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In vitro and *in vivo* anticancer activity of posterior salivary gland toxin from the cuttlefish *Sepia pharaonis*, Ehrenberg (1831)

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ABSTRACT

Posterior salivary gland (PSG) toxins are high molecular weight toxins secreted by cephalopods and gastropods which possess immense potentials in biomedical applications. In the present study, the biomedical potentials of the PSG toxin from the cuttlefish, *S. pharaonis* was determined *in vitro* and *in vivo*. The cytostatic potentials of the PSG toxin was determined by the lymphocyte migration inhibition assay. The PSG toxin (50 μg/ml) effectively inhibited the migration of lymphocytes across the agarose gel matrix under the presence of lipopolysaccharide mitogen. The cytotoxicity of the PSG toxin against cancer cell lines was determined using the MTT assay. The PSG toxin exhibited highest cytotoxicity against the MCF-7 breast cancer cells (IC_{50}-10.64 μM) followed by KB, HeLa and A549 cells. The PSG toxin also exhibited proportional release of LDH leakage by mitochondrial damage with an IC_{50}-13.85 μM against MCF-7 breast cancer cells. Flow cytometry analysis revealed that the PSG toxin induced apoptosis in MCF-7 cells by cell cycle arrest at G_0/G_1 phase. The PSG toxin (80 mg/kg b.w.) exhibited pronounced reduction (29%) in tumor growth in experimentally induced breast carcinoma in female Balb/C mice, *in vivo*. Hematological analysis illustrated the restoration of blood and biochemical parameters by the PSG toxin in mice induced with tumor. Histopathology studies also revealed the restitution of morphological features in the mammary tumor and vital organs in mice treated with the PSG toxin without any observed toxicity and adverse effects. The PSG toxin further exhibited commendable potentials in the prevention of tumor metastasis into immediate organs viz lungs, thus functioning as an anti-metastatic agent. The results of the present study showed that the PSG toxin exhibited immense promise as a potential peptide based anticancer agent, in future.

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1. Introduction

Breast cancer is the most widely diagnosed cancer among the women, contributing to a quarter of all the cancers diagnosed (140/184) and leading cause for death in women. Clinically, breast cancer is the most heterogeneous type of cancer with three distinct groups based on therapeutic strategies. The statistics for India is alarming with an age standardized rate of 25.76 per 100,000 of which 12.73 per 100,000 women lead to mortality. Indifferent to other cancer types, breast cancer is imminently treatable, if detected at early stages, primarily due to the influence of hormonal interventions and selective tumor metastasis [1]. A number of approaches are practiced towards elimination of the harmful cancerous cells and tissue from the body. Radiation therapy is one of the preliminary practices for targeted killing of cancer cells. Surgery of the diseased tumor is recommended at times of deprived penetration of radiation to the targeted tumor site. Chemotherapy is one of the alternative approaches of cancer treatment, where compounds are designed and targeted towards disrupting cancer mechanisms, preventing the continuous dividing and proliferation of cells [2]. A wide variety of signal transduction inhibitors, apoptosis inducers, angiogenesis inhibitors, gene expression modulators may function as targeted approaches towards cancer elimination. Hormone-sensitive tumors such as breast cancer and prostate cancer are

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widely treated using hormone therapies to minimize or reduce the secretion of hormones favorable for the growth of the tumor [3]. Numerous drugs approved for the treatment of breast cancer have been associated with a wide variety of adverse effects like temporary loss of hair, rashes in the skin, changes in colour and texture, loss of fingernails and toenails have been reported to be adversely affected during treatment with conventional anticancer drugs. Drugs such as vincristine and paclitaxel have also been associated with peripheral neuropathy [4]. Occurrences of reversible increase in levels of alanine transaminase and leukopenia have been reported in low dose oral cyclophosphamide and methotrexate treated hormone receptor-negative early breast cancer [5]. Hence, there is a need for alternate sources of drugs with minimized adverse effects and specific mode of action.

Marine peptide based anticancer therapy is gaining momentum due to its diverse structural features and novel mechanisms of action [6]. Among the molluscs, cephalopods and gastropods have been considered rich sources of bioactive anticancer peptides. Toxic peptides from gastropods such as cone snails have been developed into valuable macromolecules for biomedical applications. Six cyclic (Kahalalide A-F) and 1 acyclic peptide (Kahalalide G) isolated from *Elysia rufescens* has been previously reported to exhibit *in vitro* and *in vivo* anticancer activity in tumor models, against a wide range of cancers viz. breast, colon, non-small cell lung and prostate [7–9]. PSG toxins from the cuttlefishes *S. esculenta* and *S. officinalis* have been extensively characterized and studied for its toxicity and biological relevance [10,11]. However, similar studies on cephalotoxins from the Indian scenario are largely limited. We have previously reported the isolation and characterization of PSG toxin from the cuttlefish, *S. pharaonis* [12,13]. Further insights to evaluate the potential biomedical applications of the PSG toxin from *S. pharaonis* are highly warranted. Hence, in the present study the PSG toxin from *S. pharaonis is* evaluated for its anticancer activity *in vitro* and *in vivo* in mice model.

2. Materials and methods

2.1. PSG toxin isolation, purification and preliminary characterization

We have previously reported the extraction and purification of the PSG toxin from the cuttlefish, *S. pharaonis*, captured from the Chennai coast of Tamil Nadu, India. The PSG toxin was also previously estimated for its protein and carbohydrate contents and the preliminary characterization of the PSG toxin was carried out using SDS-PAGE, IR and CD spectroscopy and GC-MS, reported elsewhere [12].

2.2. In vitro cytotoxicity

2.2.1. Lymphocyte migration inhibition assay

The cytotoxicity of the purified PSG toxin against the primary cells peripheral blood mononuclear cells (PBMC) was determined using the lymphocyte migration inhibition assay in the presence of mitogen activation by lipopolysaccharide from gram negative bacteria [14].

2.2.2. In vitro anticancer activity by MTT assay

The cytotoxicity of the purified PSG toxin against selected cancer cell lines was determined by the MTT cell viability assay [15]. The anti-proliferative potentials of the PSG toxin was studied against the adherent cultures of MCF-7 breast cancer cell lines and the inhibitory concentrations (IC_{50}) were determined.

2.2.3. LDH leakage assay

The cell viability and membrane permeability of the PSG toxin against the of the MCF-7 breast cancer cells was determined using the LDH leakage assay [16].

2.2.4. Cell cycle analysis by flow cytometry

The PSG toxin from *S. pharaonis* exhibited higher anti-proliferative effects against the MCF-7 breast cancer cells and the mechanism of cell death was studied by flow cytometry. The MCF-7 cells treated with PSG toxin were suspended in 1 ml of propidium iodide (PI) solution and incubated in dark at 37 °C for 10 min. The stained cells were transferred to the Flow cytometer (BD FACS Calibur, USA) and excited at a wavelength of 536 nm. The emissions of the cells were recorded at 617 nm and the distributions of the cells in the cell cycle were analyzed [17].

2.3. In vivo anticancer activity of PSG toxin

2.3.1. Breast carcinoma induction

Breast adenocarcinoma was experimentally induced in female Balb/c mice following the ethical clearance obtained from the Institutional Animal Ethical Committee (IAEC 1/Desp.No63/Dt.09.06.14). 5×10^4 breast cancer cells were injected subcutaneously into the mammary fat pad and treatment was initiated when tumor reached 1 mm^3 in volume (day 15). 0.1 ml of purified PSG toxin (10, 20, 40 and 80 mg/kg) in 0.1 M PBS was administered intra-peritoneally (i.p.) once in 2 days for 20 days. Control mice received 0.1 ml of PBS. Paclitaxel (20 mg/kg) treated mice served as the positive control. At the end of the experimental period (day 35), the mice were euthanized and the reduction in tumor size were measured. The serum was analyzed for hematological and biochemical parameters and histological studies were carried out for the excised tumors and selected organs [18].

2.3.2. Hematological analysis

Blood was collected from the retro-orbital sinus of the mice and the hematological parameters were analyzed. The total and differential blood cell count and other parameters such as hemoglobin, MCV, MCHC and platelet count were determined in an automated hematology analyzer (Leica Biosystems, USA) following standard procedures [19]. The biochemical parameters such as plasma triglyceride, total cholesterol, total bilirubin, total protein, alanine transaminase (GPT), aspartate transaminase (GOT) and alkaline phosphatase (ALP) were measured by random access clinical chemistry analyzer Erba XL300 detected with commercial ERBA diagnostic kits (Open Source Diets, New Brunswik, NJ).

2.3.3. Histopathological analysis

Tumors of the respective induced and treated groups were collected, fixed in formalin (10%), processed, sectioned and stained (hematoxylin and eosin) in an automated processor (Leica Biosystems). The tumor and the tissue sections were observed for the presence of cytological and metastatic changes with reference to the control group [20].

2.4. Statistical analysis

The results were expressed in terms of mean ± S.D. of six animals in each group. Values within the group were statistically analyzed using one way analysis of variance (ANOVA) and Mann Whitney's *U* test using Software Package for Statistical Analysis (SPSS) *v.*21.0. A value of $P \leq 0.05$ and 0.01 were considered statistically significant.

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