Biomedicine & Pharmacotherapy 108 (2018) 216–223

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Culture filtrate ether extracted metabolites from *Streptomyces levis* ABRIINW111 increased apoptosis and reduced proliferation in acute lymphoblastic leukemia

Behnaz Valipour[a,b,c], Seyede Momeneh Mohammadi[a], Ali Abedelahi[c], Behnaz Faramarzian Azimi Maragheh[a], Elahe Naderali[a], Alireza Dehnad[d], Hojjatollah Nozad Charoudeh[e,*]

[a] Stem cell research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Department of Anatomical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[d] Agriculture Biotechnology Research Institute for Northwest and West of Iran, East Azarbijan, Tabriz, Iran
[e] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

ARTICLE INFO

Keywords:
Acute lymphoblastic leukemia (ALL)
Metabolites
Streptomyces levis ABRIINW111
Proliferation
Cell cycle

ABSTRACT

Despite the advances in the discovery of various types of anticancer drugs for curing acute lymphoblastic leukemia (ALL), their toxicity and unfavorable side effects remained as big limitations for therapeutical applications. In this regard, natural products such as *Streptomyces* -derived agents have shown potential applications as anticancer drugs. The present study deals with evaluating the anti-carcinogenic activity of the ether extracted metabolites derived from *Streptomyces* on nalm-6 and molt-4 ALL cell lines.

MTT assay was performed to evaluate the cytotoxicity effect of *Streptomyces* sp on nalm-6 and molt-4 cell lines. Apoptosis and proliferation were evaluated by Flow cytometry. Quantitative real-time RT-PCR (qRT-PCR) and western blot were performed to investigate the effect of these metabolites on the mRNA and protein expression levels of P53, Bax, and Bcl2.

In both cell lines, extracted metabolites significantly inhibited cell growth and increased apoptosis. Although P53, Bax mRNA and protein expressions were increased, Bcl-2 expression decreased in treated cells compared with control. In addition, the G0/G1 arrest of Nalm-6 cells was induced.

These findings of this work show that the ether-extracted metabolites from *Streptomyces levis* ABRIINW111 can be used as an anti-carcinogenic for acute lymphoblastic leukemia cells.

1. Introduction

Acute lymphoblastic leukemia (ALL) as a lymphoid neoplasm originates from B and T-lineage progenitor cells [1]. Although the survival rate of children improves approximately 90% with the better outcome in B -ALL than T-ALL, it remains poor in adults and reaches 45% [2].

Demand for finding novel drugs against cancers is increased typically due to relapse of chemotherapeutic drugs. In this connection, high toxicity of chemotherapy drugs and their unfavorable side effects remain a problem [3]. Nowadays, using natural products for inducing apoptosis in cancer cells without serious side effects have attracted great attention of scientists [4].

Actinomycetes as one of the most valuable prokaryotes belonging to the phylum Actinobacteria have been considered responsible for the production of secondary metabolites used as antibiotic compounds and antitumor agents [5]. Chemical compounds such as actinomycetes are responsible for providing more potent, cheaper, and safer natural anticancer compounds. *Streptomyces* sp. is one of the largest genera among actinomycetes produce about 80% of the bioactive secondary metabolites [6]. Because of these secondary metabolites, streptomycetes have been known as a source of antibiotics [7] and a resource of important clinical antitumor drugs such as doxorubicin, actinomycin, D, rapamycin, mithramycin, neocarzinostatin, antimetabolite's carzinophilin and mitomycins [8,9].

Rapamycin as an anti-cancer agent, isolated from the *Streptomyces hygroscopicus*, can induce apoptosis and arrest the cell cycle in a

* Corresponding author at: Cell Therapy Research Laboratory, Drug Applied Research Center, Tabriz University of Medical Sciences, 5166614711 Tabriz, Iran.
E-mail address: nozadh@tbzmed.ac.ir (H. Nozad Charoudeh).

https://doi.org/10.1016/j.biopha.2018.09.050
Received 7 July 2018; Received in revised form 7 September 2018; Accepted 8 September 2018

different type of cancer cells [10]. Pure cytotoxic compound (PCC) extracted from *Streptomyces* sp. SY-103 has apoptotic effects on human leukemia cell lines through activation of caspase3 and inactivation of Akt signaling [11].

Spicamycin and its derivative KRN5500, as productive compounds from *Streptomyces* genus, markedly induced apoptosis with down-regulation of Bcl-2 expression and modulation of promyelocytic leukemia (PML) protein in myeloid and lymphoid leukemia cells [12]. Streptochlorin Isolated from *Streptomyces* sp induces apoptosis in human hepatocarcinoma cells through the mitochondrial pathway [13]. Novel benzyldihydroxyoctenone derivative (F3-2-5) affects human cervical adenocarcinoma HeLa cells with a reduction in cell growth and induction in apoptosis via the p53-dependent pathway [14]. Marinomycins as polyketide compounds isolated from actinomycetes have shown potent antitumor activity against different human melanoma cell lines [15]. Nonactin, as another compound isolated from *Streptomyces* sp., has shown anticancer properties against mammalian cell lines [16]

Sadigh-Eteghad et al. reported a new strain of *Streptomyces* isolated from the Zagros Mountains Hamadan, Iran. This strain produces secondary metabolites against Gram-positive and Gram-negative bacteria. In this regard, there is a 98% similarity between *Streptomyces* sp. and ABRIINW111 with S. Levis strain NRRL B-16370 [17]. In the present study, the cytotoxic activity of ether-extracted metabolites of *Streptomyces* sp. strain ABRIINW111 was studied by investigating the cell growth, cell cycle assessment, proliferation, and induction of apoptosis. To further explore the intracellular signal transduction pathways involved in regulating apoptosis, the levels of mRNA and protein in P53, Bax, and Bcl2 were evaluated.

2. Material and method

2.1. Metabolites extraction

The isolate, molecular identification, and extraction of metabolites from *Streptomyces* strain ABRIINW111 were prepared by Sadigh-Eteghad et al., in the laboratory [17]. Briefly, the soil samples were cultivated from the northwest of Iran in 2010. For isolation of actinomycetes, serial dilution from 10^{-1} to 10^{-4} was prepared and cultured in nutrient agar medium for 7 days at 27–29℃. After 7 days, white colonies – morphological characteristics of *Streptomyces* – were cultured in nutrient agar or starch casein agar medium for 5 days at 27℃. Then, the loop full of bacteria was inoculated in ISP medium (international *Streptomyces* project medium) while agitating at 125 rpm for 3–4 days at 27°C. The turbidity of the suspension was spectrophotometrically adjusted to match the transmittance of empty medium turbidity. As previously described, the turbidity 620 nm–0.08 O.D. was considered suitable for the inoculation [17]. After fermentation time, 1 ml of preculture was used to inoculate 1000 ml Erlenmeyer flasks, each containing 150 ml of fresh Mueller Hinton Broth medium, and shacked at 70 rpm for 96 h at 27℃. Next, the bacterial culture medium was centrifuged at 4000 rpm for 20 min. The supernatant was cultivated and mixed with an equal volume (1:1) of diethyl ether. Erlenmeyer flasks were agitated in a shaker incubator for 1 h at 175 rpm at 27℃. Finally, the obtained organic extract was undertaken to be concentrated at room temperature to achieve a 0.01-gr crude extract, which was kept at 4℃ until used [17].

Extracted metabolites were analyzed by High-Pressure Liquid Chromatography (HPLC) method. Briefly, dried metabolites dissolved in acetonitrile and 1 µl solution was injected into the C18 column (250 mm × 4 mm). The mobile phase consisted of methanol, H_2O, and acetonitrile (45%, 50%, and 5%). The flow rate of solution was set as 1 ml/min for 10 min and peak responses were measured at 215 nm. Also, the sample was analyzed using Gas Chromatograph Mass Spectrometer (Agilent, 19091S-433). The column was HP-5MS capillary column (30 m long and 0.25 mm int.dia). The column temperature was programmed in an oven with 60℃ to 300℃ with an increase rate of 4.5℃/min.

2.2. Cell culture and MTT assay

Nalm-6 (B ALL) and Molt-4 (T ALL) cell lines were purchased from National Cell Bank of Iran (Pasture Institute, Tehran, Iran). RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin was used for cell culture. Cells were cultured in the T-25 flask (1.5×10^5 cells/cm^2) and incubated at 37℃ with 5% CO_2 in humidity for 7 days. The medium was replaced with a fresh medium every 3–4 days. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) test was used to determine the effective concentration of bacterial metabolites. For this purpose, 3×10^4 cells were seeded in 96-well plates with 100 µl culture medium containing different concentrations of bacterial metabolites (0.1, 0.5, 1, 2, and 5 µg/ml), with less than 0.1% Dimethyl sulfoxide (DMSO) and incubated for 24, 48 and 72 h. Afterward, the methylthiazol tetrazolium bromide (M6494/sigma, the final concentration of 5 mg/ml) was added to each well and cells were further incubated at 37℃ for 4 h. For solubilization, 100 µl of the 10% sodium dodecyl sulfate (SDS) /0.01 N HCl was included and incubated overnight. The optical density of each well was measured in an ELISA reader (Biotek ELx 808, USA) at a wavelength of 570 nm.

2.3. Antibody staining and flow cytometry analysis

Nalm-6 and Molt-4 cells were plated in 6-well culture plates at a density of 3×10^5 cells/well with different concentrations of metabolites including 1, 2, 3, 4, 5 and 6.5 µg/ml. Cell-cycle analysis was performed as follows: Cells were suspended in 1 ml phosphate-buffered saline (PBS) containing 1 µl Triton X-100, 5 µl (50 mg/ml) RNase A, and 50 µl propidium iodide (1 mg/mL) for 30 min. To evaluate Annexin V expression, cells were incubated for 20 min at room temperature with Annexin V (88-8005-11, eBiosciences, San Diago.CA). For intracellular staining for caspase3 and Ki67 expression, cells were resuspended in 0.5 ml of the Cytofix/Cytoperm solution, incubated 20 min at 4℃, washed with Perm/wash solution, and incubated with either anti-Caspase3 (5168655X, eBioscience) or anti- Ki-67 (12-5699-42, eBioscience) for 20 min at room temperature.

Flow cytometry analysis was done by BD FACS Calibur (BD Biosciences) and data were analyzed by FlowJo (7.6.1) software.

2.4. Fluorescent-based staining

Acridine orange/ethidium bromide double staining was performed to identify apoptosis following treatment with a distinct concentration of metabolites. Briefly, Nalm-6 and Molt-4 cells were seeded at a density of 3×10^5 cells/well and incubated at 37℃ in 5% CO_2 for 48 h with extracted metabolites. Then, the cells were suspended in 1 ml PBS and stained with 1 µl Acridine Orange/ethidium bromide (100 µg/ml) for 5 min. Afterward, they were transferred to glass slides and covered by glass leads. Finally, cells were observed with a fluorescent microscope.

2.5. RNA extraction, cDNA synthesis, and Real-time PCR amplification for the mRNA level expression

About 3×10^5 Nalm-6 and Molt-4 cells were incubated with a concentration of 3 and 6.5 µg/ml extracted metabolites for 48 h. Total RNA was extracted using Thermo Scientific Gene JET RNA Purification Kit (K0731) according to the manufacturer's instruction. DNase I Amplification Grade Kit (Invitrogen, Paisley, Scotland) and 1 µg RNA were used for the first strand of cDNA synthesis (K-2261-6, Bioneer, Korea) in a total volume of 20 µl. Real-time PCR was performed on the Corbett Rotor-Gene™ 6000 HRM (Corbett Research, Australia) with Power SYBR Green master mix (2x) (TaKaRa Ex Taq HS, Japan), 0.4 µM of each primer, cDNA (20 ng/µl), and nuclease-free water. The PCR cycling program was 10 min at 95℃, 15 s at 95℃, 35 s at 59℃, and 20 s