INTRODUCTION

According to the American Cancer Society one in three Americans will be diagnosed with cancer in their lifetime. Research is conducted in order to enhance the efficacy and reduce the side effects of treatments. Despite laborious works, treatments such as radiotherapy still pose significant side effects and decrease quality of life for patients. Therefore, our lab aims to sensitize medulloblastoma and colorectal cancer cells to chemotherapy drug by targeting Dicer. With the same amount of drug, the chemosensitized cancer cells will die at a faster rate. This strategy can potentially eliminate treatments that cause significant side effects to patients.

Human Dicer is a large multi domain protein about 200,000 Daltons in size.[1] There are two main functions of Dicer. First it generates short non-coding siRNAs and miRNAs, secondly it recruits DNA damage response (DDR) factors. The recruitment of DDR proteins by Dicer allow cells to undergo DNA repair. The purpose of generating non-coding siRNAs and miRNAs is to regulate post-transcriptional gene silencing.[2] Specifically, Dicer cleaves the precursor RNAs and loads them to the Argonaute protein. Then the siRNA or miRNA Argonaute complex binds to the complementary mRNA and suppress its translation.

Fascinatingly, the dsRNA cleavage mechanism was also discovered in plant cells as a defense mechanism to viral infection. The viral dsRNAs are cleaved by Dicer and are loaded to argonaute protein complex. The viral RNA fragment plus argonaute complex complimentarily bind to viral transcripts. This process inhibits translation of viral particles and suppresses its proliferation. Since this mechanism was recognized in plants, investigation is underway to discover a conserved mechanism in mammalian cells.

The cleavage of the dsRNA and pre-micro RNA occurs in the Dicer's catalytic site that contains two intermolecular RNase IIIb dimers. Other domains present in the protein such as the RNA Binding domain, dsRNA Binding Domain, and PAZ also assist in the RNA cleavage.[3] Several Mg^{+2} ions are additionally present in the catalytic site to neutralize the charge repulsion and increase the binding affinity to the RNAs.

Medulloblastoma (MB) is the most common brain tumor in children and treatment consists of surgery, chemotherapy, and radiotherapy.[4] Unfortunately, due to radiotherapy's substantial side effects such as endocrine abnormalities and long-term neurocognitive deficits, it is delayed or even avoided for patients less than 3 years of age.[5] One strategy to decrease radiotherapy treatment for MB patients would be to increase the efficacy of existing chemotherapy drugs. Interestingly, collaborative works of our lab and others have identified that full loss of the Dicer gene increased DNA damage in a mouse model, figure 1. Moreover, when the Dicer knockout (KO) cells were treated with etoposide, a commonly used chemotherapy, cell death of medulloblastoma increased substantially compared wild-type cells, figure 2. These findings indicated that Dicer knockout increases chemosensitivity in medulloblastoma cells.[3]

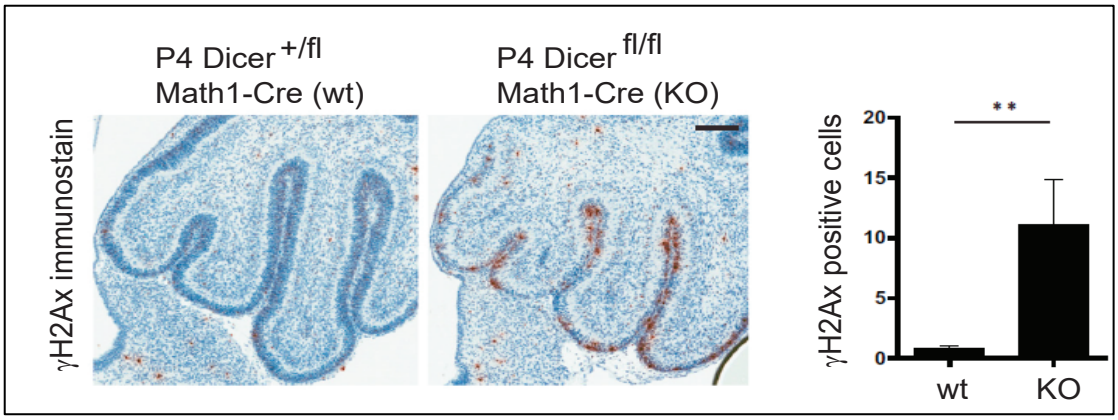

Figure 1. Cross section of mouse cerebellum, the γH2Ax immunostain indicates DNA damage in cells. Higher DNA damage, orange stain, is seen in Dicer knockout compared to wild-type cells. Image adopted from *Essential Function of Dicer in Resolving DNA Damage in the Rapidly Dividing Cells of the Developing and Malignant Cerebellum*

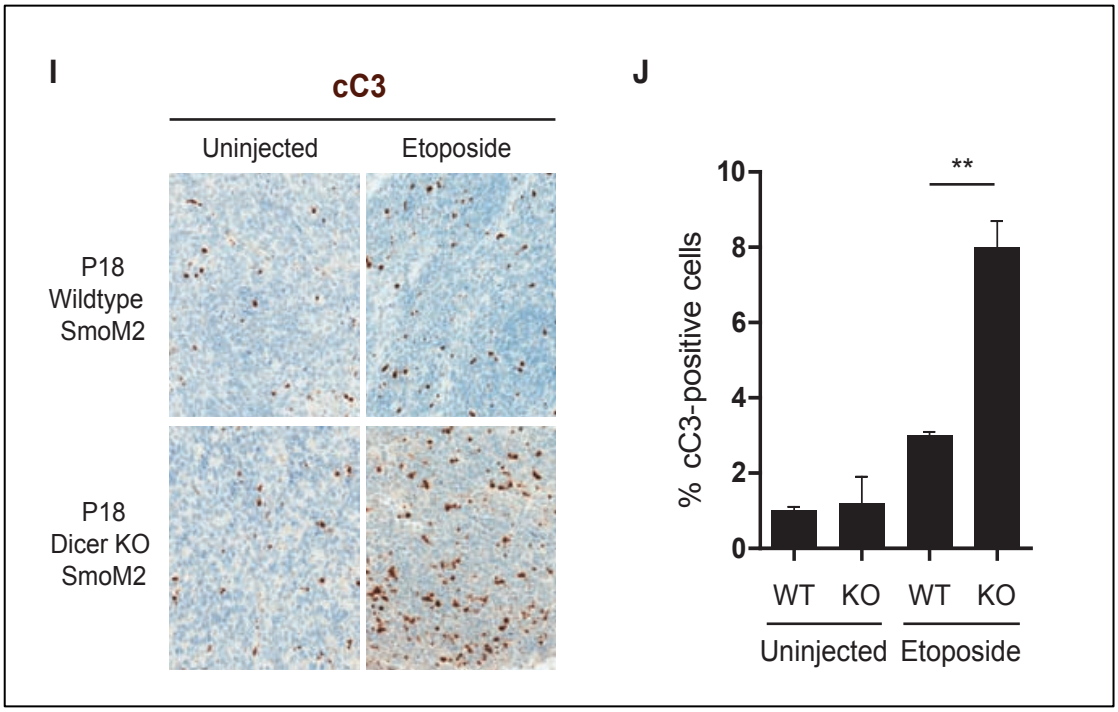

Figure 2. Etoposide injection to Dicer KO versus wildtype mouse medulloblastoma. The cC3 indicates the caspase 3, a protein that gets activated during cell death. Highest cell death was identified with Dicer KO injected with etoposide. The wild-type cells injected with etoposide shows minor cell death. Image adopted from *Essential Function of Dicer in Resolving DNA Damage in the Rapidly Dividing Cells of the Developing and Malignant Cerebellum*

Interestingly chemosensitivity in Dicer knockout was also discovered in colorectal cancer cells.[6] Therefore, our lab also focuses on chemosensitizing colorectal cancers using Dicer KO. Colorectal cancer is the "third most common cancer in men and second in women worldwide."[7] Biologically, colorectal carcinoma (HCT116) is sustained by a subpopulation of cancer-initiating cells (CICs) also referred to as cancer stem cells.[8] The CICs are thought to play a significant part in

tumor relapse and patient survival.[9] Thus, decreasing CICs proliferation can decrease tumor relapse and increase patient survival.

Recent findings have shown that epigenetic therapy such as 5-aza-2-deoxycytidine (5-AZA-CdR) can be used to target and decrease CIC proliferation rate. The drug, 5-AZA-CdR, increases transcription of endogenous retroviral transcripts. Presence of viral transcripts initiates a viral signal pathway (MDA5/ MAVS/IRF7) activates interferon, a protein that gets released when cells are infected with pathogens. Fascinatingly, this viral signal pathway decreases CICs proliferation.[10].

Also, upon presence of viral transcripts, the body can potentially utilize RNA interference (RNAi) pathway as defense mechanism. As mentioned before, the viral dsRNA may be recognized and cut by Dicer into short fragments called siRNAs or also known as viRNAS. These fragments can be loaded onto Argonaute protein and complementary bind to viral mRNA to inhibit translation.[11]

Objectives

Using the evidence that Dicer KO increases chemosensitivity, our lab aims to find small molecule that will inhibit Dicer. By inhibiting Dicer in medulloblastoma and colorectal cells this will increase chemosensitivity and cell death. Higher cell death in chemosensitized cells can potentially remove therapies that pose significant side effects to patients.

Moreover, demethylating agent 5-AZA-CdR has shown to decrease CICs proliferation by inducing the expression of endogenous viral transcripts. It is still unclear if Dicer uses the viral-RNA based immune response pathway. But if it does, Dicer inhibition can obliterate the viral-RNA based immune pathway and increase endogenous retroviral expression. Higher viral load in Dicer KO cells can further decrease the CICs proliferation rate which in turn decreases colorectal relapse and increase patient survival.

MATERIALS AND METHODS

Dicer siRNA Transfection

Lupus Erythromatosus (LE) and Daoy medulloblastoma cell lines where transfected with Dicer inhibiting siRNA. The siRNA was introduced using Lipofectamine ™ 2000 transfection reagent. For LE cells transfection was conducted around 50% cell confluency with 20 pmol of siRNA. Before the transfection, the cell medium was changed to Opti-MEM. Then Lipofectamine ™ 2000 was diluted to 1: 50 ratio with Opti-MEM and was incubated for 5 minutes at room temperature. After incubation 20 pmol siRNA was combined with diluted Lipofectamine and was incubated again for 20 minutes. Then the appropriate volume of transfection reagents was added to each well. After 6 hours, Lipfectamine and Opti-MEM was replaced with Dulbecco Modified Eagle Medium (DMEM) in 10% FBS and 1% penicillin. After 2 days of incubation in DMEM differing concentration of etoposide was added. Subsequently, the 2-day drug treated cells were stained with crystal violet to visualize cell viability. For DAOY cells transfection occurs around approximately 80% cell confluency and 20 pmol of siRNA was used. The same transfection protocol was followed as written above. After 6 hours of transfection, the cells' medium was

changed to DMEM. Cells were exposed to differing etoposide concentration for three days. DAOY cells were also stained with crystal violent and cell viability was determined.

Dicer Purification

The Sf9 insect cells were grown in a spinner flask with a magnetic stir bar at 28 ^{o}C in Grace medium (Thermo Fisher) with 10% fetal calf serum and 1% penicillin. Approximately 1.0 L Sf9 cells at concentration of 1 x10^{6} cell/ mL were infected with recombinant baculovirus with the multiplicity of infection of 1.0. After 72 hours of incubation cells were centrifuged (1,000g, 10 minutes) and resuspended with 200 mL of PBS. Cells were washed with 20 mL of W100 Buffer (Tris-HCL, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM betamercaptoethanol, 10 % glycerol, and 0.5 % Triton X-100) and one tablet of 1xEDTA-free protease mix inhibitor. The cells were passage through 2.3 gauge 5 times and was centrifuged again at 12,000g for 1 hour at 4^{o}C. The supernatants were collected and used for protein purification. Prewashed with W100 buffer, the Talon^{TM} resin and the supernatants were placed into a conical tube. The tube was placed onto a rotor for 20 minutes. Then the resin and cell mixture was placed onto a column and was washed 3 times in the order of W100, W800 (800 mM NaCl), and W100 [8]. Then ten tubes containing different Imidazole concentrations in W100 buffer (30 mM, 60 mM, 90 mM, 120 mM, 150 mM, 180 mM, 210 mM, 240 mM, 270 mM, 300 mM) were used to elute off the Dicer protein. Fractions were loaded onto 6% polyacrylamide gel and stained with Coomassie brilliant blue. Fractions that contained the Dicer were collected. Then to the prewashed Sepharose (GE Health and Life Sciences) beads imidazole eluted fractions containing Dicer were loaded. A gradient elution with differing NaCl concentrations (20 tubes with 0.05 M NaCl increase at 0.5 mL volume) was conducted. The 20 fractions were loaded onto 6% polyacrylamide gel and stained with Coomassie brilliant blue to detect total protein concentration. The fractions that contained purest concentration of Dicer (E2, E3) were collected, figure3. Western blot was conducted, figure 4, to further confirm the presence of Dicer protein. After the purification, Dicer concentration was determined using Q bit^{TM} fluorometric quantitation.

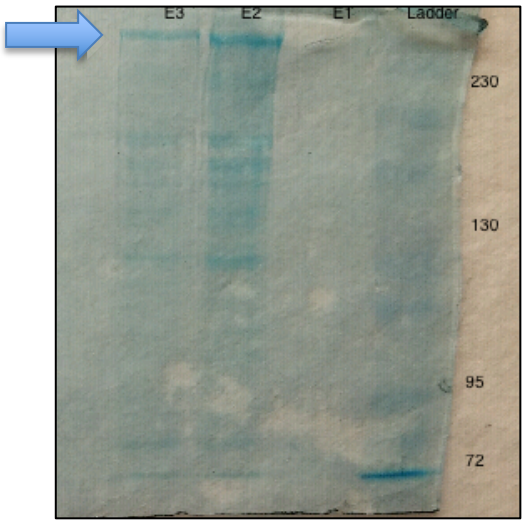

Figure 3. Purification of Dicer resolved in 6% polyacrylamide gel stained with Coomassie brilliant blue. The blue arrow on top indicates the presence of Dicer in E1, E2, and E3 lanes.

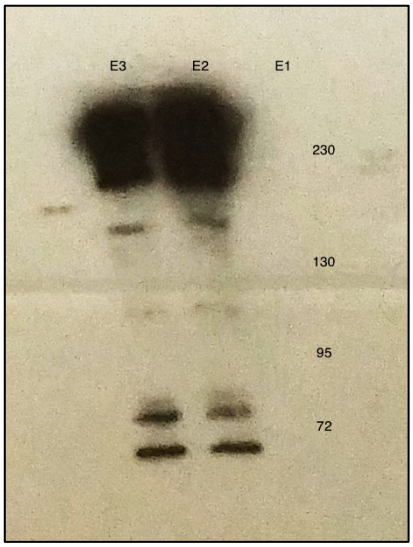

Figure 4. The Western blotting of recombinant Dicer stained with mouse monoclonal anti-His-tag antibody horseradish peroxidase. The top broad band at 230kDa is Dicer. Other smaller fragments are speculated to be degraded fragments of the Dicer protein.

High-throughput Screening (HTS): Dicer Inhibitors

To determine the Dicer's cleavage efficiency a fluorescence-based assay was used. First, the two RNA strands were annealed. One of the strands contained a fluorescent group (Cy5) whereas the other strand contained the quencher. When Dicer cleaved the dsRNA, the fluorescent group was released. The free-floating fluorescent Cy5 absorbed 640 nm and emitted 670 nm electromagnetic waves. Thus, Dicer's cleavage activity was correlated with fluorescence intensity, Figure 5. The schematic nucleotide sequence for the quencher and the fluorescent strand is outline in figure 6.

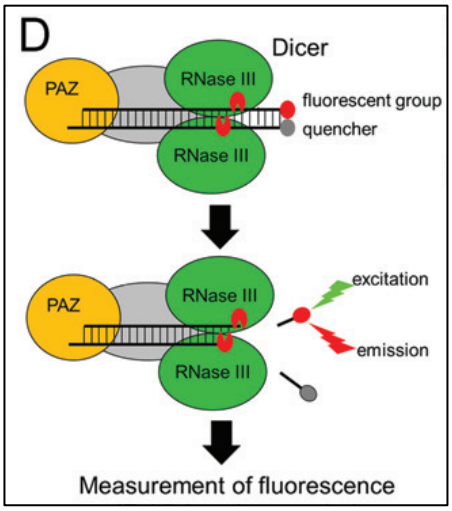

Figure 5 Image adopted from *Fluorescence-Based High-Throughput Screening of Dicer Cleavage Activity* [7], this image represents the relationship between fluorescence and Dicer cleavage activity.

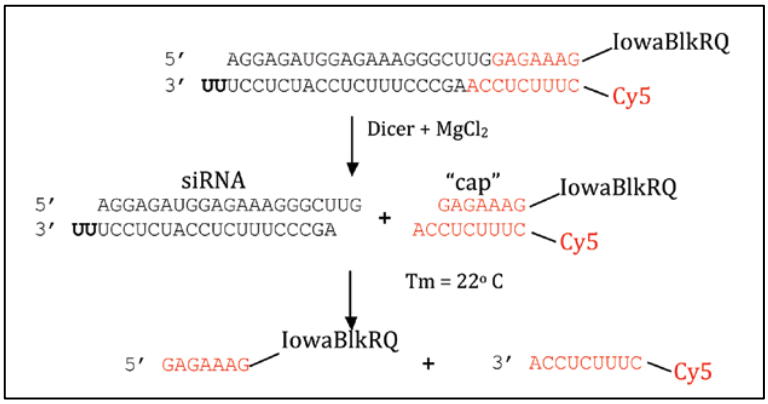

Figure 6. Image adopted from *Continuous fluorescence-based method for assessing Dicer cleavage efficiency reveals 3′ overhang nucleotide preference* [7], shows the nucleotide sequence for the Cy5 and quencher strand used for experimental fluorescence assay.

The Dicer assay was carried out with the appropriate buffer (30 mM Tris-HCl [pH 80], 50 nM NaCl, 3 mM $MgCl_2$, 0.25% Triton-X100, and 15% glycerol) in corning 96 Flat Bottom black, clear bottom Polystyrol. The assay also contained ATP, deionized water, and differing concentration of Dicer (45 nM, 454 nM) and substrate (500 nM, 50 nM, 5 nM, 0.5 nM) to reach a final volume of 25 μL. Also, positive control (Cy5 strand only) and negative control (EDTA) was used to determine the maximum and the minimum fluorescence. Tecan i-control reader system was used to measure fluorescence. Instrumental setting used in experiment is outlined in Table 1. Then 1593 small molecules from National Cancer Institute (NCI) diversity set V were introduced to the assay and Dicer activity was measured.

Table 1. Below shows the setting used in Tecan i-control to measure fluorescence in Corning 96 plate.

Target Temperature	37 °C
Kinetic Cycles	Different for each experiment
Interval Time	0:05:00 min
Excitation Wavelength	640 nm
Emission Wavelength	670 nm
Excitation Bandwidth	5 nm
Emission Bandwidth	15 nm
Gain	Optimal
Number of Flashes	100
Flash Frequency	400

Inducing Endogenous Viral Transcripts

Human colorectal HCT116 cells were plated 24 hours in DMEM (10% FBS and 1% penicillin) media prior to drug treatment. Around 30% cell confluency, cells were treated with 5-AZA-CdR. Depending on the experiment different concentration (300 nM, 900 nM, 1200 nM) and length (1 day, 5 day) of 5-AZA-CdR was introduced. Cells were washed with DMEM before replenishing with fresh media. Selected plates exposed to 300 nM of 5-AZA-CdR were also introduced with either celastrol (30 μM) or Redoxol (100 μM). Cell viability of was measured after one week of drug exposure. Also, total RNA were extracted for selected cells and reverse-transcriptase PCR was conducted.

RESULTS

Dicer siRNA Transfection

The medulloblastoma cell line Daoy was transfected with Dicer siRNA. Then the knockout and the wild type were exposed to differing etoposide concentration for approximately 60 hours. Then the cells were stained with violent blue dye to visualize the cell viability. As shown below in figure 7, Daoy cells transfected with Dicer siRNA had significant cell death compared to wild type. The difference of cell viability was prominent at lower etoposide dose.

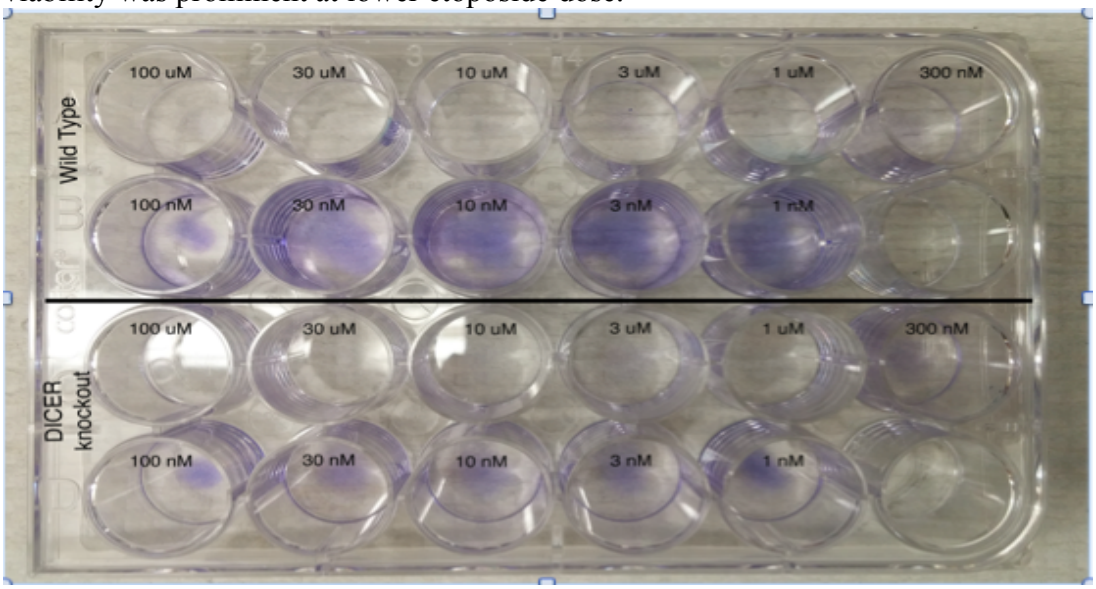

Figure 7. DAOY cell, wild type (no siRNA inhibition) or Dicer knockout (siRNA inhibition), were injected with differing concentration of etoposide. Dicer knockout cells increased chemosensitivity compare to wild type, and cell viability noticeably decreased for KO from 100 nM to 1 nM etoposide.

Drug Dosage

The medulloblastoma cell lines (Daoy, D283) were used to investigate the optimal dose of etoposide and cisplatin chemotherapy drugs. Differing concentrations of the two drugs (100 μM, 30 μM, 10μM, 3 μM, 1 μM, control) were plated into 24-welled plate. After 72 hours of incubation, the viable cells were counted. Figure 8 and 9 shows the cells viability after 12 hours of drug exposure. The dosage curve conducted for Daoy and D283 cells indicated that dosage over 3 μM of both cisplatin and etoposide pose high lethality to the cells.

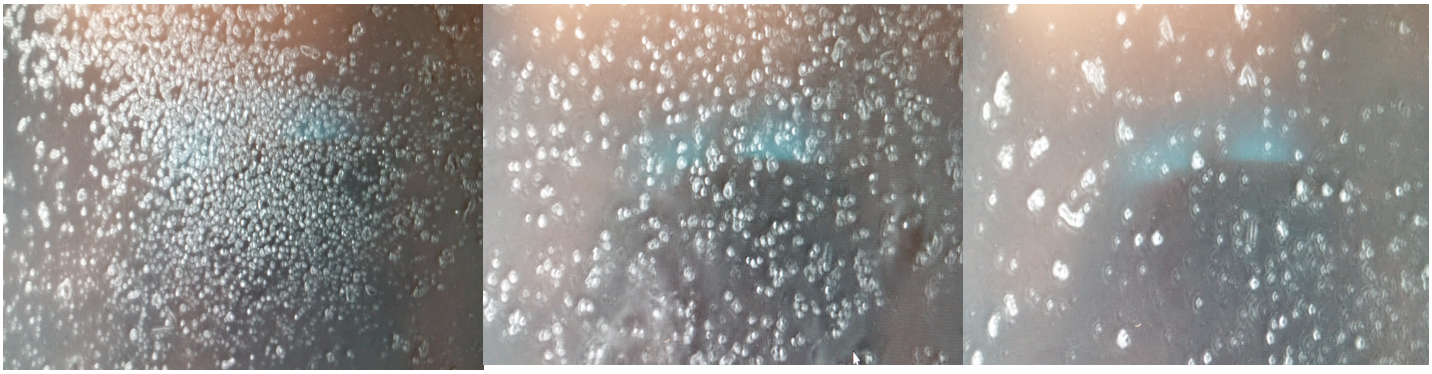

Figure 8. From left to right, D 283 control, D283 with 100 μM etoposide, D283 with 100 μM of cisplatin. Even after 12 hours of incubation, cell viability is decreased.

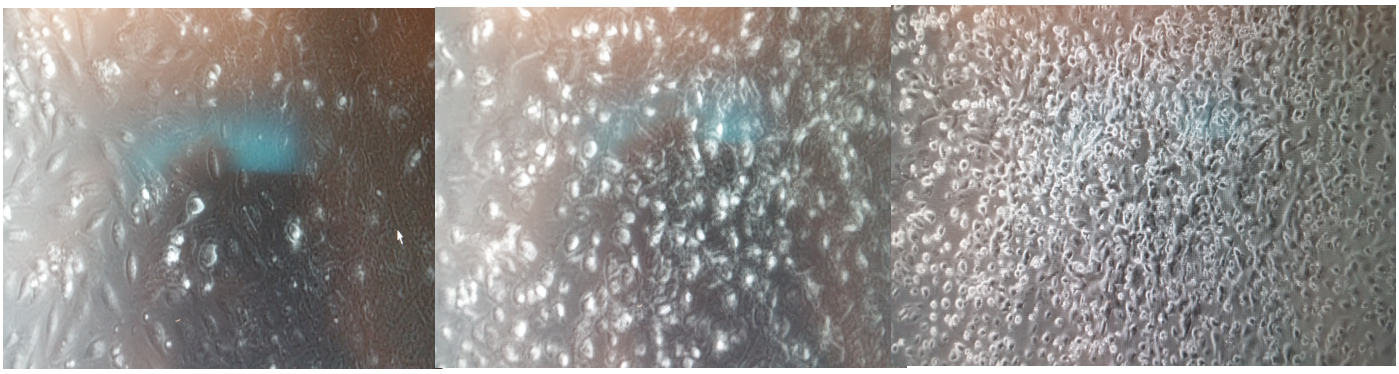

Figure 9. From left to right, DAOY control, DAOY with 100 μM etoposide, DAOY with 100 μM of cisplatin.

Protein Purification

Diverse purification techniques were optimized to obtain more purified Dicer. Specifically, gradient elution with differing concentrations of NaCl and Imidazole was used. However, the 6% polyacrylamide gel analyses showed that other protein fragments were also present in the purified protein solution. Around the 230 kDa regions a smear of bands was found which indicated that other macromolecules were also present in the solution, figure 3 and 4. Utilizing the *Methods in Enzymology*[8], overnight dialysis in W100 buffer yielded a purer Dicer.

High-throughput Screening (HTS): Dicer Inhibitors

A preliminary experiment was conducted using RNAse and EDTA as positive and negative control, respectively. Ethylenediaminetetraacetic acid (EDTA) was used as negative control since this chelating agent interferes with the Mg^{+2} in the cleavage site and prevents the dsRNA strand to bind. Concentration of 454 nm for Dicer and 60 nm of RNA substrate was determined as an optimum concentration.

A small molecule called kanamycin was tested to investigate its effect on Dicer cleavage activity. Experimental results indicated that kanamycin does decrease the Dicer activity. However, it was determined that the IC_{50} was too high to be introduced to clinical settings IC_{50} =2037 μM, figure 10.

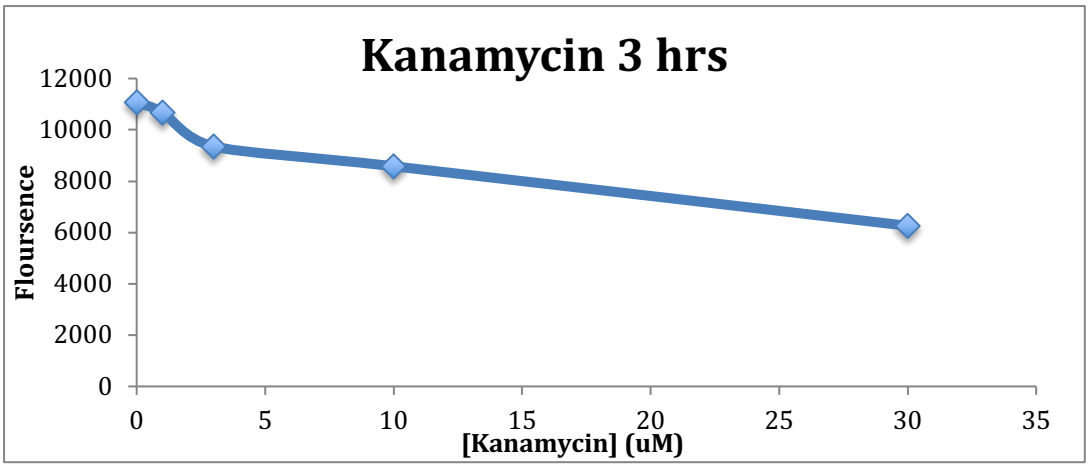

Figure 10. Differing concentration of kanamycin and its affect in Dicer RNA cleavage activity. Data collected at 3-hour exposure point.

Furthermore, assay of 1,593 compounds obtained from National Cancer Institute (NCI) diversity set V was conducted. The compounds were diluted 40 folds in dimethyl sulfoxide (DMSO). Before introducing the inhibitors, DMSO was tested to establish its impact on the fluorescent reading. Experimental data showed that DMSO does not interfere with the assay reading, figure 11. The result of all the 1,593 compounds are shown in figure 13. Compounds below the Z=4 were experimented again, figure 14. From the 29 compounds below z=4 line, six potential Dicer inhibitors compounds were discovered Figure 12.

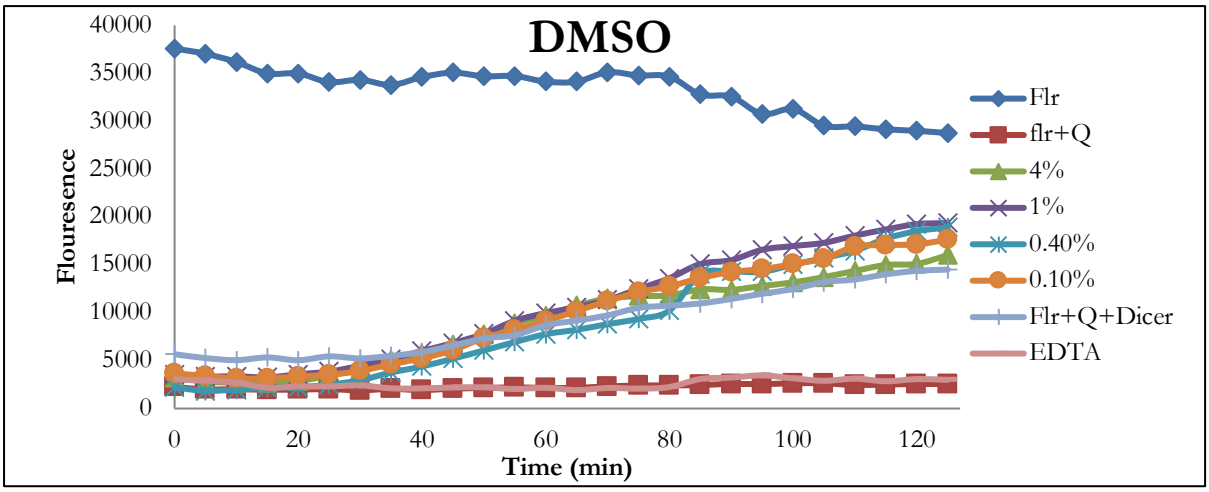

Figure 101 Different percentages of DMSO was introduced to the assay. Results showed that differing amounts of DMSO did not affect Dicer cleavage activity

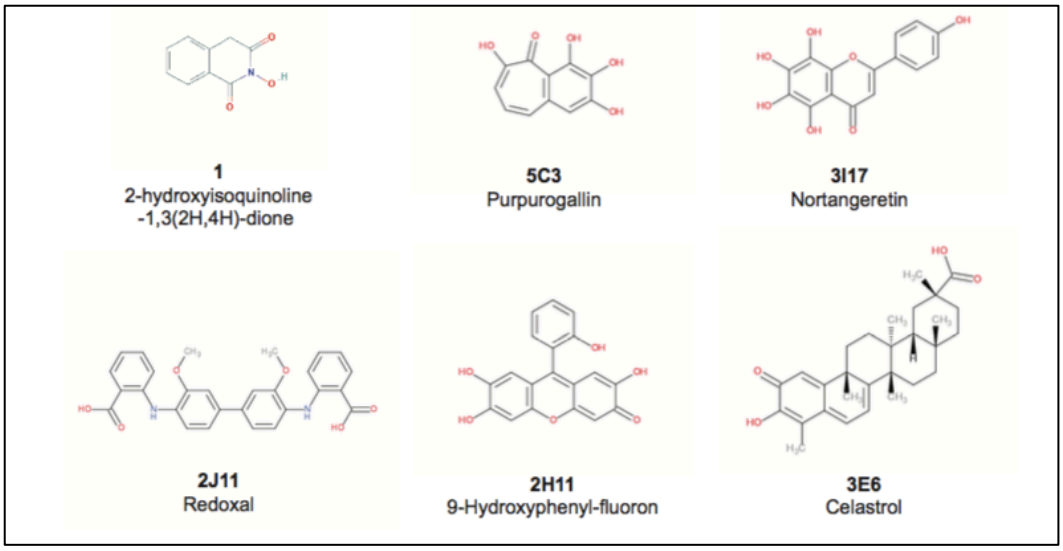

Figure 12. Six compounds experimentally found to inhibit Dicer function. Data provided by Kyle Kaufmann.

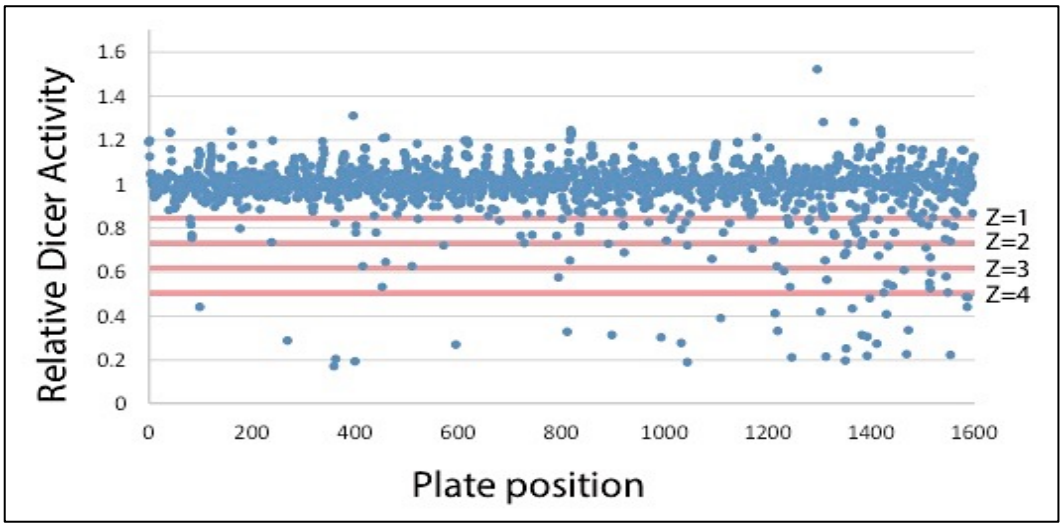

Figure 13. HTS results of 1,593 small molecule NCI diversity set V. Baseline was measured with DMSO well. The Z indicates standard deviation below the mean. Results showed significant decrease in Dicer activity for points below Z=4. Data provided by Kyle Kaufmann.

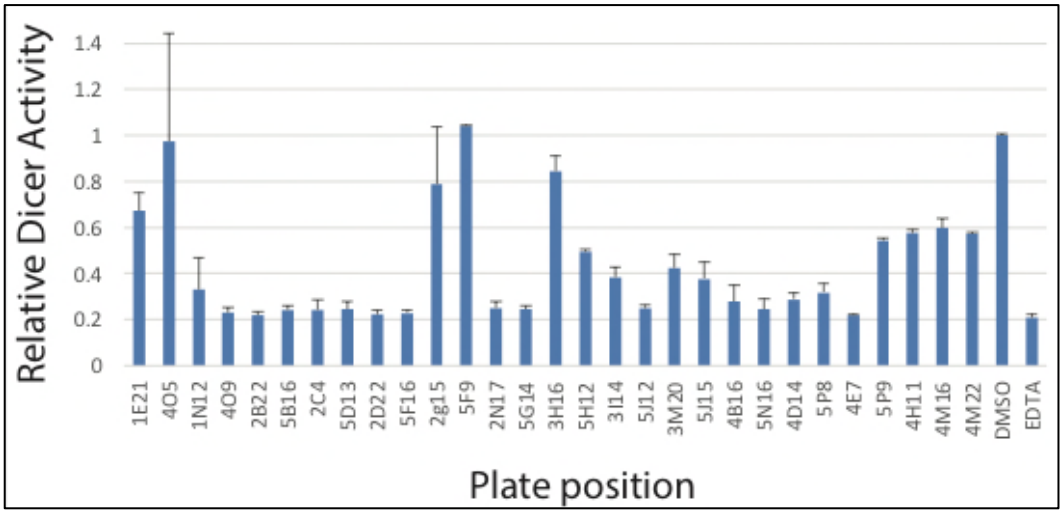

Figure 14. The 29 compounds found below the z=4 in diversity set experiment were retested. Most of the compounds showed significant decrease in Dicer activity. DMSO was used as baseline.

Inducing endogenous viral transcripts

Diverse 5-AZA-CdR treatment was conducted to optimize viral transcript induction. It was discovered that 24 hours of 300 nM 5-AZA-CdR treatment produced the highest viral transcripts. The RT-PCR results showed significant increase in viral transcripts for 24-hour 300 nM 5-AZA-CdR treatment in HCT116 cells compared to control Figure 15.

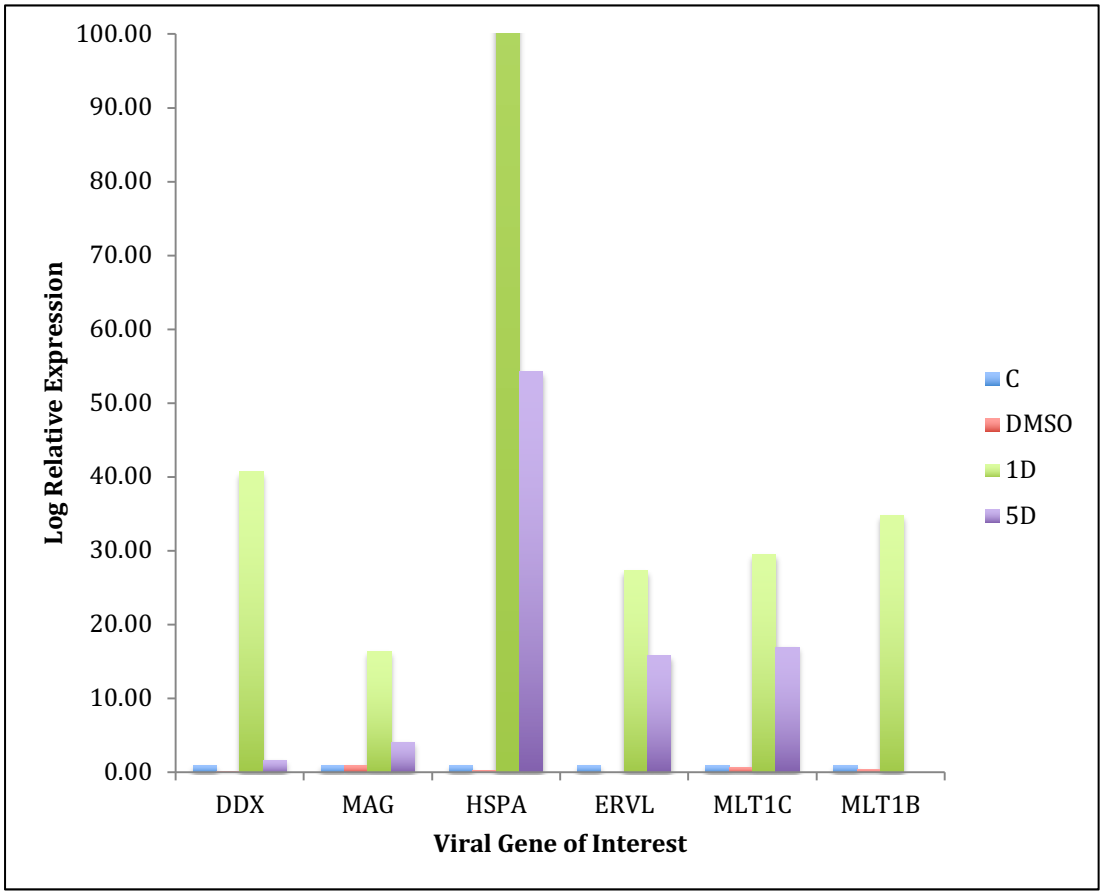

Figure 15. Relative expression of viral genes. Each transcript level was normalized by the acidic ribosomal phosphoprotein P0 (RPLPO) housekeeping gene.

Discussion

Six novel Dicer inhibitors were discovered from experimental HTS assay, figure 12. When the inhibitor putpurogallin (5C3) was directly introduced to MB or HCT116 cells the chemosensitivity did not change. Several factors such as hydrophilicity of the compound and interaction with diverse cellular components could have prevented the Dicer inhibition. However, when celestrol (3E6) and redoxal (2J11) were introduced to HCT-116, cell viability markedly reduced. Specifically, celastrol at 30 µM and redoxol at 100 µM decreased HCT-116 cell viability to 50% compared to control. Moreover, HCT-116 exposed to both 5-AZA-CdR (0.3 µM) and celastrol (30 µM) showed even higher cell death. Our lab is currently using NextGeneration sequencing to determine if this result was due to Dicer inhibition. In conclusion, not all, but some of the small molecules identified in the HTS assay showed increased cell death in colorectal cells (HCT-116).

Conclusion/ Future steps

Our prospective plan is to introduce the small molecules into animal model and determine its impacts. Moreover, we will be screening 100,000 compound diversity set in collaboration with UNC Center for Integrated Chemical Biology and Drug Discovery department.

Our lab focuses on oncological research particularly on medulloblastoma and colorectal carcinoma. Comprehensively we utilize Dicer inhibition as a potential targeted therapy. Our data indicate that small molecules discovered from HTS assay have promising outcome in increasing cell death for both colorectal and medulloblastoma cells. By utilizing these small molecules, we can decrease or potentially eliminate treatments that decrease the quality of life to patients.

Reference

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