Stellettin A Induces Oxidative Stress and Apoptosis in HL-60 Human Leukemia and LNCaP Prostate Cancer Cell Lines

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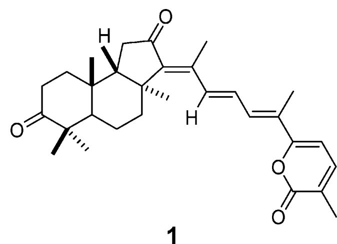
The present study has demonstrated a differential cytotoxicity of stellettin A (**1**) between human leukemia HL-60 cells (IC_{50} 0.4 μg/mL) and human prostate cancer LNCaP cells (IC_{50} 120 μg/mL). Treatment of cells with **1** revealed the activation of NADPH oxidase, the dramatic generation of reactive oxygen species, and the dissipation of mitochondrial membrane potentials, with HL-60 cells being more sensitive than LNCaP cells by an order of magnitude. Immunoblotting analysis further demonstrated a stronger upregulation of the apoptosis marker proteins, FasL and caspase-3, in HL-60 cells, and pretreatment of cells with antisense oligonucleotide for caspase-3 abolished apoptosis. All available evidence suggests that **1** induces oxidative cell death through a FasL−caspase-3-apoptotic pathway.

The marine environment is a rich source of macro- and microorganisms, estimated to contain over a million species.[1] The tremendous marine biodiversity provides a prolific source for the discovery and development of innovative therapeutic agents.[2,3] Many bioactive compounds have been isolated from marine organisms in the past decade, and about forty potential anticancer agents, including six from marine algae, have been under clinical investigation.[4−6] Many novel compounds, such as manoalide from the sponge *Luffariela variabilis*[7] and okadaic acid from *Halichondria okadai*,[8] are inhibitors of cell signaling pathways and are commercially available as biochemical probes.

Stellettins belong to a group of isomalabaricane triterpenes present in several marine sponge genera such as *Jaspis*, *Stelleta*, and *Rhabdastrella*.[9] They have also been found by our group in *Geodia japonica*.[10] These isomalabaricane triterpenoids were reported to exhibit cytotoxicity in an NCI screen,[9,11,12] showing high sensitivity toward leukemia cells and cells deficient in cyclin kinase inhibitor p21.[9] Breast and ovarian cancer cell lines were found to be less sensitive to these compounds,[12,13] yet the underlying mechanism has remained unclear. Such a selective cytotoxicity is an interesting property that warrants further investigation. In the present study, we compared the cytotoxicity of **1** against HL-60 human leukemia cells and LNCaP prostate cancer cells and demonstrated that **1** induces more potent oxidative stress in HL-60 cells than in LNCaP cells, leading to FasL−caspase-3 apoptosis.



Results and Discussion

Geodia japonica Sollas (Geodiidae) is a marine sponge indigenous to the South China Sea. Previous chemical investigations on this genus have led to the isolation of a number of secondary metabolites, including barettin, geodiamolides, geodiastatins, geodiatoxins, geodisterol, and isomalabaricane triterpenoids.[10,14−19] Stellettins A (**1**) and B, two members of the isomalabaricane

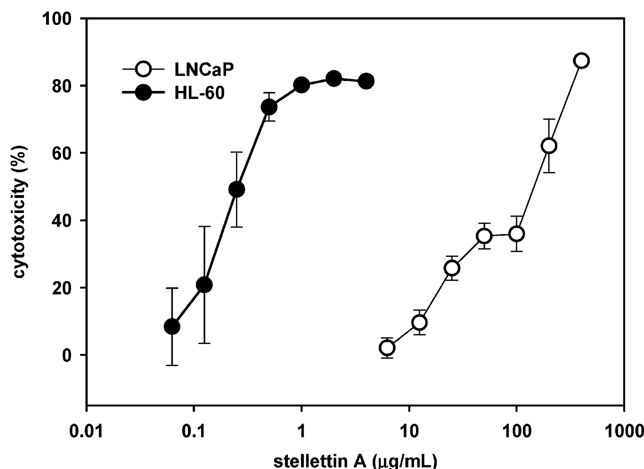


Figure 1. MTT cytotoxic assay showing HL-60 human promyelocytic leukemia cells were more sensitive than LNCaP human prostate cancer cells to the cytotoxicity of **1** by at least an order of magnitude after treatment for 48 h ($n = 3$).

triterpenoid class first reported from *Stelletta tenuis*,[20] were purified from *G. japonica* together with two new isomalabaricanes, geoditins A and B.[10] In our hands, results of the MTT assay after treatment for 48 h revealed that **1** has at least an order of magnitude stronger cytotoxic effect for HL-60 cells (IC_{50} 0.4 μg/mL) than for LNCaP cells (IC_{50} 120 μg/mL) (Figure 1). This finding is in general agreement with reports from other investigators using the NCI 60-cell-line assay panel.[9,11,21,22] The cytotoxic potency was shown to be relatively weak in ovarian tumor cells and breast cancer cells.[11]

Normal HL-60 cells are round with a central nucleus, while LNCaP cells are spindle-shaped with an oval nucleus (Figures 2a and 2c). Apoptotic bodies were observed in both cell types after treatment with **1** for 24 h (Figures 2b and 2d). A high percentage of apoptotic HL-60 cells (>50%) was counted with 4 μg/mL of **1**, but <10% apoptosis was observed in LNCaP cells with 20 μg/mL. Apoptotic death is a stress response of cells to cytotoxic agents that might be executed either through a receptor-mediated pathway involving a death ligand/receptor that activates caspase-8 or through the receptor-independent pathway that involves the cyclin-kinase inhibitors p53/p21. Both pathways would lead to a translocation of pro-apoptotic Bax protein to the mitochondria, thereby resulting in a dissipation of mitochondrial membrane potential, as well as an activation of caspase-3 and execution of the apoptotic machinery.[23] On the other hand, apoptosis can be prevented by the anti-apoptotic bcl-2 protein.[24] In a previous study, we have demonstrated that **1** induced reactive oxygen species, dissipated mitochondrial

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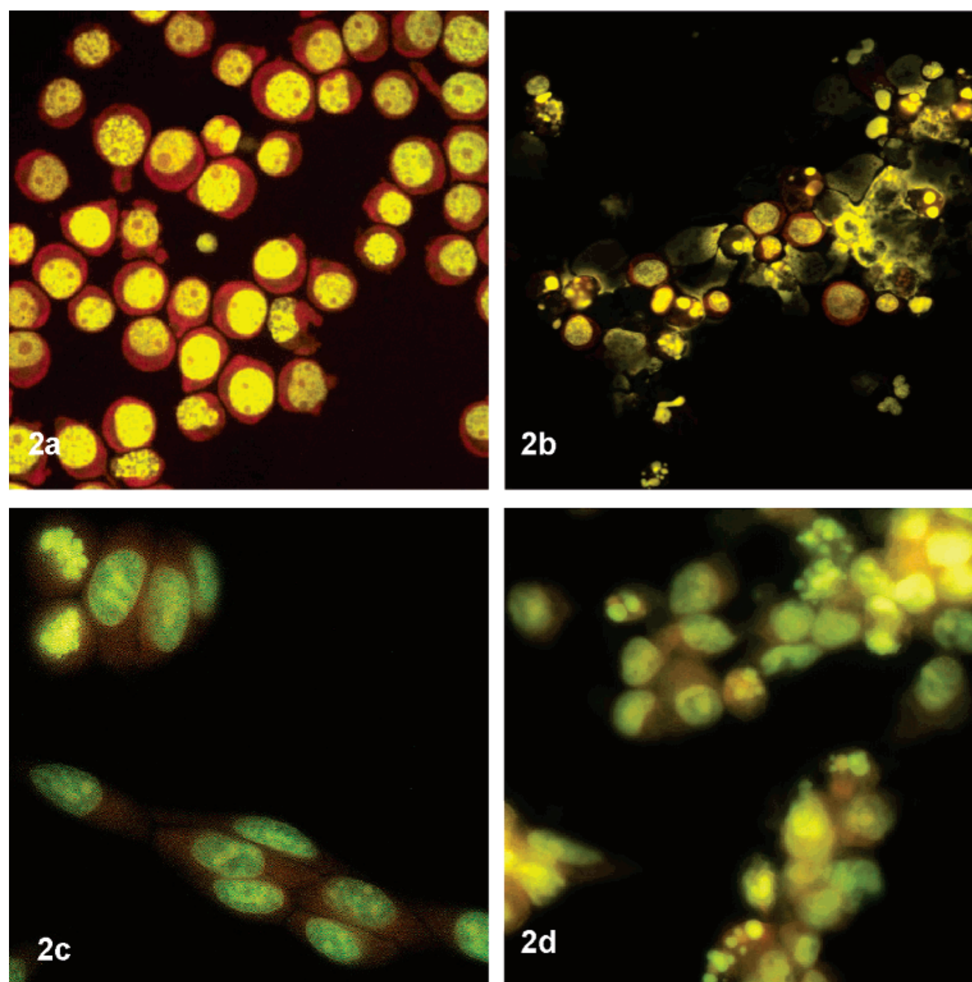

Figure 2. Fluorescence micrographs showing normal, round HL-60 cells with a central nucleus (a) and spindle-shaped LNCaP cells with an oval nucleus (c). Apoptotic bodies were prominent in HL-60 cells but not in LNCaP cells after treatment with **1** for 24 h (b and d) (300×).

membrane potential, and activated the caspase-3 apoptotic pathway in HL-60 cells.[25] This response (56% apoptosis with 4 μg/mL **1** for 24 h) was now shown to be completely inhibited by antisense caspase-3 oligonucleotide (100 nM) but not by sense caspase-3 oligonucleotide (51%), confirming the apoptotic response is mediated through a caspase-3 pathway.

Apoptotic factors are maintained in intact mitochondria, and perturbation of mitochondria would result in a dissipation of mitochondrial membrane potential (MMP) and the release of apoptotic factors (e.g., cytochrome *c*) to trigger cell apoptosis. Strong mitochondrial staining was revealed by a mitotracker to be distributed uniformly throughout the cytoplasm of both untreated HL-60 cells and LNCaP cells. However, the staining became moderate in LNCaP cells and very weak in HL-60 cells after treatment with **1** for 24 h. As a result of mitochondrial dissipation, the mean fluorescence intensity (MFI) for MMP in normal HL-60 cells dropped dramatically from 80.05 to a nearly undetectable level (3.09) at 4 μg/mL by flow cytometric analysis (Figure 3a), whereas LNCaP maintained its MMP at normal range (MFI $\approx$ 40) at doses up to 20 μg/mL (Figure 3b).

Consistent with microscopic observations, results obtained with immunoblotting analysis showed an upregulation of pro-apoptotic marker proteins, FasL and caspase-3, in both HL-60 cells and LNCaP cells after treatment with **1** for 24 h. Active caspase-3 was particularly abundant in HL-60 cells even at 1 μg/mL but was undetectable in LNCaP cells even at 20 μg/mL. The anti-apoptotic bcl-2 protein was maintained at a normal range in LNCaP but only half in HL-60 cells with 1 μg/mL of **1** (Figure 4).

Reactive oxygen species (ROS) are produced by phagocyte NADPH oxidase (phox) as a host defense against invading organisms. NADPH oxidase is composed of two membrane components, gp91phox and p22phox, and four cytoplasmic components, p47phox, p67phox, p40phox, and Rac, which upon activation are translocated to the plasma membrane for functional assembly. Increasing evidence has demonstrated that ROS are not only crucial for their bacteriocidal activities but also important in cell oxidative signaling, proliferation, differentiation, and death.[26] In addition, the generation of ROS is not a unique characteristic of phagocytes, but also a general feature in many cells, in which they are generated by phox homologues such as NOX1, NOX3, NOX4, and NOX5.[27] To study the contribution of NADPH oxidase toward the initiation of apoptosis by **1**, cells were treated with **1** for 5 h and subjected to RT-PCR analysis of transcription of NADPH oxidase components. Results shown in Figure 5 confirmed the presence of the phox enzyme system in undifferentiated human promyelocytic leukemia HL-60 cells[28] but not in prostate cancer LNCaP cells.[29] An increase of transcription of NADPH oxidase components, gp91phox, p47phox, and p67phox, was detected in HL-60 cells cultured in the presence of **1** (up to 10 μg/mL), whereas these species were undetected in LNCaP cells even at higher concentrations (up to 40 μg/mL). A mild transcriptional expression of NOX1, NOX4, and NOX5 was, however, measured in LNCaP cells but not in HL-60 cells, probably because of an absence of NOX components in the latter. The exact roles of these NOX expressions in the process of cytotoxic reaction or the cell signaling pathway remain unclear, yet a preliminary biochemical experiment

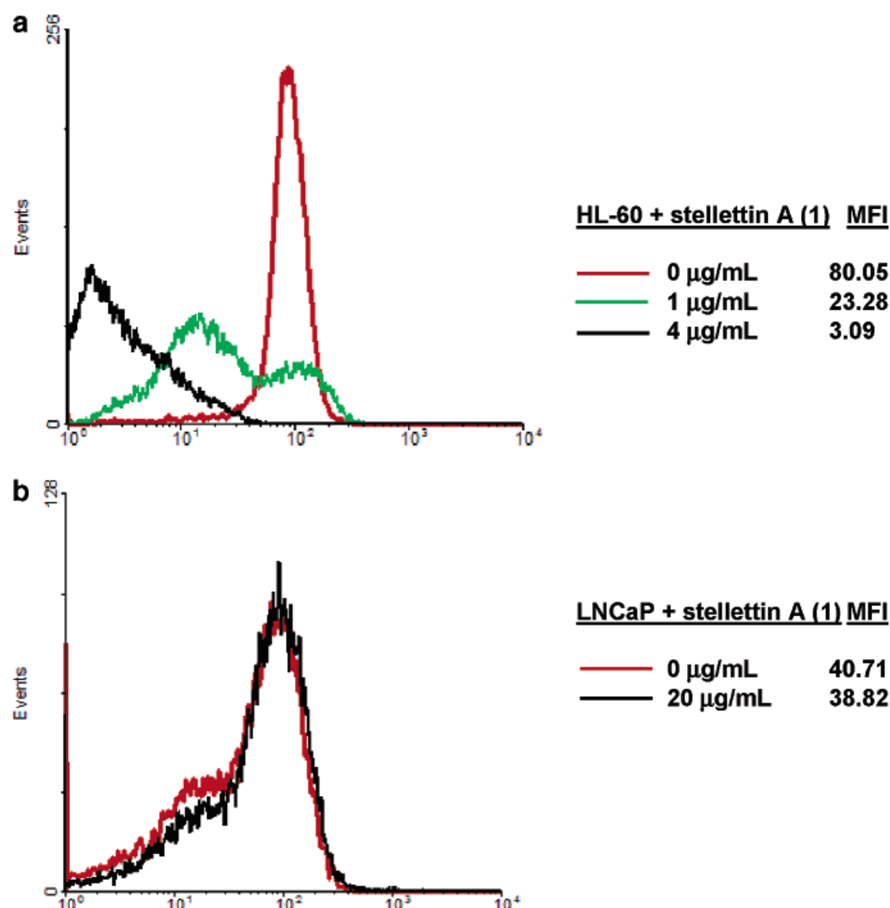

Figure 3. Results of mean fluorescence intensity (MFI) measured by flow cytometry showing a dose-dependent depolarization of mitochondrial membrane potential (MMP) in HL-60 cells treated with **1** for 24 h (a). The MMP in LNCaP cells did not change after treatment (b).

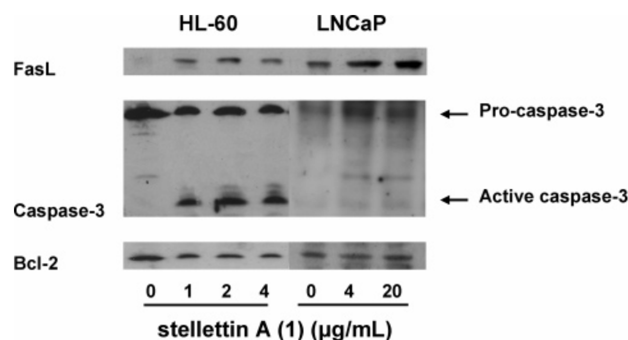

Figure 4. Immunoblotting analysis of apoptosis marker proteins shown in HL-60 cells and LNCaP cells after treatment with various concentrations of **1** for 24 h. Equal amounts of proteins in the cell lysate from each treatment group were subjected to gel electrophoresis and immunoblotted with antibodies against FasL, Bcl-2, and caspase-3. FasL protein increased in a dose-dependent manner in both cells, whereas Bcl-2 protein decreased gradually in HL-60 cells, but remained at normal levels in LNCaP cells. Abundant active caspase-3 was already demonstrated in HL-60 cells treated with **1** at 1 μg/mL, but not in LNCaP cells, even at 20 μg/mL.

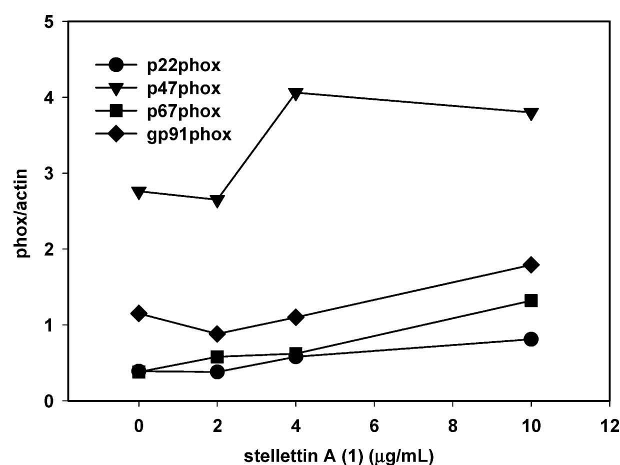

Figure 5. Dose-dependent increase of NADPH oxidase transcription (gp91phox, p22phox, p67phox, and p47phox) shown in HL-60 cells treated with **1** for 5 h. The NADPH oxidase components in LNCaP cells remained relatively unchanged (data shown in Supporting Information).

did demonstrate ROS generation in LNCaP cells by **1** (data not shown). Since the apoptotic activity of ROS is mediated through mitochondrial perturbation and activation of caspase-3, our results demonstrating a higher transcription of NADPH oxidase and apoptotic marker proteins (caspase-3 and FasL), as well as a dissipation of mitochondrial membrane potential, are in agreement that the HL-60 cells have a higher sensitivity to the cytotoxicity of **1** than LNCaP cells.

Experimental Section

Test Compound. Stellettin A (**1**) was isolated from the marine sponge *Geodia japonica*, as described previously.[10] The compound was dissolved in DMSO to make a stock solution of 40 mg/mL, which was then diluted to appropriate concentrations with culture medium before each experiment. The final concentration of DMSO did not exceed 0.5% in any experiment.

Cell Cultures. Human promyelocytic leukemic HL-60 cells (CCL-240) and the LNCaP human prostate cancer cell line (CRL-1740) were

obtained from American Type Culture Collection and routinely cultured in sterile 35 mm culture dishes with IMDM medium or RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS, GIBCO Invitrogen Co., Grand Island, NY), 100 μg/mL penicillin, and 100 IU/mL streptomycin at 37 °C in an atmosphere of 95% air and 5% CO_2.

Cytotoxicity Assay. HL-60 cells or LNCaP cells (2×10^4 cells/0.1 mL/well) were incubated in the presence of serial dilutions of **1** in 96-well culture plates (Falcon, Becton-Dickinson, Franklin Lakes, NJ) for 48 h, then reacted with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at 37 °C for 2 h. The reaction product, formazan, was extracted with dimethyl sulfoxide, and the absorbance was read at 540 nm. Results were reported as the mean values and standard deviations of triplicate samples.[25]

Fluorescence Staining for Morphological Observation. Both HL-60 cells and LNCaP cells were treated with serial concentrations of **1** for 24 h and washed briefly with phosphate-buffered saline (PBS) before they were fixed with buffered formalin. The cells were then spread onto clean glass slides, using a cytospin centrifuge (Cytospin 3, Shandon, Pittsburgh, PA), stained with 0.01% acridine orange, and differentiated with 0.1 M calcium chloride. Fluorescence micrographs were taken on a fluorescence microscope (Axioskop, Zeiss, Göttingen, Germany) with a 450−490 nm excitation block filter and a 520 nm barrier filter,[25] for the measurement of percentage apoptosis on 1000 cells.

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Cells (5×10^6) were treated with serial concentrations of **1** for 5 h before the total cellular RNA was isolated and reverse-transcribed at 42 °C for 50 min using a Superscript preamplification system (18089-011, GIBCO/BRL, Grand Island, NY). Each RT product was subjected to PCR using Thermoprime DNA polymerase and primers for members of NADPH oxidase (GIBCO/BRL)[30,31] in a 9700 Perkin-Elmer thermal cycler. The PCR products were separated on an agarose gel (1.2%), and the relative intensity against respective β-actin was measured by a densitometer with Image Quant software (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA).

Immunoblotting Analysis of Apoptosis Marker Genes. Stellettin A (**1**)-treated cells were washed with PBS twice, resuspended in lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.2% Triton X-100, 10 μg/mL aprotinin, and 0.5 mM PMSF), and centrifuged at 10 000 rpm at 4 °C for 10 min. Lysates were normalized for protein content using the protein assay reagent (500-0006, Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein samples were separated on a 12% SDS-PAGE gel and transferred onto a PVDF membrane using a semidry transfer cell (Bio-Rad). The blot was then rinsed with Tris-buffered saline containing 0.1% Tween-20 (TBS/T, pH 7.6) and soaked in blocking reagent to prevent nonspecific binding before it was reacted overnight at room temperature with polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against human FasL (sc-6237), Bcl-2 (sc492), or caspase-3 (sc7148). The blot was rinsed with TBS/T, linked with HRP-conjugated antibody, and detected using enhanced chemiluminescence blotting reagents (RPN2108, Amersham Biosciences, Little Chalfont, Bucks., UK) for 1 min and exposure to an Lumi-Film chemiluminescent detection film (11666657001 Roche Applied Science, Indianapolis, IN). The amounts of translational proteins were analyzed and normalized with actin (AB5, Neomarker, Fremont, CA) using a Fluor-Chem Imager (Alpha Innotech Corporation, San Leandro, CA).[25]

Determination of Mitochondrial Transmembrane Potential (MMP). The mitochondrial membrane potential of LNCaP cells and HL-60 cells after treatment with **1** was measured using a mitochondrial specific probe, chloromethyl-X-rosamine (CMXRos, Mitotracker Red) (M7512, Molecular Probe Inc., Eugene, OR), according to the method described by Gilmore and Wilson.[32] Briefly, cells were treated with serial concentrations of **1** for 5 h. During the last 15 min, cells were incubated with 100 nM CMXRos in culture medium at 37 °C in the dark before they were rinsed with PBS and subjected to fluorescence microscopy and flow cytometric analysis of MMP immediately with excitation at 488 nm and emission at 610 nm.[25]

Antisense Treatment. HL-60 cells were preincubated with 100 mM antisense oligonucleotides against caspase-3 (5′-GGTTAACCCGGG-TAATG-3′, Syngen Inc., Foster City, CA) in culture medium without FBS for 30 min before they were challenged with **1** (100 mM) for 24 h. The cells were washed with PBS and stained with acridine orange for apoptotic morphology evaluation and quantitation on 1000 cells from each treatment group using a fluorescence microscope. Cells treated with equimolar sense oligonucleotides for caspase-3 for 24 h were used as control.[33]

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Supporting Information Available: Primer sequences of NADPH oxidase and homologue genes; effect on apoptosis of sense and antisense caspase-3 oligonucleotides; epifluorescence microscopy of cells treated with **1** showing mitochondrial membrane potential stained with mitotracker; and effect of **1** on NADPH oxidase transcription. This information is available free of charge via the Internet at http://pubs.acs.org.

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