DNA-Drug Recognition and Effects on Topoisomerase II-mediated Cytotoxicity

A THREE-MODE BINDING MODEL FOR ELLIPTICINE DERIVATIVES*

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Cytotoxic effects and topoisomerase II-mediated DNA breaks induced in vitro by ellipticine derivatives were examined in connection with 1H NMR and circular dichroism (CD) studies on molecular structures and interactions of drugs with DNA. The compounds included four 9-hydroxyellipticines and two 7-hydroxyisoeellipticines derivatives. Structure-activity relationships indicated that a change in nitrogen atom position in the pyridinic ring greatly affected drug effects both on topoisomerase II action and cytotoxicity to L1210 cells. The four 9-hydroxyellipticine derivatives yielded bell-shaped curves in in vitro topoisomerase II-mediated DNA break assays, whereas the two 7-hydroxyisoeellipticine derivatives demonstrated an almost linear increase at the same concentration (0–10 μM). In both cases, the intensity of cleavage was modulated by the position and the degree of methylation on the pyridinic ring, and results were correlated with cytotoxic activity expressed as the in vitro ID50 values for L1210 leukemia cells. 1H NMR experiments performed on free drug molecules in solution revealed that the two protons (α and β) contiguous to the biologically important hydroxyl group were sensitive to changes in electron distribution produced by the distant chemical modifications and methylations of the pyridinic ring. A linear relationship was observed between the differences in chemical shifts of α and β protons (Δρα–β) versus ID50 values. CD experiments indicated that, at weak ionic strength (I = 0.02) and at pH 7, drugs interact with the poly[d(A-T)] duplex according to a “three-mode binding model” which is governed by the drug structure and the drug to DNA ratio. The intercalation mode was related to the induction of topoisomerase II-mediated DNA cleavage, while the external binding mode consecutive to intercalation was related to cleavage suppression. These two modes concerned the good intercalators 9-hydroxyellipticines. The third was found for the weak intercalators 7-hydroxyisoeellipticines and was characterized by self-stacked molecules bound “outside” DNA, presumably in the minor groove. Ligands either could be intercalated partially or linked at the edge of bases with a small number of molecules filling intercalation sites, for the second alternative. In addition to having different binding modes, 9-hydroxyellipticines were better inducers of DNA distortions than 7-hydroxyisoeellipticines.

The incidence of the drug binding modes on DNA-topoisomerase II recognition was discussed in connection with the in vitro cytotoxic activity exhibited by the drugs.

Research into drug action at the molecular level must be concerned primarily with the physical interaction of a drug with its receptor and then with the events which connect this interaction to the pharmacological response. Insofar as DNA can be regarded as a target for anticancer drugs, the study of its interaction with small molecules can be useful for our understanding of the mode of drug action (1–4). Thus, drug-nucleic acid interaction remains of vital interest, especially when considered within a process in which recognition of DNA by enzymes such as DNA topoisomerase II occurs (5, 6).

We are concerned with two fundamental aspects of drug-DNA interactions: the structural basis leading to a preference for either intercalation or “outside” binding and the relationship between these binding modes and the induction or inhibition of DNA-topoisomerase II cleavable complex. The drugs examined in our studies are derivatives of ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) which has been isolated from Ochrosia species (7–9) and has motivated important investigations to determine its antitumor properties (10). The derivative 2-methyl-9-hydroxyellipticinium acetate (NMHE)1 is used in the treatment of breast cancer (11–13), while the derivative 2-diethylaminoethyl-9-hydroxyellipticinium chloride hydrochloride currently is being investigated in a phase I clinical trial (14).

The mode of action of ellipticine derivatives remains unclear. Most of the ellipticines are intercalators, but other binding modes exist as well (15, 16). Structure-activity studies indicate that the hydroxy group of 9-hydroxyellipticine derivatives contributes to stabilize the intercalation complex and thereby improves antitumor activity (16, 17). Also, the 9-hydroxyellipticinium derivatives may interfere with topoisomerase II activity by stabilizing the enzyme-DNA cleavable complex (15, 18–23). The introduction or deletion of a methyl group in the ellipticine molecule also produces noticeable effects. For instance, quaternization with a methyl group at

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1 The abbreviations used are: NMHE, 2-methyl-9-hydroxyellipticinium; HE, 9-hydroxyellipticine; 1-Me-NMHE, 1,2-dimethyl-9-hydroxyellipticinium; 1-Me-isoHE, 1-methyl-7-hydroxyisoeellipticine; 1-Me-isoNMHE, 1,2-dimethyl-7-hydroxyisoeellipticinium; 1-Me-HE, 1-methyl-9-hydroxyellipticine; poly[d(A-T)]; poly[d(A-dT)]; poly[d(A-T)]; COSY, two-dimensional correlated NMR spectroscopy; NOESY, two-dimensional Overhauser effect spectroscopy.
the N-2 position increases the water solubility but, in actual fact, decreases antitumor activity (24), while the introduction of a methyl group at the N-6 position or at position 1 increases antitumor activity. In contrast, deletion of the methyl at position 5 or 11 leads to a weakly active compound (25) while the shift of the methyl group from position 11 to position 1 has little incidence on antitumor activity (26).

We focused our interest on the molecular background of the binding modes related to cytotoxic activity and the ability to induce topoisomerase II-mediated DNA breaks of selected methylated derivatives of 9-hydroxyellipticine and of its isomer 7-hydroxyisoellipticine (Fig. 1). In this paper, we report our results on (i) the electronic properties of the selected ellipticines deduced from the chemical shifts of aromatic protons in NMR spectra, (ii) the binding of drugs to the poly[d(A-T)] duplex analyzed by UV absorption and UV circular dichroism, (iii) the cytotoxic activities determined from in vitro growth inhibition of L1210 cells, and (iv) the ability of these intercalating drugs to interfere with topoisomerase II-mediated DNA effects in DNA gel electrophoresis experiments.

**EXPERIMENTAL PROCEDURES**

**Chemistry**

9-Hydroxyellipticine (HE) and 2-methyl-9-hydroxyellipticinium acetate (NMHE) were synthesized according to previously described procedures (10, 27). 1-Methyl-9-hydroxyellipticine (1-Me-HE) and 1,2-dimethyl-9-hydroxyellipticinium acetate (1-Me-NMHE) were provided by the SANOFI Co. (Toulouse, France). A detailed synthesis of 1-methyl-7-hydroxyisoellipticine (1-Me-isoHE) and of 1,2-dimethyl-7-hydroxyisoellipticinium acetate (1-Me-isoNMHE) will be given elsewhere. It must not be overlooked that 9-hydroxyellipticine and derivatives are members of the 6H-pyrrolo[3,4-b]carbazole series, whereas 7-hydroxyisoellipticine and derivatives belong to the 10H-pyrrolo[3,4-b]carbazole series. Therefore, the numbering of the ring systems 1 and 2 are different as shown in Fig. 1. Thus, positions 8, 9, and 10 in 1 correspond to positions 8, 7, and 6 in 2, respectively. For simplification and to avoid any confusion the two protons flanking the hydroxyl group in both series (protons 8 and 10 for ellipticine and protons 8 and 6 for isoellipticine) will be referred to as α and β protons, respectively, throughout this work.

**In Vitro Cytotoxicity Assay**

Exponentially growing L1210 murine leukemia cells, seeded at 5 × 10⁵ cells/ml of RPMI 1640 medium supplemented with 10% fetal calf serum were incubated in 5% C0₂ for 72 h at 37°C with serial concentrations of the compounds to be tested dissolved in water. Poorly water-soluble ellipticine derivatives were solubilized by HCl additions to a final pH 5. Stock solutions were prepared so that a maximum of 100 μl were added to 10 ml of growth medium. The total number of cells in the different cultures was determined with a Coulter Counter. The average number of cells in each duplicate treated culture was expressed as a percentage of the average number of cells in the triplicate untreated controls. The ID₅₀ value, defined as the concentration of drug that induces a 50% inhibition of cell growth, was obtained by plotting the percentage of surviving cells versus logarithm of the drug concentration and was determined by linear regression.

**Spectroscopy**

1H NMR—Ellipticine derivatives were dissolved in 99.996% D₂O (from CEA, France) and pH of solutions was adjusted to 3 with concentrated DCl (6 N). In these conditions, all samples had the same charged cationic form. The solutions were lyophilized twice and samples were made up of 0.4 ml with D₂O to obtain a final concentration of 3.3 mM. 1H NMR spectra were recorded at 60 °C on a Bruker MSL-300 spectrometer. COSY and NOESY spectra were needed in some cases to achieve the full assignment of aromatic protons. The temperature of 60 °C was chosen in order to disrupt aggregates and thus avoid intermolecular shielding effects on the proton chemical shifts (28).

UV Absorption and Circular Dichroism—Absorption spectra were recorded on an Ulvikom 860 spectrophotometer, and a Jobin-Yvon Mark IV high sensitivity dichrograph linked to a Minc digital 11 miniprocessor was used to measure the CD spectra. The poly[d(A-T)] duplex form was purchased from Boehringer Mannheim. Samples were prepared in a phosphate buffer, pH 7, at a weak ionic strength (I = 0.02) in the presence of 0.2 mM EDTA. Proportions of drug concentrations were determined spectrophotometrically by using ε₂60 = 7600 M⁻¹ (base)-cm⁻¹. Measurements were performed using quartz cells of 1 cm in path length containing 2.5 ml of poly[d(A-T)] at 65 μM concentration. For titrations small aliquots of 2 mM aqueous drug solutions were added incrementally. UV-absorption and CD spectra were recorded at 20°C.

**DNA Topoisomerase II-mediated DNA Breaks**

DNA topoisomerase II was purified from calf thymus according to previously published procedures (29, 30). Circular pBR322 DNA was purified from Escherichia coli as described by Maniatis et al. (31). The linear form was obtained by cleavage with EcoRI, followed by three phenol extractions and dialysis. The DNA topoisomerase II cleavage reaction mixture included the following: the DNA topoisomerase II enzyme (13.4 μg/ml) and circular DNA (13 μg/ml) in 100 mM KC1, 10 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 30 μg/ml bovine serum albumin, and 40 mM Tris-HCl, pH 8. The reaction tubes were incubated with 1 mM ATP in a total volume of 15 μl for 30 min at 37°C in the presence of the tested drugs at concentrations ranging from 0.3 × 10⁻⁴ to 50 × 10⁻⁶ M. Sodium dodecyl sulfate and proteinase K were added to a final concentration of 0.35% and 88 μg/ml, respectively, and the mixtures incubated for another 30 min at 50°C. A loading buffer containing 0.05% bromphenol blue, 50 mM EDTA, and 50% sucrose was added, and finally DNA was analyzed by electrophoresis at 2.5 V/cm for 16 h at room temperature in a 4.2% polyacrylamide gel in a buffer containing 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA. Gels were then stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination. The negatives were scanned and the peak areas measured with a Joyce-Loebl Chromoscan 3 (Gateshead, England). Cleavages by EcoRI and those induced by epipodophyllotoxin (VP-16, at M) were performed under the same conditions as controls.

**RESULTS**

1H NMR—The way in which electron density is affected by substituents in the aromatic molecules is important for the evaluation of the molecule's reactivity. 1H chemical shifts then depend on the distribution of electron density near the proton nuclei and may provide information on bond hybridization, bond dipole moments, and susceptibilities as well as electronic energies.

Typical 1D NMR spectra of ellipticine derivatives in D₂O are shown in Fig. 2. The assignment of aromatic resonances was achieved by an analysis of corresponding COSY and NOESY spectra. Values of the measured chemical shifts are
**9-HYDROXYElliPTICINES**

![Fig. 2](image)

**TABLE I**

| Compound | 6_α | 6_β | Δ6_α-β | ID_{so} |
|----------|-----|-----|---------|--------|
| 1-Me-HE  | 6.78| 7.03| -0.25   | 0.001  |
| HE       | 6.70| 6.84| -0.14   | 0.003  |
| 1-Me-NMHE| 6.82| 6.97| -0.15   | 0.011  |
| NMHE     | 6.78| 6.81| -0.03   | 0.021  |
| 1-Me-isoHE| 6.75| 6.72| +0.03   | 1      |
| 1-Me-isoNMHE| 6.85| 6.72| +0.15   | 1      |

Chemical shifts, 6, of α and β protons, differences of chemical shifts, Δ6_α-β, and cytotoxic activities determined from L1210 murine leukemia cell growth inhibition in culture, for several hydroxyellipticine derivatives.

See Fig. 1 for assignment of α and β protons. The experiment was performed in H2O, pH 3, 60 °C. Values were given in ppm from trimethylsilylpropionate. ID_{so} is the drug concentration in micromolar inducing 50% cell growth inhibition (72 h).

reported in Table I, together with the differences Δ6_α-β associated with chemical shifts of the α and β protons flanking the hydroxy group (for the localization of α and β see Fig. 1) and the cytotoxic activities. In the chosen experimental conditions (acidic pH and 60 °C) these proton nuclei can be regarded as good probes of the intramolecular properties of ellipticine derivatives because intermolecular stacking and edge-to-edge interaction effects (which strongly affect the chemical shifts) are disrupted (28). Thus in the hydroxyellipticine derivatives the variations of chemical shifts of the α and β protons due to long distance substitutions and modifications on the pyridinic ring could be related to intrinsic molecular effects affecting the electronic properties of the hydroxy group. As expected the difference Δ6_α-β is not constant in the two series. A remarkable feature is that methylation on position 1 decreases by ~0.1 ppm the Δ6_α-β value while methylation on position 2 induces exactly the opposite effect.
UV Absorption and Circular Dichroism—Figs. 3 and 4 show the two families of UV-absorption and CD spectra that resulted when poly[d(A-T)] was titrated with 1-Me-HE and 1-Me-isoNMHE. These two compounds were selected as representative of the 9-hydroxyellipticines and 7-hydroxyisoellipticines, respectively. CD spectra of drug-free poly[d(A-T)] at pH 7, I = 0.02, are those expected for B-DNAs. With drug addition, new signals are generated both in UV absorption and CD at wavelengths above 290 nm, and more pronounced changes are observed in the overall poly[d(A-T)] spectrum. The induced CD signals are due to the drug-bound complexes since the 9-hydroxyellipticine and 7-hydroxyisoellipticine derivatives are achiral molecules; optical activity is generated in the asymmetric environment of the DNA double helix. Such effects are observed with numerous planar and achiral dye molecules like methylene blue (33), acridine, and its derivatives (34). The signals are multishaped and multisigned and vary with the intercalator, the buffer, and the DNA or polynucleotide investigated (35-40), indicating that the so-called intercalators may interact with DNA according to multiple geometries and binding modes.

The diversity of the spectra yielded by the titration of poly[d(A-T)] with ellipticine derivatives reflects the influence of both the chemical factor and drug to DNA ratio on the binding process in that series.
In a first step, at a low drug to DNA ratio ($r' < 0.12$), the induced CD spectra of 1-Me-HE bound to poly[d(A-T)] consist in single positive bands centered at 328 nm, e.g. approximately where the red-shifted UV-absorption band (compared to the UV-absorption band of the free molecule) of 1-Me-HE bound to the polynucleotide contributes ($\lambda = 325$ nm) (Fig. 3, A and B). In the same conditions, the CD spectra of 1-Me-isoNMHE bound to poly[d(A-T)] display a bisignate pattern characterized by a disymmetrical positive signal (centered at 310 nm), a negative signal (at 294 nm), and a changing sign (at 303 nm) (Fig. 4B). The disymmetry of the positive signal is caused by a weak underlying signal contributing on its right-hand side. In the corresponding UV-absorption spectra the weakly red-shifted band of 1-Me-isoNMHE bound to poly[d(A-T)] contributes at 303 nm, too (Fig. 4A). Excitonic spectra generally are characterized by such properties (41, 42). Here again the broadness of the UV absorption band also hints at the presence of a second underlying signal.

In a second step, when the ligand to polynucleotide ratio is raised to a value above 0.12 for 1-Me-HE bound to poly[d(A-T)], an additional negative CD peak is then observed at 310 nm approximately at the point where the slightly shifted UV-absorption band of 1-Me-HE bound to the polynucleotide contributes while the positive band formerly located at 328 nm apparently has both decreased in intensity and shifted to 338 nm (Fig. 3C). The latter peak is not found in the corresponding UV-absorption spectrum (Fig. 3A), whereas the single peak, which at low ligand concentration contributed to 325 nm, persists as a shoulder on the major signal newly formed at 310 nm. The confrontation of the CD and UV spectra allows us to conclude that the peak observed at 338 nm in the CD spectrum results from the combination of the opposed signals, negative at 310 nm and positive at 328 nm. In the same conditions, e.g. from $r' = 0.12$ to $r' = 0.36$ the bisignate CD spectrum of 1-Me-isoNMHE bound to the polynucleotide exhibits a symmetric increase in its components although no concomitant wavelength change can be observed (Fig. 4B). At $r' = 0.36$, the positive and negative components display almost the same $\Delta \epsilon$ absolute value (of about 6), a feature which confirms the excitonic nature of the spectrum generated by 1-Me-isoNMHE.

**Cytotoxic Activity**—The cytotoxic effects of the selected ellipticine derivatives were tested on the proliferation of L1210 murine leukemia cells. Results expressed as ID$_{50}$ (72 h) are given in Table 1. The most potant compounds were in the 9-hydroxyellipticine series with ID$_{50}$ values ranging from $10^{-7}$ to $10^{-8}$ M. In comparison, the two 7-hydroxyisoeellipticines were characterized by remarkably low activities with an ID$_{50}$ of $\sim 10^{-7}$ and $\sim 10^{-8}$ M for 1-Me-isoHE and 1-Me-isoNMHE, respectively.

The following factors are therefore important for the manifestation of cytotoxic activity: (i) the position of the pyridinic nitrogen atom: 1-Me-HE is, for instance, ~100 times more active than its counterpart 1-Me-isoHE, and (ii) the position of the methyl group: comparatively to HE, the activity of NMHE (methylation on position 2) is 10 times lower, while that of 1-Me-HE (methylation in position 1) is ~3 times higher. Thus, among the positions submitted to methylation, it is position 1 which most increases the cytotoxic potency of 9-hydroxyellipticines.

**Topoisomerase II**—The interaction of 9-hydroxyellipticine and 7-hydroxyisoeellipticine derivatives with DNA topoisomerase II was studied by the enzyme-mediated DNA cleavage assay using circular pBR322 DNA as the substrate and calf thymus DNA topoisomerase II.

Cleavage was revealed by the addition of sodium dodecyl sulfate followed by proteinase K digestion and polyacrylamide gel electrophoresis. In this gel system, linear DNA generated by double strand cleavage of the circular form was identified clearly as a fast migrating band. Cleavage of dimeric molecules produces a small amount of linear dimers which appear in the gel as a slower band. As shown in Fig. 5A, a substantial amount of cleavage occurs in the presence of VP-16 and NMHE, whereas no detectable cleavage was observed in the absence of a drug. Fig. 5, B and C, also shows the drug-induced DNA cleavage obtained in the presence of 1-Me-HE and 1-Me-isoNMHE at various concentrations. Similar experiments were carried out with the other 9-hydroxyellipticine and 7-hydroxyisoeellipticine derivatives (not shown), and the extent of cleavage was determined and plotted in Fig. 6 against drug concentration. Two distinct types of behavior were observed: with the 7-hydroxyisoeellipticine derivatives, cleavage increases more or less steadily as a function of the drug concentration to decrease finally at high concentration (20–30 $\mu$M) for 1-Me-isoHE, whereas typical biphasic curves are already observed at low concentrations (10–20 $\mu$M) with the 9-hydroxyellipticine derivatives, in agreement with previous observations (5, 18–23).

![Fig. 5. Stimulation by ellipticine derivatives of topoisomerase II-mediated DNA cleavage.](image)

**Fig. 6. Topoisomerase II-induced DNA cleavage by ellipticine derivatives.** See "Experimental Procedures" for details. O, HE; $\Delta$, 1-Me-HE; O, NMHE; $\n$, 1-Me-NMHE; A, 1-Me-isoHE; $\n$, 1-Me-isoNMHE.
DISCUSSION

In order to establish a relationship between chemical features and biological activity, we have to identify, first, those electronic and structural properties that appear to determine the biological activity of the drug. Proton chemical shifts are very sensitive to changes in electron density distribution and are currently used as probes of molecule reactivity. The difference $\Delta \delta_{\alpha-\beta}$ was selected as a physicochemical parameter in the structure-activity relationships for hydroxyellipticines, because the $\alpha$ and $\beta$ protons flank the hydroxy group, which is essential for the biological activity of these compounds (16, 17). We demonstrated that chemical changes performed on the drug pyridinic ring promoted appreciable variations in the $\Delta \delta_{\alpha-\beta}$ parameter so that the $9\text{-hydroxyellipticine and 7\text{-hydroxyellipticine derivatives could be separated into two distinct groups characterized by negative and positive values, respectively, with a rank order similar to that found for cytotoxic activities.}$

Second, the structural and electronic properties of drug molecules may reverberate in a variety of different noncovalent interactions such as hydrogen bondings and van der Waal’s attractions, responsible for the diverse binding modes of anticancer drugs to DNAs. This aspect is relevant for our UV absorption and CD analysis on the hydroxyellipticine derivatives bound to poly[d(A-T)]. The observed appearance of induced signals at high wavelengths, subsequent to drug addition to poly[d(A-T)] is definitive evidence of the interaction of these two components. More particularly, the positive CD signal at 328 nm induced by 1-Me-HE at $r' < 0.12$ (Fig. 3B) reflects chromophore intercalation into base pairs. This is inferred from data reported by R. Lyng et al. (33) stipulating a positive Cotton effect for intercalation of methylene blue in poly[d(A-T)] with the long-axis of the chromophore oriented roughly parallel to the dyad axis of the DNA helix (see below) (43, 44). In the same conditions, 1-Me-isoNMHE induces a bisignate spectrum of the excitonic type (Fig. 4B). The respective position of the exciton components in the spectra may be interpreted according to a right-handed arrangement of 1-Me-isoNMHE molecules "outside" DNA (outside denotes here the binding of ligands either at the edge of bases or slightly inserted into base pairs); this allows interactions between close lying molecules along the DNA helix (33, 45, 46). Alternatively when molecules are oriented at the edge of bases, the intercalation of a few drug molecules must occur from the very beginning of drug addition to explain the presence of a weak corresponding signal in UV absorption and CD at about 320 nm (Fig. 4, A and B).

Another important point is that the binding of 1-Me-HE and 1-Me-isoNMHE to poly[d(A-T)] is not submitted to the same concentration dependence. For 1-Me-HE, at high drug to DNA ratio, an external binding mode follows intercalation as revealed by the newly formed CD or UV absorption signal at 310 nm (Fig. 3, C and A). The concomitant modification of the DNA spectrum below 290 nm suggests that important conformational changes in the polynucleotide facilitate or accompany this second binding step. For the binding of 1-Me-isoNMHE at a high drug to DNA ratio, the changes observed in the induced excitonic spectra (Fig. 4, A and B) are only quantitative. This allows us to conclude that 1-Me-isoNMHE still participates in a continuous and major outside binding mode, similar to that found at low concentrations. However, the minor intercalation may increase with the drug to DNA ratio.

To what extent are our results with poly[d(A-T)] representative of DNA-drug interactions? Poly[d(A-T)] has the ability to adopt a B-form in solution over a wide range of experimental conditions (47, 48). It has also been shown that drugs such as propidium interact with poly[d(A-T)] in a manner quite similar to random-sequence DNA (49). Moreover, for the binding of ellipticine and itsaza analog 6-(2-dimethylaminooethyl)-6H-indolo[2,3-b]-quinoxaline to poly[d(A-T)], poly[d(G-C)], and natural DNA no significant variations in binding affinities were observed between the three types of nucleic acids, and the overall binding constant remained slightly larger for the ellipticine toward the three nucleic acids (50). The same conclusions can be drawn for the binding affinities of the amsacrine derivatives AMSA, o-AMSA, and $m$-AMSA toward the above three nucleic acids (51, 52). Finally the present results remarkably fit our findings on the DNA-unwinding properties of hydroxyellipticines obtained in a topoisomerase I assay using a circular DNA. These confer a helix unwinding of 17° to 1-Me-HE and 11° to 1-Me-isoNMHE bound to DNA, values which reflect therefore the better intercalator properties of the former molecule compared to the second in assays performed with natural DNAs.$^2$

Thus, it was established throughout this work that the chemical modifications introduced in the pyridinic ring of hydroxyellipticine derivatives affect their electronic properties in the area of the hydroxy group, and these variations reverberate consecutively on their binding properties toward DNA. The key question then is whether a link exists between the physicochemical properties and the pharmacological action of hydroxyellipticines.

**Correlations between Cytotoxicity and Physicochemical Parameters**—Good correlations can be established when drug cytotoxic activity (ID$_{50}$ values) is plotted against selected NMR and CD parameters, e.g., $\Delta \delta_{\alpha-\beta}$ measured in the free drug molecules (Fig. 7A) and $\Delta \varepsilon$ at 328 nm induced by the

\[ \text{Correlations between Cytotoxicity and Physicochemical Parameters} \]

$^2$ Unpublished observations.
DNA-bound molecules (Fig. 7B). The linear relationships which emerge indicate that the electronic density and charge distribution surrounding the hydroxy group is the main factor mediating the binding of hydroxyllectine derivatives to DNA and subsequently the cytotoxicity observed in L1210 leukemia cells (24). A corollary is that the methyl substituents attached to the pyridinic ring do not exert a steric influence on drug-DNA complex formation. In fact, when the methyl group in position 1 or 2 of 9-hydroxyllectine derivatives is replaced by the larger ethyl group the results still fit the two correlations well (data not shown).

The behavior of the hydroxy group in some hydroxyllectine derivatives has been examined already (24, 25). Compounds deeply modified on their pyridinic ring such as the 1,2-dihydro-9-hydroxyllectine and the 1,2,3,4-tetrahydro-9-hydroxyllectine derivatives fail to generate oxiradicals from their hydroxy group and thus avoid damaging DNA compared to the lead 9-hydroxyllectine (24). This inability has been used to explain the lack of activity of such compounds toward L1210 leukemia cells (24, 53). However, since then it has been shown that DNA-intercalators such as hydroxyllectine derivatives can induce DNA strand breaks at lower concentrations than those required for oxygen radical damage, suggesting that a DNA topoisomerase enzyme might be involved in the cytotoxicity process (53–55).

**Correlations between Drug Effects on Topoisomerase II Cleavage and Physicochemical Parameters**—The mechanism by which DNA-intercalators interfere with the topoisomerase II-mediated DNA cleavable complex formation and then with DNA breaks is not yet clearly understood. For many authors, it could be the main route leading to cell death for intercalating agents (6, 56, 57). Although the relationship between cytotoxic activity and NMR and CD parameters is straightforward for hydroxyllectine derivatives, as seen above, the existence of a correlation between these parameters and topoisomerase II-mediated DNA breaks seems less evident at first glance. However, the existence of two families is manifest; on the one hand, the highly cytotoxic 9-hydroxyllectine derivatives defined as good intercalators and characterized by typical bell-shaped curves in DNA break assays, and on the other hand, the weak or noncytotoxic 7-hydroxyisoellipticine derivatives which are poor intercalators and which display almost linear dose-response curves within a wide drug concentration range (Fig. 6). Note the particular behavior of the 1-Me-isoHE derivative which induces a large amount of DNA breaks while its cytotoxicity toward L1210 leukemia cells is weak (Table I).

The above results suggest that the 9-hydroxyllectine derivatives produce two types of antagonistic effects: induction and inhibition of the topoisomerase II-DNA cleavable complex. The two effects start simultaneously, right at the beginning of the drug-DNA interaction. The resulting dose-response curves are thus composite and consequently cannot afford any precise quantitative information about the induction and inhibition effects of drugs (see "Models and Biological Effects"). Several anthracyclines and other elliphticine derivatives are leading to visible double effects (bell-shaped curves) (59–62). On the other hand, some drugs (generally those with low DNA affinities) induce DNA cleavage in a wide range of drug concentrations without apparent contradictory inhibition effects. Known examples are the two anthraquinones 5-iminodaunorubicin and 4-dimethyl-6-O-methyldoxorubicin (58) together with several amsacresins (63–66). The weak intercalator, 1-Me-isoHE belongs to this second category. There are drugs, such as 9-aminoacridine and its o-AMSA derivative (63) and 1-Me-isoNMHE in this analysis, which either induce, weakly but increasingly, the cleavable complex or others which are unable to generate the cleavable complex such as ethidium bromide (59), anthracycline derivatives (60), ditercalinium (21), and amiloride (67). The three latter compounds can however intercalate into DNA and inhibit the catalytic activity of topoisomerase II.

Despite the complexity of the dose-response curves in topoisomerase II assays, the objective of the present work, which was to provide direct evidence that hydroxyllectine derivatives activity proceeds by disturbing the DNA-topoisomerase system, is well satisfied by the experimental results. Previous reports have described a good correlation between cytotoxicity and DNA cleavage for anthracycline derivatives (58), amsacrine congeners (65), and epipodophyllotoxins derivatives as well (19, 68). Such a simple correlation does not appear with hydroxyllectine, although a careful examination of the dose response at 1 µM drug concentration suggests that the most cytotoxic derivative (1-Me-HE) is also the one showing substantial cleavage whereas, in contrast, the weaker cytotoxic derivatives (1-Me-isoHE and 1-Me-isoNMHE) display lower cleavage properties.

**Possible Models for the Binding of Ellipticine Derivatives to DNA**—All together the evidence accumulated in the present work indicates that the binding of 9-hydroxyllectine and 7-hydroxyisoellipticine derivatives to poly[d(A-T)] fits in with a "three mode binding model" similar to the one previously suggested for porphyrins by Fiel and co-workers (69–71).

The first model, as outlined above, deals with an intercalative mode and is illustrated by the binding of 1-Me-HE at a low drug to DNA ratio (r' < 0.12) to poly[d(A-T)] (Fig. 8A). This concerns the other 9-hydroxyellipticine derivatives, as well. The chromophore is inserted completely in base pairs and is oriented with its long axis parallel to the dyad axis (α = 0), the essential hydroxy group protruding in the major groove. This type of intercalation has been shown by theoretical and spectroscopic means for methylene blue (33) and also daunomycin (72–75) and confirmed by x-ray studies for daunomycin and adriamycin in crystals of oligonucleotide-drug complexes (43, 44). According to the nearest-neighbor exclusion principle which seems to apply to most good intercalators (76), only about half of all potential sites are filled by drug molecules. These are spatially too distant from each other to yield drug-drug excitonic spectra and effectively a single positive signal is induced in CD in that case (Fig. 3B).

The second model illustrates the external binding of the same 1-Me-HE molecule to the polynucleotide which occurs during the saturation of the intercalative sites, at a high drug to DNA ratio (r’ > 0.12) (Fig. 8B). It is not known whether the drugs are located at the DNA surface, although it is assumed that a large proportion of these may be distributed within the major groove (see "Models and Biological Effects"). Any group belonging to the backbone, bases, or sugars can participate in interactions with drugs as illustrated tentatively in Fig. 8B.

In the third model the drug molecules interact with DNA according to an "outside" binding mode (Fig. 8C). This mode is illustrated by 1-Me-isoNMHE and becomes dominant immediately at a low drug to DNA ratio. Nonintercalated or partly intercalated chromophores may interact with each other along DNA because, in that case, intervals between drug molecules are similar to intervals found between base pairs in DNA with a native B-type conformation. This explains the excitonic nature of the corresponding induced CD peaks (Fig. 4B). The self-stacking arrangement presumably occurs in the minor groove, although there is no spectroscopic evidence for this location (see below).
DNA-Ellipticine Recognition Effects

Models and Biological Effects—The question which then arises is to what extent the topoisomerase II-mediated DNA cleavage patterns can be explained by the above models and whether these are relevant to biological effects?

In the intercalation model (Fig. 8A), the drug is buried within base pairs, and enzyme access to DNA is not hampered. Close contacts between topoisomerase and the DNA backbone can be facilitated further by local and medium range distortions induced by the intercalated drugs (76, 77). The changes occurring below 290 nm in the CD spectra of poly[d(A-T)] upon complexation with 1-Me-HE at a low drug to DNA ratio illustrate such distortion effects (Fig. 3B). The stabilization of the DNA-topoisomerase II cleavable complex can then result either from the stiffening of DNA in the surroundings of the intercalation site or from the interaction of the intercalator via, for instance, its hydroxy group with any of the two partners of the DNA-topoisomerase complex as suggested for daunomycin on the basis of x-ray studies (43). The differences in properties between ellipticines and hydroxyellipticines have been explained by the latter hypothesis (24).

If intercalation appears advantageous for the stabilization of DNA-topoisomerase II cleavable complex, external binding shown in model II (Fig. 8B) may in contrast result in suppression of DNA cleavage. It is conceivable in that case that DNA recognition by the enzyme is hampered either by the shell of the drugs surrounding DNA or by the deeper and deeper distortions occasioned in DNA by the increasing number of intercalated drugs. Some drug molecules could also bind to the cleavage site on DNA and interfere with the enzyme action for DNA-backbone hydrolysis.

According to model III (Fig. 8C), the stacked drug molecules accommodate an “outside” mode of binding along DNA. If this arrangement takes place in the minor groove, it may be of interest from a dual point of view: first, it can either permit partial intercalation or promote intercalation of a limited number of molecules because, according to previous reports, intercalation occurs from the minor groove (43, 44); second, it does not disturb DNA recognition by topoisomerase II which, as illustrated by studies with other DNA-binding proteins, mostly concerns the major groove (76, 78). This model holds for 1-Me-isoNMHE. It is the partial intercalation or the intercalation of a limited number of molecules and the consecutive occupation of external sites, as in model II, which are responsible for the induction and then the suppression of the topoisomerase II-DNA complex.

Thus the diversity of the dose-response curves yielded by the ellipticine derivatives could be an expression of the variety of balances governing induction and suppression of the cleavable complex, balances which themselves depend on the relative occupation of intercalative sites and external sites.

As concerns the origin of the cytotoxicity demonstrated by the ellipticine derivatives in this study, it can be emphasized that only those compounds which exhibit marked induction and inhibition of the cleavable complex may have a good activity. Such is the case of 9-hydroxyellipticines which are all good intercalators at low concentration and external binders at higher concentration. Moreover, these compounds induce strong distortions in the DNA conformation which, in addition, may alter replication or transcription processes by disturbing DNA recognition by DNA and RNA polymerases (3, 79). For 7-hydroxyisoellipticines the preponderance of self-stacking along DNA is further responsible for the slow increase of external binding on intercalation ratio and therefore for the low cytotoxic activity exhibited by these compounds. In addition 7-hydroxyisoellipticines as opposed to 9-hydroxyellipticines only alter slightly the DNA conformation, which

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Fig. 8. A “three mode binding model” for ellipticine derivatives bound to poly[d(A-T)]. A, intercalative model for 1-Me-HE (at low drug to DNA ratio); B, external binding model for 1-Me-HE (at high drug to DNA ratio); C, self-stacking model for 1-Me-isoNMHE (at any concentration). See text for details.
supports the idea of a strong influence of structural effects on lethal events.

That a correlation between cytotoxicity and physicochemical parameters exists is not obvious! For instance previous studies on the acridine derivatives AMSA, o-AMSA, and m-AMSA found no correlation, either between DNA intercalation (80) or between DNA binding affinity (51). In particular the failure to detect any difference between the intercalation parameters of m-AMSA and o-AMSA (the former being highly active against tumor cells whereas the latter is essentially inactive) shows that other factors may play an important role in determining activity in vivo. The overall patterns of structure-activity relationships for such compounds and probably also for anthracyclines therefore might be a composite of metabolic factors, membrane interactions, and structural recognition effects on DNA. Yet, another important aspect is that unlike the ellipticines used in this work which are relatively simple polyaromatic derivatives, compounds such as amsacrine or anthracyclines bear large functional side-chains on their intercalating chromophores. Such side-chains may interfere both in the positioning of the chromophore within its intercalative site and in the drug-DNA binding affinity through additional interactions (or repulsions) in the DNA grooves, as well documented in the case of daunomycin and aclacinomycin molecules (43, 44). In any event it is gratifying to note that these variations, sometimes very subtle, can have strong implications for biological activity especially through their implication in the DNA-topoisomerase II-drug complex stability.

CONCLUSION

Most of the anticancer drugs are supposed to induce their effects at the DNA level. Fundamentally, effects are conveyed according to the size and shape of molecules and depend on the electron density distribution on atoms and bond polarizabilities within the potential partner molecules. In a closely related series of drug molecules, large differences can, however, be observed in the intensities of their pharmacological effects. These may be explained by different interactions displayed by the molecules with DNA and, finally, by subtle electronic differences characterizing the molecules.

The influence of intercalation on the topoisomerase II-mediated effects appears evident. This may occur either via the distortions occasioned in the DNA molecules since a good relation seems to exist between intercalation and DNA deformation or via the occupation of external sites which hinders DNA topoisomerase recognition. However, enzymes such as DNA polymerase or RNA polymerase can also be inhibited either in their rate of progression along the DNA template (3) or by termination effects at individual drug sites (79) with, in both cases, subsequent alteration of replication or transcription.

Finally, such an agreement found for all these correlations gives credence to the internal consistency of the approaches employed. It is vital to underscore the usefulness of CD and NMR parameters in testing the biological parameters of pharmacological molecules. The results of such studies could provide a valuable basis for the investigation of the models and above all the elaboration of novel anticancer compounds.

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