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Self-Immolative Polycations as Gene Delivery Vectors and Prodrugs Targeting Polyamine Metabolism in Cancer

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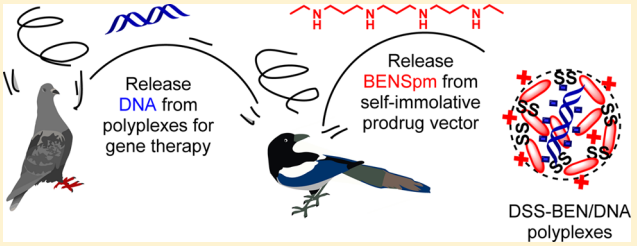
Supporting Information

ABSTRACT: Polycations are explored as carriers to deliver therapeutic nucleic acids. Polycations are conventionally pharmacological inert with the sole function of delivering therapeutic cargo. This study reports synthesis of a self-immolative polycation (DSS-BEN) based on a polyamine analogue drug N^1,N^{11}-bisethylnorspermine (BENSpm). The polycation was designed to function dually as a gene delivery carrier and a prodrug targeting dysregulated polyamine metabolism in cancer. Using a combination of NMR and HPLC, we confirm that the self-immolative polycation undergoes intracellular degradation into the parent drug BENSpm. The released BENSpm depletes cellular levels of spermidine and spermine and upregulates polyamine catabolic enzymes spermine/spermidine N^1-acetyltransferase (SSAT) and spermine oxidase (SMO). The synthesized polycations form polyplexes with DNA and facilitate efficient transfection. Taking advantage of the ability of BENSpm to sensitize cancer cells to TNFα-induced apoptosis, we show that DSS-BEN enhances the cell killing activity of TNFα gene therapy. The reported findings validate DSS-BEN as a dual-function delivery system that can deliver a therapeutic gene and improve the outcome of gene therapy as a result of the intracellular degradation of DSS-BEN to BENSpm and the subsequent beneficial effect of BENSpm on dysregulated polyamine metabolism in cancer.



KEYWORDS: *self-immolative linker, polymeric prodrugs, polyamine metabolism, gene delivery, combination therapy, bisethylnorspermine, TNFα*

1. INTRODUCTION

The combination of small-molecule drugs with therapeutic nucleic acids has emerged as a promising strategy in cancer treatment.[1] Because of the heterogeneity of cancer and the involvement of multiple genetic changes during tumorigenesis, such combination treatments offer great advantages in targeting multiple disease pathways and overcoming adaptive drug resistance. However, this potentially powerful combination modality is greatly hampered by a lack of desirable delivery systems that could accommodate various therapeutic payloads.[2] It is especially challenging to design delivery vectors for drug−nucleic acid combinations, owing to the physicochemical differences between the two agents. Among the available systems, biodegradable polycations represent promising delivery platforms suitable for delivery of nucleic acid/drug combinations.[3,4]

Natural polyamines spermine (SPM), spermidine (SPD), and their diamine precursor putrescine (PUT) are ubiquitous alkylamines that are essential for cell growth, differentiation, and survival.[5,6] Polyamines are present inside cells at millimolar concentration levels, but the majority exist in the bound form.[7,8] Most polyamines are found in polyamine−RNA complexes, thus influencing protein synthesis.[9] Intracellular levels of natural polyamines are strictly regulated by the polyamine transport system and metabolic enzymes (Scheme 1A).[10] The rate-limiting enzymes in the polyamine biosynthesis pathway include ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC). The catabolism of natural polyamines is mediated by spermine/spermidine N^1-acetyltransferase (SSAT), polyamine oxidase (APAO), and spermine oxidase (SMO). SSAT and SMO are highly inducible enzymes whose expression is regulated by a variety of stimuli, including changes in polyamine levels, cellular stress, DNA damage, or exposure to various drugs.[11,12] Dysregulation of polyamine metabolism is associated with various diseases. Polyamine depletion leads to inhibition of cell growth and acceleration of aging,[13] whereas increased levels of polyamines

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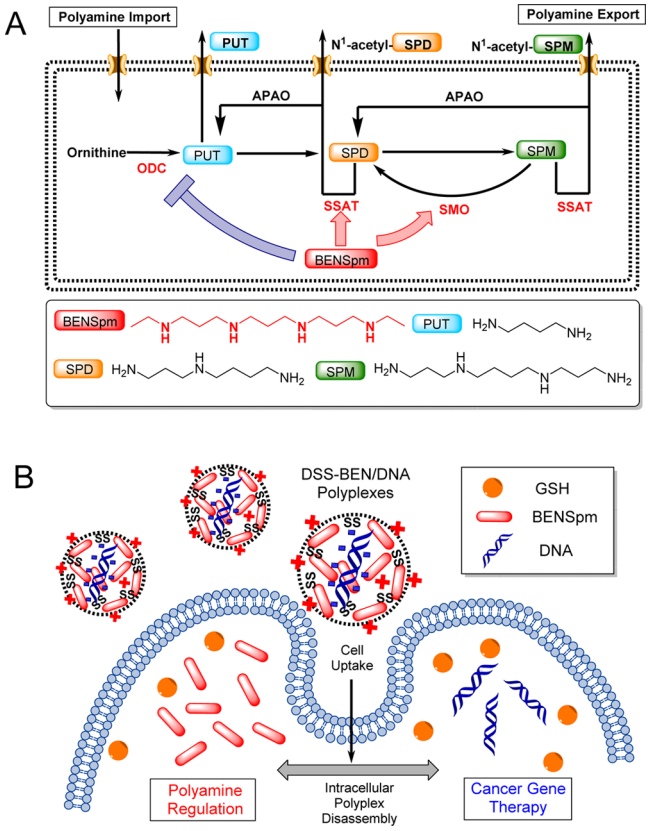
Scheme 1. (A) Effect of BENSpm on Polyamine Metabolism and (B) Proposed Mechanism of Action of DSS-BEN[a]



[a](A) BENSpm acts in multiple ways to regulate polyamine pathways and leads to the depletion of all three natural polyamines and growth inhibition of cancer cells. (B) DSS-BEN condenses DNA into polyplexes via electrostatic interactions. Upon cell uptake, the polyplexes are subjected to intracellular reduction by GSH, followed by disassembly of the polyplexes and release of the DNA for cancer gene therapy. Simultaneously, the intracellular degradation of DSS-BEN leads to release of BENSpm and inhibition of cancer cell growth due to the effect of BENSpm on polyamine metabolism.

are associated with hyper-proliferative diseases such as cancer.[14,15] In cancer, dysregulation of the enzymes involved in the polyamine pathway results in accumulation of polyamines, which promotes tumorigenesis and tumor progression. Elevation of polyamine levels enhances the malignant potential of cancer cells and decreases antitumor immunity.[16] Alteration in the polyamine pathway is also associated with poor prognosis of certain cancers.[17] Thus, the polyamine pathway represents a promising target in cancer chemotherapy and chemoprevention.[18]

Initial efforts to develop anticancer agents based on the inhibition of polyamine biosynthesis enzymes yielded limited clinical success. One of the most well-known inhibitors of polyamine biosynthesis is difluoromethylornithine, an irreversible inhibitor of ODC.[19] Alternative research focused on developing polyamine analogues that are capable of competing with natural polyamines for transport, biosynthesis, and catabolism. This line of research has proven to be more successful, as illustrated in the example of BENSpm (Scheme 1A).[5,20,21] BENSpm is among the most successful of the developed polyamine analogues. It has shown promising antitumor activity against a wide range of cancers, including melanoma, ovarian, breast, and pancreatic cancers.[22−24] BENSpm induces SSAT, downregulates ODC and AdoMetDC,[25] and ultimately causes cell growth inhibition and apoptosis.[26] Although BENSpm did not show a satisfactory clinical outcome as a single agent,[27−29] recent studies demonstrated its potential when used in combination with other chemotherapy drugs such as 5-fluorouracil and paclitaxel.[30,31]

In this study, we report the synthesis of biodegradable polycation prodrugs based on BENSpm (DSS-BEN). The synthesized prodrugs utilize bis(2-hydroxyethyl) disulfide as a self-immolative linker[32] to facilitate rapid intracellular degradation and release of BENSpm from the polycations. We report results of the intracellular BENSpm release and the effect of DSS-BEN on polyamine metabolism in cancer cells. Finally, we explore the ability of the synthesized polycations to deliver DNA and demonstrate a promising enhancement in the anticancer activity of TNFα-encoding plasmid DNA by the action of BENSpm from DSS-BEN.

2. MATERIALS AND METHODS

2.1. Materials. 1,1′-Carbonyldiimidazole (CDI), bis(2-hydroxyethyl) disulfide (BHED), spermidine (SPM) trihydrochloride, putrescine (PUT) dihydrochloride, and branched polyethylenimine (PEI, 25 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM) (99.9%, extra dry, AcroSeal), tetrahydrofuran (THF, 99.85%, extra dry, AcroSeal), 5-sulfosalicylic acid dihydrate (SSA), 1,7-diaminoheptane (DAH), dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride, 98%), and 1,6-hexanediol were from Acros Organics (Fair Lawn, NJ). L-Proline was from Alfa Aesar (Ward Hill, MA). Spermine (SPM) was from MP Biomedicals (Santa Ana, CA). BENSpm was synthesized by following a previously described procedure.[22] Plasmid DNA containing a luciferase reporter gene (gWiz-Luc) was from Aldevron (Fargo, ND), and human tumor necrosis factor (TNFα) plasmid DNA was obtained from InvivoGen (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM), Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), L-glutamine, and penicillin−streptomycin were from Thermo Scientific (Waltham, MA). RT-PCR primers were purchased from Invitrogen (Carlsbad, CA). All other reagents and chemicals were obtained from Fisher Scientific or VWR International unless otherwise stated.

2.2. Synthesis and Characterization of DSS-BEN and DCC-BEN. All reactions were performed under anhydrous conditions under nitrogen. To synthesize DSS-BEN, 403.2 mg of BHED (2.6 mmol) was dissolved in a mixture of 3.3 mL of DCM and 0.7 mL of THF on ice. Then, a solution of CDI (887.5 mg, 5.5 mmol) in 3.5 mL of DCM was added dropwise, and the reaction was kept on ice for 1 h. A solution of BENSpm (2.6 mmol, 640 mg) in DCM was then added, and the reaction was left to proceed for 18 h at 45 °C. The reaction mixture was then allowed to cool to room temperature, and the final product was precipitated in 25 mL of diethyl ether, followed by two washes with 20 mL of diethyl ether. The product was then dried under vacuum and redissolved in 0.1 mM HCl, followed by extensive dialysis (MWCO 3.5 kDa) against 0.1 mM HCl and then pure water before lyophilization. A total of 372 mg of DSS-BEN hydrochloride was obtained. To synthesize DCC-BEN, a solution of CDI (1.260 g, 7.77 mmol) in 5 mL of DCM was added dropwise into a solution of 1,6-hexanediol (437.3

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mg, 3.7 mmol) in 15 mL of DCM and 1 mL of THF. The reaction was kept on ice for 1 h, and 10 mL of BENSpm (904 mg, 3.7 mmol) in DCM was added. The reaction mixture was then refluxed for 96 h before precipitation in diethyl ether, extensive dialysis, and lyophilization following the same procedure as that described for DSS-BEN. A total of 143 mg of DCC-BEN hydrochloride was obtained.

^{1}H NMR spectra of the polymers were recorded on 400 MHz Bruker NMR spectrometer, and chemical shifts (δ) are expressed in ppm. The composition of the polymers was determined by elemental analysis from N, S, H, and Cl content (Atlantic Microlab, Inc., Norcross, GA). Weight-average molecular weight (M_w) of the polymers was determined by size-exclusion chromatography using an Agilent Technologies 1260 Infinity equipped with an isocratic pump, degasser, variable wavelength detector, thermostated column compartment, and autosampler from Agilent Technologies, Inc. (Santa Clara, CA). A Wyatt miniDWAN TREOS multiangle light scattering detector and Optilab T-rEX differential refractometer (Wyatt Technology, Santa Barbara, CA) were used as detectors, and Astra 6.1 software was used for chromatographic data processing. The refractive index increment (dn/dc) was determined experimentally as 0.1693 g/mL using a serial dilution of DSS-BEN. The analysis was conducted using single-pore AquaGel columns PAA-202 and PAA-203 from PolyAnalytik (London, ON, Canada). Sodium acetate buffer (0.3 M, pH 5.0) was used as the mobile phase at a flow rate of 0.3 mL/min.

2.3. DSS-BEN Degradation Kinetics. Degradation of DSS-BEN and release of BENSpm were evaluated using ^{1}H NMR. Briefly, DSS-BEN or DCC-BEN (10 mg) was dissolved in a 0.9 mL mixture of 0.1 M phosphate buffered D_2O and acetone-d_6 (3:2 v/v), and the solution was purged with argon for 10 min. Dithiothreitol (DTT) (15 mg, 0.097 mmol) was then added to the solution immediately before the start of NMR acquisition at 25 °C. Polymer degradation was determined from the relative decrease of the integral intensity of the BENSpm methylene protons next to the carbamate bond in the polymer (3.35−3.55 ppm in DSS-BEN and 3.23−3.50 ppm in DCC-BEN) relative to the D_2O solvent peak at 4.8 ppm.

2.4. Cell Lines. B16F10 murine melanoma cells were cultured in DMEM supplemented with 10% FBS. HepG2 cells were cultured in EMEM supplemented with 10% FBS. Human osteosarcoma U2OS cells were from Thermo Scientific (Waltham, MA) and cultured in DMEM supplemented with 2 mM L-glutamine, 10% FBS, 1% pen−strep, and 0.5 mg/mL G418. All cells were maintained in a 37 °C cell culture incubator with 5% CO_2.

2.5. Analysis of Intracellular Polyamines. B16F10 and U2OS cells were treated with 2.5 μg/mL BENSpm, 5.7 μg/mL DSS-BEN, or 5.7 μg/mL DCC-BEN for 48 or 72 h. The cells were harvested by trypsinization, and the amount of BENSpm and natural polyamines (SPM, SPD, PUT) was determined as described previously.[33] Briefly, harvested cells were homogenized in 5% SSA and centrifuged for 5 min at 12 000g. The supernatant containing the polyamines was functionalized with dansyl chloride and purified with a Bond-Elut C18 column (Agilent Technologies, Santa Clara, CA). The samples were then injected onto an Eclipse Plus C18 column (4.6 × 150 mm, Agilent Technologies) using DAH as the internal standard. A 1260 Infinity Quaternary LC System (Agilent Technologies) was used for the analysis using a gradient of acetonitrile and phosphate buffer (10 mM, pH 4.4) as the eluent system. Data were collected and analyzed using OpenLAB CDS Chemstation Edition software (Agilent Technologies). The amount of BENSpm, SPM, SPD, and PUT in the samples was quantified using standard curves constructed with the corresponding standard compound. Relative polyamine depletion (%) was calculated from polyamine concentrations found in treated vs untreated cells.

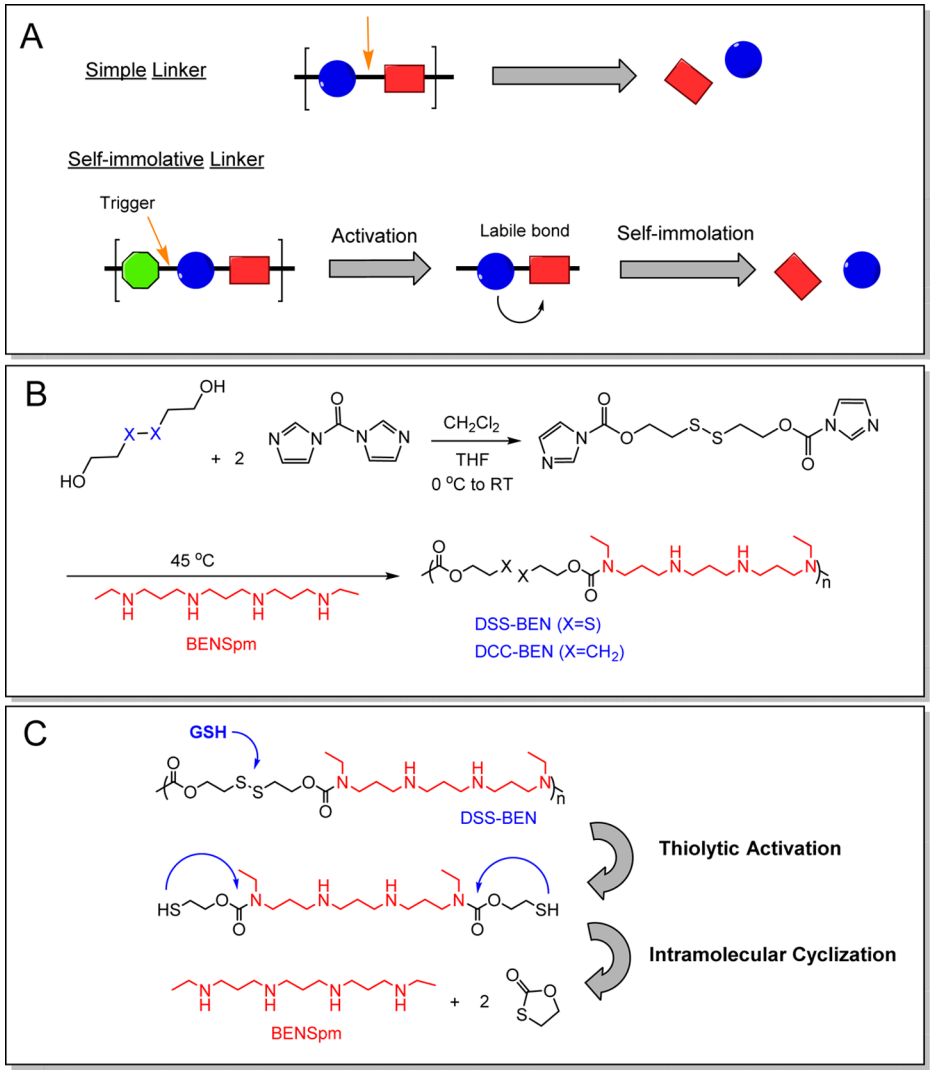
2.6. RT-PCR Analysis of the Induction of Polyamine Catabolic Enzymes. Expression of polyamine catabolic enzymes SMO and SSAT in B16F10 and U2OS cells was quantified using RT-PCR. Cells were treated with 2.5 μg/mL BENSpm, 5.7 μg/mL DSS-BEN, or 5.7 μg/mL DCC-BEN for 48 h. Total RNA (500 ng) was isolated using RNeasy mini kit (Qiagen, Valencia, CA) and reverse-transcribed to complementary DNA (cDNA) using QuantiTect reverse transcription kit (Qiagen), and the relative amount of mRNA was determined by RT-PCR (iCycler iQ real time PCR detection system, BioRad, Hercules, CA). GAPDH primer assay and QuantiFast SYBR Green PCR kit (Qiagen) were used following the manufacturer's protocol. The following primers were used: murine SMO (forward 5′-CACGTGATTGTGACCGTTTC; reverse 5′-TGGGTAGGTGAGGGTACAGTC); murine SSAT (forward 5′-CGTCCAGCCACTGCCTCTG; reverse 5′-GCAAGTACTCTTTGTCAATCTTG); human SMO (forward 5′-CGCAGACTTACTTCCCCGGC; reverse, 5′-CGCTCAATTCCTCAACCACG); and human SSAT (forward, 5′-ATCTAAGCCAGGTTGCAATGA; reverse, 5′-GCACTCCTCACTCCTCTGTTG).[30,34,35] Relative expression of the mRNA of the enzymes was calculated from the threshold values (C_T) of the target genes and the housekeeping gene GAPDH.

2.7. Preparation and Physicochemical Characterization of DNA Polyplexes. The ability of DSS-BEN and DCC-BEN to condense plasmid DNA was determined by an ethidium bromide (EtBr) exclusion assay by measuring the changes in EtBr/DNA fluorescence. A total of 1 mL of DNA solution (20 μg/mL) in 10 mM HEPES buffer (pH 7.4) was mixed with EtBr (1 μg/mL), and the raw fluorescence was set to 100% using a Quantech fluorometer (Ex 540 nm/Em 590 nm) from Thermo Scientific (Waltham, MA). Relative fluorescence (%) readings were then recorded following the stepwise addition of a polycation solution. The condensation curve for each polycation was then constructed.

DSS-BEN/DNA and DCC-BEN/DNA polyplexes were formed by adding predetermined volume of DSS-BEN or DCC-BEN to a DNA solution (20 μg/mL in 10 mM HEPES pH 7.4) to achieve the desired polymer/DNA w/w ratio, and the samples were mixed by vigorous vortexing for 10 s. Polyplexes were incubated at room temperature for 30 min prior to use. Hydrodynamic diameter and zeta potential of the polyplexes were determined by dynamic light scattering (DLS) using a ZEN3600 Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK).

DNA release from the polyplexes was analyzed by polyelectrolyte exchange with heparin. The polyplexes were prepared at a w/w ratio of 8 and incubated with increasing concentrations of heparin in the presence or absence of 20 mM glutathione (GSH) for 30 min at 37 °C. The polyplex samples were then loaded onto an 0.8% agarose gel containing 0.5 μg/mL EtBr and run for 60 min at 120 V in 0.5× Tris/Borate/EDTA running buffer. The gels were visualized under UV illumination on a KODAK Gel Logic 100 imaging system.

Scheme 2. (A) Principle of Action of Self-Immolative Linkers, (B) Synthesis of DSS-BEN and DCC-BEN, and (C) Mechanism of GSH-Triggered Intracellular Release of BENSpm from DSS-BEN



2.8. Cytotoxicity of Polymers. Cytotoxicity of DSS-BEN and DCC-BEN in B16F10, U2OS, and HepG2 cells was evaluated by MTS assay (CellTiter 96 AQueous One Solution cell proliferation assay, Promega, Madison, WI). Cells (8000, U2OS; 10 000, B16F10 and HepG2) were seeded in 96-well microplates 1 day before polymer treatment. Culture medium was replaced with 200 μL of increasing concentrations of DSS-BEN or DCC-BEN in serum-supplemented medium, and the cells were then incubated for 24 h. To measure cell viability, medium was aspirated and replaced with a mixture of 100 μL of serum-free medium and 20 μL of MTS reagent. After 1.5 h incubation, the absorbance was measured using SpectraMax M5^e multi-mode microplate reader (Molecular Devices, CA) at λ = 490 nm. The relative cell viability (%) was calculated as $[A]_{sample}/[A]_{untreated} \times 100\%$. The IC_{50} values were calculated as the polymer concentration that inhibits growth of 50% of cells relative to growth of untreated cells using GraphPad Prism, version 5.0c.

2.9. Transfection of DNA Polyplexes. Cells were seeded in 48-well plates at a density of 40 000 cells/well for B16F10 or 20 000 cells/well for U2OS 24 h prior to transfection. On the day of transfection, cells were incubated with the polyplexes containing luciferase DNA (pLuc) (0.4 μg DNA/well) in 170 μL of medium (with or without 10% FBS). After 4 h incubation, polyplexes were completely removed, and the cells were incubated in medium with 10% FBS for another 24 h. To measure luciferase expression, the medium was discarded, and the cells were lysed in 100 μL of 0.5× cell culture lysis reagent buffer (Promega, Madison, WI) for 30 min. One hundred microliters of 0.5 mM luciferin solution was then automatically injected into each well containing 20 μL of cell lysate, and the luminescence was integrated over 10 s using GloMax 96 microplate luminometer (Promega, Madison, WI). Total cellular protein in the cell lysate was determined by the bicinchoninic acid protein assay using a calibration curve constructed with standard bovine serum albumin solutions. Transfection activity was expressed as relative light units (RLU)/mg cellular protein ± SD (n = 4). Transfections of U2OS cells with TNFα plasmid DNA (pTNFα) were conducted in 96-well plates. The cells were seeded at a density of 3000 cells/well 24 h prior to polyplex addition. Transfection was performed as above using a DNA dose of 0.2 μg/well. After 4 h incubation, 115 μL of fresh medium with 10% FBS was added, and cells were incubated for another 48 h prior to measuring cell viability using MTS assay. The relative cell killing mediated by TNFα expression was normalized to the

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viability of untreated cells and expressed as the mean ± SD of triplicate samples.

3. RESULTS AND DISCUSSION

3.1. Synthesis of BENSpm-Based Polycations. The cationic character of BENSpm recently prompted us to explore its potential for use in dual-function delivery vectors suitable for combination drug/nucleic acid therapies. In proof-of-principle studies, we synthesized lipid prodrug based on BENSpm and established its utility for use in combination drug/nucleic acid delivery.[36,37] Herein, we extend the concept of dual-functioning delivery vectors that target polyamine metabolism to BENSpm-based polycations. We previously found that BENSpm has to be released from the vector in its unmodified form to exert the desired effect on polyamine metabolism.[36] Because of the presence of four secondary amines, the easiest approach to synthesize BENSpm polycations would be to polymerize BENSpm using amide or carbamate chemistry. Unfortunately, BENSpm amides and carbamates were too stable in a biological environment for the intended application.[38] As a solution, we employed self-immolative linker chemistry to synthesize rapidly degradable polycations that release unmodified BENSpm as the main degradation product. Self-immolative linkers represent an alternative strategy to traditional simple linkers used in prodrug design in situations where the linker is sterically inaccessible or, as in our case, the rates of the linker cleavage are too slow for the intended use (Scheme 2A). In the self-immolative approach, the linker uses a readily cleavable bond with an additional protecting group and a stable bond to the desired drug (i.e., BENSpm). The stable bond becomes labile when the cleavable bond is broken, and the process leads to the rapid release of a drug from the conjugate. Multiple types of self-immolative linkers have been explored in prodrug and polymer synthesis.[39−41]

In this study, we selected BHED as the self-immolative linker to synthesize the BENSpm-based polycation prodrug DSS-BEN. DSS-BEN was synthesized as shown in Scheme 2B by reacting an equimolar amount of BENSpm and the BHED linker activated with CDI. Control nondegradable polycation DCC-BEN was synthesized by reacting BENSpm with 1,6-hexanediol activated with CDI. The polymers were purified by dialysis and characterized by ^{1}H NMR, elemental analysis, and size-exclusion chromatography. The molecular weights of the prepared polymers were relatively low at M_w = 3.8 kDa for DSS-BEN and 2.8 kDa for DCC-BEN. The low molecular weight of DCC-BEN partially explained the low yield (~10%), as a significant portion of the polymer was lost during dialysis using a membrane with a molecular weight cutoff of 3.5 kDa. Polymer loss during purification also explains the low polydispersity index of the synthesized polymers, which was typically in the range 1.1−1.3. On the basis of the molecular weight, DSS-BEN contained, on average, 8 BENSpm molecules, whereas DCC-BEN contained 7 BENSpm molecules. Attempts to increase the molecular weight by exploring other solvents, temperatures, and reaction times were unsuccessful. It is likely that incomplete CDI activation of BHED and 1,6-hexanediol resulted in deviation from the assumed equimolarity and bifunctionality and thus in the low molecular weight. Results of the elemental analysis suggested that BENSpm accounts for ~44 wt % in both the DSS-BEN and DCC-BEN samples. A similar estimate was obtained from the analysis of the ^{1}H NMR spectra. A typical NMR spectrum of DSS-BEN is shown in Figure 1A. Although the polymer

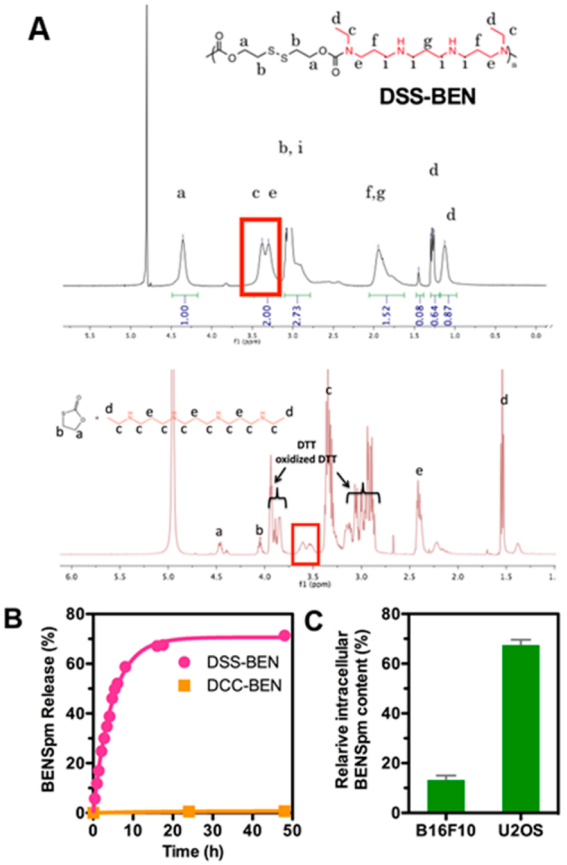

Figure 1. (A) (top) Representative ^{1}H NMR spectrum of DSS-BEN in D_2O (top). (bottom) ^{1}H NMR spectrum of DSS-BEN after 16 h degradation in DTT solution in phosphate-buffered D_2O/acetone-d_6 (peaks highlighted in the red box were used to determine polymer degradation). (B) Degradation kinetics and BENSpm release from DSS-BEN and DCC-BEN in 100 mM DTT in 100 mM phosphate-buffered D_2O (pH 7.4)/acetone (3:2) at 25 °C measured by ^{1}H NMR. (C) Intracellular release of BENSpm from DSS-BEN after 48 h incubation with B16F10 cells and 72 h incubation with U2OS cells determined by HPLC analysis (n = 3). The amount of intracellular BENSpm is expressed as a percentage relative to intracellular BENSpm content observed in cells treated with free BENSpm.

structure in Figure 1A suggests that only terminal BENSpm amines engaged in reaction with BHED, the ^{1}H NMR does not allow us to exclude the possibility that the two internal secondary amines also participated in the reaction.

3.2. Polymer Degradation and Release of BENSpm. BHED contains a disulfide bond that can be readily cleaved by intracellular thiol−disulfide exchange with free thiols such as glutathione (GSH). In DSS-BEN, the disulfide cleavage results in generation of a free thiol, which then participates in intramolecular attack of the carbamate carbonyl and cleavage of the otherwise stable carbamate bond between BENSpm and BHED (Scheme 2C). We first confirmed the proposed mechanism of polymer degradation and BENSpm release by investigating the degradation kinetics by ^{1}H NMR spectroscopy using DTT as the reducing agent (Figure 1A). BENSpm release from the polymers was calculated as the percentage of reduction in the integral intensity of the BENSpm methylene peaks (highlighted by a box in Figure 1A) relative to their integral intensity at time 0 (i.e., immediately after DTT addition). Plotting the results as the percent BENSpm release vs degradation time (Figure 1B) suggested that DSS-BEN

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degradation followed first-order kinetics. The estimated rate constant was 3.5×10^{-3} min^{-1}, and the corresponding half-life was 198 min. These results are in good agreement with previous reports on the degradation of several self-immolative polymers containing similar cyclizing spacers.[39,42] In contrast, treatment with DTT had no effect on DCC-BEN (Figure S1), confirming the stability of the carbamate bond and the benefits of the self-immolative linker in the degradation of DSS-BEN and release of free BENSpm.

Intracellular degradability and release of BENSpm from the polymers were evaluated by incubating B16F10 and U2OS cells with 5.7 μg/mL DSS-BEN or DCC-BEN or with control BENSpm (2.5 μg/mL). The polymer concentration was selected such that the cells were exposed to 2.5 μg/mL of BENSpm equivalent. The amount of intracellular BENSpm was then determined by HPLC analysis of the BENSpm in the cell lysate (Figure 1C). In order to determine how efficiently DSS-BEN can generate intracellular BENSpm when compared with treatments with free BENSpm, we expressed the results as the percent of intracellular BENSpm content in cells treated with DSS-BEN relative to the amount of BENSpm taken up by cells after incubation with free BENSpm. As shown in Figure 1C, BENSpm was detected in both tested cell lines treated with DSS-BEN. In contrast, no detectable amount of BENSpm was observed in cells treated with DCC-BEN, suggesting the high stability of the carbamate bond in the polymer and thus no intracellular degradation. We observed significant differences in the time course of intracellular BENSpm between the two cell lines, with BENSpm levels peaking at 48 h in B16F10 and 72 h in U2OS. There were also substantial differences in the achievable intracellular BENSpm concentrations between the two cell lines. In contrast to 67.5% BENSpm observed in U2OS cells, we found only 13.2% BENSpm in B16F10 cells relative to the intracellular concentration found when incubating the cells with free BENSpm. The intracellular BENSpm content when delivered by DSS-BEN is a complex function of the rate and extent of DSS-BEN uptake, intracellular trafficking, rate of degradation, and extent of excretion from the cells. We hypothesize that the differences in the intracellular reducing capacity of the two cell lines are among the most important factors behind the observed results. Overall, our findings confirm the functionality of the self-immolative linker in DSS-BEN in living cells.

3.3. Regulation of Polyamine Metabolism. BENSpm exhibits multiple functions in the regulation of intracellular polyamines. Unlike selective inhibitors of the individual enzymes in the polyamine pathway, BENSpm treatment leads to depletion of all three natural polyamines. BENSpm mimics the structure of SPM, but the alkylated terminal amines prevent its oxidation by oxidases such as APAO. BENSpm is also a potent post-transcriptional inducer of the activity of catabolic enzymes SSAT and SMO.[43−45]

After confirming that DSS-BEN is degraded into BENSpm in both tested cell lines, we evaluated the ability of the released BENSpm to regulate cellular polyamine metabolism. Because BENSpm treatment leads to depletion of all natural polyamines, we first focused on investigating the effect of DSS-BEN treatment on changes in the expression of the key catabolic enzymes SSAT and SMO using RT-PCR (Figure 2A). As expected, treatment with free BENSpm upregulated the mRNA level of both SSAT and SMO. The effect was particularly strong in U2OS cells. Overall, treatment with DSS-BEN resulted in a weaker upregulation of the catabolic enzymes than that

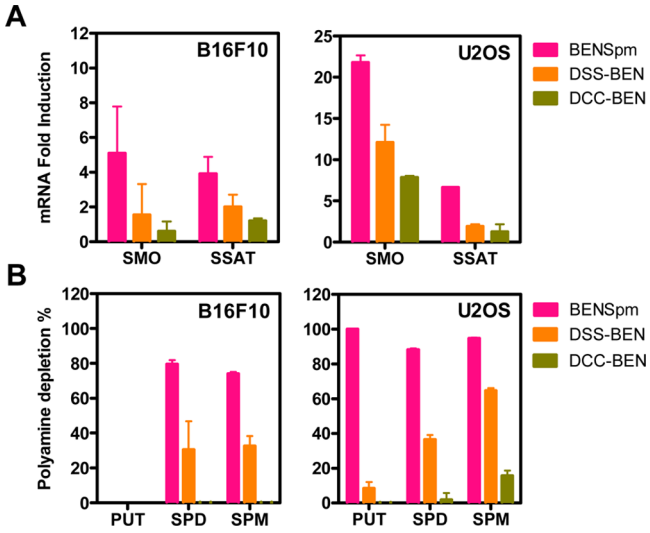

Figure 2. Effect of DSS-BEN on cellular polyamine pathways. (A) Relative changes in the expression of polyamine catabolic enzymes SMO and SSAT mRNA in B16F10 and U2OS cells. Cells were incubated with 2.5 μg/mL BENSpm or 5.7 μg/mL DSS-BEN or DCC-BEN, and mRNA levels were measured by RT-PCR. Results are expressed as the fold induction of specific mRNA in treated cells relative to that in untreated control (n = 3). (B) Effect of BENSpm, DSS-BEN, and DCC-BEN on polyamine depletion in B16F10 and U2OS cells determined by HPLC. Results are expressed as the percent polyamine depletion in treated cells relative to that of the untreated control (n = 3).

resulting from treatement with free BENSpm. For example, BENSpm increased expression of SSAT mRNA 3.9-fold in B16F10, whereas DSS-BEN increased the expression by only 2-fold. Similarly, BENSpm upregulated SMO mRNA expression 21.8-fold in U2OS, whereas DSS-BEN achieved only a 12.1-fold increase. Treatment with the nondegradable DCC-BEN had the weakest effect on induction of the two catabolic enzymes.

Having confirmed BENSpm release from DSS-BEN and its effect on the expression of catabolic enzymes, we evaluated if these findings translate into the expected effect on polyamine depletion. As above, we treated the cells with BENSpm, DSS-BEN, and DCC-BEN and measured intracellular levels of PUT, SPD, and SPM by HPLC (Figure 2B). In B16F10 cells, BENSpm treatment depleted 80% of SPD and 74% of SPM (we were unable to detect any PUT in this cell line). Treatment with DSS-BEN resulted in 30% SPD and 33% SPM depletion. Nondegradable DCC-BEN had no observable effect on the polyamine pool in B16F10 cells. In U2OS cells, BENSpm was highly effective at depleting all three natural polyamines, as suggested by the fact that it depleted 100% of PUT, 88% of SPD, and 95% of SPM. Similarly, DSS-BEN was more effective in U2OS than in B16F10, as it depleted 37% of SPD and 65% of SPM. The decreased ability of DSS-BEN to affect the cellular polyamine pool in B16F10 cells is likely related to the lower overall intracellular concentration of BENSpm in this cell line (Figure 1C). Surprisingly, only an 8% depletion of PUT was observed in the case of DSS-BEN treatment of U2OS cells. We speculate that this observation is related to the likely differences in the mechanism of cellular uptake and trafficking between DSS-BEN and free BENSpm.

3.4. Preparation and Characterization of DSS-BEN/DNA Polyplexes. To test the ability of DSS-BEN to deliver

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genes, we first investigated DNA condensation by the EtBr exclusion assay (Figure 3A). DSS-BEN was able to fully

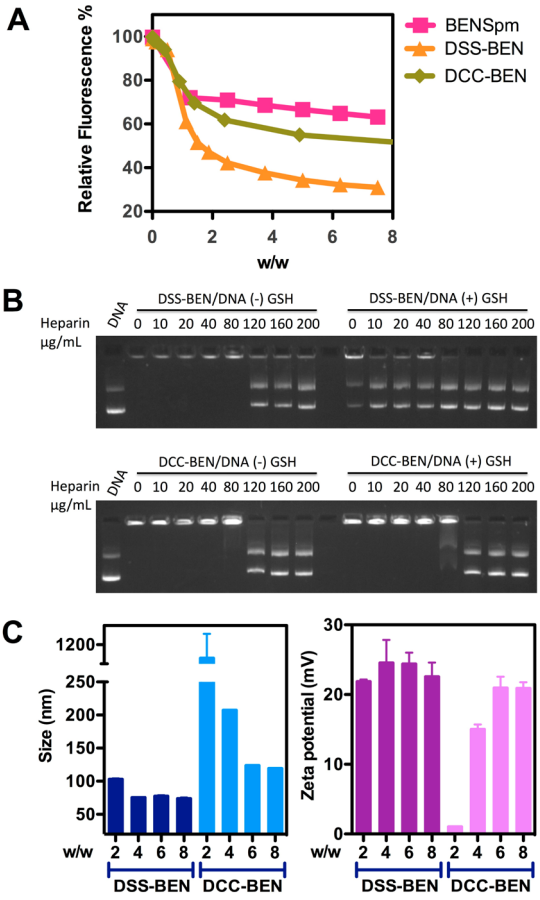

Figure 3. Preparation and characterization of DNA polyplexes. (A) DNA condensation by DSS-BEN and DCC-BEN in 10 mM HEPES buffer (pH 7.4) by EtBr exclusion assay. (B) Heparin- and GSH-induced DNA release from the polyplexes. Polyplexes were prepared at w/w 8 and incubated with increasing concentrations of heparin either with or without 20 mM GSH for 1 h. (C) Hydrodynamic size and zeta-potential of DNA polyplexes of DSS-BEN and DCC-BEN.

condense DNA above a polymer/DNA (w/w) ratio of 2.5 and displayed a typical sigmoidal condensation curve. Despite having a comparable molecular weight, DCC-BEN exhibited a less efficient DNA condensing ability than that of DSS-BEN. As expected, free BENSpm showed poor DNA condensing ability, as indicated by the fact that less than a 30% decrease in EtBr fluorescence was observed even at the highest w/w ratios used.

We expected that the intracellular cleavage of the disulfide bonds in DSS-BEN will not only lead to release of free BENSpm but also facilitate DNA release from the polyplexes because of the inability of free BENSpm to condense DNA. The reduction-triggered DNA release was studied by incubating DSS-BEN/DNA and control DCC-BEN/DNA polyplexes with 20 mM GSH and increasing concentrations of heparin. The stability of the polyplexes was characterized by the minimum heparin concentration required for polyplex disassembly and DNA release using agarose gel electrophoresis (Figure 3B). In the absence of GSH, both DSS-BEN and control DCC-BEN polyplexes were stable up to 80 μg/mL heparin. The reducing activity of 20 mM GSH selectively destabilized DSS-BEN polyplexes to a point that the DNA was released without the need for heparin. These results suggest that the self-immolative linker retains its sensitivity to reduction even when the polymer is complexed with DNA.[46]

Hydrodynamic size and zeta-potential of the DSS-BEN and DCC-BEN polyplexes prepared at different w/w ratios were measured by light scattering (Figure 3C). The sizes of DSS-BEN polyplexes ranged from 70 to 100 nm at all tested w/w ratios. In contrast, DCC-BEN required w/w > 4 to achieve similar sized polyplexes. Even then, DCC-BEN polyplexes were larger (~120−210 nm) and had a lower zeta-potential when compared with that of DSS-BEN polyplexes. These size and zeta potential differences were observed despite the structural and molecular weight similarity between DSS-BEN and DCC-BEN. A possible explanation may rest in additional stabilization of the DSS-BEN polyplexes by intrapolyplex disulfide−disulfide exchange reactions, as reported for bioreducible polyplexes recently.[47]

3.5. Cytotoxicity and Transfection Activity of DSS-BEN/DNA Polyplexes. Toxicity associated with the use of polycations remains a major hindrance for their use as gene delivery systems. One of the most effective strategies to reduce the toxicity is to incorporate degradable moieties, such as the disulfide bonds used in the present study, into the polymer backbone.[48] We evaluated the cytotoxicity of DSS-BEN and DCC-BEN by MTS assay in three different cell lines: mouse melanoma B16F10, human osteosarcoma U2OS, and human hepatocellular carcinoma HepG2. The goal of this experiment was to separate the nonspecific polycation toxicity from the toxic effects that originate specifically from the influence of BENSpm on polyamine metabolism. Thus, we assessed only acute 24 h toxicity, which we believe can be mostly attributed to the polycation character of the polymers. We included PEI (25 kDa) as a control. The IC_{50} value of each polymer was calculated from the concentration dependence of cell viability in the three cell lines tested (Figure 4A). As expected, bioreducibility of DSS-BEN contributed to a significantly decreased acute toxicity in all three cell lines when compared with those of control DCC-BEN and PEI.

After we determined the safe dosing range of DSS-BEN, the gene delivery efficiency was evaluated in B16F10 cells (Figure 4B) and U2OS cells (Figure 4C) using a luciferase reporter plasmid. The polyplexes were formulated at varying polycation/pLuc (w/w) ratios, and PEI/pLuc polyplexes (w/w 1.2) were used as an additional control. DSS-BEN polyplexes showed considerably higher luciferase transfection compared to that of DCC-BEN polyplexes. In addition, DSS-BEN polyplexes showed transfection that was not only fully comparable with that of PEI polyplexes but also exhibited low sensitivity to w/w ratio and the presence of serum in B16F10 cells.

3.6. Enhanced Anticancer Activity of TNFα Gene Delivered by DSS-BEN Polyplexes. Inhibitors of polyamine synthesis, including BENSpm, have been reported to sensitize various human and mouse cancer cells to TNF-induced apoptosis.[49] We hypothesized that using DSS-BEN as a delivery vector for pTNFα will result in improved anticancer activity due to the combined effect of polyamine depletion by BENSpm released from DSS-BEN and TNFα cancer gene therapy (Scheme 1B). To study the dual-functionality of DSS-BEN in TNFα cancer gene therapy, we used DSS-BEN to deliver pTNFα to U2OS cells and evaluated the combined anticancer activity *in vitro* (Figure 5). DSS-BEN/pTNFα polyplexes were prepared at different w/w ratios, and their cell killing activity was evaluated. In order to dissect the

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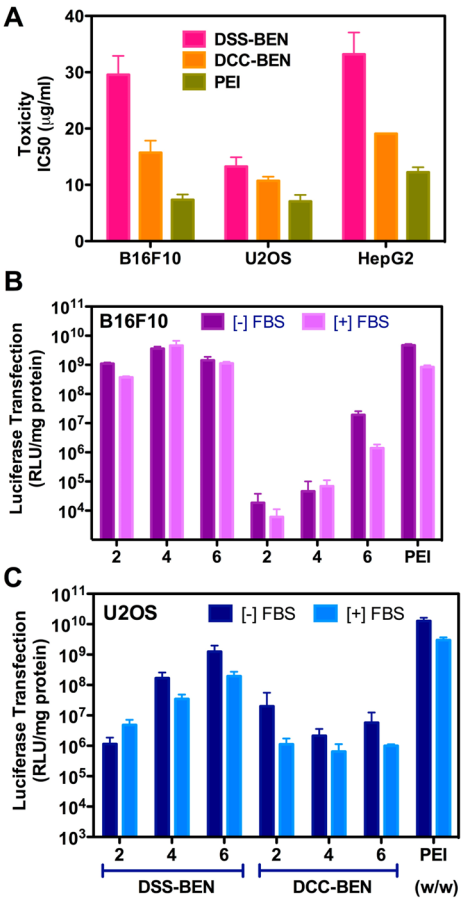

Figure 4. Cytotoxicity and transfection activity of DSS-BEN and DCC-BEN. (A) IC_{50} values of DSS-BEN, DCC-BEN, and PEI in B16F10, U2OS, and HepG2 cells based on MTS cell viability assay after 24 h incubation with polymers (n = 3). Luciferase transfection activity of DSS-BEN and DCC-BEN in (B) B16F10 cells and (C) U2OS cells. Results are expressed as RLU/mg protein ± SD (n = 4).

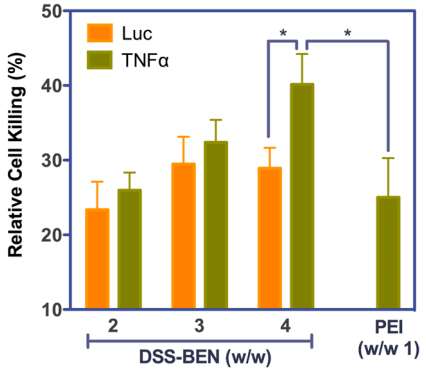

Figure 5. Enhanced anticancer activity of TNFα gene delivered by DSS-BEN polyplexes. U2OS cells were treated with DSS-BEN/pTNFα, DSS-BEN/pLuc, or PEI/pTNFα polyplexes prepared at different w/w ratios. Cell killing was measured by MTS assay. Results are normalized to the viability of untreated cells and shown as mean relative cell killing (%) ± SD (n = 3). * $P < 0.05$.

contribution of BENSpm and TNFα to the overall cell killing activity, we used DSS-BEN polyplexes with pLuc and PEI polyplexes with pTNFα as controls. As shown in Figure 5, treatment with DSS-BEN/pLuc polyplexes resulted in 23−29% cell death, which was attributed to the effect of polyamine depletion by the BENSpm released from DSS-BEN. When used to deliver pTNFα, DSS-BEN polyplexes exhibited the highest combined cell killing activity (40%) at w/w 4. At this w/w ratio, the cell killing activity was significantly higher than either the activity of DSS-BEN/pLuc polyplexes (29%) or PEI/pTNFα polyplexes (25%). It is worth noting that under the used conditions PEI showed better transfection than DSS-BEN (Figure 4C). Although the anticancer effect of TNFα cannot be fully captured *in vitro* due to the importance of the immune system for TNFα activity, the results nevertheless validate our hypothesis and confirm DSS-BEN as dual-function delivery system that can deliver a therapeutic gene and enhance its activity due to DSS-BEN intracellular degradation to BENSpm and its beneficial effect via dysregulated polyamine metabolism in cancer.

4. CONCLUSIONS

We have designed and developed self-immolative polycation DSS-BEN and confirmed its dual functionality as (i) a prodrug of the anticancer agent BENSpm that targets dysregulated polyamine metabolism in cancer and (ii) a gene delivery vector. Our results support the benefits of self-immolative linkers in the design of biodegradable polymers for use in gene delivery. The dual functionality of DSS-BEN makes it a promising delivery platform for combination anticancer therapy, which can be further expanded to deliver a variety of other therapeutic agents, including miRNA, siRNA, and proteins.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR spectra of DCC-BEN. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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