Supporting Information for

Live Cell Screening to Identify RNA-Binding Small Molecule Inhibitors of the pre-let-7–Lin28 RNA-Protein Interaction

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A. General Materials and Methods

Materials:

Chemically synthesized pre-microRNAs (deprotected, desalted and HPLC purified) containing 5-aminohexylacrylamino uridine (5-LC-N-U) modifications and biotin attached to the 5′ end of the sequence by an 18-atom spacer were purchased from Horizon Discovery Biosciences (formerly Dharmacon) and used as received for the labeling reaction. HaloTag® (HT) Succinimidyl Ester (O4) Ligands was purchased from Promega and used as received (cat #P6751) for the bioconjugation reaction as previously described.[1-3] RNAs used for RiPCA are: pre-let-7d-36-4Cl, pre-miR-21-31-4Cl, pre-let-7a1-42-2Cl, and pre-let-7g-46-2Cl from our prior report.[3] mLin28A-LgBiT plasmid was prepared as previously described.[1] The generation of Flp-In™-293 cells stably expressing SmBiT-HT was previously reported.[1] The NanoGlo® Live Cell Assay System was purchased from Promega and used as received (cat #N2012). TransIT-X2® (Mirus cat #6000) was used as received. CellTiter-Glo® (CTG) was purchased from Promega and used as received (cat #G7570). Perkin Elmer CulturPlate 384-well plates (white, tissue-culture treated) (6007680) were used for RiPCA and CTG. Streptavidin-coated High-Capacity 384-well plates (Thermo cat #15505), SuperSignal™ West Pico PLUS (Thermo cat #34578), and horseradish peroxidase (HRP) (Pierce cat #PI31491) were used as received.[4] mLin28A-HaloTag-Biotin, pre-let-7d-TCO, and mTet-HRP were prepared as previously described by our group for cat-ELCCA.[4]

Cell Culture:

Flp-In™-293 cells stably expressing SmBiT-HT[1] were cultured in DMEM (Corning cat #10-017-CV) supplemented with 10% FBS (Atlanta Biologicals S11550), L-glutamine (Gibco cat #25030081), and hygromycin B (100 μg/mL) (Gibco cat #10687010) at 37 °C with 5% CO_2 in a humidified incubator, passaged at least once before use for an experiment. T-47D cells were grown in RMPI (Gibco cat #11875-093) supplemented with 10% FBS (Atlanta Biologicals S11550), 1 mM sodium pyruvate (Sigma cat #8636-100ML), and 7 mg/mL human insulin (Santa Cruz Biotechnology cat #360248) at 37 °C with 5% CO_2 in a humidified incubator, passaged at least once before use for an experiment. Cells were passaged using Trypsin-EDTA (0.25%) (Gibco cat #25300054) approximately 10 times, and no more than 15 times, before returning to low passage stocks. To count cells, cells were harvested and 10 μL of the cell suspension was mixed with 10

μL Trypan Blue (Gibco cat #15250061) ([final] = 0.2% trypan blue) and counted using a hemocytometer.

General assay and data analysis methods:

Chemiluminescence data was collected on a PerkinElmer EnVision or BMG Labtech PHERAStar plate reader. All data was analyzed using GraphPad Prism version 9.0.0 for Mac OS X (GraphPad Software, www.graphpad.com).

B. RiPCA HTS Protocol

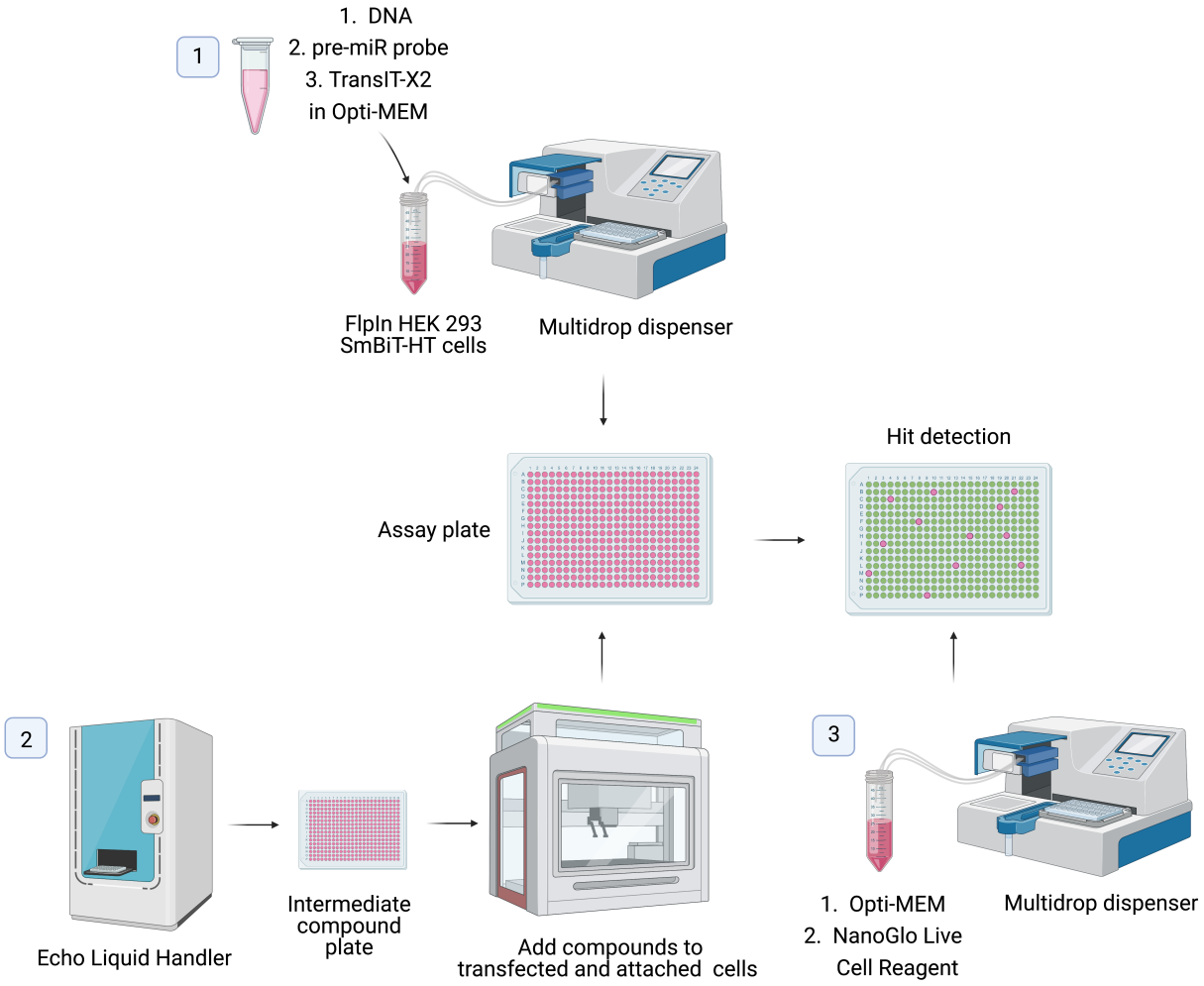

Figure S1. Overview of the RiPCA HTS workflow. (1) Batch-transfected Flp-In™-293 SmBiT-HT cells are plated in a 384-well assay plate using a Multidrop dispenser. (2) An intermediate compound plate is prepared using an ECHO liquid handler and volume is transferred to the assay plate using a Biomek liquid handler. (3) Chemiluminescence is measured after media removal and replacement with Opti-MEM™ and NanoGlo® Live Cell Reagent.

384-well Protocol (liquid handling):

A batch transfection solution was prepared by mixing Opti-MEM™, RBP plasmid, and RNA, followed by TransIT-X2 reagent. For every 50 µL of Opti-MEM™, 0.625 µL of 3.9 ng/µL pcDNA3 + mLin28A-LgBiT,[1] 0.3 µL of 50 µM pre-let-7d-Cl probe,[3] and 2.4 µL TransIT-X2 were added. The transfection solution was vortexed and briefly centrifuged, and then incubated at room temperature for ~15 min while cells were harvested. Flp-In™-293 SmBiT-HT cells[1] were harvested following standard cell culture protocols and counted using a cell counter. A cell solution of 100,000 cells/mL was prepared. For each 50 µL of transfection solution prepared, 400 µL of

cell solution was prepared. The transfection solution was added to the cell solution and mixed. Note, the batch transfection can be scaled up to a final volume of 40 mL (transfection solution + cells). The cell + transfection solution was plated at 30 µL per well using a small cassette for the Mutlidrop Combi Reagent Dispenser. To prevent striping, the tubing was primed 2× with the cell + transfection solution in the tubing for 2 min. Plates were centrifuged for 1 min at 1,000 rpm before being incubated at 37 °C with 5% CO_2 for 16 h. After 16 h, plates were removed from the incubator and 10 µL of 40 µM compound (or 0.4% DMSO) in supplemented DMEM from an intermediate compound plate (see below) was added to each well with a Biomek liquid handler. Plates were returned to the incubator for an additional 8 hr. Plates were read at 24 h after initial plating by removing the media manually and smacking the plate on a wicking or absorbent pad. Using the Multidrop, 40 µL Opti-MEM™ followed by 10 µL NanoGlo® Live Cell reagent (reconstituted according to manufacturer's recommendation) was added to each well. Plates were incubated at room temperature, covered for 30 min, and the chemiluminescence was measured by an EnVision plate reader.

Preparation of Intermediate Compound Plate:

An intermediate compound plate was prepared by adding 60 nL of 10 mM compound or DMSO to each well of a 384-well plate from a low dead volume plate using an ECHO liquid handler. Prior to addition to assay plates, 15 µL of warmed cell culture media (DMEM + 10% FBS, L-glutamine, hygromycin, and pen-strep) was added to each well. This protocol was repeated with different volumes of compound and DMSO for concentration response curve (CRC) experiments to achieve the final concentrations listed in Table S1.

Table S1. Concentrations of compound tested in RiPCA CRC.

Concentration	[Final] (µM)
1	0.391
2	0.781
3	1.563
4	3.125
5	6.25
6	12.5
7	25
8	50

384-well Protocol (multi-channel pipet):

Note: this protocol was used for pre-let-7a and pre-let-7g RiPCA. A batch transfection solution was prepared by mixing Opti-MEM™, RBP plasmid, and RNA, followed by TransIT-X2 reagent. For every 100 μL of Opti-MEM™, 2.5 μL of 3.9 ng/μL pcDNA3 + mLin28A-LgBiT,[1] 1.2 μL of 25 μM pre-miRNA-Cl probe,[3] and 4.8 μL TransIT-X2 were added. The transfection solution was vortexed and briefly centrifuged, and then incubated at room temperature for ~15 min while cells were harvested. Flp-In™-293 SmBiT-HT cells[1] were harvested following standard cell culture protocols and counted using a cell counter. A cell solution of 200,000 cells/mL was prepared. For each 100 μL of transfection solution prepared, 800 μL of cell solution was prepared. The transfection solution was added to the cell solution and mixed. The cell + transfection solution was plated at 30 μL per well (in 24 wells) using an Eppendorf multichannel repeater pipette. Plates were centrifuged for 30 sec at 1,000 rpm before being incubated at 37 °C with 5% CO_2 for 16 h. Compound was prepared by mixing 2.6 μL of Stock Compound (or 2.6 μL of DMSO) with 127.4 μL of warmed cell culture media (DMEM + 10% FBS, L-glutamine, hygromycin). After 16 h, plates were removed from the incubator and 10 μL of compound or 0.5% DMSO in supplemented DMEM was added to each well from an Intermediate Tube (see below) using an Eppendorf multichannel repeater pipette. Plates were centrifuged for 30 sec at 1,000 rpm before being incubated at 37 °C with 5% CO_2 for another 8 h. Plates were read at 24 h after initial plating by removing the media manually and smacking the plate on a wicking or absorbent pad. Using an Eppendorf multichannel repeater pipette, 40 μL Opti-MEM™ followed by 10 μL NanoGlo® Live Cell reagent (diluted 20x in the provided dilution buffer according to manufacturer's recommendation) was added to each well. Plates were centrifuged for 30 sec at 1,000 rpm, incubated at room temperature, covered for 30 min, and the chemiluminescence was measured by a BioTek Cytation3 Plate Reader.

Table S2. Concentrations of compound tested in RiPCA CRC.

Tube	[Stock] in DMSO (mM)	[Intermediate] in warmed DMEM (μM)	[Final] in assay plate (μM)
1	DMSO	0	0
2	156.25	3.125	0.781
3	312.5	6.25	1.563
4	625	12.5	3.125
5	1,250	25	6.25
6	2,500	50	12.5
7	5,000	100	25
8	10,000	200	50

C. CellTiter-Glo® Assay

Flp-In™-293 SmBiT-HT cells[1] were plated using a small cassette using a small cassette for the Mutlidrop Combi Reagent Dispenser after the tubing was primed 2× for 2 min each. Each well contained 3,000 cells in 30 μL. Plates were centrifuged at 1,000 rpm for 1 min and incubated at 37 °C and 5% CO_2 for 16 h. After 16 h, 10 μL of 40 μM compound in media was transferred to each well using a Biomek liquid handler. Plates were returned to the incubator for an additional 8 hr. Plates were read at 24 h after initial plating by removing the media manually and smacking the plate on a wicking or absorbent pad. Using the Multidrop, 25 μL Opti-MEM™ followed by 25 μL CellTiter-Glo® reagent (reconstituted according to manufacturer's recommendation) was added to each well. Plates were mixed on a plate shaker for 2 min and then incubated at room temperature, covered for 10 min and the chemiluminescence was measured by a PHERAstar plate reader.

D. cat-ELCCA

White high binding capacity streptavidin-coated 384-well plates were washed three times with 50 μL phosphate buffered saline (PBS; 100 mM phosphate, 150 mM NaCl, pH 7.0 diluted from 10× solution) using a Bio-Tek Elx405 plate washer. mLin28A was immobilized by adding 10 μL of 200 nM biotinylated mLin28A[4] in storage buffer (20 mM Tris pH 7.8, 100 mM. KCl, 0.2 mM EDTA, 10% glycerol (*v*/*v*), 0.005% Tween-20) to each well using the Multidrop. To prevent striping, the tubing was primed 2× with the mLin28A solution in the tubing for 2 min each. Plates were sealed with plate tape and centrifuged for 1 min at 1,000 rpm before being stored at 4 °C overnight. Following overnight incubation, plates were washed 3× with 50 μL PBS and smacked

against a spill pad to remove excess moisture. Compounds or DMSO were added to each well via ECHO liquid handler to achieve final concentrations listed in Table S2. Then, 10 μL of 200 nM pre-let-7d-TCO in binding buffer (50 mM Tris pH 7.6, 150 mM NaCl, 5% glycerol, 0.05% Tween-20, freshly added 1 mM $ZnCl_2$, and 10 mM β-mercaptoethanol) was added to lanes 1–23 by Multidrop. Lane 24 contained binding buffer only and was added manually with a multichannel repeater pipette. Plates were centrifuged for 1 min at 1,000 rpm and incubated for 1 h at room temperature. After incubation, wells were washed 3× with 50 μL PBS and smacked against a spill pad to remove excess moisture. Next, 10 μL of 750 nM mTet-HRP in PBS was added to each well by Multidrop and incubated for 1 h at room temperature. Finally, wells were washed 3× with 80 μL PBS-T (PBS with 0.05% Tween-20), followed by 3× with 80 μL PBS. Chemiluminescence signal was measured after the addition of 25 μL of prepared SuperSignal West Pico reagent on a PHERAstar plate reader.

Table S3. Concentrations of compound tested in cat-ELCCA for CRC

Concentration	[Final] (μM)
1	0.046
2	0.14
3	0.41
4	1.23
5	3.7
6	11.1
7	33.3
8	100

E. qRT-PCR

T-47D cells were plated in a 6-well plate at a density of 200,000 cells/mL and incubated for 24 h at 37 °C with 5% CO_2. The media was then removed and replaced with fresh media containing DMSO or 5 μM compound ([final] DMSO of 0.05%) and returned to the incubator for 48 h. Small RNAs (<200 nt) were harvested using the *mir*Vana™ miRNA Isolation Kit (Thermo cat #AM1561) following the manufacturer's protocol for enrichment of small RNAs. cDNA was synthesized from 50 ng small RNA using the Taqman™ Advanced miRNA cDNA Synthesis Kit (Thermo cat #A28007) following the manufacturer's protocol. RT-qPCR was performed in a 384-well plate with a 1:5 cDNA dilution following the Taqman™ Advanced miRNA Assay (Thermo

cat #4444557) protocol using probes for miR-16-5p, let-7a-5p, let-7d-5p, and let-7g-5p (Thermo) on a QuantStudio™5 thermocycler (Thermo) using the fast Taqman™ $\Delta\Delta C_T$ protocol. Relative fold change was calculated using the comparative threshold cycle ($\Delta\Delta C_T$) method.

F. pmiRGLO Assay

Lin28-expressing NTERA-2 cells were transfected with 100 nanograms of pmiRGLO contructs (empty vector or let-7d) using Lipofectamine LTX with PLUS reagent (Invitrogen 15338100). 5 h after transfection, cells were treated with varying concentrations of SID-415260 or the corresponding amount of DMSO. 48 h after transfection, cells were analyzed using the Dual-Glo luciferase reagent (Promega E2920) according to the manufacturer's recommendation. pmirGLO vector was ordered from Promega and let-7d was cloned in with oligos recommended by Promega.

G. Kinome Profiling

Compound SID-415260 was profiled for *in vitro* activity against protein kinases using the Invitrogen SelectScreen protein kinase profiling service:

https://www.thermofisher.com/us/en/home/products-and-services/services/custom-services/screening-and-profiling-services/selectscreen-profiling-service/selectscreen-kinase-profiling-service.html

H. Western Blot

Flp-In™-293 cells stably expressing SmBiT-HT were plated at a density of 150,000 cells/well in a clear, tissue-culture treated 6-well plate. After 16 h, media was aspirated from the cells and replaced with warmed media containing 10 µM of SID-415260 or 0.1% DMSO. 8 h after treatment, 24 h after plating, cells were harvested (rinsed once with PBS first) using 300 µL of RIPA buffer containing protease inhibitor cocktail. Lysates were sonicated at 40% Amp for 6 s and then spun down at 15,000 rpm for 5 min. Total protein was quantified using BCA, and 7 ug of total protein was loaded onto a 4–20% Tris-Glycine gel and resolved using 135 V for 90 min. The gel was transferred to a PVDF membrane in Towbin's Buffer (25 mM Tris pH 8.3, 193 mM glycine, 20% v/v methanol). The membrane was blocked in 5% non-fat milk for 1 h at 25 °C and then incubated with anti-Lin28A primary antibody (Cell Signaling Technology, 8706S) or anti-pSer200 Lin28A

(Cell Signaling Technology, 20607S) primary antibody overnight at 4 °C and secondary antibody for 1 h at 25 °C. HRP-linked anti-rabbit IgG was used as a secondary antibody (Cell Signaling Technology, 7074). Actin HRP was purchased from Santa Cruz Biotechnology (#sc-47778) and used as a standard. Clarity Max Western ECL Substrate (BIO-RAD cat #1705062) was used for detection of Lin28A and pSer200 Lin28A and Clarity Western ECL Substrate (BIO-RAD cat. #17050661) was used for detection of actin. Proteins were visualized using a Bio-Rad ChemiDoc imaging system.

I. SPR

Pre-let-7d. SPR was performed using Nicoya's automated OpenSPR-XT and their High Sensitivity Biotin-Streptavidin Sensor Kit (SEN-HS-3-STRP-KIT). The running buffer was Tris (pH 7.6), 150 mM NaCl, 1% glycerol, 1% DMSO, 0.05% Tween-20, and 1 mM $ZnCl_2$ for biotinylated pre-let7d. All injections on the SPR were 100 µL injections. The biotin sensor kit was first conditioned using 10 mM HCl over reference channel 1 and channel 2 at a flow rate of 150 µL/min. The surface was then activated using a 0.5 µM injection of streptavidin (diluted in running buffer) over channels 1 and 2 at a flow rate of 20 µL/min for a total interaction time of 5 min. The immobilization of the biotinylated pre-let7d was accomplished through injection of 1 µM at a flow rate of 15 µL/min to reach an RU of ~2,500 to saturate the sensor on channel 2 only. Compound was tested at 0, 10, 25, 50, and 100 µM concentrations at a flow rate of 30 µL/min with a dissociation time of 10 min. The chip was regenerated after each cycle with 50 mM Tris (pH 9), 260 mM NaCl, 0.5 mM EDTA, and 0.1 % Tween-20 at a flow rate of 200 µL/min followed by a dissociation time of 270 sec and an injection of running buffer at the same flow rate to flush any remaining regeneration buffer before the next injection of compound. Data was processed and the kinetics were evaluated using the TraceDrawer software using a 1:1 model where Bmax and BI were set to local fitting and the k_a and k_d were set to global fitting.

Lin28A. SPR was performed using Nicoya's automated OpenSPR-XT and their High Sensitivity Biotin-Streptavidin Sensor Kit (SEN-HS-3-STRP-KIT). The running buffer was Tris (pH 7.6), 150 mM NaCl, 1% glycerol, 1% DMSO, 0.05% Tween-20, and 1 mM $ZnCl_2$, and 1 mM β-mercaptoethanol for biotinylated HaloTag-Lin28A. All injections on the SPR were 100 µL injections. The biotin sensor kit was first conditioned using 10 mM HCl over reference channel 1

and channel 2 at a flow rate of 150 µL/min. The surface was then activated using a 0.5 µM injection of streptavidin (diluted in running buffer) over channels 1 and 2 at a flow rate of 20 µL/min for a total interaction time of 5 min. The immobilization of the biotinylated HaloTag-Lin28A was accomplished through injection of 1 µM at a flow rate of 20 µL/min to reach an RU of ~5,000 to saturate the sensor on channel 2 only. Compound was tested at 0, 10, 25, 50, and 100 µM concentrations over channcels 1 and 2 at a flow rate of 30 µL/min with a dissociation time of 10 min. The chip was regenerated after each cycle with 50 mM Tris (pH 9), 260 mM NaCl, 0.5 mM EDTA, and 0.1 % Tween-20 at a flow rate of 200 µL/min followed by a dissociation time of 270 sec. This was followed by an injection of running buffer at the same flow rate to flush any remaining regeneration buffer before the next injection of compound. Data was processed and the kinetics were evaluated using the TraceDrawer software using a 1:1 model where Bmax and BI were set to local fitting and the k_a and k_d were set to global fitting.

J. Supplemental Figures and Table

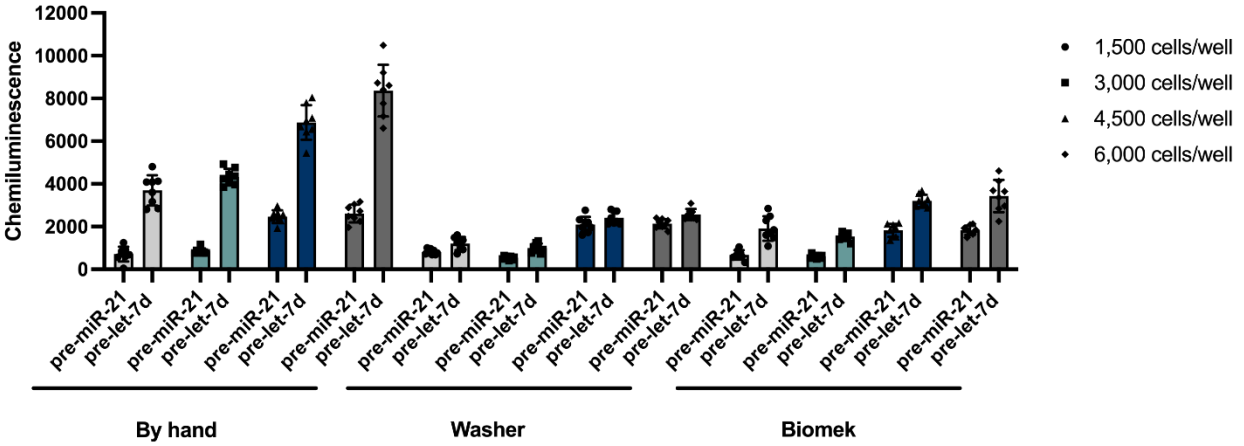

Figure S2. Optimization of number of cells per well and method of media removal prior to addition of NanoLuc® substrate. Note: media must be removed as leftover DMEM can cause an increase in signal detected. "By hand" refers to removing the media manually and smacking the plate on a wicking or adsorbent pad. "Washer" refers to aspirating with a plate washer. "Biomek" refers to aspirating with a Biomek liquid handler. Removing media by hand was found to yield the greatest signal due to low loss of cells; mechanically removing media was found to cause a loss of cells adhered to the well and thus, a loss of chemiluminescence signal detected. A pre-miR-21 probe was used as a non-binding control for the assay.

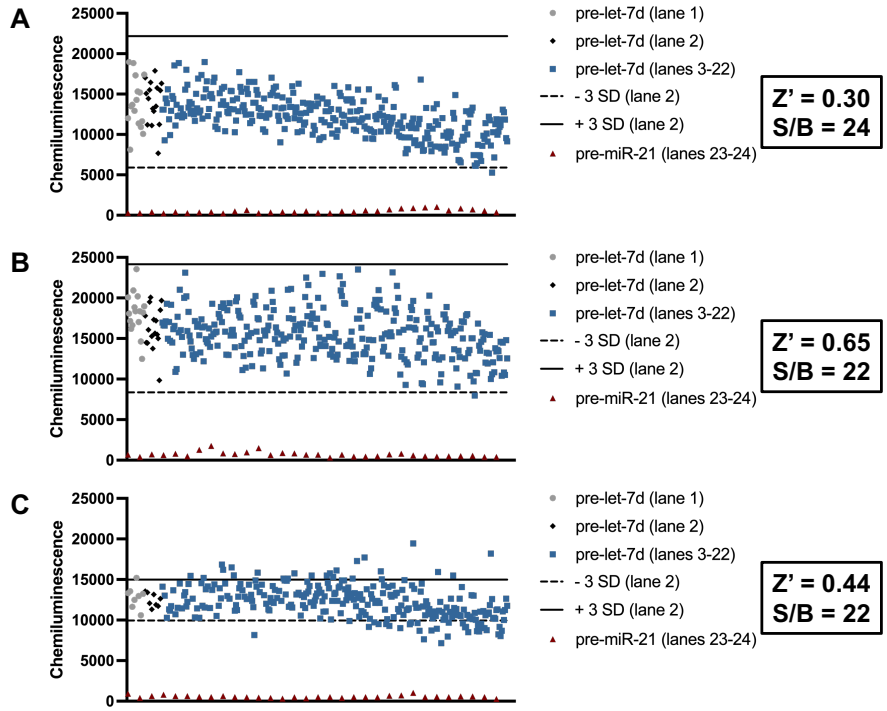

Figure S3. Reproducibility of batch transfection protocol shown in Figure S1. (A), (B), and (C) show data from 3 different plates using the 384-well RiPCA HTS protocol described herein, as well as the corresponding Z′ factor and S/B for each plate. A pre-miR-21 probe was used as a non-binding control for the assay.

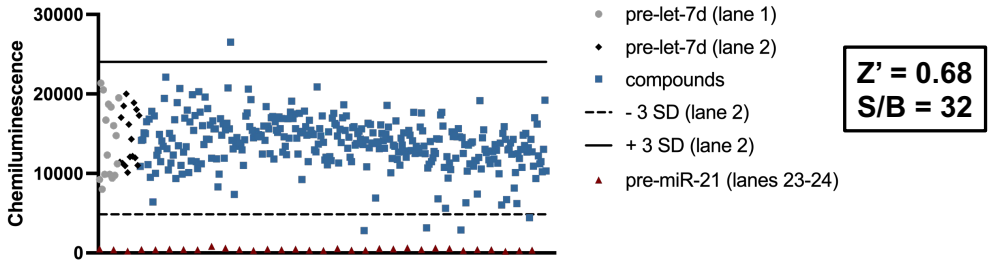

Figure S4. Pilot screening of 320 compounds from the LOPAC library. At 16 h post-transfection, 10 µL of 40 µM compound in DMEM was added to each well using a Biomek liquid handler from an intermediate compound plate prepared by the addition of 60 nL DMSO or compound by the ECHO liquid handler and 15 µL DMEM. The assay plate was returned to the incubator for 8 h and chemiluminescence signal was measured 30 min after the addition of the NanoLuc® substrate. Of the 320 compounds tested, there were four that reduced signal greater than 3 standard deviations below the mean signal for the negative control lanes (lanes 1and 2) for a preliminary hit rate of 1.25%. Hits from this pilot screen were not further investigated. A pre-miR-21 probe was used as a non-binding control for the assay. In this case, pre-miR-21 serves as a "positive control" for identifying inhibitors.

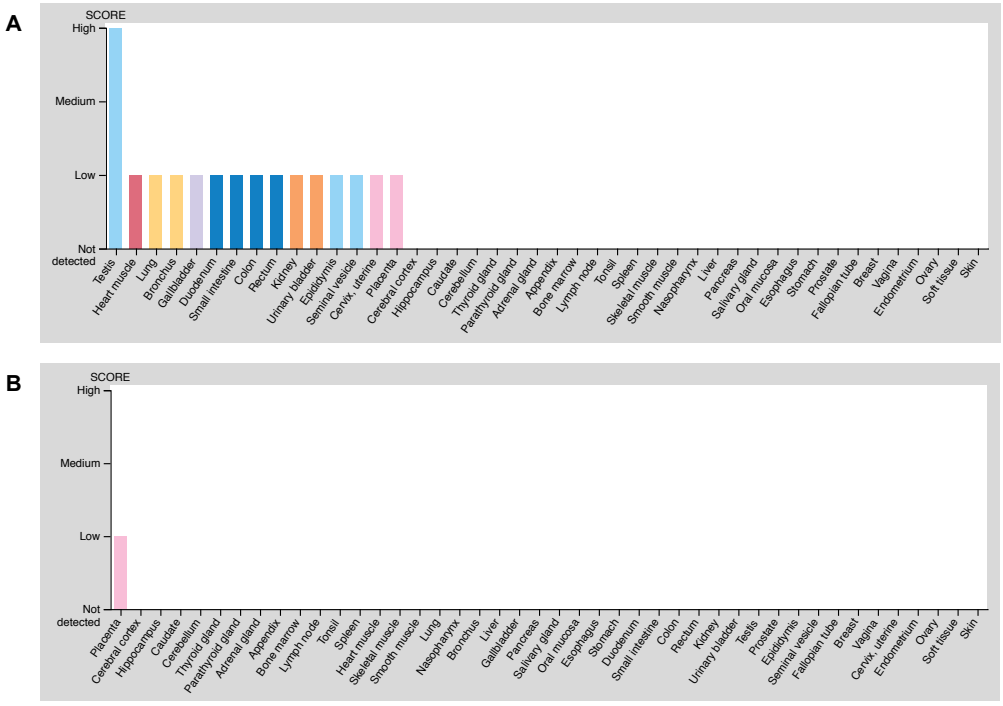

Figure S5. Tissue expression of (A) Lin28A and (B) Lin28B from The Human Protein Atlas.

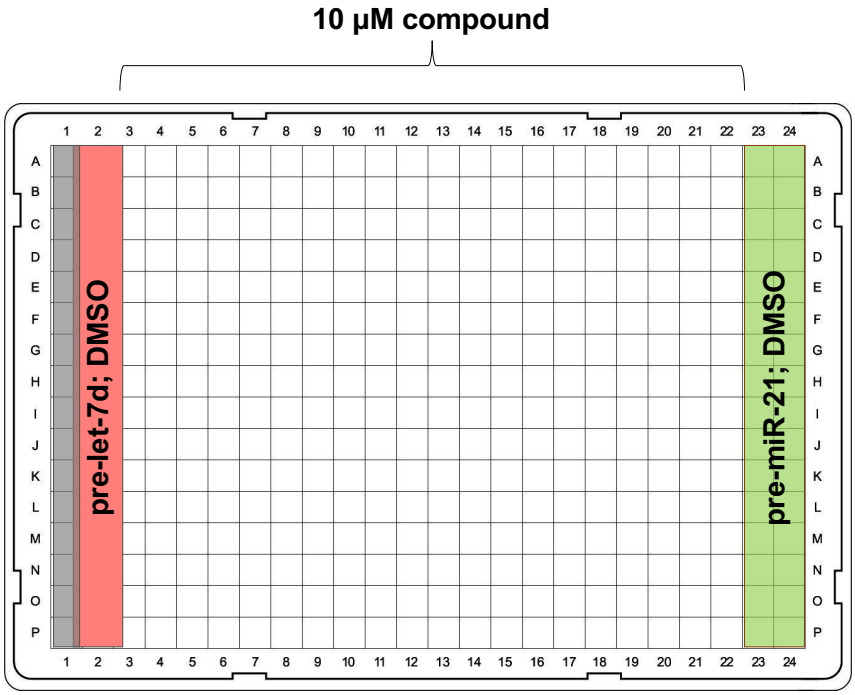

Figure S6. Plate layout for HTS. Of note, during screening, we encountered consistent data variability in lane 1 of each plate. To overcome this challenge, lane 1 was eliminated from data analysis and lane 2 (DMSO + pre-let-7d) was utilized as a negative control. A pre-miR-21 probe, a non-binding RNA substrate, was used as a positive control for delineating inhibitors (pre-miR-21 + DMSO).

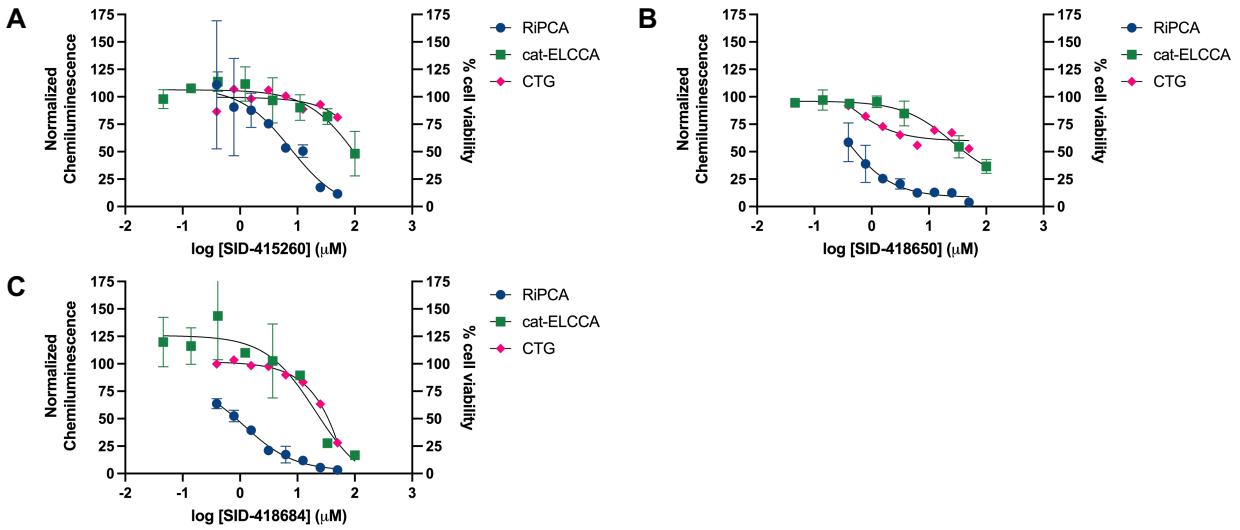

Figure S7. Dose-dependent inhibitory activity in RiPCA, cat-ELCCA, and CTG for the 3 most active hit compounds: (A) SID-415260, (B) SID-418650, and (C) SID-418684.

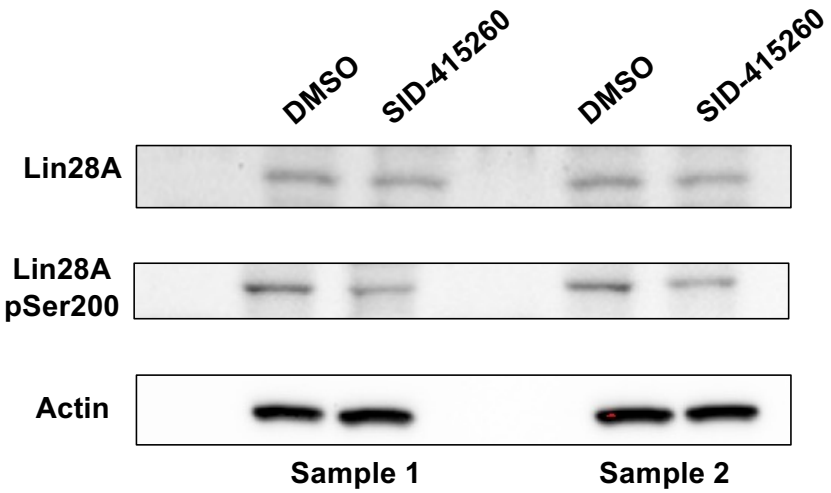

Figure S8. Protein levels of Lin28A and phosphorylated Lin28A after treatment with DMSO or SID-415260 (10 μM). Actin was used as a standard.

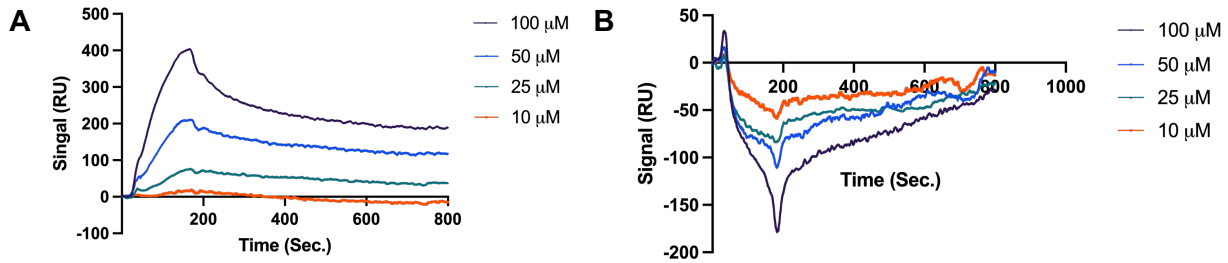

Figure S9. SPR sensorgrams for (A) pre-let-7d and (B) Lin28A.

Table S4. Kinome Profiling

Kinase	IC_{50} (μM)
WEE1	0.0352
MAP4K5 (KHS1)	0.298521688
SNF1LK2	0.330770608
MAP4K4 (HGK)	0.434830989
ABL1 E255K	0.466830014
YES1	0.467922188
PDGFRA V561D	0.491361844
MYLK2 (SKMLCK)	0.493222905
MAPKAPK3	0.50655994
MAP4K2 (GCK)	0.536016592
ABL1 Y253F	0.560817794
FGR	0.596860486
ABL1	0.720334453
MAPK14 (P38 ALPHA)	0.756649274
RPS6KA5 (MSK1)	0.768476216
CSF1R (FMS)	0.849627011
SGK (SGK1)	0.927704453
ABL1 G250E	0.929118355
LCK	0.980792697
LYN B	1.016364167
RPS6KA6 (RSK4)	1.083234603
SGKL (SGK3)	1.090316768
SRC	1.237333411
NEK4	1.302017356
BRAF V599E	1.321972531
PRKG1	1.32408321
MAP3K9 (MLK1)	1.369411763
MAPK9 (JNK2)	1.38135768
NEK2	1.425566821
MAPKAPK2	1.45905319
MELK	1.48259203
RPS6KA3 (RSK2)	1.492810419
BLK	1.495438904
AMPK A1/B1/G1	1.52274109
EPHA4	1.578887076
SGK2	1.579184045

ABL2 (ARG)	1.624356719
CAMK1 (CAMK1)	1.698008059
LYN A	1.729999408
PRKD2 (PKD2)	1.745328556
PRKX	1.757060551
FES (FPS)	1.758130446
CAMK2D (CAMKII DELTA)	1.80975324
SRMS (SRM)	1.825257531
FGFR1	1.831446972
GSK3B (GSK3 BETA)	1.902696162
EPHB4	1.972301082
PRKG2 (PKG2)	1.975519938
EPHA1	2.057783699
TYRO3 (RSE)	2.074854533
JAK3	2.118345845
PAK1	2.134861109
CDK5/P25	2.185830462
CDK9/CYCLIN T1	2.202737347
PDGFRB (PDGFR BETA)	2.225199351
MAP3K8 (COT)	2.40700055
PDGFRA (PDGFR ALPHA)	2.421208715
GRK4	2.43616798
EPHB1	2.516887998
PRKD1 (PKC MU)	2.634790679
AKT3 (PKB GAMMA)	2.644651856
RAF1 (CRAF) Y340D Y341D	2.714029648
RET Y791F	2.723138496
PRKCB1 (PKC BETA I)	2.729150683
NTRK1 (TRKA)	2.746078186
FYN	2.805313344
DAPK1	2.808066015
GRK7	2.839211933
EGFR (ERBB1) L861Q	2.880784939
MST4	2.885871549
STK25 (YSK1)	2.944440202
RPS6KA4 (MSK2)	2.983848448
STK24 (MST3)	3.108591738
PRKCB2 (PKC BETA II)	3.116205938
MET (CMET)	3.234647646

NUAK1 (ARK5)	3.245191894
AMPK A2/B1/G1	3.268988408
NEK6	3.383669222
PAK2 (PAK65)	3.401029997
AURKC (AURORA C)	3.415953216
GSK3A (GSK3 ALPHA)	3.43778306
AKT1 (PKB ALPHA)	3.597619862
CDK5/P35	3.633717268
PHKG2	3.646852027
MST1R (RON)	3.649736738
PAK3	3.738909812
PRKCG (PKC GAMMA)	4.011357196
ABL1 T315I	>10
ADRBK1 (GRK2)	>10
ADRBK2 (GRK3)	>10
AKT2 (PKB BETA)	>10
ACVR1B (ALK4)	>10
AURKA (AURORA A)	>10
AURKB (AURORA B)	>10
AXL	>10
BMX	>10
BRAF	>10
BRSK1 (SAD1)	>10
BTK	>10
CAMK1D (CAMKI DELTA)	>10
CAMK2A (CAMKII ALPHA)	>10
CAMK2B (CAMKII BETA)	>10
CAMK4 (CAMKIV)	>10
CDC42 BPA (MRCKA)	>10
CDC42 BPB (MRCKB)	>10
CDK1/CYCLIN B	>10
CDK2/CYCLIN A	>10
CDK7/CYCLIN H/MNAT1	>10
CHEK1 (CHK1)	>10
CHEK2 (CHK2)	>10
CLK1	>10
CLK2	>10
CLK3	>10
CSK	>10

CSNK1A1 (CK1 ALPHA 1)	>10
CSNK1D (CK1 DELTA)	>10
CSNK1E (CK1 EPSILON)	>10
CSNK1G1 (CK1 GAMMA 1)	>10
CSNK1G2 (CK1 GAMMA 2)	>10
CSNK1G3 (CK1 GAMMA 3)	>10
CSNK2A1 (CK2 ALPHA 1)	>10
CSNK2A2 (CK2 ALPHA 2)	>10
DAPK3 (ZIPK)	>10
DCAMKL2 (DCK2)	>10
DNA-PK	>10
DYRK1A	>10
DYRK1B	>10
DYRK3	>10
DYRK4	>10
EEF2K	>10
EGFR (ERBB1)	>10
EGFR (ERBB1) L858R	>10
EGFR (ERBB1) T790M L858R	>10
EGFR (ERBB1) T790M	>10
EPHA2	>10
EPHA3	>10
EPHA5	>10
EPHA8	>10
EPHB2	>10
EPHB3	>10
ERBB2 (HER2)	>10
ERBB4 (HER4)	>10
FER	>10
FGFR2	>10
FGFR3	>10
FGFR3 K650E	>10
FGFR4	>10
FLT1 (VEGFR1)	>10
FLT3	>10
FLT3 D835Y	>10
FLT4 (VEGFR3)	>10
FRAP1 (MTOR)	>10
FRK (PTK5)	>10

GRK5	>10
GRK6	>10
GSG2 (HASPIN)	>10
HCK	>10
HIPK1 (MYAK)	>10
HIPK2	>10
HIPK3 (YAK1)	>10
HIPK4	>10
IGF1R	>10
CHUK (IKK ALPHA)	>10
IKBKB (IKK BETA)	>10
IKBKE (IKK EPSILON)	>10
INSR	>10
INSRR (IRR)	>10
IRAK1	>10
IRAK4	>10
ITK	>10
JAK1	>10
JAK2	>10
JAK2 JH1 JH2 V617F	>10
JAK2 JH1 JH2	>10
KDR (VEGFR2)	>10
KIT	>10
KIT T670I	>10
LRRK2	>10
LRRK2 G2019S	>10
LTK (TYK1)	>10
MAP2K1 (MEK1)	>10
MAP2K2 (MEK2)	>10
MAP2K6 (MKK6)	>10
MAPK1 (ERK2)	>10
MAPK10 (JNK3)	>10
MAPK11 (P38 BETA)	>10
MAPK12 (P38 GAMMA)	>10
MAPK13 (P38 DELTA)	>10
MAPK14 (P38 ALPHA) DIRECT	>10
MAPK3 (ERK1)	>10
MAPK8 (JNK1)	>10
MAPKAPK5 (PRAK)	>10

MARK1 (MARK)	>10
MARK2	>10
MARK3	>10
MARK4	>10
MATK (HYL)	>10
MERTK (CMER)	>10
MET M1250T	>10
MINK1	>10
MKNK1 (MNK1)	>10
MUSK	>10
NEK1	>10
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NTRK2 (TRKB)	>10
NTRK3 (TRKC)	>10
PAK6	>10
PAK4	>10
PAK7 (KIAA1264)	>10
PASK	>10
PDGFRA D842V	>10
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PDK1 DIRECT	>10
PHKG1	>10
PI4KA (PI4K ALPHA)	>10
PI4KB (PI4K BETA)	>10
PIK3C2A (PI3K-C2 ALPHA)	>10
PIK3C2B (PI3K-C2 BETA)	>10
PIK3C3 (HVPS34)	>10
PIK3CG (P110 GAMMA)	>10
PIK3CD/PIK3R1 (P110 DELTA/P85 ALPHA)	>10
PIK3CA/PIK3R1 (P110 ALPHA/P85 ALPHA)	>10
PIM1	>10
PIM2	>10
PKN1 (PRK1)	>10
PLK1	>10
PLK2	>10
PLK3	>10
PRKACA (PKA)	>10
PRKCA (PKC ALPHA)	>10

PRKCD (PKC DELTA)	>10
PRKCE (PKC EPSILON)	>10
PRKCH (PKC ETA)	>10
PRKCI (PKC IOTA)	>10
PRKCN (PKD3)	>10
PRKCZ (PKC ZETA)	>10
PTK2 (FAK)	>10
PTK2B (FAK2)	>10
PTK6 (BRK)	>10
RET	>10
RET V804L	>10
ROCK1	>10
ROCK2	>10
ROS1	>10
RPS6KA1 (RSK1)	>10
RPS6KA2 (RSK3)	>10
RPS6KB1 (P70S6K)	>10
SPHK1	>10
SPHK2	>10
SRC N1	>10
SRPK1	>10
SRPK2	>10
STK22B (TSSK2)	>10
STK22D (TSSK1)	>10
STK23 (MSSK1)	>10
STK3 (MST2)	>10
STK4 (MST1)	>10
SYK	>10
TAOK2 (TAO1)	>10
TBK1	>10
TEK (TIE2)	>10
TXK	>10
TYK2	>10
ZAP70	>10

K. Characterization Data for SID-415260

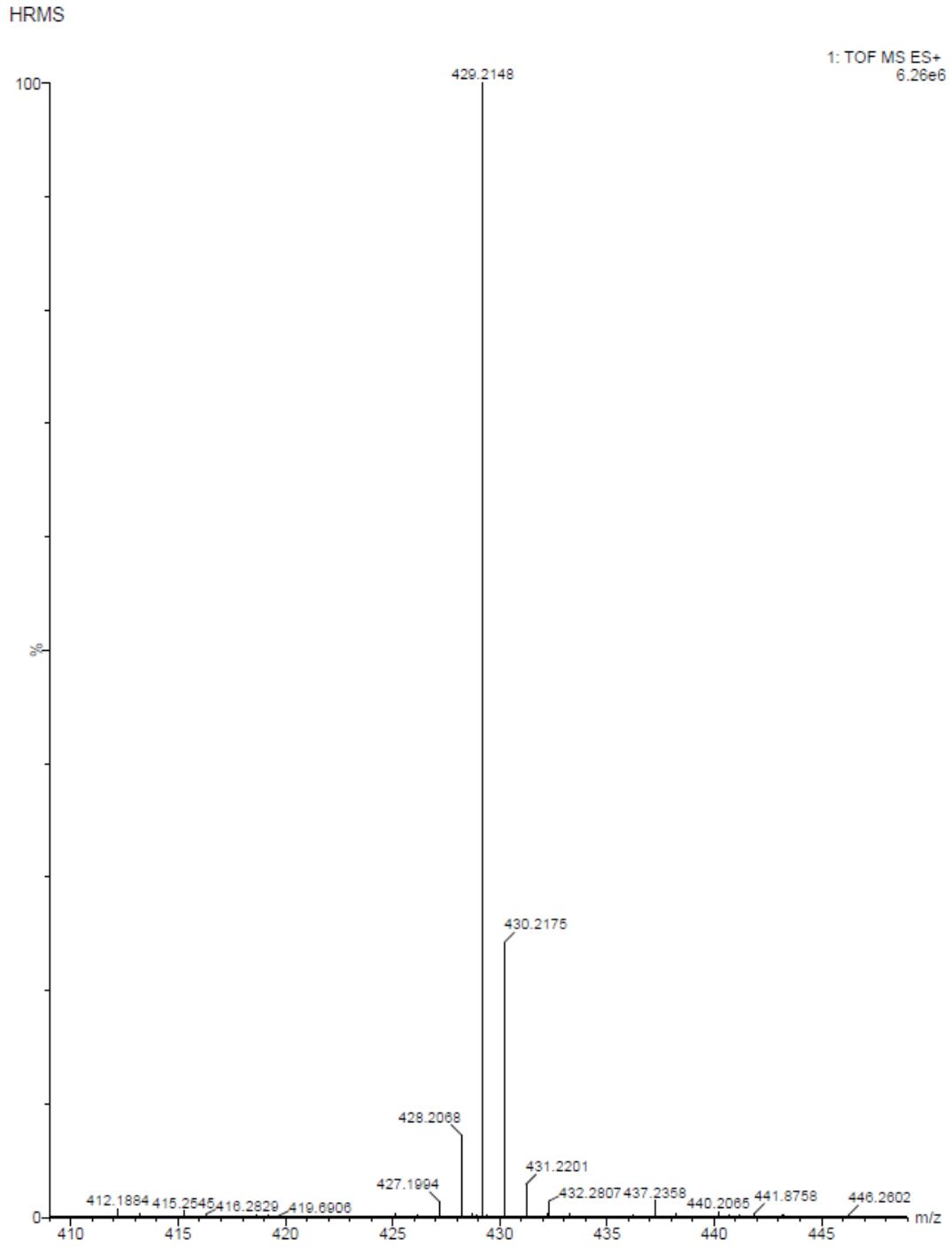


Figure S10. HRMS Data for SID-415260. High-resolution mass spectrometry (HRMS) data were recorded on a Waters Xevo G2 Q-Tof instrument in ESI^+ (electrospray) ionization mode. HRMS (ESI+) *m/z* 429.2148 [$(M+H)^+$ calc'd for $C_{23}H_{25}N_8O$: 429.2146].

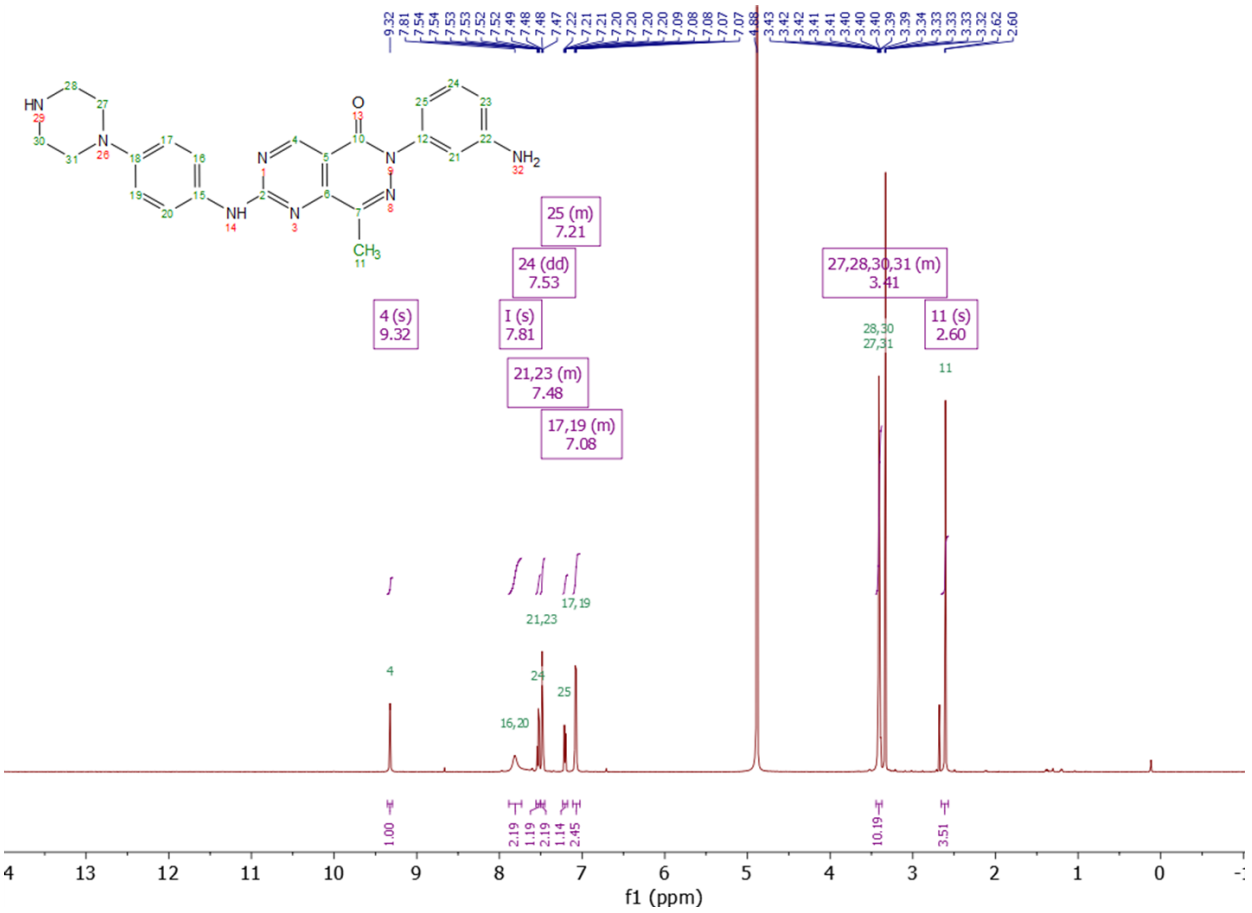


Figure S11. ^{1}H-NMR Data for SID-415260. ^{1}H NMR (600 MHz, MeOD) δ 9.32 (s, 1H), 7.81 (br s, 2H), 7.54 - 7.47 (m, 3H), 7.21 (m, 1H), 7.08 (m, 2H), 3.43 - 3.32 (m, 8H), 2.62 (s, 3H). Proton nuclear magnetic resonance spectra were recorded on a Varian 600 MHz NMR spectrometer at ambient temperature. All chemical shifts (δ) are reported in parts per million (ppm). Proton resonances are referenced to residual protium in the NMR solvent. Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (*J*) in Hertz (Hz), integration.

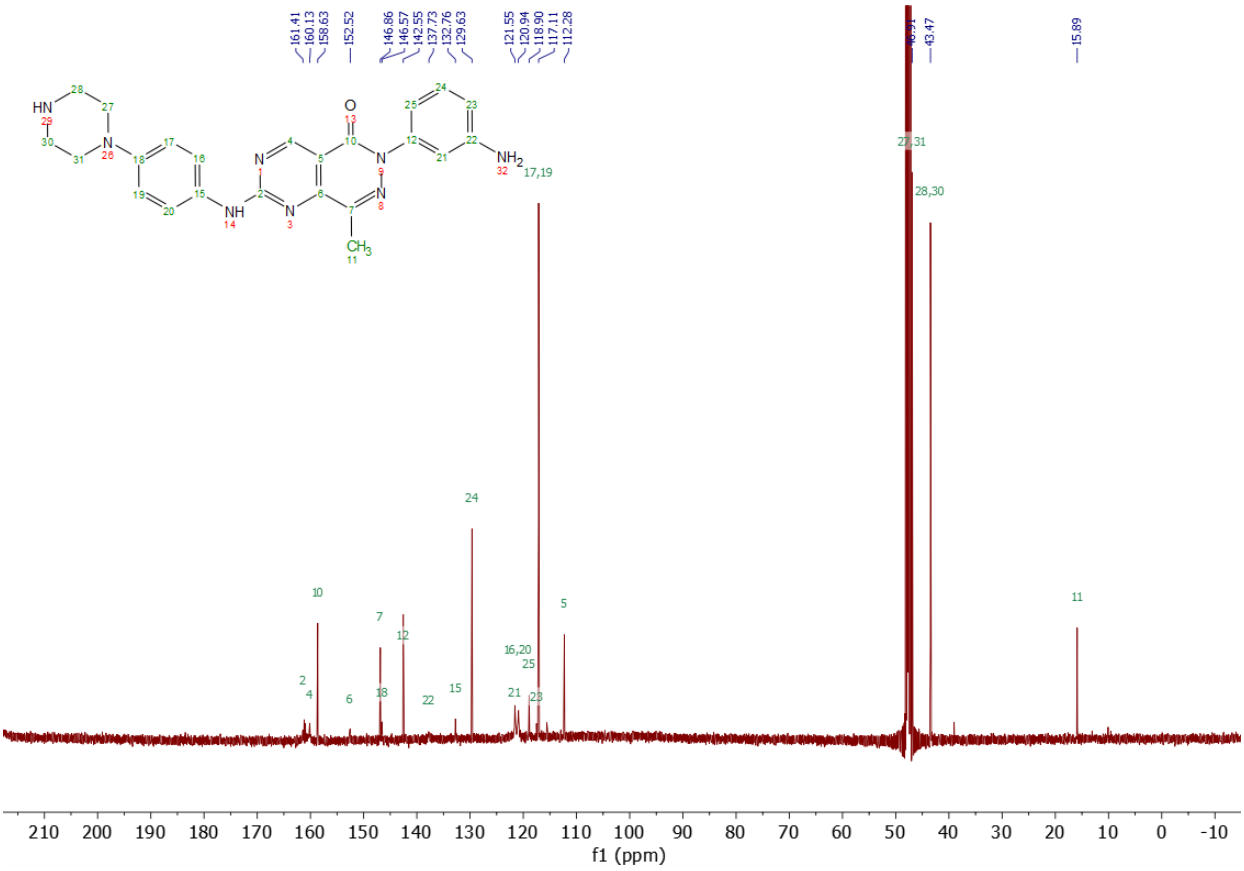
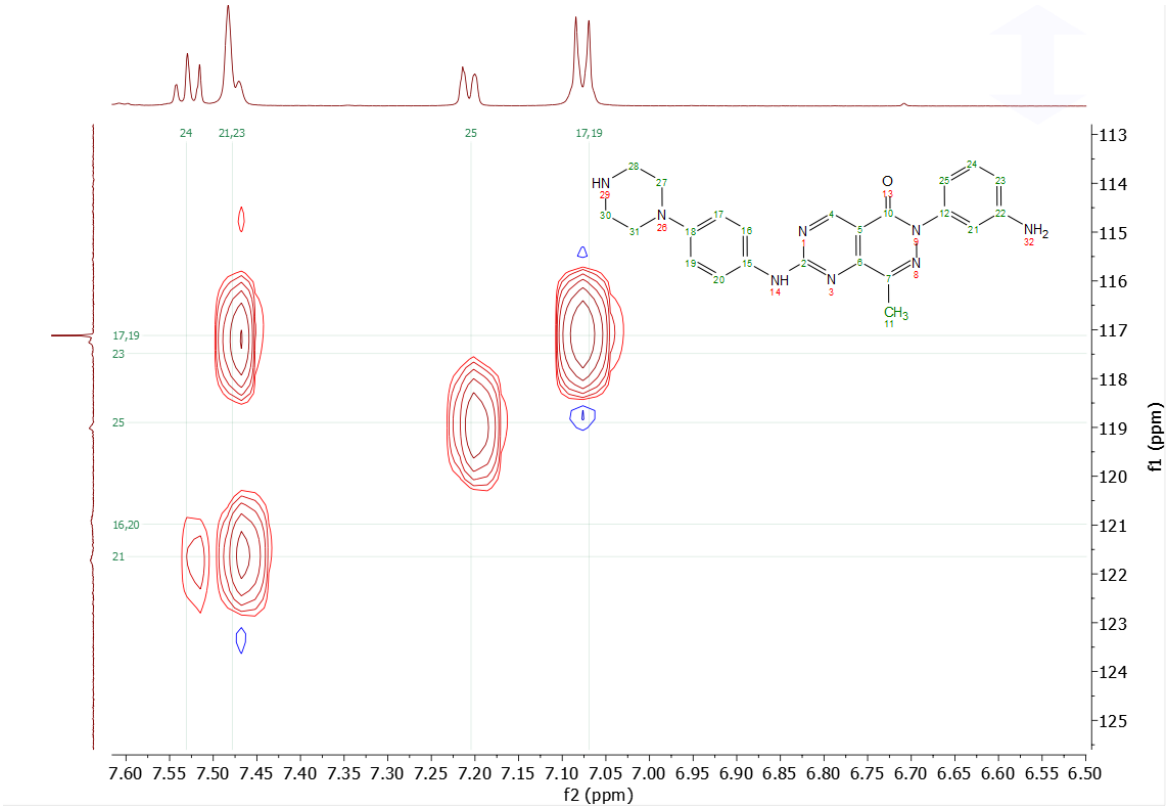
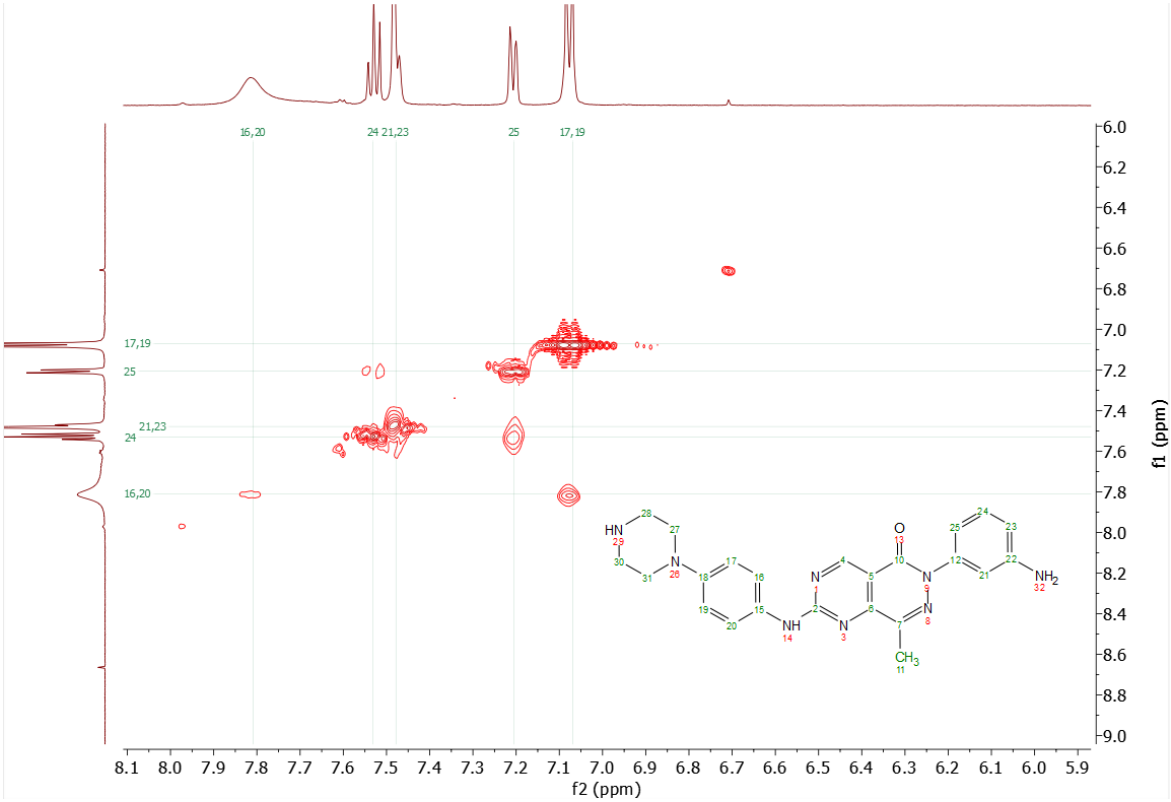


Figure S12. ^{13}C-NMR Data for SID-415260. ^{13}C NMR (600 MHz, MeOD) δ 161.4, 160.1, 158.6, 152.5, 146.9, 146.6, 142.6, 137.8, 132.8, 129.6, 121.5 (3C), 120.9, 118.9, 117.5-117.1 (3C), 112.3, 46.9 (2C), 43.5 (2C), 15.9. Carbon nuclear magnetic resonance spectra were recorded on a Varian 600 MHz NMR spectrometer at ambient temperature. All chemical shifts (δ) are reported in parts per million (ppm). Proton resonances are referenced to residual protium in the NMR solvent. Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (*J*) in Hertz (Hz), integration.


24
21,23
25
17,19
17,19
23
25
16,20
21
f2 (ppm)
f1 (ppm)


16,20
24 21,23
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17,19
17,19
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21,23
24
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f2 (ppm)
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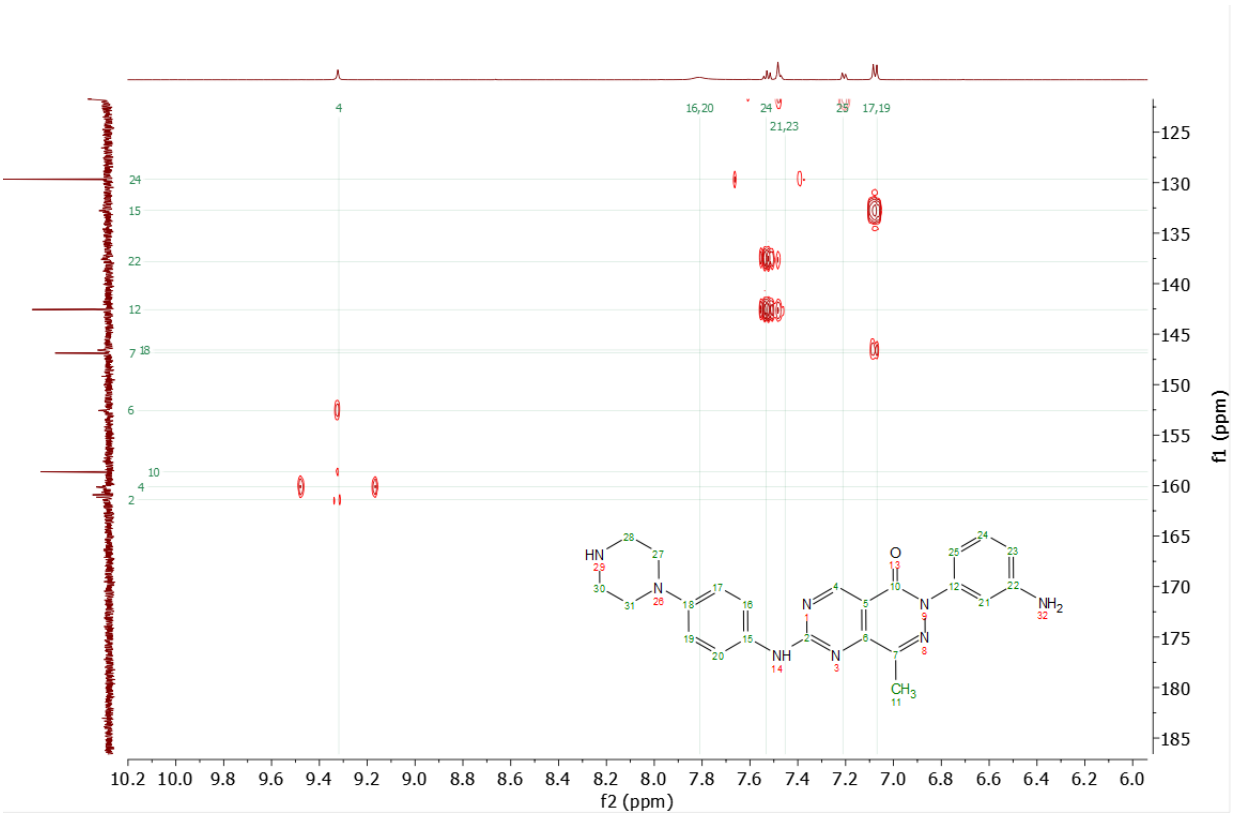

Figure S13. 2D-NMR Data for SID-415260. HSQC, COSY and HMBC, respectively.

L. References

1 Rosenblum, S. R., Lorenz, D. A. & Garner, A. L. A Live-Cell Assay for the Detection of pre-microRNA-Protein Interactions. *RSC Chem. Biol.* **2**, 241-247 (2021).

2 Rosenblum, S. R. & Garner, A. L. RiPCA: an assay for the detection of RNA-protein interactions in live cells. *Curr. Protoc. Chem. Biol.* **2**, e358 (2022).

3 Rosenblum, S. R. & Garner, A. L. Optimization of RiPCA for the live-cell detection of pre-microRNA-protein interactions. *ChemBioChem* **23**, e202200508 (2022).

4 Lorenz, D. A., Kaur, T., Kerk, S. A., Gallagher, E. E., Sandoval, J. & Garner, A. L. Expansion of cat-ELCCA for the discovery of small molecule inhibitors of the pre-let-7-Lin28 RNA-protein interaction. *ACS Med. Chem. Lett.* **9**, 517-521 (2018).