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Research paper

Induction of apoptosis in human cancer cells by a *Bacillus* lipopeptide bacillomycin D

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ABSTRACT

A newly isolated and characterized *Bacillus amyloliquefaciens* strain fiply 3A has been found to produce an extracellular cyclic lipopeptide which structurally resembled bacillomycin D, earlier reported to be produced by *Bacillus subtilis*. The lipopeptide showed a dose dependent killing of three different human cancer cell lines *viz*. A549 (alveolar adenocarcinoma), A498 (renal carcinoma) and HCT-15 (colon adenocarcinoma), while not affecting the normal cell line L-132 (pulmonary epithelial cells) when analyzed using MTT assay and FACS analysis. Staining the cells with H_2-DCFDA showed an increase in reactive oxygen species (ROS) formation in the lipopeptide treated cell population. Hoechst 33342 staining of nuclei further indicated apoptosis as a major mechanism of cell death in lipopeptide treated cells and the typical symptoms of apoptosis including cell shrinkage, nuclear condensation and fragmentation of nuclei were observed. Lipopeptide treatment induced extensive DNA damage in the treated cells, which was indicated by a TUNEL assay. Flow cytometric analysis exhibited lipopeptide concentration dependent apoptosis which was further confirmed during clonogenic assay of the lipopeptide treated cells.

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1. Introduction

Lipopeptides constitute a structurally diverse group of small molecules consisting of 7–25 amino acids (hydrophilic group), linked to β-hydroxy or β-amino fatty acids (hydrophobic group), generally in a cyclic manner [1]. Many fungal, bacterial as well as actinomycete species have been reported to produce lipopeptides with diverse biological activities. These molecules are produced non-ribosomally via large multienzyme complexes (non-ribosomal peptide synthetases) by microbes. Fungal members producing such molecules include *Aspergillus aculeatus*, and *Aspergillus nidulans*, that produce aculeacins and echinocandin B, respectively [2]. Bacterial species which produce lipopeptides belong to the genera *Bacillus*, *Pseudomonas* and *Serratia*. Lipopeptides produced by *Bacillus* species are broadly classified into 3 groups namely, surfactin, iturin and fengysin. Surfactin group includes members containing heptapeptides interlinked with a β-hydroxy fatty acid (13–15 carbon length) to form a cyclic lactone ring structure. Iturin family consists of heptapeptide molecules linked to β-amino fatty acid chain with a length of 14–17 carbons. The last group fengysin, comprises lipopeptides with an internal lactone ring in the peptidic moiety and with β-hydroxy fatty acid chain with length of 14–18 carbons [2]. Based on length and composition of fatty acid tail as well as the number and type of amino acids, lipopeptides produced by *Pseudomonas* spp. are classified into 4 major groups i.e. viscosin [9 amino acids linked to 3-hydroxy decanoic acid (3-HDA)], amphisin (11 amino acids coupled to 3-HDA), tolaasin (19–25 amino acids linked to 3-HDA) and syringomycin groups (9 unusual amino acids linked to 3-HDA) [3,4]. *Serratia marcescens* produces 3 species of lipopeptides *viz*. serrawettins W1, W2 and W3 [3]. A major class of clinically important lipopeptides is produced by the species of *Streptomyces* and *Actinomycetes*.

Lipopeptides have been shown to possess various interesting biological properties due to their amphipathic structure. These include antibacterial as well as antifungal properties [2]. Some lipopeptides show antiviral, antitumor, immunomodulating and hemolytic properties [5] while others induce the formation of ion channels in membrane lipid bilayer [2]. Iturin A is known to disrupt plasma membrane by the formation of small vesicles and the aggregation of intramembranous particles in yeast cells. Further, it also increases the electrical conductance of phospholipid biomembranes [5]. Surfactins have been reported to be involved in the inhibition of fibrin clot formation, induction of ion channel formation in lipid bilayer membranes, inhibition of cyclic

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adenosine monophosphate phosphodiesterase, inhibition of platelet and spleen cytosolic phospholipase A2 (PLA2) activity [6].

Induction of apoptosis in cancerous cells is one of the strategies of modern cancer therapeutic regimes. However, limited options are available with severe side effects. Hence, search for substances of natural origin having preferential cancer cell killing ability is of paramount importance [7]. Moreover, interestingly, different bacterial lipopeptides have shown promising results in inducing apoptosis in various cancer cell lines [8,9]. Recently, a new cyclic lipopeptide (CLP) produced by *Bacillus subtilis* natto T-2 has been shown to inhibit the growth of human leukemia K562 cells through induction of apoptosis [7]. In another study Cao et al. [10] showed that surfactin from *Bacillus natto* could kill human breast cancer MCF-7 cells through ROS mediated caspase pathway. In spite of immense potential of lipopeptide biosurfactants in the area of clinical biology, their use still remains unexplored. One of the reasons is the lack of information on their toxicity toward human system. Hence, more studies are needed to validate the use of biosurfactants in several biomedical and health related areas.

In the present study, a new *Bacillus* strain (fiply 3A) has been identified and biochemically characterized as a novel strain of *Bacillus amyloliquefaciens* (unpublished data). This strain was found to produce a lipopeptide that was characterized as bacillomycin D using LC-MS/MS analysis, and for the first time this compound has been shown to exhibit antitumor activity against three human cancer cell lines.

2. Materials and methods

2.1. Chemicals and cell lines

Human cancer cell line A549 (alveolar adenocarcinoma), A498 (renal carcinoma) and HCT-15 (colon adenocarcinoma) and normal human cell line L-132 (pulmonary epithelial cells) were obtained from National Centre for Cell Sciences, Pune, India. All the cell culture media were procured from Himedia Laboratories Pvt. Ltd. Hoechst 33342; dimethylsulfoxide (DMSO); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); and H_2-DCFDA (2′,7′-dichlorofluorescein diacetate) were procured from Sigma (St. Louis, USA). Cell permeable caspase-3 inhibitor (Z-DEVD-FMK) was procured from BD Biosciences, San Jose, CA, USA. Caspase-8 inhibitor II was obtained from EMD Biosciences, Inc., San Diego, CA, USA. Caspase-9 inhibitor and PARP inhibitor (3 aminobenzamide) were obtained from Sigma, St. Louis, MO, USA. Affinity purified biotin-conjugated, polyclonal rabbit anti-active human caspase-3 antibody was procured from PharMingen, USA. AIF and cytochrome C antibodies were obtained from Cell Signaling Technology Inc., MA, USA. Anti-caspase-8 antibody was acquired from Abcam plc Cambridge, UK. Staurosporin for positive control studies was obtained from Cell Signaling Technology Inc., MA, USA.

2.2. Isolation and characterization of the lipopeptide and its effect on human cell lines

Bacillus sp. fiply 3A was isolated from the soil samples of the Institute and was characterized as a novel strain of *B. amyloliquefaciens* using standard biochemical and molecular biology techniques (unpublished data). The strain was deposited in DSMZ culture collection under the name *Bacillus* sp. fiply 3A (DSM 22646). Lipopeptide was extracted from the culture filtrates of *Bacillus* strain grown in potato dextrose broth for 6 days at ambient temperature (26 ± 2 °C). Partial purification of the lipopeptide was achieved by ammonium sulfate precipitation (60% saturation), acid precipitation [3 N HCl at ambient temperature (26 ± 2 °C) for 2 h] and solvent [Chloroform: methanol (2:1) and methanol] extraction of the culture filtrate. Further purification of the compound was carried out using HPLC (JASCO Analytical Instruments, Easton, MD, USA) as per the method described by Kim et al. [11]. Sample was injected into an HPLC column (ODS Hypersil C18, Thermo Fisher Scientific, Switzerland) with dimensions 4.6 mm (internal diameter) × 250 mm (length), and 10 μm particle size. Mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (Solvent A) and 0.1% trifluoroacetic acid (TFA) in acetonitrile (solvent B). Sample was analyzed at a flow rate of 1.0 ml/min with a linear gradient of solvent B, increasing from 30 to 100%. The elution pattern was monitored at 225 nm. Different peaks obtained were collected separately and identified by using liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS/MS) analysis (Thermo Finnigan LCQ Deca Electrospray quadrupole ion trap mass spectrometer, ThermoElectron Co., Hemel Hempstead, Herts, UK).

As mentioned above, three different human cancer cell lines and one normal human cell line were selected to study the effect of purified lipopeptide using following assays.

2.3. Cytotoxicity assay (MTT)

In this assay, viable cells are detected using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye, which forms blue formazan crystals that are reduced by active mitochondrial dehydrogenase present in living cells. Cells were suspended at a final concentration of $\sim 10^5$ cells/ml, seeded in 96-well flat-bottom micro titre plates and treated with the purified lipopeptide at varying concentrations (15–120 μg/ml) along with vehicle (ethanol) control for 48 h. After the treatment, MTT [0.5 mg/ml in phosphate buffered saline (PBS), pH = 7.5] was added in the micro titre plates after removing the medium completely and incubated at 37 °C for 4 h. Later, the precipitated formazan was dissolved in a solubilization buffer (10% SDS in 0.1 N HCl) and the plates were incubated overnight at 37 °C. Next day the optical density (O.D.) of the control as well as treated samples was measured at 570 nm using an automated plate reader (Micro-Quant, Biotek Instruments, Singapore). The growth inhibition due to lipopeptide was calculated according to the following equation:

$$\text{Growth inhibition } (\%) = (A - B/A) \times 100, \quad \text{where,}$$

A is the average O.D. of the control group and *B* is that of treated group.

To determine the effect of lipopeptide on survival of mouse spleen cells, splenocytes were obtained by gentle homogenization of the spleen under a sterile nylon mesh. The RBCs were lysed by treatment with 0.83% NH_4Cl for 5 min. Later, the cells were centrifuged at 2000*g* at ambient temperature (26 ± 2 °C) and washed twice in ice-cold RPMI-1640 medium. This suspension of spleen cells was seeded in 96-well tissue culture plate at a final concentration of $\sim 1 \times 10^5$ cells/well in a RPMI-1640 medium. Splenocytes were treated with different concentrations (10–200 μg/ml) of purified lipopeptide for 48 h at 37 °C in a CO_2 (5%) incubator. Survival of spleen cells was quantified using MTT assay as described above. For positive control, splenocytes were treated with 1 μM staurosporin (Cell Signaling Technology Inc., MA, USA).

2.4. Quantitative analysis of apoptotic cell population by propidium iodide staining

Apoptotic cell population upon lipopeptide treatment was detected using propidium iodide (PI) staining of treated cells followed by flow cytometry to detect the sub-G1 peak [12]. Briefly, A549 cells (~50,000 cells/ml) were seeded in 6 well plate and

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