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DNA Binding Studies on Several New Anthracycline Antitumor Antibiotics II. The Importance of the Carbomethoxy-group at Position-10 of the Class II Anthracycline Molecule

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SUMMARY

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The DNA binding characteristics of the new anthracycline antitumor antibiotics, marcellomycin and rudolfomycin, were compared with those of their respective 10-descarbomethoxy analogues. The fluorescence spectra were determined and the fluorescence quenching effects caused by interactions with the four natural DNAs—calf thymus DNA, salmon sperm DNA, *Clostridium perfringens* DNA and *Micrococcus luteus* DNA—were characterized. Binding parameters were determined by Scatchard analyses of results obtained by titrating anthracycline fluorescence with increasing concentrations of DNA. Removal of the carbomethoxy-group from position 10 of marcellomycin or rudolfomycin caused a significant reduction in DNA binding ability for all four DNAs studied. These results are correlated with a marked decrease in antitumor activity, and nucleolar RNA synthesis inhibitory activity, as previously reported, and thus confirm earlier studies indicating that one subcellular target of anthracycline antitumor action is nucleolar preribosomal RNA synthesis. Additionally, the correlation between DNA binding ability and antitumor activity provides further evidence of the mechanism of action of Class II anthracyclines.

INTRODUCTION

The specificity of interaction of anthracyclines with DNA has been the subject of many studies using a variety of techniques including equilibrium dialysis (1, 2) spectrophotometric methods (1, 3, 4), and fluorescence methods (5–9). As a result, a major portion of the anthracycline-DNA interaction has be found to involve the intercalation (10) of the antibiotic between adjacent bases of the native DNA-duplex. Waring (11) and others (4, 9, 12) further supported this argument by their demonstration that, upon intercalation of anthracyclines into the native DNA-duplex, secondary structural alterations occurred in the DNA. One result is decreased template activity of the DNA molecule (13–17). The studies of Pigram and co-workers (18) have demonstrated that in addition to the strong inter-

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calative binding process, the interaction of daunomycin with DNA involves weaker electrostatic interactions between the ammonium group of the amino-sugar of daunomycin and the phosphate group of the DNA backbone.

Previous studies from this laboratory have demonstrated that anthracyclines can be divided into two classes based upon their selectivity for the inhibition of nucleolar RNA synthesis (19). Accordingly, class I anthracyclines inhibit DNA and nucleolar RNA syntheses at approximately equivalent concentrations and class II anthracyclines inhibit nucleolar RNA syntheses at drug concentrations from 200- to 1300-fold lower than those required to inhibit DNA synthesis. The structural features of the class II anthracycline molecule that may account for these differences include the presence of a carbomethoxy-group at position-10 (of the aglycone) and a glycosidic side-chain containing from two to three sugars (19).

Further studies have demonstrated the importance of the carbomethoxy-group at position-10 of the class II anthracycline molecule (20). It was shown that removal of the carbomethoxy-group from the marcellomycin (MCM[3]) and rudolfomycin (RDM) molecules resulted in a marked decrease in nucleolar RNA synthesis inhibitory potency, *in vitro* cytotoxicity, and *in vivo* antitumor activity against L-1210 leukemia. Figure 1 shows the structures of MCM, RDM and their respective descarbomethoxy-analogues.

To delineate further the possible mechanism of action of class II anthracyclines, the present study was undertaken to examine the importance of the carbomethoxy-group at position-10 of the class II anthracyclines MCM and RDM, relative to their DNA binding characteristics. This was accomplished by employing fluorescence titration studies.

[3] The abbreviations used are: MCM, marcellomycin; D-MCM, 10-descarbomethoxy-marcellomycin; RDM, rudolfomycin; D-RDM, 10-descarbomethoxy-rudolfomycin; EDTA, ethylene diaminetetracetic acid, disodium salt; A_{260}, absorbance at 260nm; A_{280}, absorbance at 280nm; K_{app}, apparent association constant; n_{app}, apparent number of binding sites per nucleotide; DMSO, dimethylsulfoxide.

MATERIALS AND METHODS

Materials. Calf thymus DNA (43% GC), salmon sperm DNA (41% GC), *Micrococcus luteus* DNA (72% GC), *Clostridium perfringens* DNA (28% GC) and EDTA were purchased from Sigma Chemical Company, St. Louis, Mo. The ratios of absorbance at 260nm (A_{260}) to the absorbance at 280nm (A_{280}) for all of the DNA preparations fell between 1.8 and 1.9. The ultraviolet-visible spectra of these drugs were determined on the Cary 14 recording spectrophotometer or on the Beckman Acta III recording spectrophotometer. The anthracycline antibiotics MCM, D-MCM, RDM, and D-RDM were all generously supplied by Bristol Laboratories, Syracuse, N.Y. Aqueous stock solutions of anthracyclines were prepared by wetting the drug crystals with DMSO, followed by addition of water. Stock drug solutions were stored at −20° and used within 2 to 4 weeks. Working stock solutions of each drug were prepared by dilution of aqueous stock solutions with DNA binding buffer (0.05 M sodium phosphate buffer, pH 6.2, 0.05 M NaCl, 0.001 M EDTA). Concentrations of anthracycline solutions were determined spectrophotometrically in methanol using molar extinction coefficients determined in this laboratory (see Table 1). DNA concentrations were determined spectrophotometrically at 260 nm using a molar extinction coefficient, with respect to nucleotides, of 6600 M^{-1}. Thus, DNA concentrations were expressed in terms of micromoles nucleotide residues/liter (μM nucleotides). All spectrophotometric determinations were made on the Zeiss PMQ-3 spectrophotometer using 1-cm path length quartz cuvettes. Spectrofluorometric measurements were made on an Aminco Bowman 4-8106 spectrophotofluorometer using a 1-cm quartz cuvette.

Fluorescence spectra and fluorescence quenching effects of DNA. Fluorescence spectra of each of the anthracycline antibiotics were determined by exciting a 1 ml solution of 5 μM drug at the visible absorption maximum of each drug (see Table 1), and varying the emission wavelength in the lower energy region of each spectrum.

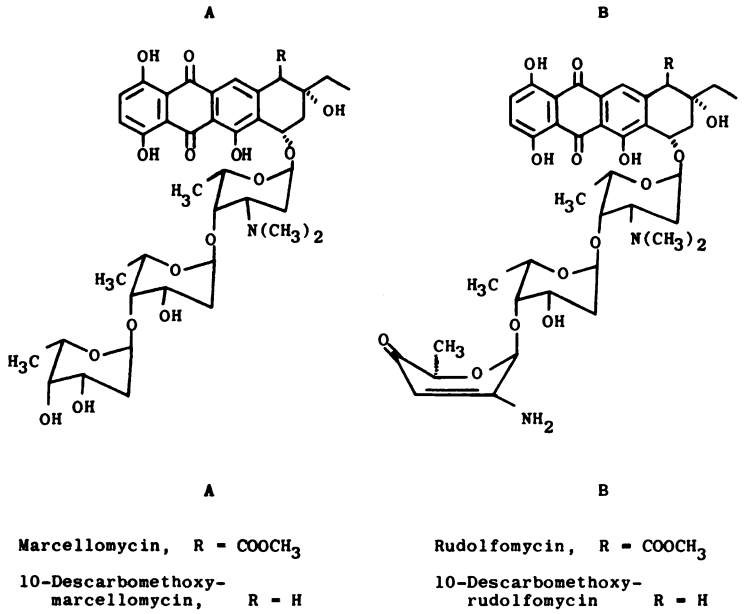

FIG. 1. *Structures of marcellomycin, 10-descarbomethoxymarcellomycin, rudolfomycin and 10-descarbomethoxyrudolfomycin*

TABLE 1

Spectral and fluorescence parameters of marcellomycin, rudolfomycin, and their 10-descarbomethoxy-analogues

Anthracycline	Absorbance[a]	Extinction coefficient (at absorbance, λ_{max})	Excitation wavelength	Fluorescence wavelength
	(nm)	*(M^{-1})*	*(nm)*	*(nm)*
Marcellomycin	490	13,400	490	555
10-descarbomethoxy-marcellomycin	448	8,600	450	550
Rudolfomycin	490	13,900	490	555
10-descarbomethoxy-rudolfomycin	490	11,100	490	550

[a] Absorbance maxima were obtained from the absorption spectra of each anthracycline: marcellomycin (23), 10-descarbomethoxymarcellomycin (20), rudolfomycin (24) and 10-descarbomethoxyrudolfomycin (20).

Quenching of drug fluorescence by DNA was measured by addition of microliter volumes of concentrated DNA solutions to the cuvette. The dilution effect caused by addition of the DNA solution was negligible. Listed in Table 1 are the fluorescence parameters, excitation and emission wavelengths, determined for each drug.

Binding measurements. All measurements were carried out at 25° in DNA binding buffer (0.05 M sodium phosphate buffer, pH 6.2, 0.05 M NaCl, 0.001 M EDTA). A minimum of two determinations of binding parameters were made for each anthracycline-DNA combination. Identical preparations of each DNA were used for all anthracyclines. Each binding determination consisted of a minimum of 11 different DNA concentrations, each of which consisted of duplicate or triplicate samples. All solutions were maintained on ice until the binding reaction was initiated.

The DNA binding of anthracycline antibiotics was measured by spectrofluorometry in a manner analogous to previous studies (7, 9, 21, 22). This was done by titrating fixed concentrations of anthracycline (3, 4, 5 or 10 μM) with increasing concentrations of DNA, thereby varying the DNA/drug ratios from 0 to 200. The DNA/drug ratio

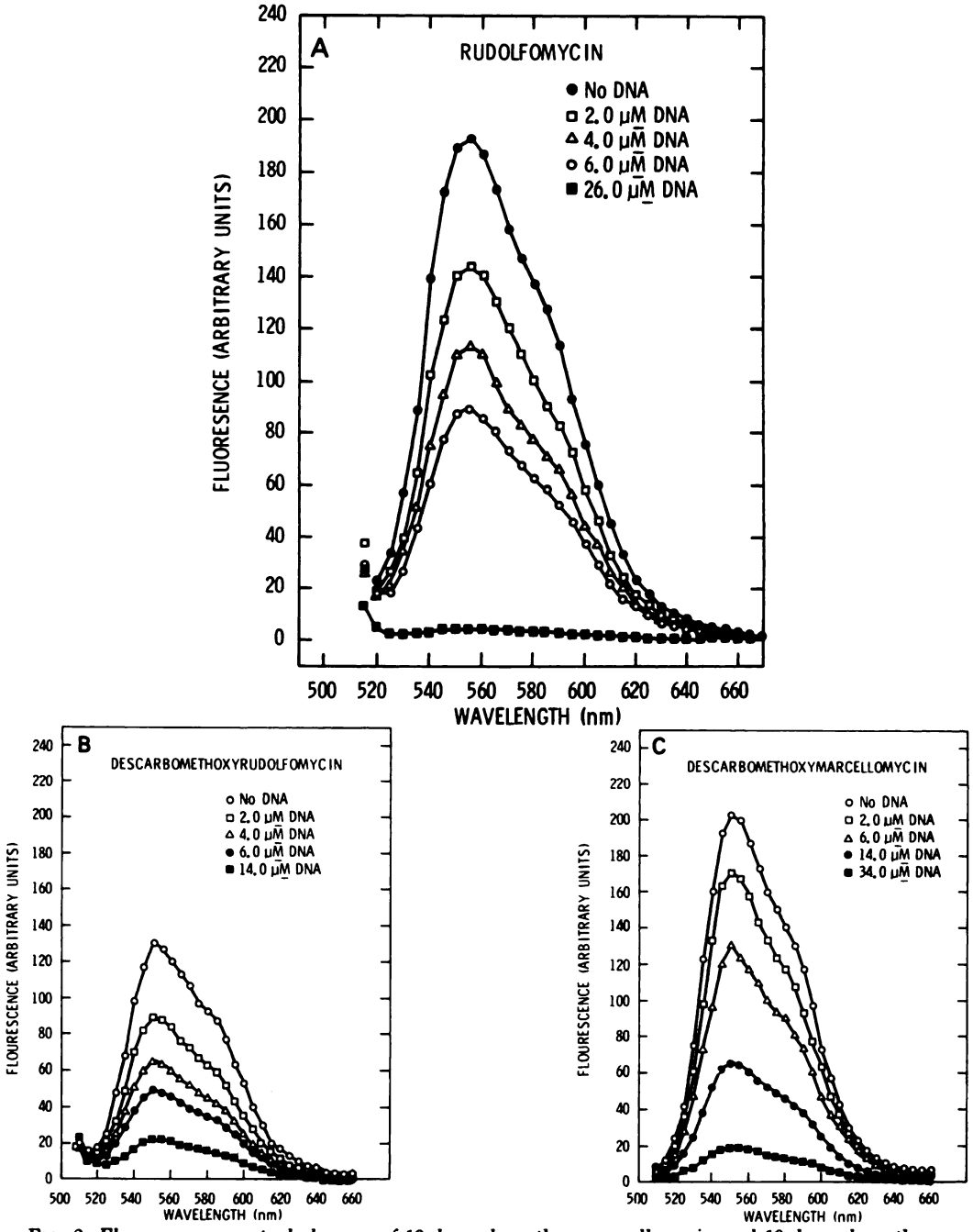

FIG. 2. *Fluorescence spectral changes of 10-descarbomethoxymarcellomycin and 10-descarbomethoxyrudolfomycin upon interaction with salmon sperm DNA*

Spectra were taken on 5 μM anthracycline solutions in 0.05 M sodium phosphate buffer, pH 6.2, 0.05 M NaCl, 0.001 M EDTA. Spectra were obtained by using the specific excitation wavelength of each anthracycline (see Table 1) and recording drug fluorescence at wavelengths in the low energy region of the spectrum (longer wavelengths). Increasing concentrations of DNA were obtained by addition of microliter volumes of a concentrated solution of salmon sperm DNA in the above buffer: (A) rudolfomycin; (B) 10-descarbomethoxyrudolfomycin; (C) 10-descarbomethoxymarcellomycin.

of 200 was taken as the endpoint in this titration assay, with the drug considered totally bound. Increasing concentrations of DNA in a total of 0.90 ml of DNA binding buffer were added to a series of acid-cleaned glass tubes. The binding reaction was initiated by addition of 0.10 ml of anthracycline working stock solution, in DNA binding buffer to each tube, followed immediately by mixing and incubation in a 25° water bath for 1 hr. The final DMSO concentration was 0.005% to 0.05%. To eliminate fluorescence due to DNA, parallel control titrations consisting of increasing concentrations of DNA in 1.00 ml of DNA binding buffer were run for each experiment. Fluorescence determinations were made for each anthracycline using the fluorescence parameters shown in Table 1.

The binding data were analyzed by the Scatchard method (26). The Scatchard variables of r (moles of ligand bound/nucleotide) and C (the molar concentration of free antibiotic) were calculated from the fluorescence data, according to the method of Peacocke and Skerrett (27). Binding parameters were determined from plots of r/C versus r, where K_{app} (apparent association constant) is the negative slope and n_{app} (the apparent number of binding sites per nucleotide) is the intercept of the curve with the x-axis.

RESULTS

Using the absorption spectra of each of the four anthracyclines MCM, RDM, D-MCM, and D-RDM, the visible absorption maximum for each compound was determined (Table 1). These parameters were used to determine the excitation wavelength and the fluorescence spectrum of each anthracycline. Figure 2 shows the fluorescence spectral changes of RDM, D-RDM and D-MCM upon interaction with DNA. Essentially equivalent results were obtained with MCM (25). Increasing concentrations of salmon sperm DNA progressively decreased the fluorescence throughout each spectrum, with no significant enhancement of any portion of the spectrum or peak shift observed. Since the greatest changes in the anthracycline fluorescence spectra, upon addition of DNA, occurred at the wavelengths indicated in Table 1, fluorescence measurements at these wavelengths yielded the most accurate determination of the fraction of the total anthracycline bound.

Figure 3 shows the quenching of fluorescence due to increasing concentrations of calf thymus DNA and salmon sperm DNA on MCM and its 10-descarbomethoxy-analogues, using the parameters indicated in Table 1. These results are typical of the

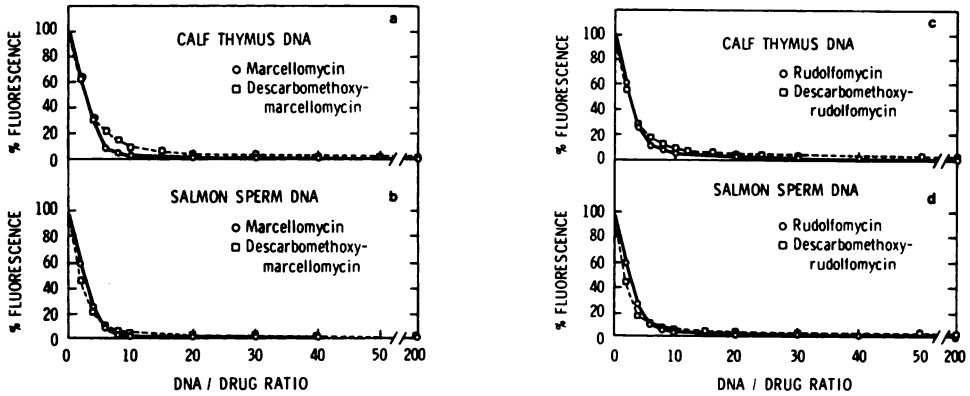

FIG. 3. *Fluorescence quenching of marcellomycin and 10-descarbomethoxymarcellomycin in the presence of increasing concentrations of calf thymus DNA and salmon sperm DNA*

To a series of glass tubes containing increasing concentrations of nucleic acid in DNA binding buffer, 0.05 M sodium phosphate buffer, pH 6.2, 0.05 M NaCl, 0.001 M EDTA, a fixed concentration of each anthracycline was added, incubated at 25° for 1 hour and fluorescence measurements taken using the fluorescence parameters indicated in Table 1. The results of duplicate experiments, each of which contained duplicate or triplicate values at each DNA concentration, are shown.

other anthracycline-DNA interactions studied. As shown, the changes in fluorescence intensity with added DNA approached zero at values of DNA/drug ratios greater than 30 for all of the drugs studied. Therefore, the DNA/drug ratio of 200 was taken as the ratio at which drug was totally bound and was used in the calculation of the Scatchard parameters r, C, and r/C.

Analysis of the results shown in Figure 3 by the method of Scatchard (26) allowed the construction of the Scatchard curves shown in Figure 4. The K_{app} and the n_{app} were obtained from the negative slope and the intercept of the curve with the x-axis, respectively. The differences in the binding behavior of the two compounds in the presence of increasing concentrations of both salmon sperm DNA and calf thymus DNA are clearly distinguished in the Scatchard analyses shown in Figure 4. MCM has a higher affinity for both DNAs than does its 10-descarbomethoxy-analogue, evidenced by the steeper slope of the MCM-curve. Similar results were obtained for RDM and D-RDM.

Because both calf thymus DNA and salmon sperm DNA contain "normal" base ratios of GC and AT, and because we have previously demonstrated nucleolar selectivity of nucleic acid synthesis inhibition by MCM (19) and RDM (20), DNAs containing altered base ratios of GC and AT were also studied. *M. luteus* DNA (72% GC) and *Cl. perfringens* DNA (28% GC) were used to compare the relative sequence specificities of MCM, RDM, D-MCM and D-RDM.

Scatchard analyses of the fluorescence titration studies of the four anthracyclines in the presence of *M. luteus* DNA and *Cl. perfringens* DNA are shown in Figure 5. The slopes obtained for D-MCM and D-RDM demonstrate the lower affinity of these analogues for both bacterial DNAs

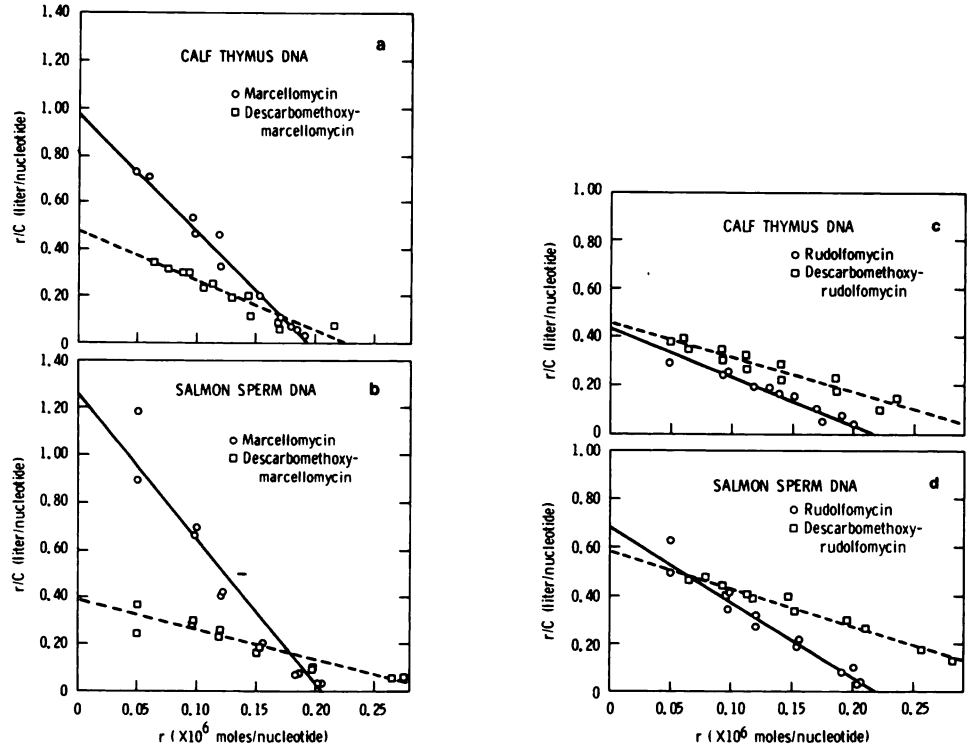

FIG. 4. *Scatchard plots of the binding data for the interaction of marcellomycin, 10-descarbomethoxymarcellomycin, rudolfomycin and 10-descarbomethoxyrudolfomycin with calf thymus DNA and salmon sperm DNA*

The Scatchard parameters r (moles ligand bound per nucleotide), C (moles per liter of free ligand) and r/C were calculated from the data similar to that presented in Figure 3.

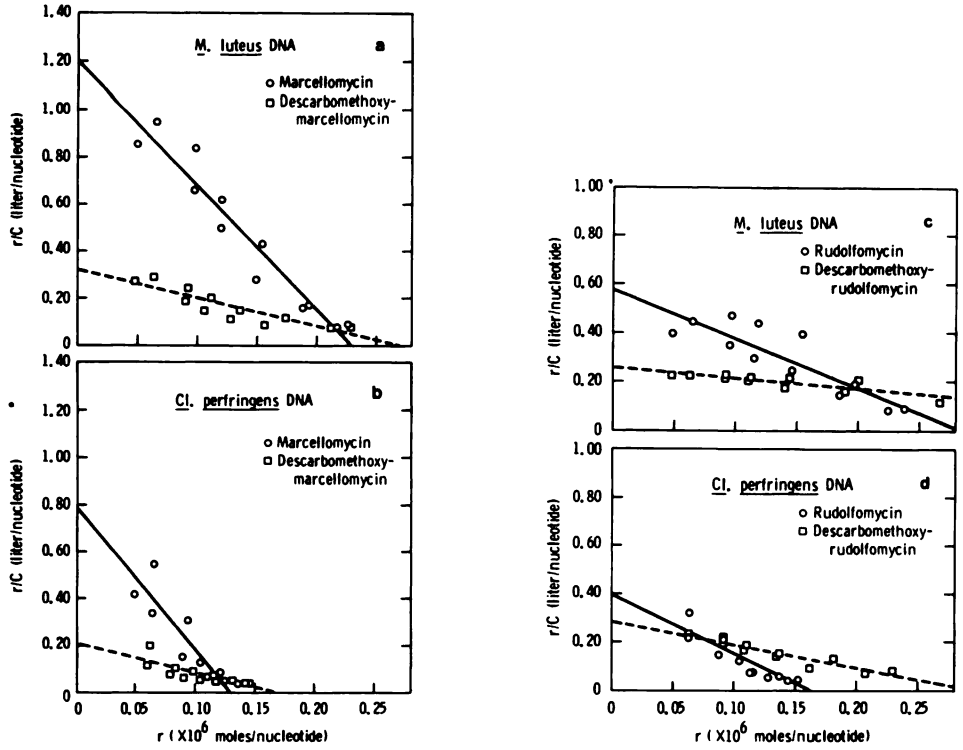

FIG. 5. *Scatchard plots of the binding data for the interaction of marcellomycin, 10-descarbomethoxymarcellomycin, rudolfomycin, and 10-descarbomethoxyrudolfomycin with M. luteus DNA and Cl. perfringens DNA*

TABLE 2

Binding parameters of marcellomycin, descarbomethoxymarcellomycin, rudolfomycin and descarbomethoxyrudolfomycin for salmon sperm DNA, calf thymus DNA, Micrococcus luteus DNA and Clostridium perfringens DNA[a]

Anthracycline	*Cl. perfringens* DNA (28% GC)		Salmon sperm DNA (41% GC)		Calf thymus DNA (43% GC)		*M. luteus* DNA (72% GC)	
	K_{app}[b]	n_{app}[c]	K_{app}	n_{app}	K_{app}	n_{app}	K_{app}	n_{app}
Marcellomycin	6.05	0.130	9.51	0.169	5.03	0.194	5.25	0.229
Descarbomethoxy-marcellomycin	1.26	0.166	1.28	0.303	2.14	0.224	1.21	0.264
Rudolfomycin	2.44	0.164	3.11	0.219	1.98	0.218	2.02	0.286
Descarbomethoxy-rudolfomycin	0.96	0.295	1.54	0.377	1.42	0.321	0.42	0.607

[a] p values were determined from tables of significance limits for correlation coefficients. All p values obtained were less than 0.001. The number of averaged values used to construct composite Scatchard curves was usually 12, but no less than 10.

[b] K_{app}, apparent association constant, in units of 10^6 M^{-1}. Values were obtained by linear regression analyses of composite Scatchard curves obtained from two or more separate experiments, each of which contained duplicate or triplicate values at each DNA concentration.

[c] n_{app}, apparent number of binding sites per nucleotide. Values are obtained as for K_{app}.

than their respective parent compounds.

Table 2 shows the results of the Scatchard analyses of MCM, D-MCM, RDM and D-RDM studied with respect to salmon sperm DNA, calf thymus DNA, *M. luteus* DNA and *Cl. perfringens* DNA. As indicated, the K_{app} for D-MCM are from 2- to 7-fold lower than those of MCM for all four of the DNAs studied. Smaller differences for RDM and D-RDM were noted for these

DNAs. The K_{app} for D-RDM are from 1- to 4-fold lower than those of RDM for the four DNAs studied. Slight increases in the n_{app} values are seen for both D-MCM and D-RDM relative to their respective parent compounds. No evidence was observed for sequence specificity of DNA binding by these anthracyclines.

DISCUSSION

Studies employing spectral (4, 27–29) as well as fluorescence (5, 7, 8) titration techniques have been used to investigate binding interactions of ligand chromaphores with nucleic acids. These techniques depend upon the demonstration of spectral alterations of the ligand upon interaction with nucleic acids (27). Such alterations occur for the anthracyclines daunomycin (3), adriamycin, pyrromycin, musettamycin, aclacinomycin, rudolfomycin and marcellomycin (25). These changes allow the quantitation of DNA-anthracycline interaction and thus facilitate their use in DNA binding studies.

Previous studies from this laboratory have demonstrated the importance of the carbomethoxy-group at position-10 of the class II anthracycline molecule (20). Removal of the carbomethoxy-group from position-10 of the RDM and MCM molecules resulted in 30- and 80-fold reductions, respectively, of nucleolar RNA synthesis inhibitory activities (20). *In vitro* cell viability inhibitory activity decreased by approximately 5-fold and over 16-fold upon removal of the carbomethoxy-group from position-10 of MCM and RDM, respectively (20). In a similar manner, *in vivo* antitumor activity against the mouse L-1210 leukemia decreased 20-fold and 80-fold upon removal of the carbomethoxy-group from MCM and RDM, respectively (20). These changes in the biological activities of these anthracyclines are correlated with a simultaneous decrease in DNA binding ability of from 1- to 7-fold, depending upon the DNA studied (Table 2). This correlation between *in vitro* isolated DNA binding and biological activities in intact cells and *in vivo* is remarkable in light of the many factors and their interactions that are thought to interplay in the production of drug-induced cytotoxicity.

The results shown in Table 2 also indicate that removal of the carbomethoxy-group from MCM and RDM caused an increase in n_{app} values for all DNAs studied. An increase in n_{app} values (expressed in units of binding sites per nucleotide) corresponds to a decrease in the number of nucleotides per binding site (size of the binding site).

Earlier studies from this laboratory demonstrated that class II anthracyclines selectively inhibited nucleolar RNA synthesis (19). One possible interpretation of these results is that class II anthracyclines may bind preferentially to GC-rich regions of native DNA, similar to the well characterized compound actinomycin D (28, 29, 30). However, no sequence specificities of DNA binding were detected in this study for MCM, D-MCM, RDM and D-RDM. Thus, alternate explanations of the nucleolar RNA synthesis inhibitory specificities of these compounds must be sought.

The decreased overall affinity of D-MCM and D-RDM for DNA, relative to their respective parent compounds, suggests an important "stabilizing" role of the carbomethoxy-group in class II anthracycline-DNA interactions. The nature of the putative interaction between the carbomethoxy-group, at position-10 of class II anthracyclines, and DNA is not known at present. Further studies employing physical and biochemical techniques to examine this aspect of anthracycline-DNA interactions are planned. Studies are also in progress using new anthracycline analogues containing other modifications at position-10 of the molecule.

The current results provide additional insights into the possible mechanism of action of anthracyclines. These findings further substantiate the hypothesis that a significant portion of the class II anthracycline antitumor activity is related to the ability of these compounds to inhibit nucleolar RNA synthesis (20).

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