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## Lipid-Modified Aminoglycoside Derivatives for *in vivo* siRNA Delivery

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## Keywords

siRNA; drug delivery; nanoparticle; gene knockdown; aminoglycoside

Since its discovery, small interfering RNA (siRNA) has demonstrated significant potential as an important tool for molecular biology,<sup>[1-3]</sup> and for therapeutics.<sup>[4-6]</sup> Following its intracellular delivery, siRNA elicits a sequence-specific degradation of a messenger RNA (mRNA) via the endogenous RNA interference (RNAi) pathway, resulting in the suppression of protein expression.<sup>[1-3, 5]</sup> siRNA may address numerous diseases, including cancers<sup>[7-9]</sup> viral infections,<sup>[10]</sup> and genetic disorders.<sup>[11]</sup> However, clinical applications of siRNA require the development of effective systemic delivery vehicles. For this reason, a wide range of non-viral siRNA delivery agents have been developed, including cationic lipids,<sup>[12-15]</sup> polymers,<sup>[7, 16-18]</sup> dendrimers,<sup>[19]</sup> and gold nanoparticles.<sup>[20-22]</sup> One promising class of delivery vehicles for siRNA are the lipid-like, or “lipidoids” materials.<sup>[23-28]</sup> Identified through combinatorial synthesis and high throughput screening, a selection of lipidoids have demonstrated significant gene silencing efficiencies *in vivo*.<sup>[24]</sup> In order to achieve the broadest clinical potentials of siRNA therapeutics, further advances in siRNA delivery should address both safety and efficacy. Many delivery vehicles are not biodegradable, and may induce toxicity after repeated administration. Furthermore, certain lipid-based nanoparticles are sequestered by the liver,<sup>[16, 24, 28]</sup> leading to acute and chronic hepatotoxicity. Therefore, there is a need to develop new delivery materials with improved tolerability. Herein, we developed a class of cationic, lipid-modified aminoglycosides (cationic liposaccharides, or CLS) for *in vivo* siRNA delivery. Materials were identified that are capable of delivering siRNA and induce target knockdown both *in vitro* and *in vivo*. The most effective compound demonstrated 50% of silencing of Factor VII expression at an siRNA dose of 0.04 mg/kg (median effective dose, EC<sub>50</sub>), without exhibiting histopathological lesions up to 5 mg/kg in mice. Therefore, this study provided a new class of materials and the leading materials identified showed attractive gene silencing activities with a wide therapeutical window.

The use of naturally occurring molecules as a basis for developing biocompatible materials has been investigated for a range of applications.<sup>[29,30]</sup> Aminoglycosides, a class of antibiotics, are natural products with three to five amino substituted sugars (Figure 1).<sup>[31,32]</sup> Their antibacterial properties derive from selective binding to bacterial ribosomal RNA (rRNA).<sup>[33,34]</sup> Aminoglycosides are also able to bind to various eukaryotic RNA molecules, and have been studied to treat genetic disorders.<sup>[35,36]</sup> We hypothesized that the affinity of aminoglycosides with RNA molecules could be exploited for the delivery of siRNA. Moreover, the multiple amino groups in the aminoglycoside structure could act as attachment points for hydrophobic lipid tails. These lipid-modified aminoglycosides would electrostatically interact with siRNA and self-assemble into nanoparticles to facilitate its delivery. Lehn *et al.* reported a few modified Kanamycin derivatives for DNA and siRNA delivery *in vitro*.<sup>[37-39]</sup> We sought to further develop the potential of aminoglycosides, as a class of natural molecules with diverse structures and attractive pharmacokinetic properties, as delivery vectors for siRNA.

The materials were prepared by a one-step reaction of epoxides with commercially available aminoglycosides, which include: neomycin (NO), amikacin (AM), paromomycin (PR), ribostamycin (RB), kanamycin (KN), hygromycin (HG), geneticin (GN), gentamicin (GT), and 2-deoxystreptamine (ST). The epoxides were selected from linear terminal aliphatic epoxides with lipid chain lengths of 10-16 carbons. The complete list of compounds synthesized in this study is shown in Table S1. The structures of two representative aminoglycosides (AM and HG) are shown in Figure 1A, along with a schematic reaction of

2-deoxystreptamine (ST) with epoxides. Stoichiometric amount of epoxides were used to ensure fully substituted structures as the major products and the reactions were complete after heated in ethanol at 60°C for 72 hrs. The crude materials synthesized were directly used for nanoparticle formulation and initial *in vitro* screening. The top-performed compounds were then purified and characterized (Table S2 and Figure S1), and were studied with a rodent model for *in vivo* transfection.

Previous studies showed that the siRNA transfection efficiencies are often difficult to predict for *in vivo* transfection in animal models.<sup>[23-28]</sup> The difference could be explained by the various barriers that the nanoparticles have to overcome after systemic administration. In this study, the same formulation method was used to improve the predictability from cell transfection to animal models. The microfluidic formulation technique previously developed in our group was used to formulate lipid nanoparticles containing synthesized CLS materials, siRNA, cholesterol, DSPC, and PEG2000-DMG.<sup>[40]</sup> A hypothetical scheme of nanoparticle is shown in Figure 1B. Measurements with dynamic light scattering indicate that the hydrodynamic diameters of these nanoparticles in phosphate buffered saline (PBS) were around 60-200 nm (Table S3). They have slightly negative surface charges (-1 – -2 mV) at 25 °C as determined by the  $\zeta$ -potentials. The entrapment ratio of siRNA within the nanoparticles was determined to be around 20-70% as quantified with a fluorescence assay using Ribogreen (Molecular Probes).<sup>[24]</sup>

The synthesized **CLS** were evaluated for their ability to facilitate *in vitro* siRNA transfections in a HeLa cell line,<sup>[24]</sup> modified with firefly *Photinus pyralis* and *Renilla reniformis* luciferases. A decrease in firefly luciferase expression relative to *Renilla* luciferase expression was correlated to protein knockdown, and *Renilla* luciferase expression was used as the internal control for cell viability. Lipofectamine 2000 reagent (Invitrogen of Life Technologies, Inc.) was used as a positive control for cellular transfection. As shown in Figure 2, many formulated nanoparticles were able to mediate significant knockdown of *firefly* luciferase in HeLa cells. The length of the lipid tails was found to be important for efficacy, with C11 and C12 tails being the most efficacious. Tails longer than C13 are often inefficient, probably due to their lower solubility and the decreased fluidity in formulated lipid nanoparticles. The core scaffold aminoglycosides are essential for maximal efficacy, and some scaffolds are strongly favored. For instance, among the five leading materials with over 85% of silencing, three were derived from HG and two were derived from GT. On the other hand, materials synthesized from AP, PR, NO, and RB generally showed weak to no silencing efficacy. It is unclear why certain structures work better than others; however, the fact that some aminoglycosides are favored indicated that structural or conformational differences of these materials play an important role in nanoparticle cellular uptake and trafficking. QSAR (quantitative structure-activity relationship) has been performed to provide more structural insights (Figure S3). No correlation was found with simple physiological parameters of these materials, such as molecular weight, hydrophobicity, or hydrogen bonds, etc. However, a significant correlation was found with topological and geometric molecular descriptors, indicating that geometric and topological features of these materials are important for their transfection performance. The *Renilla* luciferase expression was unchanged by these nanoparticles derived from HG and AM, indicating that no cytotoxicity was induced by these materials. However, mild cytotoxicity was found for materials from ST and GT at the conditions evaluated (Figure S4). In addition, the antibacterial activity were significantly abolished for these aminoglycosides with lipid-modifications (Figure S5).<sup>[42]</sup>

The liver is involved in a number of serious diseases and is a major target for siRNA therapeutics under development. The potential of CLS for systemic siRNA delivery was evaluated in a mouse model. In order to facilitate the screening of new materials and to

compare with previously developed materials, delivery to hepatocytes was examined *in vivo*. Serum enzyme FVII is chosen as the target protein because it is secreted by the liver and it can be rapidly measured with a chromogenic method.<sup>[24]</sup> Although nanoparticles can concentrate in the liver, it should be noted that sequestration of materials in the liver is different from efficacious knockdown in hepatocytes. We have observed that many nanoparticles can accumulate in the liver without knockdown in hepatocytes, even at relatively high doses (data not shown). It has been observed that nanoparticles delivered systemically can tend to accumulate first in kupffer cells, which can sequester particles away from hepatocytes. Top performing compounds with >50% *in vitro* protein silencing were selected and injected intravenously (i.v.) via the tail vein at the siRNA dose of 1 mg/kg (Figure 3A). Among the 22 materials tested, 7 of them were found to demonstrate excellent silencing efficacy (> 90%). Consistent with the *in vitro* data, four of the top seven materials were derived from HG and have lipid tails lengths of 10 - 13 carbons. For *in vivo* transfection studies, dose response studies for the three top compounds were performed and ED<sub>50</sub> values were calculated and compared to a previously published lead formulation based on C12-200 to facilitate comparison between materials (Figure 3B). Significantly, HG-C11 was found to induce excellent gene silencing, with an EC<sub>50</sub> of 0.04 mg/kg. Even though the formulation is not fully optimized, its potency is comparable to C12-200 (EC<sub>50</sub> ~0.01 mg/kg)<sup>[24]</sup> and DLin-MC3-DMA (EC<sub>50</sub> ~0.005 mg/kg)<sup>[15]</sup>, two potent materials reported in the literature.

Particle characterization revealed the common features of top-performing nanoparticles. For example, they generally have smaller sizes (~80-90 nm), and higher entrapment ratios (~70%), comparing to materials with low silencing efficacies (Table S3). These results are consistent with previous findings, which suggested that 70-75% of entrapment is necessary for efficacious compounds.<sup>[24]</sup> The structure of the most efficacious lipid nanoparticles was also studied using transmission electron microscopy (Figure 1C and Figure S2). The diameter of the nanoparticles is ~70 nm, in general agreement with DLS results (Table S3). Interestingly, a core-shell morphology was clearly observed. The core's composition exhibits higher electron density than the uniform shell layer. The outer layer is measured ~25 nm, which agrees well with the length of the PEG2000 chain (23 nm).<sup>[41]</sup> Therefore, we hypothesize that the core contains more CLS and siRNA; while the outer shell layer is relatively enriched with PEG. Even though this morphology does not directly imply a mechanism on how the core materials govern the transfection efficacy, the CLS materials within the dynamic multi-component lipid nanoparticles presumably cause differences in properties that influence one or more steps in cellular entry and trafficking.

Encouraged by the efficient siRNA silencing with these materials, biodistribution of these nanoparticles after systemic administration was evaluated with whole-body and *ex-vivo* imaging studies. siRNA labeled with Cy5.5 on the 5' end of the sense strand was formulated with CLS and injected via the tail vein of mice at an siRNA dose of 1 mg/kg. A wide fluorescent region was seen at 4 hrs post-injection, indicating the presence of nanoparticles within the body. Although strong fluorescence intensity was seen in the liver 4 hrs after injection, it was reduced and localized in kidneys and lower abdomen after 24 hrs (Figure 4A). This was further confirmed with fluorescence imaging of harvested organs 24 hrs post-injection. Compared to unformulated Cy5.5 labeled siRNA, fluorescence intensity of nanoparticles normalized by wet-mass and PBS control exhibited a significant increase in the spleen, salivary glands and lungs, and a slight increase in the heart and liver, but not in the kidneys (Figure 4B and 4C). Fluorescence seen in intestine and feces suggest excretion of some siRNA through the bile duct drainage.<sup>[43]</sup>

Body weight is a sensitive index of tolerability and toxicity and was monitored for changes. The stable body-weight (within 5%) after 48 hrs post i.v. injection at 1 mg/kg indicated that

no toxicity was detected for these materials (Figure S6). Long-term toxicity was not clearly understood at present, but no adverse effects were found within one month after injection, when the FVII levels were fully recovered to normal. Further studies were conducted to evaluate the acute toxicity of two leading materials HG-C11 and HG-C13. Mice were injected via tail vein with formulations at siRNA doses of 2 mg/kg and 5 mg/kg, and were monitored every 4 hrs for changes in health conditions. No apparent symptoms of toxicity were observed. Biomedical markers for liver damage (ALT, AST, ALP, and bilirubin) measured from serum enzymes within 48 hrs post-injection were at normal levels compared to controls, suggesting no detectable acute toxicity at these doses (Table S4). Mice were sacrificed after 48 hrs ( $n = 3$ ) and major organs (liver, spleen, and kidneys) were immediately harvested, fixed and stained with hematoxylin and eosin (H&E). Compared to PBS controls, no lesions or pathological changes were observed in liver, spleen, and kidneys after administration of nanoparticles formulated with either materials at doses of 5 mg/kg and 2 mg/kg (Figure S7 and Figure S8). At a higher dose of 10 mg/kg with HG-C11, even though both liver and kidney are normal, some mild hemosiderin deposition was found in the spleen, indicating that acute toxicity became evident at this very high dose. No statistically significant body-weight change was found at 2 mg/kg within 48 hrs. At a single siRNA dose of 5 mg/kg, mild body-weight losses ( $\sim 8\%$ ) were observed within the first 24 hrs, however, the loss was recovered to  $\sim 3\%$  within 48 hrs post injection (Figure S9). At a higher dose of 10 mg/kg, significant ( $n = 3$ ,  $p < 0.05$ ) body-weight loss was observed within 48 hrs, however, no mice died during the course of this study. These data indicate that the materials studied have no significant acute toxicity, at doses up to 5 mg/kg.

In conclusion, here we report the synthesis, efficacy, and properties of aminoglycoside-based siRNA delivery vehicles. Lipid-modified aminoglycosides readily complexed with siRNA into well-defined nanoparticles and were capable of efficient gene knockdown both *in vitro* and *in vivo*, achieving an  $EC_{50}$  of 0.04 mg/kg *in vivo*. Detailed histological analysis revealed no lesions in major target organs at siRNA doses up to 10 mg/kg, and serum biomarkers for liver toxicity were normal at 5 mg/kg compared to controls. The combination of high efficacy and low acute toxicity found in this study suggests that aminoglycoside-based materials may have promise as *in vivo* siRNA delivery agents. In future studies, we will examine the utility of these siRNA delivering materials to knockdown disease relevant targets in the liver and tumor with rodent models.

## Experimental

### Materials

Neomycin trisulfate salts hydrate (NO), amikacin hydrate (AM), paromomycin sulfate salt (PR, 98%), ribostamycin sulfate salt (RB), kanamycin B sulfate salt (KN), hygromycin B (HG), geneticin (GN), gentamicin sulfate salt (GT), 2-deoxystreptamine dihydrobromide (ST, 98%), 1,2-Epoxyoctane (C10, 96%), 1,2-epoxydodecane (C12, 95%), 1,2-epoxydodecane (C14, 95%), and 1,2-epoxytetradecane (C16, 85%) were purchased from Sigma-Aldrich Co. LLC (USA) and were used as received. 1,2-Epoxyundecane (C11) and 1,2-epoxytridecane (C13) were synthesized from the epoxidation of 1-undecene (97%) and 1-tridecene (96%) with *m*-chloroperoxybenzoic acid (mCPBA).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



## Acknowledgments

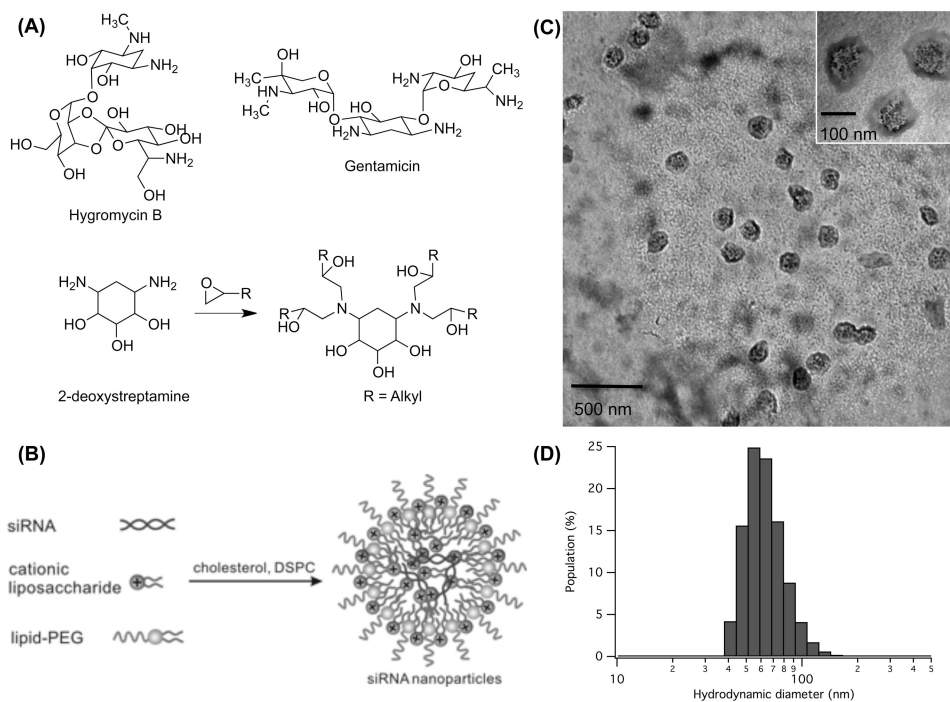
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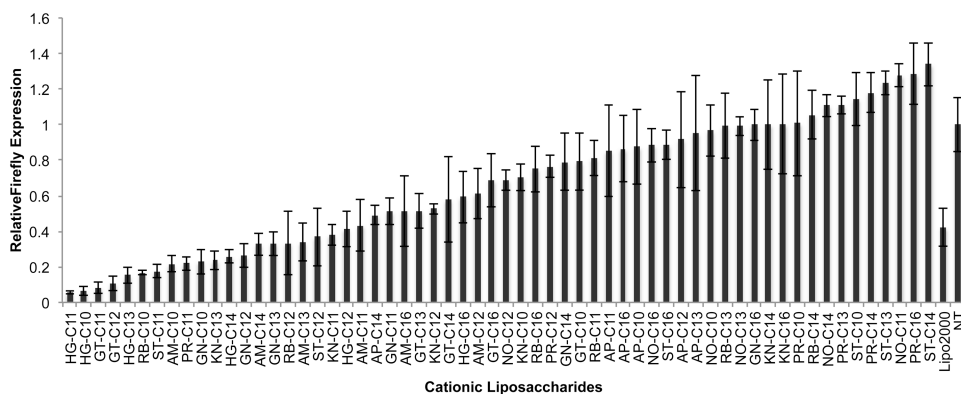
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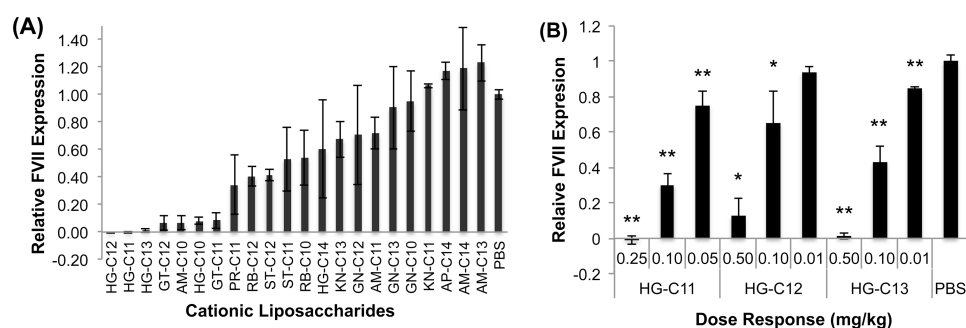
**Figure 1.**

(A) Representative structures of aminoglycosides employed in this study. A schematic reaction between aminoglycosides and epoxides is shown with 2-deoxystreptamine. (B) A scheme of nanoparticle formulation. (C) Transmission Electron Microscopy (TEM) images obtained for formulated nanoparticles to show the core-shell morphology. (D) Particle size distribution as measured by dynamic light scattering (DLS).

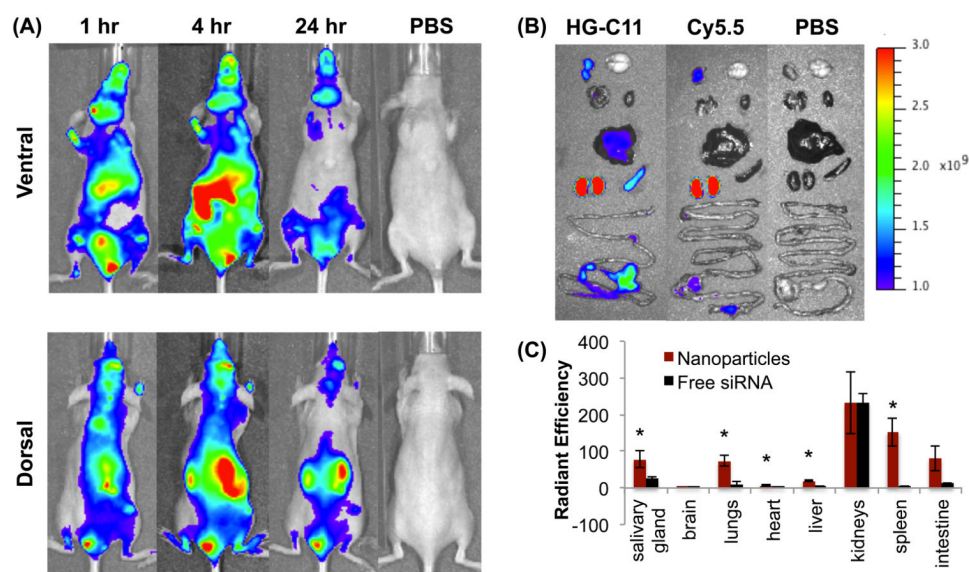


**Figure 2.**

*In vitro* knockdown of *luciferase* in HeLa cells with formulated nanoparticles. HeLa cells stably expressing both firefly and Renilla luciferase were treated with firefly targeting siRNA formulated with a microfluidic device. The relative average percent reduction of firefly versus Renilla after 24 hrs was shown as an index of transfection efficiency (s.d.,  $n = 4$ ). The concentration of siRNA was 20 nM. Lipo2000 (Lipofectamine 2000 reagent) and nontreated (NT) were used as control experiments.

**Figure 3.**

*In vivo* silencing of liver Factor VII with formulated nanoparticles. Female C57BL/6 mice were intravenously injected with formulations. The percent reduction on Factor VII levels in serum was quantified 48 hrs post injection. (A) Formulations of FVII siRNA at a dose of 1 mg/kg. (B) Dose response studies with HG-C11, HG-C12, HG-C13 (s.d.,  $n = 3$ , \*  $p < 0.05$ , \*\*  $p < 0.001$ ; t-test, single-tailed).



**Figure 4.** Biodistribution of nanoparticles formulated with fluorescent labeled (Cy5.5) siRNA and HG-C11. Whole animal imaging taken 1 hr, 4 hrs, and 24 hrs post intravenous injection. (A) Mice in ventral (top) and dorsal (bottom) postures. (B) Ex-vivo imaging of organs harvested 24 hr post injection. Organs are salivary glands (SG), brains (BR), lungs (LU), heart (HT), livers (LV), kidneys (KN), spleen (SP), and intestines (IN). (C) Radiant efficiency normalized by organ wet mass (s.d.,  $n = 3$ , \*  $p < 0.05$ , t-test, single-tailed).