 4°C in an SW 41 rotor in a Spinco Model L-2 ultracentrifuge with stabilizer (Beckman Instruments, Inc., Palo Alto, California). After centrifugation, the gradients were fractionated by an ISCO Model D Fractionator, and the optical density at 254 mju was recorded as the gradients passed continuously through an ISCO Model UA Ultraviolet Analyzer (Instrumentation Specialties Co., Lincoln, Nebraska). To each 0.5-ml fraction, 0.1 ml of bovine serum albumin (100 μg) and 0.3 ml of 15% TCA were added, and the samples were stored at 4°C overnight. The precipitates were collected on Bac-T-Flex membrane filters with interstices of 0.45 μ (Carl Schleicher and Schuell Co., Keene, New Hampshire) and were washed with 5% TCA and water. The membranes were placed into glass vials and dried, and the radioactivity was determined as described above.

To identify the components separated in the 10 to 60% linear sucrose gradients, 30 and 50 S ribosomal subunits of E. coli (purchased from Miles Laboratory, Inc., Elkhart, Indiana) were sedimented in separate gradients and fractionated as described above. The optical density profiles obtained from the E. coli subunits and the BHK-21 components were then compared. As an additional evaluation of these gradients, Fraction 14 of a linear 10 to 60% sucrose-TMK gradient containing sedimented BHK-21 cell components was examined by velocity sedimentation analysis at 30,000 rpm at 25°C in an An-D rotor in a Model E ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California). The observed sedimentation coefficient was corrected to S20,w (11).

RESULTS

The effect of mithramycin on uridine-5-3H incorporation into the nucleic acids of several different cell types in culture is presented in Table 1. After 4 hours of treatment with mithramycin (400 μg/ml), the amount of radioactive uridine incorporated into HuC cells was 45% of the amount incorporated by untreated cells. In contrast, ME and BHK-21 cells were more sensitive to mithramycin. After treatment with mithramycin (400 μg/ml) for 4 hours, the amount of uridine-5-3H incorporated into the ME and BHK-21 cells was decreased to 20% and 15% respectively of the amount incorporated by untreated cultures.

Table 1

<table>
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<tr>
<th>Mithramycin (μg/ml medium)</th>
<th>Human conjunctiva cells</th>
<th>Mouse embryo cells</th>
<th>BHK-21 cells</th>
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</tr>
<tr>
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<td>45</td>
<td>20</td>
<td>15</td>
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</table>

Effect of mithramycin on RNA synthesis. Uridine-5-3H was incorporated into the acid-insoluble components of cultures during one hour following 4 hours of treatment with mithramycin at the indicated concentrations. Each value, expressed as the percentage of controls, represents the average obtained from a minimum of 4 separate experiments.

The data presented in Chart 1 compare the effects of different concentrations of mithramycin on the synthesis of nucleic acids and proteins in BHK-21 cells. When these cells were treated with increasing concentrations of mithramycin, from 0 to 200 μg/ml, the decrease in the radioactive uridine incorporated at the different levels of the inhibitor was dose-related. Additional inhibition of uridine-5-3H incorporation was not demonstrated at mithramycin concentrations above 200 μg/ml. Radioactive amino acids and thymidine incorporation were linearly decreased by increasing concentrations of mithramycin up to 1.0 and 10 μg/ml respectively.

Chart 1. The effect of concentrations of mithramycin on nucleic acid and protein syntheses in BHK-21 cells. Cultures were treated with the indicated concentrations of mithramycin at 37°C for 4 hours. The cells were then labeled with uridine-5-3H or thymidine-2,14C for 1 hour or with a mixture of amino acids-14C for one-half hour at 37°C.
At higher mithramycin concentrations, almost no additional inhibition occurred. The effects of the duration of treatment with mithramycin (30 μg/ml) on the incorporation of radioactive uridine, thymidine, and amino acids into BHK-21 cells are presented in Chart 2. At this concentration, the primary effect of mithramycin was on RNA synthesis, and 2 hours of treatment were required to obtain maximal inhibition of RNA synthesis. Very little inhibition of protein and DNA syntheses, 12% and 6% respectively, was demonstrated during the initial 3-hour treatment period. However, after 4 hours of mithramycin treatment, protein synthesis was inhibited 20% and DNA synthesis was inhibited 30% compared to the controls.

Chart 2. The effect of duration of mithramycin treatment on RNA, DNA, and protein syntheses in BHK-21 cells. Mithramycin (30 μg/ml) was added to the media at 0 time, and the cultures were labeled with uridine-5-3H or thymidine-2-14C for 1 hour or with a mixture of amino acids-14C for one-half hour at the times indicated. The following series of experiments utilized 10 to 60% sucrose-TMK density gradients to determine the effects of mithramycin on the incorporation of uridine-5-3H into specific cell components. For the identification of cell components in this gradient, it was important to determine where the cell components sedimented. Fractions 9, 11, and 14 were found to contain 30, 50, and 68 S cellular components respectively (Chart 3). Data from spectrophotometric analysis performed on BHK-21 cellular material obtained from Fraction 14 demonstrated an absorption spectrum similar to rat liver ribosomes which have a 260 to 235 μv ratio of 1.67 (7). From these data it was concluded that single ribosomes are present in Fraction 14 of these gradients. Incorporation of radioactive uridine into the BHK-21 cellular components found in Fraction 23 suggests the presence of nuclear material in this fraction (Chart 4).

Chart 3. Sucrose density gradient analysis of the cellular components from control and mithramycin-treated (30 μg/ml for 4 hours) BHK-21 cell cultures labeled with uridine-5-3H for 1 hour at 37°C. Fractions containing 30, 50, and 68 S components are indicated by arrows.

The results of the effect of mithramycin treatment on the incorporation of radioactive uridine, thymidine, and amino acids into BHK-21 cells are presented in Chart 2. At this concentration, the primary effect of mithramycin was on RNA synthesis, and 2 hours of treatment were required to obtain maximal inhibition of RNA synthesis. Very little inhibition of protein and DNA syntheses, 12% and 6% respectively, was demonstrated during the initial 3-hour treatment period. However, after 4 hours of mithramycin treatment, protein synthesis was inhibited 20% and DNA synthesis was inhibited 30% compared to the controls. The following series of experiments utilized 10 to 60% sucrose-TMK density gradients to determine the effects of mithramycin on the incorporation of uridine-5-3H into specific cell components. For the identification of cell components in this gradient, it was important to determine where the cell components sedimented. Fractions 9, 11, and 14 were found to contain 30, 50, and 68 S cellular components respectively (Chart 3). Data from spectrophotometric analysis performed on BHK-21 cellular material obtained from Fraction 14 demonstrated an absorption spectrum similar to rat liver ribosomes which have a 260 to 235 μv ratio of 1.67 (7). From these data it was concluded that single ribosomes are present in Fraction 14 of these gradients. Incorporation of radioactive uridine into the BHK-21 cellular components found in Fraction 23 suggests the presence of nuclear material in this fraction (Chart 4).

DISCUSSION

Although mithramycin has been used in the treatment of neoplastic disease (9) and hypercalcemia (1, 5, 10), little is known about its effects at the cellular level. RNA synthesis, as determined by 32P incorporation studies in mouse ascites tumor cells (15) and in HeLa cells (14), was decreased after mithramycin treatment. In the studies briefly reported previously (6), and more extensively reported at this time, mithramycin treatment resulted in a decrease in RNA synthesis in the 3 cell types tested. HuC cell cultures were not as sensitive to the inhibitory effects of mithramycin as were ME and BHK-21 cell cultures. In these studies little difference in sensitivity was noted between secondary and continuous cell types, as represented by the ME and BHK-21 cells respectively. The significance, if any, of the apparent potentiation of RNA synthesis in ME cells at the mithramycin concentration of 0.10 μg/ml of media, is unknown at the present time.

The results of the effect of mithramycin treatment on the incorporation of radioactive uridine, thymidine, and amino acids into BHK-21 cells are presented in Chart 2. At this concentration, the primary effect of mithramycin was on RNA synthesis, and 2 hours of treatment were required to obtain maximal inhibition of RNA synthesis. Very little inhibition of protein and DNA syntheses, 12% and 6% respectively, was demonstrated during the initial 3-hour treatment period. However, after 4 hours of mithramycin treatment, protein synthesis was inhibited 20% and DNA synthesis was inhibited 30% compared to the controls.
acids into BHK-21 cells indicates that inhibition of RNA synthesis is the primary site of action of mithramycin. This is based on the fact that tritiated uridine incorporation was linearly reduced over a wide range of concentrations of mithramycin resulting in a maximum reduction of 85% under the present conditions. Under comparable conditions, protein and DNA synthesis were only reduced approximately 45%.

The early onset of inhibition of RNA synthesis compared with DNA and protein syntheses, as shown in Chart 2, offers further evidence that inhibition of RNA synthesis is the primary site of action of mithramycin.

To determine if one class of RNA was inhibited more than another by mithramycin treatment, the cellular components of BHK-21 cells labeled with tritiated uridine were analyzed in a sucrose gradient. The amount of acid-insoluble radioactive uridine found in the different fractions of control and mithramycin-treated BHK-21 cells was compared. As can be seen from the data presented above, mithramycin markedly inhibited the incorporation of uridine into all of the cellular components, which indicates that synthesis of all classes of RNA is inhibited by this antibiotic.

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