Inhibition of DNA and RNA Metabolism by Daunorubicin and Adriamycin in L1210 Mouse Leukemia

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SUMMARY

Effects of daunorubicin and its new analog, adriamycin, on nucleic acid metabolism were studied in vitro in L1210 mouse leukemia cells with labeled nucleoside precursors and were compared with the established effects of actinomycin D. L1210 ascites tumor cells incubated with daunorubicin under physiological conditions of pH, temperature, and tonicity showed significantly greater inhibition of tritiated thymidine incorporation into DNA and 14C-labeled uridine incorporation into RNA than cells treated with equimolar concentrations of adriamycin.

Investigation of cell-drug interaction showed that uptake of daunorubicin by L1210 cells was substantially greater than adriamycin uptake at 37°; also, daunorubicin was metabolized to daunorubicinol whereas adriamycin did not undergo a similar conversion. Differences in cellular uptake of drug probably play a significant role in the inhibition of nucleic acid metabolism by daunorubicin and adriamycin in L1210 cells in vitro. The importance of other mechanisms operative in determining difference in overall therapeutic efficacy in vivo remains to be established.

INTRODUCTION

Daunorubicin (daunomycin, rubidomycin, rubomycin) and adriamycin are relatively new antitumor antibiotics of the anthracycline group which differ from each other by a hydroxyl group on C-14 of the tetracycline moiety of adriamycin (1, 2) (Chart 1). Both daunorubicin and its analog adriamycin inhibit the growth of a variety of experimental tumors including L1210 mouse leukemia (20, 26). In humans, both drugs have shown efficacy against human acute leukemias and some solid tumors (5, 6, 13-15, 24, 27).

The mechanism of action of daunorubicin has been studied in bacterial systems and transplantable ascites tumors in animals (8, 9, 19, 25), but these reports of the effect of daunorubicin on nucleic acid metabolism have conflicted. Studies in which autoradiographic techniques were used depicted daunorubicin as an inhibitor of RNA synthesis while other studies emphasized its inhibitory effect on DNA metabolism. In a recent report, where autoradiographic techniques were used, adriamycin and daunorubicin have been compared (23). In the present study, the in vitro effects of daunorubicin and adriamycin on nucleic acid metabolism in mouse L1210 leukemia were assessed by measuring the incorporation of tritiated thymidine and 14C-labeled uridine into DNA and RNA, respectively. These effects were compared with the established inhibitory effects of actinomycin D (12) and were related to cellular drug uptake.

MATERIALS AND METHODS

Cells. L1210 mouse leukemia cells were grown in spinner-flask tissue culture with NIH Media 1630 (supplemented with 25% fetal calf serum), under conditions which produced exponential growth and cell concentrations of at least 2 million cells/ml; the average doubling time was less than 24 hr.

Antineoplastic Agents. Daunorubicin hydrochloride and actinomycin D were supplied by the Drug Development Branch of the Cancer Chemotherapy National Service Center, National Cancer Institute, NIH. Adriamycin hydrochloride was kindly provided by Dr. G. Mathé. For drug uptake and metabolic studies, daunorubicin and adriamycin were further purified by column chromatography (3). Solutions of daunorubicin and adriamycin with identical UV-visible absorption spectra (2) were adjusted to final equivalent molar concentrations by spectral absorption at 485 nm.

Labeled Thymidine and Uridine Incorporation Studies. L1210 cells in log phase were harvested by centrifugation at 600 x g for 10 min at room temperature and washed with 30 volumes (30 to 60 ml) of a buffered electrolyte solution consisting of NaCl, 117 mM; KCl, 5.3 mM; NaH2PO4, 1.1 mM; NaHCO3, 26.2 mM; CaCl2, 1.9 mM; and MgCl2, 1.0 mM (1.0 N HCl was added to adjust the pH to 7.45 in the presence of 5% CO2) (10). Washed cells were resuspended in this buffered electrolyte solution [supplemented with glucose (5.5 mM)] to a final concentration of 1.0 ± 0.2 million cells/ml.

For preparation of the reaction mixture that was used for all studies, 1 ml of L1210 cell suspension was added to individual siliconized glass tubes (13 x 100 mm) containing appropriate concentrations of antibiotic (final incubation volume, 1.1 ml). Samples were preincubated at 37° for 1 or 2
hr with shaking at 120 oscillations/min under an atmosphere of 5% CO₂ - 95% oxygen. After this initial incubation, either 1.0 µCi of thymidine-methyl-3H (2.0 Ci/m mole; New England Nuclear, Boston, Mass.) or 0.33 µCi of uridine-2-14C (55.6 mCi/m mole; New England Nuclear) was added in 0.01-ml volumes. The cell suspensions were incubated 1 additional hr. After the 2nd incubation, samples were chilled and the cells were immediately collected on 5-µm Millipore filters and washed with 20 volumes (20 ml) of ice-cold 0.9% NaCl solution. The filters were placed in scintillation vials with 10 ml of Aquasol scintillation mixture (New England Nuclear), and their radioactivities were measured in a Packard Tri-Carb Model 3375 scintillation spectrometer.

All experiments were done in duplicate with controls containing no antitumor drug. Cell viability was assessed prior to incubation by trypan blue dye exclusion. In all the experiments, the viability of the L1210 cells exceeded 85%. Over the course of all experiments, the pH of incubation mixtures was maintained with a physiological range at 7.45 ± 0.20.

**Uptake and Metabolism of Daunorubicin and Adriamycin by L1210 Cells.** The uptake and metabolism of daunorubicin and adriamycin by L1210 cells were investigated by incubating 1 ml of L1210 cell suspension with chromatographically pure daunorubicin or adriamycin for the appropriate times at 37° and 4° (final drug incubation concentration, 5.0 µM). After separation of cells and media by centrifugation at 1000 X g for 4 min at room temperature, 1 ml of ice-cold water was added to the cells. Daunorubicin and adriamycin were extracted from the cells and from the media by shaking each vigorously with 0.5 ml of 1-butanol for 1 min. The isolation and quantification of the drugs and metabolites have been previously described (4).

**Separation and Isolation of Radioisotopically Labeled Products.** To assure that 14C-labeled uridine was incorporated into RNA in our test system and to assure that tritiated thymidine was incorporated into cellular DNA, we extracted and isolated labeled DNA and RNA from the incubated L1210 cells. The quantity of isotope in both the trichloroacetic acid-soluble fraction and the acid-precipitable nucleic acid fractions were determined. A modification of the Schmidt-Thannhauser procedure as described by Munro and Fleck (16) was used. RNA was measured spectrophotometrically at 260 nm (16) and DNA was quantified at 490 nm after reacting with acid-indole (7). Solutions of purified yeast RNA and purified salmon sperm DNA (Schwarz/Mann Research Laboratories, Orangeburg, N.Y.) served as nucleic acid standards. The amount of tritium and 14C present in each nucleic acid fraction was determined by scintillation spectrometry.

## RESULTS

**Inhibition of Thymidine and Uridine Incorporation into L1210 Nucleic Acids.** The inhibition of incorporation of appropriate precursors into RNA and DNA of intact L1210 cells by daunorubicin, adriamycin, and actinomycin D after 1 hr of preincubation is compared in Table 1. Labeled thymidine and uridine incorporation were inhibited to virtually the same degree by daunorubicin. This was observed at all concentra-

### Table 1

<table>
<thead>
<tr>
<th>Antitumor drugs (% inhibition ± S.E.)</th>
<th>Drug concentration (µM)</th>
<th>pº</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thymidine-methyl-3H</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>16.0 ± 7.1</td>
<td>0.5</td>
</tr>
<tr>
<td>27.4 ± 3.3</td>
<td>16.3 ± 3.1</td>
<td>1.0</td>
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<td>54.9 ± 3.3</td>
<td>31.7 ± 5.8</td>
<td>3.0</td>
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<tr>
<td>74.3 ± 2.4</td>
<td>58.6 ± 4.1</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Uridine-2-14C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>76.1 ± 2.4</td>
<td>0.5</td>
</tr>
<tr>
<td>28.7 ± 4.5</td>
<td>12.4 ± 3.6</td>
<td>1.0</td>
</tr>
<tr>
<td>60.0 ± 4.1</td>
<td>35.9 ± 6.3</td>
<td>3.0</td>
</tr>
<tr>
<td>72.7 ± 4.3</td>
<td>56.6 ± 4.4</td>
<td>5.0</td>
</tr>
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</table>

a Significance of difference between daunorubicin and adriamycin; Student’s t test.
Daunorubicin, Adriamycin: Nucleic Acid Metabolism

tions tested. In a similar manner, adriamycin, the analog of daunorubicin, exerted its inhibitory effect about equally on thymidine and uridine incorporation. As expected, actinomycin D was significantly more effective in inhibiting uridine incorporation than was thymidine.

When equivalent molar concentrations of the drugs were compared, daunorubicin inhibited the incorporation of tritiated thymidine and $^{14}$C-labeled uridine into L1210 cells to a greater extent than did adriamycin. The inhibition of DNA and RNA metabolism by daunorubicin was significantly greater than adriamycin at all drug concentrations tested but was especially significant at low drug concentrations. At a drug concentration of 1 $\mu$M, daunorubicin was more than twice as effective as an inhibitor of uridine incorporation into RNA. Actinomycin D was the most effective inhibitor of RNA incorporation at the 1 $\mu$M drug concentration level. At 10 $\mu$M (Charts 2 and 3), the highest level of drug tested, the inhibitory effects of both daunorubicin and adriamycin were plateauing but a significant difference between the 2 drugs still remained; daunorubicin continued to be the more effective inhibitor of both DNA and RNA synthesis.

A parallel existed in these 1-hr incubations between the inhibitory effects of these 2 drugs. Daunorubicin at given concentrations maintained an arithmetic difference in inhibitory effect over that of adriamycin. When incubations were continued for 2 hr at the higher drug concentration (5 $\mu$M) (Table 2), the inhibitory effect of adriamycin on either DNA or RNA incorporation approached that of daunorubicin.

Chart 2. Effect of antitumor drug concentration on inhibition of tritiated thymidine ($TdR\cdot^{3}H$) incorporation into DNA. L1210 cells were incubated with drug for 1 hr; then tritiated thymidine was added and incubation was continued for 1 hr. •, daunorubicin; ○, adriamycin.

Chart 3. Effect of antitumor drug concentration on inhibition of $^{14}$C-labeled uridine ($Ur\cdot^{14}C$) incorporation into RNA. L1210 cells were incubated with drug for 1 hr; then $^{14}$C-labeled uridine was added and incubation was continued to 1 hr. •, daunorubicin; ○, adriamycin.

Table 2

<table>
<thead>
<tr>
<th>Antitumor drugs (% inhibition ± S.E.)</th>
<th>Drug concentration ($\mu$M)</th>
<th>p$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor</td>
<td>Daunorubicin</td>
<td>Adriamycin</td>
</tr>
<tr>
<td>Thymidine-methyl-$^{3}H$</td>
<td>$62.1 \pm 13.5$</td>
<td>$57.3 \pm 8.4$</td>
</tr>
<tr>
<td>Uridine-$^{14}C$</td>
<td>$89.4 \pm 4.2$</td>
<td>$50.3 \pm 7.4$</td>
</tr>
</tbody>
</table>

$^a$ Significance of difference between daunorubicin and adriamycin; Student's t test.
However, at lower drug levels (1 μM), a difference still existed so that daunorubicin remained the more effective inhibitor of RNA and DNA metabolism after 2 hr.

**Confirmation of Radioisotope Incorporation into Nucleic Acid.** Isolated RNA and DNA from incubated cells were assayed for radioactive incorporation. Results of a representative experiment are shown in Table 3. With normal L1210 cells, 99% of the acid-precipitable tritiated thymidine was incorporated into DNA, and 95% of the acid-precipitable uridine was incorporated into RNA. Consequently, this isolation technique was utilized to verify the effects of 5 μM daunorubicin or adriamycin on the RNA and DNA metabolism of L1210 cell (Table 4). The inhibitory actions of daunorubicin and adriamycin on the incorporation of labeled precursors into the isolated DNA and RNA were at levels similar to those obtained in our routine incorporation experiments. Neither daunorubicin nor adriamycin altered the amount of isotopically labeled nucleoside precursor taken into the acid-soluble fraction of the cells. The acid-soluble pool contained equal amounts of radioactivity in both control and drug-treated cells.

**Uptake of Daunorubicin and Adriamycin by L1210 Cells.** Since both our inhibition experiments and the difference in molecular structure suggested that drug uptake might be a factor in causing different inhibitory effects in vitro, experiments were performed to investigate the uptake of each drug. At 37°, the uptake of daunorubicin by L1210 cells was approximately twice that of adriamycin (Table 5). After 1-hr incubation, about 50% of the total daunorubicin was taken up by the cells, whereas 23% of the adriamycin was concentrated in the cells.

Another group of experiments carried out to determine the kinetics of drug uptake showed that the concentration of daunorubicin into L1210 cells was rapid (Chart 4). By 15 min,
38% of the daunorubicin was inside and/or bound to the cells, whereas only 9% of the adriamycin was in the cells. At 2 hr, the daunorubicin uptake still exceeded adriamycin uptake 2-fold. Low temperatures (4°) dramatically decreased uptake of both drugs; this is similar to results obtained by Kessel et al. (11) for daunorubicin. Despite the inhibition of active drug uptake, binding of adriamycin and daunorubicin to cells was observed at 4°.

During the incubation at 37°, about 3.5% of the daunorubicin was metabolized to daunorubicinol; none was found at 4°. The daunorubicinol was found in the media. No fluorescent metabolites of adriamycin were observed.

DISCUSSION

Previous reports of the effect of daunorubicin on nucleic acid metabolism have conflicted. Initially, Silvestrini et al. (21, 22) reported significant inhibition of RNA metabolism using autoradiographic techniques. Other investigators (18, 19) confirmed the effect of daunorubicin on RNA metabolism. However, widely quoted studies (25) of 32P incorporation into nucleic acids of mouse ascites 6C3HD tumor cells emphasized a greater inhibitory effect of daunorubicin on DNA metabolism.

In the present studies, we investigated the incorporation of nucleoside precursors into nucleic acids of L1210 mouse leukemia cells and showed that daunorubicin inhibited both DNA and RNA metabolism in vitro to the same degree. In our studies, we used 2 specific nucleic acid precursors in an effort to assure accurate measurement of nucleic acid metabolism (17). Moreover, we assessed the incorporation of radioisotopically labeled precursors into specific nucleic acids of L1210 cells by separating and isolating tritium-labeled DNA and 14C-labeled RNA from the L1210 cells. These differences in observations may indicate different effects of daunorubicin on various tumors.

We observed that adriamycin was less effective than daunorubicin in inhibiting nucleic acid metabolism of L1210 cells. However, adriamycin resembled daunorubicin by inhibiting DNA and RNA metabolism about equally. Neither daunorubicin nor adriamycin affected the incorporation of labeled nucleosides into the acid-soluble pool of the cells. They both differed from actinomycin D, an established antitumor antibiotic which preferentially inhibits RNA metabolism (19).

Studies of uptake of daunorubicin and adriamycin by L1210 cells in vitro showed significant differences. At 37°, the uptake of daunorubicin was more than twice that of adriamycin. The uptake of both anthracycline drugs was temperature sensitive; this suggests an energy dependence for drug uptake rather than simple diffusion into cells. However, a portion of the observed drug uptake may be due to binding (independent of temperature) since at 4° about 6% of the adriamycin and 15% of the daunorubicin was associated with the L1210 cells. Daunorubicin, a slightly less polar compound than adriamycin, could bind to membranes more readily and account for the comparative increased binding.

Another important difference between these drugs was that daunorubicin was metabolized to daunorubicinol by the L1210 cells while no similar metabolite of adriamycin could be demonstrated. Similar observations in other in vivo and in vitro experiments have been made in our laboratory. No aglycone formation was seen in these experiments. Other animal tissues (3, 4) have glycosidase activity and can cleave the anthracycline antibiotics, but the intact L1210 cells apparently do not.

When the uptake data and the inhibition data are compared, an interesting relationship is seen. Whereas daunorubicin is apparently a more potent inhibitor of nucleic acid synthesis than adriamycin, cell-bound concentration of daunorubicin is much greater at 37° than that of adriamycin. If the cell-bound concentration of drug is equivalent to intracellular concentration, at 60 min daunorubicin has a 3-fold greater intracellular concentration than adriamycin and at 120 min the concentration difference is still over 2-fold. At 120 min, however, the inhibition of thymidine or uridine incorporation by daunorubicin is only 50% greater than that of adriamycin. This indicates that the actual inhibitory effectiveness of adriamycin is as great as or greater than daunorubicin but that cellular uptake of adriamycin is slower. Nevertheless, in the intact L1210 cell, daunorubicin still exerts a more profound inhibition on nucleic acid metabolism than adriamycin; this is due, at least in part, to the increased uptake of daunorubicin by L1210 cells.

Despite apparently greater inhibition of nucleic acid metabolism by daunorubicin in vitro, animal studies (20) and preliminary clinical studies (6) indicate a higher therapeutic index for adriamycin. These 2 bodies of information, namely the in vivo effects of these drugs and our current in vitro observations, are not, however, mutually exclusive, for the differences may be due to other mechanisms that are operating in the intact animal. For example, mechanisms involving differences in rates and routes of drug excretion, differences in protein binding, different metabolic pathways, or different rates of drug metabolism in vivo for each drug probably play important roles in determining overall drug effect in vivo.

Whether the therapeutic effects of daunorubicin compared to adriamycin in vivo are due totally to different pharmacokinetic or pharmacodynamic properties or are due in part to substantially different antineoplastic activities remains to be determined. Total elucidation of in vitro and clinical actions of adriamycin and daunorubicin awaits further investigative and comparative studies.

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REFERENCES

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