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# Small Molecule Inhibition of RISC Loading

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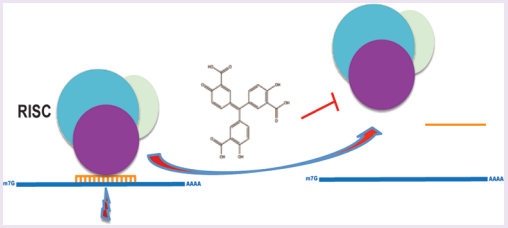
**ABSTRACT:** Argonaute proteins are the core components of the microRNP/RISC. The biogenesis and function of microRNAs and endo- and exo- siRNAs are regulated by Ago2, an Argonaute protein with RNA binding and nuclease activities. Currently, there are no *in vitro* assays suitable for large-scale screening of microRNP/RISC loading modulators. We describe a novel *in vitro* assay that is based on fluorescence polarization of TAMRA-labeled RNAs loaded to human Ago2. Using this assay, we identified potent small-molecule inhibitors of RISC loading, including aurintricarboxylic acid ($IC_{50}$ = 0.47 $\mu$M), suramin ($IC_{50}$ = 0.69 $\mu$M), and oxidopamine HCL ($IC_{50}$ = 1.61 $\mu$M). Small molecules identified by this biochemical screening assay also inhibited siRNA loading to endogenous Ago2 in cultured cells.




RNA interference (RNAi) depends on double-stranded RNA and induces sequence-specific gene silencing.[1−3] The endogenous RNAi pathway intersects with the microRNA (miRNA) machinery, which in mammals regulates gene expression by inhibiting protein synthesis and inducing mRNA decay.[4,5] Endo- and exo-siRNAs and miRNAs are processed in the cytoplasm by the RNase III enzyme Dicer and loaded to Ago proteins to form effector complexes (microRNP or RISC). Endo-siRNAs modulate innate immunity in plants,[6−8] *Drosophila*,[9,10] and *C. elegans*.[11−13] Although the function of mammalian endo-siRNAs is not yet understood, it is likely that they operate primarily in oocytes,[14,15] where miRNA function appears to be non-essential.[16] In contrast to the elusive function of mammalian endo-siRNAs, somatic miRNAs in mammals regulate numerous biological processes, and their disordered expression has been linked to human diseases.[17]

In the past decade, RNAi directed by exogenously administered small RNAs emerged as a powerful molecular tool for gene-specific studies, complementing classic genetic approaches. Like miRNAs and endo-siRNAs, exogenous siRNAs are incorporated into Ago complexes.[18] Ago2 is an RNA-guided endonuclease with the ability to form catalytically active complexes with siRNAs/miRNAs and their precursors.[18−22] In addition to its RNA binding and catalytic activity, Ago2 competes with the cellular translation machinery[23−25] to halt translation initiation. Ago2 has been implicated in the biogenesis of siRNAs[26] and of miRNA subsets, either by Dicer-independent processing of miRNA precursors[27−29] or by mechanisms that are not yet clearly understood.[30,31] Due to its role as regulator, carrier, and executor of miRNA/siRNA function, Ago2 is at the core of the RNAi, and its regulation directly affects the biology of miRNAs and siRNAs, at multiple layers.

Recently, novel approaches have emerged for the identification of chemical modulators of miRNAs. In this limited number of recent studies, cell-based assays have been the predominant methodology for large-scale screenings of compounds that enhance or inhibit miRNAs or siRNAs.[32−36] In general, these assays do not permit identification of RNAi pathway components directly affected by the modulators. With the exception of a recent report demonstrating inhibition of Dicer's function by small molecules,[33] *in vitro* assays utilizing purified recombinant RISC factors have not been previously reported.

In this study, we describe a novel method for large-scale screening of chemical compounds that interfere with RISC loading. In order to identify potential RISC modulators, we used purified recombinant Ago2 to screen two collections of small compounds: the Library of Pharmacologically Active Compounds (LOPAC) and a custom collection of compounds from the National Institute of Neurological Disorders and Stroke (NINDS). Our studies established a novel *in vitro* method that is based on fluorescence polarization (FP) of TAMRA-labeled small RNAs and identified molecules that inhibit RISC loading *in vitro*. Further testing using cell-based assays demonstrated that compounds identified by large scale *in vitro* screenings also inhibit *de novo* assembly of endogenous RISC.

## RESULTS AND DISCUSSION

**Small Molecule Inhibition of *in Vitro* RISC Reconstitution: Results of LOPAC and NINDS Compound Libraries Screening.** A total of 1,280 compounds from the LOPAC library (final screening concentration 100 $\mu$M) and a

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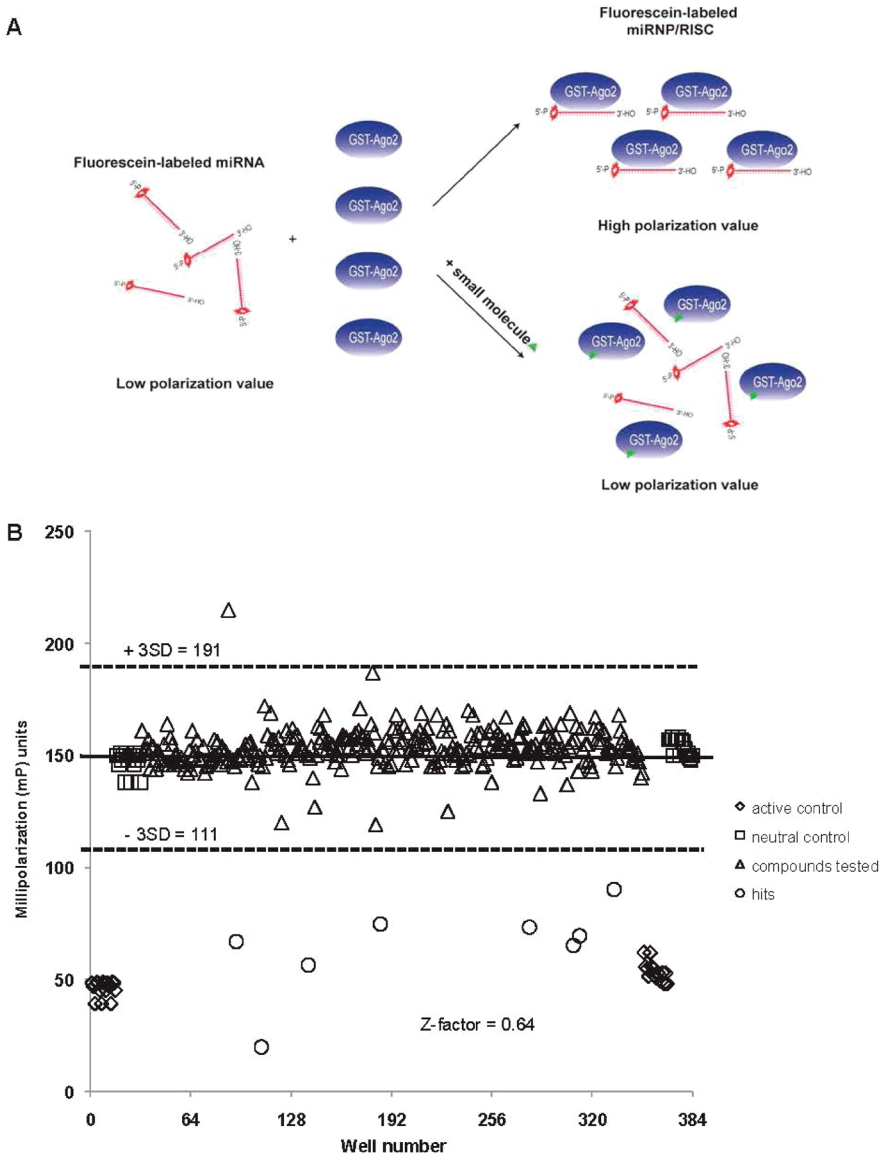


**Figure 1.** (A) Principle of the fluorescence polarization (FP) screening assay for RISC loading inhibition. TAMRA-labeled siRNA is free to rotate in the absence of Ago2, resulting in a low polarization value. The large siRNA-loaded Ago2 complex rotates more slowly, resulting in a higher polarization value. (B) Z-factor plot of one screening plate. Graphical representation of the results of one screening plate in FP HTS assay for RISC loading inhibition. Active controls (◇) were located in wells 1−16 and 353−368 (columns 1 and 23). Neutral controls (□) were located in wells 17−32 and 369−384 (columns 2 and 24). Compounds (Δ) were tested in wells 33−352 (columns 3−22). With 3 standard deviation as a cutoff point, 8 compounds were identified as hits (○) in this plate. The coefficient of variation (CV) of active and neutral control was 12.4% and 3.6% respectively, with a Z-factor of 0.64. The assay protocol is described in Methods.

custom collection of 1,040 compounds (final screening concentration 20 $\mu$M) from the National Institute of Neurological Disorders and Stroke (NINDS) were screened for potential inhibitors of miR-21 and Ago2 binding. The assay is described in Methods and illustrated in Figure 1A. The average Z-factor for the screening was ~0.6, indicating a robust assay.[37] A representative Z-factor plot is shown in Figure 1B. Setting a 40% inhibition as a cutoff point, we detected 46 hits from the LOPAC (hit rate 3.6%) and 21 hits from the NINDS library (hit rate 2%). All hits were subjected to a 16-point, 2-fold serial dilution (final concentration 50−0.0015 $\mu$M) dose−response testing to determine the $IC_{50}$ for Ago2:miR-21 binding inhibition. With confirmation dose−response testing of the high-throughput screening (HTS) hits, a total of 17 compounds from the LOPAC and 8 compounds from the NINDS library showed an $IC_{50}$ < 50 $\mu$M (confirmation rate of 37%).

**DNA Binding Assays.** To exclude nonspecific DNA-binding inhibitors, we next performed a counter screen of the candidate compounds that were confirmed in the dose−response test. In this assay, compounds were tested for competition of ethidium bromide (EtBr) binding to DNA (average Z-factor of 0.81). Compounds with $IC_{50}$ < 50 $\mu$M in the EtBr competition assay were then excluded because their activity in the Ago2:miR21 FP assay was considered a result of nonspecific nucleic acid binding. After filtering out compounds that were DNA binders, 12 confirmed hits from the LOPAC and 6 confirmed hits from the NINDS library remained. Three compounds with the lowest $IC_{50}$ values, PubChem SID 29221432 (compound **1**, aurintricarboxylic acid (ATA), Figure


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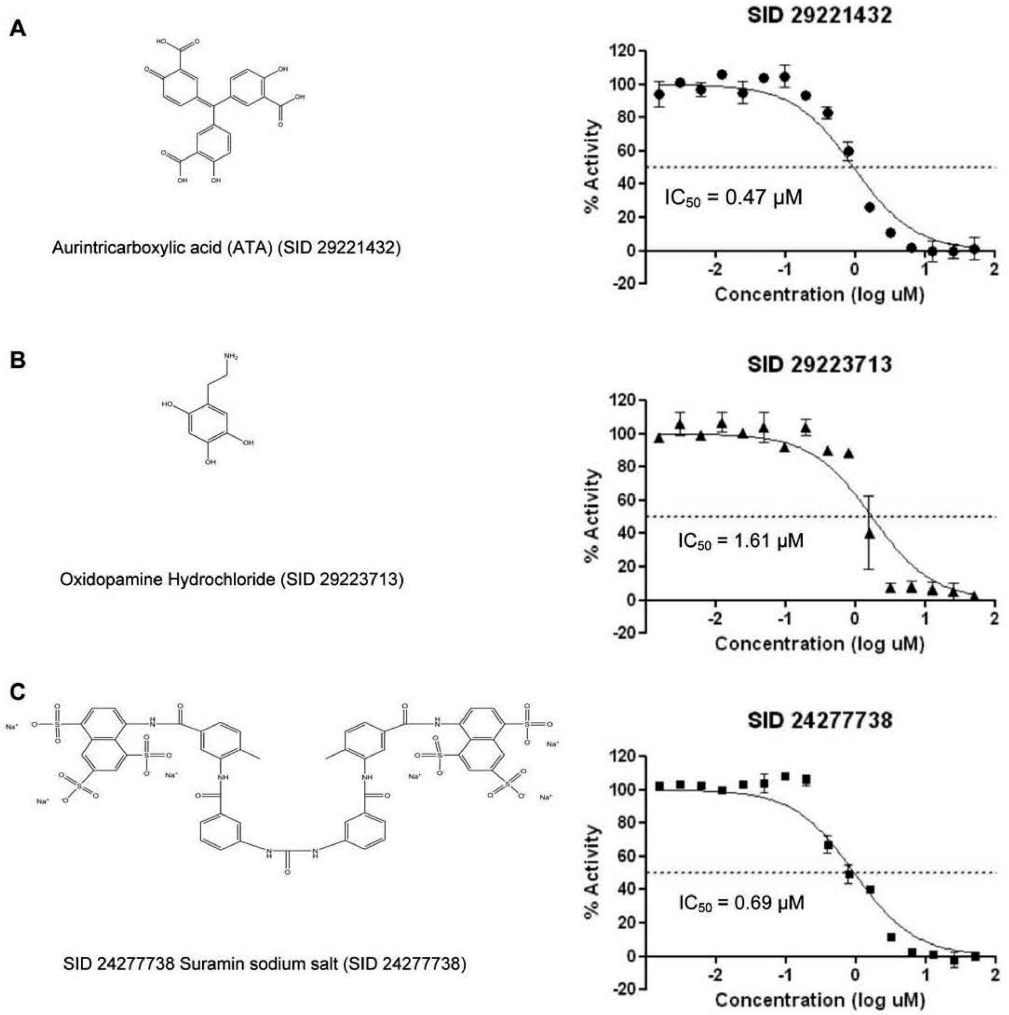


**Figure 2.** Structures and $IC_{50}$ curves of RISC loading inhibitors SID 29221432 (A), SID 29223713 (B), and SID 24277738 (C) found to inhibit miR-21 loading to Ago2 *in vitro*.

2A), SID 29223713 (compound **2**, oxidopamine hydrochloride (HCL), Figure 2B), and SID 24277738 (compound **3**, suramin sodium salt, Figure 2C) were selected for cell-based assays. $IC_{50}$ values were 0.47, 1.61, and 0.69 $\mu$M for ATA, oxidopamine HCL, and suramin, respectively.

**ATA Inhibits siRNA Function in Human Cells.** The 3 miRNA inhibitors with the lowest $IC_{50}$ values in the biochemical screening assay were subsequently tested on the function of endogenous RISC. Cells were co-transfected with Renilla (RL) and Firefly (FL) Luciferase plasmids and then treated with ATA, suramin, or oxidopamine HCL at a final concentration of 25 $\mu$M. Subsequently, cells were transfected with an siRNA targeting the coding sequence of RL (siLuc). Normalized RL expression analysis revealed a robust de-repression of RL by ATA and modest de-repression by suramin sodium salt (Figure 3A). Oxidopamine HCL in the concentration used had no effect on RL expression. Since ATA was a potent inhibitor of siRNA activity in cell-based assays, we next performed a dose–response experiment. Cells were treated with ATA at concentrations ranging from 6.25 to 50 $\mu$M. Optimal de-repression of RL was achieved at concentration of 25 $\mu$M. At higher concentrations (50 $\mu$M), the efficiency of de-repression decreased, likely indicating a saturation effect (Figure 3B). Next, we tested if ATA interferes with siRNA inhibition of an endogenous gene using an siRNA that targets RNA helicase A (RHA). We showed that in mouse embryonic fibroblasts, ATA de-represses siRNA knockdown of RHA (Figure 4). Together, these results indicate that ATA inhibits RISC activity against transiently expressed reporters and endogenous genes.

**ATA Inhibits siRNA Loading to Endogenous Ago2.** We next examined the effect of ATA on the RNA binding and nuclease activity of endogenous Ago2, in order to determine whether ATA also inhibits RNA loading to Ago2 in cultured cells. The RNA binding and catalytic activities of endogenous Ago2 were tested in 293TRex cells treated with ATA or oxidopamine HCL. The association of endogenous miRNAs to Ago2 and the RISC activity of immunoprecipitated endogenous Ago2 complexes were comparable in cells treated with ATA or oxidopamine HCL (Figure 5A and B). Interestingly, while endogenous miRNA:Ago2 complexes were not perturbed, loading of exogenous siRNA (siLuc) to Ago2 was completely inhibited by ATA (Figure 5A). These results are consistent with those from our *in vitro* screening using recombinant Ago2 and indicate that ATA inhibits RNA binding to endogenous Ago2 and *de novo* RISC assembly, without disturbing preformed complexes of Ago2 with endogenous small RNAs or inhibiting the catalytic activity of Ago2.

Recent studies have identified chemical modulators of miRNAs and siRNAs[32−36,38] utilizing cell-based assays.


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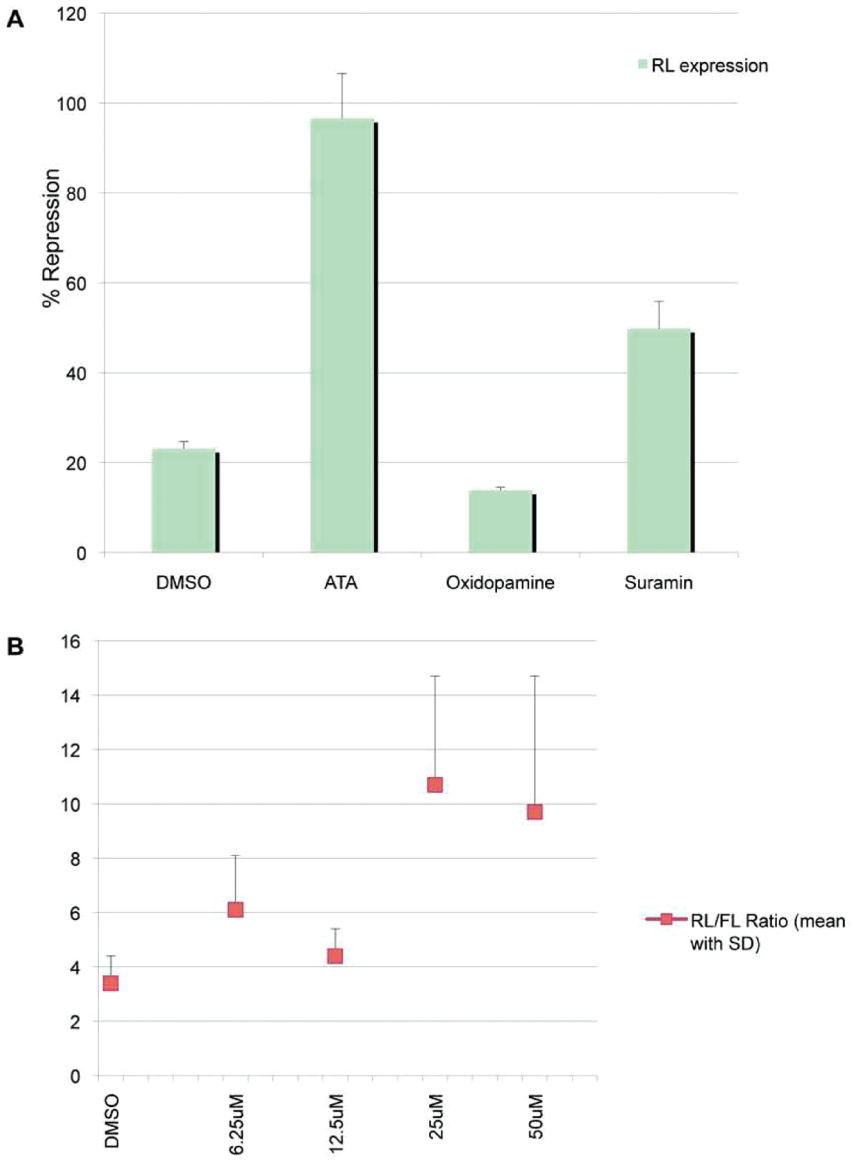


**Figure 3.** De-repression of siRNA-regulated Renilla Luciferase expression by ATA and suramin. (A) T-REx 293 cells were first co-transfected with Renilla Luciferase (RL) and Firefly Luciferase (FL) plasmids. After treatment with ATA, oxidopamine-HCL, suramin sodium salt, or DMSO for 24 h, cells were transfected with Luciferase siRNA (siLuc). Results shown are average values of normalized RL/FL expression from 3 individual experiments. (B) Dose−response curve of T-REx 293 cells co-transfected with RL and FL plasmids treated with varying amounts (DMSO/0−50 $\mu$M) of ATA, prior to siLuc transfection. The mean RL/FL ratio of 3 individual experiments is shown (error bars represent standard deviation).

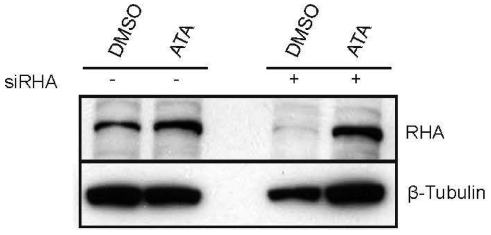


**Figure 4.** ATA de-represses siRNA inhibition of endogenous RHA. Mouse embryonic fibroblasts (MEFs) were treated with ATA or DMSO and subsequently with RHA siRNA, as described in Methods. RHA and $\beta$-tubulin were analyzed by Western blot.

Enoxacin has been shown to promote the biogenesis of miRNAs and to enhance their function.[34] In addition, inhibitors and enhancers of miR-122, a miRNA required for hepatitis C replication by the liver, have been recently reported.[36] *In vitro*, Dicer processing of synthetic shRNAs modified to contain G-quadruplexes is inhibited by porphyrazines and bisquinolinium compounds.[33] *In vitro* assays utilizing purified, minimal RISC have not been reported previously.

In our study, we undertook a novel approach by taking advantage of our previously described *in vitro* RISC reconstitution assay.[21,22] We established a robust *in vitro* methodology to screen large chemical compound libraries for modulators of RISC, by testing the binding of TAMRA-labeled miR-21, a miRNA implicated in pathways controlling cell proliferation and apoptosis,[39] to recombinant Ago2. Aurintricarboxylic acid (ATA), oxidopamine HCL, and suramin sodium salt reproducibly inhibited *in vitro* RISC reconstitution.

Suramin is a purinoceptor antagonist[40] with antihelminthic and antiprotozoal activity.[41] It inhibits the activity of deoxyribonucleic/ribonucleic acid (DNA/RNA) polymerase and human immunodeficiency virus (HIV) reverse tran-


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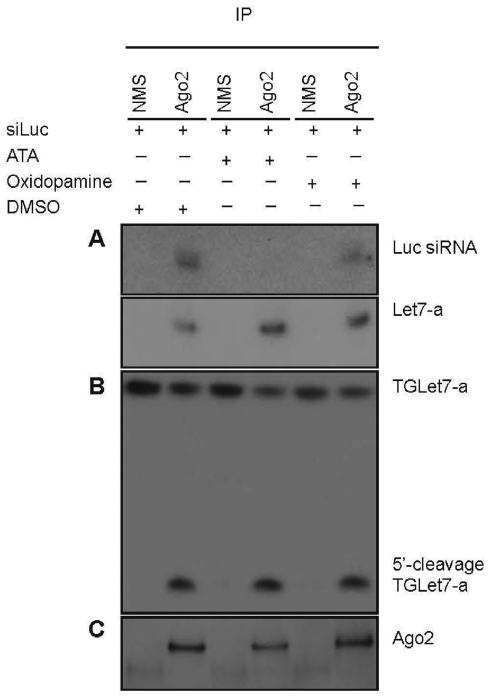


**Figure 5.** ATA inhibits *de novo* RISC loading. (A) T-REx 293 cells were first co-transfected with RL and FL plasmids for 24 h, then treated with ATA or oxidopamine-HCl or DMSO for 24 h, and then transfected with siLuc. RNA was isolated from immunoprecipitated Argonaute (Ago) complexes and analyzed by Northern blot. Probes against siLuc and Let7-a were used as indicated. (B) *In vitro* RISC assay with radiolabeled Let7-a target (TGLet7-a) perfectly complementary to Let7-a using endogenous immunoprecipitated Ago2 complexes. (C) Input T-REx 293 cell lysates used for Northern Blot (A) and immunoprecipitation (B) analyzed for Ago2 expression by Western blot.

scriptase *in vitro*.[42] In our studies, suramin inhibited the binding of miR-21 to Ago2 *in vitro* and had a modest effect in cell-based assays.

Oxidopamine HCL is an adrenergic agent that has been used extensively for the depletion of dopaminergic neurons and experimental induction of Parkinson-like syndrome in animals.[43] The molecular mechanisms of oxidopamine HCL function are not well understood.[44] Although it potently inhibited miR-21 loading to Ago2 *in vitro,* oxidopamine did not interfere with loading of siRNA to endogenous RISC in cell-based assays.

ATA is a triphenyl-methyl molecule that inhibits nucleases and nucleic acid-binding enzymes.[45,46] When administered to mice, ATA inhibits infectivity and replication of T cell viruses at concentrations that are not toxic to the host cell.[47] It was recently shown that ATA is a potent inhibitor of human flap endonuclease inhibitor 1 (FEN1).[48] *In vitro*, as well as in cell-based assays, ATA potently inhibited the loading of small RNAs to Ago2. This inhibition was not sequence-specific, as indicated by our experiments using three different RNAs (miR-21, siLuc, and siRHA). Importantly, at the concentrations used in this study, ATA blocked loading of siRNAs to endogenous Ago2 without affecting the nuclease activity of the protein or dissociating preformed Ago2 microRNPs. This implies that ATA targets RNA-free Ago2, either by inducing conformational changes or, more likely, by directly blocking the RNA binding domains of the protein. Preformed microRNPs are not affected, likely because miRNAs bound to Ago2 preclude direct interaction of ATA with the protein.

Our studies established a novel *in vitro* assay for high-throughput screening (HTS) of chemical modulators of RISC. This fluorescence polarization (FP) assay is simple, utilizes the minimal RISC components, and can be readily used for large-scale screenings. Since the assay allows for testing of individual miRNA/siRNA loading to Ago2, it can be used to identify modulators of specific miRNAs/siRNAs. RNAi is widely used as a novel tool for studies of gene expression, regulation, and function. To our knowledge, this is the first report of a tool that can be used for high-throughput screening for modulators of the RNAi executor. Furthermore, Ago proteins are also the executors of the miRNA function. Thus our novel FP assay provides a tool for HTS of modulators of a key protein in the miRNA/siRNA pathway and can potentially have broad applications in studies of the function of Ago and individual miRNAs in cell biology and human disease.

Using our novel assay we identified ATA as a potent inhibitor of *de novo* RISC assembly *in vivo* and *in vitro*, which does not affect preformed endogenous miRNA complexes and does not inhibit the catalytic activity of Ago2. Thus, ATA can be used to study individual microRNPs or miRNAs, their half-lives, and stability or to study the function of endogenous RNA-free Ago2. Such studies should be planned taking into consideration possible effects of the compounds on cell biology. For example, ATA is a generic nuclease inhibitor, with potentially deleterious effects at high concentrations. However, in the relatively low concentration of 25 $\mu$M, ATA selectively inhibits loading of siRNAs to Ago2 without affecting other functions of the protein. Previous studies have shown that ATA is associated with low toxicity in tissue cultures [49] and is well-tolerated *in vivo*.[50] For *in vitro* studies of RISC assembly using purified Ago2 and synthetic siRNAs, the direct effect of ATA on siRNA loading versus potential "adverse" effect on the nuclease activity of Ago2 can be assessed by different experimental methodologies.

In summary, we established a tool for HTS of microRNP/RISC modulators and identified compounds that can be used to block the cycle of miRNA loading to Ago2, with applications in RNAi and in studies of the biogenesis and function of miRNAs in different tissues and cell lines.

## METHODS

**Expression and Purification of GST-Ago2.** Recombinant mouse Ago2 was purified and tested for RISC activity as described in ref 22.

**High-Throughput Small Molecule Screening Assay.** Volumes of 1 $\mu$L of miRNA buffer (40 mM $CH_3COOK$, 4 mM $MgCl_2$, 1 mM DTT), 0.5 $\mu$L RNasin Ribonuclease Inhibitor, and 2.5 $\mu$L RNase-free water were dispensed into each well of 384-well assay plates. After pintool transfer of 100 nL of compound from DMSO compound plates (with pure DMSO in control columns) to the assay plate, a volume of 2 $\mu$L of TAMRA labeled miR-21 and 4 $\mu$L of GST-Ago2 were added separately to each well at a final concentration of 3.75 nM and 150 ng respectively, in the 10 mL final reaction volume. Columns 1 and 23 of each 384-well plate received 4 $\mu$L of water instead of GST-Ago2 to provide the minimum polarization value for each plate. Columns 2 and 24 received 100% DMSO instead of a chemical compound in order to determine the highest polarization signal for full binding of the fluorescent miR-21 by GST-Ago2. The means and standard deviations of the "no-Ago2" control columns 1 and 23 and the "DMSO" control columns 2 and 24 were used to calculate the Z-factor for each plate to estimate the robustness of the assay (Figure 1B). The assay plates were incubated at RT for 30 min and


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fluorescence polarization was measured (EnVision, PerkinElmer) to calculate the millipolarization units (mP) [(mP = 1000($S - GP$)/($S + GP$)]; mirror: BODIPY TMR FP Dual, excitation filter: BODIPY TMR FP 531 nm, $G$ = instrument factor, $P$ = signal from PerkinElmer BODIPY TMR FP P-pol 579 nm first emission filter, $S$ = signal from PerkinElmer BODIPY TMR FP S-pol 579 nm second emission filter). A value of 3 × standard deviation was used to set the cutoff point to select hits. Each hit from the primary HTS screen was then tested twice using a 16-point, 2-fold dilution (final concentration 50−0.0015 $\mu$M) dose−response assay to determine the $IC_{50}$ for binding inhibition of miR-21 to Ago2. Results were plotted and analyzed using an IDBS XLfit model 205 to calculate $IC_{50}$ values.

**DNA Binding Assay.** Compounds were tested for their ability to displace EtBr from DNA as an indication of the DNA-binding activity of each compound. Vector pUC19 and ethidium bromide (EtBr) were mixed to final concentrations of 8 $\mu$g/mL (pUC 19) and 5 $\mu$M (EtBr) and dispensed to the intermediate plate for columns 2−22 and 24. Columns 1 and 23 were the active control where the water and EtBr mix was dispensed instead. Columns 2 and 24 were the neutral control where 4 $\mu$L water was dispensed instead of chemical compound. For dose−response tests, a volume of 100 nL of each compound from DMSO stock plates was pintool transferred (final concentration 50−0.0015 $\mu$M) to the assay plate (columns1, 2, 23, and 24 had 100% DMSO instead of compounds). A volume of 6 $\mu$L of solution was transferred from the intermediate plate to the assay plate and incubated for 30 min at RT. The assay plate was read with EnVision from PerkinElmer (mirror: general dual, excitation filter: 535 nM, emission filter: 595 nM). The means and standard deviations of neutral and active control were used to calculate Z-factor.

**Source of Reagents Materials and Software Used for *in Vitro* Screening.** Sigma-Aldrich: Library of Pharmacologically Active Compounds (LOPAC), potassium acetate ($CH_3COOK$), magnesium chloride ($MgCl_2$), dithiothreitol (DTT). Promega: RNasin Ribonuclease Inhibitor.

Corning Incorporated: Corning 384-well low volume black round bottom polystyrene NBS microplate. Greiner Bio-One North America Inc.: polypropylene, 384 well, solid V-bottom storage microplate.

PerkinElmer Life and Analytical Sciences: EnVision Multilabel Reader, Evolution P3 liquid handler. Thermo Scientific: Microdrop 384 Reagent Dispenser. ID business solution: IDBS XLfit.

**Cell Culture, Transfection, and Luciferase Assays.** T-REx 293 (Invitrogen) cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FBS (Sigma) and L-glutamine (Mediatech). Cells were cultured in 100-mm dishes and transfected at 40% confluency with 0.5 $\mu$g of pRLTK and 2 $\mu$g of pGL3 plasmids, using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, 3 × $10^5$ cells/mL were transferred to 6-well plates and treated with 25 $\mu$M oxidopamine HCL (Sigma, H4381), aurintricarboxylic acid (ATA) (Sigma, A1895), suramin sodium salt (Sigma, S2671), or DMSO (Fisher) for additional 24 h. Treated cells were then transfected with Silencer Renilla Luciferase siRNA (AM4630) or Silencer Negative Control no. 1 siRNA (AM4611) in Opti-MEM (Invitrogen) using Lipofectamine RNAiMax (Invitrogen). Transfection media contained each of the above compounds at 25 $\mu$M concentration. Twenty-four hours after siRNA transfection, cells were harvested for Luciferase assays or immunopreciptation and Northern blot. Luciferase assays were performed as described.[51,52]

For RHA RNAi, mouse embryonic fibroblasts (MEFs) were first treated with ATA (25 $\mu$M) or DMSO for 24 h, as described above. Treated mEFs were then transfected with of RHA siRNA (25 $\mu$M, Dharmacon ON-TARGETplus SMARTpool DHX9 no. L-056729) for an additional 48 h. Cells were harvested, and RHA protein was detected by Western blot (RHA antibody: Abcam, ab26271, 1:1000). $\beta$-Tubulin was used for loading normalization (E7 ascites: Developmental Studies Hybridoma Bank, 1:2000).

**RNA Immunoprecipitation, RISC Activity Assay, and Western and Northern Blot.** For RNA immunoprecipitation (RIP), cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.4, 200 mM sodium chloride, 2.5 mM magnesium chloride, and 0.5% Triton X-100, and endogenous miRNPs were immunoprecipitated as described in ref 51. Briefly, cell lysates were first mixed with 20 $\mu$L of protein G agarose beads (Invitrogen) preincubated with 5 $\mu$L of monoclonal anti-Ago antibody 2A8[53] or 0.5 $\mu$L of nonimmune mouse serum, at 4 °C overnight. Agarose beads were then washed in lysis buffer. As described previously,[51] 10% of total protein G agarose volume was used for Western blot, 10% for *in vitro* RISC assay, and 80% for Ago2-bound RNA isolation and Northern blot. For Ago2 detection by Western blot, 2A8 was used in 1:1000 dilution. *In vitro* RISC activity assays were performed with endogenous immunoprecipitated RISC complexes and a radiolabeled RNA target perfectly complementary to endogenous Let-7a-3 (TGLet7-a), as previously described.[22,54] Northern blots were hybridized with a 5′-end radiolabeled DNA probe antisense to Renilla Luciferase siRNA (siLuc) at 37 °C overnight or LNA probe antisense to Let7a at 42 °C overnight, and hybridization signals were detected by autoradiography.

**5′-End Labeling and Sequences of RNA, DNA, and LNA Oligonucleotides.** RNA, LNAand DNA oligonucleotides were 5′-end radiolabeled with T4 polynucleotide kinase (NEB) as previously described.[51] Oligonucleotide sequences:

TGLet7a: 5′-GUAUCAACCACUAUACAACCUACUACCUCAACGUUCAAUC-3′

Renilla Luciferase siRNA (siLuc) DNA antisense: 5′- TAGGAAACTTCTTGGCACC-3′

LNA-let-7a-3: 5′-AACTATACAACCTACTACCTCA-3′

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### Author Contributions

[§]These authors contributed equally to this work.


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