2014

Optimization of a Chemical Genetic Screen to Identify Druggable Targets in U138 Cells Treated with Laromustine

Kathryn Coe
Colby College

Optimization of a Chemical Genetic Screen to Identify Druggable Targets in U138 Cells Treated with Laromustine

By Kathryn A. Coe

A Thesis Presented to the Department of Chemistry,

Colby College, Waterville, ME

In Partial Fulfillment of the Requirements for Graduation

With Honors in Chemistry

Submitted May, 2014

Optimization of a Chemical Genetic Screen to Identify Druggable Targets in U138 Cells Treated with Laromustine

By Kathryn A. Coe

Approved:

_____

(Mentor, Kevin P. Rice, Associate Professor of Chemistry)

_____Date

_____

(*Reader, Julie T. Millard, Dr. Gerald and Myra Dorros Professor of Chemistry)*

_____Date

Vitae

Kathryn Ann Coe was born on October 22, 1991 and grew up in Bedford, Massachusetts. She attended the Bedford public schools from elementary school through high school, graduating in the top 1% of her class. She matriculated at Colby College in Waterville, Maine as a Presidential Scholar. While there, Kathryn studied Chemistry with a concentration in Biochemistry and a minor in Environmental Studies. She worked in Associate Professor Kevin Rice's research laboratory during all four years of her undergraduate education. She was also a member of the Environmental Advisory Group, a council of students, faculty and staff that advises the college president on sustainability initiatives. She enjoyed being a teaching assistant for organic chemistry lab, as well. During her junior year, Kathryn received the Barry Goldwater Scholarship and was inducted into Phi Beta Kappa. In the fall of 2014, she will begin her pursuit of a PhD in Biological and Biomedical Sciences at Harvard University.

Permanent Address:

11 Bandera Drive
Bedford, MA 01730

Acknowledgments

I would like to thank Professor Kevin Rice for being such a supportive research mentor and excellent instructor during my time at Colby. His constant encouragement to seek challenges has helped me grow tremendously as a scientist, and I have thoroughly enjoyed working in the Rice Lab these past four years. I would also like to thank Tara Sargent for all of her assistance and for keeping lab operations running so smoothly. I am grateful to the rest of the Rice Lab members as well for being such a wonderful support group. Their passion for research has been truly inspiring, and I am glad to have had a chance to bond with all of them. Furthermore, I would like to thank Professor Julie Millard for her guidance in writing my thesis and for her instruction in biochemistry. I would also like to acknowledge Edmund Klinkerch for synthesizing the laromustine I used throughout my thesis work and Amy Poulin for her administrative support. This work was supported by grants from the National Center for Research Resources (5P20RR016463-12) and the National Institute of General Medical Sciences (8 P20 GM103423-12) of the National Institutes of Health and by the Colby College Division of Natural Sciences.

Table of Contents

Abstract

Laromustine is an experimental sulfonylhydrazine prodrug used in late-stage clinical studies against acute myeloid leukemia (AML) and glioblastoma multiforme (GBM). Despite initial promise for both indications, clinical trials for GBM have not been as successful as those for AML. To investigate methods for improving the effectiveness of laromustine in GBM and to learn more about the mechanism of action of laromustine, a chemical genetic screen will be conducted to identify agents that sensitize GBM cells to the anti-proliferative effects of laromustine. The library, which will include approximately 450 FDA-approved drugs, will be screened using a newly optimized high throughput assay based on the Click-iT EdU Microplate Assay kit (Molecular Probes). Optimization of the assay has required determining the proper cell seed density, drug concentration and incubation time, and fluorescent substrate concentration, among other variables. It was determined that low cell seed densities allow for maximal proliferation and a high signal-to-noise ratio. Furthermore, 50 μM laromustine was found to have little inhibitory effect on the proliferation of U138 cells, while higher laromustine concentrations yielded a sharp decrease in proliferation. These results suggest that reduced proliferation of cells exposed to 50 μM laromustine in combination with library compounds is a suitable marker for sensitization to laromustine. With these optimization data, a chemical screen can now be conducted, potentially revealing new therapeutic strategies to treat GBM.

Introduction

As the second leading cause of death in the United States, cancer continues to be a pressing biomedical problem.[1] It is estimated that a half of men and a third of women in the United States will be diagnosed with cancer at some point in their lives, leaving few people unaffected by the disease in some way.[2] In actuality, cancer is not a single disease, but a family of illnesses characterized by rapid, uncontrolled cell growth. It is often initialized by a small number of causal mutations that promote growth or impede growth suppression, leading to a wide range of further genetic and epigenetic changes as cell cycle checkpoints are bypassed. The heterogeneity of mutations encumbers treatment development, as even a single tumor can contain cells with wildly dissimilar genetic profiles. Consequently, many chemotherapeutic treatments help only a small percentage of cancer patients, and the need for scientists to identify new biological targets, to develop new treatments, and to further elucidate the bioactivities of current therapies persists.

One agent that has shown promise in preclinical and clinical studies is laromustine, a sulfonylhydrazine prodrug used in late-stage clinical studies against acute myeloid leukemia (AML) and glioblastoma multiforme (GBM). Laromustine (also known as 101M, VNP40101M, cloretazine, and Onrigin) is activated through base-catalyzed decomposition into two electrophilic components, a chloroethylating agent that can modify the O-6 position of guanine in DNA and a carbamoylating agent that is less understood (Figure 1).[3] The alkylation of guanine molecules in DNA is considered the main anticancer activity of laromustine, as it leads to G-C ethane interstrand crosslinks that disrupt DNA replication.[3,4] Laromustine is considered somewhat selective toward cancer cells because they are often deficient in O^6-alkylguanine-DNA alkyltransferase (AGT), a protein that repairs O-6 guanine alkylations.[3] Most cancer cells

maintain some AGT activity, but each AGT molecule can only repair a single guanine chloroethylation.[3,5] Thus, cells that have moderate to low levels of AGT are still sensitive to laromustine. There is also a fairly short window when AGT is effective, as AGT is able to repair the chloroethyl monoadduct but not the subsequent crosslink.[6] Moreover, laromustine has been shown to have a very favorable pharmacokinetic profile, with a longer half-life and similar DNA-crosslinking ability compared to similar compounds.[4]

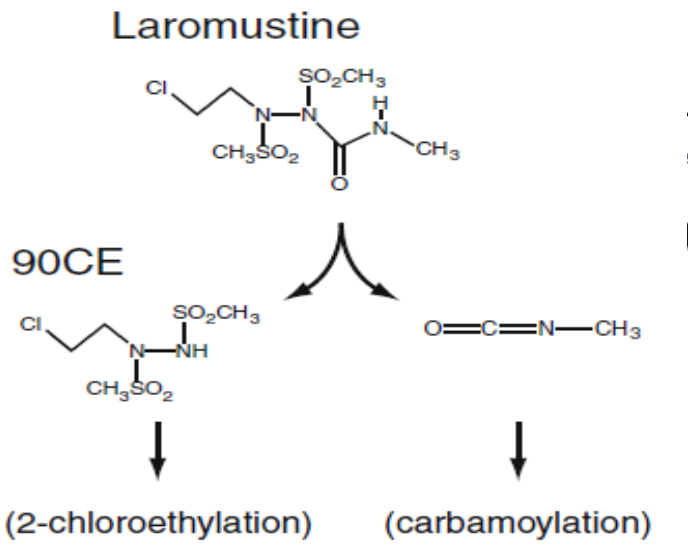


Figure 1. Base-catalyzed decomposition pathway of laromustine.[4]

Preclinical experiments in mouse models have suggested that laromustine has great therapeutic potential. One study showed that laromustine was able to cure 100% of mice at the lowest concentration (10-15 mg/kg/day for 6 days) and with the lowest toxicity (6% decrease in body weight) compared to similar sulfonylhydrazine compounds.[7] As a class of drugs, sulfonylhydrazine compounds have been shown to be effective anticancer agents.[8] Some of the sulfonylhydrazine compounds to which laromustine was compared were in clinical trials at the time the preclinical study was published, suggesting that laromustine could be even more

effective than contemporary experimental treatments. Additional studies demonstrated that laromustine could effectively cure mice of leukemia, colon carcinoma, human glioblastoma xenographs, and murine lung carcionoma.[9] The ability of laromustine to pass the blood brain barrier in mice was exceptionally impressive, as it eradicated a greater number of cranial leukemia cells than BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), one of the leading experimental central nervous system neoplasm treatments. It also had a more favorable toxicity profile than BCNU. Due to these auspicious preclinical results, laromustine was advanced to clinical trials.[10]

In the clinic, laromustine has had some success in treating AML, its primary indication. Only one third of adults with AML, the most common type of acute leukemia in adults, can be cured using current treatment.[11] AML also affects children, though the average age of diagnosis is 68 years old.[12] The current standard treatment, involving doses of daunorubicin and cytarabine, can induce complete remission in 60-80% of young and 40-60% of older newly-diagnosed patients, but many patients later suffer a relapse that is then resistant to the treatment.[11] Daunorubicin interacts with DNA–topoisomerase II and triggers apoptosis.[13] Cytabarine is a cytosine analog, inhibiting DNA synthesis.[10] Laromustine has been proposed as a potential alternative to these treatments, producing a complete response with limited extramedullary toxicity in 50% of elderly *de novo* AML patients in a phase II trial.[12] Furthermore, the effectiveness of laromustine against AML was enhanced by administering it in concert with cytarabine, and laromustine and cytarabine were effectively able to treat some patients with refractory leukemia.[10]

Clinical results for the second indication of laromustine, GBM, have been somewhat less propitious. GBM is the most common form of malignant primary brain tumor, and also one of

the most deadly.[14] It represents approximately 60% of all gliomas.[15] The current standard of treatment, radiotherapy followed by doses of temozolomide, is by no means a cure; the mean survival time is approximately 13.4 months after diagnosis.[15] Temozolomide is a DNA methylating agent that acts at several locations, the most cytotoxic of which is the O-6 position of guanine.[16] Researchers are continuing to investigate alternative treatments to prolong the lives of patients. Unfortunately, laromustine has not been very effective in the clinical studies for GBM conducted thus far. For example, laromustine was only able to produce six-month progression-free survival in 6% of adults with recurrent glioblastoma in a phase II study.[17] The median progression-free survival time was 6.3 weeks. Similar studies were also done in children with glioblastoma, with comparable results.[18] However, the clinical studies conducted were very limited and often only included patients that did not respond to initial temozolomide treatments.[17,19] Furthermore, studies to find effective partner drugs for laromustine in GBM patients have not been conducted despite clinical evidence that laromustine and temozolomide are compatible and were effective in curing some patients with refractory AML.[20]

The limited toxicity of laromustine, its compatibility with other drugs in clinical trials, and its ability to pass the blood-brain barrier suggest that it has potential for GBM treatment if researchers can optimize its use. Traditionally, the effects of laromustine on cancer cells have been studied one biological pathway or component at a time in the laboratory. However, the two active components of laromustine are both very reactive electrophiles, implying that they have the potential for broad reactivity within cells and that the mechanism of laromustine is likely very complex. As an illustration of the complexity of laromustine's action, we demonstrated that laromustine only halted reproduction in U138 human GBM cells rather than inducing acute cytotoxicity as it does in HL-60 acute promyelocytic leukemia cells (data not shown). A better

understanding of laromustine could inform clinical use of the drug, identifying the patients that will benefit most from its use and also suggesting compounds that could complement laromustine's activity.

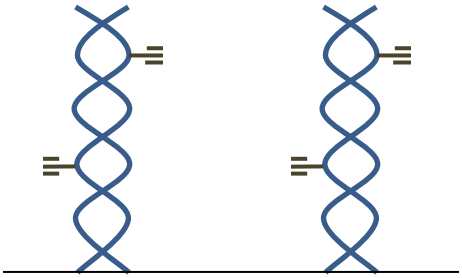
An efficient way to study multiple facets of laromustine's mechanism of action in a single experiment is to screen its anti-proliferative function against a library of well-understood small molecules. High-throughput screening has become increasingly popular in pharmacology, both to identify new therapeutic options and to further understand current treatments, as it allows researchers to study wide ranges of biochemical components and pathways simultaneously. Chemical genetic screens follow from traditional genetics experiments. However, rather than using a mutagenic agent to manipulate gene expression as in traditional genetics, chemical genetics uses small molecules to perturb biochemical pathways through interactions with proteins. In forward chemical genetics, the model system, often tissue cultures or primitive multicellular organisms, is exposed to a diverse compound library. The compounds are then evaluated on their ability to create a particular phenotype. Once the effective molecules have been identified, the molecules can be studied individually and in depth to understand how they produce the desired phenotype. Alternatively, an individual protein can be studied through reverse chemical genetics, in which the compounds are first screened for the ability to interact with a particular biomolecule. Hits are then administered in a model system to observe the resultant phenotype and decipher the function of the protein of interest.

The libraries of small molecules used in these chemical genetic screens typically include hundreds or even thousands of compounds. They are generally selected to maximize the diversity of chemical structure, providing the potential to affect varied proteins and bioprocesses. Chemical genetic screens have become even more viable as automated liquid handling systems

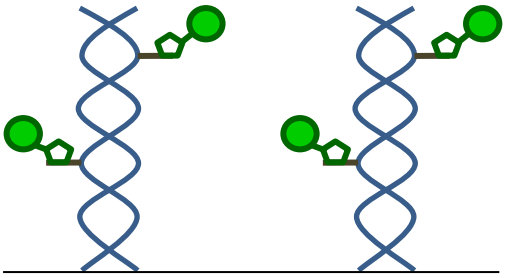
have minimized the manual labor and the imprecision inherent in working with such large collections of compounds. Chemical genetics has revolutionized the drug discovery process and has especially shown promise in the realm of orphan diseases, finally making it affordable to find treatments, if not cures, for devastating diseases that affect a relatively small percentage of the population for which there are no therapeutics currently available.[21]

Chemical genetic screens have been particularly effective when they have used libraries composed of FDA-approved compounds. The mechanisms of those compounds have already been extensively studied and are well-characterized, and thus any positive results in a screen should correspond directly to specific bioprocesses. Moreover, these screens often have extraordinary and unexpected results. In one study conducted in zebrafish, rosuvastatin, traditionally used to treat high cholesterol, was identified as an antiangiogenic compound that can suppress prostate cancer growth.[22] As a second, equally surprising example, riluzole, currently used to slow the progression of amyotrophic lateral sclerosis, was found to increase wnt/ß-catenin signaling, effectively fighting melanoma.[23]

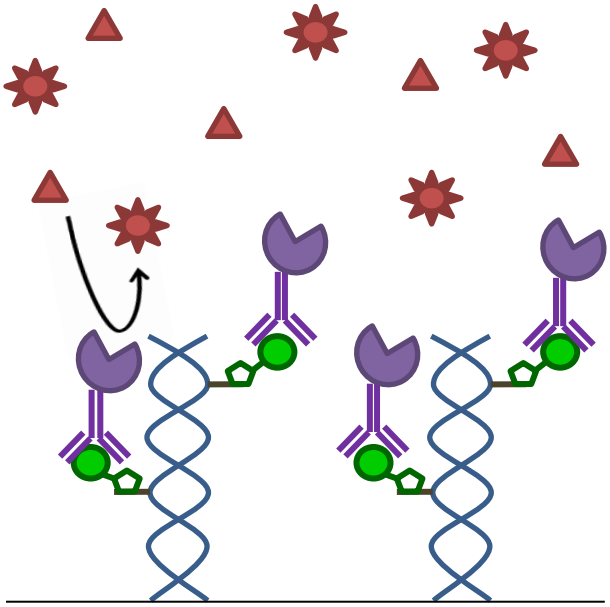
As a drug that has already shown compatibility with several other small molecules, laromustine is a suitable candidate compound to study in a chemical genetic screen. The study presented herein aims to further the current understanding of the mechanism of laromustine in GBM and to identify potential partner drugs for laromustine. To this end, a high throughput assay based on the Click-iT EdU Microplate Assay kit (Molecular Probes) was newly optimized for use in a chemical genetic screen (Figure 2). The screen will identify compounds that enhance the anti-proliferative capabilities of laromustine in U138 GBM cells. The assay development process required optimization of many parameters, including the reagent concentrations, incubation times, and cell seed density. The determined conditions produce reproducible



1. **Preparation** EdU-labeled DNA from lysed cells is fixed to the well bottoms.



2. **Click Reaction** Oregon Green 488 azide is attached to the incorporated EdU via a copper(I)-catalyzed cycloaddition reaction.



3. **Signal Amplification** Horseradish peroxidase-conjugated antibodies (purple) bind Oregon Green 488. Horseradish peroxidase catalyzes a reaction between Amplex UltraRed (red triangles) and hydrogen peroxide to create the detected fluorescent molecule (red suns).

Figure 2. Outline of the Click-iT EdU Microplate Assay used to measure cell proliferation. Cells are seeded in a multiwell plate, treated as desired, and provided with EdU (5-ethynyl-2'-deoxyuridine), which proliferating cells incorporate into new DNA. The DNA is fixed to the plate bottom (1), Oregon Green 488 is bound to the DNA (2), and the signal is amplified through a multiple step procedure (3).

results with low error and a high signal-to-noise ratio. Now that optimization has been completed, these data will soon be used to conduct the aforementioned chemical screen, potentially revealing new therapeutic strategies to treat GBM.

Materials and Methods

Cell Culture

U138 human glioblastoma cells (American Type Culture Collection, Rockville, MD) were grown in Eagle Minimum Essential Medium (Lonza, Walkersville, MD) supplemented with 0.1% gentamycin (Thomas Scientific, Swedesboro, NJ), 1% L-glutamine (Lonza), and 10% fetal bovine serum (Lonza). Cells were maintained at 45-90% confluence at 37°C and 5% CO_2. Prior to performing experiments, cells were washed twice with HEPES buffer (Lonza) and incubated with trypsin/EDTA (Lonza) at room temperature for 3 min to detach them from the flask surface. The trypsin was then neutralized with trypsin neutralizing solution (Lonza), and cells were spun at 200 *x g* for 5 min. After centrifugation, the supernatant was removed, and cells were resuspended in fresh media. Cell viability was assessed by trypan blue (Lonza) exclusion, and cells were counted using a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA).

Click-iT EdU Proliferation Assay Optimization

Spectrophotometer Signal Range

Quinine sulfate was used to determine the appropriate signal range for the spectrophotometer and to determine whether the instrument being used measures overall quantity of fluorescent molecules or their concentration. Volumes of 20, 40, 60, 80, and 100 µL of 10 µM quinine sulfate in 0.5 M H_2SO_4 were added to wells in triplicate. Fluorescence was read using a

top-reading SpectraMax M2 (Molecular Devices, Sunnyville, CA) with excitation of 320 nm and emission of 460 nm. To determine whether there was an optimal well volume for the spectrophotometer, 20 µL of 10 µM quinine sulfate in 0.5 M H_2SO_4 was added to wells and adjusted to a volume of 20, 40, 60, 80, or 100 µL with 0.5 M H_2SO_4. Fluorescence measurements were made as above. All volumes were tested in triplicate.

EdU Incubation

Prior to treatment, cells were seeded at 3,000 cells/well with 25 µL of media in a 384-well white µClear plate (Greiner Bio-One, Monroe, NC). Cells were incubated at 37°C and 5% CO_2 for 24 hr, after which time the media was aspirated and replaced with drug-treated media. The drug-treated media was prepared by diluting laromustine/DMSO solutions 1:1000 in media for final laromustine concentrations of 12.5, 25, 50, 100, and 200 µM. An equivalent volume of DMSO in media was used as the negative control. The plates were incubated for 24 hr at 37°C and 5% CO_2 prior to proliferation measurements.

The Click-iT EdU Microplate Assay (Molecular Probes, Eugene, OR) was used to measure cell proliferation after treatment, adapted to a 384-well plate by using one quarter of the manufacturer suggested well volumes for a 96-well plate. EdU (5-ethynyl-2'-deoxyuridine), a thymidine analog, was added to wells for a final concentration of 5, 10, 15, and 20 µM. Wells without EdU were used as negative controls. EdU incubation lasted 12 or 24 hr at 37°C and 5% CO_2, after which time the manufacturer's instructions were followed to develop the plate and quantify EdU incorporation. Briefly, media was removed, cells were lysed, and DNA was fixed to plate bottoms. A copper(I)-catalyzed cycloaddition reaction was performed to attach an Oregon Green 488 azide to the incorporated EdU based on the click reaction developed by Fokin

and Sharpless.[24] An anti-Oregon Green 488 antibody with horseradish peroxidase conjugate was then used to amplify the signal, using Amplex UltraRed as the substrate to generate the detected fluorescent signal. The plate was read using the SpectraMax M2 plate reader, with excitation of 544 nm and emission of 590 nm. All conditions were tested in duplicate.

Seed Density and Fluorescent Substrate Concentration

Once the optimal signal range was determined from the quinine experiments and the proper EdU quantity was identified, the cell seed density and Amplex UltraRed concentration were varied to determine the appropriate conditions for the Click-iT EdU Microplate Assay. Cells were plated at 300, 1500, 3000, and 6000 cells/well in 25 µL of media and incubated for 48 hr at 37°C and 5% CO_2. Wells with media and no cells were used as negative controls. Media was aspirated from wells and replaced with 25 µL of a 1:1000 DMSO/media solution. The plate was incubated under the same conditions for 12 hr. Then 5 µM EdU was added to each well, and the plate was incubated for 12 hr. The plate was developed as previously described except that the amount of Amplex UltraRed was varied to adjust the signal, using half, three quarters, or all of the volume suggested by the manufacturer. All conditions were tested in quadruplicate.

An additional experiment was also performed to refine the appropriate seed density range. Cells were plated at 250, 500, 750, 1000, and 1250 cells/well in 25 µL media and incubated for 48 hr at 37°C and 5% CO_2. Wells with media and no cells were used as negative controls. Media was aspirated from wells and replaced with 25 µL of a 1:1000 DMSO/media solution. The plate was incubated under the same conditions for 12 hr. Then 5 µM EdU was added to each well, and the plate was incubated for 12 hr. The plate was developed as previously

described, using the manufacturer suggested amount of Amplex UltraRed. All conditions were tested in quadruplicate or quintuplicate.

Drug Concentration

To determine the appropriate amount of laromustine to expose cells during the chemical screen, cells were plated at 500 cells/well in 25 µL of media. Wells with media and no cells were again used as negative controls. The plate was incubated for 48 hours at 37°C and 5% CO_2. Media was aspirated from wells and replaced with 0, 12.5, 25, 50, 100, or 200 µM laromustine in media, prepared as described previously, and allowed to incubate for 6 hr. EdU was then added as previously described. The manufacturer instructions were followed to measure the proliferation, again using a 12 hr EdU incubation period and using the manufacturer suggested quantity of Amplex UltraRed. All laromustine concentrations were tested in quintuplicate, while the DMSO control was performed in quadruplicate, and wells with media treated with each drug concentration but without cells were evaluated in triplicate. The experiment was then repeated to verify results, except that the DMSO control was performed in quintuplicate and conditions were tested in quadruplicate or quintuplicate.

Results and Discussion

Spectrophotometer fluorescence depends on the total quantity of fluorophore in the sample and not on its concentration

Prior to optimization, it was important to determine the range of fluorescence that is reliably detectable by the SpectraMax M2 and how fluorescence is detected. Ideally, the assay conditions used would maximize the difference between background signal and the signal from

the most proliferative cells, the DMSO control cells that are not exposed to laromustine. However, the signal from the DMSO control cannot be so high that it saturates the instrument's ability to measure fluorescence. Furthermore, it was not understood whether the fluorescent readout from the spectrophotometer was indicative of the concentration of fluorophore or the number of fluorescent molecules in the sample. Different volumes of 10 µM quinine sulfate in 0.5 M sulfuric acid were used to generate a standard curve to determine the instrument's upper limit for measuring fluorescence. Quinine sulfate is a recognized standard fluorophore that has long been used to calibrate spectrophotometers and other fluorescence instruments due to its consistent, strong fluorescence.[25] Volumes of quinine sulfate ranging from 20 to 80 µL maintained a linear relationship with the fluorescence readings (Figure 3). However, 100 µL of quinine sulfate did not follow this trend, but rather showed that the signal was beginning to

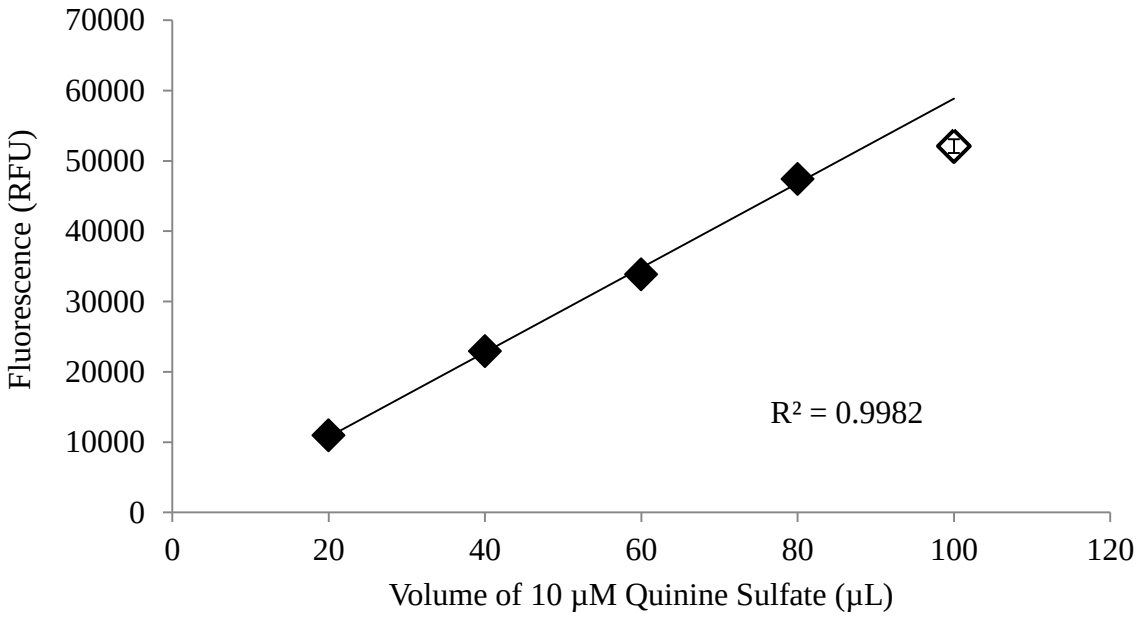


Figure 3. Fluorescence of quinine sulfate in relative fluorescent units as a function of volume of 10 µM quinine sulfate in 0.5 M sulfuric acid in wells of a 384-well plate. Data are reported in technical triplicate ± standard deviation. The trend line does not include the final data point.

saturate the instrument between $5x10^4$ and $6x10^4$ relative fluorescent units (RFU). Therefore, to obtain reliable data with the largest difference between background and maximum signal, the fluorescence readings from wells of U138 cells treated with 1:1000 DMSO in media, the negative control, should be near or below $5x10^4$ RFU.

Additionally, this experiment demonstrated that the number of fluorescent molecules, and not the concentration of the fluorophore, was the important factor in determining the fluorescent readout. All of the wells had the same concentration of quinine sulfate but provided different fluorescence. To verify this conclusion, the fluorescence of samples with the same quantity of fluorophore but concentrations ranging from 1 µM to 10 µM quinine sulfate in 0.5 M H_2SO_4 was determined. The different concentrations were tested in triplicate. Although the concentrations varied, there was no significant difference in fluorescent signal (data not shown). Another important result from these experiments is that the instrument was able to reproducibly measure the fluorescence of small volumes of quinine solutions, despite the narrowness of the wells in the 384-well plate. This was a concern for conducting the Click-iT EdU assay in a 384-well plate, as the ending volume for the assay in a 384-well plate is only 27.5 µL of the 100 µL well capacity and the kit was designed for use in 96-well plates, which have much wider wells. Based on these results, however, the 27.5 µL should be ample volume for the spectrophotometer to reliably detect the assay fluorescence.

Small EdU concentration and short incubation period provides best signal range

Several rounds of experimentation needed to be conducted to optimize the Click-iT EdU Microplate Assay for the current screen. The manufacturer recommends first adjusting the amount of EdU and the incubation time with EdU. Cells were exposed to 0 to 200 µM laromustine for 24 hours. They were then provided amounts of EdU ranging from half to double

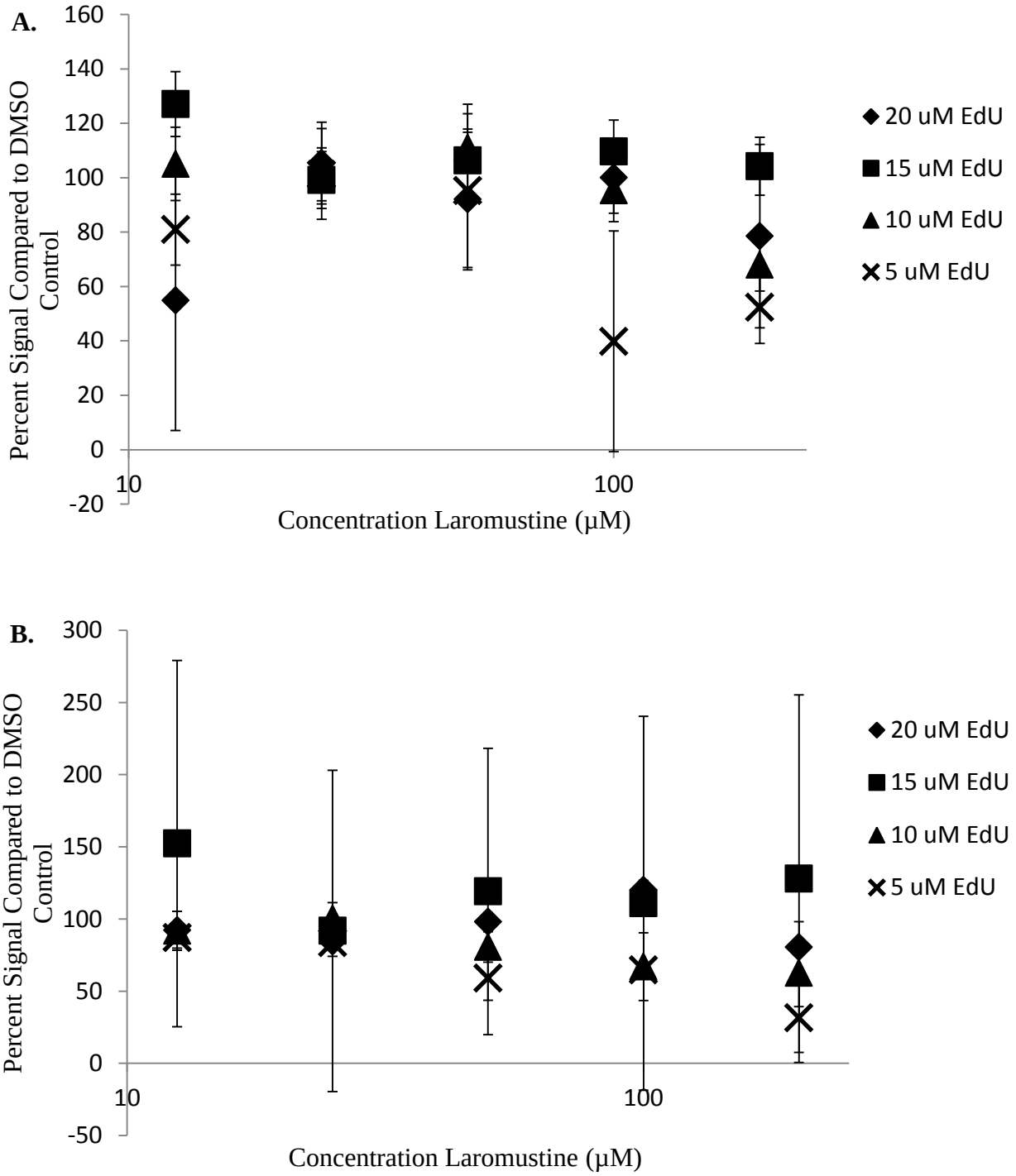


Figure 4. Proliferation of cells seeded at 3000 cells/well exposed to varying amounts of laromustine, measured 24 hr (A) or 12 hr (B) after addition of different EdU concentrations. Data are reported as the mean of duplicate experiments expressed as a percent of the proliferation of control cells treated with 1:1000 DMSO/media ± standard error.

the recommended 10 µM for 12 or 24 hrs. Many of the tested conditions showed no significant difference in proliferation between cells treated with 200 µM laromustine and the negative control cells, suggesting that the amount of EdU or the incubation time was too long to register a difference between inhibited and uninhibited proliferation (Figure 4).

With both the 12 and 24 hr incubation, however, the 5 µM EdU condition showed an adequate separation in measured proliferation between cells exposed to 0 and 200 µM laromustine (Figure 5). Because the results from the 12 and 24 hr conditions were not significantly different, a 12 hr incubation was chosen for all further experiments to make the assay maximally efficient.

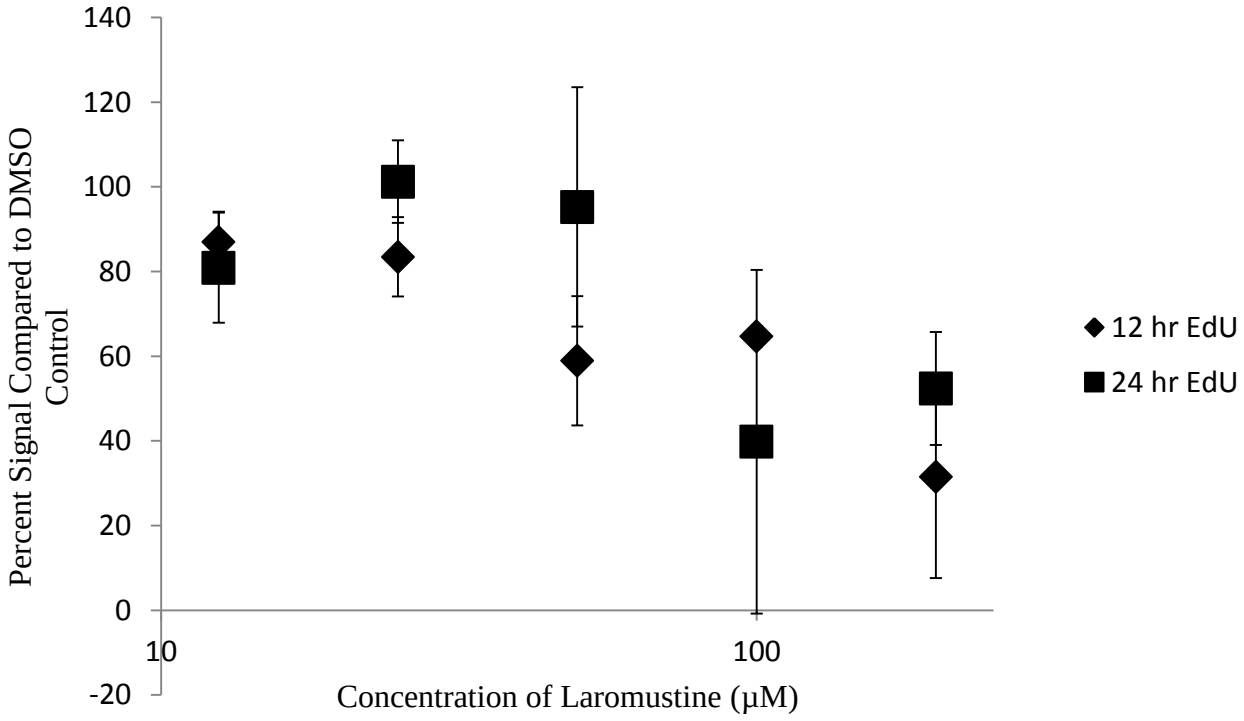


Figure 5. Proliferation of cells seeded at 3000 cells/well exposed to varying amounts of laromustine, measured 12 hr or 24 hr after addition of 5 µM EdU. Data are reported as the mean of duplicate experiments expressed as a percent of the proliferation of control cells treated with 1:1000 DMSO/media ± standard error. Data selected from those shown in Figure 4.

Recommended Amplex UltraRed quantity with low seed density gives appropriate fluorescence

The next round of optimization focused on adjusting the conditions to achieve a fluorescence signal of about $5x10^4$ RFU from the control condition, in which cells were exposed to 1:1000 DMSO in media rather than laromustine. Three main methods of adjusting the fluorescence produced in the Click-iT EdU Microplate assay are to change the amount of EdU provided to the cells, adjust the cell seed density, and modify the amount of the detected substrate that is added. The amount of anti-Oregon Green antibody with horseradish peroxidase conjugate could also be adjusted. However, it is important for the limiting factor in measuring the fluorescence to be the proliferation and the number of incorporated EdU molecules. Reducing the antibody concentration could lead to a situation in which there are incorporated EdU molecules that are not detected, and it would be difficult to distinguish between high and low levels of proliferation. Of the three other options, the EdU quantity was ruled out as it was already reduced to a level lower than the manufacturer recommended quantity. Thus, the seed density and the amount of detected Amplex UltraRed were manipulated. The seed density ranged from 300 to 6000 cells/well. Visually, a density of 300 cells/well appears sparse under the microscope, while 6000 cells/well approaches 100% confluence. All cells were provided with the 1:1000 DMSO in media control treatment. During development, the full amount, three-quarters, or half of the recommended quantity of Amplex UltraRed was pipetted to each well. All treatments were evaluated in quadruplicate.

Ultimately, it was determined that the recommended amount of Amplex UltraRed with lower seed densities was preferable to achieve the correct fluorescent signal (Figure 6). All three Amplex UltraRed data sets yielded curves with the same contour. They were also fairly evenly spaced, with about $1.5x10^4$ RFU between the 1x and 0.75x data points and between the 0.75x

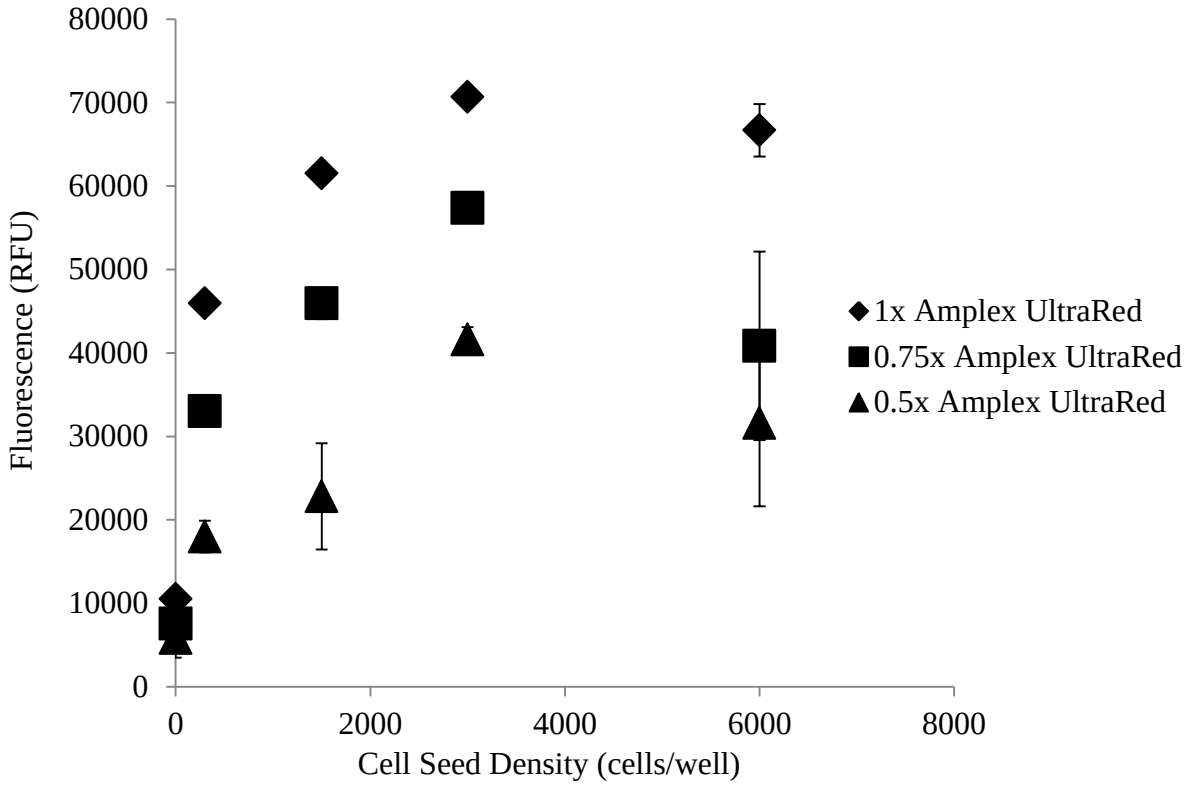


Figure 6. Proliferation of cells seeded in a 384-well plate at various densities measured 12 hr after addition of the EdU using different quantities of Amplex UltraRed, expressed relative to the manufacturer recommended amount. Data are reported in relative fluorescence units as the mean of quadruplicate experiments ± standard error.

and 0.5x data points. This was even true when the 1x Amplex UltraRed data points far exceeded the previously determined optimal signal level of $5x10^4$ RFU. These findings suggest that the Amplex UltraRed is simply a signal amplifier, with a linear relationship between Amplex UltraRed available and the fluorescence signal achieved.

The relationship between cell density and fluorescence was not linear. Instead, the curve plateaued after 1500 cells/well. Because all three Amplex UltraRed data plots exhibit the same curvature, the plateau is likely an indication of decreasing rates of proliferation rather than fluorescent signal saturation. Highly dense cell populations often proliferate at a slower rate

because resources such as nutrients and surface space on which to adhere become scarce. The large amount of standard error in the highest seed density data demonstrates that proliferation cannot be precisely determined at such a high density, possibly because some of the cells begin to die. It is also important to note that the results from the first round of experimentation, in which the appropriate EdU concentration was determined, were obtained using the second highest seed density, which is within the proliferation plateau. This may explain why such little difference was seen between the measured proliferation of the negative control cells and the cells exposed to laromustine.

To more precisely determine the seed density at which the proliferation begins to plateau, the experiment was repeated using the manufacturer recommended quantity of Amplex UltraRed and 500 to 1500 cells/well (Figure 7). The 1x Amplex UltraRed condition was chosen because it

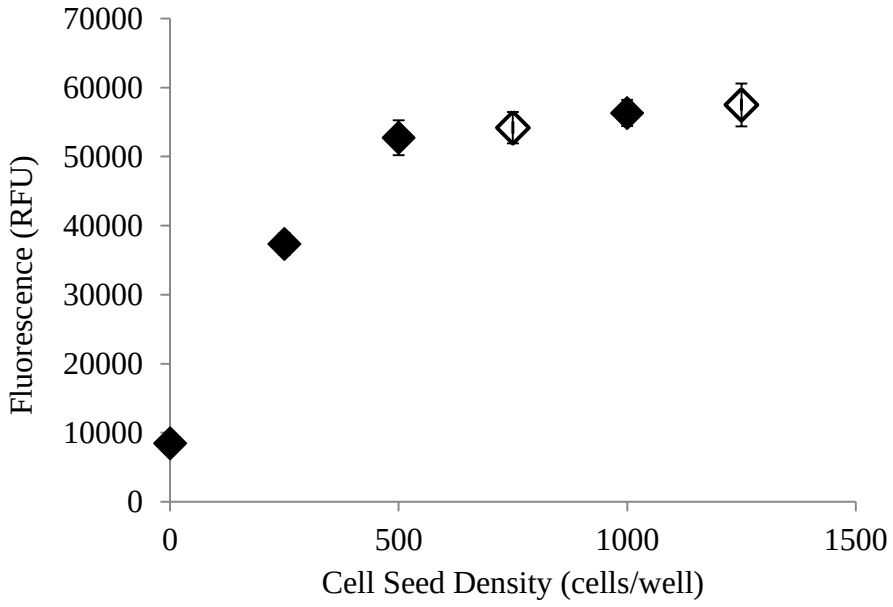


Figure 7. Proliferation of cells seeded in a 384-well plate at various low densities measured 12 hr after the addition of EdU using the manufacturer recommended quantity of Amplex UltraRed. Data are reported in relative fluorescence units as the mean of quadruplicate experiments (white points) or quintuplicate experiments (black points) ± standard error.

approached $5x10^4$ RFU even at low seed densities, allowing for the fluorescent signal to be maximized. Conditions were tested in quadruplicate or quintuplicate. The fluorescent signal plateaued even at these low seed densities. In fact, the plateau began at a seed density of 500 cells/well, suggesting that that is the greatest seed density that can be used to have the maximal fluorescence and maximal proliferation. Therefore, 500 cells/well will be used as the seed density for all future assays.

50 µM laromustine does not significantly inhibit U138 proliferation

For the screen to have the highest throughput, only one concentration of laromustine should be used. Low concentrations of laromustine will not significantly inhibit proliferation of U138 cells. However, at a certain critical concentration, laromustine begins to affect cell proliferation potently. The ideal laromustine concentration for the screen is a concentration that is slightly lower than the critical inhibitory concentration. In such a situation, the cells exposed to laromustine will show little to no inhibition, but the cells exposed to laromustine along with a library compound that sensitizes cells to laromustine will proliferate at a markedly lesser rate. To determine what this ideal concentration is in U138 cells, the Click-iT EdU assay was repeated using the previously determined conditions and laromustine concentrations ranging from 0 to 200 µM (Figure 8A). The proliferation of cells exposed to up to 50 µM laromustine was comparable to the proliferation of cells not exposed to laromustine. However, the proliferation of cells exposed to 100 µM or more was strongly inhibited, with a fluorescent signal of less than 35% of the signal from the DMSO control cells. These data suggest that 50 µM laromustine would be a suitable concentration for the chemical genetic screen.

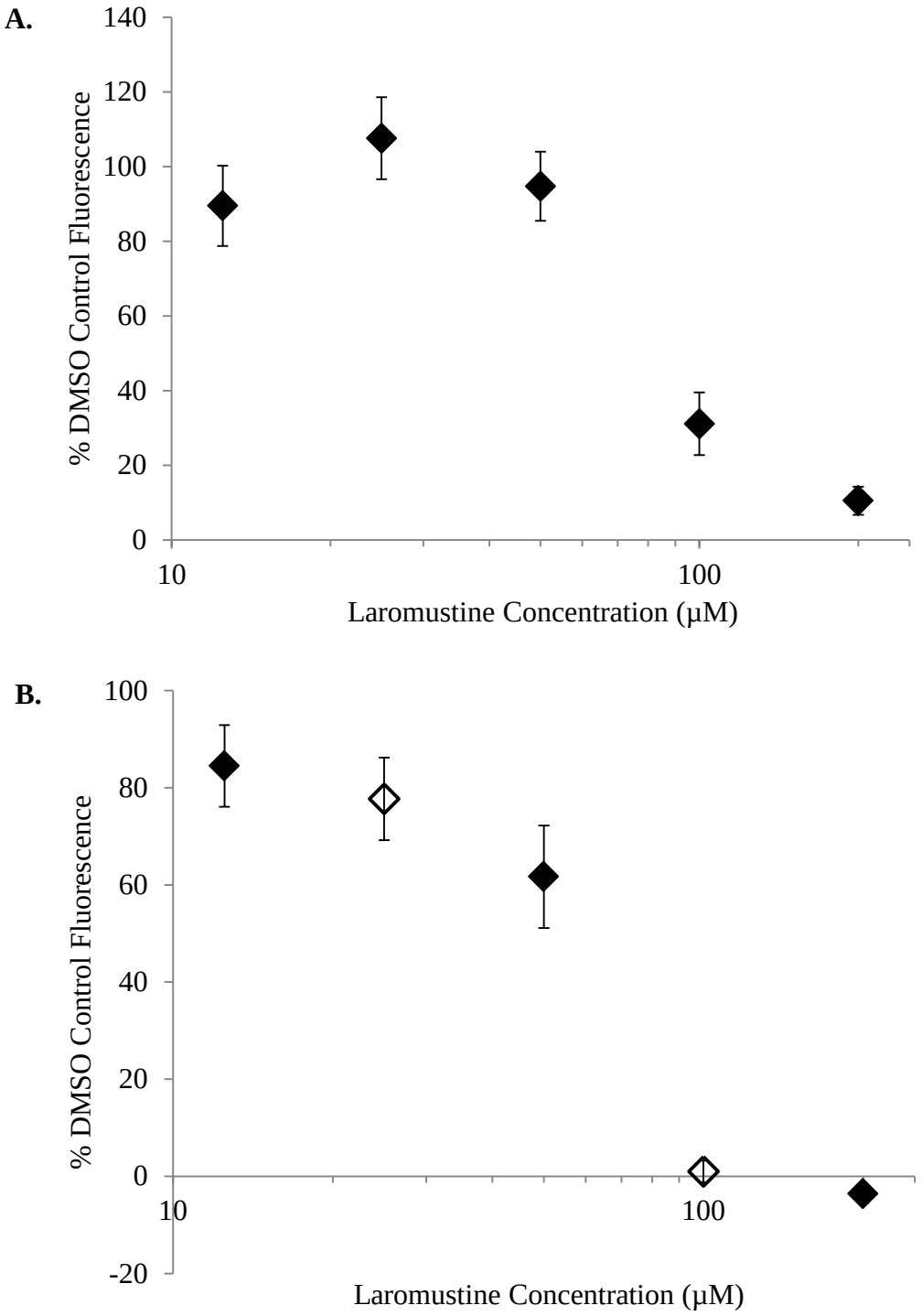


Figure 8. Proliferation of cells seeded at 500 cells/well exposed to varying amounts of laromustine, measured 12 hr after addition of 5 µM EdU. Data are reported as the mean of quadruplicate experiments (white points) or quintuplicate experiments (black points) and expressed as a percent of the proliferation of control cells treated with 1:1000 DMSO/media ± standard error. The DMSO control was performed in quadruplicate (A) or quintuplicate (B).

This experiment was then repeated to ensure that the results were reproducible. The results during this second experiment were slightly different, with all points except the 12.5 µM point appearing lower than in the first experiment (Figure 8B). Here 50 µM of laromustine seems to be enough to inhibit proliferation modestly. Nonetheless, there is still an appreciable drop in fluorescent signal between 50 and 100 µM laromustine, resulting in the same change in proliferation between the 50 and 100 µM cells in the second experiment as in the first experiment. As such, even though the numbers are not quite consistent, both trials suggest that 50 µM is the appropriate laromustine concentration for the chemical screen.

Future Work

With the Click-iT EdU assay fully optimized, a chemical genetic screen can now be performed to identify compounds that sensitize U138 GBM cells to laromustine. The screen will be conducted through collaboration with Dr. Robert Wheeler at the University of Maine in Orono using the National Clinical Collection compound library, which contains approximately 450 FDA-approved drugs. Positive hits during the screen will be defined as those that significantly inhibit the proliferation of U138 cells in concert with laromustine relative to control experiments. The positive hits will then be studied more extensively.

Compounds found to enhance the anti-proliferative effects of laromustine must be validated for their therapeutic relevance as partner drugs for laromustine. The cytotoxicity of the positive hit compounds in combination with laromustine will be evaluated in noncancerous cell lines. To be considered for therapeutic application, it is important that the admixtures of drugs demonstrate some preference for cytostatic activity in neoplastic cells relative to healthy cells. It

would also be of interest to determine whether AML cells are sensitive to the identified drug combinations, as laromustine has been used to treat AML as well.

Independent of the possibility of identifying new therapeutic strategies, the known pharmacology of any molecules that emerge in the screen will provide valuable information about the mechanism of action of laromustine. As approved drugs, the library compounds have been extensively studied and their mechanisms are largely known. Thus, the positive hits in the screen will suggest biochemical pathways that laromustine may be manipulating. Comprehensive literature research and considerable biochemical analysis will then be necessary to precisely determine how the small molecules are complementing each other within the cell.

Ultimately, this chemical genetic screen has the potential to reveal novel therapeutic strategies for a devastating human cancer. The results could suggest new combination therapies for GBM or identify heretofore unrecognized subpopulations of GBM patients in which laromustine would be particularly effective. The information will move us closer to developing a successful treatment program for GBM patients, curing them of this calamitous disease or at least prolonging their lives.

References

1. Hoyert, D. L.; Xu, J. *National Vital Statistics Reports*. **2012**, *61*, 1-52.
2. American Cancer Society. www.cancer.org (accessed Dec. 8, 2013).
3. Ishiguro, K.; Zhu, Y. L.; Shyam, K.; Penketh, P. G.; Baumann, R. P.; Sartorelli, A. C. *Biochem. Pharmacol.* **2010**, *80*, 1317-1325.
4. Penketh, P. G.; Shyam, K.; and Sartorelli, A. C. *Biochem. Pharmacol.* **2000**, *59*, 283-291.
5. Gerson, S. L. *J. Clin. Oncol.* **2002**, *20*, 2388-2399.
6. Pegg, A. E.; Byers, T. L. *FASEB J.* **1992**, *6*, 2301-2310.
7. Shyam, K.; Penketh, P. G.; Loomis, R. H.; Rose, W. C.; Sartorelli, A. C. *J. Med. Chem.* **1996,** *39*, 796-801.
8. De Vita, V. T.; Carbone, P. P.; Owens, A. H.; Gold, G. L.; Krant, M. J.; Edmonson, J. *Cancer Res.* **1965**, *25*, 1876-1881.

9. Finch, R. A.; Shyam, K.; Penketh, P. G.; Satorelli, A. C. *Cancer Res.* **2001**, *61*, 3033–3038.
10. Giles, F.; Verstovsek, S.; Thomas, D.; Gerson, S.; Cortes, J.; Faderl, S.; Ferrajoli, A.; Ravandi, F.; Kornblau, S.; Garcia-Manero, G.; Jabbour, E.; O'Brien, S.; Karsten, V.; Cahill, A.; Yee, K.; Albitar, M.; Sznol, M.; Kantarjian, H. *Clin. Cancer Res.* **2005,** *11*, 7817-7824.
11. Zhu, X.; Ma, Y.; Liu, D. *J. Hematol. Oncol.* **2010**, *115*, 453-474.
12. Giles, F.; Rizzieri, D.; Karp, J.; Vey, N.; Ravandi, F.; Faderl, S.; Khan, K. D.; Verhoef, G.; Wijermans, P.; Advani, A.; Roboz, G.; Kantarjian, H.; Bilgrami, S. F. A.; Ferrant, A.; Daenen, S. M. G. J.; Karsten, V.; Cahill, A.; Albitar, M.; Mufti, G.; O'Brien, S. *J. Clin. Oncol.* **2006**, *25*, 25-31.
13. Gewirtz, D. A. *Biochem. Pharmacol.* **1999**, *57*, 727-741.
14. Zhang, X.; Zhang, W.; Cao, W. D.; Cheng, G.; Zhang, Y. Q. *Exp. Ther. Med.* **2011**, *3*, 9-14.
15. Rock, K.; McArdle, O; Forde, P.; Dunne, M.; Fitzpatrick, D.; O'Neill, B.; Faul, C. *Br. J. Radiol.* **2012**, *85*, 729–733.
16. Chakravarti, A.; Erkkinen, M. G.; Nestler, U.; Stupp, R.; Mehta, M.; Aldape, K.; Gilbert, M. R.; Black, P. M.; Loeffler, J. S. *Clin. Cancer Res.* **2006**, *12*, 3738-4746.
17. Badruddoja, M. A.; Penne, K.; Desjardins, A.; Reardon, D. A.; Rich, J. N.; Quinn, J. A.; Sathornsumetee, S.; Friedman, A. H.; Bigner, D. D.; Herndon; J. E.; Cahill, A.; Friedman, H. S.; Vredenburgh, J. J. *J. Neuro-Oncol.* **2007**, *13*, 428-436.
18. Bouffet, E.; Tabori, U.; Huang, A.; Bartels, U. *Cancer Treatment Reviews*. **2010**, *36*, 335-341.
19. Gururangan, S.; Turner, C. D.; Steward, C. F.; O'Shaughnessy, M.; Kocak, M.; Poussaint, T. Y.; Phillips, P. C.; Goldman, S.; Packer, R.; Pollack, I. F.; Blaney, S. M.; Karsten, V.; Gerson, S. L.; Boyett, J. M.; Friedman, H. S.; Kun, L. E. *Clin. Cancer. Res.* **2008**,*14*, 1124-1130.
20. Rizzieri, D.; LoRusso, S.; Tse, W.; Khan, K.; Advani, A.; Moore, J.; Karsten, V.; Cahill, A.; Gerson, S. L. Clin. *Lymphoma, Myeloma Leuk.* **2010**, *10*, 211-216.
21. Lunn, M. R.; Stockwell, B. R. *Chem. Biol*. **2005**, *12*, 1063-1073.
22. Wanga, C.; Tao, W.; Wanga, Y.; Bikow, J.; Lu, B.; Keating, A.; Verma, S.; Parker, T. G.; Han, R.; Wen, X. Y. *Eur. Urol.* **2010**, *58*, 418-426.
23. Biechele, T. L.; Camp, N. D.; Fass, D. M.; Kulikauskas, R. M.; Robin, N. C.; White, B. D.; Taraska, C. M.; Moor, E. C.; Muster, J.; Karmacharya, R.; Haggarty, S. J.; Chien, A. J.; Moon, R. T. *Chem. Biol.* **2010**, *17*, 1177-1182.
24. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem.* **2002**, *114*, 2708-2711.
25. Fletcher, A. N. *Photochem. Photobiol*. **1969**, *9*, 439-444.