ABSTRACT

MYOUNG, SUNGHYUN SANDER. Therapeutic Potential of Small Molecule MicroRNA Inhibition. (Under the direction of Dr.Alexander Deiters).

MicroRNAs (miRNAs) play important roles in gene regulation and their misregulation has shown to be involved in various cancers. Due to their biological relevance, miRNAs are potential targets for new cancer therapeutics. The development of small molecule inhibitors of miRNAs could provide useful tools to elucidate the mechanisms of miRNA function and provide novel therapeutics for the treatment of human diseases. As a result, we have generated a stable reporter cell line for miR-125b function using lentiviral technology. We expect to identify small molecules regulators of these specific miRNAs and use these small molecules to discover new information on the regulation of miRNAs. Previously, we have discovered active miR-21 inhibitors via high-throughput screening of small molecule libraries. miR-21 has been shown to be upregulated in several human malignancies. Elevated miR-21 levels increase cell growth by inhibiting apoptosis and mediate drug resistance. We hypothesize that we could induce apoptosis and cause a reduction in cell growth in select cancer cell lines with known anti-cancer drugs in combination with our miR-21 inhibitors. Reductions in colony formation up to 80% using clonogenic assays were observed compared to non-treated cells. Thus, small molecule inhibition of miR-21 increases sensitivity to anticancer agents in several cancer cell lines. Moreover, little is known regarding the genes and pathways that are regulated by miR-21.

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Therapeutic Potential of Small Molecule MicroRNA Inhibition

by
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BIOGRAPHY

Sunghyun, Sander, Myoung was born in Incheon, South Korea. He spent his childhood in the United States from 1985 to 1994. Going back to Korea, he graduated Sogang University with a bachelor's degree in Chemistry and Life Science in 2009. After research experiences at Sogang and Hanyang University, Sunghyun entered the Chemistry graduate program at North Carolina State University in Raleigh. His research focuses on small molecules which regulate microRNA function. Under the supervision of Dr. Alexander Deiters he achieved his Masters degree in the summer of 2013.

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1. Introduction

The discovery of microRNAs (miRNAs) in 1993 by Victor Ambros' lab at Harvard University has revealed novel mechanisms by which gene expression is regulated at the post-transcriptional level.[1] miRNAs are endogenous single stranded RNAs of 21-23 nucleotides. The first indication of a short RNA was in the nematode worm *Caenorhabditis elegans* where it was shown that the lin-4 gene produces a 22-base small single stranded RNA. This RNA binds to complimentary sequences in the 3' untranslated region (UTR) of the lin-14 mRNA and represses its translation.[1] A single miRNA can bind to and regulate many different mRNA targets and several miRNAs can bind to and control a single mRNA target.[2] Bioinformatics and experimental evidence indicate that miRNAs may be involved in the regulation of up to 30% of all protein coding genes.[3] Recent studies have shown that miRNAs have important roles in regulatory pathways, including developmental timing, haematopoiesis, organogenesis, apoptosis, cell proliferation and tumorigenesis.[4]

1.1 MicroRNA biogenesis

MicroRNAs are involved in the translational control of gene expression. They are transcribed as so called primary miRNAs (pri-miRNAs) by RNA polymerase II in the nucleus. Pri-miRNAs are several kilobases long and contain one or more hairpin structures.[5] The pri-miRNAs are recognized and processed by a protein complex which consists of the RNase III type endonuclease Dorsha and its partner DGCR8. The protein complex generates a 70 nucleotide stem loop structure known as the precursor miRNA (pre-miRNA).[6] The pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5.[7] In the cytoplasm, the

pre-miRNA is further cleaved by another RNase III enzyme, Dicer, along with TAR RNA binding protein (TRBP) which excises the loop to yield the 22 nucleotide miRNA duplex.[8] After being unwound, one strand of the miRNA is selected as the biologically active mature miRNA and the other strand is rapidly degraded. The mature miRNA is loaded into the RNA induced silencing complex (RISC) which contains Argonaute (Ago) proteins and the mature miRNA. The mature miRNA allows the RISC to recognize the 3′ untranslated region (UTR) of their target mRNA via partial sequence complementarity defined by bases 2-8 of the 5' end of the mature miRNA.[9] The 5′ end of the miRNA is not only crucial for targeting the mRNA, but also for the stability and proper loading of the miRNA into the RISC complex.[10, 11] Once RISC is bound to the mRNA it can inhibit gene expression through two different mechanisms depending on the degree of complementarity to the miRNA. Perfect base pairing between the miRNA and mRNA results in RISC-mediated mRNA degradation. Imperfect complementarity, which allows short stretches of mismatched base-pairs and G-U base pairing, represses target gene expression at the level of translation by inhibiting eukaryotic initiation factor 4E (eIF4E) or cause ribosome stalling (Figure 1).[12, 13, 9]

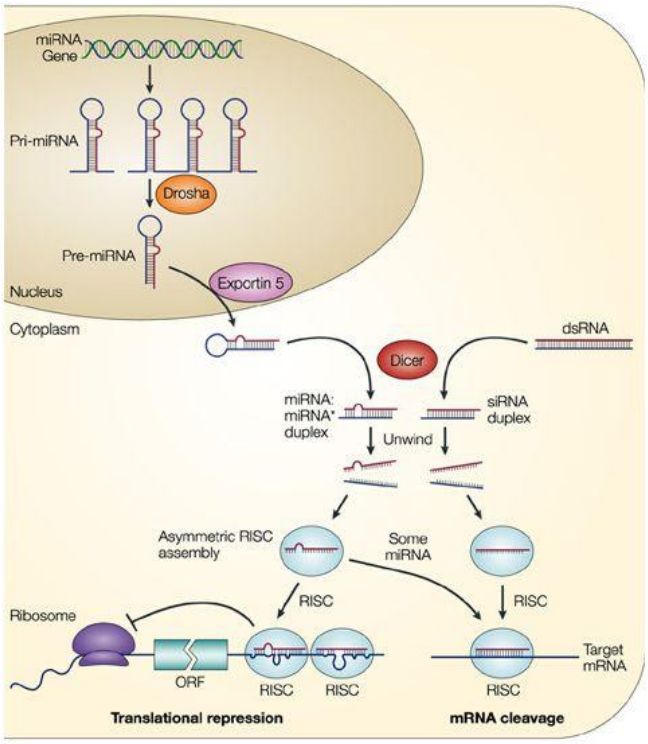


Figure 1. miRNA biogenesis pathway and post-transcriptional suppression of mRNA targets. miRNA genes are transcribed by RNA polymerase II in the nucleus to form pri-miRNA. These pri-miRNAs are then processed by Dorsha to pre-miRNA. Pre-miRNA are transported to the cytoplasm by Exportin 5 and further processed into miRNA duplexes by Dicer. One strand of the miRNA is degraded and the other strand is loaded onto RISC which targets mRNA and induces mRNA cleavage or translational repression.[13]

1.2 MicroRNAs in cancer

Cancer is caused by abnormal expression of coding and non-coding genes leading to uncontrolled cell proliferation and damage of cells resulting in tumor formation.[14] Many genes and proteins are involved in cellular proliferation and differentiation and alteration to these oncogenes or tumor suppressor genes is known to be the cause of cancer.[15] Recent studies show that miRNAs may function as oncogenes or tumor suppressors.[16] Therefore,

aberrant gene expression caused by abnormal levels of miRNAs may be the main mechanism in tumorigenesis.[17] In order to determine the involvement of miRNAs in tumor formation, miRNA profiling has revealed differentially expressed miRNAs in cancer cells compared to normal cells.[18] Out of 228 miRNA genes analyzed from six of the most frequent human tumor types, 36 were overexpressed and 21 were downregulated in cancer cells compared to normal cells.[19] Misregulated miRNA and mRNA expression in cancer results in the expression of oncogenic proteins that cause cancer phenotypes. The perturbation of miRNA expression levels can be crucial during carcinogenesis causing aberrations in the transcription of large numbers of genes. Since miRNAs are involved in the regulation of many different pathways, they also have a key role in coordinating gene networks involved in cancer development and progression. By modifying miRNA expression with small molecules it may be possible to restore homeostasis in cancer cells.[20] Table 1 shows abnormal expression of select miRNAs that are involved in various cancers.

Table 1. Selected miRNAs involved in cancer.[21]

Cancer	miRNA	Selected targets	Presumed target function
Breast	miR-21↑	TPM1, PDCD4	Tumor suppressors
	miR-27b↑	CYP1B1	
	miR-125↓	ERBB2, ERBB3	Inhibition of cell growth, decreased migration and invasion
	miR-155↑		
Prostate	miR-125b↑	Bak-1	Pro-apoptotic factors, induction of apoptosis
	miR-221/222↑		Cell cycle inhibitor, cell growth inhibitor
Pancreas	miR-221/222↑	p27	Cell cycle inhibitor, cell growth inhibitor
	miR-155↑	TP53INP1	Induction of growth arrest, apoptosis
Lung	miR-21↑	HMGA2, RAS, c-Myc	Oncogene, related to metastasis, promoter of carcinogenesis
	miR-205↑	DNMT3A/B	Oncogene activity, DNA methyltransferase
	miR-126↓	Bcl2	Anti-apoptotic factor, promoter of proliferation
	miR-17-92 cluster↑		Enhanced cell growth
	miR-29a↑		Inhibition of cell growth, increased apoptosis
Liver	miR-21↑	PTEN	Tumor suppressor, negative regulation of PI3k
	miR-122↓	Cyclin G, Bcl-w	Anti-apoptotic factor
Colon	miR-21↑	TPM1, PDCD4	Cell invasion, intravasation, metastasis
	miR-17-92 cluster↑		Increased angiogenesis
	miR-143↓	ERK5	Promoter of cell growth and proliferation
	miR-145↓	IRS1	Oncogene, strong mitogenic activity
Thyroid	miR-197,346↓		Inhibition of cell growth
Cervix	miR-218↓	LAMB3	Promoter of cell migration and tumorigenicity
Lymphoma	miR-143↓	ERK5	Promoter of cell proliferation and cell growth
	miR-155↑		
Leukemia	miR-15/16↓	Bcl2	Anti-apoptotic factor, promoter of cell growth
	miR-17-92 cluster↑		Enhanced cell growth
	miR-222↓	Kit	Proto-oncogene, tyrosine-protein kinase

Three different mechanisms can explain the cause of abnormal miRNA expression in cancer cells. First, the location of the miRNAs in the genome, as more than half of all human miRNAs are located in a particular genomic region that is prone to alterations. MiRNA genes located at minimal regions of loss of heterozygosity are thought to contain tumor suppressor

genes and minimal regions of amplification are thought to contain oncogenes.[22] Second, is the epigenetic regulation of miRNA expression. DNA hypermethylation and histone modifications can alter miRNA levels.[23, 24] In addition, transcription factors that are highly expressed in cancer cells can directly recognize promoter sequences and activate the transcription of miRNAs.[25] Last, is the alteration in miRNA-processing genes and proteins. Alteration of proteins involved in miRNA biogenesis has dramatic effects on miRNA expression.[26]

1.3 Development of small molecules for miRNA regulation

With the emergence of miRNAs as important therapeutic targets, researchers began to develop regulatory tools to control the expression of specific miRNAs. Synthetic oligonucleotides known as anti-miRNA oligonucleotides (AMO) or antagomirs bind to the complimentary mature miRNA inhibiting miRNA function.[27] These antagomirs have proven to be potent and effective. Chemical modifications of antagomirs have been developed to improve stability and lower toxicity by including 2'-O-methyl modified nucleotides, locked nucleic acids, and phosphorothioate linkages in the DNA/RNA backbone. However, problems have arisen from the high cost for *in vivo* studies, ineffective uptake to target cells, and potential off-target effects.[28]

Small molecules are less than 800 Da and can have ideal drug properties such as good solubility, bioavailability, and stability.[29] They can target proteins involved in miRNA processing and inhibit miRNA biogenesis pathway. The development of small molecule miRNA inhibitors has several advantages over antagomirs in regulating miRNA activity.

Small molecules are less expensive to synthesize and due to the small size the molecule can easily diffuse across cell membranes. Small molecules are also stable in a cellular environment. Small molecules allow temporal control because cells can be treated at any time point. Most importantly, multiple levels along the miRNA pathway can be targeted by small molecules allowing more mechanisms for controlling miRNA activity compared to antagomirs which only regulate miRNA activity via the interaction with the mature miRNA. Development of small molecules as potential therapeutics and probes for miRNA functional studies will help us learn more about the miRNA biogenesis pathway and miRNAs involved in human diseases.[30 31]

In order to identify small molecule modifiers of miRNA function, we are taking a forward chemical genetics approach, which can be applied to both the discovery of new molecular probes and the discovery of new therapeutic agents.[32] The first step of chemical genetics-based drug discovery is to develop a reporter assay system to identify compounds that induces a desired phenotype. Once a reporter assay system is developed we can use this system to perform a high-throughput screen of a large small molecule compound library (in the order of 300,000 compounds). Then secondary screens are performed to remove non-specific hits and identify compounds with desired property. Information on the chemical structure of the hit compounds are important factors for subsequent hit prioritization,[33] and structure-activity relationship (SAR) studies enable us to identify the functional groups that are responsible for the biological activity. These compounds are further used in cell models of cancer to reveal therapeutic potential as well as to conduct target identification.

Compounds that show therapeutic potential then undergo toxicological testing in animals. If the compound passes animal studies clinical trials are initiated as the final step.[34]

In previous research in the Deiters lab, miRNA-21 (miR-21) was selected as a target, because it is highly upregulated in many human malignancies including cancer. Elevated miR-21 expression was demonstrated in breast, cervical, colon, glioblastoma, hepatocellular, ovarian, prostate, and lung cancer cell lines[35] and has been shown to act as an anti-apoptotic factor enhancing cell growth by inhibiting apoptosis.[36] The miR-21 inhibitors C3 and LG157 were discovered via small molecule screening using a luciferase based miR-21 functional assay (Figure 2c). The assay is based on the natural function of mature miRNA. When the miRNA binds to the corresponding target mRNA sequence, the mRNA undergoes translational repression or induce degradation. (Figure 2a) Thus, the psiCHECK plasmid (Figure 2b) with a luciferase reporter gene and a miRNA target sequence located in the 3' UTR can act as a sensor to detect the presence of specific miRNA via the reduction in luciferase activity.

A pilot screen of over 1000 compounds from the Library of Pharmacologically Active Compounds was performed. A diazobenzene compound was discovered as an initial hit and was developed (through an structural activity relationship (SAR) study by synthesizing structurally similar molecules) into the diazobenzene analog C3.[37] Furthermore, a high-throughput screen for miR-21 inhibitors was conducted at the NIH Chemical Genomics Center and the oxadiazole compound LG157 was one of the putative hits discovered. When HeLa cells were treated with C3 or LG157 at a concentration of 10 μM, luciferase expression was increased by 1.5 fold compared to cells treated with a DMSO control (Figure 2d).

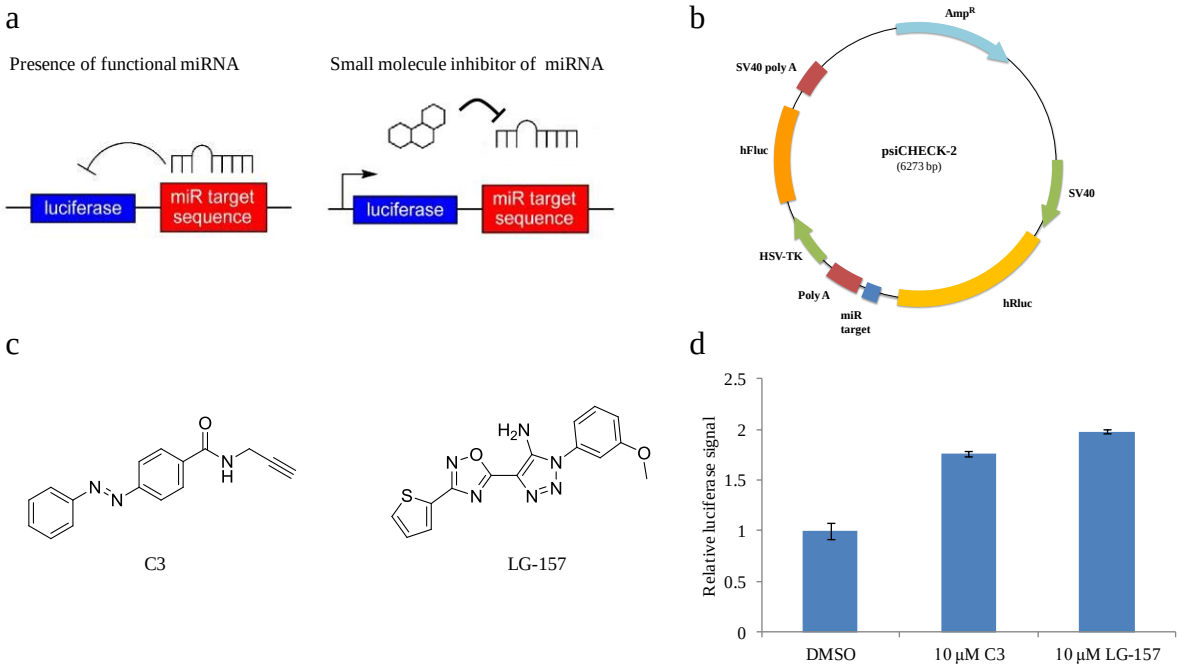


Figure 2. (a) Small molecules were discovered via small molecule screening using a luciferase based functional assay. (b) Plasmid map of psiCHECK-2 (c) The structure of the miR-21 small molecule inhibitors C3 and LG-157. (c) Luciferase assay of miR-21 inhibitors. At a 10 µM concentration of C3 and LG-157 the luciferase signal increased by 1.5-fold compared to the DMSO control.

2. Generating microRNA stable reporter cell lines using lentivirus technology

2.1 MicroRNA-155

MicroRNA-155 (miR-155) is processed from a non-coding RNA transcribed from the B-cell Integration Cluster (BIC) located on chromosome 21.[38] MiR-155 plays an important role in various pathological processes such as immunity, inflammation, cancer, cardiovascular diseases, and viral infections.

BIC/miR-155 is increased in activated B and T cells as well as in macrophages and dendritic cells. MiR-155 is required for lymphocyte development and generation of B and T cell responses.[39] Overexpression of miR-155 also plays a significant role in carcinogenesis.[40] In humans, BIC RNA levels are low in normal lymphoid tissues but increase in various B-cell malignancies such as Hodgkin's lymphoma and some types of Non Hodgkin's lymphoma.[41] [42] Hodgkin's lymphoma is characterized by the spreading of a disease from one lymph node to another developing systematic symptoms. The majority of Non Hodgkin's lymphomas do not express BIC/miR-155. BIC was observed in Primary Mediastinal B-cell Lymphoma (PMLB), Diffuse Large B-cell lymphoma (DLBCL) and in children's Burkitt's lymphoma.[43] PMLB is a rare form of Non Hodgkin's lymphoma that originates from a rare type of B-cell lymphocyte in the thymus gland. DLBCL is the most common form of Non Hodgkin's lymphoma accounting for nearly 40% of all lymphoid tumors. Burkitt's lymphoma is a fast growing form of Non Hodgkin's lymphoma. Among the different Burkitt lymphoma cell lines, the Epstein Barr virus (EBV) latency type III-positive Burkitt lymphoma cell line Raji shows both increased basal levels of BIC and miR-155. Previous

reports show that the BIC gene is a common site for viral DNA integration in virus-induced lymphomas. The infection with EBV greatly perturbs the pattern of cellular miRNA expression interfering with gene expression in the infected cells. It has been proposed that EBV initially infects naïve B cells and then induces them to undergo a period of rapid proliferation.[44 45] In particular, the EBV infection of primary B cells has been shown to strongly activate the expression of miR-155 and alter gene expression by targeting transcriptional regulatory genes such as PU.1.[46, 47, 48] Downregulation of PU.1 by miR-155 induced lymphoid tumorigenesis.[49, 50] In addition, Raji cells were found to upregulate miR-155 expression upon PKC-mediated induction of BIC RNA expression.[47, 51] The mechanism underlying the oncogenic potential or the participation in pathogenesis of miR-155 is not elucidated. However, a number of target genes implicated in differentiation, inflammation, apoptosis and transcription regulation could be controlled.

Due to miR-155's biological relevance, a reporter assay system based on the psiCHECK-2 vector was developed to screen small molecules that have relevance in miR-155 regulation (constructed by Colleen Connelly). The vector contains both a firefly and a Renilla luciferase gene. The firefly luciferase gene is used as an internal control for cell number and cell viability. The MCS (multi-cloning site) at the 3' terminus of the Renilla luciferase gene where a miRNA-target site can be inserted. The mature miR-155 complimentary sequence (5'-TCGAAC<u>ACCCCTATCACGATTAGCATTAA</u>CTCGAGTA GC-3' and 5'-GGCCGCTACTCGAGTTAATGCTAATCGTGATAGGGGTG-3; the miR-155 target site is underlined) was cloned into the MCS to generate the reporter plasmid psiCHECK-miR155. Previous experiments showed that when psiCHECK-miR-155 was

transfected into the Burkitt's lymphoma Raji cell line, which expresses high level of miR-155,[52] the luciferase activity was decreased by approximately 5-fold compared to the psiCHECK-2 control vector. The transfection of a miR-155 antagomir (20 nM) recovered the luciferase activity to the level of the control plasmid (Figure 3). The miR-155 antagomir (5'-ACCCCUAUCACGAUUAGCAUUAA-3') is a phosphorothioated RNA oligonucleotide with a 2'-OMe modification which is perfectly complimentary to the miR-155 target site.

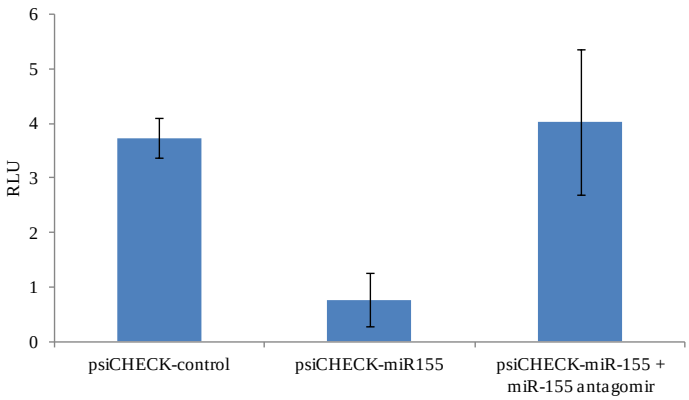


Figure 3. The psiCHECK-mi155 reporter construct was validated by transfecting the construct into Raji cells. A 5-fold decrease in luciferase acivity was observed compared to the psiCHECK-2 control. A miR-155 antagomir was co-transfected with the psiCHECK-miR155 plasmid in order to inhibit miR-155 function and recover luciferase activity. The error bars indicate the standard deviation from three independent experiments.

In order to screen for small molecule miR-155 inhibitors, we decided to generate Raji-psiCHECK-miR155stable cell line using lentiviral transduction.

Lentiviruses have high transduction efficiency due to their ability to infect both replicating and non-replicating cells.[53] Lentiviruses are composed three main genes, *gag*, *pol*, and *env*, which encode structural proteins such as surface glycoproteins and capsid as well as enzymes such as integrase, protease, reverse transcriptase and envelope proteins required for virus replication. The replication cycle of lentiviruses begins with binding to the receptor on the host cell via the surface glycoprotein ligand of the virus to a surface receptor of the host cell. This induces a fusion of the membranes of the envelope of the virus and the membrane of the host cell which allows for entry. Immediately following the entry process, uncoating and reverse transcription leads to the formation of pre-integration complex (PIC). PIC, composed of double strand DNA, integrase, and nuclear capsid, can actively enter the nucleus. Once the provirus enters the nuclear envelope, it integrates with the host genome. Cells that are infected can transcribe and translate structural viral proteins with viral RNA and form new viruses.[54] However, lentiviruses have been modified to infect target cells but not replicate within target cells. The first modification reduced the number of lentiviral genes for packaging, replication and transduction to three (gag, pol, rev). These three genes are expressed from different plasmids that lack packaging signals. Thus, the lentiviruses generated are replication-incompetent. Also, a deletion in the 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into the host genomic DNA.

First, the lentiviral expression vector was constructed by cutting out both luciferase genes from the psiCHECK-miR155 construct which includes the miR-155 target site using the restriction enzyme SpeI. The feline immunodeficiency lentiviral expression vector pCDF-

MCS1 (System Biosciences) was also cut using the restriction enzyme BglII. Using T4 DNA polymerase, the digested sticky ends were converted to blunt ends. Each of the linearized plasmids was then digested with XbaI in order to produce complementary sticky ends. The digested psiCHECK-miR155 and pCDF-MCS1 were then ligated using a sticky end-blunt end ligation to produce the pCDF-psiCHECK-miR155 plasmid (Figure 4a). The ligation reaction was transformed into NovaBlue competent cells and positive clones were selected and confirmed by DNA sequencing. The pCDF-psiCHECK-miR155 plasmid was transiently transfected into Raji cells to confirm the luciferase expression and miR-155 detection. 1 μg of DNA and 4 μL of Lipofectamine were used to transfect Raji cells in a 24-well plate format. To verify that miR-155 inhibition could restore luciferase expression, a miR-155 antagomir (100 pmol) was co-transfected with the lentivial plasmid and 48 hours post-transfection, the cells were assayed using Dual Luciferase kit. Raji cells transfected with pCDF-psiCHECK-miR155 showed a 3-fold decrease in relative luciferase compared to the pCDF-psiCHECK- control construct (Figure 4b).

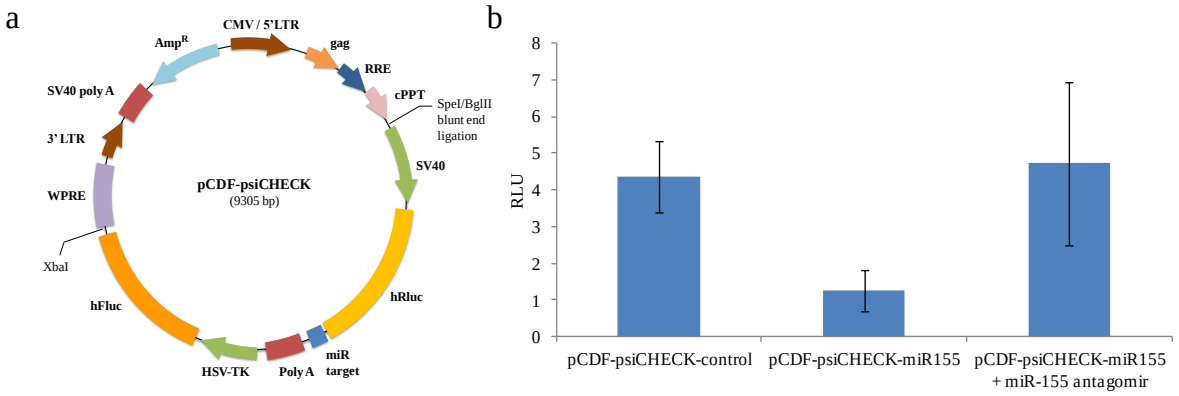


Figure 4. (a) Plasmid map of pCDF-psiCHECK (b) The pCDF-psiCHECK-mi155 reporter construct was validated by transfecting the constructs into Raji cells. A 3-fold decrease in luciferase acivity was observed for the pCDF-psiCHECK-mi155 reporter compare to the pCDF-psiCHECK-control. The error bars indicate the standard deviation from three independent experiments.

For lentivius production, our lentiviral expression construct pCDF-psiCHECK-miR155 was then transfected into the lentivirus producer cell line HEK293T with pPACKF1 packaging vectors (System Biosciences). The pPACKF1 packaging plasmids consist of two plasmids: pFIV-34N and pVSV-G. The pFIV-34N contains the structural gag, regulatory vif, gp4, rev, nef and replication pol genes to produce lentiviruses. The pVSV-G plasmid expresses the envelope glycoprotein of vesicular stomatitis virus (VSV-G) replacing env gene which enables the virus to target both mammalian and non-mammalian cells.[55] Viruses were isolated 48 hours post-transfection. The supernatant from the lentiviral producer cell line was obtained and concentrated using the PEG-it concentration reagent (System Biosciences). The next day, lenti-psiCHECK-miR155 viruses were resuspended in ice cold PBS buffer. The Raji target cells were passaged onto a 24 well plate and were transduced

with lenti-psiCHECK-miR155 using 5 or 10 multiplicities of infection (MOI). MOI is the ratio of viral particles to the number of cells being infected. 72 hours post-transduction, the viral gene should be integrated into the host genome. The transduced Raji cells were diluted and passaged into a 10 cm plate and were grown for approximately two weeks until individual colonies appeared. The colonies were isolated and tested using a Dual Luciferase kit. However, none of the colonies selected showed any luciferase expression, although lentiviruses are expected to have transduction efficiencies of up to 70-100%.[56]

We thought of four different reasons for this problem. First, the lentiviruses were not properly generated. The recommended size of the insert is 3-4 kb with reasonable packaging efficiency to produce virus. The region between the 5' and 3' LTR should not exceed 9 kb which is the size of the native HIV genome. Larger inserts will possibly package into virus, however, the efficiency of packaging may be affected negatively to the point where titer of the virus is very low. As designed, our insert is around 4 kb which in our case should be a reasonable size for packaging. Second, the Raji cells are difficult to transduce. Reports have shown that B cells are poorly transducible with VSVG-pseudotyped lentivirueses even when stimulated into proliferation in the presence of various cytokines.[57] Third, the viral gene may have integrated into a non-active site of the host genome which does not express the desired luciferase proteins. Fourth, a poly-A tail was accidentally cloned after the microRNA target in the lentiviral plasmid construct, which may led to the generation of truncated transcription products without production of lentiviruses. Therefore, to overcome these problems we decided to re-construct our lentiviral expression construct. In order to make sure our lentiviruses were produced and transduced properly, we decided to add an EGFP reporter

gene. The EGFP gene is also beneficial for selecting tranduced cells, e.g., by FACS. In order to reduce the size of the construct, only the Renilla luciferase gene with the miR-155 target was used as an insert instead of the inserting both the firefly and Renilla luciferase genes.

The lentiviral expression vector was constructed by excising the EGFP gene from the pEGFP-N1 vector using the restriction enzymes BamHI and NotI. The pCDF-MCS1 was also digested using BamHI and NotI. The two digested plamids were ligated using a sticky end ligation to form pCDF-EGFP. Next, the Renilla luciferase gene including the miR-155 target without the poly-A tail was PCR amplified from the psiCHECK-miR155 plasmid using primers purchased from IDT DNA and Phusion DNA polymerase. The primers were engineered to have SpeI restriction enzyme sites in order to install SpeI sites on the end of our Renilla luciferase insert. pCDF-EGFP and the Renilla luciferase insert were both digested with SpeI and subsequently ligated using a sticky end ligation to form the pCDF-EGFP-RLuc-miR155 plasmid (Figure 5). The ligation reaction was transformed into STBL3 competent cells (Invitrogen). The STBL3 competent cells, which have a mutation in the recA gene, are used to clone unstable lentiviral DNA plasmids containing direct repeats to prevent them from recombination. Positive clones were selected and confirmed by DNA mapping and sequencing. The pCDF-EGFP-RLuc-miR155 plasmid was transiently transfected into Raji cells to confirm EGFP expression and miR-155 detection. 1 μg of DNA and 4 μL of Lipofectamine were used to transfect Raji cells in a 24-well plate format. However, the transfection was unsuccessful using the previous conditions. We decided to proceed with making lentiviruses.

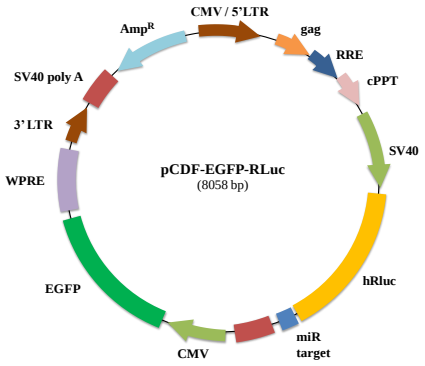


Figure 5. Plasmid map of pCDF-EGFP-RLuc-miRNA.

For lentivius production, our lentiviral expression construct pCDF-EGFP-RLuc-miR155 was transfected into the lentivirus producer cell line HEK293T with packing vectors. 72 hours post-transfection, HEK293T cells expressed EGFP indicating that lentiviruses were generated. Viruses were isolated from the supernatant of the lentiviral producer cell lineand concentrated using the PEG-it concentration reagent. The next day, lenti-EGFP-RLuc-miR155 viruses were resuspended in ice cold PBS buffer. The Raji target cells were passaged onto a 24-well plate and were transduced with lenti-EGFP-RLuc-miR155 using 5 or 10 multiplicity of infection (MOI). 72 hours post-transduction, the cells were observed using a fluorescence microscope. Transduced Raji cells showed EGFP expression, however, after additional 48 hour incubation, EGFP expression was lost in most of the cells and no stable integration was observed. However, loss of EGFP marker expression has been reported in several virally transduced cell types. [58, 59]

2.2 MicroRNA-27b

MicroRNA-27b (miR-27b) is overexpressed in highly invasive human breast cancer cell line, MDA-MB-231 subline 4175. MiR-27b promotes cell proliferation, cell migration, and cell invasion by downregulating its target protein ST14. ST14 is an epithelium-derived membrane serine protease. The expression of ST14 has been associated with several tumors, including breast, colon, prostate, and ovary.[60] MiR-27b, which binds to the 3'UTR of ST14, and ST14 show inverted expression patterns indicating that ST14 is a target of miR-27b in breast cancer cells.[61] In addition, miR-27b was also found to function as a negative regulator of adipogenesis in humans.[62] Overexpression of miR-27b interfered with the adipogenic transcription factor cascade by targeting peroxisome proliferators-activated receptor (PPARγ). PPAR are members of the nuclear receptor superfamily. PPARγ has emerged as an attractive target due to its association with many human cancers such as colon, thyroid, breast, and prostate.[63] PPARγ acts as a tumor suppressor because ligands that activate PPARγ promote growth inhibition and apoptosis. MiR-27b directly targets PPARγ and inhibits adipocyte differentiation.[64] Inhibition of PPARγ activates NHE1 expression,[65] the inflammatory response, and growth of breast cancer cells. Activation of the pH regulator NHE1 causes tumors to become more acidic extracellularly and more alkaline intracellularly during neoplastic progression, hence, NHE1 activation is tumor promoting.[66]

Similar to psiCHECK-miR155, a psiCHECK-miR27b construct was generated using the same procedure (5'-TCGAC<u>GCAGAACTTAGCCACTGTGAA</u>CTCGAGTAGC-3' and 5'-GGCCGCTACTC-GAGTT-CACAGTGGCTAAGTTCTGCG -3, miR-27b target site is underlined). The mature miR-27b complimentary sequence was cloned into psiCHECK-2 to

generate the psiCHECK-miR27b reporter plasmid (constructed by Colleen Connelly). Previous experiments showed that when psiCHECK-miR-27b was transfected into the breast cancer cell line 4175, which expresses high level of miR-27b,[61] the luciferase activity was decreased by approximately 9-fold compared to the psiCHECK control vector. The presence of a miR-27b antagomir (50 pmol, 5'-GUUCACCAAUCAGCUAAGCUCU-3') restored the luciferase activity to the level of the control plasmid (Figure 6).

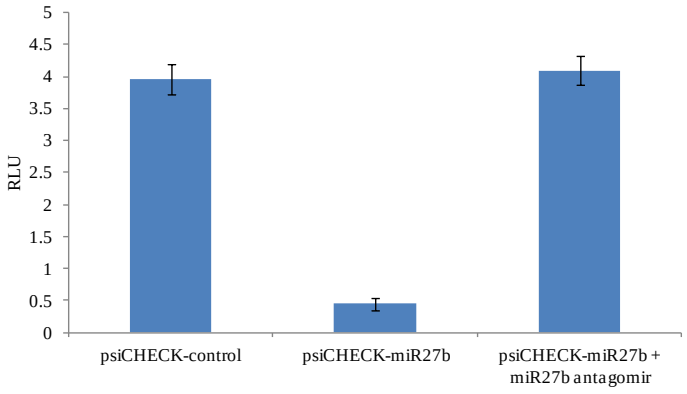


Figure 6. The psiCHECK-miR27b reporter construct was validated by transfecting the construct into 4175 cells. A 9-fold decrease in luciferase acivity was observed compare to the psiCHECK-2 control. A miR-27b antagomir was co-transfected with the psiCHECK-miR27b plasmid in order to recover luciferase activity. The error bars indicate the standard deviation from three independent experiments.

In order to screen for small molecule inhibitors of miR-27b, we decided to generate 4175-psiCHECK-miR27b stable cell line. As for miR-27b, the stable cell line was generated using lentiviruses using the same approach as discussed above for miR-155. 4175 cells

transfected with pCDF-psiCHECK-miR27b exhibited a 9-fold decrease in relative luciferase compared to the pCDF-psiCHECK control construct (Figure 7).

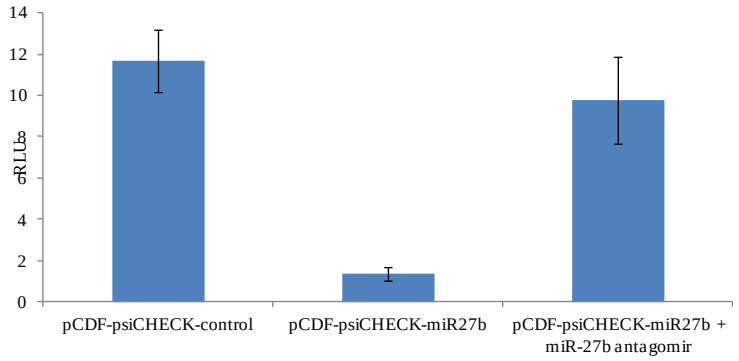


Figure 7. The pCDF-psiCHECK-mi27b reporter construct was validated by transfecting the constructs into 4175 cells. A 9-fold decrease in luciferase activity was observed compared to the pCDF-psiCHECK control. The error bars indicate the standard deviation from three independent experiments.

Lenti-psiCHECK-miR27b were generated and transduced into 4175 target cells using 5 or 10 multiplicity of infection (MOI). 72 hours post-transduction, transduced 4175 cells were diluted and passaged into a 10 cm plate and were grown for approximately two weeks until individual colonies appeared. The colonies were isolated and tested using a Dual Luciferase kit. However, none of the colonies selected expressed luciferase due to the same reasons discussed above for miR-155, e.g., early transcriptional termination due to the presence of a pre-mature poly-A site.

Therefore, we again decided to re-construct our lentiviral expression construct adding EGFP as a reporter gene in the same manner as miR-155. The pCDF-EGFP-RLuc-miR27b

plasmid without the poly-A tail was transiently transfected into 4175 cells to confirm luciferase expression. 500 ng of DNA and 2 µL of Lipofectamine were used to transfected 4175 cells in a 96-well format. 48 hours post-transfection, the cells were assayed using a Renilla luciferase kit. 4175 cells transfected with pCDF-EGFP-RLuc-miR27b showed a 7-fold decrease in luciferase activity compared to its pCDF-EGFP-RLuc control construct (Figure 8). Knowing that luciferase acivity was restored by a miR-27b antagomir co-transfected with psiCHECK-miR27b (Figure 6), additional antagomir treatment experiment with the pCDF-EGFP-RLuc-miR27b was omitted.

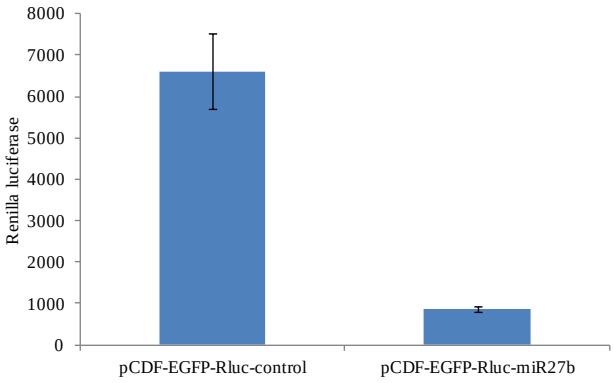


Figure 8. The pCDF-EGFP-RLuc-mi27b reporter construct was validated by transfecting the constructs into 4175 cells. A 7-fold decrease in luciferase acivity was observed for cells transfected with pCDF-EGFP-RLuc-mi27b compared to the pCDF-EGFP-RLuc control. The error bars indicate the standard deviation from three independent experiments.

Generated lenti-EGFP-RLuc-miR27b was transduced into 4175 cells using 5 or 10 multiplicity of infection (MOI). 72 hours post-transduction, the cells were observed using a fluorescence microscope. EGFP expressing cells are currently growing in the tissue culture

incubator. The cells will be diluted and passaged into a 10 cm plate. Formed colonies will be isolated and potential colonies will be assayed using a *Renilla* luciferase kit.

2.3 MicroRNA-125b

MicroRNA-125b (miR-125b) is multi-faceted, with the ability to function as a tumor suppressor or an oncogene depending on the cell type.[67] It is downregulated in ovary, bladder and breast malignancies [68, 69, 70] but is upregulated in leukemia, prostate cancer and glioma.[71, 72, 73] In prostate cancer cells, high expression levels of miR-125b stimulate androgen-independent growth that is mediated partially by downregulating Bak1.[74] MiR-125b binds to the 3' UTR of BAK1 and reduces the BAK1 mRNA and protein levels. Bak1 is a Bcl2 protein family member and functions as a pro-apoptotic regulator. In clinical studies, downregulation of Bak1 by miR-125b was shown to contribute to disease progression and resistance to therapeutic agents in prostate cancer.[75, 76 77] Repression of miR-125b activity sensitizes cells to undergo apoptosis after treatment with various therapeutic agents, including docetaxel and paclitaxel. P53 and Puma were further validated to be direct targets of miR-125b. Since p53, Puma, Bak1 are pro-apoptotic factors this suggests that miR-125b is involved in regulating apoptosis.[78]

Similar to the psiCHECK-miR155 and psiCHECK-miR27b plasmids, a psiCHECK-miR125b construct was generated using the same procedure (5'-GGCCGCTACTCGAG<u>TCCCTGAGACCCTAA-CTTGTG-A</u>G-3' and 5'-TCGACTCACAAGTTAGGGTCTCAGGGACTCGAGTAGC-3'; miR-125b target site is underlined). The mature miR-125b complimentary sequence was purchased from IDT DNA and was cloned into psiCHECK-2 to generate the psiCHECK-miR125b reporter plasmid. Previous experiments by Dustin Lockney showed that when psiCHECK-miR125b was transfected into the prostate cancer cell line PC3, which expresses high level of miR-125b,[73]

the luciferase activity was decreased by approximately 7-fold compared to the psiCHECK control vector. The presence of a miR-125b antagomir (10 pmol, 5'-UCACAAGUUAGG GUCUCAGGGA-3') restored the luciferase activity to the level of the control plasmid (Figure 9).

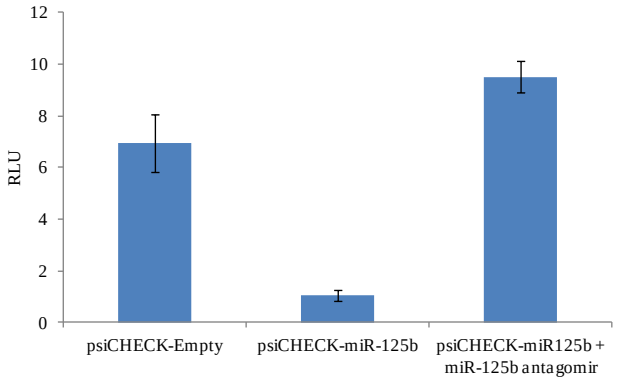


Figure 9. The psiCHECK-mi125b reporter construct was validated by transfecting the constructs into PC3 cells. A 7-fold decrease in luciferase activity was observed compare to the psiCHECK-2 control. A miR-125b antagomir was co-transfected with the psiCHECK-miR125b construct to inhibit miR-125b function and recover the luciferase activity. The error bars indicate the standard deviation from three independent experiments.

In order to screen for small molecule inhibitors of miR-125b, we decided to generate PC3-EGFP-RLuc-miR125b. The stable cell line was generated using lentiviruses. pCDF-EGFP-RLuc-miR125b with the poly-A tail was constructed. PC3 cells transfected with pCDF-EGFP-RLuc-miR125b showed EGFP expression and a 4-fold decrease in luciferase activity compared to the pCDF-EGFP-RLuc control construct (Figure 10a). From the psiCHECK-125b data above, luciferase activity was restored with a miR-125b antagomir.

Additional antagomir treatment for the pCDF-EGFP-RLuc-miR125b reporter was omitted and we proceeded with the generation of lentiviruses.

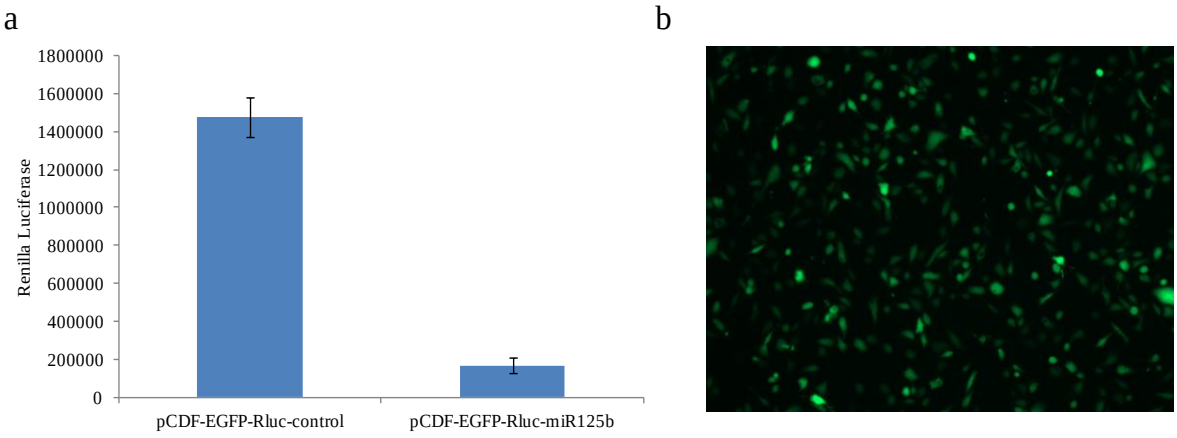


Figure 10. (a) The pCDF-EGFP-RLuc-miR125b reporter construct was validated by transfecting it into PC3 cells. A 4-fold decrease in luciferase acivity was observed compare to the pCDF-EGFP-RLuc control. The error bars indicate the standard deviation from three independent experiments. (b) PC3 cells transduced with lenti-EGFP-RLuc-miR125b displayed EFGP using a fluorescence microscope.

Lenti-EGFP-RLuc-miR125b were generated and transduced into PC3 target cells using 5 or 10 multiplicity of infection (MOI). 72 hours post-transduction, the cells were observed using a fluorescence microscope. 80% of the cells showed EGFP expression. Interestingly, EGFP was constitutively expressed unlike the Raji and 4175 mentioned above (Figure 10b). EGFP expressing clones were passaged in a less dense condition and were grown for approximately two weeks until individual colonies appeared. The colonies were isolated and tested using a *Renilla* Luciferase kit. The *Renilla* luciferase increased

approximately 4 fold when treated with miR-125b antagomir at a concentration of 10 pmol. In order to verify if our PC3-EGFP-RLuc-miR125b stable cell line is suitable for high-throughput screening, the Z' value was determined. The statistical parameter Z' factor defines the signal dynamic range and the variations associated with the signal and represent the quality of the assay.

$$Z' = 1 - \frac{(3SD_+ + 3SD_-)}{|Ave_+ - Ave_-|}$$

SD_+ is the standard deviation of the sample and SD_- is the standard deviation of the negative control. Ave_+ is the average of the sample and Ave_- is the average signal for the negative control. The Z' factor was determined by conducting an assay with our stable reporter cell lines in five individual wells in which one set of cells is transfected with an antagomir (sample data) and the other set is non-treated (negative control) in a 96 well plate format. A Z' factor in the range of 0.5-1.0 is suitable for high-throughput screening.[79] Our stable cell line provided a Z' of 0.71 which is accepted as a good assay. The variation between plates from day-to-day was 0.2% and therefore fairly small. These results validate a successful stable reporter cell lines that can be used as a robust assay for small molecule screening.

Once the stable cell lines are generated the next step is to perform a pilot screen for miR-155, miR27b, and miR-125b small molecule inhibitors using the NCI Diversity Set II

(1364 compounds). Active compounds in the initial screen that are identified as hits will be validated by a secondary screen. Also, a screen to exclude compounds that non-specifically increase Renilla luciferase activity will be conducted.

2.4 Experimental

Cell culture. Human Burkitt lymphoma cell line, Raji, Human breast cancer cell line, 4175, and Human prostate cancer cell line, PC3 (obtained from American Type Cell Collection (ATCC), Manassas, VA) were grown in DMEM and RPMI-1640 media (HyClone) supplemented with 10% fetal bovine serum, penicillin (100 units / ml), and streptomycin (100 μg / ml). All cells were incubated at 37 °C and 5 % CO_2.

Plasmid construction. The mature miRNA-155, miRNA-27b, and miRNA-125b complementary sequences were purchased from IDT DNA:

5'-TCGAACACCCCTATCACGATTAGCATTAACTCGAGTAGC-3' and

5'-GGCCGCTACTCGAGTTAATGCTAATCGTGATAGGGGTG-3. miR-155 target site is underlined.

5'-TCGACGCAGAACTTAGCCACTGTGAACTCGAGTAGC-3' and

5'-GGCCGCTACTCGAGTTCACAGTGGCTAAGTTCTGCG -3. miR-27b target site is underlined.

5'- GGCCGCTACTCGAGTCCCTGAGACCCTAACTTGTGAG -3' and

5'- TCGACTCACAAGTTAGGGTCTCAGGGACTCGAGTAGC -3. miR-125b target site is underlined.

The two oligonucleotides were hybridized and ligated into the psiCHECK-2 (Promega) plasmid digested with SgfI and PmeI restriction sites inserting the miRNA target sequence downstream of the Renilla luciferase gene.

In order to generate the lentiviral plasmid construct containing an EGFP gene, pEGFP-N1 (Clontech) was first digested with BamHI and NotI and was inserted into the pCDF-MCS1 lentiviral plasmid (System Bioscience) to form pCDF-EFGP plasmid. Next, the Renilla luciferase gene with the miRNA target site and the poly-A tail was amplified from the psiCHECK-miR-155, psiCHECK-miR-27b, and psiCHECK-miR-125b plasmids by PCR using primers engineered to have SpeI sites (5'- CGTAACTAGTAGATCTGCGCAGCACC - 3' (forward), 5'- CGTAACTAGTCGCGAGGTCCGAAGAC - 3' (reverse)). PCR reactions started with an initial denaturation at 98 °C for 30 seconds followed by an amplification cycle of 98 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds for 30 cycles total. The final extension was done at 72 °C for 10 minutes. The PCR product was inserted into the previously constructed pCDF-EGFP plasmid both digested with SpeI restriction enzyme. PCR product and both digested insert and backbone were purified using column purification. The ligation was performed using T4 DNA ligase (New England Biolabs) and at a molar ratio of 1:3 vector to insert. The ligation reaction was performed overnight at 4 °C.. Ligated lentiviral plasmid constructs were transformed into competent STBL3 cells (Invitrogen). The transformation procedure was performed by first thawing the competent STBL3 cells. Next, 1 µl of the lentiviral plasmid was added. The cells were incubated on ice for 30 minutes then heat-shocked for 45 seconds at 42 °C. Immediately the cells were placed on ice for 2 minutes. Pre-warmed super optimal broth (S.O.C.) media was added and incubated at 37 °C for 1 hour in a shaking incubator. Each transformation was then spread on selective plates and incubated overnight at 37 °C. The next day colonies were selected by plasmid mapping using AgeI and ScaI and DNA sequencing. Lentiviral plasmid constructs

were generated for all three miRNA-155, miRNA-27b, and miRNA-125b. However, only the lentiviral plasmid for miR-125b was successfully integrated generating a stable reporter cell line. Due to the inability to integrate the lentiviruses, lentiviral expression vectors were reconstructed in order to remove a premature poly-A tail from the viral transcript. Similar to the protocol above, the Renilla luciferase gene together with miRNA target site but without the poly-A tail were amplified from the psiCHECK-miR-155 and psiCHECK-miR-27b plasmids by PCR using primers engineered to have SpeI sites (5'-CGTAACTAGTAGATCT GCGCAGCACC-3' (forward), 5'-AGCTACTAGTGACCAAACCCC-3' (reverse)). The PCR product was inserted into the previously constructed pCDF-EGFP plasmid both digested with the SpeI restriction enzyme. Ligated lentiviral plasmid constructs were transformed into competent STBL3 cells. The next day colonies were selected by plasmid mapping and DNA sequencing. Lentiviral plasmid constructs were generated for miRNA-155 and miRNA-27b.

Transient Transfections. The lentiviral constructs were transiently transfected into the appropriate cells line (Raji, 4175, and PC3 cells) using Lipofectamine transfection reagent (Invitrogen). Raji cells were passaged into a 24 well plate (50,000 cells) one day before transfection. The next day 2 µg of the lentiviral construct and 4 µl of Lipofectamine reagent were used for transfection. 4175 and PC3 cells were passaged into a 96 well plate (10,000 cells) one day before transfection. The next day 250 ng of the lentiviral construct and 2 µl of Lipofectamine reagent were used for transfection. After 48 hours the cells were assayed using a Renilla luciferase kit (Promega). In order to determine if the luciferase

activity can be restored by adding a competitive inhibitor of the miRNA, antagomirs were co-transfecrted (100 pmol for miR-155, 50 pmol for miR-27b, and 10 pmol for miR-125b) together with the lentiviral construct using the same transfection conditions as above. The miR-155 and miR-27b antagomirs were purchased from Ambion (5'-ACCCCUAUCACGAUUAGCAUUAA -3' and 5'- GUUCACCAAUCAGCUAAGCUCU -3'). The miRNA-125b antagomir was purchased from IDT DNA (5'-UCACAAGUUAGGGUCUCAGGGA-3'). The antagomirs are phosphorothioate DNA oligonucleotides with a 2'-OMe modification. Their sequences are perfectly complementary to the miR-155 and miR-27b target sites.

Lentivirus production and transduction. Lentiviruses for miRNA-155, miRNA-27b, and miRNA-125b were generated in HEK293T cells. The pCDF-EGFP-RLuc-miRNA expression vectors (2 µg) along with pPACKF1 packaging vectors (20 µl, System Biosciences) were co-transfected using Lipofectamine transfection reagent into HEK293T cells. 48 hours post-transfection cell culture media containing viruses particles was collected and concentrated using the 5x PEG-it concentration reagent (System Biosciences). The appropriate amount of PEG-it reagent was added to the media and the mixture was stored at 4 °C for 24 hours. The next day, the concentrated viruses were centrifuged at 1500 g at 4 °C for 30 minutes. The virus pellet was resuspended in 100 µl of ice cold PBS buffer.

In order to determine the lentiviral titers, NIH-3T3 cells (50,000 cells / well) were plated one day before transduction. The next day cells were transduced with 0.02 µl, 0.2 µl, and 2 µl, of lentivirus in duplicates. 72 hours post-transduction the cells were lysed with 100

µl of lysis buffer. The lysates were heated at 95 °C for 2 minutes then centrifuged at 14,000 rpm for 2 minutes. The lysates are stored at -20 °C until use. The lentiviral standard titer curve was calculated by using the global ultrarapid lentiviral titer kit (System Biosciences). For each reaction, a PCR mixture was prepared which consists of 2x SYBRTaq mix, UCR1 or WPRE primer mix. For each reaction 2 µl of six control DNA calibration standards along with the lentivirus transduced cell lysates were prepared. qRT-PCR reactions started with 50 °C for 2 minutes and an initial denaturation at 95 °C for 10 minutes followed by an amplification cycle of 95 °C for 15 seconds, 60 °C for 1 minute for 40 cycles total.[80] The C_T values for UCR1 and WPRE were calculated for each standard and sample. Next, $2^{-\Delta Ct}$ was calculated where ΔC_T is the average C_T of WPRE - average C_T of UCR1. Multiplicities of infection (MOI) of standard against the $2^{-\Delta Ct}$ value was plotted to create the standard curve. Using the standard curve the MOI of the samples which corresponds to the lentiviral titer was calculated.

The appropriate cells (Raji, 4175, and PC3) were passaged into a 24 well plate (50,000 cells / well). Each cell line was transduced with the lentivirus using 5 or 10 MOIs. After 72 hours the transduced cells were observed using a fluorescence microscope. Cells expressing EGFP were diluted and passaged onto a 10 cm plate. After 2-3 weeks, colonies were grown. Colonies expressing EGFP were isolated by using cloning rings. Cloning rings were placed over the cell clones, trypsinized, and passaged to a 48 well plate and further assayed using a Renilla luciferase kit.

3. Therapeutic potential of miRNA-21 inhibition by small molecules

3.1 Chemosensitivity assays

Cellular sensitivity to anticancer agents is differs by cell type, genotype of the cell, and the dose of the agent used.[81] Determining the molar concentration that results in a 50% reduction in cell survival (IC_{50}) can be used to compare the efficiency of different drugs in one tumor system or of the same drug in different cell systems. The most commonly used assays in tissue culture can be divided into two categories, 1. Measurement of cell membrane integrity or cell metabolic assays. 2. Clonogenic cell proliferation assays.[82]

Loss of cell membrane integrity due to cell death releases cytosolic enzymes. The level of enzyme activity correlates with the amount of cell death or membrane damage. A decrease in total cell protein caused by a compound can be used to measure cell viability.[83] Sulforhodamine B (SRB) is an anionic aminoxanthene dye which forms a complex with basic amino acid residues of proteins. The color development is rapid and stable and the absorbance is measured at 560 and 580 nm.[84] While SRB measures the loss in cell viability, there are other assays which specifically measure the acute effect of compounds on cell survival. MTT assays measure the reduction of a tetrazolim dye by mitochondrial dehydrogenases resulting in a blue-purple formazan product. The colorimetric measurement reflects metabolic activity of the living cells.[85] These assays are useful in quantifying cell survival[84, 86] and measuring multiple end-points. Since drugs may affect cell size, morphology, and membrane integrity and function, multiple assays allow simultaneous measurement of several endpoints to determine cell damage. However, a drawback of the

short term assay is that along with its limited dynamic range and short signal duration, it does not differentiate between cellular damage and cell death effects. In other words, the assay regards cells as viable although they are damaged or committed to die while the plasma membrane is still intact, reducing metabolic activity. Thus, these methods work best for measuring the later stages of apoptosis. Although non-clonogenic assays can measure the potency of a cytotoxic agent, short-term assays can overestimate cytotoxicity of the drugs by not accounting for reversible damage or recovery of cells resistant to drugs in comparison with long-term assays for cell growth or colony reproducibility. It was demonstrated that clonogenic assays in established cell lines differ in results and are not always consistent with non-clonogenic assays.[87] In addition, among the multiple forms of cell death, individual cell death mechanisms occur with different rates in different cells. Thus, it is difficult to estimate the total cell number killed by drug treatment by examining a single time point such as 1-2 days after treatment. This can be problematic because it can be misinterpreted that the treatment changes the level of cell death when it actually changes the rate of cell death. Thus, clonogenic assays were developed to integrate all forms of cell death as well as the ability of cells to proliferate.[88]

Clonogenic assays were developed by Puck and Marcus in the 1950s. Single untreated or treated tumor cells are grown in soft agar petri dishes and colonies are counted after 2-3 weeks.[89] A recent study stated that the clonogenic assay is the "gold standard" for simultaneously measuring the net result of multiple forms of death and examining long-term cellular response.[90] However, there are disadvantages to the use of clonogenic assays. Along with its lack of dynamic range, it was found that only a low percentage of tumor cells are

clonogenic and would grow in agar-based media, often resulting in the need for substantial assay optimization.[91] In addition, it takes a number of weeks to yield useful data. Since cells are plated at low density it is difficult to measure the impact of cell-cell communication on cell proliferation.[92] Clonogenic assays also depend on drug exposure time. Misleading results may be obtained from cells that are continuously exposed to a drug which may change the rate of cellular proliferation or generate a resistance mechanism to the drug.[93] Clonogenic assays also have the problem of distinguishing cell death from cell cycle arrest. Thus, using a dye exclusion assay along with fluorescence activated cell sorting (FACS) can solve the problem.[94,95] Despite the caveats, clonogenic assays are the best measurement for overall cell proliferation and permanent growth inhibition. Drug cytotoxicity measured with multiple parameters can give important information on the mechanism of cell damage caused by the drug and its cytotoxic potency.[95]

3.2 Combination treatment of cancer cell lines

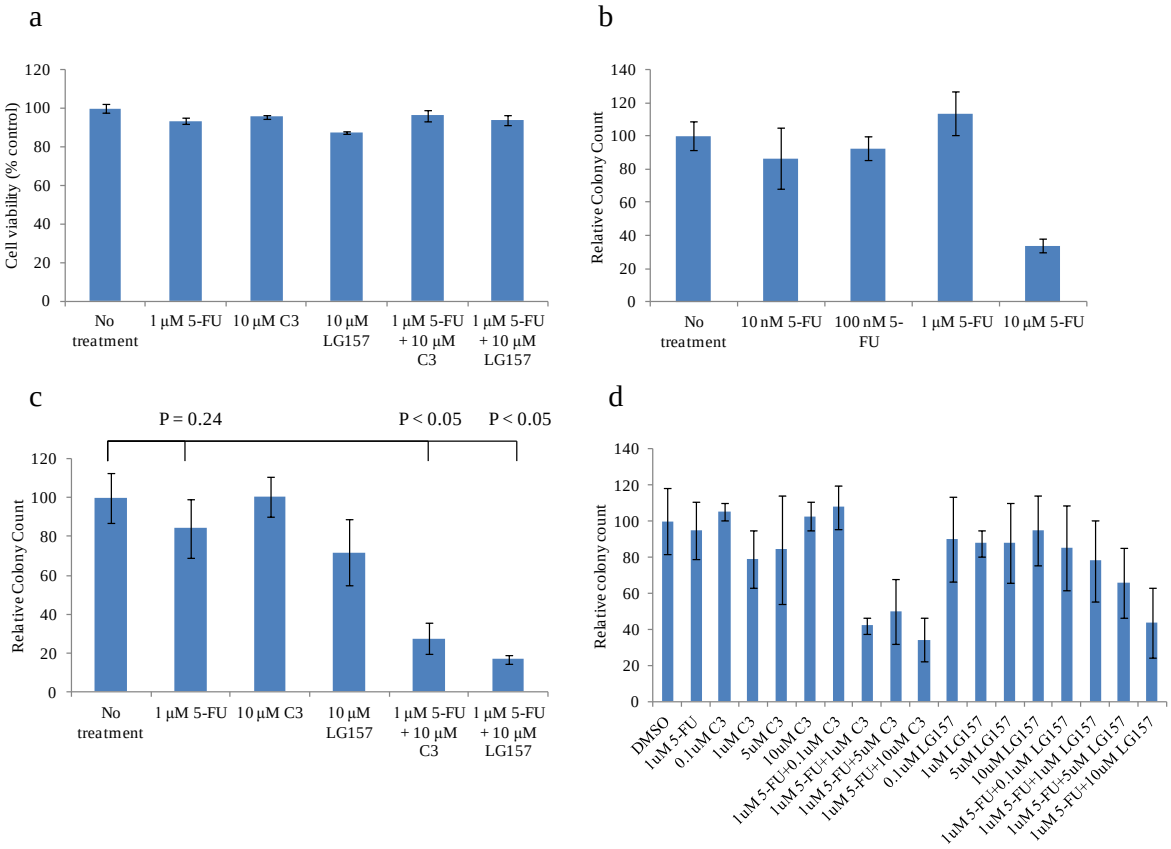
Anticancer agents have been widely used in clinical cancer treatments. However, despite all the effort and capital invested, cancer therapy has encountered many problems due to drug resistance in various cancer cells. Recently, several reports have shown miRNA-mediated drug resistance in various cancer cell lines.[96, 97, 98, 99] Among the different miRNAs, elevated miR-21 expression is known to be involved in drug resistance.[100, 101] We hypothesized that we can induce apoptosis and cause reduction in cell proliferation selectively by treating cancer cells with known anti-cancer drugs in combination with our discovered miR-21 inhibitors.

Recent reports have shown that miR-21 expression is increased in colon cancer cells after continuous exposure to the anticancer agent 5-fluorouracil (5-FU), which diminishes the apoptotic effects of the anticancer agent.[102] Also, it was discovered that overexpression of miR-21 is involved in the mismatch repair (MMR) system. When erroneous insertion, deletion, and misincorporation of bases occur during DNA replication, the proofreading exonuclease activity of the DNA polymerase edits the mistakes and corrects synthesis. Mistakes that escape the exonuclease activity are recognized and repaired by the MMR system.[103] Human mutS homolog 2 (hMSH2) functions as the core of the MMR system and forms a heterodimer with human mutS homolog 6 (hMSH6).[104] Heterodimer formation is an important initiator of DNA damage recognition. MiR-21 targets hMSH2 and hMSH6 and inhibits the formation of the heterodimer. Defects in the MMR system enhance tumor progression and resistance to 5-FU.[105] 5-FU is a widely used anticancer drug that inhibits RNA and DNA synthesis and targets thymidylate synthetase by processing to an active

metabolite which incorporates during DNA synthesis. Inhibition of a cell's ability to synthesize DNA induces cell cycle arrest and apoptosis.[106] Thus, we hypothesized that miR-21 inhibition by our small molecules will sensitize cancer cells to 5-FU treatment. In order to test this hypothesis, we first determined the IC_{50} of 5-FU in HCT-116 cells. It has been documented that the IC_{50} of 5-FU in HCT-116 cells is 17 μM.[107] First, a MTT cell viability assay[108] was performed in HCT-116 cells treated with 5-FU at a concentration of 1 μM in combination with C3 or LG157 (10 μM). However, no significant effect of the treatment was observed in the assay (Figure 11a). As the next step, a dose dependent clonogenic assay was performed in order to determine the sensitivity of HCT-116 cells to 5-FU treatment. Cells treated with 5-FU at a concentration of 1 μM showed no reduction in colony formation. Increasing the concentration of 5-FU to 10 μM reduced colony formation by 70% (Figure 11b). In our clonogenic assay we decided to use 1 μM of 5-FU to determine enhanced sensitivity towards 5-FU by selectively inhibiting high miR-21 expressing cancer cells with C3 or LG-157 leading to a reduction of colony formation and synergistic effects. Thus, 1 μM of 5-FU and 10 μM of C3 or LG-157 were used to treat the cells for the clonogenic assays. When cells were treated with 5-FU (1 μM) in combination of miR-21 inhibitor (10 μM) a significant reduction in colony formation was observed. HCT-116 colon cancer cells treated with 5-FU in combination with either C3 or LG-157 had a 70-80% reduction in colony formation compared to the non-treated cells (Figure 11c). Next, 1 μM of 5-FU and different concentrations of miR-21 inhibitors were used to treat the cells. Cells treated with the miR-21 inhibitor alone did not show a difference in colony formation at different concentrations. However, when treated in combination with 5-FU results show 60% reduction in colony

formation as less as 1 μM of C3 and 10 μM of LG-157 (Figure 11d). These results reveal the potential to selectively target cancer cells expressing high levels of miR-21 using combination therapy at lower concentrations without affecting other normal cells. Our results suggests that by inhibiting miR-21 with small molecule inhibitors, the cells became sensitive to 5-FU which had previously been shown when HCT-116 cells were treated with miR-21 antagomirs.[109]

Figure 11. (a) Cell viability assay of HCT-116 colon cancer cells treated with 1 μM of 5-fluorouracil (5-FU) in combination with 10 μM of the miR-21 inhibitors C3 and LG157 for 48 hours. (b) Dose dependent clonogenic assay of HCT-116 treated with 5-FU. (c) Clonogenic assay of HCT-116 cells treated with 1 μM of 5-FU and 10 μM of the miR-21 inhibitors C3 and LG-157 for 10-14 days. (d) Clonogenic assay of HCT-116 cells treated with 1 μM of 5-FU and different concentration of miR-21 inhibitors C3 and LG-157 for 10-14 days. The relative colony count was normalized to a DMSO negative control. The error bars indicate the standard deviation from three independent experiments.


a
Cell viability (% control)
120
100
80
60
40
20
0
No treatment
1 µM 5-FU
10 µM C3
10 µM LG157
1 µM 5-FU + 10 µM C3
1 µM 5-FU + 10 µM LG157
b
Relative Colony Count
140
120
100
80
60
40
20
0
No treatment
10 nM 5-FU
100 nM 5-FU
1 µM 5-FU
10 µM 5-FU
c
P = 0.24
P < 0.05
P < 0.05
Relative Colony Count
120
100
80
60
40
20
0
No treatment
1 µM 5-FU
10 µM C3
10 µM LG157
1 µM 5-FU + 10 µM C3
1 µM 5-FU + 10 µM LG157
d
Relative colony count
140
120
100
80
60
40
20
0
DMSO
1uM 5-FU
0.1uM C3
1uM C3
5uM C3
10uM C3
1uM 5-FU+0.1uM C3
1uM 5-FU+1uM C3
1uM 5-FU+5uM C3
1uM 5-FU+10uM C3
0.1uM LG157
1uM LG157
5uM LG157
10uM LG157
1uM 5-FU+0.1uM LG157
1uM 5-FU+1uM LG157
1uM 5-FU+5uM LG157
1uM 5-FU+10uM LG157

Past reports showed that the anti-cancer reagent docetaxel has anti-tumor activity in breast, lung, ovarian, head, neck, and gastric cancers as well as melanoma.[110,111] In addition, PC3 prostate cancer cells show an increase in miR-21 expression and IC_{50} when treated with docetaxel.[112] Based on previous reports and results of 5-FU treatment on HCT-116 cells shown above, HCT-116 cells were treated with docetaxel to determine if there is a synergistic effect with our miR-21 inhibitors. Docetaxel binds to the microtubule polymer stabilizing and enhancing tubulin polymerization.[113] Stable microtubules induce aneuploidy which leads to abnormal mitosis, cell cycle arrest, and apoptosis. It has also been found that docetaxel induces phosphorylation and downregulates the anti-apoptotic protein Bcl-2. [114] Reports have shown that the IC_{50} of docetaxel in HCT-116 cells is 46 nM.[115] HCT-116 cells treated with docetaxel at a concentration of 500 pM in combination with C3 or LG157 (10 μM) showed no significant effect in a MTT cell viability assay (Figure 12a). In order to conduct clonogenic assays, HCT-116 cells were treated with 1 nM and 500 pM of docetaxel. However, 80% reduction in colony formation was achieved by docetaxel alone for both concentrations. By lowering the concentration to 100 pM no difference in colony formation was observed compared to the non-treated cells. It appears that HCT-116 cells are sensitive to docetaxel treatment in the 100 pM to 500 pM range (Figure 12a). Thus, 250 pM of docetaxel and 10 μM of C3 or LG-157 were used to perform the clonogenic assays. At 250 pM of docetaxel alone, the HCT-116 cells showed only a 20% reduction in colony formation. However, there was a further reduction in colony formation when HCT-116 cells were treated with a combination of docetaxel (250 pM) and the miR-21 inhibitors (10 μM). HCT-116 cells treated with docetaxel in combination with either C3 or LG-157 displayed a 50%

reduction in colony formation compared to the non-treated cells (Figure 12b). By treating the cells in combination with our miR-21 small molecule inhibitors reducing miR-21 expression, cells became only moderately sensitive to docetaxel. We assume that the HCT-116 cells may show resistance to docetaxel even when miR-21 expression is reduced, or that the reduction of miR-21 in HCT-116 cells is not very pronounced when using small molecule inhibitors.

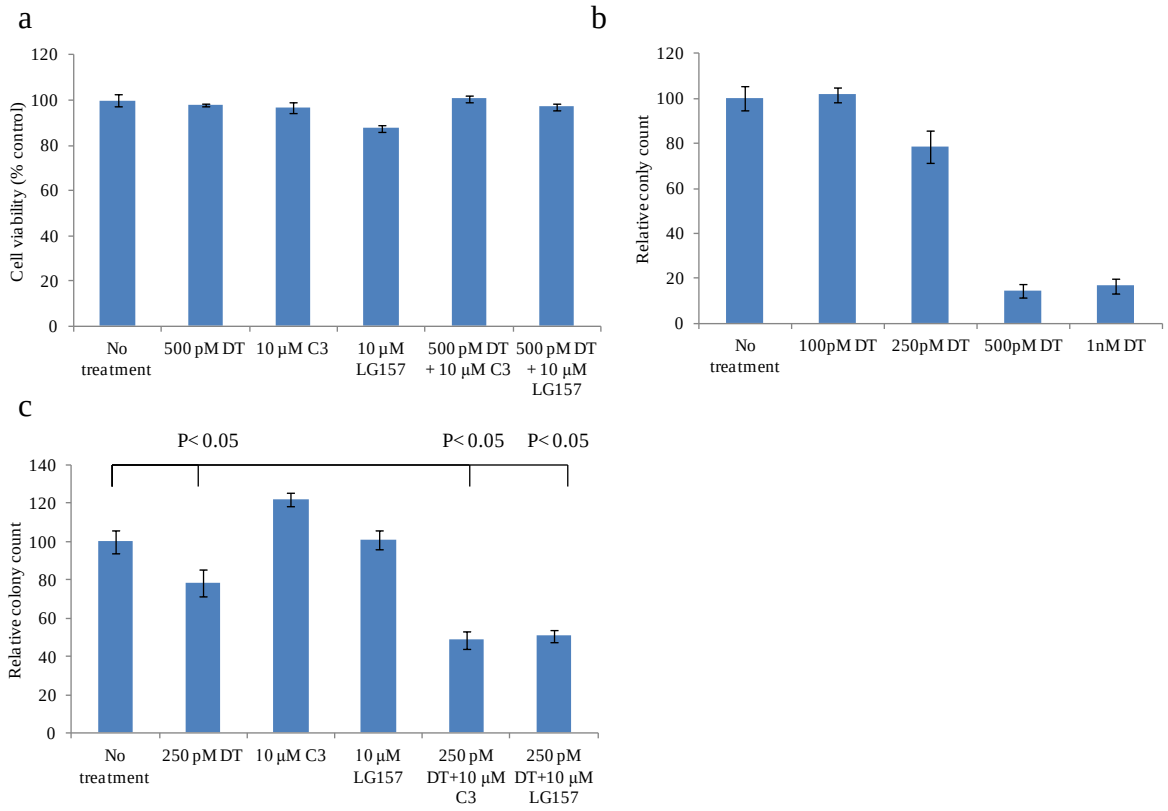


Figure 12. (a) Cell viability assay of HCT-116 cells treated with 500 pM of docetaxel (DT) in combination with 10 µM of the miR-21 inhibitors C3 and LG157 for 48 hours. (b) Dose dependent clonogenic assay of HCT-116 cells treated with docetaxel. (b) Clonogenic assay of HCT-116 cells treated with 250 pM docetaxel and 10 µM of the miR-21 inhibitors C3 and LG-157 for 10-14 days. The relative colony count was normalized to DMSO control. The error bars indicate the standard deviation from three independent experiments.

Our clonogenic assays were then expanded to different cancer cell lines. MiR-21 is highly expressed in the small lung cancer cell line A549.[116] Doxorubicin, a known anticancer agent intercalates to the topoisomerase II-cleaved DNA complex during replication, inhibiting DNA re-ligation resulting in DNA damage and eventually apoptosis.[117] Once cells are treated with doxorubicin, a significant decrease in drug potency was shown as miR-21 was increased in lung cancer cells.[118] Thus, inhibition of miR-21 in combination with doxorubicin and our small molecules can become sensitive to doxorubicin further increasing drug potency leading to selective apoptosis. Reports have shown that the IC_{50} of doxorubicin in A549 cell is 90 nM.[119] First, a dose dependent clonogenic assay was performed according to the reported IC_{50} in order to determine the sensitivity of A549 cells to doxorubicin treatment. Cells treated with 10 nM doxorubicin showed a 30% reduction in colony formation. Increased concentration of doxorubicin up to 100 nM completely reduced colony formation (Figure 13a). In order to determine synergistic effects and show a further reduction in colony formation with our miR-21 inhibitors 10 nM of doxorubicin and 10 μM of C3 or LG-157 were used to conduct clonogenic assays. By treating A549 cells with 10 nM of doxorubicin in combination with the miR-21 inhibitors significant reductions in colony formation were observed. A549 cells treated with the combination of doxorubicin and the miR-21 inhibitors showed a 50-60% reduction in colony formation compared to the non-treated cells (Figure 13b). Strangely, when treated with only the miR-21 inhibitors the cells showed an increase in colony formation in the A549 cell line. This was also seen in the colon cancer cell line HCT-116 cells, potentially due to a cellular defense mechanism.

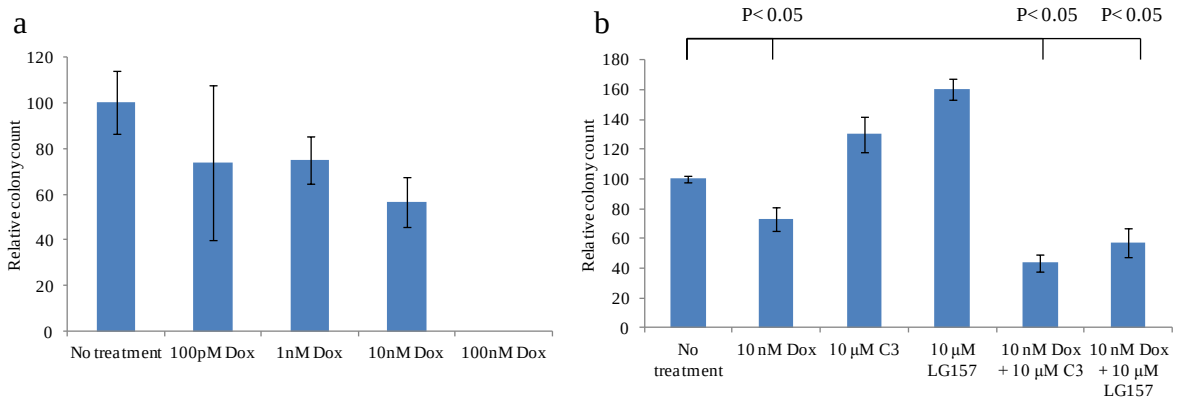


Figure 13. (a) Dose dependent clonogenic assay of A549 lung cancer cells treated with doxorubicin (Dox) (b) Clonogenic assay of A549 cells treated with 10 nM doxorubicin and 10 µM of the miR-21 inhibitors C3 and LG-157 for 10-14 days. The relative colony count was normalized to DMSO control. The error bars indicate the standard deviation from three independent experiments

Previous reports show that miR-21 is highly expressed in breast cancer cells.[69] The breast cancer cell line MCF-7 became sensitive to the anticancer agent topotecan when treated with an anti-miR-21 antagomir resulting in a decrease in cell growth.[120] In addition, the report suggested that transfection of a miR-21 antagomir leads to the downregulation of the anti-apoptotic protein Bcl-2 and thereby induces apoptosis.[120] Topotecan is a derivative of camptothecin which intercalates into the topoisomerase I (Top1)-cleaved DNA complex during replication leading to irreversible DNA damage and eventually leading to apoptosis.[121] We hypothesized that there would be a synergistic effect between topotecan and our miR-21 inhibitors in MCF-7 breast cancer cells. It was previously shown that the IC_{50} of topotecan in MCF-7 cells is > 0.3 µM [122] When MCF-7 cells were treated with topotecan at a concentration of 100 nM in combination with C3 or LG157 (10 µM) no significant effect was

observed in a MTT cell viability assay[108] (Figure 14a). Cells treated with 100 nM of topotecan showed a 70% reduction of colony formation (Figure 14b). In order to determine synergistic effects and reduce colony formation with our miR-21 inhibitors, a 10-fold lower concentration of topotecan (10 nM) and 10 μM of C3 or LG-157 were used to conduct the clonogenic assays. MCF-7 cells treated with topotecan in combination with either C3 or LG-157 displayed 50% reduction in colony formation compared to the non-treated cells (Figure 14c). However, MCF-7 cells treated with LG-157 alone showed a similar reduction in colony formation as compared to cells treated in combination with topotecan. LG-157 alone showed a 30% decrease in colony formation suggesting that LG-157 may have an additional function along with inhibiting miR-21 to decrease cell growth by regulating apoptotic proteins in MCF-7 cells. Other miR-21 inhibitors need to be evaluated in this treatmennt regiment.

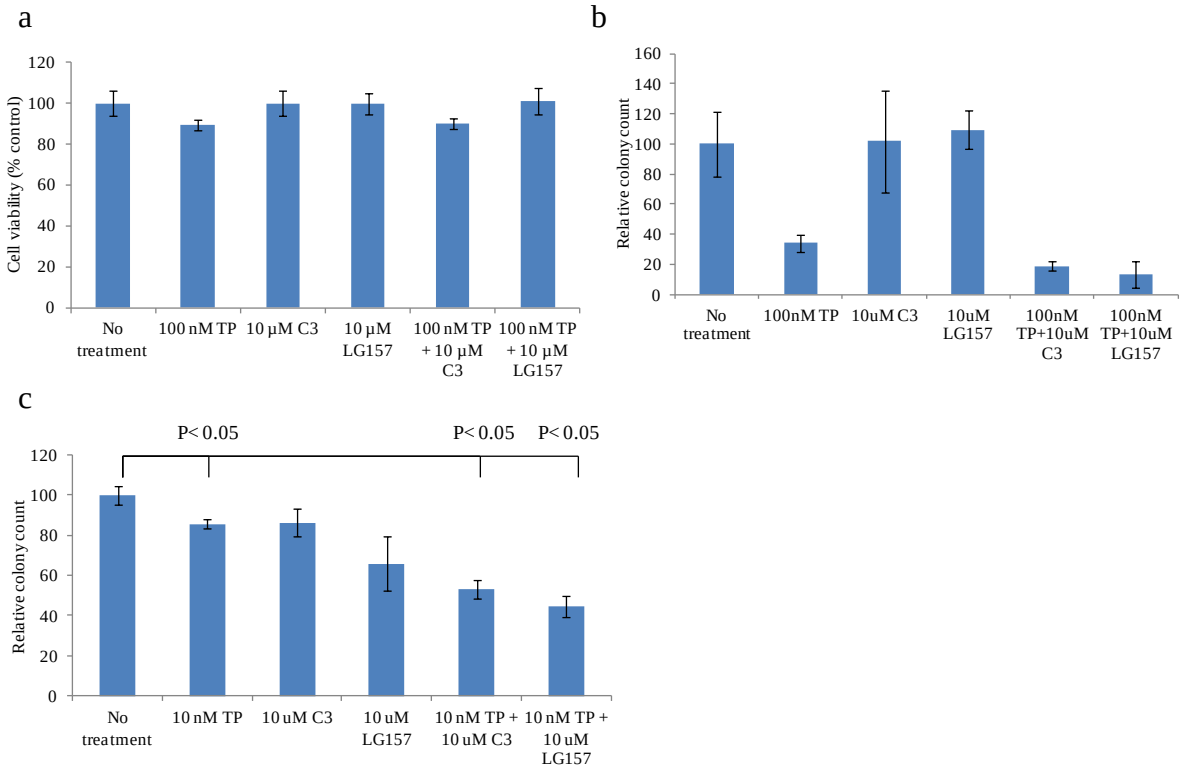


Figure 14. (a) Cell viability assay of MCF-7 breast cancer cells treated with 100 nM of topotecan (TP) in combination with 10 µM of the miR-21 inhibitors C3 and LG157 for 48 hours. (b) Clonogenic assay of MCF-7 cells treated with 100 nM topotecan and 10 µM of the miR-21 inhibitors C3 and LG-157 for 10-14 days. (c) Clonogenic assay of MCF-7 cells treated with 10 nM topotecan and 10 µM of the miR-21 inhibitors C3 and LG-157 for 10-14 days. The relative colony count was normalized to DMSO control. The error bars indicate the standard deviation from three independent experiments.

It was shown that miR-21 expression was increased in docetaxel resistant PC3 cells. Upon transfection of an anti-miR-21 antagomir, the sensitivity of the cells to docetaxel was increased as demonstrated by a decrease in the IC_{50} and a reduction in cell growth leading to apoptosis.[112] The IC_{50} of docetaxel in PC3 cells is 1 nM.[123] In a clonogenic assay, 1 nM of docetaxel alone decreased PC3 cell viability by 80%. Therefore, a lower concentration of

docetaxel (100 pM) was used to conduct our clonogenic assay. Treatment of PC3 cells with 100 pM of docetaxel alone showed a 20% reduction in colony formation (Figure 15a). When PC3 cells were treated with docetaxel (100 pM) in combination of the miR-21 inhibitors C3 or LG-157 (10 µM), no further reduction in colony formation was observed. Thus, PC3 cells treated with docetaxel (100 pM) in combination with miR-21 inhibitors showed resistance (Figure 15b).

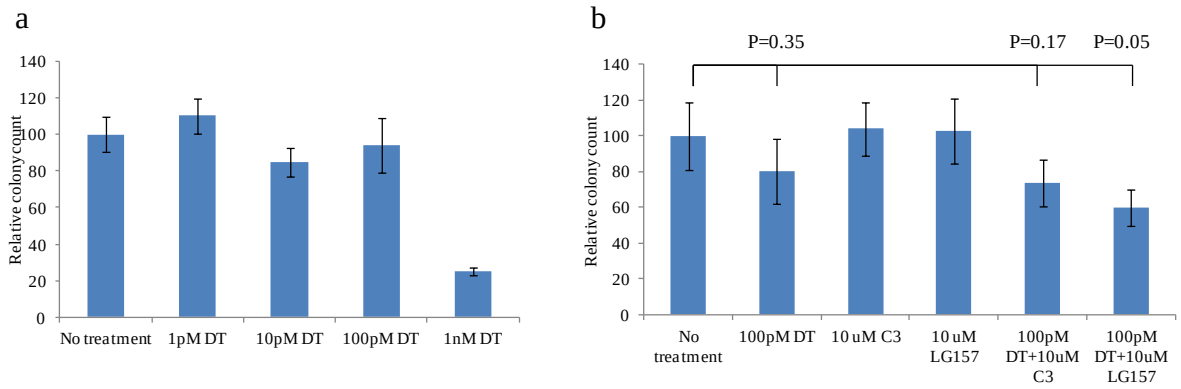


Figure 15. (a) Dose dependent clonogenic assay of PC-3 prostate cancer cells treated with docetaxel (DT). (b) Clonogenic assay of PC-3 cells treated with docetaxel (100 pM) and the miR-21 inhibitors C3 and LG-157 (10 µM) for 10-14 days. The relative colony count was normalized to DMSO control. The error bars indicate the standard deviation from three independent experiments.

Among the clonogenic assay results, HCT-116 colon cancer cells treated with 5-FU in combination with miR-21 inhibitors showed the best results (Figure 11c). Since the cells displayed reduced colony formation with the combination treatment but did not undergo

apoptosis, according to the previous MTT cell viability assay (Figure 11a), we hypothesized that the combination treatment may induce cell cycle arrest.

Cellular division is a process that can lead to differentiation or cell death[124], and misregulation of this process may result in cancer.[125] The cell cycle consists of four different phases. At the G1 phase, the cell prepares to divide. During the S phase, DNA is synthesized and replicated. Next, mitotic division is initiated in the G2 phase and followed by nuclear and cytoplasmic division which are completed in the M phase. The G1 and G2 phase act as checkpoints in response to misregulation in the cell cycle process. The cell cycle progression may be stopped or cells may undergo apoptosis to prevent mutations.[126]

5-FU induces cell cycle arrest at the transition between the G1 and S phases and later induces apoptosis independent of the p53 pathway.[127] [128] Based on the involvement in 5-FU in cell cycle arrest we wanted to determine the effect of the combination treatment on the progression of the cell cycle. In order to conduct a cell cycle experiment, HCT-116 cells were treated with 1 μM 5-FU in combination with 10 μM of C3 or LG-157 for 48 hours. Next the cells were stained with propidium iodide and analyzed by flow cytometry.

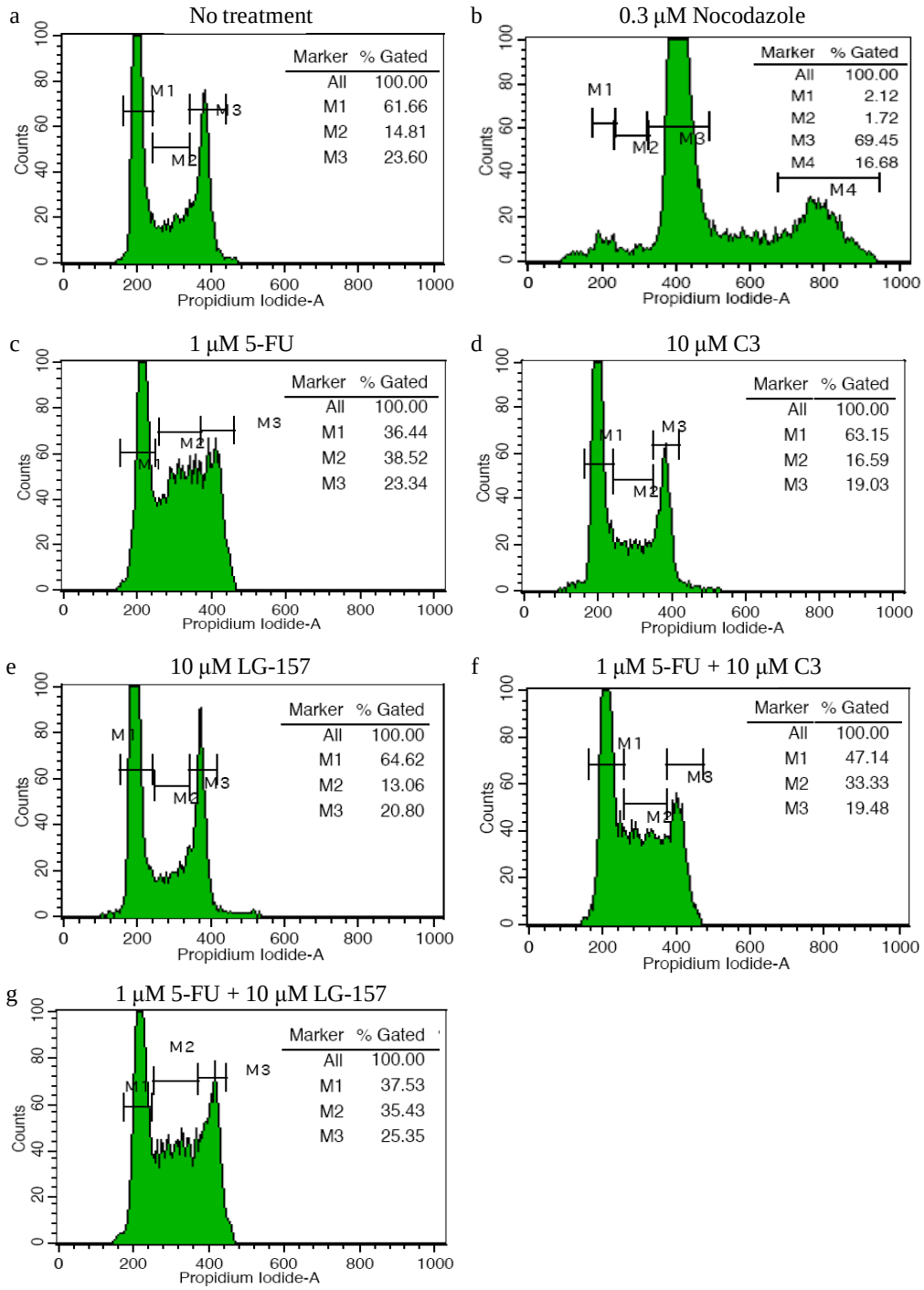


Figure 16. Flow cytometry of HCT-116 cells treated with 5-FU (1 μM) in combination with miR-21 inhibitors (10 μM). The values in each box indicates the percentages of cells in M1 (G1 phase), M2 (S phase), M3 (G2 phase), and M4 (M phase).

The results show an accumulation at the S phase when treated with 5-FU in combination with C3 (35% of cells in S phase) or LG157 (36% in S phase) compared to the non-treated cells (15% in S phase). However, cells treated with 5-FU alone also showed the same result (41% of cells in S phase) meaning that the cell cycle arrest at the S phase is not be due to the combination treatment (Figure 16). Comparing the duration of compound treatment of the cell cycle arrest experiment and clonogenic assay, the cell cycle arrest experiment is conducted right after cells were treated with 5-FU alone or in combination treatment for 48 hours which show similar results. However, in the long term clonogenic assay the results are more prevalent, showing more effective combination treatment.

Overall, selected anticancer agents in combination with our miR-21 inhibitors C3 or LG-157 showed a synergistic reduction in colony formation of certain cancer cell lines. The degree of sensitivity towards the synergistic effect was dependent on the cancer cell type. There is no clear explanation why this phenomenon occurs; however, these results provide a potential solution to selectively targeting cancer cells expressing high levels of miR-21 without affecting other normal cells. Using the same conditions effective in high miR-21 expressing HCT-116 cells, HEK293T cells which express low levels of miR-21 did not reduce colony formation verifying that inhibition of miR-21 may cause cells become sensitive to 5-FU (Figure 17). and could provide new lead compounds for the development of potential therapeutics against cancer.

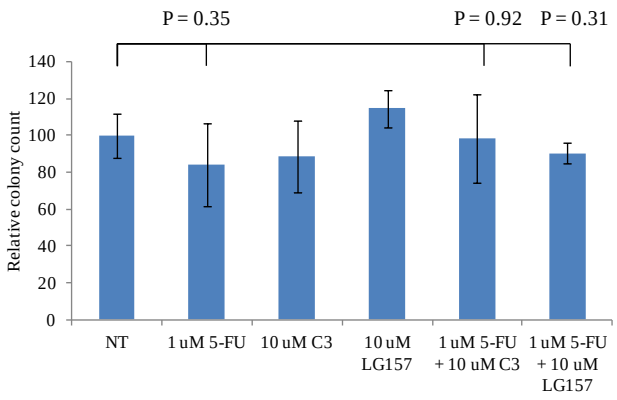


Figure 17. Clonogenic assay of HEK293T cells treated with 1 μM 5-FU and 10 μM of the miR-21 inhibitors C3 and LG-157 for 10-14 days. The relative colony count was normalized to the DMSO control. The error bars indicate the standard deviation from three independent experiments.

3.4 Experimental

Cell culture. Human colon cancer cell line, HCT-116, human lung cancer cell line, A549, human breast cancer cell line, MCF-7, and human prostate cancer cell line, PC3 (obtained from American Type Cell Collection (ATCC), Manassas, VA) were grown in DMEM and F-12K media (HyClone) supplemented with 10% fetal bovine serum, penicillin (100 units / ml), and streptomycin (100 µg / ml). All cells were incubated at 37 °C and 5 % CO_2.

MTT cell viability assay.[108] HCT-116 and MCF-7 cancer cells were plated into each well of a 96-well plate (10,000 cells / well). The cells were treated with anticancer agents 5-fluorouracil (5-FU), docetaxel, and topotecan in combination with miR-21 inhibitors. HCT-116 cells treated with 5-FU in combination with miR-21 inhibitors had a final concentration of 1 µM 5-FU and 10 µM of C3 or LG-157 (0.1% DMSO). HCT-116 cells treated with docetaxel in combination with miR-21 inhibitors had a final concentration of 250 pM docetaxel and 10 µM of C3 or LG-157 (0.1% DMSO). MCF-7 cells treated with topotecan in combination with miR-21 inhibitors had a final concentration of 100 nM topotecan and 10 µM of C3 or LG-157 (0.1% DMSO). The plates were incubated at 37 °C and 5% CO_2 for 48 hours. Media was removed and replaced 100 µl of fresh media. For each well 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 ml of PBS) was added and incubated at 37 °C for 4 hours. Next, 100 µl of the SDS-HCl solution (1 g of SDS in 10 ml of 0.01 HCl) was added and incubated at 37 °C for another 4 hours. Absorbance was read at 570 nm using a microplate reader (BioTek).

Clonogenic assay.[129] DMEM and F-12K media (HyClone) supplemented with 20% fetal bovine serum, penicillin (200 units / ml), and streptomycin (200 μg / ml) was prepared. The bottom feeder layer was first prepared by mixing equal volumes of 2x media and pre-warmed 1.2% agarose solution to a final concentration of 0.6% agarose, 1x media, and 10% FBS. The mixture was added to each well (1 ml) of a 6-well plate. The top layer was first prepared by trypsinizing and counting HCT-116, A549, MCF-7, and PC-3 cancer cells to have 500 to 1000 cells in 2x media. The appropriate cell line was then treated with anticancer agents 5-FU, docetaxel, doxorubicin, and topotecan in combination with miR-21 inhibitors. HCT-116 cells treated with 5-FU in combination with miR-21 inhibitors had a final concentration of 1 μM 5-FU and 10 μM of C3 or LG-157 (0.1% DMSO). HCT-116 cells treated with docetaxel in combination with miR-21 inhibitors had a final concentration of 250 pM docetaxel and 10 μM of C3 or LG-157 (0.1% DMSO). A549 cells treated with doxorubicin in combination with miR-21 inhibitors had a final concentration of 10 nM doxorubicin and 10 μM of C3 or LG-157 (0.1% DMSO). MCF-7 cells treated with topotecan in combination with miR-21 inhibitors had a final concentration of 100 nM topotecan and 10 μM of C3 or LG-157 (0.1% DMSO). PC-3 cells treated with docetaxel in combination with miR-21 inhibitors had a final concentration of 100 pM docetaxel and 10 μM of C3 or LG-157 (0.1% DMSO). The cell suspension with 2x media containing the compounds, pre-warmed 1.2% agarose solution, and water were mixed in a 2:1:1 ratio to give a final concentration of 0.1% DMSO, 0.3% agarose, 1x media, and 10% FBS. The mixture was added to each well (1 ml) over the bottom feeder layer. Each experiment was done in triplicate for each condition. The plates were incubated at 37 °C and 5% CO_2 for two to three weeks. The colonies were stained with

500 µl of a 0.005% crystal violet solution for 1 hour and counted using the microscope. Colonies which consists of more than 50 cells were counted using a 5x objective by moving the focus along the z position of the microscope.

Cell cycle arrest assay.[125] HCT-116 colon cancer cells were passaged into a 6 well plate (1 x 10^6 cells / well). The cells were treated with the anticancer agent 5-FU (1 µM, 0.05% DMSO) in combination with miR-21 inhibitors (10 µM, 0.05% DMSO). Nocodazole (0.3 µM, 0.1% DMSO) was used as a positive control that induces cell cycle arrest at the G2 / M phase.[130] The cells were incubated at 37 °C with 5 % CO_2. After 48 hours the cells were trypsinized, suspended in 100 µl PBS, fixed with 70% ethanol, and stained with propidium iodide solution (20 µg/ml). The DNA content was analyzed using FASCan (Becton Dickinson).

4. MicroRNA-21 and target proteins regulating signaling pathways

4.1 Target protein PTEN and PDCD4

According to recent papers, a number of tumor suppressor genes have been identified to be miR-21 targets: phosphatase and tensin homologue (PTEN), programmed cell death 4 (PDCD4), RECK, maspin, tropomyosin (TPM1), heterogeneous nuclear ribonuclear protein K (HNRPK) and Tap63.[131] Among these miR-21 targets, we focused on tumor suppressor gene PTEN and PDCD4, which have been shown to display increased levels when miR-21 was inhibited by antagomir transfection into HeLa cells.[132] Thus, we wanted to test if we can achieve the same effect with our small molecule miR-21 inhibitors C3 and LG157.

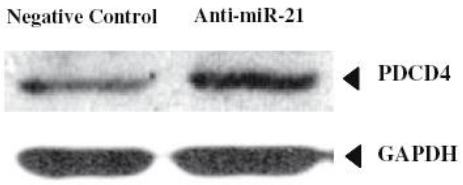


Figure 18. Inhibition of miR-21 increases its target protein PDCD4 in HeLa cells.[132]

4.2 Small molecule inhibitors of miR-21 regulating PTEN and PDCD4

In order to confirm the literature results shown in Figure 18, HeLa cells were transfected with a miR-21 antagomir (100 pmol, 5'-AUCAACAUCAGUCUGAUAAGCUA-3') using lipofectamine. At 48 hours post-transfection, the proteins were extracted and 25 μg of whole cell protein extract was loaded on a 12% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane for Western blotting. The membrane was blocked to prevent non-specific binding of the antibodies. A 1:1000 dilution of the PTEN and PDCD4 primary antibodies and a 1:2500 of the secondary antibody were used for detection. Along with the PTEN and PDCD4 primary antibodies, a GAPDH primary antibody was used as an endogenous control. However, detection of the PTEN and PDCD4 target proteins was unsuccessful. Since we were unable to detect our target proteins, the dilution of the PTEN and PDCD4 primary antibodies was increase to 1:200 dilutions and 50 μg of total protein was used. However, the detection of the target proteins was still unsuccessful.

Nuclear localized PTEN and PDCD4 is evident in inactive cells which is required for their tumor-suppressor activity while for actively dividing cancer cells, both PTEN and PDCD4 are mostly localized in the cytoplasm.[133, 134, 135, 136] In order to independently test nuclear and cytoplasmic proteins, NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific) was used followed by addition of phosphatase and protease inhibitor to stabilize the target protein. SDS-PAGE analysis and Western blots were performed. However, the detection of the target proteins was unsuccessful.

PTEN and PDCD4 antibodies from a different manufacturer (Cell Signaling Technology) provided by Dr. Yoshiaki Tsuji from the Toxicology department was then used for detection to determine if the detection was unsuccessful due to the previous antibody. Fortunately, PTEN and PDCD4 were both detected at 54 kDa as well as the endogenous control GAPDH at 37 kDa. Once the detection of the target proteins was optimized, the same procedure was used to test the effect of miR-21 inhibition by our small molecules. HeLa cells were treated with C3 and LG-157 at a concentration of 10 μM. After 48 hrs, the total protein was extracted and 50 μg was loaded on a 12% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane for Western blotting. A 1:1000 dilution of the PTEN primary antibody and a 1:200 dilution of the PDCD4 primary antibody were used for the western blot. A 1:1000 dilution of the secondary antibody was then used for detection. Along with the PTEN and PDCD4 primary antibodies, a GAPDH primary antibody was also used as an endogenous control. The intensity of the PTEN and PDCD4 bands were quantified and normalized to the GAPDH band to give the relative intensities. The expression of both PTEN and PDCD4 increased in HeLa cells transfected with miR-21 antagomir compared to non-treated cells. However, the expression of the target proteins in HeLa cells treated with C3 or LG-157 was similar to that of the non-treated cells (Figure 19a, b).

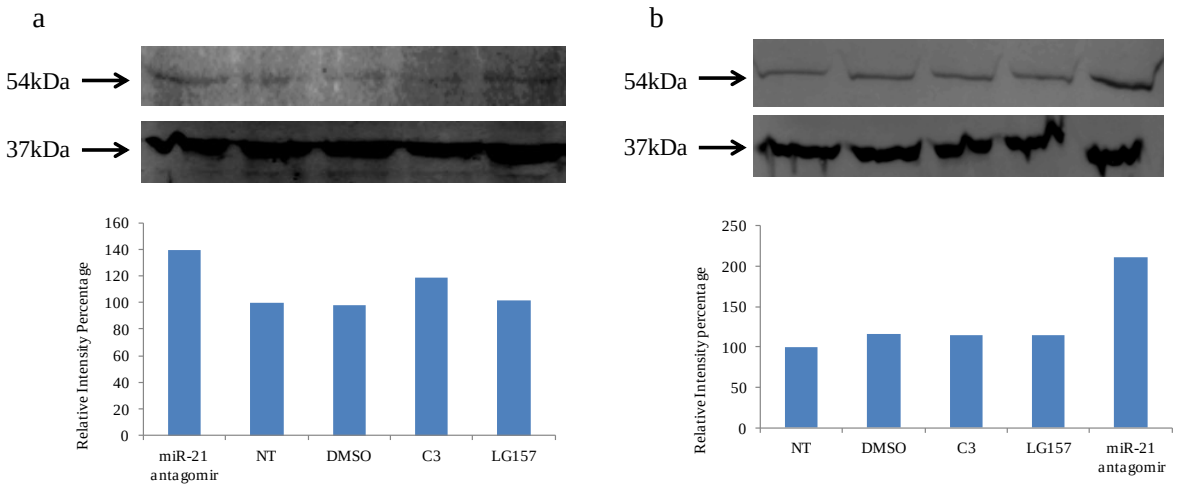


Figure 19. Expression of the miR-21 target proteins PTEN and PDCD4 in HeLa cells. Western blot was performed to detect the expression levels of the miR-21 target proteins PTEN and PDCD4. GAPDH was used as an endogenous control. (a) HeLa cells treated with our miR-21 inhibitors C3 or LG-157 at a concentration of 10 μM did not alter PTEN expression compared to non-treated cells. However, cells treated with the miR-21 antagomir showed 1.4-fold increase in PTEN expression compared to non-treated cells. (b) HeLa cells treated with our miR-21 inhibitors C3 or LG-157 at a concentration of 10 μM did not show change of PDCD4 expression as well compared to non-treated cells. Cells treated with miR-21antagomir showed a 2-fold increase in PDCD4 expression compared to non-treated cells. The intensity was measured using ImageQuantTL and calculated by quantifying the intensity of the bands for the target proteins normalized to the intensity of the GAPDH bands. The experiment was conducted in singlet.

An earlier report has shown that there was no change of mRNA levels of PTEN and PDCD4 when cells were transfected with miR-21 antagomir.[137] However, to determine if cells treated with the small molecule inhibitors alter the mRNA levels of PTEN and PDCD4 we conducted an quantitative RT-PCR (qRT-PCR) experiment. HeLa cells were either transfected with the miR-21 antagomir (100 pmol) or treated with DMSO, C3 or LG-157 (10 μM). Each treatment was conducted in triplicate for statistical validation. 48hrs after post-

transfection, the total RNA was isolated using the TRIzol reagent. Equal amounts of RNA (5 ng per reaction) and PTEN and PDCD4 primers (PTEN forward and reverse 5'-CGGCAGCATCAAATGTTTCAG-3', 5'-AACTGGCAGGTAGAAGGCAACTC-3', PDCD4 forward and reverse 5'-TATGATGTGGAGGAGGTGGATGTGA-3', 5'-CCTTTCATCCAAAGGCAAAACTAC-AC-3') at a final concentration of 500 nM were used for reverse transcription along with GAPDH primers as an endogenous control. qRT-PCR was conducted using the same primers on a BioRad MyiQ RT-PCR thermal cycler. For each qRT-PCR, C_T (threshold cycle) values were obtained. To determine the relative mRNA levels of PTEN and PDCD4 to GAPDH, the $2^{-\Delta\Delta CT}$ method was used.[80]

Since three different RNA samples were isolated, each C_T value was treated separately then averaged after three $2^{-\Delta\Delta CT}$ values were calculated. The average of the triplicate samples displays the percentage of mRNA expression for cells treated with the miR-21 antagomir or the miR-21 inhibitors normalized to the endogenous control and relative to DMSO, where DMSO is represented as 100% expression. As expected, C3 and LG-157 had no effect on the mRNA levels of *pten* or *pdcd4.* (Figure 20a, b).

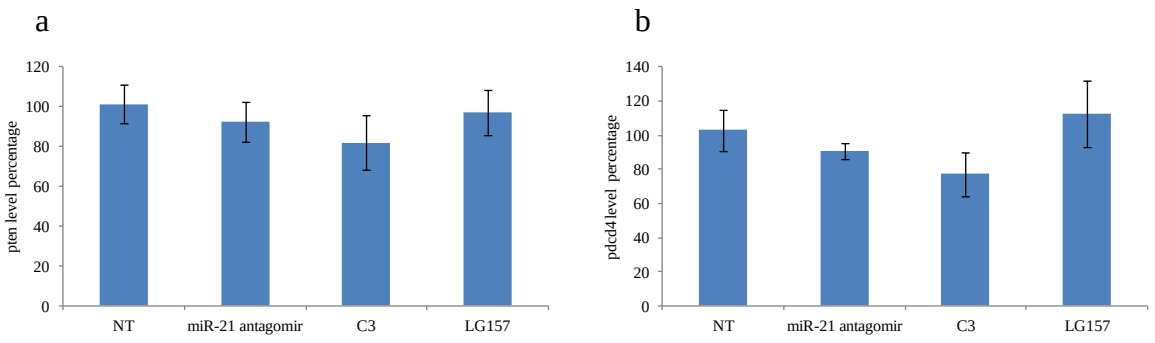


Figure 20. mRNA levels of the miR-21 targets *pten* and *pdcd4* in HeLa cells. qRT-PCR was performed to detect the mRNA levels of the miR-21 targets *pten* and *pdcd4*. *gapdh* was used as an endogenous control. The relative RNA levels were normalized to gapdh control. The error bars indicate the standard deviation from three independent experiments.

According to the results from the Western blot and qRT-PCR, cells treated with C3 or LG-157 does not alter the transcriptional as well as the translational process of the target proteins PTEN and PDCD4.

4.3 Experimental

Cell culture. Human cervical cancer cell line, HeLa (obtained from American Type Cell Collection (ATCC), Manassas, VA) were grown in DMEM media (HyClone) supplemented with 10% fetal bovine serum, penicillin (100 units / ml), and streptomycin (100 μg / ml). All cells were incubated at 37 °C with 5 % CO_2.

Transient transfection. The miRNA-21 antagomir sequences were purchased from IDT DNA.

5'-AUCAACAUCAGUCUGAUAAGCUA-3' The miR-21 antagomir is a phosphorothioated RNA oligonucleotide with a 2'-OMe modification which is perfectly complementary to the miR-21 target site. The antagomir acts as a competitive inhibitor of miRNA.

Western blotting. HeLa cells were transfected with a miR-21 antagomir (100 pmol) or treated with DMSO, C3, or LG-157 (10 μM) in 10 cm plates. After 48 hours cells were harvested, washed with PBS, and lysed with lysis buffer. Total protein (50 μg) was loaded on a 12% SDS-PAGE gel. The SDS-PAGE gel was transferred to a nitrocellulose membrane for 1 hour at 80 V (BioRad). Western blot was performed by blocking membrane for 1 hour at RT with 5% BSA solution and incubated with 1:1000 dilution of the PTEN primary antibody (Cell Signaling Technology) and 1:200 dilution of the PDCD4 primary antibody (Santa Cruz Biotechnology) overnight at 4 °C. The next day the blots were washed three times with TBS-T buffer and were incubated with 1:1000 dilution of the FITC conjugated secondary antibody (Santa Cruz Biotechnology) 1 hour and 30 minutes at room temperature. The blot was

imaged using a phosphoimager (Typhoon FLA 7000, GE Healthcare) and the intensity of the bands were measured using ImageQuantTL.

Quantitative Real-Time PCR.[80] HeLa cells were transfected with a miR-21 antagomir (100 pmol) or treated with DMSO, C3, or LG-157 (10 μM) in 6 well plates. Each treatment was conducted in triplicate for statistical validation. 48hrs after post-transfection, the total RNA was isolated using the TRIzol reagent. Equal amounts of RNA (5 ng per reaction) and PTEN (Cell Signaling Technology) and PDCD4 primers (Santa Cruz Biotechnology) were used for reverse transcription along with GAPDH primers as an endogenous control. PTEN forward and reverse: 5'-CGGCAGCATCAAATGTTTCAG-3', 5'-AACTGGCAGGTAGAAGGCAA CTC-3'; PDCD4 forward and reverse: 5'-TATGATGTGGAGGAGGTGGATGTGA-3', 5'-CCTTTCATCCAAAGGCAAAACTACAC-3'. qRT-PCR experiments were performed to determine target mRNA levels. qRT-PCR was conducted by BioRad MyiQ RT-PCR thermal cycler using the BioRad iScript one-step RT-PCR kit with SYBR green (BioRad). The PCR conditions started with cDNA synthesis at 50 °C for 10 mins. Next, initial denaturation was performed at 95 °C for 5 minutes followed by an amplification cycle of 95 °C for 10 seconds, 60 °C for 30 seconds for 40 cycles total. For each qRT-PCR, C_T (threshold cycle) values were obtained. To determine the relative mRNA levels of PTEN and PDCD4 to GAPDH, the $2^{-\Delta\Delta CT}$ method was used.

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APPENDIX

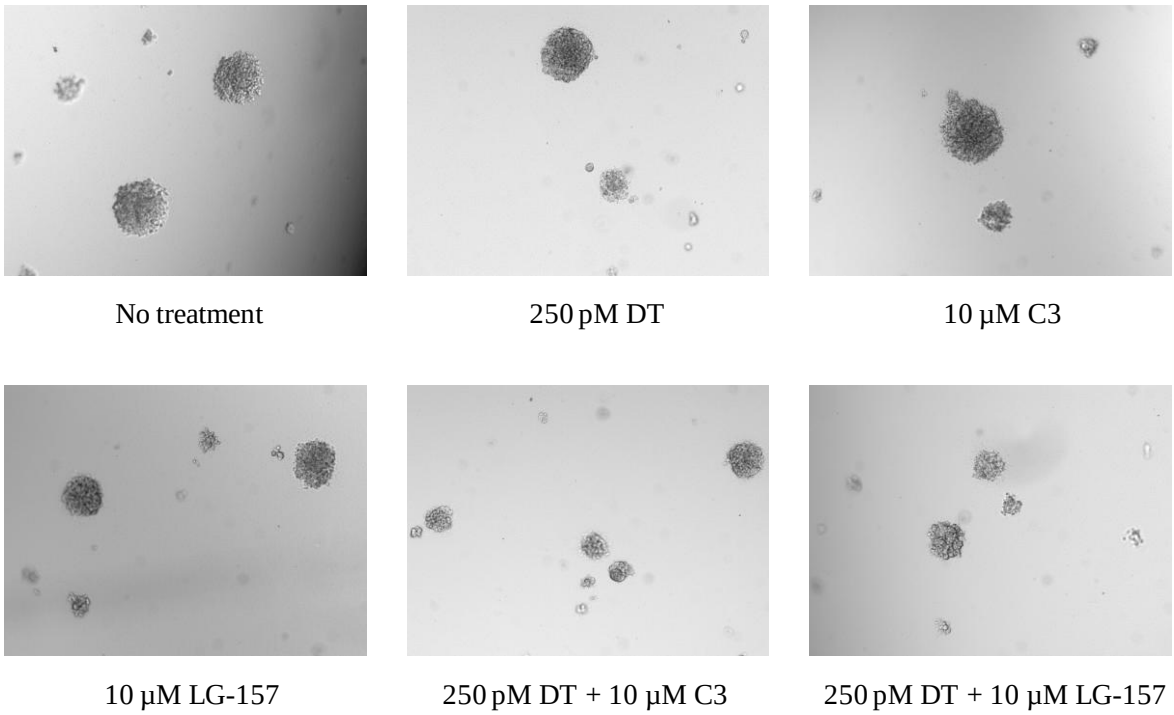

Figure 21. Micrographs of representative clonogenic assays of HCT-116 cells treated with 250 pM of docetaxel and 10 µM of the miR-21 inhibitors C3 and LG-157 for 10-14 days.

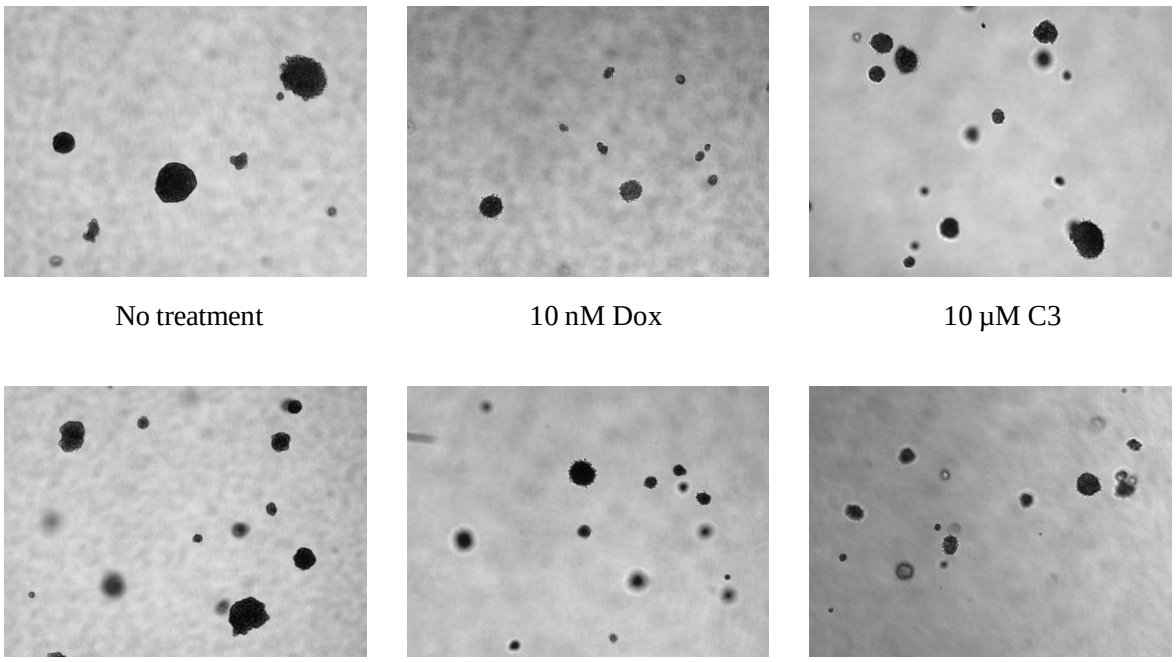

Figure 22. Micrographs of representative clonogenic assays of A549 cells treated with 10 nM of doxorubicin and 10 µM of the miR-21 inhibitors C3 and LG-157 for 10-14 days.

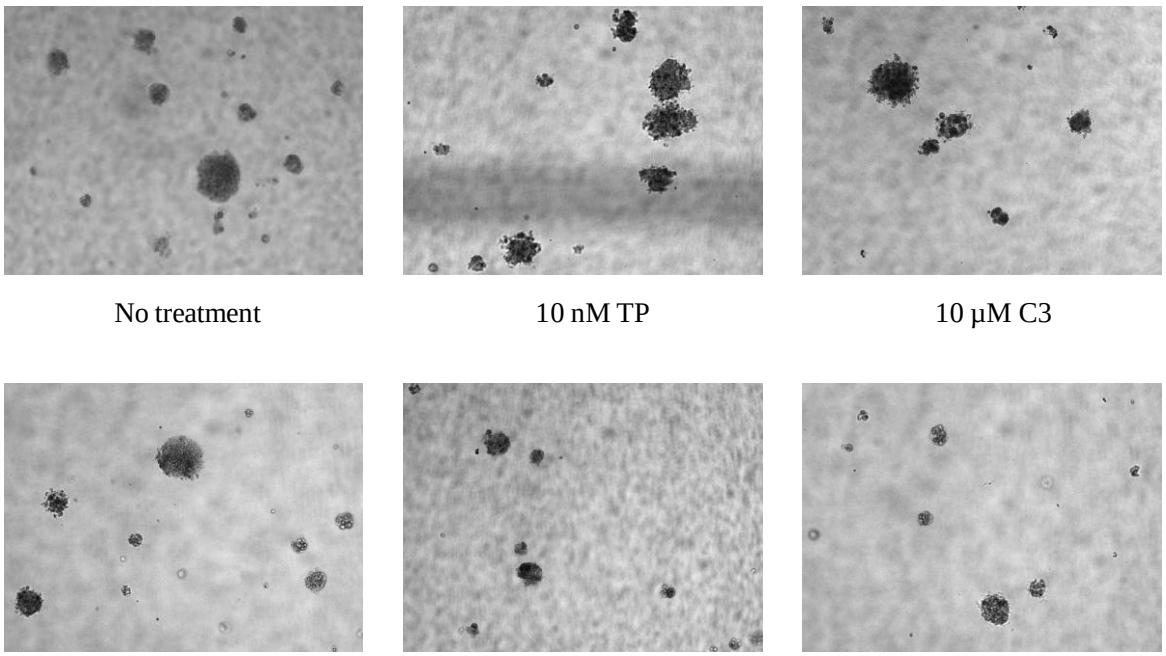

Figure 23. Micrographs of representative clonogenic assays of MCF-7 cells treated with 10 nM of topotecan and 10 µM of the miR-21 inhibitors C3 and LG-157 for 10-14 days.

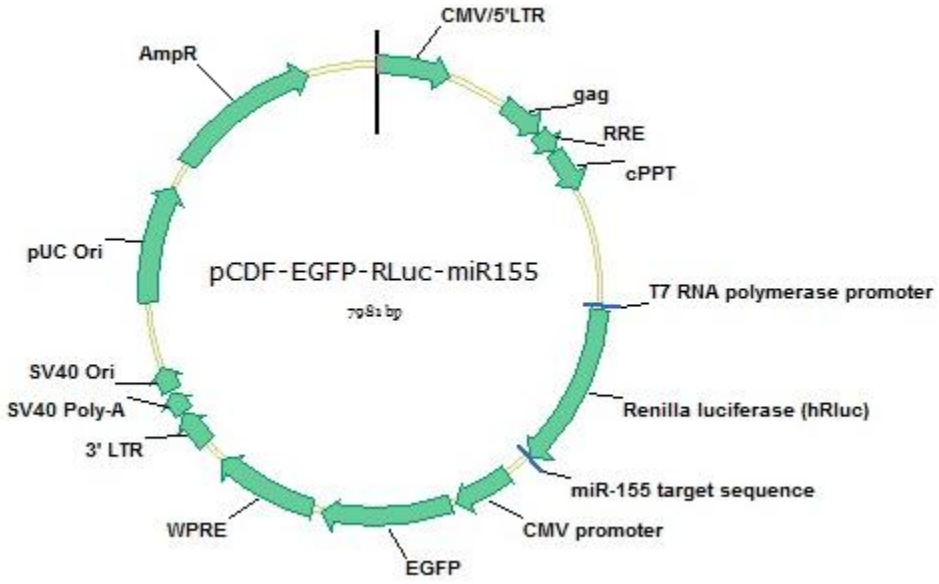

Figure 24. Plasmid map of pCDF-EGFP-RLuc-miR155.

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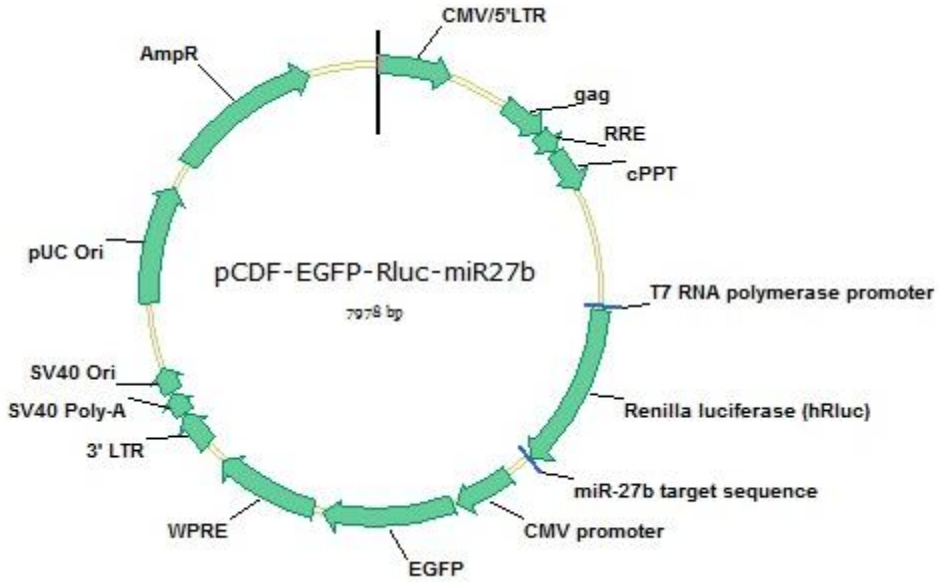

Figure 25. Plasmid map of pCDF-EGFP-RLuc-miR27b.

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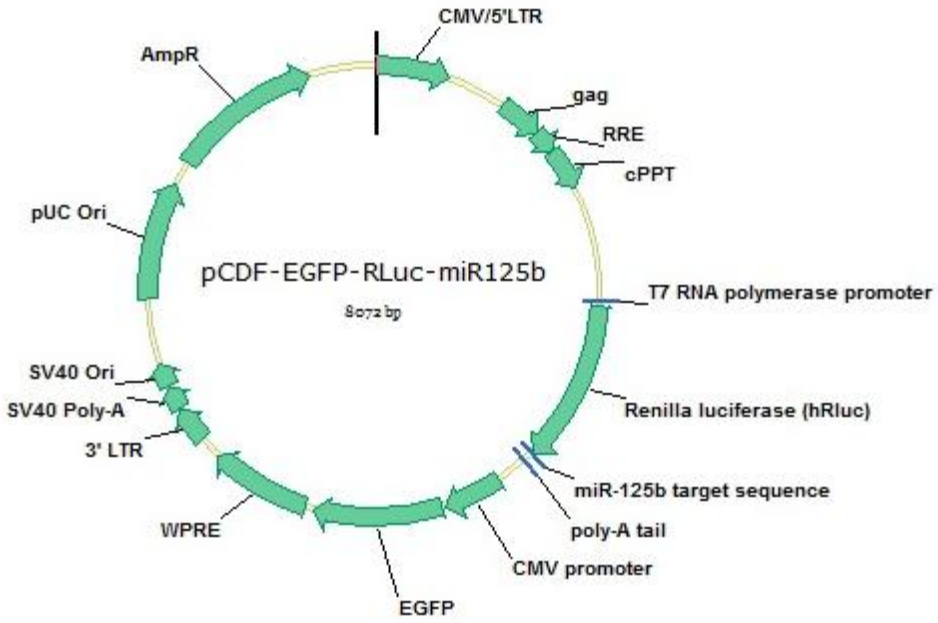

Figure 26. Plasmid map of pCDF-EGFP-RLuc-miR125b.

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