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Investigating the effects of BAG3 knockdown on the cytotoxicity of the anticancer drug Laromustine

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Vitae

Kayla Marie Gross was born on May 12, 1991 to parents Jodie L. Leen and Mark S. Gross and grew up in East Hampstead, New Hampshire. She attended high school at Phillips Exeter Academy, graduating Cum Laude before she matriculated at Colby College in Waterville, Maine. At Colby, she worked in the research laboratory of Assistant Professor Kevin P. Rice while pursuing her Bachelor of Arts degree in Chemistry: Biochemistry with a minor in Creative Writing. She was also very involved with the club Colby Dancers, serving as President for her last two years at Colby. In the fall of 2013, she will matriculate into the Integrated Studies Program as a first-year PhD candidate in the Tufts University Sackler School of Graduate Biomedical Sciences.

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ABSTRACT

Laromustine is a sulfonylhydrazine anticancer prodrug whose cytotoxicity results from the formation of interstrand cross-links caused by the synergistic action of co-generated 2-chloroethylating and carbamoylating species. The cytotoxic activities of Laromustine involve the induction of apoptosis. Described herein is an investigation into this drug's effects on apoptotic gene expression in HL-60 cells using qRT-PCR. Significant changes in the expression levels of 13 genes were observed, most dramatically in the upregulation of the bcl2-associated athanogene 3 (BAG3) gene. Given the pro-survival role of BAG3 in the cell, this investigation sought to decrease *BAG3* mRNA levels in HL-60 cells using transient shRNA transfections in conjunction with qRT-PCR analysis and cell death assays to assess the effect of *BAG3* knockdown on sensitizing cancerous cells to Laromustine-induced cell death.

INTRODUCTION

Laromustine is a sulfonylhydrazine anticancer prodrug whose main cytotoxic activity originates from its ability to interfere with DNA replication in dividing cells.[1] *In situ*, it generates two reactive electrophiles: 90CE and methyl isocyanate (Fig. 1). The 90CE component is understood to be the main contributor to the cytotoxicity of Laromustine. It inhibits DNA replication in cancerous cells by alkylating DNA at the O^6 position of guanine, and this alkylation results in the formation of harmful interstrand cross-linking of guanine with cytosine.[2] Methyl isocyanate is a carbamoylating agent that can modify sulfhydryl groups, such as cysteine residues in proteins, which can negatively affect their activity.[3] More specifically, methyl isocyanate is thought to inhibit the proteins O^6-alkylguanine-DNA alkyltransferase (AGT) and thioredoxin reductase, as well as various protein components related to base excision repair, such as DNA polymerase β and AP endonuclease.[3-5] A synergistic effect has been observed for the 90CE and methyl isocyanate components of Laromustine, though the cause of this synergism has not been definitively established.[3]

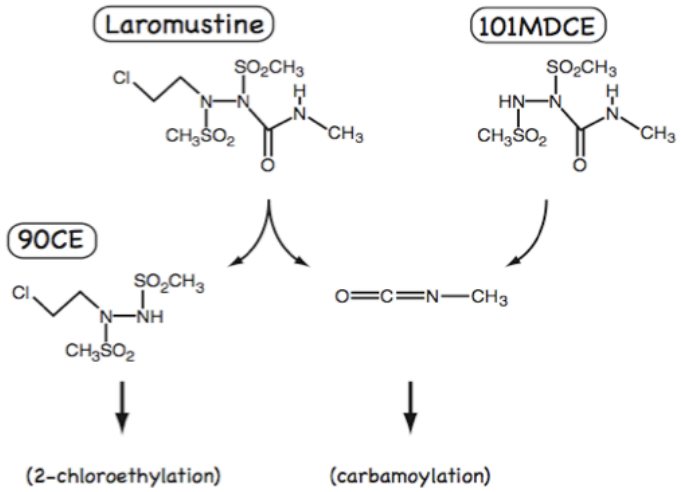

Figure 1. Chemical structures of Laromustine, 90CE, and 101MCDE.

The damage that Laromustine exerts on a cell's genetic foundation as well as its cellular machinery can result in cell death. Although initial studies suggested that Laromustine induced necrotic death, new investigations have indicated that it activates apoptotic cell death pathways.[6] While many current inquiries involving Laromustine focus on the drug's mechanism of inducing cell death, the basis for this particular investigation stemmed from wanting to more fully characterize the molecular responses of cancer cells to Laromustine in the context of apoptotic cell death. There are many pathways involved in apoptosis, each having their own triggers for induction and execution of cell death.[7] Determining what specific apoptotic components are involved in cell death caused by Laromustine may identify secondary drug targets or targets for use in clinical pre-screens of cancer patients.

A broad-spectrum investigation regarding the effect of Laromustine on apoptotic components has been completed as a precursor to this study. A library of 88 primers corresponding to genes coding for protein components involved in apoptosis was used in conjunction with quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis to measure changes in gene expression in HL-60 cells that had been exposed to Laromustine. Thirteen genes showed significantly altered levels, with the greatest change seen in levels of the bcl2-associated athanogene 3 (*BAG3*) gene, which demonstrated marked upregulation in the presence of Laromustine. Thus, this gene and its corresponding protein product was selected as a target for further investigation into the nature of Laromustine's anticancer activity in a cellular system.

BAG3, also known as CAIR-1 or Bis, is a 74-kDa protein, located in the rough endoplasmic reticulum, that is a pro-survival component of the Bcl2-associated

athanogene family, a group of proteins that have been implicated in a variety of signaling, chaperoning, and cellular death pathways.[8,9] Transcription of *BAG3* is regulated by a number of protein factors including Erg1, AibZIP, WT1, and HSF1.[10] In non-neoplastic tissue, BAG3 is only constitutively expressed in skeletal and cardiac myocytes, but sustained expression levels are significantly increased in a wide variety of cancerous tissues and cell lines.[11,12]

In general, the most important role that BAG3 plays in cells relates to promoting survival and preventing apoptosis. One of the first studies to identify BAG3 used expression cloning to isolate proteins that bound to the anti-apoptotic protein Bcl-2, and they subsequently showed that BAG3 interacted strongly with heat shock protein 70 (Hsp70) to regulate and even attenuate apoptosis in cellular systems.[13] Other members of the BAG family, particularly BAG1, have also been identified as chaperones for Hsp70 due to the BAG domain that is conserved within all members of the family.[14] Hsp70's main purpose in the cell is to regulate the folding of proteins into their native state; it has also been associated with the export and translocation of proteins as well as the rehabilitation or degradation of misfolded proteins.[15] As a chaperone for Hsp70, BAG3 is an influential component in regulating processes crucial to proper production of functional proteins and thus has a large impact on cellular survival. Specifically, BAG3 has been found to promote anti-apoptotic activity in an Hsp70-dependent manner by inhibiting degradation of polyubiquinated proteins such as Akt and cyclin D1 in cellular systems.[8]

In addition to its involvement with Hsp70, BAG3 also is believed to modulate anti-apoptotic activity within the cell by interacting with Bcl-2 family proteins. By acting

synergistically with Bcl-2, BAG3 has demonstrated the ability to prevent Bax-induced or Fas-mediated apoptosis.[16] BAG3 has also been found to prevent the translocation of Bax, a pro-apoptotic protein, to the mitochondria in glioblastoma multiforme cells.[17] Additionally, the proteins Bcl-X_L, Mcl-1, and Bcl-2 are stabilized by BAG3 at the protein turnover level, promoting cellular survival, and Bcl-X_L was shown to form a complex with BAG3 and Hsp70, further supporting the theory that BAG3 demonstrates anti-apoptotic activity through protein-protein interactions.[18] Lastly, several studies have shown BAG3 involvement in other cellular pathways, such as development, autophagy, and cell motility and adhesion, but further investigations are needed to more clearly elucidate its role in these processes.[10,12]

Because of the crucial role that BAG3 plays in regulating and maintaining cellular survival, especially in relation to protein management, this member of the BAG family has also been implicated in a number of cancers. Studies have shown that BAG3 has significant influence in the development and progression of pancreatic, thyroid, colon, and prostate cancers as well as neuroblastomas, glioblastomas, lymphomas, and leukemias.[12] In many of these cancer types—specifically pancreatic, thyroid carcinoma, glioblastoma, and leukemia—higher expression levels of BAG3 in primary tumor samples have correlated with higher tumor grades, lower patient survival rates, and higher degrees of drug resistance.[17,19-21] This relationship suggests that BAG3 may serve as a beneficial target for novel cancer treatments as it seems to have an influential impact within a broad subset of cancers.

The oncogenic capacity of BAG3 appears to be caused by hyperactivity of its normal functions, which is to sustain survival and prevent induction of cellular death.

Major advances towards understanding the exact role of BAG3 in contributing to neoplastic cellular activity have been made in the past few years, and these investigations have spanned a variety of cancer types. One of the first identifications of BAG3's cancerous activity was in osteosarcoma and melanoma systems, where BAG3 was shown to prevent IKK degradation by preventing its association with Hsp70, thereby prolonging NF-kB activation and promoting cellular survival.[22] As mentioned previously, BAG3 overexpression in glioblastoma has been shown to support pro-survival activity by blocking the translocation of Bax to the mitochondria.[17] In a study involving several thyroid carcinoma cell lines, decreasing BAG3 levels resulted in an increase of apoptosis triggered by TNF-related apoptosis inducing ligand (TRAIL), another demonstration of BAG3's ability to block induction of cellular death in cancerous systems.[21] BAG3 has also been previously shown to associate with the anti-apoptotic proteins Bcl-X_L and Bcl-2 in colon cancer cells, and more recently its association with these same pro-survival proteins has also been demonstrated in non-small cell lung cancer.[18,23] Additionally, BAG3 has been implicated in processes related to metastasis through its involvement in cellular motility and adhesion pathways, as well as to angiogenesis, which was established through demonstrating BAG3's ability to sustain ERK1/2 phosphorylation in HUVEC cells.[10,11]

BAG3 has proven to be a particularly influential player and potential therapeutic target for leukemias. The trend of BAG3 overexpression and the protein's role in preventing apoptosis in leukemic cells was demonstrated clearly by two studies in which primary cell samples from adult patients with B-cell chronic lymphocytic leukemia (B-CLL) and patients with childhood acute lymphoblastic leukemia (ALL) were tested for

BAG3 expression and then exposed to antisense oligodeoxynucleotides (ODNs) to decrease levels of BAG3.[24] In both cases, treatment with ODNs targeting BAG3 significantly increased the degree of apoptosis in the primary cell samples, even without drug treatment, and the percentage of cells undergoing apoptosis increased even further upon addition of chemotherapeutics.[25,26] Overexpression of BAG3 has also been connected to drug resistance in several leukemias, including chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), and acute myeloid leukemia (AML).[27,28] High expression levels of BAG3 have also been observed in a variety of leukemic cancer cells lines, including HL-60s, strongly supporting that the model system mimics the patient phenotype.[12]

Increased expression of *BAG3* in cancerous cell lines has also been observed to be further induced upon treatment with several anticancer drugs, which include a few proteasome inhibitors, some nucleoside analogs, and a topoisomerase inhibitor.[10,19,29-31] Similarly, we have demonstrated increased *BAG3* expression in HL-60 cells upon exposure to Laromustine. These results indicate that we may have identified alkylating agents as a new category of anticancer drugs that induce *BAG3* upregulation, although the exact link between BAG3 and Laromustine's mechanism of action remains unclear. This overexpression of *BAG3* upon exposure to anticancer drugs has been attributed to a stress response on the part of the cell to compensate for the damage that such drugs cause.[32] In many of these studies demonstrating *BAG3* upregulation in response to drug exposure, knockdown of *BAG3* prior to drug treatment has led to increased levels of cellular death in variety of cell lines.[29-31] Hence, we wish to pursue this same line of inquiry for Laromustine. Given that *BAG3* is overexpressed in many cancer types

(including leukemia and glioblastoma, which Laromustine has shown promising activity against in various clinical trials) establishing its effect as a tumor promoter may lead to ways of improving Laromustine's efficacy as an anticancer drug. Previously, AGT, an established protein target of Laromustine, has been used as a marker in pre-clinical screens to determine patient predisposition to the drug. Determining the way in which BAG3 affects the cytotoxic capacity of Laromustine may lead to a similar outcome and provide clinicians with a way of selecting patients for Laromustine treatment whose cancers will be more susceptible to the drug and therefore will respond better to the treatment.

As a first step in pursuing such a goal, this study investigates whether BAG3 knockdown can sensitize HL-60 cells to the cytotoxic effects of Laromustine. Initially, small interfering RNA (siRNA) molecules were used to achieve knockdown, but after optimization attempts, knockdown of *BAG3* in HL-60 cells was accomplished using short hairpin RNA (shRNA), with degree of attained knockdown assessed using qRT-PCR analysis. Although a trend of *BAG3* knockdown was demonstrated, further studies need to be completed in order to maximize the *BAG3* shRNA knockdown potential. Following this, cell death assays measuring cytotoxicity or cell proliferation will be used to determine the effect that *BAG3* knockdown has on sensitizing cancer cells to Laromustine-induce cell death.

MATERIALS & METHODS

Cell Culture

HL-60 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 media (Lonza, Basel, Switzerland) supplemented with

0.1% gentamycin and 10% fetal bovine serum (Fisher Scientific, Hampton, NH). Cultures were maintained between 3 x 10^5 and 7 x 10^5 cell/mL at 37°C and 5% CO_2. Cell viability was determined using trypan blue exclusion, and cell count was completed using a Cellometer (Nexcelom Biosciences, Lawrence, MA).

Small interfering RNA and short hairpin RNA

Four *BAG3* siRNA constructs were purchased as part of the Qiagen (Hilden, Germany) FlexiTube Gene Solution package. As a control to asses transfection efficiency, MAPK1 siRNA was also purchased from Qiagen. All siRNA samples were diluted with sterile, RNase-free water to appropriate concentrations for transfection.

Bacterial glycerol stock containing pLKO.1-puro vectors with a *BAG3* shRNA clone was ordered from Sigma-Aldrich (St. Louis, MO). Culturing of the clonal cell line for plasmid purification was completed using the manufacturer's recommended procedure. Briefly, ice splinters from the glycerol stock were transferred to a sterile culture tube containing 0.5 mL of LB without antibiotics and incubated for 30 min at 37°C. A sterile glass rod was used to streak the culture onto an LB agar plate containing ampicillin, and plates were incubated at 37°C for 18 hr. Several colonies were then isolated and used to inoculate cultures for plasmid purification, which was completed using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and accompanying protocol.

Transient & Stable Transfections

Purchased BAG3 siRNA or purified BAG3 shRNA was used to transiently transfect HL-60 or HeLa cells via electroporation according to established protocols for an Amaxa Nucleofector II device (Amaxa Biosystems). The protocols for each cell type

are described briefly as follows. A 100 uL volume of transfection buffer from the Nucleofector Kit V (Lonza, Basel, Switerzland) was used to transfect 2 x 10^6 HL-60 cells with 30 pmol of siRNA or 2 ug of shRNA plasmid. A 100 uL volume of transfection buffer from the Nucleofector Kit R was used to transfect 1 x 10^6 HeLa cells with 30 pmol of siRNA. Equivalent volumes of RNase-free water or elution buffer were added to negative control samples for the transfection.

To establish a stable transfection of *BAG3* shRNA in HL-60 cells, 1 x 10^6 cells were transfected with 2 ug of purified shRNA plasmid as described above. Post-transfection, cells were incubated under the previously listed conditions in T-25 culture flasks for 24 hr, after which stable transfectants were established using puromycin selection at 2 ug/mL for 3 days (Enzo Life Sciences, Farmingdale, NY). A culture of mock-transfected HL-60s was also established, in the absence of puromycin, to serve as a negative control for *BAG3* knockdown.

Drug Treatment

For transiently transfected samples, cell culture samples were incubated at the previously listed conditions in 15 mL falcon tubes for 16 hr before Laromustine was added at a final concentration of 100 μM. For stably transfected samples, the appropriate number of cells was extracted from cultures before exposure to Laromustine. Equivalent volumes of DMSO were added as a negative control for drug treatment. Treated cell samples were incubated under the previously stated conditions with drug or DMSO for 6 hr before total RNA was collected.

RNA Isolation & qRT-PCR

Total RNA was extracted as outlined in the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) protocol. After quantification by optical density using a NanoDrop 1000 (Thermo Scientific, Waltham, MA), all RNA samples were diluted using RNase-free water to a concentration of 28 ng/μL. 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 library of 88 apoptosis gene primers, 8 housekeeping gene primers, and *BAG3* primer 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 quantify the relative amount of mRNA.

RESULTS & DISCUSSION

Previous and current investigations examining the cytotoxic nature of Laromustine have indicated that the damage induced by this anticancer drug triggers the induction of apoptosis, a form of programmed cell death. In order to examine, on a broad scale, the molecular response of cancer cells to Laromustine, a primer library of 88 genes associated with apoptosis was chosen as a means to analyze the effect that the drug has on apoptotic proteins. Through screening this library, the hope was to identify genes whose role in Laromustine-induced apoptosis could be investigated to contribute to our understanding of how Laromustine exerts its cytotoxic activity.

Laromustine upregulates BAG3 mRNA in HL-60 cells

A library of primers corresponding to 88 genes associated with apoptosis was used in conjunction with qRT-PCR analyses to determine changes in gene expression levels in HL-60 cells treated with Laromustine at 100 μM for 6 hours. Several screens of the entire library were performed, and 13 targets were ultimately identified as having significant changes in gene expression upon drug exposure (Fig. 2 and Table 1). Genes were determined as having significantly altered expression if a gene's individual $\Delta\Delta C_T$ value exceeded the threshold of ±2 standard deviation from the mean $\Delta\Delta C_T$ value for all 88 genes. Within this group, the majority of genes demonstrated less than or equal to a ten-fold change in expression due to drug exposure; however, the *BAG3* gene had almost a 200-fold upregulation in the presence of Laromustine, which is a highly significant change in expression level (Fig. 2). Hence, due to this marked increase, *BAG3* was chosen as a promising target for future investigations.

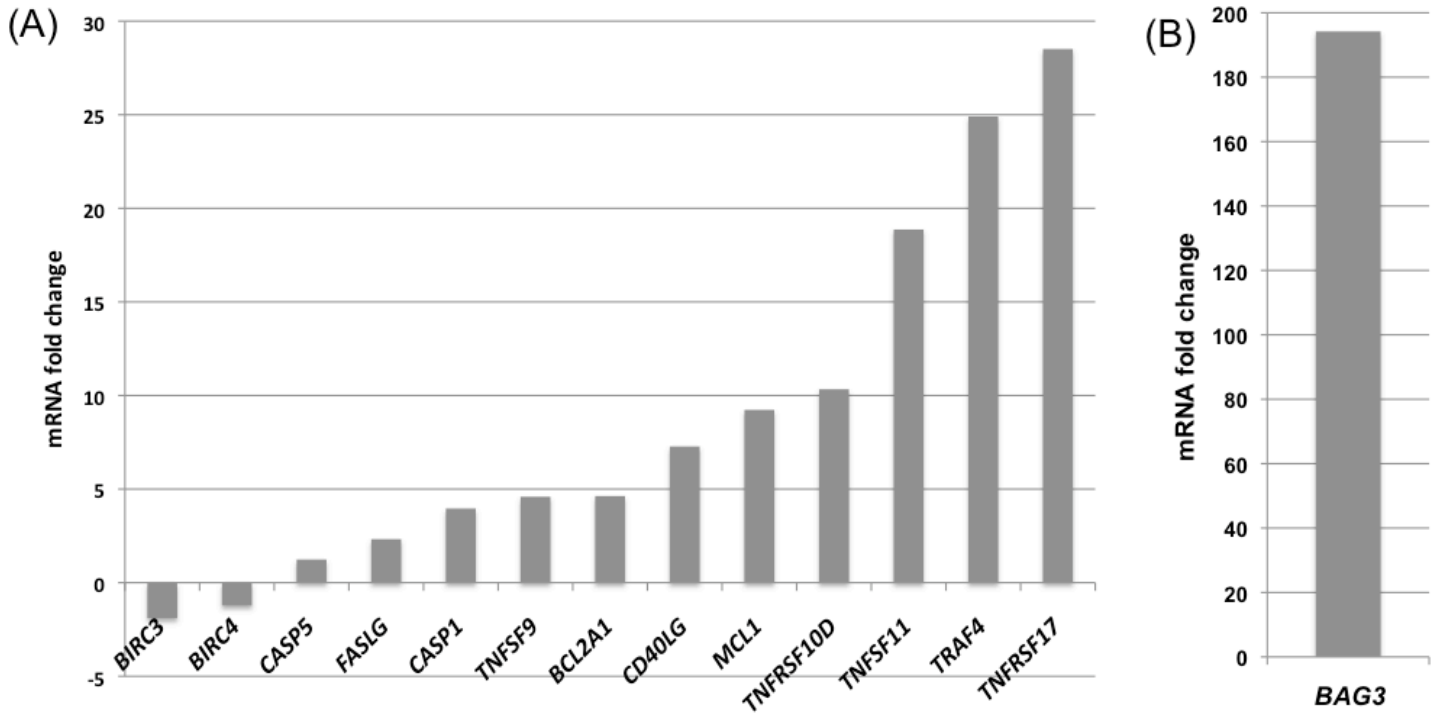

Figure 2. Apoptotic genes demonstrating significantly altered expression levels in Laromustine-treated HL-60 cells. Data was obtained from qRT-PCR analyses and fold change calculated as $2^{-\Delta\Delta Ct}$ with results shown as the mean of two trials.

Table 1. Relative quantification of mRNA levels of 88 apoptotic genes in Laromustine-treated HL-60 cells using qRT-PCR. Values were calculated via the $\Delta\Delta C_T$ method, normalized to data from endogenous control genes and vehicle-treated cells and are shown as the mean of two trials.

Gene	$\Delta\Delta C_T$	Gene	$\Delta\Delta C_T$
BAG3	-7.38	*BAX*	-0.548
MCL1	-3.38	*RIPK1*	-0.534
BCL2A1	-2.95	*BIRC5*	-0.483
TNFSF9	-2.90	*DFFA*	-0.483
CASP1	-2.68	*TRAF3*	-0.481
TNFSF11	-2.67	*TNFSF18*	-0.406
TRAF4	-2.61	*CHEK2*	-0.399
CD40LG	-2.60	*TNFRSF10A*	-0.364
TNFRSF10D	-2.59	*BAG1*	-0.361
TNFRST17	-2.41	*BCL2*	-0.341
FASLG	-2.34	*TNFSF10*	-0.327
BIRC4	-2.32	*BIK*	-0.324
CASP5	-2.21	*DFFB*	-0.269
BIRC1	-1.81	*BFAR*	-0.260
DAPK2	-1.73	*DIABLO*	-0.168
TNFRSF11B	-1.72	*GADD45A*	-0.144
TNFSF7	-1.64	*LTBR*	-0.108
DAPK1	-1.59	*CHEK1*	-0.085
TANK	-1.58	*CIDEB*	-0.073
RPA3	-1.50	*APAF1*	-0.003
TNFSF4	-1.43	*TNFSF13B*	0.022
TNFSF15	-1.36	*BIRC2*	0.056
TP73L	-1.33	*BNIP3*	0.070
CASP10	-1.28	*CASP7*	0.142
CD40	-1.25	*CRADD*	0.180
TNFRSF10C	-1.25	*TRAF6*	0.250
TNFSF8	-1.23	*BCL10*	0.254
BCL2L11	-1.18	*CASP8AP2*	0.264
TRAF5	-0.994	*TNFSF13*	0.285
CFLAR	-0.977	*CASP8*	0.303
BOK	-0.948	*TRIP*	0.304
TNFRSF8	-0.940	*CASP6*	0.327
BRE	-0.892	*TNFRSF9*	0.328
CIDEA	-0.866	*TNFRSF1A*	0.394
HRK	-0.819	*TNFRSF21*	0.460
BCL2L2	-0.800	*RIPK2*	0.511
CASP 4	-0.795	*TNF*	0.526
TP53	-0.765	*BAK1*	0.531
ATM	-0.668	*BCL2L1*	0.566
TNFRSF10B	-0.661	*FADD*	0.572
TRAF1	-0.586	*MYD88*	0.597
CASP3	-0.577	*BAG4*	0.843
BIRC6	-0.553	*CASP2*	1.01
CARD4	-0.550	*BIRC3*	3.12

siRNA knockdown of BAG3 in HL-60 cells

Previous studies investigating the role *BAG3* in various types of cancer have demonstrated a pattern of *BAG3* upregulation upon exposure to certain anticancer drugs, and they also have shown that the subsequent knockdown of *BAG3* resulted in increased sensitivity to drug-induced cell death.[29-31] The goal of this investigation was to achieve the same outcome with the anticancer drug Laromustine. Initially, transient transfection of Laromustine-treated HL-60s with small interfering RNA (siRNA) was chosen to achieve knockdown of *BAG3*. Knockdown by siRNA is a common RNAi technique used to decrease mRNA levels of a target gene in mammalian cells; however, no experimentally validated siRNA sequence existed for *BAG3* at the time of this investigation. Hence, we chose to purchase a set of four different siRNAs predicted by the vendor (Qiagen) to target *BAG3* mRNA in human cell lines so as to maximize the potential for a successful knockdown.

Prior to using these sequences, however, we attempted to validate the transfection technique—in which an Amaxa Nucleofector device was used to electroporate cells—by assessing siRNA knockdown of a validated target *MAPK1*. Although optimization ventures were attempted, we were not able to demonstrate significant *MAPK1* knockdown using a validated siRNA sequence, as qRT-PCR analysis revealed almost no change between cells transfected with siRNA and cells transfected with a vehicle control. As prior experiments had shown efficient transfection with a control GFP-containing plasmid provided by the Nucleofector vendor (Lonza), the inefficient knockdown was assumed to be due to complications with the knockdown and not the transfection technique. In a final effort to test the effectiveness of siRNA

knockdowns, one of the *BAG3* siRNAs was chosen to be transfected into HL-60s that would be subsequently treated with Laromustine, with the idea that effect of the siRNAs may be more detectable against a highly upregulated gene such as *BAG3*. While some effect was observed, it was not significant enough to demonstrate that the siRNAs were performing to their full efficiency.

shRNA knockdown of BAG3 in HL-60 cells

Since attempts at knockdown of *BAG3* with siRNA produced inconclusive results and transfection of HL-60s was successful using the GFP-containing control plasmid, we decided to change the RNAi technique to knockdown of *BAG3* via small hairpin RNA (shRNA). While there were several factors that influenced this choice, the experimental validation of the shRNA sequence we ultimately purchased was the main reason for our confidence in choosing this new approach. Initially, investigations into knockdown of upregulated *BAG3* in HL-60s due to Laromustine exposure using shRNA followed the same protocol of transient transfection via electroporation. Preliminary results using shRNA to achieve *BAG3* knockdown in this manner demonstrated an approximately 40% decrease in mRNA levels. While this is a promising trend, a more significant degree of knockdown is ultimately desired.

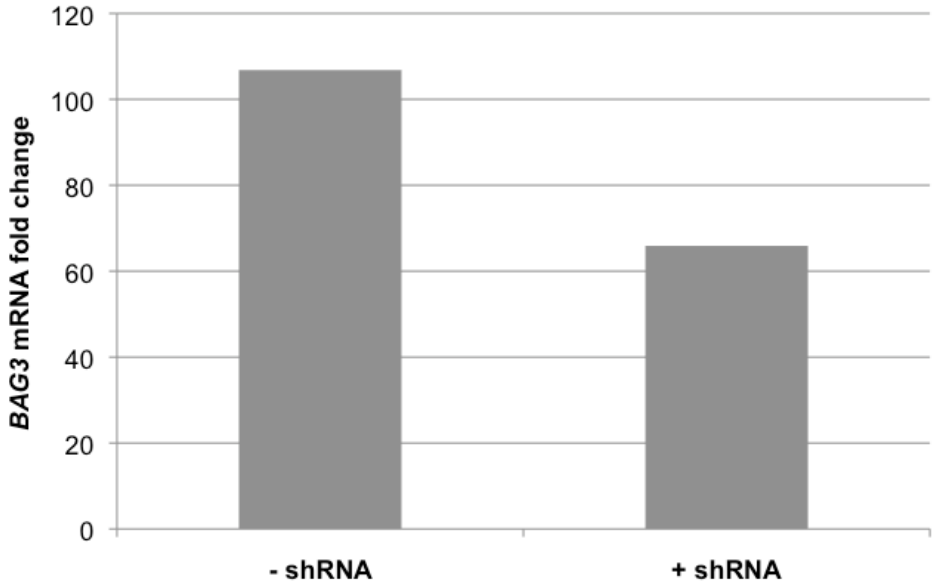

Figure 3. *BAG3* knockdown via shRNA in HL-60 cells treated with Laromustine. Data was obtained from qRT-PCR analysis and fold change expressed as $2^{-\Delta\Delta Ct}$.

Given that the shRNA plasmid purchased contained a resistance gene for selection in mammalian cells, we elected to attempt establishing a culture of HL-60s with stable expression of the *BAG3* shRNA. To do so, three groups of HL-60s were transfected—one with *BAG3* shRNA plasmid, two with equivalent volumes of the vehicle reagent—according to the previously used electroporation protocol and incubated overnight in normal growth media. At 24 hours post-transfection, the shRNA-transfected culture and one of the mock-transfected cultures were switched to media containing puromycin (4 ug/mL), with the other mock-transfected culture maintained in normal growth media. Cells were monitored until stable cultures were established, with cell count and viability data taken every 24 hours after start of the selection period. After 4 days of monitoring, the shRNA-transfected cells and mock-transfected cells maintained in the selection media showed almost no viable cells, indicating that establishing a culture that stably expressed the *BAG3* shRNA plasmid had not been successful.

FUTURE WORK

While further optimizations of transient transfection of HL-60 cells with *BAG3* shRNA will be pursued in an effort to complete this study, the focus of the investigation will shift towards establishing cell cultures that stably expresses the *BAG3* shRNA plasmid. In doing so, the consistency and degree *BAG3* knockdown should be maximized, and the process of investigating the effect of *BAG3* knockdown will be streamlined. Additional attempts at establishing stable expression in HL-60s will be completed, using a lower puromycin concentration to decrease the selection stringency, as it may have been too high previously to allow for the resistance gene in the shRNA plasmid to compensate. Also, we will turn to an alternative transfection technique, lipofection, in an attempt to establish stable *BAG3* shRNA expression in HL-60 cells as well as U138 cells, a glioblastoma cell line also used in Laromustine-related investigations.

Once stable shRNA expression in either, or both, of these cell lines results in a highly significant degree of *BAG3* knockdown, cell death assays will be used to determine the effect of decreasing *BAG3* mRNA levels on sensitizing cancer cells to Laromustine. Two assays (the ViaLight bioassay and the Click-iT EdU assay) have been chosen as appropriate methods for doing so. The ViaLight assay measures luminescence of cellular ATP to determine cell viability, hence providing cytotoxicity data. The Click-iT EdU assay uses a modified nucleoside in combination with click chemistry—in which an alkyne group on a synthetic, supplemental nucleoside reacts with an azide group on a fluorophore—to detect new DNA, thereby providing information on the degree of cell proliferation. Using both a cell death assay and a cell

proliferation assay to examine the effects of *BAG3* knockdown will produce a comprehensive examination of the degree to which cancer cells are sensitized to Laromustine. If *BAG3* knockdown does significantly increase the cytotoxic effects of Laromustine, this finding may have implications both in the lab and in the clinic as it establishes a way to improve the drug's efficacy, which would be a promising advancement in the war on cancer.

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