Published in final edited form as:

J Control Release. 2017 January 28; 246: 110–119. doi:10.1016/j.jconrel.2016.12.017.

Self-immolative nanoparticles for simultaneous delivery of microRNA and targeting of polyamine metabolism in combination cancer therapy

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Abstract

Combination of anticancer drugs with therapeutic miRNA has emerged as a promising anticancer strategy. However, the promise is hampered by a lack of desirable delivery systems. We report on the development of self-immolative nanoparticles capable of simultaneously delivering miR-34a mimic and targeting dysregulated polyamine metabolism in cancer. The nanoparticles were prepared from a biodegradable polycationic prodrug, named DSS-BEN, which was synthesized from a polyamine analog N^1, N^{11} -bisethylnorspermine (BENSpm). The nanoparticles were selectively disassembled in the cytoplasm where they released miRNA. Glutathione (GSH)-induced degradation of self-immolative linkers released BENSpm from the DSS-BEN polymers. MiR-34a mimic was effectively delivered to cancer cells as evidenced by upregulation of intracellular miR-34a and downregulation of Bcl-2 as one of the downstream targets of miR-34a. Intracellular BENSpm generated from the degraded nanoparticles induced the expression of rate-limiting enzymes in polyamine catabolism (SMOX, SSAT) and depleted cellular natural polyamines. Simultaneous regulation of polyamine metabolism and miR-34a expression by DSS-BEN/miR-34a not only enhanced cancer cell killing in cultured human colon cancer cells, but also improved antitumor activity in vivo. The reported findings validate the self-immolative nanoparticles as delivery vectors of therapeutic miRNA capable of simultaneously targeting dysregulated polyamine metabolism in cancer, thereby providing an elegant and efficient approach to combination nanomedicines.

Graphical abstract

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Keywords

Self-immolative polymer; polyplexes; nanoparticles; polyamine metabolism; miRNA; combination delivery; bisethylnorspermine; cancer

1. Introduction

Cancer is a major public health problem and a leading cause of mortality worldwide [1]. Recently, combination of small-molecule anticancer drugs with therapeutic nucleic acids, such as siRNA and miRNA, emerged as a promising strategy in cancer treatment [2–7]. Due to the heterogeneity of cancer and the involvement of multiple gene mutations during tumorigenesis and tumor progression, such combination therapies provide potentially great advantages in targeting multiple cancer-associated pathways and overcoming adaptive drug resistance. However, this promising combination approach is limited by a lack of desirable vectors for simultaneous delivery of small-molecule drugs and nucleic acids. It is a significant challenge to develop delivery systems for drug/nucleic acid combinations, due to the physicochemical differences between the two agents. Among the available delivery systems, biodegradable polymeric nanoparticles have been the most successful delivery platforms used in drug/nucleic acid combinations [8–10]. Polymeric nanoparticles are typically composed of pharmacologically inert polymer suitable for encapsulation of both types of therapeutic cargos [11]. However, the manufacturing complexity and unsatisfactory drug loading ability of the traditional nanoparticles remain a significant hurdle for their clinical translation. Alternative approaches from our lab have utilized pharmacologically active nanoparticles based on polymeric drugs and prodrugs to achieve delivery of drug/siRNA, drug/miRNA, and drug/DNA combinations [12–14]. When properly designed, such polymeric drug nanoparticles have several advantages, including simple production and high drug contents that makes them suitable for clinical translation. Moreover, mounting studies highlight self-immolative materials as powerful approach for drug or nucleic acid delivery due to smart and amplified release of cargos upon encountering a suitable trigger [15–18]. Therefore, polymeric nanoparticles composed of self-immolative polymeric prodrugs are expected to offer a promising platform for drug/nucleic acids combination.

Natural polyamines such as spermine (SPM), spermidine (SPD), and putrescine (PUT) are indispensable for cell growth, differentiation, and survival [19, 20]. The majority of polyamines is found in complexes with cellular anions including RNA, DNA, phospholipids,

and negatively charged proteins [21, 22]. Intracellular polyamine levels are tightly regulated by the polyamine transport system and metabolic enzymes [23]. The rate-limiting enzymes of polyamine biosynthesis include ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC). The catabolism of natural polyamines is regulated by spermine/spermidine *N*¹-acetyltransferase (SSAT), polyamine oxidase (PAO), and spermine oxidase (SMOX). Dysregulation of polyamine metabolism is associated with various diseases [24]. In cancer, dysregulation of the enzymes involved in the polyamine pathway causes accumulation of polyamines, which promotes tumor development and progression. Elevation of polyamine levels increases the malignancy of cancer cells and decreases antitumor immunity [25]. Thus, polyamine metabolism represents a promising target in cancer chemotherapy [26]. Available evidence supports that synthetic polyamine analogues are capable of competing with natural polyamines for transport, biosynthesis, and metabolism [27]. BENSpM is one of the most successful of the developed polyamine analogues. It has shown encouraging anticancer activity against multiple cancers [28–30]. BENSpM is able to induce SSAT, downregulate ODC and Ado-MetD [31] and its action ultimately results in cell growth inhibition and apoptosis [32]. Although BENSpM did not achieve satisfactory clinical effect as a single agent [33, 34], recent studies demonstrated its promise in combination cancer therapy with other chemotherapy drugs [35, 36].

MicroRNAs (miRNAs) are small (~22 nucleotide) noncoding RNAs that post-transcriptionally regulate gene expression [37]. MiRNAs regulate a wide range of cellular pathways and mediate the expression of nearly 30% of all human proteins [38]. Dysregulation of miRNA often results in pathological states such as cancer [39]. MiRNAs play an important role in tumorigenesis, tumor growth, angiogenesis, and metastasis [40]. Through targeting different genes simultaneously, a single miRNA enables regulation of multiple biological pathways that are critical for a cancer cell phenotype. Hence, miRNA therapy offers a highly promising approach to the treatment of cancer. As a tumor suppressor, miRNA-34a is greatly downregulated in a variety of cancers. MiR-34a functions through a complex interplay with the p53 network [41, 42]. Exogenous expression of miR-34a in cancer cells is able to induce cell apoptosis and inhibit cell migration and tumor growth by regulating multiple pathways, including those involving Bcl-2, SIRT1, E2F3, and Fra-1 [43–46]. Hence, delivery of miR-34a provides a promising approach to the treatment of cancer. As with all potential nucleic acid therapeutics, however, the clinical translation of miRNA is hindered by a lack of efficient delivery systems [47, 48].

In this study, we report the development of BENSpM-based biodegradable polycation (DSS-BEN) to simultaneously deliver miR-34a mimic and target polyamine metabolism (Scheme 1). Our previous study suggested that DSS-BEN was indeed able to function in such a dual manner [49]. Here, we hypothesized that simultaneous delivery of miR-34a and BENSpM by DSS-BEN/miR-34a nanoparticles will lead to improved combination antitumor activity (Scheme 1B) through a cooperative effect on polyamine metabolism and miR-34a-related oncogenic pathways.

2. Materials and methods

2.1. Materials

BENSpm was synthesized by following a previously described procedure [28]. The self-immolative polycation DSS-BEN (Mw = 3.8 kDa, Mw/Mn = 1.1) was synthesized and characterized as previously described [49]. BENSpm accounted for ~44 wt % in the DSS-BEN. McCoy's 5A medium, Dulbecco's phosphate buffered saline (PBS), and fetal bovine serum (FBS) were from Thermo Scientific (Waltham, MA). MiRNA-34a-5p mimic (mature miRNA sequence: 5'-UGGCAGUGUCUUAGCUGGUUGU-3'), negative control miR-NC mimic (mature miRNA sequence: 5'-UCACAACCUCCUAGAAAGAGUAGA-3') and fluorescently labeled FAM-miRNA were purchased from Dharmacon (Lafayette, CO). RT-PCR primers were purchased from Invitrogen (Carlsbad, CA). All other reagents were from Fisher Scientific and used as received unless otherwise noted.

2.2. Cell culture

The HCT116 human colorectal carcinoma cell line was kindly provided by Dr. Michael Brattain (University of Nebraska Medical Center). The cells were grown in McCoy's 5A medium supplemented with 10% FBS at 37°C with 5% CO₂ in a humidified chamber.

2.3. Preparation and physicochemical characterization of DSS-BEN/miRNA nanoparticles

The capability of DSS-BEN to condense miRNA was determined by electrophoresis in a 2% agarose gel containing 0.5 µg/mL ethidium bromide (EtBr). DSS-BEN/miRNA nanoparticles were prepared by adding predetermined volume of DSS-BEN to a miRNA solution (20 µM in 10 mM HEPES pH 7.4) to achieve the desired w/w ratio and vigorously vortexed for 10 s. Nanoparticles were then incubated at room temperature for 30 min before further use. Nanoparticles formed at various polycation-to-miRNA weight ratios were loaded (20 µL of the sample containing 0.5 µg of microRNA) and run for 15 min at 100 V in 0.5×Tris/Borate/EDTA buffer. The gels were visualized under UV illumination with a KODAK Gel Logic 100 imaging system. Hydrodynamic diameter and zeta potential of the nanoparticles were determined by dynamic light scattering (DLS) using a ZEN3600 Zetasizer Nano-ZS (Malvern Instruments Ltd., Massachusetts, USA). Morphological observation was performed under transmission electron microscopy (TEM, Tecnai G2 Spirit, FEI Company, USA) using NanoVan® negative staining (Nanoprobes, USA). MiRNA release from polyplexes was analyzed by heparin displacement assay. The nanoparticles were prepared at a w/w ratio of 10 and incubated with increasing concentrations of heparin in the presence or absence of 20 mM glutathione (GSH) for 30 min at room temperature. The samples (20 µL of the sample containing 0.5 µg of miRNA) were then analyzed by agarose gel electrophoresis as described above.

2.4. Cellular uptake and intracellular trafficking of nanoparticles

Flow cytometry analysis was conducted to study the cellular uptake of the nanoparticles. HCT116 cells (5×10^4) were seeded in 24-well plates and cultured to approximately 50% confluence. The cells were incubated at 37 °C with DSS-BEN/FAM-miRNA nanoparticles at a FAM-miRNA concentration of 200 nM for 4 h. The cells were then trypsinized, washed

with cold PBS and subjected to analysis using a BD FACSCalibur flow cytometer (BD Bioscience, Bedford, MA). The results were processed using FlowJo software. Intracellular localization was observed by confocal laser scanning microscopy. Cells were cultured on a 20-mm glass-bottom cell culture dish (Nest) using 1×10^5 cells/dish. After 24 h, the medium was replaced with fresh medium and DSS-BEN/FAM-miRNA nanoparticles were added (200 nM FAM-miRNA). After incubation for 4 h, the cells were washed twice with PBS, stained with LysoTracker® Red DND-99 (Life Technology, USA) for 30 min, and fixed with 4% paraformaldehyde for 10 min. The cells were rinsed 3 times with PBS and visualized using a LSM 710 Laser Scanning Microscope (Zeiss, Jena, Germany).

2.5. Transfection of DSS-BEN/miRNA nanoparticles

Cells were seeded in 24-well plates at 5×10^4 cells/well 24 h prior to transfection. On the day of transfection, cells were incubated with the nanoparticles containing miRNA (200 nM) in 500 μ L of FBS free medium. After 4 h incubation, nanoparticles were completely removed, and the cells were further incubated in medium with 10% FBS.

2.6. Analysis of intracellular polyamines

Cells were treated with 12.3 μ g/mL BENSp_m, DSS-BEN/miR-NC or DSS-BEN/miR-34a (w/w=10, miRNA 200 nM, DSS-BEN 28 μ g/mL) for 24 h or 48 h. The cells were harvested by trypsinization and the concentration of BENSp_m and natural polyamines (SPM, SPD, PUT) was determined as described previously [50].

2.7. Quantitative real-time PCR (qRT-PCR)

The expression levels of miR-34a were determined by TaqMan qRT-PCR. A mirVana™ miRNA Isolation Kit (Ambion™, USA) was utilized for total RNA extraction from cultured cells. Ten ng of total RNA was converted into cDNA using specific primers for miR-34a (or the internal control Z30) (Applied Biosystems) and the TaqMan miRNA reverse-transcription kit (Applied Biosystems). qRT-PCR was performed using TaqMan Universal Master Mix II, No AmpErase UNG (2 $\times$) and specific primers for miR-34a or Z30 on a Rotor-Gene Q instrument (QIAGEN) according to the manufacturer's protocols. MiRNA expression was expressed relative to the internal control using the comparative threshold cycle (Ct) method. Expression of the polyamine catabolic enzymes SMOX and SSAT in HCT116 cells was also quantified using RT-PCR. Cells were treated with 12.3 μ g/mL BENSp_m, DSS-BEN/miR-NC or DSS-BEN/miR-34a (w/w=10, miRNA 200 nM, DSS-BEN 28 μ g/mL) for 24 h or 48 h. Total RNA was isolated using mirVana™ miRNA Isolation Kit (Ambion™, USA) and reverse-transcribed to cDNA using QuantiTect reverse transcription kit (Qiagen): the relative amount of mRNA was determined by RT-PCR on a Rotor-Gene Q instrument (QIAGEN). The GAPDH primer assay and QuantiFast SYBR Green PCR kit (Qiagen) were used following the manufacturer's protocol. The following primers were used: human SMOX (forward 5'-CGCAGACTTACTTCCCCGGC; reverse, 5'-CGCTCAATTCCTCAACCACG); and human SSAT (forward, 5'-ATCTAAGCCAGGTTGCAATGA; reverse, 5'-GCACTCCTCACTCCTCTGTTG). Relative mRNA expression levels of the enzymes were calculated from the Ct values of the target genes and the housekeeping gene GAPDH.

2.8. Western blot

Cells were lysed with RIPA Lysis buffer by incubation on ice for 10 min. After centrifugation at 12,000 g for 10 min, the supernatants were collected and the concentrations of proteins were quantified by the BCA protein assay kit (Promega, USA). The protein samples were denatured by boiling for 5 min, loaded onto 10% SDS-PAGE gel for electrophoresis (at 120 V for 2 h), and then transferred (at 300 mA for 1 h) to a nitrocellulose membrane. After blocking with 5% non-fat dried milk at room temperature for 1 h, the membrane was incubated overnight at 4 °C with Bcl-2 or the internal control GAPDH rabbit monoclonal antibody (Cell Signaling Technology, USA), then washed and incubated with the secondary anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, USA) for 1 h. Finally, membranes were again washed and visualized using PierceTM ECL Western Blotting Substrate (Thermo Scientific, USA). Quantification of Western blot bands was performed using ImageJ software (National Institutes of Health), and the data were expressed as relative Bcl-2 levels vs. PBS-treated cells.

2.9. Cytotoxicity

Toxicity of the nanoparticles was evaluated by Cell Titer Blue assay in HCT116 cells. The cells were plated in 96-well microplates at a density of 5,000 cells/well. After 24 h, transfection was performed as above using PBS, DSS-BEN/miR-NC, or DSS-BEN/miR-34a with various w/w ratios at a miRNA concentration of 200 nM. After a 4-h incubation, culture medium was replaced by 200 μ L of fresh medium with 10% FBS, and cells were incubated for another 48 h prior to measuring cell viability. The medium was then removed and replaced with a mixture of 100 μ L serum-free media and 20 μ L of CellTiter-Blue reagent (CellTiter-Blue® Cell Viability Assay, Promega). After a 2-h incubation, fluorescence (560/590nm) was measured on a Synergy 2 Microplate Reader (BioTek, VT). The relative cell killing mediated by the nanoparticles was normalized to the viability of untreated cells and expressed as the means $\pm$ SD of triplicate samples.

2.10. Antitumor activity in vivo

All animal experiments followed a protocol approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Animals were placed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care upon arrival. Female athymic nude mice (5–6 weeks) were purchased from Charles River Laboratories. The xenograft tumor model was generated by subcutaneous injection of HCT116 cells (5×10^6 per mouse) into the flank region of the mouse. When the tumor volume reached 200–300 mm³, mice were randomly divided into 3 groups (n = 5) and injected intratumorally with 5% glucose, DSS-BEN/miR-NC, and DSS-BEN/miR-34a every other day (total of 4 doses) at doses of 5 mg/kg DSS-BEN and 0.5 mg/kg miRNA (w/w=10). Tumor volume was monitored by measuring the perpendicular size of the tumors using digital calipers. The estimated volume was calculated according to the following formula: tumor volume (mm³) = $0.5 \times \text{length} \times \text{width}^2$. Body weight of the mice was also recorded. At the end of the study, mice were sacrificed and all tumor tissues were collected, weighed, and used for further analysis. The total RNA was isolated using mirVanaTM miRNA Isolation Kit according to the protocol. Then, the levels of miR-34a and SMOX and SSAT mRNA in

the tumors was analyzed by qRT-PCR as described above. For Western blot analysis, tumor tissues were lysed with RIPA Lysis buffer and then were analyzed as described above. For immunohistochemistry analysis, tumors were fixed for 24 h in 4% paraformaldehyde. The tissues were embedded in paraffin and 5 mm sections were cut. The proliferation of tumor cells was determined using the Ki-67 immunohistochemical staining according to the manufacturer's instructions. Images of Ki-67 stained tumor sections were taken by EVOS xl microscope ($\times 40$). The number of Ki-67 positive cells (shown as brown dots) and the total number were counted using the ImageJ software. The percentage of Ki-67 positive cells in the samples was obtained by dividing the number of Ki-67 positive cells from the number of total cells in each microscopic field.

2.11. Statistical analysis

Data are presented as the means $\pm$ SD. The statistical significance was determined using ANOVA followed by Bonferroni *post hoc* correction with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Preparation and physicochemical characterization of DSS-BEN/miRNA nanoparticles

Polycationic prodrug DSS-BEN containing self-immolative bioreducible linker was synthesized as before and used in the present studies. The presence of 4 secondary amines in the BENSpm structure resulted in the synthesis of branched DSS-BEN polymers, although only the linear form is shown in Scheme 1. The capability of DSS-BEN to form nanoparticles with miRNA was first tested using agarose gel electrophoresis. As shown in Figure 1A and S1, DSS-BEN was able to fully condense miRNA above a DSS-BEN/miRNA (w/w) ratio of 2.5. However, partial miRNA condensation was observed at w/w ratios as low as 1 as indicated by the smear of the miRNA in the gel. Above a DSS-BEN/miRNA ratio of 2.5, the condensed miRNA was completely protected from ethidium bromide staining and no fluorescence signal was observed.

Hydrodynamic size and zeta-potential of DSS-BEN/miRNA nanoparticles prepared at various w/w ratios were measured by dynamic light scattering in 10 mM HEPES (pH 7.4). The sizes of the nanoparticles decreased gradually with increasing w/w ratios from 170 nm at w/w = 2.5 to 89 nm at w/w = 10 (Figure 1B). We hypothesize that intra-particle crosslinking via disulfide–disulfide exchange among multiple branched DSS-BEN molecules contributed to the additional stabilization and compaction of the nanoparticles, leading to the observed small sizes [51]. Low polydispersity index (< 0.15) of the formed particles further confirmed the well-controlled assembly process as did the log-normal distribution of sizes. Particle size increased to ~ 210 nm when measured in isotonic saline. TEM was used to analyze particle shape and overall morphology. As shown in Figure 1C, DSS-BEN and miR-34a formed uniform particles with spherical morphology (Figure 1C). As expected, increasing the w/w ratio used in the preparation of the nanoparticles resulted in an increase of the zeta potential, which ranged from negative (-11.5 mV at w/w = 2.5) to positive (11.8 mV at w/w = 10) (Figure 1D, E).

The cleavage of the disulfide bonds in DSS-BEN is expected not only to release the parent BEN^{Spm} but also to facilitate disassembly of the nanoparticles due to the inability of free BEN^{Spm} to condense miRNA. To investigate the reduction-triggered disassembly of the particles, we followed changes in particle size and size distribution of DSS-BEN/miRNA upon exposure to 20 mM GSH. As shown in Figure 1F, the mean diameter of the particles increased significantly with time due to the cleavage of the disulfide linkages. For example, the size increased from 89 nm to 480 nm in 4 h and reached over 800 nm in 8 h of incubation with GSH. After 24 h, the size of the particles could not be precisely measured by DLS due to disassembly of the particles and concomitant decrease in light scattering intensity. The morphological and size changes in response to GSH treatment were also reflected in TEM which showed extensive swelling and loosening of the particle structure within 4 h of GSH treatment and a complete disassembly after 24 h (Figure 1G). This reduction-triggered miRNA release was also studied directly by incubating DSS-BEN/miRNA with 20 mM GSH and increasing concentrations of a competing polyanion heparin. The stability of the nanoparticles was assessed by the minimum heparin concentration required for disassembly and miRNA release using agarose gel electrophoresis (Figure 1H and S2). In the absence of GSH, the particles were stable and released no miRNA until the heparin concentration exceeded 80 µg/mL. However, when incubated with 20 mM GSH, rapid disulfide reduction destabilized the particles, which resulted in the release of miRNA even without the addition of heparin. These results suggest that the self-immolative linker in DSS-BEN retains its redox responsiveness even when present within the DSS-BEN/miRNA nanoparticles.

3.2. Cellular uptake and intracellular trafficking

To study cellular uptake and intracellular trafficking, we prepared the nanoparticles using a fluorescently labeled miRNA (FAM-miRNA, 200 nM). HCT116 cells were incubated with DSS-BEN/FAM-miRNA for 4 h and cellular uptake was determined by flow cytometry. As shown in Figure 2A, DSS-BEN/miRNA nanoparticles were readily taken up by the HCT116 cells as indicated by the enhanced fluorescence intensity compared with control PBS-treated cells and cells treated with free FAM-miRNA. Increasing the w/w ratios in preparation of the nanoparticles resulted in enhanced cellular uptake both in terms of the mean fluorescence intensity per cell (Figure 2B) and the percentage of the cells that had internalized the nanoparticles (Figure 2C). Over 80% of the cells internalized the particles when prepared at w/w ratios above 5. Based on these uptake results, nanoparticles prepared at w/w = 10 were selected for subsequent studies.

It is important that miRNA is delivered into its site of action in the cytoplasm. Intracellular distribution and trafficking of DSS-BEN/FAM-miRNA (w/w = 10) was thus studied using confocal microscopy. HCT116 cells were incubated with the particles for 4 h and lysosomes were stained with LysoTrackerRed. As shown in Figure 2D, the fluorescence of the FAM-miRNA (green) was localized predominantly in the cytoplasm with only a limited extent of co-localization with the LysoTracker signal in lysosomes (red). This result confirmed highly efficient endosomal escape of the DSS-BEN/miRNA nanoparticles and delivery of miRNA to the cytoplasm.

3.3. Regulation of polyamine metabolism by DSS-BEN/miRNA

The ability of the nanoparticles to disassemble and degrade into BENSpm and affect polyamine metabolism was evaluated by incubating cells with either particles or free BENSpm, followed by HPLC analysis of polyamine levels in the cell lysate. As shown in Figure 3A, DSS-BEN/miRNA showed effective intracellular degradation into parent BENSpm, which was indicated by increased intracellular BENSpm concentrations at 24 and 48 h. No BENSpm was detected in untreated cells. The amount of BENSpm present in the cancer cells treated with the nanoparticles was around 40% of the levels observed in cells treated with equivalent free BENSpm. This lower intracellular accumulation of BENSpm delivered by the nanoparticles is likely a complex function of the rate and extent of cellular uptake, intracellular trafficking, rate of degradation and extent of polyamine excretion from the cells.

BENSpm exhibits multiple functions in the regulation of intracellular polyamines. BENSpm can act as a potent inducer of the expression of the key catabolic enzymes SMOX and SSAT. Here, we aimed to study the effect of DSS-BEN/miRNA treatment on changes in expression of SMOX and SSAT using RT-PCR (Figure 3B). As expected, treatment with free BENSpm upregulated the mRNA levels of both SSAT and SMOX. Overall, after a 24-h incubation, treatment with DSS-BEN/miRNA nanoparticles resulted in a smaller upregulation of the mRNA of the catabolic enzymes in comparison with treatment by free BENSpm. For example, BENSpm increased expression of SMOX mRNA 2.6-fold, whereas the DSS-BEN/miR-NC polyplexes increased the expression 2.1-fold. Similarly, BENSpm upregulated SSAT mRNA expression 3.5-fold, whereas the DSS-BEN/miR-NC nanoparticles achieved only a 1.7-fold increase. Interestingly, after a 48-h incubation, treatment with DSS-BEN/miRNA nanoparticles upregulated SMOX and SSAT mRNA levels to a similar extent as treatment with free BENSpm. This observation is likely a consequence of the delayed release of BENSpm from the nanoparticles due to the need to traffic into the cytoplasm where the disulfide reduction needed for BENSpm release takes place. Importantly, DSS-BEN/miR-34a produced no significant differences in SMOX and SSAT mRNA expression compared with DSS-BEN/miR-NC, indicating that miR-34a does not regulate SMOX and SSAT expression.

Having confirmed the release of BENSpm from the nanoparticles and its induction of polyamine catabolic enzyme expression, we evaluated the effect of the nanoparticles on intracellular polyamine depletion. As shown in Figure 3C, the nanoparticles efficiently decreased intracellular concentration of PUT, SPD and SPM after treatment for 24 and 48 h. The decrease in intracellular levels of spermidine and spermine, the substrates of SSAT and SMOX, following nanoparticle treatment are indicative of significant increases in enzymatically active SSAT and SMOX protein. These results confirmed the polyamine depletion activity of DSS-BEN/miRNA nanoparticles in cancer cells. Free BENSpm showed a better ability to induce polyamine depletion than the DSS-BEN nanoparticles. We hypothesize that this observation is associated with the likely differences in the mechanisms of cellular uptake and intracellular trafficking between DSS-BEN/miRNA nanoparticles and free BENSpm.

3.4. Delivery of miRNA by the nanoparticles

MiR-34a and its negative control (miR-NC) were used to evaluate the ability of the nanoparticles to deliver functional miRNA into cells. The miR-34a levels in HCT116 cells were measured using RT-PCR (Figure 4A). DSS-BEN/miR-34a nanoparticles showed high miRNA delivery efficiency, as indicated by more than a 12-fold increase in intracellular miR-34a when delivered in particles formed at w/w = 10. In general, increasing the w/w ratio in preparing the particles resulted in significantly enhanced miRNA delivery.

Having confirmed the capability of DSS-BEN nanoparticles to effectively deliver miR-34a to the HCT116 cells, we then studied the effect of the delivered miR-34a on one of its important downstream targets, Bcl-2 – a key oncogene regulating cancer cell death by inhibiting apoptosis. We used Western blot analysis to determine the intracellular levels of Bcl-2. As shown in Figure 4B and C, delivery of miR-34a using DSS-BEN resulted in a significant decrease (53%) of the intracellular Bcl-2 protein levels in the HCT116 cells when compared with the control DSS-BEN/miR-NC particles. This finding confirms that miR-34a was efficiently delivered by the DSS-BEN nanoparticles into the cytoplasm and released in its active state to successfully downregulate the target Bcl-2 protein.

3.5. Anticancer activity of DSS-BEN/miRNA in vitro

The above results suggest that DSS-BEN nanoparticles induced polyamine depletion through enhanced catabolism and effectively delivered miR-34a into HCT116 cells. We hypothesized that using DSS-BEN/miRNA as a delivery vector for miR-34a would result in enhanced anticancer activity due to the cooperative effect of polyamine depletion by BEN_{Spm} released from DSS-BEN and miR-34a effects on Bcl-2. To evaluate this hypothesis, DSS-BEN/miR-34a were prepared at multiple w/w ratios with constant miRNA concentration (200 nM) and their cell killing activity was determined by a cell viability assay. DSS-BEN nanoparticles prepared with miR-NC were used as a control to analyze the contribution of polyamine depletion to the overall cell killing activity. As shown in Figure 5A, increasing the w/w ratios in DSS-BEN/miR-NC nanoparticles, thereby increasing their DSS-BEN content, resulted in enhanced cell death from 10% (w/w ratio = 2.5) to 56% (w/w ratio = 10). This was most likely the effect of a more efficient polyamine depletion by free BEN_{Spm} released from DSS-BEN. In comparison with DSS-BEN/miR-NC nanoparticles, DSS-BEN/miR-34a nanoparticles exhibited significantly higher cell killing activity at w/w ratios higher than 2.5, likely due to miR-34a targeting and reduction of the Bcl-2 expression. For example, DSS-BEN/miR-34a (w/w = 10) induced 75% cell killing, which was higher than that of DSS-BEN/miR-NC particles at the same w/w ratio (56%). The cell killing activity of DSS-BEN/miR-34a nanoparticles was also confirmed by microscopic observation. DSS-BEN/miR-34a treatment decreased cancer cell number and distorted cell morphology (Figure 5B). At w/w = 2.5, DSS-BEN/miR-34a did not cause greater cell death than DSS-BEN/miR-NC due to poor miRNA delivery at low w/w ratios. These results confirmed that miR-34a delivered by DSS-BEN exhibited cooperative enhancement of anticancer activity in HCT116 cells. DSS-BEN/miR-34a nanoparticles prepared at w/w = 10 showed the highest cell killing activity of all the tested formulations and were thus selected for subsequent *in vivo* study.

3.6. Antitumor activity of DSS-BEN/miRNA nanoparticles in vivo

Antitumor efficacy of DSS-BEN/miR-34a was studied in female nude mice bearing HCT116 tumor xenografts. As shown in Figure 6, DSS-BEN/miR-NC treatment significantly inhibited the tumor growth (tumor volume inhibition rate 48%) when compared with control group given 5% glucose used as the vehicle for the nanoparticles. This effect was most likely due to the activity of BEN_{Spm} released from DSS-BEN. Importantly, greatly enhanced tumor growth inhibition efficacy was observed in animals treated with DSS-BEN/miR-34a (tumor volume inhibition rate 72%), indicating additive effect of the delivered miR-34a to the overall antitumor effect *in vivo*. At the end of the study, tumors were excised and photographed (Figure 6D). The weights of the tumors were also recorded (Figure 6E). The mean tumor weight of the DSS-BEN/miR-34a group (0.32 g) was significant lower than the weight of the control DSS-BEN/miR-NC group (0.63 g) and the glucose control group (1.05 g). These findings confirmed that DSS-BEN/miR-34a could achieve enhanced combinational *in vivo* antitumor effect as a result of the BEN_{Spm} released from DSS-BEN and miR-34a cancer gene therapy. As suggested by the unchanged body weight during the treatment, the DSS-BEN/miR-34a had no apparent signs of gross toxicity (Figure 6C). Despite the translational limitations of intratumoral injection, our results showed for the first time, the potential therapeutic benefits of combining anticancer action of miR-34a with simultaneous inhibition of polyamine metabolism.

To confirm that the mechanism of antitumor activity reflects our *in vitro* findings, mRNA expression of SSAT and SMOX in the treated tumors was evaluated by qRT-PCR (Figure 7A). Both DSS-BEN/miR-NC and DSS-BEN/miR-34a treatments significantly increased SMOX and SSAT mRNA levels in the tumor tissues with comparable potency. This finding suggests that the DSS-BEN nanoparticles targeted polyamine metabolism also *in vivo*. The miR-34a levels in the treated tumor tissues were also determined by qRT-PCR (Figure 7B). Significant upregulation of miR-34a (1.7-fold) was found in the tumors treated with DSS-BEN/miR-34a when compared with the tumors treated with the control DSS-BEN/miR-NC, indicating successful delivery of functional miR-34a into tumors *in vivo*. The downregulation of its downstream target Bcl-2 was further confirmed by Western blot analysis (Figure 7C, D). Immunohistochemical staining of Ki-67 in the tumor tissue was used to study the antiproliferative activity of the nanoparticles (Figure 7E, F). In comparison with the untreated (glucose) group (+Ki67, 75%), DSS-BEN/miR-NC treatment decreased the proliferation of cancer cells, as indicated by a lower percentage of Ki-67 positive cells (51%). DSS-BEN/miR-34a showed even higher antiproliferative activity, with only 32% of Ki-67 positive cells. This apparently greater-than-additive antiproliferative effect of DSS-BEN/miR-34a when compared with DSS-BEN/miR-NC suggests potential synergy between increased polyamine catabolism and decreased Bcl-2 expression on antitumor activity of the nanoparticles.

4. Conclusion

In this study, we have developed an innovative combination treatment strategy based on simultaneous regulation of polyamine metabolism and miRNA delivery using self-immolative polymer prodrug nanoparticles. Our results showed that upon intracellular

reduction with GSH, the self-assembled nanoparticles undergo degradation to release the parent drug BENSp_m and miR-34a. The released BENSp_m upregulates polyamine catabolic enzymes SSAT and SMOX and depletes cellular polyamine levels. Successful delivery of therapeutic miRNA by these particles was confirmed by the increase of intracellular level of miR-34a and inhibition of the targeted Bcl-2. The combination nanoparticles not only showed enhanced cell killing in HCT116 human cancer cells *in vitro*, but also achieved an improved effect on tumor growth inhibition in HCT116 xenografts *in vivo*. The self-immolative polymer nanoparticles show great promise as dual-function delivery vectors of therapeutic miRNA, providing a novel, simple and elegant approach to the design of combination nanomedicines for cancer treatment. Future studies will focus on further development of the self-immolative nanoparticles with the goal of enabling their use in systemic anticancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support from the University of Nebraska Medical Center, National Institutes of Health (EB015216, EB014570), and China Scholarship Council (YX, YW) is gratefully acknowledged.

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Figure 1.

Physicochemical characterization of DSS-BEN/miRNA nanoparticles. (A) miRNA condensation by DSS-BEN in 10 mM HEPES buffer (pH 7.4) using agarose gel electrophoresis. (B) Hydrodynamic size of DSS-BEN/miRNA nanoparticles at various w/w ratios ($n=3$). (C) Size distribution and TEM image (inset) of DSS-BEN/miRNA (w/w=10). Scale bar = 50 nm. (D) Zeta-potential of DSS-BEN/miRNA nanoparticles at various w/w ratios ($n=3$). (E) Zeta-potential distribution of DSS-BEN/miRNA (w/w=10). (F) Size distribution of nanoparticles in the presence of 20 mM GSH. (G) Morphological changes in nanoparticles in the presence of 20 mM GSH determined by TEM. (Scale bar = 200 nm). (H) Heparin- and GSH-induced miRNA release from DSS-BEN/miRNA. Nanoparticles were prepared at w/w = 10 and incubated with increasing concentrations of heparin either with or without 20 mM GSH for 30 min. ** $P < 0.01$, *** $P < 0.001$.

Figure 2. Cellular uptake and intracellular trafficking of DSS-BEN/FAM-miRNA nanoparticles in HCT116 cells. (A) Overlaid histogram of flow cytometry analysis of cells treated with DSS-BEN/FAM-miRNA nanoparticles at various w/w ratios (200 nM FAM-miRNA) after a 4-h incubation. Quantification of cellular uptake is shown by (B) mean fluorescence intensity (MFI) and (C) % of cells that had taken up the particles. Data are shown as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$, *** $P < 0.001$. (D) Intracellular trafficking of DSS-BEN/FAM-miRNA in HCT116 cells by CLSM after a 4-h incubation. (Scale bar 20 μ m)

Figure 3.

Effect of DSS-BEN/miRNA treatment on cellular polyamine metabolism. Cells were incubated with 12.3 $\mu\text{g/mL}$ BENSpm or DSS-BEN/miRNA (w/w=10, 28 $\mu\text{g/mL}$ DSS-BEN) for 24 h or 48 h. (A) Intracellular concentration of BENSpm was determined by HPLC ($n = 3$). (B) Relative changes in the expression of SMOX and SSAT mRNA in HCT116 cells. mRNA levels were measured by qRT-PCR. Results are expressed as the fold induction of specific mRNA in treated cells relative to that in the PBS- treated group ($n = 3$). (C) Intracellular polyamine concentration determined by HPLC ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4.

The ability of DSS-BEN nanoparticles to deliver functional miRNA. (A) miR-34a was measured by qRT-PCR in HCT116 cells following incubation with DSS-BEN/miR-34a or control DSS-BEN/miR-NC. (B) Effect of miR-34a delivery on the expression of Bcl-2. (C) Quantification of Western blot bands performed using ImageJ software. The results are expressed as relative Bcl-2 levels relative to untreated cells. (means $\pm$ SD, $n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5.

Anticancer activity of DSS-BEN/miRNA nanoparticles in HCT116 cells. (A) Cells were treated for 48 h with DSS-BEN/miR-NC or DSS-BEN/miR-34a prepared at different w/w ratios. Cell killing was measured by Cell Titer Blue assay. Results are normalized to the viability of untreated cells and shown as mean relative cell killing (%) $\pm$ SD (n = 3). * P < 0.05, *** P < 0.001. (B) Morphology of cells observed under light microscope ($\times$ 40) after treatment with DSS-BEN/miR-NC or DSS-BEN/miR-34a nanoparticles (w/w = 10, 200 nM).

Figure 6.

Anticancer activity of DSS-BEN nanoparticles *in vivo*. (A) Representative images of HCT116 xenograft tumors at day 8 after intratumoral injection of various formulations. Mice were administered DSS-BEN/miRNA (w/w = 10) at doses of 5 mg/kg DSS-BEN and 0.5 mg/kg miRNA once every two days. (B) Tumor growth curve. Injections are represented by black arrows. (C) Body weight during the treatment period. (D) Tumor tissues were extracted from mice and photographed. (E) Weights of tumors collected from treated mice. Data are shown as the means $\pm$ SD (n = 5). * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 7.

(A) Relative changes in the expression of polyamine catabolism enzymes SMOX and SSAT in HCT116 tumor tissues. (B) miR-34a level was detected by qRT-PCR in HCT116 tumor tissues. (C) The expression of Bcl-2 in tumor tissues determined by Western blot. (D) Quantification of Western blot bands performed using ImageJ software. (E) Immunohistochemistry analyses of tumor tissues after various treatments. The percentage (%) of Ki-67 positive cells in tumor tissues. (F) The images were taken under a light microscope ($\times 40$). Data are shown as the means $\pm$ SD ($n = 3$). * $P < 0.05$, *** $P < 0.001$.

Scheme 1.

Mechanism of action of DSS-BEN/miR-34a nanoparticles. (A) DSS-BEN condenses miRNA into nanoparticles by electrostatic interactions. (only linear form of DSS-BEN is shown but branched forms are also present) (B) Upon endocytosis and endosomal escape, the particles are subjected to cytoplasmic reduction by GSH, followed by disassembly and release of both BENSpm and miR-34a mimic. BENSpm induces expression of enzymes involved in polyamine catabolism, which reduces intracellular polyamine levels. MiR-34a mimic increases cellular miR-34a levels, which leads to Bcl-2 downregulation.