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The Biooxidation of Cytotoxic Ellipticine Derivatives: A Key to Structure-Activity Relationship Studies?

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SUMMARY

In the family of ellipticine derivatives, those with an amino-phenol or a masked amino-phenol structure are among the most cytotoxic compounds. Preliminary studies on 9-hydroxy- or 9-methoxyellipticines have shown that these molecules behave as "pro-alkylating" agents. In order to rationalize the "biooxidative alkylation" process for various ellipticine derivatives, we report in the present article (i) their electrochemical oxidation parameters, (ii) their biochemical oxidation, (iii) the ability of the oxidized forms to form adducts with nucleophiles, (iv) the biological activities, and (v) the electronic properties of oxidized forms. We present some possible correlations between the oxidizability, the electrophilicity of the oxidized derivatives, and the biological activities of the corresponding drugs.

The rational drug design of antitumor agents is very often a distant goal for many different reasons. This situation is mainly due to the current poor knowledge concerning molecular aspects of the selective toxicity of antitumor agents on normal and neoplastic cells and the exact biological role of active forms generated outside and/or inside cells. For the family of ellipticine derivatives, the first point is still an open field for biochemical research, but the results in the treatment of human cancers by one molecule of the family, elliptinium acetate (1–4), testify to the selectivity of the drug. On the second aspect, very little was done until the recent reports on the easy oxidative transformation of hydroxyellipticines by a peroxidase in the presence of hydrogen peroxide (5, 6). The enzyme used was the well known horseradish peroxidase (7) which is an efficient system for the *in vitro* modeling of oxidative transformation of various exogeneous molecules: chlorpromazine (8), aminopyrine (9), tetramethylhydrazine (10), *p*-aminophenol (11), indole-3-acetic acid (12), tetramethylbenzidine (13), acetaminophen (14, 15), phenetidine (16), nitroalkanes (17), methoxyellipticines (18), and vindoline (19). The role of metabolic activation in cellular toxicity of drugs and its understanding at a molecular level are one of the keys to a better knowledge of the mechanism of action of biologically active molecules (for a recent review, see Ref. 20).

In the case of elliptinium acetate **11**, 9-OH-NME (Fig. 1), we have previously described its oxidation to an electrophilic quinone-imine that reacts with various models of biological nucleophiles: thiols (21), amino acids (22, 23) and ribonucleosides (24, 25). The *a priori* preparation of the possible thiol conjugates of elliptinium have made easier the identification of these compounds as minor metabolites in animal (26) or human (27) bile. Furthermore, we have shown that the oxidized form of 9-OH-NME is able to bind covalently to RNA (28) and DNA (29) *in vitro* or to these same macromolecules after incubation of L1210 mouse leukemia cells with 9-OH-NME (30). In addition, a recent preliminary report indicates that such a "biooxidative alkylation" process might be responsible for the antitumor activity of elliptinium acetate (31): pretreatment of mice with *N*-acetylcysteine, known for detoxification of electrophiles, completely suppressed the drug activity.

Among the ellipticine derivatives, 9-methoxyellipticine is also active in cancer therapy (32) and can be oxidized *in vitro* (18) or *O-demethylated in vitro* (33). From all these data, one can consider 9-OH-NME and 9-methoxyellipticine derivatives as potential alkylating ("pro-alkylating") agents for which a biooxidation step is required. A possible *in vivo* activation scheme for these molecules is presented in Scheme 1. Five different important steps of the drug biotransformation are described. The quantitative evaluation of them is essential if we wish to rationalize and tentatively predict the biological activities of various molecules in the ellipticine series.

Several questions arise on the first two steps: *Step A:* Does

ABBREVIATIONS: 9-OH-NME, 9-hydroxy-*N*-methoxyellipticinium; HRP, horseradish peroxidase; CNDO, complete neglect of differential overlap; HPLC, high pressure liquid chromatography; 9-OH-N^6-Me-E, 9-hydroxy-N^6-methylellipticinium chloride; 7-OH-NME, 7-hydroxy-N^2-methylellipticinium; *m*-AMSA, 4′-(9-acridinylamino)methane-sulfon-M-anisidide, *o*-AMSA, 4′-(9-acridinylamino)methane-sulfon-*o*-anisidide.

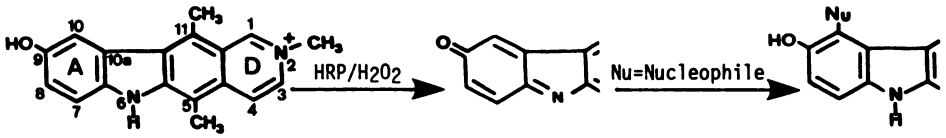
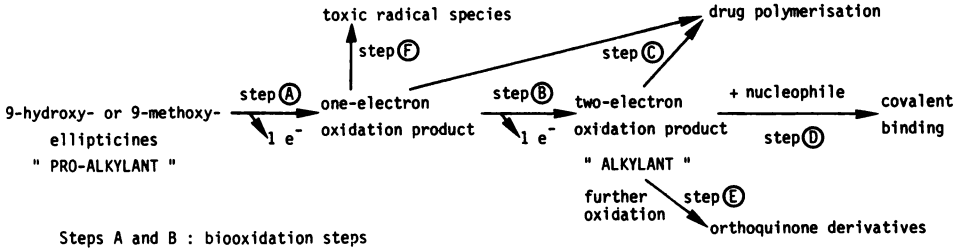


Fig. 1. Peroxidase-catalyzed oxidation of elliptinium acetate to an electrophilic quinone-imine derivative.



Scheme 1. Oxidative biotransformation of 9-hydroxy- and 9-methoxyellipticines.

the one-electron oxidation product lead directly to inactive polymers via step C or is it involved in cytotoxicity via radical species (step F)? *Step B:* Is the two-electron oxidation product a strong electrophile (step D being favored in this case) or are other side-reactions possible (polymerization, step C, or further oxidation, step E)?

In the present article, we report: (1) the electrochemical parameters and the peroxidase catalysis data on the oxidation of ellipticine derivatives in order to obtain quantitative information on the oxidizability, steps A and B, of these molecules; (ii) the ability of the peroxidase oxidation products to form adducts with nitrogen (amino acids) or oxygen (nucleosides) donors; (iii) the cytotoxic and antitumor properties of the compounds; and (iv) the possible correlation between the oxidizability, the electrophilicity of the oxidized forms, and the biological activities of the corresponding drugs. Some data on olivacine derivatives (olivacine is a natural isomer of ellipticine; see Ref. 34 and references therein for a recent article on this antitumor agent) are included in the present study. We have also studied the theoretical electron distribution (CNDO method) in the two-electron oxidized forms of some of the drugs in order to: (i) explain the unexpected regiospecific nucleophilic addition at carbon 10 of the quinone-imine and (ii) tentatively predict the electrophilic properties of oxidized forms of other derivatives which are so reactive that they cannot always be isolated *in vitro* for physical and chemical evaluation of their properties.

Materials and Methods

Elliptinium acetate and other ellipticine derivatives were kindly provided by Sanofi or were synthesized according to published procedures (35, 36) by J. Chenu in our laboratory. Horseradish peroxidase (HRP) (EC 1.11.1.7, type VI) was purchased from Sigma. Hydrogen peroxide (30%) and other chemicals were obtained from Merck and Prolabo, respectively.

Electrochemistry. A "home-made microcomputer-controlled instrument" (Laboratoire de Chimie de Coordination) with ohmic resistance compensation was used for cyclic voltammetry studies. A platinum auxiliary electrode and an Ag/AgCl (0.1 M KCl) reference electrode were used in conjunction with a platinum dish electrode (Tacussel EDI rotating electrode). All voltammograms were recorded at room temperature, at 0.02 or 0.05 V/sec. The concentration of ellipticine derivatives was 10^{-3} M in CH_3CN/phosphate buffer, 0.066 M, pH 8 (40/60, v/v). All solutions were degassed with argon for 10 min before measurements. For experiments on the influence of the solvent composition, we used 20, 35, 45, 50, 60, and 65% of organic solvent (acetonitrile or acetone) in 0.066 M phosphate buffer, pH 8.

The cell used for coulometry was a two-compartment vessel with a sintered glass dish separating anode and cathode chambers thermostated at 5°. Coulometry was conducted at a platinum gauze-working electrode with a platinum wire auxiliary electrode. Controlled potential electrolyses were carried out with a Tacussel PRT 20-2X potentiostat coupled with an I65N integrator. Aliquots of 20 μl were taken from the cell at regular intervals to determine by HPLC the remaining concentration of starting derivative.

HRP-catalyzed oxidation of ellipticine derivatives: determination of the turnover numbers. The turnover numbers were assayed by measuring the amount of H_2O_2 consumed. H_2O_2 consumption was quantitated by the formation of ferrithiocyanate from the oxidation of ferrous ammonium sulfate by H_2O_2 in the presence of potassium thiocyanate (37). For further details on the determination of kinetic parameters of the peroxidase oxidation of some of these drugs, see Ref. 18.

Ellipticine derivatives present rather small Michaelis-Menten constant values (K_m) for the HRP/H_2O_2 system ($K_m \leq 15$ μM) (18). Furthermore, excess substrate does not inhibit the rate of oxidation of this compound. For these reasons, the reaction mixtures (1.0 ml) contained 0.2 mM drug, 0.2 mM H_2O_2, 66 mM phosphate buffer (pH 7), and variable concentrations of HRP. The reactions were initiated by addition of HRP at 20° and terminated 10 min later by the addition of 0.15 ml of 60% trichloroacetic acid (during this period the oxidation rate of the ellipticine derivatives is linear according to the time). The acidified reaction mixture was centrifuged for 10 min at 2000 rpm. Of the resulting solution, 0.3 ml was diluted to 1 ml with water and then 0.2 ml of 10 mM ferrous ammonium sulfate and 0.1 ml of 2.5 mM potassium thiocyanate were added. The solutions were mixed and incubated at room temperature for 10 min and the absorbance of the resulting ferrithiocyanate complex was determined at 480 nm versus a water blank on a Beckman Acta III spectrophotometer. The absorbance readings are found to be stable for at least 60 min. H_2O_2 concentrations were determined from standard curves generated with each experiment in which known concentrations of H_2O_2 were taken through the identical procedure. All experiments were carried out in quintuplicate and included controls in which the peroxidase was omitted. The data presented are average values. The turnover numbers are expressed in μM H_2O_2 consumed/min and for 10^{-6} M HRP (or in μmol of H_2O_2 consumed/min/μmol of HRP).

Adduct formation during the peroxidase oxidation of ellipticine derivatives in the presence of model nucleophiles (L-alanine, guanosine). The reaction mixture (1 ml) contained 67 mM phosphate buffer (pH 7), 50 μM drug, 500 μM H_2O_2, and 5 mM L-alanine or guanosine. The reaction was initiated by addition of 1 μM HRP at

20° and stopped 120 min later by injecting into the HPLC. The HPLC studies were carried out on a Waters chromatograph using a μBondapak C_{18} column and a mixture of methanol/10 mM ammonium acetate (5/5 to 8/2, v/v) as eluent after acidification to pH 4.5 with acetic acid. The ellipticine derivatives and the adducts were monitored by a UV-visible spectrophotometer at 254 and 313 nm.

CNDO/2 calculations. Up to now it has not been possible to isolate in a crystalline form any quinone-imine of ellipticine derivatives. Consequently, we have generated and optimized the molecular structures of the quinone-imine compounds from known X-ray structures of stable ellipticine derivatives, namely, ellipticine (38), N^6-methylellipticine (39), 9-methoxyellipticine (40), and 9-OH-N^6-Me-E (41). Using our chain program for building molecules, MOLDESIGN (42), we have generated the refined cartesian coordinates of 26 quinone-imine derivatives with a pyrido[4,3-*b*]carbazole structure (see Table 2). Because of the number of heavy atoms (20–26) and, consequently, the large number of parameters required to describe these molecules, we have studied the electron distribution by a semiempirical method, CNDO/2 (43), which we have modified (unpublished data). For entries **1a**, **1c**, and **11**, a complementary study of the electrostatic potentials was made by the VSEM method (44, 45) which allows a bi- or tridimensional visualization of the molecular electronic potentials (see Figs. 6–8).

Biological activities. The cytotoxicity has been tested *in vitro* on murine leukemia L1210 cells according to a previously described procedure (46). All the products were dissolved in water with 1% dimethyl sulfoxide (final concentration). The inhibitory efficiency against cell multiplication is expressed in terms of ID_{50}, which represents the drug concentration that reduces the rate of cell multiplication by 50% as compared to the control.

The highest nonlethal dose (LD_0) was determined for each drug after a single intraperitoneal injection into DBA/2 or Swiss mice. The antitumoral tests were performed on DBA/2 mice that had been inoculated at J_0 with 10^5 L1210 or 10^6 P388 leukemia cells and treated at J_1 (L1210) or J_1, J_5, J_9 (P388) by the same route. Antitumor efficiency is expressed in term of *T/C* (increase in life span of treated mice over controls × 100). The therapeutic index corresponds to the ratio LD_0/dose which gives a *T/C* of 125%. Relative activity of the different compounds (see Table 1) are expressed by the symbols: −, no determination; 0, no activity, $T/C < 125\%$; +, significant activity, $T/C \geq 125\%$ and therapeutic index ≤2; ++, significant activity, $125\% < T/C < 170\%$ and therapeutic index > 2; and +++, significant activity, $T/C > 170\%$.

Results and Discussion

Electrochemical data. Using cyclic voltammetry, we have examined the electrochemical oxidation behavior of the pyridocarbazoles listed in Table 1.

The electrochemical studies of the different compounds have been done in acetonitrile/phosphate buffer mixture since: (i) all the derivatives were soluble under these conditions, and (ii) the addition of 60% acetonitrile was sufficient to greatly reduce the stacking interactions of ellipticine compounds and, consequently, the formation of a passivating film (already reported, see Ref. 47) during the anodic detection. In Fig. 2 is represented the influence of an organic solvent on the intensity of the peak current. It must be noted that the ratio Ip_a/Ip_c (anodic peak intensity/cathodic peak intensity) increases as the percentage of acetonitrile increases; as examples: $Ip_c/Ip_a = 0.43$, 0.57, and 0.73 for 20, 33, and 50% of acetonitrile in the electrolytic solution. Similar results are observed with acetone instead of acetonitrile. Typical cyclic voltammograms are illustrated by the scans shown in Fig. 3, a and b. For compound **11** (9-OH-NME, Fig. 3a), a single redox couple is observed. The forward, anodic sweep generates a peak at +0.180 V, which is ascribed to a $2e^-$ oxidation peak. The calibration of oxidation peak intensities in cyclic voltammetry or linear voltamperometry of 9-OH-NME was done with respect to the $1e^-$ oxidation of ferrocene used as standard, and the data indicate that the observed oxidation peak for 9-OH-NME corresponds to a $2e^-$ oxidation step. The easy oxidation of 9-OH-NME directly into its quinone-imine derivative is corroborated by the fact that under no conditions has it been possible to observe a noticeable concentration of compound **II** in the HRP oxidation of 9-OH-NME. Nevertheless, these oxidation data, obtained under the same conditions, represent the ability of all studied ellipticine derivatives to be oxidized and make possible their classification with respect to that property. The ratio of the cathodic and anodic peak currents ($Ip_c/Ip_a = 0.85$) indicates that, for 9-OH-NME, this is a quasi-reversible redox process. On the contrary, 7-OH-NME (Fig. 3b) gives an anodic signal at +0.295 V but no cathodic peak appears in the backward sweep, which indicates the irreversibility of the process. The behavior of the other derivatives (see Table 1) may be similar to one of these two compounds (quasi-complete reversibility or irreversibility) or intermediate between these two extremes.

The partial or total irreversibility of the electrochemical process probably results from the polymerization of the oxidized form of the drug (see below). Since, for the majority of the compounds, the electrochemical oxidation is not reversible, we have chosen the potential of the anodic signal (Ep_a) as a test of comparative oxidizability rather than the formal oxidation potential, which is not available in all cases. Under our experimental conditions (constant sweep rate, homogenous series of compounds), the values found for Ep_a can be considered as a good estimate of the oxidizability of these molecules.

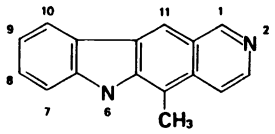
In the pH range 6–8, the peak potential (in the case of 9-OH-NME, see Fig. 4) shifted linearly with pH, with a slope $\delta Ep/\delta$ pH of −65.5 mV, showing that a fast and reversible proton transfer is also involved in the electrochemical process.

CNDO/2 calculations. During all the studies we performed on the structural determination of the different adducts resulting from the alkylation of any type of nucleophile (amino acids, thiols, or nucleosides) by the oxidized form of elliptinium, namely the quinone-imine. one intriguing fact remained as a constant: the linkage of the nucleophile heteroatom is always observed at the position 10 of the ellipticine skeleton (21, 23–27). Since this position is more hindered than two other possible candidates: positions 7 and 8, we decided to study the theoretical electron distribution to determine whether the regiospecificity was charge-controlled. Because the size of the molecule, we used a semiempirical approach (43, 48) for the calculations of the charge distribution. Furthermore, we have also studied the molecular electrostatic potentials since they can be a key factor in the control of the long distance interactions during the approach of the nucleophile to the quinone-imine itself (44).

Regioselectivity of adduct formation and electronic parameters. The main electronic characteristics of the quinone-imine form of elliptinium acetate, 9-oxo-NME, are shown in Fig. 5. The net atomic charges are reported in Fig. 5a, and we can see that the values on carbon 8 and 10 are slightly different: −0.034 at C_{10} and −0.045 at C_8. From the π-bond order values reported in Fig. 5b, it is clear that the quinone-imine structure is illustrated by the high bond order of C_7–C_8 and C_{10}–C_{10a} bonds (respective values = 0.86 and 0.81); more-

TABLE 1

Structures, physicochemical parameters, and biological activities of various ellipticine or olivacine derivatives



Registry number	Molecule abbreviation	Position of ring substitution								Electrochemical data[a]			Oxydation by HRP (turnover number at 10^{-6} M in HRP)[b]	Adduct formation		Biological activities		
		1	2	6	7	8	9	10	11	Ep_a	Ep_c	Ib/If		Alanine	Nucleoside	ID_{50}[c]	Antitumor activity[d] L1210	P388
										V						μM		
1	9-OMe-N^6-Me-E	H	–	Me	H	H	OMe	H	Me	1.0	–	–	0	no	no			
2	NME	H	Me	H	H	H	H	H	Me	0.820	–	–	18	no	no	1.03	0	–
3	9-OMe-NME	H	Me	H	H	H	OMe	H	Me	0.625	–	–	25	yes	yes	1.14	0	0
4	E	H	–	H	H	H	H	H	Me	0.600	–	–	30	no	no	1.45	+	+
5	9-OMe-E	H	–	H	H	H	OMe	H	Me	0.500	–	–	710	no	yes	1.80	+	–
6	9-OH-N^6-Me-NME	H	Me	Me	H	H	OH	H	Me	0.420	–	–	26,000	yes	yes	0.089	++	+++
7	7-OH-NME	H	Me	H	OH	H	H	H	Me	0.295	–	–	–	no	no	4.26	0	+
8	9-OH-N^6-Me-E	H	–	Me	H	H	OH	H	Me	0.280	–	–	13,500	no	yes	0.076	–	–
9	SR 95156 B	H	DEAE	H	H	H	OH	H	Me	0.260	0.125	0.86	38,500	yes	yes	0.050	+++	+++
10	9-OH-NMO	Me	Me	H	H	H	OH	H	H	0.205	0.065	0.93	13,000	yes	yes	0.29	++	++
11	9-OH-NME	H	Me	H	H	H	OH	H	Me	0.180	0.100	0.85	28,000	yes	yes	0.11	++	++
12	9-OH-7-Me-NME	H	Me	H	Me	H	OH	H	Me	0.160	0.060	0.85	–			1.19	+	++
13	8,10-diMe-9-OH-NMO	Me	Me	H	H	Me	OH	Me	H	0.130	−0.010	0.095	12,000	no	?	0.86	++	++
14	9-OH-E	H	–	H	H	H	OH	H	Me	0.100	–	–	33,500	no	yes	0.11(5)	++	–
15	8,10-diMe-9-OH-NME	H	Me	H	H	Me	OH	Me	Me	0.035	−0.100	0.61	11,000	no	?	6.04	0	+

[a] Electrochemical data: Ep_a = anodic sweep, Ep_c = cathodic sweep, Ib/If = backward intensity/forward intensity.
[b] Turnover number is measured as μmol of H_2O_2 consumed/min and per μmol of HRP.
[c] For cytotoxicity, ID_{50} (μM) = dose which reduces by 50%, after 48 hr, the L1210 cell growth as compared to controls.
[d] For antitumor activity, relative activity is expressed by the symbols: – (no determination), 0 (no activity, T/C < 125%), + (significant activity, T/C ≥ 125% and therapeutic index ≤2), ++ (125 < T/C < 170% and therapeutic index >2), and +++ (T/C > 170%).

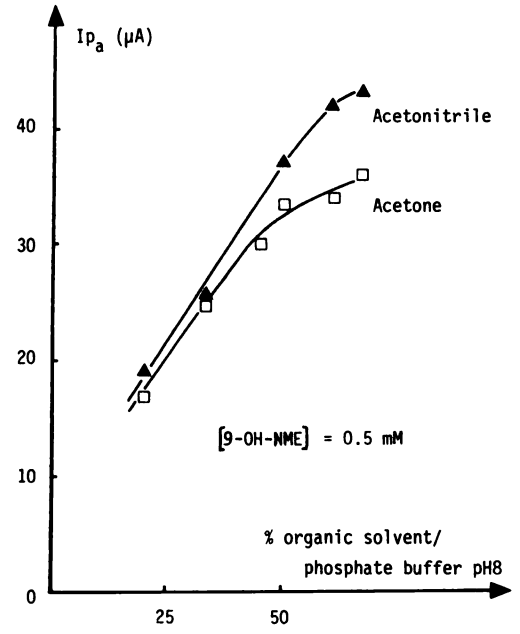


Fig. 2. Influence of the solvent on the intensity of peak current. Sweep rate constant was 0.02 V/sec.

over, the last one has a more single character and so appears more polarized than the first one. Of greater interest are the data reported in Fig. 5c on the partial πz-electronic densities. In that case, the C_{10} carbon atom exhibits a strong electronic deficiency (0.938) compared to C_7 or C_8, 0.970, and 0.984, respectively (these two atoms being the alternative sites for addition to ring A). In Table 2 a set of six data points has been reported for 26 quinone-imine derivatives: the net atomic charges and the πz-electronic density at the three possible sites for an addition to ring A. From these data, it is clear that the πz-electronic density is always lower at C_{10} compared to C_8 or C_7 for all of the oxo derivatives with a positive charge at the pyridine nitrogen (by quaternarization with a methyl group or protonation, cases a and b), suggesting that a nucleophile, which interacts more with the π-density of the molecule on the ring A, must be more attracted by C_{10} rather than C_8 or C_7. The immediate consequence of the quaternarization or protonation of the nitrogen atom is the increased difference in terms of πz-electronic density between C_7 or C_8 and C_{10}. Up to now, we have not isolated any adduct on quaternarized ellipticines with a covalent binding at a position different from C_{10}. From the recent data obtained by Sundaramonthi *et al.* (49), it is also the case for non-quarternarized quinone-imines (such as **1c**) and, even more surprisingly, for the carbazole quinone-imine (see entry **11** of Table 2). In these latter cases, the C_7 (for **1c**) or C_8 (for **11**) alkylation position might compete with C_{10} (**1c**) or C_5 (**11**, C_5 in the carbazole structure corresponds to C_{10} in the ellipticine structure) if we considered the πz-electronic density as the main regulation factor for the alkylation regioselectivity. Thus, this electronic parameter appears to be insufficient to explain the great regioselectivity of adduct formation on these compounds. The molecular electrostatic potential study (see below) constitutes a more satisfactory explanation for this phenomenon.

Regioselectivity of adduct formation and molecular electrostatic potentials. The approach of a nucleophilic species should be controlled by interaction at larger distances than the bonding distance between the nucleophile and the alkyla-

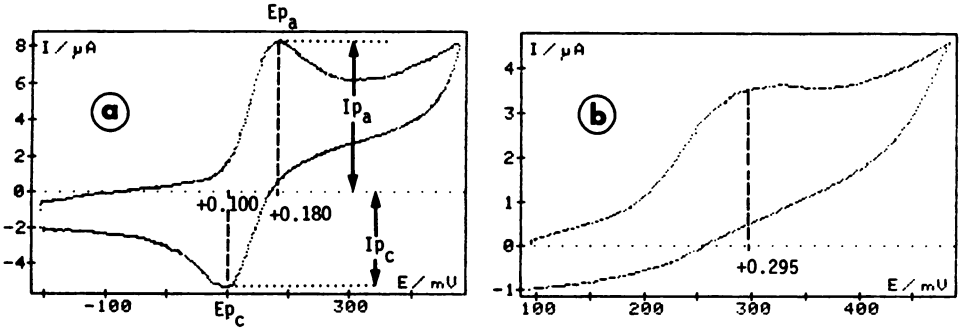


Fig. 3. Cyclic voltammetry of: a, 9-OH-NME (compound 11) and b, 7-OH-NME (compound 7) in 40% H_2O/60% CH_3CN (sweep rate 0.05 V/sec, concentration 1 mM).

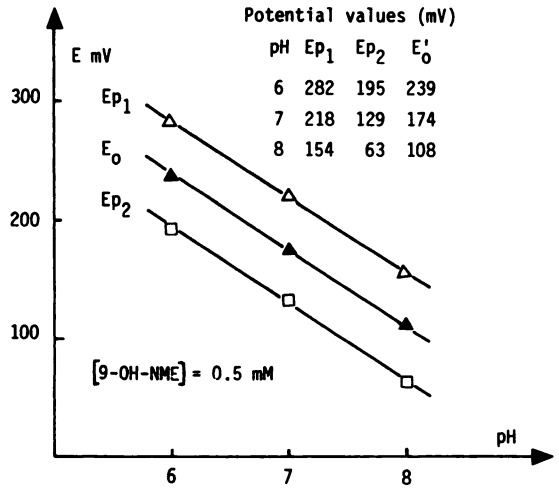


Fig. 4. Influence of the pH on the peak potential. $E'_0 = 632–65.5$ pH for 9-OH-NME in a solution mixture of acetonitrile/phosphate buffer (2:1, v/v), Ag/AgCl being the reference electrode.

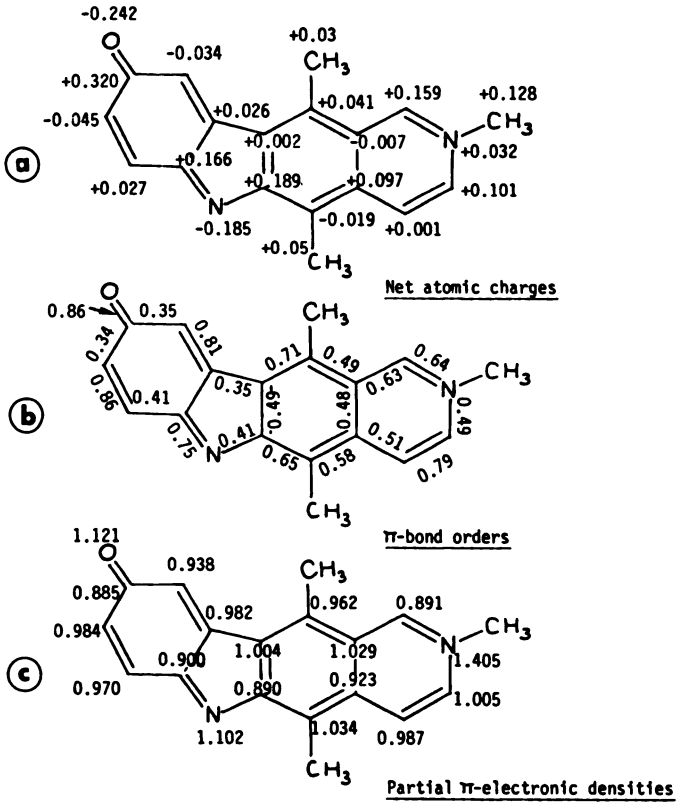


Fig. 5. CNDO/2 calculations on the quinone-imine of elliptinium acetate.

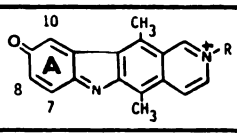
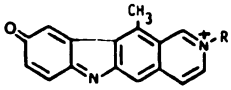
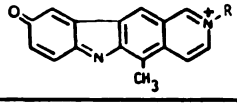
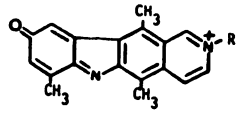
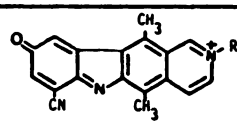
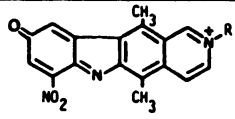
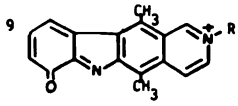
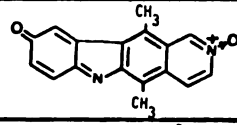
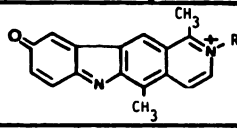
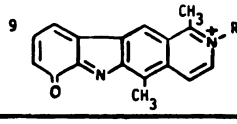
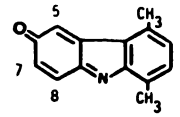
tion site. The molecular electrostatic potential is the first significant field encountered when these two molecules approach each other (50). For these reasons, the electrostatic potentials of the quinone-imines **1a**, **1c**, and **11** have been calculated by the VSEM method (45). Three maps of the electrostatic potentials are represented in Figs. 6–8 and correspond to three parallel planes at 1, 2, and 3 Å (respectively, parts a, b, and c of each figure) above the molecular plane. It is of great interest to focus our attention on Figs. 6–8, b and c. In the case of **1a** (Fig. 6), the highest positive potentials are above the ring D which is a pyridinium moiety. The counteranion interacts in this region with the quinone-imine derivative, making impossible an approach of any nucleophile. On the other side, the ring A is the more favored ring for a nucleophilic addition, and from Fig. 6, b or c, it is clear that C_{10} is at a higher potential than are the C_7 and C_8 atoms. These data could confirm the reasons why C_{10} is the preferred alkylation site of the oxidized form of elliptinium acetate. Furthermore, if we make a calculation of the energy involved at 3 Å at the vertical of C_7, C_8, and C_{10}, the energies are, respectively, 58, 54, and 63 kcal/mol. This difference of 5 or 9 kcal/mol is probably sufficient to have the complete discrimination between these three possible alkylation sites and explains why we have only observed the adduct formation at C_{10}. Similar observations may be obtained from calculations of the energy at 3 Å at the vertical of C_7 and C_8 (quinone-imine **1c**, 4 and 3.8 kcal/mol, respectively) and C_7 and C_8 (quinone-imine **11**, 5.0 and 5.2 kcal/mol, respectively), compared to C_{10} (**1c**, 5.0 kcal/mol) and C_5 (**11**, 6.3 kcal/mol). The slight difference observed (about 1 kcal/mol) between the potential values for each competitive alkylation position is likely to be a key factor in the explanation of the unexpected regioselectivity for nucleophilic addition on these uncharged derivatives.

πz electronic density as an indicator of electrophilicity. If the molecular electrostatic potential can modify the relative orientation of the two interacting molecules, we can envisage now the πz electronic density at the C_{10} position of ellipticine (and related compounds) as an indicator of electrophilicity of the reactive quinone-imines.

The lowest πz-electronic density for C_{10} atoms is observed in the case of 7-oxo-quinone-imines in both ellipticine or olivacine series (see quinone-imines **7a**, **7b**, **10a**, and **10b**, Table 2); however, 7-OH-NME appears to be a poor electrophile; this fact may result from the high percentage of polymers formed during electrochemical (51) or peroxidasic (52) oxidation (see General Discussion for 7-hydroxylated derivatives). Another powerful electrophile may be the 7-methylated derivative **4a**

TABLE 2

Calculated net atomic charges and partial π-electronic densities at C_7, C_8, and C_{10}

Entry	Molecules Quinone-imines	R	N° ([a])	Net atomic charges C_7	Net atomic charges C_8	Net atomic charges C_{10}	π-electronic density C_7	π-electronic density C_8	π-electronic density C_{10}
1 a		CH_3	11	0.027	-0.045	-0.034	0.969	0.984	0.938
b		H	-	0.020	-0.028	-0.012	0.977	0.969	0.922
c		□ ([b])	14	0.042	-0.094	-0.095	0.951	1.041	1.016
2 a		CH_3	-	0.028	-0.048	-0.041	0.968	0.984	0.945
3 a		CH_3	-	0.024	-0.038	-0.018	0.972	0.979	0.924
4 a		CH_3	12	0.052	-0.060	0.002	0.943	1.018	0.908
b		H	-	0.045	-0.053	0.008	0.948	1.010	0.902
c		□	-	0.047	-0.062	-0.014	0.946	1.038	0.954
5 a		CH_3	-	0.045	-0.037	-0.020	0.975	0.983	0.926
b		H	-	0.038	-0.020	0.0016	0.982	0.970	0.911
c			-	0.042	-0.040	-0.037	0.976	1.004	0.975
6 a		CH_3	-	0.069	-0.116	-0.128	0.990	1.011	1.012
b		H	-	0.044	-0.053	-0.062	1.015	0.962	0.962
c		□	-	0.029	-0.031	-0.056	1.027	0.966	0.996
				$(C_9)^c$			$(C_9)^c$		
7 a		CH_3	7	0.030	-0.019	0.085	0.979	0.953	0.878
b		H	-	0.032	-0.013	0.099	0.979	0.948	0.857
c		□	-	0.051	-0.070	0.028	0.956	1.022	0.962
8 a		Singulet	-	0.031	-0.055	-0.028	0.964	1.020	0.962
b		triplet	-	-0.021	-0.198	-0.234	1.041	1.182	1.229
9 a		CH_3	10	0.026	-0.044	-0.025	0.970	0.985	0.930
b		H	-	0.022	-0.032	-0.009	0.975	0.975	0.918
c		□	-	0.045	-0.091	-0.086	0.950	1.041	1.008
				$(C_9)^c$			$(C_9)^c$		
10 a		CH_3	-	0.027	-0.023	0.078	0.981	0.955	0.881
b		H	-	0.029	-0.016	0.094	0.981	0.949	0.870
c		□	-	0.052	-0.075	0.030	0.955	1.025	0.961
				$(C_8)^d$	$(C_7)^d$	$(C_5)^d$	$(C_8)^d$	$(C_7)^d$	$(C_5)^d$
11		-	-	0.018	-0.076	-0.040	0.953	1.043	0.954

[a] Registry number of the corresponding reduced form (see Table 1).
[b] Free base, i.e., no protonation of the nitrogen pyridine.
[c] Values are given for C_9 instead of C_7, which is substituted by the oxygen atom.
[d] Ring positions 8, 7, and 5 of the carbazole derivative correspond to the ring positions 7, 8, and 10 of the ellipticine (or olivacine) structure.

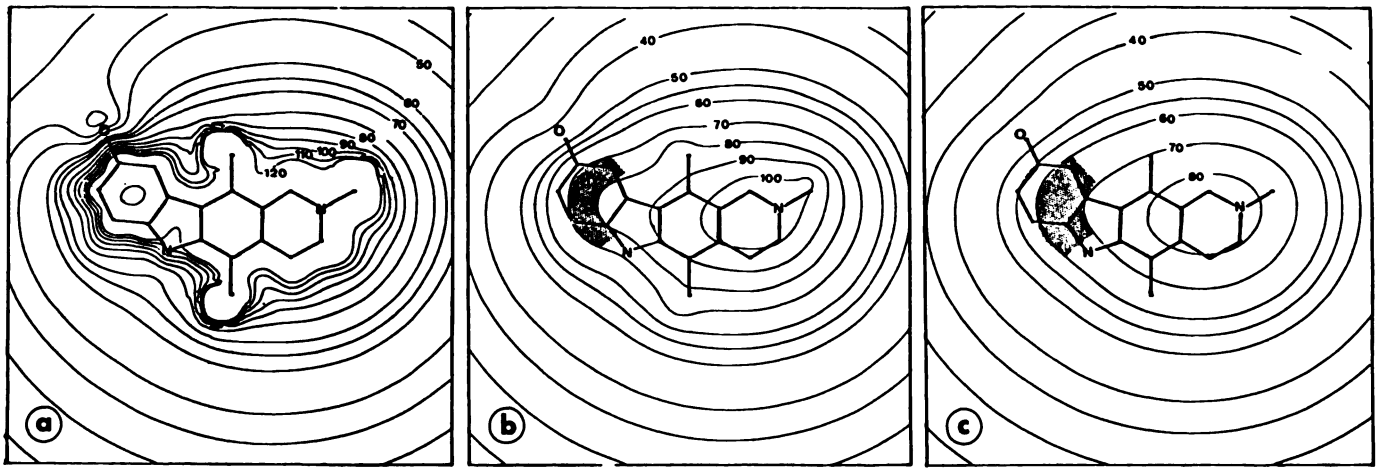


Fig. 6. Quinone-imine **1a** (structure in Table 2). Molecular electrostatic isopotential maps: a, altitude 1 Å; b, altitude 2 Å; and c, altitude 3 Å (the units are kcal/mol).

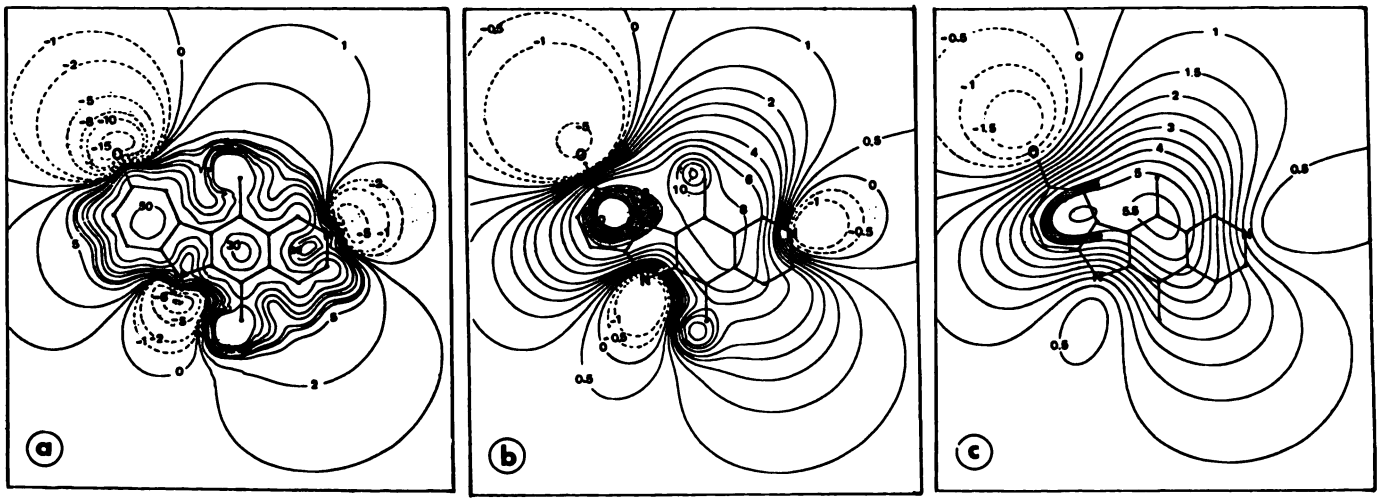


Fig. 7. Quinone-imine **1c** (structure in Table 2). Molecular electrostatic isopotential maps: a, altitude 1 Å; b, altitude 2 Å; and c, altitude 3 Å (the units are kcal/mol).

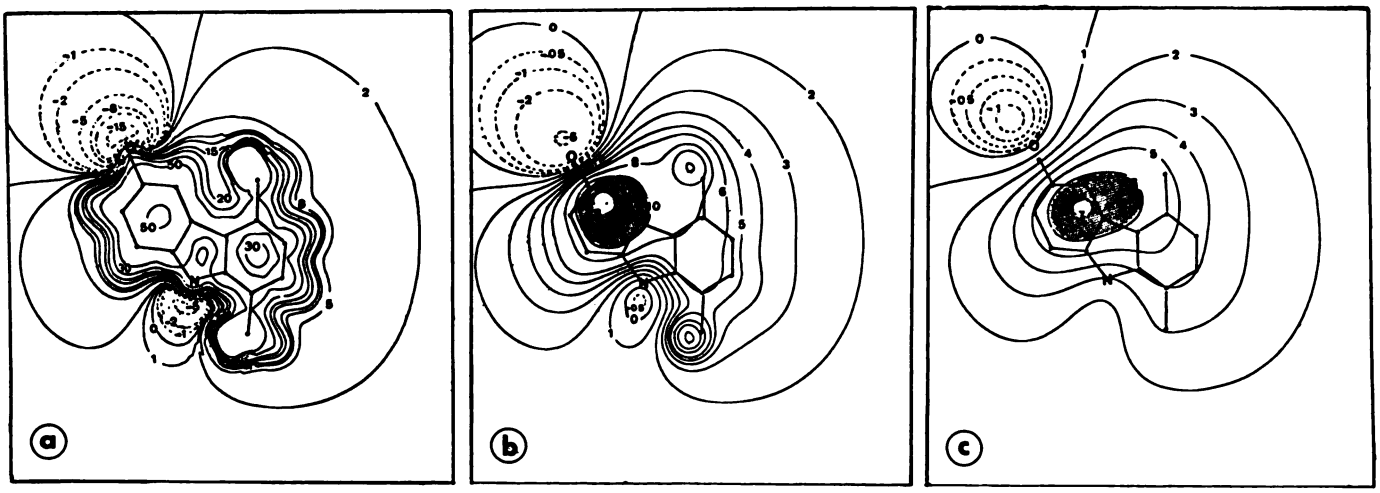


Fig. 8. Quinone-imine **11** (structure in Table 2). Molecular electrostatic isopotential maps: a, altitude 1 Å, b, altitude 2 Å; and c, altitude 3 Å (the units are kcal/mol).

(Table 2). The presence of the methyl group in the last carbon of the α,β unsaturated ketone entity (C_7-C_8-C_9-O) largely increased the difference between C_{10} and C_8 with respect to the πz-electronic density. The presence of a cyano group at C_7 does not modify the πz-electronic density at C_{10} (quinone-imine **5a** compared to **1a**). A nitro group at C_7 makes the πz-electronic density equivalent at C_{10} and C_8 (**6a**), probably because of the strong π-mesomeric effect of the electron pairs of the oxygen atoms. Compared to compounds without a positive charge at N_2, the presence of an *N*-oxide function (**8a** compared to **1c**) contributes to the increase of electrophilicity at carbon 10, and that phenomenon is only observed when the *N*-oxide is considered in the singulet state.

If we consider now the case of olivacine derivatives, we can see that the C_{10} is still more electrophilic than C_8 in the quinone-imine **9a**. The C_{10} position is also the electrophilic site for 7-oxo-quinone-imine compounds (**10a** and **10b**). But, as mentioned above for entry 7 (Table 2), the peroxidase oxidation of the 7-hydroxy-olivacinium derivative gives mainly polymers (52).

General Discussion

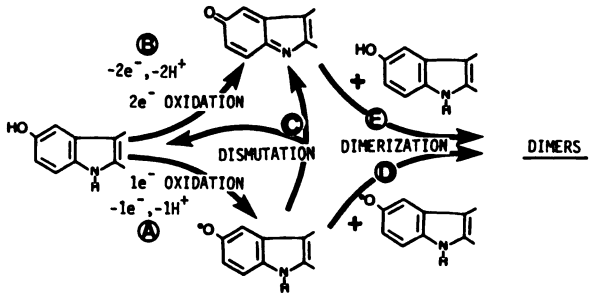
From the electrochemical data reported in Table 1, it is clear that all hydroxylated ellipticine and olivacine derivatives are easily oxidized. The oxidation peaks range from 0.035 to 0.420 V for all of the hydroxylated compounds. This easy oxidation correlates with the facile peroxidase oxidation of these molecules by the HRP/H_2O_2 system which was used to mimic a possible extrahepatic oxidation of the drug. The turnover rates are high, from 11,000 to 38,500 min^{-1}, even when the indolic N-H is blocked by a methyl group (compound **6**). When the phenolic group is blocked or absent, then the oxidation potential is higher (compounds **1–5**) and the biochemical oxidation rate is reduced but not completely stopped, except in the case where both sides of the amino-phenol structure are methylated (compound **1**).

Among these oxidizable ellipticine compounds, some of them lead to a large amount of polymeric structures, which can be considered as a waste process competing with the possible electrophilic properties of the two-electron oxidation product, namely, the quinone-imine or quinone-iminium forms (see Scheme 2 for the different pathways during the oxidation of ellipticine derivatives). The shape of the cyclic voltammograms is a good indication of the reversibility of the oxidation process. A high value for *Ib/If* (see Table 1) can be associated with a reversible or quasi-reversible oxidation in which polymers are not formed (compounds **9–13**). This is the case for 9-hydroxylated compounds. However, when the hydroxy group is at position 7, then the oxidation is not reversible (compound **7**) and leads to inert polymers. This fact is confirmed by the absence of adduct formation (so that no electrophilic species are generated during the oxidation process). It must be noted that the 7-hydroxy-ellipticines or -olivacines are inactive, and, up to now, the data reported in this paper on their oxidation behavior are the only arguments to explain their lack of biological activity. The most active molecules against L1210 and P388 leukemia cells *in vitro* or *in vivo* are in fact the 9-hydroxylated derivatives for which the electrophilicity of the oxidized form has been evidenced (compounds **9–15**). Besides this category, compound **6** represents a particular case: this N^6-methylated molecule is easily oxidized by HRP/H_2O_2 and gives polymeric structures in the absence of nucleophile. However, in the presence of such a partner, the electrophilic quinone-iminium can be trapped during the peroxidase oxidation (53), indicating that this molecule might be the origin of a strong alkylating agent. Besides these hydroxylated molecules, two particular cases of active molecules have to be discussed: ellipticine itself (**4**) and 9-methoxyellipticine (**5**). In the first case, it is known that ellipticine is easily hydroxylated *in vivo* into 9-hydroxyellipticine as main metabolite (54), and, consequently, its biological activity might be related to its main metabolite. Concerning the 9-methoxyellipticine, we have to remember that this molecule can be transformed to 9-hydroxyellipticine by liver metabolism (55) or directly oxidized in the corresponding quinone-imine by a peroxidase (18). Thus, these two derivatives can be included in the "pro-alkylating" category after a metabolic activation.

When the C_{10} alkylation site is blocked by a methyl group (compounds **13** and **15**), the oxidized form of the drug is not a strong alkylating agent (no adducts are observed with alanine, but the addition of guanosine to a quinone-imine solution of **13** or **15** gives a new compound from HPLC data (a possible adduct? the shortage of drug samples did not allow isolation of these compounds). However, the quinone-imine may act as a catalyst in redox cycle if any electron source can be associated with this oxidized form of the drug. Such a phenomenon is well known for quinone antitumor agents (20, 56, 57) and has already been observed in the ellipticine series for an orthoquinone molecule (6). In contrast to the olivacine case **13**, the corresponding ellipticine compound **15** has nearly no activity, but this latter compound is highly unstable in solution and is quickly autooxidized in various products. To be implicated in a redox cycle near the target and to induce, then, a cytotoxic event, the quinone-imine solution has to be sufficiently stable (this is not required for quinone derivatives when they act as alkylating agent, a key point in that case being the kinetic of the alkylation process, step D in Scheme 1).

The association of two simple experimental methods, cyclic voltammetry and peroxidase oxidation with or without nucleophiles, is a convenient way to collect data on the *in vitro* behavior of the oxidized form of a pro-alkylating cytotoxic molecule and might be used as a paradigm in the oxidative biotransformation of synthetic analogs of such antitumor agents.

Furthermore, the CNDO calculations on oxidized forms of the drug give an insight into the prediction of alkylation sites of the reactive quinone-imine. Because of the feasibility and low cost of this theoretical approach, the CNDO method can



Scheme 2. Possible 1 and $2e^-$ oxidation pathways for hydroxylated ellipticines.

be applied to a large number of molecules in the same series. In the present case, CNDO calculations gave the only explanation of why C_{10} is the preferred alkylation site of ellipticine quinone-imines compared to C_7 or C_8, the alternative electrophilic centers.

In conclusion, we have presented experimental evidence using electrochemical measurements, biochemical oxidations, or CNDO calculations on (i) the easy oxidation of a large number of cytotoxic ellipticine derivatives, (ii) the electrophilicity of the corresponding oxidized forms and the possible formation of adducts via alkylation, and (iii) the pro-alkylating nature of nearly all of the antitumor agents of this series.

The biooxidative alkylation mechanism has recently been supported by evidence of the covalent binding of a clinically used antitumor agent of this series (namely, elliptinium acetate) to nucleic acids of L1210 leukemia cells (30). At this point it is not possible to argue that the covalent binding of antitumor ellipticine derivatives to the target(s) is the only mechanism responsible for their cytotoxic activity. However, the results of the present study indicate that the hypothesis cannot be discarded even in the case of the recent demonstration of DNA topoisomerases as targets of intercalating antitumor agents (58, 59). In fact, the alkylation process might strenghten one aspect of the data obtained in the topoisomerase experiments. From these data, it has been noted that the most active drugs in the formation of the "cleavable complex" (DNA-topoisomerase-drug) are the ones which are known to be easily oxidized. This is a possible explanation of why ethidium bromide is inactive in causing DNA cleavage and also why *m*-AMSA is more potent than its isomer *o*-AMSA (60) [only *m*-AMSA is known to give a good electrophile after oxidation (61)]. In addition, epipodophyllotoxins (etoposide, teniposide) are also able to induce topoisomerase-mediated DNA damages (60), and they are easily activated by oxidation (62). For such pro-alkylating agents, DNA (or a protein like topoisomerase) can be considered as a potential target. Then, it may be possible that when the topoisomerase meets a DNA site where a pro-alkylating agent is covalently bound, there is formation of a cleavable complex which leads to irreversible and lethal breaks, whereas when the drug interacts with DNA only by reversible intercalation, the DNA breaks are reversible, as it has been demonstrated (60, 63, 64). Thus, the alkylation of DNA observed with elliptinium is not necessarily opposite to the topoisomerase studies, but both processes might be complementary and involved in the formation of a lethal cleavable complex responsible for the cytotoxic effects of this class of reactive intercalating agents. The possibility of a bioactivation step coupled with the topoisomerase interaction has already been mentioned for at least two compounds in the ellipticine series: 9-OH-NME and an aza-ellipticine, BD-40, for which the drug activity is better in cultured cells than in isolated nuclei (64, 65). Further studies of the nucleic acid alkylation process by cytotoxic ellipticine compounds must be undertaken with ellipticine-resistant cells in order to have a deeper insight into the mechanism of action of these antitumor agents at the molecular level.

Acknowledgments

Prof. C. Paoletti and Dr. C. Auclair are acknowledged for fruitful discussions throughout this work. We are grateful to the Sanofi Company for financial support, to J. Chenu for the synthesis of ellipticine derivatives, and to G. Francois and C. Galy for technical assistance in determination of biological activities.

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