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RESEARCH PAPER

Bisethylnorspermine Lipopolyamine as Potential Delivery Vector for Combination Drug/Gene Anticancer Therapies

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ABSTRACT

Purpose To design novel synthetic gene delivery system in which the carrier molecule functions dually as a carrier and a cytotoxic agent targeting dysregulated polyamine metabolism in cancer.

Methods Bisethylnorspermine (BENSpm) lipopolyamine was synthesized and its toxicity evaluated by MTS assay in MCF-7 and MCF-10A cells. Transfection activity was determined using luciferase plasmid DNA.

Results Asymmetrical lipid analogue of polyamine anticancer drug BENSpm was synthesized using nucleophilic ring opening of azetidinium ion. The synthesized LipoBENSpm showed cytotoxicity comparable to that of parent BENSpm in human breast cancer cell line MCF-7 but mediated 3–4 orders magnitude higher transfection activity. Importantly, cytostatic activity of BENSpm, typically in a low μM range, falls within a relevant and typical concentration range required for efficient gene delivery.

Conclusions These findings make gene delivery vectors based on BENSpm promising candidates for combination drug/gene approaches to the treatment of cancer.

KEY WORDS drug combination · drug delivery · gene delivery · lipid · transfection

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ABBREVIATIONS

BENSpm	bisethylnorspermine
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethy-lammonium methylsulfate
FBS	fetal bovine serum
SSAT	spermidine/spermine N^1-acetyltransferase

INTRODUCTION

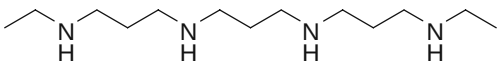
Natural polyamines spermidine, spermine, and their diamine precursor putrescine are essential factors for growth of eukaryotic cells. Polyamines play crucial roles in numerous cellular processes important for cell growth and survival, including association with nucleic acids, maintenance of chromatin conformation, regulation of specific gene expression, ion-channel regulation, maintenance of membrane stability, and free-radical scavenging (1). Given their natural function in DNA association, it is no surprise that both spermidine and spermine have been investigated for construction of synthetic gene delivery vectors (2,3). Indeed, several of widely used lipid transfection reagents, such as DOGS and DOSPA, are lipopolyamines with spermine as the DNA binding functionality. Numerous polycations based on spermine and spermidine have been also synthesized and shown to exhibit favorable transfection properties (4–6).

Involvement of naturally occurring polyamines in cell proliferation and differentiation prompted studies of the polyamine pathway as a target for chemotherapeutic intervention. Studies show that polyamine metabolism is dysregulated in many types of cancer, leading to polyamine

levels significantly higher than in normal tissues (7,8). It was also suggested that the polyamine pathway is a distal downstream target for a number of validated oncogenes and that inhibition of polyamine synthesis disrupts the action of those genes (1,9,10). The recognition that polyamines are required for cell growth and that their metabolic pathway is frequently altered in cancers resulted in the development of enzyme inhibitors for each step of the polyamine biosynthetic pathway (11). In order to overcome limitations of enzyme inhibitors, such as compensatory upregulation, an alternative strategy has been to exploit the self-regulatory nature of polyamine metabolism by developing polyamine analogues (12). A number of compounds have been developed that show anticancer activity, including symmetrically and asymmetrically 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 (13–15).

Among the most successful of the developed polyamine analogues is N^1,N^{11}-bisethylnorspermine (BENSpm, also known as DENSpm and BE333) (Fig. 1). BENSpm has shown promising antitumor activity against a wide range of cancers, including melanomas, ovarian, breast, and pancreatic cancers (16–19). BENSpm depletes intracellular polyamines, downregulates both ornithine decarboxylase and *S*-adenosylmethionine decarboxylase and upregulates polyamine catabolism by inducing spermidine/spermine N^1-acetyltransferase (SSAT). The increase in polyamine catabolism is then responsible for a mechanism of apoptotic cell death observed in cells treated with BENSpm. In addition, BENSpm has been successfully combined with standard chemotherapeutic agents to produce synergistic anticancer effect (20,21).

The long-term goal of this study is to develop a novel approach to the design of synthetic gene delivery systems in which the carrier molecule functions dually as a carrier and a cytotoxic agent targeting dysregulated polyamine metabolism in cancer. Polyamine analogues like BENSpm represent an ideal choice of the DNA-binding functionality for the preparation of such delivery systems due to their cationic nature and structural simplicity. This report describes the method of synthesis of asymmetrical lipopolyamine based on BENSpm and initial evaluation of its cytoxocity and transfection properties.



BENSpm

Fig. 1 Structure of BENSpm.

MATERIALS AND METHODS

Materials

Reagents and solvents were purchased from commercial suppliers and were used without further purification unless otherwise stated. Anhydrous THF and CH_2Cl_2 were dried and distilled according to standard procedures. Ten percent Pd/C was purchased from Aldrich (catalog No. 520888). All reactions were monitored by thin-layer chromatography (TLC), which was viewed by UV light at 254 nm and then stained with phosphomolybdic acid (PMA) or ninhydrin in ethanol. IR spectra were recorded on a Jasco FT/IR 4200 infrared spectrometer with ATR PRO450-S attachment. Selected characteristic peaks are reported in cm^{-1}. NMR spectra were recorded on a Varian FT-NMR Unity-300, Mercury-400 or Varian-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 $CDCl_3$ for ^{13}C NMR). Mass spectra were recorded on a Waters ZQ2000 single quadrupole mass spectrometer using an electrospray ionization source. gWiz™ High-Expression Luciferase (gWIZLuc) plasmid was purchased from Aldevron.

Synthesis

Synthesis of 3

The mixture of ethylbenzylamine **2** (1.35 g, 10 mmol), ethyl acrylate (1.0 g, 10 mmol) and copper acetate monohydrate (0.1 g, 0.5 mmol) in H_2O/CH_3CN (1:1) 40 mL was stirred overnight at room temperature. Ethyl acetate (30 mL) was added, and the organic layer was separated. The aqueous phase was extracted with ethyl acetate (20 mL×3). The combined organic layers were dried over anhydrous Na_2SO_4. Evaporation gave the crude product and purification by silica gel column gave the product **3** as colorless oil (2.0 g, 85%). Rf 0.60 (*n*-hexane/ethyl acetate = 3/1). FTIR (neat) 2972, 2803, 1732, 1369, 1194, 1163, 1045, 1027, 731, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.15–7.17 (m, 5H), 4.08 (q, J=7.2 Hz, 2H), 3.54 (s, 2H), 2.79 (t, J= 7.2 Hz, 2H), 2.48 (q, J=7.2 Hz, 2H), 2.42 (t, J=7.2 Hz, 2H), 1.19 (t, J=7.0 Hz, 3H), 1.00 (t, J=6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 172.2, 139.3, 128.3, 127.8, 126.4, 59.8, 57.6, 48.4, 46.8, 32.4, 13.8, 11.5.

Synthesis of 4

A solution of amino ester **3** (5.1 g, 21.7 mmol) in dry THF (10 mL) was added dropwise to a suspension of $LiAlH_4$ (1.0 g, 26.3 mmol) in dry THF (40 mL) under nitrogen at 0°C. After addition, the mixture was stirred for 3 h under

reflux (monitored by TLC). The reaction mixture was cooled to 0°C, and saturated NH_4Cl solution was added slowly. The mixture was stirred for another 30 min at room temperature and then evaporated under vacuum. The remaining aqueous solution was extracted with CH_2Cl_2 (30 mL×4). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under vacuum; the residue was purified by silica gel column to give the product **4** as colorless oil (3.93 g, 95%). Rf 0.53 (methanol/dichloromethane = 5/2). FTIR (neat) 3323, 2933, 2809, 1452, 1051, 730, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.32–7.20 (m, 5H), 5.41 (bs, 1H), 3.69 (t, J=5.6 Hz, 2H), 3.54 (s, 2H), 2.62 (t, J=5.6 Hz, 2H), 2.50 (q, J=7.2 Hz, 2H), 1.69 (quart, J=5.6 Hz, 2H), 1.06 (t, J=7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 138.2, 128.7, 128.8, 126.8, 63.8, 58.0, 53.0, 46.7, 27.6, 11.2; ESI MS (*m/z*) Calcd for C12H19NO $[M+H]^+$: 194.3, found: 194.4; $[M+Na]^+$: 216.3, Found 216.3.

Synthesis of 5 and 6

To a solution of amino alcohol **4** (3.93 g, 20.4 mmol) and Et_3N (14 mL, 102 mmol) in CH_2Cl_2 (20 mL) was added methanesulfonyl chloride (MsCl) (3.15 g, 40.8 mmol) under nitrogen at −10°C. The reaction mixture was stirred for 1 h at −10°C and diluted with 2% Et_3N in ethyl acetate (20 mL). The mixture was immediately loaded to a short deactivated silica gel column (2% Et_3N in ethyl acetate) and then eluted with 2% Et_3N in ethyl acetate. Using methanol as solvent to collect the eluate, the product of **5** (Rf 0.48, EA/Hexane = 3/1) was partly decomposed to azetidinium salt of **6** during evaporation. The product mixture of **5** and **6** was dissolved in CH_2Cl_2 (10 mL) and kept at room temperature for 24 h and almost all transformed to **6** as confirmed by TLC and NMR. Removing the solvent under vacuum and drying with diethyl ether (5 mL) gave white solid. Removal of diethyl ether under vacuum yielded the hygroscopic product **6** (4.95 g, 100%), which was kept in vacuum desiccator. mp 118–120°C. FTIR (neat) 3443, 2991, 1737, 1456, 1206, 1177, 1037, 766, 709 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.60–7.58 (m, 2H), 7.47–7.44 (m, 3H), 4.76 (q, J=8.8 Hz, 2H), 4.72 (s, 2H), 4.18 (q, J= 8.8 Hz, 2H), 3.49 (q, J=7.2 Hz, 2H), 2.77 (s, 3H), 2.60–2.53 (m, 2H), 1.44 (t, J=7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 131.6, 130.4, 129.4, 128.0, 61.9, 60.6, 54.5, 39.6, 14.1, 8.2; ESI MS (*m/z*) Calcd for azetidinium C12H18N^+: 176.3, found M^+ 176.4. Calcd for methanesulfonate CH3O3S^-: 95.0, found M^- 94.9.

Synthesis of 7 and 8

The mixture of azetidinium methanesulfonate **6** (0.542 g, 2 mmol) and mono-Boc-1,3-diamine (0.766 g, 4.4 mmol) in dry CH_2Cl_2 (10 mL) was refluxed under nitrogen for 20 h. During the heating, white precipitate appeared. After completion of the reaction, water (15 mL) was added to partition the mixture. Organic layer was separated, and aqueous layer was extracted with CH_2Cl_2 (10 mL×3). Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give the crude product, which was purified by silica gel column to give the structure of **7** (0.63 g, 90%) at Rf 0.1 and **8** (0.05 g, 5%) at Rf 0.14 (methanol) as colorless oil. Data for **7**: FTIR (neat) 3309, 2969, 2932, 2804, 1695, 1364, 1249, 1170, 730, 698 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.32–7.22 (m, 5H), 5.20 (bs, 1H), 3.53 (s, 2H), 3.18 (bq, 2H), 2.62–2.56 (m, 4H), 2.50 (q, J=7.2 Hz, 2H), 2.46 (t, J=7.2 Hz, 2H), 1.63 (m, 4H), 1.44 (s, 9H), 1.04 (t, J=7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 156.0, 139.9, 128.8, 128.1, 126.7, 78.8, 58.1, 51.3, 48.4, 47.9, 47.2, 39.4, 29.9, 28.4, 27.1, 11.7; ESI MS (*m/z*) Calcd for C20H35N3O2 $[M+H]^+$ 350.5, found: 350.5; $[M+K]^+$ 388.3, Found: 388.3. Data for **8**: FTIR (neat) 3353, 2966, 2932, 2800, 1712, 1364, 1248, 1167, 730, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.32–7.20 (m, 10H), 5.51 (bs, 1H), 3.55 (s, 4H), 3.13 (bq, 2H), 2.49 (q, J= 7.1 Hz, 4H), 2.44–2.35 (m, 10H), 1.61–1.54 (m, 6H), 1.43 (s, 9H), 1.02 (t, J=6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 156.0, 139.9, 128.8, 128.1, 126.7, 78.7, 58.1, 52.7, 52.1, 51.4, 47.3, 40.0, 28.4, 26.6, 24.5, 11.7; ESI MS (m/z) Calcd for C32H52N4O2 $[M+H]^+$ 525.4, found: 525.4; $[M+K]^+$ 563.4. Found: 563.3.

Synthesis of 9

The mixture of **7** (0.349 g, 1 mmol), mesitylenesulfonyl chloride (0.24 g, 1.1 mmol) and sodium hydroxide solid (0.1 g, 2.5 mmol) in CH_2Cl_2 (10 ml) was stirred for 24 h under nitrogen at room temperature. Water (10 mL) and sat. aq. Na_2CO_3 (5 mL) were added at 0°C. Organic layer was separated, and aqueous layer was extracted with CH_2Cl_2 (10 mL×3). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give the crude product, which was purified by silica gel column to afford **9** (0.53 g, 100%) as colorless oil. Rf 0.60 (EtOAc). FTIR (neat) 3381, 2971, 2935, 1711, 1365, 1313, 1248, 1147, 730, 698 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.29–7.21 (m, 5H), 6.91 (s, 2H), 4.60 (bs, 1H), 3.40 (s, 2H), 3.22 (t, J=6.8 Hz, 2H), 3.11 (t, J=8.2 Hz, 2H), 3.05 (m, 2H), 2.57 (s, 6H), 2.35 (q, J=7.1 Hz, 2H), 2.26–2.24 (singlet and triplet, 5H), 1.65 (quart, J=6.8 Hz, 2H), 1.54 (quart, J=7.6 Hz, 2H), 1.42 (s, 9H), 0.93 (t, J=6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 155.8, 142.2, 139.9, 139.6, 133.2, 131.8, 128.5, 128.0, 126.6, 78.8, 57.8, 50.3, 47.1, 43.8, 42.9, 37.3, 28.3, 27.5, 24.8, 22.7, 20.8, 11.4; ESI MS (m/z) Calcd for C29H45N3O4S $[M+H]^+$: 532.3, found: 532.4; $[M+K]^+$ 570.3. Found: 570.4.

Synthesis of 10 and 11

After three vacuum/hydrogen cycles to replace air inside the reaction tube with hydrogen, the mixture of **9** (0.53 g, 1 mmol) and 10% Pd/C (0.265 g, 50% *w*/*w*) in methanol (10 mL) was shaken at room temperature (22°C) under 50 psi hydrogen pressure for 2 days. The reaction mixture was filtered, and the filter cake was washed with methanol. The filtrate was concentrated to give crude product, which was purified by a short silica gel column to yield the product **10** (0.40 g, 90%) as colorless oil at Rf 0.22 and **11** (0.05 g, 10%) at Rf 0.25 (methanol). Data for **10**: FTIR (neat) 3384, 2971, 2936, 1705, 1364, 1313, 1249, 1146, 732 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) 6.95 (s, 2H), 4.57 (bs, 1H), 3.27–3.20 (m, 4H), 3.05 (q, J=6.0 Hz, 2H), 2.60 (s, 6H), 2.52 (q, J= 7.2 Hz, 2H), 2.48 (t, J=6.4 Hz, 2H), 2.30 (s, 3H), 1.72–1.62 (m, 4H), 1.42 (s, 9H), 1.03 (t, J=7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 155.7, 142.2, 139.7, 132.9, 131.7, 78.6, 46.3, 43.7, 43.1, 42.5, 37.2, 28.1, 27.4, 27.2, 22.5, 20.6, 14.9; ESI MS (*m*/*z*) Calcd for C22H39N3O4S $[M+H]^+$ 442.3, found: 442.4; $[M+K]^+$ 480.3. Found: 480.3. Data for **11**: FTIR (neat) 3390, 2974, 2937, 1698, 1365, 1313, 1249, 1147, 730 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) 6.95 (s, 2H), 4.71 (bs, 1H), 3.27–3.20 (m, 4H), 3.04 (q, J=6.0 Hz, 2H), 2.60 (s, 6H), 2.52 (q, J=6.8 Hz, 2H), 2.48 (t, J=6.4 Hz, 2H), 2.30 (s, 3H), 1.72–1.62 (m, 4H), 1.42 (s, 9H), 1.03 (t, J= 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 155.7, 142.2, 139.7, 132.9, 131.7, 78.6, 46.3, 43.7, 43.1, 42.5, 37.2, 28.1, 27.4, 27.2, 22.5, 20.6, 14.9; ESI MS (*m*/*z*) Calcd for C23H41N3O4S $[M+H]^+$: 456.3, found: 456.4; $[M+K]^+$: 494.3, Found: 494.3.

Synthesis of 12

The mixture of **10** (0.441 g, 1 mmol), mesitylenesulfonyl chloride (0.24 g, 1.1 mmol) and sodium hydroxide solid (0.1 g, 2.5 mmol) in CH_2Cl_2 (10 mL) was stirred for 24 h under nitrogen at room temperature. Water (10 mL) and sat. aq. Na_2CO_3 (5 mL) were added at 0°C. Organic layer was separated, and aqueous layer was extracted with CH_2Cl_2 (10 mL×3). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated and purified by silica gel column to give the structure of **12** as colorless oil (0.623 g, 100%). Rf 0.35 (*n*-hexane/ AcOEt = 2/1). FTIR (neat) 3393, 2975, 2937, 1710, 1313, 1247, 1145, 694 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.95 (s, 2H), 6.93 (s, 2H), 4.47 (bs, 1H), 3.13 (t, J=7.8 Hz, 4H), 3.05 (t, J=7.0 Hz, 4H), 2.99 (q, J=6.0 Hz, 2H), 2.57 (s, 6H), 2.55 (s, 6H), 2.29 (s, 6H), 1.71–1.67 (m, 2H), 1.61–1.57 (m, 2H), 1.42 (s, 9H), 0.98 (t, J=7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 155.6, 142.3, 142.1, 139.8, 139.7, 132.9, 132.8, 131.8, 131.7, 78.7, 42.9, 42.7, 42.3, 39.8, 37.1, 28.1, 27.1, 25.0, 22.5, 22.4, 20.6, 12.4; ESI MS (*m*/*z*) Calcd for C31H49N3O6S2 $[M+H]^+$ 624.3, found: 624.5; $[M+K]^+$ 662.3, Found: 662.4.

Synthesis of 13

To a solution of **12** (0.623 g, 1 mmol) in anhydrous CH_2Cl_2 (5 mL), trifluoroacetic anhydride (TFA) (5 mL) was added slowly. The mixture was stirred for 4 h at room temperature. The solvents were removed under vacuum, and the residue was neutralized by 2 N aq. NaOH, extracted with CH_2Cl_2 (15 mL×3). The combined organic layers were washed with sat. aq. Na_2CO_3, dried over anhydrous Na_2SO_4 and evaporated to give the crude product, which was purified by silica gel column to yield **13** (0.523 g, 100%) as colorless oil. Rf 0.65 (10% triethyl amine in methanol).). FTIR (neat) 2976, 2937, 1603, 1456, 1311, 1145, 732, 694 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.92 (d, 4H), 3.18 (t, J=7.2 Hz, 2H), 3.11 (q, J=7.1 Hz, 2H), 3.04 (q, J=6.8 Hz, 4H), 2.58 (t, J=7.6 Hz, 2H), 2.56 (s, 6H), 2.55 (s, 6H), 2.28 (s, 6H), 1.99 (bs, 2H), 1.70–1.66 (m, 2H), 1.59–1.55 (m, 2H), 0.97 (t, J=7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 142.2, 142.1, 139.7, 132.9, 132.8, 131.7, 131.6, 42.8, 42.3, 39.8, 38.6, 30.3, 24.9, 22.5, 22.4, 20.6, 12.4; ESI MS (*m*/*z*) Calcd for C26H41N3O4S2 $[M+H]^+$ 524.3, found: 524.4; $[M+K]^+$ 562.3, Found: 562.4.

Synthesis of 14 and 15

The mixture of **13** (0.575 g, 1.1 mmol), azetidinium methanesulfonate **6** (0.271 g, 1 mmol) and Et_3N (0.21 g, 2 mmol) in dry CH_2Cl_2 (10 mL) was refluxed for 24 h under nitrogen. After completion of the reaction, water (15 mL) and CH_2Cl_2 (10 mL) were added to partition the mixture. Organic layer was separated, and aqueous layer was extracted with CH_2Cl_2 (10 mL×3). Combined organic layers were washed with sat. aq. Na_2CO_3, dried over anhydrous Na_2SO_4, and evaporated to give the crude product, which was purified by silica gel column to give the structure of **14** (0.62 g, 88%) at Rf 0.20 and **15** (0.04 g, 5%) at Rf 0.22 (methanol/chloroform 10:1) as colorless oil. Data for **14**: FTIR (neat) 2972, 2936, 2872, 2806, 1603, 1454, 1313, 1146, 730, 696 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.30–7.22 (m, 5H), 6.91 (s, 4H), 3.52 (s, 2H), 3.12 (m, 4H), 3.04 (m, 4H), 2.55 (s, 12H), 2.49–2.45 (m, 4H), 2.41–2.36 (m, 4H), 2.28 (s, 3H), 2.27 (s, 3H), 1.70 (m, 2H), 1.55 (m, 4H), 1.03 (t, J=7.6 Hz, 3H), 0.99 (t, J=7.2 Hz, 3H); ^{13}C NMR (100 MHz, CDCl3) 142.3, 142.2, 139.9, 139.8, 133.0, 131.8, 128.7, 128.0, 126.6, 58.0, 51.1, 48.2, 47.1, 46.8, 43.5, 43.0, 42.5, 40.0, 27.6, 27.0, 25.2, 22.7, 22.6, 20.8, 20.7, 12.6, 11.6; ESI MS (*m*/*z*) Calcd for C38H58N4O4S2 $[M+H]^+$: 699.4, found: 699.6, $[M+2H]^{2+}$ 350.6. Data for **15**: FTIR (neat) 2966, 2936, 2871, 2799, 1603, 1453, 1315,

1147,730, 696 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.31–7.21 (m, 10H), 6.91 (s, 2H), 6.88 (s, 2H), 3.52 (s, 4H), 3.10 (q, $\mathcal{J}$=7.2 Hz, 2H), 3.02 (m, 6H), 2.55 (s, 6H), 2.54 (s, 6H), 2.47 (q, $\mathcal{J}$=6.4 Hz, 4H), 2.36 (t, $\mathcal{J}$=7.6 Hz, 4H), 2.29–2.25 (m and ds, 10H), 2.18 (t, $\mathcal{J}$=7.2 Hz, 2H), 1.70 (m, 2H), 1.49 (m, 6H), 1.01 (t, $\mathcal{J}$=7.2 Hz, 6H), 0.98 (t, $\mathcal{J}$=7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 142.3, 140.1, 140.0, 139.9, 133.2, 131.9, 128.8, 128.1, 126.7, 58.0, 51.8, 51.4, 51.3, 47.2, 44.1, 43.4, 42.6, 40.1, 25.5, 25.0, 24.3, 22.8, 22.7, 20.9, 20.8, 12.7, 11.7; ESI MS (*m/z*) Calcd for C50H75N5O4S2 $[M+H]^+$ 874.5, found: 874.6, $[M+2H]^{2+}$ 438.0.

Synthesis of 16

Phenol (0.235 g, 2.5 mmol) was added to the solution of **14** (0.35 g, 0.5 mmol) in dry dichloromethane (10 mL), followed by 30% HBr in glacial acetic acid (1.3 mL) under nitrogen at 0°C. The reaction mixture was stirred for 1 h at 0°C and then warmed up to room temperature (25°C). After stirring for 24 h, the color of the solution turned brown. Water (5 mL) was added at 0°C. The organic phase was separated and extracted with 5 mL of water. The combined aqueous phases were washed with dichloromethane (15 mL×5) until no more aromatic compounds were detected by TLC. The brown aqueous layer was evaporated under vacuum to get a solid residue, which was washed with methanol (30 mL) to give the light yellow solid. And then the lighter yellow solid was dissolved in water (5 mL) and stirred with activated carbon (2 g) at 50°C for 10 min. The carbon was filtered off, and the solution was evaporated under vacuum at 60°C to give the product **16** as white tetra-hydrobromide salt (0.31 g, 94%). Mp >230°C. FTIR (neat) 3445, 3017, 2970, 1738, 1365, 1228, 1217, 1204 cm^{-1}; ^1H NMR (400 MHz, D_2O) 7.52 (m, 5H), 4.39 (d, 2H), 3.28–3.08 (m, 16H), 2.14 (m, 6H), 1.34 (t, $\mathcal{J}$=7.2 Hz, 3H), 1.27 (t, $\mathcal{J}$=7.2 Hz, 3H); ^{13}C NMR (100 MHz, D_2O, spectral line broadening 0.1 Hz) 131.23, 130.56, 129.76, 129.11, 57.10, 48.64, 48.21, 44.97, 44.91, 44.88, 44.86, 44.14, 43.41, 22.99, 22.94, 20.90, 10.83, 8.47; ESI MS (m/z) Calcd for C20H38N4 $[M+H]^+$ 335.5, found: 335.5, $[M+2H]^{2+}$: 168.4.

Synthesis of 17

The mixture of **16** (0.28 g, 0.43 mmol), Boc_2O (0.37 g, 1.72 mmol) and Et_3N (0.173 g, 1.72 mmol) in dry methanol (10 mL) was stirred for 24 h under N_2. Water was added, and the mixture was evaporated under vacuum. The remaining phase was partitioned between CH_2Cl_2 (20 mL) and water. The organic layer was separated, and aqueous layer was extracted with CH_2Cl_2 (10 mL×3). Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give the crude product. Purification by silica gel column gave the product **17** as colorless oil (0.27 g, 100%). Rf 0.35 (ethyl acetate). FTIR (neat) 2973, 2931, 2802, 1686, 1414, 1364, 1248, 1153, 731 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.32–7.22 (m, 5H), 3.54 (s, 2H), 3.16 (broad m, 12H), 2.50 (q, $\mathcal{J}$=7.1 Hz, 2H), 2.42 (t, $\mathcal{J}$=7.0 Hz, 2H), 1.70 (m, 6H), 1.45 (ds, 18H), 1.43 (s, 9H), 1.09 (t, $\mathcal{J}$=7.2 Hz, 3H), 1.02 (t, $\mathcal{J}$=6.8 Hz, 3H). Coalescence peaks of carbon spectra were found at room temperature (22°C), and thus higher temperature (45°C) NMR was run on 500 MHz spectrometer. ^{13}C NMR (125 MHz, $CDCl_3$) 155.43, 155.38, 155.33, 140.01, 128.71, 128.08, 126.68, 79.29, 79.12, 79.08, 58.21, 50.84, 47.38, 45.66, 45.01, 44.52, 41.81, 28.47, 27.67, 26.31, 13.59, 11.68; ESI MS (*m/z*) Calcd for C35H62N4O6 $[M+H]^+$: 635.5, found: 635.6; $[M+K]^+$ 673.5, Found: 673.6.

Synthesis of 18 and 19

After removing the air in the reaction container with hydrogen, the mixture of **17** (0.27 g, 0.43 mmol) and 10% Pd/C (54 mg, 20% *w/w*) in methanol (10 mL) was shaken at room temperature (22°C) under 50 psi hydrogen pressure for 12 h. The reaction mixture was filtered, and the filter cake was washed with methanol. The filtrate was concentrated to give crude product, which was purified by a short silica gel column to yield the product **18** (0.21 g, 90%) as colorless oil at Rf 0.13 and **19** (0.02 g, 10%) at Rf 0.15 (10% triethylamine in methanol). Data for **18:** FTIR (neat) 3495, 2973, 2931, 1684, 1477, 1415, 1364, 1249, 1154, 771 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 45°C) 3.26–3.15 (m, 12H), 2.67–2.57 (m, 4H), 1.77–1.67 (m, 6H), 1.45 (s, 27H), 1.01 (t, $\mathcal{J}$=7.1 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$, 45°C) 155.5, 155.4, 155.3, 79.3, 79.2, 79.1, 46.9, 45, 44.8, 44.5, 44.1, 41.8, 29.0, 28.5, 27.7, 15.2, 13.6; ESI MS (*m/z*) Calcd for C28H56N4O6 $[M+H]^+$ 545.4, found: 545.4; $[M+K]^+$ 583.4. Found 583.4. Data for **19**: FTIR (neat) 2974, 2931, 2791, 1685, 1477, 1415, 1365, 1249, 1156, 731 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, 45°C) 3.23–3.15 (m, 12H), 2.39 (q, $\mathcal{J}$=7.1 Hz, 2H), 2.32 (t, $\mathcal{J}$=7.2 Hz, 2H), 2.19 (s, 3H), 1.77–1.66 (m, 6H), 1.45 (s, 27H), 1.09 (t, $\mathcal{J}$=7.1 Hz, 3H), 1.03 (t, $\mathcal{J}$=7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 45°C) 155.5, 155.4, 155.3, 79.3.79.2, 79.1, 54.7, 51.4, 45.6, 45.0, 44.5, 41.8, 41.5, 28.5, 27.7, 26.4, 13.6, 12.1; ESI MS (m/z) Calcd for C29H58N4O6 $[M+H]^+$ 559.4, found: 559.4; $[M+K]^+$ 597.4. Found 597.3.

Synthesis of 21

A suspension solution of **20** (22) (622 mg, 1 mmol) and HOBt (203 mg, 1.5 mmol) in anhydrous $CHCl_3$ (50 mL) was added to the solution of EDCI (233 mg, 1.5 mmol) in

anhydrous $CHCl_3$ (20 mL) at 0°C, followed the mixture of Et_3N (152 mg, 0.21 mL, 1.5 mmol) and **18** (545 mg, 1 mmol) in $CHCl_3$ (10 mL). The resulting mixture was stirred at room temperature for 20 h and turned to clear solution. The reaction mixture was then partitioned with water (15 mL) at 0°C. The organic layer was separated, and water layer was extracted with $CHCl_3$ (10 mL×3). Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and evaporated under vacuum to give the crude product. The residue was dry-loaded to silica gel column, and separation (Hexane/ethyl acetate 1:1) gave the product **21** (1.0 g, 87%) as colorless oil. Rf 0.3 (Hexane/ethyl acetate 1:1). FTIR (neat) 2922, 2852,1737, 1691, 1643, 1466.1452, 1414, 1364, 1159, 772 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.38–3.16 (multiple broad peak, 20H), 2.66–2.63 (bd, 4H), 1.85 (m, 2H), 1.72 (m, 4H), 1.55 (m, 2H), 1.48 (m, 2H), 1.43 (ds, 27H), 1.24 (bs, 66H), 1.08 (bt, 6H), 0.86 (t, $\mathcal{J}$=6.8 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$, 40°C) 171.7, 171.2, 155.3, 79.5, 79.2, 79.0, 47.9, 46.2, 45.6, 44.9 (multiple), 44.5, 43.8, 43.2, 41.8, 31.8, 29.6, 29.4, 29.3 (multiple), 29.2, 28.9, 28.5, 28.2, 27.7, 27.0, 22.6, 14.0, 13.6; ESI MS (*m/z*) Calcd for C68H133N5O8 $[M+H]^+$ 1149.01, Found 1148.88. $[M+K]^+$ 1186.97. Found 1186.82.

Synthesis of 1

To the solution of **21** (0.322 g, 0.28 mmol) in anhydrous CH_2Cl_2 (4 mL), TFA (2 mL) was added at 0°C. The mixture was stirred for 2 h at room temperature. Most of the solvents were evaporated under vacuum at 25°C. The remaining solution (about 1 mL) was cooled to 0°C, and 2 mL of methanol was added to form white precipitate. The white solid was obtained by centrifugation and washed with small amount of methanol. The obtained white solid was dried under vacuum to give the desired product as trifluoroacetate salt. The white solid of LipoBENSpm trifluoroacetate (0.16 g, 0.14 mmol) was suspended in 5 mL of CH_2Cl_2, and NaOH powder (0.1 g) was added at 0°C. The mixture was stirred for 2 h at room temperature, and then anhydrous Na_2SO_4 was added. The filtration was evaporated under vacuum, and methanol (2 mL), 2 N HCl (0.21 mL, 0.42 mmol) was added at 0°C. The mixture was stirred for 2 h at 0°C, and then freezing-dry and washing with anhydrous ethyl ether gave the product LipoBENSpm **1** trihydrochloride salt as white solid. (0.13 g, 100%) Mp 180°C (dec) FTIR (neat) 2954, 2917, 2849, 2753, 1742, 1641, 1464, 1428, 1365, 1215 cm^{-1}; ^1H NMR (400 MHz, CD3OD) 3.50 (m, 4H), 3.20–3.07 (m, 12H), 3.0 (t, $\mathcal{J}$= 7.1 Hz, 2H), 2.71–2.70 (bd, 4H), 2.15 (m, 4H), 1.97 (m, 2H), 1.64 (m, 2H), 1.52 (m, 2H), 1.34 (t, $\mathcal{J}$=7.2 Hz, 3H),1.29 (bs, 66H), 0.9 (t, $\mathcal{J}$=7.4 Hz, 3H); ^{13}C NMR (100 MHz, CD3OD) 174.7, 172.6, 46.3, 45 (m), 44.5, 44.1, 43.1, 42.7, 41.8, 31.9, 29.5 (m), 29.2 (m), 28.7, 28.1, 27.6 (d), 26.8, 26.7, 24.6, 23.1, 22.5, 13.2, 12.9, 10.4; ESI MS (*m/z*) Calcd for C53H109N5O2$[M+H]^+$ 848.85, found: 848.65, $[M+2H]^{2+}$ 425.06.

Cytotoxicity Evaluation

Cytotoxicity of LipoBENSpm, control BENSpm and DOTAP in human breast adenocarcinoma cell line MCF-7 and in normal human breast epithelial cell line MCF-10A was determined by MTS assay using a commercially available kit (CellTiter 96® Aqueous Cell Proliferation Assay, Promega). Four-thousand cells were seeded in a 96-well plate, and after 24 the culturing medium was removed and replaced with 200 μL of RPMI/FBS with increasing concentration of a drug. Medium was replaced every 48 h with fresh medium containing the same concentration of the respective drug. On day 6, the cell viabilities were determined by MTS assay. The incubation medium was removed, and a mixture of 100 μL of fresh medium and 20 μL of MTS reagent solution was added to each well. The cells were incubated for 1 h at 37°C in CO_2 incubator. The absorbance of each sample was then measured at wavelength 505 nm to determine cell viability. The results are expressed as mean percentage cell viability relative to untreated cells ± SEM (n=4–8). IC50 values were obtained by Boltzmann Sigmoidal nonlinear regression.

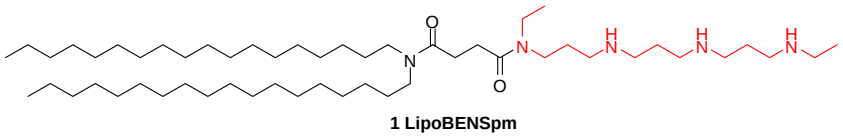
Ethidium Bromide Exclusion Assay

The ability of LipoBENSpm and BENSpm to condense plasmid DNA was confirmed by a standard ethidium bromide (EtBr) exclusion assay by measuring changes in EtBr/DNA fluorescence. Lipids were prepared in 10 mM HEPES buffer (pH 7.4) at a concentration of 5 mg/mL. Two mL of 20 μg/mL pDNA (gWiz-SEAP) solution was mixed with EtBr (1 μg/mL). The fluorescence of EtBr/DNA was measured using 540 nm excitation and 590 nm emission and set to 100%. Relative fluorescence was recorded following a stepwise addition of the lipids, and the condensation curve for each lipid was constructed.

Agarose Gel Retardation Assay

DNA condensation ability of LipoBENSpm and BENSpm was examined also by agarose gel electrophoresis. DNA complexes were formed at different N/P ratios and incubated 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 0.5X TBE running buffer. The gel was visualized under UV illumination on a Kodak Gel Logic 100 Imaging System.

Fig. 2 Structure of LipoBENSpm **1**.



Transfection activity

All transfection experiments were conducted in 48-well plates. Cells were seeded at a density of 40,000 cells/well 24 h before. On the day of transfection, the cells were incubated with the polyplexes prepared at different N/P ratios in 150 μL of medium with or without 10% FBS. The dose of luciferase pDNA was 0.4 μg per well. After 3 h of incubation, polyplexes were completely removed, and the cells were cultured in complete culture medium for 24 h prior to measuring luciferase expression. The medium was then discarded, and the cells were lysed in 100 μL of 0.5X cell lysis reagent buffer (Promega). To measure the luciferase content, 100 μL of 0.5 mM luciferin solution was automatically injected into each well of 20 μL of cell lysate, and the luminescence was integrated over 10 s using BioTek Synergy 2 Microplate Reader. Total cellular protein in the cell lysate was determined by the BCA (Bicinchoninic acid) protein assay using calibration curve constructed with standard bovine serum albumin solutions (Pierce). The transfection efficiency results are expressed as Relative Light Units (RLU) per milligram of cellular protein ± SD (n=4).

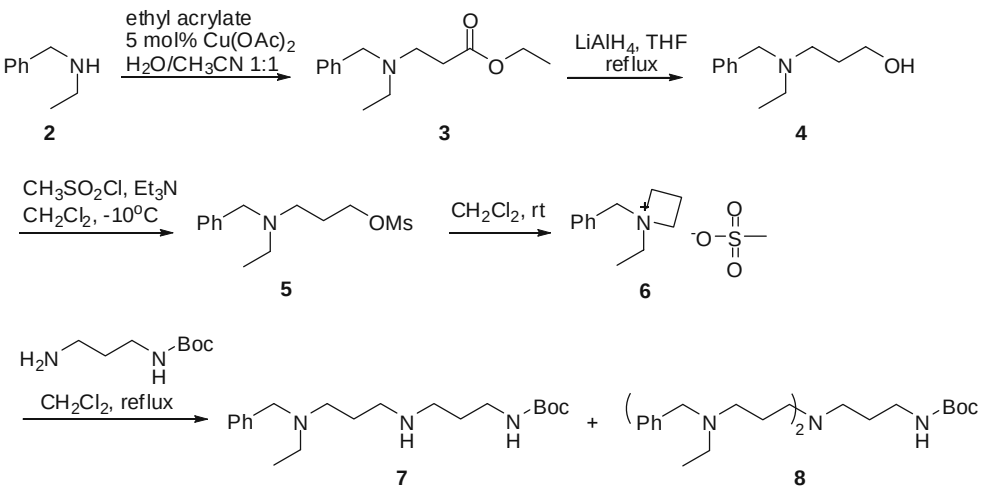
RESULTS AND DISCUSSION

Synthesis of LipoBENSpm

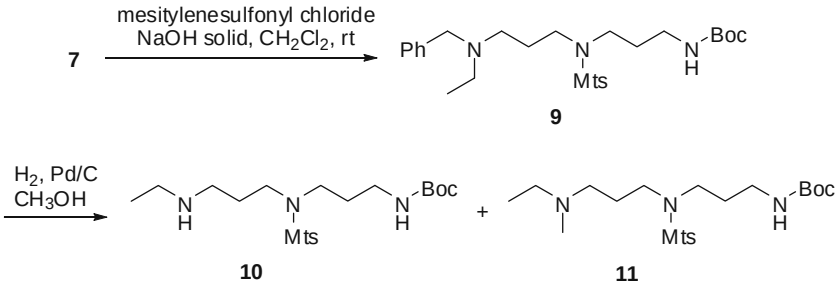
Asymmetrical lipopolyamine analogue of BENSpm, LipoBENSpm **1**, has been synthesized and characterized (Fig. 2). It consists of dioctadecylamine functionality attached to a terminal amino group of BENSpm by amide bond. The remaining three secondary amino groups are free for the purpose of electrostatically binding with DNA. The synthesis of LipoBENSpm **1** is dependent on the availability of tri-protected BENSpm. Symmetrically and asymmetrically substituted polyamine analogues have been prepared by routes involving protection of internal amino groups and asymmetrical introduction of terminal protecting groups (13–18,23–28). The application of these approaches to the synthesis of BENSpm analogues is challenged by difficulties of selectively introducing protecting groups to the terminal secondary amine in the presence of three other secondary amines with similar reactivity. Here, we proposed an effective synthesis of BENSpm with three protected amines by nucleophilic ring opening of azetidinium ion with amino group and employing appropriate amino protection and deprotection schemes.

The synthesis commenced with the Michael addition of ethylbenzylamine **2** with ethyl acrylate using copper acetate as catalyst in H_2O/acetonitrile. The reaction provided the intermediate amino ester **3** in good yield (29) (Scheme 1). Reduction of **3** with lithium aluminium hydride in tetrahydrofuran under reflux produced amino alcohol **4** in high yield. Treatment of **4** with methanesulfonyl chloride in dichloromethane in the presence of triethylamine at −10°C for 1 h gave the methanesulfonate ester **5** as colorless oil in satisfying yield on TLC analysis. However, decomposition of **5** was observed when the reaction mixture was kept at room temperature, and a

Scheme 1 Synthesis of intermediate **7**.



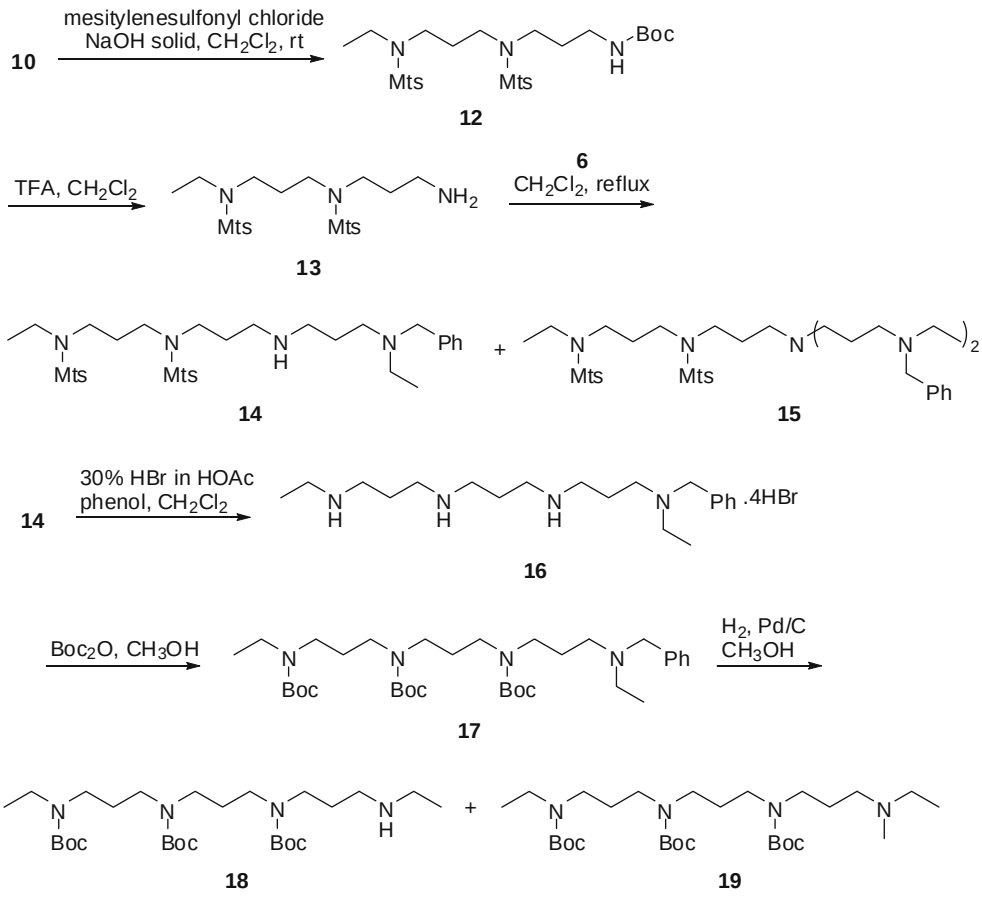
Scheme 2 Synthesis of intermediate **10**.



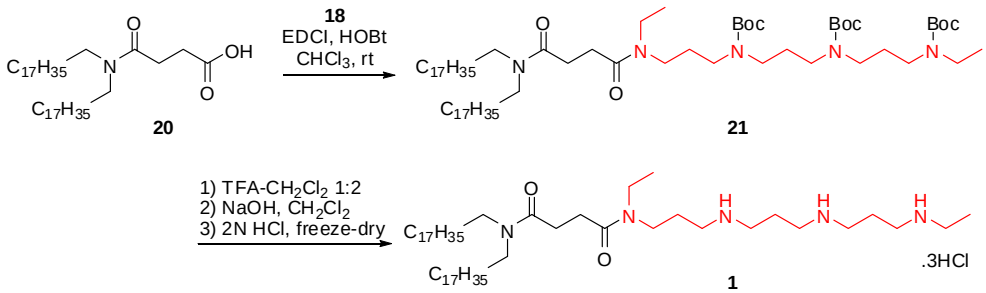
complete decomposition was observed within 24 h of storage. Literature reports suggest that compounds containing 3-aminopropyl methanesulfonate are unstable on prolonged storage as free bases (30). Azetidinium methanesulfonate is facile to form by intramolecular cyclization reaction when treating hydroxypropyl amine with methanesulfonyl chloride (31). Moreover, azetidinium four-membered ring was also found in the salts of triflate (32,33) and iodide (34). NMR spectroscopy of the conversion compound shows the formation of four-membered ring of azetidinium methanesulfonate **6**, which was also confirmed by ESIMS with an M+ signal at m/z 176.4 of cationic azetidinium moiety and M− at 94.9 of methanesulfonate anion.

Azetidinium ion ring opening with nucleophiles such as halides, alkoxides, phenoxides, amines and cyanide is reported to be facile (35–37). Nucleophilic reaction of azetidinium ring of **6** with mono-Boc-1,3-propanediamine (38,39) in dichloromethane under reflux resulted in intermediate **7** in 90% yield, with about 5% of **8** separated chromatographically. Considering the high polarity of polyamines, the next step was to introduce a mesitylenesulfonyl (Mts) protection group to the secondary amine **7** for improved purification purposes (Scheme 2). The reaction of **7** with mesitylenesulfonyl chloride (MtsCl) afforded **9** in quantitative yield. Catalytic debenzylation of **9** with 10% Pd/C (0.5:1, w/w) and hydrogen (50 psi) in methanol provided 100% conversion with the isolation of 90% yield of

Scheme 3 Synthesis of tri-Boc BENSpm **18**.



Scheme 4 Synthesis of LipoBENSpm **1**.



10 and 10% yield of methylation product **11**. We found that more catalyst (10% Pd/C, 1:1 *w*/*w*) and longer reaction time (4 days, 50 psi H_2) increased the amount of **11** to 50%. It was reported that *N*-methylation was observed in hydrogenolysis of benzyl group in an alkaloid synthesis (40–42). The proposed mechanism is that the catalyst of palladium (40) or other precious metals such as ruthenium (43) and iridium (44) can oxidize the adsorbed alcohol to the corresponding aldehyde, which can be trapped by free amine and then be reduced by hydrogen to *N*-alkylated products. The hydrogenolysis was also run in ethanol and trifluoroethanol solvents. At the same reaction conditions to that in methanol, these two reactions were slower. Ten percent yield of ethylation product was found in ethanol, and a mixture including de-Boc products was found in trifluoroethanol. No further efforts were extended to improve this reaction. Methanol was used as hydrogenolysis solvent in this work.

Next, another Mts group was introduced by treatment of **10** with MtsCl in dichloromethane to give the intermediate **12** quantitatively (Scheme 3). Subsequent removal of the Boc group of **12** with trifluoroacetic acid gave the corresponding disulfonamide **13** with free terminal amine. Another nucleophilic ring opening reaction of azetidinium ion of **6** with **13** in dichloromethane under reflux afforded intermediate **14** in 88% yield and 5% yield of **15**.

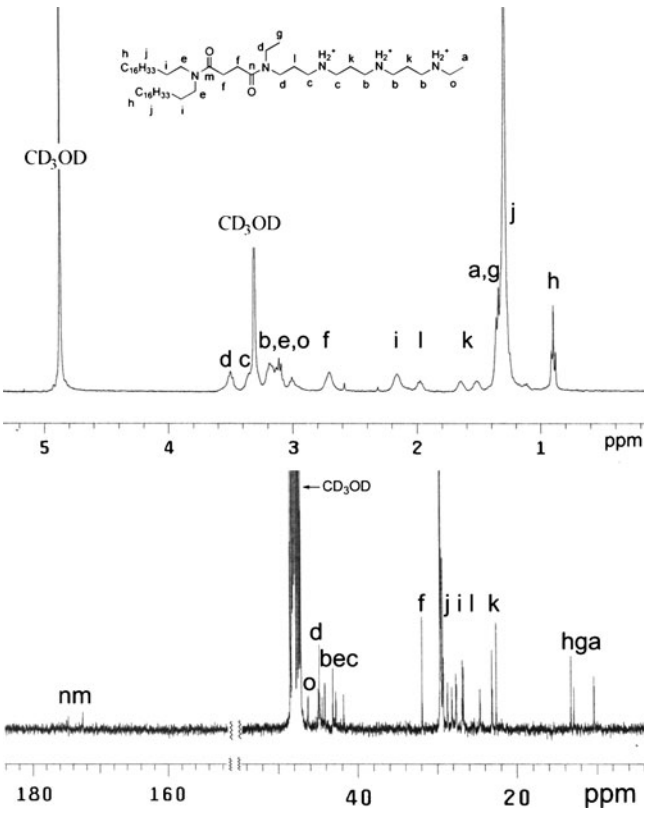


Fig. 3 ^{1}H-NMR and ^{13}C-NMR of LipoBENSpm.3HCl in CD_3OD.

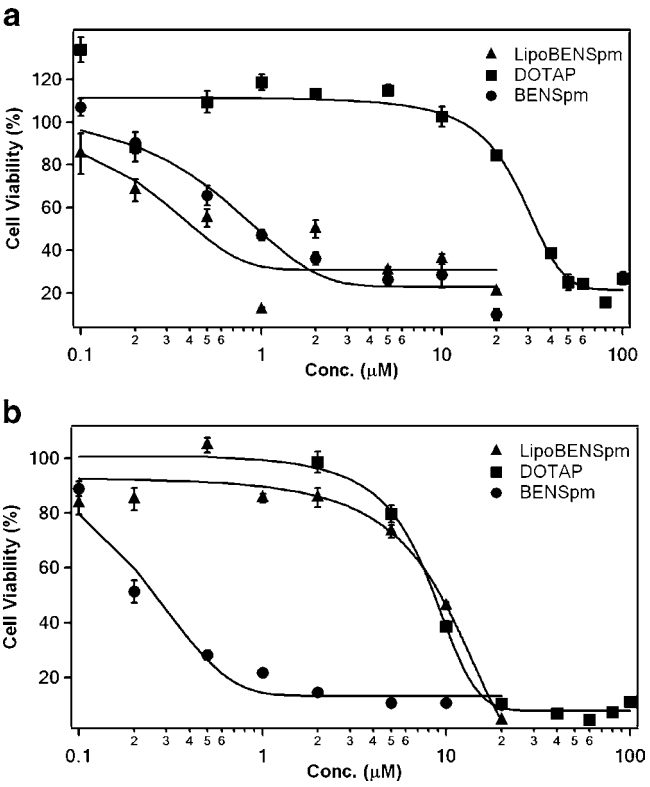


Fig. 4 Cytotoxicity of LipoBENSpm in (**a**) human breast cancer MCF-7 cells and (**b**) control normal MCF-10A breast cells. Metabolic activity was measured by MTS assay following 144 h incubation of the cells with increasing concentration of LipoBENSpm, BENSpm, and DOTAP (mean ± S.D., $n = 3$).

Deprotection of Mts groups of **14** with 30% HBr in acetic acid and phenol in dichloromethane gave **16** as HBr salt in 94% yield. Subsequent protection of the three amino groups using di-*tert*-butyl dicarbonate produced tri-Boc-protected structure **17**. ^{13}C-NMR of **17** shows broad coalesced resonance peaks when measured at 22°C. Performing the NMR analysis at elevated temperature (45°C) improved both the carbon and proton spectra resolution. Catalytic debenzylation of **17** under H_2 (10% Pd/C, 0.2:1, *w*/*w*) yielded target structure tri-Boc BENSpm **18** in 92% yield, with 8% of undesired methylation structure **19**. Similar to structure **17**, coalescence of NMR spectra of **18** and **19** were found at room temperature, and well-resolved peaks were obtained at 45°C.

N-succinyl-dioctadecylamine **20** was obtained by the reaction of dioctadecylamine with succinic anhydride (22). The combination of **20** and tri-Boc BENSpm **18** under typical peptide-forming conditions (EDCI, HOBt) gave the tri-Boc BENSpm lipid conjugate **21**, which underwent deprotection of Boc group (TFA, CH_2Cl_2) to yield the desired structure **1** (Scheme 4). Considering its stability, the final LipoBENSpm **1** was converted to hydrochloride salt.

The structure of LipoBENSpm **1** was confirmed by NMR in deuterated methanol (CD_3OD) (Fig. 3). The ^{1}HNMR peaks marked with letters *a*–*k* are assigned to the corresponding hydrogen atoms of the lipid tail and cationic head group. Due to the formation of the hydrochlorides, the CH3 peak *a* of terminal amino group is shown as expected down field at about 1.34 ppm, which overlapped with the strong signal *j* of CH2 group of the lipid tail. At the same time, another CH3 peak *g* of BENSpm mixed with the CH2 peaks at about 1.3 ppm. The ^{13}CNMR spectra of LipoBENSpm shown in Fig. 4 indicate two amide carbon atoms *n* and *m* (174.7 and 172.6 ppm). Three CH3 groups in the structure are shown as *a*, *g*, *h* in the carbon spectra (13.2, 12.8 and 10.4 ppm).

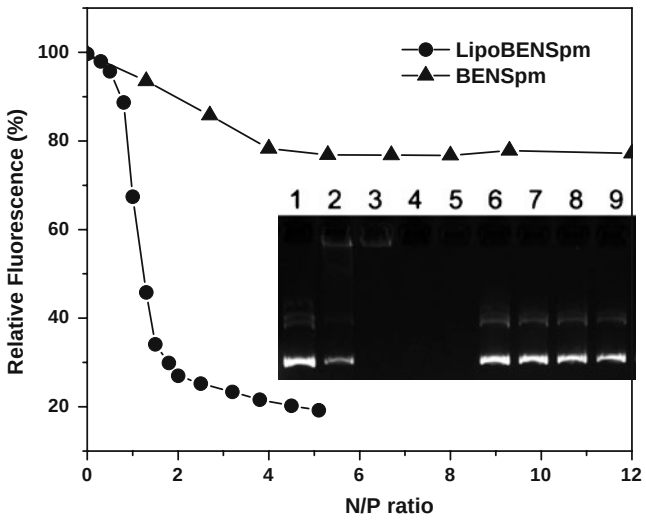


Fig. 5 DNA condensation by LipoBENSpm. (inset: gel retardation assay; lane 1: free DNA; lanes 2–5: LipoBENSpm/DNA complexes prepared at N/P 0.8, 1.6, 3.2, 6.4, respectively; lanes 6–9: BENSpm/DNA complexes prepared at N/P 4, 8, 16, 32, respectively).

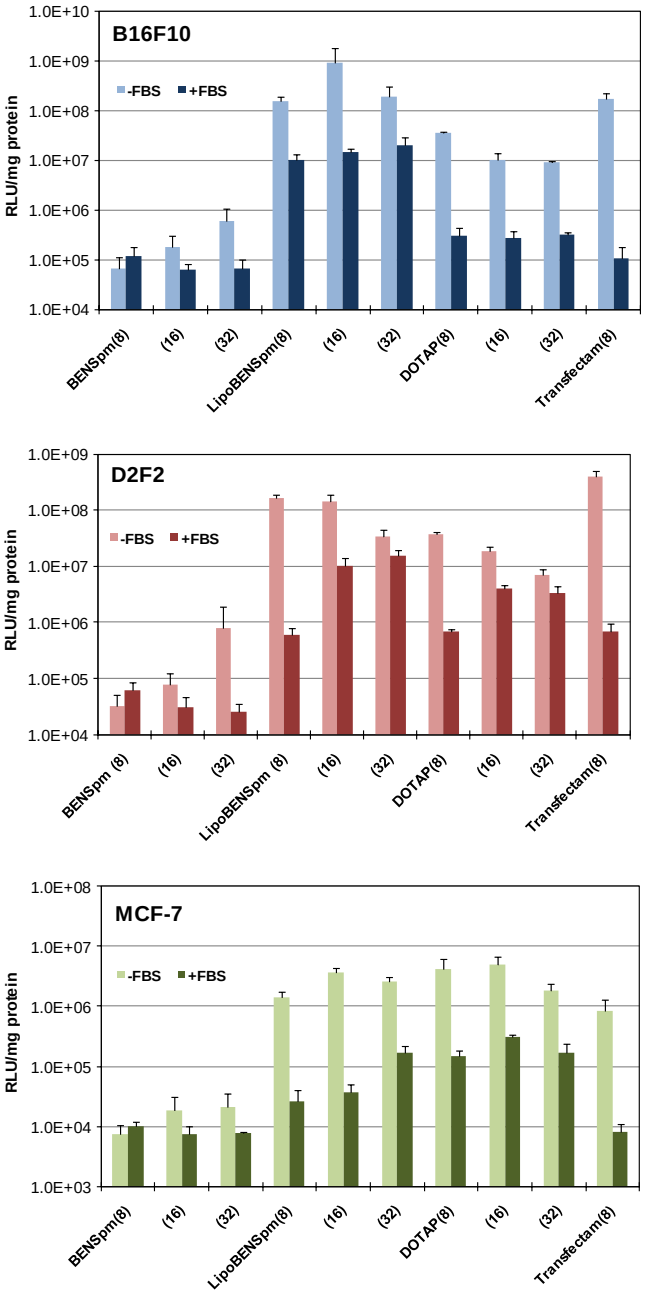


Fig. 6 Transfection activity of LipoBENSpm in a panel of cancer cell lines in the presence (+FBS) or absence (−FBS) 10% serum. Luciferase activity was measured 24 h after 3 h incubation of cells with DNA complexes (2.6 μg DNA/mL). Results are shown as mean RLU/mg of cellular protein ± S.D. (n=3).

Gene Delivery Activity of LipoBENSpm

The main goal of this study was to develop a method of synthesis of asymmetrical BENSpm lipopolyamines and to confirm their ability to effectively deliver genes. First, we investigated cytotoxicity of LipoBENSpm in human breast cancer cell line MCF-7 and in normal human breast epithelial cell line MCF-10A (Fig. 4). LipoBENSpm exhibited slightly enhanced cytotoxicity compared to BENSpm in MCF-7 cells (IC50 0.36±0.04 μM *vs.* 1.03± 0.10 μM) (Fig. 4a) and a significantly reduced cytotoxicity in the normal MCF-10A control cells (8.73±0.60 μM *vs.* 0.19±0.01 μM) (Fig. 4b). In comparison, control lipid DOTAP showed significantly lower cytotoxicity than LipoBENSpm and BENSpm in both cell lines tested (IC50 33.84±1.84 μM in MCF-7 and 8.33±0.24 μM in MCF-10A). The data in Fig. 4 do not allow reaching conclusion if the observed effect of LipoBENSpm on cell viability and the differences between LipoBENSpm and BENSpm in the two cell lines are the result of a specific selective effect on polyamine metabolism or demonstration of general toxicity of cationic lipids. Available structure-activity studies suggest that BENSpm may need to be released from the lipopolyamine in order to exert its intracellular effect. Detail evaluation of the effect of BENSpm release on polyamine catabolism is beyond the scope of this work and will be the subject of our upcoming study.

The ability of LipoBENSpm to condense DNA was investigated by ethidium bromide exclusion and agarose gel retardation assays (Fig. 5). As expected, LipoBENSpm efficiently condensed DNA above N/P ratio 2, while the parent BESNpm could not retard DNA migration in the gel or effectively exclude ethidium bromide even at N/P 16.

Transfection activity of the luciferase DNA complexes of LipoBENSpm was measured in B16F10 mouse melanoma, D2F2 mouse breast cancer, and MCF-7 human breast cancer cell lines (Fig. 6). Transfection experiments were conducted at three N/P ratios, and the results with LipoBENSpm and BENSpm complexes were compared with transfection activities of control DOTAP and Transfectam lipoplexes. Even though total concentrations of BENSpm and LipoBENSpm used in the transfection experiments were above their IC50 values (Fig. 4), a combination of the short incubation time (3 h *vs.* 144 h in the cell viability experiment) and measuring the transfection 24 h after the incubation of complexes with cells ensured that no significant toxicity was observed in the timeframe of the transfection experiments. LipoBENSpm complexes showed consistently high transfection activity in all three cell lines both in the presence and absence of serum. As expected based on the limited DNA condensation ability, BENSpm complexes mediated low transfection activity, typically 3–4 orders of magnitude lower than transfection of LipoBENSpm. Transfection activity of LipoBENSpm complexes was either higher or comparable to transfection activity of complexes of both control lipids, DOTAP and the structurally similar Transfectam.

CONCLUSIONS

In conclusion, we developed a method of synthesis of asymmetrical analogues of polyamine anticancer drug BENSpm. The synthesized LipoBENSpm shows cytotoxicity comparable to that of parent BENSpm but mediates 3–4 orders of magnitude higher transfection activity. Importantly, cytostatic activity of BENSpm falls within a relevant and typical concentration range required for efficient gene delivery. These findings make gene delivery vectors based on BENSpm promising candidates for combination drug/gene approaches to the treatment of cancer.

ACKNOWLEDGEMENTS

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