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Development of a Chemical Genetic Screen to Identify Compounds that Enhance Laromustine Cytotoxicity

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Development of a Chemical Genetic Screen to Identify Compounds that Amplify Laromustine Cytotoxicity

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Vitae

Xiaoou (Alice) Wang was born on February 21, 1997 and grew up in Beijing, China. She graduated from Beijing National Day School in 2015. She matriculated at Colby College in Waterville, ME. While at Colby, Alice was a double major in chemistry-biochemistry and mathematics. She joined the research lab of Professor Kevin Rice in the summer after freshman year as a research assistant and continued her research with Prof. Rice until graduation. Additionally, she volunteered in the Day Surgery and Emergency Department in Inland Hospital for 3 years. She was also a teaching assistant for the Honors Calculus, Probability, and Statistical Inference classes. Alice also helped in the Chemistry Help Center. During the summer of 2017, Alice worked as a research assistant for Dr. Greg Feero in Maine-Dartmouth Family Medicine Residency program. During the summer of 2018, she was a summer research scholar at Harvard Medical School in the laboratory of Professor Don Coen. In the fall of 2019, she will join the Biomedical Informatics master's program at Harvard Medical School.

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Abstract

Laromustine is a chemotherapeutic sulfonylhydrazine prodrug used in clinical trials against acute myeloid leukemia. Though laromustine showed some success in clinical trials, more experiments are needed to understand the hematological toxicity and the molecular mechanisms of patients' resistance. This project aims to develop a strategy to identify compounds synergistic with laromustine in cultured leukemia cells from a library of 450 FDA-approved compounds through a forward chemical genetic screen. To optimize the screen, the cell seeding density, doubling time, and dose response curves were determined. The optimized concentration of HL60 cells in these experiments was determined to be between 25,000 and 40,000 cells/well in 384 well-plates for 12 or 24 hr before the measurement. The LD_{50} of laromustine was determined to be 159 μM. A concentration of 50 μM was identified as optimal for the chemical genetic screen because when tested with temozolomide, a compound with a similar mechanism of action to laromustine, 50 μM laromustine and 500 μM temozolomide showed synergistic effects. Following those optimized conditions, the chemical genetic screen should have the potential to find compounds that enhance laromustine's cytotoxicity, which, in the future, would help identify new molecular targets for laromustine's mechanism of action.

Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia, which is a type of fast-growing cancer of the blood and bone marrow.[1] According to NCI's PDQ cancer information summary, patients with this type of cancer have abnormal myeloblasts, red blood cells, or platelets. Those myeloid blasts cannot become healthy white cells, and when abnormal cells build up in the bone marrow, less room remains for the healthy cells.[2] AML is a disease that primarily affects elderly patients with a median age of presentation at around 68 years old.[3] The standard of care treatment for AML typically involves chemotherapy, radiation therapy, or stem cell transplant. Among these, chemotherapy is the most common method to treat AML patients.

Laromustine (cloretazine; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino) carbonyl]hydrazine) is a chemotherapeutic sulfonylhydrazine prodrug used in clinical trials against AML. Laromustine is a prodrug because the compound is not pharmacologically active until it decomposes into its active forms. Laromustine generates two reactive electrophiles, methyl isocyanate and 90CE, by base catalysis, as shown in Figure 1.[4]

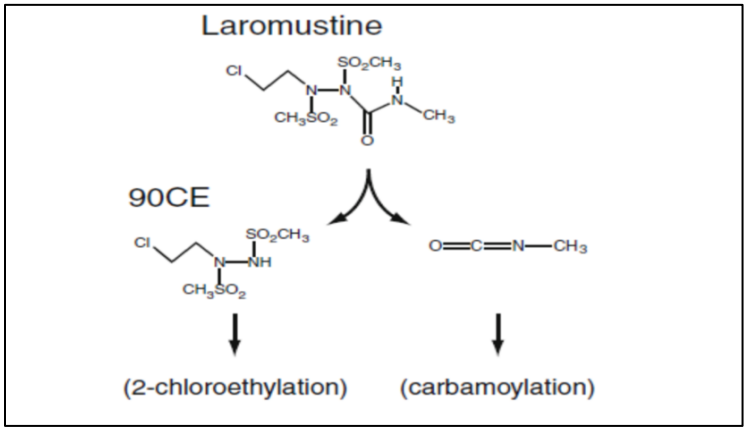


Figure 1: Decomposition of laromustine to 90CE and methyl isocyanate.[4]

Both species have critical functions, but the *in situ* mechanisms and features of 90CE have been studied the most. When laromustine yields its two active subspecies, 90CE is responsible for 2-chloroethylation activity on DNA at the O^6 position of guanine (Figure 2).[5] The subsequent formation of an inter-strand crosslink with cytosine is believed to be the reason that laromustine can stop the DNA replication and thus the proliferation of AML and glioblastoma multiforme (GBM) cancer cells[4,6].

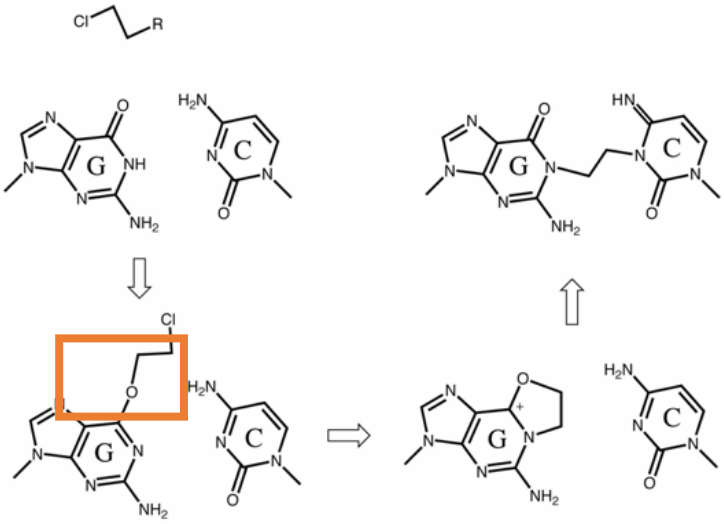


Figure 2. The 90CE 2-chloroethylation and the formation of the interstrand crosslink. (The orange box highlights the 2-chloroethylation activity at the O^6 position of guanine.)

A particular Phase I study demonstrated that laromustine had significant antileukemic activity towards refractory leukemia patients. In this study, laromustine was combined with cytarabine (1-beta-d-arabinofuranosylcytosine, ara-C). However, some patients did not respond to this compound. The activity of O^6-alkylguanine-DNA alkyltransferase (AGT) might be a predictor of responsiveness to laromustine.[7] A study by Finch *et al.* also discusses this possibility. In their study, they found that cells with high AGT

expression prevented the formation of the cross-links by transferring the alkyl adduct from guanine to a cysteine moiety in the protein.[8] In this situation, AGT is serving as a DNA repair protein, and its existence in tumor tissues affects the effectiveness of the chemotherapeutic drugs.

According to another phase II clinical study, laromustine has significant success in treating elderly patients with poor-risk AML.[9] Though some progress was observed by an increase in the survival for those patients who were able to respond to this treatment, hematological side effects, such as myelosuppression, were detected in patients.[9] The study suggested further studies using a combination of laromustine with standard treatment regimens. It remains challenging for scientists to decide whether laromustine should be part of the treatment of AML.

A very recent study by Penketh *et al.* compared the combined treatment with chloroethylating and carbamoylating prodrugs with laromustine.[10] This study demonstrates an excellent tumor selectivity of laromustine. Another recent analysis by Nassar *et al.* conducted a pharmacokinetic analysis of laromustine. This study estimates that the mean volume of distribution at steady state exceeds the amount of the total body water, which means that laromustine has distributed to the peripheral tissues. Also, laromustine has a short half-life, less than an hour, which reveals a rapid clearance.[11] Following to the insights brought by those two studies, further experiments are in need to improve the performance of laromustine. The phase II study previously mentioned specifically suggested to combine laromustine with other drugs.[12]

Based on the suggestions from the clinical trials, this project attempts to identify compounds synergistic with laromustine in cultured leukemia cells. This study is first going

to access the combined effects of laromustine with temozolomide (TMZ). TMZ is another compound used clinically against GBM, which is one of the most aggressive brain cancers, and TMZ targets the same position on DNA as laromustine. It functions through O^6 guanine methylation, which differs from the chloroethylation and crosslinking activity of laromustine.[13] Methylation of O^6 guanine triggers mismatch-repair mechanisms and can lead to cytotoxicity.[14] Moreover, TMZ can deplete AGT in tumor tissue and peripheral blood mononuclear cells, which is thought to occur via irreversible methyl transfer to AGT from TMZ-methylated guanine O^6.[15–17] Therefore, this study hopes to observe that TMZ increases laromustine's cytotoxicity against cultured cancer cells by depleting AGT.

Besides temozolomide, this project is also going to test the combined effect between laromustine and Olaparib. Olaparib is a poly-(ADP-ribose) polymerase (PARP) inhibitor, which was approved by the FDA in the treatment of BRAC1 or BRAC2 mutated ovarian cancer and HER2-negative metastatic breast cancer. PARP is an enzyme involved in DNA repair processes, particularly base excision repair.[18] Due to Olaparib's ability to inhibit DNA damage repair, research has demonstrated that the combination of chemotherapy and PARP inhibition may benefit PARP1 SNP rs1805407 (single nucleotide polymorphism) carriers (especially in ovarian cancers).[19] Consider the results shown by the combination of chemotherapy and PARP inhibition, this project is also going to test the combination of laromustine and Olaparib, which should increase laromustine's cytotoxicity.

Finally, this experiment sought to identify new molecular targets for laromustine's mechanism of action by testing compounds from a library of 450 US Food and Drug Administration (FDA) approved drugs using a forward chemical genetic screen. A chemical genetic screen would be able to conduct in the future when all the screening conditions are

optimized, and Figure 3 shows its basic schematic. Different from a genetic screen or mutagenesis, a chemical genetic screen uses small molecules to perturb biological processes, testing hundreds of compounds simultaneously in a cell-based assay.[20] A reverse chemical genetic screen is usually a target-based approach, but a forward chemical genetic screen is a phenotype-based approach.[20] Forward chemical genetic screens have been successfully applied in several fields. For instance, Child *et al.* performed a chemical genetic screen against toxoplasma Gondii and identified compounds that significantly enhance infectivity, which helped them understand the mechanisms under the induction of invasion.[21]

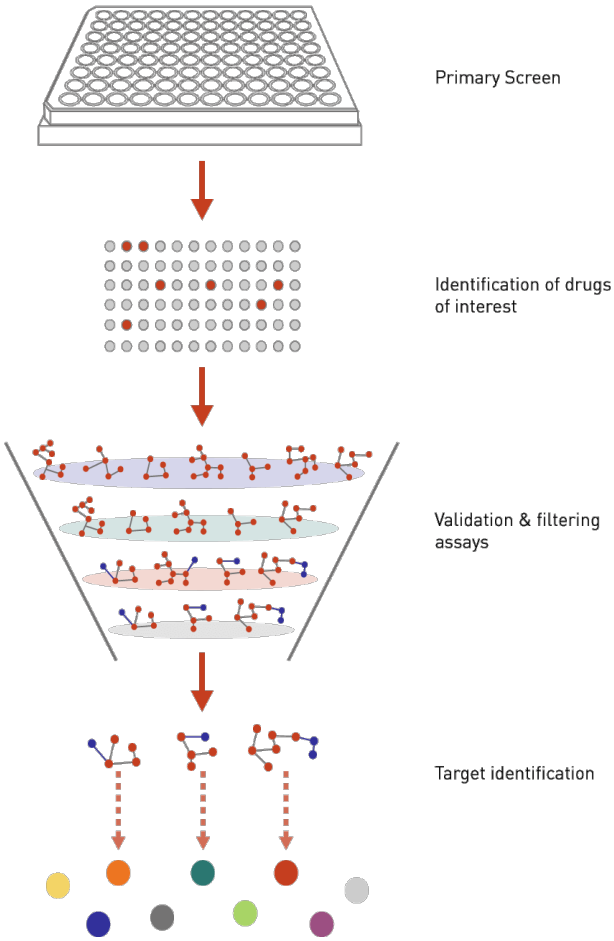


Figure 3. From a chemical genetic screen to drug discovery. A cell-based assay is performed to figure out the compounds that can induce a particular cellular phenotype (cell death for this project). Once the hits from the primary screen are identified and validated, the cellular targets of those hits could be further analyzed by various genetic assay.[22]

In this experiment, the "phenotype" would be cell death, which means the combination of a particular compound and laromustine induces more cell death as compared to their effects individually. As shown in Figure 3, a forward chemical genetic screen usually involves three stages. First, a primary screen identifies molecules of interest. In this case, this screen should find compounds that can amplify laromustine's cytotoxicity in this cell-viability assay. Then, cellular targets that interact with the particular chemicals, which in most cases are proteins, are identified.[20] A common method to confirm those targets would be an affinity pull-down assay using the protein that interacts with the isolated compounds. This step could potentially suggest new molecular targets for laromustine's mechanism of action. Because the FDA-approved compounds have well-studied molecular pathways, they are going to quickly bring insights into the new mechanism of laromustine's action. The final step consists of validation of the molecular targets, and characterization of their specificity as small molecules usually show multiple but unrelated effects due to low specificity.[20] According to the suggestions, to test the specificity of the target, a genetic test using a modified cell line for the identified cellular target and a competition assay should be applied.[20]

Material and Methods

HL60 Cell Culture:

HL60 human acute myeloid leukemia cells were maintained between 1 x 10^5 and 8 x 10^5 cells/mL in Roswell Park Memorial Institute (RPMI) 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. All incubations described herein are in these conditions unless specified otherwise.

Determination of HL60 Optimum Seeding Concentration:

The optimum cell seeding concentration was determined by seeding cultured HL60 cells in triplicate at concentrations ranging from 156 cells/well to 40,000 cells/well (from 6 cells/μL to 1,600 cells/μL) in a 384-well plate (25μL/well). The negative controls lacked cells with an equal volume of RPMI media. After 30 min, 25 μL of CellTiter-Glo reagent (CellTiter-Glo Luminescent Cell Viability Assay, Promega, WI) was added to each well. The luminescence signal was detected using a SpectraMax M5 plate reader (Molecular Devices) after 10 min. Data were analyzed as the average luminescence by triplicates, and errors were measured by the standard deviation.

Determination of HL60 Doubling Time and Maximum Cells per Well

The incubation time that HL60 cells needed to double themselves in a 384-well plate was tested by seeding cultured HL60 cells in different concentrations. Initial cell concentrations ranged from 2,000 – 10,000 cells/well and were compared to the negative control wells without cells. 25 μL of CellTiter-Glo luminescent reagent was added to each

well at specified time points ending at 120 hr, then measured using the SpectraMax M5 plate reader. Data were analyzed as the average luminescence, and errors were measured as standard deviations.

Generation of Laromustine and Temozolomide Dose Response Curves

2,500 HL60 cells were seeded in 25 µL of RPMI at 25,000 cells per well in wells of a 384-well plate. Two-fold serial dilutions of laromustine and temozolomide were made in DMSO with 4% DMSO and media as the controls. At 24 hr, 25 µL of the CellTiter-Glo reagent was added to each well, and the relative luminescence signals were measured using the plate reader. The best fit curve fit and LD-50 were generated by the Excel Solver tool, fit curve: $\frac{1}{1+(\frac{[compound]}{LD_{50}})^{a}}$.

Determination of Incubation Period of Temozolomide with Lethal Dose Treatment

HL60 cells were seeded in at 2,500 cells/well to determine the necessary exposure time for temozolomide (TMZ) to result in cell death. Cells were treated with 2 mM of TMZ and cellular viability was measured at indicated time points up to 24 hr using the CellTiter-Glo assay as previously described. Positive control wells lacked TMZ but contained an equivalent concentration of DMSO (4% w/v). Negative control wells contained only RPMI.

Verification of the Assay Optimization using Temozolomide

The conditions prepared for the chemical genetic screen were verified by testing temozolomide for its synergism with laromustine. HL60 cells were seeded in the format of a 24-well plate with 1 x 10^6 cells/mL, 1 mL/well, and the cells were treated with various

combinations of laromustine and temozolomide. The control groups were treated with 2% DMSO, laromustine alone, and temozolomide alone. After 12 or 24 hr, the cell mixtures in each well were mixed by pipetting, then 25 μL of the mixture was transferred to wells of a 384-well plate. After 25 μL of the CellTiter-Glo reagent was added to each well, the luminescence was measured using the plate reader. Data were analyzed for the average luminescence and errors were measured by the standard deviation.

Results

Optimal conditions for the high-throughput CellTiter-Glo assay are 40,000 cells/well incubated for 24 hours prior to drug treatment

In order to give sufficient opportunity for HL60 cells to grow before drug treatment, the optimal cell seeding concentration was determined. This optimal concentration should not impede the growth of cells, and also this density should generate a stable luminescence signal to measure. The best concentration is one that could generate a large light signal that accurately reflects the different numbers of cells in each well. Cells were seeded in a concentration ranging from 156 cells/well to 40,000 cells/well, and the signal was obtained 30 min after seeding (Figure 4). The seeding of 40,000 cells/well gave the largest luminescence signal, just over 50,000 RLU and also had a significant difference from the seeding of 20,000 cells/well. The concentrations ranging from 156 cells/well to 20,000 cells/well showed a relatively linear relationship between the concentration at seeding and the luminescence signal.

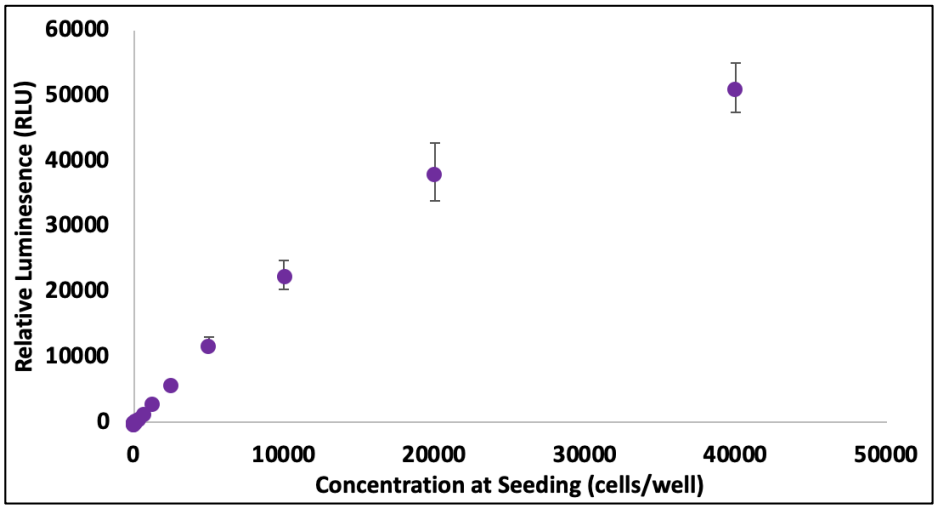


Figure 4. Cells were seeded in concentration from 156 cells/well to 40,000 cells/well. CellTiter-Glo reagent was added to each well 30 min later, and the signal was detected by SpectraMax M5 (n=4).

In order to determine the time HL60 cells needed to multiply in 384-well plates, the doubling time experiment was carried out. This experiment tried to observe the changes of the cell growth from between 0 and 120 hr. According to Figure 5, the cells of different concentrations generated stable signal before 24 hr. At 48 hr, huge error bars were observed, so 48 hr would not be the ideal time point to take any measurement. To confirm this finding, the same experiment was repeated, and the luminescence signal was detected over a longer time period (0 – 120 hr). Cells grew in the first 48 hr (Figure 6) but were considered not metabolically viable after that. After 48 hr, the number of cells reflected by the luminescence signal was doubled, but with significant error. According to the previous experiments on this project, laromustine results in significant loss of viability after 6 hr.[23] Therefore, measurements should take place 6 - 24 hr after the treatment. To make sure that cells yield a significant luminescence signal with minimal error, a 24 hr incubation period for HL60 cells was selected for this screen. This result is confirmed in Figure 7. After 24 hr, different concentrations of cells provide significant amount of luminescence signals with only modest error that can accurately represent the difference between different concentrations at seeding.

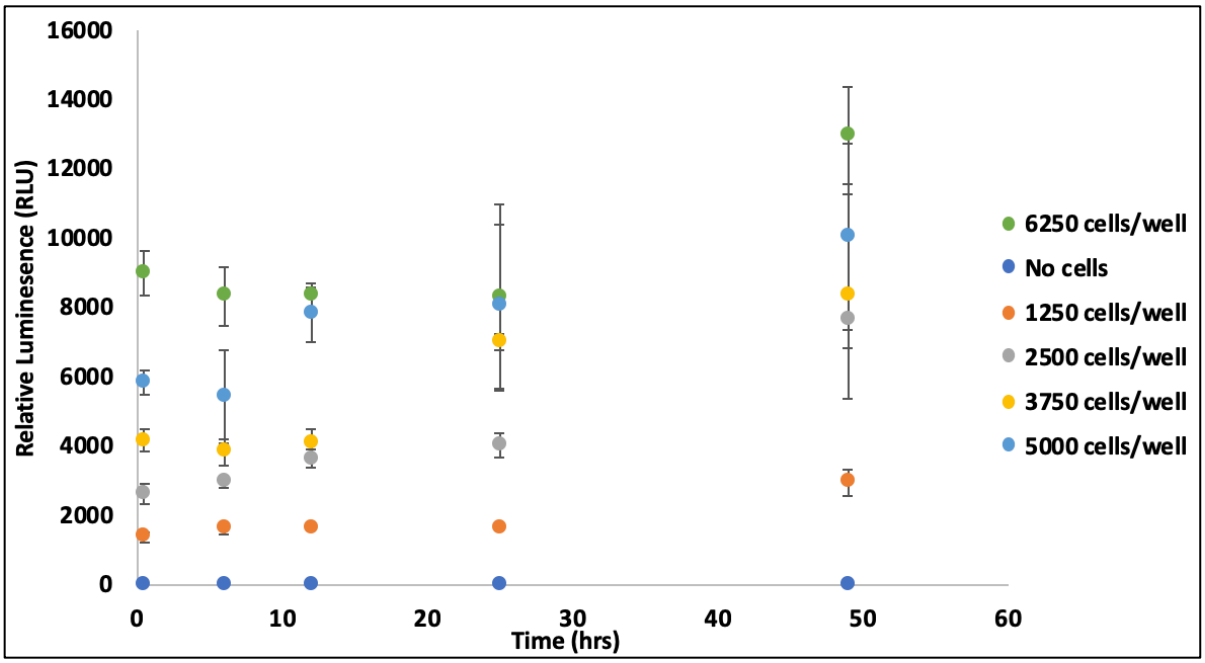


Figure 5. Determination of doubling time of HL60 cells. Wells were seeded with different concentration of cells, and luminescence signal was detected from 0 to 48 hr (n=4).

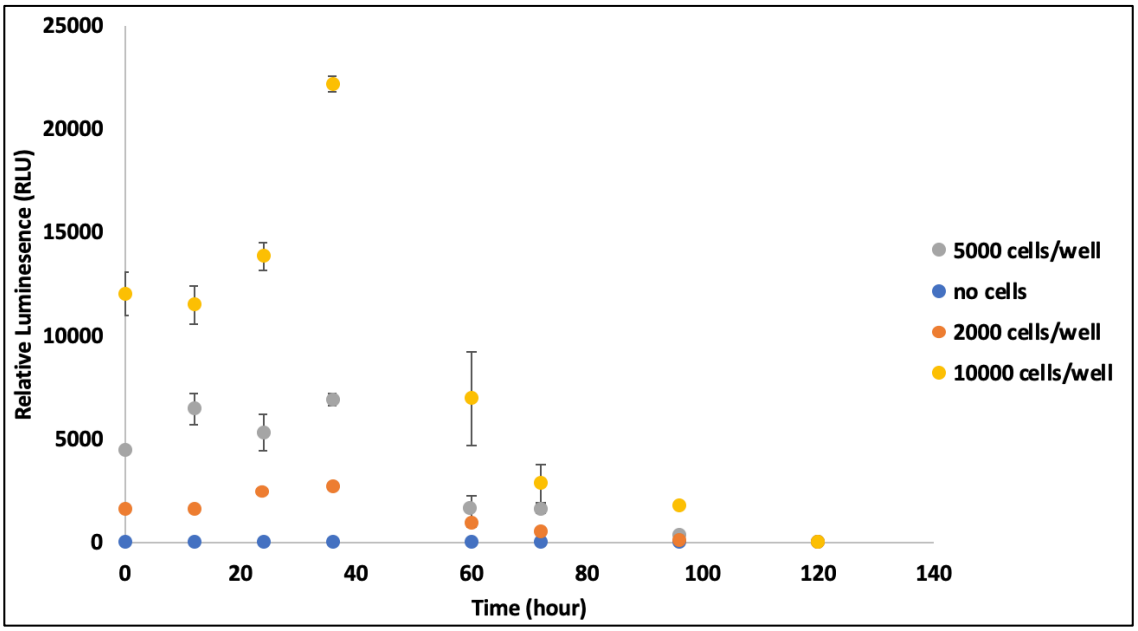


Figure 6. Determination of doubling time of HL60 cells. Wells were seeded with different concentration of cells, and luminescence signal was detected from 0 to 120 hr (n=4).

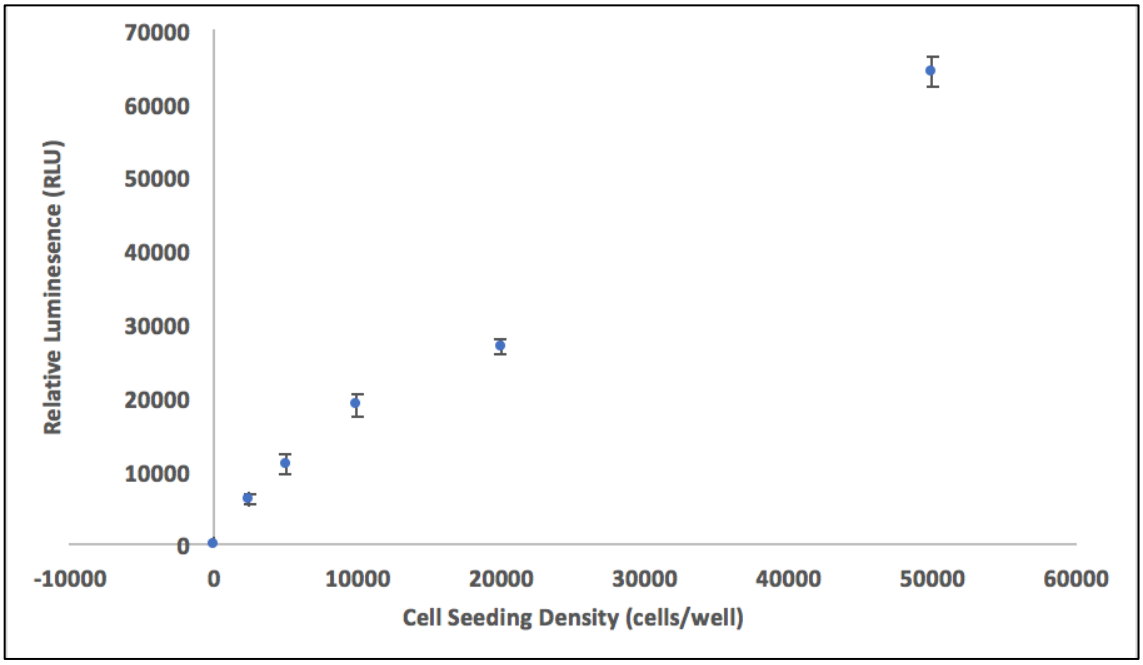


Figure 7. Determination of doubling time of HL60 cells. Wells were seeded with different concentration of cells, and luminescence signal was detected at 24 hr (n=4).

Temozolomide treatment results in significant loss of viability in HL60 cells after 24 hours.

When temozolomide enters cancer cells, cell death should be reflected in a change in luminescence signal. The series of events will not happen immediately. The amount of time for the drug to significantly impact cells must be experimentally determined.[23] The viability of HL60 cells was tested from between 0 and 48 hr post TMZ treatment with repeatable results. In this experiment, HL60 cells showed a stable decrease in viability in the experimental time frame. In the time trial experiment (Figure 8), the viability of HL60 cells decreased from 0 to 24 hr.

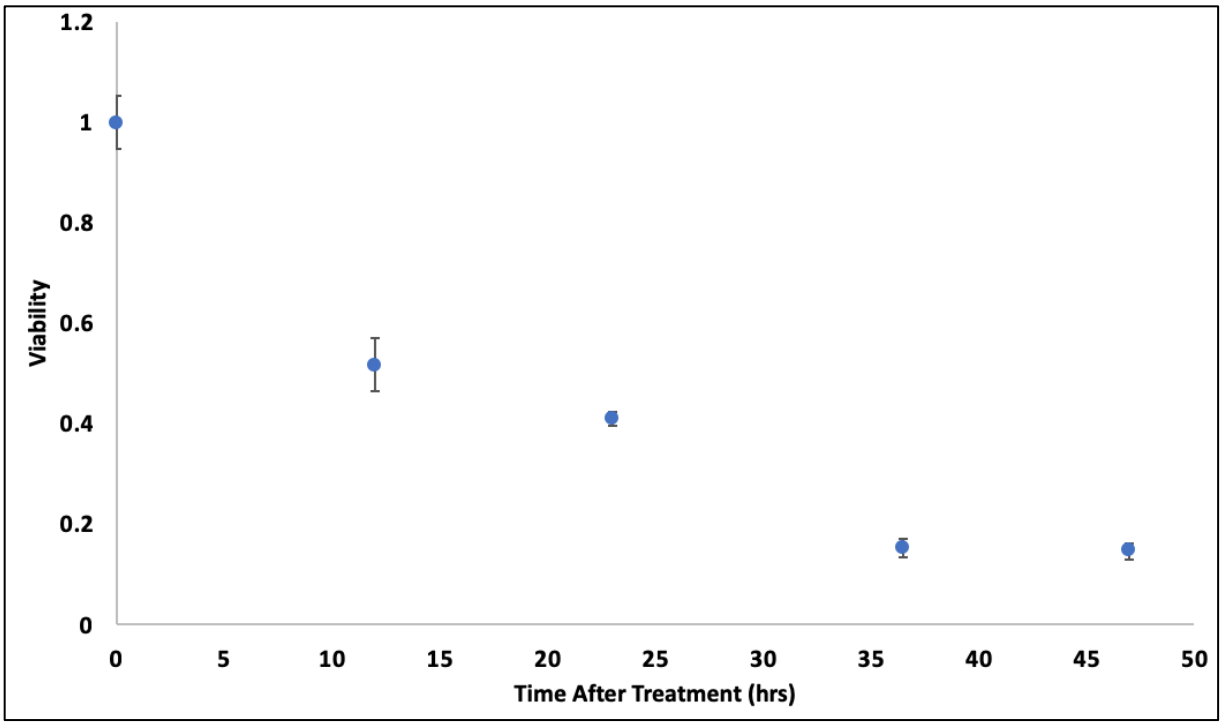


Figure 8. Determination of lethal dose time response of temozolomide using HL60 cells. Data were collected from t = 0 to t = 48 hr. Data were analyzed for average luminescence by quadruplicates, and error was measured by standard deviation (n=4).

The Median Lethal Dose of Laromustine in Treating HL60 cells is 159μM

When the HL60 cells were treated with laromustine at concentrations from 250 μM to 2000 μM, HL60 cells lost most of their viability. When the cells were treated with 0.244 μM to 4 μM laromustine, most of the HL60 cells remained similarly viable to those of the DMSO control group (Figure 8). The LD_{50} was calculated to be 159 μM. In order to test laromustine's synergistic effect with other compounds, a specific concentration needed to be selected, such that most of the cells in the well would still be alive, but such that a slight increase in the concentration of the drug would result in significant cell death. Laromustine concentrations of 200 μM and 20 μM were selected as lethal and near-lethal doses, respectively, for the further steps.

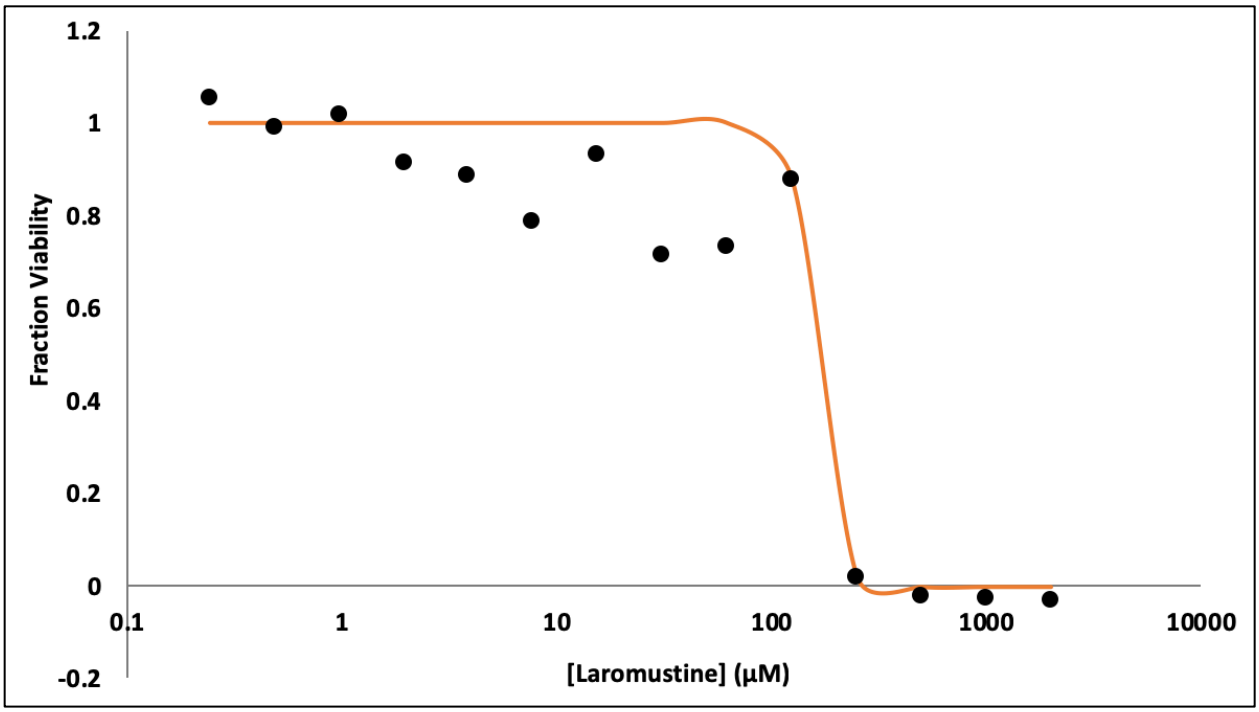


Figure 9. The laromustine dose response curve for HL60 cells seeded at the concentrations of 25,000 cells/well, in the format of 384-well plates. Concentrations of the drugs ranged from 2000 μM to 0.24 μM in a two-fold serial dilution with the DMSO as the control, and the LD_{50} = 159 μM (n=3). The fraction viability was calculated by the average, and the orange curve represents the fitted curve for this dose response curve.

The Median Lethal Dose of Temozolomide in Treating HL60 cells was 981 μM

As opposed to laromustine treatment, a 24 hr temozolomide treatment with concentrations ranging from 0.244 μM to 62.5 μM yielded very little cell death. Cell viability started to significantly decrease at concentrations above 125 μM. The lethal dose of 1000 μM was established (Figure 9). For the synergism analyses, temozolomide concentrations of 1000 μM and 100 μM were selected as lethal and near-lethal doses, respectively.

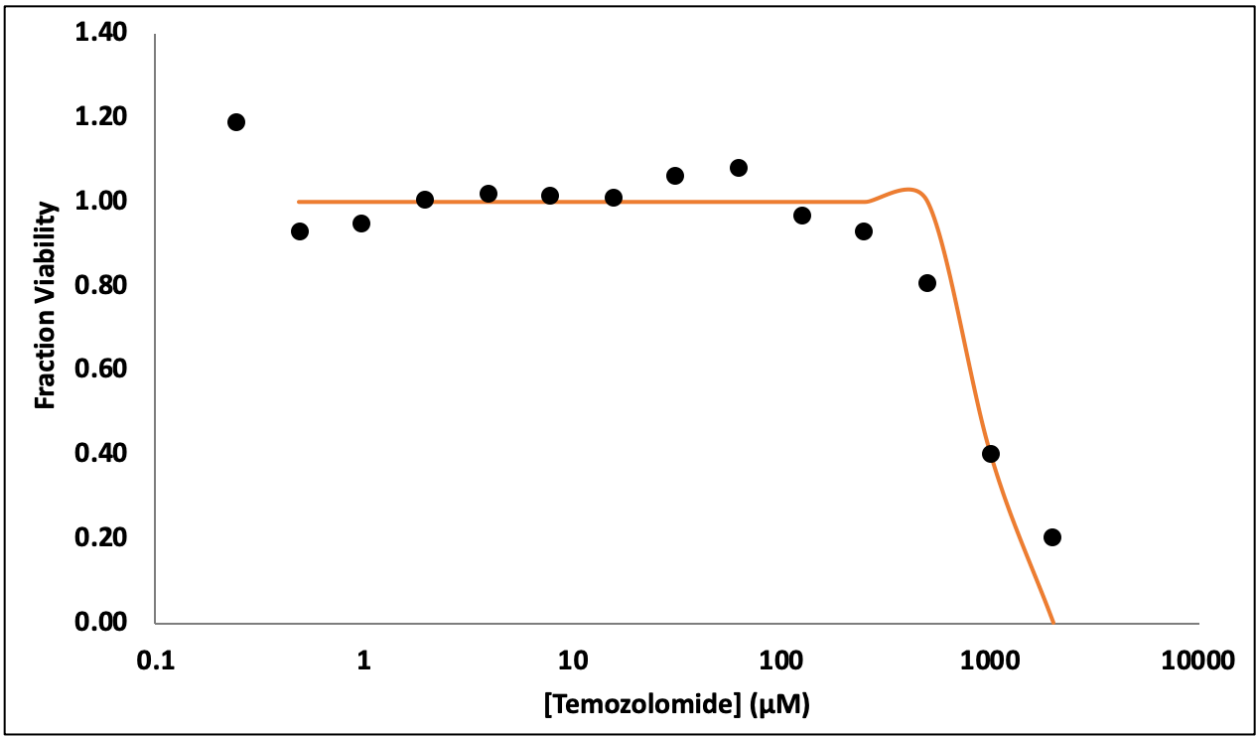


Figure 10. The temozolomide dose response curve for HL60 cells seeded at the concentrations of 25,000 cells/well, in 384-well plates. Concentrations of the drugs ranged from 0.24 to 2,000 μM in a two-fold serial dilution with DMSO as the control, and the LD_{50} = 981 μM (n=3). The fraction viability was calculated by the average, and the orange curve represents the fitted curve for this dose response curve.

For both laromsutine and temozolomide, concentrations that could be applied for the chemical genetic screen were estimated from Figure 9 and Figure 10. The concentrations selected should induce some but not all the cell death. A high concentration would impede the observation for synergism effects, and a low concentration might not be able to yield any observable changes. Initially, 20 μM laromustine and 100 μM temozolomide were chosen, and they were compared with 200 μM laromustine and 1000 μM temozolomide, which were 10x the chosen concentrations. Those 10x concentrations were expected to induce a significant amount of cell death. Different concentrations of laromustine and temozolomide were tested for luminescence after 24 hr of treatment. The control groups (treated by DMSO)

worked as expected. For laromustine, a sharp decrease in cell viability was observed when the concentration increased from 20 μM to 200 μM (Figure 11). As expected, a concentration of 200 μM resulted in significant loss of HL60 cell viability. A 20 μM concentration showed a luminescence signal lower than that of the control but still high enough to show the potential influence of the other compound. Thus, concentrations of 20 μM and 50 μM laromustine were selected for the synergism experiments. As for temozolomide, the luminescent signal generated by cells exposed to 1000 μM temozolomide was significantly lower than that of cells treated with 100 μM temozolomide (Figure 11). However, the 100 μM treatment produced a luminescent signal higher than the controls. Therefore, 200 μM and 500 μM temozolomide were chosen to be tested with laromustine.

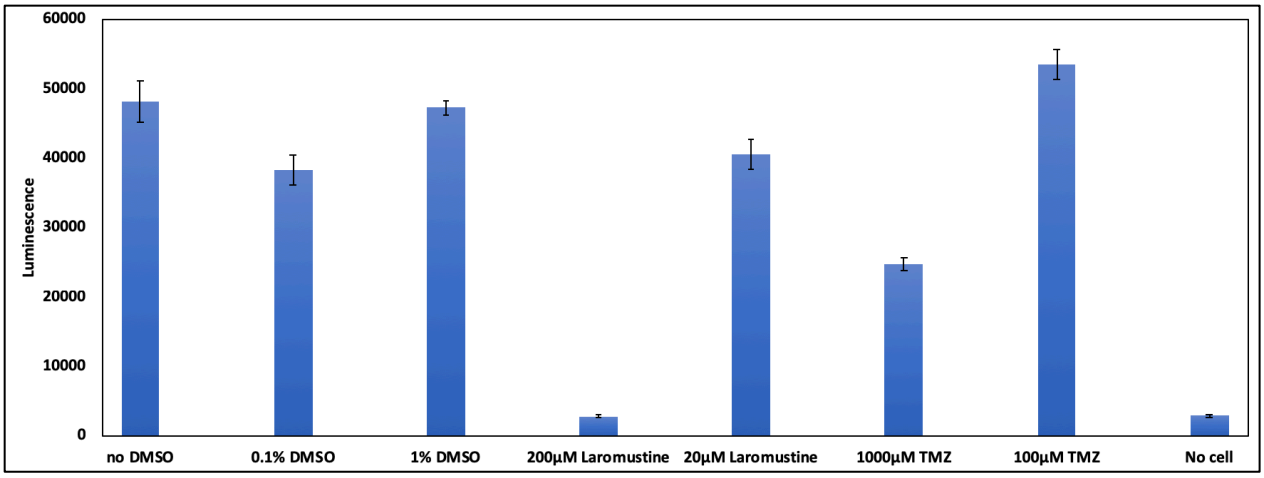
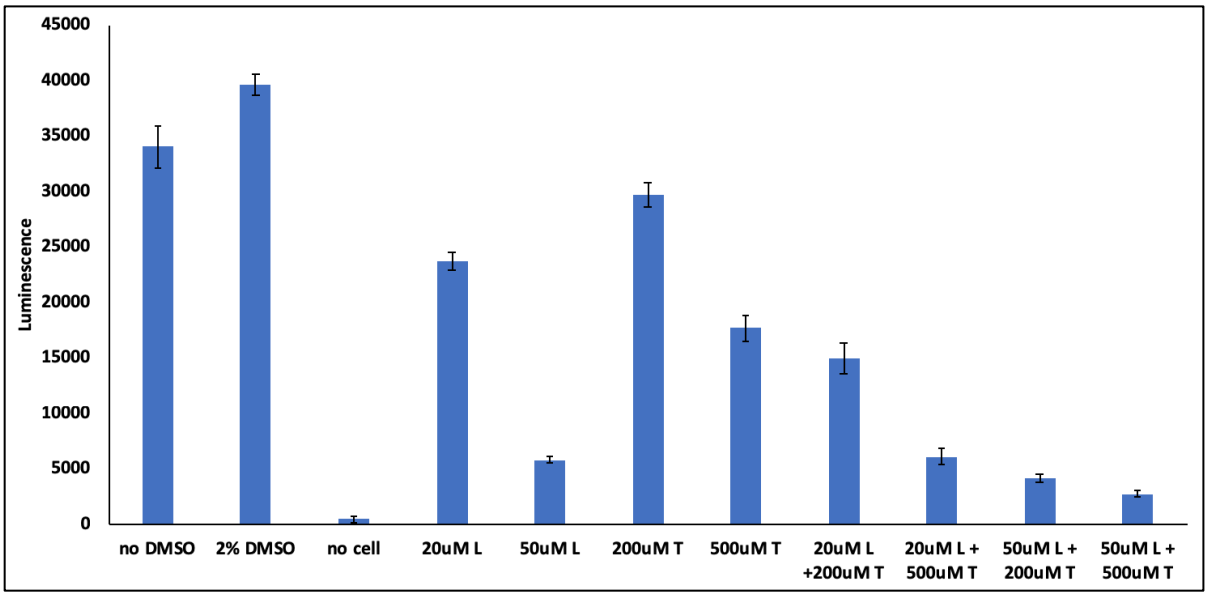


Figure 11. HL60 cells were seeded at the concentration of 40,000 cells/well with different compound concentrations, including DMSO as the control. Data were analyzed after 24 hr for average luminescence (n=8).

Laromustine (50μM) and Temozolomide (200μM) Worked Synergistically to Induce HL60 Cell Death

In order to test the optimization results of the chemical genetic screen, temozolomide was tested for its synergistic effect with laromustine. Among all four combinations, cells treated with 50 μM laromustine and 500 μM temozolomide showed some synergistic toxicity (Figure 12.A). This result was significant relative to the control DMSO group. The other combinations also showed signal results lower than that of the control group, but those results looked more additive than synergistic. However, the results from Figure 12.A were not repeatable at 24 hr post treatment. The reason for this observation might be that the long treatment period made the cells continue to grow under pressure. In another experiment (Figure 12.B), cells were treated for only 12 hr. As shown in Figure 12.B, the combination of 50 μM laromustine and 500 μM temozolomide still demonstrated some synergism.

A



B

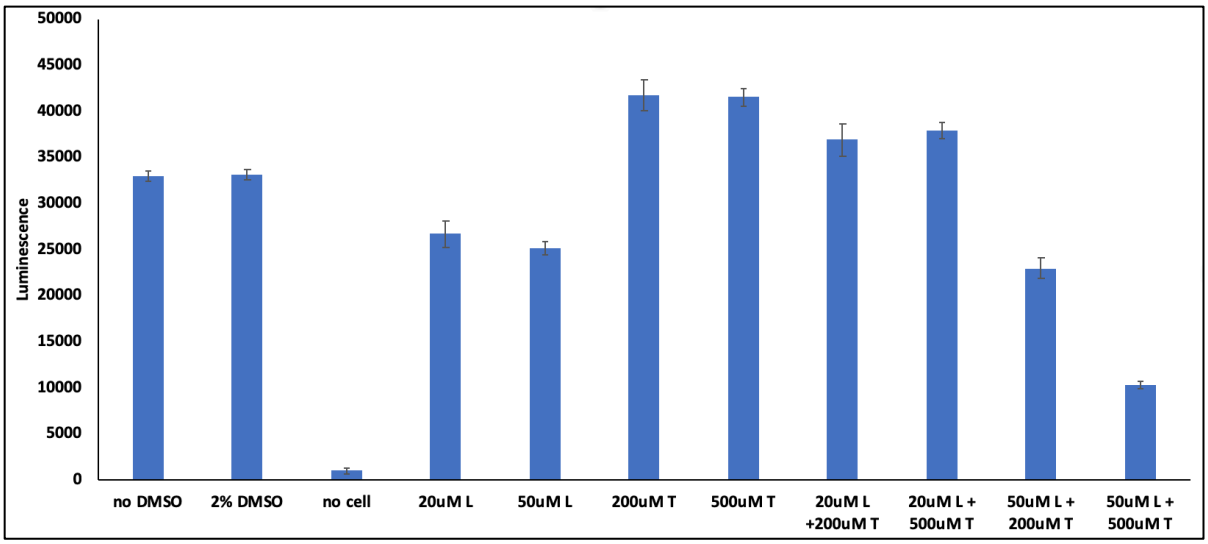


Figure 12. Cell viability at different concentrations of temozolomide (T), laromustine (L), and their combinations, as compared to the control group with the treatment of 2% DMSO. **A** showed the light signal detected after 24 hr of treatment (n=8). **B** showed the luminescence signal measured after 12 hr of treatment (n=4).

Discussion

As a result of these experiments, the optimal seeding density for HL60 cells was determined to be 25,000 – 40,000 cells/well in a 24-well plate, with 2% DMSO as the control. Those seeding densities showed stable luminescence signal and could accurately reflect the number of viable cells in each well. Then, the cells should be transferred to the 384-well plates, 25 µL/well, to be measured for luminescence signal by the CellTiter-Glo reagent after 12 hours or 24 hours of treatment. Though in the experiment with temozolomide, the synergism effect was not able to be observed again at 24 hr post treatment, both 12 hr and 24 hr should be considered in the next step experiments. When testing the combined effects between laromustine and temozolomide, some synergism was observed between 50 µM laromustine and 500 µM temozolomide. When being tested for the chemical genetic screen, 50 µM of laromustine was selected as the optimal concentration.

At this point, all the conditions (cell seeding density, treatment period, and laromustine concentration) for the chemical genetic screen were optimized. This project should be able to move to the screening process. Before that, the cytotoxicity for the combination of laromustine and Olaparib, the PARP inhibitor, should be tested. The project is going to collaborate with Professor Robert Wheeler at the University of Maine, Orono to test laromustine with the collection of 450 FDA-approved compounds. Compounds and cellular targets identified from this chemical genetic screen will be verified individually and independently.

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