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Cholesterol conjugated spermine as a delivery modality of antisense oligonucleotide

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The major issue in the development of nucleic acid based therapeutics is the inefficient delivery of these agents into cells. We prepared cholesterol conjugated spermine and evaluated its usefulness as a delivery modality for antisense oligonucleotides in HeLa-Luc cells. A 2'-O-methyl antisense oligonucleotide sequence, designed to correct splicing at an aberrant intron inserted into a normal luciferase reporter gene, was used for complex formation with cholesterol conjugated spermine. Effective delivery of this antisense agent into nucleus would results in the expression of a luciferasereporter gene product. The cholesterol-spermine formed stable complexes with the antisense oligonucleotide and showed modest delivery activity. Furthermore, this delivery activity was maintained even in the presence of serum proteins, mimicking in vivo conditions. Cholesterol-spermine thus has potential as a delivery system for antisense oligonucleotides into cells.

Key words: cholesterol conjugated spermine, antisense oligonucleotide, delivery

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Introduction

Oral cavity is an attractive site for the local delivery of nucleic acid drug and may provide the excellent opportunity for the therapeutic application of antisense agent. One of the major issues in the development of nucleic acid drugs as therapeutics is the inefficient delivery of oligonucleotides, siRNA or plasmid DNA into cells or tissues. Over the past decades a variety of non-viral agents were investigated to enhance the delivery efficiency of nucleic acid agents into cells or target tissues. This includes cationic liposomes [1-3], cationic lipid complexes [4,5], polypeptides [6,7], dendrimers [8] and surfactants [9] and other cationic polymers [10-12]. Unfortunately, however, most of these agents were not sufficiently enough in delivering oligonucleotides to cells or tissues, especially *in vivo* condition [13]. The common approach among these agents involves complex formation with oligonucleotide to enhance intracellular delivery of antisense. For example, Lipofectin, one of the most wildly used delivery agents, is known to form cationic lipid complex with oligonucleotides, internalize by endocytosis and release from the endosome [14-17]. However, the large size and high surface charge density of the lipid complex interrupted its cellular uptake and release from endosome, preventing efficient transfer of oligonucleotides into cytoplasm or nucleus of cells. In addition, the large size of complex made difficult its wide penetration into tissue compartment of target organ [18]. From this point, we were interested in the

preparation of delivery agent with small size, which might be of benefit to the access of oligonucleotides to disease sites such as solid tumor or inflammation [19-22].

Spermine is a polyamine with positively charged at physiological pH, which is found in all eukaryotic cells and a wide variety of tissues. It has been known that spermine interacts with DNA, stabilizing helical structure via nonspecific electrostatic interaction and hydrogen bonds [23-25]. Conjugating cholesterol to spermine may increase hydrophobicity of the molecule and contribute to cellular uptake of spermine by shielding non-specific interaction with cell membrane proteins. Also cholesterol conjugated spermine may have less interaction with serum proteins in blood, thus increasing circulation time *in vivo*. Here, we prepared cholesterol conjugated spermine and evaluated its usefulness as a delivery modality of antisense oligonucleotide in HeLa-Luc cells. Cholesterol conjugated spermine formed stable complexes with antisense oligonucleotide [26] without inducing cytotoxicity and showed its function as a delivery modality for delivery of antisense oligonucleotide into HeLa-Luc cells.

Materials and methods

Materials

Spermines were purchased from Aldrich (Milwaukee, WI, USA). A phosphorothioate 2'-O-methyl oligonucleotide (5'-CCUCUUACCUCAGUUACA-3') was purchased from the Midland Certified Reagent Company (Midland, TX). DMEM and Opti-MEM were from Life Technologies (Gaithersburg, MD). All other chemicals were from Sigma Chemical (St. Louis, MO, USA). HeLa cells stably transfected with plasmid pLUC/705 were a generous gift of Dr RL. Juliano (University of North Carolina, Chapel Hill, NC).

Cholesterol conjugation to spermine

Cholesterol conjugated spermine (e.g., 5:1 mole ratio of cholesterol to spermine) was synthesized by following procedure: cholesteryl chloroformate in methylene chloride was added dropwise to a solution of spermine in methylene chloride and N,N-diisopropyl ethylamine. The reaction was stirred at room temperature for 3 hr. The unreacted free cholesteryl chloroformate was removed from the reaction product by using ethyl ether. The remaining crude product, cholesterol conjugated spermine, was purified by chromatography on silica gel as an eluent. column using methanol : methylene chloride (1:1, v/v) as eluent. The final products were obtained by evaporation of the solvent and stored at -20 °C.

Cytotoxicity assay

Cells in a 48-well plate were incubated with cholesterol-spermine, in serum free DMEM medium, or in DMEM medium containing 30 % serum, for 24 hr, rinsed twice with phosphate-buffered saline (PBS), incubated for a further 24 hr. The surviving fraction was determined by the MTT dye assay. MTT dye solution (0.5 μg/ml) was added to each well and incubation continued for another 30 min. Absorbance at 540 nm was quantified with an automated microplate reader (MULTISCAN® EX, THERMO).

Antisense splicing correction assay

HeLa cells transfected stably with a reporter gene construct were plated in 6-well plates at a density of 4 x 10^5 cells per well in 3 ml of 10 % FBS/DMEM and antibiotics. Cells were maintained for 24 hr at 37 °C in a humidified incubator (5 % CO_2 / 95 % air). A 100 μl of aliquot of oligonucleotide at a given concentration in Opti-MEM was mixed with 100 μl of Opti-MEM containing various concentrations of cholesterol-spermine. The preparation was left undisturbed at room temperature for 10 min, followed by dilution to 1 ml with Opti-MEM before being layered on the cells. The cells were incubated for 6 hr and subsequently the medium was replaced with 10 % FBS/DMEM. After further 18 hr, the cells were rinsed with PBS and lysed in 100 μl of lysis buffer (200 mm Tris-HCl, pH 7.8, 2 mM EDTA, 0.05 % Triton X-100) on ice for 15 min. Following centrifugation (1300 rpm, 2 min), 5 μl of supernatant cell extract was mixed with 100 μl of luciferase substrate (1 mM D-luciferin). Luciferase activity was determined by using luminoplate and expressed as a relative light units (RLU) per well [27]. The light emission was normalized to the protein concentration of each sample, which was determined by bicinchonic acid assay [28].

Results

Cytotoxicity evaluation

Cholesterol conjugated spermine (e.g., 5:1 mole ratio of

cholesterol to spermine) was synthesized as shown in the reaction scheme of Figure 1. The toxic effects of cholesterol-spermine or cholesterol-spermine/antisense complexes in cell were assessed by a MTT assay, after treating cells in serum-free or 30 % serum containing DMEM medium (Fig. 2). The toxicity of cholesterol-spermine or cholesterol-spermine/antisense oligonucleotide complexes was markedly reduced when cells were treated in the presence of serum while cholesterol-spermine/antisense oligonucleotide and cholesterol - spermine alone showed toxicity in serum-free condition.

Cellular delivery of antisense oligonucleotide

The ability of cholesterol-spermine to deliver antisense oligonucleotide into cell was examined by a luciferase reporter gene assay (splicing correction assay) which utilizes antisense oligonucleotide to permit aberrant splicing into correct splicing in the nuclei of viable cells. The detection of luciferase activity, after splicing correction by the binding of antisense to an overlapping site of a mutated intron, provides direct evidence that pharmacologically active oligonucleotide not only entered into cells, but also reached to nucleus of cells, since splicing phenomena occur only in the nucleus of cell. The measured luciferase activity by the treatment with cholesterol-spermine/oligonucleotide complexes in HeLa-Luc cells showed moderate delivery efficiency in serum-free condition. The delivery efficiency was improved as the cholesterol-spermine concentration increased with maximum luciferase activity at the concentration of 10 μg/ml (Fig. 3). In the absence of serum, oligonucleotide complexes with cholesterol-

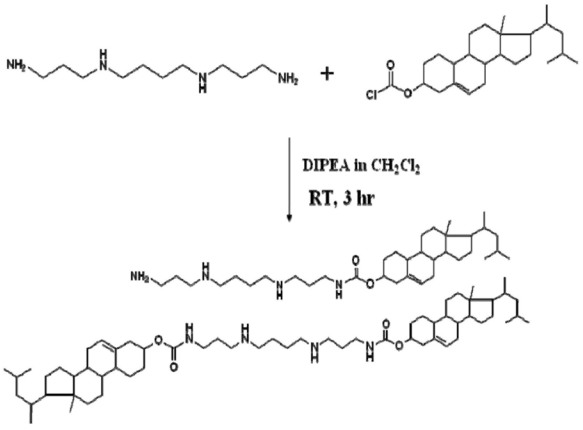

Fig 1. Synthetic scheme of cholesterol conjugated spermine. Cholesteryl chloroformate in methylene chloride was added to a solution of spermine in methylene chloride using N,N-diisopropyl ethylamine as a catalyst.

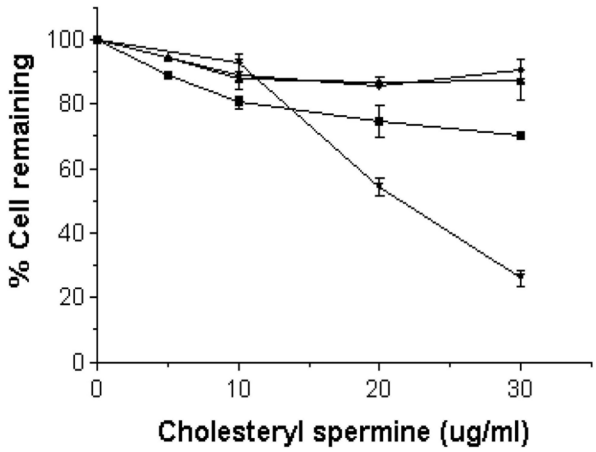

Fig 2. Acute toxicity assay of cholesterol conjugated spermine. Cells were treated with cholesterol conjugated spermine alone or cholesterol conjugated spermine/oligonucleotide complexes in the presence / absence of serum. Cell viability was expressed as percent cell remaining compared to untreated cells. Filled square, cholesterol conjugated spermine/oligonucleotide complexes in serum free medium; filled triangle, cholesterol conjugated spermine/oligonucleotide complexes in 30 % serum containing medium; filled reversed triangle, cholesterol conjugated spermine alone in serum free medium; filled diamond, cholesterol conjugated spermine alone in 30 % serum containing medium. Vertical bars represent means and standard errors (n>3).

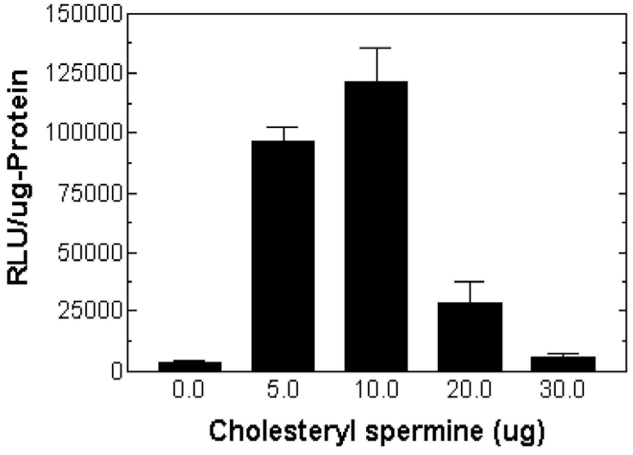

Fig 3. Activation of a luciferase reporter by cholesterol conjugated spermine /antisense oligonucleotide complexes. HeLa -Luc cells were treated with various doses of cholesterol conjugated spermine at a fixed concentration (0.15 μM) of 2'-O-methyl phosphorothioate oligonucleotide. RLU: relative luminescence unit. Vertical bars represent means and standard errors (n=3).

spermine showed much higher delivery efficiency than spermines and comparable with the commercial cytofectins, Lipofectin and Lipofectamine (Fig. 4).

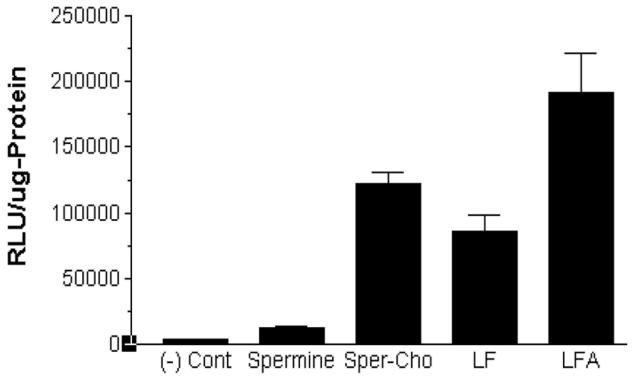

Fig 4. Comparison of luciferase activity of cholesterol conjugated spermine with commercial cytofectins at optimized concentrations of delivery agents and oligonucleotides. 10 μg of cholesterol conjugated spermine were complexed with 2'-O-methyl phosphorothioate oligonucleotide (0.15 μM) for the cellular delivery. Treating cells with commercial cytofectins were followed manufacturer's instructions and used as a positive control. Negative control samples underwent the same procedure, but in the absence of delivery agent. Vertical bars represent means and standard errors (n=3).

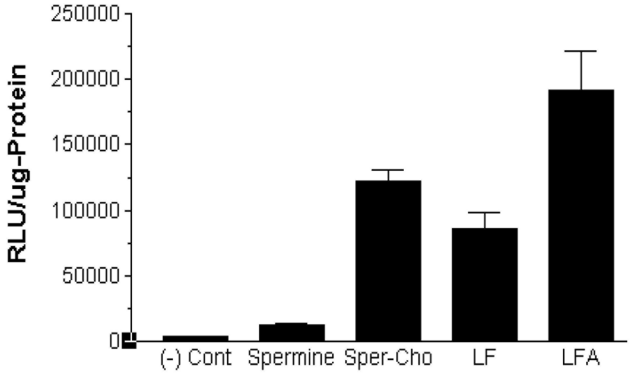

Fig 5. Serum dependence of antisense oligonucleotide delivery by cholesterol conjugated spermines. The concentration of oligonucleotide was 0.15 μM. Vertical bars represent means and standard errors (n=3). LFA: Lipofectamine, LF: Lipofectin.

Serum dependence on antisense delivery by cholesterol-spermines

The serum dependence of oligonucleotide delivery by cholesterol-spermine was compared at an optimized condition of oligonucleotide/cholesterol-spermine complexes (0.15 μM for oligonucleotide, 10 μg for cholesterol-spermine). Cholesterol-spermine as well as commercial cytofectins (Lipofectin and Lipofectamine) showed excellent activity in the absence of serum, but lost the delivery activity dramatically in the presence of serum. Both Lipofectin and Lipofectamine displayed almost no activity in the presence of 10 % or 30 % serum. Compared to these commercial cytofectins, cholesterol-spermine maintained relatively higher degree of efficiency even in the presence of 30 % of serum (Fig. 5).

Discussion

This study was initiated to evaluate the potential use of cholesterol conjugated spermines as a delivery modality for antisense. The delivery efficiency of cholesterol-spermine was evaluated *in vitro* HeLa cell culture by using an excellent assay system, splicing correction assay, for antisense activity and its delivery. In this assay only active oligonucleotide reaching nuclei of viable cells induces correct splicing, thereby resulting in positive read-out of luciferase activity. High luciferase activity indicates a direct evidence of nuclear delivery of antisense oligomer in the cell. The complex formed by cholesterol-spermine with antisense oligonucleotide showed no considerable cytotoxicity to HeLa cells and was pharmacologically active in the reporter gene assay. In serum-free condition, cholesterol-spermine showed substantially high degree of efficiency for the delivery of antisense oligonucleotide, and the efficiency was reduced to moderate level under the standard cell culture condition containing 10 % serum. The degree of efficiency in the cellular delivery of antisense oligomer by cholesterol-spermine was comparable to commercial cytofectins in serum-free medium. In addition, cholesterol-spermine maintained its delivery activity even in the presence of 30 % serum, which is distinguishable from other commercial cytofectins and makes cholesterol-spermine as a promising *in vivo* delivery agent [28,29]. As indicated in Figure 1, the reaction product of cholesterol conjugate to spermine may be the mixture of two types of cholesterol adducts. Unfortunately, in this study, we were not able to identify the presence of individual products due to the experimental difficulty.

The main advantage of cholesterol-spermine might be the size of the complex since small complexes penetrate to tumors or inflammatory disease sites more efficiently and increase enhanced permeability and retention (EPR) effects *in vivo*. Further studies are necessary to understand the mechanism of action of cholesterol-spermine on the cellular uptake and delivery of antisense agents as a pharmacologically active complex.

In conclusion, our study showed that the conjugate of

cholesterol-spermine is a promising delivery agent for antisense oligonucleotide *in vitro*. In addition, the observed delivery efficiency, especially in the presence of serum in media, makes this agent attractive for the therapeutic application.

Acknowledgements

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