Cytoskeleton alterations induced by Geodia corticostylifera depsipeptides in breast cancer cells

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1. Introduction

Studies on marine life forms in the last few years have led to the discovery of a variety of organic compounds with known or novel pharmacological and toxic activities on mammalian species. Available evidence suggests that the sea offers a rich source of new organic molecules which, either structurally modified or not, may be used as medicines, or as biochemical, physiological or pharmacological tools in biomedical research [17, 29, 40].

Chemical defense through synthesis or accumulation of large amounts of toxic or deterrent natural products is usually found in Porifera [5]. Many of the compounds isolated from marine sponges exhibit neurotoxic, bactericidal, ichthyotoxic, cytotoxic, haemolytic and other toxic properties [37].

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Bioassay guided fractionation is the most frequently used technique to isolate sponge peptides, which are usually cyclic and lipophilic [18]. The depsipeptides isolated from marine sponges or associated organisms are usually described as cytotoxic substances, such as jasplamide, or jasplakinolide [14,45,46], geodiamicolides [10,12,13,15,43], hemiasterlins and criamides [12], halicilindramides [23]. Nevertheless, there are sponge depsipeptides which presented anti-inflammatory activity, as the halipeptins A and B [36], and anti-HIV activity, as papuamides A and microspinomidas [16,44].

From the genus Geodia, compounds other than depsipeptides have also presented interesting biological activities; for example, the brominated cyclopeptides from the marine sponge Geodia barretti, barretin and 8,9-dihydrobarretin, which showed potent antifouling effects inhibiting the settlement of cyprid larvae of the barnacle Balanus improvisus [41]. A Geodia species collected from southern Australian waters of the Great Australian Bight has yielded a potent new in vitro nematocidal agent identified as geodin A Mg salt (1), a new macrocyclic polyketide lactam tetramic acid magnesium salt [9]. Additionally, two proteins from Geodia mesotriaena, named geodiastatins 1 and 2, presented antineoplastic activity against the murine P388 lymphocytic leukemia [33], and geodiatoxins 1 and 2, related proteins from the same species, were very toxic to mice (i.p.) [34].

The present is focused on the anti-proliferative compounds from the marine sponge Geodia corticostylifera (Porifera, Demospongiae). This species can be found in Southeastern Brazilian coast and Venezuela [21]. Its orange color, the apparent lack of predators and other sponge species nearby, suggests the presence of chemical defense mechanisms. Previous studies indicated that the crude extracts of G. corticostylifera displayed bactericidal and fungidical activities [30], and cytotoxicity to sea urchin eggs, neurotoxicity to crab sensory nerve, and haemolytic activity in mice erythrocytes [38]. Neurotoxic and haemolytic activities were recently related to a pore-forming substance in the extract, which incorporates channels on artificial lipid bilayers. These channels have small conductance levels, are rectifiers and cation selective [37].

The cyclic depsipeptides geodiamolides A (1), B (2), H (3) and I (4) were previously isolated and characterized from the Caribbean marine sponge Geodia sp. [10,43]. Geodiamolides A and B presented antifungal activity [10], while geodiamolide H was active against cancer cell lineages (lung, HOP 92; central nervous system, SF-268; ovarian, OV car-4; kidney, A498 and UO-31; breast, MDA-MB-231/ATCC and HS 578T), although geodiamolide I was considered inactive in the same screening [43]. Compounds 1–4 were also isolated from the marine sponge G. corticostylifera, collected on the Brazilian coast, during our studies on purifying the pore-forming substance, and the anti-proliferative effects of these peptides were investigated against sea urchin eggs (Lytechinus variegatus), and T47D and MCF7 human breast cancer cells lineages.

Using fluorescence techniques and confocal microscopy the effects of the geodiamolides A, B, I and H on cancer cell cytoskeleton and nucleus were observed. In an attempt to give evidence for the biomedical potential of these compounds, the cytoskeleton proteins of two different normal cell lineages (human fibroblasts and rat liver cells) were also analyzed under confocal microscopy after incubation with the sponge depsipeptides.

2. Materials and methods

2.1. Extraction and isolation of compounds

Specimens of G. corticostylifera (2.7 kg) were collected by Dr. Marcio dos Reis Custodio on June 2001 off the coast of Sao Paulo State, Brazil, then homogenized in methanol (1:3, w/v) and filtered. The filtered material was evaporated and partitioned with water/methylene chloride (1:1, v/v). The non-polar fraction volume was reduced in a vacuum evaporator and partitioned in methanol–water (9:1, v/v) and n-hexane (1:2, v/v). The methanol–water fraction was fractionated by a Sep Pak Vac C18 cartridge with step-wise elution of 20, 50 and 90% CH3CN in water. Successive purification of the 50–90% acetonitrile fractions by reversed-phase HPLC using CAPCELL PAK C18 (10 mm x 250 mm) with isocratic elution of 45% CH3CN/H2O at a flow rate of 2.5 ml/min over 40 min, monitored by UV absorption at 215 nm yielded the anti-proliferative compounds geodiamolide A (8.8 mg), B (4.6 mg), H (12.2 mg) and I (5.9 mg).

2.2. Spectroscopic analysis

The compounds purified by HPLC were detected by positive electrospray ionization (ESI) mass spectrometry. Typical conditions were a capillary voltage of 2.4 kV, a cone voltage of 32 V, and a desolvation gas temperature of 150 °C. The experiments were done with a Q-Time mass spectrometer (Micromass, UK) in Qq-orthogonal time-of-flight configuration. The spectra were recorded by the use of MassLynx 4.0 software (Micromass).

NMR spectra were recorded on a JEOL EX-400 (at 400 MHz) or a Bruker DMX-750 spectrometer (at 750 MHz) in CD3OD or CDCl3.

2.3. Sea urchin eggs development experiments

Antimitotic activity was monitored initially as the ability of the geodiamolides A, B, H and I to inhibit the first cleavage of L. variegatus sea urchin eggs. The animals were collected in Sao Sebastiao, off the north coast of Sao Paulo State, Brazil. Germ cell release was induced by KCl injections (0.5 M, up to 3 ml) into the perivisceral cavity of the sea urchins. The eggs were washed three times in filtered seawater to remove the jelly coat. The sperm was maintained under refrigeration and not diluted until it was used. The geodiamolides A, B, H and I were diluted in filtered sea/water/MeOH (19:1; v/v) in different concentrations. The final volume was 200 μl of seawater/MeOH mixture, plus 200 μl of egg suspension (prepared with 50 ml of washed eggs + 50 μl of sperm and observation of formation of fertilization layer). Control tubes contained 200 μl of sea water/MeOH mixture. When
the first division in control tubes occurred, the material was
fixed in formaldehyde 10%, observed under microscope and
photographed [25].

The results were analyzed according to their logarithms of
mean and respective standard errors (n = 3). Dose response
curves were plotted, and the EC50 values were calculated by
means of non-linear regression.

2.4. Breast cancer cells experiments

The T47D and MCF7 human breast cancer cells were grown
in Sigma culture medium (DMEM) with 10% fetal bovine
serum (Cultilab) in cellular culture multiplates (24 wells)
with an initial density of 5 × 10^4 cells/well and incubated
during 24 h. After this period, the medium was changed to a
new one with the geodiamolides A, B, H and I. After 48 h the
medium was removed, each well was washed with PBS, and
trypsin was added to release the cells from the bottom. Then
the trypsin was neutralized with medium, and 20 μl of
Trypan Blue were added. The living cells were counted in a
Neubauer chamber.

The results were analyzed according to their mean log and
respective standard errors (n = 3). Dose–response curves were
plotted and EC50 were calculated by means of non-linear
regression.

For the fluorescence techniques, cells were plated on
coverslips within culture Petri dishes. After 48 h incubating
with either geodiamolides or control, the medium was
removed, and the cells were fixed with formaldehyde (3.7%).
After washing with PBS, the cells were treated with RNAase,
stained with phalloidin-FITC (actin), and propidium
iodide (nuclei). Monoclonal anti-tubulin and secondary anti-
mouse-CY were used in immunofluorescence reactions. The
preparations were mounted on slides with anti-fading (Vecta-
Shield, Vector). The fluorescent images were obtained by
confocal laser scanning microscope (Zeiss LSM 510) with lasers
of argon (458, 488 and 514 nm), helium–neon 1 (543 nm) and
helium–neon 2 (633 nm) connected to an inverted fluorescence
microscope, Zeiss Axiovert 100 M [26].

The effect of geodiamolides A and H (50 ng/ml) on micro-
filaments of T47D cells was also observed at different incubation
times (2, 4, 8, 12 and 48 h), using phalloidin-FITC stained cells
and confocal laser scanning microscope, as described above.

2.5. Normal cells experiments

Primary culture human fibroblasts and BRL3A rat liver
epithelial cells were tested against geodiamolides A and H,
stained with phalloidin-FITC and observed under a confocal
laser scanning microscope, as described above.

3. Results

3.1. Extraction and isolation of compounds

Reversed-phase HPLC purification of Sep Pak Vac prepared
extract fractions GC503 and GC901 yielded four anti-mitotic
peaks (screened in sea urchin eggs), named 1–4. Further mass
spectrometry and NMR analysis of these peaks as well as
comparison with literature revealed that the structures of
GC503/901-1, -2, -3, and -4 were identical to geodiamolides B
(2), A (1), I (4), and H (3), respectively, cyclic depsipeptides
previously described [10,43].

3.2. Biological activities

In the experiments with L. variegatus sea urchin eggs, the
geodiamolides A, B, H and I inhibited the first cleavage in a
dose-dependent form (Fig. 1), and the geodiamolides A and B
acted at much lower concentrations than H and I (Table 1).
Light microscope images of sea urchin eggs revealed a
particularity of the depsipeptides anti-mitotic effect: nuclei
duplications without complete cytokinesis at lower concen-
trations, and cells deformations at high concentration treat-
ment (Fig. 2).

In the breast cancer cells experiments, the values of EC50
for the geodiamolides A, B, H and I were also obtained in
nM range (Table 1). Geodiamolides A and H were more
effective against T47D cells, while geodiamolides B and A
had a stronger effect on MCF7 cells. T47D and MCF7 cells
growth inhibition curves by the compounds are shown in
Fig. 3.

The observation of T47D cells stained for actin filaments
and nuclei in confocal microscope showed that geodiamo-
lides A, B, H and I act upon F-actin, disorganizing the
filaments and gathering them in the cytoplasm, in a dose-
dependent manner (Fig. 4). At the concentration of 100 ng/ml
(135–170 nM), nuclei were displaced from central position in
the cytoplasm and their shape changed when compared to
control cells (Fig. 4). Notwithstanding, microtubule organi-
ization remained unchanged, as observed in immunofluor-

Table 1 – EC50 values of geodiamolides (in nM) tested in L.
variegatus sea urchin eggs, and in T47D and MCF7 human
breast cancer cell lineages

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sea urchin eggs</th>
<th>T47D</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geodiamolide A</td>
<td>101.2</td>
<td>18.82</td>
<td>17.83</td>
</tr>
<tr>
<td>Geodiamolide B</td>
<td>98.8</td>
<td>113.90</td>
<td>9.82</td>
</tr>
<tr>
<td>Geodiamolide H</td>
<td>595.6</td>
<td>38.36</td>
<td>89.96</td>
</tr>
<tr>
<td>Geodiamolide I</td>
<td>620.0</td>
<td>115.30</td>
<td>65.70</td>
</tr>
</tbody>
</table>
Fig. 2 – Light microscope observation of sea urchin eggs first cleavage in the absence of (control (A)) and under treatment with the geodiamolides A ((B) and (C)) (0.1 and 15 μg/ml), B ((D) and (E)) (0.1 and 15 μg/ml), H ((F) and (G)) (0.5 and 50 μg/ml) and I ((H) and (I)) (0.5 and 15 μg/ml).
escence preparations under confocal microscope, showing that the geodiamolides do not disassemble microtubules (Fig. 5).

Disorganization of microfilaments of T47D cells induced by the geodiamolides A and H was perceived within 2 h of the treatment (the first time interval chosen in our experiment), and progressed along the incubation time (Fig. 6).

In our experiments using normal cells lines, only geodiamolide A induced a slight disorganization of the human fibroblasts microfilaments at 100 ng/ml concentration, while geodiamolide H did not cause cytoskeleton alterations (Fig. 7(A)–(C)). The same concentration of both peptides had no effect on rat liver epithelial cells (BRL-3A) F-actin (Fig. 7(D)–(F)).

4. Discussion

Cyclic peptides and depsipeptides are metabolite classes that have not been reported in sponges until recent years. These metabolite classes were described in species of four different orders: Axinellida, Choristida (to which the genus Geodia belongs), Halichondrida and Lithistida. A number of peptides and depsipeptides are characterized by the presence of new amino acids [39]. Depsipeptides, besides presenting unusual amino acids in their molecules, are also characterized by ester bonds and carboxylic acid.

According to Tinto et al. [43], who first described the structures of the geodiamolides H and I, only geodiamolide H presented cytotoxic activity, inhibiting the growth of tumor cells in vitro, while geodiamolide I was completely devoid of activity. Our results with sea urchin eggs (Figs. 1 and 2) showed that both peptides are active under similar concentrations. Additionally, geodiamolides A and B from G. corticostylifera inhibited first division at smaller concentrations than geodiamolides H and I (Fig. 1).

Observation under light microscope of sea urchin eggs showed that at higher concentrations, the geodiamolides induced cell deformation, and at lower concentrations multinucleated cells were present (Fig. 2). Inhibition of sea urchin egg cleavage may occur in several processes in different stages of the cell cycle; for example, DNA, RNA or protein synthesis. Nevertheless, initial cleavage inhibition in these cells is due to either DNA synthesis blockade or inhibition of cytoskeleton protein organization [19], since in the first period of development the embryo does not synthesize RNA once all the mRNA it needs comes from the oocyte [6]. When there is microtubule disorganization, spots corresponding to nuclei duplication are perceptible in the cytoplasm, without having fulfilled mitosis [19]. Moreover, actin filaments sustain the cytoplasm, and form contractile rings during cell division [1]. Thus the effects induced by the geodiamolides in the sea urchin eggs seem to be due to a disorganization of cytoskeleton, specially the microfilaments.

Similar results were found when the macrolides, isolated from an unidentified nudibranch whose chemical structures are similar to swinholide A produced by the Red Sea sponge Theonella swinhoei, inhibited the development of starfish embryos, producing multinucleated cells and unusually shaped nuclei [19]. Also, the diterpenoids of the sponge...
Strongylophora strongylata from Japan inhibited the maturation of starfish oocytes Asterina pectinifera, and this effect may be due to either cyclinB/cdc2 or microtubule assembly inhibition [24].

Though a series of cyclic depsipeptides geodiamolides have been found and described as in vitro cytotoxic substances [10,12,13,15,43], their mechanism of action was not investigated in as much detail as jaspamide (5) [7,8,11,28,31,35].

Fig. 4 – Laser scanning confocal microscope images of T47D cells stained with phalloidin-FITC (actin, green) and propidium iodide (nuclei, red); controls, and after 48 h incubation with the geodiamolides A, B, H and I.
Fig. 5 – Laser scanning confocal microscope images of T47D cells stained with phalloidin-FITC (actin) and Cy-5 (tubulin); controls, and treated with 100 ng/ml of the geodiamolides A, B, H and I.
Fig. 6 – Laser scanning confocal microscope images of T47D cells stained with phalloidin-FITC (actin), treated with 50 ng/ml of geodiamolides A and H and observed at different time intervals: ((A) and (G)) control; geodiamolide A: (B) 2 h, (C) 4 h, (D) 8 h, (E) 12 h, (F) 24 h; geodiamolide H: (H) 2 h, (I) 4 h, (J) 8 h, (K) 12 h, (L) 24 h.
The cytoskeleton is one of the main targets of substances in the tests applied to search for new compounds with potential anti-tumor activity [2,27]. It is known that jaspamide, isolated from the sponge *Jaspis johnstoni*, exerts its cytotoxic activity through actin filament stabilization, competing with phalloidin binding to F-actin [7,8,32]. Other cytotoxic marine depsipeptides, isolated from the sea hare *Dolabella auricularia*, dolastatin 11 [4] and doliculide [3], also stabilize actin filaments, in a way similar to jaspamide. More recently, bistramide A, a cyclic polyether from the ascidia *Lissoclinum bistratum* was found to induce actin depolymerization by direct binding to F-actin [42]. Laser scanning confocal microscope analysis of T47D cells stained for actin and nucleus showed that the geodiamolides A, B, H and I act by affecting the cell microfilaments, disorganizing them and forming aggregates in the cytoplasm, in a dose-dependent manner (Fig. 4). In addition, under high doses the nucleus shapes were altered, as well as their location in the cytoplasm. In the present work α- and β-tubulin...
immunofluorescence was utilized to show that the geodi-
molides do not affect microtubule assembly (Fig. 5).

Usually, the microtubule and microfilament networks
interact during a variety of cellular processes, including
vesicle and organelles transport, cleavage orientation, cell
migration control, mitotic spindle rotation, and nuclear
migration. Thus, substances that specifically affect micro-
tubules or microfilaments may impair the processes accom-
plished by the cooperation of both of them [20]. Since the
geodiamolides A, B, H and I disorganize actin microfilaments,
they must be impairing the mechanisms involved in mitosis,
and may be inducing cell death by apoptosis, like jaspamide
does [11,28,31,35].

The disassembly of microfilaments of T47D cells induced
by the geodiamolides A and H (Fig. 6) was observed within
2 h of the treatment, and progressed along the incubation
time. Jasplamide effect was evident after 2 h incubation, and
continued to grow until 24 h of treatment [8]. Other peptides
can affect the microfilaments in shorter incubation time,
such as doliculide [3] and dolastatin 11 [4], which start to
disrupt actin filaments of kangaroo rat kidney epithelial
cells (PtK1 and 2) within 30 min of the beginning of
treatment. The results of our experiments using normal cells lines
(Fig. 7) are encouraging, considering that at the same
concentration of the geodiamolides 70% of the breast cancer
cells died in culture, and at 25 ng/ml a microfilaments
disorganization was very clear (Fig. 4), thus indicating the
biomedical potential of these marine depsipeptides.

It is noteworthy that the structure–activity relationships
of these compounds seem to vary according to the cell type.
For instance, in sea urchin eggs, geodiamolides A and B are
much more potent than geodiamolides H and I, a difference
which apparently resides in the structural difference of
alanine versus β-tyrosine. In contrast, this structural
difference does not matter with T47D cells, but the halogen
substituent X in the phenol ring of N-methyltyrosine moiety
is crucial in this case because geodiamolides A and H (X = I)
are much more potent than geodiamolides B and l (X = Br).
More interestingly, in case of MCF7 cells, the trend is similar
to that of sea urchin eggs, but distinct from that of T47D
cells, despite the fact that these are mammalian cancer cell
lines similar to T47D cells. Thus, we found that small
structural alteration in geodiamolides largely affects the
rank order of potency in each cell line, indicating that there
would be different cellular sensitivity depending on its
phenotype.

Disruption of cytoskeleton elements such as microtubule
and microfilament has been shown to interfere with the
invasiveness and adhesion of tumor cells during the initial
phases of metastasis formation [22]. New drugs acting in
specific manner of this process may contribute to establishing
new therapeutic approaches focusing on different phases of
tumor progression.

5. Conclusions

The geodiamolides A, B, H and I presented anti-proliferative
activity against breast cancer cells. This effect is related to the
actin depolymerization, as observed in the confocal micro-
scopy analysis of stained cells. Normal cell lines, however, did
not show cytoskeleton alterations after treatment with the
peptides, which is beneficial to the biomedical potential of
such compounds. Interestingly, differences in peptide poten-
cies are associated with an amino acid substitution or with the
presence of bromide or iodide.

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