Supplementary Information

Materials and Methods

Materials

Gramine, 4-pyridinecarboxaldehyde, *N*,*O*-bis(trimethylsilyl)acetamide (BSA), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), dithiothreitol (DTT) and propidium iodide (PI) were obtained from Sigma-Aldrich. Bromoacetic acid, 2-bromoethanol and bis(2-hydroxyethyl) disulfide (*ca.* 50% in water) were obtained from TCI. Iodomethane, 5-fluorouracil (5-FU), 2-bromoethylamine hydrobromide and 1,3-dibromopropane were obtained from Energy Chemical. Tributylphosphine, 4-dimethylaminopyridine (DMAP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Aladdin. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. MitoTracker® Red CM-H_2XRos and Hoechst 33342 were purchased from Invitrogen. Annexin V PE Apoptosis Detection Kit was purchased from eBioscience. Dihydroethidium (DHE) was purchased from Beyotime. All other reagents were of analytical reagent grade, and all solutions were prepared with aseptic double-distilled water. Double-distilled water was prepared from a Milli-Q-RO4 water purification system (Millipore).

All ^{1}H NMR spectra were performed on Varian Unity Inova 400 MHz spectrometer (Varian). Mass spectra were analyzed by Agilent-7890A/5975C ESI-MS System (Agilent Technologies), LCMS-2010 (Shimadzu) and AXIMA-TOF^2 (Shimadzu). All

fluorescence spectra were recorded on LS-55 spectrofluorophotometer (Perkin Elmer) equipped with 1.0 cm quartz cells and a thermostat bath. The UV-visible absorption spectra were recorded at room temperature on a UV-6100 UV-vis Double Beam Spectrophotometer (MAPADA) equipped with 1.0 cm quartz cells. MTT assays were performed by ELx800 Absorbance Microplate Reader (BioTek). Cells were analyzed by BD Accuri C6 flow cytometer (Becton Dickinson). In colocalization assay, the fluorescent signal was observed by NOL-LSM 710 laser scanning confocal microscope (Carl Zeiss).

Synthesis of F16-5-FU

F16 and F16-5-FU were prepared according to our previous reports and the procedures were shown in Scheme S1.[1]

(*E*)-3-(2-(pyridine-4yl)vinyl)-1*H*-indole (PVI, compound 1, yield: 37.7%): ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 11.494 (s, 1H), 8.469 (d, J = 4.9 Hz, 2H), 8.050 (d, J = 7.2 Hz, 1H), 7.727 (d, J = 15.7 Hz, 2H), 7.526 (d, J = 4.9 Hz, 2H), 7.447 (d, J = 7.7 Hz, 1H), 7.215-7.125 (m, 2H), 7.060 (d, J = 16.5 Hz, 2H). ESI-MS: calcd for $C_{15}H_{12}N_2$: 220.10; found: 221.2 ($[M + H]^+$), 219.3 ($[M – H]^-$).

(*E*)-4-(1*H*-indol-3-ylvinyl)-*N*-methylpyridinium iodide (F16, compound 2, yield: 63.5%): ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 12.050 (s, 1H), 8.686 (d, J = 6.3 Hz, 2H), 8.241 (d, J = 16.2 Hz, 1H), 8.171-8.104 (m, 3H), 7.968(s, 1H), 7.519 (d, J = 7.4 Hz, 1H), 7.317-7.207(m, 3H), 4.174(s, 3H). ESI-MS: calcd for $C_{16}H_{15}N_2^+$ (M^+): 235; found: 235.

FU-Br (compound 3, yield: 27.4%): ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 11.774 (s,

1H), 8.058 (d, *J* = 6.8 Hz, 2H), 3.729 (t, *J* = 13.6 Hz, 2H), 3.535 (t, *J* = 13.2 Hz, 2H), 2.190-2.101 (m, 2H). ESI-MS: calcd for $C_7H_8BrFN_2O_2$: 250.0; found: 500.6 ($[2M]^-$).

F16-5-FU (compound 4, yield: 97.4%): ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 12.073 (s, 2H), 8.800 (d, *J* = 5.7 Hz, 2H), 8.284 (d, *J* = 16.2 Hz, 1H), 8.158 (s, 4H), 7.979 (s, 1H), 7.526 (d, *J* = 6.8 Hz, 1H), 7.337-7.253 (m, 3H), 4.467 (s, 2H), 3.747 (s, 2H), 2.273-2.255 (m, 2H). GC-MS: calcd for $C_{22}H_{20}FN_4O_2^+$ (M^+): 391.2; found: 391.2.

Synthesis of F16-OOC-FU

FU-COOH (compound 5): Compound 5 was prepared according to reported procedure.[2] Briefly, KOH (1.68 g, 30 mmol) and 5-FU (1.3 g, 10 mmol) were dissolved in 8 mL of water at 70 ºC, then 4 mL aqueous solution of bromoacetic acid (2.08 g, 15 mmol) was added slowly. The mixture was stirred at 70 ºC for 2.5 h, and the pH value of the reaction mixture was kept at 9-10 by adding aqueous solution of KOH. Then the mixture was cooled and adjusted to pH 5-6 with hydrochloric acid. The precipitates was removed by filtration, then the pH value of the mixture was adjusted to 2. The mixture was put under the temperature of 4 ºC, and the compound 5 was seperated from the aqueous solution as white solid at a yield of 40.8%. ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 13.270 (s, 1H), 11.948 (s, 1H), 8.088 (d, *J* = 6.0 Hz, 1H), 4.359 (s, 2H). ESI-MS: calcd for $C_6H_5N_2O_4$: 188.0; found: 187.0 ($[M - H]^-$), 374.8 ($[2M - H]^-$).

FU-COO-Br (compound 6): Compound 5 (188 mg, 1 mmol) was dissolved in 6 mL anhydrous THF, then 2-bromoethanol (90 μL, 1.2 mmol) and DMAP (13 mg, 0.1 mmol) were added. After stirring for several minutes under a N_2 atmosphere, EDC (288 mg, 1.5 mmol) in THF was added. The mixture was stirred at room temperature, and

the reaction was monitored by thin layer chromatography (TLC). When the reaction was completed, the mixture was quenched with water and diluted with ethyl acetate. The organic layer was washed with 0.5 M HCl, saturated solution of $NaHCO_3$ and saturated solution of NaCl, respectively. Then the organic layer was dried with Na_2SO_4 and evaporated to give the crude product. The crude product was purified by silica column to give the product as white solid (yield: 24.7%). ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 12.022 (s, 1H), 8.108 (d, *J* = 6.7 Hz, 1H), 4.534 (s, 2H), 4.440 (t, *J* = 10.7 Hz, 2H), 3.698 (t, *J* = 10.7 Hz, 2H). ESI-MS: calcd for $C_8H_8BrFN_2O_4$ (M^-): 294; found: 293 ($[M - H]^-$) .

F16-OOC-FU (compound 7): Compound 1 and equivalent amount of compound 6 were dissovled in acetonitrile and refluxed overnight. When reaction was completed, the mixture was cooled and separated by neutral alumina column to give the product as reddish-brown solid (yield: 54.9%). ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 12.304 (s, 1H), 8.744 (d, *J* = 6.5 Hz, 2H), 8.279 (d, *J* = 16.2 Hz, 1H), 8.161 (t, *J* = 14.8 Hz, 3H), 7.986 (s, 1H), 7.531 (d, *J* = 7.8 Hz, 1H), 7.343-7.246 (m, 3H), 4.476 (d, *J* = 16.2 Hz, 3H), 3.834 (s, 2H), 3.679 (s, 1H). MOLDI-TOF: calcd for $C_{23}H_{20}FN_4O_4^+$ (M^+): 435.15; found: 435.85.

Synthesis of F16-NHOC-FU

FU-CONH-Br (compound 8): Compound 5 (188 mg, 1 mmol), EDC (384 mg, 2 mmol) and NHS (60 mg, 0.5 mmol) were dissolved in 2.5 mL DMF and stirred for 30 min. Then 2-bromoethylamine hydrobromide (306 mg, 1.5 mmol) in 1.5 mL DMF was added, and the mixture was stirred at room temperature. The reaction was monitored

by TLC. When the reaction was completed, the mixture was quenched with water and diluted with ethyl acetate. The organic layer was washed with saturated solution of $NaHCO_3$ twice and saturated solution of NaCl. Then the organic layer was dried with anhydrous Na_2SO_4 and evaporated to give the crude product. The crude product was purified by silica column to give the product as white solid (yield: 10.2%). ^{1}H NMR (300MHz, CD_3OD, δ): 7.783 (d, J = 6.2 Hz, 1H), 4.407 (s, 2H), 3.605 (t, J = 12.3 Hz, 2H), 3.468 (t, J = 12.5 Hz, 2H). ESI-MS: calcd for $C_8H_9BrFN_3O_3$ (M^-): 293; found: 292 ($[M - H]^-$).

F16-NHOC-FU (compound 9) Preparation of compound 9 was conducted with the procedure similar to F16-OOC-FU at a yield of 69.1%. ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 11.632 (s, 2H), 8.723 (d, J = 6.0 Hz, 3H), 8.276 (d, J = 6.1 Hz, 1H), 8.166 (s, 1H), 8.119-8.060 (m, J = 17.6 Hz, 3H), 7.993 (s, 1H), 7.530 (d, J = 7.0 Hz, 1H), 7.331-7.249 ((m, J = 24.7 Hz, 3H), 4.502 (s, 2H), 4.296 (s, 2H), 3.619 (s, 2H). MOLDI-TOF: calcd for $C_{23}H_{21}FN_5O_3^+$ (M^+): 434.16; found: 434.00.

Synthesis of F16-SS-FU

Bis-(2-bromoethyl)disulfide (Br-SS-Br, compound 10): Compound 5 was prepared according to reported procedure with minor modification.[3] Concentrated H_2SO_4 (30 mL) was slowly added to a stirred 48% HBr aqueous solution (20 mL) at 0 ºC, then bis(2-hydroxyethyl) disulfide (*ca.* 50% in water) (1.8 g, 6 mmol) was added dropwise. After stirred for 24 h at room temperature, the mixture was heated on a steam bath for 3 h. Dichloromethane (30 mL) was added to the cooled reaction mixture to extract the product. The organic layer was separated, dried with anhydrous Na_2SO_4, then

evaporated under reduced pressure to give compound 10 (yield: 42.6%). ^{1}H NMR (300MHz, $CDCl_3$, δ): 3.623 (t, *J* = 15.6 Hz, 4H), 3.100 (t, *J* = 15.5 Hz, 2H). ESI-MS: calcd for $C_4H_8Br_2S_2$ (M^+): 278; found: 279 ($[M + H]^+$).

FU-SS-Br (compound 11): 5-FU (325 mg, 2.5 mmol) and BSA (1.2 g, 6mmol) were dissolved in 8 mL acetonitrile. Then compound 10 (2.4 g, 8.5 mmol) and KI (83 mg, 0.5 mmol) were added. The mixture was refluxed under a N_2 atmosphere. The reaction was monitored by TLC. When the reaction was completed, the mixture was diluted with ethyl acetate. The organic layer was washed with water twice and dried with anhydrous Na_2SO_4. Removal of the solvent under reduced pressure followed by silica column to give the compound 11 (yield: 35.5%). ^{1}H NMR (300MHz, $CDCl_3$, δ): 8.474 (s, 1H), 4.031 (t, *J* = 12.4 Hz, 2H), 3.631 (t, *J* = 15.3 Hz, 2H), 3.112 (t, *J* = 15.3 Hz, 2H), 3.014(t, *J* = 12.3 Hz, 2H). ESI-MS: calcd for $C_8H_{10}BrFN_2O_2S_2$ (M^-): 328; found: 327 ($[M - H]^-$).

F16-SS-FU (compound 12): Compound 1 and equivalent amount of compound 11 were dissovled in acetonitrile and refluxed overnight under a N_2 atmosphere. When reaction was completed, the mixture was cooled and separated by neutral alumina column to give the compound 12 (yield: 60.0%). ^{1}H NMR (300MHz, $(CD_3)_2SO$, δ): 12.424 (s, 1H), 11.881 (s, 1H), 8.840 (d, *J* = 5.1 Hz, 2H), 8.345 (d, *J* = 16.0 Hz, 2H), 8.192 (s, 4H), 8.008 (s, 1H), 7.541 (d, *J* = 16.4 Hz, 1H), 7.356-7.244 (m, *J* = 33.4 Hz, 3H), 4.757 (s, 2H), 3.962 (s, 3H), 3.083 (s, 3H). MOLDI-TOF: calcd for $C_{23}H_{22}FN_4O_4S_2^+$ (M^+): 469.12; found: 470.67.

Cell Lines and Cell Culture

Human fetal gastric epithelial (GES-1) and human gastric carcinoma (SGC-7901) cell

lines were obtained from Prof. Xiang Zhou (Wuhan University, Wuhan, China). Human breast (HBL-100) and human breast cancer (MCF-7) cell lines were obtained from Prof. Wenhua Li (Wuhan University, Wuhan, China). Cells were cultured in DMEM medium supplemented with 10% FBS at 37 ºC/5% CO_2.

MTT Experiment

In MTT assay, 2×10^4 cells were seeded in each well of 96-well plate. After 24 h, the synthesized compounds at various concentrations were added to each line. The treatment lasted for 48 h or 72 h. In the experiment of enhancing the toxicity of F16-SS-FU, DTT was added 4 h after the addition of F16-SS-FU. Cells were incubated with 0.5 mg mL^{-1} MTT for 4 h before measuring the absorbance at 490 nm.

Detection of cellular uptake

SGC-7901 cells were incubated with 3 μM F16, 5 μM F16-5-FU, 5 μM F16-OOC-FU, 5 μM F16-NHOC-FU or 5 μM F16-SS-FU for various hours. Then the treated cells were collected and resuspended in PBS and immediately analyzed by flow cytometer.

Colocalization assay

2 μM F16 or 5 μM F16-OOC-FU was incubated with MCF-7 cells for 24 h, while 5 μM F16-5-FU, 5 μM F16-NHOC-FU or 5 μM F16-SS-FU was treated with SGC-7901 cells for 24 h. MitoTracker® Red CM-H_2XRos and Hoechst 33342 were added to stain mitochondria and nuclei, respectively. The fluorescent signals were observed by laser scanning confocal microscope.

Detection of Apoptosis and Cell Cycle

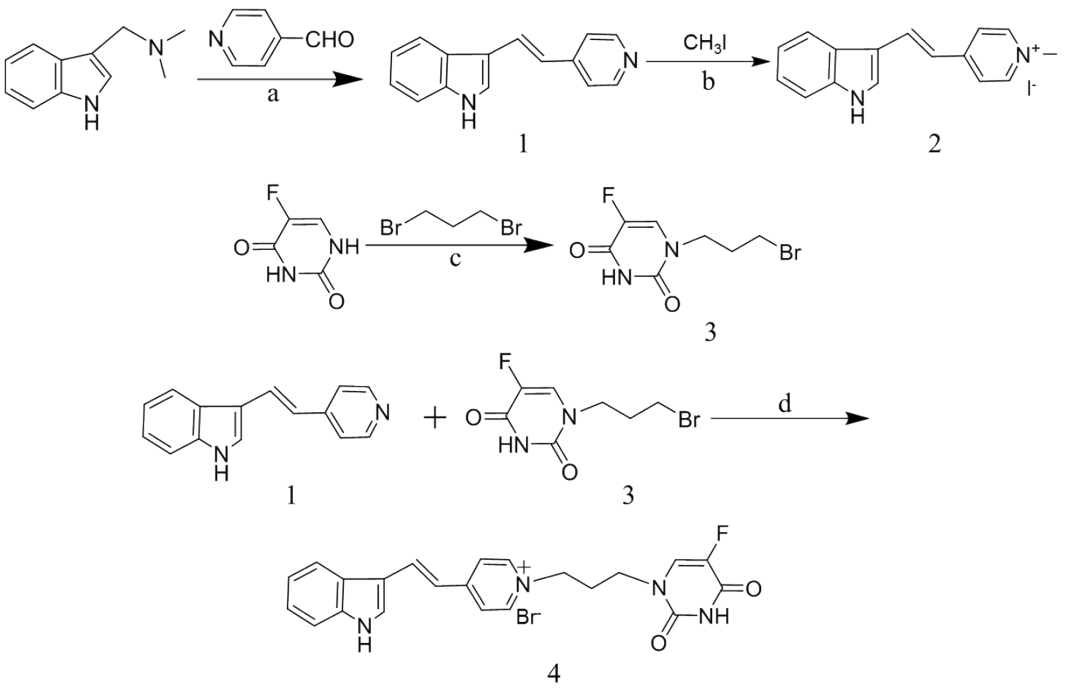
SGC-7901 cells untreated or treated with various concentrations of F16-OOC-FU for

48 h were stained with AnnexinV-PE and 7-AAD following manufacturer's procedure. Then the labeled cells were analyzed by flow cytometer.

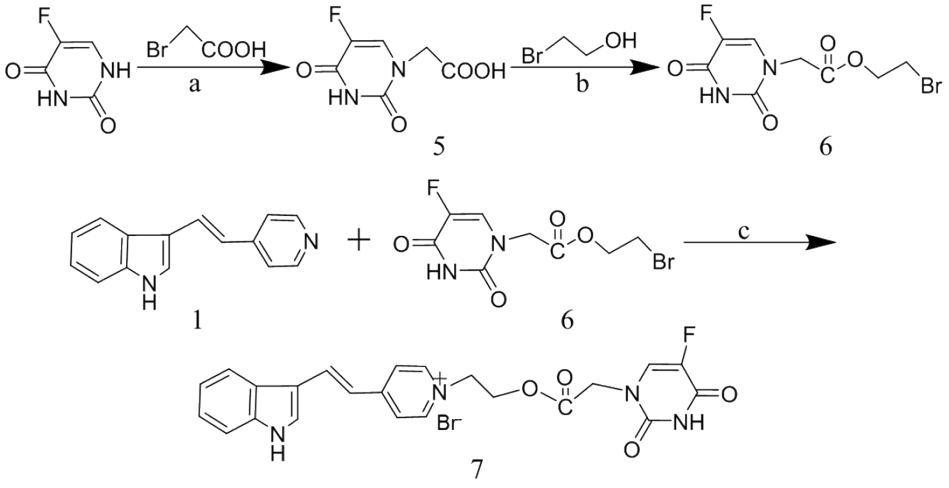
In cell cycle assay, SGC-7901 cells were seeded in 6-well plate. After adhering to the surface of wells, cells were serum starved for 24 hours to arrest them in G0 phase of the cell cycle, after which they were treated with various concentrations of F16-OOC-FU in DMEM complete medium for 24 hours. Cells were collected, fixed in 70% ethanol and stained with PI before analyzed by flow cytometer.

Measurement of Intracellular Oxidation Level

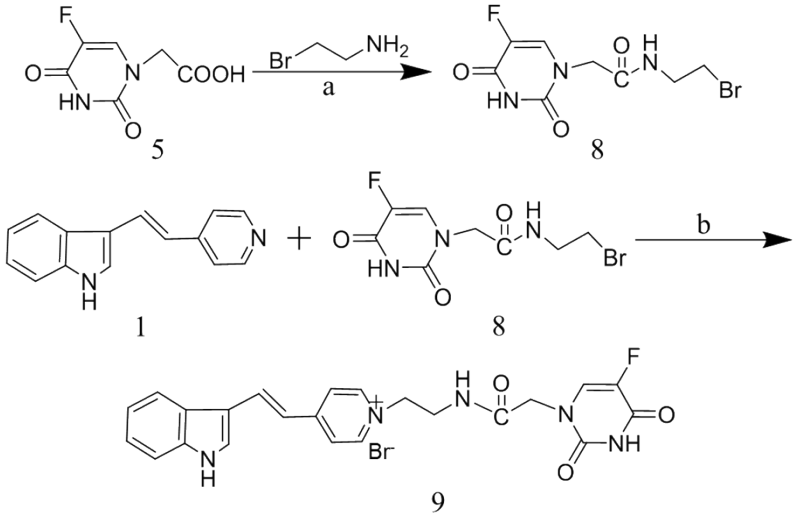
SGC-7901 cells were grown in the absence or in the presence of F16-OOC-FU for 48 h. Then cells were incubated in PBS contained 5 μM DHE or 1 μM MitoTracker® Red CM-H_2XRos at 37 ºC for 30 min. Next cells were collected and resuspended in PBS and immediately analyzed by flow cytometer.



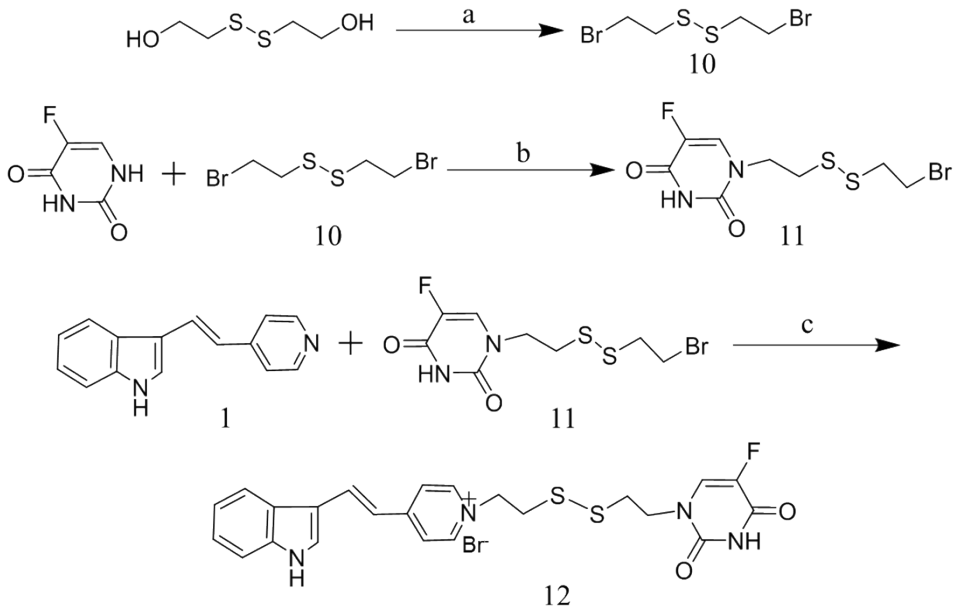
Scheme S1 Synthesis of F16 and F16-5-FU. Reagents and conditions: (a) n-Bu_3P, CH_3CN, 85 ºC; (b) CH_2Cl_2/CH_3OH, rt; (c) BSA, KI, CH_3CN, 86 ºC; (d) CH_3CN, 86 ºC.



Scheme S2 Synthesis of F16-OOC-FU. Reagents and conditions: (a) KOH (aq), 70 ºC; (b) EDC, DMAP, THF, rt; (c) CH_3CN, 86 ºC.



Scheme S3 Synthesis of F16-NHOC-FU. Reagents and conditions: (a) EDC, NHS, THF, rt; (b) CH_3CN, 86 ºC.



Scheme S4 Synthesis of F16-SS-FU. Reagents and conditions: (a) HBr, H_2SO_4, 0 ºC to rt; (b) BSA, KI, CH_3CN, 86 ºC; (c) CH_3CN, 86 ºC.

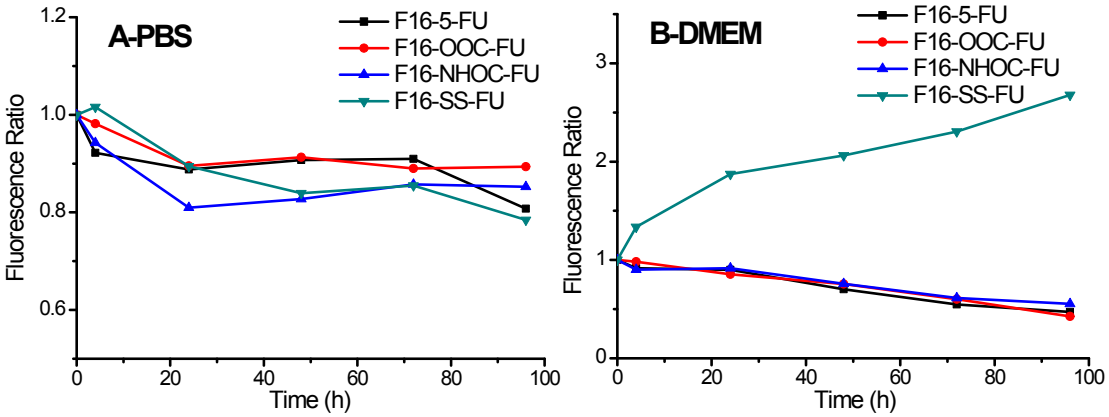


Fig. S1 Stability studies of these conjugated compounds. F16-5-FU, F16-OOC-FU, F16-NHOC-FU and F16-SS-FU were incubated in PBS (A) or DMEM medium (supplemented with FBS, B) for 0, 4, 24, 48, 72 and 96 h, and the fluorescence intensity was compared to that of 0 h.

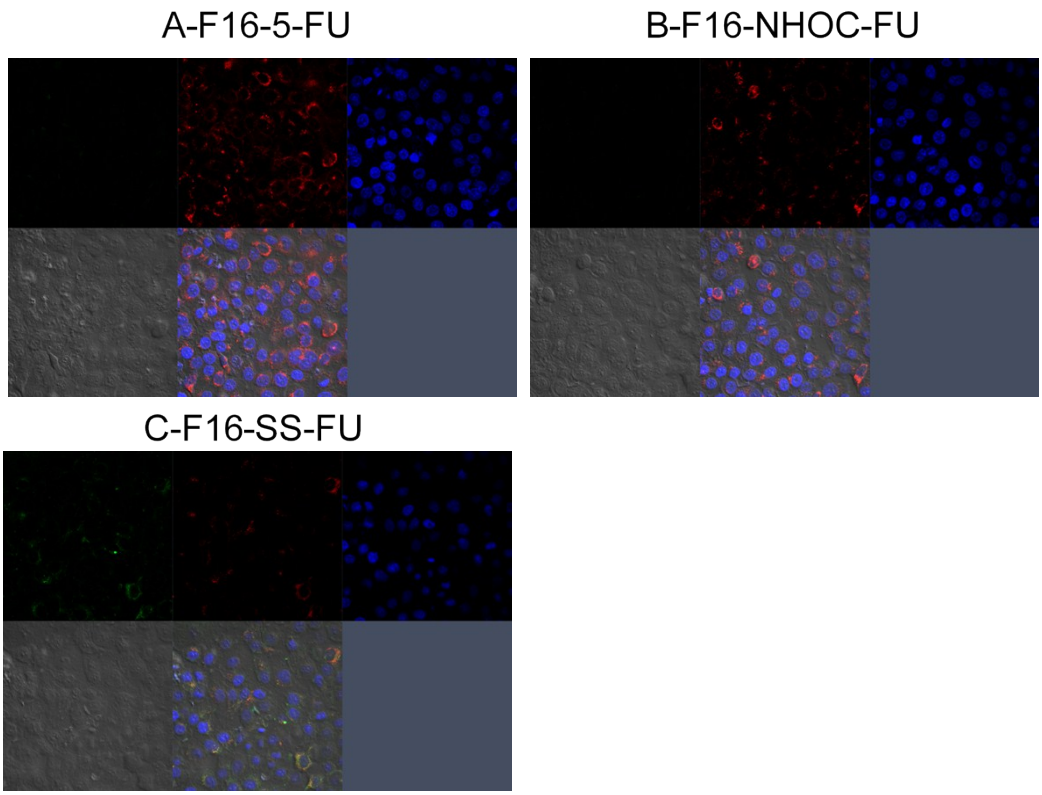


Fig. S2 SGC-7901 cells were incubated with (A) 5 μM F16-5-FU, (B) 5 μM F16-NHOC-FU or (C) 5 μM F16-SS-FU, then were stained with MitoTracker® Red CM-H_2XRos and Hoechst 33342.

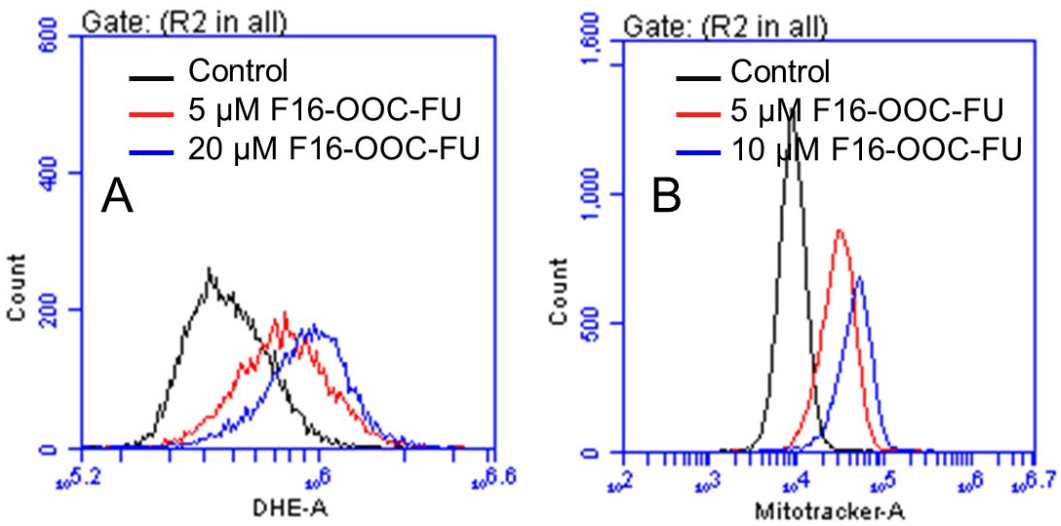


Fig. S3 Comparison of intracellular reactive oxygen species levels in SGC-7901 cells with and without the treatment of F16-OOC-FU. Flow cytometry analysis of ethidium bromide-DNA fluorescence resulting from oxidation of dihydroethidium (DHE) (A) and fluorescence induced by oxidation of MitoTracker® Red CM-H_2XRos (B).

Table S1 Cytotoxicity of F16-OOC-FU on different cell lines. Values represent the mean ± SD values of at least three independent experiments.

Cell lines	SGC-7901	MCF-7	GES-1	HBL-100
IC_{50} (μM)	35.04 ± 9.18	28.61 ± 3.03	71.31 ± 6.68	112.98 ± 3.22

References

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