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Antitubercular activity of the pigment from forest soil *Streptomyces* sp SFA5

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Abstract

Extracellular pigment from the forest soil *Streptomyces* sp SFA5 was produced by submerged fermentation using yeast extract malt extract broth. Crude pigment from the medium was extracted using ethyl acetate. Antitubercular activity of the pigment was tested against *M. tuberculosis* H37Rv by microplate alamar blue assay and luciferase reporter phage assay. The pigment was also tested for inhibitory activity against *M. tuberculosis* lysine aminotransferase by colorimetric method. In both microplate alamar blue and luciferase reporter phage assay, the crude pigment showed activity against *M. tuberculosis* H37Rv at 125 and 250 µg/mL concentration, respectively. The *M. tuberculosis* lysine aminotransferase was inhibited at the IC_{50} value of 4.5 µg/mL concentration.

Introduction

The incidence of emerging infectious diseases in humans has increased within the recent past or threatens to increase in the near future. *Mycobacterium tuberculosis* still remains a deadly pathogen two decades after the announcement of tuberculosis as a global health emergency by the World Health Organization. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360,000 of whom were HIV-positive (WHO report, 2014). In last few years new drug combinations have shown promising potential to significantly shorten tuberculosis treatment times. However, there are very few new chemical entities being developed to treat this global threat (Riccardi and Pasca, 2014). Hence, there is a dire need for new chemical entities to develop as potential antitubercular molecules.

Microorganisms of both terrestrial and marine origins have proven to be excellent sources of novel natural products. Programs aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years (Busti et al., 2006). Among the microbial sources, Actinobacteria are the well-explored source in terms of secondary metabolites especially antibiotics. The diversity of Actinobacteria in various natural including extreme and man-made environments is well documented. They are the most economically valuable prokaryotes which are well-known to produce chemically diverse metabolites with a wide range of biological activities (Berdy, 2015; Velho-Pereira and Kamat, 2013). From January 1990 to December 2012, in total 949 anti-mycobacterium natural products were reported in the literature (Dashti et al., 2014). Recently the rate of discovering new compounds from terrestrial actinobacteria has decreased but the rate of re-isolation of known actinomycetes and antibiotics is on the increase.

Hence researchers are now searching rare ecosystems, instead of normal terrestrial sources, such as marine, mountain and forest ecosystems for Actinobacteria for potentially new biosynthetic diversity (Lam, 2006; Berdy, 2012). With this view, the present study was

attempted to study the antitubercular activity of forest soil *Streptomyces* sp SFA5.

Materials and Methods

Description of Streptomyces sp *SFA5*

Streptomyces sp SFA5 was isolated from the soil sample collected from Sabarimalai forest (Western Ghats), Kerala, India using starch casein agar medium. The soluble yellow pigment produced by the strain SFA5 on yeast extract malt extract agar showed good activity against bacterial pathogens. The viability of strain SFA5 was maintained on ISP2 agar slants as well as in 30% glycerol broth.

Production and extraction of pigment

Spores of *Streptomyces* sp SFA5 was transferred into 50 mL of YEME broth and incubated in a rotary shaker at 28°C for 48 hours. About 10% of inoculum was transferred into each 100 mL of YEME broth and incubated at 28°C for 120 hours. After incubation, the cell-free supernatant was prepared by centrifugation at 5,000 rpm for 30 min. The pigment present in the cell free supernatant was extracted by liquid-liquid extraction using ethyl acetate at 1:1 ratio. Then the ethyl acetate portion was concentrated and dried using rotary evaporator (Radhakrishnan et al., 2007).

Microplate alamar blue assay

Briefly, the inoculum was prepared from fresh LJ medium resuspended in 7H9 medium, adjusted to a McFarland tube number 1, and diluted 1:20; 100 µL was used as inoculum. Each drug stock solution was thawed and diluted in 7H9-S at 4-fold the final highest concentration tested. Serial 2-fold dilutions of crude pigment were prepared 6.125 to 500 µg/mL concentration directly in a sterile 96-well microtiter plate using 100 µL 7H9-S. A growth control containing no antibiotic and a sterile control was also prepared on each plate. Sterile water was added to all perimeter wells to avoid evaporation during the incubation. The plate was covered, sealed in plastic bags and incubated at 37°C in normal atmosphere. After 7 days of incubation, 30 µL of alamar blue solution was added to each well, and the plate was re-incubated overnight. A change in color from blue (oxidized state) to pink (reduced) indicated the growth of bacteria, and the MIC was defined as the lowest concentration of drug that prevented this change in color (Samala et al., 2014).

Luciferase reporter phage assay

Antitubercular activity of pigment was also studied against standard laboratory strain *M. tuberculosis* H37Rv by adopting luciferase reporter phage assay (Radhakrishnan et al., 2014). About 350 µL of G7H9 broth supplemented with 10% albumin dextrose complex and 0.5% glycerol was taken in cryovials and added with 50 µL of crude extract in order to get the final concentration of 250 µg/mL and 500 µg/mL. One hundred microliter of *M. tuberculosis* cell suspension was added to all the vials. DMSO (1%) was also included in the assay as a solvent control. All the vials were incubated at 37°C for 72 hours. After incubation, 50 µL of high titre phage phAE129 and 40 µL of 0.1M $CaCl_2$ solutions were added to the test and control vials. All the vials were incubated at 37°C for 4 hours. After incubation, 100 µL from each vial was transferred to luminometer cuvette. About 100 µL of D-luciferin was added and relative light unit (RLU) was measured in luminometer. RLU reduction by 50% or more when compared to control was considered as having antitubercular activity.

%RLU reduction = Control RLU − Test RLU / Control RLU × 100

Lysine aminotransferase enzyme inhibition assay

MTB lysine aminotransferase activity was determined by the detection of piperidine 6-carboxylate. The crude pigment was tested at <1 to 100 µg/mL concentration. Briefly, 15 mM enzyme solution was added to 1.0 mL 200 mM phosphate buffer pH 7.2 containing 1 mM L-lysine–HCl, 1 mM a-ketoglutarate and 15 mM PLP. The mixture was incubated at 310 K for 1 hour. The reaction was terminated by the addition of 500 mL 10% trichloroacetic acid in ethanol. Piperidine 6-carboxylate was detected by measuring the color intensity of its adduct with o-aminobenzaldehyde spectroscopically at 465 nm (Samala et al., 2014).

Results

Streptomyces sp SFA5 showed good growth with soluble yellow pigment production on YEME broth. In microplate alamar blue assay, the growth of *M. tuberculosis* H37Rv was inhibited at 125 µg/mL concentration. In luciferase reporter phage assay, the crude pigment showed 60 and 74% reduction in RLU at 250 and 500 µg/mL concentration, respectively. Results of both the method showed that the yellow pigment produced by *Streptomyces* sp SFA5 was active against *M. tuberculosis* H37Rv. Standard drugs isoniazid and rifampicin showed more than 95% reduction in RLU in luciferase reporter phage assay. In enzyme inhibition assay, the MTB-LAT enzyme was inhibited by 4.98 µg/mL concentration of crude pigment.

Discussion

The majority of natural product collections usually start as crude extracts of fresh or dried material processed by different methods using various chemical solvents. In the present study, antitubercular activity exhibited by the crude pigment extracted from the cell-free

supernatant indicated its extracellular nature. Most of the actinobacterial compounds are extracellular in nature (Radhakrishnan et al., 2011). Further, action-bacterial pigments are reported to exhibit wide range of biological activities such as antibiotic, anti-cancer, antifungal etc.

The inability to convert target inhibition into growth inhibition and eventually to bacterial cell death, which bedevils the target-driven approach, is circumvented by identifying compounds with potent antitubercular activity by whole-cell screening, which is clearly a feasible starting point (Manjunatha and Smith, 2015). In the present study, crude pigment exhibited good antitubercular activity in both rapid and whole-cell screening based methods such as microplate alamar blue assay and luciferase reporter phage assay. Previously there are many authors reported the anti-tubercular activity of natural products by microplate alamar blue assay. However, the luciferase reporter phage assay was rapid which produced results in 3 days when compared to microplate alamar blue assay which took 7-10 days to produce results.

Lysine aminotransferase in *M. tuberculosis* is an important enzyme for its long-term persistence. It is implicated in mycobacterial stress response and is up-regulated approximately 40-fold in nutrient-starved models designed to mimic the persistent/latent state of tuberculosis. It catalyzes an overall reaction involving the transfer of the epsilon-amino group of L-lysine to a-ketoglutarate to yield L-glutamate and alpha-amino-adipate-D-semialdehyde and then to piperidine 6-carboxylate. In this study, lysine aminotransferase inhibition activity of crude pigment was determined by the detection of reaction product piperidine 6-carboxylate by measuring the color intensity of its adduct with o-aminobenzaldehyde (Samala et al., 2014). The low IC_{50} value exhibited by the crude pigment indicated that this pigment will be a promising candidate to fight against latent tubercle bacilli also.

Crude natural product extracts are complex mixtures of perhaps hundreds of different compounds working together in synergy when the extract is administered as a whole. Discovery of natural product hits and their progression towards development includes extraction of the crude extract from the source, concentration, fractionation and purification to yield a single bioactive compound (Nguta et al., 2015).

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