Cytotoxicity and Inhibition of Lymphocyte Proliferation of Fasciculatin, a Linear Furanosesterterpene Isolated from Ircinia variabilis Collected from the Atlantic Coast of Morocco

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Abstract: Fasciculatin, a furanosesterterpene isolated from the marine sponge Ircinia variabilis from the Atlantic Coast of Morocco, has been evaluated for its influence on a mitogen-induced proliferation of human lymphocytes and growth of human tumor cell lines.

Keywords: Porifera, Ircinia variabilis, cytotoxicity, human tumor cell lines, inhibition of lymphocyte proliferation.

Introduction

Linear sesterterpenes incorporating furanyl and tetronic acid termini are characteristic of a large number of metabolites isolated from the marine sponges especially of the genera Ircinia, Sacrotragus,
and Psammocinia [1, 2, 3, 4]. These furanosterterpenes have been shown to possess a wide range of biological activity including antiviral, antibacterial, anti-inflammatory, antitumor, and protein phosphatase inhibitory activity [4, 5].

In the course of our investigation on bioactive secondary metabolites from the marine organisms from the Atlantic Coast of Morocco, we have isolated fasciculatin (1) from the chloroform extract of the marine sponge Ircinia variabilis, collected from the Atlantic coast of Morocco. We have also investigated the effect of this compound on the mitogenic response of human lymphocytes to phytohemagglutinin (PHA) as well as on the in vitro growth of the human tumor cell lines: MCF-7 (breast), NCI-H640 (lung) and SF-268 (CNS).

Results and Discussion

Fasciculatin (1) has first been isolated from the marine sponge Ircinia fasciculata, collected from the Bay of Naples [2] and later, together with palinurin (2), from Ircinia variabilis collected from the Mediterranean Sea [6]. However, there is no report on its biological activity.

The identity of fasciculatin (1) was confirmed by comparison of its $^{13}$C NMR spectrum as well as its rotation with those reported previously [2, 6]. Full assignments of the $^1$H and $^{13}$C NMR spectra were achieved through the use of COSY, HSQC, HMBC and NOESY spectroscopy. The chemical shift values of C-20 and C-21 corroborated that the configuration of the C-20, 21 double bond is Z [7]. The NOESY spectrum showed crosspeaks between H-11 ($\delta$ 6.10 dd, $J$=15.0, 10.7) and CH$_3$-9 (1.65d, $J$=0.9) as well as between H-10 (5.70 d, $J$=10.8) and H-7 (1.99t, $J$=7.4), H-12 (5.36 dd, $J$=15.0, 7.9) confirming the $E$ configuration of both C-13 and C-18.
The effect of fasciculatin on the in vitro growth of MCF-7, NCI-H460 and SF-268 cell lines was evaluated after a continuous exposure for 48 h. Results showed that fasciculatin exhibited a dose-dependent growth inhibitory effect which is equivalent in potency against all the three cell lines (Table 1). However, no activity was detected on the proliferation of human lymphocytes induced by PHA. The absence of antiproliferative effect on normal peripheral blood lymphocytes at concentrations that inhibit growth of tumor cell lines may represent a tumor specific sensitivity of this compound.

Table 1. Effect of fasciculatin (1) on the growth of human tumor cell lines and proliferation of human lymphocytes. Results are expressed as GI50 (concentrations of compound that cause 50% inhibition of tumor cell growth) or IC50 (concentration that cause 50% inhibition of lymphocyte proliferation) and are means ± SEM of 3-6 independent experiments performed in duplicate. Doxorubicin and cyclosporin were used as positive controls on tumor cell growth assay and lymphocyte proliferation assay, respectively.

|                | GI50 (µM) | IC50 (µM) |
|----------------|-----------|-----------|
|                | MCF-7     | NCI-H460  | SF-268 (CNS) | Human lymphocytes |
| Fasciculatin (1) | 47.11 ± 0.93 | 64.49 ± 0.84 | 72.45 ± 2.19 | >125 |
| Doxorubicin a | 42.82 ± 8.21 | 94.01 ± 8.72 | 93.02 ± 7.02 | _ |
| Cyclosporine | _ | _ | _ | 0.34 ± 0.04 |

a Data from doxorubicin are expressed in nM.

Conclusion

Fasciculatin was the major secondary metabolite of the marine sponge Ircinia variabilis, collected from the Atlantic coast of Morocco. Like many other sesterterpenes isolated from the marine sponges, fasciculatin showed a moderate cytotoxicity and no selectivity on the three cancer cell lines. Though the immunomodulatory activity of fasciculatin has never been investigated, we have found that it was inactive on human lymphocyte proliferation.

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of Amsterdam for sponge classification, Dr. Graham Eaton, Department of Chemistry, Leicester University, UK for HRMS, Prof. Artur Silva, Department of Chemistry, University of Aveiro for providing NMR spectra and NCI (USA) for kindly providing the tumor cell lines.

**Experimental**

**General**

$^1$H and $^{13}$C NMR spectra were recorded at ambient temperature on a Bruker AMC instrument operating at 300.13 and 75.47 MHz, respectively. Rotation was determined on a Polax-2 L instrument. HRMS spectra were acquired using +FAB ionization with Xe gas at 6 kV on a Kratos Concept III 2 sector mass spectrometer. Silica gel for column chromatography was Si Gel 60 (0.2-0.5 mm Merck), for analytical and preparative TLC Si Gel G-60 254 Merck.

**Biological Material**

The marine sponge *Ircinia variabilis* (Pallas, 1766) was collected in March 2003 at the litoral Atlantic of El-Jadida city, Morocco. The sponge was identified by Dr. Rob van Soest, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam. A voucher of specimen (ZMA Por. 17787) was deposited in the collections of the Zoological Museum Amsterdam. The collected material was immediately frozen at -20°C for one night prior to extraction.

**Extraction and Isolation of the constituents**

The sample (720 g wet weight) was thawed, homogenized with acetone (500 mL), allowed to stand in a dark chamber for 24 hours and filtered. The residue on the filter paper was again extracted with acetone (3x500 mL). The aqueous acetone extracts were combined, evaporated at reduced pressure until the final volume of the aqueous solution was ca. 100 mL and then extracted with CHCl$_3$ (3 x 100 mL). The CHCl$_3$ extracts were combined and concentrated at reduced pressure to give a viscous crude CHCl$_3$ extract (7.5 g) which was applied to a silica gel 60 column (70 g) and eluted with petrol-CHCl$_3$ and CHCl$_3$ – acetone, 250 mL frs being collected as follows: frs 1-64 (petrol – CHCl$_3$, 1:1), 65-95 (petrol – CHCl$_3$, 3:7), 96-141 (CHCl$_3$, acetone 9:1), 142-170 (CHCl$_3$,acetone 4:1). The ratios refer to v/v.

Fractions 3 and 4 (1.56 g) were combined, applied over Silica gel 60 (15 g) and eluted with petrol-CHCl$_3$, 200 mL subfrs being collected as follows: subfrs 1-5 (petrol – CHCl$_3$, 7:3), 6-9 (petrol – CHCl$_3$, 1:1), 10-15 (petrol – CHCl$_3$, 3:7). Purification of subfrs 5-9 (265 mg) by TLC (Si gel, CHCl$_3$-acetone-HCO$_2$H, 98:2:0.1) gave 174 mg of fasciculatin (1) which was identified by HRMS, $^1$H and $^{13}$C NMR measurement ($^1$H, $^{13}$C, DEPTs, COSY, HSQC, HMBC and NOESY) as well as comparison of its specific rotation with that reported in the literature [2].
**Fasciculatin** (I): yellow oily mass; HRMS: 397.23793 (M-H), calcd for C_{25}H_{34}O_{4}-H, 397.23788. $[^{17}\alpha]D = -12^\circ$ (CHCl$_3$, c = 0.43 g/100 mL). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.27 t ($J$=1.6, H-1), 7.13 t ($J$=1.0, H-4), 6.19 dd ($J$=1.7, 0.7, H-2), 6.10 dd ($J$=15.0, 10.7, H-11), 5.70 d ($J$=10.7, H-10), 5.36 dd ($J$=15.0, 7.9, H-12), 5.23 d ($J$=10.1, H-20), 2.71 m (H-18), 2.32 t ($J$=7.6, H-5), 2.08 br (H-13), 1.99 t ($J$=7.4, H-7), 1.73 s (Me-25), 1.65 d (J=0.9, CH$_3$-9), 1.61 t (J=8.0, H-6), 1.1-1.3 m (H-15, H-16, H-17), 0.97 d (J=6.7, Me-19), 0.90 d (J=6.7, Me-14). $^{13}$C NMR (75.74 MHz, CDCl$_3$) $\delta$ 171.95 (C-24), 162.13 (C-22), 142.84 (C-21), 142.58 (C-1), 138.75 (C-4), 138.45 (C-12), 135.99 (C-8), 125.03 (C-10), 124.99 (C-3), 124.69 (C-11), 115.83 (C-20), 110.96 (C-2), 99.08 (C-23), 39.28 (C-7), 37.12 (C-17), 37.05 (C-15), 36.84 (C-13), 30.79 (C-8), 28.04 (C-6), 25.07 (C-16), 24.32 C-5), 20.65 (C-19), 20.59 (C-14), 16.45 (C-9), 6.16 (C-25).

**Biological activity**

**Material and Methods**

A stock solution of fasciculatin (I) was prepared in DMSO (Sigma Chemical Co) at 400 times the desired final maximum test concentration and stored at –20 ºC. The frozen sample was freshly diluted with the cell culture medium just prior the different assays. Final concentrations of DMSO (≤ 25%) did not interfere with any of the biological activities tested.

**Tumor cell growth assay**

The effect of fasciculatin on the growth of tumor cell lines were evaluated according to the procedure adopted by the National Cancer Institute (NCI, Bethesda, U.S.A.) in the in vitro anticancer drug discovery screen that uses the protein-binding dye sulforhodamine B (SRB, Sigma Chemical Co) to assess cell growth [8]. Three human tumor cell lines were used, namely MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and SF-268 (CNS cancer). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium (Gibco BRL) supplemented with 5% heat-inactivated fetal bovine serum (Gibco BRL), 2 mM glutamine (Sigma Chemical Co) and 50 µg/ml of gentamicin (Sigma Chemical Co) at 37°C in a humidified atmosphere containing 5% CO$_2$. The optimal plating density of each cell line, that ensures exponential growth throughout all the experimental period, was the same as originally published [9] and was respectively $1.5 \times 10^5$ cells/ml to MCF-7 and SF-268 and $7.5 \times 10^4$ cells/ml to NCI-H460. Cells in 96-well plates were allowed to attach overnight and then exposed for 48 hours to five concentrations of fasciculatin. Following this incubation period the adherent cells were fixed in situ, washed and dyed with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (EAR 400, STL-Labinstruments). A dose-response curve was generated and the growth inhibition of 50% (GI$_{50}$), corresponding to the concentration of fasciculatin that inhibits 50% of the net cell growth, was
calculated as described [9]. Doxorubicin (Sigma Chemical Co) used as a positive control was tested in the same manner.

**Lymphocyte proliferation assay.**

The effect of fasciculatin on the proliferation of human lymphocytes induced by the mitogen PHA (10 µg/ml, Sigma Chemical Co) was evaluated using a modified colorimetric MTT assay [10] previously described by our group [11]. Human mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by Histopaque-1077 (Sigma Chemical Co) density centrifugation. Human mononuclear cells were adjusted to 2-3 x 10⁶ cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine and 50 µg/ml of gentamicin. In a flat-bottom 96-well plate, human mononuclear cells were allowed to proliferate in the presence of seven serial concentrations of fasciculatin for 4 days at 37°C in a humidified atmosphere containing 5% CO₂. Following this incubation period, the MTT solution (1 mg/ml, Sigma Chemical Co) was added. After 4 h of incubation, the MTT formazan products were solubilized with the SDS/DMF solution (20% SDS in a 50% solution of DMF, pH 4.7) overnight at 37°C. Absorbance of the colored solution was measured at 550 nm in a plate reader (EAR 400, STL-Labinstruments). The concentration giving 50% inhibition in the test system (IC₅₀) was calculated. Cyclosporin A (Sigma Chemical Co) used as a positive control was tested in the same manner.

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Sample availability: Not available.

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