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Systemic Delivery of Bc12-Targeting siRNA by DNA Nanoparticles Suppresses Cancer Cell Growth

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Conflict of interest

The authors declare no conflict of interest.

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Abstract

Short interfering RNA (siRNA) is a promising molecular tool for cancer therapy, but its clinical success is limited by the lack of robust in vivo delivery systems. Rationally designed DNA nanoparticles (DNPs) have emerged as facile delivery vehicles because their physicochemical properties can be precisely controlled. Nonetheless, few studies have used DNPs to deliver siRNAs in vivo, and none has demonstrated therapeutic efficacy. Herein, we constructed a number of DNPs of rectangular and tubular shapes with varied dimensions using the modular DNA brick method for the systemic delivery of siRNA that targets anti-apoptotic protein Bcl2. The siRNA delivered by the DNPs inhibited cell growth both in vitro and in vivo, which suppressed tumor growth in a xenograft model that specifically correlated with Bcl2 depletion. This study suggests that DNPs are effective tools for the systemic delivery of therapeutic siRNA and have great potential for further clinical translation.

Keywords

Bcl2; cancer therapy; DNA nanotechnology; nanoparticles; siRNA delivery

Short interfering RNAs (siRNAs) are able to specifically silence mRNA translation,^[1] which has emerged as a promising therapeutic tool in targeted cancer therapy.^[2] However, limited success has been achieved in the systemic administration of siRNA^[3] because of a series of hurdles including kidney filtration, clearance by phagocytes, aggregation with serum proteins, and degradation by nucleases before reaching the target cells.^[3b,d] Nano-carriers are promising tools for the targeted delivery of siRNAs, however, conventional delivery systems (for example, inorganic and organic nanoparticles) face significant challenges, such as toxicity and limited tuning capacity of particles physicochemical properties (for example, size, shape, and surface chemistry) to achieve optimal delivery effect.^[4] Therefore, the lack of an efficient and versatile siRNA delivery system is a critical issue in limiting its clinical translation. Structural DNA nanotechnology has unprecedented ability to construct DNA nanoparticles (DNPs) with well-controlled size, shape, and surface functionality,^[5] which have been utilized in a variety of fields,^[6] including drug delivery.^[7] The homogeneous and versatile nature of DNPs affords great potential for the systemic delivery of siRNAs. To our best knowledge, few studies have explored the use of DNPs for the in vivo delivery of siRNAs, though several reports on using DNA origami nanostructures for the systemic delivery of small molecule drugs (for example, doxorubicin) have been published.^[7j] Lee et al. utilized a DNA tetrahedral structure for the tumor-targeted delivery of siRNAs silencing luciferase.^[7f] However, no therapeutic efficacy (that is, tumor growth inhibition) was demonstrated in this study. Moreover, the reported small tetrahedral structure offers limited programmability in physicochemical properties, in comparison to the delicate structures designed by the DNA origami^[5b] or DNA brick^[5c] methods.

Herein, we designed DNPs with rectangular or tubular shapes of varied dimensions using the modular DNA brick method and studied their cellular uptake in cancer cells to pinpoint the optimal design toward in vitro and in vivo siRNA delivery. DNA brick nanostructures are self-assembled from short DNA strands, and can achieve high yields of target structures. In addition, their modularity makes it relatively facile to design and construct multiple nanostructures of different sizes. These properties make the DNA brick method suitable for rapid screening studies toward identifying structures with potent delivery capabilities and holds better practical applicability because of its ease of preparation. This study focused on nanorectangles and nanotubes because DNA nanostructures of similar shapes have demonstrated robust capability for in vitro^[7b,e,h,i] and in vivo^[7j] delivery of cargos such as doxorubicin.^[7e,j] Bcl2 was targeted because the Bcl2 family are central regulators of programmed cell death.^[8] Overexpression of Bcl2 is implicated in tumor initiation, progression, and resistance to therapy.^[9]

Eight DNPs were designed (Figure 1a and the Supporting Information, Figure S1 and Table S1), including a 6-helix-wide, 96-bp-long rectangle (6Hx96BP-Rect), a 6-helix-wide, 96-bp-long tube (6Hx96BP-Tb), a 6Hx192BP-Rect, a 6Hx192BP-Tb, a 12Hx96BP-Rect, a 12Hx96BP-Tb, a 12Hx192BP-Rect, and a 12Hx192BP-Tb. As shown in Figure 1a and the Supporting Information, Figure S2, AFM images confirmed the successful assembly of DNPs with expected dimensions and morphologies. For the tubular structures, opened rectangular structures were observed occasionally, which may be attributed to the AFM sample preparation and tip interference during scanning. Native agarose gel electrophoresis verified the assembly of DNPs (Figure 1b). Discrete bands were observed for DNPs, with the larger ones exhibiting slower mobility, suggesting the formation of the designed structures. Single-stranded handles that protrude from the surface of DNPs were used for loading siBcl2 through hybridization between handles and overhangs extending from siBcl2 (Figure 1c). The successful loading of siBcl2 onto 6Hx96BP-Rect was confirmed by gel electrophoresis since the loading of siBcl2 led to slower mobility (Figure 1d). Cy5-conjugated single-stranded DNAs were loaded by using the same strategy for the cellular uptake studies (Supporting Information, Figure S3). We also examined DNP resistance to nuclease digestion in 10% fetal bovine serum (FBS) at 37°C with varied incubation times. All DNPs remained intact after 4 h of incubation, with larger DNPs surviving as long as 8 h (Supporting Information, Figure S4). Overall, DNPs exhibited strong resistance to nuclease digestion similar to that reported for DNA nano-structures,^[7a] making them potentially suitable for siRNA delivery in vitro and in vivo.

We next examined the cellular internalization of DNPs in a small cell lung carcinoma (SCLC) cell line (DMS53) and a non-small cell lung carcinoma (NSCLC) cell line (H1299). Cy5-DNAs were anchored onto DNPs to track their cellular uptake (Figure S3). A time-course study was conducted to compare the internalization efficiency of DNPs. Flow cytometry demonstrated that all DNPs, regardless of size or shape, had higher internalization efficiency than Cy5-DNA alone in both H1299 and DMS53 cells at all time points ($P < 0.05$), indicating that DNPs served as delivery vehicles to transport Cy5-DNA into cells (Figure 2a and the Supporting Information Figure S5). In general, small DNPs exhibited slightly higher internalization efficiency than large DNPs in both cell lines. To compare with Cy5-DNA-tagged DNPs, Cy5-conjugated siBcl2 were loaded onto 6Hx96BP-Rect. Flow

cytometry revealed similar cell internalization efficiency, with siBcl2-loaded 6H×96BP-Rect having slightly lower uptake (Supporting Information, Figure S6), which may be caused by siBcl2's lower loading efficiency onto DNPs given its relatively larger size than Cy5-DNA (steric hindrance and electrostatic repulsion).

We then visualized the cellular uptake of DNPs by confocal microscopy (Figure 2b and the Supporting Information, Figure S7). Strong Cy5 fluorescence was observed within the cytoplasm, indicating DNPs were successfully internalized into the cells. Both studies revealed that small DNPs accumulate in cells efficiently and could be considered a better vehicle for siRNA delivery. Scavenger receptors are known to mediate the endocytosis of nucleic acids structures in multiple mammalian cell lines.^[9c] To examine the involvement of scavenger receptors in the uptake of DNPs, polyinosine (Poly-I) was used to competitively bind and saturate scavenger receptors. All DNPs showed 90% reduction upon Poly-I treatment (Figure 2c), indicating that scavenger receptors are critical for the uptake of DNPs by DMS53 cells. Based on cell internalization studies, 6H×96BP-Rect was selected for subsequent in vitro and in vivo delivery of siBcl2. We first examined whether the siBcl2 used in this study can specifically silence Bcl2 in DMS53 cells. In both western blotting and RT-PCR analysis (Supporting Information, Figure S8), siBcl2 showed significant silencing effect against Bcl2, while a control siRNA had no apparent effect, indicating the high specificity of siBcl2. DMS53 cells were then treated with varying concentrations of siBcl2-loaded 6H×96BP-Rect. Western blotting showed that 6H×96BP-Rect loaded with siBcl2 can inhibit Bcl2 expression by up to 90% (Figure 2d). On the mRNA level, a circa 70% silencing efficiency was observed by RT-PCR (Figure 2e). 6H×96BP-Rect alone showed Bcl2 inhibition by western blotting but not by RT-PCR, implying that the DNPs might have certain unknown interactions with Bcl2 proteins. 6H×96BP-Rect-siBcl2 induced significant cell growth inhibition, which was not observed for vehicle or naked siBcl2 (Figure 2 f,g).

The anti-cancer therapeutic potential of siBcl2 delivered by 6H×96BP-Rect was studied in mice bearing DMS53 tumor xenografts. Five groups of drugs were administered through the tail vein on days 1, 4, 8, 11, and 15. Dosages were determined based on the siRNA amount. Significant tumor growth inhibition was observed in mice treated with 6H×96BP-Rect-siBcl2 (5 mgkg⁻¹ or 10 mgkg⁻¹) relative to the three control groups (Figure 3a and the Supporting Information, Figure S9 and Table S2). After 17 d, the average tumor size of mice treated with these two groups was significantly smaller (ca. 2-fold, **P* < 0.05) than that of the control groups. Furthermore, there was no evidence of toxicity for any treatment as judged by body weight (Supporting Information, Figure S10), behavior of mice, or pathologic examination of major organs (Figure 3b and the Supporting Information, Figure S11). Immunohistochemical staining revealed a significant reduction in Bcl2 protein in tumor tissues treated with 6H×96BP-Rect-siBcl2 (5 mgkg⁻¹ or 10 mgkg⁻¹), compared to controls (Figure 3b,c). Tumors with reduced Bcl2 protein levels also exhibited growth suppression and elevated levels of apoptosis, as revealed by Ki67 staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays (Figure 3b,c), respectively. Overall, these results demonstrated that pharmacologically active amounts of siBcl2 were delivered to the cytoplasm of cells in the tumor using achievable doses of 6H×96BP-Rect-siBcl2 to knockdown Bcl2 protein, induce apoptosis, inhibit cell

proliferation, and eventually suppress tumor growth, though DNPs may be subject to faster degradation profiles in vivo (Supporting Information, Figure S12).

In summary, our studies show that all DNPs exhibited efficient uptake in cancer cells. The siBcl2 delivered by one DNP showed high efficiency as evidenced by circa 90% knockdown of Bcl2 protein, circa 70% silencing of Bcl2 mRNA, and subsequent cellular growth inhibition in vitro. More importantly, we demonstrated that systemic delivery of siBcl2 in mice by 6Hx96BP-Rect exhibited effective tumor growth inhibition and no toxicity. In the current work, DNPs have no targeting moieties to minimize off-target delivery of siRNAs to healthy tissues. The DNA nanoparticles enter cell using the scavenger receptors, there are possibilities to trigger release of various cytokines and immune stimulation. In conjunction with targeting ligands (for example, folic acid or transferrin), we believe DNP-based siRNA or other therapeutics delivery will continue to overcome barriers and play an important clinical role in future cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design, construction, and characterization of DNPs. a) Schematics (top) and AFM images (bottom) of 8 DNPs. Scale bars=50 nm. Color bars are from -1 to 3 nm to indicate the height of DNPs. b) Native agarose gel electrophoresis of DNPs. Major distinct bands indicate the successful formation of DNPs with high yield. c) Loading mechanism of siBcl2 onto DNPs. siBcl2 are loaded through single-stranded handles extended from the surface of DNPs. d) Native agarose gel electrophoresis shows the successful loading of siBcl2 onto 6H×96BP-Rect as retarded mobility was observed after loading of siBcl2.

Figure 2.

Cellular internalization and growth inhibition of DNP in DMS53 cells. a) Flow cytometry analysis of DNP cellular uptake. Cells with no treatment (NT), Cy5-DNA as negative control, and eight different structures of DNP-Cy5. DNP and Cy5 concentrations were 16.7 nM and 250 nM, respectively. All DNPs exhibited better uptake than controls, $*P < 0.05$. b) Confocal microscopy imaging of cells treated with DNP-Cy5 for 8 h. Red= Cy5 fluorescence, blue= nuclei stained with DAPI. Scale bars=50 μm . c) Flow cytometry analysis of DNP uptake in cells treated with Poly-I, $*P < 0.05$. d) Western blotting of Bcl2 expression in cells treated with no treatment (NT); treatment 1, 6H $\times$ 96BP-Rect vehicle with concentration equivalent to treatment 6; treatment 2, siBcl2 with concentration at 2.0 μM ; treatments 3–6, 6H $\times$ 96BP-Rect-siBcl2 with siBcl2 concentration at 0.3, 0.6, 1.2, and 2.0 μM . The relative ratio of Bcl2 and Actin is shown below the gel, which is normalized based on NT. The corresponding 6H $\times$ 96BP-Rect concentration is 25-fold lower than the indicated siBcl2 concentration because one 6H $\times$ 96BP-Rect loads 25 siBcl2. e) Quantitative RT-PCR analysis of Bcl2 mRNA level. Treatments 5 and 6 have significantly better effects on silencing Bcl2 mRNA than control groups, $*P < 0.05$. f) Cell viability under different treatments. Scale bars= 200 μm . g) Quantitative analysis of cell viability. All data represent mean $\pm$ SD ($n=3$). Cell-growth inhibition in treatments 5 and 6 are statistically significant compared to other groups, $*P < 0.05$.

Figure 3.

Bcl2 knockdown-induced tumor-growth suppression in a xenograft tumor mice model by intravenous administration of 6H×96BP-RectsiBcl2. a) Tumor growth curve of xenograft tumor-bearing mice. Five groups were studied including PBS buffer, 6H×96BP-Rect alone (equivalent to 10 mgkg⁻¹), siBcl2 alone (10 mgkg⁻¹), 6H×96BP-Rect-siBcl2 (5 mgkg⁻¹) and 6H×96BP-Rect-siBcl2 (10 mgkg⁻¹). Error bars are standard errors of five mice in each group. Representative photos of harvested tumors on day 17 are shown on the right, presented in the same order as the growth curves. b) Immunohistochemical and toxicity analysis. On day 17, mice were sacrificed. Tumor and major organs were processed for immunohistochemical (Bcl2 protein, Ki67, TUNEL) and H&E stain, respectively. Representative images from each group are shown. c) Quantitative analysis of immunohistochemical studies of tumor tissues. The color of the bar matches the color of

labeling for each group as shown in (a, b). 6H×96BP-Rect-siBcl2 (10 mgkg⁻¹) performed statistically better than all other groups, **P*<0.05.

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