**Using Resveratrol to Induce Apoptosis in B16 Melanoma Cells Through Changes in p53, Bcl-2, and Caspase-9**

**Introduction**

Current methods for treating melanoma are becoming more harmful than helpful. Although high-dose interleukin-2 (HDIL-2) is a very effective treatment for melanoma, it causes endothelial cell injury and vascular leak syndrome (VLS). However, when mice with melanoma were treated with resveratrol, it prevented the endothelial cell injury and VLS from occurring, while reducing the relative tumor area (Guan et al. 2012).

A specific strain of melanoma, B16, is also resistant to a popular chemotherapy drug, doxorubicin. An in vitro study presented to inhibit the growth of B16 melanoma cells when in the presence of resveratrol (Gatouillant 2010).

Resveratrol is a trihydroxy derivative of stilbene (3,5,4’-trihydroxystilbene) and is a naturally occurring antioxidant primarily found in grapes, peanuts, and red wine. A daily dose of 40 mg per kg of body weight of resveratrol also increased the survival rate of mice with melanoma from 0% to 70% (Baur & Sinclair 2006). It is a very successful agent in the treatment of melanoma because of its anti-proliferative and photo-protective effects. Resveratrol can cause a G1-phase arrest of the cell cycle and apoptosis as well as induce apoptosis (Ndiaye et al. 2011). Resveratrol imposes an artificial checkpoint at G1-S phase, causing this arrest, leading to apoptosis (Gatouillant 2010).

Apoptosis can occur as a defense mechanism against disease or noxious agents. DNA damage caused by irradiation or drugs used for chemotherapy can cause apoptosis through a p53-dependent pathway. Bcl-2 proteins and Caspase-9 are both intrinsic, or mitochondrial pathways, to apoptosis. The Bcl-2 family of proteins has the ability the determination of the cell committing to apoptosis or not, by its ability to alter mitochondrial membrane permeability. Caspases are widely expressed in an inactive form, but when activated, can cause a cascade of caspases, amplifying the apoptotic signaling pathway (Elmore 2007).

The p53 protein is a transcription factor that speeds up the rate of transcription. In the presence of DNA damage, p53 activity heightens. More than 50% of all human cancers contain mutations in this gene. In skin cancers, a p53 mutation occurs early in pre-malignant lesions. In some cases, p53 mutations have resulted in shorter disease-free survival and lower total survival of patients (Levine 1997).

The Bcl-2 proteins inhibit cell death (Cory & Adams 2002). Bcl-2 family members that are anti-apoptotic inhibit cytochrome c release by blocking the activation of BAX and BAK (Fesik 2005). Bcl-2 successfully demonstrates this by increasing the time-to-death and cell-to-cell variability. Protein levels vary and thus alter the commitment to death of the cell (Skommer et al. 2010).

Caspase-9 is required in most cases of apoptotic cell death. It acts downstream of the same mitochondrial pathway as Bcl-2. It has an important role in initiating apoptosis in cells that are damaged. In tumor tissues, caspase-9 activity is significantly decreased (Wurüstle et al. 2012). In general, caspases inactivate proteins that protect living cells from apoptosis. They induce DNA fragmentation, chromatin condensation, membrane bleeding, cell shrinkage, and disassemble apoptotic bodies (Thornberry & Lazebnik 1998).

Normal B16 melanoma cells should express high levels of p53 and low levels of Bcl-2 and Caspase-9. However, when the melanoma cells are treated with resveratrol, expression of p53 and Caspase-9 should increase, and Bcl-2 expression should decrease. It is hypothesized that this will provide a successful treatment to induce apoptosis.

**Methods**

*RNA Isolation*

B16 murine melanoma cells (5 x 10^6 cells) were treated with resveratrol (25 µM) for 24 hours. Cells were snapped frozen at -80 degrees Celsius. RNA was isolated from B16 cells treated and not treated with resveratrol. To isolate the RNA from the melanoma cells, SV- Total RNA Miniprep isolation system was utilized from Promega. The spin protocol was utilized from this manual. First, EtOH was added to the lysate, followed by transferring the lysate to the spin basket assembly, washing, treating with DNase, centrifuging, and finally eluting RNA. RNA concentration and quality was measured using a nanodrop.

*cDNA Synthesis*

Next, cDNA was created from the RNA, using GoTaq 2-step RT-PCR System from Promega. The RNA from each treated type (with resveratrol or not) was combined with reverse transcription primer in a reaction tube. A ten µl solution of RNA, primer, and nuclease free water was obtained for each treatment group, followed by a denaturing of the RNA and reverse transcription primer at 70 degrees Celsius for five minutes and chilled at four degrees Celsius for five minutes.

The GoScript Reaction Mix was then combined, on ice, to each treatment group of RNA and reverse transcription primer in the reaction tubes. The mixture contained a ten µl solution of nuclease-free water, 5X reaction buffer, MgCl2, PCR nucleotide mix, recombinant RNasin ribonuclease inhibitor, and reverse transcriptase, combined in that order.

Ten µl of the GoScript reaction mix was mixed with ten µl of each treatment group of RNA. Finally, the reaction processed through five minutes of annealing at 25 degrees Celsius, one hour of extending at 42 degrees Celsius, and a 15-minute period of inactivating at 70 degrees Celsius. The cDNA samples were stored at -20 degrees Celsius until sequencing.

*RT-PCR*

After the cDNA was created from reverse transcription, RT-PCR was then ready to be performed with the primers for the genes of interest. Ten µl of each RNA treatment group were placed in each well in a multicell plate. 25 µl of GoTaq qPCR Master Mix, 10 µl of nuclease-free water, and five µl of the appropriate primers were paced into each cell. (p53: forward – TCACAGCGTCTGTTGACATTT, reverse – ACCAAGCTCATTACCCTGACA; Bcl-2: forward – GAGCCTGTGAGAGACGTGG, reverse – CGAGTCTGTGTATAGCAATCCCA; Caspase-9: forward – GGCTGTTAAACCCCTAGACCA, reverse – TGACGGGTCCAGCTTCACTA; B-actin: forward – GTGTGATGGTGGGAATGGGTCAGA, reverse - TACGACCAGAGGCATACAGGGACA). Each treatment group was combined with B-actin to act as a housekeeping gene, p53, Bcl-2, and Caspase-9 in three trials for each primer sequence. The multicell plate was ran through the PCR instrument to undergo standard qPCR (GoTaq Hot Start Polymerase activation, denaturation, annealing/extension, and dissociation).

*Data Analysis*

After the cDNA and its respective primers underwent standard qPCR, the number of cycles that it took the gene to cross over the threshold to display expression was quantified and analyzed in comparison to the housekeeping gene. The delta delta Ct method was utilized. First, p53, Bcl-2, and Caspase-9 values of expression were normalized to the house-keeping gene values, B-actin (delta Ct). Next, the normalized values were used to compare the untreated (media) B16 samples to the treated, resveratrol samples (delta delta Ct). A two-tailed, homoscedastic t-test was conducted for each gene comparison in excel to determine significance.

**Results**

Resveratrol significantly increased expression of p53 and Caspase-9, and decreased expression of Bcl-2. Expression of p53 and Caspase-9 in resveratrol treated B16 melanoma samples was significantly higher than media samples (p<0.01 for both analyses). Expression of Bcl-2 in resveratrol treated B16 melanoma samples was significantly lower than media samples (p<0.01).

In resveratrol treated B16 melanoma samples, expression of p53 was increased by approximately 6-fold (Figure 1-A); expression of Bcl-2 was decreased by approximately 275-fold (Figure 1-B); expression of Caspase-9 was increased by approximately 8-fold (Figure 1-C).

**Figure 1. Change in expression levels of p53 (A), Bcl-2 (B), and Caspase-9 (C) in B16 melanoma samples (media) and resveratrol treated samples (res).** Fold changes were determined by calculating 2^delta delta Ct. The error bars represent standard deviation from the mean values.

**Discussion**

The hypothesis was supported; resveratrol increased expression of genes that are responsible for inducing apoptosis, p53 and Caspase-9, and decreased expression of a gene that inhibits apoptosis, Bcl-2. Inducing apoptosis in cancer cells to kill the cancer without damaging other normal cells is the main goal in cancer therapies. Specifically, decreasing the activity of Bcl-2 is so critical for treatment of cancer. In cancer cells, Bcl-2 protects the cells from undergoing apoptosis by the removal of growth factors, and gives the cells a resistance to chemotherapy drugs. Cells that lack Caspase-9 display a delayed appearance of an apoptotic phenotype, however, it did not affect the number of cells that ultimately died. P53 causes apoptosis by transcriptionally activating pro-apoptotic genes (Gerl & Vaux 2005).

Although all genes have been to be significantly impacted by resveratrol, expression of Bcl-2 drastically varied from the media to resveratrol samples the most, by approximately 275-fold. This translates that it is more successful for cancer therapies to decrease the function in genes that inhibit apoptosis. This is feasible because drug therapies should not drastically increase the expression of genes that induce apoptosis because the drug will travel into all cells, thus killing normal cells that are essential for human function.

The main problem with treatment of melanoma with resveratrol is its poor *in vivo* bioavailability. Resveratrol is metabolized by the intestine and liver and very quickly metabolized. Because of this, it is difficult for resveratrol to be used as a drug treatment because it disappears from the plasma in such a short amount of time. Future studies should focus on discovering a viable mechanism for resveratrol to treat melanoma without having to be digested (Ndiaye et al. 2011).

**Works Cited**

Baur J. & Sinclair D. Therapeutic potential of resveratrol: the *in vivo* evidence. Nature Reviews. 2006; 5 (493-506).

Cory S. & Adams J. The Bcl2 family: Regulators of the cellular life-or-death switch. Nature. 2002; 2 (647-656).

Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicologic Pathology. 2007; 35 (495-516).

Fesik S. Promoting apoptosis as a strategy for cancer drug discovery. Nature. 2005; 5 (876-885).

Gatouillant G., et al. Resveratrol Induces Cell-Cycle Disruption and Apoptosis in Chemoresistant B16 Melanoma. Journal of Cellular Biochemistry. 2010; 110 (893-902).

Gerl R. & Vaux D. Apoptosis in the development and treatment of cancer. Carcinogenesis. 2005; 26 (263-270).

Guan H., et al. Resveratrol Prevents Endothelial Cells Injury in High-Dose Interleukin-2 Therapy against Melanoma. PloS One. 2012; 7 (4).

Levine A. p53, the Cellular Gatekeeper for Growth and Division. Cell. 1997; 88 (323- 331).

Ndiaye M., et al. The Grape Antioxidant Resveratrol for Skin Disorders: *Promise, Prospects, and Challenges.* Archives of Biochemistry and Biophysics. 2012; 508 (164-170).

Skommer J., et al. Bcl-2 inhibits apoptosis by increasing the time-to-death and intrinsic cell-to-cell variations in the mitochondrial pathway of cell death. Apoptosis. 2010; 15 (1223-1233).

Thornberry N. & Lazebnik Y. Caspases: Enemies Within. Science. 1998; 281 (1312- 1316).

Wurüstle et al. The central role of initiator caspase-9 in apoptosis signal transduction and the regulation of its activation and activity on the apoptosome. Environmental Cell Research. 2012; (1213-1220).