Effects of *Agelas oroides* and *Petrosia ficiformis* crude extracts on human neuroblastoma cell survival

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Abstract. Among marine sessile organisms, sponges (Porifera) are the major producers of bioactive secondary metabolites that defend them against predators and competitors and are used to interfere with the pathogenesis of many human diseases. Some of these biological active metabolites are able to influence cell survival and death, modifying the activity of several enzymes involved in these cellular processes. These natural compounds show a potential anticancer activity but the mechanism of this action is largely unknown. In this study, we investigated the effects of two Mediterranean sponges, *Agelas oroides* and *Petrosia ficiformis* on the viability of human neuroblastoma cells. Upon treatment with the methanolic extract of *Petrosia ficiformis*, a marked cytotoxic effect was observed at any concentration or time of exposure. In contrast, a time- and dose-dependent effect was monitored for *Agelas oroides* that induced the development of apoptotic features and ROS production in LAN5 cells. These events were suppressed by calpeptin or zVAD and by vitamin C suggesting that the cell death caused by *Agelas oroides* was calpain- and caspase-dependent and of oxidative nature. Comet assay showed that this methanolic extract was not able to produce a genotoxic effect. Future studies will be applied to investigate the effect of isolated bioactive compounds from crude extract of this sponge which are potentially useful for cancer therapeutics.

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Introduction

Sponges (Porifera) are a type of marine fauna that produce bioactive molecules to defend themselves from predators or spatial competitors (1,2). It has been demonstrated that some of these metabolites have a biomedical potential (3) and in particular, Ara-A and Ara-C are clinically used as antineoplastic drugs (4,5) in the routine treatment of patients with leukaemia and lymphoma.

Moreover, *in vitro* studies have attributed to sponge metabolites several biological activities such as antimicrobial, antifungal, antiviral, neurotoxic and cytotoxic properties (6-11). Some of these bioactive compounds are able to influence cell survival and death, modifying the activity of several enzymes involved in these cellular processes. In fact, it has been found that a polyketide quinone compound, isolated from the marine sponge *Xestospongia carbonaria*, inhibits protein tyrosine kinase and is cytotoxic for several malignant cell lines (12). Another enzyme inhibitor, okadaic acid, from the marine sponge *Halichondria okadai*, strongly inhibits a protein phosphatase, inducing cytoskeletal disruption and is a long-known tumour-promoting compound (13,14). Moreover, many authors have attributed to some of these compounds a potential anticancer activity. In particular, Halichondrin B (15-17) has shown a marked *in vitro* and *in vivo* anticancer activity against murine melanoma and leukaemia (18) and Dictyostatin-1, isolated from *Spongia* sp., is cytotoxic for adenocarcinoma and breast cancer cells (19). Laulimalide and Discodermolide, isolated from *Cacospongia mycofijiensis* and *Discodermia dissoluta*, respectively (20,21) have demonstrated tubulin hyperstabilizing properties (22) similar to that of paclitaxel used to cure breast, lung and ovarian cancers (23,24).

The Mediterranean sponge *Agelas oroides* (Schmidt, 1864; Agelasida, Agelasidae) contains pyrrole imidazole alkaloids such as oroidin, clooroidin and taurodispacamide A (25) with a good antihistaminic activity and the brominated compounds agelorin A and B with an antibiotic activity against *Bacillus subtilis* and *Micrococcus luteus* (26).

Petrosia ficiformis (Poiret, 1789; Haplosclerida, Petrosiidae), another Mediterranean sponge, is known to

contain a number of highly toxic and anti-HIV active polyacetylenes such as the petrosyformynes and petrosynol (27), and its cytotoxicity has been demonstrated on human red blood cells (10).

The aim of the present work is to analyse the effect of methanolic crude extracts from the Mediterranean sponge species *A. oroides* and *P. ficiformis* on two human neuroblastoma (NB) cell lines: LAN5 and SK-N-BE(2)-C.

We found that *A. oroides* extract showed a pro-apoptotic effect in LAN5 cells and investigations have been performed to elucidate some molecular mechanisms of this apoptotic pathway.

Materials and methods

Chemicals. Polyvinylidene difluoride (PVDF) membrane, enhanced chemiluminescence Western blot analysis system, and secondary conjugated horseradish-peroxidase antibodies, which binds first rabbit or mouse antibodies, were supplied by Amersham International (Buckinghamshire, UK).

Rabbit polyclonal antibodies reacting with PKCδ and NF-L were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibodies reacting with PKCα and ε were supplied by AbCam (Cambridgeshire, UK) and anti-α fodrin was from Chemicon International (Germany). Goat anti-rabbit FITC-conjugated antibody was supplied by Upstate cell signaling solutions (NY, USA). The Annexin V-FITC apoptosis detection kit was from Biovision Research Products (Mountain View, CA, USA). Protein G-sepharose and dithiothreitol (DTT) were supplied by Pharmacia Biotech (Uppsala, Sweden). [γ-^{32}P]-ATP (specific activity 3,000 Ci/mmol) was from Perkin-Elmer Life Sciences. All other reagents were from Sigma Chemicals Co. (St. Louis, MO, USA).

Sponge collection and extraction procedure. Some individuals of the Mediterranean sponges *Agelas oroides* and *Petrosia ficiformis* were collected during Winter 2003 from Portofino's Promontory (Ligurian Sea, Italy) from depths of 10 to 20 m and then frozen at -20˚C. Samples were then thawed and placed in 0.9% NaCl at room temperature. Sponges were then homogenised and centrifuged at >10,000 rpm for 30 min. The supernatant was filtered, lyophilised and frozen again at -20˚C. Samples were extracted with methanol (1:1) on a stirrer for 3 h or overnight. The supernatant was filtered through a ceramic filter and the solid material was placed again in the solvent for the second and third extraction. The supernatant was then dried using a Rotovapor at 40˚C with 5-10 ml of distilled water to obtain the crude extract of the two sponge species.

Cell culture. Our experimental model was represented by two human neuroblastoma cell lines: LAN5 and SK-N-BE(2)-C. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% penicillin/streptomycin, 1% sodium piruvate, 1% non-essential aminoacid solution, and 1% antimycotic solution. Cells were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 and were splitted and seeded in new flasks (75 cm^2) every two days to maintain them in log-phase. Cells were treated for 15 and 30 min with 5, 10, 20 and 50 ppm of *A. oroides* or *P. ficiformis* methanolic crude extracts. Some samples of LAN5 cells were pre-treated for 30 min with 250 μM vitamin C, 10 μM calpeptin, 20 μM z-VAD-fmk.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. This assay is based on the ability of Annexin-V to bind to the phosphatidylserine exposed on the surface of cells undergoing apoptosis and the capacity of propidium iodide to enter cells that have lost their membrane integrity (28). Cells were grown and treated on chamber slides (Iwaki Seiyaku Co., Tokyo, Japan), the medium was discarded, and the cells were incubated in the dark for 5 min at room temperature with 200 μl of 1X binding buffer, 0.5 μg/ml FITC-labelled recombinant Annexin-V, and 0.5 μg/ml propidium iodide. The cells were observed and counted (5-fields of ~60 cells) under a fluorescence Leica DIMRB microscope (Leica Microsystems AG, Wetzlar, Germany) using a dual filter set for FITC and rhodamine. To evaluate apoptotic process, we considered the percentage of Annexin-V-positive/propidium iodide-negative cells.

Measurement of reactive oxygen species (ROS) production. After sponge extract exposure, cells were incubated for 20 min at 37˚C with 20 μM 2'-7'-dichlorofluorescein-diacetate (DCFH-DA), a cell-permeable, nonfluorescent precursor of DCF that can be used as an intracellular probe for oxidative stress (29,30). Accumulation of DCF in the cells was measured by an increase in fluorescence at 530 nm when the sample was excited at 485 nm. Observations were made with a Leica DIMRB microscope, and a standard set of filters for fluorescein (excitation 460-500 nm, emission 510-560 nm) was used.

NF-L detection. After treatments cells were fixed for 15 min in 4% paraformaldehyde (PFA) in 10 mM PBS. Then, cells were permeabilized with 0.1% Triton X-100 in PBS, and washed three times in 10 mM PBS. Anti-NF-L primary antibody (1:200 dilution in PBS) was added and incubated at room temperature. After washing in PBS, FITC-conjugated secondary antibody was added (1:300) and the cells were incubated for 30 min in a humidified atmosphere. Slides were mounted with Mowiol 4-88 (Calbiochem, Darmstadt, Germany) and analyzed by Leica DIMRB microscope, using 60x oil-immersion objective.

Single cell gel electrophoresis (Comet assay). We used formamidopyrimidine DNA glycosylase (Fpg)-modified comet assay (31) to evaluate DNA oxidative damage. This test employes the Fpg enzyme, a glycosylase that recognizes and specifically cuts the oxidized bases, principally 8-oxo-guanine, from DNA producing apurinic sites converted in breaks by the associated AP-endonuclease activity. The procedure of Tice and Strauss (32) has been followed with minor modifications. The comet assay protocol was carried out under dim light to prevent any additional DNA damage. After treatments, cells were trypsinized and cell suspensions ($15x10^3$ cells) were mixed with low melting point agarose (0.5% in PBS) and spread on slides with a thin layer of normal melting point agarose (1.5% in PBS). The slides were washed three times in enzyme buffer (50 mM Na_3PO_4, 10 mM EDTA, 100 mM

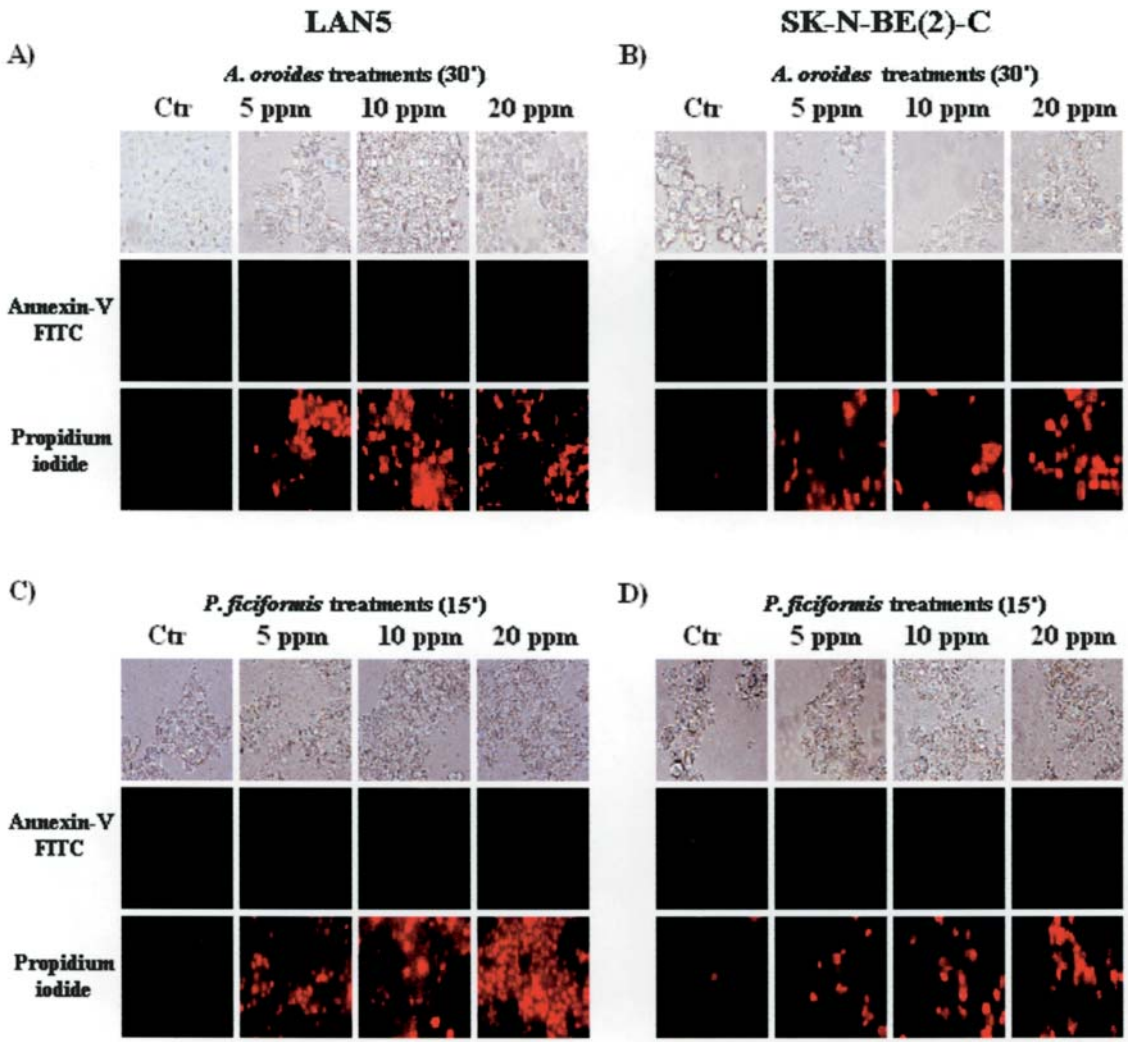

Figure 1. *A. oroides* and *P. ficiformis* methanolic crude extracts differentially influenced human neuroblastoma cell viability. Apoptotic or necrotic changes were tested by fluorescence microscopy in LAN5 (A and C) and SK-N-BE(2)-C cells (B and D). Panels A and B show NB cells treated for 30 min with 5, 10 and 20 ppm of *A. oroides* extract. Panels C and D show NB cells treated for 15 min with 5, 10 and 20 ppm of *P. ficiformis* extract. Apoptosis or necrosis were assessed by counting the number of Annexin-V- or propidium iodide-positive cells. The lower subpanels show representative images obtained by fluorescence microscopy analysis of propidium iodide-positive cells in all conditions of treatment. The middle subpanels are representative of images obtained by fluorescence microscopy analysis of Annexin-V-FITC-positive cells. The upper subpanels show images of cells observed by standard filters.

NaCl, pH 7.5), drained and incubated with 50 μl of either buffer or Fpg (1 μg/ml in enzyme buffer) in the dark for 30 min at 37˚C. The slides were placed in a horizontal gel box near the anode end, and covered with electrophoretic buffer (300 mM NaOH, 1 mM EDTA, pH>13.0); after 30 min, slides were subjected to an electric field of 300 mA for 40 min. Finally, slides were coated with neutralisation buffer (0.4 M Tris-HCl pH 7.5), dried and incubated for 10 min in absolute ethanol. Slides were then stained with 50 μl 1X ethidium bromide staining solution, covered with a cover slip and analysed by means of a fluorescence microscope (Leica DIMRB) with an excitation filter of 515-560 nm and a barrier filter of 590 nm.

Total protein extraction. After washing, cells were treated with a lysis-buffer containing protease inhibitors and 0.25% Triton. Cells were detached and then collected in tubes, sonicated and finally centrifuged at 40,000 rpm for 30 min at 4˚C. Total proteins were determined by means of the Lowry method and using bovine serum albumin as standard.

Immunoblot analysis. Proteins (50 μg) were denatured in 3.5X Laemmli buffer and then subjected to 7% and 8% SDS-polyacrylamide gel electrophoresis for α-fodrin and PKCs respectively, followed by electroblotting (100 V for 1 h) onto a PVDF membrane. Subsequently, the PVDF membrane was washed in distilled water and marked with Ponceau solution to verify the blotting. Immunodetection was performed using specific antibodies. After incubation with secondary antibody, the immunoblots were detected by means of an enhanced chemiluminescence system. Changes in protein levels were estimated by densitometric analysis.

PKC activity assay. Classic and novel PKC isoforms were immunoprecipitated with specific antibodies and protein G-sepharose using 50 μg of protein sample. The beads were washed three times in a washing buffer containing 10 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM DTT. The activity assay of classic isoenzymes was performed by adding 15 μl of washing buffer supplemented with 0.1 mM ATP, [γ-^{32}P]ATP (2 μCi per sample), 1 μg of phosphatidyl-

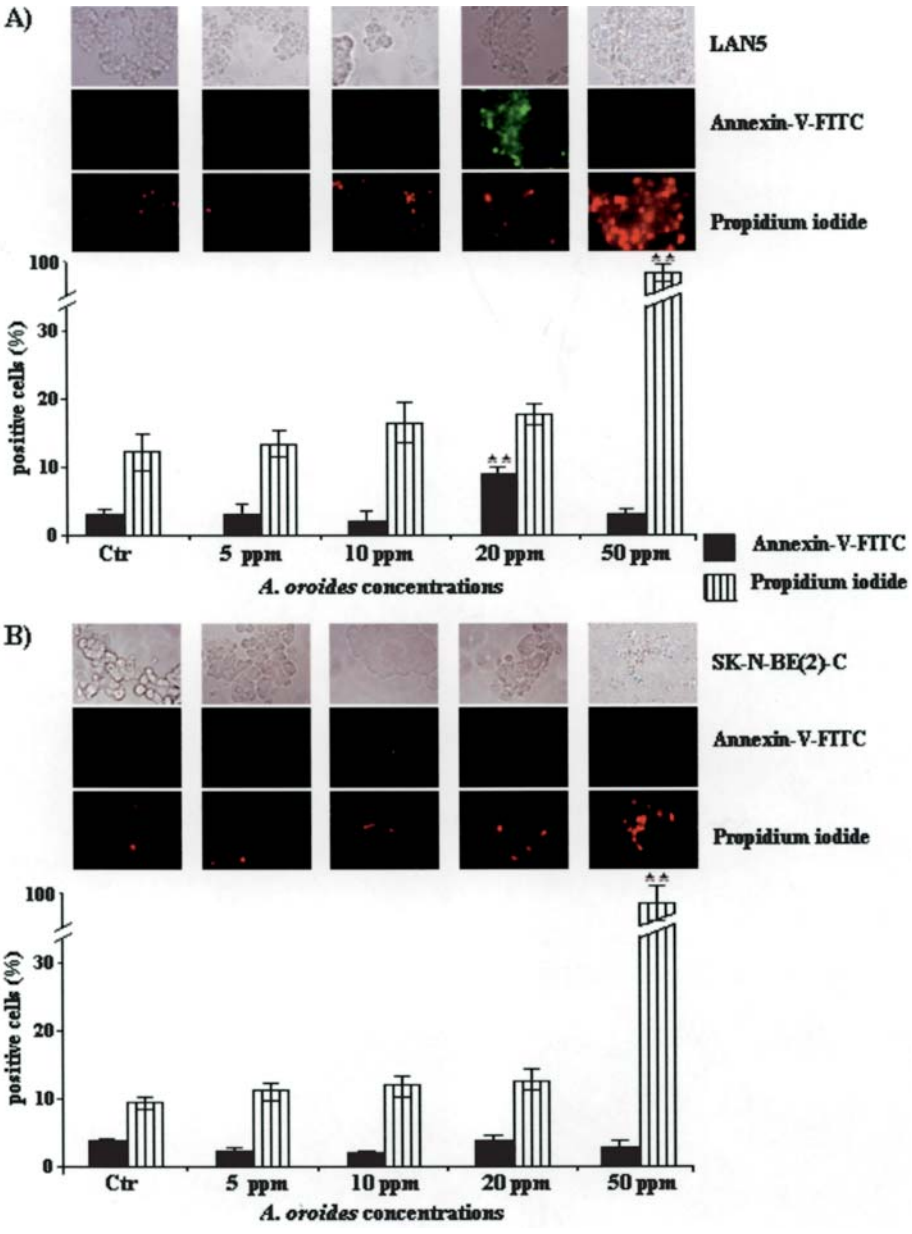

Figure 2. The 20 ppm concentration of *A. oroides* extract induced apoptosis of LAN5 cells. Apoptosis and necrosis were tested by fluorescence microscopy in LAN5 (A) and SK-N-BE(2)-C cells (B). Neuroblastoma cells were treated for 15 min with 5, 10, 20 and 50 ppm of *A. oroides* extract. Apoptosis and necrosis were assessed by counting the number of Annexin-V- and propidium iodide-positive cells. The lower panels show representative images obtained by fluorescence microscopy analysis of propidium iodide-positive cells in all conditions of treatment. The middle panels are representative of images obtained by fluorescence microscopy analysis of Annexin-V-FITC-positive cells. The upper panels show images of cells observed by standard filters. The histograms are representative of three independent experiments. $^{**}p<0.01$ vs. control (Ctr).

serine, 0.4 μg of dioleoylglycerol, 0.5 mM $CaCl_2$ and 10 μg of H1 histone as substrate (33). A reaction mixture without calcium was used to evaluate the activity of novel PKC isoforms. The reaction was carried out at 30˚C for 10 min using histone as a substrate, then stopped by adding 3.5X Laemmli buffer. The reaction mixtures were loaded onto 12.5% SDS-polyacrylamide gel, which was dried and then exposed to an autoradiographic film for 24 h at -80˚C. The relative intensity of phosphorylated substrates was measured by densitometric analysis.

Statistical analysis. Results were expressed as mean ± SD from at least three independent experiments. The statistical significance of parametric differences among sets of experimental data was evaluated by One-way ANOVA and Bonferroni's test for multiple comparisons.

Results

A. oroides and P. ficiformis methanolic crude extracts differentially influence human neuroblastoma cell survival. To evaluate the impact of *A. oroides* and *P. ficiformis* methanolic crude extracts on cell survival, we have treated two human neuroblastoma cell lines, LAN5 and SK-N-BE(2)-C, with 5, 10 and 20 ppm of the sponge extracts for 15 and 30 min.

The concentrations of 5, 10 and 20 ppm of *A. oroides* were highly cytotoxic for both cell lines exposed for 30 min (Fig. 1A and B) and these effects were compared to those

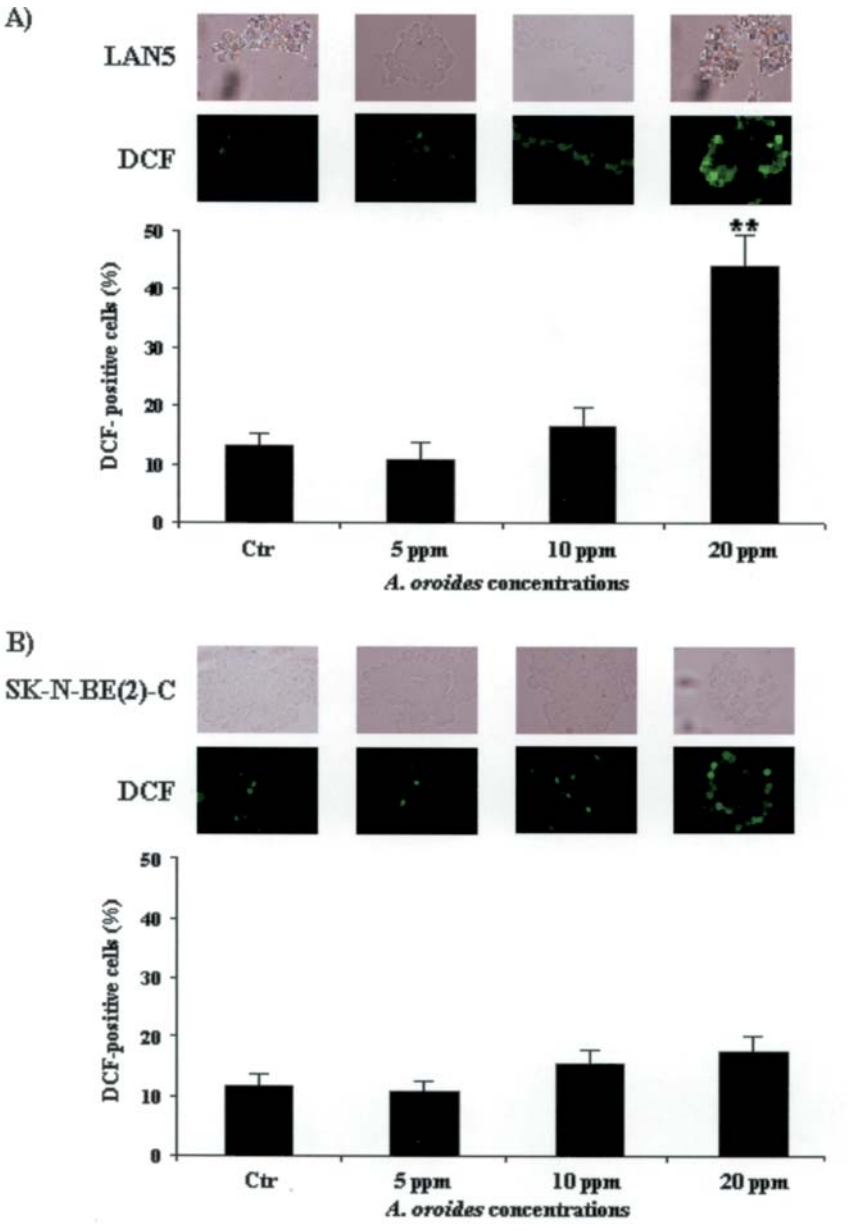

Figure 3. *A. oroides* extract increased ROS production in LAN5 cells. ROS levels were measured by fluorescence microscopy in LAN5 (A) and SK-N-BE(2)-C (B) untreated cultures and in cells after 15 min of incubation with different concentrations (5-20 ppm) of *A. oroides* extract. ROS generation was estimated by counting the number of DCF-positive cells. The lower panels show representative images obtained by fluorescence microscopy analysis of DCF-positive cells in all conditions of treatment. The upper panels show images of cells observed by standard filters. The histograms are representative of five independent experiments. Data are given as mean ± SD. $^{**}p<0.01$ vs. control cells compared by ANOVA, followed by the Bonferroni's test.

determined by *P. ficiformis* which had already induced necrosis of both NB cell lines after 15 min of treatment (Fig. 1C and D). As expected, a marked cell death of these NB cells was found also after 30 min treatment with *P. ficiformis* (data not shown).

Interestingly, we observed in both cell lines a time- and dose-dependent effect of *A. oroides* extract after 15 min of cell exposure (Fig. 2): in particular, 10 and 20 ppm concentrations induced a light increase of necrotic cells versus the basal conditions, while 50 ppm induced a marked enhancement of propidium iodide-positive cells comparable to that observed at the longest time (30 min) of treatment with 20 ppm.

In addition, while no changes in apoptotic marker were detected in SK-N-BE(2)-C cells (Fig. 2B), a 20 ppm concentration of *A. oroides* extract induced a 1.5-fold increase (vs. control) in Annexin-V-positivity of LAN5 cells (Fig. 2A).

A 20-ppm concentration of A. oroides extract causes ROS overproduction in LAN5 cells. It has been widely demonstrated that sponge crude extracts can modulate cell oxidative state (34,35) and for this reason we monitored ROS levels by measuring the number of DCF-positive cells.

Both NB cells were characterised by basal ROS production (12-13%) and 15 min exposure to 5 ppm sponge extract did not change the control redox state. The concentration of 10 ppm induced a light enhancement of ROS levels (Fig. 3) that became 2.5 fold increased after exposure of LAN5 to 20 ppm (Fig. 3A).

ROS overproduction and apoptosis of LAN5 cells is prevented by vitamin C or calpain or caspase inhibition. The percentages of DCF-positive and Annexin-V-positive LAN5 cells exposed

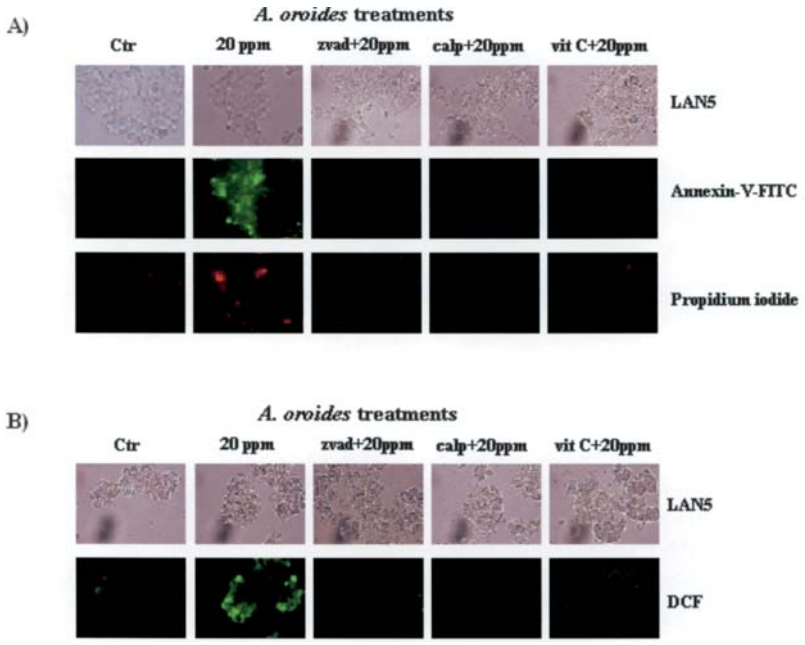

Figure 4. Pre-treatments of LAN5 cells with calpain or caspase inhibitors or with vitamin C totally prevented cell death and ROS overproduction induced by *A. oroides* extract. Apoptosis, necrosis (A) and ROS generation (B) were evaluated by fluorescence microscopy in untreated cultures of LAN5, in cells after 15 min incubation with 20 ppm of *A. oroides* and in cultures pre-treated for 30 min with 10 μM calpeptin, or 20 μM z-VAD-fmk or 250 μM vitamin C. Apoptosis and necrosis were assessed by showing Annexin-V- and propidium iodide-positive cells. ROS generation was estimated by showing DCF-positive cells. In panel A the lower subpanels are representative images obtained by fluorescence microscopy analysis of propidium iodide-positive cells. The middle subpanels are representative images obtained by fluorescence microscopy analysis of Annexin-V-FITC-positive cells. The upper sub-panels show cells observed by standard filters. In panel B the lower sub-panels are representative images obtained by fluorescence microscopy analysis of DCF-positive cells in all conditions of treatment. The upper subpanels show cells observed by standard filters.

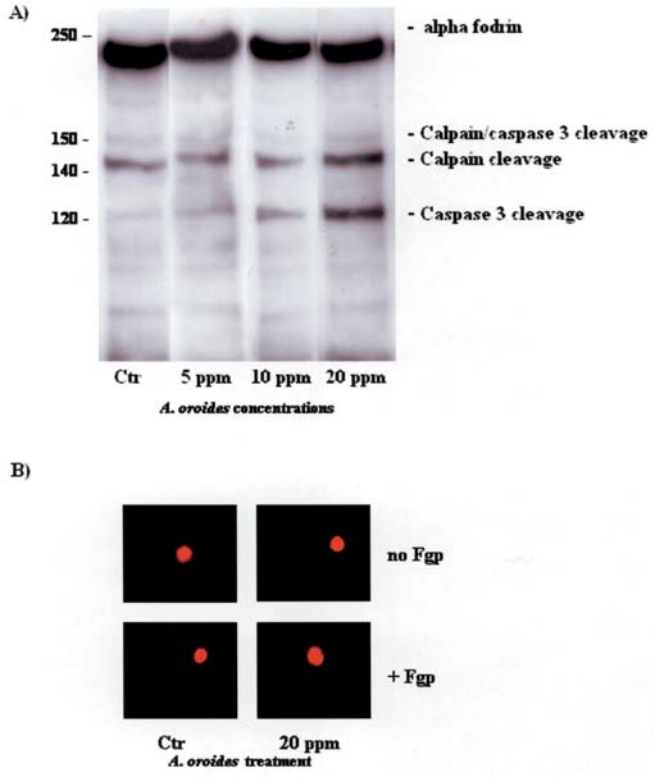

Figure 5. *A. oroides* extract induced the activation of calpain- and caspase-dependent pathways without oxidative or non-oxidative DNA damage. LAN5 cells were treated for 15 min with 5, 10 and 20 ppm of methanolic crude extracts of *Agelas oroides*; then, cells were harvested and protein extracts were subjected to immunoblot analysis. Panel A shows the determination of calpain and caspase activities by analysis of α-fodrin. The immunoblot shown is representative of three independent experiments. Panel B shows fgp-modified comet assay in LAN5 cells treated for 15 min with 20 ppm of *A. oroides*.

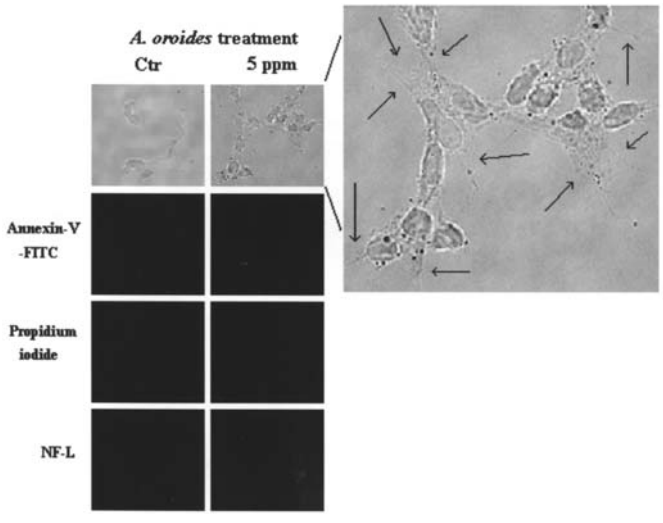

Figure 6. The 5-ppm concentration of *A. oroides* extract induced morphological changes in LAN5 which are not associated to apoptosis, necrosis or NF-L labelling. Apoptotic or necrotic changes were assessed by showing Annexin-V or propidium iodide staining, the NF-L labelling was tested by fluorescence microscopy analysis. The first panels from the top are representative images of cells observed by standard filters, the second and third panels are representative images of Annexin-V and propidium iodide staining respectively. The fourth panel shows images of NF-L labelling.

to 20 ppm of *A. oroides* extract were drastically suppressed by 250 μM vitamin C or 10 μM calpeptin, a calpain inhibitor or 20 μM zVAD, a pan-caspase inhibitor (Fig. 4).

A. oroides extract induces apoptosis without DNA oxidative damage. To confirm the apoptotic process induced in LAN5

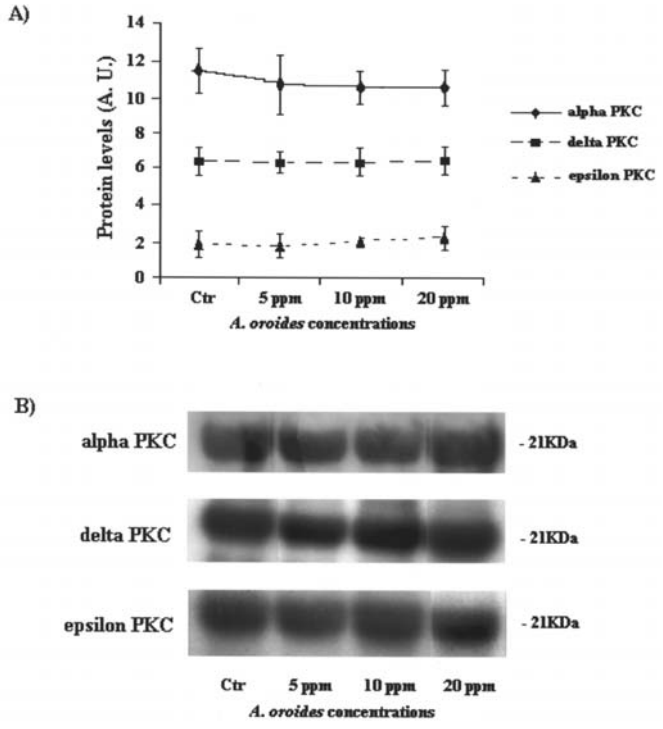

Figure 7. *A. oroides* extract did not modify PKC expression levels and functional activities. LAN5 cells were treated with *A. oroides* extract at different concentration (5, 10, 20 ppm); cells were then harvested and protein extracts were subjected to immunoblot analysis (A) and activity assay (B) of PKC isoforms. Panel A shows the graph of PKCα, δ and ε protein levels obtained after densitometric analysis of immunoblots. The values are the mean ± SD of three determinations. Panel B shows the autoradiographs of H1 histone phosphorylated by PKCα, δ and ε. The autoradiographs shown are representative of three independent experiments.

cells exposed to 20 ppm of *A. oroides* extract we have measured the expression levels of α-fodrin. This cytoskeletal substrate can be degraded by caspase 3 into smaller fragments of 150 and 120 kDa and by calpain into fragments of 150 and 145 kDa (36,37). In the present study conditions, a 20 ppm concentration caused a marked formation of the fragments of 145 and 120 kDa as consequence of calpain and caspase cleavage (Fig. 5A). Comet assay showed that apoptosis of LAN5 cells was not accompanied by oxidative or non-oxidative DNA damage (Fig. 5B).

A 5 ppm concentration of A. oroides extract causes membrane alterations in LAN5 cells. Interestingly, 5 ppm induced the formation of some membrane extensions in LAN5, whose phenotype was associated neither to Annexin-V-FITC staining nor propidium iodide labelling (Fig. 6). To investigate whether these membrane extensions were the result of neuronal differentiation, the expression level of NF-L was examined and we found that this event was not accompanied by NF-L labelling (Fig. 6).

A. oroides does not influence PKC expression levels and activities. The expression levels of PKC-α, -δ and -ε, whose protein levels are more expressed in this kind of tumour, were not significantly modified after sponge extract treatment (Fig. 7A). Enzymatic activities of the three isoforms tested were not affected by *A. oroides* extract treatment (Fig. 7B).

Discussion

Considering the pharmaceutical potential of many bioactive molecules, a wide number of studies have been carried out to examine the effects of marine sponge crude extracts and isolated molecules on several kinds of tumoural cell lines as suitable bioassays. Screening of extracts from marine sponges is a common approach to identify possible compounds of biomedical importance (38,39).

In the present study, the effects of the methanolic crude extracts of *A. oroides* on SK-N-BE(2)-C and LAN5 human NB cell lines have been investigated and compared to those produced by *P. ficiformis*. We have found that 10 and 20 ppm concentrations of *A. oroides* or *P. ficiformis* extracts were able to induce a marked cell death of NB lines but at quite different times of exposure.

In fact, comparing the impact of the same concentrations of the two sponge extracts on NB cells tested, we have observed that the cytotoxic effect induced by *P. ficiformis* was already evident after 15 min and at lower concentration (5 ppm); while, a similar effect was appreciable for *A. oroides* only after 30 min of treatment.

Moreover, *A. oroides* had a time- and dose-dependent cytotoxic effect inducing an increase in apoptosis of LAN5 cells after 15 min of exposure to 20 ppm.

Recently, it has been reported that Petrotetrayndiol A, a polyacetylene isolated from the sponge *Petrosia* sp., induced caspase-dependent apoptosis in SK-MEL-2 human melanoma cells (40), Lembehyne A, from *Haliclona* sp., led differentiation of Neuro 2A cells (41) and Labuanine A, from *Biemna fortis*, caused in Neuro 2A cells multipolar neuritogenesis and arrested cell cycle at the G2/M phase (42). Many studies in the scientific literature have therefore investigated the effects of isolated molecules from sponge extracts on murine and less frequently on human neuroblastoma cells (43).

Our previous reports have demonstrated that the apoptotic process of human NB cells is strictly related to intracellular ROS overproduction (44) and also in this context, we have found that a 2.5-fold increase of ROS levels was accompanied by the appearance of apoptotic features in LAN5 cells treated with 20 ppm of *A. oroides*.

The present study shows that the apoptosis of LAN5 triggered by *A. oroides* extract is of oxidative nature, since the prevention of this phenomenon is obtained after pretreatment with antioxidant vitamin C. Moreover, these apoptotic features are abolished by a calpain or caspase inhibitors, which are also able to counteract ROS generation.

Inhibition of caspase activation has been recently demonstrated to block ROS production (45), suggesting that the generation of oxidant species during apoptosis may be caspase-dependent.

Therefore, our results demonstrate that 20 ppm of *A. oroides* induces calpain- and caspase-dependent apoptosis of LAN5 cells and these findings are confirmed by the cleavage of α-fodrin that leads the formation of 120 and 145 kDa fragments, markers of calpain and caspase 3 activation (36,37).

A. oroides-induced oxidative death of LAN5 cells is not accompanied by DNA oxidative damage thereby suggesting a potential anticancer activity of this extract since it shows apoptotic but not genotoxic properties. In fact, we observe

in LAN5 cells the translocation of phosphatidylserine to the outer cell membrane and the proteolytic activation of caspases as marker events of apoptosis but we don't detect any DNA single-strand breaks.

On the other hand, the lower and not cytotoxic concentration (5 ppm) of *A. oroides* induced morphological changes in LAN5 cells exposed for 15 min and these membrane extensions were not associated to apoptosis or necrosis.

Many authors have reported that long-time treatment (24-48 h) with several sponge crude extracts induces alterations of cell morphology and microtubule organization (41,42,46). In the present conditions, membrane alterations were a more precocious event than that observed by other studies (18,42); for this reason, we hypothesize that *A. oroides* extract might specifically modify cytoskeletal proteins but further analyses are needed. Certainly, we have excluded that these morphological changes are the result of neuronal differentiation because the event is not characterized by NF-L presence.

Most bioactive metabolites isolated from sponges are inhibitors of key enzymes, which mediate or produce mediators of intracellular or intercellular messengers involved in the pathogenesis of human diseases (12,14,47).

It has been demonstrated that some isolated sponge compounds are inhibitors of PKCs (48) and PKC inhibitors have attracted interest worldwide as there is evidence that high levels of PKC enzyme are involved in the pathogenesis of many human diseases and in tumour development (49).

Our previous studies have demonstrated that PKC isoforms are specifically affected by intracellular ROS generation and apoptosis of NB cells was mediated by an increase in the activity of δ and a concomitant inactivation of α isoform (50).

In the present study, no changes were observed in protein levels and activities of PKC isoenzymes mainly expressed in neuroblastoma. Additional study is in progress to identify the action of single bioactive compounds from the methanolic crude extract of *A. oroides* responsible for the morphological changes observed in LAN5 cells. These studies might provide evidence of marine sponges as a promising therapeutic approach for this kind of tumour.

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