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Cytotoxic effect of a 3-(4-chlorophenyl)-5-(4-methoxybenzyl)-4*H*-1,2,4-triazole derivative compound in human melanoma cells

Abstract. Melanoma represents the most forceful derm cancer with a high rate of mortality. Although chemotherapy has been commonly used in the treatment of melanoma, drug resistance and side effects of conventional chemotheurapeutics negatively affect the continuity and success the treatment. 1,2,4 triazole derivatives are popular compounds of recent years with anti-microbial, anti-inflammatory, analgesic, anti-viral, anti-proliferative and COVID-19 associated anti-fungal activities. However, studies that revealed the effects of 1,2,4 triazole compounds on melanoma cells are limited. The aim of this study was to investigate the effects of a 3-(4-chlorophenyl)-5-(4-methoxybenzyl)-4*H*-1,2,4-triazole derivative (B9) compound on cytotoxicity and cell cycle using MTT and flow cytometry, respectively and the inhibitory effect on tyrosinase in human melanoma (VMM917) cells using colorimetric assay for melanin content. The compound B9 exhibited a selective cytotoxic effect (4.9-fold) on VMM917 cells compared to normal cells. B9 induced cell cycle arrest at the S phase and also decreased the amount of melanin in the cells. The results suggest a novel candidate drug in melanoma therapy. The detailed investigation of the molecular mechanism of this selective cytotoxic effect will provide understanding on the usability of B9 as an alternative chemotherapeutic agent in melanoma.

Key words: apoptosis, cell cycle, cytotoxicity, melanoma, triazole compound.

Introduction

Today cancer is the second disease with the highest mortality in the world. This also threatens the future with the increasing number of cases as the average number of five-year cancer diagnosis reported to be 43.8 million. Skin cancer has increased rapidly in the last 20 years and the number of melanoma cases is expected to increase gradually each year [1; 2]. This increase is supposed to be associated with environmental conditions such as UV light, and the increase in global warming and the changes in climate change around the globe also affect the level of UV exposure [3; 4]. Despite the rapid increase in the number of cases, skin cancer is the second as to have a high chance of survival among cancer types. Skin cancer is derived from malignant cells in the surface layer of the skin [2, 3]. Melanoma is specifically characterized with an increase in the amount of melanin within melanocytes [5]. Tyrosinase (oxidoreductase, EC 1.14.18.1) is the lock enzyme responsible for the biosynthesis of melanin pigment in melanocytes [6; 7]. There are a range of therapeutic approaches in the treatment of melanoma including chemotherapy, photodynamic therapy, immunotherapy, targeted therapy or enzyme inhibition therapy. Conventional chemotherapy has been frequently used especially in the form of advanced melanoma, but the development of resistance against chemotherapeutic drugs over time and/or the possible mutagenic effect of chemotherapeutics on healthy cells can decrease the success of therapy [3; 8]. The side effects of chemotherapeutic drugs used in cancer treatment on patients constantly lead researchers to the discovery of new drugs. In recent years, a line of drug design studies has been focused on triazole compounds regarding their potential to be used in the treatment of various diseases. In particular, 1,2,4 triazole derivatives have widely pharmacological treatmentes, such as anti-inflammatory, analgesic, anti-microbial, anti-viral, anti-cancer, anti-proliferative, and COVID-19 associated anti-fungal [9-13]. The imidazole portion of the triazole compounds guides the successful use of various medicinal agents [13]. However, studies examining the effects of 1,2,4 triazole compounds on melanoma cells are limited. Bekircan *et al.* reported that asymmetric 3,5-diaryl-4*H*-1,2,4-triazole derivatives have cytotoxic effects on melanoma cell lines, but they have not addressed the mechanism of this effect [14].

In our previous study, the inhibition potential of some triazole derivative compounds including B9 (4-{[4-(Fluorophenyl)methylidene]amino}-2-(4-methylpiperazin-1-yl)-5-{[3-(4-chlorophenyl)-5(4-methoxybenzyl)-*H*-1,2,4-triazol-4-yl]methyl}-2,4-dihydro-3*H*-1,2,4-triazol-3-thion)on) tyrosinase activity was evaluated in terms of IC_{50} value. B9 was found the most potent inhibitor and inhibition type and Ki value for this molecule was determined. Molecular modeling programs were also used to theoretically calculate the binding energy and to demonstrate the interactions of this compound with the enzyme [15]. In this study, we aimed to investigate (i) the cytotoxic effect of this molecule on melanoma cell line by Annexin-PI method, (ii) the effect on cell cycle arrest and (iii) the function for *in vitro* inhibition of melanin synthesis.

Materials and methods

Chemicals and reagents. Dimethyl sulfoxide (DMSO), hydrogen peroxide, cisplatin and kojic acid were bought from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). All chemicals and solutions used in the cell culture studies were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), Gibco (NY, USA) and Sigma-Aldrich (St. Louis, MO, USA). All kits used in the flow cytometric analysis of apoptosis (BD, 556547) and cell cycle (BD, 340242) experiments were purchased from Becton Dickinson (San Diego, CA, USA).

Cell culture. Human melanoma (VMM917, CRL-3232) and healthy foreskin fibroblast (BJ, CRL-2522) cells were purchased from ATCC (Manassas, VA, USA). Both cells were cultured in Rosewell Park Memorial Institute-1640 Medium (RPMI-1640) with 1% penicillin/streptomycin and 10% heat inactivated fetal bovine serum (FBS). Cells were incubated at 37°C supplied with 5% CO_2 [16; 17].

Drug preparation and treatment. Cisplatin, kojic acid and B9 were dissolved in DMSO. Cisplatin was used as a positive control to confirm the experimental setup in cytotoxicity studies [16]. Kojic acid was used as a positive control due to is a standard tyrosinase inhibitor [15]. Final solvent concentrations were adjusted to maximum 0.1% in the medium during cell culture [17].

Measurement of cell viability. Cell viability was evaluated with a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [18]. Briefly, $5x10^3$ and $2.5x10^3$ cells VMM917 and BJ cells were seeded into each well of sterile 96-well cell culture plates. After 24h incubation, the media on the plates were replaced with fresh media and the cells were used either with various concentrations of B9 molecule (1.95-500 μM) or kojic acid (0.5-72 μM) or cisplatin (0.3-40 μM) for 24 h. Later, 10 μL of MTT dye (0.25 mg/mL) was added to each well for 2 h. After 2h, the dye was removed and then the formazan crystals in order to dissolve was added to 200 μL of DMSO each well. The absorbance of the purple color formed was then read on a microplate reader (Molecular Devices Versamax, CA, USA) at 570 nm [19]. Consequently, absorbans were used to evaluate viabilities % of treated cells to compare to control cells. A logarithmic plot of log concentrations against cell viability was plotted to determine the IC_{50} value. The IC_{50} values of B9, kojic acid and cisplatin in VMM917 and BJ cell lines were used to calculate selectivity index with the Formula I [17].

$$\text{Selectivity Index} = \text{BJ cells } IC_{50}/\text{VMM917 cells } IC_{50}$$

Cell cycle analysis. VMM917 cells were treated with 8, 16, and 32 μM of B9 for 24 h followed by the removal of media and wash twice with 1xPBS buffer. Preparation of cells was performed according to the manufacturer's recommendations (BD Biosciences, Cat No: 340242, San Diego, CA). At least $3x10^4$ cells per sample were run by flow cytometer (BD Accuri C6, MI, USA). The percentages of cells in cycle phases were determined using MODFIT 3.0 verity software. Data were finally compared to untreated counterpart cells.

Assessment of cell apoptosis by Annexin V-FITC/PI staining assay. VMM917 cells were used with 8, 16, and 32 μM concentrations of B9 for 24 h followed by the performing apoptosis analysis using a commercial kit (BD Pharmingen™, Cat No: 559763, SanDiego, CA, USA) Cells were washed first and were suspended with 100 μL 1xbinding buffer, and 5 μL of Annexin V and after 5 μL of PI. Finally, the volume of the sample was completed to 500 μL with a 1xbinding buffer and $1x10^4$ cells were collected and analyzed by flow cytometer. Cells were gated on the FSC-SSC (forward scatter-side scatter) plot with respect to untreated cells, and split into four regions [19; 20]. The percentages in each region were calculated and compared with untreated counterparts.

Measurement of melanin contents. The melanin content of cells was analyzed according to method of Hu [21] with minor modifications. Briefly, 25×10^3 of VMM917 cells were seeded in T-25 culture flasks and then incubated for 24 h. Cells were then treated with various concentrations of B9 compound (0.25-512 μM) or kojic acid (0.56-72 μM) for 6 days. At the end of the 6-day incubation, cells were harvested by trypsinization, and washed with 2xPBS buffer. Cells were incubated

with trypan blue and cell viability was determined using automated cell counter [20]. The cells were lysed with 1 mL 2 M NaOH at 100°C for 60 min, and then were transferred to 96-well plates [6]. Finally, absorbance was evaluated at 400 nm with a microplate reader (Molecular Devices Versamax, California, USA). Meanwhile absorbances of a serial dilution of synthetic melanin (ng/mL) were read and the melanin calibration graph was drawn using these absorbance values. The levels of melanin were expressed as percentages compared to the control group [19; 21].

Statistical analysis. All experiments were performed as at least three independent repeats and the results were given as arithmetic mean ± standard deviation. Statistical analyzes were carried out using SPSS (Statistics Program for Social and Science) software (Version13.0.1). The suitability of the data to normal distribution was evaluated with the Kolmogorov-Smirnov test. One-Way ANOVA test was used for parametric data and then post-hoc Tukey test for multiple comparisons between groups. p value less than 0.05 was considered as statistically significant.

Results and discussion

The B9, a 1,2,4 triazole compound, was designed and synthesized for the first time and its inhibitory potential on tyrosinase activity was investigated using *in silico* and *in vitro* biochemical assays in our previous studies [15, 22]. This study revealed the significant effect of compound B9 on cell death and tyrosinase inhibition in melanoma cells. B9 induced cytotoxicity in both VMM917 and BJ cells in a dose-dependent manner (Figure 1A). But healthy BJ cells were more resistant to compound B9 at each dose compared to VMM917 cancer cells. During the cytotoxicity studies, cisplatin [16, 17], an anti-cancer drug known as a positive control, was used, while kojic acid [23] was used as the tyrosinase inhibitor. Cytotoxicity studies of these molecules on VMM917 and BJ cells are given in Figure 1B, 1C. However, the proportion of dead cells was more in melanoma cells than in normal fibroblasts after B9 treatment at 7.8-500 μM ($p<0.05$). There was no significant difference between the cells at 3.9 μM, and also expectedly no difference between untreated cells. This suggests the selective effect of novel B9 on the cytotoxicity of melanoma cells. The selectivity index of the tested compounds was calculated using the formula described in the materials and method. The IC_{50} values obtained for each cell by MTT method are shown in Table 1. The selective cytotoxic effect of B9 on VMM917 cells was higher than cisplatin. Even at 72 μM, the highest concentration used for kojic acid, the IC_{50} value could not be calculated because the cell viability did not decrease by 50%. Therefore, the selectivity index value for kojic acid could not be determined.

Cell viabilities (%) are shown in each cell after treatment with B9 at various concentrations. Error bars are the representative of standard deviation value of independent experiments (n=3). * $p<0.05$.

Selectivity is one of the most important criteria for a drug candidate to be evaluated as a chemotherapeutic [17]. The most important disadvantageous of current chemotherapeutics is to have a mutagenic effect on normal cells. At this point development of selectivity is one of the main approaches focused on cancer studies. There are a range of drugs developed chemically or using biotechnological tools. One of the group of chemicals are 1,2,4-triazole compounds and drug development studies have been focused on the design and synthesize these compounds due to their clinical importance [15; 24-26]. There are some studies investigating the cytotoxic effects of various compounds containing 1,2,4-triazole on leukemia, lung, prostate, breast, ovary, and colon and melanoma cancer cells [24-27]. Bekircan *et al.* [14] reported that asymmetric 3,5-diaryl-4*H*-1,2,4-triazole derivatives have cytotoxic effects on various melanoma cell lines and IC_{50} values varied in the range of 6.18-19.6 μM. Georgiyants *et al.* [24] also demonstrated that the new 1,2,4-triazole (4*H*) derivatives exhibited cytotoxic effects on melanoma cells at different range. Novel furan C-2 quinoline coupled 1,2,4-triazole analogs were also shown to have a cytotoxic effect on melanoma (A375) cells and the IC_{50} values changed between 2.9 and 207.1 μg/mL [26]. 5-mercapto-1,2,4-triazole derivatives exhibited moderate cytotoxic effect on melanoma (A375 and B164A5) cells [25]. Recently, Quattrini *et al.* [27] reported that a 1,2,4-triazole core compound (6a) shows a cytotoxic effect on human melanoma (A2058) cell line. However, these studies did not include an evaluation with normal cells so that it is hard to conclude the selective effect of compounds experienced. The findings of current study confirm the previous studies that showed 1,2,4-triazole compounds can cause cytotoxicity in melanoma cells, but also declare the selective index of compound B9 for cancer treatment. This study also reveals the cellular effects of 1,2,4 triazole compounds on melanoma cells such as cell cycle status and apoptotic/necrotic pattern which have not been reported before. All the concentrations of B9 significantly increased the cell arrest at sub-G_1 ($p<0.01$) and at S phase ($p<0.05$) compared to untreated control cells (Figure 2).

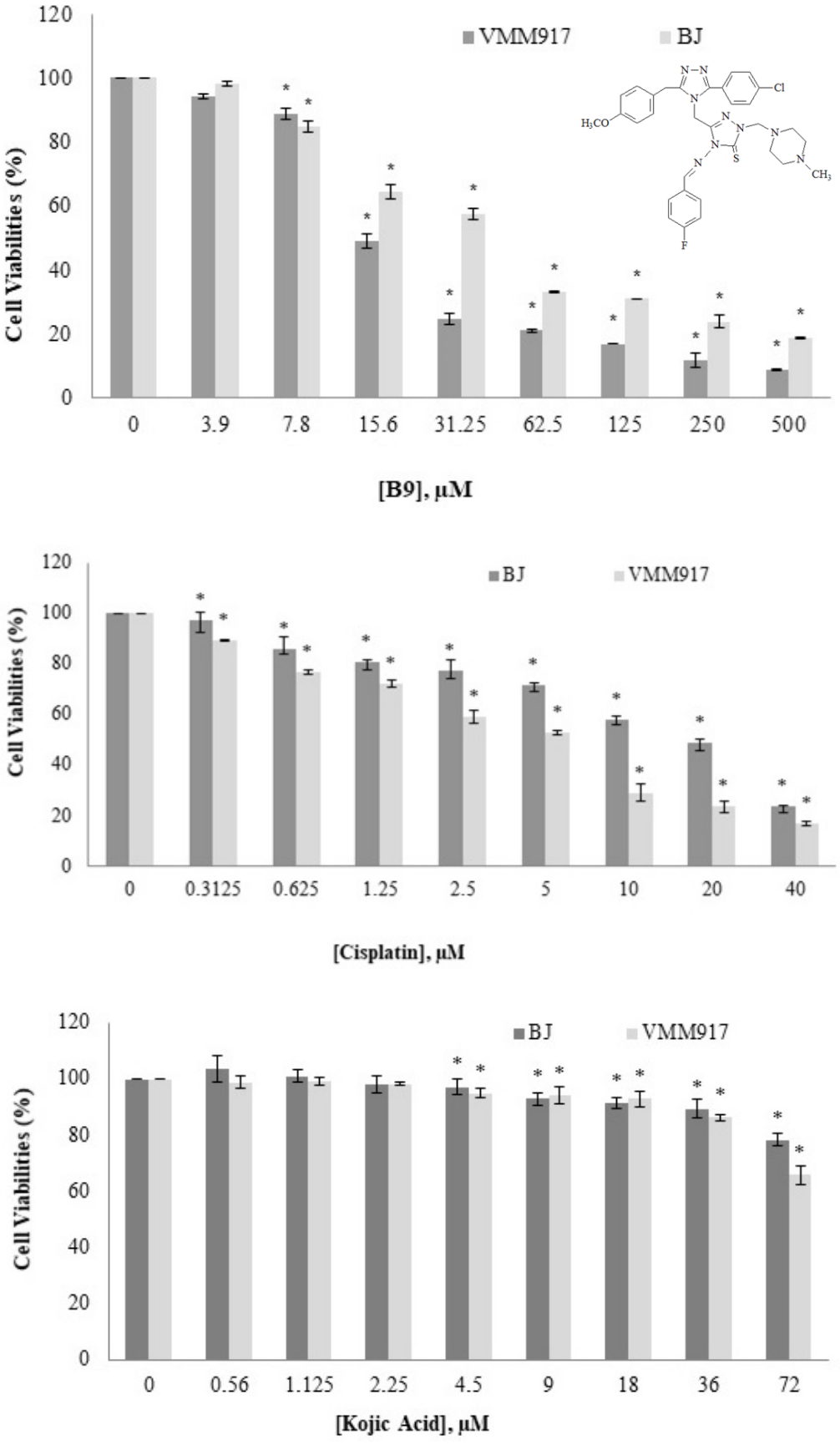

Figure 1 – The cytotoxic effect of B9 compound, cisplatin and kojic acid on VMM917 and BJ cells.

Table 1 – IC_{50} and selectivity index values (μM) calculated for B9, cisplatin and kojic acid on VMM917 and BJ cell lines (n=3)

Test Compounds	VMM917	BJ	Selectivity index
B9	16.8±0.7	82.59±6.43	4.91
Cisplatin	8.02±0.64	17.1±1.1	2.08
Kojic acid	>72	>72	N.D.*
*N.D.: Not determined			

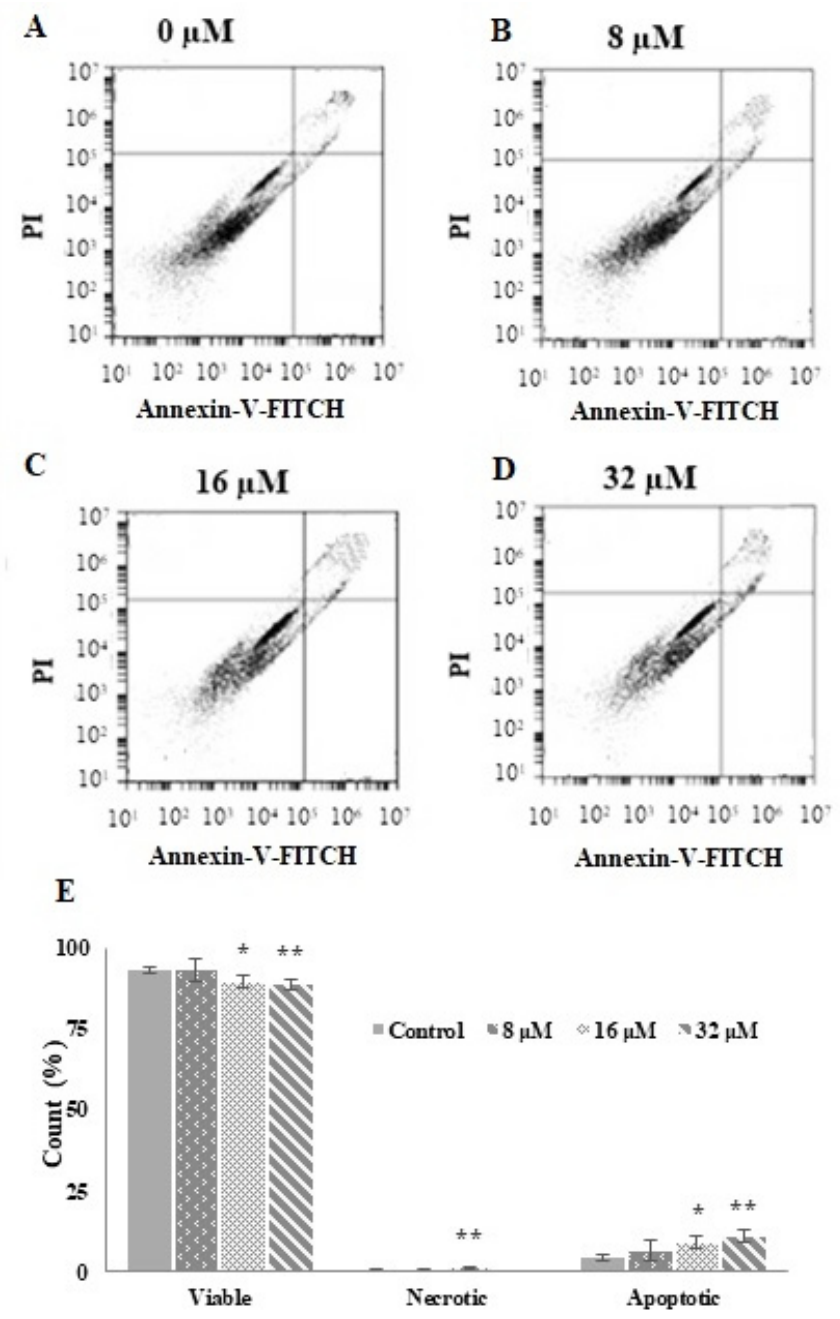

Figure 2 – The effects of B9 on cell cycle in VMM917 cells.
Cells were treated with B9 (8, 16, 32 μM) for 24 h, then, stained with PI and analyzed by flow cytometry (A, B, C, D).
Data were presented as mean ± SD, n = 3 (E). *$p < 0.05$ and **$p < 0.01$ compared with control.

Similarly, S-substituted 1*H*-5-mercapto-1,2,4-triazole derivative compounds and bis-1,2,4 triazole derivatives induced the arrest of cells at sub-G_1 in human colon cancer (HT-29) [25] and in leukemia (MOLT-4) cells, [28] respectively. However, treatment of cells with B9 at 16 µM and 32 µM significantly decreased the number of cells arrested at G_1 phase (p<0.01). The results showed that the novel compound B9 arrested cells at DNA synthesis of cell cycle (S phase) and also cells at the preparation of cells for mitosis (G_1 phase) which some DNA damage, such single-strand breaks, are recognized and repaired. This suggests that B9 molecule specifically targets G_1 and S phases and this can be considered as a cell cycle inhibitor in melanoma therapy. It is an important finding because abnormalities of cell cycle are one of the characteristics of malignancy as normal cells are controlled by cell cycle checkpoints during cell proliferation. Therefore, the cell cycle of cancer cells has been accepted as one of the target mechanisms of cancer treatment [29].

On the other hand, 16 and 32 µM of B9 molecule significantly increased the number of apoptotic cells (p<0.05) (Figure 3). Although 8 µM concentration of B9 molecule increased the number of apoptotic and necrotic cells numerically, these increases were not statistically significant (p>0.05).

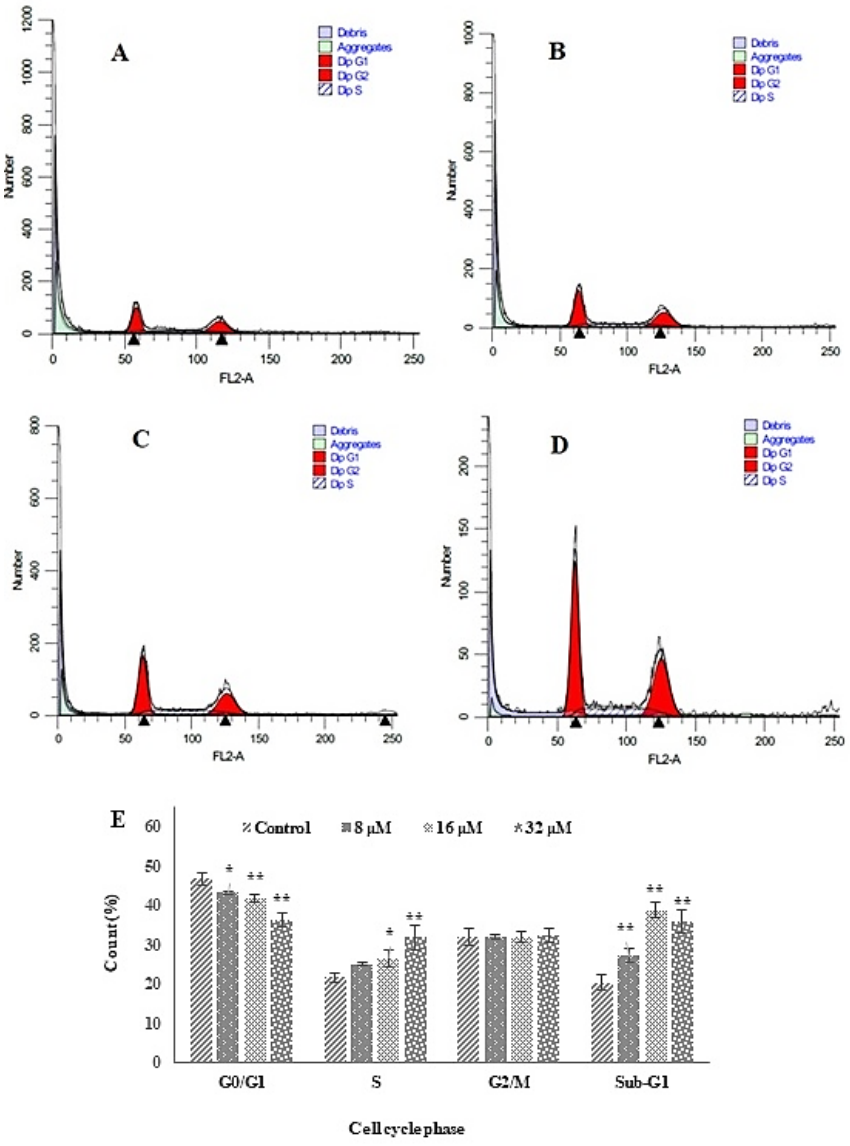

Figure 3 – The effects of B9 on apoptosis in VMM917 cells. Cell were treated with B9 (8, 16, 32 µM) for 24 h, the apoptotic cells were stained by Annexin-V/PI and analyzed by flow cytometry (A, B, C, D). Data was presented as mean ± SD, n=3 (E). *p<0.05 and **p<0.01 compared with control.

The findings revealed apoptotic profile of melanoma cells after treatment with 1,2,4-triazole compounds for the first time. Apoptosis of some other cancers such as prostate, colon, breast and lung were induced by different 1,2,4-triazole derivative compounds [30-32]. 1,5-disubstituted 1,2,4-triazole compounds were shown to induce apoptosis and cell cycle arrest at the G_2/M phase in cervix (HeLa) and leukemia (Jurkat) cancer cells [33]. A 7-hydroxy-4-phenylchromen-2-one-linked triazole compound exhibited cytotoxic effect in human gastric cancer (AGS) cells through inducing the number of annexin-V positive cells and cell cycle arrest at the G_2/M phase [34]. Similarly, a novel compound carrying 1,2,4-triazole ring induced apoptosis and cell cycle arrest at the G_2/M phase in human lung cancer (A549) cells [9]. These indicate that the ability of the triazole derivatives for controlling cell cycle checkpoints depends on their structural features.

Melanoma has been reported to cause the deaths of approximately 15.000 people every year in Europe, and it is the form of skin cancer with a high risk of death among cancer types [4]. Melanin is the key biopolymer to color skin, hair, gums and eye [35]. This pigmentation protects the tissue from external conditions, such sun light [36]. In addition, melanin plays important roles in the elimination of free radicals produced in the cytoplasm to prevent DNA damage by protection from various types of ionizing radiation, such UV light. But mutations in melanocytes can induce the excessive production of melanin pigment in skin, eyes and inner ear so that may cause various dermatological disorders such as melasma, freckles, age spots, actinic damage and melanoma [37]. This study also revealed *in vitro* inhibitory effect of this novel 1,2,4 triazole compound on tyrosinase activity that is the key enzyme for melanin production. B9 compound decreased the content of melanin in VMM917 cells in a dose dependent manner (Figure 4).

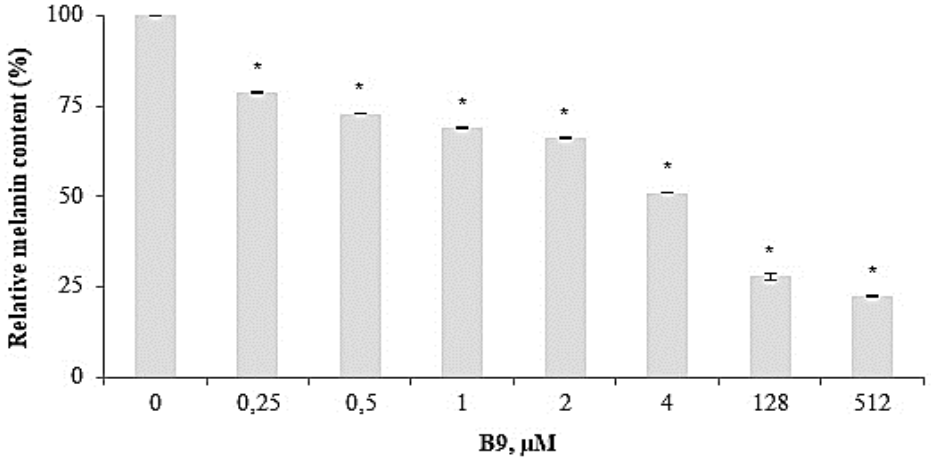

Figure 4 – The effect of B9 compound on the melanin content of VMM917 cells.
Note: * Statistically significant difference compared to untreated control cells (p<0.05).

As a result of melanin content analysis, IC_{50} values for B9 and kojic acid compound were calculated as 4 μM and 72 μM, respectively. These results showed that B9 compound is more effective on the inhibition of tyrosinase than kojic acid. It is previously reported that kojic acid derivatives known as tyrosinase inhibitors were used to reduce the amount of melanin in melanoma cells and the calculated IC_{50} values vary between 5-200 μM [5, 7]. Excessive enzyme activities are common in cancers [38, 39]. This leads researchers to focus on enzyme inhibition studies for cancer therapy. The purpose of all drug discovery attempts is to effectively treat human diseases and develop safe therapeutics [40]. The use of enzyme inhibition compared to chemotherapeutic agents in cancer treatment seems much more advantageous in terms of toxicity [41]. These such compounds can be also considered to be combined with chemotherapeutics since multiple drug use are more effective than in single drug administration on recovering cancer.

Conclusion

Melanoma appears an increasing cancer around the globe. Although there are many studies on

melanoma, the functions of triazole ring compounds on cytotoxicity and melanin inhibition in VMM917 cell have not been reported before. The novel compound also appears as a selective drug due to its high selectivity index. This study should be extended for other melanoma cell lines and *in vivo* studies with B9 compound will make a broader conclusion for its use as a chemotherapeutic.

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