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Electronic supplementary information

Acid-labile boronate-bridged dextran-bortezomib conjugate with up-regulated hypoxic tumor suppression

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1. Materials

Dextran (Dex; weight-average molecular weight (M_w) = 1 × 10^4 Da) was purchased from J&K Scientific Ltd. (Beijing, P. R. China). Bortezomib (BTZ) was obtained from Beijing HuaFeng United Technology Co., Ltd. (Beijing, P. R. China). Cell culture products including Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by Gibco (Gland Island, NY, USA). Penicillin and streptomycin were obtained from Huabei Pharmaceutical Co., Ltd. (Shijiazhuang, P. R. China). BCA Protein Assay Kit was purchased from Pierce Biotechnology (Rockford, IL, USA). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased Sigma-Aldrich (Shanghai, P. R. China). NF-κB p65 antibody was purchased from Beyotime Biotechnology Co., Ltd. (Nanjing, P. R. China), and CHOP antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The second antibody, *i.e.*, goat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated F(ab') 2, was purchased from Boster Biological Engineering Co., Ltd. (Wuhan, P. R. China).

2. Methods

2.1. Synthesis of Dex-BTZ

As shown in Scheme 1 in the text, BTZ (384.3 mg, 1 mmol, 0.2:1 (mol:mol) with respect to the glucose unit in Dex) and Dex (810.8 mg) were transferred to a 50 mL vial and dissolved in anhydrous dimethyl sulfoxide (DMSO; 10.0 mL). 4 Å molecular sieves (2000 mg) were added as a water-absorbing agent, and the reaction was stirred for 24 h at 60 °C. The reactant was filtered using 0.45 polytetrafluoroethylene membrane, and Dex-BTZ was obtained by the precipitation in cold ethanol (50.0 mL). Then the product was filtered, and washed with ethanol 3 times (10.0 mL × 3). The drug conjugation efficiency (DCE) was calculated by Equation (1).

$$\text{DCE}\ (\%) = \frac{\text{mole of drug in prodrug}}{\text{mole of glucose unit in prodrug}} \times 100 \quad (1)$$

In Equation (1), the mole of BTZ in prodrug was determined using ultraviolet-visible (UV-vis) spectrophotometry with λ_{ab} = 280 nm (UV-1800, Shimadzu, Kyoto, Japan).

2.2. Proton nuclear magnetic resonance (^{1}H NMR) and Fourier-transform infrared spectroscopy (FT-IR) detections

^{1}H NMR spectra were recorded on a Bruker AV 600 NMR spectrometer in deuterated dimethyl sulfoxide (DMSO-d_6) and water (D_2O) (1:1, V/V). FT-IR spectra were recorded on a Bio-Rad Win-IR instrument using potassium bromide method.

2.3. Dynamic laser scattering (DLS) and transmission electron microscopy (TEM) measurements

DLS measurements were performed with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology). TEM assays were performed on a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 kV.

2.4. *In vitro* BTZ release

The BTZ release from Dex-BTZ was operated in phosphate-buffered saline (PBS) at different pHs (*i.e.*, 5.0, 6.8, and 7.4), and in PBS with different glucose concentrations (*i.e,*. 0.1 and 5.0% (W/V), mimicking the preprandial blood glucose level in healthy human body and the glucose concentration in clinical isotonic glucose injection, respectively) at pH 7.4, 37 °C. Dex-BTZ was weighted and dissolved in release medium with a concentration of 0.2 mg mL^{-1}. In detail, 10.0 mL of diluted solution was transferred into a dialysis bag (molecular weight cut off (MWCO) = 3500 Da). The release experiment was initiated by placing the end-sealed dialysis bag into 100.0 mL of release medium at 37 °C with 75 rpm constant shaking. At preselected time intervals, 2.0 mL of release medium was taken out and replenished with an equal volume of fresh one. The amount of released BTZ was determined using UV-vis spectrophotometer.

2.5. Cellular uptakes

Human hepatocarcinoma HepG2 cells were cultured in complete DMEM supplemented with 10% (V/V) FBS, penicillin (50.0 IU mL^{-1}), and streptomycin (50.0 IU mL^{-1}) in conventional culture condition (5%, V/V, carbon

dioxide (CO_2), 37 °C). The cells were seeded in 6-well plates with a density of 2.0 × 10^5 cells per well in 2.0 mL of complete DMEM and conventionally cultured for 12 h. Subsequently, the preconditioning in normoxia and hypoxia were respectively implemented for 12 h as follows: i) preconditioning in normoxia, the cells were cultured in conventional culture condition for 12 h; ii) preconditioning in hypoxia, the cells were cultured in a hypoxic environment (95% nitrogen (N_2), 4% CO_2, and 1% oxygen (O_2), (V/V), 37 °C) for 12 h. The original medium was replaced with Dex-BTZ or BTZ solution in complete DMEM at a final BTZ concentration of 10.0 μM. The cells were further conventionally incubated for 2 h, and then washed with PBS 5 times. After adding equal volume of distilled water, the repeated freezing and thawing cycles (−80 °C and 37 °C, 6 cycles) were operated toward cells for obtaining the solutions of broken cells. The amount of BTZ and protein in cells were detected by inductively coupled plasma-mass spectrometry (ICP-MS) and BCA Protein Assay Kit, respectively. The cellular uptakes of BTZ after the incubation with various BTZ formulations were quantified in the form of μg mg^{-1} protein.

2.6. Cytotoxicity assays

The cytotoxicities of Dex-BTZ and BTZ were evaluated by a MTT assay. HepG2 cells with a density of 7,000 cells per well were seeded in 96-well plates in 150.0 μL of complete DMEM and incubated for 12 h. The normoxic and hypoxic preconditioning were implemented for another 12 h, respectively. The culture medium was replaced with 200.0 μL of fresh medium containing Dex-BTZ and BTZ with BTZ concentrations up to 10.0 μM. The cells were subjected to MTT assay after being conventionally incubated for another 48 h, and then 100.0 μL of a stock solution containing 0.05 mg of MTT in PBS was added and incubated for further 4 h. And then, the medium was replaced with 100.0 μL of DMSO. The absorbance of above solution was measured on a BioTek ELx808 microplate reader at 490 nm. The cell viability was calculated based on Equation (2).

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (2)$$

2.7. Cell apoptosis analyses

The cells were seeded in 6-well plates with a density of 2.5 × 10^5 cells per well. The same normoxic or hypoxic preconditioning was operated as mentioned above. The original medium was replaced with Dex-BTZ and BTZ solution in complete DMEM at a final BTZ concentration of 10.0 nM. The cells were further conventionally incubated for 48 h and harvested gently. Then the Annexin V-FITC (AnV-FITC) and propidium iodide (PI) were used to label cells. The apoptosis analyses of HepG2 cells were determined by Flow cytometry (FCM, BD Biosciences, San Jose, CA, USA).

2.8. Cell immunohistochemical staining

The same pre-treatment was carried out as the cell apoptosis analysis, besides that a coverslip was placed in each well before the start of experiment. At the same time interval, the cells was washed and fixed with precooled acetone at −4 °C for 20 min at room temperature. The immunohistochemical stainings (*i.e.*, NF-κB and CHOP) were performed according to the routine method as follows: i) antigen retrieval; ii) serum closure; iii) adding primary antibody; iv) adding second antibody. The photos were taken by confocal laser scanning microscopy (CLSM; Carl Zeiss, LSM 780, Jena, Germany).

2.9. Animal procedures

The male BALB/c mice were obtained from the Laboratory Animal Center of Jilin University, and used at 7 weeks of age. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Animal Care and Use Committee of Jilin University. The tumor-bearing mice were prepared through the subcutaneous injection in the armpits of right anterior limbs with 0.1 mL of cell suspension containing 1.5 × 10^6 mouse hepatocarcinoma H22 cells in PBS.

2.10. *In vivo* antitumor assays

The antitumor tests were performed when the tumor volumes grew up to around 80 mm^3 (n = 8 for each group). The mice xenografted with H22 hepatocarcinoma were treated with Dex-BTZ and BTZ with an

equivalent BTZ dose of 2.0 mg kg^{-1} body weight, and normal saline as control (CON) by intravenous administration on Day 2, 5, and 8. The tumor volume and tumor inhibition rate were calculated using Equation (3) and (4), respectively.

$$V\,(\mathrm{mm}^3) = \frac{L \times S^2}{2} \tag{3}$$

$$\text{Tumor inhibition rate}\,(\%) = \frac{V_c - V_e}{V_c} \times 100 \tag{4}$$

In Equation (3), L and S (mm) were the largest and smallest diameters of tumor, respectively. In Equation (4), V_e and V_c were denoted as the tumor volumes in the experimental and control groups, respectively.

2.11. Histopathological and immunohistochemical analyses

After all the treatments, the tumors were collected and cut into ~ 5 and ~ 7 μm slices. The ~ 5 and ~ 7 μm slices were stained with hematoxylin and eosin (H&E) and immunohistochemistry (*i.e.*, NF-κB and CHOP), respectively. The methods were operated as previously described in Section 2.8. The H&E microimages were taken by the microscope (Nikon Eclipse *Ti*, Optical Apparatus Co., Ardmore, PA). Three observation fields were repeated to get an average value of relative necrosis area with the area of total observation field as "100%". The immunohistochemical microimages were taken by CLSM. The optical density of CON group was defined as "1", and the relative optical density was calculated by the optical density ratio of sample and control groups. The ImageJ software was used in the above operation for analyzing microimages.

2.12. Statistical Analysis

All tests were implemented at least three times, and the data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). $^{*}p < 0.05$ was considered statistically significant, and $^{**}p < 0.01$ and $^{\#}p < 0.001$ were considered highly significant.

3. Supplementary figures

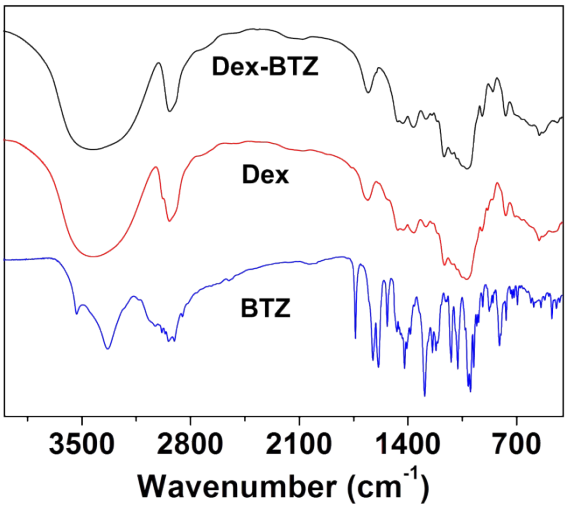

Fig. S1 FT-IR spectra of Dex-BTZ, Dex, and BTZ.

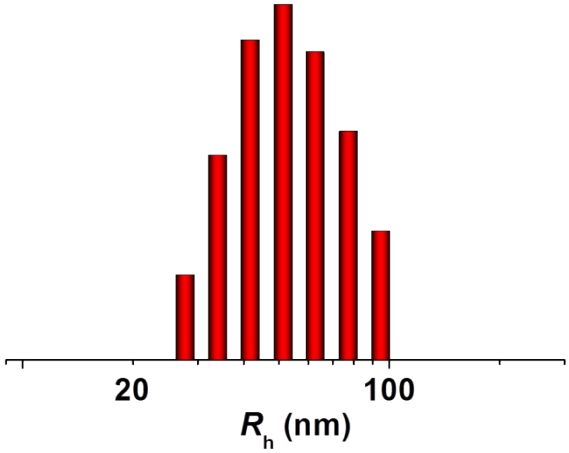

Fig. S2 Hydrodynamic radius (R_h) of Dex-BTZ micelle.

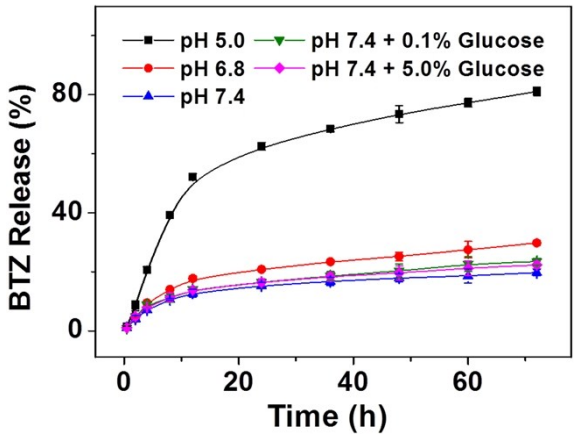

Fig. S3 pH- and time-dependent BTZ release profiles of Dex-BTZ in PBS at various pHs, and in PBS with different glucose concentrations (*i.e,*. 0.1 and 5.0% (W/V)) at pH 7.4, 37 °C. The data were represented as mean ± SD (*n* = 3).

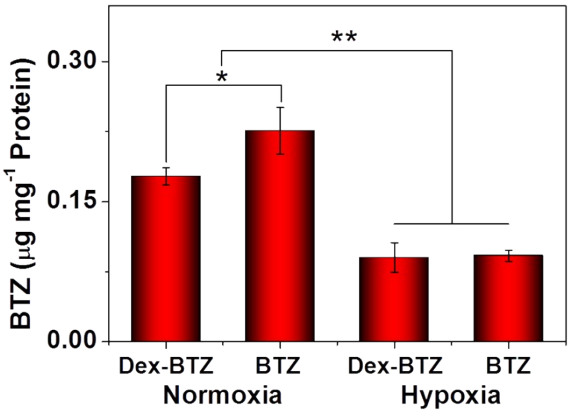

Fig. S4 Cellular uptakes of BTZ after incubation with Dex-BTZ and BTZ toward HepG2 cells pretreated with normoxia and hypoxia for 2 h. The data were represented as mean ± SD (n = 3, *p < 0.05, **p < 0.01).

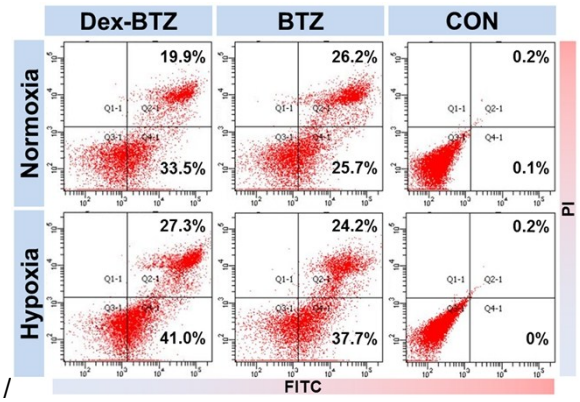

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Fig. S5 Apoptosis analyses of HepG2 cells after incubation with Dex-BTZ and BTZ for 48 h.

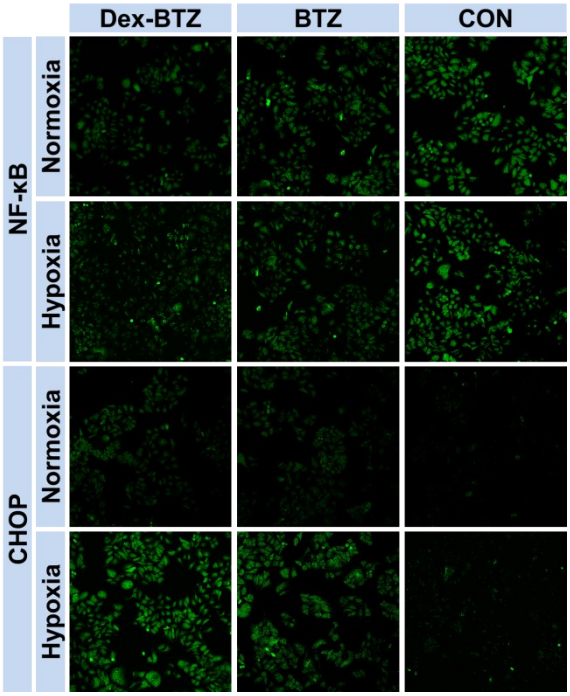

Fig. S6 Immunohistochemical analyses (*i.e.*, NF-κB and CHOP) of HepG2 cells preconditioned in normoxia and hypoxia after co-cultured with Dex-BTZ and BTZ for 48 h. Magnification: 100 ×.

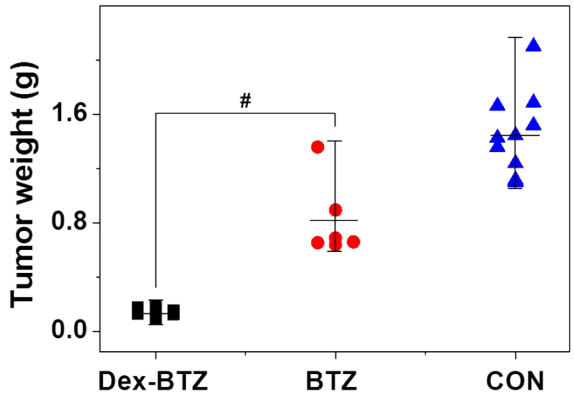

Fig. S7 Tumor weight of H22 hepatocarcinoma-xenografted mice after treatment with Dex-BTZ, BTZ, or NS. The data were presented as mean ± SD (n = 8, $^{\#}p < 0.001$).

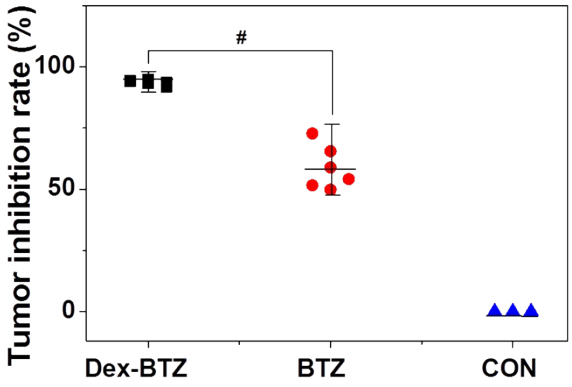

Fig. S8 Tumor inhibition rate of H22 hepatocarcinoma-xenografted mice after treatment with Dex-BTZ, BTZ, or NS. The data were presented as mean ± SD (n = 3, $^{\#}p < 0.001$).

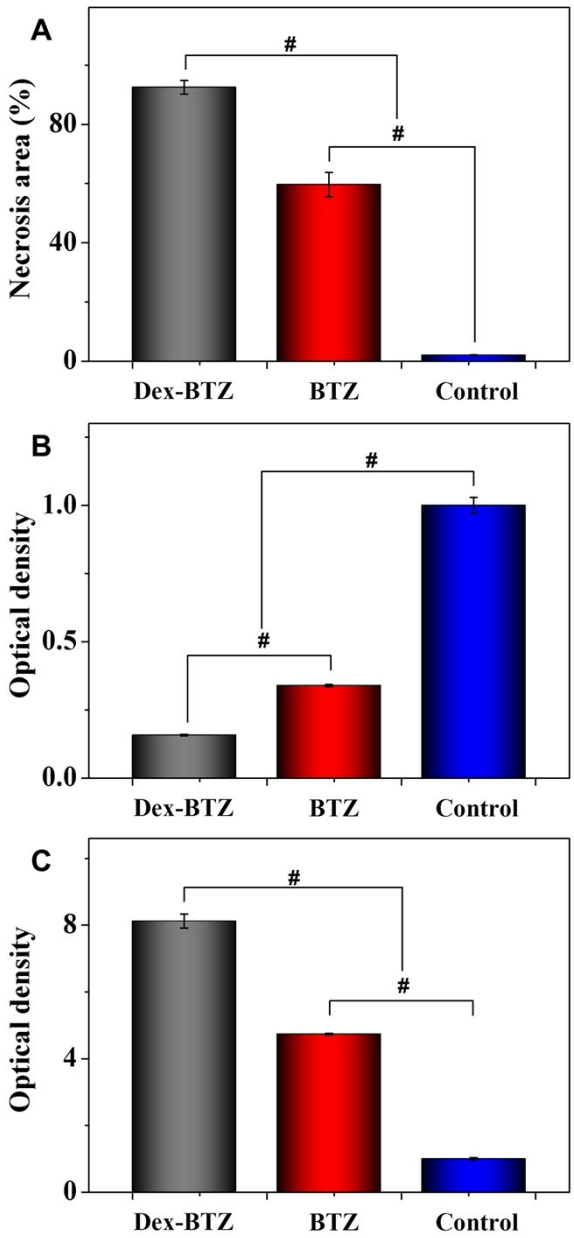

Fig. S9 Relative necrosis area of tumor section from H&E staining (A), and relative optical densities of tumor sections from NF-κB (B) and CHOP staining (C) after treatments with Dex-BTZ, BTZ, or NS. The data were presented as mean ± SD (n = 3, $^{\#}p < 0.001$).