Supporting Information

Bifunctional Chimera for Ligand-directed Photo-degradation of Oncogenic microRNA

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Materials and Methods

1. General materials and methods

All chemicals and solvents were purchased from J&K chemicals or Sigma-Aldrich. High glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penincilin/streptomycin were purchased from Life Technologies. MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All aqueous solutions were treated by diethypyrocarbonate (DEPC) before use. UV-Vis absorption and fluorescence spectra were recorded at room temperature on Ocean Optics Maya 2000 and Shimadzu RF-5301PC spectrofluorophotometers with a 1 cm standard quartz cell, respectively.

^{1}H NMR and ^{13}C NMR spectra were obtained on a 400 MHz Bruker AVANCE III-400 spectrometer. Chemical shifts are reported in δ (ppm) relative to the solvent residual peak. High resolution mass spectrometry was performed on Thermo Fisher Q Exactive LC-MS. HPLC was carried out on Thermo UltiMate 3000 with CH_3CN/H_2O (0.1 M CH_3COONH^+) as eluents.

2. Synthesis and characterization of the compounds

Synthesis of TP-1

N-(4-formylphenyl) acetamide (2 g, 12.3 mmol) was slowly added to the mixed solvent of 60 mL H_2SO_4: HNO_3 (5:1, v:v) under the condition of ice water bath, and the reaction system reacted at 0 ℃ for 5 min. Then, the solution was poured into ice water to filter out the precipitation. The precipitation was dissolved with DCM, washed with saturated salt water and dried by rotation to afford compound TP-1 as yellow solid (1.82 g, yield 71%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.62 (s, 1H), 10.00 (s, 1H), 8.47 (d, J = 1.8 Hz, 1H), 8.17 (dd, J_1 = 8.4, J_2 =1.8 Hz, 1H), 7.91 (d, J = 8.4 Hz, 1H), 2.14 (s, 3H). ^{13}C

NMR (101 MHz, DMSO-d_6): δ 191.3, 169.3, 141.9, 136.6, 133.8, 132.4, 127.5, 125.2, 24.2.

Synthesis of TP-2

5-(4-methylpiperazin-1-yl)-2-nitroaniline (1 g, 4.23 mmol) was dissolved in 200 mL ethanol and 500 mg Pd-C was added to the system. The reaction system was stirred in hydrogen for 12 h and monitored by TLC. After the reaction of raw materials is completed, diatomite is filtered and the filtrate is dried. Due to the instability of the TP-2, the next step was directly carried out without further purification.

Synthesis of TP-3

To a solution of TP-1 (500 mg, 2.4 mmol) and TP-2 (494 mg, 2.40 mmol) in nitrobenzene (10 mL) at 140 ℃ and stirred for 12 h. After completion of the reaction monitored by TLC, the mixture was concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography to obtain the desired product TP-3 (482 mg, yield 51%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.87 (s, 1H), 10.43 (s, 1H), 8.74-8.58 (m, 1H), 8.37 (s, 1H), 7.81 (t, J = 8.0 Hz, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.08-6.72 (m, 2H), 3.13 (m, 4H), 2.52-2.50 (m, 4H), 2.24 (s, 3H), 2.12 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 169.1, 149.0, 148.1, 142.7, 138.3, 136.5, 132.1, 131.3, 127.5, 125.9, 122.4, 119.6, 115.9, 114.4, 111.9, 105.5, 97.5, 55.3, 50.0, 46.2, 24.0.

Synthesis of TP-4

To a solution of TP-3 (400 mg, 1.01 mmol) in 10 mL MeOH:H_2O (4:1, v:v), NaOH (48 mg, 1.2 mmol) was added, then the mixture was stirred at 70 ℃ for 6 h. After completion of the reaction monitored by TLC, the mixture was concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography to obtain the desired product TP-4 (312 mg, yield 87%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.75 (s, 1H), 8.24 (d, J = 8.5 Hz, 1H), 7.15 (d, J = 8.4 Hz, 1H),

6.97 (d, J = 8.7 Hz, 1H), 6.87 (s, 1H), 6.49 (d, J = 8.3 Hz, 1H), 2.99 (s, 4H), 2.51-2.48 (m, 4H), 2.23 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 159.2, 148.8, 145.4, 144.5, 144.9, 135.2, 130.9, 127.8, 121.5, 118.9, 115.7, 110.5, 104.0, 55.8, 52.01, 49.0, 46.4.

Synthesis of TP-5

To a solution of TP-4 (300 mg, 0.85 mmol) in EtOH (50 mL), was added 150 mg Pd-C under hydrogen, then the mixture was stirred for 12 h. After completion of the reaction monitored by TLC, the mixture was filtered with diatomite and then concentrated under reduced pressure to afford the product TP-5, the next step was directly carried out without further purification.

Synthesis of TP-6

To a solution of 3-hydroxybenzaldehyde (1.22 g, 10 mmol) and K_2CO_3 (4.14 g, 30 mmol) in DMF (60 mL) was added ethyl 4-bromobutanoate (4 g, 20 mmol) drop by drop. After stirred for 12 h at 70 ℃, the solvent was removed under reduced pressure. The obtained residue was resolved in DCM and washed with brine (50 mL×3). The organic layer was dried over and concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography to obtain the desired product TP-6 (1.46 g, yield 62%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.99 (s, 1H), 7.56-7.50 (m, 2H), 7.44-7.41 (m, 1H), 7.29-7.26 (m, 1H), 4.12-4.05 (m, 4H), 2.49 (t, J = 7.3 Hz, 2H), 2.07-1.95 (m, 2H), 1.19 (t, J = 7.1 Hz, 3H).

Synthesis of TP-7

To a solution of TP-5 (250 mg, 0.78 mmol) and TP-6 (184 mg, 0.78 mmol) in nitrobenzene (5 mL) at 140 ℃ and stirred for 12 h. After completion of the reaction monitored by TLC, the mixture was concentrated under reduced pressure to afford the crude product, which was purified by silica gel chromatography to obtain the desired product TP-7 (189 mg, yield 45%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.49 (d, J = 1.1 Hz, 1H), 8.07 (dd, J = 8.5, 1.6 Hz, 1H), 7.92 (d, J = 8.5 z, 1H), 7.87-7.80 (m, 2H), 7.73

(d, J = 9.0 Hz, 1H), 7.52 (t, J = 8.0 Hz, 1H), 7.34 (dd, J_1 = 9.1, J_2 = 2.1 Hz, 1H), 7.26 (d, J = 2.0 Hz, 1H), 7.14 (dd, J = 8.2, 1.9 Hz, 1H), 4.18-4.03 (m, 4H), 3.92 (s, 2H), 3.59 (s, 2H), 3.25 (s, 2H), 3.09 (s, 2H), 2.94-2.88 (m, 3H), 2.51-2.50 (m, 4H), 2.05 (m, 2H), 1.20 (t, J = 7.1 Hz, 3H).

Synthesis of TGP-210

To a solution of TP-7 (150 mg, 0.28 mmol) in 20 mL MeOH:H_2O (4:1, v:v), KOH (63 mg, 1.12 mmol) was added, then the mixture was stirred at 70 ℃ for 6 h. After completion of the reaction monitored by TLC, the mixture was concentrated under reduced pressure and the pH value of residual solution was adjusted to 6 with dilute HCl (0.1 M). The mixture was purified by HPLC to obtain the desired product TGP210 (114 mg, 80% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.48 (s, 1H), 8.07 (d, J = 8.5 Hz, 1H), 7.91 (d, J = 8.5 Hz, 1H), 7.86 – 7.78 (m, 2H), 7.72 (d, J = 9.0 Hz, 1H), 7.50 (t, J = 8.0 Hz, 1H), 7.33 (dd, J_1 = 9.1, J_2 =1.9 Hz, 1H), 7.26 (s, 1H), 7.13 (d, J = 10.1 Hz, 1H), 4.12 (t, J = 6.3 Hz, 2H), 3.90 (s, 2H), 3.61 (s, 2H), 3.17 (d, J = 63.3 Hz, 4H), 2.91 (s, 3H), 2.45 (t, J = 7.3 Hz, 2H), 2.01 (dd, J_1 = 13.6, J_2 = 6.7 Hz, 2H).

Synthesis of TGP-210-NH_2

To a solution of TGP-210 (114 mg, 0.22 mmol) and ethane-1,2-diamine (16 mg, 0.26 mmol) in DMF (5 mL), HBTU (109 mg, 0.29 mmol), HOBt (39 mg, 0.29 mmol) and DIEA (109.09 μL, 0.66 mmol) were added. After stirred overnight at room temperature, the solution was poured into ice water mixture and extracted with ethyl acetate. After drying over sodium sulfate, the product was purified by chromatography (3-5% MeOH/CH_2Cl_2) to yield a solid TGP-210-NH_2 (106 mg, yield 87%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (d, J = 8.4 Hz, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.71 (t, J = 4.5 Hz, 1H), 7.54 (dt, J_1 = 10.5, J_2 =7.8 Hz, 1H), 7.41 (dd, J_1 = 8.2, J_2 =2.0 Hz, 1H), 7.31 (d, J = 9.0 Hz, 1H), 7.14 (dd, J_1 = 8.2, J_2 =2.0 Hz, 1H), 4.13 (t, J = 6.3 Hz, 2H), 3.31 (dd, J_1 = 12.2, J_2 =6.2 Hz, 2H), 3.08 (s, 1H), 2.97-2.81 (m, 2H), 2.35 (t, J = 7.5 Hz, 1H), 2.09-1.94 (m, 1H).

Synthesis of the targeted compound TGP-210-Ppa

To a solution of TGP-210-NH_2 (106 mg, 0.19 mmol) and pheophorbide-a (135 mg, 0.23 mmol) in DMF (5 ml), HBTU (94 mg, 0.25 mmol), HOBt (34 mg, 0.25 mmol) and DIEA (94 μL, 0.57 mmol) were added. After stirred overnight at room temperature, the solution was poured into ice water mixture and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and purified by chromatography (3-5% MeOH/CH_2Cl_2) to yield a solid TGP-210-Ppa (167 mg, 78% yield). HRMS calcd for $C_{66}H_{70}N_{12}O_6$ 1126.5541, [M+H^+], found 1127.5612.

3. Cell culture

MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum in a humidified environment at 37 ℃ which contains 5% CO_2 and 95% air.

4. MTT assay

MDA-MB-231 cells were seeded on a 96-well plate with 5,000 cells per well and incubated with different concentration of TGP-210-Ppa (0-25 μM) for 24 h. Then 20 μL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added for 4 h. After removing the remaining MTT, 150 μL DMSO was added to dissolve the formazan. The absorbance of each well at 570 nm was measured by a microplate reader, the cell viability was calculated using the follow formula: (a-b)/(c-b) × 100%, where a, b and c are absorbance of treated wells, control wells and untreated wells, respectively.

5. Measurement of $^1O_2$ generation capacity *in vitro*.

TGP-210-Ppa (500 nM) in PBS was mixed with SOSG (5 μM). The mixtures were irradiated with 670 nm light (70 mW/cm^2) for 0-20 min. Fluorescence emission of SOSG at 530 nm was acquired

with excitation at 488 nm.

6. Cellular fluorescence imaging

MDA-MB-231 cells were incubated with TGP-210-Ppa, after each incubation procedure, the cells were washed with PBS buffer three times. A red laser (640 nm) was used to excite the PS and its fluorescence was detected at wavelengths >670 nm (APC channel). Then, cells were stained with Hoechst (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol. Images were captured using OLYMPUS SpinSR Rotary confocal superresolution microscope.

7. qRT-PCR

MDA-MB-231 cells (5×10^6 cells) were seeded on a 12-well plate. Cells were cultured under normaxia (21% O_2) or hypoxia (1% O_2). To evaluate the light-dependent cytotoxicity, cells were irradiated with 670 nm light (70 mW/cm^2) for 5 min. Total RNA of the samples was separated by Trizol reagent (Invitrogen, Shanghai, China) following the manufacturer's protocols. Reverse transcriptase reactions were performed using HiScript® III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). RT-qPCR was done using Quantitative PCR with ChamQTM Universal SYBR QPCR Master Mix (Vazyme, Nanjing, China). Eventually, the product was analyzed on the Applied Biosystem Step One Plus Real-Time PCR system. The quantitative measures were obtained using the ΔΔCT statistical method and was normalized to GAPDH mRNA levels. Primers:

GAPDH (F): 5'-CGAGCCACATCGCTCAGACA-3'

GAPDH (R): 5'-GTGGTGAAGACGCCAGTGGA-3'

pre-miR-210 (F): 5'-GCAGCCCCTGCCCACCGCACACT-3'

pre-miR-210 (R): 5'-CCGCTGTCACACGCACAG-3'

HIF1α (F): 5'-CGCGAACGACAAGAAAAAG-3'

HIF1α (R): 5’-AAGTGGCAACTGATGAGCAA-3’

GPD1L (F): 5’-CGCGAACGACAAGAAAAAG-3’

GPD1L (R): 5’-GGCAGCTTGTGTCCAGGAA-3’

hsa-miR-210 (F): 5’-CTGTGCGTGTGACAGCGGCTGA-3’

hsa-miR-21 (F): 5’-TAGCTTATCAGACTGATGTTGA-3’

hsa-miR-24 (F): 5’-TAGCTTATCAGACTGATGTTGA-3’

hsa-miR-192 (F): 5’-CTGACCTATGAATTGACAGCC-3’

Universal Reverse: 5’-GAATCGAGCACCAGTTACGC-3’

8. Gel-shift assay

600 μM of TGP-210-Ppa and 5 μM pre-miR-210 was incubated together for 2 h at room temperature. The mixture was exposed to visible light for 0, 5, 10, 15, 20 and 25 min respectively, then subjected to electrophoresis on 4% agarose gels stained with ethidium bromide. The light source used in the experiment is 670 nm light (70 mW/cm^2).

9. Measurement of Intracellular $^1O_2$

A total of 1×10^5 MDA-MB-231 cells were seeded on 35 mm glass-bottom tissue culture dishes. When reaching 60% confluency, cells were treated with TGP-210-Ppa (500 nM). Prior to detection, cells were washed with fresh medium three times and treated with H_2DCFDA (20 μM) for 20 min. At the 4 h time points, cells were irradiated with 670 nm light (70 mW/cm^2) for 0–5 min. After irradiation, cells were washed with PBS three times, and the fluorescence signals from H_2DCFDA were acquired on an Olympus IX73 fluorescent inverted microscope. The fluorescence intensities were analyzed using ImageJ.

10. $^1O_2$ Quantum Yield Measurements

9,10-anthracenedipropanoic acid (ADPA) was used as the $^1O_2$-trapping agent, and MB was used as the standard photosensitizer. In the experiments, the 670 nm laser was employed as the irradiation source. To eliminate the inner-filter effect, the absorption maxima of MB and the TGP-210-Ppa were adjusted to ≈0.2 OD. The absorption of ADPA at 378 nm was recorded at various irradiation times to obtain the decay rate of the photosensitizing process. The $^1O_2$ quantum yield of the TGP-210-Ppa in water was calculated using the following formula:

$$\Phi_{TGP-210-Ppa} = \Phi_{MB}\frac{k_{TGP-210-Ppa} \times A_{MB}}{k_{MB} \times A_{TGP-210-Ppa}}$$

where k is the slope of the absorbance versus irradiation time. A represents the light absorbed by TGP-210-Ppa or MB at 670 nm. ΦMB is the $^1O_2$, quantum yield of reference MB, which is 0.52 in water.

11. Microscale Thermophoresis (MST) Binding Measurements

MST fluorescent measurements were performed on a Monolith NT.115 system (NanoTemper Technologies) using the fluorescence of a 5' Cy5 labeled miR-210 Hairpin RNA: 5' – Cy5 CGCACACUGCGCUGCCCCAGACCCACUGUGCG), a 5' Cy5 miR-210 Mutant RNA: 5' - Cy5 CGCACAGUGCGCUGCCCCAGACCCACUGUGCG) which were synthesized by K&A series DNA/RNA/LNA nucleic acid synthesizer with RNase-free HPLC purification and used without further purification. The RNA (5 nM) was prepared in 1× MST Buffer (8 mM Na_2HPO_4, 190 mM NaCl, 1mM EDTA, and 0.05% (v/v) Tween-20) and folded by heating at 60°C for 5 min, and slowly cooling to room temperature. Compounds (TGP-210 or TGP-210-Ppa) were diluted in 1× MST Buffer and then were added to a final concentration of 20 μM, followed by 1:2 dilutions in 1× MST Buffer containing 5 nM RNA. Samples were incubated for 30 min at room temperature and then loaded into premium-coated capillaries (NanoTemper Technologies). The data were analyzed by thermophoresis analysis, and fitted by the quadratic binding equation

in MST analysis software (NanoTemper Technologies). Dissociation constants were then determined by curve fitting using a single-site model.

12. Clonogenic Assay.

MDA-MB-231 cells (3×10^4 cells) were seeded into 12-well tissue culture plates. After 20 h, cells were treated with TGP-210, Ppa or TGP-210-Ppa for 4 h, cells were irradiated with 670 nm light (70 mW/cm^2) for 5 min. The plates were then washed with PBS and stained with the solution containing 0.5% (w/v) crystal violet for 30 min. The plates were then washed with running water until cell colonies were clear without background color and dried at room temperature. Olympus IX73 Inverted Fluorescence Microscope was used for the acquisition of the images.

13. Statistical analysis

All data shown in the manuscript are mean ± SEM. Statistical analyses were performed using a two-tailed unpaired Student's t-test with GraphPad Prism 6.0 software (GraphPad Software Inc., USA). All of the assays were performed at least three times. *$*P < 0.05, **P < 0.01, and ***P < 0.001$* were considered statistically significant.

Supporting Figures

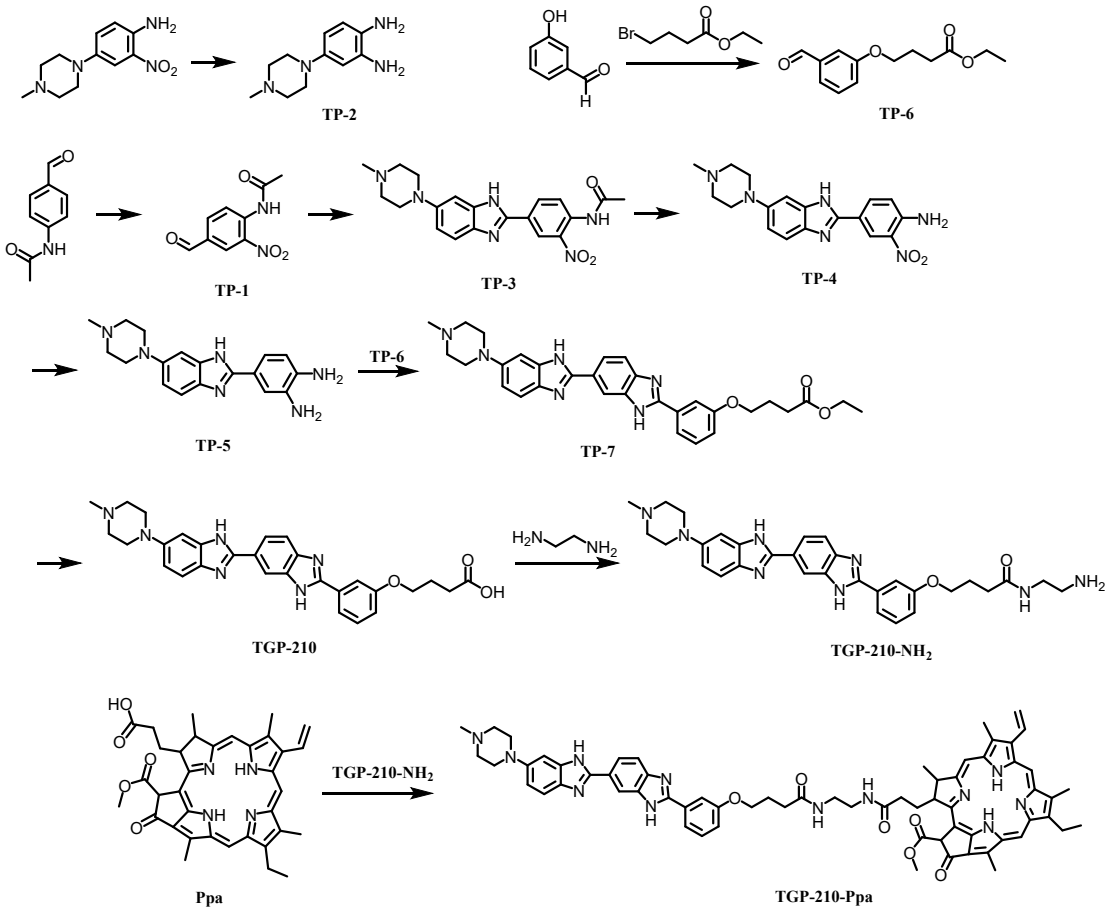


Figure S1. The synthetic routes for TGP-210-Ppa. TGP-210 was synthesized according to the method reported by Disney *et al*.[1]

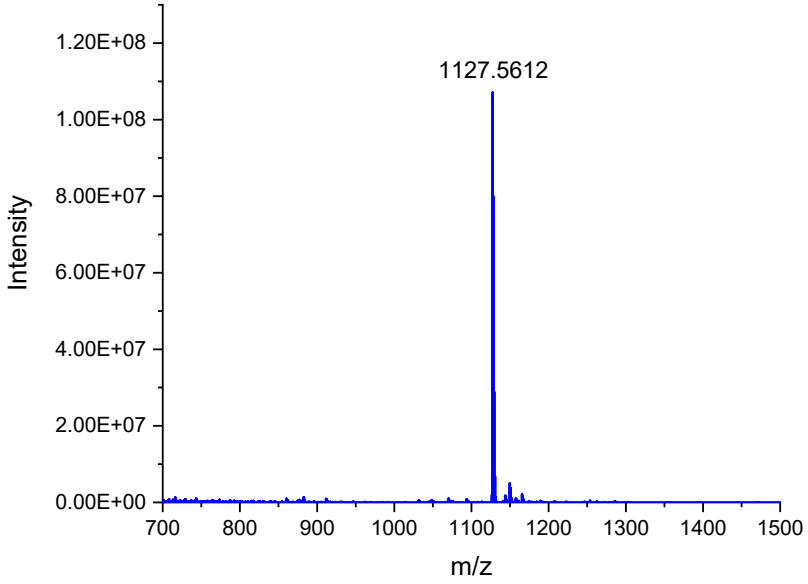


Figure S2. The electrospray ionization-mass spectrometry of TGP-210-Ppa.

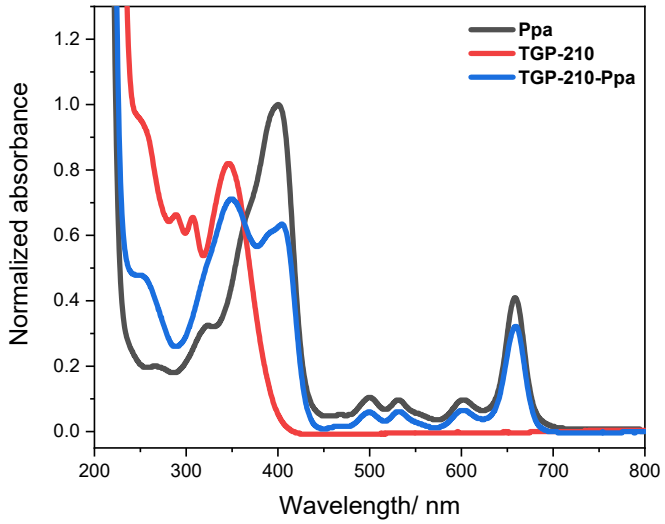


Figure S3. The normalized UV/vis absorption spectra of TGP-210, Ppa, TGP-210-Ppa.

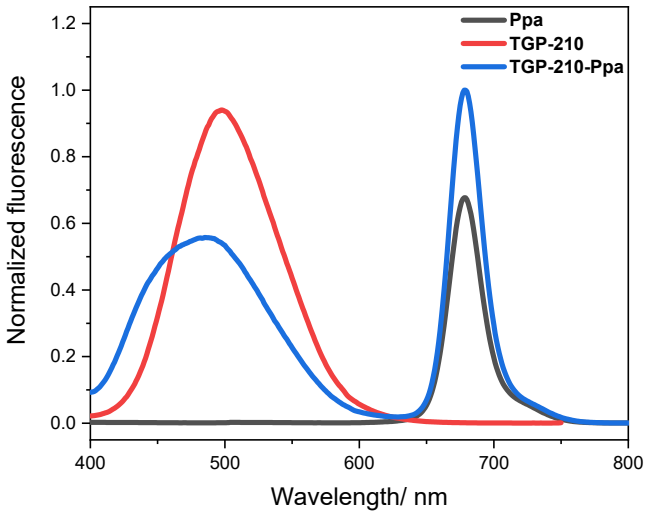


Figure S4. The normalized fluorescence spectra of TGP-210, Ppa, TGP-210-Ppa.

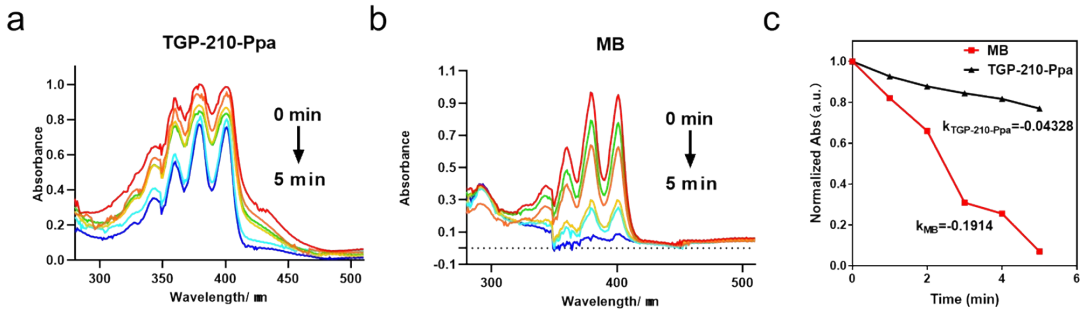


Figure S5. Photodegradation of ADPA with (a) TGP-210-Ppa and (b) MB under 670 nm laser irradiation. (c) Decay curves of ADPA absorption at 378 nm as a function of time in the presence of TGP-210-Ppa and MB under 670 nm laser irradiation.

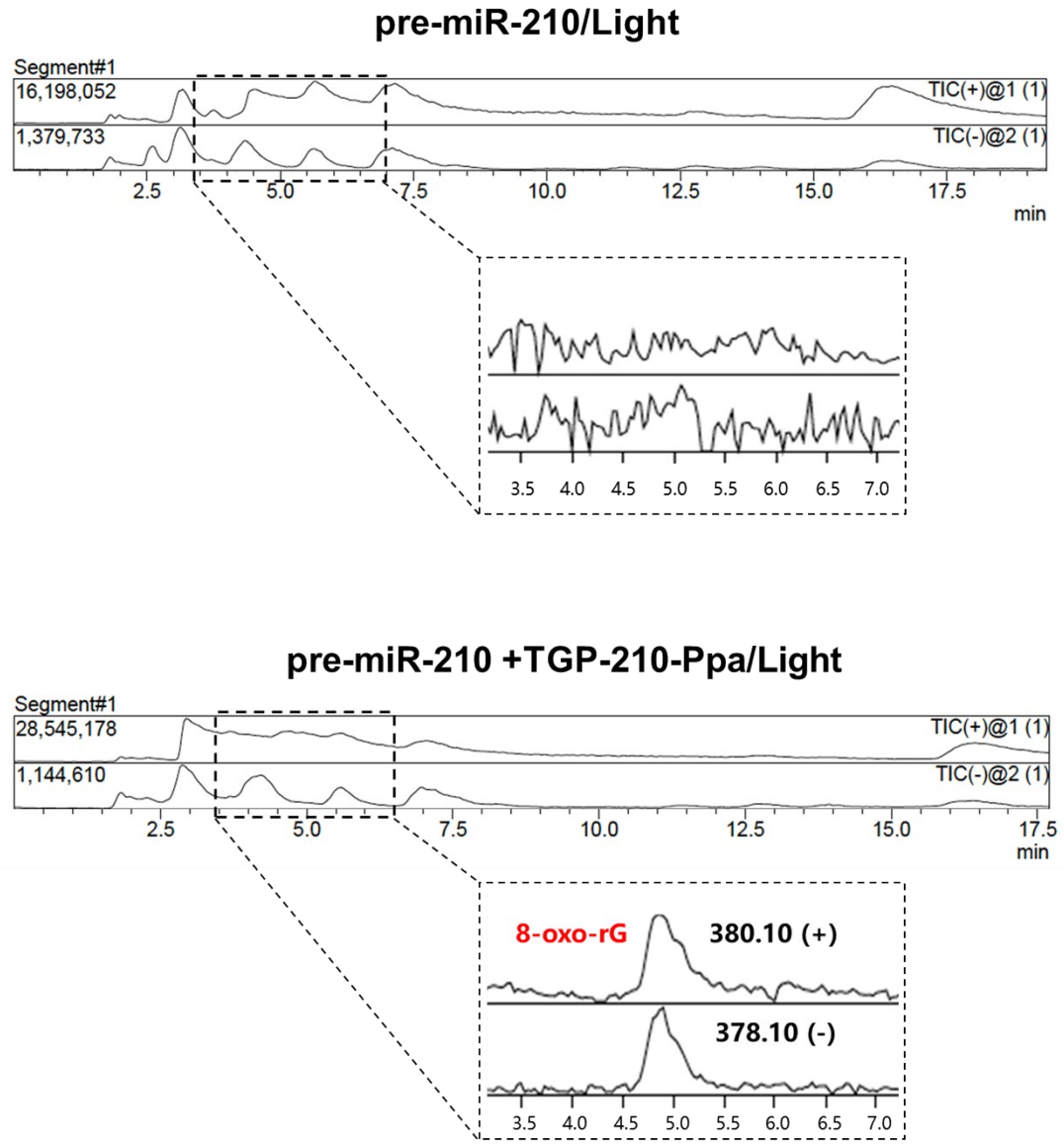


Figure S6. The hydrolysis products of pre-miR-210 with or without TGP-210-Ppa under light irradiation were analyzed by the LC-ESI-MS/MS. The concentration ratio of TGP-210-Ppa and pre-miR-210 was 10:1. After irradiation at 670 nm for 20 min, 1 unit of nuclease P1 was added to hydrolyze RNA into a single nucleotide. Meanwhile, nuclease P1 was added to the pre-miR-210 solution without any treatment as the control.

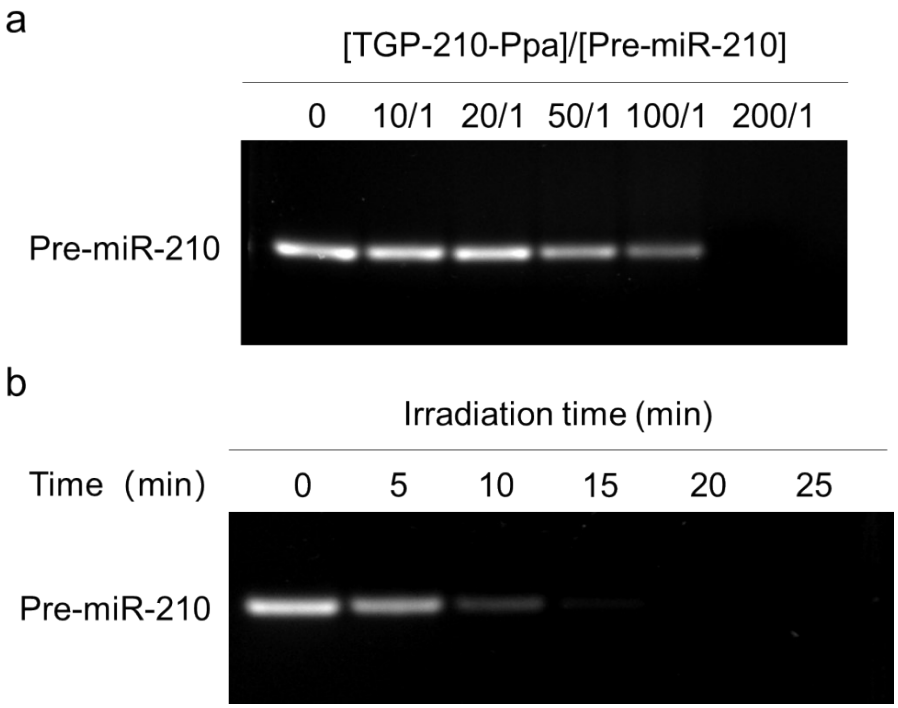


Figure S7. (a) Gel shift analysis of TGP-210-Ppa and pre-miR-210 at different concentration ratio with 20 mins' 670 nm light irradiation. (b) Gel shift analysis by the presence of 200 equivalent of TGP-210-Ppa to pre-miR-210, and irradiation with 670 nm light (70 mW/cm^2) for 0-25 min.

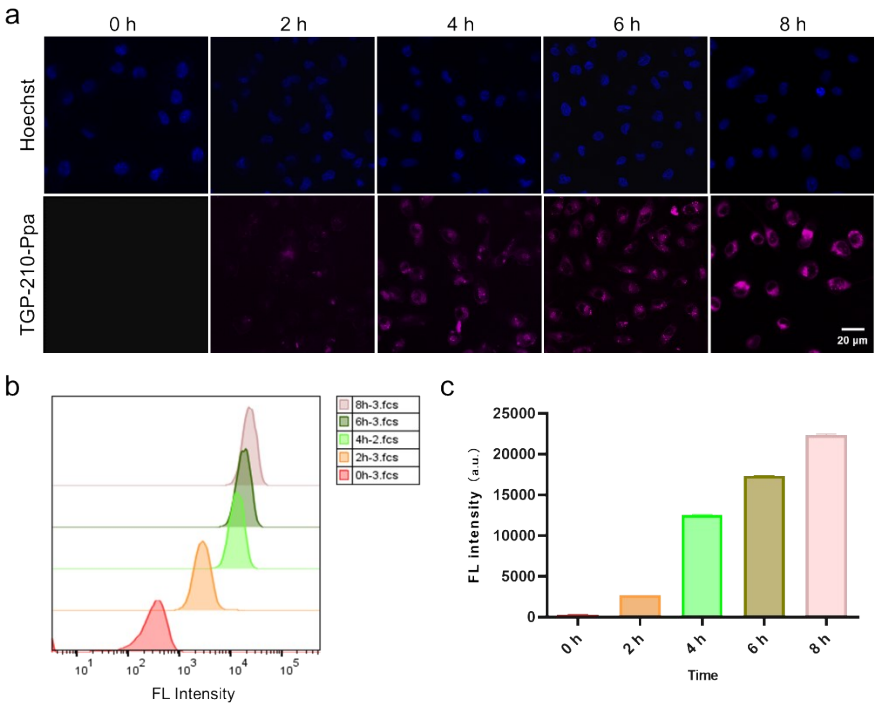


Figure S8. Fluorescence images of MDA-MDB-231cells after an incubation with TGP-210-Ppa (500 nM) for 0-8 h. All images share the same scale bar. The celluar uptake of the probe was further quantified by flow cytometry.

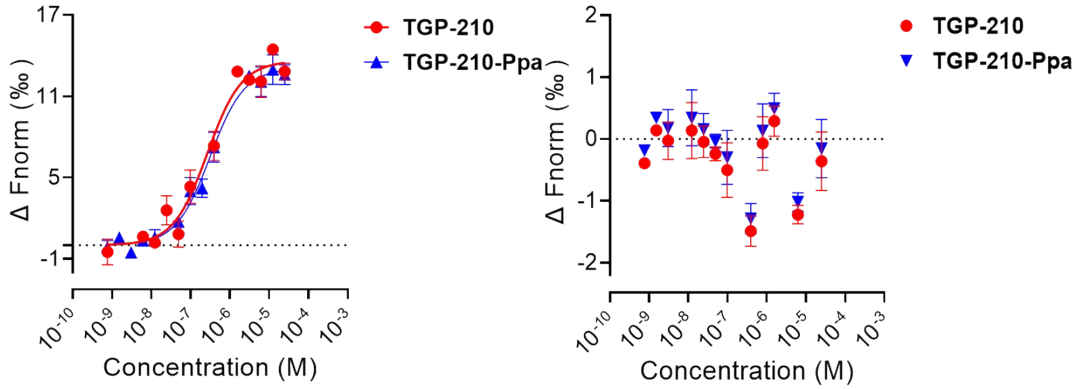


Figure S9. The binding affinity of TGP-210 and TGP-210-Ppa to 5' Cy5-labeled miR-210 Hairpin RNA and miR-210 Mutant RNA by microscale thermophoresis (MST) analysis. Data represent means ± SEM of triplicates.

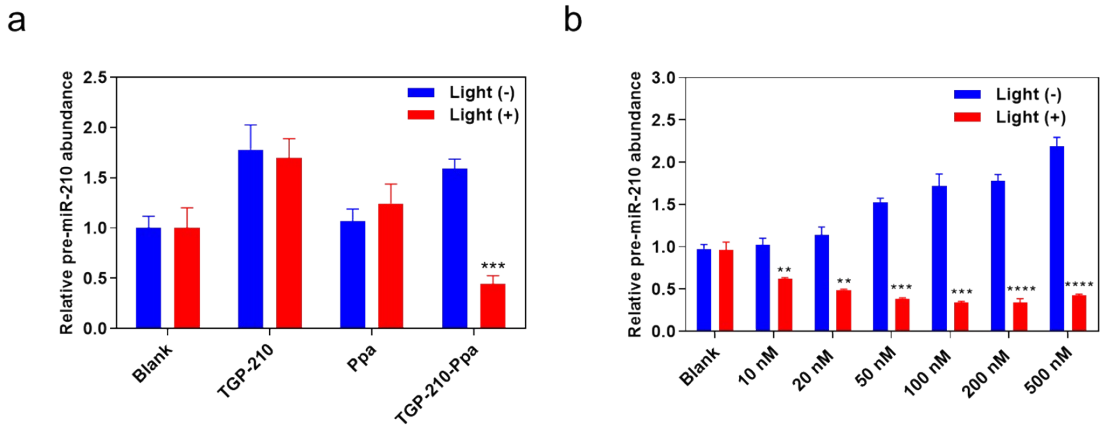
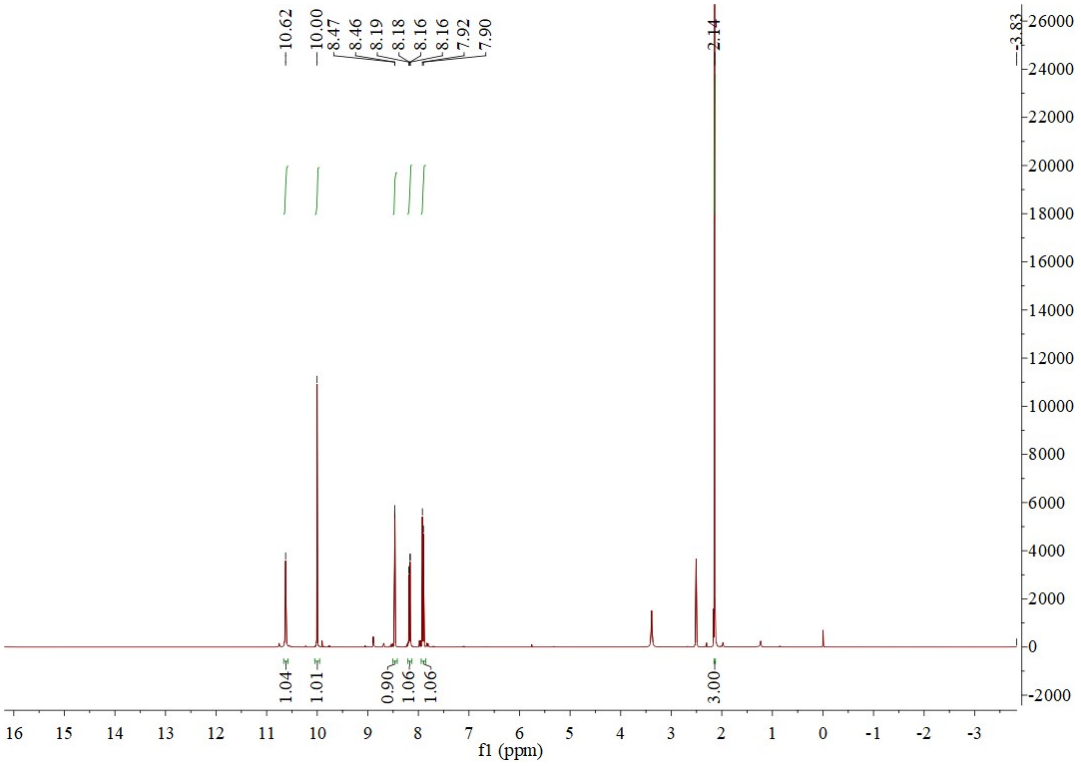


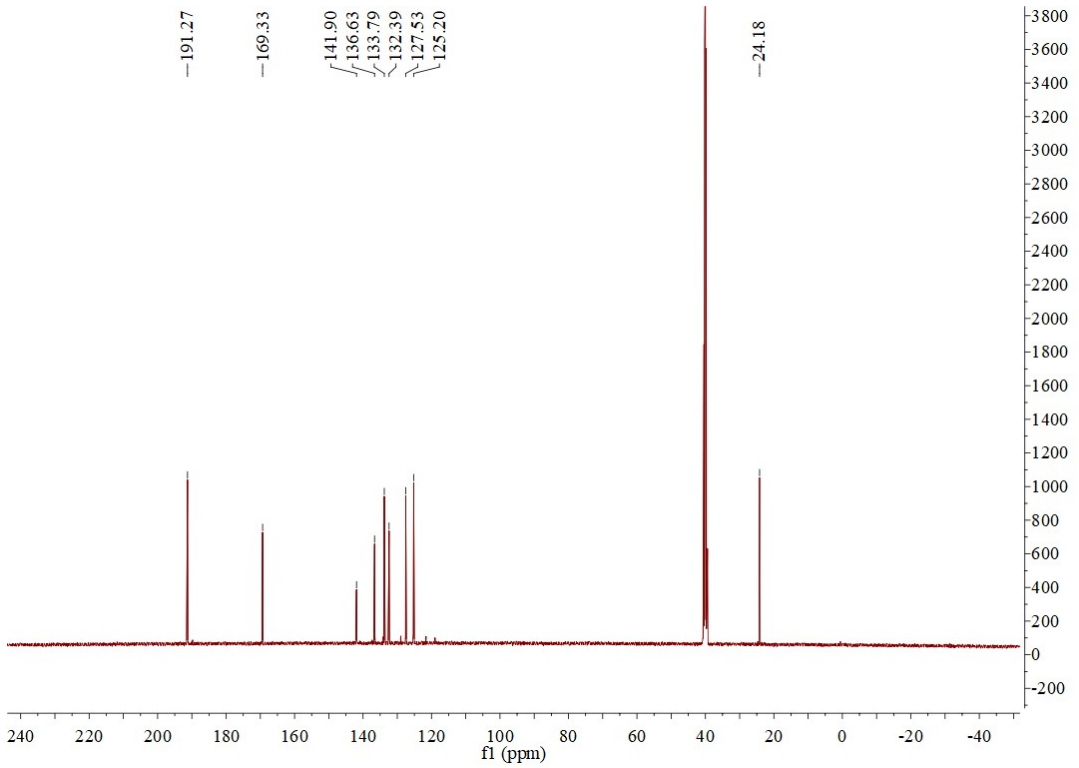
Figure S10. (a) qRT-PCR analysis of pre-miR-210 in MDA-MB-231 cells treated with TGP-210 (500 nM), Ppa (500 nM) or TGP-210-Ppa (500 nM) for 4 h. Cells were irradiated with 670 nm light (70 mW/cm^2) for 5 min. (b) The relative expression levels of miR-210 in the different concentrations of TGP-210-ppa treated group with or without 670 nm light (70 mW/cm^2). Data are shown as mean ± SEM (n=3). **P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.*

NMR Spectra

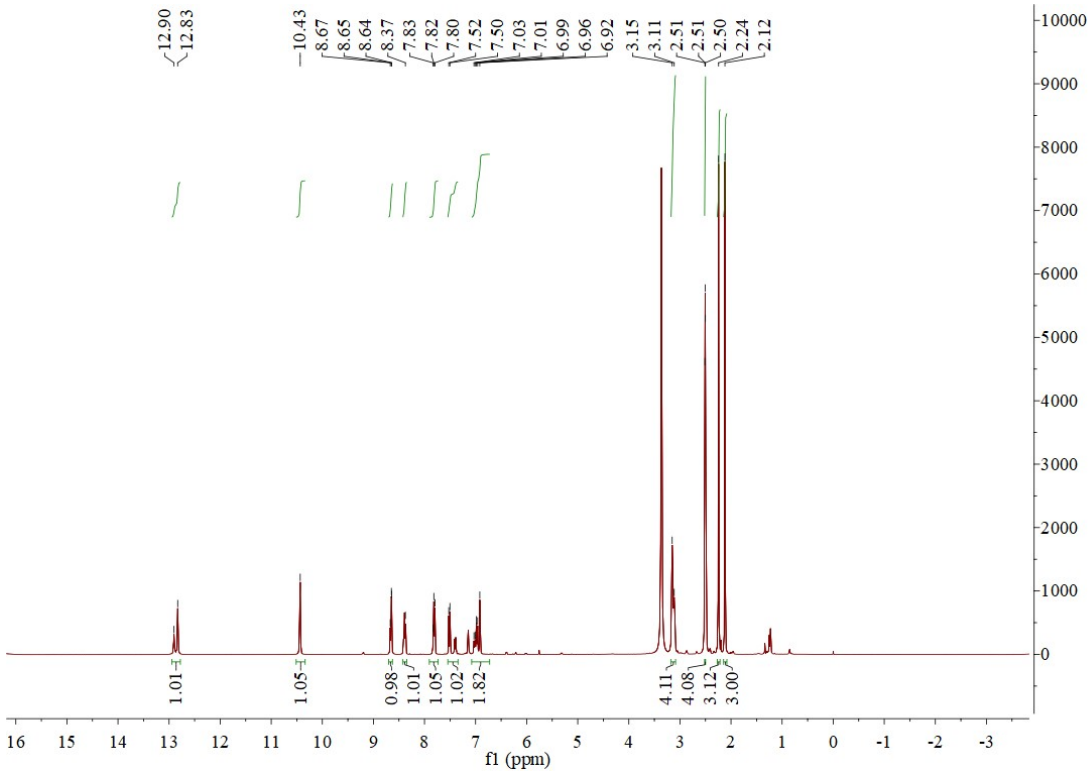
^{1}H NMR of TP-1



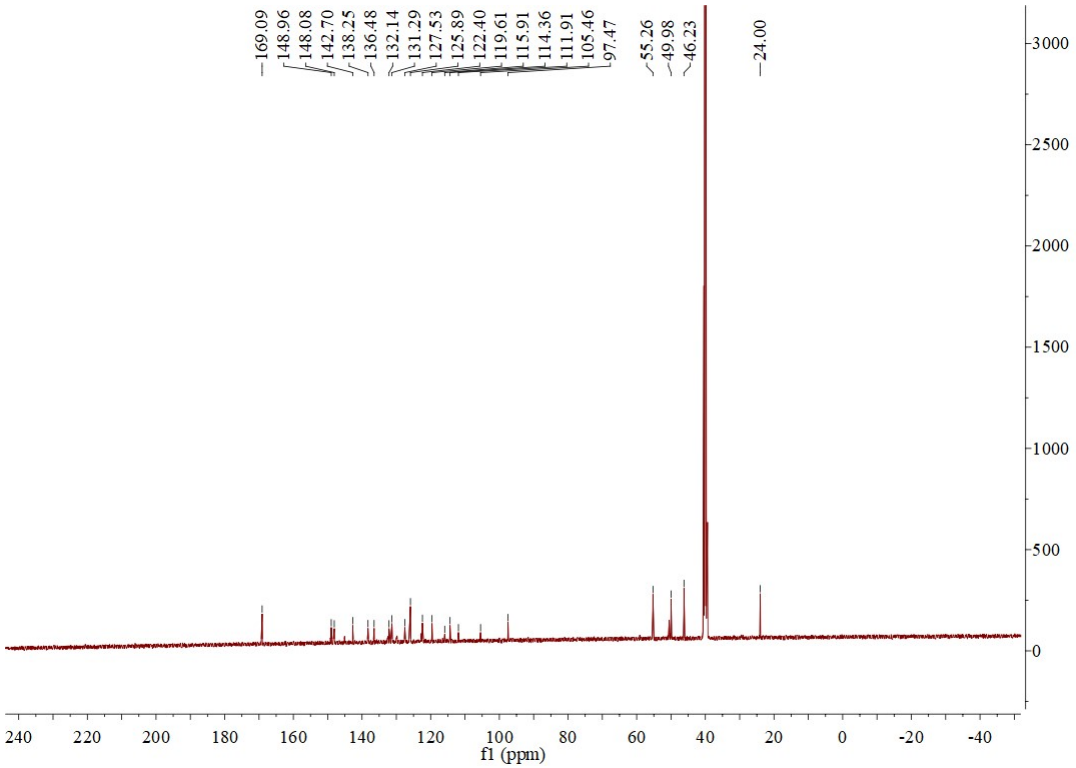
^{13}C NMR of TP-1



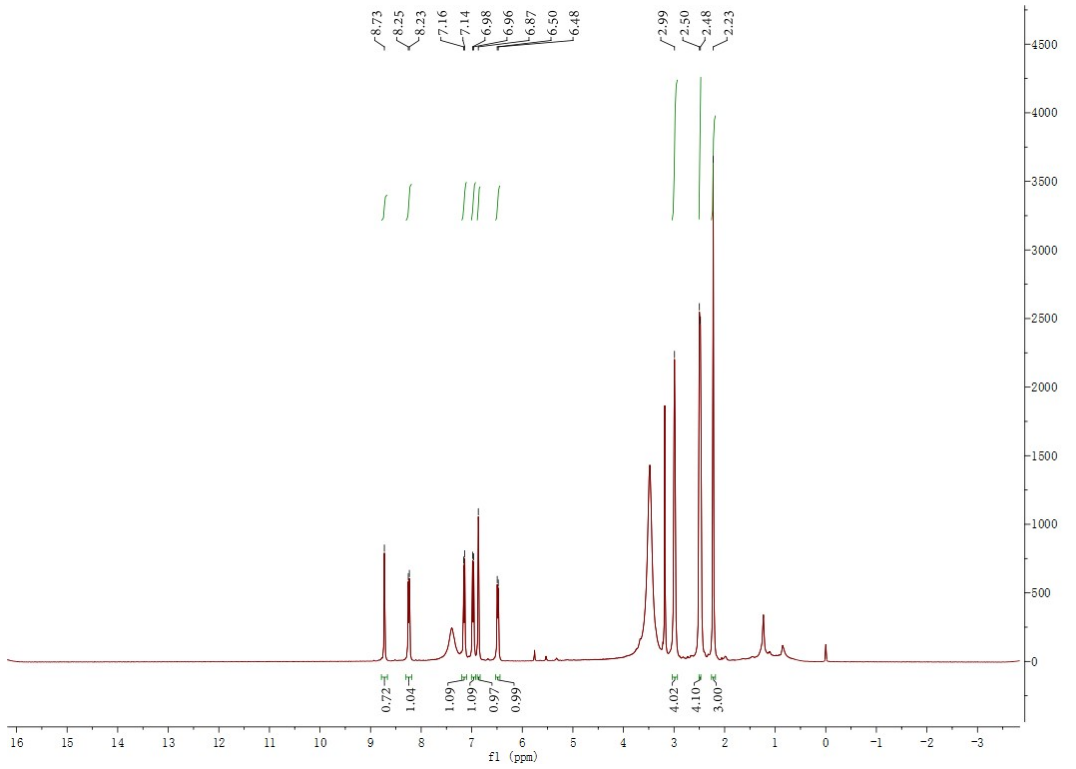
^{1}H NMR of TP-3



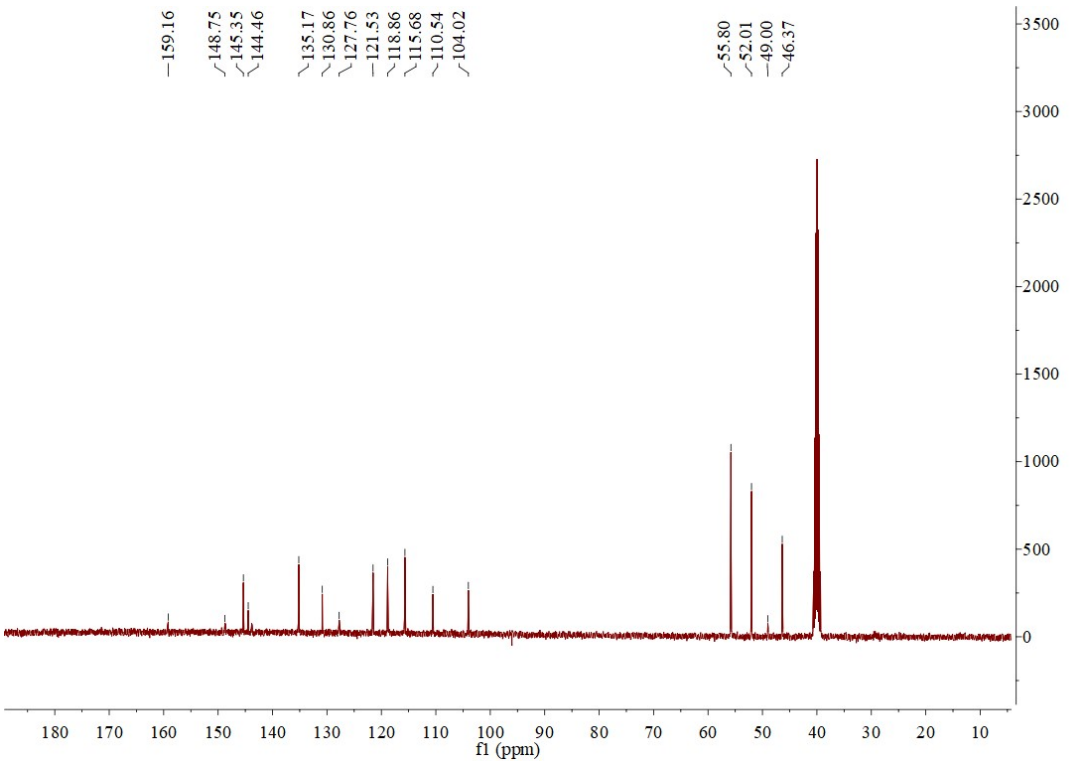
^{13}C NMR of TP-3



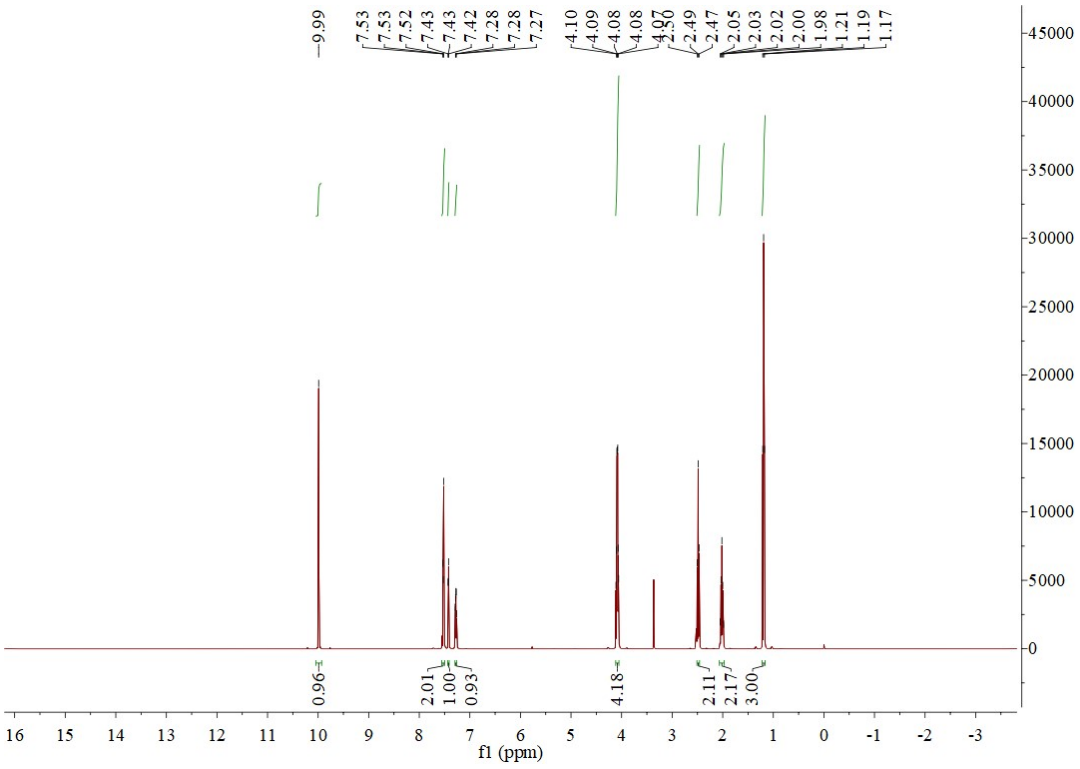
^{1}H NMR of TP-4



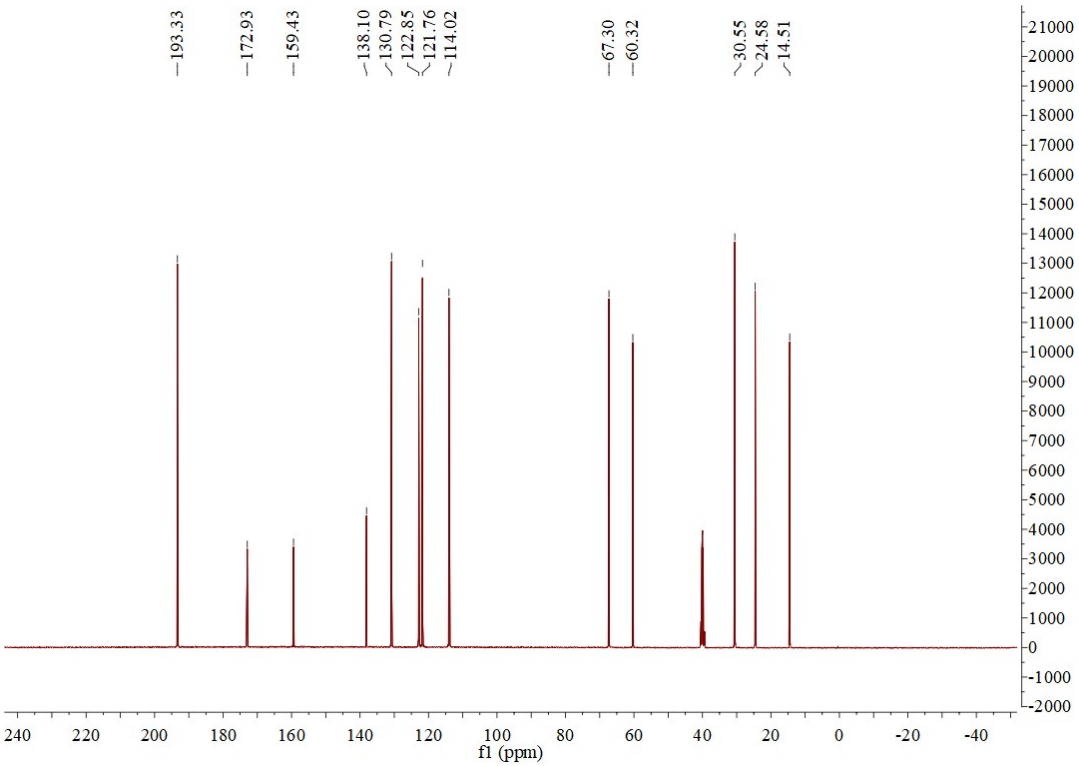
^{13}C NMR of TP-4



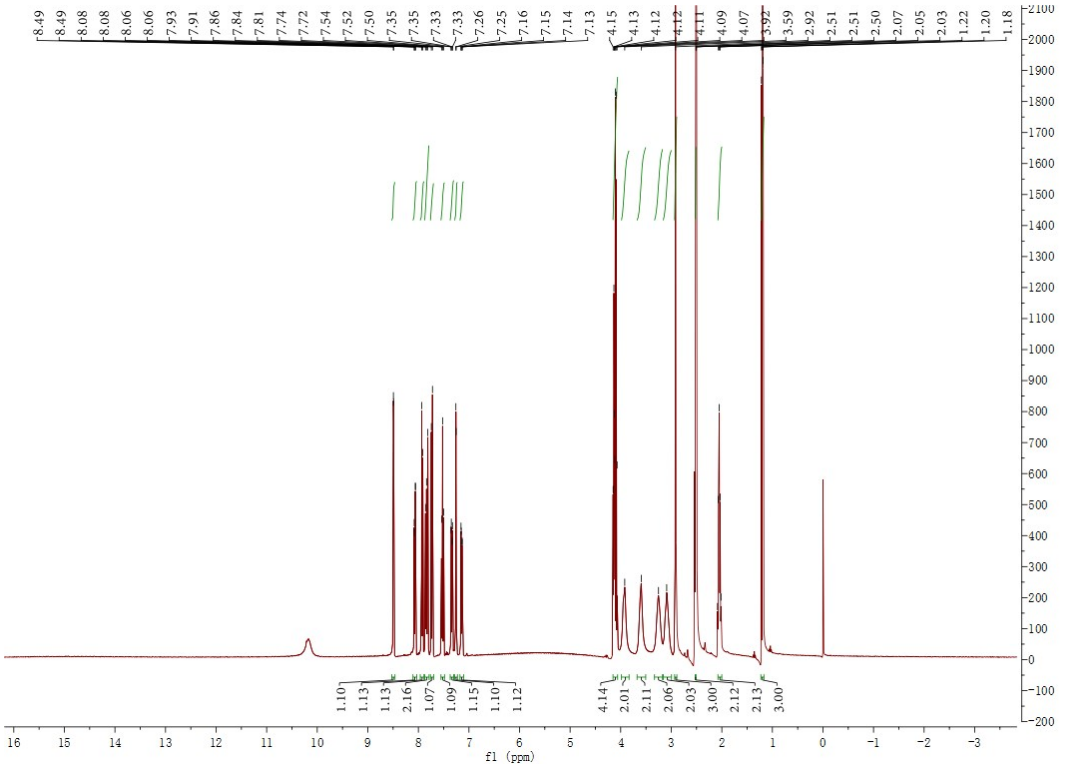
^{1}H NMR of TP-6



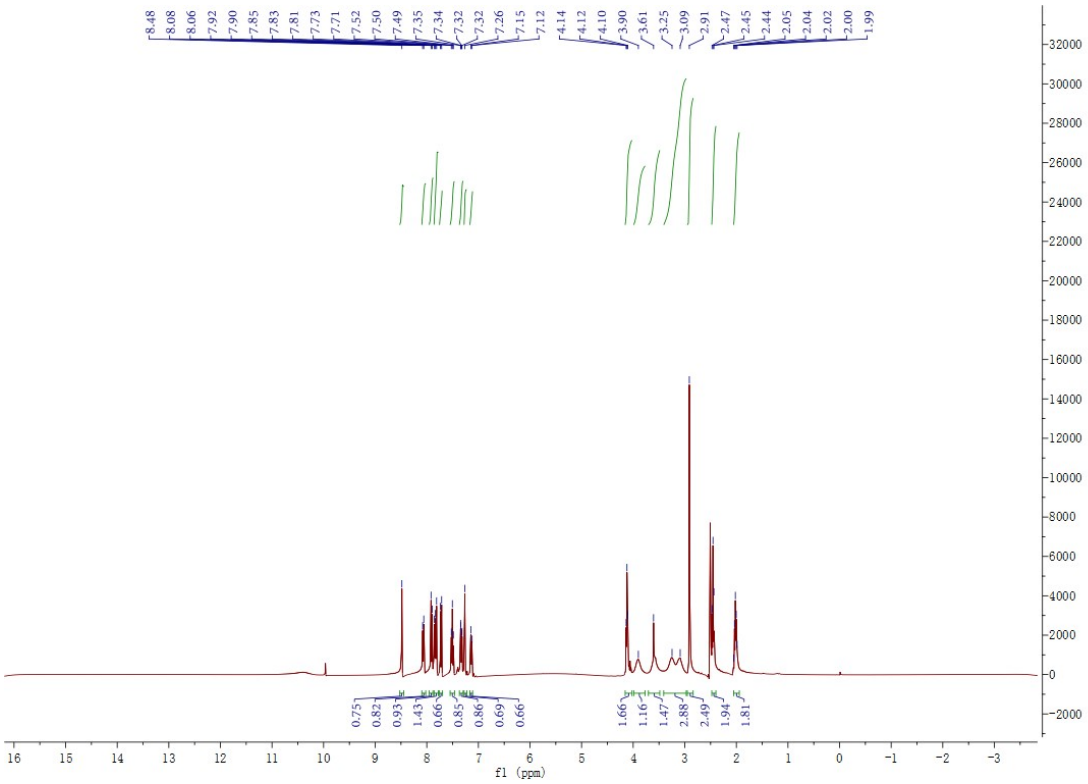
^{13}C NMR of TP-6



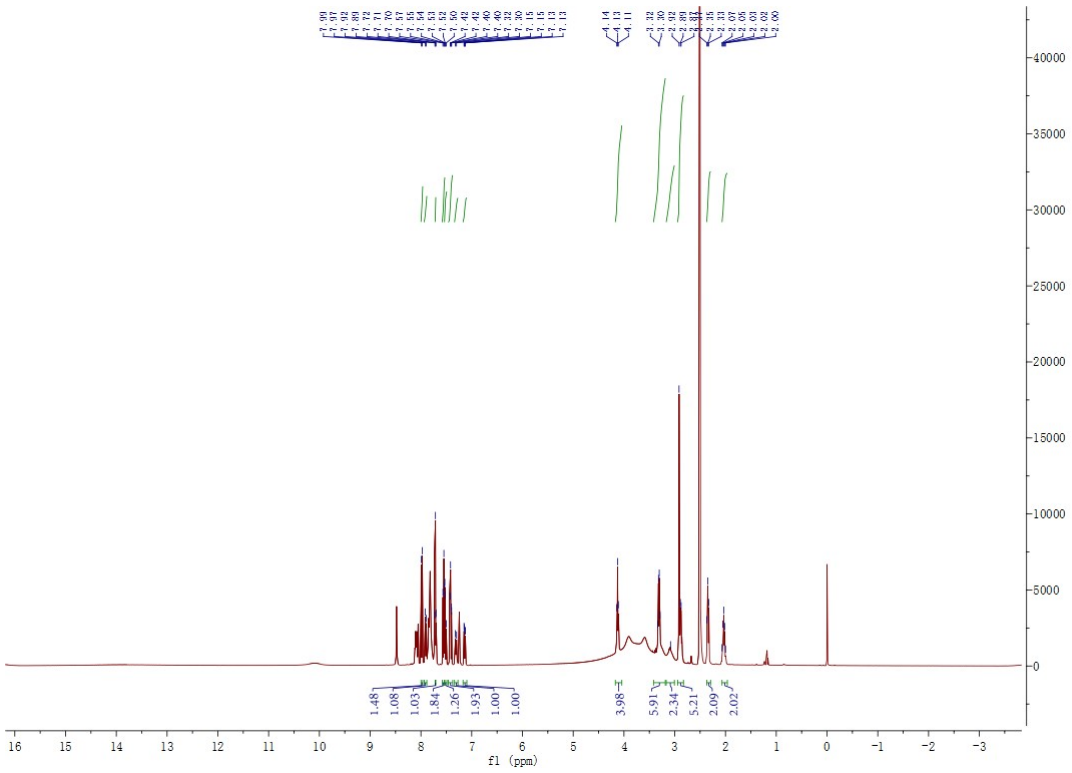
^{1}H NMR of TP-7



^{1}H NMR of TGP-210

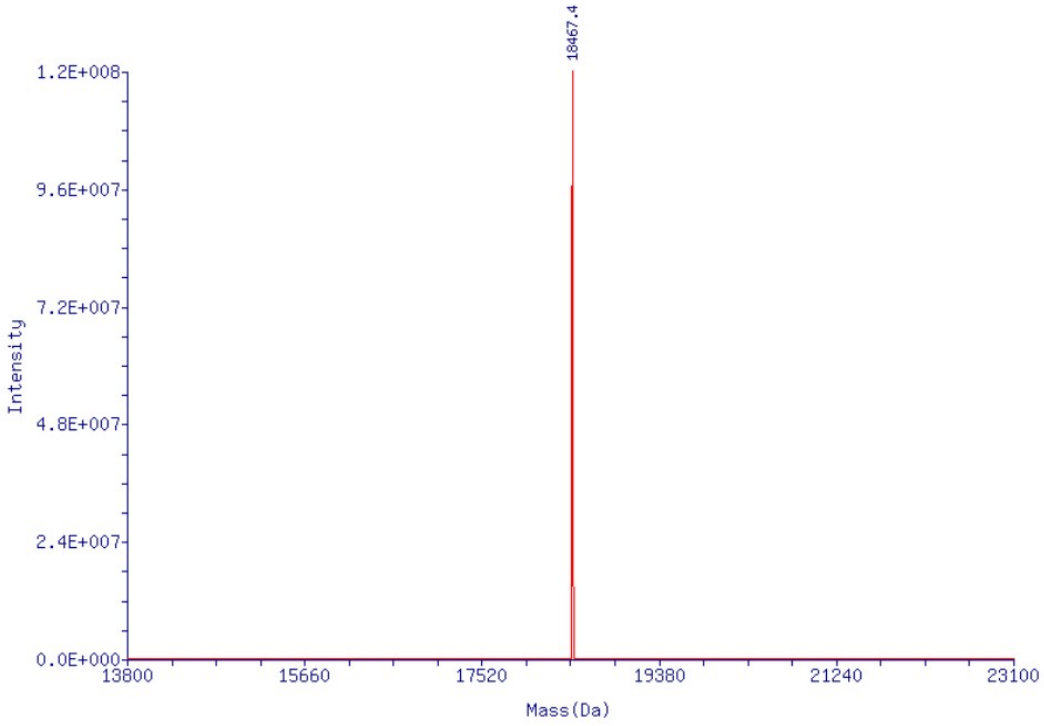


^{1}H NMR of TGP-210-NH_2



MS Spectra

Pre-miR-210



Reference

1. D. D. Young, C. M. Connelly, C. Grohmann and A. Deiters, *J Am Chem Soc*, 2010, **132**, 7976-7981.