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A Search for Potential Antitumor Agents: Biological Effects and DNA Binding of a Series of Anthraquinone Derivatives

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SUMMARY

In an effort to establish a relationship between mechanism of binding and affinity to DNA, cytotoxic activity, and genotoxic activity, we have studied four new anthracenedione derivatives bearing charged side chain groups at various positions of the polycyclic aromatic system. Cytotoxicity, genotoxicity, and thermodynamic DNA binding parameters were shown to be directly related, indicating the polynucleotide as an important target for drug action, rather than a minor, subterminal interacting site. This finding was further supported by the observation of extensive anthraquinone accumulation occurring in the nuclear compartment. The relative binding affinities of the drugs are discussed in terms of nature and position of side-chain substituents. Different binding modes were found: three compounds intercalate into the nucleic acid double helix, and one interacts with the exterior of the macromolecule. The biological results suggest that the mode of complex formation plays a less relevant role than DNA binding efficiency.

The anthracycline antibiotics, doxorubicin and daunorubicin, are recognized to be among the most important cancer chemotherapeutic drugs discovered to date (1). However, their efficacy is limited by severe cumulative and irreversible cardiac toxicity. In a search for less toxic anticancer agents, several analogues have been studied with side chains containing a positively charged amino group (2, 3). These compounds strongly interact with DNA, mainly by intercalation into the polynucleotide double helix. Antitumor anthracycline-DNA interactions and their relevance to cytotoxicity have been the object of several investigations (4–9). Many authors believe that nuclear complex formation with DNA represents the prime mode of action of chemotherapeutic agents containing planar rings in their structure. Others, however, consider drug binding to DNA just a late subterminal event. With the aim of developing new anticancer drugs and of contributing to a better understanding of their mechanism of action, we investigated interaction with DNA and biological effects of four new 9,10-anthracenedione derivatives (Fig. 1), bearing different charged side chains at positions 1, 2, and 4.

Materials and Methods

Chemical Compounds

The synthesis and characterization of compounds I–IV have been reported previously (10). Doxorubicin hydrochloride was a generous gift of Farmitalia (Milan, Italy). [methyl-^{3}H]Thymidine (specific activity 40–60 Ci/mmol) was purchased from Amersham (Bucks, UK). Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, MO) and purified as described (11). Sonicated DNA from calf thymus, with an average molecular weight of 4.3×10^5, was obtained according to literature procedures (12, 13). It had the following properties: $A_{260}/A_{280} = 1.94$, $A_{260}/A_{230} = 2.38$, and hyperchromism 38.8%.

The following extinction coefficients were used: 6,600 M^{-1} cm^{-1} for calf thymus DNA at 260 nm; 4,980 M^{-1} cm^{-1} for compound I at 400 nm; 7,950 M^{-1} cm^{-1} for compound II at 310 nm; 13,100 M^{-1} cm^{-1} for compound III at 470 nm; 12,200 M^{-1} cm^{-1} for compound IV at 439 nm; and 11,200 M^{-1} cm^{-1} for doxorubicin at 480 nm.

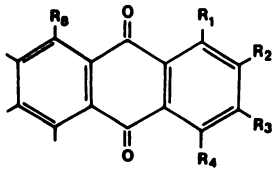
Cell Lineages

HeLa, HeLa S_3, WiL-2 and P-388 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum (Grand Island Biological Co., Grand Island, NY), antibiotics and L-glutamine (complete medium). Cells were cultured at 37° in a moist atmosphere of 5% CO_2 in air. They were routinely checked for mycoplasma contamination by Hoechst 33250 fluorochrome staining (14) and by aerobic and anaerobic culture techniques.

Cell Cytotoxicity

The cytotoxic activity of anthracenediones I–IV was determined with exponentially growing cells in complete medium over a 3-day period (15). For this purpose, cells were originally seeded at $5–7 \times 10^4$ cells in 20-mm Costar wells, incubated with variable amounts of the tested compounds, and counted at the end of the 72-hr growth period in a hemocytometer with trypan blue staining. The effect of anthra-

ABBREVIATION: EDTA, ethylenediaminetetraacetate.



COMPOUND

	I	II	III	IV
R_1	Y	Y	OH	OH
R_2	H	H	H	X
R_3	OCH_3	OCH_3	H	H
R_4	$NHCOCH_3$	NH_2	X	H
R_5	H	H	OH	OH

X = $-NHCO(CH_2)_2N(C_2H_5)_2 \cdot HCl$

Y = $-O(CH_2)_3N(C_2H_5)_2 \cdot HCl$

Fig. 1. Chemical structures of compounds I–IV.

quinone drugs on DNA synthesis was also evaluated on exponentially growing cells incubated in the presence of increasing drug amounts and 1 μCi/ml [methyl-^{3}H]thymidine for a period of 16 hr. Trichloroacetic acid-precipitable radioactivity was then measured on cell lysates using a liquid scintillator spectrometer (Packard Instrument Co., Downers Grove, IL). The amount of substance required to reduce by 50% cell growth or DNA synthesis was derived from the best linear plot of a least squares fit relating surviving cells (or ^{3}H-methylthymidine incorporation) to anthraquinone concentration (on a molar basis) (15).

Uptake and Intracellular Distribution of Anthraquinone Derivatives

HeLa S_3 cells adapted to growth in suspension were incubated in complete medium (0.5–1.2 × 10^6 cells/ml) with biologically active concentrations of compounds I–IV and doxorubicin. Drug concentrations were in the range of their ED_{50} values. Doxorubicin was used at a 1 μM concentration, which slightly exceeded its ED_{50}, due to detectability limits of the drug under our experimental conditions. After an incubation period of about 2 hr, cells were washed twice in saline in order to remove most of the free drug, as shown by fluorometric assay of the supernatant fluid. Measurements were carried out with whole cells to determine total drug uptake, by resuspending the washed pellet in 2 ml of 0.3 N HCl in 50% ethanol (16). Drug contents were evaluated by fluorescence readings using appropriate drug standard and drug-free control material for comparison. The excitation wavelengths were 420 nm for compound I, 500 nm for compound II, 490 nm for compound III, 460 nm for compound IV, and 470 nm for doxorubicin.

To determine the subcellular locale of accumulated drug, cells were fractionated essentially as described previously (17). Briefly, cells were suspended at 3–4 × 10^7 cells/ml in ice-cold 10 mM Tris, 1 mM $MgCl_2$, pH 7.45, for 10 min. After this period, tonicity was restored and cell membranes were disrupted by rapidly forcing the suspension through a tuberculin syringe fitted with a 26 gauge needle. Microscopic evaluation of trypan blue-stained isolated nuclei was also performed in order to establish whether cell lysis was properly accomplished (17). Cellular constituents were then fractionated into a low speed sediment (nuclei), which contained all detectable DNA (18), cytosol, and a high speed sediment (100,000 × g pellet). The resulting material was treated as reported for whole cells, and drug contents were evaluated by fluorescence readings.

Efflux Experiments

After incubation with a 1 μM concentration of drug for 2 hr, HeLa cells were washed twice with saline and resuspended at 2 × 10^6 cells in 5 ml of drug-free medium. After varying periods at 37°, cells were sedimented and assayed for intracellular drug content as previously described.

Alkaline Elution

HeLa S_3 cells were labeled with [methyl-^{3}H]thymidine (1 μCi/ml) by overnight incubation in complete medium. Subsequently they were exposed to a 10 μM concentration of derivatives over a period of 2 hr. Samples were also irradiated with a UVS-54 ultraviolet lamp (Ultraviolet Products Inc., San Gabriel, CA) at a radiation dose of 1500 erg/mm^2. The procedure of Kohn *et al.* (19) was then followed. This includes collection of cells onto 2-μm disc BS filters (Millipore Corp., Bedford, MA) positioned on cylindrical light-shielded funnels and then washing at a flow rate of about 20 ml/min. Washing and subsequent elution were performed by using a multichannel peristaltic pump. Lysis was accomplished by passing through the filters a solution containing 0.2% (w/v) Sarcosyl (Ciba-Geigy, Milan, Italy), 2 M NaCl, 40 mM EDTA, pH 10.

The eluting solution consisted of 20 mM EDTA (acid form) plus tetrapropylammonium hydroxyde (Fluka) added to yield a pH of 12.2. The flow rate during this step was maintained at 0.04 ml/min. Radioactivity of eluted fractions was measured on a liquid scintillator spectrometer.

Dye Titrations of Covalently Closed Supercoiled DNA Analyzed by Agarose Gel Electrophoresis

Electrophoretic titration of anthraquinone derivatives was carried out according to the method described by Espejo and Lebowitz (20) using pBR322 as the source of DNA. Briefly, 15 × 0.5 cm agarose gels were formed in glass tubes with one end wrapped with parafilm. Agarose was dissolved at 1% in a buffer solution containing 50 mM Tris, 20 mM sodium acetate, 18 mM NaCl, 2 mM EDTA adjusted to pH 8 with glacial acetic acid (TANE buffer). The melted solution contained different amounts of compounds I–IV (0–850 ng/ml). Samples consisting of 0.25 μg of covalently closed supercoiled (form I) and open circular DNA (form II) in 20 μl of loading buffer (TANE with 7% sucrose and bromophenol blue) were applied to the top of the gels and run at 60 V (about 2.5 ma/tube) at 20° for 3–4 hr. The gels were then extruded from the tubes, stained with a 0.5 μg/ml ethidium bromide solution, and illuminated with a long wavelength UV lamp system (Ultraviolet Products Inc.).

Photographs were taken with a Polaroid MP4 Land camera.

Binding Studies

Spectrophotometric experiments. Spectrophotometric measurements were performed in a Perkin Elmer Lambda 5 instrument, equipped with a Haake F3C thermostat. Experiments were usually performed by addition of known amounts of DNA to solutions containing a given concentration of the ligand. Alternatively, known amounts of drug were added at constant DNA concentrations. The amounts of bound and free ligand (C_b and m, respectively) were determined at a given wavelength according to the equations:

$$C_b = (A_f - A/A_f - A_b)C_o$$
$$m = C_o - C_b \qquad (1)$$

where A_f is the absorption of free drug, A_b is the absorption of bound drug, A is the absorption of a mixture of free and bound drug, and C_o is the total amount of drug present.

Spectrophotometric experiments were considered quantitatively only in the presence of an isosbestic point.

Equilibrium dialysis. DNA solutions (3–7 ml) were dialyzed against equal volumes of solvent containing the compound under investigation. Thomas dialysis tubing with a 12,000-Da cutoff were used. Equilibrium was usually reached within 18 hr with no appreciable binding of the drug to the membrane. The concentration of free drug in the dialysate was measured directly from absorbance readings at the maximum wavelength, with reference to a standard curve obtained from solutions of compounds I–IV at known concentrations. The amount of total drug in the retentate was determined directly by absorbance at the isosbestic point or by dissociating the drug/DNA

complex by addition of equal volumes of 0.1 M lithium chloride in methanol and measuring the absorbance at the maximum wavelength. A calibration curve for compounds I–IV in the aqueous-alcoholic medium was determined by absorption measurements. Polyelectrolyte concentrations were always low enough to minimize Donnan effects.

Circular dichroism. Circular dichroism (cd) studies were carried out in a Jasco J 500 A spectropolarimeter equipped with a Jasco model DP-501 data processor. Four to eight scans were accumulated for each sample.

Fluorescence. Fluorescence measurements were performed with a Perkin Elmer MPF 2A or MPF 66 instrument equipped with a Perkin Elmer 7300 computer instrument. One to 40 scans were accumulated according to sample response and concentration.

Unless stated otherwise, the physicochemical measurements were carried out at 25° in aqueous 10 mM Tris buffer, pH 7.0, containing 1 mM EDTA and known amounts of sodium chloride to adjust ionic strength to the desired value. DNA and ligand concentrations were determined by absorption measurements.

Experimental binding data were plotted according to the method of Scatchard (21) and analyzed using the neighbor exclusion model, which describes the binding of a ligand to a homogeneous lattice, according to the equation (22):

$$r/m = K_i\,(1 - nr)\,\{(1 - nr)/[1 - (n - 1)r]\}^{n-1} \qquad (2)$$

where r is the number of ligand molecules bound per DNA phosphate, m is the free ligand concentration, K_i is the intrinsic binding constant, and n is the number of consecutive lattice residues covered by a ligand molecule. A best fitting least squares procedure was used.

Results

Cell growth inhibition. The cytotoxic activity of the tested compounds was measured on exponentially growing cells over a period of time that enabled at least three cell divisions. Doxorubicin was always used as the reference cytotoxic drug. The results are presented in Table 1. It is immediately evident that compound I is rather poor as a cytotoxic agent. In fact, as much as a 55 μM concentration of drug is needed to inhibit HeLa cell growth by 50%. A quite different behavior is shown in all cell lines tested by derivative II, which is derived from compound I by hydrolysis of the acetamide moiety at position 4 (see Fig. 1). ED_{50} values for this derivative are about 20 times lower than the corresponding values for its congener. Compound IV is slightly but significantly less toxic than compound II, whereas compound III is endowed with the most prominent growth-inhibitory properties.

Its cytotoxicity is well comparable to that of doxorubicin, which appears to be slightly less effective on P-388 cells. In turn, doxorubicin is more potent than compound III in WiL-2 cells.

Effects on DNA synthesis. Incorporation of [methyl-^{3}H] thymidine into cells during a 16-hr assay period is inhibited by our anthraquinones in a manner reflecting their antiproliferative potential. In particular, derivative III is again the most effective of the group, its activity being close to that of doxorubicin (see Table 2).

A cell count with trypan blue at the end of the 16-hr period of treatment with the ED_{50} concentrations showed that the number of nonviable cells present was always less than 5% of the total cell population for all tested compounds. This observation would suggest that loss of viable cells, unrelated to DNA synthesis inhibition, is not a major cause for the observed cytotoxicity.

Results from these experiments therefore indicate that impairment of DNA template function might well represent an essential feature in the mechanisms of action of the investigated anthraquinones, as is true for the anthracycline antibiotic (23).

Cellular accumulation and disposition of drugs. Doxorubicin, together with tested compounds, was employed on HeLa S_3 cells adapted to growth in suspension. A 2-hr incubation time was chosen in analogy with other studies concerning L-1210 cell influx of doxorubicin (24). Longer incubation periods did not show an appreciable increase in drug uptake. This was slightly higher for compound III than for doxorubicin and the others (see Table 3). In addition, all compounds were extensively accumulated in the nucleus, except compound I, which was more evenly distributed. Since a HeLa cell genome contains about 2×10^{10} base pairs (25), it turns out that at least one drug molecule is bound to a number of base pairs from 150 to 300 according to drug dose and tested compound. This could reasonably lead to an altered gene function and, consequently, to the observed inhibition of cell growth and DNA metabolism *in vivo*.

It is noticeable, however, that up to 25% of incorporated drug is also found in the 100,000 × *g* pellet.

In contrast, the cytosol fraction possesses a low amount of drug. Thus, either a few macromolecular acceptors are present in this compartment, or they exhibit low affinity for the tested compounds. In both cases the drugs are easily removed by extensive washing. Although the distribution values are not significantly different among studied compounds, it should be considered that accumulation data might be affected by redistribution phenomena during the fractionation procedures.

TABLE 1
Cell growth inhibition in the presence of tested compounds

Compound	Cell lines (ED_{50})		
	P-388	HeLa	WiL-2
		μM	
I	21.7 ± 4.5	54.2 ± 9.8	17.3 ± 1.1
II	2.3 ± 0.1	2.3 ± 1.1	0.69 ± 0.46
III	0.07 ± 0.04	0.09 ± 0.06	0.11 ± 0.02
IV	9.5 ± 1.1	5.3 ± 1.9	1.4 ± 0.2
Doxorubicin	0.13 ± 0.06	0.08 ± 0.03	0.01 ± 0.01

TABLE 2
Inhibition of DNA synthesis after addition of tested compounds

Compound	Cell lines (ED_{50})		
	P-388	HeLa	Wil-2
		μM	
I	ND[a]	91.2 ± 9.6	ND
II	3.7 ± 0.9	2.8 ± 0.4	1.4 ± 0.3
III	1.2 ± 0.02	0.97 ± 0.24	0.24 ± 0.12
IV	14.3 ± 2.6	9.5 ± 1.7	4.8 ± 1.9
Doxorubicin	0.34 ± 0.17	1.0 ± 0.3	0.03 ± 0.01

[a] ND, not determined.

The results of efflux studies after varying periods of incubation are reported in Fig. 2. It appears that compound III, which is more efficiently incorporated into cells after 2 hr, effluxes quite rapidly in drug-free medium. A very similar efflux pattern is observed for compound II which is, by the way, less well absorbed by the cells. Finally, doxorubicin exhibits a slightly slower efflux kinetics, quite comparable to the results reported by Egorin *et al.* (24) for daunorubicin in L-1210 cells.

In conclusion, differences exhibited in cellular accumulation and disposition of drugs are unlikely to be responsible for the observed variation in cytotoxicity.

Alkaline elution. The damage produced on cellular DNA following administration of compounds I–IV and doxorubicin was evaluated by alkaline elution (26). Results of this study are reported in Fig. 3. It has to be noted that derivative III is the most effective in inducing single strand breaks of the polynucleotide. Its activity is comparable to the effects produced by UV irradiation.

Compounds II and IV cause much less DNA damage than compound III although they still retain a certain activity, somewhat greater for the former drug. As might be expected from the affinity data, compound I does not cause appreciable single strand breaks in the nucleic acid.

Mode of interaction. Spectrophotometric, fluorimetric, chiroptical, and electrophoretic drug titration techniques were used to evaluate the mode of interaction of compounds I–IV with DNA. Comparative results are shown in Table 4 and Fig. 4.

Substantial spectral modifications are observed upon addition of the polynucleotide to buffered solutions of the examined compounds. A red shift of the maximum absorption occurs, along with relevant hypochromicity for compounds I, III, and IV. The results are different for compound II, for which increasing amounts of DNA cause smaller hypochromicity without apparent change in the maximum wavelength (Table 4).

Fluorescence measurements confirm a different response for compound II. In fact, important quenching effects take place in the presence of DNA for compounds I, III, and IV, whereas the fluorescence of compound II is almost unaffected even after addition of a large DNA excess (Table 4).

Circular dichroism experiments further indicate changes in interaction stereochemistry when comparing the results relative to compound II to those concerning compounds I, III, and IV. The latter cause an increase in the 275-nm positive band of DNA, which can be ascribed to shielding of base pair transitions due to ligand intercalation. No changes in the maximum wavelength are observed. Upon addition of compound II, however, a relevant blue shift occurs, along with an almost 2-fold increase in rotational strength (Table 4).

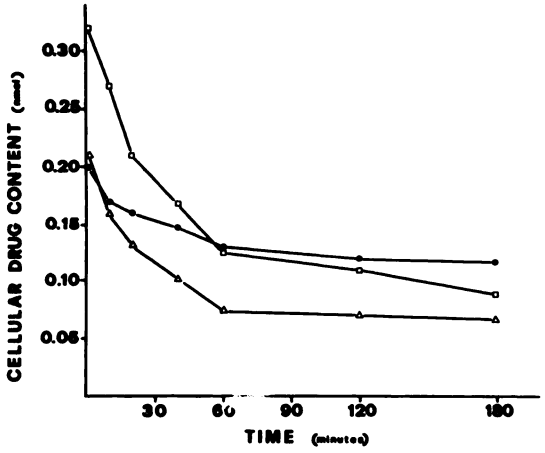


Fig. 2. Time course of efflux of compound II (△), compound III (□), and doxorubicin (●) from HeLa S_3 cells. Cells (2×10^6) were incubated at 37° with 1 μM drug for 2 hr. After being washed twice in saline, cultures were resuspended in drug-free medium at 37° and assayed for intracellular drug content after varying periods of incubation. The assay conditions are reported in the text. Each experimental point represents the mean of a triplicate experiment.

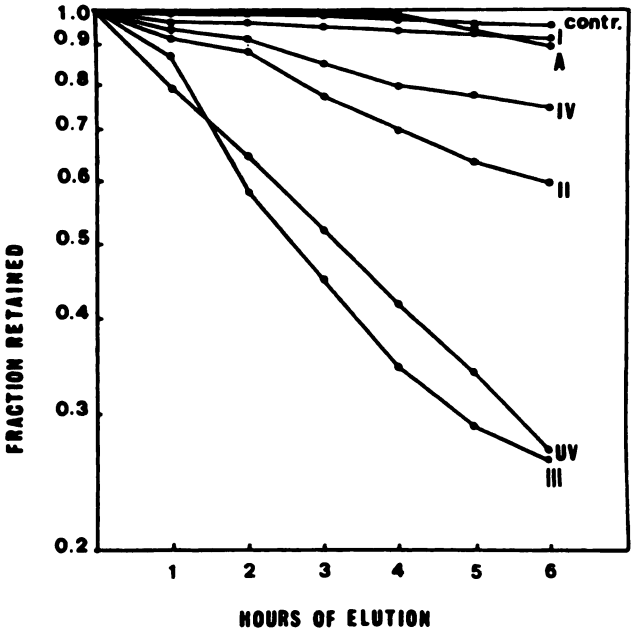


Fig. 3. Alkaline elution patterns of DNA from HeLa S_3 cells on filters. Effects of treatment with compounds I–IV, doxorubicin (*A*), and UV radiation are shown.

TABLE 3
Uptake and distribution of tested compounds into cell compartments

	Compound				
	I	II	III	IV	Doxorubicin
Drug incorporated into whole cells (nmol/10^6 cells)	0.17 ± 0.05	0.21 ± 0.05	0.32 ± 0.05	0.22 ± 0.05	0.20 ± 0.04
Drug incorporated into nuclei (nmol/10^6 cells)	0.08 ± 0.04	0.12 ± 0.05	0.22 ± 0.05	0.13 ± 0.05	0.14 ± 0.04
Drug incorporated into cytosol (nmol/10^6 cells)	0.03 ± 0.02	0.02 ± 0.002	0.011 ± 0.002	0.02 ± 0.007	0.004 ± 0.002
Drug incorporated into 100,000 × *g* pellet (nmol/10^6 cells)	0.05 ± 0.02	0.05 ± 0.005	0.06 ± 0.005	0.06 ± 0.005	0.05 ± 0.01

TABLE 4
Effects of DNA binding on the physicochemical properties of tested compounds

Compound	Spectrophotometric changes in the visible band of the drug		Drug fluorescence quenching	Circular dichroism of the DNA-drug complex	
	Peak shift	Hypochromicity		$\Delta\epsilon$	λ_{max}
	nm	%	%		*nm*
I	+18	21	80	+3.4	275
II	~0	10	5	+4.8	258
III	+22	26	94	+3.5	275
IV	+20	24	87	+3.5	275

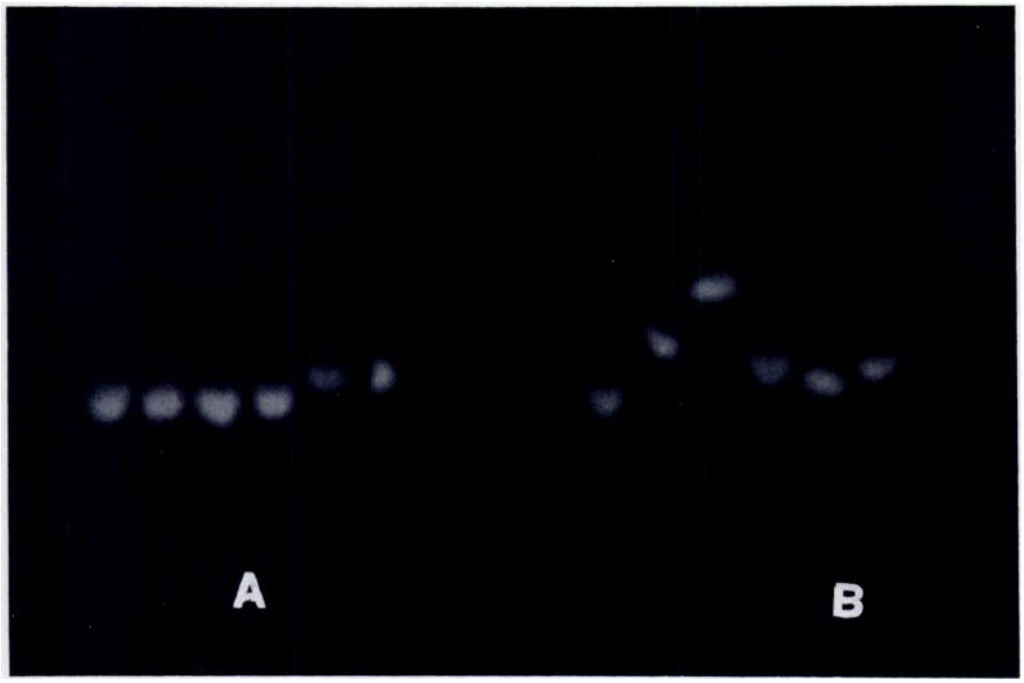


Fig. 4. Electrophoretic dye titration of pBR322 DNA. Electrophoresis was performed in gels containing 250 ng of pBR322 DNA (form I and form II) and increasing concentrations of compounds II (A) and III (B) (from right to left, 0, 1, 5, 50, 100, and 150 ng/ml). The upper, slower moving band represents unwound supercoiled pBR322 DNA.

Electrophoretic drug titration methods were additionally used to assay the hydrodynamic properties of closed circular double stranded pBR322 DNA in the presence of the examined compounds at varying concentrations. Using drug III, the supercoiled DNA band moves slower, initially, as a consequence of its unwinding, and gets close to the open circular form. Increasing the drug concentration in the gel brings back the original migration rate (Fig. 4B). Similar results were obtained using compounds I and IV (not shown). This behavior reflects the typical changes in superhelical density which are produced by intercalative agents. On the contrary, only minor modifications in the electrophoretic pattern are observed with compound II under identical experimental conditions (Fig. 4A).

Thermodynamic DNA-binding parameters. For a quantitative evaluation of the affinity of the examined derivatives for DNA, spectrophotometric and equilibrium dialysis experiments were performed. The former were considered only in the presence of an isosbestic point, to correctly evaluate the amount of free and bound drug (27).

The results are reported as the Scatchard plot in Fig. 5. The corresponding binding parameters (neighbor exclusion) at 0.017 M ionic strength are summarized in Table 5. Compound III exhibits the highest affinity to DNA, followed by compounds II, IV, and I, respectively. The latter drug interacts with the macromolecular rather poorly.

Increasing ionic strength leads to a decreased binding affinity in all cases. This fact confirms the importance of electrostatic interactions between charged groups of the drug and polynucleotide backbone in stabilizing both intercalated and non-intercalated complexes. Due to the similar ionic strength dependence of the DNA binding affinity for the examined drugs (compound II appears to be only slightly more sensitive than the others), we are confident that the reactivity scale observed at low salt concentration holds even under physiological conditions.

Since cooperativity parameters rather close to 1 are obtained, the DNA binding sites are independent for all examined drugs. The number of base pairs involved in complex formation appears to be close to 2 for compounds III and IV and about 3 for compound I. The externally interacting drug II "covers" three nucleotide moieties per complex unit.

Discussion

The four new anthraquinone derivatives reported in Fig. 1 cover a wide range of DNA-interacting ability and exhibit substantial differences in degree of cytotoxic and genotoxic activities. Physicochemical studies indicate that compounds I, III, and IV interact with the nucleic acid through an intercalative mechanism (20, 28–32). From the quite different responses given by compound II, it is concluded that this drug exhibits predominantly external binding to the macromolecule.

The intrinsic binding constant values are in decreasing order of magnitude going from compound III to compounds II, IV, and I. These data can be explained in terms of molecular

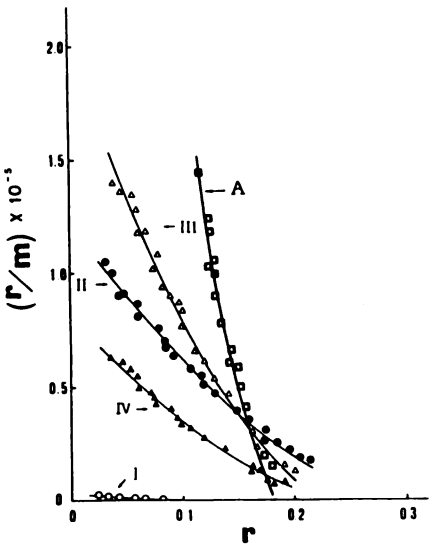


Fig. 5. Scatchard plots for the interaction of compounds I–IV and doxorubicin (*A*) with DNA at 25° and 0.017 M ionic strength, pH 7.0. *r* represents the ratio between bound drug and total DNA on a phosphate basis; *m* is the free drug concentration. *Solid lines* correspond to the theoretical plots using the binding parameters reported in Table 5.

TABLE 5
Thermodynamic DNA binding parameters for compounds I–IV at 25° and 0.017 M ionic strength (pH = 7.0) obtained from spectroscopic and equilibrium dialysis measurements

Compound	$K_i \times 10^{-5}$ (M^{-1})	*n*
I	0.03 ± 0.01	5.6 ± 0.2
II	1.2 ± 0.1	3.1 ± 0.2
III	2.0 ± 0.2	3.8 ± 0.2
IV	0.8 ± 0.1	3.6 ± 0.2

structure of the above substances. In fact, derivative I, being sterically the most hindered, exhibits the lowest DNA affinity. Hydrolysis of the acetamido moiety at position 4 gives compound II, raising binding ability up to 30 times. The presence in this drug of an extra free amino group results in the prevalence for external grafting with respect to intercalative binding. This probably can be ascribed to hydrogen-bond formation and electrostatic interactions which overcome the loss of free energy due to lack of insertion of the aromatic chromophore into DNA. This mode of binding is reminiscent of the one presented by low molecular weight polyamines such as spermine and spermidine (33, 34).

Examination of the isomeric derivatives III and IV leads to the appreciation that substitution at position 2 exerts a destabilizing effect on intercalative complex formation. These findings are in agreement with theoretical studies suggesting that groups bound to this position of the anthraquinone moiety interfere sterically with the polynucleotide backbone. (35).

The biological activity matches quite well with the above described values for DNA affinity, a result consistent with evidence that DNA is indeed an important target for drug action. Besides, this fact rules out the possibility, at least for anthraquinone derivatives, of drug-DNA interaction being just a subterminal event as proposed in the literature for anthracycline compounds (36, 37). Further evidence is obtained by evaluating the drug distribution patterns into cell compartments. It is clearly shown that nuclear accumulation is a prominent feature of these compounds, the relative nuclear uptake being related to DNA affinity. Conversely, uptake and efflux studies on drugs II and III as models for intercalating and non-intercalating anthraquinones indicate that membrane permeability is not a discriminating factor for biological activity. The inactive compound I gets into cells in amounts comparable to those of its active congeners. We can thus exclude poor access into cells as an additional cause for lack of cytotoxicity in this case. The observed relationship between DNA affinity scale and biological activity does not rule out the possibility that other metabolic pathways (such as membrane effects and/or free radical production) might be simultaneously affected, in so contributing to the observed cytotoxicity as proposed for doxorubicin (38). It is intriguing in this connection that cell growth and DNA synthesis inhibition show somewhat different levels of response. Relevant alkaline elution of DNA is found for compound III. This finding is rather unique in comparison with anthracycline antibiotics which are quite inactive in the absence of proteinase K (39). An explanation for this behavior might rest on the fact that compound III is not able to induce DNA-protein cross-links which are reported to occur in the presence of doxorubicin (40). In any event, compound III is, by far, more active than the other anthraquinone derivatives we investigated in inducing DNA single strand breaks. It is not clear whether this finding has to be related to the rather high DNA binding affinity exhibited by compound III or to some more complex mechanisms, including drug metabolism. Repair of DNA damage, as monitored by the elution technique, also occurs to a certain level in the absence of intercalation, as shown by compound II. This may represent an interesting point since it was suggested that external binding to DNA would cause a lower genotoxic action and consequently lead to a safer drug (41). However, our data produce evidence for a direct relationship between cytotoxicity and alkaline elution response, therefore suggesting that mutagenic potential is likely to be combined with chemotherapeutic activity in DNA-binding drugs.

Although the present approach is a useful one in the search for potential antitumor agents, the *in vitro* findings do not necessarily imply that the anthracenedione derivatives we investigated exhibit antitumor activity *in vivo*. Nonetheless, preliminary results indicate that compound III is a promising one after being tested against P-388 and L-1210 leukemias in BDF-1 mice. More detailed *in vivo* investigations on the subject are under way.

In conclusion, the results thus far presented point to a more crucial role played by DNA affinity, rather than by the mode of interaction, in affecting this target. It appears that, whichever mechanism is active, either template function inhibition or free radical strand scission, the key feature is to bring as much drug as possible in close contact with the nucleic acid. To confirm this interpretation we are currently investigating a number of other derivatives exhibiting different DNA affinities and binding stereochemistry.

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