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Synthesis of Bisethylnorspermine Lipid Prodrug as Gene Delivery Vector Targeting Polyamine Metabolism in Breast Cancer

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Abstract

Progress in the development of nonviral gene delivery vectors continues to be hampered by low transfection activity and toxicity. Here we proposed to develop a lipid prodrug based on a polyamine analogue bisethylnorspermine (BSP) that can function dually as gene delivery vector and, after intracellular degradation, as active anticancer agent targeting dysregulated polyamine metabolism. We synthesized a prodrug of BSP (LS-BSP) capable of intracellular release of BSP using thiololytically sensitive dithiobenzyl carbamate linker. Biodegradability of LS-BSP contributed to decreased toxicity compared with nondegradable control L-BSP. BSP showed a strong synergistic enhancement of cytotoxic activity of TNF-related apoptosis-inducing ligand (TRAIL) in human breast cancer cells. Decreased enhancement of TRAIL activity was observed for LS-BSP when compared with BSP. LS-BSP formed complexes with plasmid DNA and mediated transfection activity comparable to DOTAP and L-BSP. Our results show that BSP-based vectors are promising candidates for combination drug/gene delivery.

Keywords

gene delivery; plasmid DNA; cationic lipid; bisethylnorspermine; TRAIL

INTRODUCTION

Successful implementation of novel gene therapy protocols requires the development of strategies to effectively deliver nucleic acids to disease targets. Cationic lipids and polymers

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continue to gain strength as viable alternatives to viral delivery vectors. Progress in the development of these vectors continues to be hampered by their low transfection activity and toxicity of the cationic molecules. The classical biomaterial design paradigm—preparing vectors that are biodegradable into nontoxic low-molecular weight byproduct—yielded many cationic lipids and polymers with acceptable toxicity. Although the toxicity can be decreased to some extent by using biodegradable molecules, amplifying transfection activity is the more difficult problem to solve because it involves complex delivery across multiple barriers. One way to overcome the low transfection is to combine the therapeutic gene with traditional small-molecule drugs that enhance the gene's therapeutic activity.^{1,2} Such drug/gene combination therapies can be accomplished by a simple combination of gene therapy protocols with existing drugs. Alternatively, synthetic gene delivery vectors can be designed that not only deliver a gene but also augment the activity of the gene by exerting their own pharmacologic effect.

There is a growing number of successful examples of drug and gene delivery vectors that combine the delivery function with a pharmacologic activity. For example, Huang et al. developed a novel cationic lipid capable of delivering siRNA while simultaneously enhancing siRNA antitumor effect by downregulating pERK.³ Pluronic copolymers have been shown to chemosensitize multidrug resistant cancers by inhibiting P-glycoprotein and decreasing cellular ATP pools.⁴ Peptides with intrinsic proteasome inhibitory function have been shown to deliver and enhance transfection activity of plasmid DNA.⁵ Cyclodextrins, widely used as excipients and as parts of drug delivery vectors, have been shown to have activity in treatment of a lysosomal storage disorder.⁶

Natural polyamines spermidine, spermine, and their diamine precursor putrescine are essential factors for growth of eukaryotic cells. Polyamines play crucial roles in numerous cellular processes important for cell growth and survival, including association with nucleic acids, maintenance of chromatin conformation, regulation of specific gene expression, ion-channel regulation, maintenance of membrane stability, and free-radical scavenging.⁷ We have recently proposed dually functioning cationic gene delivery vectors based on a class of drugs called polyamine analogues.⁸ These agents exploit the self-regulatory nature of the metabolism of cellular polyamines and have multiple targets in the polyamine pathway. Polyamine metabolism is frequently dysregulated in cancer and other hyper-proliferative diseases.⁷ The polyamine pathway is a distal downstream target for a number of oncogenes, and inhibition of polyamine synthesis disrupts the action of those genes.^{7,9,10} All these factors make polyamine analogues attractive building blocks in the design of delivery vectors that can not only deliver a therapeutic gene but also augment the activity of the gene by exerting their own pharmacologic effect. A number of compounds have been developed that show anticancer activity, including terminally alkylated polyamine analogues that can suppress the polyamine biosynthetic enzymes, greatly induce the polyamine catabolic enzymes, deplete natural polyamine pools, and inhibit cell growth.^{11–13} Among the most successful of the developed polyamine analogues is *N*¹,*N*¹-bisethyl norspermine (BSP) (Figure 1). BSP has shown promising antitumor activity against a wide range of cancers, including melanomas, ovarian, breast, and pancreatic cancers.^{14–17} BSP induces a polyamine catabolic enzyme, spermidine/spermine *N*¹-acetyltransferase (SSAT), downregulates ornithine decarboxylase and *S*-adenosylmethionine decarboxylase,¹⁸ and ultimately causes cell growth inhibition and apoptosis.^{19–21} BSP has also been successfully combined with standard chemotherapeutic agents to produce synergistic anticancer effect.^{22,23}

The goal of this study was to develop biodegradable lipid prodrug based on the polyamine analogue BSP that can function dually as gene delivery vector and, after intracellular degradation, as active pharmacologic agent that synergistically augments the activity of a

therapeutic gene in cancer. We have tested the hypothesis that BSP will enhance activity of TNF-related apoptosis-inducing ligand (TRAIL) in breast cancer.

MATERIALS AND METHODS

Materials

Diocetadecylamine, 4-mercaptobenzoic acid, lithium aluminum hydride, 1-hydroxybenzotriazole (HOBt), 1-(3-*N,N*-dimethylaminopropyl)-3-ethylcarbodiimide chlorohydrate (EDCI), 3,3'-dithiodipropionic acid, zinc powder, carbomethoxysulfonyl chloride, *p*-nitrophenyl chloroformate, trifluoroacetic acid (TFA), dithiothreitol (DTT), hydroxylamine monochloride, spermidine, and ethidium bromide (EtBr) were purchased from Sigma-Aldrich (St. Louis, MO). [¹⁴C]Acetyl-CoA was purchased from PerkinElmer (Waltham, MA). Biodegradable scintillation cocktails Bio-Safe NA for nonaqueous samples and Bio-Safe II for aqueous samples were purchased from RPI Corp. (Mount Prospect, IL). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-like enzyme (TrypLE Express), and Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco (Invitrogen, Carlsbad, CA). RPMI 1640 medium was purchased from Thermo Scientific-Hyclone (Logan, UT). Plasmid DNA, gWiz high-expression luciferase, containing luciferase reporter gene was from Aldevron (Fargo, ND). Luciferase assay system with reporter lysis buffer was purchased from Promega (Madison, WI). Recombinant human TRAIL/Apo2L protein was purchased from PeproTech (Rocky Hill, NJ). L-BSP was synthesized as described in our recent publication.⁸ All other reagents and solvents were purchased from commercial suppliers and were used without further purification unless otherwise stated.

Synthesis

NMR spectra were recorded on a Varian FTNMR Unity-300, Mercury-400 or Mercury-500 MHz spectrometer. Chemical shifts (δ) are expressed in ppm and are internally referenced (0.00 ppm for TMS for ¹H NMR and 77.0 ppm for CDCl₃ for ¹³C NMR). Mass spectra were recorded on a Waters ZQ2000 single quadrupole mass spectrometer using an electrospray ionization source.

Synthesis of 3—A suspension of 3,3'-dithiodipropionic acid **2** (105 mg, 0.5 mmol) and HOBt (203 mg, 1.5 mmol) in anhydrous CHCl₃ (30 mL) was added to the solution of EDCI (233 mg, 1.5 mmol) in anhydrous CHCl₃ (10 mL) at 0 °C, followed by addition of the mixture of Et₃N (152 g, 0.21 mL, 1.5 mmol) and diocetadecylamine **1** (522 mg, 1 mmol) in CHCl₃ (10 mL). The resulting mixture was stirred at room temperature for 12 h, when it turned to a clear solution. The reaction mixture was then partitioned with water (15 mL) at 0 °C. The organic layer was separated, and the water layer was extracted with CHCl₃ (3 × 10 mL). Combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and evaporated under vacuum to give the crude product. The residue was dry-loaded to a silica gel column, and separation (eluent: CHCl₃/ethyl acetate 4:1) gave the product **3** (0.58 g, 95%) as a white solid. *R*_f 0.85 (CHCl₃). Mp 38–39 °C. ¹H NMR (400 MHz, CDCl₃): 3.29 (bt, *J* = 7.8 Hz, 4H), 3.22 (bt, *J* = 7.6 Hz, 4H), 2.96 (t, *J* = 7.0 Hz, 4H), 2.73 (t, *J* = 7.4 Hz, 4H), 1.55–1.50 (m, 8H), 1.26 (bs, 112H), 0.88 (t, *J* = 6.6 Hz, 12H). ¹³C NMR (100 MHz, CDCl₃): 170.2, 47.9, 46.0, 33.5, 32.7, 31.9, 29.65, 29.60 (m), 29.55, 29.51, 29.4, 29.3, 29.1, 27.7, 27.0, 26.9, 22.6, 14.0. ESI MS (*m/z*): calcd for C₇₈H₁₅₆N₂O₂S₂ [M + H]⁺ 1218.16, found 1217.99; [M + Li]⁺ 1224.17, found 1224.00; [M + K]⁺ 1256.12, found 1255.96.

Synthesis of 4.²⁴—To a solution of **3** (0.55 g, 0.79 mmol) in acetic acid (10 mL) was added zinc powder (0.51 g, 7.9 mmol) at 0 °C. The mixture was refluxed for 40 min and monitored by TLC (viewed by UV and stained by DTNB). The mixture was filtered over

Celite and washed with CHCl_3 . The obtained organic solution was evaporated under vacuum. The residue was flushed with nitrogen to remove excess acetic acid. The white solid was dissolved in CHCl_3 (10 mL) and washed with water to neutrality. The combined organic layers were then washed with brine, dried over anhydrous Na_2SO_4 , and concentrated under vacuum. The residue was purified by silica gel column (eluent: CHCl_3 /ethyl acetate 4:1) to give the product **4** (0.55 g, 100%) as a white solid. R_f 0.52 (CHCl_3). Mp 44–45 °C. ^1H NMR (400 MHz, CDCl_3): 3.30 (bt, $J = 7.8$ Hz, 2H), 3.20 (bt, $J = 7.8$ Hz, 2H), 2.80 (bq, $J_1 = 7.6$ Hz, 2H), 2.63 (t, $J = 6.6$ Hz, 4H), 1.72 (t, $J = 8.4$ Hz), 1.53 (m, 4H), 1.26 (bs, 56H), 0.88 (t, $J = 7.0$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3): 169.9, 47.7, 45.9, 37.0, 31.8, 29.60, 29.56 (m), 29.5, 29.4, 29.3, 29.2, 28.9, 27.7, 26.9, 26.8, 22.6, 20.2, 13.9. ESI MS (m/z): calcd for $\text{C}_{39}\text{H}_{79}\text{NOS}$ $[\text{M} + \text{H}]^+$ 610.59, found 610.51; $[\text{M} + \text{Li}]^+$ 616.60, found 616.52; $[\text{M} + \text{K}]^+$ 648.55, found 648.47.

Synthesis of 5—The solution of **4** (0.53 g, 0.87 mmol) in methanol (5 mL) was added dropwise to a solution of carbomethoxysulfonyl chloride (0.11 g, 0.87 mmol) in methanol (10 mL) and CHCl_3 (20 mL) at 0 °C. The mixture was stirred for 30 min at 0 °C, then allowed to reach room temperature, and after 4 h evaporated under vacuum. The obtained residue was dissolved in CHCl_3 (10 mL) and purified on silica (CHCl_3 /ethyl acetate 4:1). Pure product **5** was obtained as a colorless oil, which turned to a white solid when kept in a freezer (0.55 g, 90%). R_f 0.39 (CHCl_3). Mp 36–37 °C. ^1H NMR (400 MHz, CDCl_3): 3.88 (s, 3H), 3.29 (bt, $J = 7.8$ Hz, 2H), 3.20 (bt, $J = 7.8$ Hz, 2H), 3.01 (t, $J = 7.2$ Hz, 2H), 2.72 (t, $J = 7.0$ Hz, 4H), 1.52 (m, 4H), 1.26 (bs, 56H), 0.88 (t, $J = 7.0$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3): 170.1, 169.7, 55.2, 47.8, 46.0, 35.1, 32.7, 31.9, 29.63, 29.59, 29.52 (m), 29.5, 29.3, 29.2, 29.0, 27.7, 26.9, 26.8, 22.6, 20.2, 14.0. ESI MS (m/z): calcd for $\text{C}_{41}\text{H}_{81}\text{NO}_3\text{S}_2$ $[\text{M} + \text{H}]^+$ 700.57, found 700.57; $[\text{M} + \text{Na}]^+$ 722.55, found 722.54; $[\text{M} + \text{Li}]^+$ 706.58, found 706.58; $[\text{M} + \text{K}]^+$ 738.52, found 738.51.

Synthesis of 6—A solution of 4-mercaptobenzoic acid (0.77 g, 5 mmol) in dry THF (10 mL) was added dropwise to a suspension of lithium aluminum hydride (0.57 g, 15 mmol) in dry THF (10 mL) under nitrogen at 0 °C.²⁵ The mixture was stirred overnight at room temperature. Water (5 mL) was added, followed by aqueous HCl (1 N, 5 mL) at 0 °C, and the mixture was stirred for 5 min. The solution was extracted with diethyl ether (3 × 10 mL). The organic layer was washed with H_2O (20 mL) and brine (10 mL), dried over anhydrous Na_2SO_4 , and then concentrated under vacuum at room temperature. The residue was purified by a short silica gel column to give the product as a white solid (0.56 g, 80%). R_f 0.28 (hexane:EA 2:1) Mp 49–51 °C. ^1H NMR (400 MHz, CDCl_3): 7.26–7.20 (m, 4H), 4.60 (s, 2H), 3.45 (s, 1H), 2.00 (bs, 1H). ^{13}C NMR (100 MHz, CDCl_3): 138.3, 129.9, 129.5, 127.8, 64.7.

Synthesis of 7—A solution of **6** (0.32 g, 2.28 mmol) in $\text{CH}_3\text{OH}/\text{CHCl}_3$ (5 mL/10 mL) was added to a solution of **5** (0.53 g, 0.76 mmol) in CH_3OH (5 mL) at 0 °C under nitrogen. The mixture was stirred for 1 h at 0 °C and then warmed to room temperature. After 3 days, the mixture was evaporated and the obtained residue was dissolved by CHCl_3 and purified by a short silica gel column (hexane/ethyl acetate 3:1). Compound **7** was obtained as a colorless oil, which turned to a white solid in the freezer (0.42 g, 75%). R_f 0.38 (hexane/ethyl acetate 3:1). Mp 40–41 °C. ^1H NMR (400 MHz, CDCl_3): 7.47 (d, $J = 8.4$ Hz, 2H), 7.28 (d, $J = 8.4$ Hz, 2H), 4.61 (s, 2H), 3.35 (bs, 1H), 3.24 (bt, $J = 7.6$ Hz, 2H), 3.10 (bt, $J = 7.8$ Hz, 2H), 2.96 (t, $J = 7.0$ Hz, 2H), 2.66 (t, $J = 7.2$ Hz, 2H), 1.47 (m, 4H), 1.26 (bs, 56H), 0.88 (t, $J = 7.0$ Hz, 6H). ^{13}C NMR (100 MHz, CDCl_3): 170.1, 140.2, 135.9, 127.6, 127.4, 64.1, 47.8, 46.0, 34.0, 32.4, 31.8, 29.58, 29.54, 29.47 (m), 29.4, 29.3, 29.2 (m), 28.9, 27.6, 26.9, 26.7, 22.6, 14.0. ESI MS (m/z): calcd for $\text{C}_{46}\text{H}_{85}\text{NO}_2\text{S}_2$ $[\text{M} + \text{H}]^+$ 748.60, found

748.61; $[M + Na]^+$ 770.59, found 770.60; $[M + Li]^+$ 754.62, found 754.63; $[M + K]^+$ 786.57, found 786.59.

Synthesis of 8—*p*-Nitrophenyl chloroformate (0.23 g, 1.12 mmol) was added to a solution of **7** (0.42 g, 0.56 mmol) in anhydrous $CHCl_3$ at 0 °C under nitrogen, followed by Et_3N (0.234 mL, 1.68 mmol). The mixture was stirred for 30 min and then warmed to room temperature. After stirring for 24 h, the mixture was evaporated and the obtained residue was dissolved in $CHCl_3$ and purified by a short silica gel column (hexane/ethyl acetate 5:1). Recrystallization from ethyl acetate gave the pure product **8** as a white solid (0.36 g, 70%). R_f 0.37 (hexane/ethyl acetate 5:1). Mp 52–53 °C. 1H NMR (400 MHz, $CDCl_3$): 8.27 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.8 Hz, 2H), 7.41–7.37 (m, 4H), 5.27 (s, 2H), 3.28 (bt, J = 7.8 Hz, 2H), 3.14 (bt, J = 7.8 Hz, 2H), 3.04 (t, J = 7.0 Hz, 2H), 2.71 (t, J = 6.8 Hz, 2H), 1.48 (m, 4H), 1.26 (bs, 56H), 0.88 (t, J = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 169.9, 155.4, 152.4, 145.4, 138.7, 132.6, 129.4, 127.2, 125.2, 121.7, 70.3, 47.8, 46.1, 34.2, 32.5, 31.9, 29.66, 29.62, 29.6 (m), 29.4, 29.3, 29.0, 27.7, 27.0, 26.8, 22.6, 14.1. ESI MS (m/z): calcd for $C_{53}H_{88}N_2O_6S_2$ $[M + H]^+$ 913.62, found 913.72; $[M + Na]^+$ 935.60, found 935.69; $[M + Li]^+$ 919.62, found 919.74.

Synthesis of LS-BSP—BSP-4HBr (0.705 g, 1.25 mmol) was stirred with solid sodium hydroxide (1.0 g, 25 mmol) in anhydrous $CHCl_3$ (20 mL) for 3 h at room temperature. Anhydrous Na_2SO_4 (1.0 g) was added, and the mixture was stirred for another 30 min. Filtration and washing with $CHCl_3$ (10 mL) gave a solution that contained free base BSP (1.25 mmol). The obtained BSP solution in $CHCl_3$ was cooled down to 0 °C, and a solution of **8** (0.23 g, 0.25 mmol) in anhydrous $CHCl_3$ (15 mL) was added dropwise under nitrogen. After addition, the reaction mixture was stirred at 0 °C for 2 h to reach completion based on TLC. The reaction mixture was purified by a short silica gel column (methanol/ethyl acetate 1:1) to remove *p*-nitrophenol, followed by elution with CH_2Cl_2/CH_3OH /sat. NH_3 (25:10:0.5). The collected eluent containing product was neutralized with 5 M acetic acid to pH 7.4. Evaporation under reduced pressure at 25 °C gave a solid residue. Residual ammonium acetate was removed under vacuum at room temperature to give the product as acetate salt (0.36 g, 70%, kept at –40 °C). R_f 0.46 (CH_2Cl_2/CH_3OH /sat. NH_3 25:10:1). Mp 36–40 °C. 1H NMR (400 MHz, $CDCl_3/CD_3OD$ 10:1): 7.54 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 5.09 (s, 2H), 3.36 (m, 6H), 3.22 (bt, 2H), 2.99 (t, J = 6.4 Hz, 2H), 2.85 (m, 4H), 2.76 (m, 6H), 1.95 (s, 3H), 1.79 (bm, 4H), 1.51 (bm, 4H), 1.26 (bs, 59H), 1.14 (t, J = 6.8 Hz, 3H), 0.88 (t, J = 6.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 177.3, 170.0, 137.2, 135.2, 128.8, 127.4, 66.0, 47.9, 46.1, 34.1, 32.6, 31.9, 29.7, 29.6 (m), 29.4, 29.3, 29.0, 27.7, 27.0, 26.9, 25.2, 24.1, 23.2, 22.6, 14.1. ESI MS (m/z): calcd for $C_{60}H_{115}N_5O_3S_2$ $[M + H]^+$ 1018.85, found 1018.89; $[M + 2H]^{2+}$ 509.93, found 510.23; $[M + Li]^+$ 1024.86, found 1024.94.

Synthesis of 9—Di-*tert*-butyl dicarbonate (Boc_2O) (0.2 g, 0.92 mmol) was added to a solution of LS-BSP (0.11 g, 0.11 mmol) in anhydrous $CHCl_3$ (5 mL) at 0 °C under nitrogen. The mixture was stirred for 1 h at 0 °C and then warmed to room temperature. After stirring for 12 h, the mixture was purified by a short silica gel column to give pure product **9** as colorless oil (0.11 g, 75%). R_f 0.30 (hexane/ethyl acetate 5:1). 1H NMR (400 MHz, $CDCl_3$): 7.52 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.0 Hz, 2H), 5.09 (s, 2H), 3.30–3.10 (m, 22H), 3.02 (t, J = 6.8 Hz, 2H), 2.72 (t, J = 6.8 Hz, 2H), 1.74 (m, 6H), 1.49–1.45 (mul, 31H), 1.26 (bs, 56H), 1.09 (t, J = 6.8 Hz, 2H), 0.88 (t, J = 7.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 170.1, 155.8, 155.4, 152.4, 145.3, 138.6, 132.6, 129.4, 127.2, 125.2, 121.7, 70.3, 47.8, 46.1, 34.2, 32.5, 31.9, 29.66, 29.62, 29.6 (m), 29.4, 29.3, 29.0, 27.7, 27.0, 26.8, 22.6, 14.1. ESI MS (m/z): calcd for $C_{75}H_{139}N_5O_9S_2$ $[M + Na]^+$ 1340.99, found 1341.42; $[M + 2Na]^{2+}$ 681.99, found 682.54; $[M + K]^+$ 1356.96, found 1357.39.

EtBr Exclusion Assay

The ability of the cationic lipids to condense DNA was determined by measuring changes in EtBr/ DNA fluorescence. DNA solution at a concentration of 20 µg/mL was mixed with EtBr (1 µg/mL), and fluorescence was measured and set to 100% using $\lambda_{exc}/\lambda_{em}$ 540/590 nm. Fluorescence readings were taken following a stepwise addition of the lipid solution prepared in 10 mM HEPES pH 7.4, and the condensation curve for each lipid was constructed.

Agarose Gel Retardation Assay

The condensation ability of the lipids and the redox stability of the lipoplexes were examined by agarose gel electrophoresis. Lipid/DNA complexes were formed at specified N/P ratios and incubated with or without 20 mM GSH and/or with or without increasing concentration of heparin for 30 min. Samples were loaded onto a 0.8% agarose gel containing 0.5 µg/mL EtBr and run for 60 min at 120 V in 0.5× TBE running buffer. The gel was visualized under UV illumination on a Gel Logic 100 imaging system.

Particle Size and Zeta Potential Measurement

The hydrodynamic diameters and zeta potentials of lipid/DNA complexes prepared in 10 mM HEPES (pH 7.4) were determined using ZetaPlus Particle Size and Zeta Potential analyzer (Brookhaven Instruments) (Table 2). Scattered light was detected at 90° angle and 25 °C.

Cell Lines

B16F10 mouse melanoma cells were cultured in DMEM supplemented with 10% FBS. MCF-7 human breast adenocarcinoma cells were cultured in RPMI medium with 10% FBS. MDA-MB-231 breast cancer cells were cultured in RPMI with 10% FBS and 1% penicillin/streptomycin. All cells were cultured at 37 °C in incubator with 5% CO₂.

Cell Viability Assay

Cytotoxicity of the cationic lipids in MCF-7 and MDA-MB-231 was determined by MTS assay using CellTiter 96 Aqueous Cell Proliferation Assay Kit (Promega). 4,000 cells were seeded in a 96-well plate and allowed to attach overnight. Medium was then removed and replaced with 200 µL of fresh medium with increasing concentration of BSP, L-BSP, and LS-BSP. Medium was replaced every 48 h with fresh medium containing the same concentration of the tested agents. Cell viability was determined by MTS assay after 120 h treatment according to the manufacturer's protocol. The results are expressed as mean percentage cell viability relative to untreated cells $\pm$ SEM ($n = 4-8$). IC₅₀ values were calculated using Prism 5 (Graphpad Software, Inc.) using Sigmoidal nonlinear regression.

Luciferase Transfection

Complexes were prepared by adding a predetermined amount of cationic lipids to the solution of plasmid DNA in 10 mM HEPES pH 7.4 to achieve a final DNA concentration of 32 µg/mL and N:P ratios of 2, 4, 8, 16, 32. Mass of 325 per one phosphate group of DNA was assumed in the calculations. All transfection studies were performed in 96-well plates with cells plated 24 h before transfection at the seeding density of 12,000 cells/well. The cells were incubated with DNA complexes for 3 h in 150 µL of medium without FBS at 2 µg DNA/mL. Luciferase expression was measured after 24 h and expressed as mean relative light units (RLU) per mg of cellular protein measured by the bicinchoninic acid (BCA) assay $\pm$ SD of quadruplicate samples.

SSAT Assay

SSAT activity in MDA-MB-231 cells was determined using [^{14}C]acetyl-CoA substrate as previously described.^{26,27} Cells were treated for 72 h with IC_{50} doses of BSP, L-BSP and LS-BSP. The cells were harvested immediately after the incubation, resuspended in SSAT breaking buffer (5 mM HEPES, 1 mM DTT, pH 7.2) at 2×10^7 cells/mL and centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant (25 μL) was used to measure total cellular protein concentration using BCA protein assay. Another 20 μL of the supernatant was mixed with a reaction mixture containing 10 μL 0.5 M HEPES pH 7.8, 5 μL 30 mM spermidine, 0.5 μL [^{14}C]acetyl-CoA, and 14.5 μL of water. The mixture was incubated in 37 °C water bath for 5 min, 20 μL 0.5 M hydroxylamine was added, and the reaction was stopped by boiling for 3 min. Fifty microliters of each sample was then pipetted to p81 Whatman filter, and put into a wash system with continuous water flow for 30 min and suspended in BioSafe counting fluid. Each sample was counted for 1 min using 1209 RackBeta liquid scintillation counter (Perkin-Elmer).

Analysis of the Combination Treatment with TRAIL

The median effect/combination index (CI) model was used to determine synergy of the combination treatments of the cationic lipids with TRAIL. Cells were treated for 120 h with increasing concentration of TRAIL, BSP, L-BSP, LS-BSP, or their combinations at a constant molar ratio equal to the ratio of their IC_{50} . The following molar ratios were used: BSP:TRAIL 14,700:1; L-BSP:TRAIL 1,372:1; and LS-BSP: TRAIL 19,600:1. Cell viability was measured by MTS assay as described above. Results were analyzed by CompuSyn software (ComboSyn, Inc. Paramus, NJ) to quantitatively determine whether the combination treatment was synergistic.²⁸ $\text{CI} < 0.90$ is an indication of synergy.

RESULTS AND DISCUSSION

We have previously proposed that BSP can serve as a suitable building block of cationic lipids and polymers that can function dually as gene delivery vectors and active pharmacologic agents targeting dysregulated polyamine metabolism in cancer. To that end, we reported synthesis of a lipid derivative of BSP (L-BSP, Figure 1) and demonstrated its ability to deliver genes in vitro.⁸ The cytotoxic effects of BSP result, in part, from inducing polyamine catabolic enzymes such as SSAT. Here we measured whether the SSAT inducing capability is preserved in L-BSP by measuring SSAT activity in human breast cancer cells MDAMB-231 (Figure 2). The results show that modification of BSP with the lipid moiety results in the loss of its SSAT-inducing activity. This result motivated the present study and provided a strong rationale for adopting a prodrug approach to the synthesis of BSP-based gene delivery vectors.

Synthesis of LS-BSP Prodrug

We have considered multiple prodrug strategies in our approach. Ester prodrugs are widely used in drug design because of easy degradation by ubiquitous plasma esterases or chemical hydrolysis. Prodrug strategies for amine drugs like BSP, however, are limited by the high stability of amide bonds. For dual-functioning BSP-based gene delivery vectors, spatiotemporal considerations of the prodrug activation were crucial. Successful gene delivery requires the delivery vector to remain intact until the DNA is transported into the target cell. Therefore, enzymatically activated amine prodrug approaches, such as carbamates and *N*-acyloxyalkyl derivatives, in which activation typically occurs in the plasma, were not considered suitable. Other strategies that limit prodrug activation to intracellular enzymes, such as peptide spacers activated by lysosomal cathepsins,²⁹ were excluded because of the unfavorable intracellular location of the activation (lysosomal activation and release of DNA is considered premature). Functional groups like -

aminoketones, *N*-Mannich bases, and enaminones offer easy nonenzymatic hydrolytic transformation of amine drugs from their prodrug forms.^{30–32} The possible ways to control spatiotemporal localization of the activation in these approaches are limited. However, it is well established that disulfide reduction is localized predominantly to the cytoplasm and nucleus, which is highly beneficial for gene delivery.^{33–38} Based on these considerations, we selected reducible dithiobenzyl carbamate linker^{39,40} to synthesize LS-BSP prodrug that can be activated by intracellular thiolysis of the disulfide bond (Scheme 1).

The target compound LS-BSP was synthesized using a six step synthesis, depicted in Scheme 2. Despite the four amines in BSP, a single product LS-BSP was detected by ESI-MS and TLC. ESI-MS of the reaction mixture gave the expected m/z 1018.97 and 510.26 peaks, corresponding to m/z $(M + H)^+$ and $(M + 2H)^{2+}$ of LS-BSP (Figure 3a). Excess BSP (MW 244.26) was seen at m/z $(M + H)^+$ and $(M + 2H)^{2+}$ 245.47 and 123.33 in Figure 3a. Purification of LS-BSP was confronted with difficulties caused by easy decomposition during drying due to susceptibility to disulfide–disulfide exchange reactions. Purification of the reaction mixture by silica gel chromatography gave the expected LS-BSP in the eluent. Isolated LS-BSP was neutralized with 5 M acetic acid and the solvent was removed to give the desired compound as acetate salt mixed with ammonium acetate. The ammonium acetate could be removed slowly under reduced pressure but some decomposition was still observed (Figure 3b,c).

Finally, we confirmed that, due to steric effects, it was the terminal and not the internal amine of BSP that was substituted in LS-BSP. The purified LS-BSP was reacted with Boc_2O to give fully Boc-protected compound **9** (Scheme 3). Reductive degradation of **9** with DTT gave tri-Boc-protected BSP **10**. The ^1H NMR and ^{13}C NMR of compound **10** were found to agree with the known NMR spectra of 3,7,11-tri-Boc BSP reported in our previous study.⁸ This result confirmed that the location of BSP substitution in LS-BSP shown Figure 1 and Scheme 2 is correct. We have also attempted synthesis of LS-BSP by reaction of **8** with **10**, but this approach failed due to decomposition of compound **9** during Boc deprotection with TFA.

Reductive Release of BSP from LS-BSP and Restoration of SSAT Inducing Activity

Intracellular reductive or thiolytic breakage of the disulfide in LS-BSP by GSH is expected to lead to an unstable *p*-mercaptobenzyl urethane intermediate, followed by breakdown via 1,6-elimination and decarboxylation, and ultimately resulting in the regeneration of the original BSP molecule and a GSH-conjugated lipid (Scheme 1).^{41,42} For success of our approach, it was necessary to verify that LS-BSP is transformed to free BSP following cleavage of the disulfide bond. LS-BSP was therefore treated with excess of a strong reducing agent (100 mM DTT) in PBS at 37 °C, and progress of the reaction was monitored by TLC and ESI-MS for 24 h. The TLC showed complete disappearance of LS-BSP after 10 min of treatment with DTT and appearance of a mixture of intermediates that disappeared after 4 h (not shown). ESI-MS analysis shown in Figure 4 confirmed the TLC results by showing complete decomposition of LS-BSP and release of BSP as indicated by the disappearance of the peak at 1018 and appearance of the peak at 632.64, corresponding to m/z $[M + \text{Na}]^+$ of regenerated compound **4** (MW 609.58) and the peaks of free BSP shown at m/z $(M + H)^+$ and $(M + 2H)^{2+}$ at 245.47 and 123.33. The activation of the LS-BSP prodrug and release of functional BSP was subsequently confirmed in MDA-MB-231 cells by demonstrating restored SSAT-inducing activity when compared with nondegradable L-BSP (Figure 2). This result clearly suggests that the dithiobenzyl carbamate linker is cleaved in the intracellular environment and that BSP is released in its active form.

Decreased Toxicity of LS-BSP and Synergistic Enhancement of TRAIL Activity

Toxicity of cationic vectors is a major concern in their use in gene delivery. We expected that degradability of LS-BSP not only will restore BSP activity as shown above but also will result in decreased toxicity of the lipid. We measured the toxicity using MTS assay in MDA-MB-231 cells (Table 1). The results confirmed that LS-BSP is significantly less toxic than the nondegradable L-BSP. Similar results were observed also in MCF-7 cells, where L-BSP showed $IC_{50} = 1.40 \pm 0.40 \mu M$ while LS-BSP showed decreased toxicity with $IC_{50} = 17.2 \pm 1.9 \mu M$. Our results confirm previous studies that show that cationic lipids and polymers degradable by intracellular disulfide reduction exhibit reduced toxicity compared to nondegradable analogues.^{43–45} The fact that LS-BSP had lower IC_{50} than the parent BSP is a reflection of the general toxicity of cationic lipids and not a result of enhanced antiproliferative activity of BSP as suggested by similar SSAT-inducing activity of LS-BSP and BSP (Figure 2).

The main objective of this study was to design BSP-based lipids capable of functioning dually as (i) gene delivery vectors and (ii) active anticancer agents targeting dysregulated polyamine metabolism in cancer. We selected TRAIL as a suitable therapeutic agent that could benefit from enhancing activity of BSP. TRAIL is attractive in cancer treatment because it selectively induces apoptosis in cancer cells and its activity can be enhanced by various anticancer drugs.^{1,46,47} To allow determination of synergy using the Chou–Talalay method, all TRAIL combination experiments were performed at constant BSP:TRAIL or BSP lipid:TRAIL ratios (Figure 5) determined from the ratios of the IC_{50} values of the individual agents. Five to six doses were used in serial dilutions covering the activity range of BSP, L-BSP, LS-BSP, and TRAIL, and the fraction of killed cells (F_a) was determined by MTS assay. A combination index (CI) was calculated by the Chou–Talalay method using CompuSyn software.⁴⁸ We first tested whether the parent BSP enhances activity of the TRAIL protein in triple-negative breast cancer cells MDA-MB-231 (Figure 5a). The observed CI values were <0.04 across the entire studied F_a range, indicating a strong synergy between BSP and TRAIL and supporting our choice of TRAIL as the therapeutic gene. Less potent but still strong synergy between BSP and TRAIL was found also in the estrogen-dependent MCF-7 cells (not shown). We then evaluated whether the enhancing effect of BSP on TRAIL activity is preserved in LS-BSP (Figure 5b). Combination treatment with LS-BSP and TRAIL was indeed more effective than either of the agents alone, but the calculated CI values were higher than in the case of BSP/TRAIL combination, indicating a weaker synergism and a simple additive effect at lower F_a range. A similar outcome was obtained when the cells were treated with L-BSP/TRAIL combination (Figure 5c). We hypothesize that the transition from a strong synergism in BSP/TRAIL treatment to a weaker synergism and even more additive effect of the BSP lipids is the result of nonspecific toxicity associated with the cationic lipids and not a specific effect of BSP on polyamine metabolism. The effect of the nonspecific lipid toxicity is prominent even in LS-BSP despite its degradability. We propose that, in order to take full advantage of the BSP/TRAIL synergism that is based on a specific mechanism of action of BSP on the polyamine metabolism, toxicity of the BSP-based delivery vectors will have to be decreased even beyond the decrease achieved with LS-BSP.

Initial Evaluation of DNA Complexes of LS-BSP

The ability to efficiently deliver genes is an important requirement for the dually functioning BSP-based delivery vectors. We have therefore conducted preliminary studies to determine their ability to deliver reporter gene in vitro. We first confirmed the ability of L-BSP and LS-BSP to condense DNA using EtBr exclusion assay (Figure 6a). Both LS-BSP and L-BSP efficiently condensed DNA, while the parent BSP showed poor condensation ability with only 20% decrease in fluorescence intensity at N/P 8. L-BSP showed a more efficient DNA

condensing ability as indicated by the fact that full DNA condensation was achieved at N/P < 2, while N/P 5 was required in the case of LS-BSP.

We expect that the intracellular cleavage of the disulfide bond in LS-BSP is mediated by thiol/disulfide exchange reactions with small redox molecules like GSH. The reduction is expected not only to release the active form of BSP but also to facilitate release of the DNA from LS-BSP/DNA complexes. Cellular GSH is predominantly present in the cytoplasm (1–11 mM), which is also the principal site of GSH biosynthesis.^{49–51} However, nuclear GSH concentrations are typically even higher and can reach up to 20 mM.^{52–54} As shown above, the reduction of the disulfide bond in LS-BSP resulted in the release of BSP. The effect of disulfide reduction on the DNA release was investigated by incubating LS-BSP/DNA and control L-BSP/DNA complexes with 20 mM GSH and by analyzing the concentration of heparin required to destabilize the complexes and release free DNA (Figure 6b). The control nonreducible L-BSP/DNA complexes were stable in the absence of GSH up to 100 µg/mL heparin. At heparin concentrations 100 µg/mL and above, signs of free DNA were observed but most of the DNA was confined to the start of the gel. In the reducing environment of 20 mM GSH, first signs of DNA release from the L-BSP/DNA complexes were observed at 80 µg/mL heparin. Strong responsiveness to reducing conditions was observed for LS-BSP/DNA complexes, suggesting that the employed prodrug strategy not only results in the release of an active form of BSP but also facilitates DNA release from the complexes. We predict that the fact that cancer cells often exhibit elevated levels of GSH will further increase the effectiveness of the thiolitically activated gene delivery vector.³⁵ No DNA release was observed in oxidizing conditions up to 120 µg/mL heparin, while only 40 µg/mL heparin was required to cause DNA release in the reducing conditions of 20 mM GSH.

Initial transfection activity of the BSP-based DNA complexes was determined in a panel of three cell lines using luciferase reporter plasmid (Figure 7). DOTAP/DNA complexes were used as controls. DNA complexes of the nondegradable L-BSP showed consistently higher transfection activity than bio-reducible LS-BSP/DNA complexes in all three cell lines. As expected, transfection of BSP/DNA complexes was several orders magnitude lower than transfection of any of the BSP lipid complexes across all tested N/P ratios. Overall, transfection of the LS-BSP complexes showed a stronger dependence on the N/P ratio, in particular in the B16F10 and MDAMB-231 cells. Between the lowest and highest tested N/P ratio, the transfection of LS-BSP complexes increased 192-, 15-, and 6,760-fold in the B16F10, MCF-7, and MDA-MB-231 cells, respectively. For comparison, the corresponding increases for L-BSP complexes were 4-, 6-, and 270-fold. The strong N/P dependence is likely the result of premature disulfide breakage in LS-BSP. It suggests that LS-BSP may be partly degraded already before cell uptake by extracellular thiols, thus requiring larger lipid excess to achieve comparable transfection as L-BSP complexes. Differences among the three cell lines are likely the result of different amounts of secreted thiols from different cells as suggested previously.⁵⁵

CONCLUSIONS

We described synthesis of degradable BSP prodrug capable of functioning dually as a gene delivery vector and an active anticancer agent targeting dysregulated polyamine metabolism in cancer. The used dithiobenzylcarbamate linker in the LS-BSP prodrug is cleavable by thiolysis, which leads to the release of free BSP and restoration of SSAT-inducing activity of BSP. BSP demonstrated a strong synergy with TRAIL in triple negative breast cancer cells MDA-MB-231. Although LS-BSP also enhanced TRAIL activity, the results were confounded by nonspecific lipid-related toxicity that diminished the synergistic enhancement that originated in the specific mechanism of action of BSP on the polyamine pathway. In conclusion, we demonstrated that BSP can serve as a suitable building block for

the design of gene delivery vectors with dual functionality. BSP-based delivery vectors should be especially effective in TRAIL-based cancer gene therapies due to the strong synergistic effect of BSP on TRAIL cytotoxic effect. The use of BSP-based delivery vectors for delivery of TRAIL gene and anticancer TRAIL gene therapy is under investigation.

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BSP **L-BSP** **LS-BSP**

Figure 1.
Structure of BSP and its lipid derivatives.

Figure 2. SSAT induction by BSP and its lipid derivatives. MDA-MB-231 cells were treated with IC50 dose of indicated sample for 72 h. Results are shown as mean pmol of N^1 -acetylspermidine/mg of protein/min $\pm$ SD, $n = 3$. One-way ANOVA with Tukey's multiple comparison test ($p < 0.05$ untreated vs BSP, untreated vs LS-BSP, and L-BSP vs LS-BSP).

Figure 3. ESI-MS and ^1H NMR spectra of LS-BSP prodrug. (a) ESI-MS of unpurified LS-BSP after reaction of **8** with BSP. (b) ESI-MS of LS-BSP after purification. (c) ^1H NMR of LS-BSP in CDCl_3 .

Figure 4.
ESI-MS after reductive degradation of LS-BSP.

Figure 5.
Effect of BSP, LS-BSP, and L-BSP on antiproliferative activity of TRAIL in MDA-MB-231 cells.

Figure 6. DNA condensation and reduction-triggered DNA release from LS-BSP complexes. (a) DNA condensation by BSP, L-BSP, and LS-BSP assessed by EtBr exclusion assay. (b) Effect of disulfide reduction on susceptibility of LS-BSP/DNA complexes to polyelectrolyte exchange reactions with heparin. L-BSP/DNA (N/P 8) and LS-BSP (N/P 20) were treated with increasing concentration of heparin (0, 20, 40, 60, 80, 100, 120, 140 $\mu\text{g/mL}$) either alone or in combination with 20 mM GSH.

Figure 7. Transfection activity of BSP and its lipid derivatives in (a) B167F10 cells, (b) MCF-7 cells, and (c) MDA-MB-231 cells. Results are expressed as luciferase expression in RLU/mg of protein (mean $\pm$ SD, $n = 4$).

Scheme 1.
Mechanism of Thiolytic Activation of LS-BSP Prodrug

Scheme 2.
Synthesis of LS-BSP Prodrug

Scheme 3.
Synthesis and Degradation of Compound 9

Table 1

Cytotoxicity of TRAIL, BSP and Its Derivatives in MDA-MB-231

sample	IC ₅₀ ^a (μM)
BSP	91.4 ± 37.6
LS-BSP	21.4 ± 2.9
L-BSP	6.84 ± 0.69
TRAIL	0.0051 ± 0.2

^aIC₅₀ values were determined by MTS assay after 120 h treatment.

Table 2

Hydrodynamic Diameter and Zeta Potential of L-BSP/DNA (N/P 8) and LS-BSP/DNA (N/P 20) Complexes

sample	size (nm)	zeta (mV)
LS-BSP	98.6 ± 0.9	30.9 ± 3.1
L-BSP	86.2 ± 0.8	41.0 ± 1.1