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Lipid-like Nanomaterials for simultaneous gene expression and silencing *in vivo*

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Abstract

New lipid-like nanomaterials were developed to simultaneously regulate expression of multiple genes. Self-assembled nanoparticles are capable of efficiently encapsulating pDNA and siRNA. These nanoparticles were shown to induce simultaneous gene expression and silencing both *in vitro* and *in vivo*.

Keywords

lipid-like nanomaterials; siRNA; plasmid DNA; gene silencing; gene expression

Aberrant gene expression underlies thousands of human diseases, including inherited genetic disorders.^[1, 2] Gene therapy offers the promise of treatment through delivery of an exogenous gene.^[3, 4] Due to concerns regarding the carcinogenesis, immunogenicity, and

toxicity of viral delivery systems, a number of non-viral systems have been developed for the delivery of plasmid DNA (pDNA) encoding a functional and therapeutic gene.^[3, 5, 6] Alternatively, the delivery of RNA interference elements offer the potential to down-regulate expression of specific target genes.^[7-9] pDNA and siRNA therapeutics have the potential for treating disease through the direct alteration of target biological pathways currently not addressed by other therapeutic options.^[3, 10] A number of clinical trials are in progress using pDNA or siRNA, and viral therapies have just started to become approved therapeutics, such as Glybera for the treatment of lipoprotein lipase deficiency.^[10-14]

In general, non-viral studies have focused on regulating therapeutic targets through either pDNA or siRNA alone. However, many genetic diseases are caused by mutations in more than one gene;^[2] and complex diseases such as cancer and type 2 diabetes can be caused by malfunction of multiple genes.^[15, 16] The simultaneous control of pathogenic genes in different biological pathways could result in efficacious management of these diseases.^[17] In order to regulate multiple signaling pathways, several groups pursued the co-delivery of pDNA and siRNA in human cells using polymer-based nanoparticles.^[18-22] However, to our knowledge, efficacious delivery of both pDNA and siRNA using synthetic vectors in animals has not yet been demonstrated.

Previously, a library of lipid-like materials (termed as lipidoids) was reported for siRNA delivery.^[23, 24] Lead materials have shown significant silencing effects *in vivo*.^[23-25] Recently, Xu *et al* developed related materials for intracellular delivery of pDNA.^[26] Chemical synthesis of these materials is based on efficient synthetic routes such as Michael additions and epoxide ring-opening reactions.^[23, 24] Formulation of lipidoids with additional delivery excipients can allow the encapsulation of nucleic acid payloads. We sought to develop materials capable of co-delivery of siRNA and pDNA. To this end, we synthesized novel 1,3,5-triazinane-2,4,6-trione derivatives (TNT or TNT2-TNT10, Figure 1) composed of a six-membered ring and multiple lipid tails. Herein, we report the development of these lipidoid nanomaterials for simultaneous control of multiple genes both *in vitro* and *in vivo* (Figure 1b).

We synthesized 9 TNT lipid derivatives, TNT2-TNT10 through a microwave irradiation reaction in ethanol using TNT1 and various amines as the starting materials (Figure 1a). The structures of TNT2-TNT10 were confirmed through high resolution LC-MS and NMR spectroscopy (Supporting information). First, we evaluated the delivery efficiency of green fluorescent protein (GFP) pDNA and firefly luciferase siRNA separately in HeLa cells. We formulated TNT2-TNT10 with PEG-lipid, DSPC, cholesterol, and green fluorescent protein (GFP) pDNA or firefly luciferase siRNA as reported previously.^[27] TNT6 precipitated under the conditions used for nanomaterial formulation and thus was not tested in the assay. The transfection efficiency of GFP-pDNA was quantified through the proportion of cells expressing GFP as determined by fluorescence-activated cell sorting (FACS) analysis.^[28] As shown in Figure 2a, over 80% of cells expressed GFP after transfection with TNT4-pDNA (TNT4-D, dose= 50 ng pDNA/well). Other materials transfected fewer than 10% of cells. Meanwhile, we investigated the silencing activity of TNT2-TNT10 formulated with firefly luciferase siRNA in HeLa cells stably expressing both firefly and *Renilla* luciferase. In accordance with previous reports, silencing in treated groups was determined by

measuring the ratio of firefly to *Renilla* luciferase expression and normalizing to that of PBS-treated cells.^[23] Interestingly, TNT4 showed over 80% silencing of firefly luciferase expression at a dose of 50 ng siRNA/well, while other materials displayed moderate silencing activity (Figure 2b). The structural features of TNT4 are consistent with those of previously identified lead materials: (i) tail length in the range of 8-12 carbons; (ii) more than two alkyl chains.^[23] As such, TNT4 was selected as a lead material for further study.

To characterize the TNT4 nanoparticles formulated with various nucleic acid payloads, we measured their z-average diameter by dynamic light scattering (Figure 2c). The rank order of size for TNT4-siRNA (TNT4-R), TNT4-pDNA (TNT4-D), and TNT4-siRNA/pDNA (TNT4-RD) was TNT4-D (185.6±8.7, PDI= 0.134±0.017) > TNT4-RD (150.5±5.9, PDI= 0.129±0.004) > TNT4-R (88.5±2.2 nm, PDI= 0.058±0.005). We also evaluated the entrapment efficiency of TNT4-R, TNT4-D, and TNT4-RD using gel electrophoresis and quantified the entrapment percentage using siRNA and pDNA standard curves (Figure S1). The results showed that the entrapment for siRNA was over 99% and the entrapment for pDNA was around 87%. The relative size difference of these molecules may explain the higher siRNA entrapment efficiency. When we loaded 50% pDNA/50% siRNA (weight/weight) in the same mass of nanoparticles, the overall nucleic acid entrapment efficiency was over 99%.

To visualize the cellular uptake of siRNA and pDNA, TNT4 was formulated with Alexa-647 labelled siRNA and/or Cy3-labelled pDNA. The membranes were stained with WGA-AF488 and the nuclei were stained with DAPI (Figure 3a). The confocal images showed that whereas TNT4-R and TNT4-D mediated intracellular delivery of siRNA and pDNA, respectively, TNT4-RD yielded delivery of both siRNA and pDNA within cells (Figure 3a). To evaluate the functional potency of TNT4-RD, we further performed a dose-response study for TNT4 using GFP pDNA and luciferase siRNA. TNT4-D and TNT4-RD showed dose dependent transfection of GFP (ED₅₀~ 10 ng/well, Figure 3b). A similar phenomenon was also observed for luciferase silencing with TNT4-R and TNT4-RD (ED₅₀~ 6.25 ng/well, Figure 3c). As such, TNT4-RD delivered both pDNA and siRNA to the same cells and regulated two genes simultaneously *in vitro*, expressing GFP protein and silencing luciferase expression (Figure 1b).

To further examine the potential of simultaneous delivery of pDNA and siRNA *in vivo* using TNT4 based lipidoid materials, we utilized firefly luciferase pDNA and Tie2 siRNA (Tie2 is a class of receptor tyrosine kinases expressed in various organs^[29]). First, we formulated TNT4 with luciferase pDNA to identify the target organs for transgene expression in mice. TNT4-D was injected intravenously via the tail vein. 8h after injection of TNT4-D, we injected D-luciferin and dissected five representative organs (liver, lungs, spleen, heart, and kidney). As shown in Figure 4a, we observed significant luciferase expression in the lungs and spleen as measured using an IVIS imaging system. Luciferase expression was minimal in the liver. No significant signal was observed in the heart and kidney (Figure 4a). TNT4-D and TNT4-RD displayed similar luciferase expression patterns in these organs at a dose of 1.25 mg/kg (pDNA). Subsequently, we explored the silencing activity of TNT4-R and TNT4-RD formulated with Tie2-siRNA and Tie2-siRNA/luciferase pDNA, respectively (Figure 4b). Both TNT4-R and TNT4-RD significantly reduced the expression of Tie2 in the

lung, liver, and spleen compared with the PBS control groups at a dose of 1.25 mg/kg (siRNA). Taken together, these results demonstrate the feasibility of using these novel nanomaterials to achieve co-delivery of pDNA and siRNA and the simultaneous control of two different genes in mice.

Malfunctions of multiple genes are the origins of many types of diseases such as cancer and diabetes.^[3, 30] Appropriate regulation of multiple disease-causing genes may afford optimal therapeutic effects.^[17, 31] The co-administration of pDNA and siRNA therapeutics represents an attractive strategy to specifically counteract the effects of such mutations, but this approach is currently hindered by inefficient methods to achieve intracellular delivery. The TNT lipidoid nanoparticles described in this paper were shown to mediate co-delivery of pDNA and siRNA both *in vitro* and *in vivo*, simplifying the formulation method, transporting the nucleic acids to the same target location, and maximizing the desired therapeutic functions.

In summary, to simultaneously regulate multiple genes, we developed new lipidoid nanomaterials to co-deliver pDNA and siRNA. Among the 9 TNT derivatives synthesized here, TNT4 showed dose-dependent delivery of both pDNA and siRNA in HeLa cells. Moreover, this lead material efficiently entrapped both pDNA and siRNA (>99%) and achieved gene expression and silencing in the same cell type. Importantly, when loaded with luciferase pDNA and Tie2 siRNA, TNT4 demonstrated the feasibility of this concept in mice as well. To our knowledge, these nanomaterials represent the first *in vivo* delivery system to simultaneously express and silence multiple genes.

Experimental methods

Materials

1,3,5-triazinane-2,4,6-trione was provided by Nissan Chemical America Corporation. Amines were purchased from Sigma-Aldrich (St.Louis, MO) and TCI America (Portland, OR). Alexa Fluor® 488 Wheat Germ Agglutinin conjugates, DAPI, and Prolong gold anti-fade mounting medium were all purchased from Life Technologies (Grand Island, NY).

Lipidoid synthesis and formulation—A mixture of 1,3,5-triazinane-2,4,6-trione (TNT1) and amines (a ratio of 1.5:1 epoxides/ amine) in EtOH was irradiated in the microwave oven at 150 °C for 5 h. The reaction mixture was purified by flash column chromatography. TNT2-TNT10 were formulated with cholesterol, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), mPEG2000-lipid (weight ratio of 68:18:9:5) and siRNA/pDNA (TNT:siRNA/pDNA = 5:1) via a microfluidic based mixing device.^[27] Formulations were then dialyzed against PBS in 3,500 MWCO dialysis cassettes overnight.

In vitro transfection of pDNA and siRNA—HeLa cells, stably expressing firefly luciferase and *Renilla* luciferase, were seeded (14,000 cells/well) into each well of an opaque white 96-well plate (Corning-Costar) and allowed to attach overnight in growth medium. Growth medium was composed of 90% phenol red-free DMEM and 10% FBS (Invitrogen). TNT lipidoid nanoparticles were formulated with TNT2-TNT10 and luciferase siRNA and/ or GFP pDNA. Cells were transfected with pDNA and/ or siRNA by addition of

formulated particles to growth medium. Transfections were performed in quadruplicate. Cells were allowed to grow at 37°C and 5% CO₂ (1 d for siRNA and 2 d for pDNA). Cells were then analyzed for GFP and luciferase expression. Firefly and *Renilla* luciferase expression was analyzed using Dual-Glo assay kits (Promega). Luminescence was measured using a Victor3 luminometer (Perkin Elmer). GFP signal was measured by FACS analysis.

FACS analysis—After aspirating conditioned medium and washing cells with PBS, cells were detached using 25 µl per well of 0.25% trypsin-EDTA (Invitrogen). Following a 5 min incubation at 37°C, ice-cold FACS running buffer comprising 2% v/v FBS in PBS (50 µl) was added to the cells, which were mixed thoroughly and then transferred to a 96-well round-bottom plate. The cells were then immediately subjected to FACS analysis using a BD LSR II (Becton Dickinson, San Jose, CA), and at least 10,000 cells per well were analyzed. Gating and GFP expression analysis were performed using FlowJo v8.8 software (TreeStar, Ashland, OR). 2D gating was used to separate increased auto-fluorescence signals from increased GFP signals to more accurately count positively expressing cells.

In vitro confocal imaging—50,000 HeLa cells were plated on each of 24 mm glass cover slips and allowed to attach for 24 h. Cells were transfected for 3 h and then washed with PBS and fixed in 4% formaldehyde (Polysciences Inc., Warrington, PA) for 30 min. Cells were then washed 3 times with PBS, and stained with DAPI (3 µM), and membrane stain WGA-AF488 (5 µg/ml). Cover slips were then mounted onto microscope slides using Prolong Gold antifade mounting medium. Prepared slides were then imaged using an LSM 700 point scanning confocal microscope (Carl Zeiss Microscopy, Jena Germany) equipped with an 40 × oil immersion objective. Images were acquired using optical Z sectioning and presented as maximum intensity projections. Obtained images were adjusted linearly for presentation using Photoshop (Adobe Inc. Seattle, WA).

Gel retardation assay—TNT4-R, TNT4-D, and TNT4-RD were diluted to a concentration of approximately 62.5 ng nucleic acids/well before loading on the gel. DNA and siRNA standards (250, 125, 62.5, 31.2, 15.6 ng) were run to comprise a standard curve. 10 µl of each sample, in either PBS or PBS with 2% v/v triton, was loaded into each lane of a pre-cast 0.8% agarose E-gel (Invitrogen) stained with ethidium bromide. The gel was run using the E-gel electrophoresis system (Invitrogen) for 15 mins at RT, and the bands were visualized with a Bio-Rad Gel Doc XR+ gel imager (Hercules, CA) and quantified using Bio-Rad Image Lab software.

Luciferase expression in mice—C57BL/6 mice (Charles River Labs) were used for luciferase pDNA expression experiments. Prior to injection, formulations were diluted in PBS at pDNA concentrations such that each mouse was administered a dose of 0.01 mL/g body-weight. Formulations were administered intravenously via tail vein injection. After 8 h, mice were injected with D-luciferin and euthanized 8 mins after injection by CO₂ asphyxiation, and lung, liver, heart, kidney, and spleen tissues were harvested and immediately imaged by IVIS Series Pre-clinical In Vivo Imaging Systems.

Tie2 silencing activity in mice—Endothelial silencing was examined 72 h after injection (n=6 for PBS group and n=3 for treated groups). Mice were euthanized by CO₂

asphyxiation, and lung, liver, and spleen tissues were harvested and immediately frozen in liquid nitrogen. Frozen tissues were pulverized, and tissue lysates were prepared in Tissue and Cell Lysis Buffer (Epicentre) supplemented with 0.5 mg/ml Proteinase K (Epicentre). Tie2 silencing was evaluated in lysates from all 3 tissues collected using a branched DNA assay (QuantiGene 2.0 Reagent System, Affymetrix). A standard curve for each tissue and target gene was constructed using samples from PBS-treated mice. The relative silencing in treated groups was determined by measuring each individual target gene/GAPDH level and normalizing to the corresponding ratio for PBS-treated mice controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Synthetic routes to TNT2-TNT10. The structures of TNT2-TNT10 were confirmed through high resolution LC-MS and ^1H NMR spectroscopy. MW, microwave. b) A hypothesized scheme for simultaneous regulation of multiple genes using pDNA and siRNA.

Figure 2.

In vitro screening of TNT lipidoid nanoparticles in HeLa cells. a) Transfection efficiency of GFP pDNA. Over 80% of cells expressed GFP after transfection with TNT4-pDNA (dose= 50 ng pDNA/well). b) Firefly luciferase silencing. TNT4 showed over 80% silencing of firefly luciferase expression at a dose of 50 ng siRNA/well. c) Particle sizes of TNT4-siRNA (TNT4-R), TNT4-pDNA (TNT4-D), and TNT4-siRNA/pDNA (TNT4-RD). PC: positive control.

Figure 3.

Simultaneous gene expression and silencing in HeLa cells. a) Cellular uptake of TNT4-siRNA (TNT4-R), TNT4-pDNA (TNT4-D), and TNT4-siRNA/pDNA (TNT4-RD). TNT4 was formulated with Alexa-647 labelled siRNA and/or Cy3-labelled pDNA. Cell membranes were stained with WGA-AF488 and the nuclei were stained with DAPI. The confocal images showed that whereas TNT4-R and TNT4-D mediated intracellular delivery of siRNA and pDNA, respectively, TNT4-RD yielded delivery of both siRNA and pDNA within cells. b) Transfection efficiency of GFP pDNA with TNT4-D and TNT4-RD. TNT4-D and TNT4-RD showed dose dependent transfection of GFP. c) Firefly luciferase silencing with TNT4-R and TNT4-RD. TNT4-R and TNT4-RD showed dose dependent silencing of luciferase.

Figure 4.

Simultaneous luciferase expression and Tie2 silencing *in vivo* following intravenous injection. a) Luciferase expression in the liver, lung, spleen, kidney, and heart with TNT4-D and TNT4-RD. We observed significant luciferase expression in the lungs and spleen as measured using an IVIS imaging system. Luciferase expression was minimal in the liver. No significant signal was observed in the heart and kidney. TNT4-D and TNT4-RD displayed similar luciferase expression patterns in these organs. b) Tie2 silencing in the lung, liver, and spleen with TNT4-R and TNT4-RD. Both TNT4-R and TNT4-RD significantly reduced the expression of Tie2 in the lung, liver, and spleen compared with the PBS control groups. Control, phosphate-buffered saline (PBS). Data points represent group mean $\pm$ s.d. (n=3, *, $P < 0.05$; t-test, double-tailed).