DNA-Binding Specificity and RNA Polymerase Inhibitory Activity of Bis(aminoalkyl)anthraquinones and Bis(methylthio)vinylquinolinium Iodides

WILLIAM O. FOYE ˣ, OPA VAJRAGUPTA*, and SISIR K. SENGUPTA‡

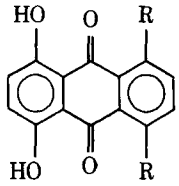
Received November 21, 1980, from the *Samuel M. Best Research Laboratory, Massachusetts College of Pharmacy and Allied Health Sciences, Boston, MA 02115.* Accepted for publication June 9, 1981. *Present address: School of Pharmacy, Mahidol University, Bangkok, Thailand. ‡Present address: Boston University Medical Center, Boston, MA 02118.

Abstract □ The determination of DNA-binding specificities for a series of bis(methylthio)vinylquinolinium iodides and two bis(aminoalkyl)-anthraquinones was accomplished by spectral analysis, equilibrium dialysis, elevation of melting temperature, and inhibition of DNA function as a template for *Escherichia coli* RNA-polymerase transcription activity *in vitro*. Studies of complex formation were carried out by comparison of difference spectra of the compounds in the presence of native double-stranded DNA and separated-strand DNA. Base specificity of the interaction between DNA and the compounds was demonstrated for both series, particularly for the anthraquinones, for the guanine–cytosine base pair. Comparison of the difference spectra of the compounds in the presence of DNA with varied base-pair ratios showed a strong preference of the anthraquinones for the guanine–cytosine base pair, but the quinolinium compounds showed no preference. The linear-binding isotherm for the quinolinium compounds indicated one type of binding site, while two types of binding sites were apparent for the anthraquinones. Since only one anthraquinone was active in leukemia tests, factors other than DNA binding must account for the activity of the antileukemic derivative.

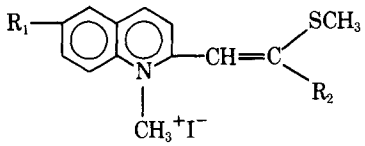
Keyphrases □ DNA—binding specificity of bis(aminoalkyl)anthraquinones and bis(methylthio)vinylquinolinium iodides □ Bis(aminoalkyl)anthraquinones—DNA binding specificity and RNA polymerase inhibition □ Bis(methylthio)vinylquinolinium iodides—DNA binding specificity and RNA polymerase inhibition

A series of 1-methylquinolinium-2-dithioacetic acid zwitterions (1, 2) and derivatives (3) showed appreciable anticancer activity against P-388 lymphocytic leukemia growth in mice. Since the structures of these compounds differ in several respects from those of other antileukemic agents, no obvious clue to a possible mechanism of anticancer action was apparent. However, the planar nature of the quinoline ring suggested binding to DNA as a possible factor in their action, and accordingly, DNA binding studies were carried out. At the same time, two structurally similar anthraquinone derivatives (4), one having good antileukemic activity and the other inactive, were measured and examined for any differences in their binding ability. Chloroquine, a known intercalating agent for DNA, was used as a control substance.

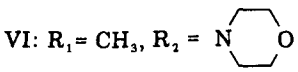
The methods used to determine DNA binding ability involved spectral analysis, equilibrium dialysis, elevation of melting temperature, and *in vitro* inhibition of cell-free RNA transcription. In addition, studies of complex formation of the compounds with DNA were carried out using native double-strand and single-strand DNA, and also using DNA from various sources with different base-pair ratios. Absorption spectra of the compounds in the presence of DNA with different base-pair ratios were compared to determine any base-pair specificities in the DNA binding. It was thought that use of several different techniques could provide more information on DNA binding than one method alone.



I: R = $NH(CH_2)_2NH(CH_2)_2OH \cdot 2\,HCl \cdot 5\,H_2O$
II: R = $NH(CH_2)_4N(CH_3)_2$



III: R_1 = H, R_2 = SCH_3
IV: R_1 = CH_3, R_2 = SCH_3
V: R_1 = OCH_3, R_2 = SCH_3



EXPERIMENTAL[1]

Materials — 5,8-Dihydroxy-1,4-bis[2-(hydroxyethyl)aminoethyl] amino-9,10-anthracenedione (I) and 5,8-dihydroxy-1,4-bis[4-(dimethylaminobutyl)amino]-9,10-anthracenedione(II) were used. 1-Methyl-2-bis(2-methylthio)vinylquinolinium iodide(III), 1,6-dimethyl-2-bis(2-methylthio)vinylquinolinium iodide(IV), 1-methyl-6-methoxy-2-bis(2-methylthio)vinylquinolinium iodide(V), and 1,6-dimethyl-2-[2-methylthio-2-(1-morpholino)vinyl]quinolinium iodide (VI) were previously reported (3). Chloroquine (VII), calf thymus DNA type I sodium salt [43% guanosine–cytidine (G–C)], *Escherichia coli* DNA type VIII (50% G–C), *Clostridium perfringens* DNA type XII (27% G–C), *Micrococcus lysodeikticus* DNA type XI (72% G–C), bovine serum albumin, RNA polymerase type I from *E. coli*, adenosine 5′-triphosphate disodium salt (ATP), cytidine 5′-triphosphate sodium salt type III (CTP), uridine 5′-triphosphate sodium salt type III (UTP), and guanosine 5′-triphosphate sodium salt type III (GTP) were all obtained commercially[2]. Tromethamine[3] and [8-^{14}C]adenosine 5′-triphosphate tetrasodium salt[4], [8-^{14}C]ATP, (40 Ci/mmole) were also obtained commercially.

Spectral Analysis—Stock solutions of the compounds were made 1 × 10^{-4} *M* in 5 m*M* tromethamine hydrochloric acid buffer, pH 7.4. Beer's law curves were prepared from the regressed absorbance values, and solutions of 1.5–3.5 × 10^{-5} *M* were scanned in the UV and visible regions. Molar absorptivities are listed in Table I. Spectra were observed in the presence of calf thymus DNA solutions at concentrations of 1.5 × 10^{-5}–5 × 10^{-4} *M*, and are shown in Figs. 1 and 2 for the anthraquinones.

Equilibrium Dialysis—These experiments were carried out using an earlier method (5) with 5-ml glass dialysis cells and a membrane with

[1] Absorption spectra were taken with a Beckman DB spectrophotometer. Melting temperatures were measured with a Gilford 250 spectrophotometer equipped with a baseline reference compensator, analog multiplexer No. 6046 and a No. 2527 thermoprogrammer. Measurement of inhibition of DNA function as a template for *E. coli* RNA polymerase was done using a Packard Tricarb LSC model 3320 liquid scintillation spectrometer.

[2] Sigma Chemical Co., St. Louis, Mo.

[3] Trizma compounds 8.2 and 7.5, Sigma Chemical Co., St. Louis, Mo.

[4] New England Nuclear Corp., Boston, Mass.

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Table I—Molar Absorptivities [a]

Compound	Absorption Maximum, nm	ϵ_{max}
I	608	18,630
II	610	18,490
III	402	25,480
IV	402	16,870
V	404	20,060
VI	434	30,245
VII	338	13,225

[a] Observed in 5 m*M* tromethamine hydrochloric acid buffer (pH 7.4).

a molecular weight limit of 12,000[5]. Solutions of calf thymus DNA ranging from 1.0×10^{-5} to 1.5×10^{-3} *M* were made in 5 m*M* tromethamine hydrochloric acid buffer (pH 7.4), and a concentration of 1×10^{-4} *M* of compound was prepared in the same medium. Concentrations at equilibrium (24 hr, 25°) were measured by absorption spectra and determinations for each compound were made in duplicate or triplicate. Concentrations of free and bound species were calculated as previously described (6, 7). The correlation coefficient for each compound tested was >0.91. Binding parameters K_{app} and B_{app} are listed in Table II.

Melting Temperature Determination—The temperature in a heated cell was increased at the rate of 1°/min. The concentration of calf thymus DNA was 1.0×10^{-4} *M* nucleotide phosphorus, and the buffer was 5 m*M* tromethamine hydrochloric acid (pH 7.4). Concentrations of compounds used were 1×10^{-4} and 1×10^{-5} *M* for the anthraquinones and 1×10^{-4} *M* for the quinolinium compounds. Absorbance was measured at 260 nm. The temperature at which 50% hyperchromicity is attained (T_m) for the compounds tested is recorded in Table III.

Inhibition of *E. coli* RNA Polymerase—Stock solution H was prepared from: tromethamine hydrochloric acid (1.376 g); potassium chloride (2.795 g); 2-mercaptoethanol (0.195 g); magnesium chloride (0.508 g); bovine serum albumin (0.125 g); and distilled water to make 100 ml.

Stock solution I was prepared from: ATP sodium salt (0.117 g); CTP sodium salt (0.109 g); UTP sodium salt (0.105 g); GTP sodium salt (0.114 g); and distilled water to make 10 ml.

Stock solution J contained [8-^{14}C]ATP (0.01 mCi, 0.13 mg/0.5 ml, 30 μl), and distilled water to make 200 μl.

Stock solution K consisted of 100 units of *E. coli* RNA polymerase diluted with 60% glycerol–40% tromethamine hydrochloric acid buffer containing 0.2 m*M* 1,4-dithiothreitol, 0.01 m*M* ethylenediaminetetraacetic acid, and 10 m*M* magnesium chloride to give 2 units/10 μl. It was stored below 0°.

Stock solution L was prepared from calf thymus DNA solution, 5 m*M* in nucleotide phosphorus (absorption coefficient at 260 nm of 6600 M^{-1}/cm, 0.083 g); ethylenediaminetetraacetic acid (0.056 g); and 5 m*M* tromethamine hydrochloric acid buffer (pH 7.4) to make 50 ml. Solution L was stored below 0°. Other DNA solutions were prepared in the same way.

The liquid scintillation solution contained 2,5-diphenyloxazole (6.000 g); 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.200 g); toluene (1400 ml); and methanol (600 ml).

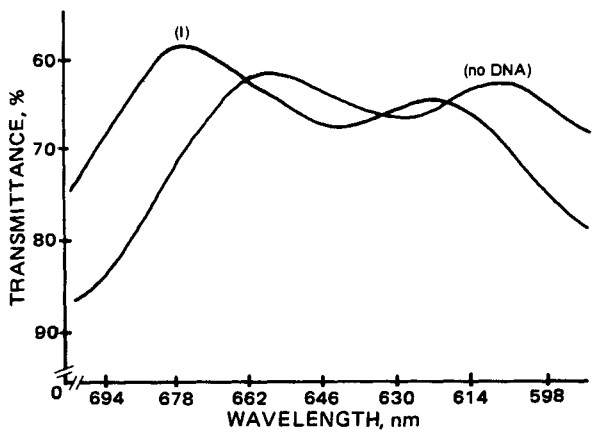

Figure 1—*Influence of DNA on the visible spectrum of I. DNA was used at a concentration of* 25.0×10^{-5} M.

[5] UM-10 Diaflo, Amicon Corp.

Table II—Equilibrium Constants for Binding of I–VII to Calf Thymus DNA [a]

Compound	K_{app}	B_{app}
I	1.31×10^6	0.341
II	2.61×10^6	0.341
III	7.47×10^4	0.063
IV	1.31×10^3	0.075
V	1.05×10^3	0.085
VI	8.70×10^4	0.077
VII	8.08×10^5	0.138

[a] Determined in 5 m*M* tromethamine hydrochloric acid buffer (pH 7.4).

The gain setting for maximum efficiency of the scintillation spectrometer was determined at 40%. The activity of RNA polymerase was determined by measuring the amount of [8-^{14}C]ATP rendered acid insoluble as determined by the filter paper disk assay of Bollum (8).

The assay mixture contained in 0.125 ml consisted of pH 8.0 tromethamine hydrochloric acid buffer (5.000 μmoles); magnesium chloride (1.250 μmoles); potassium chloride (18.750 μmoles); 2-mercaptoethanol (1.250 μmoles); CTP sodium salt (0.019 μmole); GTP sodium salt (0.019 μmole); UTP sodium salt (0.019 μmole); [8-^{14}C]ATP tetrasodium salt (6,500 cpm) (0.019 μmole); calf thymus DNA, (5.000 μg); and bovine serum albumin, (62.500 μg).

DNA (5.0 μg) solutions were preincubated with each compound (1.0×10^{-5}–5.0×10^{-5} *M*) in 25 μl of 5 m*M* tromethamine hydrochloric acid buffer (pH 7.4) for 10 min at 37° prior to the assay of RNA polymerase activity. The reference blank contained DNA in buffer solution.

The assay mixture was prepared by adding 50 μl of solution H, 10 μl of solution I, 10 μl of solution J, 20 ml of distilled water, 5.0 μl of preincubated DNA and the test compound in 25 μl, and 10 μl of *E. coli* RNA polymerase solution (2 units). The mixture was incubated for 10 min at 37°, and the enzyme reaction was terminated by the addition of 25 μl of cold 5% trichloroacetic acid containing 1% tetrasodium pyrophosphate and by chilling in an ice water bath for 15 min.

The acid insoluble material from a 100-μl aliquot of each incubation mixture was spotted uniformly on a 2.5-cm circular millipore filter (0.45 μm pore size), kept at room temperature for 15 min, and washed three times by being swirled in 10 ml of 5% trichloroacetic acid containing 1% tetrasodium pyrophosphate, followed by two washings each of 2.5% trichloroacetic acid and 95% ethanol. Filters were dried and placed in 15 ml of scintillation liquid. Radioactivity was counted in the scintillation spectrometer and all assays were run in triplicate. Inhibition of RNA polymerase activity by the test compounds was recorded (Tables IV and V).

Comparison of Absorption Spectra of Compounds in the Presence of Native Double-Stranded DNA and Separated-Strand DNA—One milliliter of a test compound solution (2.0–5.0×10^{-4} *M*, in tromethamine hydrochloric acid buffer, pH 7.4) was added to 1 ml of either native double-stranded calf thymus DNA (1.25×10^{-4}–5.0×10^{-4} *M*) or to 1 ml of separated-strand DNA solution. Strand separation was done by heating calf thymus DNA solution at 98° for 30 min and immediately

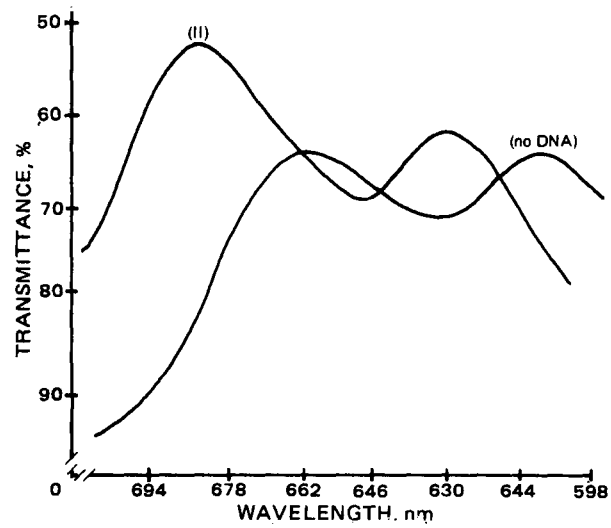

Figure 2.—*Influence of DNA on the visible spectrum II; DNA was used at a concentration of* 40.0×10^{-5} M.

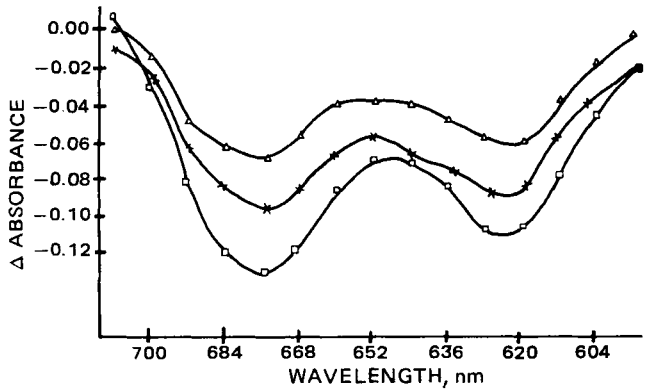

Figure 3—*Difference spectra of free and DNA-bound test compounds. The concentration used was 2.0 × 10⁻⁵ M I and the DNA concentration used was 1.25 × 10⁻⁵ M. Key:* △, C. perfringens *DNA; X, calf thymus DNA; and* □, M. lysodeikticus *DNA.*

cooling the solution at 0°. The control solution used was 5 m*M* tromethamine hydrochloric acid buffer, pH 7.4. Difference spectra were recorded in the visible region.

Inhibition of DNA Function as Template for *E. coli* RNA Polymerase with Varied Base Ratio DNA–DNA from the following sources was used: *C. perfringens* (27% G–C); calf thymus (43% G–C); *E. coli* (50% G–C); and *M. lysodeikticus* (72% G–C). RNA polymerase activity was measured as described and results are tabulated in Table VI. Concentrations used were 1×10^{-4} *M* for the anthraquinones and 1.5×10^{-3} *M* for the quinolinium compounds.

Comparison of Absorption Spectra of Compounds in the Presence of Varied Base Ratio DNA. One milliliter of a test compound solution (2.0×10^{-5}–5.0×10^{-4} *M* in 5 m*M* tromethamine hydrochloric acid buffer, pH 7.4) was added to 1 ml of the various DNA solutions (1.25×10^{-5} *M*, based on phosphorus). The buffer (5 m*M*, pH 7.4) was used as a control. Difference spectra were recorded in the visible region, and the data are plotted in Figs. 3 and 4 for the anthraquinones.

DISCUSSION

Spectral Analysis—The interaction of the compounds with calf thymus DNA resulted in a consistent bathochromic/hypochromic shift in the visible spectrum around 20–30 nm for the anthraquinones and 20–50 nm for the quinolinium compounds. In comparison, chloroquine showed an average shift of 10 nm in the presence of calf thymus DNA.

Equilibrium Dialysis—These experiments using calf thymus DNA were done at ambient temperature at pH 7.4. For determining binding parameters, Scatchard plots were employed using the expression:

$$r/C_f = K_{app}(B_{app} - r) \qquad \text{(Eq. 1)}$$

where r is the ratio of bound compound to DNA nucleotide phosphorus, C_f is the concentration of free compound, K_{app} is the apparent binding constant, and B_{app} is the apparent number of binding sites per nucleotide phosphorus. The binding parameters K_{app} and B_{app} are shown in Table II. These values were obtained from the nearly linear portion of the isotherm at small values of r for a strong binding.

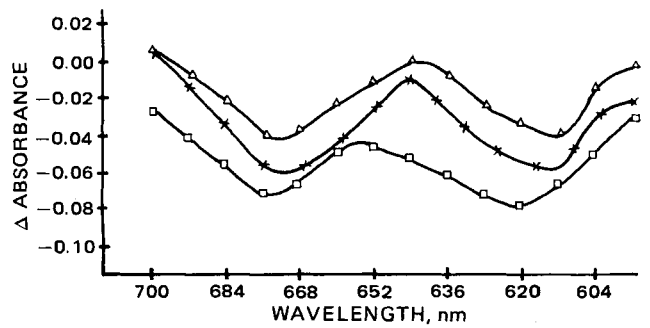

Figure 4—*Difference spectra of free and DNA-bound test compounds. The concentration used was 2.0 × 10⁻⁵ M II and the DNA concentration used was 1.25 × 10⁻⁵ M. Key:* △, C. perfringens *DNA; X, calf thymus DNA;* and □, M lysodeikticus *DNA.*

Table III—Melting Temperature Determination [a]

Compound	T_m	ΔT_m
DNA (calf thymus) [b]	62.0°	
I [b]	72.0°	10.0°
II [b]	74.0°	12.0°
I [c]	>100.0°	>38.0°
II [c]	>100.0°	>38.0°
III [b]	69.5°	7.5°
IV [b]	71.5°	9.5°
V [b]	71.0°	9.0°
VI [b]	70.0°	8.0°
VII [b]	76.0°	14.0°

[a] Measured at 260 nm with a temperature rise of 1°/min. [b] 1.0×10^{-4} *M*. [c] 1.0×10^{-5} *M*.

Table IV—Inhibition of *E. coli* RNA Polymerase by the Anthraquinones on Calf Thymus DNA as Substrate

Concentration, *M*	Inhibition, % I	Inhibition, % II
2.0×10^{-4}	89.01	90.26
1.0×10^{-4}	79.03	79.20
6.0×10^{-5}	40.39	68.38
2.0×10^{-5}	31.80	35.92
1.0×10^{-5}	26.69	10.44
1.0×10^{-6}	0.00	0.00

Table V—Inhibition of *E. coli* RNA Polymerase by the Methylthiovinylquinolinium Iodides on Calf Thymus DNA as Substrate

Concentration, *M*	Inhibition, % III	IV	V	VII	VIII
5.0×10^{-3}					60.00
4.0×10^{-3}	50.08	67.44	52.18	47.08	
2.0×10^{-3}	35.28	57.66	26.05	35.98	35.12
1.0×10^{-3}	27.40	41.47	18.41	3.37	19.28
1.0×10^{-4}	17.02	8.03	13.28	0.00	0.00
1.0×10^{-5}	13.18	0.00	0.00	0.00	0.00
1.0×10^{-6}	0.00	0.00	0.00	0.00	0.00

Scatchard plots of the binding data for the anthraquinones yielded biphasic curves, which indicates that two binding sites are involved, a stronger and a weaker (Figs. 5 and 6). The stronger binding data were derived from the linear region of the plot at small values of r. The other slope at higher values of r indicates the presence of a weaker binding site. The strong binding is most likely associated with intercalation of the anthraquinone ring into DNA base pairs (9, 10). The weaker binding may be associated with the secondary binding of the side chains by electrostatic interaction with the anionic exterior of the DNA helix.

Association constants for the anthraquinone derivatives are of the same order of magnitude as those found for daunorubicin and doxorubicin (9, 10), lower than that for dactinomycin (11, 12), and greater than that for chloroquine (13). The apparent number of binding sites per nucleotide obtained for the strong binding site is 0.34, indicating that binding of these compounds involves approximately three adjacent phosphate groups on the DNA.

The linear binding isotherms of the quinolinium compounds indicate that only one type of binding site is involved on the DNA helix. The association constants are lower than those obtained for either the anthraquinones or chloroquine. The compounds of this group bind at approximately one of every contiguous 8–12 phosphate groups on the DNA. The binding abilities of the compounds of this type were essentially the same, indicating little effect from the 6-substituents.

Melting Temperature Elevation—Thermal denaturation studies were done at pH 7.4, using calf thymus DNA. The results shown in Table III indicate that all of the compounds bind to and stabilize the DNA helix toward temperature denaturation. Thermal stabilization did not vary much between the members in each series, but the anthraquinones caused a greater T_m increase than either the quinolinium compounds or chloroquine, and much greater than the usual tetracyclic intercalating agents (9). This suggests the possible formation of a covalent bond *via* a radical reaction induced at higher temperature (14).

Inhibition of *E. coli* RNA-Polymerase Activity—Inhibition of DNA function as a template for *E. coli* RNA polymerase was carried out *in vitro* using graded concentrations of the compounds. Results are listed in

Table VI—Inhibition of *E. coli* RNA Polymerase Using Varied DNA Base Ratios

DNA (% G–C)	Inhibition, %						
	I	II	III	IV	V	VI	VIII
C. perfringens (27%)	46.43	41.17	20.79	18.18	14.39	25.21	5.05
Calf thymus (43%)	79.74	78.68	26.52	35.66	26.17	22.58	25.78
E. coli (50%)	81.12	89.49	33.84	36.66	28.24	41.42	45.19
M. lysodeikticus (72%)	97.53	93.86	48.93	40.17	48.94	46.54	51.51

Tables IV and V. The anthraquinones were the stronger inhibitors, giving 80–90% inhibitions at concentrations of 10^{-4} *M*. The quinolinium compounds gave 47–67% inhibitions at concentrations of 10^{-3} *M*, which were comparable to the inhibitory ability of the same concentrations of chloroquine (13).

Studies of Complex Formation—Comparison of the visible absorption spectra of the compounds in the presence of native double-stranded and separated-strand DNA (calf thymus) was made. The difference spectra showed marked quenching in absorptivities in the spectrum of the double-stranded DNA (Figs. 3 and 4) but very little change in the spectrum of the separated-strand DNA. The requirement for double-stranded DNA with all the compounds tested indicated that the double-helical structure is necessary for strong binding, and that both strands are involved.

Base specificity for the interaction between DNA and the compounds tested was clearly demonstrated by the inhibition of DNA function as measured by inhibition of *E. coli* RNA-polymerase activity with different base ratios of guanosine and cytidine. The extent of transcription inhibition is shown in Table VI. It is apparent that there is a preference for the G–C base pair, since the degree of inhibition is greatest, with both series of compounds, with the DNA source having the highest G–C base pair ratio. The anthraquinones appeared to have a greater preference for this base pair than the quinolinium compounds, as indicated by extent of inhibition.

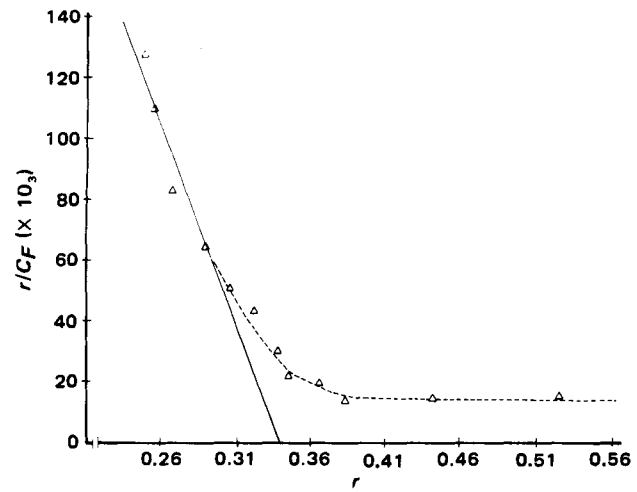

Figure 5—*Scatchard plot of I.*

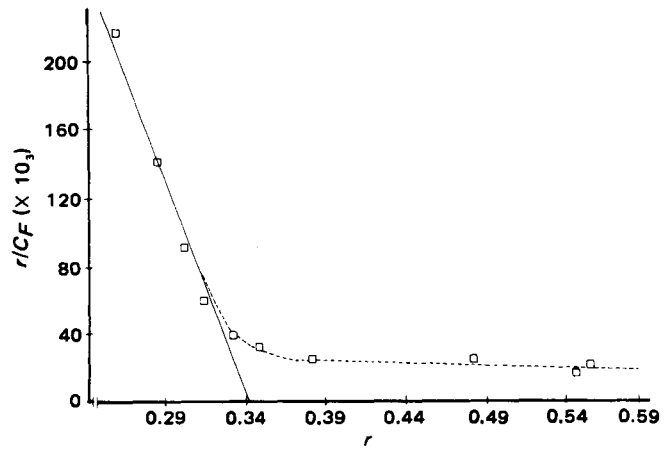

Figure 6—*Scatchard plot of II.*

Comparison of the absorption spectra of the compounds in the presence of the varied DNA base ratios was also carried out to further delineate the tendency toward base-pair specificity in DNA binding. The difference spectra for the anthraquinones (Figs. 3 and 4) revealed a proportionality to the G–C contents of the DNA species used, but the quinolinium derivatives showed no difference in depressive effect on the extinction coefficients in the spectra, indicating no G–C specificity. This illustrates the advantage of employing more than one method of measurement.

A probable mode of action of the methylthiovinylquinolinium iodides is an inhibition of nucleic acid biosynthesis with formation of a molecular complex with DNA. Only one type of binding site is apparently involved. The complex formed involves both strands of the DNA double helix and may result from the insertion of the planar ligand between base pairs of the helix, the intercalation model of Muller and Crothers (15) and Lerman (16).

The binding parameters of the four compounds in this series are of the same order of magnitude. Although a marginal preference of G–C base-pair binding was shown by the extent of inhibition of RNA transcription, this was not detectable by comparison of the visible absorption spectra of the compounds in the presence of varied DNA base ratios. One can assume that the biochemical technique is definitely more sensitive and meaningful than the spectral methods for distinguishing those agents with borderline DNA base-pair specificities of binding. The presence of a 6-substituent has little effect on the extent of DNA binding.

The binding studies of the two anthraquinones indicate that both compounds bind to DNA with comparable strength. Binding isotherms indicate two binding sites on DNA for these compounds. The stronger binding is presumed to be intercalation between successive base pairs; the weaker binding is considered to be an electrostatic interaction involving DNA phosphate groups and the amino side chains. Comparison of absorption spectra and the extent of RNA transcription inhibition with varied DNA base ratios show a preference for binding with the G–C base pair. Since the binding parameters of the two anthraquinones are essentially the same, the strong antileukemic activity of the active compound (I) could be due to additional factors such as *in vivo* metabolic activation, pharmacokinetic behavior of biologically active components, or alternative action sites in the cell apparatus other than nuclei (14).

It is apparent from these *in vitro* methods of studying DNA binding ability that some differences in anticancer activity, such as between the anthraquinones and the quinolinium compounds, may be correlated with the relative binding strengths to DNA. On the other hand, these methods may fail to distinguish between active and inactive compounds, as seen with the anthraquinones, where other biological factors may become determinant.

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Antitumor Agents XLVII: The Effects of Bisbrusatolyl Malonate on P-388 Lymphocytic Leukemia Cell Metabolism

IRIS H. HALL ˣ, Y. F. LIOU, K. H. LEE, M. OKANO, and STEPHEN G. CHANEY *

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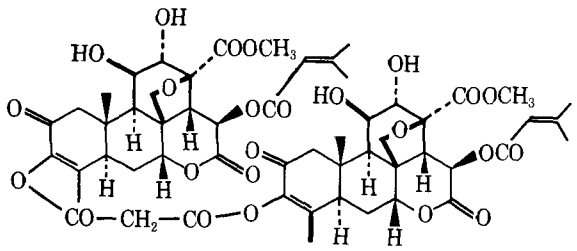
Abstract □ Bisbrusatolyl malonate, which was shown previously to be active against P-388 lymphocytic leukemia cell growth, was investigated for inhibitory effects on nucleic acid and protein synthesis. DNA and RNA synthesis as well as protein synthesis were markedly inhibited at 10, 25, and 50 μmole final concentrations *in vitro*. The major sites of inhibition of nucleic acid synthesis appeared to be DNA polymerase, messenger and transfer RNA polymerases, orotidine-5′-monophosphate decarboxylase, phosphoribosyl pyrophosphate amino transferase, and dihydrofolate reductase. Moderate inhibition of nucleotide kinase activities and oxidative phosphorylation processes occurred after drug treatment. Cyclic adenosine monophosphate levels were reduced. Protein synthesis was inhibited during the elongation step of peptide synthesis. The data suggested that bisbrusatolyl malonate interfered with the peptide bond formation. However, the ongoing polypeptide synthesis must be completed before the drug can bind to the ribosome effectively.

Keyphrases □ Bisbrusatolyl malonate—effects on P-388 lymphocytic leukemia cell metabolism □ Antitumor agents—effects of bisbrusatolyl malonate on P-388 lymphocytic leukemia cell metabolism □ RNA—synthesis, effect of bisbrusatolyl malonate, P-388 lymphocytic leukemia cells □ DNA—synthesis, effect of bisbrusatolyl malonate, P-388 lymphocytic leukemia cells □ Protein synthesis—effect of bisbrusatolyl malonate, P-388 lymphocytic leukemia cells

Antineoplastic activity against P-388 lymphocytic leukemia growth was established previously for bisbrusatolyl esters (1, 2). One of these derivatives, bisbrusatolyl malonate at 0.6 mg/kg/day ip, gave T/C % values of 271, 197, and 188 in BDF_1 male mice (2) in a P-388 tumor model sensitive to quassinoids (2). This agent was shown to suppress *in vitro* DNA synthesis by 45%, RNA synthesis by 48%, and protein synthesis by 83% at 10 μM concentration after 90 min incubation. A number of enzymes involved in nucleic acid metabolism, *e.g.*, DNA polymerase and dihydrofolate reductase, were inhibited *in vitro* by this ester (2). A detailed study of the effects of bisbrusatolyl malonate on P-388 lymphocytic leukemia cellular metabolism was conducted and its effects on nucleic acid and protein metabolism are now reported.

EXPERIMENTAL

Bisbrusatolyl malonate (I) was synthesized and characterized previously in the literature (2). The P-388 lymphocytic leukemia tumor line was maintained in DBA/2 male mice (~20 g). For the *in vitro* studies,



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P-388 cells were harvested from the peritoneal cavity 10 days after administering 10^6 P-388 lymphocytic leukemia cells intraperitoneally into BDF_1 male mice (~20 g) on day 0 (3). In the *in vivo* studies, BDF_1 male mice were inoculated with 10^6 P-388 cells intraperitoneally, and on days 7, 8, and 9 the mice were administered 0.6 mg/kg/day bisbrusatolyl malonate intraperitoneally. The biochemical studies were performed on cells harvested from the peritoneal cavity on day 10.

The *in vitro* incorporation studies (4) were conducted using 1 μCi [6-^{3}H]thymidine (21.8 Ci/mmole), [6-^{3}H]uridine (22.4 Ci/mmole), or [4,5-^{3}H(N)]L-leucine (56.5 Ci/mmole) with 10^6 P-388 cells in minimum essential medium, pH 7.2, in a total volume of 1 ml, incubated for 60 min at 37°. Thymidine incorporation into DNA was terminated with perchloric acid containing pyrophosphate which was filtered on glass fiber paper by vacuum suction. RNA and protein assays were terminated with trichloroacetic acid and collected on nitrocellulose membranes by vacuum suction. The acid-insoluble precipitates on the filter papers were placed in scintillation vials and counted. *In vivo* thymidine incorporation into DNA was determined by injecting into the animal 10 μCi of [^{3}H]-methylthymidine (24.7 Ci/mmole) intraperitoneally 1 hr before sacrifice. The DNA was isolated (5) and the [^{3}H]thymidine content determined in scintillation fluid[1] and corrected for quenching. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as the standard. The results were expressed as dpm/mg of DNA isolated. Uridine incorporation into RNA was determined in an analogous manner with 10 μCi of [^{3}H]uridine (20 Ci/mmole) and the RNA extracted (6). Leucine incorporation into protein was determined by the method of Sartorelli (7) with 10 μCi[^{3}H]L-leucine (56.5 Ci/mmole). The control values for DNA synthesis were 202,098 dpm/mg of DNA, 235,360 dpm/mg of RNA for RNA synthesis, and 99,102 dpm/mg of protein isolated for leucine incorporation.

On day 10 after *in vivo* administration of the drug, the number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer (8). The *in vitro* UV binding studies were conducted with I (50 μg/ml) incubated with DNA (38 μg/ml) in 0.1 *M* phosphate

[1] Fisher Scintiverse.

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