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TOPOISOMERASE II-MEDIATED DNA CLEAVAGE ACTIVITY INDUCED BY ELLIPTICINES ON THE HUMAN TUMOR CELL LINE N417

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Abstract—Ellipticine derivatives have been shown to induce DNA strand breaks by trapping DNA–topoisomerase II (Topo II) in an intermediary covalent complex between Topo II and DNA which could be related to their cytotoxic effects. We report here that Celiptium® and Detalliptinium®, two ellipticine derivatives clinically used for their antitumoral properties against breast cancer, exhibit the highest *in vitro* activity on Topo II DNA cleavage reaction and decatenation among a series of 14 ellipticine derivatives. The *in vitro* cleavage site specificity in pBR 322 plasmid DNA and in a human c-*myc* gene inserted in a lambda phage DNA is identical for both ellipticines, but different from *m*-AMSA, another Topo II related antitumoral agent. Recently, it has been shown that the ellipticine derivative Celiptium® presents a strong cytotoxic activity *in vitro* on different human tumors including small cell lung carcinoma (SCLC). However, the studies that involved Topo II as a target for ellipticine derivatives have been performed only by using animal tumor cell lines. Therefore we have studied the *in vivo* DNA cleavage activity of Celiptium® and Detalliptinium® on a human SCLC cell line, NCI N417, comparatively to that obtained with *m*-AMSA. The respective IC_{50} on cell growth are 9, 8 and 1 μM for Celiptium®, Detalliptinium® and *m*-AMSA, respectively. Using the alkaline elution technique, we have observed that Celiptium® and Detalliptinium® exhibit a weak cleavage activity on genomic DNA from whole cells. The ellipticines are about 50 times less potent than *m*-AMSA in inducing DNA strand breaks. Analysis of *in vivo* c-*myc* gene cleavage by Southern blot hybridization also demonstrates a lack of activity of the ellipticine derivatives as no gene cleavage could be detected up to 50 μM of the drug. With *m*-AMSA, c-*myc* gene cleavage is detected at a concentration of 0.2 μM, which indicates that this methodology is less sensitive in detecting DNA strand breaks than is the alkaline elution. Further studies of the drug effect on isolated nuclei by alkaline elution also show that the DNA cleavage activity of Celiptium® and Detalliptinium® is increased when compared to whole cells. Our data indicate that these two drugs have a weaker cytotoxic effect than *m*-AMSA on NCI N417 cell line, due to a limited access to the cell nucleus rather than to a lack of activity on Topo II as assessed by *in vitro* and isolated nuclei experiments.

It is now well established that many DNA intercalating agents (acridines, ellipticines, anthracyclines) and one class of non-intercalating agents (epipodophyllotoxins) interact with mammalian topoisomerase II (Topo II),‡ an enzyme that alters DNA conformation through a concerted breaking and rejoining of both strands of the DNA backbone (for a review, see Ref. 1). *In vitro*, these drugs trap the enzyme in an intermediary reversible complex with DNA, named the "cleavable complex", which prevents the final rejoining step of the reaction. Treatment of the complex with a denaturing agent such as SDS leads to a single- or double-strand breakage of DNA and to the covalent linking of one Topo II subunit to each 5′ phosphoryl end of the broken DNA strand [2]. *In vivo*, these drugs have been shown to interact according to that mechanism on overall genomic DNA, by the alkaline elution method, and also on specific regions of some genes by the Southern blot technique [3].

Topo II activity has been reported to be highly increased in cancer cells [4, 5]. These data suggest that the formation of Topo II–DNA complexes could be one of the major causes of the drug cytotoxic action *in vivo*. However, the mechanism by which the cleavable complex could result in cell death is not well understood. A correlation between the number of double-strand breaks (DSB) formed by a particular agent and its cytotoxicity was reported in different studies [6–8], showing, however, great variations between the different classes of drugs. Many authors have also reported a correlation between the drug resistance developed *in vitro* and Topo II activity, i.e. quantitative and/or qualitative modification of Topo II accompanying the drug resistance [9, 10].

Studies on Topo II-induced cleavage were usually undertaken on tumor cell lines of animal origin or occasionally on HL60 [11], a human promyelocytic leukemia cell line. In human clinical practice, most

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‡ Abbreviations used: EthBr: ethidium bromide; SSB: single-strand break; DSB: double-strand break; PBS: phosphate-buffered saline; Topo II: DNA topoisomerase II; SCLC: small cell lung carcinoma; IC_{50}: inhibitory concentration 50; RPMI 1640: Roswell Park Memorial Institute medium 1640; SDS: sodium dodecyl sulfate; EDTA: ethylene diamine tetra-acetic acid; Tris: tris(hydroxymethyl)aminomethane.

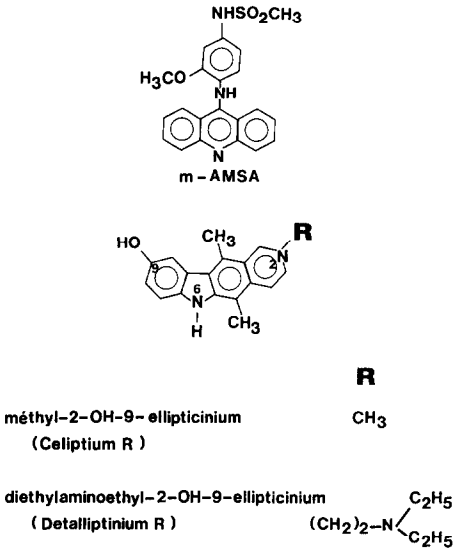

Fig. 1. Structure of *m*-AMSA and ellipticine derivatives.

solid tumors cause real therapeutic problems with chemicals. Therefore it is of interest to test drug cleavage activity in cell lines originating from solid human cancer. In this report we have studied the drug cleavage activity in N417 cells, a human cell line originating from a small cell lung carcinoma. Moreover, N417 genomic DNA contains more than 45 copies of the c-*myc* protooncogene also highly transcribed [12].

On the other hand, we have suggested in previous reports that the c-*myc* gene, which is involved in cell proliferation and in tumor progression, could be an important target for drugs which interact with Topo II. We have shown both *in vitro* and *in vivo* that the cleavable complex formation induced by *m*-AMSA and VM26 preferentially occurs in the 5′ non-coding end of the c-*myc* gene close to DNase I hypersensitive cleavage sites [3, 13]. The 5′ end of the c-*myc* gene has been shown to be important for c-*myc* regulation and several control elements have been localized near the *m*-AMSA- and VM26-induced Topo II cleavage sites [14].

In the present paper we compared drug-induced Topo II cleavage data obtained by alkaline elution and Southern blot hybridization of the c-*myc* gene, when N417 cells were treated by *m*-AMSA and the two ellipticinium derivatives. *m*-AMSA is the most potent antitumoral derivative of the acridine series. Celiptium® and Detalliptinium® (Fig. 1), two ellipticine derivatives, were studied because they are antitumoral in experimental systems and in tumors, including SCLC.

MATERIALS AND METHODS

DNA substrates. kDNA was prepared from *Trypanosoma cruzi* as previously described [15]. Supercoiled DNA from plasmids pUC13 and pBR322 were a gift from Dr E. May (Institut de Recherches sur le Cancer, Villejuif, France). Lambda k76 DNA which contains the complete human c-*myc* gene [16] was a gift from C. J. Larsen (INSERM U 301, Hôpital Saint Louis, Paris, France). End-labelling of pBR322 DNA on Hind III site with (α-^{32}P)dCTP was performed as previously described [2]. 3′ End labelled DNA was then obtained by digestion with *Eco*R1.

Purification of Topo II and drug assay. Topo II was purified from calf thymus as previously described [17]. SDS–polyacrylamide gel electrophoresis of the purified enzyme had revealed a major component (>80%) with an apparent molecular weight of 140 kDa. The specific activity of the enzyme preparation was 1.5×10^5 units/mg of protein. One unit was defined as the quantity of enzyme which fully decatenated 0.1 μg of kDNA in 30 min at 37°.

Inhibition of decatenation has been described in detail elsewhere [18]. Briefly, kDNA substrate (0.1 μg) was incubated for 15 min at 37°, with variable drug concentrations, before addition of enzyme (10 ng) to the reaction mixture (20 μl) containing 20 mM Tris–HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM EDTA, 0.5 mM DTT, 15 μg/ml BSA. The reaction was incubated for 30 min at 37° and samples were electrophoresed on horizontal 2% agarose slab gel. Double-strand DNA cleavage assay has been characterized previously [2]. Topo II (100 ng) was incubated at 37° for 10 min with the drug at various concentrations. The reaction mixture (20 μl) contained 20 mM Tris–HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM EDTA, 0.5 mM DTT, 15 μg/ml BSA and 0.1 μg of supercoiled pUC13 DNA or 0.025 μg of lambda k76 DNA (*Eco*R1 fragment) or 0.025 μg of pUC13 (α-^{32}P) 3′ end-labelled DNA. The reaction was stopped by addition of 5 μl of 5% SDS, 4 mg/ml proteinase K, 25% glycerol, 0.02% Bromophenol Blue. The mixture was incubated 45 min at 50° and DNAs electrophoresed in 1.2% agarose gel.

Cells and radioactive labelling. NCI N417 small cell lung carcinoma cell line was provided by Dr D. Carney (Mater Misericordiae Hospital, Dublin, Ireland). Cells were grown in suspension at 37°, in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin, 5% CO_2. Cellular DNA was radioactively labelled with methyl-[^{14}C]-thymidine (0.02 μCi/ml) in exponentially growing cells.

Drug treatment and irradiation of cells. m-AMSA and *o*-AMSA were provided by Dr B. Baguley (Auckland Medical School, New Zealand) and ellipticine derivatives by Dr C. Paoletti (IGR, Villejuif, France). Cells were treated with drug for 30 min at 37° and then drug effect stopped by a 20-fold dilution in PBS at 0°. Cells were irradiated at ice temperature with a ^{60}Co gamma-ray source (Eldorado, Atomic Energy of Canada Limited) at a dose rate of about 3 Gy/min.

Determination of N417 cell growth inhibition. IC_{50} of the drugs (*m*-AMSA, Celiptium®, Detalliptinium®) were determined under the following conditions: exponential growing cells were submitted to drug treatment for 30 min at 37° and then the drug effect was stopped by two successive washings in PBS. Four $\times 10^5$ cells were grown in normal conditions and, after 5 days, viable cells were numbered using the Trypan Blue exclusion method.

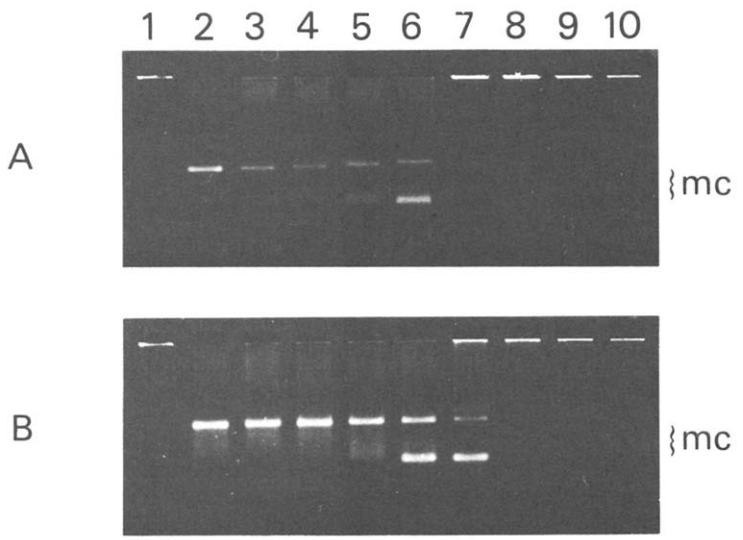

Fig. 2. Inhibition of the decatenation reaction catalyzed by purified calf thymus Topo II. Electrophoresis in 2% agarose slab gel. Lane 1: native kDNA control (100 ng). Lane 2: DNA + Topo II (10 ng). Lanes 3–10: same as lane 2 + 0.2, 0.4, 0.8, 1.6, 3.1, 6.2, 12.5 and 25 μM drug. A: Celiptium®; B: Detalliptinium®. mc: minicircle.

The IC_{50} represents the concentration of drug which induces 50% reduction of the cell viability in the conditions of the assay.

Alkaline elution assays. Single-strand DNA breaks were determined as described by Kohn *et al.* [19]. Briefly, the cells were deposited on polycarbonate filters 2.0 μm pore (Nuclepore Corporation) in cold PBS and lysed with 5 ml of 2% SDS, 25 mM EDTA pH 9.7. When all the cells were lysed, the filter holders were connected to the peristaltic pump, 2 ml of lysis solution containing 0.5 mg/ml proteinase K were added and allowed to stand for 30 min before elution. The eluting buffer was added when the proteinase K reached the neck between the syringe barrel and the filter holder. The flow rate was adjusted to about 2.15 ml/hr and the fractions were collected every 3 hr. The fractions and the filters were processed as indicated by Kohn except that the fractions were adjusted to pH 9.6 with Tris–HCl and 1.5 vol. of scintillator (Instagel Packard) was added.

Identification of DNA cleavage sites on c-myc gene from N417 cells. After drug exposure, DNA extraction and gel electrophoresis were carried out as previously described [20]. Briefly, N417 cells were washed with 50 mM Tris pH 7.9, 1 mM EDTA, and immediately lysed with 2% SDS, 50 mM Tris pH 7.9, 20 mM EDTA. Proteinase K was added to a final concentration of 1 mg/ml for 4 hr at 50°. The lysate was then treated twice with phenol and DNA extracted with ether, precipitated with ethanol, dried and then resuspended in 10 mM Tris pH 7.9, 0.1 mM EDTA. Samples of cell DNA were digested to completion by *Eco*R1 digestion endonuclease and electrophoresed in horizontal 1.2% agarose slab gel. The DNA fragments were denatured, transferred onto a Gene-Screen-Plus membrane (New England Nuclear) and hybridized with the human c-*myc* probe (*Eco*R1-*Cla*1 fragment, encompassing the third exon). The probe was labelled with [^{32}P]-dCTP (2000–3000 Ci/mmol) to a specific activity of 2–4 × 10^8 cpm/μg using the nick-translation technique [21]. Hybridizations were performed in stringent conditions [22] and hybrids were revealed by autoradiography on a Kodak XAR 5 film. Lambda phage Hind III DNA fragments and ΦX174-*Hae* III DNA fragments were used as gel calibration markers.

RESULTS

Inhibition by the antitumor drugs m-*AMSA, Celiptium® and Detalliptinium® of the decatenation reaction catalyzed by Topo II*

The *Trypanosoma cruzi* kinetoplast DNA (kDNA) consists, in a huge network, of thousands of catenated minicircles, 1450 base pairs each [15]. The native complex kDNA network (Fig. 2, lane 1) was resolved in free minicircles by Topo II in the presence of ATP (Fig. 2, lane 2). As previously reported, the decatenation reaction of Topo II can be used as an attractive model to test the *in vivo* inhibitory effect of intercalating and non intercalating drugs [23, 24, 17]. The inhibition of the decatenation reaction by increasing concentrations of drugs results in a progressive disappearance of free minicircles.

Celiptium® and Detalliptinium® provide an inhibition of 100% of the decatenation reaction at about 5 and 3 μM respectively (Fig. 2, lanes 7 and 8). The acridine derivative *m*-AMSA presents a weak inhibitory effect since 77 μM drug are needed to inhibit 100% of the reaction. Furthermore, Celiptium® and Detalliptinium® are two of the most potent inhibitors of the decatenation reaction when compared to different chemical compounds from the series of ellipticines, epipodophyllotoxins and acridines [25, 17].

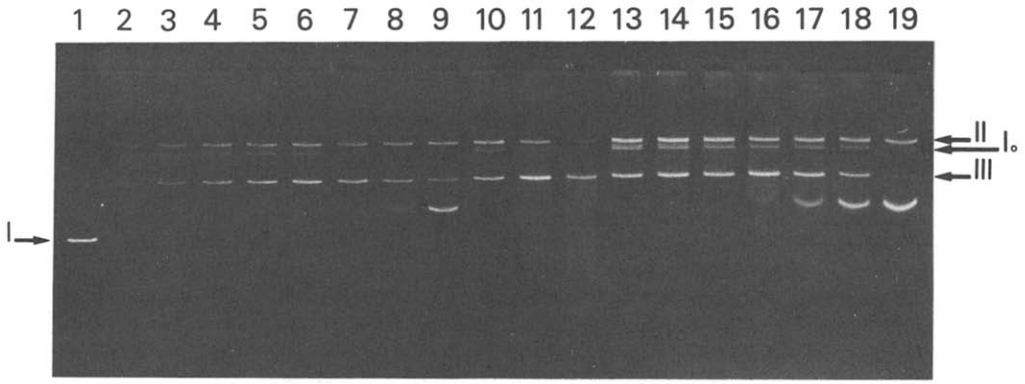

Fig. 3. Stabilization of the Topo II–DNA cleavable complexes by the drugs. Electrophoretic analysis in 1.3% agarose slab gels containing 60 μg/ml EthBr. Lane 1: pUC 13 plasmid DNA control (100 ng). Lane 2: DNA + purified calf thymus Topo II (100 ng); Topo II at this high concentration induces single- and double-strand DNA breakage (forms II and III) and also catenates circular DNA molecules as observed at the top of the gel. Lanes 3–9: same as lane 2 + 0.07, 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 μM Detalliptinium®. Drugs stimulate Topo II–DNA cleavage (form II and III) with a maximum of intensity at 0.6 μM (lane 6). For higher concentrations, DNA cleavage is partially inhibited; note the apparition of supercoiled DNA in lanes 8 and 9. Lanes 10–12: same as lane 2 + 0.1, 1 and 10 μM *m*-AMSA. DNA cleavage is so intense with *m*-AMSA that numerous sites are produced, revealed by a DNA smear (lane 12). Lanes 13–19: same as lane 2 + 0.07, 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 μM Celiptium®. Single- and double-strand cleavage is maximum at 0.3 μM (lane 15) then inhibited at higher drug concentrations. Bands I, Io, II and III correspond to supercoiled, relaxed, nicked and linearized circular DNA respectively.

Stimulation by the drugs of the in vitro *stabilization of the Topo II–DNA-cleavable complexes*

As previously shown by Tewey [26], some anti-tumor drugs may act on Topo II by a mechanism which involves the stabilization of an intermediary cleavable complex of the Topo II reaction. This complex includes drug, DNA and Topo II. Addition of detergent such as SDS leads to the dissociation of this complex and creates a protein-linked DNA double-strand break. In this report, we have used the same *in vitro* procedure with purified calf thymus DNA Topo II and pUC 13 DNA as a substrate to compare the stimulation of the cleavable complex formation by Detalliptinium®, Celiptium® and *m*-AMSA.

When increasing concentrations of Detalliptinium® were added to the reaction mixture and DNA was analyzed in an EthBr–agarose gel, several observations were made.

(1) Topo II induces the formation of oligomeric catenanes which migrate as a smear close to the top of the gel, so that the total amount of DNA seems to vary in the different lanes (Fig. 3). Detalliptinium® strongly stimulates the formation of linear DNA (form III). The DNA cleavage activity is optimal at

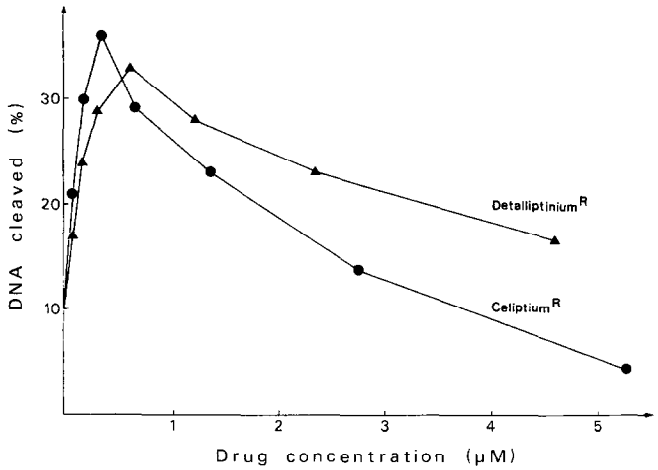

Fig. 4. Quantitation of the stabilization of the Topo II–DNA cleavable complexes by Celiptium® and Detalliptinium®. The percentage of DNA linearized (form III) by Topo II in the presence of the drugs was determined by scanning the gels with a laser densitometer (Ultroscan LKB).

0.6 μM of drug (Fig. 3, lane 6). At higher concentrations, the intensity of the linear DNA band decreases and the cleavage reaction is partially inhibited at 4.8 μM (lane 9). This biphasic stimulation, then inhibition, of the cleavable complex formation was previously described for ellipticine derivatives [17, 26]. With Celiptium® the drug activity is optimal at 0.30 μM and is almost completely abolished at 4.8 μM. With *m*-AMSA, the formation of the cleavable complex is proportional to the drug concentration up to 20 μM [27].

(2) The amount of linearized DNA (form III) was measured by scanning the gels with a laser microdensitometer (LKB Ultroscan). The quantitative data provided by Celiptium® and Detalliptinium® are presented in Fig. 4. The maximal amounts of linear DNA generated with Celiptium® and Detalliptinium® corresponds to 37 and 32% of the total DNA respectively. The amount of DNA cleaved with *m*-AMSA is much higher and cannot be quantified by microdensitometry. Indeed, with 10 μM *m*-AMSA (Fig. 3, lane 12), several cleavage sites were present in the same pUC 13 DNA molecule, producing short-size DNA fragments revealed by a smear after electrophoresis.

(3) In addition to the linear DNA (form III), we have also detected the formation of relaxed DNA (form Io) or nicked DNA (form II). To distinguish between form Io and form II, we tested the DNA cleavage reaction products in EthBr–agarose gels. EthBr induces positive supercoils in relaxed DNA which migrates faster than nicked DNA. Figure 3 shows a form II DNA band whose intensity increases with Celiptium® or Detalliptinium® concentration. The single- and double-strand breaks are simultaneously produced and are inhibited at high drug concentrations.

In vitro *drug-induced Topo II cleavage sites on pBR322 DNA*

In order to map the cleavage sites, linearized pBR322 DNA labeled at only one 3′ end (see Materials and Methods) was used as DNA substrate. The cleavage reaction products were analyzed by agarose gel electrophoresis (Fig. 5). As already described [17, 26], the electrophoretic patterns of the untreated sample (Fig. 5, lane 2) revealed the presence of discrete lower bands corresponding to Topo II DNA cleavage products. *m*-AMSA (lane 3), Celiptium® (lane 5) and Detalliptinium® (lane 6) stimulate the cleavage reaction. This can be appreciated by the progressive disappearance of the 4.3 kb plasmid DNA band. The pattern of cleavage generated by *m*-AMSA corresponds to preferential stimulation of some of the sites which are cleaved by the enzyme alone [17, 26]. *o*-AMSA, a biologically inactive isomer of *m*-AMSA stimulates to a lesser extent the DNA cleavage reaction but with an identical DNA cleavage pattern (lane 4). Celiptium® and Detalliptinium® (lanes 5 and 6) also present a common DNA cleavage pattern which is, however, different from those of *m*-AMSA and *o*-AMSA (lanes 3 and 4).

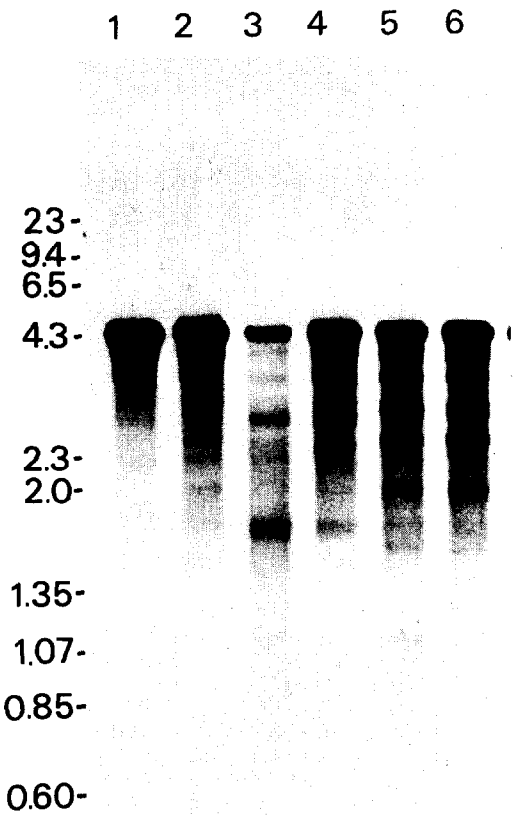

Fig. 5. *In vitro* cleavage of pBR 322 plasmid DNA. The DNA was radioactively labelled at the 3′ end as described in Materials and Methods. After electrophoresis, the gel was dried overnight and autoradiographed. Lane 1: pBR 322 plasmid DNA control (25 ng); lane 2: same as lane 1 + 100 ng purified calf thymus Topo II; lane 3: same as lane 2 + *m*-AMSA 2 μM; lane 4: same as lane 2 + *o*-AMSA 2 μM; lane 5: same as lane 2 + Celiptium® 0.4 μM; lane 6: same as lane 2 + Detalliptinium® 1.2 μM.

Localization of in vitro *drug-induced Topo II DNA cleavage sites in the c-*myc *protooncogene*

We have analyzed the DNA cleavage sites stimulated by Celiptium® and Detalliptinium® in a human c-*myc* gene cloned in a lambda K76 phage DNA [16]. Southern blot hybridization shows that Celiptium® (Fig. 6, lanes 3–7) and Detalliptinium® (lanes 8–12) increases the production of cleaved DNA bands generated by purified Topo II alone (lane 2). The progressive decrease of intensity of the native 13 kb *Eco*R1 fragment of lambda k76 DNA (lane 1) shows that the DNA cleavage activity increases with drug concentration for both drugs up to a maximum at 0.5–1 μM (lanes 5–6, 10–11) and decreases for higher concentrations (lanes 7 and 12). The lambda k76 DNA cleavage patterns are very similar for both

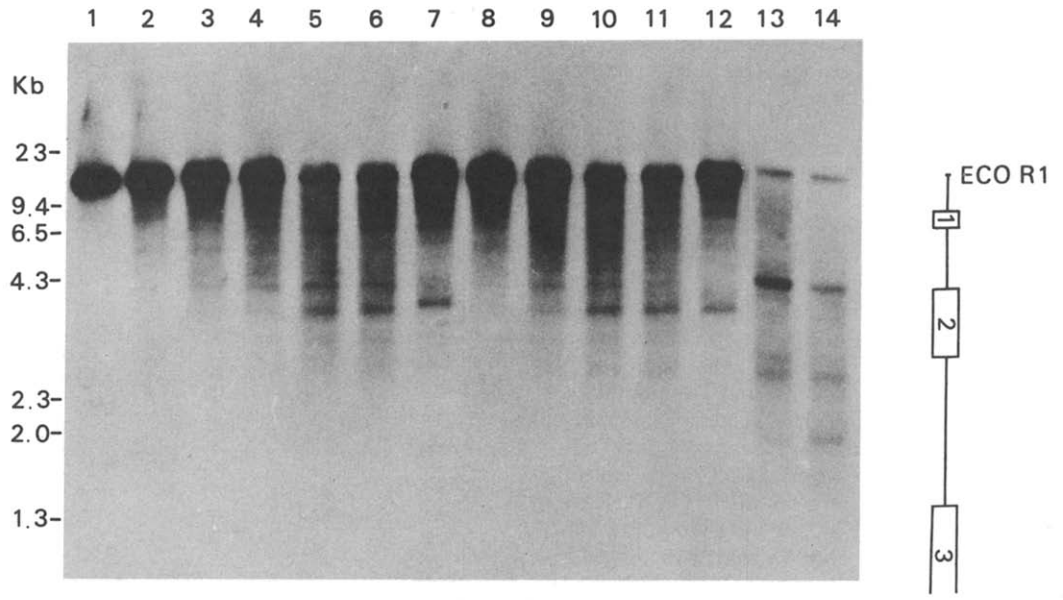

Fig. 6. *In vitro* drug-induced DNA cleavage of c-*myc* protooncogene inserted in lambda DNA. Lane 1: 25 ng control DNA (*Eco*R1 fragment) hybridized with the third exon of the c-*myc* gene; lane 2: same as lane 1 + 100 ng Topo II; lanes 3–7: same as lane 2 + 0.05, 0.1, 0.5, 1 and 5 μM Celiptium®; lanes 8–12: same as lane 2 + 0.05, 0.1, 0.5, 1 and 5 μM Detalliptinium®; lanes 13–14: same as lane 2 + 1 and 10 μM *m*-AMSA; note that the total amount of DNA in these last two lanes seems to be less important than that of other lanes; this is due to the formation of small fragments which migrate as a smear in the bottom of the gel. The sizes of the introns and exons of the c-*myc* protooncogene are not represented on a linear scale but according to the distances of electrophoretic migration as indicated by the DNA size markers (kb).

ellipticine derivatives but different from those of *m*-AMSA (lanes 13 and 14). The major differences between these two classes of drugs are: (1) the lack of stimulation of a cleavage site in the first intron, and (2) the additional presence of one site in the second exon with the ellipticine derivatives (see map of the c-*myc* gene reported in Fig. 6). Similar results were obtained with 9-hydroxy-ellipticine and BD-40, an aza-ellipticine derivative [28].

Inhibitory drug effect on N417 cell growth

Drug treatments were performed as indicated in Materials and Methods. The IC_{50} of *m*-AMSA, Celiptium® and Detalliptinium® are 1, 9 and 8 μM respectively (Fig. 7).

N417 cell genomic DNA cleavage

Exponentially growing N417 cells were exposed to different drug concentrations for 30 min and immediately processed by alkaline and neutral filter elution techniques [19, 29]. The alkaline elution curves show that 0.2 μM *m*-AMSA is equivalent to about 10 μM Celiptium® or Detalliptinium® in inducing SSB (Fig. 8). The formation of SSB and DSB seems to be proportional to *m*-AMSA concentration up to 0.2 μM for SSB and 1 μM for DSB (Fig. 9). For higher drug concentrations, the elution curves are not precise enough to establish a dose–effect relationship. Such a relationship can neither be established with Celiptium® nor with Detalliptinium® (see error bars, Fig. 8). However, the formation of DSB by these two drugs is stimulated up

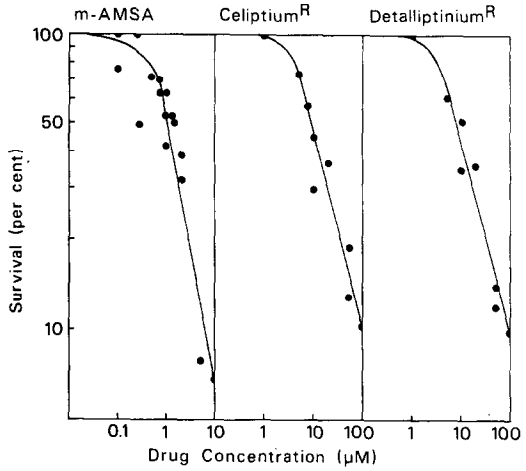

Fig. 7. N417 cells growth inhibition by *m*-AMSA, Celiptium® and Detalliptinium®. IC_{50} were 1, 8 and 9 μM for *m*-AMSA, Celiptium® and Detalliptinium® respectively.

to 50 μM drug (Fig. 9), in contrast to the *in vitro* experiments, where an inhibition of the DNA cleavage is observed at lower concentrations. In order to determine whether the weak activity of the two ellipticine derivatives could be related to a limited cell uptake, drugs were tested in isolated nuclei. Because of high levels of DNA cleavage in untreated nuclei at 37°, as was previously shown [30], the drug-induced SSB could not be measured. By contrast,

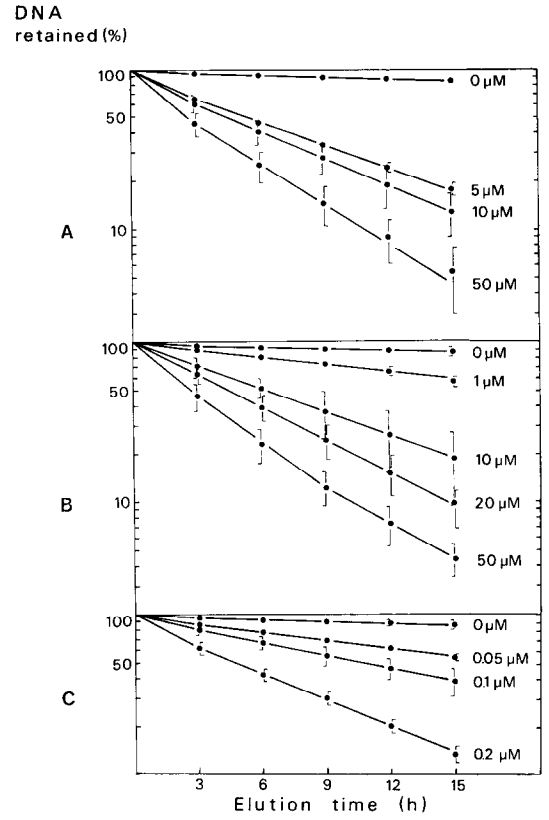

Fig. 8. *In vivo* drug-induced DNA SSB in N417 whole cells. The cells were exposed to drugs at different concentrations for 30 min before lysis and the DNA was analyzed by alkaline filter elution. A: cells treated with Celiptium®; B: cells treated with Detalliptinium®; C: cells treated with *m*-AMSA.

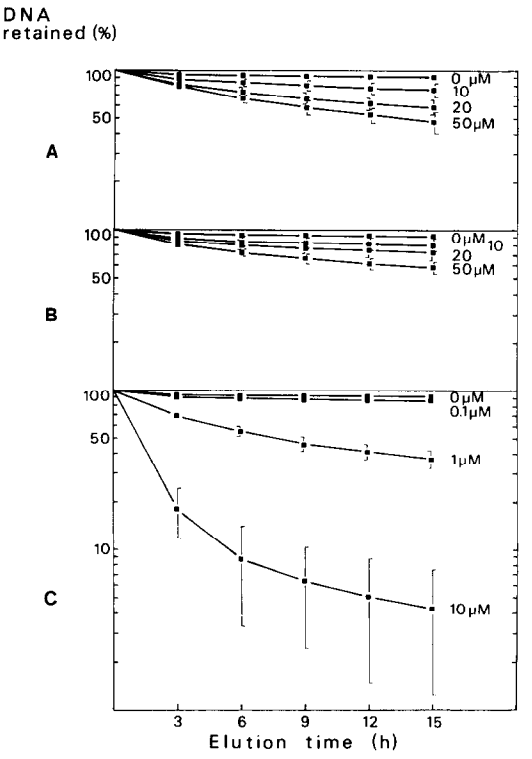

Fig. 9. *In vivo* drug-induced DNA DSB in N417 whole cells. The cells were exposed to drugs at 37° for 30 min before lysis and the DNA was analyzed by neutral filter elution. A: cells treated with Celiptium®; B: cells treated with Detalliptinium®; C: cells treated with *m*-AMSA.

drug-induced DSB could be analyzed in isolated nuclei. The results show that the ellipticines at low concentrations (10 μM) produce more DSB on isolated nuclei than on whole cells (Fig. 10A). Furthermore, there is a partial inhibition of the DNA cleavage reaction with higher drug concentrations (50 μM) (Fig. 10B) as previously described for Celiptium® in isolated nuclei [31]. By contrast, *m*-AMSA is less efficient on isolated nuclei than on whole cells (Fig. 10).

In vivo *drug-induced Topo II cleavage sites in the c-myc gene from N417 cell line*

Exponentially growing N417 cells were treated by *m*-AMSA, Celiptium® or Detalliptinium® for 3 hr at doses previously shown to induce the formation of double-stranded DNA breaks by alkaline elution. Purified DNA preparations were digested to completion with *Eco*R1 endonuclease and analyzed by Southern blot hybridization using a human c-*myc* probe encompassing the third exon. Genomic DNA pattern revealed the presence of a germline 13 kb band (Fig. 11, lane 1). A minor band, located between 6.5 and 4.3 kb appears in the DNA from control cells as well as in the DNA from treated cells. This minor band was not found in repeated

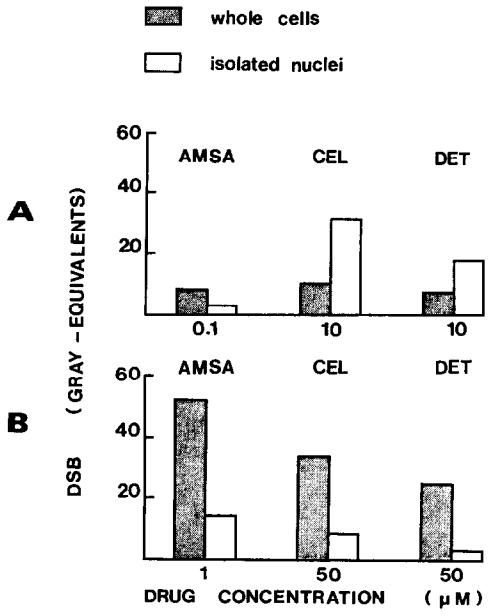

Fig. 10. Comparison of DSB induced by *m*-AMSA (AMSA), Celiptium® (CEL) and Detalliptinium® (DET) in whole cells and isolated nuclei.

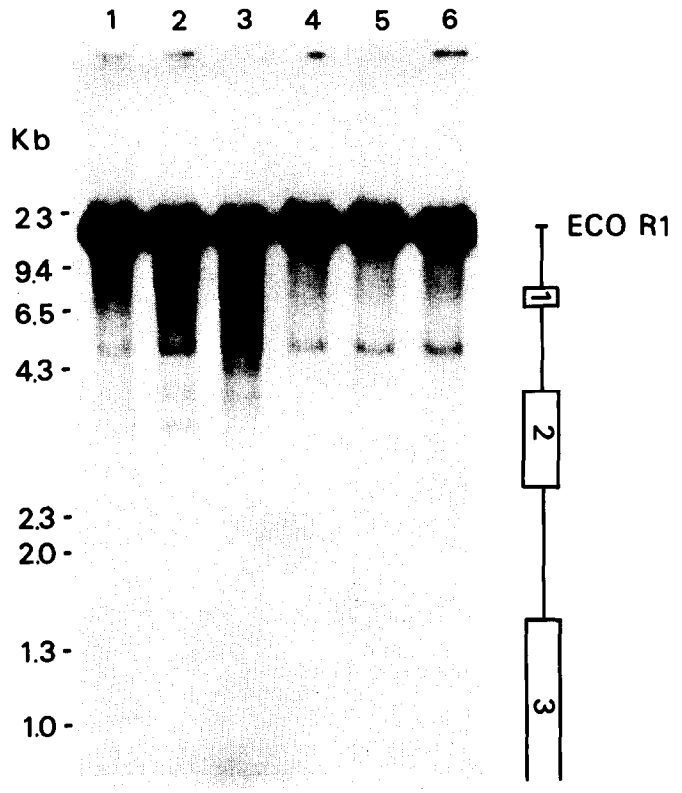

Fig. 11. *In vivo* drug-induced c-*myc* protooncogene DNA cleavage. The genomic cell DNA was extracted after 3 hr of drug exposure, *Eco*R1 digested and analyzed by Southern hybridization with the third exon of the c-*myc* gene (see Materials and Methods). Lane 1: 10 μg DNA from untreated cells; lanes 2–6: 10 μg DNA from cells treated with different drugs; lane 2: 20 μM *m*-AMSA; lane 3: 20 μM teniposide; lanes 4–5: 5 and 20 μM Celiptium®; lane 6: 20 μM Detalliptinium®. The sizes of the introns and exons of the c-*myc* protooncogene are represented as in Fig. 6.

experiments and could be due to an occasional nuclease action of our DNA preparation during cell lysis, as the position of this band in the c-*myc* map corresponds to a major DNAse I hypersensitive site already described [3]. Experiments were repeated using Celiptium® and Detalliptinium®. A unique DNA cleavage band was observed with Celiptium® (lanes 4 and 5) and Detalliptinium® (lane 6), corresponding to the minor band in the DNA from control cells. Other DNA intercalating agents, 9-OH-ellipticine, BD-40, and adriamycin, also have no effect on this gene *in vivo* (data not shown). In a few cases, the c-*myc* gene cleavage was detected, but at a very low level as compared to that of *m*-AMSA- (lane 2) or VM 26- (lane 3) treated cells.

DISCUSSION

N417 cell line was recently established from a SCLC obtained from an untreated patient [32]. They grow *in vitro* as small clumps of floating aggregates and are tumorigenic in the nude mouse. Small cell lung carcinomas are usually sensitive to chemo- and radiotherapy but become rapidly resistant to all anticancer drugs. It was recently shown that Celiptium® is cytotoxic *in vitro* against different human tumors including SCLC [33].

In vitro *DNA cleavage induced by ellipticines*

Recent studies have shown that Topo II could be an important target for antitumor drugs. In this report we have used *in vitro* and *in vivo* experiments on N417 cells to examine the mechanism of action of two ellipticine derivatives, Celiptium® and Detalliptinium®. Experiments were performed by comparison with the antitumor acridine derivative *m*-AMSA. We have examined *in vitro* the effects of Celiptium® and Detalliptinium®, on purified Topo II. These two drugs present the most potent inhibitory effect on the decatenation and cleavage reactions among ellipticine derivatives. Similar results were found with Celiptium® on *Plasmodium* Topo II [18]. Celiptium® and Detalliptinium® have a higher inhibitory effect than *m*-AMSA on the decatenation reaction catalyzed by Topo II. By contrast, *m*-AMSA stimulates more efficiently the DNA cleavage reaction. It is noticeable that *in vitro* these two ellipticines induce not only DSB, described as being predominant in L1210 whole cells [34], but also appreciable amounts of SSB. The limited DNA cleavage activity of the ellipticine compounds is due to a secondary inhibition of the cleavable complex formation involving drug intercalation to DNA [26]. Detalliptinium® presents a weaker secondary inhibitory effect on the DNA cleavage reaction than does

Celiptium®. Detalliptinium® differs from Celiptium® by the presence of a hydrophobic diethylaminoethyl side chain and a weaker inhibitory effect is also observed when Celiptium® is substituted by other aliphatic side chains in the methyl-2 position (results not shown). This difference of activity between Detalliptinium® and Celiptium® could be explained by a greater affinity of Detalliptinium® for DNA but in a non-intercalating mode [35].

We have also analyzed the *in vitro* distribution of drug-induced Topo II cleavage sites in a plasmid DNA pBR322 and in a human c-*myc* gene inserted in a lambda vector. Detalliptinium® and Celiptium® stimulate the same Topo II cleavage sites as those found with other ellipticine derivatives [3, 17]. By contrast, noticeable differences with the ellipticines cleavage patterns could be detected when *m*-AMSA or *o*-AMSA were used. These results indicate that the cleavage patterns in plasmidic or genomic DNA are specific for each series of drugs [26].

In vivo *DNA cleavage induced by ellipticines*

Many attempts to demonstrate *in vivo* a correlation between the drug cytotoxicity and the number of SSB or DSB induced in the genomic DNA after a short (1 hr) drug treatment have provided some contradictory results. In many cases, the cytotoxicity could be correlated with DSB or SSB [6, 36]. Other experiments have shown a clear discordance between SSB or DSB when different classes of drugs were compared [34]. Rowe *et al.* [7] and Denny *et al.* [37] have suggested that the amount of SSB was correlated with the cytotoxicity of derivatives in a particular class of drugs. The different patterns of DNA cleavage may reflect different molecular mechanisms of action.

In order to exclude differences in drug uptake and/or metabolism between *m*-AMSA, Celiptium® and Detalliptinium®, we have studied the drug-induced DNA cleavage activity in isolated nuclei. These results confirm the observations of Pommier *et al.* [31] for Celiptium® in L1210 nuclei. However, the drug-induced SSB could not be measured in isolated nuclei because the production of SSB in the control was too intense. Such high SSB formation was also described by Li and Kaminskas [30] in permeabilized cells. This observation suggests that the permeabilization of cells may cause a release of degradative enzymes. Our experiments are in favour of this explanation as, in isolated nuclei, the c-*myc* gene was cleaved in small fragments and no difference between controls and DNAse I treated nuclei was observed (results not shown).

The number of DSB induced by *m*-AMSA in isolated nuclei is less important than that observed in whole cells as described in L1210 cells [38]. Different explanations could be suggested: (i) Topo II activity could be partially reduced during the nuclei isolation, (ii) *m*-AMSA could be concentrated or activated in the cell. Several fluorescent ellipticine derivatives are overconcentrated in different cell lines but retained in the cytoplasm so that only a small amount can reach the nucleus [39]. If this hypothesis is valid for the N417 cell line, it could explain why the drug is more active on isolated nuclei than on whole cells. The different ellipticine derivatives are metabolized in liver but no metabolite could be evidenced in different cell lines in culture [40] (A. Gouyette, personal communication).

We have previously shown that the c-*myc* gene was cleaved *in vivo* by *m*-AMSA but not by BD-40, an aza-ellipticine [28]. Our present data show that the two most potent inhibitors of Topo II activity among the ellipticine derivatives also fail to induce detectable c-*myc* gene DNA cleavage *in vivo*, whatever the drug concentration used. This result could also be due to the sensitivity of the technique used. Because of the different mechanisms of action suggested from *in vitro* experiments, each class of drugs could act preferentially on different genes *in vivo*. The c-*myc* protooncogene could be a target of particular importance because of its implication in the cell proliferation, but the ellipticines induce usually a G2 arrest in the cell cycle. This block can be produced by many lesions in other parts of the genome and it has to be kept in mind that the DNA breaks are most probably not cytotoxic *per se* but responsible for other lesions such as illegitimate recombination [41, 42].

Finally, we would like to emphasize the notion that, since some ellipticine derivatives are mainly retained in the cytoplasm, Topo II could not be the only target for this class of drugs.

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