Table V. Antihypertensive Activity of Quinazoline Derivatives in Spontaneously Hypertensive Rats

compd	dose, μmol/kg po	fall in systolic BP, mmHg, at the following times after dosing: 1 h	3 h	5 h
6m	1	11	NS	NS
	10	29	29	26
	100	52	66	83
6r	1	13	14	NS
	10	40	36	27
6s	0.3	NS	11	11
	1	18	20	21
	3	34	34	35
	10	45	54	50
6t	1	15	20	NS
	10	NS	54	51
6y	1	12	17	19
	10	25	30	38
prazosin	1	29	30	26
	10	38	36	34
	100	38	30	26

[a] Values in the table indicate statistically significant decreases in blood pressure ($p \leq 0.05$) relative to control values. NS = nonsignificant change in blood pressure. The average systolic blood pressure before the administration of the compounds ranged in various experiments from 175 to 190 mmHg. Five rats were used to study the effect of each dose.

yielding 23.3 g (90%): ^{1}H NMR ($CDCl_3$) δ 1.45–1.95 (m, 7 H), 2.40–3.60 (m, 10 H), 2.55 (s, 1 H).

1-Benzyl-4-cyanopiperidine (10). A mixture of 1-benzylpiperidine-4-carboxamide (14.3 g, 0.066 mol) in thionyl chloride (115 mL) was refluxed for 3 h. Excess thionyl chloride was evaporated in vacuo, and the residue was basified with sodium hydroxide solution and extracted with dichloromethane. The solvent was evaporated, and the residue was distilled in vacuo, yielding 9.6 g (73%) of a viscous oil: bp 130–132 °C (0.4–0.5 mmHg); ^{1}H NMR ($CDCl_3$) δ 1.6–2.2 (m, 4 H), 2.2–2.8 (m, 5 H), 3.42 (s, 2 H), 7.21 (s, 5 H).

1-Benzyl-4-(cyclopentylcarbonyl)piperidine. To a solution of cyclopentylmagnesium bromide, prepared in the usual way from magnesium (4.8 g, 0.2 mol) and bromocyclopentane (29.8 g, 0.2 mol) in THF (100 mL), was added, with stirring and cooling (0–5 °C), **10** (34.0 g, 0.17 mol) in THF (100 mL). The mixture was refluxed for 3 h and allowed to stand overnight at room temperature. Hydrochloric acid (5 N, 300 mL) was added, and THF was evaporated in vacuo. The aqueous solution was made basic with 50% sodium hydroxide and extracted with dichloromethane. The solvent was evaporated, yielding 31.4 g (68%) of a viscous oil: ^{1}H NMR ($CDCl_3$) δ 1.4–3.0 (m, 18 H), 3.40 (s, 2 H), 7.17 (s, 5 H).

4-(Cyclopentylcarbonyl)piperidine (3s). A solution of 1-benzyl-4-(cyclopentylcarbonyl)piperidine (18.7 g, 0.069 mol) in methanol (270 mL) and 1 N hydrochloric acid (85 mL) was hydrogenated at normal pressure and at room temperature in the presence of 10% Pd/C (5.0 g) until the theoretical amount of hydrogen was consumed. The solution was filtered, and the filtrate was evaporated to dryness in vacuo. The residue was made basic with 5 N sodium hydroxide and extracted with dichloromethane. The solvent was evaporated, yielding 12.5 g (87%): mp 32–33 °C; ^{1}H NMR ($CDCl_3$) δ 1.5–1.9 (m, 10 H), 1.97 (s, 1 H), 2.4–3.2 (m, 8 H). Anal. ($C_{11}H_{19}NO$) C, H, N.

Cyclopentyl-4-piperidinylcarbinol (3x). To a solution of **3s** (4.7 g, 0.026 mol) in methanol (50 mL) was added, with stirring and cooling (0–5 °C), sodium borohydride (1.3 g) in water (15 mL). The mixture was stirred for 2 h at room temperature. The solvent was evaporated in vacuo, water was added to the residue, and the solution was extracted with chloroform. The solvent was evaporated yielding 3.4 g (72%) of a crystalline solid: mp 123–125 °C. Anal. ($C_{11}H_{21}NO$) C, H, N.

Registry No. **1**, 19216-56-9; **2**, 43091-89-0; **3e**·HCl, 86542-85-0; **3g**, 86542-86-1; **3i**, 86542-87-2; **3ii**, 86542-88-3; **3j**, 35090-95-0; **3k**, 63214-58-4; **3l**, 86542-89-4; **3m**, 86542-90-7; **3n**, 86542-91-8; **3o**, 86542-92-9; **3p**, 86542-93-0; **3q**, 86542-94-1; **3r**·HCl, 86542-95-2; **3s**, 86542-96-3; **3t**, 86542-97-4; **3u**, 86542-98-5; **3v**, 24152-50-9; **3w**, 86542-99-6; **4a**, 86543-00-2; **4b**, 86543-01-3; **4c**, 86543-02-4; **4cc**, 86543-03-5; **4d**, 86543-04-6; **4e**, 86543-05-7; **4f**, 86543-06-8; **4g**, 86543-07-9; **4i**, 86543-08-0; **4ii**, 86543-09-1; **4j**, 86543-10-4; **4k**, 86543-11-5; **4l**, 86543-12-6; **4m**, 86543-13-7; **4n**, 86543-14-8; **4o**, 86543-15-9; **4p**, 86543-16-0; **4q**, 86543-17-1; **4r**, 86543-18-2; **4s**, 86543-19-3; **4t**, 86543-20-6; **4u**, 86543-21-7; **4v**, 86543-22-8; **4w**, 86543-23-9; **4x**, 86543-24-0; **5a**, 86543-25-1; **5b**, 86543-26-2; **5c**, 80030-55-3; **5cc**, 80030-56-4; **5d**, 80024-64-2; **5e**, 80030-53-1; **5f**, 80030-57-5; **5g**, 80030-58-6; **5i**, 80030-61-1; **5ii**, 80342-07-0; **5j**, 80030-52-0; **5k**, 80030-54-2; **5l**, 80030-60-0; **5m**, 80030-69-9; **5n**, 80030-62-2; **5o**, 80030-59-7; **5p**, 80030-63-3; **5q**, 80030-66-6; **5r**, 80030-67-7; **5s**, 86543-27-3; **5t**, 80030-68-8; **5u**, 86543-28-4; **5v**, 86543-29-5; **5w**, 86543-30-8; **5x**, 86543-31-9; **6a**, 86543-32-0; **6b**, 23673-00-9; **6c**, 86543-33-1; **6cc**, 86543-34-2; **6d**, 80024-65-3; **6e**, 80024-67-5; **6f**, 80024-72-2; **6g**, 86543-35-3; **6h**, 86543-36-4; **6i**, 80024-76-6; **6ii**, 80024-77-7; **6j**, 80024-63-1; **6k**, 86543-37-5; **6l**, 80024-75-5; **6m**, 80024-83-5; **6n**, 80024-78-8; **6o**, 80024-74-4; **6p**, 80030-64-4; **6q**, 80024-80-2; **6r**, 80024-81-3; **6s**, 86543-38-6; **6t**, 80024-82-4; **6u**, 65189-42-6; **6v**, 86543-39-7; **6w**, 86543-40-0; **6x**, 86543-41-1; **6y**, 86543-42-2; **10**, 62718-31-4; **11** (R_3 = cyclopentyl), 86542-84-9; 1-(4-pyridylcarbonyl)pyrrolidine, 86542-83-8; 1-benzylpiperidine-4-carboxamide, 62992-68-1; bromocyclopentane, 137-43-9.

Autoxidation of the Antitumor Drug 9-Hydroxyellipticine and Its Derivatives[1]

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In aqueous solution and using molecular oxygen as electron acceptor, the antitumor drug 9-hydroxyellipticine (9-OH-E) undergoes a spontaneous oxidation to give hydrogen peroxide (H_2O_2), the quinone imine 9-oxoellipticine (9-oxo-E), and a dimer of 9-OH-E (9-OH-E_2). Electron paramagnetic resonance (EPR) experiments performed either in alkaline Me_2SO or in phosphate buffer in the presence of the spin trap 5,5-dimethylpyrroline 1-oxide (DMPO) suggest that the oxidation process involves the initial formation of superoxide anion ($O_2^{-}\cdot$) and the free radical of the drug. In aqueous medium, this step is followed by the dismutation of both $O_2^{-}\cdot$ and free radicals of the drug generating, respectively, H_2O_2 and 9-oxo-E. 9-Oxo-E further reacts with the 9-OH-E remaining in the solution to form the dimer 9-OH-E_2 as the terminal product. The autoxidation process is strongly enhanced by superoxide dismutase and manganese ions. In the ellipticine series, all drugs that have an OH group in position 9 exhibit the ability to transfer one electron on molecular oxygen to generate $O_2^{-}\cdot$. This property may be involved in the cytotoxic activities of these drugs.

Two main hypotheses have been proposed concerning the mechanisms of action of the antitumor drugs derived from ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole; see Table I). First, the possible interaction of these drugs

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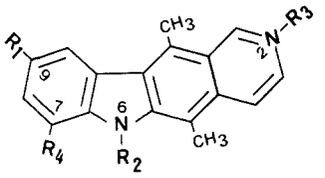
Table I. Structures of Ellipticine Derivatives



no.	R_1	R_2	R_3	R_4	name	abbrev
1	H	H		H	ellipticine	E
2	H	H	CH_3	H	ellipticinium	N^2-Me-E
3	OH	H		H	9-hydroxyellipticine	9-OH-E
4	OH	H	CH_3	H	9-hydroxyellipticinium	N^2-Me-9-OH-E
5	OH	CH_3	CH_3	H	N^6-methyl-9-hydroxyellipticinium	N^2,N^6-Me-9-OH-E
6	H	H		OH	7-hydroxyellipticine	7-OH-E

with DNA through an intercalation process may result in cytotoxic events;[2] second, the oxidation of these drugs may lead to chemically reactive products endowed with potential toxicity.[3] In agreement with the latter hypothesis, using a peroxidase–hydrogen peroxide system as a model of bioactivation, we demonstrated that 9-hydroxyellipticine derivatives (Table I) may undergo an oxidation through a one-electron transfer process, yielding free radicals of the drugs as intermediates and quinones as terminal products of the reaction.[3] Both free radicals and quinones were found to be very reactive oxidizing molecules. In the ellipticine series, the most cytotoxic drugs were those that were easily oxidized in this system. In the present paper, we show that 9-hydroxyellipticine derivatives undergo a spontaneous oxidation, using molecular oxygen as electron acceptor (autoxidation). Such a process is responsible for the generation of the reactive species of the drugs and the toxic forms of oxygen that are possibly involved in the mechanism of the cytotoxicity of these molecules.

Results

Autoxidation of 9-OH-E in Aqueous Solutions. A number of phenols undergo autoxidation at alkaline pH, and there is general agreement that the phenolate form of these molecules is the efficient electron donor.[4] We investigated the behavior of 9-OH-E in carbonate medium (pH between 10 and 11.20). As shown in Figure 1, the addition of 9-OH-E in air-saturated carbonate solution (pH 11.20) resulted in the spontaneous transformation of the drug to a red-colored compound (increase in absorbance in the 500-nm range). After extraction with methylene chloride and purification, the red compound was identified (see Experimental Section) as the two-electron oxidized product of 9-OH-E, and quinone imine 9-oxoellipticine (9-oxo-E) (Figure 2). This compound is identical with the one obtained during the peroxidase-catalyzed oxidation of 9-OH-E. As indicated in Figure 3, the oxidation of 9-OH-E to 9-oxo-E requires the presence of oxygen, which is the rate-limiting factor in air-saturated solution. Moreover, lowering the pH from 11.20 to 10 resulted in a decrease of the 9-OH-E oxidation rate, and below pH 10 no appreciable oxidation occurs, as detected by spectrophotometry. During the oxidation process, molecular oxygen was reduced by the transfer of two electrons from

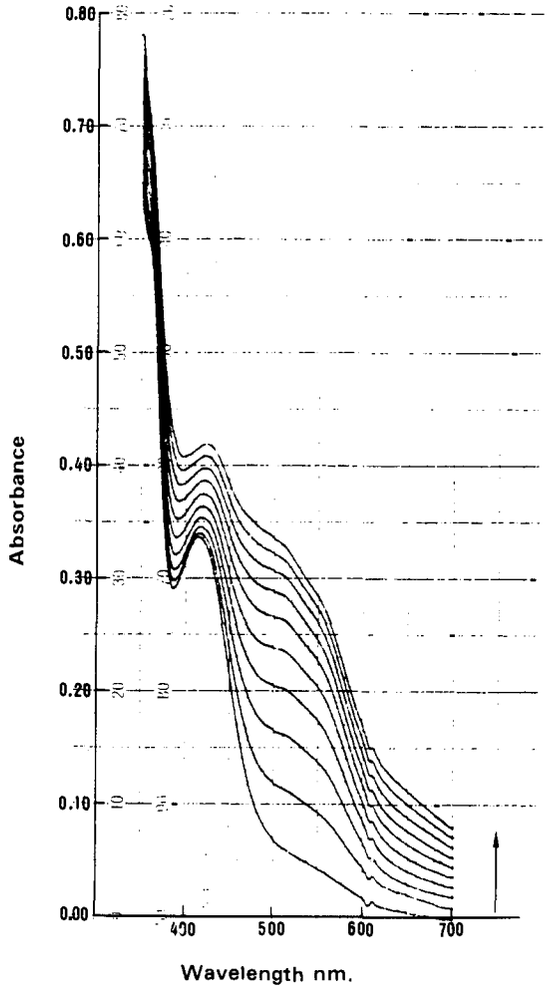


Figure 1. Change in absorbance associated with 9-OH-E autoxidation. Spectra were recorded each minute after the addition of 10^{-4} M 9-OH-E in a carbonate medium (pH 11.20) containing 0.05 M Na_2CO_3 and 10^{-4} M EDTA, at 37 °C. Accumulation of the spectra occurs as a function of time, as indicated by the arrow.

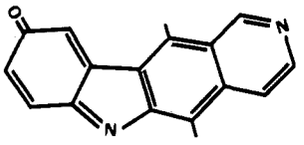


Figure 2. Structure of 9-oxoellipticine. The structure has been recently identified by NMR as indicated in ref 3.

9-OH-E, resulting in the production of hydrogen peroxide (H_2O_2) (see Experimental Section). Determination of the amount of 9-oxo-E generated at pH 11.20 by HPLC (see Experimental Section) indicated that during the first

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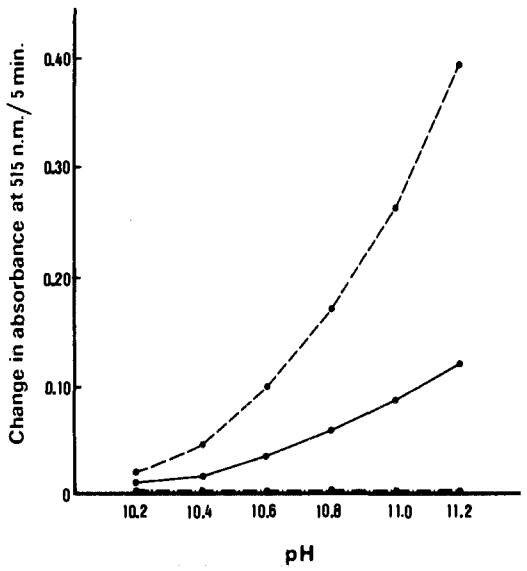

Figure 3. Effect of pH and oxygen tension on 9-OH-E autoxidation rate. Experiments were performed in a mixture containing 0.05 M Na_2CO_3, 10^{-4} M EDTA, 10^{-4} M 9-OH-E, at 37 °C. The pH's were adjusted by the addition of HCl: (•·····•) oxygen-saturated solutions; (•—•) air-saturated solutions; (•-·-·-•) argon saturated solutions.

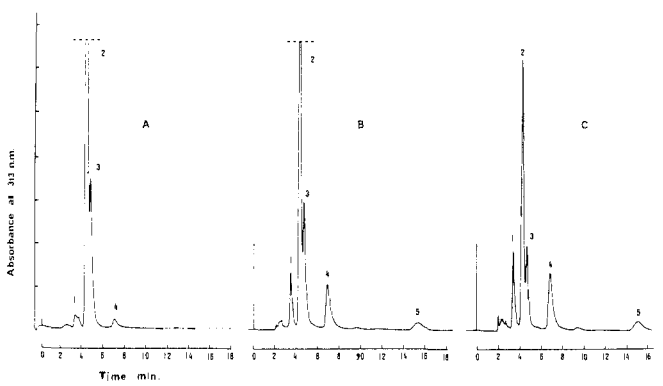

Figure 4. HPLC of aliquots of a carbonate mixture (pH 11.20) containing initially 10^{-4} M 9-OH-E: A, chromatogram obtained after 1-min incubation at 37 °C; B, obtained after 5 min; C, obtained after 10 min. Mobile phase: methanol/water, 70:30, containing 1 g of heptanesulfonate and 1 mL of acetic acid. Flow rate: 1.2 mL/min. Peaks: 1 and 5, not identified; 2, 9-OH-E; 3, 9-oxo-E; 4, 9-OH-E_2.

minute of the reaction, 9-oxo-E and H_2O_2 were produced in stoichiometric amounts (1:1), suggesting that the oxidation of 9-OH-E to 9-oxo-E occurs as shown in eq 1–3.

$$\text{9-OH-E} + OH^- \rightarrow \text{9-O}^-\text{-E} + H_2O \quad (1)$$

$$\text{9-O}^-\text{-E} + O_2 \rightarrow \text{9-oxo-E} + HO_2^- \quad (2)$$

$$HO_2^- + H_2O \rightarrow H_2O_2 + OH^- \quad (3)$$

HPLC experiments (Figure 5) indicate that secondary products were generated. Purification of the various fractions so obtained (see Experimental Section) led to the identification of the major secondary product (peak number 4 in Figure 4) as a dimer of 9-OH-E, which exhibits the structure shown in Figure 5. Moreover, results indicated in Figure 4 suggest that the dimer is generated by the direct reaction between 9-O^--E and 9-oxo-E, 9-O^--E acting as nucleophile as shown in eq 4.

$$\text{9-O}^-\text{-E} + \text{9-oxo-E} \rightarrow \text{9-OH-E}_2 \quad (4)$$

Thus, at alkaline pH, 9-OH-E undergoes an autoxidation reaction that generates H_2O_2, the quinone 9-oxo-E, and the

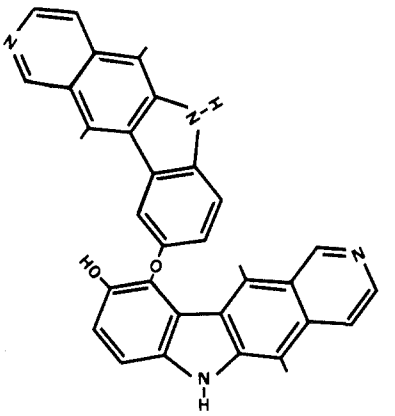

Figure 5. Structure of the dimer 9-OH-E_2. The structure of this compound has been previously identified by NMR as indicated in ref 25.

Table II. Effect of Oxygen Tension on the Generation Rate of the Dimer 9-OH-E_2 at Various pH's[a]

	9-OH-E_2 generation rate, nmol/h		
	pH 6.00	pH 7.40	pH 8.60
argon	0	0	0
air	0	1.03	2.80
oxygen	0	1.72	5.20

[a] Experiments were performed at 37 °C in 0.05 M phosphate buffer containing 10^{-4} M EDTA and 10^{-4} 9-OH-E. Amounts of 9-OH-E_2 generated were estimated by HPLC (see Experimental Section) after 2-h incubation.

dimer 9-OH-E_2. Below pH 10, the accurate determination of the 9-OH-E oxidation rate is not possible by using either the determination of oxygen uptake (which is too low) or by the measurement of 9-oxo-E produced (as it reacts with 9-OH-E to form 9-OH-E_2). Fortunately, 9-OH-E_2 remains stable in solution, and it is possible to determine the HPLC (see Experimental Section) the amount of dimer generated. This is an exact measure of the 9-OH-E autoxidation rate. Results obtained by this method are given in Table II. 9-OH-E_2 is effectively generated at physiological pH, in air-saturated phosphate buffer. Moreover, the dimer is not generated at acidic pH or in the absence of oxygen. In this pH range, the 9-OH-E oxidation rate remains very low, in agreement with the fact that the phenolate form of the drug (9-O^--E) is the electron donor.

Involvement of One-Electron Transfer in the 9-OH-E Autoxidation Process. During 9-OH-E autoxidation, electrons are transferred from 9-O^--E to oxygen. This step may occur either through one-electron transfer, generating superoxide anion ($O_2^{-\cdot}$) and the free radical of the drug as intermediates, or though two-electron transfer, generating directly H_2O_2 and the quinone 9-oxo-E. We have performed a series of experiments in alkaline Me_2SO, which allows the formation of a great amount of the phenolate form of 9-OH-E. In the presence of oxygen, the reaction shown in eq 5 may occur.

$$\text{9-O}^-\text{-E} + O_2 \rightarrow O_2^{-\cdot} + \text{9-O}\cdot\text{-E} \quad (5)$$

In a proton-deficient solution such as alkaline Me_2SO, the $O_2^{-\cdot}$ and 9-O·-E possibly generated remain stable in solution and can be detected by EPR spectroscopy. The free radical of the drug can be detected at room temperature, whereas $O_2^{-\cdot}$ can be identified at low temperature from the appearance of a spectrum that exhibits $g_\parallel$ near 2.09 G and an unusual temperature dependance. Figure 6 shows the EPR spectra obtained at room temperature (I) and at low temperature (II) with alkaline Me_2SO in the absence (spectra a) and in the presence of 9-OH-E (spectra

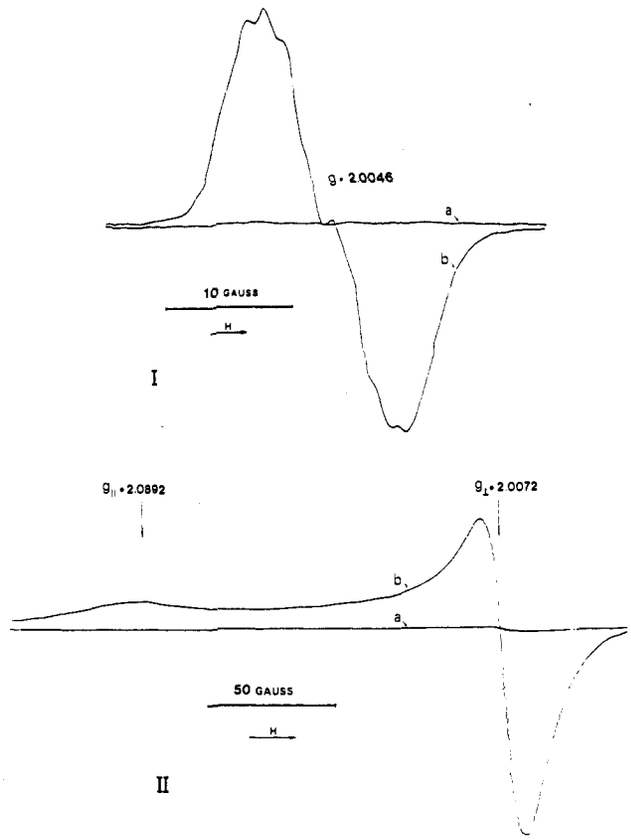

Figure 6. EPR spectra generated in alkaline Me_2SO. Spectra in I were recorded at room temperature, whereas spectra in II were recorded at 110 K. Spectra a (I and II) were obtained with Me_2SO containing 0.55 M water and 5×10^{-3} M NaOH. Spectra b (I and II) were obtained in similar mixture but in the presence of 10^{-4} M 9-OH-E. In all cases, the microwave frequency was 9.753 GHz, with 1 G modulation and 250-mW microwave power.

b). The unresoluted spectrum Ib is related to the free radical of the drug 9-O·-E, whereas spectrum IIb is the typical spectrum of $O_2^{-}\cdot$ showing a very broad $g_{\parallel}$ in the region of $g = 2.09$. In air-saturated Me_2SO, a nonnegligeable amount of $O_2^{-}\cdot$ is generated in the absence of drug.[5] However, this amount remains very low as compared to that obtained in the presence of 9-OH-E.

Thus, 9-O$^-$-E may transfer one electron on molecular oxygen to generate 9-O·-E and $O_2^{-}\cdot$. This assumption can be confirmed in aqueous solution near physiological pH by the use of the spin trap DMPO as a probe. The incubation of 9-OH-E in phosphate buffer (pH 7.40) in the presence of 50 mM DMPO resulted in the spectra of Figure 7a,b. This 1:2:2:1 quartet ($g = 2.0060$, $a^N = a^H = 15.0$ G) is comparable to the spectrum attributed to the hydroxyl radical (OH·) spin adduct of DMPO.[6] When superoxide dismutase (SOD) was added to the reaction mixture, the signal was suppressed (Figure 7c), indicating that $O_2^{-}\cdot$ is involved in the generation of this DMPO adduct. An identical signal is obtained (Figure 7d) by the addition of K^+, $O_2^{-}\cdot$ in the phosphate medium. The presence of the DMPO/OH· spin adduct instead of the expected DMPO/$O_2^{-}\cdot$ can be explained either by the secondary production of OH· through a Fenton reaction[7] or by the breakdown of the DMPO/$O_2^{-}\cdot$ to DMPO/OH· as previously described.[8] However, the addition of catalytic amounts of catalase or 40 mM of the OH· scavenger benzoate in the reaction mixture did not affect the nature of the signal, suggesting that OH· is not involved in the formation of the DMPO/OH·. We conclude that at physiological pH, 9-OH-E spontaneous transfers one electron to molecular oxygen.

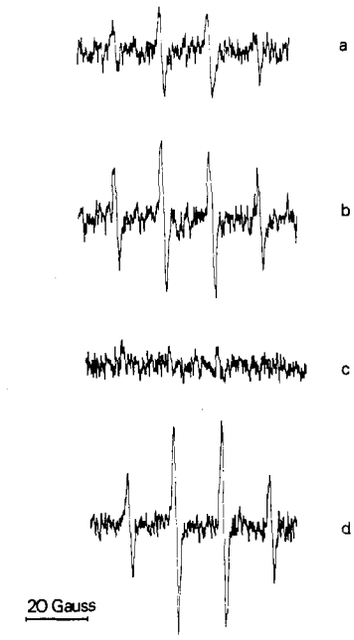

Figure 7. EPR spectra of DMPO spin adducts. Spectra were recorded at room temperature by using a microwave frequency of 9.670 GHz, 1.25-G modulation, and 100-mW microwave power. Spectra a and b were obtained in a mixture composed of 0.05 M phosphate buffer (pH 7.40), 10^{-4} M EDTA, 50 mM DMPO, and 10^{-4} M 9-OH-E. Spectrum a was recorded after 1-h and spectrum b after 2-h incubation at 37 °C. Spectrum c was obtained in the same manner as spectrum b, except that 1 μg/mL of SOD was added in the mixture. Spectrum d was obtained in the same manner as spectrum b, except that 9-OH-E was replaced by $K^+O_2^{-}$.

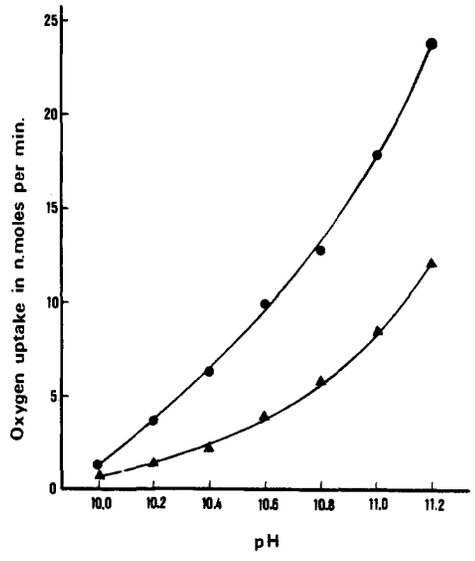

Figure 8. Effect of SOD on 9-OH-E autoxidation rate at various pH's. Experiments were performed in 0.05 M Na_2CO_3 containing 10^{-4} M EDTA and 10^{-4} M 9-OH-E at 37 °C: (▲–▲) experiments performed in the absence of SOD; (●–●) experiments performed in the presence of 2 μg/mL of SOD.

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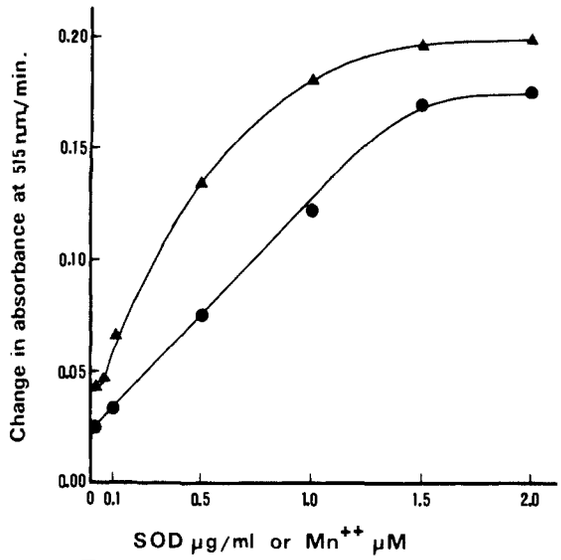

Figure 9. Effect of SOD and manganese ion on the 9-OH-E autoxidation rate. Experiments were performed in 0.05 M Na_2CO_3 (pH 11.20) (37 °C) containing 10^{-4} M EDTA (expected for Mn^{2+} assay) and 10^{-4} M 9-OH-E: (●–●) SOD; (▲–▲) Mn^{2+}.

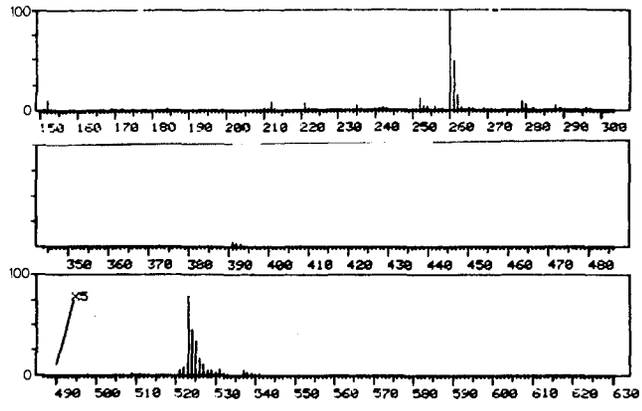

Figure 10. Desorption chemical ionization (DCI) spectra of 9-oxo-E (m/z 260) and 9-OH-E_2 (m/z 523). 9-Oxo-E and 9-OH-E_2 were purified from a carbonate mixture (pH 11.20) containing initially 10^{-3} M 9-OH-E.

Effect of Superoxide Dismutase and Manganese Ions on the 9-OH-E Autoxidation Rate. The autoxidation of diphenols involves $O_2^{-}\cdot$ as the chain-propagating species.[4,9,10] The addition of SOD results in a marked inhibition of the oxidation rate of these molecules. In contrast with this model, the addition of SOD in carbonate mixtures containing 9-OH-E resulted in a strong increase in the 9-OH-E oxidation rate, as determined by both oxygen consumption and generation of the quinone (see Figures 8 and 9). This effect is also obtained with metals having SOD-like activities, such as manganese ions[11] (Figure 10). In phosphate buffer, both SOD and manganese ions increase the 9-OH-E oxidation rate, as observed by the increase in the generation rate of 9-OH-E_2 (Table III). The chelating agent EDTA strongly decreases the 9-OH-E oxidation rate, indicating that traces of metal ions present in the buffer may act as a catalyst of the reaction. The increasing effect of SOD and metal ions on the 9-OH-E autoxidation rate can be explained by the displacement toward the right of the equilibrium in eq 5

Table III. Effects of Manganese and SOD on the 9-OH-E_2 Generation Rate at various pH's[a]

	9-OH-E_2 generation rate, nmol/h		
	pH 6.00	pH 7.40	pH 8.60
-EDTA	0.30	2.70	7.40
+EDTA (10^{-4} M)	0	1.03	2.80
-EDTA + Mn^{2+} (0.1 mM)	0.30	7.80	20.0
+EDTA + SOD (2 μg/mL)	0.40	3.80	10.60

[a] Experiments were performed in phosphate buffer 0.05 M (37 °C) containing 10^{-4} M 9-OH-E. Amounts of 9-OH-E_2 generated were estimated by HPLC (see Experimental Section) after 2-h incubation.

leading to a decrease of $O_2^{-}\cdot$ concentration at the steady state. Thus, the sequence of reactions leading to the oxidation of 9-OH-E can be written as shown in eq 5–7.

$$9\text{-}O^{-}\text{-}E + O_2 \rightleftarrows 9\text{-}O\cdot\text{-}E + O_2^{-}\cdot \quad (5)$$

$$O_2^{-}\cdot + O_2^{-}\cdot + 2H^{+} \xrightarrow{SOD,\ Mn^{2+}} H_2O_2 + O_2 \quad (6)$$

$$9\text{-}O\cdot\text{-}E + 9\text{-}O\cdot\text{-}E \rightarrow 9\text{-oxo-}E + 9\text{-}O^{-}\text{-}E \quad (7)$$

A similar mechanism has been previously proposed for the SOD-inhibitable reduction of nitroblue tretrazolium dye (NBT),[12] as well as for the SOD-inhibitable reduction of methemoglobin by the menadione semiquinone.[13]

Autoxidation of Various Ellipticine Derivatives. We have tested (1) the ability of ellipticine derivatives to consume oxygen in carbonate medium (pH 11.20) in the presence and in the absence of SOD and (2) the ability of the drugs to generate $O_2^{-}\cdot$ in phosphate buffer (pH 7.40). The results obtained are summarized in Table IV and indicate that only drugs having an OH group in position 9 may transfer electrons on molecular oxygen. These drugs are those that can be oxidized by the peroxidase–H_2O_2 system.[3] However, at pH 11.20 and in the absence of SOD, both N^2-Me-9-OH-E and N^2,N^6-Me_2-9-OH-E are not oxidized (lack of oxygen consumption). A slight oxygen uptake occurs in the presence of SOD, and $O_2^{-}\cdot$ can be detected both in alkaline Me_2SO and in phosphate buffer. These results suggest that in aqueous medium these drugs generate a very slight amount of $O_2^{-}\cdot$ through reaction 5 and that addition of SOD or DMPO displaces the equilibrium by consumption of $O_2^{-}\cdot$.

Discussion

In addition to the interaction with nucleic acids through intercalation, it has been suggested that the cytotoxic activity of hydroxyellipticine derivatives may be due to their possible transformation to reactive and toxic metabolites. In this way, using a peroxidase–H_2O_2 system as a model of biological oxidation, we have previously demonstrated that drugs hydroxylated at the C-9 position may undergo one- and two-electron oxidation, yielding electrophilic quinones. Here, we show that 9-OH-E may undergo similar transformation through an autoxidation process (i.e., using molecular oxygen as electron acceptor). The autoxidation reaction initially involves a one-electron transfer from the drugs to molecular oxygen, generating $O_2^{-}\cdot$ and the free radical of the drug. Unfortunately, because of the poor resolution of the EPR spectrum of the 9-OH-E radical, it is not possible to characterize the structure of this radical. However, from the results ob-

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Table IV. Occurrence of the Autoxidation of Some Ellipticine Derivatives in Various Systems

no.	drug	O_2 consumption (pH 11.20), nmol/min		production of $O_2^{-}\cdot$	
		−SOD	+SOD	DMSO/OH^-	DMPO, pH 7.40
1	E	0	0	−	−
2	N^2-Me-E	0	0	−	−
3	9-OH-E	13.10	23.50	+	+
4	N^2-Me-9-OH-E	0	1.02	+	+
5	N^2,N^6-Me_2-9-OH-E	0	0.44	+	+
6	7-OH-E	0	0	−	−

Table V. Cytotoxic Activities and Possible Formation of Reactive Compounds of Ellipticine Derivatives

no.	drug	ID_{50},[a] μM	reactive compds generated in the presence of peroxidase/H_2O_2[b]	reactive compds generated during autoxidation
1	E	0.99	none	none
2	N^2Me-E	1.68	none	none
3	9-OH-E	0.015	quinone	$O_2^{-}\cdot$, free rad, quinone
4	N^2-Me-9-OH-E	0.050	quinone	$O_2^{-}\cdot$, free rad, unidentified
5	N^2,N^6-Me_2-9-OH-E	0.041	free radical	$O_2^{-}\cdot$, free rad, unidentified
6	7-OH-E	5.44	none	none

[a] Dose that reduced by 50%, after 48 h, the L1210 cell growth as compared to controls; results taken from ref 26. [b] Results taken from ref 3.

tained by Perez-Reyes and Mason[14] with 5-hydroxyindole and related compounds, it is reasonable to think that the semiquinone imine free radical of 9-OH-E was generated. In aqueous medium, $O_2^{-}\cdot$ and the free radical of the drug dismutate, yielded, respectively, H_2O_2 and the quinone 9-oxo-E. The quinone further undergoes a nucleophilic attack from the 9-OH-E remaining in the solution to form the dimer 9-OH-E_2. An interesting feature is that SOD markedly increases the 9-OH-E oxidation rate. This catalytic activity can be considered as an oxidase reaction, and SOD acts in this particular case as an indophenol oxidase. A metal ion such as manganese, which exhibits an SOD-like activity, catalyzes the 9-OH-E oxidation. In the ellipticine series, all drugs having an OH group at the C-9 position exhibit the ability to transfer one electron to molecular oxygen. This was obvious from the generation of $O_2^{-}\cdot$ observed both in a strong oxidizing medium, such as alkaline Me_2SO, and in phosphate buffer, at physiological pH, in the presence of the spin trap DMPO. However, in contrast to 9-OH-E, the quaternarized drugs N^2-Me- and N^2,N^6-Me_2-9-OH-E exhibit, even at alkaline pH, a very low rate of oxidation. At pH 11.20, an oxygen uptake can only be detected in the presence of SOD. This indicates that the quaternarized drugs can be oxidized through a similar process as 9-OH-E but that the initial step, as indicated in the following reaction (eq 8), is

$$N^2\text{-Me-9-O}^{-}\text{-E} + O_2 \rightleftarrows N^2\text{-Me-9-O}\cdot\text{-E} + O_2^{-}\cdot \quad (8)$$

strongly displaced toward the left. Both SOD and the spin trap DMPO can displace this equilibrium toward the oxidation of drugs.

From a pharmacological point of view, two distinct phenomena must be considered. The first is the generation of $O_2^{-}\cdot$ as an intermediate of the oxidation of the drugs, and the second is the generation of reactive metabolites of the drugs. It is well known that $O_2^{-}\cdot$ is an unstable compound directly or undirectly cytotoxic.[15] Many investigators have suggested that $O_2^{-}\cdot$ formed from semiquinone radicals of mitomycin C, daunorubicin, adriamycin, and streptonigrin may be responsible for the toxic action of these compounds.[16] In fact, $O_2^{-}\cdot$ is a normal byproduct of the reduction of oxygen, and SOD is thought to protect aerobic cells against $O_2^{-}\cdot$ mediated injury.[15] It has been claimed that tumor cells could be more sensitive to the toxic effects of $O_2^{-}\cdot$.[17] In this case, drugs generating $O_2^{-}\cdot$ inside the cells would exhibit a potential selective toxicity toward tumor cells. In the ellipticine series, only 9-OH-E may generate a significant amount of $O_2^{-}\cdot$, and this drug exhibits the most cytotoxic activity on cultured L1210 leukemia cells (Table V). In addition to the generation of $O_2^{-}\cdot$, the aerobic oxidation of 9-hydroxyellipticine derivatives generates free radical and quinone products of the drugs. Both free radicals and quinones derived from hydroxyellipticines have been demonstrated to be strongly electropholic molecules and are therefore potentially cytotoxic. A striking feature is that an unusually high proportion of anticancer agents, including adriamycin, daunorubicin, rubidazone, mitomycin C, streptonigrin, and lapachol, contains quinone groups. The reduction of these compounds seems to be a common prerequisite for the development of their cytotoxic effects.[18,19] The one-electron reduction of these drugs generates strong reducing semiquinone radicals, which can transfer their single electron to oxygen to form $O_2^{-}\cdot$,[16] whereas a two-electron reduction generates the hydroquinone products, leading to the formation of a carbocation-containing quinone.[19] Hydroxyellipticines can be considered as a reduced form of the corresponding quinone. The initial reduction step is not required, since, unlike the anthraquinone derivatives, their stable form is the phenolic product. The aerobic oxidation of 9-hydroxyellipticines can be catalyzed by a metalloenzyme, such as SOD, or by free manganese, whereas myeloperoxidase, which is an iron-containing enzyme, catalyzes the oxidation of these drugs in the presence of H_2O_2 as electron acceptor. These observations indicate that 9-hydroxyellipticine derivatives can be oxidized to reactive compounds inside the cell through various metalloenzyme-catalyzed reactions. The results summarized in Table V indicate that the most cytotoxic drugs are those that can be oxidized to reactive compounds. However, the

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occurrence of such processes in cells remains to be demonstrated.

Experimental Section

Absorption spectra, as well as other spectrophotometric studies, were performed on a Beckman Acta III spectrophotometer. Mass spectra were performed on a Riber R-10-10 apparatus by using the DCI (NH_3) method. EPR studies were performed on a Bruker B-ER 420 apparatus. Oxygen tension was measured by an oxygen electrode from Yellow Springs Instruments, recorded with a Gilson Oxygraph. HPLC was performed on a Waters apparatus using a μBondapak C18 column.

Chemicals. Superoxide dismutase from bovine red blood cells was purified according to McCord and Fridovich.[20] The spin trapping agent 5,5-dimethylpyrroline 1-oxide (DMPO) was purchased from Aldrich Chemical Co. and purified according to Buettner and Oberley.[21] The concentration of the stock solution was determined spectrophotometrically by using a molar extinction of 7700 M^{-1} cm^{-1} at 234 nm.

Ellipticines Derivatives. Ellipticine (E), ellipticinium (N^2-Me-E), 9-hydroxyellipticine (9-OH-E), 9-hydroxyellipticinium (N^2-Me-9-OH-E), and N^6-methyl-9-hydroxyellipticinium (N^2,N^6-Me_2-9-OH-E) were synthesized according to Dalton et al.[22] 7-Hydroxyellipticine (7-OH-E) was synthesized according to Lallemand et al.[23] and was a gift of Dr. Mansuy.[24] 9-Oxoellipticine (9-oxo-E) was prepared by the oxidation of 9-OH-E in the presence of peroxidase–H_2O_2 as previously described.[3] The dimer of 9-OH-E (9-OH-E_2) was purified from the industrial source of 9-OH-E as described by Muzard[25] or prepared as follows: A solution of 50 mg 9-OH-E in 10 mL of Me_2SO was added to a solution of 25 mg of 9-oxo-E in 10 mL of Me_2SO. The mixture was stirred overnight at room temperature and then applied to a Biobeads SM2 column. Me_2SO was removed by extensive elution with water. The remaining compounds were eluted with pure methanol. This solution was evaporated, and the solid material was dissolved in ethanol–ammonium acetate buffer, 0.1 M, pH 6.5, 60:40. After filtration, the mixture was applied on a Servachrom XAD-2 (100 μm) resin column. 9-OH-E was eluted by the same mixture as described in ref 25, whereas the remaining compound was eluted by methanol–ammonium acetate buffer 0.1 M, pH 6.5, 60:40, and identified as 9-OH-E_2 by spectrophotometry, mass spectrum [m/z 522 (M^+)], and HPLC.

Autoxidation of 9-OH-E and Purification of the Oxidized Products. Autoxidation of 9-OH-E was carried out in a carbonate medium (pH 11.20) composed of 0.05 M Na_2CO_3 and 10^{-4} M EDTA. 9-OH-E·HCl, dissolved in water, was added to a final concentration of 1 mM, and the solution was allowed to stand at room temperature for 30 min. An insoluble red-colored material was extracted with methylene chloride. This extract was dried under vacuum at 30 °C. The remaining material was dissolved in chloroform–ethyl acetate (v/v), applied to a silica gel column, and eluted with the chloroform–ethyl acetate mixture. The aqueous phase was evaporated, and the remaining material was redissolved in methanol–ammonium acetate buffer, 0.1 M, pH 6.5, 40:60. After filtration, the solution obtained was applied to a Servachrom XAD-2 column. The first compound was eluted as a single peak by the same solvent, whereas a second compound was eluted by methanol–ammonium acetate buffer, 0.1 M, pH 6.5, 60:40.

Identification of the Oxidized Products. The compound obtained from the methylene chloride extract exhibits a mass spectrum [m/z 260 (M^+) (Figure 10)] and a HPLC retention time in agreement with the quinone imine 9-oxo-E. The first compound eluted from the XAD-2 column exhibits a mass spectrum [m/z 262 (M^+)] and a HPLC retention time in agreement with 9-OH-E. The second compound eluted from the XAD-2 column exhibits a mass spectrum [m/z 522 (M^+) (Figure 10)] and a HPLC retention time identical with the one obtained with the authentic dimer 9-OH-E_2.

Determination of the Concentration of 9-Oxo-E and 9-OH-E_2. Determination of the 9-oxo-E concentration was performed by HPLC using a μBondapak C18 column with an elution mixture composed of acetonitrile–ammonium acetate buffer, 0.002 M, pH 4.5. A calibration curve was obtained by using pure 9-oxo-E. The 9-OH-E_2 concentration was also determined by HPLC by using an elution mixture composed of methanol–water, 70:30, containing 1 g of heptanesulfonate and 2 mL of acetic acid. A calibration curve was obtained with pure dimer. In all cases, the eluted compounds were detected at 313 nm, and the areas under the peaks were computed by an integrator Sigma 10 (Perkin-Elmer).

Oxygen Consumption and H_2O_2 Production. Oxygen consumption and H_2O_2 production were carried out in a cylindrical glass chamber (1 mL volume) equipped with a magnetic stirrer and Clark electrode. In air-saturated solutions, the oxygen concentration was estimated to be 0.20 nmol/mL at 37 °C. The H_2O_2 concentration was determined by the amount of oxygen released in the presence of catalase, an enzyme that catalyzes the decomposition of H_2O_2 according to the reaction shown in eq 9.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (9)$$

Cytotoxic Test. Inhibition of cell growth was determined with L1210 lymphocytic leukemia cells as previously described.[26] The inhibitory efficiency against cell multiplication is expressed in terms of ID50, which represents the drug concentration that reduces the rate of cell multiplication by 50% as compared to the control. The values presented in this work have already been published.[26]

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Registry No. 2, 69467-91-0; 3, 51131-85-2; 4, 58337-34-1; 5, 56501-52-1; 6, 67484-39-3; 9-oxo-E, 76004-29-0; 9-OH-E_2, 86542-48-5; $O_2^{-\cdot}$, 11062-77-4; SOD, 9054-89-1.

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