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THE ANTILEUKEMIC ALKALOID FAGARONINE IS AN INHIBITOR OF DNA TOPOISOMERASES I AND II

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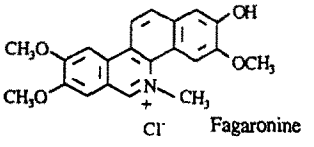
Abstract—The antileukemic alkaloid, fagaronine, is a potent differentiation inducer of various hematopoietic cell lines. We show here that fagaronine is a DNA base-pair intercalator with a K_{app} of 2.1×10^5 M^{-1} for calf thymus DNA. Fagaronine inhibits the catalytic activity of purified calf thymus topoisomerase I as shown by relaxation of supercoiled plasmid DNA followed by electrophoresis in neutral as well as in chloroquine-containing gels. The catalytic activity of topoisomerase I is inhibited at concentrations above 30 μM. Fagaronine also inhibits the catalytic activity of purified calf thymus topoisomerase II at concentrations above 25 μM as shown by decatenation of kinetoplast DNA. Fagaronine stabilizes the covalent DNA–enzyme reaction intermediate (the cleavable complex) between topoisomerase I and linear pBR322 DNA at concentrations up to 1 μM. Further increase of the fagaronine concentration leads to a progressive decrease in the cleavable complex formation, which is totally inhibited at 100 μM. In contrast, up to 1 μM fagaronine has no effect on cleavable complex formation between purified calf thymus topoisomerase II and linear pBR322 DNA, whereas cleavable complex formation is inhibited at higher concentrations. Exposure to fagaronine results in an increase in DNA–protein complex formation in intact P388 murine leukemia cells. P388CPT5 cells, which have an altered topoisomerase I activity, are 4-fold resistant to the growth inhibitory effects of fagaronine compared to the parental cell line. Similarly, DC-3F/9-OH-E Chinese hamster fibrosarcoma cells, which have an altered topoisomerase II activity, are about 5-fold resistant to the growth inhibitory effects of fagaronine. We conclude that fagaronine is an inhibitor of both DNA topoisomerase I and II and propose that this might play a role in the cytotoxic activity.

Fagaronine (2-hydroxy-3,8,9-trimethoxy-5-methylbenzo [*c*] phenanthridine) (Fig. 1) was isolated from the roots of *Fagara zanthoxyloides* Lam. (Rutaceae), a small tree indigenous to West Africa [1]. Fagaronine is bactericidal on *Salmonella typhimurium* and cytotoxic towards L1210 and KB cells [1, 2]. This activity seems in both cases to depend on the presence of the quaternary nitrogen since the desmethyl congener is inactive [2]. No mutagenic effects are observed in *Salmonella typhimurium* while fagaronine is found to be weakly clastogenic in *Drosophila melanogaster* [2, 3]. Fagaronine shows potent antitumor activity towards P388 and L1210 lymphocytic leukemias *in vivo* and towards colon 26 [1, 4]. More recently, fagaronine has been shown to induce differentiation in murine erythroid Friend cells, human K562 erythroleukemia cells and human promyelocytic HL60 cells [5–7].

In vitro, fagaronine has been shown to interact with DNA as well as with double stranded regions of ribonucleic acids such as tRNA [2]. In cell-free systems, fagaronine strongly inhibits *Escherichia coli* DNA polymerase I and RNA polymerase activities as well as protein synthesis [8, 9]. Reverse transcriptase from avian myeloblastosis virus, simian sarcoma virus or murine leukemia virus is also inhibited by fagaronine [9, 10]. In KB cells, DNA, RNA and protein synthesis are inhibited with practically the same effectiveness [8], and it is suggested, that fagaronine may exert its cytotoxic activity by at least two different mechanisms; inhibition of nucleic acid synthesis due to interaction with DNA, and by inhibition of the elongation step of protein synthesis [8].



Fagaronine

Fig. 1. Structure of fagaronine.

The present study was undertaken in an attempt to elucidate the mechanism of action of fagaronine. The results show that fagaronine is an inhibitor of both DNA topoisomerase I and II, and that this may play a role in the cytotoxic activity of the compound.

MATERIALS AND METHODS

Drugs and chemicals. Camptothecin (CPT¶) was

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¶ Abbreviations: mAMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; 9-OHE, 9-hydroxyellipticine; CPT, camptothecin; SDS, sodium dodecyl sulfate.

a kind gift from Christine Jaxel (Orsay, France), while 4'-(9-acridinylamino)methanesulfon-*m*-aniside (mAMSA) was obtained from Substantia Laboratories (Courbevoie, France). Fagaronine (NSC 157995) was extracted from the roots of *Fagara xanthoxyloides* Lam. (Rutaceae) as described previously [1]. [α^{32}P]dATP, L-[^{14}C(U)]leucine and [6-^{3}H]thymidine were purchased from Amersham (Amersham, U.K.). All other chemicals were of reagent grade.

DNA substrates. Supercoiled plasmid pBR322 (>95% form I) and calf thymus DNA were purchased from Boehringer Mannheim (Germany). Highly catenated kinetoplast DNA was purified from *Trypanosoma cruzi* (kindly provided by Guy Riou, Villejuif, France) after DNA extraction and sucrose sedimentation [11]. Linear α^{32}P end-labeled pBR322 was prepared as described previously [12].

Enzymes. Restriction endonucleases, *E. coli* DNA polymerase (Klenov fragment) and proteinase K were purchased from Boehringer Mannheim. DNA topoisomerases I and II were purified from calf thymus using an adaptation of procedures described previously [13, 14]. Briefly, nuclei were extracted with 0.35 M NaCl and the nucleic acids precipitated with polymine P (0.1% final concentration). The supernatant was passed through a phosphocellulose column and assayed for topoisomerase I and II activities. Fractions containing topoisomerase I or II were pooled and applied separately to a hydroxylapatite column and subjected to glycerol gradient centrifugation. The purified enzymes were stored at −70° in 10 mM Tris–HCl, pH 7.5, 20 mM β-mercaptoethanol, 0.5 mM EDTA, 100 μg/mL bovine serum albumin and 50% glycerol. One topoisomerase I or II unit corresponds to the minimal amount of enzyme needed to relax/decatenate 50% of 200 ng of supercoiled pBR322 DNA/kDNA at 37° for 30 min.

DNA binding studies. Viscosimetric measurements were performed at 25° using sonicated calf thymus DNA in 0.1 M NaCl, 0.1 M Tris–HCl, pH 7.4 in the presence of increasing drug concentrations. The increase in length of DNA upon drug binding was calculated as described by Saucier *et al.* [15]. DNA binding constants were determined by measuring the drug's ability to compete with the binding of ethidium bromide according to Le Pecq and Paoletti [16]. The DNA binding of ethidium bromide itself was measured by spectrofluorometry. K_{app} is the DNA binding constant of the various compounds when measured in 0.1 M NaCl, 0.1 M Tris–HCl, pH 7.4 at 25°.

Relaxation assay. The reaction mixture contained 50 mM Tris–HCl, pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol and 200 ng of supercoiled pBR322. The reaction was initiated by the addition of DNA topoisomerase I (2 units) and allowed to proceed at 37° for 30 min. Reactions were terminated by the addition of a loading buffer containing EDTA, bromophenol blue and sucrose (10 mM, 0.02% and 8% final concentrations, respectively). The samples were electrophoresed in 0.8% agarose gels at 2 V/cm for 18 hr in Tris/borate/EDTA buffer at pH 8. Some gels also contained chloroquine (5 μM). Photographic negatives of the ethidium bromide-stained agarose gels were scanned with a Joyce-Loebl Chromoscan 3 densitometer and the peak areas of supercoiled DNA determined.

Decatenation. Reaction conditions were as described above except that the reaction buffer also contained 10 mM $MgCl_2$ and 1 mM ATP and that 200 ng of kinetoplast DNA was used as substrate instead of pBR322. Electrophoresis was in 1.2% agarose gels at 5 V/cm for 4 hr. Liberated minicircles were quantified by densitometric scanning of photographic negatives.

Topoisomerase I cleavage reactions. The reaction mixture contained 20 mM Tris–HCl, pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 2×10^4 dpm of 3'-end-labeled pBR322 DNA and the indicated drug concentrations. The reaction was initiated by the addition of DNA topoisomerase I (20 units in 20 μL reaction volume) and allowed to proceed at 37° for 10 min. Reactions were terminated by the addition of sodium dodecyl sulfate (SDS) and proteinase K (0.25% and 250 μg/mL final concentrations, respectively) and incubated for 30 min at 50°. The samples were denaturated by the addition of 10 μL 0.45 M NaOH, 30 mM EDTA, 15% w/v sucrose, 0.1% bromocresol green and loaded on a 1% agarose gel in Tris/borate/EDTA buffer with 0.1% SDS. The samples were electrophoresed at 2 V/cm for 18 hr and autoradiographed with hyperfilm MP (Amersham) for 1 or 2 days.

Topoisomerase II cleavage reactions. The reaction mixture contained 20 mM Tris–HCl, pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 2×10^4 dpm of 3'-end-labeled pBR322 DNA and the indicated drug concentrations. The reaction was initiated by the addition of DNA topoisomerase II (20 units in 20 μL reaction volume) and allowed to proceed for 10 min at 37°. Reactions were terminated by the addition of SDS and proteinase K (0.25% and 250 μg/mL final concentrations, respectively) and incubated for 30 min at 50°. Loading buffer (5 μL) was added and the samples were electrophoresed and autoradiographed as described for the topoisomerase I cleavage reaction. Alternatively, the samples were denaturated with NaOH after addition of SDS and proteinase K and processed as described above for topoisomerase I.

Filter binding assay. The DNA–protein binding was measured by a filter binding assay as described [17]. Briefly, 40 ng of ^{3}H-labeled pBR322 was incubated with DNA topoisomerase II in the absence or presence of drug for 15 min at 30°. Reactions were stopped with 20 mM Na_2EDTA, pH 10, at 4° and applied to a polyvinyl chloride filter. Filters were washed with a solution containing 0.2% sarkosyl, 40 mM Na_2EDTA, 2 M NaCl, pH 10. The effluents, washes and filters were collected separately and processed for radioactivity determination.

Cells and culture medium. The Chinese hamster cell line DC-3F and the 9-hydroxyellipticine resistant subline DC-3F/9-OH-E have been described previously, as have the media and growth conditions [18]. Murine P388 leukemia cells were grown at 37° in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium containing 10% fetal calf serum, 0.01 mM β-mercaptoethanol, 10 mM L-glutamine,

100 IU/mL penicillin, 2 mg/mL streptomycin, 50 mg/mL gentamycin and 50 mg/mL nystatin. The camptothecin resistant subline P388CPT5 is a clone isolated in soft agar from P388CPT0.3 [19]. The resistant subline was grown in the presence of 15% fetal calf serum.

Formation of covalent topoisomerase II–DNA complexes in intact P388 cells. This assay was carried out essentially as described previously [20]. Briefly, cellular DNA and protein were metabolically labeled by incubating approx. 4×10^5 P388 cells with both [^{14}C]leucine (0.2 μCi/mL) and [^{3}H]thymidine (0.6 μCi/mL), in RPMI 1640 medium containing 10% fetal bovine serum. The cells were then centrifuged at 400 *g* for 5 min and resuspended to approx. 8×10^5 cells/mL in sterile phosphate-buffered saline (PBS). The indicated concentrations of fagaronine or camptothecin were added to the cell suspensions, and the cells were incubated for an additional 30 min at 37°. After incubation with drug, each sample was divided into 0.5-mL aliquots, and reactions were stopped by adding 0.5 mL of a solution containing 2.5% SDS, 10 mM EDTA (pH 8.0) and 0.8 mg/mL salmon sperm DNA. Cell lysates were passed 10 times through a 22-gauge needle and then heated to 65° for 10 min. KCl (250 mM final concentration) was added to each tube. The tubes were vortexed for 10 sec, put on ice for 5 min, and then centrifuged at 10,000 *g* for 10 min at 4°. Each pellet was washed three times with 1 mL of a solution containing 10 mM Tris–HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, pH 8.0 and 0.1 mg/mL salmon sperm DNA. The pellets were then dissolved in 0.5 mL water at 65° for 15 min and centrifuged for 10 sec at 10,000 *g* at room temperature, and 0.5 mL was transferred to a vial for scintillation counting. Results are expressed as the ratio of [^{3}H]DNA/[^{14}C]-protein, with the dpm of protein precipitated as the internal control for all samples.

Growth inhibition assays. All experiments were carried out with exponentially growing cells. Briefly, $2–5 \times 10^4$ DC-3F or DC-3F/9-OH-E cells were plated in 24-well dishes (Falcon, Lincoln Park, NJ, U.S.A.) with various concentrations of drug. After a 72-hr incubation at 37° the cells were trypsinized and the number of cells per well counted. The ED_{50} of each cell line was determined from the exponential part of the dose–response curve. Alternatively, 2×10^4 P388 or P388CPT5 cells were seeded in 96-well microculture plates with various drug concentrations. After a 96-hr incubation at 37° the cells were incubated with 0.02% neutral red for 16 hr and washed and lysed with 1% SDS. The incorporation of the dye reflecting cellular growth and viability was evaluated by measuring the optical density at 540 and 346 nm for each well using a titertek multiwell spectrophotometer. Each experiment was done at least twice in quadruplicate. SE was <10% of the mean in all cases.

RESULTS

Fagaronine is a DNA intercalator

DNA intercalating agents cause an increase in the length of DNA due to their insertion between the base pairs. The length increase can be measured by viscosimetric titration of sonicated DNA in the presence and absence of drug. The theoretical treatment of viscosimetric data has shown that the slope of the curve representing the relative increase in DNA contour length vs the drug/nucleotide ratio is expected to be near 2.2 for monointercalating agents such as ethidium bromide [15]. Both the slope and the DNA association constant (K_{app}) of fagaronine (Table 1) are comparable to the values found for ethidium bromide (Table 1) and consistent with a binding of fagaronine occurring according to an intercalation mode.

Table 1. DNA binding properties of fagaronine

Compound	Lengthening* (slope)	K_{aff} (M^{-1})†
Fagaronine	2.33	2.1×10^5
Ethidium bromide	2.19	2.0×10^5

* Viscosimetric lengthening determined on sonicated calf thymus DNA in 0.2 M Tris–HCl, pH 7.4, 25°.

† Competition with ethidium bromide on native calf thymus DNA in 0.2 M Tris–HCl, pH 7.4, 25°.

Fagaronine inhibits the catalytic activity of DNA topoisomerase I

The effect of fagaronine on the catalytic activity of DNA topoisomerase I was assayed by two methods. Neutral agarose gel electrophoresis (Fig. 2) suggests that fagaronine totally inhibits the relaxation of supercoiled DNA by topoisomerase I starting at 7.5 μM (lane e). However, when DNA is relaxed in the presence of an intercalating agent and then transferred to a drug-free medium (such as a neutral agarose gel), the intercalated molecules are removed, and the DNA can return to the supercoiled state. Therefore, the experiment shown in Fig. 2 does not allow us to deduce whether the drug inhibits topoisomerase I, unwinds the supercoiled DNA or both. Such a distinction becomes possible when the DNA is electrophoresed in gels where an unwinding agent such as chloroquine has been added [21]. Chloroquine was used at a concentration of 5 μM under which conditions the control supercoiled DNA is migrating at a relaxed position [relaxed state (R) in Fig. 3, lane DNA] between the nicked DNA (N) and the supercoiled DNA (S). When the supercoiled DNA is incubated with topoisomerase I it becomes relaxed and the DNA appears in the chloroquine gel in a highly positive supercoiled state (S^+, lane E). In the presence of low concentrations of fagaronine (0.1–1 μM, lanes a to c) the DNA becomes less and less supercoiled as compared to lane E, and at 3 μM (lane d) chloroquine and fagaronine has an equal effect and the DNA remains in the relaxed state (R). As the concentration of the drug in the reaction mixture is further increased (10 and 30 μM, lanes e and f), the DNA becomes increasingly more supercoiled during electrophoresis (S^-). At 100 μM fagaronine (lane g) a partial inhibition of the relaxation reaction is detected since a portion of the DNA remains in the negatively supercoiled state (S^-) and the other part presents the same topological conformation as the control (R). Further increase of the fagaronine concentration

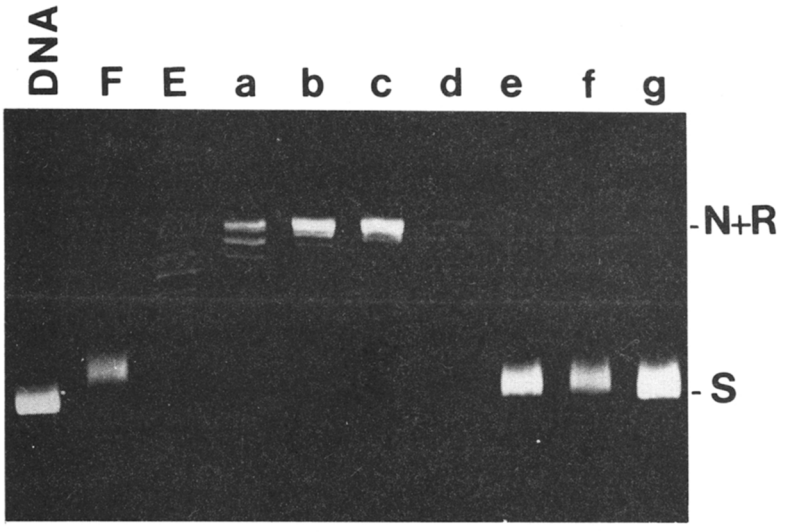

Fig. 2. Inhibition of the catalytic activity of purified calf thymus DNA topoisomerase I as measured by relaxation. Supercoiled pBR322 DNA (lane DNA) was relaxed by topoisomerase I in the absence (lane E) or presence of 1, 2, 3, 5, 7.5, 10 and 15 μM fagaronine (lanes a to g). Plasmid pBR322 was also incubated with 100 μM fagaronine in the absence of topoisomerase I (lane F). S, supercoiled; N, nicked; R, relaxed.

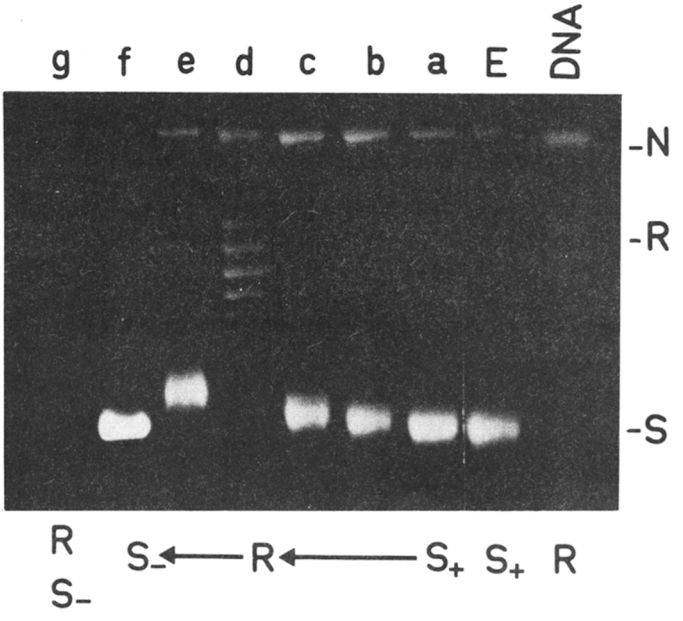

Fig. 3. Inhibition of the catalytic activity of purified calf thymus DNA topoisomerase I as measured by relaxation and electrophoresis in chloroquine-containing gels. Supercoiled pBR322 DNA (lane DNA) was relaxed by topoisomerase I in the absence (lane E) or presence of 0.1, 0.3, 1, 3, 10, 30 and 100 μM fagaronine (lanes a to g). S, supercoiled; N, nicked; R, relaxed.

leads to a complete inhibition of the relaxation catalysed by topoisomerase I (results not shown). These results show that fagaronine both unwinds the DNA and inhibits the catalytic activity of topoisomerase I.

Fagaronine inhibits the catalytic activity of DNA topoisomerase II

The effect of fagaronine on the catalytic activity of DNA topoisomerase II was measured by decatenation as shown in Fig. 4. In the absence of drug, decatenated minicircles (mc) are formed when kinetoplast DNA (kDNA) is incubated with topoisomerase II. The decatenation reaction is inhibited by fagaronine starting at 50 μM (lane f). At 100 μM (lane h) the reaction is totally inhibited.

Fagaronine stimulates the topoisomerase I cleavage reaction

The ability of fagaronine to stabilize the covalent DNA–enzyme reaction intermediate (the cleavable complex) is evaluated by incubating calf thymus topoisomerase I with α^{32}P-labeled linear pBR322 DNA in the absence and presence of drug followed

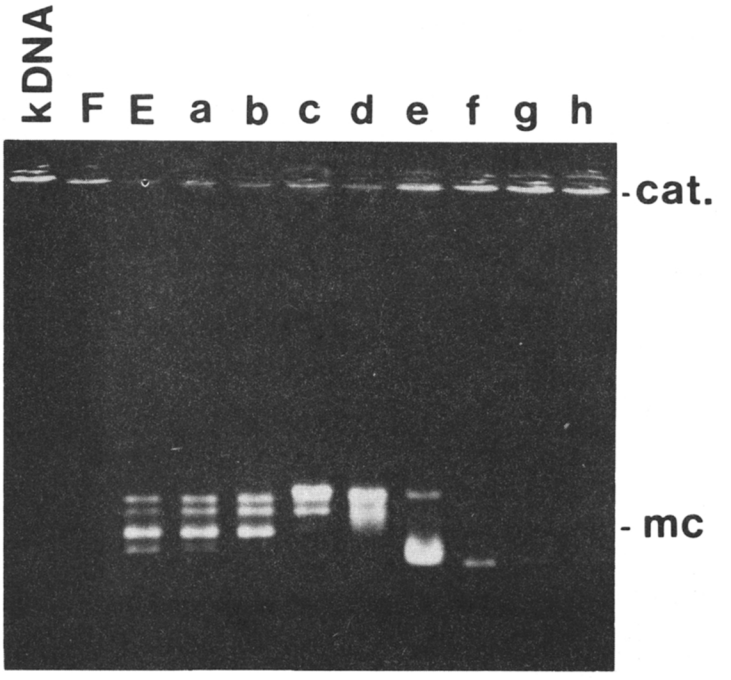

Fig. 4. Inhibition of the catalytic activity of purified calf thymus DNA topoisomerase II as measured by decatenation. Kinetoplast DNA (lane kDNA) was decatenated by topoisomerase II in the absence (lane E) or presence of 0.5, 1, 2, 5, 10, 25, 50 or 100 μM fagaronine (lanes a to h). Kinetoplast DNA was also incubated with 100 μM fagaronine in the absence of topoisomerase II (lane F). Cat, kinetoplast DNA; mc, decatenated minicircles.

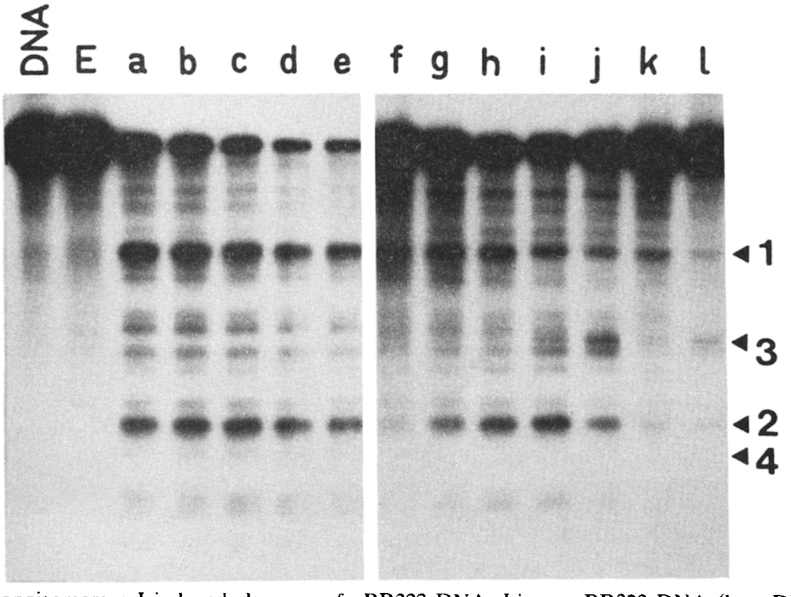

Fig. 5. Topoisomerase I induced cleavage of pBR322 DNA. Linear pBR322 DNA (lane DNA) was incubated with purified calf thymus DNA topoisomerase I in the absence (lane E) or presence of 0.1, 0.3, 1, 3 and 10 μM CPT (lanes a to e) or 0.1, 0.3, 1, 3, 10, 30 and 100 μM fagaronine (lanes f to l), followed by treatment with SDS and proteinase K.

by treatment with SDS and proteinase K. Cleavable complex formation is revealed by the appearance of DNA single strand breaks when the sample is analysed by agarose gel electrophoresis under denaturing conditions. Figure 5 shows a typical experiment. Control DNA migrates as a single band whereas some cleavable complex formation is observed in the presence of topoisomerase I (lane E). The addition of increasing concentrations of fagaronine results in a biphasic response curve. At concentrations up to 1 μM (lanes f to h) fagaronine induces a dose-dependent formation of the cleavable complex as judged by the appearance of new low molecular weight bands and an associated decrease

of the native DNA band. Further increase of the fagaronine concentrations (lanes i to l) leads to a progressive decrease ending in total disappearance of the cleavage products. In order to estimate the potency of the fagaronine-induced cleavage reaction, the remaining amount of native DNA is measured by scanning the autoradiogram with a densitometer. The maximum DNA cleavage is found for 1 μM fagaronine and corresponds to 45% of the native DNA. In comparison, CPT, a known topoisomerase I inhibitor, stimulates cleavable complex formation at all doses tested between 0.1 and 10 μM (Fig. 5, lanes a to e). At 0.1 μM CPT (lane a) the amount of cleaved DNA corresponds to 51% of the native DNA as measured by densitometry. These results indicate that in terms of concentration, fagaronine is at least 10 times less potent than CPT in stabilizing topoisomerase I cleavable complexes.

Fagaronine stimulates both the formation of pre-existing topoisomerase I cleavage sites and new cleavage sites not observed with the enzyme alone. The cleavage pattern generated by fagaronine and CPT are quite similar, especially as the two major cleavage sites are common for the two drugs (arrows 1 and 2). However, some differences are observed at the minor cleavage sites (arrows 3 and 4).

Fagaronine inhibits the topoisomerase II cleavage reaction

The topoisomerase II cleavage reaction is assayed with calf thymus DNA topoisomerase II and linear pBR322 followed by treatment with SDS and proteinase K. The cleavable complex is revealed by the appearance of DNA double strand breaks during agarose gel electrophoresis under neutral conditions. Figure 6 shows, that at low concentrations (0.1–1 μM, lanes h to j) fagaronine had no effect on cleavable complex formation as compared to the cleavage induced by topoisomerase II in the absence of drug (lane E). At concentrations higher than 1 μM (lanes k to n) the endogeneous cleavable complex formation was inhibited by fagaronine. Under the same experimental conditions, the topoisomerase II inhibitor mAMSA induces a dose-dependent increase of the cleavage reaction (lanes a to g). Similar results are obtained in cleavage-assays where the ability of fagaronine to stimulate topoisomerase II-induced single strand breakage is assessed (results not shown).

Finally, the effect of fagaronine on the overall binding of DNA topoisomerase II to pBR322 DNA was analysed by a filter binding assay. No effect on the retention of DNA is observed in the presence of fagaronine (10–200 μM) as compared with the background value (results not shown).

Formation of covalent topoisomerase–DNA complexes in intact P388 cells

Fagaronine-stimulated cleavable complex formation was determined in intact P388 murine leukemia cells by the SDS-KCl precipitation assay [20]. Figure 7 shows that incubation of cells with fagaronine in the concentration range of 1–10 μM results in a dose-dependent increase in DNA–protein complex formation. This last concentration results in a 2-fold stimulation of cleavable complexes as compared to untreated control cells which represents a plateau in the dose–response curve, since a further increase in the concentration of fagaronine does not change the level of DNA–protein complexes. A 7.9-fold stimulation of DNA–protein complexes is observed in the presence of 10 μM CPT. Therefore, at this concentration CPT is about four times more potent than fagaronine.

Cells with altered topoisomerase I and II activity are cross-resistant to fagaronine

The growth inhibitory effects of fagaronine on CPT resistant P388CPT5 murine leukemia cells [19] are examined. The P388CPT5 cells have a rearranged topoisomerase I gene, reduced topoisomerase I transcription and show reduced drug-induced complex formation *in vitro* as well as in living cells [19]. This cell line is about 80-fold resistant to CPT, 400-fold resistant to topotecan and 70-fold resistant to SN38 (the active metabolite of CPT11). In contrast, no change is observed with respect to the topoisomerase II inhibitors etoposide (VP-16) and amsacrine (mAMSA) (J. F. Riou, unpublished results). The results (Fig. 8) show, that the P388CPT5 cell line is four times resistant to fagaronine as compared to the parental line (IC_{50} P388:2 μM; IC_{50} P388CPT5:8 μM).

The growth inhibitory effects of fagaronine are also examined on 9-hydroxyellipticine (9-OHE) resistant DC-3F/9-OH-E Chinese hamster fibrosarcoma cells. These cells have an altered topoisomerase II activity, which leads to a reduction of drug-induced cleavable complex formation *in vitro* as well as in living cells [22–24]. DC-3F/9-OHE cells are about 140 times resistant to amsacrine while the sensitivity to CPT is unchanged (A. K. Larsen, unpublished results). The results (Fig. 9) show that DC-3F/9-OH-E cells are about five times resistant to fagaronine as compared to the parental DC-3F cells line (IC_{50} DC-3F:0.9 μM; IC_{50} DC-3F/9-OH-E:4.5 μM).

DISCUSSION

The present study was undertaken in an attempt to elucidate the mechanism(s) of action of fagaronine. Our results show that fagaronine interacts with calf thymus DNA with a k_{app} of 2.1×10^{-5} M. It has previously been observed that the addition of DNA leads to the occurrence of at least two isosbestic points in the UV-absorption spectrum of fagaronine [2] suggesting that DNA and fagaronine may interact in different ways. We show here that fagaronine extends the DNA helix and unwinds supercoiled DNA which is consistent with intercalation as being the dominant mode of interaction. Another mode of interaction of fagaronine with DNA is most likely by external binding. Interestingly, a three-mode DNA drug binding model has previously been suggested for ellipticine derivatives, which like fagaronine are planar aromatic compounds with a quaternary nitrogen. This includes intercalation, external binding and self-stacking of the drug molecules along the DNA [25].

Fagaronine inhibits the catalytic activities of both DNA topoisomerase I and II at concentrations above

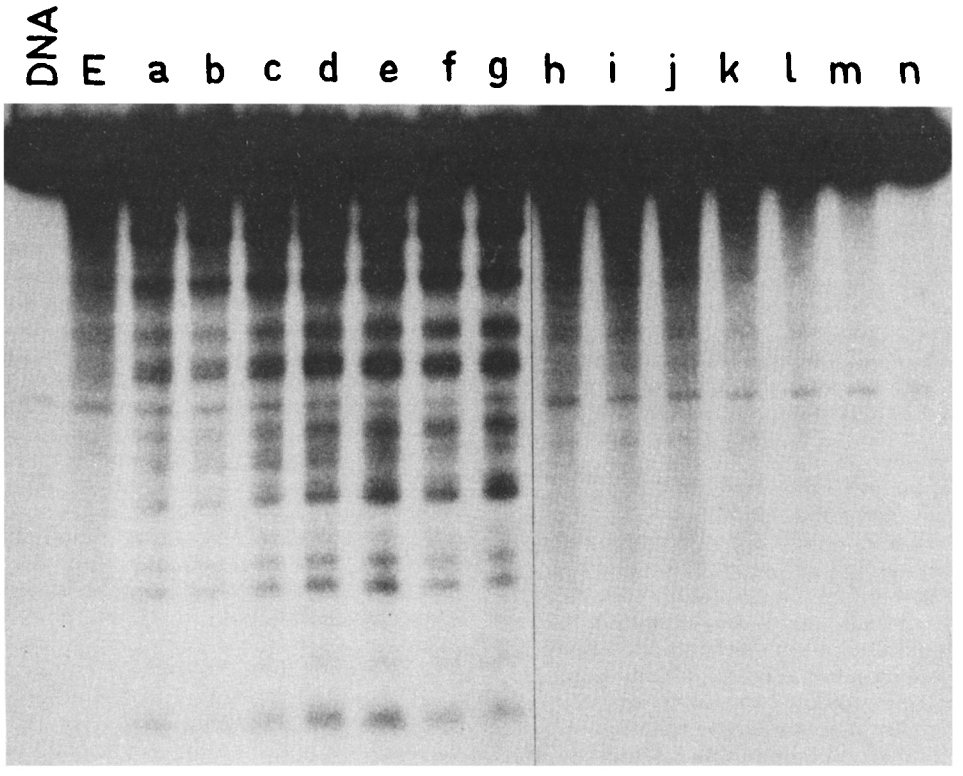

Fig. 6. Topoisomerase II-induced cleavage of pBR322 DNA. Linear pBR322 DNA (lane DNA) was incubated with purified calf thymus DNA topoisomerase II in the absence (lane E) or presence of 0.1, 0.3, 1, 3, 10, 30 and 100 μM mAMSA (lanes a to g) or 0.1, 0.3, 1, 3, 10, 30 and 100 μM fagaronine (lanes h to n) followed by treatment with SDS and proteinase K.

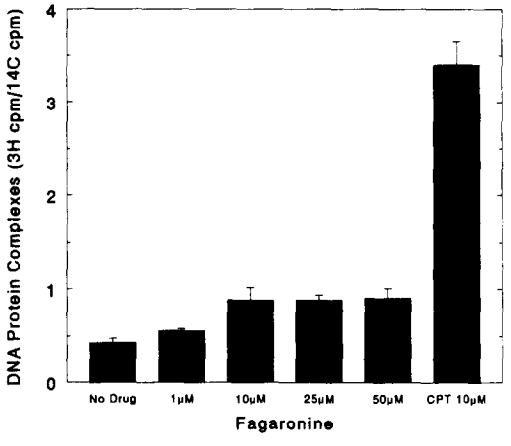

Fig. 7. Effects of fagaronine and CPT on DNA–protein complex formation in intact P388 cells. $^{3}H/^{14}C$-labeled cells were incubated for 30 min with the indicated concentration of fagaronine or CPT and DNA–protein complex formation determined as described in Materials and Methods.

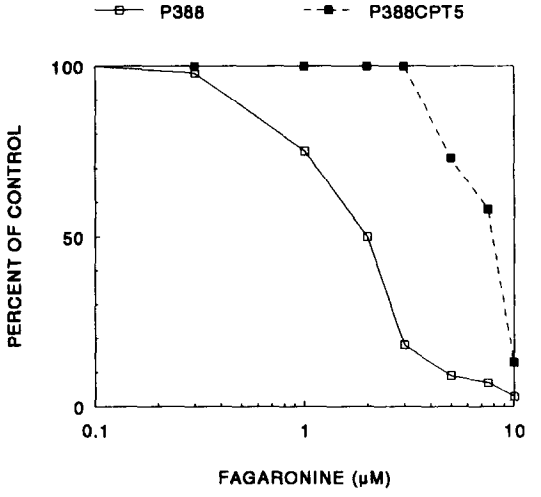

Fig. 8. Effect of fagaronine on the growth of P388 (□) and P388 CPT5 (■) murine leukemia cells.

25–30 μM. At low concentrations (below 1–3 μM) fagaronine stimulates the formation of topoisomerase I-mediated DNA cleavage, whereas no changes are observed with respect to topoisomerase II-mediated DNA cleavage. Higher concentrations of fagaronine resulted in a dose-dependent global suppression of both topoisomerase I and II mediated DNA cleavage.

Suppression of topoisomerase II-mediated DNA cleavage has been observed for most DNA intercalators at elevated drug concentrations and has been attributed to high drug intercalation resulting in extensive distortions of the DNA structure which may prevent topoisomerase binding [17, 26, 27]. Alternatively, extensive external binding may occur at elevated drug concentrations creating a shell of drug molecules around the DNA which would hamper enzyme binding to DNA sites [24].

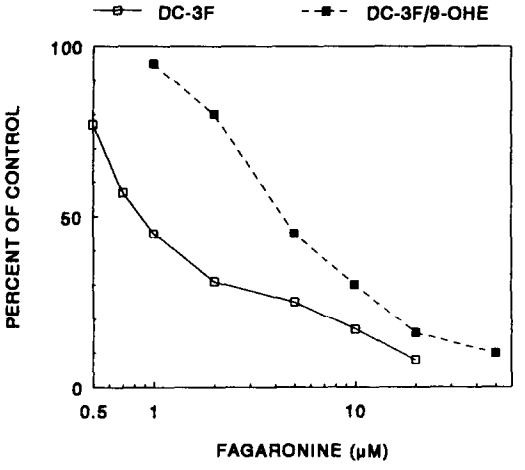

Fig. 9. Effect of fagaronine on the growth of DC-3F (□) and DC-3F/9-OH-E (■) Chinese hamster fibrosarcoma cells.

Intercalators such as ethidium bromide and 9-amino-acridines have also been reported to inhibit DNA topoisomerase I [27, 28]. In this regard, it is particularly interesting to notice that mAMSA is a potent inhibitor of both DNA topoisomerase I and II [27]. However, in contrast to the inhibition of topoisomerase II, the inhibition of topoisomerase I is not associated with enzyme-linked DNA strand breaks [27].

Therefore, the DNA intercalators might inhibit topoisomerases I and II in four different ways. First, both topoisomerases may be inhibited without formation of cleavable complexes as has been described for ethidium bromide [25, 27]. Second, the drug may stimulate topoisomerase I-, but not II-, mediated DNA strand breakage as reported here for fagaronine. Third, the drug may stimulate topoisomerase II-, but not I-, mediated DNA strand breakage as has been observed for mAMSA [28]. Finally, the drug may stimulate both topoisomerase I- and II-mediated DNA cleavage as has been described for actinomycin D and saintopin [29, 30]. This differential effect on topoisomerase-induced DNA cleavage may at least in part by explained by a degree of DNA sequence selectivity of the various intercalators [31] which could either favor or inhibit the formation of topoisomerase–DNA complexes.

Both CPT-resistant P388 cells and 9-OHE-resistant DC-3F cells are cross-resistant to the growth-inhibitory effects of fagaronine, but, to a lesser degree than that observed for the drugs with which the cell-lines were selected. It has been shown, that topoisomerase I, but not topoisomerase II, is altered in P388CPT5 cells while the opposite is the case for DC-3F/9-OHE cells. This is compatible with the observed cross-resistance patterns towards "pure" topoisomerase I or II inhibitors such as CPT, etoposide and amsacrine. Therefore, the cross-resistance studies presented here strongly suggest that fagaronine may exert its cytotoxic activity through either topoisomerase I or II, although the relative contribution of each enzyme on the cytotoxic effects of fagaronine on sensitive cells can not be determined with certainty. This would also explain why the cytotoxic effects of the drug increases with concentration, although a plateau of topoisomerase I-associated cleavable complexes is reached at 10 μM, since an additional increase of fagaronine would result in further inhibition of topoisomerase catalytic functions. Topoisomerase II is an essential enzyme which is required for the separation of intertwined chromosomal DNA molecules during mitosis [32, 33]. Interestingly, chromosomal clumping and abnormalities were observed in a high percentage of bone marrow cells after exposure to high doses of another intercalator, 9-OHE [34]. Therefore, in this case, the cytotoxic effects of the drug was most likely exerted through direct inhibition of topoisomerase II function.

Numerous topoisomerase I and II inhibitors are able to induce a partial differentiation of various human and rodent leukemia cell lines. In addition to fagaronine, this includes CPT, novobiocin, genistein, doxorubicin, aclacinomycin, elsamicin, etoposide, teniposide, suramin and 3-nitrobenzothiazole (3,2-a) quinolinium [35–45]. Although most of these inhibitors induce enzyme-linked DNA strand breaks, others (novobiocin, suramin, aclacinomycin [46–48]) act on different steps of the catalytic cycle. Therefore, the important therapeutic effects of topoisomerase inhibitors towards leukemias and lymphomas may be explained by a combination of cell growth inhibition and induction of cell maturation. In this regard, it might be important that apoptotic cell death has been shown to be the normal end for at least some differentiated leukemia cells [49].

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