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With the discovery, in the last few years, of thousands of genes that produce small non-coding RNAs transcripts with no significant open reading frame named microRNAs (miRNAs), it has become evident that the genomic complexity of the cancer cell is far greater than expected. Breast cancer (BC) is the most common cancer in women and over a third of women with BC will develop metastatic diseases. The broad goal of our research program is to develop an innovative approach of a novel microRNA-based targeted therapy in BC, on the hypothesis that small molecule inhibitors could target miRNAs and this targeting has functional consequences in malignant cells. This proposal focuses on the identification of novel small molecule inhibitors targeting miRNAs using virtual high-throughput screening (vHTS) approaches. We already accomplished the first aim, to identify "in silico" small molecules that target miRNA transcripts. The second aim is under development, the identification of the functional consequences of the interaction between miRNAs and small molecules in BC cells, including cell proliferation, cell death and invasion capacity. Our project already generate preliminary data useful for the basis of a new type of miRNA-based targeted therapy that will benefit BC patients mainly with metastases, by focusing now on hits that target all six proposed miRNAs. We also developed a new method to increase the amount of tested hits, namely a "sensor" vector for each of the miRNAs proposed to be tested that detects the amount of specific miRNA after cell treatment with the small molecule.

**15. SUBJECT TERMS**
non-coding RNAs, small molecules, therapy, metastases

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## INTRODUCTION

MicroRNAs (miRNAs) are non-coding RNAs (ncRNAs) that regulate gene expression. In this project we propose an innovative approach to develop a novel microRNA-based targeted therapy for BC, on the hypothesis that small molecule inhibitors could target miRNAs. We hypothesize that overexpressed miRNAs can be targeted for breast cancer therapy by small molecules, which can be identified and designed in silico. The targeted inhibition will have biological consequences culminating with death of BC cells. The key to this proposal is the close interplay among computer simulation, medicinal synthesis and biological testing, and the scope of this collaboration is that we will develop new potential breakthrough drugs targeting miRNAs and open up a brand new era of drug design for the therapy of breast cancer.

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## BODY

During the first two years of funding we performed experiments related to the first two tasks described in SOW and we accomplished the followings:

**Task 1. In silico identification of small molecules that target *miR-10b, miR-16, miR-21, miR-155, miR-373,* and *miR-520c*.** This task was performed in collaboration with Dr Shuxing Zhang, the co-PI of the project. In the 24 months from the start of the project, we have achieved our goals by constructing the 3D structures models of overexpressed miRNAs in BC and identified sets of their potential hits using our newly developed bioinformatics and cheminformatics integrated high-throughput docking method to screen databases including 10 million compounds. These initially selected hits have been further evaluated in silico using multiple docking/scoring packages, and the final selected hits have been tested for experimental testing. These studies have resulted in one publication in *The Clinical Pharmacology and Therapeutics* (1) and several other manuscripts in preparation (2-4). The detailed work is as follows:

**(1-1) Structural modeling of precursor microRNAs (pre-miRNAs)**

Human ***miR-10b, miR-16, miR-21, miR-155, miR-373,* and *miR-520c*** single-stranded sequences were obtained from miRBase (www.mirbase.org). MC-fold was used to model the secondary structure prior to tertiary structural prediction so as to establish constraints such as canonical/non-canonical base pairings, following with MC-Sym and RosettaRNA to generate diversity pools of coarse models based upon the best scored secondary model for each miRNA. After the structural refinement and relaxation via simulated annealing, the top 10 models were selected based on several RNA-specific scores, such as Score, P-score and radius of gyration, to evaluate the overall energy, likelihood of A-form helix and compactness, respectively.

As expected, initial structural prediction with MC-fold/MC-sym (3) followed by the mechanic structural refinement approach can reproduce the pre-miRNA basic A-form helix that can be observed in miR-30a and let-7 crystal or NMR structures (3A6P and 2KPV). The superimposition exhibits the conserved antiparallel RNA A-helix fold among the selected pre-miRNA targets. Furthermore, 3D characteristics including Watson-Crick base pairs and wobble base pairs, bulges and stem loops generally match the predicted secondary structures in miRBase. Heavy-atom-based RMSD between the predicted pre-miR-30a and its crystal structure (43nt) is as low as 2.55Å. This result confirms the compatibility of using our approaches as the primary miRNA structural prediction tools. For instance, miR-21 A17 stretches towards the solvent and hereby forms a bulge behind the Dicer docking surface,

whereas two successive bulges made by miR-155 C15 and A19 bend the duplex structure about 35°. The diverse chemical environment around different pre-miRNA bulges may provide selectivity for the discovery of novel drugs targeting miRNAs with greater specificity. The pocket created by these mismatches in major or minor groove also provides additional specificity against RNAs other than miRNAs. Compared with the relatively uniformed helix part, the structure of stem loop exhibits significantly variability among 10 decoys of each miRNAs. This may provide even further possibility to design selective miRNA inhibitors. To date we have predicted structures of miR-21, miR-16, miR-155, miR-10b and miR-373. Fig 1 demonstrates a predicted structure of miR-10b in surface representation.

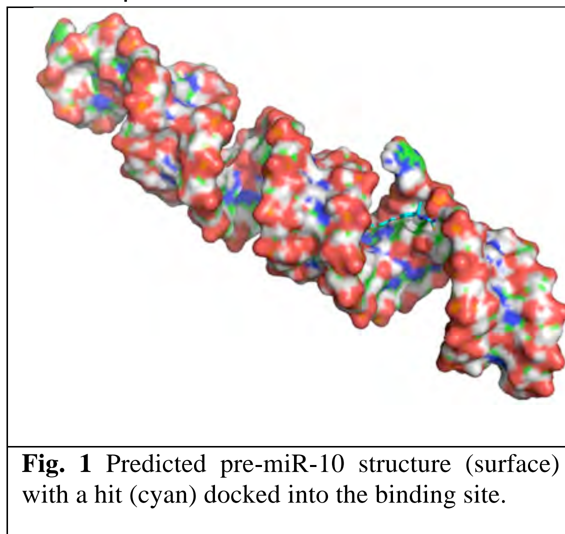

**Fig. 1** Predicted pre-miR-10 structure (surface) with a hit (cyan) docked into the binding site.

**(1-2) Validation of docking programs and scoring functions against RNA targets**

As most existing docking programs are designed for protein targets, it is crucial to perform an assessment to validate the compatibility of these classical docking programs with RNA targets and provide hints to develop miRNA specific approaches. We collected a total of 34 RNA-ligand complex structures from PDB, including 22 high-resolution crystal structures and 9 NMR structures, most of which have been used to evaluate the accuracy of various docking programs against RNA targets (4-11). RNA receptors in this dataset cover rRNA, HIV-1 TAR RNA, riboswitch, aptamer, etc, whereas the ligands contains 17 flexible aminoglycosides and 17 other relatively rigid small compounds. Unfortunately there are no available miRNA/pre-miRNA structures complexed with any small molecule ligands. Five popular docking programs, AutoDock 4.0, GOLD 4.0, Glide 5.6, FRED 2.2.5, and Surflex 2.3 were used in this study. Here we used RMSD value of 3.0Å and top 5-ranked conformations as the cutoff to determine the ability of pocket identification and pose sampling for each docking suite.

Apparently, GOLD stands out for successfully docking 79% of the ligands back to their cognate complexes. The success rate of GOLD for flexible aminoglycosides is surprisingly as high as 82%, which is statistically significantly higher than any other docking software. The rank for the correct poses for the successful cases is $1.64\pm0.39$ ($\alpha=0.05$) for GOLD. Glide ranks 2$^{nd}$ and has comparable performance with GOLD on docking small molecules. While GOLD seems to have difficulties in sampling and scoring the orientation of acidic tail, such as 2Z74, 2Z75, 2GDI, 1F27, this can be solved by rescoring the docked decoys by other scoring functions like GlideScore or Surflex-Dock Score. With this approach, we have identified an active hit which is docked into the pre-miR-10b (Fig. 1).

**(1-3) Virtual screening for hit identification against miRNA/pre-miRNA targets**

A diverse set of our HiPCDock and GOLD against the miRNA/pre-miRNA targets, whose overexpression can be frequently observed in breast cancer. The top 1000 hits were selected based on HiPCDock score and GoldFitness ranking and manually visualized to choose drug-like compounds. Most selected compounds were docked into the major groove, forming H-bonding

interaction with the several bases (e.g. G18, A19, C20, G54, U55, C56 or G57). The channel created by A17 pentose and A19 base is usually preferred by high ranked molecules. In addition to H-bonding interaction, we also take into account the aromatic effects like π-coplanarity and π -π stacking. The docked conformation of one hit is showed as an example in Fig. 1e. To address the miRNA structural flexibility, normal mode analysis has been employed to generate the most probably target conformations for docking. This simplified molecular dynamics-based approach will help us to study the dynamics of the target structures and perform flexible docking. Additionally cross docking (dock each hit to several miRNA targets) was conducted to study the binding specificity. As expected many initial hits could bind to multiple miRNAs but indeed we observed series of active hits specific to a particular miRNA targets. **Table 1** has listed our hits and they have been submitted for experimental evaluations.

| Test_ID | miR-10b | | miR-21 | | miR-155 | | miR-373 | | Promiscuity | MW | LogP | No. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SZC-6 | 76.36 | -5.34 | 63.51 | | 66.7 | | 67.14 | -5.14 | 1 | 496.57 | 3.53 | 1 |
| SZC-9 | 61.41 | | 68.84 | | 63.77 | | 71.71 | -5.51 | 1 | 497.97 | 4.96 | 2 |
| SZC-11 | 69.93 | -7.07 | 57.69 | | N/A | | 56.7 | | 1 | 371.42 | 4.08 | 3 |
| SZC-13 | 75.74 | -4.28 | 66.36 | | 73.77 | -5.59 | 76.22 | -5.12 | 1 | 493.59 | 5.87 | 4 |
| SZC-15 | 68.35 | -6.11 | 67.16 | -6.60 | 72.46 | -7.53 | 66.6 | -6.50 | 1 | 491.64 | 3.83 | 5 |
| SZC-18 | 66.35 | | 68.07 | | 66.74 | | 77.13 | -7.27 | 1 | 456.54 | 3.54 | 6 |
| SZC-19 | 69.53 | | 73.2 | -5.75 | 70.92 | -6.59 | 71.08 | -5.83 | 2 | 478.60 | 3.71 | 7 |
| SZC-21 | 64.46 | | 64.07 | | 66.62 | | 70.84 | -5.16 | 1 | 432.50 | 3.16 | 8 |
| SZC-22 | 77.41 | -4.06 | 64.92 | | 65.49 | | 64.19 | | 1 | 423.51 | 2.36 | 9 |
| SZC-24 | 74.73 | -5.34 | 69 | | 72.51 | -6.95 | 61.74 | | 2 | 450.54 | 4.37 | 10 |
| SZC-26 | 68.89 | | 72.36 | -4.09 | 75.96 | -4.83 | 66.1 | | 1 | 489.60 | 4.73 | 11 |
| SZC-27 | 68.96 | | 71.65 | -5.15 | 70.98 | -7.19 | 72.07 | -5.42 | 1 | 412.54 | 3.04 | 12 |
| SZC-28 | 70 | -6.46 | 66.56 | -7.14 | 67.47 | -8.61 | 67.01 | -6.74 | 2 | 484.60 | 1.73 | 13 |
| SZC-30 | 65.64 | -6.50 | 69.12 | -6.88 | 77.79 | -8.28 | 65.82 | -7.00 | 2 | 441.54 | 5.66 | 14 |
| SZC-31 | 73.45 | -5.94 | 60.37 | | 66.72 | | 64.65 | -6.22 | 1 | 379.51 | 3.72 | 15 |
| SZC-32 | 55.8 | | 64.94 | -7.52 | 60.94 | | 55.58 | | 1 | 420.49 | 2.39 | 16 |
| SZC-33 | 59.96 | | 76.72 | -4.56 | 66.95 | | 57.42 | | 1 | 481.64 | 5.16 | 17 |
| SZC-34 | 63.56 | | 67.42 | -6.25 | 72.51 | -7.33 | 59.24 | | 1 | 440.96 | 2.10 | 18 |
| SZC-36 | 61.66 | | 63.02 | | 67.07 | -8.40 | 63.46 | -7.22 | 1 | 427.48 | 4.21 | 19 |
| SZC-37 | 65.25 | | 72.07 | -4.17 | 66.2 | | 71.73 | -4.10 | 1 | 402.54 | 3.26 | 20 |
| SZC-38 | 72.75 | -4.04 | 60.84 | | 65.96 | | 71.54 | -4.44 | 1 | 401.51 | 3.61 | 21 |
| SZC-39 | 62.32 | | 72.51 | -5.60 | 65.92 | | 58.81 | | 1 | 418.47 | 4.03 | 22 |
| SZC-41 | 77.31 | -4.61 | 67.54 | | 69.38 | | 66.33 | | 1 | 472.61 | 4.62 | 23 |
| SZC-42 | 66.14 | | 72.18 | -6.15 | 67.72 | -7.60 | 63.76 | -6.19 | 1 | 496.65 | 2.41 | 24 |
| SZC-43 | 66.14 | | 71.5 | -5.97 | 57.48 | | 61.13 | | 1 | 403.93 | 2.82 | 25 |
| SZC-46 | 61.89 | | 70.07 | -7.82 | 67.38 | -8.18 | 62.32 | | 1 | 497.60 | 1.04 | 26 |
| SZC-50 | 65.32 | -6.32 | 67.92 | -7.16 | 57.6 | | 59.49 | | 1 | 387.38 | 1.00 | 27 |
| SZC-55 | 59.42 | | 67.49 | -7.08 | 70.8 | -7.40 | 62.33 | | 1 | 499.64 | 1.60 | 28 |
| SZC-61 | 72.25 | | 59.66 | | 63.94 | -9.18 | 57.92 | | 1 | 414.49 | 2.36 | 29 |
| SZC-64 | 70.21 | -3.99 | 72.08 | -5.51 | 67.16 | | 69.99 | -5.41 | 1 | 435.55 | 2.32 | 30 |
| SZC-69 | 65.26 | | 67.72 | | 76.87 | -5.88 | 66.36 | | 1 | 388.45 | 2.66 | 31 |
| SZC-70 | 79.01 | | 72.72 | -5.67 | 66.4 | | 59.4 | | 1 | 430.50 | 4.53 | 32 |
| SZC-72 | 59 | | 76.2 | -4.56 | 70.15 | | 62.98 | | 1 | 431.58 | 4.21 | 33 |
| SZC-74 | 69.28 | | 73.19 | -4.20 | 76.55 | -5.90 | 64.64 | | 1 | 467.64 | 4.20 | 34 |
| SZC-84 | 59.76 | | 65.68 | -7.64 | 61.40 | | 60.49 | -7.91 | 2 | 483.58 | 2.72 | 35 |
| SZC-88 | 71.49 | -4.66 | 64.89 | -6.13 | 73.84 | -6.86 | 69.75 | -5.06 | 1 | 379.49 | 1.46 | 36 |
| SZC-89 | 78.52 | -4.17 | 62.74 | | 65.14 | | 69.25 | -4.74 | 1 | 420.52 | 5.03 | 37 |
| SZC-91 | 59.15 | | 57.25 | | 64.03 | -8.27 | 53.00 | | 1 | 438.46 | 1.67 | 38 |
| SZC-92 | 78.72 | -6.15 | 66.71 | -6.13 | 70.15 | -6.82 | 65.52 | -6.70 | 1 | 433.58 | 2.81 | 39 |
| SZC-93 | 69.35 | | 70.43 | -6.09 | 73.26 | -7.15 | 67.69 | -4.88 | 1 | 471.56 | 3.18 | 40 |
| SZC-94 | 76.56 | -7.18 | 68.27 | -6.85 | 60.38 | | 67.04 | -6.57 | 1 | 406.85 | 1.80 | 41 |
| SZC-96 | 60.31 | | 60.50 | | 69.48 | -7.93 | 60.56 | -6.82 | 1 | 388.41 | 2.11 | 42 |
| SZC-99 | 73.07 | -3.78 | 69.93 | -5.53 | 69.84 | | 76.85 | -4.02 | 1 | 490.59 | 3.39 | 43 |
| SZC-100 | 65.53 | -6.94 | 66.50 | -6.67 | 73.17 | -7.09 | 61.99 | -6.07 | 1 | 506.56 | 3.35 | 44 |
| SZC-102 | 76.59 | -4.22 | 81.04 | -4.62 | 74.85 | -5.74 | 66.30 | -6.09 | 1 | 457.58 | 2.69 | 45 |
| SZC-103 | 61.68 | | 75.26 | -5.70 | 66.09 | | 64.51 | | 1 | 437.52 | 3.87 | 46 |
| SZC-104 | 73.03 | -5.47 | 64.87 | | 67.71 | | 61.68 | | 1 | 440.52 | 3.95 | 47 |
| SZC-106 | 79.63 | -5.70 | 73.11 | -5.74 | 74.71 | -7.15 | 65.85 | -6.24 | 3 | 462.58 | 2.36 | 48 |
| SZC-108 | 70.21 | -5.33 | 65.89 | | 78.64 | -4.27 | 67.40 | -5.08 | 1 | 470.53 | 2.32 | 49 |
| SZC-110 | 61.88 | -7.01 | 62.02 | -6.49 | 69.40 | -7.91 | 59.65 | -6.54 | 2 | 405.52 | 3.44 | 50 |
| SZC-113 | 67.29 | | 75.30 | -4.16 | 80.39 | -5.46 | 60.70 | | 2 | 486.48 | 4.23 | 51 |

**Table 1.** Identified hits for 4 miRNAs. Yellow means non-selective inhibitors. Blue for miR-10b, purple for miR-373, green for miR-155, and red for miR-21.

**Task 2. Experimental validation of identified small molecule hits and discovery of the functional consequences of miRNA–small molecules interaction in BC cells.** As we generated preliminary data prior to the start of this funding, we were able to initiate at full speed this task during the first year. Calin's laboratory already analyzed 50 compounds in year 1 and additional 50 compounds in year 2 for each miR-10b, miR-16, miR-21, and miR-155 (for a total of 400 compounds) in two breast cancer cell lines, the non-metastatic BC cell line BT474 and in the high metastatic MCF7 cells.

This was done by real time PCR using specific microRNA probes commercially available as previously reported in Calin's publications (see ref 12 for miR-21 and miR-155 amplification methods as examples). We initially used two time points (24 and 72 hours) and two concentrations (25 and 100 micromolar), but find out that these conditions were toxic for cells as the majority of cells were apoptotic after 72 hours in the treated cells independent of the reduction in the levels of specific miRNA. Therefore, after analyzing the first set of data obtained in the two cell lines for miR-10b, miR-16, and miR-21 with five compounds each, we decided to

use two smaller concentration (12.5 and 50 micromolar) and three time points (1hr, 12hr and 48hr). In this way we identified 2 compounds (HTS00375M and PD00612, out of the 200 analyzed till now) that significantly reduce the levels of miR-10b and miR-21, respectively (see **Figure 2** – the reduction is significant by using t test, P<0.05; the data represent the mean of two experiments performed in different days). Of note, the compounds did not have cross-effects, otherwise HTS00375 did not reduce the levels of miR-21 and PD00612 did not affect the levels of mR-10b). We expanded these data also in BT20 and CAMA1 cell lines. Furthermore, we tested the mechanism of this downregulation, by profiling not only the active molecule of miRNA but also the precursor and the primary transcript and we identified that in the case of the two positive hits the effects occur at the active molecule. We are also performing additional experiments for the identification of apoptosis and migration effects due to the reduction of miR-10b and mR-21 in BT474 and MCF7, respectively, and we are modulating various technical factors such as time of treatment and type of assay to use. Also, we are working with small molecules against miR-16 and miR-373 and miR-520c too, so we have under testing now all the six miRNAs that were under pipeline in Zhang's laboratory.

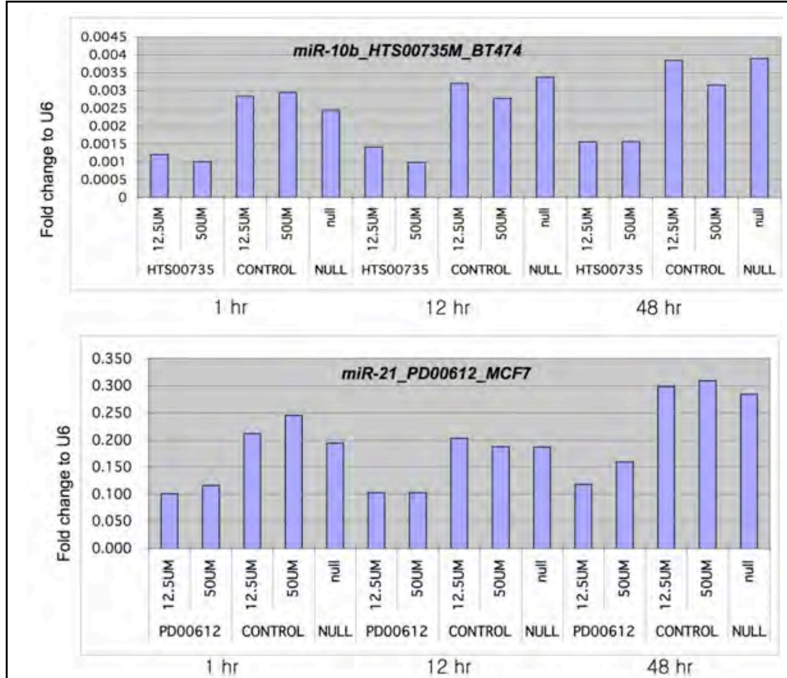


**Fig. 2** Examples of expression reduction of specific miRNA after small molecule transfection in breast cancer cells.

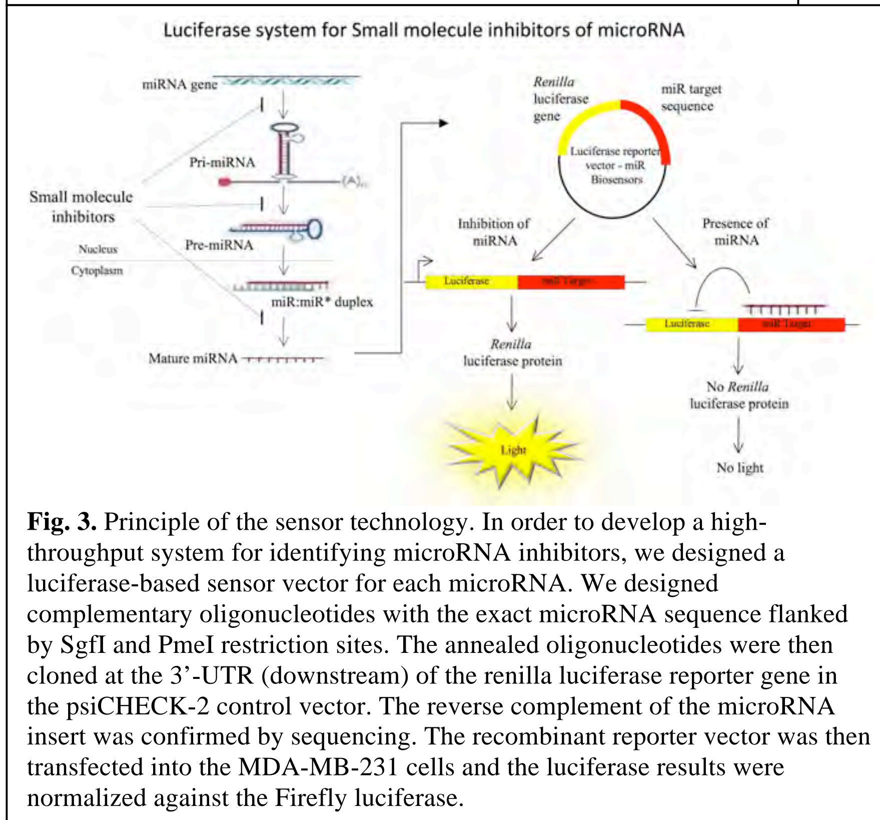


**Fig. 3.** Principle of the sensor technology. In order to develop a high-throughput system for identifying microRNA inhibitors, we designed a luciferase-based sensor vector for each microRNA. We designed complementary oligonucleotides with the exact microRNA sequence flanked by SgfI and PmeI restriction sites. The annealed oligonucleotides were then cloned at the 3'-UTR (downstream) of the renilla luciferase reporter gene in the psiCHECK-2 control vector. The reverse complement of the microRNA insert was confirmed by sequencing. The recombinant reporter vector was then transfected into the MDA-MB-231 cells and the luciferase results were normalized against the Firefly luciferase.

A development from year 2 is the fact that after screening 400 compounds in total by quantitative RT-PCR we realized that the PCR approach is very time consuming and also expensive. Therefore we decided to increase the "speed" of the screen by the construction of the "sensor" vectors for miR-10b, miR-21 and miR-16 (**Figure 3** for the technology and **Figure 4** for results). These are composed by luciferase vectors in which we cloned upstream the antisense specific sequence for the miRNA – in this way, when we co-transfect the "sensor" vector with the small molecule we are performing a simple luciferase assay in a 96-well automated machine. This is an experiment done day-by-day in Calin's laboratory for the identification of direct interaction between miRNAs and targets and therefore the lab personnel has good skills in performing this (see ref 13). As the new approach eliminate the step of RNA extraction and also the expensive replenishment of cDNA preparation kits and the qRT probes, this will increase the number of hits that we can analyze per unit of time, as due to the efficiency of the bioinformatics pipeline developed by Zhang/s laboratory we are now able to predict and further experimentally analyze a higher number of hits as initially proposed in the grant (600 hits in total). With this technology we can screen 12 compounds at 2 time points and in duplicate wells with the use for the additional 48 wells from the DMSO controls. As we are performing in two independent days each luciferase assays, we anticipate to be able to screen 12 compounds in all proposed four cells lines each two to three weeks. The positive hits from this assay will be tested by the initially proposed qRT-PCR method, and therefore in this way we will increase the significance of the positive findings.

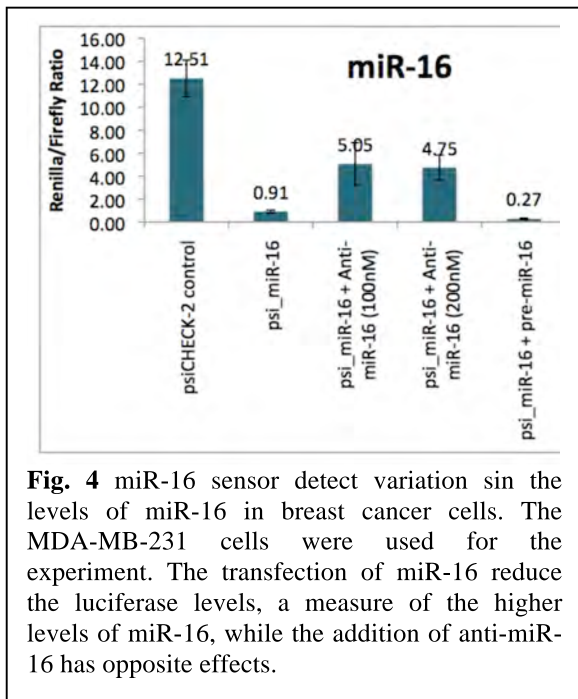


**Fig. 4** miR-16 sensor detect variation sin the levels of miR-16 in breast cancer cells. The MDA-MB-231 cells were used for the experiment. The transfection of miR-16 reduce the luciferase levels, a measure of the higher levels of miR-16, while the addition of anti-miR-16 has opposite effects.

**Pitfalls and alternative approaches**. Two problems we had to solve in the first two years of this application. The first one was the fact that the majority of tested compounds were loosing the effect while maintained dissolved for a longer period of time. Therefore, we decided to perform the duplicate experiments with freshly dissolved compounds; during this critical step we were assisted by Dr. Izabela Fogt from Dr. Priebe laboratory, a chemist with long experience in synthesis of small molecules. This pitfall as well as the identification of the best working conditions (time of treatment and concentration of compound) was solved and therefore we have a much higher efficiency in testing the hits at present time. The second was the necessity of increasing the number of hits analyzed per unit of time and this was solved by the production of 'sensor' vector for 3 out of the 6 proposed miRs and we continue with the production of the remaining 3 "sensors". With this technology we can screen 12 compounds in 2 time points and in duplicate wells with the use for the additional 48 wells fro the DMSO controls.

Furthermore, during the first tow years of funding, as we gained invaluable experience in the in silico modeling of 3D structures of miRNAs and on prediction of hits, we presented this in a Commentary entitled "**Targeting microRNAs with small molecules: Between Dream and Reality"** to a high impact journal from the pharmacology field – *The Clinical Pharmacology and Therapeutics* (IF = 9, ISI 2008) in which we acknowledged the support from the Department of Defense (see Appendices). In this manuscript we focused on the novel idea of using small

molecules to target overexpressed miRNAs as a new therapy of human diseases, in particular cancer and presented the double potential impact: a) Development of a new type of cancer therapies targeting miRNAs and b) Development of new tools to explore the function of miRNAs (Zhang et al, 2010).

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## KEY RESEARCH ACCOMPLISHMENTS

**1 – development of a bioinformatics pipeline for hits prediction;**

**2 – identification of additional specific hits by in silico prediction tools for six miRNAs;**

**3 – experimental proof that two hits downregulates significantly the expression of the active molecule of miR-10b and miR-21, respectively;**

**4 – development of a high-throughput method of experimentally confirmation of hits by using the "sensor" method.**

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## REPORTABLE OUTCOMES

1 – publication of the first review ever discussing about the SMIRS – small molecules targeting microRNAs in which we set up the rational bases of the proposed approach;
2 – preparation of a various other manuscripts reporting the findings from the first year and part of the second year of this project (see refs 2 to 4); one of these discussing the bioinformatics pipeline and the initial experimental hits identifications is close to initial submission;
3 – preparation of reagents ("sensor" vectors) for the generation of additional data;
4 – generation of large amount of preliminary data that will be used for an RO1 application to NIH after the publication of the second manuscript.

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## CONCLUSION

Although high risk, the proposed research is generating encouraging results by the identification of small molecules already available on the market that can be used to inhibit the expression of miR-10b and miR-21, two genes involved in metastatic breast cancer. The generation of "sensor" vectors will highly increase the amount of small molecules screened in the last year of this grant

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## APPENDICES

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# Targeting MicroRNAs With Small Molecules: From Dream to Reality

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**Currently, one out of four deaths in the United States is due to cancer; therefore, every major therapeutic advance in cancer has the potential to save many lives. One of the most fascinating discoveries in the area of molecular oncology in the past decade is that, in cancer, alterations in both protein-coding genes and noncoding RNAs, known as microRNAs (miRNAs), complement each other. In this article, we focus on a novel idea involving the use of small molecules to target overexpressed miRNAs as a new therapy for diseases, particularly cancer, in humans.**

## MicroRNAs AND THEIR FUNCTION

MicroRNAs (miRNAs) are noncoding RNAs that regulate gene expression.[1] Structurally, they are 19–24 nucleotides in length and are processed from much longer primary transcripts (100–1,000 nucleotides) arising from hairpin loop structures after successive enzymatic maturation steps (by Drosha in the nucleus and Dicer in the cytoplasm). miRNAs are involved in a variety of biological processes, from development, differentiation, apoptosis, and proliferation to senescence and metabolism. Functionally, miRNAs regulate gene expression in a sequence-specific fashion after being incorporated into the multiprotein complex RISC (RNA-induced silencing complex), primarily by upsetting the translation and/or stability of messenger RNAs (mRNAs).[2] Overall, the effect of miRNAs is to silence the expression of the target mRNAs, either by mRNA cleavage or by translational repression. However, it has been discovered that miRNAs can also increase the expression of a target mRNA.[3] Each miRNA may target several different transcripts; for instance, it has been demonstrated that a cluster of two miRNAs (namely, *miR-15a* and *miR-16*) can affect the expression of ~14% of the human genome in a leukemic cell line.[4] Conversely, a single mRNA can be targeted by several miRNAs.

## miRNAs AS TARGETS FOR CANCER THERAPY

Alterations in miRNA are involved in a variety of human diseases, including cancer, immune disorders, and cardiovascular disorders.[5] Abnormalities in miRNAs are linked to the initiation, progression, and metastases of human cancers (**Table 1**).[6,7] The main molecular alterations are represented by variations in gene expression, usually mild, but with consequences for a vast number of target protein-coding genes. The causes of the widespread differential expression of miRNA genes in malignant cells as compared with normal cells can be explained by the location of these genes in cancer-associated genomic regions, the presence of epigenetic mechanisms, and alterations in the miRNA processing machinery. miRNA expression profiling of human tumors has identified signatures associated with diagnosis, staging, progression, prognosis, and response to treatment.[6,7] In addition, profiling has been exploited to identify miRNA genes that may represent downstream targets of activated oncogenic pathways or that are targeting protein-coding genes involved in cancer. With respect to human cancers specifically, it was found in at least two independent reports published between 2004 and 2009 that 192 miRNAs were abnormally expressed in cancer cells, including 168 overexpressed miRNAs; this means that a high expression of miRNAs is a hallmark of a malignant phenotype. Furthermore, findings from two mouse models strongly suggest that alterations in miRNA expression alone can cause a cell to become neoplastic: the *miR-155* transgenic mouse overexpressing the oncogenic *miR-155* developed acute lymphoblastic/high-grade lymphoma,[8] whereas the knockout model of the tumor-suppressor cluster *miR-15/16* developed, as in humans, chronic lymphocytic leukemia.[9]

RNA inhibition (defined as the blocking of messenger RNA production or function) finds application in the therapy of human disorders via either (i) the use of miRNAs as therapeutic drugs against the messenger RNAs of genes proven to be involved in the pathogenesis of a disease or (ii) direct targeting of noncoding RNAs that participate in the pathogenesis of a disease. To date, the main RNA inhibition agents used in preclinical and clinical studies include antisense oligonucleotides, ribozymes and the DNAzymes, small interfering RNAs and short hairpin RNAs, and anti-miRNA agents such as antisense oligonucleotides, locked nucleic acids, and antagomirs (**Table 2**).[10]

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**Table 1 Examples of oncogenic microRNAs in human cancers**

| Human microRNA | Deregulation in tumors | Molecular mechanisms and targets | Proven targets[a] | Diagnostic and prognostic markers |
|---|---|---|---|---|
| *miR-17/18a/19a/20a/92* cluster (13q31.3, intron 3 C13 or f25) | Overexpression in lung and colon cancers, lymphomas, multiple myelomas, and medulloblastomas | *Molecular mechanism:*<br>• *miR-17*, *-18a*, *-19a*, *-20a*, and *-19b-1* accelerate tumor growth and increase tumor vascularization<br>• *miR-20a* has an anti-apoptotic role; it causes lymphoproliferative disease and autoimmunity in transgenic *miR-17/92* cluster mice with increased expression in lymphocytes | AIB1 AML1, BIM1, CTGF, CDKN1A, E2F1, E2F2, E2F3, HIF-1A PTEN, TGFBR2, TSP1, Rb2/P130 | *Diagnosis:*<br>• High levels in plasma help to distinguish patients with CRC from normal individuals and patients with gastric cancer |
| *miR-21 (17q23.1,* 3′ UTR TMEM49) | Overexpression in glioblastomas; breast, lung, prostate, colon, stomach, esophageal, and cervical carcinomas; uterine leiomyosarcomas; DLBCL; and head and neck cancers | *Molecular mechanism:*<br>• *miR-21* knockdown induces apoptosis in glioblastoma cells<br>• *miR-21* induces invasion and metastasis in colorectal cancers | BCL2, MASPIN, PDCD4, PTEN, TPM1, RECK, RASA1 | *Poor prognosis:*<br>• High expression of *miR-21* (in colon, breast, and pancreatic cancers)<br>*Good prognosis:*<br>• High expression of *miR-21* in *de novo* DLBCL<br>*Drug resistance:*<br>• *miR-21* affects the potency of chemotherapy in NCI60 cells |
| *miR-155 (21q21.3,* exon 3 ncRNA BIC) | Overexpression in pediatric BL, Hodgkin's disease, primary mediastinal lymphomas, and DLBCL, and in breast, lung, colon, and pancreatic cancers | *Molecular mechanism:*<br>• Pre-B cell proliferation and lymphoblastic leukemia/ high-grade lymphoma in *miR-155* transgenic mice | AGTR1, AID, IKBKE, TP53INP1 | *Poor prognosis:*<br>• High expression of *miR-155* (in lung cancers, DLCBL, and aggressive CLL) |

CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; DLBCL, diffuse large B-cell lymphoma.

[a]Target names as in National Center for Biotechnology Information database at http://www.ncbi.nlm.nih.gov/gene.

However, it is known that there are challenges involved in the delivery of these non-small-molecule agents, and their pharmacodynamics and pharmacokinetics properties are not ideal for the application.

## A NEW WAY TO TARGET miRNAs: THE USE OF SMALL MOLECULES

Given the intriguing fact that miRNAs play crucial roles in cancer and other diseases, and in view of the challenges involved in the use of nucleotide analogs, the development of small-molecule drugs targeting specific miRNAs (which we named SMIRs) and modulating their activities would be a promising approach. We anticipate that it will open a new avenue for targeted cancer therapy.

### Challenges and promises of the approach

As compared with proteins, RNA molecules have long been neglected as drug targets because of their structural flexibility and highly electronegative surfaces. In particular, the targeting of miRNA with small molecules (**Figure 1**) is a new approach and is perceived as challenging because of the paucity of X-ray or nuclear magnetic resonance structures of miRNAs for *in silico* drug design as well as the limited availability of miRNA-Dicer or RISC complex structures.[11] However, successful examples of ligands designed to target RNA molecules, such as ribosome rRNAs, mRNAs, and viral transactivation response RNAs, may provide signposts for developing miRNA-specific drugs for therapeutic purposes.[12]

From the perspective of their secondary structures, miRNAs appear to be "druggable." The formation of stem loops found in pre-miRNAs and the bulges in miRNAs facilitate targeting by small molecules.[13] These structural features not only enlarge the major groove for drug entry but also partially uncover the internal bases, scattering the local electronegative distribution and providing a basis of specificity for structure-based drug design; it has been found that highly positive compounds targeting RNA can easily reach nanomolar (nmol/l) binding affinities.[13] Furthermore, it was shown that miRNAs can target not only messenger RNAs but also DNA, and, more recently, it was shown that they can target proteins. *MiR-373* was found to target promoter sequences and induce gene expression,[14] whereas Eiring *et al.* reported a novel function for miRNAs called "decoy activity": *miR-328* interacts with a heterogeneous ribonucleoprotein, hnRNP-E2, to regulate RNA-binding protein function.[15]

Despite these challenges, the findings from some recently published studies are very promising and have revealed insights into miRNA-targeted drug discovery. Gumireddy *et al.*, after screening more than 1,000 compounds and carrying out structure–activity relationship analyses, identified diazobenzene and its derivatives as effective inhibitors of pri-miR-21 formation.[16] It was also reported that the small-molecule enoxacin (Penetrex) can enhance small interfering RNA–mediated mRNA degradation and promote the biogenesis of endogenous miRNAs.[17] Unfortunately, both inhibitory and enhancing mechanisms, such as drug binding site and specificity, remain poorly understood. However, these studies undoubtedly provide proof of concept

**Table 2 Main characteristics of the principal types of RNA-inhibiting drugs**

| RNA-inhibiting drugs | Definition | Mechanism of action | Drug development phase |
|---|---|---|---|
| Small molecules that target miRNAs (SMIRs) | Small synthetic organic molecules that can bind directly to miRNAs. Their molecular weight is usually <800 Da, and they have ideal drug properties, including good solubility, bioavailability, PK/PD, and metabolism | SMIRs bind to the grooves and pockets on the surfaces of miRNAs and interact with them directly, thereby interfering with the biological functions of targeted miRNAs; alternatively, processing of miRNAs can be inhibited | None |
| Antisense oligonucleotides (ASOs) | An ASO is a single-stranded, chemically modified DNA-like molecule 17–22 nt in length, designed to complement a selected messenger RNA and thereby specifically inhibit expression of that gene | Formation of an mRNA–ASO duplex through Watson–Crick binding, leading to RNAse-H-mediated cleavage of the mRNA of the target gene. The ASOs also inhibit transcription, splicing, and mRNA maturation, as well as ribosomal readthrough | Phase II and III clinical trials |
| AMOs, LNAs anti-miR and antagomirs | AMOs are single-stranded, chemically modified DNA-like molecules 17–22 nt in length, designed to complement a selected microRNA and thereby specifically inhibit expression of that gene<br>The LNA anti-miRNAs represent LNA-modified ASOs<br>Antagomirs are single-stranded 23-nucleotide RNA molecules, complementary to a targeted microRNA, that have been modified to increase the stability of the RNA and protect it from degradation. The modifications include a partial phosphorothioate backbone in addition to 2′-*O*-methoxyethyl | AMOs are ASOs against miRNAs and therefore produce ASO–miRNA duplexes through Watson–Crick binding, leading to RNAse-H-mediated cleavage of the target miRNA<br>The LNA anti-miRNAs have the same mechanism as ASOs/AMOs<br>The antagomirs silence miRNA through a mechanism that has not yet been completely elucidated; the miRNA–antagomir duplexes induce degradation of the miRNA and recycling of the antagomir | Preclinical studies |
| Ribozymes or DNAzymes | A ribozyme, or RNA enzyme, is an RNA molecule that can catalyze a chemical reaction. A DNAzyme, or deoxyribozyme, is a catalytic DNA that site-specifically cleaves the target RNA | Watson–Crick base pairing to a complementary target sequence, then site-specific cleavage of the substrate, and finally release of the cleavage products | Phase I and II clinical trials |
| siRNAs | A siRNA is a double-stranded (ds) RNA, homologous to an mRNA of a target gene | The siRNAs are incorporated into a multiprotein RNA-induced silencing complex (RISC), leaving the antisense strand to guide this complex to its homologous mRNA target for endonucleolytic cleavage of messenger RNA | Phase I clinical trials |

AMO, antisense microRNAs; LNA, locked nucleic acids; siRNA, small interfering RNA.

for modulation of miRNA activity by small molecules. Instead of using these pathway-based approaches, in which the exact mechanisms are not clear, we targeted specific miRNAs of interest by employing our integrated drug discovery platform with synergistic collaboration among computational modelers, miRNA biologists, and medicinal chemists. This innovative and synergistic approach can help build the 3D structures of miRNAs and use structure-based design methods to perform lead identification and optimization.

### *In silico* discovery of small-molecule inhibitors of RNAs

Drug discovery and development is an expensive and time-consuming process. However, computer-aided approaches[18] offer promise because they can improve the efficiency of the drug pipeline dramatically in a cost-effective way as compared with traditional strategies for RNA-targeted lead identification.[19–21] Two docking programs, AutoDock and Dock, have been thoroughly assessed for their capacity to reliably predict the binding sites and affinities of known ligands in RNA systems in which receptor flexibility is considered.[19,21,22] Various RNAs can be targeted with small molecules, including ribosomes, tRNAs, and mRNAs. For instance, by a process of structure-based virtual screening followed by multiple *in vitro* binding assays, inhibitors that target HIV-1 transactivation response RNA and the bacterial ribosomal A-site have been identified.[23] These agents—including but not limited to the marketed drug erythromycin, aminoglycoside derivatives, and neamine mimics—are being investigated in clinical studies. Similar approaches may be applicable for discovering small molecules that target miRNAs for the purpose of developing therapeutic agents for cancer.

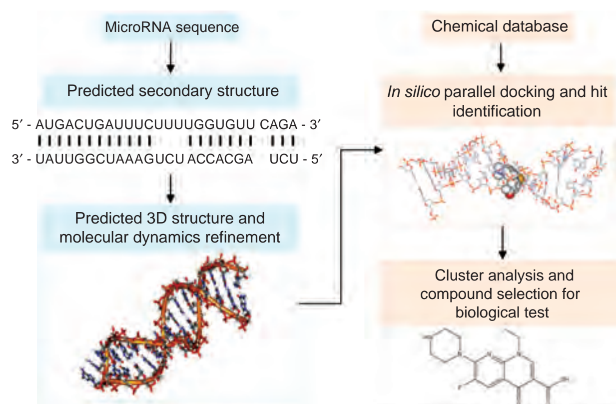


**Figure 1** *In silico* discovery of small-molecule inhibitors targeting microRNAs; such inhibitors can be used as anticancer therapeutics.

### Discovery of small-molecule inhibitors targeting miRNAs

To identify lead compounds that target miRNAs through structure-based approaches, the accurate determination or prediction of the 3D structures of miRNAs is the top priority. Although RNA crystallography poses a challenge, *in silico* 3D structure prediction has undergone significant advances in recent years due to the availability of new experimental data, along with enhanced computer power and improved modeling methodologies.[24] MC-fold/MC-Sym,[25] for example, has been used successfully to accurately predict the double-helix region of several pre-miRNAs (*let-7c*, *miR-19*, and *miR-29a*), and an energy-based *de novo* approach was able to recapitulate noncanonical base pairs observed in native RNA structures.[26] Once the miRNA structure is obtained, molecular docking–based virtual high-throughput screening (vHTS) techniques will be used to enhance the miRNA drug discovery process based on RNA-compatible scoring functions in which it is necessary to re-evaluate the electrostatic interaction and solvation terms in accordance with experimental statistics.[27] **Figure 1** demonstrates this idea in relation to the discovery of novel miRNA small-molecule inhibitors.

## THE POTENTIAL IMPACT OF USING SMALL-MOLECULE THERAPY

Overexpression of miRNAs has been reported in various health conditions, including heart-related diseases and autoimmune diseases; therefore, the development of new small molecules as targeted therapeutics and as probes for miRNA functional analysis can greatly advance public health.

### Development of a new type of cancer therapy targeting miRNAs

Targeting miRNAs for anticancer therapeutics development is a very innovative and promising approach. It is expected to extend the ambit of research into miRNAs and move the field from a hypothesis-driven science toward clinical application through a synergy of innovative miRNA studies, medicinal synthesis, and the development of state-of-the-art computational drug discovery approaches. The impact of this work is multifold. First, the identification of potent small-molecule inhibitors targeting miRNAs can lead to drug development for targeted cancer therapy. Second, the computational effort will help to develop an integrated drug discovery platform, which will be made publicly available (database, methodology, and predictors) for knowledge dissemination. Third, the accumulated knowledge and the drug discovery platform can be readily applied to the discovery of other miRNA-targeted (or, in general, RNA-targeted) small molecules. We estimate that this development will be of immense help to the scientific community involved in drug discovery, miRNA biology, computational modeling, biological signaling pathway studies, and many other related areas. A logical approach toward better outcomes for cancer patients is to exploit the tremendous advances in our understanding of the genetic nature of cancer and the molecular pathways involved in malignant transformation.

Using these advances and the impressive spectrum of new molecular drugs, it is logical to start designing regimens based on combinations of old and new agents. One way to accomplish this—the "multiplex RNA inhibition targeting" strategy—is to target various molecular defects in the multistep pathways of specific cancers by using different RNA inhibition approaches. For example, in aggressive forms of chronic lymphocytic leukemia, both *miR-21* and *miR-155* are overexpressed;[28] therefore, for these patients a combination of small molecules specifically targeting these transcripts could be envisioned, in addition to the actual chemotherapy regimens. The second way—the "sandwich RNA inhibition" strategy—is to focus multiple agents onto a major molecular alteration that is clearly linked to the pathogenesis of a disease. This strategy aims at keeping a specific target under multiple destructive pressures through various mechanisms. An example is *miR-372/373* cluster overexpression in testicular germ cell tumors;[29] here, the targeting of these genes with small molecules as well as with antagomirs could represent a new approach to treatment.

### Development of new tools to explore the function of miRNAs

Understanding the roles of miRNAs in cancer cells is of even greater importance today, in view of the large body of evidence suggesting the involvement of several hundred miRNAs in human cancers. The tools available for exploring the function of overexpressed miRNAs include anti-miRNA oligonucleotides (antisense microRNAs), which are antisense oligonucleotides targeting miRNAs; locked nucleic acid anti-miRNAs that constitute a new class of bicyclic high-affinity RNAs targeting miRNAs; and a novel class of chemically engineered oligonucleotides named "antagomirs." Although good results have been reported with each of these agents, the major drawback for *in vivo* studies is the high cost. For example, the use of an antagomir in mouse models of cancer costs several thousands of dollars for an experiment with two groups of five mice each (treated vs. untreated) for weekly administration of the agent for 3–5 weeks. Therefore, the use of much less expensive small molecules that are already available but not known to target miRNAs, or of new small molecules derived from known ones, will represent an alternative approach for the scientific community. The identified active hits (or the potential drugs) can be used as probes to study the response of miRNA to the compounds, as well as the biological consequences of such a response. The characteristics of specific small molecules with respect to pharmacokinetics and distribution in tissues are already known from previous studies (and are relatively easy to determine for new molecules). This will clearly help in carrying out *in vitro* and *in vivo* experiments.

### Pitfalls in the discovery of small molecules as miRNA inhibitors

On the basis of current knowledge, researchers are still far from being able to design novel and potent molecules modulating miRNA pathways with a clear understanding of their mechanisms. Clearly, more information is needed in regard to the structure of miRNAs and the thermodynamics of miRNA–small molecule interactions; for instance, it is essential to elucidate their 3D structures and describe in detail the mechanisms

through which miRNAs regulate gene expression. The fact that even nanomolar binders display poor specificity implies the necessity to introduce novel concepts and strategies when targeting miRNAs using small molecules. The cost-effective computational approaches employed in our work can certainly help to achieve this task and accelerate the discovery process.

## CONCLUSION

Cancer represents a serious threat for a significant percentage of the population. Although the existing drugs show promise in early treatment, most metastatic cancers are still considered incurable. Hence, there is an urgent unmet need to develop potential "breakthrough drugs" for cancer treatment. miRNAs have been found to play important roles in the development and metastasis of various cancers. We propose a synergistic and innovative approach to developing a novel miRNA-based technology with wide applications for functional studies and targeted therapy, on the hypothesis that small-molecule inhibitors can target miRNAs and that the targeted inhibition will have biological consequences culminating in the death of cancer cells. This approach links two exciting areas of research: the involvement of miRNAs in human cancers and computational discovery of small molecules that inhibit targets that are important in tumorigenesis.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest.

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