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Could eumelanin molecule from *Streptomyces parvus* BSB49 strain be a potential anticancer agent for lung cancer?

Hamit Emre Kizil[1], Sinan Bayram[1], Serdar Ozturk[2], Umit Karakas[3]

[1]*Bayburt University, Vocational School of Health Services, Department of Medical Services and Techniques, Bayburt, Türkiye*
[2]*Bayburt University, Vocational School of Demirozu, Department of Veterinary Medicine, Bayburt, Türkiye*
[3]*Bayburt University, Vocational School of Health Services, Department of Pharmacy Services, Bayburt, Türkiye*

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Abstract

Melanins, which are divided into five different subgroups as eumelanin, pheomelanin, allomelanin, neuromelanin and pyomelanin, are a heterogeneous and amorphous biopolymer family. Although melanin is produced in many different ways today, it is more practical, and more economical to produce melanin using bacterial strains. The eumelanin pigment, which is the subject of this study, was obtained using *Streptomyces parvus* BSB49 strain, and its antiproliferative effect at various concentrations against small cell (DMS-114) and non-small cell (H-460) lung cancer was determined. According to our results, the synthesized eumelanin molecule was statistically cytotoxic on non-small cell lung cancer cell line (H-460) at concentrations of 3.125 and 1.56 µg/mL after 24 hours of treatment (IC50=8 µg/mL) and only at a concentration of 3.125 µg/mL after 48 hours of treatment (IC50=8.4 µg/mL). In the small cell lung cancer cell line (DMS-114), no statistically cytotoxic concentration was detected ($p<0.05$).

Keywords: Eumelanin, H-460, DMS-114, lung cancer

Introduction

Smoking and tobacco use are two of the main risk factors for developing lung cancer, which is the most common cause of cancer-related deaths globally [1,2]. The etiology of disease is also significantly influenced by genetic factors, nutrition, air pollution, and occupational exposure [3-5]. Clinical, histopathological, and neuroendocrine traits are used to separate lung cancer into two groupings. Between 15% and 20% of all lung tumors are small cell lung cancers (SCLC), which are aggressive. Patients with SCLC have a terrible prognosis, with a 5-year survival rate of fewer than 5%. The majority of lung cancers (80–85%) are non-small cell lung cancers (NSCLC), which are further categorized into big cell carcinomas and squamous cell carcinomas [6]. Adenocarcinoma, which makes up 60% of NSCLC, is the most prevalent kind, while squamous cell carcinoma, which makes up about 25% to 30% of lung malignancies, is the second most prevalent type. A rare and aggressive tumor with the potential for quick development and spread is large cell carcinoma [7,8].

Melanins are a family of heterogeneous and amorphous biopolymers. Commonly found in microorganisms, plants and animals, these organic pigments include 5.6-Dihydroxyindole (DHI), 5.6-dihydroxyindole-2-carboxylic acid (DHICA), 1.8-dihydroxy naphthalene (1.8-DHN), catechol, caffeic acid, chlorogenic acid, protocatechuic acid, gallic acid, and homogentisic acid (HGA). A lot of research has been done, up to a point, to synthesize natural pigments instead of synthetic pigments. Plants, animals and microorganisms have all been used for this purpose, but numerous studies have found microorganisms to be much more suitable and compatible than other living things. Microbial pigments have fundamentally great

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Corresponding Author: Hamit Emre Kizil, Bayburt University, Vocational School of Health Services, Department of Medical Services and Techniques, Bayburt, Türkiye
Email: ekizil@bayburt.edu.tr

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production properties such as repeatability and manageability as well as lasting regardless of geographic boundaries and season [9-11]. This pigment group is divided into five different subgroups as eumelanin, pheomelanin, allomelanin, neuromelanin and pyomelanin and different biochemical pathways are used in the production process of these organic pigments [12]. One of the main functions of this high molecular weight pigment group is to protect living organisms from the negative effects of low wavelength rays such as X-rays and UV rays. These organic pigments, which are composed of phenolic and indolic monomers, also reduce the negative effects of free radicals caused by these rays. Melanins play a role in the reduction of reactive oxygen species (ROS) and prevention of DNA damage thanks to their antioxidant properties [13]. Melanins determine hair, eye and skin color in humans. In animals, these organic pigments contribute to body temperature regulation [14,15] and play a critical role in camouflage [16].

Many studies to date have shown that melanins have antioxidant, anti-microbial, antiviral, anti-inflammatory, anti-proliferative, radioprotective, photoprotective, and liver-protective properties which is the potential to be widely used in fields such as cosmetics, medicine and pharmacology [9,17-19]. In addition to this feature, as their ability to absorb X-rays at high levels, melanin pigments have the potential to be used to prevent or minimize DNA damage during radiotherapy [10,20-22].

In this context, we aimed to determine the antiproliferative effect of six different concentrations of eumelanin pigment purified from *Streptomyces parvus* BSB 49 strain on lung cancer cells (H-460 and DMS-114).

Material and Methods

Ethical approval

In this study, no living tissue was isolated from humans or animals, and the cells used were commercially purchased. Ethical approval was approved by the Bayburt University Non-Invasive Clinical Research Ethics Committee (2023-8/2).

Eumelanin production

Extracellular eumelanin pigment was produced using *Streptomyces parvus* strain BSB49. For this purpose, this strain was inoculated in ISP 2 medium by streak plate method and incubated for 48 hours at 35°C. At the end of the incubation period, samples taken from a single colony were transferred to ISP 1 (Tryptone-Yeast Extract Broth) medium. This sample was incubated for 24 hours at 35°C and used as an inoculant in the later stages of the study. For pigment production, 150 mL of sterile ISP 1 (Tryptone-Yeast Extract Broth) medium was prepared in 250 mL flasks. These prepared media were cooled at room temperature to approximately 35°C. With the help of a sterile micropipette, 1 mL of the previously prepared inoculant was taken and transferred to the ISP 1 medium (prepared before in 250 mL flasks). After these procedures, the inoculated flasks were incubated for one week in an orbital shaker (35°C, 200 rpm) (Heidolph | Memmert – Germany). At the end of the one week incubation period, it was observed that the samples turned dark black and produced extracellular eumelanin pigment. These eumelanin solutions obtained were transferred to sterile 50 ml Falcon tubes and centrifuged at 10.000 rpm for 10 minutes (Beckman Coulter | Life Sciences – USA). After centrifugation, the bacterial biomass was separated from the solution and the same centrifugation was repeated for the second time. Then, the pH value was adjusted to 2 by adding 6M HCl to the supernatant. For the polymerization of the eumelanin pigment, the solution was left at room temperature for 24 hours. After 24 hours, this solution was centrifuged once again for 10 minutes at 10.000 rpm (Beckman Coulter | Life Sciences – USA), the supernatant was once more discarded, and the resultant eumelanin was twice rinsed with distilled water. Final centrifugation of the eumelanin sample was performed at 10,000 rpm for 10 minutes. Finally, the purified eumelanin was lyophilized and stored at -20°C [17,20].

Cell culture and viability analysis

H-460 (Non-small cell lung cancer) and DMS-114 (Small cell lung cancer) cell line was selected for cell culture and purchased (American Type Culture Collection | ATCC–USA). The medium created by adding RPMI 1640 medium (Gibco | Thermo Fisher Scientific - USA) and 10% FBS (Gibco | Thermo Fisher Scientific - USA) in 25 ml flasks was used for cell proliferation. During cell transfer, trypsin-EDTA solution (Gibco | Thermo Fisher Scientific - USA) was used after washing with PBS (Merck | KGaA-Germany) buffer in order to obtain viable cells at the base of the flask. The flasks were incubated for 24-48 hours in an incubator containing 5% CO_2 at 37°C. Cells were checked for viability with trypan blue dye (Gibco | Thermo Fisher Scientific-USA) before each passage. After the cells in the flask were centrifuged, the suspension was poured and the cells were counted on the thoma slide by staining with trypan blue dye. Tetrazolium salts are organic compounds of heterocyclic structure. These salts are reduced by gaining electrons and transform into formazan structure, creating a color change, this change occurs as a result of the active mitochondria breaking the tetrazolium rings [23]. Since this ability is not present in dead cells, there is no color change formation [24]. As a result, the quantity of formazan dye generated is closely correlated with the quantity of live cells. Dehydrogenases in cells degrade WST-8 to a substance that is orange in color and soluble in cell culture media. To this end, the commercially available kit (CVDK-8 | Ecotech Biotechnology® -Turkey) was used to analyze WST-8 a tetrazolium salt [25].

Statistical evaluation

The data from the cytotoxicity research, which was conducted three times, are presented as mean standard deviation. The Student's t test was used to examine the data by the GraphPad Prism statistical program, and $p<0.05$ was considered significant.

Results

The goal of this study was to show that our previously synthesized eumelanin molecule had lethal effects at low doses on both small

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cell lung cancer (DMS-114) and non-small cell lung cancer (H-460) cell lines. The concentrations tested were 25, 12.5, 6.25, 3.125, 1.56 µg/mL. Although 24 hours of eumelanin treatment resulted in a decrease in cell viability at all concentrations in the H-460 cell line, statistically only concentrations of 3.125 and 1.56 µg/mL were considered cytotoxic ($p<0.05$). With the cell viability, IC50 concentration of eumelanin was determined as 8 µg/mL. In DMS-114 cell line, it was statistically determined that none of the concentrations tested were cytotoxic ($p<0.05$) (Table 1).

Table 1. Cell viability results of 24th hour treatment (Viability%)

24th hour	H-460	DMS-114
	Mean±SD	Mean±SD
Control	100±0.8	100±9.1
25 µg/mL	91.18±5.3	158.25±4.0
12.5 µg/mL	79.58±3.9	125.75±4
6.25 µg/mL	64.50±6.4	128.20±2.8
3.125 µg/mL	59.86±8.0*	109.50±9.6
1.56 µg/mL	65.66±3.9*	96.93±3.2

SD±: standart deviation, viability%: cell viability detection Kit-8 (CVDK-8) results, Mean: the average of the test results performed with 3 repetitions, (*$p<0.05$)

In the 48-hour eumelanin treatment, only a concentration of 3.125 µg/mL was statistically tested as cytotoxic in the H-460 cell line ($p<0.05$). With the cell viability, IC50 concentration of eumelanin was determined as 8.4 µg/mL. No cytotoxic concentration was found in the DMS-114 cell line (Table 2).

Table 2. Cell viability results of 48th hour treatment (Viability%)

48th hour	H-460	DMS-114
	Mean±SD	Mean±SD
Control	100±5	100±6
25 µg/mL	112.07±3.5	99.05±4.3
12.5 µg/mL	95.78±3.8	109.61±0.4
6.25 µg/mL	101.52±3.5	106.46±7.3
3.125 µg/mL	88.63±2*	113.87±4.3
1.56 µg/mL	93.78±3	95.26±7.6

SD±: standart deviation, viability%: cell viability detection Kit-8 (CVDK-8) results, Mean: the average of the test results performed with 3 repetitions, (*$p<0.05$)

Discussion

The most prevalent kind of melanin, eumelanin, is a pigment found naturally in human skin, hair, and eyes [26]. All living things have melanin, an old pigment, since very early on. Melanin is frequently praised for its exceptional capacity to absorb a variety of radiations. Moreover, many creatures that live in poor environmental conditions are thought to use melanization as a survival tactic. Due to the pigment's versatility, it has been found to be effective as an organic semiconductor, an antioxidant, a radical scavenger, a photoprotector that effectively absorbs and dissipates solar radiation as heat, and an absorber that chelates metals. In addition to serving these purposes, melanin is produced naturally by the majority of species, making it eco-friendly and biocompatible [27]. Melanin pigment-producing microorganisms have the ability to survive in extreme environments that emit high levels of radiation and use this radiation to perform radio-synthesis [28]. They have the ability to absorb a wide part of the electromagnetic spectrum and the ability to absorb X-rays, UV rays, visible region rays and radio waves gives this pigment a great advantage for use in nanotechnology and materials science.

In the literature, it is seen that many research teams have obtained melanin molecules using microorganisms and tried to elucidate the biological activities of this molecule.

Researchers discovered that melanin pigment obtained from *Aspergillus bridgeri* ICTF-201 strain exhibited high free radical scavenging activity in 2011 [29]. In another study, it was found that melanin pigment obtained from *Pseudomonas stutzeri* strain had antiproliferative effect on A549 and HeLa cell lines [30]. In 2017, a new strain identified as *Streptomyces glaucescens* NEAE-H was isolated and the *in vitro* anticancer activity of the melanin pigment obtained from this strain was determined against skin cancer and the IC50 value was calculated as 16.34±1.31 µg/ml [31]. In another study, melanin molecule obtained from *Schizophyllum commune* species was found to have antioxidant, antibacterial and cytotoxic effects against Human Epidermoid Larynx Carcinoma Cell Line (HEP-2) [32]. Bayram, Dengiz [9] were assessed eumelanin's *in vitro* antiproliferative properties on the HeLa cell line. Cell vitality, cell index, and mitotic index were among the parameters evaluated during the course of these investigations. 10 µM eumelanin IC50 concentration was found using cell viability and cell index data. Although there are a few biological activity studies in the literature, there is no study on the cytotoxic activity of eumelanin pigment synthesized from *Streptocyses parvus* BSB49 strain in small cell and non-small cell lung cancer cell lines. This study is valuable in terms of showing that eulmelanin has cytotoxic activity in H-460 cell line.

In the literature, there are very few studies on obtaining melanin from *Streptocyses parvus* BSB49 strain. One of the most valuable studies on this subject is a toxicology study on experimental animals. It was shown that eumelanin molecule synthesized from *Streptocyses parvus* BSB49 strain had a protective effect against diethylnitrosamine-induced liver damage in rats [17].

Conclusion

Supporting this discovered cytotoxic effect of the eumelanin molecule obtained from *Streptocyses parvus* BSB49 strain with *in vivo* anticancer studies and conducting activity studies in different cancer lines will increase the medical value of this molecule over the years.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Ethical approval was approved by the Bayburt University Non-Invasive Clinical Research Ethics Committee (Protocol No: 2023-8/2).

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