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Evaluation of genotoxic biomarkers in extracts of marine sponges from Argentinean South Sea

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ABSTRACT

Marine sponges are a rich source of structurally and biologically active metabolites of biomedical importance. We screened polar and non-polar samples of crude extracts obtained from marine sponges collected in different locations of Argentinean south sea coast, as a novel approach for their characterization.
The evaluation was performed using cytotoxic and genotoxic biomarkers such as mitotic index (MI), cell proliferation kinetics (CPK) and sister chromatid exchanges (SCE), monitored *in vitro* using peripheral blood lymphocytes. Statistical analysis was performed using two-way analysis of variance (ANOVA). The extracts evaluated belonged to: *Callyspongia flabellata* (BURTON, 1932) *(Callyspongiidae)*; *Plicatellopsis sp.(Suberitidae)*; *Callyspongia fortis* (RIDLEY, 1881) *(Callyspongiidae)*; *Clathria (Microciona) antarctica* (TOPSENT, 1917) *(Microcionidae)*; *Spongia* (Spongia) *magellanica* (THIELE, 1905) *(Spongiidae)*; *Halicnemia papillosa* (THIELE, 1905) *(Axinellidae)*; *Cliona chilensis* (THIELE, 1905) *(Clionidae)*; *Haliclona sp.* 1; *Haliclona sp.* 2*(Chalinidae)*.
Genotoxicity studies revealed that the evaluated sponge extracts did not exhibit cytotoxic activity measured from mitotic index MI and cell proliferation kinetics(CPK). In contrast, sister chromatid exchanges (SCE) showed that the non-polar extract of *Callyspongia fortis* and the polar extract of *Cliona chilensis* presented significant differences in SCE frequency ($p<0.001$), when compared with control cultures. These results emphasize the need to set up a standard battery of "in vitro" genotoxicity testing for new chemicals, pharmaceutical and drugs.

1. Introduction

Marine natural products provide a source of valuable chemical diversity that can be used to design and develop new and potentially useful therapeutic agents. Pharmaceutical interest in sponges was aroused in the early 1950's by the discovery of nucleosides in the marine sponge *Cryptotethya crypta* (Bergmann and Feeney, 1950, 1951). The unknown nucleosides spongothymidine and spongouridine were the basis for the synthesis of 1-β-d-arabinofuranosyl cytosine (ara-C) as anticancer agent currently used in the routine treatment of patients with leukaemia and lymphoma and the antiviral drug 1-β-d-arabinofuranosyl adenine (ara-A) used for herpes simplex virus infection (Proksch et al., 2002).

Most bioactive compounds in advanced pre-clinical testing can be classified as anti-inflammatory, antitumor, immunosuppressive or neurosuppressive, antiviral, antimycotic, haemolytic, hemaglutinating, ichthyotoxic, analgesic, antiprotozoan, antimalarial, nematocidal, anti-angiogenic, antiacetilcholinesterase, antibiotic, antifouling, anti-asthmatic and others. However, some of them may cause side effects due to their low selectivity (Bjarnason et al., 1993; Borchard, 1998; Rahden-Staron, 2002; De Rosa, 2002; Rifai et al., 2005).

The marine environment displays an enormous biodiversity, and sponges show a circumpolar distribution and inhabit all seas. South America's Brazilian coast sponges, were studied by screening extracts for novel pharmaceuticals (Rangel et al., 2001; Monks et al., 2002) and cytotoxicity studies through "in vitro" assays were developed (Scuteri, 2002; Prado, 2002; Torres et al., 2002; Aiub et al., 2002).

In Argentina, especially in the South Atlantic, Porifera biodiversity knowledge is fragmentary, particularly the continental shelf of Rio Negro and Chubut Provinces (López Gappa and Landoni, 2005) and remains chemically unexplored. Novel alkaloids were found from

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Cliona sp (Palermo et al., 1996), *Cliona chilensis* (Palermo et al., 1998) and *Clathria sp* extracts (Zuleta et al., 2002).

It is well a known fact that physical, chemical and biological agents can act, directly or indirectly, over individual genetic information, generating shifts in cellular function, which are in turn responsible for medium or long term effects on health. Since bioactive compounds of marine sponge extracts have been evaluated only through cytotoxicity assays (usually cell death), it is from this perspective that the analysis of these extracts and their influence in genome constitution becomes relevant, and genetic and cytotoxic biomarkers are excellent tools to aid in this analysis (Grandjean, 1995).

The aim of this work is the evaluation of organic and aqueous extracts from nine sponge species from Argentinean south sea coast in order to know their potential genotoxic effect using a non-cytotoxic concentration on peripheral blood lymphocytes against biological endpoints such as mitotic index (MI), cell proliferation kinetics (CPK) and sister chromatid exchange (SCE).

2. Materials and methods

2.1. Collection and extraction procedures

Nine specimens of marine sponge were collected during the 2006 campaign by wading at low tide and scuba diving at a depth of between 0 and 10 m in Rio Gallegos, Puerto Deseado and Playa Ciega, Santa Cruz Province and Punta Pardelas, Península de Valdez, Chubut Province, Argentina. The samples were stored in ethanol 70% until processed. The sponges were ground up and extracted with EtOH (500 ml). The extracts were filtered and the residue extracted once again at room temperature with EtOH (500 ml) and EtOAc (500 ml). The combined extracts were evaporated and the resulting aqueous suspension was exhaustively extracted with EtOAc. The organic extract was partitioned between cyclohexane and MeOH/H_20 (9:1) yielding lypophyllic (cyclohexane layer) and polar (aqueous Me0H layer) extracts. Voucher specimens were deposited at MNRJ - Porifera collection of Museu Nacional of Universidade Federal do Rio de Janeiro, Rio de Janeiro and at MCN-POR - Porifera collection of Museu de Ciências Naturais, Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, Brazil.

2.2. Lymphocyte Cultures from Human Peripheral Blood

Heparinized blood samples were obtained from two healthy donors with no recent history of exposure to mutagens. Briefly, 0.8 ml of each of the blood samples were placed in a sterile flask containing 7.5 ml RPMI culture medium supplemented with 1.5 ml foetal bovine serum (Gibco), 0.1 ml phytohemagglutinin (PHA) (Gibco) and 0.1 ml bromodeoxyuridine (BrdU) (Sigma). Then, 0.1 ml sponge extracts were added at the beginning of the cultures, which were then incubated for 72 h at 37 °C. The chosen concentration was performed considering a cytotoxicity screening that we developed in peripheral blood lymphocytes through mitotic index evaluation. The experimental design included from 1 to 48 μg/ml and we found that 6 μg/ml culture was the first assayed dose where we can analyze the chosen biomarkers (sister chromatid exchanges, cell proliferation kinetics and mitotic index) with good response and resolution.

Negative control was developed by adding distilled water (100 ul) to each donor's culture. Two hours before harvesting eliminate, 0.2 ml of Colcemid (10 μg/ml; Sigma) was added to each culture flask. For harvesting, cells were centrifuged at approximately 800-1000 rpm for 10 min. The supernatant was removed and 5 ml of a pre-warmed (37 °C) 0.075 M KCl hypotonic solution were added. Cells were re-suspended and incubated at 37 °C for 45 min. The supernatant was removed by centrifugation, and 5 ml of a fixative (methanol:glacial acetic acid, 3:1) were added. The fixative was removed and the procedure was repeated twice. To prepare the slides, five drops of the fixed cell suspension were dropped on clean glass slides and air-dried. Cells were stained following a modified Fluorescence plus Giemsa (FPG) technique (Perry and Wolff, 1974). Slides were stained for 20 min in a 0.05% (w/v) Hoechst 33258 solution, rinsed with tap water and placed under a near-UV lamp for 90 min, covered with Sorensen's buffer, pH 6.8, and stained with a 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min.

2.3. Microscopic Evaluation

2.3.1. Sister Chromatid Exchanges (SCE)

The frequency of Sister Chromatid Exchanges (SCE) was counted in 50 metaphases, all with 46 centromeres per sponge polar and non polar extract. The results were expressed as the frequency of SCE per metaphase.

2.3.2. Mitotic Index (MI)

The mitotic index was estimated as the proportion of mitotic cells in 2000 cells counted for each preparation sponge polar and non polar extract and donor in Giemsa stained slides (Miller and Adler, 1989).

2.3.3. Cell Proliferation Kinetics (CPK)

The proportion of first (M_1), second (M_2) and third (M_3) division cells was scored in 100 consecutive metaphases from each duplicate 72 hr culture for each experimental point. Replication index (RI) was calculated as: $RI=(M_1 x\ 1)+(M_2 x\ 2)+(M_3 x\ 3) / 100$ (Rojas et al., 1993).

2.3.4. Statistical Analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) randomised block design for each biomarker (Graphpad Instat) and Mann Whitney U-test for not paired samples with two tails. A statistically significant difference was set at $P<0.05$.

3. Results

Most screenings of secondary metabolites of biomedical importance from marine sponge extracts reported an inhibitory effect that turned out to be strongly cytotoxic in our system. Taking into account these findings, we developed our screening with several dose extracts and selected 6 μg/ml as chosen dose in our experimental design because it showed no cytotoxic effect in peripheral blood lymphocytes.

When potential cytotoxicity was analyzed through mitotic index and cell proliferation kinetics in the non polar extracts of *C. flabellata, Plicatellopsis sp., C. fortis and Clathria antarctica*, we could not find significant modifications after treatment on lymphocytes cultures. However, SCE frequency analysis showed that *C. fortis* extract presented a statistically significant increase when compared with control values ($p<0.04$) (Fig. 1).

Non polar extracts of *Spongia magellanica, Halicnemia papillosa, Cliona chilensis, C. fortis, Haliclona sp 1 and 2*, did not induce modifications either in mitotic index or in cell proliferation kinetics or SCE, when compared with control cultures (Fig. 2).

When we developed the same protocol for polar extracts (Fig. 3), we could observe no cytotoxic effect as well as no modifications in cell proliferation kinetics; however, *Cliona chilensis* extract was considerably more active since a significant increase ($p<0.0005$) in SCE frequency was observed when chromosomal instability was measured.

4. Discussion

The progress in molecular and cell biology in the last decades has enabled a rational exploitation of the natural resources of the secondary metabolites and biomaterials from sponges and their associated micro-organisms.