

AN ABSTRACT OF THE THESIS OF

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Title: Effect of Antioxidants Co-enzyme Q10 and β -carotene on the Cytotoxicity of Vemurafenib against Human Malignant Melanoma

Thesis Chair: _____

Abstract approved: _____

(Thesis Advisor Signature)

Melanoma is a type of highly invasive skin cancer derived from melanocyte with a poor prognosis. Clinical studies have shown that the most common mutation in malignant melanoma is BRAF^{V600E}. Vemurafenib (PLX4032) is a clinically approved targeted drug for BRAF mutant melanoma that has a high therapeutic efficiency and significantly prolongs overall survival rate. However, most patients will become drug resistant and suffer from melanoma relapse within one year. Antioxidants have been widely used as supplements for cancer prevention and decreasing the side effects of cancer therapy. However, antioxidants could also protect cancer cells from oxidative stress and promote cell growth. This study aims to examine the effect of coenzyme Q10 and β -carotene on cell growth and invasion, and on the cytotoxicity of Vemurafenib against both Vemurafenib-sensitive and Vemurafenib-resistant human malignant melanoma cell lines.

Results showed that coenzyme Q10 alone significantly reduced the viability and migration of human malignant melanoma cells. In addition, coenzyme Q10 alone inhibited the apoptosis induction in Vemurafenib-sensitive SK-MEL-28 cells. β -carotene alone did not affect the viability and apoptosis induction of melanoma cells; however, it inhibited cell mobility and invasiveness. Coenzyme Q10 enhanced the ability of Vemurafenib to decrease viability and mobility of melanoma cells. In contrast, β -carotene alleviated the cytotoxicity of Vemurafenib and mitigated the inhibitory effect of Vemurafenib on cell viability, mobility and invasion. Both coenzyme Q10 and β -carotene protected melanoma cells from undergoing apoptosis induced by Vemurafenib. β -carotene enhanced the suppression of Ras-Raf-Mek-Erk intracellular signaling pathway activation, which may contribute its inhibitory effect on cell mobility and invasiveness. Therefore, since it increases the cytotoxicity of Vemurafenib, coenzyme Q10 can potentially serve as an adjunct used together with anti-melanoma chemotherapy. However, due to alleviating anti-melanoma activities of Vemurafenib, β -carotene may not be appropriate supplements for melanoma patients who concurrently receive Vemurafenib as a targeted therapy.

Keywords: Coenzyme Q10, β -carotene, Vemurafenib, Melanoma, and BRAF

Effect of Antioxidants Co-enzyme Q10 and β -carotene on the Cytotoxicity of
Vemurafenib against Human Malignant Melanoma

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PREFACE

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1. Introduction

Melanoma is a type of highly invasive and metastatic skin cancer with a poor prognosis. Incidence rates of melanoma have been growing in all regions of the world [1-5]. In 2017, the estimated new cases of melanoma in the United State is 87110, and the estimated deaths are 9730 [6]. The mortality of metastatic melanoma is very high. About 50% to 80% of advanced melanoma patients have liver metastases, and 8% to 46% of melanoma patients have brain metastases [7]. The median survival of patients is only about 6 months; the 5-year survival rate is less than 5% [8]. Melanoma derives from melanocytes which protect skin from ultraviolet radiation (UVR) [9,10]. Risk factors of melanoma include family history, UVR, abnormal moles, and severe sunburns [1,3,8,9,11-13].

Mainstream treatments of melanoma include immunological therapy and chemotherapy. Ipilimumab is a monoclonal antibody that can activate tumor specific T cells by targeting immune-suppressant cytotoxic T lymphocytes antigen 4 (CTLA-4), thus allowing T cells to regain the ability to recognize cancer cells [4,14]. However, the side effects of immunological therapy are too severe to be ignored. High proportion of liver damage and immune-related adverse events such as colitis, dermatitis, and pituitary lesions limit the success of use of ipilimumab in some patients [15].

Chemotherapy is a common treatment of melanoma even though it is not the frontline option for advanced melanoma [4,16-21]. Studies showed that a single-agent chemotherapy drug, such as dacarbazine, did not improve overall survival. Therefore, a number of studies focus on the combination of two chemotherapy agents or the combination of chemotherapy and other treatments such as immunotherapy, called biochemotherapy, which yields better clinical performance but similar overall survival rate [4,6,16]. Different chemotherapy agents have different side effects. From mild to severe, main side effects include hair-loss, headache, nausea and vomiting, and myelosuppression [16-18,21].

BRAF is a gene that regulates cell growth. The B-Raf protein encoded by *BRAF* is a kinase involved in cell signaling associated with cell proliferation [18,22-24]. Phosphorylated B-Raf can activate downstream MEK protein, MEK1 and MEK2, which in turn phosphorylates ERK protein [19,25]. This process is MAPK signaling pathway, which regulates cell proliferation and survival [21]. When *BRAF* is mutated, it becomes constitutively activated and subsequently it continuously activates and induces cell growth and survival. Mutated *BRAF* can be found in some human cancer cells [22]. *BRAF* mutation is the most common mutation in melanoma. About 50% of melanoma patients harbor *BRAF* mutations, of which 80% to 90% occur at the 600th codon known as BRAFV600E [17,26].

A few BRAF kinase inhibitors have been developed, of which dabrafenib and Vemurafenib targeting BRAFV600E were approved by the Food and Drug Administration (FDA). Clinical trials showed that Vemurafenib (PLX4030) produces as high as an 80% response rate on BRAFV600E melanoma patients [17,27,28]. However, after 6 months treatment, melanoma cells become Vemurafenib-resistant and patients suffer melanoma relapse. In addition, common side effects are joint pain, rash, photosensitivity dermatitis, fatigue, hair loss, and itching [17-19].

Oxidative pressure is one important factor that causes genetic instability. For example, reactive oxygen species (ROS) such as superoxide anion radicals, hydroxyl radicals, hydroxyl radicals, and hydrogen peroxides are endogenous harmful by-products of cell metabolisms [29]. In fact, living cells always suffer from endogenous or exogenous oxidative pressure. For instance, in inflammatory reactions, neutrophils and macrophages can produce free radicals, molecules with unpaired electrons, which are highly reactive [29,30]. Once these free radicals are produced, these molecules are ready to catch electrons from other molecules, and the disruption of these functional molecules will in turn produce more free radicals [29-31]. This process can cascade, and free radicals can be continuously generated. As a result, such free radicals at high levels will attack normal functional molecules such as DNA, lipids, and proteins [32,33]. DNA damages caused by ROS or other free radicals result in elevated mutation rates. Hence, genetic instability, which leads to cell death or tumorigenesis, will accumulate in the cells. In addition to endogenous free radicals, however, it is noteworthy that environmental exposure also contributes to the oxidative pressure. For example, smoking, UV light, and pollution can

increase the production of free radicals [29]. At the same time, free radicals are vital for cell proliferation and differentiation [34]. Therefore, the roles of free radicals in cellular processes are quite complex.

To control ROS level, antioxidant defense system is responsible for balancing oxidative pressure [33]. Antioxidant is a chemical that is able to reduce or slow the process of oxidation. Thiols and polyphenols are typical antioxidants. Antioxidant system includes many antioxidants such as vitamin C, vitamin E, and glutathione (GSH: reductive form, GSSG: oxidative form). A high GSH/GSSG ratio can decrease oxidative pressure by neutralizing free radicals. On the contrary, a low level of antioxidants will result in interruption of biochemical reaction and lead to cell death or mutation. Antioxidants such as vitamin C and vitamin E, in the diet or as supplements, can enhance the antioxidant system, which balances oxidative pressure level and protects molecules from being attacked by free radicals. It has been a common belief that antioxidants-rich fruits and vegetable such as blueberries, blackberries, and small red beans can prevent and even treat some diseases [32,35-39]. Previous studies reported that antioxidants were able to inhibit cancer progression. However, some research showed that antioxidants could increase cancer risks. At present, the effects and working mechanism of antioxidant supplements on the progression of cancers are poorly understood [29,34]. The contradictory results about the effect of antioxidants on the progression of cancers warrant further investigation.

Antioxidants can inhibit the initiation of cancer by scavenging free radicals that cause DNA damages and mutations of tumor suppressor genes. Therefore, antioxidants have been advocated as potent and safe supplements for cancer prevention. Antioxidants can also repair normal tissue damage caused by free radicals generated by radiotherapy and chemotherapy [35,37,39,40]. Thus, antioxidants have been widely used to decrease the side effects of cancer therapy. However, antioxidants could likewise protect cancer cells from oxidative stress, inhibit apoptosis, and promote the cell cycle progression. Therefore, there is heated debate on whether antioxidants are recommended for cancer patients, especially for patients who receive free-radical-producing radiotherapy or chemotherapy.

It is reported that some antioxidants such as N-acetylcysteine (NAC) and Trolox can increase melanoma metastasis in mice by two-fold compared with the control group [41] in an *in vivo* experiment in mice harboring melanoma. When treated with NAC, the tumor numbers per mouse and tumor size did not change compared with the control group. However, lymph node metastasis doubled in the NAC-administration group. Lung metastases also increased dramatically when treated with NAC [41]. The researchers also quantified the amount of GSH and the GSH/GSSG ratio. The results showed that in primary tumors, the same amount of GSH and GSH/GSSG ratio were found in both the NAC-treated group and control group. However, surprisingly, the GSH amount and GSH/GSSG ratio were much higher in lymph node metastases [32,34]. In addition, a similar effect of NAC and Trolox on cultured human melanoma cells was found.

Coenzyme Q-10 (CoQ10) physiologically serves as a component of the electron transport chain in mitochondria [42,43]. Declined levels have been verified in abnormal myocardium and in Parkinson disease [44]. Research showed that low plasma CoQ10 levels could be an independent prognostic factor for melanoma progression [45]. CoQ10 is widely used as a dietary supplement because of its anti-oxidative property. CoQ10 has been reported to be beneficial in treating hypertension, congestive heart failure, statin myopathy, and ameliorating side effects of chemotherapy [44-49]. Recent studies showed that CoQ10 has no effect on the viability of some B-RafV600E melanoma cells [48].

β -carotene has been widely used as pigment in foods, cosmetics, and drugs [50-52]. β -carotene contains two retinyl groups, which is broken down in human small intestine by enzyme β -carotene 15, 15'-monooxygenase to retinal, a form of vitamin A. Toxicity studies showed no mutagenicity or cytotoxicity [50,53-56]. It was reported that β -carotene inhibits angiogenesis and activation of transcription factor in mouse melanoma cells [57]. An *in vivo* experiment showed β -carotene inhibits lung metastasis induced by B16 melanoma in mice [58]. However, an epidemiological study showed that a higher incidence of lung cancer was found in those who took β -carotene than in the placebo group [50,59].

Antioxidants can prevent cellular components from being damaged by free radicals and decrease the mutations that cause tumorigenesis. However, since cancer cells carry about a high level of metabolic activity and suffer from more oxidative stress, antioxidants

could potentially promote the growth and progression of cancers by removing free radicals from cancer cells. In fact, our previous research has shown that antioxidant vitamin C exerted inhibitory and stimulatory effects at pharmacological and physiological concentration, respectively [60]. In this work, we hypothesize that antioxidant Coenzyme Q-10 and Beta-carotene have an effect on the growth and invasion of human malignant melanoma cells. In addition, in a previous study our lab has shown that BRAF kinase inhibitor Vemurafenib, a FDA-approved anti-melanoma drug, increased the oxidative stress in human malignant melanoma cells [60]; thus antioxidants may interfere with the cytotoxic effect of Vemurafenib on melanoma cells by removing the free radicals. In this work, we hypothesize that Coenzyme Q-10 and Beta-carotene affect the cytotoxic effect of Vemurafenib on human malignant melanoma cell lines.

Therefore, this study will determine (1) the effect of Coenzyme Q-10 and Beta-carotene on the growth, migration and invasion, and apoptosis induction of human malignant melanoma cell lines; and (2) the effect of Coenzyme Q-10 and Beta-carotene on the cytotoxicity of vemurafenib on human malignant melanoma cells. This study aims to better understand the biological effects and the working mechanism of antioxidants Coenzyme Q-10 and Beta-carotene on malignant melanoma and help clinicians make decisions on adding antioxidant supplements to the cancer treatment regimen.

2. Materials and Methods

2.1 Chemicals

PLX4032 was purchased from Selleck Chemicals (Houston, TX). Coenzyme Q10 and Beta carotene were purchased from Sigma (St. Louis, MO).

2.2 Cell Type and Culture Conditions

SK-MEL-28 and A2058 human melanoma cell lines were purchased from the American Type Culture Collection (ATCC). SK-MEL-28 and A2058 were cultured in Eagle's Minimum Essential Medium (EMEM) and Dulbecco's Modified Eagle's medium (DMEM), respectively, and were supplemented with fetal bovine serum (10%) and penicillin/streptomycin (0.1%). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂.

2.3 Cell Viability Assay

Cells were seeded into 96-well plates (3000 cells/well) (Corning Costar, Cambridge, MA) and incubated for 12h to allow cells to attach. To test the effect of Coenzyme Q10

on cell viability, the cells in the experimental groups were treated with Coenzyme Q10 at final concentrations of 1 μ M, 5 μ M, and 10 μ M, respectively. The cells in control groups were treated with the drug vehicle dimethyl sulfoxide (DMSO) (Fisher Bioreagents, Pittsburgh, PA).

To test the effect of Coenzyme Q10 on cytotoxicity of PLX4032 against melanoma cell proliferation, the SK-MEL-28 cells were treated with PLX4032 at 2 μ M together with Coenzyme Q10 at 1 μ M, 5 μ M, and 10 μ M. The cells in control groups were treated with the drug vehicle dimethyl sulfoxide (DMSO) (Fisher Bioreagents, Pittsburgh, PA).

Concentration of PLX4032 for A2058 was 20 μ M. After incubation for 48 h, 10 μ M MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (Promega, Madison, WI) was added into each well and incubated for 3h at 37 °C. The absorbance of solubilized dye was measured by a micro-plate reader (BioTek Instrument, Winooski, VT) at 490 nm. Three independent experiments were performed and the results were reported as means \pm SD.

2.4 Wound Healing Assay

2×10^5 cells were seeded into 12-well plates and incubated for 24 h at 37 °C. Then the layer of cells were scraped with a 200 μ l micropipette tip to create a wound. Plates were washed with Hanks' Balanced Salt Solution (HBSS, Sigma, St. Louis, MO) and replaced with fresh medium. The assay was performed in eight groups. Group 1 was the control

group, which was treated with drug vehicle (DMSO). Groups 2, 3, and 4 were treated with Coenzyme Q10 or β -carotene at final concentrations of 1 μ M, 5 μ M, and 10 μ M respectively; groups 6, 7, and 8 were treated with PLX4032 at a final concentration of 2 μ M and Coenzyme Q10 or β -carotene at final concentrations of 1 μ M, 5 μ M, and 10 μ M, respectively. Group 5 was treated with 2 μ M PLX4032 alone. The images of the wounds were captured at 24h at 40-fold magnification under an inverted microscope and the average wound distances were calculated using Image Pro Plus software.

2.5 Transwell Cell Invasion Assay

3×10^4 cells were re-suspended in 200 μ L serum-free Eagle's Minimum Essential Medium in the upper chamber of a 24-well plate. Culture medium (7000 μ L) containing 20% fetal bovine serum was applied to the lower chamber. The assay was done with eight groups. Group 1 was the control group, which was treated with drug vehicle (DMSO). Groups 2, 3, and 4 were treated with Coenzyme Q10 at final concentrations of 1 μ M, 5 μ M, and 10 μ M, respectively; groups 5, 6, and 7 were treated with PLX4032 at a final concentration of 2 μ M and Coenzyme Q10 at final concentrations of 1 μ M, 5 μ M, and 10 μ M, respectively. Group 8 was treated with 2 μ M PLX4032 alone. After incubation for 18 h at 37 $^{\circ}$ C in a 5% CO₂ incubator, cells were fixed by formaldehyde (3.7% in PBS) and stained by Giemsa stain. Non-migrated cells were scraped off by cotton swabs, and migrated cells were counted under the microscope.

2.6 Apoptosis Analysis

Apoptosis was examined using an Annexin V-FITC-PI dual staining kit (Biolegend, San Diego, CA) followed by flow cytometry analysis according to the manufacturer's instructions. The assay was done with eight groups. Group 1 was the control group, which was treated with drug vehicle (DMSO). Groups 2, 3, and 4 were treated with Coenzyme Q10 at final concentrations of 1 μ M, 5 μ M, and 10 μ M, respectively; groups 5, 6, and 7 were treated with PLX4032 at a final concentration of 2 μ M and Coenzyme Q10 at final concentrations of 1 μ M, 5 μ M, and 10 μ M, respectively. Group 8 was treated with 2 μ M PLX4032 alone. After 24h, cells were harvested by trypsinization, being washed with ice-cold BioLegend cell staining buffer (Biolegend, San Diego, CA), and being re-suspended in binding buffer (Biolegend, San Diego, CA) at a density of 1 \times 10⁶ cells/ml. Cell suspension was stained with Annexin V-FITC and PI and analyzed by the Accuri C6 Flow Cytometer System.

2.7 Western Blotting Analysis

After 48 h treatment, cells were trypsinized and washed three times with PBS and then lysed in lysis buffer for 30 min at 4 °C. The proteins were extracted in the supernatant after centrifugation at 13,000 rpm for 20 min at 4°C, and the concentration of protein was detected using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Proteins were mixed into a Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% 2-Mercapto-

ethanol (β -ME) (Sigma, St. Louis, MO) at the ratio of 1:1 and boiled for 10 min before being electrophoresed on an AnyKD SDS-PAGE gel (Bio-Rad, Hercules, CA). The protein was transferred to a nitrocellulose membrane at 350 mA for 1 h in transfer buffer. The membrane was blocked with 5% non-fat milk in TBST for 2 h at room temperature with shaking. The membrane was then rinsed three times with TBST and then immunoblotted with primary antibody for rabbit anti-GAPDH, anti-phospho-P44/42 MAPK, anti-phospho-BRAF, (Cell Signaling Technology, Danvers, MA) with a dilution of 1: 10000, 1: 1000, and 1: 1000, respectively, at 4 °C overnight. Signals were developed by incubating with HRP-linked secondary antibody (1: 1000) for 2 h at room temperature and then with Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA) for 5 min. The intensity of the signals was determined by the FluorChem™ E system (Protein Simple, Santa Clara, CA).

Statistical analysis

All values are represented as mean \pm standard deviation (SD) from at least three independent experiments. Statistical differences between groups was measured by using Student's T-test. P-values in all experiments were considered significant at less than 0.05.

3. Results

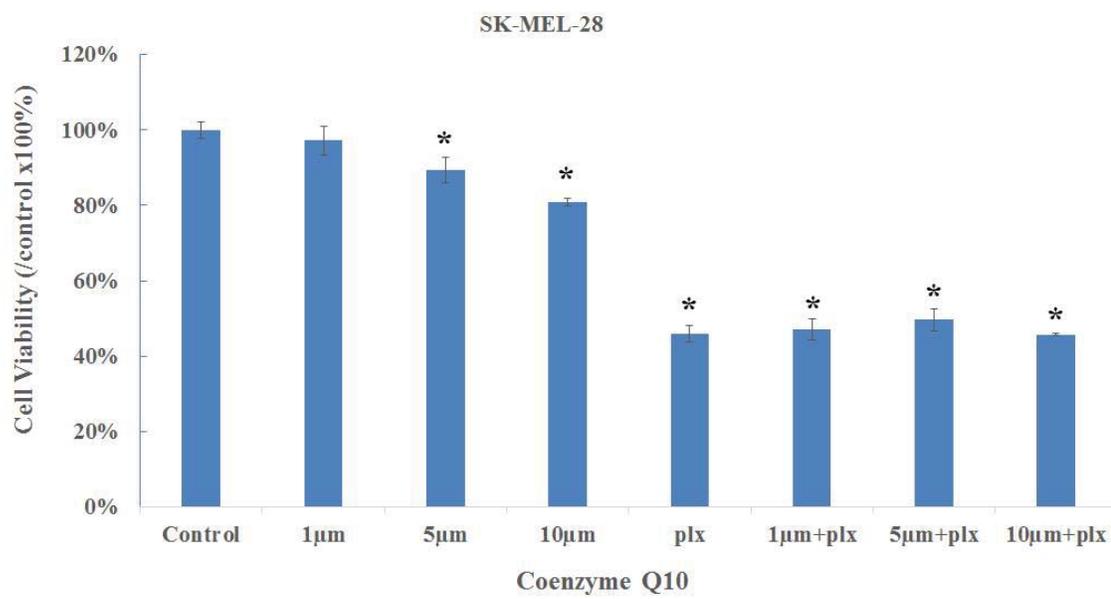
3.1 Coenzyme Q10 and β -carotene alleviated the cytotoxicity of PLX against melanoma cells.

The cytotoxic effect of coenzyme Q10 and β -carotene were examined by MTS assay in two malignant melanoma cell lines. In SK-MEL-28 cell line, which is a Vemurafenib-sensitive cell line, coenzyme Q10 decreased the cell viability and displayed cytotoxicity at 5 and 10 μ M but did not affect the cytotoxicity of PLX (Figure 1A). In A2058 cell line, which is a Vemurafenib-resistant cell line, coenzyme Q10 did not display cytotoxicity; however, coenzyme Q10 increased the cytotoxicity of PLX in a dose dependent way (Figure 1B). In both SK-MEL-28 and A2058 cell lines, beta-carotene did not display cytotoxicity (Figure 1C, 1D). However, surprisingly, beta-carotene relieved (decreased) the cytotoxicity of PLX in both cell lines (Figure 1C, 1D).

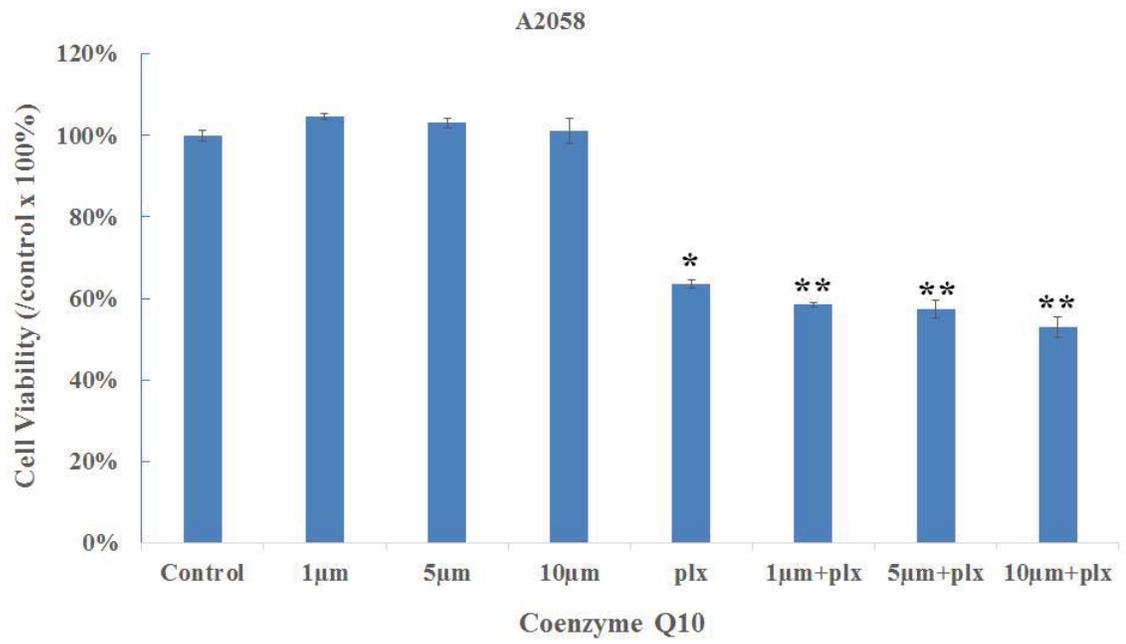
Figure 1. Coenzyme Q10 and β -carotene alleviated the cytotoxicity of PLX against melanoma cells.

The effects of Coenzyme Q10 (Figure 1A, 1B) and β -carotene (Figure 1C, 1D) on cell viability of SK-MEL-28 and A2058 melanoma cells and effect of these two antioxidants on the cytotoxicity of PLX4032 against SK-MEL-28 and A2058 melanoma cells were determined by MTS assay after 48 hours treatment. Each experiment was repeated four times with quadruplicate reactions in each treatment. The results are represented as mean \pm SD. “*” indicates a significant difference between control group and treatment group ($P<0.05$). “**” indicates a significant difference between control group and treatment group and between combined treatment group and PLX alone group ($P<0.05$).

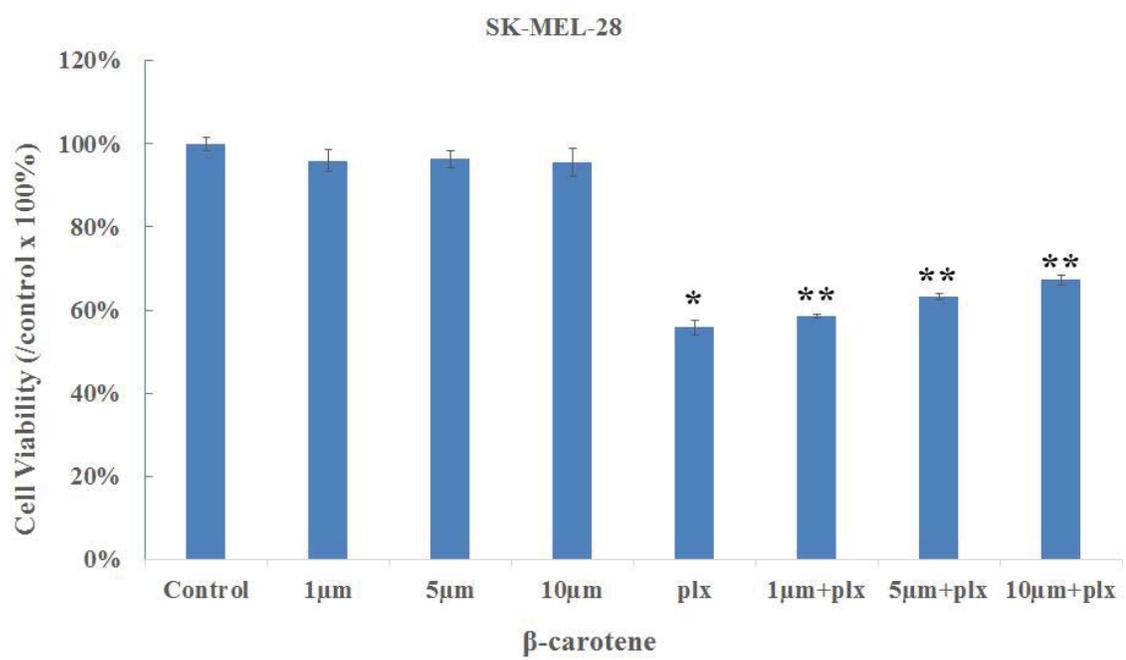
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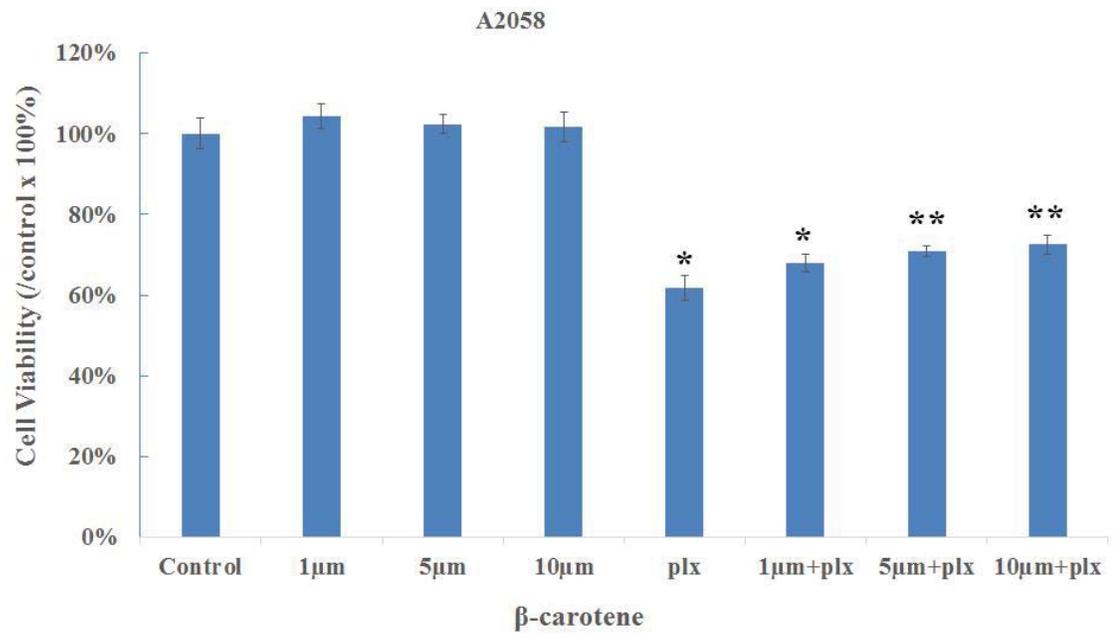
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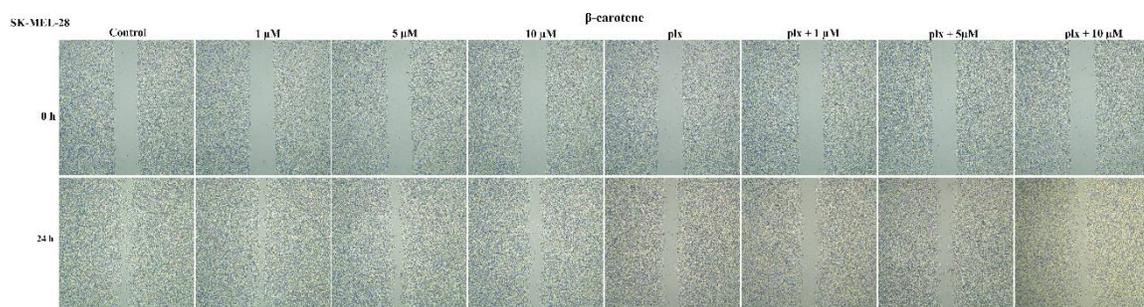
3.2 Coenzyme Q10 and β -carotene inhibited mobility but alleviated the inhibition of mobility of melanoma cells by PLX4032.

The migration ability of SK-MEL-28 and A2058 was examined by wound healing assay. β -carotene inhibited mobility of SK-MEL-28 cells (Figure 2A, 2B) but showed no effect on A2058 cells (Figure 2C, 2D). Interestingly, β -carotene alleviated the inhibition effect of PLX on mobility of both SK-MEL-28 (Figure 2A, 2B) and A2058 (Figure 2C, 2D) cells. Coenzyme Q10 inhibited mobility of both SK-MEL-28 (Figure 2E, 2F) and A2058 (Figure 2G, 2H) cells. In contrast to β -carotene, coenzyme Q10 at 10 μ M enhanced the inhibition of SK-MEL-28 cell mobility by PLX (Figure 2E, 2F) and at 5 μ M and 10 μ M enhanced the inhibition of A2058 cell by PLX (Figure 2G, 2H).

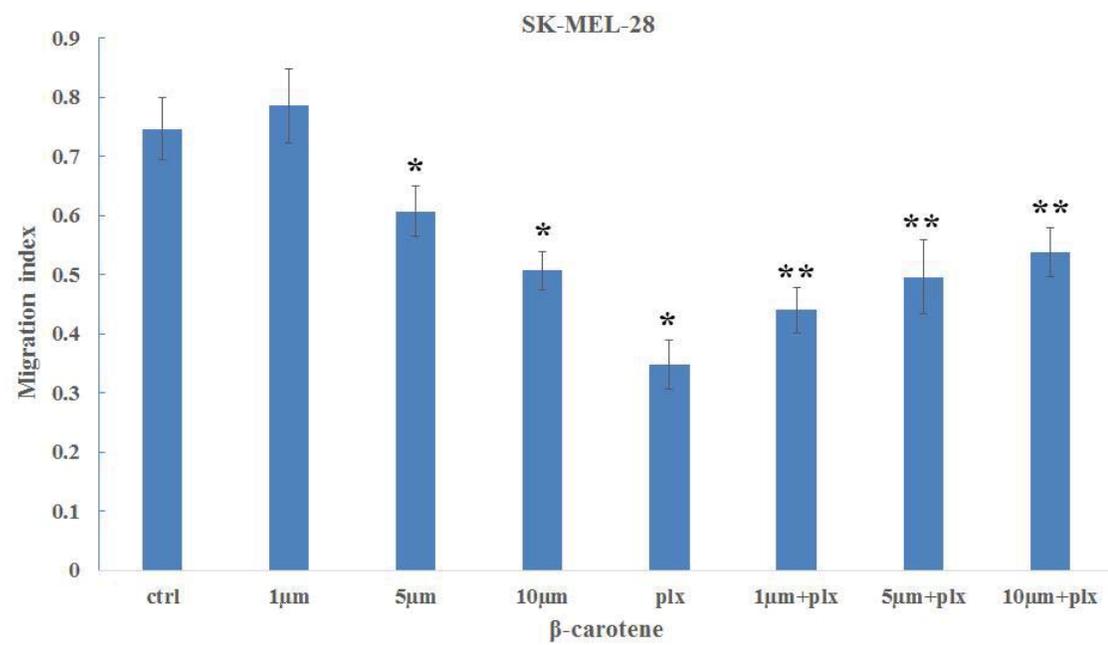
Figure 2. Coenzyme Q10 and β -carotene inhibited mobility and alleviated the inhibition of mobility of melanoma cells by PLX.

Wound healing assay was used to determine the effect of coenzyme Q10 and β -carotene on cell migration and on the inhibitory effect of PLX on cell migration of SK-MEL-28 (Figure 2A, 2B, 2E, 2F) and A2058 (Figure 2C, 2D, 2G, 2H) melanoma cells. Cells were grown to confluence and the wound was scraped with a 200 μ l pipette tip. The migration of the cells are observed under the microscope after 24h growth (Figure 2A, 2C, 2E, 2G). The wound healing index was measured by ImageJ software and is shown in bar graph (Figure 2B, 2D, 2F, 2H). Experiments were repeated three times independently.

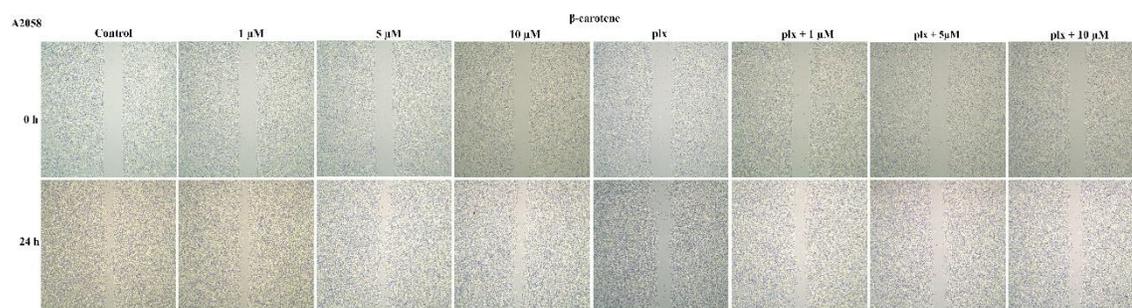
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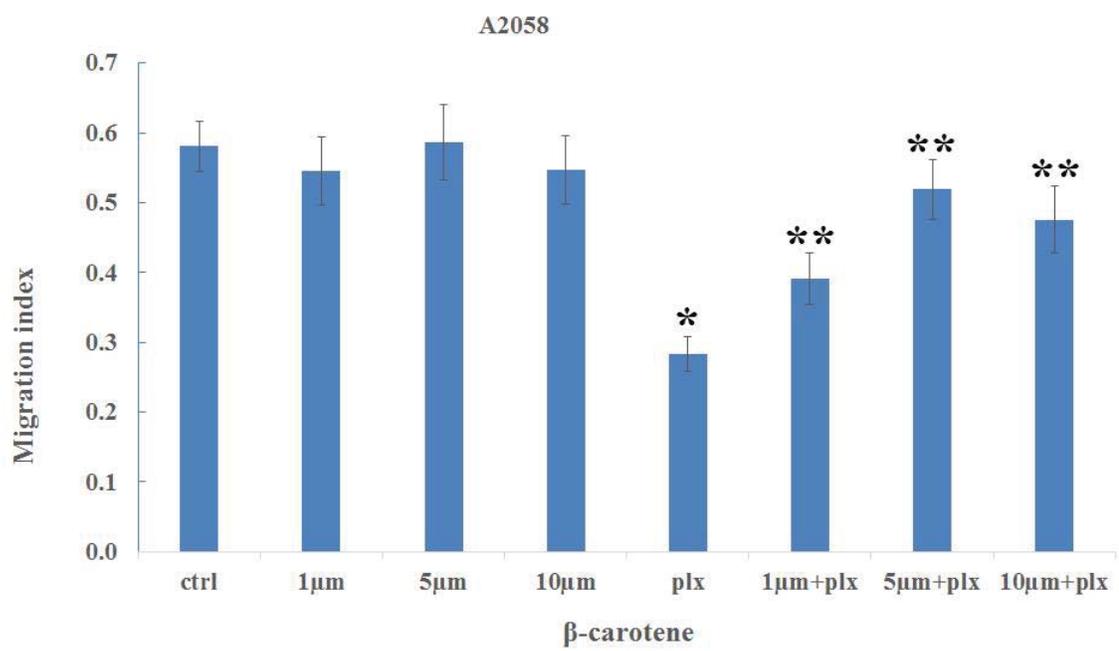
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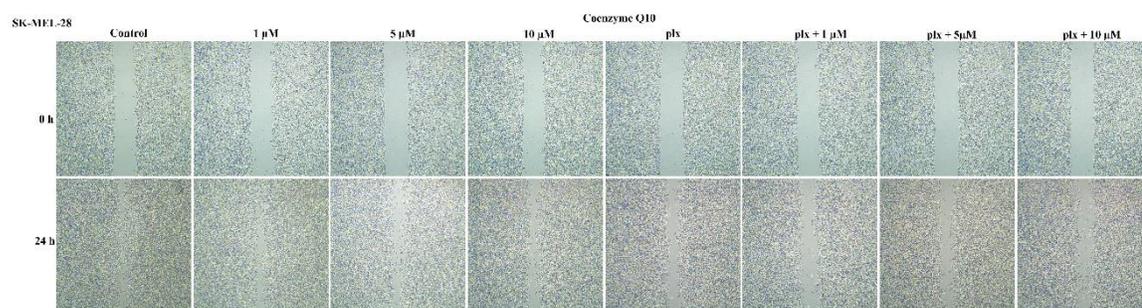
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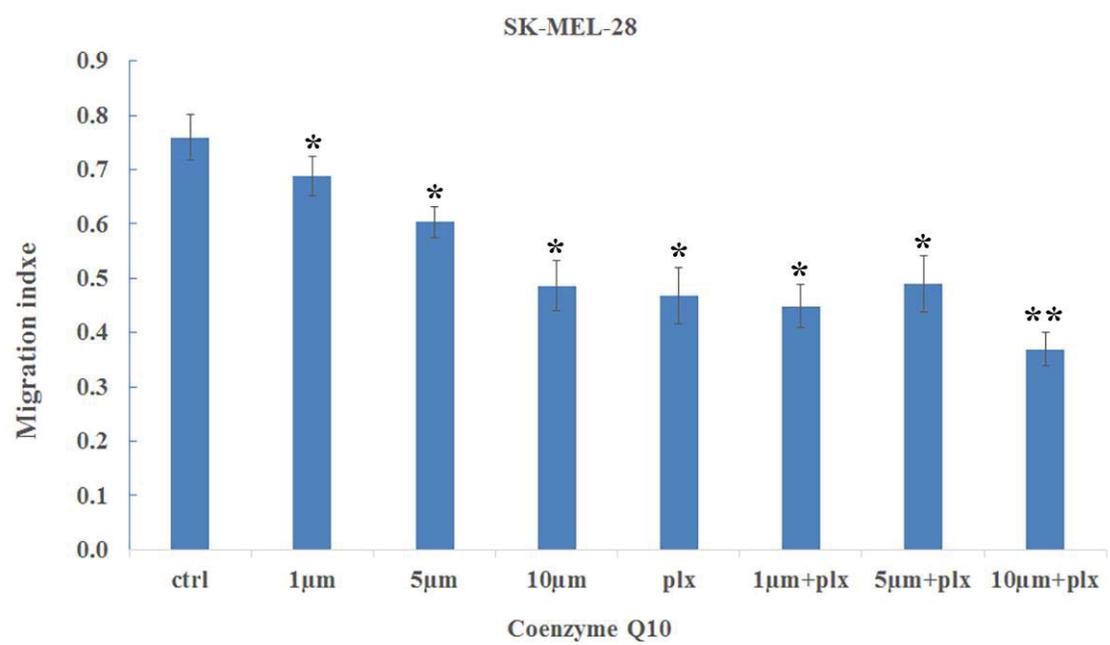
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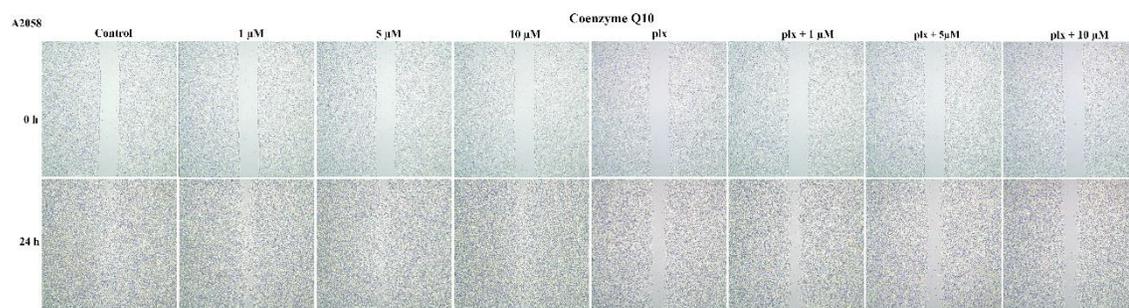
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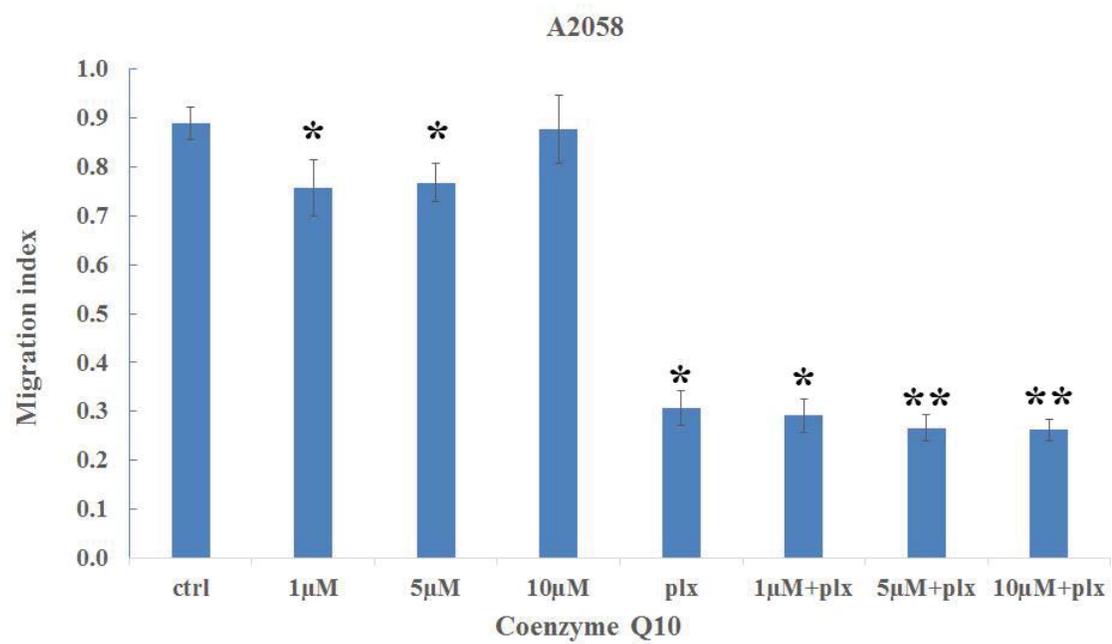
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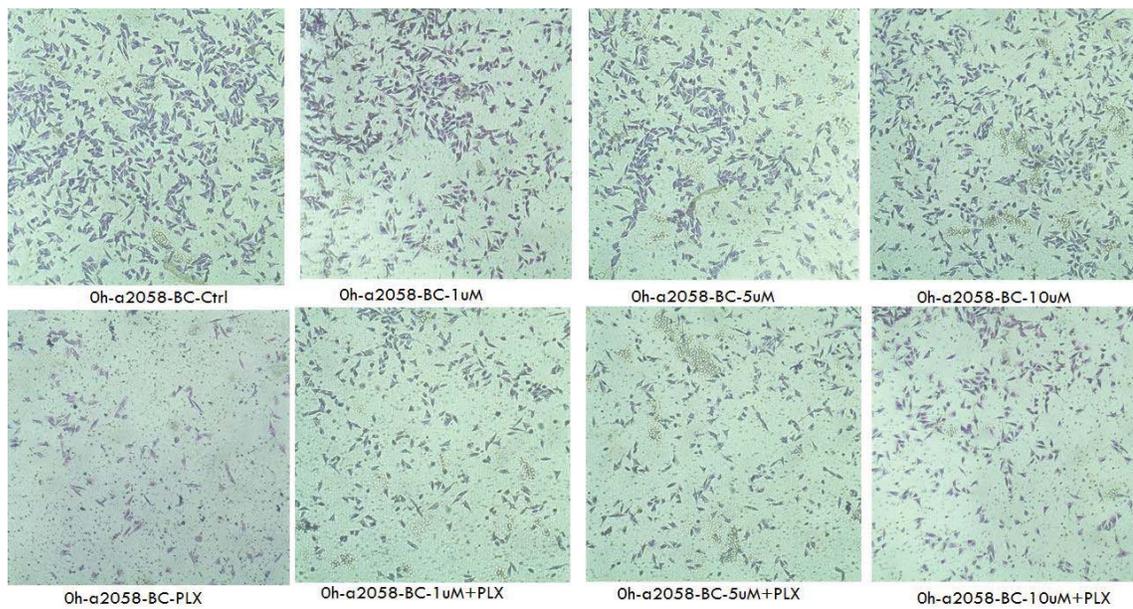
3.3 β -carotene inhibited cell migration/invasion and alleviated the inhibitory effect of PLX on cell migration/invasion

The effect of β -carotene on cell invasion ability and the effect of β -carotene on the inhibitory effect of PLX4032 on cell invasion ability were examined using a Transwell cell migration/invasion assay. β -carotene alone at 5 μ M and 10 μ M significantly ($P < 0.05$) decreased cell migration/invasion across the base membrane by 6% and 17%, respectively. Interestingly, β -carotene alleviated the inhibitory effect of PLX on cell migration/invasion in a dose dependent manner (Figure 3A, 3B).

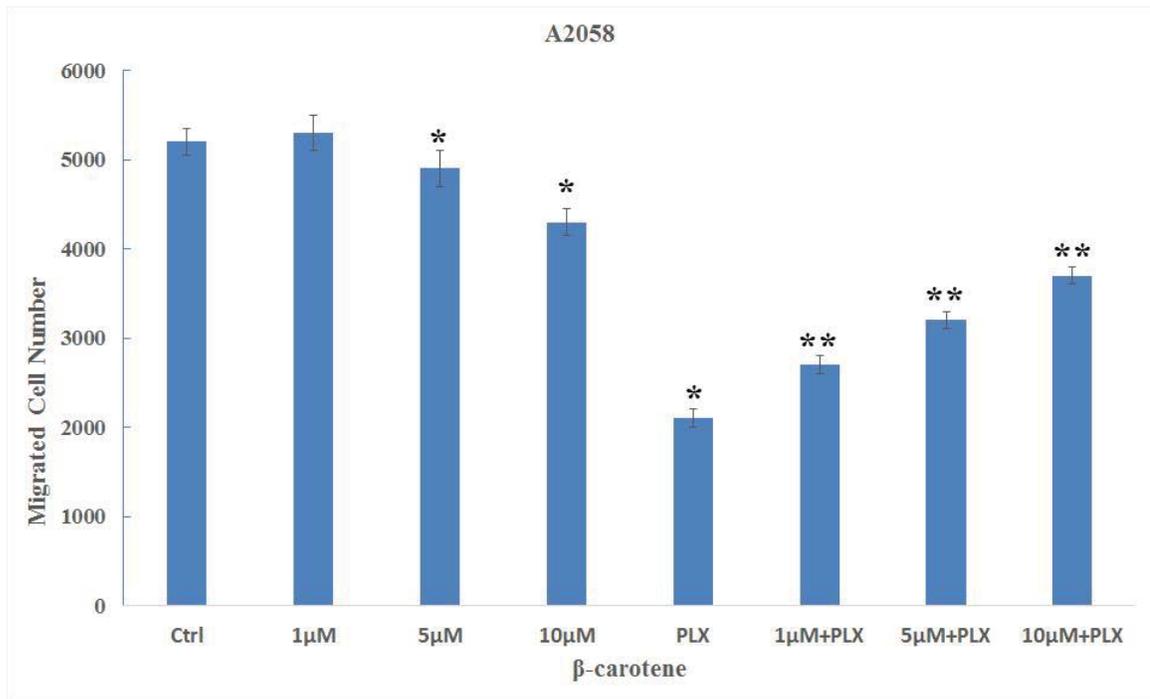
Figure 3. β -carotene inhibited cell migration/invasion and alleviated the inhibitory effect of PLX on cell migration/invasion.

A. 30000 cells were seeded in the upper chamber of 24-well Transwell plates. After 24 hours incubation, migrated cells were counted under the microscope. Representative microscopy images (100 \times magnification) showed the inhibitory effect of β -carotene alone and PLX together with β -carotene. B. Migrated cell numbers in control group and different treatment groups. Data represent three independent experiments, and results are mean \pm SD. “*” indicates a significant difference between control group and treatment group ($P < 0.05$). “**” indicates a significant difference between control group and treatment group and between combined treatment group and PLX alone group ($P < 0.05$).

A.



B.



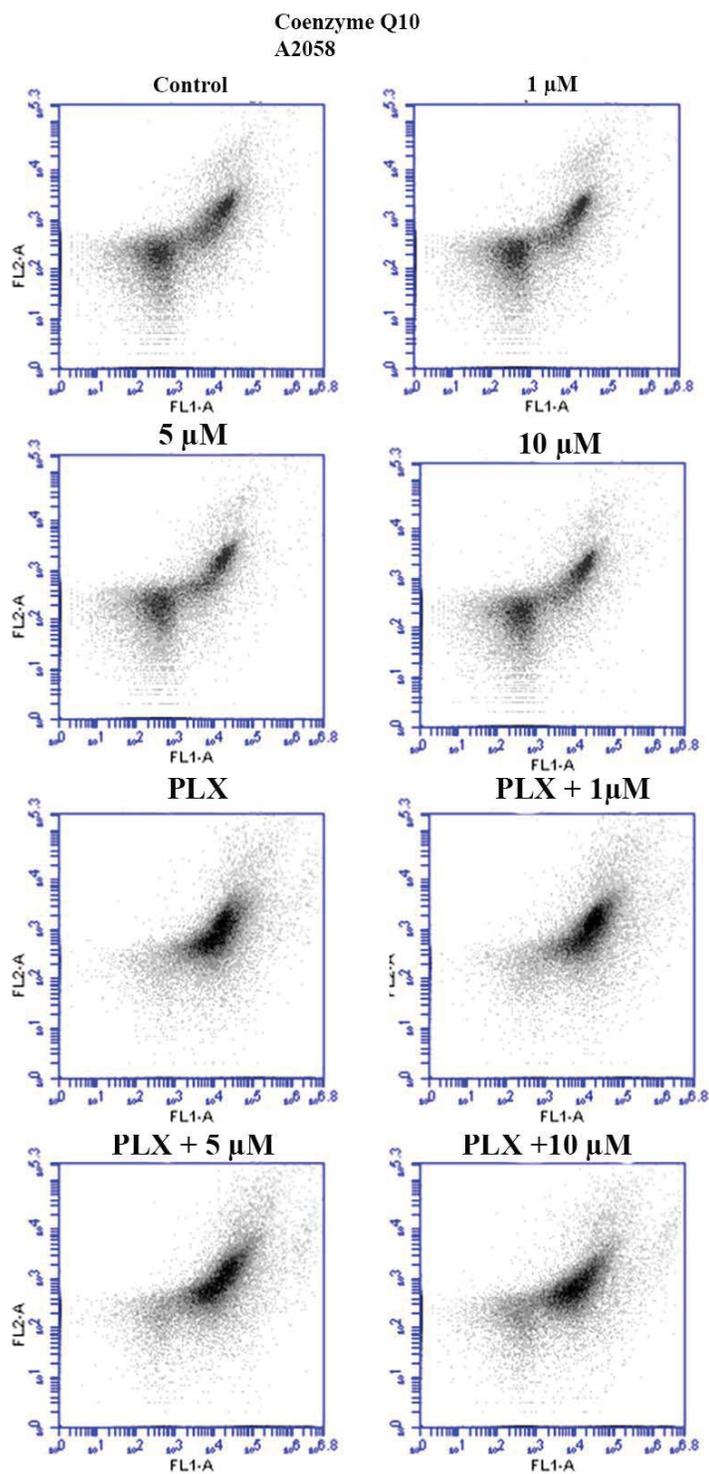
3.4 Coenzyme Q10 and β -carotene inhibited the apoptosis induced by PLX

To determine whether coenzyme Q10 and β -carotene induce apoptosis and whether coenzyme Q10 and β -carotene affect apoptosis induced by PLX, SK-MEL-28 and A2058 were treated by coenzyme Q10 or β -carotene alone, PLX alone, or PLX together with coenzyme Q10 or β -carotene. Apoptosis induction was analyzed by C6 flow cytometry as described in Materials and Methods. In A2058 and SK-MEL-28 melanoma cells, coenzyme Q10 at 10 μ M inhibited the apoptosis induced by PLX (Figure 4A, 34B). Coenzyme Q10 inhibited the apoptosis in SK-MEL-28 cells (Figure 4C, 4D). In A2058 cells, β -carotene at 10 μ M protected cells from undergoing apoptosis induced by PLX (Figure 4E, 4F). β -carotene did not induce apoptosis but protected SK-MEL-28 cells from apoptosis induced by PLX (Figure 4G, 4H).

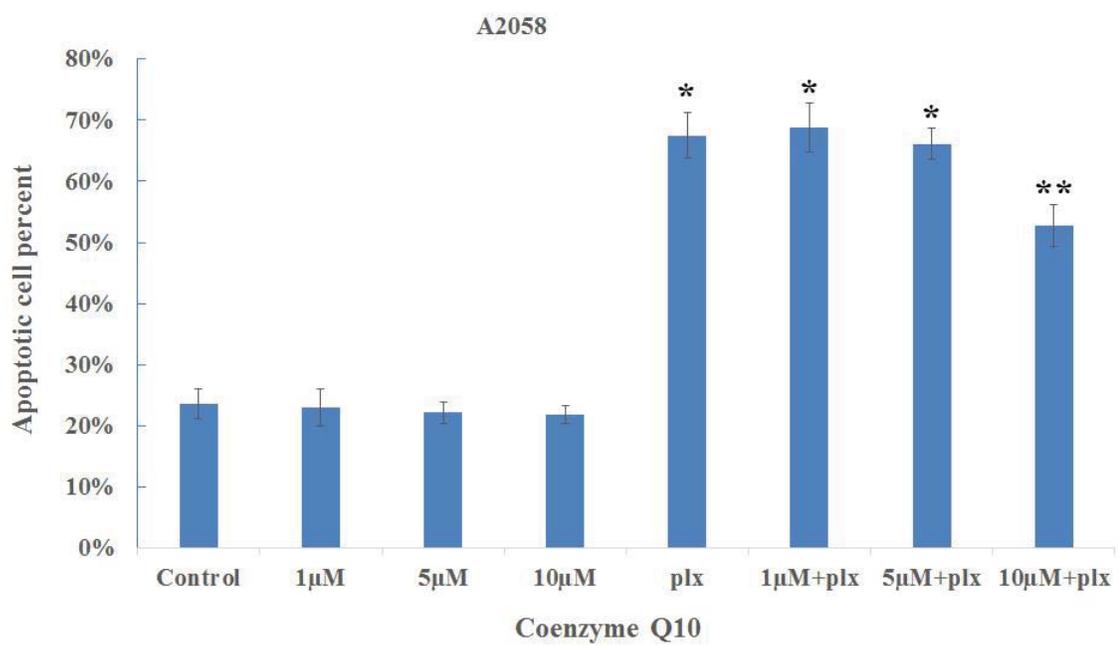
Figure 4. Coenzyme Q10 and β -carotene protect cells from apoptosis induced by PLX.

Cells were treated with coenzyme Q10 or β -carotene alone, PLX alone, PLX together with coenzyme Q10 or β -carotene, or DMSO vehicle for 48 hours. SK-MEL-28 and A2058 cells were stained with Annexin V-FITC and PI, and the fluorescence intensity was measured by C6 flow cytometry (Figure 4A, 4C, 4E, 4G). The percent of Annexin-V positive cells is represented in bar graph (Figure 4B, 4D, 4F, 4H). “*” indicates a significant difference between control group and treatment group ($P < 0.05$). “**” indicates a significant difference between control group and treatment group and between combined treatment group and PLX alone group ($P < 0.05$).

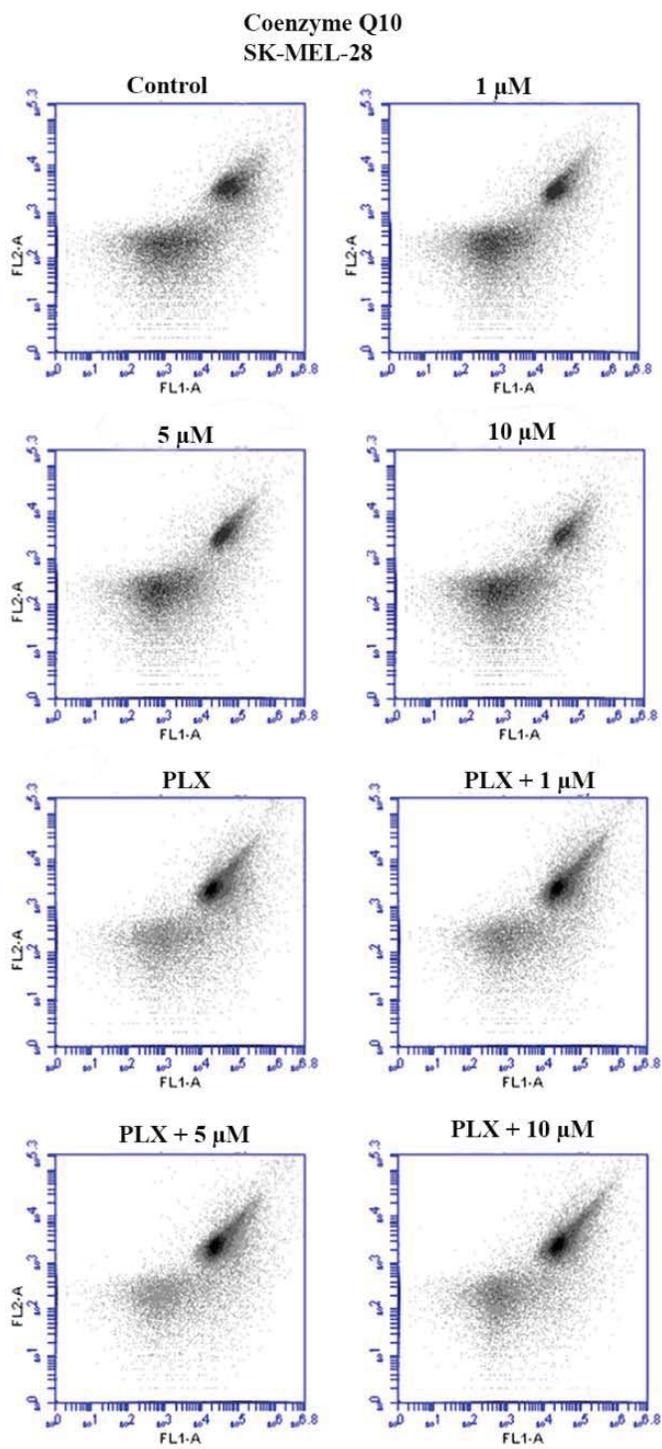
A.



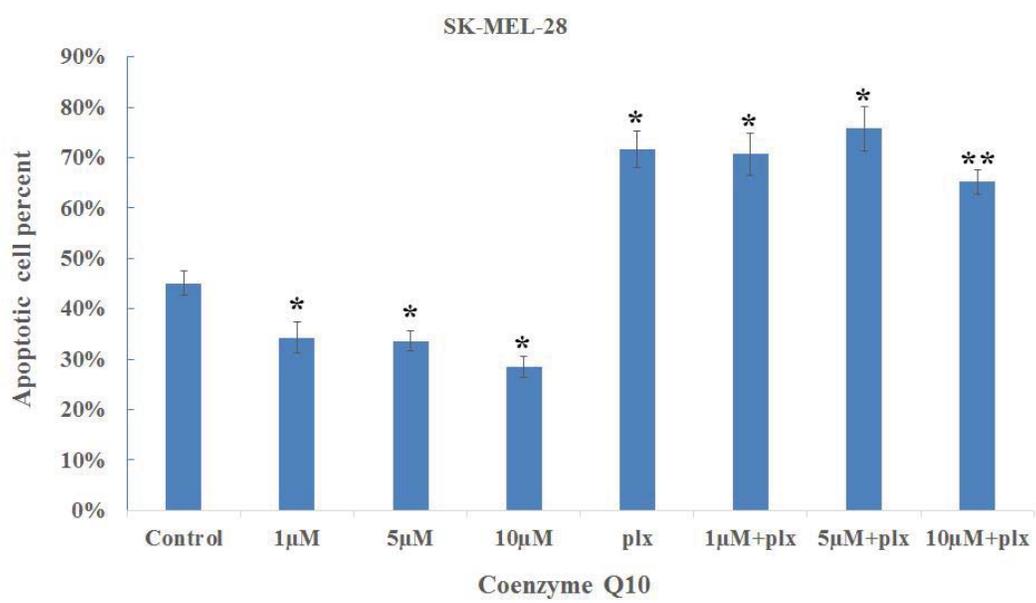
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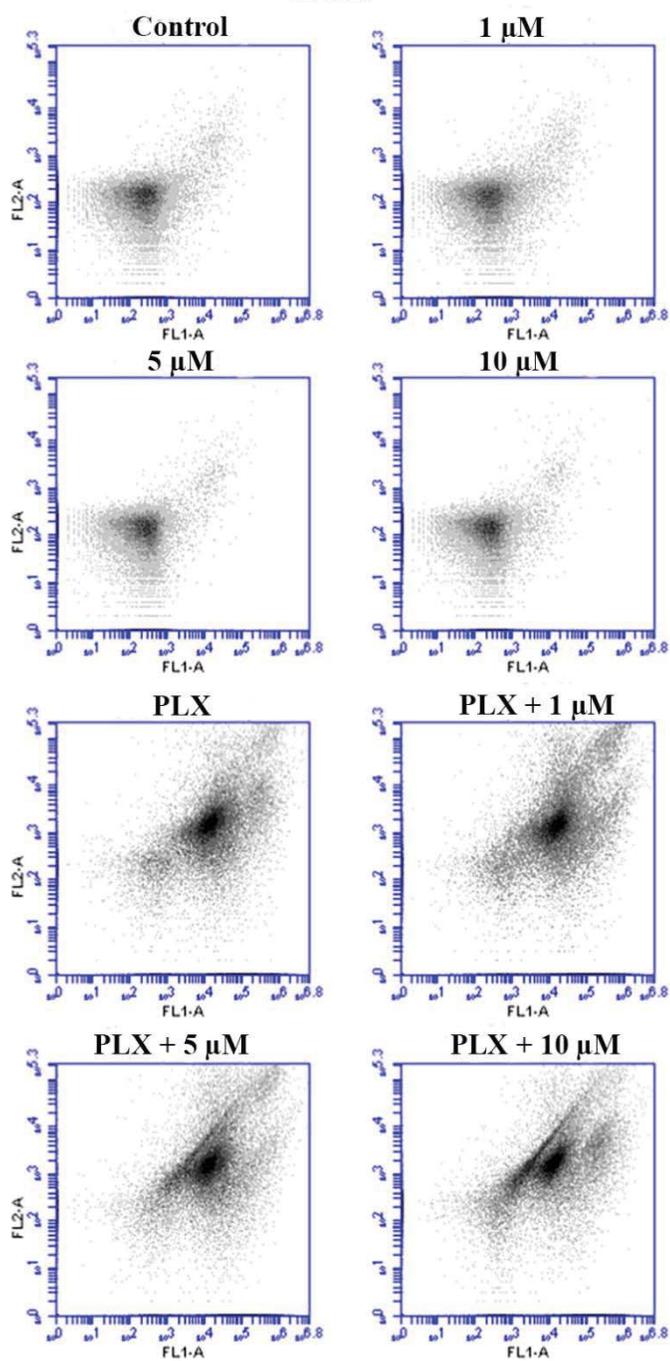
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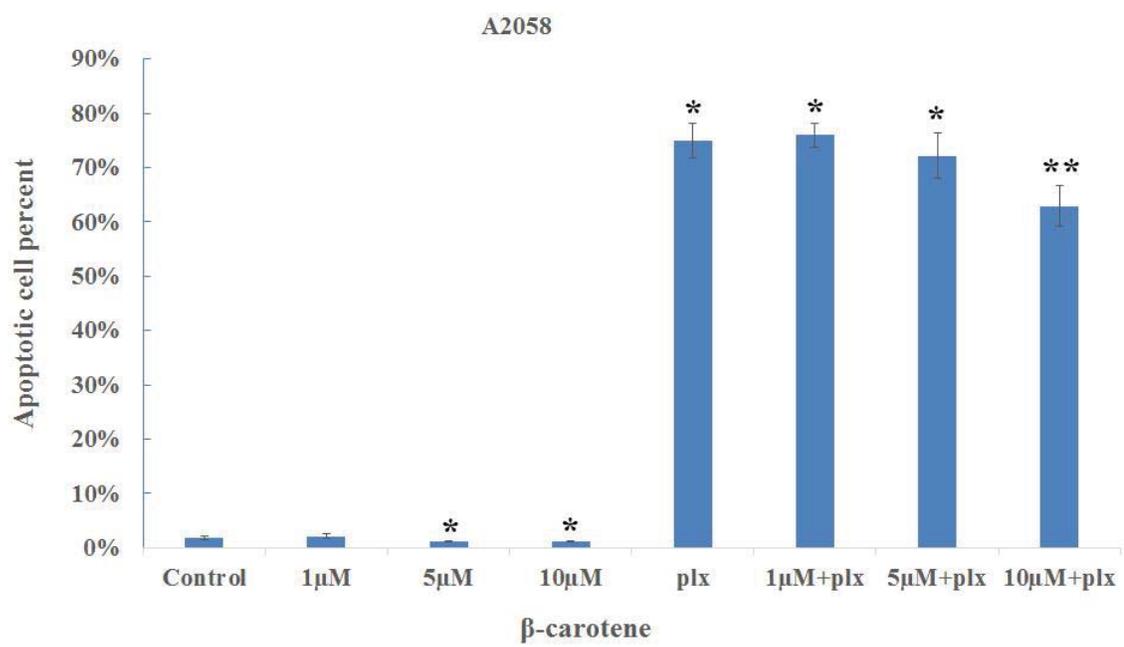
D.



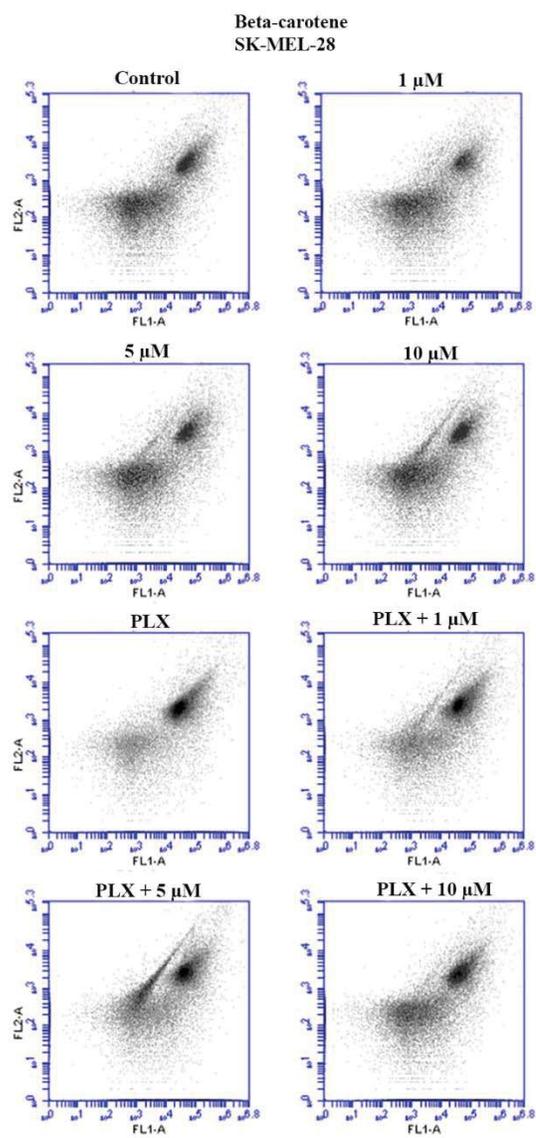
E.

**Beta-carotene
A2058**

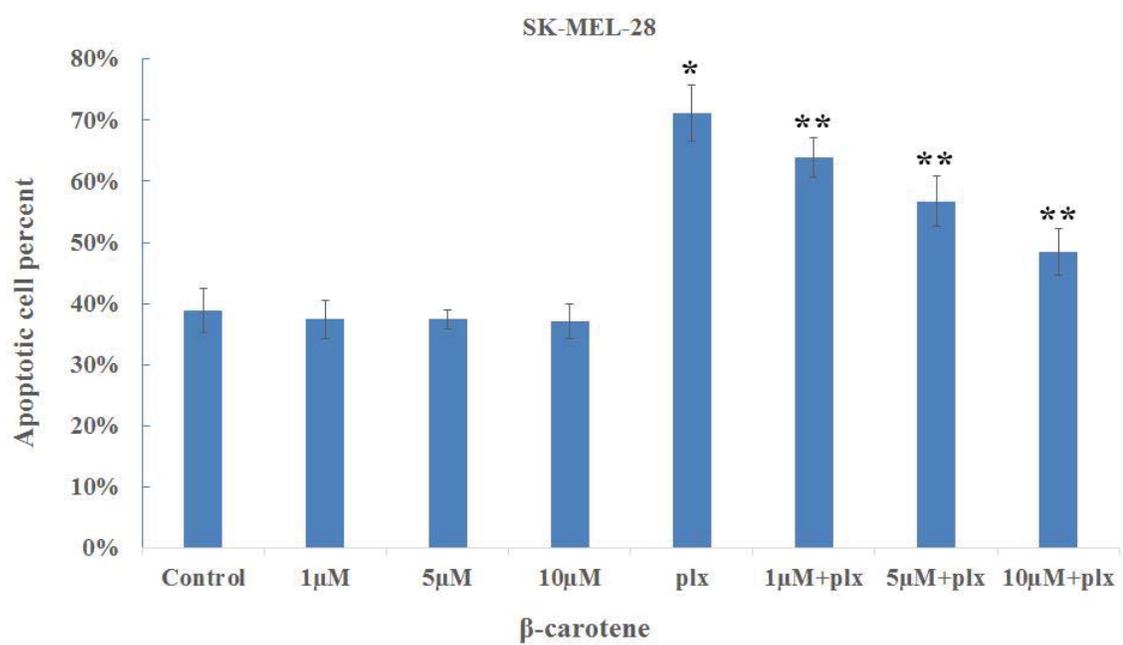
F.



G.



H.



3.5 β -carotene worked synergistically with PLX to suppress the Ras-Raf-Mek-Erk pathway

Ras-Raf-Mek-Erk is one of the most important intracellular cell growth signaling pathways. Since both SK-MEL-28 and A2058 harbor B-raf activating mutations, we examined the effect of β -carotene on activation of Ras-Raf-Mek-Erk signaling pathway. A2058 cells, which are melanoma resistant, were treated with β -carotene alone, PLX alone, PLX together with β -carotene, and DMSO vehicle. After 48h incubation, Western-blot analysis showed that β -carotene had no effect on BRAF or ERK expression (Figure 5B). β -carotene alone did not have an effect on the activation of this pathway. However, β -carotene at a physiological concentration (1 μ M) synergistically worked with Vemurafenib to suppress the activation of BRAF and the downstream Erk1/2 (Figure 5A).

Figure 5. β -carotene showed no effect on BRAF expression and activation.

Western-blot analysis of BRAF, phosphorylated BRAF, ERK, and phosphorylated ERK in A2058 cells. GAPDH was used as the internal loading control. This experiment was repeated three times and representative figure is shown.

4. Discussion

Malignant melanoma is the most aggressive type of cutaneous malignant tumor and is often a fatal disease [61]. The incidence of melanoma has been rising faster than any other cancer in men and is second only to lung cancer in women. It is the fifth most common cancer in the US, accounting for over 60,000 new cases each year [62]. It is estimated that one out of 63 persons in the United States will develop malignant melanoma in his or her lifetime [63,64]. Invasive melanoma is refractory to the current therapeutic strategy, and the 5-year survival rate is less than 5-15% [4]. Current strategies focused on systemic therapy for treatment of melanoma have shown no effect on long term survival.

ROS, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorite ($HClO^-$), are a group of chemically reactive oxygen-containing molecules. Even though ROS play crucial roles in cell signaling and homeostasis [65], ROS produces various kinds of DNA damages including base modifications, single strand breaks, and DNA strand crosslink [66]. DNA damages induced by ROS lead to gene mutations that directly contribute to cancer initiation and development. It is interesting to know that ROS have contradictory roles in cancer progression. While ROS can promote cancer cell survival and growth by mutating and deactivating tumor suppressor genes (i.e. p53), activating growth factors [67], and increasing the production of inflammatory factors, they can also inhibit cancer initiation and progression by increasing expression of tumor suppressor

genes, inducing apoptosis [68], and maintaining the activation of cell cycle inhibitors [69].

Antioxidants are molecules that remove free radicals including ROS and thus relieve the oxidative stress of cells. Humans and animals maintain complex systems of various types of antioxidants including glutathione, vitamin A, vitamin E, vitamin C, coenzyme Q, carotenoids, and uric acid. Insufficient level of antioxidants causes increased oxidative stress that is intimately involved in aging and many diseases including cancers.

Antioxidants can prevent ROS from causing cellular damages to DNA, proteins, and cell membrane. Specifically, antioxidants affect tumor initiation and development through the following potential mechanisms: (1) antioxidants inhibit formation of carcinogen by quenching carcinogen activation; (2) antioxidants decrease levels of DNA mutations by reducing DNA oxidation; (3) antioxidants decrease tumor cell proliferation by shutting down growth-related signal transduction pathways and causing cell cycle arrest; and (4) antioxidants decrease cancer metastasis by inhibiting cell migration and invasion [70].

Therefore, people have generally believed that taking antioxidant supplementation is beneficial for the prevention and treatment of cancers. Since radiotherapy and some chemotherapies produce ROS that cause damage to the normal tissues, the capacity of scavenging ROS and repairing tissue damage by antioxidants adds the enthusiasm for using antioxidant supplements to alleviate the side effects of therapy. Many research articles advocate antioxidants as cancer fighters [71] and report that high doses of dietary antioxidants often inhibit the growth of cancer cells without affecting the growth of normal cells [72,73]. A population-based prospective cohort study showed that the use of

antioxidants vitamin E and C in the first six months of diagnosis significantly reduced the mortality and recurrence of invasive breast cancer [74]. Antioxidants have been used as beneficial adjuncts to the conventional cancer therapy in clinical studies [75,76]. However, there has been increasing evidence showing that antioxidants are not necessarily our friends in combating cancers. It was reported that antioxidants stimulated tumor cell growth [77]. Also, the use of antioxidants vitamin E and β -carotene concurrently with radiotherapy in head and neck cancer patients significantly increased recurrence and cancer-specific mortality [78]. Our recently published work showed that antioxidant vitamin C is a double-edged sword in melanoma treatment, showing cytotoxic or growth-promoting effects at different concentrations. Therefore, whether the use of antioxidants in cancer prevention and treatment is recommendable, and whether antioxidants exert a synergistic or antagonistic effect with chemotherapy, deserve close scrutiny.

Coenzyme Q10 is a free radical-scavenging antioxidant due to its capacity to act as both a two-electron carrier and a one-electron carrier. Since no large-scale strictly-controlled clinical trials of coenzyme Q10 in cancer treatment have been done, the correlation between coenzyme Q10 and cancers is not well understood. However, the American Cancer Society has concluded that, "CoQ₁₀ may reduce the effectiveness of chemo and radiation therapy, so most oncologists would recommend avoiding it during cancer treatment." Research showed that an imbalance in the antioxidant system can be detected in melanoma cells and in a percentage of normal melanocytes from melanoma patients [30], and low plasma levels of Coenzyme Q10 could be a prognostic factor for

melanoma progression [45]. And due to the low concentration of coenzyme Q10 in melanoma cell lines, and in sera of melanoma patients, coenzyme Q10 was used in combination with an optimized dose of recombinant interferon α -2b in a 3-year trial, which showed that this combination significantly reduced the recurrence rate [79].

Hong et al. reported that mitochondrial-targeted carboxy-proxyl coenzyme Q10, but not coenzyme Q10, suppressed the B-RafV600E melanoma cell line [48]. However, when we examined the effect of Coenzyme Q10 on the viability of two human malignant melanoma cell lines, coenzyme Q10 modestly but significantly reduced the viability of SK-MEL-28 cells, which is a PLX-sensitive melanoma cell line. For A2058 cell line, which is a PLX-resistant cell line, coenzyme Q10 alone did not display cytotoxicity. However, it increased the cytotoxicity of the FDA-approved BRAF inhibitor Vemurafenib. It adds to the evidence that coenzyme Q10 can potentially be a good adjunct to targeted chemotherapy or immunotherapy against melanoma.

This study also found that coenzyme Q10 significantly reduced the mobility of both SK-ME-28 and A2058 cells in “wound healing” assay. This is the first work reporting that coenzyme Q10 inhibits the mobility of cancer cells. It was reported that a functional dietary supplement containing Coenzyme Q10, branched-chain amino acids and L-carnitine, completely inhibited metastasis of melanoma to the lung [49]. In addition, exogenous coenzyme Q10 reduced matrix metalloproteinases 2 (MMP-2) activity in breast cancer cell line, suggesting the importance of coenzyme Q10 on the cell invasion

effector molecules [80]. Therefore, coenzyme Q10 may inhibit metastasis of melanoma by directly inhibiting cell mobility and reducing the MMP-2 activity that helps cancer cells break through the intracellular matrix and facilitate the metastasis.

This work also examined the effect of coenzyme Q10 on induction of apoptosis, a programmed cell death that plays vital roles in tumor survival and progression. Our data shows that coenzyme Q10 significantly reduced the percentage of apoptotic cells. In addition, coenzyme Q10 alleviated the apoptosis induced by Vemurafenib. This finding is in agreement with previous reports that coenzyme Q10 protects cells from undergoing apoptosis induced by cytotoxic chemicals in both cancerous [81] and non-cancerous cells [82]. Therefore, apoptosis induction is not a mechanism by which coenzyme Q10 exerts its cytotoxic effect against melanoma cell lines.

The association of β -carotene with cancers has been under heated debate. A trial showed that neither β -carotene nor vitamin A supplement showed any beneficial effect in preventing cancer. Instead, increased risk of lung and prostate cancers was found in participants who consumed β -carotene and had lung irritation from cigarette smoking or asbestos exposure [83]. Another study showed that, in addition to lung cancer, the incidence of gastric cancers was also significantly increased in individuals who take beta-carotene at 20-30 mg day [55]. Moreover, meta-analysis suggests that intake of vitamin A or beta-carotene is not associated with reduced risk of melanoma [59]. However, some research obtained results that showed anti-cancer activities of β -carotene. β -carotene at

low physiological concentration inhibited cell viability and induced apoptosis in human breast cancer MCF-7 cell line [84]. In addition, in a published study, β -carotene inhibited lung metastasis in mice [58]. β -carotene was also reported to inhibit angiogenesis and activation of transcription factor in mouse melanoma cells [57]. This work aims to further characterize the effect of β -carotene on human malignant melanoma cells.

Our data shows that β -carotene do not show inhibitory or promoting effects on the viability of SK-MEL-28 and A2058 cells. However, in both cell lines, β -carotene mitigated the cytotoxic effect of Vemurafenib, suggesting that intake of β -carotene may decrease the therapeutic effect of Vemurafenib.

In “wound healing” assay, β -carotene significantly reduced cell mobility. Similarly, β -carotene reduced the ability of cells to move across the matrix basement, as seen in Transwell cell migration/invasion assay. These findings are consistent with a published work reporting that β -carotene inhibited migration of human umbilical vein endothelial cells and down-regulated the expression of MMP-2 and MMP-9, and up-regulated the expression of tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 [57]. Our data support that β -carotene may inhibit the metastasis of melanoma. However, in both “wound healing” assay and Transwell cell migration/invasion assay, β -carotene significantly relieved the inhibition of cell migration and invasion caused by Vemurafenib in both SK-MEL-28 and A2058 cell lines. These data raise a concern that β -carotene may suppress the anti-metastatic effect of Vemurafenib.

β -carotene was reported to induce apoptosis by decreasing the expression of anti-apoptotic proteins Bcl-2 and PARP, and the survival protein NF- κ B [84]. However, β -carotene did not significantly induce apoptosis in human malignant melanoma cell lines. However, similar to the cell viability assay and cell migration/invasion assays, β -carotene alleviated the cytotoxic effect, in this case, apoptosis induction, in both SK-MEL-28 and A2058 cells. Again, this suggests that β -carotene may decrease the anti-melanoma effect of Vemurafenib.

Ras-Raf-Mek-Erk is a vital cell-signaling pathway that drives cell proliferation. Since both SK-MEL-28 and A2058 harbor B-raf activating mutation, we examined the effect of β -carotene on activation of Ras-Raf-Mek-Erk signaling pathway. β -carotene alone did not have effect on the activation of this pathway; however, it is interesting to find that β -carotene at physiological concentration (1 μ M) synergistically worked with Vemurafenib to suppress the activation of Braf and the downstream Erk1/2. This result is consistent with a published data showing that β -carotene inhibited Erk1/2 in breast cancer MCF-7 cells [84]. Suppressing effect of β -carotene on Ras-Raf-Mek-Erk intracellular signaling pathway can help explain its inhibitory effect on cell migration and invasion.

In summary, regardless of displaying its ability to protect melanoma cells from apoptosis induction, coenzyme Q10 showed an inhibitory effect on cell proliferation and migration/invasion when used individually or in combination with Vemurafenib. The

cytotoxic effects of coenzyme Q10 make it a good candidate adjunct for existing therapy. In contrast, β -carotene suppressed the anti-melanoma effects of Vemurafenib, including its anti-proliferative effect, anti-invasive effect, and apoptosis-inducing effect. Caution should be taken when β -carotene is used concurrently with anti-melanoma chemotherapy.

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Effect of Antioxidants Co-enzyme Q10 and β -carotene on the Cytotoxicity of Vemurafenib

against Human Malignant Melanoma

Title of Thesis

Signature of Graduate School Staff

Date Received