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Vývoj chemických regulátorů drah mikroRNA a RNAi

Development of chemical regulators of microRNA and RNAi pathways

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Souhrn v českém jazyce

MikroRNA jsou nekódující RNA navozující sekvenčně specifickou inhibici genové exprese na posttranskripční úrovni. MikroRNA představují hlavní skupinu malých endogenních RNA v savčích buňkách. V současnosti je známo více než 2500 lidských mikroRNA, které potencionálně regulují více než 60% lidských genů kódujících proteiny. MikroRNA se účastní většiny buněčných procesů a změny v jejich expresi byly popsány u různých patologií, včetně rakoviny.

V současnosti neexistuje žádná malá chemická sloučenina schopná efektivního ovlivnění aktivity dráhy mikroRNA. Nicméně, malé chemické sloučeniny představují skvělé nástroje pro výzkum procesů, ve kterých dráhy sekvenčně specifického umlčování RNA (RNA silencing) hrají roli. Navíc by mohly být využitelné i pro biotechnologické aplikace a mohly by mít i značný terapeutický potenciál. Tato práce je součástí širokého projektu, jehož konečným cílem je: (i) najít soubor malých molekul umožňujících stimulaci a inhibici drah sekvenčně specifického umlčování RNA a (ii) identifikovat případy, kdy se tyto dráhy vzájemně ovlivňují s jiných buněčnými drahami. Tato práce shrnuje výsledky prvních dvou fází projektu, vývoj vysoce výkonných metod testování s vysokou propustností a vysoce výkonné testování s vysokou propustností (high-throughput screening, HTS) dostupných knihoven malých sloučenin.

Abychom mohli monitorovat aktivitu dráhy mikroRNA, vyvinuli jsme a optimalizovali jednu biochemickou *in vitro* metodu založenou na měření změny fluorescence po modulaci aktivity enzymu Dicer a dále několik metod založených na měření změny luminiscence po modulaci dráhy mikroRNA přímo v buňkách. Naší strategií bylo získat data umožňující třídit výsledky z HTS na základě různých parametrů tak, abychom mohli zohlednit například buněčně-specifické efekty, mikroRNA specifické efekty, popřípadě určit v jaké fázi jsou dráhy sekvenčně specifického umlčování RNA ovlivněny. S pomocí optimalizovaných metod byl proveden HTS ~30,000 malých chemických sloučenin. Kombinací dat ze všech HTS jsme získali desítky potencionálně zajímavých sloučenin, které je potřeba dále ověřit. Vybrané sloučeniny budou dále analyzovány řadou metod s cílem určit jejich farmakokinetické vlastnosti, místo jejich působení na dráhy sekvenčně specifického umlčování RNA a jejich regulační potenciál v různých modelových systémech.

Abstract

MicroRNAs are noncoding RNAs inducing sequence-specific posttranscriptional inhibition of gene expression and represent the major class of small endogenous RNAs in mammalian cells. Over 2,500 of human microRNAs potentially regulating more than 60% of human protein-coding genes have been identified. MicroRNAs participate in the majority of cellular processes, and their expression changes in various diseases, including cancer.

Currently, there is no efficient small chemical compound available for the modulation of microRNA pathway activity. At the same time, small chemical compounds represent excellent tools for research of processes involving RNA silencing pathways, for biotechnological applications, and would have a considerable therapeutic potential. The presented work represents a part of a broader project, whose ultimate goal is: (i) to find a set of small molecules allowing for stimulation or inhibition of RNA silencing and (ii) to identify crosstalks between RNA silencing and other cellular pathways. This thesis summarizes results from the first two phases of the project, the development of high-throughput screening assays and the high-throughput screening (HTS) of available libraries of small compounds.

To monitor the microRNA pathway activity, we developed and optimized one biochemical fluorescence-based *in vitro* Dicer assay and several cell-based luciferase assays. Our strategy was to generate data allowing sorting HTS results according to different parameters, such as cell-type specific effects, miRNA specific effects, or sorting compounds affecting different steps of RNA silencing pathway. Optimized assays were used for HTS of ~30,000 small chemical compounds. The combination of data from all HTS generated tens of interesting hits that need to be further validated. Selected compounds will undergo series of assays to characterize their pharmacokinetic properties, their mode of action, and their regulatory potential in different model systems.

1. Introduction

1.1. RNA silencing

RNA silencing is a common term for pathways utilizing short RNA molecules (21-25 nt long). Short RNAs form ribonucleoprotein complexes and guide sequence-specific silencing e.g. transcriptional silencing, mRNA degradation and inhibition of translation [1]. In the following text, RNA silencing refers to two mammalian pathways: microRNA (miRNA) and RNA interference (RNAi).

The bulk of mammalian short RNAs represent miRNAs. They originate as polymerase II-produced long pri-miRNAs, from which the nuclear Microprocessor complex releases short hairpin precursors (pre-miRNAs). Cytoplasmic pre-miRNAs are cleaved by Dicer and resulting mature miRNAs are loaded on effector complexes, which typically interact with 3'UTRs and inhibit translation. miRNAs function as inhibitors of endogenous genes where networks of miRNAs provide another level of control of gene expression.

RNAi is initiated by dsRNA, which is processed into short interfering RNAs (siRNAs) that guide mRNA cleavage in the middle of the basepaired sequence. Natural siRNAs are often derived from repetitive sequences. RNAi acts in maintaining genome integrity and invertebrate immunity. Mammalian RNAi has no antiviral role and is mostly restricted to the female germline cells.

Mammalian RNAi and miRNA pathways are initiated by different RNA molecules but utilize a single Dicer nuclease to produce miRNAs and siRNAs. Dicer belongs to an RNase III class characterized by two RNase III domains. Several other domains are found in Dicer-like proteins in *Metazoa*. These include N-terminal DEAD-like and helicase superfamily C domains, piwi/argonaute/zwille (PAZ) domain, domain of unknown function DUF283, and C-terminal doublestranded RNA binding domain (dsRBD) [2]. The PAZ domain binds the 3' protruding overhang of dsRNA. Two RNase III domains form a single processing center producing short dsRNA with 2 nt 3' overhang, where each domain cleaves one strand of the duplex5. Thus, Dicer acts as a molecular ruler, measuring the substrate length from PAZ domain to RNase III domains. Dicer and the downstream components of RNAi and miRNA pathways in mammals are shared. Both, siRNAs and miRNAs, are loaded onto an Argonaute-containing effector ribonucleoprotein complex, often referred to as RISC (RNA-Induced Silencing Complex) that targets cognate mRNAs. Argonaute proteins have two characteristic domains: the central PAZ domain and the C-terminal PIWI domain. The PAZ domain binds the 3' end of a short RNA while its 5' end is bound by a pocket between the PAZ and MID domains (reviewed in [3]). Structural studies of Argonaute homologs revealed that the PIWI domain has an RNase H-like fold and that Argonaute is the "Slicer",

i.e. the enzyme catalyzing the mRNA cleavage in the canonical RNAi pathway. Mammalian miRNAs and siRNAs are bound by any of four Argonaute proteins (AGO1 through AGO4), all of which were implicated in translational repression but only AGO2 in RNAi-like mRNA cleavage. Whether a short RNA will cause an endonucleolytic cleavage or a block of translation thus depends on the degree of complementarity and the AGO protein, rather than on the origin of the short RNA. The cleavage requires perfect base pairing, while imperfect base-pairing results in translational repression.

1.2. Experimental modulation of RNA silencing pathways

There are several available strategies for modulating RNA silencing in mammalian cells. Globally, RNA silencing can be inhibited by knocking-down or knocking-out components, such as Dicer or AGO2, or by plant virus-encoded repressors [4]. Conversely, RNA silencing can be globally enhanced by eliminating natural inhibitory mechanisms [5]. Individual miRNAs can be inhibited selectively using modified antisense oligonucleotides (antagomirs [6]), and specific miRNA-target interactions can be prevented using target protectors [7]. Enhanced activity of individual miRNAs can be achieved by overexpression or by transfecting cells with miRNA mimics. However, available methods suffer from various limitations. For example, knock-down or knock-out approaches are limited by the targeted protein-product half-life, thus the inhibitory effect cannot be rapidly induced. This is a problem, for example, when addressing the role of miRNAs in rapidly changing systems, such as zygotic genome activation or during early phases of ES cell differentiation.

Chemical biology is an interdisciplinary approach spanning the fields of chemistry and biology that employs compounds produced through synthetic chemistry for the study and manipulation of biological systems. So far, chemical biology has not been systematically applied to explore RNA silencing and data on small compound modulators of RNA silencing are scarce (reviewed in [8]). Chiu et al. reported screening of a small library of dihydropteridinones that yielded ATPA-18, an inhibitor of RNAi acting at the level of passenger strand unwinding or earlier [9]. A screen of 2,000 small compounds (which are represented in our collection as well) for modulators of RNAi identified an enhancer enoxacin [10]. Polylysine (PLL) and trypaflavine (TPF) were identified in a small screen (530 compounds) as suppressors of miRNA-RISC activity [11]. Interestingly, these two compounds also neutralized tumor growth in a cell-based model [11]. In addition, several additional modulators [12] and miRNA-specific inhibitors (reviewed in [13]) have been described. Altogether, research of RNA silencing modulators represents an interesting and still relatively unexplored area.

1.3. Research plan overview

Our research strategy is to use chemical biology to study and manipulate RNA silencing in mammals. The research goal of the entire project is (i) to provide a set of small compounds allowing for direct stimulation or inhibition of RNA silencing and (ii) to identify crosstalks between RNA silencing and other pathways.

Assay development – Cell based assays

The cell-based assays that generated the bulk of HTS data, were developed to monitor endogenous miRNA activity in cells in order to discover both general and miRNA-specific small molecule modifiers of RNA silencing. The cell-based assays are based on plasmid reporters carrying miRNA binding sites in 3'UTRs. The principle of the assay is described below. Under normal conditions, endogenous mature miRNAs bind a target sequence on the reporter mRNA and silence it as follows: (i) In case of the reporter containing perfectly complementary binding site(s) ("perfect" reporter), miRNA mediates direct cleavage of the reporter mRNA through AGO2 slicer activity. The minimal requirement for the cleavage is the small RNA bound to AGO2, which form so-called holo-RISC. The perfect reporter thereby reflects the miRNA biogenesis, the efficiency of miRNA loading on AGO2, and the holo-RISC function. (ii) In case of the reporter containing imperfectly complementary binding site(s) (bulged reporter), the central mismatches interfere with short RNA-guided slicer activity of AGO2, and the reporter is repressed by translation repression [14, 15]. In addition to AGO and small RNA, this requires additional accessory proteins in the so-called full-RISC. Thus, the bulged reporter monitors the capability of endogenous miRNAs to induce translational repression by full-RISC and indirect target mRNA degradation on top of miRNA biogenesis. As a result, any interference with miRNA biogenesis or function is detected as an increase in the reporter activity.

Assay development – Fluorescence based *in vitro* Dicer cleavage assay

Activity of Dicer can be monitored *in vitro* by the Dicer cleavage of dsRNA substrate [16]. Typically, cleavage assays are based on processing of radiolabeled dsRNA substrates by recombinant Dicer and a detection of cleaved products by electrophoresis [17, 18]. However, this type of assay is not suitable for HTS. To solve this problem, an assay yielding fluorescence upon cleavage by Dicer [19, 20] was developed. Notably, biochemical assays allow for identification of Dicer modulators regardless of their cellular uptake or cytotoxicity, thus enabling a discovery of compounds elusive in cell-based assays.

We developed an assay to monitor the activity of recombinant Dicer by fluorescence as follows. As a substrate, we used short (27 bp) perfect RNA duplex containing a 2-nt 3′ overhang at one terminus and a blunt end at the other one. At the blunt end, one RNA strand carries a fluorescent group and the other strand a quencher. During the Dicer-mediated cleavage, the 2-nt 3′ overhang is recognized by the PAZ domain, and the cleavage releases

a short duplex carrying the fluorophore and the quencher. Subsequent separation of the fluorophore from the quencher can be monitored as fluorescence increasing over time. To adapt the assay for HTS, we tested several fluorophore/quencher pairs, reaction conditions and volume, concentrations of Dicer and the substrate, and timing of data collection.

High-throughput screening (HTS)

After assay the establishment and optimization, we performed five HTS of ~30,000 compounds: four (HTS-I – HTS-IV) using cell-based luciferase reporter assays and one (HTS-V) using biochemical fluorescence-based *in vitro* Dicer cleavage assay. All HTS were performed in collaboration with Petr Bartunek group, IMG AS CR, CZ-OPENSCREEN (particularly with David Sedlak), which provided equipment and the original collection of compounds from 15 libraries including Sigma LOPAC Library, Prestwick Library, NIH Clinical Trial Collection, and several proprietary sublibraries. Our strategy was to generate data allowing for thorough filtering HTS results (see results and discussion).

Compounds classification, analysis and further development

After initial HTS data analysis, the cherry-picked compounds will be assessed using various reporters transiently or stably expressed in cell lines to validate their activity and provide initial kinetic data (concentration and time dependence). To gain insights into the mechanism of action of individual cherry-picked compounds, several secondary assays will be run to follow two research aims. The first aim is to identify a small list (<10) of lead compounds, which will form the basis for obtaining efficient miRNA inhibitors and stimulators with low toxicity and good cellular uptake. If needed, lead compounds will be further modified to improve their pharmacokinetic properties and to reduce toxicity. Strategies for derivation will be decided either by Michal Hocek (IOCB) or Tomas Martinu (UCT). The second aim will be the investigation of mechanisms regulating the miRNA pathway in mammalian cells. Since many compounds used in HTS have defined effects on the specific signaling or metabolic pathways, data mining should uncover mechanisms regulating the RNA silencing. Although such compounds likely regulate the miRNA pathway indirectly, they will point to specific mechanisms regulating the miRNA pathway, which is of great interest.

To classify the cherry-picked compounds according to their mode of action on RNA silencing, several strategies, including (i) testing effects on pre-miRNA, pri-miRNA, and mature miRNA levels, (ii) rescue-based classification of inhibitory compounds, (iii) biochemical assays (Radioactive Dicer assay, RISC loading assay) and (iv) others, will be used.

2. Aims and significance of the project

Potential of chemical biology in the field of RNA silencing has not been fully explored yet. At the same time, small chemical compounds might represent great tools for: (i) a direct modulation of miRNA/RNAi pathways and (ii) a discovering crosstalks between RNA silencing and other cellular pathways.

The direct modulation of RNA silencing offers novel applications in various fields of research. In basic research, small compound modulators would be an excellent tool for studying RNAi and miRNA pathways *in vivo* and *in vitro* and could help to determine essential roles of RNA silencing pathways in different processes in various cell types, tissues, and animal model systems. Moreover, inhibition or activation of RNAi can increase potential of the RNAi technology. In biotechnology, inhibition of RNA silencing could be useful for applications that aim at global reprogramming of gene expression and require a relief of repression of miRNA targets in a differentiated cell type. In therapy, Dicer stimulators are candidates for treatment of age-related macular degeneration, in which Dicer deficiency was implicated [21]. As various miRNAs have been implicated as potential causative agents with opposing roles in tumor suppression and tumor induction [13, 22], modulation of miRNAs can be considered for cancer therapy. As demonstrated by histone-deacetylase-inhibitors (HDIs), global modulation of miRNA pathway might be valuable strategy. While histone deacetylases cause global and complex changes in gene expression, HDIs are used to treat different types of cancer [23-26]. Currently, there is a growing number of evidence that small RNA pathways participate in the antiviral response in mammals [27-29]. Therefore, it is tempting to speculate that boosting of RNAi effect by small compound activators could contribute to antiviral therapy.

Many small chemical compounds have been implicated in regulation of specific cellular pathways. Therefore, detailed HTS data analysis is expected to uncover existing mechanisms that regulate the RNA silencing indirectly through signaling, metabolic and other pathways. For the research of such interactions between RNA silencing and other processes, chemical biology provides an excellent choice.

The presented work represents a part of a broader project whose ultimate goal is to obtain a set of small molecules allowing for stimulation or inhibition of RNA silencing. This thesis summarizes the results from the first two phases of the project - (i) development of high-throughput screening assays and (ii) high-throughput screening of available libraries of small compounds.

Specific aims of this thesis were:

Development and validation of fluorescence-based biochemical assay for HTS of the Dicer cleavage activity.

Development and validation of cell-based reporter assays for monitoring the RNA silencing activity in HTS.

HTS of the unique selection of ~30,000 small compounds and initial data analysis.

3. Overview of the most important materials and methods

Preparation of the recombinant Dicer

Competent DH10Bac cells were transformed (Heat shock method). Recombinant bacmid DNA isolated from transformed cells was used for transfection of insect Sf9 cells (Calcium phosphate method). Obtained baculoviral stock was amplified and plaque-purified. Recombinant human Dicer containing 6xHis on C terminus was isolated from insect cells by Talon^{TM} affinity resin (*BD Biosciences Clontech*). The presence and purity of recombinant Dicer was tested by western blotting.

Fluorescence based *in vitro* Dicer cleavage assay

To prepare the dsRNA substrate for Dicer, fluorophore- and quencher–labeled RNA oligonucleotides were annealed together by heating the mixture at 90 °C for 10 min followed by slow cooling. The assay was performed in Dicer assay buffer in black microplates. The assay was usually performed under single-turnover conditions (Dicer in molar excess over its substrate). Samples were incubated at 37 °C under 100% humidity. Fluorescence was measured at indicated timepoints using the multilabel reader EnVision (*PerkinElmer*) equipped with a Bodipy TMR optimized filter set for particular fluorophores.

Cell culture

Human cell lines (HeLa, HEK293, U2OS), and mouse NIH/3T3 cells were used for transfections and for generation of stable lines with stable expression of *Renilla* luciferase reporter, firefly luciferase reporter, or both reporters.

Bioluminiscence assays

Transfected cells were collected 48 h post-transfection. Luciferase reporter activity was assessed either using the Dual-Luciferase Reporter Assay (*Promega*) or ONE Glo, eventually Renilla Glo Luciferase assay system (*Promega*) according to the manufacturer's instructions. Luminiscence intensity was measured either by Modulus Microplate Multimode Reader (*Turner Biosystems*) or by EnVision plate reader (*PerkinElmer, Inc.*). Data were normalized to the total protein amount in lysates and to control transfection.

HTS

HTS was performed on a fully automated robotic platform cell::explorer (*Perkin Elmer*) in a 394-well or 1536-well format. For dispensing of cell suspension and luciferase substrate, or Dicer and Dicer substrate, Multidrop Combi liquid dispenser (*Thermo Scientific*) was used. Fluorescence or luminiscence intensity was recorded with multimode plate reader Envision (*Perkin-Elmer*).

4. Results and discussion

The presented work is a part of a large scientific project that aims to obtain a set of small molecules allowing for stimulation or inhibition of RNA silencing. This thesis summarizes the first two phases of the project: (i) development of high-throughput screening assays and (ii) high-throughput screening (HTS) of a library of ~30,000 small compounds. Highly efficient, low-cost, safe, and robust assays represent a prerequisite for a successful HTS. During the first part of the thesis project, one biochemical and several cell-based assays were developed to monitor the miRNA pathway activity. Both types of assays, as well as the results of HTSs, are discussed below.

4.1. Aspects of fluorescence-based *in vitro* Dicer cleavage assay

To identify compounds modulating the Dicer activity, we developed a fluorescence-based *in vitro* Dicer cleavage assay. As a substrate for Dicer we designed a short (27-nt) RNA duplex that was easy to synthesize and resembled an artificial siRNA structure described earlier [30]. The sequence of the substrate was derived from a well-characterized human let-7a miRNA, hence allowing for combining our assay with other tools developed for let-7 analysis.

An important feature of the RNA substrate for Dicer is the optimal combination of the fluorophore and the quencher. I tested three combinations: (i) 5(6)-FAM fluorescent donor and 3-Dab quencher (*Exiqon, Vedbaek, Denmark*), (ii) Cy5 fluorescent donor and IowaBlackRQ (IB-RQ) quencher (*Integrated DNA Technologies, Coralville, IA*), and (iii) Cy5 fluorescent donor and BHQ2 quencher (*Sigma-Aldrich*). Of them, the Cy5/IB-RQ combination was clearly superior to others. Notably, a similar substrate for fluorescence-based Dicer cleavage assay was independently designed and used by DiNitto *et al.* (2010) [20] yielding comparable results.

The reaction buffer for our *in vitro* Dicer assay was adopted from Kolb *et al.* (2005) [17] with only minor modifications. We performed fluorescent Dicer cleavage assay under the single-turnover conditions, when the rate of the product formation is not limited by the product release, since each enzyme molecule reacts with one substrate molecule at most. There were several reasons for the selection of this option: (i) insufficient amount of available dsRNA substrate stock, (ii) a high background activity of the dsRNA substrate at concentrations over ~100 nM and (iii) insufficient activity of Dicer at concentrations bellow ~50 nM. The HTS reaction was downscaled to 5 μl, and 35 nM substrate and 70 nM Dicer was used. This combination yielded an acceptable signal-to-noise ratio and a relatively slow substrate processing rate ($t_{1/2}$~4 h), which provided enough flexibility for the robust monitoring of a large number of reactions at multiple timepoints in a continuous assay.

The screen of a library of ~30,000 compounds was performed in a kinetic manner. Importantly, the number of selected hits is influenced by both time at which the reading was taken, and the selected cut-off conditions. Under the conditions when the readings were taken at time 6 and 15 h, 207 compounds significantly inhibiting the assay and 72 compounds potentially activating the assay were identified A dose-response assay performed on 2,816 selected potential Dicer inhibitors originating from the library containing annotated bioactive compounds validated 22 compounds (reaction time 4 h from the initiation of the assay) [31]. Interestingly, three common scaffolds were found among the validated compounds, which suggests that some common themes exist in the manner by which compounds interfere with the assay. Inhibitory compounds could interfere with the Dicer cleavage in many ways. At this point it is difficult to distinguish between the compounds inhibiting Dicer specifically and the compounds which affect the assay but are not Dicer-specific inhibitors. Such non-specific assay modulators could be, for example: (i) compounds that nonspecifically denature or aggregate proteins, (ii) compounds that affect the substrate structure (intercalating compounds), or (iii) compounds that interfere with the fluorescence. Regarding the Dicer-specific compounds, future experiments will validate them and describe their mode of action.

The fact that our results did not show any overlap with previous analyses is not surprising. While we used a biochemical assay directly aimed at compounds modulating Dicer, the previous screens were mainly cell-based and monitored performance of an entire RNA silencing pathway [8]. In addition, our assay would poorly detect inhibitors with IC50>15 μM, such as kanamycin. Taken together, the fluorescence-based Dicer cleavage assay was adapted for HTS and successfully used for identifying candidate modulators of the key step in miRNA and RNAi pathways.

4.2. Aspects of cell-based assays

To identify potential global small-molecule modulators of RNA silencing pathways, especially the miRNA pathway, several cell-based assays were developed. These assays are based on reporters carrying miRNA binding sites in 3´UTR. In the assay, the endogenous miRNA suppresses reporter activity, thus reflecting the activity of selected miRNA levels. In the presence of a small molecule inhibitor, the reporter repression is relieved, resulting in the increased reporter signal. Since this reporter system monitors an increase of reporter signal in the presence of an active inhibitor, the majority of false positives due to the compound cytotoxicity should be excluded. Importantly, although data processing after HTS included analysis of both miRNA pathway inhibitors and activators, the presented cell-based assays are primarily aimed to screen for miRNA pathway inhibitors and are suboptimal for identification of miRNA pathway stimulators.

To perform a HTS, stable reporter cell lines were established to reduce the number of manipulations and to increase the reproducibility and robustness of the assay. Interestingly, the assay development went through several turns and twists and sometimes lad us to dead ends. To select a suitable reporter, an EGFP-based reporter was examined first because EGFP fluorescence requires no additional substrates or cofactors and the EGFP fluorescence can be easily and cheaply detected by a fluorometer. Although functionality of the EGFP reporter cell-based assay was confirmed, due to a low fluorescence signal induction upon the miRNA pathway inhibition and a relatively high background fluorescence, the assay was found not suitable for HTS.

As the second option during our search for an optimal reporter system for HTS we examined luciferase reporters. At that time, a bidirectional luciferase-based reporter system containing both an experimental *Renilla* luciferase and an independently transcribed firefly luciferase reporter gene was established in our laboratory. Therefore, the first choice was the establishment of a bidirectional luciferase-based reporter in which the binding sites for an endogenous miRNA were inserted downstream of *Renilla* CDS, and the firefly reporter was used for normalization purposes to account for variation in transfection efficiency and cell viability. However, despite our so far good experience with small-scale bidirectional luciferase assays employing *Renilla* luciferase as the experimental target, the use of this system for HTS turned out to be problematic for the following reasons: (i) the EnVision plate reader, which was used for measurement of luminescence signal during HTS experiments, did not allow to measure *Renilla* and firefly luciferase signals sequentially and (ii) the usage of Renilla-glo (*Promega*) steady substrate for *Renilla* luciferase was not established and optimized well at that time. Furthermore, the pilot HTS with Renilla-glo substrate showed a poor reproducibility. The reasons of inconsistent results were not further studied. Instead, we decided for firefly luciferase-based reporter system, because tens of cell-based HTSs employing firefly luciferase reporters were already successfully performed in the collaborating group of Petr Bartunek [IMG AS CR, CZ-OPENSCREEN], providing a large volume of existing data for data comparison.

During the assay development, tens of reporters varying in type of promoters (PGK, TK, SV-40), polyA sites and miRNA biding sites (perfect vs. bulged), eventually number of miRNA binding sites (1-4), were tested. Such reporters were repressed to different levels and had a different sensitivity to repression and stimulation of RNA silencing. The different types and number of miRNA binding sites allowed to: (i) distinguish compounds affecting translation repression and acting upstream of it and (ii) select a reporter yielding the best dynamic range of the assay. Finally, reporters containing binding sites for two endogenous miRNAs, let-7 and miR-30, were tested.

To test a selectivity and a robustness of the assay in the HTS format and to verify a feasibility of the HTS to estimate the hit rate, we first performed a proof-of concept HTS of a part of the collection (~10,000 compounds). For the pilot HTS, the assay based on

HeLa stable cell line carrying the firefly luciferase reporter with three perfect let-7 binding sites was used for its good dynamic range and a higher basal activity compared with other stable cell lines tested, potentially enabling to discover both inhibitors and activators of the miRNA pathway. Surprisingly, the pilot HTS produced a satisfactory hit rate. The hit rate is dependent on the cut-off value chosen for data analysis. Due to a relatively high amount of compounds stimulating the firefly activity, we decided to set 5xB-score cut-off value for potential miRNA pathway inhibitors. On this cut-off value, 180 potential miRNA inhibitors were identified. In contrast, due to a relatively poor amount of compounds inhibiting the firefly activity, we decided to set -1,8xB-score cut-off value for potential miRNA pathway activators. On this cut-off value, 27 potential miRNA activators were identified, however 22 of them were removed due to their cytotoxicity. Notably, tens of identified hits were already annotated bioactive compounds. Subsequent data analysis revealed the diversity in terms of the compound association with various cellular processes. There were for example compounds causing DNA damage, blocking translation, blocking cell cycle, corticosteroids or compounds interfering with different signaling pathways. Importantly, identification of compounds interfering with miRNA pathway indirectly would enable to discover crosstalks between RNA silencing and other cellular pathways.

The proof-of-concept HTS validated the selectivity and robustness of the firefly reporter cell-based assay. To complement the proof-of-concept HTS we performed HTS of the rest of the library (~20,000 compounds) collection with the same reporter assay. In addition, three additional HTSs of ~30,000 compounds using (i) NIH-3T3 stable cell line carrying the firefly luciferase reporter with three perfect let-7 binding sites, (ii) HeLa stable cell line carrying the firefly luciferase reporter with four bulged let-7 binding sites and (iii) HeLa stable cell line carrying the firefly luciferase reporter with four bulged miR-30 binding sites were performed. This allows us to filter the HTS data according to the following parameters: (i) cell type- specific vs. cell type-non-specific modulators of miRNA pathway, (ii) let-7 miRNA-specific vs. global modulators of miRNA pathway, and (iii) miRNA pathway-specific compounds affecting translation repression vs. compounds affecting the upstream steps of RNA silencing pathways. The cut-off values for hit rate determination were set to 5xB-score value for potential miRNA pathway inhibitors and -5xB-score value for potential miRNA pathway stimulators. We identified: (i) 227 potential general miRNA inhibitors and 15 potential general miRNA pathways activators, (ii) 21 potential let-7 specific inhibitors and 1 potential let-7-specific activator, or (iii) 27 putative general inhibitors and 10 putative activators of translation repression.

HTSs generated several groups of interesting hits. First, global small compound modulators would be a great tool for studying RNA silencing pathways *in vivo* and *in vitro* and they would have also a therapeutic potential. miRNAs are associated with cancer and can act as oncogenes or tumorsuppresors [22]. Finding that miRNAs inhibit senescence in cancer cells [32] indicates that cancer cells could be more sensitive to the global inhibition

of miRNA pathway then the normal cells, which opens a possible strategy for cancer therapy. Moreover, as exemplified by histone-deacetylase inhibitors, the global modulation of the highly complex miRNA pathway is valuable. While histone deacetylases cause global and complex changes in gene expression, histone-deacetylase inhibitors are used to treat different types of cancer [23-26]. Interestingly, Dicer activators are candidates for the therapy of age-related macular degeneration, in which Dicer deficiency has been implicated [21]. Next, some biotechnological applications may benefit from the inhibition of RNA silencing pathways. let-7 inhibits the induction of pluripotent stem cells and vice versa the inhibition of let-7 miRNA in differentiated cells contributes to a higher efficiency of iPS formation [33]. Therefore let-7-specific inhibitors would have a great potential in the research of pluripotency and stem cells. Finally, potential modulators of miRNA-mediated translation repression may contribute to the functional analysis of the full-RISC complex.

The data on small compound modulators of RNA silencing are scarce. However, several studies have identified small compound modulators of RNA silencing [8-12, 34, 35], utilizing both cell-based assays and biochemical *in vitro* assays. Several compounds identified in previous HTSs were in our collection as well, enabling the data comparison. First, enoxacin has been shown to promote the miRNA biogenesis and to enhance the miRNA function in a dose-dependent manner, with a median effective concentration (EC50) ~30 μM [10]. Importantly, enoxacin did not show any remarkable activity in our hands. It is not surprising as we used fifty times lower compound screening concentration than was the concentration used in the study of Shan *et al.* (2008) [10]. Moreover, Shum *et al*. [34] showed that enoxacin is inactive at lower screening concentration (10 μM) [34]. A recent biochemical HTS has identified three potent small-molecule inhibitors of the RISC loading: (i) aurintricarboxylic acid, (ii) suramin sodium salt, and (iii) oxidopamine hydrochloride [35]. Of them, the first two were present in our library as well. Although suramin sodium salt did not show any activity in our HTSs at screening concentration hundred times lower than the screening concentration used in study of Tan *et al.* (2012) [35], aurintricarboxylic acid [35] was identified as a potential Dicer inhibitor in our Dicer HTS. Finally, five of six compounds identified as inhibitors of the miRNA-21 biogenesis in the recent study utilizing an image-based biosensor assay [34] were also present in our collection. Four of them (N,N-dipropyldopamine hydrobromide, 8-hydroxy-DPAT hydrobromide, deoxycorticosterone and flutamide) were inactive in our HTS, in which the screening concentration was ten times lower than that used in the study of Shum *et al.* (2012) [34]. Interestingly, one of them, neurotoxin "6-Hydroxy-DL-DOPA" [34], was identified as a potential Dicer inhibitor in our Dicer HTS as well.

The analysis of the data from the pilot HTS revealed known inhibitors of particular signaling pathways, potential inducers of the stress response, or modulators of specific metabolic pathways. It is unlikely that these compounds regulate the miRNA pathway directly. Instead, they point to other mechanisms that can regulate the miRNA pathway.

For instance, it was reported that after the stress induction the miRNA pathway is blocked and the AGO2 with the miRNA is relocalized from P bodies or cytoplasm into the stress granules indicating that there is a link between the stress induction and inhibition of miRNA pathway [36, 37]. Therefore, primary candidates on the secondary modulation of miRNA pathway would be those inducing cellular stress. Surprisingly, analysis of ~200 compounds that were cherry-picked based on the results from the proof-of-concept screen revealed that only 4% of compounds induced the formation of the stress granules, and therefore the majority of identified compounds inhibits the miRNA pathways by other mechanisms than stress induction. The fact that one of the compounds capable of the stress granules induction was emetine, which has been linked to stress induction in the past [38], confirmed the cell-based assay robustness and credibility of HTS data.

As mentioned before, several reports have already used chemical biology to study the RNA silencing. However, there are some technical differences between HTSs performed so far and our HTSs. First, in contrast to earlier studies, which were performed on relatively small libraries comprising several hundreds to ~7,000 compounds [8-12, 34, 35], we screened an extensive collection of ~30,000 compounds that varied in terms of their structure. Second difference between our HTSs and HTSs, which were done in the past, is the compound concentration. Whereas we used 1 μM compound concentration enabling us to identify only very specific modulators of RNA silencing, the majority of previously identified RNA silencing modulators showed considerable activity only at much higher concentrations [8-12, 34, 35]. Therefore, it is not surprising that the majority of compounds, which were identified in previous HTSs and which were in our collection as well, did not show any remarkable activity in our HTSs.

5. Conclusion

In summary, one biochemical fluorescence-based *in vitro* Dicer cleavage assay focused on searching for Dicer modulators and four cell based assays focused on searching for modulators of RNA silencing pathways were developed and used for HTS of ~30,000 small chemical compounds. These HTSs were unique in terms of extensiveness of the compound library and the low compound screening concentration, thus promising the identification of very specific and potent small compound modulators of RNA silencing. Moreover, no assay similar to the fluorescence-based *in vitro* Dicer cleavage assay has been used in such an extensive kinetic HTS so far. Although our HTSs generated a lot of interesting data, first it is necessary to verify them in dose- and time-dependent experiments, and subsequently the best candidates need to be characterized in various secondary assays to find out their mode of action. Taken together, the aims of the dissertation were completely achieved, and the project can enter into the next stage – validation and characterization of the cherry-picked compound.

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List of publications

During my Ph.D., I co-authored three impacted publications:

(i) A review summarizing the use of RNAi *in vivo*:

Podolska, K., Svoboda, P., 2011. Targeting genes in living mammals by RNA interference. Briefings in functional genomics 10, 238-247, (IF = 3.427)

(ii) Reporters, which I developed for HTS were used in the following study, in which I monitored miRNA activity upon inhibition of Lsm8.

Novotny, I., Podolska, K., Blazikova, M., Valasek, L.S., Svoboda, P., Stanek, D., 2012. Nuclear LSm8 affects number of cytoplasmic processing bodies via controlling cellular distribution of Like-Sm proteins. Mol Biol Cell 23, 3776-3785, (IF = 5.98)

(iii) An article summarizing development of Dicer HTS:

Podolska, K., Sedlak, D., Bartunek, P., Svoboda, P., 2014. Fluorescence-based high-throughput screening of dicer cleavage activity. Journal of biomolecular screening 19, 417-426, (IF = 2.012)

In addition, a manuscript summarizing cell-based assay results is currently in preparation.

Curriculum vitae

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Research experience

2008 – Present:	Petr Svoboda group
	Department of Epigenetic Regulations
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	Main project: *Development of chemical regulators of microRNA and RNAi pathways*
2004 – 2008:	Jitka Forstova group
	Laboratory of Virology, Department of Genetics and Microbiology, Faculty of Science, Charles University
	Main project: *Preparation of monoclonal antibodies and expression plasmids for study of properties of polyomavirus BK structure proteins*

Education

2008 – Present:	Ph.D. study
	Department of developmental and cellular biology
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2011 – 2014:	Complementary pedagogical study of biology (finished by certificate)
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