MicroRNA Therapeutics

-Overview and Cutting Edge of Small Molecule-based Approach-

Literature Seminar on 13th, May, 2017

D3 Kai Kitamura

Outline

- Introduction of miRNA
- Overview of miRNA therapeutics
- Main topic:

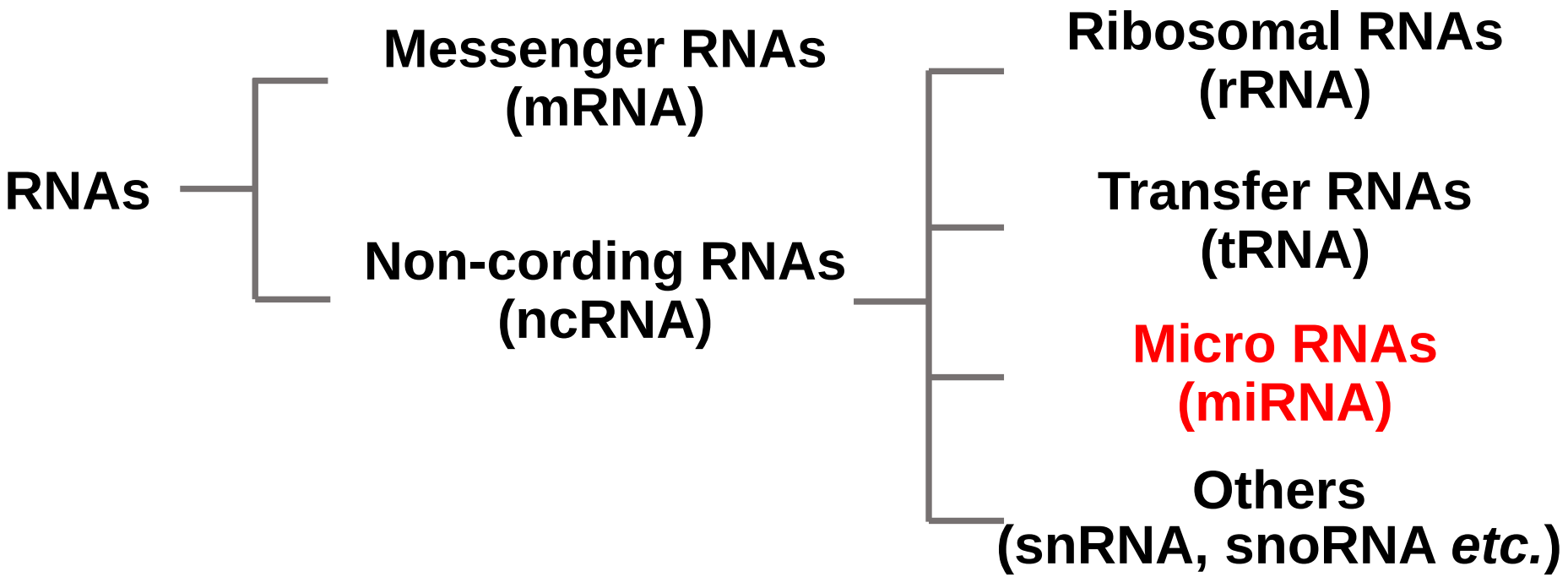
  Rational Design of Small Molecule Targeting Noncoding RNAs

  (Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205)
- Subtopic:

  Regulating miRNA by Bifunctional Small Molecule

  (Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987)

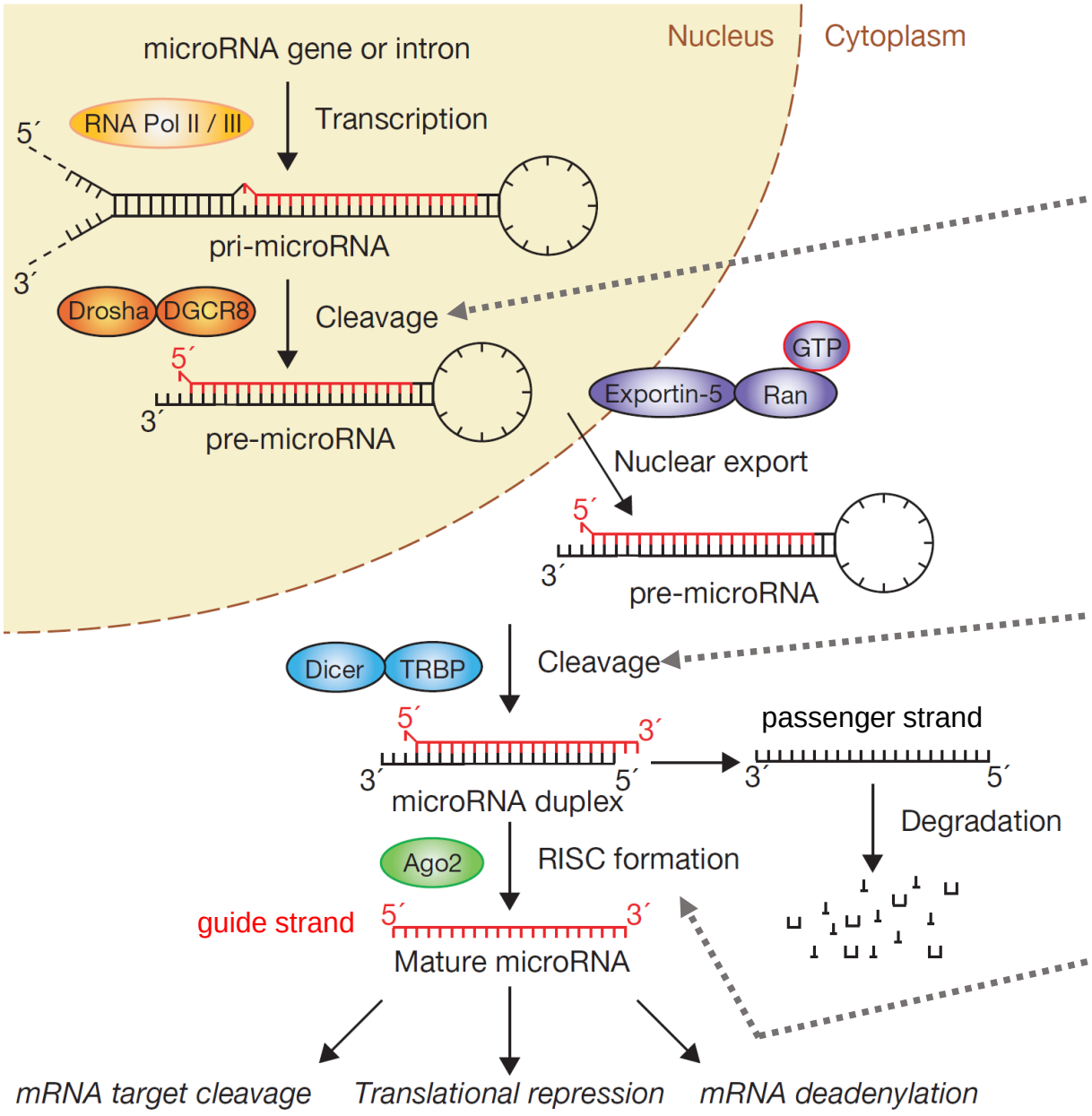
MicroRNAs (miRNAs)



- ✓ *Small non-coding RNAs consist of 21-25 nucleotides*
- ✓ *Encoded in the genomes of plants and animals, and highly conserved*
- ✓ *Regulating the expression of genes by binding to mainly the 3'-untranslated regions (3'-UTR) of specific mRNAs*

Lee, R.C. *et al Cell* **1993**, *75*, 843; Lau, N. C*. et al Science* **2001**, *294*, 858.

Biogenesis of miRNAs



The Microprocessor complex

Drosha:
double-stranded (ds)
RNA-specific
Endoribonucleases

DGCR8:
the double-stranded
RNA binding protein
/essential for
Drosha activity

Dicer:
double-stranded (ds)
RNA-specific
Endoribonucleases

TRBP:
a double strand RNA binding protein required
for the recruitment of Ago2 to
the miRNA bound by Dicer

RNA induced silencing complex (RISC)

Ago2:
The Argonaute protein plaing a central
role in RNA silencing processes, as
essential components RISC.

Winter, J. *et al Nat. Cell Biol.* **2009**, *11*, 228.

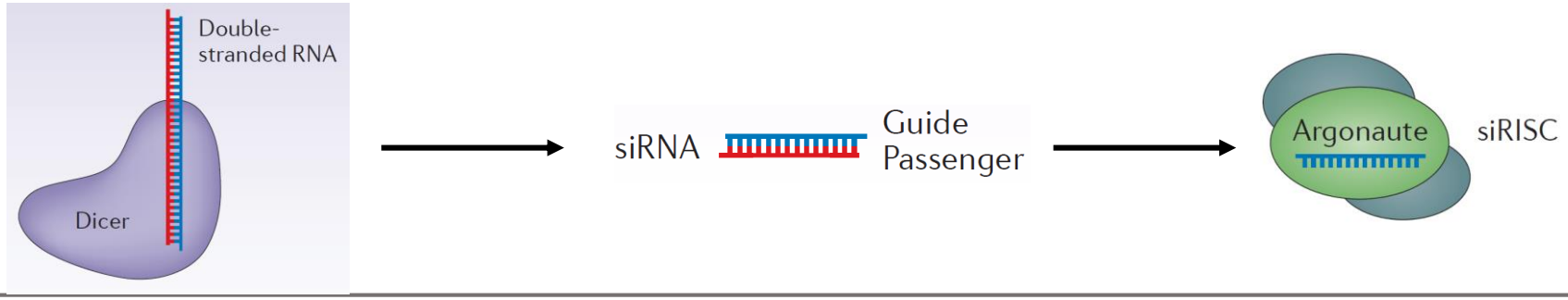
Difference between miRNAs and siRNAs

Similarities

- ✓ *Processed by Dicer*
- ✓ *Formation of RISC (RNA-induced silencing complex)*
- ✓ *degrade mRNAs bearing fully complementary sequences*

Differences

- ✓ *miRNAs are endogenously encoded small noncoding RNAs, derived by processing of short RNA hairpins*
- ✓ *siRNAs are derived by processing of long double-stranded RNAs and are often of exogenous origin*
- ✓ *miRNA can inhibit the translation of mRNAs bearing partially complementary target sequences*



Meister, G. *Nat. Rev. Genet.* **2013**, *14*, 447.

The role of miRNA in diseases

■ The role of miRNAs in cancer

✓ Dysregulation of miRNA biogenesis enzymes

(mutations, transcriptional changes *etc.* in Drosha, Dicer, *etc.*)

✓ Dysregulation of tumor-suppressive miRNAs

(miR-34 family, let-7 family, miR-200 family, miR-15/16 *etc.*)

✓ Dysregulation of miRNAs with oncogenic function

(miR-21 = anti-aptoptic role, miR-155, miR-210, *etc.*)

■ Other diseases relevant to miRNAs

✓ Hepatitis C infection (miR-122: upregulating the replication)

✓ Cardiovascular disease (miR-21, miR143/145, miR-1 etc.)

✓ Atherosclerosis (miR-33: downregulating)

✓ Diabetes (miR-200, miR192, miR-29 family)

✓ Scleroderma (miR-29)

Rupaimoole, R. and Slack, F. J. *Nat. Rev. Drug Discov.* **2017**, *16*, 203.

Outline

- Introduction of miRNA
- Overview of miRNA therapeutics
- Main topic:

  Rational Design of Small Molecule Targeting Noncoding RNAs

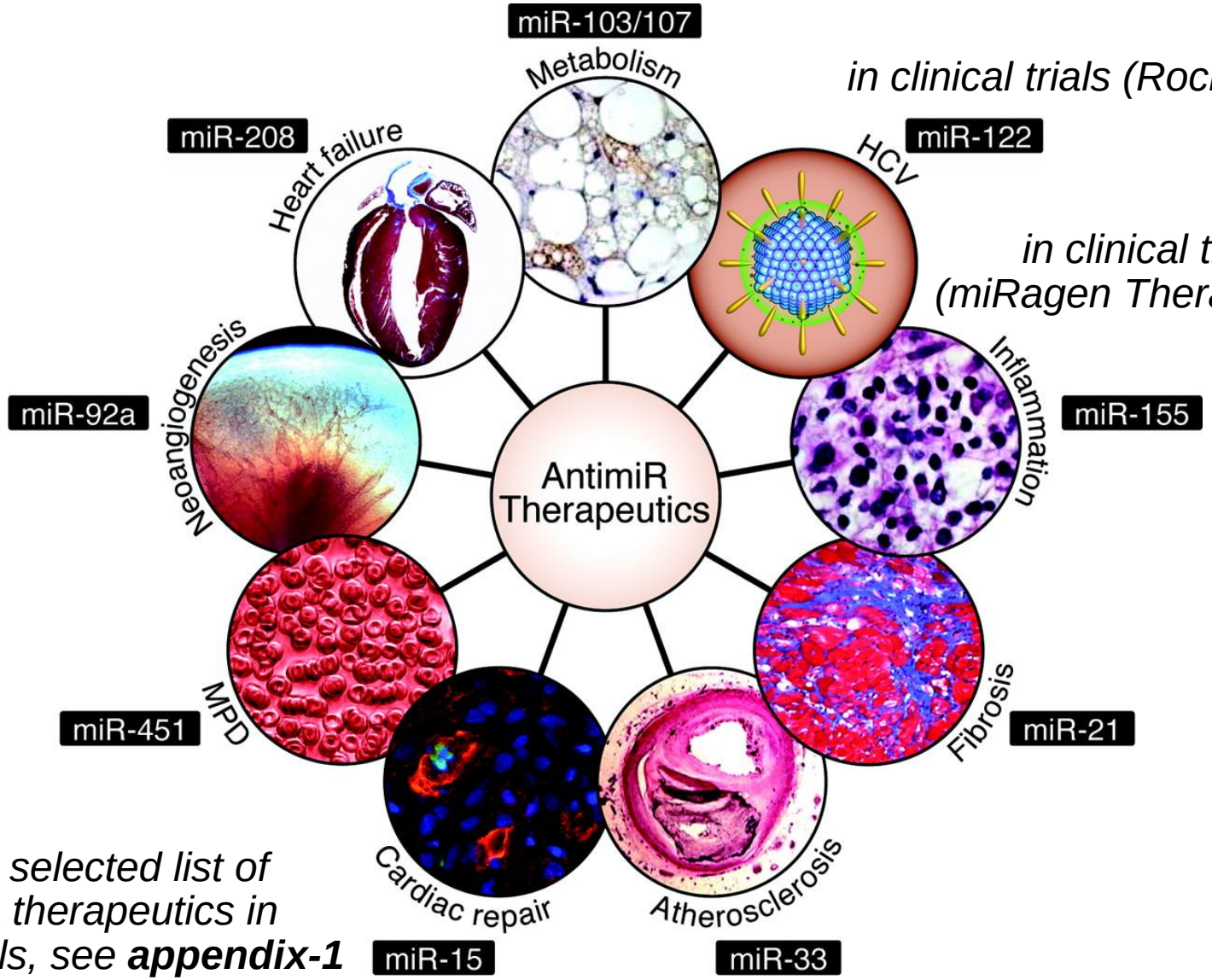
  (Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205)
- Subtopic:

  Regulating miRNA by Bifunctional Small Molecule

  (Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987)

miRNA therapeutics

in clinical trials (Regulus Therapeutics)



in clinical trials (Roche etc.)

in clinical trials (miRagen Therapeutics)

for the selected list of miRNA therapeutics in clinical trials, see ***appendix-1***

Rooij, E. van *et al. Circ. Res.* **2012**, *110*, 496.

Strategies in miRNA therapeutics

■ Micro RNA therapeutics:

1. Compensation for miRNA dysfunction
2. Inhibition of miRNA activity

→ RNAs themselves seem to be promising drug candidates

Problems in using RNAs as drug candidates

✓ Instability in bloodstream

✓ Poor delivery to the target site (poor cell permeability)

Strategies

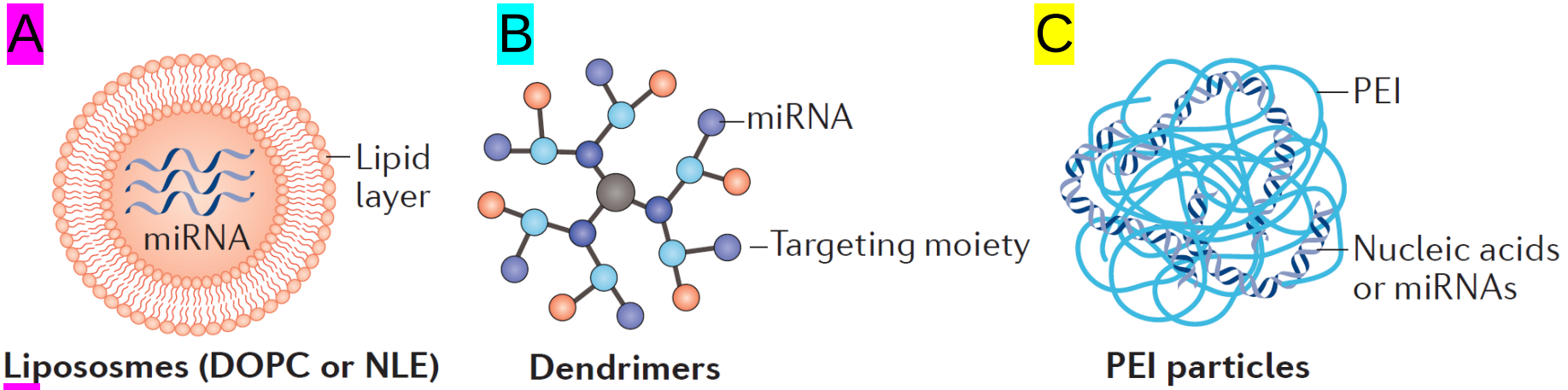
✓ Delivery vehicle to encapsulate RNAs

✓ Chemical modifications to the nucleotide backbone

✓ Small molecules to control biogenesis of RNAs (Topic)

Rupaimoole, R. and Slack, F. J. *Nat. Rev. Drug Discov.* **2017**, *16*, 203.

RNA delivery technologies



A: Neutral lipid emulsions (NLEs) consist of DOPC (Dioleoyl-phosphatidylcholine)

Neutral charge but low efficiency of delivery to tumor

B: Dendrimers consist of poly(amideamine)- or poly(propylene-imine)-nucleic acid conjugate

C: Proton-sponge with polyethyleneimine (PEI)

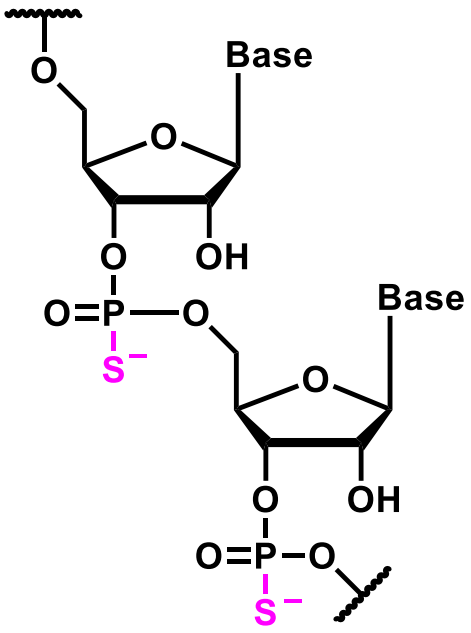
High efficiency of delivery but often toxic due to cationic property

D: Poly(ethyleneglycol) (PEG)-RNA conjugate via disulfide bond

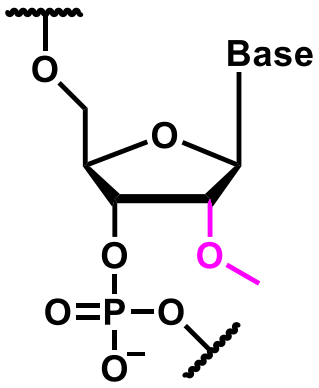
One of the most advanced system currently in clinical trials

Rupaimoole, R. and Slack, F. J. *Nat. Rev. Drug Discov.* **2017**, *16*, 203.

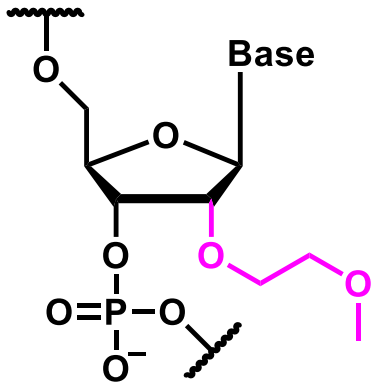
1st and 2nd generation chemical modifications of RNA (phosphorothionate, 2'-*O*-alkylation)



phosphorothioate
(the oxidation step of phosphoramidite method is replaced by sulfur transfer reaction)



2'-*O*-methylnucleotide

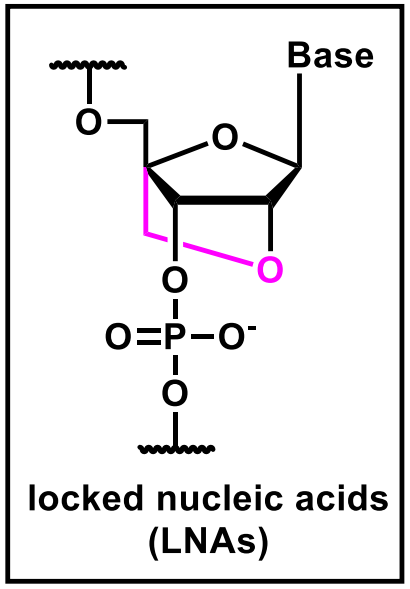


2'-*O*-methoxyethyl

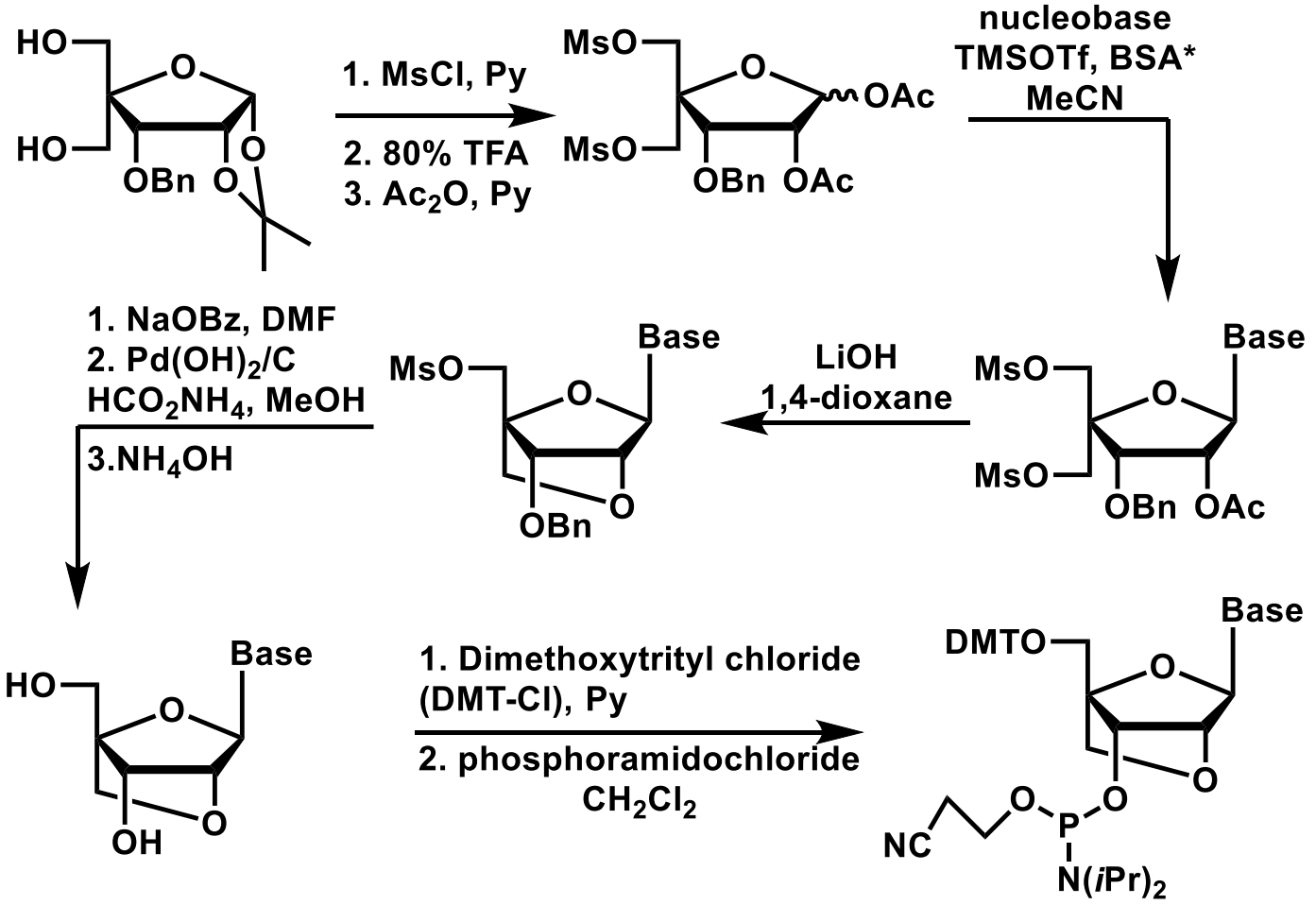
- ✓ Only the (*S*)-P phosphorothioate diastereomer is nuclease resistant
- ✓ 2'-*O*-alkylation modification improves nuclease resistance and binding affinity

Rupaimoole, R. and Slack, F. J. *Nat. Rev. Drug Discov.* **2017**, *16*, 203.
Scotson, J. L. *et al. Org. Biomol. Chem.* **2016**, *14*, 10840.

3rd generation-1 (LNAs)



locked nucleic acids (LNAs)



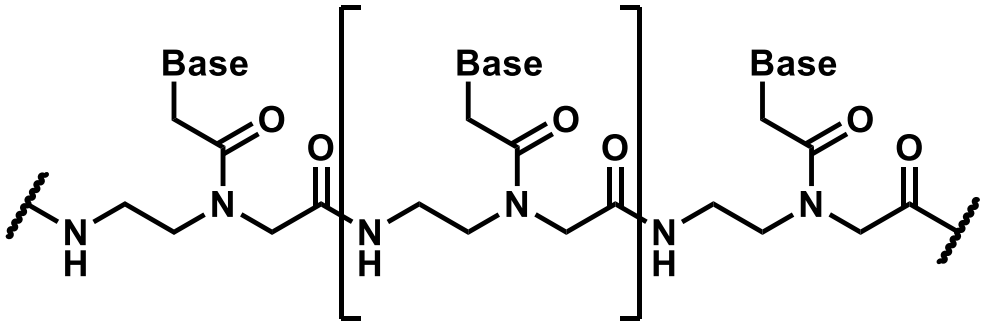
ASOs = antisense oligonucleotide

BSA* = *N*,*O*-bis(trimethylsilyl)acetamide

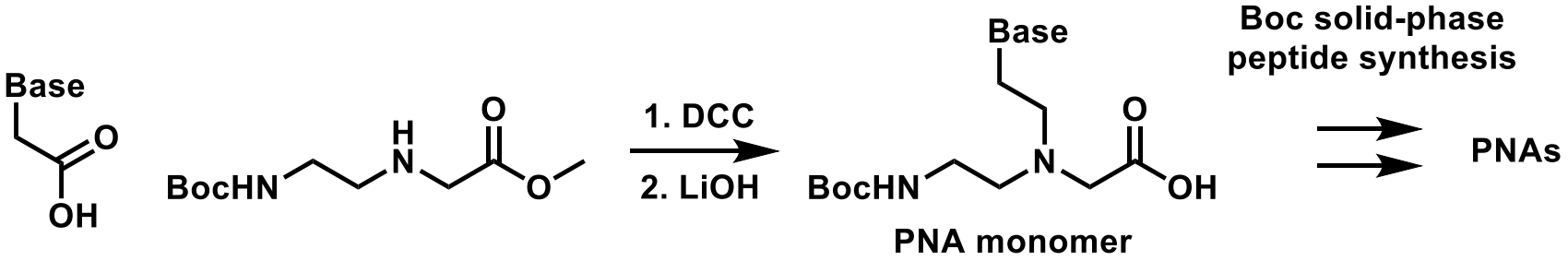
- ✓ LNAs improve sequence-specificity and stability of ASOs
- ✓ LNAs shows resistance to degradation by 3'-exonucleases

Sharma, V. K. *et al. Sustainable Chemical Processes* **2015**, *3*, 2.
Campbell, M. A. and Wengel, J. *Chem. Soc. Rev.* **2011**, *40*, 5680.

3rd generation-2 (Peptide nucleic acid)



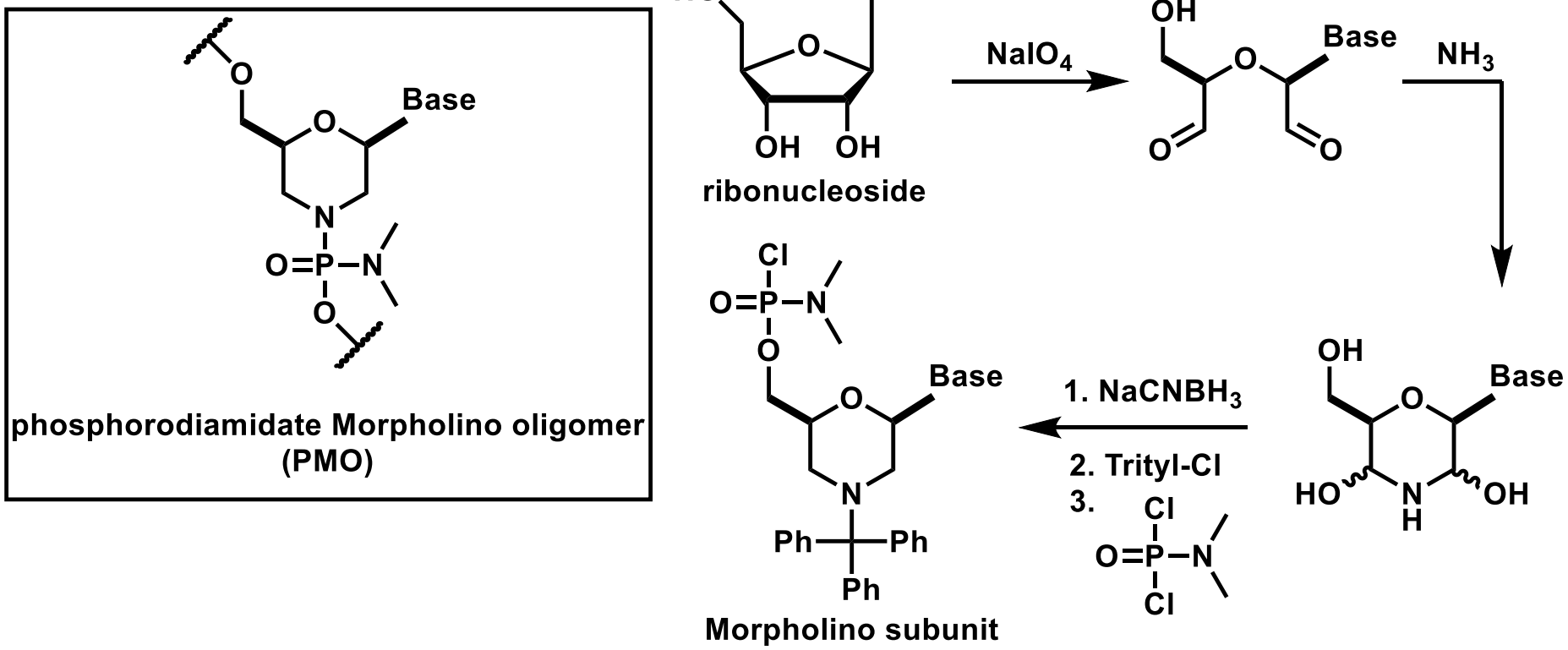
peptide nucleic acid (PNA)



- ✓ No negative charge to bind DNA more strongly than DNA
- ✓ High resistance to proteases and nucleases
- ✓ Stability in a wide range of pH

Hyrup, B. *et al. Bioorg. Med. Chem. Lett.* **1996**, *4*, 5.
Cheng, C. J. *et al. Nature* **2015**, *518*, 107. (conjugate with tumor-targeting cell-penetrating peptide)

3rd generation-3 (Morpholino oligomer)



- ✓ Morpholinos do not degrade their target RNA molecules
- ✓ Acting by "steric blocking", binding to a target RNA
- ✓ Morpholinos are not recognized by cellular proteins
- ✓ Up to 18% of Morpholinos appear to induce nontarget-related phenotypes

Summerton, J. *et al. Antisense Nuc. Acid Drug Develop.* **1997**, *D7*, 187.

Outline

- Introduction of miRNA
- Overview of miRNA therapeutics
- Main topic:

  Rational Design of Small Molecule Targeting Noncoding RNAs

  (Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205)
- Subtopic:

  Regulating miRNA by Bifunctional Small Molecule

  (Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987)

Small molecule-based approach with informatics

Prof. Matthew D. Disney

B.S., Chemistry, University of Maryland, College Park, 1997
M.S., Chemistry, University of Rochester, 1999
Ph.D., Biophysical Chemistry, University of Rochester, 2003 (Prof. Edwin L. Turner)
2002-2005 Postdoctoral Fellow, Swiss Federal Institute of Technology Zurich (ETH) (Prof. Peter H. Seeberger)
2005-2010 Assistant Professor (Principle Investigator), University at Buffalo, New York
2011- Professor, Department of Chemistry, Graduate Program Faculty Member, Kellogg School of Science and Engineering (THE SCRIPPS FLORIDA)

RNA is a highly desirable target for small molecule modulators

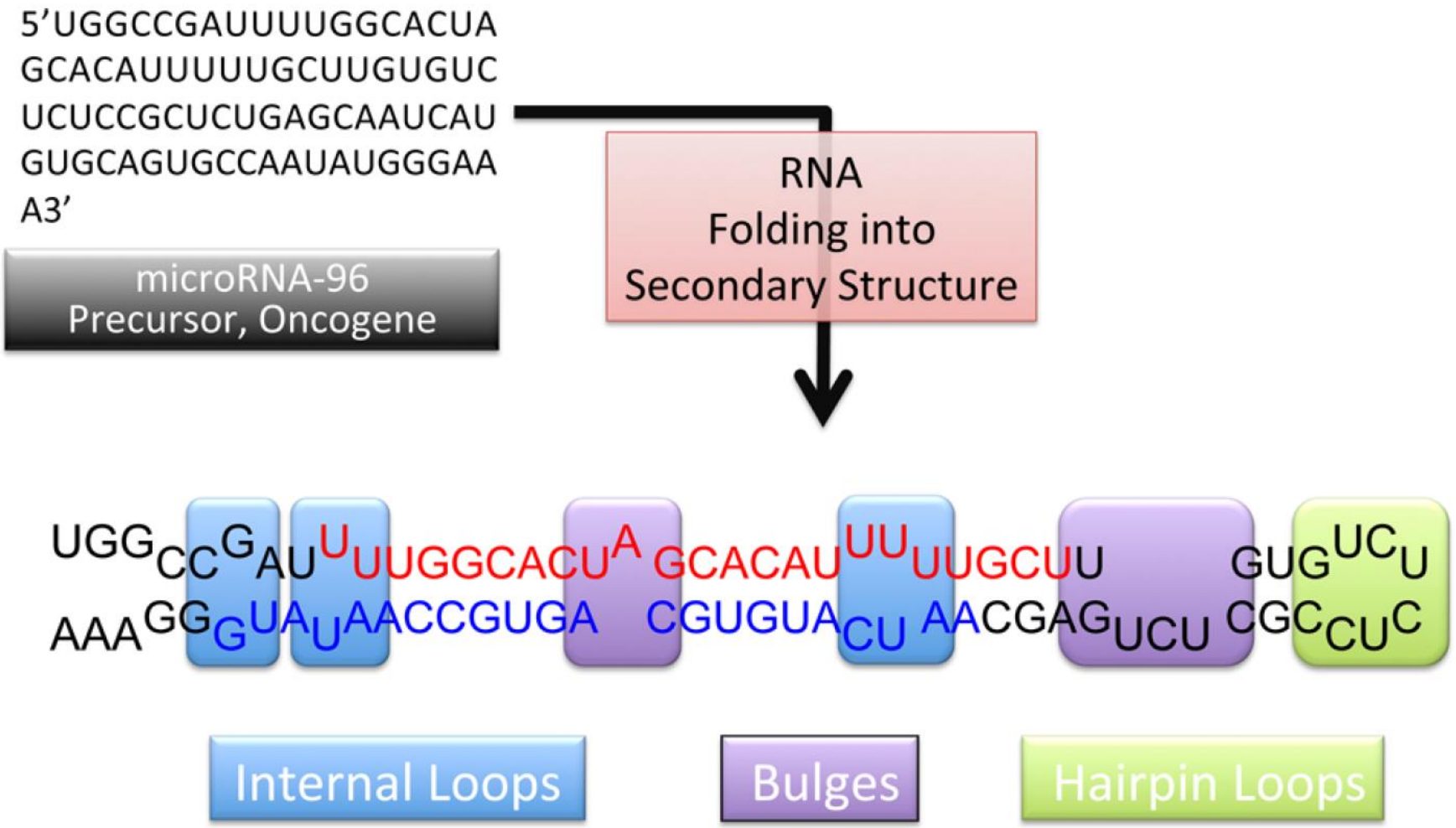
- ✓ Only 15% of all proteins are 'druggable' with small molecule, whereas 85% are not
- ✓ RNAs encode proteins are only a small portion (1-2%) and RNAs encode 'undruggable' proteins may be 'druggable'

Is RNA 'druggable' with small molecules?

- ✓ No understanding about the RNA secondary structural motifs which can be binding site of small molecules
- ✓ Few small molecule elicit their effects by modulating RNA yet

Disney, M. D. and Angelbello, A. J. *Acc. Chem. Res.* **2016**, *49*, 2698.

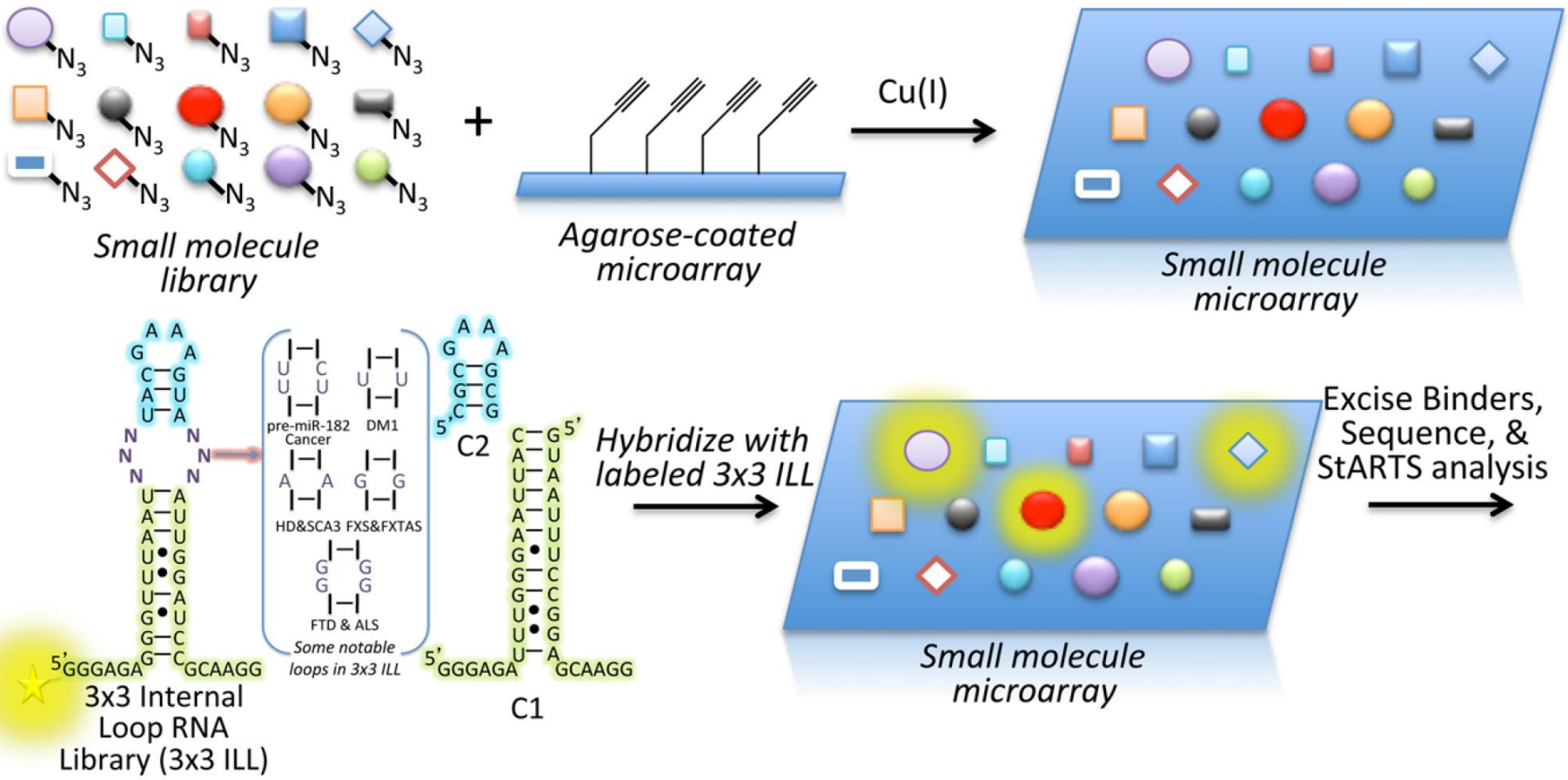
Targeting noncanonical structures in RNAs



- ✓ These secondary structures can be deduced from sequences

Disney, M. D. and Angelbello, A. J. *Acc. Chem. Res.* **2016**, *49*, 2698.

Two-dimensional combinatorial screening (2DCS)

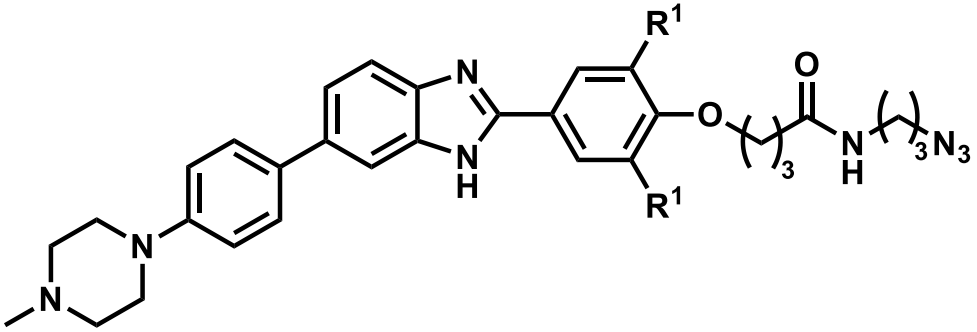


- ✓ A library vs library method to probe the small molecule-RNA motif bindings
- ✓ Resulting data comprise a database of binding partners

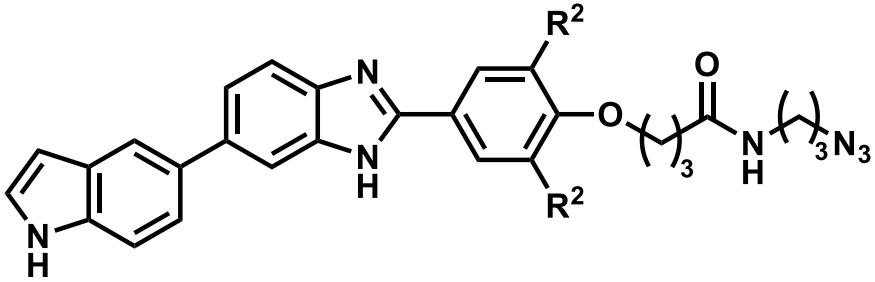
Disney, M. D. and Angelbello, A. J. *Acc. Chem. Res.* **2016**, *49*, 2698.

2DCS of an RNA-focused small molecule library

- An RNA-focused small molecule library

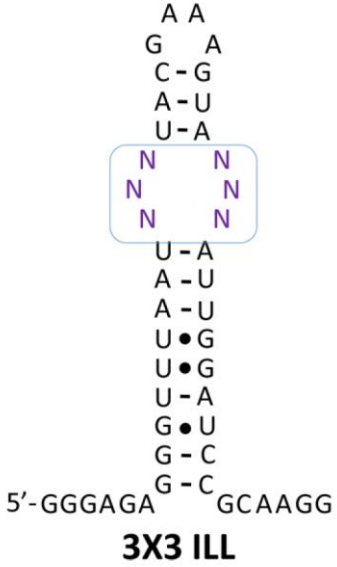


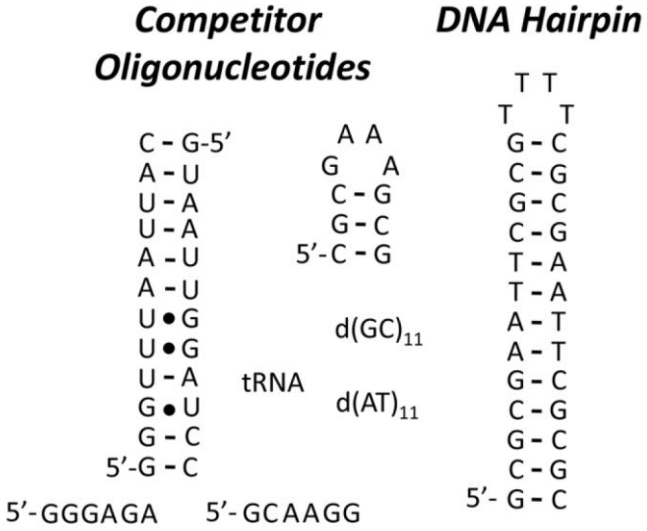
R^1 = H (1), Me (2), *i*-Pr (3), *t*-Bu (4)



R^2 = H (5), Me (6), *i*-Pr (7), *t*-Bu (8)

- An 3 × 3 nucleotide internal loop library (3 × 3 ILL)



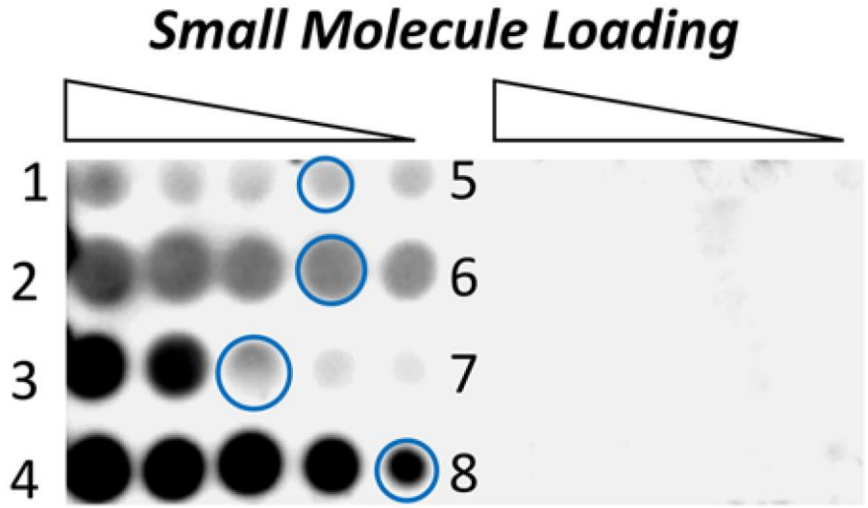


- ✓ ^{32}P-labeled
- ✓ 4^6 = 4096 members

8 × 4096 = 32768 interactions were investigated

Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

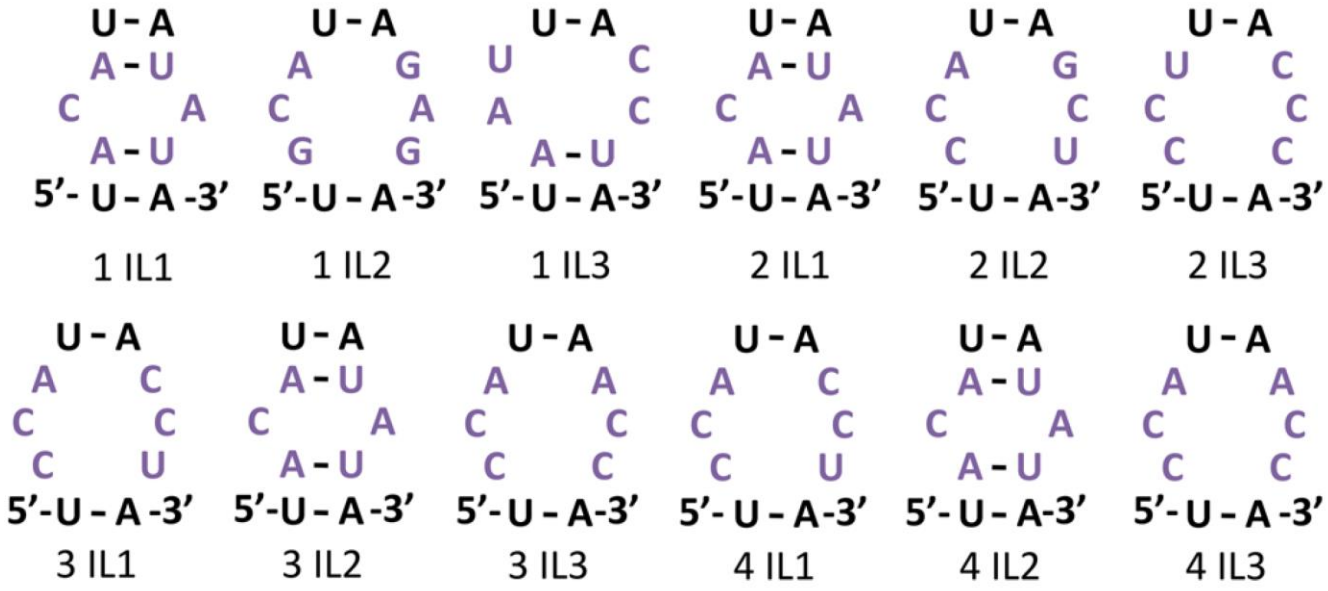
Results of 2DCS using 3 × 3 ILL



Immobilized molecules:
840, 560, 370, 250, 170
(picomoles)

Harvested and identified by RNA-seq

- The top three selected RNA motif binding to **1-4**

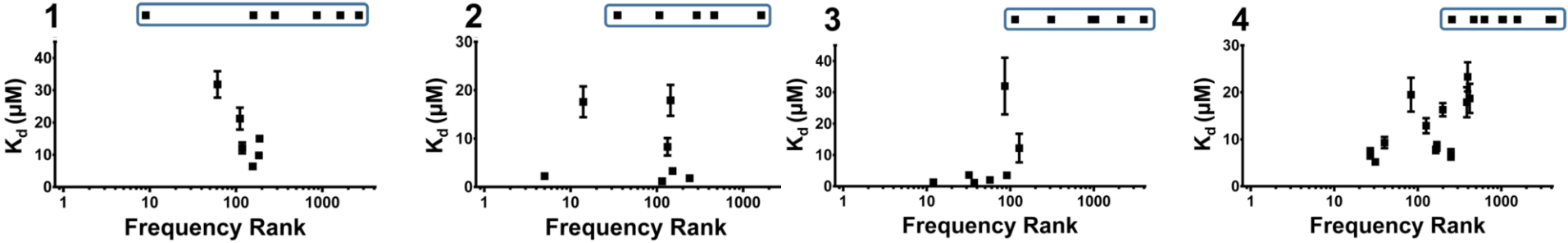


Binding affinities (K_ds) of the identified pairs were measured

Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

Investigation into global analysis of the interactions

- Plots of frequency rank *vs* experimentally determined K_d



Due to the biases that occur during transcription and sequencing?

- High Throughput Structure-Activity Relationship (HiT-StARTS) (Introduction of Z_{obs} rank to estimate the affinity accurately)

Pooled population comparison (*Z*-test)

(1) $$\phi = \frac{n_1 p_1 + n_2 p_2}{n_1 + n_2}$$

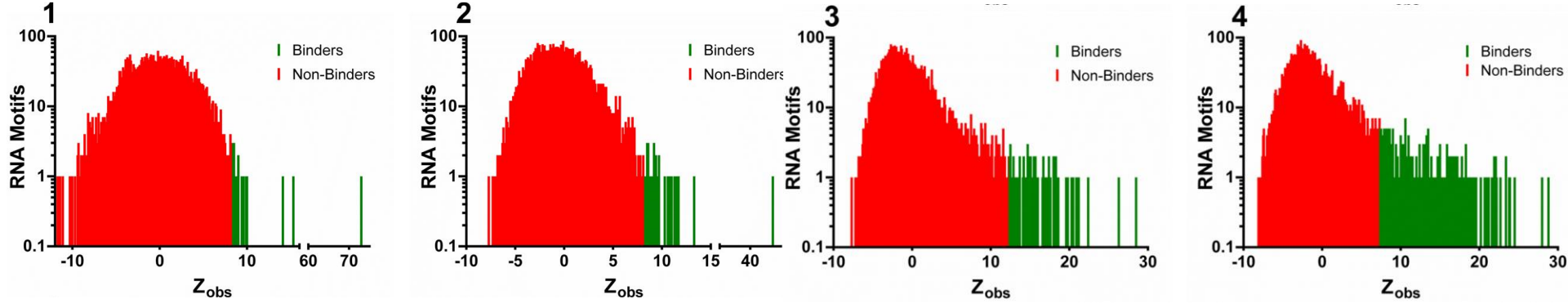
(2) $$Z_{obs} = \frac{p_1 - p_2}{\sqrt{\phi(1-\phi)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

- ✓ n_1 is the size of population 1 (number of reads for a selected RNA)
- ✓ n_2 is the size of population 2 (number of reads for the same RNA from sequencing of the starting library)
- ✓ p_1 is the observed proportion of population 1
- ✓ p_2 is the observed proportion of population 2

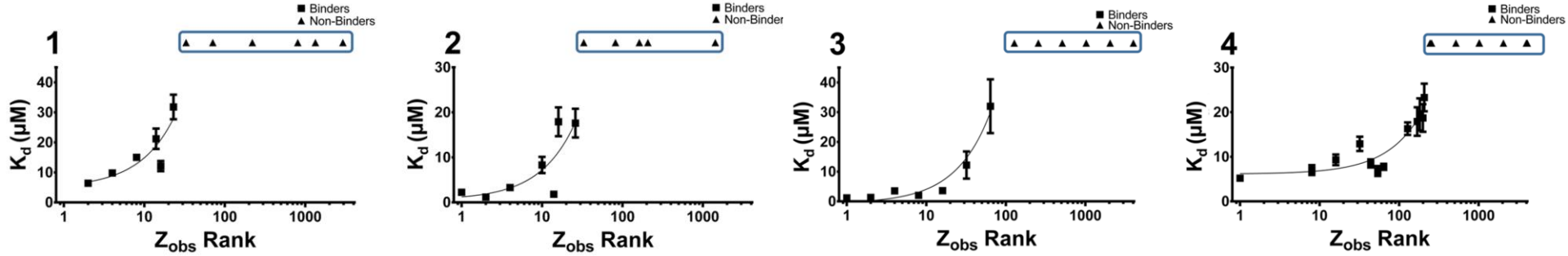
Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

Results of HiT-StART

- Plots of frequency of RNA motifs as a function of Z_{obs}



- Plots of Z_{obs} rank *vs* experimentally determined K_d



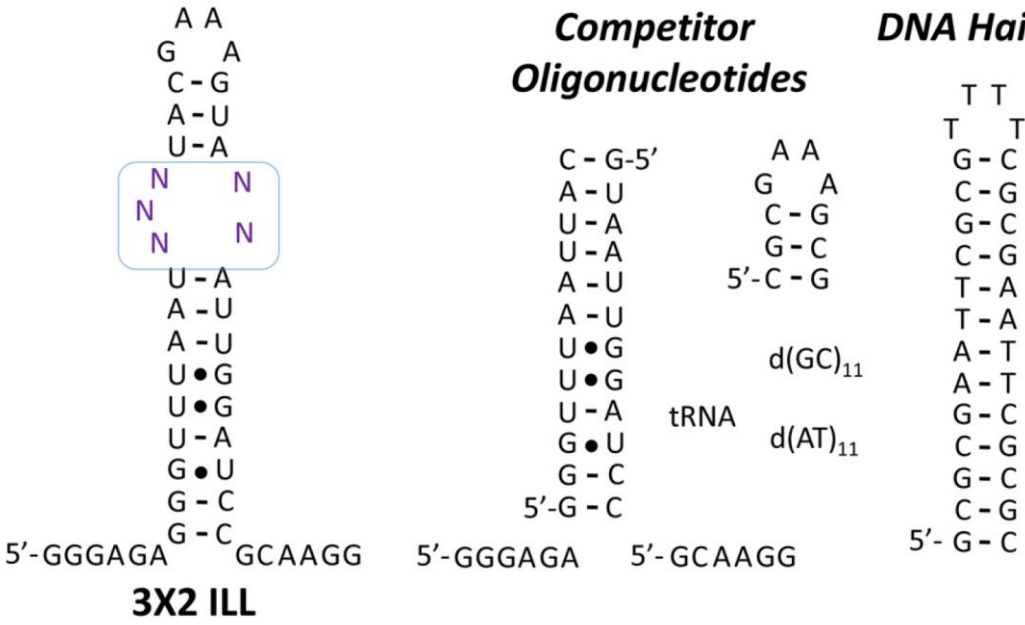
Excellent correlations were found between two values

- ✓ HiTStART analysis enables to estimate binding affinities rapidly and accurately
- ✓ Z_{obs} values can be normalized to *fitness score* (the most statistically significant RNA binder = 100% fitness)

Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

HiT-StART applied to other RNA motif libraries

- An 3 × 2 nucleotide internal loop library (3 × 2 ILL)

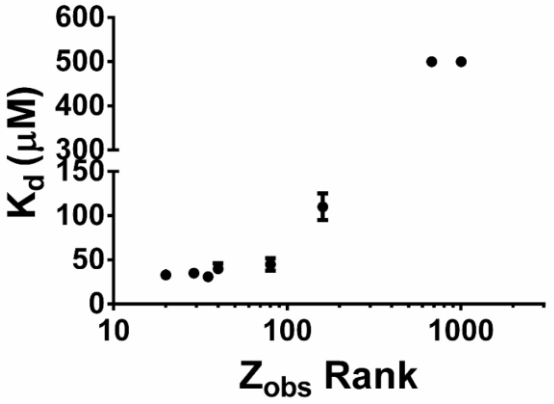


- ✓ ^{32}P-labeled
- ✓ 4^5 = 1024 members
- ✓ vs the same small molecule library (**1-8**)

8 × 1024 = 8192 interactions were investigated

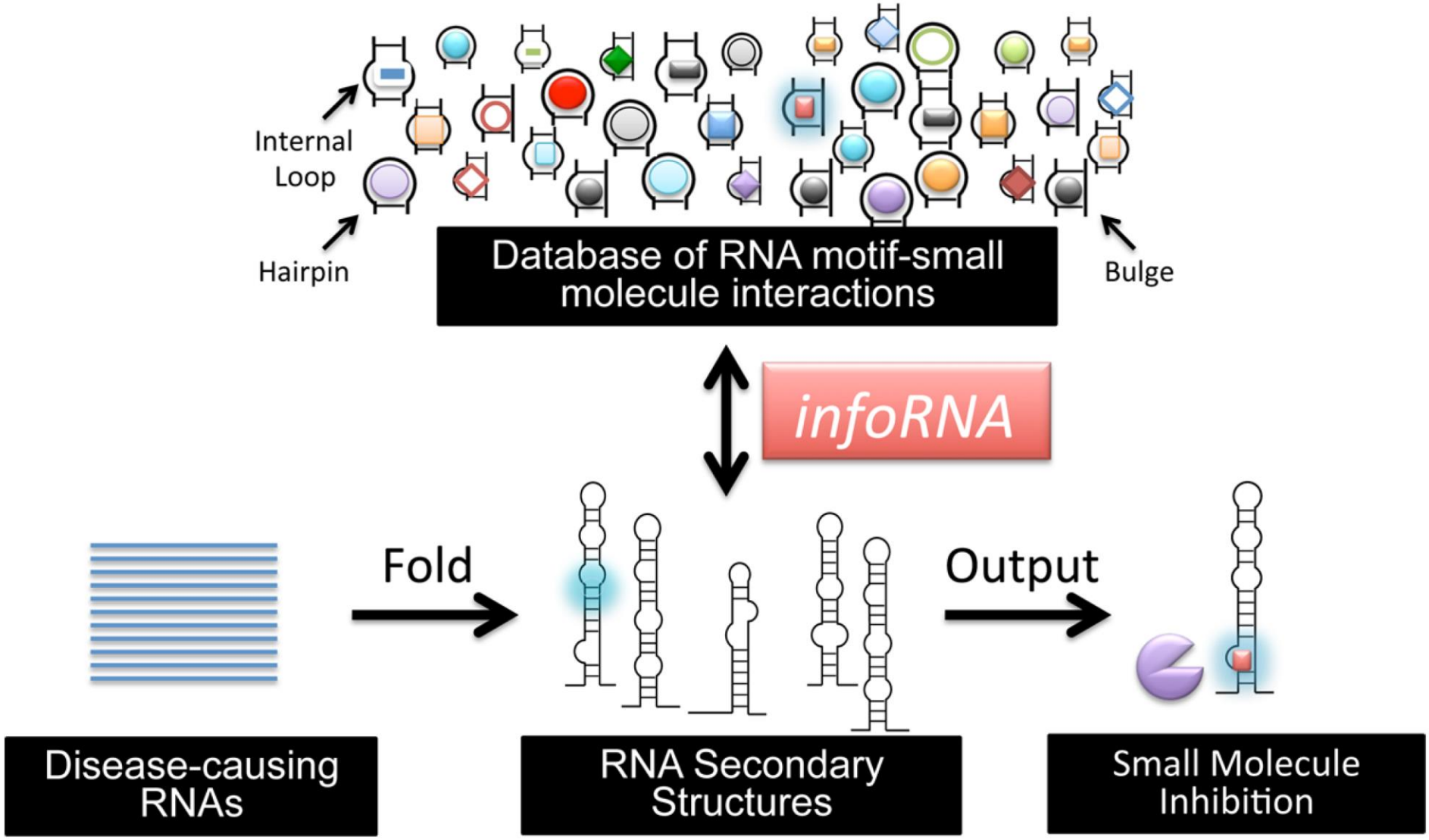
- ✓ Only compounds **1-4** bind members of the RNA-library
- ✓ The interactions were well estimated by Z_{obs} value (→)

3 × 2 ILL and compound **4** interactions



Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

InfoRNA

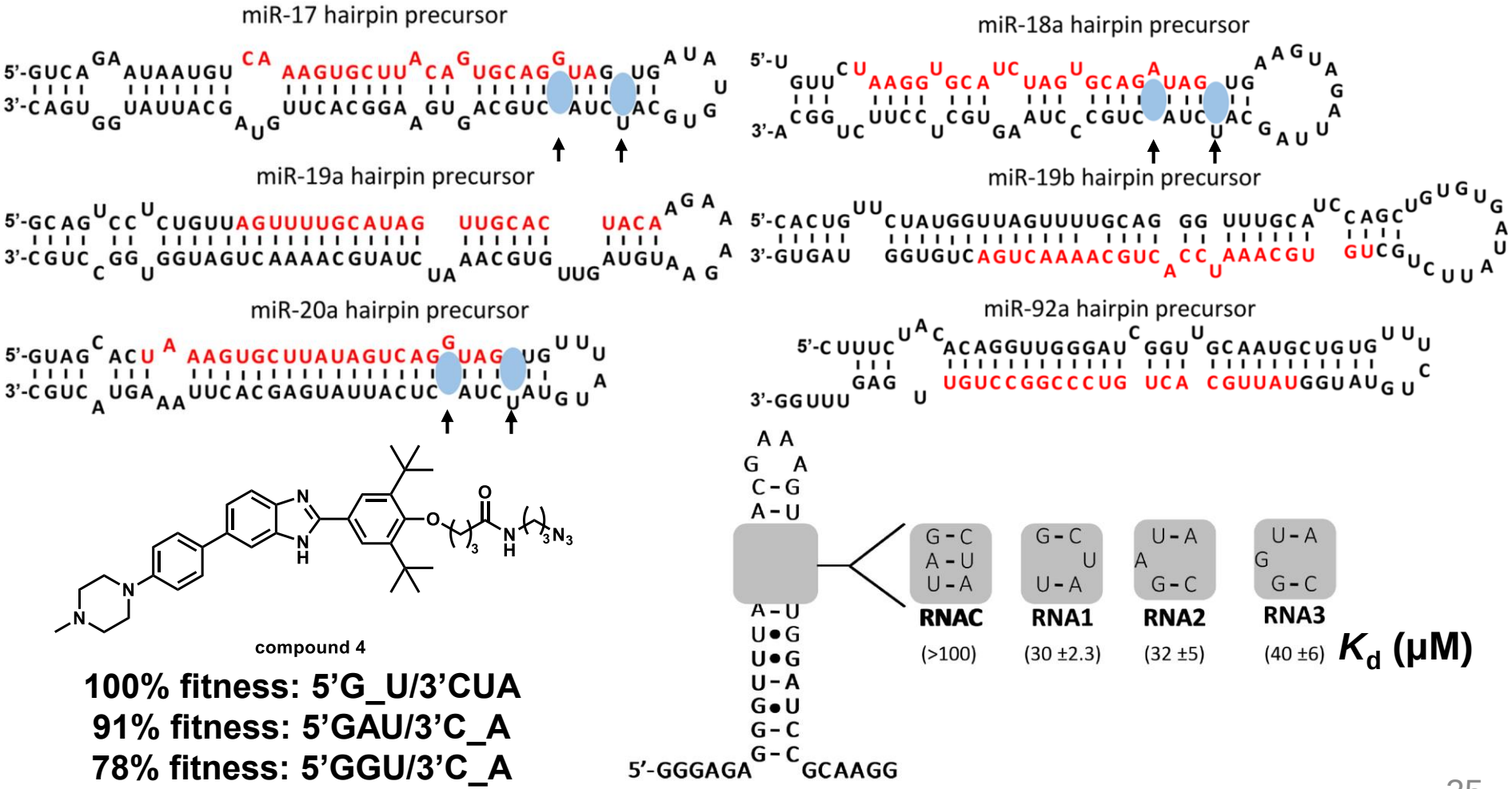


Disney, M. D. *et al. ACS Chem. Biol.* **2016**, *11*, 1720.

Identification of biologically important RNAs that can be targeted (InfoRNA analysis)

- Analysis focusing on miRNA associated with disease that have targetable motifs in Dicer/Drosha processing site

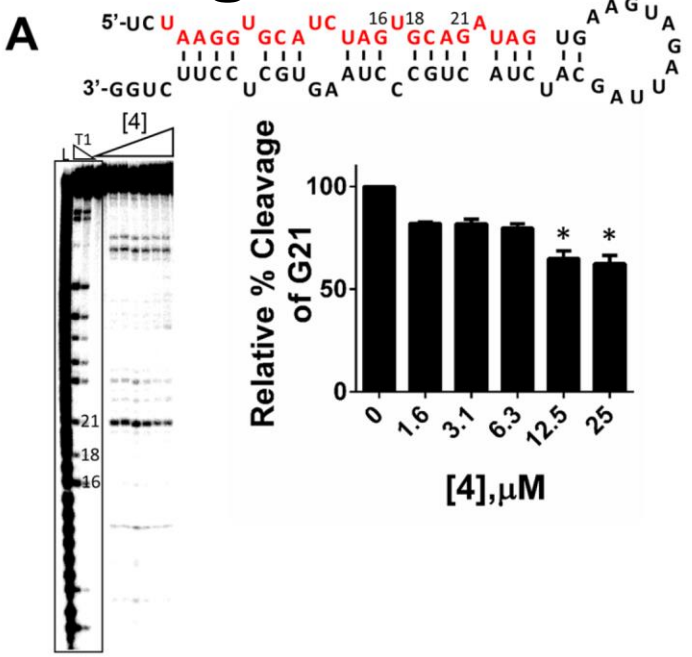
The oncogenic miR-17-92 cluster *Red = mature miRNA

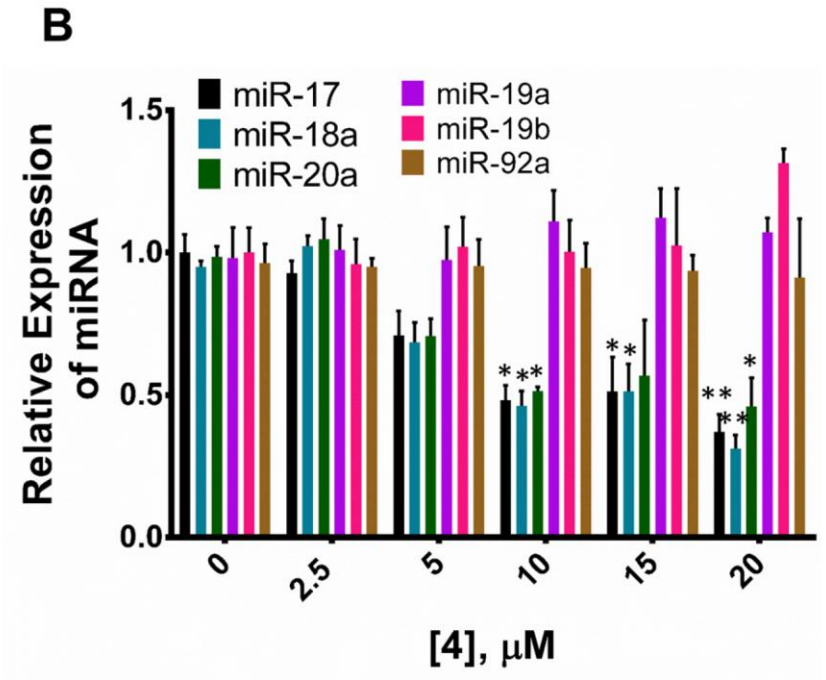


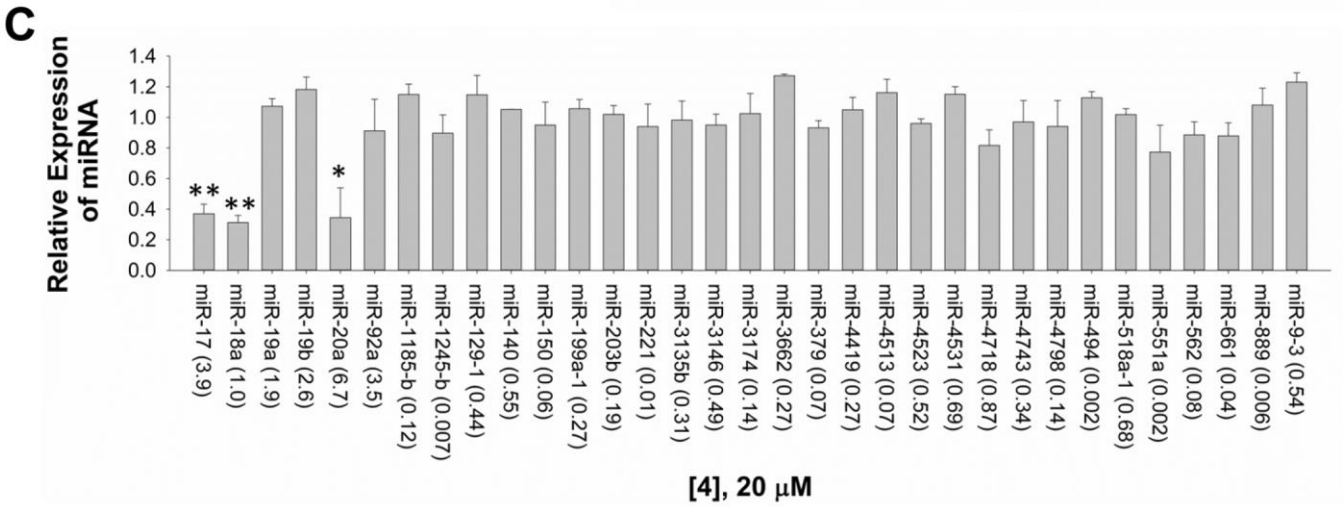
100% fitness: 5'G_U/3'CUA
91% fitness: 5'GAU/3'C_A
78% fitness: 5'GGU/3'C_A

Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

Inhibition of processing miRNA in the oncogenic miR-17-92 cluster by compound 4

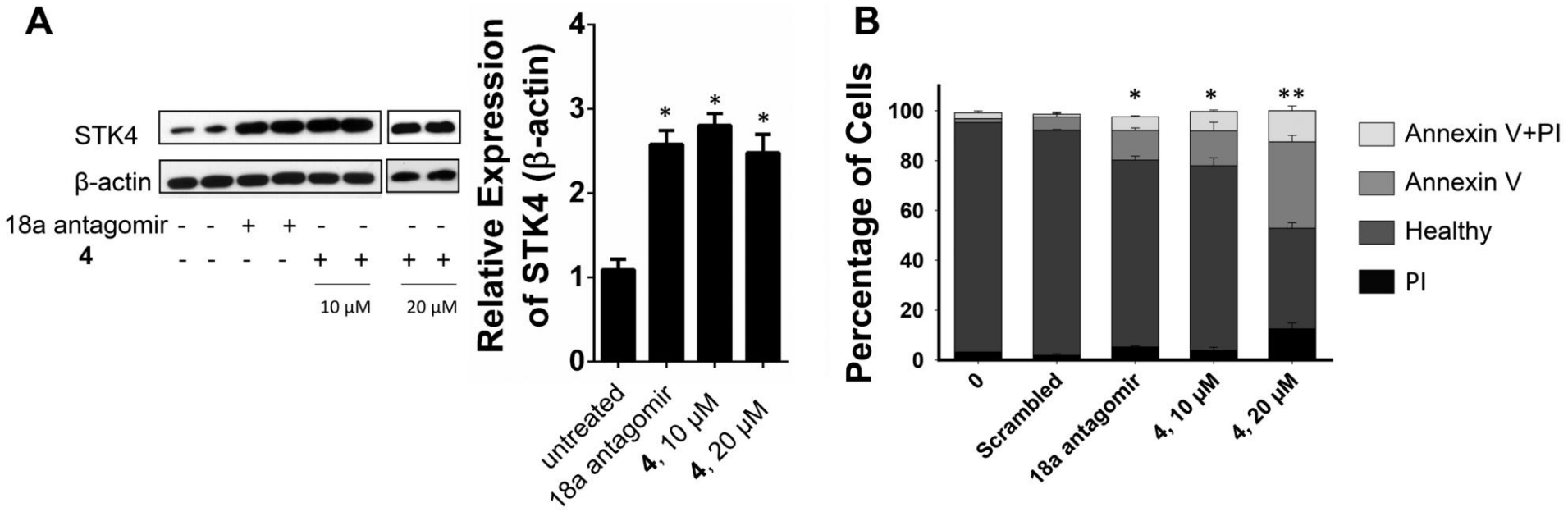




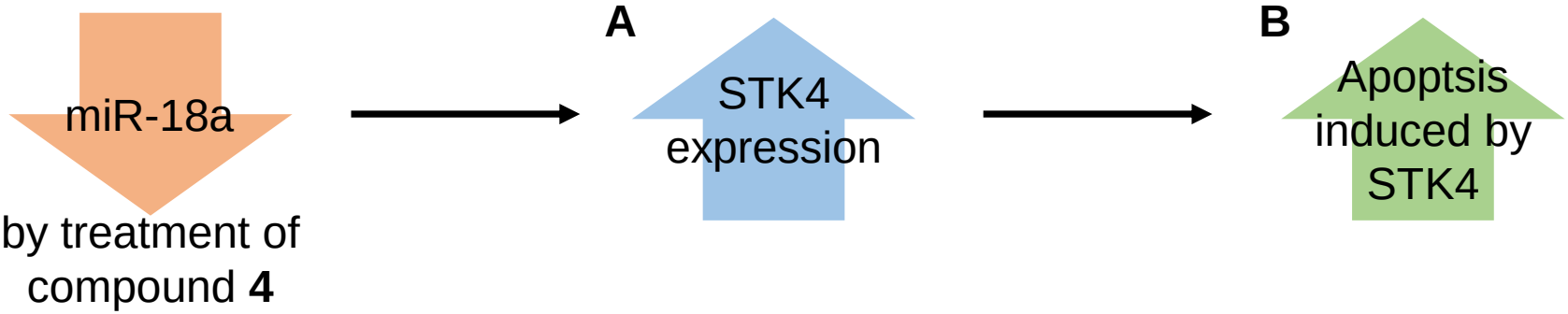


Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

Downstream protein analysis and apoptosis detection



- ✓ Expression of serine/threonine protein kinase 4 (STK4) is repressed by miR-18a
- ✓ STK4 is a tumor suppressor in prostate cancer cells



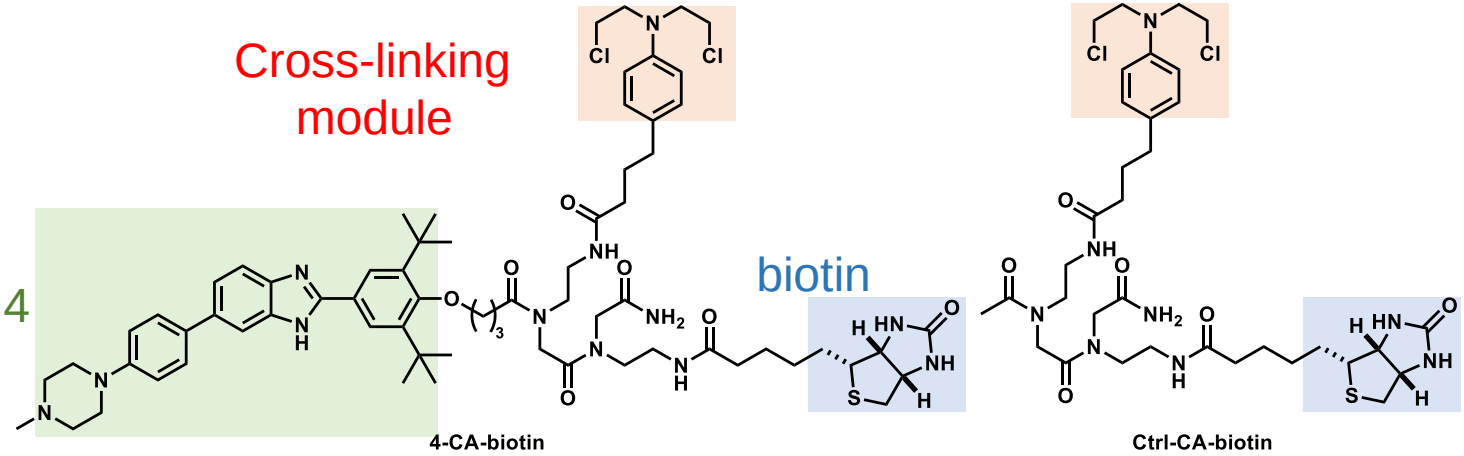
Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

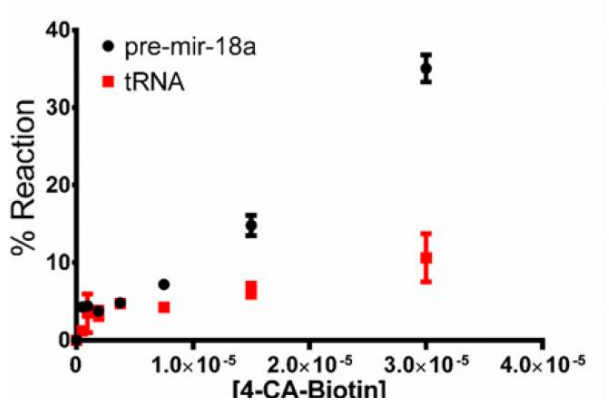
Chem-CLIP to study small molecule engagement

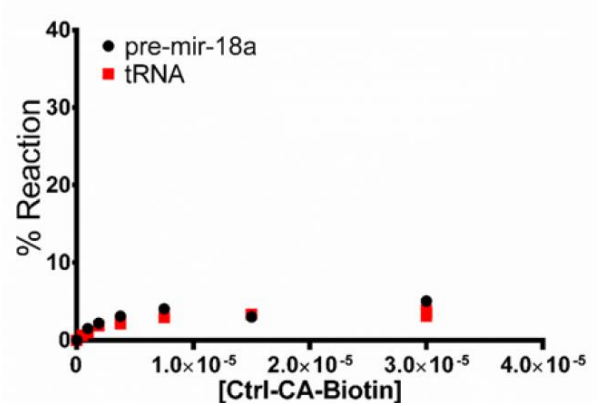
Cross-linking module

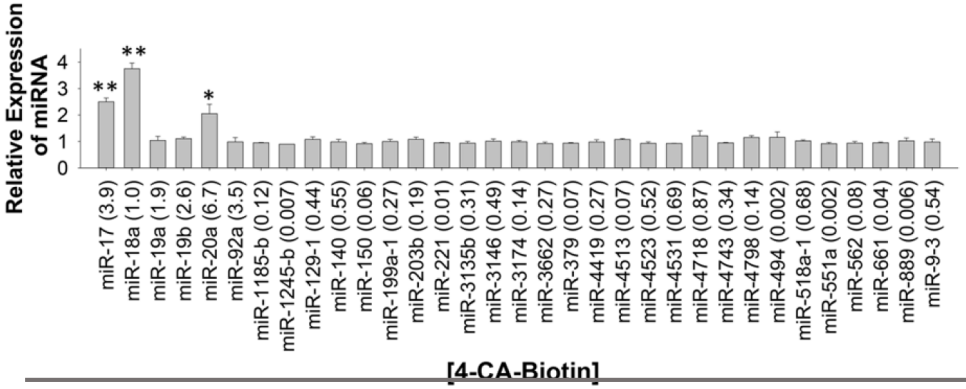
Compound 4 structure

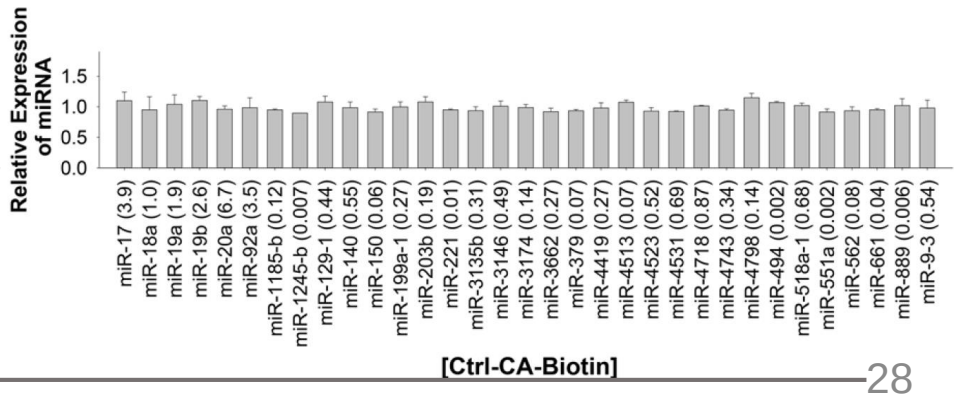
biotin





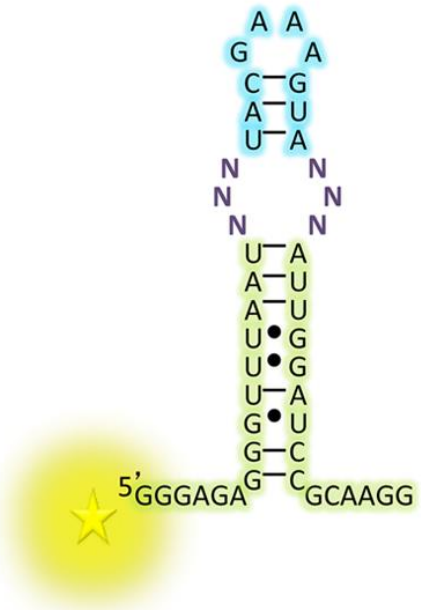




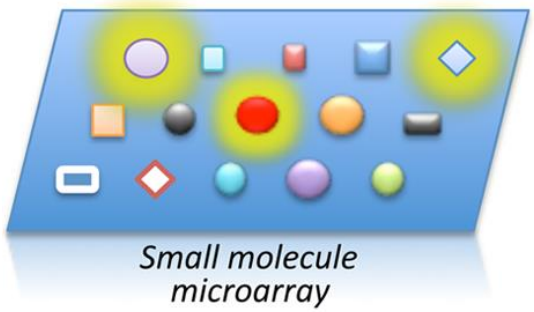


Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

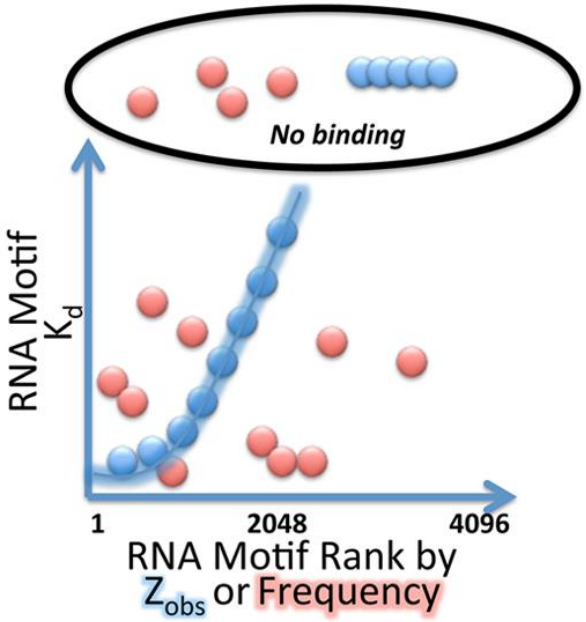
Short summary

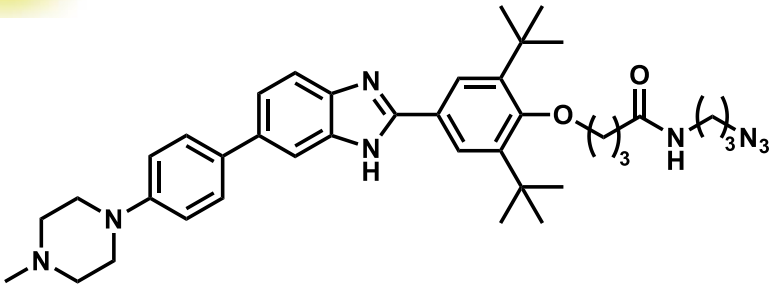


2DCS



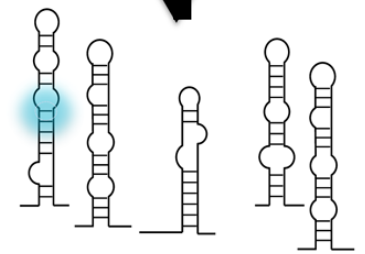
Excise Binders, Sequence, & HiT-StARTS analysis





compound 4

infoRNA



RNA Secondary Structures

Selective inhibition of biogenesis of miR-17, 18a, 20a

Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

Outline

- Introduction of miRNA
- Overview of miRNA therapeutics
- Main topic:

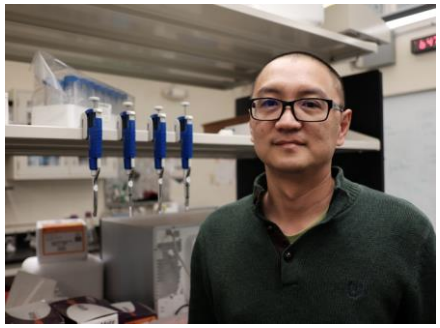
  Rational Design of Small Molecule Targeting Noncoding RNAs

  (Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205)
- Subtopic:

  Regulating miRNA by Bifunctional Small Molecule

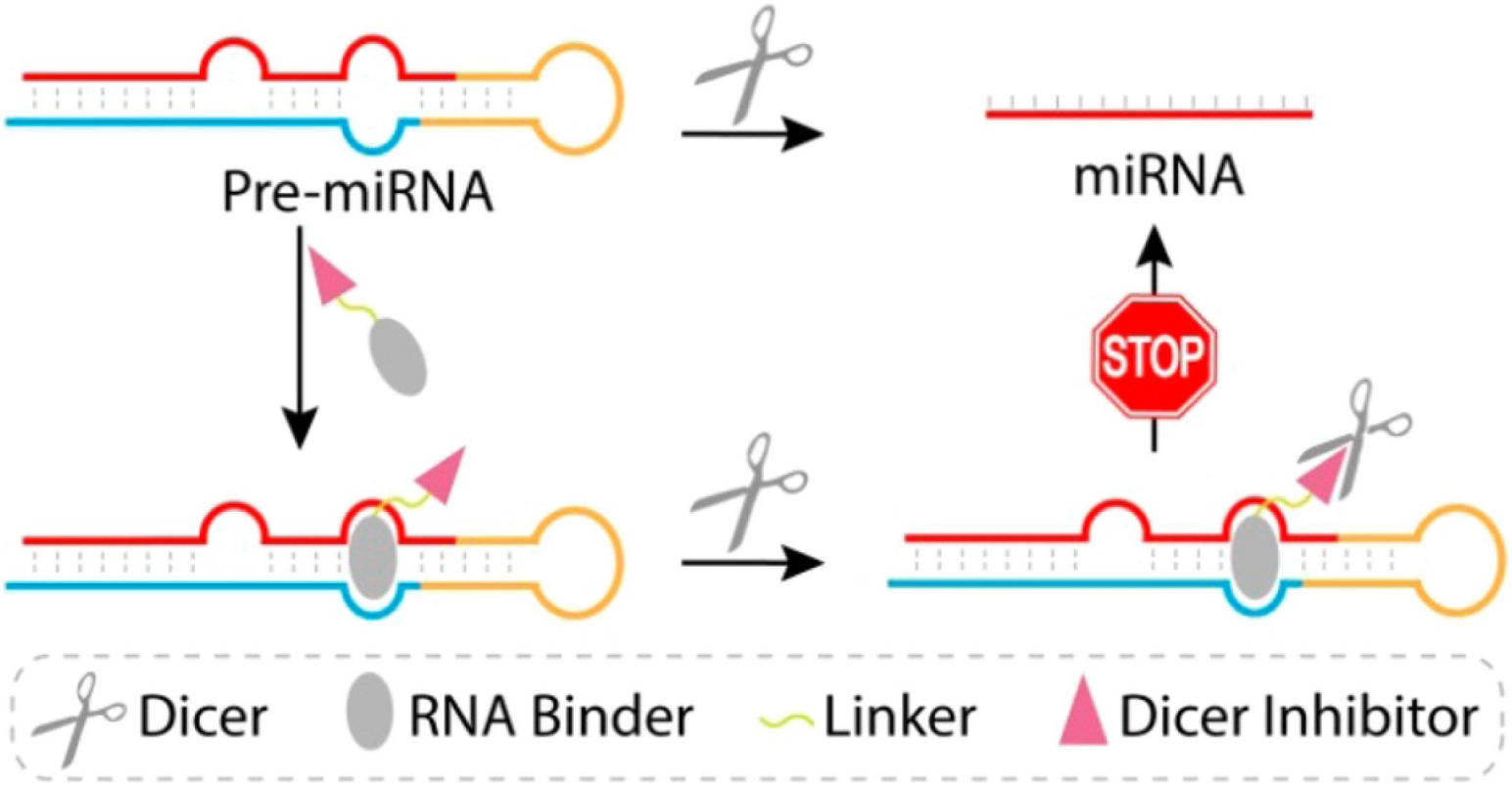
  (Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987)

Bifunctional strategy to inhibit miRNA biogenesis



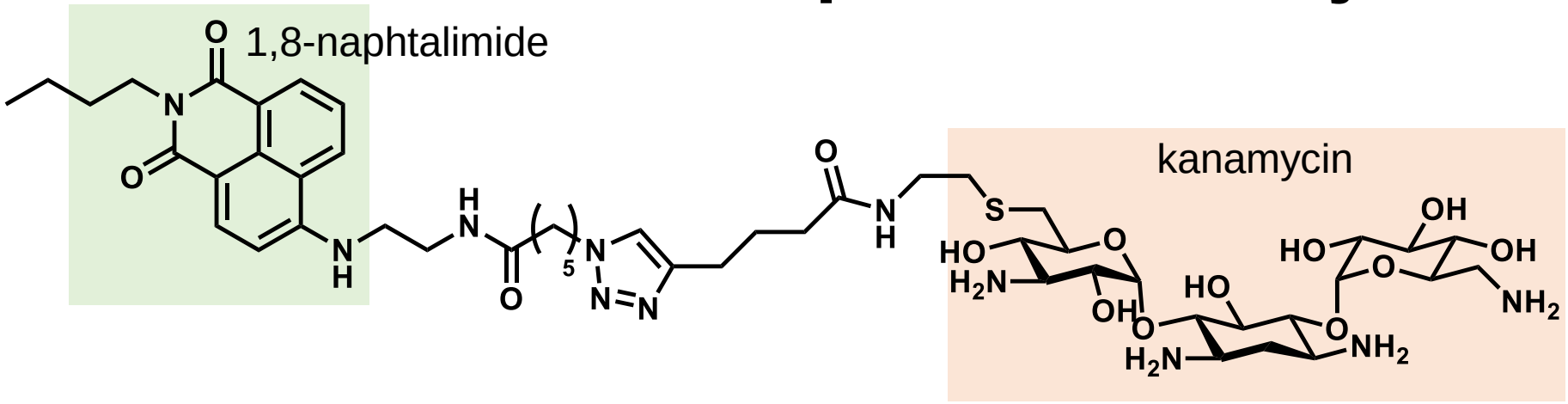
Dr. Fu-Sen Liang

1994 BS in Chemistry, National Taiwan University
1996 MS in Organic Chemistry, National Chiao Tung University
2005 Ph.D in Bioorganic Chemistry, The Scripps Research Institute, La Jolla, CA (Prof. Chi-Huey Wong)
2005-2011 Postdoc in Chemical Biology, Stanford Medical School (Gerald R. Crabtree)
2012- Assistant professor (independent research group), University of New Mexico

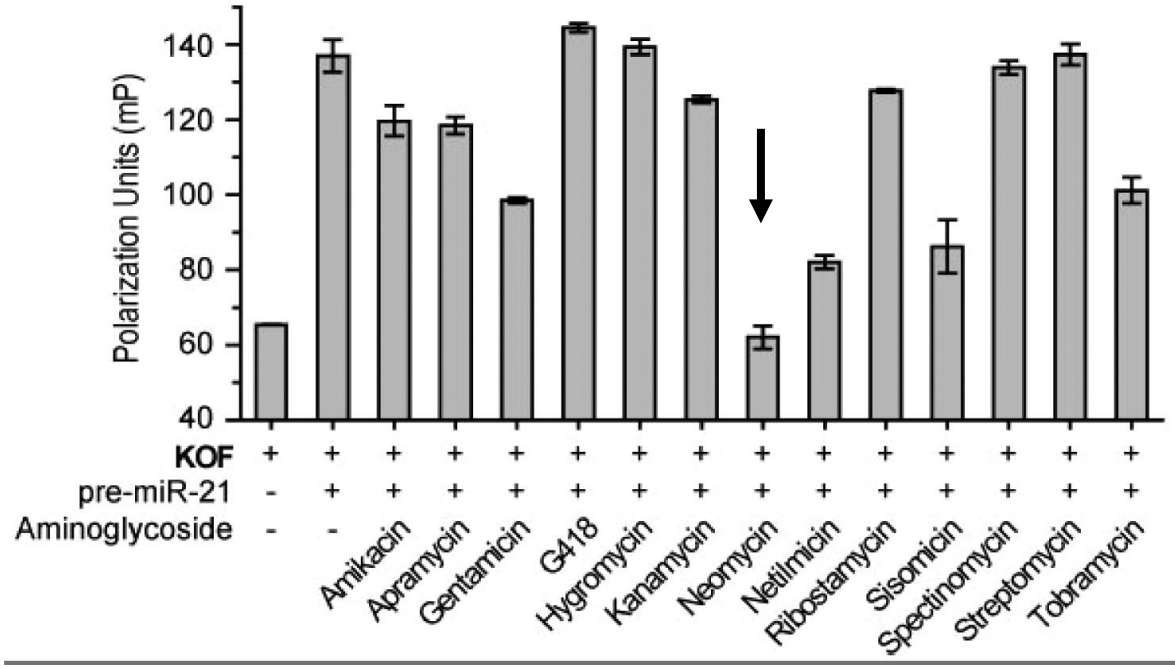


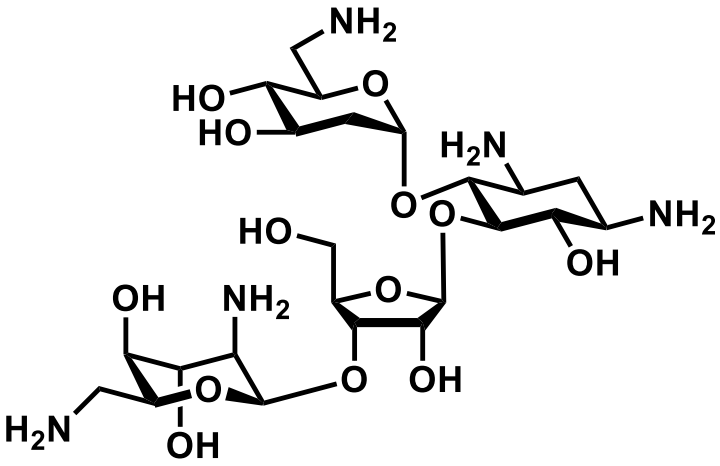
Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987

Screening of aminoglycosides binding to pre-miR-21 with a fluorescence polarization assay



KOF (Kd = 419 ± 83 nM)

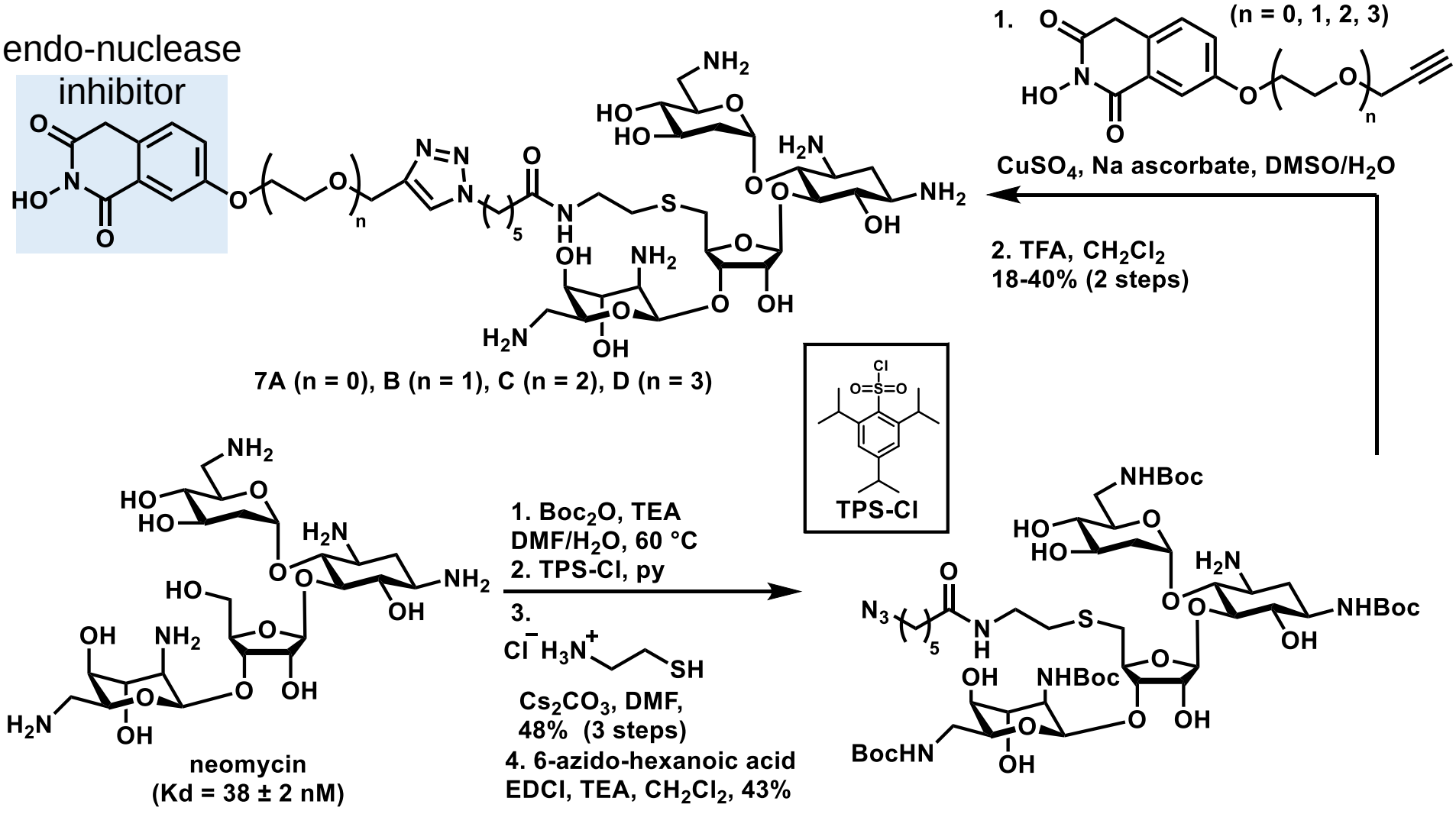




neomycin
(Kd = 38 ± 2 nM)

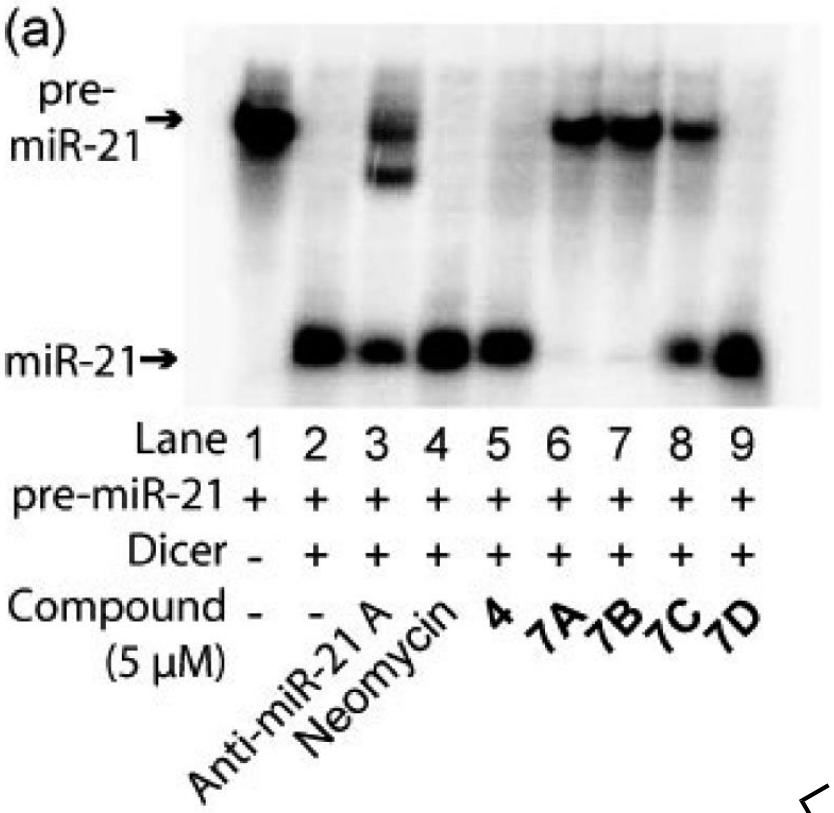
Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987

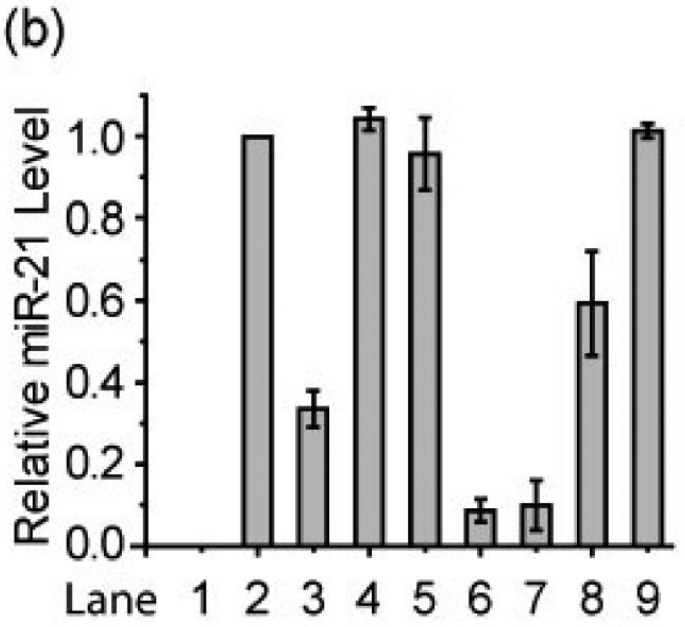
Design and synthesis of bifunctional small molecules

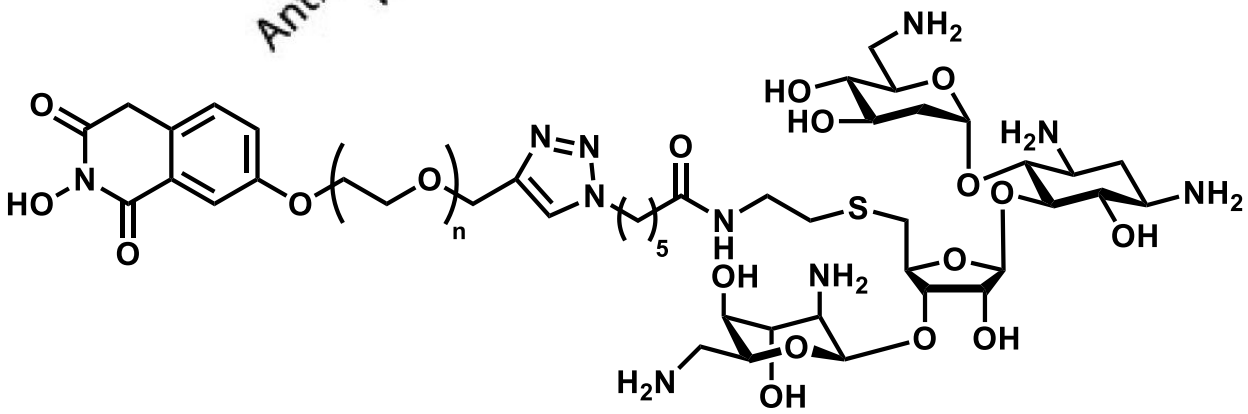


Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987

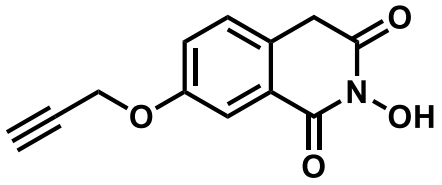
In vitro Dicer-mediated pre-miR-21 cleavage







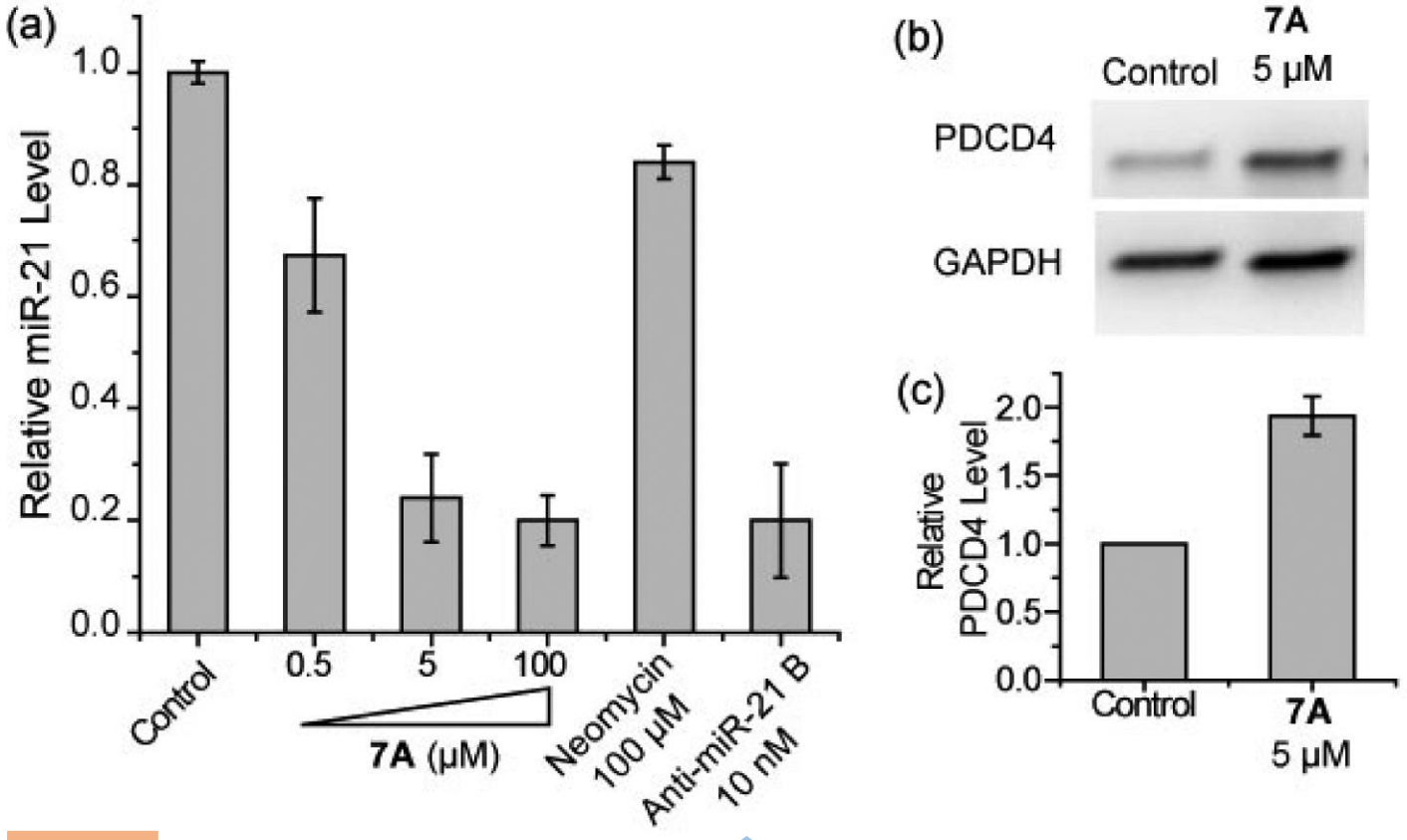
7A (n = 0), B (n = 1), C (n = 2), D (n = 3)



compound 4

Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987

In cell analysis of mature miR-21 expression levels and the downstream protein (PDCD4)



miR-21

by treatment of compound **7A**

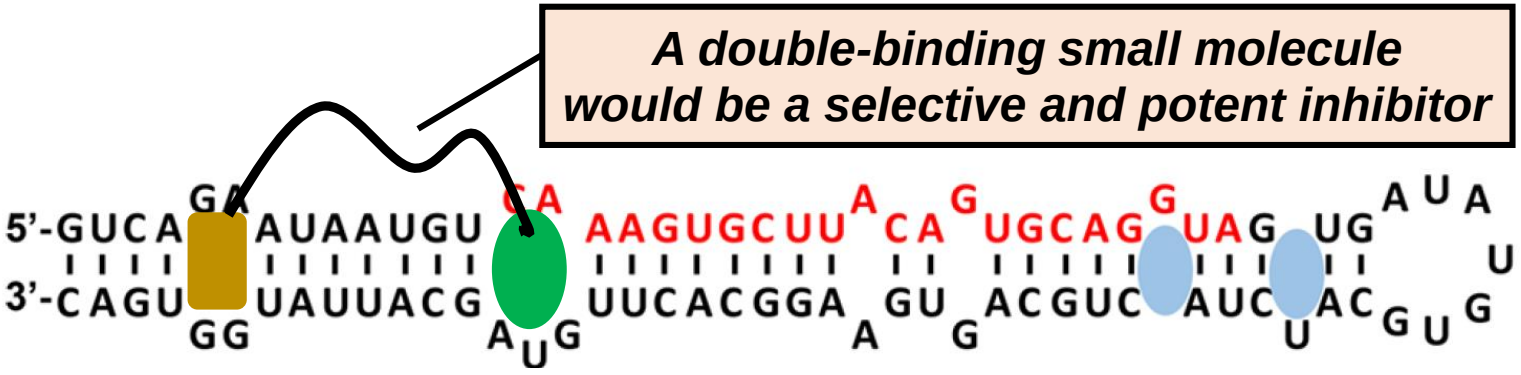
PDCD4 expression

PDCD4: Programmed Cell Death Protein 4

Yan, H. *et al. J. Am. Chem. Soc.* **2017**, *139*, 4987

Summary

- miRNAs are desirable therapeutic target
  - ✓ Modified antisense oligonucleotides with drug delivery systems are the main stream of strategies
  - ✓ The small molecule-based approach is a new field and has a great potential
- Problems and a proposal in small molecule-based approach
  - ✓ Small molecules seem to have a low affinity (up to 100 μM) to RNAs (maybe due to the small binding site on RNAs)
  - ✓ Off-target effects have to be well considered
  - ✓ Can combination of the two topics make the affinity and the selectivity much higher?



Appendix-1

Table 2 | **Selected list of miRNA therapeutics in clinical trials**

Name (company)	Therapeutic agent	Delivery system	Target diseases	Trial details	ClinicalTrials.gov identifier
miRNA-based therapeutics					
Mirvirasen (Santaris Pharma A/S and Hoffmann-La Roche)	AntimiR-122	LNA-modified antisense inhibitor	Hepatitis C (chronic infections included)	Single-centre phase I, completed	NCT01646489
				Multicentre phase II, completed	NCT01200420
				Multicentre phase II, ongoing	NCT01872936
				Single-centre phase II, ongoing	NCT02031133
				Single-centre phase II, ongoing	NCT02508090
RG-101 (Regulus Therapeutics)	AntimiR-122	GalNAc-conjugated antimiR	Chronic hepatitis C	Phase I, completed	–
				Multiple phase II, ongoing	–
RG-125/ AZD4076 (Regulus Therapeutics)	AntimiR-103/107	GalNAc-conjugated antimiR	Patients with type 2 diabetes and non-alcoholic fatty liver diseases	Single-centre phase I, ongoing	NCT02612662
				Single-centre phase I/IIa, ongoing	NCT02826525
MRG-106 (miRagen Therapeutics)	AntimiR-155	LNA-modified antisense inhibitor	Cutaneous T cell lymphoma and mycosis fungoides	Multicentre phase I, ongoing	NCT02580552
MRG-201 (miRagen Therapeutics)	miR-29 mimic	Cholesterol-conjugated miRNA duplex	Scleroderma	Single-centre phase I, ongoing	NCT02603224
MesomiR-1 (EnGeneIC)	miR-16 mimic	EnGeneIC delivery vehicle	Mesothelioma, non-small cell lung cancer	Multi-centre Phase I, ongoing	NCT02369198
MRX34 (Mirna Therapeutics)	miR-34 mimic	LNPs (Smarticles)	Multiple solid tumours	Multicentre phase I, terminated	NCT01829971

DOPC, 1,2 dioleoyl-*sn* glycero-3 phosphatidylcholine; eIF, eukaryotic initiation factor; GalNAc, *N*-acetyl-D-galactosamine; HBV, hepatitis B virus; LNA, locked nucleic acid; LNPs, lipid nanoparticles; miRNA, microRNA; PEI, polyethylenimine; RSV, respiratory syncytial virus.

Rupaimoole, R. and Slack, F. J. *Nat. Rev. Drug Discov.* **2017**, *16*, 203.

Appendix-2: synthesis of compound 4 in main topic

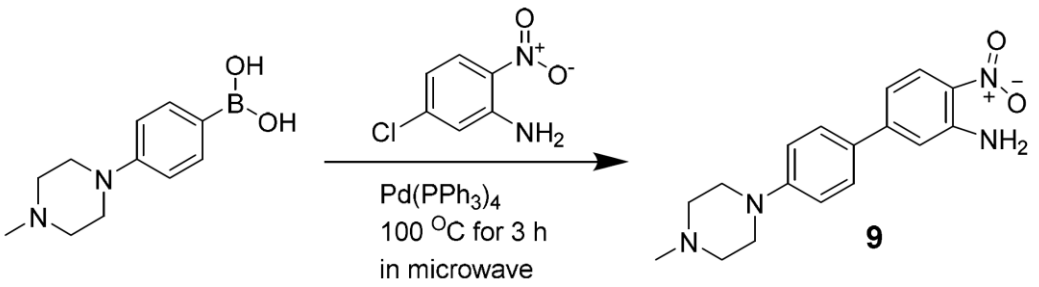


Figure S-1: Synthetic scheme of 4'-(4-methylpiperazin-1-yl)-4-nitro-[1,1'-biphenyl]-3-amine (**9**)

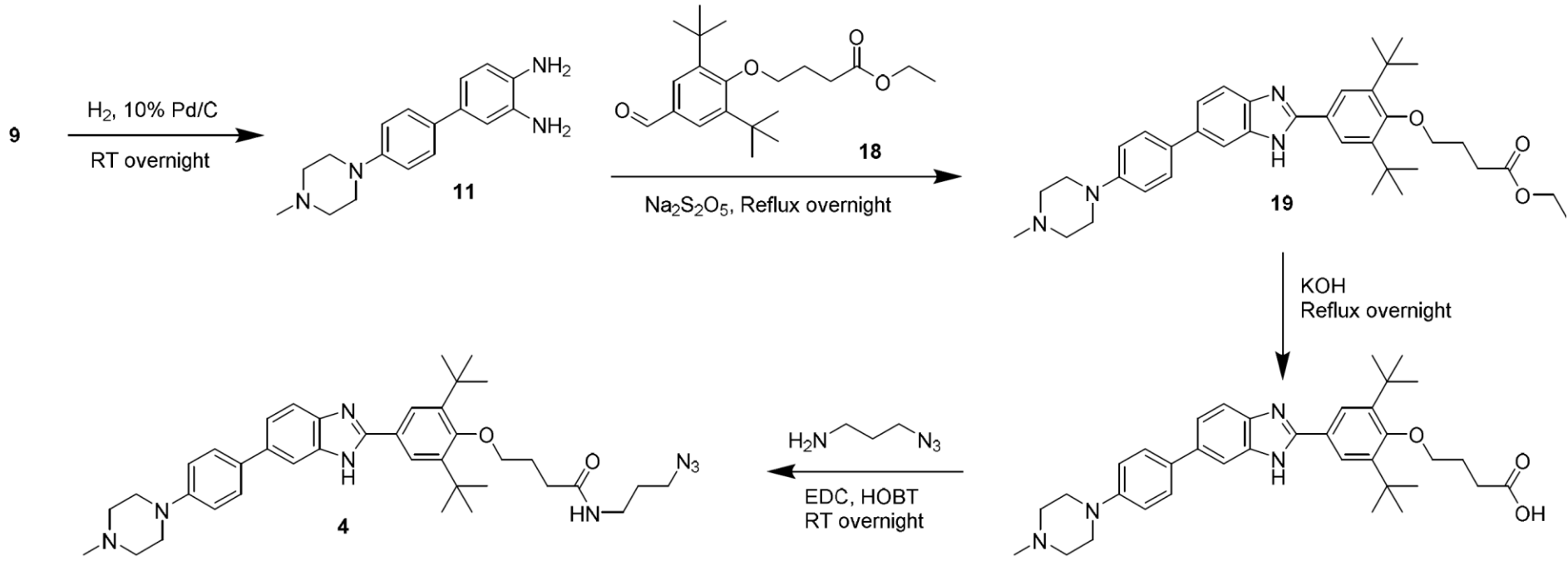
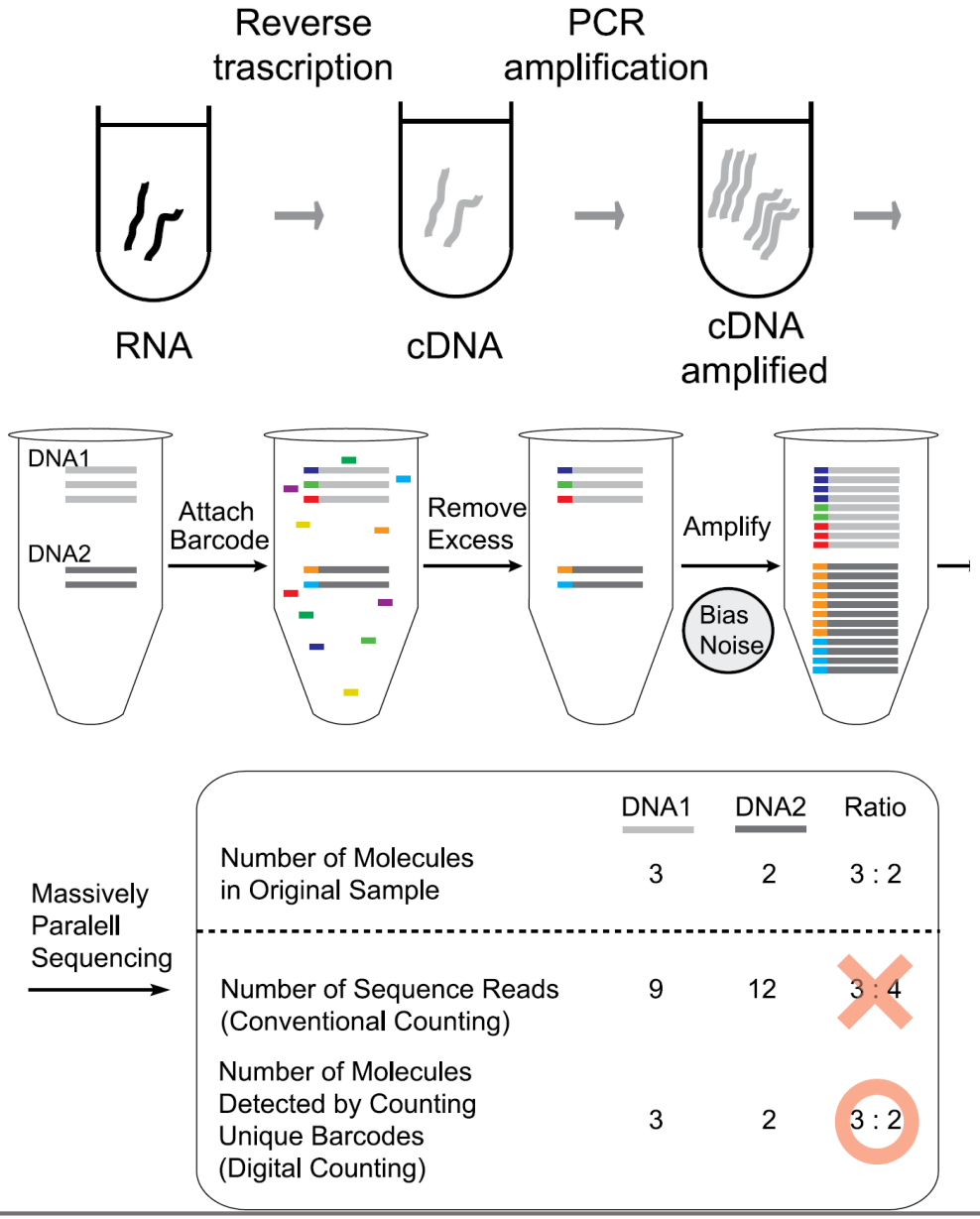


Figure S-22: Synthetic scheme of **4**

Velagapudi, S. P. *et al. ACS Cent. Sci.* **2017**, *3*, 205

Appendix-3: quantitative RNA-seq



城口克之 生物物理 2013, 53 (6), 290-294