Lamellarin D: A Novel Potent Inhibitor of Topoisomerase I

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ABSTRACT

We report the identification and characterization of a novel potent inhibitor of DNA topoisomerase I: lamellarin D (LAM-D), initially isolated from a marine mollusk, Lamellaria sp., and subsequently identified from various ascidians. This alkaloid, which displays potent cytotoxic activities against multidrug-resistant tumor cell lines and is highly cytotoxic to prostate cancer cells, bears a 6H-[1]benzopyrano[4’;3’;4,5]pyrrolo[2,1-i]isoquinolin-one pentacyclic planar chromophore, whereas its synthetic 5,6-dehydro analogue, LAM-501, has a significantly tilted structure. DNA binding measurements by absorbance, fluorescence, and electric linear dichroism spectroscopy show that LAM-D is a weak DNA binder that intercalates between bp of the double helix. In contrast, the nonplanar analogue LAM-501 did not bind to DNA and failed to inhibit topoisomerase I. DNA intercalation may be required for the stabilization of topoisomerase I-DNA complexes by LAM-D. In the DNA relaxation assay, LAM-D strongly promoted the conversion of supercoiled DNA into nicked DNA in the presence of topoisomerase I. The marine product was ~5 times less efficient than camptothecin (CPT) at stabilizing topoisomerase I-DNA complexes, but interestingly, the two drugs exhibited slightly distinct sequence specificity profiles. Topoisomerase I-mediated DNA cleavage in the presence of LAM-D occurred at sites common to CPT, but a few specific sites identified with CPT but not with LAM-D or conversely unique sites cleared by LAM-D but not by CPT were detected. The distinct specificity profiles suggest that LAM-D and CPT interact differently with the topoisomerase I-DNA interface. A molecular modeling analysis provided structural information on the orientation of LAM-D within the topoisomerase I-DNA covalent complex. The marine alkaloid did not induce DNA cleavage by topoisomerase II. Immunoblotting experiments revealed that endogenous topoisomerase I was efficiently trapped on DNA by LAM-D in P388 and CEM leukemia cells. P388/CPT5 and CEM/C2 cell lines, both resistant to CPT and expressing a mutated top1 gene, were cross-resistant to LAM-D. Collectively, the results identify LAM-D as a novel lead candidate for the development of topoisomerase I-targeted antitumor agents.

INTRODUCTION

Fifteen years of efforts in targeting topoisomerase I for the discovery of anticancer agents have led to the identification of several families of compounds capable of stabilizing DNA-topoisomerase I covalent complexes (1–3). The lead series is clearly the CPT3 family, with two drugs, topotecan and irinotecan, approved for cancer treatment and several second (e.g., lurtotecan and exatecan) and third (e.g., dilitromotecan) generations of CPT analogues in clinical trials at present (4–6). However, apart from the CPTs, only a few topoisomerase I poisons have reached Phase I clinical trials. Promising results have been reported with glycosyl indolocarbazoles (7); to date, however, there are still no non-CPT topoisomerase I poisons in advanced clinical trials. Continuous efforts in the optimization of indolocarbazoles, indenoisoquinolines, and benzimidazoles will hopefully lead to effective candidates for clinical development, but the need for new series of topoisomerase I poisons remains pressing (8, 9). Here we report our successful attempt at discovering one such a new series of potent topoisomerase I poisons: the lamellarins.

LAM-D (Fig. 1) is a hexacyclic marine alkaloid initially isolated from a prosobranch mollusk of the genus Lamellaria (10) and subsequently found in ascidians (11). More than 30 lamellarins have been isolated, and interestingly, some of them, including LAM-D, show equally potent cytotoxic activities against both multidrug-resistant tumor cell lines and their corresponding parental cell lines (12). LAM-D displays a pronounced selective cytotoxicity for tumor types, but its mechanism of action was totally unknown. On the basis of its chemical structure, we postulated that LAM-D can bind to DNA and interfere with the catalytic activities of human topoisomerases. A molecular modeling analysis (Fig. 1) suggested that the 6H-[1]benzopyranono[4’;3’;4,5]pyrrolo[2,1-i]isoquinolin-one planar chromophore of LAM-D can intercalate between DNA bp and that the appended methoxyphenol substituent oriented at a right angle with respect to the main chromophore may serve as a hook to trap proteins. These considerations prompted us to analyze the interaction of LAM-D with DNA and its effect on topoisomerases by use of a panel of complementary biochemical, biophysical, and cell assays. The pharmacological properties of LAM-D were compared with those of LAM-501, a synthetic lamellarin derivative that lacks the C5–C6 double bond in the quinoline B ring (Fig. 1). Collectively, the results presented here identify LAM-D as a potent topoisomerase I poison. Cytotoxicity measurements in two pairs of leukemia cell lines sensitive or resistant to CPT indicate that topoisomerase I plays a role in the antiproliferative activity of LAM-D. The synthetic analogue LAM-501 was found to be inactive against topoisomerase I and considerably less cytotoxic than the natural product, indicating that the planarity of the hexacyclic chromophore is essential for DNA binding and topoisomerase I inhibition.

MATERIALS AND METHODS

Drugs. LAM-D and LAM-501 (provided by PharmaMar, Spain) were synthesized according to a described process (13). CPT was purchased from Sigma Chemical Co. The drugs were dissolved in DMSO at 5 mM. Drug stock solutions were kept at ~20°C and freshly diluted with water to the desired concentration immediately before use.

Absorbance Spectroscopy and Melting Temperature Studies. Absorbance spectra and melting curves were measured in an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. Titrations of the drug with DNA, covering a large range of P:D ratios, were performed by adding aliquots of a concentrated DNA solution to a drug solution at constant ligand concentration (20 μM). The Tm measurements were performed in BPE buffer (pH 7.1; 6 mM Na2HPO4, 2 mM NaH2PO4, 1 mM EDTA) at 2°C/min. The temperature inside the cuvette (10-mm pathlength) was increased over the range 20–100°C with a heating rate of 2°C/min.
...with 1,5-mm electrode separation. The samples were oriented under an electric field strength varying from 1 to 14 kV/cm. The drug being tested was incorporated into Prism 3.0. Fluorescence titration data were fitted directly to get the binding constant by use of a fitting function (one-site binding model) incorporated into Prism 3.0.

**ELD.** Calf thymus DNA (Pharmacia) was deproteinized with SDS (protein content <0.2%) and extensively dialyzed against 1 mM sodium cacodylate buffered solution (pH 7.0). ELD measurements were performed with a computerized optical measurement system according to previously outlined procedures (14). All experiments were conducted with a 10-mm pathlength Kerr cell with 1.5-mm electrode separation. The samples were oriented under an electric field strength varying from 1 to 14 kV/cm. The drug being tested was present at 10 µM, and the DNA was present at 200 µM unless otherwise stated. This electro-optical method senses only the orientation of the polymer-bound ligand: free ligand is isotropic and does not contribute to the signal (15).

**DNA Relaxation Experiments.** Recombinant topoisomerase I protein was produced and purified from baculovirus-infected Sf9 cells (16). Some experiments were also performed with the commercially available enzyme (TopoGen, Inc.). Supercoiled pLAZ3 DNA (0.5 µg) was incubated with 4 units of human topoisomerase I at 37°C for 1 h in relaxation buffer [50 mM Tris (pH 7.8), 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA] in the presence of various concentrations of the drug under study. Reactions were terminated by the addition of 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 at room temperature for 2 h at 120 V in 1% agarose gels containing ethidium bromide (1 µg/ml). After electrophoresis, gels were washed and photographed under UV light (17).

**Purification of the DNA Restriction Fragment and Radiolabeling.** The 198-bp DNA fragment was prepared by [3²⁺³²P] end labeling of the HindIII-SacI double digest of the pMS2 plasmid (kindly provided by Professor K. R. Fox, University of Southampton, Southampton, UK) using [α²⁺³²P]-dATP (3000 Ci/mmol; Amersham) and AMV reverse transcriptase (Roche). The labeled digestion products were separated on a 6% polyacrylamide gel under nondenaturing conditions in Tris-borate-EDTA buffer [89 mM Tris-borate (pH 8.3), 1 mM EDTA]. After autoradiography, the gel containing the requisite band of DNA was excised, crushed, and soaked in water overnight at 37°C. This suspension was filtered through a 0.22 µm Millipore filter, and the DNA was precipitated with ethanol. After washing with 70% ethanol and vacuum drying of the precipitate, the labeled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 and containing 10 mM NaCl.

**Sequencing of Topoisomerase I-Mediated DNA Cleavage Sites.** Each reaction mixture contained 2 µl of [3²⁺³²P] end-labeled DNA (~1 µg), 5 µl of water, 2 µl of 10× topoisomerase I buffer, and 10 µl of drug solution at the desired concentration (1–50 µM). After a 10-min incubation to ensure equilibration, the reaction was initiated by addition of 2 µl (20 units) of calf thymus topoisomerase I. Samples were incubated for 45 min at 37°C before the addition of SDS to 0.25% and proteinase K to 250 µg/ml to dissociate the drug–DNA–topoisomerase I cleavable complexes. The DNA was precipitated with ethanol and then resuspended in 5 µl of formamide-Tris-borate-EDTA loading buffer, denatured at 90°C for 4 min, then chilled in ice for 4 min before loading on the sequencing gel. DNA cleavage products were resolved by polyacrylamide (8%) gel electrophoresis under denaturing conditions (8 M urea). After electrophoresis, gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper, and dried under vacuum at 80°C. A

![Fig. 1. Structures and conformations of LAM-D and LAM-501 (top and side views). The softwares HyperChem 5.01 and Alchemy 2000 were used to construct the energy-minimized models.](image-url)

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Cell Growth Inhibition Assay: Screening. A colorimetric assay using SRB was adapted for quantitative measurement of cell growth and viability, following a previously described method (18, 19). Cells were seeded in 96-well microtiter plates, at 5 × 10^3 cells/well in 195-μl aliquots of RPMI medium, and were allowed to attach to the plate surface by incubation in drug-free medium for 18 h. Afterward, samples were added in 5-μl aliquots (dissolved in DMSO–H_2O; 3:7, v/v). After 72 h of exposure, the antitumor effect was measured by the SRB methodology as follows: cells were fixed by addition of 50 μl of cold 50% (w/v) trichloroacetic acid and incubation for 60 min at 4°C. Plates were washed with deionized water and dried; 100 μl of SRB solution (0.4% w/v in 1% acetic acid) were added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, and bound stain was solubilized with Tris buffer. Absorbances were read on an automated spectrophotometer plate reader at a single wavelength of 490 nm. Data analysis were generated automatically by Laboratory Information Management System implementation. Using control absorbance values (C), test absorbance values (T), and time zero absorbance values (T_0), we calculated the drug concentration that causes 50% growth inhibition (GI_50 value) from the equation: 100 × [(T − T_0)/C − T_0] = 50.

Cell Cultures and Survival Assay: Cross-Resistance. Human CEM and CEMC2 leukemia cells were obtained from the American Tissue Culture Collection. P388 and P388CPT5 murine leukemia cell lines sensitive and resistant to CPT, respectively, were kindly provided by Dr. J- F. (University of Reims, France). The P388CPT3 cell line, which is resistant to CPT, was derived from a stable clone of the P388CPT0.3 cell line obtained at the 42nd passage (20). The leukemia cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine C supplementation. Using control absorbance values (C), test absorbance values (T), and the electric field strength (E), the DNA:drug (P:D) ratio (B), and the DNA:drug (P:D) ratio (B), the DNA:drug (P:D) ratio (B), and the DNA:drug (P:D) ratio (B), respectively, we calculated the drug concentration that causes 50% growth inhibition (GI_50 value) from the equation: 100 × [(C − T_0)/C − T_0] = 50.

Immunoblot Assay of Topoisomerase I DNA Complexes in Cells. The in vivo topoisomerase I link kit from TopoGEN, Inc. (Columbus, OH) was used, and the recommended protocol was followed with a few modifications. Briefly, 10^9 exponentially growing P388 cells in 5 ml of serum-free RPMI 1640 were treated with the test drug at 50 μM for 1 h at 37°C. Cells were pelleted by centrifugation (1000 rpm for 5 min) and rapidly resuspended in 0.8 ml of the lysis buffer [10 mm Tris-HCl (pH 7.5), 1 mm EDTA, 1% sarkosyl]. The lysed cell mixture was then overlaid on a CsCl density gradient containing four different density steps (0.8 ml of CsCl at 1.82, 1.72, 1.50, and 1.37 g/ml). The tubes were centrifuged in a Beckman SW60 rotor at 31,000 rpm (130,000 × g) for 15 h at 25°C. The top of the gradient, 12 fractions of 330 μl were collected. The DNA content in each fraction was estimated by absorbance measurements at 260 nm. For the immunoblot analysis, 50 μl of each fraction were diluted with 100 μl of 25 mM PBS (pH 6.5) before the diluted solution was applied in the slot blot unit under a mild vacuum. PBS-washed Hybond-C nitrocellulose membranes (Amersham) cut to fit the vacuum slot-blot device (Life Science, Cergy-Pontoise, France) were loaded with the diluted samples, washed briefly with PBS, and then soaked for 2 h in TBST-B [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20, 1% BSA] supplemented with 5% nonfat dried milk. The membranes were washed three times (10 min/wash) with TBST before incubation for 1 h at room temperature with the antitopoiosomerase I antibody (1:10,000 dilution in 25 ml of TBST). After three successive washes (10 min with TBST), the membranes were incubated with a goat antirabbit antibody conjugated to horseradish peroxidase (1:1000 dilution in 25 ml of TBST; Amersham LifeSciences) for 30 min. After four successive washes (10 min each with TBST), the Western blot chemilu-
RESULTS

DNA Interaction. Melting temperature measurements showed that the marine compound weakly stabilizes duplex DNA to reduce heat denaturation of the double helix. The $T_m$ of the polynucleotide poly-(dAT)$_2$ increases from 42°C to 46°C in the presence of LAM-D at a 1:1 nucleotide:drug ratio in BPE buffer (Fig. 2A). There was no shift of the $T_m$ value with calf thymus DNA, but slight changes of the absorbance spectrum of the drug were also observed on titration with DNA (a weak hypochromic effect), and the fluorescence of the drug chromophore increased significantly on binding to DNA, as shown in Fig. 2B. We exploited the intrinsic fluorescence of the pentacyclic chromophore to evaluate the binding strength. The fluorescence emission at 441 nm is weak when the drug is free in solution, but it is markedly enhanced when the drug is bound to DNA. Nonlinear least-squares analysis of the fluorescence titration curves yielded a binding constant of 29.3 (±1.3) $10^4$ M$^{-1}$ for LAM-D in 1 mM sodium cacodylate buffer at neutral pH. Similar experiments were performed with CPT and LAM-501, but in both cases no binding to DNA could be detected.

ELD measurements were performed to estimate the orientation of LAM-D on binding to DNA. The results of these experiments, performed in a low-salt buffer [1 mM sodium cacodylate (pH 7.0)] because of the high electric field voltage, are shown in Fig. 3. The ELD spectrum (Fig. 3A) for DNA–LAM-D complexes shows that the reduced dichroism $Δg/A$ is negative in the drug absorbance band centered at 370 nm and the amplitude of the ELD signal is similar to that measured with DNA alone in the 260 nm region. The dependence

\[ \text{ELD spectrum} \]

\[ \text{Concentration [log (M)]} \]

\[ \% \text{DNA form II} \]

\[ \text{Log (M)} \]

\[ \text{Concentration} \]

\[ \text{ELD} \]

\[ \text{Binding} \]

\[ \text{Orientation} \]

\[ \text{Drug} \]

\[ \text{DNA} \]

\[ \text{LAM-D} \]

\[ \text{CPT} \]

\[ \text{Absorbance} \]

\[ \text{Fluorescence} \]

\[ \text{Binding constant} \]

\[ \text{Fluorescence emission} \]

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\[ \text{Drug absorbance} \]

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of the reduced dichroism $\Delta A/A$ as a function of the electric field strength was similar for DNA alone and its complex with the drug (Fig. 3C). This reflects the orientation of the ligand perpendicular to the helix axis (or electric field direction). The ELD data are entirely consistent with an orientation of the planar hexacyclic chromophore parallel to the DNA bp, as expected for an intercalative binding. Stabilization of the $\Delta A/A$ values was observed only at a high DNA/drug ratio (Fig. 3B). Therefore, LAM-D must be considered a low-affinity DNA intercalator.

**Topoisomerase I Poisoning: Efficacy and Site Selectivity.** In the relaxation assay, negatively supercoiled plasmid pLAZ3 was incubated with topoisomerase I in the presence of increasing concentrations of LAM-D, from 0.01 to 50 $\mu M$. Parallel experiments were performed with CPT as a reference inhibitor. DNA samples were treated with SDS and proteinase K to remove any covalently bound protein and resolved in 1% agarose gels containing ethidium bromide. As shown in Fig. 4A, supercoiled DNA is fully relaxed by topoisomerase I in the absence of drug (compare Lanes DNA and Topol). In the presence of LAM-D, the intensity of the slowest-migrating band, corresponding to the nicked form of DNA, increased significantly in a manner similar to that for CPT. This effect reflects the stabilization of topoisomerase I–DNA cleavable complexes. Band intensities from different gels were quantified, and the extents of cleavage detected with LAM-D and CPT were compared (Fig. 4B). LAM-D clearly induced potent dose-dependent stimulation of DNA cleavage by topoisomerase I. From the concentration analysis, we calculated $C_{50}$ values of 0.42 and 0.087 $\mu M$ for LAM-D and CPT, respectively. Although the drug concentration required to convert supercoiled DNA into the relaxed form was ~5 times higher with LAM-D compared with CPT, it is clear that the marine product can generate a high level of single-strand breaks. At 2 $\mu M$, LAM-D and CPT were equally efficient and converted ~70% of the DNA plasmid into single-strand breaks.

To investigate further the topoisomerase I poisoning activity of LAM-D, we performed DNA cleavage experiments with a 198-bp DNA restriction fragment. The *HindIII–SacI* double-digest of the pMS2 plasmid was uniquely end-labeled at the $3'$ end at the *HindIII* site and used as a substrate for the topoisomerase I cleavage reactions. The cleavage products were analyzed on sequencing polyacrylamide gels (Fig. 5). The results confirmed that LAM-D promotes the cleavage of DNA by topoisomerase I. Several LAMD-induced cleavage sites could be easily identified and their positions on the gel could be compared with those observed with CPT under identical conditions. The cleavage profiles obtained with LAM-D were distinct from those seen with CPT. In some cases, such as at site b in Fig. 5 (A $^1$ T79), the two drugs stabilized the same topoisomerase I–DNA complexes. These common cleavage sites were frequent. At a few other sites, however, clear differences between the two drugs were evident. For example, site a was specific to CPT; there was almost no cleavage at position T $^1$ G81 with LAM-D. On the other hand, site c was specific to LAM-D. The A $^2$ G65 site was well stabilized by LAM-D, whereas CPT showed no effect at this site. Altogether the results of the DNA relaxation (Fig. 4) and cleavage (Fig. 5) experiments demonstrate that LAM-D is a potent topoisomerase I poison.

**Molecular Modeling.** The topotecan–DNA–topoisomerase crystallographic model published recently (22) provides a very useful structural basis to elucidate the mode of interaction between LAM-D and the DNA–topoisomerase I covalent complex. Topotecan was replaced with LAM-D, and the structure of the resulting ternary complex was minimized. As shown in Fig. 6, LAM-D intercalates at the site of DNA cleavage, stabilized with both the +1 (C-G) and −1 (A-T) bp forming stacking interactions. Hydrogen bonds between the drug and specific amino acid residues of the protein further stabilize the ternary structure. According to this model, the phenol ring on the C-1 position (Fig. 1) is pointing out to the protein–DNA interface and does not have any direct interaction with the protein. It may serve to recruit other protein partners. It will be useful to see whether this extra ring is dispensable. The C-8 and C-20 hydroxyl groups are at a hydrogen bond distance from the Asn$^{222}$ and Glu$^{356}$ residues of the enzyme, respectively whereas the keto group is interacting with the Arg$^{364}$ residue. This configuration is in perfect agreement with the known structure–activity relationships in the lamellarin series. Indeed,
plexes in CEM human leukemia cells. We incubated $10^7$ exponentially growing CEM cells with CPT or LAM-D at 5 $\mu$g/ml for 1 h at 37°C. The lysates were applied to a CsCl gradient and centrifuged overnight. Twelve fractions were collected and analyzed by the slot-blot method described in “Materials and Methods.” Fractions 1–4 contain free topoisomerase I. DNA–topoisomerase I covalent complexes can be detected in fractions 7–9 of the drug-treated samples.

A previous study revealed that the presence of hydroxyl groups at the C-8 and C-20 positions is essential for cytotoxicity, whereas the hydroxyl group at C-14 and the two methoxy groups at C-13 and C-21 are less important (26). Interestingly, the observation that the hydroxyl group at C-8 interacts with the Asn$^{722}$ residue of the enzyme is entirely consistent with the experimental data showing that CEM/C2 cells resistant to CPT are cross-resistant to LAM-D (see below). As for CPT, LAM-D may not be able to efficiently stabilize DNA–topoisomerase I complexes in these cells, which express a modified topoisomerase I with an Asn→Ser mutation. The molecular model gives an experimentally coherent picture of the drug binding to the enzyme–DNA complex.

Stabilization of Topoisomerase I–DNA Complexes in Cells. We set up an immunoblot assay to identify the drug-stabilized topoisomerase I–DNA complexes directly in the CEM leukemia cells. This assay, commonly referred to as the “in vivo link assay,” has previously been used to investigate the topoisomerase I poisoning activity of drugs such as actinomycin D (27) and the homocamptothecin derivative hCPT (28). CEM leukemia cells were incubated with 5 $\mu$M LAM-D or CPT for 1 h, and the cell extracts were fractionated by centrifugation with a CsCl gradient (Fig. 7). In the control samples with no drug, topoisomerase I was found exclusively at the top of the CsCl gradient as free protein. In sharp contrast, with cells treated for a short time with a low concentration of LAM-D, topoisomerase I was found in the top fractions as well as in the fractions (fractions 6–9) near the bottom of the gradient, where the nucleic acids were localized (as judged from absorbance measurements at 260 nm). Although the immunoassay was essentially qualitative, the drug-trapped covalent complexes were at least equally abundant with LAM-D and CPT. These results indicate that the marine compound stabilizes topoisomerase I–DNA complexes in cells, thus supporting the molecular data to show that we have identified a new topoisomerase I poison. Similar immunoblotting experiments showed that endogenous topoisomerase I can be trapped on DNA by LAM-D in P388 leukemia cells (data not shown). Here again, no effect was observed with LAM-501.

Cytotoxicity. A panel of 12 human tumor cell lines was used to evaluate the cytotoxic potential of LAM-D and LAM-501. A conventional colorimetric assay was set up to estimate GI$_{50}$ values, i.e., the drug concentration that causes 50% cell growth inhibition after 72 h continuous exposure to the test molecule. Histograms summarizing the data are presented in Fig. 8. The cell lines tested could be classified in three groups according to their sensitivity to LAM-D. The marine alkaloid was weakly toxic to colon cancer cells. It showed only a modest activity against the HT29 human colon carcinoma cell line and the LoVo human colon lymph node metastasis cell line as well as LoVo-Dox cells resistant to doxorubicin. A more pronounced growth-inhibitory effect was detected with the two ovarian cell lines sensitive or resistant to otezolastatin-743 (IGROV and IGROV-ET), the melanoma cell line MEL-28, the pancreatic carcinoma cell line PANC-1, the lung carcinoma cell line A-549, and the breast cancer cell line SK-BR3. In each case the GI$_{50}$ values were in the order of 100 nM or below. Interestingly, LAM-D was particularly efficient against the prostate tumor cell lines LNCaP and DU-145, with GI$_{50}$ values in the 10–20 nM range. DU-145 metastatic cells, which are androgen-insensitive, were very sensitive to LAM-D compared with the hormonally responsive but nonmetastatic LNCaP cells. The growth of human K562 erythroid cells was also strongly inhibited by LAM-D. It is important to note that the synthetic analogue LAM-501, which has no effect on topoisomerase I, is poorly cytotoxic and showed no selectivity for prostate cancer cells. Thus, LAM-D is clearly a good candidate for in vivo evaluation.

Role of Topoisomerase I in the Cytotoxic Activity of LAM-D. We further evaluated the cytotoxicity of LAM-D, using two pairs of leukemia cells sensitive and resistant to CPT. On the one hand, we used P388 and P388CPT5 marine leukemia cells, which are sensitive and resistant to CPT, respectively. 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 (20). These P388CPT5 cells carry two point mutations in conserved regions of the topoisomerase I gene (Gly$^{361}$Val and Asp$^{709}$Tyr) responsible for the high resistance to CPT (29). On the other hand, we used CEM and CEM/C2 human leukemia cells, available from the ATCC. The resistance of CEM/C2 cells to CPT has been attributed to a single mutation of Asn$^{722}$ to a Ser residue adjacent to the active site Tyr$^{723}$ residue of the human topoisomerase I enzyme (30). The
antiproliferative activity for LAM-D was evaluated by a tetrazolium-based assay, and the IC_{50} values and RRI were calculated for LAM-D and compared with those obtained with CPT under strictly identical conditions. As indicated in Table 1, the cytotoxic activity of LAM-D was significantly reduced when the leukemia cell line expressed a mutated topoisomerase I. With both the P388CPT5 and CEM/C2 leukemia cell lines, the mutations of the topoI gene that render the enzyme highly resistant to CPT apparently conferred cross-resistance to LAM-D, at least partially. CEM/C2 cells were 70 times less sensitive to LAM-D than the parental CEM cell line, which expresses a nonmutated topoisomerase I. The difference was less pronounced with P388 cells, but nevertheless the parent cells were one order of magnitude more sensitive to LAM-D than the cells expressing a mutated topoI gene. The use of these two pairs of cell lines, sensitive or resistant to CPT, provided another line of evidence that topoisomerase I is involved in the cytotoxicity of LAM-D. The fact that the RRI measured with the marine compound was significantly lower than those calculated with CPT may arise from the superior effects of CPT in terms of topoisomerase I inhibition compared with CPT or from the fact that CPT and LAM-D probably interact differently with the DNA–topoisomerase I interface. Alternatively, it is possible that topoisomerase I is one but not the unique target for LAM-D in cells. Nevertheless, from the molecular data and the cell studies, we can conclude that a new potent inhibitor of topoisomerase I has been identified from a marine organism. The observation that topoisomerase I represents at least one of the cellular target for LAM-D opens a completely new avenue for the design of anticancer agents affecting the integrity of DNA.

LAM-501 was 42 times less cytotoxic than LAM-D against P388 murine leukemia cells (Table 1). Surprisingly, LAM-501 was more cytotoxic to CPT-resistant P388/CPT5 cells than to the CPT-sensitive parental cells. This character, as characterized by a saturated C5–C6 bond (instead of the C5–C6 double bond of LAM-D) was totally inactive against topoisomerase I and failed to bind to DNA. The difference between the two lamellarin analogues can be explained from the structural models presented in Fig. 1. The planar conformation of the hexacyclic chromophore of LAM-D is ideally suited for intercalation into DNA. In contrast, the reduction of the C5–C6 bond introduces a large kink into the main chromophore, and this must seriously alter and perhaps abolish the capacity of LAM-501 to insert between two consecutive bp.

### DISCUSSION

The discovery of the main target for an anticancer agent is an essential element to better understand its mechanism of action and to guide the development of clinically useful analogues. To illustrate this, one can refer to CPT, discovered in the early 1960s but successfully developed only a quarter of a century later when its main, and perhaps unique, molecular target, topoisomerase I, was identified. The observation in 1985 that CPT stabilizes DNA–topoisomerase I complexes provided the starting point for the rational development of safe CPT analogues, which culminated in the mid 1990s with the approval of topotecan and irinotecan for the treatment of ovarian and colon cancers.

The search for non-CPT topoisomerase I poisons has been very active for the past 10 years, but only a limited number of potent topoisomerase I poisons have been discovered. To date, very few topoisomerase I poisons have been isolated from marine organisms. This is the case for ecteinascidin-743, the active tumor-alkylating agent extracted from Ecteinascidia turbinata (31), and for the bispyrrolominoquinone metabolite wakayin, isolated from the ascidian Clavelina in Fiji (32), and ascididemin, extracted from the Mediterranean ascidian Cystodytes dellechiajei (33). The latter pyridoacridine derivative also inhibits topoisomerase II, whereas LAM-D shows absolutely no effect on topoisomerase II-mediated DNA cleavage (data not shown). LAM-D inhibits only the DNA relaxation/cleavage activity of topoisomerase I; the drug has no effect on the kinase activity of the enzyme. The two marine compounds wakayin and ascididemin were found to intercalate into DNA and to enhance the formation of DNA–topoisomerase I covalent complexes, but they have no structural similarities with LAM-D. Among the many naturally occurring topoisomerase I poisons identified to date from microorganisms and plants, we found none that showed a direct structural analogy with LAM-D. This marine product should be considered as a new pharmacophore for topoisomerase I targeting. The structure of LAM-D is relatively simple, and the syntheses of different lamellarin derivatives have been described previously (26, 34–36). The work reported here could be very useful to guide the development of more potent analogues of LAM-D that target topoisomerase I.

Despite the profound structural differences between CPT and LAM-D, their mechanisms of action are apparently similar. However, the distinct DNA cutting profiles observed in the in vitro assay suggest that, at least at a few specific sites, LAM-D recognizes structural elements of the topoisomerase I–DNA covalent complex different from those recognized by CPT. The modeling analysis suggests that the two drugs are positioned differently at the DNA–topoisomerase I interface. Sequence specificity profiles different from that of CPT have been observed previously with an indolocarbazole derivative (37) and with the indenoisoquinoline NSC314622 (38), but these two drugs are not structurally related to LAM-D, although there may be similarities in size and electronic distribution as noted for other classes of topoisomerase I poisons (9, 39). A recent X-ray analysis of the topotecan–DNA–topoisomerase I ternary complex confirmed that the CPT derivative is intercalated at the DNA cleavage site (22). In terms of DNA binding, the behavior of LAM-D is reminiscent of that of topotecan, which also binds weakly to DNA in the absence of the enzyme (40). Hopefully, the development of the lamellarins as antitumor agents will be as fruitful as that of the CPTs.

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TOPOISOMERASE I INHIBITION BY LAM-D


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