

Isolation and characterization of *Streptomyces tauricus* from Thajiwas glacier—a new source of actinomycin-D

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Abstract The aim of the present study was to isolate potent anticancer compound from actinomycetes strain *SRP18* isolated from soil of high altitude region of Thajiwas glacier, Sonamarg Kashmir—India. The potential isolate *SRP18* was identified as *Streptomyces tauricus* on the basis of 16S rDNA sequence technique. The large scale cultivation of potent strain, *Streptomyces tauricus SRP18* and subsequent isolation and purification by a series of chromatographic techniques resulted in isolation of potent anticancer compound. Structure elucidation of potent compound by using various spectroscopic techniques including NMR was confirmed to be actinomycin-D. As such, this is the first report of a strain of *S. tauricus* capable of producing this bioactive compound. The isolated compound was evaluated for its cytotoxicity potential against four human cancer cell lines like HeLa, PC-3, THP-1, and Caco-2 and showed a potent activity with IC₅₀ range of 4.91–7.25 μM.

Keywords Thajiwas glacier Kashmir · *Streptomyces tauricus SRP-18* · 16S rDNA · Actinomycin-D · Cytotoxicity

Introduction

Cancer has emerged as a major public health threat worldwide. World population growth and aging imply that the cancer burden will increase in the future (Lin et al. 2009). Most antitumor secondary metabolites that have been identified are produced by microorganisms. It is widely accepted that actinobacteria are prolific producers of numerous antitumour metabolites with a variety of structures. These compounds are unrivalled and unmatched in medical significance. The upcoming threat of cancer and the development of resistance by pathogens to multiple drugs have brought research on actinomycetes into focus again during the previous years. The genus *Streptomyces* have been reported as the producers of two-thirds of the microbial antibiotics known today (Newman et al. 2003). The particular capacity of streptomyces is to produce large variety of different bioactive compounds that have a wide spectrum of activity (Zin et al. 2007). In our on-going research on actinomycetes, we report an actinomycin-D from a new source *Streptomyces tauricus*. The strain was isolated from the soil sample collected from high altitude region of Thajwas glacier Kashmir—India. The actinomycins are chromopeptide lactone antibiotics of which more than 30 native and many synthetic variants are known. *Streptomyces parvulus* is used for the commercial production of actinomycin-D (Sousa et al. 2002). Actinomycin-D has been studied most extensively and is widely used for the treatment of malignant tumours such as Wilms' tumour and childhood rhabdomyosarcoma (Womer 1997). The purpose of this study was isolation, purification, and structure elucidation, biological activity of actinomycin-D from *Streptomyces tauricus*.

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Materials and methods

Isolation and selection of potent strain

The soil sample were collected from high altitude region of Thajiwas glacier in Sonamarg region of Kashmir, India and transported to the laboratory under aseptic conditions. One gram of air dried soil sample was suspended in 9 ml sterile water and mixed by shaking vigorously for 30 min. The supernatant was diluted serially (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) with sterile water, 100 μ l of each dilution was spread onto starch-casein agar (Kuster and Williams 1964) and International *Streptomyces* Project medium (ISP-2) medium recommended by Shirling and Gottlieb (Moncheva et al. 2002), which were supplemented with nalidixic acid and cycloheximide. The plates were incubated at 30 °C for 7–10 days and the resulting colonies were purified and maintained on ISP-2 agar slants. Isolated actinomycete strains were screened their cytotoxic activity. Among active isolates, the strains coded as SRP18 showed significant cytotoxic activity and was subsequently preserved in 25% glycerol at -80 °C.

Identification of potent strain

The selected isolate SRP18 was identified by 16S rDNA technique. The DNA was isolated by using modified cetyltrimethylammonium bromide method (Doyle and

Doyle 1987). 16S rRNA gene was amplified by using universal bacterial primers, 27F (5-AGAGTTTGATC MTGGCTCAG-3) and 1525R (5-AAGGAGGTGWTCC ARCC-3). The PCR reaction mixture (50 μ l) contained 25 μ l of PCR master mix (Genei, Bangalore, India), 1 pmole of each primer, and 100 ng of template DNA and the amplification was carried out in 0.2 ml PCR tubes, using Master cycler personal (Eppendorf). Thermal cycling conditions were as follows: initial denaturation (3 min at 95 °C), followed by 30 cycles of denaturation (94 °C for 15 s), annealing (55 °C for 30 s), and extension (72 °C for 45 s), with a final extension step for 7 min at 72 °C. The purified amplified product was sequenced by ABI genetic analyser 3130XL.

Phylogenetic analysis

The 16S rDNA sequence of the strain was aligned to sequences retrieved from NCBI databases, using multiple sequence alignment software (CLUSTAL W). A BLAST search of the GenBank database showed sequence similarity to many species of the genus *Streptomyces*. Phylogenetic tree was constructed based on neighbour-joining (NJ) method using MEGA software ver. 4.0 (Tamura et al. 2007). The NJ method was used to infer the evolutionary history of the isolates and the bootstrapping was carried out using 1000 replications. *Streptomyces tauricus* was taken as

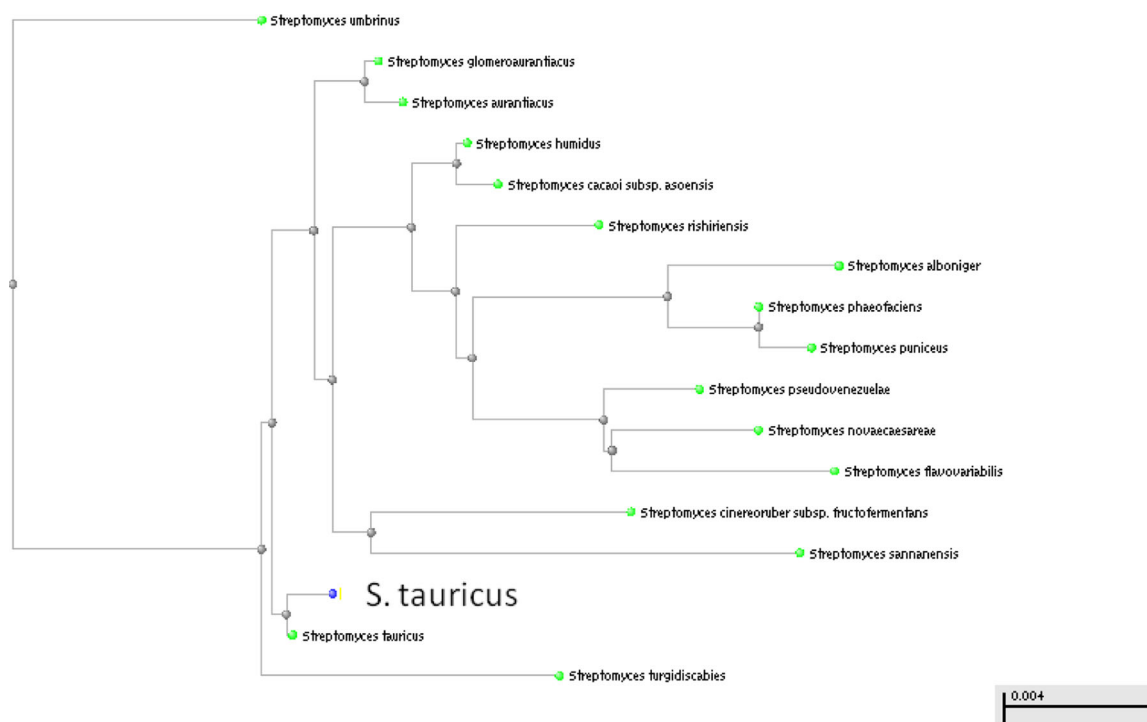


Fig. 1 Phylogenetic tree constructed from sequence alignment of 16S rRNA genes of a *Streptomyces tauricus* and respective related strains (color figure online)

out group (Fig. 1). The sequence was submitted to NCBI GenBank (Accession No KC355418).

Fermentation and organic metabolite extraction

The colonies of the *Streptomyces tauricus* (SRP18) were inoculated into a 250 ml flask containing 100 ml of ISP-2 media. Before sterilization the pH of the medium was adjusted at 7.2 using 1N NaOH solution. This preculture after incubating at 28 °C for 4 days in an incubator with shaking at 180 rpm, was used to inoculate (5% v/v) a total volume of 5 L culture medium having the same composition as the preculture (250 ml in 1 L Erlenmeyer flasks). The flasks were incubated under similar conditions for 10 days.

The culture broth was homogenized with ethyl acetate (1 L) and filtered through a pad of Celite, which was further extracted with ethyl acetate (1 L × 2). The crude extract was passed through the Na₂SO₄ and concentrated under reduced pressure. The concentrated extract (0.3 g) was subjected to column chromatography on silica gel (100–200 mesh), using a stepwise gradient of methanol in chloroform to give enriched fractions (1–5), which was further purified by preparative thin layer chromatography (MeOH:CHCl₃, 10:90) afforded 12.9 mg (yield 4.3% of crude extract) of isolated compound that was biologically active. The purity of the isolated compound was 96% and was subjected for identification.

Cytotoxic activity

The cell lines (HeLa, PC-3, THP-1 and Caco-2) were purchased from European Collection of Cell Culture, fetal bovine serum, Roswell Park Memorial Institute—1640 medium (RPMI-1640), minimum essential medium (MEM), penicillin G, streptomycin, and trypsin—ethylenediamine-tetraacetic acid were obtained from Invitrogen Corporation (USA). 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Aldrich Corp. (St. Louis MO). Cells were cultured in RPMI-1640/MEM containing 10% fetal bovine serum in the presence of 70 mg/L penicillin and 0.1 g/L streptomycin and were incubated at 37 °C with 95% air and 5.0% carbon dioxide. All cells were used in experiments during the linear phase of growth. All the assays were carried out using staurosporine as standard and dimethyl sulfoxide (DMSO) as vehicle.

The cell viability was determined by standard MTT dye uptake method (Koppikar et al. 2010). HeLa, PC-3, THP, and Caco-2 cells (3×10^3) cells/well were plated into a 96-well tissue culture plate and treated with different concentration of the isolated compound in triplicates so that the final concentration of DMSO solvent was 0.2%. After 48 h incubation, MTT solution was added and cells were cultured for another 4 h at 37 °C in 5.0% CO₂ incubator. The

amount of coloured formazan derivative was determined by measuring optical density using TECAN microplate reader (Infinite M200 PRO) at 570 nm. The percentage viability was determined according to the protocol described, and the IC₅₀ was calculated by software graph pad prism (Garg et al. 2008).

Clonogenic assay

In this assay 6-well tissue culture grade plates were used and the HeLa cells were plated at a seeding density of (1×10^3 cells/well). The culture medium was changed after 24 h and new medium was added and cells were exposed to various concentrations of isolated compound along with vehicle (DMSO) for 5 days at 37 °C incubator in 5% CO₂. Later on, the obtained colonies were fixed with 4% paraformaldehyde and were stained with 0.5% crystal violet solution. The colonies from the plates were counted and averaged from the observed fields randomly ($n = 3$) and photographed with Olympus c-7070 wide 700 M inverted microscope camera (Lotan et al. 1985).

Scratch motility (wound healing) assay

The assay performed was same as described above, except HeLa cells were seeded in a 6-well plate at a concentration of (5.5×10^5 cells/well) and allowed to form a confluent monolayer for 24 h, it was then serum starved for 24 h. After that the monolayer was scratched with a sterile pipette tip, washed with serum free medium to remove floated and detached cells and photographed (time 0 h). Cells were successively treated in medium containing low serum (1.0%) in the presence of different concentrations of isolated compound (2.5, 5.0, and 7.5 μM) along with vehicle (DMSO) for 24 h. Wounded areas were progressively photographed with Olympus c-7070 with 700 M camera (1009 magnification). The percentage of wound closure was estimated by the following equation: wound closure % = $[1 - (\text{wound area at } t_1 / \text{wound area at } t_0) \times 100]$, where t_1 is the time after wounding and t_0 is the time immediately after wounding (Agarwal et al. 2005; Holeiter et al. 2008).

Results and discussion

In our on-going research programme, screening of actinomycetes strains isolated from the soil samples collected from Thajiwas glacier Kashmir-India for their anticancer activities. A potent actinomycete strain coded as SRP18 were selected for the isolation of active principle. The *Streptomyces* strain was identified by 16S rDNA gene sequence alignment with the sequences retrieved from NCBI public databases (<http://www.ncbi.nlm.nih.gov>),

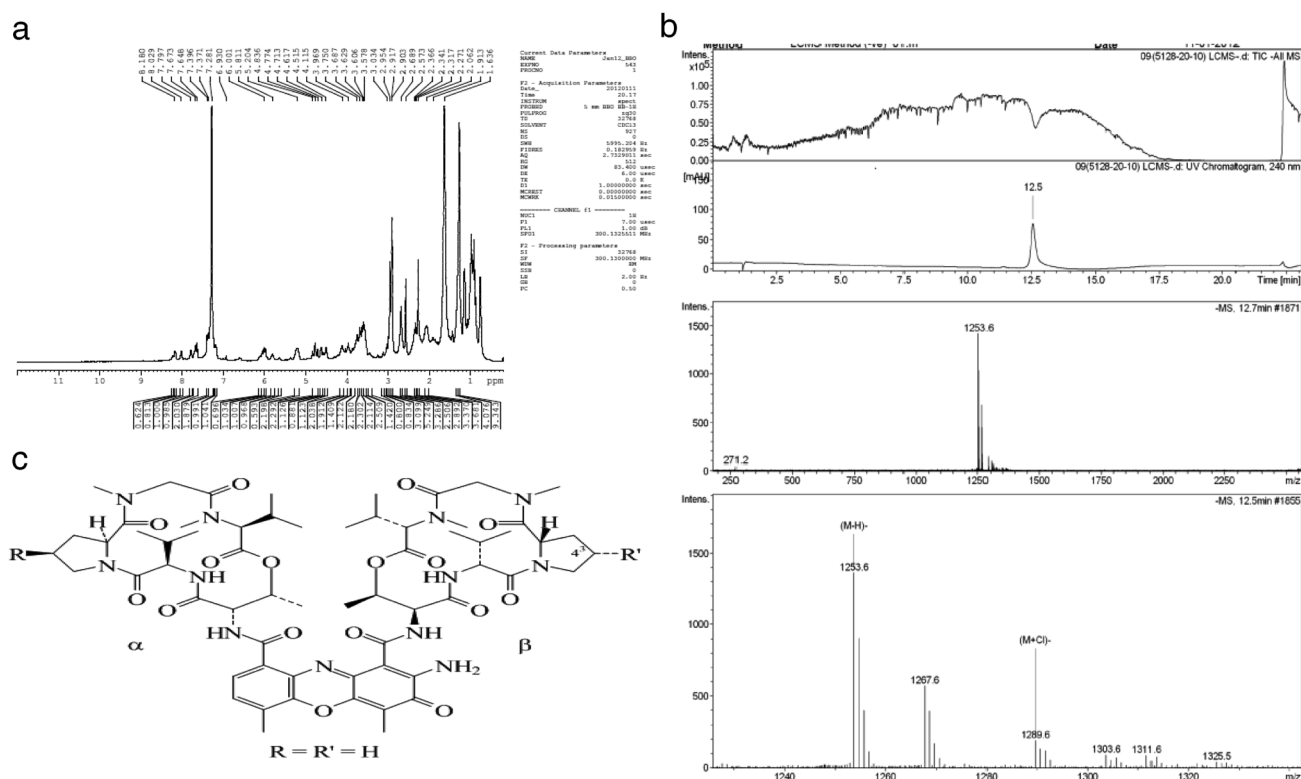


Fig. 2 **a** 300 MHz ^1H NMR of actinomycin D. **b** IR,HPLC, LS-MS/MS of Compound actinomycin D. **c** Structure of isolated compound

analysis indicated that the strain belongs to *Streptomyces tauricus*.

Determination of structure of active compound

In the present study a potent molecule, was isolated from ethyl acetate extract of selected *Streptomyces* strain. The active Compound was characterised by using extensive spectroscopic data. Active Compound was isolated as yellow amorphous powder. The ESIMS of compound yielded molecular ions with a peak at (m/z 1253.6 $[\text{M}-1]^+$), which was further supported by ^1H NMR established as $\text{C}_{62}\text{H}_{86}\text{N}_{12}\text{O}_{16}\text{S}$ showed molecular mass similar to actinomycin-D. Infrared absorptions implied the presence of hydroxyl (3399 cm^{-1}) and carbonyl (1654 cm^{-1}) functionalities (Figs. 2a, b, c). Furthermore similar ^1H NMR spectral data of isolated compound which confirmed the structure of isolated compound was actinomycin-D on the basis of existing literature (Booth et al. 1976).

The cytotoxic activity of compound was evaluated for percentage in vitro growth inhibition against four human cancer cell lines, namely, prostate (PC-3), cervical (HeLa), Leukemia (THP-1), and colon cancer (Caco-2) by using the MTT assay. The results are expressed as the concentration inhibiting 50% of cell growth (IC_{50}). The cytotoxicity

Table 1 Antiproliferative activity of the isolated compound on different cell lines by MTT assay

Cell line	IC_{50} (μM)
HeLa (Cervical Cancer)	4.91
PC-3 (Prostate Cancer)	5.63
THP-1 (Leukemia)	7.0
Caco-2 (Colon Cancer)	7.25

results indicated that the compound is most active on cervical cancer cell line (Hela) with IC_{50} 4.91 μM as compared to other cell lines (Table 1). Collectively, antiproliferative results demonstrate that compound is a prospective cytotoxic compound obtained from *Streptomyces tauricus*.

Isolated compound inhibits cell motility and colony formation of HeLa cells

To evaluate the antimetastatic activity of compound, we assessed the effect of compound on the migration by the wound healing assay. Wound healing assays were used to determine whether sub-toxic concentration of isolated compound could inhibit motility of Hela cells. After 48 h, cell monolayer's were wounded, the vehicle (DMSO)

Fig. 3 a, b isolated compound (actinomycin-D) inhibits migration of HeLa cells in a dose-dependent manner measured by using wound healing assay. HeLa cells were treated with different concentrations of isolated compound and observed for migration of cells under microscope after 48 h (color figure online)

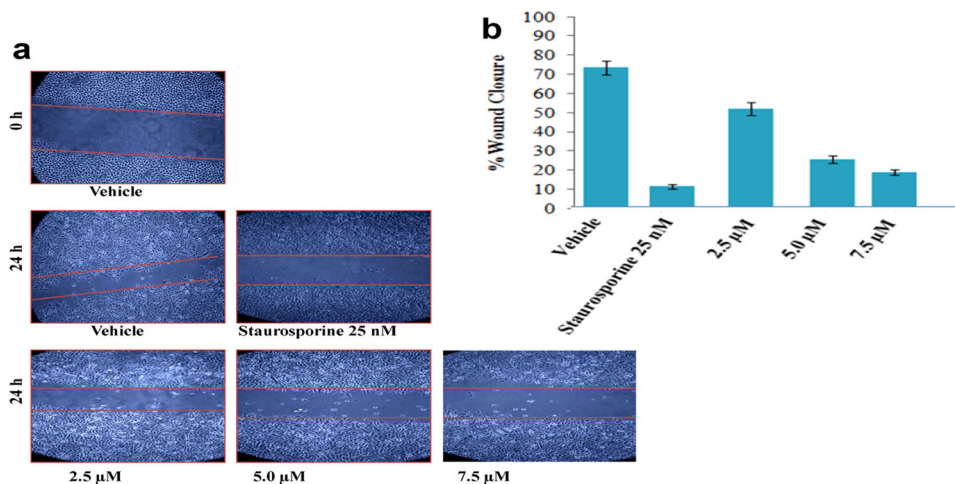
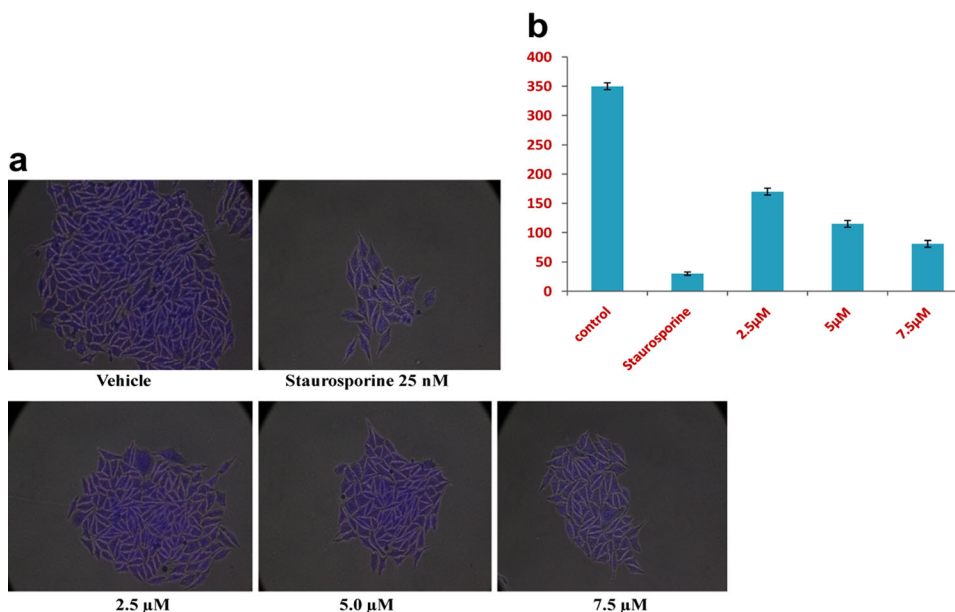


Fig. 4 a, b Isolated compound inhibits the colony formation of HeLa cells in a dose dependent manner. Cells treated with indicated concentration of isolated compound and colony-forming assay was performed as described in materials and methods section (color figure online)



treated cells had completely filled in the cleared area, whereas treatment with 2.5, 5.0, and 7.5 μM of isolated compound significantly ($p < 0.05$) abrogates motility and invasion potential of HeLa in a dose dependent manner as compared to untreated control (Figs. 3a, b).

One of the hallmark traits of cancer cells is their ability to undergo isolated colonial growth. The cytotoxic activity of isolated compound was further shown using a colony formation assay on HeLa cells. Isolated compound causes significant ($p < 0.05$), dose-dependent inhibition of colony formation of cells as compared untreated control (Figs. 4a, b). Thus, isolated compound has distinct cytotoxic cell killing activity in HeLa cells. The compound significantly exhibited cytotoxic effect in a panel of cell lines tested, having IC_{50} within a range of 5.39–8.86 μM (Fig. 5). Collectively, these results (Table 1) demonstrate that compound

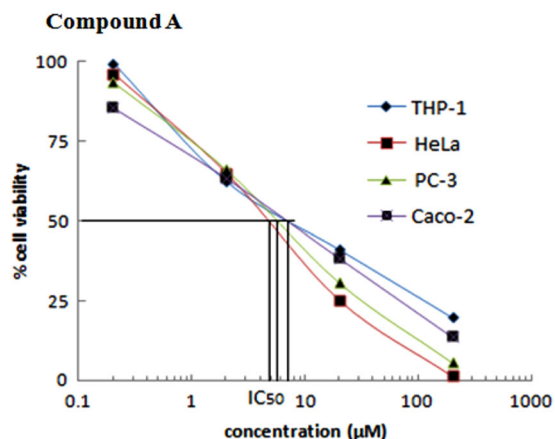


Fig. 5 MTT assay of isolated compound at different concentrations against different cell lines (color figure online)

is a prospective cytotoxic compound derived from microbial source.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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