Supplementary materials

Cellular uptake and transport mechanism of 6-mercaptopurine nanomedicines for enhanced oral Bioavailability

Yaru Zou[1,2], Wei Gao[3], Huizhen Jin[2], Chenmei Mao[2], Yi Zhang[1], Xiaoling Wang[1], Dong Mei[1]*, Libo Zhao[1,4]*

[1] Department of Pharmacy, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing, 100045, China

[2] Department of Pharmacy, Children's Hospital of Soochow University, Suzhou, Jiangsu, 215025, China.

[3] Department of Pharmaceutical Sciences, College of Pharmacy, Rogel Cancer Center, University of Michigan, Ann Arbor, MI 48109, USA.

[4] Department of Pharmacy, Peking University Third Hospital, Beijing, 100191, China.

* Correspondence: Dong Mei; Libo Zhao; Email: meidong11290926@126.com; libozhao2011@163.com.

Methods

Cell cultrue

Caco-2 cell culture

Caco-2 cells were cultured with DMEM containing 25 mM glucose, 10% (v/v) FBS, 100 U/mL penicillin, 100 U/mL streptomycin and 1% (v/v) NEAA and maintained in5% CO2 at 37°C. The media were changed every 2 days. After the proliferation of the density of 80-90%, Caco-2 cells were trypsinized by 0.25% trypsion.

Caco-2 monolayer culture

To establish the cellular transport model *in vitro*, Caco-2 cells were seeded at a density of 4×10^5 cells per well onto a permeable polycarbonate membrane with pores of 3μm diameter and cultured for 21 days. The media were refreshed every other day during first two weeks and were replaced every day afterward. Transepithelial electrical resistance (TEER) was measured using an electrical resistance meter (Millicell ERS-2, Millipore) to confirm the integrity of the cell monolayers. Only when a TEER of value is between 500 and 1000 Ω/cm^2 can the monolayers be used during the studies.

Raji cell culture

Human Burkit's lymphoid Raji B cells were cultured in RPMI 1640 media supplemented with 1% dual antibody, 1% NEAA and 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin.

FAE cell monolayer culture

An *in vitro* model of FAE monolayers was prepared to study nanomedicines transport by the microfold

cells (M cells). The Caco-2 cell has the unique property to differentiate *in vitro* into polarized mature enterocytes and to form an impermeable monolayer. Caco-2 cells (1×10^6 cells/well) were grown on upper chambers of the transwell and the media were changed every other day. After incubation for 15 days, Raji B cells (1×10^6 cells/well) were then added to basolateral compartments and these co-cultures were maintained for 6 days. The Caco-2 cells were authenticated by their ability to differentiate spontaneously into a polarized epithelium and to transdifferentiate into M cells. Meanwhile, the transwell nested plate after 15 days was continuously monitored for transmembrane resistance to investigate the changes in TEER of FAE monolayers.

Effects of different endocytosis and transport inhibitors on Caco-2 Cell viability

Since the uptake of 6-MPNs by cells is closely related to the activity of Caco-2 cells, in order to exclude the effect of various concentrations of endocytosis and transport inhibitors on the activity of Caco-2 cells, this study carried out endocytosis and transport experiments. Cell viability was previously measured with different concentrations of inhibitors to ensure that the various inhibitors did not reduce the viability of Caco-2 cells during the co-culture time.

The effect of different inhibitors on the viability of Caco-2 cells was determined by CCK-8 assay. Caco-2 cells were seeded in 96-well plates at a density of 1×10^4 cells/mL, 100 μL per well, and used for cell viability assay 48 h after seeding. First, discard the culture medium in the 96-well plate, wash three times with PBS buffer, add 200 μL of prepared dispersions of different inhibitors to the corresponding well plate, and add serum-free medium containing 5‰ DMSO and no Serum-free medium with any inhibitor was used as a blank control group, and the 96-well plate was incubated at 37°C in a 5% CO_2 incubator for 4 h. After the incubation, discard the medium containing the inhibitor

in each well, add 100 μL of fresh serum-free medium to each well, and add 10 μL of CCK-8 solution to each well at 37°C. Incubate for 1 h in a 5% CO_2 incubator, and measure the OD value of each well with a microplate reader at 450 nm.

Tables

Table S1. Concentrations of the endocytosis and transmembrane inhibitors

Inhibitors	Concentrations	Function	Refs
MβCD	10 mM	Deplete cholesterol, inhibit lipid raft/caveolae mediated endocytosis	16
Dynasore	80 μg/mL	Intercepts formation of pinched off vesicles, inhibit lipid raft/caveolae and clathrin mediated endocytosis	17
CPZ	50 μM	Inhibitor of clathrin-mediated pathway	18
Genistein	100 μM	Inhibitor of protein tyrosine kinase	21
Cyt D	0.5 μM	Disrupt actin filaments	20
EIPA	40 μM	Inhibitor of macropinocytosis pathway	19
BFA	25 μg/mL	Blocks transport from ER to Golgi complex	22
Monensin	32.5 μg/mL	Blocks transport from Golgi complex to plasma membrane	23
Baf-A1	100 nM	Inhibitor of endosomal acidification	24
Nocodazole	10 μg/mL	Inhibits microtubules	25

Table S2. The ratio of alkaline phosphatase activity of AP side and BL side in Caco-2 monolayer at different culture time (mean ± SD, n = 3)

Time	ALP activity (AP/BL)
9 d	2.396 ± 0.092
15 d	3.529 ± 0.465
21 d	5.365 ± 0.241

Figures

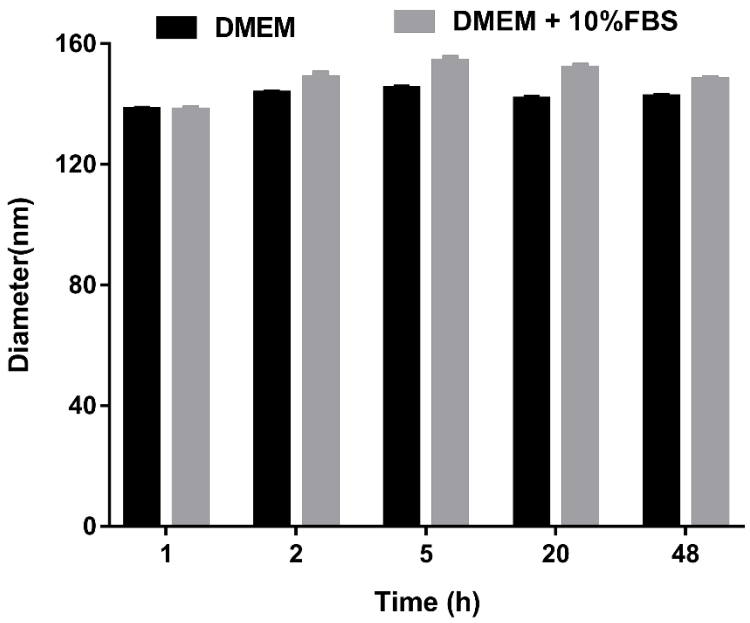

Figure S1 The dispersion stability of 6-MPNs in different media including DEME and DMEM with 10% FBS, monitored by particle size distribution over 48 h.

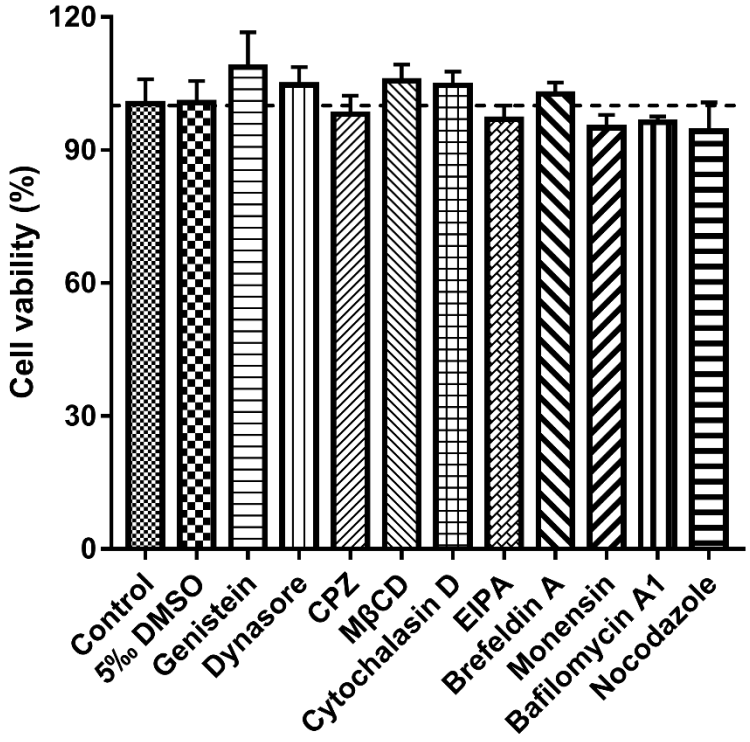

Figure S2. Cytotoxicity analysis of Caco-2 cells incubated with various inhibitors at their working

concentration for 4 h by CCK-8 method.

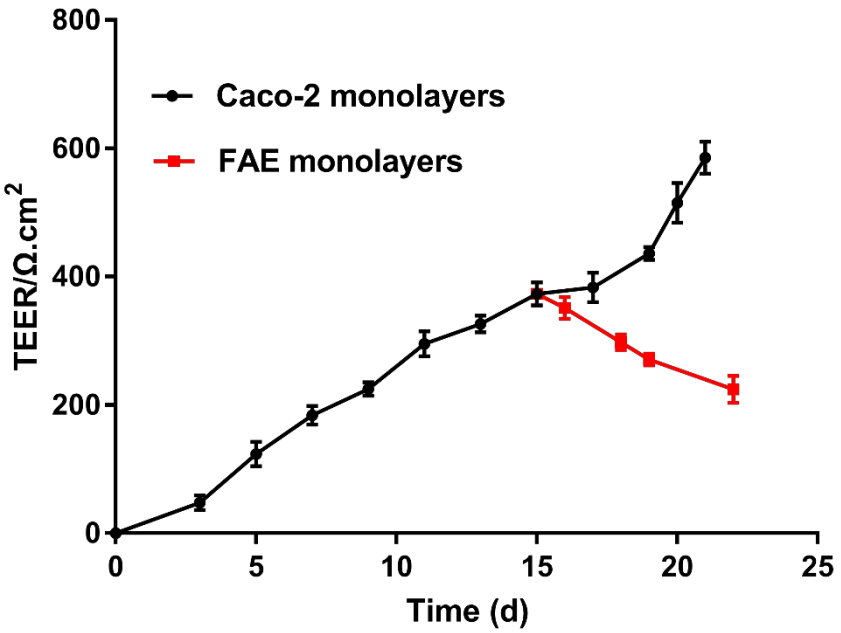

Figure S3. TEER values of FAE monolayer cells and Caco-2 monolayers at different culture time.