



Streptomyces puniceus strain AS13., Production, characterization and evaluation of bioactive metabolites: A new face of dinactin as an antitumor antibiotic

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ABSTRACT

A highly active actinobacterial strain isolated from untapped areas of Northwestern Himalayas and characterised as *Streptomyces puniceus* strain AS13 by 16S rRNA gene sequencing was selected for production of bioactive metabolites. The bioassay-guided fractionation of microbial cultured ethyl acetate extract of the strain, led to isolation of macrotetrolide compound **1** (Dinactin) and compound **2** (1-(2,4-dihydroxy-6-methylphenyl)-ethanone). Structures of the isolated compounds were elucidated interpretation of NMR and other spectroscopic data including HR-ESI-MS, FT-IR. These compounds are reported for first time from *Streptomyces Puniceus*. Compound **1** exhibited strong anti-microbial activity against all tested bacterial pathogens including *Mycobacterium tuberculosis*. The MIC values of compound **1** against Gram negative and Gram positive bacterial pathogens ranged between 0.019 – 0.156 $\mu\text{g ml}^{-1}$ and 1 $\mu\text{g ml}^{-1}$ against *Mycobacterium tuberculosis* H37Rv. Dinactin exhibited marked anti-tumor potential with IC_{50} of 1.1– 9.7 μM in various human cancerous cell lines and showed least cytotoxicity ($\text{IC}_{50} \sim 80 \mu\text{M}$) in normal cells (HEK-293). Dinactin inhibited cellular proliferation in cancer cells, reduced their clonogenic survival as validated by clonogenic assay and also inhibited cell migration and invasion characteristics in colon cancer (HCT-116) cells. Our results expressed the antimicrobial potential of dinactin and also spotted its prospective as an antitumor antibiotic.

1. Introduction

Microbial sources have long been recognised as important reserves for isolation of lead molecules in drug discovery paradigms (Gullo et al., 2006; Harvey, 2007). Actinomycetes have robust capacity to biosynthesise the potent bioactive molecular scaffolds with broad array of biological activities (Bérdy, 2012). A little portion of the existing ecosystems have systematically been explored for isolation of microbial diversity (Monciardini et al., 2014). Although, many molecules have been isolated from actinomycetes but few have been evaluated for diverse biological activities. At present, the world is in dire need for the discovery and development of new drugs with safer therapeutic potential. So far only 1% of actinomycetes have been cultured, thus bio-prospection of actinomycetes from distinct habitats for production of

biologically potent metabolites becomes an imperative aspect (Baltz, 2008; Subramani and Aalbersberg, 2013). In course our search for the potential actinobacterial strains by exploring the Himalayan habitats (Hussain et al., 2017a), as part of our program, a potent actinomycete isolate characterised as *Streptomyces puniceus* strain AS13 was selected for further investigation. This study besides the fermentation, isolation, structural elucidation of the isolated compounds (**1** and **2**), also describes their antimicrobial spectrum and antitumor efficiency. The compound **1** characterised as dinactin (a macrotetrolide) (Gerlach et al., 1967; Xie et al., 2014), exhibited potent biological activity. The literature investigation on the dinactin (**1**) did not reveal special report on its anti-tuberculosis MIC and *in-vitro* nontoxic character. So this encouraged us to evaluate the compound for its possible role in the anti-proliferation. The dinactin proved to be a potent anticancer agent,

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inhibited cellular proliferation characteristics in colon cancer (HCT-116) cells. Although macrotetrolides are known family of molecules, but in our study high antibacterial and specific anticancer activity with low toxicity profile of dinactin on normal cells (HEK-293) could decide on its new application as antitumor antibiotic.

2. Material and methods

2.1. General

IR spectra were recorded on PerkinElmer spectrum two FT-IR spectrometer (PerkinElmer, Waltham, MA, USA). ^1H and ^{13}C NMR spectra were obtained with a Bruker –400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer (Bruker, Bremen, Germany). Chemical shifts (δ) are expressed as parts per million and coupling constant (J) in Hertz. The ^1H NMR and ^{13}C NMR spectral assignments were supported by ^1H - ^1H COSY, HSQC, HMBC and NOESY experiments. High-resolution electron spray ionization mass spectroscopy (HR-ESI-MS) data were taken on Agilent technologies spectrometer 6450 UHD Accurate Mass Q TOF LC/MS (Santa Clara, USA).

2.2. Isolation of strain

The actinobacterial strain AS-13 was isolated from soil sample collected from Sonamarg mountains located in north western Himalayas, Jammu and Kashmir, India on CYP5 medium, supplemented with nystatin ($50\ \mu\text{g ml}^{-1}$) and polymyxin B sulphate ($5.0\ \mu\text{g ml}^{-1}$). The isolate was purified and maintained on CYP5 medium slants and stored in 20% glycerol at $-20\ ^\circ\text{C}$.

2.3. Optimization of conditions

The actinomycete strain was inoculated into three different types of media for optimization of metabolite production. The strain was inoculated in 1000-ml Erlenmeyer flasks, each containing 250 ml of different medium and incubated at $28\ ^\circ\text{C}$ for 14 days at $180\ \text{rev min}^{-1}$. The fermented broths of each medium were subjected to centrifugation (Hitachi CR22GIII) at $3860\ \text{g rev min}^{-1}$ for 10 min and followed by their filtration. The supernatant filtrates were exhaustively extracted by ethyl acetate (2X) and the respective organic layers were concentrated on the rotary evaporator (Rotavapor R-215, BUCHI). The concentrated and dried extracts were quantified and checked for their bioactivity. The three types of media (with pH-7.2) used included: CYP5 medium (10 g starch, 10 g casein, 1 g peptone and 1 g yeast extract in 1L water), SCP-1 medium (1 l sterile water containing 5 g casein and 3 g Yeast Extract) and SC medium (10 g soluble starch, 2 g KNO_3 , 2 g K_2HPO_4 , 2 g NaCl, 0.3 g tryptone-I, 0.05 g MgSO_4 , 0.02 g CaCO_3 and 0.01 g FeSO_4 in 1 l sterile water).

Duplicate cultures of the strain were grown at temperatures: $4\ ^\circ\text{C}$, $16\ ^\circ\text{C}$, $28\ ^\circ\text{C}$ and $37\ ^\circ\text{C}$ in CYP5 medium (5 ml) for 21 days to optimize the temperature conditions of the strain. Dry weight of the biomass of one of the cultures at intervals of every 3rd day was estimated by lyophilising the sample. The process was continued for other samples to complete the period of 21 days.

2.4. Molecular characterization and phylogenetic analysis

Genomic DNA was isolated by CTAB method as devised by Magarvey et al. (2004). The amplification of 16S rRNA gene sequence by PCR (Master cycler personal Eppendorf) was carried by using universal primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACT T-3'). The PCR reaction was carried as per procedure (Shah et al., 2016) using Taq polymerase (Fermentas) with amplification profile: initial denaturation at $95\ ^\circ\text{C}$ (3mins), followed by 30 cycles of denaturation at $95\ ^\circ\text{C}$ (15s), annealing at $59\ ^\circ\text{C}$ (30s), and extension at $72\ ^\circ\text{C}$ (45s) and final extension at $72\ ^\circ\text{C}$ (7 min).

The amplified reaction products were purified by Qiagen gel extraction kit as per the manufacturer instructions and sequenced by ABI genetic analyser 3130XL (Applied Biosystems) using gene specific primers (Edwards et al., 1989). Search for homology was carried using BLAST Search algorithm and 16S RNA gene sequence alignment with other selected sequences was performed by using Clustal W program version 1.81 (Thompson et al., 1994). The phylogenetic tree was generated by the neighbour-joining (NJ) algorithm using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (Tamura et al., 2013). The numbers on major branches indicate bootstrap percentages for 1000 replicate analyses.

2.5. Fermentation, extraction and isolation

The strain was pre cultivated on SC medium agar plates at $28\ ^\circ\text{C}$ for 14 days. Fermentation involved the generation of a seed culture in SC medium. The colonies of strain were inoculated into 1 l Erlenmeyer flask containing 250 ml of SC medium and culture was grown for 4 days at $28\ ^\circ\text{C}$ with shaking at $180\ \text{rev min}^{-1}$. The seed culture obtained was used to inoculate total volume of 10 l SC media in 1 l Erlenmeyer flasks, each containing 250 ml medium (pH 7.2). All the flasks were incubated at $28\ ^\circ\text{C}$ for 14 days with shaking at $180\ \text{rev min}^{-1}$. The fermented broth was subjected to centrifugation at $3860\ \text{g}$ for 10 mins and the supernatant was extracted twice with equal volume of ethyl acetate. The ethyl acetate layer was concentrated using the rotary evaporator (Rotavapor R-215, BUCHI, Switzerland), which yielded 2.5 g of a red coloured oily extract. The extract was fractionated using silica gel column chromatography (60–120 mesh, Merck; $100 \times 3\ \text{cm}$ column) and eluted with ethyl acetate: hexane in percentages of 25%, 50%, 75% and finally with 30% methanol: chloroform. Based on TLC screening, the collected fractions were pooled to form four major fractions (I, II, III and IV). All the fractions were screened against various test bacteria. The most active (fraction II) on further purification by employing silica gel column chromatography afforded the compound 2 (6 mg) in gradient of 20% ethyl acetate: hexane and compound 1 (15 mg) in gradient of 30% ethyl acetate: hexane.

2.6. Anti-bacterial activity

2.6.1. Test microorganisms

The panel of test bacteria, *Mycobacterium tuberculosis* (ATCC 25177), *Klebsiella pneumonia* (ATCC BAA-2146), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 11774), *Micrococcus luteus* (ATCC 10240), *Enterococcus faecalis* (ATCC 51299), *Pseudomonas aeruginosa* (ATCC 10145) and *Escherichia coli* (ATCC 10536) used in the study were acquired from American Type Culture Collection (ATCC), USA.

2.6.2. Minimum inhibitory concentration (MIC) measurements

The MICs of the test compounds against non-mycobacterial and mycobacterium strains were determined by the broth microdilution method described previously (Hussain et al., 2017b; Shah et al., 2017), prescribed under the guidelines of Clinical and Laboratory Standards Institute (CLSI) procedures, M07-A9 (2012). The stock solutions ($50\ \text{mg ml}^{-1}$) of the test compounds were prepared in DMSO, then sequentially diluted either with Muller Hinton broth (MHB) for non-mycobacterial or with Middlebrook 7H9 (MB7H9) broth for mycobacterial strains to working concentrations. Briefly, a range of concentrations of the test compounds as well as reference drugs (INH, RIF, EMB and LVX for mycobacterial and AMK and LVX for non-mycobacterial pathogens) were made by two fold serial dilution in 200 μl volumes and bacterial inoculum (50 μl) was added in each well of a 96-well plate with the final cell density of 1×10^5 CFUs/ml. The plates were incubated at $37\ ^\circ\text{C}$ and read after 24 h for non-mycobacterial pathogens and 14 days for H37Rv strain of *M. tuberculosis*. The MIC was defined as the lowest concentration which prevented the visible

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