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An Investigation of BAG3 Knockdown and its Effects on the Cytotoxicity of Laromustine

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**An Investigation of BAG3 Knockdown and its Effects on the
Cytotoxicity of Laromustine**

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A Thesis Presented to the Department of Chemistry

Colby College, Waterville, ME

In Partial Fulfillment of the Requirements for Graduation

With Honors in Chemistry

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VITAE:

Colin Timothy Sheehan was born on December 15, 1992 to parents Lisa and Timothy Sheehan and grew up in Chester Springs, Pennsylvania. He attended The Hill School in Pottstown, Pennsylvania graduating Cum Laude before matriculating to Colby College in Waterville, Maine. At Colby he worked as a research assistant for Kevin Rice, Associate Professor of Chemistry while pursuing his Bachelor of Arts degrees in Chemistry: Biochemistry and Mathematical Science. In the fall of 2015, he will matriculate to Dartmouth College's Molecular and Cell Biology program as a first-year PhD candidate.

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ABSTRACT:

Laromustine is a sulfonylhydrazine anticancer prodrug whose main cytotoxic arises from its ability to interfere with DNA replication of dividing cells. Multiple studies have suggested that Laromustine induces a form of cell death known as apoptosis. In a previous study investigating the mechanism of apoptosis, bcl2-associated athanogene 3 (BAG3) demonstrated significant upregulation in the presence of Laromustine. Given its anticancer ability, we selected BAG3 as a target for further investigation. BAG3 knockdown through transient shRNA transfections was performed in U138 glioblastoma multiforme cells and verified using qRT-PCR analysis. Finally, cell death assays were used to assess Laromustine's cytotoxic effect on BAG3 knockdown cells.

INTRODUCTION:

Laromustine is a sulfonylhydrazine anticancer prodrug that possesses a broad spectrum of anticancer activity, and its cytotoxicity arises from its ability to interfere with DNA replication of dividing cells (1). In clinical studies, Laromustine has shown a significant ability to treat acute myelogenous leukemia (AML) and glioblastoma multiforme (2). In early clinical trials Laromustine displayed significant activity in older patients with previously untreated acute myeloid leukemia or high-risk myelodysplastic syndrome, for which there is no standard treatment (3-5). In these studies, few extramedullary toxicities were observed, indicating that Laromustine is less intensive than alternate treatments, which is promising for elderly individuals (3). Unfortunately, the pharmaceutical company supporting Laromustine through clinical trials went bankrupt so Laromustine is currently an orphaned drug. Multiple research groups are still performing studies into the nature of Laromustine, so hopefully Laromustine will be sponsored again.

Laromustine is a prodrug that generates two reactive species *in situ*: 90CE and methyl isocyanate (Figure 1). 90CE is a short-lived chloroethylating agent and is thought to be the main contributor to the cytotoxicity of Laromustine (6,7). 90CE produces a high yield of electrophiles that are able to react with many different nucleophiles such as O⁶ and N⁷ of guanine (7). While 90CE can alkylate many other nucleophiles, the alkylation of O⁶ of guanine is believed to be responsible for the therapeutic effects of 90CE (7). The O⁶-(2-chloroethyl)guanine lesions caused by 90CE ultimately lead to highly cytotoxic 1-(N³-cytosinyl),-2-(N¹-guaninyl)ethane DNA interstrand cross-links (Figure 2). These interstrand linkages are detrimental to DNA binding and thus cause damage to the cell if not repaired. In recent studies, it was shown that 90CE is highly selective towards tumors with decreased levels of O⁶-alkylguanine-DNA

alkyltransferase (MGMT), the protein responsible for O⁶-alkylguanine repair (7,8). The formation of DNA-DNA interstrand cross-links likely accounts for the majority of the anticancer activity of 90CE; however, a recent study found a new pathway in which 90CE decomposes into soft thiophilic electrophile rather than a hard oxophilic electrophile (8). This pathway is subject to general acid/base catalysis with inorganic phosphate (P_i), phosphomonoesters, and phosphodiester (8). In regular cellular conditions it is likely that 10-25% of 90CE decomposes into the soft thiophilic electrophile (8).

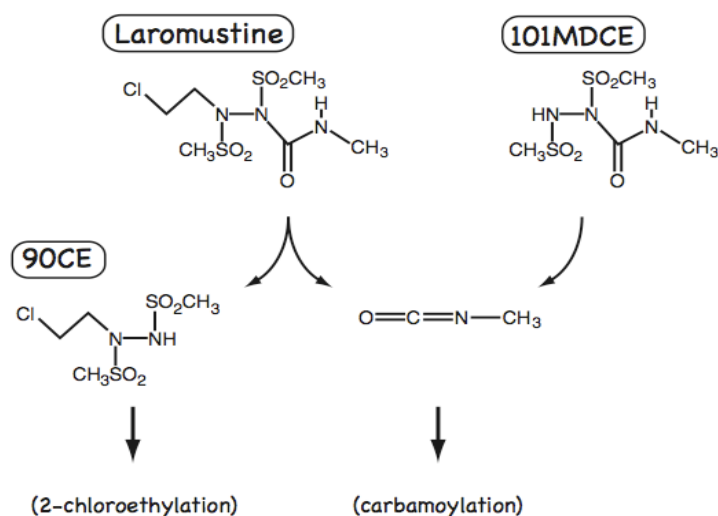


Figure 1. The generation of 90CE and methyl isocyanate from Laromustine, as well as 101MDCE, an analog of Laromustine.

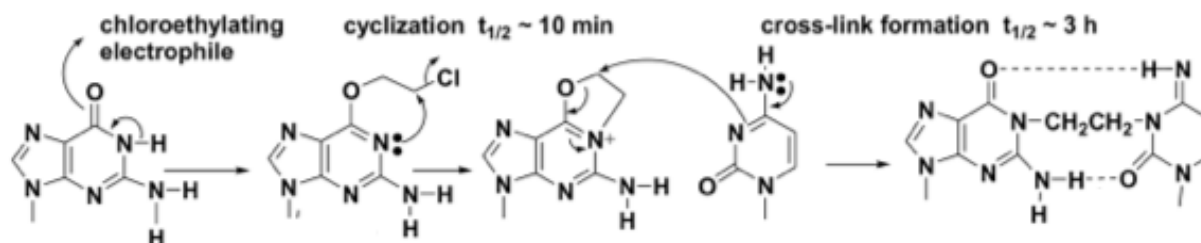


Figure 2. Mechanism of 2-chloroethylation at the O⁶ position of guanine forming interstrand crosslink with cytosine. (8).

Methyl isocyanate is a carbamoylating species that is thought to inhibit proteins involved in DNA base excision repair by modifying sulfhydryl groups, like those on cysteine residues in proteins (9). In particular, methyl isocyanate is thought to inhibit the proteins of O⁶-alkylguanine-DNA alkyltransferase and thioredoxin reductase, and other proteins crucial to base excision repair (9-11). Recently, it was found that methyl isocyanate might contribute further to the anticancer activity of Laromustine by interfering with tumor angiogenesis, the process by which new blood vessels form from existing ones (12). In particular, methyl isocyanate was found to induce the dissociation of ASK1 and Trx1 via directly carbamoylating critical cysteine groups in the ASK1-Trx1 complex or indirectly inhibiting TrxR1 (12). The DNA damage from Laromustine is thought to induce apoptosis, a form of cell death (9,13).

Apoptosis is a process in cell lifecycles, and can be defined as programmed cell death due to the energy dependence and control of the process (14). Apoptosis involves a cascade of caspases that activate one another (15). These activated caspases are then able to activate other proteins that begin to dismantle the cell quickly and neatly (15). This process is well monitored with multiple regulators, one such being the Bcl-2 family proteins which regulate the activation of procaspases, the precursors to caspases. Some members of this family inhibit apoptosis, while others promote procaspase activation (15). Initially, Laromustine was thought to induce necrosis in cells, but the current theory in the Rice Lab is that Laromustine causes apoptosis. Apoptosis is a complex mechanism involving many different pathways and proteins, thus it is difficult to identify how exactly Laromustine causes apoptosis. Thus to better understand Laromustine's cytotoxic effect, more investigations into how Laromustine activates and inhibits the different proteins and cellular machinery involved in the apoptosis mechanism is necessary to gain a complete understanding of Laromustine's cytotoxic ability.

Previously in the Rice Lab, a study was performed that looked at differential gene expression in response to exposure to Laromustine. The Rice Lab wanted to better understand Laromustine's apoptotic effect, and in particular examine which genes are activated or deactivated in the presence of Laromustine. A library of 88 primers corresponding to transcripts coding for proteins involved in apoptosis was used in conjunction with quantitative real-time reverse transcriptase PCR (qRT-PCR) to measure gene expressions levels in HL-60 cells that were exposed to Laromustine. Several genes exhibited different expression levels, while one gene, bcl2-associated athanogene 3 (BAG3), exhibited significant upregulation in the presence of Laromustine as seen in Figure 3 (16). Further research into the primary literature demonstrated that BAG3 is an anti-apoptotic gene. We believe that upon exposure to the cytotoxic Laromustine, HL-60 cells are activating and overproducing BAG3 to block the apoptotic signals from Laromustine. The cell is activating BAG3 as a survival mechanism to withstand the harsh conditions induced by Laromustine. This survival response from the cell is the basis of further investigations in the nature of BAG3 and its interaction with Laromustine.

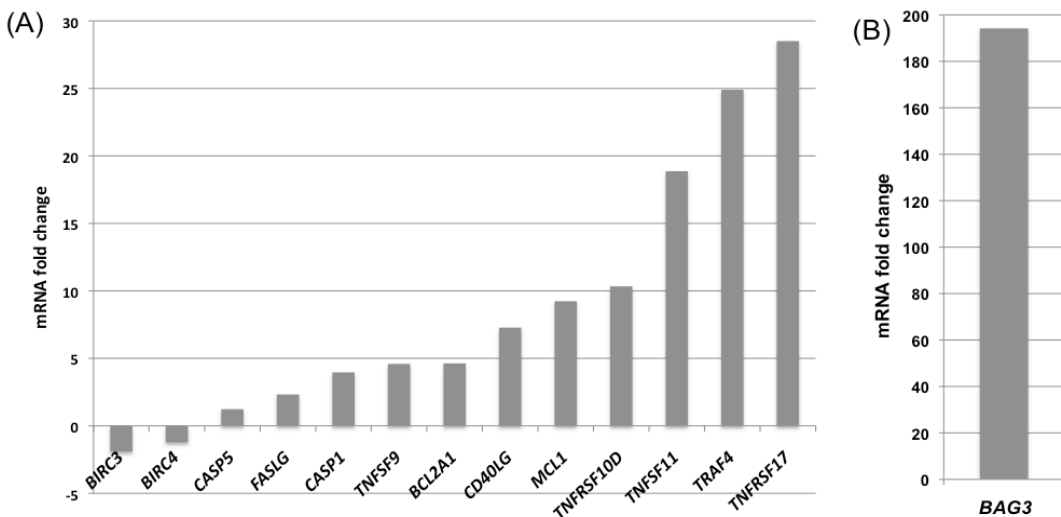


Figure 3. Apoptotic genes demonstrating significantly altered expressions levels in Laromustine-treated HL-60 cells.

BAG3, also known as CAIR-1 or Bis, is a 74-kDa protein located in the rough endoplasmic reticulum. It is a pro-survival component of the bcl2-associated athanogene family, and so has been connected to cell death pathways (17,18). However, its most important role is to promote cell survival, thus preventing apoptosis. An early study showed that BAG3 interacted heavily with heat shock protein 70 (Hsp70) to regulate and attenuate apoptosis in cellular systems (19). More recently, BAG3 has been shown to interact with the SH3 domain of SRC, and thus mediates the effects of HSP 70 on SRC signaling (20). The interactions between Hsp70 and BAG3 have also been implicated in the regulation of multiple transcription and translation factors, as well as cell-cycle regulators p21 and survivin (20). It is evident that BAG3 has a strong control of the signaling networks, and thus, cell survival. The five other BAG family members also regulate, both positively and negatively, the function of Hsp70, as well as form complexes with a various transcription factors that are able to modulate processes like apoptosis (21). This similarity in function can be attributed to the BAG domain, which is conserved among the members of the BAG family (21). Hsp70 is an important chaperone that stabilizes the folding and conformation of mature proteins, and thus is a therapeutic target for cancer treatments (22). Due to BAG3's involvement as a chaperone to Hsp70, BAG3 must play a key role in protein folding and therefore has a larger impact on cell survival.

Along with its connection with Hsp70, BAG3 is also believed to induce anti-apoptotic activities through its interactions with other BAG family proteins. One effect is due to the interaction between BAG3 and Bcl-2 that has been shown to prevent Bax-induced and Fas-mediated apoptosis (23). BAG3 has also been shown to prevent translocation of Bax, a pro-apoptotic protein, to the mitochondria in glioblastoma multiforme cells (24). In addition, the proteins Bcl-X_L, Mcl-1, and Bcl-2 are stabilized by BAG3 at the protein turnover level, which

promotes cell survival (25). Bcl-X_L has also been shown to form a complex with BAG3 and Hsp70, which further supports the theory that BAG3 induces apoptosis via protein-protein interactions (25).

BAG3 is crucial to regulating cell survival, especially due to its role in protein folding, and this protein has been implicated in a number of cancers. Multiple studies have shown that BAG3 plays a significant role in the survival, growth, and invasiveness of multiple different cancer types including pancreatic, thyroid, colon, and prostate cancers as well as neuroblastomas, glioblastomas, lymphomas, and leukemias (26,27). In most of these cancer types, higher expression levels of BAG3 from primary tumor samples have correlated with higher tumor grades, lower patient survival rates, and higher degrees of drug resistance (24,27-30). Two studies have also shown that BAG3 also regulates epithelial-mesenchymal transition (EMT), the process by which tumors grow and metastasize (31, 32). Due to its significant influence in many cancer lines, BAG3 may serve as a beneficial target for novel cancer treatments or pre-screening tests.

The oncogenic phenotype of BAG3 appears to be a hyperactive version of its normal activity, this change is to promote survival as well as prevent the induction of cell death. In a recent study, it was shown that normal lung tissue did not express BAG3 while high expression levels were detected in multiple small cell lung cancer tumors (27). It is believed that BAG3 production is related to the growth and progression of tumors, but we are still uncertain how this connection occurs. Major advances towards understanding the role and function of BAG3 in maintaining normal cell activity have been made in the past few years, and these investigations have spanned a variety of cancer types (24-25, 30, 33-35). In an early investigation into the role of BAG3, it was shown that BAG3 prevents IKK degradation by preventing its association with

Hsp70, and thus prolonging NF- κ B activation and promoting cell survival (33). In a different study, the overproduction of BAG3 in glioblastomas was shown to block translocation of Bax to the mitochondria and thus promotes survival (24). In a study involving thyroid carcinoma, the researchers decreased BAG3 levels, which resulted in an increase in apoptosis triggered by TNF-related apoptosis, this again shows that BAG3 has the ability to impede apoptosis in cancerous cells (30). In previous studies involving colon cancer, it has been shown that BAG3 associates with Bcl-X_L and Bcl-2, anti-apoptotic genes, but recently this same association has been proven in non-small cell lung cancer (25, 34). In a recent study involving cervical cancer, researchers were able to show that decreasing BAG3 expressions levels sensitized HeLa cells to PEITC treatment which in turn promoted recovery of p53, a natural tumor suppressing protein (35). The increase of p53 within the cells directly correlated to an increase in the rate of apoptosis.

BAG3 was proven to be a significant influence and potential therapeutic target for leukemias. The overexpression of BAG3 and the protein's anti-apoptotic ability was demonstrated in two different studies in which primary cell samples from adult patients with B-cell chronic lymphocytic leukemia (B-CLL) and patients with childhood acute lymphocytic leukemia (ALL) were tested for BAG3 expression and then exposed to antisense oligodeoxynucleotides to decrease levels of BAG3 (36). In both studies, the rate of apoptosis significantly increased in the primary cell samples that were treated with the oligodeoxynucleotides targeting BAG3, and upon further treatment with chemotherapeutics, the percentage of apoptosis increased further (37, 38). The overexpression of BAG3 has also been linked to drug resistance in several leukemias including chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and chronic lymphocytic leukemia (CLL) (39, 40). A recent effort to combat drug resistance has been successful as one study showed that BAG3

downregulates miR-29b to induce anticancer drug resistance in ovarian cancer (41). However, the study also demonstrated that BAG3 knockdown upregulates miR-29b and thus makes the ovarian cancer cells more sensitive to anticancer drugs (41).

The overexpression of BAG3 has been attributed to a stress response of the cell due to the toxic nature of chemotherapeutic drugs (45). A recent study even showed the BAG3 expression levels were minimum or non-existent in healthy cells, this further confirms the theory that BAG3 is crucial to tumor survival, and it is over produced due to the cytotoxicity of chemotherapeutic drugs (45, 27). Many studies have shown that knockdown of BAG3 prior to drug treatment results in a greater level of cell death in multiple cell lines (42-44). As our previous studies have shown, BAG3 expressions levels in HL-60 cells are significantly increased in the presence of Laromustine. We hope to establish a procedure for decreasing BAG3 levels, which may ultimately help improve the efficacy of Laromustine. Increasing the efficacy, and thus cytotoxicity of Laromustine, could prove beneficial in clinical settings due to patients being pre-screened for BAG3 expression levels, with those patients who do not overexpress BAG3 being recommended for Laromustine treatment. An alternate way to increase the cytotoxicity of Laromustine would be to potentially create a concomitant therapeutic drug that would decrease BAG3 levels within the patient.

To test this hypothesis, this study will investigate whether BAG3 knockdown can sensitize U138 cells to the Laromustine. We will use RNA interference to perform this knockdown. RNA interference is a natural process that cells use to monitor protein levels. The cell produces a ssRNA that binds complementary to a specific mRNA and cleaves the target mRNA before it is able to be transcribed into a protein. We can hijack this mechanism by introducing our own ssRNA molecules into the cell that are designed to bind complementary to

only the mRNA for BAG3. Thus BAG3 levels should be decreased after this treatment. We hope to show a correlation between decreased BAG3 expression levels and increased cytotoxicity of Laromustine. To achieve this knockdown, shRNA will be transfected into HL-60 cells. Subsequent BAG3 expression levels will be analyzed using qRT-PCR. Once a stable knockdown of BAG3 can be demonstrated, then cell death and proliferation assays will be performed to analyze how the knockdown affects Laromustine's cytotoxic effect.

MATERIALS AND METHODS:

Cell Culture

U138 cells from American Type Culture Collection (Rockville, MD) were grown in EMEM complete media (Lonza, Basel, Switzerland) supplemented with 0.1% gentamycin, 10% fetal bovine serum, and 1% L-Glutamine (Fisher Scientific, Hampton, NH). Cells were split approximately every five days and maintained at 40% - 80% confluence. To split the cells, they are first washed with HEPES-BSS to remove serum and protein from monolayer. Next, they are trypsinized for 5 minutes to detach them from the culture flask. Cell suspension is then spun to pellet cells, and resuspended with fresh media to desired concentration.

Processing of Short Hairpin RNA (shRNA)

A bacterial glycerol stock with pLKO.1-puro vectors with a BAG3 shRNA clone was obtained from Sigma-Aldrich (St Louis, MO). Clonal cell line culturing was performed in accordance with the manufacturer's procedure. Ice splinters were removed from the stock and placed in 0.5 mL of LB without antibiotics and incubated at 37°C with shaking for 15-30

minutes. Using the incubated culture, a plate containing LB agar and carbenicillin, 100 ug/mL, was streaked and incubated for 15-20 hours at 37°C. A single colony was then isolated to be used in plasmid purification with Qiagen QIAprep Spin Miniprep Kit (Hilden, Germany). Manufacturer's suggested protocol was followed for the purification of plasmid.

Transfection of U138 cells

Purified BAG3 shRNA plasmids were then used to transfect U138 cells through lipofection using the Effectene Transfection Reagent (Qiagen, Hilden, Germany). In brief, 5.0×10^5 U138 cells were transfected with 0.4 ug of plasmid, 3.2 uL of Enhancer, and 10 uL of Effectene Reagent (Qiagen, Hilden, Germany). Post transfection cells were incubated in culture flasks for 24 hours before the addition of 0.5 ug/mL puromycin (Enzo Life Sciences, Farmingdale, NY) for selection of cells containing transfection plasmid.

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from transfected and/or drug-treated cell culture samples as outlined in the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) protocol and quantified by optical density using a NanoDrop 1000 (Thermo Scientific, Waltham, MA). All qRT-PCR assays were carried out using the QuantiFast SYBR Green RT-PCR Kit (Qiagen) on an Applied Biosystems StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA). The BAG3 primers were obtained from Real Time Primers (Elkins Park, PA). Analysis of gene expression data was calculated using the comparative ΔC_T method to generate $\Delta\Delta C_T$ and fold change values as a way to relatively quantify the amount of mRNA that was in the original samples.

Apoptosis Assays

U138 cells were incubated in Laromustine at desired concentration to induce apoptosis. After, cells were incubated for 15 minutes in suspension containing FITC annexin V and PI. Stained cells were then analyzed by flow cytometry, measuring fluorescence emission at 530 nm (FL1) and >575 nm (FL3). Populations of cells were then defined by three categories: healthy, early apoptotic, and late apoptotic.

RESULTS AND DISCUSSION:

Previous studies into the nature of Laromustine's anticancer effect suggest that Laromustine induces apoptosis in cancer cells (1, 6-8, 10). Analyzing the effect Laromustine has on genes associated with apoptosis, we found that BAG3 is significantly upregulated in the presence of Laromustine (16). BAG3 is an anti-apoptosis gene, so the cancer cells are likely overproducing this gene as a form of defense against the harsh Laromustine treatment. Therefore we will knockdown BAG3 levels then analyze Laromustine's anticancer effect on cells with decreased BAG3 levels.

Laromustine upregulates BAG3 mRNA in U138 cells

Previously we utilized HL-60 leukemia cells, but with this current study we decided to use U138 glioblastoma multiforme cells instead. Since we switched to the U138 cell line, we first needed to recreate the BAG3 upregulation result that was initially seen in HL-60 cells. To do this we incubated the U138 cells in Laromustine and then analyzed BAG3 expressions levels using qRT-PCR. Comparing the untreated and treated U138 cultures, the ΔC_t value is 5.45 as

seen in Figure 4. The fold change can then be calculated using equation $2^{\Delta Ct}$, and we see a BAG3 mRNA fold change of 43.7. While this value is not of the same scale displayed in HL-60 cells, the result is still consistent with the results we saw initially in HL-60 cells.

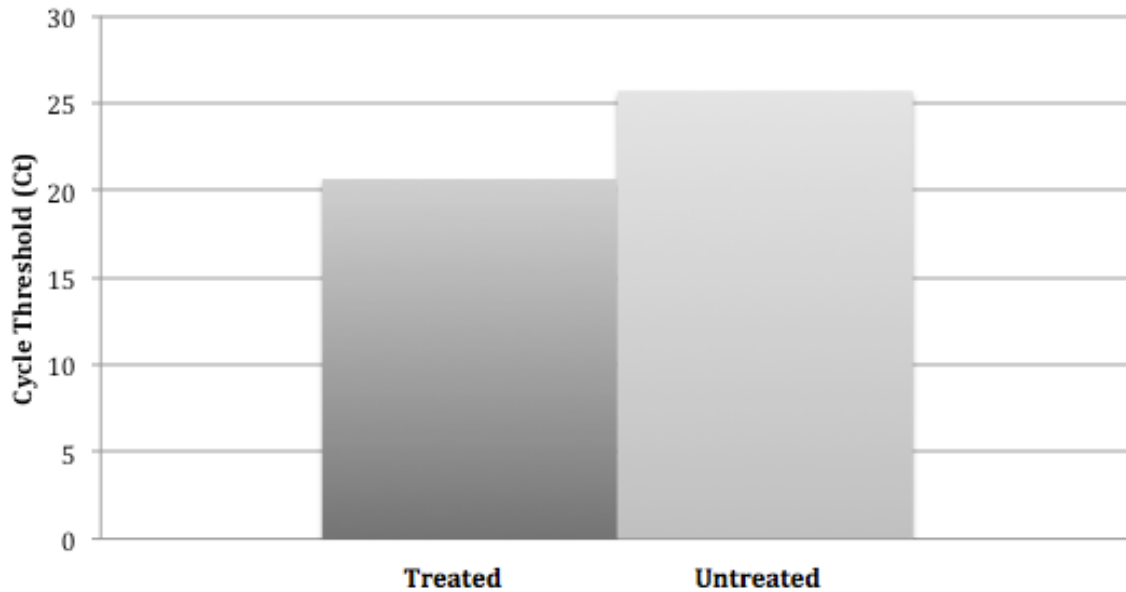


Figure 4. BAG3 expression levels in U138 cells for treatment with Laromustine and control.

Transient shRNA knockdown of BAG3 in U138 cells

After verifying that Laromustine-treated U138 cells upregulate BAG3, we transiently transfected the shRNA plasmid for BAG3 into the U138 cells using the Effectene Reagent. The shRNA plasmid was first purified from *E. coli* using the Qiaprep spin MiniPrep kit from Qiagen. To verify that we successfully digested the correct plasmid, we performed a restriction enzyme digest using *Pvu II*. We expected bands three bands with molecular weights of 3803bp, 2513bp, and 776bp, which is consistent with the gel seen in Figure 5. We then performed the transient transfection followed by treatment with Laromustine or DMSO for 6 hours, and analyzed BAG3 expressions levels again using qRT-PCR. The resulting data shows an increase in cycle threshold

for the Laromustine treated cells when you compare those cells with the plasmid to those without it (Figure 6). This increase in cycle threshold correlates to a decrease in BAG3 expression in these cells. The ΔC_T value for the cells receiving Laromustine was 1.28 as seen in Figure 7, which correlates to a fold change of -2.43 using the equation $-2^{\Delta C_T}$. When we combine the resulting from the control trials using DMSO, we see a $\Delta\Delta C_T$ value of 1.27 and a BAG3 fold change of -2.41 using the equation $-2^{\Delta\Delta C_T}$. This result depicts a decrease in BAG3 expression with a fold change less than zero. These data show that the transfection was successful and with the predicted result of decreased BAG3 expression.

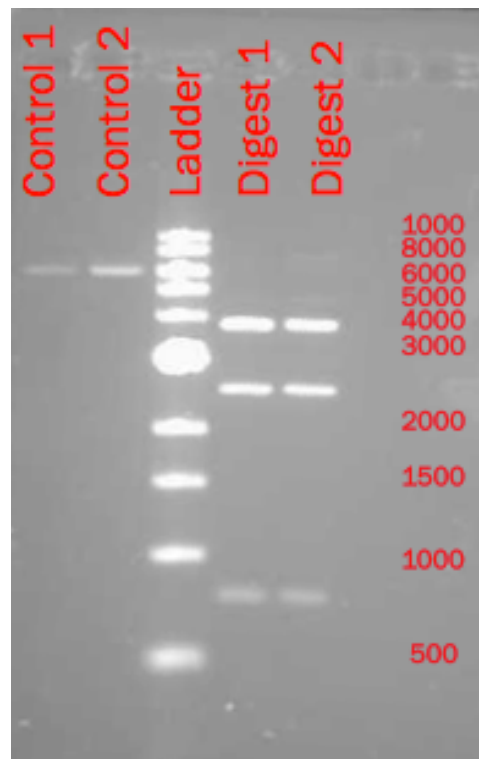


Figure 5. *Pvu II* restriction enzyme digest of purified shRNA plasmid. Bands in digests are 3803bp, 2513bp, and 776bp in size.

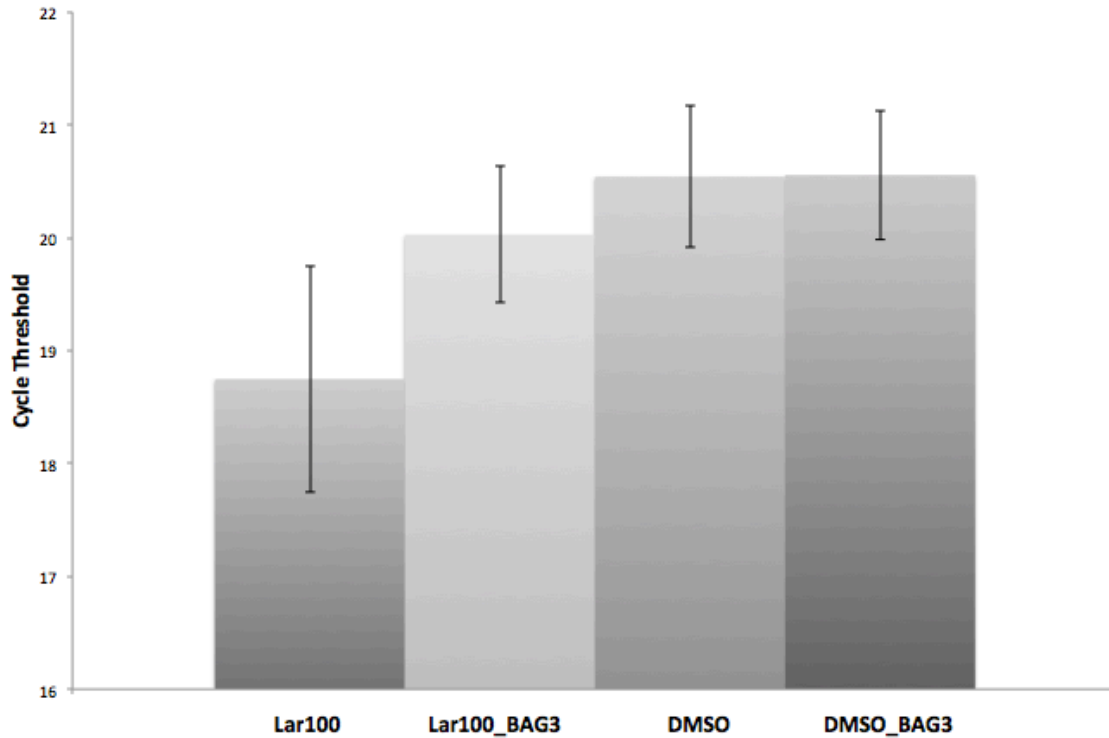


Figure 6. BAG3 knockdown via transfection of shRNA plasmid and subsequent treatment with either 100 μ M Laromustine or DMSO. Data obtained from qRT-PCR analysis.

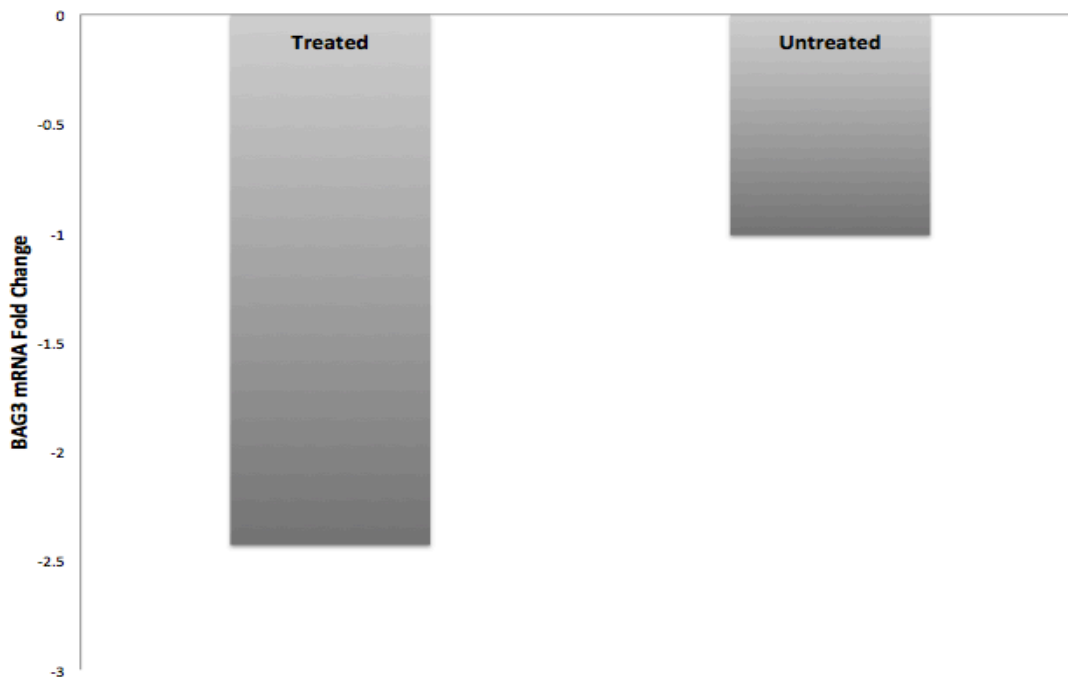


Figure 7. BAG3 knockdown via shRNA transient transfection and treatment with either 100 μ M Laromustine or DMSO. Data obtained from qRT-PCR analysis and fold change expressed as $-2^{\Delta Ct}$.

FUTURE WORK:

Given the success of the transient transfection in decreasing BAG3 expression, we will transfect again to create a stable U138 cell culture containing the BAG3 shRNA plasmid. The shRNA plasmid contains a resistance gene for puromycin. We can use this resistance gene to select for those cells that successfully received the plasmid. This will create a new population of U138 cells that all contain the BAG3 shRNA plasmid which can be used for future assays. Again, BAG3 knockdown will be confirmed using qRT-PCR. The newly created U138 cells will be subjected to Annexin V/PI staining to assay for apoptosis levels. In brief, Annexin V is a fluorescent molecule that binds to phosphatidylserine, a phospholipid found on the inner membrane of cells. In healthy cells, Annexin will be unable to bind phosphatidylserine; however, during early apoptosis phosphatidylserine is translocated to exterior membrane and thus is able to bind Annexin V, which can be detected using a flow cytometer. Propidium iodide (PI) is a red-fluorescent molecule that only fluoresces when bound to double-stranded DNA. PI is membrane impermeant and thus does not fluoresce in normal cells. But during late apoptosis the plasma membrane is destroyed and thus PI can bind dsDNA and fluoresce. Again this can be detected using a flow cytometer. We will use these two fluorescent molecules to determine the rate of Laromustine-induced apoptosis in our newly created U138 population compared to normal U138 cells. We hope that the newly created BAG3 knockdown cell line will display greater levels of apoptosis when treated with Laromustine as compared to normal Laromustine-treated U138 cells.

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