0026-895X/95/010030-09$3.00/0

MOLECULAR PHARMACOLOGY, 30:30–38 (1995).

Topoisomerase II DNA Cleavage Stimulation, DNA Binding Activity, Cytotoxicity, and Physico-chemical Properties of 2-Aza- and 2-Aza-oxide-anthracenedione Derivatives

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Received January 18, 1995; Accepted April 17, 1995

SUMMARY

The cytotoxic activity of mitoxantrone and related anthracenediones has been ascribed to the ability of these compounds to interfere with DNA topoisomerase II function, resulting in DNA cleavage stimulation. The molecular details of enzyme inhibition by these intercalating agents remain to be defined. In an attempt to identify the structural determinants for optimal activity, the molecular and cellular effects of a series of heteroanalogues bearing different side-chains were examined in relation to the physico-chemical and DNA binding properties of these compounds. The results indicated that substitution of a pyridine ring for the dihydroxyphenylene ring in the planar chromophore caused a marked reduction of cytotoxic activity and of the ability to stimulate topoisomerase II-mediated DNA damage in intact cells and with simian virus 40 DNA *in vitro*. Although all tested derivatives were shown to intercalate into DNA, their DNA binding affinities were appreciably lower than that of mitoxantrone. The behavior of 2-aza derivatives more closely resembled that of ametantrone, suggesting that the potency of agents of this class is influenced more by the presence of hydroxyl groups than by the phenylene ring. The observation that a dramatic reduction (or loss) of the ability of aza derivatives to stimulate DNA cleavage is associated with a marked reduction of cytotoxic potency supports a primary role of topoisomerase II-mediated effects in the mechanism of action of the effective agents of this class. Because appreciable cytotoxic activity and significant *in vivo* antitumor efficacy are retained by compounds inactive (or poorly active) in inhibition of topoisomerase II, these results are consistent with multiple effects of anthracenediones at the cellular level.

Mitoxantrone and ametantrone are antitumor 1,4-bis[(aminoalkyl)amino]anthracene-9,10-diones that were discovered by molecular simplification of the anthracycline pharmacophore (1–5). In particular, mitoxantrone is a 5,8-dihydroxylated derivative that proved to be a potent cytotoxic agent and is clinically useful in cancer chemotherapy (6). Similarly to the DNA-intercalating anthracyclines, mitoxantrone is a topoisomerase II inhibitor (7). The drug stimulates topoisomerase II-mediated DNA cleavage by inhibiting the DNA religation step of the enzyme catalytic reaction. Nevertheless, the molecular basis of drug-DNA-enzyme interactions is still unclear, and the structural requirements for mitoxantrone activity remain to be elucidated.

Numerous anthracenedione analogues have been prepared, mainly exploiting variations in the nature of the side-chains and repositioning of the hydroxy substituents and/or the side-chains (1–5). Heteroanalogues of anthracenediones have been receiving increasing attention. In particular, analogues have been studied in which the dihydroxyphenylene ring of mitoxantrone, which is possibly involved in the cardiotoxic side effects of this drug (8), was replaced by an azine (9–11) or diazine (12) ring. This effort resulted in the identification of BBR 2778, a benzo[*g*]isoquinolinedione (2-aza-anthracenedione) that is effective in experimental tumor models and devoid of cardiotoxic effects (11).

The study of molecules structurally related to antitumor anthracenediones is expected to provide useful information about the importance of critical structural determinants in the biological activity of these compounds and a rational basis for further analogue development of this class of anticancer agents. In an attempt to better delineate the possible mechanism of action of heteroanalogues of anthracenediones related to mitoxantrone and ametantrone, the present study

This work was partially supported by Consiglio Nazionale delle Ricerche (Finalized Project "Applicazioni Cliniche della Ricerca Oncologica") and by Associazione Italiana per la Ricerca sul Cancro.

ABBREVIATIONS: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SSB, single-strand breaks; SV40, simian virus 40.

was undertaken to examine the importance of (a) the presence of a dihydroxyphenylene, phenylene, pyridine, or pyridine-*N*-oxide ring in the planar chromophore and (b) the nature of the basic side-chains in influencing the cytotoxicity, ability to stimulate topoisomerase II-mediated DNA cleavage, and physico-chemical properties relevant for DNA binding. In this study, mitoxantrone, ametantrone, and the known anthracenedione BBR 1722 [namely 1,4-bis[(2-aminoethyl)amino]anthracene-9,10-dione] were examined for the aforementioned effects, in comparison with structurally related analogues of the 2-aza-anthracene-9,10-dione (BBR 2378, BBR 2778, and BBR 2945) and 2-aza-anthracene-9,10-dione-2-oxide (BBR 2867 and BBR 2868) families (Fig. 1).

Experimental Procedures

Materials. The 2-aza-anthracene-9,10-diones BBR 2378 [6,9-bis[(2-dimethylaminoethyl)amino]benzo[*g*]isoquinoline-5,10-dione], BBR 2778 [6,9-bis[(2-aminoethyl)amino]benzo[*g*]isoquinoline-5,10-dione dimaleate], and BBR 2945 [6,9-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]benzo[*g*]isoquinoline-5,10-dione dimaleate] and the 2-aza-anthracene-9–10-dione-2-oxides BBR 2867 [6,9-bis[(2-dimethylaminoethyl)amino]benzo[*g*]isoquinoline-5,10-dione-2-oxide] and BBR 2868 [6,9-bis[(2-aminoethyl)amino]benzo[*g*]isoquinoline-5,10-dione-2-oxide dimaleate] were synthesized according to the method of Krapcho *et al.* (11). The known anthraquinone derivative BBR 1722 [1,4-bis[(2-aminoethyl)amino]anthracene-9,10-dione] was prepared according to the method of Greenhalgh and Hughes (13). Mitoxantrone (NSC 301739-NB) and ametantrone (NSC 287513-NA) were provided by the National Cancer Institute (Bethesda, MD). DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures (14), and strand-passing activity was determined with the P4 DNA unknotting assay, as already described (14). Poly(dG·dC) (200–500 kDa), poly(dA·dT) (200–500 kDa), and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO).

Self-aggregation studies. Inasmuch as the 2-aza-anthraquinones BBR 2778 and BBR 2378 exhibit a nonlinear absorbance-concentration response, they undergo aggregation phenomena similarly to their congeners mitoxantrone and ametantrone (15). Absorption spectra of the test compounds were recorded using a wide range of concentrations (5–80 mM) between 0.1 and 0.5 M ionic strength. The molar extinction coefficients of the monomer species were calculated by extrapolation of the apparent extinction coefficients to zero concentration for each compound. The calculations were performed according to the method of Schwartz *et al.* (16) (previously used for mitoxantrone and ametantrone) (17) and allowed an evaluation of K_d, the dimerization constant of the drug. Using this formalism, the extinction coefficient of the pure aggregate is not required *a priori*. Moreover, by considering the equation (16) $\log K_o = \log K_d - IS^{0.5}/(1 + IS^{0.5})$, it is possible to calculate the thermodynamic aggregation constant K_o as a function of K_d and the ionic strength (IS).

DNA binding studies. The measurements were carried out at 25° in 10 mM Tris·HCl, pH 7.0, 1 mM EDTA, with known amounts of NaCl. Binding was monitored spectrophotometrically in the ligand absorption region (500–750 nm). At a drug concentration sufficiently low to avoid self-aggregation phenomena (below 20 mM for aza-anthraquinones) and with 0.1–0.5 M NaCl, the presence of isosbestic points during titration with DNA allowed an evaluation of free and DNA-bound drug. To avoid large systematic inaccuracies due to experimental errors in extinction coefficients, the range of bound drug fractions was 0.15–0.85. The data were evaluated according to the method of McGhee and Von Hippel (18), to obtain K_i, the intrinsic binding constant, and n, the exclusion parameter. No cooperativity appeared to occur. Spectrophotometric measurements were performed with a Perkin-Elmer Lambda 5 apparatus equipped with a Haake F3-C thermostat.

CD studies. Chiroptical studies were carried out with the complexes of each test drug with poly(dG·dC) or poly(dA·dT) in the visible absorption region, where induced rotational strength can be easily measured because the drugs themselves are not optically active. The experiments were performed using a Jasco J-500 spectropolarimeter interfaced to a J-500 N computing station. Four to eight scans were accumulated for each measurement.

Cyclic voltammetry. Cyclic voltammetry measurements were performed at room temperature, using an Amel scanning potentiostat at the following settings: initial potential, −0.1 V; switching potential, 1 V; scan rate, 0.4 V/sec. A three-electrode system was used with a reference saturated calomel electrode and a platinum

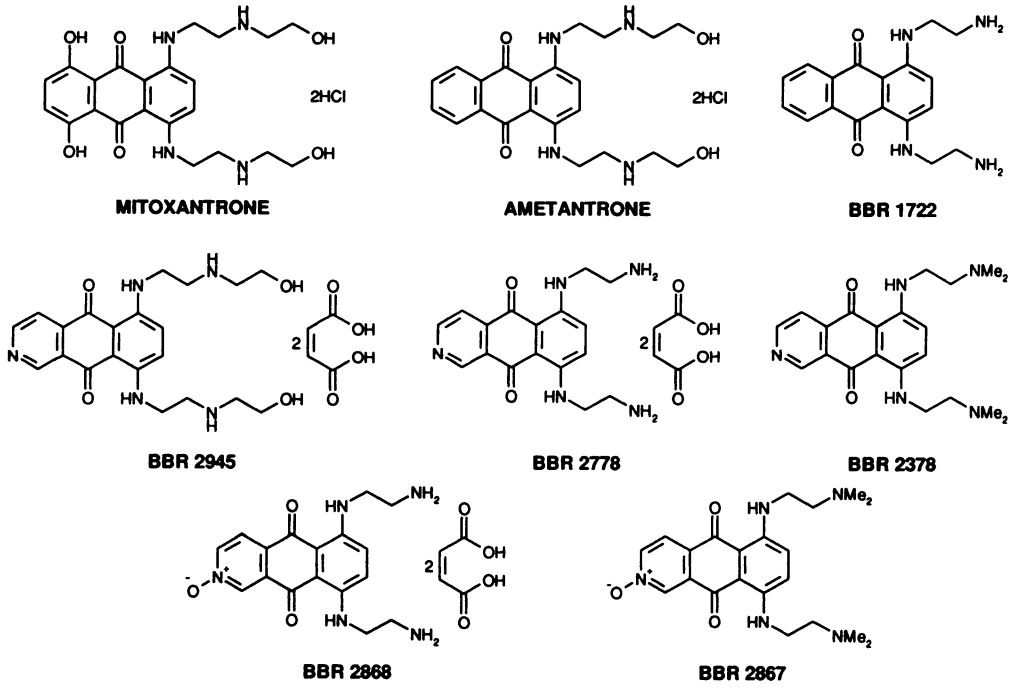

Fig. 1. Chemical structures of the studied anthracenedione derivatives.

wire counter electrode; a hanging mercury drop electrode (Metrohm) was the working electrode.

Cell lines and cytotoxicity assay. Human promyelocytic leukemia HL-60 cells (19) and the human small-cell lung carcinoma cell line NCI-H187 (20) were cultured in RPMI 1640 medium (Flow Laboratories, Irvine, UK) plus 10% fetal calf serum (Flow Laboratories). Drug treatments were for 1 hr at 37°, using exponentially growing cells. HL-60 cells were centrifuged after drug treatments, washed twice, resuspended in drug-free medium, cultured for 72 hr, and then counted. NCI-H187 cells were cultured in drug-free medium for 96 hr, and cell survival was then determined by means of the MTT method (21).

Cellular drug uptake. Exponentially growing NCI-H187 cells (10^7) were incubated for 1 hr at 37° with 10 μM drug. After two washings with phosphate-buffered saline, cells were sonicated and then suspended in 1 ml of borax buffer (0.1 M sodium tetraborate, pH 9.4). Mitoxantrone (200 ng) was added as the internal standard. The aqueous phase was then extracted with 5 ml of dichloromethane. The mixture was shaken for 5 min and allowed to stand until complete phase separation occurred (20 min). The dichloromethane phase was then transferred into glass vials and evaporated to dryness under nitrogen flow. The residue was resuspended in a water/acetonitrile (70:30, v/v) mixture containing 0.5 ml/liter trifluoroacetic acid and 2 mM hexanesulfonic acid. The same mixture was used as the eluent in chromatography. Ion-pair, reverse phase, liquid chromatography was used for the quantitative determination of the tested compounds. Separation was obtained at room temperature using a 30- × 0.4-cm Bondapak C_{18} column (Waters; Millipore, Milford, MA). The flow rate was 1.0 ml/min. Spectrophotometric detection of the samples was performed at 600 nm.

Alkaline elution assay. NCI-H187 cells were labeled with 0.1 mCi/ml [^{14}C]thymidine for 90 hr at 37°. The labeled nucleoside precursor was removed 24 hr before drug treatments, by centrifugation and resuspension of cells in fresh medium. Alkaline elution procedures were as reported by Kohn *et al.* (22) and extensively described elsewhere (23, 24).

Topoisomerase II-mediated DNA cleavage. SV40 DNA fragments were uniquely 5′-end-^{32}P-labeled as described previously (25). Briefly, SV40 DNA was restricted with the indicated enzyme, dephosphorylated, and ^{32}P-labeled with T4 kinase. Then, DNA was subjected to a second enzyme digestion to generate uniquely 5′-end-labeled fragments, which were separated by agarose gel electrophoresis and purified by electroelution and ethanol precipitation. DNA cleavage reactions were performed in a volume of 20 ml of 0.04 M Tris·HCl, pH 7.5, 0.08 M KCl, 0.01 M $MgCl_2$, 0.005 M dithiothreitol, 1 mM ATP, 15 mg/ml bovine serum albumin. The DNA fragment was incubated for 30 min with approximately 32 units of topoisomerase II (200 ng of protein in 2 ml of storage buffer), in the presence or absence of drug. Reactions were stopped by the addition of sodium dodecyl sulfate (1%) and proteinase K (0.25 mg/ml), and the mixtures were incubated at 42° for an additional 45 min. DNA was then precipitated with ethanol, resuspended in 2.5 ml of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue), heated at 90° for 2 min, chilled in ice, and then loaded onto an 8% polyacrylamide gel in Tris/borate/EDTA buffer (0.09 M Tris-borate, 0.002 M EDTA) containing 7 M urea. Gels were run at 80 W for 2 hr, dried, and autoradiographed with Amersham Hyperfilm MP.

Results

Modifications in Anthracenedione Derivatives

The anthracenedione derivatives examined in this study (Fig. 1) are characterized by two types of modifications. For chromophore modifications, the dihydroxyanthraquinone chromophore was replaced either with the 2-aza-anthracenedione chromophore, to give BBR 2378, BBR 2778, and the 2-aza-isoster of ametantrone BBR 2945, or with the 2-aza-anthracenedione-2-oxide chromophore, to give BBR 2867 and BBR 2868. By modification of the basic side-chain, the effect of the presence of the 2-aminoethylamino (BBR 2778 and BBR 2868), 2-dimethylaminoethylamino (BBR 2378 and BBR 2867), and 2-[[(2-hydroxyethyl)amino]ethyl-]amino (BBR 2945) side-chains was examined. The aforementioned heteroanalogues were studied in comparison with the anthracenediones mitoxantrone, ametantrone, and BBR 1722, with the latter being the carbocyclic analogue of BBR 2778 and BBR 2868.

Physico-chemical Properties and DNA Binding Activity

Thermodynamics of the drug self-aggregation process. Self-aggregation in aqueous solutions is a characteristic property of mitoxantrone and ametantrone (15, 26) and may be relevant for understanding drug availability and binding to target sites. Spectrophotometric data showed that the molar extinction coefficient of 2-aza-anthracenediones is a function of drug concentration and ionic strength of the medium (Fig. 2). Hence aggregation phenomena occurred also with compounds of this class. This behavior was particularly remarkable for the derivatives BBR 2378 and BBR 2778 (data not shown), whereas their *N*-oxide analogues showed modest changes in extinction coefficients at high (0.5 M) ionic strength only (data not shown). Linear diagrams were found at each ionic strength, and the extrapolated K_d (drug dimerization constant) values are reported in Table 1. Increasing ionic strength shields charge repulsions (the examined drugs are charged under physiological conditions) and stabilizes dimer formation. The values of K_0 (Table 1) for BBR 2778 and BBR 2378 confirm aggregation phenomena to an extent comparable to those for ametantrone but substantially reduced in comparison with those for mitoxantrone. In contrast, self-aggregation was negligible for the *N*-oxide derivatives.

Thermodynamics of DNA binding activity. At sufficiently low drug concentrations (to avoid important interference from self-aggregation phenomena), an isosbestic point was generally observed in the spectrophotometric titration experiments (Fig. 3). The only exception was compound BBR 2378, for which the isosbestic point was never as clear as it

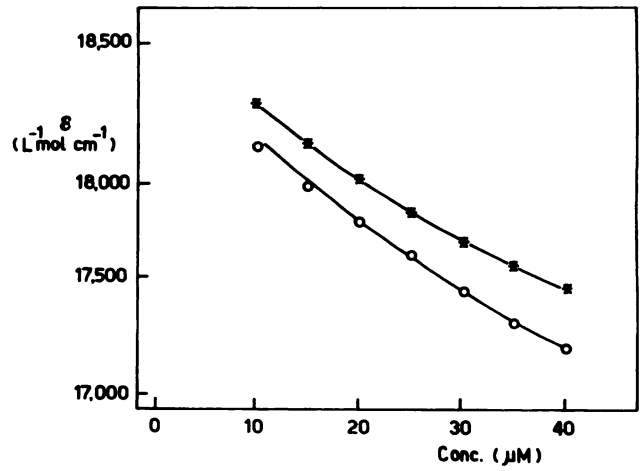

Fig. 2. Effects of drug concentration on the molar extinction coefficient of BBR 2378 in 10 mM Tris, 1 mM EDTA, with NaCl to adjust the ionic strength to 0.1 M (*) or 0.5 M (O), at pH 7.0 and room temperature.

TABLE 1

Self-aggregation parameters for 2-aza-anthracenediones, 2-aza-oxide-anthracenediones, mitoxantrone, and ametantrone

Compound	Ionic strength	$K_d \times 10^{-4}$ [a]	K_o [b]
	M	M^{-1}	M^{-1}
Mitoxantrone	0.017	2.5 ± 0.3	18,000
	0.25	3.9 ± 0.3	
	0.75	4.5 ± 0.4	
	2.00	6.9 ± 0.4	
Ametantrone	0.15	0.76 ± 0.09	4,000
	0.5	1.04 ± 0.16	
BBR 2778	0.1	0.54 ± 0.03	2,300
	0.25	0.55 ± 0.04	
	0.5	0.59 ± 0.09	
BBR 2378	0.022	0.16 ± 0.06	1,650
	0.1	0.32 ± 0.01	
	0.5	0.43 ± 0.01	
BBR 2868	0.5	Negligible	Negligible
BBR 2867	0.5	Negligible	Negligible

[a] K_d, drug dimerization constant. Values were obtained at room temperature in 10 mM Tris·HCl, 2 mM NaCl, 1 mM EDTA, pH 7.0 at the indicated ionic strength.
[b] K_o, self-aggregation constant (see Experimental Procedures for details).

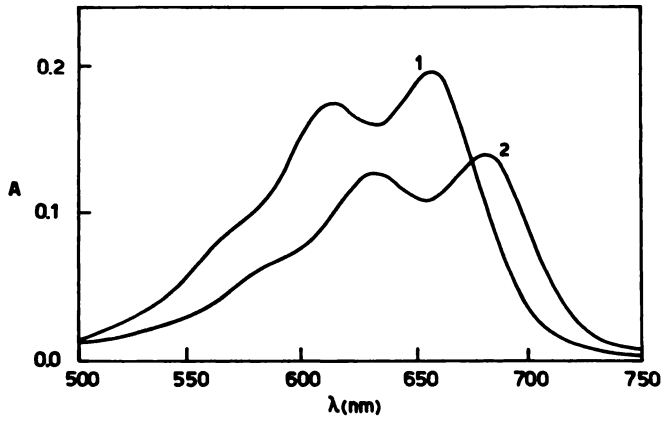

Fig. 3. Spectral properties of BBR 2868 (*spectrum 1*) and of its complex with calf thymus DNA (*spectrum 2*) in 10 mM Tris, pH 7.0, 1 mM EDTA, with NaCl to adjust the ionic strength to 0.1 M. Measurements were performed at room temperature. In titration experiments, an isosbestic point was observed at 675 nm.

was for the other compounds. Possibly in this case more than one type of drug-DNA interaction occurred. However, BBR 2778 and BBR 2868 exhibited substantially higher affinity for DNA than did BBR 2378 and BBR 2867, respectively, with behavior similar to that of ametantrone (Table 2). The binding site at physiological ionic strength is generally constituted by two base pairs, which is consistent with intercalation of 2-aza-anthraquinones and their *N*-oxides into DNA (Table 2). This binding mechanism was further supported by the bathochromic and hypochromic shifts observed in the spectrophotometric titrations of each drug with DNA and was substantiated by linear dichroism experiments, which indicated that the bound chromophore was perpendicular to the helix axis with reference to DNA bases (data not shown). An increase in ionic strength caused a dramatic drop in the binding constant, indicating the participation of charged residues in complex formation (15, 26). According to Manning's theory (27), the slope of a plot of log K_i versus log ionic strength gives the number of ion pairs formed per complex unit. The values (Table 2) fall in the range of 1.3–2. Thus, both side-chains of 2-aza-anthraquinones and their *N*-oxides are involved in ionic interactions when bound to DNA.

TABLE 2

Thermodynamic parameters for the binding of aza-anthracenediones, mitoxantrone, and ametantrone to calf thymus DNA in 10 mM Tris, 1 mM EDTA, pH 7.0, at 23° and different ionic strengths

Compound	Ionic strength	$K_i \times 10^{-4}$ [a]	n [b]	$\delta \log K_i / \delta \log$ ionic strength [c]
	M	M^{-1}		
Mitoxantrone	0.25	120.00 ± 30.0	2.2 ± 0.05	1.0
	0.75	31.00 ± 6.00	2.3 ± 0.05	
	2	15.00 ± 5.00	2.4 ± 0.05	
Ametantrone	0.15	34.03 ± 3.37	1.75 ± 0.05	1.5
	0.5	5.71 ± 0.26	3.8 ± 0.15	
BBR 2778	0.1	30.42 ± 1.44	1.85 ± 0.05	2.0
	0.25	2.74 ± 0.09	2.35 ± 0.05	
	0.5	1.13 ± 0.01	1.55 ± 0.05	
BBR 2378	0.1	6.73 ± 0.46	2.0 ± 0.05	1.3
	0.25	1.93 ± 0.04	2.5 ± 0.05	
	0.5	0.89 ± 0.02	2.7 ± 0.05	
BBR 2868	0.1	24.31 ± 1.83	2.7 ± 0.05	2.0
	0.25	2.73 ± 0.04	3.3 ± 0.05	
	0.5	0.96 ± 0.02	5.4 ± 0.1	
BBR 2867	0.1	6.12 ± 0.12	2.15 ± 0.05	1.7
	0.25	1.32 ± 0.01	3.6 ± 0.05	
	0.5	0.47 ± 0.00	3.9 ± 0.05	

[a] K_i, intrinsic constant for the binding of the first ligand to DNA.
[b] n, number of adjacent base pairs forming the binding site of the drug.
[c] Parameter that gives the number of ion pairs formed per complex unit.

Stereochemistry of DNA binding activity. It is quite difficult to accurately interpret induced CD spectra in terms of drug orientation in the intercalated system (28). Thus, measurements were made using the alternating polynucleotides poly(dA·dT) and poly(dG·dC), because a careful theoretical analysis of CD induction by intercalated chromophores was recently carried out with the aforementioned polynucleotides (29). The spectra obtained using BBR 2378 and BBR 2868 are presented in Fig. 4. All derivatives (Table 3), including ametantrone and mitoxantrone, generated positive CD when bound to poly(dA·dT). The results were different using poly(dG·dC). Similarly to mitoxantrone, BBR 2867 and BBR 2868 (i.e., *N*-oxide derivatives) exhibited positive rotational strength, whereas BBR 2778 and BBR 2378 were similar to ametantrone and induced negative CD.

Redox potentials. The anthracenedione ring system is known to undergo redox processes, which at least in part have been related to the known undesired side effects, such as cardiotoxicity (6). The redox potentials (Fig. 5; Table 3) for 2-aza-anthracenediones and their *N*-oxide derivatives appeared to be 0.2–0.3 V lower than for the carbocyclic analogues, which means that reduction was substantially easier for the former drugs. The potentials of drugs bound to DNA could not be evaluated due to precipitation of the complexes under the experimental conditions of cyclic voltammetry.

Biological Activity

Cytotoxic activity. The cytotoxic activities of the tested drugs were examined in the human small-cell carcinoma cell line NCI-H187 and in the human leukemia cell line HL-60 (Table 4) after 1 hr of drug exposure. NCI-H187 cells were less sensitive to anthracenediones than were HL-60 cells, as observed previously (17). In NCI-H187 cells, all studied derivatives were markedly less cytotoxic than mitoxantrone. BBR 1722, BBR 2945, and BBR 2378 were more potent than ametantrone. BBR 2778 and the two *N*-oxide compounds showed cytotoxic activities comparable to that of amet-

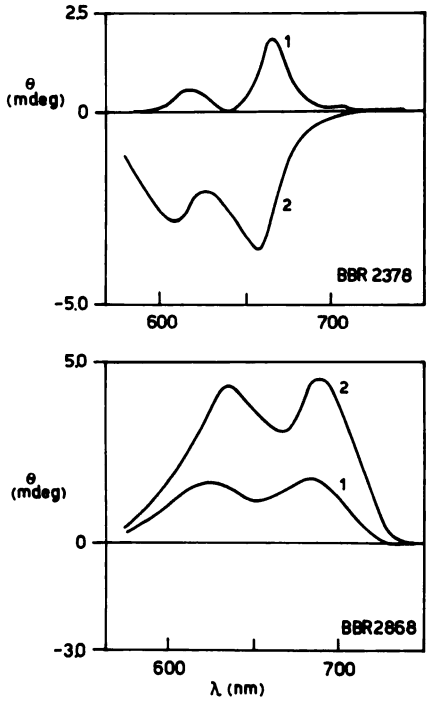

Fig. 4. Induced CD in the ligand absorption region for the complexes of BBR 2378 and BBR 2868 with poly(dA·dT) (*spectra 1*) and poly(dG·dC) (*spectra 2*) in 10 mM Tris, pH 7.0, 1 mM EDTA, with NaCl to adjust the ionic strength to 0.1 M, at room temperature.

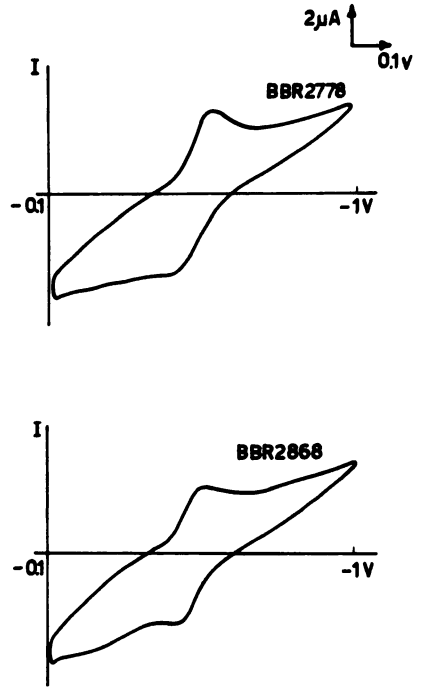

Fig. 5. Cyclic voltammetry of BBR 2778 and BBR 2868 in 10 mM Tris, pH 7.0, 1 mM EDTA, with NaCl to adjust the ionic strength to 0.1 M, at room temperature.

TABLE 3

Induced CD and half-wave reduction potential ($E_{1/2}$) values of 2-aza-anthracenediones, 2-aza-oxide-anthracenediones, mitoxantrone, and ametantrone

Compound	CD		$E_{1/2}$
	Poly(dG·dC)	Poly(dA·dT)	
			V
Mitoxantrone	Positive	Positive	−0.74
Ametantrone	Negative	Positive	−0.68
BBR 2778	Negative	Positive	−0.54
BBR 2378	Negative	Positive	−0.54
BBR 2868	Positive	Positive	−0.43
BBR 2867	Positive	Positive	−0.52

antrone. The cytotoxic activities of the 2-aza- and 2-aza-oxide-anthracenediones were increased after methylation of the amino group of the side-chains (i.e., BBR 2378 and BBR 2867) (Table 4).

Cellular drug uptake. For 2-aza compounds able to stimulate low levels of SV40 DNA cleavage (i.e., BBR 2778, BBR 2378, and BBR 2945), drug accumulation was studied in NCI-H187 cells after 1-hr exposure to the drug. No correlation could be found for these compounds between cytotoxic potency and topoisomerase II-mediated effects. The most notable result was the 10-fold reduction in the cellular uptake of BBR 2778, compared with other 2-aza analogues (BBR 2378 and BBR 2945), mitoxantrone, and ametantrone (Table 5). This finding supports a critical role of the side-chains in the cellular pharmacokinetics of these agents.

Formation of DNA SSB. DNA SSB produced by the 2-aza and 2-aza-oxide derivatives were determined in NCI-H187 cells after a 1-hr drug exposure (Fig. 6). All of the derivatives produced markedly smaller numbers of DNA breaks than did

TABLE 4

Cytotoxic effects of the studied anthracenedione derivatives

Cells were exposed to drugs for 1 hr. Drug cytotoxicity was determined with the MTT assay in NCI-H187 cells and by the cell-counting method in the HL-60 cell line. Values are the mean ± standard error of two to four independent determinations.

Compound	IC_{50}[a]	
	NCI-H187	HL-60
	μM	
Mitoxantrone	1.4 ± 0.7	0.013 ± 0.009
Ametantrone	112 ± 29	0.44 ± 0.04
BBR 1722	34 ± 14	ND[b]
BBR 2945	60 ± 13	7.1 ± 5.1
BBR 2778	110 ± 74	7.7 ± 6.9
BBR 2378	17 ± 6	2.9 ± 2.4
BBR 2868	157 ± 31	ND
BBR 2867	86 ± 66	ND

[a] IC_{50}, drug concentration required for 50% inhibition of cell growth.
[b] ND, not determined.

TABLE 5

Cellular drug accumulation

NCI-H187 cells were exposed to anthracenedione derivatives for 1 hr at 37°, and then drug uptake was measured as described in Experimental Procedures. Values are the mean of two experiments.

Compound	Uptake
	nmol/10^7 cells
Mitoxantrone	13
Ametantrone	10
BBR 2945	21
BBR 2778	1.1
BBR 2378	42

mitoxantrone. Among the 2-aza-anthracenediones, BBR 2778 was the most effective, stimulating DNA breaks at concentrations above 2 μM, as observed for ametantrone.

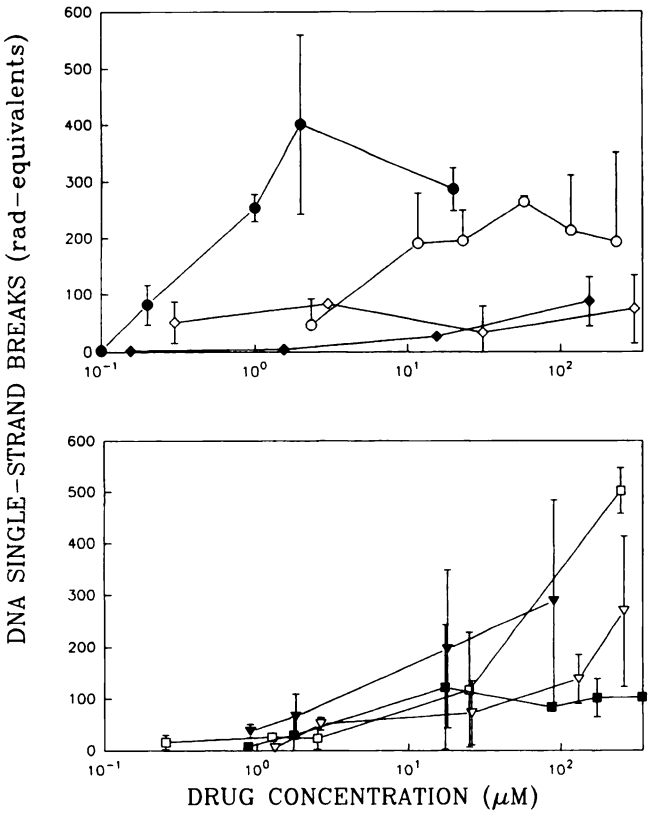

Fig. 6. DNA SSB induced by anthracenedione derivatives in NCI-H187 cells. Cells were exposed to drugs for 1 hr at 37°, lysed on the filters in the presence of proteinase K, and eluted at pH 12.15. See Experimental Procedures for details. *Standard error bars* are shown when values were derived from two to four independent determinations. ●, Mitoxantrone; ○, ametantrone; ◇, BBR 1722; ◆, BBR 2945; ▼, BBR 2778; ▽, BBR 2378; ■, BBR 2868; □, BBR 2867.

BBR 2867 and BBR 2378 stimulated a significant number of SSB only at very high concentrations (Fig. 6). A comparison of the effects of ametantrone and its 2-aza derivative (BBR 2945) clearly indicated that the presence of the heteroatom markedly reduced the ability of the drug to induce DNA SSB.

Drug stimulation of SV40 DNA cleavage induced by topoisomerase II. The relative drug activity in stimulating topoisomerase II-mediated DNA breaks was studied using ^{32}P-labeled SV40 DNA as a substrate for murine topoisomerase II (Fig. 7). BBR 2778 and BBR 2378 stimulated levels of DNA cleavage similar to that produced by ametantrone (Fig. 7) but were markedly less effective than mitoxantrone. DNA cleavage was dose dependent, with maximal stimulation for all tested compounds at about 1 μM. BBR 2945 and BBR 1722 were also efficacious in stimulating topoisomerase II-mediated DNA cleavage, compared with ametantrone, but were somewhat less potent, producing maximal stimulation in the range from 1 to 10 μM. The stimulation of DNA breaks was negligible for the 2-aza-oxide derivatives (BBR 2868 and BBR 2867). With the exception of BBR 2378, at pharmacologically relevant drug concentrations ($\leq$100 μM) the relative potency of the compounds in stimulating cleavage of SV40 DNA was similar to that for the observed stimulation of DNA cleavage in NCI-H187 cells. Cleavage suppression was observed at high drug concentrations for all of the studied drugs. The patterns of DNA cleavage stimulated by the studied compounds were similar to those of ametantrone and mitoxantrone (Fig. 7). Thus, the structural modifications in this series did not significantly influence the sequence specificity of DNA cleavage stimulation.

Discussion

The results of the present study indicated that the introduction of a nitrogen atom in the anthraquinone chromophore led to compounds characterized by very low levels of cytotoxic activity. The effects of 2-aza-anthracenedione derivatives at the molecular and cellular levels more closely resemble those of ametantrone than those of mitoxantrone (Table 6). It is likely that this behavior reflects the absence of the hydroxyl groups rather than substitution of a carbon atom for the nitrogen at position 2 of the planar ring system. A comparison of the cytotoxic effects of ametantrone and the 2-aza derivative with the same side-chains (BBR 2945) indicated variable effects of the heteroatom in the tested human tumor cell systems, because an appreciable reduction of cytotoxic potency was found with HL-60 leukemia cells but not with NCI-H187 cells. A comparison of the cytotoxic effects of ametantrone and BBR 1722 and their 2-aza derivatives (BBR 2945 and BBR 2778, respectively) suggested a contribution from the presence of the heteroatom to the reduction of cytotoxic potency. Among 2-aza derivatives, BBR 2378 was found to be more potent than other 2-aza derivatives, partly as a result of greater intracellular accumulation (Table 5) due to the presence of lipophilic methyl groups. Cellular uptake was substantially modulated by the nature of the side-chains, because the *N*-substituted compounds BBR 2378 and BBR 2945 penetrated the cells far better than did the corresponding nonsubstituted derivative BBR 2778. Clearly, the former are more hydrophobic and may cross membrane barriers more efficiently. This conclusion is also supported by the somewhat different cytotoxic potencies of the *N*-oxide derivatives BBR 2867 and BBR 2868.

The new derivatives were shown to intercalate into DNA but did not exhibit DNA binding affinities as prominent as that of mitoxantrone, again more closely resembling ametantrone. The dimethyl amino-substituted derivatives BBR 2378 and BBR 2867 were markedly less effective than the corresponding amino derivatives BBR 2778 and BBR 2868. This points to hydrogen-bonding contributions from the latter, which add up to the stabilizing effects arising from charge interactions. In addition, the introduction of an *N*-oxide moiety did not appear to affect the interaction with DNA to an appreciable extent. In contrast, drug orientation in the intercalation pocket appeared to be quite sensitive to the presence of the *N*-oxide moiety, because BBR 2867 and 2868 exhibited a clear-cut change in complex geometry, compared with BBR 2778, BBR 2378, and BBR 2945. This probably reflects the change in electron density generated by the linking of oxygen to nitrogen in the *N*-oxide derivatives, which requires a rearrangement of the drug-nucleic acid contacts to optimize the energy of interaction.

Interestingly, 2-aza-anthracenediones and their 2-aza-oxide derivatives were characterized by less negative half-wave reduction potential values. Hence, they undergo reversible reduction more easily than do anthracenediones, which facilitates free radical-mediated DNA damage. The cytotoxic properties of these compounds appeared to be more dependent on the ability to accumulate in cells than on DNA

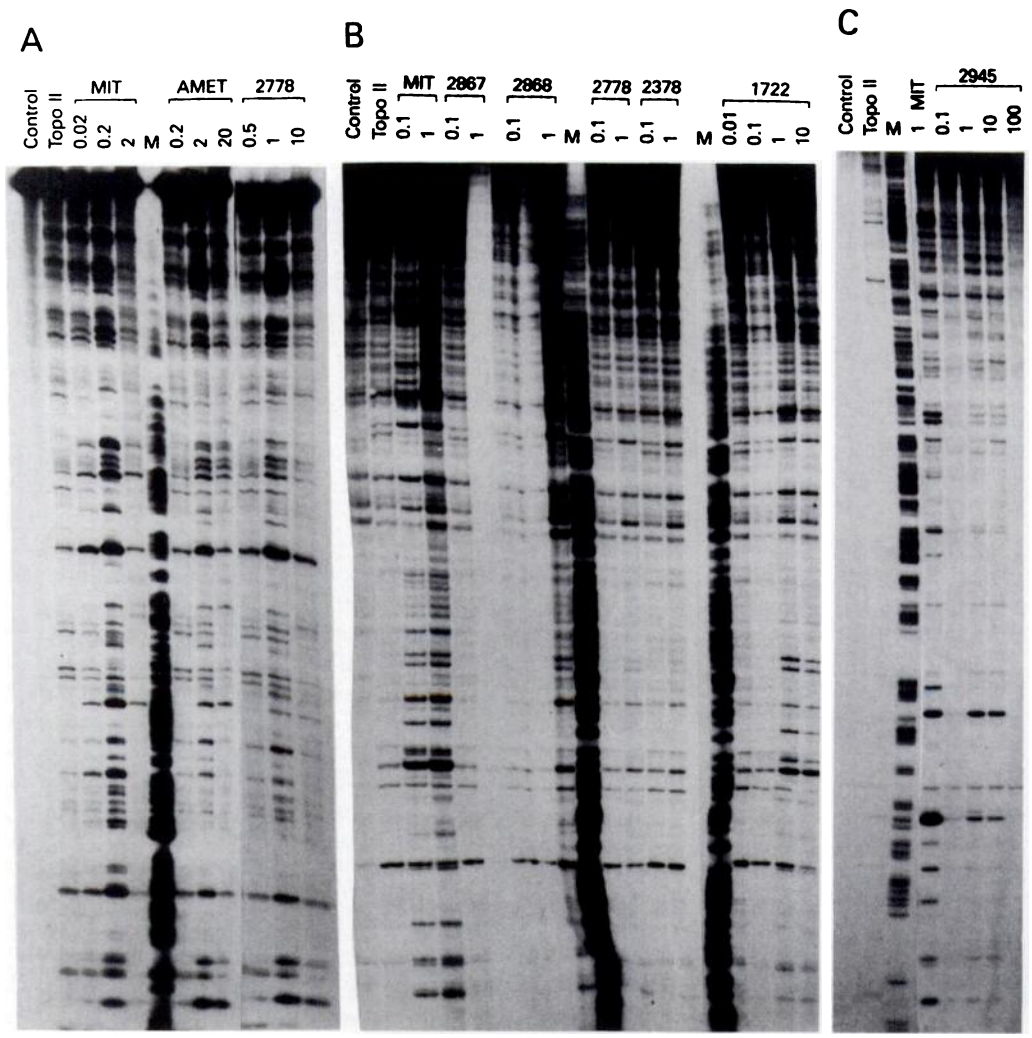

Fig. 7. Topoisomerase II-mediated DNA cleavage stimulated by anthracenediones. SV40 DNA (*Control*) was incubated at 37° with 120 ng of topoisomerase II without drug (*Topo II*) or with the indicated concentration (in μM) of compound. See Experimental Procedures for details. *MIT*, mitoxantrone; *AMET*, ametantrone; *2778*, BBR 2778; *2867*, BBR 2867; *2868*, BBR 2868; *2378*, BBR 2378; *1722*, BBR 1722; *2945*, BBR 2945; *M*, markers. A, An *Xho*II-*Taq*I SV40 DNA fragment, 5′-end-labeled at the *Xho*II site, was used. B, A *Bam*HI-*Acc*I SV40 DNA fragment, 5′-end-labeled at the *Bam*HI site, was used. C, An *Acc*I-*Kpn*I SV40 DNA fragment, 5′-end-labeled at the *Acc*I site, was used.

TABLE 6

Summary of the rank order of the potencies of the studied analogues in each study

Self-aggregation	DNA binding	Redox potential	Cytotoxicity[a]	Uptake	DNA cleavage	
					In vivo	*In vitro*
MXT[b]	MXT	2868	MXT	2378	MXT	MXT
AMT	AMT, 2778, 2868	2867	2378	2945	AMT, 2778	AMT
2778	2378, 2867	2778, 2378	1722	MXT, AMT	2378, 2867	2778, 2378
2378		AMT	2945, 2867	2778	2868	2868, 2867
2868, 2867		MXT	AMT, 2778			
			2868			

[a] Cytotoxic potency in the NCI-H187 cell line.
[b] MXT, mitoxantrone; AMT, ametantrone; numbers refer to BBR code number.

binding affinity. This was particularly evident for the derivatives BBR 2378 and BBR 2778. In fact, the latter exhibited a 5-fold increased DNA binding constant, compared with the former, but was about 40 times less effective in entering the cells. These findings point out that cytotoxicity derives from the balance of several factors related to physico-chemical properties of drug molecules.

In general, no clear correlation appears to emerge when DNA binding activity, DNA damage, cytotoxicity, and cellular uptake are considered for this class of compounds. This makes even more speculative the rationalization of the observed biological activity simply on the basis of the aforementioned single properties. This is particularly evident for BBR 2778. Although from the results presented here this compound would appear to be endowed with unfavorable cellular pharmacokinetics and molecular pharmacology, in compari-

son with mitoxantrone, it does show excellent *in vivo* activity against a number of experimental tumors, especially disseminated lymphomas, suggesting that it is a promising candidate for clinical development (11, 30).

Although the structural features of anthracenediones suggest that their mechanisms of action should be similar to those of anthracyclines, significant differences have been found between these classes of antitumor intercalating agents. Mitoxantrone and ametantrone are known to induce topoisomerase II-mediated DNA cleavage (7). Although this type of DNA damage is recognized as a primary lesion (6), other cellular effects of anthracenediones have been described, including aggregation and condensation of DNA and free radical generation, with consequent formation of non-protein-associated DNA strand breaks (6). Alkaline elution experiments indicated a very low ability (or loss of ability) of 2-aza-anthracenedione derivatives to produce protein-associated DNA strand breaks. The frequency of these DNA lesions correlated with cell-killing effects of mitoxantrone, ametantrone, and BBR 2778. However, the mechanism by which 2-aza-anthracenediones exert cytotoxic activity does not seem to be primarily related to stimulation of topoisomerase II-mediated DNA damage. This conclusion is also supported by the lack of ability of *N*-oxide derivatives to stimulate DNA cleavage *in vitro*. Thus, it is likely that anthracenediones and their 2-aza derivatives exert multiple effects at a cellular level, as proposed for mitoxantrone. Taken together, these observations support a primary role of topoisomerase-mediated DNA lesions in determining the cytotoxic potential of mitoxantrone, because a loss or dramatic reduction of the ability to induce DNA strand breaks is associated with a marked reduction of cytotoxic potency, as detected with the aza derivatives.

Another interesting observation of this study is the finding that the reduced ability of drugs to interfere with topoisomerase II function as a result of carbon replacement of nitrogen at the 2-position is not related to a marked change in DNA binding affinity or to a different cleavage pattern, as suggested by *in vitro* effects on SV40 DNA. Thus, it is likely that drug interaction in the ternary complex with enzyme and DNA is a more critical determinant of cytotoxic activity than is the strength of the drug interaction with DNA.

Finally, modulation of a number of pharmacologically relevant physico-chemical properties, including self-aggregation, complex formation with DNA, and redox potential, can account at least in part for marked changes in cellular uptake, cytotoxic potential, and mechanism of action. Together with the results obtained using other aza-substituted anthracenediones (11), these results provide valuable information for the rational development of new drugs of this class.

Acknowledgments

The authors wish to thank L. Zanesi and B. Johnston for editorial assistance.

References

1. Zee-Cheng, R. K.-Y., and C. C. Cheng. Antineoplastic agents: structure-activity relationship study of bis(substituted aminoalkylamino)anthraquinones. *J. Med. Chem.* **21**:291–294 (1978).
2. Cheng, C. C., and R. K.-Y. Zee-Cheng. The design, synthesis and development of a new class of potent antineoplastic anthraquinones. *Prog. Med. Chem.* **20**:83–118 (1983).
3. Zee-Cheng, R. K.-Y., and C. C. Cheng. Anthraquinone anticancer agents. *Drugs Future* **8**:229–248 (1983).
4. Cheng, R. Y.-K., E. G. Podrebarac, C. S. Menon, and C. C. Cheng. Structural modification study of bis(substituted aminoalkylamino)anthraquinones: an evaluation of the relationship of the [2-[(2-hydroxyethyl)amino]ethyl]amino side chain with antineoplastic activity. *J. Med. Chem.* **22**:501–505 (1979).
5. Murdock, K. C., R. G. Child, P. F. Fabio, R. B. Angier, R. E. Wallace, F. E. Durr, and R. V. Citarella. Antitumor agents. 1. 1,4-Bis[(aminoalkyl)amino]-9,10-anthracenediones. *J. Med. Chem.* **22**:1024–1030 (1979).
6. Faulds, D., J. A. Balfour, P. Chrisp, and H. D. Langtry. Mitoxantrone: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in the chemotherapy of cancer. *Drugs* **41**:400–449 (1991).
7. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **58**:351–375 (1989).
8. Shipp, N. G., R. T. Dorr, D. S. Alberts, B. V. Dawson, and M. Hendrix. Characterization of the experimental mitoxantrone cardiotoxicity and its partial inhibition by ICRF-187 in cultured neonatal rat heart cells. *Cancer Res.* **53**:550–556 (1993).
9. Krapcho, A. P., J. J. Landi, M. P. Hacker, and J. J. McCormack. Synthesis and antineoplastic evaluation of 5,8-bis[(aminoalkyl)amino]-1-aza-anthracene-9,10-diones. *J. Med. Chem.* **28**:1124–1126 (1985).
10. Krapcho, A. P., M. E. Petry, J. J. Landi, J. Stallman, J. F. Polsenberg, M. P. Hacker, S. Spinelli, A. Oliva, R. Di Domenico, and E. Menta. The synthesis of 6,9-difluorobenzo[*g*]quinoline-5,10-dione: displacements of the fluorides by diamines which lead to 6,9-bis[(aminoalkyl)amino]benzo-[*g*]quinoline-5,10-diones. *J. Heterocycl. Chem.* **30**:1565–1569 (1993).
11. Krapcho, A. P., M. E. Petry, Z. Getahun, J. J. Landi, J. Stallman, J. F. Polsenberg, C. E. Gallagher, M. J. Maresch, M. P. Hacker, F. C. Giuliani, G. Beggiolin, G. Pezzoni, E. Menta, C. Manzotti, A. Oliva, S. Spinelli, and S. Tognella. 6,9-Bis[(aminoalkyl)amino]benzo[*g*]isoquinoline-5,10-diones: a novel class of chromophore-modified antitumor anthracenediones: synthesis and antitumor evaluations. *J. Med. Chem.* **37**:828–837 (1994).
12. Krapcho, A. P., M. J. Maresch, A. L. Helgason, K. E. Rosner, M. P. Hacker, S. Spinelli, E. Menta, and A. Oliva. The synthesis of 6,9-bis[(aminoalkyl)-amino] substituted benzo[*g*]quinoxaline, benzo[*g*]quinazoline and benzo[*g*-]phthalazine-5,10-diones via regiospecific displacements. *J. Heterocycl. Chem.* **30**:1597–1606 (1993).
13. Greenhalgh, C. W., and N. Hughes. The reaction of leucoquinizarins with alkylenediamines. *J. Chem. Soc. Sect. C Org. Chem.* 1284–1288 (1968).
14. De Isabella, P., G. Capranico, M. Binaschi, S. Tinelli, and F. Zunino. Evidence of DNA topoisomerase II-dependent mechanisms of multidrug resistance in P388 leukemia cells. *Mol. Pharmacol.* **37**:11–16 (1990).
15. Kapuscinski, J., and Z. Darzynkiewicz. Interactions of antitumor agents ametantrone and mitoxantrone (novantrone) with double strand DNA. *Biochem. Pharmacol.* **24**:4203–4213 (1985).
16. Schwartz, G., S. Klose, and W. Balthasar. Cooperative binding to linear biopolymers. 2. Thermodynamic analysis of the proflavine-poly(L-glutamic acid) system. *Eur. J. Biochem.* **12**:454–460 (1970).
17. De Isabella, P., G. Capranico, M. Palumbo, C. Sissi, A. P. Krapcho, and F. Zunino. Sequence selectivity of topoisomerase II DNA cleavage stimulated by mitoxantrone derivatives: relationships to drug DNA binding and cellular effects. *Mol. Pharmacol.* **43**:715–721 (1993).
18. McGhee, J. D., and P. H. Von Hippel. Theoretical aspects of DNA-protein interactions: cooperative and non cooperative binding of large ligands to a one-dimensional homogeneous lattice. *J. Mol. Biol.* **86**:469–489 (1974).
19. Collins, S. J., R. C. Gallo, and R. E. Gallagher. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature (Lond.)* **270**:347–349 (1977).
20. Gazdar, A. F., D. N. Carney, M. M. Nau, and J. D. Minna. Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological, and growth properties. *Cancer Res.* **45**:2924–2930 (1985).
21. Alley, M. C., D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski, D. L. Fine, B. J. Abbott, J. G. Mayo, R. H. Shoemaker, and M. R. Boyd. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **48**:589–601 (1988).
22. Kohn, K. W., R. A. G. Ewing, L. C. Erickson, and L. A. Zwelling. Measurements of strand breaks and cross-links by alkaline elution, in *DNA Repair: A Laboratory Manual of Research Procedures* (E. C. Friedberg and P. C. Hanawalt, eds.). Marcel Dekker, New York, 379–401 (1981).
23. Capranico, G., T. Dasdia, and F. Zunino. Comparison of doxorubicin-sensitive and -resistant P388 murine leukemia cells. *Int. J. Cancer* **37**: 227–231 (1986).
24. Capranico, G., P. De Isabella, S. Penco, S. Tinelli, and F. Zunino. Role of DNA breakage in cytotoxicity of doxorubicin, 9-deoxydoxorubicin, and 4-demethyl-6-deoxydoxorubicin in murine leukemia P388 cells. *Cancer Res.* **49**:2022–2027 (1989).
25. Capranico, G., F. Zunino, K. W. Kohn, and Y. Pommier. Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: relationship with DNA binding affinity and cytotoxicity. *Biochemistry* **29**:562–569 (1990).
26. Lown, J. W., A. R. Morgan, S. F. Yen, Y.-H. Wang, and W. D. Wilson. Characteristics of the binding of the anticancer agents mitoxantrone and ametantrone and related structures to the deoxyribonucleic acids. *Biochemistry* **24**:4028–4035 (1985).

27. Record, M. T., Jr., C. F. Anderson, and T. M. Lohman. Thermodynamic analysis of ion effects on the binding and conformational equilibria of proteins and nucleic acids: the roles of ion association or release, screening, and ion effects on water activity. *Q. Rev. Biophys.* **11**:103–178 (1978).
28. Lyng, R., T. Hard, and B. Norden. Induced CD of DNA intercalators: electric dipole allowed transitions. *Biopolymers* **26**:1327–1345 (1987).
29. Lyng, R., A. Robger, and B. Norden. The CD of ligand-DNA systems. 1. Poly(dG-dC) B-DNA. *Biopolymers* **31**:1709–1720 (1991).
30. Pezzoni, G., G. Beggiolin, C. Manzotti, S. Spinelli, S. Tognella, and F. C. Giuliani. BBR 2778: a novel aza-analog of anthracenediones endowed with preclinical anticancer activity. *Proc. Am. Assoc. Cancer Res.* **34**:373 (1993).

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