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Pro-apoptotic activity of lipidic α-amino acids isolated from *Protopalythoa variabilis*

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ABSTRACT

Lipidic α-amino acids (LAAs) have been described as non-natural amino acids with long saturated or unsaturated aliphatic chains. In the continuing prospect to discover anticancer agents from marine sources, we have obtained a mixture of two cytotoxic LAAs (**1a** and **1b**) from the zoanthid *Protopalythoa variabilis*. The anti-proliferative potential of 14 synthetic LAAs and **1a/1b** were evaluated on four tumor cell lines (HCT-8, SF-295, MDA-MB-435, and HL-60). Five of the synthetic LAAs showed high percentage of tumor cell inhibition, while **1a/1b** completely inhibited tumor cell growth. Additionally, apoptotic effects of **1a/1b** were studied on HL-60 cell line. **1a/1b**-treated cells showed apoptosis morphology, loss of mitochondrial potential, and DNA fragmentation.

1. Introduction

Lipidic α-amino acids (LAAs) are classically described as non-natural amino acids with long saturated or unsaturated aliphatic chains.[1,2] In our continuing search to discover anticancer agents from marine sources,[3–6] we have isolated a cytotoxic LAAs mixture (**1a** and **1b**) (Fig. 1) from the zoanthid *Protopalythoa variabilis* Duerden 1898 collected at a northeast Brazilian beach.[7] **1a/1b** has a longer aliphatic chain than any other known synthetic LAA. Although highly lipophilic, LAAs also have a polar behavior, showing chemical and conformational characteristics of amino acids and peptides.[8,9]

The utility of these compounds in the industry is wide: as lubricants,[10] cosmetics,[11] and polishes.[12,13] LAAs may also have therapeutic application, as they have been shown to inhibit both pancreatic[14] and platelet phospholipase A2,[15] being potential anti-inflammatory agents. LAAs and their oligomers have been used in several drug delivery systems.[16] In addition, a LAAs based Lipid-Core-Peptide system was designed and used for the development of self-adjuvanting peptide vaccines against various diseases.[17,18] Despite their wide use, the cytotoxicity of LAAs has not been addressed. Herein we have investigated the cytotoxic activity of 14 synthetic LAAs along with the anti-proliferative and pro-apoptotic effects of **1a/1b** isolated from *P. variabilis* on tumor cells.

2. Materials and methods

2.1. Chemicals and reagents

Propidium iodide (PI) and rhodamine 123 (rh123) were purchased from Sigma–Aldrich. Doxorubicin (Doxolen) was purchased from Zoadiac. All reagents were of analytical grade.

2.2. Lipidic α-amino acids (general procedures)

The isolation of **1a/1b** (Fig. 1) from the zoanthid *P. variabilis* was performed as previously described.[7] Its structures were well characterized by ^{1}H and ^{13}C NMR spectral data and HR-ESI-TOFMS. The ^{1}H NMR spectra showed a signal at δ 3.57 (br t) characteristic of a nitrogenated methine, an intense signal at δ 1.27 (br s) for several methylene groups, and a triplet at δ 0.88 characteristic of the terminal methyl of fatty acids. The ^{13}C NMR (CPD and DEPT 135) spectra exhibited a signal at δ 54.7 (CH), which in the HMBC spectrum showed correlation with the signal at δ 3.57, compatible with an amino acid moiety. In addition, several methylene signals in the range of δ 32.5–23.3, and a carbon signal at δ 14.6 for the terminal methyl group were observed. The HR-ESI-TOFMS spectrum showed two peaks at *m/z* 490.4566 $[M+Na]^{+}$ indicating the molecular

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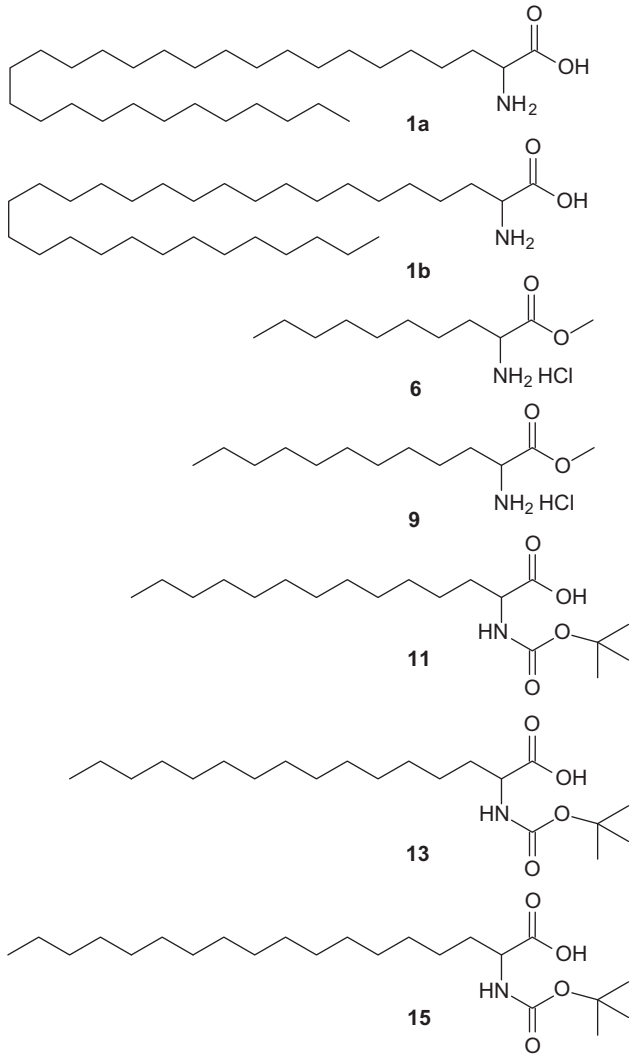


Figure 1. Structures of the most active synthetic lipidic α-amino acids (**6**, **9**, **11**, **13**, and **15**), including the lipidic α-amino acids (**1a**/**1b**) from *Protopalythoa variabilis*.

formula $C_{30}H_{61}NO_2$ (**1a**), while the ion peak at *m/z* 504.4706 $[M+Na]^+$ suggested the molecular formula $C_{31}H_{63}NO_2$ (**1b**). The aforementioned spectroscopic data and functionalities suggested that compounds **1a**/**1b** were indeed two lipidic α-amino acids.

All the other LAAs tested (**2**–**15**), for their in vitro anti-proliferative properties, were synthesized by Gibbons et al. 1990,[8] as shown in Scheme 1. The synthetic procedure includes the reaction catalyzed by EtONa of diethyl acetamidomalonate with 1-bromo alkanes, under reflux overnight, followed by the reaction with HCl to give α-amino acids with an alkyl side chain (**2**, **4**, **7**, **10**, **12**, and **14**). These lipoamino acids could be *N*-Boc protected by reacting with Boc_2O to afford the derivatives **3**, **5**, **8**, **11**, **13**, and **15**, or further O-methylated by reacting with $SOCl_2$ in MeOH to yield **6** and **9**.

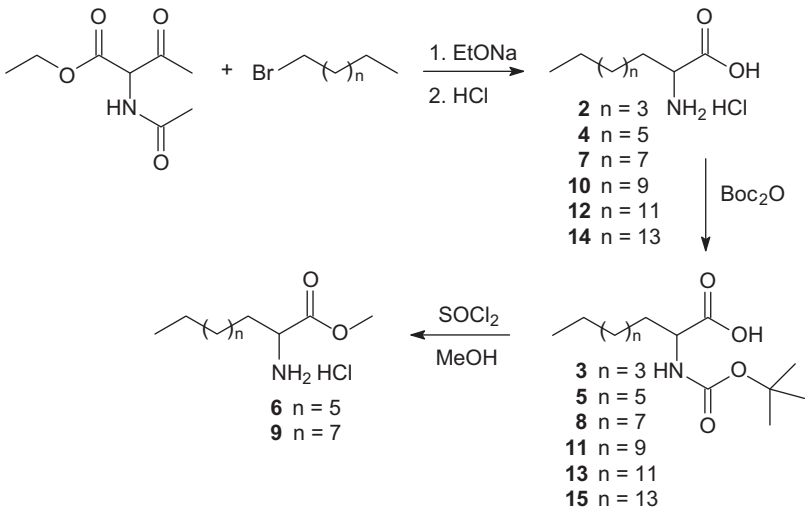
2.3. Cell culture

Anti-proliferative activity of all LAAs was evaluated for four human tumor cell lines obtained from the National Cancer Institute (Bethesda, MD, USA): HL-60 (promyelocytic leukemia), MDA-MB-435 (melanoma), SF-295 (CNS glioblastoma), and HCT-8 (colon carcinoma). Cells were grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in a 5% CO_2 atmosphere. The cell cultures were regularly split to keep them in a logarithmic phase of growth.

Pro-apoptotic effects of **1a/1b** were evaluated against the HL-60 cell line (3×10^5 cells/ml). The 24 h incubation time chosen is sufficient for the cells to complete at least one cell cycle. **1a/1b** was diluted in sterile DMSO and stored at −20 °C. Cell membrane integrity, evaluated by flow cytometry (EasyCyte™ Mini System, Guava Technologies, CA, USA), was used to assess cell viability. All experiments were performed with cell viability above 95%. Five-thousand events were evaluated per experiment and cellular debris was omitted from analysis. Experiments were performed three to five times in triplicate. Doxorubicin (Dox) at a concentration of 0.3 μg/ml was used as a positive control.

2.4. Anti-proliferative assay

The effect of 5 μg/ml concentrations of **1a/1b** and synthetic LAAs (**2–15**) on the tumor cell growth following 72 h incubation was evaluated in vitro using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay, as described by Mosmann.[19] The inhibitory concentration mean (IC_{50}) of **1a/1b** (test concentrations ranging from 0.019 to 10 μg/ml) against



Scheme 1. Synthesis of lipidic α-amino acids **2**–**15**.

HL-60 cells was also obtained using the MTT assay with 24 h incubation. The IC_{50} value was, then, used to drive the concentration choice for the flow cytometry analysis.

2.5. Hemolytic assay

Direct membrane disruption potential of **1a/1b** (test concentrations ranging from 1.56 to 200 μg/ml) was assayed using a 1% mouse erythrocyte (*Mus musculus* swiss) suspension in 0.85% NaCl containing 10 mM $CaCl_2$. The hemolytic activity was measured colorimetrically (at 540 nm) after different incubation times (1, 2, and 4 h), as previously described.[3]

2.6. Cell viability

Cell viability was estimated on the basis of membrane integrity, and evaluated by measurement of the exclusion of PI. Cell fluorescence was determined by flow cytometry using a 680 nm filter (log scale) and Cytosoft software. Cells were diluted to about 300 cells/μL and 5000 events were evaluated per experiment. Cellular debris was omitted from the analysis.[20]

2.7. Analysis of morphological changes

HL-60 cells that were untreated, treated with Dox or treated with **1a/1b** were examined for morphological changes by light microscopy (Olympus, Tokyo, Japan) and flow cytometry. To visualize nuclear and cell membrane morphology microscopically, cells were harvested, transferred to cytospin slides, fixed with methanol for about 1 min and stained with May-Grünwald-Giemsa. To evaluate size changes and nuclear condensation, light-scattering characteristics (linear scale) of 5000 events were analyzed by flow cytometry using FCS express software (De Novo Software, Ontario, CAN).[20]

2.8. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (ψ_m) was determined by the retention of rhodamine 123 (rh123) in HL-60 cells. 1×10^5 cells

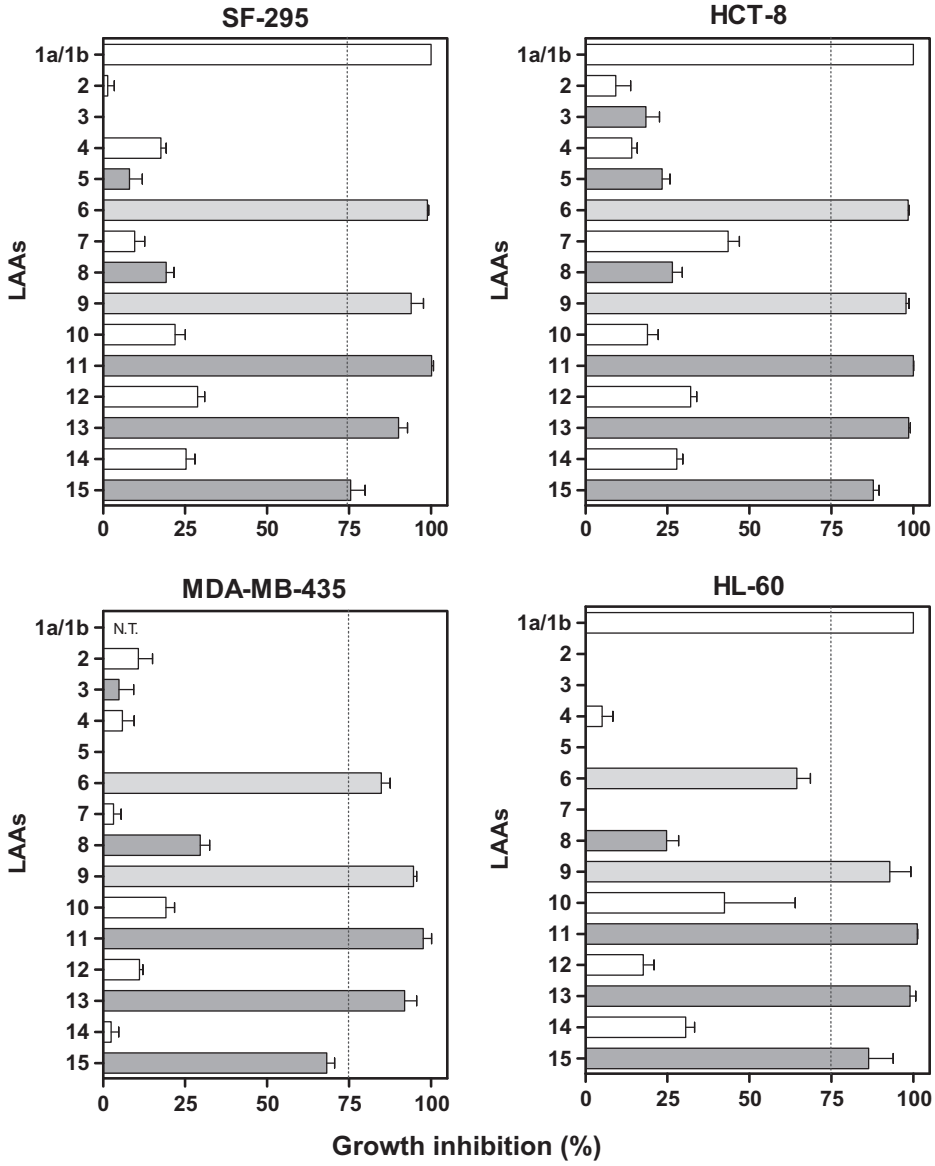


Figure 2. Single dose cytotoxic activity of **1a**/**1b** and synthetic LAAs evaluated against SF-295 (glioblastoma), HCT-8 (colon cancer), MDA-MB-435 (melanoma), and HL-60 (promyelocytic leukemia) cell lines (at 5 μg/ml) using the MTT assay after 72 h incubation. Data obtained from three to four experiments and presented as a percentage of growth inhibition relative to untreated cells. N.T. not tested.

were incubated with rh123 (1 μg/ml) at 37 °C in a 5% CO_2 atmosphere for 15 min in the dark. Cells were then centrifuged, the supernatant aspirated and the pellet incubated in PBS at room temperature for 30 min in the dark. Fluorescence was measured using a 625 nm filter (log scale) and analyzed using Cytosoft software.[21]

2.9. Internucleosomal DNA fragmentation

HL-60 cells (3×10^5) were incubated in the dark for 30 min at room temperature in a buffered stain/lysis solution containing 0.1% citrate, 0.1% Triton X-100, and PI. DNA fluorescence was then determined by flow cytometry using a 680 nm filter (log scale) and analyzed using Cytosoft software.[21]

2.10. Statistical analysis

For the MTT assay, the IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by non-linear regression. For all other assays performed by flow cytometry, the differences between control and experimental groups were determined by analysis of variance (ANOVA) followed by the Dunnett's test. All statistical analyzes were performed using the GraphPad Prism software version 5 (Intuitive Software for Science, San Diego, CA) and the minimal significance level for ANOVA and *t*-test analysis was set at $p < 0.05$.

3. Results

3.1. Anti-proliferative activity

As shown in the Figure 2, the LAAs, including both natural and some synthetic ones, were able to inhibit tumor cell growth. The natural compounds **1a/1b** were more active, completely inhibiting tumor cell growth at 5 μg/ml, while among the synthetic LAAs only compounds **6**, **9**, **11**, **13**, and **15** could be considered active. In fact, previously published IC_{50} values of **1a/1b**, for 72 h incubation, were as low as 0.05, 0.07, and 0.13 μg/ml on HCT-8, SF-295, and HL-60, respectively.[7] Herein it was demonstrated that after 24 h, **1a/1b** still strongly inhibited the growth of HL-60 cells, but with an IC_{50} four times higher (0.53 μg/ml, with CI 95% ranging from 0.48 to 0.60 μg/ml). Unfortunately, the IC_{50} for the synthetic LAAs could not be obtained due to the poor solubility and amphiphilic character of these compounds.

3.2. Hemolytic activity

The hemolysis assay is a useful test to establish if the cytotoxic activity is related to direct damage on the cell membrane. No lytic effect was observed on mice erythrocytes after 1, 2, and 4 h treatment with **1a/1b** at the highest concentration tested (200 μg/ml) (data not shown).

3.3. Morphological changes induced by 1a/1b

May-Grünwald-Giemsa staining showed important morphological changes in treated cells. In the untreated cells, the nuclei were stained homogeneously and there were few vacuoles in the cytoplasm (Fig. 3A). Most of the Dox-treated cells showed intense nuclear fragmentation accompanied by substantial cell shrinkage. Treatment with **1a/1b** induced chromatin condensation with a half-moon or ring-like appearance, DNA fragmentation, and membrane blebbing (Fig. 3C and D). All of these morphological changes present in both the Dox and **1a/1b**-treated cells are considered characteristic of apoptosis (Fig. 3B–D).

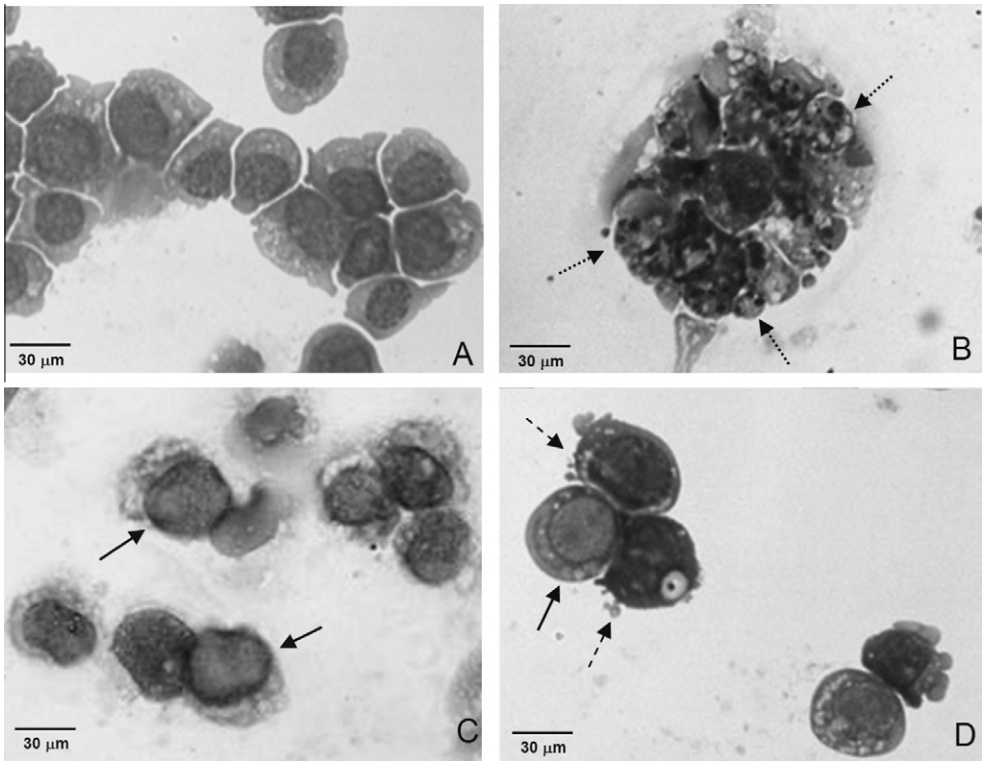


Figure 3. Morphological changes caused by **1a**/**1b** treatment. Photomicrographs of HL-60 cells untreated (A), treated with doxorubicin at 0.3 μg/ml (B), or treated with **1a**/**1b** at 0.1 and 0.25 μg/ml (C and D, respectively), after 24 h, stained with May-Grünwald-Giemsa (400×). Arrow patterns: solid line denotes half-moon or ring-like nuclear chromatin condensation; dotted line denotes nuclear fragmentation; dashed line indicates membrane blebbing.

The changes in light scattering properties were quantified by flow cytometry. Cell shrinkage is related to a decrease in the FSC value which was detected in the R2 region (Fig. 4A–E). Untreated cells showed approximately 5% cell shrinkage, whereas **1a/1b**-treated cells showed increased cell shrinkage (p <0.05) in a dose dependent fashion, ranging from 10% to 70% (Fig. 4C–F). Possible nuclear condensation was also investigated. It is related to a FSC decrease and a SSC increase simultaneously, which was detected in the R3 region.[20] Dox treatment also increased cell shrinkage to 60% (Fig. 4B). There was no considerable increase of nuclear condensation as observed by the low cell population related to region 3 (Fig. 4A–E).

3.4. 1a/1b effect on cell viability

Determination of cell concentration using flow cytometry was performed based on the light-scattering characteristics of a group of healthy cells from the untreated group. As expected, the control cells more than doubled their population from 3×10^5 to about 7×10^5 cells/ml after 24 h. Treatment with **1a/1b** decreased the cell count in a dose-dependent manner. At the highest **1a/1b** concentration, as well as for the Dox treatment, the number of cells remained near the initial plated cellular density (Fig. 5A).

The percentage of viable cells was evaluated by their ability to exclude PI. Membrane permeability to this dye is directly related to already dead or late-staged apoptotic cells. Viable cells show low fluorescence intensity due to their ability to exclude the dye. Untreated cells and 0.1 and 0.25 μg/ml **1a/1b**-treated cells showed minimal membrane disruption. However, cells treated with **1a/1b** at 0.5 μg/ml showed approximately 30% of non-viable cells (Fig. 5B). Dox-treated cells did not show any membrane disruption.

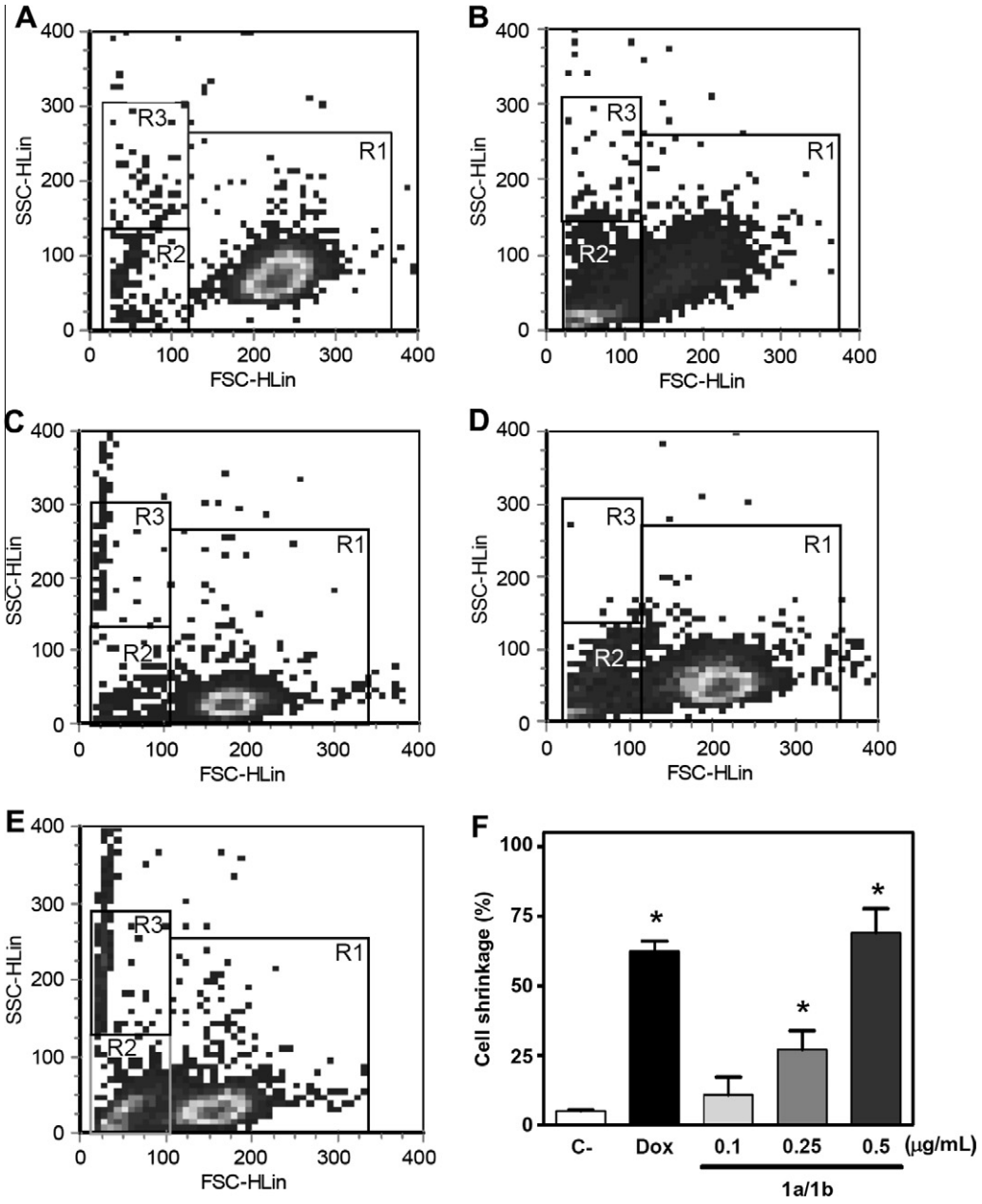


Figure 4. Light-scattering features of **1a**/**1b**-treated cells. (A) Untreated cells; (B) cells treated with doxorubicin at 0.3 μg/ml; (C, D, and E) treated with **1a**/**1b** at 0.1, 0.25, and 0.5 μg/ml after 24 h incubation. (F) Cell shrinkage presented as a percentage relative to control. R1, gated region of viable cells; R2, gated region of cell shrinkage; and R3, gated region of nuclear condensation. *p <0.05 compared to negative control by ANOVA followed by Dunnett's test.

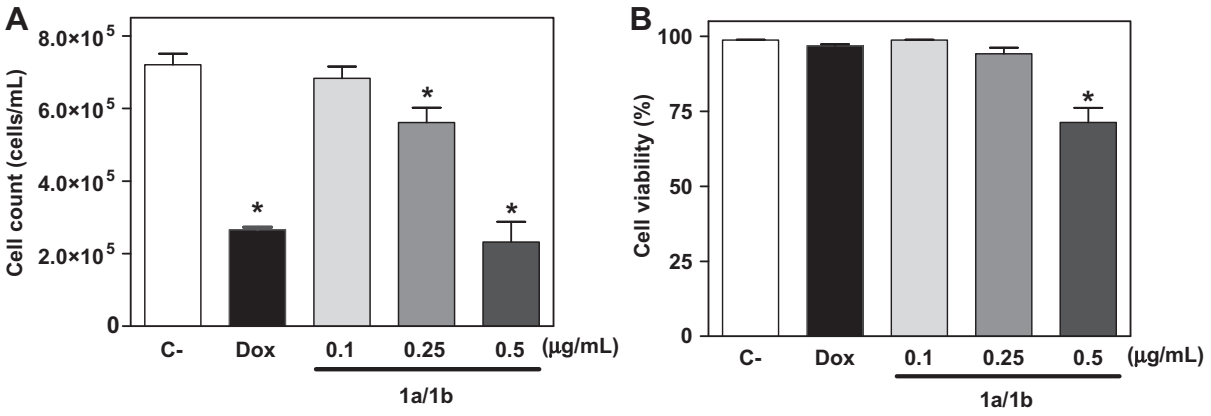


Figure 5. Anti-proliferative effects of **1a**/**1b**. Cell count (A) and cell viability (B) of HL-60 cells untreated (C−) or treated with doxorubicin at 0.3 μg/ml (Dox) or **1a**/**1b** at 0.1, 0.25, and 0.5 μg/ml, after 24 h incubation, by the exclusion of propidium iodide (50 μg/ml). The graphs show the percentage of viable cells obtained from five experiments performed in triplicate by flow cytometric analysis. *p <0.05 compared to negative control by ANOVA followed by Dunnett's test.

3.5. Loss of mitochondrial membrane potential

To investigate the involvement of the mitochondrion in the LAAs-induced apoptosis, dissipation of $\Delta\psi_m$ was evaluated. The percentage of depolarized $\Delta\psi_m$ is shown in Figure 6A. Control cells elicited higher fluorescence, suggesting that these cells were intact. **1a/1b**-treated cells showed $\Delta\psi_m$ dissipation detected by decreased fluorescence intensity (Fig. 6A and B). The untreated cells showed only 9% of $\Delta\psi_m$ dissipation, while **1a/1b** at 0.1 μg/ml increased dissipation to 22% and to almost 50% at 0.5 μg/ml. Dox treatment induced 60% of $\Delta\psi_m$ depolarization.

3.6. Internucleosomal DNA fragmentation

The internucleosomal DNA fragmentation of HL-60 cells was assessed by flow cytometry. The sub-G0/G1 count was considered to represent fragmented DNA, whereas debris was omitted from the analysis. Control cells showed 4% of fragmented DNA. **1a/1b-**treated cells showed no increase in DNA fragmentation at the lowest concentration tested, however, in the following concentrations DNA fragmentation did increase as shown in the Figure 7. Dox-treated cells showed 47% DNA fragmentation. Cell cycle analysis was also performed, however no changes were observed in the **1a/1b**-treated cells (data not shown).

4. Discussion

This study has shown the cytotoxic and the apoptosis-inducing properties of the natural LAAs isolated from *P. variabilis*. This class of molecules (LAAs) share a double behavior, showing chemical and conformational characteristics of lipid and amino acids.[9] Despite the wide use of LAAs including their therapeutic applications since the 90s,[9,14] the first cytotoxic Letter of these compounds was only recently published by our group.[7]

1a/**1b** was isolated as a LAAs mixture through a cytotoxic-guided fractionation from the zoanthid *P. variabilis* collected in Ceará State, on the northeast Brazilian coast. They showed potent activity against three tumor cell lines with IC_{50} values in the nanomolar range.[7] Herein this activity was confirmed using a shorter incubation time, although with a lower potency, suggesting a time-dependence for the observed cytotoxicity.

LAAs display an amphiphilic character, as observed to sphingolipids, that present free amide-group linked to a long alkyl chain.[22,23] Accordingly, the critical aggregative concentrations of sphingolipids is in the nano- or picomolar range,[23–25] and the amount of free monomers in solution is low mainly at high concentrations. Additionally, during cytotoxicity assays these amphiphilic molecules could also interact with proteins from the fetal calf serum, what drastically reduces the cell uptake.[25] These structural

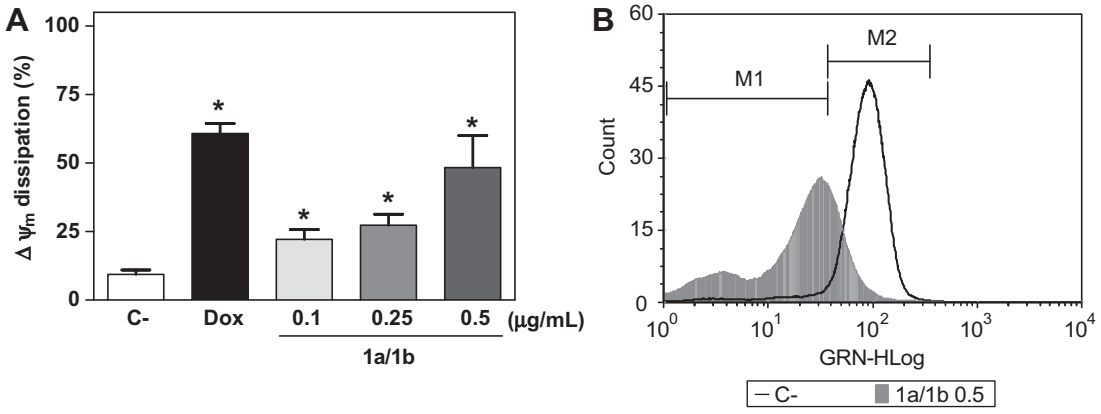


Figure 6. **1a**/**1b** treatment induces mitochondrial membrane potential ($\Delta\psi_m$) loss. $\Delta\psi_m$ depolarization of HL-60 cells, after 24 h incubation, determined by the retention of rhodamine 123 (1 μg/ml) dye. (A) Depolarized cells, obtained from five experiments performed in triplicate. *p <0.05 compared to the negative control (C−) by unpaired t-test. Doxorubicin (0.3 μg/ml) was used as a positive control (Dox). (B) Mitochondrial fluorescence histogram overlay of cells untreated (C−, black outline) and treated with **1a**/**1b** at a concentration of 0.5 μg/ml (shadow). Five thousand events were acquired in each replicate.

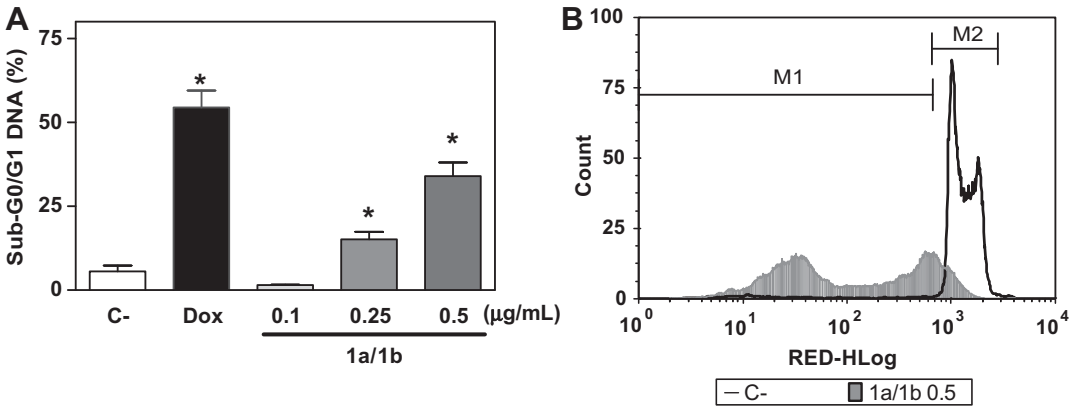


Figure 7. Internucleosomal DNA fragmentation induced by **1a**/**1b** treatment. DNA fragmentation assessed by flow cytometry on HL-60 cells, after 24 h incubation, analyzed by nuclear fluorescence using propidium iodide (50 µg/ml), Triton X-100 (0.2%), and citrate (0.1%). (A) Sub-G0/G1 DNA content of cells obtained from five experiments performed in triplicate. *p <0.05 compared to the negative control (C−) by unpaired t-test. Doxorubicin (0.3 µg/ml) was used as a positive control (Dox). (B) DNA fluorescence histogram overlay of cells untreated (C−, black outline) and treated with **1a**/**1b** at 0.5 µg/ml (shadow). Five thousand events were acquired in each replicate.

similarities may explain the difficulties in the IC_{50} calculation, since the MTT experiments using serial dilutions of the synthetic LAAs did not result in a concentration–response relationship; thus, the IC_{50} value could not be obtained. Since the natural compounds are much stronger active, the assay was conducted in lower concentrations. Even though, the preliminary data on the cytotoxic effects of the 14 synthetic LAAs (**2**–**15**) suggest that lipoamino acids with shorter aliphatic chains, which can be easily synthesized, would also demonstrate anticancer activity. Present findings do not support any considerations on the structure–activity requirements for this unusual class of compounds, however they provide the opportunity for rational design of lipoamino acid-based anticancer agents.

Additionally, further studies were performed with **1a/1b** investigating the mechanisms underlying its anti-proliferative effects on the promyelocytic leukemia cell line, HL-60. Taking together all the results, it could be noticed that treated cells at the lowest concentration are dying through apoptotic process characterized by $\Delta\psi_m$ dissipation and peripheral nuclear condensation followed by DNA fragmentation, membrane blebbing at an intermediate concentration and, finally, at the highest concentration membrane disruption. Apoptosis is an essential physiological process to maintain tissue homeostasis and proper function of multicellular organisms. Cell apoptosis is regarded as the preferred way to eliminate undesired cells and its induction is an important strategy in cancer therapies.[26]

Due to the lack of previous studies on the cytotoxic activity of LAAs, it is impracticable to compare our findings with others of the same chemical class. However, as previously mentioned LAAs share important structural characteristics with sphingolipids. Sphingosine, a precursor and breakdown product of sphingolipids and related compounds, was found to inhibit protein kinase C (PKC), an enzyme involved in cell replication, oncogenesis, tumor promotion, and signal transduction.[22] Structure–activity relationship studies showed that a long hydrophobic chain and a free amino group were the structural requirements for the inhibition of PKC.[27] Spisulosine (ES-285) is a natural cytotoxic marine product that resembles both LAAs and sphingosine. ES-285 acts on PKC via an atypical cell death pathway,[28] and it is currently undergoing phase 1 clinical studies for cancer treatment under the sponsorship of PharmaMar.[29] Additionally, LAAs inhibit PLA_2 because they are potential anti-inflammatory agents. Since sphingolipid analogs are cytotoxic (inhibiting PKC) with an anti-inflammatory effect (in vivo and in vitro inhibiting PLA_2) associated with the presence of a long alkyl chain and free amino group,[30] it can be further speculated that their mechanisms should be similar. Hence, further studies investigating the likely function of LAAs as inhibitors of sphingolipid metabolism would be a good way to explain the mechanism of action by which **1a/1b** triggers apoptosis.

In summary, the cytotoxicity of 14 synthetic LAAs were evaluated on several tumor cell lines, as well as the anti-proliferative and pro-apoptotic effects of a cytotoxic LAAs mixture isolated from the marine zoanthid *P. variabilis*. This is the first Letter on the cytotoxic activity of synthetic LAAs. Additionally, present findings indicate that **1a/1b** induces apoptosis, however further studies are still needed to clarify the exact mechanism of action involved with **1a/1b**-induced apoptosis.

Conflict of interest statement

None.

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