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Structure-Activity Relationships of Anthracyclines Relative to Effects on Macromolecular Syntheses

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SUMMARY

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The anthracyclines studied may be divided into two classes on the basis of their effects on DNA and RNA syntheses. Class I anthracyclines—adriamycin, carminomycin, and pyrromycin—inhibit DNA, whole cell RNA, and nucleolar RNA syntheses at approximately comparable concentrations. Class II anthracyclines—aclacinomycin, marcellomycin, and musettamycin—inhibit whole cellular RNA synthesis at 6–7-fold lower concentrations than those required to inhibit DNA synthesis, and nucleolar RNA synthesis at 170–1250-fold lower concentrations than necessary to inhibit DNA synthesis. Structure-activity relationship studies suggest that the presence of di- or trisaccharides confers nucleolar RNA synthesis-inhibitory specificity on anthracyclines. None of the anthracyclines studied had demonstrable effects on processing of preribosomal RNA.

INTRODUCTION

Anthracyclines have been reported to intercalate between adjacent nucleotides in native DNA, resulting in significant alterations in its secondary structure, its sensitivity to degradation by nucleases, and altered template activity (1–5). Exposure of intact cells to anthracyclines has been reported to result in inhibition of DNA and RNA syntheses, but in only minimal inhibition of protein synthesis (6–9).

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Although variability resulting from differences in cell type and culture conditions has been noted (10), most anthracyclines, with two exceptions, have been reported to inhibit bulk RNA synthesis at concentrations approximately equivalent to or greater than the concentrations necessary to inhibit DNA synthesis (6–9, 11). Cinerubin and aclacinomycin A have been reported to inhibit RNA synthesis at concentrations 10-fold less than those required to inhibit DNA synthesis (12, 13). The structure-activity relationships that may account for the differences between cinerubin and aclacinomycin and the other anthracyclines have not been explored fully.

Adriamycin, daunomycin, and carminomycin have been reported to inhibit nucleolar preribosomal RNA synthesis at 2–5-fold lower concentrations than required to inhibit whole cell RNA or DNA synthe-

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sis (9, 14). The effects of cinerubin and aclacinomycin on nucleolar RNA synthesis have not been reported. Moreover, the effects of anthracyclines on the processing of nucleolar preribosomal RNA have not been fully studied (12).

Since several new anthracyclines with structural similarities to aclacinomycin and cinerubin have recently been isolated and purified, the studies presented in this publication were designed (a) to determine the concentrations of the new anthracyclines required to inhibit DNA, RNA, and protein syntheses, (b) to determine the concentrations of the newer anthracyclines necessary to inhibit nucleolar RNA synthesis relative to that of adriamycin, (c) to determine the effects of several anthracyclines on preribosomal RNA processing, and (d) to elucidate the structure-activity relationships that may result in anthracyclines that inhibit nucleolar RNA synthesis at concentrations significantly lower than those required to inhibit DNA synthesis.

MATERIALS AND METHODS

Drugs and isotopes. Adriamycin (Adria Laboratories, Wilmington, Del.) was a generous gift from Dr. M. Lane. Carminomycin, marcellomycin, musettamycin, and pyrromycin were provided by Bristol Laboratories (15, 16). Aclacinomycin was provided by Sanraku-Ocean Company, Ltd., Fujisawa, Japan. All drugs were shown to be at least 95% pure by high-pressure liquid chromatography. Tritiated uridine, thymidine, and valine were obtained from Schwarz/Mann. Carrier-free $^{32}P_i$ was obtained from Union Carbide.

Novikoff hepatoma cells. Novikoff hepatoma ascites cells type N_1S_1-73, were maintained in monolayer cultures at 37° in sealed glass prescription bottles with Roswell Park Memorial Institute type 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin A. For drug studies, Novikoff hepatoma ascites cells were grown in shaker culture (New Brunswick Gyrorotary shaking incubator) at 37° in Gibco-type 500-ml liquid media bottles in RPMI-1640 medium with 0.2% methylcellulose.

Novikoff hepatoma ascites cells used as "carrier" cells were obtained from rats bearing ascites cells as previously described (17).

Effects on whole cell macromolecular syntheses. Cells were grown to a density of approximately 8×10^5–1.0×10^6/ml, harvested by centrifugation at $800 \times g$ for 10 min at room temperature, and resuspended in fresh RPMI-1640 medium. The cell density was adjusted to 6×10^5–8×10^5/ml, and the cells were incubated in a 37° shaking water bath with gentle agitation for 30–60 min in closed vessels containing 5-ml aliquots of cell suspension. Then 0.05 ml of aqueous drug solution was added, and the cells were incubated with drugs for 15 min, followed by addition of [^{3}H]uridine, [^{3}H]thymidine, or [^{3}H]valine to a final concentration of 5×10^6 dpm/ml. The cells were then incubated for 2 hr with gentle agitation and chilled on ice.

The total radioactivity with which the cells 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 had been washed three times with 1-ml aliquots of NKM medium (0.14 M NaCl, 0.005 M KCl, and 0.008 M $MgCl_2$). TCA[3]-precipitable radioactivity was determined by filtering 1.0 ml of cell suspension through a glass fiber filter, after which the filter was washed three times with 1 ml of NKM medium, then twice with 1.0 ml of 5% TCA solution. The radioactivity remaining on the filter was then determined. The percentage of the total radioactivity and of the cellular radioactivity that was incorporated into macromolecules was then determined for each sample. All assays were performed in triplicate.

The radioactivity present in each sample was determined using 10 ml of scintillation fluor containing toluene, 1,4-dioxane, ethylene glycol monoethyl ether, naphthalene, 2,5-diphenyloxazole, and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]ben-

[3] The abbreviations used is: TCA, trichloracetic acid.

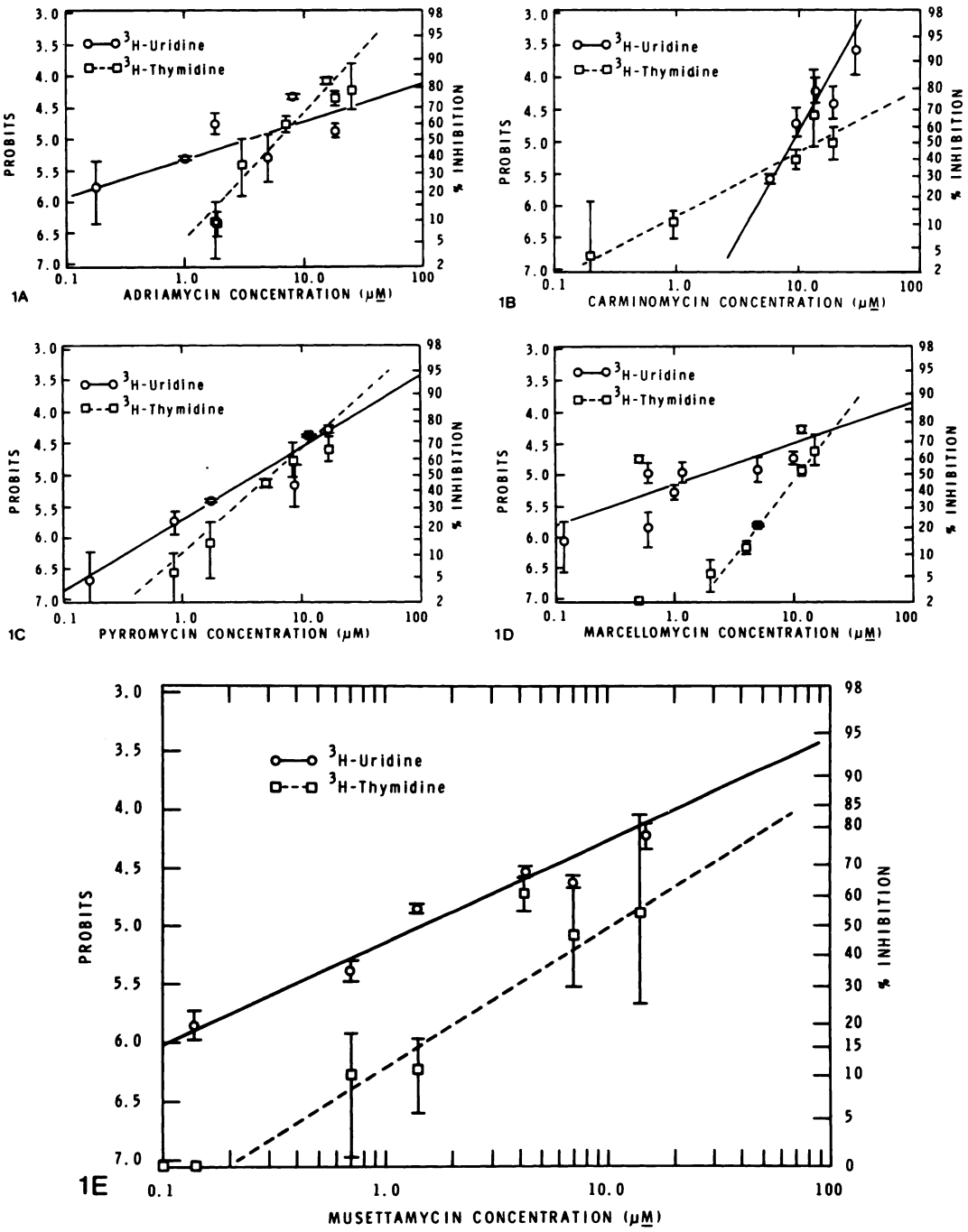

FIG. 1. *Probit analysis of effects of anthracyclines on DNA and RNA syntheses determined by measuring incorporation of labeled precursors into acid-insoluble material*

Cells were prepared and experiments performed as indicated in MATERIALS AND METHODS. The data were calculated as percentage of cellular radioactivity incorporated into TCA-insoluble radioactivity. A. Adriamycin. B. Carminomycin. C. Pyrromycin. D. Marcellomycin. E. Musettamycin. F. Aclacinomycin.

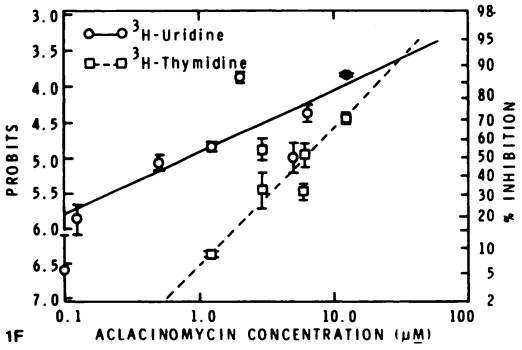

FIG. 1 F

zene in disposable polyethylene vials and counting in a Packard Tri-Carb model 3385 scintillation spectrometer.

Nucleolar RNA labeling, isolation, and fractionation. Novikoff hepatoma ascites cells were incubated for 30–40 hr in RPMI-1640 medium with 0.01 mCi/ml of [^{3}H]uridine (specific activity, 40–60 mCi/mmole). Cells were then collected by centrifugation at 850 × *g* for 10–15 min at room temperature, washed once with "phosphate-free" Earle's basic modified Eagle's medium (17), and resuspended with the same medium to a concentration of approximately 0.6–1.0 × 10^6 cells/ml.

After preliminary incubation for 30 min, the drug was added to the cell culture. Thirty minutes later, $^{32}P_i$ was added to a final concentration of 0.2 mCi/ml. The cells were incubated with isotope for 60 min and then chilled on ice. After washing twice with NKM medium, the cells were combined with 10–20-fold (by weight) of unlabeled "carrier" cells. Nucleoli were prepared as previously described (18).

RNA was extracted from isolated nucleoli by the sodium dodecyl sulfate–phenol method at 65° (18, 19). The RNA was redissolved in 4–8 ml of sterile deionized water and reprecipitated with 2 volumes of 95% ethanol with 2% potassium acetate for approximately 4 hr at −20°. The RNA was then resuspended in 1.0 ml of sterile deionized water and layered on a 33-ml linear sucrose gradient (5–40%) containing 0.1 ml of NaCl, 0.01 M sodium acetate (pH 5.1), and 0.001 M disodium EDTA. Centrifugation was carried out in an SW 27 rotor at 26,000 rpm for 16 hr at 4° in a Beckman model L2-65B ultracentrifuge. The gradients were fractionated into 0.6- or 1-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 above.

RESULTS

Effects on whole cellular macromolecular syntheses. Figure 1 shows a probit analysis of the effects of various anthracyclines on DNA and RNA syntheses as determined by the incorporation of radioactive precursors into TCA-precipitable products. Table 1 summarizes the results of the probit analyses for adriamycin, carminomycin, pyrromycin, marcellomycin, musettamycin, and aclacinomycin and compares the values determined in these studies with those obtained by other investigators employing different cell lines. The IC_{50} values for adriamycin, carminomycin, and pyrromycin for DNA synthesis were less than twice the IC_{50} values for RNA synthesis. For the anthracyclines marcellomycin, musettamycin, and aclacinomycin, the IC_{50} values for DNA synthesis were 6.6–7.6 times greater than their respective IC_{50} values for RNA synthesis. None of the anthracyclines inhibited protein synthesis to a significant extent.

TABLE 1

IC_{50} values of anthracyclines for DNA and RNA syntheses

The IC_{50} (50% inhibitory concentration) values were determined using probit analyses as indicated in Fig. 1. Numbers in parentheses are values obtained from the literature.

Compound	IC_{50}	
	DNA	RNA
	μM	μM
Adriamycin	6.1 (0.8)[a]	3.2 (0.9)[a]
Carminomycin	14.7 (0.04)[b]	8.9 (0.2)[b]
Pyrromycin	5.7	4.5
Marcellomycin	11.3	1.7
Musettamycin	10.0	1.5
Aclacinomycin	6.3 (1.2)[a]	0.83 (0.12)[a]

[a] From Henry (12).

[b] Studied in *Micrococcus lysodeikticus* (20).

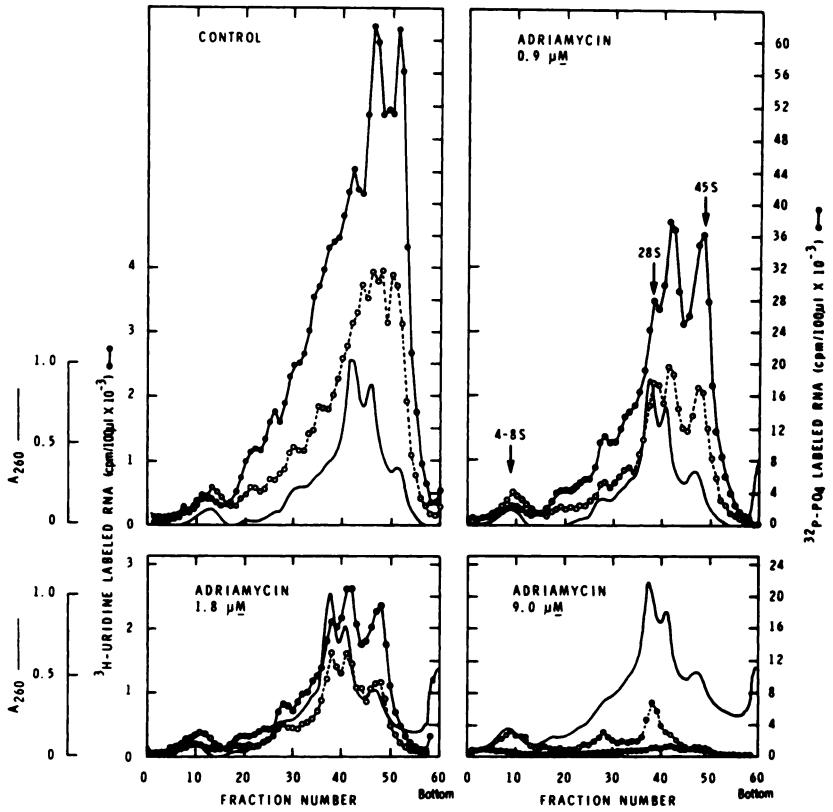

FIG. 2. *Sedimentation profiles of nucleolar RNA extracted from control and adriamycin-treated cells*
Cells were prepared, treated, and labeled as indicated in MATERIALS AND METHODS. Nucleolar RNA was extracted according to Steele *et al.* (19) and analyzed on linear 5–40% sucrose density gradients, also as indicated in MATERIALS AND METHODS. Gradients were fractionated, 0.6-ml fractions were collected, and absorbance at 254 nm was monitored by means of a flow cell and recording ultraviolet monitor. Radioactivity was determined as described in the text. 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.

On the basis of results shown in Table 1, the anthracyclines can be tentatively divided into two classes. Class I anthracyclines—adriamycin, carminomycin, and pyrromycin—inhibited DNA and RNA syntheses at approximately equivalent concentrations. Class II anthracyclines—marcellomycin, musettamycin, and aclacinomycin—inhibited RNA synthesis at 6–7-fold lower concentrations than those required to inhibit DNA synthesis.

Effects of anthracyclines on nucleolar RNA synthesis. Since very limited amounts of the newer anthracyclines were available, a double-label technique was employed to assess the specific activity of 45 S RNA in each sucrose density gradient. Because the cells were labeled for 30 hr with [^{3}H]uridine, the [^{3}H]uridine radioactivity represents uniformly labeled nucleolar RNA. To determine the degree of inhibition of newly synthesized 45 S preribosomal RNA, the ratio of ^{32}P in the 45 S peak to [^{3}H]uridine in the 28 S peak was determined at each drug concentration and compared with the value observed in untreated cells. The IC_{50} for 45 S preribosomal RNA synthesis was then estimated from probit analyses of these ratios.

Figure 2 shows sucrose density gradient absorbance and radioactivity profiles of nucleolar RNA obtained from cells treated with various concentrations of adriamycin. Increasing concentrations of adriamycin progressively inhibited incorporation of ^{32}P into 45 S preribosomal RNA.

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