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STIMULATION BY γ-CARBOLINE DERIVATIVES (SIMPLIFIED ANALOGUES OF ANTITUMOR ELLIPTICINES) OF SITE SPECIFIC DNA CLEAVAGE BY CALF DNA TOPOISOMERASE II

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Abstract—γ-Carbolines are tricyclic aromatic compounds which intercalate into DNA base pairs and exhibit significant cytotoxic and antitumor activities. These compounds which are structurally related to ellipticine by deletion of an aromatic ring, induce DNA breaks in cultured L1210 cells. Since the mechanism of cytotoxic activity of ellipticines involves DNA topoisomerase II, this enzyme might also be a target for γ-Carbolines. We have tested this hypothesis using an *in vitro* system containing purified enzyme and pBR322 DNA. The ability of nine derivatives to stabilize the DNA–enzyme covalent complex was studied and compared to their cytotoxicity. The four less cytotoxic compounds do not induce cleavable complex to a significant extent. In contrast, the two most cytotoxic γ-Carbolines are the most efficient stabilizers of the cleavable complex. The last three compounds exhibit an intermediate cytotoxicity and cleavage activity. In the presence of γ-Carbolines, cleavage occurs predominantly at a single site in pBR322 which is one of the cleavage sites observed with ellipticines. The cleavage position was determined at the nucleotide level. The increased DNA cleavage specificity observed with γ-Carbolines suggests that a tricyclic system is as efficient as ellipticines for DNA topoisomerase II cleavage at DNA sequences involved specifically in cytotoxic response. The data presented support the hypothesis that DNA topoisomerase II is a target involved in the mechanisms of action of antitumor γ-Carbolines.

1-Amino-substituted-4-methyl-5*H*-pyrido[4,3-*b*]indoles (γ-Carbolines§) are tricyclic analogues of ellipticines. Several compounds of this series exhibit *in vitro* cytotoxic activity on L1210 cultured cells, and *in vivo* antitumor activity [1]. Most active γ-Carbolines bind to DNA by intercalation (binding constants determined at room temperature pH 7.4, in the presence of 0.11 M Na^+, *ca.* 2×10^5/M), but there is no clear correlation between DNA affinity and biological properties [1, 2]. However, Pierson *et al.* [2] using the alkaline elution technique have observed that active γ-Carbolines like other antitumor intercalating drugs (Adriamycin®, ellipticine and *m*-AMSA) [3, 4] induce DNA cleavage in L1210 cultured cells. Furthermore, the induction of DNA breaks is strongly correlated to the cytotoxicity of the drugs [2]. Single strand or double strand DNA breaks induced by intercalating agents are associated with DNA protein cross-links resulting from the trapping of topoisomerase II–DNA complexes by the drugs [5, 6]. DNA topoisomerases II are enzymes composed of two identical subunits which bind covalently to the 5′ ends of both strands of DNA during the enzymatic reaction [7]. *In vitro* studies using purified enzyme and DNA have demonstrated that antitumor intercalating agents and epipodophyllotoxin derivatives stabilize the enzyme–DNA covalent complex termed "cleavable complex" [8, 9]. Treatment of this complex with a protein denaturant results in DNA double strand (or single strand) breaks which can be revealed by gel electrophoresis of the DNA fragments. Probably, active γ-Carbolines like other antitumor intercalating drugs stabilize topoisomerase II–DNA complexes. To investigate this problem, we tested the ability of several γ-Carboline derivatives (structures shown in Fig. 1) to stimulate *in vitro* the cleavage of pBR322 DNA by calf topoisomerase II. We then searched to find if a correlation exists between the structure of these drugs and their ability to induce pBR322 DNA cleavage. Since γ-Carbolines are structurally related to ellipticines we have compared DNA cleavage patterns induced by γ-Carbolines and ellipticines in order to determine if the deletion of a ring changed the site-specificity of cleavage.

MATERIALS AND METHODS

Drugs. γ-Carboline derivatives were synthesized as previously described [1] and their purity was checked by thin layer chromatography on silica gel and alumina plates with at least three solvent systems. 2-*N*-Methyl-9-hydroxyellipticinium (NMHE) was a gift from SANOFI (France). These

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§ Abbreviations used: *m*-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; NMHE, 2-*N*-methyl-9-hydroxy-ellipticinium; VP-16, etoposide; γ-Carbolines, 1-amino-substituted-4-methyl-5*H*-pyrido[4,3-*b*] indoles.

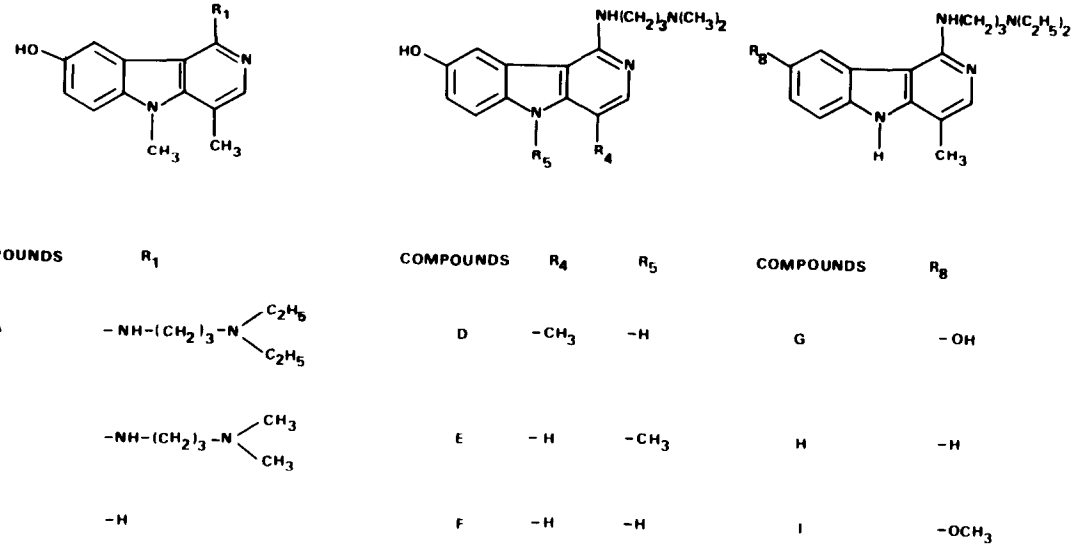


Fig. 1. Structures of the γ-Carboline derivatives.

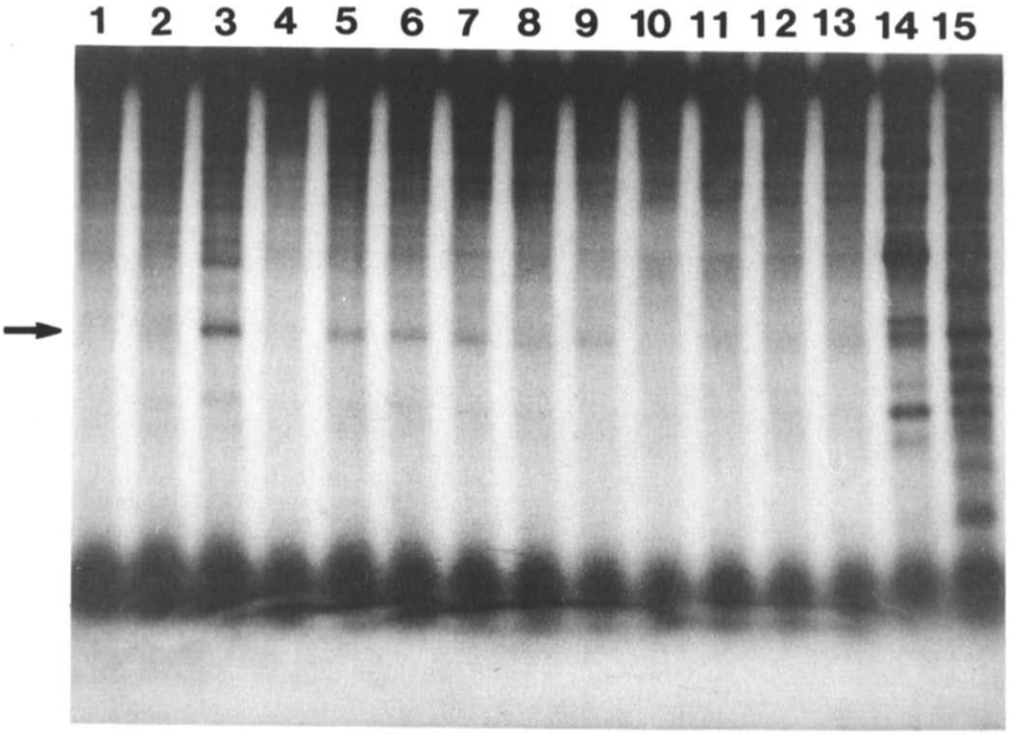
drugs were dissolved in distilled water at a concentration of 2 mM just before use. The epipodophyllotoxin derivative VP-16 was kindly given by Dr Kiechel (Laboratories Sandoz, Rueil-Malmaison, France). *m*-AMSA (4′-(9-acrydinylamino)-methanesulfon-*m*-anisidide) was provided by National Cancer Institute (U.S.A.). Both drugs were dissolved in dimethyl-sulfoxide at a concentration of 10 mM and stored at −20°.

Enzymes and biochemicals. Restriction endonucleases, T4-polynucleotide kinase were purchased from New-England Biolabs (Beverly, MA). Calf intestine alkaline phosphatase, *Escherichia coli* DNA polymerase I (Klenow enzyme) and proteinase K came from Boehringer Mannheim (Hamburg, F.R.G.). Reagents for Maxam-Gilbert sequencing were from N.E.N. Du Pont De Nemours Products (Boston, MA). Adenosine 5′-[γ-^{32}P]triphosphate and [α-^{32}P]dNTP came from Amersham (Bucks, U.K.). DNA topoisomerase II was purified from calf thymus using previously published procedures [10, 11]. The enzyme activity was assayed using kinetoplast DNA from *Trypanosoma cruzy* as substrate. One μg of enzyme decatened 38 μg of free minicircles from the networks per min at 30° in standard conditions [11]. Circular pBR322 was purified from *E. coli* (*hsd* R, *hsd* M, *rec* A13) as described by Maniatis *et al.* [12].

Preparation of [^{32}P]*-end-labeled pBR*322 *fragments.* Circular pBR322 DNA was cut with *Hind*III restriction endonuclease and then labeled with [α-^{32}P]dATP by using the large fragment of *E. coli* DNA polymerase I and three other unlabeled deoxyribonucleoside triphosphates at 20° for 20 min. The labeled DNA was purified by two cycles of ethanol precipitation and was further cut with *Eco*RI restriction endonuclease at position 0 of the genome [13]. This procedure generates one large fragment (4333 base pairs) and one small fragment (29 base pairs) which were labeled at one end. These two DNA fragments were present in the reaction of topoisomerase II mediated DNA cleavage and DNA fragments longer than 30 base pairs could be localized into the pBR322 genome. Size markers were obtained by double digestion of pBR322 with restriction endonucleases *Eco*RI and *Pvu*I (3734 and 629 bp), *Ava*I and *Bam*HI (3314 and 1049 bp), *Eco*RI and *Ava*I (2939 and 1424 bp) and *Eco*RI and *Pvu*II (2298 and 2065 bp). These DNA fragments were labeled as described above. In order to prepare the 232 bp fragment labeled at one end, pBR322 DNA was cut with *Nar*I and dephosphorylated with calf alkaline phosphatase. The 5′-ends were phosphorylated by polynucleotide kinase with adenosine 5′-[γ-^{32}P]triphosphate and the [^{32}P]-end-labeled DNA was cut with *Nru*I restriction endonuclease. The 232 bp fragment labeled at the *Nar*I site was then isolated by electrophoresis on a 6% polyacrylamide gel followed by electroelution and ethanol precipitation. The 232 bp fragment was submitted to sequencing reactions carried out as described by Maxam and Gilbert [14].

DNA cleavage assay. Cleavable complex was formed by incubation for 15 min at 37° of DNA (3–5 × 10^4 cpm) and topoisomerase II (1.1 μg/mL) in the presence of drug in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM Na_3-EDTA, 40 mM Tris–HCl pH 7.5, 30 μg/mL bovine serum albumin and 1 mM ATP. Sodium dodecylsulfate (SDS) and proteinase K were added to a final concentration of, respectively, 0.4% and 0.1 mg/mL and the mixture was incubated at 50° for 30 min.

In the case of analysis by agarose gel electrophoresis, cleavage reactions were made in 15 μL and after proteinase K digestion, 5 μL of loading buffer (0.05% bromophenol blue, 50 mM Na_3-EDTA, 50% sucrose) was added to samples which were loaded into a 1% agarose gel. In the case of analysis by sequencing gel, cleavage reactions were made in 60 μL and after proteinase K digestion, extraction with phenol–chloroform and ethanol precipitation were performed. The samples were



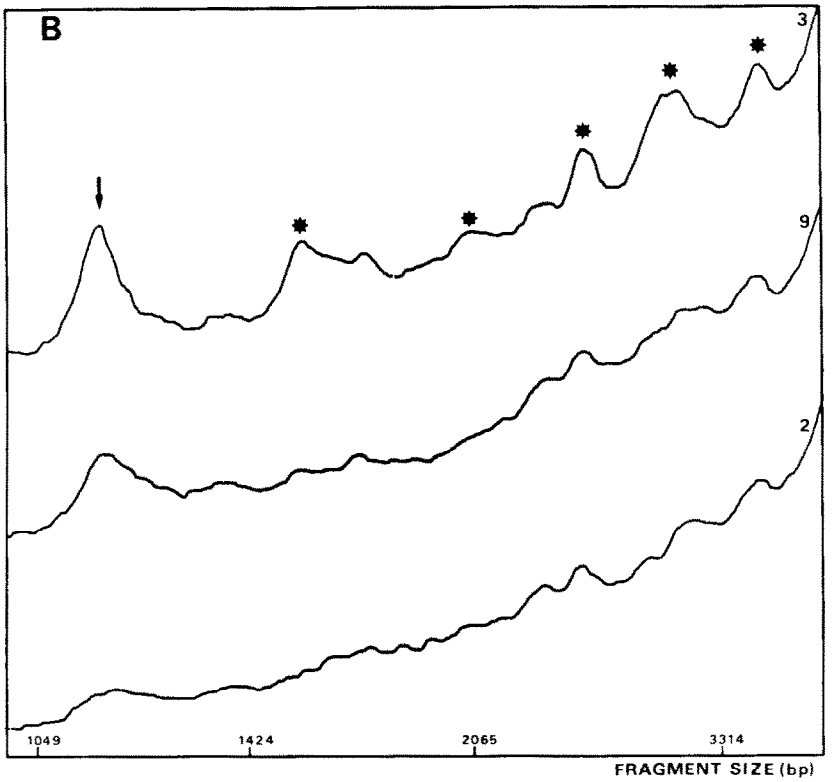


Fig. 2. γ-Carboline stimulated DNA double strand cleavage mediated by DNA topoisomerase II. (A) pBR322 DNA labeled with [^{32}P] at its *Hind*III end was incubated in the presence of DNA topoisomerase II and the γ-Carbolines at a concentration of 4 μM. After proteinase K digestion in the presence of SDS, the samples were analysed by agarose gel electrophoresis as described in Materials and Methods. (1) Control without enzyme and drug; (2) Control without drug; (3) 2 μM NMHE; (4) γ-Carboline A without enzyme; (14) 20 μM *m*-AMSA; (15) 100 μM VP-16; γ-Carboline A (5), B (6), D (7), E (8), G (9), I (10), H (11), C (12), F (13). The major site of γ-Carbolines induced cleavage is indicated by an arrow. (B) Lanes 2, 3 and 9 of the gel autoradiography were scanned. The position of different size markers obtained with several restriction enzymes is shown at the bottom of the figure. The arrow indicates the position of cleavage site induced by compound G and NHME. The symbol * indicates NMHE induced specific cleavage sites.

resuspended into 3 μL of loading buffer [80% (v/v) formamide, 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol]. The samples were heated at 100° for at least 3 min before electrophoresis.

Agarose gel electrophoresis. The electrophoresis buffer contained 89 mM Tris, 89 mM boric acid, 2.5 mM Na_3-EDTA. Electrophoresis was carried out at 2.5 V/cm in 1% agarose gel for *ca* 15 hr at 11°. At the end of electrophoresis, gel was dried on a 3 MM paper and autoradiographed with Fuji R-X film.

Polyacrylamide gel electrophoresis. The same buffer as for agarose electrophoresis was used. Resolution of cleavage patterns was achieved by electrophoresis on 0.4 mm thick, 40 cm long, 8% polyacrylamide sequencing gels containing 7 M urea. Electrophoresis was carried out at 1800 V. At the end of electrophoresis, gel was autoradiographed with Fuji R-X film.

Densitometer scanning. Autoradiography films of agarose gels were scanned by a double beam recording Microdensitometer MKIIIC, Joyce, Loebl and Co. Ltd (U.K.). Peak area of the band obtained in the presence of γ-Carbolines was determined by weighing tracing paper.

RESULTS

Calf DNA topoisomerase II *cleaves pBR*322 *DNA in the presence of* γ*-Carbolines*

To test whether DNA breaks in L1210 cells induced by γ-Carbolines involve DNA topoisomerase II we incubated [^{32}P]-end-labeled pBR322 DNA with calf DNA topoisomerase II in the presence of the drugs. As previously reported [9, 15], Fig. 2A shows that DNA cleavage patterns induced by *m*-AMSA, VP-16 and NMHE are different. In the absence of DNA topoisomerase II and presence of compound A no DNA cleavage was detectable (Fig. 2A, lane 4). On the other hand, in the presence of DNA topoisomerase II cleavage of pBR322 DNA was significantly stimulated by γ-Carbolines at a single site only (see Fig. 2A band indicated by an arrow). DNA cleavage patterns induced by the different γ-Carbolines were similar (Fig. 2A, lanes 5–9). In other experiments using pBR322 DNA linearized at different restriction sites (*Eco*RI and *Nde* I) and labeled at one end with [^{32}P], we did not find cleavage sites induced by γ-Carbolines other than the cleavage site previously

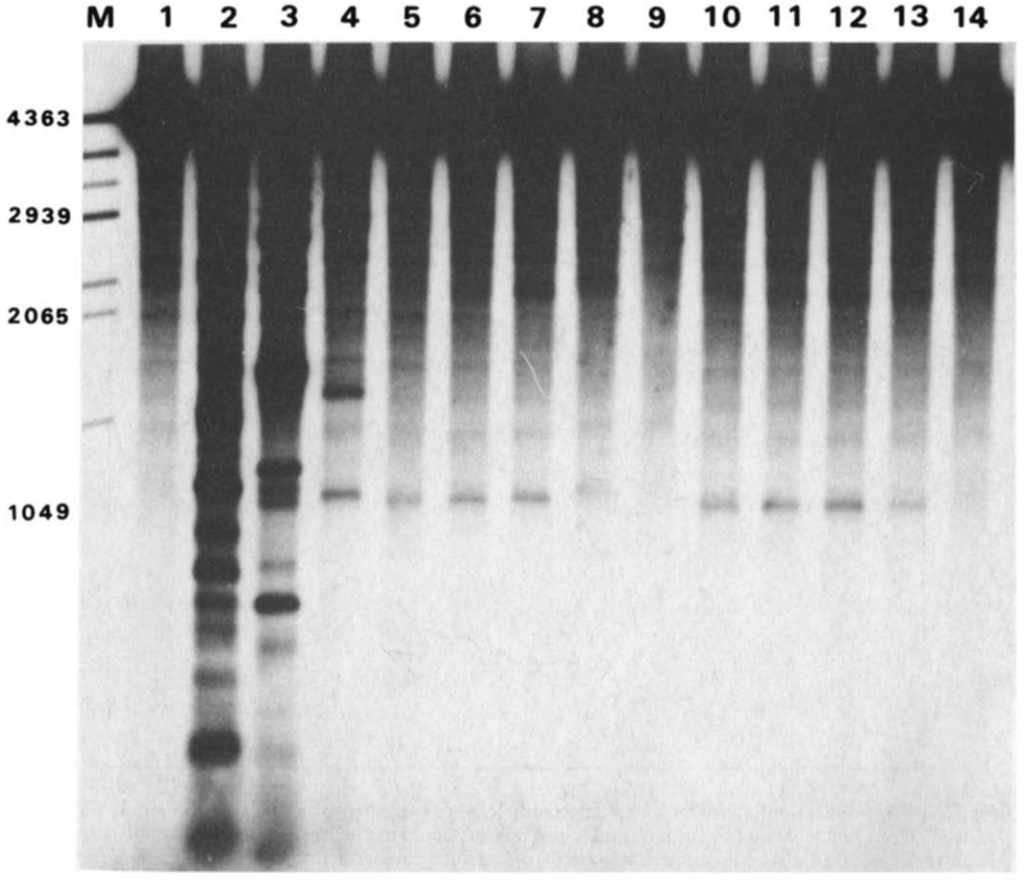


Fig. 3. Effect of drug concentration on the pBR322 DNA double strand breakage mediated by topoisomerase II. 3′-End-labeled pBR322 DNA was incubated with topoisomerase II as described in Fig. 2 in the presence of compound A (lanes 5–9) or compound B (lanes 10–14). Drug concentrations were 1.25, 2.5, 5, 10 and 20 μM. Controls were 100 μM VP-16 (lane 2), 20 μM *m*-AMSA (lane 3), 2 μM NMHE (lane 4) or no drug (lane 1). Size markers in base pairs are in lane M.

observed in Fig. 2A (data not shown). A comparison of the distribution of cleavage sites clearly indicated that the single cleavage site stimulated by γ-Carbolines was one of cleavage sites induced by NMHE (see Fig. 2A and B). Densitometric tracing of the gel autoradiography indicated that five cleavage sites stimulated by NMHE were not stimulated by γ-Carboline derivatives (Fig. 2B). Figure 3 shows that the breakage of pBR322 DNA by topoisomerase II was dependent upon drug concentration and was inhibited at high concentrations of compounds A or B. In order to estimate the intensity of the band generated by cleavage of pBR322 DNA according to γ-Carbolines concentration we have scanned the autoradiography films of agarose gels (see Materials and Methods). Since NMHE also stabilized the cleavable complex at this site we have arbitrarily defined as 100% of cleavage, the intensity of the band obtained at the concentration of NMHE which gave the maximum of pBR322 DNA cleavage. Maximum stimulation of pBR322 DNA cleavage occurred at 4 μM for compounds B and G and the inhibition of DNA cleavage was strong above 8 μM (Fig. 4). We have found the same biphasic curve for cleavable complexes stabilized by γ-Carbolines A, D and E (data not shown). Previous studies have established that this type of dose–response curve was a feature of strong intercalators [15–18]. γ-Carbolines stimulated the pBR322 DNA cleavage by topoisomerase II over a larger concentration range than NMHE (Fig. 4). However, our data indicate that γ-Carbolines are less potent and less effective than NMHE.

Mapping and sequencing of the cleavage site induced by γ-Carboline derivatives

The coefficients of the regression line of the logarithm of the size of the markers versus the distance of migration were calculated (correlation coefficient >0.99) and were used to calculate the size of the DNA fragment corresponding to the observed band in the presence of γ-Carboline derivatives (Figs 2 and 3). The γ-Carboline-induced topoisomerase II-mediated breakage site, was localized at position 1100 (±40 base pairs) in pBR322. In order to determine more precisely the position of γ-Carboline induced cleavage site, a DNA fragment of pBR322 (between *Nar*I and *Nru*I restriction sites) containing the 1100 site was isolated for analysis by sequencing gel. In this DNA fragment (Fig. 5) no difference was detectable in the DNA cleavage pattern between NMHE (lane 3) and compound B (lane 4). This gel confirms that the γ-Carboline derivatives and NMHE stabilized the cleavable complex at the same site in

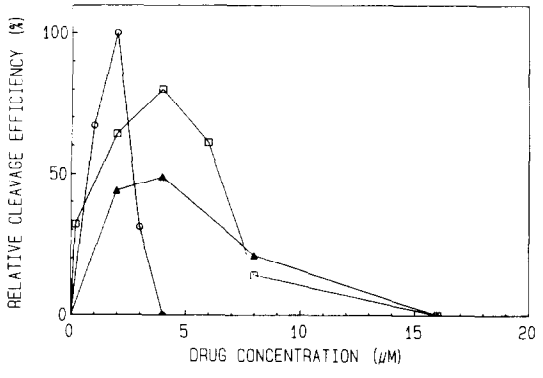


Fig. 4. Inhibition of pBR322 DNA cleavage at high drug concentrations. Reaction conditions and the quantitative evaluation of pBR322 DNA cleavage are described in Materials and Methods. (□) Compound B; (▲) compound G; (○) NMHE.

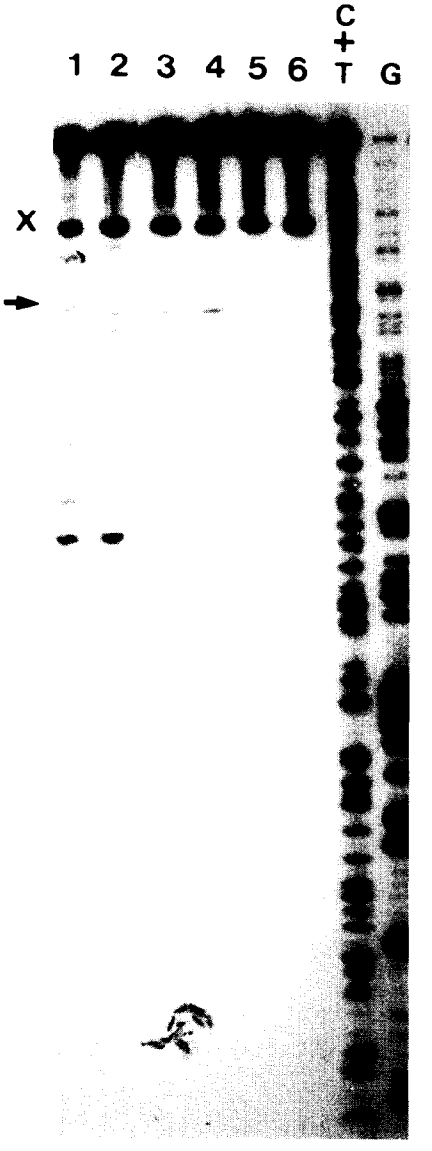


Fig. 5. DNA nucleotide sequencing of the cleavage site induced by compound B. The 232 bp DNA fragment (between *Nar*I and *Nru*I sites in pBR322) with its 5'-end-labeled at *Nar*I site was isolated and used in the cleavage reaction. The DNA fragment (lane 6) was incubated with topoisomerase II in the absence (lane 5) or in the presence of different drugs: lane 4, compound B (4 μM); lane 3, NMHE (2 μM); lane 2, m-AMSA (20 μM); lane 1, VP-16 (100 μM). The DNA from cleavage reaction was analysed by electrophoresis in 8% polyacrylamide, 7 M urea sequencing gel. Sequence ladders are products of Maxam and Gilbert chemical degradation reactions. The arrow indicates the cleavage site induced by compound B. X is an impurity.

this region of pBR322. A topoisomerase II cleavage fragment having a 3'-hydroxyl [19, 20] exhibits an electrophoretic mobility which is slightly different of that of the chemical sequence marker which contains a 3'-phosphoryl end [14]. We could therefore localize the break produced by topoisomerase II in the presence of compound B on one DNA strand (Fig. 5) at position 1082 of pBR322 (arrow) in the following DNA sequence: 5'-GCTGT/CCCTGAT-3' (cut indicated by /). The opposite strand was not analysed on sequencing gels but we could predict the cut because one cleavage event by the same enzyme molecule at one site corresponds to two cuts separated by four base pairs on opposite strands [19, 20]. VP-16 and m-AMSA stabilized the cleavable complex at other sites than those induced by compounds B and NMHE (see Fig. 5). Nevertheless, it may be noted that m-AMSA stimulated also the cleavage at position 1082 and VP-16 at position 1081. No cleavage occurred in the absence of drug (Fig. 5, lane 5) because in our conditions we probably were not at saturating topoisomerase II activities.

The intense band noted X was independent of the cleavage reaction (see lane 6 of Fig. 5) and perhaps was due to a star activity of restriction enzyme *Nru*I. Other experiments have not detected a drug-induced topoisomerase II cleavage site at this site (data not shown).

Structure–activity relationships

Nine γ-Carboline derivatives were evaluated for their ability to stimulate the cleavable complex formation (Table 1). All compounds which stabilized the cleavable complex presented a maximum cleavage activity at 4 μM. Among these compounds, A and B were the most effective to stimulate pBR322 DNA cleavage. Compounds A and B are highly cytotoxic and induced more single strand breaks in L1210 cells than other compounds. It must be noted that compounds which were less cytotoxic (F, C, I and H) lacked the ability to stimulate the DNA cleavage *in vitro* over a wide range of drug concentrations (0.2–32 μM). A dimethylaminopropyl or diethylaminopropyl side chain at the 1-position was an important determinant for the DNA cleavage activity (compare compound C versus A and B). A CH_3 at the 4- or 5- position was required for cleavage activity (compare compound F versus D and E), but no significant difference was observed when a single methyl group was present at the 4- or 5- position (compare D and E). Compounds having methyl groups at both 4- and 5- positions were more efficient to stimulate pBR322 DNA cleavage (compare compounds A and G; B and D; B and E). Finally, comparison between compounds G, H and I shows that an 8-hydroxy substituent was essential for DNA breakage activity *in vitro*.

DISCUSSION

The observation by Pierson *et al.* [2] that several γ-Carboline derivatives induce DNA strand breaks in L1210 cells raises the possibility that these breaks result from the trapping of topoisomerase II–DNA complexes by the drugs as this has been established for various DNA intercalating drugs (Adriamycin®, ellipticine and *m*-AMSA) [8, 15]. The aim of this work was to determine if DNA–topoisomerase II is indeed a target for γ-Carbolines. First, we showed that double strand breaks are produced in pBR322 DNA in the presence of both DNA–topoisomerase II and γ-Carbolines, but not in the absence of either one. Secondly we observed that the extent of cleavage determined *in vitro* as a function of the drug concentration with γ-Carboline derivatives follows a typical biphasic curve similar to the concentration–response curves observed in cultured cells by the alkaline elution technique [2]. Finally, the γ-Carbolines structural requirements are identical for *in vitro* DNA cleavage mediated by DNA–topoisomerase, induction of DNA breaks *in vivo* [2] and cytotoxicity [1]. Taken together, these data indicate that topoisomerase II is the primary target for the cytotoxic action of γ-Carbolines.

The following observations emerge from our structure–activity relationship study: a dimethylaminopropyl or diethylaminopropyl side chain at the 1-position, an 8-hydroxy substituent and a methyl

Table 1. Relationship between DNA cleavage activity and cytotoxicity produced by nine γ-Carboline derivatives

Compound	ID_{50} (μM)*	SSB (Rad Equiv.)†	% of cleavage‡
B	0.01	1194	77
A	0.03	1274	70
G	0.06	ND	48
D	0.09	480	50
E	0.19	371	54
H	0.22	ND	0
I	0.30	ND	0
C	9.20	0	0
F	11.20	145	0

* From Refs 1 and 2. ID_{50} is the concentration of drug that, when added to cultures L1210 for a 48hr period, reduces the growth rate by 50%.

† From Ref. 2 SSB is the abbreviation of single-strand breaks.

‡ Present study. 100% of cleavage represents the intensity of pBR322 DNA cleavage at 1100 site in the presence of 2 μM NMHE. Each value is the mean of two or three determinations at 4 μM drug concentration.

ND: not determined.

group at the 4- or 5- positions are essential for DNA cleavage activity; two methyl groups at both 4- and 5- positions enhance DNA cleavage activity. The importance of a dibasic side chain at the 1- position to determine the cytotoxicity of γ-Carbolines has been pointed out by Pierson *et al.* [2]. Such a side-chain increases by a factor of about 10 the binding constant of γ-Carbolines favouring the intercalation into DNA of the chromophore. However, intercalation is not sufficient by itself to induce cleavable complex [9], and one can assume that the side-chain interacts directly with DNA topoisomerase II thereby stabilizing the ternary complex responsible for DNA cleavage. Previous studies with congeners of podophyllotoxin [21] or ellipticine derivatives [22] have shown that a free hydroxyl group is required for DNA cleavage activity. Since these hydroxyl groups are oxidizable, stabilization of the DNA–enzyme complex may be related to redox properties of antitumor drugs. γ-Carbolines which are ellipticine analogues by deletion of a ring, induce the DNA cleavage with a higher specificity than the ellipticines do. However, the fact that the single cleavage site stimulated by γ-Carbolines is one of the major sites involved in the ellipticine series indicates that a tricyclic intercalating chromophore such as γ-Carbolines nucleus interacts more specifically than tetracyclic compounds.

Active γ-Carbolines are weaker inducers of cleavable complex than NMHE whereas no significant difference is observed for cytotoxic activity between active γ-Carbolines [1] and active ellipticines [22]. One possible explanation is that the toxicity of the cleavable complex varies according to its genomic location. Therefore, γ-Carbolines would preserve the capacity to induce cleavable complex in some genome regions which would be mainly involved in the mechanisms of cytotoxicity. In agreement with this hypothesis is the fact that different chemical classes of intercalating agents induce different levels of DNA breaks in L1210 cells for a same level of cytotoxicity [5].

In conclusion, it would be interesting to modify the aromatic nuclei of other antitumor agents in order to determine if the cytotoxic activity is generally related to DNA cleavage specificity. The ultimate aim would be to identify some genes where the cleavable complex triggers the cell death, which is still an unexplained phenomenon.

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