

Cytotoxic and apoptotic activity of leinamycin produced by *Streptomyces atroolivaceus* THS-44 isolate from Iraqi soil

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ABSTRACT

Leinamycin is a thiol dependent DNA alkylating agent which shows very potent activity against various cancer cell lines. This natural compound forms guanine adducts (N7) in DNA which are converted into a basic sites and simultaneously generates Reactive Oxygen Species (ROS), to produce DNA strand breaks in human cancer cells. In present study, eight different strains isolated from Iraqi soils were taxonomically assigned as Streptomyces.atroolivaceous. Remarkably the strain named as THS-44 was distinguished in productivity in comparison with other strains; the amount of leinamycin was 50.98 mg/l. In this study, we assessed the cytotoxic activity of leinamycin against RD and ANM3 cancer cell line in compare with REF cell line as a normal control. Leinamycin revealed a potent activity against growth of both RD and AMN3 cell lines with inhibition rate (93.56% and 86.67%) respectively at 100mg/ml as a highest concentration. Moreover, leinamycin from THS-44 strain was mediate apoptotic effects on cancer cells without affecting on the normal cell line REF; a clear cut difference in the percentage of fragmented DNA was found between both RD and AMN3 malignant cells, and REF normal cell line (89.16, 83.1466, and 13.75; $p < 0.001$) respectively after exposure to 4.5 μ g/ml of leinamycin for 24hrs of incubation period.

Key words: Leinamycin, *S.atroolivaceous*, Antiproliferation, Natural product, Apoptosis.

INTRODUCTION

For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases [1]. Among 43,000 bioactive natural products, approximately 10,000 are bioactive metabolites produced by produced by Actinomycetes. It was reported that 6,550 are produced by *Streptomyces* species [2, 3]

Leinamycin (LNM) is natural product produced by several *Streptomyces atroolivaceus* species which is characterized by an unusual 1,3-dioxo-1,2-dithiolane moiety that is spiro-fused to a 18-member macrolactam ring, a molecular architecture that has not been found to date in any other natural product [4]. Early studies by the discoverers of leinamycin showed that attack of the thiol on the 1, 2-dithiolan-3-one 1-oxide in leinamycin initiates conversion of heterocyclic to a 1, 2-oxathiolan-5-one, which undergoes further rearrangement to an episulfonium ion [5]. This episulfonium ion associates no covalently with duplex DNA and alkylates the N7-position of guanine residues in double-stranded DNA in highly efficiency [6].

In addition to the DNA alkylation activity described above, attack of a thiol on leinamycin releases a persulfide intermediate (RSSH) that can mediate generation of reactive oxygen species (ROS) in vitro [7], intracellular generation of ROS cause DNA strand cleavage and lead to cell death via general oxidative stress. Cells that are actively respiring such as tumor cells, appear to be more susceptible to reactive oxygen species (ROS) generated by leinamycin [8]. Thus, this activity makes leinamycin an important and promising compound in biotherapy. Accordingly, this work was aimed to isolate leinamycin-producing wild type strain of *S. atroolivaceous* THS-44 and investigating its toxicity on RD cell line.

MATERIALS AND METHODS

Bacteria

Streptomyces atroolivaceous THS-44 strain was isolated from soil samples collected at various area of Baghdad - Iraq.

Taxonomic Studies: Growth characteristics and carbohydrate utilization were determined by methods of International Streptomyces Project (ISP-4). Color codes were assigned to the substrate and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago) [4].

Culture condition: Fermentation studies were conducted by using a high producing strain, THS-44. Seed media (glucose 10g, soluble starch 10g, Bacto Tryptone 5g, yeast extract 5g, beef extract 3g, CaCO₃2g per liter of deionized water, pH 7.2 prior to sterilization) was inoculated with a loopful of vegetative mycelium from starch-casine agar slant. The seed medium was incubated at 22°C for 48 hours then transferred into a fermentation medium. Basal components of the fermentation media were KH₂PO₄0.5 g, MgSO₄-7H₂O 0.5g, ZnSO₄-7H₂O 20mg, vitamin B12 0.1 mg and CaCO₃ 5g per liter of deionized water. The pH of media was adjusted to 7.0 prior to sterilization [9]. Filtration was carried out through cotton wool and followed by centrifugation at 5000 rpm for 15 minutes [10]. Culture growth was evaluated by centrifuging of untreated fermentation broth in 10-ml conical tubes at 1,200 x g for 10 minutes. The packed cell solids were reported as % of total broth volume.

Extraction: The clear filtrate was adjusted at PH value (2) and extraction process was carried out using Ethyl acetate solvent at the level of 1:1 (v/v), the mixture was shaken vigorously for 1 hour for complete extraction. The organic phase was concentrated to dryness under vacuum using a rotary evaporator at a temperature not exceeding 50°C [11].

Purification by Column Chromatography: purification of the antimicrobial compound was carried out using silica gel column (2.5 X 50) chromatography. Chloroform and Methanol 10:2 (v/v), was used as an eluting solvent. The column was left overnight until the silica gel (Prolabo) was completely settled. One-ml crude extract to be fractionated was added on the silica gel silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antimicrobial activities [12].

Cell Culture

A plevic rhabdomyosarcoma is a human cancer cell line (RD) with 239 passages and Mouse mammary adenocarcinoma cancer cell line (AMN3) were used throughout this study, it was propagated and maintenance on minimal essential medium (MEM) (US Biological, USA). Rat Embryo Fibroblast (REF), was used as as a normal control. The propagation and maintenance of REF carried on RPMI-1640 medium [13].

Measurement of Cytotoxicity of leinamycin

The cytotoxic activity of leinamycin produced by THS-44 strain was examined according to the percent of inhibition rate (%IR) [14]. 2×10^4 of exponentially growing cells were seeded in 96- well microcultureplates with various LNM concentrations (0.781, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µg/l) in a volume of 100µl. After incubation period (24, 48, and 72hrs) at 37°C, a 20µl of cell's pigment was added to each well, and then the plates were incubated at 37°C. The absorbance of each concentration was carried out in triplicate in addition to untreated cell as a control. The inhibitory rate of cell proliferation was calculated according following equation: %IR= A-B/A×100; A where represents the absorbance of control, while B represents the absorbance of treatment [15, 16].

Determination of IC 50

The results of inhibitory rate (%IR) were plotted on X- axis. While the concentrations of leinamycin were plotted on Y-axis. From the linear scale we could be measure the IC₅₀ of leinamycin [17].

Quantitation of DNA Fragmentation

5×10^5 cell/ml density of both RD and AMN3 cells were treated with selected concentration of leinamycin that obtained from IC₅₀ and incubated 48hrs. Then resuspended in 0.8ml of 10mM phosphate buffer saline (PBS), PH.4, and 0.7ml ice cold lysis buffer. The lysate cells transferred to microfuge tubes and centrifuged at 13000g at 4°C for 15min to separate fragmented DNA from high molecular weight DNA. Thereafter, the fragmented DNA was suspended in 1.5ml TE buffer; also 10% of TCA was added and incubated at 25°C for 10min. The centrifugation was done again at 500g for 15min and calculate the supernatant. Then, the precipitate was resuspended in 10% TCA and boiled at 100°C for 15min. Dimethylamine reagent (1ml) was added to the supernatant and incubated at 30°C for 18hrs. The absorbance was measured at 600nm. Finally the percentage of DNA fragmentation was measured according to formula: OD_{600} of the supernatant / [OD_{600} of supernatant + OD_{600} of pellet] x 100 [18].

RESULTS

Leinamycin producing strain was identified as *S. atroolivaceus* depending on morphological and biochemical properties according to International Streptomyces Projects (ISP) [19]. The vegetative mycelium of the isolates grew abundantly on starch-casine agar media, no soluble pigment was produced. The aerial mycelium was gray, white and well developed, branched but not fragmented. It bore chains of 10 to 30 or more spores, which were Rectiflexible. The ability of THS-44 to produce of leinamycin was investigated. An onion white crystal (leinamycin) isolated from 30 liters of culture broth extract. Then, the amount of this agent was determinate; and the results indicated that the wild type strain THS-44 is able to produce 50.98 mg/l of leinamycin.

The cytotoxicity of leinamycin produced by THS-44 strain of *S. atroolivaceus*, against RD, AMN3 cancer cell lines, and REF as a normal cell line was examined. Fig I, shows that the low concentrations (1.56, 3.125, and 6.25 µg/ml) of leinamycin had cytotoxic effect on RD cells, and the inhibition rate was ranged between 42.64-59.98% after 72hrs of incubation period. Whereas increasing concentration of leinamycin results in an increasing inhibitory effect of this antibiotic on the viability of RD cells. At 12.5 µg/ml concentration the IR% was ranged between 76.24%, and 82.83%, after 48hrs, and 72hrs of exposure time respectively, moreover when the concentration was increased up to (25 µg/ml), the IR% increases to 84.55%, and 90.71%, after 48 and 72hrs respectively.

In addition, when the highest concentrations of purified leinamycin (50 and 100 µg/ml) were achieved a significant inhibition on RD growth was seen, the inhibition rate IR% was 93.01% and 93.56%, respectively after 72hrs of incubation period.

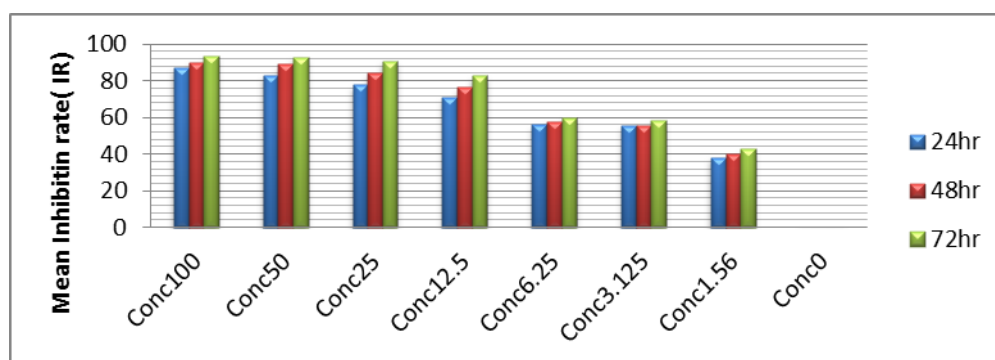


Fig. I. Cytotoxicity of Various Concentrations of leinamycin Produced by *S. atroolivaceus* THS-44 at Different Periods of Incubation on Cancer Cell Line RD

On the other hand, at lower concentrations (1.56 µg/ml) leinamycin had low cytotoxicity 42.66% on viability of AMN3 cancer cell line (Fig II). Whereas at highest concentration of leinamycin the IR% was increased significantly and reach up to 86.67% at 100 µg/ml, after 24hrs of exposure time.

Notable, the cytotoxicity of leinamycin against AMN3 was decreased with increasing the period of exposure time; IR% was 37.25%, and 30.18%, at 1.56 µg/ml, after 48, and 72hrs of incubation period, respectively.

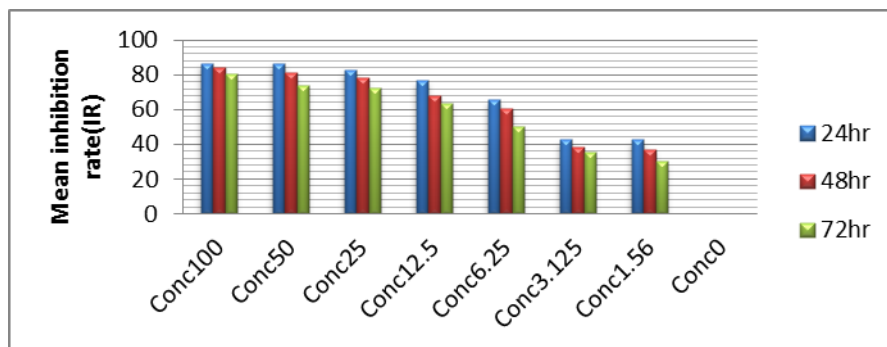


Fig. II. Cytotoxicity of Various Concentrations of leinamycin Produced by *S.atroolivaceus* THS-44 at Different Periods of Incubation on Cancer Cell Line AMN3

In contrast, this agent produced by THS-44 had no effectiveness on the viability of REF cells at all period of incubation (Fig. III).

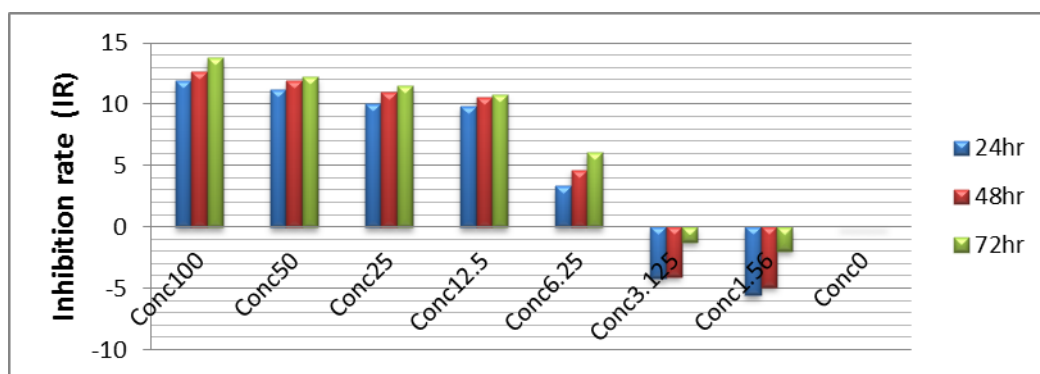


Fig. III. Cytotoxicity of various Concentrations of leinamycin Produced by *S.atroolivaceus* THS-44 at Different Periods of Incubation on Normal Cell Line REF

When leinamycin IC₅₀ dose was calculated, we found that the THS-44 strain IC₅₀ dose was 4.5µg/ml, after 24hrs of incubation. Thus this concentration was used to evaluate the apoptotic activity of leinamycin. The results indicated anti-proliferative activity leinamycin produced by THS-44 achieved high percentage of fragmented DNA in RD and AMN3 malignant cells than normal cell line; the percentage of fragmented DNA was 89.16%, 83.1466% , and 13.75% respectively , keeping in mid this differences was significant according to statistical analysis ($p < 0.001$) (Fig IV, V, and VI).

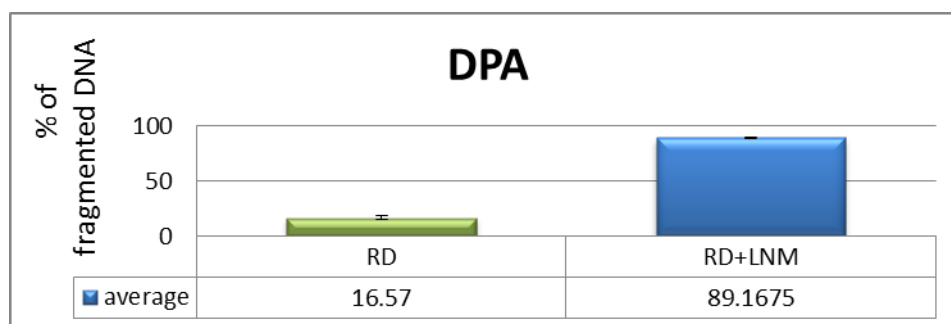


Fig. IV. The Percentage of DNA Fragmentation of leinamycin Produced by *S.atroolivaceus* THS-44 on cancer cell line RD

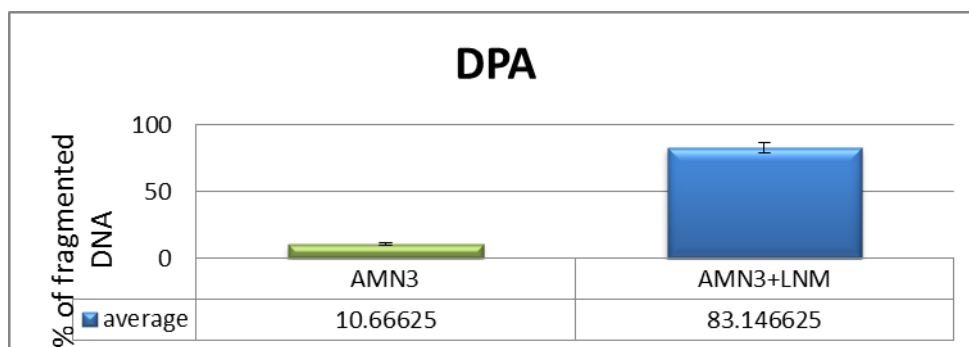


Fig. V. The Percentage of DNA Fragmentation of leinamycin Produced by *S.atroolivaceous* THS-44 on cancer cell line AMN3

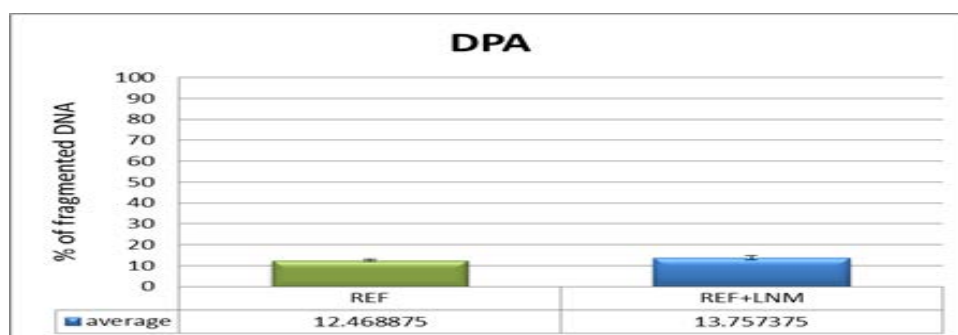


Fig. VI. The Percentage of DNA Fragmentation of leinamycin Produced by *S.atroolivaceous* THS-44 on normal cell line REF

DISCUSSION

In the present study, about nine isolates were found to produce leinamycin, THS44 produce LNM better than other isolates and it kept production in large amounts constantly, as well as this isolate was found to have a better growth, and was stable in the production in solid and liquid media in comparison with other isolates. Indeed, such differences in the productivity may return to the genes encoding leinamycin which might be more active in this strain than other isolates, as well as, soil nature and its component because THS44 was isolated from dry soil, as well as the temperature, and pH of soil also play a role in this variation [20, 21], for this reason THS44 isolate was chosen to conduct other steps in this study. Since, current study revealed that leinamycin produced by THS-44 had significant toxic effect on RD and AMN3 cells ($p < 0.001$), at concentrations ranged between (6.25 to 100 $\mu\text{g/ml}$) and, the inhibition rate (IR) at higher concentration (100 $\mu\text{g/ml}$) was (93.5% and 86.67%, respectively). Thus, our findings indicated that leinamycin induced highly DNA breaks in cancer cell line (RD and AMN3) in comparison with normal cell line REF.

As shown above, the inhibition activity of leinamycin on both cell lines was varied due to several reasons such as sensitivity of treated cells used in this study, and the ability of cancer cell lines to adsorb leinamycin according to the type of cells and metabolism behaviors. Moreover, we can speculate that, the reduction in the percentage of inhibition rate in AMN3 cell line after long incubation period might be related to dividing of unaffected cells when the killing activity of leinamycin was diminished.

The mechanism responsible for this antitumor activity of leinamycin could be explained according to different mechanisms. One of such mechanisms is a remarkable rearrangement reaction, in which leinamycin was initially converted by reaction with cellular thiol to the oxathiolanone intermediate molecule. This molecule is responsible for covalent modification of DNA [22]. The electrophilic sulfur of the oxathiolanone group then reacts with the C6-C7 alkane of the antibiotic's 18-membered macrocycle to yield an episulfonium ion, which enters cells and triggers DNA damage [22]. Moreover, leinamycin is capable of forming guanine adducts (N7) in DNA that are converted into basic sites and simultaneously generate reactive oxygen species (ROS) to produce DNA strand breaks, which are considered to be the primary mechanisms of action responsible for its antitumor effect of leinamycin [23]. As well as thiol-independent DNA alkylation by leinamycin is initiated by attack of water (or hydroxide) on the C3'-carbonyl of the antibiotic lead to a cascade of reactions finally leading to yield the episulfonium ion. Which causes conformational change in the cancerous cell DNA. Since, leinamycin is capable of forming guanine adducts (N7) in DNA that are converted into basic sites and simultaneously generate reactive oxygen species (ROS) to produce

DNA strand breaks [24, 25], which considered to be the primary mechanisms of action responsible for its antitumor effect of leinamycin [26].

CONCLUSION

In this study *Streptomyces atroolivaceus* was successfully isolated from Iraqi soil. THS-44 strain was recorded to have high productivity of leinamycin. Our findings confirm that Purified leinamycin has potent cytotoxicity on RD and AMN3cancer cell lines, and its effect was found to be concentration dependent manner. Leinamycin mediated cytotoxicity by promoting the activation of leinamycin into its DNA reactive form. Furthermore, we have established a strong link between the DNA-damaging properties of leinamycin and its apoptotic activity through inducing DNA fragmentation in RD and AMN3cancer cell lines.

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