Supporting information

Evaluation of drug combination to glioblastoma based on an intestine-liver metabolic model on microchip

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Chemicals and reagents

CPT-11 and TMZ were purchased from Sigma (St. Louis, MO, USA). CP was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Cell culture medium (RPMI 1640), fetal bovine serum (FBS) and phosphate buffer saline solutions (PBS, 0.01 M, pH 7.4), trypsin-EDTA were provided by CALGibco (Grand Island, NY). Modified polyethersulfone (mPES) HFs (molecular weight cut off (MWCO) 100 kD; lumen size 0.5 mm) were purchased from Spectrum Labs (Rancho Dominguez, CA). Dihydroethidium (DHE) and 2, 3-naphthalenedicarboxaldehyde (NDA), Calcein AM, ethidium homodimer-1 (EthD-1) and Hoechst 33342 were purchased from Invitrogen (CA, USA). SU-8 2050 negative photoresist with the developer was purchased from MicroChem Corp (Newton, MA, USA). Poly dimethyl siloxane (PDMS) prepolymer and its curing agent were obtained from Dow Corning (Sylgard 184, Midland, MI, USA). 50 or 100 mg/mL aliquots of drugs in DMSO were stored at -20°C, thawed, and diluted just before use. Methanol and acetonitrile of HPLC grade (JT Baker, Phillipsburg, NJ, USA) and purified water (>18 MΩ·cm) were used. All other reagents were of analytical reagent grade and used without further purification.

Caco-2, HepG2, and U251 cell lines were obtained from Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China) and cultured in RPMI1640 supplemented with 10 % heat-inactivated FBS. Cells were maintained in an incubator with a humidified atmosphere containing 5 % CO_2 at 37°C.

Fabrication of integrated microdevice

To fabricate the integrated microdevice, SU-8 2075 negative photoresist was spin-coated on the silica wafer at a speed of 1000 rpm for three times, each time 60 s to get a height of 400 μm for the chambers. The silica wafer was then baked at 65 °C for 45 min every time, exposed to UV light for 2 min and baked again for 10 min at 65 °C before being developed to complete the SU-8 structures. After that the SU-8 mould was silanized to make the surface hydrophobic. Then the PDMS prepolymer and the

curing agent were mixed at a ratio of 10: 1. The mixture was poured onto the SU-8 mould and degassed under vacuum for 20 min to remove the air bubbles in the mixture. The PDMS was polymerized after being placed in an oven at 65 °C for 4 h and then the SU-8 mould was peeled off. Polydimethylsiloxane (PDMS) microdevices were fabricated using standard soft lithography and replica molding techniques.[1]

The length of the top chamber and bottom chambers were 35 and 45 mm, respectively, and the widths of the top chamber and bottom chambers were 15 and 21 mm, respectively (Fig. S1B). The depths of the upper and lower chambers were 400 mm each. The length, width and height of connection array channels were 4.5 mm, 0.2 mm and 50 μm, respectively.

SEM imaging of Caco-2 cultured into the HF

The fibers were taken out from the microchip and soaked in 2.5% glutaraldehyde solution and fixed for more than 2 h at room temperature. After removal the solution, slices, 0.5-2 cm, were cut from the HF tube with a razor blade resulting in ring-like shape, and then the slices were obliquely cut into small pieces to expose the inner surface or by first cutting 2-3 cm slices from the tubes and subsequently slicing the tubes lengthwise into half pieces to obtain longitudinal samples. The fibers were rinsed three times with phosphate-buffered saline (PBS) for 10 minutes followed by eluting in a gradient ethanol of 50, 70, 80, 90 and 100%. Afterwards, the fibers were critical point dried by Leica EM CPD 300 system (German) for 1 h followed by sputtering with gold and the inner surface features of HFs were observed using a Hitachi SU8010 SEM (Tokyo, Japan) at an accelerating voltage of 15 keV. The chamber was kept in vacuum to avoid surface charging during the observation.

Cell proliferation assay in drug combination and monotherapy

Cells were plated at a density of 5000 cells per well in 96-well plates with cell culture medium RPMI 1640. Different concentrations of CPT-11, TMZ and CP at 6.25, 12.5, 25, 50, 75 and 100 μg/mL were added into the cell culture wells singly or in

combination. Counting Kit-8 assays were performed at 4, 8 and 12 h after drug combination and monotherapy, respectively. At the end of drug action, 10 μl of the cell proliferation reagent WST-8 was added to each well and incubated for 3 h at 37°C. Viable cell numbers were estimated by measurement of optical density at 450 nm. The amount of the formazan dye, generated by the activities of dehydrogenises in cells, is directly proportional to the number of living cells. Culture medium without drugs treatment was conducted at the same time for control in each experiment. The viability of cells in the experiments was calculated by the ratio of drug treated values to the control values.

Calculation of cell viability curve and drug combination index

Combination index (CI) was used to quantitatively describe the efficiency of drug combination, which has been widely used in conventional drug combination studies.[2, 3] First, the experimental data of cell viability vs dose concentration for a definite single drug was fitted to the median-effect equation: [4]

$$f_a = \frac{1}{1 + \left(\frac{Dm}{D}\right)^m} \quad (1)$$

where D is the concentration of the drug, Dm is the IC50 value for the drug, m is the coefficient describing the shape of the curve cell viability vs concentration, and f_a is the normalized cell viability at drug concentration D. On the basis of equation 1, the Dm (IC50) and m values can be obtained by fitting the data of cell viability vs dose concentration. With the calculated Dm and m, a curve of cell viability vs dose concentration was obtained by equation 1. With the Dm and m values for the three single drugs, the CI value for different drug combination can be expressed as [5]

$$CI = \frac{D'_1}{(D_m)_1\left[f'_a/\left(1-f'_a\right)\right]^{1/m_1}} + \frac{D'_2}{(D_m)_2\left[f'_a/\left(1-f'_a\right)\right]^{1/m_2}}$$

(2)

where D'_1 and D'_2 are the concentration of the two kinds of drugs used in the drug combination and f_a' is the normalized cell viability at the drug combination experiment of concentration D'_1 and D'_2. CompuSyn software was used to calculate the CI value automatically.

Cell cycle assay for drug combination evaluation

After a series of treatments of fixation and rinsing, the cell collection was resuspended in 500 μL PBS containing 50 μg/mL propidium iodide (PI), 50 μg/ mL RNase A and 3.8 mM sodium citrate. The cells suspension was incubated at 37 °C for half an hour, then rinsed and resuspended in PBS. Afterwards, the labeled cells were processed in flow cytometry (BD FACSCalibur, BD Company, USA) to analyze cell cycle. The control experiment was performed with untreated U251 cells.

Cell apoptosis assay of drug combination and monotherapy

From a pharmacological view, cell apoptosis after anti-cancer medicine treatment is an index to evaluate drug efficiency. U251 cells viability at various concentrations of combination drugs was examined by Calcein AM and EthD-1 after 4 h. Hoechst 33342 (10 mM) was the specific fluorescent probe to detect cell apoptosis observed by a confocal laser scanning microscopy. The data were analyzed by program Image-Pro Plus 6.0 to get to know cell proliferation index at various concentrations of CPT-11 and TMZ after 4 h treatment.

LC-MS analysis of CPT-11 and TMZ metabolites

HepG2 and U251 cell medium were collected into the centrifuge tube and centrifuged at 225 g speed for 3 min to remove dead cells and debris. The supernatant of cell medium was collected and added methanol to precipitate and remove protein by centrifugation at 10,000 × g, 4 °C for 10 min. The cells were trypsinized into PBS

solution, and collected into the centrifuge tube. Then, cells suspension was centrifuged at 225 g speed for 3 min and cell precipitates were transferred into aqueous solution to be crushed by ultrasonic disruptor. Then, the supernatant was collected after 10,000 × g centrifugation. The metabolite in the supernatants of crushed cells and culture medium was concentrated by SPE, respectively.[6]

The analysis of the samples was performed on a liquid chromatograph/mass spectrometer (LCMS-IT-TOF, Shimadzu, Japan). The analytes were separated on a C18 column (4.6 mm × 150 mm, 5 μm, Hitachi). The mobile phase for CPT-11 and SN-38 was the mixture of acetonitrile/methanol/5 mM ammonium formate buffer containing 0.1% formic acid (3:4:3, v: v: v) at 0.8 mL/min flow rate. For TMZ and MTIC, the mobile phase consists of methanol (30%) and 1 mM ammonium acetate in 0.1% formic acid (70%) at a flow rate of 1.0 ml mL/min.[7] With the positive electrospray ionization (ESI) mode, CPT-11, TMZ, SN-38 and MTIC were simultaneously detected. Standards of CPT-11 and SN-38 were obtained from J&K Chemical Co. Ltd. Working standards of CPT-11 and TMZ at the serial concentrations of 0.5, 1, 5, 10, 50, and 100 μg/mL, and SN-38 and MTIC at the concentrations of 5, 25, 50, 75, and 100 ng/mL were prepared in blank cell culture medium samples for quantitative standard curves. Due to the chemical instability of MTIC, all reagents were maintained ice-cold; sample preparation and processing were minimized. All calibration standards and actual samples were processed one at a time and immediately analyzed and LC-MS conditions were porformed by our previous work.[6]

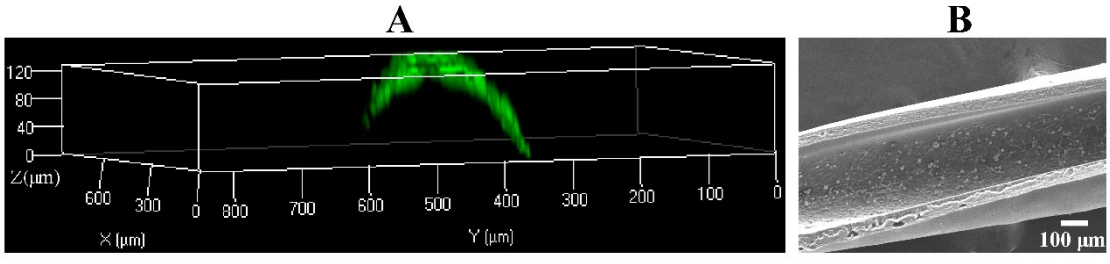

Fig. S1 Fluorescence image and fabrication of the HF integrated microfluidic device. (A) 3D confocal fluorescence microscopy photograph of HF cultured with Caco-2 cells. (B) SEM image of Caco-2 cells cultured in the lumen of HF.

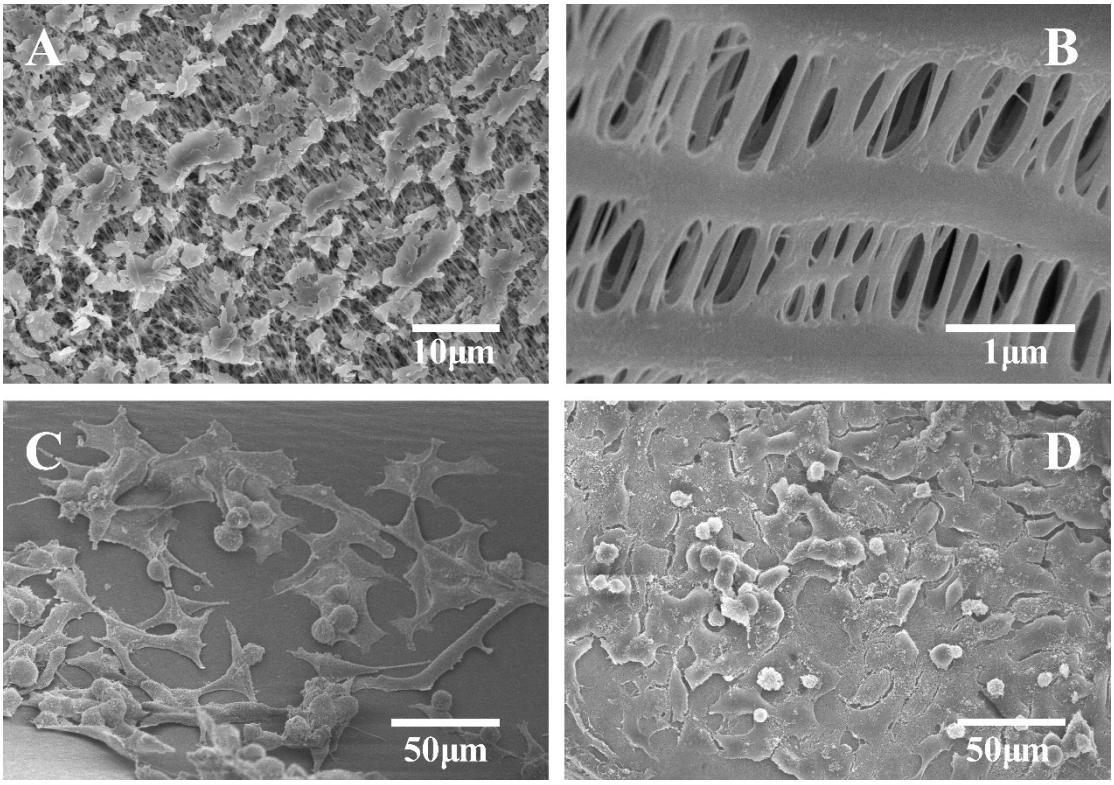

Fig. S2 SEM images of mPES HF and time-dependent morphology of Caco-2 cell layers on the inner surface of mPES HF. (A) Inner surface of mPES HF. (B) Inner edge of the cross-section. (C) Cell layers at 4 h. (C) Cell layers at 12 h.

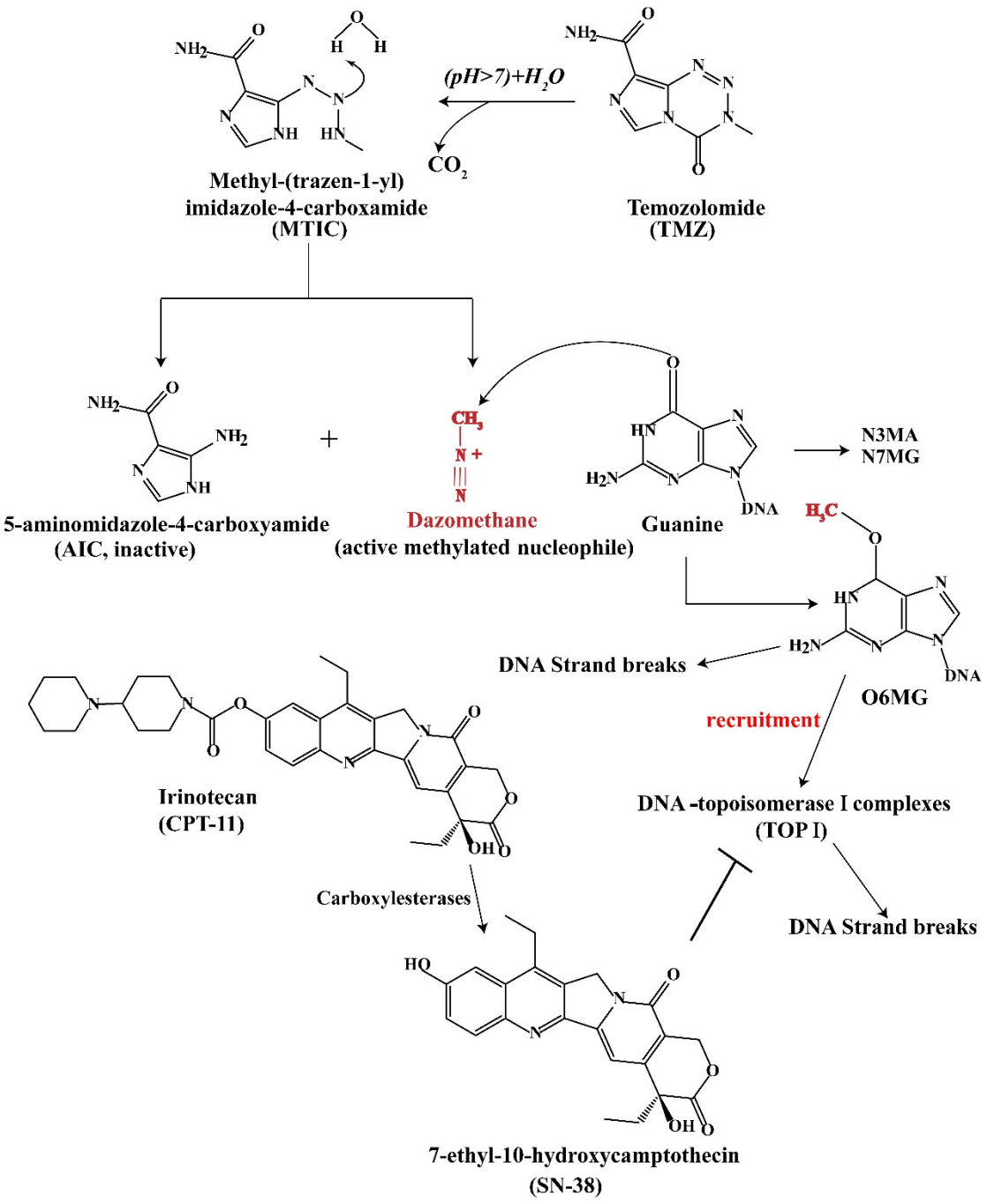

Fig. S3 Mechanism of action of CPT-11 and TMZ, illustrating the release of diazomethane, which alkylates DNA, further breaks DNA strand and trigger of apoptosis. Meanwhile, modification to DNA recruits TOPI and potentially increases the probability of CPT-11 stabilizing the DNA-enzyme covalent complex, thus enhances the probability of inducing CPT-11-mediated damage.[8]

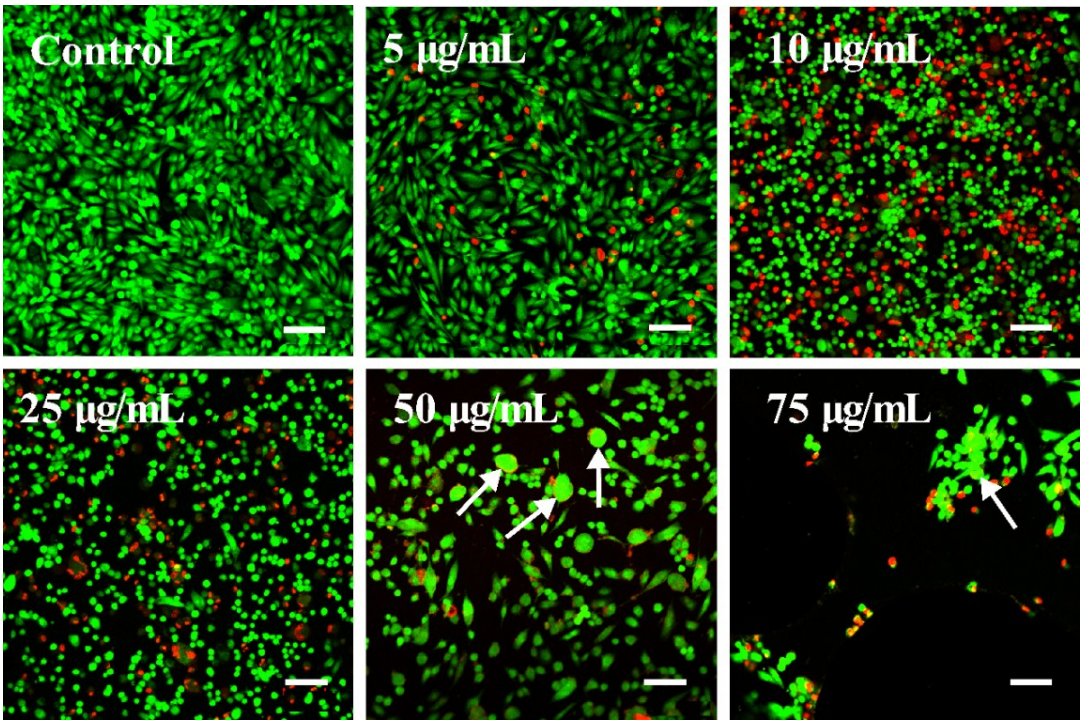

Fig. S4 Fluorescent images of U251 cell apoptosis under different concentration of combined drug treatment stained by Calcein AM and EthD-1 probes (scale bar: 100 μm).

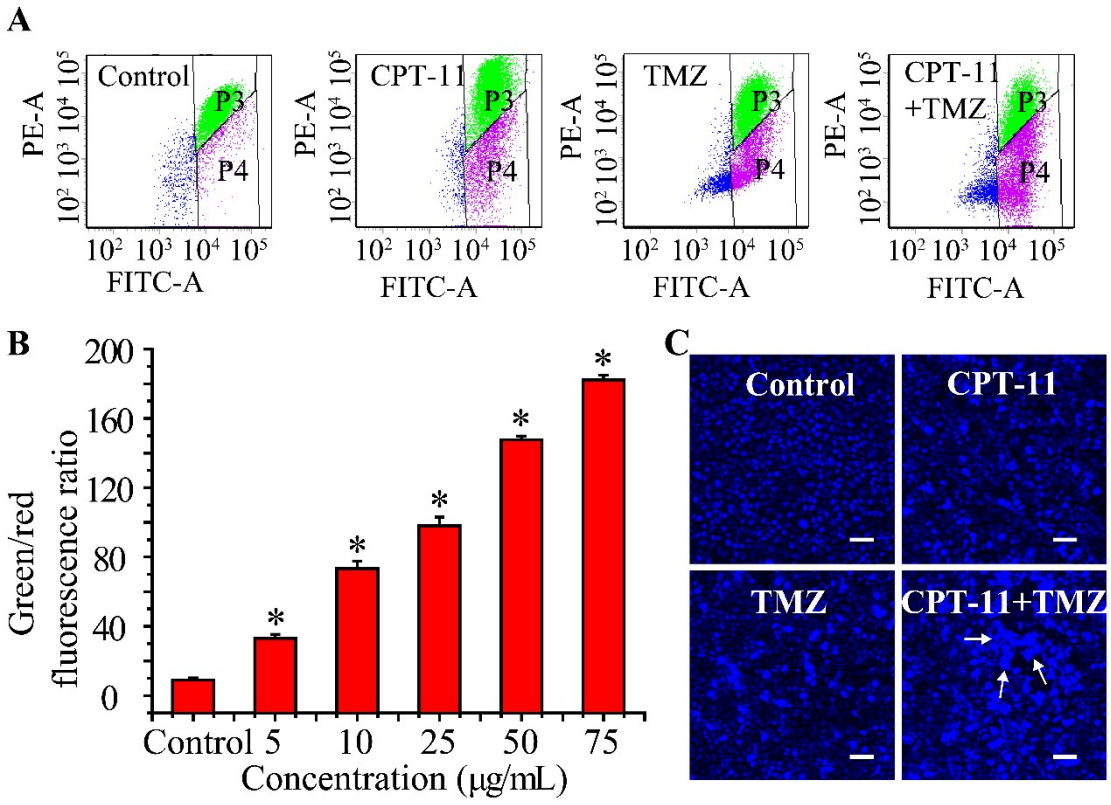

Fig. S5. Combined drug-induced dissipation of ΔΨm and apoptosis effect in U251 cells. A. Red fluorescence P3 represents the mitochondrial aggregate form of JC-1, indicating intact mitochondrial membrane potential. Green fluorescence P4 represents the monomeric form of JC-1, indicating dissipation of ΔΨm. (B) Quantitative analysis of the shift of mitochondrial red fluorescence to green fluorescence among groups. (C) Fluorescent images of U251 cells stained by Hoechst 33342 under monotherapy and drug combination. All values are denoted as means ± S.E.M. from three independent photographs shot in each group. Significant differences are indicated as: **P* < 0.05 compared with control cells cultured in RPMI 1640 medium.

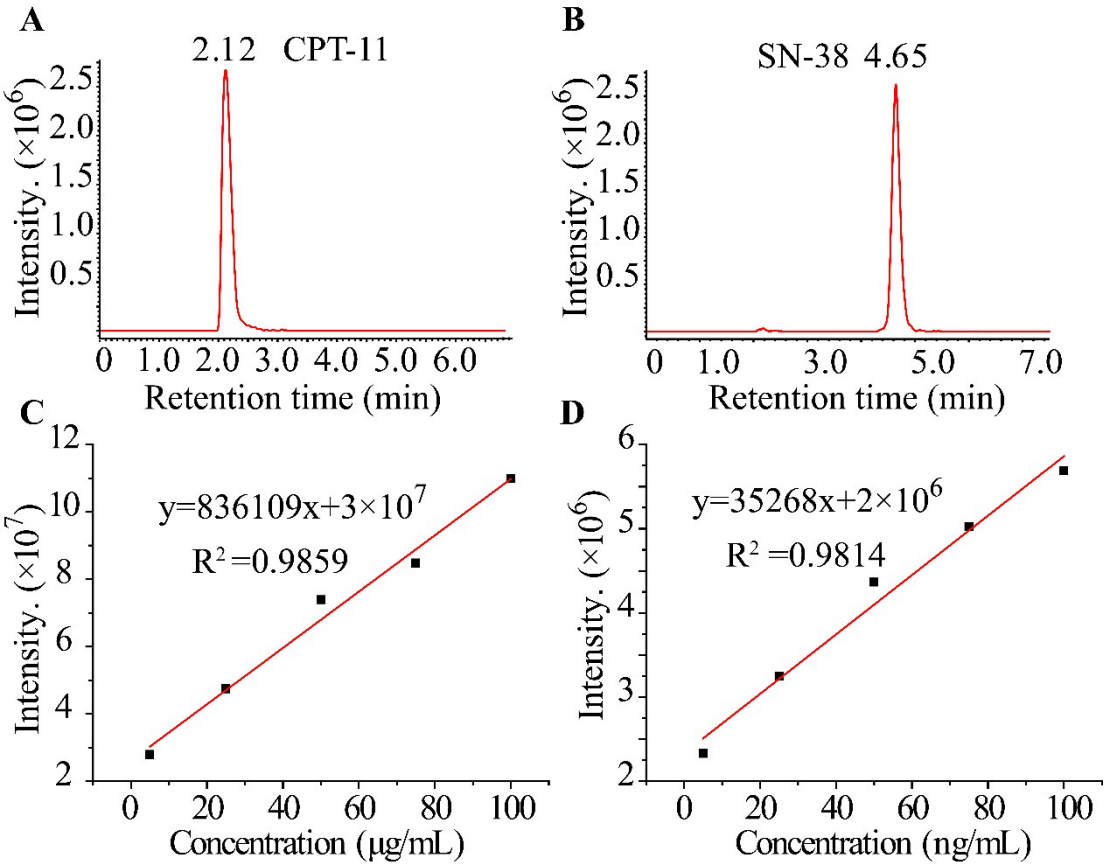

Fig. S6 Quantitation in intracellular CPT-11 and SN-38 in HepG2 cells and calibration curves of CPT-11 and SN-38. The concentrations are 17.8 μg/mL and 65.4 ng/ml for CPT-11 (A) and SN-38 (B) in HepG2 cells, respectively. (C) Calibration curve of CPT-11 in the range of 5 to 100 μg/mL. (D) Calibration curve of SN-38 in the range of 5 to 100 ng/mL.

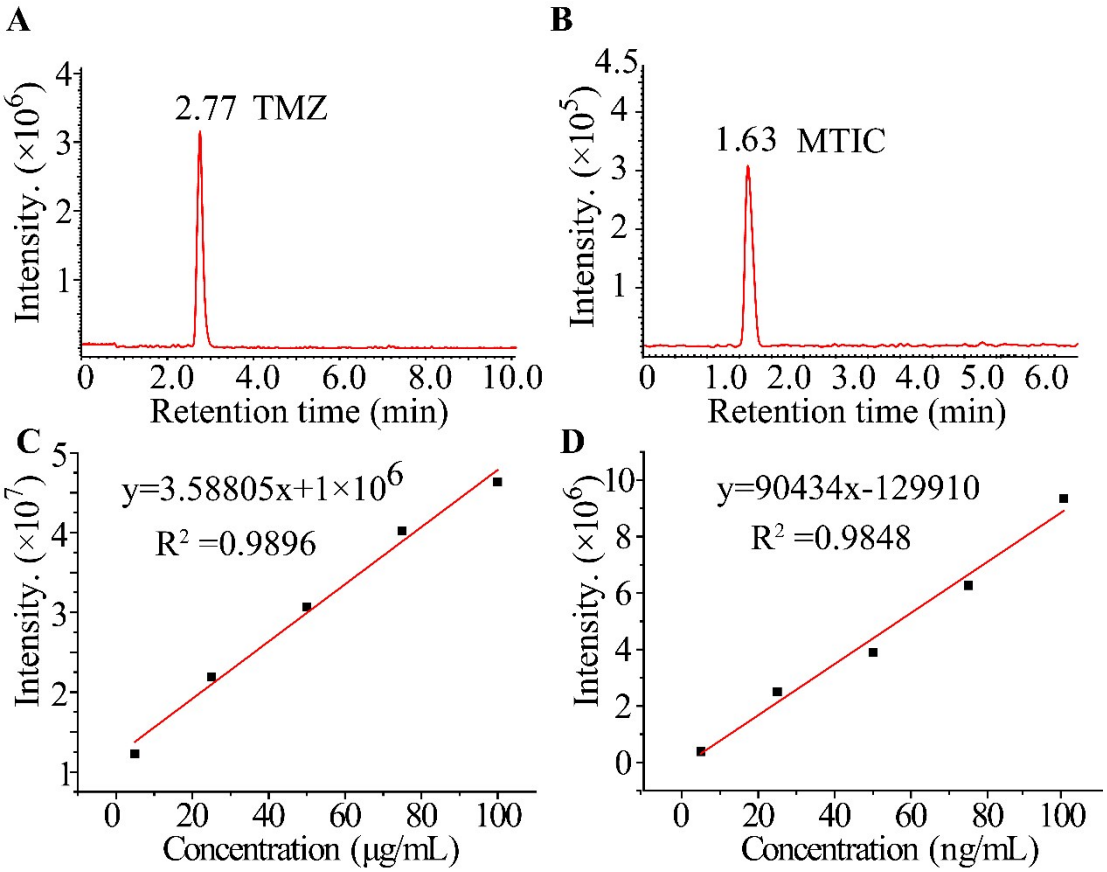

Fig. S7 Quantitation in intracellular TMZ and MTIC in U251 cells and calibration curves of TMZ and MTIC. The concentrations are 10.4 μg/mL and 22.7 ng/ml for TMZ (A) and MTIC (B) in U251 cells, respectively. (C) Calibration curve of TMZ in the range of 5 to 100 μg/mL. (D) Calibration curve of MTIC in the range of 5 to 100 ng/mL.

Table S1 Concentrations of drugs used in single drug experiments.

Concentration group	CPT-11 (μg/mL)	TMZ (μg/mL)	CP (μg/mL)
1	6.25	6.25	6.25
2	12.5	12.5	12.5
3	25	25	25
4	50	50	50
5	75	75	75
6	100	100	100

Table S2 Combinations of concentrations of drugs used under the drug combination.

Combination group	CPT-11 (μg/mL)	TMZ (μg/mL)	CP (μg/mL)
1	6.25	6.25	6.25
2	12.5	12.5	12.5
3	25	25	25
4	50	50	50
5	75	75	75
6	100	100	100

Table S3 The population percentage of cells distributed in different cell cycle phases based on the drug combination.

Cell cycle	Control cells (%)	CPT-11 treated cells (%)	TMZ treated cells (%)	CPT-11+TMZ treated cells (%)
G0/G1	73.08	65.56	60.24	57.44
S	0.08	29.85	27.14	42.48
G2/M	26.83	4.59	12.62	0.07

References

1. J. Zhang, J. Wu, H. Li, Q. Chen and J. M. Lin, *Biosens. Bioelectron.*, 2015, **68**, 322.
2. T. C. Chou, *Pharmacol. Rev.*, 2006, **58**, 621.
3. G. S. Du, J. Z. Pan, S. P. Zhao, Y. Zhu, J. M. den Toonder and Q. Fang, *Anal. Chem.,* 2013, **85**, 6740-6747.
4. T. C. Chou, *JTBio*, 1976, **59**, 253-276.
5. T. C. Chou and P. Talalay, *Trends Pharmacol. Sci.*, 1983, **4**, 450-454.
6. M. Jie, H.-F. Li, L. Lin, J. Zhang and J.-M. Lin, *RSC. Adv.,* 2016, **6**, 54564-54572.
7. S. K. Chowdhury, D. Laudicina, N. Blumenkrantz, M. Wirth and K. B. Alton, *J. Pharm. Biomed. Anal.*, 1999, **19**, 659-668.
8. R. Rai, M. Banerjee, D. H. Wong, E. Mccullagh, A. Gupta, S. Tripathi, E. Riquelme, R. Jangir, S. Yadav and M. Raja, *Bioorg. Med. Chem. Lett.*, 2016, **26**, 5103-5109.