MOLECULAR PHARMACOLOGY, 21:196–203

The Antitumor Effects of Anthracyclines

II. The Stereospecificity of the Carbomethoxy Group at Position 10 of the Class II Anthracycline Molecule

VIRGIL H. DUVERNAY,[1] DAVID EUBANKS,[1] RICHARD PERALES,[1] ARCHIE W. PRESTAYKO,[1,2] AND STANLEY T. CROOKE[1,3]

Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030, Bristol Laboratories, Syracuse, New York 13201, and Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101

Received April 13, 1981; Accepted August 28, 1981

SUMMARY

The effects of marcellomycin, musettamycin, and their respective position 10 epimers mimimycin and collinemycin were compared in several systems both *in vivo* and *in vitro*. The results of these studies showed that the epimerization of the carbomethoxy-group at position 10 of the Class II anthracycline aglycone resulted in a 3-fold to 20-fold decrease in DNA-binding ability. The reduced DNA binding ability of these compounds is correlated with a 2-fold to 17-fold decrease in whole cellular nucleic acid synthesis inhibitory potency, a 2-fold to 4-fold decrease *in vitro* antitumor potency, and a 4-fold to 32-fold reduction in *in vivo* antitumor potency. These results further support the concept that a major portion of the antitumor activity of Class II anthracyclines is related to their avidity for DNA interactions and resultant disruption of normal template function. Previous studies from this laboratory have demonstrated that the 10-carbomethoxy group is essential for nucleolar RNA synthesis inhibition and Class II anthracycline antitumor activity. The current study further demonstrates that the stereochemical orientation of the carbomethoxy group at position 10 of the aglycone is also important in conferring Class II anthracycline antitumor activity.

INTRODUCTION

Previous studies from this laboratory have reported on the characterization of a series of new anthracycline antitumor antibiotics, including the two nucleolar-selective (Class II) anthracyclines MCM[4] and MSM (1–4). This classification scheme was based on the ability to inhibit selectively No-RNA synthesis (1, 2). The initial studies have demonstrated several structural features governing nucleolar selectivity, including the length and composition of the glycosidic side chain (1, 5). Subsequent studies have demonstrated the importance of the carbomethoxy group at position 10 of the Class II anthracycline molecule with respect to No-RNA synthesis inhibitory potency (2), antitumor activity *in vivo* and *in vitro* (2), and DNA-binding ability to four naturally occurring DNAs (4). Thus, removal of the 10-carbomethoxy group from the two Class II anthracycline molecules MCM and RDM significantly reduced activity in each of the above systems (2, 4, 5).

Recently, Doyle and co-workers (6) have reported on the isolation and structural characterization of several new pyromycinone-based anthracycline analogues, two of which are modified at position 10 of the aglycone. These are the position 10 epimers of the Class II anthracyclines MSM and MCM, and correspond to the analogue CLM and MIMI, respectively (6). The chemical structures of these four anthracyclines are shown in Fig. 1. In an effort to characterize further the importance of the carbomethoxy group at position 10 of the Class II anthracycline molecule, and to better understand the stereochemical requirements at this portion of the aglycone regarding Class II anthracycline activity, the current investigation was undertaken. The present study examines the effects of the four analogues on whole cellular nucleic acid synthesis inhibition, cytotoxicity *in vitro*, and antitumor activity *in vivo*. In addition, studies characterizing the DNA-drug interactions of these four

This work was supported in part by a grant from Bristol Laboratories and by National Institutes of Health Grant CA-10893-P12 (to S. T. C.).

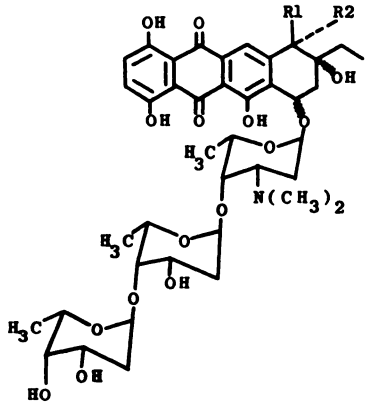
[1] Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine.

[2] Bristol Laboratories.

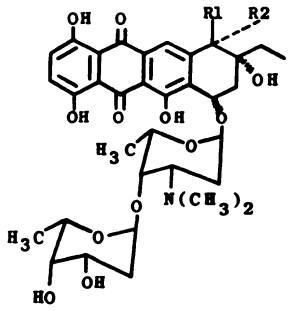
[3] Smith Kline & French Laboratories.

[4] The abbreviations used are: MCM, marcellomycin; MSM, musettamycin; No-RNA, nucleolar pre-ribosomal RNA; RDM, rudolfomycin; CLM, collinemycin; MIMI, mimimycin; RPMI-1640, Roswell Park Memorial Institute medium, Type 1640; NHAC, Novikoff hepatoma assites cells; ADM, adriamycin; DNM, daunomycin.

0026-895X/82/010196-08$02.00/0



	R1	R2
MARCELLOMYCIN	$COOCH_3$	H
MIMIMYCIN	H	$COOCH_3$



	R1	R2
MUSETTAMYCIN	$COOCH_3$	H
COLLINEMYCIN	H	$COOCH_3$

FIG. 1. *Structures of MCM, MSM, and their respective position 10 epimers CLM and MIMI*

anthracyclines are currently under investigation. The DNA-binding affinities of these agents are presented here, and additional studies will appear elsewhere.[5] The results suggest that the activity of these compounds is a result of the occurrence of a particular configuration of the carbomethoxy group at position 10 of the aglycone and not due to its presence alone.

MATERIALS AND METHODS

Materials. Gelman Type E glass-fiber filters were obtained from Curtin-Matheson Scientific Company (Houston, Tex.). RPMI-1640, sterile fetal calf serum, glutamine solution, and penicillin-streptomycin solution (10,000 units; 10,000 μg) were obtained from Grand Island Biological Company (Grand Island, N.Y.). Tritiated precursors [^{3}H]thymidine and [^{3}H]uridine) were obtained from Schwarz/Mann (Orangeburg, N.Y.). Calf thymus DNA (43% GC), salmon sperm DNA (41% GC), and EDTA were purchased from Sigma Chemical Company (St. Louis, Mo.). 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 of nucleotide residues per liter (micromolar nucleotides).

The ratios of absorbance at 260 nm (A_{260}) to the absorbance at 280 nm (A_{280}) for all of the DNA preparations varied between 1.8 and 1.9. All spectrophotometric determinations were made on the Zeiss PMQ-3 spectrophotometer using 1-cm path length quartz cuvettes.

The anthracycline antibiotics MCM, MIMI, MSM, and CLM were isolated from the bohemic acid complex as previously described (6, 8). All four compounds were supplied by Bristol Laboratories (Syracuse, N.Y.). Aqueous stock solutions of anthracyclines were prepared by wetting the drug crystals with dimethyl sulfoxide, followed by addition of water. Stock drug solutions were stored at −20° and used within 1–2 weeks. The molar extinction coefficients, determined in methanol for MCM, MSM, CLM, and MIMI were 13,400 $M^{-1}cm^{-1}$ at 490 nm; 12,500 $M^{-1}cm^{-1}$ at 490 nm; 10,500 $M^{-1}cm^{-1}$ at 490 nm; and 8,900 $M^{-1}cm^{-1}$ at 490 nm, respectively (6). For DNA-binding studies, 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, and 0.001 M EDTA]. Dilutions of aqueous stock for cell viability studies were made in ethanol. Spectrofluorometric measurements were made on an Aminco Bowman 4-8106 spectrophotofluorometer using a 1-cm quartz cuvette.

Cell culturing. Cultured NHAC, type N_1S_1-73, were grown in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin A (100 μg/ml). NHAC were maintained in monolayer cultures, as previously described (1). For drug studies, log-phase NHAC were grown in shaker culture (New Brunswick Gyrotary shaking incubator) at 37° in 500 ml in liquid media bottles.

Effects on whole cellular nucleic acid synthesis. Anthracycline-induced whole cellular nucleic acid synthesis effects were determined using a filter assay method as reported previously (1). The 50% inhibitory concentrations (IC_{50} values) were obtained by probit analysis of the data (1, 2, 9).

Colony survival studies. The IC_{50} values for drug-induced cell viability inhibition were determined as previously reported (2). The results were displayed as the log of the survival fraction versus drug concentration and IC_{50} values obtained by probit analysis (9).

In vivo antitumor effects. Anthracycline *in vivo* antitumor activities against mouse L-1210 leukemia cells were determined as previously reported (2, 10).

[5] V. H. DuVernay, D. Eubanks, S. T. Crooke, and A. W. Prestayko. DNA binding studies on several anthracycline antitumor antibiotics. III. The stereospecific orientation of the carbomethoxy groups at position 10 of the class II anthracycline molecule. Submitted for publication.

Fluorescence spectra and fluorescence quenching effects of DNA. Fluorescence spectra of each of the anthracycline antibiotics were determined as previously described (3, 4). Fluorescence spectral characteristics of MCM, MSM, CLM, and MIMI, as well as the DNA quenching effects, were previously described (3–5). Drug solutions were excited at 490 nm and emission was read at 555 nm.

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). At least 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 reactions was initiated. The DNA binding of anthracycline antibiotics was measured by spectrofluorometry in a manner described previously (3, 4).

The binding data were analyzed by the Scatchard method (11). The Scatchard variables of r (moles of ligand bound per nucleotide) and C (the molar concentration of free antibiotic) were calculated from the fluorescence data according to the method of Peacocke and Skerrett (12). 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.

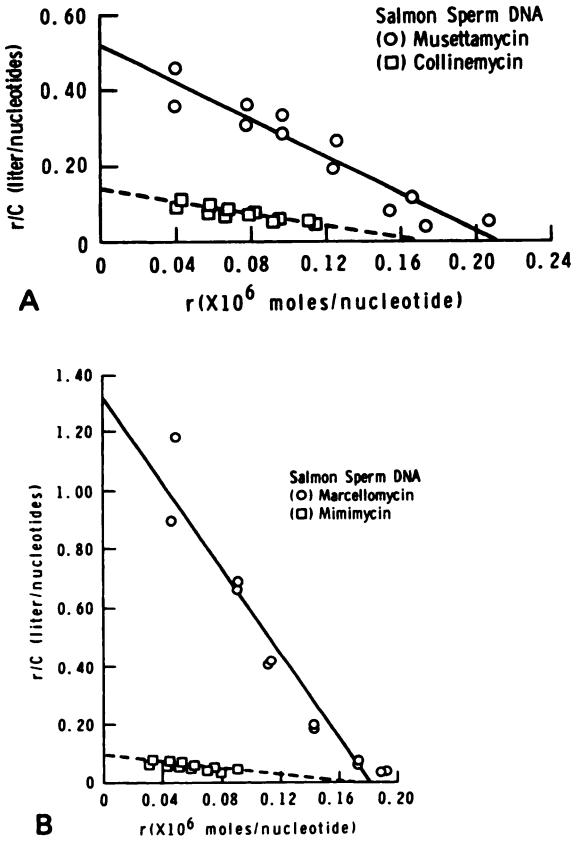


FIG. 2. *Scatchard plots of the binding data for the interaction MSM and CLM (A) and MCM and MIMI (B) with salmon sperm DNA*

The Scatchard parameters r (moles ligand bound per nucleotide), C (moles per liter of free ligand) and r/C were calculated from fluorescence titration data as previously described (3, 4, 12). DNA binding was measured at 25° in DNA-binding buffer (0.05 M sodium phosphate buffer (pH 6.2), 0.05 M NaCl and 0.001 M EDTA) described under Materials and Methods. The results of duplicate experiments, each of which contained duplicate or triplicate values at each DNA concentration, are shown.

TABLE 1

DNA binding characteristics of MCM, MIMI, MSM, and CLM

Data analysis and statistical calculations were carried out as previously described (3, 4, 13). Each value represents the mean of a minimum of duplicate determinations. Each curve represent a minimum of duplicate experiments and consists of 12 values.

Anthracycline	Calf thymus DNA		Salmon sperm DNA	
	K_{app}[a]	n_{app}[b]	K_{app}	n_{app}
	$\times 10^6$ M		$\times 10^6$ M^{-1}	
MCM	6.21 ± 0.42	0.17 ± 0.06	7.22 ± 0.55	0.18 ± 0.08
MIMI	0.62 ± 0.08	0.16 ± 0.01	0.55 ± 0.07	0.16 ± 0.01
MSM	3.17 ± 0.27	0.16 ± 0.04	2.47 ± 0.22	0.21 ± 0.03
CLM	0.67 ± 0.06	0.19 ± 0.01	0.82 ± 0.05	0.17 ± 0.01

[a] Apparent association constant. 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.

[b] Apparent number of binding sites per nucleotide. Values were obtained as for k_{app}.

RESULTS

DNA binding characteristics. The fluorescence spectra of MCM, MSM, CLM, and MIMI as well as the fluorescence spectral changes which occur upon interaction of these compounds with DNA have been described elsewhere.[5]

Fluorescence titration studies were performed as described previously (3, 4) by titrating fixed concentrations of each anthracycline with increasing concentrations of calf thymus DNA and salmon sperm DNA (data not shown). These results were analyzed by the method of Scatchard (11), and typical results are shown in Fig. 2. These results clearly show a higher affinity of MCM for salmon sperm DNA than is shown by its position 10 epimer, MIMI, as evidenced by the steeper slope of the MCM curve. Similar results were obtained for MSM and its position 10 epimer CLM. The results obtained with calf thymus DNA (data not shown) parallel those obtained with salmon sperm DNA. These results are summarized in Table 1, which shows K_{app} and n_{app} values for the interaction of both parent compounds and their epimers with calf thymus DNA and salmon sperm DNA. The k_{app} values of CLM were 3-fold to 5-fold lower than those of MSM, and the K_{app} values of MIMI were 10-fold to 13-fold lower than those of MCM. No significant changes in the n_{app} values were observed.

Effects on whole cellular macromolecular synthesis. The effects of MCM, MIMI, MSM, and CLM 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 described previously (1, 2, 13). The percentage inhibition of incorporation of cellular-associated radio-

activity into trichloroacetic acid-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 by linearizing the center region (near the 50% inhibition point) of a dose-percentage inhibition curve, thereby allowing for a straight line to be fitted by the weighted least-squares method (2, 9, 13). Linear regression analysis was therefore applied to the data points, and the IC_{50} values estimated from each curve.

Figure 3 shows a representative probit analysis of the effects of MCM and MIMI on whole cellular DNA synthesis in cultured NHAC, using a filter assay method previously described (1, 2, 13). The IC_{50} values obtained for the incorporation of [^{3}H]thymidine into acid-insoluble material were 14.8 μM and 43.9 μM for MCM and MIMI, respectively. Thus, MIMI is approximately 3-fold less potent than MCM when inhibiting whole cellular DNA synthesis. Similar differences in potencies were observed for MSM and CLM, with IC_{50} values of 13.5 μM and 31.9 μM, respectively. This reflects a 2-fold difference in potency toward DNA synthesis inhibition.

Similar trends are observed for whole cellular RNA synthesis inhibition. Figure 4 shows a representative probit analysis of the effects of MCM and MIMI on the incorporation of [^{3}H]uridine into acid-insoluble material. The IC_{50} values obtained for MCM and MIMI were 0.76 μM and 13.0 μM, respectively. This represents a 17-fold difference in potencies for these analogues. Similarly, for MSM and CLM, IC_{50} values of 1.4 μM and 3.6 μM, respectively, were obtained for whole cellular RNA synthesis inhibition. Thus, CLM is only 2.5-fold less potent than MSM when measuring [^{3}H]uridine incorporation.

These results indicate that, upon epimerization of the carbomethoxy group at position 10 of the MCM or MSM molecules, a significant loss of ability to inhibit macromolecular synthesis occurs. This effect is more pronounced for the analogues MCM and MIMI, but is consistent across both pairs of analogues.

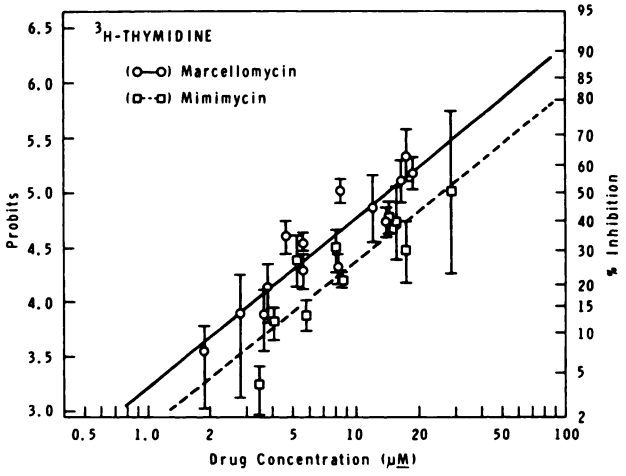


FIG. 3. *Effects of MCM and MIMI on the incorporation of [^{3}H] thymidine into acid-insoluble material.*

Results were obtained by using a filter assay method as previously described (1, 2). The means of a minimum of triplicate determinations are shown, and the curves represent the results of a minimum of triplicate experiments. Linear regression analysis was applied to each curve and the IC_{50} values were obtained as previously described (1, 2, 9). The p values of <0.001 were obtained for both MCM and MIMI. Similarly, for MSM and CLM, when comparing their effects on [^{3}H] thymidine incorporation (data not shown), p values of <0.01 were obtained.

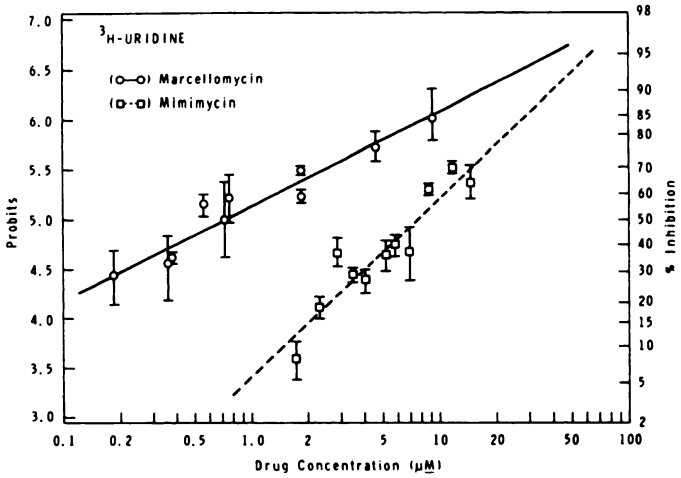


FIG. 4. *Effects of MCM and MIMI on the incorporation of [^{3}H] uridine into acid-insoluble material.*

Results were obtained using a filter assay method as previously described (1, 2). The means of a minimum of triplicate determinations are shown, and the curves represent the results of a minimum of triplicate experiments. Linear regression analysis was apllied to each curve, and the IC_{50} values were obtained as previously described (1, 2, 9). The p values obtained for both MCM and MIMI were <0.001. Similarly, for MSM and CLM, when comparing their effects on [^{3}H] uridine incorporation (data not shown), p values of —0.001 were obtained.

Effects on cell survival. To compare the effects of MCM, MIMI, MSM, and CLM on cell viability, colony survival studies were employed, as indicated previously (2, 13). Plates containing fixed numbers of cells were treated with drug, washed, supplemented with fresh media, and incubated for 7–14 days to allow cells to proliferate. 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 by probit analysis (2, 13).

The results presented in Fig. 5A show the effects of MCM and MIMI on cell viability. Estimates of IC_{50} values for MCM and its position 10 epimer MIMI were 0.92 μM and 4.33 μM, respectively. These results show a 4-fold loss in the cytotoxicity of MCM upon epimerization of the carbomethoxy group at position 10 of the aglycone. Similar results, shown in Fig. 5B, were obtained for MSM and CLM. In this case, IC_{50} values for MSM (0.65 μM) and CLM (1.35 μM) differed by a factor of 2, again reflecting a loss in cytotoxicity upon epimerization of the carbomethoxy group at position 10 of the aglycone.

Antitumor effects in vivo. The four anthracyclines MCM, MSM, CLM, and MIMI were tested *in vivo* against mouse L1210 leukemia cells, similar to previous reports (2, 8, 10, 13), and the results are listed in Table 2. When administered as a single dose, and when comparing minimum effective doses (percentage T/C values of approximately 127–133), MIMI (51.2 mg/kg per injection) was approximately 32-fold less potent than MCM (1.6 mg/kg per injection). Similarly, CLM (12.8 mg/kg per

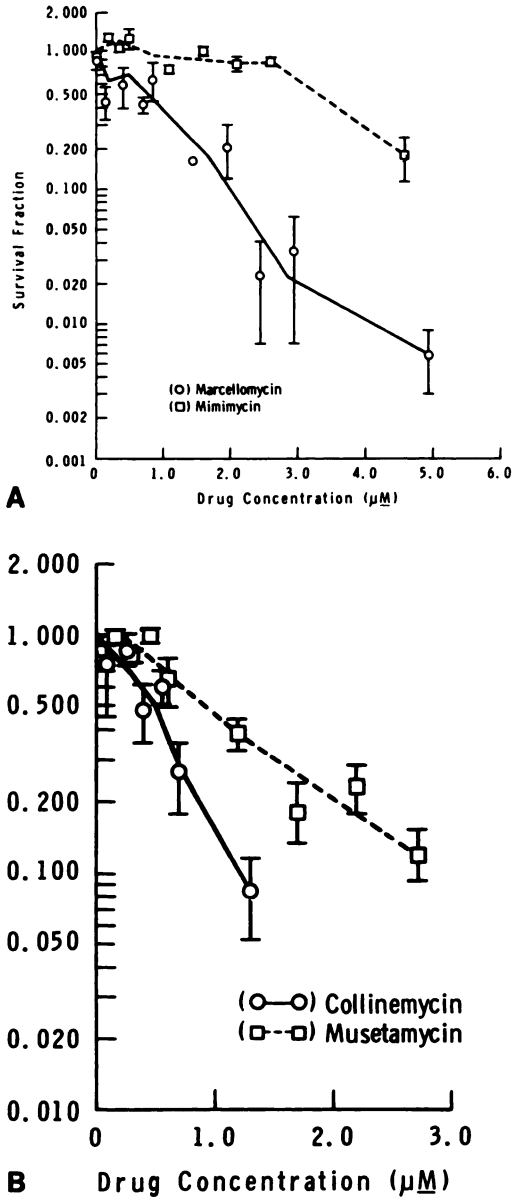


FIG. 5. *Effects of MCM and MIMI (A) and CLM and MSM (B) on the viability of NHAC.*

Petri plates containing approximately 1000 cells were treated with drugs for 15 min and washed with sterile saline followed by incubation with fresh medium for 7 to 14 days (2, 13). Resulting colonies were fixed, stained, and dired, and those containing 50 or more cells were counted. The means of a minimum of triplicate determinations are shown and the curves represent a minimum of duplicate or triplicate experiments. The IC_{50} values were obtained by probit analysis of the above results (data not shown). Linear regression analysis of the transformed data yielded p values of <0.001, <0.05 <0.001, and <0.01 for MCM, MSM, CLM, and MIMI, respectively.

injection) was approximately 4-fold less potent than MSM (3.2 mg/kg per injection).

The results indicate that epimerization of the carbomethoxy group at position 10 of the MCM molecule results in a significant (32-fold) reduction in antitumor potency *in vivo*. Similar studies with MSM resulted in only a 4-fold reduction in antitumor potency *in vivo*. These findings are in qualitative agreement with the *in vitro* cytotoxicity results.

DISCUSSION

Anthracyclines represent a major class of DNA-active antitumor antibiotics, of which ADM and DNM are the two prototype compounds. Both ADM and DNM have been shown by a number of workers to inhibit rapidly DNA and whole cellular RNA syntheses *in vivo* (14–16) in tissue culture cells (1, 15) and *in vitro* in cell-free systems (17–19). The effects of ADM on nucleolar preribosomal RNA synthesis have also been reported (1). These effects have been postulated to result from specific anthracycline-DNA interactions (3, 4, 14, 20–24) which involve an intercalative mechanism (25) as well as an electrostatic interaction (24). The results of this interaction are a decreased template activity (17, 18) and an altered sensitivity to nucleases (23). Thus, a major source of the mechanism of action of anthracyclines is thought to be due to DNA-binding and subsequent perturbation of normal nucleic acid metabolism.

Other activities have been reported to account for anthracycline antitumor activity, including cell surface effects (26, 27) and effects on mitochondrial function and

TABLE 2

In vivo antitumor activity MCM, MIMI, MSM, and CLM on L1210 leukemia cells

Tumor inoculum was 10^6 ascites cells implanted i.p. Hosts were female BDF (C57 BL/6 × DBA 2 F_1) mice. Drug was administered i.p. once on Day 1 only.

Material and dose (mg/kg)	MST[a]	Effective MST	Average wt. change	Survivors on Days 5[c]
	days	*% T/C*[b]	*g*	
MCM				
6.4	6.0	80	−5.8	6/6
3.2	10.5	140	−3.4	6/6
1.6	9.5	127	−2.5	6/6
0.8	9.0	120	−1.9	5/6
0.4	8.0	107	−0.2	6/6
0.2	7.5	100	+0.1	6/6
MSM				
12.8	10.0	133	−3.8	5/6
6.4	9.5	128	−2.3	6/6
3.2	10.0	133	−3.2	6/6
1.6	8.0	107	−0.6	6/6
0.4	7.0	93	+0.8	6/6
MIMI				
51.2	10.0	133	−3.2	5/6
25.6	8.6	107	+0.1	6/6
12.8	8.5	113	−0.3	6/6
6.4	9.0	120	−1.3	6/6
3.2	8.0	107	−0.3	6/6
1.6	7.5	100	+0.5	6/6
CLM				
51.2	11.0	147	−2.5	5/6
25.6	11.0	147	−4.2	6/6
12.8	10.0	133	−2.3	6/6
6.4	8.0	107	−1.2	6/6
3.2	8.0	107	+1.3	6/6
1.6	8.0	107	−0.3	6/6
Control Saline	7.5	—	+0.2	10/10

[a] MST, median survival time.

[b] *%T/C* MST-treated/MST control × 100. A *%T/C* ≥125 is considered to reflect a significant antitumor effect.

[c] Toxicity, <4/6 survivors on Day 5.

energy metabolism (28). Murphree and co-workers (26) reported essentially complete inhibition of the growth of Sarcoma 180 cells in culture by ADM at concentrations which had no effect on the rate of incorporation of [^{3}H] thymidine and [^{3}H]uridine into nucleic acids. Their findings indicated a correspondence in the concentrations of ADM needed for both cytotoxicity and cell surface phenomena. In addition, the finding that ADM increased the rate of cellular agglutination by concanavalin A, at low (i.e., 10^{-7} M) drug concentrations, suggested a direct membrane action of ADM (26). These studies were extended with the demonstration of effects on membrane fluidity by ADM (27). Decreased fluidity was observed in cardiolipin-containing lipid bilayers, indicating differential sites of action of ADM. A number of other studies have implicated a direct interaction of anthracyclines with cell membranes and cytoskeleton (5, 14, 15). These studies suggest an action of ADM at the membrane level affecting the movement of molecules in the membrane itself.

Although some analogues of ADM have been postulated to exert their antitumor effects without significant DNA-interactions, e.g., carcinomycin (13) and *N*-trifluoroacetyladriamycin-14-valerate (5, 15, 29), the antitumor effects of MCM and its congeners have been shown to correlate to a significant extent with the specific inhibition of No-RNA synthesis (1, 2, 5, 19). The biochemical changes have also been confirmed by electron microscopic studies demonstrating nucleolar morphological alterations induced by MCM and its analogues (30). Additionally, these nucleolar effects of MCM and other Class II anthracyclines have been shown to be dependent upon DNA-anthracycline interactions (2, 4, 5). Thus, reduced DNA-binding ability of these compounds was shown to correlate with decreased No-RNA synthesis inhibition and antitumor activity both *in vivo* and *in vitro* (2, 4).

The molecular pharmacology of Class II anthracyclines has been characterized as a result of several studies from this laboratory (1–5, 13, 19). Accordingly, several structural features have been shown to be important for optimal Class II activity. These include the presence of a glycosidic side chain with two to three sugar residues (1), and the presence of the carbomethoxy group at position 10 of the aglycone (2). The present study suggests the importance of the additional requirement regarding the specific stereochemical orientation of the carbomethoxy group present in the MCM or MSM molecule. As summarized in Table 3, significant decreases in activity occur in the five parameters studied upon epimerization of the carbomethoxy group at position 10 of MSM and MCM.

Although a similar trend is observed when comparing the activity changes for both MCM versus MIMI pair and the MCM versus CLM pair, the decreases in activity observed for the former pair were generally significantly greater than those of the latter pair (Table 3). Accordingly, epimerization of the carbomethoxy group at position 10 of the MCM molecule resulted in a 10-fold to 13-fold reduction in DNA-binding ability, a 2-fold reduction in DNA synthesis inhibitory potency, a 10-fold reduction in RNA synthesis inhibitory potency, a 5-fold reduction in *in vitro* cytotoxicity, and a 32-fold reduction in *in vivo* antitumor potency. Epimerization of the carbomethoxy group at position 10 of the MSM molecule resulted in generally smaller decreases in DNA-binding ability (3- to 5-fold), DNA and RNA synthesis inhibitory potency (2-fold), *in vitro* cytotoxicity (2-fold), and *in vivo* antitumor potency (4-fold). The small change in DNA synthesis inhibitory activity obtained for both MIMI and CLM suggests that this process is probably not a target of the Class II anthracyclines.

These studies confirm and extend the observation that for Class II anthracyclines a principal intracellular target is DNA, and variations in DNA binding result in variations in *in vitro* and *in vivo* activities. Given the fact that the comparisons involved are relatively simple isolated DNA assays of complex *in vivo* systems, the conclusions

TABLE 3

Summary of the nucleic acid synthesis inhibitory effects, DNA-binding characteristics, and in vivo and in vitro antitumor activities of MCM, MIMI, MSM, and CLM

Anthracycline	K_{app}[a]		*In vitro* IC_{50} values[b]			*In vivo* antitumor activity in L1210 leukemia cells
	Calf Thumus DNA	Salmon Sperm DNA	DNA synthesis	RNA synthesis	Cell viability	
	$\times 10^6\ M^{-1}$			μM		*mg/kg/injection*
MCM	6.21	7.22	13.77	0.71	0.86	1.6
MIMI	0.62	0.55	25.37	7.51	2.50	51.2
-Fold decrease in activity[d]	10.0	13.0	1.87	10.58	2.91	32.0
MSM	3.17	2.47	10.88	1.17	0.52	3.2
CLM	0.67	0.82	22.79	2.59	0.96	12.8
-Fold decrease in activity	4.73	3.0	2.09	2.21	1.85	4.0

[a] K_{app} values were obtained from Table 1.

[b] IC_{50} values wre determined using probit analysis as indicated in Figs. 3 and 4.

[c] Determined following single-dose administration in the mouse L1210 leukemia system, monitoring MST of treated relative to control as indicated in Table 2. Minimum effective doses are indicated, i.e., those producing a $\%T/C$ value of 127–133.

[d] Ratio of the analogue/parent compound for each parameter studied (e.g., MIMI/MCM) except for DNA-binding characteristics (K_{app}) in which case the ratio of the parent compound/analogue was obtained.

are impressive. Nevertheless, such correlations, even when coupled with our previous observations, do not exclude the possibility that other mechanisms may contribute to the cytotoxicity of these drugs.

Previous studies have shown that enhanced DNA binding ability is correlated with increased length of the glycosidic side chain of the Class II anthracyclines (3). Thus, the trisaccharide MCM exhibits higher K_{app} values for DNA binding than does the disaccharide MSM or the monosaccharide pyrromycin (3). This relationship does not appear to obtain when the carbomethoxy group at position 10 is epimerized. As shown in Table 3, the trisaccharide MIMI has a lower K_{app} value for both DNAs than does disaccharide CLM. In fact, removal of the carbomethoxy group, as in 10-descarbomethoxy-MCM (4), resulted in a smaller decline in DNA-binding ability than does epimerization of this moiety (e.g., MIMI) when these two analogues are compared with MCM. These observations suggest that the stereochemical configuration of the carbomethoxy group at position 10 is of major importance with regard to DNA template interactions. Results obtained from parallel DNA binding studies employing viscometric titrations suggest that CLM and MSM differ significantly when comparing intercalative interactions with DNA.[5] In addition, model studies of the four anthracyclines MCM, MIMI, MSM, and CLM reveal that marked molecular conformational changes occur upon epimerization of the 10-carbomethoxy group.[5] Using the numbering sequence of Doyle and co-workers (6), the rings of the anthracycline aglycone are lettered from left to right, when viewing the structure as shown in Fig. 1. Thus, the three fused aromatic rings correspond to rings A, B, and C. The nonaromatic ring, to which the glycosidic side chain is attached, is the D-ring. It is this D-ring which undergoes conformational alterations dependent upon the types of substituents attached to positions 7, 8, 9, and 10. In the conformation which is present in MCM and MSM, the 10-carbomethoxy group is above the plane of the aglycone and the glycosidic side chain, attached to position 7, is below the plane of the aglycone, and forms an angle of near 90° with the aglycone.[5] This conformation facilitates the intercalation of the aglycone between adjacent nucleotide bases of the DNA helix, and the sugar side chain is able to stabilize this binding by interacting with the ribose-phosphate backbone of DNA.[5] However, epimerization of the 10-carbomethoxy group, as for CLM or MIMI, results in the positioning of the glycosidic side chain to lie more nearly in line with the plane of the aglycone.[5] In this configuration, the sugar side chain is much less able to stabilize the intercalation of the aglycone by interacting with the ribose-phosphate backbone of DNA.[5]

The molecular model studies offer some explanation of the decreased DNA-binding ability of MIMI and CLM as compared with MCM and MSM. These molecular observations can be extended to offer some explanation of the decreased biological activities of the position 10 epimers. Since previous studies have demonstrated the high degree of correlation between DNA-binding ability, No-RNA synthesis inhibitory activity and antitumor potency (1, 2, 4, 5) for the Class II anthracyclines, it is not too surprising that the marked decrease in antitumor potency exhibited by the position 10 epimers as compared with their parent compound is correlated with a marked reduction in K_{app} values (Table 3). Parallel studies examining the nature of the anthracycline-DNA interactions for MCM, MIMI, MSM, and CLM will be published elsewhere.[5] The results of these studies should allow a better understanding of the requirements for Class II anthracycline-DNA interactions. These results are valuable for future antitumor drug design because they allow a further characterization of the nature of the Class II anthracycline DNA receptor and thus specify the drug-receptor interactions which are of importance in the mechanism of antitumor action.

ACKNOWLEDGMENTS

The authors are indebted to Ms. B. Biddle, Ms. Paulette Dixon, and Ms. Sally VanCamp for assistance in the preparation of the manuscript. We also thank Ms. Clara Gibson for her assistance in the culturing and preparation of cells. We are indebted to Drs. W. Rose and W. T. Bradner for the *in vivo* antitumor data and assistance in the preparation of Table 2. The support and guidance of Dr. Harris Busch are most appreciated.

REFERENCES

1. Crooke, S. T., V. H. DuVernay, L. Galvan, and A. W. Prestayko. Structure-activity relationships of anthracyclines relative to effects on macromolecular syntheses. *Mol. Pharmacol.* **14**:290–98 (1978).
2. DuVernay, V. H., J. M. Essery, T. W. Doyle, W. T. Bradner, and S. T. Crooke. The antitumor effects of anthracyclines: the importance of the carbomethoxy-group at position-10 of marcellomycin and rudolfomycin. *Mol. Pharmacol.* **15**:341–336 (1979).
3. DuVernay, V. H., J. A. Pachter, and S. T. Crooke. DNA binding studies on several new anthracycline antitumor antibiotics. I. Sequence preference and structure activity relationships of marcellomycin and its analogs as compared to adriamycin. *Biochemistry* **18**:4024–4030 (1979).
4. DuVernay, V. H., J. A. Pachter, and S. T. Crooke. DNA binding studies on several new anthracycline antitumor antibiotics. II. The importance of the carbomethoxy-group at position-10 of marcellomycin and rudolfomycin. *Mol. Pharmacol.* **16**:623–632 (1979).
5. DuVernay, V. H., S. Mong, and S. T. Crooke. Molecular pharmacology of anthracyclines: demonstration of multiple mechanistic classes of anthracyclines, in *Anthracyclines: Current Status and New Developments* (S. T. Crooke and S. D. Reich, eds.). Academic Press, New York, 61–123 (1980).
6. Doyle, T. W., D. E. Nettleton, R. E. Grulich, D. M. Balitz, J. L. Johnson, and A. L. Vulcano. Antitumor agents from the bohemic acid complex. IV. Structures of rudolfomycin, mimimycin, collinemycin and alcindoromycin. *J. Am. Chem. Soc.* **101**:7041–7049 (1979).
7. Deleted in proof.
8. Nettleton, D. E., Jr., W. T. Bradner, J. A. Bush, A. B. Coon, J. E. Moseley, R. W. Myllymaki, F. A. O-Herron, R. H. Schreiber, and A. L. Vulcano. New antitumor antibiotics: musettamycin and marcellomycin. *J. Antibiotics* **30**: 525–529 (1977).
9. Litchfield, J. T., Jr., and F. Wilcoxon. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* **96**:99–113 (1949).
10. Bradner, W. T., and D. J. Hutchinson. Neocarzinostatin (NSC-69856): an antitumor antibiotic effective against ascitic leukemia L-1210 in mice. *Cancer Chemother. Rep.* **50**:79–84 (1966).
11. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
12. Peacocke, A. R., and J. N. H. Skerrett. The interaction of aminoacridines with nucleic acids. *Trans. Faraday Soc.* **52**:261–279 (1956).
13. DuVernay, V. H., J. A. Pachter, and S. T. Crooke. Molecular pharmacological differences between carcinomycin and its analog, carminomycin-11-methyl ether, and adriamycin. *Cancer Res.* **40**:387–394 (1980).
14. DeMarco, A., F. Arcamone, and F. Zunino. 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.). Springer-Verlag, Berlin, 101–128 (1975).
15. Henry, D. W. Adriamycin, in *Cancer Chemotherapy* (A. C. Sartorelli ed.). American Chemical Society Division of Medicinal Chemistry Symposium, Washington, D. C. 15–57 (1976).
16. Skovsgaard, T., and N. I. Nissen. Adriamycin, an antitumor antibiotic: a

review with special reference to daunomycin. *Danish Med. Bull.* **22**:62–73 (1975).
17. Barthelemy-Clavey, V. C., Molinier, G. Aubel-Sadron, and R. Maral. Daunorubicin inhibition of DNA-dependent RNA polymerases from Ehrlich ascites tumor cells. *Eur. J. Biochem.* **69**:23–33 (1976).
18. Momparler, R. L., M. Karon, S. E. Siegel, and F. Avila. Effect of adriamycin on DNA, RNA and protein synthesis in cell-free systems and intact cells. *Cancer Res.* **36**:2891–5 (1976).
19. DuVernay, V. H., and S. T. Crooke. Effects of several anthracycline antitumor antibiotics on the transcriptional activity of isolated nucleoli. *J. Antibiotics* **33**:1048–1053 (1980).
20. Zunino, F., R. Gambetta, A. DiMarco, G. Luoni, and A. Zaccara. Effects of the stereochemical configuration on the interaction of some daunomycin derivatives with DNA. *Biochem. Biophys. Res. Commun.* **69**:744–750 (1976).
21. DiMarco, A., A. M. Casazza, T. Dasdia, A. Necco, G. Pratesisi, P. Rivolta, A. Velcich, A. Zaccara, and F. Zunino. Changes of activity of daunorubicin, adriamycin and stereoisomers following the introduction or removal of hydroxyl groups in the amino sugar moeity. *Chem. Biol Interact.* **19**:291–302 (1977).
22. Gabbay, E. J., D. Grier, R. E. Fingerle, R. Reimer, R. Levy, S. W. Pearce, and W. D. Wilson. Interaction specificity of the anthracyclines with deoxyribonucleic acid. *Biochemistry* **15**:2062–2069 (1976).
23. Tsou, K. C., and K. F. Yip. Effect of deoxyribonucleases on adriamycin-polynucleotide complexes. *Cancer Res.* **36**:3367–3373 (1976).
24. Pigram, W. J., W. Fuller, and L. D. Hamilton. Stereochemistry of intercalation: interaction of daunomycin with DNA. *Nature New Biol.* **235**:17–19 (1972).
25. Lerman, L. S. Structural considerations in the interaction of DNA and acridines. *J. Mol. Biol.* **3**:18–30 (1961).
26. Murphree, S. A., L. S. Cunningham, K. M. Hwang, and D. C. Sartorelli. Effects of adriamycin on surface properties of sarcoma 180 ascites cells. *Biochem. Pharmacol.* **25**:1227–31 (1976).
27. Tritton, T. R., S. A. Murphree, and A. C. Sartorelli. Adriamycin: a proposal on the specificity of drug action. *Biochem. Biophys. Res. Commun.* **84**:802–805 (1978).
28. Andrieini, G., C. M. Beretta, and O. Sonzogni. Antimitochondrial activity of adriamycin and daunomycin on *Saccharomyces cerevisiae*. *Pharmacol. Res. Commun.* **9**:155–64 (1977).
29. Krishan, A., M. Israel, E. J. Modest, and E. Frei, III. Differences in cellular uptake and cytofluorescence of adriamycin and *N*-trifluoracetyl/adriamycin-14-valerate. *Cancer Res.* **36**:2114–2116 (1976).
30. Daskal, Y., C. Woodward, S. T. Crooke, and H. Busch. Comparative ultrastructural studies of nucleoli of tumor cells treated with adriamycin and the newer anthracyclines, carcinomycin and marcellomycin. *Cancer Res.* **38**:467–473 (1978).

Send reprint requests to: Dr. Virgil H. DuVernay, Bristol-Baylor Laboratory, Department of Pharmacology, Baylor College of Medicine, Houston, Tex. 77030.