Biological and Biochemical Effects of Chartreusin on Mammalian Cells


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ABSTRACT

Chartreusin (CT), an antibiotic produced by Streptomyces chartreusis, was recently found to be active against experimental tumors B16 melanoma and L1210 and P388 leukemia. This report describes the correlation between its biological and biochemical effects on mouse L1210 and P388 leukemia.

CT was growth inhibitory to L1210 cells and other mammalian cells tested (drug concentration required for 50% inhibition of cell growth in culture, between 0.22 and 0.87 μg/ml). The inhibition of L1210 cell growth was rapid and dose dependent. The inhibition could not be eliminated after a 30-min exposure to 2.5-μg/ml drug doses and was not affected by the simultaneous addition of various metabolites. CT inhibited RNA synthesis greater than it did DNA synthesis and had least effect on protein synthesis. At 2.5 μg/ml it inhibited about 60% RNA synthesis, and the inhibition could no longer be reversed after a 30-min exposure to the drug. Again, this was a dose- and time-dependent phenomenon. These results closely correlated to those of growth inhibition, suggesting that inhibition of polynucleotide synthesis by CT played an important role in its action against mouse leukemia.

CT showed no effect on deoxyribonucleoside or ribonucleoside kinases but significantly inhibited DNA and RNA polymerase isolated from L1210 leukemia in culture and in vivo. The inhibition of highly purified DNA polymerase α and RNA polymerase II by CT varied with the template used. The latter correlated well with the interaction between CT and DNA polymers such as calf thymus, DNA, poly(dA-dT):poly(dT-dA), and poly(dG-dC):poly(dC-dG), demonstrated by circular dichroism measurements, suggesting that the biochemical and biological activities of CT were manifested in part by its binding to DNA. CT also caused significant damage to DNA.

INTRODUCTION

CT³ (NSC 5159), an antibiotic produced by Streptomyces chartreusis (10), was recently found to be active against several experimental tumors, such as B16 melanoma and L1210 and P388 leukemia (14), and is currently undergoing preclinical evaluation by the National Cancer Institute. This report primarily describes the correlation between its bio-

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3 The abbreviations used are: CT, chartreusin; dThd, thymidine; poly(dA-dT), poly(dA-dT); poly(dG-dC), poly(dG-dC); poly(dC-dG), poly(dC-dG); BSA, bovine serum albumin; RPMI, Roswell Park Memorial Institute; ID₅₀, drug concentration required for 50% inhibition of cell growth in culture; CD, circular dichroism.

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logical and biochemical effects on L1210 leukemia growing in culture in an attempt to elucidate its mechanism of action. The structure of CT is shown in Chart 1.

MATERIALS AND METHODS

Materials. CT was supplied by The Upjohn Co., Kalamazoo, Mich. [methyl-¹⁴C]dThd (53 μCi/mmol) was obtained from Amersham/Searle Corp., Arlington Heights, Ill.; [2,8-³H]adenosine (30 to 50 Ci/mmol), [5-H]uridine (27 Ci/mmol), [methyl-²H]dThd (2.2 Ci/mmol), [8,5-⁵H]dGTP (20 to 50 Ci/mmol), [methyl-²H]dTTP (10 to 20 Ci/mmol), [5-³H]UTP (>20 Ci/mmol), and dL-[¹-¹⁴C]leucine (40 to 60 mCi/mmol) were purchased from New England Nuclear, Boston, Mass.; Escherichia coli, RNA (16S + 23S mixture), and poly(dA-dT) were supplied by Miles Laboratories, Inc., Elk hart, Ind.; poly(dG-dC) was purchased from P-L Biochemicals, Milwaukee, Wis.; calf thymus DNA and BSA were obtained from Calbiochem, San Diego, Calif., and Armour Pharmaceutical Co., Kanakee, Ill., respectively. a-Amanitin and yeast RNA were purchased from Sigma Chemical Co., St. Louis, Mo.; RPMI 1629 and 1634 media were supplied by Associated Biomedic Systems, Buffalo, N. Y.

Mammalian Cell Growth in Culture. The basal media for growing mouse leukemia cells, human acute myelogenous leukemia RPMI 6410, and human normal lymphocyte RPMI 1788 were RPMI 1634 with 5% fetal calf serum, RPMI 1634 with 10% fetal calf serum, and RPMI 1629 with 10% fetal calf serum and 1% human serum, respectively. A mixture of penicillin (0.1 mg/ml of medium) and streptomycin (50 μg/ml of medium) was added to each of the basal media.

For growth studies 4 ml of cells (approximately 5 x 10⁶ cells/ml for L1210 cells and 1 x 10⁷ cells/ml for either human acute myelogenous leukemia RPMI 6410 or human lymphocyte RPMI 1788 cells) were pipetted into each sterile plastic culture tube with screw cap (16 x 125 mm; Falcon Plastics, Oxnard, Calif.). The experiment was initiated by the addition of 0.5 ml of medium, metabolites, and/or drug. Cell number was determined with a Coulter counter (Coul ter Electronics, Hialeah, Fla.). Three days later, after incubation at 37°, the ID₅₀ and drug concentration required for 90% inhibition of cell growth were calculated (5).

For time-course studies logarithmically growing L1210 cells (approximately 5 x 10⁷ cells/ml) were incubated with CT for a given time (30 to 120 min). At the termination of each incubation, drug was removed by centrifugation; cells were washed twice and then resuspended in fresh medium at a concentration of 5 x 10⁶ cells/ml. Five ml of the washed cells were pipetted into each culture tube and incubated at 37° for 3 days. Cell number was determined.

Macromolecular Synthesis. Cells used in the incorporation studies were in the logarithmic growth phase. Cells (23 ml) were incubated at 37° simultaneously with 1 ml of CT at a given concentration and 1 ml of a labeled metabolite such
as deoxyribonucleoside, ribonucleoside (2.5 \mu Ci/12 \mu mol/ml of cells), or DL-[1-14C]leucine (0.5 \mu Ci/200 \mu mol/ml) for up to 4 hr with gentle shaking. Aliquots (6 ml) removed at different times (1, 2, and 4 hr) were then pipetted into a centrifuge tube containing 1 ml of a corresponding unlabeled metabolite at 10 mg/ml and kept at 4°C. One-half ml of the aliquots was used for cell counting, and 2 ml were applied onto a glass fiber paper disc (Whatman GF/C; 2.4 cm presoaked in saturated phosphate) supported by a Millipore 3025 sampling manifold (Millipore Corp., Bedford, Mass.). Samples (in triplicate) were then washed 4 times with 4 ml of ice-cold 10% trichloroacetic acid and 3 times with 4 ml of absolute ethanol. For DNA and RNA extraction, the dried discs were placed individually in the counting vials and were extracted with 0.5 ml of 0.5 n perchloric acid at 70°C for 60 min. After cooling, 15 ml of Diotol (Burick & Jackson Laboratories, Inc., Muskegon, Mich.) were added. For protein uptake each disc was placed in a vial, 5 ml of Diotol were added, and the radioactivity was determined (11). The specific activity for the incorporation of a given labeled metabolite was expressed as cpm/10^8 cells. The initial rate of incorporation (cpm/10^8 cells/min) was obtained from linear regression analysis of the specific activities for 30-, 60- and 120-min incubation. The Pearson's coefficients (r) in this analysis were generally around 0.99. The percentage of inhibition was calculated from the initial rate of incorporation with and without addition of drug.

For time-course studies 1 ml of solvent or CT at a given concentration was added to 99 ml of L1210 cells (approximately 5 x 10^6 cells/ml) and was mixed well. Five-ml aliquots were pipetted into each culture tube and incubated at 37°C for 30, 60, and 120 min. At the termination of each incubation, drug was removed by centrifugation and cells were washed twice with 5 ml of medium, and were resuspended in 4 ml of medium (37°C). One ml of a labeled metabolite was added to the cells, and the aliquots were then incubated at 37°C for 40 min. The cell pellet was collected on paper discs, and the radioactivity was determined.

Enzyme Assays. The isolation of DNA polymerase of ascites cells of BALB/c x DBA/2 F1 (hereafter called CD2F1) mice carrying L1210 leukemia was essentially according to the procedure of Mantsavos and II were purified from isolated normal mouse liver nuclei (17) except that dithiothreitol (1 \mu mol/ml) and spermine phosphate (0.25 \mu mol/ml) were added to the isolation medium further to protect the nuclei. The DNA polymerase activity was determined by the method described by Babcock and Rich (1). Actinomycin D was used as a positive control.

Purification of DNA Polymerases. DNA polymerase a of cultured L1210 cells was purified according to the procedure of Graham et al. (7) with some modifications. Briefly, 12 g of L1210 cells were sonically dispersed and extracted twice with 0.8 M KCl buffer. After ammonium sulfate fractionation (65% saturation), the aliquot was dialyzed against buffer composed of 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol and was passed through a DEAE-cellulose column that was eluted with buffer containing 0.3 M KCl to remove nucleic acids. Fractions containing DNA polymerase activity were pooled, dialyzed, applied onto a phosphocellulose column, and eluted with buffer containing 0 to 0.05 M KCl. After the removal of most of the nonenzymatic proteins, the column was further eluted with a buffered KCl gradient from 0.1 to 0.7 M. Although both DNA polymerases a and b were collected, only DNA polymerase a was used for subsequent studies.

Assay of DNA Polymerase a Activity. The enzyme activity was assayed in 100-\mu l volumes containing 5 \mu g of activated calf thymus DNA or 0.1 A260 unit of poly(dA-dT) or poly(dG-dC); 50 mM Tris-HCl buffer (pH 7.8); 1.5 mM MgCl_2; 25 mM KCl; 5 mM dithiothreitol; 80 mM each dATP, dCTP, and dGTP; 7 \mu M [3H]TTP (7400 cpm/pmol); and 25 \mu l of DNA polymerase a. The reaction was incubated at 37°C for 40 and 40 min. After incubation acid-insoluble material was collected on a glass fiber paper disc, and radioactivity was determined as described previously.

Purification of RNA Polymerases. RNA polymerases I and II were purified from isolated normal mouse liver nuclei (17) essentially according to the procedure of Blair (3). Briefly, nuclear RNA polymerase was solubilized by sonic extraction of the nuclei in buffer [50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM MgCl_2, and 25% glycerol] containing 0.3 M (NH_4)_2SO_4. The enzyme was precipitated with (NH_4)_2SO_4 (0.42 g/ml of extract), dissolved in buffer containing 0.5 M (NH_4)_2SO_4, and dialyzed with buffer. After centrifugation at 105,000 x g for 1 hr, the supernatant was applied onto DEAE-Sephadex A-25 column and eluted with a linear gradient of buffered (NH_4)_2SO_4.
solution (0.05 to 0.5 \text{m}). Although both RNA polymerases I and II were isolated, Enzyme I was relatively a minor component in our enzyme preparation.

**Assay of RNA Polymerase II Activity.** The enzyme activity was assayed in 200-\text{\mu}l volumes containing 40 mg calf thymus DNA or 0.2 {A}_{260} unit of poly(dA-dT) or poly(dG-dC); 62.5 \text{mm} Tris-HCl (pH 7.9); 2 \text{mm} MnCl\text{\textsubscript{2}}; 85 \text{mm} (NH\textsubscript{4})\text{SO\textsubscript{4}}; 0.25 \text{mm} each ATP, CTP, and GTP; 2.5 \mu\text{M} [5-3\text{H}]UTP (20 \mu\text{Ci}/\text{pmol}); and 10 to 20 \mu\text{l} of RNA polymerase II. The reaction mixture was incubated at 37° for 20 min, acid-insoluble fraction was collected, and the radioactivity was determined.

**Interaction with Macromolecules.** The interaction between CT and calf thymus DNA, yeast RNA, *Escherichia coli* RNA (16 and 23S mixture), BSA, poly(dA-dT), or poly(dG-dC) was studied at 25° by absorbance measurements on a Cary 15 spectrophotometer and by CD measurements on a Cary 60 spectropolarimeter with a Model 6003 CD attachment (Varian Associates, Palo Alto, Calif.). The Cary 60 was calibrated with 10-camphorsulfonic acid (9).

The interaction between CT and calf thymus, poly(dA-dT), or poly(dG-dC) was also determined by the change of T\textsubscript{m} of DNA polymers in a recording thermospectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, Ohio).

**DNA Damage.** The DNA damage possibly caused by CT was determined according to the alkaline elution procedure of Swenberg et al. (16). Briefly, cultured L1210 cells were labeled with [\textsuperscript{3}\text{H}]dThd (0.01 to 0.04 \mu\text{Ci/ml of cells}) at 37° for 24 hr, and the radioactive medium was removed by centrifugation at 800 x g for 5 min. The cells were resuspended in regular medium, incubated for another 4 to 24 hr, and then exposed to CT for a given period (30 min, 2 hr, and 4 hr). Following that the cells were collected by centrifugation, resuspended in 0.5 ml of medium (2 to 4 x 10\textsuperscript{6} cells/ml), and subjected to alkaline elution analysis as described elsewhere (16).

**RESULTS**

**Effect of CT on Mammalian Cell Growth in Culture.** CT was growth inhibitory to several mammalian cells tested (Table 1). The ID\textsubscript{50} value of the drug ranged between 0.22 to 0.67 \mu\text{g/ml} when cells were continuously in contact with the drug for 3 days. Human acute myelogenous leukemia RPMI 6410 was slightly more inhibited by CT than was human lymphocyte RPMI 1788. Similarly, mouse leukemia P388 ascites cells growing in culture in the presence of mercaptoethanol (10 \mu\text{mol/ml}) appeared to be slightly more sensitive to CT than were mouse leukemia L1210 ascites cells. The difference was, however, not dramatic.

The growth inhibition of cultured L1210 cells by CT was not prevented or reversed by the simultaneous addition of a purine-pyrimidine mixture (75 \mu\text{g/ml of each component}), by tricarboxylic acid cycle intermediates (125 \mu\text{g/ml}), by a vitamin (and coenzyme) mixture (5-fold concentration of Eagle's basal medium), or by an amino acid mixture (5-fold concentration of Eagle's basal medium) (data not presented).

When cultured L1210 cells were exposed to different concentrations of CT for a given period (30 to 240 min) and the drug was removed by centrifugation and washings, the growth inhibition by CT could no longer be eliminated. The phenomenon was, however, dose related. A typical result is illustrated in Chart 2. At 10 \mu\text{g/ml} a marked inhibition (approximately 60%) was observed after a mere 30-min exposure, but at 1 \mu\text{g/ml} the inhibitory effect was not detected even after a 120-min incubation with cells.

**Drug Effects on Macromolecular Synthesis.** The macromolecular synthesis was measured as the incorporation of radioactive precursors into the acid-insoluble fraction. The results (Chart 3) indicate that RNA synthesis (measured as the incorporation of adenosine or uridine) appeared to be more inhibited by CT than by DNA synthesis (measured as the incorporation of dThd). Protein synthesis (incorporation of leucine) was least affected. If the percentage of inhibition was plotted against the logarithm of drug concentrations up to 10 \mu\text{g/ml}, the ID\textsubscript{50}'s could be calculated. The ID\textsubscript{50}'s for adenosine and uridine incorporation were 2.3 and 2.1 \mu\text{g/ml}, respectively, 4.4 \mu\text{g/ml} for dThd incorporation, and about 10 \mu\text{g/ml} for leucine incorporation.

When L1210 cells were exposed to CT (2.5 or 10 \mu\text{g/ml}) for a short period (30, 60, and 120 min) and the drug was removed by washing, the residual effect of CT was mea-
drug was removed, the DNA and RNA synthesis did not fully recover 24 hr later. Inhibition was 30% lower (Chart 5), which might reflect the irreversible damage, possibly cell death, caused by the drug.

Inhibition of Enzymes by CT. In an attempt to understand the mechanism of the inhibition of macromolecular synthesis, the effect of CT on several enzymes involved in DNA and RNA synthesis was investigated. At up to 100 μg/ml, CT was not inhibitory to either dThd or uridine kinase of cultured L1210 cells (data not presented) but significantly inhibited DNA polymerase of both cultured and ascites L1210 leukemic cells and RNA polymerase of liver nuclei isolated from either normal or leukemic CD2F1 mice (Table 2). The percentage of inhibition of DNA polymerase of cultured cells was derived from the initial rate of reaction for up to 120-min incubation. A typical result is illustrated in Chart 6. At 25 μg/ml CT inhibited approximately 50 and 60% of DNA polymerase of cultured and ascites L1210 cells, respectively (Table 2). Twenty-eight % inhibition of DNA polymerase of ascites L1210 cells was detected at a concentration as low as 5 μg/ml. CT also showed significant inhibitory effect on RNA polymerase of liver nuclei isolated from either normal CD2F1 or L1210 leukemic mice (Table 2). At 50 μg/ml CT inhibited about 30% of the enzyme activity of normal mice and approximately 40% of that from leukemic mice.

Interaction between CT and Macromolecules. The interaction between CT and calf thymus DNA, heat-activated calf thymus DNA, E. coli rRNA, yeast tRNA, BSA, poly(dA-dT), or poly(dG-dC) was studied by absorption and CD measurements (Chart 7). Difference spectra were determined by subtracting the spectra of CT and macromolecule in separate cells from the spectrum of the mixture of CT and macromolecule obtained in the same cell. Hence, a negative difference response means that the absorbance or CD of the mixture of CT and macromolecule is less than the sum of the absorbance or CD of the components. Yeast tRNA at 40 μg/ml and BSA at up to 3.5 mg/ml showed little or no interaction with CT (1.54 × 10⁻⁸ M). The CD difference spectra between 500 and 218 nm for both rRNA and BSA were measured by pulse-labeling (40 min) with the respective radioactive precursors (dThd, uridine, or leucine). These results are shown in Chart 4. At 2.5 μg/ml the drug effect on protein synthesis was not detected until CT was in contact with cells for more than 1 hr, but its effect on RNA synthesis could no longer be eliminated after a 30-min contact period, and an approximate 20% inhibition was observed. The inhibition increased to 50% after a 2-hr incubation.

Results in Chart 4A indicate that at 2.5 μg/ml drug effects on DNA and protein synthesis lagged behind those on RNA synthesis. This was, however, a dose-related phenomenon. At 10 μg/ml (Chart 4B), the inhibition of protein synthesis by CT also persisted after a mere 30-min contact time. Approximately 20% inhibition was noted, and the inhibition increased with time. A 50% inhibition was recorded at the end of a 2-hr period. Again, RNA synthesis was more inhibited than DNA synthesis, and protein synthesis was least affected.

When L1210 cells were exposed to CT for 2 hr and the
Table 2
Inhibition of DNA and RNA polymerase of L1210 leukemia by CT

<table>
<thead>
<tr>
<th>Drug concentration (μg/ml)</th>
<th>DNA polymerase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RNA polymerase&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cultured L1210 cells</td>
<td>Ascites Li210 cells</td>
</tr>
<tr>
<td>5</td>
<td>47-49</td>
<td>28</td>
</tr>
<tr>
<td>25</td>
<td>56-65</td>
<td>34</td>
</tr>
<tr>
<td>50</td>
<td>74-86</td>
<td>34</td>
</tr>
<tr>
<td>100</td>
<td>96</td>
<td>60-62</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Percentage of inhibition of DNA polymerase was calculated from the initial rate of incorporation of [3H]TTP. For the cultured L1210 cells, the rate of enzyme reaction was linear up to 120 min. For ascites Li210 cells, the rate of enzyme reaction was linear up to 120 min. The reaction mixture contained 0.03 μmol [3H]dTTP when poly(dA-dT) (50 μg) was used as template; 0.1 μmol each dATP, dCTP, and dGTP, and 0.03 μmol [3H]dTTP (167 mCi/mmol) were added when heat-activated calf thymus DNA (50 μg) was used as template; 0.1 μmol each dATP and 0.03 μmol [3H]dTTP were added when poly(dA-dT) (50 μg) was used as template; 0.1 μmol dCTP and 0.3 μmol [3H]dGTP (87 mCi/mmol) were added when poly(dG-dC) (50 μg) was used as template.

<sup>b</sup> RNA polymerase of liver nuclei was assayed according to the procedure of Babcock and Rich (1). The assay system (0.1 ml) contained 10 mmol Tris-HCl buffer (pH 7.9), 16 μmol MgCl<sub>2</sub>, 1 μmol 2-mercaptoethanol, 4 μmol EDTA, about 250 μg enzyme protein, 0.1 μmol each dATP, dCTP, and dGTP, and 0.03 μmol [3H]dTTP (167 mCi/mmol) were added when heat-activated calf thymus DNA (50 μg) was used as template; 0.1 μmol dATP and 0.03 μmol [3H]dTTP were added when poly(dA-dT) (50 μg) was used as template; 0.1 μmol dCTP and 0.3 μmol [3H]dGTP (87 mCi/mmol) were added when poly(dG-dC) (50 μg) was used as template.

DISCUSSION

Since CT was inhibitory to several mammalian cells tested (Table 1), cultured L1210 leukemic cells were chosen for the subsequent studies. The inhibition of cultured L1210 cell growth by CT could not be prevented or reversed by the simultaneous addition of a variety of metabolites. Hence, no apparent antidote would be available for this drug. Time-course study (Chart 2) indicated that the action of CT was rather rapid but required a higher concentration (>10 μg/ml) to achieve complete inhibition of cell growth that correlated closely to the results of cell kill by CT (14). CT appeared to inhibit RNA synthesis more than DNA synthesis, and protein synthesis was least affected (Chart 3). The incorporation of radioactive-labeled adenosine or concentration by a factor of 2 over that given in Chart 7 (data not presented). The absorbance and CD difference spectra of heat-activated DNA and CT as well as the CD difference spectrum of E. coli rRNA and CT were comparable to those of calf thymus DNA and CT (Chart 7), but the absorbance difference spectrum of E. coli rRNA and CT was less (data not presented).

The interaction between CT and calf thymus DNA, poly(dA-dT), or poly(dG-dC) was also investigated by T<sub>m</sub> determination. Surprisingly, the T<sub>m</sub> of these 3 macromolecules were not altered in the presence of CT (10 μg/ml) (data not presented).

The interaction between CT and DNA was further investigated by determining the drug effect on DNA (or chromosome) structure and integrity. After the incubation of [14C]dThd-labeled cultured L1210 cells with CT for 30 min, 2 hr, and 4 hr, possible chromosome damage was assessed by alkaline elution method (16). The results (Table 3) indicate that CT did cause DNA damage in cultured L1210 cells, and the effects were dose and time dependent. At 2.5 μg/ml CT caused little damage to DNA after a 30-min exposure to the cells, but it caused over 50% damage after a 4-hr exposure.

Inhibition of DNA Polymerase α and RNA Polymerase II by CT (Template Study). With calf thymus DNA, heat-activated calf-thymus DNA, poly(dA-dT), or poly(dG-dC) as the template, the effects of CT on the highly purified DNA polymerase α of cultured L1210 cells as well as on RNA polymerase II of mouse liver were compared. For DNA polymerase α, 2 representative experiments were reported here, and the results (Table 4) indicate that the enzyme activity was inhibited about 20 to 30% by CT at 2.5 μg/ml and about 50% at 10 μg/ml. There appeared to be little difference in inhibition whether calf thymus DNA, denatured calf thymus DNA, or poly(dA-dT) was used as the template. However, inhibition was somewhat lower when poly(dG-dC) was used as the template.

The pattern of inhibition of RNA polymerase II by CT was, however, different from that of DNA polymerase α. The results (Table 5) indicate that CT inhibited RNA polymerase II to a greater extent when poly(dG-dC) or poly(dA-dT) was used than when calf thymus DNA was used as the template. At 2.5 μg/ml the percentage of inhibition was 5, 46, and 87 with calf thymus DNA, poly(dA-dT), and poly(dG-dC) as the template, respectively.
Chart 7. Interaction between CT and DNA polymers. Absorbance (upper) and circular dichroism difference spectra (mixture spectrum minus spectrum of separate components of CT (10 μg/ml or 1.54 × 10^{-9} M) and calf thymus DNA (---), poly(dA-dT) (-- -- --), and poly(dG-dC) (-----), all 1.02 × 10^{-4} M for nucleotides, in 0.01 M phosphate buffer (pH 7.2). Ellipticity has been defined as arc tangent of the ratio of minor to major axes of the elliptical polarization.

Inhibition of highly purified DNA polymerase α of cultured L1210 cells by CT

<table>
<thead>
<tr>
<th>Template</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drug concentration (μg/ml)</td>
<td>Rate (cpm/min)</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>2.5</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>98</td>
</tr>
<tr>
<td>Calf thymus (denatured)</td>
<td>10.0</td>
<td>47</td>
</tr>
<tr>
<td>Poly(dA-dT)</td>
<td>2.5</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>64</td>
</tr>
<tr>
<td>Poly(dG-dC)</td>
<td>2.5</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>4.7</td>
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</table>

The condition for the enzyme assay was described in the text. Reaction was carried out at 37° for 20 and 40 min. The initial rates of reaction were analyzed by linear regression and cell lines passed through the origin. The Pearson coefficients (r) were between 0.99 and 1.00.

DNA damage studies with CT

<table>
<thead>
<tr>
<th>Inhibition time (min)</th>
<th>CT concentration (μg/ml)</th>
<th>% radioactivity retained</th>
<th>% DNA damage</th>
<th>% radioactivity retained</th>
<th>% DNA damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 0 (control)</td>
<td>2.5</td>
<td>64.9</td>
<td>16.7</td>
<td>73.1</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>44.2</td>
<td>43.3</td>
<td>62.9</td>
<td>21.3</td>
</tr>
<tr>
<td>120 0</td>
<td>2.5</td>
<td>64.9</td>
<td>16.7</td>
<td>73.1</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>44.2</td>
<td>43.3</td>
<td>62.9</td>
<td>21.3</td>
</tr>
<tr>
<td>240 0</td>
<td>2.5</td>
<td>64.9</td>
<td>16.7</td>
<td>73.1</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>44.2</td>
<td>43.3</td>
<td>62.9</td>
<td>21.3</td>
</tr>
</tbody>
</table>

DNA damage was assessed by alkaline elution method (16). The procedure was described briefly in the text. Percentage of radioactivity retained on the membrane was calculated as follows:

\[
\frac{\text{cpm retained on the membrane}}{\text{cpm on membrane + cpm in filtrate}} \times 100
\]

\[
\frac{\text{% DNA damage = (1 - \text{radioactivity retained on membrane from drug-treated cells})}}{\text{radioactivity retained on membrane from control cells}} \times 100
\]

uridine was inhibited more or less equally by CT, suggesting that CT might block the final stages of polynucleotide synthesis. This hypothesis was indirectly supported by the evidence that neither thymidine kinase nor uridine kinase activity was affected by CT at concentrations of up to 100 μg/ml. Time-course study showed that the inhibition pattern of macromolecular synthesis (Chart 4) paralleled that of cell growth (Chart 2), suggesting that these biochemical effects of CT are an important aspect in its action against L1210 cells and possibly other mammalian cells.

Further studies indicated that CT significantly inhibited DNA polymerase of both in vitro and in vivo ascites leukemic L1210 cells and RNA polymerase of liver nuclei isolated from either normal CD4Fi or leukemic L1210 mice (Table 2). In an attempt to understand the mechanism of inhibition of DNA and RNA polymerase, the possible interaction between
CT and macromolecules was investigated by means of difference spectrum determination with spectrum absorption and CD measurements. Although CT did not show significant interaction with either yeast RNA or bovine serum albumin, it did show small but significant interaction with E. coli rRNA. These results might support the findings of Gregg and Heintz (8) that CT bound to the reticulocyte ribosome and inhibited eukaryotic aminoacyltransferase I activity. In addition, we found that CT also showed significant interaction with calf thymus DNA as well as poly(dG-dC) or poly(dA-dT) (Chart 7).

The interaction between CT and DNA polymers was also evaluated with $T_m$ determination. To our surprise no apparent effect on the $T_m$ of calf thymus DNA, poly(dA-dT), or poly(dG-dC) was observed. Later we found that the interaction was thermally labile. For instance, the difference CD band at 435 nm decreased upon heating the mixture of CT and the polymer and was restored upon cooling of the mixture. This result might explain why the $T_m$'s of these DNA polymers were not significantly affected by CT. Apparently, the binding of CT to the DNA polymer was dissociated before the $T_m$'s of these polymers were reached. Hence, the interaction between DNA polymers and CT appeared to be relatively weak as compared to that with, for example, actinomycin D.

The relationship between CT interaction with DNA and its biochemical effects was further evaluated through 2 highly purified polymerases with calf thymus DNA, poly(dA-dT), or poly(dG-dC) as the template. DNA polymerase $\alpha$ was chosen because the enzyme played a large role in DNA replication (2) and reflected the proliferation rate of the cells. DNA polymerase $\alpha$ was a predominant component in rapidly dividing cells as compared to DNA polymerase $\beta$ (4). This is also the case in L1210 cells. This enzyme was significantly inhibited by CT (Table 4). The magnitude of its inhibition coincided quite well with that of DNA synthesis (Chart 4). Current evidence suggests that RNA polymerase I was localized within the nucleolus and mainly involved in transcription of rRNA genes. RNA polymerase II, on the other hand, was localized in the nucleoplasmic fraction and was mainly responsible for the transcription of mRNA genes (15). We found that, as reported in the literature (15), only RNA polymerase II was sensitive to $\alpha$-amanitin and was markedly inhibited by CT (Table 5). Its inhibition was similar to that of RNA synthesis (Chart 4). Furthermore, the pattern of enzyme inhibition by CT paralleled the drug interaction with DNA as measured by CD (Chart 7). In other words, RNA polymerase II was more inhibited when poly(dG-dC) or poly(dA-dT) rather than when calf thymus DNA was used as the template (Table 5). This suggested that the enzyme inhibition was in part mediated by CT binding to DNA.

As evidenced by the results obtained, the action of CT is certainly a complex one. In addition to CT binding to E. coli rRNA as described here and to reticulocyte ribosome as reported by Gregg and Heintz (8), we also found that CT interacted with a variety of DNA polymers, causing considerable DNA (chromosome) damage (Table 3). The inhibition of cell growth and cell kill (14) by CT paralleled that of polynucleotide synthesis, particularly RNA synthesis which, in turn, correlated closely to that of highly purified RNA polymerase II. The enzyme inhibition correlated with the ability of CT to bind to different DNA polymers. The under- line of these findings was that, although the mechanism of action of CT could not be precisely pinpointed, its ability to interact with DNA and its effects on the DNA structure and integrity would certainly play a major role in determining its biochemical and cytotoxic effects as well as its antitumor activity.

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