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THE *IN VITRO* INVOLVEMENT OF TOPOISOMERASE II IN THE ACTIVITY OF AZA-ELLIPTICINE ANALOGUES IS NOT CORRELATED WITH DRUG ACTIVITY ON ISOLATED NUCLEI

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Abstract—Aza-ellipticines are DNA intercalative ellipticine analogues with antitumor activity that induce protein-linked DNA breaks in NIH 3T3 cells in culture The effects of two aza-ellipticine congeners (BD-40 and BR-76) on the activity of purified Calf Thymus type II topoisomerase were studied using pUC13 DNA as substrate DNA cleavage was stimulated by both molecules at those doses required for inducing lethal effects in cells (DE5O) This effect was reversed by high salt treatment, indicating that it was actually mediated by Topo II Mapping of cleavage sites on linearized and 3′ end-labelled pUC13 DNA showed that ellipticine and aza-ellipticines stimulated the same sites, which differed from those stimulated by m-AMSA Decatenating activity of Topo II on *Trypanosoma cruzi* kDNA was both inhibited by ellipticine and BD-40 at concentrations much higher than DE50 concentrations

Activity of aza-ellipticines was also investigated on isolated nuclei Unlike ellipticine which promoted DNA-breaking activity, BD-40 and BR-76 were repeatedly inactive Prior treatment of DNA by Proteinase K did not reveal hidden breaks which are formed in intact cells treated with BD-40 (Vilarem *et al*., 1984, *Nucleic Ac Res* **12**, 8653) Concordant with these data, BD-40 did not impair DNA-synthetic activity in isolated nuclei, while Ellipticine largely decreased it

These results indicate that lesions induced in DNA by Aza-ellipticines are mediated by Topo II The absence of effect of these drugs on isolated nuclei compared to that of Ellipticine may be due to some specific features of the association between Topo II and Aza-ellipticines or reflect a bioactivation step as a prerequisite for *in vivo* activity

Among DNA intercalative agents commonly used in Cancer Chemotherapy, ellipticine and its derivatives have recently gained an increasing role The strong intercalator ellipticinium is currently used with some success in the treatment of breast tumors [1]. To improve the antitumor activity of ellipticine, a series of ellipticine analogs otherwise named aza-9 ellipticines (or pyrido-pyrrolo-isoquinoline) was recently designed [2]. One member of this series, BD-40§ (N9C-327471D in the National Cancer Institute nomenclature) has shown a promising oncostatic activity in several experimental tumors, while it presented less toxicity than ellipticine and almost no mutagenicity [3, 4] In addition, an interesting activity to human tumors was observed during a phase I clinical study [5]

On animal cells grown *in vitro*, BD-40 displays properties very similar to those of other intercalative agents. Like m-AMSA or ellipticine, it delays the progression of cells in their cycle and eventually blocks them in G2 [6]. This overall effect is most likely a direct consequence of the single- and double-strand breaks induced by the drug in DNA [7] However, the DNA-protein links (DPLs) accompanying DNA breaks were found to differ from those induced by ellipticine [8], because they maintained the apparent size of DNA unless a proteolytic DNA treatment revealed hidden DNA breaks [7] To explain this result, we have postulated that BD40-induced DPLs are in the form of intrastrand DNA-protein bridges [7]

Another property distinguishing ellipticine from aza-ellipticine derivatives was recently observed on cells recovering from a serum deprivation [9] While ellipticine immediately exerted its cytotoxic effects and suppressed cell viability, aza-ellipticine derivatives were completely devoid of both activities during the first cycle following growth resumption

K. Kohn and his group were the first to implicate topoisomerases in models explaining the biological activity of intercalative agents [10, 11], and this hypothesis is currently supported by data in several important papers [12–15] Briefly, the drugs interfere with the nicking-closing reaction of topoisomerase II

§ Abbreviations used BD-40 (NSC-327471D in the nomenclature of the National Cancer Institute, 10-diethylaminopropylamino-6-methyl-5H pyrido 3′,4′ 4,5 pyrrolo 2,3-g isoquinoline, Topo II, type II topoisomerase, DPL, DNA-protein links, NMHE, 2 methyl-9-hydroxyellipticinium, m-AMSA, 4′-(9-acridinylamino)-methanesulfon-m-anisidide

BP 35 17-C

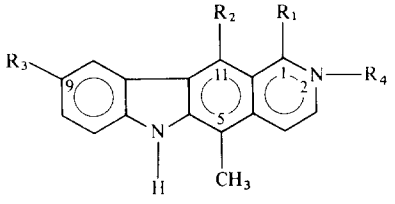
(Topo II, for a general review, see [16]) and stabilize a cleavable complex which results in DNA breaks upon protein denaturant treatment, and covalent linking of one Topo II subunit to each 5′ phosphoryl end of the broken DNA. At the moment, it is not clear whether the intercalation is involved in this process, as non-intercalative drugs such as epipodophyllotoxins also induce topoisomerase-mediated DNA breaks [17, 18] Rather, a direct interaction between drug and enzyme could be the cornerstone of the whole mechanism.

In this report, we show that BD-40 and BR-76, another aza-ellipticine, stimulate *in vitro* cleavage activity of purified calf thymus Topo II and also inhibit the decatenation of *Trypanosoma cruzi* kinetoplast DNA (kDNA) by the same enzyme These results indicate that Topo II is certainly involved in the biological activity of these drugs. Nevertheless, they do not explain the lack of activity of aza-ellipticines on isolated nuclei, while under the same conditions ellipticine is active

MATERIALS AND METHODS

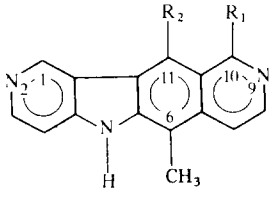
Drugs Structures of ellipticine and aza-analogue derivatives are shown in Figs. 1A and 1B respectively BD-40 and BR-76 obtained from Dr E Bisagni (Institut Curie, Orsay, France), were dissolved in distilled water (100 μM) and stored at −20° in colored flasks. Ellipticine (NSC 71795) and 2 methyl-9-hydroxyellipticinium acetate (NMHE) were a gift from Dr J B Le Pecq (Laboratoire de Physicochimie Macromoléculaire, Institut Gustave Roussy, Villejuif, France) Ellipticine was dissolved in HCl 0 1 N at 2 mM m-AMSA (NSC 249 992) provided by Dr Baguley (Auckland Medical School, New Zealand) It was dissolved in DMSO at 40 mM Teniposide VM-26 was a gift from Sandoz Laboratories It was dissolved in dimethylsulfoxide at 3 mM

A



	R_1	R_2	R_3	R_4
Ellipticine	H	CH_3	H	
NMHE	H	CH_3	OH	CH_3

B



	R_1	R_2
BD - 40	$NH-(CH_2)_3-N(C_2H_5)_2$	H
BR - 76	H	CH_3

Fig 1 Chemical structure of ellipticine (A) and aza-ellipticine derivatives (B)

Cell cultures and synchronization NIH-3T3-D55 murine fibroblasts were grown as previously described [19] Synchronization was performed using a thymidine double block [6]. Cell synchrony was monitored by flow cytometry [6] DNA labelling was performed by incubating exponentially growing cells in Fisher medium with 1 μCi/ml of ^{3}H-thymidine for 18–24 hr at 37°

Isolation of nuclei NIH-3T3-D55 cells were harvested by trypsinization, centrifuged and washed in buffer (10^{-2} M Tris, 10^{-3} M $MgCl_2$ 15 10^{-3} M NaCl) at 4°, sedimented, resuspended in a loose-fitting Dounce homogenizer at a concentration of 10^7 cells/ml in 2 ml of 10^{-2} M Tris–HCl pH 6 4 10^{-3} M $MgCl_2$, 10^{-3} M phenyl methyl sulphate fluoride (PMSF), and homogenized (omission of non ionic detergent treatment at this step was critical to prevent any trapping of cytoplasmic or extracellular BD-40 by nuclei). After centrifugation at 800 *g* for 10 min, nuclear pellets were resuspended in the nuclei buffer (15.10^{-3} M NaCl, 10^{-3} M KH_2PO4, 5 10^{-3} M $MgCl_2$, 10^{-3} M EDTA, 10^{-4} M dithiothreitol, pH 6 4) containing an appropriate concentration of ellipticine or BD-40. The nuclei were protected from exposure to fluorescent light The treatment was performed for 30 min at 37° and stopped by transferring the tubes to ice

Alkaline sucrose gradient sedimentation ^{3}H-labelled nuclei (3 10^6) were gently resuspended without pipeting in saline solution containing 2% Na dodecyl sulfate (SDS) in the presence of 0 5 mg/ml proteinase K (Sigma) After 1 hr incubation at 37°, homogenates were layered onto the top of sucrose gradient solution as previously described [7]

DNA synthetic activity of isolated nuclei Nuclei (5 10^6) from cells arrested by thymidine block at the G_1/S step were suspended in 100 μl of replication buffer at 37°. 2.10^{-2} M Tris–HCl pH 7 4, 5 10^{-2} M NH_4Cl, 5 10^{-2} M glucose, 10% glycerol (v/v) 10^{-3} M, $MgCl_2$, 2 10^{-3} M 2-mercaptoethanol, 2 10^{-3} M EDTA, 10^{-3} M ATP, 3 10^{-4} M dATP, 3 10^{-4} M dCTP, 3 10^{-4} M dGTP, 10^{-4} M dTTP and 0 5 μCi of ^{32}P-dCTP (spec act 3000 Ci/mmole) At 1 hr intervals, nuclei were sedimented by centrifugation for 5 min at 800 *g* (4°) and solubilized in 0 1 N NaOH Trichloracetic acid (TCA) was added to a 40% final concentration and precipitates were collected by filtration onto Whatman fiber glass filters which were washed successively twice in cold 10% TCA and 80% aqueous ethanol, dried and counted in a liquid scintillation spectrometer (Packard)

Preparation of Topo II. The DNA topoisomerase II (Topo II) was purified from calf thymus and the decatenating activity was monitored throughout the purification steps using kinetoplast DNA of trypanosomes as DNA substrate [20, 21] The main advantage of this method results in eliminating most of

Topoisomerase I (Topo I) contamination at the first steps of the purification. Residual topo I activity of the final topo II preparation was estimated by monitoring relaxation of supercoiled pBR322 DNA in the absence of ATP. Less than 1% of ATP-independent relaxing activity was found.

Briefly: nuclei were prepared, lysed with 35 10^{-2} M NaCl and nucleic acids were precipitated by addition of final polymine P 0.1% The supernatant was chromatographed through a phosphocellulose column and active fractions were successively chromatographed through hydroxylapatite, Sephacryl S-200, hydroxylapatite and DNA cellulose columns. SDS polyacrylamide gel electrophoresis of the purified enzyme had revealed a major component (> 80%) with an apparent molecular weight of 140 K DNA Topo II was efficiently stored, without detectable loss of activity at −20° in a conservation buffer (10^{-2} M Tris HCl pH 7 9, 2 10^{-2} M 2-mercaptoethanol, 0.5 10^{-3} M EDTA, 50% glycerol) 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°.

Preparation of DNA substrates kDNA was prepared from *Trypanosoma cruzi* as previously described [22] Supercoiled DNA from plasmids pUC13 and pBR322 were a gift from Dr E May (Institut de Recherche sur le Cancer, Villejuif, France).

3′ end labelling of pUC13 DNA. Plasmid pUC13 DNA was 3′ end labelled with (α-^{32}P) dCTP on *Bam H1* site as previously described [23] Then 3′ end-labelled pUC13 DNA was obtained by digestion with *Eco*RI, whose restriction site is located at 18 bp from a *Bam* HI site

Drug assays. Inhibition of decatenation has been described in detail elsewhere [30]. 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). The reaction was incubated for 30 min at 37° and samples were electrophoresed on horizontal 2% agarose gel

Double-strand DNA cleavage assay has been characterized previously [13].

Topo II (450 ng) was incubated at 37° for 10 min with drug at various concentrations. The reaction mixture (20 μl) contained 2.10^{-2} M Tris HCl pH 7 9, 5.10^{-2} M KCl, 10^{-2} M $MgCl_2$, 5 10^{-4} M ATP, 5 10^{-4} M EDTA, 5.10^{-4} M dithiothreitol, 15 μg/ml of BSA and 0 1 μg of supercoiled pUC13 DNA or 25 ng of pUC13 (α-^{32}P)3′ end-labelled DNA.

The reaction was stopped by addition of 5 μl of 5% SDS, 4 mg/ml of proteinase K, 0.02% of bromophenol blue, 25% glycerol. The mixture was incubated 45 min at 50° and DNA electrophoresed in 1.2% agarose gel

Reversion of double-strand cleavage was performed by addition of salt (5 10^{-1} M NaCl) to the reaction mixture before addition of protein denaturant treatment as previously described [13].

RESULTS

(1) Aza-ellipticine derivatives BD-40 and BR-76 interfere with the breakage-reunion reaction of DNA Topo II

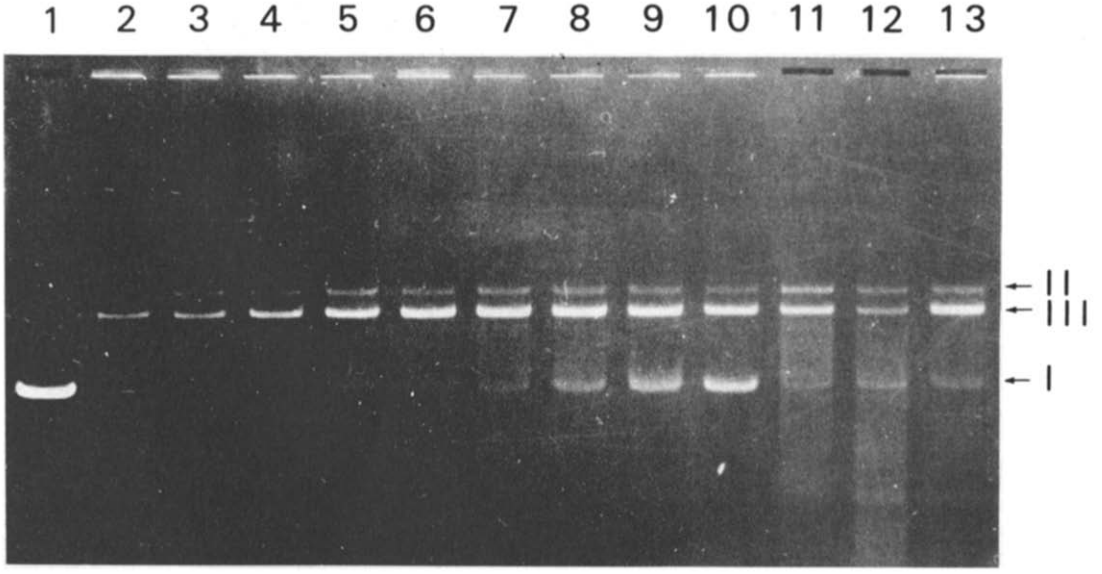


Fig 2 Stimulation of DNA cleavage by calf thymus type II topoisomerase (Topo II) The reaction mixtures (20 μl) containing 0 1 μg of supercoiled pUC13 DNA and 450 ng of topo II, were incubated for 10 min at 37° in the presence of various concentrations of BD-40, m-AMSA or NMHE and analyzed by 1 2% agarose slab gel electrophoresis Lane 1 control DNA, I ane 2 DNA + Topo II, Lanes 3–10. same as lane 2 + BD-40 (0 04 μM, lane 3, 0 08 μM, lane 4, 0 16 μM, lane 5, 0 32 μM, lane 6, 0 64 μM, lane 7, 1 25 μM, lane 8, 2 5 μM, lane 9, 5 μM, lane 10), lanes 11–12, same as lane 2 + mAMSA (10 μM, lane 11; 50 μM, lane 12), Lane 13, same as lane 2 + 0 34 μM NMHE At 50 μM mAMSA, pUC13 DNA from III was degraded into smaller pieces resulting in a smear

Table 1 Stimulation of the Topo II-mediated pUC13 DNA cleavage by ellipticines and aza-ellipticines

Agent	Maximal concentration* (μM)	Cleavage percentage of DNA† (form III)	Stimulatory effect‡
Topo II alone		10	1
Ellipticines			
Ellipticine base	1 42	25%	2 5
NMHE	0 34	36%	3 9†
Aza-Ellipticine			
BD-40	1 25	31 5%	3 4§
BR-76	0 64	28%‖	2 1

* Drug concentrations generating the maximal cleavage percentage
† Estimated by laser densitometry (see Materials and Methods)
‡ Expressed as the ratio of the cleavage percentage in the presence of the drug versus the cleavage percentage in control
§ Average of two experiments
‖ In this experiment, the cleavage of Topo II alone was 13%

Experiments were designed to test the hypothesis of Topo II mediated DNA cleavage by Aza-ellipticines The cleaving activity of a highly purified enzyme from calf thymus was stimulated using supercoiled pUC13 DNA as substrate Figure 2 illustrates the results with BD-40 and shows that the closed circular form (form I) is progressively converted to open circular form (form II) and linear form (form III) as the drug concentration increases from 0 4 μM to 5 μM (lanes 3–10). m-AMSA (lanes 11–12) and NMHE (lane 13) generated identical patterns In the conditions used for the cleavage assay, ie a high concentration of Topo II and in the presence of ATP, catenanes are formed without any aggregant cofactor added (lane 2) as previously described by Hsieh *et al.* [35] This catenating activity is fully abolished in the presence of 10 μM mAMSA (lane 11)

Quantitative data provided by ellipticine and Aza-ellipticines are presented in Table 1 and Fig 3 ellipticine and BR-76 stimulated the enzyme cleaving activity in a similar fashion (2 5- and 2 1-fold

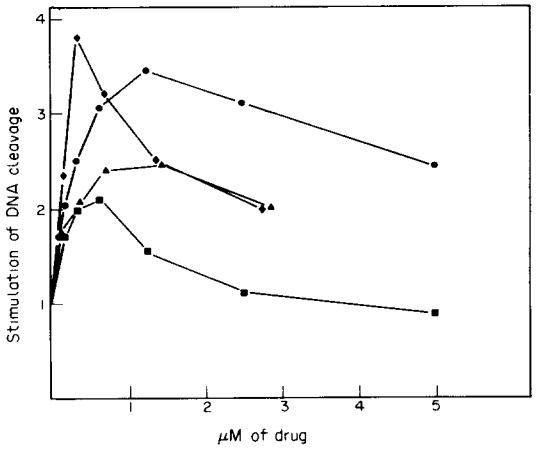


Fig 3 Effects of ellipticine and aza-ellipticine on double stranded DNA cleavage induced by Topo II Cleavage reactions were performed as described under "Materials and Methods" The extent of double-stranded DNA cleavage was calculated from areas under peaks obtained by scanning the negative photograph of the gel with a laser microdensitometer Controls were also done using α-^{32}P-labelled pUC13 DNA Results were in agreement using both unlabelled and labelled DNA substrates Results are presented with regard to the natural cleavage activity of the Topo II which has been given as a reference value of one (Y axis on the figure) DNA cleavage induced by BD-40 (●—●), NMHE (◆—◆), BR-76 (■—■), and ellipticine (▲—▲) Higher concentrations of BD-40 and BR-76, like NMHE or ellipticine were found to inhibit the enzyme activity

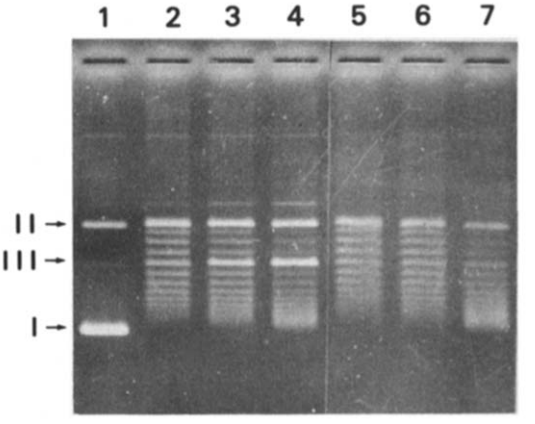


Fig 4 Reversion of DNA double-strand breakage by high salt Double strand DNA cleavage was performed as described in Materials and Methods Topo II and drug contained in the reaction mixture were incubated at 37° for 10 min The reaction was stopped by addition of SDS and proteinase K and the incubation was extended for 45 min at 50° before electrophoresis Lane 1, pBR322 DNA control (0 1 μg), Lane 2 + Topo II (450 ng), Lanes 3–4, same as lane 2 + BD-40 (0 32 μM, lane 3, 1 25 μM, lane 4) Reversion of DNA breakage by salt 0 5 M NaCl was added for 15 min at 37° before SDS and proteinase K, lanes 5–7, Lane 5, same as in lane 2, Lanes 6–7 same as in lanes 3–4

respectively) whereas NMHE and BD-40 stimulated it 3.8- and 3.5-fold Since BD-40 and BR-76 mainly differ in their structures by the presence of a diethylaminopropylamino side chain, this substitution appears to be important for enhancing the *in vivo* DNA cleavage by Topo II

Several pieces of evidence indicated that the DNA cleavage reaction was due to the formation of a complex between calf thymus Topo II and DNA rather than to nucleases or chemical degradation. First, the cleavage process occurred within seconds following the mixing of enzyme, DNA substrate, and drugs, and did not significantly increase during prolonged incubation. In addition, increasing the salt concentration at the end of the assay, reversed, as expected the cleavage process For BD-40, this salt reversal was almost complete even after 15 min as judged by disappearance of form III (Fig. 4, lanes 6, 7), consistent with the notion that DNA breakage was the consequence of the exposure of the drug-stimulated cleavable complex to protein denaturants as described elsewhere [13]

(2) Mapping of ellipticine and aza-ellipticine-stimulated cleavage sites

In order to map the cleavage sites, linearized pUC13 DNA labelled only at one 3′ end (see Materials and Methods) was used as a substrate in the reaction and cleavage sites were mapped by agarose gel electrophoresis (Fig 5) The pattern of the

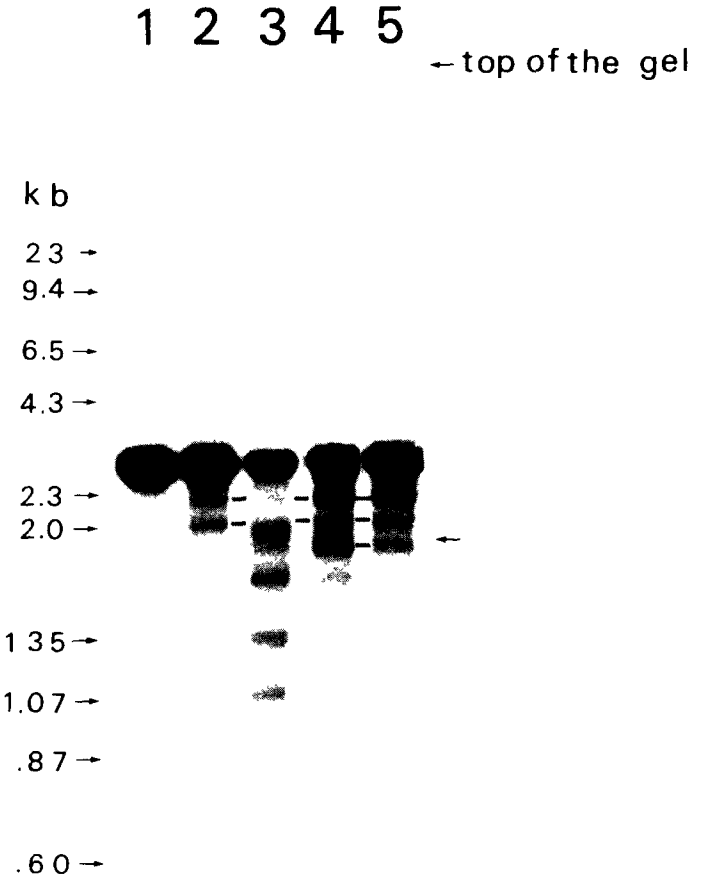


Fig 5 Mapping of Topo II cleavage sites on pUC13 DNA in the presence of m-AMSA, NMHE and BD-40 In each reaction mixture (20 μl) 25 ng of 3′-end labelled pUC13 and 450 ng of Topo II were present After 10 min at 37°, the reactions were terminated by the addition of SDS (1% final) 800 μg/ml of proteinase K Incubation was continued for another 45 min at 50° before being loaded onto 1 2% native agrose gel Lane 1, control DNA (no Topo II), Lane 2, Topo II no drug, Lane 3, 2 μM of mAMSA, Lane 4, 0 4 μM of NMHE, Lane 5, 1 25 μM of BD-40 The sizes of fragment were deduced from migration rates of phage λ Hind III- and φ × 174 Hae III-DNA fragments used as gel calibration markers The origin of the faint band observed in the lane of control DNA (Lane 1) around nucleotide 2020 is unknown, in any case, its intensity was consistently weaker than that of the Topo II-treated DNA (Lane 2)

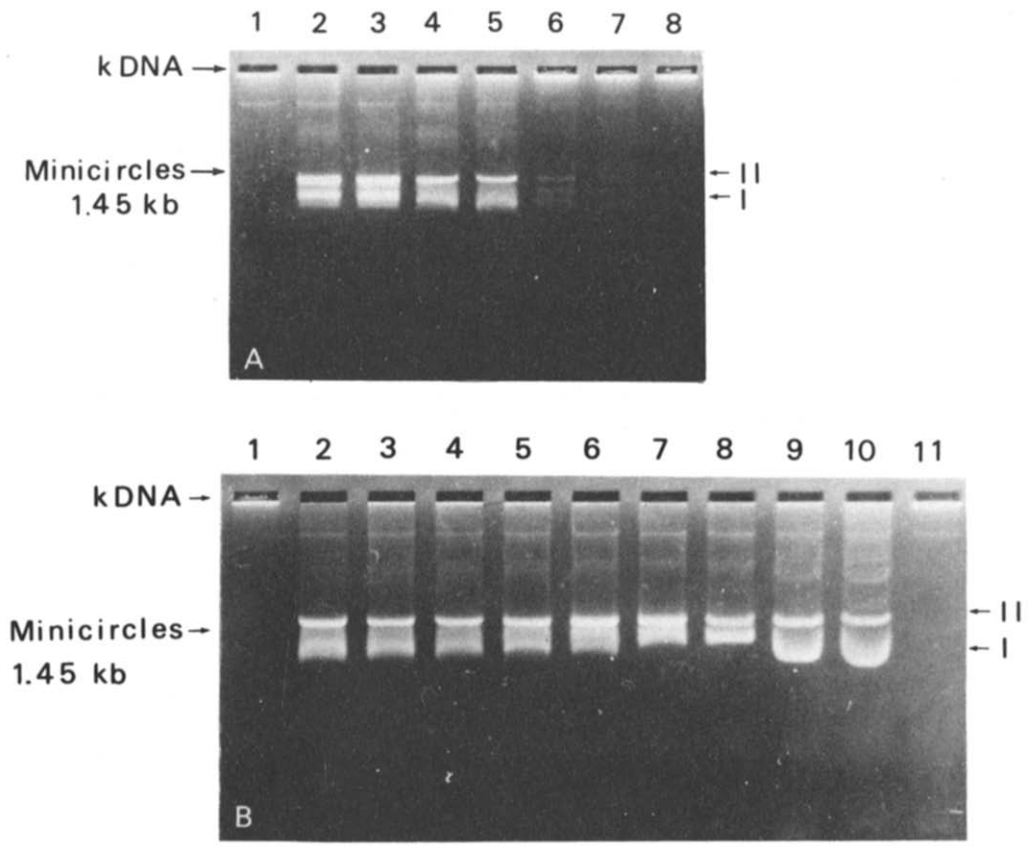


Fig 6 Inhibition of the decatenating activity of calf thymus topoisomerase II by VM26 (A) and BD-40 (B) The kDNA decatenating activity was monitored as described in Materials and Methods (A) Lane 1, kDNA control, Lane 2, kDNA + Topo II (10 ng), Lanes 3–8, same as Lane 2 + VM26 (1 μM, lane 3; 5 μM, lane 4, 10 μM, lane 5, 25 μM, lane 6, 50 μM, lane 7, 100 μM, lane 8) VM26 inhibits the decatenating reaction at 50 μM Form I is the covalently closed minicircle (negatively supercoiled) Form II is the closed relaxed minicircle (nicked or closed) (B) Lane 1, kDNA, control, Lane 2 kDNA + topo II (10 μg), Lane 3–11, same as lane 2 + BD-40 (0 05 μM, lane 3, 0,1 μM, lane 4, 0 25 μM, lane 5, 0 5 μM, lane 6, 1 25 μM, lane 7, 2 5 μM, lane 8, 5 μM, lane 9, 7 5 μM, lane 10, 10 μM, lane 11) In this experiment, BD-40 inhibited the decatenating reaction at 10 μM Following BD-40 treatment, form I minicircle was converted to positively supercoiled form (lanes 9–10)

untreated sample (containing only the enzyme + DNA – lane 2) revealed the presence of two major sites around nucleotides 2320 and 2020 (These values are only indicative as the lack of precision of the method precludes accurate measurements) These two sites were found in patterns of samples treated with NMHE (lane 4), BD-40 (lane 5) and mAMSA (lane 3) Another major site was observed with NMHE and BD-40, but not (or very weakly) with m-AMSA (arrow) In addition, other major sites were observed in m-AMSA pattern, which were absent in BD-40 and ellipticine patterns Thus, in spite of their structural differences, ellipticine and BD-40 stimulated the same sites, which differed from those activated by m-AMSA. This is consistent with previously published results showing that Topo II sites generated by antitumor drugs from different classes are different [24].

(3) Trypanosome kDNA decatenation by Topo II is inhibited in the presence of ellipticine and aza-ellipticine

In the presence of Topo II and in an ATP-dependent fashion, kDNA which exists in its native form as a multicatenane [25] is converted into supercoiled and relaxed monomers (Fig 6, lane 2) This decatenating activity can be inhibited by different agents, resulting in a progressive disappearance of the monomers in the reaction mixture [26] For example, with the epipodophyllotoxin VM26 (Teniposide form), a non intercalative agent that induces Topo II-mediated DNA single- and double-strand breaks [27, 28], these effects were visible at a 5 μM dose and complete at 50 μM (Fig 6A, lanes 3–8) With BD-40 (Fig 6B, lanes 3–11), BR-76, ellipticine and NMHE (data not shown) the decatenating process was completely inhibited at various drug concentrations (respectively 10, 20, 20 and 2 8 μM) which did not correlate well (except for NMHE) to D55 cell cytotoxic activity (DE50 are respectively 0 2, 0 5, 3, 1 μM) Furthermore other studies with different chemical compounds from the series of ellipticine, epipodophyllotoxin and acridine on the decatenating reaction show that NMHE and BD-40 are two of the most potent inhibitors of this reaction [29]

The topological structure of the decatenated mini-

circle appeared to be modified by BD-40 intercalation With low doses of drug (lanes 3–7), closed circular minicircles (form I) were relaxed. With higher concentrations (lanes 8–10), before the complete inhibition of the reaction, closed circular minicircles became positively supercoiled This phenomenon has been observed with all intercalative agents so far tested that inhibited the decatenating reaction [30]. In contrast, VM26 did not cause this phenomenon.

(4) DNA-breaking activity of ellipticine and aza-ellipticine on isolated nuclei

We have previously shown that two aza-ellipticine derivatives (BD-40 and BR-76) induced DNA breaks associated to DPLs in intact cells incubated with cytotoxic drug concentrations [7] Identical effects were observed with ellipticine [8] However, while DPLs generated by ellipticine did not prevent a DNA size reduction observed by alkaline sucrose gradient centrifugation, BD-40-induced breaks were not revealed unless a proteinase K treatment was included prior to centrifugation This result indicated that DPLs induced by aza-ellipticines are under the form of SDS- and alkaline-resistant bridges which maintain cohesiveness of DNA termini and prevent any DNA size reduction

To further compare the effects of ellipticine and BD-40 on isolated nuclei, cells were labelled with ^{3}H-thymidine for 24 hr and nuclei were isolated and incubated for 10 min with ellipticine (2 μM) or BD-40 (0 05 μM or 0.5 μM). Nuclei were always submitted to digestion with proteinase K before centrifugation.

DNA from isolated nuclei treated with ellipticine sedimented less rapidly than control DNA, indicating a size reduction (Fig 7). Omission of the proteinase K treatment resulted in the same profile (not shown) In contrast, DNA from BD-40-treated nuclei did not undergo any reduction of its apparent molecular weight and superimposed to control DNA

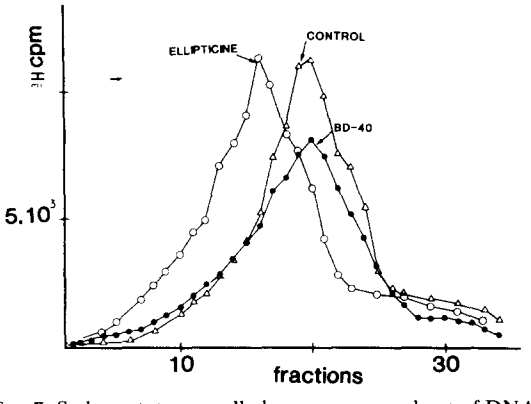


Fig 7 Sedimentation in alkaline sucrose gradient of DNA from ellipticine and BD-40-treated isolated nuclei D55 isolated nuclei containing labelled DNA were incubated with ellipticine (2 μM) or BD-40 (0 05 μM or 0 5 μM) for 10 min DNA size was analysed by alkaline sucrose gradient sedimentation after proteinase K treatment Arrow indicates the direction of the sedimentation

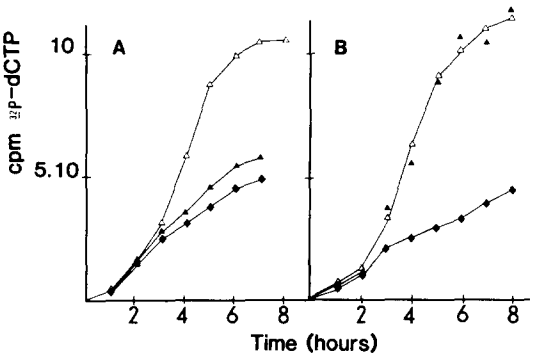


Fig 8 DNA synthesis by isolated from synchronized cells (see Materials and Methods) (A) DNA synthesis by nuclei isolated from control cells (△—△), cells treated with 2 μM ellipticine (♦—♦) and cells treated with 0 5 μM BD-40 (▲—▲) (B) DNA synthesis by isolated nuclei control (△—△), nuclei treated with 2 μM ellipticine (♦—♦) and 0 5 μM BD-40 (▲—▲) Intermediate concentrations produced the same results

(same data with 0.5 μM, not shown). An identical result was obtained with BR-76 (not shown).

To summarize, while ellipticine-induced breaks in DNA of intact cells as well as of isolated nuclei, BD-40 did not appear to efficiently act on isolated nuclei in spite of a readily discernible effect on whole cells. This apparent failure was not related to any impairment in the drug penetration: a fluorescence microscope study indicated that the fluorescence intensity of nuclei incubated for 5 min with 0.05 μM was equivalent to that observed in the nuclei of intact cells incubated for 4 hr (at this time, drug uptake is at equilibrium) with 0.5 μM

(5) Action of ellipticine and aza-ellipticine on DNA synthetic activity by isolated nuclei

Ellipticine and BD-40 greatly decreased the incorporation of ^{3}H-thymidine into acido insoluble material when they were added to synchronized intact cells (Fig 8A), indicating a diminished rate of DNA synthesis in agreement with the lengthening of the S phase noticed in the presence of drugs [9]

Isolated nuclei also exhibited a decrease in DNA synthetic activity, when they were released from synchronized cells incubated with ellipticine or BD-40 (Fig. 8A) This effect persisted when synchronized nuclei were treated *in vitro* with ellipticine but did not appear more in the presence of BD-40 (0.05 or 0.5 μM). These data confirmed that BD-40 efficiently acted on intact cells similarly to ellipticine but was devoid of activity on nuclei purified prior to any drug treatment

DISCUSSION

In this report, we present data showing that aza-ellipticines stimulate the cleavage activity of purified calf thymus Topo II The cleavage activity can be reversed in high-salt conditions that are not favorable for nuclease and ligase-type enzymes. According to Chen *et al* [18], this reaction is not ATP-dependent but is stimulated by ATP (data not shown). Fur-

thermore, as judged by mapping experiments, cleavage sites generated by BD-40 are the same as those stimulated by NMHE or ellipticine These results support the conclusion that Topo II is directly involved in the action of these ellipticine analogues. It is unlikely that a Topo I was implicated in this reaction because, (1) residual Topo I activity in the Topo II preparation was low, and (2) only high Topo I concentrations can induce double strand-like breaks on complementary DNA strands [31].

It is worth noting that BD-40 concentrations stimulating Topo II are close to DE50 concentrations in cultured cells [19]. In the *in vitro* enzyme test, the first drug concentration that converts the pUC supercoiled DNA form to the linear form is 0.04 μM (Fig 3). A 0 05 μM concentration has been shown to inhibit cell growth and induce transient DNA breaks [7] Thus, a good correlation seems to exist between drug activity on Topo II and cell effects. In contrast, there is no evident dose–activity relationship as far as the inhibition of kDNA decatenation by drugs is concerned BD-40, the most potent inhibitor of the decatenating Topo II activity in aza-ellipticine series, required 50-fold higher doses than cytotoxic ones (10 μM versus 0 2 μM) to interfere with the enzyme. A possible explanation might be that all available intercalation sites are not saturated by lower doses and that Topo II is active as long as specific cleavage sites are not occupied by the drug Results with teniposide (VM26) are consistent with this hypothesis as inhibition of decatenation activity has been observed although VM26 does not intercalate into DNA In that case, a VM26-Topo II interaction is sufficient to explain the inhibition by the drug Moreover, Et Br which is a strong intercalator but does not induce Topo II-mediated DNA breaks has been reported to inhibit DNA cleavage by agents interacting with Topo II [34]. Therefore, evidence exists that drug intercalation can interfere with Topo II activity when conditions are favorable.

It is also notable that BD-40 stimulates Topo II more efficiently than its congener BR-76 which structurally differs by a diethylaminopropylamino side chain in C_1 and is also substituted by a methyl group in C_{11} This suggests that the side chain plays an important role in the activity and has to be put together with the highest *in vivo* activity of BD-40 compared to its congeners. A systematic study of the role of this chain should be initiated

Ellipticine and aza-ellipticine do not appear to act identically on isolated nuclei in which chromatin is assumed to be in a conformation close to its native state Since ellipticine efficiently reacted under our experimental conditions, it is rather unlikely that Topo II was released from nuclei during purification or inactivated (it can be emphasized that reaction conditions included use of a buffer adapted at best to Topo II activity) On the other hand, BD-40 uptake by nuclei is not diminished. According to the data published in the literature, lesions induced in DNA by intercalative or non intercalative drugs are assumed to occur via Topo II–drug complexes [13, 16, 18, 32] Our present results strongly suggest that a similar complex is formed between BD-40 and Topo II *in vitro* Therefore, in isolated nuclei, this complex might not be formed or might be inactive A similar phenomenon could explain the apparent absence of NMHE activity on isolated nuclei [33], while it is active on intact cells [34]

An alternative hypothesis might be that aza-ellipticines require a bio-activation step before being functional and that this step cannot occur in isolated nuclei because it is not the proper place where the process normally takes place This metabolic activation model is supported by experimental evidence BD-40 toxicity on yeast has been shown to be greatly stimulated by oxygen [4]. The validity of this model will be only verified by isolation of active aza-ellipticine metabolites in cells

Finally, since it is reasonable to assume that differences in DPLs induced by each chemical series may reflect a different type of DNA-Topo II–drug ternary complex, a test tube study using purified enzyme should be valuable to understand the exact nature of the cleavable complex

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