Catechol Polymers for pH-Responsive, Targeted Drug Delivery to Cancer Cells

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Supporting information

Materials and General Procedures:

CH_3O-PEG-NH_2 (MW 550) and mono-protected di-amine terminated tert-butoxycarbonyl-NH-PEG-NH_2 (tBoc-NH-PEG-NH_2, MW 2000) were purchased from LaysanBio, INC (Arab, AL). Fmoc protected amino acids, synthesis resins and d-biotin were purchased from Anaspec, INC. (Fremont, CA). Bortezomib was purchased from ChemieTek (Indianapolis, IN). All other chemicals, unless stated otherwise, were purchased from Sigma-Aldrich. Nuclear magnetic resonance (NMR) spectra were recorded on AVANCE III 500 MHz with direct cryoprobe. Spectra were recorded in $CDCl_3$ or D_2O solutions at 293K. LC-MS analysis was performed on an Agilent 6520 quadrupole time-of-flight (Q-TOF) mass spectrometer. MALDI-TOF MS analysis was performed in positive mode on Bruker Apex III MALDI TOF mass spectrometer.

1. Synthesis of PEG-catechol (compound 1, **PEG-Cat**)

   100 mg (0.2 mmol) of CH_3O-PEG-NH_2 (average MW: 528) was dissolved in 4 ml dimethylformamide (DMF), to which a solution of 72 mg (0.4 mmol) 3-(3,4-dihydroxyphenyl)propionic acid (DHPA), 177mg (0.4 mmol) benzotriazol-1-yloxy) tris(dimethylamino) phosphonium hexafluorophosphate (BOP), 54 mg (0.4 mmol) 1-hydroxybenzotriazole (HOBT) and 350 μl *N,N*-diisopropylethylamine (DIPEA) in 1ml DMF was added dropwise. The mixture was stirred at room temperature for 8 hours. Completion of the amide formation between CH_3O-PEG-NH_2 and DHPA was confirmed by a negative ninhydrin

test. 30 ml cold ether was added to precipitate the crude product, which was then centrifuged at 0 °C to remove supernatant to give an oil-to-wax-like residue. 5ml H_2O was added to dissolve the residue and the solution was filtered through 0.45 μm pore size membrane filter. Lyophilization of the filtrate gave the product as a pale-yellow wax.

1H NMR ($CDCl_3$, 500 MHz) δ (ppm): 6.5~6.7 (m, 3H), 3.50~3.70 (bm, 56H), 2.81 (dd, 2H), 2.68 (dd, 2H)

MS $(M+1)^+$: average MW 692.6

2. Synthesis of biotin-PEG-NH_2

0.1 mmol (200mg) of tBoc-NH-PEG-NH_2 (average MW 1990.2) was dissolved in 500 μl DMF and 0.2 mmol (42mg) biotin was dissolved separately in 500μl dimethyl sulfoxide (DMSO). The two solutions were mixed, to which 0.2 mmol BOP, 0.2 mmol HOBT and 0.25 mmol DIPEA in 500 μl DMF were added. The reaction mixture was stirred at room temperature for 12 hours. A negative ninhydrin test confirmed completion of the amide formation between (tBoc)NH-PEG-NH_2 and biotin. 30 ml cold ether was added to precipitate the crude product, and the mixture was centrifuged at 0 °C to remove the supernatant and gave a waxy residue. Without further purification, deprotection of tBoc group on the crude product was carried out as follows. About 220 mg crude product (tBoc)NH-PEG-NH-biotin was dissolved in a mixture of 5 ml dichloromethane (DCM) and 5ml trifluoroacetic acid (TFA). The solution was stirred at room temperature for 2 hours and the solvent was evaporated under vacuum. 2ml TFA was added to dissolve the oily residue and 30 ml cold ether was added to precipitate the crude product. Centrifugation of the mixture at 0 °C gave a soft wax-like product. 5ml H_2O was added to dissolve the residue and the solution was filtrated through a 0.45 μm pore size membrane filter. Lyophilization of the filtrate gave the product biotin-PEG-NH_2 • TFA as a white, solid wax.

MS $(M+1)^+$: average MW 2117.0

3. Synthesis of biotin-PEG-FITC (compound 2)

   50 mg (0.024mmol) TFA salt of biotin-PEG-NH_2 was dissolved in 1 ml DMF and neutralized with 100 μl DIPEA. A solution of 15 mg (0.039 mmol) fluorescein 5-isothiocyanate in 500 μl DMF was added and the mixture was stirred for 4 hours at room temperature. The mixture was diluted in 20 ml DCM and extracted with 5ml 0.1 N HCl in H_2O, 5 ml H_2O and 5 ml saturated NaCl in H_2O. The DCM solution was dried over $MgSO_4$ and evaporation of the solvent under vacuum gave the product as a yellow oil. (39 mg, yield 80%).

   MALDI-TOF MS $(M+Na)^+$: average 2504.1

4. Synthesis of fully protected peptide Ac-$[Lys(tBoc)]_4$-Gly–OH

   Standard solid phase peptide synthesis was carried out using Fmoc protected amino acids with BOP/HOBT as coupling agents. Briefly, 200 mg glycine-loaded 2-chlorotrityl resin (0.8 meq/g) was washed and swelled in DMF. 225mg (0.48 mmol, 3 eqv.) Fmoc-Lys(tBoc)-OH, 212mg (0.48 mmol) BOP, 65mg (0.48 mmol) HOBT and 90 ul DIPEA were dissolved in 4 ml DMF and added to the resin. The reaction vessel was vigorously rocked for 4 hours and negative ninhydrin test indicated the coupling was complete. The resin was washed with DMF 3 times and a solution of 20% piperidine in DMF was used to remove the Fmoc group and expose the free amine groups on the peptide-conjugated resin for the next coupling cycle. Such coupling and deprotection cycle was repeated 4 times. After exposure of amine groups on $[Lys(tBoc)]_4$-Gly–resin, a solution of 1ml acetyl anhydride and 300 μl DIPEA in 4 ml DMF was used to cap the α-amine of the last lysine residue. The resin was washed thoroughly in DMF and then DCM prior to drying under vacuum overnight. The dried resin was suspended in 10 ml of a DCM solution containing 1% TFA and gently stirred at room temperature for 1 hour. The suspension was filtered through a glass filter to remove the resin, and the filtrate was concentrated to about 1ml under vacuum. Cold

H_2O was added to precipitate the product peptide acid and suction filtration gave the product as a white solid powder. Without further purification, this crude product was directly used to synthesize Ac-$(Lys)_4$-Gly-PEG-biotin.

MALDI-TOF MS $(M+Na)^+$: 1052.7, $(M-1)^-$: 1028.5

5. Synthesis of Ac-$(Lys)_4$-Gly-PEG-biotin

110 mg (~ 0.05 mmol) compound 2 was dissolved in 2 ml DMF and neutralized with 100 µl DIPEA. 42 mg (~0.04 mmol) Ac-[Lys(tBoc)]$_4$-Gly–OH, 22 mg (0.05 mmol) BOP, 7 mg (0.05 mmol) HOBT and 25µl IPEA was mixed in 1ml DMF and the mixture was added to the neutralized solution of compound 2. The final reaction mixture was stirred at room temperature for 16 hours. 20ml diethyl ether was added to precipitate the crude product and centrifugation of the mixture at 0 °C gave a waxy residue. 10ml of a solution containing 50% DCM and 50%TFA was used to dissolve the residue and stirred at RT for 3 hours to remove the tBoc groups at the side chains of lysine on the PEG-peptide conjugate. The reaction mixture was concentrated under vacuum to about 2 ml and 25 ml cold ether was added. After centrifugation of the mixture at 0 °C, crude product was obtained. 10 ml H_2O was added to dissolve the residue and lyophilization of the solution gave the product compound 4 as white, solid wax/powder, which was further purified by RP-HPLC.

MALDI-TOF MS $(M+1)^+$: average 2728.4

6. Synthesis of Ac-[Lys(ε-NH-catechol)]$_4$-Gly-PEG-biotin (BPC)

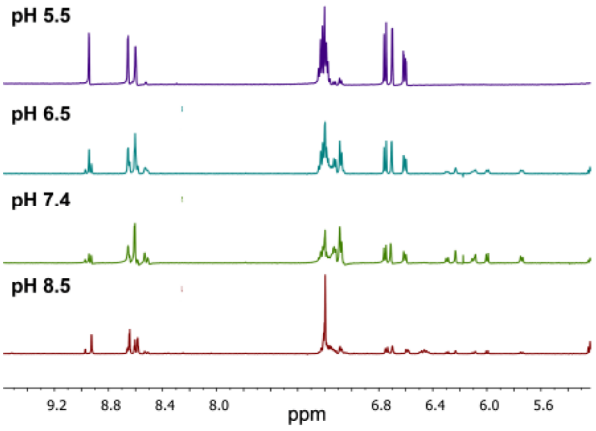
50 mg (~ 0.018 mmol) Ac-$(Lys)_4$-Gly-PEG-biotin was dissolved in 1 ml DMF and neutralized with 50 µl DIPEA. 37 mg (0.2mmol) DHPA, 0.2 mmol DBTU, 0.2 mmol HOBT and 130 µl DIPEA were mixed in 2 ml DMF and the mixture was added to the neutralized solution of Ac-$(Lys)_4$-Gly-PEG-biotin. The final reaction mixture was stirred at room temperature for 20 hours.

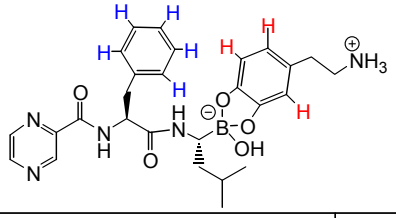
25ml diethyl ether was added and centrifugation of the mixture at 0 °C gave a light brown, waxy residue. This crude product was further purified by RP-HPLC.

MALDI-TOF MS $(M+Na)^+$: average 3406.7

7. H^1-NMR analysis of pH-dependent dissociation of bortezomib-catechol conjugates

Bortezomib (BTZ) and dopamine (DA) hydrogen chloride were dissolved separately in d^6-DMSO at 0.2 M concentration. The two solutions were mixed to give a stock solution of DA-BTZ conjugate at 0.1 M in DMSO. A solution of 0.1 M monosodium phosphate in D_2O was used to dilute the DA-BTZ stock to 1 mM, and pH of such solutions were adjusted with 4N NaOH in D_2O to 5.5, 6.5, 7.4 and 8.5. These solutions were analyzed an hour after preparation on AVANCE III 500 MHz and peak integrals in the range of 7.2 to 5.5 ppm were used for quantifying the degree of dissociation of DA-BTZ presented in Figure 2B.





	Number of H			
pH	7.2 ppm	6.6-6.8 ppm	5.5-6.5 ppm	(DA-BTZ)%
	(reference)	(free DA, I)	(DA-BTZ, II)	= I/(I+II)
5.5	5	3	0	0%
6.5	5	1.875	0.75	29%
7.4	5	1.5	1.5	50%
8.5	5	1.125	1.525	58%

Figure SI 1 Characterization of pH-dependent association of the dopamine-bortezomib conjugate (DA-BTZ) by H^1-NMR. The peaks around 7.2 ppm correspond to H atoms on the phenyl ring of bortezomib (highlighted in blue). The peaks between 6.6 and 6.8 ppm represent Hs on the catechol ring of DA (highlighted in red). In DA-BTZ conjugates, Hs on the catechol ring shifted to the range of 6.5~5.5 ppm. Peak integrals from the ranges above were used for calculating degree of DA-BTZ association.

8. The amphiphilicity of **BPC** gave micelle-like structures in the range of 30~50 nm in diameter in aqueous solutions (pH 7.4), and the size of microstructure increased to 50~80 nm after the conjugation of BTZ.

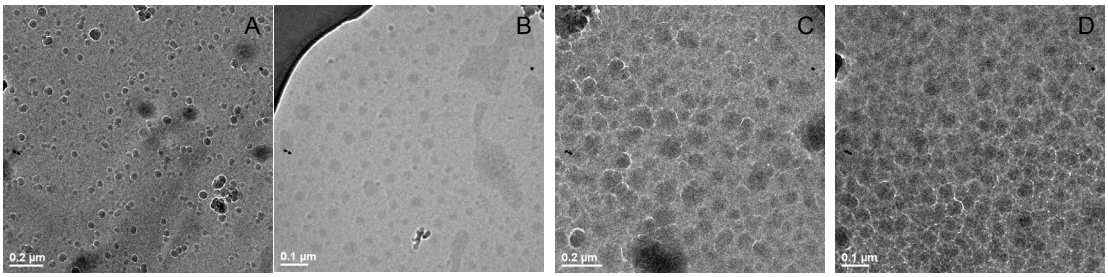


Figure SI 2 Cryo-TEM analysis was performed on Hitachi H-8100 transmission electron microscope using solutions containing 0.25 mM **BPC** in 0.1M phosphate monosodium buffer before (A and B) and after (C and D) conjugation of 1 mM bortezomib at pH 7.4. The scale bars are 0.2 μm in A and C and 0.1 μm in B and D.

9. Cell culture and biotin-mediated cell uptake of PEG-FITC

MDA-MB-231 and MCF-7 breast cancer cells were originally purchased from ATCC and cultured in MEM containing 10 mM L-glutamine, nonessential amino acids, 10 mM HEPES, 100 U/ml streptomycin, 100 U/ ml penicillin and 10% FBS at 37°C under 5% CO_2. Passages between 9 and 12 were used for all the cell experiments in our work. MCF-10A-Vector and MCF-10A-H-RasV12 cells[1] were cultured in DMEM-F12 media supplemented with 20 ng/ml EGF, 10 μg/ml insulin, 0.5μg/ml hydrocortisone, 100 ng/ml cholera toxin, 100 U/ml streptomycin, 100 U/ ml penicillin and 5% FBS. Cells were seeded in 24-well plates at a density of 1×10^5 cells/ml and cultured for 24 hours. Cells were washed with PBS containing 0.91 mM Ca^{2+} and 0.56 mM Mg^{2+} and treated with solutions of 1 μM compound 2 or 3 in PBS for 15 minutes at 37°C. Cells treated with PBS only were used as control. Varying concentrations of biotin in buffer were used to inhibit the cellular uptake of biotin-

PEG-FITC. Cells were then washed with PBS and imaged under an Eclipse TE2000U inverted fluorescence microscope at 10x magnification (Figure SI 3).

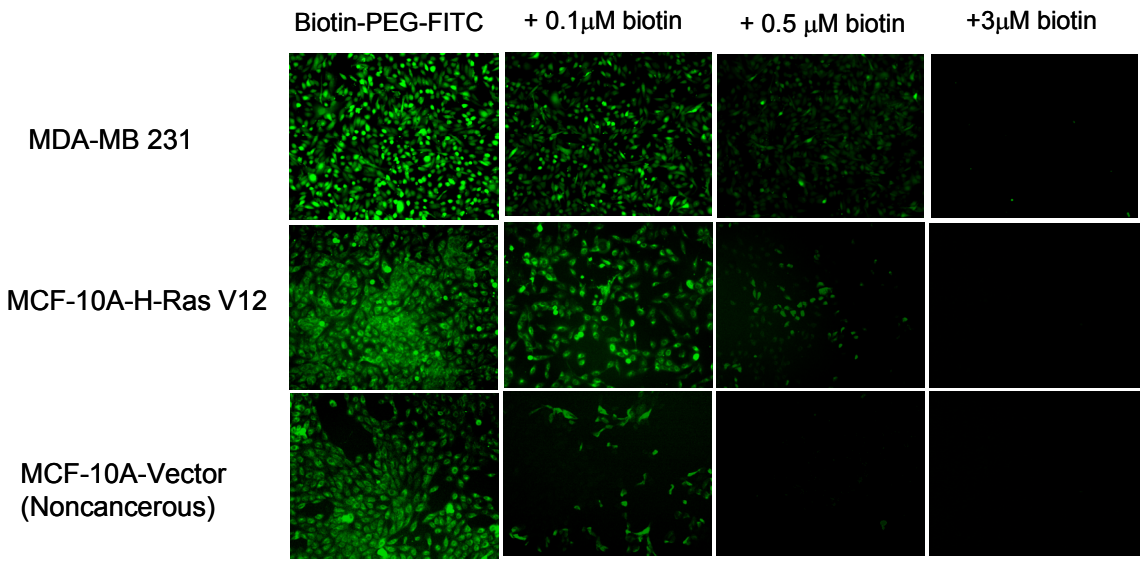


Figure SI 3 Dose-dependent inhibition of cellular uptake of biotin-PEG-FITC by free biotin. Although cancerous and noncancerous cells were able to take up the biotinylated fluorescent polymer, higher concentrations of free biotin were needed to inhibit polymer uptake by cancer cells compared to noncancerous cells, implying higher expressions of biotin receptor on cancer cell surface. All images were taken after 15 minute-treatment.

10. Time-dependent release of BTZ from catechol polymers

BTZ and the catechol polymer (BPC or PC) were separately dissolved in DMSO at 0.1 M. The stock solutions are mixed at volume ratio 4:1, which were further diluted in PBS to achieve a 0.2 mM BTZ conjugated to 0.05 mM polymer (BPC or PC). This and a solution of 0.05 mM polymer (BPC or PC) only were mixed at varying ratios to give series of solutions containing 0.05 mM polymer (BPC or PC) and BTZ concentration ranging from 0~0.2 mM at defined pH. UV absorbance at 260 nm was measured for each solution to plot standard curves. 120 ul aliquots of

the above BTZ-polymer solutions were put in Slide-A-Lyzer MINI Dialysis Device Floats (Thermo Scientific, USA) with a MWCO of 2000 g / mol in preparation for release experiments at multiple time intervals. The tubes were placed in 1.0 L of 10 mM buffer solutions with pH at 5.0, 6.5 and 7.4. Temperature was held constant at 37°C throughout the experiments, mimicking physiological conditions in the body. The sampling time intervals were 0, 0.5, 1.5, 3, 6, 12 and 24 hrs. At each time interval, a sample of 100 μL was withdrawn from a tube for UV absorbance analysis at 260 nm. Three repetitions of each release experiment were conducted and amount of released BTZ was calculated and converted to percentage of total BTZ loaded to BPC or PC.

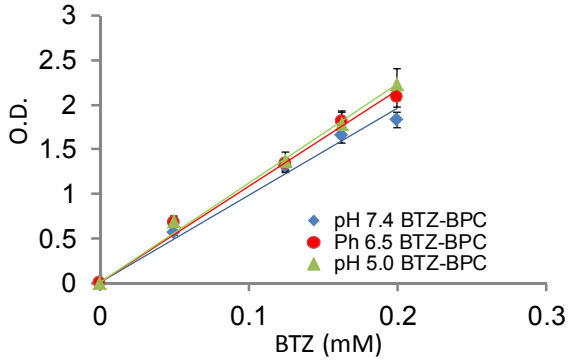


Figure SI 4. Standard curves of concentration-dependent BTZ-BPC absorbance at 260 nm at pH 5.0, 6.5 and 7.4, which was used to calculate BTZ release presented in Figure 4.

11. Preparation of catechol polymer-bortezomib conjugates for cytotoxicity assays

For all the experiments where polymer-bortezomib conjugates were tested, the following sample preparation method was used. Bortezomib and catechol polymers including PC and BPC were separately dissolved in DMSO at 0.1 M. Bortezomib-polymer conjugates were prepared by mixing solutions of 0.1M bortezomib and 0.1M PC or BPC in DMSO at volume ratio 4:1. These stock solutions were then diluted in PBS (containing Ca^{2+} and Mg^{2+}) to achieve a 0.1mM concentration of each compound, which were further diluted with the cell assay buffers (cell growth media containing 1% serum) to obtain final solutions used in cytotoxicity studies.

12. Proteasome activity assay

MDA-MB231 breast carcinoma cells were seeded in 96-well plates at 1×10^5 cells/ml and cultured for 24 hours. Cells were washed with PBS and treated with solutions of bortezomib, catechol-containing polymers and polymer-bortezomib conjugates at defined concentrations for 6 hours at 37°C under 5% CO2. Cells treated with the assay buffer only were used as control. Cells were then washed with PBS and treated with reagents from a proteasome activity assay kit (Cayman Chemicals, Ann Arbor, MI) following the manufacture's protocols. The fluorescence measurement was carried out at excitation 360 nm, emission 480nm.

13. Cell viability assays

MDA-MB231 cells were seeded in 96-well plates at 3,000 cells / well and cultured for 24 hours. Cells were washed with PBS and treated with solutions of bortezomib, catechol-containing polymers and polymer-bortezomib conjugates at defined concentrations in cell growth media containing 1% serum for 48 hours at 37°C under 5% CO_2. Cells treated with media only were used as control. Cells were then washed with PBS and cell viability was analyzed using the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen) (live cells stained as green and red cells stained as red) followed by calculation of cell death using the equation to plot data for Figure 4. Cell death% = number of dead cells/ (total number of live and dead cells) × 100%.

14. Cell apoptosis assays

Cell apoptosis and necrosis were measured by fluorescence-activated cell sorting (FACS) using Annexin-V, Alexa Fluor 67 conjugate (Invitrogen) as apoptosis indicator and 4',6-diamindino-2-phenylindole (DAPI) as a dead-cell indicator, following manufacturer's protocol. MCF-10A vector and MCF-10A-H-RasV12 cells were treated with BPC-BTZ (10 or 25 nM), BPC (25 nM)

and media only for 48 h prior to analysis at the Robert H. Lurie Cancer Center Flow Cytometry Core Facility at Northwestern University. The data were plotted with red dots representing histogram of cells that exhibit a particular combination of Annexin-V/DAPI fluorescence.

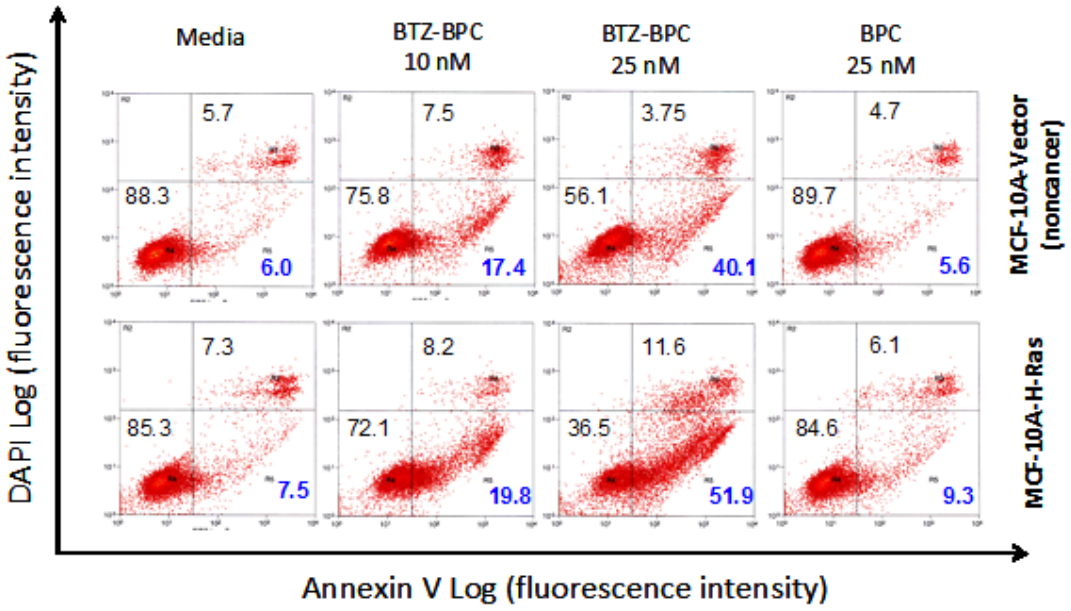


Figure SI 5 Cell death induced by BPC-BTZ conjugate was measured using flowcytometry with Annexin-V/DAPI staining. In each panel, the lower-left (Annexin-V^-, $DAPI^-$), lower-right (Annexin-V^+, $DAPI^-$), and upper-right (Annexin-V^+, $DAPI^+$) quadrants represent the populations of live cells, apoptotic cells, and necrotic/dead cells, respectively. The average % population in each quadrant is indicated by the numbers at the corners of the panels. These data show that non-cancerous MCF-10A breast epithelial cells stably expressing empty vector (*i.e.*, control cells) were less sensitive than MCF-10A cells stably transformed with the H-RasV12 oncogene (*i.e.*, cancerous cells) to BPC-BTZ -induced cell apoptosis and necrosis.

15. Cell proliferation assays

MCF-10A vector and MCF-10A-H-RasV12 cells were seeded in 96-well plates at 10,000 cells / well and cultured for 24 hours. The media in the wells were then replaced with a prepared growth media containing bortezomib, BPC and BPC-BTZ at defined concentrations in DMEM-F12. Treated cells were further incubated for 48 hours at 37°C under 5% CO_2. Cells were then washed with PBS and cell survival was determined by MTS assay, and the relative cell survival percentages compared to the drug-free control were plotted against the total BTZ concentration in logarithmic scale. The dose-response curves were obtained using sigmoldal logistic fitting (SigmaPlot version 10) to calculate the half-maximal inhibitory concentration (IC_{50}) values presented in Figure 5C.

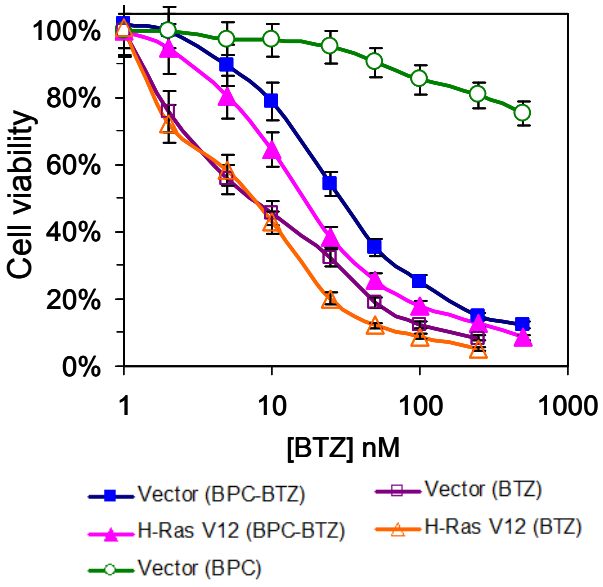


Figure SI 6 Dose-dependent cytotoxicity of BPC-BTZ, free BTZ, or polymer only (BPC) against MCF-10A-vector and MCF-10A-H-RasV12 cells. SigmaPlot software was used for sigmoidal logistic fitting to obtain IC_{50} values shown in Figure 4C.

References:

(1) Moyano, J. V.; Evans, J. R.; Chen, F.; Lu, M. L.; Werner, M. E.; Yehiely, F.; Diaz, L. K.; Turbin, D.; Karaca, G.; Wiley, E.; Nielsen, T. O.; Perou, C. M.; Cryns, V. L. *J Clin Invest* **2006**, *116*, 261.