Cancer Cell Mitochondria Are Direct Proapoptotic Targets for the Marine Antitumor Drug Lamellarin D

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Abstract
Lamellarin D is a marine alkaloid with a pronounced cytotoxicity against a large panel of cancer cell lines and is a potent inhibitor of topoisomerase I. However, lamellarin D maintains a marked cytotoxicity toward cell lines resistant to the reference topoisomerase I poison camptothecin. We therefore hypothesized that topoisomerase I is not the only cellular target for the drug. Using complementary cell-based assays, we provide evidence that lamellarin D acts on cancer cell mitochondria to induce apoptosis. Lamellarin D, unlike camptothecin, induces early disruption of the inner mitochondrial transmembrane potential (Δψm) in the P388 leukemia cell line. The functional alterations are largely prevented by cyclosporin A, an inhibitor of the mitochondrial permeability transition (MPT), but not by the inhibitor of caspases, benzylxycarbonyl-Val-Ala-Asp(0me)-fluoromethylketone. Δψm disruption is associated with mitochondrial swelling and cytochrome c leakage. Using a reliable real-time flow cytometric monitoring of Δψm and swelling of mitochondria isolated from leukemia cells, we show that lamellarin D has a direct MPT-inducing effect. Furthermore, mitochondria are required in a cell-free system to mediate lamellarin D–induced nuclear apoptosis. The direct mitochondrial effect of lamellarin D accounts for the sensitivity of topoisomerase I–mutated cell lines to P388CPT5 cells resistant to camptothecin. Interestingly, a tumor-active analogue of lamellarin D, designated PM031379, also exerts a direct proapoptotic action on mitochondria, with a more pronounced activity toward mitochondria of tumor cell lines compared with nontumor cell lines. Altogether, this work reinforces the pharmacologic interest of the lamellarins and defines lamellarin D as a lead in the search for treatments against chemoresistant cancer cells. (Cancer Res 2006; 66(6): 3177-87)

Introduction
Lamellarins are marine pyrrole alkaloids initially isolated from a prosobranch mollusk of the genus Lamellaria (1) and subsequently found in a variety of organisms, mostly ascidians. More than 30 lamellarins have been isolated thus far, but only a few show interesting bioactive properties (2). Certain lamellarins and derivatives (e.g., storniamide) function as multidrug resistance (MDR) reversal agents (3), whereas others (e.g., lamellarins K and M) are highly potent cytotoxic compounds. However, the best known member in the series is lamellarin D, which we have characterized previously as a potent inhibitor of topoisomerase I endowed with antitumor activities (4). Like camptothecin, lamellarin D stabilizes topoisomerase I-DNA covalent complexes and induces DNA damages responsible for cell death. The alkaloid was shown to exert remarkable in vitro cytotoxicity against a large panel of human cancer cell lines (4). Precise structure-activity relationships have been delineated in the lamellarin D series (5), and some of the key molecular determinants responsible for topoisomerase I poisoning and cytotoxicity have been recently identified, thus facilitating the design of synthetic analogues developed as anticancer agents (6). A subset of synthetic lamellarins showing potent antitumor activities in human tumor xenografts has been selected (7, 8), and a few compounds are currently being profiled for inclusion in clinical trials.

We have recently shown that lamellarin D triggers apoptosis in P388 murine leukemia cells sensitive and resistant to camptothecin (9). Interestingly, the compound was found to be almost equally toxic to drug-sensitive and MDR tumor cells. Specifically, our work identified lamellarin D as a potent inducer of apoptosis insensitive to P-glycoprotein-mediated drug efflux, suggesting that the marine compound is a useful lead in the search for treatments against chemoresistant cancers. The intimate mechanism leading to apoptosis was not detailed, but an activation of the mitochondrial pathway was evidenced (9), as it is generally the case with all topoisomerase I poisons. However, the exact mechanism by which lamellarin D exerts its antitumor activity is not fully understood at present. Although there is no doubt that lamellarin D is able to inhibit topoisomerase I at the molecular level and in culture tumor cells, additional experiments with cell lines sensitive or resistant to known topoisomerase I poisons indicated that this enzyme is not the only cellular target for lamellarin D. Indeed, topoisomerase I–mutated cell lines exhibit a reduced but still significant level of chemosensitivity toward lamellarin D and analogues (4, 6). These observations prompted us to investigate further the mechanism of action of lamellarin D at the cellular level, with the goal to identify targets or pathways implicated in or responsible for lamellarin D–induced apoptosis.

The mitochondrion, due to its pivotal role in cell apoptosis, has recently emerged as a novel pharmacologic target for anticancer chemotherapy (10–13). Experimental drugs that act on mitochondrial targets present therapeutic activities in preclinical mouse models. This applies, for instance, to Bcl-2 or Bcl-XL ligands (HA-14), lipophilic cations (F16), or the synthetic retinoid-related derivatives (e.g., storniamide) function as multidrug resistance (MDR) reversal agents (3), whereas others (e.g., lamellarins K and M) are highly potent cytotoxic compounds.
molecules, CD437/AlHPN (10–13). Several molecules acting on mitochondrial targets are currently under clinical evaluation, such as oblimersen sodium (Genesense, phase III), GX-015-070 (Bel-2 antagonist, phase II), ALS-357 (betulinic acid, Bel-2 antagonist, phase II), SP-5000 (benzodiazepine receptor antagonist phase II), gossypol (phase I), etc.

Using complementary cell-based assays, particularly powerful real-time methods, to dissect the drug effects, we came to the unexpected and highly stimulating conclusion that lamellarin D exerts direct effects on mitochondria ultrastructure and functions. This work reinforces further the pharmacologic interest of the lamellarins and provides robust information essential to the development of tumor-active compounds in this series. The main cellular circuits activated during lamellarin D–induced apoptosis have now been properly characterized; all together, the results reported here further establish that lamellarin D stands as a unique, outstanding lead for the development of anticancer agents. The interest of this work goes well beyond the specific case of the lamellarins, as it provides distinct opportunities to think of different types of anticancer agents directly interfering with mitochondrial functions.

Materials and Methods

Chemicals and modulation of apoptosis. Camptothecin was purchased from Sigma (St. Louis, MO), and lamellarin D and the derivative PM031379 were synthesized at PharmaMar (Madrid, Spain). Cells were cultured with these reagents alone or in combination with the inhibitor of mitochondrial permeability transition (MPT) cyclosporin A at 5 μmol/L (Sandoz, Hanover, Germany). Benzoyloxy carbonyl-Val-Ala-Asp(Ome)-fluoromethyl ketone (z-VAD.fmk; Bachem, Basel, Switzerland) was used as a general inhibitor of caspases activity.

Cell lines, culture conditions, and morphologic assessment of apoptosis. The following cell lines were grown in DMEM supplemented with antibiotics and 10% heat-inactivated FCS: H9C2, embryonic rat heart-derived cell line; MCF-7, human breast adenocarcinoma; A549, human lung carcinoma; and NIH3T3 murine fibroblast. All cells were treated with lamellarin D, camptothecin, or PM031379 under strictly identical conditions in the exponential phase of growth. Subconfluent cells were seeded in six-well dishes and incubated with the test drug in the specific medium containing 10% heat-inactivated FCS. Both floating and adherent cells were used for experiments. The P388 mouse leukemia cell line and the P388/CPT5 topoisomerase I–mutated subclone resistant to camptothecin (14) were routinely cultivated in RPMI 1640 supplemented with t-glutamine, antibiotics, and 10% heat-inactivated FCS. For the morphologic assessment of apoptosis, cells were stained with the standard Papanicolaou procedure and examined by light microscopy. The precise morphology of the nuclei was evaluated by fluorescence microscopy after staining with the dye Hoechst 33258.

Cytotoxicity analysis in intact cells. The frequency of hypoploid cells (sub-G1 cells) was assessed as described previously (15). To evaluate inner mitochondrial transmembrane potential (ΔΨm) in real time, cells (5 × 10^3/mL) were incubated with 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; 1 μmol/L in PBS; Molecular Probes, Eugene, OR) for 15 minutes at 37°C and then were immediately analyzed on a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ). JC-1 exists as a monomer at low values of ΔΨm (green fluorescence; ε_{527 nm} = 0.55 nm), whereas it forms aggregates at high ΔΨm (orange fluorescence; ε_{590 nm} = 990 nm). In both cases, the fluorescence was recorded in the logarithmic amplifier mode. Time scale was divided into periods of 1 second. Basal ΔΨm was registered for 5 minutes, drugs were added to samples when indicated, and variation in fluorescence was recorded for the following 15 minutes. Alternatively, drug-treated cells were incubated for 15 minutes at 37°C with tetramethylrhodamine methyl ester (TMRM; 125 μmol/L in PBS) to measure ΔΨm in fixed time by cytofluorometry (ε_{573 nm} = 573 nm). Samples were stored in ice before the cytofluorometric analysis on a FACSscan cytofluorometer. Control experiments were done in the presence of 10 μmol/L carbamoyl cyanide m-chlorophenylhydrazone (mCCP; 15 minutes at 37°C), an uncoupling agent that abolishes the ΔΨm.

Purification of isolated mitochondria. Mitochondria were purified from P388 cells by differential centrifugation as described previously (16).

Real-time flow cytometric analysis of isolated mitochondria. Real-time experiments were done on mitochondrial samples containing 2 μg proteins in 500 μL swelling buffer (200 mmol/L sucrose, 10 mmol/L Tris-HCl, 10 mmol/L MOPS, 1 mmol/L NaN3, 10 mmol/L EGTA, 5 mmol/L sodium succinate, and 2 μmol/L rotenone). To evaluate ΔΨm variations, mitochondria were stained with JC-1 (1 μmol/L; 15 minutes at 37°C) and JC-1 orange fluorescence was collected over the time. Mitochondrial size was analyzed through the light scattered by the mitochondria at low angle using the forward scatter channel (FSC) as described previously (17). Basal signals (ΔΨm and FSC) were registered for 5 minutes, drugs were added to samples, and variations in signal intensities were recorded for the following 15 minutes.

Mitochondrial analyses were done after appropriate settings of the side scatter and forward scatter detectors. Discrimination of mitochondria from cellular debris and background was done by labeling mitochondria with the ΔΨm-insensitive dye Mitotracker Green (200 mmol/L; 15 minutes at 37°C; Molecular Probes).

Cell sorting and immunofluorescence. P388 cells were incubated for 30 minutes at 37°C with lamellarin D (3 μmol/L) before being washed and stained with TMRM as described above. Next, cells were sorted into subpopulations of cells with normal (ΔΨm, high) and decreased ΔΨm (ΔΨm, low) in an ALTRA flow cytometer (Beckman Coulter, Fullerton, CA). The calibration was done using cells treated with the protonophore agent mCCCP to select the subpopulation with a decreased ΔΨm. At the end of the sorting, subpopulation of cells were fixed with 4% paraformaldehyde and 0.19% picric acid in PBS for 60 minutes and washed in PBS for 10 minutes. The cells were further permeabilized with 0.1% SDS in PBS plus 1% FCS for 5 minutes before incubation with 2 mg/mL of the 6H2-B4 mouse IgG1 anti–cytochrome c antibody (PharMingen, San Diego, CA) for 1 hour. After three washes in PBS plus 1% SVF, cells were incubated in 1:125 FITC-conjugated anti-mouse [Fab′]2 Sigma for 30 minutes. The cells were washed three times in PBS plus 1% FCS followed by washing in 80% glycerol in PBS. All samples were viewed using fluorescence microscope (DMR Leica), and images were processed using the Leica software (Leica Qfluoro).

Immunoblot analysis. After indicated treatments, P388 intact cells (1 × 10^7 for each sample) were resuspended in extraction buffer and the supernatants were prepared as described previously (16). For each experiment, 25 μg proteins from each sample of the cytosolic fraction were loaded on a 12% polyacrylamide gel. After electrophoresis, the gels were blotted onto nitrocellulose membrane (Amersham Life Science, Piscataway, NJ), which was then probed with a mouse monoclonal antibody (mAb) THB.2C12 (1:1,000; PharMingen) specifically recognizing the denatured form of cytochrome c. Activated caspase-3 was detected using an antibody (1:1,000; Cell Signaling Technology, Inc., Danvers, MA), which recognizes the activated caspase-3 (17 kDa) as described previously (15). Immunoblot with anti-Hsc70 mouse mAb (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) was used for standardization of protein loading. Primary antibodies binding was detected with horseradish peroxidase–conjugated goat anti-mouse (1:5,000; Sigma) and visualized by enhanced chemiluminescence following the manufacturer’s instructions (Amersham).

Cell-free system of apoptosis. Apoptotic activity in the supernatant of lamellarin D–treated mitochondria was tested on Jurkat T leukemia cell nuclei purified as described previously (16). Purified mitochondria (0.5 mg/mL final concentration) in the presence of 300 μg P388 cell cytosol were incubated in the presence or absence of lamellarin D (5 μmol/L) alone or in combination with cyclosporin A (5 μmol/L) for 1 hour at 37°C. After centrifugation, supernatants were added to purified Jurkat nuclei (10^6) at 37°C for 90 minutes in a volume of 20 μL buffer containing 10 mmol/mL HEPES, 2.5 mmol/mL KH2PO4, 50 mmol/mL NaCl, 2 mmol/mL MgCl2, 1 mmol/mL DTT, 0.2 mmol/mL EGTA, 2 mmol/mL ATP, 10 mmol/mL phosphocreatine, and 50 μg/mL creatine kinase. Nuclei were then stained with 5 μmol/L Hoechst 33188.
Transmission electron microscopy. P388 cells or isolated mitochondria were treated with lamellarin D for 1 hour or 15 minutes, respectively. Cells or isolated mitochondria (20 μg mitochondrial proteins) were prepared for transmission electron microscopy as described previously (15).

Measurements of cytochrome c release by ELISA Quantikine kit. Mitochondria (50 μL of 0.4 mg protein/mL) were incubated in 250 μL swelling buffer with gentle shaking at 37°C. After 15 minutes, the suspension was centrifuged at 15,000 × g for 5 minutes and supernatants (50 μL) were used to measure cytochrome c release from isolated P388 cells mitochondria following the original protocol provided by the manufacturer (R&D Systems, Minneapolis, MN). Reading was realized in a microplate reader Digiscan (Asys Hitech, Eugendorf, Austria) at 450 nm (correction wavelength was done at 540 nm).

Topoisomerase I–mediated DNA cleavage. Topoisomerase I inhibition was investigated with a 32P-labeled 160-bp DNA fragment from a previously described procedure (18). Briefly, each reaction mixture contained 2 μL of 3′–end 32P-labeled DNA (~1 μm/L), 5 μL water, 2 μL of 10× topoisomerase I buffer, and 10 μL of the drug solution (20 μm/L, final concentration). After 15 minutes of incubation, to ensure equilibration, the reaction was initiated by addition of 2 μL (20 units) human topoisomerase I. Samples were incubated for 45 minutes at 37°C before adding SDS to 0.25% and proteinase K to 250 μg/mL. The DNA was precipitated with ethanol, resuspended in 5 μL formamide-Tris-borate EDTA loading buffer, denatured at 90°C for 4 minutes, and chilled in ice for 4 minutes before loading on to the sequencing gel. DNA cleavage products were resolved by polyacrylamide (8%) gel electrophoresis under denaturing conditions (8 mol/L urea).

Results

Lamellarin D, unlike camptothecin, induces early mitochondrial signs of apoptosis. Lamellarin D and the prototype inhibitor of topoisomerase I, camptothecin, were studied for their ability to induce apoptosis in the leukemia cell line P388. After 24 hours of incubation, lamellarin D and camptothecin at 5 μm/L induced significant chromatin condensation and nuclear fragmentation, common features of late apoptosis (ref. 9; data not shown). The morphologic nuclear changes were accompanied by the appearance of a hypoploid DNA content (sub-G1) peak in lamellarin D– and camptothecin-treated cells (Fig. 1A). However, although camptothecin induced cell cycle arrest in G2-M, no major cell cycle perturbations were observed in lamellarin D–treated cells before the manifestation of hypoploidy (data not shown), suggesting the possibility of differences in the mechanisms of action of these two inhibitors of topoisomerase I. Caspase-3, an important effector caspase, is involved in drug-induced apoptosis because an increase in DEVDase activity was detected after lamellarin D and camptothecin exposure (data not shown). Caspase inhibition led to a significant inhibition of DNA fragmentation induced by lamellarin D or camptothecin (Fig. 1A), indicating that lamellarin D, like camptothecin, induced caspase-dependent apoptosis. Lamellarin D induced apoptosis in a concentration-dependent manner with a significant increase in apoptotic cells observed only at the micromolar range (Fig. 1B).

Many anticancer drugs activate caspases through a mitochondria-mediated signaling. Hallmarks of the mitochondrial pathway of apoptosis are the release of proapoptotic factors, such as cytochrome c, from the mitochondrial intermembrane space and the dissipation of the electrochemical gradient (Δψm) on the inner mitochondrial membrane. Thus, we compared the appearance of these mitochondrial signs of apoptosis in lamellarin D– and camptothecin-treated cells. First, we did a kinetic analysis of the Δψm disruption measured by the reduction of the uptake of the potential-sensitive dye TMRM in P388 cells. As shown in Fig. 1C (left), lamellarin D induced a rapid (within 4 hours) Δψm disruption in P388 cells. In contrast to lamellarin D, camptothecin did not induce a loss in Δψm after a short incubation time but displayed a late hyperpolarization profile followed by a dissipation of Δψm. The early Δψm disruption was observed only when lamellarin D was used at the micromolar range (Fig. 1C, right). Interestingly, the effects of lamellarin D on Δψm (Fig. 1C) and apoptosis (Fig. 1B) occurred over the same concentration range, suggesting that the proapoptotic effect of lamellarin D depends on the functional alterations of mitochondria.

The early involvement of mitochondria in lamellarin D–induced apoptosis requires the monitoring of rapid Δψm responses to lamellarin D exposure. Therefore, we developed a real-time monitoring of JC-1 fluorescence by flow cytometric analysis, in which cells are first labeled with the potential-sensitive dye JC-1 to detect Δψm and then treated with lamellarin D, and the JC-1 fluorescence is monitored over the time. Real-time flow cytometry monitoring reveals that lamellarin D–treated P388 cells, compared with DMSO-exposed cells, exhibited a quasi-immediate (within 2 minutes) reduction of JC-1 aggregation (orange JC-1 fluorescence) and a concomitant increase in JC-1 monomers (green JC-1 fluorescence; Fig. 1D). Thus, lamellarin D–treated cells exhibited the same pattern of fluorescence as cells treated with the protophore mCCCP, a substance that dissipates the Δψm (Fig. 1D). Consistent with the above data, cells maintained high Δψm after short time treatment with camptothecin (data not shown).

Next, we tested the release of mitochondrial proapoptotic factors into the cytosol of drug-treated cells. The early loss of Δψm in lamellarin D–treated cells was accompanied by the release of cytochrome c into the cytosol. Indeed, immunoblot analysis shows a significant increase of cytochrome c in the cytosolic fraction evident only 15 minutes after stimulation (Fig. 1E, left). In striking contrast, camptothecin triggered a late release of cytochrome c detected only 18 hours after exposure (Fig. 1E, left). The same kinetics of release were also observed for the mitochondrial protein apoptosis-inducing factor, suggesting that lamellarin D activates both caspase-dependent and caspase-independent pathways (data not shown).

Lastly, to investigate the correlation between cytochrome c release from mitochondria and dissipation of Δψm, cells were incubated with lamellarin D for 30 minutes, stained with TMRM, separated into Δψm high and Δψm low fractions by cell sorting, and then stained for cytochrome c. Immunofluorescence detection of cytochrome c in lamellarin D–treated cells indicates that almost all of the cells sorted for Δψm low (95 ± 5%) showed a bright spotted cytoplasmic staining consistent with a mitochondrial location, whereas the immunostaining of many of the lamellarin D–treated cells sorted for Δψm high (35 ± 10%) exhibited diffuse staining, indicating cytochrome c release from mitochondria (Fig. 1E, right). Thus, these results show that the reduction in Δψm induced by lamellarin D is associated with the release of cytochrome c into the cytosol. Taken together, these data suggest that lamellarin D, in contrast to camptothecin, induces a quasi-immediate commitment of the mitochondrial pathway of apoptosis.

Lamellarin D induces the MPT pore opening in intact cells. Decrease in the Δψm is reminiscent of the opening of the MPT pore, a key phenomenon in cell death by apoptosis and necrosis. The extensive and prolonged opening of the pore responsible of MPT causes the dissipation of the Δψm as well as swelling of the mitochondrial matrix (19). Because lamellarin D induces a
disruption of the Δψm, we investigated whether lamellarin D–provoked Δψm changes were dependent on MPT pore opening by pharmacologic modulation using the reference inhibitor of MPT, cyclosporin A. As shown in Fig. 2A, cyclosporin A inhibited the lamellarin D–induced Δψm loss. The caspase inhibitor z-VAD.fmk that suppressed nuclear apoptosis (Fig. 1A) did not prevent the Δψm disruption provoked by lamellarin D (Fig. 2A), indicating that caspases must act downstream of the mitochondrion.

MPT pore opening is also responsible for mitochondrial swelling and subsequent rupture of the outer mitochondrial membrane (20).

Therefore, we examined by electron microscopy the ultrastructural changes in P388 cells after stimulation with 5 μmol/L lamellarin D. A 1-hour exposure to lamellarin D promoted cytoplasmic and nuclear shrinkage in P388 cells as represented in Fig. 2B (c). Moreover, as depicted in Fig. 2B, cells treated with lamellarin D showed mitochondria that appear bigger with lower electron density compared with untreated cells, suggesting a swelling of the mitochondrial matrix. In some cases, mitochondrial membrane ruptures were also observed (Fig. 2B, d). Thus, these results suggest that lamellarin D may be a potent inducer of MPT pore opening in intact cells.

Figure 1. Apoptosis in P388 cells exposed to lamellarin D or camptothecin. A, cells were treated with camptothecin (CPT; 5 μmol/L) or lamellarin D (Lam-D; 5 μmol/L) for 24 hours before cell cycle analysis. When indicated, drug-treated cells were also cultured in the presence of the broad-spectrum caspase inhibitor z-VAD.fmk (50 μmol/L) for 24 hours. Percentages of sub-G1 cells quantitated by flow cytometry. Columns, mean of four independent experiments; bars, SD. B, dose response of the lamellarin D–induced apoptosis in P388 cells. Cells were incubated with increasing concentrations (0.01, 0.1, 0.2, 1, and 5 μmol/L) of lamellarin D for 24 hours and stained with the standard Papanicolaou procedure for the morphologic assessment of apoptosis. Cells (n = 200) were counted for each point. Points, mean of three independent experiments; bars, SD. C, mitochondrial changes observed in lamellarin D– and camptothecin-treated P388 cells. Left, kinetics of the mitochondrial depolarization induced by lamellarin D (5 μmol/L; ○) or camptothecin (5 μmol/L; ■) in P388 cells. Cells were treated in the presence of the test drugs; at the indicated times, the mitochondrial potential was measured by flow cytometry using the TMRM fluorescent dye. Points, relative mean fluorescence (MFI) of three independent experiments; bars, SD. Right, dose-response curve represents the mitochondrial potential of P388 cells treated with increasing concentrations (0.01, 0.1, 0.2, 1, and 5 μmol/L) for 4 hours. D, mitochondrial changes observed in lamellarin D– and camptothecin-treated P388 cells. Real-time flow cytometry monitoring of lamellarin D–induced Δψm disruption of P388 cells. Basal levels of JC-1 green fluorescence (JC-1 monomers) and JC-1 orange fluorescence (JC-1 aggregates) were recorded for 5 minutes. Then, 5 μmol/L lamellarin D (or DMSO as control) was added (arrows, addition of drugs), and fluorescence variations were monitored for the following 20 minutes. For the control, cells were exposed to the uncoupling agent mClCCP (10 μmol/L). Black line, mean fluorescence values. E, mitochondrial changes observed in lamellarin D– and camptothecin-treated P388 cells. Left, time course of mitochondrial release of cytochrome c. At the indicated time intervals after the treatment with lamellarin D (5 μmol/L) or camptothecin (5 μmol/L), P388 cell lysates were fractionated and the cytosolic fractions were analyzed by Western blot using an anti–cytochrome c antibody. Hsc70 served as loading control, and a mitochondrial fraction was used as a positive control (data not shown). Right, cytochrome c (cyt-c) distribution in Δψm or Δψm pop subpopulations. P388 cells were treated with lamellarin D (3 μmol/L) for 30 minutes, stained with TMRE, and subjected to flow cytometric sorting using the ALTRA. Cells were separated in two distinct subpopulations showing high and low Δψm based on TMRE staining. After sorting, each subpopulation of cells was fixed and incubated with anti–cytochrome c antibody followed by FITC secondary antibody before examination by fluorescence microscopy. Original magnification, ×630.
**Lamellarin D provokes the MPT pore opening of isolated mitochondria.** Despite the importance of the mitochondria for the induction of lamellarin D–mediated apoptosis, our results did not explain how lamellarin D activates the mitochondrial pathway. The early involvement of the MPT pore opening in lamellarin D–induced apoptosis prompted us to investigate whether lamellarin D might have direct effects on isolated mitochondria. Because it has been shown that flow cytometry is a reliable tool to evidence mitochondrial swelling through FSC (size) detection (17), we applied a real-time flow cytometric approach to analyze simultaneously ΔΨm changes (Fig. 3A) and mitochondrial size (Fig. 3B) on isolated mitochondria purified from the cancer cells P388.

Atractyloside, which is a specific ligand of the adenine nucleotide translocator that causes MPT pore opening (21), was used as a control to induce a cyclosporin A–inhibited ΔΨm loss (Fig. 3A) and swelling (Fig. 3B), confirming that the real-time flow cytometry is reliable to detect MPT-dependent events on isolated mitochondria.

When treated with lamellarin D, the mitochondrial population was subjected to a rapid and significant decrease in ΔΨm (Fig. 3A) and a concomitant size increase (Fig. 3B) similar to that observed with the MPT inducer atractyloside. The lamellarin D–triggered mitochondrial size increase has been confirmed by electron microscopy (Fig. 3C). Ultrastructural examination of isolated mitochondria exposed to lamellarin D revealed an important swelling of mitochondria (Fig. 3C, b), which was consistent with the mitochondrial alterations observed in lamellarin D–treated cells (Fig. 2B, d). The size of mitochondria almost doubled on treatment with lamellarin D: their diameter, measured from the electron micrographs (including >200 mitochondria), increased from 0.53 ± 0.06 μm in the control–untreated samples to 0.93 ± 0.10 μm in the treated samples (P < 0.05, Student’s t test).

As expected from the fact that cyclosporin A prevents lamellarin D–induced ΔΨm dissipation in intact cells (Fig. 2A), cyclosporin A inhibited the lamellarin D–triggered ΔΨm reduction (Fig. 3A) and FSC increase (Fig. 3B) of isolated mitochondria. It should be noted that simultaneous detection of ΔΨm changes and mitochondrial size did not allow us to define the hierarchy of these related events, which seem to appear simultaneously. In contrast, camptothecin was unable to promote MPT of isolated mitochondria (Fig. 3A and B). Hence, lamellarin D has the unique property of exerting a direct effect on isolated mitochondria that involves MPT pore opening. **Lamellarin D promotes the MPT-dependent release of cytochrome c from isolated mitochondria.** MPT pore opening is considered a predominant mechanism mediating the release of proapoptotic mitochondrial molecules, such as cytochrome c (22). Therefore, we tested this hypothesis on isolated mitochondria. Isolated mitochondria were treated with lamellarin D alone or in combination with cyclosporin A or z-VAD.fmk and then centrifuged. The mitochondrial supernatants were recovered to measure cytochrome c by ELISA capable of detecting <0.5 ng cytochrome c/mL. Lamellarin D treatment resulted in significant cytochrome c release from mitochondria (Fig. 3D). This amount of cytochrome c was detected immediately after addition of lamellarin D, and further incubation did not produce additional cytochrome c release. The addition of the MPT inhibitor cyclosporin A reduced the release of cytochrome c by lamellarin D–treated mitochondria, suggesting the involvement of a MPT-dependent mechanism in the lamellarin D–induced cytochrome c release. Contrasting with the effects of the MPT inhibitor cyclosporin A, z-VAD.fmk did not affect the mitochondrial cytochrome c release induced by lamellarin D (Fig. 3D). We also compared lamellarin D and atractyloside on cytochrome c releasing in isolated mitochondria. Lamellarin D induced cytochrome c release more efficiently and at a lower concentration than did the MPT inducer, atractyloside (Fig. 3D). Thus, lamellarin D seems to be a direct inducer of the MPT pore opening. **Lamellarin D requires mitochondria to induce apoptosis in a cell-free system.** To further correlate the direct effect of lamellarin D on MPT and nuclear apoptosis, we used a cell-free system of apoptosis (23). We first added to purified nuclei the cytosols of cells treated with lamellarin D alone or in combination with cyclosporin A and determined their effect on chromatin condensation (Fig. 4A). Confirming the results above, the cytosols of lamellarin D–treated cells, but not those of cells treated with lamellarin D in combination with cyclosporin A, induced nuclear apoptosis of purified nuclei. We next tested the direct effect of lamellarin D alone or in the presence of cytosols from control cells on isolated nuclei (Fig. 4B). Lamellarin D on its own is unable to promote apoptosis of isolated nuclei. Moreover, the addition of cytosol without mitochondria is unable to restore the apoptogenic effect of lamellarin D on isolated nuclei. Finally, isolated mitochondria were added in this cell-free system of apoptosis (Fig. 4C). In the presence of mitochondria, lamellarin D was able to induce apoptosis of isolated nuclei, indicating that lamellarin D–induced nuclear apoptosis requires mitochondria at least in a cell-free system. Again, this effect was not detectable when mitochondria were pretreated with cyclosporin A. Together, these results suggest that lamellarin D did not require any premitochondrial activation to induce apoptosis and that the apoptogenic effect of lamellarin D is mediated, at least in part, by its direct effect on MPT.

**Lamellarin D induces apoptosis of topoisomerase I–mutated cells through its effect on MPT.** We recently reported that lamellarin D maintains a significant level of cytotoxicity in the topoisomerase I–mutated cells (P388CPT5) resistant to camptothecin (9). Here, we addressed the question whether the mitochondrial effects of lamellarin D could explain its death-inducing activity in camptothecin-resistant cells. Kinetic studies revealed that lamellarin D provokes a rapid loss in ΔΨm in P388CPT5 (Fig. 5A), similar to that observed in P388 cells (Fig. 1B). In contrast, camptothecin was totally devoid of any mitochondrial effect in the topoisomerase I–mutated cells (Fig. 5A).

Addition of cyclosporin A to P388CPT5 cells largely prevented both the ΔΨm reduction and the nuclear signs of apoptosis induced by lamellarin D (Fig. 5B), confirming that MPT pore opening is involved in lamellarin D–induced apoptosis. Preincubation of cells with z-VAD.fmk failed to inhibit the lamellarin D–induced ΔΨm dissipation but prevented the appearance of hypolipid cells (Fig. 5D), indicating that MPT pore opening precedes the activation of caspases in lamellarin D–mediated apoptosis. Collectively, these data suggest that lamellarin D induces apoptosis of topoisomerase I–mutated cells via its effect on MPT pore.

**Targeting of mitochondria with the tumor-active analogue of lamellarin D PM031379.** As mentioned in Introduction, a few synthetic analogues of lamellarin D have been selected as preclinical drug candidates based on their in vitro efficacy against a panel tumor xenograft models and acceptable ADME profiles. One of the prominent candidates is the amino derivative PM031379 that only differs from lamellarin D by the substitution of an aminopropine side chain for the methoxyphenol ring of lamellarin D branched on the pentacyclic chromophore (Fig. 6A). This derivative has revealed little toxicity toward nontumor cells in vitro (7) and displays potent anticancer activities in vivo in a human colon tumor xenograft model (8). Unlike lamellarin D, PM031379 does not stimulate DNA cleavage
by topoisomerase I (Fig. 6B), therefore suggesting that this compound does not stabilize topoisomerase I-DNA covalent complexes. However, as shown in Fig. 6C, PM031379 produced a dose-dependent increase in tumor cell death through a mitochondrial-dependent pathway. Indeed, on treatment of cells with PM031379, a rapid ΔΨm disruption was accompanied by a substantial release of cytochrome c from mitochondria into the cytosol followed by the proteolytic activation of caspase-3 as shown.
by a Western blot analysis. A similar increase in the percentage of sub-G1 apoptotic cells was observed following exposure to various doses of PM031379. Apoptosis was confirmed morphologically because PM031379-treated cells exhibited classic features of nuclear apoptosis (Fig. 6C, bottom).

Our discovery that lamellarin D exerts a direct proapoptotic effect on mitochondria prompted us to investigate the capacity of the tumor-active candidate PM031379 on the Δψm of isolated P388 mitochondria using our newly developed real-time flow cytometry method with the JC-1 fluorescent probe as described for lamellarin D in Fig. 3A. The effect of PM031379 is directly comparable with that of lamellarin D; the drug decreases the formation of JC-1 aggregates (decrease of orange JC-1 fluorescence; Fig. 6D) and a concomitant increase of the forward scattering (Fig. 6D) in a dose-dependent manner. Furthermore, as observed with lamellarin D, PM031379 promotes a significant release of cytochrome c from isolated mitochondria (Fig. 6D). These effects are equally potent and rapid with PM031379 compared with lamellarin D, suggesting that mitochondria are the target of both compounds.

Finally, we investigated the mitochondrial effects of PM031379 on a panel of cell lines using the same JC-1 flow cytometry method. As shown in Fig. 6E, a short treatment (1 hour) with the drug leads to cell depolarization. The effect is clearly dose dependent and occurs in all cell lines tested. However, it is remarkable to observe that the extent of depolarized cells is higher in tumor cells, including P388 leukemia, A549 lung cancer, and MCF-7 breast cancer, than in nontumor cells, such as NIH3T3 fibroblasts and H9C2 cardiomyocytes. At a concentration of 40 μmol/L (1-hour...
that this particular derivative is inactive against topoisomerase I. PM031379 may play a role in its antitumor activity, all the more observation suggests that the direct mitochondrial effects of fluorescent dye. mitochondrial potential was measured by flow cytometry using the TMRM Cells were cultivated in the presence of the test drugs; at the indicated time, the bars, experiments; TMRM and propidium iodide, respectively. Columns, mean of four independent experiments; bars, SD.


Discussion

This study discloses an entirely novel facet of the mechanism of action of the antitumor drug lamellarin D. This marine alkaloid was previously characterized as a potent inhibitor of topoisomerase I, stabilizing topoisomerase I-DNA covalent complexes, as it is the case with camptothecin (4). However, we then discovered that cells resistant to the reference topoisomerase I poison camptothecin were only partially resistant to lamellarin D. The drug maintains a significant level of cytotoxicity in P388CPT5 topoisomerase I–mutated cells, for example (9). We first suspected that the limited resistance could reflect an efflux mechanism, but this hypothesis was invalidated when we found that lamellarin D was insensitive to P-glycoprotein-mediated drug efflux (9). During a study aimed at characterizing the topoisomerase I–dependent downstream signaling pathway activated by lamellarin D, we observed that the drug acted as a potent inducer of apoptosis in P388 cells and preliminary evidences implicating mitochondria in the apoptotic cascade were outlined (9). This observation prompted us to investigate more deeply the involvement of mitochondria during lamellarin D–mediated apoptosis.

Here, we have set up a novel real-time flow cytometry approach to follow the effect of drugs on mitochondrial dysfunction using both intact cells and purified mitochondria from leukemia cells. This approach, well integrated with other experimental settings in a multifaceted study using electron microscopy, Western blot analysis, and fluorescence imaging, was useful to characterize the kinetics of the mitochondrial damages following lamellarin D exposure and allowed us to discover the unexpected direct effect of lamellarin D on cancer cell mitochondria. Indeed, as shown in this article, lamellarin D is likely to induce apoptosis by a direct effect on mitochondria in a MPT-dependent manner.

Increasing evidence shows that marine organisms are a rich source of unique compounds with antitumor properties (24). Several compounds have been isolated that induce apoptosis of cancer cells affecting various cellular components, including tubulin, protein kinases or phosphatases, and topoisomerases (24). This study defines mitochondria as a new proapoptotic target for marine antitumor drugs.

Furthermore, our observations have multiple consequences. First, they provide a much clearer picture of the mechanism of action of the marine alkaloid lamellarin D, with, in particular, a rational basis to explain why lamellarin D maintains a cytotoxic potential toward camptothecin-resistant cells. Lamellarin D was previously regarded as a conventional topoisomerase I poison. This view is too restrictive, and the drug must now be considered as a bifunctional pharmacologic effector: targeting topoisomerase I and acting on mitochondria in targeting a specific mitochondrial protein (or protein complexes) that remains to be identified. At submicromolar concentrations, lamellarin D exerts its effect on topoisomerase I at the nuclear level, which also induces marked cell cycle perturbations (4, 9). However, at higher concentrations (at the micromolar range), direct mitochondrial effects take place to contribute to the extensive level of apoptosis induced by the drug. The two phases likely contribute to the antitumor activity of lamellarin D. At the pharmacologic level, this work thus positions lamellarin D in a very different mechanistic situation compared with camptothecin, with a more challenging perspective in terms of drug design and development. Second, the present discovery of a direct activity of lamellarin D on mitochondria opens novel opportunities for the selection of clinical candidates and backup compounds. Several series of lamellarin D derivatives have been synthesized, and each compound is being characterized for its targeting of topoisomerase I versus mitochondria. The real-time flow cytometry assay described here is sufficiently solid and flexible to allow comparative analyses and to delineate further structure-activity relationships in the lamellarin series. Using this cytofluorometric technology, compounds selective of mitochondria, such as the example reported here, PM031379, and molecules inhibiting topoisomerase I without affecting mitochondria, have been recently selected and profiled. This chemical accessibility of

Figure 5. Apoptosis-related changes in camptothecin-resistant cells on lamellarin D treatment. A, kinetics of the mitochondrial depolarization induced by lamellarin D (5 μmol/L; ■) or camptothecin (5 μmol/L; □) in P388CPT5 cells. Cells were cultivated in the presence of the test drugs; at the indicated time, the mitochondrial potential was measured by flow cytometry using the TMRM fluorescent dye. Points, relative MFI of three independent experiments; bars, SD. B, pharmacologic inhibition of lamellarin D–induced apoptosis. P388CPT5 were cultivated in the presence of 5 μmol/L lamellarin D alone or in combination with cyclosporin A (5 μmol/L) or z-VAD.fmk (50 μmol/L). After 24 hours, ΔΨm and the percentage of sub-G― cells were determined by flow cytometry using TMRM and propidium iodide, respectively. Columns, mean of four independent experiments; bars, SD.

In preparation.
the lamellarins, their relatively well-understood mechanism of action, and the validation of their antitumor activity in vivo (8) make this series a very promising reservoir of anticancer drug candidates with the exciting goal to position at least two compounds in two distinct categories of topoisomerase I inhibitor and/or mitochondria-targeted agents. Third, targeting directly mitochondria could be of significant therapeutic relevance to bypass the resistance of cancer cells to proapoptotic signals. Indeed, unlike lamellarin D, most agents used in conventional chemotherapy have no direct effects on mitochondria, and the response of cells to these anticancer agents is frequently determined by signal transducing events upstream of mitochondria. On theoretical grounds, agents that directly target mitochondria act on a more downstream level of apoptosis control and may be advantageous for treating cancers, in which such signal transducing systems are interrupted (e.g., mutations of p53).

Therefore, the identification of compounds that directly affect mitochondria is of substantial clinical interest (25, 26). Lamellarin D can trigger apoptosis and the MPT, either via an indirect effect after topoisomerase I inhibition or via a direct action on mitochondria, as reported here. This latter characteristic might provide lamellarin D with advantages to overcome drug resistance because we showed that lamellarin D is a particularly useful cancer cell death inducer when the topoisomerase I inhibitor camptothecin, requiring a nuclear step, fails to act as a therapeutic agent. Thus, our study validated the notion that mitochondria are viable intracellular targets for new chemotherapeutic agents.

However, drugs that specifically compromise the structural and functional integrity of mitochondria raise novel opportunities to combat cancer cell proliferation, providing that these molecules can be selectively delivered to tumor sites or that they present a more pronounced effect on cancer cells versus nontumor cells. Tumor-specific molecular mechanisms show promise as new means to provide tumor-selective treatment options. In this respect, mitochondria now appear as reservoirs of potential targets for anticancer therapy and various approaches to interfere with the mitochondrial structures and functions in cancer cells have been proposed (25, 27). Indeed, the composition and function of mitochondria are different in normal and malignant cells. Thus, mitochondria of tumor cells display higher $\Delta\psi_m$ (28) and are characterized by an increased anaerobic glycolysis that is not influenced by the oxygen concentration (the Warburg effect; ref. 29), and some components of the MPT pore complex are

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**Figure 6.** Effect of the tumor-active lamellarin D derivative PM031379 on topoisomerase I and mitochondria. A. Structures of PM031379 and lamellarin D. B, inability of PM031379 stimulates DNA cleavage by topoisomerase I. The 3'-end-labeled 160-bp DNA fragment (lane 1, DNA) was incubated in the absence (lane 2, Topo I) or presence of lamellarin D, or PM031379 (20 μmol/L each). Topoisomerase I cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel. C, PM031379 induces cell death through a mitochondrial pathway. P388 cells were treated with the indicated concentrations of PM031379. Four hours after treatment, the cytotoxic fraction of cells was analyzed by Western blotting with cytochrome c antibodies and the mitochondrial potential was measured by flow cytometry using the TMRR fluorescent dye. Percentages of $\Delta\psi_m$ (depolarized cells) were determined using flow cytometry as described in Materials and Methods. Columns, mean of three independent experiments; bars, SD ($< 10\%$). Eight hours after treatment, an immunoblot analysis of active caspase-3 was done on P388 cell lysates. The percentage of apoptotic cells (sub-G1 cells) was determined in P388 cells exposed to PM031379 for 48 hours. Columns, mean of three independent experiments; bars, SD ($< 10\%$). The morphology of the nuclei was visualized by fluorescence microscopy after staining with the DNA-binding dye Hoechst 33258 of P388 cells treated or not with PM31379 at 10 μmol/L for 12 hours. Original magnification, ×100. D, effects of PM031379 on isolated mitochondria. Left, monitoring of mitochondrial $\Delta\psi_m$ and swelling by real-time flow cytometry. Mitochondria isolated from P388 cells were monitored for $\Delta\psi_m$ by real-time incorporation of JC-1 and measurements of FSC variations. Basal JC-1 orange fluorescence and basal levels of FSC were recorded for 2 minutes. Then, isolated mitochondria were exposed (arrows) to DMSO or PM31379 at 1.5, and 10 μmol/L, and fluorescence variations (JC-1) or morphologic variations (FSC) were monitored for the following 5 minutes. Right, effect of lamellarin D on the release of cytochrome c from isolated mitochondria. Purified mitochondria from P388 cells were incubated at 37 °C in the presence or absence of 20 μmol/L PM031379 for 15 minutes. Then, the supernatants were assayed for the determination of cytochrome c content by ELISA. E, mitochondrial effect of PM031379 was assayed on two nontumoral cell lines, H9C2 cardiac myocytes and NIH3T3 fibroblasts, and on tumor cell lines A549 (lung), MCF-7 (breast), and P388 (leukemia). Cells were cultivated in the presence of PM31379 at the indicated concentration for 1 hour, and the mitochondrial potential was measured by flow cytometry using the JC-1 fluorescent dye. Percentages of $\Delta\psi_m$ (depolarized cells) were determined using flow cytometry as described in Materials and Methods. Columns, mean of three independent experiments.
differentially expressed in tumor cells (10). Several tumor-active mitochondrial dioxiglucoreceptors have been identified, such as compound F16 endowed with marked tumor cell growth-inhibitory properties (30, 31) and the inhibitor of mitochondrial F1F0-ATP synthase, aconitidin, which is one of the most cell line selective cytotoxic agents tested against the 60–cell line panel of the National Cancer Institute and a potent proapoptotic product (32). The results reported here are also reminiscent to those recently published with jasmonates, which act directly on mitochondria derived from cancer cells in a permeability transition pore complex-mediated manner (33, 34). This family of mitochondria-targeted products becomes increasingly studied as anticancer agents (11). In this respect, compound PM031379 has revealed interesting preferential activities on mitochondria of tumor cells (Fig. 6), suggesting that the effect of this preclinical candidate on cancer mitochondria may account for its antitumor activity, as the drug is devoid of action against topoisomerase I. The mechanism of the selectivity of PM031379 for tumor versus normal cells mitochondria is under investigation. It has been known for some time that the constitutively higher ΔΨm of cancer cells is the driving force that selectively accumulates lipophilic cations, such as the small-molecule F16 (30, 31), in their mitochondria (35, 36). Thus, it can be assumed that the protonation of the amino side chain of PM031379 reinforces the mitochondriophilic potential of lamellarin D.

Micromolar concentration of lamellarin D or PM031379 is required to observe the direct effect on mitochondria and apoptosis. However, this level of concentrations is entirely compatible with an in vivo situation. The pharmacokinetics of PM031379 after a single i.v. bolus dose (1 mg/kg) was investigated in CD-1 mice. This dose level was selected as 1/10th of the maximum tolerated dose (MTD; 10.3 mg/kg) after single i.v. bolus administration of PM031379 in mice. Plasma levels of PM031379 showed a Cmax value of 1.64 μmol/L. This strongly suggests that attainable plasma concentrations in the range of 5 to 20 μmol/L might be likely when PM031379 is given at higher doses (close to the MTD) in mice. Therefore, the mitochondrial effect of the drug can be considered pertinent to its the antitumor activity.

We provided evidences that the lamellarin D–induced mitochondrial alterations may involve MPT. Although MPT pore opening is not the unique mechanism of mitochondrial membrane permeabilization, our study and others suggest that the MPT pore is an interesting proapoptotic target for some anticancer drugs. In recent years, various cytotoxic molecules, such as arsenic trioxide, lonidamide, betulinic acid, and CD437 acting directly on the MPT pore, have been identified (12). Our future experiments will focus on the identification of the mitochondrial targets of lamellarin D and the exact functional consequences of the target activation. Components of the MPT pore complex are potential targets of lamellarin D (12). Among them, adenine nucleotide translocator and peripheral benzodiazepine receptor are direct targets for several anticancer agents (13, 37). However, at this stage, the possibility exists that opening of the MPT pore is a result of an indirect effect and other targets, such as the respiratory chain complexes or even mitochondrial topoisomerase I, cannot be excluded. These pursuits are fully in the realm of cellular pharmacology, and we are currently designing novel experimentations to unravel the networks, interactions, and pathways that lamellariins can use to alleviate cancer processes.

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**References**


Cancer Cell Mitochondria Are Direct Proapoptotic Targets for the Marine Antitumor Drug Lamellarin D

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