Supporting Information for

Tetracyclines as Inhibitors of pre-microRNA Maturation: A Disconnection Between RNA Binding and Inhibition

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A. General Materials and Methods

General assay methods. Gels were imaged on a ProteinSimple Fluorchem M Gel Imager. Chemiluminescence data was collected on either a BioTek Cytation3 or PHERAstar FS plate reader.

General chemistry methods. Preparative reverse-phase HPLC was performed using an Agilent 1260 Infinity HPLC equipped with a PrepHT XDB-C18 column (21.2 × 150 mm; 5 μm) at a flow rate of 18 mL/min using 10–100% water/acetonitrile as a mobile phase and detection at 254 nm. Mass spectrometry (HRMS) was performed using an Agilent 6520 Accurate-Mass Q-TOF LC-MS spectrometer using ESI ionization with less than 5-ppm error for all HRMS analyses. NMR spectra were recorded on a 400 MHz Bruker instrument and calibrated using a solvent peak as an internal reference. Spectra were processed using MestReNova software.

Data and statistical analysis. All data was analyzed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, www.graphpad.com). Graphs show mean ± standard deviation. IC_{50} values from Figure 1B are presented as the average from triplicate analyses performed using automated liquid handling as previously described.[1] IC_{50} values from Figures 2, 4 and 5 are presented as 95% confidence intervals from duplicate assays; the assay was run using a multi-channel pipette. Inhibition data shown in Figure 7 is from triplicate assays; the assay was run using a multi-channel pipette.

Materials. Chemically synthesized pre-miR-21 and pre-let-7d (deprotected, desalted and HPLC purified), containing biotin and aminoallyl uridine modifications and an 18-atom spacer, were purchased from Dharmacon and used as received. RNA labeling with *trans*-cyclooctene (TCO)-PEG4-NHS (cat #A137; Click Chemistry Tools) was performed as previously described.[2] Horseradish peroxidase (HRP), streptavidin-coated 384-well plates (cat #15407), and SuperSignal West Pico Chemiluminescent substrate kit were purchased from Pierce. HRP labeling with methyltetrazine (mTet)-NHS (cat #1128; Click Chemistry Tools) was performed as previously described.[2] SYBR® Gold was purchased from Life Technologies. RNA ladders were purchased from New England Biolabs. *E. coli* RNase III was purchased from Life Technologies (cat #AM2290). All reagents were used as received without further purification. Human Dicer was prepared as previously reported and stored at -20 °C in 20 mM Tris pH 7.5, 100 mM NaCl, 1.0 mM $MgCl_2$, 50% glycerol, and 0.1% Triton X-100.[2]

Tetracyclines. Methacycline HCl (PHR1594-1G), Meclocycline Sulfosalicylate salt (M1388-500MG), Minocycline HCl (M9511-25MG), Demeclocycline HCl hydrate (46161-100MG), Oxytetracyline HCl (O5875-10G), Tigecycline hydrate (PZ0021-5MG), Tetracycline HCl (T7660-5G), were purchased from Sigma-Aldrich. Chlortetracycline HCl was purchased from Chem Impex (01588-5G). Doxycycline Hyclate was purchased from Alfa Aesar (J60579-14). Epitetracycline HCl (19470) and Anhydrotetracycline HCl (10009542) were purchased from Cayman Chemical. All were confirmed to be >90% purity via HPLC and LC-MS and used as received.

B. Dicer cat-ELCCA

Black, standard capacity streptavidin-coated 384-well plates were washed with phosphate buffer (50 μL, 100 mM, pH 7.0 (PB7); 3×). 5 μL of biotinylated pre-miRNA substrate (500 nM final) was then dispensed into the plate, followed by plate centrifugation for 1 min at 1,000 RPM (223 × g). The plate was sealed with plate tape and incubated overnight at 4 °C. Following RNA incubation, plates were

washed 3× with PB7 (50 µL), followed by the addition of Dicer digest buffer (5 µL, 20 mM Tris, 12 mM NaCl, 2.5 mM $MgCl_2$, 1 mM fresh DTT, and 4.5% DMSO) and centrifugation. Compounds were added into the sample wells, and the plates were incubated at 25 °C for 15 min before addition of digest buffer containing 217 µg/nL Dicer (5 µL, 108 µg/mL Dicer, 5% glycerol and 0.01% Triton X-100 final, excess with respect to pre-miRNA). For the positive control wells, digest buffer without Dicer was added. The plates were centrifuged again and resealed before being placed in a 37 °C incubator for 5 h. After Dicer cleavage, plates were washed with PB7 (50 µL; 3×). mTet-HRP in PB7 (10 µL, 750 nM final) was subsequently dispensed into each well, and the plates were centrifuged, sealed, and incubated at 25 °C for 2 h. Plates were washed with wash buffer (50 µL, 2 mM imidazole, 260 mM NaCl, 0.5 mM EDTA, 0.1% Tween-20, pH 7.0; 3×), followed by washing with PB7 (50 µL; 3×). Finally, SuperSignal West Pico was added (25 µL), the plates were incubated at 25 °C for 5 min, and chemiluminescence signal was detected.

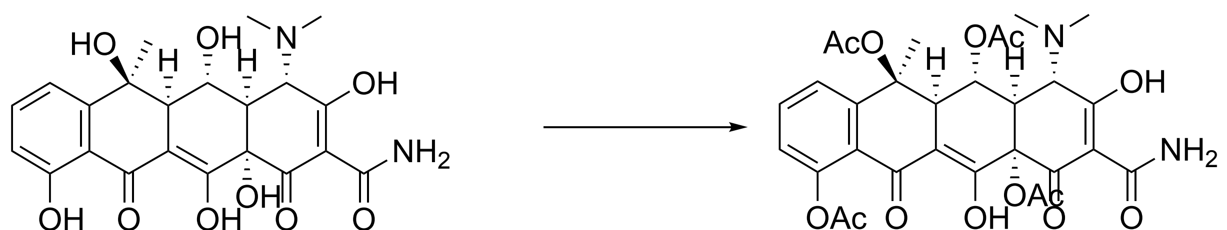
C. In-Solution Dicer Digestion

pre-miRNA (500 nM final) was treated with Dicer (1.0 µL, 1.3mg/ml) in buffer (20 mM Tris-HCl, pH 7.4, 12 mM NaCl, 2.5 mM $MgCl_2$, 40 U/mL RNase Out, 1.0 mM fresh DTT) at 37 °C for 3 h (10-µL total volume).[1] Digests were analyzed using a 12.5% TBE-Urea gel and visualized using SYBR® Gold. Data is representative from at least biochemical triplicate assays.

D. Surface Plasmon Resonance (SPR)

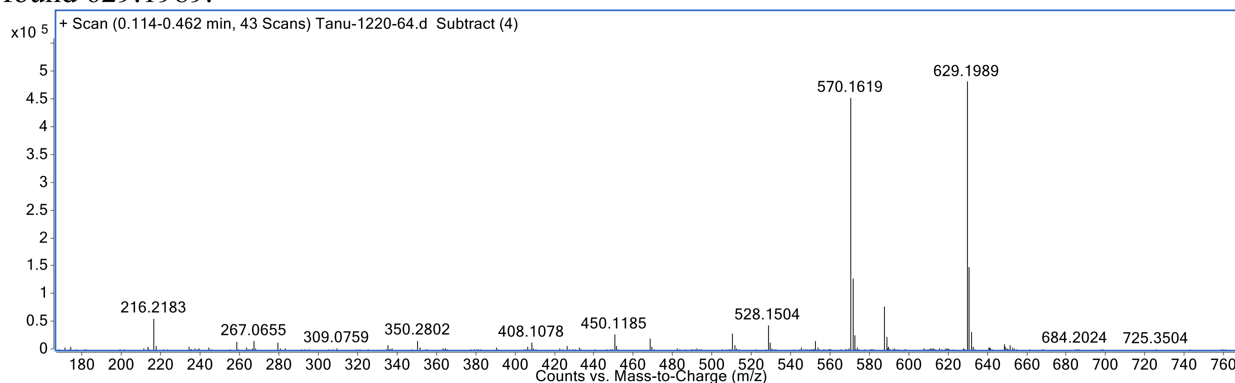
SPR was performed using a SensiQ Pioneer instrument and a BioCap biosensor. The chip was conditioned with 30 µL injections of 1 M sodium chloride in 50 mM sodium hydroxide, 0.5% sodium dodecyl sulfate, and running buffer with 1 M sodium chloride. Biotinylated pre-miR-21 or pre-let-7d was immobilized at 2300 RU. Compounds were dissolved in running buffer (50 mM Tris (pH 7.6), 150 mM sodium chloride, 5% glycerol, 0.05% Tween-20, 1 mM magnesium chloride, and 5% DMSO) to 50, 25, 12.5, and 6.25 µM and tested with an injection volume of 30 µL and a flow-rate of 30 µL/min. Regeneration was performed with a 10 µL injection of 50 mM Tris (pH 7), 260 mM sodium chloride, 0.5 mM EDTA, and 0.1% Tween-20. Data was processed using QDAT analysis software. Triplicate experiments were performed for each analysis.

E. Synthesis of Acetylated Tetracycline



(5S,5aR,6S,6aR,7S,10aS)-9-carbamoyl-7-(dimethylamino)-8,11-dihydroxy-5-methyl-10,12-dioxo-5,6,6a,7,10,12-hexahydrotetracene-1,5,6,10a(5aH)-tetrayl tetraacetate:

The synthesis of acetylated oxytetracycline was carried out by dissolving the oxytetracycline (1.0 equiv) in dry dichloromethane (2 mL), and subsequently cooling the reaction to 0 °C using an ice bath. After 5 min, acetic anhydride (4.4 equiv) and *cat.* dimethylaminopyridine (0.2 equiv) were added, and the resultant mixture was stirred at 25 °C for 1.5 h. The reaction was then quenched with water (~5 mL), and the organic layers were extracted with dichloromethane (3 × 20 mL). The organic layers were dried over anhydrous sodium sulfate and concentrated under reduced pressure *in vacuo*. The crude reaction mixture was purified by preparative reverse-phase HPLC to yield the corresponding product in 70% yield. White solid (72%); HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{32}N_2O_{13}$ $[M + H]^+$ 629.1983, found 629.1989.



F. Cellular Assay

HeLa cells were transfected with pmirGLO empty or pmir21 vector (100 ng) using Lipofectamine 2000. 5 h after transfection, cells were treated with the tetracyclines (100 μM) 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. The pmirGLO vector was ordered from Promega and miR21 was cloned in using oligos recommended by Promega.

G. Supplemental Figures

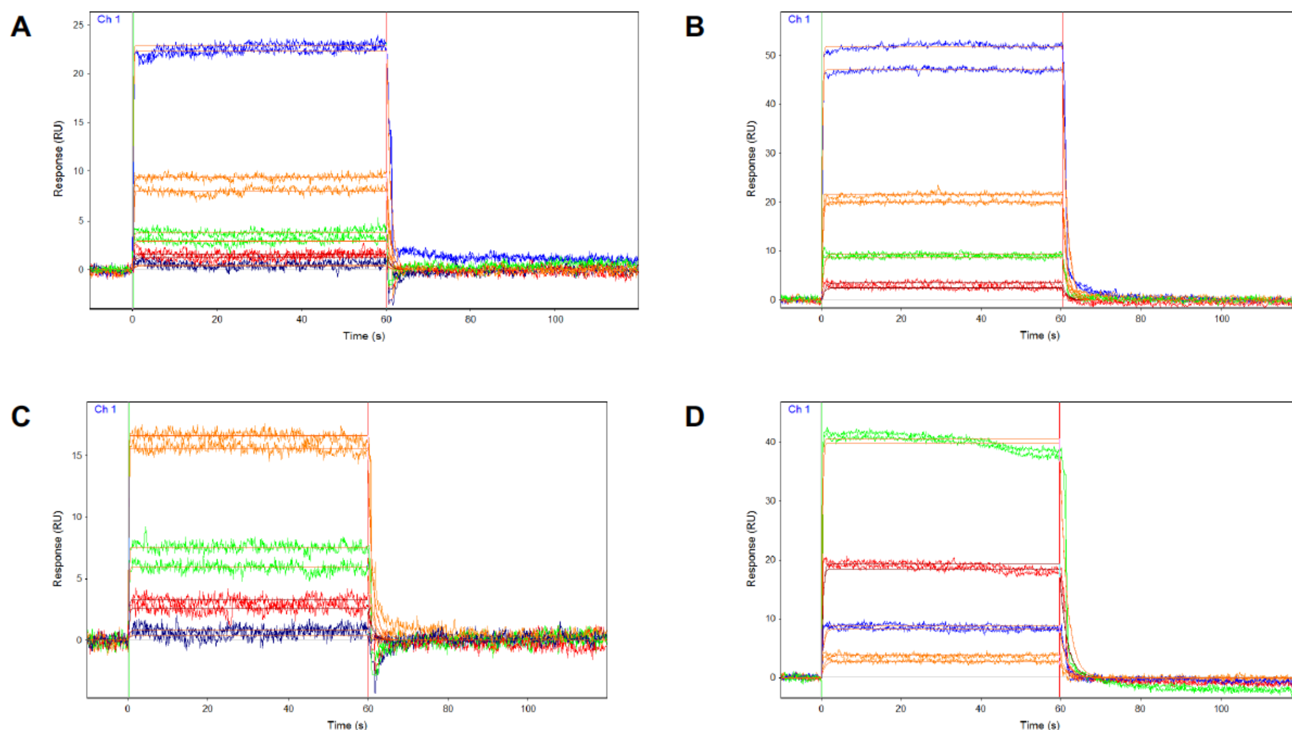


Figure S1. Direct binding of methacycline and meclocycline to pre-miR-21 and pre-let-7d measured via SPR.* (A) Binding of methacycline to pre-miR-21 (k_a = 1.1 ± 0.04 × 10^5 $M^{-1}s^{-1}$; k_d = 1.1 ± 0.01 s^{-1}; K_d = 9.9 ± 0.4 μM). (B) Binding of methacycline to pre-let-7d (k_a = 5.0 ± 0.07 × 10^4 $M^{-1}s^{-1}$; k_d = 0.84 ± 0.004 s^{-1}; K_d = 17 ± 0.3 μM). (C) Binding of meclocycline to pre-miR-21 (k_a = 3.9 ± 0.2 × 10^5 $M^{-1}s^{-1}$; k_d = 0.81 ± 0.009 s^{-1}; K_d = 2.1 ± 0.1 μM). (D) Binding of meclocycline to pre-miR-21 (k_a = 9.4 ± 0.2 × 10^4 $M^{-1}s^{-1}$; k_d = 0.54 ± 0.004 s^{-1}; K_d = 5.8 ± 0.1 μM). *Data not shown for the other compounds, as no binding was observed.

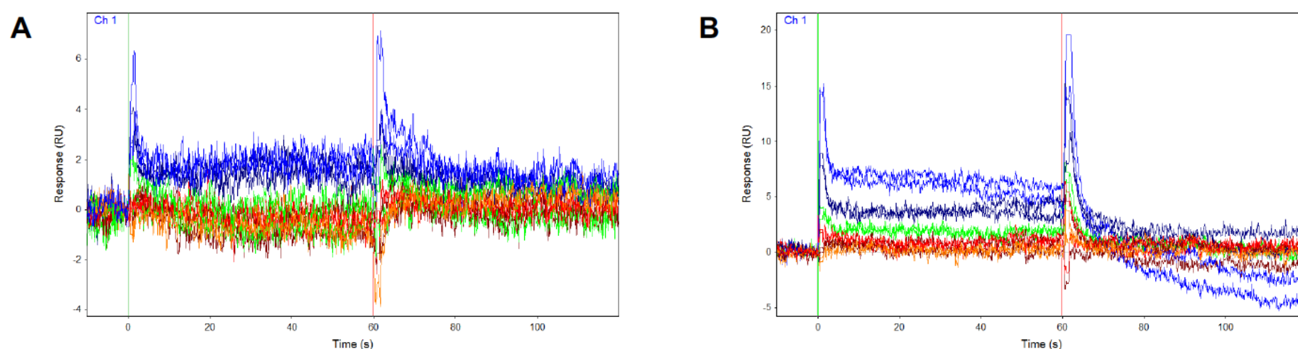


Figure S2. Direct binding of CMT-3 to (A) pre-miR-21 and (B) pre-let-7d. No binding was observed with this compound.

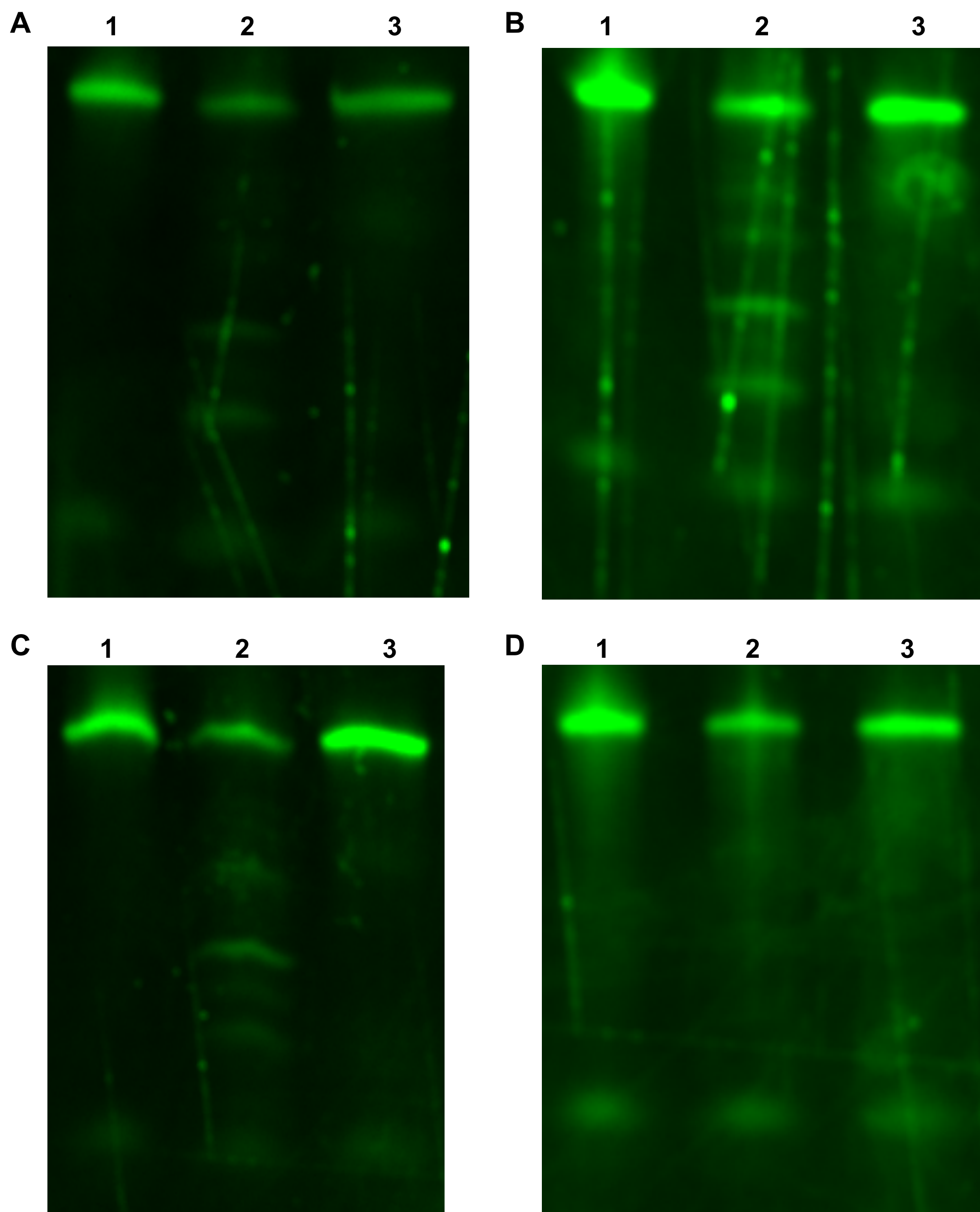


Figure S3. Processing of pre-miR-21 (500 nM) by Dicer in the presence of varying concentrations of metal ions. (A) 1 mM $MgCl_2$. (B) 3 mM $MgCl_2$. (C) 1 mM $MgCl_2$ plus 1 mM $CaCl_2$. (D) 2 mM $MgCl_2$ plus 4 mM $CaCl_2$. Lane 1 = pre-miR-21; Lane 2 = pre-miR-21 incubated with Dicer; Lane 3 = pre-miR-21 incubated with Dicer and methacycline (1.0 mM).

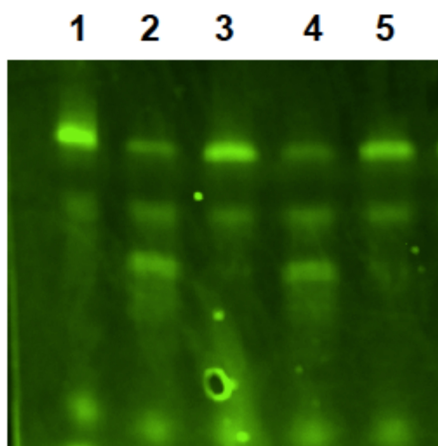


Figure S4. Processing of pre-miR-21 (500 nM) by *E. coli* RNase III (1 unit). Lane 1 = pre-miR-21; Lane 2 = pre-miR-21 incubated with enzyme for 0.5 h; Lane 3 = pre-miR-21 incubated with enzyme and methacycline (1.0 mM) for 0.5 h; Lane 4 = pre-miR-21 incubated with enzyme (1 unit) for 1.0 h; Lane 5 = pre-miR-21 incubated with enzyme and methacycline (1.0 mM) for 1.0 h.

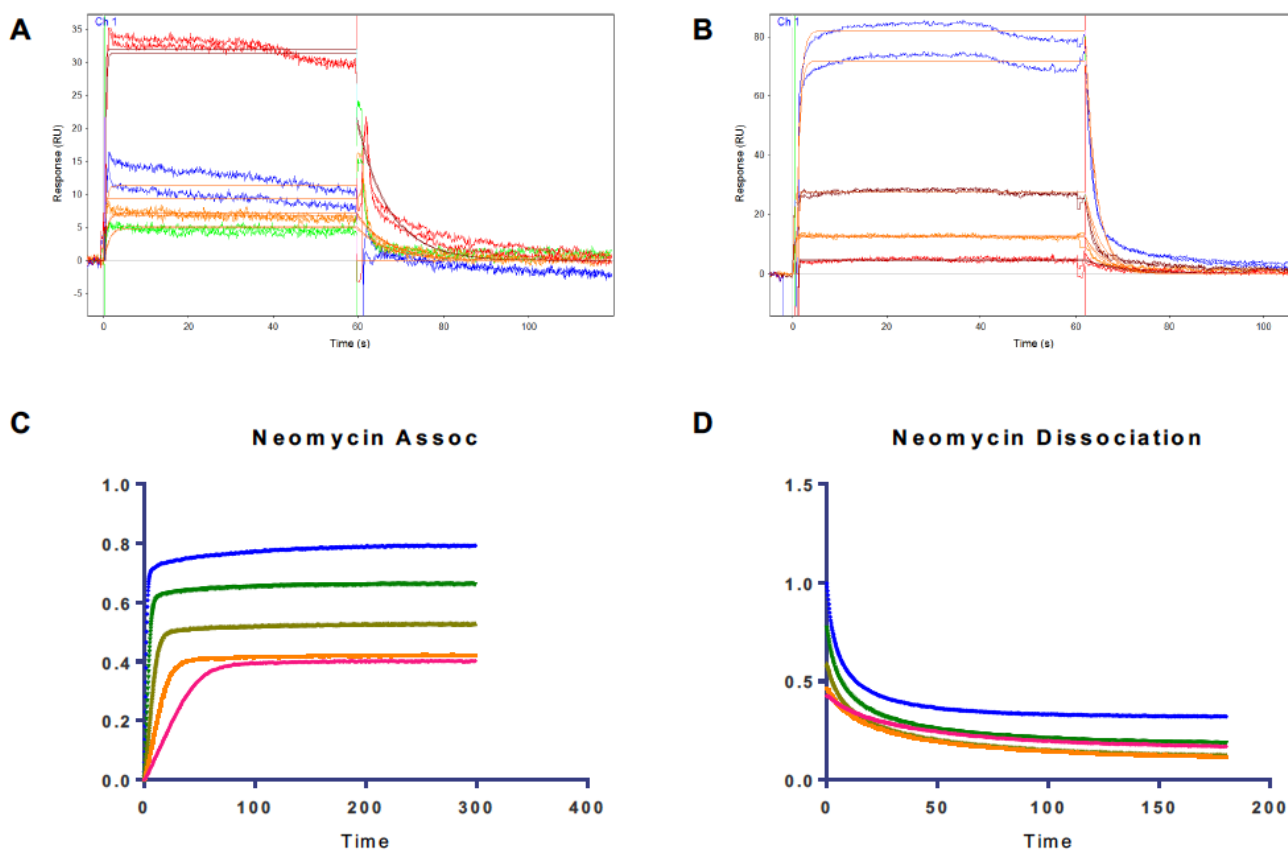


Figure S5. Direct binding of Hoechst 33258 and neomycin to pre-miR-21 measured via SPR and Octet Red, respectively. (A) Binding of Hoechst 33258 to pre-miR-21 (k_a = 2.1 ± 0.3 × 10^5 $M^{-1}s^{-1}$; k_d = 0.13 ± 0.004 s^{-1}; K_d = 0.7 ± 0.1 μM). (A) Binding of Hoechst 33258 to pre-let-7d (k_a = 1.6 ± 0.07 × 10^6 $M^{-1}s^{-1}$; k_d = 0.21 ± 0.01 s^{-1}; K_d = 0.13 ± 0.1 μM). (C), (D) Association and dissociation, respectively, of

neomycin binding to pre-miR-21 as observed via Octet Red. From this analysis, two binding sites were observed with K_d values of 100 nM and 10 μM.

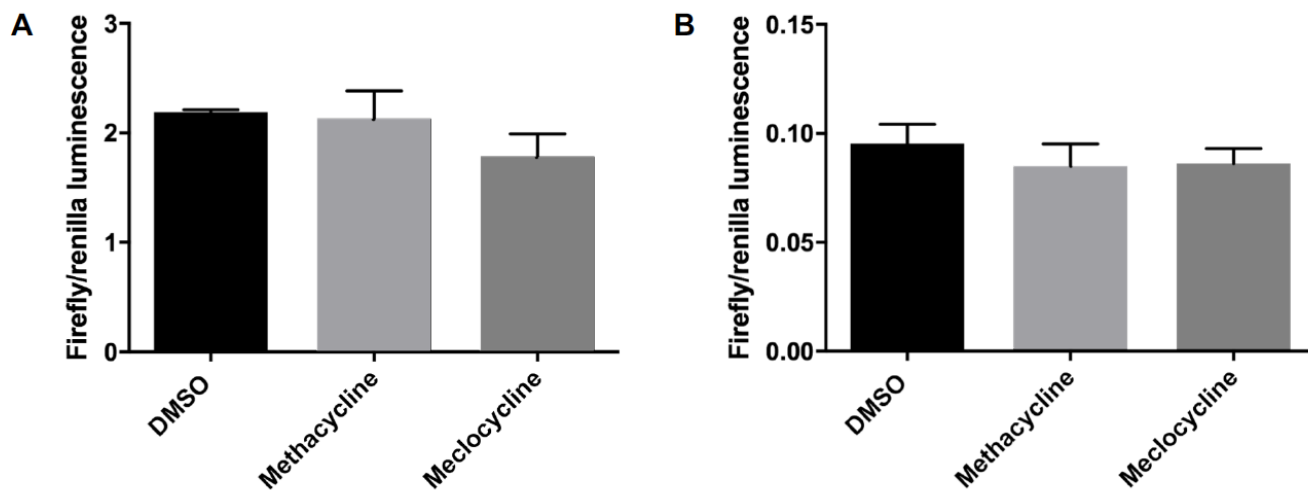
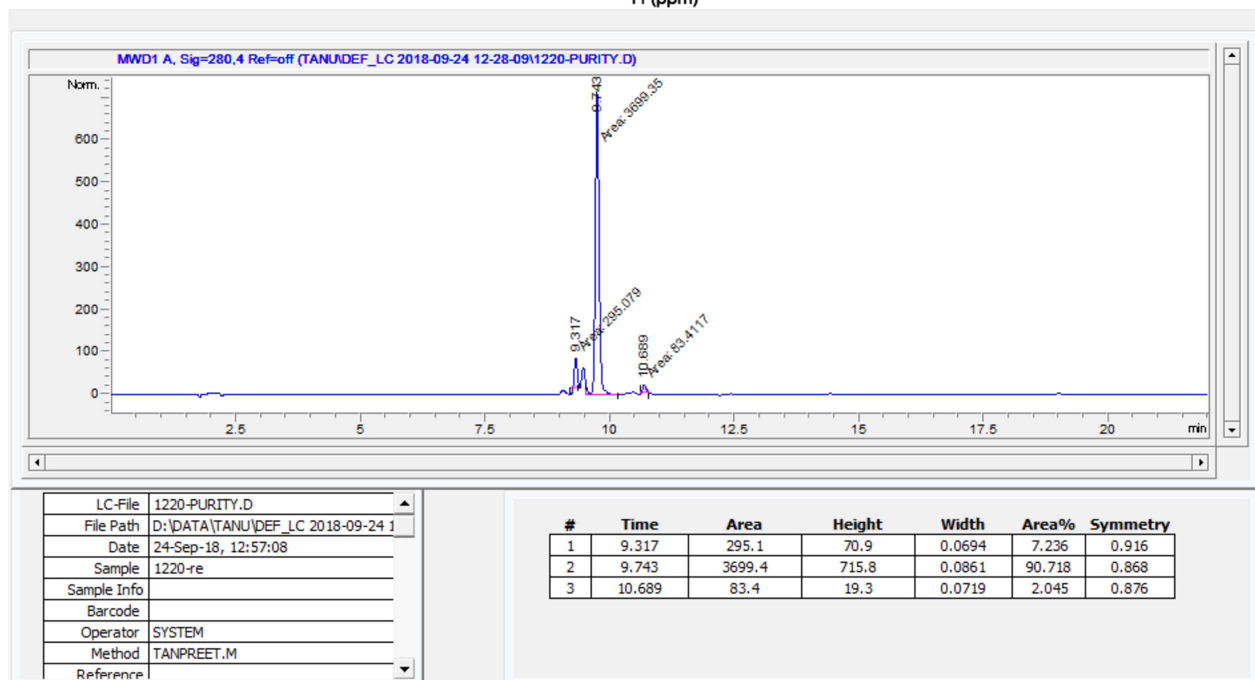


Figure S6. Activity of methacycline and meclocycline in a cell-based phenotypic assay of miR-21 activity. (A) Empty vector. (B) pmir21 vector.

I. NMR and Purity Spectra



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2	9.743	3699.4	715.8	0.0861	90.718	0.868
3	10.689	83.4	19.3	0.0719	2.045	0.876

H. References

1. Lorenz, D. A.; Vander Roest, S.; Larsen, M. J.; Garner, A. L., Development and implementation of an HTS-compatible assay for the discovery of selective small-molecule ligands for pre-microRNAs. *SLAS Disc.* **2018,** *23*, 47-54.
2. Lorenz, D. A.; Garner, A. L., A click chemistry-based microRNA maturation assay optimized for high-throughput screening. *Chem. Commun.* **2016,** (52), 8267-8270.