Supporting Information:

"Watson-Crick G≡C"-inspired supramolecular nanodrug of methotrexate and 5-fluorouracil for tumor microenvironment-activatable self-recognizing synergistic chemotherapy

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Materials and methods

Methoxy (polyethylene glycol)-2000 ($mPEG_{2000}$-OH), 4-carboxybenzaldehyde (*p*-CBA), octadecylamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), 4-dimethylaminopyridine (DMAP), and methotrexate (99%) was obtained from Sigma-Aldrich Co., Ltd (America). 5-Fluorouracil (99%) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (China). 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258,

and Annexin V-FITC/PI were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) and dichloromethane (CH_2Cl_2) were purchased from Xiamen Lvyin Reagent Glass Instrument Co., Ltd. (China). Dulbecco's modified Eagle media (DMEM), trypsin/ethylenediamine tetra-acetic acid (EDTA) and fetal bovine serum (FBS) were obtained from Hyclone (USA). Cyanine5.5 (Cy5.5, 95%) was obtained from Molecular Probes Inc. (Eugene, OR, USA).

Characterization

The ^1H nuclear magnetic resonance (NMR) spectrum was recorded on NMR Spectrometer (Avance II 400M, Bruker, Switzerland) with deuterium oxide (D_2O): dimethylsulfoxide-d_6 (DMSO-d_6) = 1:9 (v/v). The Fourier transform infrared (FT-IR) spectrum was detected on Bruker IFS-55 infrared spectrometer (Bruker, Switzerland). The ultraviolet-visible (UV-vis) spectrum was tested through Shimadzu UV-2550 (China). Dynamic light scattering (DLS) was measured with a Malvern Zeta-sizer Nano-ZS (Malvern Instruments, UK). The test of transmission electron microscope (TEM) and scanning electron microscope (SEM) were carried out on a JEOL-1400 (Japan) operated at 100 kV and SU-70 (Japan) operated at 10 kV, respectively. The cellular uptake was performed by using Leica TCS SP5 confocal laser scanning microscopy (CLSM, Leica Microsystems, Germany) and Flow cytometry (ATTUNE NXT, Thermo, USA).

Experimental section

Preparation of MTX-5-FU nanoparticles.

The MTX-5-FU nanoparticles were prepared by anti-solvent method. Briefly, 0.5 mL of MTX (14 mg/mL) and 0.5 mL of 5-FU (4 mg/mL) solution in DMSO were prepared respectively. Then, the two solutions (1 mL) were mixed and sonicated at room temperature for 30 minutes (Sheng Yan SCQ 3201, 40 kHz, power Output 200 W). Subsequently, 1 mL of the ultrasonically mixed solution was added dropwisely

into a 9 mL of deionized water and stirred slightly for 45 minutes. 10 mL of mixed solution was dialyzed against deionized (DI) water for 24 h (MWCO = 1.0 kDa), and the water was renewed every 4 h. Finally, a self-assembled MTX-5-FU nanoparticles dispersion was obtained by lyophilization.

Synthesis of $mPEG_{2000}$-benzoic-imine-octadecyl

$mPEG_{2000}$-CHO was synthesized from $mPEG_{2000}$-OH and *p*-CBA by esterification reaction under EDC/DMAP conditions. Subsequently, the synthesized $mPEG_{2000}$-CHO was coupled with octadecylamine to obtain $mPEG_{2000}$-benzoic-imine-octadecyl**.** In brief, a mixture $mPEG_{2000}$-OH (0.4 g, 1 equivalent) and *p*-CBA (0.06 g, 2 equivalent) in CH_2Cl_2 (10 mL) was stirred at room temperature for 24 h under the catalysis of EDC (0.0767 g, 2 equivalent) and NHS (0.0244 g, 5 equivalent). After completion of the reaction, the resulting solution was washed with saturated $NaHCO_3$ aqueous solution (100 mL × 3) to get crude product. Next, the organic phase was extracted, separated, and dried over anhydrous sodium sulfate. Then, CH_2Cl_2 was removed under reduced pressure. Finally, the acquired white solid was dispersed in DI water for dialyzing against DI water followed by lyophilization.

Subsequently, 0.543 g of octadecylamine (1 equivalent) and 0.146 g of $mPEG_{2000}$-CHO (2 equivalent) were mixed in 10 mL of CH_2Cl_2, and then stirred at room temperature for 24 h. After completion of the reaction, CH_2Cl_2 was dried under reduced pressure. Next, the white product was dispersed in DI water for dialyzing against DI water followed by lyophilization.

Preparation of PEG-MTX-5-FU nanoparticles.

The trace amounts of $mPEG_{2000}$-benzoic-imine (w/w= 1/20) was added into 1 mg/mL of MTX-5-FU nanoparticles dispersion. The dispersion was then sonicated for 30 minutes, and stirred slightly at ambient temperature for 2 h. Subsequently, the collected dispersion was continuously extruded through polycarbonate membranes with 0.8 μm, 0.45 μm, and 0.2 μm of pore size. Finally, a self-assembled PEG-MTX-

5-FU nanoparticles dispersion was obtained, lyophilized, and stored at 4°C for future experiment.

Calculation of drug-loading content

The UV-vis absorbance of MTX or 5-FU (DMSO as solvent) was measured to establish the concentration-absorbance standard curve of MTX and 5-FU at 301 nm and 267 nm, respectively. And the UV-vis absorbance of 1 mg/mL of MTX-5-FU nanoparticles or PEG-MTX-5-FU nanoparticles was evaluated. Then, the concentration of MTX and 5-FU was determined according to the standard curve. The drug-loading content (DLC) was calculated with the equation below:

$$DLC(\%) = \frac{V \times C}{M_{nanoparticles}} \times 100\%$$

V: Volume of Solution (mL); *C:* Concentration of MTX or 5-FU in solution (mg/mL); $M_{nanoparticles}$: Total mass of nanoparticle (mg).

Measurement of critical micelle concentration (CMC) of MTX-5-FU nanoparticles.

Pyrene was used as a CMC measurment fluorescent probe for MTX-5-FU nanoparticles. A series of solutions with different concentration (1.2 μg/mL to 120 μg/mL) were prepared in advance. Next, 20 μL of an acetone solution of pyrene (0.22 g/L) was dropped to the tubes and the acetone was evaporated. Then, the previously prepared dispersion of MTX-5-FU nanoparticles were added to the tubes and sonicated for 30 minutes in an ice bath, maintained at 60°C for 30 minutes. Next, the temperature of dispersion dropped to 40°C and kept overnight. All samples were tested by using a fluorescence spectrometer (a FluoroMax-4 Spectrofluorometer, HORIBA Jobin Yvon, USA) at an excitation wavelength of 335 nm. The values of the emission wavelength at 373 nm and 384 nm were recorded.

Evaluation of size and morphology of nanoparticles.

The morphology of MTX-5-FU and PEG-MTX-5-FU nanoparticles was

visualized by TEM (JEOL-1400, Japan) operating at an acceleration voltage of 100 kV and SEM (SU-70, Japan) operating at an acceleration voltage of 10 kV. Moreover, the size distribution and surface potential change were obtianed by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) on a Malvern Zeta-sizer Nano-ZS (Malvern Instruments, UK). Besides, the time-dependent storage stability of nanoparticles in water, DMEM, and RPMI 1640 were evaluated by monitoring the changes in hydrodynamic diameters within certain period of time.

***In vitro* drug release study**

The release amounts of MTX and 5-FU from MTX, 5-FU, MTX-5-FU nanoparticles, and PEG-MTX-5-FU nanoparticles were determined by dialysis technique. In brief, A total 3 mL of drug formulation with the same concentration of drug (MTX or 5-FU) was transferred into a dialysis bags (MWCO 3.5 KDa) which was immersed into 17 mL of external phosphate buffer solution (pH 7.4, 6.5 or 5.0), then dialysis bags was gently shaken at 100 rpm/min (37°C). At predetermined time points, 3 mL of released medium was replaced with an equal volume of fresh phosphate buffer solution. The content of MTX or 5-FU in the released medium was determined by UV-vis absorbance measurement with reference to the standard curve of MTX or 5-FU at 301 nm and 267 nm, respectively.

Cell lines and mice.

HeLa cells (human cervical carcinoma cell line), A549 cells (human lung cancer cell line), 4T1 cells (murine Breast cancer cell line) and L02 cells (normal human hepatocytes cell line) were used in following experiments. Cells were grown in DMEM or RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin in an incubator (Thermo Scientific) at 37°C under a 5% CO_2 atmosphere. Male Kunming mice (20 ± 2 g) were purchased from Xiamen University Laboratory Animal Center and all procedures followed the guidelines outlined in Institutional Animal Care and Committee approved procedure at Xiamen University. For the tumor model, the

Kunming mice were injected subcutaneously in the right flank region with 200 μL of cell suspension containing 4×10^6 4T1 cells

***In vitro* cellular uptake study.**

Cellular uptake was investigated in HeLa, A549, and L02 cells by CLSM and flow cytometry respectively.

NBD-labeled MTX, 5-FU, MTX-5-FU nanoparticles, PEG-MTX-5-FU nanoparticles were prepared by mixing NBD-Cl and MTX, 5-FU respectively in DMSO, with the catalysis of $CaCO_3$ and stirred at room temperature. The resulting solution was dialyzed against DI water followed by lyophilization. Finally, MTX^{NBD} and $5\text{-}FU^{NBD}$ were used to synthesize MTX^{NBD}-5-FU nanoparticles and PEG-MTX^{NBD}-5-FU nanoparticles.

CLSM: HeLa, A549, and L02 cells were seeded on 6-well plates (3×10^5 cells per well) for 24 h at 37°C. Then, medium was removed and replaced with fresh serum-free culture medium containing NBD-labeled MTX, 5-FU, MTX-5-FU nanoparticles (with FA pretreatment for 30 min or not), and PEG-MTX-5-FU nanoparticles after incubation of 1 or 4 h. Subsequent, the HeLa cells were rinsed three times with cold PBS and fixed with 4% paraformaldehyde. Thereafter, the cells were washed with PBS and the nucleus was stained with Hoechst 33258. The fluorescence of cells was visualized with a Leica TCS SP5 confocal laser scanning microscope (CLSM, Leica Microsystems, and Mannheim, Germany).

Flow cytometry analysis: HeLa cells were seeded on 6-well plates (3×10^5 cells per well) and incubated for 24 h at 37°C. Then, the NBD-labeled MTX, 5-FU, MTX/5-FU, MTX-5-FU nanoparticles (with FA pretreatment for 30 min or not), and PEG-MTX-5-FU nanoparticles dispersed in fresh serum-free culture medium were added into wells at the same MTX or 5-FU concentration and the cells were further cultured for 4 h. Afterwards, the cells was washed three times with PBS, digested with trypsin, resuspended, and then analyzed by a flow cytometry (Beckman Coulter ALTRA Epics, CA, USA).

***In vitro* cytotoxicity assay study.**

The cytotoxic effect of PEG-MTX-5-FU nanoparticles was investigated against HeLa, 4T1, and L02 cells by a MTT assay. HeLa cells were seeded on 96-well plates (1×10^4 cells per well) and incubated at 37°C for 24 h. The culture medium was replaced with a fresh medium containing serial concentrations of MTX, 5-FU, the MTX/5-FU, MTX-5-FU nanoparticles, or PEG-MTX-5-FU nanoparticles for 24 h. Thereafter, 10 μL of solution of MTT assay (5 mg/mL) was added to each well. After another 4 h of incubation, the old medium was removed carefully, and 150 μL of DMSO was added into each well to dissolve blue formazan crystals. The absorbance was measured on a microplate reader (Biotek, USA) at a wavelength of 490 nm.

CompuSyn software developed by Chou and Talalay was used to study the synergistic effects of cytotoxicity. The combination index (CI) represents the cytotoxic synergy of the drug. In general, CI > 1 antagonistic, CI= 1 additive, and CI < 1 synergistic.

Cellular apoptosis study.

Apoptosis inducing ability was characterized using Annexin V-FITC/PI apoptosis detection and analyzed by flow cytometry. HeLa cells were seeded on 6-well plates (1.0×10^6 cells per well) and incubated for 24 h. The cells were treated with MTX, 5-FU, the MTX/5-FU, MTX-5-FU nanoparticles, or PEG-MTX-5-FU nanoparticles at the same concentration (50 μM) for 24 h. HeLa cells without any treatment were set as a control. Subsequently, all cells were harvested, rinsed thrice with cold PBS, and stained with Alexa Fluor® 488 Annexin V and PI. Finally, the result was analyzed by a flow cytometry (Beckman Coulter ALTRA Epics, CA, USA).

Biodistribution analysis.

To evaluate the biodistribution of PEG-MTX-5-FU nanoparticles**,** 4T1 tumor-bearing mice were injected with the Cy5.5-labeled PEG-MTX-5-FU nanoparticles at

an equivalent Cy5.5 concentration via the tail vein. The free Cy5.5-injected mice were used as a control. At pre-scheduled post-injection time intervals, the real-time biodistribution and tumor accumulation ability of Cy5.5, Cy5.5-labeled MTX-5-FU nanoparticles and Cy5.5-labeled PEG-MTX-5-FU nanoparticles were recorded by *in vivo* imaging system (IVIS Spectrum 200, Perkin-Elmer Co., MA, USA). Mice were sacrificed after 48 h post-injection. Tumors and normal organs (liver, lung, spleen, kidneys, and heart) were collected, washing with saline. The resulting data were used to identify, separate, and remove the contribution of auto fluorescence in the analyzed images.

***In vivo* antitumor activity.**

In vivo antitumor activity was evaluated by using 4T1 tumor-bearing mice models. After the inoculated tumor volume measure up to 150 mm^3, the 4T1 tumor-bearing Kunming mice were randomly separated into 6 groups (n= 5). 200 μL of saline, free MTX, free 5-FU, MTX/5-FU, MTX-5-FU nanoparticles, and PEG-MTX-5-FU nanoparticles (containing 5 mg/kg of MTX, the mass ratio of MTX and 5-FU was 1: 1) were injected through the tail vein at 3-day interval. During treatment, the tumor size calculated by using the formula $V= 1/2*A*B^2$ (A, long diameter; B, short diameter). Body weight was recorded at a certain time interval. After 21 days, all of mice were sacrificed. Then the tumors and major organs (heart, liver, spleen, lung, and kidney) were harvested, rinsed with PBS, and fixed in 4% formaldehyde for histological examination. Finally, the samples was embedded in paraffin, stained with hematoxylin and eosin (H&E), and observed by an optical microscopy (DM5500B, Leica).

Statistical analysis.

Data are presented as means ± standard deviation of at least three experiments. The differences among groups are analyzed using one-way ANOVA Analysis followed by Tukey's post-test, in which P value < 0.05 (*) is considered significant,

P< 0.01 (*) is considered very significant, and P< 0.001 (***) is considered highly significant.

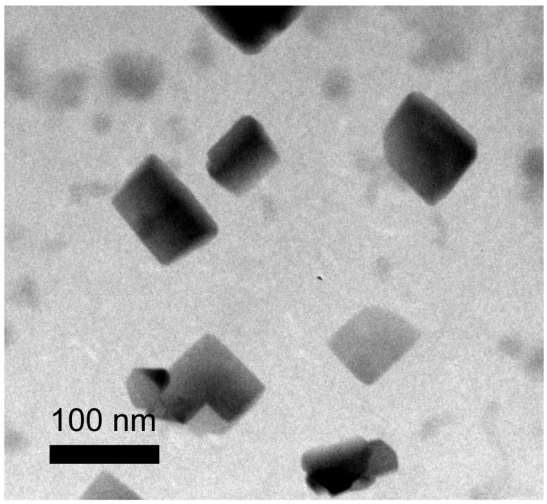

Fig. S1 TEM images of pristine MTX.

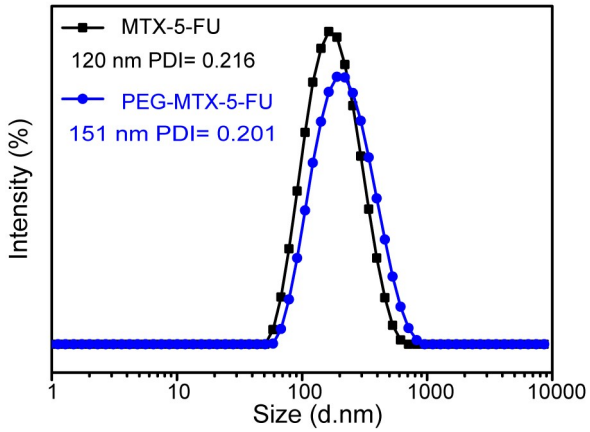

Fig. S2 D_h distribution of PEG-MTX-5-FU and PEG-MTX-5-FU nanoparticles ..

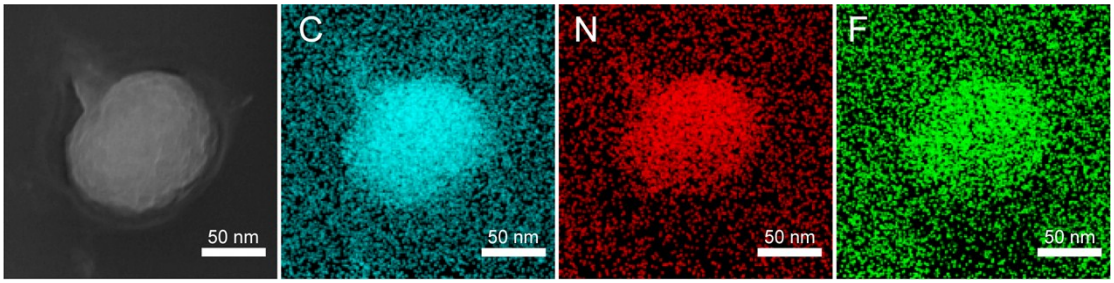

Fig. S3 SED-EDS mapping of PEG-MTX-5-FU nanoparticles.

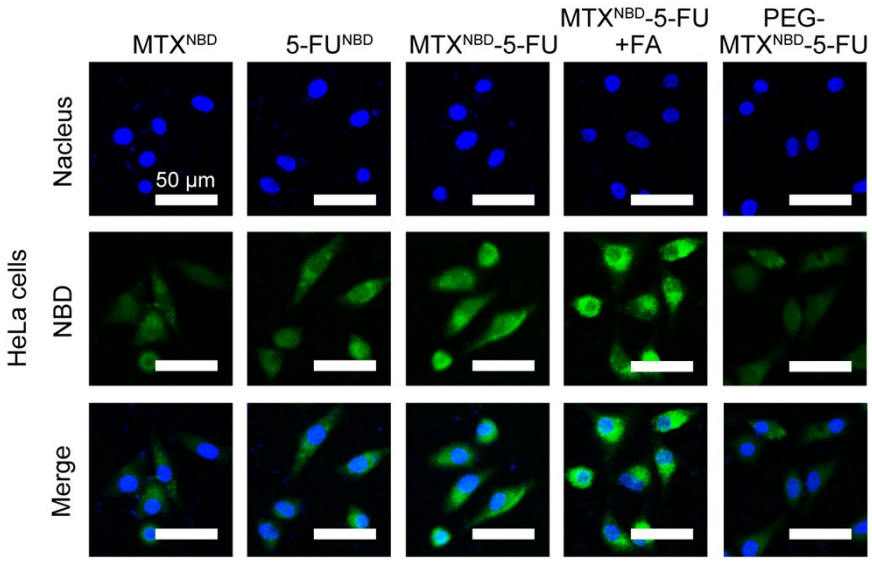

Fig. S4 CLSM images of HeLa cells incubated with MTX, 5-FU, MTX-5-FU, MTX-5-FU with pretreatment of free FA, and PEG-MTX-5-FU upon tumor acidic condition for 1 h.

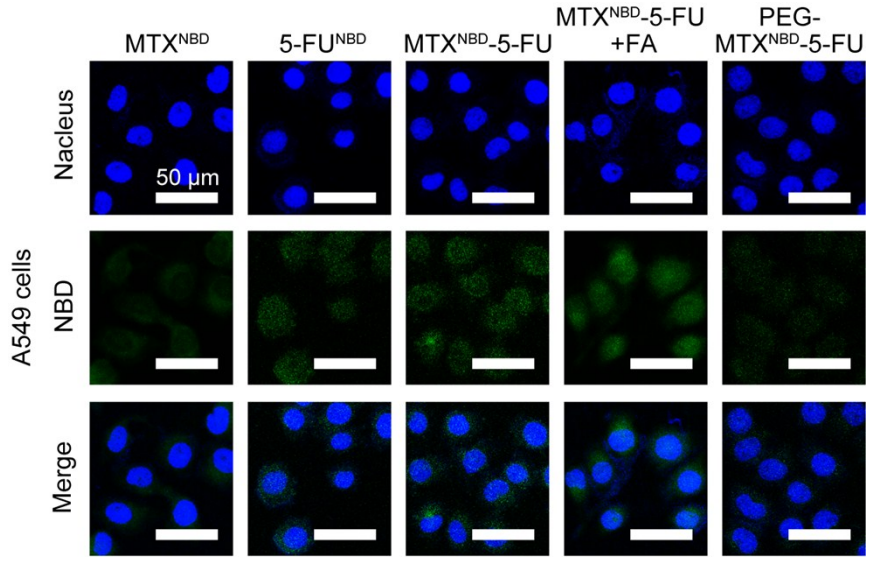

Fig. S5 CLSM images of A549 cells incubated with MTX, 5-FU, MTX-5-FU, MTX-5-FU with pretreatment of free FA, and PEG-MTX-5-FU upon tumor acidic condition for 1 h..

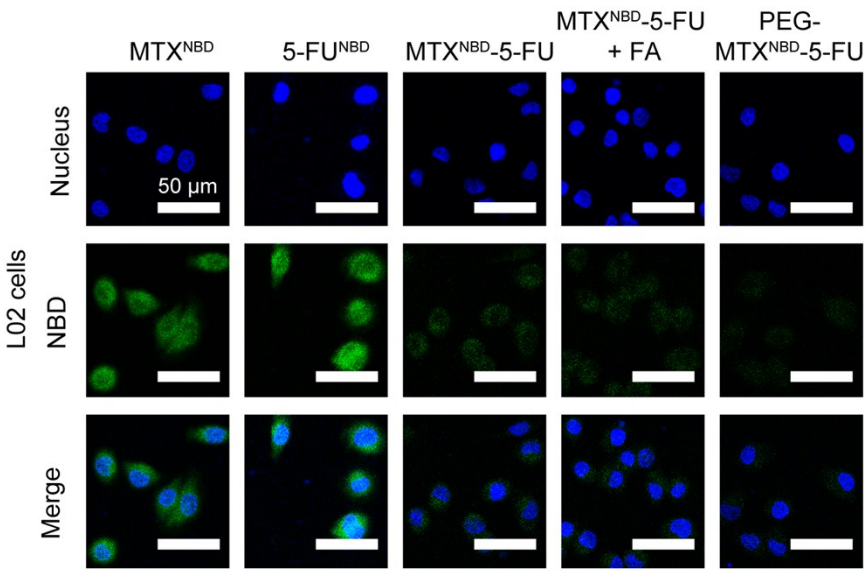

Fig. S6 CLSM images of L02 cells incubated with MTX, 5-FU, MTX-5-FU, MTX-5-FU with pretreatment of free FA, and PEG-MTX-5-FU upon tumor acidic condition for 1 h..