Biochemical Pharmacology, Vol. 60, pp. 527–537, 2000.


ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00351-8

# Inhibition of Topoisomerase II by the Marine Alkaloid Ascididemin and Induction of Apoptosis in Leukemia Cells

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**ABSTRACT.** Ascididemin (ASC) is a pentacyclic DNA-intercalating agent isolated from the Mediterranean ascidian *Cystodytes dellechiajei*. This marine alkaloid exhibits marked cytotoxic activities against a range of tumor cells, but its mechanism of action remains poorly understood. We investigated the effects of ASC on DNA cleavage by human topoisomerases I and II. Relaxation assays using supercoiled DNA showed that ASC stimulated double-stranded cleavage of DNA by topoisomerase II, but exerted only a very weak effect on topoisomerase I. ASC is a conventional topoisomerase II poison that significantly promoted DNA cleavage, essentially at sites having a C on the 3′ side of the cleaved bond (−1 position), as observed with etoposide. The stimulation of DNA cleavage by topoisomerase I in the presence of ASC was considerably weaker than that observed with camptothecin. Cytotoxicity measurements showed that ASC was even less toxic to P388 leukemia cells than to P388CPT5 cells resistant to camptothecin. In addition, the marine alkaloid was found to be equally toxic to HL-60 leukemia cells sensitive or resistant to mitoxantrone. It is therefore unlikely that topoisomerases are the main cellular targets for ASC. This alkaloid was found to strongly induce apoptosis in HL-60 and P388 leukemia cells. Cell cycle analysis showed that ASC treatment was associated with a loss of cells in the G1 phase accompanied with a large increase in the sub-G1 region. Cleavage experiments with poly(ADP-ribose) polymerase (PARP) revealed that caspase-3 was a mediator of the apoptotic pathway induced by ASC. The DNA of ASC-treated cells was severely fragmented. Collectively, these findings indicate that ASC is a potent inducer of apoptosis in leukemia cells. BIOCHEM PHARMACOL **60**;4:527–537, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** ascididemin; topoisomerase; DNA cleavage; apoptosis; cell cycle; marine products; antitumor agent

ASC§ (Fig. 1) is a pentacyclic aromatic alkaloid isolated from the mediterranean ascidian *Cystodytes dellechiajei* collected near the Balearic Islands (Spain) [1] as well as from Okinawan tunicate *Didemnum* species [2]. It is a cytotoxic metabolite structurally related to a number of biologically active marine products such as meridine, 2-bromoleptoclinidone, shermilamines A and B, and the cystodytins A, B, and C [3–6]. These pyridoacridine alkaloids are highly toxic toward human cancer cells. ASC itself was found to be highly toxic to human colon (HCT116) and breast (MCF7) cancer cell lines [7] and to different human leukemic cell lines [1]. Most importantly, we found that ASC was almost equally toxic to drug-sensitive and multidrug-resistant cell lines [1]. The drug is not sufficiently safe for use in man but because of its unusual cytotoxicity profile, ASC and its congeners constitute an interesting series of antitumor agents that may warrant further development. ASC-based drugs may provide a new avenue of approach to treatment of chemoresistant tumors.

Our knowledge of the molecular mechanism of action of ASC remains very sparse. Although earlier studies showed that the alkaloid affects the release of calcium from sarcoplasmic reticulum [2], the main target for the drug is DNA. Spectroscopic measurements provided evidence that ASC intercalates into DNA, preferentially at GC-rich sequences [1]. Intercalation into DNA of various planar pyridoacridine derivatives has been well characterized; however, it is unlikely that the biological effect of the drug arises solely from intercalation into DNA. The literature is replete with DNA-intercalating agents, but only a few exhibit useful therapeutic properties. Moreover, the biological properties vary considerably: some intercalators are potent anticancer drugs (e.g. daunomycin, actinomycin D, amsacrine), whereas others exhibit antiparasitic (e.g. lucanthone, py-

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§ *Abbreviations:* ASC, ascididemin; CPT, camptothecin; TUNEL, TdT-mediated dUTP nick end labeling; HL-60, human leukemia 60 cells; and PARP, poly(ADP-ribose) polymerase.

Received 22 June 1999; accepted 14 February 2000.

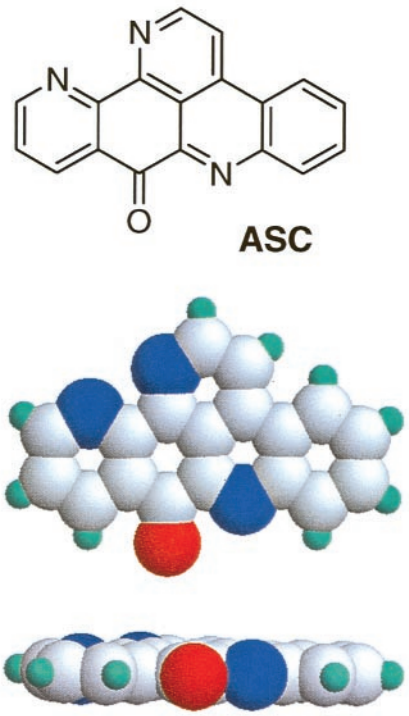


**FIG. 1. Chemical structure of ascididemin. An energy-minimized structure of the drug is shown. The softwares HyperChem™ 5.01 and Alchemy 2000® were used to construct the structure.**

ronaridine), antiviral (e.g. tilorone), or antimicrobial (e.g. ethidium) activities [8]. Other potent intercalators are carcinogenic (e.g. certain polycyclic aromatic hydrocarbons). There is mounting evidence suggesting that the antitumor activities of intercalating drugs are not due to interaction with DNA *per se* but is, at least in part, the result of the inhibition of enzymes that regulate DNA topology: the topoisomerases [9, 10].

In most cases, topoisomerase poisons inhibit the reaction of topoisomerases with DNA by stabilizing an abortive reaction intermediate, termed the cleavable (covalent) complex, whereby the DNA is cleaved but cannot be readily resealed. The cytotoxicity of clinically used intercalating drugs such as mitoxantrone and daunomycin is closely related to breakage of double-stranded DNA, for example as a result of the collision between replication forks and enzyme–DNA–drug ternary complexes. Intercalation into DNA and the ensuing poisoning of topoisomerases affect the genetic stability and integrity of chromosome structure and thus contribute to the cytotoxic action of the drugs. The vast majority of DNA-intercalating agents inhibit topoisomerase II, which cleaves both strands of DNA [11]. However, in recent years a variety of intercalators inhibiting the single-strand cleaving enzyme topoisomerase I have been reported. These included plant alkaloids such as bulgarein and coralyne and indolocarbazole derivatives related to the antibiotic rebeccamycin [12–14]. A few intercalating drugs are capable of poisoning both topoisomerases I and II: actinomycin D, intoplicine, saintopin, and TAS-103 [15–18].

There is some evidence suggesting that ASC exerts its biological activity via an inhibition of topoisomerase II. On the one hand, certain pyridoacridine alkaloids related to ASC (e.g. diplamine, cystodytin J) have been shown to interfere with topoisomerase II [19]. On the other hand, it was reported that ASC is extremely toxic to DNA double-strand break repair-deficient xrs-6 cells [7] thus raising the possibility that ASC might affect topoisomerase II functions in tumor cells. In contrast, there was no direct indication suggesting that topoisomerase I might also be a potential target for ASC. However, we were intrigued by a recent study showing that the ascidian alkaloid wakayin isolated from *Clavelina* species stimulates DNA cleavage by topoisomerase I [20]. Wakayin is a bispyrroloiminoquinone structurally distinct from ASC, but these two marine metabolites may be biosynthetically related because they both contain an iminoquinone core. These are the reasons why we investigated the effects of ASC on the catalytic activities of both topoisomerases I and II.

## MATERIALS AND METHODS

### *Drugs*

The procedures for the extraction and isolation of ascididemin have been reported previously [1]. The total synthesis of the alkaloid has also been published [21]. The drug was dissolved in dimethylsulfoxide at 2 mM and then further diluted with water. The stock solution of the drug was kept at −20° and freshly diluted to the desired concentration immediately prior to use. Etoposide and camptothecin were from Sigma Chemical Co. All other chemicals were analytical grade reagents.

### *Chemicals and Biochemicals*

Nucleoside triphosphates labeled with [$^{32}$P] (α-dATP and γ-ATP) were obtained from Amersham. Restriction endonucleases *Ava*I, *Pvu*II, *Hin*dIII, and *Eco*RI, alkaline phosphatase, T4 polynucleotide kinase, and AMV reverse transcriptase were purchased from Boehringer and used according to the supplier's recommended protocol in the activity buffer provided. All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionized, Millipore filtered water.

### *DNA Purification and Labeling*

Plasmids pBS (Stratagene), pKMp27 [22], and pLAZ3 [23] were isolated from *Escherichia coli* by a standard SDS–sodium hydroxide lysis procedure and purified by banding in CsCl–ethidium bromide gradients. Ethidium was removed by several isopropanol extractions followed by exhaustive dialysis against Tris–EDTA–buffered solution. The purified plasmid was then precipitated and resuspended in appropriate buffered medium prior to digestion by the

restriction enzymes. The two pBS DNA fragments were prepared by $^{32}$P-3′ end labeling of the *Eco*RI–*Pvu*II double digest of the plasmid using [$\alpha$-$^{32}$P]dATP and AMV reverse transcriptase or by $^{32}$P-5′ end labeling of the *Eco*RI/alkaline phosphatase-treated plasmid using [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase followed by treatment with *Pvu*II. Similarly, the 155- and 178-mer fragments were prepared by 3′-end labeling of the *Eco*RI–*Hin*dIII and *Eco*RI–PvuII digests, respectively, of plasmid pLAZ3. The 160-bp *tyr*T fragment was prepared by 3′ or 5′ end labeling of the *Eco*RI–*Ava*I digest of plasmid pKMp27. In each case, the digestion products were separated on a 6% polyacrylamide gel under native conditions in TBE-buffered solution (89 mM Tris–borate pH 8.3, 1 mM EDTA). After autoradiography, the band of DNA was excised, crushed and soaked in water overnight, at 37°. This suspension was filtered through a Millipore 0.22 μM filter and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the labeled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

### *DNA Relaxation Experiments*

Supercoiled pKMp27 DNA (0.5 μg) was incubated with 6 units human topoisomerase I or II (TopoGen) at 37° for 1 hr in relaxation buffer (50 mM Tris pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/mL. DNA samples were then added to the electrophoresis dye mixture (3 μL) and electrophoresed in a 1% agarose gel at room temperature for 2 hr at 120 V. Gels were stained with ethidium bromide (1 mg/mL), washed, and photographed under UV light. Similar experiments were performed using ethidium-containing agarose gels.

### *Topoisomerase I DNA Cleavage Reaction*

The plasmid pKMp27 was linearized with *Eco*RI and labeled with [$\alpha$-$^{32}$P]dATP in the presence of AMV reverse transcriptase or the Klenow fragment of DNA polymerase I. The labeled DNA was then digested to completion with *Ava*I. The cleavage reaction mixture contained 20 mM Tris–HCl pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, $2 \times 10^4$ dpm of [$\alpha$-$^{32}$P]-labeled DNA, and the indicated drug concentrations (in 20-μL reaction volume). The reaction was initiated by the addition of 20 units topoisomerase I (Life Sciences) and allowed to proceed for 45 min at 37°. Reactions were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 250 μg/mL, followed by incubation for 30 min at 50°. Samples were denatured by the addition of 5 μL buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green prior to loading onto 1% agarose gel in TBE buffer containing 0.1% SDS. Electrophoresis was conducted at 2 V/cm for 18 hr.

### *Topoisomerase II DNA Cleavage Reaction*

The cleavage 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, 500 cps of [$\gamma$-$^{32}$P]pBS DNA, and the indicated drug concentrations. The reaction was initiated by the addition of human topoisomerase II (20 units in 20-μL reaction volume, p170 form from TOPOgen) and allowed to proceed for 30 min at 37°. Reactions were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 250 μg/mL, followed by incubation for 30 min at 50°. Five microliters of loading buffer (30 mM EDTA, 15% [w/v] sucrose, 0.1% electrophoresis dye) were added to each sample prior to loading onto a 1% agarose gel in TBE-buffered solution containing 0.1% SDS. Electrophoresis was conducted at 2 V/cm for 18 hr.

### *Sequencing of Topoisomerase-Mediated DNA Cleavage Sites*

Each reaction mixture contained 2 μL of $^{32}$P-labeled DNA (~1 μM), 5 μL of water, 2 μL of 10X topoisomerase buffer, and 10 μL of drug solution at the desired concentration (1–100 μM). After 10-min incubation to ensure equilibration, the reaction was initiated by the addition 2 μL (20 units) bovine thymus topoisomerase I (Life Sciences) or 5 μL (10 units) human topoisomerase II (TOPOgen). Samples were incubated for 45 min at 37° prior to adding SDS to 0.25% and proteinase K to 250 μg/mL to dissociate the drug-stabilized cleavable complexes. The DNA was precipitated with ethanol, resuspended in 5 μL of formamide–TBE loading buffer, denatured at 90° for 4 min, and then chilled in ice for 4 min prior to loading onto the sequening gel. DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After electrophoresis (about 2.5 hr at 60 watts, 1600 V in TBE buffer, BRL sequencer model S2), gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper, and dried under vacuum at 80°. A Molecular Dynamics 425E PhosphorImager was used to collect data from the storage screens exposed to dried gels overnight at room temperature. Base line- corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the DNA fragment by comparison of its position relative to sequencing standards generated by treatment of the DNA with dimethylsulfate followed hy piperidine-induced cleavage at the modified guanine residues.

### *Cell Cultures and Survival Assay*

Human HL-60 and HL-60/MX2 promyelocytic leukemia cells were obtained from the American Tissue Culture Collection. Cells were grown at 37° in a humidified atmosphere containing 5% $CO_2$ in RPMI-1640 medium, supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 UI/mL), and streptomycin (100 μg/mL). P388 and P388CPT5 murine leukemia cell lines sensitive and resistant to CPT, respectively, were kindly provided by Dr J.-F. Riou (Rhône-Poulenc Rorer, France). The P388CPT5 cell line resistant to campthotecin was derived from a stable clone of the P388CPT0.3 cell line obtained at the 42nd passage [24]. Both cell lines were grown in RPMI-1640 medium containing 0.01 mM 2-mercaptoethanol, 10 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 IU/mL of penicillin, 2 μg/mL of streptomycin, 50 μg/mL of gentamycin, and 50 μg/mL of nystatin at 37° in a humidified atmosphere containing 5% $CO_2$. The cytotoxicity of ASC was assessed using a cell proliferation assay developed by Promega (CellTiter 96™ $AQ_{ueous}$ one solution cell proliferation assay). Briefly, $2 \times 10^4$ exponentially growing cells were seeded in 96-well microculture plates with various drug concentrations in a volume of 100 μL. After 72-hr incubation at 37°, 20 μL of MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopheenyl)-2*H*-tetrazolium, inner salt) [25] was added to each well and the samples were incubated for a further 2 hr at 37°. Plates were analyzed on a Labsystems Multiskan MS (type 352) reader at 492 nm. For the microscopic observations, cells were stained with the standard Pap method [26].

### *Cell Cycle Analysis*

For flow cytometry analysis of DNA content, $5 \times 10^5$ HL-60 cells in exponential growth were treated with 5 μM ASC for 18 hr and then washed 3 times with citrate buffer. The cell pellet was incubated with 125 μL of trypsin-containing citrate buffer for 10 min at room temperature and then with 100 μL of citrate buffer containing a trypsin inhibitor and RNase (10 min) prior to adding 100 μL of propidium iodide at 125 μg/mL. Samples were analyzed on a Becton Dickinson fluorescence-activated cell sorter (FACScan) flow cytometer using the LYSYS II software that is also used to determine the percentage of cells in G1, S, and G2/M phases.

### *PARP Cleavage*

Briefly, $7 \times 10^5$ exponentially growing HL-60 cells in a serum-free medium were treated with the test drug at the indicated concentration for 24 hr at 37°. Cells were pelleted by centrifugation, resuspended in 3 mL of lysis buffer containing 25 mM PBS, 0.1 mM PMSF, and the protease inhibitors chymostatin, leupeptin, aprotinin, and pepstatin A (5 μg/mL each). After centrifugation, the pellet was resuspended in the loading buffer containing 50 mM Tris–HCl pH 6.8, 15% sucrose, 2 mM EDTA, 3% SDS, and 0.01% bromophenol blue. The mixture was sonicated for 30 sec at 4° and then boiled to 100° for 3 min. For Western blotting, the cell lysates were fractionated on a 7.5% polyacrylamide gel containing 0.1% SDS, then transferred onto a Hybond-C nitrocellulose membrane (Amersham) for 40 min at 150 mA using a Semidry transfer system. Membranes were blocked with 10% non-fat milk in PBST (0.1% Tween 20, 25 mM phosphate buffer, pH 7.4) for 30 min followed by incubation with anti-PARP monoclonal antibody (Clontech) (dilution 1:10,000 in PBST supplemented with 0.1% non-fat milk) for 30 min. The blots were washed three times (5 min each with PBST) and incubated with a goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham Life Sciences, 1:10,000 dilution in PBST containing 0.1% non-fat milk) for 30 min. After three successive washes with PBST, the Western blot chemiluminescence reagent from NEN was used for the detection. Bands were vizualized by autoradiography.

### *Detection of DNA Fragmentation*

HL-60 cells at a density of about $10^6$ cells/mL were treated with various concentrations of ASC for the indicated periods and then collected by centrifugation at $2500 \times g$ for 5 min. The resultant cell pellets were resuspended in PBS buffer containing 5 mM $MgCl_2$ and lysed in 500 μL of TE buffer containing 0.1% SDS and proteinase K (1.5 mg/mL) overnight at 37°. After two successive extractions with phenol/chloroform, the aqueous layer was transferred to a new centrifuge tube. The DNA was precipitated with ethanol, resuspended in water (100 μL), and treated with RNase A (400 μg/mL) for 2 hr at 37°. Electrophoresis was performed in 1% agarose gel in Tris–borate buffer at about 2 V/cm for approximately 14 hr. After electrophoresis, the gel was stained with ethidium bromide (1 mg/mL), washed, and photographed under UV light.

### *Apoptosis Detection System in Fluorescence Microscopy*

The TUNEL assay developed by Promega was used to identify ASC-induced apoptotic HL-60 cells. The supplier's recommended protocol was followed. Briefly, cells were incubated with fluorescein-12-dUTP in the presence of terminal deoxynucloeotidyl transferase (TdT) to label 3′-OH ends of fragmented DNA [27]. All cells were stained with propidium iodide. Samples were photographed using a Zeiss Axiophot 2 microscope. Images were captured using the software Quips Smart Capture™ (Vysis).

## RESULTS

### *Topoisomerase II Inhibition*

ASC inhibited the catalytic activity of topoisomerase II. Closed circular DNA was treated with human topoisomerase II in the absence and presence of increasing concentrations of ASC (Fig. 2). The supercoiled DNA was relaxed by

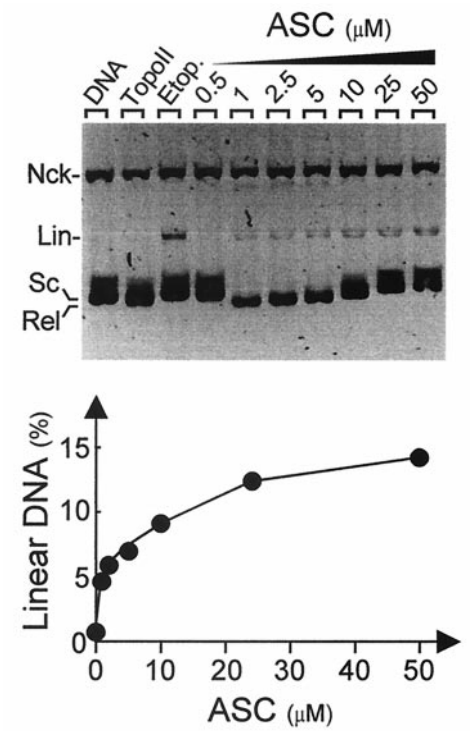


**FIG. 2. Effect of increasing concentrations of ASC on the relaxation of plasmid DNA by human topoisomerase II. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated with 4 units topoisomerase II in the absence (lane Topo II) or presence of drug at the indicated concentration (μM). Etoposide (lane Etop.) was used at 50 μM. Reactions were stopped with SDS and treatment with proteinase K. DNA samples were separated by electrophoresis on an agarose gel containing ethidium bromide. The gel was photographed under UV light. Nck, nicked; Lin, linear; Rel, relaxed; Sc, supercoiled. The graph shows the formation of linear DNA (form III) as a function of the ASC concentration. Data were compiled from quantitative analysis of two gels and are to be considered as a set of averaged values.**

the enzyme in the absence of the drug. In the presence of etoposide, a band corresponding to linear DNA (form III) could be clearly seen, attesting to the fact that the epipodophyllotoxin inhibited the religation of DNA once the double helix had been cleaved by the enzyme. The same band could be detected with ASC, but its intensity was much weaker. The extent of DNA cleavage was proportional to the drug concentration, but the band of linear DNA never exceeded 15% of the DNA products even when high concentrations of ASC were used. This first set of data suggests that ASC is a weak inhibitor of topoisomerase II. The topoisomerase II inhibitory properties of ASC were investigated further using radiolabeled DNA substrates. The *Eco*RI-*Hin*dIII restriction fragment of pLAZ3 was $^{32}$P-labeled at the 5′-end of the *Eco*RI site. The DNA cleavage products were analyzed by neutral agarose gel electrophoresis. A phosphorimage of a typical gel obtained after treatment of the 3.1-kbp DNA substrate with topoisomerase II in the presence of ASC is shown in Fig. 3. For comparison, the two well-established topoisomerase II inhibitors amsacrine and etoposide were used as positive controls. The effect seen with ASC was weaker than that obtained with etoposide. There is no doubt that ASC stimulates DNA cleavage. ASC effectively functions as a topoisomerase II poison stabilizing DNA–topoisomerase II covalent complexes. A few cleavage sites were sequenced (Fig. 4). The 117-and 265-bp *Eco*RI-*Pvu*II restriction fragments from pBS were incubated with ASC prior to cleavage by topoisomerase II. The cleavage patterns observed with ASC were different from those seen with etoposide. However, the majority of the cleavage sites were identical. For a typical cleavage site on the 117-mer fragment, one can see that the cleavage site identified on one strand was paired with the corresponding site, staggered with the expected 5′ overhang of four bases, on the complementary strand (gels at the bottom of Fig. 4). Sequence analysis showed that most of the cutting sites had a C at positions −1, i.e. on the 3′ side of the breaks.

### *Topoisomerase I Inhibition*

DNA relaxation experiments were performed with topoisomerase I. Negatively supercoiled plasmid pKMp27 was incubated with human topoisomerase I and concentrations

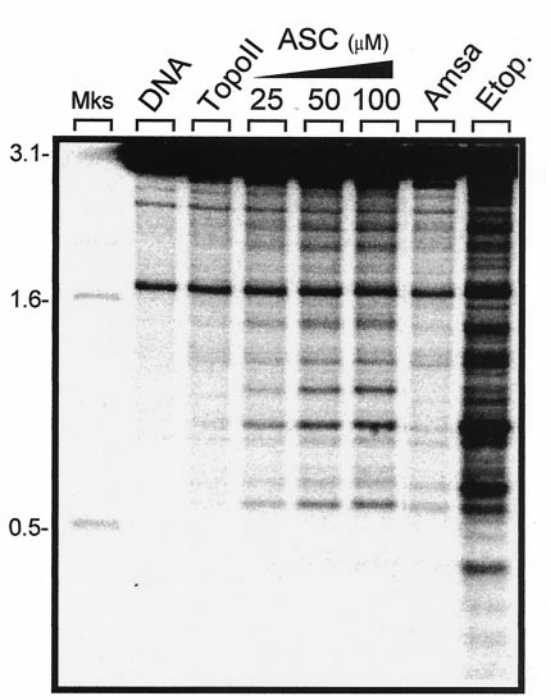


**FIG. 3. Topoisomerase II-mediated cleavage of radiolabeled DNA in the presence of ASC. Purified human p170 topoisomerase II (20 units) was incubated with the *Eco*RI–*Hin*dIII restriction fragment from pLAZ3 (3.1 kb, 5′ end $^{32}$P-labeled at the *Eco*RI site) in the absence (lane Topo II) and the presence of the drug. Double-stranded DNA fragments were analyzed on a 1% neutral agarose gel in the TBE buffer. Amsacrine (Amsa) and etoposide (Etop.) were used at 25 and 50 μM, respectively. The size of the molecular weight markers (Mks in kilobase) is indicated.**

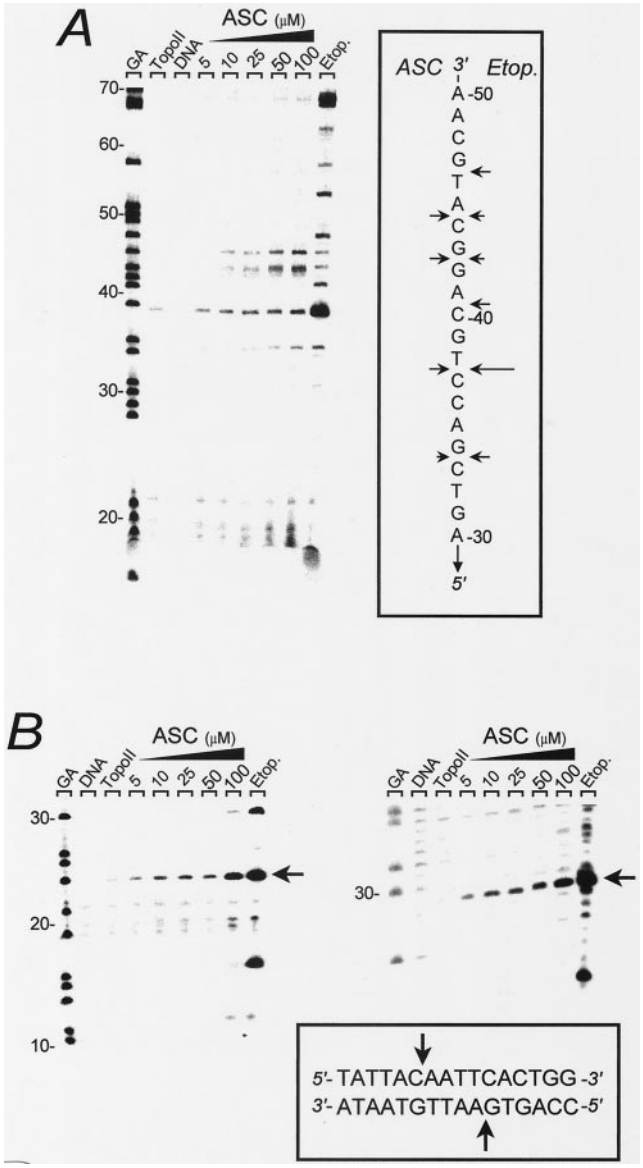


**FIG. 4. Sequence analysis of the topoisomerase II cleavage sites stimulated by ASC. The gels show the results obtained with (A) the 265-mer and (B) the 117-mer DNA fragments from pBS. In both cases, the 5′-end labeled DNA was incubated in the absence (lane Topo II) or presence of the test drug at the indicated concentration (μM). Etoposide was used at 50 μM. Topoisomerase II cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel as described in Materials and Methods. Numbers at the left side of the gels refer to the nucleotide position of cleavage sites, The arrows on the sequence point to the sites of cleavage stimulated by ASC or etoposide. For the 117-mer (B), the DNA was 5′-end labeled on one or the other of the two complementary strands, The tracks labelled G + A represent formic acid–piperidine markers specific for guanine and adenine residues.**

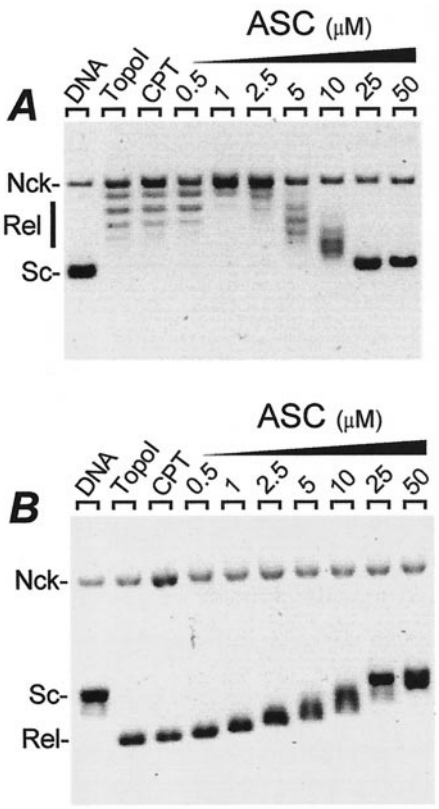


**FIG. 5. Effect of increasing concentrations of ASC on the relaxation of plasmid DNA by human topoisomerase I. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated with 4 units topoisomerase I in the absence (lane Topo I) or presence of drug at the indicated concentration (μM). Camptothecin (lane CPT) was used at 20 μM. Reactions were stopped with SDS and treatment with proteinase K. DNA samples were separated by electrophoresis on an agarose gel (A) without or (B) containing ethidium bromide. The gel was photographed under UV light. Nck, nicked; Rel, relaxed; Sc, supercoiled.**

of ASC ranging from 0.5 to 50 μM. The gel shown in Fig. 5A indicates that, at low ASC concentrations (1–2.5 μM), the intensity of the slowest migrating band (corresponding to nicked + fully relaxed DNA) increased significantly, as observed with the reference topoisomerase I poison, CPT. At higher ASC concentrations (>5 μM), the relaxation of DNA was inhibited, most likely as a result of the intercalation of the alkaloid into DNA. To rule out a non-specific effect due to direct interaction of ASC with DNA, the relaxation assay was repeated on an ethidium bromide-containing agarose gel, as shown in Fig. 5B. Under these conditions, the level of nicked DNA molecules was considerably increased in the presence of CPT, whereas it was very weakly increased with ASC. The subsequent experiments with radiolabeled DNA substrates confirmed that ASC had minimal effect on topoisomerase I. In Fig. 6, the 3.1-kbp DNA fragment (labeled at the 3′ end of the *Eco*RI site) was incubated with 50 μM ASC or 10 μM CPT. Prominent cutting sites could easily be detected with CPT, whereas only faint bands were seen with ASC under identical conditions. Although ASC weakly stimulated DNA cleavage by topoisomerase I, the extent of stimulation was so weak that it is best not to consider the marine alkaloid as a topoisomerase I poison. The cytotoxicity measurements confirmed this view.

To determine whether topoisomerase I is an intracellular target for ASC, we compared the cytotoxicity of the drug

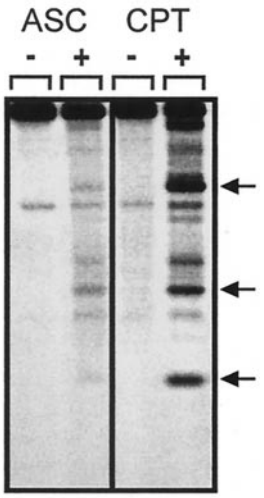


**FIG. 6. Cleavage of a radiolabeled DNA by human topoisomerase I in the absence (−) or the presence (+) of the test drug. ASC was used at 50 μM and CPT at 10 μM. The 3.1-kbp *Eco*RI *Hin*dIII restriction fragment from pLAZ3 was $^{32}$P-labeled at the 3′ end of the *Eco*RI site. Topoisomerase I cleavage reactions were analyzed on a 1% agarose gel.**

with two murine leukemia cell lines sensitive (P388) and resistant (P388CPT5) to CPT. The resistance of the P388CPT5 cells has been attributed to the expression of a deficient form of topoisomerase I as a result of a mutation in the *top1* gene of these cells [24]. Under the experimental conditions used (3 days of continuous exposure), ASC, surprisingly, proved to be less toxic to P388 cells than to P388CPT5 cells (Table 1). The latter exhibited marked resistance to CPT (78-fold), but were absolutely not resistant to ASC. We can therefore conclude that topoisomerase I is surely not a cellular target for ASC.

Similarly, we compared the cytotoxic activity of ASC towards HL-60 human leukemia cells sensitive (HL-60) or resistant (HL-60/MX2) to the antitumor drug mitoxantrone. HL-60/MX2 cells display atypical multidrug resistance with the absence of P-glycoprotein overexpression and altered topoisomerase II catalytic activity and reduced levels of topoisomerase IIα and IIβ [28]. We found that ASC was equally toxic to both cell lines ($IC_{50}$ = 0.48 and 0.65 μM for HL-60 and HL-60/MX2, respectively), thereby suggesting that topoisomerase II does not contribute to the cytotoxic action of ASC. We also tried to detect ASC-stabilized DNA–topoisomerase II covalent complexes in HL-60 cells using an *in vivo* link assay. We have recently developed such an immunoblot assay to show that endogenous topoisomerase II can be trapped onto DNA by topoisomerase II-targeted antitumor drugs such as etoposide and cryptolepine [29]. With ASC, we could not detect DNA–topoisomerase II covalent complexes in HL-60 leukemia cells, reinforcing the idea that topoisomerase II is not a privileged cellular target for this marine alkaloid.

**TABLE 1. Cytotoxicity ($IC_{50}$, μM)**

| Cpd | P388* | P388CPT5* |
|---|---|---|
| CPT | 0.07 | 5.5 |
| ASC | 2.4 | 0.05 |

*P388 and P388CPT5 murine leukemia cells are sensitive and resistant to camptothecin, respectively. $IC_{50}$ values refer to the concentrations of drugs giving 50% of growth inhibition.

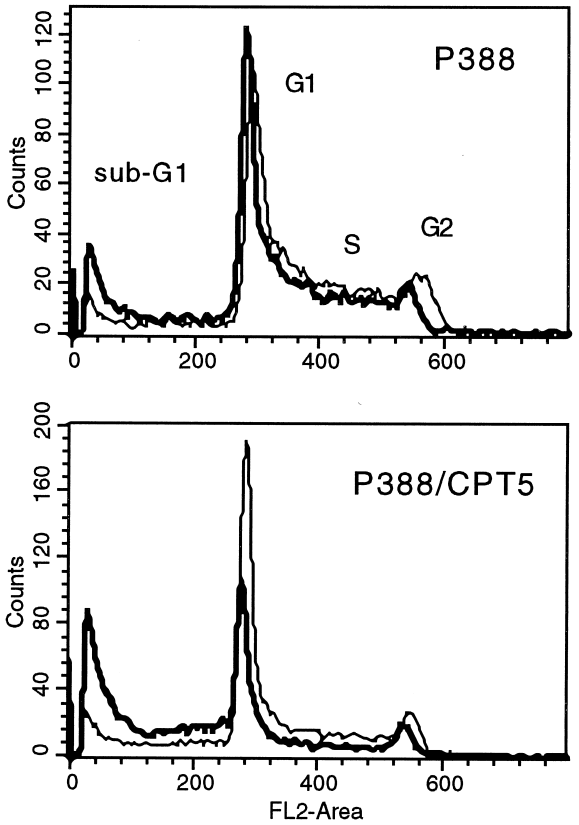


**FIG. 7. Cell cycle analysis of untreated (solid line) and ASC-treated (dashed line) P388CPT5 cells. Cells were incubated with 1 μM ASC for 18 hr prior to analysis with the FACScan flow cytometer as described in Materials and Methods. The sub-G1, G1, S, and G2+M populations represent 2, 34, 42, and 21% of the cells in the control and 54, 27, 13, and 6% in the ASC-treated cells, respectively.**

Microscopic examination of the P388 leukemia cells (sensitive or resistant to CPT) showed alterations of the nuclear and cytoplasmic membrane morphology. Cell cycle analysis revealed that, after an overnight exposure to ASC, a significant proportion of the cells showed an important reduction of the G1 phase accompanied by an increase in the sub-G1 region (Fig. 7). These observations prompted us to investigate the possibility that ASC might induce the programmed cell death pathway. Antitumor drugs inhibiting topoisomerase II, such as etoposide and amsacrine, are known to rapidly induce apoptosis [30, 31].

### *ASC-Induced Apoptosis of HL-60 Leukemia Cells*

ASC is highly toxic to human HL-60 leukemia cells [1]. The concentration of ASC required to kill 50% of the cells is 0.48 μM ($IC_{50}$). We sought to determine the mechanism

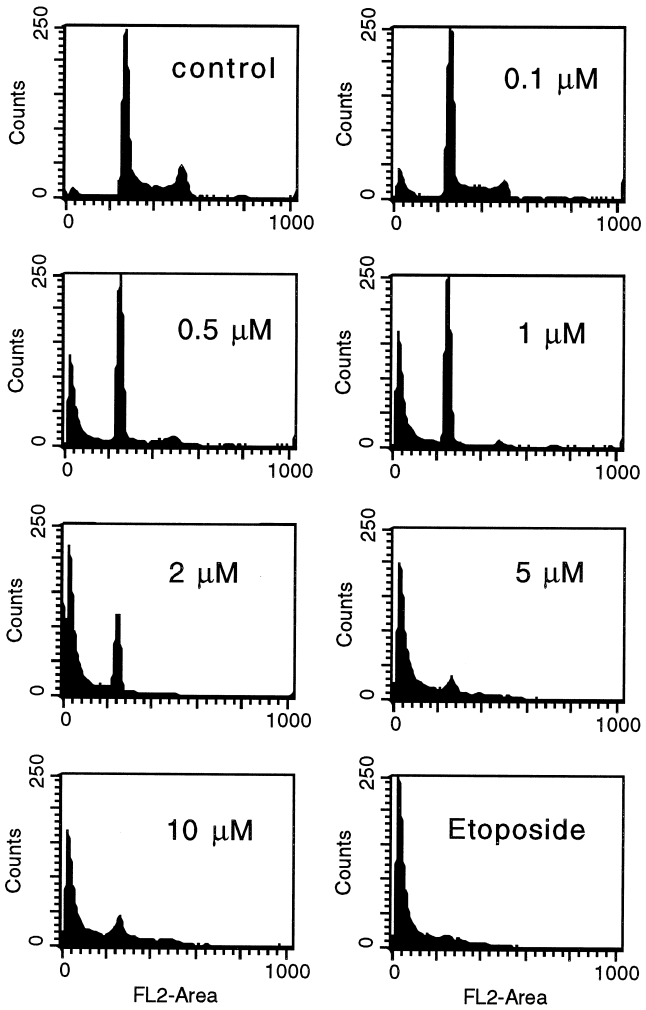


**FIG. 8. Cell cycle distribution determined by flow cytometry in HL-60 cells treated with ASC. Cells were exposed to increasing concentrations of ASC (0.1–10 μM) or etoposide (10 μM) for 18 hr prior to the FACScan analysis.**

by which ASC kills these leukemia cells, which are p53 null and prone to enter apoptosis. Most topoisomerase II inhibitors can induce apoptosis in leukemia cells. For example, it is well known that etoposide, amsacrine, and mitoxantrone induce programmed cell death in HL-60 cells [32]. Certain topoisomerase I poisons, such as CPT and SN38, also activate the programmed cell death pathway in HL-60 cells [31, 33, 34].

To investigate whether or not ASC promoted apoptosis in HL-60 cells, the cultures were treated overnight (18 hr) with increasing concentrations of ASC (0.1–10 μM). Cell cycle analyses show that a prominent sub-G1 population appeared in ASC-treated cells (Fig. 8). The increase in the sub-G1 fraction was accompanied by a loss of cells in the G1 and G2 phases. HL-60 cells incubated with ASC showed a cell cycle distribution similar to that observed upon continuous exposure to etoposide. The sub-G1 fraction represented only 3% of cells in the control and reached about 70% upon treatment with ASC. Concomitantly, the G1 population markedly decreased from 45% to 10%. The effects of the drug on the cell cycle of HL-60 cells sensitive or resistant to mitoxantrone were roughly equivalent (Fig. 9). The apoptotic cells (sub-G1 population) were also identified after staining. The morphology of non-apoptotic cells could easily be distinguished from that of HL-60 cells undergoing apoptosis (Fig. 10). The non-apoptotic cells were usually convoluted and pseudopods frequently seen. Their chromatin was not well stained, whereas HL-60 cells engaged in the apoptotic pathway was rather round and generally presented several clumps of condensed chromatin.

In parallel to the cell cycle analyses, we investigated the effect of ASC on caspase-3, which is a key executioner of apoptosis mediated by various antitumor agents [35]. A Western blot procedure was used to determine the effect of ASC on the cleavage of PARP, an enzyme involved in DNA repair, by caspase-3. The blot in Fig. 11 shows that the 116-kDa PARP protein was cleaved into its characteristic 89-kDa fragment upon treatment of the cells with ASC. With both ASC and etoposide, an almost quantitative cleavage of PARP was observed after treatment for 24 hr with 0.5 μM drug. We can therefore conclude that the induction of apoptosis is associated with an activation of caspase-3.

The DNA of the cells treated with ASC was extracted and analyzed by electrophoresis on agarose gels. The gel presented in Fig. 12 shows unambiguously that the genomic DNA of ASC-treated cells was severely fragmented, even when using a low drug concentration (0.5 μM). The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis [36]. Patterns of DNA internucleosomal fragmentation were observed with ASC as well as with etoposide. Another detection system, the TUNEL assay, was also employed to characterize ASC-induced apoptosis. The method is based on the fluorescein labeling

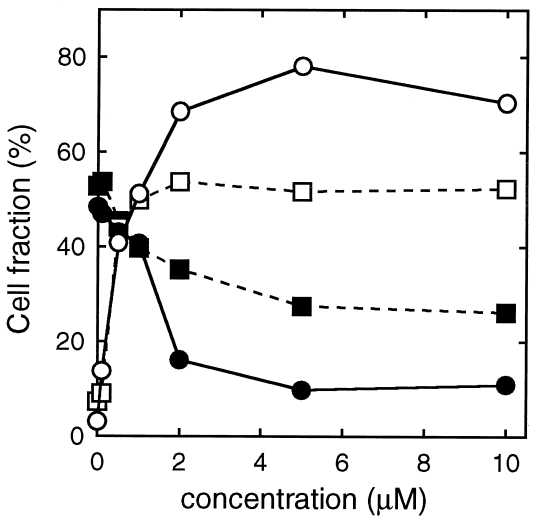


**FIG. 9. Variation of the fraction of HL-60 (circles) and HL-60/MX2 (squares) cells in the sub-G1 (open symbols) or G1 (filled symbols) phases of the cell cycle as a function of the ASC concentration. Each drug concentration was tested in duplicate.**

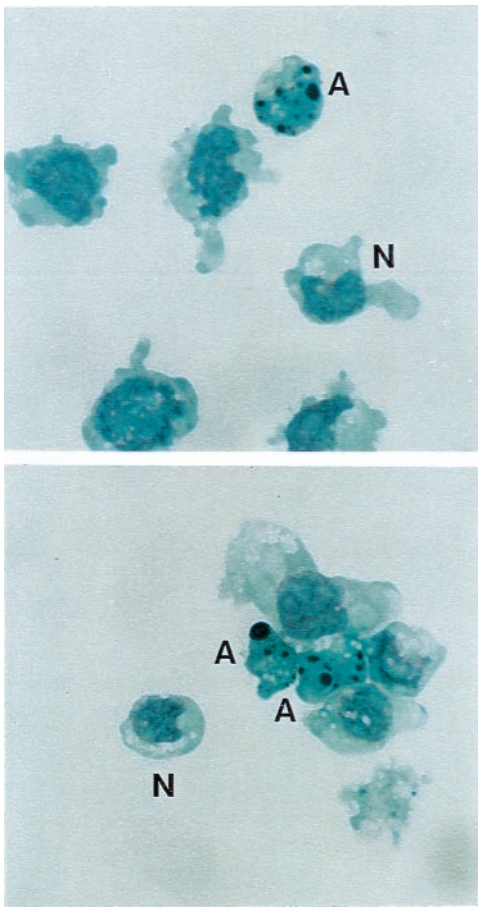


**FIG. 10. Morphology of ASC-treated HL-60 cells undergoing apoptosis. Cells were treated with 2 μM ASC for 18 hr at 37° before fixing and staining. (N) Non-apoptotic cells are convoluted with pseudopods and coarse chromatin. (A) Apoptotic cells are often round with dense clumps of chromatin.**

of apoptotic DNA fragments. Control cells containing intact genomic DNA appeared in red (due to the staining with propidium iodide), whereas about 60% of apoptotic cells colored in green were detected by fluorescence microscopy within the population of cells treated with ASC (5 μM for 18 hr) (data not shown). There was relatively little differences whether the HL-60 cells were treated with 1 or

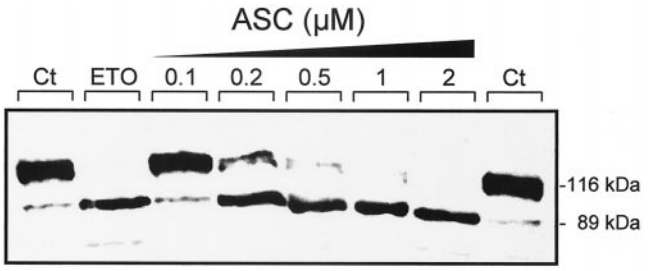


**FIG. 11. Induction of PARP cleavage by ASC. Western blot was used to detect cleavage of full-length PARP [116-kDa band] into the 89-kDa fragment in untreated cells (Ct) and cells treated with the drug at the indicated concentration (μM) for 24 hr. Whole cell lysates were subjected to SDS–PAGE followed by blotting with an anti-PARP monoclonal antibody. Etoposide (ETO) was used at 0.5 μM.**

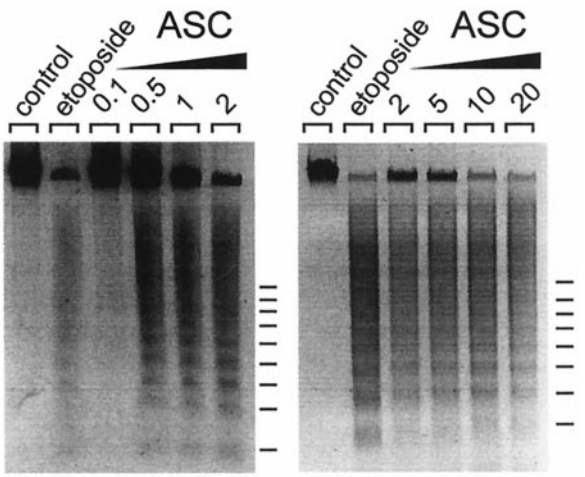


**FIG. 12. Agarose gel electrophoresis of DNA extracted from untreated HL-60 cells (control, Ct) and cells treated with different concentrations (μM) of ASC for 18 hr. DNA was stained with ethidium bromide after agarose gel electrophoresis. Oligonucleosome-sized DNA fragmentation can be seen in cells treated with etoposide and ASC (arrows).**

10 μM ASC: brightly fluorescent green cells were always detected. Therefore, the cell cycle analysis, DNA laddering, and TUNEL assays concur that ASC induces apoptosis in HL-60 cells.

## DISCUSSION

The results indicate that ASC behaves as a site-specific topoisomerase II inhibitor, as reported with related marine products such as meridine and diplamine [4, 19]. ASC bears a structural analogy with liriodenine, a cytotoxic oxoaporphine plant alkaloid isolated from *Cananga odorata*, which was shown to inhibit topoisomerase II [37]. The dibenzoquinolinone core common to ASC and liriodenine may be responsible for the inhibitory activity against topoisomerase II. Recently, the related aporphine alkaloid dicentrine was also shown to inhibit topoisomerase II [38], as is the case with other natural products such as saintopin [39] and fagaronine [40]. The ascidian alkaloid stimulates DNA cleavage by topoisomerase II preferentially at sites having a C on the 3′ side of the cleaved bond. A similar preference for a C at the −1 position has been observed with various anticancer drugs, including the epipodophyllotoxins, etoposide and teniposide, and the anthraquinone derivative, mitoxantrone [41].

Unlike the ascidian alkaloid wakayin [20], ASC has minimal, if any, effects on topoisomerase I. Surprisingly, we found that the CPT-resistant P388CPT5 cell line is 50-fold more sensitive to ASC than the parental cell line. A possible explanation for this important degree of collateral sensitivity could be that ASC preferentially targets the mutated form of topoisomerase I present in the CPT-resistant cells.

Although ASC acts as a poison for purified topoisomerases, our studies with different cell lines sensitive or

resistant to known topoisomerase I/II poisons indicate that neither topoisomerase I nor topoisomerase II can be considered as a potential cellular target for ASC. We found that ASC was equally toxic to HL-60 and HL-60/MX2 cells resistant to mitoxantrone. Both cell lines undergo a similar extent of apoptosis upon treatment with ASC. Because HL-60/MX2 cells contain reduced levels of topoisomerase II, it is unlikely that the inhibition of this enzyme by ASC represents the initial stimulus that triggers a series of events responsible for the induction of a cellular suicide process. An alternative mechanism of cytotoxicity should be considered. Recently, it was reported that ASC-mediated DNA cleavage can occur via the production of reactive oxygen species [42]. Oxygen radical-dependent DNA cleavage may be responsible, at least in part, for the anticancer activity of the drug. If the efficacy of ASC is confirmed in xenograft models, this DNA-intercalating agent might turn out to be the first in a promising series of antitumor drugs of marine origin.

*This work was supported by research grants (to C.B.) from the Ligue Nationale Française Contre le Cancer. L.D. is currently supported by a fellowship from the Association pour la Recherche sur le Cancer. The authors thank Céline Facompré for help with the cytotoxicity and cell cycle measurements and the Service Commun d'Imagerie Cellulaire de l'IFR22 for access to the fluorescence microscope.*

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