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Biological Evaluation of FDA-Approved Drugs as miRNA-31 Inhibitors using Real Time QRT-PCR and a Luciferase Assay

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PROPOSED BIOLOGICAL EVALUATION OF FDA-APPROVED DRUGS AS MIRNA-31 INHIBITORS USING REAL TIME QRT-PCR AND A LUCIFERASE ASSAY

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Abstract: Overexpression of miRNA-31 has been shown to play an essential role in the genesis and development of colorectal cancer (CRC), as well as drug resistance to approved CRC drug therapies (5-FU). The goal of this research was to identify a small molecule inhibitor of miRNA-31 expression by repurposing an FDA approved drug. The compounds were identified using the Psmir database which predicts associations between small molecule compounds and miRNA expression. 14 FDA-Approved drugs were selected and methods for their evaluation were proposed. A luciferase assay for the primary evaluation of miRNA-31 expression still requires optimization, but the expression and purification of the miRNA-31 pmirGLO and empty vector were completed. An optimized qRT-PCR assay for secondary evaluation of miRNA-31 expression was completed, finding miRNA-16 and miRNA-93 as suitable housekeeping genes for qRT-PCR normalization.

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by

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Disclaimer: Due to the abrupt shut-down of labs during the Coronavirus pandemic, parts of the experiments were unable to be completed. Future steps will be discussed in each chapter.

CHAPTER I: INTRODUCTION

1.1. MicroRNAs

MicroRNAs (miRNAs) are a group of post-transcriptional, negative regulators of gene expression, consisting of ~19-24 nucleotides. They were first discovered in 1993 at Harvard University when researchers were investigating the *lin-4* gene in *C. elegans*.[1] The first miRNA observed in mammals was in the *let-7* gene of *C.elegans*.[2] After a search using the basic local alignment search tool (BLAST), the same sequence was revealed to be an exact match to mature *let-7* in *D. melanogaster* and human genomes.[3] *Let-7* is not often studied, as its interaction with LIN28B, a proto-oncogene, is still unknown.[4] miRNAs function by binding to the 3' untranslated region (3' UTR) of a target mRNA, resulting in either destabilization or complete degradation of the mRNA and subsequent inhibition of translation. MicroRNAs have been detected in a wide variety of animal models and considerably conserved across species.[5-9]

The non-canonical pathways are grouped into Drosha/DGCR8-independent and Dicer-independent pathways. Those pre-miRNAs that are generated via the Drosha/DGCR8-pathway resemble Dicer substrates, such as mirtrons.[10-11] Dicer-independent pre-miRNAs are processed via Drosha from short hairpin RNA (shRNA) transcripts. These require AGO2 to be fully processed, as they are too small to be Dicer substrates.[12] This pathway also results in mature miRNA, which is then used to regulate gene expression.

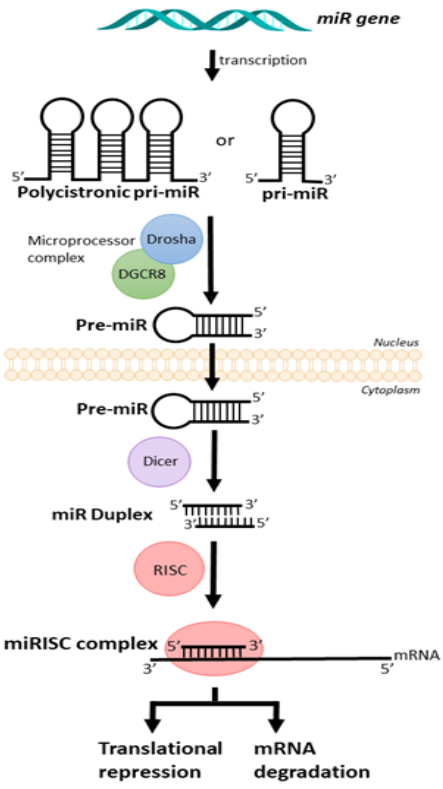


Figure 1.1. Canonical biogenesis pathway of miR. (borrowed from Van Meter, E.N, *et al.*, 2020)

miRNAs regulate gene expression via the miRNA-induced silencing complex (miRISC). It recognizes specific miRNAs via miRNA response elements (mRNEs). A perfectly complementary

interaction results in AGO2 endonuclease activity, resulting in mRNA cleavage.[13-15] In humans, most of the interactions between miRISC and mRNEs are not perfectly complementary. Consequently, the miRISC complex operates as a meditator of RNA interference.

1.2. miRNA-31

miRNA-31 is a highly conserved transcriptional regulator that has been found to play a role in many cellular processes. Its gene is located on the 9p21.3 (chr9:21502114–21502184) near the p16-Arf-p15 locus. The p16 gene is considered a tumor suppressor gene and has antiproliferative biological activity. Mutation and/or deletion of this essential gene have been evaluated in many different types of cancers.[16-18] MiRNA-31's role in cancer cell proliferation is context dependent, meaning it can act as either as a tumor-suppressor or oncogenic gene. As seen in **Figure 1.2**, the normal function of tumor suppressor miRNAs is to target mRNA that produce proteins involved in cancer promoting pathways. If tumor suppressor miRNAs are decreased in the tissue due to dysregulation of the cell, then malignancies will occur. Conversely, oncogenic miRNA inhibits the production of proteins that stop cancer growth, therefore when overexpressed they promote tumor growth and invasion to surrounding tissue.

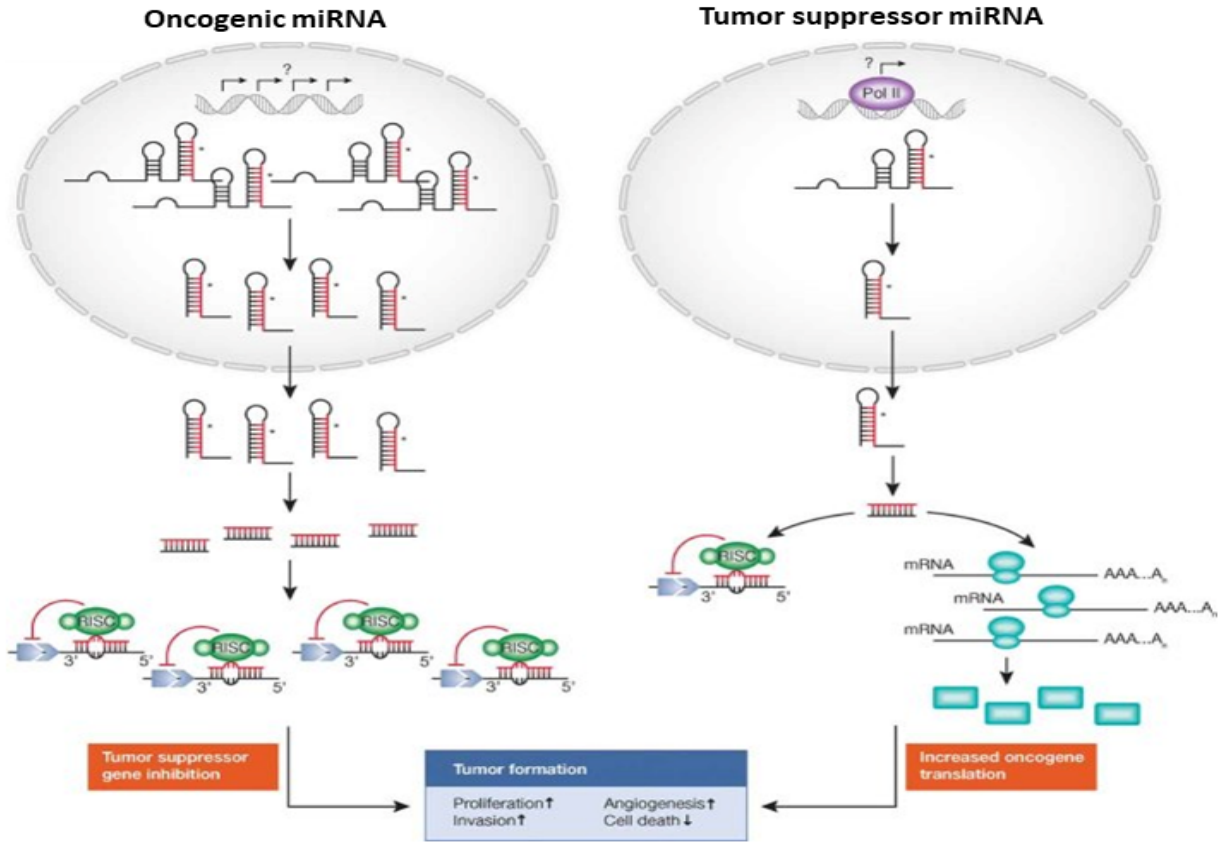


Figure 1.2. Comparison of oncogenic miRNA and tumor suppressor miRNA. (borrowed from Cailin, *et. al*, 2006)

In breast cancer, miRNA-31 is heavily down-regulated and acts as a tumor suppressor.[19] miR-31 prohibits various pathways essential to the survival of cancer. For instance, miR-31 target genes *WASF3*, *RHOA*, and *ITGB* produce proteins that enable cell motility; while *GNA13* and *PKC* allows for cell invasion.[20-23] It is also downregulated in certain leukemias, glioblastomas, hepatocellular carcinoma, ovarian cancer, and bladder cancer.[24-29] In other cases, as in the case with colorectal cancer, miRNA-31 acts as an oncogene and has been shown to be upregulated in many cell types. In one study, miRNA-31 was found to be up-regulated in 91% of a set of 98 colorectal cancer samples.[30]

1.3 Colorectal Cancer and miRNA-31

Currently, colorectal cancer (CRC) is the third leading cause of cancer-related death in the United States and the third most common cancer in men and women.[31] Due to the improved early detection methods, the five-year survival rate for localized colorectal cancer in the United States is at 90%. However, if the disease is not detected early and metastasizes (distant), the five-year survival rate falls to 14%.[32] The current treatment for the disease includes excision of the tumor site, chemotherapy, and radiation. For chemotherapy, 5-fluorouracil (5-FU) has been the choice drug in treating the disease. 5-FU functions as a thymidylate synthase (TS) inhibitor to interrupt the action of this enzyme (citation). By doing so, it blocks the synthesis of the pyrimidine thymidine, a nucleoside required for DNA replication. The side effects of this treatment include hair loss, nausea and vomiting, sores of the mouth, and decreased a decreased white cell count. As patients are continually exposed to the medication, it often results in chemoresistance leading to a relapse in the colorectal cancer.

Recent findings have suggested that miRNAs play a significant role in every stage of CRC initiation, progression, and development, as well as induce resistance to chemotherapy drugs like 5-FU.[33] In particular, research has shown that increased levels of miRNA-31 cause 5-FU resistance in CRC and that suppression of miRNA-31 expression will increase the sensitivity of CRC cells to 5-FU increasing the likelihood of survival of patients, especially in the case of relapse in the cancer. [34-35]

1.4 Drug Discovery and miRNA

Currently, there are multiple strategies in order to disrupt miRNA function. These include miRNA sponges, antisense oligonucleotides (ASOs), CRISPR and small molecule modulators. Each therapeutic strategy reaps its own benefits and disadvantages in terms of disrupting miRNA activity.

miRNA Sponges

miRNA sponges are RNA transcripts that can associate with specific miRNA's via high affinity binding sites, which prevent them from interacting their target mRNAs. Generally, the miRNA binding sites (MBS) are separated by 4-6 nucleotides. These sites are either antisense to the specific miRNA or contain a bulge. The sponges that contain the central bulge are most effective, as perfectly antisense sequences are prone to interference and degradation.[36]

The benefit of this sort of technology is the ability to inhibit multiple miRNAs, possibly a whole family, with multiple binding sites. Furthermore, it presents specific advantages over something like an oligonucleotide. Generally, oligonucleotides are difficult to uptake by the cell and require specific reagents and tedious optimization. Sponges can easily be introduced by a viral vector. The vector can include a selectable marker, making it simple to choose cells that have uptake the vector. Moreover, continuous dosing of oligonucleotides is necessary for long-term efficacy. However, given sponges operate via a vector, they can continuously be generated within the cell.

Given the large number of binding sites, and the relatively small number of nucleotides for miRNAs, there is the potential for nonspecific interactions with miRNAs that were not intended to be sequestered. Careful optimization is required to make sure that nonspecific miRNA binding does not occur.[37] The most significant factor is concentration. If the miRNA concentration is very high, titrations of the sponge must also be considerably large.

These sponges have been successfully used to study the role of miRNA-31 in certain cancers. In a study by Valstyn et al., experimenters transduced nonmetastatic breast cancer cells with retroviral eGFP sponges for miR-31 in mouse mammary fat pads.[38] Compared to the negative control, the miR-31 sponge containing tumors metastasized to the lungs of the mouse, forming 10x more lesions, measured by GFP fluorescence. This led researchers to conclude that miRNA-31 in nonmetastatic breast cancer was a tumor suppressor.

Antisense Oligonucleotides (ASOs)

ASOs are a fairly practical approach to miRNA modulation. Essentially, the oligonucleotides are designed with a sequence that is the complement to the miRNA of interest. The target miRNA then becomes tightly bound to the oligonucleotides, where it will eventually be degraded by the cell.

Although seemingly straightforward, there are many limitations to the use of antisense oligonucleotides. ASOs require the use of expensive transfection reagents to introduce them to cells. Furthermore, AOSs are chemically unstable in the cell and require tedious optimization in order to determine the correct concentration, and more importantly the time of the inhibitor exposure. Although ASOs are successful at inhibiting mature miRNA, they are unable to inhibit precursor miRs, limiting them to a single step biogenesis pathway to exert activity. As a drug, ASOs suffer from poor delivery efficiency and tissue distribution. In human subjects, they have been found to have more serious side effects due to off-target effects.[39-40] Improvement of ASO specificity is an active research topic. In particular specialized formulations enable precise delivery of the ASO to a specific tissue/mRNA target has helped increase the therapeutic relevance of ASOs.[41] Several ASOs therapies have since been approved by the FDA for various diseases.[42] As of 2017, these include Vitravene, Macugen, Kynamro, Eteplirsenn, Defitelio, and Spinraza. These ASOs have been used to treat a wide variety of conditions, including cytomegalovirus (CMV) retinitis, age-related macular degeneration (AMD), homozygous familial

hypercholesterolemia (HoFH), Duchenne muscular dystrophy (DMD), severe hepatic veno-occlusive disease (sVOD), and infant spinal muscular atrophy, respectively.

CRISPR

The use of CRISPR (clustered regulatory interspaced short palindromic repeats) technology has gained widespread attention for its potential use in treating genetically related disorders. The system for gene editing is composed of CRISPR, *Cas9*, an endonuclease from S*. pyrogenes,* and a guide RNA. The system operates by guide RNA binding to a specific sequence on the target DNA, along with binding to Cas9. Cas9 then cuts the DNA at the targeted location. Then, using the cell's own repair machinery, the DNA sequence can be changed, deleted, or replaced by a customize sequence. Research has shown that this technology can be used in order to knock down miRNA expression levels both *in vivo* and *in vitro*.[43] This is accomplished by targeting the terminal loop, or 5' region of the pre-miRNA, or by targeting the secondary loop structures of primary miRNAs. The CRISPR/cas9 system has been shown to decrease the expression levels stably in cells for up to 30 days.

The most significant disadvantages of this technology are the ethical concerns raised by genome editing and the issue of "off-target" modifications. Because the cells use their own repair machinery, additional mutations, duplications, or deletions could potentially affect the DNA sequence that is being targeted.[44-45] In humans it is unknown what affect these modifications could have on the complex biochemical processes that occur in the body. Mosaicism is also a significant concern.[46] This occurs when some cells retain the edit, but other cells do not.

Small Molecules

Small molecule compounds are viewed as an ideal solution for developing inhibitors. These compounds have many distinct advantages, over the previously mentioned miRNA-targeting therapeutic strategies. Small molecules drugs can be easily designed to be easily delivered and permeate the cell

membranes through traditional medicinal chemistry efforts. Small molecules can target any part of the miRNA biogenesis pathway either through direct engagement to the stem-looped pri- or pre- miRNA structures or auxiliary proteins that facilitate miRNA biogenesis.[47-48] Much is still being learned about the design of small molecules that modulate miR activity. For one, pri- and pre- miRNA do not have complex tertiary structure like proteins, therefore, the medicinal chemistry "rules" for designing drug-like molecules that target the secondary structure motifs of miRNA are still being defined.

1.5 Current Status of miRNA-31 targeting small molecules

Currently, there are few compounds that have been shown to modulate miRNA-31 activity. Among these compounds are aminosulfonylarylisoxazole analogues, as identified by Im *et. al*, shown in **Figure 1.3**.

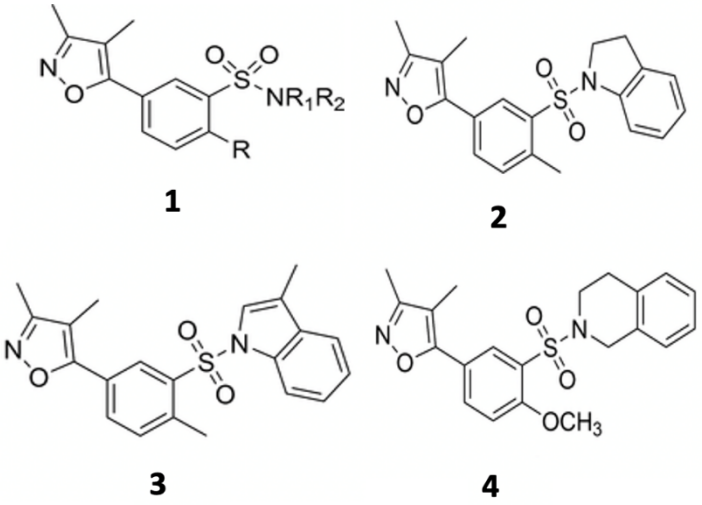


Figure 1.3. Aminosulfonlyarylisoxazole analogues. "R" and "R1R2" represent areas in which additional functional groups may be added (borrowed from Im et. al, 2017)

Three analogues of this compound have been shown to selectively inhibit miRNA-31 activity, as shown in **Figure 1.3.** Compound 1 represents the aminosulfonlyarylisoxazole scaffold upon which compounds 2-4 were synthesized. Compounds 2 and 4 specifically inhibited the expression of miRNA-31, while not affecting other miRNAs tested which included. Compound 3 significantly lowered the

expression levels of miRNA-21. Reduction in the target mRNAs of miRNA-31 including *STK40*, *E2F2*, and *PPP2R2A* was also shown. It was found that these compounds affected the maturation of miRNA-31 into mature miRNA from pri- and pre-miRNA-31. Similar inhibitory effects were observed when treated with a miRNA-31 mimic, when compared to the compounds.[49]

1.6 Drug Repurposing

Drug repurposing (repositioning) involves redeveloping a drug compound for use in a different disease.[50] The repurposing of these compounds is advantageous, as FDA-approved drugs have detailed information relating to their pharmacology, formulation, dose, and toxicity.[51] Moreover, these drugs are generally more accessible for purchase, rather than having to be synthesized directly. The differentiated use of these compounds also results in decreased cost and less development time.

The targeting of RNAs as a therapeutic strategy has become increasingly understood. RNA is a key drug target, as it is upstream of many pathological mechanisms involved in disease states.[52-53] In relation to miRNA, drug repurposing is especially relevant. Numerous FDA drugs that have been designed to target protein to promote a biological effect are typically evaluated for off-target effects and selectivity against similar proteins. However, it has become increasingly evident that these protein-targeting drugs also affect miRNA expression. For instance, Tamoxifen, Cisplatin, 5-FU, and Docetaxel have been shown to modulate miRNA levels in drug resistant breast cancer cell lines.[54]

CHAPTER II: IDENTIFICATION OF POTENTIAL MIRNA-31 INHIBITORS USING Psmir COMPUTATIONAL TOOL

2.1 Introduction: Psmir Base

Psmir is a large-scale database that uses computational mathematics to predict small molecule-miRNA associations via comparison of current gene expression profiles. The database was created by Fanlin Meng *et. al* at Harbin Medical University.[55] The database predicts associations of small molecules

with specific miRNA and scores them from-1.0 to 1.0. The closer the number is to -1.0, the more potent of an inhibitor of miRNA-31. In order to calculate these values, the researchers collected gene expression profiles using keywords including “miRNA transfection” and “microRNA transfection” from the Gene Expression Omnibus (GEO) database. Moreover, small molecule-perturbed gene expression profiles were obtained from the Connectivity Map (cmap). Probes were screened if they could be perturbed by one small molecule or one miRNA, which then translated to the degree of probes perturbed by one small molecule, or miRNA over the entire expression profile.

The size of the score itself related to the similarity between the small molecule and the miRNA. These scores were combined between the same small molecule and miRNA. This data was then compiled into the Psmir databse. Each listed compound includes the chemical name, whether it is FDA approved, the miRNA it interacts with, the gene the miRNA regulates, the score, p-value, and a link for further details. An example of a compound is shown in **Figure 2.1** below.

Figure 2.1. Example of information provided by psmir during searches. Aminophenazone was identified as a miRNA-31 inhibitor, is FDA approved, with a score close to -1 meaning it is a good inhibitor based on the program.

2.2. Results using Psmir

Compounds were selected based on their ability to inhibit miRNA-31, along with FDA approval. **Table 1** below outlines the compounds selected, with their current FDA approved uses.

Table 1. List of miRNA-31 inhibitors identified by psmir, their FDA approved purpose, scores, and NCI numbers.

Compounds	Current Role	*Score Value	NCI #
Aminophenazone	Analgesic	-0.619	4993
Chlorpromazine	Antipsychotic	-0.302	167745

Clotrimazole	Antifungal	-0.585	257473
Hydroxyzine	Anxiety disorders and Allergic Conditions	-0.511	169188
Methantheline bromide	Antimuscarinic agent (anticholinergic)	-0.589	32145
Metyrapone	Glucocorticoid synthesis inhibitor	-0.615	25265
Nitrofurantoin	Antibiotic	-0.568	44150
Phenazone	Non-narcotic analgesic, antipyretic and NSAID	-0.794	7945
Physostigmine	Anticholinergic agent	-0.717	30782
Procyclidine	Muscarinic antagonist	-0.607	169103
Raloxifene	Selective estrogen receptor modulator	-0.468	747974
Rescinnamine	Antihypertensive	-0.645	15628
Riluzole	Antidepressant	-0.562	759823
Trimethadione	Anticonvulsive and antiepileptic	-0.599	169503

*Scores closer to -1 estimated as more potent inhibitors of miR-31

The small molecule compounds identified in the Psmir database were requested from the Developmental Therapeutics Program though the National Institutes of Health National Cancer Institute (NIH NCI). Not all compounds were available through this program, resulting in 14 compounds for analysis. They arrived in solid form and were serially diluted at a 1:3 for future studies. Structures for the compounds are shown in **Figure 2.2**.

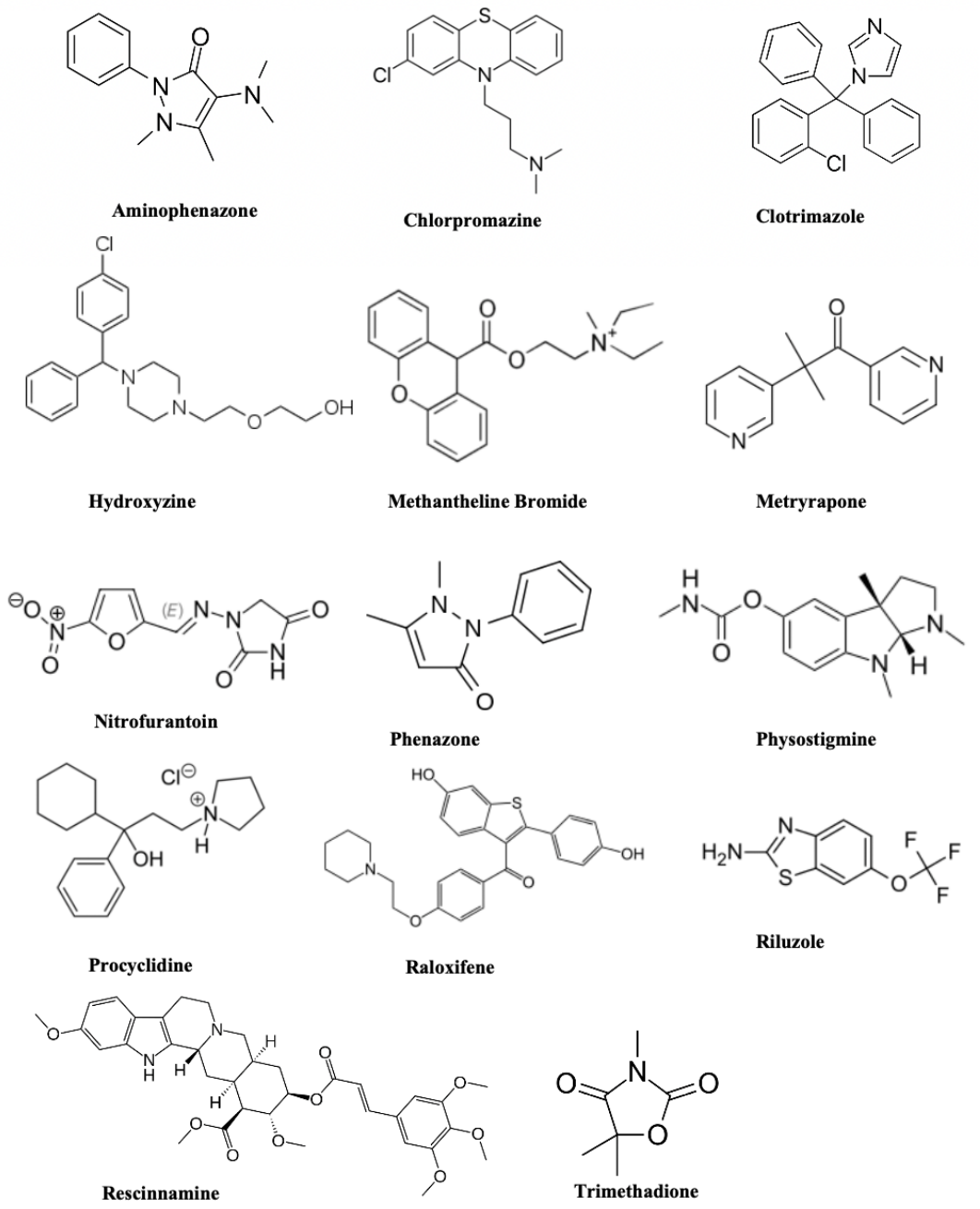


Figure 2.2. Compound structures identified at miR-31 inhibitors in psmir. Compounds will be tested in cell-based assays in the future.

2.3. Conclusions

The compounds listed in **Figure 2.2** were chosen based on their perceived ability to inhibit miRNA-31, FDA-approval, and their availability from NCI. Although there are no biochemical assays that

evaluated their potency, the mathematical relationship that exists between these compounds and their ability to inhibit miRNA-31 is the foundation for what this project explores. Although the data from biochemical assays has yet to be understood, these compounds will serve as the test compounds for future experiments, as outlined in this paper. The compounds are expected to show activity based on the theoretical approach taken by the creators of psmir base. As showcased in **Figure 2.2**, every compound has a nitrogen containing region. This generates a positive electrostatic charge, which would allow them to interact with the negatively charged miRNA.

CHAPTER III: EVALUATION OF SMALL MOLECULE COMPOUND MODULATION OF miRNA-31 EXPRESSION LEVELS USING A LUFICERASE ASSAY

3.1. Background

The luciferase assay has been used for the detection of mature miRNA since its first use by Deiters *et. al* in 2008. However, Deiters *et. al* utilized the psiCHECK vector, whereas this experiment uses the pmirGLO vector. Briefly, the reverse complement of the miR of interest was inserted downstream of a firefly luciferase gene, which contains the miR binding sequence (**Figure 3**). Without an inhibitor, the miRNA of interest can bind to the miR-binding sequence in the reporter plasmid thus inhibiting translation of the luciferase gene (**Figure 3.1.A**). Without the luciferase enzyme being produced, bioluminescence will not be observed even in the presence of luciferase substrates. In the presence of an inhibitor (**Figure 3.1.B**), the miRNA does not bind to the miRNA-binding sequence, therefore, allowing for translation and production of luciferase and a measurable signal of bioluminescence when substrates are introduced.

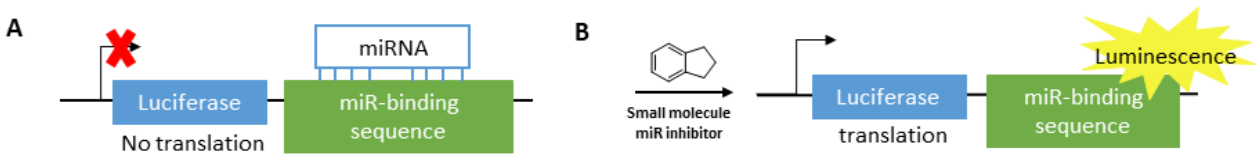


Figure 3.1 Luciferase-based assay for detecting miR expression. **A)** Upon the binding of miRNA-31 to its miRNA response element on its target mRNA, the luciferase gene is unable to be translated. **B)** A small molecule inhibitor of miRNA-31 allows for luciferase translation and the measurement of luminescence.

This use of luciferase technology for miRNA expression analysis allows for investigation of small molecule that may act upon any part of the miRNA biogenesis pathway. This means a small molecule could be inhibiting the expression of miRNA-31 by targeting a protein along the pathway or directly interacting with the stem-looped structure of pri- or pre-miRNA-31.

We have chosen to use the pmirGLO vector (Promega) to construct our pmirGlo-miRNA-31 reporter plasmid because it contains both *Firefly* and *Renilla* luciferase genes which allows for normalization of signal account replicates and account for extraneous variables affecting the validity of each assay. As seen in **Figure 3.2**, the Firefly luciferase reporter enzyme requires D-luciferin, ATP, O_2, and Mg^{2+} to produce bioluminescence and resulting products (oxyluciferin, PP_i, AMP, and CO_2) while the *Renilla* luciferase enzyme needs coelenterazine and O_2 to produce bioluminescence and coelenteramide and CO_2 products. This works to our advantage because the Firefly luciferase substrates can be added first and bioluminescence read followed by the addition of *Renilla* luciferase substrate and the quantification its light product. Our compounds should only affect the translation of the reporter gene, *firefly* luciferase, and not *Renilla* luciferase*. Note:* Due COVID-19 and the shutting down of research labs, the experimental procedures discussed below is proposed based on literature and will be tested by future researchers.

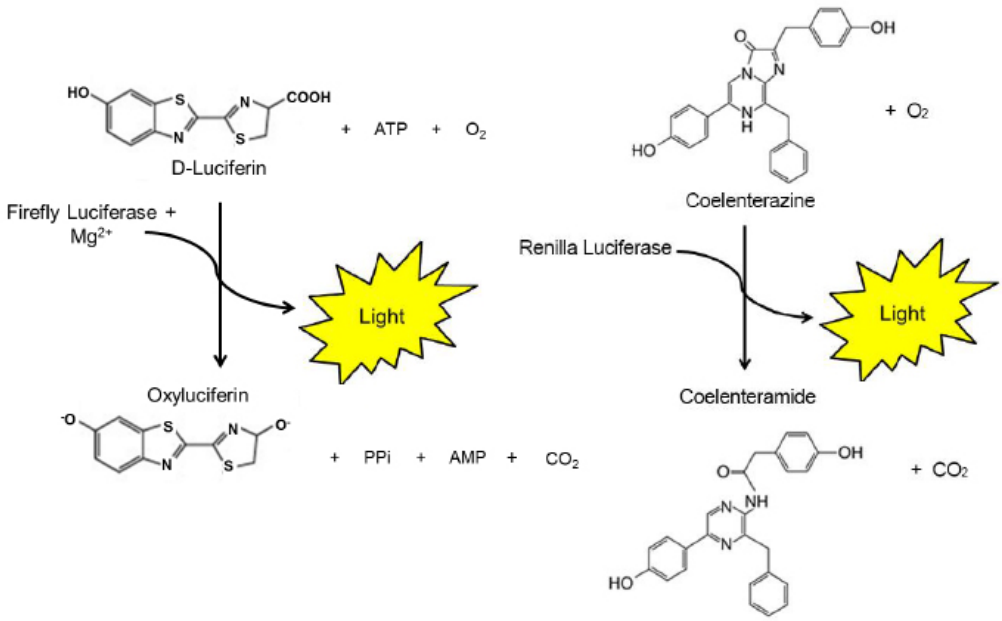


Figure 3.2. Firefly luciferase substrates vs Renilla luciferase substrates. The Firefly luciferase enzyme requires different substrates than the Renilla luciferase making them ideal for normalization in a Dual Luciferase study. (Image borrowed from Promega)

3.2. Experimental Procedure

3.2.1. General reagents and instrumentation for experiments *(proposed)*

Small molecules identified in Psmir database were requested from the Developmental Therapeutics Program though the National Institutes of Health National Cancer Institute (NIH NCI). Small molecules (5 mg) were diluted in DMSO to make 10 mM stock solution that were stored in -20 ^{o}C. 1:3 serial dilutions were done in 96-well polypropylene plates with DMSO and placed in 384-well opaque compound plates with concentrations ranging from 2mM to 0.10uM. Compound plates were sealed with aluminum tape and stored at -20 ^{o}C to avoid degradation.

HCT-116 colorectal cancer cells were chosen as our cell model because they have been shown to overexpress miRNA-31 and are easily transfectable.[56] Cells were purchased from ATCC and initially cultured in 75-cm^2 flasks (Falcon) using McCoy's 5A modified media (Gibco) supplemented with 10% FBS and incubated at 95% humidified air under 5% CO_2 at 37°C. Cells were transitioned to DMEM High Glucose Media (Gibco) supplemented with 10% FBS prior to use in this assay.

The pmirGLO Dual-Luciferase miRNA target expression vector was purchased from Promega and the reverse complement of the 5p mature miRNA-31 strand was cloned in by GenScript (sequence: 5' AGCTATGCCAGCATCTTGCCT 3', obtained from miRBase).The empty pmirGlo vector and pmirGlo-miRNA-31 plasmid will be transfected into HCT-116 colorectal cancer cells (ATCC) at a final concentration of 0.016 µg/µL using Lipofectamine 2000 and Opti-MEM reduced serum medium (Gibco) according to manufacturer's protocols. The empty vector will represent 100% translation of the luciferase gene and will be used as a positive control. Other controls for the assay will be 1% DMSO (negative control) and IDT miR-31 inhibitor (positive control) at a final concentration of 15 nM. Upon arrival, 5 nmols of IDT miRNA-31 inhibitor will be diluted in 50 uL autoclaved TE buffer (pH = 8) to 100 uM concentration, aliquoted into 10 uL portions, and stored at -20 C.

Dual-Glo Luciferase Assay System was purchased from Promega (cat. no. E2920) and used to quantify both firefly and *Renilla* luciferase bioluminescence signals according to manufacturer's protocols. Assays will be conducted in 384-well white optical bottom plates (Nunc, cat. no. 142762) and white tape was placed on the bottom before reading the plates for bioluminescence in a Tecan Spark multimode plate reader.

3.2.2. Transformation of pmirGLO miRNA-31 and purification *(completed)*

The pmirGLO-miRNA-31 plasmid (**Figure 3.3**) was transformed a JM109 competent cell line (Promega) via heat shock. An isolated colony was selected using ampicillin plates. The culture was grown up to 500 mL and purified using the *Endofree Plasmid Mega Kit* (Qiagen, cat. no. 12381). The vectors were stored in 1X TAE buffer in the -20C freezer. For plasmid verification,

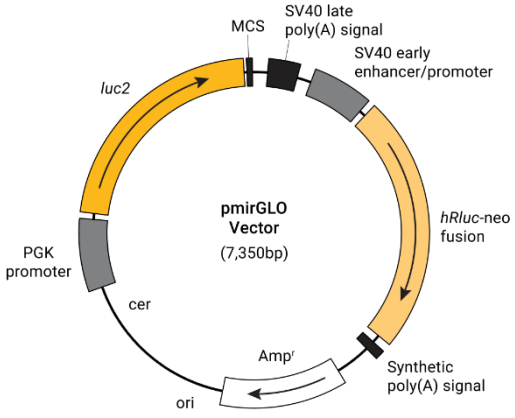


Figure 3.3. pmirGLO vector construct. The inverse complement of miRNA-31-5p was inserted between Sac1 and Sal1 cut sites downstream of the *luc2* (*firefly* gene). (Image borrowed from Promega)

the constructs were cut using HI EcoRI for 1hr at 37C, resulting in a 7350bp strand of linear plasmid. Once digested, the vectors were run on a 0.8% agarose gel with SYBR Gold and visualized using a Bio-Rad gel imaging system.

3.2.3. Dual-Glo Luciferase Assay protocol *(proposed)*

HCT-116 cells will be grown in a 75-cm^2 flask and 6-well plates (Falcon) using DMEM (Gibco) supplemented with 10% FBS (Gibco) until 70-80% confluence (5% CO_2 at 37°C). Once cells reach the desired confluence, the T-75 flask will be transfected with the pmirGLO-miRNA-31 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. One well in the 6-well plate will be transfected with the empty vector and another with pmirGLO-miRNA-31 and an ASO for miR-31 using Lipofectamine 2000. After 24 hours, HCT-116 cells will be harvested and plated into 384-well white optical bottom plates pretreated with Matrigel using the assay media DMEM without phenol red and FBS. Each well will contain 10,000 cells. Plates will be centrifuged at 1000 rpm and incubated (5% CO_2 at 37°C) for 4-5 hours or until they are fully adhered to the plates. Small molecules identified through Psmir will be introduced at to each well in a dose-responsive manner. Using a pintool (V&P Scientific, Inc.), 100 nL of each compound will be transferred to each well containing 20 µL of media. We will do two transfers (200 nL) to obtain a final maximum compound concentration of 20 µM and minimum concentration of 1 nM. Because DMSO is toxic to cells, two transfers ensure a safe limit of 1% DMSO. After 24 hours, Dual-Glo Luciferase substrate (Promega), which contains the substrates required for Firefly luciferase and *Renilla* luciferase, will be added according to manufacturer's protocols (1:1 media to reagent ratio). Plates will be read at t = 10 minutes to ensure full lysis of cells.

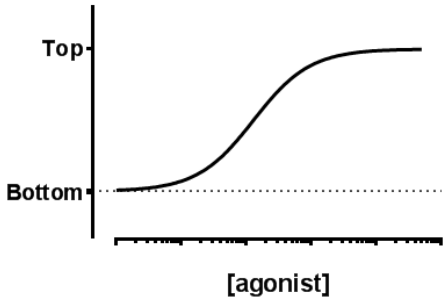
Controls will be measured in each plate to determine Z factor (**Equation 1**) and to enable data normalization.

$$Z' = 1 - \frac{3(\sigma_p - \sigma_n)}{|\mu_p - \mu_n|}$$

Equation 1. Z' factor equation. 'p' subscript indicates a positive control, whereas 'n' subscripts indicate negative controls.

Three independent experiments will be performed in quadruplicate and data will be analyzed using nonlinear regression with variable slope (GraphPad Prism 8, **Equation 2**).

$$Y = \frac{Bottom + (X^{hillslope})(Top - Bottom)}{(X^{hillslope} + EC50^{hillslope})}$$



Equation 2. Nonlinear regression equation. EC_{50} is the concentration of agonist (in our case antagonist) that gives a response half-way between Bottom and Top. Hillslope is the steepness of the family of curves and is typically 1. Top and Bottom are plateaus in the units of the Y axis.

3.2 Results and Discussion

3.2.1. Transformation and purification results *(completed)*

The digested pmiRGlo purification products were run in a 1.0% agarose gel and stained with SYBR Gold to be visualized. As shown in **Figure 3.4**, all products appear at 6-10 kilobases (kb), although ranging concentrations. In the future, Lane 2, 4, 5, should be re-tested at lower concentrations in order to determine more accurately their size. However, this showcased that the products were free of contaminating DNA, along with being in right range of molecular weight (7350bp). As showcased in Figure 3.4, miR-18a was also

tested, alongside miR-31. The "scramble" vectors, as labeled in Figure 3.4 are pmirGLO vectors of which the insert was generated incorrectly. Instead of the reverse complement being inserted into the pmirGLO vector, the exact sequence of the miRNA was inserted. Therefore, there would be no miRNA binding. However, due to the same number of bp, the vectors for the scramble and true pmriGLO vectors are the exact same size. Moreover, the mir-18a pmirGLO vector will be used in future luciferase experiments and is not relevant to the project being discussed. However, like the scramble vector, the mir-18a pmirGLO vector is similar in size to the mir-31 pmirGLO vector (7350bp), plus or minus a few base pairs. The empty vector contains the pmirGLO construct but does not have the miRNA binding site insert. The concentrations of the vectors are listed in the Table 2.

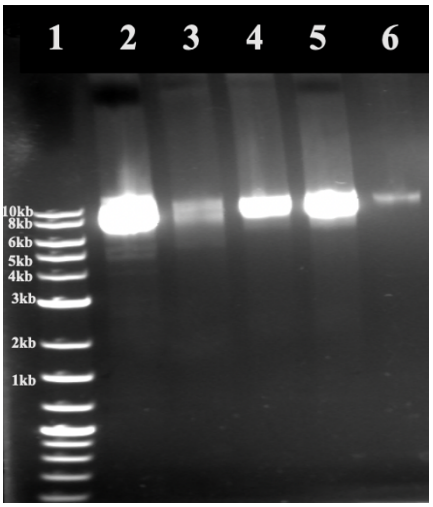


Figure 3.4. Gel confirming pmirGlo-miRNA-31 transformation and purification. Purple DNA Ladder (1), Scramble pmirGLO- miRNA-31 (2), Scramble pmirGLO- miRNA-18a (3), pmirGLO- miRNA-31 (4), pmirGLO-miRNA-18a (5), pmirGLO-Empty vector (6)

Table 2. Concentrations of pure pmirGLO plasmids.

Sample	Amount (ug/uL)
miRNA-31 pmirGlO (1)	0.5038
miRNA-31 pmirGLO (2)	0.1941
Empty pmirGLO	0.2879

3.2.2. Luciferase assay results *(not completed)*

No results were obtained due to time conflicts and the Covid-19 shutdown.

3.3 Future experiments and conclusions

The transformation and purification of the pmirGlo-miRNA-31 and empty plasmids were successful, although a new gel with lower and consistent concentrations of DNA loaded should be

performed. Vectors were pure and yielded working concentrations that were easily manipulated for future experimentation. The assay itself was not optimized due time and Covid-19, however, based on the manufacturer's protocols and the literature I am confident that future researchers will have success with my proposed protocol. Once optimized, future researchers will be able to screen the 14 psmir database compounds for miR-31 inhibitory activity. This will serve as the primary assay for testing of miRNA-31 inhibitors and that can be used with other miRNA investigated in the Teske group (i.e miRNA-18a). In summary, although I did not have an opportunity to optimize my proposed luciferase assay protocol, I was successful in transforming the designed vectors and purifying them for future studies.

CHAPTER IV: EVALUATION OF SMALL MOLECULE INHIBITION miR-31 EXPRESSION USING qRT-PCR

4.1. Background

Real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is an effective method used to measure the amount of RNA (coding or noncoding) in a given sample. Essentially, qRT-PCR operates by first isolating the RNA from a given sample, in this case HCT-116 cells. Once the RNA is purified, it is reverse transcribed using a reverse transcriptase and highly specific primers for miRNA to generate cDNA. That cDNA can then be used in qRT-PCR. Fluorescent probes bind to a sequence on the RNA, and during each amplification step, are released, generating fluorescence. These fluorescence values can be used to quantitatively determine the amount of nucleic material in a given sample. It is particularly useful in screening small molecule inhibitors. In our case, an inhibitor would decrease the amplification levels of miRNA therefore generating lower fluorescence values.

Because miRNA are short nucleotide strands (18-22 nt), the reverse transcription (RT) step becomes difficult with the typical linear primers. Because these primers can be non-specific with such short strands, amplification of other RNA products in addition the miRNA of interest can occur. This has since been remedied by using stem-looped forward primer which increases the length of the miRNA

nucleotide strand from about 22 bp to 60 bp (**Figure 4.1**).[57] This decreases the likelihood of nonspecific binding. Stability of the miRNA is also an issue, especially in the reverse transcription protocol. A pulsed RT transcription protocol is typically applied to fix that issue (see section 4.2.2). Essentially, this decreases the likelihood of miRNA degradation, due to prolonged thermal exposure. Furthermore, it allows multiple opportunities for the miRNA to bind to the stem-loop primers, resulting in increased copy DNA (cDNA) levels. Other precautions, including avoiding multiple freeze-thaw cycles of the isolated miRNA, and keeping samples on ice, can also be instituted.

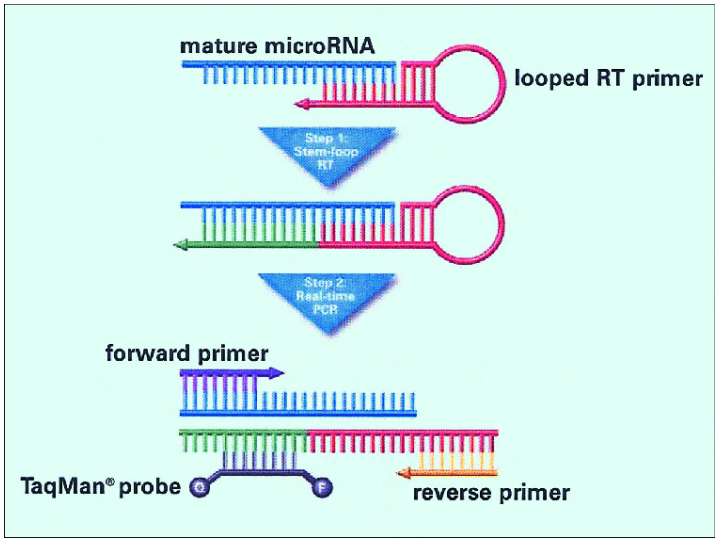


Figure 4.1. Taqman qRT-PCR for miRNA. This technology for evaluating miRNA expression uses a stem-looped RT primer to ensure specific amplification. (borrowed from Springer, 2005)

Selecting suitable housekeeping genes (HKG) for qRT-PCR analysis is also a challenge because amplification levels of miRNAs vary based on the cell type and environmental conditions. A suitable HKG is one that is stable across sample types, has relatively high amplification levels, and is biochemically similar to the target. Candidate HKGs were selected based on a thorough literature search focused on HCT-116 cells. The four HKGs that were selected to be tested included miRNA-93, miRNA-25, SNORD44 (RNU44), and miRNA-16. Both miRNA-93 and miRNA-25 were evaluated to determine the most suitable HKGs by Niu *et al.* in HCT116 and MCF-7 (breast cancer) cells.[58] RNU44 was found by Morata Torifa *et al.* to be an adequate candidate for comparing healthy cells to cancerous cells as a normalizer.[59] miRNA-16 was find by Rinnerthaler *et al.* to be a suitable HKGs.[60] Furthermore, miRNA-16 recommended by Applied Biosystems as to be one of the most stably expressed miRNAs among 59 of NCI's 60 cell lines, which includes HCT116.[61]

Note: Due to Covid-19, the psmir compounds were not tested for miRNA-31 inhibition in qRT-PCR.

4.2 Experimental Procedure

4.2.1 General reagents and instrumentation

HCT-116 cells were purchased from ATCC and cultured in 75-cm^2 flasks (Falcon) using McCoy's 5A modified media (Gibco) with 10% FBS (Gibco) and incubated at 95% humidified air under 5% CO_2 at 37°C. The *mirVana* miRNA Isolation Kit (Invitrogen, cat. no. AM 1560), Taqman microRNA Reverse Transcription Kit (Applied Biosystems, cat. no. 4366596), and Taqman Fast Advanced Master Mix (Applied Biosystems, cat. no. 4444556) were utilized for this study. Probes included the HKGs: hsa-miRNA-25-3p, hsa-miRNA-93-5p, RNU44 (hsa-SNORD44), and hsa-miRNA-16-1*-3p; and hsa-miRNA-31-5p (gene of interest [GOI]). All probes were purchased from Thermofisher Scientific. RT to produce cDNA was accomplished using Applied Biosystems Veriti 60 Well Thermocycler. qRT-PCR was performed on an Applied Biosystems QuantStudio 3 qRT-PCR system. Data was analyzed using GraphPad Prism 8.

4.2.2. Real Time qRT-PCR Protocol for analysis of HKGs *(completed)*

Cell culture for qRT-PCR

Using a 70-80% confluent 75-cm^2 flask, HCT-116 cells were plated in a 24-well plate at 100,000 cells/well in 400 µL McCoy's 5A media supplemented with 10% HI FBS. 400 µL of media was added to empty wells to prevent evaporation while cells incubated overnight at 37 ^oC and 5% CO_2. After 24 hrs, growth media was removed and 400 µL of Assay Media (McCoy's 5A without HI FBS) was placed in each well. The cells were incubated again overnight at 37 ^oC and 5% CO_2. We chose to plate cells in FBS containing media and then replace it with media without FBS to ensure proper cell adherence and growth prior to drugging. Although, we did not drug cells with small molecules in this section it was important to do the same procedure while testing HKGs.

miRNA isolation form HCT-116 cells

After overnight incubation to FBS-free media, miRNA was isolated using the *mirVana™* miRNA isolation kit following the manufacturer's protocol. Media was aspirated from each well and 500 µL of

cold PBS was added carefully as to not disrupt the cells. The PBS was removed and 600 µL of the Lysis/Binding solution was added to each well and mixed thoroughly. The lysates were transferred to 2 mL microcentrifuge tubes. 60 µL (1/10 volume) of miRNA homogenate additive was added to each tube containing the lysate and pipetted in order to generate a homogenous mixture. Microcentrifuge tubes were then placed on ice for 10 minutes. 600 µL of an Acid-Phenol: Chloroform was added to each microcentrifuge tube and vortexed for 30-60 seconds. The tubes were then centrifuged for 5 mins at 10,000 x g at room temperature. The upper (aqueous phase) was removed and transferred to a fresh tube. Elution solution provided in the kit or nuclease-free water was heated to 95 ^{o}C before being used. 750 µL of 100% ethanol was added to the aqueous phase containing the isolated miRNA. Tubes were mixed by inversion. A filter cartridge was placed into labeled collection tubes for each sample and the lysate-ethanol mixture was added to the filter cartridge (700 µL maximum). The collection tubes were centrifuged for 15 seconds at 10,000 x g to pass the mixture through the filter and was repeated until all the liquid had passed through. 700 µL of miRNA Wash Solution 1 was added to the filter cartridge and centrifuged for 5-10 seconds to pass the solution through the filter. Two portions of 500 µL of Wash Solution 2/3 were added to the cartridges, centrifuging at 10, 00 x g for 1 min after each portion. After the washes were completed, the tubes were centrifuged at 10,000 x g for 1 min in order to remove residual fluid. The filter cartridge was then transferred to a fresh, labeled collection tube and 100 µL of preheated Elution Solution (or nuclease-free water) was applied to the filter. The cap was closed and centrifuged at 10,000 x g for 1 minute. RNA was stored for up to one week at -20C.

Reverse Transcription

Reverse Transcription of the miRNAs was carried out using the TaqMan MicroRNA Reverse Transcription Kit. The reverse transcription components were thawed on ice and centrifuged in order to collect on the bottom. The RT master mix was prepared with 0.15 µL of 100 mM dNTPs, 1.00 µL of 50 U/nL MultiScribe Reverse Transcriptase, 1.50 µL of 10X RT Buffer, 0.19uL of 20 U/nL RNase Inhibitor and

4.16 µL of Nuclease Free Water. 7uL of the Reaction Mix was placed in a RT tube along with 5 µL of the isolated miRNA. 3 µL of 5X RT Primer was added to the tube, specific for the miRNA or HKGs, to make a final solution of 15 µL. Thermal Cycling was carried out using the Applied Biosystems Veriti 60 Well Thermocycler according to the Pulse RT protocol in Table 3. cDNA products were not stored, as a stability experiment has not been performed.

Table 3. Pulsed RT protocol used in Applied Biosystems Veriti 60 Well Thermocycler.

Step	Temperature	Time	Cycles
Reverse Transcription (1)	16 ^{o}C	30 mins	1
Reverse Transcription (2)	30 ^{o}C for 30s 42 ^{o}C for 30s 50 ^{o}C for 1s	30 mins	60
Stop Reaction	85 ^{o}C	5 mins	1
Hold	4 ^{o}C	Hold	N/A

qRT-PCR

qRT-PCR was performed using the TaqMan Fast PCR Master Mix. The master mix was inverted, centrifuged, and placed on ice. qRT-PCR was run with the GOI (miRNA-31) and HKG (hsa-miRNA-25-3p, hsa-miRNA-93-5p, RNU44 (hsa-SNORD44), and hsa-miRNA-16-1*-3p), a nuclease-free water control, a no reverse transcriptase (miRNA) control, and a no template control (master mix). Each PCR reaction contained 0.50 µL of the TaqMan miRNA Assay (probe), 5.00 µL of the TaqMan Universal PCR Master Mix, 3.50 µL of Nuclease Free Water, and 1.00uL of the cDNA Template. The PCR plate was kept on ice during this process in order to prevent degradation. The master mix, cDNA, and probe were all mixed into a single tube before transferring to the PCR plate, making sure to add the probe last. After all components were mixed together, 10 µL of each reaction was added into the appropriate wells, making

sure to avoid creating bubbles. The plates were sealed with optical adhesive film and centrifuged at 1000 x g for two minutes. Samples were run according to the protocol listed in Table 4 in an Applied Biosystems QuantStudio 3 qRT-PCR system. Each sampled was tested in triplicate.

Table 4. qRT-PCR protocol used for Taqman assays in the Applied Biosystems QuantStudio 3 qRT-PCR system.

Step	Temperature	Time	Cycles
UNG Activation	50 ^{o}C	2 mins	1
Enzyme Activation	95 ^{o}C	20 secs	1
Denature	95 ^{o}C	1 sec	45
Anneal	60 ^{o}C	20 secs	45

4.3 Results and Discussion

4.3.1 Determination of suitable HKG *(completed)*

Multiple HKG candidates were chosen based on thorough literature search. A suitable HKG is one that is biochemically similar to the target, has relatively high expression levels, and is stable across sample types. hsa-miRNA-25-3p, hsa-miRNA-93-5p, RNU44 (hsa-SNORD44), and hsa-miRNA-16-1*-3p and GOI hsa-miRNA-31 were all evaluated using the qRT-PCR protocol listed in the experimental section. The comparative C_t method used to evaluate the paper by Silver *et. al* to evaluate the variability of HKGs within a given assay.[62] The calculations for this graph were performed in GraphPad Prism v8 and calculated in the following manner as showcased in **Equations 3 and 4**.

$$\Delta Ct(HGX) = C_t\,(gene\ of\ interest) - C_t\,(housekeeping\ gene) \qquad \textbf{Equation 3}$$

$$\frac{\Delta C_t(HG1)}{\Delta C_t(HG2)} \qquad \textbf{Equation 4}$$

Equation 3 and 4. ΔC_T calculation. ΔC_T calculated by determining the difference between the target gene (miRNA-31) and the HKG being evaluated. These values where then used to generate ratios between two HKGs.

These values were then used in order to generate two graphs. **Figure 4.2** showcases the C_t values for the given genes at various cell counts. This demonstrates the C_t values for the given genes based on cell count. As cell count increases, the number of amplicons should increase, therefore decreasing the C_t value. This happens in most of the genes evaluated, with the exception of RNU44, specifically, within 40,000 cells. A possible reason for this is that the 20,000 cell sample, and the 40,000 cell sample were improperly placed, therefore, resulting in higher C_t values for an larger number of amplicons. If the 20,000 cell sample and the 40,000 cell sample were to be switched, the C_t would decrease along a predicted path. Other possible reasons for this include pipetting errors, and the high variability of RNU44. **Figure 4.3**, the lines below and above the box represent the standard deviation of each sample and the boxes represent the range of values for a given ratio. This plot showcases which HKGs exhibit the highest variability in a given assay of the same cell type. For example, miRNA-25 showcases relatively low variability when compared to the other HKGs, except when exposed to RNU44, this is also the same for miRNA-93 and miRNA-16. From this, it can be reasonably concluded that RNU44 is variable across the assay, indicating a wide range of expression levels. This should be eliminated as an HKG candidate, due to the fact that the relative expression levels vary so greatly. It was from this data that miRNA-16 and miRNA-93 were chosen as suitable HKGs. miRNA-25 was not selected as a candidate for housekeeping studies, as miRNA-16 was established to be one of the most stably expressed genes in 59 of NCI-60 cell lines. Furthermore, miRNA-93 had a slightly lower C_t value than miRNA-25.

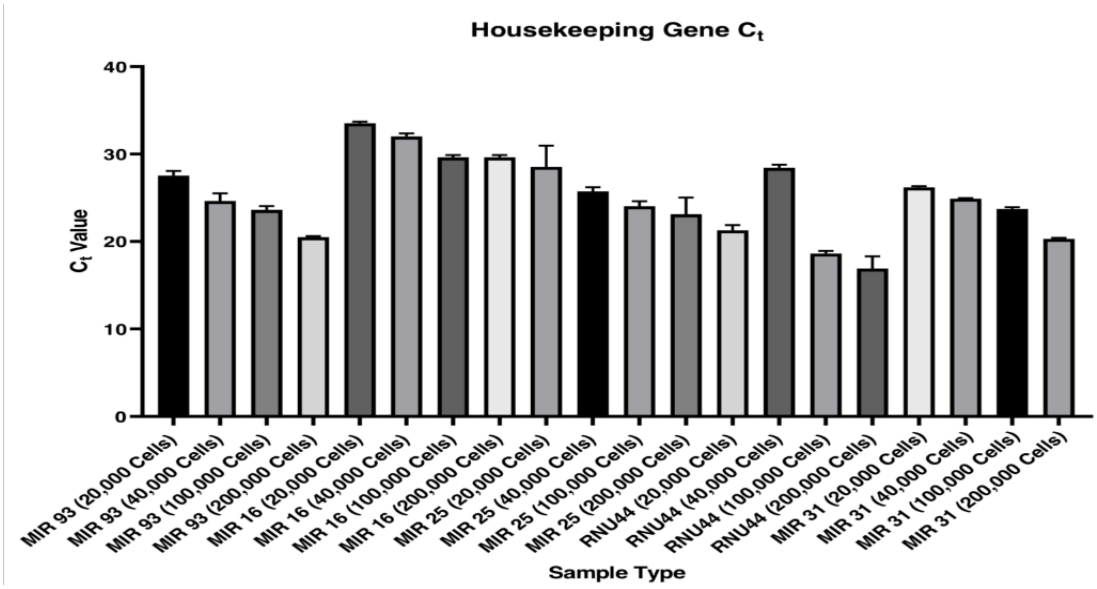


Figure 4.2. C_t values obtained during evaluation of HKGs for miR-31 analysis.
Expression levels of each HKG and GOI were evaluated from different increasing amounts of cells.

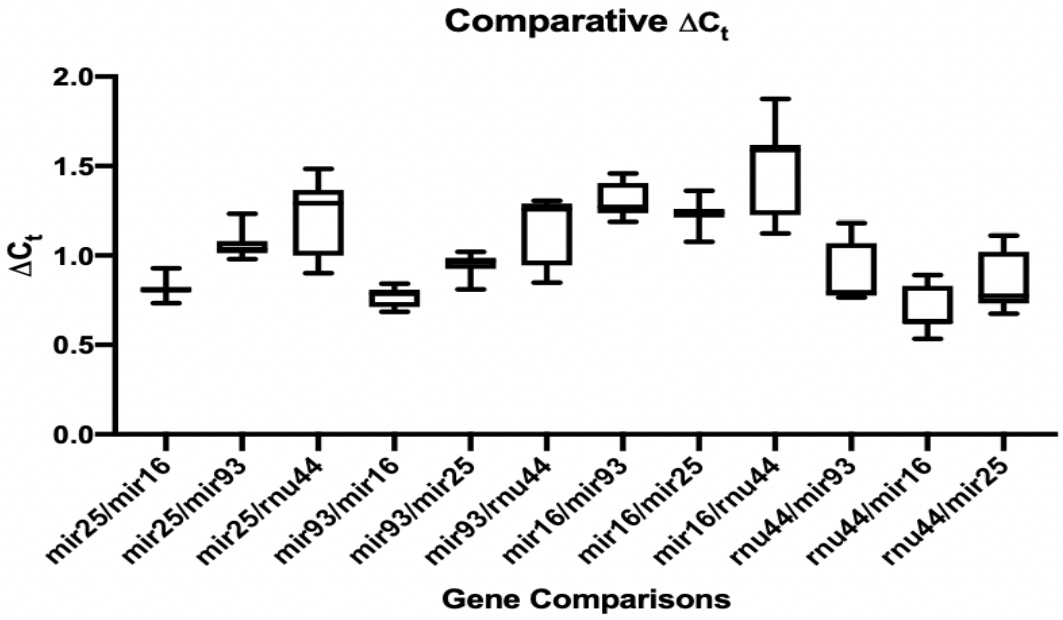


Figure 4.3. Variability assessment via comparative ΔC_t. The HKG were compared by ΔC_T values leading to the conclusion that miRNA-25, miRNA-16, or miRNA-93 would be suitable HKGs for studying miRNA-31 expression.

4.3.2. Small molecule compounds effect on miR-31 expression in real time qRT-PCR *(not completed)*

No results were obtained due to conflicts in time and the Covid-19 shutdown.

4.4. Future experiments and conclusions

Future experiments will include introducing the psmir small molecules to the HCT-116 cells and evaluating miR-31 expression levels. Drug solutions will be introduced using dose-response concentrations ranging from 20 µM – 1 nM and will be incubated for 24 hours.

The determination of suitable HKGs for qRT-PCR normalization was successful. This experimentation resulted in miRNA-31 and miRNA-16 as being selected for adequate normalization. Furthermore, the qRT-PCR assay was optimized, and works adequately under the current methods. As stated in section 3.3, the miRNA-31 qRT-PCR assay will be used as a secondary assay for the verification of the luciferase assay results. Moreover, this assay will be used as a secondary assay for other projects, including that of miRNA-18a. Notably, one freeze-thaw step was tested for the assay and did not have a significant impact on amplification levels. The recommendation was not exceeded. Moreover, it was determined that the stability of the miRNA had a significant impact on amplification levels. Initially, the standard PCR mix with a run time of two hours was being used and under these conditions, C_t values always exceeded 32. With the switch to the fast PCR protocol, reduction to one-freeze thaw step, and shift to a pulsed reverse transcription protocol, the C_t values reached appropriate levels.

CHAPTER V: CONCLUSION

The steps taken in terms of literature searches and preparing reagents proves that these studies can be accomplished in the future. The psmir base established a relationship between various FDA-approved compounds and their possible inhibitory effect on miRNA-31. Based on this, and fundamental connection between their structures, it is believed that the molecules will have an inhibitory effect on miRNA-31. Whether the level of inhibition will be respective to their relative score values is unknown but will be evaluated in future experiments.

The experimentation through the qRT-PCR led to an optimized standard operating protocol and the determination of HKGs. Through this experimentation, it is perceived that miRNA-16 and miRNA-93

will serve as reliable genes for assay normalization. Moreover, the data seems to indicate that a reduced number of cells could be used for future experiments, based on the relatively high C_t values. This is further supported by the manufacturer's protocol which states as low as 100 cells can give reproducible results. Stability of the miRNA was found to be a significant issue in the initial stages of qRT-PCR. It is now understood that a single freeze-thaw, along with the fast PCR protocol, contributed to increased amplicon levels.

The necessary vectors for the luciferase assay were produced and successfully purified. The use of a 500 mL culture, and the *EndoFree Plasmid Mega Kit* proved to be a suitable method for generating and isolating the pmirGLO vectors. Future testing and optimization are needed in order for the assay to be in working condition.

Overall, the conclusion drawn from these experiments set the foundation for investigating the miRNA-31 inhibition via the compounds selected from psmir.

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