Isolation and Identification of A Novel Aporphine Alkaloid SSV, An Antitumor Antibiotic from Fermented Broth of Marine Associated Streptomyces sp. KS1908

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Abstract

A marine actinomycete, Streptomyces sp. KS1908 was isolated from a marine sediment sample collected in the Bay of Bengal, India and identified based on morphological, cultural, physiological, biochemical characteristics, along with the cell wall analysis and 16S rRNA gene sequence analysis. Strain KS1908 has more similarities with Streptomyces albus but some variations were observed. A novel aporphine alkaloid SSV was isolated according to bioactivity guided fractionation of fermented broth through solvent extraction and chromatography. Chemical structure of the aporphine alkaloid SSV was elucidated on the basis of spectroscopic analysis, including two-dimensional (2D) NMR and HR-ESI-MS data. Aporphine alkaloid SSV showed significant antibacterial, antifungal activity and also possesses considerable anticancer activity against human larynx (HEp-2), cervical (HeLa), leukemia HL-60 and MCF-7 breast cancer cell lines.

Keywords: Antitumor antibiotic; Streptomyces sp. KS1908; Aporphine alkaloid SSV; Fermented broth

Introduction

Streptomyces have been shown to possess the ability to synthesize antibacterial, antifungal, insecticidal, antitumor [1-5], anti-inflammatory, anti-parasitic, antiviral, anti-infective, antioxidant and herbicidal compounds [1,2,6-9]. Hence, these are widely recognized as industrially important microorganisms [10]. Moreover, approximately 60% of the antibiotics discovered in the year 1990 and most of the antibiotics are from the genus Streptomyces [11]. These characteristics make this genus an important research area. Earlier literature suggests that many antimicrobial molecules have been isolated from Streptomyces albus. Salinomycin, a new polyether antibiotic was produced by strain of Streptomyces albus ATCC 21838 [12]. A new macromolecular peptide antibiotic, named AN-1 was isolated from the culture broth of Streptomyces albus AJ 9003 [13]. An antibiotic complex identical to Paulomycins A and B active against multiple resistant strains of staphylococci and other gram-positive bacteria was isolated from cultures of Streptomyces albus G [14]. In present study, bioactive actinomycete was collected from marine sediment and identified as Streptomyces sp. KS 1908 further responsible antibiotic was isolated and spectroscopic assignment of structure was demonstrated here.

Isolation and Taxonomy

Marine sediment samples were collected at Bay of Bengal near Gangavaram Coast, Visakhapatnam, India. Sample serially diluted to isolate as pure culture on a starch casein agar (SCA-soluble starch, 10.0 g; vitamin free casein, 0.3 g; KNO3, 2.0 g; NaCl, 2.0 g; K2HPO4, 2.0 g; MgSO4•7H2O, 0.05 g; CaCO3, 0.02 g; FeSO4•7H2O, 0.01 g; agar, 20.0 g; sterilized natural aged sea water, 1.0 L; pH 7.2; supplemented with rifampicin 25 µg/ml and cycloheximide 75 µg/ml to inhibit bacterial and fungal contamination, respectively) plate, which had been seeded with a sediment sample suspension and incubated at 28°C for 14 days [10]. The isolated actinomycete colonies being filamentous, compact, often leathery giving a conical appearance, and maintained on YEME (Yeast Extract Malt Extract) slants at 4°C and as a glycerol suspension (20%, v/v) at −20°C [15]. This pure culture was later used for taxonomic and bioactivity studies.

Taxonomic identification was done by physiological conditions, biochemical tests, chemotaxonomic investigations and molecular characterization. Morphological observation through macroscopic based on growth pattern on different media like yeast malt extract agar (ISP-2), Oatmeal agar (ISP-3), Inorganic salts starch agar (ISP-4), Glycerol asparagines agar (ISP-5), tryptone yeast glucose agar, peptone agar, and nutrient agar. The color of aerial mycelium, substrate mycelium and soluble pigment were observed by naked eye. Optical and Scanning electron microscopies (JSM-6610LV, JEOL Ltd.) were used for microscopic observations. Organism growth conditions were studied on SCA at various pH, Temperature and NaCl levels for physiological characterization [16]. Organism was biochemically characterized using the tests viz., Enzymes, H2S production tests, Carbon and nitrogen utilization tests using different substrates12. Cell wall chemical composition was demonstrated according to the procedures of Lechevalier [17] for chemotaxonomic investigation17. Molecular characterization was done by 16s rRNA gene sequencing [16,18,19].

Fermentation

Sporulated isolate prepared with sterile water according to 0.5Mc Farland standard. The resulted spore suspension at 10% level was transferred aseptically into a 250 ml Erlenmeyer flask containing 45 ml of the inoculation medium [8]. The flasks were inoculated and incubated at 28°C for 48 hrs at 120 rpm. Thoroughly washed pellet containing cell

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mass was suspended in sterile 0.9% sodium chloride solution and used as inoculum was then transferred to the modified production medium [8] and incubated at 30°C for 96 hrs at 160 rpm on a rotary shaker. The fermented broth was used for extraction of the active principle.

Bioactivity guided fractionation and purification

The fermented broth was aseptically collected in a sterile centrifuge tube and centrifuged at 4000 rpm for 15 min at 4°C. The culture filtrate (supernatant) and mycelial pellet obtained were extracted separately for identification of the active principle source. Antibiotics from the cell mass were isolated usually by extraction with polar and non-polar solvents while that from the fermented medium were extracted by solvent extraction only when the antibiotic has a reasonably high degree of solubility in non-polar organic solvents. Bioautography was performed to identify bioactive fraction. The other alternative technique for the separation of bioactive principle from the culture filtrate is the adsorption of the compound on some inert material like silica [20].

Compound identification and Structure elucidation

Thin Layer Chromatography (TLC) was analyzed on the glass percolated silica gel plates GF254, and spots were checked by UV light, Iodine and spraying with 10% sulfuric acid in methanol followed with heating. The melting point was determined on Fisher-Johns melting point apparatus. FT-IR spectra were recorded on a Perkin-Elmer spectrophotometer with KBr pellet. The sample was scanned between 400 and 4000 cm⁻¹ wave number. High Resolution Mass Spectrum (HRMS) was recorded on QSTAR XL. HYBRID MS System and EI MS was recorded on VG 7070H (70 eV). The NMR data of purified compound was acquired using an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). ¹H NMR spectra were obtained at 400.13 MHz and ¹³C NMR spectra were obtained at 100.6 MHz. All NMR spectra were recorded in DMSO-d₆. The chemical shifts were expressed in δ (ppm) using DMSO-d₆ as solvent and TMS as internal reference. Dragendorff’s reagent test was used for the identification of alkaloid [21].

Antimicrobial assay

Anti-microbial studies were carried out on clinical isolates of human pathogenic bacteria and dermatophytic fungi, Salmonella typhi, Vibrio cholerae, Shigella dysenteriae, Enterococcus faecalis are gastrointestinal pathogens, which were collected at King Gorge Hospital, Visakhaptnam, India. Proteus vulgaris NCIM 2813, Pseudomonas aeruginosa NCIM 5031 cultures were collected from NCL, Pune, India. Staphylococcus aureus MTCC 7443, Bacillus subtilis MTCC 8141, Aspergillus niger MTCC 6484, Aspergillus awamori MTCC 7711, Candida albicans MTCC 1346, Trichophyton rubrum. MTCC 3272 cultures were collected from Indian microbial technology, India. Clinical isolate of Candida albicans was collected from skin lesions. Zone of inhibitions were determined using agar well diffusion method ad Minimum Inhibitory Concentration (MIC) was done by broth dilution assay. Microbial broth cultures (Mueller Hinton broth for bacteria, Sabouraud Dextrose broth for fungi) were adjusted to the absorbance to 0.6 (Optical Density at 620 nm) in Spectrophotometer according to CLSI guidelines. These cultures were used as Inoculums. The agar plates were prepared by pour plate method using 20 ml of sterilized agar medium (MH agar for bacteria, SD agar for fungi). The sterile agar medium was cooled to 45°C and mixed thoroughly with 1ml of growth culture of concerned test organism (inoculum) and then poured into the sterile petri dishes and allowed to solidify. Wells of 6 mm size were made with sterile cork borer and test compounds were added. The agar plates were incubated at for 4 days at 28°C for fungi, 24 hours at 37°C for bacteria. Zone of inhibitions were measured by Himedia milli meter zone reader. MIC was performed on broth media (10 ml) containing 1000-1 µg/ml of test compound prepared by 10 fold dilution. 0.1 ml of culture inoculums was added. The MIC was determined at which concentration of compound causes nil absorbance (no growth) in the spectrophotometer at 620 nm. All the experiments were conducted according to Clinical Laboratory Standard Institute. Ciprofloxacin (for bacteria) and fluconazole (for fungi) were antibiotics used as positive control [22-26].

MTT assay for anticancer activity

The cytotoxic activities of the compound (SSV) isolated from Streptomyces sp. KS1908, were tested against human larynx carcinoma cells HeLa, cervical cancer (HeLa), human leukemia HL-60 and MCF-7 breast carcinoma cell lines. This cell line was obtained from TRIMS and National Centre for Cell Sciences (NCCS), India further cultured at 37°C with 5% CO₂, using MEM(minimum essential medium) medium. MTT assay [8] was used to study the cytotoxic properties of the sample. 200 µl of cell culture (2x10⁴ cells/ml) was added in each well containing 100 µl MEM medium in a 96 well plate. After 24 hrs of incubation 20 µl of different test concentrations like 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml were added to the respective wells. After incubation of 4 days at 37°C temperature and 5% CO₂ in a CO₂ incubator. 20 µl of MTT (3-(4,5- dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide) reagent 5mg/ml concentration, was added to each well and incubated for 4hrs. Then the medium was carefully discarded and the Formazan complexes formed in the cells were dissolved in 200 µl of DMSO (Dimethyl sulfoxide). The rate of color appearance was measured at 570 nm in a spectrophotometer. The percent of cell inhibitions were calculated based on control and test absorbance values. Results were expressed as IC₅₀, means concentration of compound where 50% of cancer cell growth inhibition occurred. The experimental measurements were made in five replicates. Camptothecin was used as the standard [27,28].

Molecular docking

Topoisomerase I (PDB ID 1T8I) and II (PDB ID 2XCT) crystal structures of proteins were obtained from Protein Data Bank. Co-crystallized ligands and water molecules are removed from target protein using Argus lab. Ligands are prepared using Chemoffice (Cambridge). Energy minimization was done using molecular mechanics. The minimized was executed until root mean square value reached smaller than 0.001 Kcal/mol. Such energy minimized ligands and receptor used for docking studies using Molegro Virtual Docker [29].

Results

Isolation and Taxonomy of isolated marine actinomycete

The isolated bioactive actinomycete colonies being filamentous, compact, often leathery giving a conical appearance, dry surface on SCA, which can easily be distinguished from fungi and non filamentous bacteria. Morphological and cultural observations of the isolate grown on different ISP media given in Table 1, revealed that vegetative mycelium showed yellow-brown color, aerial hyphae were abundant, well-developed with white color on different test media and substrate mycelium with pale yellow color. It didn’t produce any pigments but faint yellow color pigmentation on Yeast-malt extract agar (ISP-2). The scanning electron micrograph of the strain KS1908 revealed that aerial mycelia were monopodially branched with compact spirals of sporophore terminating in long open coils. Each spore chain consisted
of 8-20 white, oblong to cylindrical shaped spores, 0.6 ÷ 0.7 x 0.8 ÷ 0.9 µm in size, having smooth surface (Figure 1). The chemotaxonomic investigations revealed that the cell wall peptidoglycan of isolate contained L-diaminopimelic acid and glycine. This indicates that isolate belongs to cell wall type I which is characteristic of the genus Streptomyces.

16S rRNA gene sequence analysis (Genbank accession no. KC556777) and other cultural, biochemical physiological, chemotaxonomic characteristics revealed that Strain KS1908 has close similarities with Streptomyces albus [12] (Figure 2 and Tables 1 and 2) but some variations were observed so named as Streptomyces SP. KS 1908.

Bioactivity guided fractionation and purification

Fermented broth of sterptomyces sp. KS1908 was extracted using various solvents but only ethyl acetate extract showed bioactivity. Then ethyl acetate extract was run by chromatography using silica gel to obtain bioactive fraction II (255 mg), which was further fractionated with Sephadex LH-20 column and separated into five major fractions that included with fraction IIc. Preparative reverse phase HPLC was used to get light brown colored pure bioactive compound SSV in FIic2 fraction. Schematic representation of detailed fractionation and purification was given in Figure 3.

Structural elucidation of aporphine alkaoid SSV

Compound SSV was obtained as light brown needles having melting point 68–72°C; [α]D 28.0 (c = 1.5, MeOH). Dragendorff’s reagent test showed positive [16]. The molecular formula of SSV was assigned as C82H80NO4 from its elemental and mass spectral analyses (HRMS: M/z 326.1386 [M+H]+, 348.1206 [M+Na]+). This was corroborated by the decoupled [13] C-NMR spectrum which showed signals for all the nineteen carbones of the molecule (Table 3). The mass fragmentation pattern of compound SSV showed typical of aporphine alkaoid [17]. The IR absorption bands at 3430 (OH), 945 (-O-CH2-O-) cm-1 indicated the presence of hydroxyl and methylenedioxy groups.

The proton NMR spectrum of compound SSV showed three, one proton singlet peaks at δ 7.55, 6.75 and 6.57 corresponding to H-11, H-8 and H-3 respectively of an aporphine alkaoid (Table 3). The two, one proton singlet peaks at δ 6.11 and 5.97 indicated the presence of one methylenedioxy group on C1-C2 and two, three proton singlet peaks at δ 3.77 and 2.42 attributed to one methoxyl group and one N-methyl group, respectively. The proton NMR spectrum also showed a one proton singlet peak at δ 9.19 attributed to hydroxyl group. Further the correlations observed in HMQC, HMBC and HSQC confirmed that methylenedioxy group was present at C-1 and C-2 carbons, methoxyl group was present on C-9 and free hydroxyl group was present on C-10 (Table 3). Thus from the foregoing spectral studies, the structure of compound SSV was established as 10-hydroxy-9-methoxy-1,2-methylenedioxy-6-methyl-4,5,6,6a-tetrahydro-7H,6-azabenzanthrene (Figure 4).

Antimicrobial and anticancer activities of aporphine alkaoid SSV

Aporphine alkaoid SSV showed good antimicrobial activity especially on multi drug resistant clinical isolates including bacteria and fungi. Aporphine alkaoid SSV was more effective against bacteria than fungi.

As shown in Table 4, zone of inhibition found to be between 9-14 mm at 30 µg of compound. MIC range found to be between 1-100 µg/ ml. 14 mm was the inhibitory zone showed by SSV on S. typhi and with lowest MIC of 1 µg/ml. Aporphine alkaoid SSV showed potent antibacterial activity against both gram positive and gram negative bacteria. Compound SSV showed very effective activity against gastrointestinal pathogenic bacteria (S. typhi, V. cholerae, E. faecalis and E. coli). Dermatophytic fungi (T. rubrum and C. albicans) showed slight resistance. Aporphine alkaoid SSV showed comparable antimicrobial potency with ciprofloxacin and flucanozole (antibiotics).

Anticancer activity of aporphine alkaoid SSV

Anticancer activity of compound SSV performed by MTT assay and found significant cytotoxic activity on human larynx (HEp-2), cervical (HeLa), human leukemia HL-60 and MCF-7 breast cancer cell lines with the IC50 values of 1.10, 1.13, 2.85 and 4.44 µg/ml, respectively. Aporphine alkaoid SSV showed comparable potency with camptothecin (Table 5).

Docking of aporphine alkaoid SSV

Molecular docking studies of aporphine alkaoid SSV were on topoisomerasuses using Molegro Virtual Docker. Docked energy or binding energy was inversely proportional to affinity of compounds towards enzyme. Lower binding energy indicated higher binding affinity. -98.4 kcal was the binding energy of aporphine alkaoid SSV on topo II, which was less than docked score of ciprofloxacin (-79.7 Kcal/mol). Aporphine alkaoid SSV showed comparable binding energy (-78.6 Kcal/mol) with camptothecin (-77.1 Kcal/mol) on topoisomerase I. Figure 4, demonstrated that Compound SSV binding interactions
Figure 2: Phylogenetic tree of obtained by distance matrix analysis of 16s rRNA gene sequence of closely related streptomyces sps. obtained from BLAST results and constructed using neighbor-joining method, showing phylogenetic position of Streptomyces sp. KS1908 with yellow marking.

Figure 3: Isolation and purification procedure of compound SSV.
isolated aporphine alkaloid SSV can be promising agent for treatment characterized through advanced spectroscopic data. Therefore, the isolated from bioactive fraction of fermented broth and chemically and characterized from marine associated actinomycetes further isolated from the culture filtrate of II inhibition [30]. Pimprinine, an extracellular alkaloid has been special class of antitumor antibiotics which act through topoisomerase II inhibition [30]. Pimprinine, an extracellular alkaloid has been isolated from the culture filtrate of Streptomyces CDRIL-312 [31]. Alkaloid group of aporphine antibiotics are topoisomerase I inhibitors from Streptomyces sp [32].

In conclusion, bioactive streptomyces sp. KS1908 was isolated and characterized from marine associated actinomycetes further novel aporphine alkaloid SSV is an antitumor antibiotic which was isolated from bioactive fraction of fermented broth and chemically characterized through advanced spectroscopic data. Therefore, the isolated aporphine alkaloid SSV can be promising agent for treatment of cancer and microbial infections.

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