Biochemical Pharmacology, Vol. 28 pp. 345–350

0006-2952/79/0201-0345 $02.00/0

ELLIPTICINE AND DERIVATIVES INDUCE BREAKAGE OF L1210 CELLS DNA *IN VITRO*

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(*Received* 3 *May* 1978; *accepted* 13 *June* 1978)

Abstract—Part of the radioactive DNA extracted from prelabelled L1210 cells exposed *in vitro* to ellipticine, 9-hydroxyellipticine, 2-methyl-9-hydroxyellipticinium and 9-aminoellipticine, migrate more slowly into an alkaline sucrose gradient. These effects occur in less than one hr of exposure, and can be detected at drug concentrations which lie respectively around 1000, 20, 100 and 500 ng per ml whereas the drug concentrations which inhibit by 50 per cent the rate of the *in vitro* cell growth are 242, 3.9, 13 and 53 ng per ml. The DNA breaks become rapidly undetectable when the cells' exposure to the drugs is discontinued.

Ellipticine (5,11-dimethyl-(6H)-pyrido[4,3-b] carbazole) and a number of its derivatives are cytotoxic agents, some of which are very active at concentrations of about 10^{-8} M [5]. These substances are endowed with antitumor properties [1, 2] and one of them, 2-methyl-9-hydroxyellipticinium has been submitted to preliminary trials on human cancers [3]. They also bind strongly to DNA and most of them intercalate into DNA base pairs [2, 4]. They inhibit *in vivo* DNA synthesis [5, 6]. Moreover, it has been established that some ellipticines are mutagenic, even in the absence of microsomes, using the Ames *Salmonella* test and provided that the 2N pyridinic nitrogen is not quaternarized [7]. These data suggest that DNA could provide a biological target involved when cells are exposed to the ellipticines.

It is known that some other antitumor drugs, also belonging to the DNA intercalating group, such as actinomycin D [8] and anthracycline derivatives [9], are able to induce *in vivo* fragmentation of DNA in eucaroytic cells. It could therefore be of interest to look for a similar effect with the ellipticines (Table 1). This paper demonstrates that L1210 cells exposed *in vitro* to ellipticine (E), 9-hydroxyellipticine (9 OHE) (NSC 210717) 2-methyl-9-hydroxyellipticinium (2-CH_3 9 OHE) (NSC 264137) or 9-aminoellipticine (9 NH_2E) yield a more fragmented DNA than untreated cells after lysis in detergent–alkaline sucrose. L1210 cells were chosen as the biological material because they can be bio-assayed into mice and lend themselves to quantitative assessments of their viability and of their cytotoxic damage.

MATERIALS AND METHODS

L1210 cells§ [10] were propagated in sterile R.P.M.I. medium 1640 (Gibco) supplemented by 20 per cent heat inactived horse serum, L. glutamine 2 mmol/ml, penicillin 200 U/ml and streptomycin 50 μg/ml. The cultures were repeatedly tested and shown to be free of contaminating bacteria or mycoplasma. Cell counts were performed using a hemocytometer after dilution. Viability was estimated by the ability of cells to exlude Trypan Blue.

The average doubling time was about 12 hr (Fig. 1). The inhibitory dose 50 (ID_{50}) is the drug concentration which reduces by 50 per cent the rate of cell multiplication relative to control.

Cell DNA was labelled prior to drug treatment by growing the cells (2×10^5 cells/ml) for approximately 20 hr in the presence of [methyl-^{14}C]thymidine (specific activity 53.9 mCi/mmol, CEA, France) 0.06 μCi/ml. The cells were spun down at 1500 rev./min. for 5 min. They were suspended again to their original volume in a fresh medium, for 1 hr prior to exposure to the drugs. Then 2 ml aliquots of these cells were incubated at 37° with and without drugs for periods ranging from one to eight hr. At the end of each incubation, the cells were centrifuged at 1500 rev./min. for 5 min, suspended in 200 μl of NaCl–EDTA solution pH 7.4 (75 mM NaCl–24 mM EDTA) and layered on top of a linear 5 to 20 per cent alkaline sucrose gradient containing 0.5 M NaCl, 0.3 M NaOH and 0.01 M EDTA. The top of the gradients contained 0.5 ml of a lysing solution consisting of 1% sarkosyl and 2.5% alkaline sucrose. After a 20 hr lysis, the runs were performed at 20° in a Spinco centrifuge in conditions described under the legends of each figure.

After centrifugation, 9 drops fractions were collected from the top of the gradient. 100 μg of carrier DNA (calf thymus) were added to each fraction, and precipitated with 2 ml of cold 5% trichloroacetic acid. After remaining at 4° for at least 30 min the samples were filtered through Whatman GF/C glass fiber filters, washed with 10 ml 5% TCA at 4° with 95%

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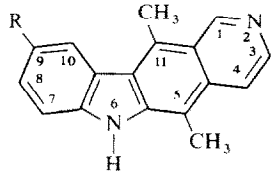
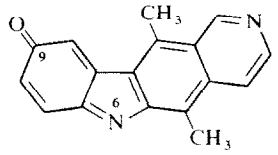
§L1210 cells were received through the courtesy of Dr. I Gresser (I.R.S.C. Villejuif).

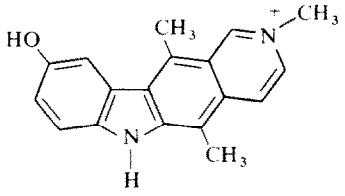
Table 1. Structure of ellipticine and derivatives



R = H: Ellipticine (E)
R = OH: 9-hydroxyellipticine (9 OHE)
R = NH_2: 9-aminoellipticine (9 NH_2E)



quinone imine structure derived from the 9-hydroxylated derivative of ellipticine



2-methyl-9-hydroxyellipticinium (2-CH_3 9 OHE)

ethanol and air dried. The radioactivity was measured by liquid scintillation counting. The recovery yield was usually around 80 per cent.

Oxygen uptake was assayed at 22° using a Gilson oxygraph equipped with a Clark oxygen electrode. The assay medium contained 6.16 mM KCl, 9.35 mM Na_2HPO_4, 1.65 mM NaH_2PO_4 and 0.9% NaCl pH 7. The cell concentration was 15.10^6 cells/ml.

Ellipticine (5,11-dimethyl(6H)pyrido[4,3-b-carbazole) was synthesized according to Dalton *et al.* [1]. 9-hydroxyellipticine and 9-aminoellipticine were prepared as formerly described [11]. These drugs were kindly provided by P. Lecointe. 2-methyl-9-hydroxyellipticinium was a generous gift from Dr. N. Dat Xuong (CNRS, Gif-sur-Yvette). The purity of these compounds was checked by HPLC and was found to be better than 95 per cent.

Each of the four ellipticines was dissolved in sterile distilled water. Antinomycin D was supplied by the Sigma Chemical Co.

RESULTS

The growth rate of the cell population at the starting concentration, about 10^5 cells per ml, remains rather constant for 48 hr (Fig. 1) in presence of increasing concentrations of the drugs under study. This observation will be further documented in another report [12].

It has been verified that the cells submitted for 48 hr

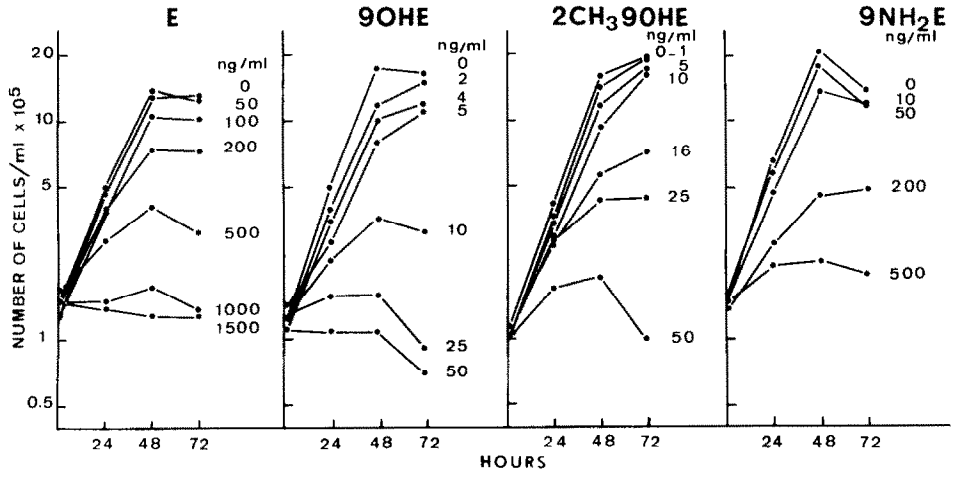


Fig. 1. Effects of ellipticine and its derivatives on L1210 cell growth in culture. Cells (approx. 1.10^5/ml) were incubated at 37° in 5% CO_2 with drug. Viable cell counts were determined as described in Materials and Methods.

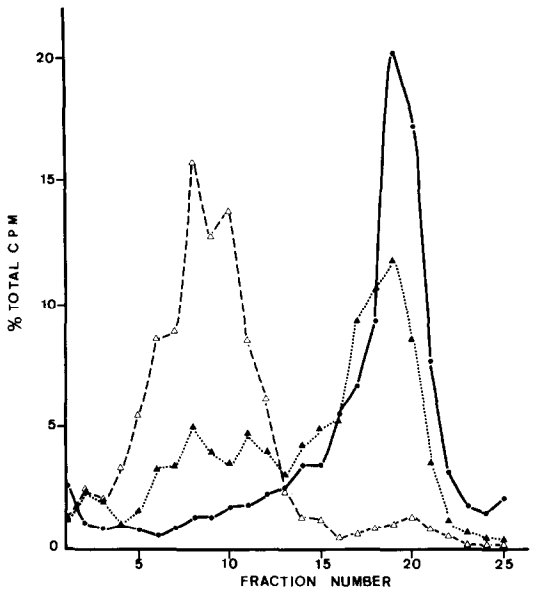


Fig. 2. Sedimentation of DNA [^{14}C]thymidine-prelabelled L1210 cells treated by actinomycin D and 9 OHE. 5 × 10^5 cells/ml were treated for 1 hr with(●) control, (△) actinomycin D, 10 μg/ml, (▲) 9 OHE 1 μg/ml. A SW27.1 rotor was used in a Beckman ultracentrifuge at 23,000 rev./min. for 4.5 hr at 20° Total c.p.m. found in control gradient are 49,320 c.p.m. Fractionation was from top (left) to bottom (right).

to each of the four drugs, are able to exclude the vital dye Trypan Blue. Under our experimental conditions, the proportion of cells which are permeable to this dye is around 3 per cent, whatever the treatment to which they are submitted. The oxygen consumption of the L1210 cells is about 5 μmol per min per 10^6 cells. Neither of the four drugs modify it in the range of concentration assayed here.

After treatment with 9 OHE, [^{14}C]thymidine-prelabelled L1210 cells yield radioactive DNA, a noticeable part of which migrates less rapidly into an alkaline sucrose gradient than the peak of the material extracted from untreated cells. Figure 2 gives one example of this effect compared to that obtained after exposure of the L1210 cells to actinomycin D. The fragmentation of DNA by actinomycin D is complete as demonstrated previously [8] whereas only a part of the DNA extracted from cells treated by 9 OHE migrates at higher speed through the sucrose gradient. However, the experimental conditions used for lysing and centrifuging the DNA display a critical influence on the size of the slowly moving peak. It was verified that no degradation of DNA occurs when the drugs are added to the lysis medium after layering control cells.

The breakdown of cellular DNA by 9 OHE and 2-CH_3 9 OHE was examined using a wide range of drug concentrations (Fig. 3). The L1210 cells in culture were incubated either for 1 hr with 9 OHE in doses ranging from 8 to 800 ng/ml (Fig. 3A) or for

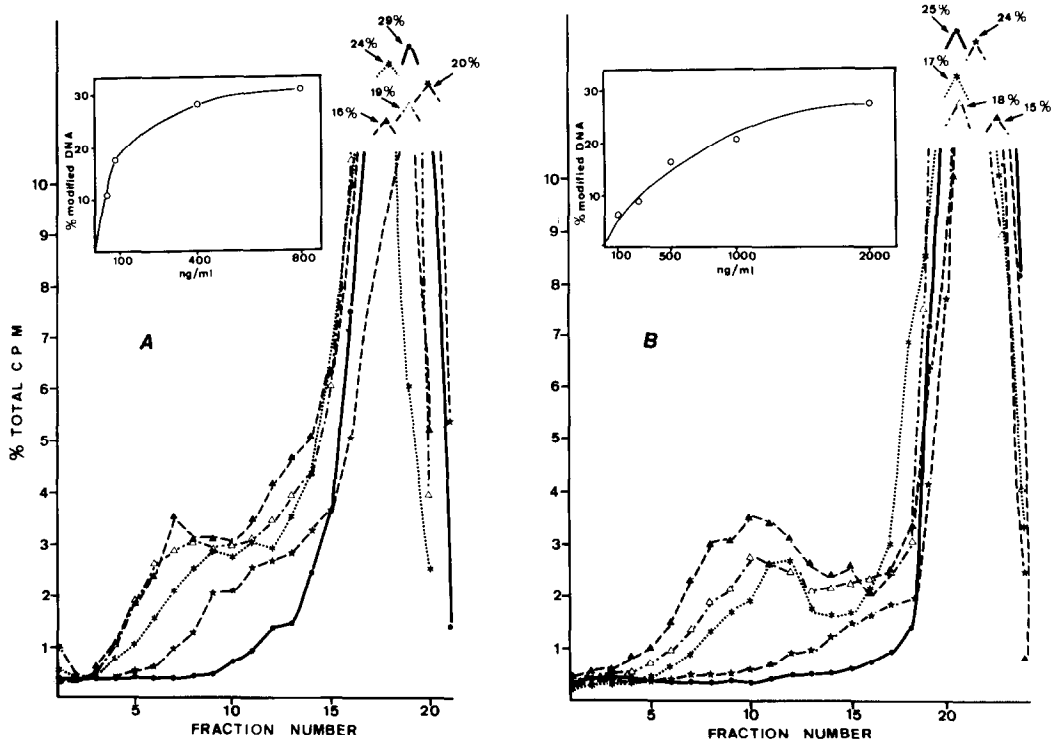


Fig. 3. Sedimentation of DNA from [^{14}C]thymidine-prelabelled L1210 cells treated by increasing amounts of 9 OHE and 2-CH_3 9 OHE. (A) 9 OHE, 5 × 10^5 cells incubated with (●) control, (★) 40 ng/ml, (*) 80 ng/ml, (△) 400 ng/ml, (▲) 800 ng/ml at 37° for 60 min. (B) 2-CH_3 9 OHE, 5 × 10^5 cells/ml incubated with (●) control, (★) 100 ng/ml, (*) 500 ng/ml, (△) 1000 ng/ml, (▲) 2000 ng/ml at 37° for 75 min. A SW50.1 rotor was used in a Beckman ultracentrifuge at 40,000 rev./min. for 45 min at 20°. Total c.p.m. found in control gradients are, (A) 43,850 c.p.m. and (B) 31,600 c.p.m. Fractionation was from top (left) to bottom (right).

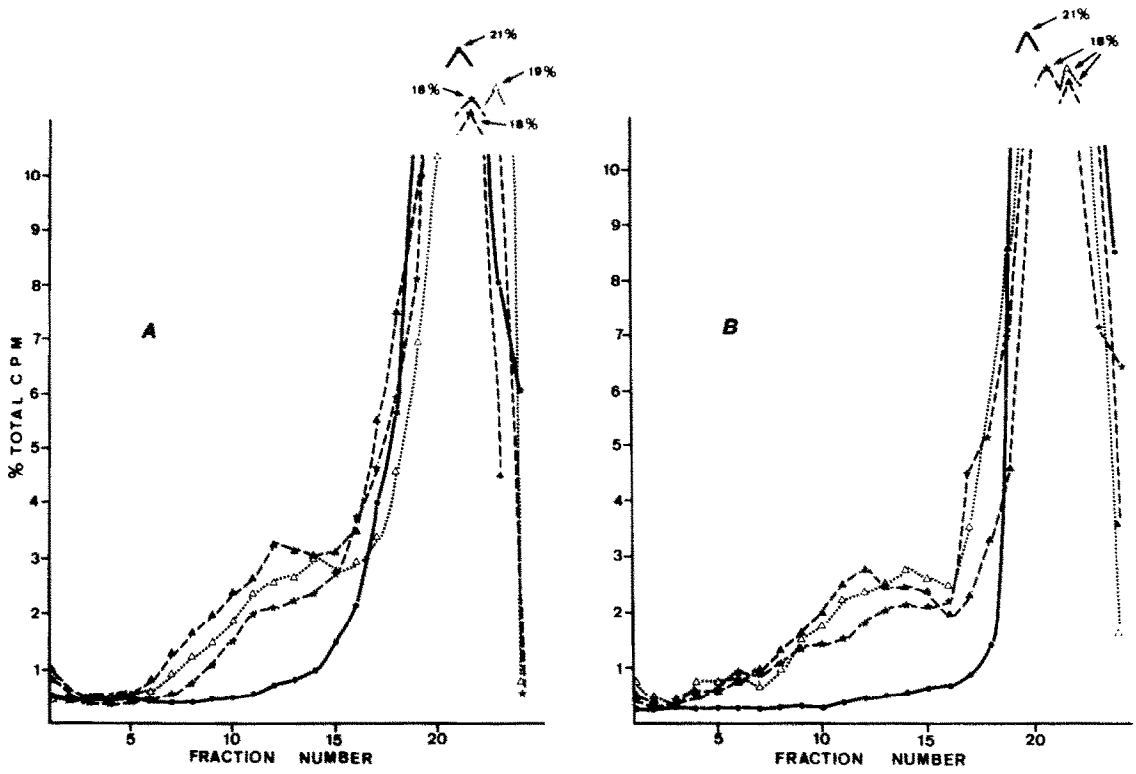


Fig. 4. Sedimentation of DNA from [^{14}C]thymidine prelabelled L1210 cells treated for varying times by 9 OHE and 2-CH_3 9 OHE. (A) 9 OHE: 200 ng/ml of drug. (B) 2-CH_3 9 OHE: 250 ng/ml of drug (●) 0 time; (★) 2 hr; (△) 4 hr; (▲) 8 hr. A SW50.1 rotor was used in a Beckman ultracentrifuge at 40,000 rev./min. for 45 min at 20°. Total c.p.m. found in control gradients are (A) 39,550 c.p.m.; (B) 55,536 c.p.m. Fractionation was from top (left) to bottom (right).

75 min with 2-CH_3 9 OHE in doses ranging from 100 to 2000 ng/ml. In these experimental conditions, the minimal doses at which some changes in the DNA sedimentation pattern can be detected lie around 20 ng/ml of 9-OHE and 100 ng/ml of 2-CH_3 9 OHE. The amount of the more rapidly sedimenting DNA increases with higher drug concentrations until about 400 ng/ml (inset Fig. 3A) in the case of 9 OHE or about 2000 ng/ml in the case of 2-CH_3 9 OHE (inset Fig. 3B). Larger doses of 9-OHE or 2-CH_3 9 OHE do not significantly modify the profiles of the gradients further.

The action of E and 9 NH_2E on the DNA of L1210 cells was studied under the same conditions stated above using doses from 100 to 5000 ng/ml and results similar to those described for 9 OHE and 2-CH_3 9 OHE were obtained for both drugs (not shown); the effects on DNA were first detected for E concentrations of around 1000 ng/ml and for 9 NH_2E concentrations of around 500 ng/ml.

Figure 4 shows the results of a time course study; the cells were exposed to each drug for one to eight hr at 9 OHE concentrations and 2-CH_3 9 OHE concentrations of 200 ng/ml and 250 ng/ml respectively. The fragmentation of DNA occurs within less than one hr of drug action; if the contact duration is increased, the fraction of slowly moving DNA in the alkaline sucrose gradients is not significantly enhanced.

In order to compare the threshold doses which induce biochemical modifications in DNA of L1210 cells *in vitro*, and the doses which modify the physiological behaviour of cultured cells, we determined the ID_{50} for each of the four drugs (Fig. 1 and Table 2). These determinations were carried out after growing the cells for 24 and 48 hr. Due to the rather constant—though reduced—rate of growth of the cells exposed to these drugs, whatever the time of exposure to the drugs, the measured ID_{50} is roughly independent of the exposure time. The fragmentation of DNA became apparent at drug concentrations several-fold higher than the ID_{50}; it occurred after the cell division came almost to a complete stop. We were unable to detect any break in DNA at drug concentrations close to ID_{50}. For each of the four drugs, the ratio of the minimal effective doses (in terms of biochemical modification of the DNA) to ID_{50}, was about the same, although the ID_{50} values varied from 1 (9 OHE) to about 60 (E).

Table 2. *In vitro* comparative effect of four ellipticine derivatives on the cellular growth rate (ID_{50}) and on the breakage of DNA (mice L1210 Leukemia)

Compound	ID_{50} (ng/ml)	Minimal effective DNA breaking dose (ng/ml)
Ellipticine	242	1000
9-Hydroxyellipticine	3.9	20
2-Methyl-9-hydroxyellipticinium	13.8	100
9-Aminoellipticine	53	500

The removal of the DNA breaks is very rapid after the exposure of the cells is discontinued; for instance, there are no detectable breaks in DNA extracted from cells maintained for three hr after the end of the exposure of L1210 cells to 9 OHE at 500 ng/ml in a fresh medium free of drug; when the drug concentration is increased to 1000 ng/ml, the rapidly sedimenting DNA found in the same experimental conditions is less than 2 per cent instead of about 30 per cent in DNA obtained from cells not allowed to recover.

DISCUSSION

E, 9 OHE, 2-CH_3 9 OHE and 9 NH_2E induce breaks on the DNA of L1210 cells exposed to them *in vitro*. There is little radioactive material in the top gradient fractions where mononucleotides and oligonucleotides would sediment. This result indicates that the breaking of DNA must have resulted from an endonucleolytic mode of attack rather than from an exonucleolytic one which would have yielded these small products.

The degradation of DNA cannot be attributable to a cellular lysis because there is no significant decrease in cell number during the drug exposure time whereas these cells keep their ability to exclude a vital dye and to consume oxygen. However the ellipticine treated cells are no longer able to kill mice after grafting them back [12].

The mechanisms of the cellular action of each of the four ellipticines and the nature of their targets could be the same in spite of marked differences in the physicochemical properties and metabolic fates of the drugs, because the first appreciable DNA damages occur at drug concentrations (Table 2) where ratios are close to the ratios of the ID_{50}.

No DNA damage has been recorded at drug concentrations which inhibit the leukemic cells growth and their tumorigenic ability. Although the sensitivity of the alkaline sucrose gradient for detection of DNA nicking is not precisely known, this result suggests that the DNA breaking could be cell-mediated, i.e. not actually initiated by a direct DNA–drug interaction, but, on the contrary, triggered by some level of cell damages caused by the drugs. Along this line, Williams *et al.* [13] have shown that an endonucleolytic type of DNA degradation is constantly associated with widely varying types of trauma on mammalian cells. Such an hypothesis might explain why so many chemically unrelated drugs are able to fragment cell DNA. That is the case, for instance, for bleomycin [14, 15], neocarzinostatin [16, 17], nitrosoureas [18], streptonigrin [19], mitomycin C [20] and acridine derivatives [21].

One interesting observation made in this work is the limited extent of DNA degradation (less than 30 per cent of total DNA) whatever the amount of the ellipticines (Fig. 3) or the time of exposure to them (Fig. 4).

Several explanations can be put forth for interpreting these data: (a) DNA degradation might depend on a specific cellular process which the drugs would be able to both trigger and inhibit. The final pattern of DNA modification would depend on the balance between these triggering and inhibiting actions. Along this line, it has been established that ellipticine induces the microsomal cytochrome P450 mixed monooxygenases in rats [22] whereas it strongly inhibits the same enzymes *in vitro* [23]; (b) the ellipticines while participating in the DNA breaking process, might concomitantly activate some DNA repair mechanisms; the rapid disappearance from treated cells of the slow migrating DNA after drug removal implies that the L1210 cells are active in the repair of ellipticine degraded DNA; or (c) the ellipticines might generate some reactive cytotoxic intermediates during their metabolic modification by some endogenous compound(s) present in limited amounts. These intermediates could be responsible for the DNA breaking effect; their concentration level would only be partially dependent on the ellipticine concentrations or on the duration of exposure to them; it would rather be controlled by the intracellular amounts of the ellipticines metabolizing compounds. Along this line, it has been proposed that several antitumor drugs, for instance anthracycline, streptonigrin, mytomycin C, submitted to intracullular oxydo-reduction processes, express their cytotoxicity after transformation into reduced reactive intermediates (see general review in [24]); it is likely that the 9-hydroxy derivatives in the ellipticines series share these mechanisms of action with these drugs because they are easily oxidized into a quinone–imine compound (unpublished work) (Table 1) during a one-electron transfer process which generates highly reactive free radicals. Ellipticine itself is expected to be no exception to this rule since it has been shown to be hydroxylated *in vivo* [25] as well as *in vitro* [26] by the microsomal cytochrome P450 hydroxylases; according to this expectation, this drug has been shown to break down cell DNA.

The influence of the oxydo-reduction characteristics of several ellipticines on their antitumor properties and their ability to degrade DNA *in vivo* as well as *in vitro* is presently under study in our laboratories.

Acknowledgements—We thank Dr. M. Guerineau for helpful discussion and Mr A. Rouquie for oxygen concentrations determinations. The technical assistance of G. François is gratefully acknowledged. This work was aided by CNRS-ATP "Pharmacologie des médicaments anticancéreux et immunomodulatuers".

Note added in proof: While this paper was in press, another report appeared by W. E. Ross *et al.*, *Biochim. biophys. Acta* **519**, 23 (1978).

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