Electronic Supplementary Information (ESI)

A Hairpin DNAs-Fueled Nanoflare for Simultaneous Illuminating Two MicroRNAs in Drug-Induced Nephrotoxic Cells with Target Catalytic Recycling Amplification

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Experimental Section

Chemicals and Materials. All the oligonucleotides used in this study were custom-synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), trisodium citrate, sodium dodecyl sulfate (SDS), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), levodopa (L-DOPA), triptolide (TPL), doxorubicin (DOX), citrinin (CTN), 5-fluorouracil (5-FU), aristolochic acid Ⅰ (AAⅠ), tetracycline (TE), rifampicin (RIF), cisplatin (CDDP), 4-aminophenol (4-AP), ibuprofen (IBU) and cyclophosphamide (CTX) were purchased from Aladdin Industrial Corporation (Shanghai, China). SYBR Gold nucleic acid stain (10000 × in DMSO) was purchased from Invitrogen (Grand Island, NY, USA). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco (Gaithersburg, USA). The CellTiter Glow (CTG) luminescent cell viability assay kit was purchased from Promega (Madison, Wisconsin). PureZOL RNA Isolation Reagent was purchased from Bio-Rad (California, USA). The miRNA 1st Strand cDNA Synthesis Kit and miRNA Universal SYBR qPCR Master Mix were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). Ultrapure water was prepared using a Millipore Simplicity System (Millipore, Bedford, USA). All other reagents used in this study were of analytical grade and used directly without further purification.

Apparatus. Absorption spectra were measured on a UV-2550 UV-VIS spectrophotometer (Shimadzu Company, Japan). Fluorescence spectra, fluorescence intensity and CTG assay were recorded on Varioskan Flash (Thermo Scientific, USA). Transmission electron microscopy (TEM) measurements were performed using JEOL JEM-200CX TEM operated at 200 kV. The dynamic light scattering (DLS) measurement was performed using a Mastersizer 2000 particle size analyzer. Zeta potential measurement was displayed at 25 °C on a Malvern Zeta sizer-Nano Z instrument. The gels were scanned with a BIO-RAD Molecular Imager (USA). All the nucleic acids concentrations were measured with NanoDrop (Thermo Scientific, USA). qPCR procedure was performed by using

QuantStudio™ 3 Real-Time PCR Instrument (Thermo Scientific, USA). The isolation of extracellular vesicles (EVs) was operated by Beckman Preparative Ultracentrifuges (L-80XP) (USA).

DNA Structure. The potential secondary structure of DNA was designed by using Analysis on http://www.nupack.org/. The website indicated that the "stem and loop" conformation for DNA has formed.

Native Polyacrylamide Gel Electrophoresis (PAGE). Hairpin DNA pairs H_1 and F_1 were dissolved at 100 μM in Tris-acetate/EDTA/Mg^{2+} (TAE/Mg^{2+}) buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 12.5 mM magnesium acetate, pH 8.0). These hairpin DNA pairs were heated to 95 ºC for 5 min and then allowed to cool to 25 °C in 2 h before use. A mixture of target and hairpin DNAs at a final concentration of 0.5 μM was incubated at 37 °C for 2 hours. A 1 mm 12% polyacrylamide gel was prepared using 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gel was pre-run at 100 V for 30 min and then was run at 80 V for 90 min in 1 × TAE buffer, followed by staining with SYBR Gold for 25 min. The target and hairpin DNA products were visualized using BIO-RAD ChemiDoc XRS+ imaging system.

Preparation of Gold Nanoparticles (AuNPs). Before preparing AuNPs, all the glassware were soaked in aqua regia (HCl/HNO_3, 3: 1) overnight, then rinsed with water and oven-dried prior to use. AuNPs were prepared using the citrate reduction of $HAuCl_4$ method reported before.[1] The specific method was as follows: an aqueous solution of $HAuCl_4$ (1 mM, 100 mL) was brought to reflux while heating and stirring. After boiling, 10 mL 38.8 mM trisodium citrate solution was quickly added. The color of the solution changed from the initial pale yellow to light gray, then turned to purple-black, and finally became a stable burgundy. At this time, boiling for additional 10 min, then stopped heating and continued stirring for 15 min. After the solution was slowly cooled to room temperature, subsequently filtered through a 0.45 μm Millipore membrane filter, and stored at 4 ºC. The prepared gold nanoparticles were characterized by TEM for size and morphology.

Quantitation of Hairpin DNA per AuNP. To measure the average number of the hairpins on each AuNP, the 20 mM 2-mercaptoethanol (2-ME) was added into the nanoflare solution (1 nM) and placed

in the dark. After overnight incubation with stirring at room temperature, the hairpin H_1 and H_2 were released. The solution was centrifuged at 13500 g for 30 min to precipitate the AuNPs. The AuNPs precipitate was resuspended in Tris-HCl buffer for the following agarose gel electrophoresis analysis. The fluorescence intensity of released hairpins was measured by a fluorescence microplate reader. The fluorescence of FAM-labeled hairpin H_1 was excited at 492 nm and measured at 518 nm and the fluorescence of Cy5-labeled hairpin H_2 was excited at 648 nm and measured at 668 nm. The fluorescence was converted to molar concentration of hairpin by interpolation from a standard linear curve measured by known concentrations of FAM- or Cy5-labeled hairpin with the same pH buffer, ionic strength, and concentration of 2-ME. By dividing molar concentration of each hairpin by the original nanoflare concentration, the number of hairpins per AuNP was calculated.

Agarose Gel Electrophoresis. 20 μL naked AuNPs, nanoflare and AuNPs precipitate (2-ME-treated nanoflare centrifugated) were loaded into 1% agarose gel, respectively. The agarose gel electrophoresis was carried out in 1 × TAE buffer at 100 V for 5 min at room temperature. After electrophoresis, the agarose gel was photographed by a digital camera. Then the gel was stained with SYBR Gold in 1 × TAE buffer for 25 min, and visualized by using Bio-Rad ChemiDoc XRS+ imaging system.

Characterization of Nanoflare. TEM was performed with a JEOL JEM-200CX TEM at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 5 nM naked AuNPs and 5 nM nanoflare on copper grids, respectively, and dried for 3 min. Then the residual solution was blotted off using filter paper.

DLS and zeta potential measurements of naked AuNPs and nanoflare were performed at 25 °C on Mastersizer 2000 particle size analyzer and Malvern Zeta sizer-Nano Z instrument. The samples were dispersed in double distilled water and the pH value of the solution was adjusted to 7.2 before the analysis.

Fluorescence Kinetics. The miR-21 and miR-200c with a concentration of 250 nM were used in this procedure. The nanoflare (5 nM) was mixed with miR-21and miR-200c at 37 °C. The fluorescence

intensities were examined with increasing time (0, 5, 10, 15, 20, 30, 40, 60, 80, 100, 120, 140 minutes). Then the fluorescence was excited at 492 nm and measured at 518 nm for FAM and excited at 648 nm and measured at 668 nm for Cy5. All the experiments were repeated at least three times.

Sequence Specificity Experiment. The complementary, single-base mismatched, single-base inserted and single-base deleted DNA target with hairpin DNA H_1 and F_1 were added to 500 μL hybridization buffer, respectively. A mixture of target with H_1 and F_1 at a final concentration of 0.5 μM was incubated at 37 °C for 2 hours. Detailed experiments refer to the above operation procedure of PAGE.

Selectivity Analysis. To investigate the specificity for miRNAs detection, the complementary, single-base mismatched, single-base inserted, single-base deleted and non-complementary miRNAs were used to react with nanoflare, respectively. The combined solution was incubated at 37 ºC for 1 h, including 5 nM nanoflare and 250 nM miRNA target for each group. The complementary, single-base mismatched, single-base inserted, single-base deleted and non-complementary miRNA at a final concentration of 250 nM was added into 5 nM nanoflare, respectively. Then the mixed solution was incubated at 37 ºC for 1 h, and the fluorescence was measured at 492 nm excitation and 648 nm excitation, respectively. All the experiments were repeated at least three times.

Nuclease Stability Assay. Two groups of nanoflare (5 nM) were designed for nuclease stable assay. One group was mixed with 1 μL of 2 U/L DNase I and the other was set as control. Both of the two groups were placed in a 96-well fluorescence microplate. Then the two groups were incubated at 37 °C and the fluorescence were monitored for 12 consecutive hours, during which time the fluorescence intensities were recorded at intervals. Then miR-21 and miR-200c (250 nM) were added into the two groups of nanoflare with incubation for 1 h at 37 ºC, and the fluorescence intensities were measured when the solution was cooled to room temperature. All the experiments were repeated at least three times.

Cell Culture. Renal proximal tubule epithelial cells (RPTECs) were obtained from ATCC (Manassas, VA). According to the manufacturer's instructions, RPTECs were cultured in DMEM/F12 in a 35

mm^2 Petri dish, containing 10% fetal bovine serum and 100 U/ml 1% antibiotics penicillin/streptomycin. Human normal liver L-02 cells, human cervical carcinoma HeLa cells, human mammary cancer MCF-7 cells were obtained from KeyGEN Biotech Co., Ltd (Nanjing, China) and cultured in DMEM containing 10% fetal bovine serum and 100 U/ml 1% antibiotics penicillin/streptomycin. These cells were grown in a 100% humidified atmosphere containing 5% CO_2 at 37 °C.

Cell Viability Assay. CTG luminescent cell viability assays were carried out to evaluate the cytotoxicity of L-DOPA, TPL, DOX, CTN, 5-FU, AA Ⅰ, TE, RIF, CDDP, 4-AP, IBU and CTX to RPTECs, respectively. RPTECs were dispersed in 96-well microtiter plates at 1×10^4 cells per well in a total volume of 200 μL. The plates were maintained in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. After the original medium has been discarded, all the compounds with 0.1% DMSO at final concentrations of 1 ~ 1000 μM was diluted in fresh medium, then the medium was added to the RPTECs respectively, and incubated for 24 h. Subsequently, RPTECs were washed three times with PBS buffer and 100 μL CellTiter-Glo® reagent was added to each well, and mixed for 2 minutes on an orbital shaker to induce cell lysis, then incubated at room temperature for 10 minutes to stabilize the luminescent signal. The cell viability was measured and dose-response curves were generated by Origin 2018. The luminometric intensity (LI) was measured with a fluorescent microplate reader. The cell viability was then assessed as: Cell viability (%) = $100\times(LI_1\text{-}LI_2)/(LI_3\text{-}LI_2)$, where LI_1, LI_2, and LI_3 were LI value of drug treatment group, LI value of blank group, and LI value of control group, respectively. All the experiments were repeated at least three times.

EVs Isolation by Differential Centrifugation. EVs isolation from different cell (RPTECs, L-02 cells, HeLa cells and MCF-7 cells) culture media was based on differential centrifugation at 4 °C.[2] The collected supernatant (100 mL) was first centrifuged at 300 g for 10 min, 2000 g for 20 min and 11000 g for 50 min to remove intact cells, dead cells, cells debris, protein and so forth. Then the supernatant was ultracentrifuged at 110000 g for 4 h to obtain sediment EVs. The collected EVs were resuspended in PBS and stored in −80 °C for later use.

MiRNA Isolation. For simultaneous detection of miRNAs in media, RPTECs were seed in 6-well plates with a density of 1×10^7 cells per well and treated with 11.81 μM TPL, 16.57 μM DOX, 31.51 μM CTN, 33.31 μM 5-FU, 41.27 μM AAI, 45.46 μM TE, 62.06 μM RIF, 82.97 μM CDDP, 100.69 μM 4-AP, 164.52 μM IBU, 473.89 μM CTX and 500 μM L-DOPA, respectively. After 24 h treatment, the cell culture medium was removed and centrifuged twice (10 min 1600 g then 10 min 16000 g). The resulting supernatant was used for extracting total RNA (including miRNA). According to the manufacturer's protocol, all operations were performed in an RNase-free environment. For RNA extraction in supernatant, 500 μL of the resulting supernatant was mixed with 1 mL of PureZOL reagent and incubated for 5 min. After adding chloroform, tubes were centrifuged to separate the upper aqueous phase, which was carefully collected into a fresh tube. Then adding isopropanol to the aqueous phase for 5 min followed by centrifugation at 12000 g at 4 °C for 10 min. The RNA precipitate was washed with 75% ethanol (prepared in diethylpyrocarbonate (DEPC)-treated water) and centrifuged at 7500 g at 4 °C for 5 min. Then the ethanol was discarded and the purified RNA was resuspended in 50 μL DEPC-treated water. For intracellular RNA extraction, 1 mL of PureZOL reagent was added into each well of 6-well plates, and repeated the above extraction steps.

qRT-PCR Procedure for miRNA Analysis. Total intracellular RNA and supernatant RNA for reverse transcription (RT) reaction were extracted from L-02 cells, HeLa cells, MCF-7 cells, normal RPTECs, and drug-treated RPTECs, respectively. The cDNA samples were prepared by using miRNA 1st Strand cDNA Synthesis Kit. qPCR analysis was performed with miRNA Universal SYBR qPCR Master Mix. The relative expression of miR-21 and miR-200c were calculated using the $2^{-\Delta\Delta Ct}$ method. All the primers used in this procedure were described in Table S1, and all the qRT-PCR reactions were performed in triplicate.

Statistical Analysis. Data were presented as means ± SD. Student's *t* test was applied to compare the treatment effects with that of a control experiment. Statistical analysis was calculated by statistics software (Origin 2018 Software). A *P* value less than 0.05 ($P < 0.05$) was considered statistically significant.

Supplementary Tables

Table S1. Detailed DNA and RNA sequences employed in this research.

Name	Sequence (5' to 3')
miRNA-21	UAG CUU AUC AGA CUG AUG UUG A
miRNA-200c	UAA UAC UGC CGG GUA AUG AUG GA
single-base mismatched miRNA-21	UAG CUU AUC AUA CUG AUG UUG A
single-base inserted miRNA-21	UAG CUU AUC AGUA CUG AUG UUG A
single-base deleted miRNA-21	UAG CUU AUC A_A CUG AUG UUG A
single-base mismatched miRNA-200c	UAA UAC UGC CGU GUA AUG AUG GA
single-base inserted miRNA-200c	UAA UAC UGC CGGU GUA AUG AUG GA
single-base deleted miRNA-200c	UAA UAC UGC CG_ GUA AUG AUG GA
miRNA-16	UAG CAG CAC GUA AAU AUU GGC G
miRNA-155	UUA AUG CUA AUC GUG AUA GGG GU
U6_forward primer	CTC GCT TCG GCA GCA CA
U6_reverse primer	AAC GCT TCA CGA ATT TGC GT
miR-21_forward primer	GCG CGT AGC TTA TCA GAC TGA
miR-21_reverse primer	AGT GCA GGG TCC GAG GTA TT
miR-21_reverse transcription primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAA CA
miR-200c_forward primer	CGC GTA ATA CTG CCG GGT AAT
miR-200c_reverse primer	AGT GCA GGG TCC GAG GTA TT
miR-200c_reverse transcription primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CCA TC
single-base mismatched	TAG CTT ATC ATA CTG ATG TTG A

$C_{miR\text{-}21}$DNA	
single-base inserted $C_{miR\text{-}21}$DNA	TAG CTT ATC AGTA CTG ATG TTG A
single-base deleted $C_{miR\text{-}21}$DNA	TAG CTT ATC A_A CTG ATG TTG A
$C_{miR\text{-}21}$DNA	TAG CTT ATC AGA CTG ATG TTG A
hairpin H_1-FAM (1-2-3-4-3*-2*)	HS-TTTTTTTTTT_TCAACAT_CAGTCTG_ATAAGCTA_CCATGTGTAGA_TAGCTTAT_CAGACTG-FAM
hairpin F_1 (3-4*-3*-2*-4)	ATAAGCTA_TCTACACATGG_TAGCTTAT_CAGACTG_CCATGTGTAGA
hairpin F_1-TMR	ATAAGCTA_TCTACACATGG_TAGCTTAT_CAGACTG_CCATGTGTAGA-TMR
hairpin H_2-Cy5 (5-6-7-8-7*-6*)	HS-TTTTTTTTTT_TCCATCA_TTACCCGG_CAGTATTA_CCATGTGTAGA_TAATACTG_CCGGGTAA-Cy5
hairpin F_2 (7-8*-7*-6*-8)	CAGTATTA_TCTACACATGG_TAATACTG_CCGGGTAA_CCATGTGTAGA

Table S2. Analytical performance of reported methods and proposed method for miRNA detection.

Limit of detection	Linear range	Strategy	References
1.1 nM	None	Electrochemiluminescence	Shamsi *et al.*[3]
1 nM	None	Surface plasmon resonance platform	Loo *et al.*[4]
500 pM	0.5 nM–200 nM	Fluorometric assay by molecular beacon	Song *et al.*[5]
400 pM	1 nM–10 μM	Quartz crystal microbalance	Palaniappan *et al.*[6]
91 pM	0.5 nM–50 nM	Isothermal exponential amplification	Zhao *et al.*[7]
8.5 pM	0.01 nM–10 nM	Fluorescence polarization	He *et al.*[8]
18.1 pM	0.05 nM–30 nM	Target catalytic recycling amplification	This work

Supplementary Figures

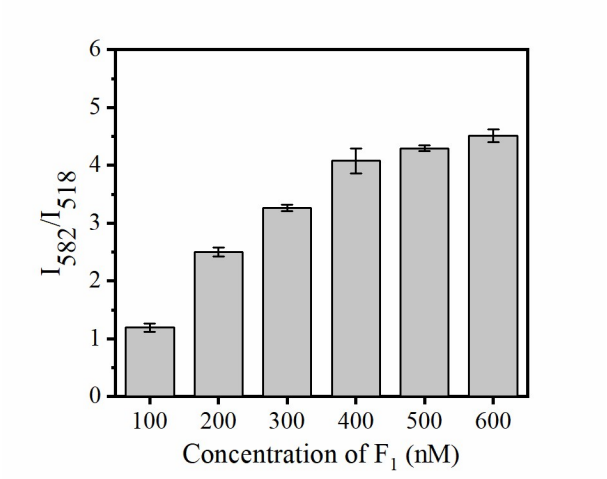

Fig. S1 Optimized concentration of hairpin DNA F_1 (100 ~ 600 nM) relative to constant H_1 concentration (100 nM). Data represent means ± SD (n = 3).

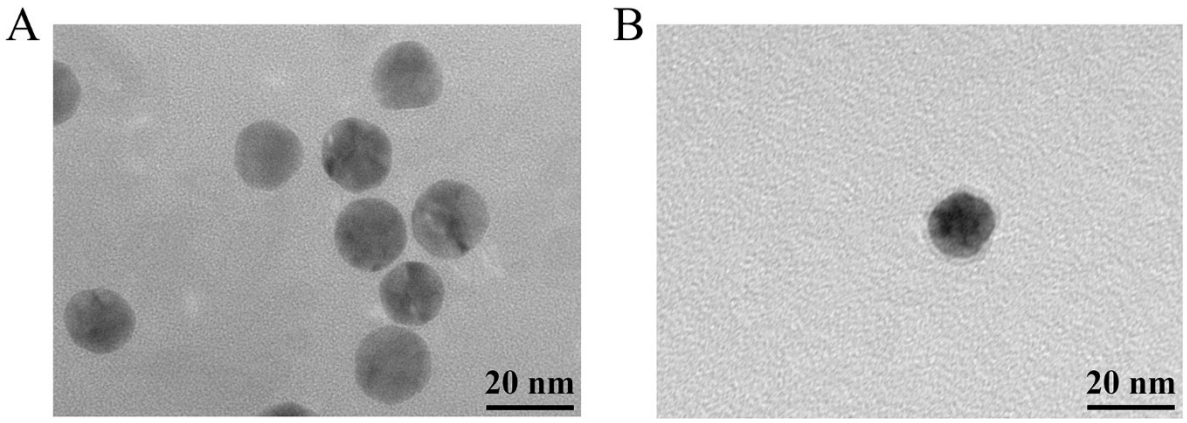

Fig. S2 TEM images of AuNPs (A) and nanoflare (B).

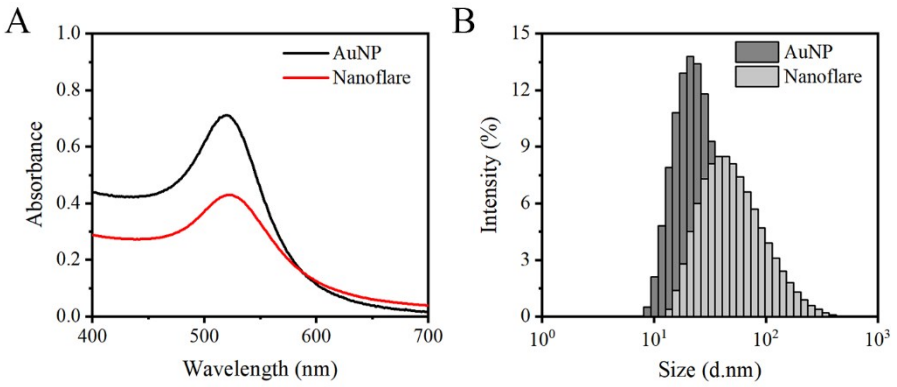

Fig. S3 UV-vis spectra (A) and DLS analysis (B) for AuNPs and nanoflare.

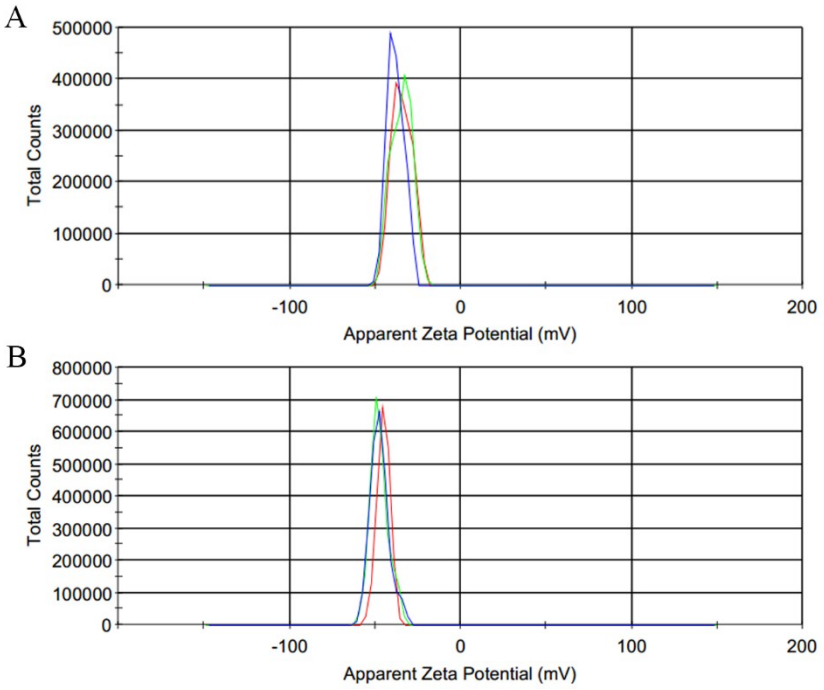

Fig. S4 Zeta potential of AuNPs (A) and nanoflare (B).

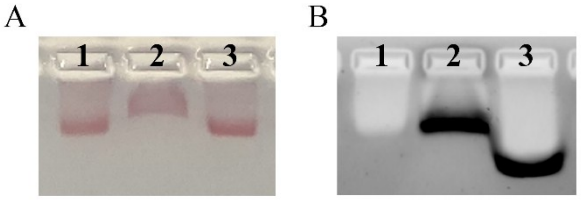

Fig. S5 Photos of agarose gel electrophoresis taken by digital camera (A) and BIO-RAD ChemiDoc XRS+ imaging system (B). Lane 1: naked AuNPs, Lane 2: nanoflare, Lane 3: 2-ME-treated nanoflare.

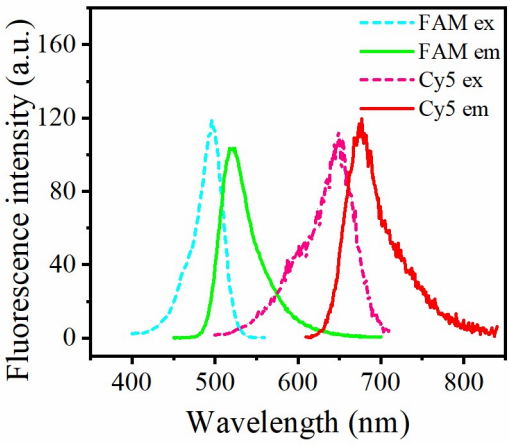

Fig. S6 Excitation spectra and emission spectra of FAM and Cy5.

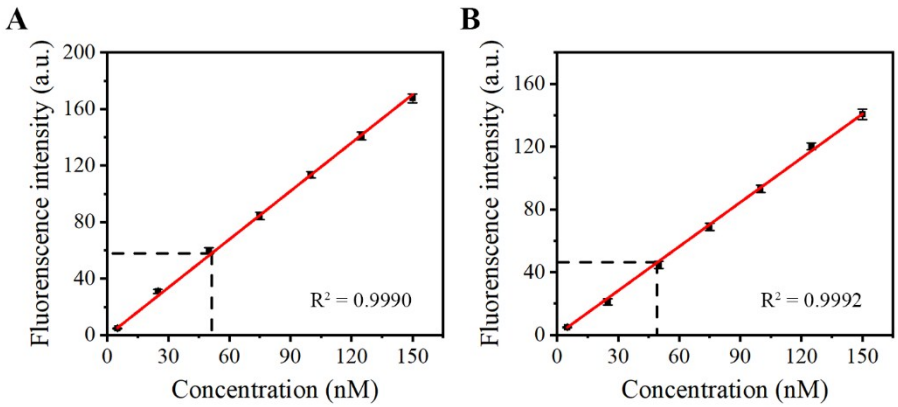

Fig. S7 Standard linear calibration curves of H_1-FAM (A) and H_2-Cy5 (B). Data represent means ± SD (n = 3).

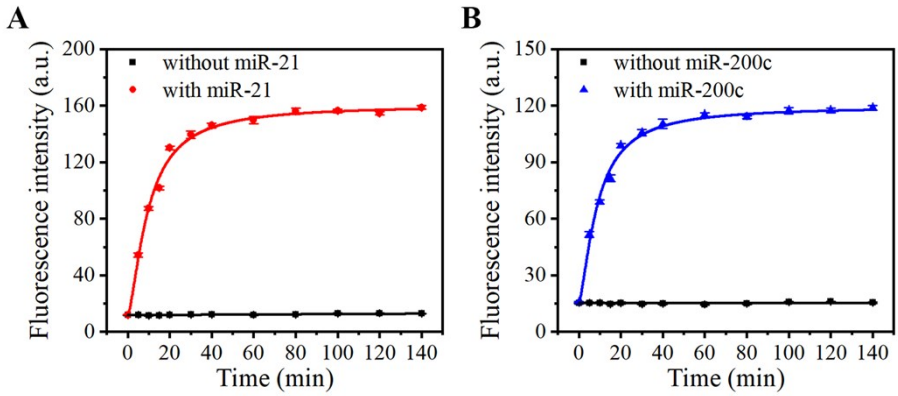

Fig. S8 Influence of the incubation time for fluorescence recovery at (A) 518 nm and (B) 668 nm. Data represent means ± SD (n = 3).

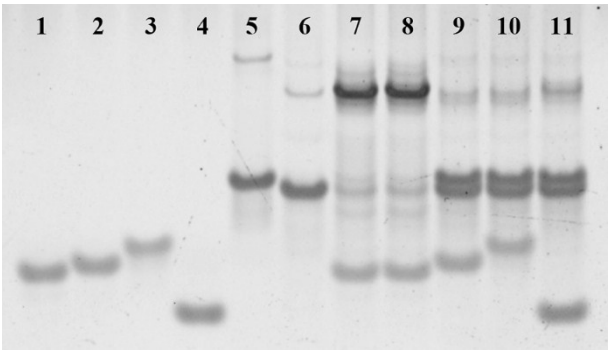

Fig. S9 Sequence specificity test. Lane 1: perfectly-matched $C_{miR\text{-}21}$DNA; Lane 2: single-base mismatched $C_{miR\text{-}21}$DNA; Lane 3: single-base inserted $C_{miR\text{-}21}$DNA; Lane 4: single-base deleted $C_{miR\text{-}21}$DNA; Lane 5: H_1; Lane 6: F_1; Lane 7: annealing of perfectly matched $C_{miR\text{-}21}$DNA plus H_1 and F_1; Lane 8: perfectly matched $C_{miR\text{-}21}$DNA plus H_1 and F_1 incubated for 2 h at 37 ℃; Lane 9: single-base mismatched $C_{miR\text{-}21}$DNA plus H_1 and F_1 incubated for 2 h at 37 ℃; Lane 10: single-base inserted $C_{miR\text{-}21}$DNA plus H_1 and F_1 incubated for 2 h at 37 ℃; Lane 11: single-base deleted $C_{miR\text{-}21}$DNA plus H_1 and F_1 incubated for 2 h at 37 ℃.

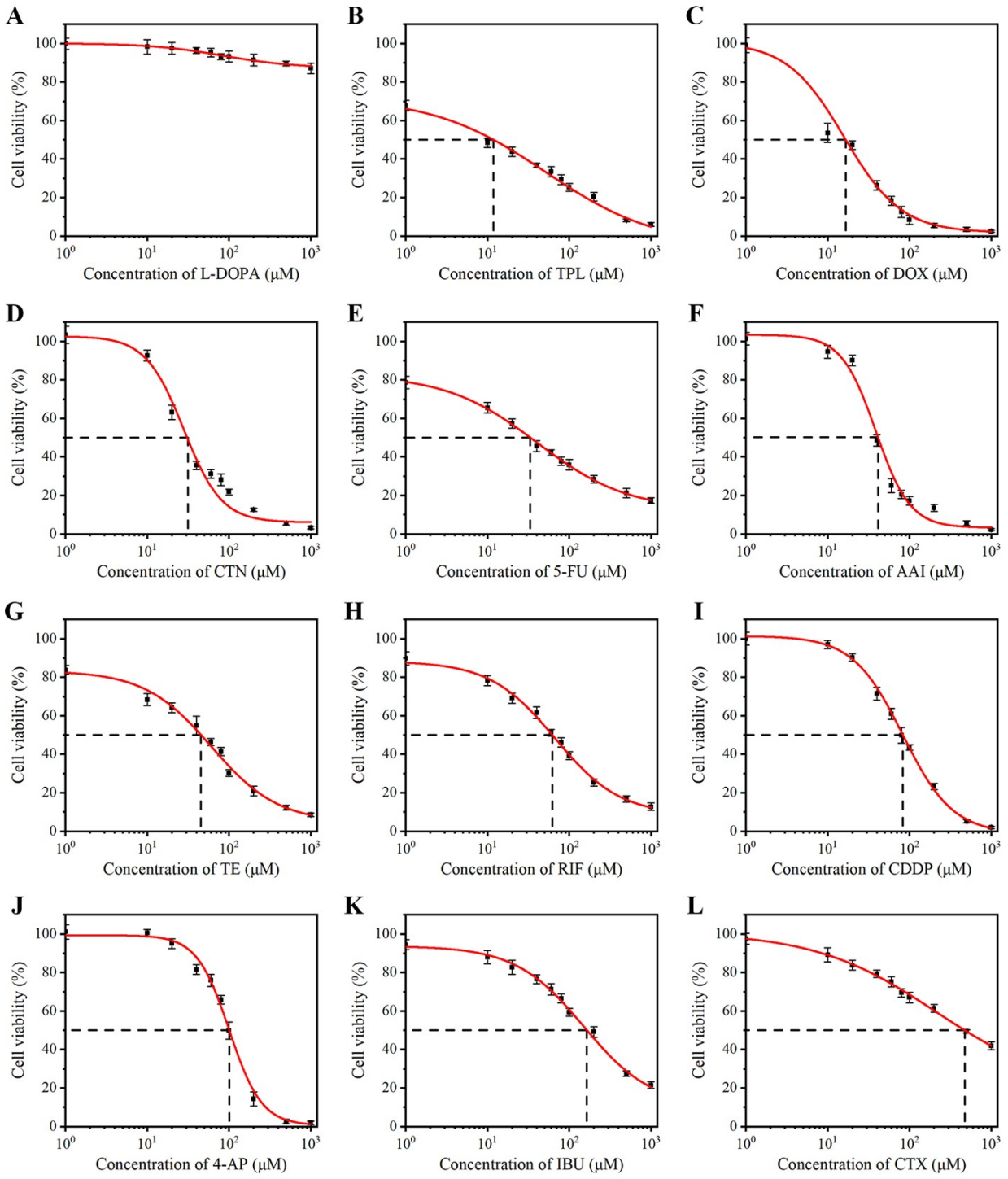

Fig. S10 CTG assay for the cell viability of RPTECs incubated with several drugs at different concentrations. Dose-response curves of RPTECs after 24 h treatment with 1 ~ 1000 μM (A) L-DOPA, (B) TPL, (C) DOX, (D) CTN, (E) 5-FU, (F) AAI, (G) TE, (H) RIF, (I) CDDP, (J) 4-AP, (K) IBU, (L) CTX, respectively. Data represent means ± SD (n = 5).

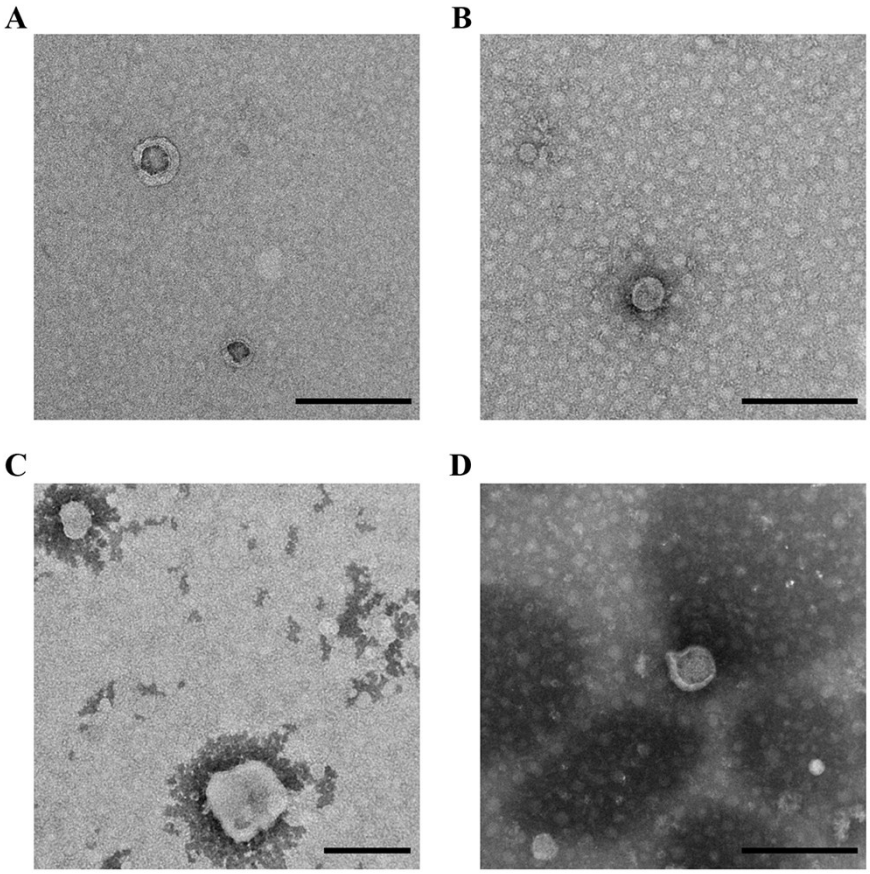

Fig. S11 TEM images of (A) HeLa EVs, (B) L-02 EVs, (C) MCF-7 EVs and (D) RPTECs EVs stained with 2% sodium phosphotungstate. Scale bar: 200 nm.

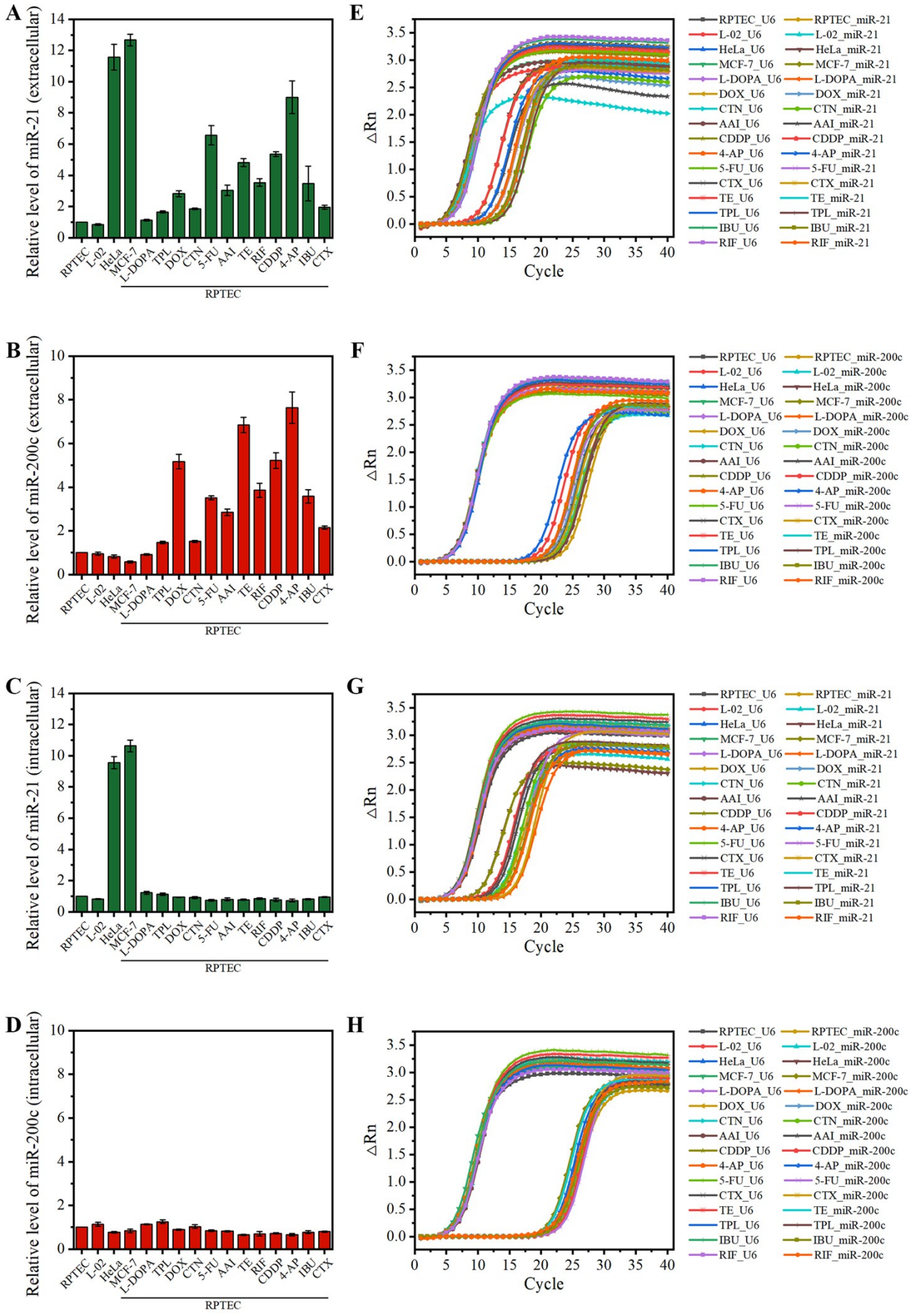

Fig. S12 qRT-PCR analysis of relative expression levels of miR-21 and miR-200c in (A, B)

extracellular incubation media and (C, D) intracellular media, respectively. Fluorescence amplification curves E, F, G and H corresponding to the qRT-PCR results A, B, C and D, respectively.

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