Notes

Anthracene-9,10-diones as Potential Anticancer Agents: Bacterial Mutation Studies of Amido-Substituted Derivatives Reveal an Unexpected Lack of Mutagenicity

Stanley Venitt,[†] Christopher Crofton-Sleigh,[†] Mavis Agbandje,[‡,§] Terence C. Jenkins,[†,▽] and Stephen Neidle*[,‡]

Section of Molecular Carcinogenesis and Cancer Research Campaign Biomolecular Structure Unit, The Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surrey SM2 5NG, U.K.

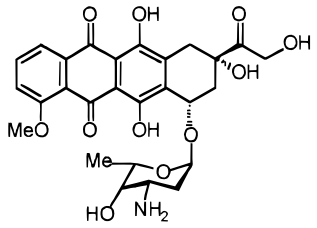
Received March 18, 1998

Fifteen anthracene-9,10-dione ("anthraquinone") derivatives with (ω-aminoalkyl)carboxamido substituents at the 1-, 2-, 1,4-, or 2,6-ring positions were tested for bacterial mutagenicity in reverse-mutation assays using *Salmonella typhimurium* frameshift strains TA1538, TA98, and TA97a, in the presence and absence of a metabolic activation system prepared from the livers of rats treated with Aroclor 1254. Six of the compounds were also tested in *S. typhimurium* TA100 and *Escherichia coli* WP2*uvr*ApKM101 strains, which carry mutations particularly sensitive to reversion by DNA base-pair substitution. Two structurally related compounds, mitoxantrone and bisantrene, were tested in parallel as positive controls. Mitoxantrone was mutagenic to *S. typhimurium* TA1538 and TA98, whereas bisantrene was weakly mutagenic to both these strains but strongly mutagenic toward the TA97a variant. By contrast, although they are also DNA-binding intercalators, none of the amide-functionalized anthracene-9,10-diones of the present study showed significant mutagenic activity in any of the bacterial strains examined. Further, neither substituent position nor systematic alterations in the nature of attached side chains appeared to induce mutagenicity with these agents, although other studies have shown that such structural factors markedly influence their cytotoxic potencies toward mammalian cells in vitro.

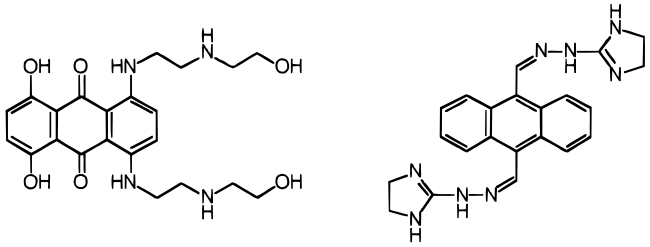
Introduction

The anthracene-9,10-dione (9,10-anthraquinone) group forms the basis of a number of clinical and experimental anticancer drugs, notably the natural antibiotic doxorubicin and the synthetic compound mitoxantrone (Figure 1). Although these two drugs in particular have established clinical activity toward a number of human cancers,[1] they have markedly reduced efficacy in resistant disease and against slowly growing tumors. There is continuing interest in the development of new agents that retain the core anthracene-9,10-dione moiety yet exhibit different spectra of potency, together with reduced overall toxicity.[2,3]

This general class of compound is believed to act by initially binding to cellular DNA sequences, which results in formation of a ternary complex with DNA topoisomerase II.[4] This process in turn produces double-strand breaks in the DNA, and ultimately apoptosis and cell death. The structural characteristics of the anthracene-9,10-dione molecule are appropriate for intercalative binding between adjacent DNA base pairs, as has been shown by numerous physicochemical studies of mitoxantrone, doxorubicin, and their analogues,



doxorubicin (adriamycin)



mitoxantrone **bisantrene**

Figure 1. Structures of two clinically used anthracene-9,10-diones and bisantrene, a putative DNA-threading difunctionalized anthracene derivative.

* Address correspondence to: Professor S. Neidle, Cancer Research Campaign Biomolecular Structure Unit, F-Block, The Institute of Cancer Research, Cotswold Road, Sutton, Surrey SM2 5NG, U.K. Tel: (+044) 181 643 8901, ext 4251. Fax: (+044) 181 643 1675. E-mail: steve@iris5.icr.ac.uk.

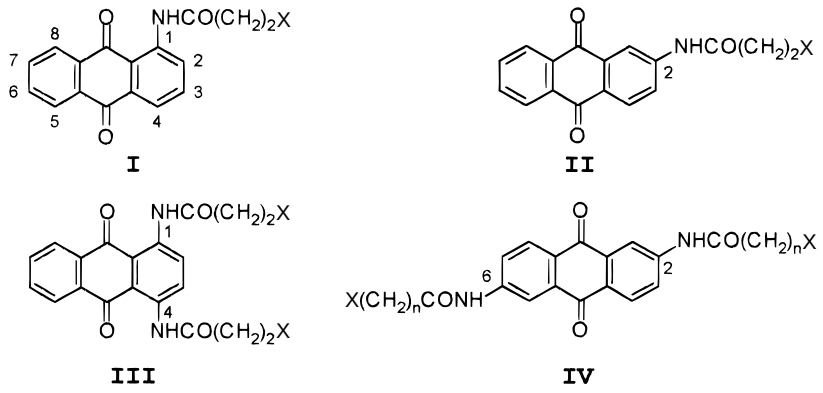
[†] Section of Molecular Carcinogenesis.

[‡] Cancer Research Campaign Biomolecular Structure Unit.

[§] Present address: Department of Biological Sciences, University of Warwick, Warwick CV4 7AL, U.K.

[▽] Present address: School of Chemical and Life Sciences, The University of Greenwich, London SE18 6PF, U.K.

S0022-2623(98)00167-8 CCC: $15.00
Published on Web 08/07/1998



X =	—NEt$_2$	—N	—N O	—N	—N NMe
type **I** (1-mono) :		**1**		**2**	
type **II** (2-mono) :		**3**		**4**	
type **III** (1,4-bis) :	**5**	**6**	**7**	**8**	
type **IV** (2,6-bis)					
n = 1 :	**9**		**10**		**11**
n = 2 :	**12**	**13**	**14**	**15**	

Figure 2. Structures of the amide-functionalized anthracene-9,10-diones examined in this study, showing the 1-mono- (type-I), 2-mono- (type-II), 1,4-bis- (type-III), and 2,6-bis(*ω*-aminoalkyl)carboxamide (type-IV) derivatives. The ring-numbering system is indicated.

together with crystallographic and NMR studies of anthracycline−oligonucleotide complexes.

The introduction of amide substituents into the anthracene-9,10-dione ring system has been the subject of several studies.[2,5−9] The rationales developed for using such groups, rather than the amine functions present in mitoxantrone and its analogues, have been that (i) the planar amide group extends the overall conjugation of the planar chromophore and thereby leads to more effective DNA-binding affinity and (ii) such chemistry can facilitate the attachment of secondary peptide-based moieties. The majority of synthetic anthracene-9,10-diones, including amine- and amide-bearing derivatives, have exploited substitution at the 1,4-ortho-ring positions (cf., numbering system in Figure 2), by direct analogy with mitoxantrone.

In contrast, we have developed an alternative series of (*ω*-aminoalkyl)carboxamide anthracene-9,10-dione derivatives substituted at the symmetric 2,6-ring positions.[9] These compounds have distinct properties in several ways: (i) they bind to duplex DNA by a 'threading' mechanism and have reduced DNA affinity compared with their 1,4-regioisomers, in accord with kinetic and thermodynamic behaviors[10,11] and predictions from molecular modeling,[9,12] (ii) they bind to and differentially stabilize triple-stranded (triplex) DNA,[11,13] and (iii) they show considerably reduced cytotoxicity in vitro compared with their regioisomers.[14] Further, one 2,6-difunctionalized compound has recently been demonstrated to behave as an effective inhibitor of human telomerase, possibly by interacting with a folded DNA guanine-tetrad structure.[15]

It is almost axiomatic that DNA-binding agents are mutagens,[16] and frameshift mutagenicity is a characteristic of many intercalators. Indeed, this property is likely to be a major factor in the carcinogenicity of such agents and is an adverse property that should be eliminated by rational drug design, if possible. This study examines the mutagenicity in bacterial systems of structurally related 1-monosubstituted (**1** and **2**; type-I compounds, see Figure 2) and 2-monosubstituted (**3** and **4**; type-II) amide derivatives and their 1,4-disubstituted (**5**−**8**; type-III) and 2,6-disubstituted (**9**−**15**; type-IV) counterparts. Further, comparison is made with bisantrene, an established DNA-threading agent, and mitoxantrone, a clinical agent that is a paradigm for the present generation of anthracene-9,10-dione antitumor drugs.

Results

Mitoxantrone and Bisantrene. The results obtained for mitoxantrone and bisantrene are shown in Figure 3 and are summarized for the frameshift mutants TA1538, TA98, and TA97a in Table 1. Both agents produced statistically significant dose-related increases in revertants in *Salmonella typhimurium* TA1538 and TA98. Mitoxantrone was about 10-fold more potent than bisantrene in both strains, giving slopes (revertants/*μ*g) in the linear portion of the dose−response curve of 3.52 (TA1538) and 3.04 (TA98) compared with values of 0.2 and 0.33, respectively, for bisantrene. In contrast, bisantrene was convincingly mutagenic to *S. typhimurium* TA97a, whereas mitoxantrone produced no increase in revertants. Both compounds were mutagenic in the absence of S9 rat liver homogenate, in agreement with earlier reports showing that these agents do not require exogenous metabolic activation to exert their mutagenic effects.[17,18]

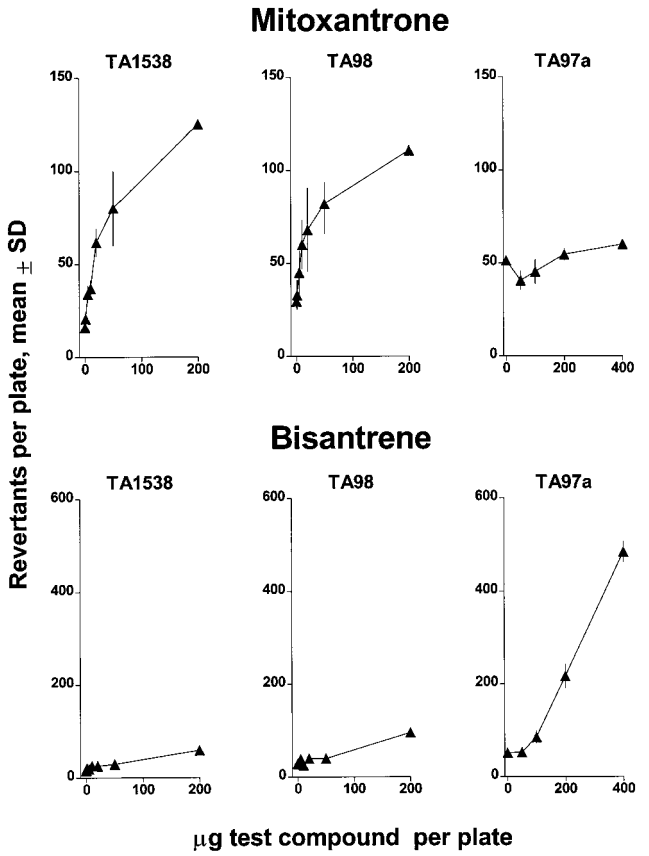


Figure 3. Dose−response curves for mitoxantrone and bisantrene with *S. typhimurium* strains TA1538, TA98, and TA97a. Each point represents the mean ± SD from three plates tested at each drug dose.

In an initial experiment mitoxantrone induced a dose-related increase in revertants in the base pair-substitution mutant *Escherichia coli* WP2*uvr*ApKM101. However, at no dose was there a doubling in the background value, and further experiments showed that this effect was not reproducible (data not shown). Bisantrene was toxic to this *E. coli* strain, as shown by the marked reduction in revertant colonies which was accompanied by drastic thinning of the background lawn. Neither mitoxantrone nor bisantrene was mutagenic to *S. typhimurium* TA100 (a base pair-substitution mutant) in the range of doses tested (data not shown).

Amide-Functionalized Anthracene-9,10-diones. Experiments were performed with derivatives **1**−**15** at doses ranging between 20 and 2000 μg/plate. None of these compounds showed mutagenic activity in assays in which structurally related positive controls (200 μg/plate mitoxantrone for TA1538 and TA98 and 400 μg/plate bisantrene for TA97a) produced at least 4-fold increases in revertants per plate. (A table of data is available as Supporting Information.) The maximum doses used were those that elicited toxic responses, as judged by reductions in revertant colonies, accompanied by a marked thinning of the background lawn.

Discussion

Under the conditions of the experiments reported in this paper, we have found no evidence that any of the amide-functionalized anthracene-9,10-dione derivatives examined are mutagenic to the bacteria that we used. That the frameshift *his* loci in TA1538, TA98, and TA97a are susceptible to reversion by compounds that are thought to intercalate but not bind covalently to DNA was demonstrated by using mitoxantrone and bisantrene as positive controls; both drugs are structurally related to the compounds under investigation. In this study, we have confirmed previous reports[18,19] that mitoxantrone can induce point mutations in several frameshift mutants of *S. typhimurium*. We have also shown that bisantrene is mutagenic to several strains of *S. typhimurium*, the most sensitive being TA97a. It has previously been reported that bisantrene gives positive results in *S. typhimurium* TA1537, TA98, and TA100.[18]

These results suggest that (ω-aminoalkyl)amide substitution at either the 1,4- or 2,6-ring positions of the anthracene-9,10-dione system results in a markedly reduced ability to form complexes with DNA that can undergo frameshifts, in striking contrast to the behavior of the anthracene-9,10-diones functionalized with 1,4-amine groups, typified by mitoxantrone. Molecular modeling and solution binding studies[8,9,12] have shown that 1,4 derivatives disubstituted with either amine or amide moieties interact with duplex DNA in a similar way to, for example, proflavine, whereas the isomeric 2,6 compounds bind in a distinct manner. Thus, the 1,4 compounds (type-III, Figure 2) are restricted to intercalative binding so that substituents are positioned in the major groove of a B-DNA duplex, whereas the 2,6 isomers (type-IV) effectively 'thread' through the intercalation site with one substituent simultaneously located in each of the minor and major grooves. Biophysical support for these distinct models is provided by data from kinetic[10] and calorimetric experiments,[11] which highlight clear contrasts for DNA complexation by the two isomeric compound families. By direct analogy, the 1- and 2-monofunctionalized amide compounds (type-I and -II, respectively) would be predicted to act as "classical" DNA-intercalating agents, particularly on the basis of their kinetic profiles for binding to duplex-form nucleic acids.[10]

Our data indicate that side-chain length, determined by the number of methylene groups separating the anthracene-9,10-dione chromophore and the remote protonatable amine groups in the pendant arms [e.g., $n = 1$ (**9**−**11**) or 2 (**12**−**15**) in the type-IV compounds], appears to have no effect upon mutagenicity. This result contrasts with a marked loss of both cytotoxic

Table 1. Summary of Mutagenicity Data for Mitoxantrone and Bisantrene in *S. typhimurium* TA1538, TA98, and TA97a

	S. typhimurium TA1538			*S. typhimurium* TA98			*S. typhimurium* TA1538		
compound	dose range[a]	slope ± SE (mutants/μg)	p[b]	dose range[a]	slope ± SE (mutants/μg)	p	dose range	slope ± SE (mutants/μg)	p
mitoxantrone	0−5	3.52 ± 0.51	<0.001	0−10	3.04 ± 0.65	<0.001	0−400	0.01 ± 0.02	NS
bisantrene	0−200	0.20 ± 0.01	<0.001	0−20	0.33 ± 0.02	<0.001	0−400	1.83 ± 0.10	<0.001

[a] μg/plate. [b] Linear regression.

potency and DNA-binding affinity upon shortening the side chain.[9]

The molecular basis of the lack of mutagenicity shown by the amidoanthraquinones examined in this study remains to be determined. Aminoanthraquinones are generally mutagenic and genotoxic,[18−22] and the aminoanthraquinone drugs mitoxantrone and doxorubicin are also inhibitors of DNA topoisomerase II.[23−25] Frameshift mutagenesis is generally associated with DNA replication forks, possibly with transient bulged or looped-out regions being stabilized by mutagenic agents.[26] A more recent model proposes that mutagens first stabilize topoisomerase II DNA-cleavable complexes, leading to DNA double-strand breaks. In the bacteriophage T4, the preferred sites of acridine mutagenesis correspond to topoisomerase-nicked sites; it has been shown that the processing of these nicks by exonuclease and polymerase activities results in these mutations.[27] A recent report that amido analogues of mitoxantrone do not produce topoisomerase-associated DNA cleavage[28] is consistent with our own preliminary observations[14] on the compounds presented here. This suggests that amidoanthraquinone-DNA intercalation complexes possess distinctive structural (and possibly electronic) features which are not recognized by DNA topoisomerase II.

Conclusions

The lack of mutagenicity seen for the anthracene-9,10-dione compounds **1**−**15** suggests that amide-functionalized derivatives of this class warrant further study as potential anticancer agents, particularly as they do not show (at least in bacterial systems) the mutagenic activity associated with current DNA-binding cytotoxic drugs of clinical application,[22,29] which is of particular concern when drugs are used in long-term administration with children and young adults. For example, molecules active against the novel anticancer target telomerase (cf. 2,6- and 1,4-disubstituted members of this series)[15] will require chronic administration over prolonged periods of time in order for tumor cell growth arrest (senescence) to occur. The lack of mutagenicity shown by the compounds in this study indicates that they may be especially suitable for this particular target.

Experimental Section

Chemicals. Mitoxantrone (NSC-301739; Lederle Pharmaceuticals) and bisantrene (Figure 1), a gift from Upjohn & Co., were used as aqueous solutions of their hydrochloride salts. The (ω-amino)acetamido- (**9**−**11**) and (ω-amino)propionamido-substituted anthracene-9,10-dione derivatives (**1**−**8** and **12**−**15** in Figure 2) were prepared as their water-soluble hydrochloride or acetic acid addition salts using general literature procedures.[8,9] These derivatives were selected to examine the effects of (i) substituent position(s) in the chromophore, (ii) side-chain length, and (iii) basicity and increasing alkyl load for the attached protonatable tertiary amine functions. Full synthetic and characterization details for novel compounds will be published elsewhere.[14] All compounds were homogeneous by chromatography, and solutions were prepared in either dimethyl sulfoxide (DMSO) or *N*,*N*-dimethylformamide (DMF).

Mutagenicity Assays. Bacterial phenotypes were checked and mutagenicity assays were conducted by methods described previously,[19] using *S. typhimurium* TA1538, TA98, TA100, and TA97a and *E. coli* WP2*uvr*ApKM101. Assays were conducted with and without a source of exogenous metabolic activation, in the form of a 9000/*g* supernatant (S9) prepared from the livers of 6-week-old male CB-hooded rats induced for 5 days with Aroclor 1254. S9 mix contained 10% S9 and necessary cofactors as described.[19] All assays were performed using 3 plates/dose. The activity of S9 preparations used in these experiments was checked using benzo[*a*]pyrene (2 and 5 μg/plate) in mutagenicity assays with *S. typhimurium* TA100.[19] Table 2 (Supporting Information shows the dose ranges tested and details of replicate experiments. Revertant colonies were counted after 48−72-h incubation, using a New Brunswick Biotran II model C111 automatic colony counter. An assay was considered positive if there was at least a 2-fold increase over the control in the mean number of revertants per plate accompanied by a dose response. Each experiment included assays of 200 μg of mitoxantrone/plate (*S. typhimurium* TA1538 and TA98) and 40 μg of bisantrene/plate (*S. typhimurium* TA97a) as positive controls.

Acknowledgment. We are grateful to the Cancer Research Campaign for support (program grants to S.V. and S.N.).

Supporting Information Available: Summary of mutagenicity tests (Table 2) (1 page). Ordering information is given on any current masthead page.

References

(1) Lown, J. W., Ed. *Anthracycline and Anthracenedione-based Anticancer Agents*; Elsevier: Amsterdam, 1988.
(2) Gatto, B.; Zagotto, G.; Sissi, C.; Cera, C.; Uriarte, E.; Palù, G.; Capranico, G.; Palumbo, M. Peptidyl anthraquinones as potential antineoplastic drugs: Synthesis, DNA binding, redox cycling, and biological activity. *J. Med. Chem.* **1996**, *39*, 3114−3122.
(3) Gandolfi, C. A.; Beggiolin, G.; Menta, E.; Palumbo, M.; Sissi, C.; Spinelli, S.; Johnson, F. Chromophore-modified antitumor anthracenediones: Synthesis, DNA binding and cytotoxic activity of 1,4-bis[(aminoalkyl)amino]benzo[*g*]phthalazine-5,10-diones. *J. Med. Chem.* **1995**, *38*, 526−536.
(4) Capranico, G.; de Isabella, P.; Tinelli, S.; Bigioni, M.; Zunino, F. Similar sequence specificity of mitoxantrone and VM-26 stimulation of in vitro DNA cleavage by mammalian DNA topoisomerase II. *Biochemistry* **1993**, *32*, 3038−3046.
(5) Palù, G.; Palumbo, M.; Antonello, C.; Meloni, G. A.; Marciani Magno, S. A search for potential antitumor agents: biological effects and DNA-binding of a series of anthraquinone derivatives. *Mol. Pharmacol.* **1986**, *29*, 211−217.
(6) Palumbo, M.; Palù, G.; Gia, O.; Ferrazzi, E.; Gastaldi, S.; Antonello, C.; Meloni, G. A. Bis-substituted hydroxyanthracenediones: DNA binding and biological activity. *Anti-Cancer Drug Des.* **1987**, *1*, 337−346.
(7) Zagotto, G.; Uriarte, E.; Antonello, C.; Conconi, M. T.; Marciani Magno, S.; Palumbo, M. New hydroxy-amido-anthraquinones as potential antineoplastic drugs. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 659−662.
(8) Collier, D. A.; Neidle, S. Synthesis, Molecular modeling, DNA binding, and antitumor properties of some substituted amidoanthraquinones. *J. Med. Chem.* **1988**, *31*, 847−857.
(9) Agbandje, M.; Jenkins, T. C.; McKenna, R.; Reszka, A. P.; Neidle, S. Anthracene-9,10-diones as potential anticancer agents. Synthesis, DNA-binding, and biological studies on a series of 2,6-disubstituted derivatives. *J. Med. Chem.* **1992**, *35*, 1418−1429.
(10) Tanious, F. A.; Jenkins, T. C.; Neidle, S; Wilson, W. D. Substituent position dictates the intercalative DNA-binding mode for anthracene-9,10-dione antitumor drugs. *Biochemistry* **1992**, *31*, 11632−11640.
(11) Haq, I.; Ladbury, J. E.; Chowdhry, B. Z.; Jenkins, T. C. Molecular anchoring of duplex and triplex DNA by disubstituted anthracene-9,10-diones: calorimetric, UV melting and competition dialysis studies. *J. Am. Chem. Soc.* **1996**, *118*, 10693−10701.
(12) Neidle, S.; Jenkins, T. C. Molecular modeling to study DNA intercalation by antitumor drugs. *Methods Enzymol.* **1991**, *203*, 433−459.
(13) Fox, K. R.; Polucci, P.; Jenkins, T. C.; Neidle, S. A molecular anchor for stabilizing triple-helical DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7887−7891.
(14) Jenkins, T. C.; Polucci, P.; Reszka, A. P.; Kelland, L. R.; Neidle, S. To be published.
(15) Sun, D.; Thompson, B.; Cathers, B. E.; Salazar, M.; Kerwin, S. M.; Trent, J. O.; Jenkins, T. C.; Neidle, S.; Hurley, L. H. Inhibition of human telomerase by a G-quadruplex-interactive compound. *J. Med. Chem.* **1997**, *40*, 2113−2116. Perry, P. J.; Gowan, S. M.; Reszka, A. P.; Polucci, P.; Jenkins, T. C.; Kelland, L. R.; Neidle, S. *J. Med. Chem.*; in press.

(16) Glickman, B. W.; Kotturi, G.; de Boer, J.; Kusser, W. Molecular mechanisms of mutagenesis and mutational spectra. In: *Environmental Mutagenesis*; Phillips, D. H., Venitt, S., Eds.; Bios Scientific Publishers: Oxford, 1995; pp 33−59.
(17) Venitt, S.; Crofton-Sleigh, C.; Forster, R. Bacterial mutation assays using reverse mutation. In *Mutagenicity. A Practical Approach*; (Venitt, S., Parry, J. M., Eds.; IRL Press: Oxford, 1984; pp 45−98.
(18) Au, W. W.; Butler, M. A.; Matney, T. S.; Loo, T. L. Comparative structure-genotoxicity study of three aminoanthraquinone drugs and doxorubicin. *Cancer Res.* **1981**, *41*, 376−379.
(19) Ferguson, L. R.; Baguley, B. C. Verapamil as a co-mutagen in the Salmonella/mammalian microsome mutagenicity test. *Mutat. Res.* **1988**, *209*, 57−62.
(20) Harrington-Brock, K.; Parker, L.; Doerr, C.; Cimino, M. C.; Moore, M. M. Analysis of the genotoxicity of anthraquinone dyes in the mouse lymphoma assay. *Mutagenesis* **1991**, *6*, 35−41.
(21) Tikkanen, L.; Matsushima, T.; Natori, S. Mutagenicity of anthraquinones in the Salmonella preincubation test. *Mutat. Res.* **1993**, *116*, 297−304.
(22) Ferguson, L. R.; Baguley, B. C. Mutagenicity of anticancer drugs that inhibit topoisomerase enzymes. *Mutat. Res.* **1996**, *355*, 91−101.
(23) Zunino, F.; Capranico, G. DNA topoisomerase II as the primary target of anti-cancer anthracyclines. *Anti-Cancer Drug Des.* **1990**, *5*, 307−317.
(24) Capranico, G.; Palumbo, M.; Tinelli, S.; Mabilia, M.; Pozzan, A.; Zunino, F. Conformational drug determinants of the sequence specificity of drug-stimulated topoisomerase II DNA cleavage. *J. Mol. Biol.* **1994**, *235*, 1218−1230.
(25) Anderson, R. D.; Berger, N. A. Mutagenicity and carcinogenicity of topoisomerase-interactive agents. *Mutat. Res.* **1994**, *309*, 109−142.
(26) Ferguson, L. R.; Denny, W. A. Frameshift mutagenesis by acridines and other reversibly binding DNA ligands. *Mutagenesis* **1990**, *5*, 529−540.
(27) Kaiser, V. L.; Ripley, L. S. DNA nick processing by exonuclease and polymerase activities of bacteriophage T4 DNA polymerase accounts for acridine-induced mutation specificities in T4. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2234−2238.
(28) Zagotto, G.; Moro, S.; Uriarte, E.; Ferrazzi, E.; Palù, G.; Palumbo, M. Amido analogues of mitoxantrone: physicochemical properties, molecular modeling, cellular effects and antineoplastic potential. *Anti-Cancer Drug Des.* **1997**, *12*, 99−112.
(29) Ferguson, L. R.; Pearson, A. E. The clinical use of mutagenic anticancer drugs. *Mutat. Res.* **1996**, *355*, 1−12.

JM980167R