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Supplementary Information

Folate-targeted pH-sensitive Bortezomib Conjugates for Cancer Treatment

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1. MATERIALS AND METHODS

1.1. Materials

Chloroform, dichloromethane, methanol, acetonitrile (ACN), and fluorescein isothiocyanate (FITC) were purchased from Thermo Fisher Scientific (Rockford, IL). Folic acid (FA), dimethyl sulfoxide (DMSO), dopamine, bortezomib (BTZ), trifluoroacetic acid (TFA), triethylamine (TEA), N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), thylenediamine were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Retinoic acid was purchased from Beantown Chemical, Inc. (Hudson, NH). L-cysteine ethyl ester hydrochloride (L-Cys) was purchased from Alfa Aesar (Haverhill, MA). Agarose RA™ was purchased from Amresco (Solon, OH). Maleimide-PEG (MW 3,400 Da)-succinimidyl valerate was purchased from Laysan Bio, Inc. (Arab, AL). Thin-layer chromatography (TLC) plate (silica gel 60 F254) was from EMD Biosciences (La Jolla, CA). Dialysis tubing (MWCO 2,000 Da) was purchased from Spectrum Laboratories, Inc. (Houston, TX). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI)-1640, penicillin streptomycin solution, Hoechst 33258, and trypsin-EDTA were purchased from Invitrogen Corporation (Carlsbad, CA). Female mouse plasma was purchased from BioIVT (Westbury, NY).

The human cervical cancer cells (HeLa), breast cancer cells (MDA-MB-231), human breast cancer (MCF-7), mouse melanoma (B16F10), mouse macrophage (RAW 264.7), and mouse fibroblast (NIH/3T3) were grown in the complete growth media (DMEM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% FBS) at 37 ºC with 5% CO_2. The non-small cell lung cancer cells (A549) were grown in complete growth media (RPMI1640 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% FBS) at 37 °C with 5% CO_2.

1.2. Synthesis, purification and characterization of FA-Cat-BTZ and FA-PEG-Cat-BTZ.

1.2.1 Synthesis of FA-Cat

Folic acid (441 mg, 1 mmol) was activated by DCC (247 mg, 1.2 mmol) and NHS (230 mg, 2 mmol) in 20 mL of DMSO at 50 °C for 6 h. The resulting folate-NHS was mixed with dopamine (1.9 g, 10 mmol) and the mixture was stirred at room temperature for 5 h. The reaction was monitored by the TLC (chloroform/methanol, 3/7 v/v). The product was precipitated by addition of 20 mL of acetonitrile and centrifuged at 1000 rpm. The precipitate was washed with diethyl ether for three times before drying under vacuum. A fine yellow powder (folate-catechol linker, FA-Cat) was obtained. The yield was ~ 55%. The FA-Cat was characterized by ^{1}H-NMR (Bruker 300 MHz) using DMSO-d_6 as a solvent.

1.2.2 Synthesis of FA-Cat-BTZ

FA-Cat (5.94 mg, 0.01 mmol) was reacted with BTZ (3.84mg, 0.01mmol) in 1 mL of DMSO at 25 °C overnight. The reaction was monitored by the TLC (chloroform/methanol, 7/3 v/v). The final product (FA-Cat-BTZ) was purified by the preparative chromatography. The yield was ~ 80%. The product was characterized by the Advion Expression L Mass Spectrometer in atmospheric pressure chemical ionization (APCI) mode and ^{1}H-NMR using DMSO-d_6 as a solvent.

1.2.3 Synthesis of FA-PEG-Cat-BTZ

Maleimide-PEG-succinimidyl valerate (MAL-PEG3400-SVA) (6.8 mg, 2.2 µmol) was reacted with dopamine (0.45 mg, 2.2 µmol) in 2 mL of DMF in the presence of a trace amount of TEA at room temperature overnight. The crude was purified by the dialysis (MWCO 2,000 Da) against water for 48 h, followed by the freeze-dry to get the MAL-PEG-Cat as a fine white powder (yield: ~ 95%). Folic acid

(441 mg, 1 mmol) was reacted with DCC (247 mg, 1.2 mmol) and NHS (230 mg, 2 mmol) in 20 mL of DMSO at 50 °C for 6 h. The resulting folate-NHS was mixed with L-cysteine ethyl ester hydrochloride (Cys) (1.85 g, 10 mmol) and the mixture was stirred at 25 °C for 5h. The reaction was monitored by the TLC (chloroform/methanol, 7/3 v/v). The product folate-cysteine ethyl ester (FA-Cys) was precipitated by addition of 20 mL of acetonitrile and centrifuged. The precipitate was washed for three times with diethyl ether before drying under vacuum (yields: ~ 55%). The MAL-PEG-Cat and FA-Cys (1:1, molar ratio) were reacted in 2 mL DMSO in the presence of a trace amount of TEA at room temperature overnight to obtain folate-PEG-catechol linker (FA-PEG-Cat) (a fine yellow powder, yield: ~ 85%). The FA-PEG-Cat was reacted with BTZ using the methods of the synthesis of FA-Cat-BTZ. The FA-PEG-Cat-BTZ (yield: ~ 78%) was characterized by ^{1}H-NMR using DMSO-d_6 as a solvent.

1.3. Determination of BTZ by RP-HPLC

The BTZ content was quantitated by RP-HPLC [23] on a reverse-phase C18 column (250 mm × 4.6 mm, 5 µm) (Agilent) by a Waters HPLC system. The chromatographic conditions were: solvent A, ACN; solvent B, water. 0 - 11 min: 30% - 70% A, 12 - 15 min: 70% A. The sample was eluted at a flow rate 1.0 mL/min at room temperature and detected at 270 nm. The BTZ standard curve was: $y_{(BTZ)} = 718.63x - 320.45$, $R^2 = 0.999$.

1.4. pH-dependent cleavage and drug release

Two methods were used to analyze the pH-sensitive cleavage of the BTZ conjugate. For the TLC, 2 mg/mL of FA-Cat-BTZ was incubated with 1% TFA in DMF at room temperature for 0.5 h. The TLC was performed in chloroform/methanol (8/2, v/v) and visualized under UV 254 nm. Furthermore, the molecular weights of the spots were determined by the TLC/MS. For the ^{1}H NMR, 2 mg/mL of FA-Cat-BTZ was incubated with 1% TFA in DMSO-d_6 at room temperature for 0.5 h. The samples were directly analyzed by ^{1}H NMR. To study the cleavage kinetic /drug release, the Cat-BTZ (1 mM) was incubated with the PBS at various pHs (5.5, 6.0, 7.4, and 8.5) and in the 100% mouse plasma. The drug release (dissociation of Cat-BTZ) was monitored over 2 h by the RP-HPLC.

1.5. Cellular uptake determined by fluorescence-activated cell sorting (FACS)

The cells were seeded in 24-well plates at 1×10^5 cells/well 24 h before experiments. The FA-FITC (synthesized by the reported methods [24]) or FITC (12 µM) were incubated with the cells and retinoic acid pretreated (1 µM, 48 h) HeLa cells in the serum-free medium for 1.5 h. Then, the cells were trypsinized and collected by centrifugation at 1000 rpm for 2 min. After washing with ice cold PBS, the cells were re-suspended in 200 µL of PBS and immediately applied on a BD Accuri™ C6 flow cytometer (BD Biosciences). The data was collected (2.5×10^4 cell counts) and, upon acquisition, the forward vs. side scatter gating was used to exclude cell debris and dead cells.

1.6. Cellular uptake determined by fluorescence microscopy

The cells were seeded in 12-well plates at 2×10^5 cells/well 24 h before experiments. The FA-FITC or FTIC (12 µM) were incubated with the cells and retinoic acid pretreated (1 µM, 48 h) HeLa cells in the serum-free medium for 1.5 h. The cells were fixed by 4 % paraformaldehyde for 15 min at room temperature. To visualize cell nuclei, the cells were incubated with 2 µM of Hoechst 33258 in the dark at room temperature for 1 min. The photos were taken with a Nikon Ti Eclipse confocal microscope system and analyzed using the NIS-Elements BR software.

1.7. Drug uptake determined by HPLC

The cells were seeded at 4×10^5 cells/well in 6-well plates 24 h before treatments. The cells were incubated with BTZ or FA-Cat-BTZ at 10 μM for 4 h, and then washed with PBS for 5 times to remove the uninternalized drugs. The cells were lyzed by 200 μL of 1% triton x-100 in PBS. The drug was extracted by mixing with DMSO/ ACN mixture (1/4, v/v), followed by the centrifugation (10,000 rpm, 10 min) to remove cellular proteins. The internalized BTZ was quantitated by HPLC and normalized by the total protein content determined by the Pierce™ BCA protein assay (Thermo Fisher Scientific).

1.8. Cell viability assay

To study the cytotoxicity of the BTZ conjugates, the cells were seeded at 2×10^3 cells/well in 96-well plates 24 h before treatments. The BTZ, BTZ-mannitol, FA-Cat-BTZ and FA-PEG-Cat-BTZ were incubated with the cells and retinoic acid pretreated (1 μM, 48 h) HeLa cells for 48 h in complete growth media, respectively, followed by the MTT cell viability assay. Briefly, 20 μL of 5 mg/mL MTT reagent was diluted with 180 μL of the complete growth medium per well and incubated with the cells at 37 °C for 4 h. Then, the medium was removed and 150 μL DMSO was added. Thereafter, the absorbance intensity was recorded at λ_{abs} 570 nm on a microplate reader.

1.9. Proteasome activity assay

For the proteasome activity assay, the cells were seeded in 96-well plates at 4×10^3 cells/well and cultured for 24 h. The cells were treated with the BTZ, FA-Cat, FA-Cat-BTZ, and FA-PEG-Cat-BTZ at defined concentrations at 37 ºC for 6 h, respectively. The cells were then washed with PBS and analyzed by the proteasome activity assay kit (Cayman Chemical). The cells treated with the assay buffer only were used as the control.

1.10. Caspase activity assay

The cells were seeded in 96-well plates at 4×10^3 cells/well and cultured for 24 h. The cells were washed with PBS and incubated with the BTZ or FA-Cat-BTZ at 10 and 100 nM at 37 °C for 12 h. The caspase-3/7 activity was measured by the Apo-ONE® Homogeneous Caspase-3/7 Assay kit (Promega).

1.11. Apoptosis analysis

The cell apoptosis was analyzed by the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). Briefly, the cells were incubated with BTZ or FA-Cat-BTZ at 10 nM in the complete growth medium for 12 h or 24 h. The treated cells were trypsinized and collected by centrifugation at 1,000 rpm for 2 min. The cells were resuspended in 100 μL of the binding buffer and stained with 10 μL annexin V-FITC and 10 μL propidium iodide (PI) for 15 min at room temperature in the dark. Then, the stained cells were analyzed by the FACS.

1.12. Establishment of 3D multicellular cancer spheroids

The cancer cell spheroids were established by a well-developed method [25, 26]. Briefly, the 96-well flat-bottom plate was pre-coated with 50 μL of the culture medium containing 1.5% agarose (w/v). The cells were seeded in the plate at 7×10^3 cells per well. The plate was then centrifuged for 20 min at 2000 rpm and incubated at 37 °C and 5% CO_2 for the spheroid formation.

1.13. Cancer spheroid penetration

The 4-5-day spheroids were incubated with 6 μM FITC or FA-FITC for 1.5 h. After treatments, the

medium were removed by pipet and gently washed with PBS three times. Then, the spheroids were observed under a confocal microscope. The Z-stack images were obtained at an interval of 20 μm. The fluorescence intensity of each image was analyzed by the ImageJ software. The mean fluorescence intensity was plotted against the distance from the spheroid periphery.

1.14. Cytotoxicity in cancer spheroids

The cancer spheroids were incubated with the BTZ or FA-Cat-BTZ at 10, 100, and 1000 nM in the complete growth medium for 48 h. The cell viability was determined by the CellTiter-Blue® Cell Viability Assay (Promega). Briefly, 20 μL of the reagent was diluted with 200 μL of the complete growth medium per well and incubated with the spheroid at 37°C for 12 h [25, 26]. The fluorescence intensity was recorded at λ_{ex} 560 nm and λ_{em} 590 nm on a microplate reader.

1.15. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and processed with the SigmaPlot 12.5. T-test was used for statistical analyses. $P < 0.05$ is considered to be significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2. SUPPORTING DATA

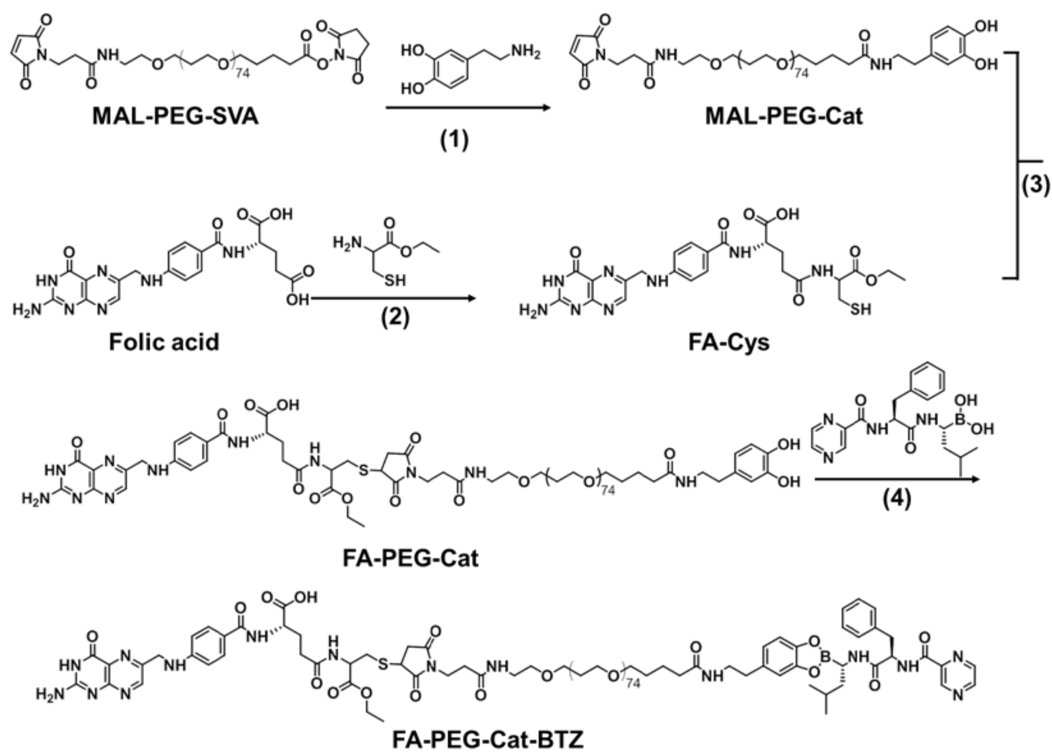
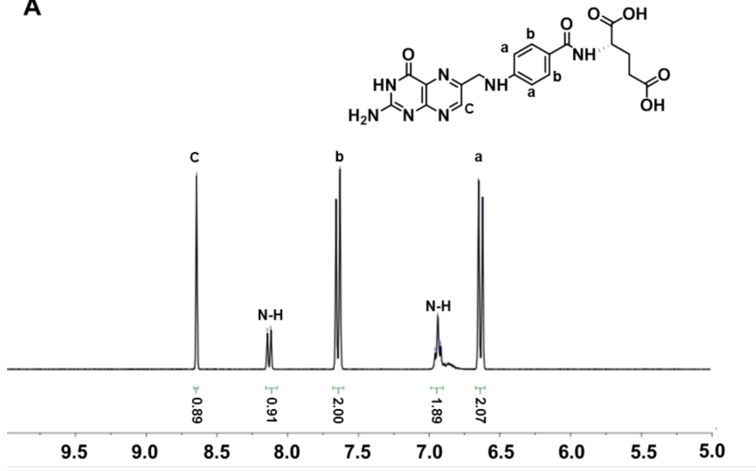


Figure S1. Synthesis of FA-PEG-Cat-BTZ. (1) DMF, room temperature; (2) DMSO, DCC/NHS, 50°C; (3) DMF, room temperature; (4) DMSO, room temperature. The average molecular weight of the PEG: 3,400 Da.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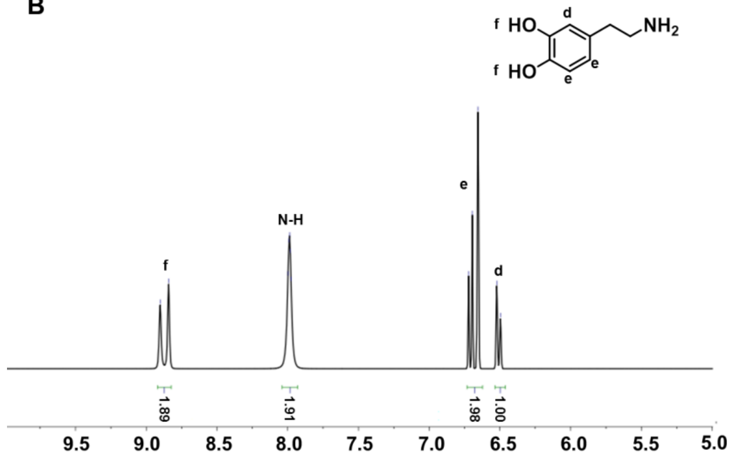
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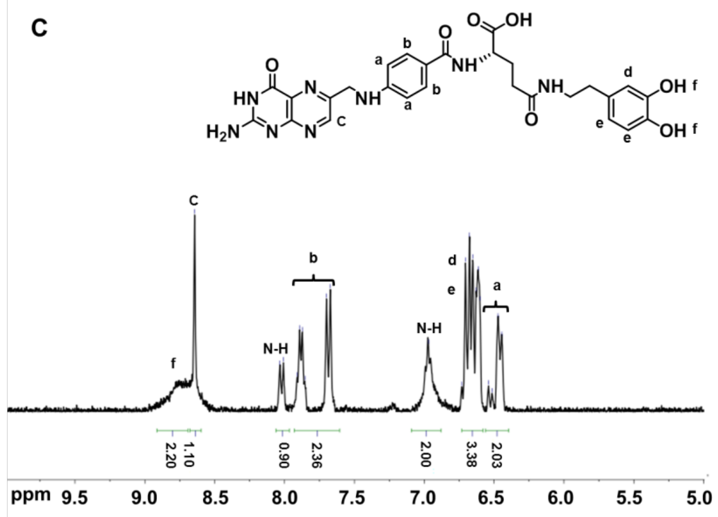
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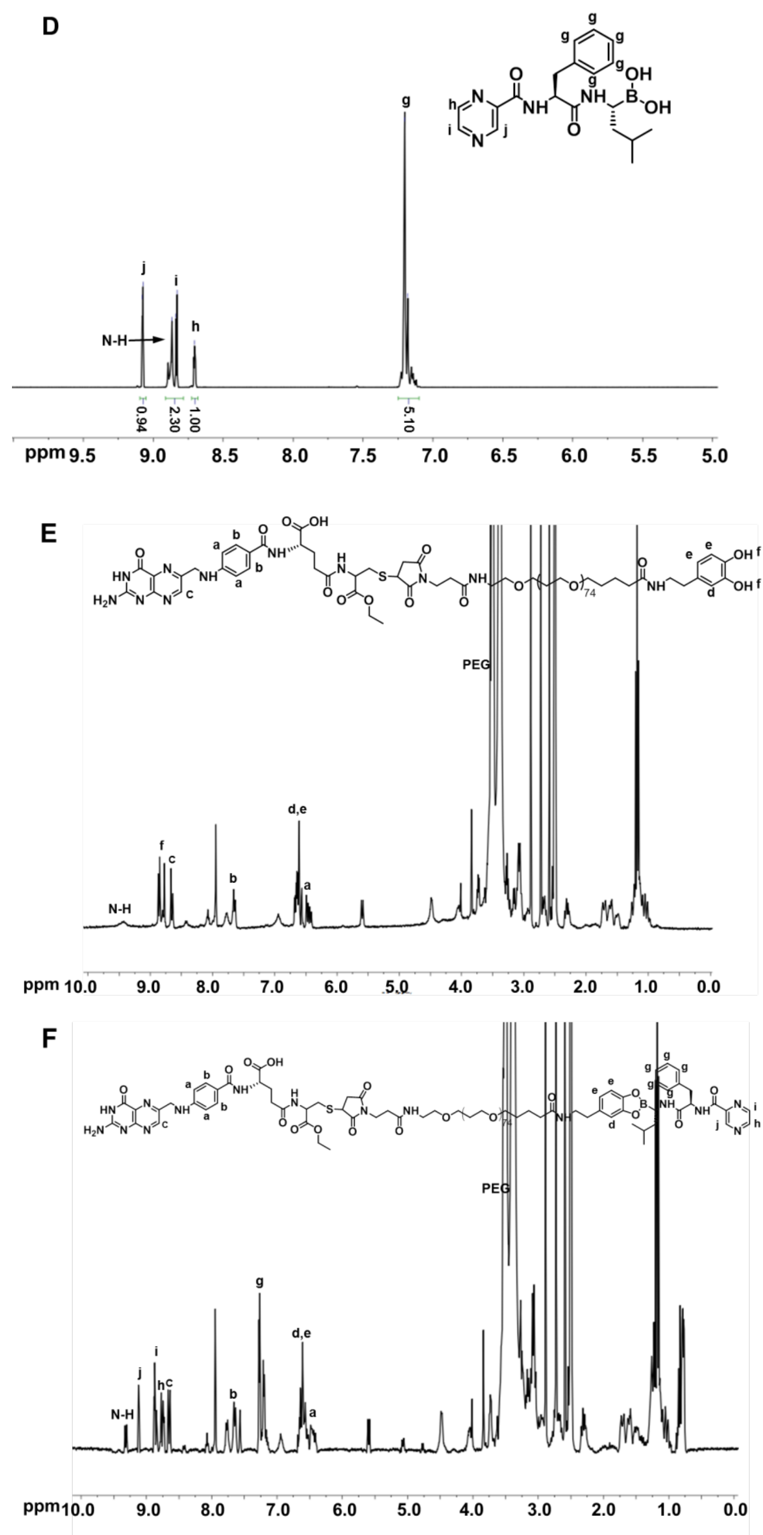

Figure S2. ^{1}H-NMR of FA (A), dopamine (Cat) (B), FA-Cat (C), BTZ (D), FA-PEG-Cat (E), and FA-PEG-Cat-BTZ (F) in DMSO-d_6.

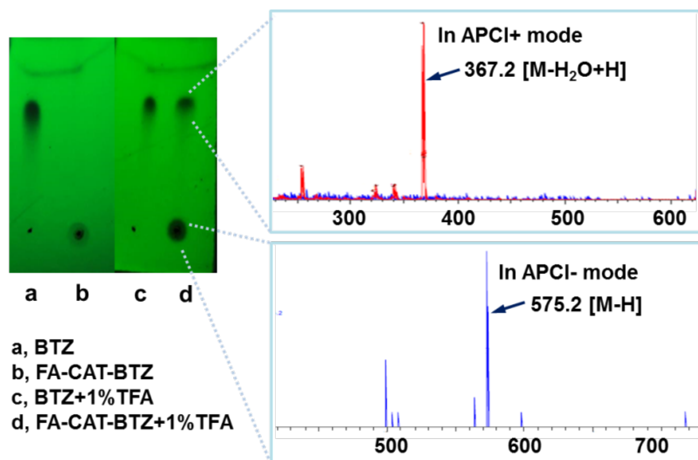

Figure S3. pH-sensitive drug dissociation of FA-Cat-BTZ determined by TLC and TLC/MS.

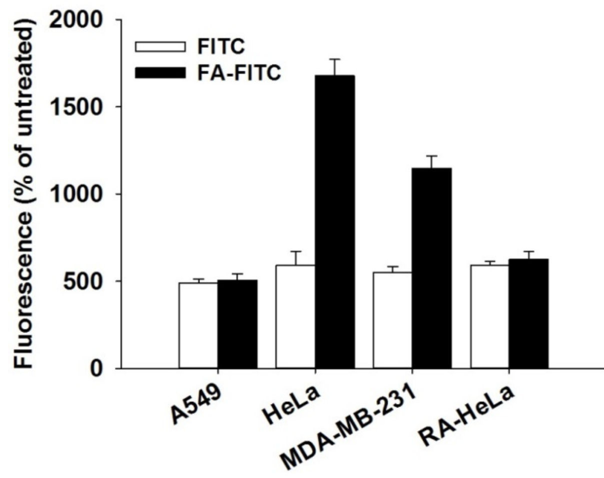

Figure S4. Cellular uptake of FA-FITC and FITC. Incubation time: 1.5h. RA-HeLa, the HeLa cells were pretreated with 1 μM retinoic acid (RA) for 48h before the experiment.

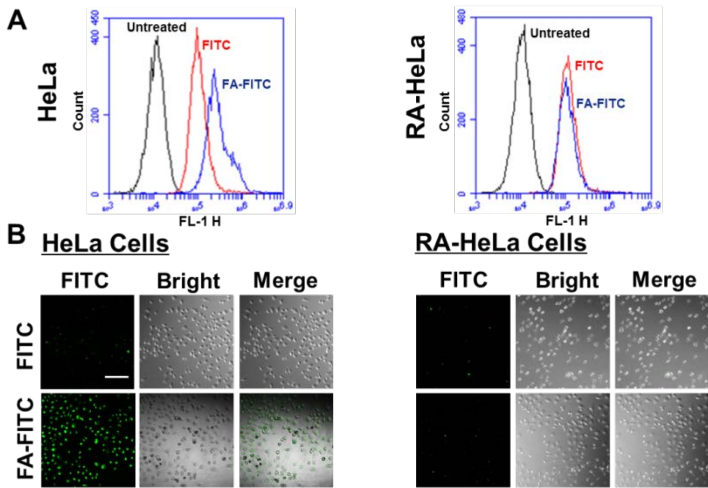

Figure S5. Folate-mediated cellular uptake in HeLa cells and retinoic acid (RA)-pretreated HeLa cells (1 μM, 48h). (A) Cellular uptake determined by FACS. (B) Cellular uptake determined by fluorescence microscopy. The cells were incubated with FA-FITC or FITC for 1.5 h. Scale bar: 100 μm.

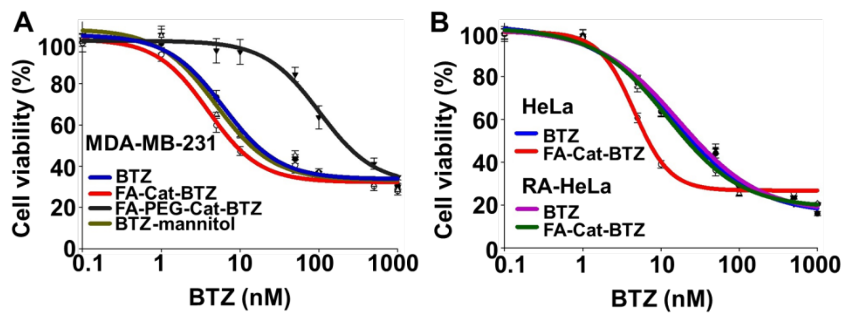

Figure S6. Cytotoxicity of the BTZ conjugates in the (A) MDA-MB-231 cells and (B) retinoic acid-pretreated HeLa cells (RA-HeLa). Incubation time: 48 h. Cell viability was determined by the MTT Assay.

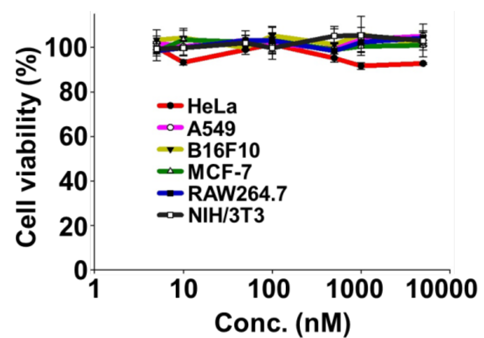

Figure S7. Cytotoxicity of non-drug component, FA-Cat, in the HeLa, A549, B16F10, MCF-7, RAW 264.7, and NIH/3TS cells (MTT Assay). Incubation time: 48 h.

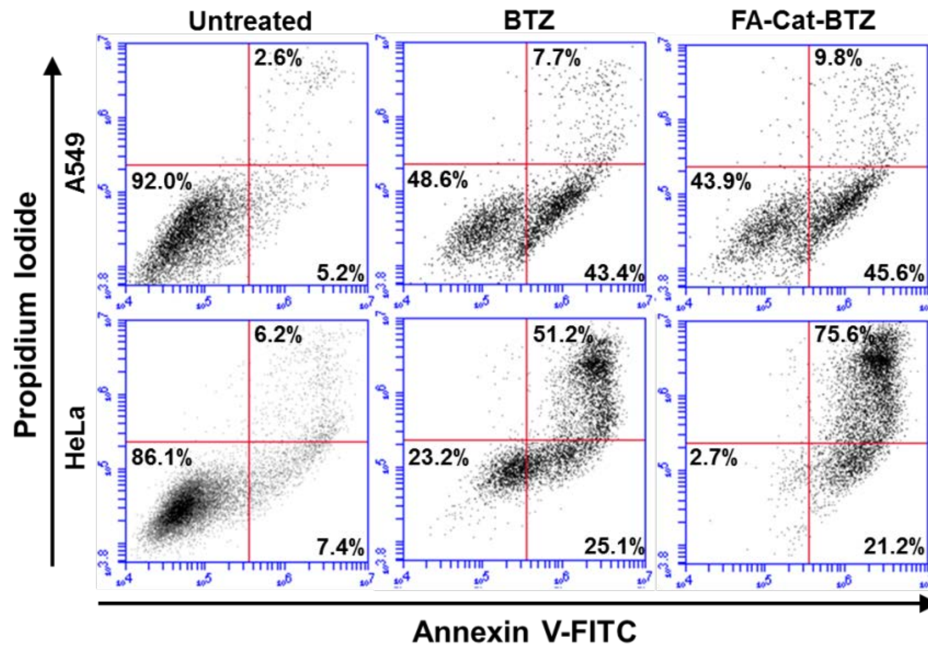

Figure S8. Cell apoptosis and death were analyzed by the annexin V and PI double staining. The results were obtained at 24 h after treatments.

Table S1 The IC_{50} of the BTZ and its conjugates in cancer cells (nM).

	A549	HeLa	MDA-MB-231
BTZ	38.9±1.2	31.0±1.5	23.9±0.9
FA-Cat-BTZ	42.7±2.1	6.9±0.2	12.4±0.3
FA-PEG-Cat-BTZ	320±12.4	206.6±11.4	273.7±13.4
BTZ-mannitol	62.4±3.4	15.3±3.4	21.6±1.2