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Effect of Anthracycline Analogues on the Appearance of Newly Synthesized Total RNA and Messenger RNA in the Cytoplasm of Erythroleukemia Cells

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SUMMARY

Effects of the structural analogues, adriamycin (ADM), daunomycin (DNM), carminomycin (CMM), 4-demethoxydaunomycin (4D-DNM), pyrromycin (PYM), marcellomycin (MCM), and aclacinomycin (ACM) upon total cell RNA synthesis and the appearance of total RNA and poly(A)$^+$-RNA in the cytoplasm of uninduced Friend erythroleukemia cells were investigated. The anthracyclines inhibited cellular RNA synthesis with IC_{50} values of 1–3 μM (ADM, DNM), 0.3–0.5 μM (CMM, 4D-DNM, PYM), and 0.06 μM (MCM, ACM). IC_{50} values for the appearance of total RNA in the cytoplasm were consistently 2–3 times lower than those for total cell RNA synthesis for each anthracycline. IC_{50} values for the inhibition of poly(A)$^+$-RNA in the cytoplasm by ADM, DNM, and CMM were equivalent to those for total RNA synthesis. The values for MCM and ACM were 2–3 times higher than those for total RNA synthesis. The kinetic actions of drug-induced inhibition of poly(A)$^+$-RNA appearance in the cytoplasm and inhibition of total RNA synthesis were equivalent for ADM, DNM and CMM, whereas the other anthracyclines showed different kinetics. These studies confirm the greater sensitivity of nucleolar RNA synthesis to Class II anthracyclines in erythroleukemia cells and suggest that inhibition of post-transcriptional events may occur in cells exposed to PYM, MCM, and ACM but at higher concentrations than are required for inhibition of RNA synthesis.

INTRODUCTION

The cytotoxicity of anthracyclines has been attributed to their ability to inhibit DNA-dependent nucleic acid synthesis by intercalation into DNA (1–3). Exposure of cells in culture to anthracycline drugs at low concentrations (0.1–10 μM) produces marked inhibition of both DNA and RNA synthesis without appreciable effects on protein synthesis at comparable doses (4–13). In general, pre-ribosomal RNA synthesis is thought to be more sensitive to inhibition by anthracyclines than total cell RNA synthesis (5, 11–14).

This laboratory has evaluated the effect of structurally different anthracyclines upon the synthesis of DNA, total RNA, and nucleolar RNA. The results have led to the grouping of anthracyclines into two mechanistic classes: Class I, those anthracyclines that yield similar IC_{50} values for inhibition of DNA, total RNA, and nucleolar RNA synthesis, and Class II, those that are selective for the inhibition of nucleolar RNA synthesis (11).

Although it is recognized that anthracyclines intercalate into and bind to DNA (1–3, 15–21), there have been no structure-activity studies relating anthracyclines to inhibition of messenger RNA synthesis or possible intercalation of anthracyclines into double-stranded RNA to the extent that it may interfere with RNA processing and transport. An early observation by Crook et al. (9) that DNM,[2] while exerting little inhibition of heterogeneous nuclear RNA synthesis, appeared to alter physical characteristics of the hnRNA after drug treatment. Such a change may manifest itself by the interference of mRNA appearance in the cytoplasm without actually inhibiting RNA synthesis. Zähringer *et al.* (22) reported that the myocardial content of total RNA was decreased by 23% whereas mRNA was decreased by 52% in ADM-treated rats.

To elucidate further the mechanism of action of the anthracyclines we chose to investigate the likelihood that some anthracyclines may have a preference for the inhibition of either RNA synthesis, RNA processing to rRNA and mRNA within nuclei, or the transport of processed mRNA to the cytoplasm. We have approached this question by determining the effects of structurally different anthracycline analogues on the appearance of newly syn-

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[2] The abbreviations used are: DNM, daunomycin; ADM, adriamycin; CMM, Carminomycin; PYM, pyrromycin; MCM, marcellomycin; ACM, aclacinomycin; 4D-DNM, 4-demethoxydaunomycin; SDS, sodium dodecyl sulfate.

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thesized total RNA and mRNA in the cytoplasm of uninduced Friend erythroleukemia cells.

MATERIALS AND METHODS

Chemicals. The anthracycline antibiotics ADM, CMM, PYM, MCM, and ACM were supplied by Bristol Laboratories (Syracuse, N.Y.); DNM was purchased from Calbiochem (San Diego, Calif.), and 4D-DNM was a gift from Dr. Aurelio DiMarco (Farmitalia, Italy). Uridine, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, Tris, EDTA, and SDS were purchased from Sigma Chemical Company (St. Louis, Mo.); chloroform and aqueous phenol were from Fisher Scientific Company (Fair Lawn, N. J.); oligo(dT)-cellulose was obtained from P-L Biochemicals (Milwaukee, Wisc.); and 5,6-[^{3}H]uridine (33–42 Ci/mM) was a product of Schwarz-Mann (Orangeburg, N. J.).

Cell culture. Friend erythroleukemia cells (clone no. 745) were generously provided by Dr. Art Skoultchi (Albert Einstein College of Medicine, Bronx, N. Y.) and were grown for experimentation and maintenance in Dulbecco's minimal essential medium (4.5 g/liter of glucose) (Grand Island Biological Company, Grand Island, N. Y.) supplimented with 10% fetal calf serum (Grand Island Biological Company). Each liter of medium contained 100,000 units of potassium penicillin G (Sigma Chemical Company) and 100 mg of Kanamycin (Grand Island Biological Company). For each experiment, cultures were initiated in plastic flasks 2 days in advance to yield 400 ml of cells between 1.0×10^6 and 1.5×10^6 cells/ml in logarithmic growth. Incubation was carried out at 37° in 10% CO_2 in air atmosphere.

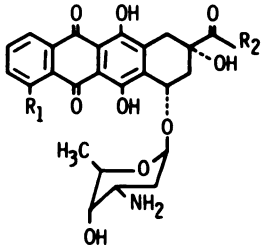
Incorporation of [^{3}H]uridine. Cells were diluted to 1.0×10^6 cells/ml with fresh, warm medium containing 10% fetal calf serum, and 50 ml of cell suspension were added to each of eight 50-ml polystyrene tubes (Falcon). The tubes were sealed and incubated for 30 min at 37° prior to drug addition. The anthracyclines (approximately 2 mg of each) were dissolved in 0.1 ml of dimethyl sulfoxide, followed by the addition of 0.9 ml of water and 10 μl of 500 mM sodium acetate (ph 4.5). The actual anthracycline concentrations were determined from absorbance in methanol obtained with a Hitachi 110 double-beam spectrophotometer and calculated from the molar extinction coefficients of Pachter *et al.* (21) These working solutions were generally made the day of the experiment but were never stored longer than 2 weeks. Serial dilutions of the drugs were made in water, and equal aliquots of these or water alone were added to eight tubes each containing 50 ml of cells. The incubation continued for 30 min, at which time 20 μl of a 10 mM solution of unlabeled uridine and 75 μl of [^{3}H]uridine were added to each tube. The incubation continued for 2 hr with periodic inversion of the tubes to ensure a uniform suspension of cells.

Isolation of cytoplasmic fractions and extraction of RNA. At the end of the 2-hr incubation, the cells were harvested and washed two times by centrifugation in cell wash buffer [5% sucrose, 100 mM KCl, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (pH 7.0), and 1.5 mM $MgCl_2$] at 250 × *g* for 10 min at 3°. All further steps were conducted in the cold with samples on ice. The washed cell pellets were resuspended in 5 ml of RNA-lysis buffer [100 mM KCl, 10 mM EDTA, and 10 mM Tris (pH 7.5)] by pipetting up and down in plastic pipettes. Aliquots were then removed and counted directly to obtain whole cell counts or added to 10% trichloroacetic acid for trichloroacetic acid-insoluble incorporation of label by whole cells. Cells were lysed by the addition of a 20% solution of Triton X-100 in water to the suspensions to a final concentration of 2% after mixing by inversion. Detergent-treated suspensions were centrifuged at 250 × *g* for 10 min to sediment nuclei, and the resulting supernatants were transferred by pipette to 50-ml polypropylene tubes. Aliquots were removed and added to 10% trichloroacetic acid for determination of trichloroacetic acid-insoluble incorporation of radioactivity into the cytoplasmic fraction. The remaining cytoplasmic fractions were then immediately mixed by vortexing, first with the addition of 0.025 volume of 20% SDS then with 1.0 volume of redistilled, water-saturated phenol. The RNA extraction continued at room temperature according to the procedure of Palmiter (23), involving addition of 1 volume of chloroform, mixing, and centrifugation. The interphase and aqueous layers were extracted once more with phenol-chloroform and twice with chloroform. Precipitation of RNA was accomplished by the addition of 2 volumes of ethanol and storage overnight at −10°.

Oligo(dT)-cellulose column chromatography. The procedure used for the isolation of poly(A)$^+$-RNA was essentially that described by Singer and Penman (24) with the exception that approximately 100 mg (dry weight) of oligo(dT)-cellulose was hydrated in application buffer [400 mM NaCl, 10 mM Tris (pH 7.5), and 0.5% SDS] and distributed among eight 1-ml disposable Pipetman tips with tips plugged by fine glass wool. Elution was expedited by centrifugation of columns, fitted with rubber sleeves, over glass tubes (13 × 100 mm) in a Beckman TH-6 centrifuge run at 500 rpm for 5 min at room temperature. Cytoplasmic RNA pellets were dissolved in 1.0 ml of application buffer with 0.2 ml (~$3A_{260}$ units) added to each column. The elution sequence was as follows: 2.0 ml of application buffer, 3.0 ml of elution buffer I [100 mM NaCl, 10 mM Tris (pH 7.5), and 0.5% SDS], and 3.0 ml of 0.1 N NaOH. The columns were regenerated with application buffer. All glassware was baked, plasticware and water were autoclaved, and handling was performed with plastic gloves.

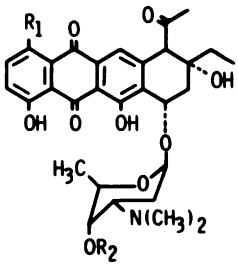
Quantification. Extracted RNA was quantified by calculation from A_{260}, and the extinction coefficient for RNA, $E^{1\%}_{260}$ nm = 250 at pH 7.5. Radioactivity in aliquots of washed cells and column fractions was measured directly by addition of 10 ml of ACS (Amersham, Arlington Heights, Ill.). Trichloroacetic acid-precipitated samples were washed twice in cold 10% trichloroacetic acid, suspended in 0.5 ml of 10% trichloroacetic acid, heated in boiling water for 15 min, cooled, and centrifuged at 1000 × *g* for 10 min. A 0.4-ml aliquot of each sample was counted in 10 ml of ACS. Radioactivity was obtained as counts per minute from a Beckman LS 7500 liquid scintillation counter with counting efficiencies of 24–32% for tritium.

Calculations. Experimental values, obtained as counts per minute for each fraction from cells exposed to a given drug concentration, were expressed as a percentage of



General Structure of the Daunomycinone Class of Anthracyclines

ANTHRACYCLINE	ABREVIATION	R_1	R_2
Adriamycin	ADM	OCH_3	CH_2OH
Daunomycin	DNM	OCH_3	CH_3
Carminomycin	CMM	OH	CH_3
4-Demethoxy-Daunomycin	4D-DNM	H	CH_3



General Structure of the Aklavinone-Pyrromycinone Class of Anthracyclines

ANTHRACYCLINE	ABREVIATION	R_1	R_2
Pyrromycin	PYM	OH	H
Marcellomycin	MCM	OH	deoxyfucose-deoxyfucose
Aclacinomycin	ACM	H	deoxyfucose-rednosamine

FIG. 1. *Structural analogues of the anthracyclines*

the average counts per minute of two control samples after correction for differences in precursor uptake by cells at different drug concentrations. It should be noted that the drugs at the doses used had little effect on precursor uptake. IC_{50} values (median effect doses) were determined by two procedures: probit analysis and median-effect plot (25). Probit analysis linearizes the median region of a log dose-response (as percentage of control) curve by conversion of percentage values to probit values using probit transformation tables (26). A median-effect plot linearizes the median region by conversion of percentage values to log $[(\%\text{ of control})^{-1} - 1]$ (25). Thus, linear regression analysis was applied to both probit vs. log [drug] and log $[(\%)\ \text{control}^{-1} - 1]$ versus log [drug] for each drug, and best-fit lines were calculated, yielding IC_{50} values, slopes, and correlation coefficients of 0.90–0.999.

RESULTS

The drugs studied in this investigation include the Class I anthracyclines ADM, CMM, and PYM, and the Class II anthracyclines MCM and ACM (Fig. 1). In order for the inhibitory effects of a drug upon the appearance of RNA in the cytoplasm to be assessed using a radioactive RNA precursor, it is important that the incorporation of radioactivity into RNA increases in a linear

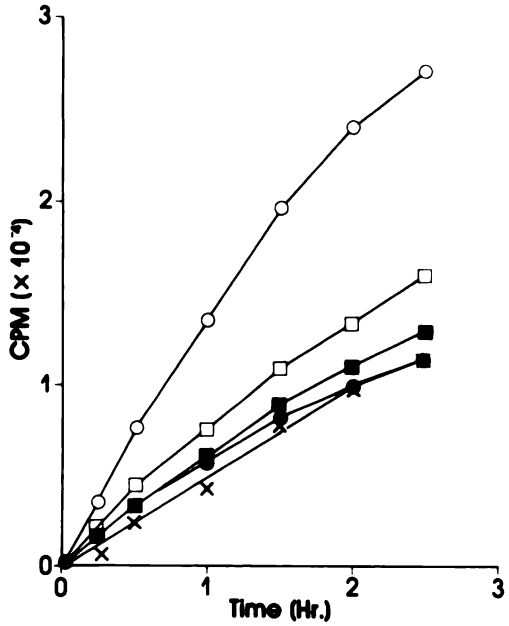


FIG. 2. *Kinetics of [^{3}H]uridine incorporation into total cellular RNA*

Cells were incubated for 30 min at 37° without drugs or with anthracyclines at the appropriate concentrations to produce a 50% inhibition of RNA synthesis (1.2 μM for DNM, 0.3 μM for CMM and PYM, and 0.06 μM for ACM). Unlabeled and tritium-labeled uridine were then added and triplicate 0.5-ml aliquots were removed at the designated times for processing and quantification of trichloroacetic acid-precipitable radioactivity as described under Materials and Methods. Average of duplicate controls of untreated cells, (○——○); DNM (●——●), CMM (□——□); PYM (■——■); and ACM (×——×).

manner during the entire study period. Under the described incubation conditions but without the addition of unlabeled uridine, the incorporation of radioactive uridine reached a plateau within 20 min[3]. However, the addition of 0.2 μmole of unlabeled uridine to the incubation medium resulted in linear incorporation of [^{3}H]uridine for more than 2 hr for both untreated cells and cells treated with median-effect concentrations of anthracyclines (Fig. 2). Table 1 shows that under these conditions 20% of the added radioactivity was taken up by the untreated cells after 2 hr of incubation, of which 84% was incorporated into trichloroacetic acid-precipitable products. Approximately 36% of the radioactive RNA in the cells was contained in the cytoplasm, and, of the cytoplasmic RNA, 7.5% of the radioactivity was found in poly(A)$^+$-RNA.

Figure 3 shows the effects of the different anthracycline analogues upon total cell RNA synthesis, appearance of total RNA into the cytoplasm, and appearance of poly(A)$^+$-RNA into the cytoplasm. Each drug was tested at six different concentrations and compared with the average of two controls in three separate experiments. It can be seen in Fig. 3 that there are greater differences, for the most part, between the effects of different drugs than are exhibited by the same drug upon the different RNA populations. This is more evident when IC_{50} values are compiled (Table 2). The Class II anthracyclines,

[3] B. H. Long, C. E. Willis, A. W. Prestayko, and S. T. Crooke, unpublished results.

TABLE 1

Percentage distribution of radioactivity in untreated erythroleukemia cells after 2-hr incubation with [^{3}H]uridine

Values represent a composite of 34 controls from 17 experiments with resulting mean values and standard deviation. Experiments were conducted as described under Materials and Methods.

Fraction	% Total added [^{3}H]uridine	% Total cell [^{3}H]uridine	% Total cell [^{3}H]RNA
Whole cells	19.7 ± 5.8	—	—
RNA in whole cells	16.4 ± 5.5	84.0 ± 12.3	—
RNA in cytoplasm	5.8 ± 2.0	29.9 ± 3.9	35.6 ± 14.6
Poly(A$^+$)-RNA in cytoplasm	0.42 ± 0.12	2.2 ± 0.6	2.6 ± 0.7

MCM and ACM, inhibited RNA synthesis by 50% at 60 nM, which is 20–50 fold lower than that of the Class I anthracyclines, DNM and ADM.

TABLE 2

Median effect plot determination of IC_{50} values of drugs upon RNA synthesis and appearance of total RNA and poly(A$^+$)-RNA into the cytoplasm

Values represent micromolar concentrations of drug that exert a median effect. Calculations for the determination of values are described under Materials and Methods.

Drug	Total cell RNA synthesis	Appearance into cytoplasm		
		Total RNA	Poly(A$^+$)-RNA	Ratio Poly(A$^+$)-RNA/total RNA
ADM	2.88	1.34	2.34	1.7
DNM	1.19	0.37	1.19	3.2
CMM	0.33	0.14	0.38	2.7
4 D-DNM	0.45	0.13	0.77	5.9
PYM	0.33	0.12	0.46	3.8
MCM	0.06	0.03	0.18	6.0
ACM	0.06	0.02	0.11	5.5

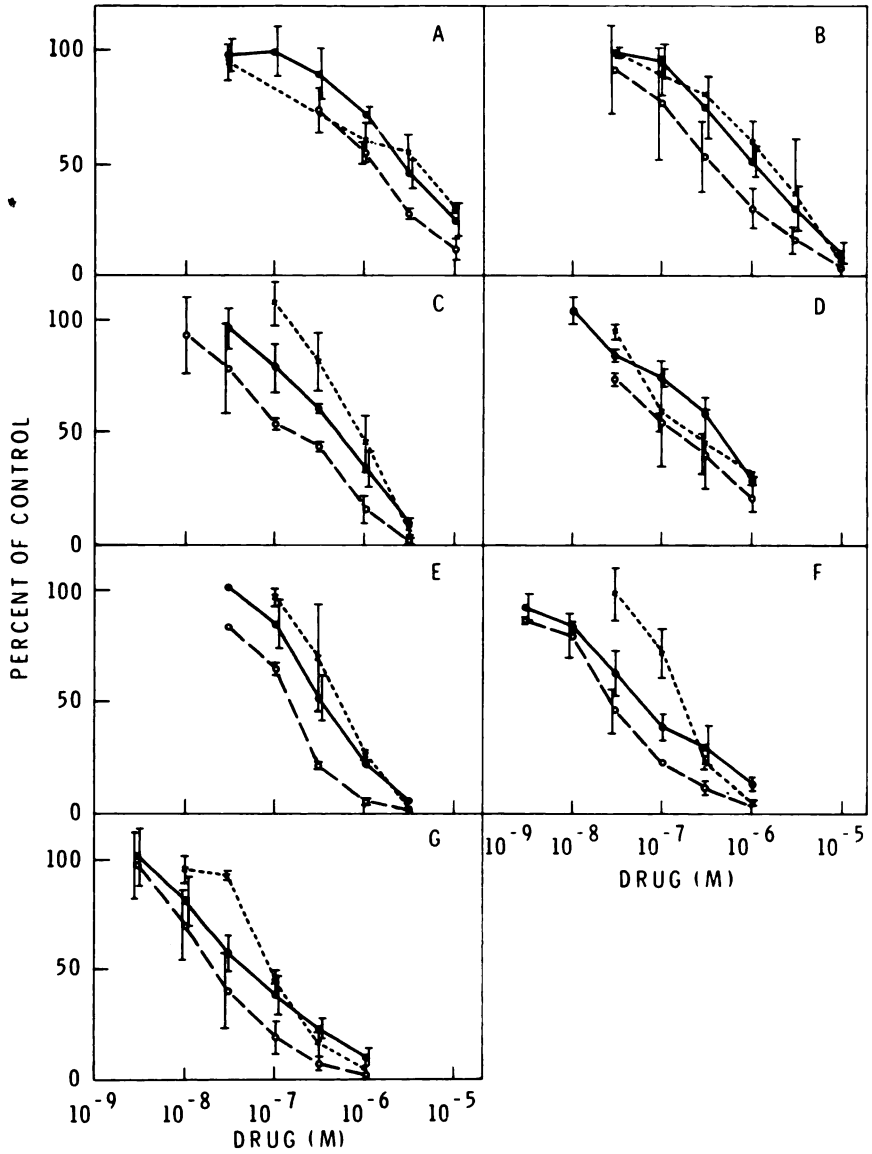


FIG. 3. *Effect of the anthracycline analogues upon RNA synthesis and the appearance of total RNA and poly(A)$^+$-RNA in the cytoplasm*

The values shown represent the mean with standard deviations for three separate experiments of results expressed as percentage of the average of two controls from each experiment, conducted as described under Materials and Methods, for each drug. Cellular RNA synthesis (●——●), total RNA appearance in the cytoplasm (○– –○), and poly(A)$^+$-RNA appearance in the cytoplasm (×-----×). A, ADM; B, DNM; C, 4D-DNM; D, CMM; E, PYM; F, MCM; and G, ACM.

Another prominent feature of Fig. 3 is that the inhibition of the appearance of poly(A)$^+$-RNA in the cytoplasm closely parallels the inhibition of total RNA synthesis for the Class I anthracyclines ADM, DNM, and CMM, whereas the appearance of poly(A)$^+$-RNA is less sensitive to inhibition by the Class II drugs. A comparison of the IC_{50} values in Table 2 for the appearance of poly(A)$^+$-RNA in the cytoplasm with cellular RNA synthesis results in a ratio of 1 for ADM, DNM, and CMM, 1.6 for 4D-DNM and PYM, and 2–3 for MCM and ACM. The preferential inhibition of preribosomal RNA synthesis by the Class II anthracyclines (11) is reflected in Fig 3F and G, where both MCM and ACM, at concentrations inhibiting total cellular RNA synthesis by 50%, had essentially no effect upon the appearance of poly(A)$^+$-RNA in the cytoplasm. The different slopes observed in Fig. 3 for the effects of 4D-DNM, MCM, and ACM upon the appearance of poly(A)$^+$-RNA in the cytoplasm are probably a result of the concerted action of the drugs upon both hnRNA synthesis and post-transcriptional events. However, the effects upon the latter require higher drug concentrations than necessary for action upon pre-mRNA synthesis.

IC_{50} values for the inhibition of the appearance of total RNA into the cytoplasm were consistently one-half to one-third of the respective IC_{50} values for total cellular RNA synthesis inhibition. This observation could be construed to suggest that at least one possible factor in the effects of these anthracyclines is alteration of post-transcriptional events and that all anthracyclines studied seem to exert a similar inhibition upon post-transcriptional modifications and/or transport to the cytoplasm of RNA.

DISCUSSION

Early investigations of the action of ADM and DNM upon RNA synthesis demonstrated that these drugs exhibited a preference for the inhibition of pre-rRNA synthesis (4, 5, 9, 14). This slight preference by ADM was observed in Novikoff hepatoma cells when RNA extracted from isolated nucleoli was examined, but a striking preference for the inhibition was observed by anthracyclines with a pyrromycinone or alklavinone ring structure containing a two- or three-sugar side chain (11, 13). This observation led to the grouping of anthracyclines into Class I (nucleolar nonselective) and Class II (nucleolar selective) (11, 13), a distinction that has been confirmed by electron microscopic observations revealing alterations of nucleolar morphology produced by the Class II drugs similar to that produced by low concentrations of actinomycin D (27).

In addition to activity against RNA synthesis there exists evidence indicating involvement of anthracyclines in post-transcriptional events. The report by Zähringer *et al.* (22) of a 52% decrease in mRNA with only a 23% decrease in total RNA content of rat myocardial tissue following ADM treatment at clinically relevant doses suggested that anthracyclines may also play a significant role in inhibiting appearance of mRNA in the cytoplasm. Sinha and Sik (28) reported finding a preferential binding of anthracyclines to RNA *in vitro*, which may account for the altered physical properties exhibited by RNA after extraction from DNM-treated cells (9). The existence of double-stranded, helical regions in nuclear RNA formed either by intramolecular or intermolecular hybridization affords a possibility for anthracycline intervention of RNA metabolism, i.e., binding to double-stranded RNA, although it had been suggested that intercalation is not likely in double-stranded RNA (29). Proflavine, another drug recognized for its ability to intercalate into double-stranded nucleic acids, has recently been shown to inhibit processing of SV-40 hnRNA, probably by intercalation into double-stranded RNA (30). If such is the case for anthracyclines, the obvious possibility exists that RNA processing or transport from nuclei may be affected. Any effects of the anthracyclines upon post-transcriptional events at or less than those concentrations affecting transcription or preferential effects upon pre-rRNA transcription would be manifest by lower IC_{50} values for the appearance of RNA in the cytoplasm than for RNA synthesis. The expression of median-effect (IC_{50}) values for the inhibition of appearance of poly(A)$^+$-RNA as a ratio of the inhibition of appearance of total RNA in the cytoplasm provides a rational comparison of effects produced by the different drugs upon mRNA and rRNA appearance (Table 2).

In erythroleukemia cells several observations can be made with regard to the effects of anthracyclines upon RNA metabolism. There are three different levels of activity toward the inhibition of cellular RNA synthesis: low activity, with IC_{50} values of 1–3 μM (ADM, DNM); intermediate activity, with IC_{50} values of 0.3–0.5 μM (CMM, 4D-DNM, and PYM); and high activity, with IC_{50} values of 0.06 μM (MCM, ACM). This grouping is followed with respect to the appearance of RNA in the cytoplasm. When the ratios of IC_{50} values for inhibition of the appearance in the cytoplasm of mRNA/rRNA are considered, again three groups are found: ADM, DNM, and CMM have ratios of 2–3; PYM has a ratio of 4; and 4D-DNM, MCM, and ACM have ratios of 6. Thus, ADM and DNM consistently behave as Class I anthracyclines and MCM and ACM behave as Class II. PYM, CMM, and 4D-DNM behave as Class I, Class II, or intermediate, depending upon the system studied. For example, in a recent study by Pachter *et al.* (21) of the binding of different analogues to DNA under high salt conditions favoring intercalation, ADM and CMM yielded unwinding angles of 13.1°, whereas PYM behaved like other Class II anthracyclines with unwinding angles around 10.5°.

Although IC_{50} values for mRNA and rRNA appearance in the cytoplasm were obtained, it was not possible to dissect the contribution of pre-mRNA and pre-rRNA synthesis effects in this study to determine the actual contribution of post-transcriptional inhibition, other than to say that such activity plays a minor role toward inhibiting the appearance of RNA in the cytoplasm relative to activity displayed at the transcriptional level. It is possible that the anthracycline drugs MCM and ACM have a post-transcriptional effect upon the appearance of poly(A)$^+$-RNA with median effect values approximately those for hnRNA synthesis, which may account for steeper inhibition curves produced by these drugs. However, this could be determined more accurately by

observing the effect of the drugs upon the synthesis of hnRNA and the appearance of mRNA of a specific gene product.

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