SciMedCentral

Journal of Cancer Biology & Research

Research Article

Synthetic Compounds That Inhibit Melanoma Growth and Invasiveness by Reducing Cancer Stem Cell Population

Nicholas M. Schlaeger[1], Carmen Berkan[1], Ingrid Albu[1], Aaron P. Monte[2], and Cheng-chen Huang[1]*

[1]Department of Biology, University of Wisconsin-River Falls, USA

[2]Department of Chemistry and Biochemistry, University of Wisconsin-La Crosse, USA

*Corresponding author

Cheng-chen Huang, Biology Department, University of Wisconsin-River Falls, 410 South Third Street, River Falls 54022, Wisconsin, USA, Tel: 1-715-425-4276; Fax: +1-715-425-0378; Email: cheng-chen.huang@uwrf.edu

Submitted: 21 February 2020

Accepted: 8 March 2020

Published: 09 March 2020

ISSN: 2373-9436

OPEN ACCESS

Keywords

- Melanoma; Cancer stem cell; Metastasis; Differentiation therapy

Abstract

Within melanoma tumors are a small subpopulation of cells called cancer stem cells (CSC). They can be categorized by two fundamental properties, self-renewal and differentiation. CSC play a vital role in metastasis, tumor relapse and chemotherapeutic resistance. Here we identified compounds that seem to specifically target the melanoma CSC population. B16F10 mouse melanoma cells treated with synthetic compounds SK0408 or SK0459 first showed reduction of proliferation in the MTT and anti-phophohistone H3 immunostaining assays. Western blotting revealed that SK0408 and SK0459 has no effect on phosphorylated MAPK level but significant decrease of phophorylated Akt and increase of phosphorylated b-catenin. Using melanoma stem cell markers CD133, CD271, and CD20, we found that SK0408 and SK0459 indeed cause a 54-79% reduction in the melanoma stem cells population. Cell invasion assay showed reduced metastatic potential in treated cells. Furthermore, we observed an increase of the epithelial marker E-cadherin but a decrease in the mesenchymal marker vimentin in the treated cells. Additionally, western blotting and RT-qPCR showed an elevated level of differentiation gene expression of Pax-3, MITF, DCT, and tyrosinase. Our results demonstrate the therapeutic potential of these compounds in suppressing melanoma cell growth and metastasis by reducing melanoma stem cells through promoting melanocyte differentiation.

ABBREVIATION

CSC: Cancer Stem Cell; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-qPCR: Reverse Transcription-quantitative Polymerase Chain Reaction; Pax-3: Paired box gene 3; MITF: Microphthalmia-associated Transcription Factor; DCT: Dopachrome Tautomerase; MAPK: Mitogen Activated Protein Kinase; EMT: Epithelial-Mesenchymal Transition

INTRODUCTION

The most lethal type of skin cancer, known as melanoma, has shown evidence of enhanced metastasis, chemotherapeutic resistance, and relapse following some conventional melanoma treatments. Melanoma with the BRAF mutation is commonly treated with drugs that target and inhibit the Mitogen Activated Protein Kinase (MAPK), signaling pathway. These include BRAF inhibitors, such as vemurafenib and dabrafenib, or MEK inhibitors, such as trametinib and cobimetinib. Studies show that melanoma may develop resistance to chemotherapy by activating resistance pathways and altering gene expression. For example, melanoma treated with vemurafenib up-regulates the tumor micro-environment modulator matrix metalloproteinase-2 (MMP-2), which degrades extracellular matrix proteins that keep cells stationary and prevent them from migrating [1]. Thus, vemurafenib enhances melanoma metastasis by making melanoma cells more mobile and readily invasive. In addition to promoting metastasis, vemurafenib resistant cells tend to express stem cell markers such as CD271 and fibronectin [2]. Initial responses to BRAF and MEK inhibitors may appear promising, but side effects are a major complication associated with these medications. The cancer stem cell hypothesis states that there is a rare subpopulation of tumor cells with stem cell characteristics that bring about metastasis, resistance, and relapse.

Cancer stem cells (CSC) are a small population of cancer cells that possess stem cell properties [3]. They are slow in dividing and able to self-renew and differentiate into many cell types. In melanoma, melanocyte precursors possess stem cell properties. Clinical and basic research strongly suggest that CSC are the source of metastasis, which is the main contributor to cancer lethality [4,5]. A metastatic case of cancer involves anchorage independent CSC that detach from primary tumors, cause invasion through the vascular system, and initiate tumor formation throughout the body. Therefore, treating melanoma with therapeutics that reduce the stem cell population would decrease the probability of metastasis, and other problems related to CSC, such as tumor relapse and chemotherapeutic resistance [6]. However, efficient CSC targeting drugs have yet to be discovered and are not commercially available for melanoma

patients. CSC have the capability of initiating tumor growth and tumor relapse [7]. Relapse occurs when a patient treated with chemotherapy has a positive initial result of remission, but the tumors later reappear, and are often resistant to the previously used chemotherapy. Relapse and resistance often go hand in hand. The connection of CSC to metastasis, relapse, and resistance make them an excellent target for treating melanoma. Although BRAF and MEK inhibitors increase the risk of metastasis, relapse, and resistance development, they may still prove useful in mixed treatments [8].

A novel mixed treatment for melanoma might include a known BRAF or MEK inhibitor, as well as a drug that targets CSC. The BRAF or MEK inhibitors would act as a cytotoxic agent to kill melanoma cells and slow tumor growth, while the CSC targeting drug would prevent the deleterious effects of BRAF or MEK inhibitors. The CSC targeting drug within the chemotherapeutic cocktail could, at the very least, prolong tumor relapse, while reducing the chance of metastasis and resistance development. The most desirable CSC targeting drug would also aid in the reduction of cell growth and proliferation. This would allow the BRAF or MEK inhibitor to be administered at lower concentrations and thereby reduce toxicity and side effects. This study identifies two novel CSC reducing small molecules that diminish melanoma cell proliferation by what appears to be an enhancement of differentiation.

It has been hypothesized that one way to reduce the effects of CSC is to enhance differentiation, called differentiation therapy [9]. Differentiated melanoma cells and melanocytes are slow to proliferate and are not invasive like the stem cell precursors [10]. The main purpose of differentiated melanocytes is to produce melanin. A number of genes participate in the biosynthesis of melanin and specify fully differentiated melanocytes. Some genes that specify differentiation progression in melanocytes include paired box 3 (Pax3), microphthalmia transcription factor (MITF), dopachrome tautomerase (DCT), and tyrosinase. Pax3 is a transcription factor gene that can code for multiple proteins through alternate splicing (Dye). Differentiation of melanocytes is dependent on Pax3, because Pax3 activates the melanocytic modulator MITF, which activates a cascade of melanogenic genes. MITF activates transcription of many genes including tyrosinase and DCT. Both take part in the biosynthesis of melanin. Tyrosinase is the enzyme that initiates the synthesis of melanin. It oxidizes the phenolic amino acid tyrosine into dopaquinone. After additional chemical reactions take place, DCT catalyzes the synthesis of quinone molecules which polymerize to form a network of aromatic molecules [11]. Aromatic structures are good at absorbing ultraviolet light, so this function is utilized by differentiated melanocytes in the skin, hair, iris and retina to protect underlying tissues from UV radiation.

In this study, we identified two small molecules, SK0408 and SK0459, that can inhibit melanoma proliferation. More interestingly, these compounds demonstrated the ability to reduce the melanoma stem cell population. We further showed that the biological functions of SK0408 and SK0459 are correlated with decrease of Akt and wnt signaling activities and increase of melanocyte differentiation. All together our results suggest that SK0408 and SK0459 could be novel therapeutics for treating melanoma by targeting melanoma stem cells.

MATERIAL AND METHODS

Cell culture

The mouse melanoma cell line B16F10 (ATCC CRL-6475), was purchased from ATCC (American Tissue Culture Center), and maintained in DMEM supplemented with 10% fetal bovine serum. Trypsinization and subculture were performed following the protocols found on the ATCC website. For chemical treatment, approximately 14,000 melanoma cells and 30,000 normal melanocytes were seeded in each well of the 24-well plate with 0.5ml of culture medium. Cells were set up triplicate and allowed to grow two days for melanoma or three days for normal melanocytes before chemical treatment. After 24 hours of chemical incubation, cells were first washed with PBS once and trypsinized. Equal volume of cell suspension and trypan blue were mixed in an eppendorf tube before the cells were counted with a hemacytometer.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT), assay is a reliable method of measuring cellular metabolic activity and proliferation. 10,000 melanoma cells were plated into individual wells of a 96-well plate with 200µL of growth medium as specified in the *Cell culture* section. The cells were allowed to incubate for 24 hours after the plating procedure. After the incubation period, dimethyl sulfoxide (DMSO), SK0408, and SK0459 were all administrated at 30µM concentration, and MEK inhibitor (MEK-I), was administered at 10µM concentration. Compound-treated cells were allowed to incubate for an additional 24 hours before the MTT reaction. The compound concentrations and incubation periods apply to all of the subsequent compound experiments in this study. MTT is reduced by mitochondrial reductases into a purple colored crystal called formazan and SDS-HCL solution is used to dissolve the formazan for analysis. Dissolved formazan is analyzed spectrophotometrically with a Model 680 microplate reader (Bio-Rad, Foster City, CA), at 570nm and the absorbance reading is correlated with the quantity of cell growth and replication. Results were plotted as means ± standard deviation.

Immunohistochemistry

For mitosis detection, 200 cells were seeded onto Teflon-coated slides (HTC Super- cured, 4-well, 10mm from Cell-Line/Thermo Fisher), with 50µL of growth medium. Cells were allowed to grow for 24 hours before the medium was refreshed with or without compound. After 24 hrs of chemical incubation, cells were fixed with 4% paraformaldehyde (PFA) for 24 hours at 4°C. Phosphate buffered saline (PBS), is used to wash the cells several times throughout the procedure. Next, blocking buffer was administered at room temperature for an hour followed by incubation with anti-phosphorylated-Histone H3 (Ser10), Rabbit mAb-Alexa Fluor 555 conjugate (Cell Signaling Technology, 1:100 dilution in blocking buffer). For apoptosis analysis, cells were prepared the same way, but stained with the cleaved caspase-3 (Asp175) (5A1E), rabbit mAb (Cell Signaling Technology, 1:1000 dilution) overnight followed by Alex594 conjugated goat- anti rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc, 1:1000 dilution), for two hours at room

temperature. Lastly, a drop of ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technology), was applied to the stained cells. The number of positively stained melanocytes was manually counted and graphed. Student T-test was performed to determine the statistical significance.

Detection of cancer stem cells using flow cytometry

To detect melanoma cancer stem cells, B16F10 cells were seeded in 6-well plates, 200,000 cells/well. Cells were allowed to grow for 24 hours before compound treatment. After 24 hours of incubating with the compound treatments, cells were trypsinized and fixed in 4% PFA overnight. The cells were washed with PBS and stained with the following antibodies in 4°C overnight: CD133 monoclonal antibody (13A4), FITC (Thermo Fisher Scientific, 1:100 dilution); CD271 monoclonal antibody (ME20.4), PE (Thermo Fisher Scientific, 1:100 dilution); CD20 monoclonal antibody (AISB12), PE (Thermo Fisher Scientific, 1:100 dilution). After staining, cells were suspended in PBS and run through the BD Accuri C6 Flow Cytometer (BD Biosciences, Inc).

Western blot

Cells were prepared for western blotting in a 6-well plate. To harvest the proteins, we first added 400µL of freshly prepared lysis buffer (0.2% NP-40, 100mM Tris, 150mM NaCl, 8mM of EDTA, pH 7.4), with protease inhibitor cocktail (P8340, Sigma), to the wells. We used a cell scraper to detach the cells from the surface of the wells. The cells were lysed in 1.5mL microcentrifuge tubes for 30 minutes on a shaker at 4°C. The lysate was then centrifuged at 12,000 rpm at 4°C for 20 minutes. After the centrifugation, the protein supernatant was transferred to a clean tube and stored in -80°C. Using the Pierce BCA Assay Kit (23227, Thermo Scientific), protein concentrations were analyzed. 10-20µg of protein were loaded into a 12% SDS-PAGE gel (Biorad), for gel electrophoresis. The western blotting was performed using the Pierce Fast Western Blot Kit (35050, Thermo Scientific). Antibodies were purchased from Cell Signaling Technology. The anti-MITF antibody (MITF D5G7V rabbit antibody), recognizes all the MITF isoforms and thus detected the total MITF proteins. E-Cadherin (4A2), mouse mAb and vimentin (D21H3), XP Rabbit mAb are purchased from Cell Signaling Technology. Phospho-SMAD4 (Thr276), polyclonal antibody was purchased from ThermoFisher and the anti-Twist rabbit polyclonal antibody (ab49254), was from Abcam.

Reverse transcription-Qpcr

In 6-well plates, 100,000 cells were seeded in 2mL of growth medium and allowed to incubate for 24 hours before compound treatment. After chemical treatment an additional 24-hour incubation period took place. The cells were washed with PBS and RNA was extracted with Trizol following the commercial instructions (Invitrogen, Carlsbad, CA). RNA concentration and quality were determined using the Genesys 10S UV-VIS ectrophotometer (Thermo Scientific, USA). 5µg of total RNA was then used to generate the cDNA library using the Superscript III kit (Invitrogen, Carlsbad, CA). Quantitative PCR (RT-qPCR) was set up using the Power SYBR Green PCR kit (Biorad, Foster City, CA), and run on the Mx300P qPCR thermocycler (Agilent Technologies, Santa Clara, CA). Relative quantities of mRNA were determined and the data analyzed and graphed using the MxPro qPCR software provided by the manufacturer. The GAPDH gene used as a normalizer. Primers used for RT-qPCR are:

GAPDH L, 5' TGCACCACCAACTGCTTAGC 3'

GAPDH R, 5' TCTTCTGGGTGGCAGTGATG 3'

Pax3 L, 5' TCGGCCTTGCGTCATTTC 3'

Pax3 R, 5' CAGGATCTTAGAGACGCAACCA 3'

MITF L, 5' TGCCTTGTTTATGGTGCCTTCT 3'

MITF R, 5' TCCCTCTACTTTCTGTAATTCCAATTC 3'

DCT L, 5' CCGGCCCCGACTGTAATC 3'

DCT R, 5' GGGCAGTCAGGGAATGGATAT 3'

Cell invasion assay

The cell invasion assay kit was purchased from Corning, Inc (Product #354880, BioCoat Matrixgel Invasion Chambers with 8.0µm PET membrane). Cells were prepared in growth medium and seeded in the invasion chamber, 40,000 cells per chamber with or without compound. In the TC companion plate, 0.75ml of complete medium with 10% fetal bovine serum was placed below the invasion chamber. Cells were allowed to migrate overnight in a humidified tissue culture incubator at 37°C, 5% CO_2 atmosphere. On the next day, the cells that remained in the apical side of the invasion chamber were carefully cleaned up using cotton swabs and tooth picks. The cells on the lower surface of the PET membrane were fixed with 100% methanol and stained with 1% toluidine, 2 minutes in each solution. After staining, the cells were washed with distilled water twice, 2 minutes each. The PET membrane was then cut off and mounted on a slide with DAPI-containing mounting medium. Cells were counted with a compound microscope.

Ames test

The Ames test Muta-ChromoPlate Kit was purchased from the Environmental Bio-detection Products Inc. (Ontario, Canada). This kit is a modified Ames test that utilizes a mutated *Salmonella typhimurium* TA100 strain for detection of mutagenic activity. As many mutagens must first be metabolized into their reactive form by enzymes, two experiments were set up to test the mutagenicity of compounds (without rat liver extract), or compound metabolites through bioactivation using rat liver extract following the manufacture's instruction. In brief, lyophilized salmonella bacteria were refreshed overnight in 37°C oven. On the next day, the reaction mix which contain Davis-Mingioli salts, D-glucose, Bromocresol purple, biotin, L-Histidine was prepared. Test compounds were prepared in sterilized, double-stilled water at 10-30µM. After mixing the bacteria, reaction mix, and test compounds, the solution was aliquoted into a 96-well plate and incubated for several days. Revertants, which will turn the medium from purple to yellow, typically appear from day 2 to day 6. The number of yellow wells was recorded daily.

RESULTS AND DISCUSSION

SK0408 and SK0459 reduce B16F10 melanoma proliferation

This project was designed to look for compounds that

can inhibit melanoma cell proliferation using the widely used quantitative biochemical method called MTT assay which measures the activity of mitochondrial dehydrogenases that have been shown to correlate with cell proliferation accurately [12]. After testing a small library of about fifty compounds, we identified several that showed significant inhibition of cell proliferation, SK0381, SK0408, and SK0459 (Figure 1A). While SK0381 somehow failed to show reproducible effect, SK0408 and SK0459 repeatedly showed significant inhibition on B16F10 cells. Thus, we concentrated our study on SK0408 and SK0459. To actually examine cell proliferation, we performed immunostaining to detect the phosphorylated histone H3 (PH3), which is a marker for DNA replication and cell proliferation. As histone H3 is phosphorylated when cells undergo mitosis, we expected to see fewer cells labeled for PH3 following treatment with SK0408 or SK0459. The results show that both SK0408 and SK0459 caused significant reduction of PH3 stained cells compared to the DMSO control (Figure 1B and 1C). Out of 600 melanoma cells, 7% were stained with anti-pH3 in the DMSO control, 8% in MEK-I, 4% in SK0408, and 3% in SK0459. In addition, we tested whether SK0408 and SK0459 induced apoptosis in melanoma cells by using an antibody for cleaved caspase 3, which is a known marker for apoptosis. Cleaved caspase 3 was stained in our melanoma cells and three out of 200 melanoma cells displayed cleaved caspase 3 after treatment with SK0459 and MEK-I separately and 2 out of 200 cells treated with DMSO presented active cleaved caspase 3. As we hypothesized, MEK-I, which is a common targeted melanoma chemotherapy used today [13], also revealed a decrease in melanoma proliferation. From these results, we deduced that the cell proliferation rate was being suppressed by the administration of our chemical treatments as well. Our results also showed that SK0408 and SK0459 reduce melanoma proliferation even more so than MEK-I.

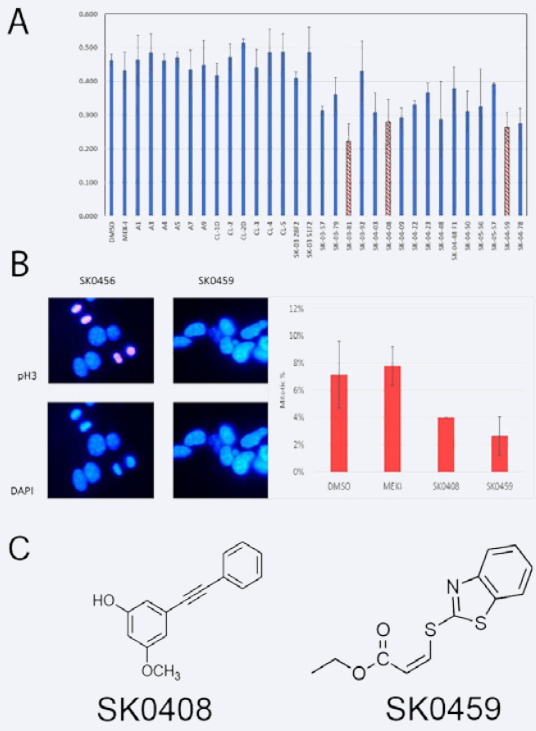


Figure 1 SK0408 and SK0459 reduce B16F10 melanoma proliferation. (A) The MTT assay was used as a screening tool to identify compounds (at 10 μM) that reduce the proliferation of B16F10 melanoma cells. Several compounds did in fact reduce the proliferation significantly, including SK0357, SK0381, SK0409, SK0422, and SK0459. We choose to focus on SK0408 and SK0459 because of their ability to reduce the stem cell population in further experiments. These experiments were repeated, and SK0408 does show consistent reduction, albeit non-significant using Student's *t*-test. *P < 0.05 by Student's *t*-test.

(B) Another way to visually observe the reduction of cell division is by immunohistochemistry. In this experiment we used anti-phosphorylated histone H3 (pH3) which is an indicator of mitosis. SK0456 did not reduce cell proliferation, i.e. mitotic cells are stained pink for the pH3 antibody. The nuclei of cells were stained with DAPI for enhanced observance of all the cells.

(C) chemical structures of SK0408 and SK0459.

SK0408 and SK0459 have no effect on the MAPK signaling pathway but show a reduction of phosphorylated Akt expression

Since the Ras-Raf-MEK-MAPK signaling pathway is one of the major pathways that promote cell proliferation as well as the pathway targeted by several melanoma therapeutic drugs, such as MEK inhibitor cobimetinib and BRAF inhibitor vemurafenib, we wondered if SK0408 and SK0459 would inhibit melanoma proliferation by inhibiting MAPK activity. To our surprise, western blotting showed relatively no difference in phosphorylated MAPK (p-MAPK) level after the treatment with SK0408 or SK0459 (Figure 2A). We then examined the other cell proliferation promoting pathway, Akt. The same western blotting technique was used to detect phosphorylated Akt (pAkt). We observed a significant reduction in the level of pAkt following SK0408 and SK0459 treatment (Figure 2B). Thus, SK0408 and SK0459 inhibit melanoma proliferation by inhibiting Akt, but not MAPK, signaling activity.

SK0408 and SK0459 promote melanoma differentiation

Studies have shown that Akt activity is also required for stem cell maintenance [14]. We then asked whether the lowered p-Akt level in response to SK0408 and SK0459 might affect melanoma stem cells. More specifically we performed immunostaining in conjunction with flow cytometry to examine whether or not the melanoma stem cell population was reduced by SK0408 and SK0459. Cancer stem cells have been identified in many cancer types based on glycoproteins expressed on the CSC surface [15]. Melanoma stem cells can be distinguished by the presence of cell surface glycoproteins, such as CD133 and CD271 [16,17]. Examination of these cell surface glycoproteins in compound treated cells can provide insight as to whether or not the compounds decrease the CSC population. A reduction in the expression of these cell surface glycoproteins is correlated with a reduction in CSC. We chose three widely accepted melanoma stem cell markers: CD133, CD271, and CD20. B16F10 cells were treated with DMSO, SK0408, or SK0459 for 24 hours and fixed for immunostaining with either FITC-conjugated anti-CD133 and PE-conjugated anti- CD271 antibodies or FITC-conjugated anti-CD133 and PE-conjugated anti-CD20 for overnight. The

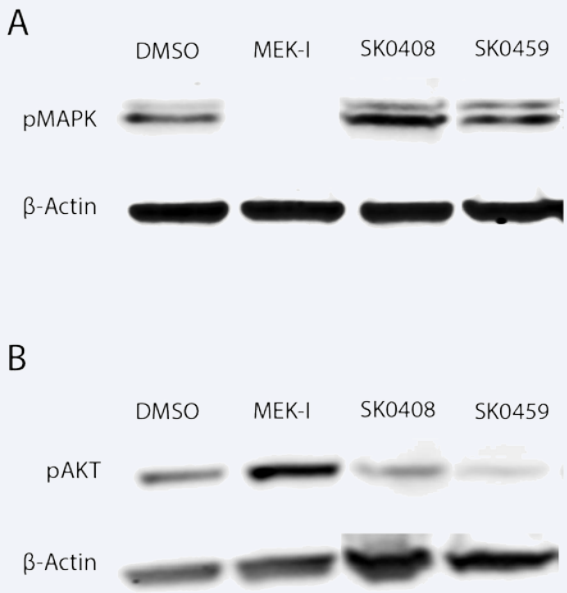


Figure 2 SK0408 and SK0459 have no effect on the MAPK signaling pathway but reduce the level of phosphorylated Akt. Western blotting for proliferation signaling proteins from B16F10 cells treated with our compounds show that SK0408 and SK0459 might have a different mechanism than most targeted melanoma therapies that are currently in use. Antibodies used were anti-phosphorylated MAPK, phosphorylated Akt, and β-Actin (as an internal control). **(A)** As expected, MEK-I inhibits pMAPK entirely, whereas SK0408 and SK0459 appear to have little or no effect on pMAPK level. **(B)** SK0408 and SK0459 do show significant reduction of pAkt indicating that these compounds might be inhibiting cell division through this signaling pathway instead.

stained cells were analyzed with a flow cytometer to measure the percentage of CD133+; CD271+ or CD133+; CD20+ cells. Our results revealed that in both SK0408 and SK0459 treated B16F10 cells, the melanoma stem cell population was significantly reduced (Figure 3A). In the experiment using CD133 and CD271, we observed 2.4% of double positive cells in the DMSO control group but only 0.5% and 1.1% in the cells treated with SK0408 and SK0459, respectively. These numbers translate into a nearly 80% and 55% melanoma stem cell reduction by SK0408 and SK0509, respectively. In the experiment using CD133 and CD20, we observed similar reduction in the stem cell population by both compounds (not shown). There appears to be more of this specific population of cells in the melanoma cultures, but both tests show a sizeable decrease after administering SK0408 and SK0459.

Reduced stemness of melanoma cells by SK0408 and SK0459

The lower level of pAkt and reduced melanoma stem cell population in response to SK0408 and SK0459 treatment strongly suggest that treated melanoma cells are losing their stemness, to evaluate the stemness of melanoma stem cells, we examined canonical wnt signaling. It has been shown that wnt signaling maintains the stemness of many cell types, including cancer stem cells [18]. In particular, phosphorylation of β-catenin leads to the degradation of β-catenin and shutdown of wnt signaling leading to the loss of stemness in stem cells. We performed western blotting using an antibody specific for phosphorylated β-catenin. The results showed an increase in phosphorylated β-catenin in the SK0408 and SK0459 treated melanoma cells (Figure 3B), indicating that our compounds caused a decrease in wnt signaling and a likely loss of stemness in melanoma cells.

It has been shown that the canonical Wnt signaling pathway promotes stem cell maintenance by preventing the degradation of b-catenin and allowing it to activate Wnt mediated genes [19]. In differentiated cells, Wnt signaling is absent and b-catenin is transported to a destruction complex composed of ATC and AXIN, easing its phosphorylation by casein kinase 1 (CK1) and GSK3β. Phosphorylated b-catenin is then ubiquitylated and destroyed by a proteasome complex [20]. In the presence of wnt signal, GSK is inhibited by disheveled exiting b-catenin, which is then translocated to the nucleus where it activates the transcription of target genes. Genes that are activated by beta catenin enhance the viability of stem cells. This pathway has been heavily studied because it has been hypothesized that Wnt signaling can be

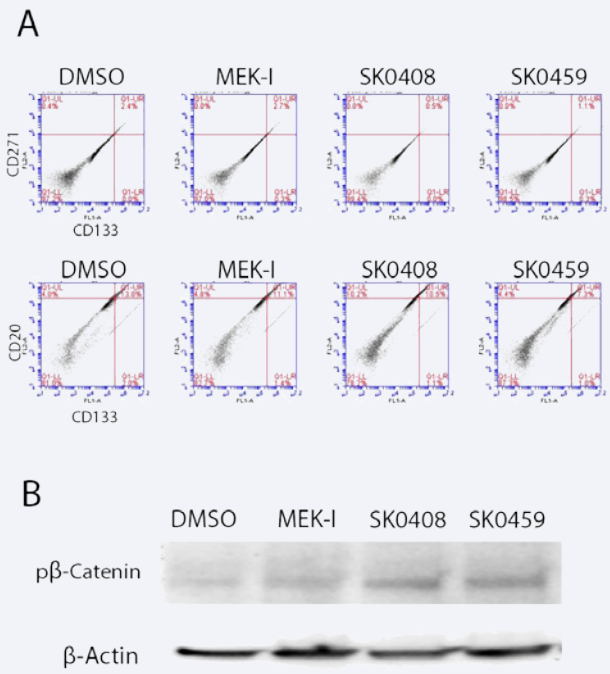


Figure 3 Reduced stemness of melanoma cells by SK0408 and SK0459. (A) Flow cytometry analysis of CD133+; CD271+ (top) and CD133+; CD20+ (bottom) double positive melanoma cells revealed a decrease in the stem cell population after the administration of SK0408 and SK0459. As a result of our compound treatment, we observed a 55-80% decrease in CD133+; CD271+ melanoma cells (0.5% in SK0408 and 1.1% in SK0459 vs 2.4% in DMSO). Likewise, a similar but less significant reduction of 20-45% was observed in CD133+; CD20+ melanoma cells (10.5% in SK0408 and 7.3% in SK0459 vs 13% in DMSO). As a comparison, MEK-I has no effect on the melanoma stem cell population (2.7% of CD133+; CD271+ and 11.1% of CD133+; CD20+). **(B)** Using anti-phosphorylated β-Catenin in a western blot analysis, we found that SK0408 and SK0459 increased pβ-Catenin expression and likely shut down the wnt signaling pathway.

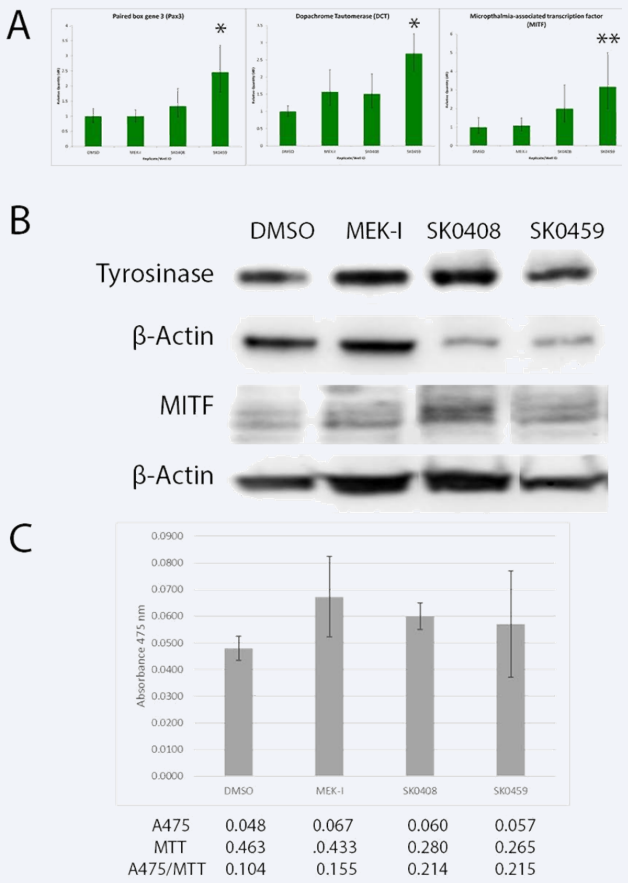
targeted for cancer treatment [21]. In patients with high levels of b-catenin, a drug called pentoxifylline has been shown to inhibit of Wnt signaling in a combinational therapy. This treatment lowered the activity of Wnt transcription factor TCF/LEF and nuclear localized b-catenin [18]. Our results show that both SK0408 and SK0459 increase the level of phosphorylated b-catenin, which then inhibits the Wnt pathway and likely decreases the stemness of melanoma stem cells. In addition, we found that SK0408 and SK0459 also inhibit Akt signaling, indicating that these compounds might regulate stem cell maintenance through inhibiting Akt as well. It would be interesting to know if SK0408 and SK0459 can affect these two pathways independently. One way to test this idea would be to synthesize many analogs and determine if any of them affect the wnt and/or Akt pathways. The ultimate test would be to identify receptor(s), for SK0408 and SK0459.

SK0408 and SK0459 increased expression of melanocyte differentiation genes

If the SK0408 and SK0459 treated melanoma stem cells are losing their stemness, they may become differentiated melanocytes. To test this hypothesis, we examined the expression of MITF, which is a known melanocyte differentiation gene. Using RT-qPCR, we found that SK0408 and SK0459 treated B16F10 cells increased the expression of MITF gene by 2-3 folds compared with the DMSO control (Figure 4B). The other melanocyte differentiation gene, Pax-3 was also upregulated by our compounds. Consistent with the gene expression results, we observed an increase in MITF protein in compound-treated cells (Figure 4B). If the compound- treated cells were induced to differentiate, we expected to see elevated expression of the genes involved in melanogenesis, such as DCT and tyrosinase. As predicted, we observed elevated expression of DCT and higher level of tyrosinase protein in SK0408 and SK0459 treated B16F10 cells (Figure 4A,B). These results suggest that SK0408 and SK0459 enhance melanoma cell differentiation. We then determined if SK0408 and SK0459 increased melanin production. We observed a slight increase in melanin production in the compound-treated cells, but it was not significant. Considering that SK0408 and SK0459 both cause reduction of cell proliferation, the total melanin may not truly reflect the melanin production in individual cells. Therefore, we tried to assess the melanin production in each cell by calculating the A475/MTT ratio (using the A475 absorbance for total melanin and the MTT reading from Figure 1 for cell number). We found that the A475/MTT ratios of SK0408 and SK0459 treated melanoma cells are more than double than that of control cells, suggesting a significant increase of melanin in individual cell.

Decrease of cell invasiveness by SK0408 and SK0459

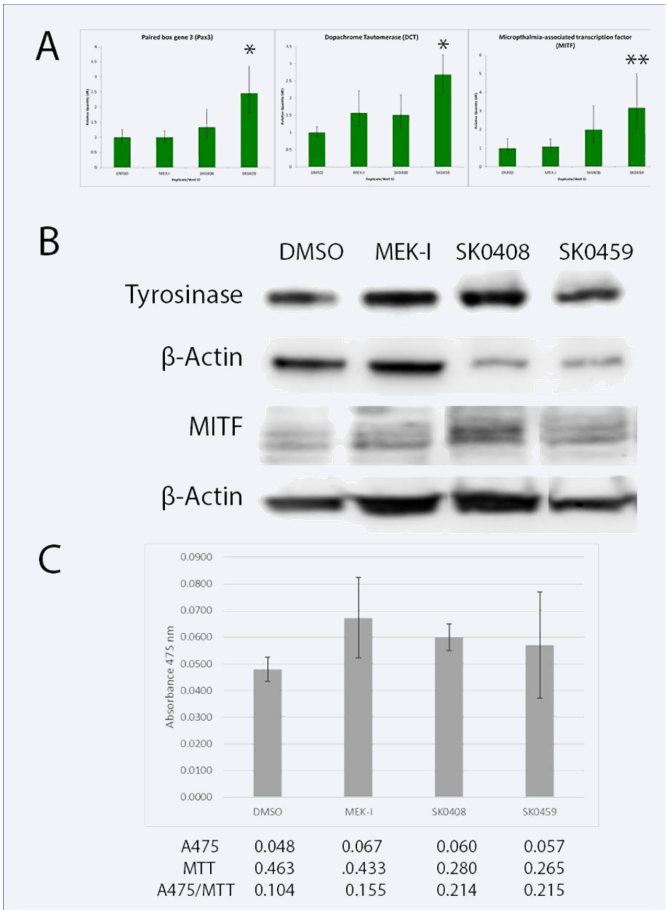
As stated earlier, cancer stem cells are the source of cancer metastasis and cancer relapse [22], Reduction of cancer stem cells should, therefore, result in fewer or the absence of metastatic cells. From the above results, we hypothesized that SK0408 and SK0459 might also decrease the invasiveness of melanoma. To test this idea, we first adapted an in vitro cell invasion assay (Corning Inc.). In this experiment, cells were seeded in the collagen coated supportive in the presence of SK0408 or SK0459 for 24 hours. After setting 100% cell invasiveness in DMSO control solvent as a normalizer, the results showed a significant decrease in the proportion of cells that migrated through collagen in the SK0408 and SK0459 treated groups, approximate 65% and 62% respectively (Figure 5A,B). These results strongly suggest that melanoma cells lost their invasiveness or metastatic ability in response to SK0408 and SK0459 treatment. Interestingly, MEK-I seemed to enhance melanoma invasiveness in our experiments which is consistent with the report by Sandri [1]. Next, we examined the mesenchymal cell marker vimentin, which is a marker associated with epithelial-mesenchymal transition (EMT)



A475	0.048	0.067	0.060	0.057
MTT	0.463	.0.433	0.280	0.265
A475/MTT	0.104	0.155	0.214	0.215

Figure 4 Increased expression of melanocyte differentiation genes by SK0408 and SK0459. (A) RT-qPCR revealed an up regulation of melanocyte transcription factors MITF and Pax3, and the melanogenic enzyme DCT. These genes were up-regulated in SK0408 treated cells, but this increase was not statistically different than the control. However, the SK0459 treated cells show a significant increase in all three of these genes that are involved in melanocyte differentiation. *P < 0.05 and **P < 0.01 by Student's *t*-test. **(B)** Western blotting results for MITF and tyrosinase both showed heightened expression. The elevated expression of both genes suggests that melanoma cells produce more proteins that are specific to differentiated melanocytes after being exposed to SK0408 and SK0459. **(C)** To determine if the melanoma cells are producing more melanin, we measured the absorbance of cell lysate at 475 nm. Top, We observed a slight increase in total melanin production in the compound treated cells. Bottom, Assessment of the melanin production in individual cell by A475/MTT ratio showed that the A475/MTT ratios of the SK0408 and SK0459 treated cells are much higher than that of control.

and metastasis [23]. We found that vimentin protein level was significantly decreased in the SK0408 treated B16F10 melanoma (Figure 5C). The vimentin level in SK0459 treated cells is difficult to evaluate due to the loading error (referring to the dark band of b-actin). E-cadherin, which is a marker for epithelial cells, was not affected by treatment with either SK0408 or SK0459.



A475	0.048	0.067	0.060	0.057
MTT	0.463	.0.433	0.280	0.265
A475/MTT	0.104	0.155	0.214	0.215

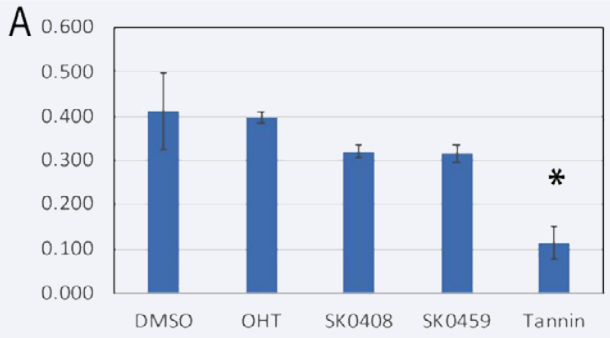
Figure 5 SK0408 and SK0459 decreased cell invasiveness. (A) A cell invasion assay using a Matrigel revealed less invasive melanoma cells after chemical treatment with SK0408 and SK0459. Smaller clusters of compound-treated cells were observed using microscopy. In our controls, DMSO and MEK-I, many large clusters of cells are observed on the external side of the Matrigel. In general, the SK0408 and SK0459 treated cells produced smaller invasive clusters that contain less cells. **(B)** Quantitative data of the cell invasion assay showed that while MEK-I seemed to cause a significant increase in invasive cells, which correlated with the metastatic risk posed by this type of chemotherapy, SK0459 treated cells showed a significant decrease in invasiveness. Although the reduction by SK0408 is not significantly different than the control due to the high standard deviation, the experiment was repeated and we observed consistent and reductive results. *P < 0.05 by Student's *t*-test. **(C)** We used western blot analysis to assess the expression of genes involved in cell migration and invasiveness. There did not appear to be a substantial difference in E-Cadherin expression after our compound treatments. Vimentin, on the other hand, was almost completely inhibited in SK0408 treated cells. The slightly darker band in SK0459 is due to a higher concentration of total protein loaded into the gel (referring to the β-Actin control) not a real increase of vimentin by SK045**(D)** TGF-b signaling pathway and the transcription factor Twist are known to promote EMT. Both the TGF-b signaling activity (indicated by the amount of phosphorylated SMAD4, p-SMAD4) and the expression of Twist are reduced in the SK0408- and SK0459-treated cells.

To further confirm the inhibition of EMT and cell invasiveness of melanoma by SK0408 and SK0459, we examined the TGF-b signaling activity, which is a known EMT-promoting signaling pathway. We found that the level of phosphorylated SMAD4 (pSMAD4), which is an indicator of TGF-b signaling activity, is reduced in SK0408 and SK0459 treated melanoma cells. We also examined the level of Twist protein, which is a known EMT-promoting transcription factor. Our results showed reduction of Twist in SK0408 and SK0459 treated cells as well (Figure 5D). All these results together strongly suggest that the invasiveness of melanoma cells is inhibited by our compounds.

Metastasis accounts for 90% of all cancer death and remains the main cause of this cancer's lethality [22]. The high mortality rate associated with metastatic cancer cells makes this biological phenomenon the main target for effective melanoma treatments. However, metastasis remains one of the most difficult targets for cancer therapy due to the robustness displayed by CSC. Metastasis typically involves epithelial-mesenchymal transition (EMT). One of the important mesenchymal markers is vimentin. Our compounds decreased the vimentin protein level, indicating that treated melanoma cells are less mesenchymal and more differentiated. Consistent with this finding, our cell invasion assay showed a decrease in the invasiveness of melanoma following treatment with SK0408 and SK0459. While these results strongly suggest that our compounds have the potential to prevent cancer metastasis, their effect on in vivo tumorigenesis needs to be assessed in order to further prove this hypothesis. Since CSC only constitutes a very small percentage of the total cell population, ideally we should sort CSC out using a Fluorescence-activated cell sorter (FACS), for tumorigenesis test. Since there are not many drugs that show effective inhibition of metastasis and the few FDA-approved anti-metastasis drugs are primarily trying to inhibit metallomatrix proteases which cancer cells use to promote migration and invasion, our compounds provide a new way to combat metastasis.

One of the current medications for melanoma specifically targets biomolecules present in the MAPK pathway due to high percentage of melanoma patients with the BRAF mutation [20]. However, these drugs are associated with high risk of resistance, relapse, and metastasis [1]. SK0408 and SK0459 have no effect on the MAPK pathway, and, therefore, provide another chemotherapeutic option. They should be good candidate for combined therapies with other agents that target the MAPK signaling pathway or the immune response.

One important question concerns whether or not SK0408 and SK0459 also affect normal stem cells. Since not all the CSC surface markers are expressed on normal stem cells [15], it is possible that our compounds have little or no effect on normal stem cells. We will eventually need to test this possibility using embryonic stem cells or even developing embryos. If our compounds do cause reduction of normal stem cells, we might see phenotypes of premature aging and defects in wound healing and other biological and developmental processes that are heavily dependent on stem cells. In our test experiments using zebrafish embryos, we have not observed any morphological defects in



B

Treatment	without liver extract	With liver extract (S9)
Blank	0/48	0/48
Background	9/48	18/48
Positive control	46/48 (sodium azide)	45/48 (2-aminoanthracene)
SK0408	8/48	8/48
SK0459	10/48	15/48

Figure 6 Specificity and mutagenicity tests for SK0408 and SK0459. (**A**) MTT assay was performed to test if SK0408 and SK0459 also inhibited breast cancer cell proliferation using MCF-7 cell line. No significant inhibition was observed with either SK0408 or SK0459 treatment. OHT: 4-hydroxyl-tamoxifan at 3 μM. DMSO at 0.3% and Other compounds at 30 μM. *Tannin is a compound that strongly inhibits MCF-7 proliferation (unpublished data). (**B**) Ames tests showed that neither SK0408 nor SK0459 is a mutagen. Numbers in the chart are: # of yellow wells/total wells. Representative results were recorded at day 5. The blank group contained no bacteria, while the background group contained bacteria but no test chemical which showed spontaneous mutation.

the SK0408- and SK0459-treated embryos (unpublished data), suggesting that these compounds possibly have little or no effect on normal stem cells. Differentiation enhancement might be a safe method of targeting cancer stem cells because instead of being cytotoxic, our compounds might only alter gene expression. By inhibiting melanoma growth through differentiation, the targeted cytotoxic drugs in a combinational therapy could be administered at a lower concentration thereby making cancer treatment more tolerable and reducing the chances of future mutations from the chemotherapy.

Aggressive melanoma shows a high degree of heterogeneity and plasticity. Genetic studies of melanoma disclose cellular dexterity in surviving various microenvironments by displaying vascular mimicry or either an embryonic or stem cell phenotype [24]. Invasive melanoma subpopulations express the embryonic signaling pathway Nodal, which has been shown to be associated with CD133+ cells. Some combinatorial treatments that use dacarbazine (DTIC) and antibodies for Nodal have been successful in inhibiting melanoma growth and metastasis [25,26]. Our small molecules could have the same effect when used in a combinational therapy. The benefit of a small molecule drug over an antibody is that the small molecule drug could be formulated into an orally administered pill, rather than having to be injected like most protein-based medicines. Small molecule drugs are also synthesis based and could be manufactured at a lower cost [27-31].

No effect of SK0408 and SK0459 on MCF-7 breast cancer cells

Finally, we asked if SK0408 and SK0459 also affected MCF-7 breast cancer cells. Our MTT assay showed no significant inhibition of MCF-7 viability or proliferation by SK0408 or SK0459 (Figure 6A), suggesting that our compounds are fairly specific for melanoma. Finally, we performed the Ames test to determine if SK0408 and SK0459 are potential mutagens. We set up the Ames test with or with liver extract and showed that neither SK0408, nor SK0459 nor their metabolites were mutagens (Figure 6B).

CONCLUSION

This project has identified two novel synthetic compounds that have great potential as melanoma treatment agents. Our results suggest that these compounds can reduce melanoma growth by targeting the stem cells. These compounds also inhibited cancer metastasis in the in vitro cell invasion assay. As melanoma is a highly metastatic cancer, our compounds could be an effective tool for the treatment of melanoma. To our knowledge, there is no single chemotherapeutic agent that has been shown to target cancer stem cells. Our results not only open an exciting door for melanoma therapy but also a hopeful avenue for cancer therapy which is to inhibit cancer growth and metastasis by targeting cancer stem cells.

REFERENCES

1. Sandri S, Faiao-Flores F, Tiago M, Pennacchi PC, Massaro RR, Alves-Fernandes DK, et al. Vemurafenib resistance increases melanoma invasiveness and modulates the tumor microenvironment by MMP-2 upregulation. Pharmacoll Res. 2016; 111: 523-533.
2. Zubrilov I, Sagi-Assif O, Izraely S, Meshel T, Ben-Menahem S, Ginat R, et al. Vemurafenib resistance selects for highly malignant brain and lung-metastasizing melanoma cells. Cancer Lett. 2015; 361: 86-96.
3. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001; 414: 105-111.
4. Dalerba P, Clarke MF. Cancer stem cells and tumor metastasis: First steps into uncharted territory. Cell Stem Cell. 2007; 1: 241-242.
5. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell. 2007; 1: 313-323.
6. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 2005; 65: 10946-10951.
7. Kumar D, Gorain M, Kundu G, Kundu GC. Therapeutic implications of cellular and molecular biology of cancer stem cells in melanoma. Mol Cancer. 2017; 16: 7.
8. Li Y, Atkinson K, Zhang T. Combination of chemotherapy and cancer stem cell targeting agents: Preclinical and clinical studies. Cancer Lett. 2017; 396: 103-109.
9. Greaves M. Leukaemia "firsts" in cancer research and treatment. Nat Rev Cancer. 2016; 16: 163-172.
10. Takeuchi T, Harukazu N. Cell proliferation and development. Development, Growth & Differentiation. 2014; 56: 323-323.
11. Solano F. Melanins: Skin pigments and much more - types, structural models, biologicalfunctions, and formation routes. New J Sci. 2014; 1-28.

12. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol Methods. 1983; 65: 55-63.

13. Grimaldi AM, Simeone E, Festino L, Vanella V, Strudel M, Ascierto PA. MEK inhibitors in the treatment of metastatic melanoma and solid tumors. Am J Clin Dermatol. 2017; 18: 745-754.

14. Segrelles C, Garcia-Escudero R, Garin MI, Aranda JF, Hernandez P, Ariza JM, et al. Akt signaling leads to stem cell activation and promotes tumor development in epidermis. Stem Cells. 2014; 32: 1917-1928.

15. Kim WT, Ryu CJ. Cancer stem cell surface markers on normal stem cells. BMB Rep. 2017; 50: 285-298.

16. Boiko AD, Razorenova OV, Rijn MVD, Swetter SM, Johnson DL, Ly DP, et al. Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. Nature. 2010; 466: 133-137.

17. Grasso C, Anaka M, Hofmann O, Sompallae R, Broadley K, Hide W, et al. Iterative sorting reveals CD133 and CD133- melanoma cells as phenotypically distinct populations. BMC Cancer. 2016; 16: 726.

18. Talar B, Gajos-Michniewicz A, Talar M, Chouaib S, Czyz M. Pentoxifylline inhibits WNT signaling in β-catenin high patient-derived melanoma cell populations. PLoS One. 2016; 11: e0158275.

19. Dye DE, Medic S, Ziman M, Coombe DR. Melanoma biomolecules: Independently identified but functionally intertwined. Front Oncol. 2013; 3: 252.

20. Shang S, Hua F, Hu ZW. The regulation of β-catenin activity and function in cancer: Therapeutic opportunities. Oncotarget. 2017; 8: 33972-33989.

21. Prasad CP, Mohapatra P, Anderson T. Therapy for BRAFi-resistant melanomas: Is wnt5a the answer? Cancers. 2015; 7: 1900-1924.

22. Seyfried TN, Huysentruyt LC. On the origin of cancer metastasis. Crit Rev Oncog. 2013; 18: 43-73.

23. Satelli A, Li S. Vimentin as a potential molecular target in cancer therapy. Cell Mol Life Sci. 2011; 68: 3033-3046.

24. Hendrix MJC, Seftor EA, Margaryan NV, Seftor REB. Heterogeneity and plasticity ofmelanoma: Challenges of current therapies. Cutaneous melanoma: Etiology and therapy.Brisbane (AU): Codon Publications; 2017. Chapter 4.

25. Hardy KM, Strizzi L, Margaryan MV, Gupta K, Murphy GF, Scolyer RA, et al. Targeting nodal in conjunction with dacarbazine induces synergistic anticancer effects in metastatic melanoma. Mol Cancer Res. 2015; 13: 670-680.

26. Justus CR, Leffler N, Ruiz-Echevarria M, Yang LV. *In vitro* cell migration and invasion assays. J Vis Exp. 2014; 88: e51046.

27. Nusse R, Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell. 2017; 169: 985-999.

28. Parmiani G. Melanoma cancer stem cells: Markers and functions. Cancers. 2016; 8: 34.

29. Takahashi-Yanaka F, Kahn M. Targeting wnt signaling: Can we safely eradicate cancer stem cells? Clin Cancer Res. 2010; 16: 3153-3162.

30. Virginia T, Desiderio V, Paino F, Papaccio G, De Rosa M. Methods for cancer stem cell detection and isolation. Somatic Stem Cells. Methods Mol Biol. 2012; 513-529.

31. Wellbrock C, Imanol A. Microphthalmia-associated transcription factor in melanoma development and MAP-kinase pathway targeted therapy. Pigment Cell Melanoma Res. 2015; 28: 390-406.

Cite this article

Schlaeger NM, Berkan C, Albu I, Monte AP, Huang CC (2020) Synthetic Compounds That Inhibit Melanoma Growth and Invasiveness by Reducing Cancer Stem Cell Population. J Cancer Biol Res 8(1): 1126.