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Anticancer activity of a low immunogenic protein toxin (BMP1) from Indian toad (*Bufo melanostictus*, Schneider) skin extract

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ABSTRACT

Earlier, a protein (BMP1, MW-79kDa) had been isolated from Indian toad (*Bufo melanostictus*) skin aqueous extract possessed anticancer activity against EAC bearing mice (Bhattacharjee et al., 2011). In the present study, the anti-proliferative and apoptogenic activities of BMP1 have been evaluated in leukemic (U937 and K562) and hepatoma (HepG2) cells. BMP1 dose dependently inhibited U937 and K562 cell growth having IC_{50} values of 49 μg/ml and 30 μg/ml respectively. The anti-proliferative activity of BMP1 was observed in MTT assay, proliferating cell nuclear antigen (PCNA) expression and cell cycle arrest study. Flow-cytometric data revealed that BMP1 arrested cell cycle in U937 and K562 cells at Sub-G1 and G1 phases. The BMP1-induced dose dependent expressions of CDKIs ($p21^{cip1}$ and $p27^{kip1}$) and inhibition of CDK2 and PCNA expression in HepG2 cells support the inhibition of cell proliferation due to G1 arrest. BMP1-induced apoptosis analyzed by annexin-V binding study and the DNA fragmentation by comet assay were correlated with the sub-G1 arrest. The parallel induction of bax and p53 expression in HepG2 cells and the up-regulation of caspase 3 and caspase 9 due to BMP1 treatment indicated the involvement of p53-dependent intrinsic pathway of apoptosis. BMP1 was found to be low immunogenic in nature.

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1. Introduction

Toad's granular gland secretions contain biogenic amines, steroids, peptides and proteins (Clarke, 1997), not only contain toxic defense molecules but also possess potent therapeutic activities against microbial infection, diabetes, cardiovascular disorder and cancer (Gomes et al., 2007a). Chan Su, the traditional Chinese medicine preparation from the dried white secretion of the auricular and skin glands of toad (*Bufo bufo gargarizans*) induced apoptosis in T24, human bladder carcinoma cell line (Ko et al., 2005). Bufalin and other steroid molecules isolated from Chan Su, showed anticancer property against leukemia, carcinoma, melanoma and other cancer cells (Zhang et al., 1992; Kamano et al., 1998; Cunha-Filho et al., 2010). Cinobufocini injection, a preparation containing certain components of Chan Su, showed anticancer effects in clinical and experimental studies (Wang et al., 2005; Qi et al., 2010). The anticancer activity of aurein peptides

Abbreviations: AO, acridine orange; BMP1, *Bufo melanostictus* protein 1; CDKI, cyclin-dependent kinase inhibitor; DAPI, 4′,6-diamidino-2-phenylindole; EAC, Ehrlich ascites carcinoma; EtBr, ethidium bromide; 5FU, fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PCNA, proliferating cell nuclear antigen; TSAE, toad skin aqueous extract.

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was established by the National Cancer Institute, USA (Rozek et al., 2000). Citropin 1.1, and its other analogous synthetic peptide, A4K14-citropin 1.1, showed anticancer activity on 60 different human cell lines as tested by US National Cancer Institute (Doyle et al., 2003). Maximins, isolated from skin secretions of *Bombina maxima*, showed cytotoxicity to tumor cells, but at the same time it was toxic to mice (Lai et al., 2002).

Earlier from this laboratory, it was found that the skin extract of common Indian toad (*Bufo melanostictus*, Schneider) possessed significant antineoplastic activity on EAC cells and human leukemic cell lines (Das et al., 1998; Giri et al., 2006). Later, a non-protein crystalline compound, BM-ANF1 had been isolated from *Bufo melanostictus* that possesses anticancer properties (Gomes et al., 2007b). Very recently from this laboratory, a protein toxin (BMP1) had been isolated which was demonstrated to be anti-proliferative and apoptogenic against mouse Ehrlich ascites carcinoma with limited toxicity (Bhattacharjee et al., 2011). In the present communication, further detail studies have been carried out on the anticancer activities of BMP1 against leukemic (U937 and K562) and hepatoma (HepG2) cancer cells.

2. Materials & methods

2.1. Materials

U937, K562 and HepG2 cells were procured from National Center for Cell Sciences (Pune, India), Acridine orange (Sigma, USA), annexin-V (Sigma, USA), DMSO (SRL, India), Bradford reagent (Sigma, USA), EDTA (Sigma, USA), ethidium bromide (Sigma, USA), 5-Fluorouracil (Sigma, USA), heparin (Sigma, USA), low melting point agarose (Promega, USA), methanol (Spectrochem, India), normal melting point agarose (Promega, USA), propidium iodide (Sigma, USA), PVDF (Pall, USA), RPMI 1640 (HiMedia, India), Triton X-100 (SRL, India) and Trypan blue (SRL, India) were used. All antibodies are from Santa Cruz Biotechnologies Inc., USA and Cell Signaling Tech., USA. The other chemicals were purchased locally and were analytical grade unless otherwise mentioned.

2.2. Animals

Male Swiss albino mice (20 ± 2 g) were used for lethality study and New Zealand strain male rabbits (1.6 ± 0.1 kg) were used for antiserum development. Animal experiments were approved by the University Animal Experimental Ethics Committee, and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India.

2.3. Collection and preparation of toad skin aqueous extract (TSAE)

Adult toads (*Bufo melanostictus*, Schneider) of both sexes (70 ± 10 g) were collected commercially. After pithing the toad, its skin was taken out leaving the head region including parotid gland intact. The skin was taken into a ceramic glass mortar and grinded with sea sand and distilled water. The extract was filtered, centrifuged. The concentration of the freshly prepared toad skin aqueous extract (TSAE) was expressed in terms of its protein content.

2.4. Anti-proliferation study

2.4.1. U937 and K562 cell count and determination of IC50

One hundred microliters of U937 and K562 cell suspension containing 10^5 cells/ml of sterile RPMI 1640 was added to each well in a sterile 96-well plate. BMP1 was dissolved in sterile RPMI medium and cancer cells were cultured in the presence and absence of BMP1 (10–200 μg/ml). Ara-C (100 mg/ml) and Imatinib mesylate (100 mg/ml) were used as standard drugs. The number of viable cells was counted after 24 h and 48 h by trypan blue dye exclusion method using a phase contrast microscope (Olympus, CK40), and the percentage inhibition of cell growth was calculated as compared to control (Agrawal et al., 1989) ($n = 6$). The IC_{50} of BMP1, defined as the concentration of compound that causes 50% reduction in viable cell count in 24 h, was calculated (Reed and Muench, 1938).

2.4.2. MTT assay

MTT assay of control and BMP1-treated (50 μg/ml) cells was done to confirm the cytotoxic effect (Kawada et al., 2002). Control and treated cells were cultured in sterile 96-well plates incubated at 37 °C in 5% CO_2 incubator for 24 h ($n = 6$). 20 ml MTT (5 mg/ml) was then added in each well and allowed to incubate further in same condition for 3 h. 100 μl of DMSO was added to each well to dissolve the formazan crystal formed. The OD was recorded at 570 nm with microplate reader (Merck-MIOS Mini, Model No. 309). Percentage growth inhibition was equal to [1-(OD of treated/OD of control)] $\times$ 100.

2.4.3. Flow-cytometric analysis of cell cycle arrest

To assay the stage of cell cycle arrest in a flow-cytometer, control and BMP1 (IC_{50} dose, 24 h) treated U937 and K562 cells were fixed in ethanol overnight, washed and treated with DNase free RNase A (10 μg/ml) at 37 °C for 30 min and stained with 200 μl propidium iodide (50 μg/ml) and kept at dark for 15 min. Intracellular DNA content was measured by the amount of red fluorescence in a flow-cytometer (Becton Dickinson FACS caliber single laser cytometer) using 488 nm argon laser light source and 623 nm band pass filter and analyzed by Modfit software (Becton Dickinson) (Giri et al., 2009).

2.4.4. Western immunoblotting

The effect of BMP1 on the expression of cell cycle-related proteins (PCNA/p21/p27/p53) and pro-apoptotic bax was evaluated through Western blotting. HepG2 cells were treated with BMP1 (15, 30, 60 μg/ml) for 48 h and were seeded onto 60-mm plates, washed with PBS at termination and lysed in lysis buffer containing protease inhibitor mixture (Roche Applied Science, Indianapolis, IN), 1 mM phenylmethylsulfonyluoride, 10 mM NaF, 1 mM Na_3VO_4, and 50 mM b-glycerophosphate and phosphatase

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