Cytotoxic Activity of Pisosterol, a Triterpene Isolated from *Pisolithus tinctorius* (Mich.: Pers.) Coker & Couch, 1928

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Pisolithus tinctorius (Basidiomycete) is an ectomicorrhizal fungus found in the roots and soil surrounding of many species of eucalyptus and pine trees. The present work verified the cytotoxic potential of pisosterol, a triterpene isolated from *P. tinctorius* collected in the Northeast region of Brazil, on three different animal cell models: mouse erythrocytes, sea urchin embryos and tumor cells. Pisosterol lacked activity on mouse erythrocytes as well as on the development of sea urchin eggs, but strongly inhibited the growth of all seven tumor cell lines tested, especially the leukemia and melanoma cells (IC_{50} of 1.55, 1.84 and 1.65 μg/ml for CEM, HL-60 and B16, respectively). The results found for pisosterol were compared with those of doxorubicin and etoposide.

Key words: Pisolithus tinctorius, Pisosterol, Cytotoxicity

Introduction

Microbial secondary metabolites represent a large source of compounds endowed with ingenious structures and potent biological activities. Many of the products currently used for human or animal therapy are produced by microbial fermentation, or are derived from chemical modification of a microbial product. Thus, new bioactive substances continue to be identified from microbial sources, in regard of the large variety of existing strains (Donadio *et al.*, 2002).

Pisolithus tinctorius (Basidiomycete) is a fungus found in the roots and soil surrounding of many species of eucalyptus and pine trees. It has acquired an important economic interest since the basidiospore inoculum forms specific ectomycorrhizae which aid the host plant in obtaining the basic minerals for its survival, enabling the creation of artificial foresting zones (Baumert *et al.*, 1997; Marx and Kenney, 1982). Thus, to the ectomycorrhizae, the function of protecting the host plant against microbial invaders is also attributed, as it was demonstrated by Tsantrizos *et al.* (1991) with the isolation of two antibiotic compounds, pisolitine A and B, from cultures of *P. tinctorius*.

The aim of the present study was to evaluate the cytotoxicity of pisosterol, a triterpene isolated from *P. tinctorius* (Gill *et al.*, 1989) collected in the Northeast region of Brazil. The cytotoxic activity was measured as the ability to inhibit the sea urchin eggs development and tumor cell lines proliferation and to cause lysis of mouse erythrocytes. It is worthwhile to mention that there are not much data available concerning pharmacological aspects of pisosterol, which highlight the significance of this work.

Materials and Methods

Isolation of pisosterol

Pisosterol was isolated from the ectomycorrhizal fungus *Pisolithus tinctorius* collected in an eucalyptus plantation at Brejo, Maranhão, Northeast Brazil. The air-dried powdered material (370 g) of *P. tinctorius* was exhaustively extracted with acetone at room temperature to give 21 g of the extract after evaporating the solvent under reduced pressure. The acetone extract (4 g) was chromatographed over silica gel, eluted with acetone/methanol as a binary mixture (100:0–0:100) to yield pisosterol as the major component (120 mg). The

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structure of pisosterol (Fig. 1) has been determined by spectroscopy means, including one and two dimensional NMR such as COSY, HMQC, HMBC, physical properties and comparison with data from literature.

Hemolytic assay

The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method described by Jimenez *et al.* (2003). Pisosterol was tested at concentrations ranging from 3.9 to 1000.0 μg/ml. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectrophotometrically as the absorbance at 540 nm.

Assay of sea urchin eggs

The assay was performed following the method described by Jimenez *et al.* (2003). Adult sea urchins (*Lytechinus variegatus*) were collected at Lagoinha beach, on the northeastern coast of Brazil. Gamete elimination was induced by injecting 3.0 ml of 0.5 M KCl into the urchin's coelomic cavity. For fertilization, 1 ml of a sperm suspension (0.05 ml of concentrated sperm in 2.45 ml of filtered sea water) was added to every 50 ml of egg solution. The assay was carried out in 24-multiwell plates. Pisosterol was added immediately after fecundation (within 2 min) to get concentrations of 1, 3, 10, 30 and 100 μg/ml in a final volume of 2 ml. Doxorubicin (Doxolem®, Zodiac Produtos Farmacêuticos S/A, Brazil) and etoposide (Etosin®, Asta Medica Ltda, Brazil) were used as positive controls. At appropriate intervals, aliquots of 200 μl were fixed in the same volume of 10% formaldehyde to obtain first and third cleavages and blastulae. One hundred eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal cells.

MTT assay

The cytotoxicity of pisosterol was tested against CEM (human leukemia) and HL-60 (human leukemia), B16 (murine melanoma), HCT-8 (human colon cancer), MCF-7 (human breast cancer), PC-3 (human prostate cancer) and SF-268 (human neuroblastoma) cell lines obtained from the National Cancer Institute, Bethesda, MD, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin, and incubated at 37 °C with a 5% CO_2 atmosphere. For experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.5×10^5 cells/well for suspended cells in 100 μl of medium). After 24 h, pisosterol (0.39 to 25.0 μg/ml) dissolved in DMSO 1% was added to each well and incubated for 3 d (72 h). Control groups received the same amount of DMSO. Doxorubicin and etoposide were used as positive controls. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a purple formazan product (Mosmann, 1983). At the end of the incubation, the plates were centrifuged and then, the medium was replaced by fresh medium (200 μl) containing 0.5 mg/ml MTT. 3 h later, the MTT formazan product was dissolved in 150 μl DMSO, and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). The drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm.

Statistical analysis

Data are presented as mean ± S. E. M. The IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA).

Results and Discussion

The present study evaluated the cytotoxic activity of pisosterol (Fig. 1), a triterpene isolated from *Pisolithus tinctorius*, in mouse erythrocytes, developing embryos of sea urchin and tumor cell lines.

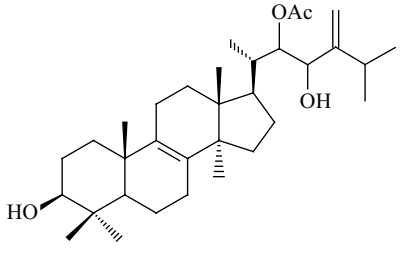

Fig. 1. – Molecular structure of pisosterol isolated from *Pisolithus tinctorius*.

Table I. Antimitotic activity of pisosterol, doxorubicin and etoposide (positive controls) on sea urchin (*Lytechinus variegatus*) egg development. Data are presented as IC_{50} (μg/ml) values and their 95% confidence interval (CI 95%) for the first and third cleavages and the blastulae obtained by non-linear regression.

Substance	1st cleavage IC_{50} [μg/ml (μM)]	3rd cleavage IC_{50} [μg/ml (μM)]	Blastulae IC_{50} [μg/ml (μM)]
Pisosterol	> 100.0 (194.5)	> 100.0 (194.5)	> 100.0 (194.5)
Doxorubicin	6.28 (10.83) 4.34–9.09	0.34 (0.59) 0.16–0.73	0.54 (0.93) 0.27–1.07
Etoposide	7.85 (13.33) 1.21–48.84	0.30 (0.51) 0.20–0.46	0.85 (1.44) 0.53–1.37

Moreover, the activity of this compound was compared to doxorubicin and etoposide, two clinically used drugs in cancer chemotherapy.

To verify whether pisosterol acts through membrane disruption, it was tested for the ability to induce lysis of mouse erythrocytes and the results showed that it does not cause membrane damage.

In the sea urchin eggs assay, pisosterol did not inhibit the development of the embryos even at the highest concentration tested (100 μg/ml), differently to what was observed for both of the chemotherapeutic agents tested (Table I). On the other hand, pisosterol, as well as the chemotherapeutic agents, inhibited the proliferation of the seven cell lines in a dose-dependent manner analyzed through the MTT assay. It presented a stronger activity against the leukemia and melanoma cells, however, in a slightly lower potency than that of doxorubicin and etoposide, as confirmed through the IC_{50} values (Table II).

Generally, cytotoxic substances tested in the sea urchin eggs and tumor cells have shown to be active in both assays, although compounds may present a higher IC_{50} value in the sea urchin eggs than in tumor cells, as observed herein for doxorubicin and etoposide (Jimenez *et al.*, 2003; Costa-Lotufo *et al.*, 2003).

The study of alterations in sea urchin egg development is a suitable model for detecting cytotoxic, teratogenic and antineoplastic activities of new compounds (Jacobs and Wilson, 1986; Costa-Lotufo *et al.*, 2002). This assay method can detect such selective agents as DNA and RNA synthesis inhibitors, protein synthesis inhibitor and inhibitors of microtubule assessment (Fusetani, 1987). A wide number of chemotherapeutic drugs have been assayed on the sea urchin eggs, to mention a few, 5-fluorouracil, dactinomycin, taxol, vincristine, cycloheximide. Drugs that affect DNA or protein synthesis, as 5-fluorouracil and cycloheximide, respectively, may show their effects very early, during the first cleavage of the egg (Fusetani, 1987). Doxorubicin and etoposide are topoisomerase II inhibitors and act disrupting the DNA synthesis process (Chabner *et al.*, 2001). In this case, it was noticeable that the cell divisions

Cell line	Pisosterol IC_{50} [μg/ml (μM)]	Etoposide IC_{50} [μg/ml (μM)]	Doxorubicin IC_{50} [μg/ml (μM)]
SF-268	12.42 (24.16) 10.35–14.91	2.53 (4.30) 1.54–4.14	0.11 (0.19) 0.11–0.18
B16	1.65 (3.21) 1.42–1.91	0.11 (0.19) 0.07–0.18	0.03 (0.05) 0.02–0.04
PC-3	4.96 (9.65) 4.37–5.63	> 5.80 (9.85)	0.24 (0.41) 0.21–0.27
MCF-7	4.86 (9.46) 4.05–5.85	> 5.80 (9.85)	0.20 (0.34) 0.17–0.24
HCT-8	4.34 (8.44) 3.55–5.30	0.11 (0.18) 0.08–0.15	0.04 (0.07) 0.03–0.05
HL-60	1.84 (3.58) 1.26–2.69	0.01 (0.02) 0.01–0.02	0.02 (0.03) 0.01–0.02
CEM	1.55 (3.01) 1.24–1.96	0.03 (0.05) 0.02–0.04	0.02 (0.03) 0.01–0.02

Table II. Inhibitory effect on cultured cell growth of pisosterol, etoposide and doxorubicin (positive controls) on 7 different tumor cell lines. Data are presented as IC_{50} (μg/ml) values and their 95% confidence interval (CI 95%) obtained by non-linear regression.

were compromised since the first cleavage. If the test substance acts on RNA synthesis, like dactinomycin, the development of the egg continues freely until the morulae stage before any abnormality can be seen (Brandshort, 1985). Whereas, when the substance disrupts microtubule assemble, as taxol or vincristine, clear spots corresponding to nucleus duplication can be observed in a continuous, undivided cytoplasm (Jacobs *et al.*, 1981; Jacobs and Wilson, 1986).

Being the sea urchin egg a large, low pigmented cell which holds great similarities to the human cell cycle, it became a trustworthy and useful model to infer about the action mechanisms of the substances tested (Fusetani, 1987). However, among the major differences, which separate both cellular models, there is the briefness of the G2 phase in the sea urchin cycle, in comparison to human cells, and the inexistence of phase G1. Therefore, drugs that interfere on the G1 phase will show no activity on the sea urchin eggs (Jacobs and Wilson, 1986).

The selectivity of pisosterol towards mammal cells, suggests a cycle-dependent activity or inquires about a mechanism of action that would involve a specific structure of pathway different to those described for typically cytotoxic substances. Further studies are already in progress for the elucidation of pisosterol's mechanism of cytotoxicity against the tumor cells.

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