

Exploring Baltic Sea cyanobacteria for small-molecule inhibitors of microRNA function: a project description

January 2018 – January 2021

Decision No. DEC-2017/25/B/NZ9/00202, National Science Centre of Poland

Principal investigator: Prof. Dr. Eng. Paweł BRZUZAN

Paweł Brzuzan¹, Hanna Mazur Marzec², Filip Stefaniak³, Maciej Woźny¹, Maciej Florczyk¹

¹Department of Environmental Biotechnology, Faculty of Environmental Sciences, University of Warmia and Mazury in Olsztyn, Poland

²Faculty of Oceanology and Geography, University of Gdańsk, Gdańsk, Poland,

³Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology, Warsaw, Poland

Corresponding author: Paweł Brzuzan, Department of Environmental Biotechnology, Faculty of Environmental Sciences, University of Warmia and Mazury in Olsztyn, Stoleczna 45G, 10-709 Olsztyn, Poland; Phone +48 895234187; Fax: +48 89 5234131; E-mail brzuzan@uwm.edu.pl

Key words: cyanobacteria, luciferase reporter cell line, microRNA, small-molecule inhibitor

ABSTRACT

Cyanobacteria constitute a rich source of biologically active and structurally diverse compounds. The pharmacological potential of these compounds resides among others in their ability to control the proliferation and growth of cancer cell lines and potent disease-causing microbial agents. Despite recent scientific advances, the way these compounds interact with the body's molecular structure are still unclear and science still has to discover how the cyanobacterial metabolites interact with cell structures and how cells react to them. In this project, we will study yet unexamined cyanobacterial metabolites, especially the compounds which act as chemical ligands for microRNA (miRNA) -binding sites, making them promising regulators (inhibitors) of gene networks that are involved in various diseases. We will first develop a stable cell line that

constitutively expresses a unique miRNA reporter system. Then, we will conduct a screen on chemical compounds discovered in Baltic cyanobacteria to identify small molecules with inhibitory activity and specificity to MIR92b-3p, which has a significant impact on liver cell behavior in humans. We assume that a successful MIR92b-3p inhibitor will bind to the precursors of MIR92b-3p miRNA, disabling the action of either of the two processing enzymes involved in the biogenesis of any miRNA in a cell (Drosha or Dicer), thus affecting the MIR92b function. The discoveries made with these inhibitory chemical molecules could provide insight into the role of the MIR92 pathway in liver diseases and cancer, and possibly, if promising results appear, they may facilitate a strategy for treating some human diseases in the future.

ABBREVIATIONS

antimir – microRNA antisense oligonucleotide (inhibitor)
Huh7 – human hepatocellular carcinoma cell line
LucMIR92b-3p – MIR92b-3p reporter system based on luciferase activity
MIR92 - microRNA 92 precursor family
MIR92b-3p – microRNA92b-3p
miRNA - microRNA

EXPLORING BALTIC SEA CYANOBACTERIA FOR SMALL-MOLECULE INHIBITORS OF MICRORNA FUNCTION

Cyanobacteria constitute a rich source of biologically active and structurally diverse compounds. Many of these metabolites exhibit important biological properties, such as interfering in signal transduction, inducing apoptosis, or inhibiting activity of important proteins, such as enzymes, membrane transporters, or receptors. The pharmacological potential of these compounds resides in their ability to control the proliferation and growth of cancer cell lines and potent disease-causing microbial agents. Surprisingly, the biological properties of extracts or compounds from Baltic Sea cyanobacteria are largely unexplored. A part of the cyanobacterial metabolites identified by our partner institution (University of Gdańsk) belong to new analogues of known classes of peptides (e.g. anabaenopeptins, spumigins, aeruginosins, cyanobactins, cyanopeptolins, nostocyclopeptides, nodularins), while others cannot be classified to any of the groups. Some of them represent pharmacophore structure and biological activity which increase the probability for the development into novel medical treatments. Recent studies show that these compounds may hold promise for anticancer agents. For example, Oftedal et al. (2010) have found that extracts from *Anabaena* sp. (Nostocales order) from the Baltic Sea induced apoptosis of acute myeloid leukemia cells. More recently, Felczykowska et al. (2015) have documented selective inhibition of cancer cells' proliferation by compounds included in extracts from Baltic Sea cyanobacteria. Further studies have shown that many of the peptides inhibit activity of proteases that are involved in key metabolic pathways (Spoof et al., 2016; Häggqvist et al., 2016; Mazur-Marzec et al., 2015).

However, despite these recent advancements, molecular details underlying regulatory mechanisms of anticancer activity of these compounds still remain elusive. With molecular abilities to modulate regulatory elements of cell processes (RNA, protein), the bioactive cyanobacterial metabolites that are uncovered would be important tools for clarifying the mechanisms of these cell processes and serve as lead structures for the development of new therapeutic agents. MicroRNAs (miRNAs), probably the best-characterized subclass of noncoding RNAs, have key roles in the molecular control of cell development and function. miRNAs are ubiquitously expressed short RNAs of approximately 22 nucleotides in length that bind to recognition sequences on 3'-untranslated regions (3'-UTRs) of mRNAs and target them for degradation, translational repression, or decay (Fig. 1). Since miRNAs control global transcriptome, they are particularly suitable for studying grids of molecular processes underlying pathological alterations of cells and tissues at the gene and protein levels. Indeed, miRNAs have been shown to regulate a large variety of molecular functions, and have been associated with a number of diseases (Hata and Lieberman, 2015). Due to that, the RNA has become an increasingly important target for therapeutic interventions and for chemical probes that dissect and manipulate its cellular function. Emerging targets

include human RNAs that have been shown to directly cause cancer, metabolic disorders, and genetic disease (Childs-Disney and Disney, 2016).

In this project, University of Warmia and Mazury in Olsztyn will develop, validate and apply a cellular screen for miRNA-pathway inhibitors, bioactive cyanobacterial metabolites identified by our partner institution, University of Gdańsk. Another partner of the project, International Institute of Molecular and Cell Biology, will explain the observed activity of the analyzed candidate compound using computational modeling of RNA 3D structure (Boniecki et al, 2016).

Our experimental model employs a Huh7 human hepatoma cell line stably transfected with a Renilla luciferase sensor for endogenous MIR92b-3p. In human hepatoma cell line this miRNA is abundantly expressed (thousands of copies per cell). The MIR92 family plays important roles in cell proliferation, and several members confer an antiapoptotic phenotype. Importantly, it was found that MIR92b antisense oligomers (antimirs), that decrease levels of the miRNA in humans, could inhibit proliferation, migration, and tumor cell invasion, and promote apoptosis (Zhuang et al., 2016). These promising results suggest that MIR92b-3p could be a viable target for diseased liver cell therapy using yet unknown small-molecule MIR92b-3p inhibitors.

The project has two main objectives:

- I. To develop a robust reporter assay for small-molecule inhibitors of microRNA92b function, a stable human hepatoma cell line that constitutively expresses an MIR92b-3p reporter system (Huh7/LucMIR92b-3p).
- II. To screen cyanobacterial compound library for molecules that inhibit biogenesis of the miRNA, and to evaluate their inhibition selectivity and specificity towards miRNA-92b in liver cells (in silico and in vitro approach).

Our hypothesis is that Drosha and Dicer (ribonucleases) processing sites of the miRNA represent crucial elements in modulation of miRNA expression by cyanometabolites, mainly peptides; the binding of the compounds to these sites could inhibit miRNA biogenesis, and therefore affect miRNA function. Accordingly, we will test the following hypotheses:

Hypothesis #1:

The developed assay based on cellular reporter system(s), that can detect changes in the cell endogenous MIR92b-3p abundance, will identify cyanobacterial compounds that decrease MIR92b-3p levels. Specifically, exposure of the Huh7/LucMIR92b-3p stable cells to increasing concentrations of the inhibitor will lead to a dose-dependent restoration of the luciferase signal. This will negatively correlate with the luciferase signal in transiently transfected cells containing the 3' UTR of SMAD7, the downstream target of the MIR92b-3p.

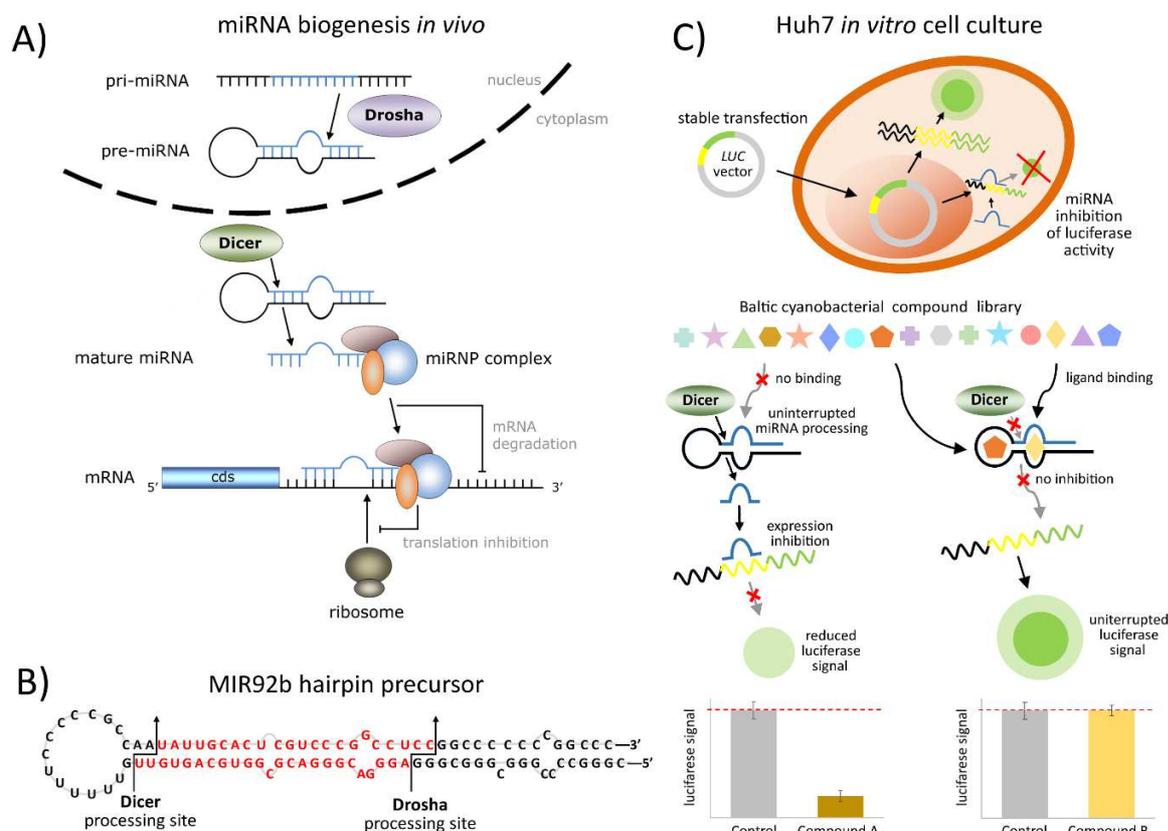


Figure 1. Cellular reporter system for identification of cyanobacterial compounds targeting Mir92b. A) miRNA processing and target recognition in the cell *in vivo*. MiRNAs are transcribed by RNA polymerase II or III as primary transcripts (pri-miRNAs). The pri miRNA is processed by the Drosha enzyme to a stem loop structured miRNA precursor molecule (pre-miRNA). The pre-miRNAs is transported to the cytoplasm where the Dicer enzyme cleaves off the double stranded (ds) portion of the hairpin and generates the mature miRNA, which is incorporated into miRNA protein complexes (miRNPs). The mature miRNA binds to partially complementary recognition sequences on 3'-UTRs of mRNAs and targets them for decay or translational repression. B) Secondary structure of MIR92b precursor, with indicated Drosha and Dicer processing sites. The mature miRNA sequences, MIR92b-3p and MIR92b-5p are indicated with red lettering. C) The developed luciferase reporter (in vitro assay) should detect the presence of a functional mature MIR92b-3p through repression of the Renilla luciferase signal (on left). In the presence of a small-molecule inhibitor of MIR92b-3p biogenesis (cyanobacterial metabolite such as peptide) or a MIR92b-3p antimir, luciferase expression is restored (on right).

Hypothesis #2:

The decrease in MIR92b-3p levels will be due to inhibition of biogenesis and not transcriptional inhibition. To test this, we will determine how the cyanobacterial compound affects levels of pri- and pre-MIR92b. If, for example, it binds to the Drosha site and inhibits processing, an increase in levels of pri-MIR92b and a decrease in pre-MIR92b levels are expected.

Hypothesis #3:

Down-regulation of MIR92b-3p, through delivery of the candidate inhibitor, will impact MIR92b-3p functional targets in the liver hepatoma cells, and induce pathway-specific

phenotype changes in the cells (e.g. apoptosis, cell cycle arrest). Little effect (if any) on expression of other miRNAs will suggest that the compound is pathway-selective.

Obtaining results that support or disprove these hypotheses will constitute an important step in investigating the potential pharmacological utilization of cyanobacterial metabolites as possible miRNA inhibiting (silencing) factors, which has been relatively neglected until now (Brzuzan et al., 2016). With these results, we will learn which chemical structures enable these metabolites to bind directly to and perturb MIR92b functions. The discoveries made with these

chemical molecules could provide insight into the role of the MIR92 pathway in liver diseases and cancer, and possibly, if promising results appear, they may facilitate a strategy for treating some human diseases in the future. Small-molecule inhibitors of MIR92b-3p with enhanced activity that are identified in the project could be used as unique probes to uncover the currently unknown biogenesis and regulation of MIR92b-3p in the liver of vertebrates. Furthermore, chemical modulators found in this study that do not bind directly to MIR92b and inhibit its function, but regulate important cellular processes such as liver cell apoptosis could be examined in the future to determine their other mechanism(s) of action. As for the screening technique that will be developed in this study, using a stable reporter cell line instead of a transient transfection will not only be more cost efficient, less time-consuming, and remove variation associated with transient transfection efficiency and additional manipulations, but the reporter assay that will be developed could be readily extended to other miRNAs and other compounds.

ACKNOWLEDGEMENTS

The project is funded by the National Science Centre of Poland (decision number: DEC-2017/25/B/NZ9/00202) between January 2018 and January 2021.

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