12-2015

Targeting oncogenic miRNAs with Small Molecules for Breast Cancer Therapy

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A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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July,2015

Dedication

I would like to dedicate my dissertation to my family, specifically to my parents who have supported me immensely throughout all of my years of studying.

This is for my mother, who has been there at every single moment that I have needed her, and has been very detached to all her duties, when it comes to situations where I have requested her help. Mom, I know that you have sacrificed yourself and other members of our family to help me achieve my goals, mostly because you believe in me more than I do in myself. Simply thank you!

To my father, who has constantly reminded me that he holds me very close to his heart and that he loves me very much! Dad, thanks for all your prayers and for all your phone calls, and for letting me know I could always count on you.

To my sisters, who have been extremely supportive throughout all of these years.

Finally, I want to dedicate this work to my loving husband. Who is simply the most patient man in the world! Thank you, first of all for waiting; secondly, for understanding how important my career is for me; and thirdly for simply being there in all the possible ways.

I love you all!

Acknowledgements

First of all, I would like to thank God, for providing me with the gift of life, and for allowing me to pursue a very challenging career with Him by my side. To Him I owe everything, and I have promised to give back to others all the blessings I have received.

Secondly I would like to thank my mentor, Dr. George A. Calin, for his patience, guidance, and support throughout my Ph.D. He accepted the challenge of taking me in his laboratory, and more so he inspired me to work as hard as I could every day in order to make great discoveries. I am very proud to say that his continuous happiness and positive energy/attitude is what kept me going during the difficult times. He always believed in me and in this project, and I am eternally grateful to call him my mentor. More than my boss he has been a very good father figure. I have to say I admire him deeply.

I would also like to thank the members of my supervisory committee: Drs. Diana Milewicz, Geoffrey Bartholomeusz, Shuxing Zhang and Zahid Siddik who dedicated their time and contributed with their expertise in order to guide me throughout my years here. Without your support and guidance I would not be in the place I am today. Additionally I would like to extend my gratitude to Dr. Gabriel Lopez-Berestein who has also been a very good mentor to me, and who has supported me tremendously throughout the years.

My deepest appreciation is extended to all my colleagues in Dr. Calin's laboratory: including Roxana Redis, Hui Ling and Maitri Shah who have in one way or another been an important part of my journey here. I would also like to mention my dear friend and colleague Cristina Ivan; thank you for all the work you have done with me, and for being there for me as a very special friend. Your work and dedication are very admirable.

To my family, there are truly no words I can put together to let you know how much your support has meant to me. Thank you for all your unconditional love, support, and encouragement. My parents and my sisters have always found a special way of motivating me even with a simple phone call to keep me moving forward. Finally thanks to Ruben Martinez, for being the most perfect husband and for trusting me and believing in me when things were tough and difficult.

Last, but not least I would like to thank the University of Puerto Rico Medical Science Campus for allowing the medical students from Puerto Rico to be part of such a prestigious combined degree MD./Ph.D. program. Special thanks to Ilka Rios for being in support of all the U54 students in this program. And also thanks to the University of Texas and UT MD Anderson Cancer Center. I am blessed with all the circumstances that brought me to Houston to advance my career as a physician scientist.

Targeting oncogenic miRNAs with Small Molecules for Breast Cancer Therapy

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Abstract

The crucial role of microRNAs (miRNAs) in cancer pathobiology has driven the introduction of new drug development approaches such as miRNA inhibition. In order to advance miRNA-therapeutics, there is a need to develop screening strategies that can target tumors in a specific way. Small molecule inhibitors represent an attractive approach to pursue this. However, the absence of molecular structures for most of the miRNAs makes it very difficult to predict which inhibitors can bind to them. Herein we designed a strategy to screen for small molecules by assesing whether they could directly bind/ interact with miR-10b/miR-21. As part of our results, we found a new mechanism of action for the multi-tyrosine kinase inhibitor Linifanib (5-6A); it inhibits miR-10b *in vitro* in breast cancer (BC) models. Furthermore, we confirmed that Linifanib (5-6A) interacts with the precursor sequence of miR-10b through nuclear magnetic resonance (NMR). Overall, our findings demonstrate an effective strategy to screen for small molecule inhibitors of miRNAs (SMIRs), one that is applicable for any disease type in which miRNA overexpression promotes pathology. More so, we provide a first-in-class lead compound for further development in cancer therapeutics.

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List of Abbreviations

AAV	Adenoassociated virus
AMOs	Anti-mRNA oligonucleotides
ANKRD46	ankyrin repeat domain 46
ASOs	Anti-sense oligonucleotide
BC	Breast Cancer
cDNA	complementary DNA
CL	Claudin low
CLL	Chronic lymphocytic leukemia
CRC	Colorectal cancer
DCGR8	DiGeorge critical region 8
DCIS	Ductal Carcinoma in situ
DFS	Disease free survival
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
E2F1	E2F Transcription Factor 1
EC50	Half maximal effective concentration
ENCODE	The encyclopedia of DNA elements
EphA2	Ephrin type A receptor 2
ER	Estrogen Receptor
FDA	Food and drug administration
FLT3	Fms-Related Tyrosine Kinase 3
FP	Fluorescent polarization
HCC	Hepatocelular carcinoma

HCV	Hepatitis C virus
HER2	Human Epidermal Growth Factor Receptor 2
HIF1-α	Hipoxia induced factor 1 alpha
HOXD10	Homeobox D10
HSP90	Heat shock protein 90
HTS	High-througput screening
IC50	Half maximal inhibitory concentration
IPA	Ingenuity pathway analysis
LCIS	Lobular Carcinoma in situ
LNA	Locked nucleic acid
miRNA	micro-RNA
mRNA	Messenger RNA
MT1-MMP	Membrane type 1-matrix metalloproteinase
MTg-AMO	multiple target anti-miRNA antisense oligodeoxiribonucleotide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
ncRNA	Non-coding RNA
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
NSCLC	Non-cmall cell lung cancer
OD	Optical density
ORR	Objective response rate
OS	Overall survival
PAM50	Prediction analysis for microarrays of 50 genes
PDCD4	Programmed Cell Death 4
PDGFR	Platelet derived growth factor receptor
PEG	Polyehtylene glycol

PK	Pharmacokinetics
PR	Progesterone Receptor
Pre-miRNA	precursor-microRNA
pri-miRNA	Primary-miRNA
qRT-PCR	Quantitative real-time polymerase chain reaction
RAN-GTP	RAs-related Nuclear protein - Guanosine-5'-triphosphate
RDC	Residual dipolar coupling
RHOC	Ras homologue gene, family member C
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RR	Response rate
RTK	Receptor tyrosine kinase
siRNA	Small interfering RNA
SMIR	Small molecule inhibitor of RNA
STAT3	Signal transducer and activator of transcription 3
TCGA	The Cancer Genome Atlas
TGFB4	Transforming growth factor B4
TIMP3	TIMP Metallopeptidase Inhibitor 3
TNBC	Triple negative breast cancer
TPM1	Tropomyosin 1
TTP	Time to progression
TWIST	Twist-related protein 1
UPAR	Urokinase receptor
UTR	Untranslated regions
VEGFR	Vascular endothelial growth factor receptor

CHAPTER I: Introduction

(Parts of this section were adapted with permission in part from: Monroig, P et al., MicroRNA and Epigenetics: Diagnostic and Therapeutic Opportunities, 2013; Monroig, P et al., Small molecule compounds targeting miRNAs for cancer therapy, 2015; and Berindan-Neagoe, I and Monroig P et al., MicroRNAome genome: A Treasure for Cancer Diagnosis and Therapy 2014.)

Breast Cancer

Breast cancer (BC) is the most common non-cutaneous cancer among American women, accounting for approximately 1 in every 3 cancers diagnosed. There are more than 1,300,000 cases and 450,000 deaths each year worldwide, making BC the second leading cause of cancer death in females (exceeded only by lung cancer) [1-3]. The overall worldwide burden of BC has doubled from the 1980's until the present representing an enormous threat to women's health due to the number of patients being constantly diagnosed [4]. The vast majority of the cases are seen in women over 40 years of age, who have aggressive tumor types or that present at an advanced disease stage [5].

BC can be categorized under two main subgroups: sarcomas and carcinomas. The former, are extremely rare types of histologically heterogeneous cancers that arise from connective tissue components within the breast (such as myofibroblasts, lymph or blood vessels). The latter, are tumors arising from the breast epithelium that consist on cells that line the lobules as well as the ducts.

Carcinomas are the most common type of breast cancer, comprising malignancies that are further categorized as either in situ or invasive. Carcinomas in situ are considered a premalignant lesions composed of abnormal cells growing in their normal place, at a point where they are not yet invading the breast tissue. They are clinically relevant because they have been demonstrated to have the potential of progressing and transforming into an invasive cancer. It is known that 15-53% of ductal carcinomas in situ (DCIS) could become invasive over a period of 10 years if they remain untreated [6]. Similarly, lobular carcinoma in situ (LCIS) has been associated with an 8-11 fold increase in the relative risk of breast cancer [7].

Invasive carcinomas, on the other hand represent the most aggressive presentation of carcinoma, where cancer cells infiltrate the lobules and ducts and begin to grow and spread into the breast connective tissue (Figure 1). Once invading the surrounding tissue, the tumor can spread to distant body sites such as lymph nodes and other organs. The majority of the invasive carcinomas are from ductal origin, accounting for approximately 80% of the cases, followed by lobular carcinomas with 10-15% of them [8]. The remaining cases are segregated as part of another group, in which they are individually characterized by their pathological findings and are known to have different prognosis as well as treatment implications. Some of these include tubular, colloid, medullary, papillary and micropapillary.

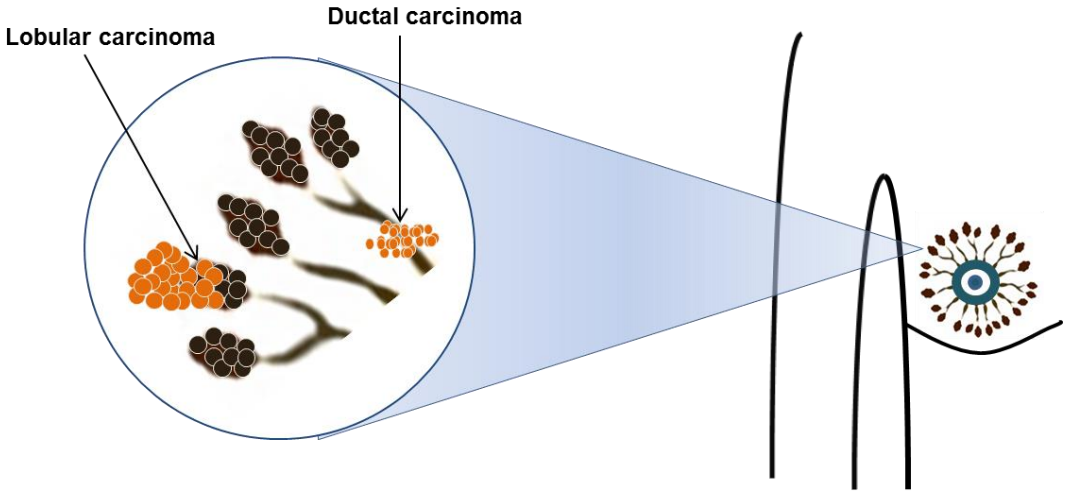

Figure 1: Anatomical localization of invasive breast carcinomas

Lobular carcinoma in situ (LCIS) is an uncommon condition in which abnormal cells form in the lobules or milk glands in the breast. Similarly, ductal carcinoma in situ (DCIS) is a non-invasive cancer where abnormal cells surround the lining of the breast milk duct.

Molecular and Histological Categorization of BC tumors

BC is a clinically heterogeneous disease which is categorized according to immunohistochemistry/fluorescence in situ hybridization (IHC/FISH) profile, and is further divided into different therapeutic groups based on the presence of estrogen receptor (ER), progesterone receptor (PR) and HER2 (also known as ERBB2). More so, the analysis of gene expression patterns identified by Perou and colleagues described four signatures: luminal, HER2-enriched, basal-like, and normal breast-like subtype [9, 10]. Further along, luminal tumors where separated into types A and B. Studies of these patterns have demonstrated correlations between patient relapses, clinical outcomes, survival etc [11, 12]. These characterizations have led researchers to develop gene classifiers such as PAM50 (Prediction Analysis of Microarray of 50 genes), which are able to define the major intrinsic subtypes to which a tumor belongs to [13].

Recently, the addition of another molecular subtype has been described as “claudin low” (CL), in tumors lacking tight junction proteins including claudin 3 and E-cadherin [14]. Additionally, the proliferation marker Ki-67 was associated with common histopathological parameters, but was also another independent prognostic parameter for disease free survival (DFS) and overall survival (OS) in BC patients [15]. Altogether these descriptions and most recent findings underlie the importance of molecular and histological characterizations in guiding therapeutic approaches as well as prognostic predictions for breast cancer patients.

BC therapies

Over the past decade, we have witnessed a significant progress in early BC detection and diagnosis. The favorable outcomes are in their majority attributed to the enhancement of mammography screenings, which have reduced breast-cancer related deaths by approximately 15-25% [16]. In addition, different therapeutic approaches have been effectively used among patients, with surgery being one of the principal methods of eliminating localized tumors. Alongside, multiple modalities including conventional chemotherapy, molecular targeted therapy and endocrine therapy are being applied individually or in combination with surgical approaches to target BC tumors [17].

Regarding the surgical management of breast cancer, several changes have occurred over the past years. Breast conserving therapies, followed by radiation have been validated for patients presenting with early tumor stages. These types of surgeries are also used in cases of patients with locally advanced tumors, after preoperative chemotherapy is given to decrease the tumor size. Sentinel node biopsy and axillary lymph node dissection are considered “debatable approaches” with benefits and unresolved issues on both. Finally, radical mastectomy remains a valid alternative in selected cases [18].

In addition to surgical approaches, targeted therapies have been validated, developed and used extensively over the past years in BC patients. The treatment of choice for individual patients relies on the molecular markers characteristic of the tumors themselves. Tumors with positive ER +/-PR, benefit from adjuvant endocrine therapy and chemotherapy. In addition, the HER2 receptor status defines if a patient is subjected to Trastuzumab (HER2 positive) or not. In patients with ER and PR negative tumors,

chemotherapy is the standard treatment (with Trastuzumab in HER2+ cases). Nevertheless patients with TNBC tumors, the treatment guidelines are chemotherapy, followed by surgery and lymph node dissection (www.nccn.org).

Even with aggressive, multidisciplinary approaches such as hormone therapy, chemotherapy, HER2 targeted therapy and surgery; studies have demonstrated that there are an unfortunate 30-50% of patients that relapse after their treatment (regardless of their disease stage at the time of diagnosis) [19, 20]. For example, in patients with TNBC, studies involving the administration of chemotherapy before surgery suggest that this treatment is very effective only in the minority of women with this diagnosis (who have a complete pathological response and thus an excellent outcome). In contrast, the outcome for the majority of them is relatively poor, due to the presence of residual disease [21]. Additionally, some of the therapies being used have exhibited serious side effects and toxicities [22]. Hence, there is an urgent unmet need to develop novel breakthrough of drugs for BC therapy with higher safety profiles and therefore patient compliance and therapeutic efficacy. With the aims of developing both local treatment of macroscopic tumors and systemic therapies of microscopic disease, current researchers aim to prolong the time of remission in patients and decrease the number of metastatic cases.

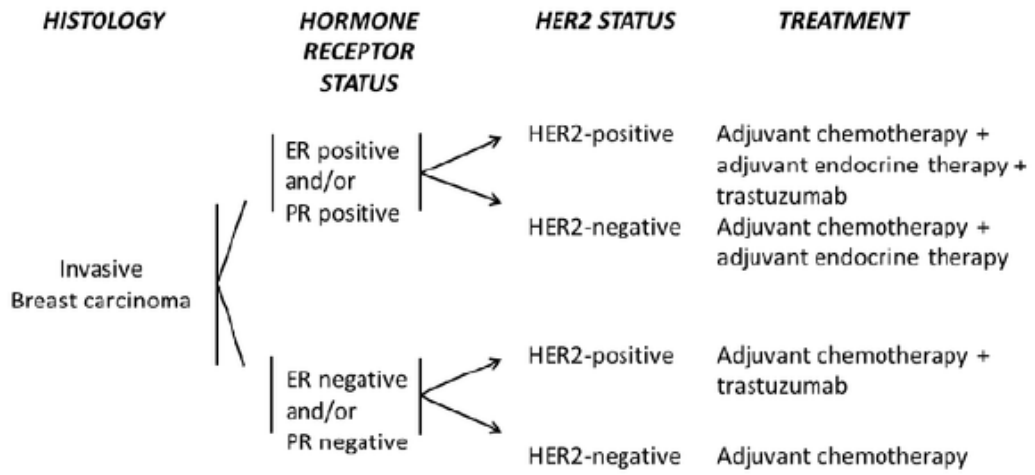

Figure 2: Summary of treatment guidelines for invasive breast carcinoma based on receptor status (ww.nccn.org).

Non coding RNAs – A shift in the genetic paradigm

Years ago, researchers constantly emphasized that protein-coding genes were the only relevant products of our genetic profile. This principle was propagated to very recent times, until ncRNAs were discovered [23]. Today, a more complex view is emerging: instead of focusing on genes, we have widened our research to non-coding genomic regions that are actually transcribed from what was originally designated as the “junk pile”. Furthermore, molecular biology has evolved and redefined dogmatic terms in the field, nowadays considering the atomic unit for genetics the RNA transcript (not the gene per se).

Important research projects focused on the human genome have been developed over the years by scientists all over the world. Most of them have evolved around the transcriptome encode by genes in humans. One of the most important studies was ENCODE (The Encyclopedia of DNA Elements), which identified functional elements demonstrating the fact that RNA can be dually processed yielding both, short and long RNAs [24]. Alongside, it demonstrated that even though over 90% of the human genome can be transcribed, only about 2% of it actually codifies for proteins [25]. Thus, a vast number of transcripts, are non-protein coding RNAs (ncRNAs), that can function in many ways, one of which is regulating transcription or translation of protein-coding genes [26, 27].

MicroRNAs

For over ten years, non-coding RNAs (ncRNAs) have been demonstrated to be involved in many regulatory mechanisms involving cellular physiological processes and other biological ones.[28]. In the early 1990's the understanding of lin-14, a gene in C. elegans by Ambros and colleagues, guided the findings of a type of non-protein coding RNA-transcript that negatively regulated translation through an antisense RNA-RNA interaction. These elements were later referred to as microRNAs (miRNAs) [29]. However, it was not only until the year 2000 that the characterization of a second RNA sequence repressing protein expression elucidated the existence of a wider phenomenon concerning an unknown genomic regulatory elements: microRNAs (miRNAs) [30].

MiRNAs are a type of non-coding RNAs of about 18-22 nucleotides (nt) in length which have been validated to regulate genetic expression in a post transcriptional manner. They are initially transcribed by RNA polymerase as independent genes, or as introns of protein-coding genes [31]. Their widely known for targeting messenger RNAs (mRNAs) causing critical changes that can directly inhibit translation, or that can cause mRNA instability and therefore degradation. The majority of the protein coding genes (approximately 60%), have been proven to be targeted by miRNAs through pairing complementarity [32].

MiRNAs are transcribed from their particular coding gene, as a long primary transcript which can fold upon itself further forming a doblue stranded hairpin (pri-miRNAs). An endonuclease, as well as a microprocessor complex (Drosha and DCGR8) cleave the primary transcript forming a precursor sequence of approximately 70 nt in length (pre-miRNAs). Proteins such as Exportin 5 and RAN-GTP promote the nuclear

translocation of the precursor sequences into the cytoplasm where another nuclease called Dicer further cleaves it. Subsequently, the precursor sequences shortens to a double stranded RNA sequence of 18-22 nt in length, which then separates into a guide and passenger strand [33]. The guide strand promotes the association of a group of proteins reffered to as an RNA-induced silencing complex (RISC) with the complementary 3’ unstranslated region of the mRNA (s) targeted by the specific miRNA. Even though their main function is to target mRNAs, microRNAs have also been known to have many other functions, such as regulating promoter regions, targeting cellular receptors, increasing the stability of additional mRNAs, influencing cell signaling pathways etc [34].

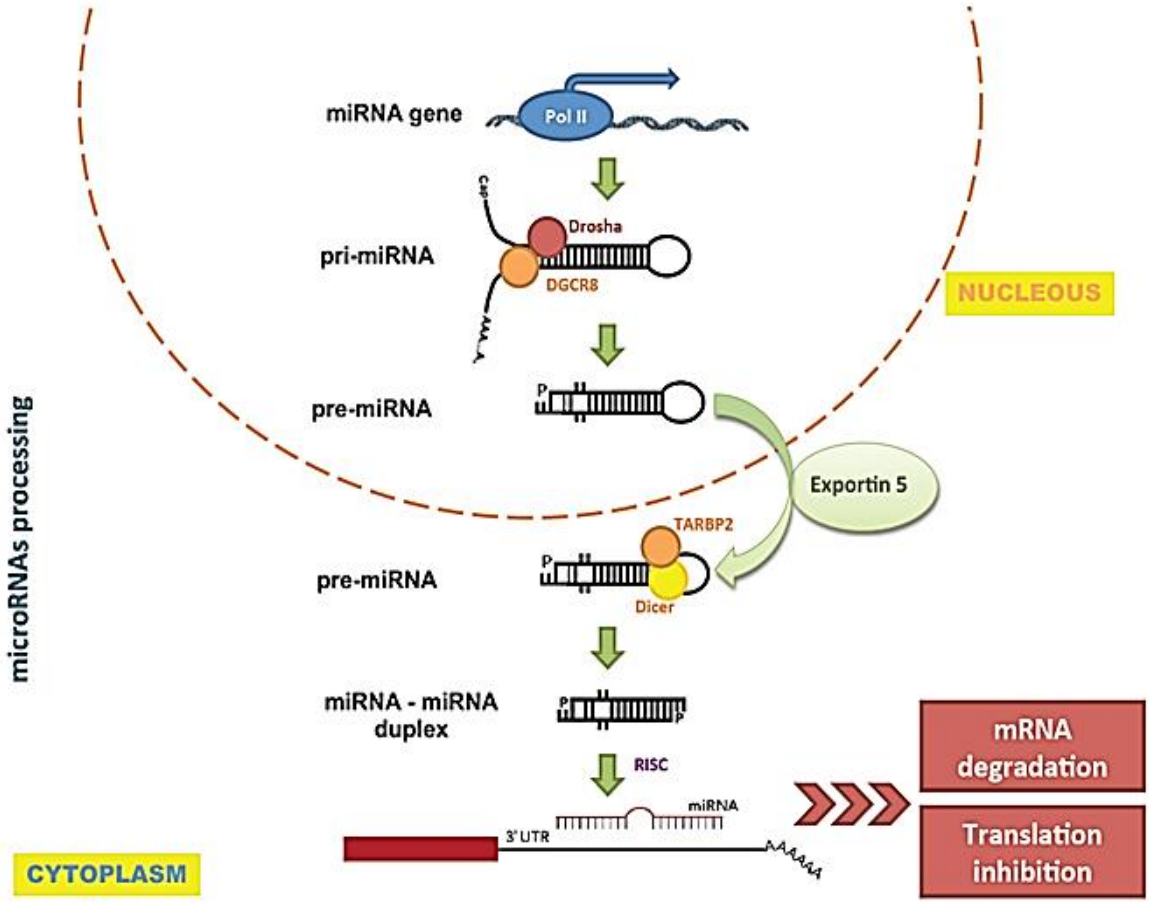

Figure 3: MicroRNA biogenesis

MiRNA-coding genes are initially transcribed by an RNA polymerase II or III, creating a primary transcript (pri-miRNAs), which can be hundreds or thousands of nt long. It is further processed, into a 100 nt precursor transcript. The precursor sequence is then exported to the cytoplasm, where it undergoes a series of processing events, prior to achieving maturation. Once in the cytoplasm, mature single-stranded miRNAs are integrated into a number of proteins that compose the RNA-induced silencing complex (RISC), and thereafter they interact by sequence complementarity with the messenger RNA (mRNA). In this way miRNAs inhibit translation or alternatively, they can increase mRNA instability (consequently causing its degradation).

MiRNA functions and role in cancer

The initial link between miRNA levels and cancer development was the miR-15 and miR-16 dysregulation in B cell CLL, as a result of chromosome 13q14 deletion [35]. Further studies have described the fact that many miRNA genes are located in fragile genomic sites (regions more susceptible to mutations, rearrangements and loss of heterozygosity), which are frequently found in tumorigenesis and cancer development [36]. To date, miRNAs have been linked to angiogenesis, uncontrolled proliferation, insensitivity to anti-growth signals, and every single one of the cancer hallmarks (Figure 4) [34]. They do so by tightly regulating tissue processes such as morphogenesis, apoptosis, or others [37, 38]. Thus, in this way, the alteration of important messenger RNAs (mRNAs) are involved in tumor initiation and progression, as these non-protein coding genes behave as oncogenes or tumor suppressor genes [39].

Tumor suppressive miRNAs reduce the levels expression of oncogenes, and can therefore delay carcinogenesis, or disrupt tumor maintenance. They are typically downregulated in cancer. On the other hand, oncogenic miRNAs are typically overexpressed or amplified in cancer, and they reduce the levels of important tumor suppressor proteins. One example of each follows.

MiR-34a has been known as a classic example of a tumor suppressive miRNA. It has been shown to be downregulated in several cancer types such as prostete. In prostate cancer cells purified from xenograft and primary tumors, its expression was shown to inhibit clonogenic expansion, and tumor regeneration as well as the process of metastasis because of its targeting of CD44 (a cell- surface glycoprotein involved in cell–cell interactions, cell adhesion and migration).

A representative example of an oncogenic miRNA is miR-*224* in colorectal cancer (CRC). MiR-224 expression increases consistently with tumor burden and enhances CRC metastasis *in vitro* and *in vivo*. SMAD4 was identified as a translational regulator targeted by this miRNA as demonstrated in clinical samples. Patients with high miR-224 levels had shorter overall survival in multiple CRC cohorts and shorter metastasis-free survival [40].

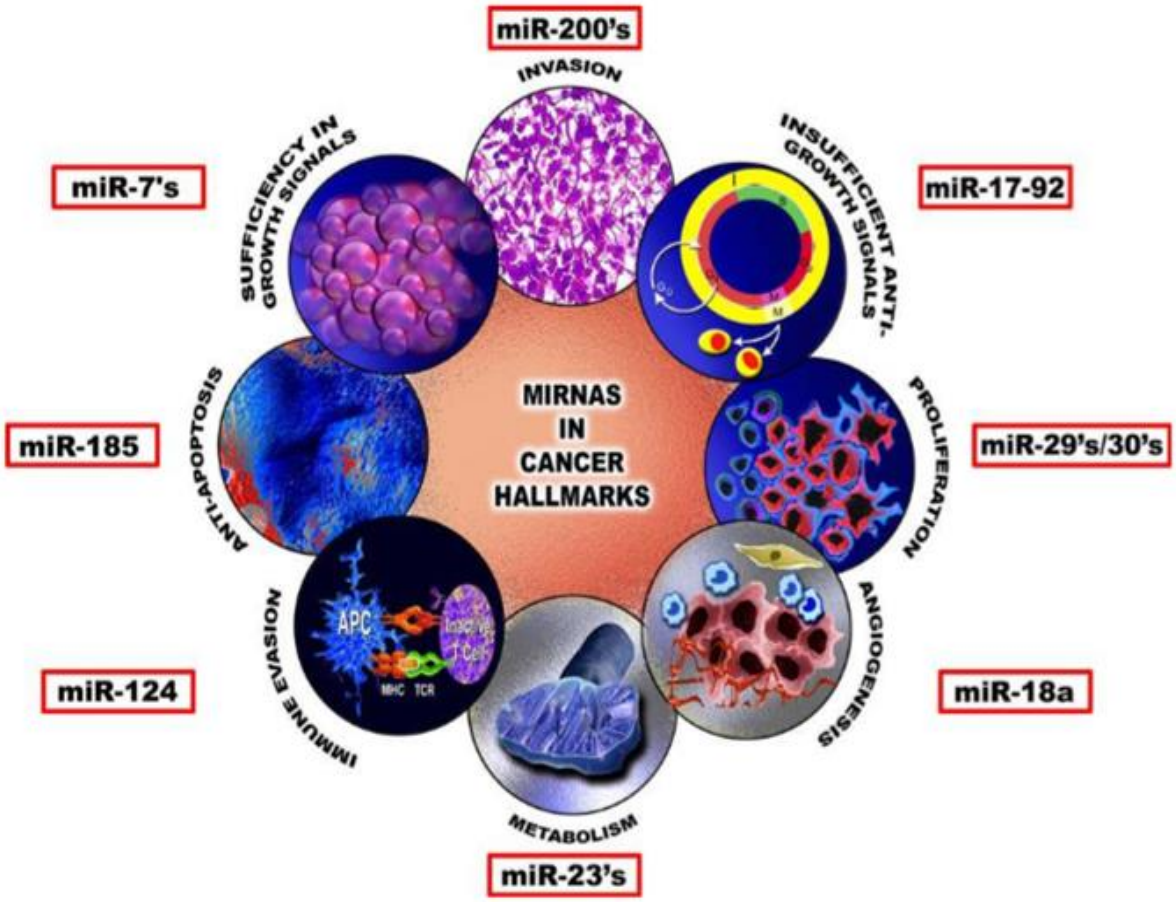

Figure 4: Examples of MicroRNAs Involved in the Cancer Hallmarks.
This figure is used with permission and originally published by Berindan-Neagoe, I., Monroig, P., Pasculli, B., and Calin, G. in 2014 in A Cancer Journal for Clinicians (http://onlinelibrary.wiley.com/doi/10.3322/caac.21244/epdf)

MiRNA-based therapeutic opportunities in cancer

Two important strategies have guided the use of these molecules as novel therapies. First, miRNA molecules have been modified to achieve a prolonged half-life and efficiency *in vivo* (e.g. anti-miRNA and locked nucleic acid-modified oligonucleotides, and antagomiRs). Second, the development of miRNA transgenic mice (such as the miR-155, and miR17~92) and knockouts (such as miR-15, miR-16, miR-146 and miR-29) have offered valuable information that has guided therapeutic opportunities for cancer patients [41]. The strategies are based on the following principles: targeting oncogenic miRNAs to decrease their levels expression; and restoring tumor suppressive miRNAs to rescue their levels expression (Table 1).

i. Inhibiting Oncogenic miRNAs

Cancer cells contain many genetic and epigenetic abnormalities, but despite their complexity, their growth and survival can often be impaired by inactivating a single oncogene. This phenomenon, called “oncogene addiction,” provides a rationale for molecular targeted therapy [42]. Correlations between regulatory miRNAs and cancer have revealed that this concept applies to miRNA dysregulation in patients. Thus, regarding the first principle, therapies against oncogenic miRNA focus on decreasing miRNA levels by inhibiting them through complementary base pairing.

Because of the diversity of mechanisms by which miRNA levels contribute to tumor initiation and progression, several therapeutic models have been developed to target these processes. Therapies to decrease the effect of a specific miRNA have been proposed by using antisense oligonucleotides (ASOs) that inhibit targets by adhering to the miRNA forming miRNAanti-miRNA binding complexes. Several types have been

RNA therapeutic drug	Definition	Mechanism of Action
Antisense oligonucleotides (ASOs)	A single-stranded chemically modified DNA-like molecule 17 to 22 nucleotides in length designed to be complementary to a selected messenger RNA (mRNA).	Specifically inhibits expression of that gene mainly through formation of an mRNA-ASO duplex by sequence complementarity, leading to cleavage of the mRNA of target gene.
MicroRNA mimics	A microRNA (miRNA) mimic is a small single-strand 19 to 24 nucleotide RNA with identical sequence to the miRNA of interest (to be re-expressed).	Mimic the effects of an endogenous miRNA with consequent inhibition of protein production by either transcriptional inhibition or translational block or both.
ASOs/AMOs, LNAs, and antagomiRs	The ASOs/AMOs are single-stranded, chemically modified DNA-like molecules that are 17 to 22 nucleotides in length and designed to be complementary to a selected miRNA and specifically inhibits its expression. The LNAs anti-miRNAs represent LNA-modified ASOs. The antagomirs are single-stranded 23-nucleotide RNA molecules complementary to the targeted miRNA that have been modified to increase the stability of the RNA and protect it from degradation.	AMOs are ASOs against miRNAs, and therefore produce ASO-miRNA duplex through sequence complementarity, leading to RNAse-H mediated cleavage of the target miRNA gene. The LNA anti-miRNA have the same mechanism as the ASO/AMO. The miRNA/antagomir-duplexes induce degradation of the miRNA and recycling of the antagomir in a way still not completely known.
Small interfering RNAs (siRNAs)	A double-strand RNA homologous to an mRNA of a target gene.	The siRNAs are incorporated into a multiprotein RNA-induced silencing complex, leaving the antisense strand to guide this complex to its homologous mRNA target for endonucleolytic cleavage of mRNA.

Table 1: Principle Types of RNA-based therapeutic drugs

described: antagomiRs, locked nucleic acids (LNAs) and ASOs with chemical alterations to optimize efficacy (AMOs) (Table) [43-45]. With the use of antagomiRs, Fontana and colleagues demonstrated that tumor growth was inhibited when injecting the antagomiR-17-p in therapy resistant neuroblastoma cell lines [46]. In reference to the chemically modified, Ma and colleagues used AMOs to intravenously inhibit miR-10b in a mammary mouse tumor model, and observed that metastasis was inhibited [47]. Park and colleagues tested another chemically modified antagomiR, chol-anti-miR-221. In their orthotopic mouse model of hepatocellular carcinoma (HCC), they proved that chol-antimiR- 221 significantly reduced miR-221 levels in the liver, and that this, correlated with a reduction in tumor cell proliferation, an increase in apoptosis markers and cell cycle arrest [48]. LNA's, (another type of ASO) have also proven to be efficient, as a liver regeneration mouse model tested by Sapra and colleagues demonstrated that using a locked nucleic acid antisense oligonucleotide against survivin (an apoptosis inhibitor), reduced its mRNA levels in 80%. In addition, researchers have recently engineered a single subunit termed "multiple-target anti-miRNA antisense oligodeoxyribonucleotide" (MTg-AMO), through which simultaneous silencing has been achieved [49]. MTg-AMO's have been proven to allow the restoration of dysregulated miRNA levels by targeting several key aspects of the biology of cancer cells in tumor tissue at once. For example, Lu and colleagues demonstrated that the MTg-AMO targeting miR-155 and miR-17-5p produced a greater inhibitory effect on cancer cell growth, compared with the regular single-target AMOs [50].

Another therapeutic approach to decrease miRNA levels involves expressing competitive inhibitors of their function. The typical example are "microRNA sponges" which are vectors containing multiple artificial miRNA binding sites that are placed under the control of strong promoters to produce large quantities of transcript [51]. These transcripts express multiple tandem sites to a miRNA of interest [52]. MiRNA sponges were used in

metastatic breast cancer mouse model, where *in vivo* experiments demonstrated that the downregulation of an over-expressed breast cancer miRNA (miR-19) could be achieved, along with metastasis inhibition [53].

ii. Restoring tumor suppressive miRNAs

Although miRNAs are mostly known to be overexpressed in cancer tissue, they can also be downregulated in tumors [54]. This fact recalls the second principle mentioned above (regarding miRNA-therapeutics): restoring the levels of tumor suppressive miRNAs. In the past, global repression of miRNA expression has been proven to increase tumorigenesis in both *in vitro* and *in vivo* models [55]. For tumors with reduced expression of miRNAs, restoring their basal levels is the key strategy, which can be achieved through miRNA mimetics, which are synthetic small RNAs that contain the exact sequence of the endogenous ones. To achieve the delivery of a stable molecule, miRNA's are delivered as perfectly complementary duplexes, similar in architecture to siRNAs [56].

The majority of the therapies that have tried to restore tumor suppressive miRNA with mimetics have achieved their goal by administering them locally. However, nowadays the challenge of developing a systemic delivery in a tissue/cell-type specific manner has been proven to be achievable through different delivery systems. The lead candidate in this field has been MRX34 from *Mirna Therapeutics* [57]. The liposome-encapsulated miR-34 mimic for patients with unresectable primary liver cancer has evolved into a (currently open) clinical trial. It is a phase I, open-label, multicenter dose-escalation study to investigate the safety, pharmacokinetics and pharmacodynamics of the MRX34 (ClinicalTrials.gov Identifier: NCT01829971).

Oncogenic miRNAs miR-21 and miR-10b, and their role in BC

Over the past years, the molecular mechanisms that underlie breast cancer pathogenesis gave been extensively researched. However many challenges prevail when managing patients. Some of these include: relapses, resistance to known treatments, and metastasis. As regulators of tumor suppressor protein-coding genes, miRNAs where thought to be attractive oncogenic agents to target in BC therapeutics. In the year 2005, miRNA dysregulation was associated with BC for the first time [58]. Since then, many studies have identified aberrantly expressed / clinically relevant miRNAs [59-62]. Two examples of these are miR-10b and miR-21.

MiR-10b has been demonstrated to initiate tumor invasion and metastasis by positively regulating cell migration and invasion in BC [63, 64]. It does so by indirectly causing the activation of the pro-metastatic gene *RHOC* through suppression of *HOXD10. HOXD10* is a transcriptional repressor involved in cellular migration and extracellular modelling (such as *RhoC, uPAR, α3-integrin* and *MT1-MMP*) [65]. Furthermore, miR-10b has been shown to be therapeutically relevant in mouse mammary tumor models, where its silencing inhibited metastasis development [47]. Its clinical relevance has been seen in patient samples, where miR-10b expression levels are lower in all of the breast carcinomas from metastasis-free patients; but in contrast, the majority of metastasis-positive patients have elevated miR-10b levels in their primary tumors [66]. This effect has been demonstrated to be independent of the tumor subtype. Notably, overexpression of miR-10b in otherwise non-metastatic breast cancer cells confers invasive and metastatic abilities on these cells when they are growing (as proven in xenografts *in vivo*) [66]. Overall these facts thoroughly exalt the importance of targeting miR-10b in BC therapeutics.

On the other hand, miR-21 has been found consistently upregulated in BC carcinoma in situ compared to normal tissue [67]. In patients, levels of serum miR-21 has been found to be significantly higher compared to controls, suggesting its usage as marker

for early stage BC detection [68]. Its overexpression has been correlated with specific BC bio-pathologic features such as advanced tumor stage, lymph node metastasis and also with poor patient survival [69]. Several tumor suppressor proteins have been validated as targeted by miR-21; some of these include TIMP3, ANKRD46, TPM1, PDCD4 and Maspin [70-72]. Knockdown of *miR-21* in BC cells has been demonstrated to inhibit migration in vitro as well as growth *in vitro* and *in vivo* [71]. Therefore, the finidngs to this date also suggest that miR-21 is another attractive miRNA to target in BC.

Interestingly, both miRNAs -10b and -21 have been identified as promoters of metastatis progression (Figure 4) [72] .Thus, the discovery of a therapeutic agent that could directly interact with miR-10b/miR-21 would open a new window of treatment opportunities to explore against cancer; both, as a single agent or in combinatorial therapies.

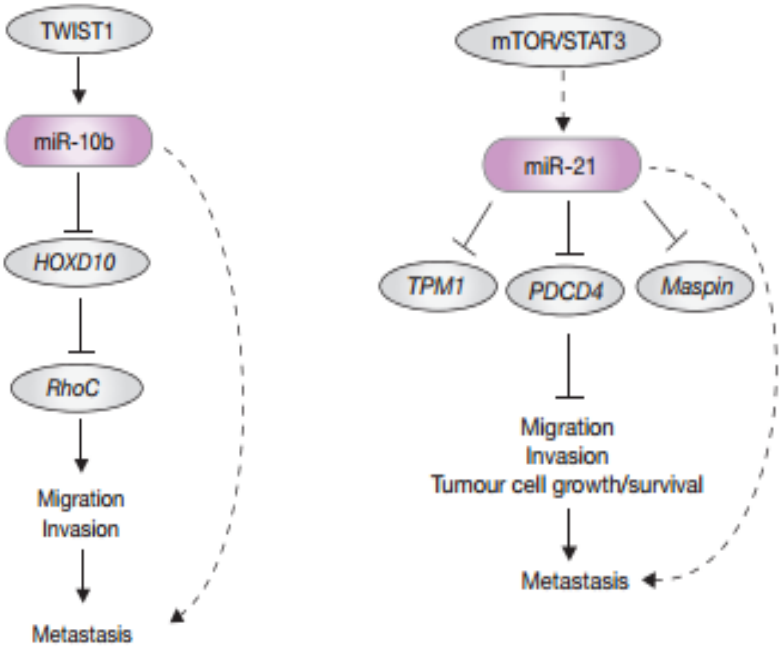

Figure 5: MiRNAs -10b and -21 are involved in metastasis progression in BC patients.
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Delivery Mechanisms

The main focus of miRNA-therapeutics is to be able to deliver the agents *in vivo* (both locally and systemically). Local approaches such as direct site injection have several evident advantages. Some of these are evasion of nuclease degradation, decrease uptake by non-targeted tissues, and increase bioavailability at the tumor targeted site. Nevertheless they are applicable to a limited number of tissues such as ocular and brain [73]; and even in these tissues, not all targeted tumor cells are reached by the miRNA-therapeutic agents [74]. On the other hand, a systemic delivery consisting of a bloodstream injection, theoretically should achieve a more efficient delivery to targeted sites. Neverrtheless, this mechanisms have to overcome several barriers (*in vivo*) such as nuclease degradation and non-specific targeting of tissues that are reached by blood vessels nearby (eg. liver, jejunum, kidney) [74].

As an approach to surpass some of these obstacles, changes such as chemical modifications, encapsulations and conjugations have aimed to protect miRNA-based therapeutic agents so they can be stably conserved while in the bloodstream (where they are required to maintain intact). In this way, they are able to traverse cellular membranes and more importantly reach the cytosol, where they perform their main function [75]. Thus, overall, the development of efficient carriers remains a challenge since synthesizing a stable, biodegradable and biocompatible miRNA-therapeutic agent is important, but ensuring an adequate cell penetration and molecular delivery is also crucial.

Current known carriers can be separated into 2 groups: viral and non-viral. Both groups target tumor tissues and aim to evade immunological reactions / (cause toxicity). Viral-based strategies have been used in the past with vectors such as lentivirus,

adenoviruses and adenoassociated virus (AAVs), and through specific genetic manipulation they have been able to target the tissues of choice [76, 77]. However, even though preclinical *in vivo* models (using virus as vector-mediated delivery) have shown promising results, they continue to present flaws that need to be addressed before advancing into the clinic (such as undesired immunogenic responses) [75].

Non-viral strategies, such as liposomes, have been thoroughly used and have proved to induce tumor suppression *in vivo* [78-80]. Their success has been accomplished due to the evasion of the oligonucleotides from being degraded by nucleases, and due to their ability to increase the circulating half-life when systemically delivered [73]. Nevertheless, some of these systems have had adverse effects *in vivo*, mostly related to the positive charge of the lipid component (which also triggers undesired immune responses) [73, 81].

A strategy that has begun to overcome these toxic effects has been the development of neutrally charged liposomes, based on 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) [82]. In this way, nanoliposomes deliver microRNA-based therapeutic agents into tumor cells much more efficiently *in vivo*. Several studies have proven that this approach is effective. For example, in a mouse model for lung cancer, Trang and colleagues systemically delivered the tumor suppressor miR-34a in a neutral lipid emulsion and obtained a 60% reduction in tumor area compared to controls [83]. In an additional model, mice with ovarian cancer tumors were treated with neutral-DOPC liposomes thich incorporated siRNA to target the oncogene *EphA2*. Through their experiments Landen and colleagues demonstarated that they could efficiently achieve a reduction in tumor growth (compared to control) [84]. Interestingly, another approach includes coating cationic bilayers with polyethylene glycol (PEG), demonstrated

effectiveness and safety by decreasing immunogenic responses [85]. Finally the addition of biodegradable synthetic or natural polymers could also represent an effective alternative to facilitate sustained delivery *in vivo*, although once again, toxicity remains a continuos challenge. Overall, the findings described above are still under development and have not yet reached their primary endpoint completely. More so, the fact of having to produce a delivery agent to administer therapy will continue to remain a burden, and are consuming both: time and money.

Small Molecule compounds targeting miRNAs for cancer therapy

Drug discovery and development are extremely long processes that take approximately 10–15 years. In addition, drug production results in an incredible economic burden that "boosts" their final overall cost, and patients end up having to pay exaggerated prices for their treatments. Thus, time and cost are considered the main obstacles in achieving new therapeutic alternatives for cancer treatment. Nonetheless, over the years, the study of different types of proteins as well as other types of molecules, has led to the discovery and identification of many inhibitors that are currently being used in the clinic. More so, several therapeutic approaches using miRNAs have also begun to be developed. However, even know some miRNA-based therapeutic strategies have been truly exciting, there are challenges involved in the delivery of these non-small-molecule agents, and even more, their pharmacodynamic and pharmacokinetic properties are not ideal for clinical application [73, 81]. For this reason, there is still a need of developing new alternative therapeutic approaches to inhibit oncomiRs, and decrease their activity.

Over the past years, significant attention has been focused on overexpressed oncomiRs, and several studies have been published regarding the initiation and development of a novel therapeutic approach for miRNA inhibition: the use of small molecule inhibitors (Figure 6). Small molecules have been thoroughly used with clinical applications for numerous diseases, but also specifically for cancer [86, 87]. The use of chemical compounds that are already FDA approved to treat a specific disease ("X"), would accelerate the process of completing toxicological studies and clinical trials in order to apply it to another disease ("Y"). If a compound has already been through thorough pre-clinical testing and FDA-related studies (10 or more years), then using the exact

compound for another disease would eliminate the need of going through the process again (shortening both money expenses and time consuming processes).

On the other hand, small molecule compounds could facilitate the identification of specific motifs from a lead compound. Then, by modifying the known compound rather that designing a new one (“from scratch”), researchers could also decrease the time it takes to synthesize and test a molecule de novo.

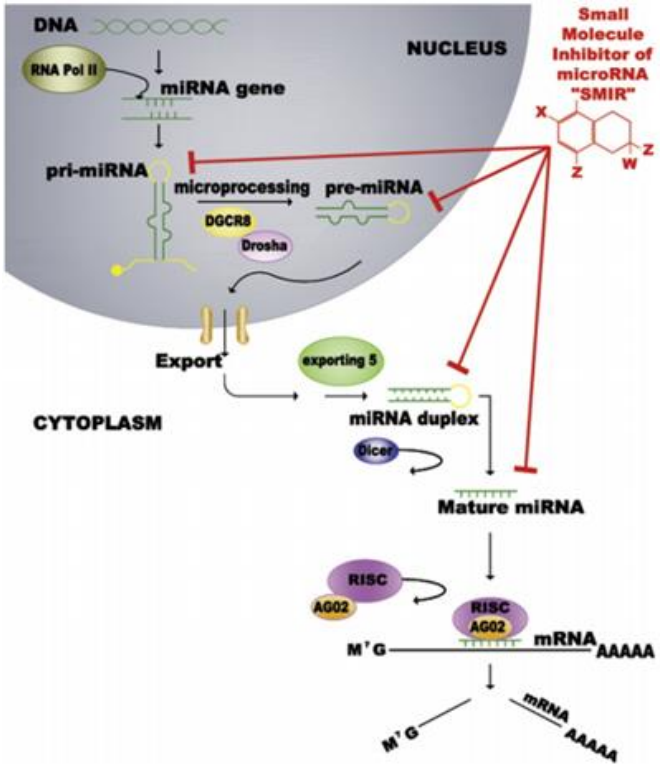

Figure 6: Small molecule compounds targeting microRNAs
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i. A novel approach with new challenges

The interaction of small molecules and miRNAs was termed for the first time as "SMIR" by Melo, Calin, and colleagues [88, 89], referring to small molecules inhibitors of RNAs. The novel concept represented an attractive modality to eventually develop more targeted cancer therapies. The idea of targeting RNAs, (in our case miRNAs specifically) with small molecules seems very convenient in the sense that it is considered to be a way of selecting a "fast-track pathway" in the drug developing race, reducing the time it takes to develop, produce and approve drugs, not to mention the manufacturing (and overall) cost of the process. If successful, targeting miRNAs with small molecules from which pre-clinical / clinical trials have previously been perfomed, could definitely result in an effective drug, reaching the patient's bedside in an incredibly short period of time.

Although interesting, the idea behind targeting miRNAs with small molecules involves embracing many risks and challenges. For example, compared to proteins, RNA transcripts have never before been considered drug targets due to their electronegativity and structural flexibility. More so, the absence of molecular structures of the majority of the miRNAs, as well as the limited availability of miRNA-Dicer or RISC complex structures makes their discovery increasingly difficult [90]. Nonetheless, recent findings suggest that indeed miRNAs are good genetic elements to target [88]. The secondary structures of immature miRNA transcripts, specifically in the pre-miRs, actually form stem loops and bulges that facilitate their targeting by small molecule inhibitors. Thus, in this way, they represent attractive "druggable" elements [91].

ii. Developing a new concept

A few years ago, Deiters and colleagues described that there are 3 basic processing stages which miRNAs require in order to perform their main function. More

so, they envisioned that small molecules could indeed block miRNA maturation at all 3 of them: pre-transcriptionally, at the level of transcription, or post-transcriptionally [92]. They determined that several ideas were possible; herein we briefly discuss them.

First of all, they perceived that pre-transcriptionally, small molecules could decrease the levels of miRNA expression as well as their function [92]. By altering miRNA promoter regions, small molecules where speculated to regulate miRNA expression in an indirect way. Also, small molecules were thought to potentially inhibit deacetylation or promote hypermethylation and thereby decrease the expression of miRNA-coding genes [93, 94].

Secondly (but in a similar way), transcription factors were also thought as plausible elements to be targeted by small molecules. By inhibiting their function, small molecules could (in a way) decrease or inhibit completely the expression of genes coding for miRNAs. For example, c-Myc has been demonstrated to activate the expression of 2 miRNAs from a cluster: miR-17-5p and miR-20a. Both of these are known to negatively regulate the expression of E2F1, thereby promoting G1-to-S phase progression [95]. Thus, the idea behind this rationale was that a small molecule could target oncogenic transcription factors such as E2F1, and in this way inhibit the expression of oncogenic miRNAs (miR-17-5p and miR-20a) resulting in recovery of tumor suppressor proteins.

Thirdly, small molecules were also speculated to target the process of miRNA biogenesis (post-transcriptionally), by inhibiting RNA-endonucleases (e.g. Drosha/Dicer), or other important proteins [96-99]. These crucial elements, (required to produce mature miRNA sequences), were thought to be potential targets to inhibit with small molecule compounds in order to decrease the expression of oncogenic miRNAs.

Overall, by comparing the different steps of miRNA regulation, research groups speculated that targeting transcription was the most effective way of inhibiting miRNAs [92]. However, the truth of the matter was that after different screenings were completed, it was clear that targeting transcription factors was not a way of targeting miRNA expression in a specific way. The rationale behind it is that transcription factors can ubiquitously affect promoter regions of many genes simultaneously, including oncomiRs, tumor suppressor miRs or genes coding for miRNAs that regulate cellular homeostasis.

Nowadays, after developing a deeper understanding of the field, the main goal of the SMIR-based approach, is to discover compounds that can potently bind, and decrease the levels of miRNAs in the most specific way possible [100]. The aim is for a small molecule, to target a mature miRNA sequence by binding to it, or any of its upstream precursors. If a small molecule inhibitor could be proven to bind to any of these sequences directly, it could be used in any cancer type (or even disease) that overexpresses that specific oncogenic miRNA. In order to achieve this, the initial step is determining which miRNAs are important oncogenic targets. Following this, an efficient screening technique needs to be developed and validated, in order to search for small molecule compounds from chemical libraries.

iii. Screening techniques to identify SMIRs

Screening chemical compound libraries is a drug-discovery approach broadly used in research for many types of studies. The process usually involves “quick” methods to assess the biological or biochemical activity of numerous compounds, in the shortest amount of time with the highest precision possible. The identification of hit compounds through screening techniques tipically provides a basis/starting point for future drug design. Screening techniques have been used previously by several research groups in

order to identify small molecule inhibitors of miRNAs [101-103]. The initial SMIR-screening approaches can be done either *in vitro* or *in silico*; and both of these types of techniques have been recently explored [103-109]. Herein we describe several assays used *in vitro*. For a summary of in silico approaches to target SMIRs please refer to [100].

Assays detecting fluorescence

An example of an assay using fluorescence to detect changes in miRNA levels are fluorescent beacons. These are oligonucleotide hybridization probes that can report the presence or the absence of specific nucleic acids (such as miRNAs) in solutions. They are hairpin shaped molecules, composed of a quencher, as well as a fluorophore that can emit fluorescence when interacting to its complementary target sequence. The technique has been proven useful in past research studies [106, 107]. It is performed by synthesizing a doubly-labeled precursor beacon containing a 5′ fluorescence emitter, and a 3′ quencher. Dicer-mediated hydrolysis of the precursor results in a dissociation of the fluorescence emitter and quencher, generating a Dicer-dependent increase in the fluorescence. If a small molecule is not effective in inhibiting the miRNA, the miRNA itself would bind its complementary sequence in the beacon and fluorescence emission would be high. On the other hand, an effective SMIR would not allow the miRNA to bind the beacon, therefore there would be less fluorescence (Figure 7A).

Alternatively, another research group developed a DNA molecular beacon that is independent of the precursor sequence and complementary

to the mature miRNA produced after the Dicer cleavage (and in this way Dicer cleavage is not expected to be altered in any way) [103]. .

Luciferase reporter constructs

Luciferase biosensors assays are an efficient / rapid screening technique to determine the levels of miRNAs *in vitro*. These vectors are usually assembled by introducing the complementary sequence of the miRNA of interest. An additional vector with a control sequence, downstream of a luciferase reporter gene is used alongside (to blank artifact effect). The reporter constructs are then used as sensors to determine the presence of specific mature miRNAs. The plasmids are cloned and further sequenced, validated, and transfected into cells. The system has been tested and proved efficient in detecting minor changes in endogenous miRNA levels. The basis of the assay is as follows: in the presence of a SMIR that efficiently targets the miRNA of interest, the mature sequence is decreased, and the luciferase gene is expressed constitutively. If the compounds tested do not inhibit the miRNA, then the miRNA will bind to its target sequence and inhibit the expression of the luciferase gene. Herein we briefly describe 2 examples.

- pEZX-MT05 plasmid

The pEZX-MT05 vector from (GeneCopoeia) also offers the opportunity of miRNA target identification. Similarly, miRNA complementary 3′ UTR sequences are inserted downstream of the

secreted Gaussia luciferase (GLuc) reporter gene in its respective vector system, driven by SV40 promoter for expression in. Besides using GLuc as the miRNA 3′ UTR target reporter, a secreted Alkaline Phosphatase (SEAP) reporter driven by a CMV promoter, is also cloned and it serves as the internal control. In addition to the GLuc/SEAP dualreporter vector system, a firefly/Renilla Duo-Luciferase reporter vector (pEZX-MT01) has also been validated and is currently available for SMIR screening [108].

- psiCHECK-2 reporter plasmid

The psiCHECK2 vector (Promega) has been a useful tool to screen for SMIRs *in vitro*. It is a reporter plasmid in which researchers clone a miRNA target sequence downstream of a Renilla luciferase (Rluc) gene. The presence / absence of a specific miRNA are directly proportional to changes in the levels of expression of Rluc. This vector provides a very significant advantage: it contains a Firefly luciferase (F-luc) gene which is constitutively expressed. Thus, there is no need of co-transfecting a control luc-gene (Figure 7B).

Stable cell lines

During the past years, many research groups have focused on developing more efficient high-throughput screening techniques in order to identify SMIRs. It has been proposed that using a stable cell line that constitutively expresses a luciferase reporter, (rather than a transient transfection), would be an approach more cost efficient and less time-consuming. More so, it would eliminate variations associated with transfection efficiency and other technical manipulations. This approach

was tried by Connelly and colleagues; and in their study, they recorded the steps needed to create a stable cell line with a constitutively expressed vector [102]. In it they validated the Huh 7 cell line with a constitutive expression of psiCHECK2 vector with the miR-122 target sequence [102]. This technique could be applied to any other cell line as well as any other miRNA.

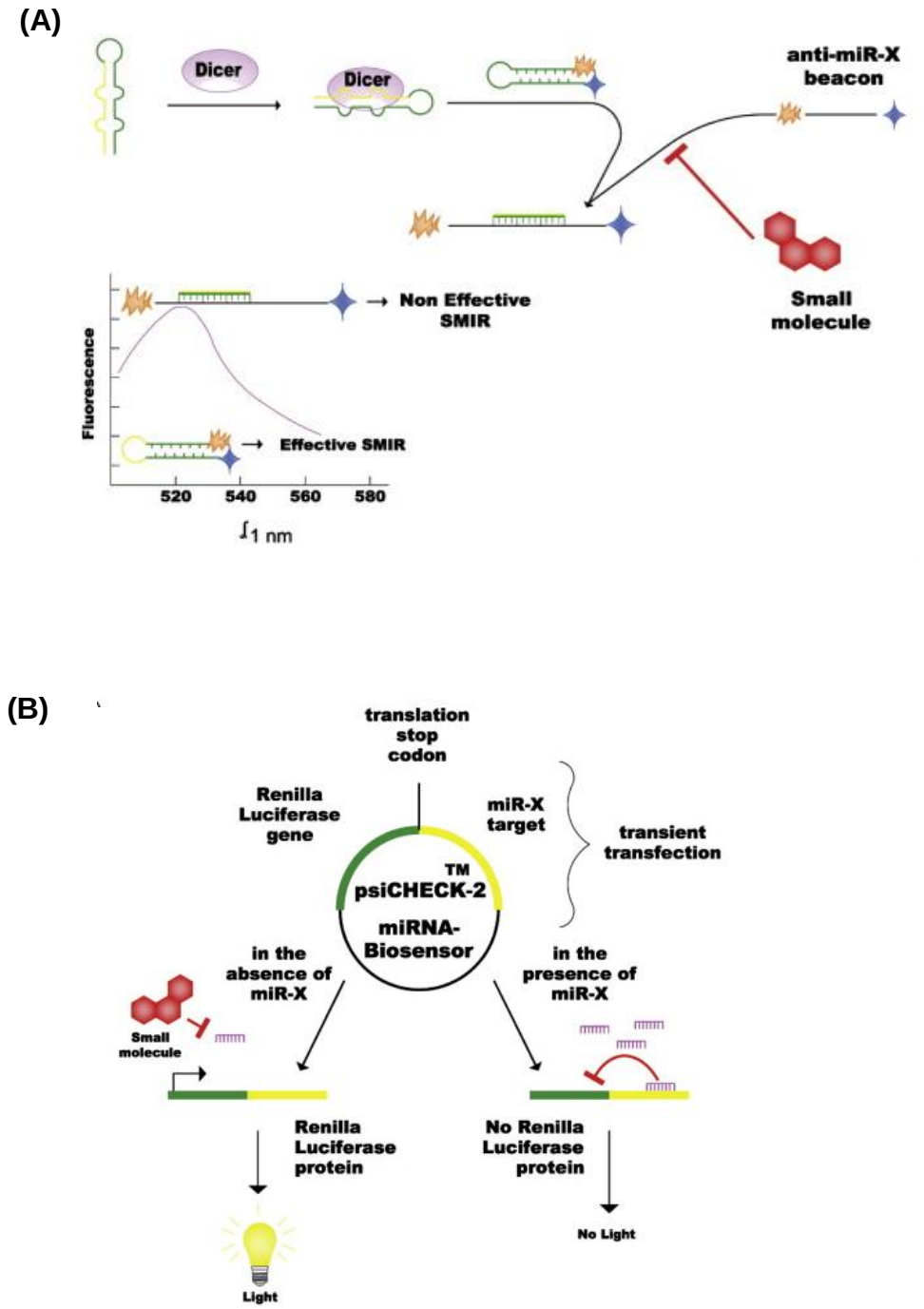

Figure 7: Examples of high-throughput screening *(in vitro)* techniques for SMIRs

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Hypothesis and Aims of the Study

Even though miRNAs are small RNA molecules that do not codify for proteins, they can regulate their expression in a significant manner. Due to their mechanism of action, miRNAs have been demonstrated to be very important in contributing to the progress of diseases such as cancer. This has compelled the progress of drug development approaches such as the inhibition of oncogenic miRNA.

MiRNA expression in BC has proven to induce tumor initiation, progression and metastasis. Death rates from BCs have decreased only slightly in the last decade, and new therapeutic approaches are under scrutiny. To identify new therapeutic targets for BC patients, we proposed to study oncogenic miRNAs that have been proven to target tumor suppressor proteins in BC (miR-9, miR-10b, miR-21, miR-155, miR-181a, and miR-181b). Among the miRNAs studied, two of them proved to be expressed at a significantly higher level compared to the others: miR-10b and miR-21. Thus, for this project, we envisioned the usage of small molecules to target and inhibit the function of these miRNAs.

Screening for small molecules inhibitors of miRNAs represents enormous challenges Two of the most important ones are: (1) mature miRNAs are single stranded and unstructured, and (2) there are few high resolution structures of immature miRNAs. Thus, structure-based discovery is very difficult.

Encouragingly, we studied the *in vitro* strategies employed in a handful of trials performed over the past few years (designed to identify small molecules inhibitors of miRNAs through different approaches). Herein we proposed to generate a high-

throughput screening system, to screen for small molecule compounds that can directly interact and inhibit oncomir-10b / oncomiR-21.

Overall hypothesis:

Overexpressed oncogenic miRNAs can be inhibited by small molecules for BC treatment. The targeted inhibition will have biological consequences in cancer cells.

We tested this hypothesis with the following specific aims:

Aim 1: To identify oncogenic miRNAs with clinical relevance in breast cancer, and determine a strategy to screen for small molecule inhibitors that can target them.

Aim 2: To perform a high-throughput screening, and determine positive hit candidates that can inhibit the identified oncogenic miRNAs.

Aim 3: To determine the efficiency, potency and specificity of the small molecule inhibitors of the oncogenic miRNAs selected as targets; and to demonstrate the type of interaction between the molecule and the miRNA.

CHAPTER II: Methods

Integrative computational analysis and patient data selection. We performed a literature review and selected a group of 6 miRNAs that were validated to have tumor suppressor targets in breast cancer (through microRNA arrays, *in vitro* and *in vivo* models, and/or patient cohort studies) [47, 53, 58, 63, 64, 66, 69, 71, 110-138]. Then, we obtained clinical information and miRNA expression (miRSeq) from patients with invasive breast carcinoma (BRCA), from "the Cancer Genome Atlas Project" (TCGA) available through the associated files of the paper: Comprehensive molecular portraits of human breast tumors, Nature, September 27, 2012 (https://tcgadata.nci.nih.gov/docs/ publications/) [3]. For these patients, we also downloaded their most recent clinical information from the TCGA portal (http://tcgadata.nci.nih.gov/). We merged the clinical information from both sources. All statistical analyses were performed in R (version 3.0.1). All tests were 2-sided and considered statistically significant at the 0.05 level.

Kruskal-Wallis test was applied to assess the relationship among miRNA expression levels in tumors and a box-and-whisker plot was used to visualize the miRSeq data. Further along, the Shapiro-Wilk test was applied to determine whether data followed a normal distribution. The t-test or analysis of variance (depending on the number of groups considered) was applied to normally distributed data; otherwise the Mann-Whitney-Wilcoxon test or again the Kruskal-Wallis test was applied to assess the relationship between miRNA expression levels and clinical parameters. A box-and-whisker plot (Box plot represents first (lower bound) and third (upper bound) quartiles, whiskers represent 1.5 times the interquartile range) was used to visualize the miRSeq data.

For each miRNA, we checked for a relation with the survival as follows. Patients were grouped into percentiles according to the miRNA expression. The Log-rank test was employed to determine the association between miRNA expression and overall survival

and the Kaplan-Meier method was used to generate survival curves. Cut-off points to significantly split (log-rank test p-value <0.05) the samples into low/high miRNA groups were recorded. The cut-off to optimally separate the patients in high/low (min p-value) was chosen.

Cell Culture. Cell lines were obtained from the American Type Culture Collection and grown as suggested by the supplier. Experiments were performed using The BC cells MCF-7 cultured in DMEM plus 10% FBS, and MDA-MB-231 and MDA-MB-468 cultured in RPMI. We also used 293FT cells which were grown in DMEM at 10% FBS. In addition we used MiaPaCa-2, Hey-A8, HepG2, and DLD1 cells corresponding to pancreatic cancer, ovarian cancer, hepatocellular carcinoma and colorectal cancer cells. The medias used were the same ones suggested by ATCC, at 10%FBS. All cells where maintained at 37° C in a humidified atmosphere of 5% CO_2.

miRNA correlation analysis. The Spearman's rank-order correlation test was applied to measure the strength of the association between miR-10b and miR-21. Statistical analysis and scatter plot graph were done using R (version 3.0.1) (http:///www.r-project.org/) and the statistical significance were defined as a p-value less 0.05. We imposed also a cut-off of functional relevance on the Spearman correlation coefficient in absolute value of 0.2 based on the method published previously [Integrated genomic analyses of ovarian carcinoma, Nature 474 (2011), pp 609-615, (Table S7.1)] [139].

Reporter Plasmid Construction. To develop a high-throughput screening system for identifying microRNA inhibitors, we acquired the Psi-CHECK 2 luciferase reporter vector (Promega). We then designed and acquired complementary oligonucleotides

(Thermo Scientific) for hsa-miR-21 and hsa-miR-10b based on the mature miRNA sequences for both (Appendix - Table 2). In the design of the exact microRNA target sequence, we added several base pairs to have two different restriction sites recognizable by the enzyme *SgfI* at one side, and *PmeI* on the other (both of the restriction sites were present in the vector as well). In addition, the insert was designed with a restriction site for *SacI* enzyme in order to have an additional site to digest the vector when monitoring colonies for the presence/absence of the insert.

The designed oligonucleotides were annealed as follows. Complementary strands were mixed at a 1:1 molar ratio in a microcentrifuge tube. The mixture was diluted to a fnial concentration of 1pmol/µl with a buffer solution of 10mM Tris, 1mM EDTA, 50mM NaCl (pH 8.0). The annealing reaction was prepared the same way for both miRNAs. In brief, the reaction consisted of 1ul of 100µM oligo#1, 1uL of 100µM of oligo#2, 5µL of 0.5M NaCl and 43µl of TE buffer. Both mixes were incubated at 95° C for 5 minutes, then the heat was reduced gradually for 70 minutes, until reaching 4° C. Subsequently the reaction was aliquoted and stored at -20° C.

The annealed oligonucleotides were digested along with the Psi-CHECK2 vector with the enzymes were digested with *SgfI* and *PmeI* according to the manufacturer's protocols for the enzymes (New England Biolabs/NEB). In brief, 1µg of DNA was digested with 1µl of each of the enzymes for 1 hour at 37° in a total reaction volume of 50µL. Digested products where purified with a DNA purification kit (Qiagen). Vector and inserts where ligated at the 3'-UTR (downstream) of the Renilla luciferase reporter gene in the psiCHECK-2, using T4 ligase and buffer (NEB), at a ratio of 1:40 vector: insert (to ensure insertion). Ligation was left for 2 hours at room temperature. Vector ligated without any insert (oligonucleotide sequence) was used as control.

Ligation reactions where transformed into One Shot Top 10 *E. coli* cells (Life Technologies). Cells where initially thawed on ice and 2.5 µl of ligation reaction was added to 25µl of competent cells and mixed gently by tapping. Mixes where then incubated on ice for 30 minutes. Then reactions where "heat shocked" for 30 seconds at 42° C, followed by a 2 minute incubation on ice. Subsequently, 250µl of SOC medium (at room temperature) was added to each and incubated for 1 hour at 37° C shaking (200rpm). Each reaction was then spread on pre-warmed plates with LB and ampicillin. Colonies were then selected, and grown in LB broth overnight at 37° C shaking (200rpm). Vector constructs were extracted from the bacteria using a mini-prep kit (Qiagen), according to the manufacturer's protocol. Insert sequences were further confirmed two ways. Firstly, by enzymatic digestion (according to the manufacturer's protocol - NEB), which was done with *SacI,* for the restriction site we designed in the insert and *BamHI,* for a restriction site contained in the vector (digestion with these two enzymes should theoretically give 2 products of approximately 1600bp and 4600bp). Secondly, the sequences were confirmed by sequencing, with primer sequences described previously [140].

Sensitivity Assays for reporter vector constructs. A total of (2.0×10^4 MCF7-cells/well) were plated the evening prior to transfection and treatment with a final volume of media of 100µl. Cells where transfected with the psi-CHECK2 vector containing the miR-10b target sequence, either alone or in combination with pre-miR-10b, or antagomiR10b (acquired from Life Technologies). Concurrently, scramble sequences were used as controls. All transfections were performed in triplicates for statistical analysis. The exact protocol was used for miR-21 cloned vector as well. The final concentration of the vector transfected was 25ng/100µl (250ng/mL), while for the precursors and antagomiRs they were 50nm and 100nm respectively. Six hours after the transfection, media was completely removed and replaced with new one. After 48 hours

of incubation the media was removed, and cells were lysed and assayed with a Dual Luciferase Assay (Promega) was performed using a Vi-Tech luminometer. The ratio of Renilla to Firefly luciferase expression was calculated for each of the triplicates and the data was analyzed.

Molecular Luciferase-Based Small Molecule Inhibitor Screening. An inhibitor screening library of lyophilized and dissolved in DMSO was purchased (Selleckchem Chemicals). Compounds from this company have been used in the past by different research groups [141, 142]. MCF-7 cells were seeded 24 hours prior to transfection, in 96 well plates (2.0×10^4 cells/well). All transfections were performed in triplicates for statistical analysis. The cells were incubated at 37° C for 6 hours, followed by replacement of the transfected media with fresh media containing the small molecule inhibitors to screen at a concentration of 10 μM each. After 48 hours of incubation the media was removed, and cells were lysed and Dual Luciferase Assay (Promega) was performed using a Vi-Tech luminometer. The ratio of Renilla to Firefly luciferase expression was calculated for each of the triplicates and the data was analyzed. Results of the complete library after transfection with the luciferase construct and small molecule treatment were merged together in 2 groups (according to the targeted miRNA -10b or 21. The top 5% of compounds with the highest luciferase fold change were considered positive hits and further studied.

RNA extraction. The media was removed from the plates containing the cells. Cells were washed with PBS, and RNA was isolated using Trizol Reagent (Invitrogen). RNA extraction was performed according to manufacturer's instructions. RNA quantity was assessed with NanoDrop ND-1000 (Thermo Fisher Scientific) and the integrity was analyzed by gel electrophoresis.

cDNA synthesis and quantitative real time PCR analysis. Positive hits from the luciferase-based screening assay were selected to further confirm their potential as SMIR's. Their inhibition was tested in MCF-7 cells at the same concentration and time point but through a different technique: RT-PCR. Cells were seeded in 6 well plates the evening prior treatments at a confluency of 50-60%. They were then treated with the small molecule compound hits, at a concentration of 10 μM and RNA was collected after 24 and 48 hours. MicroRNA expression was tested using TaqMan microRNA assay (Applied Biosystems). The cDNA was synthesized using TaqMan Reverse Transcription Reagents kit (Applied Biosystems) and employed for quantitative RT-PCR analysis together with TaqMan probes and SsoFast Supermix (Bio-rad) according to the manufacturer's protocol. Primers and probes were purchased for hsa-miR-10b, hsa-miR-21, hsa-pri-miRNA-10b and hsa-pri-21 and U6 snRNA (which was used as an internal control) (Appendix - Table 3). Experiments were performed in triplicates; treated samples were compared to the DMSO solvent and normalized to the reference gene (snRNA U6) as an experimental control. Relative expression levels were calculated using the $2^{-\Delta Ct}$ method by Michael W. Pfaffl [143].

To detect the levels of the precursor sequences, no commercial taqman probes are available. Thus, cDNA was synthesized using the SuperScript III cDNA kit (Invitrogen), and diluted cDNA was used for RT-PCR analysis using iQ SYBR Green Supermix (Bio-Rad) with the appropriate primers (Table 4) according to the manufacturer's protocol. Relative expression levels were calculated using the $2^{-\Delta Ct}$ method by Michael W. Pfaffl [143].

Computational model of pre-mir-10b (third fragment) in complex with Linafinib. The secondary structure of pre-mir-10b (third fragment) was predicted by CentroidFold

[144, 145]. RNA tertiary structure was predicted by RNAComposer using the predicted secondary structure as the constraints [146]. Molecular docking was performed using rDock2006 [147, 148]. We explored the binding site using the geometrical center of UG:AGU internal loop as sphere center, 15Å radius, and RbtCavityGridSF site searching scoring function. A total of 200 separate runs were performed using rDock_solv scoring function. We selected the binding mode that received the best rDock_solv score for demonstration.

Lentivector-based MicroRNA Precursor Constructs. Primers for the precursor sequences of each microRNA were designed (OligoPerfect^{TM} Designer) including *BamH1* and *Ecor1/Not1* restriction sites (Appendix- Table 4). The amplicon was subsequently confirmed by sequencing in the pCR2.1-Topo Vector (according to the manufacturer's protocol). We then digested the pCDH-CMV-MCS-EF1-copGFP vector (System Biosciences), along with the vector containing our insert. Subsequently, we ligated and purified the construct already containing the precursor sequences of our interest with a kit for DNA purification (QIAGEN) according to the manufacturer's protocol.

For the lentiviral production, 293FT cells were grown in DMEM supplemented with 10% FBS and "splitted" when they were 80% confluent. Cells were transfected in 10cm dishes when 60% confluent with 8.0µg of transfer vector (pMirna – System Biosciences), 5.2µg of the packaging vector (CDH-CMV MCS-EF1-copGFP) and 2.8µg of the envelope vector (pMD.G – System biosciences). After 48 hours of incubation the supernatant containing the virus was collected and filtered (0.45 micrometers). MCF7 cells were then taken to infect with the virus supernatant, containing either the empty vector, or the vector containing sequences for pre-miR10b or -21. When 60% confluent, 2mL of viral supernatant was added to the cells, along with Polybrene/Sequabrene antibiotic, at a final

concentration of 8µg/mL. The plate was then centrifuged 1500-1800g for 90 minutes, and incubated at 37° C for 30 minutes. Finally the media was removed and replaced with fresh one. Transfection efficiency was monitored through GFP, as well as RT-PCR.

Dose-Dependent Cell-based assays. The luciferase-reporter vector construct was used to test the half maximal effective concentration (EC50) at which each small molecule inhibitor induced a response halfway between the baseline and maximum after a specified exposure time. Cells were plated at a 50-60% confluence the evening prior transfection. Transfection was performed as initially done for the screening, and effectiveness of small molecule inhibitors was tested at three separate time-points 24 and 48 hours. A total of 5 different concentrations were tested in serial dilutions and the EC-50 was determined for each compound using MCF7 cells.

Cell Viability. Cell viability and IC50 were determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-^{2}H-tetrazolium bromide (MTT assay). Briefly, 2.0 x 10^{4} MCF7-cells/well were plated the evening prior to treatment in 96 well microculture plates. After cells were adherent, 5 different doses of the drugs were added to the supernatant. After 24 and 48 hours, the MTT reagent (Sigma) was added to each well and incubated for 3 hours at 37° C. The optical density (OD) was read at 570nm on a microplate spectrophotometer and growth values (%) were calculated as (OD treated cells / OD untreated cells) x 100. The experiments were performed in quadruplicates after 24 and 48 hours.

In vitro migration and invasion assay. For migration, 8 micron translucent inserts for 24 well plates were covered with gelatin (100µL) were put to gently shake/rotate for 2 hours. Excess solution was discarded, and plates were left to dry under sterile conditions

for 1 hour. Following this, 50,000 cells were seeded onto the top of gelatin-coated insert and 500 µl of media with 10% FBS was added to the bottom of each well. Cells were left to migrate 24 hours. The cells that migrated to the bottom of the well were fixed, stained (HEMA 3 stain set – according to manufacturer's instructions), and counted using a microscope. For each well, 5 different fields were counted and the average number of cells was determined. The experiments were performed in triplicates. The same protocol was used for invasion assay with a top coat containing collagen and laminin. Results were normalized for proliferation

Proliferation assay: Cell proliferation was determined using a colorimetric assay were the highly water-soluble tetrazolium salt WST-8 (Dojindo Molecular Technologies) is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, (generated by the activities of dehydrogenases in cells, and directly proportional to the number of living cells) was measured after 3 hours of incubation. The OD was read at 450 nm on a microplate spectrophotometer and growth values (%) were calculated as (ODtreated cells /ODuntreated cells) x 100. The experiments were performed in triplicate.

Statistical analysis The qRT-PCR, luciferase dosage dependent analysis, migration and invasion assays were performed in triplicates. A (two-sided) t-test was applied to compare the mean between control and treated samples. Analyses were carried out in GraphPad (Prism 6). The statistical significance was defined as a p-value less 0.05. *MTT assay:* data and analysis The MTT experiments were performed in quadruplicates. A (two-sided) t-test was applied to compare the mean between solvent control and treated samples. Analyses were carried out in GraphPad (Prism 6). The statistical significance was defined as a p-value less 0.05.

Nuclear Magnetic Resonance. The human microRNA sequences were obtained from miRBase database (http://www.mirbase.org/). RNA molecules were prepared by *in vitro* transcription using T7 RNA polymerase (approximately 2 ml reaction) using 15N isotope labeled 5'-NTPs (nucleoside triphosphates) and purified over PAGE gels using established procedures [149, 150]. Resonance assignments for the RNA hairpins containing the target motifs were be obtained using standard homonuclear and heteronuclear multidimensional NMR techniques (e.g. HCCH-TOCSY, HNN-COSY, and ^{13}C- or ^{15}N-edited 3D NOESY-HMQC) [151]. Inter-proton distances were derived from ^{1}H-^{1}H NOESY and $^{13}C/^{15}N$-edited 3D NOE-based spectra. Residual dipolar couplings (RDC's) were generated by Pf1 phage-induced weak alignment of the RNA molecules and incorporated into the structure determination [152-155]. Assignment of constraint values and the structure calculation protocols were similar to those in the following manuscripts [152, 155-157].

Spectrums of the RNA and the DMSO (solvent) were used as controls. Peaks in the samples with the RNA and small molecules were compared to the control spectrums to predict miRNA-compound interactions. To confirm peak changes in any positive 2D NMR, we designed RNA Oligonucleotide sequences (Dharmacon) to create shorter segments of the primary transcript of miR-10b of approximately 50 base pairs (with some bases overlapping each other between sequences). We then read a 1D NMR spectrum to determine in a more precise way in which segment of the primary the molecule was binding to.

Western Blot. Proteins were lysed with Lysis buffer (Cell Signalling Technology), diluted from 100X up to 1x, as well as phosphatase and protease inhibitors (also diluted from 100X to 1X). Cells were washed with PBS to remove residual medium. A total of 50 – 100µl of cold 1x LB was added, and cells were mixed by pipetting. Subsequently cells

were incubated on ice for minimum 10 minutes with vortex every other minute during the time. Then lysate was centrifuged at maximum speed for 10 minutes at 4°C. Supernatant where transferred to a new tube and stored at -80°C until used.

For protein quantification the equipment used was: Smartspec Plus Spactrophotometer (BioRad) and the BioRad Dye Reagent was used to dilute the proteins (1:1000). Criterion pre-casted gels (4-20% Tris-HCl 1.0mm) were used (BioRad), at a constant mAmp of 35 and a voltage of 160. “Trans-blot Turbo Transfer packs” were used to transfer the proteins to 0.2 µM nitrocellulose membranse, for a ten minutes (dry transfer). Membranes were blocked with 5% milk for one hour. Primary antibodies were left overnight, and secondary antibodies were left for 1-2 hours.

Ingeuity Pathway Analysis (IPA). A detailed description of IPA can be found at http://www.Ingenuity.com. Genes of interest were entered into Path Designer using “My Pathway” function. Path Explorer tool was used to identify the shortest pathways among the genes using both Ingenuity pathway analysis knowledge base and external databases. Grow tool under “Build Tab” was also used to simultaneously explore direct or indirect relationships for either down-stream or up-stream molecules of the miRNA genes of interest.

Identification of miRNA/mRNA target interactions /functional profiling. A large number of target prediction programs and databases on experimentally validated information have been developed for miRNAs. For miR-10b and miR-21, we retrieved data on miRNA-mRNA interaction predictions and miRNA-mRNA validated interactions from miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/). miRWalk is a comprehensive

database on miRNAs, which hosts predicted miRNA-mRNA interactions (given by 8 algorithms), as well as validated miRNA-mRNA binding sites. We also downloaded information on validated miRNA - target interactions from miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/). The data base miRTarBase accumulates miRNA-target interactions (MTIs), which are collected by manually surveying literature (the collected MTIs are validated experimentally by reporter assay, western blot, or microarray experiments with overexpression or knockdown of miRNAs). For further analyses we chose mRNA targets predicted by at least 4 programs and/or validated by multiple assays. We used then KEGG Database Resource (http://www.genome.jp/kegg/) and PANTHER (http://pantherdb.org/) for a functional profiling of miRNA targets.

CHAPTER III: Results

Evaluation of the clinical significance of validated oncomiRs in BC

Initially we carried out a literature review of recently validated oncomiRs in BC. The majority of the studies used where published within the last 5 years. We then selected a total of 6 microRNAs which were found to be highlighted as oncogenic in different BC studies *in vitro* / *in vivo,* and also in patient samples. These were: miR-9, miR-10b, miR-21, miR-155, miR-181a and miR-181b. Following the selection of miRNAs, we carried out an integrative computational analysis to identify their clinical relevance within tumor samples from invasive BC patients. Using The Cancer Genome Atlas (TCGA) patient datasets of invasive breast carcinoma tumors, we found that miR-10b and miR-21 were overexpressed at statistically significant level when compared to all the others (both with a P<0.0001) (Figure 8). Thus, we selected these 2 oncomiRs for the purposes of our studies.

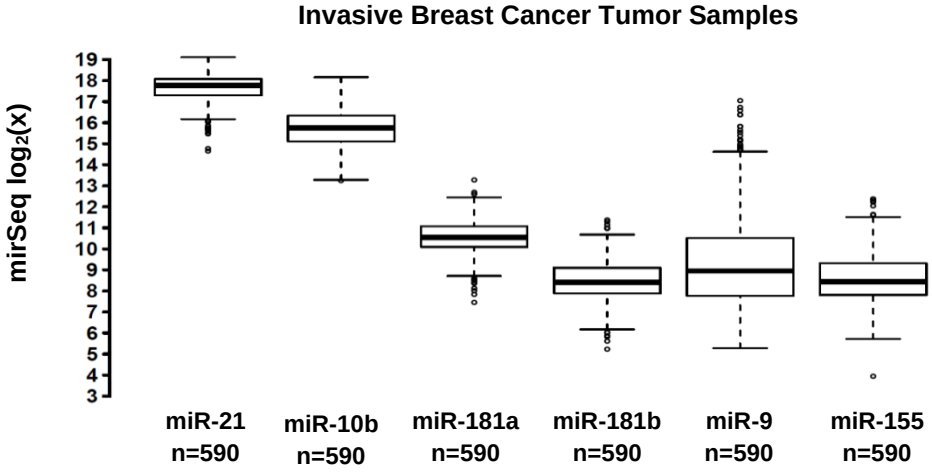


Figure 8: Oncomirs -21 and -10b are highly expressed in invasive breast carcinoma tumors.

Using TCGA patient data sets of all BC tumor types, a comparison between the levels of expression of miRs: -9, -10b, -21, -155, -181a, -181b, yield that miR-21 and miR-10b are expressed at a statistically significant higher level.

The expression of oncomiRs -10b and -21 are not correlated in BC patient samples.

Within the TCGA patient dataset, we investigated if the levels of expression of both of these miRNAs were correlated. Initially we tested the complete datasate of invasive breast carcinoma tumors (n=590). Within the pooled set of samples, we found a correlation coefficient of -0.25; which, although negative, suggested statistical but not biological significance.

We then separated the patient samples into 2 groups. As part of our first group, we selected patients with tumors types between stages I and II. In the second group we pulled together the patients with tumor between stages III and IV. Within these groups we did not find any correlation in the expression of miR-21 and miR-10b (Figure 9). We also divided patient samples by specific receptor status (ER, PR, TNBC), and found no significant correlation between the expression levels of both miRNAs *(data not shown)*. Thus, we concluded their expression is not correlated in BC.

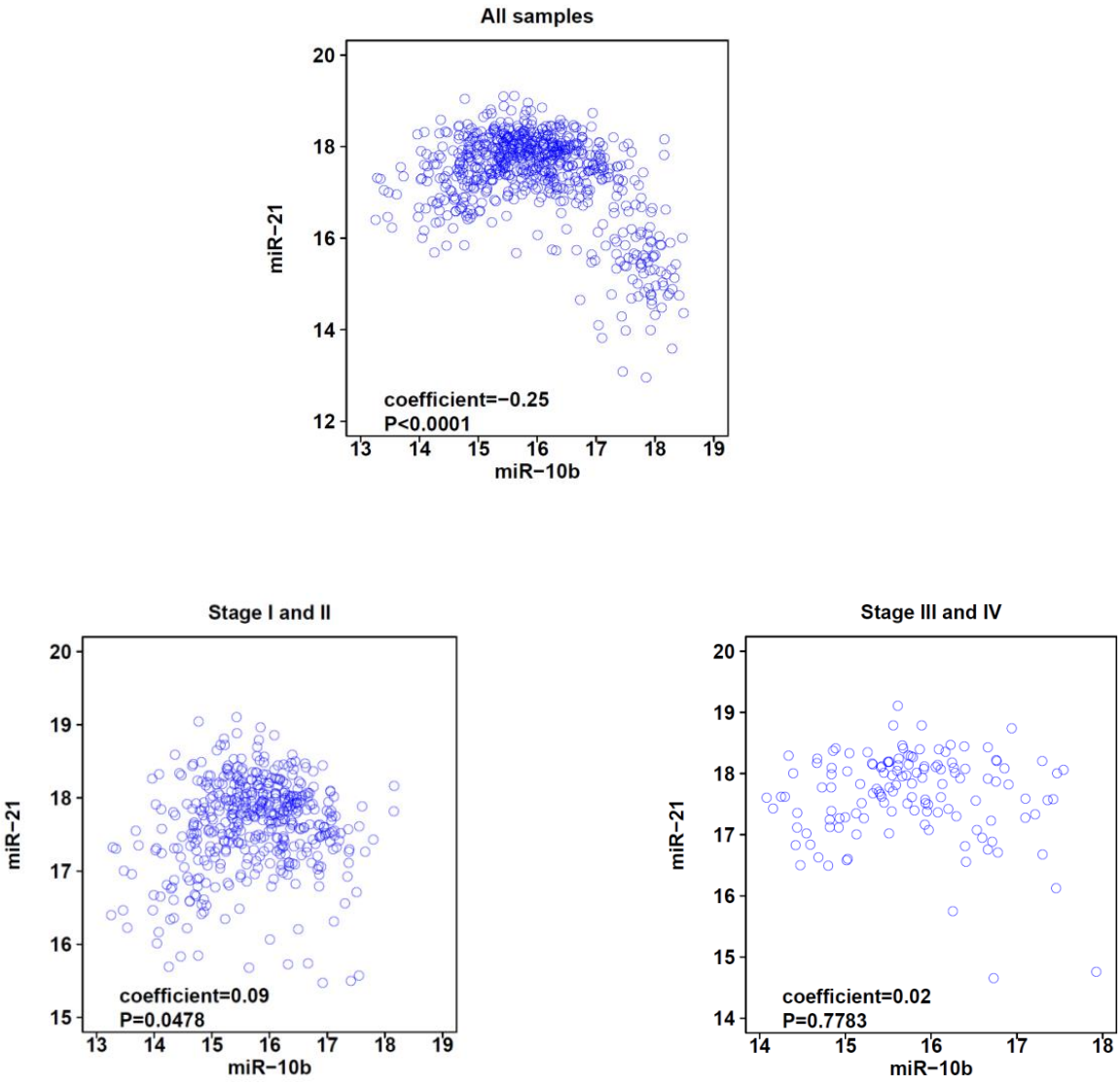


Figure 9: The expression of oncomirs -21 and -10b is not correlated in BC patients

Using TCGA patient data sets of all BC tumor types, we investigated if the expression of miRNAs -21 and -10b was correlated. Comparison between all the samples resulted in a small negative correlation (coefficient of -0.25). Although statistically significant these results did not suggest a significant biological correlation. More so, dividing the patients by specific tumor stages did not yield any correlation between the expression of miR-10b and -21.

Individual assessment of miR-10b/-21 levels of expression within specific BC tumor subtypes

In order to better understand the significance of the miR-10b and miR-21 overexpression in invasive breast carcinoma samples from patients (TCGA database), se grouped them according to their estrogen, progesterone and HER2 receptor status. We found a significant increase in the levels of expression of miR-10b, in ER+ subtypes compared to their negative counterparts (Figure 10A). Similarly, PR+ patient tumor samples had higher miR-10b levels compared to negative tumor types (Figure 10B). We further confirmed this tendency by studying samples from patients with TNBC tumors, and found that the levels of miR-10b were significantly lower in the group of patients with TNBC tumor types (compared to non-TNBC) (Figure 10C). We thus confirmed for the first time to our knowledge, that high levels of miR-10b are characteristic of ER^+PR^+ subtypes of breast cancer tumors.

Within the same group of tumor samples (grouped by the expression of ER, PR and HER2), we analyzed the expression levels of miR-21. Interestingly there was no difference in the levels of miR-21 between ER/PR +/- tumor samples (Figure 11A and 11B), nor so in TNBC or non-TNBC (Figure 11C). We thus confirmed, not only that miR-21 is overexpressed in invasive breast carcinoma samples, but that its overexpression seems to be independent of the molecular classification of the tumor type (ER/PR/HER2).

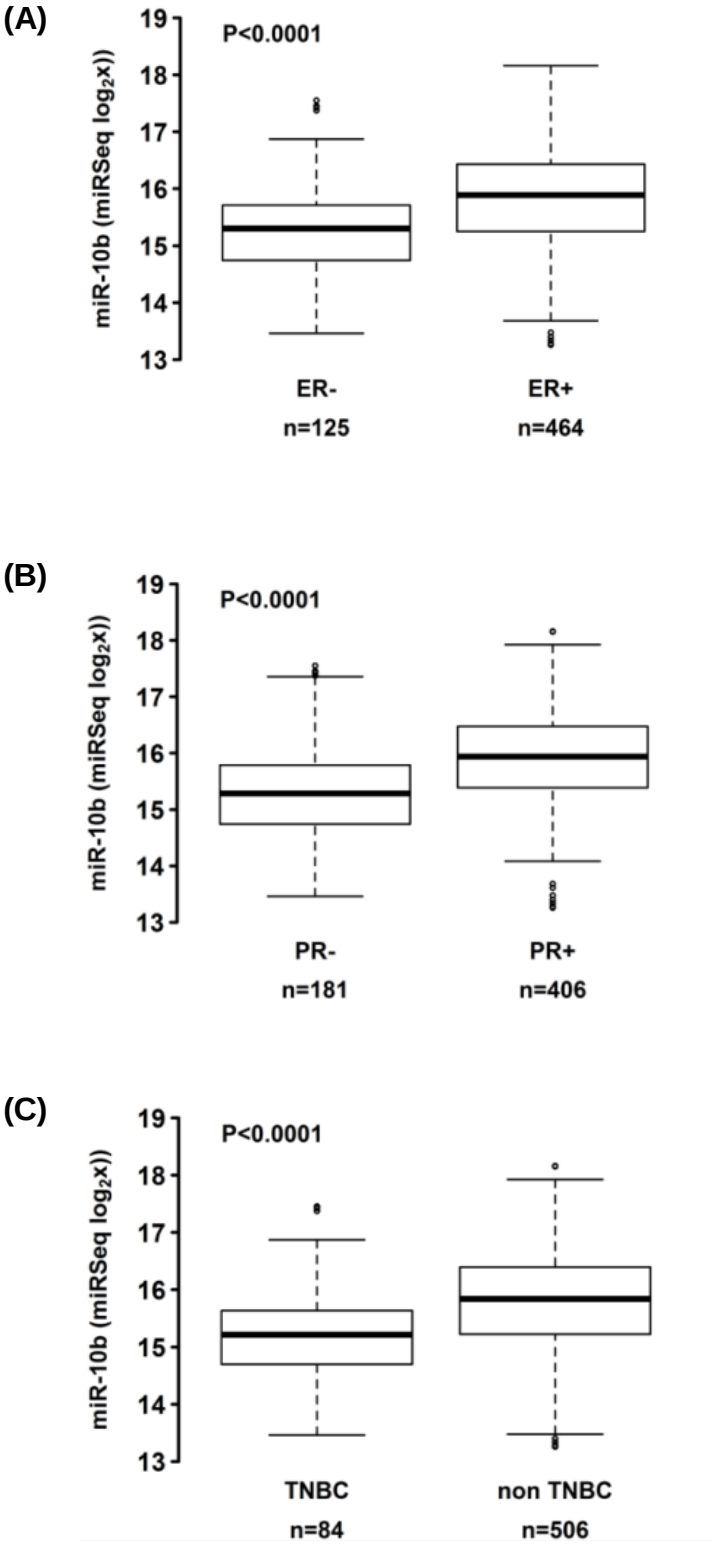


Figure 10: Levels of expression of miRNA -10b within subgroups of BC patients classified by estrogen, progesterone and HER2 receptor status.

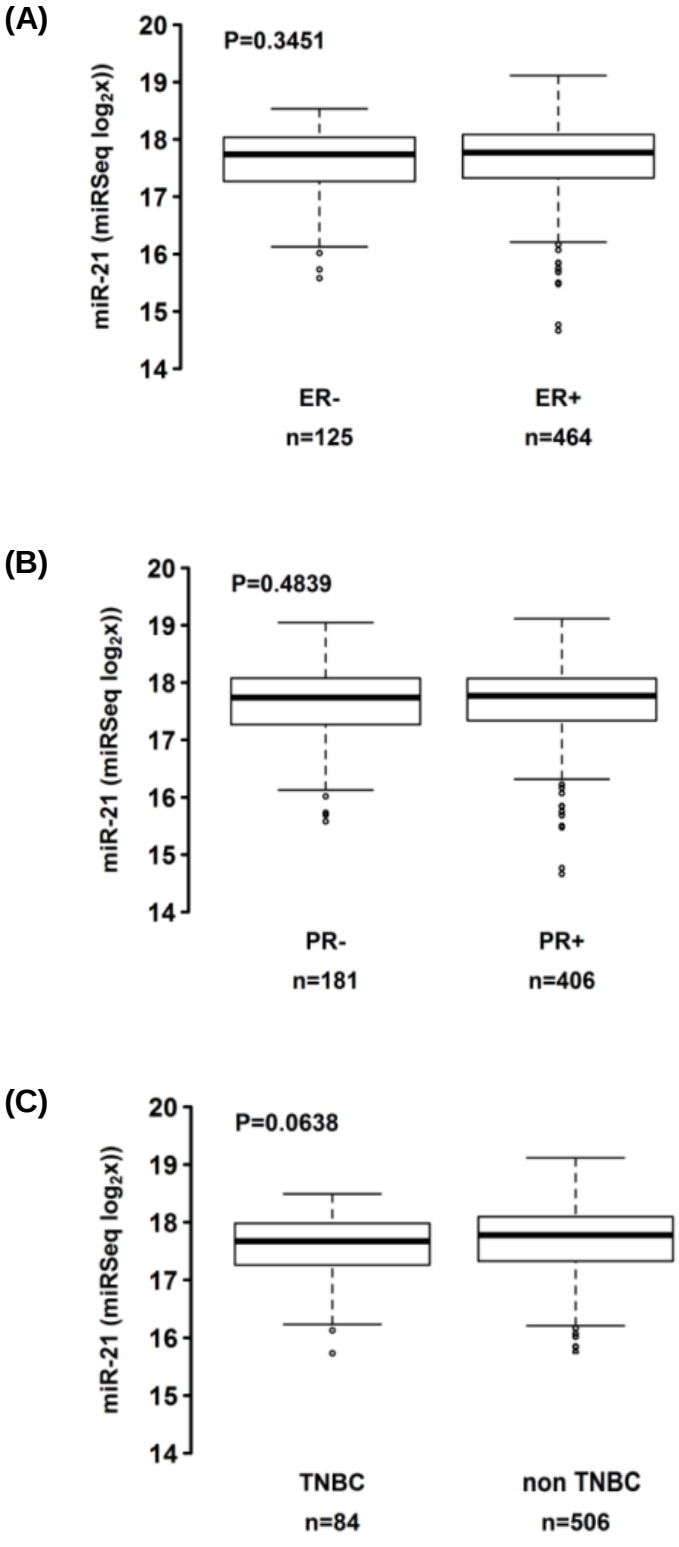


Figure 11: Levels of expression of miRNA -21 within subgroups of BC patients classified by estrogen, progesterone and HER2 receptor status.

Expression of miR-10b and miR -21 influence overall survival of ER$^+$ BC patients

We determined to identify the influence of miR-10b and miR-21 overexpression in the overall survival (OS) of BC patients. Once again, using TCGA patient datasets, we analyzed the clinical correlation of the expression levels of miR-10b and miR-21 in different BC tumor subtypes. Our results demonstrated that miR-10b is associated with decreased OS, specifically in early tumor stages (I-II) of patients with ER+ tumor types (Figure 12A). Similarly, miR-21 overexpression correlated with OS in ER+ tumors; however, it was statistically significant only at later tumor stages (III-IV) (Figure 12B). Thus, we confirmed that both oncomirs are clinically relevant, but also, we identified a specific group of BC patients that could benefit from a miRNA-targeted therapy.

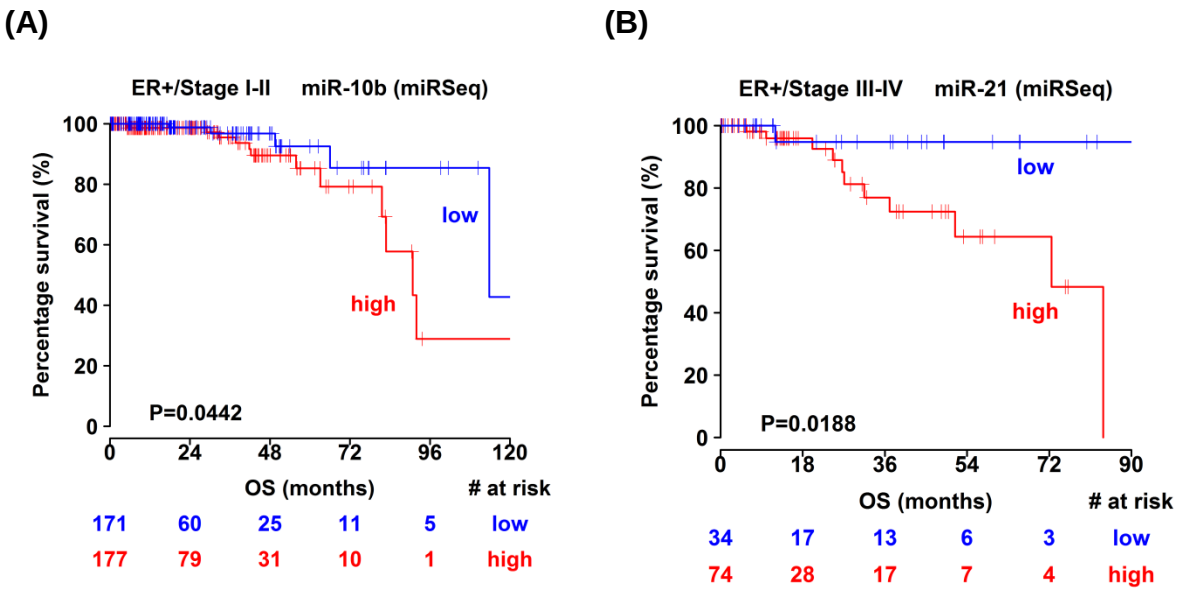


Figure 12: High levels of expression of miRNAs -10b and -21 correlate with decreased OS in BC patients.

(A) High levels of expression of miR-10b are associated with decreased OS, of patients within early stages (I-II) of ER+ tumors. **(B)** Similarly, high levels of expression of miR-21 also correlate with decreased OS in patients within tumor stages III-IV.

High-throughput screening of small molecule inhibitors targeting miR-10b/ miR-21

With the aims of discovering novel therapeutic approaches for BC patients, we designed a step-wise approach to screen, detect and validate SMIRs targeting miR-10b or miR-21 through different assays (Figure 13).

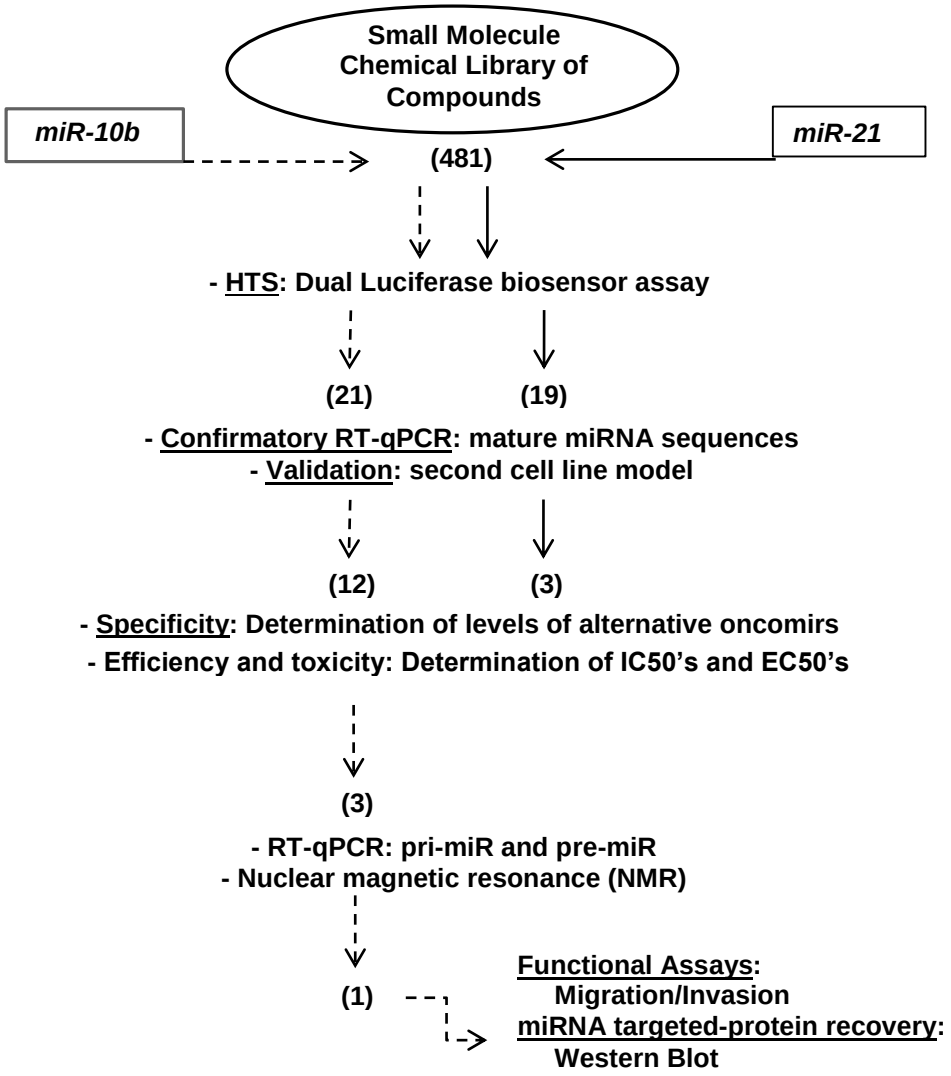


Figure 13: Screening Design

Summary of the steps used to determine if small molecule inhibitors targeted miR10b/-21. The number of compounds remaining in the study after each step is shown.

Initially we constructed a reporter vector designed with complementary oligonucleotides for miR-10b, and another one for miR-21. We cloned these sequences on a psi-CHECK2 vector, downstream of a Renilla luciferase reporter gene (Figure 14A). We then tested the effectiveness of the cloning, by sequencing and also by verifying the presence of the insert in both of the vectors constructed. For the latter, we digested the vectors with *SacI* and *BamH1* enzymes, for which restriction sites were present in the insert and vector (respectively). In the presence of the insert, the construct was cleaved at 2 sites (yielding 2 separate bands seen on agarose gels). On the other hand, if the vectors where empty (ligated upon themselves) only 1 band could be seen in the agarose gels (Figure 14B and 14C).

Further along we tested the sensitivity of the reporter in detecting changes in the levels of expression of the miRNA for which they had the complementary target sequence. To do this, we transfected them into MCF-7 cells and alongside, we also added antagomiR-10b/-21 or pre-miR10b/-21, to verify that the assay was sensitive in detecting the changes in their levels. In the presence of antagomiRs, luciferase expression increased significantly. On the other hand, in the presence of precursors, it decreased (when tested 48 hours after transfection) (Figure 15A and 15B). Given the fact that the assay proved sensitive, we used it as the initial step of our screening (refer to Figure 13).

Given the fact that miR-10a varies only by 1 in comparison with miR-10b, we also performed the sensitivity assay with antagomiRs and precursors for miR-10a for the psi-CHECK2 vector containing the miR-10b target sequence. We found no statistically significant changes in luciferase expression when altering levels of miR-10a (Appendix-Figure 38). Our results once again suggested sensitivity and specificity, only towards changies in miR-10b levels.

We screened a library of 450 small molecule inhibitors. A total of 6/96-well plates with scattered solvent controls were tested. The results of all six plates were merged, and are reported (Figure 15C). For results representing individual plates per compound, refer to the Appendix- Figures 39, 40 and 41. Compounds representing the top 5 % of compounds with the highest luciferase fold change expression were considered miR-10b small molecule inhibitor hits, and submitted to further testing.

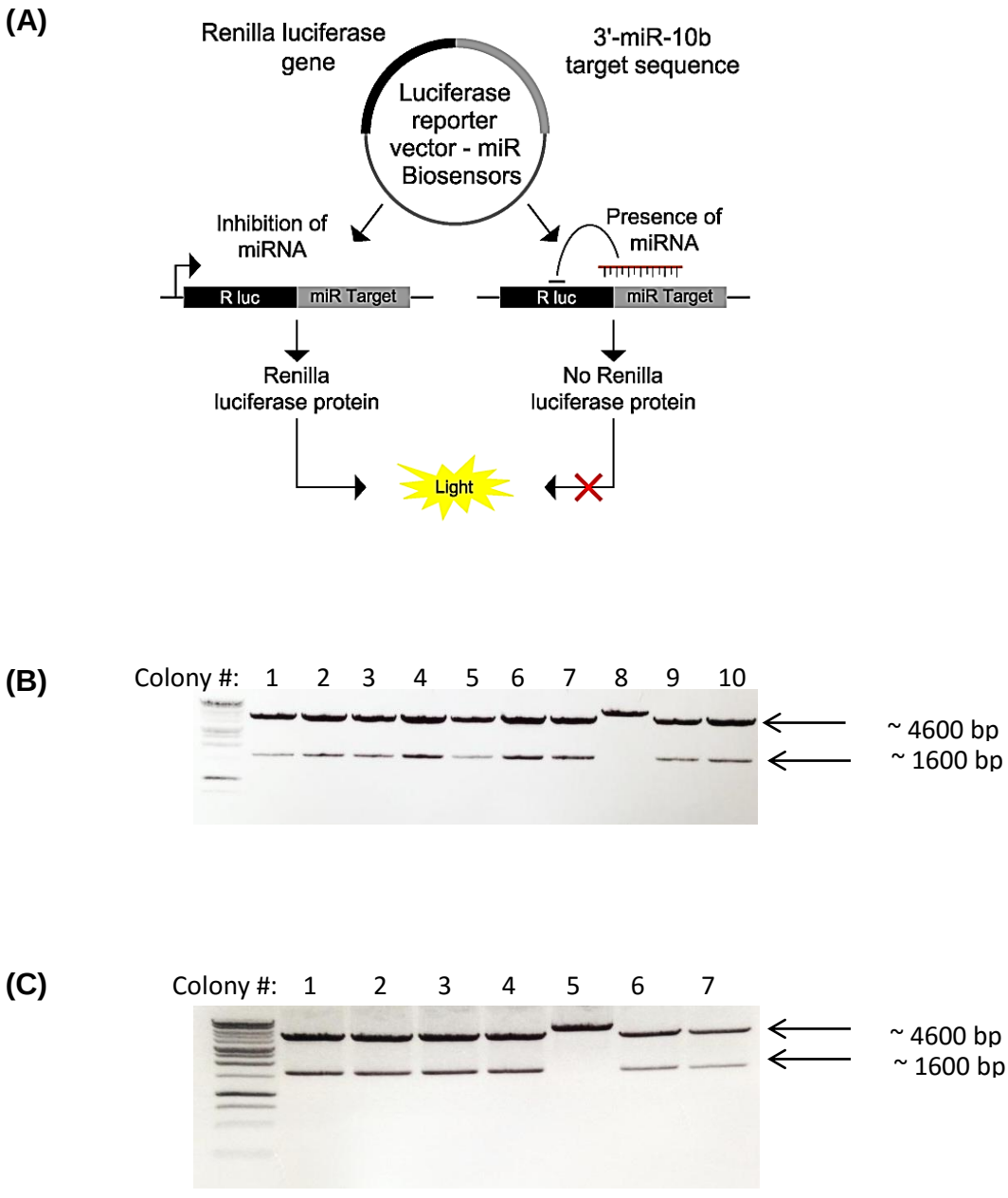


Figure 14: Cloning of Psi-CHECK2 Luciferase vector constructs

(A) Psi-CHECK-2 vector was used to clone the miR-10b / miR-21 target sequences downstream of a Renilla luciferase (Rluc) gene. After the transformation, vectors were extracted from *E. coli* colonies, and digested with *SacI* and *BamHI* enzymes to confirm the presence or absence of the insert. **(B)** Representative colonies from psi-CHECK2+ miR10b target sequence. **(C)** Representative colonies from psi-CHECK2+ miR21 target sequence.

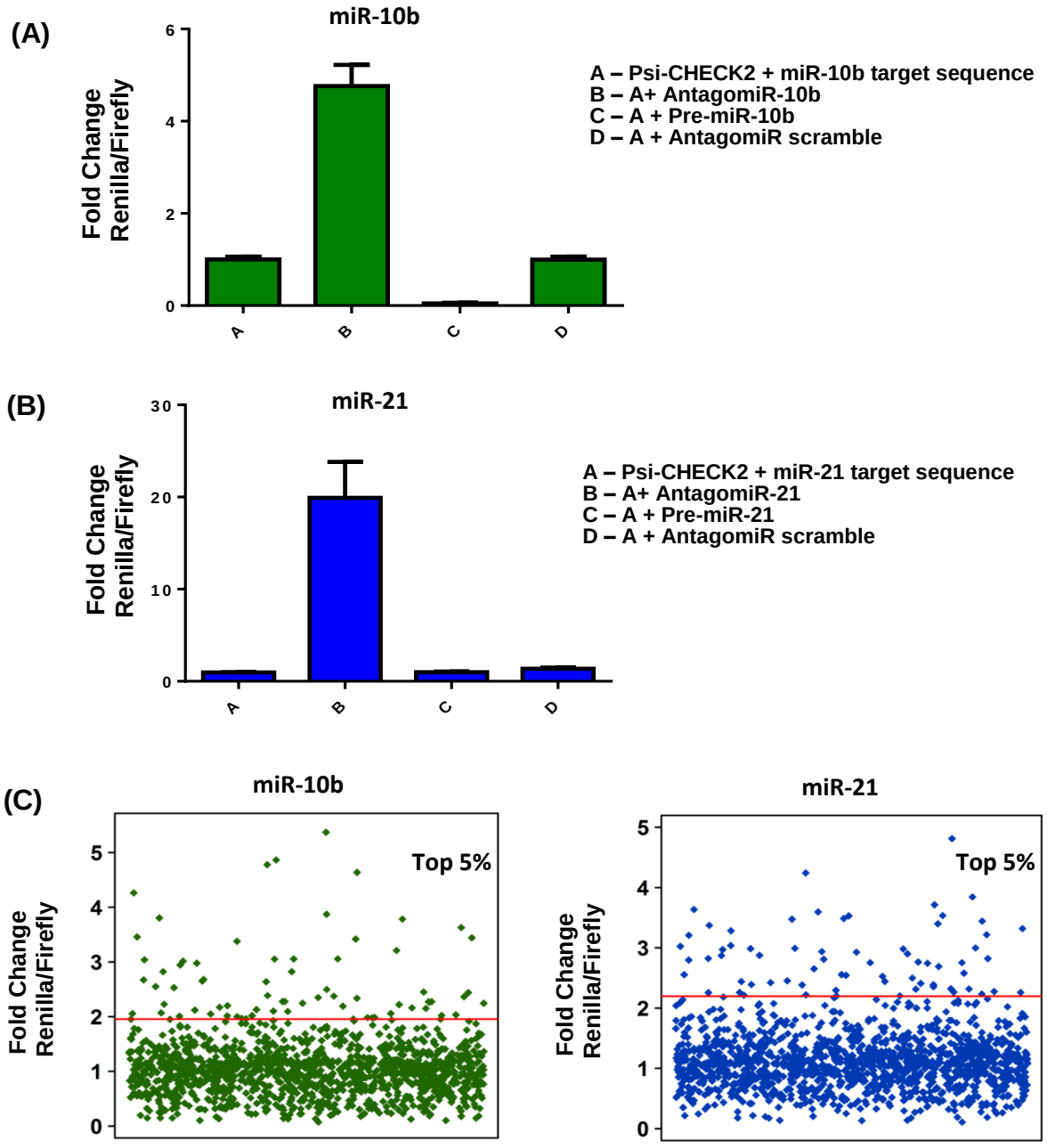


Figure 15: Luciferase-based microRNA biosensor assay detects changes in the expression levels of miR-10b and miR-21.

(A and B) To test the sensitivity of the reporter vector construct, we transfected the vectors into MCF-7 cells, along with antagomirs/precursor sequences for miR-10b / miR-21. Luciferase expression increased significantly in the presence of antagomiRs, and decrease concurrently with the addition of precursor sequences. **(C)** Screening results of the acquired library revealed potential SMIR-10b/-21 candidates. Approximately 20 compounds were selected from the top 5% with the highest luciferase expression. These were determined to be positive hits, and thus were chosen to perform confirmatory assays.

Validation of SMIR-10b and SMIR-21 candidates

As a second step in our screening, we tested the positive hits from the luciferase-based reporter assay with a technique that increased sensitivity: real time-quantitative polymerase chain reaction (RT-qPCR). MiR-10b / -21 inhibitions were evaluated by RT-qPCR in MCF-7 cells at the same time point and under the same conditions at which the luciferase assay was performed (48 hours). Compounds confirmed as hits in the RT-PCR were considered “true positives”. Non-confirmed hits were considered “false-positives”, and therefore eliminated from our screening. In all, a total of 13 compounds were confirmed to decrease the levels of miR-10b, and 2 where confirmed to decrease miR-21 (Figures 16A and 16B respectively).

To further validate specific hits, we decided to verify whether the inhibition could also be achieved in one additional BC cell line model (MDA-MB-231 or MDA-MB-468, both triple negative). For this, we selected each of the validated hits, and repeated the exact same treatment conditions in one additional cell line, at two time points (24 and 48 hours). We observed that all the SMIR-10b and SMIR-21 validated hits inhibited miR-10b and miR-21 respectively in at least 1 additional cell line model under the same conditions (Figure 17 – Panels A, B and C). These results confirmed our previous findings in MCF-7 cells, and demonstrated that the inhibition was not cell line-specific.

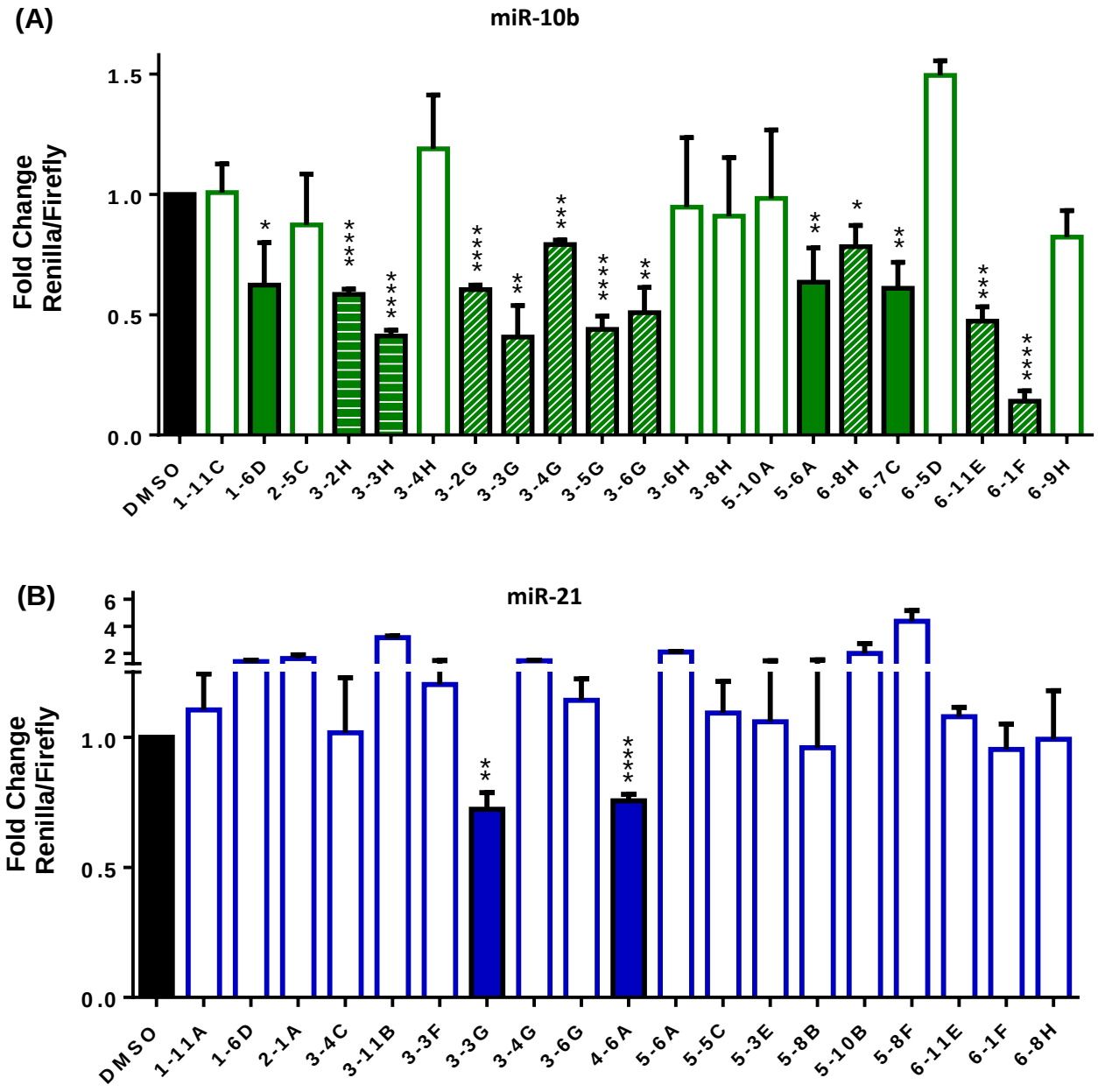
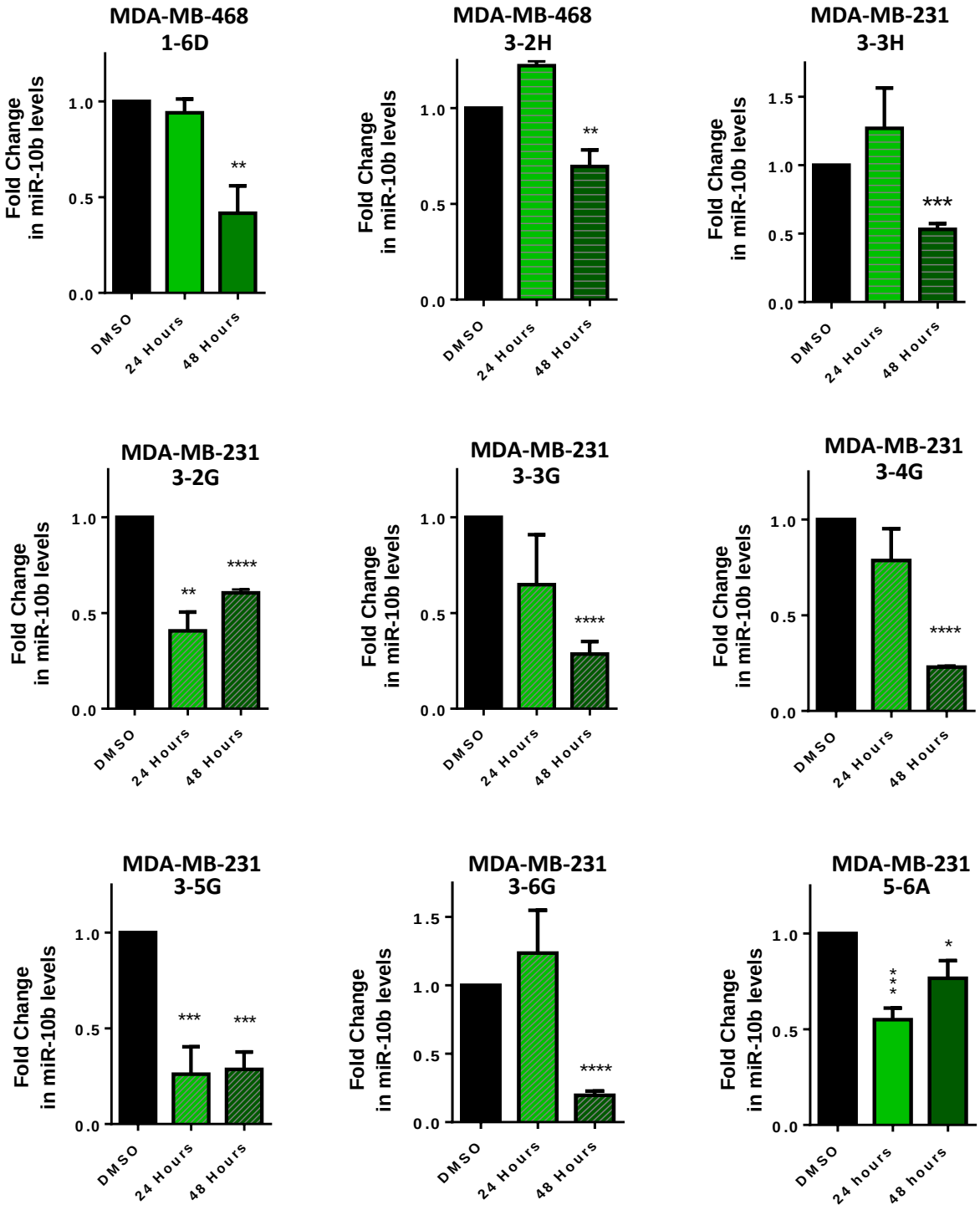


Figure 16: Validation of SMIR candidates using qRT-QPCR

qRT-PCR was used as a confirmatory assay to increase the sensitivity of our screening results. We evaluated miR-10b/miR-21 inhibition in MCF-7 cells at the same time point at which the luciferase assay was performed (48 hours). Compounds confirmed to be positive hits in the RT-PCR were considered “true positives” (shown as filled bar graphs). Non-confirmed hits were considered “false-positives” (shown as empty bar graphs). Several hit compounds belonged to the same family of inhibitors (shown as filled bar graphs with different patterns - per family type).

(A)



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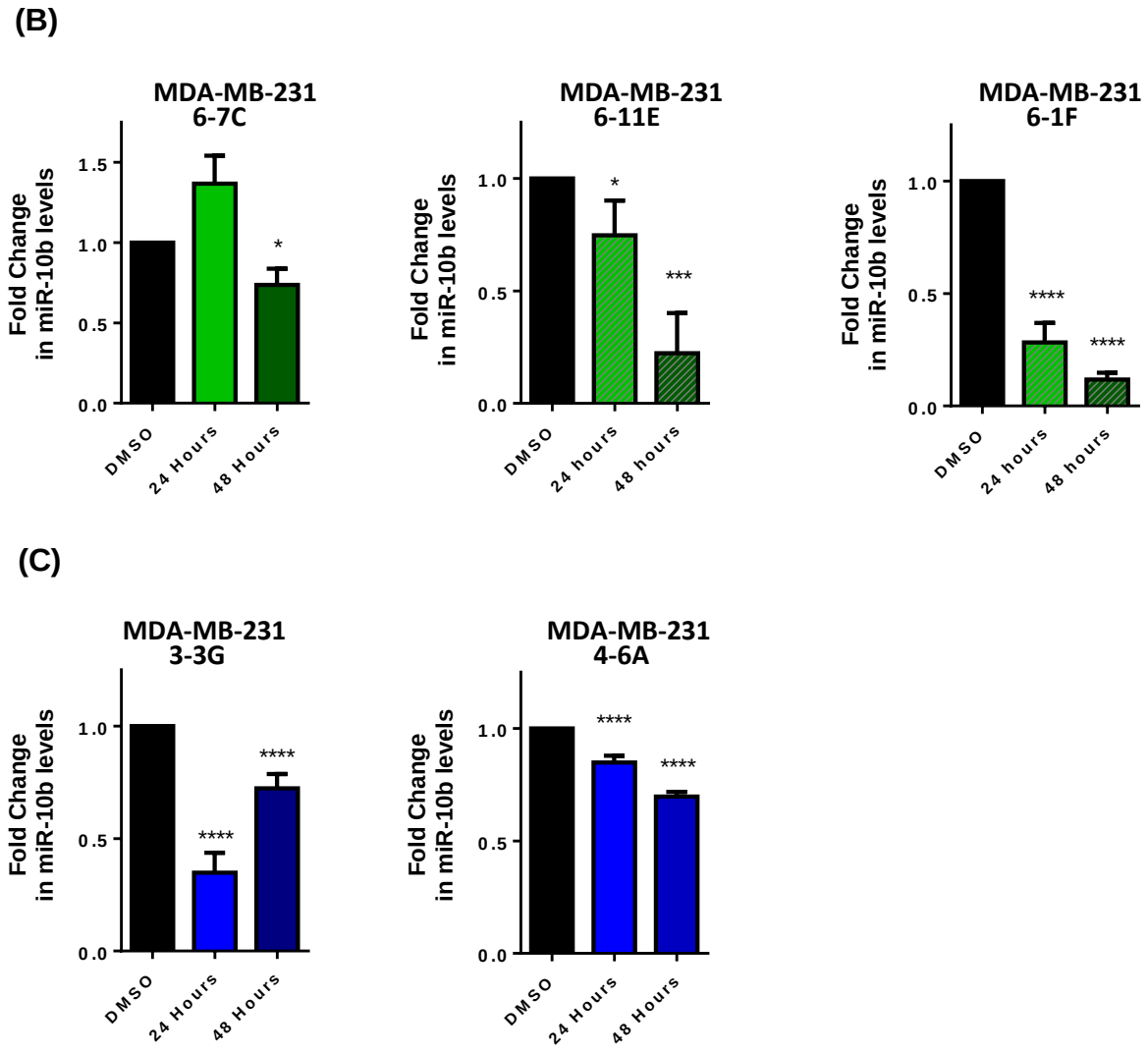
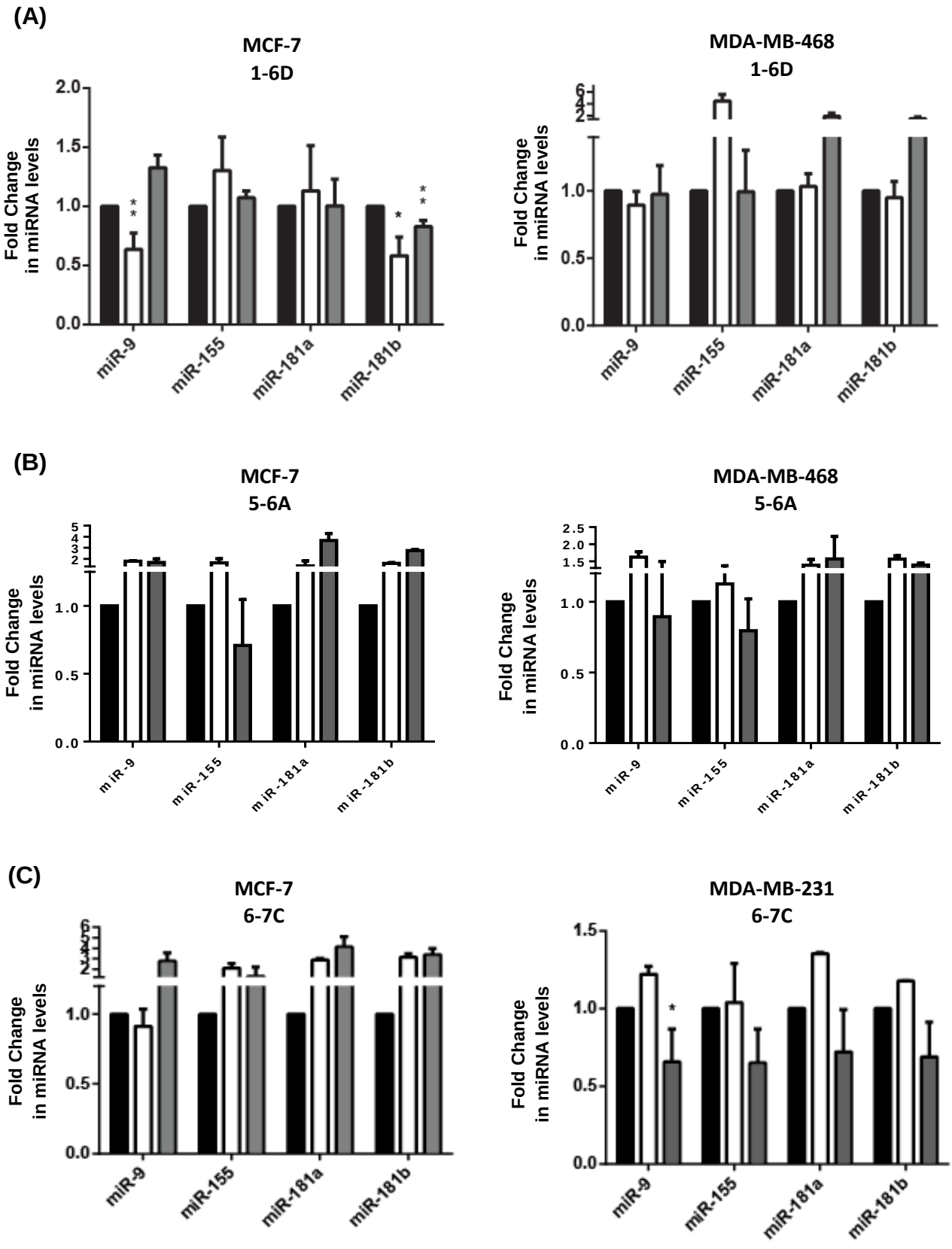


Figure 17: True positive SMIR compounds validated in TNBC cell lines (MDA-MB-231 or MDA-MB-468). Panels A and B - All of the "true positive" SMIR-10b hits were treated at two time-points (24 and 48 hours), and all of them decreased miR-10b levels. The results were statistically significant when treated under the same conditions used in the initial screening assay. **Panel C** – The 2 "true positive" SMIR-21 hits were treated at two time-points (24 and 48 hours). They both decreased miR-10b levels, and the results were statistically significant after 24 and 48 hours of treatment.

Determining the specificity of oncomiR inhibition by SMIR candidates

As the following step of our screening, we determined to identify possible off-target effect of the SMIRs, such as inhibiting other oncomiRs (not-10b / -21). For this, we used RNA from MCF-7 cells that had been previously treated with the SMIR hits (for both miRNAs). We then verified the levels of expression additional oncogenic miRNAs, to determine the specificity of the molecules (Figure 18A-F and Figure 19A-B respectively). We selected 4 oncogenic miRNAs from the group of 6 which we had initially found to have validated tumor suppressor targets in our literature review (-9, -155, -181a, -181b) [53, 110, 111, 158]. For compounds of the same family (inhibitors of the same protein), we only tested 1-2 compounds, and the rationale behind this decision was that if compounds of the same family of inhibitors are decreasing the levels of miR-10b or -21, it is highly probable that the mechanism of action (mechanism of miRNA inhibition) is an indirect one (and not a direct one, which is the focus of our study).

After verifying the levels other oncogenic miRNAs we divided the compounds into one of two groups: a “specific hits” group (defined as compounds that only altered the levels miR-10b, and not additional miRNAs), or “non-specific” group (defined as groups that decreased the levels additional miRNAs). The RT-PCR analysis of 4 oncomirs allowed the definition of a spectrum of specificity for each of the candidates. For example, compound 6-11E from the family of HSP90 inhibitors was considered part of the group of “non-specific hits” (Figure 18E). On the other hand, compounds: 1-6D, 5-6A and 6-7C were considered “specific hits”, because treatment with them didn’t affect the levels of additional miRNAs tested (Figures 18A, 18B and 18C).



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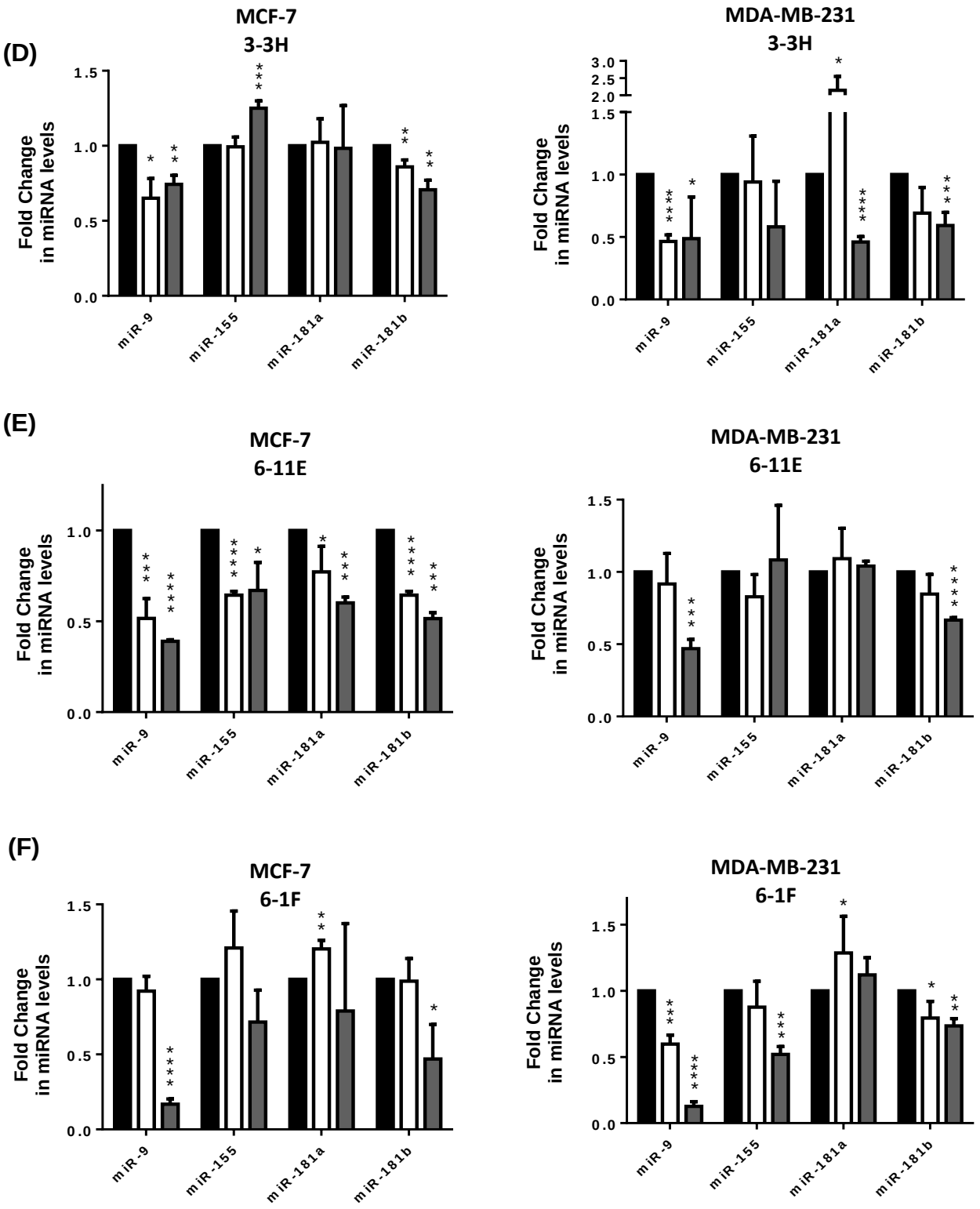


Figure 18: Levels of oncogenic miRNAs (-9, -155, -181a, -181b) in BC cells treated with SMIR-10b compound hits.

For SMIR-10b hit compounds, we tested the levels of expression of alternative oncomiRs after treatment with the SMIR-10b hits **(A)** 1-6D, **(B)** 5-6A, **(C)** 6-7C, **(D)** 3-3H and **(E)** 6-11E, **(F)** 6-1F.

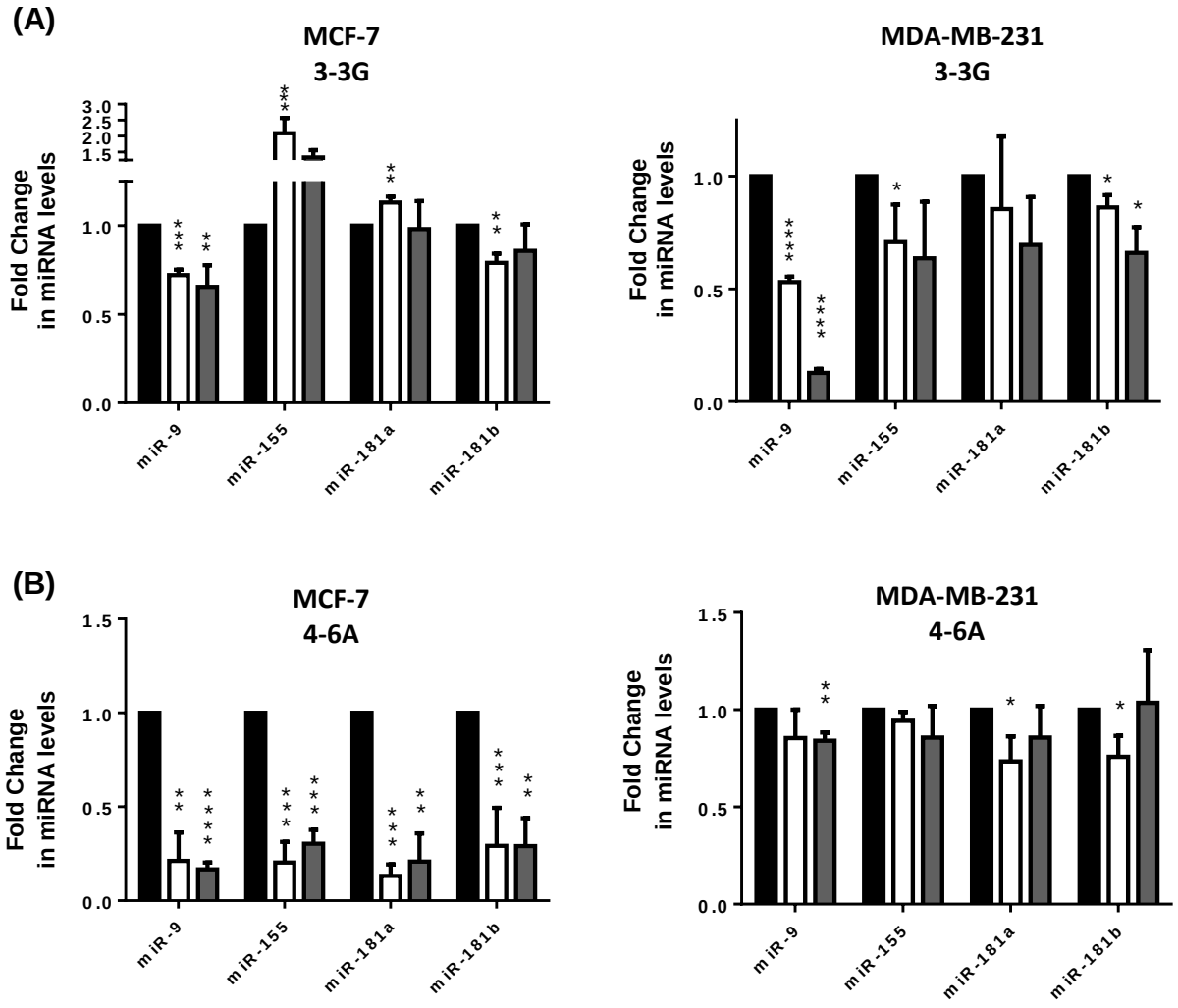


Figure 19: Levels of oncogenic miRNAs (-9, -155, -181a, -181b) in BC cells treated with SMIR-21 compound hits.

For SMIR-21 hit compounds, we tested the levels of expression of alternative oncomiRs after treatment with the SMIR-21 hits **(A)** 3-3G and **(B)** 4-6A.

Non-specific hits reveal a role of Heat Shock Protein 90 in regulating miR-10b levels

A total of 8 molecules, (out of the 13 confirmed miR-10b positive hits) belonged to the same type of family: *inhibitors of heat-shock protein 90.* Out of the 8 compounds, the RNA of 3 was tested to determine if the treatment also inhibited other oncogenic miRNAs in two different cell line models (Figures 18E, 18F). Since they also inhibited other miRNAs we grouped them in the “non-specifics” group of hits. Nevertheless, we observed that these, the inhibition of mIR-10b seemed to be strong, and the results were consistent among them. Thus, we selected the 2 most potent representatives from the family, 6-1F and 6-11E to pursue further studies.

For third time, we tested for miR-10b inhibition in MCF-7 cells after 24 and 48 hours of treatment (Figure 20 – left panel). More so, compound 6-1F decreased the levels of miR-10b in MDA-MB231 after both: 24 and 48 hours of treatment, by approximately 65% and 80% respectively (Figure 20 – bottom panel / right). Similar results were obtained with compound 6-11E decreased miR-10b levels after 24 and 48 hours of treatment by 25% and 50% respectively in an additional cell line tested (MDA-MB-231) (Figure 20 – bottom panel / left). Since the inhibition proved to be strong, we continued studying alongside, the role of HSP90 in regulating miR-10b in other cancer types.

The 8 HSP90 inhibitors that formed part of our library had different chemical structures (Appendix Table 6). Furthermore, they were previously used in an array of clinical trials for different diseases such as cardiovascular disease, asthma, depression and cancer.

However, their most significant common feature is the protein they target: HSP90. We therefore hypothesized that indeed it was the inhibition of the HSP90 protein itself what was causing the down-regulation of *miR-10b* (rather than a direct interaction). To further explore the mechanism, we tested for the levels of the primary and precursor transcripts of *miR-10b*, to determine if the inhibition was achieved by targeting miR-10b biogenesis/ maturation. We found both the primary and precursor transcripts were also significantly decreased in MDA-MB-231 cells (Figure 21).

We also investigated the possibility of validating our results in other cancer models *in vitro*. We thus tested 2 additional cancer types that have been recently linked to miR-10b, ovarian and pancreatic cancer [159-161]. We treated the ovarian and pancreatic cell lines HeyA8 and MiaPaca2 with two representative inhibitors of the HSP90 family. Our results demonstrated that miR-10b was inhibited in a time dependent manner at a standard dose of 10 µM (Figure 22). Thus, the miR-10b inhibition mediated by HSP90 inhibitors proved to be independent of the cancer type.

Finally, using Ingenuity Pathway Analysis, (IPA), we predicted that HSP90 inhibitors could be regulating the transcription process of miR-10b, through the transcription factor TWIST (Figure 23). The validation of these predictions could result in future alternative pathways to target miR-10b.

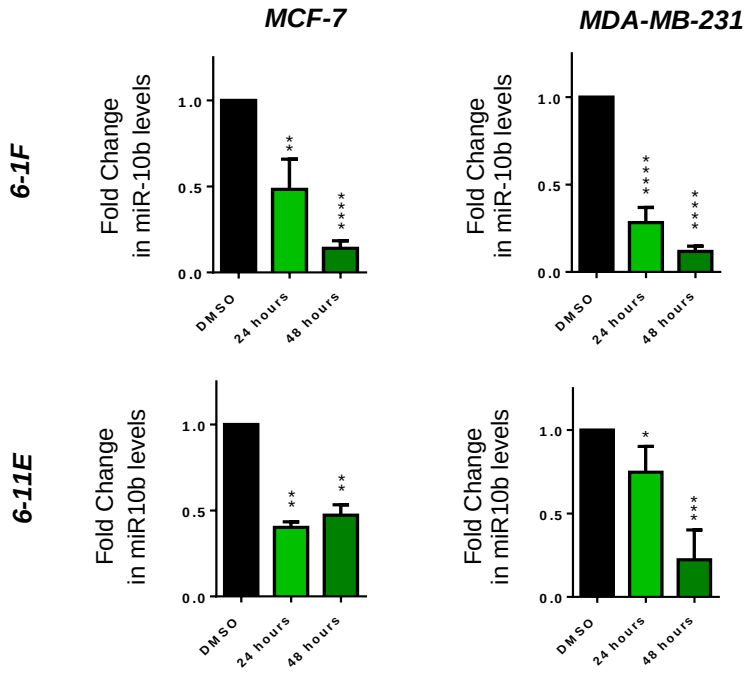


Figure 20: HSP90 inhibitors decrease miR-10b levels in BC cells after both 24 and 48 hours of treatment.

The 2 compounds 6-1F and 6-11E (representative the HSP90 inhibitor family), decreased the levels of miR-10b after two different time-points of treatment: 24 and 48 hours. The results were consistent in both: MCF-7 and MDA-MB-231 cells.

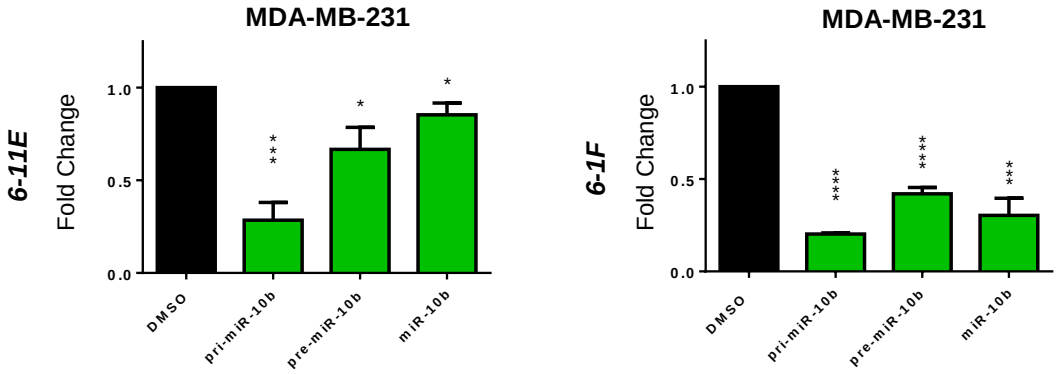


Figure 21: HSP90 inhibitors decrease the levels of expression of all three miR-10b transcripts: primary, precursor and mature sequence

Treatment with compounds 6-1F and 6-11E for 24 hours decreased the levels of pri-miR-10b, pre-miR-10b and miR-10b in MDA-MB-231 cells.

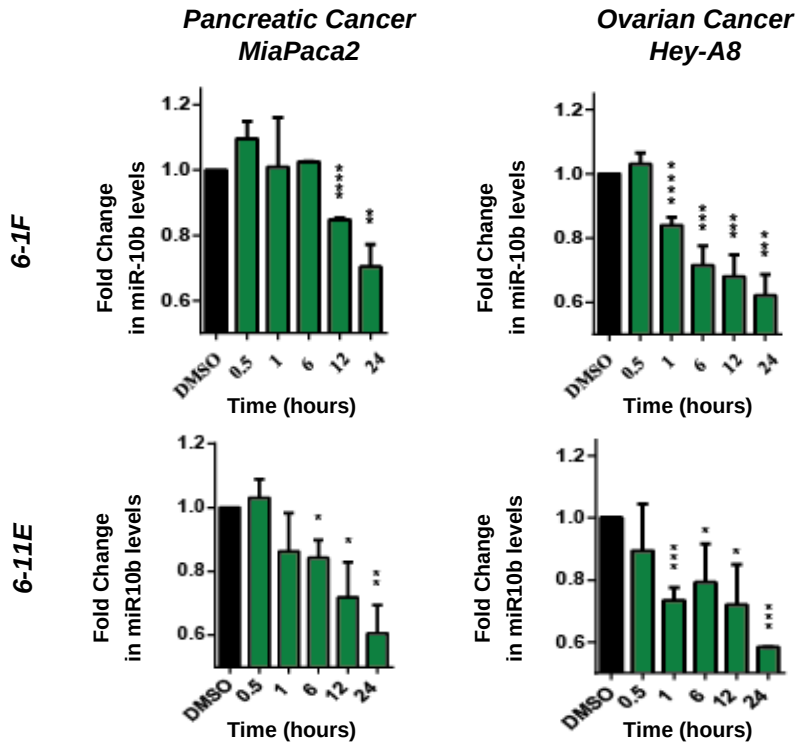


Figure 22: HSP90 inhibitors decrease the levels of miR-10b *in vitro* in pancreatic and ovarian cancer cell lines.

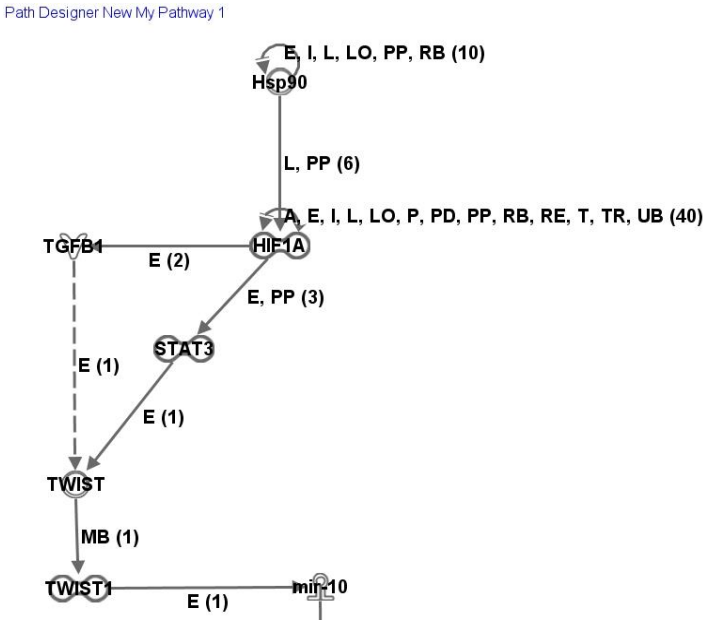


Figure 23: Ingenuity Pathway predictions of HSP90 regulating miR-10b transcription Dosage dependence and MTT assays reveal the inhibitory potency of the "specific hits", as well as their half maximal inhibitory concentration of cell death

Three of the compounds were confirmed as “positive-specific hits” in our screening: 1-6D-Bosutinib, 6-7C-Clomifene Citrate and 5-6A-Linifanib. For all of them we determined the potency of the miR-10b inhibition by performing a dosage dependence assay. Using the previously cloned psiCHECK2 vector, we tested five different concentrations (ranging from .1µM – 50µM), at two different time-points (24 and 48 hours). Two of the compounds 1-6D-Bosutinib and 6-7C-Clomifene Citrate, increased the luciferase expression 3 to 4 fold after 48 hours of incubation at concentrations as low as .1 and 1µM (Figure 24A and 24C). For both we determined the IC50’s after 24 hours, and 48 hours of treatment with the compounds in MCF-7 cells: 15.74µM, 8.3µM for compound 1-6D, and 15.2µM, 13.5µM for compound 6-7C respectively (Figure A and 25C). Similarly, we tested compound 5-6A and calculated the IC50’s in MCF7 cells, which were 42µM after 24 hours and 9.3µM after 48 hours (Figure 24B). Moreover, compound 5-6A-Linifanib increased the luciferase expression from 6-fold, at 24 hours, to up to 10-fold, after 48 hours of incubation at a standard dose of 10µM (Figure 25B).

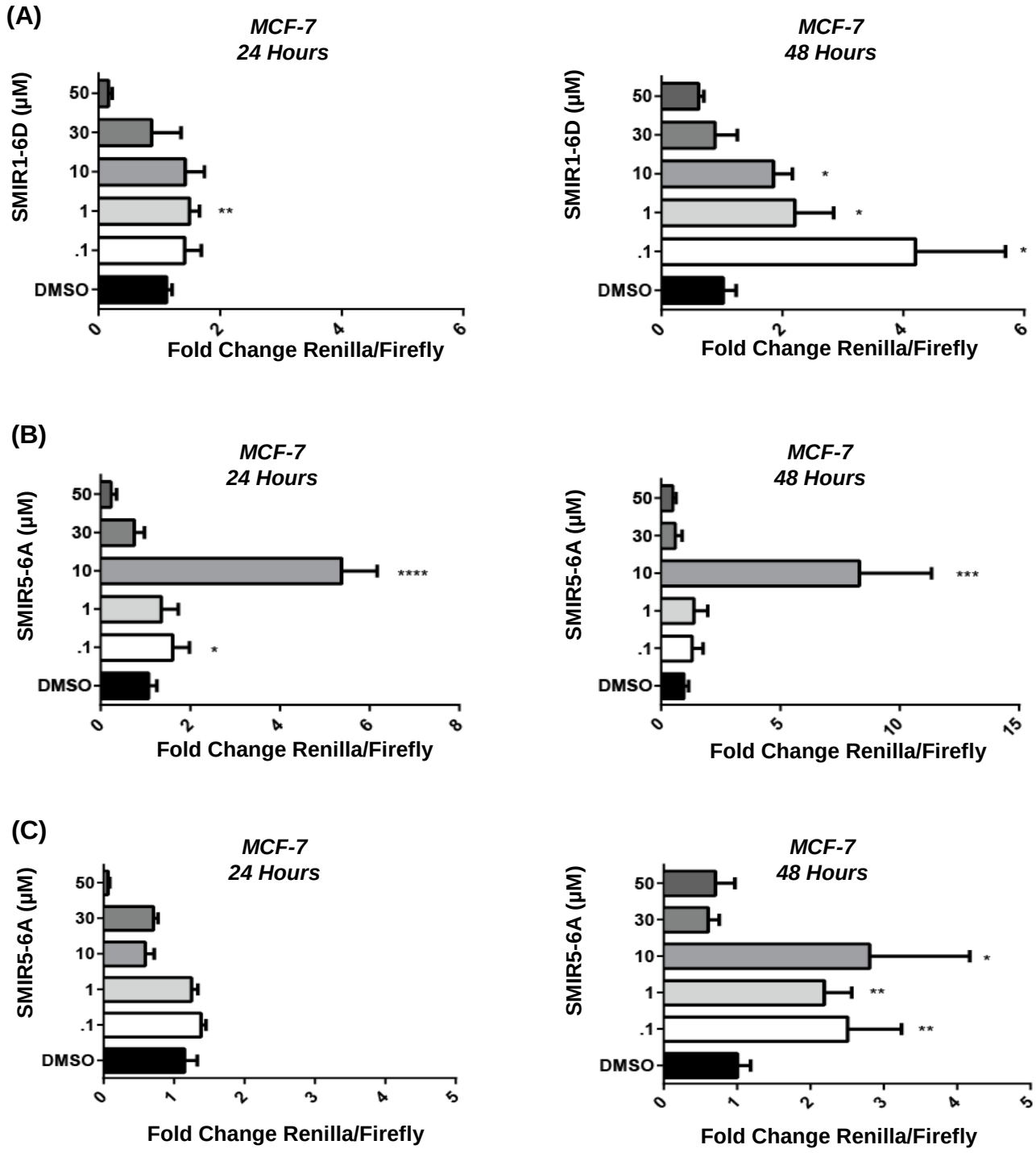


Figure 24: Dosage Dependence Assay

The three compounds from the group designated “specific hits” for miR-10b, were tested for a dosage dependence assay in MCF-7 cells. A total of 5 concentrations were used (ranging from .1 - 50 uM). The assay was performed at two different time-points: 24 and 48 hours. **(A)** SMIR 1-6D, **(B)** SMIR 5-6A and **(C)** SMIR 6-7C.

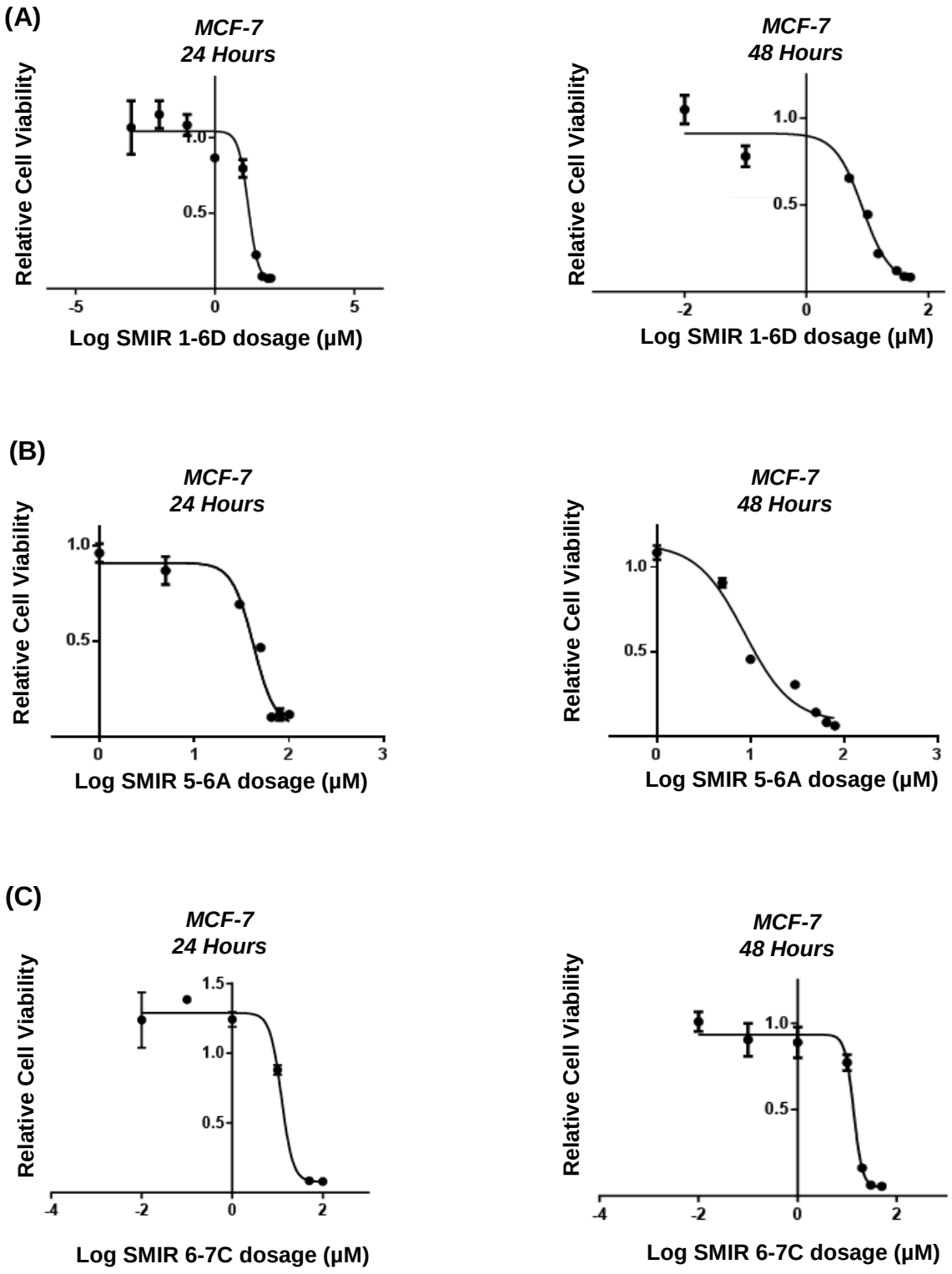


Figure 25: Determination of IC50's of "specific hits" through MTT Assay

Half maximal inhibitory concentration (for death) was calculated for all three compounds constituting the "non-specific group". Experiments were done in MCF-7 cells at 24 and 48 hour time-points. **(A)** SMIR 1-6D, **(B)** SMIR 5-6A and **(C)** SMIR 6-7C.

Nuclear magnetic resonance (NMR) analysis suggests small molecules 5-6A / Linifanib directly binds to miR-10b

We proceeded next to determine if the top 3 compounds were validated to target miR-10b using NMR. An RNA hairpin corresponding to pre-miR-10b was synthesized to screen for the RNA binding activity of the compounds by monitoring NMR chemical shift displacement and resonance broadening in the imino (NH) region of the RNA spectrum. Peaks in the NH region arise from base pairing and other stable base-mediated intra-molecular hydrogen bond interactions. DMSO at 11% was used as a co-solvent for the compounds and was determined to have minimal effect on the NMR spectra. Although the size of the RNA molecule and the presence of non-canonical features in the stem of pre-miR-10b limited the resolution of even the two-dimensional NH spectrum, no spectral changes could be observed for compounds 1-6D-Bosutinib and 6-7C-Clomifene Citrate (*data not shown*). However, compound 5-6A-Linifanib was found to cause small but reproducible chemical shift changes for peaks in the spectrum, suggesting a specific interaction between the molecule and the immature miRNA (Figure 26).

(A)

pre-miR-10b

```
cca      guaa     u        a    g     c          -ug   u
   gagguu    cguug cuauauau cccu uagaa cgaauuugug   gua c
   ||||||    ||||| |||||||| |||| ||||| ||||||||||   |||
   cuucaa    guagc gguauaua gggg aucuu gcuuagacac   uau c
--a      aaac     u        a    -     a          uga   g
```

(B)

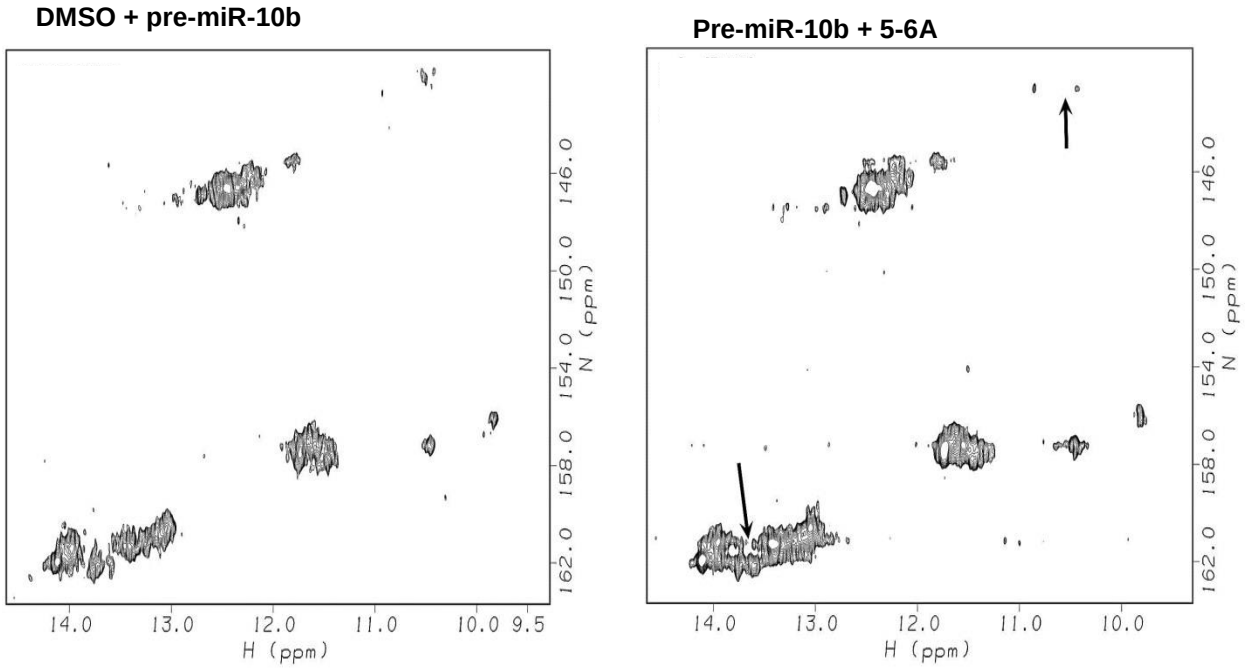


Figure 26: 2D NMR of pre-miR-10b and SMIR 5-6A / Linifanib

(A) Precursor sequence of miR-10b used for in vitro transcription. **(B)** 2D-NMR spectrum of pre-miR-10b RNA (synthesized in vitro) in 10% DMSO. This spectrum was the control. **(C)** 2D-NMR spectrum of pre-miR-10b RNA and SMIR 5-6A / Linifanib. Changes in the spectrum are pointed out in the arrows.

In order to narrow down the possible site(s) of interaction and to reduce spectral complexity, the stem of the pre-miR-10b RNA molecule was divided into three overlapping segments of approximately 50-58 nt each (designated I, II and III) (Figure 27) and analyzed individually (for a detailed description of the designed models, refer to Appendix-Figure 42). A UUCG tetraloop was used to terminate stem segments I and II and the UCCG tetraloop native to pre-miR-10b was used to terminate stem segment III. The NH (1D) spectra of stem segments I and II, which correspond to the base and central regions of the pre-miR-10b hairpin stem, respectively, were unchanged after addition of 5-6A-Linifanib, indicating the compound lacks affinity for these segments (*data not shown*). Peaks in the imino and downfield amino regions of the NMR spectrum of stem segment III, however, are altered by the presence of 5-6A, indicative of binding (Figure 29 – spectra on the left). Additional peak changes are observed in the base (C6H6/C8H8) region of the 2D ^{13}C-^{1}H HSQC (*Heteronuclear Single Quantum Coherence*) spectrum (Figure 28). The regions of sequence overlap between stem segments II and III and the peak perturbations in the NH and base spectra indicate that binding occurs near the loop end of the RNA molecule, which is adjacent to nucleotides that are part of the mature 5p and 3p forms of miR-10b *(*refer to Figure 27- RNA III*)*.

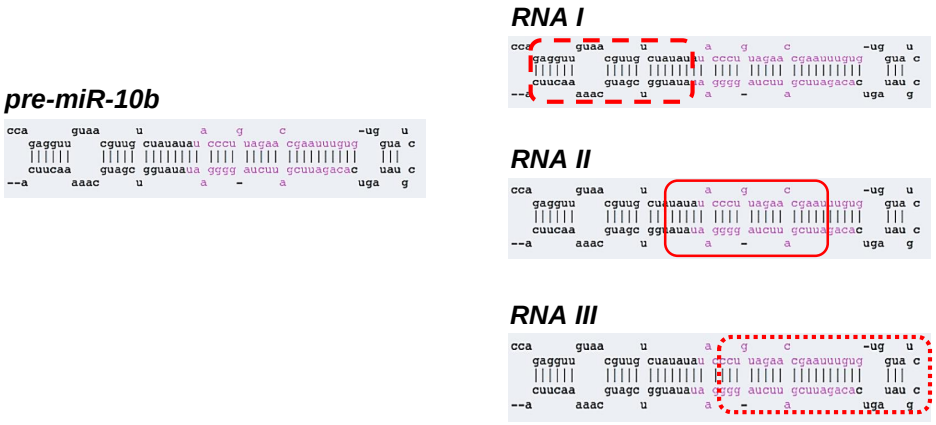


Figure 27: Design of oligonucleotide segments of pre-miR-10b

The nucleotide sequence of the pre-miR-10b (miRbase.org), along with the designed oligonucleotide sequences accounting for three separate regions of the precursor sequence (as indicated by the selected regions as RNAI, II and III.

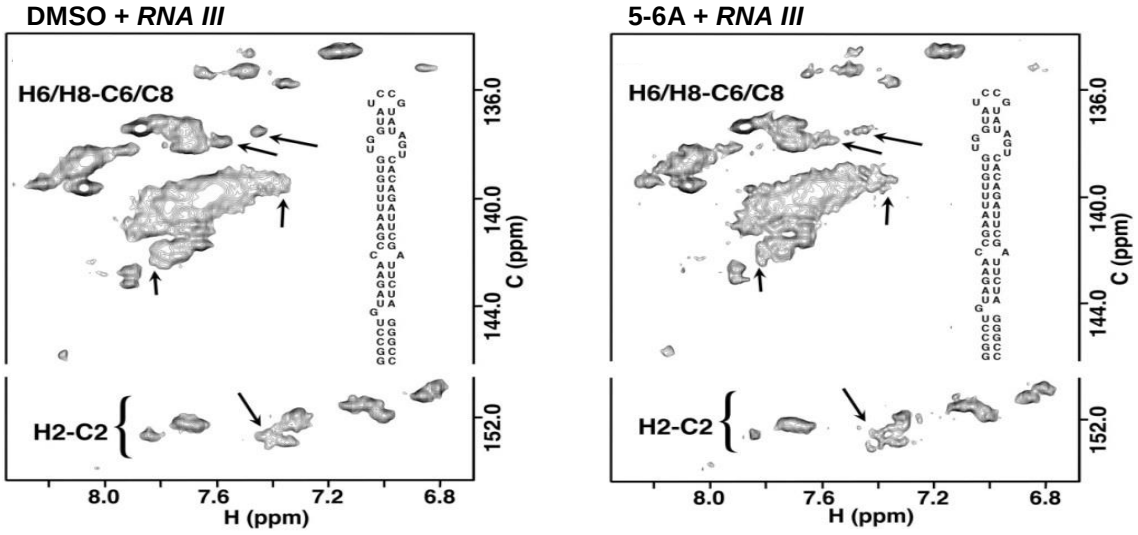


Figure 28: 2D-NMR spectrum of RNA III and 5-6A / Linifanib

Base regions of the ^{13}C-^{1}H HSQC spectra of RNA hairpin III in the absence (upper) and presence (lower) of 0.05 mM Linifanib/5-6A. The broad appearance of the peaks in the spectra is indicative of the dynamic nature of the molecule.

To further assess the specificity of this interaction, we designed a double mutant of the stem segment III: U50C, A62C. These substitutions extend the Watson-Crick secondary structure to the base of the UCCG tetraloop region. The 1D NH spectrum of this variant is unchanged by the addition of up to 65 μM 5-6A-Linifanib, indicating that the compound no longer binds the stem segment III (Figure 29- specta on the right). Overall, these experiments support a direct interaction between 5-6A-Linifanib with pre-miR-10b that localizes to the (UG/UGA) internal loop proximal to the cleavage site of Dicer.

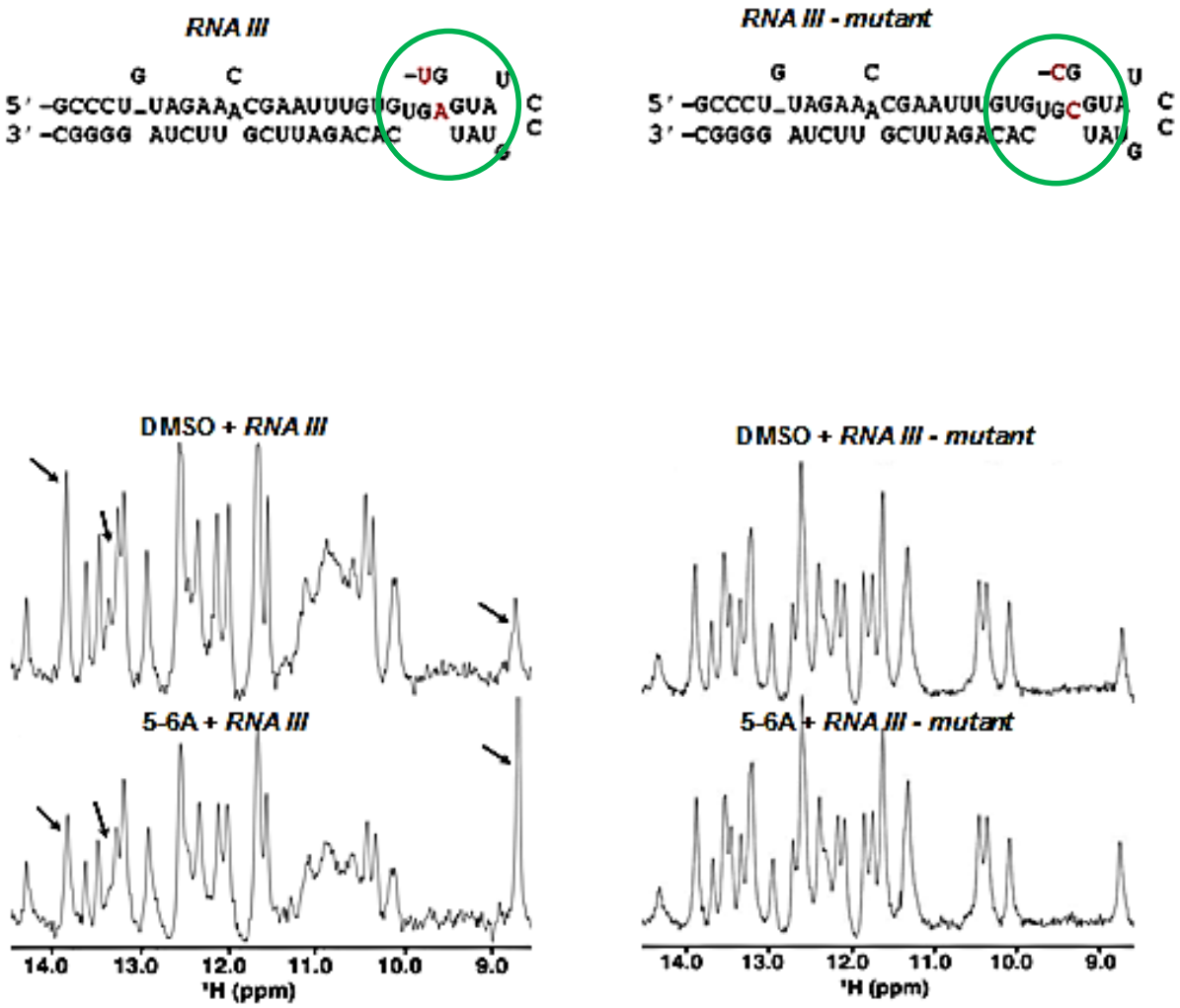


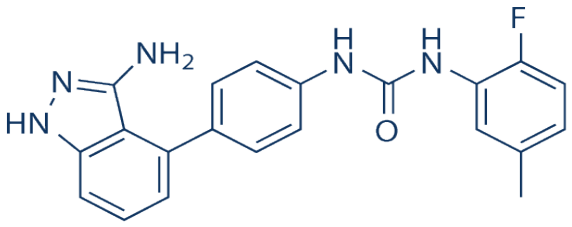
Figure 29: NMR (1D) with RNA segment III of the pre-mIR10b designed oligonucleotide

The detailed structures of RNAIII is presented (top left panel), with the base paris where the molecule interacts with it exalted (green circle). The muted version of RNAIIII is presented (top right panel), with the mutated based paris exalted (green circle). Imino ^{1}H spectra of stem segment III RNA hairpin in the absence (upper) and presence (lower) of 0.05 mM Linifanib/5-6A. Some of the peaks whose chemical shifts or intensities are altered are indicated. Imino ^{1}H spectra of a double mutant of the stem segment III RNA hairpin in the absence (upper) and presence (lower) of 0.05 mM Linifanib/5-6A. The spectra display no differences indicating Linifanib/5-6A does not interact with the mutant RNA molecule.

Computational model of the stem loop region of pre-miR-10b in complex with 5-6A/ Linafinib

With the known chemical structure of compound Linifanib/5-6a (Figure 30A), and a confirmed interaction to the stem loop region of miR-10b (through NMR), we generated an *in silico* model of Linifanib in complex with the UG:AGU internal loop (in stem segment III) using molecular docking. Figure 30B depicts the possible binding mode for 5-6A within the stem loop region of pre-miR-10b. Due to the non-canonical base pairing between G51 and G63, the loop region was predicted to bend ~30° with respect to the stem, and A62 formed a bulge which created an extra pocket for the urea phenyl group to bind. The 3-amino-indazol moiety, moreover, formed aromatic stacking with G51 base and the amine group interacted with U61 base through hydrogen bonding. Recognition of this unique conformation of UG:AGU internal loop is consistent with extended Watson-Crick pairing and loss of Linifanib binding in the U50C, A62C double mutant.

(A)



(B)

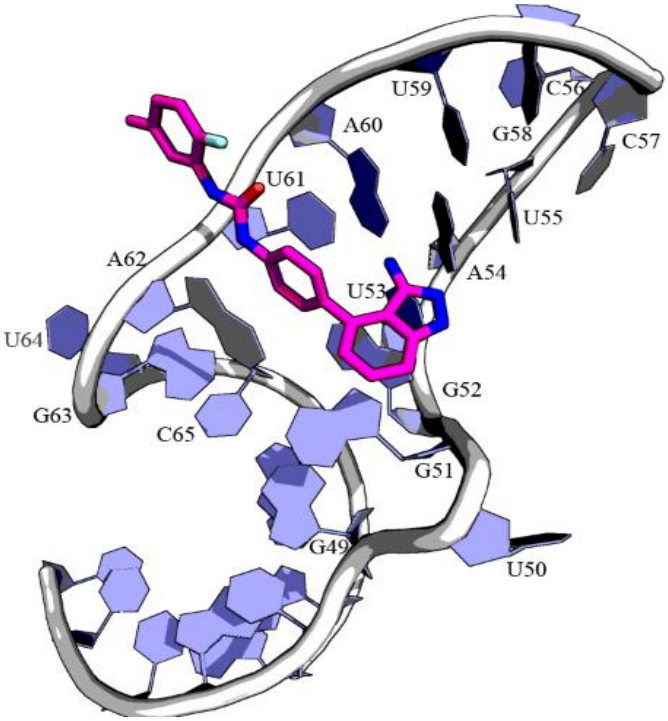


Figure 30: Predicted molecular docking model of 5-6A / Linifanib and miR-10b

(A Chemical Structure of Linifanib/5-6A. **(B)** Computational model of the stem loop region of pre-miR-10b in complex with Linafinib (using rDock). The majority of RNA-small molecule interactions occurs around UG:AGU internal loop, consistent to NMR data.

Linifanib /(5-6A) binds to the precursor sequence of miR-10b and inhibit its maturation process *in vitro*

To strengthen the biophysically detected interaction between Linifanib/(5-6A) and pre-miR-10b, we tested the levels of expression of pri-, pre-, and miR-10b mature form, in the RNA of Linifanib/(5-6A)-treated MCF7-cells. Indeed we found that the levels of the primary transcript were increased 2-3 fold in the cell model tested. However, the levels of mature miR-10b decreased significantly. When testing for the pre-miR-10b sequences, we were not able to detect it in our RNA samples by qRT-PCR (Figure 31).

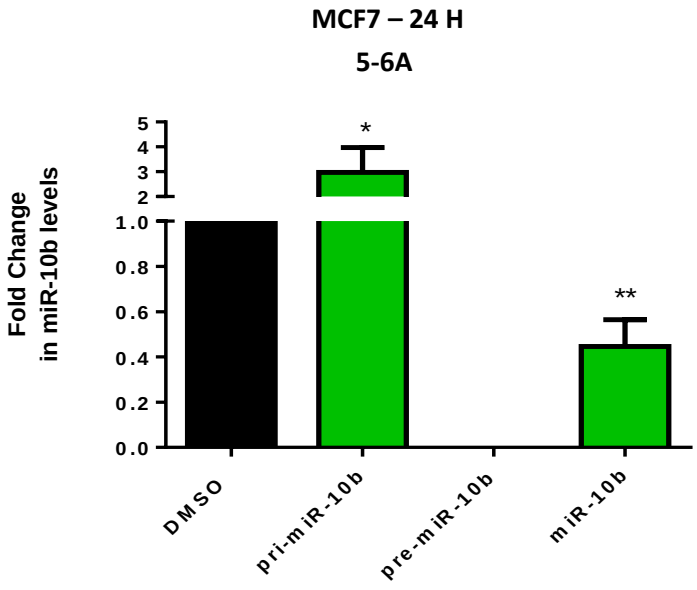


Figure 31: Assesment of levels of expression of pri-, pre- and mature-miR-10b transcripts
MCF7 cells treated with linifanib/5-6A reduce the levels of mature miR-10b sequences (as tested by qRT-PCR), but increase the levels of its primary transcripts. Pre-miR-10b sequences were undetectable by this method. These results suggest an interruption of the miR-10b maturation process.

Treatment with Linifanib/(5-6A) recovers levels of expression of HOXD10 by targeting miR-10b without affecting miRNA biogenesis

Additionally, cells were treated with Linifanib, and after 48 hours we verified the levels of the miR-10b targeted tumor suppressor protein HOXD10. Our results demonstrated a significant recovery of the HOXD10 levels (Figure 32A). Under the same conditions we tested for the levels of the two most important proteins required for miRNA biogenesis and maturation: Dicer and Drosha. With our experiments we demonstrated that treatment with Linifanib did not reduce the levels of any of these, suggesting miRNA biogenesis is not significantly altered (Figure 32B).

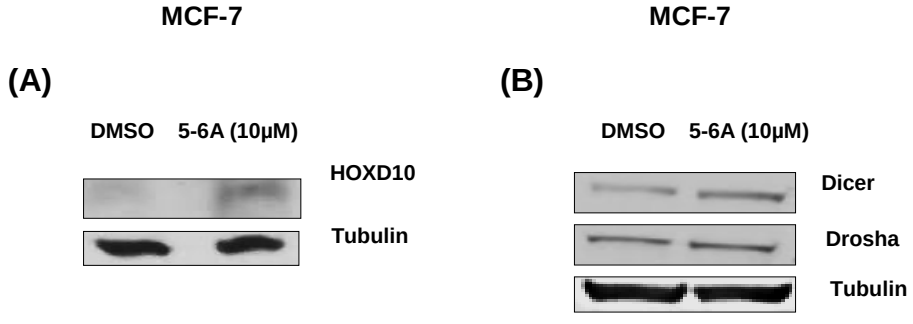


Figure 32: Assessment of protein levels after Linifanib treatment (10uM) in MCF7 cells.

(A) Linifanib treatment in MDA-MB-231 cells recovers tumor suppressor target HOXD10. **(B)** At 10 μM dosage, Linifanib does not decrease the levels of Dicer, nor Drosha.

Linifanib/(5-6A) decreases miR-10b levels in MCF7 overexpressing clones

We evaluated the potency of Linifanib in inhibiting the miR-10b in MCF7 clones overexpressing the precursor sequence of the miRNA. Once again we found that Linifanib was able to significantly decrease the levels of miR-10b, in conditions where the miRNA was overexpressed with a fold change of over 1000X.

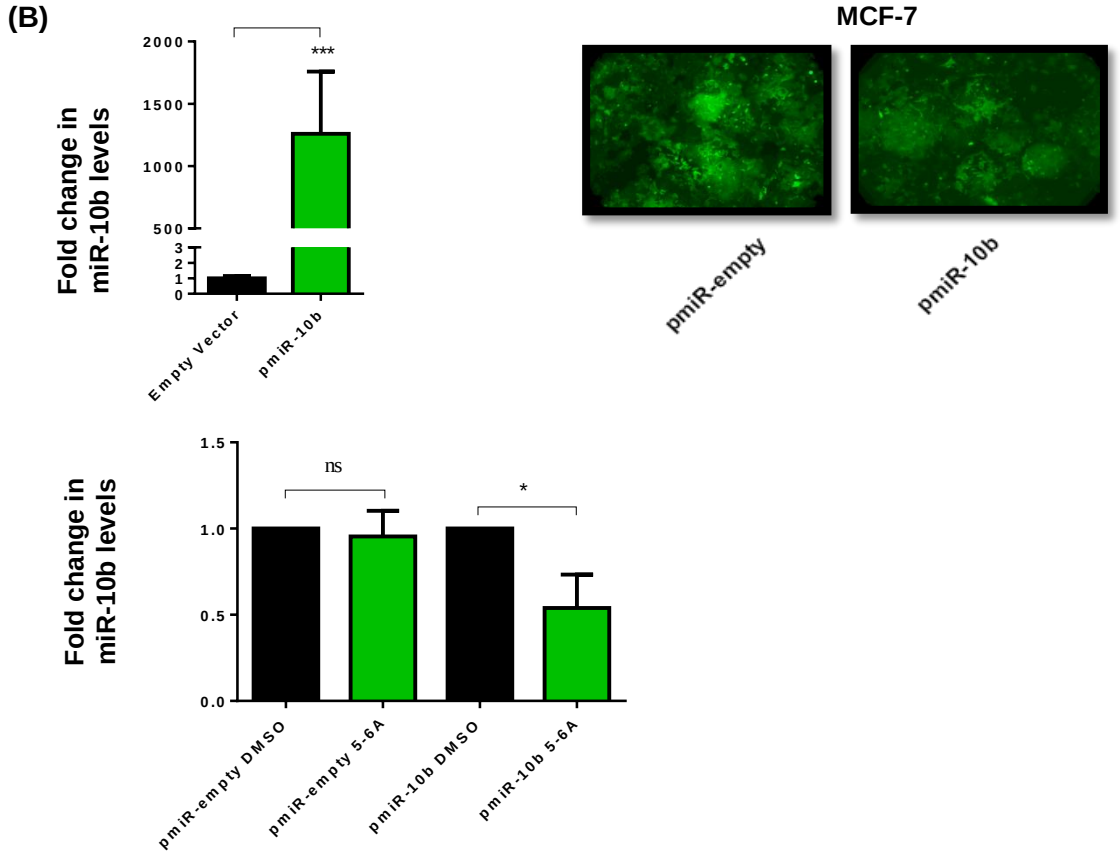


Figure 33: MCF7-miR-10b overexpressing clones

MCF7 cells were transfected with a vector containing a constitutively expressed promoter (labeled pmiR). Downstream of the promoter we cloned the DNA sequence codifying for pre-mIR-10b. An empty vector was transfected as a control. **(A)** Transfection efficiency was monitored through GFP. **(B)** To test the empty vector vs. pmiR-10b, we extracted RNA from the cells transfected with both, and performed qRT-PCR for miR-10b levels. **(C)** Linifanib treatment after 24 hours reduced miR-10b levels in MCF-7-miR-10b overexpressing clones.

Linifanib /(5-6A) binds to the precursor sequence of miR-10b and inhibit its oncogenic functions *in vitro*

We also tested for a reversal in the tumor-like phenotype that has been correlated by overexpression of *miR-10b.* BC cells with high basal levels of miR-10b were used to perform a wound healing assay. Alongside we compared Linifanib treatment with antagomiR-10b. Upon treatment with Linifanib/(5-6A) migration was reduced compared to solvent/DMSO, and the reduction was comparable to antagomiR-10b.

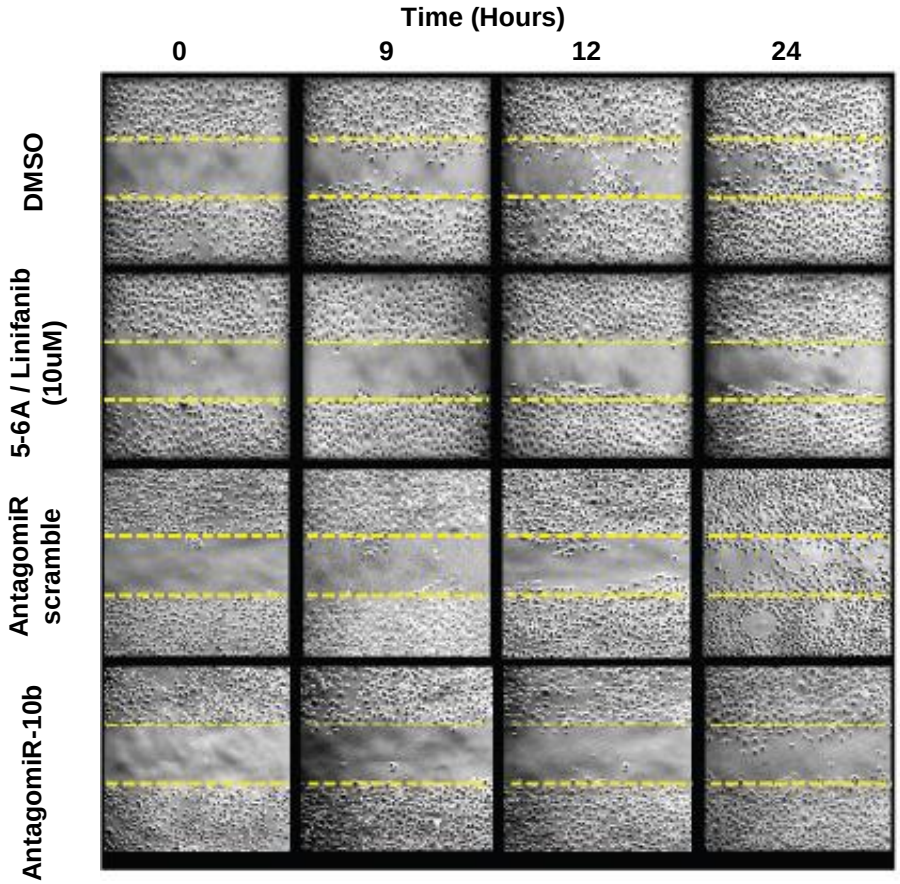


Figure 34: Wound healing assay demonstrates that Linifanib decreases migration

In MDA-MB-231 cells, treatment with Linifanib/(5-6A) reduced the migration capabilities (top panel). The inhibition of migration was comparable to cells transfected with antagomiR-10b (bottom panel).

Mir-10b has been demonstrated to affect migration and invasion. Nevertheless it has not been associated with influencing proliferation. Thus, we tested the effect of Linifanib/(5-6A) on proliferation in BC cells. We found that Linifanib strongly affects proliferation at approximately 63% after 24 hours of treatment. We assumed this mechanism was independent of the miR-10b inhibition, and for further functional assays where it was relevant, we normalized for proliferation.

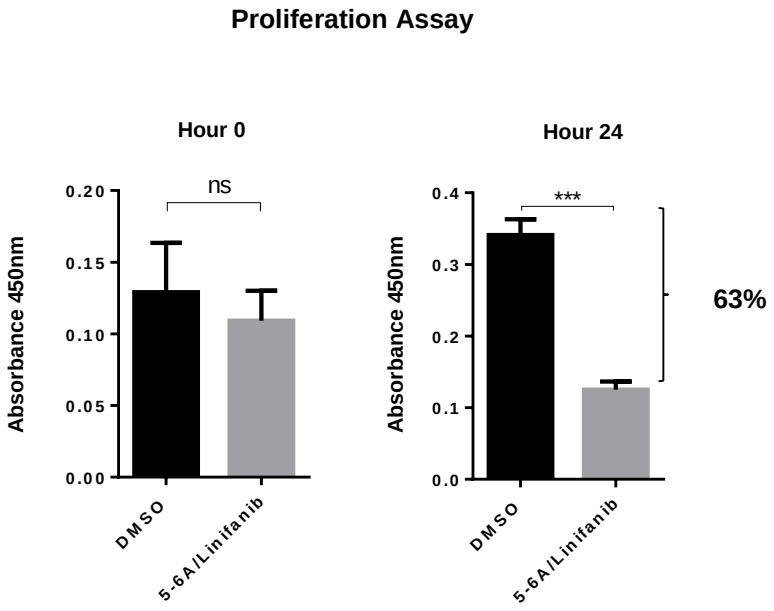


Figure 35: Linifanib inhibits proliferation in breast cancer cells

MDA-MB-231 cells treated with Linifanib/(5-6A) reduced their proliferation in 63% after 24 hours.

We then proceed to perform migration and invasion assay in BC cells with inserts coated with either gelatin or collagen. Cells treated with *Linifanib* markedly inhibited migration as well as invasion in BC cells. Our results were normalized for proliferation (by subtracting the 63% of the effect in cells treated with Linifanib), and still both (migration and invasion) were reduced significantly.

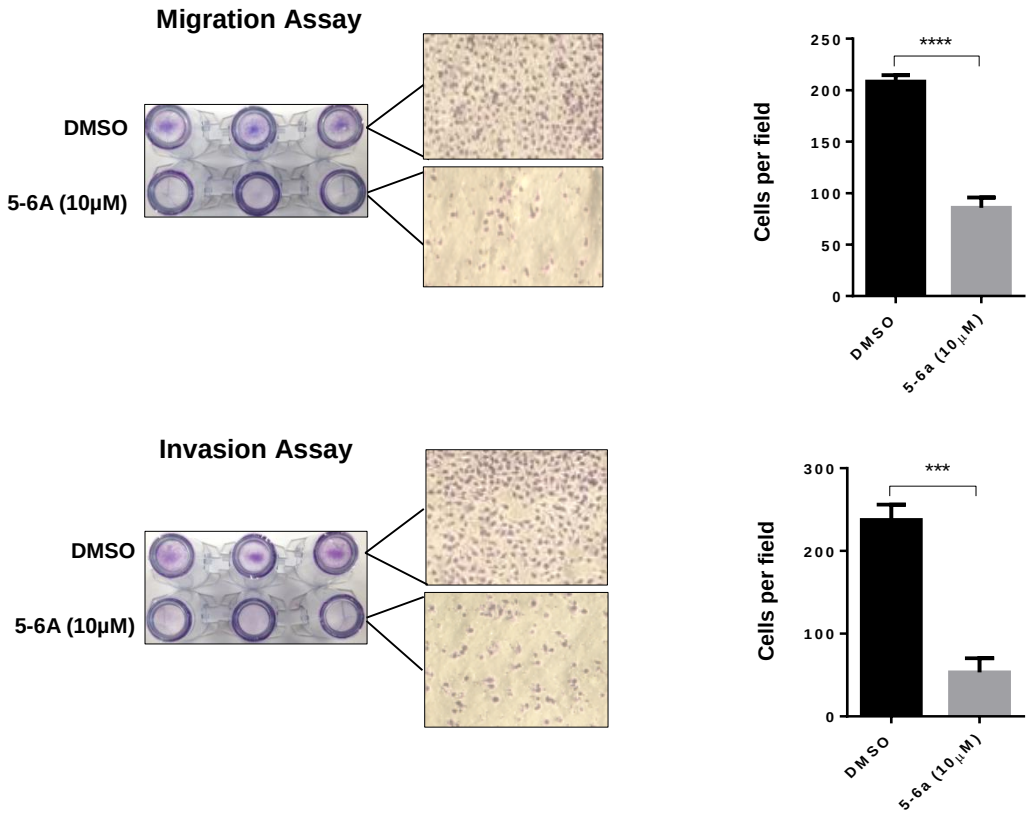


Figure 36: Linifanib/(5-6A) treatment decreases migration and invasion in BC cells

Linifanib/(5-6A) treatment decreases migration and invasion in MDA-MB-231 cells after 24 hours of treatment.

Linifanib decreases miR-10b levels in hepatocellular and colorectal cancer cells *in vitro*

We hypothesized that the inhibition of mIR-10b would be consistent among different cancer cell lines. In this regard, we opted to test cell lines of lung, liver and colorectal cancers. We selected these cancer types in particular because Linifanib was used in clinical trials for all of them. After a 24 hour treatment, we observed that the reduction in miR-10b levels was consistent among different cancer types such as: non-small cell lung cancer (NSCLC), HCC, and CRC (Figure 33).

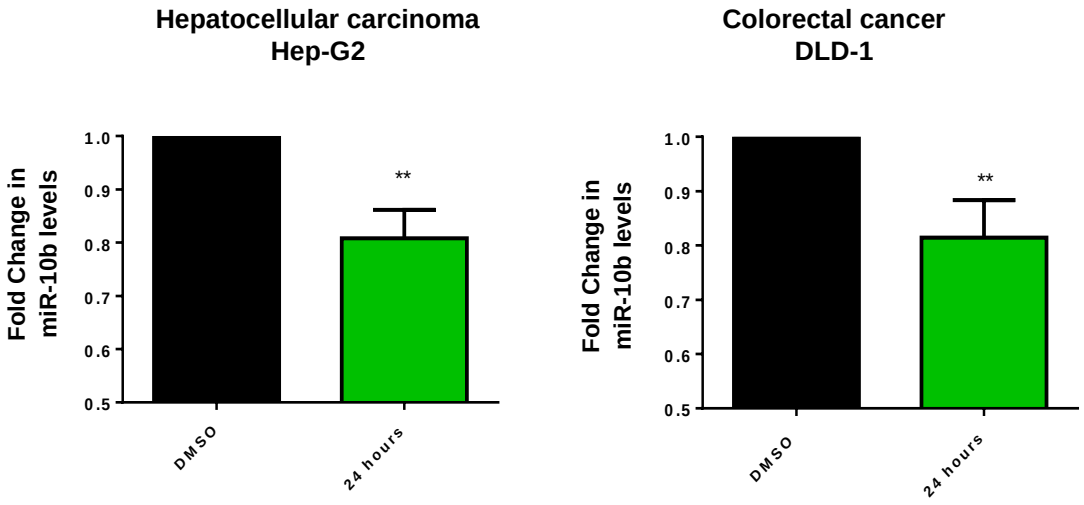


Figure 37: Linifanib decreases miR-10b levels in HCC and CRC cells in vitro

CHAPTER IV: Discussion

Summary

Breast cancer is the second most common cancer in the world (following lung cancer), and the leading cause of cancer death in women [162]. Its burden exceeds all other cancers and the incidence rates are consistently increasing [163]. One of the main reason for this is the fact that the heterogeneity of BC tumors makes them a challenging one to treat [164].

Regarding their molecular characterization, hormonal therapy has improved the outcome of patients with ER/PR positive tumors [165]. Herceptin has also significantly influenced treatment response and overall survival in patients with HER2 positive tumors [166]. However, taken together, these parameters have not achieved the realization of an extremely effective individualized targeted therapy for patients, and resistance to therapy continues to be frequently encountered in the clinic [12, 167, 168]. Furthermore, molecular subtypes such as TNBC tumors are not efficiently treated with the approaches above mentioned, and the prognosis of these patients is significantly low [169, 170]. Hence, new systemic therapies are desperately needed, and the study and development of personalized cancer therapy, represents a strategic approach in aiming to overcome therapeutic inefficiency and/or resistance [171].

MiRNAs are a type of non-coding RNA transcript that can regulate oncogenic processes, and thus, they are considered crucial elements in fields such as: “personalized cancer therapeutics”. MiRNAs-based research throughout the years has emphasized that specific miRNAs are up- or down-regulated, concurrently, in cancer [73]. Their dysregulation, results in the activation/inactivation of oncogenes or tumor suppressor

genes respectively [73]. Therefore, from a therapeutic point of view, miRNA targeting possesses anti-cancer potential, based on the fact that the cancer phenotype can be changed by targeting miRNA expression. Furthermore, miRNA signatures and profiles in BC have been thoroughly studied in the past, and strong evidence has already been established regarding their therapeutic potential [3, 172, 173]. In this regard, the development of a therapy to target specific oncogenic mIRNAs represents a significantly important approach for new treatment options.

In this study, we decided to focus on testing small molecules that have undergone clinical trials or that are already FDA approved (with adequate pharmacokinetic/dynamic properties for in-human delivery), to target microRNAs. We hypothesized that already-available small molecules could bind specific RNA motifs and inhibit the function of oncogenic miRNAs. If proven effective, this would expand their applicability in the clinic, greatly accelerating the lengthy process of drug discovery [100]. In our approach, we combined techniques such as bioinformatics, non-coding RNA technologies, chemical compound library screening and structural chemistry, with the aim of identifying oncogenic miRNAs to target in BC. We also designed and validated a step-wise approach that can be used to discover small molecule compounds that directly bind oncogenic miRNAs and inhibit their functions *in vitro*.

The key finding from this study is that Linifanib, the receptor tyrosine kinase inhibitor, directly interacts with the precursor sequence of miR-10b (a known oncogenic miRNA in BC). More so, we found that the interaction of Linifanib with the miRNA results in the recovery of tumor suppressor proteins (e.g. *HOXD10*) and can reverse the cancer phenotype *in vitro*.

Overall, we have identified a number of assays with high sensitivity and specificity, which allow an adequate identification of molecules that can target oncomiRs. Our techniques are reproducible and applicable to a broad range of other types of cancer, or to any other pathology that involves miRNA overexpression.

Small molecule compounds targeting miRNAs

Compared to the currently known treatment regimens, miRNA-based therapeutics offers the advantage of targeting multiple genes with different roles within a cancer network. Previous strategies have involved the use of oligonucleotides or viral-based constructs; however, early clinical trials have showed that their delivery can be ineffective or toxic [100]. Recent focus regarding miRNA-based therapeutics has centered on the possibility of using small molecule inhibitors to target miRNAs directly. Nevertheless, to date, very few studies have successfully been able to find small molecules to target oncomirs in an effective manner. Herein we review the most important findings regarding small molecule compounds targeting miRNAs.

The first screening assay to detect small molecules targeting miRNA processing was done by Davies and colleagues in 2006. Using pre-let-7 RNA from *Drosophila melanogaster*, they developed a doubly-labeled pre-miRNA beacon with a fluorescent emitter and quencher able to detect Dicer processing (hydrolysis) through fluorescence emission. In this way, they established the first homogeneous fluorescence assay to detect miRNA inhibitors [106, 107]. However, their main focus was to find molecules that targeted the miRNA biogenesis process (in a non-specific way).

In 2008, a study done by Gumireddy and colleagues; the group claimed to have found an inhibitor of a miRNA [104]. They selected miR-21 as the target miRNA because of its known tumorigenic properties in cancer [38, 69, 174, 175]. They then developed a lentiviral reporter construct with the miR-21 target sequence, downstream of a luciferase reporter gene. Diazobenzene, was found as positive hit in the study; however a thorough analysis revealed that the compound actually targeted the transcription of the miR21-coding gene [104]. In another similar study, Young and colleagues developed a project

focused on targeting miR-122, a miRNA crucial for hepatitis C virus (HCV) infection and replication [176]. A luciferase based psiCHECK-2 reporter plasmid was used to screen for SMIRs [176], and 2 hits were identified. Nevertheless, both of the compounds targeted the transcription of the miRNA-coding gene upon treatment (againproving to have an indirect mechanism of inhibition) [176].

Following this, Watashi and colleagues developed a very extensive screening for SMIRs that could target oncomiRs -93 and -130b [177]. Collectively their results suggested that one of their compounds reduced pre-miRNA association with Dicer, while the other reduced the miRNA association with AGO2 (enzymes important for miRNA biogenesis) [90,[177] . Even though both treatments demonstrated to reverse the cancer phenotype *in vitro*, their mechanisms of action were indirect ones [177]. In a similar approach, Bose et.al; screened for SMIRs against miR-21, using a luciferase-based reporter was used (pEZXMT01 plasmid) with the complementary 3'UTR sequence [108]. In the screening, Streptomycin was found as a hit inhibitor in a BC model *in vitro,* by interfering with the Dicer processing of the miRNA [108]. However, when testing the levels of additional miRNAs to determine the specificity of the inhibition, out of a total of 10 tested, miR-27a was proven to be downregulated as well [108].

The idea of targeting miRNAs with small moelcules continued expanding in 2013 when Bose and colleagues used a fluorescent probe (Refer to Figure 7A) to screen a total of 14 compounds, and test if any of them inhibited miR-27a, a miRNA proven to be over-expressed in several cancer types [103]. They found that a set of them inhibited Dicer-catalyzed miR-27a maturation [103]. Their high-throughput beacon-based assay compared favorably to other previously described methods, as it proved to be cost-effective, sensitive and robust. Unfortunately their results were validated *in vitro* only in 1

cell model (which doesn't rule out that the compound-mediated inhibition is cell-specific manner)

With the aims of achieving SMIR against miR-21 with a higher specificity, Shi and colleagues designed an *in silico* technique [101]. By using the three-dimensional structure of the Dicer binding site on the pre-miR-21, they conducted an *in silico* screening of 1990 compounds to find molecules that could block miR-21 maturation using MC-Fold/MC-Sym and Auto Dock programs [101]. Through this method, they identified a SMIR termed AC1MMYR2, which blocked the ability of Dicer to process the pre-miR-21. Its potency was tested and validated *in vitro* [101]. More so, the compound demonstrated a decrease in tumor growth, invasiveness, and metastasis, thereby increasing overall host survival *in vivo* [101]. Their approach was very successful in the fact that they actually managed to thoroughly evidence a reversal of the cancer phenotype. Nevertheless, 5/11 alternative miRNAs tested, were also significantly reduced upon treatment [101]. More so, they did not perform assays to demonstrate a direct interaction between the structure of the molecule and the pre-miR-21.

On another note, two important miRNAs in gastric cancer: miR-372 and miR-373, were also the focus of investigation when searching for SMIR candidates. In their screening, Vo and colleagues reported the first example of multimodal RNA ligands aiming to inhibit the biogenesis of oncomiRs [178]. The multimodal ligands were composed of an artificial nucleobase design that recognizes the pre-miRNA, and an aminoglycoside known to interact in a potent manner with stem-loop RNAs (with high affinity) [178]. Some of their multimodal ligands inhibited Dicer precursor cleavage by binding to these stem-loop structured RNAs [178]. More so, one of their hits proved to be an efficient miR-372 and -373 inhibitor *in vitro*, demonstrating a dose dependent decrease of the miRNAs [178].

Their approach demonstrated to be successful for targeting these particular oncomiRs; however, they identified a significant decrease in the levels of at least 3 other oncogenic miRNAs as well, upon treatment.

Recently, a research group developed “Inforna”, a novel approach that was validated to design lead compunds that target miRNA precursors previously identified through sequencing and functional studies [179]. Initially they downloaded the sequences of all the miRNA hairpin precursors in the human transcriptome, and subsequently, their secondary structures were predicted *in silico*. Inforna created the output of the targetable motifs in each RNA, and the corresponding lead small molecules that could potentially bind them [179]. Their results showed a strong interaction between benzimidazole and the precursor sequence for miR-96 [179]. Unfortunately these results were only validated *in vitro* in a single cell line model.

Finally, Murata and colleagues studied the synthesis and structure–activity relationships of xanthone and thioxanthone derivatives as fluorescent indicators of interactions between small molecules and RNA sequences [180]. In their study they demonstrated that the xanthone and thioxanthone derivative X2S-N, N-diMe inhibited premiR-29a maturation in a specific manner by binding to the nucleotides that form part of the internal loop/bulge (but not by any interaction with the processing enzyme Dicer) [180]. However, the off target effect of this compound (inhibition of other miRNAs) was not investigated [180].

Current challenges

Compared to proteins, miRNAs (and all RNA molecules in general) have been quite neglected as promising drug targets. This is mostly due to the absence of defined molecular structures for the majority miRNAs; a fact that increases the difficulty of predicting which inhibitors can bind them. However, the truth of the matter is that miRNAs are indeed “druggable”, due to the formation of stem loop in precursor sequences and bulges in miRNAs [88]. Their structural features not only enlarge the major groove for drug entry, but also partially disclose the internal bases, scattering the local electronegative distribution [88]. These advantages suggest that it would be feasible to target them with small molecules.

Only a handful of studies have been published over the last 10 years, regarding small molecules targeting miRNAs. Although advancements in screening techniques and validation of targeted hits have been seen, there are still weaknesses in the studies performed, and thus several challenges to overcome. In view of this, we aimed to identify the most important ones, to determine what changes could be implemented when screening for SMIRs. Altogether, our primary endpoint was to find drugs that could be specific by targeting oncomiRs directly; and as the initial step when doing so, we determined to address several flaws (which will briefly be discussed).

Firstly, we determined that it is important to test if our compounds cause any alterations of the miRNA biogenesis pathway machinery. The reason for this is because when the concept of targeting miRNAs with small molecules began, one of the ideas was targeting their maturation process in cells; and several research groups performed screenings focusing on finding inhibitors of the miRNA biogenesis pathway. However, this approach proved inefficient, due to the fact that by inhibiting miRNA

biogenesis/maturation, the expression of all miRNAs was targeted / affected. MiRNAs crucial for cellular homeostasis could be altered in this manner, not to mention many tumor suppressor miRNAs would be affected as well. Thus, we determined it was crucial to test if the small molecule treatments have an effect in elements (such as enzymes like Dicer, Drosha) that are required for the processing of miRNAs (their maturation process).

Secondly, but on the same line, we believe that it is very important to determine if treatment with the small molecules affects the transcription of the miRNA-coding gene (of our miRNA of interest). This could be done by testing changes in methylation of promoter sequences of the specific miRNA-coding gene; or more easily, by verifying the levels of primary and precursor sequences (along with the mature miRNA itself). The later because when small molecules target transcription, the levels of all three miRNA transcripts are decreased upon treatment.

On a separate note (thirdly), the levels of additional miRNAs should be tested under the same conditions done in which the screenings are performed. Unfortunately in several of the studies done to date, the levels of additional miRNAs were not tested after treatment, a fact that doesn't allow a "prediction" of the degrees of specificity of the inhibition. More so, those that claim to do so "randomly" select miRNAs to test in addition to their target one, but their selections include miRNAs that are not considered oncogenic, or that have even been validated to be tumor suppressors (not oncomiRs that might have similar functions in cancer). Additionally (fourthly), *in vitro* models should include at least two different cell lines with different properties, in order to demonstrate that the small molecule inhibition is not cell-specific, but miRNA-specific.

Finally (fifthly), one of the most significant weaknesses of the SMIR studies to date is that none of them have demonstrated enough evidence describing a direct interaction

between a small molecule and the miRNA of interest. The closest to it, was when a research group demonstrated through an *in silico*-predicted docking model, that the small molecule AC1MMYR2 bound to the pre-miR-21 and blocked its maturation [101]. However, their models were not validated through techniques such as nuclear magnetic resonance or mass spectroscopy; and thus there is a level of uncertainty regarding the interaction itself. This uncertainty arises due to the fact that miRNAs (the majority) are not available as a crystal structure, and thus the 3D computerized models actually use a prediction of their crystal structure when searching for candidates molecules that can target them. In other words, they are actually developing a model that is based on: "a prediction of a prediction". Because of this, very few molecules identified through computerized programs as: "likely to bind a specific miRNA", have an actual interaction that is able to be validated *in vitro* an *in vivo*. From this perspective, the biggest challenge is not quite to find a compound that evidently inhibits an oncogenic miRNA, but more so proving that it does so through a direct interaction.

To this extent, we believe that a specific SMIR:miRNA interaction could be thoroughly evidenced by addressing all of the mentioned criteria. In summary, these criteria include proving that the small molecule does not alter the following: miRNA biogenesis /processing, the levels of expression of additional oncogenic miRNAs, or the transcription of the specific miRNA-coding gene. To achieve an even more precise SMIR:miRNA interaction, results should be validated in at least two cellular models (with different characteristics. Additionally, it is crucial to demonstrate a direct interaction through structural analysis such as crystallography or nuclear magnetic resonance.

Small molecule screening for SMIRs targeting miR-10b or miR-21

Chemical compound libraries offer the advantage of allowing screenings of known therapeutic agents, the majority of which have been tested in pre-clinical and clinical trials, some even approved by the FDA for different diseases. As a result of screening libraries of chemical compounds, new potent inhibitors have been discovered for and array of diseases such as infections, neurological disorders and cancer [181-183]. In this study, as proof of concept, we used approximately 450 compounds from a chemical library of known small molecule inhibitors to test their ability to interact with, and inhibit oncomir-10b or -21. The results of our screening demonstrated that using a combination of assays with high specificity and sensitivity, we were able to find a small molecule that directly interacts with, and inhibits an oncogenic miRNA (miR-10b).

Role of heat shock protein 90 in regulating the transcription of miR-10b

As part of our screening design, we performed a series of assays to test if the small molecules where specific on their miRNA inhibition. As a result, we eliminated compounds that altered the levels of expression of any of the oncogenic miRNAs tested after treatment (besides the targeted one). Interestingly we found a group of compounds belonging to the same family of inhibitors: *inhibitors of heat-shock protein 90.* Even though these inhibitors proved to downregulate two or more miRNAs in a non-specific manner, they demonstrated to inhibit miR-10b very potently (even though they had very different molecular structures). More so, they demonstrated to decrease the levels of all three: primary, precursor and mature miR-10b transcript.

With the aims of establishing a link between HSP-90 inhibitors and decrease in miR-10b levels, we used Ingenuity Pathway Analysis, (IPA), to predict the correlation between HSP90 inhibitors and miR-10b. In our prediction we found that HSP90 enhances

the activity of HIF1-alpha, which promotes both TGFB4 and STAT3. Furthermore, TGFB4 and STAT3 were both predicted to enhance TWIST, a transcription factor known to increase the expression of miR-10b (Figure 23).

In the past, several studies had already proven to link several of these relationships, although (to our knowledge), no one has validated the complete pathway from HSP90 to miR-10b. For example, for years a direct correlation between HSP90 protein and HIF1-alpha has been known. A research group in 1999, demonstrated thoroughly for the first time, that Hsp90 activity is essential for HIF-1 activation, by demonstrating that using the HSP90 inhibitor geldanamycin, they achieved potent inhibition of HIF-1alpha [184]. Since then, several research groups have validated the interaction between both proteins extensively [185, 186].

Regarding elements further down the pathway, it has been demonstrated that TGFβ triggers SMAD-dependent induction of 2 important transcription factors: SNAIL1 and TWIST [187]. Additionally, several studies have indicated that STAT3 transcriptionally induces *Twist*, which plays an important role in promoting migration, invasion, and anchorage-independent growth [188, 189]. Finally, the transcription factor TWIST has been thoroughly demonstrated to increase the transcription of miR-10b, which is also predicted in our IPA model (refer to Figure 23) [63, 64] .

Unfortunately, from the correlations of our IPA-predicted pathway there have not been any studies that validate hif1-alpha as a regulator of TGB4 or STAT3. Interestingly STAT3 has indeed been validated to regulate HIF1alpha expression [190, 191]. Thus, the majority of the predictions from the IPA model have been individually proven in the past; which strongly suggest the pathway has a high probability of being validated altogether. The

confirmation of these predictions could lead to alternative pathways to target in diseases where miR-10b is an overexpressed miRNA contributing to pathology.

Small molecule Linifanib targets miR-10b through a direct interaction

In our screening, we identified 5-6A-Linifanib as a specific SMIR which blocks miR-10b processing by binding to the precursor sequence near the stem loop region. More so, we validated the direct interaction thoroughly using NMR techniques to assess the molecular dynamics of the small molecule in the presence of pre-miR-10b.

Previous studies

Linifanib is a novel potent inhibitor with selectivity for the VEGFR and PDGFR family of receptor tyrosine kinases (RTKs) [63, 64, 192]. In nonclinical *in vivo* studies, Linifanib demonstrated potent inhibition of tumor growth in xenograft models of over 15 different tumor types [75]. In preclinical studies, it was also proven to have antitumor activity as a single agent or in combination with known chemotherapies [77] . To date, Linifanib has proven to be clinically active in patients with an acceptable safety profile; therefore, a total of 18 clinical trials have been initiated in diseases including solid tumors such as BC [193], CRC, non-small cell lung cancer (NSCLC) [194], renal cell carcinoma (RCC) [195], but to a more significant extent in HCC [77]. Specifically, in a phase 1 clinical trial, it demonstrated potent antitumor effects as a single-agent in patients with refractory solid malignancies [196]. More so, Linifanib has also shown antitumor activity in phase 2 studies in patients with NSCLC, HCC or RCC [194, 195].

The most significant studies in which Linifanib has been used as a therapeutic agent have been performed in HCC. Remarkable results have been achieved (several years ago) in both: nonclinical, and preclinical trials; and thus, it became evident that linifanib could be considered a potential new threatment for patients HCC [75, 77]. In order to do so, linifanib was compared with the standard of care treatment for patients with HCC: the multitargeted tyrosine kinase inhibitor sorafenib, which blocks the activity of Raf serine/threonine kinase isoforms, VEGFR-2 and -3, PDGFR β, c-KIT, FLT-3, and RET. By doing so, sorafenib inhibits tumor angiogenesis and cell proliferation [26, 27, 197-201]. It was chosen as standard of care for patients with advanced HCC based on results from 2 large randomized trials, both which showed an improvement in overall survival (OS) compared to placebo [200].

In order to compare their efficiencies, both inhibitors were compared in (2014), in a randomized phase 3 trial in patients with advanced HCC [200]. The primary endpoint of the trial was comparing OS between treatments of linifanib vs. sorafenib. The results of the trial demonstrated that there was a slight difference in the median OS of patients treated with Linifanib vs Sorafenib, of 9.1 months and 9.8 months respectively [200]. However these results were not statistically significant. More so, regarding the secondary outpoints of the study: the time to progression (TTP) of patients receiving linifanib were significantly longer (P=.001), and the objective response rate (ORR) of these patients was also significantly higher (P=0.018) [200]. Furthermore the response rates (RR) for linifanib compared favorably to previous phase 3 trials of sorafenib in advanced HCC (13% vs 6.9%) [198, 199]. Nevertheless, improvements in all these did not translate into increase in OS. Thus, the study failed to achieve its primary endpoint, and sorafenib continues to be the treatment of choice for these patients.

New mechanistic role of Linifanib

In our screening we observed and validated a new mechanistic role for Linifanib: the direct inhibition of miR-10b. In BC models we observed that Linifanib decreased the levels of miR-10b, and the sensitivity of the inhibition was consistent among three different cell line models, suggesting that the effect was independent of the molecular markers/histological tumor subtypes. By restoring the levels of the tumor suppressor target HOXD10, Linifanib decreased invasion and migration of BC cells. Since previous studies have demonstrated both *in vitro* and *in vivo,* that silencing of miR-10b markedly suppresses formation of BC metastasis [47, 63], linifanib represents an attractive agent to target BC disease progression (and spread) by inhibiting, the “oncmiR-10b addiction” of these tumor types.

The fact that linifanib physically interacts with, and inhibits miR-10b, opens up a window of therapeutic opportunities, not only for BC, but for other cancer types as well. As previously described, linifanib treatment has been tested (and is currently being tested) in several clinical trials, of cancer types such including CRC, NSCLC and HCC. Interestingly, all of these cancer types have been proven to worsen upon increased miR-10b expression. For example, high level miR-10b expression was found to be significantly associated with high incidence of lymphatic invasion and poor prognosis in CRC patients and has been shown to confer resistance to chemotherapeutic agents *in vitro* [202]. Furthermore, in NSCLC miR-10b expression levels are significantly positively correlated with the tumor stage and regional lymph node involvement, and patients with higher levels of miR-10b have significantly poorer survival compared to those with lower expression of this miRNA [203, 204]. More importantly, miR-10b is highly expressed in metastatic HCC tissues inducing invasion and migration; and patients with higher miR-10b expression have significantly poorer OS [137, 205].

The evidence of miR-10b having a crucial role in cancer types for which linifanib has been explored as a possible treatment option, suggests that there might be a link between overexpression of miR-10b and linifanib treatment inefficiency. Supporting this are the results of the phase 3 randomized clinical trial evaluating linifanib treatment in patients with advanced HCC. Results demonstrate that initially (first 3/4s of the treatment period) linifanib proofs more effective than the standard of care (with a higher progression free probability) compared to the standard treatment (sorafenib). Additionally, OS seems to be equal between both treatments during the initial 3/4s of the treatment period, and only separates from the standard-of-care treatment at the very end of the treatment period. These results, alongside the fact that miR-10b expression increases upon metastatic HCC and is associated with decreased OS, suggest that high levels of miR-10b can overcome the "miR-10b targeting" by linifanib and contribute to disease progression/relapse. Thus, in clnical trials of patients with advance HCC, where linifanib did not impove the OS compared to Sorafenib, we speculate that poor responders to linifanib are most likely patients with enhanced miR-10b overexpression to begin with, or developed along a period post-treatment to overcome the effect of linifanib.

In this regard, we believe that the fact that Linifanib inhibits miR-10b (along with its known targets), could be contributing to define the therapeutic efficacy of preclinical models and clinical trials of different cancer types, and that the combination of linifanib with additional agents could increase the positive outcomes of cancer patients. To achieve this (and as a subsequent translational approach of our findings), we believe that by evaluating the chemical functional groups of Linifanib that target motifs of the pre-miR-10b, we will have a basis to develop a series of small molecules with variable levels of affinity towards RTKs, and more towards binding miR-10b. We predict that SMIRs against miR-10b could offer potential therapeutic benefits in additional tumor types that have been proven to be

oncomiR-10b addictive, not only breast but also, HCC, NSCLC, CRC, pancreatic cancer and glioblastoma [160, 161, 206]. Furthermore, our study validates the fact that by upscaling screenings such as this one to a high throughput manner, many potential SMIRs could be discovered and used as targeted therapy for cancer patients.

CHAPTER V: Future Directions

Structure analysis of small molecule inhibitors of miRNA (SMIRs) maturation

MiRNA precursor sequences have array of internal loops and non-canonical pairings in combinations that are unique for each miRNA, offering target specificity. However, structural based hit discovery highly relies on precise high-resolution structures, and with miRNAs, their inherent flexibility limits the number of structures available (approximately 1% in Protein Data Bank). Only a handful of structures have been elucidated for miRNA fragments and none of them are in complex with small molecules. Furthermore, *in silico* modeling techniques for RNAs have not yet reached the capacity of those for protein targets. Limitations on direct structure determination and computational modeling have significantly restricted drug discovery efforts against RNAs.

One of the approaches to overcome these challenges and barriers is developing an integrated platform for miRNA structural studies and inhibitor discovery by combining cutting-edge *in silico* modeling and direct structure methodologies. Dedicated computational methods are in the need of being employed, in order to build initial structure models of miRNAs. Validation of these methods through solution NMR is critical in order to investigate their conformational dynamics, and generate an ensemble of structures that accurately reflect their conformational distributions.

Medicinal synthesis and testing of newly synthesized compounds to establish structure-activity relationship and drive lead optimization

Derived knowledge from miRNA structural studies can be used to design new more potent derivatives. Each of the scaffolds can be synthesized followed by experimental evaluations for biological activity *in vitro* and *in vivo.* The process of lead optimization would most definitely be a synergistic and iterative process involving modeling, synthesis, and biological testing.

To complement the optimization of linifanib (5-6A) (or other prospective compounds), a structure-based strategy can be implemented in order to identify compounds specific for non-Watson-Crick features in the stem of pre-miR-10b (Figure 26C). Heteronuclear NMR spectroscopy can be used to generate ensembles of structures that accurately reflect the conformational distribution sampled by the centrally located C-A and A-A mismatches and the GUAA/AAAC internal loop at the base of the hairpin. RNAs I and II (Figure 27) , which contain these features, can be used. These data could serve as the input for structure-based virtual HTS hit identification and alternative SMIR design.

In vivo studies to characterize optimized lead compounds

Expanding the pharmacokinetic (PK) experiments would be an important path to continue directing this project. Studies including absorption and distribution of the administered drug, as well as the rate at which a drug action begins, the duration of the effects, the chemical changes of the substance in the body (e.g., by enzymes), and the effects and routes of excretion of the metabolites of the drug are significantly important. PK studies should be done using only rodent at single, multiple, or cassette dosing. They should include preclinical non-compartmental pharmacokinetics, compartmental pharmacokinetics, ascending dose (assessment of dose proportionality), repeat dose (assessment of multiple-dose linearity), etc.

The toxicity of the modified compounds should also be assessed through gross observation, blood chemistry, and selected tissues taken for histological examination. Concentrations should be determined through techniques such as high performance liquid chromatography. The plasma area under the concentration time curve of the molecules should be determined, as well as other pharmacokinetic parameters, including half-life ($T_{1/2}$), apparent volume of distribution (V_d), clearance (Cl_{int}), etc.

Drug physicochemical properties are critical for their stability, formulation, bioavailability, and other pharmacokinetic behavior. Several pharmacodynamic properties are also important to be studied to determine the gastrointestinal absorption and the compound's oral bioavailability [207]. Lipophilicity, is also a key determinant of the

pharmacokinetic behavior of drugs that should also be assessed , as well as the compound's chemical stability [208].

Completion of PK and pharmacodynamic studies, would clearly lead to assessing the anti-tumor activity of lead compounds *in vivo* independently and in combination with either anti-miR10b, LNAs or with standard chemotherapy regimens. The main objective of such studies would be to investigate *in vivo* antitumor activity of our lead compound against breast tumor xenografts in *scid* mice. The primary endpoint of the experiments would be overall survival (OS), defined as the time from the malignant cells administration to the time of death or to the time of sacrifice. By targeting overexpressed miR-10b with a specific SMIR, we expect that apoptosis will be induced, and more so, invasion and migration will be reduced. We believe that altogether the anti-tumor activity will be significant in animal studies.

Appendix

Table 2: Oligonucleotide design for Psi-CHECK2 vector inserts

miRNA / Mature sequence	Designed oligonucleotides	
hsa-miR-10b UACCCUGUAGAACCGAAUUUGUG	**# 1** **# 2**	5' - CGCAGTAGAGCTCTAGTCACAA ATTCGGTTCTACAGGGTAGTTT - 3' 5' - AAACTACCCTGTAGAACCGAATT TGTGACTAGAGCTCTACTGCGAT - 3
hsa-miR-21 UAGCUUAUCAGACUGAUGUUGA	**# 1** **# 2**	5' - CGCAGTAGAGCTCTAGTTCAAC ATCAGTCTGATAAGCTAGTTT - 3' 5' - AAACTAGCTTATCAGACTGATGT TGAACTAGAGCTCTACTGCGAT - 3'

Nomenclature: 5' **SgfI site** – **linker** – **miR-10b binding site** – **PmeI site**– 3'

Table 3: Taqman Real-Time PCR Assays

miRBase ID	**Assay Name**	**Assay ID**	**Catalogue #**
hsa-miR-10b-5p	hsa-miR-10b	002218	4427975
hsa-miR-21-5p	hsa-miR-21	00397	4427975
n/a	U6 snRNA	001973	4427975
miRBase ID	**Stem loop accession #**	**Assay ID**	**Catalogue #**
hsa-miR-10b	MI0000267	Hs03302879_pri	4427012
hsa-miR-21	MI0000077	Hs03302625_pri	4427012

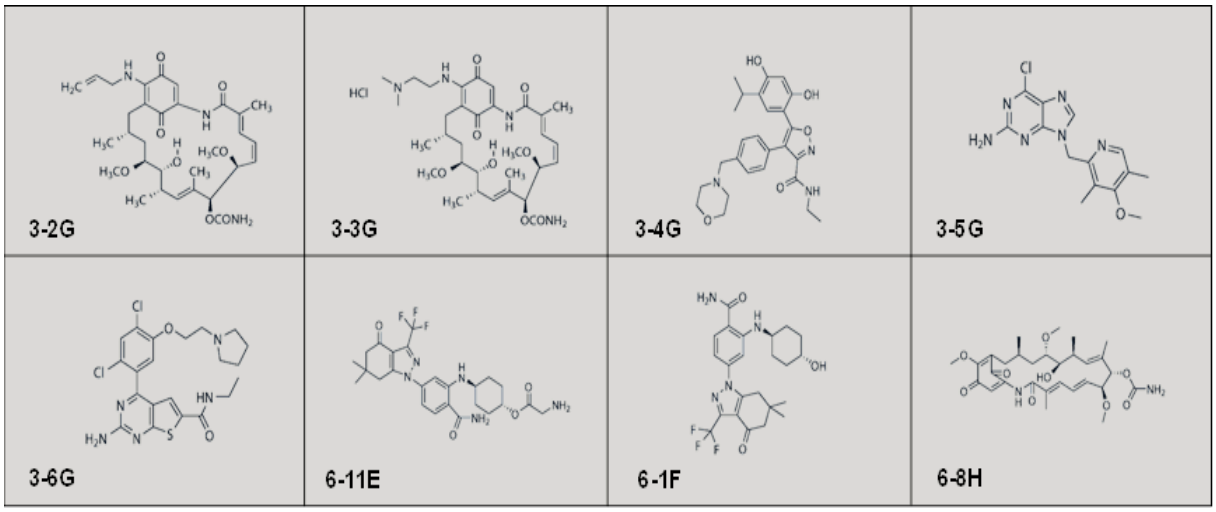
Table 4: Primers designed for this study

Primer name:	Type:	Sequence:	Experiment:
pre-miR-10b	Forward	CCCTGTAGAACCGAATTTGTG	RT-PCR of premiR sequence
pre-miR-10b	Reverse	TGAAGTTTTTGCATCGACCA	RT-PCR of premiR sequence
pre-miR-21	Forward	TGTCGGGTAGCTTATCAGAC	RT-PCR of premiR sequence
pre-miR-21	Reverse	TGTCAGACAGCCCATCGACT	RT-PCR of premiR sequence
U6-normalizer	Forward	CTCGCTTCGGCAGCACA	RT-PCR reference gene (control)
U6-normalizer	Reverse	AACGCTTCACGAATTTGCGT	RT-PCR reference gene (control)
pre-miR-10b	Forward	CGGGATCCTCCTTGGGATGGA	Clone-premiR-DNA coding region
pre-miR-10b	Reverse	CGGGATCCAGGAAAAGCTGCT	Clone-premiR-DNA coding region
pre-miR-21	Forward	CGGGATCCGTTTTTGATTGAA	Clone-premiR-DNA coding region
pre-miR-21	Reverse	CGGGATCCTTTATTTGTGGTC	Clone-premiR-DNA coding region

Table 5: Antibodies

Antibody Name	Manufacturer	Catalogue ID
Vinculin	Santa Cruz	sc-7649
HOXD10	Abcam	ab90704
Dicer	Cell Signaling Technologies	5325
Drosha	Cell Signaling Technologies	3364S
α-Tubulin	Sigma Aldrich	T9026

Table 6: Molecular Structures of the Heat Shock protein-90 inhibitors included in the study



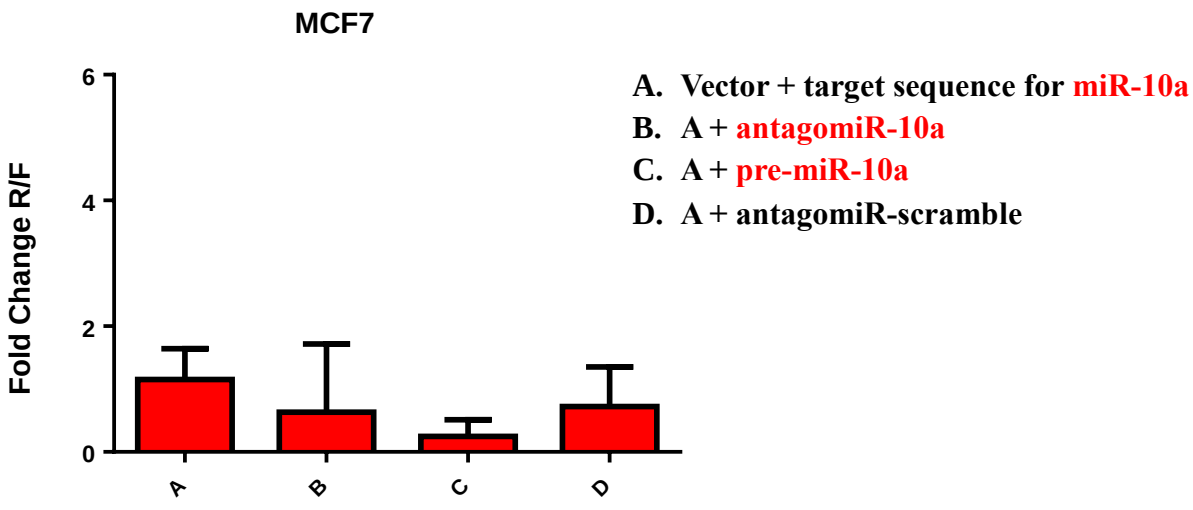
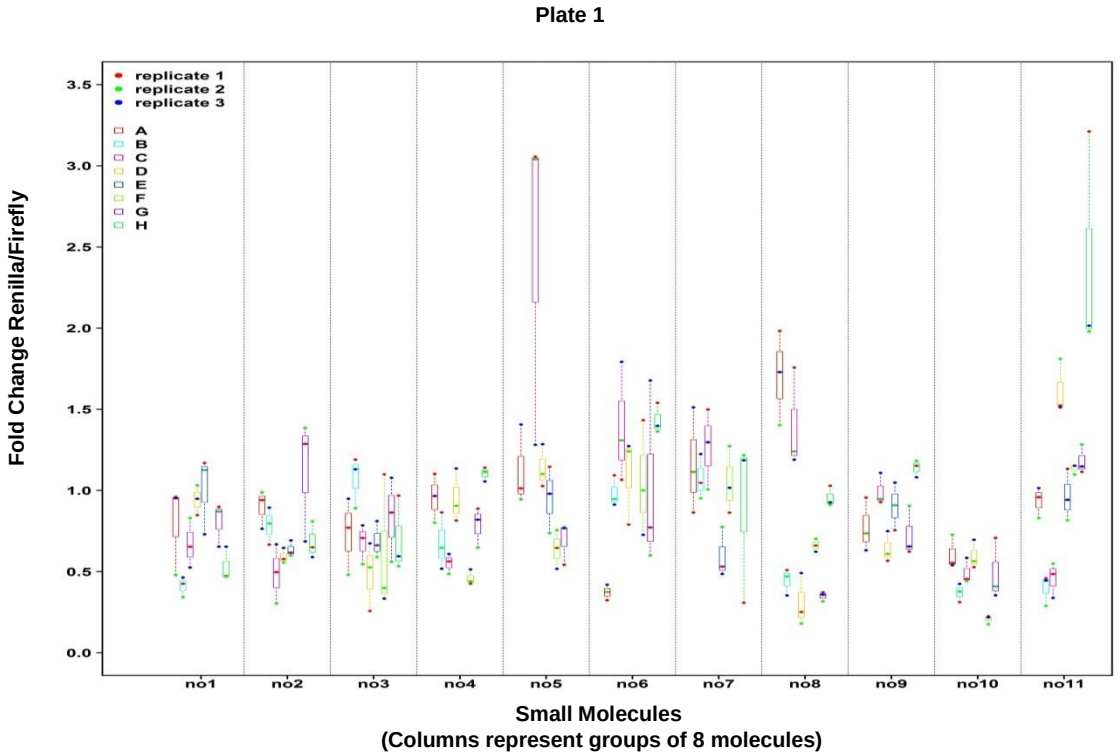


Figure 38: **Luciferase-based reporter for miR-10b levels tested in the presence of different levels of miR-10a**

Figure 39: **Luciferase-based screening for miR10b:** ***Plates 1-6.***



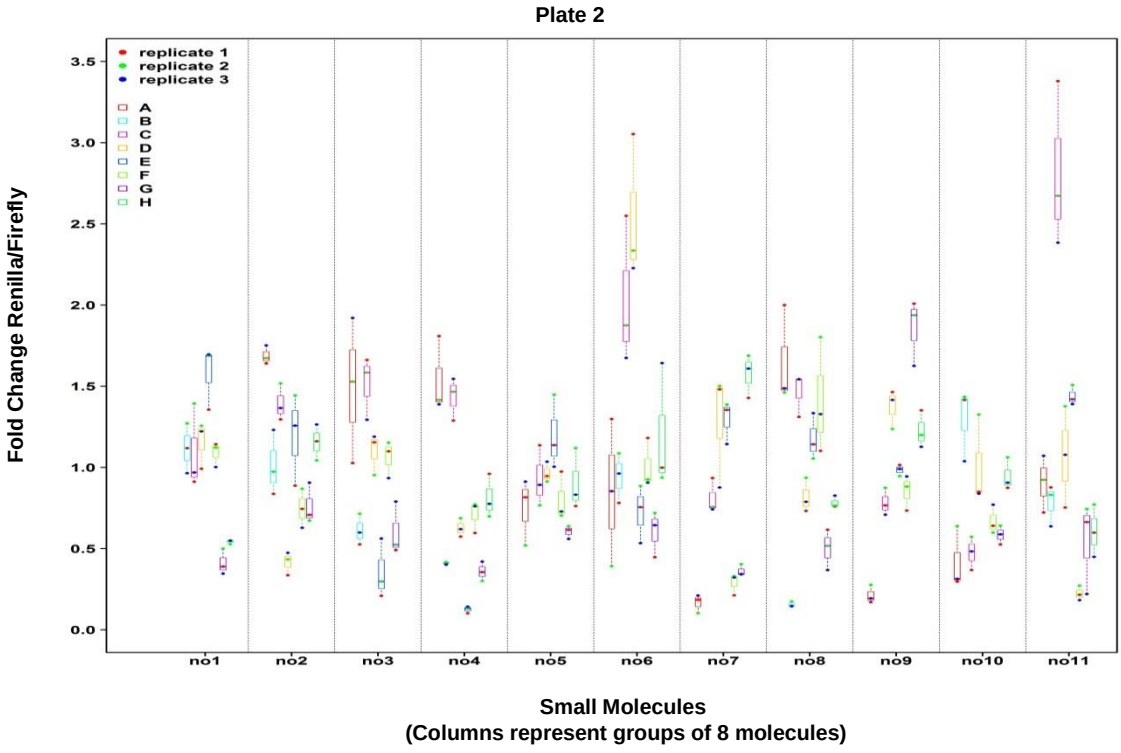


Plate 3

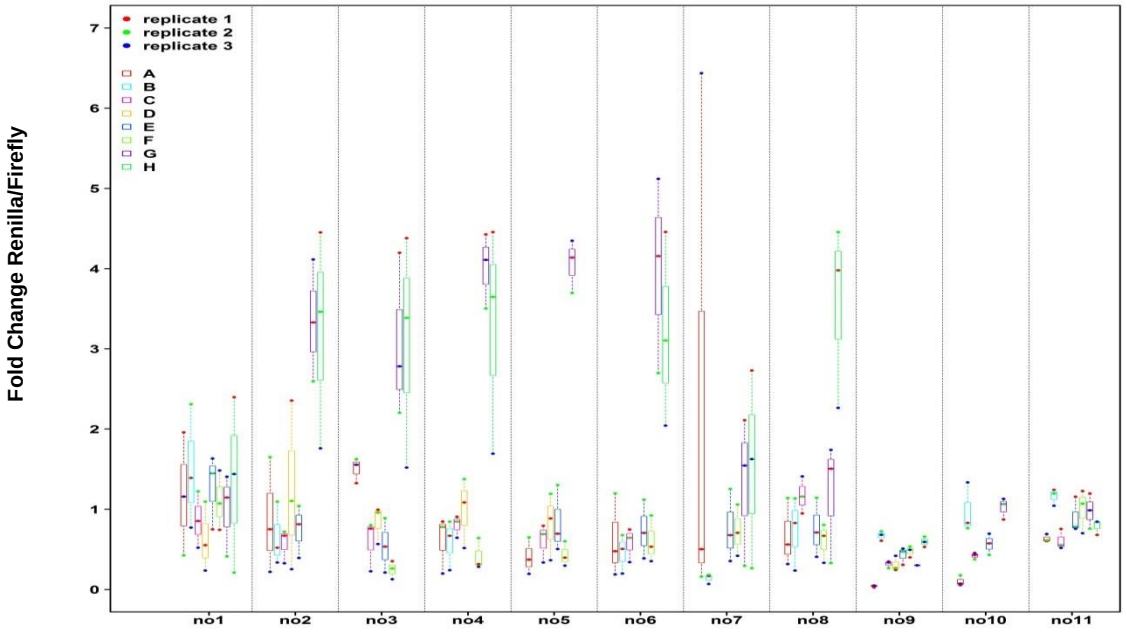


Plate 4

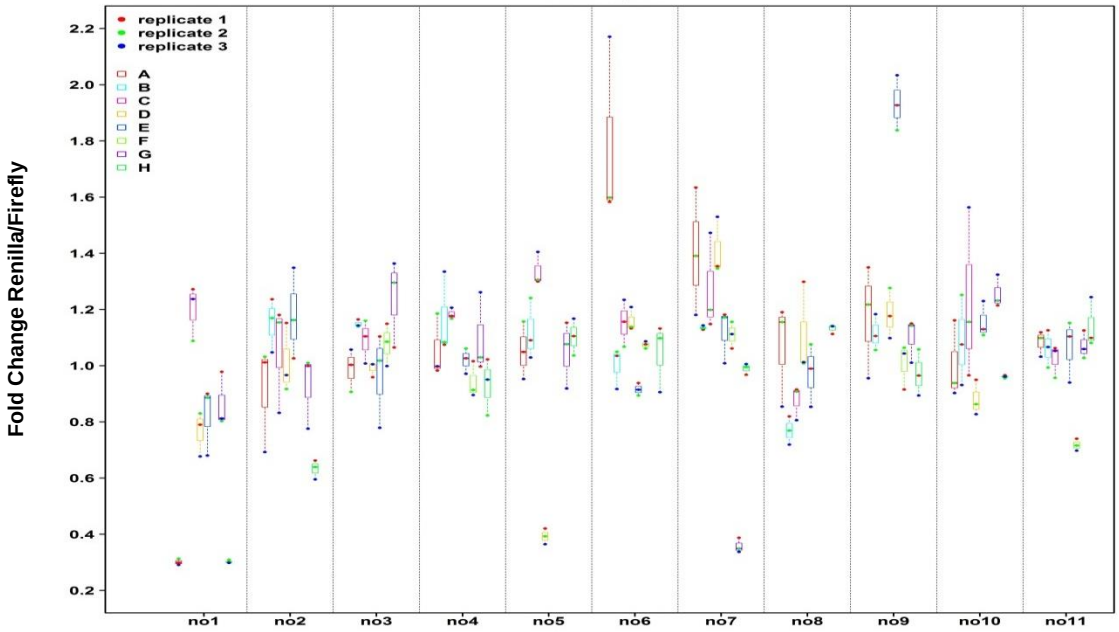


Plate 5

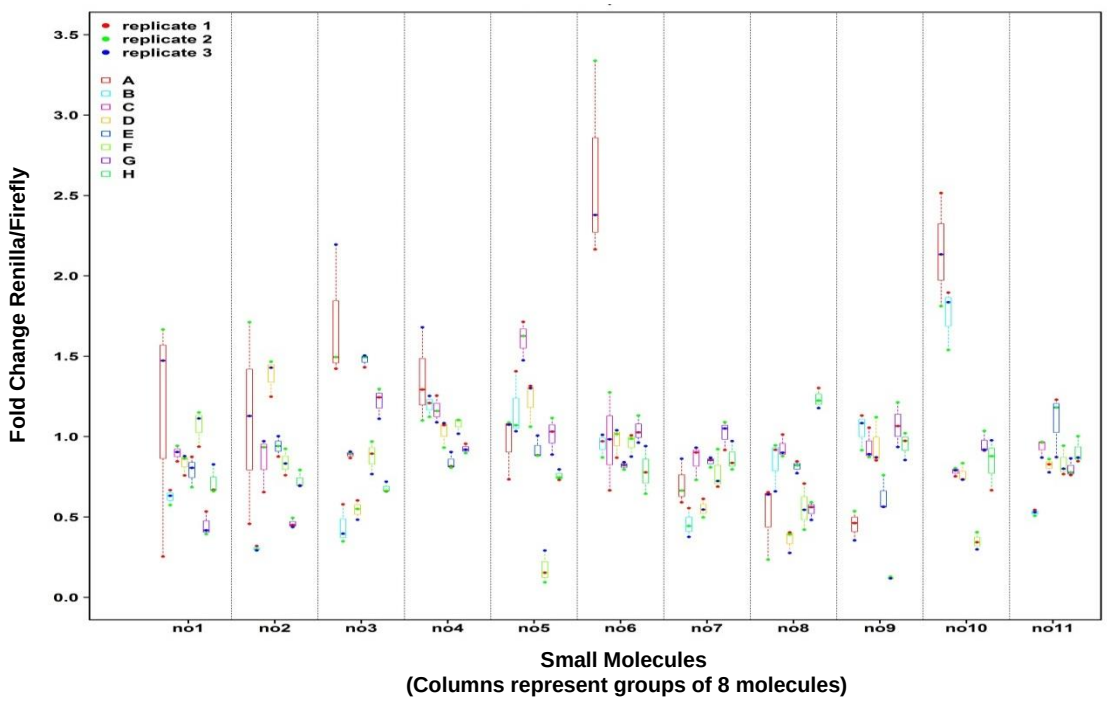


Plate 6

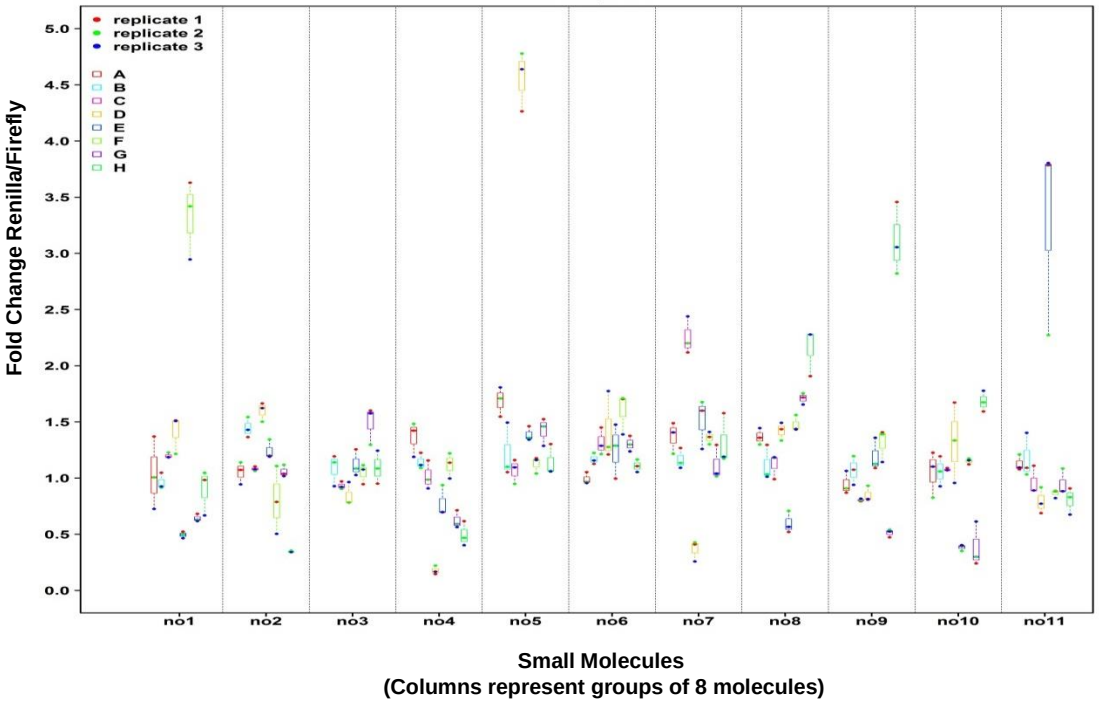
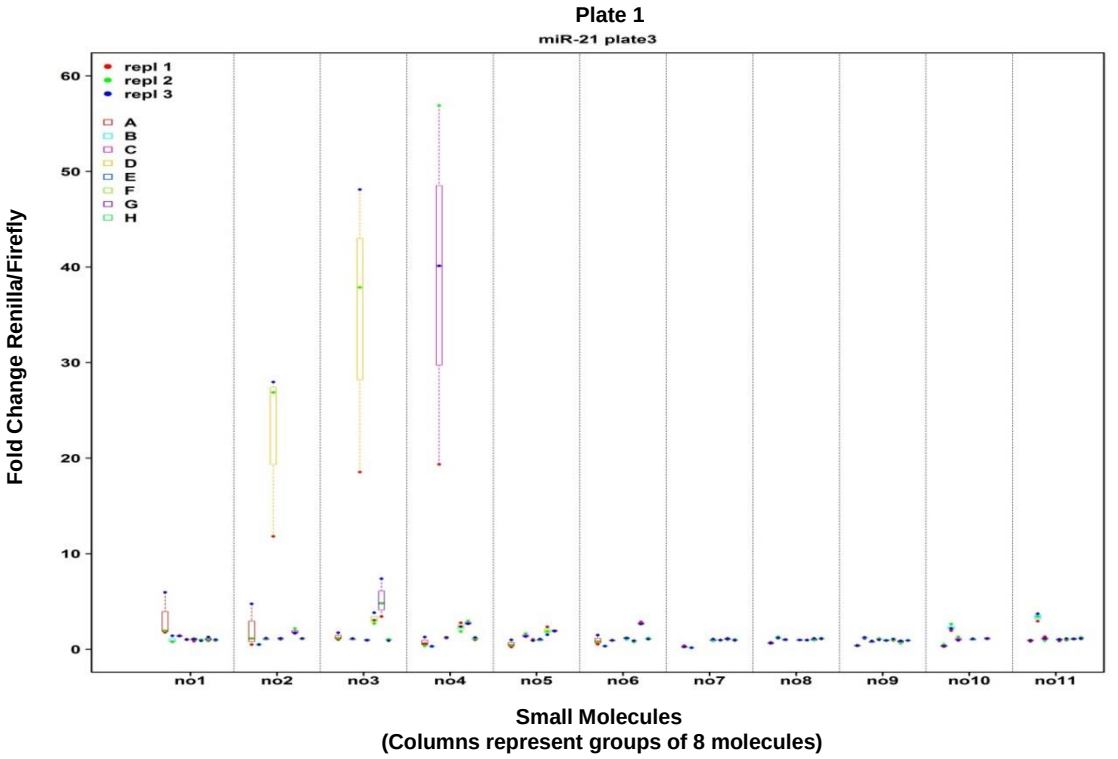


Figure 40: **Luciferase-based screening for miR21: *Plates 1-6.***



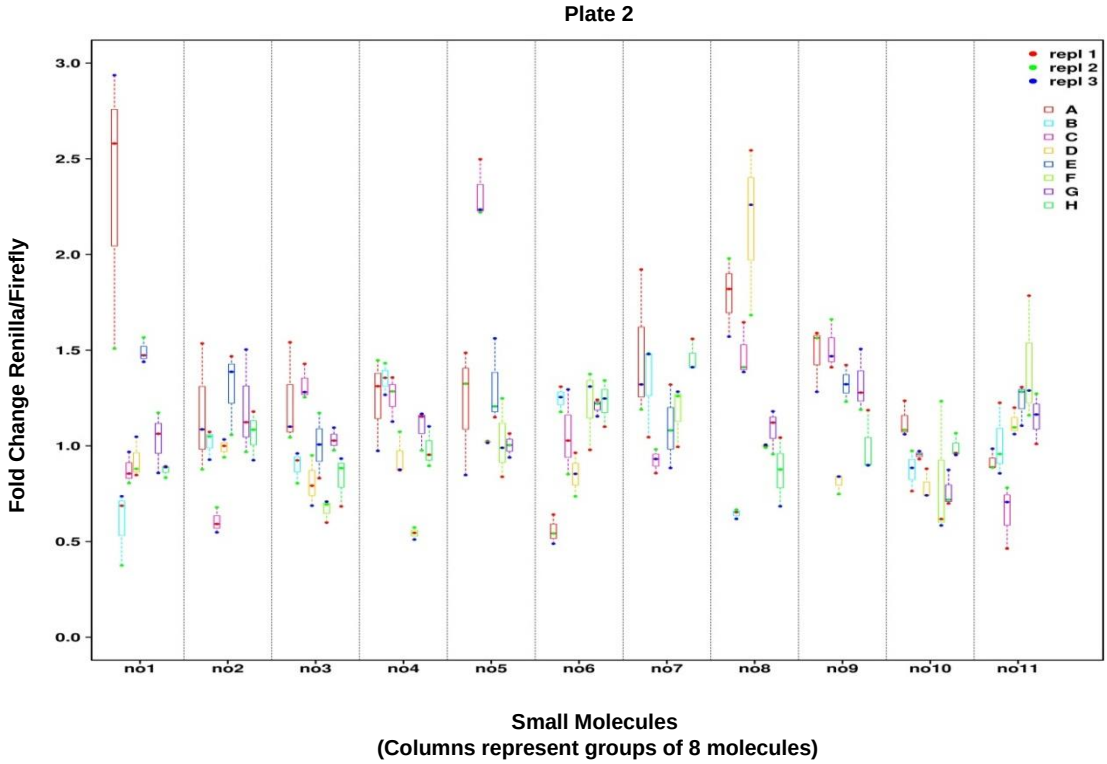
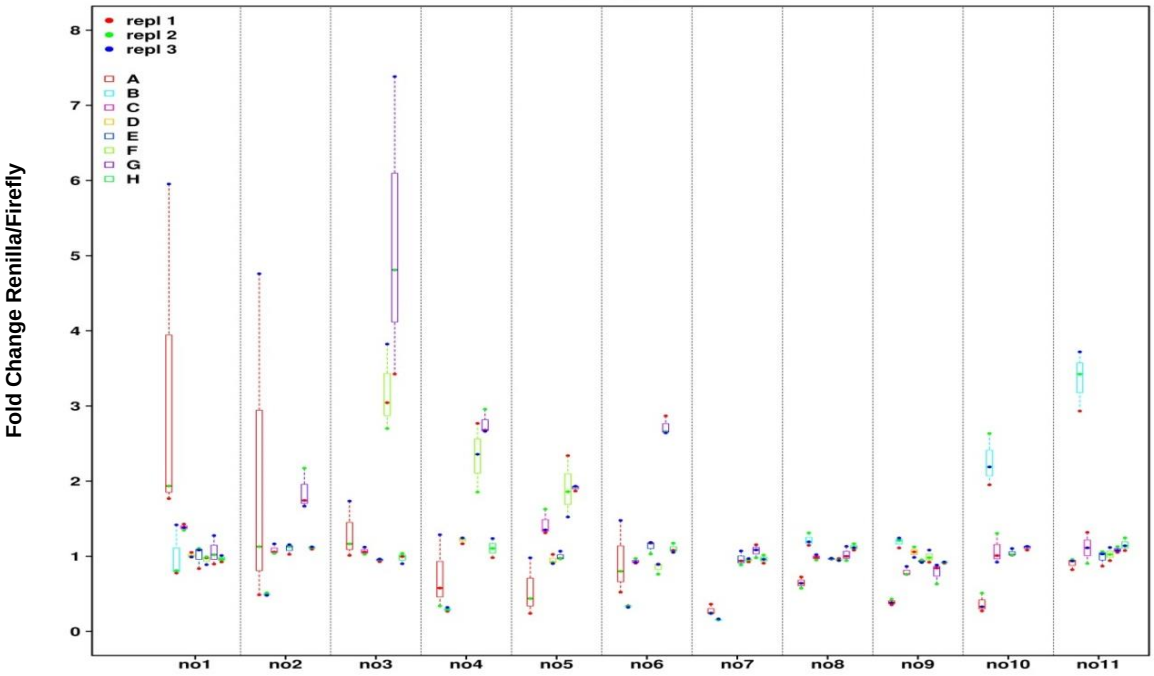
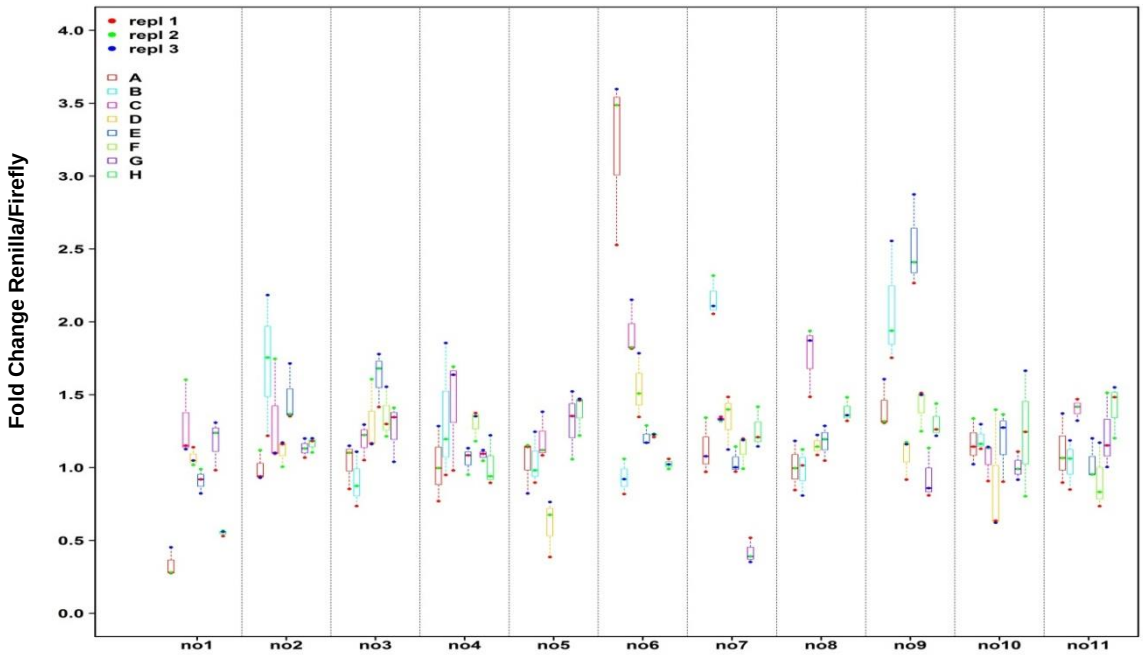


Plate 3



Small Molecules
(Columns represent groups of 8 molecules)

Plate 4



Small Molecules
(Columns represent groups of 8 molecules)

Plate 5

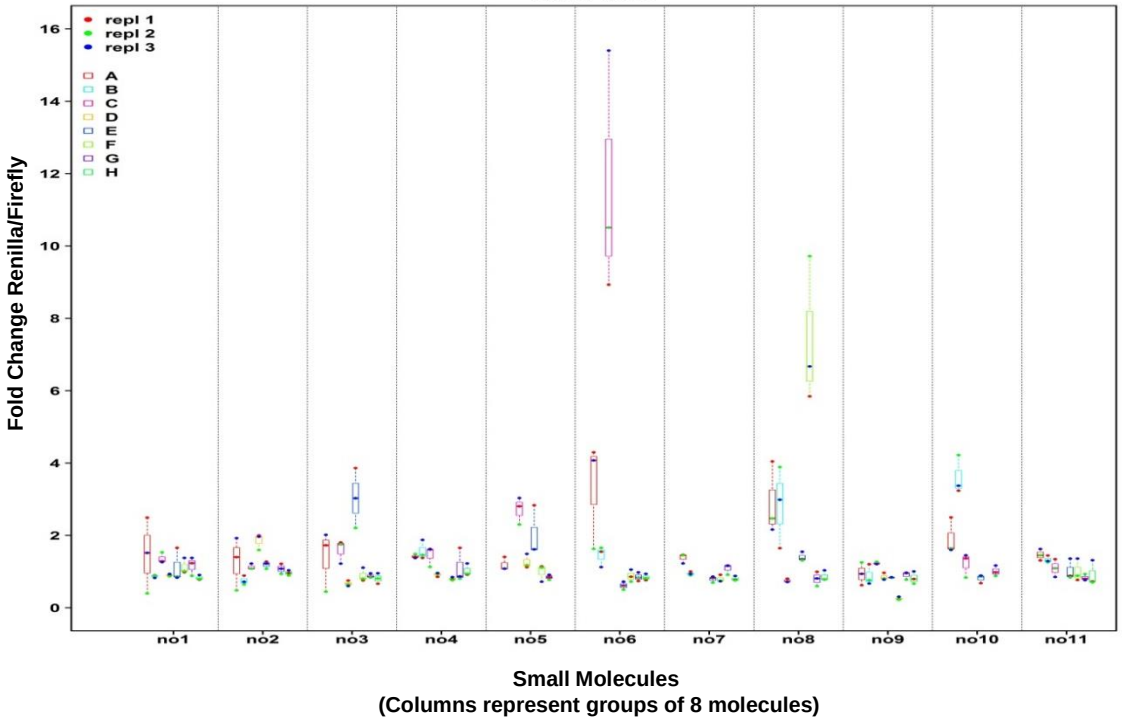


Plate 6

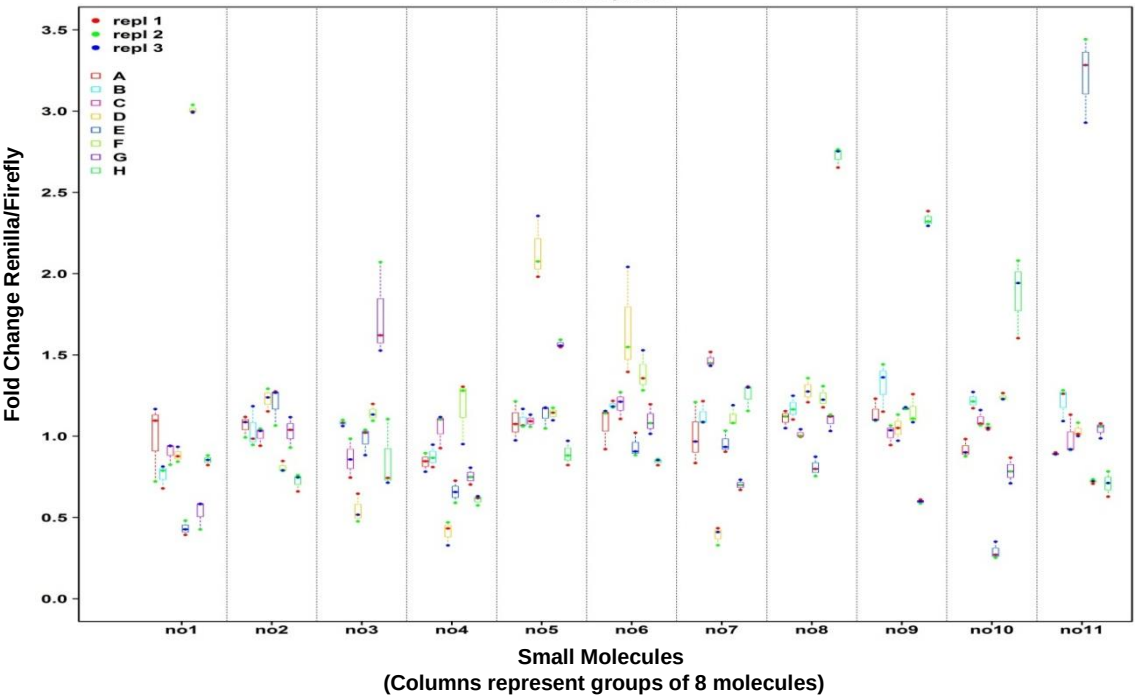
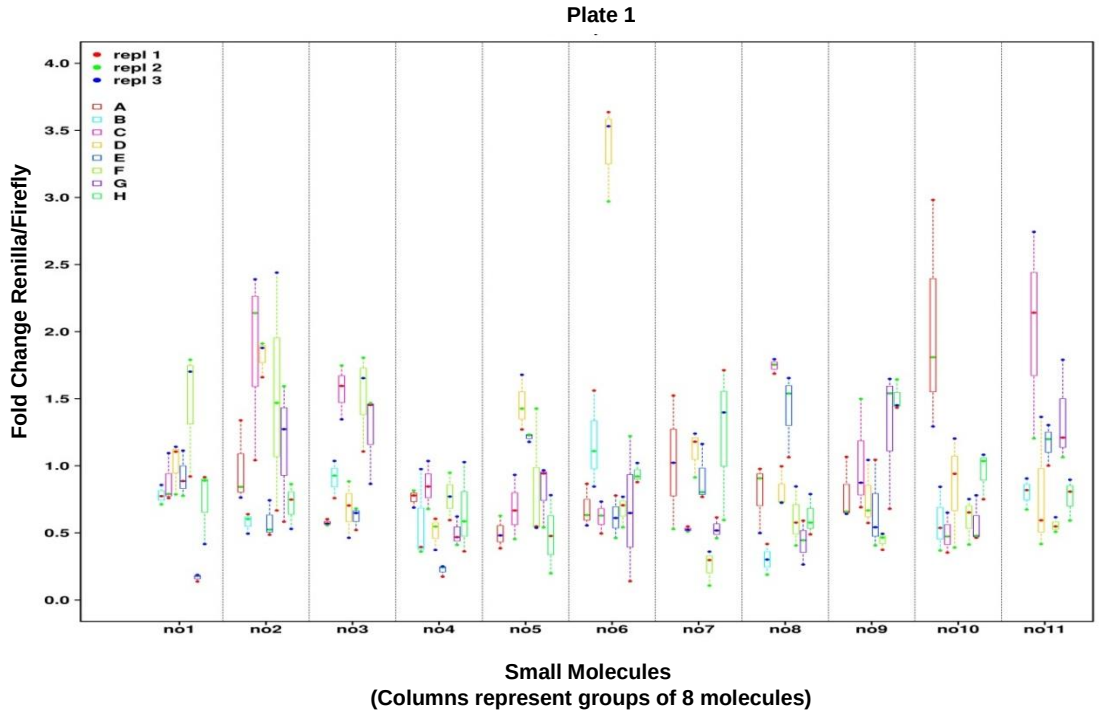


Figure 41: **Luciferase-based screening for miR21: *Plates with excluded outliers.***



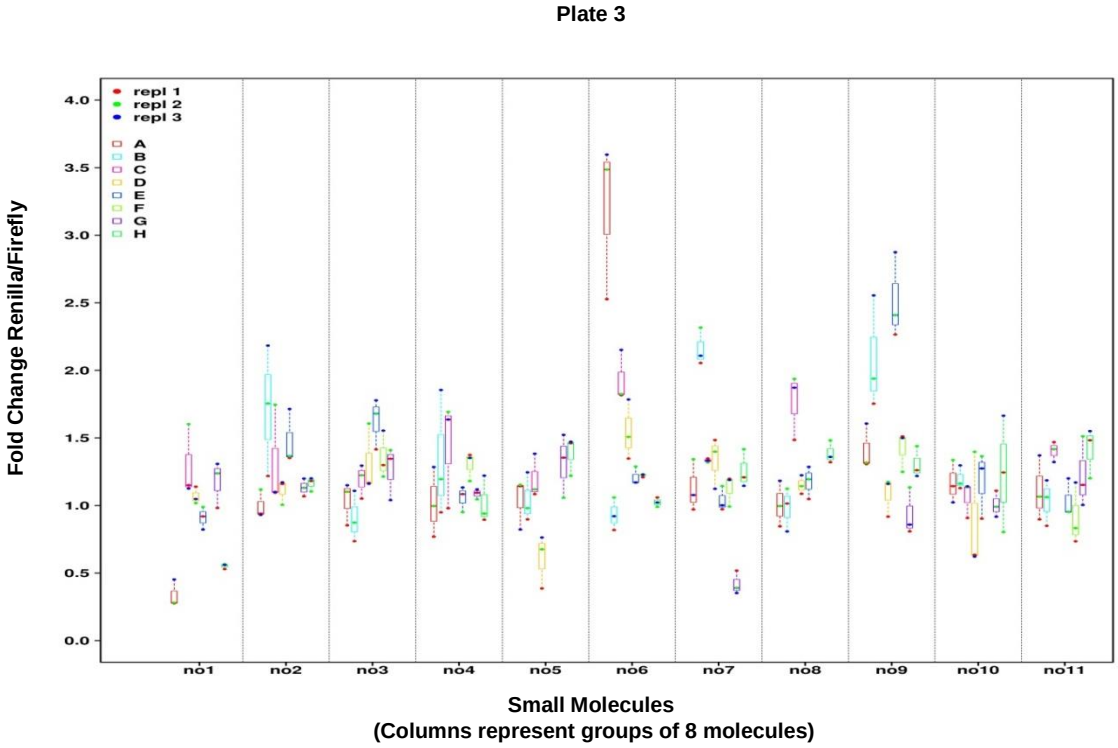
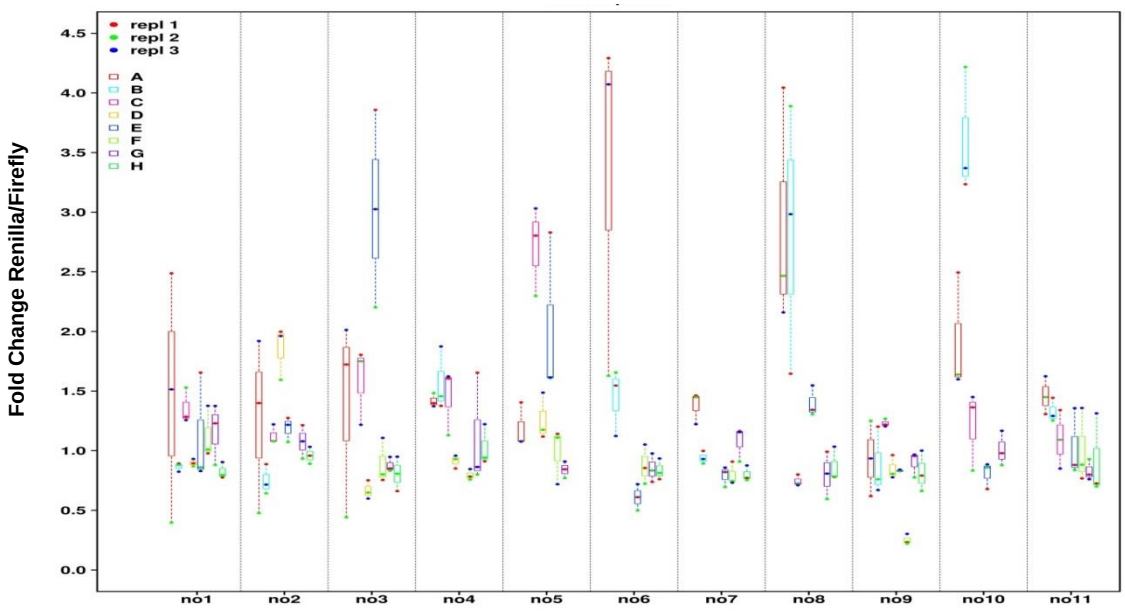


Plate 5



Fold Change Renilla/Firefly

Small Molecules
(Columns represent groups of 8 molecules)

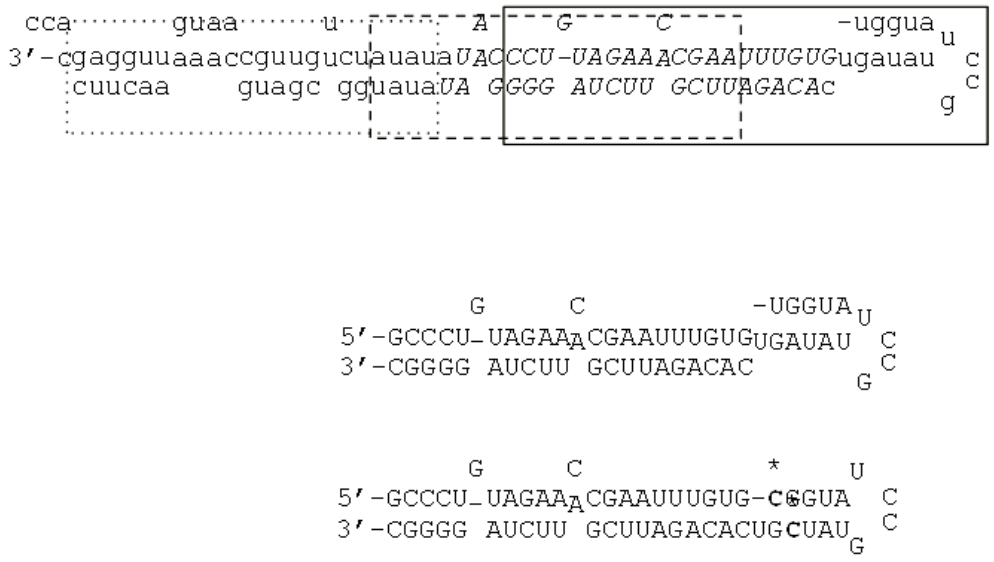


Figure 42: Detailed design of oligonucleotide sequences for NMR studies.

The nucleotide sequence of the pre-miR-10b (miRbase.org). Three guanine nucleotides were added to the 5' end to facilitate in vitro transcription. The mature 5p and 3p miR-10b sequences (capitalized and italicized) are released after processing by Dicer. Boxed regions show segments of the stem that were prepared as three separate RNA hairpins. Helical segments I (dotted) and II (dashed) were capped by UUCG tetraloop sequence to facilitate folding of the RNA molecules. The UCCG loop sequence of segment III (solid) was left unchanged. Cross-strand pairing in the terminal loop exends the helix and introduces an internal loop (UG/UGA).

References

[1] J. Ferlay, H.R. Shin, F. Bray, D. Forman, C. Mathers, D.M. Parkin, Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008, Int J Cancer, 127 (2010) 2893-2917.
[2] C. DeSantis, R. Siegel, P. Bandi, A. Jemal, Breast cancer statistics, 2011, CA Cancer J Clin, 61 (2011) 409-418.
[3] N. Cancer Genome Atlas, Comprehensive molecular portraits of human breast tumours, Nature, 490 (2012) 61-70.
[4] P. Boyle, A. Howell, The globalisation of breast cancer, Breast Cancer Res, 12 Suppl 4 (2010) S7.
[5] H.A. Assi, K.E. Khoury, H. Dbouk, L.E. Khalil, T.H. Mouhieddine, N.S. El Saghir, Epidemiology and prognosis of breast cancer in young women, J Thorac Dis, 5 Suppl 1 (2013) S2-8.
[6] E. Dereere, K. Papadimitriou, W. Tjalma, S. Altintas, Ductal Carcinoma In Situ: A disease entity that merits more recognition, Minerva Chir, (2015).
[7] M.R. Flanagan, M.H. Rendi, K.E. Calhoun, B.O. Anderson, S.H. Javid, Pleomorphic Lobular Carcinoma In Situ: Radiologic-Pathologic Features and Clinical Management, Ann Surg Oncol, (2015).
[8] G.L. Menezes, M.A. van den Bosch, E.L. Postma, M.A. El Sharouni, H.M. Verkooijen, P.J. van Diest, R.M. Pijnappel, Invasive ductolobular carcinoma of the breast: spectrum of mammographic, ultrasound and magnetic resonance imaging findings correlated with proportion of the lobular component, Springerplus, 2 (2013) 621.
[9] C.M. Perou, T. Sorlie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, C.A. Rees, J.R. Pollack, D.T. Ross, H. Johnsen, L.A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S.X. Zhu, P.E. Lonning, A.L. Borresen-Dale, P.O. Brown, D. Botstein, Molecular portraits of human breast tumours, Nature, 406 (2000) 747-752.
[10] T. Sorlie, C.M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, T. Thorsen, H. Quist, J.C. Matese, P.O. Brown, D. Botstein, P.E. Lonning, A.L. Borresen-Dale, Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications, Proceedings of the National Academy of Sciences of the United States of America, 98 (2001) 10869-10874.
[11] H. Kennecke, R. Yerushalmi, R. Woods, M.C. Cheang, D. Voduc, C.H. Speers, T.O. Nielsen, K. Gelmon, Metastatic behavior of breast cancer subtypes, J Clin Oncol, 28 (2010) 3271-3277.
[12] R. Rouzier, C.M. Perou, W.F. Symmans, N. Ibrahim, M. Cristofanilli, K. Anderson, K.R. Hess, J. Stec, M. Ayers, P. Wagner, P. Morandi, C. Fan, I. Rabiul, J.S. Ross, G.N. Hortobagyi, L. Pusztai, Breast cancer molecular subtypes respond differently to preoperative chemotherapy, Clin Cancer Res, 11 (2005) 5678-5685.
[13] J.S. Parker, M. Mullins, M.C. Cheang, S. Leung, D. Voduc, T. Vickery, S. Davies, C. Fauron, X. He, Z. Hu, J.F. Quackenbush, I.J. Stijleman, J. Palazzo, J.S. Marron, A.B. Nobel, E. Mardis, T.O. Nielsen, M.J. Ellis, C.M. Perou, P.S. Bernard, Supervised risk predictor of breast cancer based on intrinsic subtypes, J Clin Oncol, 27 (2009) 1160-1167.
[14] R. Sabatier, P. Finetti, A. Guille, J. Adelaide, M. Chaffanet, P. Viens, D. Birnbaum, F. Bertucci, Claudin-low breast cancers: clinical, pathological, molecular and prognostic characterization, Mol Cancer, 13 (2014) 228.

[15] E.C. Inwald, M. Klinkhammer-Schalke, F. Hofstadter, F. Zeman, M. Koller, M. Gerstenhauer, O. Ortmann, Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry, Breast Cancer Res Treat, 139 (2013) 539-552.
[16] M. Loberg, M.L. Lousdal, M. Bretthauer, M. Kalager, Benefits and harms of mammography screening, Breast Cancer Res, 17 (2015) 63.
[17] M. Colozza, E. de Azambuja, N. Personeni, F. Lebrun, M.J. Piccart, F. Cardoso, Achievements in systemic therapies in the pregenomic era in metastatic breast cancer, The oncologist, 12 (2007) 253-270.
[18] G. Franceschini, A.M. Sanchez, A. Di Leone, S. Magno, F. Moschella, C. Accetta, M. Natale, D. Di Giorgio, A. Scaldaferri, S. D'Archi, L. Scardina, R. Masetti, Update on the surgical management of breast cancer, Ann Ital Chir, 86 (2015) 89-99.
[19] Y.C. Cheng, N.T. Ueno, Improvement of survival and prospect of cure in patients with metastatic breast cancer, Breast Cancer, 19 (2012) 191-199.
[20] H. Liu, Y. Liu, J.T. Zhang, A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction, Mol Cancer Ther, 7 (2008) 263-270.
[21] W.D. Foulkes, I.E. Smith, J.S. Reis-Filho, Triple-negative breast cancer, The New England journal of medicine, 363 (2010) 1938-1948.
[22] M.S. Ewer, D.J. Lenihan, Is trastuzumab associated with adverse cardiac effects in patients with breast cancer?, Nat Clin Pract Oncol, 5 (2008) 192-193.
[23] R. Spizzo, M.I. Almeida, A. Colombatti, G.A. Calin, Long non-coding RNAs and cancer: a new frontier of translational research?, Oncogene, 31 (2012) 4577-4587.
[24] E.T.P. Affymetrix, E.T.P. Cold Spring Harbor Laboratory, Post-transcriptional processing generates a diversity of 5'-modified long and short RNAs, Nature, 457 (2009) 1028-1032.
[25] B.T. Wilhelm, S. Marguerat, S. Watt, F. Schubert, V. Wood, I. Goodhead, C.J. Penkett, J. Rogers, J. Bahler, Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution, Nature, 453 (2008) 1239-1243.
[26] S.M. Wilhelm, C. Carter, L. Tang, D. Wilkie, A. McNabola, H. Rong, C. Chen, X. Zhang, P. Vincent, M. McHugh, Y. Cao, J. Shujath, S. Gawlak, D. Eveleigh, B. Rowley, L. Liu, L. Adnane, M. Lynch, D. Auclair, I. Taylor, R. Gedrich, A. Voznesensky, B. Riedl, L.E. Post, G. Bollag, P.A. Trail, BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis, Cancer research, 64 (2004) 7099-7109.
[27] J.M. Llovet, S. Ricci, V. Mazzaferro, P. Hilgard, E. Gane, J.F. Blanc, A.C. de Oliveira, A. Santoro, J.L. Raoul, A. Forner, M. Schwartz, C. Porta, S. Zeuzem, L. Bolondi, T.F. Greten, P.R. Galle, J.F. Seitz, I. Borbath, D. Haussinger, T. Giannaris, M. Shan, M. Moscovici, D. Voliotis, J. Bruix, S.I.S. Group, Sorafenib in advanced hepatocellular carcinoma, The New England journal of medicine, 359 (2008) 378-390.
[28] J. Ferdin, T. Kunej, G.A. Calin, Non-coding RNAs: identification of cancer-associated microRNAs by gene profiling, Technol Cancer Res Treat, 9 (2010) 123-138.
[29] R.C. Lee, R.L. Feinbaum, V. Ambros, The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, Cell, 75 (1993) 843-854.
[30] B.J. Reinhart, F.J. Slack, M. Basson, A.E. Pasquinelli, J.C. Bettinger, A.E. Rougvie, H.R. Horvitz, G. Ruvkun, The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans, Nature, 403 (2000) 901-906.

[31] J. Krol, I. Loedige, W. Filipowicz, The widespread regulation of microRNA biogenesis, function and decay, Nature reviews. Genetics, 11 (2010) 597-610.
[32] R.C. Friedman, K.K. Farh, C.B. Burge, D.P. Bartel, Most mammalian mRNAs are conserved targets of microRNAs, Genome research, 19 (2009) 92-105.
[33] A.M. Denli, B.B. Tops, R.H. Plasterk, R.F. Ketting, G.J. Hannon, Processing of primary microRNAs by the Microprocessor complex, Nature, 432 (2004) 231-235.
[34] I. Berindan-Neagoe, C. Monroig Pdel, B. Pasculli, G.A. Calin, MicroRNAome genome: a treasure for cancer diagnosis and therapy, CA: a cancer journal for clinicians, 64 (2014) 311-336.
[35] G.A. Calin, C.D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich, C.M. Croce, Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia, Proceedings of the National Academy of Sciences of the United States of America, 99 (2002) 15524-15529.
[36] G.A. Calin, C. Sevignani, C.D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini, C.M. Croce, Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers, Proceedings of the National Academy of Sciences of the United States of America, 101 (2004) 2999-3004.
[37] M. Inui, G. Martello, S. Piccolo, MicroRNA control of signal transduction, Nature reviews. Molecular cell biology, 11 (2010) 252-263.
[38] Y. Wang, C.G. Lee, MicroRNA and cancer--focus on apoptosis, J Cell Mol Med, 13 (2009) 12-23.
[39] M. Negrini, M.S. Nicoloso, G.A. Calin, MicroRNAs and cancer--new paradigms in molecular oncology, Current opinion in cell biology, 21 (2009) 470-479.
[40] H. Ling, K. Pickard, C. Ivan, C. Isella, M. Ikuo, R. Mitter, R. Spizzo, M.D. Bullock, C. Braicu, V. Pileczki, K. Vincent, M. Pichler, V. Stiegelbauer, G. Hoefler, M.I. Almeida, A. Hsiao, X. Zhang, J.N. Primrose, G.K. Packham, K. Liu, K. Bojja, R. Gafa, L. Xiao, S. Rossi, J.H. Song, I. Vannini, F. Fanini, S. Kopetz, P. Zweidler-McKay, X. Wang, C. Ionescu, A. Irimie, M. Fabbri, G. Lanza, S.R. Hamilton, I. Berindan-Neagoe, E. Medico, A.H. Mirnezami, G.A. Calin, M.S. Nicoloso, The clinical and biological significance of MIR-224 expression in colorectal cancer metastasis, Gut, (2015).
[41] A.L. Kasinski, F.J. Slack, Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy, Nature reviews. Cancer, 11 (2011) 849-864.
[42] C.J. Cheng, F.J. Slack, The duality of oncomiR addiction in the maintenance and treatment of cancer, Cancer journal, 18 (2012) 232-237.
[43] J. Elmen, M. Lindow, S. Schutz, M. Lawrence, A. Petri, S. Obad, M. Lindholm, M. Hedtjarn, H.F. Hansen, U. Berger, S. Gullans, P. Kearney, P. Sarnow, E.M. Straarup, S. Kauppinen, LNA-mediated microRNA silencing in non-human primates, Nature, 452 (2008) 896-899.
[44] C.C. Esau, Inhibition of microRNA with antisense oligonucleotides, Methods, 44 (2008) 55-60.
[45] J. Krutzfeldt, N. Rajewsky, R. Braich, K.G. Rajeev, T. Tuschl, M. Manoharan, M. Stoffel, Silencing of microRNAs in vivo with 'antagomirs', Nature, 438 (2005) 685-689.
[46] L. Fontana, M.E. Fiori, S. Albini, L. Cifaldi, S. Giovinazzi, M. Forloni, R. Boldrini, A. Donfrancesco, V. Federici, P. Giacomini, C. Peschle, D. Fruci, Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM, PloS one, 3 (2008) e2236.

[47] L. Ma, F. Reinhardt, E. Pan, J. Soutschek, B. Bhat, E.G. Marcusson, J. Teruya-Feldstein, G.W. Bell, R.A. Weinberg, Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model, Nature biotechnology, 28 (2010) 341-347.
[48] J.K. Park, T. Kogure, G.J. Nuovo, J. Jiang, L. He, J.H. Kim, M.A. Phelps, T.L. Papenfuss, C.M. Croce, T. Patel, T.D. Schmittgen, miR-221 silencing blocks hepatocellular carcinoma and promotes survival, Cancer research, 71 (2011) 7608-7616.
[49] P. Sapra, M. Wang, R. Bandaru, H. Zhao, L.M. Greenberger, I.D. Horak, Down-modulation of survivin expression and inhibition of tumor growth in vivo by EZN-3042, a locked nucleic acid antisense oligonucleotide, Nucleosides, nucleotides & nucleic acids, 29 (2010) 97-112.
[50] Y. Lu, J. Xiao, H. Lin, Y. Bai, X. Luo, Z. Wang, B. Yang, A single anti-microRNA antisense oligodeoxyribonucleotide (AMO) targeting multiple microRNAs offers an improved approach for microRNA interference, Nucleic acids research, 37 (2009) e24.
[51] M. Esteller, Non-coding RNAs in human disease, Nature reviews. Genetics, 12 (2011) 861-874.
[52] J. Stenvang, S. Kauppinen, MicroRNAs as targets for antisense-based therapeutics, Expert opinion on biological therapy, 8 (2008) 59-81.
[53] L. Ma, J. Young, H. Prabhala, E. Pan, P. Mestdagh, D. Muth, J. Teruya-Feldstein, F. Reinhardt, T.T. Onder, S. Valastyan, F. Westermann, F. Speleman, J. Vandesompele, R.A. Weinberg, miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis, Nat Cell Biol, 12 (2010) 247-U252.
[54] A. Gaur, D.A. Jewell, Y. Liang, D. Ridzon, J.H. Moore, C. Chen, V.R. Ambros, M.A. Israel, Characterization of microRNA expression levels and their biological correlates in human cancer cell lines, Cancer research, 67 (2007) 2456-2468.
[55] M.S. Kumar, J. Lu, K.L. Mercer, T.R. Golub, T. Jacks, Impaired microRNA processing enhances cellular transformation and tumorigenesis, Nature genetics, 39 (2007) 673-677.
[56] T.M. Love, H.F. Moffett, C.D. Novina, Not miR-ly small RNAs: big potential for microRNAs in therapy, The Journal of allergy and clinical immunology, 121 (2008) 309-319.
[57] A. Bouchie, First microRNA mimic enters clinic, Nature biotechnology, 31 (2013) 577.
[58] M.V. Iorio, M. Ferracin, C.G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J.P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G.A. Calin, P. Querzoli, M. Negrini, C.M. Croce, MicroRNA gene expression deregulation in human breast cancer, Cancer research, 65 (2005) 7065-7070.
[59] J. Lu, G. Getz, E.A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B.L. Ebert, R.H. Mak, A.A. Ferrando, J.R. Downing, T. Jacks, H.R. Horvitz, T.R. Golub, MicroRNA expression profiles classify human cancers, Nature, 435 (2005) 834-838.
[60] C. Blenkiron, L.D. Goldstein, N.P. Thorne, I. Spiteri, S.F. Chin, M.J. Dunning, N.L. Barbosa-Morais, A.E. Teschendorff, A.R. Green, I.O. Ellis, S. Tavare, C. Caldas, E.A. Miska, MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype, Genome biology, 8 (2007) R214.
[61] M. Riaz, M.T. van Jaarsveld, A. Hollestelle, W.J. Prager-van der Smissen, A.A. Heine, A.W. Boersma, J. Liu, J. Helmijr, B. Ozturk, M. Smid, E.A. Wiemer, J.A. Foekens, J.W. Martens, miRNA expression profiling of 51 human breast cancer cell

lines reveals subtype and driver mutation-specific miRNAs, Breast cancer research : BCR, 15 (2013) R33.
[62] F. Mar-Aguilar, J.A. Mendoza-Ramirez, I. Malagon-Santiago, P.K. Espino-Silva, S.K. Santuario-Facio, P. Ruiz-Flores, C. Rodriguez-Padilla, D. Resendez-Perez, Serum circulating microRNA profiling for identification of potential breast cancer biomarkers, Disease markers, 34 (2013) 163-169.
[63] L. Ma, J. Teruya-Feldstein, R.A. Weinberg, Tumour invasion and metastasis initiated by microRNA-10b in breast cancer, Nature, 449 (2007) 682-688.
[64] L. Ma, Role of miR-10b in breast cancer metastasis, Breast cancer research : BCR, 12 (2010) 210.
[65] N.V. Vardhini, P.J. Rao, P.B. Murthy, G. Sudhakar, HOXD10 expression in human breast cancer, Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine, 35 (2014) 10855-10860.
[66] P. Parrella, R. Barbano, B. Pasculli, A. Fontana, M. Copetti, V.M. Valori, M.L. Poeta, G. Perrone, D. Righi, M. Castelvetere, M. Coco, T. Balsamo, M. Morritti, F. Pellegrini, A. Onetti-Muda, E. Maiello, R. Murgo, V.M. Fazio, Evaluation of microRNA-10b prognostic significance in a prospective cohort of breast cancer patients, Molecular cancer, 13 (2014) 142.
[67] L. Chen, Y. Li, Y. Fu, J. Peng, M.H. Mo, M. Stamatakos, C.B. Teal, R.F. Brem, A. Stojadinovic, M. Grinkemeyer, T.A. McCaffrey, Y.G. Man, S.W. Fu, Role of deregulated microRNAs in breast cancer progression using FFPE tissue, PloS one, 8 (2013) e54213.
[68] J. Gao, Q. Zhang, J. Xu, L. Guo, X. Li, Clinical significance of serum miR-21 in breast cancer compared with CA153 and CEA, Chinese journal of cancer research = Chung-kuo yen cheng yen chiu, 25 (2013) 743-748.
[69] L.X. Yan, X.F. Huang, Q. Shao, M.Y. Huang, L. Deng, Q.L. Wu, Y.X. Zeng, J.Y. Shao, MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis, Rna, 14 (2008) 2348-2360.
[70] B. Song, C. Wang, J. Liu, X. Wang, L. Lv, L. Wei, L. Xie, Y. Zheng, X. Song, MicroRNA-21 regulates breast cancer invasion partly by targeting tissue inhibitor of metalloproteinase 3 expression, Journal of experimental & clinical cancer research : CR, 29 (2010) 29.
[71] L.X. Yan, Q.N. Wu, Y. Zhang, Y.Y. Li, D.Z. Liao, J.H. Hou, J. Fu, M.S. Zeng, J.P. Yun, Q.L. Wu, Y.X. Zeng, J.Y. Shao, Knockdown of miR-21 in human breast cancer cell lines inhibits proliferation, in vitro migration and in vivo tumor growth, Breast cancer research : BCR, 13 (2011) R2.
[72] N. Pencheva, S.F. Tavazoie, Control of metastatic progression by microRNA regulatory networks, Nat Cell Biol, 15 (2013) 546-554.
[73] R. Garzon, G. Marcucci, C.M. Croce, Targeting microRNAs in cancer: rationale, strategies and challenges, Nature reviews. Drug discovery, 9 (2010) 775-789.
[74] A.G. Bader, D. Brown, J. Stoudemire, P. Lammers, Developing therapeutic microRNAs for cancer, Gene Ther, 18 (2011) 1121-1126.
[75] J. Zhou, B.C. Goh, D.H. Albert, C.S. Chen, ABT-869, a promising multi-targeted tyrosine kinase inhibitor: from bench to bedside, Journal of hematology & oncology, 2 (2009) 33.
[76] H. Otsuka, T. Fujii, U. Toh, N. Iwakuma, R. Takahashi, M. Mishima, M. Takenaka, T. Kakuma, M. Tanaka, K. Shirouzu, Phase II clinical trial of metronomic chemotherapy with combined irinotecan and tegafur-gimeracil-oteracil potassium in metastatic and recurrent breast cancer, Breast cancer, 22 (2015) 335-342.

[77] H.C. Toh, P.J. Chen, B.I. Carr, J.J. Knox, S. Gill, P. Ansell, E.M. McKeegan, B. Dowell, M. Pedersen, Q. Qin, J. Qian, F.A. Scappaticci, J.L. Ricker, D.M. Carlson, W.P. Yong, Phase 2 trial of linifanib (ABT-869) in patients with unresectable or metastatic hepatocellular carcinoma, Cancer, 119 (2013) 380-387.
[78] Y. Akao, Y. Nakagawa, I. Hirata, A. Iio, T. Itoh, K. Kojima, R. Nakashima, Y. Kitade, T. Naoe, Role of anti-oncomirs miR-143 and -145 in human colorectal tumors, Cancer gene therapy, 17 (2010) 398-408.
[79] D. Pramanik, N.R. Campbell, C. Karikari, R. Chivukula, O.A. Kent, J.T. Mendell, A. Maitra, Restitution of tumor suppressor microRNAs using a systemic nanovector inhibits pancreatic cancer growth in mice, Molecular cancer therapeutics, 10 (2011) 1470-1480.
[80] K. Rai, N. Takigawa, S. Ito, H. Kashihara, E. Ichihara, T. Yasuda, K. Shimizu, M. Tanimoto, K. Kiura, Liposomal delivery of MicroRNA-7-expressing plasmid overcomes epidermal growth factor receptor tyrosine kinase inhibitor-resistance in lung cancer cells, Molecular cancer therapeutics, 10 (2011) 1720-1727.
[81] C.V. Pecot, G.A. Calin, R.L. Coleman, G. Lopez-Berestein, A.K. Sood, RNA interference in the clinic: challenges and future directions, Nature reviews. Cancer, 11 (2011) 59-67.
[82] Y. Gutierrez-Puente, A.M. Tari, R.J. Ford, R. Tamez-Guerra, R. Mercado-Hernandez, M. Santoyo-Stephano, G. Lopez-Berestein, Cellular pharmacology of P-ethoxy antisense oligonucleotides targeted to Bcl-2 in a follicular lymphoma cell line, Leukemia & lymphoma, 44 (2003) 1979-1985.
[83] P. Trang, J.F. Wiggins, C.L. Daige, C. Cho, M. Omotola, D. Brown, J.B. Weidhaas, A.G. Bader, F.J. Slack, Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice, Molecular therapy : the journal of the American Society of Gene Therapy, 19 (2011) 1116-1122.
[84] C.N. Landen, Jr., A. Chavez-Reyes, C. Bucana, R. Schmandt, M.T. Deavers, G. Lopez-Berestein, A.K. Sood, Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery, Cancer research, 65 (2005) 6910-6918.
[85] P. de Antonellis, C. Medaglia, E. Cusanelli, I. Andolfo, L. Liguori, G. De Vita, M. Carotenuto, A. Bello, F. Formiggini, A. Galeone, G. De Rosa, A. Virgilio, I. Scognamiglio, M. Sciro, G. Basso, J.H. Schulte, G. Cinalli, A. Iolascon, M. Zollo, MiR-34a targeting of Notch ligand delta-like 1 impairs CD15+/CD133+ tumor-propagating cells and supports neural differentiation in medulloblastoma, PloS one, 6 (2011) e24584.
[86] M.R. Arkin, J.A. Wells, Small-molecule inhibitors of protein-protein interactions: progressing towards the dream, Nature reviews. Drug discovery, 3 (2004) 301-317.
[87] J. Zhang, P.L. Yang, N.S. Gray, Targeting cancer with small molecule kinase inhibitors, Nature reviews. Cancer, 9 (2009) 28-39.
[88] S. Zhang, L. Chen, E.J. Jung, G.A. Calin, Targeting microRNAs with small molecules: from dream to reality, Clinical pharmacology and therapeutics, 87 (2010) 754-758.
[89] S. Melo, A. Villanueva, C. Moutinho, V. Davalos, R. Spizzo, C. Ivan, S. Rossi, F. Setien, O. Casanovas, L. Simo-Riudalbas, J. Carmona, J. Carrere, A. Vidal, A. Aytes, S. Puertas, S. Ropero, R. Kalluri, C.M. Croce, G.A. Calin, M. Esteller, Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing, Proceedings of the National Academy of Sciences of the United States of America, 108 (2011) 4394-4399.

[90] H.W. Wang, C. Noland, B. Siridechadilok, D.W. Taylor, E. Ma, K. Felderer, J.A. Doudna, E. Nogales, Structural insights into RNA processing by the human RISC-loading complex, Nat Struct Mol Biol, 16 (2009) 1148-1153.
[91] J.R. Thomas, P.J. Hergenrother, Targeting RNA with small molecules, Chem Rev, 108 (2008) 1171-1224.
[92] A. Deiters, Small molecule modifiers of the microRNA and RNA interference pathway, AAPS J, 12 (2010) 51-60.
[93] M.S. Pop, N. Stransky, C.W. Garvie, J.P. Theurillat, E.C. Hartman, T.A. Lewis, C. Zhong, E.K. Culyba, F. Lin, D.S. Daniels, R. Pagliarini, L. Ronco, A.N. Koehler, L.A. Garraway, A small molecule that binds and inhibits the ETV1 transcription factor oncoprotein, Molecular cancer therapeutics, 13 (2014) 1492-1502.
[94] S.J. Haggarty, K.M. Koeller, J.C. Wong, C.M. Grozinger, S.L. Schreiber, Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation, Proceedings of the National Academy of Sciences of the United States of America, 100 (2003) 4389-4394.
[95] K.A. O'Donnell, E.A. Wentzel, K.I. Zeller, C.V. Dang, J.T. Mendell, c-Myc-regulated microRNAs modulate E2F1 expression, Nature, 435 (2005) 839-843.
[96] Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, V.N. Kim, The nuclear RNase III Drosha initiates microRNA processing, Nature, 425 (2003) 415-419.
[97] R.I. Gregory, K.P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, R. Shiekhattar, The Microprocessor complex mediates the genesis of microRNAs, Nature, 432 (2004) 235-240.
[98] J. Han, Y. Lee, K.H. Yeom, Y.K. Kim, H. Jin, V.N. Kim, The Drosha-DGCR8 complex in primary microRNA processing, Genes Dev, 18 (2004) 3016-3027.
[99] G. Hutvagner, J. McLachlan, A.E. Pasquinelli, E. Balint, T. Tuschl, P.D. Zamore, A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA, Science, 293 (2001) 834-838.
[100] P.D. Monroig, L. Chen, S. Zhang, G.A. Calin, Small molecule compounds targeting miRNAs for cancer therapy, Advanced drug delivery reviews, 81C (2015) 104-116.
[101] Z. Shi, J. Zhang, X. Qian, L. Han, K. Zhang, L. Chen, J. Liu, Y. Ren, M. Yang, A. Zhang, P. Pu, C. Kang, AC1MMYR2, an inhibitor of dicer-mediated biogenesis of Oncomir miR-21, reverses epithelial-mesenchymal transition and suppresses tumor growth and progression, Cancer research, 73 (2013) 5519-5531.
[102] C.M. Connelly, M. Thomas, A. Deiters, High-throughput luciferase reporter assay for small-molecule inhibitors of microRNA function, J Biomol Screen, 17 (2012) 822-828.
[103] D. Bose, G.G. Jayaraj, S. Kumar, S. Maiti, A molecular-beacon-based screen for small molecule inhibitors of miRNA maturation, ACS chemical biology, 8 (2013) 930-938.
[104] K. Gumireddy, D.D. Young, X. Xiong, J.B. Hogenesch, Q. Huang, A. Deiters, Small-molecule inhibitors of microrna miR-21 function, Angew Chem Int Ed Engl, 47 (2008) 7482-7484.
[105] G.S. Tan, C.H. Chiu, B.G. Garchow, D. Metzler, S.L. Diamond, M. Kiriakidou, Small molecule inhibition of RISC loading, ACS chemical biology, 7 (2012) 403-410.
[106] B.P. Davies, C. Arenz, A fluorescence probe for assaying micro RNA maturation, Bioorg Med Chem, 16 (2008) 49-55.
[107] B.P. Davies, C. Arenz, A homogenous assay for micro RNA maturation, Angew Chem Int Ed Engl, 45 (2006) 5550-5552.

[108] D. Bose, G. Jayaraj, H. Suryawanshi, P. Agarwala, S.K. Pore, R. Banerjee, S. Maiti, The tuberculosis drug streptomycin as a potential cancer therapeutic: inhibition of miR-21 function by directly targeting its precursor, Angew Chem Int Ed Engl, 51 (2012) 1019-1023.
[109] Y. Li, J. Shen, X. Sun, W. Li, G. Liu, Y. Tang, Accuracy assessment of protein-based docking programs against RNA targets, Journal of chemical information and modeling, 50 (2010) 1134-1146.
[110] R. Albulescu, TGF-beta upregulates miR-181a expression to promote breast cancer metastasis, Biomarkers in medicine, 7 (2013) 204.
[111] A. Bisso, M. Faleschini, F. Zampa, V. Capaci, J. De Santa, L. Santarpia, S. Piazza, V. Cappelletti, M. Daidone, R. Agami, G. Del Sal, Oncogenic miR-181a/b affect the DNA damage response in aggressive breast cancer, Cell Cycle, 12 (2013) 1679-1687.
[112] M.A. Taylor, K. Sossey-Alaoui, C.L. Thompson, D. Danielpour, W.P. Schiemann, TGF-beta upregulates miR-181a expression to promote breast cancer metastasis, The Journal of clinical investigation, 123 (2013) 150-163.
[113] Y. Wang, Y. Yu, A. Tsuyada, X. Ren, X. Wu, K. Stubblefield, E.K. Rankin-Gee, S.E. Wang, Transforming growth factor-beta regulates the sphere-initiating stem cell-like feature in breast cancer through miRNA-181 and ATM, Oncogene, 30 (2011) 1470-1480.
[114] A.C. Godfrey, Z. Xu, C.R. Weinberg, R.C. Getts, P.A. Wade, L.A. DeRoo, D.P. Sandler, J.A. Taylor, Serum microRNA expression as an early marker for breast cancer risk in prospectively collected samples from the Sister Study cohort, Breast cancer research : BCR, 15 (2013) R42.
[115] M. Sochor, P. Basova, M. Pesta, N. Dusilkova, J. Bartos, P. Burda, V. Pospisil, T. Stopka, Oncogenic microRNAs: miR-155, miR-19a, miR-181b, and miR-24 enable monitoring of early breast cancer in serum, BMC cancer, 14 (2014) 448.
[116] M. Ouyang, Y. Li, S. Ye, J. Ma, L. Lu, W. Lv, G. Chang, X. Li, Q. Li, S. Wang, W. Wang, MicroRNA profiling implies new markers of chemoresistance of triple-negative breast cancer, PloS one, 9 (2014) e96228.
[117] G. Mansueto, F. Forzati, A. Ferraro, P. Pallante, M. Bianco, F. Esposito, A. Iaccarino, G. Troncone, A. Fusco, Identification of a New Pathway for Tumor Progression: MicroRNA-181b Up-Regulation and CBX7 Down-Regulation by HMGA1 Protein, Genes & cancer, 1 (2010) 210-224.
[118] S. Li, X. Yang, J. Yang, J. Zhen, D. Zhang, Serum microRNA-21 as a potential diagnostic biomarker for breast cancer: a systematic review and meta-analysis, Clinical and experimental medicine, (2014).
[119] G. Dong, X. Liang, D. Wang, H. Gao, L. Wang, L. Wang, J. Liu, Z. Du, High expression of miR-21 in triple-negative breast cancers was correlated with a poor prognosis and promoted tumor cell in vitro proliferation, Medical oncology, 31 (2014) 57.
[120] M.F. Wu, J. Yang, T. Xiang, Y.Y. Shi, L.J. Liu, miR-21 targets Fas ligand-mediated apoptosis in breast cancer cell line MCF-7, Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban, 34 (2014) 190-194.
[121] T.H. Huang, F. Wu, G.B. Loeb, R. Hsu, A. Heidersbach, A. Brincat, D. Horiuchi, R.J. Lebbink, Y.Y. Mo, A. Goga, M.T. McManus, Up-regulation of miR-21 by HER2/neu signaling promotes cell invasion, The Journal of biological chemistry, 284 (2009) 18515-18524.

[122] B. Qian, D. Katsaros, L. Lu, M. Preti, A. Durando, R. Arisio, L. Mu, H. Yu, High miR-21 expression in breast cancer associated with poor disease-free survival in early stage disease and high TGF-beta1, Breast cancer research and treatment, 117 (2009) 131-140.
[123] Y. Lu, J. Yao, J. Yu, Q. Wei, X. Cao, The association between abnormal microRNA-10b expression and cancer risk: a meta-analysis, Sci Rep, 4 (2014) 7498.
[124] Z. Lu, M. Liu, V. Stribinskis, C.M. Klinge, K.S. Ramos, N.H. Colburn, Y. Li, MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene, Oncogene, 27 (2008) 4373-4379.
[125] S. Zhu, M.L. Si, H. Wu, Y.Y. Mo, MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1), The Journal of biological chemistry, 282 (2007) 14328-14336.
[126] S. Volinia, G.A. Calin, C.G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R.L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C.C. Harris, C.M. Croce, A microRNA expression signature of human solid tumors defines cancer gene targets, Proceedings of the National Academy of Sciences of the United States of America, 103 (2006) 2257-2261.
[127] J. Kluiver, S. Poppema, D. de Jong, T. Blokzijl, G. Harms, S. Jacobs, B.J. Kroesen, A. van den Berg, BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas, The Journal of pathology, 207 (2005) 243-249.
[128] W. Kong, L. He, M. Coppola, J. Guo, N.N. Esposito, D. Coppola, J.Q. Cheng, MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer, The Journal of biological chemistry, 285 (2010) 17869-17879.
[129] A. Parikh, C. Lee, P. Joseph, S. Marchini, A. Baccarini, V. Kolev, C. Romualdi, R. Fruscio, H. Shah, F. Wang, G. Mullokandov, D. Fishman, M. D'Incalci, J. Rahaman, T. Kalir, R.W. Redline, B.D. Brown, G. Narla, A. DiFeo, microRNA-181a has a critical role in ovarian cancer progression through the regulation of the epithelial-mesenchymal transition, Nature communications, 5 (2014) 2977.
[130] J.M. Gwak, H.J. Kim, E.J. Kim, Y.R. Chung, S. Yun, A.N. Seo, H.J. Lee, S.Y. Park, MicroRNA-9 is associated with epithelial-mesenchymal transition, breast cancer stem cell phenotype, and tumor progression in breast cancer, Breast cancer research and treatment, 147 (2014) 39-49.
[131] X. Zhou, C. Marian, K.H. Makambi, O. Kosti, B.V. Kallakury, C.A. Loffredo, Y.L. Zheng, MicroRNA-9 as potential biomarker for breast cancer local recurrence and tumor estrogen receptor status, PloS one, 7 (2012) e39011.
[132] J. Krell, A.E. Frampton, J. Jacob, L. Pellegrino, L. Roca-Alonso, D. Zeloof, C. Alifrangis, J.S. Lewis, L.R. Jiao, J. Stebbing, L. Castellano, The clinico-pathologic role of microRNAs miR-9 and miR-151-5p in breast cancer metastasis, Molecular diagnosis & therapy, 16 (2012) 167-172.
[133] Y. Khew-Goodall, G.J. Goodall, Myc-modulated miR-9 makes more metastases, Nat Cell Biol, 12 (2010) 209-211.
[134] Y. Sun, J. Wu, S.H. Wu, A. Thakur, A. Bollig, Y. Huang, D.J. Liao, Expression profile of microRNAs in c-Myc induced mouse mammary tumors, Breast cancer research and treatment, 118 (2009) 185-196.
[135] T.A. Farazi, H.M. Horlings, J.J. Ten Hoeve, A. Mihailovic, H. Halfwerk, P. Morozov, M. Brown, M. Hafner, F. Reyal, M. van Kouwenhove, B. Kreike, D. Sie, V. Hovestadt, L.F. Wessels, M.J. van de Vijver, T. Tuschl, MicroRNA sequence and expression analysis in breast tumors by deep sequencing, Cancer research, 71 (2011) 4443-4453.

[136] A. Ahmad, S. Sethi, W. Chen, R. Ali-Fehmi, S. Mittal, F.H. Sarkar, Up-regulation of microRNA-10b is associated with the development of breast cancer brain metastasis, Am J Transl Res, 6 (2014) 384-390.
[137] Q.J. Li, L. Zhou, F. Yang, G.X. Wang, H. Zheng, D.S. Wang, Y. He, K.F. Dou, MicroRNA-10b promotes migration and invasion through CADM1 in human hepatocellular carcinoma cells, Tumour Biol, 33 (2012) 1455-1465.
[138] C.H. Moriarty, B. Pursell, A.M. Mercurio, miR-10b targets Tiam1: implications for Rac activation and carcinoma migration, The Journal of biological chemistry, 285 (2010) 20541-20546.
[139] N. Cancer Genome Atlas Research, Integrated genomic analyses of ovarian carcinoma, Nature, 474 (2011) 609-615.
[140] S.F. Tavazoie, C. Alarcon, T. Oskarsson, D. Padua, Q. Wang, P.D. Bos, W.L. Gerald, J. Massague, Endogenous human microRNAs that suppress breast cancer metastasis, Nature, 451 (2008) 147-152.
[141] P.K. Mazur, N. Reynoird, P. Khatri, P.W. Jansen, A.W. Wilkinson, S. Liu, O. Barbash, G.S. Van Aller, M. Huddleston, D. Dhanak, P.J. Tummino, R.G. Kruger, B.A. Garcia, A.J. Butte, M. Vermeulen, J. Sage, O. Gozani, SMYD3 links lysine methylation of MAP3K2 to Ras-driven cancer, Nature, 510 (2014) 283-287.
[142] E. Rognoni, M. Widmaier, M. Jakobson, R. Ruppert, S. Ussar, D. Katsougkri, R.T. Bottcher, J.E. Lai-Cheong, D.B. Rifkin, J.A. McGrath, R. Fassler, Kindlin-1 controls Wnt and TGF-beta availability to regulate cutaneous stem cell proliferation, Nature medicine, 20 (2014) 350-359.
[143] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic acids research, 29 (2001) e45.
[144] T. Puton, L.P. Kozlowski, K.M. Rother, J.M. Bujnicki, CompaRNA: a server for continuous benchmarking of automated methods for RNA secondary structure prediction, Nucleic acids research, 41 (2013) 4307-4323.
[145] K. Sato, M. Hamada, K. Asai, T. Mituyama, CENTROIDFOLD: a web server for RNA secondary structure prediction, Nucleic acids research, 37 (2009) W277-280.
[146] K.J. Purzycka, M. Popenda, M. Szachniuk, M. Antczak, P. Lukasiak, J. Blazewicz, R.W. Adamiak, Automated 3D RNA structure prediction using the RNAComposer method for riboswitches, Methods Enzymol, 553 (2015) 3-34.
[147] S. Ruiz-Carmona, D. Alvarez-Garcia, N. Foloppe, A.B. Garmendia-Doval, S. Juhos, P. Schmidtke, X. Barril, R.E. Hubbard, S.D. Morley, rDock: a fast, versatile and open source program for docking ligands to proteins and nucleic acids, PLoS computational biology, 10 (2014) e1003571.
[148] L. Chen, G.A. Calin, S. Zhang, Novel insights of structure-based modeling for RNA-targeted drug discovery, Journal of chemical information and modeling, 52 (2012) 2741-2753.
[149] E.P. Nikonowicz, A. Sirr, P. Legault, F.M. Jucker, L.M. Baer, A. Pardi, Preparation of 13C and 15N labelled RNAs for heteronuclear multi-dimensional NMR studies, Nucleic acids research, 20 (1992) 4507-4513.
[150] J.F. Milligan, D.R. Groebe, G.W. Witherell, O.C. Uhlenbeck, Oligoribonucleotide synthesis using T7 RNA Polymerase and Synthetic DNA Templates, Nucleic acids research, 15 (1987) 8783-8789.
[151] T. Dieckmann, J. Feigon, Assignment methodology for larger RNA oligonucleotides: application to an ATP-binding RNA aptamer, Journal of Biomolecular NMR, 9 (1997) 259-272.
[152] P. Bayer, L. Varani, G. Varani, Nuclear magnetic resonance methods to study RNA-protein complexes, Methods in enzymology, 317 (2000) 198-220.

[153] E. Miclet, E. O'Neil-Cabello, E.P. Nikonowicz, D. Live, A. Bax, 1H-1H dipolar couplings provide a unique probe of RNA backbone structure, Journal of the American Chemical Society, 125 (2003) 15740-15741.
[154] E.T. Mollova, A. Pardi, NMR solution structure determination of RNAs, Curr Opin Struct Biol, 10 (2000) 298-302.
[155] J. Wang, E.P. Nikonowicz, Solution structure of the K-turn and Specifier Loop domains from the Bacillus subtilis tyrS T-box leader RNA, Journal of molecular biology, 408 (2011) 99-117.
[156] A.T. Chang, E.P. Nikonowicz, Solution nuclear magnetic resonance analyses of the anticodon arms of proteinogenic and nonproteinogenic tRNA(Gly), Biochemistry, 51 (2012) 3662-3674.
[157] A.P. Denmon, J. Wang, E.P. Nikonowicz, Conformation effects of base modification on the anticodon stem-loop of Bacillus subtilis tRNA(Tyr), Journal of molecular biology, 412 (2011) 285-303.
[158] S.A. Jiang, H.W. Zhang, M.H. Lu, X.H. He, Y. Li, H. Gu, M.F. Liu, E.D. Wang, MicroRNA-155 Functions as an OncomiR in Breast Cancer by Targeting the Suppressor of Cytokine Signaling 1 Gene, Cancer research, 70 (2010) 3119-3127.
[159] I. Nakayama, M. Shibazaki, A. Yashima-Abo, F. Miura, T. Sugiyama, T. Masuda, C. Maesawa, Loss of HOXD10 expression induced by upregulation of miR-10b accelerates the migration and invasion activities of ovarian cancer cells, International journal of oncology, 43 (2013) 63-71.
[160] H. Ouyang, J. Gore, S. Deitz, M. Korc, microRNA-10b enhances pancreatic cancer cell invasion by suppressing TIP30 expression and promoting EGF and TGF-beta actions, Oncogene, 33 (2014) 4664-4674.
[161] T. Setoyama, X. Zhang, S. Natsugoe, G.A. Calin, microRNA-10b: a new marker or the marker of pancreatic ductal adenocarcinoma?, Clinical cancer research : an official journal of the American Association for Cancer Research, 17 (2011) 5527-5529.
[162] L. Hutchinson, Breast cancer: challenges, controversies, breakthroughs, Nature reviews. Clinical oncology, 7 (2010) 669-670.
[163] A. Jemal, R. Siegel, J. Xu, E. Ward, Cancer statistics, 2010, CA: a cancer journal for clinicians, 60 (2010) 277-300.
[164] L.G. Martelotto, C.K. Ng, S. Piscuoglio, B. Weigelt, J.S. Reis-Filho, Breast cancer intra-tumor heterogeneity, Breast cancer research : BCR, 16 (2014) 210.
[165] O.C. Freedman, G.G. Fletcher, S. Gandhi, M. Mates, S.F. Dent, M.E. Trudeau, A. Eisen, Adjuvant endocrine therapy for early breast cancer: a systematic review of the evidence for the 2014 Cancer Care Ontario systemic therapy guideline, Current oncology, 22 (2015) S95-S113.
[166] M.C. Figueroa-Magalhaes, D. Jelovac, R.M. Connolly, A.C. Wolff, Treatment of HER2-positive breast cancer, Breast, 23 (2014) 128-136.
[167] D. Gajria, S. Chandarlapaty, HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies, Expert review of anticancer therapy, 11 (2011) 263-275.
[168] C.K. Osborne, R. Schiff, Mechanisms of endocrine resistance in breast cancer, Annual review of medicine, 62 (2011) 233-247.
[169] C.K. Anders, L.A. Carey, Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer, Clinical breast cancer, 9 Suppl 2 (2009) S73-81.
[170] R. Dent, M. Trudeau, K.I. Pritchard, W.M. Hanna, H.K. Kahn, C.A. Sawka, L.A. Lickley, E. Rawlinson, P. Sun, S.A. Narod, Triple-negative breast cancer: clinical features and patterns of recurrence, Clinical cancer research : an official journal of the American Association for Cancer Research, 13 (2007) 4429-4434.

[171] F. Meric-Bernstam, G.B. Mills, Overcoming implementation challenges of personalized cancer therapy, Nature reviews. Clinical oncology, 9 (2012) 542-548.
[172] F.J. Esteva, G.N. Hortobagyi, Prognostic molecular markers in early breast cancer, Breast cancer research : BCR, 6 (2004) 109-118.
[173] H. Dvinge, A. Git, S. Graf, M. Salmon-Divon, C. Curtis, A. Sottoriva, Y. Zhao, M. Hirst, J. Armisen, E.A. Miska, S.F. Chin, E. Provenzano, G. Turashvili, A. Green, I. Ellis, S. Aparicio, C. Caldas, The shaping and functional consequences of the microRNA landscape in breast cancer, Nature, 497 (2013) 378-382.
[174] Y. Lou, X. Yang, F. Wang, Z. Cui, Y. Huang, MicroRNA-21 promotes the cell proliferation, invasion and migration abilities in ovarian epithelial carcinomas through inhibiting the expression of PTEN protein, International journal of molecular medicine, 26 (2010) 819-827.
[175] F. Sicard, M. Gayral, H. Lulka, L. Buscail, P. Cordelier, Targeting miR-21 for the therapy of pancreatic cancer, Molecular therapy : the journal of the American Society of Gene Therapy, 21 (2013) 986-994.
[176] D.D. Young, C.M. Connelly, C. Grohmann, A. Deiters, Small molecule modifiers of microRNA miR-122 function for the treatment of hepatitis C virus infection and hepatocellular carcinoma, J Am Chem Soc, 132 (2010) 7976-7981.
[177] K. Watashi, M.L. Yeung, M.F. Starost, R.S. Hosmane, K.T. Jeang, Identification of small molecules that suppress microRNA function and reverse tumorigenesis, The Journal of biological chemistry, 285 (2010) 24707-24716.
[178] D.D. Vo, C. Staedel, L. Zehnacker, R. Benhida, F. Darfeuille, M. Duca, Targeting the Production of Oncogenic MicroRNAs with Multimodal Synthetic Small Molecules, ACS chemical biology, 9 (2014) 711-721.
[179] S.P. Velagapudi, S.M. Gallo, M.D. Disney, Sequence-based design of bioactive small molecules that target precursor microRNAs, Nature chemical biology, 10 (2014) 291-297.
[180] A. Murata, T. Fukuzumi, S. Umemoto, K. Nakatani, Xanthone derivatives as potential inhibitors of miRNA processing by human Dicer: targeting secondary structures of pre-miRNA by small molecules, Bioorganic & medicinal chemistry letters, 23 (2013) 252-255.
[181] J. Davenport, M. Balch, L. Galam, A. Girgis, J. Hall, B.S. Blagg, R.L. Matts, High-throughput screen of natural product libraries for hsp90 inhibitors, Biology, 3 (2014) 101-138.
[182] R.S. Atwal, C.R. Desmond, N. Caron, T. Maiuri, J. Xia, S. Sipione, R. Truant, Kinase inhibitors modulate huntingtin cell localization and toxicity, Nature chemical biology, 7 (2011) 453-460.
[183] R.E. Morgan, S. Ahn, S. Nzimiro, J. Fotie, M.A. Phelps, J. Cotrill, A.J. Yakovich, D.L. Sackett, J.T. Dalton, K.A. Werbovetz, Inhibitors of tubulin assembly identified through screening a compound library, Chemical biology & drug design, 72 (2008) 513-524.
[184] E. Minet, D. Mottet, G. Michel, I. Roland, M. Raes, J. Remacle, C. Michiels, Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction, FEBS letters, 460 (1999) 251-256.
[185] Y.V. Liu, J.H. Baek, H. Zhang, R. Diez, R.N. Cole, G.L. Semenza, RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O(2)-independent and HSP90 inhibitor-induced degradation of HIF-1alpha, Molecular cell, 25 (2007) 207-217.
[186] F. Koga, S. Tsutsumi, L.M. Neckers, Low dose geldanamycin inhibits hepatocyte growth factor and hypoxia-stimulated invasion of cancer cells, Cell Cycle, 6 (2007) 1393-1402.

[187] J. Massague, TGFbeta signalling in context, Nature reviews. Molecular cell biology, 13 (2012) 616-630.
[188] C. Zhang, F. Guo, G. Xu, J. Ma, F. Shao, STAT3 cooperates with Twist to mediate epithelial-mesenchymal transition in human hepatocellular carcinoma cells, Oncology reports, 33 (2015) 1872-1882.
[189] G.Z. Cheng, W.Z. Zhang, M. Sun, Q. Wang, D. Coppola, M. Mansour, L.M. Xu, C. Costanzo, J.Q. Cheng, L.H. Wang, Twist is transcriptionally induced by activation of STAT3 and mediates STAT3 oncogenic function, The Journal of biological chemistry, 283 (2008) 14665-14673.
[190] M.R. Pawlus, L. Wang, C.J. Hu, STAT3 and HIF1alpha cooperatively activate HIF1 target genes in MDA-MB-231 and RCC4 cells, Oncogene, 33 (2014) 1670-1679.
[191] Q. Xu, J. Briggs, S. Park, G. Niu, M. Kortylewski, S. Zhang, T. Gritsko, J. Turkson, H. Kay, G.L. Semenza, J.Q. Cheng, R. Jove, H. Yu, Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways, Oncogene, 24 (2005) 5552-5560.
[192] Linifanib, Drugs R D, 10 (2010) 111-122.
[193] H. Asahina, Y. Tamura, H. Nokihara, N. Yamamoto, Y. Seki, T. Shibata, Y. Goto, M. Tanioka, Y. Yamada, A. Coates, Y.L. Chiu, X. Li, R. Pradhan, P.J. Ansell, E.M. McKeegan, M.D. McKee, D.M. Carlson, T. Tamura, An open-label, phase 1 study evaluating safety, tolerability, and pharmacokinetics of linifanib (ABT-869) in Japanese patients with solid tumors, Cancer chemotherapy and pharmacology, 69 (2012) 1477-1486.
[194] E.H. Tan, G.D. Goss, R. Salgia, B. Besse, D.R. Gandara, N.H. Hanna, J.C. Yang, R. Thertulien, M. Wertheim, J. Mazieres, T. Hensing, C. Lee, N. Gupta, R. Pradhan, J. Qian, Q. Qin, F.A. Scappaticci, J.L. Ricker, D.M. Carlson, R.A. Soo, Phase 2 trial of Linifanib (ABT-869) in patients with advanced non-small cell lung cancer, Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer, 6 (2011) 1418-1425.
[195] N.M. Tannir, Y.N. Wong, C.K. Kollmannsberger, M.S. Ernstoff, D.J. Perry, L.J. Appleman, E.M. Posadas, D. Cho, T.K. Choueiri, A. Coates, N. Gupta, R. Pradhan, J. Qian, J. Chen, F.A. Scappaticci, J.L. Ricker, D.M. Carlson, M.D. Michaelson, Phase 2 trial of linifanib (ABT-869) in patients with advanced renal cell cancer after sunitinib failure, European journal of cancer, 47 (2011) 2706-2714.
[196] C.I. Wong, T.S. Koh, R. Soo, S. Hartono, C.H. Thng, E. McKeegan, W.P. Yong, C.S. Chen, S.C. Lee, J. Wong, R. Lim, N. Sukri, S.E. Lim, A.B. Ong, J. Steinberg, N. Gupta, R. Pradhan, R. Humerickhouse, B.C. Goh, Phase I and biomarker study of ABT-869, a multiple receptor tyrosine kinase inhibitor, in patients with refractory solid malignancies, Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 27 (2009) 4718-4726.
[197] S. Wilhelm, C. Carter, M. Lynch, T. Lowinger, J. Dumas, R.A. Smith, B. Schwartz, R. Simantov, S. Kelley, Discovery and development of sorafenib: a multikinase inhibitor for treating cancer, Nature reviews. Drug discovery, 5 (2006) 835-844.
[198] A.L. Cheng, Y.K. Kang, Z. Chen, C.J. Tsao, S. Qin, J.S. Kim, R. Luo, J. Feng, S. Ye, T.S. Yang, J. Xu, Y. Sun, H. Liang, J. Liu, J. Wang, W.Y. Tak, H. Pan, K. Burock, J. Zou, D. Voliotis, Z. Guan, Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial, The Lancet. Oncology, 10 (2009) 25-34.
[199] J. Bruix, J.L. Raoul, M. Sherman, V. Mazzaferro, L. Bolondi, A. Craxi, P.R. Galle, A. Santoro, M. Beaugrand, A. Sangiovanni, C. Porta, G. Gerken, J.A. Marrero,

A. Nadel, M. Shan, M. Moscovici, D. Voliotis, J.M. Llovet, Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial, Journal of hepatology, 57 (2012) 821-829.
[200] C. Cainap, S. Qin, W.T. Huang, I.J. Chung, H. Pan, Y. Cheng, M. Kudo, Y.K. Kang, P.J. Chen, H.C. Toh, V. Gorbunova, F.A. Eskens, J. Qian, M.D. McKee, J.L. Ricker, D.M. Carlson, S. El-Nowiem, Linifanib versus Sorafenib in patients with advanced hepatocellular carcinoma: results of a randomized phase III trial, Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 33 (2015) 172-179.
[201] F. Carlomagno, S. Anaganti, T. Guida, G. Salvatore, G. Troncone, S.M. Wilhelm, M. Santoro, BAY 43-9006 inhibition of oncogenic RET mutants, Journal of the National Cancer Institute, 98 (2006) 326-334.
[202] N. Nishida, S. Yamashita, K. Mimori, T. Sudo, F. Tanaka, K. Shibata, H. Yamamoto, H. Ishii, Y. Doki, M. Mori, MicroRNA-10b is a prognostic indicator in colorectal cancer and confers resistance to the chemotherapeutic agent 5-fluorouracil in colorectal cancer cells, Annals of surgical oncology, 19 (2012) 3065-3071.
[203] Y. Liu, M. Li, G. Zhang, Z. Pang, MicroRNA-10b overexpression promotes non-small cell lung cancer cell proliferation and invasion, Eur J Med Res, 18 (2013) 41.
[204] J. Zhang, L. Xu, Z. Yang, H. Lu, D. Hu, W. Li, Z. Zhang, B. Liu, S. Ma, MicroRNA-10b indicates a poor prognosis of non-small cell lung cancer and targets E-cadherin, Clin Transl Oncol, 17 (2015) 209-214.
[205] C.G. Liao, L.M. Kong, P. Zhou, X.L. Yang, J.G. Huang, H.L. Zhang, N. Lu, miR-10b is overexpressed in hepatocellular carcinoma and promotes cell proliferation, migration and invasion through RhoC, uPAR and MMPs, J Transl Med, 12 (2014) 234.
[206] K. Nakata, K. Ohuchida, K. Mizumoto, T. Kayashima, N. Ikenaga, H. Sakai, C. Lin, H. Fujita, T. Otsuka, S. Aishima, E. Nagai, Y. Oda, M. Tanaka, MicroRNA-10b is overexpressed in pancreatic cancer, promotes its invasiveness, and correlates with a poor prognosis, Surgery, 150 (2011) 916-922.
[207] C.A. Lipinski, Drug-like properties and the causes of poor solubility and poor permeability, J Pharmacol Toxicol Methods, 44 (2000) 235-249.
[208] R. Mannhold, The impact of lipophilicity in drug research: a case report on beta-blockers, Mini Rev Med Chem, 5 (2005) 197-205.