Synergistic Effects of d-δ-Tocotrienol And Xanthorrhizol on Murine B16 Melanoma Cells

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ABSTRACT

SYNERGISTIC EFFECTS OF \(d-\delta\)-TOCOTRIENOL AND XANTHORRHIZOL ON MURINE B16 MELANOMA CELLS

by

Darren Chan

**Background:** Skin cancer has become the most commonly diagnosed cancer in the United States, of which melanoma is the most lethal form. Conventional chemotherapy treatments come with a host of side effects. \(d-\delta\)-Tocotrienol and xanthorrhizol have shown potential in treatment of cancers. Tocotrienol, an isomer of vitamin E, has demonstrated apoptotic and cell cycle arrest effects on cancer cells. Xanthorrhizol, a sesquiterpenoid, has demonstrated anticancer effects through both hormonal and nonhormonal pathways. It is unknown whether combinations of \(d-\delta\)-tocotrienol and xanthorrhizol provide greater growth-suppressive activity than these agents alone.

**Methods:** The proliferation of murine B16 melanoma cells was measured using MTT assay with CellTiter 96 Aqueous One Solution (Progmega, Madison WI, USA). Cell cycle distribution was examined by flow cytometry. Protein expression was detected by Western blot analysis with chemiluminescent imaging.

**Results:** Xanthorrhizol inhibited cell proliferation in a dose-response manner. The IC\(_{50}\) of xanthorrhizol, or the concentration of xanthorrhizol required to suppress the cell proliferation by 50\%, was estimated to be 65 \(\mu\)M. A combination of 16.25 \(\mu\)M xanthorrhizol and 10 \(\mu\)M \(d-\delta\)-tocotrienol inhibited cell proliferation to a greater extent than those by individual compounds \(P<0.01\), suggesting a synergistic effect. Cell cycle analysis showed trends towards G1 cell-cycle arrest, though these did not reach statistical
significance. Western blot analysis did not demonstrate significant changes in protein expression across treatment groups in cyclin D1, cyclin-dependent kinase 4, or procaspase-3.

**Conclusion:** Xanthorrhizol and *d*-tocotrienol demonstrate additive and possibly synergistic effects on the viability of murine B16 melanoma cells. The underlying mechanisms of action for this finding, including apoptosis and cell cycle, warrant further investigation.
SYNERGISTIC EFFECTS OF \textit{d-δ-TOCOTRIENOL} AND XANTHORRHIZOL ON MURINE B16 MELANOMA CELLS

by

Darren Chan

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# Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>CSD</td>
<td>chronically sun-damaged</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>mL</td>
<td>milliliter</td>
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<td>µL</td>
<td>microliter</td>
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<td>M</td>
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<td>µM</td>
<td>micromolar</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC3</td>
<td>Procaspaase-3</td>
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<tr>
<td>PBS-T</td>
<td>phosphate buffered saline with Tween 20</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride membrane</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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CHAPTER I
SYNERGISTIC EFFECTS OF $d$-$\delta$-TOCOTRIENOL AND XANTHORRHIZOL ON MURINE B16 MELANOMA CELLS

Introduction

Skin cancer has become the most prevalent type of cancer in the United States (Gershenwald & Guy, 2016; Guy et al., 2015), within which melanoma is ranked third in prevalence (CDC, 2017). While melanomas are the least commonly diagnosed skin cancer, they are the most lethal (CDC, 2017; Gershenwald & Guy, 2016). Melanoma incidence doubled between 1982 and 2011 (Guy et al., 2015). Due to its rising prevalence, developing effective treatments has become a priority. While chemotherapeutic drugs are developed to combat progression of cancers, they are often derived from natural products: an estimated 40% of medicine is derivative of natural components (Lahlou, 2013). Natural compounds have demonstrated antimicrobial, anti-inflammatory, and anticancer effects (Oon et al., 2015). Two such compounds are xanthorrhizol, a sesquiterpenoid (Oon et al., 2015), and $d$-$\delta$-tocotrienol, an isomer of vitamin E (Eitsuka, Tatewaki, Nishida, Nakagawa, & Miyazawa, 2016). The purpose of this study is to investigate the effects of these compounds, individually and in conjunction with each other, on murine B16 melanoma cells.
CHAPTER II

Literature Review

The epidermis contains roughly 1,500 melanocytes per square millimeter. These cells serve to produce pigment, dividing when ultraviolet radiation damages the DNA of keratinocytes. Melanocytes that have undergone abnormal growth patterns, termed melanocytic neoplasms, can either become benign or malignant lesions (Shain & Bastian, 2016b). Malignant lesions, termed melanomas, demonstrate uninhibited growth of cells that produce pigment (Weir et al., 2011). Melanomas are divided into two categories based on sun exposure, chronic sun-damaged skin (CSD) and otherwise (non-CSD). Melanomas are categorized based on signs of damage due to ultraviolet radiation (Shain & Bastian, 2016b). Other risk factors for melanoma include family history, predisposition to freckles, a weakened immune system, and the existence of melanocytic naevi (Weir et al., 2011). Naevi are cases of melanocyte proliferation that, while benign, contribute to instances of melanoma due to their sheer number: the average Caucasian will have approximately 25 naevi exceeding 2 mm in diameter, though this number has a significant genetic component (Shain & Bastian, 2016a). Prior studies suggest that manipulation of cell cycle progression could yield control over melanocyte proliferation (Halaban, 2000).

Xanthorrhizol is found in the essential oil of Curcuma xanthorrhiza Roxb., or Java turmeric (Noomhorm et al., 2014; Oon et al., 2015). The rhizomes are found throughout Southeast Asia (Noomhorm et al., 2014; Oon et al., 2015). Once extracted, it is dissolved in ethanol (Oon et al., 2015). It has historically been used to tighten the
uterus, especially right after a woman delivers a child to alleviate swelling (Noomhorm et al., 2014).

Xanthorrhizol has demonstrated anticancer properties through a number of proposed mechanisms, including antibacterial, anti-inflammatory, and antimicrobial capabilities (Oon et al., 2015). While the leading cause of skin cancer is ultraviolet radiation (CDC, 2017), skin cancer is among carcinomas correlated with microbial agents (Kuper, Adami, & Trichopoulos, 2000). Xanthorrhizol has shown signs of antibacterial (Hwang, Shim, Baek, & Pyun, 2000; Hwang, Shim, & Pyun, 2000; Rukayadi & Hwang, 2006), anticandidal (Rukayadi & Hwang, 2013; Rukayadi, Yong, & Hwang, 2006), and antifungal (Rukayadi & Hwang, 2007a, 2007b) capabilities. The proposed mechanism of xanthorrhizol as an antimicrobial agent suggests it also has anti-inflammatory (Chung et al., 2007; Devaraj, Esfahani, Ismail, Ramanathan, & Yam, 2010; Lee et al., 2002; Lim et al., 2005) capacities. These anti-inflammatory properties may contribute to cancer prevention (Galdiero, Marone, & Mantovani, 2017). Xanthorrhizol also demonstrates antioxidant (Jantan, Saputri, Qaisar, & Buang, 2012; Lim et al., 2005), antihyperglycemic (Kim, Kim, Song, & Hwang, 2014), antihypertensive (Campos et al., 2000; Ponce-Monter, Campos, Aguilar, & Delgado, 1999), antiplatelet (Jantan et al., 2008), estrogenic, and anti-estrogenic (Anggakusuma, Yanti, Lee, & Hwang, 2009; Noomhorm et al., 2014) properties. Its estrogenic activity was investigated in relation to breast cancer, in which it seemed to compound the impact of hormone therapy (Noomhorm et al., 2014).

Tocotrienol and tocopherol are the two vitamin E classes, within which there are four analogues for each (Aggarwal, Sundaram, Prasad, & Kannappan, 2010). These isoforms are designated α, β, γ, and δ. These forms are differentiated by the presence of
three double bonds in the carbon tail in tocotrienols (Aggarwal et al., 2010), which characterizes tocotrienols as isoprenoids (Aggarwal et al., 2010; Mo, 2013). Both tocopherols and tocotrienols demonstrate antioxidant activity, and tocotrienols have anticancer properties (Aggarwal et al., 2010; Mo, 2013). Tocotrienol, including d-δ-tocotrienol specifically, has been studied with respect to various forms of cancer (Aggarwal et al., 2010; Fernandes, Guntipalli, & Mo, 2010; He, Mo, Hadisusilo, Qureshi, & Elson, 1997; Hussein & Mo, 2009; Mo, 2013; Mo & Elson, 1999; Qureshi, Mo, Packer, & Peterson, 2000). As with xanthorrhizol, anti-inflammatory properties may be at least partially responsible for the anti-cancer capacities of d-δ-tocotrienol (Mo, 2013). The effects of tocotrienol on various cancers has been studied in the past, causing cells to demonstrate cell cycle arrest and apoptosis (Fernandes et al., 2010; He et al., 1997; Hussein & Mo, 2009; McAnally, Gupta, Sodhani, Bravo, & Mo, 2007; Mo & Elson, 1999). d-δ-Tocotrienol demonstrated inhibition of cell growth through the CDK4 and procaspase-3 pathways in human melanoma cells (Fernandes et al., 2010).

CDK4 is a member of the cyclin-dependent kinase (CDK) family of genes, which are responsible for cell proliferation through management of cell-cycle progression and transcription (Sheppard & McArthur, 2013). There are four stages to the life cycle of a cell, all of which center around its replication. G1 is the stage before DNA synthesis, S is the stage during which DNA is synthesized, G2 is the stage before cellular division, and M is the stage of cellular division (Hamilton & Infante, 2016). CDK4, and its homolog CDK6, regulate the G1-S transition in mammalian cells (Hamilton & Infante, 2016; Sheppard & McArthur, 2013). CDK4 phosphorylates the retinoblastoma protein (RB1), which allows for RNA polymerases I and III to transcribe ribosomal RNA.
Phosphorylated RB1 results in cells progressing from G1 to S phases and blocks senescence (Sheppard & McArthur, 2013). The CDK4 pathway has been implicated in a number of cancers, including melanoma, and is a target for pharmaceutical treatments (Hamilton & Infante, 2016; Sheppard & McArthur, 2013). Ribociclib inhibits CDK 4 and 6, to be used alongside hormone therapy in breast cancer (Lopez-Tarruella, Jerez, Marquez-Rodas, Echavarria, & Martin, 2017). Current CDK4/6 inhibitors used to treat melanoma include LEE01 and palbociclib. They are used alongside mitogen-activated protein kinase kinase (MEK) inhibitors binimetinib and trametinib, respectively (Reddy, Miller, & Tsao, 2017).

Cyclin D1 is a protein closely related to CDK4 in its role, mediating the transition from the G1 to S phase of cell cycle progression (Alao, 2007; Ramirez, Guitart, Rao, & Diaz, 2005). In order for DNA to replicate, cyclin D1 must be degraded. It has been implicated in a number of cancers (Alao, 2007), including melanomas (Reddy et al., 2017). Cyclin D1 binds to CDK 4 and 6, which stimulates phosphorylation of RB1 (Reddy et al., 2017). While this action stimulates the G1 to S transition (Sheppard & McArthur, 2013), during which cyclin D1 levels rise steadily, progression beyond the S phase has been associated with depletion of the protein (Alao, 2007). Conversely, overexpression of cyclin D1 was associated with increases in cell proliferation (Ramirez et al., 2005); where cyclin D1 levels usually fall after the S phase transition, cells that proliferate abnormally have correspondingly irregular cyclin D1 levels.

Caspases are a family of proteins that regulate apoptosis. Among the caspases, caspase-3 is commonly known for its role in cell death (Porter & Janicke, 1999). Caspase enzymes begin as zymogens with low activity. Caspase-3 is the active form of
procaspase-3 (Peterson et al., 2009). With respect to cancer cell lines, activation of caspase-3 would ideally bring cell proliferation back down to more normal levels. Due to its role as a precursor to caspase-3, procaspase-3 has become a target for cancer treatments seeking to induce apoptosis. Procaspe-3 activating compound 1 (PAC-1) and its derivatives are being investigated as potential treatments (Peterson et al., 2009).
CHAPTER III

Materials

Murine B16 melanoma cells were received as a gift from the Georgia State University Department of Biology (Atlanta, GA, USA). Trypsin, Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS) penicillin-streptomycin, gentamycin, fluconazole, Hank’s Balanced Salt Solution (HBSS), 75 cm$^2$ tissue culture flasks, 96 well tissue culture plates, CellTiter 96® Aqueous One Solution, and pure ethanol were purchased from Fisher Scientific LLC (Houston, TX, USA). Xanthorrhizol and tocotrienol were gifts from Dr. Barrie Tan at American River Nutrition, Inc (Hadley, MA, USA).

Methods

Cell Growth Assay

Murine B16 melanoma cells were cultured in a 75-cm$^2$ tissue culture flask with DMEM modified to contain 10% FBS, 1% penicillin-streptomycin, 1% gentamycin, and 0.01% fluconazole (growth media) until 80-85% confluent. The flask was incubated in a humidified environment at 37°C, and five percent CO$_2$. The media was then aspirated, washed with Hank’s Balanced Salt Solution (HBSS), and five mL trypsin was added. Once the cells were lifted off the flask, about two minutes in the incubator, five mL of ~37°C growth media was added to stop the reaction. The cells were spun down at 2000 RPM for five minutes at 23°C. They were then seeded at 1,200 cell/0.1 mL medium per well in the 96-well tissue culture plates. Six wells were allotted for each planned
treatment group. After 24 h, the medium from each well was aspirated and treatment media was added. Treatments were added to growth media at 0.1% volume. Treatments for the concentration titration were: an ethanol control and xanthorrhizol 200, 100, 50, 25, 12.5, and 6.25 µM. The IC$_{50}$ of xanthorrhizol was calculated from these data. 48 hours after treatment (72 h total), the treatment media was aspirated and wells were washed with warm HBSS. 0.1 mL DMEM without the growth media additions. CellTiter 96® Aqueous One Solution (20 µL) was added to each well, and the plate was incubated for two hours. The plate was then read by a Synergy HT plate reader and Gen5™ software from Biotek® Instruments, Inc (Winooski, VT, USA). The cell proliferation assay for combination of xanthorrhizol and $d$-δ-tocotrienol was carried out using the same protocol.

**Western Blot**

Murine B16 melanoma cells cultured in six-well plates with 9.6-cm$^2$ per well at $3.2 \times 10^5$ cells/cm$^2$ were incubated with a growth medium for 24 hours. The growth medium was comprised of Dulbecco’s Modification of Eagle’s Medium fortified with fetal bovine serum, penicillin/streptomycin, gentamycin, and fluconazole. After 24 hours, the growth medium was aspirated and replaced with treatment medium. The treatment medium consisted of growth medium with 0.1% by volume of the treatments. Treatments were xanthorrhizol at 16.25 and 32.5 µM, $d$-δ-tocotrienol at 5 and 10 µM, and combinations of the two compounds. Treatments were made by diluting stock solutions of each compound with ethanol. Treatment media was aspirated after 24 hours, and the cells were washed once with 2 mL warm HBSS. Each well was then treated with 75-150 µL of lysis buffer
(1% v/v Nonidet P40 Substitute, 1% w/v sodium deoxycholate, 0.05% v/v SDS, 0.81% NaCl, 0.25% v/v of 1M Tris at pH 6.8, 5% v/v 0.5 M EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate pentahydrate, 2.5 mM sodium pyrophosphate, and 1% v/v protease inhibitor cocktail (VWR International, Suwanee GA, USA)), depending on confluence within the wells, and incubated for 30 minutes on ice. Each treatment was allocated into two 1.5 mL centrifuge tubes and spun down at 13.3x10^3 RPM and 4°C for 20 minutes. The supernatant was then collected and protein estimation was performed (specify the type of protein assay used). 10 µg of protein after mixing with a loading buffer at 1:3 ratio (v/v) was then loaded into each well of a 12% SDS gel for Western blot. The loading buffer was made by adding a stock solution of 270 µL 4x Laemmli buffer to 30 µL 2-mercaptoethanol. Gels were run at 80 V for room temperature, until the samples cleared the stacking gel. After this, the chamber was moved to 4°C. The voltage was then increased to 100 V for 20 minutes, and 150 V afterwards until the loading dye reached the bottom of the gel. The gels were then removed and soaked, without the stacking gel, in a 1X transfer buffer solution with 1% SDS. Polyvinylidene difluoride (PVDF) transfer membranes were soaked for 30 seconds in ethanol and then transferred to 1X transfer buffer. Sponges and filter papers were pre-soaked in the transfer buffer. Transfer buffer was a 1X Tris glycine solution, in which 20% of the final volume was ethanol. Transfer was accomplished by a Bio-Rad Trans-Blot Turbo machine (Hercules CA, USA), using 25 V and 1.0 A for 30 minutes. The membranes were then blocked in a PBS-T solution with 5% nonfat dry milk powder for 30 minutes at room temperature. They were then briefly washed with PBS-T. Primary antibody solutions were then added, during which membranes were shaken at 4°C
overnight. Primary antibody for β-actin (Santa Cruz Biotechnology, Dallas TX, USA; 1:1,000 dilution), CDK4 (Santa Cruz Biotechnology, Dallas TX, USA; 1:500 dilution), procaspase 3 (Sigma-Aldrich Corporation, St. Louis MO, USA; 1:500 dilution), or cyclin D1 (Cell Signaling Technology, Danvers MA, USA; 1:500 dilution) was added to 4 mL PBS-T. The membranes were then subjected to three washings with PBS-T for ten, five, and five minutes respectively. Secondary antibody solutions were then applied at a 1:3000 dilution. The membranes were then washed again with PBS-T three times for ten, five, and five minutes respectively. Membranes were then stripped using Restore PLUS Western Blot stripping buffer from Thermo Scientific (Waltham MA, USA). Ten mL of stripping buffer was added to each membrane for ten minutes, after which they were washed briefly with PBST and blocked again. The membranes could then be probed again with different primary antibodies. Membranes were exposed to 1 mL of mixed ECL (Thermo Scientific, Waltham MA, USA) solution for 30 seconds prior to imaging with an ImageQuant LAS4000 chemiluminescence imager.

**Cell Cycle Distribution**

B16 cells were seeded in 25-cm² flasks at 1x10⁶ cells/flask with 3 mL medium/flask. After incubating for 24 hours, the medium was changed out for one containing 0.1% (v/v) of treatment solutions. Treatments included xanthorrhizol at 32.5 and 65 µM, δ-tocotrienol at 5 and 10 µM, combinations of xanthorrhizol and δ-tocotrienol, and an ethanol control. The cells were then harvested after an additional 24 hours of treatment. One sample of 0-hour cells without any treatment was also harvested. Cell collection was achieved through trypsinization and spun down with centrifugation for cell pellets. These
were fixed in 1 mL of 70% ethanol at -20°C. These cells were kept at this temperature for at least 8 hours and up to two weeks before analysis. Samples were washed with 1 mL phosphate buffered solution (PBS) twice and resuspended in 500 µl PBS containing 0.5 mg RNase A (Roche Diagnostics, Indianapolis IN, USA). They were incubated at 37°C for 30 min, after which 100 µL of propidium iodide solution was added. The propidium iodide solution was made by mixing 1 mg of propidium iodide (Sigma-Aldrich, St. Louis MO, USA) into 1 mL of PBS with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis MO, USA). The cells were then kept in the dark for 15 minutes before performing analysis.

Statistics

Western Blot bands were quantified using ImageJ (NIH, Bethesda MD, USA). Cell cycle files (.fcs) were analyzed using FCS Express 6 (De Novo Software, Glendale CA, USA). Data was analyzed using the Prism software by GraphPad (La Jolla CA, USA) to perform a one-way analysis of variance (ANOVA) with post-hoc Tukey Test. Significance was achieved at $P<0.05$. Values are presented as mean±SEM.
CHAPTER IV

Results

The IC$_{50}$ value, or concentration required to inhibit cell viability by 50%, of $d$-$\delta$-tocotrienol was estimated to be 10 µM from prior studies (He et al., 1997; McAnally et al., 2007). Xanthorrhizol (0 – 200 µM) demonstrated a dose-dependent inhibition of B16 cell viability. The IC$_{50}$ value of xanthorrhizol was investigated during this experiment, and estimated to be 65 µM. Cell viability was inhibited by 8.7 ± 5.2%, 16.1 ± 4.7%, 28.8 ± 7.8%, 62 ± 6.3%, and 79.1 ± 6.0% for concentrations of 12.5, 25, 50, 100, and 200 µM, respectively. Significant differences were seen between control and xanthorrhizol 50 µM ($P<0.01$), control and xanthorrhizol 100 µM ($P<0.01$), control and xanthorrhizol 200 µM ($P<0.01$), xanthorrhizol 12.5 µM and 100 µM ($P<0.01$), 12.5 µM and 200 µM ($P<0.01$), 25 µM and 100 µM ($P<0.01$), 25 µM and 200 µM ($P<0.01$), 50 µM and 100 µM ($P<0.01$), and 50 µM and 200 µM ($P<0.01$) (Figure 1A). Initial tests with a combination of $d$-$\delta$-tocotrienol and xanthorrhizol at concentrations of their IC$_{50}$ values found it too cytotoxic. Instead, xanthorrhizol at half and a quarter of its IC$_{50}$ value were tested both alone and in combination with $d$-$\delta$-tocotrienol at half and one IC$_{50}$ value. The results of cell viability tests are summarized in Figure 1.

Xanthorrhizol at 16.25 and 32.5 µM produced an 11.1 ± 4.6% and 72.5 ± 2.6% reduction in viable cells, respectively. $d$-$\delta$-Tocotrienol increased cell viability by 12.0 ± 5.2% and 2.2 ± 4.4% at doses of 5 and 10 µM, respectively, although these were non-significant increases. Combinations of xanthorrhizol (16.25 µM) and $\delta$-tocotrienol...
(5 and 10 µM) inhibited cell viability by 18.3 ± 3.3% and 54.7 ± 7.4%, respectively, exceeding the sum of inhibitions induced by individual agents. Combinations of xanthorrhizol (32.5 µM) with d-δ-tocotrienol (5 and 10 µM) reduced cell viability by 76.7 ± 2.7% and 81.6 ± 2.2%, respectively. The control group was significantly different from xanthorrhizol 32.5µM (P<0.01), combination of xanthorrhizol (16.25 µM) and d-δ-tocotrienol (10 µM) (P<0.01), and both combinations with xanthorrhizol 32.5 µM (P<0.01). Synergy between xanthorrhizol and d-δ-tocotrienol could not be computed by CompuSyn due to the effect of d-δ-tocotrienol treatments. However, the combination of 16.25 µM of xanthorrhizol and 10 µM d-δ-tocotrienol suggested a synergistic effect.

The effect of the combinations on procaspase-3 was evaluated to show whether caspase-3 activation is involved in the growth inhibition induced by the compounds. d-δ-Tocotrienol did not appear to induce apoptosis by way of procaspase-3 in B16 melanoma cells as shown in this preliminary study. A prior study demonstrated that d-δ-tocotrienol causes a concentration-dependent reduction in procaspase-3 expression in human melanoma cells (Fernandes et al., 2010). However, there was no trend demonstrated by any of the treatments used in this study (Figure 2).

Figure 3 shows the effect of the individual and combined treatments on the percentage of cells in the G1 (Figure 3A), S (Figure 3B) and G2 (Figure 3C) phases of cell cycle. The 0-hour control had 60.7 ± 1.0 % of cells in G1, which fell slightly to 57.7 ± 1.3 % in the 24-hour control (P>0.05). Treatment with xanthorrhizol resulted in 67.0 ± 1.4 % (16.25 µM) and 79.6 ± 2.2 % (32.5 µM) of cells in G1. The 16.25 µM xanthorrhizol group was significantly different from the higher 32.5 µM group (P=0.048), suggesting a dose-dependent effect of xanthorrhizol on cell cycle progression.


d-δ-Tocotrienol treatments yielded 64.4±2.0% (5 µM) and 63.3±5.9% (10 µM) of cells in G1. Combination treatments resulted in 69.7±1.0% (16.25 µM xanthorrhizol and 5 µM d-δ-tocotrienol), 71.9±1.9% (16.25 µM xanthorrhizol and 10 µM d-δ-tocotrienol), 68.5±4.1% (32.5 µM xanthorrhizol and 5 µM d-δ-tocotrienol), and 85.4±2.3% (32.5 µM xanthorrhizol and 10 µM d-δ-tocotrienol), respectively, of the cells in G1 phase. Xanthorrhizol at 32.5 µM and the combination of xanthorrhizol (32.5 µM) and d-δ-tocotrienol (10 µM) increased the G1% (P<0.01), suggesting a G1 arrest. There was also a trend of non-significant decreases in the S% in these treatment groups. The G1% in other treatment groups did not reach statistical significance in comparison to that in the control group. The combination of xanthorrhizol (32.5 µM) and d-δ-tocotrienol (10 µM) remained significantly different from the other combination treatments, including xanthorrhizol at 16.25 µM and d-δ-tocotrienol at 5 µM (P<0.01), xanthorrhizol at 16.25 µM and d-δ-tocotrienol at 10 µM (P=0.0232), and xanthorrhizol at 32.5 µM and d-δ-tocotrienol at 5 µM (P<0.01), suggesting a dose effect in combinations.

S-phase cell cycle data was similarly inconclusive (Figure 3B). The percentages of cells in S phase were 29.9 ± 1.3 % (0-hour control), 8.9 ± 2.1 % (24-hour control), 29.4 ± 0.6 % (xanthorrhizol 16.25 µM), 12.8 ± 1.5 % (xanthorrhizol 32.5 µM), 26.8 ± 1.1 % (d-δ-tocotrienol 5 µM), 30.7 ± 6.1 % (d-δ-tocotrienol 10 µM), 22.2 ± 1.1 % (xanthorrhizol 16.25 µM and d-δ-tocotrienol 5 µM), 15.7 ± 3.0 % (xanthorrhizol 16.25 µM and d-δ-tocotrienol 10 µM), 18.3 ± 1.9 % (xanthorrhizol 32.5 µM and d-δ-tocotrienol 5 µM), and 8.7 ± 2.1 % (xanthorrhizol 32.5 µM and d-δ-tocotrienol 10 µM). The 0-hour control was different from the 24-h control (P<0.01), suggesting that cells are transitioning through the cell cycle in the time period. The S% in the combination group
consisting of 16.25 µM xanthorrhizol and 10 µM d-δ-tocotrienol was significantly lower than those in groups with either compounds alone ($P<0.01$), suggesting a cumulative effect. Similar differences were found in the combination group consisting of 32.5 µM xanthorrhizol and 10 µM d-δ-tocotrienol.

As with the cell cycle data, western blot analysis of associated proteins were inconclusive. CDK4 and cyclin D1 demonstrated no changes suggestive of cell cycle alteration (Figure 4).
CHAPTER V
Discussion

This study investigated the anti-proliferative effects of \( d-\delta \)-tocotrienol (IC\(_{50}\)=10 \( \mu \)M) (He et al., 1997; McAnally et al., 2007) and xanthorrhizol, alone and in combination, on murine B16 melanoma cells. The cell viability data shown here suggest that there may be a synergistic effect between xanthorrhizol and \( d-\delta \)-tocotrienol at the lower xanthorrhizol dose of 16.25 \( \mu \)M. However, the 32.5 \( \mu \)M dose of xanthorrhizol inhibited cell viability so drastically that the addition of \( d-\delta \)-tocotrienol did not produce a compounded effect. Cell viability at both 24 h and 48 h were so low when both compounds were used at their IC\(_{50}\) values in the combination that the concentrations necessitated reduction. Additionally, the increased cell proliferation, although non-significant from that of the control cells, at both doses (5 \( \mu \)M and 10 \( \mu \)M) of \( d-\delta \)-tocotrienol prohibited computation of synergistic effects using the CompuSyn program as initially planned.

Cell cycle analysis provided inconclusive results. The data shown in this study did not confirm cell cycle arrest at the G1 phase as shown in prior studies with murine B16 cells (Fernandes et al., 2010) and other cell lines (Hussein & Mo, 2009; Mo & Elson, 1999). While this study suggests a trend towards G1 cell-cycle arrest, which would be in line with prior studies, this trend did not achieve statistical significance. This could have been due to several factors. Analysis showed a significant proportion of aneuploid cells in some groups, and only diploid cell data was used when considering the proportion of
cells in each phase. There may be an insufficient number of viable cells in the treatment groups as a result of either treatment concentrations or duration. Future studies could alter the number of events recorded by the flow cytometer, either in general or between G1 and S phases, or decrease the treatment time.

Western blot analysis was also inconclusive with respect to the proteins investigated. Prior studies demonstrated effects of $d$-$\delta$-tocotrienol on cyclin D1, CDK4, and procaspase-3 (Fernandes et al., 2010; Ji et al., 2012). Some studies showing the effect of tocotrienols on procaspase-3 and CDK4 had the same cell line as in this study (Fernandes et al., 2010), while others showing the effect on cyclin D1 used lung cancer cells (Ji et al., 2012). Several factors could have contributed to the discrepancies between this and prior studies. First, doses of the compounds could be adjusted. In this study, there were very few cells after 24 hours of treatment with the higher doses of xanthorrhizol and combinations due to the inhibition of cell proliferation. Second, a shorter treatment period may detect the changes that occurred earlier than the measured time point. A prior study established that cyclin D1 and CDK4 expression could be altered in as little as 12 hours (Katuru et al., 2011). Third, only monolayers of cells remaining attached to the culture plate were harvested for cell cycle and protein analyses. However, many cells detached as floaters due to the effect of the higher dose of xanthorrhizol, and these cells were not part of the analysis. The sourcing of primary antibodies also proved slightly problematic. All three primary antibodies were purchased from separate companies, and provided differing qualities in imaging. Both the CDK4 and procaspase-3 primary antibody products yielded images with much more background and nonspecific protein binding when compared to that of cyclin D1. Future studies could
investigate the effects of reduced treatment time, thus diminishing the cytotoxic effects of the treatments and increasing the possibility of observing altered protein expressions in cyclin D1, CDK4, and procaspase-3 at a different time point.

While the majority of results in this study were inconclusive, there were some positive yields. The IC$_{50}$ value of xanthorrhizol for murine B16 melanoma cells was calculated to be approximately 65 µM. The IC$_{50}$ value for $d$-$\delta$-tocotrienol in the same cell line has been established in prior studies (He et al., 1997; McAnally, Jung, & Mo, 2003). Differences in compound preparation and cell culture conditions may have contributed to the discrepancies between this (Figure 1B) and prior studies. The inhibition of cell viability induced by the xanthorrhizol and $d$-$\delta$-tocotrienol may have impacted the cell cycle and especially Western blot results. There were very few cells adhering to flasks and plates after the 24-hour treatment time. Since cyclin D1 and CDK4 expression can be affected in as little as 12 hours (Katuru et al., 2011), a shorter treatment time could be an area for future studies with respect to harvesting cells for cell cycle and Western blot analyses.

Furthermore, a number of alternative proteins exist to regulate both cell cycle and apoptosis. Tocotrienols in general have been implicated in the regulation of a number of cell cycle and apoptosis modulators (Aggarwal et al., 2010). This study investigated expression of proteins present in whole-cell lysates, but there are a number of proteins that could be further evaluated in fractionated cells. Ras is a gene that encodes for proteins found in the membrane fraction of cells when activated (Adjei, 2001) and whose expression has been shown to be affected by $d$-$\delta$-tocotrienol (Fernandes et al., 2010). Ras protein acts as a gatekeeper to the expression of several other proteins governing cell
cycle regulation, proliferation, and differentiation (Adjei, 2001). Investigation of Ras could also open a window to the mevalonate pathway, as it is one of many protein types that requires prenylation for activity. Prenylation involves covalent addition of farnesyl or geranylgeranyl to the protein of interest (Adjei, 2001). The farnesyl and geranylgeranyl groups are derived from mevalonate that is in turn converted from 3-hydroxy-3-methylglutaryl (HMG)-CoA by HMG-CoA reductase (HMGCR) (Buhaescu & Izzedine, 2007). d-δ-Tocotrienol has already been suggested to act upon the mevalonate pathway in cancer cells (Eitsuka et al., 2016; Fernandes et al., 2010; Hussein & Mo, 2009; McAnally et al., 2007) through down-regulation of HMGCR. While HMGCR is the main regulator of the mevalonate pathway, there are a number of other points of control (Buhaescu & Izzedine, 2007). These other proteins may warrant investigation as well, since xanthorrhizol clearly demonstrated its ability to inhibit cell viability in addition to the effects induced by d-δ-tocotrienol.

In summary, xanthorrhizol and d-δ-tocotrienol demonstrated combinational effect on the viability of B16 melanoma cells with used in doses lower than their IC_{50} values. Their effects on cell cycle arrest and apoptosis remain inconclusive. However, the combination may hold potential in the prevention or treatment of melanoma pending further studies on their mechanisms of action.
Figure 1. (A) The dose-dependent effect of xanthorrhizol on murine B16 melanoma cell viability. IC$_{50}$ value for xanthorrhizol was calculated using Prism 7. (B) Effect of combinations of xanthorrhizol and d-$\delta$-tocotrienol on cell viability. The combination of xanthorrhizol at 16.25 $\mu$M and 10 $\mu$M d-$\delta$-tocotrienol reduced cell viability to a greater extent than those induced by individual agents, suggesting synergy. Combinations of d-$\delta$-tocotrienol and 32.5 $\mu$M xanthorrhizol did not produce synergy. Values are mean ± SEM, n = 18. Significance levels: * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001.
Figure 2. (A) Representative blots showing the impact of xanthorrhizol (16.25 μM and 32.5 μM) and d-δ-tocotrienol (5 μM and 10 μM) on procaspase-3 expression. (B) Quantification of procaspase-3 bands shown in (A). The intensity of procaspase-3 band was normalized by that of β-actin. X is an abbreviation for xanthorrhizol, while T is an abbreviation for d-δ-tocotrienol. Dosages are included without the μM unit.
Figure 3. The effect of xanthorrhizol and d-δ-tocotrienol, individually and in combination, on the distribution of B16 cells in the G1 (A), S (B), and G2 (C) phases. Significance levels:  * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001.
Figure 4. (A) Representative blots showing the impact of xanthorrhizol (16.25 μM and 32.5 μM) and d-δ-tocotrienol (5 μM and 10 μM) on CDK4 and cyclin D1 expression. Quantification of (B) CDK4 and (C) Cyclin D1 Western blot bands. Xan is an abbreviation for xanthorrhizol, while Toco is an abbreviation for d-δ-tocotrienol. Dosages are included without the μM unit. Values are mean ± SEM, n = 18.
References


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