Peptidyl Anthraquinones as Potential Antineoplastic Drugs: Synthesis, DNA Binding, Redox Cycling, and Biological Activity

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A series of new compounds containing a 9,10-anthracenedione moiety and one or two peptide chains at position 1 and/or 4 have been synthesized. The amino acid residues introduced are glycine (Gly), lysine (Lys), and tryptophan (Trp), the latter two in both the L- and D-configurations. The peptidyl anthraquinones maintain the ability of intercalating efficiently into DNA, even though the orientation within the base-pair pocket may change somewhat with reference to the parent drugs mitoxantrone (MX) and ametantrone (AM). The interaction constants of the mono-, di-, and triglycyl derivatives are well comparable to those found for AM but 5−10 times lower than the value reported for MX. On the other hand, the glycyl-lysyl compounds bind DNA to the same extent as (L-isomer) or even better than (D-isomer) MX. As for the parent drugs without peptidyl chains, the new compounds prefer alternating CG binding sites, although to different extents. The bis-Gly-Lys derivatives are the least sensitive to base composition, which may be due to extensive aspecific charged interactions with the polynucleotide backbone. As far as redox properties are concerned, all peptidyl anthraquinones show a reduction potential very close to that of AM and 60−80 mV less negative than that of MX; hence, they can produce free-radical-damaging species to an extent similar to the parent drugs. The biological activity has been tested in human tumor and murine leukemia cell lines. Most of the test anthraquinones exhibit cytotoxic properties close to those of AM and considerably lower than those of MX. Stimulation of topoisomerase-mediated DNA cleavage is moderately present in representatives of the glycylanthraquinone family, whereas inhibition of the background cleavage occurs when Lys is present in the peptide chain. For most of the test anthraquinones, the toxicity data are in line with the DNA affinity scale and the topoisomerase II stimulation activity. However, in the lysyl derivatives, for which lack of cytotoxicity cannot be related to poor binding to DNA, the steric and electronic properties of the side-chain substituent must impair an effective recognition of the cleavable complex.

Introduction

The anthraquinone ring system is often found in antitumor drugs, such as anthracyclines, mitoxantrone (MX), and anthrapyrazoles.[1−4] Its planarity allows an intercalation between base pairs of DNA in the B conformation, while its redox properties are linked to the production of radical species in biological systems. The chemical and biological activity exhibited by anthraquinone compounds is greatly affected by the different substituents of the planar ring system.[5−8] Side chains bear usually one or two positive charges, in order to establish an electrostatic interaction with the phosphate backbone of the polynucletotide. In addition they must meet the requirements for effective poisoning of topoisomerase II.[9−12] In fact the mechanism of action appears to be mainly related to drug binding to the so-called "cleavable complex" formed by DNA and topoisomerase II,[13−17] each drug having unique DNA-recognition and enzyme-recognition features.[18−20] In particular, it appears that the relative location of the planar and side-chain groups plays a major role in affecting enzyme function and sequence specificity.[21,22]

In MX and active congeners, the substituents are linked to the anthraquinone structure *via* amino linkages. We have recently started to explore the changes in the physicochemical and biological properties of substituted anthraquinones, exhibiting an amide linkage between the planar ring system and the side-chain substituents.[23−29] Since some of the compounds retain a remarkable biological activity, this class appears to be worthy of further examination.

Besides other substituents, formation of amide bonds with the anthraquinone group allows an easy introduction of peptide residues into the drug structure. The presence of a peptide sequence linked to a pharmacophoric group might prove very useful in terms of drug specificity. In fact, it is well known that precise recognition of defined DNA sequences in biological systems is mediated by enzymes and proteins having appropriate structural motifs.[30] Indeed, since one of the major drawbacks of DNA-directed antineoplastic agents is lack of selective targeting of cancer cells, the introduction of specific recognition elements for mutated oncogenes into presently available drugs would represent a basic advance in cancer chemotherapy.

A few encouraging reports are available in the recent literature on amsacrine-peptide adducts to increase

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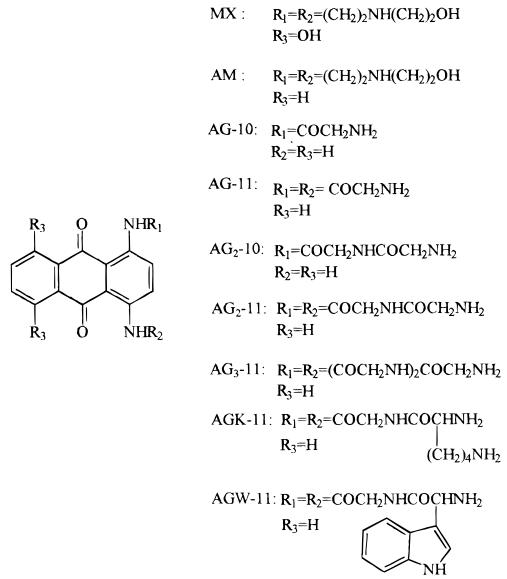


Figure 1. Chemical structures of test peptidyl anthracenediones.

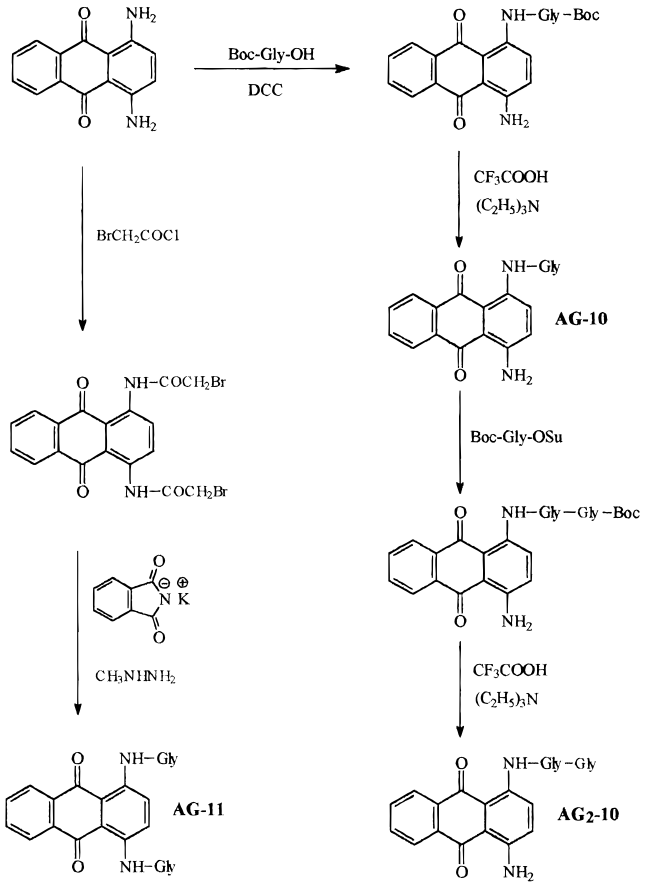
DNA affinity and on copper-chelating peptides linked to anthracenedione to yield DNA-cleaving agents endowed with cytostatic activity.[31−34] A theoretical work on the DNA specificity of an oligopeptide linked to MX has also been published.[35] In addition, anthracenyl−amino acid conjugates have shown topoisomerase I and II inhibition properties.[36]

In the present paper we will present our results on a number of peptidyl anthraquinones containing one or two side chains at positions 1 and 4 of the ring system, up to three amino acids long. Glycine (Gly) was placed in the first position, as it is nonchiral and conformationally rather flexible; in addition to Gly, lysine (Lys) or tryptophan (Trp) was then incorporated, both in the L- and D- configuration, as suitable representatives for polar and apolar residues, which could affect both drug uptake and delivery, as well as affinity and specificity for target DNA.

Chemistry

The compounds synthesized are reported in Figure 1. The labels used to identify them are according to the following rationale: The first letter (**A**) stands for 1,4 diamino-9,10-anthracenedione, the following letters are the one-letter symbols for the amino acids introduced in the side chain starting from the C terminus and the final numbers indicate the presence (**1**)/absence (**0**) of a given side chain at positions 1 and 4 of the planar ring system. When using chiral amino acids, their absolute configuration is also mentioned as L or D. The synthetic route is presented in Schemes 1 and 2. The preparation of **AG-11** was based on the method of Gabriel,[37] involving formation of *ω*-(bromoacetamido)-1,4-diaminoanthracene-9,10-dione using the acid halide and subsequent reaction with potassium phthalimide followed by hydrazinolysis of the phthalyl derivative with methylhydrazine. Direct condensation of the substrate with (*tert*-butyloxycarbonyl)glycine using dicyclohexylcarbodiimide yields a large amount of the monosubstituted anthracenedione because the nucleophilicity of the second amino group remarkably decreases after the first one has reacted. The peptidyl chains of **AG-10** and **AG-11** were elongated using hydroxysuccinimide esters for

Scheme 1



L-amino acids and dicyclohexylcarbodiimide for the D-enantiomers, for which activated esters were not commercially available. Gly, α- and ϵ-protected Lys, or α-protected Trp was linked to the Gly residue of **AG**. All reactions were followed by TLC, and intermediates were characterized by NMR and elemental analysis. Removal of the protecting groups was achieved by treatment with piperidine in dimethylformamide for the Fmoc, while for the Boc, trifluoroacetic acid containing 10% water in the presence of traces of *p*-cresol was used. When removing protection groups from Trp, the treatment in alkali requires a careful control of the reaction conditions in order to avoid partial hydrolysis of the aromatic amide bond, observed by NMR. All final products were characterized by NMR, IR, mass spectrometry, amino acid analysis, and elemental analysis.

DNA-Binding Studies

The interaction of the peptidyl anthraquinones with DNA has been studied by means of fluorescence and circular and linear dichroism. All compounds examined interact with the polynucleotide, albeit with different affinity and degree of sequence specificity.

Thermodynamics. Formation of the DNA−anthraquinone complex causes almost total quenching of the fluorescence signal for all of the compounds. The data, represented as Scatchard plots, have enabled us to evaluate the thermodynamic parameters of binding, which are reported in Table 1. All the anthraquinones examined exhibit a remarkable affinity for DNA (*K* in the range of 10^5 M^{-1}), close to that found for AM but clearly lower than that for MX. Particularly effective are the lysyl derivatives characterized by doubly charged

Scheme 2

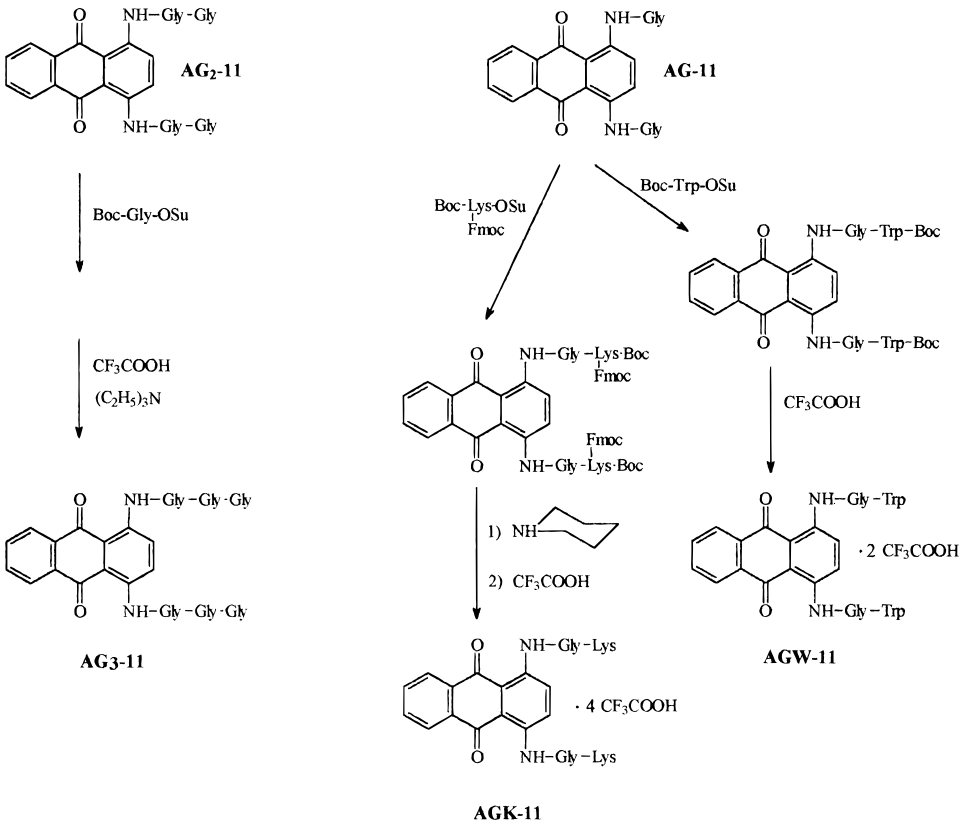


Table 1. Thermodynamic Parameters for the Binding of the Test Compounds to Calf Thymus DNA at Physiological Ionic Strength, pH 7.0 and 25 °C

compd	$K \times 10^{-5}$ (M^{-1})[a]	n[b]
AG-10	3.6 ± 0.3	2.1 ± 0.2
AG-11	2.2 ± 0.3	2.3 ± 0.2
AG_2-10	1.7 ± 0.4	2.2 ± 0.2
AG_2-11	2.4 ± 0.4	2.5 ± 0.2
AG_3-11	2.4 ± 0.3	2.4 ± 0.2
AGW-11(L)	nd[c]	nd[c]
AGW-11(D)	nd[c]	nd[c]
AGK-11(L)	17.5 ± 2.4	2.3 ± 0.3
AGK-11(D)	33.0 ± 3.2	2.4 ± 0.3
AM	3.4 ± 0.2	2.7 ± 0.1
MX	18.5 ± 2.0	2.7 ± 0.2

[a] Intrinsic binding constant. [b] Exclusion parameter (base pairs). [c] Not determined.

sidechains: The L-isomer compares well with MX, while the D-isomer is even more effective than the reference anthraquinone. The complex with the polynucleotide always involves between two and three base pairs, suggesting a similar stoichiometry for mono- and bispeptidyl compounds. No cooperativity is observed at physiological conditions with any of the test anthraquinones. **AGW-11(L)** and **AGW-11(D)** are very poorly soluble at physiological conditions. For this reason, data for the interaction of these compounds with DNA are not available.

Experiments with **AG-10** and **AG-11** showed a slow change of the fluorescence response as a function of time, with formation of a sparingly soluble material. This phenomenon can be attributed to an intramolecular cyclization involving the terminal amino group of glycine and the carbonyl group of the anthraquinone, as indicated by NMR data (not shown). Nonetheless, quenching of the fluorescence signal by DNA and thermal stabilization of the double helix by the compounds strongly suggest a binding mechanism similar to the other peptidyl anthraquinones. In any event, reproducible binding data could be obtained at physiological ionic strength and neutral pH, performing the experiments immediately after dissolution of the drugs in the aqueous medium.

Sequence Specificity. Fluorescence experiments have been performed using DNAs at different contents of CG in order to investigate the DNA sequence preference or specificity of the peptidyl anthraquinones. The results of these measurements are summarized in Figure 2, where the ratio between the binding constant to a given DNA and the binding constant to calf thymus DNA is reported as a function of CG content. Mono-, di-, and triglycyl derivatives show a distinct preference for CG-rich sequences as many anthraquinones do,[38] the most specific being **AG_3-11** and **AG_2-11**. They compare well to AM and MX. The lysyl compounds show a lesser degree of selectivity, although they still slightly prefer CG-rich sequences.

Geometry of the Drug−DNA Complex. The presence of a negative band in the visible region of linear dichroism spectra supports an intercalative mechanism for the interaction of the examined compounds with DNA.[39]

Induced circular dichroism is observed for the test compounds in the visible region (Table 2). In all cases induced rotational strength is negative, which suggests a common geometry of intercalation for the peptidyl anthraquinones, similar to that of AM and MX. Interestingly the DNA complexes of both **AGK** enantiomers exhibit almost identical dichroic features, which indicates that their intercalation geometry is not affected by the opposite chirality of Lys residues in the side

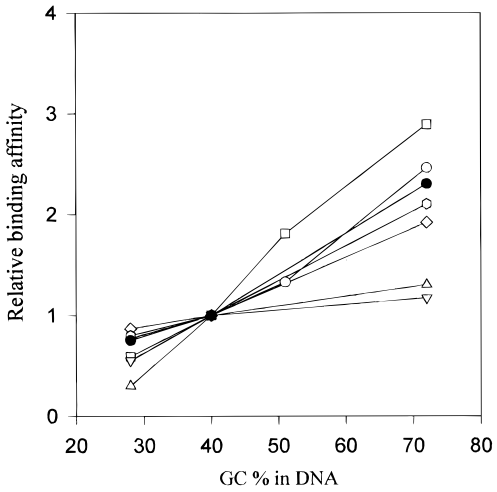


Figure 2. Effects of DNA composition on the binding affinity of test anthraquinones. The relative binding affinity for each drug has been calculated as the ratio between the binding constant to a given DNA and the binding constant to calf thymus DNA (CG = 40%). The symbols are as follows: ◇, **AG-10**; ⬡ **AG_2-10**; ○, **AG_2-11**; □, **AG_3-11**; △, **AGK-11(L)**; ▽, **AGK-11(D)**, ●, MX.

Table 2. Maximum Intensity of Induced Circular Dichroism in the Visible Region for the Complex of the Test Anthraquinones with Calf Thymus DNA (0.1 M NaCl, pH 7.0, 25 °C)

compd	$[\Theta] \times 10^{-3}$ (deg cm^2/dmol)
AG-10	−1.87
AG-11	−1.70
AG_2-10	−1.51
AG_2-11	−2.26
AG_{3-11}	−2.34
AGK-11(L)	−3.99[a]
AGK-11(D)	−3.97[a]
AM	−4.87 and −4.67[b]
MX	−4.60 and −5.07[b]

[a] Difference between the ellipticity of the complex and the ellipticity of the free drug. [b] Two dichroic bands are observed.

chains. The circular dichroism spectra of intercalated dyes have been correlated to the relative orientation of the ligand molecule and base pairs.[40,41] However, it has been shown that the induction signal depends not only upon orientation but also upon sequence context and displacement of the intercalated molecule with reference to the base-pair vertical axis.[42,43] Hence it is difficult to deduce from the above dichroic data the absolute orientation of the intercalated chromophores.

Redox Properties

The anthracenedione moiety is known to undergo redox processes, which could directly produce cytotoxic effects.[1,44−48] Redox behavior is also important for assessing the relevance of undesired side effects, such as cardiotoxicity.[49] The results of cyclic voltammetry measurements in aqueous media are practically identical with that of AM ($E_{1/2} \approx -0.67$ V).

The redox process is reversible for all compounds; hence 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.

Biological Results

Cell Cytotoxicity. The cytotoxicity of the peptidyl anthraquinones was tested in three tumor cell lines: HL60, HeLa, and L1210. The results are reported in Table 3. The toxicity data for AM and MX are also reported for comparison. In general, all derivatives have potencies similar to that of AM. However, in the HeLa cell line the Gly derivatives compare well to MX. It is interesting to note that both Lys derivatives are practically devoid of activity, whereas both Trp compounds, notwithstanding their low solubility, are cytotoxic. No significant difference is evident between anthraquinones bearing L- or D-amino acids.

Table 3. ED_{50} Values[a] of the Examined Peptidyl Anthraquinones on Different Tumor Cell Lines

	cell line ED_{50} (mM)		
compd	HL60	HeLa	L1210
AG-10	2.0 ± 0.3	2.7 ± 0.2	0.31 ± 0.09
AG_2-10	2.0 ± 0.3	2.7 ± 0.4	0.24± 0.08
AG-11	1.9 ± 0.3	1.6 ± 0.3	0.9 ± 0.1
AG_2-11	2.3 ± 0.4	2.4 ± 0.3	0.8 ± 0.2
AGK-11(L)	>100	>100	nd[b]
AGK-11(D)	>100	>100	nd[b]
AGW-11(L)	30 ± 11	nd[b]	nd[b]
AGW-11(D)	28 ± 14	nd[b]	nd[b]
MX	0.04 ± 0.01	0.9 ± 0.1	0.01 ± 0.005
AM	0.88 ± 0.14	3.1 ± 1.7	0.1 ± 0.06

[a] Drug concentration that inhibited 50% of cell growth. [b] Not determined.

Drug Stimulation of DNA Cleavage Induced by Topoisomerase II. The relative drug activity in stimulating topoisomerase II-mediated DNA cleavage was studied using ^{32}P-labeled SV40 DNA as a substrate for murine topoisomerase II. The derivatives of the **AG_n** and **AGW** type showed a moderate tendency to stimulate DNA breaks, which occurred at locations where also MX and AM cause stimulation. On the contrary, the **AGK** compounds, while not showing any tendency to stimulation, abolished background cleavage even at 1 μM concentration. Representative examples are reported in Figure 3.

Discussion

Introduction of peptidyl side chains into the anthracene-9,10-dione structure causes important effects in the physicochemical, DNA-binding, and biological responses when compared to the parent anthraquinones AM and MX. First of all, DNA affinity can be modulated as a function of the sequence in the side chain. In our case changes of more than 1 order of magnitude in the binding constant were monitored. Increasing the basicity of the amino acid residues increases the efficiency of the interaction with the nucleic acid. Indeed the **AGK** derivatives are about 10 times more effective than the parent AM and comparable to, if not better than, MX in this respect. Interestingly, the D-isomer proved to be superior to the L-isomer. This might represent an advantage for the design of new drugs, as D-peptide linkages are resistant to protease activity and do not undergo fast degradation processes *in vivo*.

In addition to affinity, drug binding specificity also plays a role in eliciting biological response. The glycyl derivatives (**AG_n**) appear to behave very closely to AM (and MX), exhibiting a clear preference for CG steps in the polynucleotide chain. Less specific proved to be the lysyl derivatives which distribute more evenly along the nucleic acid chain. This is conceivable bearing in mind that a very high electrostatic contribution to the binding

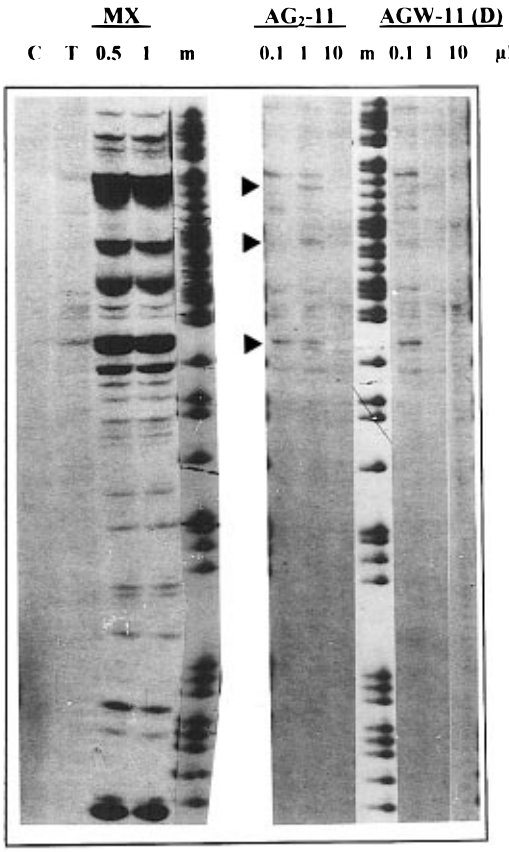


Figure 3. DNA cleavage intensity patterns stimulated by MX and peptidyl anthraquinones. Symbols are as follows: C, control DNA; T, topoisomerase II only; and m, purine molecular markers. MX, **AG$_2$-11**, and **AGW-11(D)** were previously defined. Numbers above the lanes indicate the drug concentrations used. Arrows indicate major common stimulation sites.

process derives from side-chain- and main-chain-protonated amino groups of the drug. Charged interactions are clearly occurring irrespective of base-pair composition, as they arise from the negative charge density generated by the phosphate groups of the DNA backbone. Hence, in this case, high affinity is not coupled with high specificity.

Overall drug orientation in the intercalation pocket does not appear to be substantially modified by the nature of side-chain substituents, as negative rotational strength in the anthracenedione absorption region is always observed in circular dichroism measurements. However, different values of induced molar ellipticity point to slightly different arrangements of peptidyl anthraquinones when inserted between base pairs.

Peptidyl anthraquinones exhibit reduction potentials almost identical with those of AM and somewhat higher than those of MX. As a consequence of redox cycling, production of radical species including superoxide and hydroxyl[46] by all new compounds will be comparable to the reference anticancer drugs and will probably contribute to cell killing in a similar way.

Cell cytotoxicity is correlated to the nature of the amino acids in the side chains rather than to the presence of one or two of them at positions 1 and/or 4 of the anthracenedione ring. All glycyl derivatives exhibit cell-killing properties close to those of AM in all tested cell lines but are clearly much less active than MX. It is worth recalling that the examined compounds, like AM, lack the 5,8-dihydroxy substituents in the

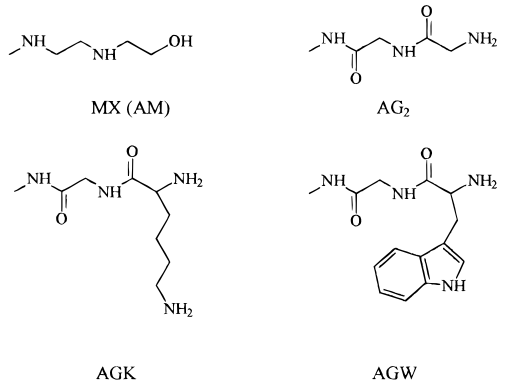


Figure 4. Comparison of the chemical structures of the side chains of AM (MX) and selected peptidyl anthraquinones.

planar ring system which are present in MX. Our data confirm the relevance of these hydroxyl groups in anticancer activity and suggest the synthesis of peptidyl derivatives exhibiting this structural feature. In any event, peptidyl substitution is still compatible with anthracenedione activity when glycines only are present in the side chains. Unfortunately, this is not the case for Gly-Lys derivatives, which are devoid of detectable cytotoxicity.

The pharmacological activity data match well with stimulation of topoisomerase II-mediated DNA cleavage. Indeed, the anthracenediones **AG$_n$** and **AGW**, which are remarkably less effective than MX in the stimulation process, exhibit also a comparably reduced cytotoxicity. On the other hand, the Gly-Lys derivatives **AGK** inhibit, rather than stimulate, topoisomerase II functions, which suggests competition between the drug and the enzyme in DNA template occupancy. Accordingly, they are devoid of cell-killing properties, which indirectly points to the importance of topoisomerase II-mediated mechanisms in anticancer activity.

A possible rationalization of the experimental results can be attempted considering the chemical structure of the side-chain residues of the test anthracenediones (Figure 4). A remarkable similarity between the AM (MX) and **AG$_2$** side chains can be observed. Both are linear, with atoms of similar electronegativity and hydrogen-bonding capability placed at almost coincident positions, the main difference resting in the relative loss of flexibility and basicity when amide replaces amine in the middle of the side chain. Accordingly, both physicochemical and biological properties are close to each other, and interference with the topoisomerase−DNA cleavable complex may occur in a similar fashion. When a bulky side-chain group is introduced as the second amino acid residue in the peptidyl anthraquinone as in **AGW** and **AGK**, a drop in biological response and topoisomerase poisoning occur. The worse situation appears to be represented by the lysyl chain, which, besides being relatively long, bears an additional protonated amino group. This residue cannot probably be accommodated in the topoisomerase II−DNA complex to stabilize the cleavable intermediate. In addition to that, the pharmacokinetic properties of a highly charged compound, such as **AGK**, might well be unfavorable and further contribute to loss of cytotoxicity. This is consistent with the reduced activity shown by a number of MX congeners bearing bulky substituents and additional positive charges in the side chains.[2,5]

Experimental Section

The purity of all products and reaction intermediates was determined by TLC, HPLC (Perkin-Elmer Model 3B, C18 μBondpak reverse phase Waters column 25 × 4 cm, Perkin-Elmer LC-75 flow spectrophotometer, 45−65% acetonitrile gradient eluant system), ^{1}H-NMR (Varian Model Gemini-200), IR (Perkin-Elmer Model 580-B), amino acid analysis (Carlo Erba), and elemental analysis (Carlo Erba). NMR data are reported in δ units.

Mitoxantrone and ametantrone were obtained from Lederle and Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, respectively. They were used without further purification. DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures[50] and stored at −20 °C in 20 mM KH_2PO_4, pH 7.0, 50% glycerol, 0.5 mM PMSF, 0.1 mM EDTA, and 1 mM β-mercaptoethanol. One unit of strand-passing activity was defined as the smallest amount of protein that completely unknotted 0.2 μg of knotted P4 DNA at 37 °C in 30 min.[50] SV40 DNA, T4 polynucleotide kinase, agarose, and polyacrylamide were purchased from GIBCO-BRL (Basel, Switzerland). [γ-^{32}P]ATP was purchased from Amersham (Milan, Italy). Calf intestinal phosphatase and restriction endonucleases were purchased from New England Biolabs (Taunus, Germany). DNAs from calf thymus, *Clostridium perfringens*, *Escherichia coli*, and *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. (St. Louis, MO) and phenol-extracted before use. All other reagents and solvents were purchased from either Aldrich or Fluka and used without further purification.

1-(Glycylamino)-4-aminoanthracene-9,10-dione (AG-10). Dicyclohexylcarbodiimide (DCC) (8.9 g, 43 mmol) was dissolved in 100 mL of anhydrous tetrahydrofuran (THF); 6.7 g (38 mmol) of (*tert*-butyloxycarbonyl)glycine and 2.0 g (8.4 mmol) of 1,4-diaminoanthracene-9,10-dione were added, and the mixture was stirred 24 h at room temperature. The dicyclohexylurea was filtered out, and the solvent was evaporated under vacuum. The crude product was purified by silica gel column chromatography eluting with $CHCl_3$ to give 2.04 g of 1-[(Boc-glycyl)amino]-4-aminoanthracene-9,10-dione, 61% yield, as dark red crystals: mp 179−181 °C. ^{1}H NMR ($CDCl_3$): 12.81 (s, 1H), 7.87 (d, 1H), 7.73 (dd, 2H), 7.65 (dd, 2H), 7.02 (d, 1H), 4.01 (d, 2H), 1.63 (s, 9H). IR (1% KBr, cm^{-1}): 3412, 3305, 3076, 2977, 1702, 1629, 1602, 1501, 13833, 1369, 1277, 1168, 733. Anal. ($C_{21}H_{21}N_3O_5$) C, H, N.

1-[(Boc-glycyl)amino]-4-aminoanthracene-9,10-dione (1.4 g, 3.5 mmol) and 0.97 g (8.9 mmol) of *p*-cresol were dissolved in trifluoroacetic acid (TFA) containing 10% water. The solution was stirred for 2 h at room temperature and poured in 150 mL of ethyl ether; a fine dark precipitate formed immediately and was separated by filtration and resuspended in 200 mL of H_2O. The aqueous suspension was extracted with diethyl ether (Et_2O) and the solvent removed; the resulting trifluoroacetate salt was resuspended in ethyl acetate and neutralized with triethylamine. The product was precipitated with petroleum ether and dried (0.50 g, 47% yield of **AG-10,** mp 139−141 °C). ^{1}H NMR (D_2O): 7.55 (dd, 1H), 7.38 (dd, 2H), 7.13 (m, 2H), 6.30 (s, 1H), 3.69 (s, 2H). IR (1% KBr, cm^{-1}): 3412, 3298, 1682, 1633, 1605, 1503, 1539, 1275, 720. Anal. ($C_{16}H_{13}N_3O_3$) C, H, N.

1-[(Glycyl-glycyl)amino]-4-aminoanthracene-9,10-dione (AG_2-10). Triethylamine, 0.284 mL (2.04 mmol), and 0.306 g (1.12 mmol) of Boc-Gly-OSu were added to 0.301 g of **AG-10** (1.02 mmol) dissolved in 25 mL of anhydrous THF. The solution was stirred at room temperature for 24 h, and the solvent was removed under vacuum. The crude product (**AG_2-10**-Boc, mp 130−132 °C) was crystallized from ethanol−petroleum ether and dried under vacuum (0.216 g of 1-[(Boc-glycyl-glycyl)amino]-4-aminoanthracene-9,10-dione, 46% yield). ^{1}H NMR ($CDCl_3$): 8.85 (d, 1H), 8.21 (m, 2H), 7.74 (m, 2H), 7.01 (d, 1H), 4.23 (d, 2H), 4.16 (d, 2H), 1.51 (s, 1H). IR (1% KBr, cm^{-1}): 3411, 3301, 3071, 2980, 1780, 1678, 1608, 1502, 1384, 13367, 1277, 1171, 730. Anal. ($C_{23}H_{24}N_4O_6$) C, H, N.

1-[(Boc-glycyl-glycyl)amino]-4-aminoanthracene-9,10-dione (0.188 g, 0.419 mmol) and 0.112 g (1.04 mmol) of *p*-cresol were dissolved in 5 mL of trifluoroacetic acid containing 10% water, reacted for 30 min at room temperature, and then poured in 80 mL of diethyl ether. The oily product that separated was dissolved in water and extracted with ethyl acetate. The organic phase was reduced in volume and neutralized with Et_3N. The purple precipitate was collected, recrystallized from petroleum ether, and dried under vacuum (0.095 g of **AG_2-10**, 65% yield, mp 135−137 °C). ^{1}H NMR (D_2O): 7.48 (d, 1H), 7.23 (dd, 2H), 6.98 (dd, 2H), 6.10 (d, 1H), 3.79 (s, 2H), 3.73 (s, 2H). IR (1% KBr, cm^{-1}): 3410, 3000, 1682, 1609, 1505, 1277, 1026, 726. Anal. ($C_{18}H_{16}N_4O_4$) C, H, N.

1,4-Bis(glycylamino)anthracene-9,10-dione (AG-11). Pyridine (4.3 mL, 53 mmol) and 5.2 mL (62 mmol) of bromoacetyl chloride were added to 5.0 g (21 mmol) of 1,4-diaminoanthracene-9,10-dione dissolved in 100 mL of warm dimethylformamide. The mixture was heated at 100 °C for 90 min. The solution was then cooled, and 50 mL of pyridine was added; then the mixture was poured in 700 mL of diethyl ether. The precipitate was filtered and dried under vacuum at 40 °C (8.4 g of 1,4-bis(ω-bromoacetamido)anthracene-9,10-dione, 80% yield). ^{1}H NMR (DMSO-d_6): 12.60 (s, 2H), 8.95 (s, 2H), 8.12 (m, 2H), 7.97 (m, 2H), 4.40 (s, 4H). IR (1% KBr, cm^{-1}): 3148, 2949, 1681, 1640, 1584, 1489, 1257, 724. Anal. ($C_{18}H_{12}N_2O_4Br_2$) C, H, N.

Potassium phthalimide (0.96 g, 5.2 mmol) was added to 1.0 g (2.1 mmol) of 1,4-bis(ω-bromoacetamido)anthracene-9,10-dione dissolved in 50 mL of dimethylformamide and stirred for 4 h at room temperature. The solvent was removed under vacuum, and the residue was dissolved in 100 mL of dichloromethane and washed thrice with water. The solvent was removed under vacuum and the residue dissolved in 180 mL of warm 9:1 ethyl acetate−acetic acid. The solution was then refrigerated at 4 °C for overnight. The product was filtered and dried under vacuum at 40 °C (1.13 g of 1,4-bis(ω-phthalimidoacetamido)anthracene-9,10-dione, 89% yield). ^{1}H NMR ($CDCl_3$): 12.89 (s, 2H), 9.08 (s, 2H), 8.05−7.75 (m, 12H), 4.65 (s, 4H). IR (1% KBr, cm^{-1}): 3478, 3440, 3122, 2935, 1717, 1638, 1588, 1502, 1260, 715. Anal. ($C_{34}H_{20}N_4O_8$) C, H, N.

Methylhydrazine (10 mL, 0.18 mol) was added to 0.98 g (1.6 mmol) of 1,4-bis(ω-phthalimidoacetamido)anthracene-9,10-dione in 100 mL of dichloromethane and stirred at room temperature for 6 h. The solvent was removed under vacuum and the product dissolved in 85 mL of diethyl ether. The solution was cooled at −20 °C overnight. The precipitate was filtered and dried under vacuum at 40 °C (0.52 g of **AG-11**, 93% yield). ^{1}H NMR (CF_3COOD): 9.04 (s, 2H), 8.38 (m, 2H), 8.01 (m, 2H), 4.58 (s, 4H). IR (1% KBr, cm^{-1}): 3409, 3347, 3152, 2909, 1686, 1644, 1587, 1493, 1259, 1067, 729. Anal. ($C_{18}H_{16}N_4O_4$) C, H, N.

1,4-Bis[(Glycyl-glycyl)amino]anthracene-9,10-dione (AG_2-11). To 0.225 g (0.63 mmol) of **AG-11** dissolved in 10 mL of anhydrous THF were added 0.50 g (1.8 mmol) of Boc-Gly-OSu and 0.1 mL of triethylamine. The reaction mixture was stirred for 4 h at room temperature, and the solvent was evaporated under vacuum. The residue was dissolved in ethanol, and the resulting solution was poured in water. The precipitate was collected and dried under vacuum (0.359 g of 1,4-bis[(Boc-glycyl-glycyl)amino]anthracene-9,10-dione, 84% yield). ^{1}H NMR ($CDCl_3$): 8.85 (s, 2H), 8.21 (m, 2H), 7.74 (m, 2H), 4.23 (d, 4H), 4.16 (d, 4H), 1.51 (s, 18H). IR (1% KBr, cm^{-1}): 3412, 3301, 2980, 1783, 1678, 1609, 1502, 1384, 1367, 1277, 1171, 730. Anal. ($C_{32}H_{38}N_6O_{10}$) C, H, N.

The above Boc derivative (0.300 g, 0.445 mmol) was suspended in 2 mL of trifluoroacetic acid containing 10% water and 0.010 g (0.09 mmol) of *p*-cresol. After stirring for 20 min at room temperature, 8 mL of diethyl ether was added; the precipitate was collected, crystallized from ethanol, and dried under vacuum (0.158 g of **AG_2-11** as bis(trifluoroacetate), 51% yield). ^{1}H NMR (CF_3COOD): 9.10 (s, 2H), 8.36 (dd, 2H), 8.01 (dd, 2H), 4.60 (s, 4H), 4.52 (s, 4H). IR (1% KBr, cm^{-1}): 3410, 3002, 1680, 1613, 1506, 1277, 1027, 723. Anal. ($C_{26}H_{24}N_6O_{10}F_6$) C, H, N.

1,4-Bis[(Glycyl-glycyl-glycyl)amino]anthracene-9,10-dione (AG_3-11). **AG_2-11** (0.120 g, 0.173 mmol) and 0.50 g (1.8 mmol) of Boc-Gly-OSu were dissolved in 10 mL of anhydrous THF, and 0.2 mL of triethylamine was added. The reaction mixture was stirred for 4 h at room temperature and then the

solvent evaporated under reduced pressure. The residue was dissolved in ethanol and the product precipitated with water. The crystals were collected and dried under vacuum (0.117 g of 1,4-bis[(Boc-glycyl-glycyl-glycyl)amino]anthracene-9,10-dione, 87% yield). ^{1}H NMR ($CDCl_3$): 8.87 (s, 2H), 8.23 (m, 2H), 7.71 (m, 2H), 4.23 (d, 4H), 4.16 (d, 4H), 4.08 (d, 4H), 1.51 (s, 18H). IR (1% KBr, cm^{-1}): 3411, 3309, 2980, 1782, 1678, 1608, 1502, 1386, 1367, 1277,1171, 735. Anal. ($C_{36}H_{46}N_8O_{12}$) C, H, N.

1,4-Bis[(Boc-glycyl-glycyl-glycyl)amino]anthracene-9,10-dione (0.100 g, 0.128 mmol) was added to a mixture of 2 mL of TFA containing 10% water and 0.01 g of *p*-cresol. The reaction mixture was stirred for 30 min at room temperature and the reaction quenched with 10 mL of diethyl ether. The crystalline product was filtered and dried (0.097 g of **AG$_3$-11** as bis(trifluoroacetate), 94% yield). ^{1}H NMR (CF_3COOD): 9.64 (s, 2H), 8.55 (dd, 2H), 8.30 (dd, 2H), 5.19−4−88 (m, 12H). IR (1% KBr, cm^{-1}): 3413, 3004, 1679, 1606, 1509, 1276, 1023, 726. Anal. ($C_{30}H_{30}N_8O_{12}F_6$) C, H, N.

1,4-Bis[(L-lysyl-glycyl)amino]anthracene-9,10-dione [AGK-11(L)]. To 0.103 g (0.29 mmol) of **AG-11** in 10 mL of anhydrous THF were added 0.080 mL of anhydrous triethylamine and 0.293 g (0.66 mmol) of Boc-Lys(Boc)-OSu. The reaction mixture was stirred at room temperature for 1 day and the solvent evaporated under vacuum. The product was purified on silica gel, acetonitrile−ethanol gradient. The fractions containing the product were pooled together and dried (0.045 g of 1,4-bis[(Boc-L-lysyl(Boc)-glycyl)amino]anthracene-9,10-dione, 15% yield). ^{1}H NMR (DMSO-d_6): 9.03 (s, 2H), 8.12 (m, 2H), 7.75 (m, 2H), 4.24 (m, 6H), 2.93 (m, 1H), 1.87 (m, 4H), 1.72 (m, 4H), 1.35 (s, 36H). IR (1% KBr, cm^{-1}): 3370, 3343, 3302, 3076, 2978, 1688, 1668, 1636, 1593, 1501, 1392, 1366, 1259, 1026, 729. Anal. ($C_{50}H_{72}N_8O_{14}$) C, H, N.

To remove the protecting groups, 0.094 g (0.093 mmol) of 1,4-bis[(Boc-L-lysyl(Boc)-glycyl)amino]anthracene-9,10-dione was suspended in 0.8 mL of trifluoroacetic acid containing 10% water and 0.020 g (0.18 mmol) of *p*-cresol. After stirring for 20 min at room temperature, 8 mL of diethyl ether was added; the crystals were collected and dried under vacuum at 40 °C (0.098 g of **AGK-11(L)** as tetrakis(trifluoroacetate), 98% yield). ^{1}H NMR (CD_3OD): 9.05 (s, 2H), 8.31 (m, 2H), 7.92 (m, 2H), 4.22 (m, 6H), 3.00 (t, 3H), 2.06 (m, 4H), 1.77 (m, 4H), 1.64 (m, 4H). IR (1% KBr, cm^{-1}): 3405, 2963, 1677, 1639, 1591, 1500, 1263, 1203, 1027, 723. Anal. ($C_{30}H_{40}N_8O_6$·4TFA) C, H, N.

1,4-Bis[(L-tryptophanyl-glycyl)amino]anthracene-9,10-dione [AGW-11(L)]. Triethylamine (0.060 mL, 0.43 mmol) and 0.262 g (0.653 mmol) of Boc-Trp-OSu were added to 0.100 g (0.284 mmol) of **AG-11** dissolved in 10 mL of anhydrous THF. The solution was stirred for 24 h at room temperature. Evaporation of the solvent afforded a crude product that was purified on silica gel eluting with acetonitrile (0.044 g of 1,4-bis[(Boc-L-tryptophanyl-glycyl)amino]anthracene-9,10-dione, 17% yield). ^{1}H NMR (DMSO-d_6): 8.98 (s, 2H), 7.80 (m, 2H), 7.55 (m, 4H), 6.88 (m, 2H), 6.77 (s, 2H), 6.70 (m, 4H), 4.63 (m, 4H), 4.25 (d, 4H), 3.86 (m, 4H), 1.47 (s, 18H). IR (1% KBr, cm^{-1}): 3337, 3059, 2932, 1691, 1662, 1590, 1497, 1392, 1367, 1258, 1169, 1027, 743. Anal. ($C_{50}H_{52}N_8O_{10}$) C, H, N.

The protecting groups were removed as reported above affording **AGW-11(L)** as bis(trifluoroacetate) (0.102 g, 99% yield). ^{1}H NMR (CD_3OD): 9.12 (s, 2H), 8.20 (m, 2H), 7.77 (m, 4H), 7.39 (m, 2H), 7.28 (s, 2H), 7.13 (m, 4H), 4.92 (m, 2H), 4.51 (q, 2H), 4.21 (d, 2H), 3.74 (m, 4H). IR (1% KBr, cm^{-1}): 3242, 2931, 1674, 1589, 1500, 1261, 1202, 1029, 722. Anal. ($C_{40}H_{36}N_8O_6$·2TFA) C, H, N.

1,4-Bis[(D-lysyl-glycyl)amino]anthracene-9,10-dione [AGK-11(D)]. Fmoc-D-Lys(Boc)-OH (0.301 g, 0.642 mmol) and 0.100 g (0.284 mmol) of **AG-11** were added to 0.180 g (0.872 mmol) of dicyclohexylcarbodiimide dissolved in 10 mL of anhydrous THF. The mixture was stirred for 15 h at room temperature. Evaporation of the solvent gave the crude product that was purified on silica gel using CH_2Cl_2−ethanol (15:1) as eluant. The fractions containing the product were pooled together, and the solvent was evaporated under vacuum (0.54 g of 1,4-bis[(Fmoc-D-lysyl(Boc)-glycyl)amino]anthracene-9,10-dione, 15% yield).

To remove the protecting groups, 40 μL of piperidine was added to 0.047 g (37 μmol) of 1,4-bis[(Fmoc-D-lysyl(Boc)-glycyl)-amino]anthracene-9,10-dione was suspended in 1 mL of DMF. The solution was stirred at room temperature for 9 min; then 15 mL of diethyl ether was added. The precipitate was centrifuged, washed twice with diethyl ether, treated with trifluoroacetic acid, and dried under vacuum (0.031 g of **AGK-11(D)** as tetrakis(trifluoroacetate), 65% yield). ^{1}H NMR (CD_3-OD) 9.05 (s, 2H), 8.31 (m, 2H), 7.92 (m, 2H), 4.22 (m, 6H), 3.00 (t, 3H), 2.06 (m, 4H), 1.77 (m, 4H), 1.64 (m, 4H). IR (1% KBr, cm^{-1}):3396, 2942, 1676, 1639, 1589, 1504, 1261, 1203, 1026, 723. Anal. ($C_{30}H_{40}N_8O_6$· 4TFA) C, H, N.

1,4-Bis[(D-tryptophanyl-glycyl)amino]anthracene-9,-10-dione [AGW-11(D)]. Boc-D-Trp-OH (0.100 g, 0.329 mmol) and 0.050 g (0.142 mmol) of **AG-11** were added to 0.087 g (0.42 mmol) of dicyclohexylcarbodiimide dissolved in 5 mL of anhydrous THF. The mixture was stirred at room temperature for 15 h and the solvent evaporated under vacuum. The crude product was purified on silica gel eluting with acetonitrile. The fractions containing the product were combined and dried (0.035 g of 1,4-bis[(Boc-D-tryptophanyl-glycyl)amino]-anthracene-9,10-dione, 27% yield).

The protecting groups were removed as reported above affording the product (0.016 g of **AGW-11(D)** as bis(trifluoroacetate), 46% yield). ^{1}H NMR (CD_3OD): 9.12 (s, 2H), 8.20 (m, 2H), 7.77 (m, 4H), 7.39 (m, 2H), 7.28 (s, 2H), 7.13 (m, 4H), 4.92 (m, 2H), 4.51 (q, 2H), 4.21 (d, 2H), 3.74 (m, 4H). IR (1% KBr, cm^{-1}): 3393, 2936, 1673, 1590, 1501, 1260, 1202, 1012, 722. Anal. ($C_{40}H_{36}N_8O_6$· 2TFA) C, H, N.

Spectrophotometric Studies. Fluorescence spectra were recorded on a Perkin-Elmer MPF 66 spectrometer interfaced with a Perkin-Elmer 7500 data processor. The excitation wavelength was chosen corresponding to the isosbestic point observed in absorption spectra. All measurements were performed in a 10 mm path length quartz cuvette. Small amounts (10−40 μL) of a stock solution of compound were added to a concentrated solution of DNA (0.5−2.0 mM). A second solution at the same drug concentration was prepared by diluting the stock solution with buffer alone. By mixing different amounts of these two solutions, different DNA/anthraquinone ratios were obtained at constant anthraquinone concentration. The measurements were carried out at 25 °C in aqueous 10 mM Tris buffer, pH 7.0, containing 1 mM EDTA and known amounts of NaCl to adjust ionic strength to the desired value. For MX and AM, which were not fluorescent, binding was followed spectrophotometrically (Perkin Elmer Lambda 5 apparatus) in the ligand absorption region (500−750 nm). At a drug concentration sufficiently low to avoid self-aggregation phenomena (below 10^{-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 due to experimental errors in extinction coefficients, the range of bound drug fractions was 0.15−0.85. The binding data obtained from fluorometric and spectrophotometric titrations were evaluated according to McGhee and Von Hipppel[51] to obtain *K*, the intrinsic binding constant, and *n*, the exclusion parameter.

Circular and linear dichroism spectra were recorded on a Jasco 500A spectropolarimeter interfaced with a Jasco 501N data processor. Experimental data were analyzed on a Compaq MT 4/66 personal computer; 10 mm path length cells were used, and four to eight scans were recorded for each measurement. Linear dichroism spectra were recorded on the same spectropolarimeter equipped with a cylindrical cell with 1 mm optical path length. Orientation of DNA was achieved by rotating the inner cell wall at 900 rpm.

Cyclic Voltammetry. Cyclic voltammetry (CV) measurements were performed using an Amel scanning potentiostat at the following settings: initial potential, −0.1 V; switching potential, 1 V; scan rate, 0.4 V/s. In CV measurements a hanging mercury drop electrode (HMDE; Metrohm) was used as 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 in 10 mM Tris, 100 mM NaCl, pH 7.0.

The potentials of drugs bound to DNA could not be evaluated due to precipitation of the complexes in the experimental conditions of cyclic voltammetry.

Stimulation of Topoisomerase II-Mediated DNA Cleavage. SV40 DNA fragments were uniquely 5′-end-labeled as already described.[50] DNA cleavage reactions were performed at 37 °C for 20 min in 40 mM Tris-HCl, pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 1 mM ATP, and 15 μg/mL bovine serum albumin, in the presence of 32 topoisomerase II units and increasing (0.1−10 μM) drug concentrations. Reactions were stopped with SDS and proteinase K (1% and 0.1 mg/mL, respectively), and samples were incubated at 42 °C for 45 min. DNAs were then ethanol-precipitated, resuspended in 2.5 μL of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, heated at 95 °C for 2 min, chilled on ice, and then loaded onto a 8% polyacrylamide denaturing gel. Gels were run at 70 W for 2 h. Autoradiograms of dried gels were on Amersham hyperfilms.

Cell Lines and Cytotoxicity Assay. HL60 cells were cultured in RPMI-1640 medium (Flow Laboratories, Irvine, U.K.) plus 10% fetal calf serum (Flow). HeLa and L1210 cells were grown in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY) supplemented with 5% fetal calf serum, antibiotics, and L-glutamine. Drug treatments were carried out for 1 h at 37 °C on exponentially growing cells. In cytotoxic experiments where cell survival was determined by means of the MTT assay,[52] cells were centrifuged after drug treatment, washed, resuspended, and cultured in drug-free medium for 96 h. Drug cytotoxicity determined with the cell-counting method was evaluated after 72 h culture.

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