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Development of a Chemical Genetic Screen to Determine Synergistic Compounds with Laromustine in Treating Glioblastoma Multiforme Cultured Cells

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Vitae

Ryan Adam Weeks was born on September 30, 1993 and grew up in Framingham, Massachusetts. He graduated from Framingham High School in 2012 in the top 1% of his class. He matriculated at Colby College in Waterville, ME as a William D. Adams Presidential Scholar. While at Colby, Weeks double majored in chemistry with a concentration in biochemistry and mathematical sciences. He joined Associate Professor Kevin Rice's biochemistry laboratory during his first year as a Colby Academic Research Assistant and continued research with Prof. Rice until graduation. Additionally, he was a four-year student-athlete as a member of the varsity swimming and diving team, a Co-President of Hillel, and a CCAK mentor in Winslow, ME. During 2015 summer, Weeks was a visiting research assistant at Dana-Farber Cancer Institute in the laboratory of Assistant Professor Timur Yusufzai studying chromodomain helicases. In the fall of 2016, he will join the Chemistry-Biology Interface Program at Johns Hopkins University in his pursuit of a Ph.D. in Chemical Biology.

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Abstract

Laromustine is a chemotherapeutic sulfonylhydrazine prodrug used in clinical trials to treat acute myeloid leukemia (AML) and glioblastoma multiforme (GBM). While treatment of AML with laromustine has more demonstrative clinical success, there are enough promising data against GBM to pursue additional pre-clinical and clinical experiments. To determine the synergistic effects caused by treating cultured GBM cells with laromustine and a library of FDA-approved compounds, a chemical genetic screen was developed. To optimize the screen, optimal cultured GBM cell seed density, growth period and maximum well capacity were determined. The treatment period for a lethal dose of laromustine in cultured GBM cells was found to be 6 hours; causing acute cell death in half as much time as the treatment with a lethal dose of Temozolomide, the current GBM treatment. The LD_{50} for laromustine in cultured GBM cells was observed to be approximately 700 μM when treated for 6 hours. Using these standards of optimization for maximum reproducibility, a chemical genetic screen will be used to determine the synergistic effects of laromustine with a library of characterized small molecules.

Introduction

Despite numerous advances made in the treatment of cancer, it still remains as one of the leading causes of death in the United States. Cancer is categorized by ailments caused by uncontrolled and unregulated growth of abnormal cells in the body. Some of the main treatment plans for cancer in the United States include radiation therapy, surgical resection, and chemotherapy. Chemotherapy is the treatment of cancer through the introduction of chemicals that damage or destroy cancer cells, often insulting normal cells in the process. While existing chemotherapy is successful in many cases, there are still numerous treatments left to be discovered, with over 4775 active clinical trials ranging from phase I to phase IV across the United States (2)

Glioblastomas are highly malignant tumors generally found in the cerebral hemispheres of the brain. They arise from astrocytes, star-shaped adhesive cells found in the supportive tissue of the brain. (3). Glioblastomas multiforme, or grade 4 glioblastoma, is the most common, yet most aggressive form of glioblastoma in humans. Because of the tumors' aggressive form and low survival rate of less than a year, successful treatments have been evading researchers for the last 50 years (4). Current treatments involve immediate surgical resection of the tumor, followed by treatment within 3-4 weeks with radiation therapy and concurrent or adjuvant chemotherapy (5). However, treatment remains difficult because of the complex nature of glioblastoma multiforme in aspects ranging from varying forms microscopically, grossly, and genetically (4). Research has estimated that glioblastoma multiforme cells may have mutations in any gene at a rate of 1 in 1,000 cells, making it extremely difficult to target due to its mutating nature (6). Current research for treatment has spanned many approaches, including immunologic avenues,

gene therapy, as well as additional chemotherapeutic possibilities, though there has been little success in extending the mean survival (7,8).

Laromustine (Cloretazine, Onrigin, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl] hydrazine), is an experimental chemotherapeutic agent used in clinical trials to combat acute myeloid leukemia (AML) and glioblastoma multiforme (GBM). Laromustine, a sulfonylhydrazine prodrug, undergoes base-catalyzed activation to produce 90CE (1,2- bis(methylsulfonyl)-1-(2-chloroethyl)-hydrazine), a chloroethylating species, and methyl isocyanate, which can carbamoylate thiols and primary amines (Figure 1) . Research demonstrates that 90CE chloroethylates DNA at the O^6 position of guanine, which leads to an interstrand crosslink with cytosine on the opposite strand; these

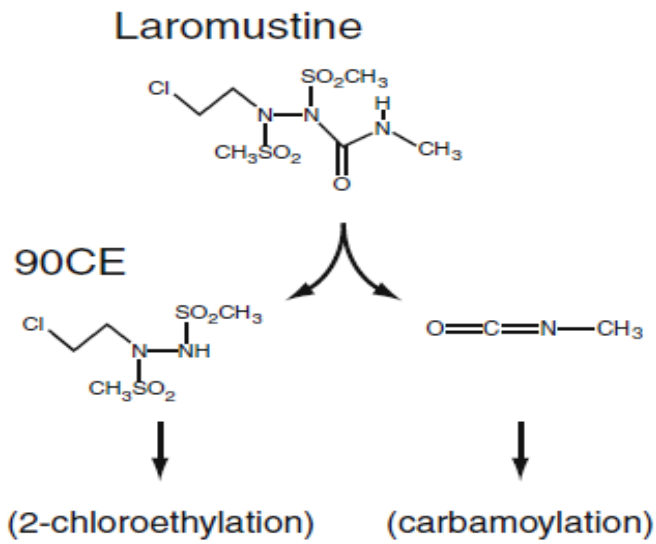

Figure 1: Decomposition of laromustine to 90CE and methyl isocyante (1)

linkages are considered the lethal lesions that disrupt DNA replication and cause cell death (9). Data suggest that laromustine produces more than twice the molar yield of DNA cross-

links compared to common nitrosoureas, lipophilic DNA alkylating agents containing nitroso and urea groups that are often used in treating gliomas (10,11).

One of the main processes of DNA repair is base excision repair in which enzymes remove erroneous nitrogenous bases and replace them with the correct base. Laromustine's carbomoylating activities has been shown to inhibit the activity of the repair enzyme DNA polymerase β (Pol β), which is involved in base excision repair (12). Cells deficient in Pol β have shown hypersensitivity to some crosslinking agents, creating a possible synergism between laromustine's 2-chloroethylating species and carbomoylating species (12,13). At the same time, methyl isocyanate is hypothesized to interfere with tumor angiogenesis by inducing dissociation of ASK1 from thioredoxin (1,14). Additionally, it is thought that methyl isocyante may promote cross-linking by 90CE, perhaps through the inhibition of the DNA repair protein O^6- alkylguanine-DNA- alkyltransferase (AGT) or other DNA repair processes (12,15).

Laromustine has yielded promising preclinical data in cultured neoplastic cells and *in vivo* activity against AML. It induces a dose-dependent inhibition of proliferation, reduction in cell viability and an increase in apoptosis in all samples, effects only enhanced when combined with the other AML drugs cytarabine or daunorubicin (16,17). In treating patients with AML, laromustine has been used in phase II trials, which suggest possible benefits over other chemotherapy drugs. For example, in a trial with 85 poor-risk elderly patients with previously untreated AML, the overall response rate was 32%; however, there was no randomized setting during the study which made it impossible to determine if laromustine was more successful than current treatment strategies (10,18,19). In a separate study, laromustine combined with cytarabine showed a 37% response rate while

the control of just cytarabine showed only a 19% response rate. The study was stopped due to high level of death from myelosuppression with the intent of continuing the trial with lower dosages due to its response success (16). Clinical trials suggest that laromustine should be investigated further with other non-traditional cytotoxic agents with a lack of toxicity that may synergistically extend the benefits of laromustine (16).

Currently, the median survival for an adult with glioblastoma is 14.6 months with concurrent radiation therapy and treatment with temozolomide, an alkylating chemotherapeutic agent that passes through the blood-brain barrier (20). Previous research has been done regarding the treatment of glioblastoma patients with laromustine; however, the results have not proven to be conclusive. Patients who did not respond to radiation and temozolomide treatment were given laromustine and showed a 6-month survival rate of 6% with a median progression free survival rate of 6.3 weeks (21,22). Despite the modest success, laromustine is still being investigated for treatment of GBM due to its ability, like temozolomide, to pass the blood-brain barrier. Additionally, although evidence suggests that laromustine and temozolomide are effective in treating patients with refractory AML, further studies have not been done to examine the effects of partner drugs with laromustine in GBM treatment (23).

High-throughput screening has become an important tool for researchers, as it creates an efficient way for them to develop new therapeutic compounds and study their effects with other known drugs as well as across numerous biochemical pathways. A chemical genetic screen incorporates the canonical genetics rationale to solve a biochemical problem. In a typical forward genetic experiment, random mutations are introduced into a population that is then screened for the desired phenotype. In chemical

genetics, small molecules that inhibit the activity of gene products are used, instead, to interfere with biochemical phenomena, and the population is screened for a desired phenotype. As with traditional genetics experiments, there are two forms of a chemical genetic screen, forward and reverse. A forward screen can be used to determine which molecules, and therefore which gene products acted upon by the compounds, produce a desired phenotype; a library of molecules is examined for a certain phenotype and individual molecules are then studied to understand the determined phenotype (24). Reverse screens, alternatively, can be used to validate drug targets; proteins are screened for their affinity to library compounds then used to observe biological phenomena (25,26).

The results of a chemical genetic screen can provide researchers with information as to how certain pathways, treatments, and processes react to the introduction of foreign species with known interactions. Chemical genetic screens have proven successful in many model systems from cultured tumor cells to zebrafish. To determine inhibitors of the PI3K/PTEN/Akt signal transduction pathway, a chemical genetic screen was performed on PTEN lipid phosphatase, a tumor suppressor and negative regulator of PI3K/Akt pathway, null cells using a library of compounds. Successful inhibitors from the first round of treatment were studied in depth to determine a set of molecules that successful inhibits the PI3K/Akt pathway in PTEN null cells, exemplifying the use of the chemical genetic screen to identify known compounds for novel uses (27). In zebrafish, a chemical genetic screen was used to identify known compounds that had not been previously known to have cell cycle activity. The library of 320 compounds was used to find alterations in the mitotic marker phosphor-histone H3: revealing 14 compounds that may be useful in studying cell cycle biology and in developing chemotherapeutic agents (28). Most similar to the desired

results with laromustine, a chemical genetic screen was developed to show synergistic cytotoxicity in nine different melanoma cell lines. Among the 300 drug combinations tested, synergy between two known drugs, sorafenib, a multikinase inhibitor, and diclofenac, a nonsteroidal anti-inflammatory drug, exhibited the most cytotoxicity (29). The study shows that it is possible to identify previously unknown uses and targets for already known drugs as well as shows that the discovery of synergistic molecules is possible using a chemical genetic screen.

To aid researchers in creating screens with a large number of compounds the National Institutes of Health developed the NIH Clinical Collection from the NIH Small Molecular Repository. The collection consists of approximately 450 clinically tested, US Food and Drug Administration approved, compounds. The library has been tested to allow researchers access to a large number of clinically approved and understood molecules for high-throughout screening. It has been used in screens to identify previously unknown uses for the known drugs, such as observing unknown inhibitory effects on adenylyl cyclase isoforms; experts have expressed medium to high levels of confidence in the performance of the collection (30,31).

To examine the possibly undiscovered synergistic effects in treating glioblastoma multiforme between laromustine when paired with compounds from the NIH Clinical Collection, a forward chemical genetic screen will be performed in which viability of cultured cells treated with laromustine and the library of compounds is assessed (Figure 2). While laromustine likely has multiple mechanisms of action, as discussed, no bias will be placed on a particular of the cytotoxic effects; but instead on the effects as a whole. If molecules are identified, they will be studied more in depth to determine the mechanisms

behind their synergism with laromustine. Results from this screen could lead to a better understanding of glioblastoma multiforme and to an improved treatment for the deadly cancer.

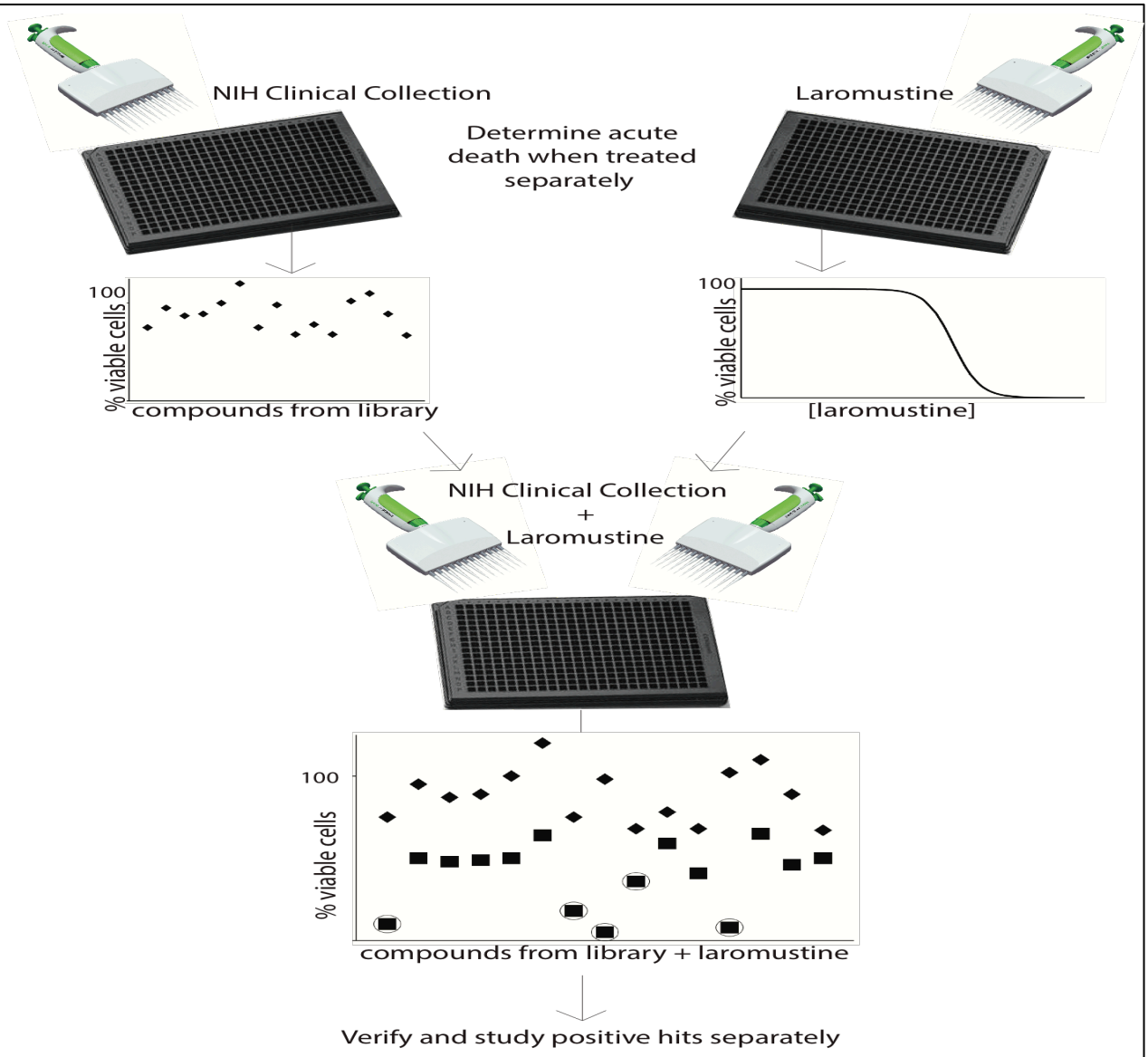

Figure 2: A schematic depicting the overall procedure of the chemical genetic screen to determine synergistic compounds with laromustine. Cells will be treated with laromustine and the NIH Clinical Collection separately, and acute cell death will be determined. The library and laromustine will then be combined for treatment and combinations that show greater cell death will be studied in depth. (Note: All graphs are predictions. In graph predicting cell death with library + laromustine, diamond points represent cell death with just library compound, square represents death with library compound and laromustine, circled squares represent positive hits for increased cell death.

Materials and Methods

U138 Cell Culture Between 25-75% Confluence:

U138 human glioblastoma cells were grown in Eagle Minimum Essential Medium with 0.1% gentamycin, 1% L-glutamine, and 10% fetal bovine serum. Cells were grown at 37˚C with 5% CO_2 and 100% relative humidity.

U138 Cell Seeding

Before each experiment, 80 – 90% confluent U138 cells were washed twice with 0.12 mL/cm^2 of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline solution (150 mM NaCl, 20 mM HEPES, pH 7.4) and subjected to trypsin/EDTA (Ethylenediaminetetraacetic acid) for 5 min to detach the cells from the flask. The trypsin was neutralized using twice the volume of media, and detached cells were centrifuged at 1100 rpm for 5 min. The supernatant was removed, and the cells were re-suspended in 3 mL fresh media. The cells were counted with a Cellometer Auto T4 and viability was determined using the Trypan blue exclusion assay. Detached U138 cells were incubated in a 384-well white μClear plate with 25 μL/well at the indicated seed densities for two days under the aforementioned conditions.

Determination of Optimum Seeding Concentration:

To determine the optimum cell count for the assay, cultured U138 cells were seeded in quadruplicate at concentrations ranging from 250 cells/ well to 25,000 cells/well (3,000 cells/cm^2 – 300,000 cells/cm^2) and a negative control without cells, and incubated for two days. At t = 48 hr, 25 μL of CellTiter-Glo reagent (CellTiter-Glo Luminescent Cell Viability Assay, Promega, Madison,WI) was added to each well and relative luminescence was obtained using a Molecular Devices Spectra Max M5 Microplate reader. Data were analyzed

for average luminescence by quadruplicate and error measured by standard deviation of quadruplicates.

Determination of U138 Doubling Time and Maximum Cells per Well

To assess the incubation period for U138 cells to double in wells and the maximum capacity of cells in each well, U138 cells were seeded in triplicate at concentrations ranging from 250 cells/ well to 25,000 cells/well (3,000 cells/cm^2 – 300,000 cells/cm^2) and incubated for two days with a negative control without cells. At time t = 48 hr, 60 hr, 74 hr, 84 hr, 96 hr, 120 hr, 144 hr, and 168 hr, 25 µL of CellTiter-Glo reagent was added to each well and relative luminescence was obtained. Data were analyzed for average luminescence by triplicates and error measured by standard deviation of triplicates.

Determination of Incubation Period of laromustine with lethal dose treatment:

To determine the amount of time needed for lethal dose laromustine to cause acute cell death U138 cells were seeded in triplicate at 5,000 cells/well (60,000 cells/cm^2) as described previously for two days with a negative control without cells. At t = 48 hr, culture media was aspirated and cells were treated with 25 µL of media containing 1 mM laromustine, 1 mM temozolomide or the corresponding volume of 1% DMSO by volume. At each time t = 10 min, 20 min, 30 min, 1 hr, 2 hr, 3 hr, 6 hr, 9 hr, 12 hr, 18 hr, and 24 hr, 25 µL of CellTiter-Glo reagent was added to the wells and relative luminescence was obtained. Data were analyzed by average luminescence by triplicates and error measured as a standard deviation of triplicates. Average luminescence across the timescale were compared to determine time needed for laromustine to cause acute cell death in cultured U138 cells.

Determination of 50% Lethal Dose:

To determine the median dose of laromustine is needed to cause 50% acute cell death, U138 cells were seeded in triplicate at 2500 cells/well (30,000 cells/cm^2) as described previously and incubated for two days with a negative control without cells. At t = 48 hr, media was aspirated and cells were treated with 25 μL media containing a concentration of laromustine ranging from 2000 μM to 1 μM in a two-fold serial dilution with the comparative volume of 1% DMSO by volume as a control. At time t = 6 hr after seeding, 25 μL of CellTiter-Glo reagent was added to wells and relative luminescence was obtained. Data were analyzed by average luminescence of triplicates over three trials and error measured by standard deviation of triplicates. Curve fit analysis was used to determine the LD50 for laromustine when using cultured U138 cells.

Verification of assay optimization using Temozolomide:

To verify the assay conditions before beginning the screening process, Temozolomide was used to assess synergistic effects with laromustine. Cultured U138 cells were seeded in triplicate at 2500 cells/well (30,000 cells/cm^2) as described previously and incubated for two days with a negative control without cells. At t = 48 hr, media was aspirated and cells were treated with 25 μL media containing a concentration the following conditions: 1000 μM Temozolomide with 500 μM laromustine and without laromustine, 100 μM Temozolomide with 500 μM laromustine and without laromustine, 100 μM Temozolomide with 500 μM laromustine and without laromustine, 500 μM laromustine and a control of 3% DMSO by volume. At time t = 6 hr and t= 12 hr after seeding, 25 μL of CellTiter-Glo reagent was added to wells and relative luminescence was obtained. Data were analyzed by average luminescence of triplicates and error measured by standard

deviation of triplicates. Average luminescence were compared between data for Temozolomide/laromustine treatments and Temozolomide and laromustine treatments separately.

Results and Discussion

Cells should be seeded at 2500 cells/well and incubated for 48 hours prior to drug treatment

To ensure that cultured U138 cells are given the sufficient space and time to grow before drug treatment, the optimal cell seeding concentration was determined. This would ensure that the total number of cells per well was not impeding the cells' ability to divide comfortably, but also to ensure that there were enough cells per well to achieve a stable luminescence signal. Ideally, the transparent bottom of each well would be maximally covered such that the largest number of cells per area could be treated. Concentrations ranging from 250 cells/well to 25,000 cells/well (3,000 cells/cm^2 – 300,000 cells/cm^2) were seeded and viability after 48 hours of incubation was compared by luminescence (Figure 3). Wells containing more than 7500 cells/well at seeding produced luminescence of approximately 3.5 x 10^4 RLU. As the relative luminescence peaked at this value, despite the increasing cell concentration, it is likely that the wells either became too dense for survival or the CellTiter-Glo was a limiting agent. As it is preferred for the relative luminescence to be on the threshold such that the number of cells treated is as high as possible without impairing growth, the relative luminescence of wells seeded at 250-1250 cells/well was too low. Wells seeded at 2500 cells/well and 5000 cells/well resulted in luminescence just before the luminescence threshold at approximately 3.0 x 10^4 RLU and 3.3 x 10^4 RLU, respectively.

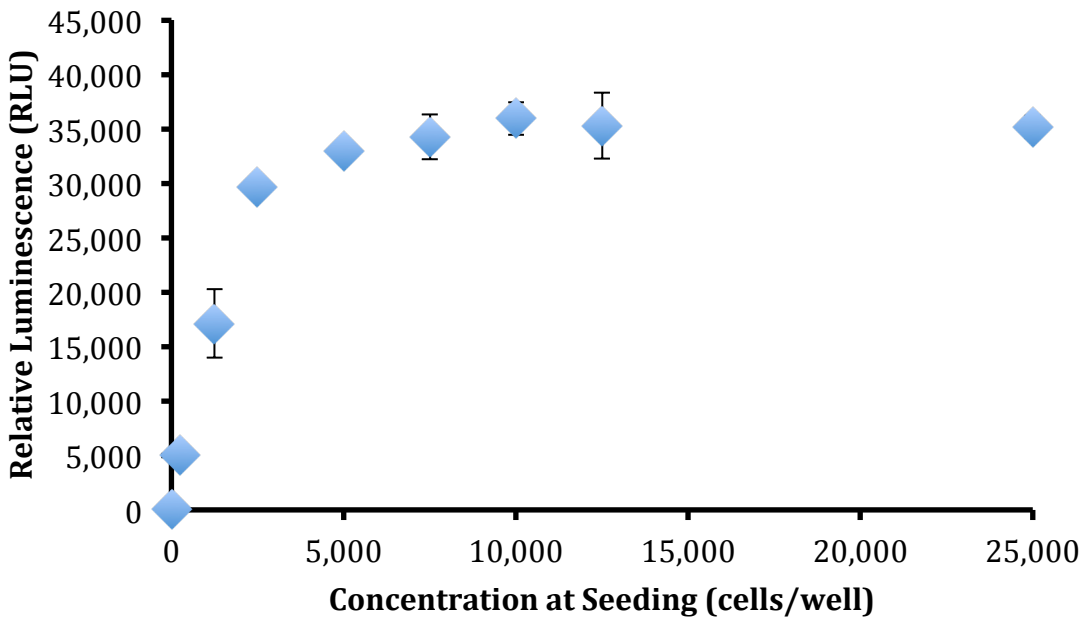

Figure 3: Seed concentration of U138 cultured cells. Cells were lysed 48 h after seeding and luminescence recorded. Error is standard deviation of quadruplicates.

Because the luminescence signal plateaued at 3.5 x 10^4 RLU, it was necessary to ensure that the CellTiter-Glo was not the limiting agent in each reaction mixture. Volumes of 25 – 50 µL of CellTiter-Glo reagent were added to wells containing 1.0 x 10^5 cells/well at the time of seeding. Luminescence results show an inversely proportional relationship between the volume of CellTiter-Glo and relative luminescence (Figure 4). Increasing the volume of CellTiter-Glo may cause unnecessary competition or the added luciferase may interfere with luminescence in some way. The results confirm that the 25 µL of CellTiter-Glo is the optimal volume to produce peak luminescence.

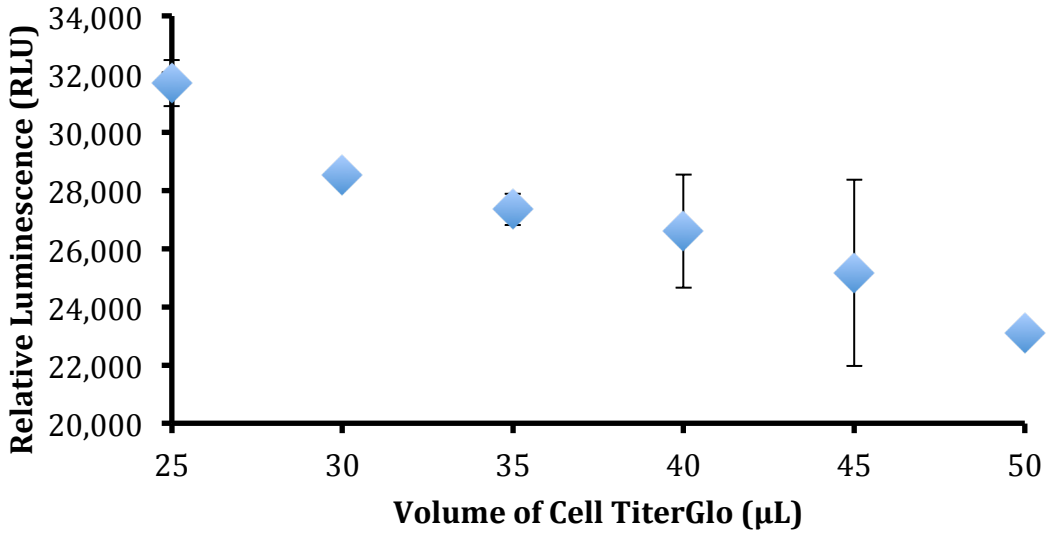

Figure 4: Determination of optimal volume of CellTiter-Glo used to lyse cells and show luminescence relative to viability of cells in each well. Error is standard deviation of triplicates.

Based on previous work, cells were incubated for 48 hours, as it takes approximately 24 hours for cells to adhere to the well bottom, providing an additional 24 hours for cells number to double at least once. To confirm that 48 hours provides ample time for cells to adhere and divide, wells were seeded with 1250 cells/well to 6250 cells/ well, as to try to avoid the threshold luminescence at 3.5×10^4 RLU. For up to 48 hours, cells were lysed and luminescence recorded every 12 hours, then every 24 hours following until 144 hours after seeding (Figure 5). The data appear to show that for lesser concentrations, at 48 hours, luminescence is approximately double the luminescence at 0 hours, while for higher concentrations the luminescence is approximately 1.5 times the luminescence at 0 hours. This may have resulted because it took longer for cells in wells with higher concentrations to adhere to the well bottom due to competition for space and nutrients to divide. At periods beyond 48 hours, error amongst triplicates became larger as

luminescence varied by greater amounts, suggesting that cells were dividing at different rates and the conditions in each well could not be controlled to maintain consistency.

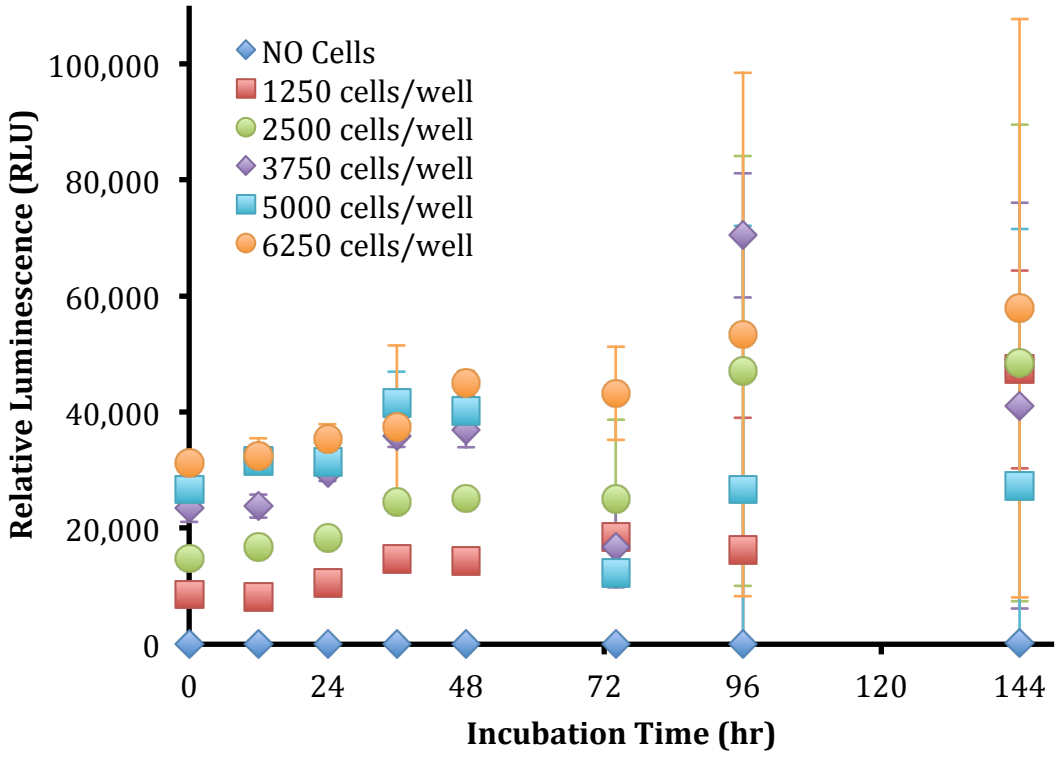

Figure 5: Determination of doubling time of U138 cultured cells. Wells were seeded with various concentrations of cells and luminescence measured at multiple time points. Error is standard deviation of triplicates.

However, despite error, the data suggest that cell doubling still occurs roughly every 48 hours until wells become too dense with cells. The wells seeded at 2500 cells/well show approximate doubling after 48 hours, with considerably low error. After 48 hours, the relative luminescence of the wells seeded at 2500 cells/well was 2.5 x 10^4 RLU, similar to the relative luminescence emitted when determining the optimal seeding concentration. The cells seeded in wells at 2500 cells/well duplicate in approximately 48 hours and error increases markedly after 48 hours of incubation; seeding cells at 2500 cells/well with 48

hours of incubation prior to treatment produces the most precise conditions for the chemical genetic screen.

At lethal dose, laromustine results in significant loss of viability in U138 cultured cells after 6 hours

The exact time after treatment for laromustine to enter cells and cause marked loss in cell viability in U138 cultured cells is important to assess before completing the chemical genetic screen. The drug requires time to enter the cell and begin to cause its deleterious effects. The cell should respond to the treatment by trying to assuage the drug's effects. The series of events will not happen immediately, thus the amount of time for the drug to significantly affect cells must be determined. This is done using a lethal dose of drug and measuring the viability at various times after treatment. The viability of U138 cultured cells was measured at different times from 10 min to 24 hours after treatment with lethal doses of laromustine and temozolomide (Figure 6, 7). Within 6 hours of treatment, there was significant loss of viability in the cells treated with laromustine compared to the DMSO control, from 1.43 times within 3 hours of treatment to 0.20 times the control at 6 hours after treatment. The viability of cells treated with laromustine continued to decrease in subsequent hours, until the cells showed approximately 1% viability relative the DMSO control. Similar results were observed in the cells treated with temozolomide, though decreases in viability occurred 12 hours after treatment. Viability of cells treated with temozolomide decreased to 10% relative the DMSO control after 12 hours but remained at approximately 10% during subsequent hours.

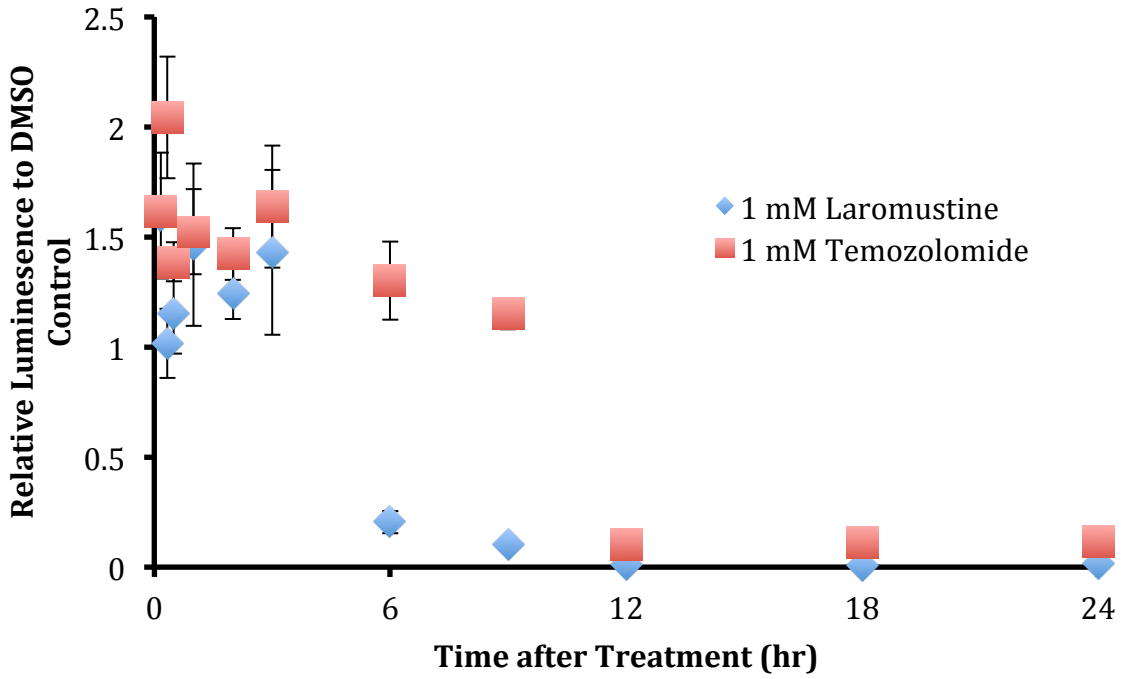

Figure 6: Determination of lethal dose time response of laromustine compared to temozolomide. U138 cells were treated with 1000 µM laromustine and 1000 µM temozolomide and luminescence determined at varying time points over course of 24 hours. Error is standard deviation of triplicates.

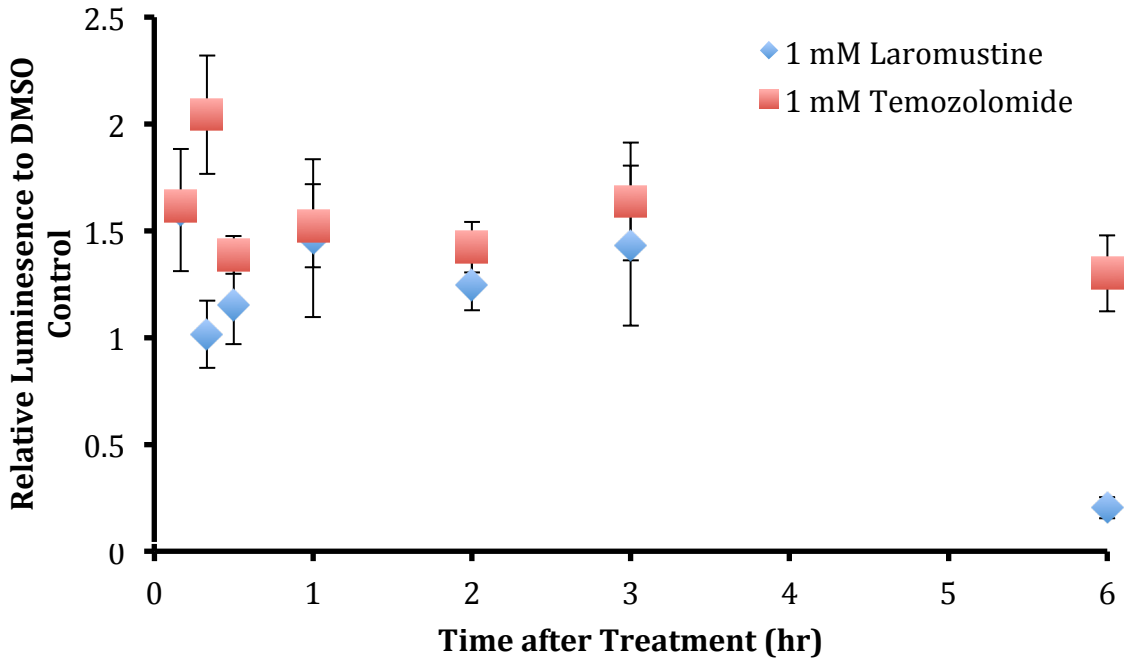

Figure 7: Determination of lethal dose time response of laromustine compared to temozolomide. Shows data from Figure 5a in greater detail from 0 to 6 hours after treatment. Error is standard deviation of triplicates.

The cells treated with laromustine showed significant loss of viability within 6 hours of treatment. This provides evidence that laromustine requires 6 hours of incubation following treatment to drastically affect cells and impede their ability survive and duplicate. Laromustine may be causing loss of viability in U138 cells in both direct and indirect ways. The high dose of the drug is cytotoxic to the cell, though the nature of the drug's acute toxicity is unknown. The drug may affect cells via its known method of crosslinking DNA, impeding cells from replicating and forcing them into apoptosis. Similarly, temozolomide causes significantly loss of viability within 12 hours of treatment. At lethal doses, laromustine affects viability in half the amount of time, indicating that laromustine is able to enter cells and cause harmful effects quicker than temozolomide. Additionally, laromustine causes an increased loss of viability compared to temozolomide. At 24 hours after treatment, laromustine showed 1.5% viability relative to the DMSO control while temozolomide remained at 11.5% viability relative to the DMSO control. This preliminary 10-fold difference in viability, within error, implies that laromustine may be more effective in treating U138 cultured cells, though more results are necessary to confirm.

The median lethal dose of laromustine in treating U138 cultured cells for 6 hours is 400 μM

A lethal dose of laromustine is shown to cause loss of viability such that cells remain as little as 1% viable compared to DMSO control. In order to assess the effective synergistic effects between laromustine and other compounds, a concentration of laromustine must be found such that more than 1% viability remains. Ideally, a median lethal dose is found such that cells are 50% viable after 6 hours of treatment relative to the DMSO control. Using a median lethal dose allows for comparison of loss of viability among cells treated with solely

laromustine and cells treated with both laromustine and a separate compound. The median lethal dose was determined by treating U138 cultured cells with a two fold dilution of laromustine from 2000 µM to 1 µM and allowing 6 hours of incubation before lysing and recording luminescence (Figure 8). Significant doses over 1000 µM are effective in

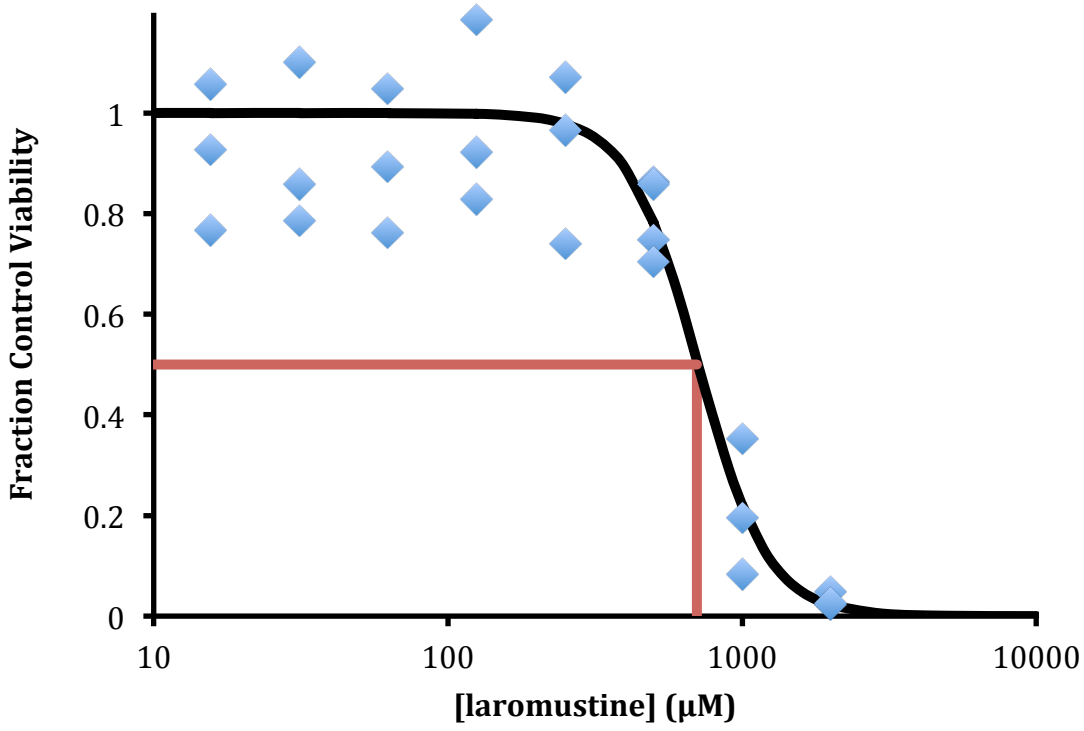

Figure 8: Determination of LD50 of laromustine in treating cultured U138 cells. Curve fit analysis shows LD50 of 708 µM. Data shown are of three trials.

drastically affecting cell viability, while doses below 250 µM were less effective. Data analysis shows a sigmoidal curve with a median lethal dose of 708 µM. Previous studies demonstrate that laromustine is effective at concentrations lower than 50 µM in treating an AML cell line, HL60. The ability for the cell to protect itself against low levels of laromustine provides motivation for the chemical genetic screen. It is plausible, that with an additional

infecting agent, laromustine may be more effective in decreasing the viability of cultured brain cancer cells at a lower dose.

Though not confirmed by laboratory work, laromustine and Temozolomide should act synergistically in inducing cell death of U138 cells

As previously mentioned, Temozolomide should show synergistic effects with laromustine due its ability to interference with AGT enzyme function. Decreasing the effectiveness of AGT should allow for a larger molar quantity of crosslinking by laromustine. Preliminary tests have shown that there is not an increased cell death when using both laromustine and temozolomide together in relation to when using them separately. While there is drastic cell death, results show that it is most likely related solely to the action of laromustine, as the Temozolomide control of 1000 μM, a lethal dose, is not proving to induce marked cell death. These results are still forthcoming and work will continue to be done in the future. Despite a lack of confirmation with Temozolomide, the chemical genetic screen has been fully optimized to compare cell death between laromustine and laromustine in conjunction with a library of small molecules. Using the data obtained from the screen, compounds showing synergistic effects with laromustine can be verified, laromustine's mechanisms of actions can be better understood, and the possibility of more successful treatments of glioblastoma multiforme with laromustine can be investigated.

Future Work

Little work remains to complete this project. First, the small-scale combination with Temozolomide must be completed. It is expected that Temozolomide and laromustine will have synergistic effects due to their determinedly similar mechanisms of action and Temozolomide's ability to inhibit AGT. Once the test combination is completed, the standard death for each compound in the NIH Clinical Collection will be determined, as written below. Then, with the assistance of Professor Robert Wheeler at the University of Maine Orono, the chemical genetic screen will be run to determine the cell death when laromustine and compounds from the library are used in conjunction. The chemical genetic screen will be conducted as described below. Based on the results of the screen, further research can be done into the mechanisms of laromustine and the effectiveness of the compounds that showed synergistic effects from the screen.

Determination of Standard Death for NIH Clinical Collection

Cells will be seeded at 2500 cells/well (30,000 cells/cm^2) with 25 µL/well as previously described in methods section and incubated for two days. At time t = 48 hr, media will be aspirated and media containing one of each of the compounds from the NIH Clinical Collection will be added to each well. At time t = 6 hr after seeding, 25 µL of CellTiter-Glo reagent will be added to each well and relative luminescence obtained.

Chemical Genetic Screen using Laromustine and NIH Clinical Collection

Cells will be seeded at 2500 cells/well (30,000 cells/cm^2) with 25 µL/well as previously described in the methods section and incubated for two days. At time t = 48 hr, media will be aspirated and media containing LD_{50} concentration of laromustine as well as

one of each of the compounds from the NIH Clinical Collection is to be added to each well. At time t= 6 hr after seeding, 25 μL of CellTiter-Glo reagent will be added to each well and relative luminescence obtained. Combinations of drugs that show a decreased luminescence than when laromustine and the NIH Clinical Collection separately will be tested again as described, though in triplicate, to ensure validity of data.

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