Studies on the Mechanism of Inhibition by Polyenic Antibiotics of Nucleic Acid Biosynthesis in Ascites Tumor Cells

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SUMMARY

The polyenic antibiotic lucensomycin caused a severe inhibition of uridine or thymidine incorporation into RNA or DNA of ascites tumor cells. The inhibition appeared to be related to the ability of the polyene to interact with the cell membrane, the permeability of which was increased; simple leakage of precursors or of enzymes from the cells did not fully account for the kinetics of inhibition.

Lucensomycin did not affect DNA or RNA synthesis, starting from triphosphonucleotides, by isolated nuclei. In the presence of the polyene, the cells became permeable to externally added nucleotides, which they could utilize for RNA or DNA synthesis. Nucleosides, even at high concentrations, could not substitute for nucleotides.

INTRODUCTION

In previous work from this laboratory (20–22), oxygen consumption and DNA synthesis by Novikoff hepatoma cells and Ehrlich ascites tumor cells were inhibited, although to a different extent, by 2 polyenic antibiotics, filipin and lucensomycin. These findings were tentatively correlated (21) to an increase in permeability of the cell membranes caused by the 2 polyenes, which are known to act on phospholipid-cholesterol membranes. Regenerating liver cells, which were used as “normal” (i.e., nonmalignant) controls for Novikoff hepatoma cells, were relatively insensitive to the 2 antibiotics. Lucensomycin, more selective than filipin, had no effect, even at high concentrations, on oxygen uptake by tumor cells.

This paper reports the results of experiments aimed at elucidating the mechanism by which lucensomycin inhibits DNA and RNA synthesis in ascites tumor cells. Ascites cells were preferred to cells from solid tumors because ascites cells were accessible, their population is relatively homogeneous, and the cell membrane is reliably intact. Besides Ehrlich ascites tumor cells from mice, which had been the subject of our previous work (21), Yoshida ascites tumor cells from rats were also studied, since they could be obtained in larger amounts from a small number of animals (and therefore with a low heterogeneity of the cell population) and could be better compared to normal liver cells (25).

MATERIALS AND METHODS

Chemicals

Eagle's minimal essential medium, Medium 109, was obtained from Grand Island Biological Co., Grand Island, N. Y.; Hanks' balanced salts solution and fetal calf serum were from Microbiological Associates, Bethesda, Md.; uridine-5-3H (specific activity, 5 Ci/m mole), thymidine-methyl-3H (specific activity, 5 Ci/m mole), UMP-4-14C (specific activity, 16.7 mCi/m mole), and UTP-4-14C (specific activity, 51 to 53 mCi/m mole) were from the Radiochemical Centre, Amersham, England; TTP-methyl-3H (specific activity, 10 to 13 Ci/m mole) was from New England Nuclear, Boston, Mass.; UTP, ATP, GTP, and CTP were from Boehringer und Soehne, Mannheim, Germany.; TTP, dATP, dCTP, dGTP, and fluorescein diacetate were from Koch-Light Laboratories, Colnbrook, England; Nonidet P-40 was from British Drug Houses, Poole, England; PPO and POPOP were from Nuclear-Chicago Corp., Des Plaines, Ill.; and Soluene was from Packard Instrument Co., Downers Grove, Ill. Other chemicals were obtained from E. Merck, Darmstadt, Germany, or from British Drug Houses.

Lucensomycin (etruscomycin) and N-acetyllucensomycin, in nitrogen-filled, sealed vials each containing 10 mg of the polyene, were kindly supplied by Professor F. Arcamone and Professor M. Ghione of Farmitalia, Milan, Italy. The vials were kept at 4°C, opened just before use, and dissolved in 0.6 to 1.0 ml DMSO.

Cell and Nuclei Preparation

Yoshida ascites tumor cells and Ehrlich ascites tumor cells were harvested 6 to 8 days after i.p. transplantation of 4 to 7 million cells in male Wistar rats or Swiss mice, respectively. Cell viability was checked by the dye exclusion method, with the use of trypan blue, 1 mg/ml, in 0.9% NaCl solution, according to the method of Tennant (29). Only nonhemorrhagic cell samples containing more than 90% viable cells were used. The cells were washed in the cold by centrifugation in KRB and were submitted to low-speed centrifugation (1500 rpm), resuspended in KRB, passed

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through an 80 mesh nylon net and an 18-gauge needle, counted in a Burker chamber, diluted if necessary, and kept in ice until use, i.e., for not more than 5 hr.

For preparation of isolated nuclei, 2 different procedures were used, Procedure A and Procedure B. In Procedure A, Ehrlich ascites cells, sedimented by low-speed centrifugation in the cold, were resuspended in 10 volumes of a buffer containing 0.25 M sucrose, 50 mM Tris, 20 mM KCl, 5 mM MgCl₂, and 0.2 mM CaCl₂, pH 7.4 (Solution A), equilibrated for 20 min in a pressure bomb with nitrogen at 65 atmospheres, and then rapidly expelled through a needle valve. The cavitation caused by the rapid release of nitrogen dissolved in the suspension was effective in disrupting the plasma membrane of the cells (26), while preserving most nuclei in an intact state (2).

The suspension was submitted to 10 strokes in a Teflon Potter-Elvehjem homogenizer at low speed (400 rpm), to free the nuclei from cytoplasmic residues, and then layered over an equal volume of 1 M sucrose and centrifuged at 700 X g for 30 min. The pellet, resuspended in 2.4 M sucrose containing 1 mM MgCl₂, was centrifuged twice at 50,000 X g for 60 min in the SW25 rotor of the Spinco Model 40 ultracentrifuge (31) and was then resuspended in a buffer containing 50 mM Tris, 30% (v/v) glycerol, and 10 mM mercaptoethanol, pH 7.8 (Buffer B) (11). Light microscopy on either fresh preparations or those after Papanicolaou staining showed that the final pellet thus obtained consisted of naked nuclei, sometimes over a sparse background of nuclear debris, with no whole cells present.

Nuclei from Yoshida ascites cells were prepared similarly, but in order to increase the yield, it was necessary to subject the cells to hypotonic shock prior to cell disruption. The sedimented cells were therefore resuspended in 8 volumes of H₂O; after 20 min in the cold, 4 times concentrated Solution A was added to regain isosmolarity, and high-pressure nitrogen was applied as already described.

In Procedure B, the cells, suspended in 8 volumes of Solution A, were mixed with an equal volume of solution A to which the surface-active agent Nonidet P-40 had been added. The optimal final concentration of Nonidet P-40 for maximal lysis of cell plasma membranes and minimal disruption of nuclei was 0.5% for Ehrlich ascites cells and 1% for Yoshida ascites cells. For optimal yield, the latter cells were subjected to hypotonic shock, as indicated above, prior to addition of Nonidet. After 5 min at room temperature, centrifugation through 1 M sucrose and then in 2.4 M sucrose was performed as described for Procedure A.

Procedure B, as compared to Procedure A, gave higher yields of pure nuclei with low amounts of nuclear debris and practically no cytoplasmic contamination. Nuclei prepared by the 2 methods behaved in an identical manner as far as triphosphoribonucleotide incorporation into polynucleotide was concerned.

Evaluation of Cell Permeability

Fluorescein Efflux. The cells were loaded with fluorescein by incubation with fluorescein diacetate, essentially as described by Celada and Rotman (4).

As shown by Rotman and Papermaster (24) for single cells, the concentration of fluorescein within the cells decreases exponentially with time at rates proportional to the concentration remaining in the cells. If \( c_t \) is the concentration of fluorescein in the supernatant at the various times and \( c_\infty \) is the concentration after at least 60 min at 45°, plotting \( \ln(c_\infty - c_t) \) versus time yields, therefore, a straight line (Chart 1). Its slope \( k \), which is the rate constant of the exit process, allows an estimate of the diffusion coefficient of fluorescein throughout the cell membrane (A. Scioscia Santoro, R. Strom, and C. Crifo. An Appraisal of Some Methods for Evaluating Cell Membrane Permeability, submitted for publication).

Leakage of Enzymes from the Cells. The amount of LDH in the extracellular compartment was evaluated by the following, in 3-ml cuvets at 340 nm, pH 7.0, and 25°: the oxidation of 2 X 10⁻⁴ M β-NADH by 7.5 X 10⁻⁶ M pyruvate in the presence of 50 μl of the supernatant from a cell suspension in KRB.

Incorporation of Labeled Nucleosides and Nucleotides into Nucleic Acids. The cells, suspended in KRB, were added to 9 volumes of KRB at 30° containing either thymidine-³H or uridine-³H, at concentrations varying between 0.2 and 6 μCi/ml and the appropriate amount of lucensomycin in DMSO. Controls were always run with addition of DMSO alone, although the low amounts used (1 to 10 μl/ml) were without effect. Use of Hanks' balanced salts solution or Eagle's minimal essential medium with or without fetal calf serum did not, under our experimental conditions, modify the rate of incorporation of either uridine or thymidine.

The incubation mixture was kept at 30° with moderate shaking; repetitive samples were taken at 1- to 4-min intervals and were mixed immediately with 7 volumes of ice-cold 0.4 M HClO₄. In a few experiments, sampling was begun before addition of lucensomycin (for further details, see Chart 2, legend). The precipitate was then either washed by centrifugation, defatted according to Tyner et al. (30), and dissolved in 1 ml of formamide at 140° for 5 to 8 hr, as previously described (20, 21), or collected by filtration on Whatman GH 83 glass fiber filters, extensively washed with cold HClO₄, and then digested with 1 ml of Soluene at 55° for 20 min. Toluene (10.4 ml) containing 6 g PPO and 0.1 g POPOP per liter were added (together with 8.6 ml of 95% ethanol for the formamide-dissolved samples), and the vials were counted on a Packard Tri-Carb Model 3380 liquid scintillation counter. Correction of cpm to dpm was done by automatic external standardization but was always controlled by a channel ratio method.

Autoradiography was performed by smearing microscope slides with the cells from the incorporation experiments; after fixation in ethanol:acetic acid (3:1), the slides were passed through cold 70% ethanol, dried, thoroughly washed with tap water and then with distilled water, dried again, and finally covered with L4 emulsion from Ilford Ltd., Ilford, England. After a week at room temperature in the dark, the autoradiographs were developed, with 0.45% diaminophenol (3). Its slope \( k \), which is the rate constant of the exit process, allows an estimate of the diffusion coefficient of fluorescein throughout the cell membrane (A. Scioscia Santoro, R. Strom, and C. Crifo. An Appraisal of Some Methods for Evaluating Cell Membrane Permeability, submitted for publication).

DNA synthesis by isolated nuclei was studied by addition of the nuclear suspension, kept at 0° in Buffer B, to 4 to 6 volumes of a solution, equilibrated at 30°, and containing 3 to
RESULTS

Effect of Lucensomycin on Nucleoside Incorporation by Cells. Under the experimental conditions used, both thymidine and uridine incorporation into DNA or RNA of either Ehrlich or Yoshida ascites cells exhibited a lag period of about 5 min, after which in the absence of lucensomycin the uptake of labeled nucleoside was linear for more than 20 to 30 min. Only with large amounts of cells and low concentrations of nucleoside was deviation from linearity observed after 15 to 20 min, generally when more than one-third of the nucleoside had been incorporated. Table 1 indicates how the incorporation of labeled uridine depended upon the extracellular nucleoside concentration. If we assume that the intracellular pool of uridine and of uridine nucleotides is small enough to be negligible, it is possible to convert the dpm to pmoles of uridine incorporated. Half-maximal incorporation was obtained for extracellular uridine concentrations around 0.4 to 0.8 μM.

Different batches of cells could vary by as much as an order
of magnitude in the rate of uridine or thymidine incorporation. The values reported for the experiment given in Table 1 are, for instance, rather low, and might be higher even by a factor of 6 with other batches of Ehrlich ascites cells. The reasons for such a variability have not been investigated.

Table 1

Dependence of uridine incorporation on extracellular nucleoside concentration in intact Ehrlich ascites cells

Ehrlich ascites cells (13 × 10⁶) suspended in 6 ml KRB, were incubated at 30° with 5 μCi of uridine-³H and different concentrations of "cold" uridine. The results are expressed as increase/min of radioactivity incorporated into the RNA from 10⁶ cells (Δ dpm/min/10⁶ cells), or, assuming that the intracellular nucleoside pool can be neglected, as pmoles uridine incorporated per min per 10⁶ cells.

<table>
<thead>
<tr>
<th>Total uridine concentration (μM)</th>
<th>Specific activity of uridine (μCi/μmole)</th>
<th>dpm/min/10⁶ cells</th>
<th>Uridine incorporated (pmoles/min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>5000</td>
<td>756</td>
<td>0.069</td>
</tr>
<tr>
<td>1.07</td>
<td>1111</td>
<td>291</td>
<td>0.119</td>
</tr>
<tr>
<td>8.57</td>
<td>138.9</td>
<td>41</td>
<td>0.135</td>
</tr>
<tr>
<td>83.54</td>
<td>14.3</td>
<td>5.4</td>
<td>0.172</td>
</tr>
</tbody>
</table>

Preliminary autoradiographical investigation indicated that under our experimental conditions most cells (> 80%) were able to incorporate the labeled nucleoside. With thymidine-³H, after short incubation times (3 min), the label was found mainly at the periphery of the nucleus (Fig. 1). This observation can probably be compared to that of Comings and Kakefuda (6), but its importance in the mechanism of DNA synthesis requires more detailed investigation.

In all the systems investigated, addition of lucensomycin produced inhibition of nucleoside incorporation (Chart 2). It can be shown that the percentage of inhibition did not depend on the concentration of the polyene in the medium but on the ratio between the amount of lucensomycin and the number of cells (Table 2); a similar behavior, as has been shown for other polyenes (17, 18), is also valid for lysis of bovine erythrocytes.

Thymidine and uridine incorporation by Ehrlich ascites cells were inhibited to a comparable extent by equal amounts of lucensomycin (Chart 2, A and B). Inhibition of uridine incorporation by Yoshida ascites cells instead required somewhat higher amounts of lucensomycin per cell (Chart 2C); this may be correlated to the microscopic observation of the larger size of these cells as compared to Ehrlich ascites cells.

Autoradiographic experiments confirmed that in the presence of lucensomycin the cells became unable to incorporate ³H-labeled nucleosides into their nuclei. Moreover, the cells appeared swollen and with enlarged nuclei (Fig. 2), as has previously been described (21) in fresh, nonfixed cells.

Chart 2. Kinetics of inhibition by lucensomycin or metabolic inhibitors, of uridine or thymidine incorporation by Ehrlich or Yoshida ascites cells. All assays at 30° were initiated by addition of cells to KRB containing the labeled nucleoside (2 μCi/ml). After 15 min, during which repetitive sampling was performed, more KRB, containing equal concentrations of labeled nucleoside and either DMSO alone, 2 μl/ml (shown as L = 0), or lucensomycin in DMSO (the figure after L indicating the lucensomycin/cell ratio, as μg lucensomycin/10⁶ cells) or NaCN (5 × 10⁻⁴ M, final concentration or 2,4-dinitrophenol (DNP) (5 × 5⁻³⁸ M final concentration). The incorporation values are given as dpm in the acid-insoluble residue from 10⁶ cells. A and D, Ehrlich ascites cells, thymidine incorporation; B, Ehrlich ascites cells, uridine incorporation; C, Yoshida ascites cells, uridine incorporation. Arrows, addition of lucensomycin; values on the ordinate should be divided by the negative exponentials to obtain the correct dpm values.
Table 2  
Dependence of inhibition of nucleoside incorporation and erythrocyte lysis upon lucensomycin concentration and the number of cells

Uridine incorporation by Ehrlich ascites cells was determined as indicated in Chart 2, and the percentage of the residual rate of incorporation was evaluated from comparison of the rates before and after the addition of lucensomycin. Hemolysis of bovine erythrocytes was evaluated by the amount of hemoglobin in the supernatant 20 min after the addition of lucensomycin.

<table>
<thead>
<tr>
<th>Lucensomycin (µg/ml)</th>
<th>Ehrlich ascites cells (% residual uridine incorporated)</th>
<th>Bovine erythrocytes (% hemolysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.1 x 10^6 cells/ml</td>
<td>11 x 10^6 cells/ml</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>99</td>
</tr>
<tr>
<td>5.1</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>8.4</td>
<td>1</td>
<td>60</td>
</tr>
</tbody>
</table>

Chart 3. Effect of lucensomycin upon fluorescein efflux from Ehrlich ascites tumor cells. The cell concentration was 4 x 10^6 cells/ml at 29.8°. Lucensomycin/cell ratios (as µg lucensomycin/10^6 cells) are indicated, Curve C being the control without lucensomycin. Other symbols are as indicated in “Materials and Methods” or in the legend to Chart 1. The 1st-order rate constants of efflux, k, had values of 3.0, 6.0, and 48.0 hr⁻¹ for C, L = 1.6, and L = 3.3, respectively.

preparations; fixation makes this phenomenon even more evident.

When lucensomycin was added to the cells during uridine or thymidine incorporation, inhibition reached its final value within the 1st min after addition of the polyene (Chart 2, A to C). This behavior, which was true also for low lucensomycin/cell ratios, is quite different from the pattern observed after the addition of agent which inhibit incorporation either by blocking cellular oxidations (NaCN) or by uncoupling oxidative phosphorylation (2,4-dinitrophenol); in such cases, as shown in Chart 2D, inhibition was slight at first and became more severe rather slowly.

Variations of Cell Membrane Permeability. Addition of lucensomycin to the cells caused an immediate and remarkable increase of fluorescein efflux from fluorescein-loaded cells. In all cases, the efflux followed 1st-order kinetics without any indication of heterogeneity of the cell population (Chart 3). The addition of lucensomycin also effected an increase in cell permeability to high-molecular-weight compounds, such as the cytoplasmic enzyme LDH (M.W., 140,000). Leakage of the enzyme from the cells followed 1st-order kinetics without any sign of heterogeneity of the cell population (Chart 4).

A certain heterogeneity was apparent, however, when the effects of lucensomycin were evaluated by observation of...
Chart 4. Effect of lucensomycin on LDH leakage from Ehrlich ascites cells. Cells were incubated with or without lucensomycin (polyene/cell ratios, as µg lucensomycin/10⁶ cells, are shown on the chart) at 30°. At different time intervals, samples were centrifuged, and the supernatant was assayed for LDH activity. Values are expressed as decrease of absorbance at 340 nm/min (ΔA/min), after addition of 50 µl of the supernatant to the pyruvate-NADH mixture under the conditions indicated in "Materials and Methods."

The sensitivity of these membrane properties appeared, in some experiments, to be slightly lower compared to uridine or thymidine incorporation; a certain variability between different batches of cells makes this small difference doubtful, so that the increase in cell membrane permeability and the inhibition of nucleic acid biosynthesis appear to be strictly correlated.

A further parallelism between the action of polyene on cell membrane permeability and its ability to inhibit nucleoside incorporation into nucleic acids of ascites tumor cells was found by investigations on the N-acetyl derivative of lucensomycin. This compound, which is slightly acidic and more soluble in water than the amphoteric lucensomycin, is approximately 40 times less effective on a molar basis than lucensomycin in increasing cell membrane permeability, and had, at concentrations up to 10 µg/10⁶ cells (i.e., 14 nmol/10⁶ cells), no effect on uridine incorporation by Ehrlich ascites cells.

The structural formula of lucensomycin is (14):

\[
\text{N-Acetyl derivatives of mycosamine-containing polyenes show less biological activity (as antifungal antibiotics) than the parent compounds (23).}
\]

Incorporation of Nucleotides into Nucleic Acids by Isolated Nuclei and by Lucensomycin-treated Cells. Incorporation of the uridylic moiety of labeled UTP by nuclei isolated from either Ehrlich or Yoshida cells in the presence of appropriate concentrations of all 4 triphosphoribonucleotides was not single cells by phase-contrast microscopy or in the presence of trypan blue, 1 mg/ml. The cells appeared enlarged to a variable extent, and dye uptake by the nucleus and by the cytoplasm (21) was neither simultaneous nor uniform in all cells. The experimental conditions (time, temperature, oxygenation) could not, however, be carefully controlled during the observation, and the heterogeneity observed, never very marked, is therefore of doubtful significance.

The structural formula of lucensomycin is (14):
Table 3

Binding capacity for lucensomycin of whole Ehrlich ascites cells and isolated nuclei

The amount of lucensomycin required for 50% inhibition of uridine incorporation was determined in the presence of different amounts of Ehrlich ascites cells or of isolated nuclei from Ehrlich ascites cells.

<table>
<thead>
<tr>
<th>Cells/ml</th>
<th>Nuclei/ml</th>
<th>Lucensomycin (µg/ml) for 50% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x 10⁶</td>
<td></td>
<td>1.18</td>
</tr>
<tr>
<td>6 x 10⁶</td>
<td></td>
<td>7.1</td>
</tr>
<tr>
<td>1 x 10⁶</td>
<td>28 x 10⁶</td>
<td>2.4</td>
</tr>
</tbody>
</table>

significantly modified by addition of lucensomycin (Chart 5).

Similar results were also obtained for the incorporation of the thymidyl moity of labeled TTP into DNA of nuclei from Ehrlich ascites cells (Chart 6). The procedure used for the isolation of the nuclei (nitrogen cavitation or Nonidet P-40) had no influence upon the results.

Since in a well-chosen range of lucensomycin concentrations it is possible to evaluate the amount of free lucensomycin by its effect on nucleoside incorporation by intact cells, this assay isolated nuclei and of whole cells to bind the polyene. As shown in Table 3, it is possible to calculate that 1 µg (i.e., 1.5 nmol) lucensomycin is bound by at least 23 million nuclei and by only 0.5 million cells.

Lucensomycin-treated cells became capable of incorporating the uridylic moiety of UTP into RNA (Chart 7). Under our conditions, the rate of this incorporation was (with UTP between 10 and 200 µM) remarkably constant, i.e., around 0.33 pmole UMP incorporated per min per 1 million Ehrlich ascites cells. Paper chromatography confirmed that the label was contained in a nucleoside monophosphate moiety, which could be freed by mild alkaline hydrolysis.

The presence of all 4 triphosphoribonucleotides was not mandatory, labeled UMP being able in the presence of ATP, GTP, and CTP to substitute for labeled UTP without any appreciable decrease in the rate of incorporation. As shown in

Table 4

Dependence of uridine incorporation on extracellular nucleoside concentration in lucensomycin-treated Ehrlich ascites cells

The procedure was the same as that described for Chart 7a, except for uridine concentration. The final number of cells was 4.8 x 10⁶/ml. Samples of 0.5 ml were used. The rate of incorporation in the presence of lucensomycin is expressed as percentage of controls without the polyene. In the uridine range considered, all controls had similar incorporation values.
Table 4, under these conditions even uridine, at very high concentrations, could be incorporated into RNA of lucensomycin Ehrlich ascites cells at a rate comparable to that of intact cells. Lucensomycin-treated cells could also perform the incorporation of the thymidylic moiety of TTP into DNA.

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