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Physicochemical Properties, Cytotoxic Activity and Topoisomerase II Inhibition of 2,3-Diaza-Anthracenediones

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ABSTRACT. The physicochemical, cytotoxic and pharmacological properties of 2,3-diaza-anthracenedione derivatives were examined to gain insight into the structure–activity relationships in this class of compounds. Spectrophotometric, chiroptical and voltammetric measurements were performed, along with cell cytotoxicity, alkaline elution, topoisomerase II-mediated DNA cleavage and cellular drug-uptake determinations. In comparison with classic anthracenediones such as mitoxantrone and ametantrone, the aza derivatives were characterized by less negative reduction potentials, lower affinity for DNA and modified geometry of intercalation. The biological effects of the new compounds were also profoundly affected by bioisosteric N for C replacement. Stimulation of topoisomerase II-mediated DNA cleavage was not observed, whereas other mechanisms of cell cytotoxicity, possibly involving oxidative DNA damage, appeared to be operative. The inability to generate protein-associated strand breaks could be explained by an unfavorable orientation of the drug in the intercalation complex rather than by a reduced binding to DNA. Geometry of drug intercalation may have a critical influence on the formation of the ternary complex. In turn, the onset of a different DNA-damaging pathway is likely to be related to easy redox cycling of the 2,3-diaza-substituted anthracenedione derivatives, which could produce radical species to a remarkably greater extent than could the carbocyclic parent drugs. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:161–169, 1997.

KEY WORDS. diaza-anthracenedione; physicochemical properties; cytotoxicity; DNA damage; topoisomerase II

The cytotoxic effects of conventional antitumor DNA-intercalating agents have been related to their ability to interfere with the nuclear enzyme topoisomerase II [1]. These agents impair the DNA cleaving–rejoining ability of the enzyme, thereby stimulating DNA cleavage. Although stabilization of the topoisomerase II–DNA cleavable complex is the common mechanism of action of several antitumor agents (including nonintercalators) [2], topoisomerase inhibitors may differ in their interaction with DNA or with the enzyme–DNA complex. In fact, different antitumor agents stimulate enzyme-mediated cleavage of DNA at different sites in a sequence-specific manner [2]. Moreover, the persistence of the trapped cleavable complex is critically dependent on the nature of the inhibitor, and drug accessibility to critical genomic sites could reasonably influence cytotoxic potency and antitumor efficacy. In addition, the pharmacological drug profile could reflect other drug interactions at the cellular level. Mitoxantrone and other anthracenedione derivatives are important examples of DNA intercalators able to poison topoisomerase II, thereby generating protein-associated DNA-strand breaks that eventually lead to drug-induced cell death [3]. The precise role of the intercalating moiety in their molecular and pharmacological effects remains to be defined. In an attempt to obtain new active drugs exhibiting less pronounced side effects and to provide a thorough insight into structure–activity relationships, the development of second-generation anthraquinones has led to the synthesis of analogues with one or two C → N bioisosteric substitutions in the anthracenedione planar system [4–9]. The introduction of hetero atoms into the anthracene-9,10-dione chromophore was expected to modify a number of physicochemical properties of the planar ring system, which may be relevant for drug interaction with DNA and may influence drug bioactivity.

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In a previous paper [10], we examined a series of 2-aza derivatives and demonstrated that the physicochemical, biological and pharmacological properties of the new drugs were remarkably affected by the presence of the heteroatom in the intercalating moiety of the drug. To understand the structural determinants of cellular response and the role of DNA-binding affinity in the inhibition of topoisomerase II, we examined the 2,3-diaza derivative compounds presented in Fig. 1. The properties of diaza derivatives at molecular and cellular levels were examined and compared with those of the parent compounds, mitoxantrone and ametantrone.

MATERIALS AND METHODS

Materials

The 2,3-diaza anthracenediones [6,9-bis[(2-dimethylaminoethyl)amino] benzo[g]phthalazine-5,10-dione] (BBR 2853) and 6,9-bis[(2-aminoethyl) amino]benzo[g]phthalazine-5,10-dione trihydrochloride] (BBR 2894) (Fig. 1) were synthesized according to the method of Krapcho *et al.* [7]. Mitoxantrone (NSC 301739-NB) and ametantrone (NSC 287513-NA) were provided by the National Cancer Institute (Bethesda, MD, USA). Poly (dG.dC) and poly-(dA.dT) (200–500 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures [11] and was stored at −20°C in 20 mM KH_2PO_4, pH 7, 50% glycerol, 0.5 mM PMSF,[‖] 0.1 mM EDTA, 1 mM β-mercaptoethanol.

DNA binding studies

The measurements were carried out at 25°C in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA and known amounts of NaCl to adjust ionic strength to the desired value. Binding was followed spectrophotometrically in the ligand absorption region (500–750 nm). In the ionic strength range of 0.1–0.5 M, the presence of isosbestic points during titration with DNA allowed an evaluation of free and DNA-bound drug. To avoid large systematic inaccuracies from experimental errors in extinction coefficients, the range of bound drug fractions was 0.15–0.85. The data were evaluated according to the equation of McGhee and Von Hippel for noncooperative ligand–lattice interactions [12].

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

where r is the molar ratio of bound ligand to DNA, m is the free ligand concentration, K_i is the intrinsic binding constant and n is the exclusion parameter. Spectrophotometric measurements were performed with a Perkin-Elmer Lambda 5 apparatus equipped with a Haake F3-C thermostat.

Circular and linear dichroism

Circular dichroism studies were carried out for the complexes of each 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. For linear dichroism experiments, DNA was oriented in a rotating quartz cell (600–800 rpm). Measurements were carried out at pH 7.0 in Tris 10 mM, EDTA 1 mM, and NaCl to adjust ionic strength to 100 mM.

Cyclic voltammetry

Cyclic voltammetry measurements were performed using an Amel scanning 222 potentiostat at the following settings: initial potential −0.1 V, switching potential 1 V, scan rate 0.4 V/sec. In cyclic voltammetry measurements, a hanging mercury drop electrode (HMDE, Metrohm) was used as the working electrode. In all measurements, a three-electrode system was used with a reference saturated calomel electrode and a platinum wire counter electrode. All experiments were performed at room temperature.

Cell lines and cytotoxicity assay

Human promyelocytic leukemia HL-60 cells [13] and the human small-cell lung carcinoma cell line NCI-H187 [14] were cultured in RPMI-1640 medium (Flow Laboratories, Irvine, UK) plus 10% fetal calf serum (Flow). Drug treatments were carried out for 1 hr at 37°C on exponentially growing cells. In cytotoxicity experiments where cell survival was determined by means of the MTT‡ assay [15], cells were centrifuged after drug treatment, washed, resuspended and cultured in drug-free medium for 96 hr. Drug cytotoxicity determined with the cell counting method was evaluated after 72 hr in culture in drug-free medium.

Cellular drug uptake

NCI-H187 cells (10^7) were incubated for 1 hr at 37°C in the presence of 10 μM drug. Following two washings with PBS, cells were lysed by sonication, suspended in 1 mL of a water/acetonitrile (70:30, v/v) mixture containing 0.5 ml/L trifluoroacetic acid and 2×10^{-3} M hexanesulfonic acid and centrifuged. The clear supernatant was used for the quantitative analysis of the amount of drug uptaken. This solvent mixture was used as the eluent in chromatography. Ion-paired reverse-phase liquid chromatography was used for the quantitative determination of diaza anthraquinones. Separation was obtained at room temperature using a 30- × 0.4-cm Bondapak C18 column (Water Millipore, Milford, MA, USA). The flow rate was 1.0 mL/min. Spectrophotometric detection of the samples was performed at 600 nm.

[‖] *Abbreviations:* MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide; DTT, dithiothreitol; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SSB, single-strand breaks; SV40, simian virus 40; TBE, tris/borate/EDTA buffer.

Alkaline elution assay

NCI-H187 cells were labeled with 0.1 μCi/mL of [^{14}C]thymidine for 90 hr at 37°C. The labeled nucleoside precursor was removed 24 hr before drug treatments by centrifugation and resuspension of cells in fresh medium. HL60 cells were labeled with 0.1 μCi/mL of [^{14}C]thymidine for 24 hr at 37°C, and the labeled nucleoside precursor was removed 12 hr before drug treatments. Alkaline elution procedures were as reported by Kohn *et al.* [16] and extensively described elsewhere [17, 18].

Topoisomerase II-mediated DNA cleavage

SV40 DNA was uniquely 5′-end-labeled as previously described [19]. The DNA fragment was reacted for 30 min at 37°C with 32 units of topoisomerase II and drugs in 40 mM Tris-HCl, pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 15 μg/mL bovine serum albumin. Reactions were stopped by adding 1% SDS and 0.1 mg/mL proteinase K and incubated at 42°C for an additional 45 min. DNA was then ethanol precipitated, resuspended in 2.5 μL of 80% formamide, 10 mM NaOH, 1 mM EDTA and 0.1% dyes, heated at 90°C for 2 min, chilled in ice and then loaded onto an 8% denaturing polyacrylamide gel [29:1, acrylamide:bis(acrylamide) ratio; 7 M urea in TBE buffer]. Electrophoresis was performed at 80 W for approximately 2 hr. Gels were dried and autoradiographed with Amersham Hyperfilm MP.

RESULTS

The new anthracenedione derivatives examined in this study (BBR 2853 and BBR 2894; see Fig. 1) were obtained by exploiting two types of structural manipulations of mitoxantrone and ametantrone. In comparison with mitoxantrone, both BBR 2853 and BBR 2894 lack the 1,4-dihydroxyphenylene ring, which has been replaced by a pyridazine ring. This modification represents a bioisosteric C → N substitution of carbons 2 and 3 of mitoxantrone (and ametantrone). In addition, the compounds have amino-substituted side chains different from mitoxantrone, i.e. the 2-(amino)ethylamino side chain for BBR 2894 and the 2-(dimethylamino)ethylamino side chain for BBR 2853. However, the new compounds retain the tricyclic planar arrangement and the quinone moiety of the parent drugs.

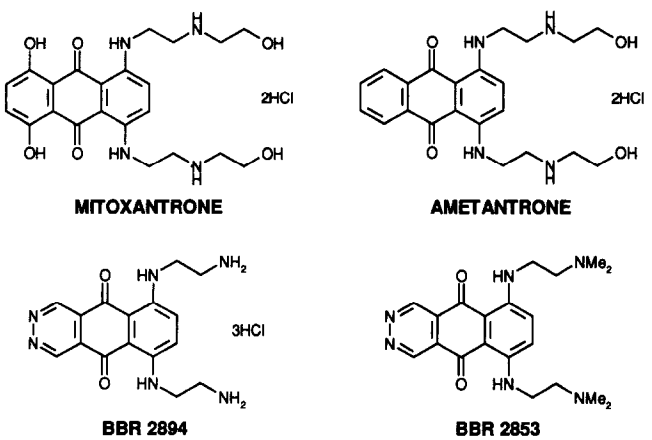

FIG. 1. Chemical structures of the studied anthracenedione derivatives.

Physicochemical properties and DNA-binding

Physicochemical studies performed on compounds BBR 2853 and BBR 2894 and reference compounds mitoxantrone and ametantrone were concerned with the following elements: (1) redox potentials, (2) thermodynamics and (3) stereochemistry of DNA binding. These properties are relevant in defining structure–activity relationships because they are involved in the pharmacological activity of antitumor anthracenediones.

REDOX STUDIES. The anthracenedione moiety undergoes redox processes, which could be partly responsible for undesired side effects such as cardiotoxicity [20]. Hence, an evaluation of the tendency for aza anthraquinone to be reduced to the semiquinone could provide useful insight into its potential to generate free radicals. In fact, the semiquinone can be re-oxidized *in vivo*, thereby producing DNA-damaging free radicals [20]. The quinone reduction–reoxidation process can be monitored by cyclic voltammetry. Representative results obtained in aqueous media are reported in Fig. 2 and summarized in Table 1 in terms of reduction potentials. The redox process was reversible for all compounds; thus, permanent modifications of drug molecules as a result of redox cycling are not expected unless the intermediates are trapped in their reactive form by enzymatic systems. The reduction potentials for diaza anthracenediones appeared to be approximately 0.3 V less negative than for the carbocyclic analogues, which means that reduction was substantially easier for the anthracenedione drugs. The potentials of drugs bound to DNA could not be evaluated because of precipitation of the complexes under the experimental conditions of cyclic voltametry.

DRUG BINDING TO DNA. The DNA-binding parameters obtained at different ionic strength conditions are summarized in Table 2. In all cases, an isosbestic point was observed in the spectrophotometric titration experiments. BBR 2894 and BBR 2853 exhibited a modestly lower affinity for DNA than for ametantrone. In turn, mitoxantrone was markedly more efficient in binding to the nucleic acid. A decrease in the DNA-binding constant was observed with increasing ionic strength. Hence, charged side chains form efficient electrostatic contacts with the DNA phosphate backbone. The ionic-strength dependence of the binding process is reported in Table 3. The slope $d(\log K_i)/d(\log \text{ionic strength})$ is proportional to the number of charged interactions occurring per complex unit [21]. For diaza anthraquinones, we found values very close to 2.0, which is consistent with an involvement of both charged

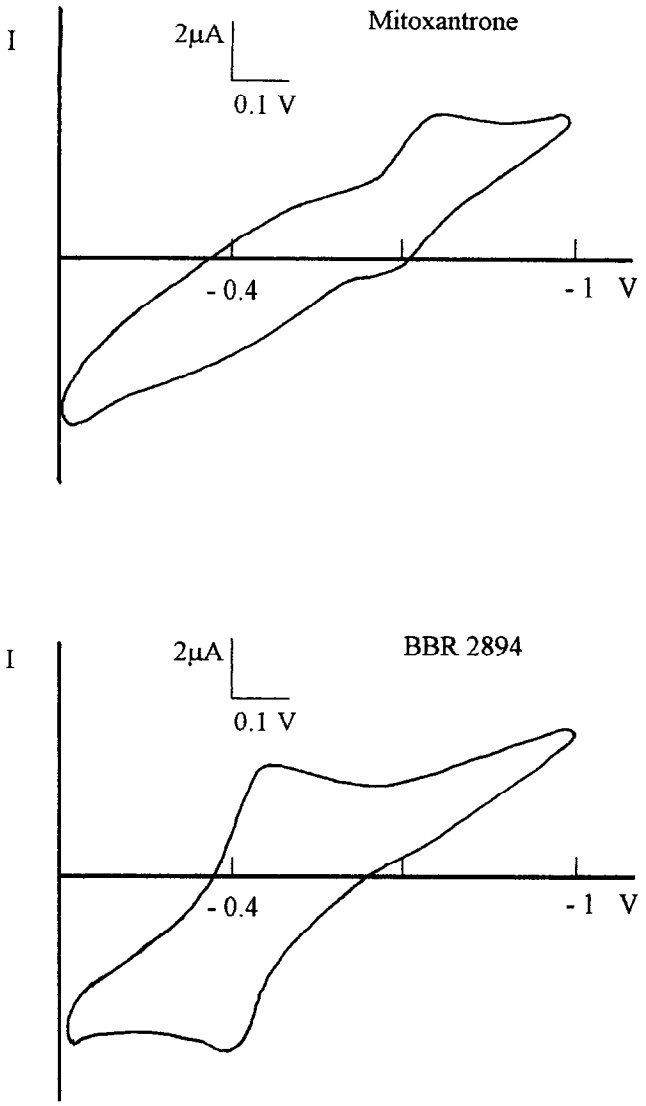

FIG. 2. Cyclic voltametry of mitoxantrone and BBR 2894 in 10 mM Tris, pH 7, 1 mM EDTA and NaCl to adjust ionic strength to 0.1 M.

aminoethylamino side chains in the complex. For mitoxantrone and ametantrone, ionic effects were less prominent, as indicated by the low dependence of the binding constant on salt concentration. The values of the exclusion parameters (n) generally increased with increasing ionic strength, which is probably related to salt-induced compaction effects of the double helix structure [22], which reduce accessibility of the drug chromophore for intercalation.

TABLE 1. Half-wave reduction potentials ($E_{1/2}$) of diaza anthracenediones mitoxantrone and ametantrone in 10 mM Tris, 100 mM NaCl, pH 7.0

Compound	$E_{1/2}$ (v)
Mitoxantrone	-0.74
Ametantrone	-0.68
BBR 2894	-0.42
BBR 2853	-0.41

TABLE 2. Thermodynamic parameters for the binding of diaza anthracenediones, mitoxantrone and ametantrone to calf thymus DNA in 10 mM Tris, 1 mM EDTA, pH 7.0, 23°C and different ionic strength

Compound	Ionic strength (M)	$K_i \times 10^{-4}$* (M^{-1})	n†
Mitoxantrone	0.25	120.00 ± 30.00	4.4 ± 0.1
	0.75	31.00 ± 6.00	4.6 ± 0.1
	2	15.00 ± 5.00	4.8 ± 0.1
Amentantrone	0.15	34.03 ± 3.37	3.5 ± 0.1
	0.5	5.71 ± 0.26	7.6 ± 0.3
BBR 2894	0.1	20.60 ± 0.84	4.4 ± 0.1
	0.25	3.08 ± 0.04	5.6 ± 0.1
	0.5	1.25 ± 0.03	6.2 ± 0.3
BBR 2853	0.1	12.91 ± 0.30	5.0 ± 0.1
	0.25	2.09 ± 0.03	6.0 ± 0.1
	0.5	0.63 ± 0.01	3.8 ± 0.1

* K_i, intrinsic constant for the binding of the first ligand to DNA.
† n, number of bases forming the binding site of the drug.

MODE AND STEREOCHEMISTRY OF DIAZA-ANTHRAQUINONE BINDING TO DNA. Linear dichroism measurements make it possible to evaluate the angle between bound drug and DNA axis [23]. In these experiments, we always found values close to 90°, which is consistent with an intercalation mechanism of interaction and with previous results obtained with the closely related 1,4 bis-substituted benzo-[g]phthalazine-5,10 diones [8]. In addition to the mode of DNA binding, the orientation of the intercalated chromophore might be relevant in affecting drug activity. In fact, the main mechanism of action of mitoxantrone and ametantrone involves interaction with a topoisomerase II–DNA cleavable complex because the drug-binding site is very close to (or overlaps) the enzyme cutting–rejoining site [2]. Different orientations of the drug chromophore in the intercalation pocket might generate different drug–enzyme contacts in the cleavable complex and consequently change the pharmacological response. Circular dichroism studies give useful insight into this matter [24]. Because it is quite difficult to interpret induced circular dichroism spectra safely in terms of drug orientation in the intercalated system [25], we performed measurements using the alternating polynucleotides poly(dA-dT) and poly(dC-dG) rather than random DNA sequences because a theoretical analysis of circular dichroism induction by intercalated chromophores has been recently carried out with

TABLE 3. Ionic strength dependence of K_i, the binding constant of the test anthraquinone derivatives to calf thymus DNA

Compound	$\delta(\log K_i)/\delta$ (log ionic strength)
Mitoxantrone	1.3
Ametantrone	1.5
BBR 2894	1.8
BBR 2853	1.9

these polynucleotides [26, 27]. The spectra are presented in Fig. 3. BBR 2894 (not shown) and BBR 2853 exhibited two negative dichroic bands in the absorption region of the drug chromophore, whereas the opposite sign was observed in the presence of mitoxantrone using either polynucleotide. This change in CD sign can be principally related to two structural factors: (1) modified orientation of the chromophore with reference to the base-pair longest dimension and (2) displacement of the intercalated chromophore from the DNA axis [26, 27]. Our results indicate that the average arrangement of the diaza anthracenediones in the intercalation pocket differs markedly from that of the reference compound. It is not possible from the CD data to decide whether one or both of these factors are modified in the diaza-anthraquinone–DNA interaction.

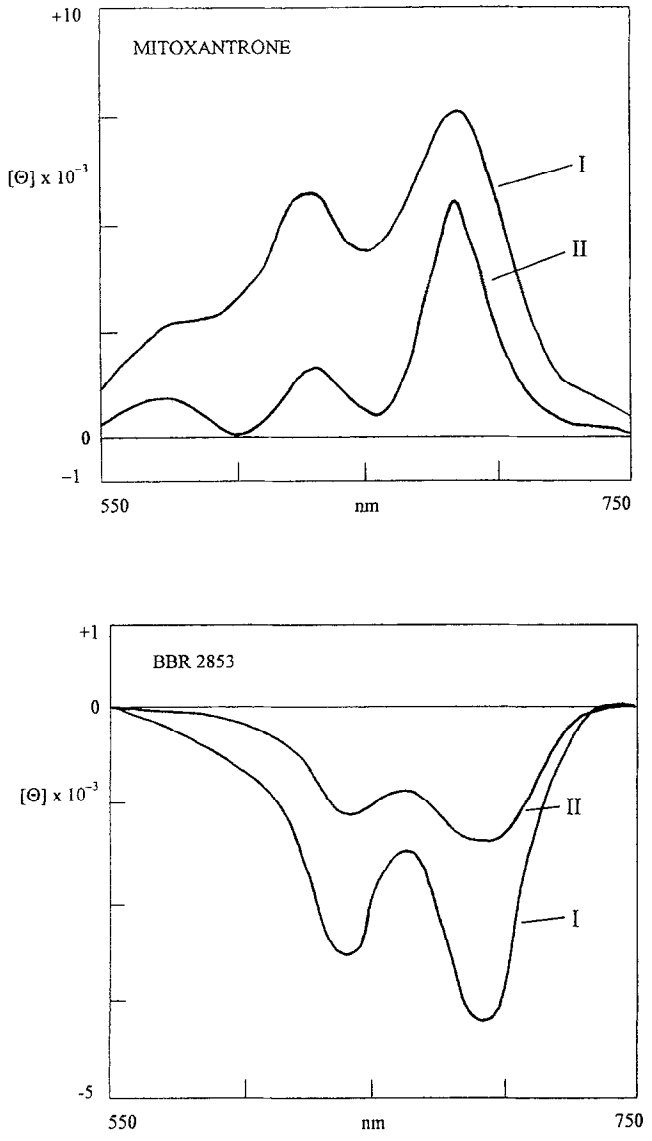

FIG. 3. Induced circular dichroism (deg · cm² · dmoL⁻¹) in the ligand absorption region for the complexes of mitoxantrone and BBR 2853 with poly(dA-dT) (II) and poly(dG-dC) (I) in 10 mM Tris, pH 7, 1 mM EDTA and NaCl to adjust ionic strength to 0.1 M.

Cytotoxic activity

Cytotoxic activity was examined in the human small-cell carcinoma NCI-H187 and leukemia HL60 cell lines (Table 4) following 1 hr exposure. In NCI-H187 cells, BBR 2894 was poorly cytotoxic, similarly to ametantrone, whereas BBR 2853 showed a cytotoxic activity comparable to that of mitoxantrone. As previously observed [3, 10], HL60 cells were more sensitive than NCI-H187 cells to anthracenediones, whereas BBR 2853 was remarkably less cytotoxic than mitoxantrone in the leukemia cells.

Formation of DNA SSB

DNA-SSB produced by the diaza derivatives were determined after a 1-hr drug exposure. The profile of DNA breaks produced by mitoxantrone and BBR 2853 in NCI-H187 cells was quite different (Fig. 4). In the range of drug concentrations below 2 μM, mitoxantrone was more potent than the BBR 2853. However, the derivative caused an increased extent of DNA damage above this concentration, whereas mitoxantrone reached a plateau with a trend to a biphasic (i.e. bell-shaped) behavior. The dose–response curve of mitoxantrone is typical of intercalating DNA topoisomerase II inhibitors [28, 29]. BBR 2894 stimulated a number of SSB comparable to ametantrone, up to 23 μM. Again, a plateau was not observed for the former compound above this concentration. To verify whether DNA breaks stimulated by BBR 2853 were associated with covalently bound proteins, the formation of DNA-SSB was evaluated in the presence or absence of proteinase K in both NCI-H187 and HL-60 cell lines. The results (Table 5), showed that enzymatic deproteinization was not necessary to detect the breaks because in both cell systems DNA-SSB formed by BBR 2853 were not protein associated, unlike other anthracenediones [3]. A different range of drug concentrations was used because HL60 exhibited an extreme sensitivity to mitoxantrone (Table 4). At doses causing approximately 50% inhibition of cell growth in the NCI-H187 cell line (1 and 2 μM for mitoxantrone and BBR 2853, respectively), a comparable extent of DNA-SSB was found (ca. 400–500 rad equivalents) (Table 5). A similar correlation was observed when comparing the effects of IC_{50} (approximately 0.01 and 10 μM for mitoxantrone and BBR 2853,

TABLE 4. Cytotoxic activity of the studied anthraquinone derivatives*

Drug	IC_{50} (μM)	
	NCI-H187	HL60
Mitoxantrone	1.3 ± 0.7	0.013 ± 0.009
Ametantrone	112 ± 29	0.44 ± 0.09
BBR 2894	84 ± 58	>230
BBR 2853	2 ± 1.3	13.30 ± 9.9

* Cells were exposed to drugs for 1 hr at 37°C. Drug cytotoxicity was determined with the MTT assay in NCI-H187 cells and with the cell-counting method in the HL60 cells. See Materials and Methods for details.

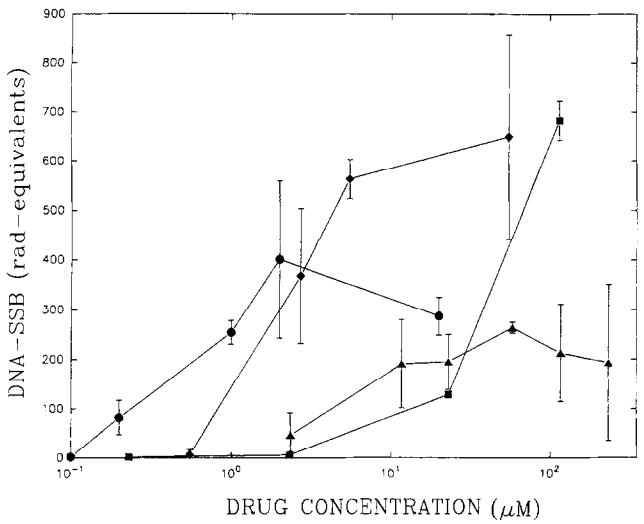

FIG. 4. DNA-SSB induced by anthracenedione derivatives in NCI-H187 cells. Cells were exposed to drugs for 1 hr at 37°C, lysed on the filter in the presence of proteinase K and eluted at pH 12.15. See Materials and Methods for details. Standard error deviation bars are indicated when values were derived from two to four independent determinations. Circle, mitoxantrone; triangle, ametantrone; diamond, BBR 2853; square, BBR 2894.

respectively) in terms of DNA-SSB induced in the HL60 cell line (ca. 100 rad equivalents). However, no correlation could be found between the level of DNA-SSB and cytotoxicity in the two cell systems. A lack of correlation between drug-induced bulk DNA damage and growth inhibition has been reported [30].

Drug stimulation of SV40 DNA cleavage induced by topoisomerase II

The relative drug activity in stimulating topoisomerase II-mediated DNA breaks was studied by using ^{32}P-labeled SV40 DNA as a substrate for murine topoisomerase II (Fig. 5). The results showed that stimulation of DNA breaks by

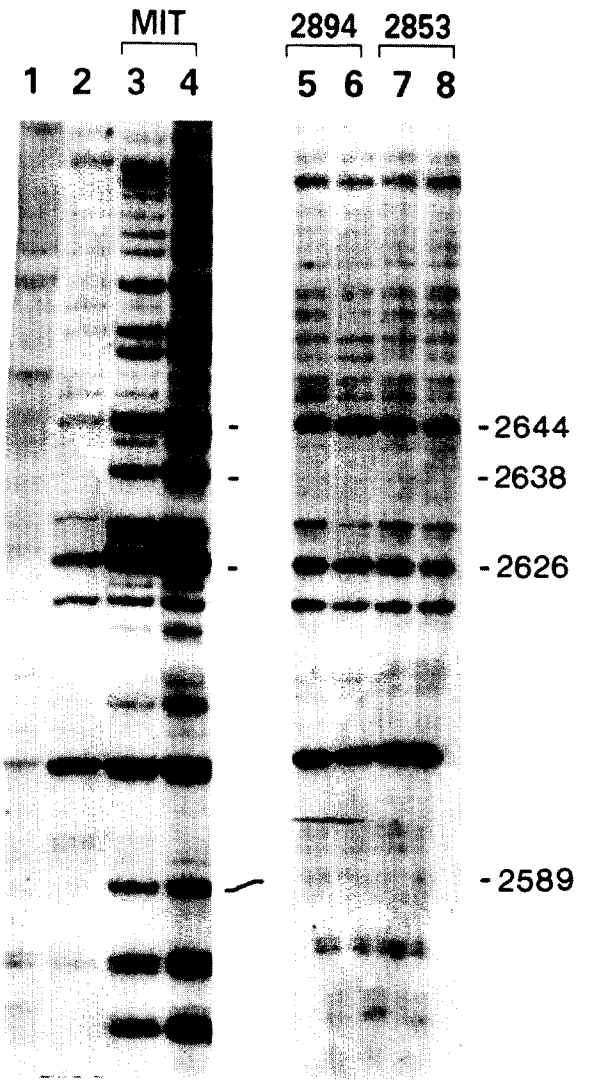

FIG. 5. Topoisomerase II-mediated DNA cleavage stimulated by anthracenedione derivatives. A BamHI-AccI SV 40 DNA fragment, 5′-end labeled at BamHI (lane 1, control) was incubated at 37°C with 32 units of topoisomerase II without drug (lane 2) or with 0.1 μM (lanes 3, 5, 7) or 1 μM (lanes 4, 6, 8) of the indicated compounds. See Materials and Methods for details.

TABLE 5. DNA single-strand breaks produced by BBR 2853 and mitoxantrone‡

	DNA-SSB (rad equivalents)					
	H187			HL60		
Drug	Concentration (μM)	+Proteinase K	-Proteinase K	Concentration (μM)	+Proteinase K	-Proteinase K
Mitoxantrone	1.1	422 ± 135*	15 ± 9*	0.018	80	n.d.†
	2.25	519 ± 210*	121 ± 91*	0.18	237 ± 75*	n.d.
	22.5	369	100	0.9	355	n.d.
				1.8	402	n.d.
BBR 2853	1.3	221	288	2.6	35	n.d.
	2.6	422 ± 78*	510 ± 140*	15	125	152
	5.5	1234 ± 809*	1005 ± 592*	52.3	92	212

‡ Cells were exposed to drug for 1 hr lysed on the filters in the presence (+) or absence (−) of proteinase K and eluted at pH 12.15, as described in Materials and Methods.
* Mean ± SE of two independent experiments.
† n.d., not detectable.

the two diaza derivatives was very weak under these conditions. Some cleavage sites induced by the enzyme without drugs (sites 2626, 2644; Fig. 5) were strongly stimulated by mitoxantrone but only slightly stimulated by diaza-anthracenedione derivatives. In addition, other sites induced in the presence of mitoxantrone (sites 2638, 2589) were not induced by the studied compounds.

Cellular accumulation

To determine whether the different responses observed with the tested anthracenedione derivatives were related to cellular pharmacokinetics, we measured drug uptake in the H187 and HL60 cell lines (Table 6). In H187 cells, the derivative BBR 2853 was incorporated into cells twice as efficiently as were mitoxantrone and ametantrone and 10 times better than BBR 2894. In HL60 cells, the accumulation of BBR 2853 was approximately four times that of mitoxantrone.

DISCUSSION

This study documents that the introduction of two nitrogen atoms, at positions 2 and 3 of the anthracenedione chromophore, caused marked changes in the physicochemical and DNA-binding properties and in the biological response of the above intercalators. The main differences between diaza derivatives and the reference anthraquinone compound mitoxantrone can be summarized as follows.

1. The diaza derivatives exhibit a ≈0.3 V less negative reduction potential, which allows easier redox cycling, with production of radical species including oxygen superoxide and hydroxyl.
2. Because the binding parameters of aza derivatives are comparable to those of ametantrone (which lacks OH groups of mitoxantrone), the major factor impairing the efficiency of intercalating into DNA is probably the absence of hydroxyl groups rather than C → N substitution.
3. The intercalation geometry of diaza derivatives and mitoxantrone differs considerably in terms of chromophore orientation within the base-pair pocket, as shown by circular dichroism measurements. The CD response of ametantrone exhibits intermediate chiroptical properties [3]. Thus, the intercalation geometry, besides the presence/absence of hydroxyl groups, is also sensitive to changes in electron distribution occurring when replacing carbon with nitrogen in the anthraquinone moiety. A change in drug orientation should substantially modify the location of the side chain groups in diaza-anthraquinone–DNA complexes, thus affecting recognition of the topoisomerase II–DNA cleavable complex, which represents the main target for carbocyclic analogues.

TABLE 6. Cellular drug accumulation†

Compound	Uptake* (nmol/10^7 cells)	
	H187	HL60
Mitoxantrone	13	3.5
Ametantrone	10	
BBR 2894	2.5	
BBR 2853	27	16

† Cells were exposed to anthracenedione derivatives (10 μM) for 1 hr at 37°C, and drug accumulation was then measured as described in Materials and Methods.
* Mean of two experiments.

The changes in physicochemical properties of the examined compounds are accompanied by dramatic biological and pharmacological effects. The cytotoxic potency of BBR 2853 is substantially reduced in the HL60 cell line compared with mitoxantrone, in spite of increased drug accumulation. In addition, loss of antitumor activity against P388 murine leukemia is observed (unpublished results). These findings correlate well with the drop in stimulation of topoisomerase II-mediated DNA cleavage observed in the presence of diaza anthracenediones, which indirectly confirms the importance of this type of DNA lesion in the mechanism of anticancer activity. At the molecular level, the examined diaza compounds may not be able to recognize the enzyme–DNA cleavable complex efficiently. To confer activity, a well-defined orientation of the drug is needed with reference to topoisomerase in the enzyme–DNA–drug ternary complex. In particular, the relative location of the planar and side chain groups seems to play a major role in affecting enzyme function and sequence specificity [31]. Hence, changes in geometry of intercalation, as seen for BBR 2853 and BBR 2894, may well be responsible for dramatically impairing drug activity. Therefore, we suggest that an altered mode of drug binding, rather than reduced affinity for DNA, affects drug ability to inhibit DNA topoisomerase II.

An unexpected finding of this study was a significant cytotoxic activity of BBR 2853 against the H187 cell line. In this small-cell lung cancer cell line, cytotoxic activity was comparable to that of mitoxantrone, and the IC_{50} value was lower than that found in the treatment of HL60, a cell system very sensitive to topoisomerase II inhibitors. This pattern of cell response is consistent with a different mechanism of action of BBR 2853 with reference to mitoxantrone. On the basis of these observations, a comparative analysis of DNA-damaging ability of the two compounds was carried out on the H187 cell line. In spite of a comparable extent of DNA damage, the different profile of dose–response curves (biphasic for mitoxantrone vs. monophasic for BBR 2853) and the different nature of DNA breaks (protein-associated for mitoxantrone vs. non-protein-associated for BBR 2853) clearly indicated a different mechanism of induction of DNA lesions. A free radical mechanism may be responsible for the DNA damage produced by BBR 2853. As mentioned above, generation of free radicals is facilitated by the less negative redox po-

tential (Table 1) and by increased drug accumulation (Table 5).

In conclusion, 2,3-diaza substitution in the anthraquinone chromophore caused a marked reduction in cytotoxic potency and efficacy in tumor systems (e.g. leukemias) responsive to classic topoisomerase II inhibitors of the anthraquinone family. However, 2,3-diaza anthracenediones are able to use another mechanism of cytotoxicity, probably related to oxidative damage following drug activation. The therapeutic potential of this alternative mechanism remains to be assessed. Again, the loss of ability of diaza derivatives to interfere with topoisomerase II supports a primary role of enzyme-mediated DNA cleavage in the mechanism of action of intercalating agents.

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