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A CD study of interactions of ellipticine derivatives with DNA

Relations with the in vitro cytotoxicity

Monique Monnot, Olivier Mauffret, Valérie Simon, Elie Lescot, Bernard Psaume, Jean-Marie Saucier, Jean Belehradek Jr and Serge Fermandjian

Institut Gustave Roussy, PRII, Laboratoire de Biochimie-Enzymologie, URA147 CNRS, U140 INSERM, 94800 Villejuif, France

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UV-absorption and circular dichroism (CD) experiments showed that ellipticine derivatives may interact with DNA according to 3 possible binding modes depending on their structure and concentration. The first mode concerned intercalation of 1-methyl-9-hydroxyellipticine (1-Me-HE) with its long axis perpendicular to the long axis of base pairs. The same drug was able to bind to external sites (second mode) once the intercalation sites were saturated at high concentration. The third mode illustrated by 1,2-dimethyl-9-hydroxyisoellipticinium (1-Me-isoNMHE), concerned self-stacked molecules interacting at the surface of DNA. Biological significance of these different binding modes was then discussed in connection with in vitro cytotoxic activity of compounds.

Ellipticine; UV spectroscopy; CD spectroscopy; Binding model; Cytotoxic activity, in vitro

1. INTRODUCTION

Research into drug action mode at the molecular level must be first concerned with the interaction of the drug with its receptor. Then come the events which link that interaction with the pharmacological response. As long as DNA can be regarded as the main target for anticancer drug action [1-3], the spectroscopic study of drug-DNA interaction remains highly important for a good understanding of drug action mechanism [4].

We report results on the interaction of two derivatives of ellipticine (5,11-dimethyl-6H-pyrido-[4,3-b]carbazole) [5-11], 1-MeHE and 1-Me-isoNMHE (Fig. 1) with poly d(A-T) · poly d(A-T) chosen as a B-type DNA model. These compounds were selected as representatives of the 9-hydroxyellipticines and 7-hydroxyisoellipticines. The two molecules bear thus an important hydroxy group on their leftern ring [12,13]. They differ however by the position of their pyridinic nitrogen atom. UV-absorption and circular dichroism experiments as well as in vitro cytotoxicity assays on L1210 leukemia cells were performed for this comparative work.

2. MATERIALS AND METHODS

Absorption and CD spectra were recorded on a Uvicon 860 spectrophotometer and on a Jobin-Yvon Mark IV high sensitivity dichrograph associated to a Minc Digital 11 miniprocessor, respectively. Poly d(A-T) · poly d(A-T) was obtained from Boehringer. We used an extinction coefficient of $\epsilon_{260} = 6700\ M^{-1}\ (_{Base}) \cdot cm^{-1}$. Samples of poly d(A-T) · poly d(A-T) were prepared in 10 mM sodium phosphate buffer, e.g. at weak ionic strength ($I = 0.02$), pH 7 with 0.2 mM EDTA. Titrations were performed by incrementally adding aliquots of 2 mM aqueous drug solutions to 2.5 ml of 65 μM poly d(A-T) · poly d(A-T) solutions, in 1 cm path-length quartz cells, and following UV-absorbance and CD signals at different wavelengths at 20°C.

For cytotoxicity assays, exponentially growing L1210 murine leukemia cells seeded at 5×10^3 cells/ml were incubated in 5% CO_2 for 72 h at 37°C in RPMI 1640 medium supplemented with 10% foetal calf serum, and drugs added at different concentrations. The results were expressed in ID_{50} (drug concentration inducing 50% cell growth inhibition).

Correspondence address: S. Fermandjian, Institut Gustave Roussy, PRII, Laboratoire de Biochimie-Enzymologie, 94800 Villejuif, France

3. RESULTS AND DISCUSSION

Fig. 2A and B show the UV absorption spectra obtained when poly d(A-T) · poly d(A-T) is titrated with 1-Me-HE and 1-Me-isoNMHE, respectively. In each figure, we inserted the corresponding free drug absorption spectra.

At low drug to DNA ratio, the signal originally observed at 305 nm is shifted at 323 nm ($\Delta\lambda = +18$ nm) for 1-Me-HE, and from 300 to 315 ($\Delta\lambda = +15$ nm) for 1-Me-isoNMHE. Such an induced change is consistent with a binding occurring according to an intercalation mode (1-4). This signal grows up to $r' = 0.12$ for 1-Me-HE (r' corresponds to the ratio of ligand to polynucleotide expressed in Base) while it reaches its maximum at $r' = 0.04$ in the case of 1-Me-isoNMHE. Beyond these two r' values a new peak arises at $\lambda = 305$ nm, e.g. at about the same wavelength where the free ellipticine derivatives themselves contribute. See for

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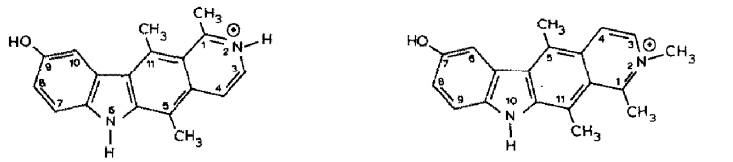

1 - Me - HE
1 - méthyl - 9 - hydroxyellipticine

1 - Me - isoNMHE
1,2 - diméthyl - 7 - hydroxyisoellipticinium

Fig. 1. Structure of ellipticine derivatives

comparison the inserted free drug spectra in Fig. 2A and B. Thus, because of the possible superposition of two contributions, this latter peak does not tell us too much on the binding ability of these two drugs at high r' values.

We resort then to CD experiments which can help to discriminate between the free molecules in solution and the molecules bound to DNA. The 1-Me-HE and 1-Me-isoNMHE derivatives lack chirality but optical activity can be conferred by insertion of the chromophore in the asymmetric environment of the DNA double helice while the unbound molecules remain optically silent.

Fig. 3 shows the CD spectra obtained when poly d(A-T) · poly d(A-T) is titrated with 1-Me-HE (a) and with 1-Me-isoNMHE (b).

In the case of 1-Me-HE a signal is induced, for r' = 0–0.12, at λ = 328 nm, e.g. about the same wavelength as in the corresponding UV spectra (Fig. 2A). Thus, the CD and UV measurements conclude to an intercalative binding mode for 1-Me-HE at low drug to DNA ratio. The simplicity of the positive CD signal (λ = 328 nm) suggests that 1-Me-HE intercalates with its chromophore plane parallel to the base pair planes and its long axis oriented perpendicularly to the long axis of the base pairs as demonstrated for intercalation of Methylene blue by Lyng et al. [14,15]. Existence of such a geometry is further proved by X-ray analysis of daunomycin and adriamycin in their crystallized complexes with an hexanucleotide [16].

At a higher value ($r' > 0.12$) a negative signal is induced at λ = 310 nm proving the binding of 1-Me-HE to a new site. The signal position which appears only weakly shifted compared to that detected for the free drug in UV-absorption suggests an outside binding mode at high concentration for 1-Me-HE (Figs 3A and 2A).

Addition of 1-Me-isoNMHE to poly d(A-T) · poly d(A-T), after a very weak signal induction near 320 nm, generates rapidly excitonic spectra characterized by equally intense positive and negative signals at λ = 310 nm and λ = 295 nm, respectively, and a change in sign at λ = 303 nm where the corresponding UV absorption signal is observed (Fig. 2B). These features may suggest

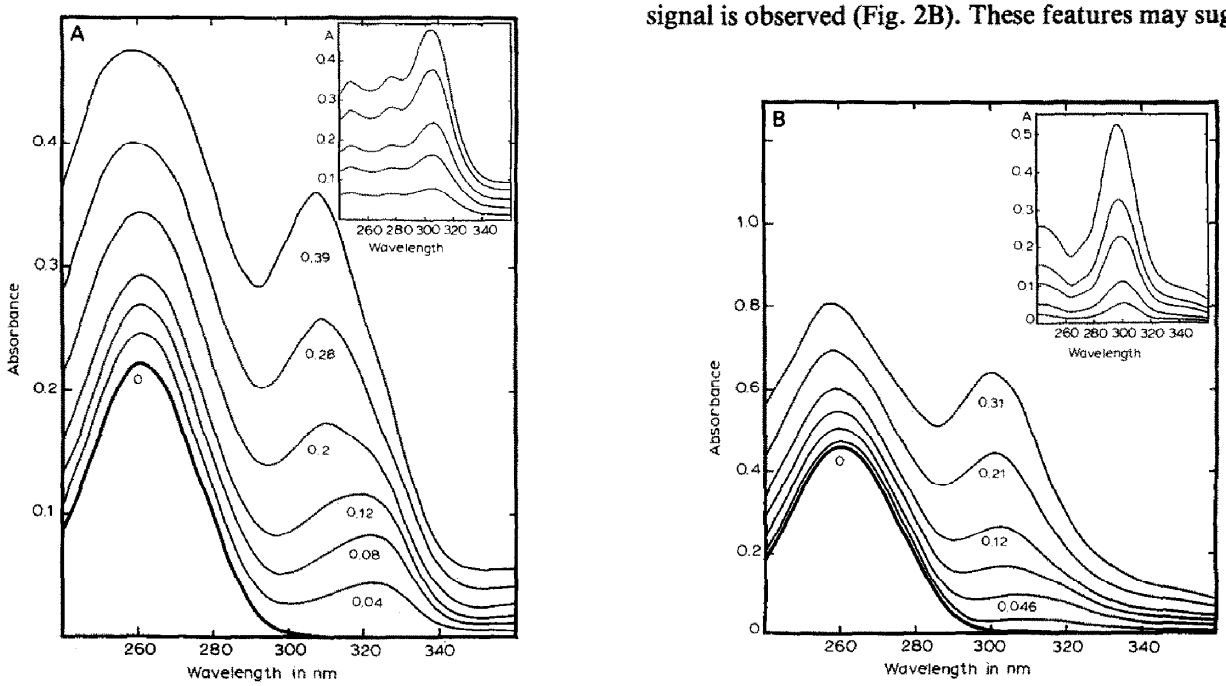

Fig. 2. UV-absorption spectra of poly d(A-T) · poly d(A-T) titrated with: (A) 1-Me-HE at r' = 0; 0.04; 0.08; 0.12; 0.2; 0.28; 0.39; and (B) 1-Me-isoNMHE at r' = 0; 0.016; 0.046; 0.08; 0.12; 0.21; 0.31 (See text for definition of r' and experimental conditions).

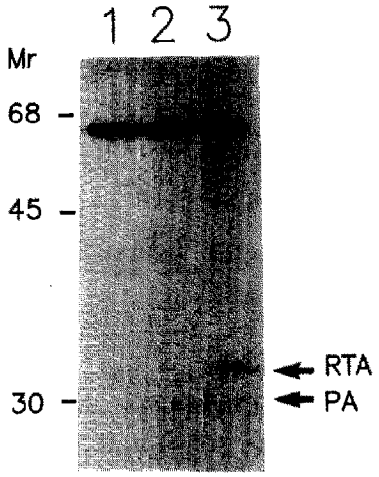

Fig. 5. Electrophoretic analysis of RTA-DA-PA before (lane 1) or after (lane 2 and 3) brief trypsin treatment in the presence (lanes 1 and 3) or the absence (lane 2) of dithiothreitol. The arrows indicate the positions of the release RTA and PA bands.

3.3. *Cytotoxicity*

Recombinant RTA-PA and PA-RTA premixed with rabbit anti-human Kappa light chain (RAHk) were not cytotoxic to Daudi cells. Their IC_{50} values were greater than that of free RTA (10^{-7} M or greater) (Fig. 4a). In contrast, RTA-DT-PA premixed with RAHk was cytotoxic to Daudi cells ($IC_{50} = 8\times10^{-11}$ M). Trypsin treatment of RTA-DT-PA increased its toxicity approximately 4-fold ($IC_{50} = 2\times10^{-11}$ M). Thus inclusion of the DT sequence and cleavage site in the recombinant fusion protein significantly enhances cytotoxicity. In the presence of antibody, the IC_{50} for the trypsin-treated chimeric protein approached that of whole ricin (6×10^{-12} M (Fig. 4a)). The toxicity of RTA-DT-PA premixed with RAHk for Daudi cells was specific since RTA-DT-PA applied alone was several 100-fold less toxic ($IC_{50} = 3\times10^{-8}$ M for non-cleaved RTA-DT-PA and 6×10^{-9} M for cleaved RTA-DT-PA (Fig. 4a)) and RTA-DT-PA mixed with a mouse IgG2a antibody (which was also able to bind PA) was no more cytotoxic than RTA-DT-PA added alone (data not shown).

In an alternative cytoxicity assay RTA-DT-PA premixed with mouse anti-CD7 antibody specifically killed $CD7^+$ Jurkat cells (Fig. 4b), although the potency was low ($IC_{50} = 10^{-9}$ M) and it was only 10-fold higher than that observed with a control mouse IgG2a antibody of irrelevant specificity (Fig. 4b). Trypsin cleavage did not increase the potency of RTA-DT-PA in the presence of anti-CD7 antibody suggesting, as did the experiment shown in Fig. 4a, that cellular proteolytic cleavage of RTA-DT-PA was occurring.

The DT loop was specifically susceptible to trypsin digestion. After digestion, the treated product ran with an apparent molecular weight indistinguishable from undigested product when electrophoresed under non-reducing conditions (Fig. 5, lanes 1 and 2). Under reducing conditions, two smaller bands were observed in the digested sample (Fig. 5, lane 3, arrowed) which were the expected apparent size for the RTA and PA components of the fusion protein. The bands released by limited trypsin digestion were difficult to visualize, but more prolonged trypsin treatment resulted in proteolysis of the released RTA and PA polypeptides. The RTA band was positively identified by Western blotting using anti-recombinant RTA antibodies, which also bound to a second band whose apparent size was that expected for PA (data not shown).

Further evidence that proteolytic cleavage of RTA-DT-PA liberated a potentially toxic RTA fragment was obtained using RTB-mediated cytotoxicity assays in which RTB reassociated with the liberated RTA to form a ricin-like adduct. Recombinant RTA-DT-PA was not toxic to Vero cells, even in the presence of 10^{-8} M purified RTB (TAble I). After trypsin treatment, however, RTA-DT-PA became potently cytotoxic in the presence of RTB (Table I).

The fusion proteins RTA-PA and PA-RTA were not cytotoxic to target cells in the presence of an appropriate cell-reactive antibody. This accorded with the finding with chemically-linked antibody-RTA conjugates that a disulfide linkage between the two protein components is necessary for cytotoxicity [22]. This suggested that the lack of toxicity of the RTA-PA and PA-

Table I

Effects of trypsin treatment of RTA-DT-PA on RTB-mediated inhibition of protein synthesis in Vero cells

Addition	Trypsin treatment	(% control)
None (control)	No	100
10^{-8} M RTB	No	111
1.5×10^{-7} M RTA-DT-PA	No	102
1.5×10^{-7} M RTA-DT-PA + 10^{-8} M RTB	No	98
1.5×10^{-9} M RTA-DT-PA + 10^{-8} M RTB	Yes	84
1.5×10^{-8} M RTA-DT-PA + 10^{-8} M RTB	Yes	24
1.5×10^{-7} M RTA-DT-PA + 10^{-8} M RTB	Yes	8

RTA-DT-PA, with or without trypsin treatment, was mixed with RTB before incubation with Vero cells. The ability of the cells to incorporate [^{35}S]methionine into protein was determined. Each assay was performed in triplicate. 10^{-8} M RTA plus 10^{-8} M RTA gave 98% inhibition of protein synthesis.

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