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The Antitumor Effects of Anthracyclines

The Importance of the Carbomethoxy-Group at Position-10 of Marcellomycin and Rudolfomycin

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SUMMARY

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The effects of four anthracyclines on DNA, RNA and nucleolar RNA syntheses were compared to their *in vitro* cytotoxicity and *in vivo* antitumor activity. The results of these studies show that removal of the 10-carbomethoxy group from two class II anthracyclines, rudolfomycin and marcellomycin, resulted in a marked reduction in *in vivo* antitumor activity. The reduction in antitumor activity correlated with a loss of *in vitro* cytotoxicity, and a marked reduction in potency of nucleolar RNA synthesis inhibitory activity. This study supports the concept that a significant portion of the antitumor activity of class II anthracyclines is related to their ability to inhibit nucleolar RNA synthesis. These studies also demonstrate that the 10-carbomethoxy group is essential for nucleolar RNA synthesis inhibition by class II anthracyclines.

INTRODUCTION

Previous studies have suggested that a major contribution to the mechanism of action of anthracyclines is made by the interaction of these agents with DNA, thereby interfering with normal nucleic acid synthesis (1-11). Other studies have demonstrated that anthracyclines induced significant alterations in membranes, characterized by altered cellular agglutination ability and mitotic arrest at concentrations below those required to inhibit nucleic acid synthesis (3-8, 12-14).

Adriamycin and daunomycin (Fig. 1) have been shown to interact with DNA *in vitro* by intercalating between adjacent nu-

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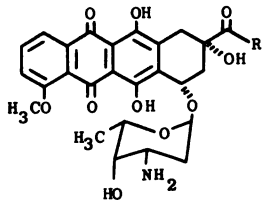
cleotide base pairs (2, 3, 4, 12, 15). Further, they have been shown to inhibit DNA and RNA syntheses at approximately equivalent concentrations and nucleolar preribosomal RNA (No-RNA)[2] synthesis at concentrations 2–5 fold lower than those required to inhibit DNA synthesis (5, 8, 10, 16).

Previous studies from this laboratory have demonstrated that anthracyclines may be divided into two classes on the basis of their effects on DNA and RNA syntheses (11). Class I anthracyclines such as adriamycin were shown to inhibit No-RNA synthesis at a concentration approximately equal to the concentration required to inhibit DNA synthesis. Class II anthracyclines such as marcellomycin were shown to inhibit No-RNA synthesis at concentrations 200–1500 fold lower than those required to inhibit DNA synthesis (11). These studies suggested that the presence of a di- or tri-saccharide glycosidic side chain conferred No-RNA synthesis inhibitory specificity (11).



Adriamycin, R = CH_2OH

Daunomycin, R = CH_3

FIG. 1. *The structures of adriamycin and daunomycin, typical of class I anthracyclines*

[2] The abbreviations used are: MCM, marcellomycin; D-MCM, 10-descarbomethoxy-marcellomycin; RDM, rudolfomycin; D-RDM, 10-descarbomethoxy-rudolfomycin; NHAC, Novikoff hepatoma ascites cells; RPMI, Roswell Park Memorial Institute type 1640 medium; IC_{50}, 50% inhibitory concentration; TCA, tri-chloroacetic acid; A_{254}, absorbance at 254 nm; E-BME, Earle's Basic Modified Eagle's Medium; EDTA, ethylene diaminetetraacetic acid, disodium salt; NKM, 140 mM NaCl, 5 mM KCl, 8 mM $MgCl_2$; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(2-(4-methyl-5-phenyloxasolyl) benzene; NMR, nuclear magnetic resonance; IR, infrared; KBr, potassium bromide; $CDCl_3$, deuterated chloroform; UV, ultraviolet; Me_4Si, tetramethylsilane; TLC, thin-layer chromatography; ppm-parts per million; MST, median survival time; T/C, (MST treat/MST control) × 100, expressed as a percentage value; ip, intraperitoneal; No-RNA, nucleolar RNA.

To characterize further the relationships between No-RNA synthesis inhibition and antitumor activity, the effects of two semisynthetic anthracyclines were compared to the effects of their parent compounds. The compounds, shown in Fig. 2, include MCM, D-MCM, RDM and D-RDM.

The present study compares the effects of four analogues on macromolecular syntheses, cell colony survival and *in vivo* antitumor activities. The results provide additional evidence that the antitumor activities of class II anthracyclines are related to their effects on No-RNA synthesis. Further, the present studies define additional structure activity relationships for class II anthracyclines. The carbomethoxy group at position-10 of class II anthracyclines is also an important locus for antitumor activity in addition to the glycosidic sidechain which has been previously described (11).

MATERIALS AND METHODS

Materials. Gelman type E glass fiber filters were obtained from Curtin-Matheson Scientific Company, Houston, Texas. RPMI-1640 medium; sterile fetal calf serum; sterile glutamine solution; sterile penicillin-streptomycin solution (10,000 units: 10,000 mcg) were all obtained from Grand Island Biological Company, Grand Island, New York. Tritiated precursors (^{3}H-thymidine and ^{3}H-uridine) and RNase-free sucrose were obtained from Schwarz/Mann, Orangeburg, New York. Carrier-free ^{32}P-inorganic phosphate was obtained from Union Carbide, Oak Ridge, Tennessee. Melting points were determined in capillary tubes in a Mel-Temp apparatus and are uncorrected. IR spectra were recorded on a Beckman IR-9 spectrophotometer as KBr disks and maxima are reported in cm^{-1} units. NMR spectra were run in $CDCl_3$ at 100 MHZ with a Varian XL-100 instrument using Me_4Si as an internal standard. Chemical shift values are reported in ppm of the total applied magnetic field. UV-visible spectra were recorded in methanol on a Beckman Acta III spectrophotometer.

Descarbomethoxy-marcellomycin. The

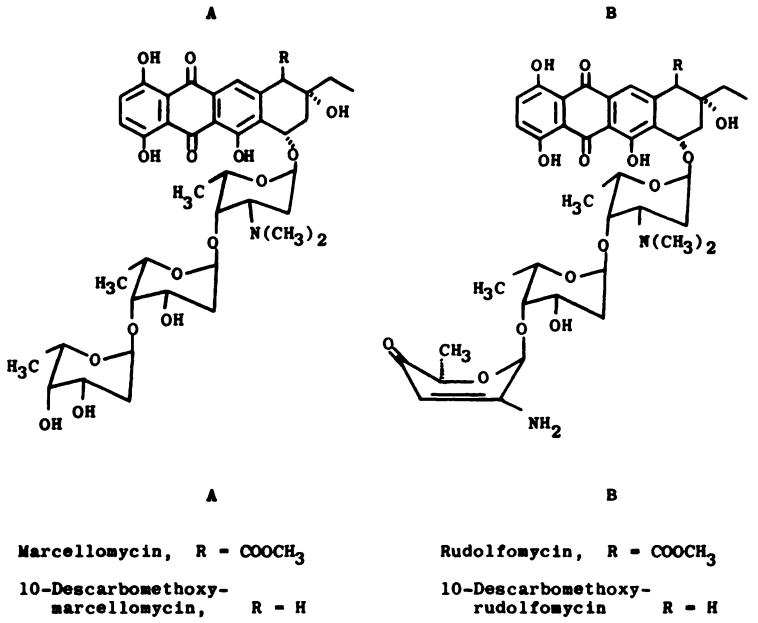


FIG. 2. *The structures of marcellomycin, 10-descarbomethoxy-marcellomycin, rudolfomycin, and 10-descarbomethoxy-rudolfomycin*

bohemic acid complex has been shown to produce various anthracycline antitumor agents including marcellomycin (17) and rudolfomycin (18). Using the procedure of Wiley *et al.* (19), a solution of 904 mg of marcellomycin in 10 ml of 0.53 N potassium hydroxide was stored for 17 hours at room temperature. The solution was diluted with 10 ml of water and acidified to pH 6.2 with 2 N hydrochloric acid. Lyophilization gave a red solid which was dissolved in 25 ml of N,N-dimethylformamide, and the solution was stored for 17 hours at room temperature. The solvent was removed under reduced pressure at 30° and the gummy residue was chromatographed on a dry column of 15 g of acid-washed silica gel containing 10% by weight of water. Chloroform was used as the solvent and fast-moving impurities were removed in 85 ml of eluate. The solvent was changed to chloroform:methanol 80:20 and the product was eluted in a 55 ml fraction. Evaporation of the solvent provided 392 mg of red solid which was rechromatographed on a short column (10 cm × 8 mm) of silica gel containing 10% by weight of water. A chloroform:methanol 95:5 mixture was employed and 5 ml fractions were collected and assayed by TLC (silica gel using chloroform:methanol 9:1). Fractions showing only a single zone at Rf 0.41 were combined and evaporated, and the residue was triturated with skellysolve B containing a trace of ether. The red solid was collected by filtration and dried under vacuum to afford 50 mg (6%) of the title compound, mp 175–7°. IR 1600, 1455, 1220, 1010; UV-visible λ_{max} 231 nm (ϵ22900), 257 (14200), 287 (6200) 448 (8600), 506 (sh) (7250), 520 (sh) (6150); NMR 7.65 (IH, s, H_{11}), 7.30 (2H, s, H_2, H_3), 5.50, 5.24 and 5.00 (4H, broad singlets, anomeric protons and H_7), 3.00 (2H, AB quartet, H_{10}), 2.18 (6H, s, N-methyl), 1.0–1.4 (m, C-methyls), 1.5–2.5 (m, methylene protons), 3.5–4.6 (m, sugar methine protons). Analysis Found: C 60.64, H 6.82, N 1.50. Calculated for $C_{40}H_{53}NO_{15}$: C 60.98, H 6.78, N 1.78.

Descarbomethoxy-rudolfomycin. Using the same procedure and purification as above, 830 mg of rudolfomycin was converted to 195 mg (24%) of the title compound, mp 187–9°. IR 1600, 1445, 1225, 1020; UV-visible λ_{max} 232 nm (ϵ 30,100), 259 (21800), 280 (21500), 490 (11100), 508 (sh) (9500), 521 (sh) (8100); NMR 7.64 (IH, s, H_{11}), 7.31 (2H, s, H_2, H_3), 5.52, 5.32, 5.27, 5.04 (5H, singlets, anomeric proton s, ole-

finic proton and H_7), 3.00 (2H, AB quartet, H_{10}), 2.20 (6H, s, N-methyls), 1.42 (3H, d, unsaturated sugar methyl), 1.00–1.36 (m, C-methyls), 1.56–2.30 (m, methylene protons), 3.64–4.70 (m, sugar methine protons). Analysis Found: C 58.27, H 6.63, N 3.08. Calculated for $C_{40}H_{50}N_2O_{14}\cdot 2H_2O$: C 58.66, H 6.65, N 3.42.

Novikoff hepatoma cells. NHAC, type N_1S_1-73, were maintained in monolayer cultures at 37° in sealed tissue culture flasks with RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mcg/ml streptomycin A. For drug studies, NHAC were grown in shaker culture (New Brunswick Gyrorotary shaking incubator) at 37° in GIBCO-type 500 ml liquid media bottles in the above medium.

NHAC used as "carrier" cells were obtained from rats bearing ascites cells as previously described (20).

Effects on whole cell macromolecular synthesis. NHAC were grown to a density of approximately 8.0×10^5 to 1.0×10^6 cells/ml, harvested by centrifugation at 800 $\times$ *g* for 10 min at room temperature and resuspended in fresh RPMI-1640 medium supplemented with fetal calf serum and antibiotics as indicated above. The cell density was then adjusted to 6×10^5 cells/ml with supplemented RPMI-1640 medium. The NHAC were then incubated in a 37° shaking water bath with gentle agitation for 30–60 minutes in closed vessels containing 5 ml aliquots of cell suspension. After preincubation, 0.05 ml of aqueous drug solution was added. Cells were incubated with drugs for 15 minutes followed by addition of ^{3}H-uridine or ^{3}H-thymidine to a final concentration of 0.225 mCi/ml. NHAC were then incubated for 2 hr with gentle agitation and then chilled on ice.

The total radioactivity with which NHAC aliquots were incubated was determined by counting 0.05 ml aliquots of the cell suspension. Total cellular radioactivity was determined by collecting 0.5 ml aliquots of cells on glass fiber filters (type E) and determining the radioactivity remaining on the filters after they were washed two times with 10 ml aliquots of NKM (0.14 M NaCl, 0.005 M KCl, 0.008 M $MgCl_2$). TCA precipitable radioactivity was determined by filtering 1.0 ml of cell suspension through a glass fiber filter, after which the filter was washed two times with 10 ml NKM, then twice with 10 ml 10% TCA solution followed by two washes with 10 ml of 5% TCA solution. The radioactivity remaining on the filter was then determined. The percent of the total radioactivity and of the cellular radioactivity which was incorporated into macromolecules was then determined for each sample. All assays were performed in triplicate. The radioactivity present in each sample was determined by using 5 ml of scintillation fluor containing toluene, 1,4-dioxane-ethylene glycol monoethylether, naphthalene, PPO and POPOP in disposable polyethylene vials and counting in a Packard Tri-Carb model 3385 scintillation spectrometer.

Nucleolar RNA labeling, isolation and fractionation. NHAC were incubated for 30–40 hr in supplemented RPMI-1640 medium with 0.01 mCi/ml of ^{3}H-uridine (specific activity 40–60 mCi/mmole). Cells were then collected by centrifuging at 850 $\times$ *g*, 10–15 min at room temperature, washed once with "phosphate-free" E-BME medium (20) (International Biological Labs., Rockville, Md.) and resuspended with the same medium to a concentration of approximately $6.0–10.0 \times 10^6$ cell/ml.

After preincubation for 30 min, the drug was added to the cell culture. Thirty minutes later, ^{32}P-inorganic phosphate was added to a final concentration of 0.2 mCi ^{32}P/ml. NHAC were incubated with isotope for 60 min and then chilled on ice. After washing twice with NKM, the cells were combined with 10–20 fold (by weight) of unlabeled "carrier" NHAC. Nucleoli were prepared as previously described (21).

RNA was extracted from isolated nucleoli by the SDS-phenol method at 65° (21, 22). The RNA was redissolved in 4–8 ml of sterile deionized water and re-precipitated with 2 volumes of 95% ethanol containing 2% potassium acetate for approximately 4 hr at −20°. The RNA was then resuspended in 1.0 ml sterile deionized water and layered on a 33 ml linear sucrose gradient (5–40%) containing 0.1 M NaCl, 0.01 M sodium acetate (pH 5.1) and 0.001

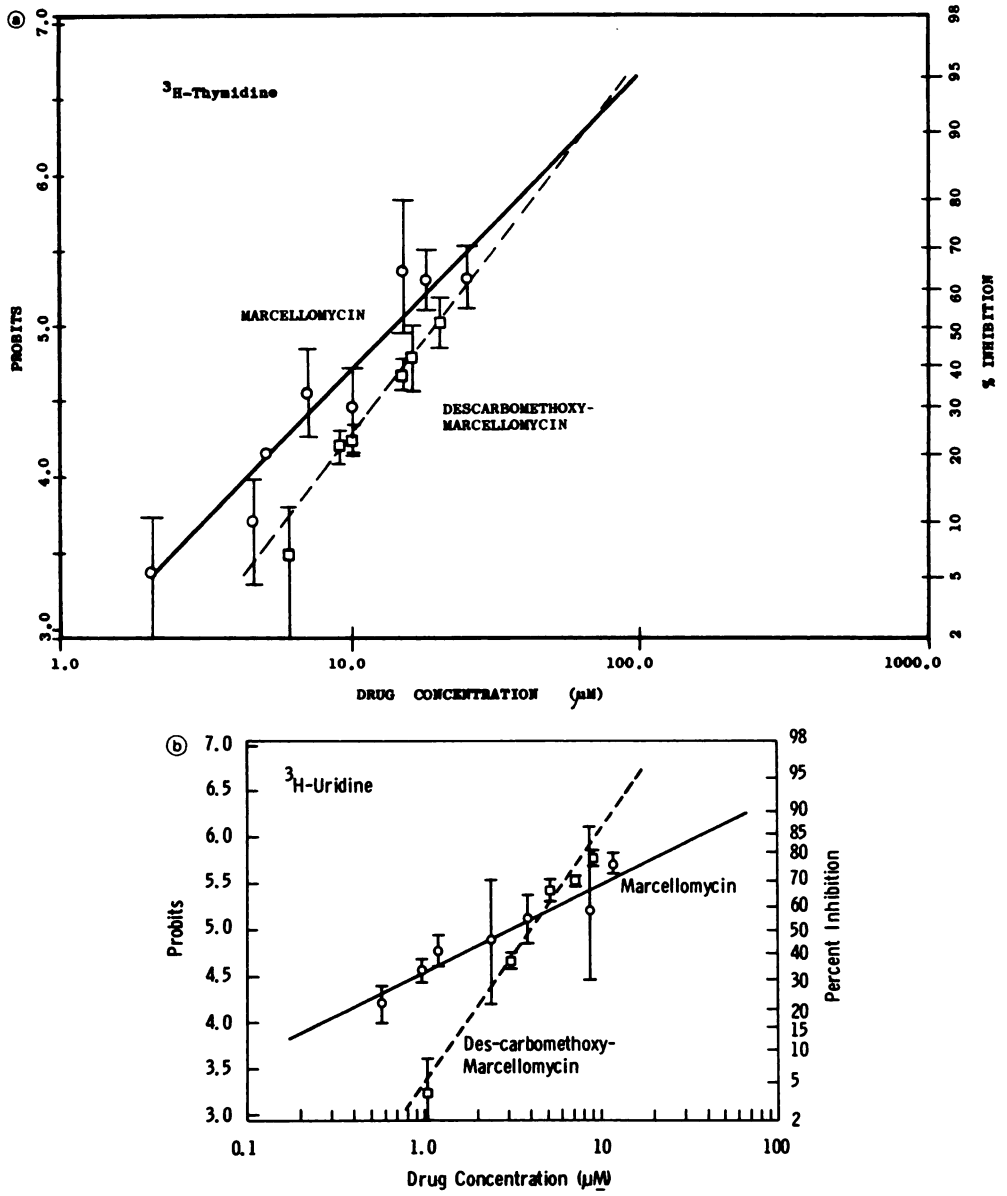


FIG. 3. *Comparison of the inhibitory effects of MCM and D-MCM on thymidine and uridine incorporation*
The experiments were performed by measuring the incorporation of labeled precursors into acid-insoluble material as indicated in MATERIALS AND METHODS. The data presented are the result of triplicate or quadruplicate experiments. a, inhibitory effects on the incorporation of thymidine; b, inhibitory effects on the incorporation of uridine. (O——O), MCM; (□----□), D-MCM. Linear regression analysis was applied to each curve, and coefficient of correlation values of 0.952 and 0.989 were obtained for MCM and D-MCM, respectively, for thymidine incorporation. Values of 0.956 and 0.988 were obtained for MCM and D-MCM, respectively, for uridine incorporation.

M EDTA. Centrifugation was carried out in a Beckman SW27 rotor at 24,000 to 26,000 rpm for 16–20 hr at 4° in a Beckman model L5-65 ultracentrifuge. The gradients were fractionated into 0.6 ml, 1 ml or 1.2 ml fractions and the absorbance profile at 254

nm was recorded with an ISCO density gradient fractionator. The radioactivity in 0.1 ml aliquots of the sucrose gradient fractions was determined as described previously in MATERIALS AND METHODS.

Cell culture techniques. NHAC were grown exponentially in suspension culture as indicated previously. Asynchronous pop-

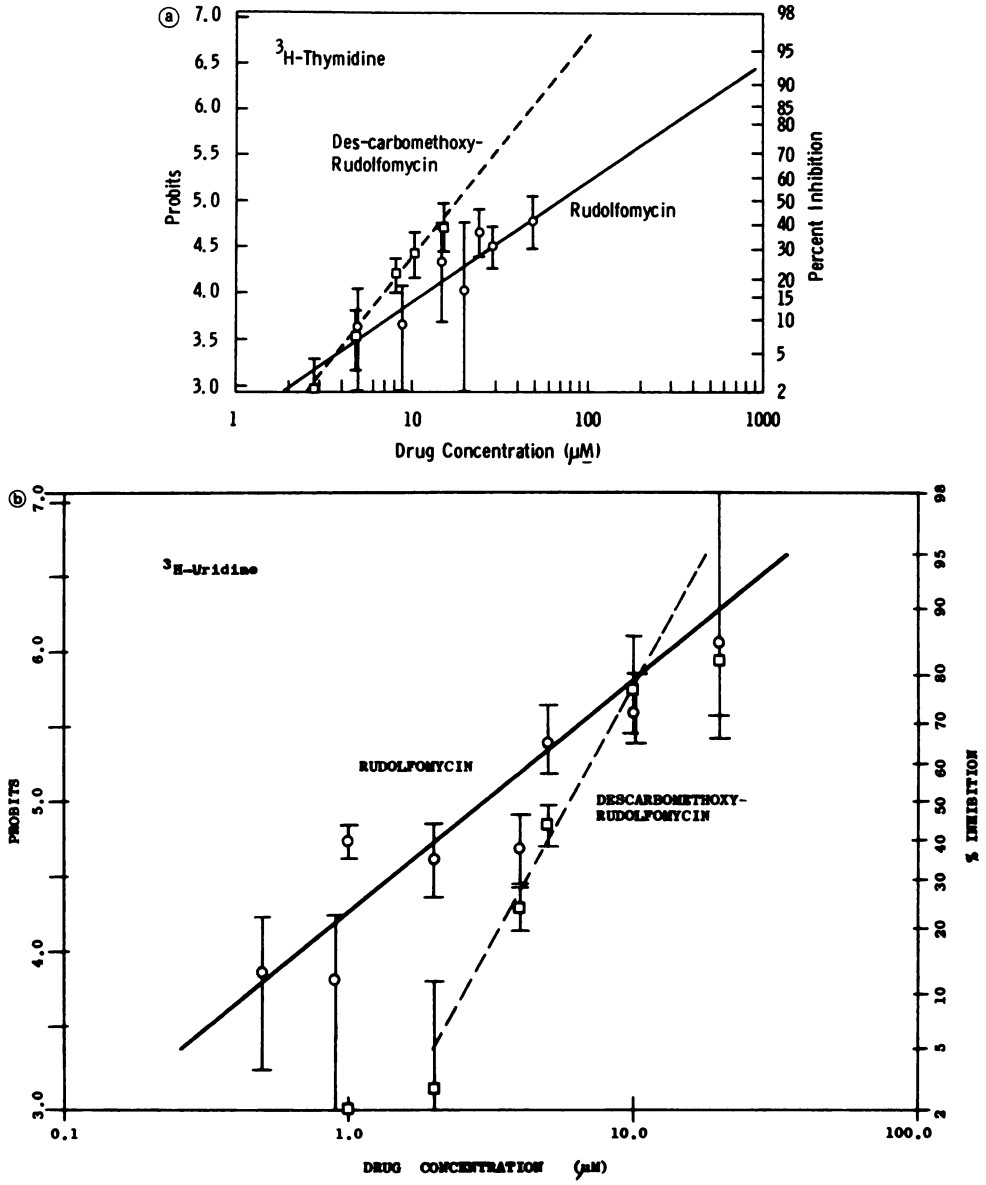


FIG. 4. *Comparison of the inhibitory effects of RDM and D-RDM on thymidine and uridine incorporation*
The experiments were performed by measuring the incorporation of labeled precursors into acid-insoluble material as indicated in MATERIALS AND METHODS. The data presented are the result of duplicate or triplicate experiments. a, inhibitory effects on thymidine incorporation; b, inhibitory effects on uridine incorporation. (○——○), RDM; (□----□), D-RDM. Linear regression analysis was applied to each curve, and coefficient of correlation values of 0.926 and 0.988 were obtained for RDM and D-RDM, respectively, for thymidine incorporation. Values of 0.936 and 0.962 were obtained for RDM and D-RDM, respectively, for uridine incorporation.

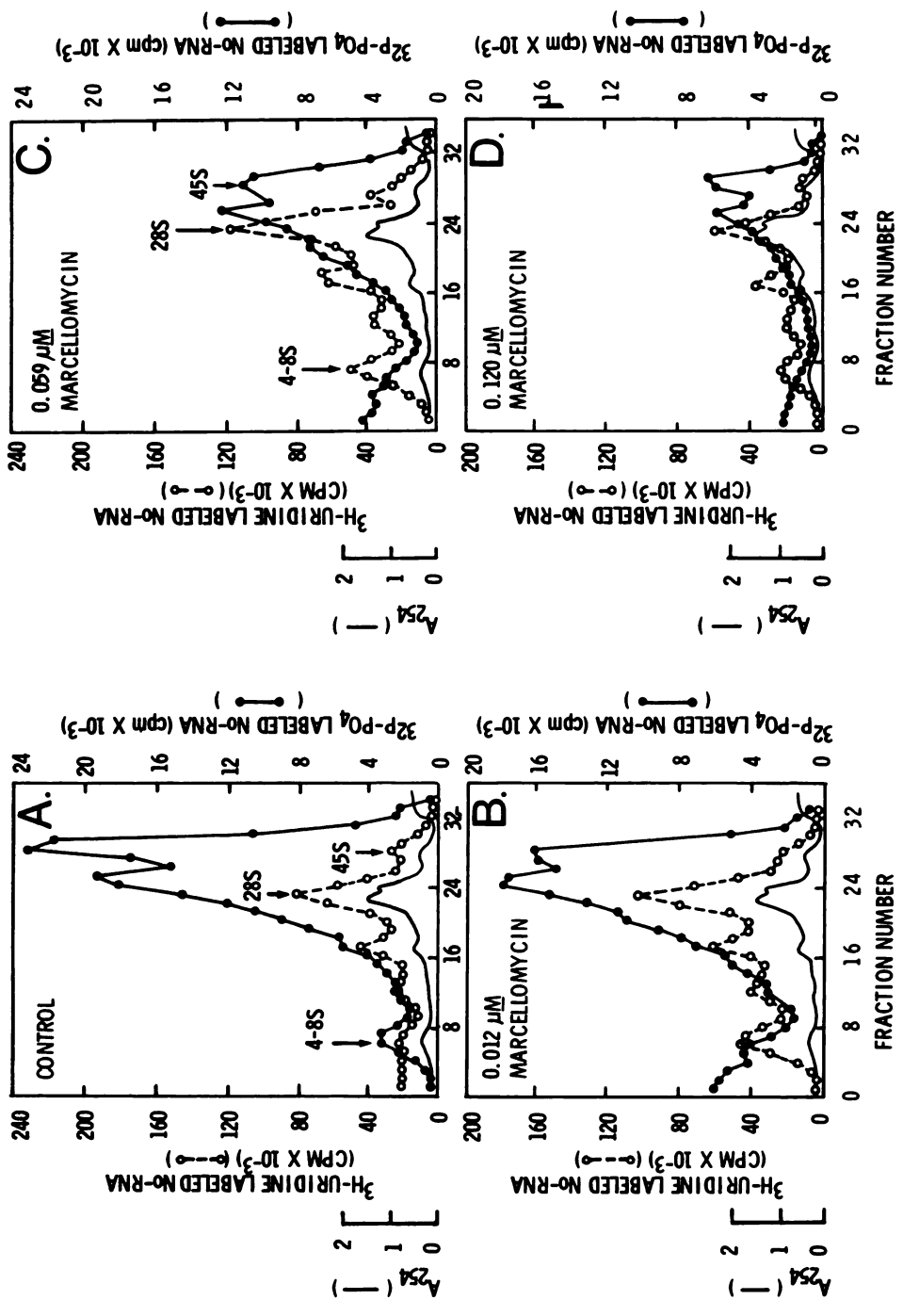


FIG. 5. *Sucrose density gradient sedimentation profiles of nucleolar RNA extracted from control and MCM-treated NHAC*

Procedures used are as indicated in MATERIALS AND METHODS. Centrifugation was for 16–18 hr at 26,000 rpm and gradients were fractionated, collecting 1.0 ml fractions. Patterns are displayed with the bottoms of the gradients to the right of each pattern. The four patterns represent the results of a single experiment.

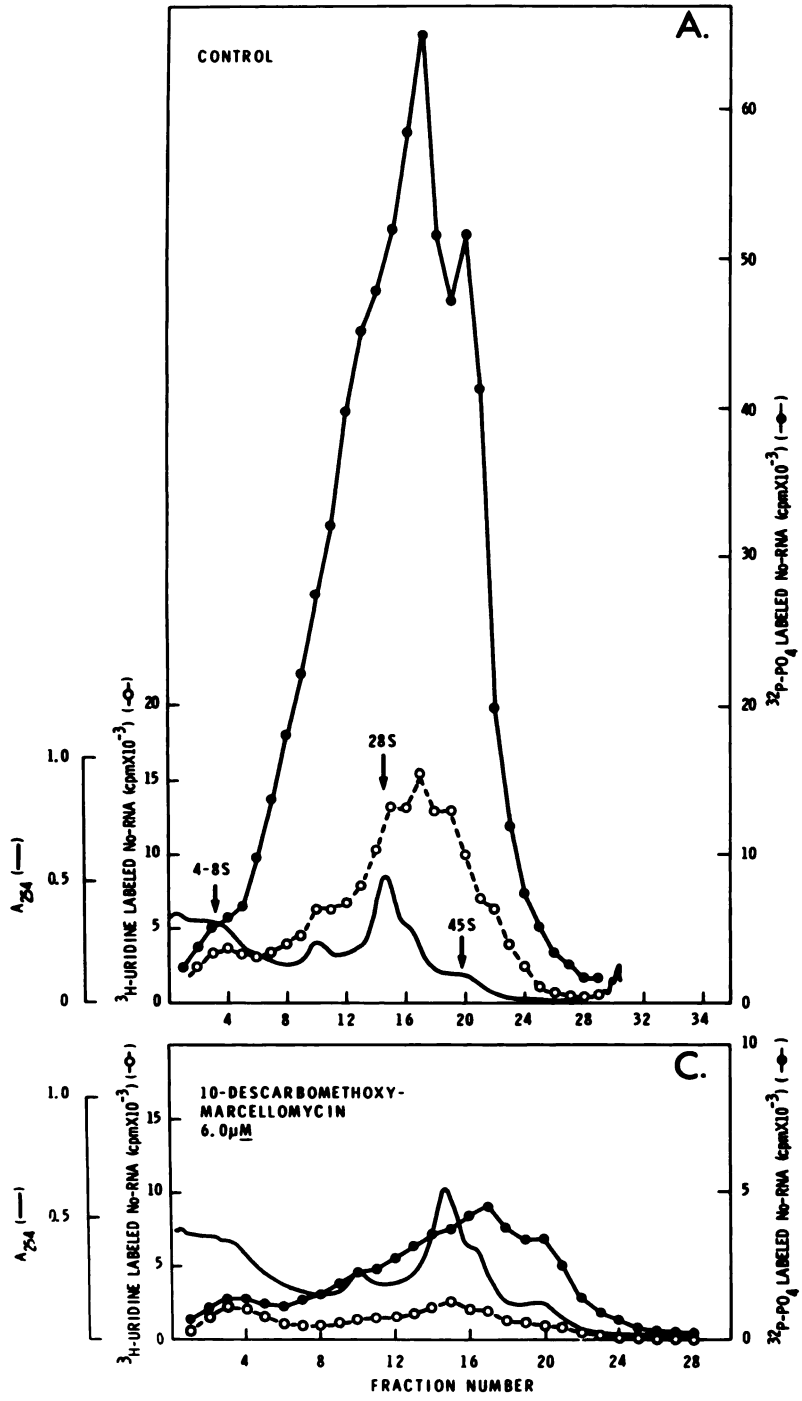
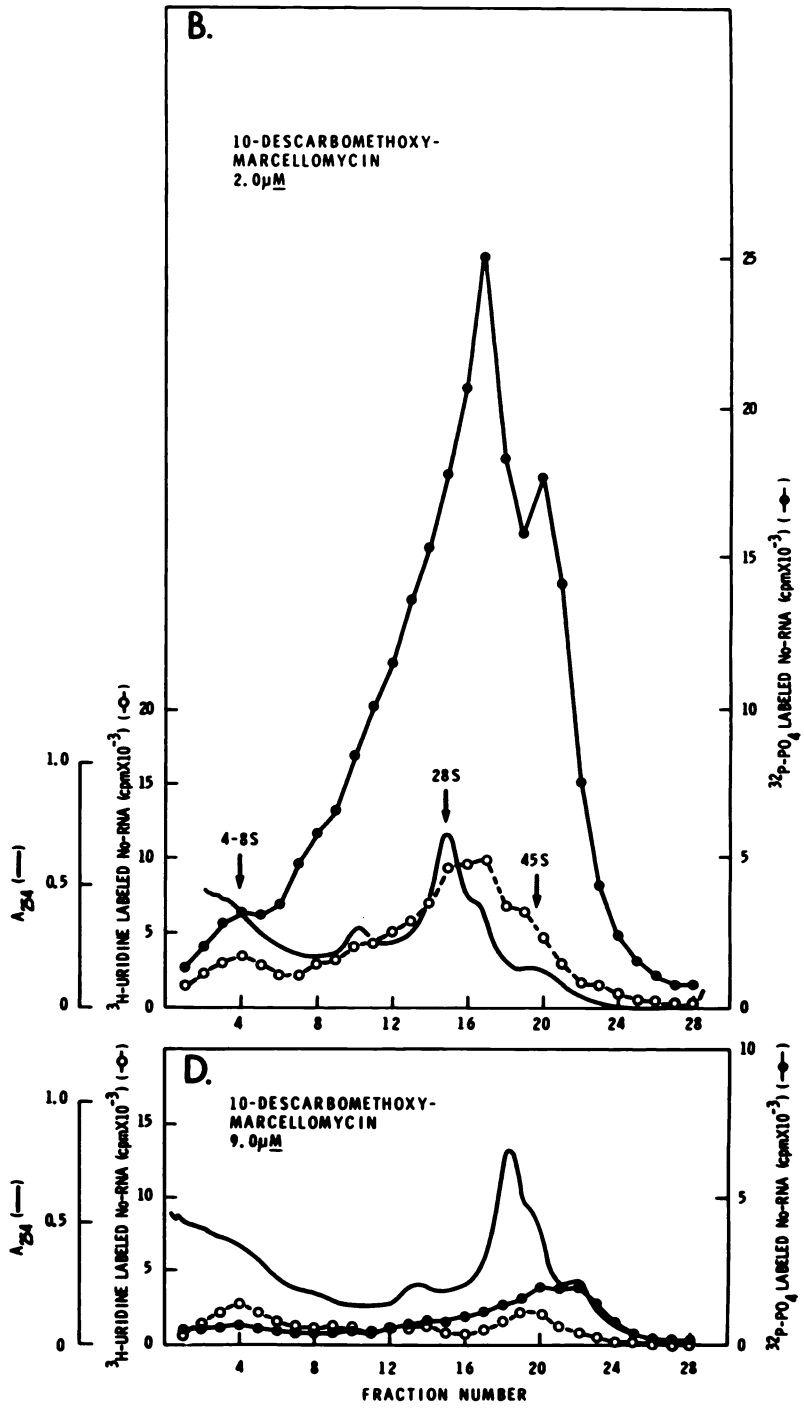


FIG. 6. *Sucrose density gradient sedimentation profiles of nucleolar RNA extracted from control and D-MCM-treated NHAC*

Procedures used are as indicated in MATERIALS AND METHODS. Centrifugation was for 18–20 hr at 24,000 rpm and gradients were fractionated, collecting 1.2 ml fractions. Patterns are displayed with the bottoms of the gradients to the right of each pattern. The four patterns represent the results of a single experiment.



ulations of cells in logarithmic growth phase were used. Cells were plated at 1000–2000 cells/plate in sterile 60 mm × 15 mm petri plates using RPMI-1640 medium containing 10% fetal calf serum, 1% penicillin-streptomycin and 1% glutamine. Plates were incubated overnight at 37° in a CO_2-incubator to allow attachment of cells to the plate.

Cells were treated with drugs under sterile conditions, allowed to react for 15 minutes followed by aspiration of medium. Plates were washed one time with 5 ml of sterile NKM solution, followed by the addition of 3 ml of fresh medium. Plates were incubated for 7–14 days at 37° in a CO_2-incubator. Viability was measured by the ability of a cell to form a colony of greater than 50 cells. Colonies were fixed with 0.5% crystal violet in 95% ethanol. The plates were dried and colonies were counted. Mean and standard deviation of triplicate samples were determined for each drug concentration. The data were analyzed by plotting the log of the survival fraction (number of colonies in each sample/number of colonies in the control) versus the drug concentration.

In vivo antitumor effects. The compounds were tested, as previously described (23), against the transplanted leukemia L-1210 in BDF_1 female mice inoculated with 10^6 ascitic cells implanted ip. The drugs were administered ip once daily on days 1 through 5 and the MST in days was evaluated. The reported effect is % T/C = (MST treated/MST control) × 100, with T/C ≥ 125 considered significant tumor inhibition (prolongation of host survival).

RESULTS

Effects on whole cellular macromolecular synthesis. The effects of MCM, D-MCM, RDM and D-RDM on DNA and RNA synthesis were determined by measuring the incorporation of radioactive precursors, ^{3}H-thymidine and ^{3}H-uridine, respectively, into acid-insoluble products as indicated in MATERIALS AND METHODS. The percentage inhibition of incorporation of cellular-associated radioactivity into TCA precipitable radioactivity, relative to the control, was then plotted versus the log of the drug concentration for probit analysis as shown in Figs. 3 and 4. Probit analysis permits the accurate quantitation of the drug-induced inhibitory effects of the anthracyclines tested. This analysis linearizes the center region (near the 50% inhibition point) of a dose-percent inhibition curve, thereby allowing for a straight line to be fitted by the weighted least-squares method (24). Linear regression analysis was therefore applied to the probit plots of each drug and the best-fit lines for each drug were constructed. The coefficients of correlation obtained varied from 0.92 to 0.99.

Figures 3a and 3b show a comparison of the inhibitory effects of MCM and D-MCM on DNA and RNA synthesis, respectively. The IC_{50} values for DNA synthesis for MCM (13.52 μM) and D-MCM (18.99 μM) are approximately equivalent. Similarly for RNA synthesis, the IC_{50} value for MCM (3.03 μM) approximates that of D-MCM (4.07 μM). The differences in slopes obtained for the dose-percent inhibition curves for MCM and its analogue for both DNA and RNA synthesis (Figs. 3a and 3b) suggest differences in mechanisms of action for the two drugs. As such, a comparison of the IC_{50} values obtained from non-parallel dose-response curves may not be valid but reflect a convenient point of comparison for the several agents studied.

Figures 4a and 4b show a comparison of the inhibitory effects of RDM and D-RDM on DNA and RNA synthesis, respectively. The IC_{50} value for DNA synthesis for RDM (69.70 μM) is nearly 4-fold higher than that of D-RDM (18.37 μM). This effect, although not seen with the MCM/D-MCM pair, suggests that the removal of the carbomethoxy group at position-10 of the RDM molecule confers increased DNA synthesis inhibitory activity. The IC_{50} value for RNA synthesis for RDM (3.65 μM) is 2-fold lower than that of D-RDM (7.24 μM). A comparison of the nucleic acid inhibitory effects of RDM and MCM shows that MCM had greater activity toward DNA and RNA synthesis inhibition and that the difference in DNA synthesis inhibitory activity is significant.

Effects on nucleolar pre-ribosomal RNA synthesis. The effects of MCM, D-MCM, RDM and D-RDM on No-RNA synthesis were determined by extracting No-RNA from the nucleoli of control and drug-treated cells, and analyzing the No-RNA on sucrose density gradients. The gradients were fractionated while monitoring absorbance at 254 nm and radioactivity, with the resulting sedimentation patterns shown in Figs. 5 and 6. Typically, increasing concentrations of anthracyclines progressively decreased the level of ^{32}P-PO_4 incorporation

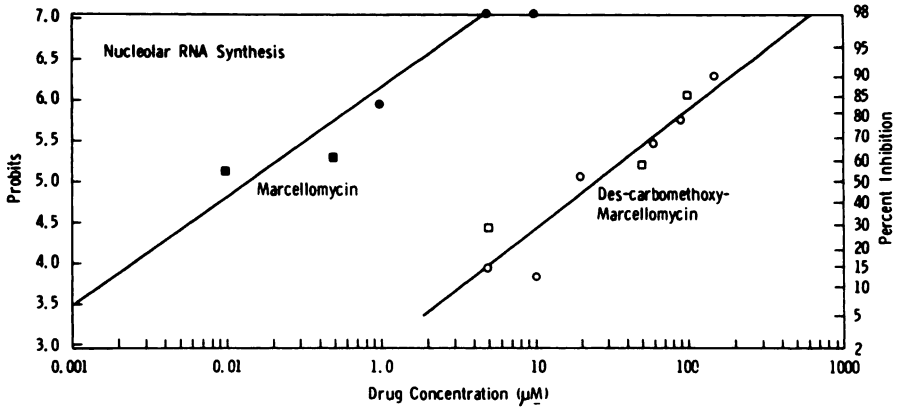


FIG. 7. *Probit analysis of the effects of MCM and D-MCM on nucleolar RNA synthesis*

The drug-induced inhibitory effects on nucleolar RNA synthesis were estimated by determining the ratio of the ^{32}P-PO_4 cpm in the 45S RNA peak (see Figs. 5 and 6) to the ^{3}H-uridine cpm in the 28S RNA peak/each drug concentration. These ratios were then compared to that of the control, converted to percent inhibition and plotted versus the log of the drug concentration. Linear regression analysis was then applied to each curve as indicated in Fig. 3. The coefficient of correlation values are 0.953 for MCM and 0.942 for D-MCM. Each curve represents the results of two separate experiments.

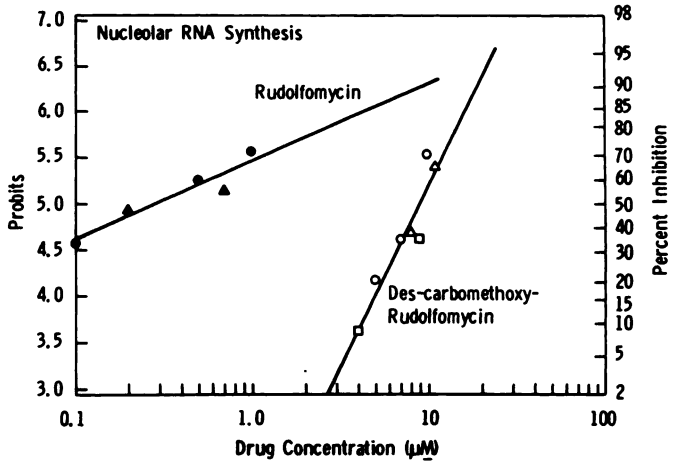


FIG. 8. *Probit analysis of the effects of RDM and D-RDM on nucleolar RNA synthesis*

The percent inhibition values were obtained as indicated in Fig. 7, and linear regression analysis applied to each curve as indicated in Fig. 3. The coefficient of correlation values obtained for RDM and D-RDM are 0.947 and 0.925, respectively. Each curve represents the results of two (RDM) or three (D-RDM) separate experiments.

into 45S No-RNA. The degree of inhibition of newly synthesized 45S No-RNA was determined (as reported previously) (11) by obtaining the ratio of ^{32}P-cpm in the 45S peak to the ^{3}H-uridine cpm in the 28S peak at each drug concentration, and comparing that with the value obtained for the control sample. The IC_{50} values for 45S No-RNA synthesis were then estimated from probit analyses of these ratios versus the log of the drug concentration as shown in Figs. 7 and 8. The IC_{50} values determined are a result of duplicate or triplicate experiments.

Figures 5 and 6 show sucrose density gradient absorbance and radioactivity profiles of No-RNA extracted from cells treated with varying concentrations of MCM and D-MCM, respectively. As indicated, increasing concentrations of MCM and D-MCM inhibited 45S No-RNA synthesis. Similar experiments were performed for RDM and D-RDM. Probit analyses of

the effects of the four anthracyclines on No-RNA synthesis are shown in Fig. 7 (MCM and D-MCM) and Fig. 8 (RDM and D-RDM). Probit analyses of these experiments demonstrated that the IC_{50} values for No-RNA synthesis were 0.014 μM, 2.56 μM, 0.29 μM, and 9.13 μM for MCM, D-MCM, RDM and D-RDM, respectively. Thus, the ratio of the IC_{50} values for No-RNA synthesis of D-MCM and MCM was 183, and the ratio for D-RDM and RDM was 31 (see Table 2). Clearly the removal of the 10-carbomethoxy group markedly reduced the effectiveness of these agents in inhibiting No-RNA synthesis.

Effects on cell survival. To compare the effects of MCM, D-MCM, RDM and D-RDM on cell viability, colony survival studies were employed, as indicated in MATERIALS AND METHODS. Plates containing fixed numbers of cells were treated with drug, washed, fresh media added and cells allowed to proliferate over 7 to 14 days. Retention of cell viability was measured by the ability of cells to form colonies of 50 or more cells. The log of the survival fraction was then plotted versus drug concentration, and IC_{50} values were estimated.

Figure 9a shows the results of the effects of MCM and D-MCM on cell viability. The IC_{50} value of D-MCM (3.80 μM) was approximately 5-fold higher than that of MCM (0.75 μM), thus reflecting a 5-fold loss in cytotoxicity upon removal of the carbomethoxy group at position-10 of the MCM molecule. Similar results are seen in Fig. 9b

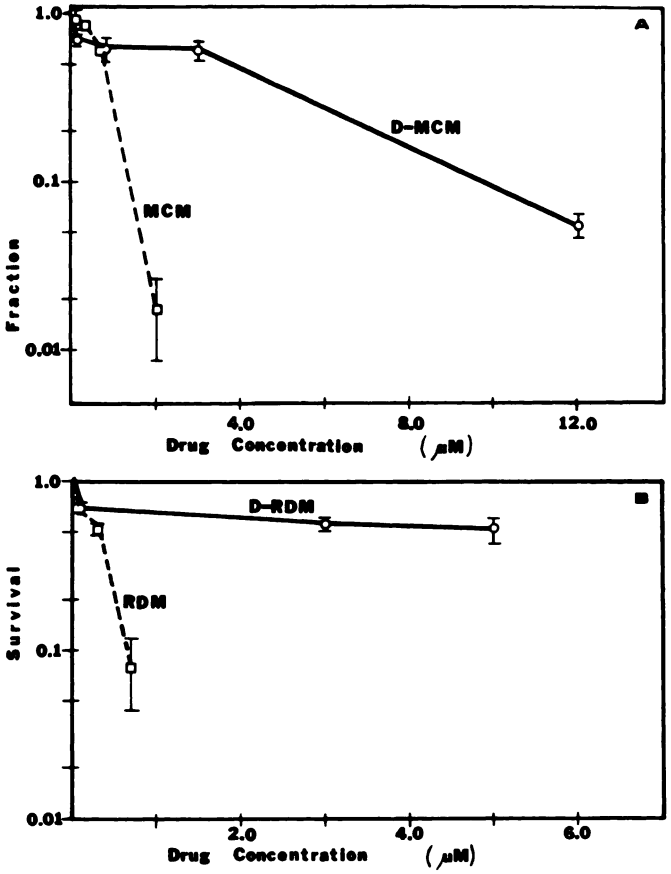


FIG. 9. *Fraction of cells surviving 7-14 days after treatment*

A) Treatment with different concentrations of MCM and D-MCM. The above represents the results of triplicate samples in each of two experiments. B) Treatment with different concentrations of RDM and D-RDM. The above represents the results of triplicate samples in each of two experiments.

TABLE 1

In vivo antitumor activity of marcellomycin, descarbomethoxy-marcellomycin, rudolfomycin and descarbomethoxy-rudolfomycin on L-1210 leukemia

The tumor inoculum was 10^6 ascites cells implanted ip. The hosts were BDF_1 (C57BL/6 × DBA/2 F_1) female mice.

Drug	Days treated	Total injection schedule	Dose	MST[a]	Effect[b] MST	Average weight change	Survivors day 5
			(mg/kg/inj)	*(days)*	*(% T/C)*	*(g)*	
Marcellomycin	QD 1–5[c]	5	3.2	6.0	86	−2.7	3/6
			1.6	7.5	107	−2.4	4/6
			0.8	10.5	150	+0.9	6/6
			0.4	10.0	143	+0.6	5/6
			0.2	9.0	129	+2.4	6/6
			0.1	8.0	114	+1.7	6/6
Descarbomethoxy-marcellomycin	QD 1–5	5	16.0	8.5	121	−0.9	6/6
			8.0	9.0	129	−0.3	6/6
			4.0	9.0	129	+0.9	6/6
			2.0	8.0	114	+2.2	6/6
			1.0	8.0	114	+2.6	6/6
Rudolfomycin	QD 1–5	5	3.2	9.0	129	−0.6	4/6
			1.6	9.0	129	−0.4	5/6
			0.8	10.0	143	+0.3	6/6
			0.4	9.0	129	+0.9	6/6
			0.2	9.0	129	+2.2	6/6
			0.1	8.0	114	+0.8	6/6
Descarbomethoxy-rudolfomycin	QD 1–5	5	32.0	9.5	136	+1.3	6/6
			16.0	9.0	129	+2.7	6/6
			8.0	8.0	114	+1.3	6/6
			4.0	7.5	107	+1.8	6/6
			2.0	7.0	100	+2.1	6/6
			1.0	7.0	100	+3.2	6/6
Control			Saline	7.0	—	+2.6	10/10

[a] MST = median survival time.

[b] Effect: % T/C = (MST treated/MST control) × 100. T/C ≥ 125 considered significant antitumor.

[c] QD 1–5: Daily administration of drug ip on days 1 to 5.

comparing the effects of RDM and D-RDM on cell viability. The IC_{50} value of D-RDM (~5.0 μM) is over 16-fold greater than that of RDM (0.31 μM), again reflecting a loss of cytotoxicity upon removal of the carbomethoxy group.

The *in vitro* data are in qualitative agreement with the *in vivo* antitumor activity in the mouse L-1210 leukemia system as shown in Table 1. When administered in multiple dose schedules (5 injections), and when comparing minimum effective doses (T/C values of 129) D-MCM (4 mg/kg/injection) was approximately 20-fold less potent than MCM (0.2 mg/kg/injection). Similarly, D-RDM (16 mg/kg/injection) was approximately 80-fold less potent than RDM (0.20 mg/kg/injection). These results indicate that the descarbomethoxy analogues are considerably less active against L-1210 leukemia *in vivo* than their parent compounds.

DISCUSSION

Previous studies from this laboratory demonstrated that anthracyclines may be

TABLE 2

Summary of the 50% inhibitory concentrations (IC_{50} values) for MCM, D-MCM, RDM, and D-RDM for DNA, RNA, and No-RNA synthesis, and cell viability in comparison with in vivo antitumor data

	In vitro IC_{50} values[a]			Cell[b] viability	*In vivo*[c] antitumor activity in L-1210 leukemia
	DNA synthesis	RNA synthesis	No-RNA[d] synthesis		
	(μM)	*(μM)*	*(μM)*	*(μM)*	*(mg/kg/inj)*
Marcellomycin (MCM)	13.52	3.03	0.014	0.75	0.20
10-descarbomethoxy-marcellomycin (D-MCM)	18.99	4.07	2.56	3.80	4.0
Ratio D-MCM/MCM	1.40	1.34	183.0	5.10	20.0
Rudolfomycin (RDM)	69.70	3.65	0.29	0.31	0.20
10-descarbomethoxy-rudolfomycin (D-RDM)	18.37	7.24	9.13	>5.0	16.0
Ratio D-RDM/RDM	0.264	1.98	31.0	>16.0	80.0

[a] IC_{50} values were determined using probit analysis as indicated in Fig. 3.

[b] Cell viability IC_{50} values were determined from plots similar to those shown in Fig. 9.

[c] *In vivo* antitumor activity determined in multiple dose schedule (5 total doses) in the L-1210 leukemia system monitoring median survival time of treated relative to control. Doses represent those producing a T/C of 129. See Table 1.

[d] The IC_{50} values were determined by probit analysis as shown in Figs. 7 and 8.

TABLE 3

The inhibition of No-RNA synthesis relative to whole cellular DNA and RNA syntheses by four anthracyclines

Drug	IC_{50} DNA[a]/IC_{50} whole cell RNA	IC_{50} DNA[a]/IC_{50} No-RNA
Marcellomycin (MCM)	4.46	966
10-descarbomethoxy-marcellomycin (D-MCM)	4.67	7.42
Rudolfomycin (RDM)	19.10	240
10-descarbomethoxy-rudolfomycin (D-RDM)	2.54	2.01

[a] Values obtained from Table 2.

divided into two classes. Class II anthracyclines were shown to inhibit No-RNA synthesis at concentrations much lower than those required to inhibit DNA synthesis. The studies suggested that No-RNA synthesis inhibition may be an important biochemical effect accounting for the mechanism of antitumor action of class II anthracyclines. The studies reported in the present communication provide additional support for this concept.

Table 2 summarizes the data on inhibition of DNA, RNA and No-RNA syntheses, and cell viability for MCM, D-MCM, RDM, and D-RDM. Also included are *in vivo* antitumor data in the mouse L-1210 leukemia system. A comparison of the ratios of the activities of the 10-descarbomethoxy analogues to those of the parent compounds shows that removal of the 10-carbomethoxy moiety resulted in 1) a significant reduction in *in vivo* antitumor activity, 2) a significant reduction in *in vitro* cytotoxicity to NHAC, and 3) a significant reduction in the No-RNA synthesis inhibitory activity.

Based on the current results, it is clear that for both RDM and MCM, removal of the carbomethoxy group at position-10 significantly decreased No-RNA synthesis inhibitory activity. It is also clear that there was a simultaneous decrease in cytotoxicity as determined by *in vitro* and *in vivo* techniques. Since no decrease in DNA synthetic inhibitory activity or whole cellular RNA synthetic inhibitory activity was observed, this suggests a direct relationship between nucleolar preribosomal RNA synthesis and cytotoxicity. Although additional studies are required to exclude other factors such as effects on mRNA synthesis or on membrane synthesis and function, the relationship described is striking and supports the observations previously reported (11).

In addition to D-MCM and D-RDM,

RDM is a new anthracycline which was recently isolated and chemically characterized (18). The studies reported in the present communication allow classification of RDM on the basis of structure activity relationships previously reported (11), and confirm those observations with studies on an additional compound. Table 3 summarizes the information on the four compounds relative to No-RNA synthesis inhibitory specificity. Inasmuch as rudolfomycin is a trisaccharide, previously reported structure activity relationships suggested that it should be classified as a class II anthracycline. The ratio of the IC_{50} for DNA synthesis to the IC_{50} for No-RNA synthesis clearly demonstrates that rudolfomycin, as predicted, is a class II anthracycline. Moreover, the present studies suggest that the 10-carbomethoxy moiety is also important in determining No-RNA synthesis inhibitory specificity.

Although RDM is a class II anthracycline, it is significantly less potent than MCM, another trisaccharide, as an inhibitor of DNA or No-RNA synthesis. Further, the ratio of DNA to No-RNA synthesis IC_{50} values demonstrates that it is less No-RNA selective than MCM. These results and previously reported results (11) suggest that presence of a terminal deoxy-fucose in the glycosidic side chain, e.g., MCM or musettamycin (17, 11), confers additional specificity for the inhibition of No-RNA synthesis.

Finally, it should be noted that although MCM inhibits No-RNA synthesis at significantly lower concentrations than RDM, they have approximately equipotent *in vitro* and *in vivo* cytotoxic activities. This suggests that although No-RNA synthesis may account for a major portion of the antitumor activities of class II anthracyclines, other activities may also be important. This also confirms the necessity of comparing analogues in this series which differ in only one characteristic when attempting to develop structure activity relationships.

To characterize further the actions of class II anthracyclines, studies are in process in this laboratory to determine other important sites of anthracycline antitumor action, including DNA binding studies, subcellular distribution studies, studies on the effects of these compounds on membrane synthesis and function, and studies of the effects on other types of RNA species.

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REFERENCES

1. Waring, M. (1970) Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: Evidence for molecular models involving intercalation. *J. Mol. Biol.*, **54**, 247–279.
2. DiMarco, A., Arcamone, F. & Zunino, F. Daunomycin and adriamycin and structural analogues: Biological activity and mechanism of action, in *Antibiotics III-Mechanism of Action of Antimicrobial and Antitumor Agents.* (J. W. Corcoran and F. E. Hahn eds.), 101–128. Springer-Verlag, Berlin, 1975.
3. Gabbay, E. J., Grier, D., Fingerle, R. E., Reimer, R., Levy, R., Pearce, S. W. & Wilson, W. D. (1976) Interaction specificity of the anthracyclines with deoxyribonucleic acid. *Biochemistry*, **15**, 2062–2069.
4. Tsou, K. C. & Yip, K. F. (1976) Effect of deoxyribonuclease on adriamycin-polynucleotide complexes. *Cancer Res.* **36**, 3367–3373.
5. Theologides, A., Yarbro, J. W. & Kennedy, B. J. (1968) Daunomycin inhibition of DNA and RNA synthesis. *Cancer*, **21**, 16–21.
6. Rusconi, A. & DiMarco, A. (1969) Inhibition of nucleic acid synthesis by daunomycin and its relationship to the uptake of the drug in HeLa cells. *Cancer Res.*, **29**, 1507–1511.
7. Meriwether, W. D. & Bachur, N. R. (1972) Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukemia. *Cancer Res.*, **32**, 1137–1142.
8. Danø, K., Frederiksen, S. & Hellung-Larsen, P. (1972) Inhibition of DNA and RNA synthesis by daunorubicin in sensitive and resistant ehrlich ascites tumor cells *in vitro*. *Cancer Res.*, **32**, 1307–1314.
9. Henry, D. W. Adriamycin, in *Cancer Chemotherapy* (Sartorelli, A. C. ed.), 15–57, 169th meeting Amer. Chem. Soc. Div. Med. Chem. Symp., April 7, 1975, Philadelphia, PA, 1975.
10. DiMarco, A., Silvestrini, R., DiMarco, S. & Dasdia,

T. (1965). Inhibiting effect of the new cytotoxic antibiotic daunomycin on nucleic acids and mitotic activity of HeLa cells. *J. Cell Biol.* **27**, 545–550.

11. Crooke, S. T., DuVernay, V. H., Galvan, L. & Prestayko, A. W. (1978) Structure-activity relationships of anthracyclines relative to effects on macromolecular syntheses. *Mol. Pharmacol.*, **14**, 290–298.
12. Pigram, W. J., Fuller, W. & Hamilton, L. D. (1972) Stereochemistry of intercalation: Interaction of daunomycin with DNA. *Nature New Biol.*, **235**, 17–19.
13. Murphree, S. A., Cunningham, L. S., Hwang, K. M. & Sartorelli, A. C. (1976) Effects of adriamycin on surface properties of sarcoma 180 ascites cells. *Biochem. Pharmacol.*, **25**, 1227–1231.
14. Murphree, S. A., Tritton, T. R. & Sartorelli, A. C. (1977) Differential effects of adriamycin on fluidity and fusion characteristics of cardiolipin containing liposomes. *Fed. Proceed.*, **36**, 303.
15. Zunino, F., Gambetta, R., DiMarco, A., Velcich, A., Zaccara, A., Quadrifuglio, F. & Crescenzi, V. (1977) The interaction of adriamycin and its β-anomer with DNA. *Biochim. Biophys. Acta*, **476**, 38–46.
16. Tong, G., Lee, W. W., Black, D. R. & Henry, D. W. (1976) Adriamycin analogs. Periodate oxidation of adriamycin. *J. Med. Chem.*, **19**, 395–398.
17. Nettleton, D. E. Jr., Bradner, W. T., Bush, J. A., Coon, A. B., Moseley, J. E., Myllymaki, R. W., O'Herron, F. A., Schreiber, R. H. & Vulcano, A. L. (1977) New antitumor antibiotics: Musettamycin and marcellomycin from bohemic acid complex. *J. Antibiotics*, **30**, 525–529.
18. Doyle, T. W., Grulich, R. E., Nettleton, D. E. & Essery, J. M. New anthracyclines from the bohemic acid complex. Presented at the 61st Canadian Chemical Conference and Exhibit, Winnipeg, Manitoba, June 4–7, 1978, in press.
19. Wiley, P. F., Johnson, J. L. & Houser, D. J. (1977) Nogalamycin analogs having improved antitumor activity. *J. Antibiotics*, **30**, 628–629.
20. Mauritzen, C. M., Choi, Y. C. & Busch, H. (1971) Preparation of macromolecules of very high specific activity in tumor cells *in vitro*. *Methods in Cancer Res.*, **6**, 253–282.
21. Muramatsu, M. & Busch, H. (1967) Isolation, composition and function of nucleoli of tumors and other tissues. *Methods in Cancer Res.*, **2**, 303–359.
22. Steele, W. J., Okamura, N. & Busch, H. (1965) Effects of thioacetamide on the composition and biosynthesis of nuclear and nucleolar RNA. *J. Biol. Chem.*, **240**, 1742–1749.
23. Bradner, W. T. & Hutchison, D. J. (1966) Neocarzinostatin (NSC-69856): An antitumor antibiotic effective against ascitic leukemia L1210 in mice. *Cancer Chemoth. Reports*, **50**, 79–84.
24. Litchfield, J. T., Jr. & Wilcoxon, F. (1949) A simplified method of evaluating dose-effect experiments. *J. Pharm. & Exptl. Therapeut.* **96**, 99–113.