Supporting Information

Modifying mesoporous silica nano-particle to avoid the metabolic deactivation of 6-mercaptopurine and methotrexate in combinatorial chemotherapy

1. Synthesis and Characterization

1.1 Synthesis of MSNSN

1.0 g (2.74 mmol) of CTAB (cetyl-trimethylammonium bromide) was dissolved in 480 mL of deionized water under vigorous stirring, and then 3.5 mL of aqueous sodium hydroxide (2.0 M) was added. After the solution temperature sustained at 353K, to CTAB solution the mixture of 5.0 mL (22.5 mmol) of TEOS (tetraethylorthosilicate), 0.9 mL (4.76 mmol) of MPTMS (3-mercaptopropyltrimethoxysilane) and 0.1 mL (0.54 mmol) of APTMS (3-aminopropyltrimethoxysilane) was added dropwise. The reaction mixture was vigorously stirred for 2 h. The resulted colourless solids were centrifuged, washed with water and ethanol for several times and dried under vacuum for 24 h. To remove the surfactant template CTAB, the colourless solids were refluxed in a solution of 2 mL of HCl (37%) and 200 mL of methanol for 12 h, and then the surfactant-removed material was centrifuged, with ethanol washed for several times and dried under vacuum for 24 h to produce purified MSNSN as colourless solids.

1.2 Synthesis of MSNSN-MTX

To 95.7 mg (0.21 mmol) of methotrexate a solution of 285 mg (1.49 mmol) of EDC (N-ethyl-N'-(3-dimethylaminopropyl)carbodi-imide hydrochloride) in 10 mL of anhydrous DMSO was added. Into this solution 275 mg of MSNSN was added and the suspension was stirred at room temperature for 6 h. The resulted product MSNSN-MTX was centrifuged and successively washed with DMSO, water, and ethanol till no methotrexate could be detected (which was monitored at 303nm with absorption spectroscopy on a Shimadazu UV-2550 spectrophotometer), and then dried under vacuum.

1.3 Synthesis of 6-MP-MSNSN-MTX

To 15mL dimethylsulfoxide (DMSO) solution of mercaptopurine (176.3mM), iodine solution (104.9mM) was added dropwise to oxidize mercaptopurine. The oxidation was monitored with absorption spectroscopy until 1,2-di(purin-6-yl)disulfane is prominent in the solution. Then the powder MSNSN-MTX was suspended in the mixture, stirred vigorously at room temperature to undergo the disulfide bond exchange reaction. The resulting 6-MP-MSNSN-MTX was centrifuged, washed thoroughly with DMSO, water and ethanol till no mercaptopurine to be detected in the supernatant (which is monitored at 331nm with absorption spectroscopy on a Shimadazu UV-2550 spectrophotometer), and dried under vacuum for 24 h. With elemental analyses 6-MP and MTX grafted on the silica nanoparticles 6-MP-MSNSN-MTX were evaluated to 2.0×10^{-4} and 6.1×10^{-5} mol/g, respectively.

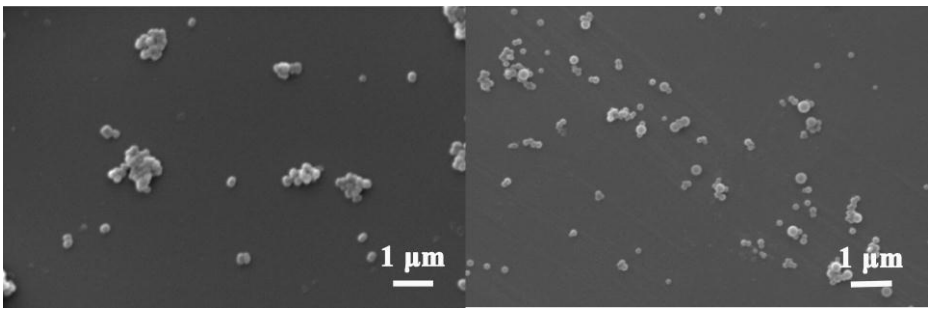

Fig. S1 The SEM images of 6-MP-MSNSN-MTX (left) and MSNSN(right)

1.4 The size and charge of nanoparticles

The size of MSNSN nanoparticles is 236.0±3.8 with PDI 0.282±0.009. The zeta-potential data of the nanoparticles MSNSN, MSNSN-MTX, and 6-MP-MSNSN-MTX are as listed in Table S1.

Table S1. Zeta-potential of the nanoparticles

MSNSN	MSNSN-MTX	6-MP-MSNSN-MTX
17.68±1.54	0.35±1.63	14.76±1.16

2. Release Measurement

The release assessment was performed in a physiological buffer solution (PBS, pH = 7.4, 37°C). 20.0 mg 6-MP-MSNSN-MTX was dispersed in 50.0 mL of PBS with DTT (dithiolthreitol, 1.58 mM). In order to alleviate the limitation of the delivering rate by external diffusion constraints, a constant rotation speed was maintained during the assays. The PBS buffer was collected and measured at desired intervals.

3. In vivo Measurement

The study described herein was performed according to a protocol reviewed and approved by the ethics committee of Capital Medical University. The committee assures that the welfare of the animals was maintained in accordance with the requirements of the animal welfare act and according to the guide for care and use of laboratory animals.

The metabolites of mercaptopurine and methotrexate in the blood were examined with anticoagulated plasma collected from SD rat. The anticoagulated plasma was cultured with mercaptopurine and methotrexate at 37°C for 15, 30, 60, 75, 90, 120, and 240 min, and then 100μl chromatographic methanol was added. After oscillated for 5min the plasma was centrifuged at 4°C for 15min, and then the supernatant was examined with mass spectrometer(Quattro microTM API MS, ESI$^+$V: 30). The anticoagulated plasma cultured with 1μl methanol was set as a blank control. The groups of mercaptopurine and methotrexate cultured with 100μl methanol instead of the anticoagulated plasma were set as positive control experimental.

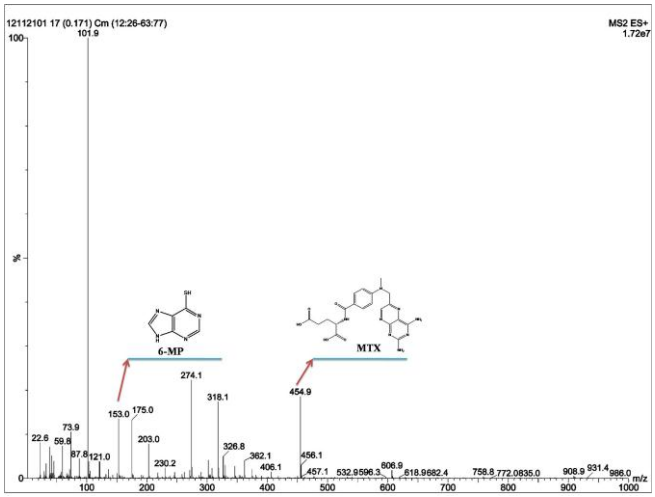

Fig. S2 The mass spectrum of mercaptopurine and methotrexate in methanol as positive control

Table S2. The summary of mass data of the metabolites of mercaptopurine and methotrexate

	6-MP	MTX
Positive Control	153 $[M+1]^+$	455 $[M+1]^+$
Plasma	--	-
15 min	191 $[M+Na]^+$	326 $[M+1]^+$
30 min	191 $[M+Na]^+$	326 $[M+1]^+$
45min	191 $[M+Na]^+$	326 $[M+1]^+$
60min	191 $[M+Na]^+$	326 $[M+1]^+$
75min	191 $[M+Na]^+$	326 $[M+1]^+$
90min	191 $[M+Na]^+$	326 $[M+1]^+$
120min	191 $[M+Na]^+$	326 $[M+1]^+$
240min	191 $[M+Na]^+$	326 $[M+1]^+$

4. In vitro Measurement

4.1. Cellular Viability Assay

The cellular viability of K562 cells after incubation with nano-particles was evaluated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazo-lium bromide) assay. Briefly, HeLa cells were cultured in 96-well plates (Corning, Inc., Corning, NY) at a density of 2×10^3 cells per well in growth medium DMEM (Dubelco Modified Eagle's Medium) containing 10% FBS (fetal bovine serum). After 24 h incubation, 30 μg/mL of 6-MP-MSNSN-MTX was loaded onto the 96-well plates. K562 cells cultured with mercaptopurine and methotrexate acted as positive controls. After 72 h incubations, the inhibitory rate of K562 cells was determined separately. To each well 10 μL of MTT was added and incubated for 4 h till purple crystals to be visible. After removal of the MTT solution, the purple formazan crystals were dissolved in 200 μL of DMSO and the absorbance was measured at 570 nm on a microplate reader (Thermo, USA). All assays were done with six parallel samples.

4.2. Fluorescence Microscopy Study

K562 cells (1.0×10^5 cells per well) were seeded into 6-well culture plates and incubated with mercaptopurine and methotrexate at 2 μg/mL and with 6-MP-MSNSN-MTX at 30 μg/mL for 24 h. The blank group was treated with drug-free culture media. After washing with PBS for 3 times, the cells were collected and stained with PI (propidium iodide) for 15 min at room temperature. The morphological changes were observed with a Leica TCS SP5 confocal fluorescence microscope system.

Fig. S3 Morphologies of K562 cells treated with MSNSN and 6-MP-MSNSN-MTX separately. The phase contrast pictures are shown for each treatment, with left in light field and right in dark field

4.3. Annexin V-FITC/PI Double Staining Assay

The transversion of phosphatidyl serine from the inner to outer plasma membrane leaflet, an initial event in the apoptotic pathway, was assessed by dual dye staining using Annexin V-FITC/PI kit. K562 cells with different treatments were incubated for 24 h. At the end of the treatment, the control (untreated) and treated cells were harvested and washed with cold PBS. Then 1×10^6 cells were collected with 1 mL of annexin binding buffer and incubated with 5 μL of Annexin V-FITC and 1 μL of PI (100 μg/mL) in the dark for 15 min. Finally, 400 μL of binding buffer was added into the samples before analysis on FAC-Scan flow cytometer (Becton Dickinson, USA).

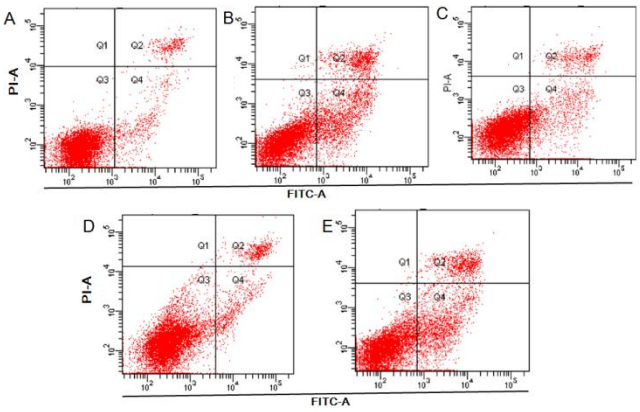

Fig. S4 Effect of MSNSN, 6-MP, MTX and 6-MP-MSNSN-MTX on the apoptosis of K562 cell. A) Untreated K562 cells (Blank); B) 6-MP treated K562 cells; C) MTX treated K562 cells; D) MSNSN treated K562 cells, and E) 6-MP-MSNSN-MTX treated K562 cells.

4.4. Flow cytometry measurement

Flow cytometry was performed on Becton-Dickinson FACS Aria cytometer. After 24 h incubation with fluorescein isothiocyanate FITC labled MSNSN (MSNSN-FITC), the intracellular fluorescent intensity of K562 cells was recorded and analyzed with BD-FACS Diva 4.1 software.