Cytotoxic effects of Fisturalin-3 and 11-Deoxyfisturalin-3 on Jurkat and U937 cell lines

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Background. Fisturalines are bromotyrosine compounds isolated from marine sponges. Previous studies have shown antineoplasic, antiviral and antibacterial effects *in Vitro*; however, the possible effects of these compounds in hematologic malignancies have not been assessed.
Methods. In the present study, the antiproliferative and pro apoptotic effects of Fistularin-3 (F) and 11-Deoxyfistularin-3 (DF) were assessed using the MTT method and annexin V/propidium iodide by flow cytometry using the cell lines: Jurkat E6.1 and U937. In addition, the cell cycle was assessed by flow cytometry.
Results. Inhibition of the proliferative response was concentration and time dependent. The IC50 of F was 7.39 and 8.10 μM for Jurkat E6.1 and U937 respectively. At 24 and 48 h, in the U937 cell line, but not in the Jurkat cell line, both compounds induced up to 35% annexin V increase. Necrosis was not observed in any case. Compound F induced, in both cell lines, a decrease in the number of cells in the S phase and increase in the G0/G1 phase. In the Jurkat cell line only, there was an increase in the number of cells in the G2/M phase. Compound DF was not as effective as F.
Conclusions. F is more active than DF in repressing the cell cycle and inducing apoptosis. Both compounds are potentially useful in the development of new drugs to treat hematologic malignancies.

Key words: Fistularin-3, 11-Deoxyfistularin-3, leukemia, cancer, bromotyrosine, quercetin, *Aplysina fistularis*

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INTRODUCTION

Sponges of the phylum Porifera are marine invertebrates that have developed efficient mechanisms of defense against for example viruses, bacteria and eukaryotic organisms. For this reason, and consequently they are a natural source of interesting metabolites which are potential drugs for therapy[1-6]. Some are being tested in clinical trials with relevant success; however, researchers are still encountering new compounds from marine species that could become leader compounds in future therapy[3-6].

Sponges of the *Aplysinidae* family are a rich source of microorganisms. They also contain high concentrations of brominated alkaloids[2,7-13]. Of these alkaloids Compagnone RS et al.[9] identified 11-Deoxyfistularin-3 (DF) and Fistularin (F), (the structures are illustrated in Fig. 1). The compounds have been shown to have wide biological activity including anti bacterial and anti viral effects[7-13]. In addition, DF has been reported to inhibit cell proliferation of MCF-7 cells with an IC_{50} of 17 μM; however, the mechanism by which the decrease in cell viability occurs is not known[9]. There are no reports on their effects on hematological malignancies. The aim of the present study was to ascertain the effect of DF and F on cell viability in two different cell lines Jurkat and U937.

MATERIAL AND METHODS

Compounds

The compounds F and DF were obtained from the sponge *Aplysina fistularis insulares*, as described previously[9], (the structures are illustrated in Fig. 1). Quercetin was purchased from Sigma-Aldrich. The solutions of F, DF and quercetin were prepared in DMSO at a concentration of 0.1 M and the final concentration of DMSO in cell culture was never higher than 0.1% w/v.

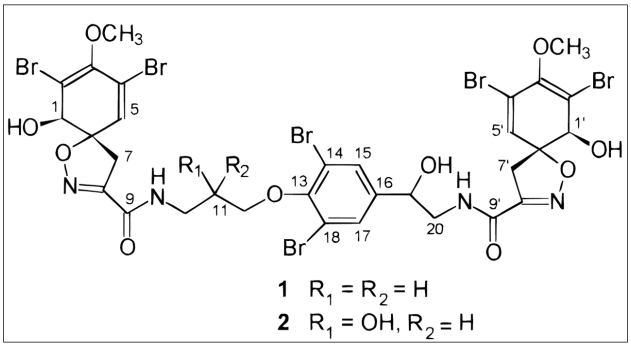

Fig. 1. Chemical structures of 11-Deoxyfistularin-3 (1) and Fistularin-3 (2).

Cell Lines

Jurkat, Clone E6-1, and U937 cell lines were maintained in 10% FBS (Hyclone) in RPMI-1640 (ATCC) supplemented with 100 units/mL penicillin and 0.1 mg/mL streptomycin (Sigma-Aldrich). The cells were kept in a humidified atmosphere, at 37 °C, containing 5% CO_2. For all experimental designs, the cells were then seeded at a cell density of 3×10^5 per mL. All experimental assays were performed five times in triplicate.

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) viability assay

The assay was performed as described previously by Suárez AI et al.[14] which is a modification of Mossman's protocol[15]. Briefly, the cells were plated at $5x10^4$ cells per well in 200 µL of complete culture medium containing 0, 1, 5, 10, 25, 50 and 100 µM concentrations of DF or F, in 96-well microtiter plates in sextuplicate for 24, 48 and 72 h, at 37 °C in a humidified incubator. At the end of the incubation, MTT (0.50 mg/mL in phosphate buffered saline) was added to each well and the plates were incubated for 4 h. Then, the plates were centrifuged at 1800 x *g* for 5 min at 4 °C and washed with PBS to remove excess MTT. Finally, the formazan crystals were dissolved with DMSO and the plates were read by a microplate reader at 540 nm. The effect of DF and F on growth inhibition was assessed as per cent cell viability where vehicle-treated cells were taken as 100% viable. The IC50 value was defined as the concentration of compound required to induce a 50% reduction of absorbance compared with control, non-treated, cells. Quercetin was used as a positive control in the assay.

Table1. Effect of Fisturalines on cell viability assessed by the MTT method (µM).

Compound	U937		Jurkat E6.1	
	24h	48h	24h	48h
Fistularin-3	16.01	8.10	13.18	7.39
11-Deoxyfistularin-3	>50	32.39	40.58	15.77
Quercetin	36.26	15.19	6.25	5.26

The effect of the Fisturalin-3, 11-Deoxyfistularin-3, and quercetin (control) on cell viability by the MTT assay is represented. The IC50 were calculated based on the results of five independent experiments.

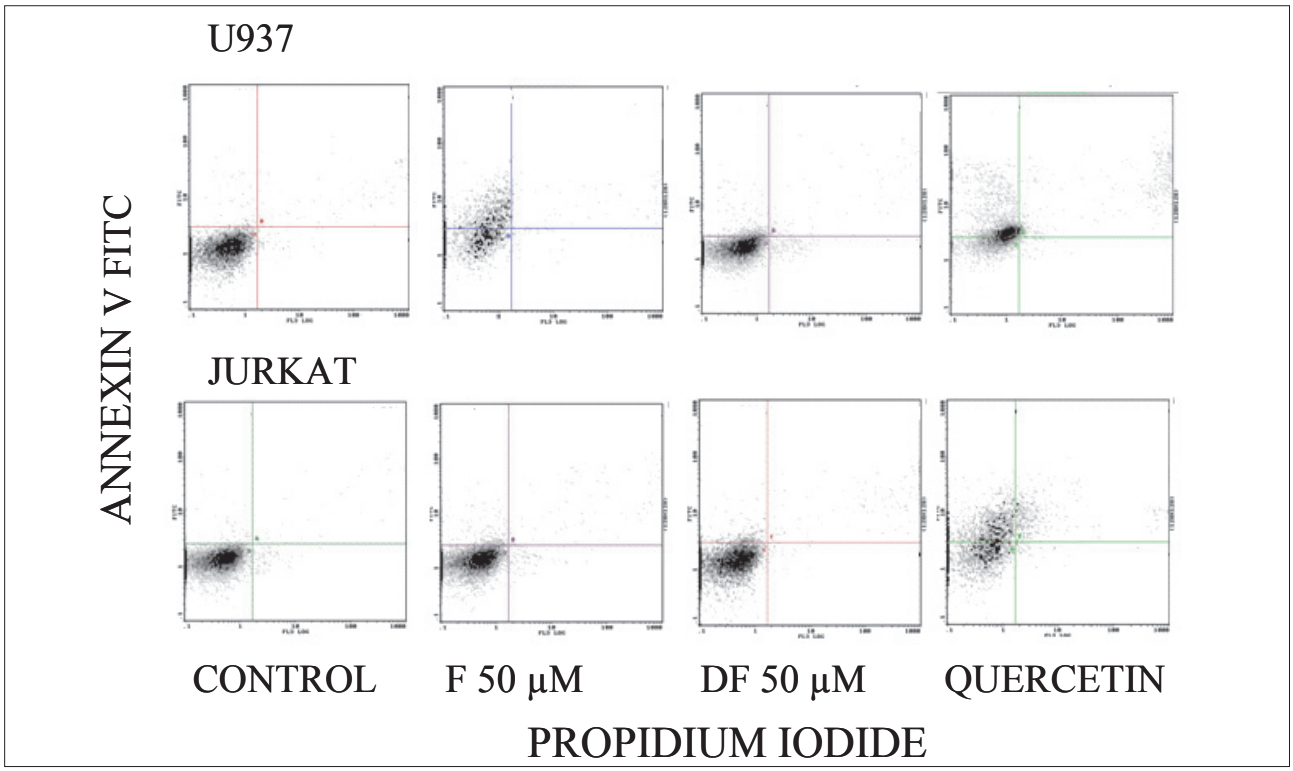

Fig. 2. Effect of DF and F on the expression of annexin V and propiudium idodide upon 24 hr of treatment.
The figure represents a typical experiment in which the cells were treated with vehicle (control), brominated marine metabolites, and quercetin for 24 h and then apoptosis and necrosis were determined by flow cytometry as described in the Material and Methods section.

Table 2. Effect of Fisturalines on apoptosis and necrosis.

	A ANNEXIN-V			
	U937		Jurkat E6.1	
Compound	24h	48h	24h	48h
	0.3 ± 0.1	0.8 ± 0.4	0.2 ± 0.1	0.6 ±0.1
Fistularin-3 5 μM	1.4 ± 0.2	2.6 ± 0.2	1.5 ± 0.5	3.9 ± 0.3
10 μM	2.9 ± 0.3	3.9 ± 0.4	1.9 ± 0.4	4.5 ± 0.5
25 μM	20.5 ±1.8	25.4 ± 0.4	2.1 ± 0.3	4.3 ± 0.4
50 μM	35.4 ±1.2	31.8 ± 0.8	3.6 ± 0.4	6.2 ± 0.8
11-DF -3 5 μM	1.6 ± 0.1	4.3 ± 0.4	1.9 ± 0.8	3.8 ± 0.3
10 μM	1.3 ± 0.4	13.5 ± 0.7	1.5 ± 0.2	4.4 ± 0.4
25 μM	4.4 ± 0.6	21.5 ± 1.1	2.4 ± 1.0	4.3 ± 0.5
50 μM	15.9 ± 1.9	34.2 ± 1.2	3.2 ± 0.4	5.2 ± 0.8
Quercetin 5 μM	5.3 ± 0.6	10.8 ± 0.7	5.5 ± 0.9	2.5 ± 0.3
10 μM	15.3 ± 0.5	13.9 ± 0.8	25.5 ± 0.5	4.4 ± 0.5
25 μM	28.8 ± 1.2	39.8 ± 0.6	45.8 ± 2.4	35.5 ± 1.3
50 μM	50.9 ± 1.9	60.5 ± 0.5	64.2 ± 1.4	66.2 ± 1.4
B PROPIDIUM IODIDE UPTAKE (%)				
	24h	48h	24h	48h
	0.5 ± 0.1	1.7 ± 0.5	0.4 ± 0.2	1.5 ± 0.4
Fistularin-3 5 μM	0.2 ± 0.1	0.8 ± 0.4	0.5 ± 0.2	0.4 ± 0.2
10 μM	1.1 ± 0.2	0.7 ± 0.1	0.8 ± 0.3	0.5 ± 0.1
25 μM	1.4 ± 0.4	0.7 ± 0.3	0.8 ± 0.4	0.7 ± 0.3
50 μM	0.9 ± 0.3	3.9 ± 0.4	0.6 ± 0.1	0.7 ± 0.4
11-DF -3 5 μM	0.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
10 μM	0.3 ± 0.1	0.7 ± 0.2	0.4 ± 0.2	0.5 ± 0.2
25 μM	0.2 ± 0.1	1.8 ± 0.9	0.4 ± 0.2	0.3 ± 0.3
50 μM	0.1 ± 0.1	1.9 ± 0.7	1.0 ± 0.1	0.9 ± 0.2
Quercetin 5 μM	0.6 ± 0.2	1.3 ± 0.4	0.6 ± 0.1	2.3 ± 0.4
10 μM	1.6 ± 0.3	1.1 ± 0.3	1.3 ± 0.3	3.2 ± 0.3
25 μM	1.3 ± 0.4	1.4 ± 0.4	2.5 ± 0.4	2.3 ± 0.5
50 μM	1.3 ± 0.4	1.9 ± 0.4	3.8 ± 0.4	2.7 ± 0.4

The effect of the compounds on apoptosis and necrosis is represented. The numbers represent the mean and standard deviation of the total positiveness of five different experiments for annexin V (apoptosis) and propidium iodine (necrosis).

Annexin V/Propidium iodine labeling

U937 and Jurkat cells were treated as indicated, then washed twice with PBS and resuspended in Annexin V binding buffer (0.01 M HEPES, 0.14 M NaCl and 2.5 mM $CaCl_2$); then, annexin V-FITC conjugate (Santa Cruz Biotechnology) and propidium iodide (20 μg/mL) were added to the cells for 15 min apart in the dark, at room temperature, following the protocol suggested by the manufacturer. The cells were analyzed using and Epics XL cytometer (Beckman Coulter) using as negative control the untreated cells and positive control the cells treated with Quercetin.

Cell cycle

Treated cells were subjected to DNA content analysis. Briefly, the cells were harvested and washed twice with PBS and fixed with cold 70% ethanol for at least 24 h. The ethanol was removed and followed by two PBS washes. Cells were stained in the following solution: PBS supplemented with 0.1% Triton X-100, 0.1% Na_3-citrate, 30 μg/mL RNase and 20 μg/mL propidium iodide. After incubation in the dark for 30 minutes at room temperature, the cells were analyzed on Epics XL (Beckman Coulter) and analyzed by the Multicycle program® (University of Washington 1994, distributed by Phoenix Flow Systems, Inc).

Statistical analysis

All experiments were performed at least three times. The results are expressed as mean ± SD. ANOVA tests were performed. Only post hoc Dunnet test $P<0.01$ was considered to be statistically significant. The values of

Table 3. Effect of Fisturalines on cell cycle phases assessed by flow cytometry (%).

Compound	U937			Jurkat E6.1		
	G0/G1	G2/M	S	G0/G1	G2/M	S
Control	52.6 ± 2.2	6.7 ± 1.9	40.6 ± 2.2	42.4±2.1	10.3±0.6	47.4±1.4
Fistularin-3						
25 μM	61.9 ± 3.3*	9.2 ± 0.8	28.9 ± 1.1*	64.4 ± 2.2*	24.3±0.5*	12.3±1.2*
50 μM	81.8± 2.1*	9.9 ± 1.1	9.1± 1.7*	70.8 ± 1.1*	24.4±0.8*	4.8±1.7*
11-deoxy-Fistularin-3						
25 μM	52.7 ± 2.2	9.3 ± 1.2	37.9 ± 1.5	47.3 ± 1.3	8.8 ± 0.9	44.0±1.3
50 μM	67.7 ± 2.5*	11.0± 0.9	21.2 ±1.3*	52.5 ± 2.4	17.5 ± 2.3	30.0±1.5*
Quercetin						
25 μM	60.3 ± 2.5	10.5± 0.8	29.5± 1.5*	48.8±1.5	12.6±0.6	38.6±1.6
50 μM	72.5± 2.8*	15.5± 0.9	12.0± 1.4*	75.5±1.9*	10.5±0.8	14.0±1.9*

The effect of the compounds on cell cycle kinetics is represented in the table. The percentage of cells in the different phases was compared and in some cases was significant (* P<0.01).

IC50 were determined by non-linear regression of individual experiments, using the program Graph Pad Prism®, version 5.

RESULTS

The effect of fisturalins on U937 and Jurkat cell lines MTT viability assays is illustrated in Table 1. Fisturalin-3 inhibited cell viability in a similar fashion in both cell lines. The viability of the Jurkat cell line markedly decreased in the presence of DF only at 48 h. The control, Quercetin, decreased Jurkat cell viability at lower concentrations than the U937 cell line.

In order to assess the possible mechanism of cell death, the cells were cultivated with the metabolites and at the end of the incubation, 24 or 48 h; were then washed and labeled with Annexin V/propidium iodide to ascertain apoptosis of necrosis. Fig. 2 represents a typical flow cytometry assay cell viability using both cell lines. The major differences in the treatment are represented by the increase in Annexin V without major increase in propidium iodide positivity. The results of all assays are shown in Table 2A and 2B. Interestingly, the expression of Annexin V in the treated cell lines differed. In U937 cells treated with F, the maximum effect was reached, at 50 μM, which was lower than 50% independently of the incubation time. DF was significantly (P<0.05) less effective at 24 h, than 48 h. There was no significant increase in the Annexin V Jurkat cell line with any of the treatments except for quercetin, the positive control. The effects of the compounds on propidium iodide expression are illustrated in Table 2B. The tested compounds did not induce necrosis as can be seen in Fig. 2.

In Table 3, analysis of the cell cycle reveals important changes in the number of cells in different cell cycle stages. In the U937 cell line, the presence of F induced a significant (P<0.01) and concentration dependent increase in number of cells in the G0/G1 phase with a marked decrease in the S phase without affecting the G2/M phase. The effect of the compound in the Jurkat cell line was similar; however, the number of cells in the G2/M stage increased. The compound DF was significantly less effective in decreasing the number of U937 cells in the S stage compared to F and it effects on the Jurkat cell line tended to be similar at the highest dose. In similar fashion, the effect of DF on cell cycle modulation was very mild. The effect of Quercetin was similar to that reported in the literature[16].

DISCUSSION

The brominated natural products purified from the *Aplysinidae* family of sponges have been shown to be potential candidates in cancer therapy. Most studies have dealt with cancer cell lines originating from solid tumors; however, there is increasing interest in studying the effects in cells from hematological malignancies. In the present report, two different cell lines were used to assess the effect of brominated natural products. Even though, in principle, the effect of the compounds on both cell lines seem to be related to a decrease in cell viability, using the MTT method, the mechanism by which this effect occurs differed in the cell lines. Fisturalin-3 and 11-Deoxyfisturalin induced a mild increase in apoptosis in the U937 cell line, but they do not have any effect in the Jurkat cell line. The effect of fisturalin-3 was seen earlier (at 24 h) than the deoxy counterpart. Nevertheless, none of the compounds induced necrosis.

The decrease in cell viability, monitored by the MTT method, could be due to a cytostatic effect of the compounds. A reduction in cell cycle kinetics induces a decrease in cell number and consequently resembles fewer cells than in the untreated control. The remarkable increase in number of cells in the G0/G1 phase, parallel to

the remarkable decrease in cell numbers in the S phase by both compounds, supports this conclusion. Nevertheless, a decrease in cell cycle kinetics may also be due to induction of cell differentiation which in turn will make the cell more prone to die.

In the literature, reviewed in 17, remarkable compounds purified from the sponge *Dolabella auricularia* have been shown to be inhibitors of tubulin and consequently are good cytostatic compounds. These cytostatic compounds are currently used in clinical studies for several types of cancer. It is possible, after more studies on the mechanisms of the brominated compounds, that these structures could be considered for new therapeutic developments.

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CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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