A New Antitumoral Agent: 9-Hydroxyellipticine. Possibility of a Rational Design of Anticancerous Drugs in the Series of DNA Intercaling Drugs

[cancer chemotherapy/ethidium bromide/DNA unwinding/pyrido (4-3b) carbazole]

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ABSTRACT  The designing of DNA intercalating drugs with high DNA affinity in the series of elliptine has led to a new antitumoral agent, 9-hydroxyellipticine, which has a high DNA affinity, a high activity on L 1210 mouse leukemia, and a lack of toxicity at therapeutic dose. The possible correlations among chemical structure, DNA reactivity, and pharmacological activity of DNA intercalating drugs are discussed.

Except for the hormonal products, which can prevent specific cells from proliferating, and drugs acting on the immunological system, most of the presently used anticancerous agents are cytotoxic compounds acting preferentially on tumor cells. Presently used antitumoral agents are already highly specific. For instance, it has been shown in an appropriate experimental model that a cancerous cell could be about a million times more sensitive to bis-chlorethylnitrosourea than a normal cell (1). Further progress in specificity seems, therefore, very difficult, especially since the basis at the molecular level of this specific toxicity is poorly understood. A rigorous approach in the design of new anticancerous compounds seems, therefore, an almost insuperable task. Nevertheless, the mechanism of the cytotoxicity property itself can in some cases be understood; that is mainly true for drugs acting on DNA structure or metabolism, among which are most of the anticancerous agents. If cytotoxic compounds could be rationally designed, one could hope to have better chance of finding among them anticancerous agents, since at the same time one could study the specificity of their action and try to understand it.

Among products susceptible to such an approach are DNA intercalating drugs, because DNA intercalation is a rather well-understood mechanism and because it is the only case where the structure of what is thought to be the pharmacological receptor is known at atomic resolution (2). On the other hand, there belong already to this class of compounds some of the most powerful anticancerous agents, such as actinomycin D, daunomycin, and adriamycin. Our reasoning was, therefore, very simple. If DNA is the real receptor of these drugs and if we are able to design DNA intercalating compounds with the highest possible affinity for DNA, we would have a much better chance of finding active anticancerous drugs among these compounds. If this reasoning is correct, for this class of compounds high DNA reactivity is a condition necessary for anticancerous activity but not a condition that is sufficient; high DNA reactivity is necessary for conferring a potential cytotoxicity but is insufficient for conferring a specificity directed towards the cancerous cells.

With these assumptions, the approach to be taken is clear. First, DNA intercalating drugs with increasing DNA affinities must be synthesized. Second, the correlation between the DNA reactivity and the pharmacological activity must be studied. The ability of drugs to intercalate in DNA is conditioned by their stereochemical parameters, such as size, shape, and planarity, as well as by their specific electronic configuration, which controls the size of the interaction energy with DNA (3). The drug must be in some way a mold of the cavity formed in DNA when two adjacent base pairs unstake as a consequence of the dynamic structure of DNA (4, 5). Derivatives of 6-H-pyrido (4-3b) carbazole have most of the required characteristics. In particular, the size and the arc-shaped form of these molecules is remarkably suited for DNA intercalation. It has been effectively recognized that one derivative of this series, 9-methoxyllepticine could intercalate in DNA (6). Two plant alkaloids (ellipticine and 9-methoxyllepticine) belonging to this series were active on experimental tumors (7, 8). 9-Methoxyllepticine was reported, in preliminary studies, to be active in human myeloblastic leukemia (9). We, therefore, decided to study the relation between the physicochemical properties of these derivatives and their ability to intercalate in DNA in order to design new compounds of higher DNA affinity. At the same time, the various changes of DNA conformation, as measured by the unwinding angle of the DNA helix, were studied. Our understanding of the intercalation process made effectively possible the synthesis of new derivatives with higher DNA affinity. The anticancerous activities of the various derivatives were measured on L 1210 mouse leukemia, an experimental tumor that would have permitted the selection of most of the compounds active in human cancer (10). These data allow us to propose and discuss in this series some relation between the structure of the pyrido-carbazole derivatives, their reactivity for DNA, and their antineoplastic action. This study led to the selection of a new antitumoral agent, 9-hydroxyellipticine, which has high DNA affinity, a high activity on L 1210 mouse leukemia, and a lack of toxicity at therapeutic dose. The anticancerous activity of this compound was reported in a preliminary communication (11).

MATERIALS AND METHODS

The structures of the various derivatives studied are shown in Fig. 1 and Table 1. 9-Hydroxyellipticine, 9-aminoellipticine,
N-6-isopentylellipticine, N-6-isopentyl-9-methoxyellipticine, nor-11-methylellipticine, and nor-5,11-dimethylellipticine have been synthesized by original procedures that will be described in detail in a later publication. Other ellipticine derivatives were synthesized according to Dalton et al. (7).

Calf thymus DNA was prepared in the laboratory according to Aubin et al. (12). Bacteriophage PM2 (strain from Dr. Espejo) and PM2 DNA, a covalently closed circular DNA (13), was prepared as described (13, 14).

pK values were deduced from the changes of light absorption spectra as a function of pH.

DNA binding constants of the various ellipticine derivatives were determined by measuring their ability to compete with the binding of ethidium bromide according to Le Pecq and Paoletti (15). The DNA binding of ethidium bromide itself was measured by spectrofluorometry (14, 15), with a photon-counting spectrofluorometer built in this laboratory (16). $K_{ap}$ is the DNA binding constant of the various ellipticine derivatives when measured in 0.1 M NaCl-0.1 M Tris-HCl buffer (pH 7.4) at 25°C. From the variations of $K_{ap}$ as a function of pH, $K_{E^+}$ (the DNA binding constant of the protonated form of the ellipticine derivatives) can be computed (17) as follows:

$$\log K_{ap} = \log K_{E^+} - \log \left(1 + \frac{K_{H^{-1}}}{[H^+]} \right) + \log \left(1 + \frac{1}{\alpha [H^+]} \right)$$

where $K_{H^{-1}}$ is the dissociation constant of the equilibrium between the protonated and nonprotonated drug and $\alpha$ is the ratio of the DNA binding constants of the protonated and neutral form of the drug.

The unwinding angle of the DNA helix caused by the intercalation of each molecule was measured with covalently closed circular DNA from PM2 phage DNA as described (18, 19). The relaxation of the supercoiled DNA was followed by viscosimetry as described by Revet et al. (20). This determination is valid only if one is sure that all the molecules bound to the circular DNA are intercalated. This can be controlled, as recently shown (19), by measuring the increase in length of short DNA helices in solution as a function of the amount of bound drug. This determination was performed by viscosimetry of sonicated DNA (18). Viscosimetric determinations were done with a capillary viscosimeter as described by Revet et al. (20), with a laboratory-built instrument where flow times are measured electronically with an accuracy of ±0.001 sec.

Anticancerous activity of the different ellipticine derivatives was determined with the L 1210 leukemia, an experimental tumor of mice (21), as described by Skipper et al. (1). A given number of cells was injected intraperitoneally into DBA/2 female mice about 2 months old. The animals were randomly divided into two groups (20–30 animals in each). One day later, a group received an intraperitoneal injection of the product to test, and the other received an injection of the solvent used in the first group. This last group served as a control. The death of the animals was recorded each day at the same time. Since a single L 1210 cell leads to the animal's death in 15 days, animals surviving more than 45 days were considered cured (1). The percentage of cells killed by the therapy is calculated according to Skipper et al. (1) from the increase of life span when there are no survivors or from the percentage of survivors. To evaluate the therapeutic index, we express the dose of the injected compound as a fraction of the LD$_{50}$ (dose killing 50% of the animals) or of the sublethal dose (dose that never kills any animal). The statistical significance of the results was determined by the methods of Mann-Whitney and Wilcoxon (36). The toxicologic and pharmacologic data that are not directly correlated with the anticancerous property have been reported elsewhere (22, 23).

**RESULTS AND DISCUSSION**

**Relation Between the Physicochemical Characteristics of the Ellipticine Derivatives and Their Affinity for DNA at Physiologic pH.** As shown previously (17), the DNA binding affinity of the cationic form of the drug $K_E$ is about 30 times larger than the DNA affinity of the neutral form. In solution at physiologic pH 7.4, both protonated and neutral forms can coexist and the apparent binding constant that is measured under these conditions ($K_{ap}$) is dependent on the respective fractions of protonated and neutral forms, that is, of the $pK_a$ of the molecule. The $pK_a$ appears, therefore, as one of the main factors controlling the affinity of those molecules for DNA in solution at physiologic pH. To study, independent of this effect, other factors affecting the DNA affinity, we must consider only the value of $K_{E^+}$ (affinity of the cationic form of the drug).

**Table 1. Structure of the ellipticine derivatives (see Fig. 1)**

<table>
<thead>
<tr>
<th></th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$R_4$</th>
<th>$R_5$</th>
</tr>
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<tr>
<td>N-Isopentylellipticine</td>
<td>CH₃</td>
<td>H</td>
<td>Isopentyl</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>N-Isopentylmethoxy-ellipticine</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>Isopentyl</td>
<td>CH₃O</td>
</tr>
<tr>
<td>Nor-5,11-dimethylellipticine</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Nor-11-methylellipticine</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Methoxyellipticine</td>
<td>CH₃</td>
<td>CH₂</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Ellipticine</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>9-Bromoellipticine</td>
<td>CH₂</td>
<td>CH₂</td>
<td>H</td>
<td>Br</td>
<td></td>
</tr>
<tr>
<td>N-Methylellipticine</td>
<td>CH₃</td>
<td>CH₂</td>
<td>H</td>
<td>CH₃O</td>
<td></td>
</tr>
<tr>
<td>N-Aminoellipticine</td>
<td>CH₃</td>
<td>CH₂</td>
<td>H</td>
<td>NH₃</td>
<td></td>
</tr>
<tr>
<td>N-Methoxymethoxyellipticine</td>
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<td>CH₂</td>
<td>H</td>
<td>CH₃O</td>
<td></td>
</tr>
<tr>
<td>9-Hydroxyellipticine</td>
<td>CH₂</td>
<td>CH₂</td>
<td>H</td>
<td>OH</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1. Structure of the ellipticine derivatives.**
Table 2. Physicochemical properties and anticancerous activity of ellipticine derivatives.

<table>
<thead>
<tr>
<th>Product</th>
<th>pK</th>
<th>$K_{sp}$ (pH 7.4)</th>
<th>log $K_{B+}$</th>
<th>Unwinding angle</th>
<th>Pharmacologic activity (% of L 1210 cells killed) by a third of the LD₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Isopentylellipticine</td>
<td>4.7</td>
<td>&lt;10⁴*</td>
<td>6.3</td>
<td>8.8†</td>
<td>0</td>
</tr>
<tr>
<td>N-Isopentylmethoxylellipticine</td>
<td>4.5</td>
<td>&lt;10⁴*</td>
<td>6.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Nor-5,11-dimethylellipticine</td>
<td>6.35</td>
<td>1.0 × 10⁴</td>
<td>5.08</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Nor-11-methylellipticine</td>
<td>6.30</td>
<td>2.4 × 10⁴</td>
<td>5.52</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9-Methoxylellipticine</td>
<td>6.8</td>
<td>1.0 × 10⁴</td>
<td>5.7</td>
<td>6.8</td>
<td>90</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>9.1</td>
<td>1.5 × 10⁶</td>
<td>5.2</td>
<td>9</td>
<td>94</td>
</tr>
<tr>
<td>9-Bromoellipticine</td>
<td>6.1</td>
<td>4.0 × 10⁶</td>
<td>6.92</td>
<td>10.2</td>
<td>92</td>
</tr>
<tr>
<td>N-Methylellipticine</td>
<td>6.1</td>
<td>4.0 × 10⁶</td>
<td>6.92</td>
<td>10.2</td>
<td>92</td>
</tr>
<tr>
<td>9-Aminoellipticine</td>
<td>9.8</td>
<td>1.2 × 10⁶</td>
<td>6.08</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>N-Methyl-9-methoxylellipticine</td>
<td>6.45</td>
<td>2.0 × 10⁶</td>
<td>7.3</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>9-Hydroxyellipticine</td>
<td>9.8</td>
<td>2.0 × 10⁶</td>
<td>6.15</td>
<td>12</td>
<td>99.96</td>
</tr>
</tbody>
</table>

* Those compounds are insoluble at pH 7.4. Log $K_{B+}$ is deduced from $K_{sp}$ measurement done at pH 5.0 (0.1 M NaCl-0.1 M acetate buffer, pH 5.0).
† Measurement done at pH 5.0 (0.1 M NaCl-0.1 M acetate buffer, pH 5.0). All other unwinding angle determinations were done in 0.1 M NaCl-0.1 M Tris-HCl (pH 7.4) at 25°.
‡ 9-Bromoellipticine does not intercalate.

$K_{sp}$ are measured at 25° in 0.1 M NaCl-0.1 M Tris-HCl buffer (pH 7.4) with calf thymus DNA.

$K_{B+}$ is the pK of the molecule at pH 7.4. The values of $K_{B+}$ and $pK_{a}$ of the molecule are no longer a critical factor of the $K_{sp}$ and the pK of the molecule is no longer a critical factor.

The values of $K_{sp}$, $K_{B+}$, and $pK_{a}$, as well as anticancerous activity of the various derivatives, are reported in Table 2, where the compounds have been ordered by increasing value of $K_{sp}$.

A striking illustration of the influence of $pK_{a}$ on the intercalation between ellipticine derivatives and DNA is provided by the comparison of ellipticine and nor-11-methylisopentylellipticine, on one hand, and by the comparison of ellipticine and $N$-isopentylellipticine on the other. In the first case, the removal of a single methyl group from ellipticine in position 11 causes a $pK_{a}$ drop of 3 units and, consequently, a 10-fold decrease of the DNA binding constant at pH 7.4. The $K_{B+}$ values of the two derivatives are similar. It is, therefore, concluded that in this case the variation of $pK_{a}$ is the only intervening factor to account for the change of DNA affinity.

Prediction of the $pK_{a}$ of the ellipticine derivatives is, therefore, of much interest. Such prediction has been attempted by quantum mechanical calculations (17). Quite an interesting result came out of this study. The effect of the substituent on the isoquinoline part of the ellipticine ring could well be explained, as it was done previously in 6-aminoisoquinoline (24). But to explain the effect of the N-6 and 9-OH substitutions, a special hydration at these positions must be taken into account. The complex effect of N-6 substitution could then be understood. For instance, methylation of this position, as in N-methylellipticine, would cause a drop of $pK_{a}$ by making this specific hydration impossible. At the same time one could understand why such substitution would increase the affinity of the cationic form very much. As a matter of fact, it can be reasonably assumed that when ellipticine intercalates in DNA, a water molecule attached by a hydrogen bond at the sixth position would be expelled out of the DNA helix. As a consequence of this necessary dehydration of the molecule, the solvation energy of the ellipticine would be lost on DNA binding and the binding free energy would be decreased by this same solvation energy. Molecules methylated at this position would no longer present this effect, and the DNA binding free energy would, therefore, be larger, as observed. Methylation acts at this position in two opposite ways. It decreases the DNA affinity at pH 7.4 by causing a drop of the $pK_{a}$ of the molecule, but on the other hand it increases the binding affinity of the cationic form. If such a $pK$ drop could be compensated by another substitution elsewhere on the molecule, a binding constant of the order of $10^{-10}$ M⁻¹ could be expected.

Substitutions at position 9 were introduced to place an electrophilic substituent at a position that would permit a direct interaction, possibly through a hydrogen bond, with the negatively charged oxygen of the DNA phosphate group. 9-Hydroxyellipticine and 9-aminoellipticine were synthesized for this purpose. As expected in both cases, a 10-fold increase of $K_{B+}$ is observed. Because these substitutions also cause a $pK_{a}$ increase, a high value of $K_{sp}$ is attained. At variance when an electronegative substituent like Br is at position 9, intercalation is no longer observed and the binding occurs through another mechanism.

As predicted (19), the change of DNA conformation upon intercalation as measured by the unwinding angle is not a constant. A complete range of values between 12° and 4° is observed. A possible correlation between the quadrupole direction of these molecules and the unwinding of the DNA helix after their intercalation (17) could shed some light on the mechanism of this DNA conformational change. The fact that there is not a single type of intercalation complex but may be a large variety may be of biologic and pharmacologic importance.

Relation Between the DNA Reactivity of Ellipticine Derivatives and Their Anticancerous Properties. Such a relation is obviously very difficult to establish with certainty, because,
as pointed out earlier, antitumoral activity is expected to be related only indirectly to DNA reactivity, the DNA reactivity being probably a necessary but not a sufficient condition for antitumoral activity. On the other hand, such a relation could be partially hidden by an eventual metabolic modification of the drugs in vivo. For instance, for a long time such an effect has prevented the observation of the mutagenicity of the carcinogens (25). Therefore, to establish such a relation it would be necessary to study thoroughly a very large number of molecules, active as well as inactive. In particular, the study of the metabolic modifications of these molecules would be critical. Nevertheless, in our opinion, the results obtained in this study tend to give support to our belief that such a relation exists. The fact that all anticancerous intercalating drugs of natural origin, e.g., actinomycin D, daunomycin, and adriamycin, have a high DNA affinity \( K \geq 10^6 \text{ M}^{-1} \) was already an indication that the relation exists.

As expected, no compound with no or low affinity for DNA has ever been found. Many inactive compounds have been synthesized by others (7, 26–31). It can safely be predicted that most of them are unable to react with DNA for very obvious reasons, either because they are not planar (reduced molecules or open cycles) or because they derive from the low pK derivatives of ellipticines (demethylated at position 11, for instance). Because metabolic activation could arise in vivo, such a rule must not been made absolute, especially in the absence of metabolic data.

Of the different compounds studied here, and which are listed in Table 2, the last three of them were synthesized because a high DNA affinity was expected. These compounds reach an DNA affinity that is of the same order of magnitude as the DNA affinity of the natural anticancerous DNA intercalating drugs, actinomycin, daunomycin, and adriamycin (32, 33).

9-Aminoellipticine has no anticancerous activity, but its toxicity is about four times lower than the toxicity of the other derivatives of the same series. (Sublethal dose in mice of 9-aminoellipticine is 200 mg/kg, as compared to 50 mg/kg for 9-hydroxyellipticine.) This suggests strongly that this compound is metabolically inactive. 9-Methoxy-6-N-methyl-ellipticine has a poor anticancerous activity, but it appears to be a more powerful trypanocidal drug (Benard and Riou, personal communication). This observation is of much interest because basically the mechanism of action of trypanocidal and anticancerous drugs is the same. They must be compounds cytotoxic for only one specific kind of cell. This compound is more hydrophobic, and it has long been known that in other series such a property was important for trypanocidal action (34). In this context it is interesting to notice that ethidium bromide, a DNA intercalating drug selected for its trypanocidal action many years ago, possesses a nonnegligible anticancerous activity (35).

9-Hydroxyellipticine, the molecule that has the highest DNA affinity of our series, is a strong anticancerous, drug as shown on L 1210 mice leukemia.

Anticancerous Properties of 9-Hydroxyellipticine. The dose–activity relation of 9-hydroxyellipticine, as observed with L 1210 mice leukemia, is represented in Fig. 2. If this result is compared with the data obtained under the same conditions for compounds commonly used in the therapy of human cancer (1), the following comments can be made: (i) This compound has an extremely high activity at low doses.

![Fig. 2. Relation between dose and anticancerous activity of 9-OH-ellipticine.](image)

99% of the cancerous cells are killed with a single dose 10 times lower than the sublethal dose. (ii) At higher doses, this compound is relatively less active because the dose–activity relation is not linear. (iii) The lack of linearity of this dose–activity relation raises very interesting problems. One of the most attractive hypotheses to explain this phenomenon is that leukemic cell populations are heterogeneous as far as drug sensitivity is concerned. This heterogeneity is often related to a dependence of the drug activity with the different phases of the cell cycle. But other types of heterogeneity, mainly genetic, can also be inferred. The comprehension of such problems is very important for selection of the correct association of anticancerous compounds, on which depends, finally, the real usefulness of a drug.

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