In vitro Characterization of a Lipidic Delivery System for Anti-angiogenic siRNA

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ABSTRACT SUMMARY

In this study, Anginex lipoplexes (AxL) were developed for intracellular delivery of non-coding RNA (ncRNA) to endothelial cells of tumor vasculature. Physicochemical characteristics, stability, cell binding and cell uptake of this system were investigated in HUVEC. Upon transfection of HUVEC with AxL containing siVEGFR2, considerable gene silencing was achieved.

INTRODUCTION

Since the discovery of RNA interference (RNAi) as a gene regulation process, successful delivery of ncRNAs appears as the major challenge for therapeutic activity. ncRNAs are mediators of the RNAi phenomenon and are involved in various processes in cells among which cell proliferation and cell migration play a central role in angiogenesis. Prevention of angiogenesis inhibits tumor growth as it restricts the access of tumor cells to oxygen and nutrients. Endothelial cell (EC) activation is the first step in angiogenesis and is influenced by various effectors. Vascular endothelial growth factor (VEGF) is a major trigger in EC activation. Therefore, siRNA silencing VEGF receptor 2 (VEGFR2) gene of EC seems as an effective tool to inhibit tumor progression. Although different polymeric and lipidic delivery systems have already been investigated, efficient delivery to EC has remained a challenge. Here, we developed lipoplexes targeted to tumor EC for delivering siRNA against VEGFR2. Anginex, a 33-mer peptide, was used to target galectin-1 receptors the expression of which is upregulated upon EC activation.

EXPERIMENTAL METHODS

Lipoplexes were formed by complexation of siRNA with protamine in 20mM Hepes Buffer containing 5% glucose at pH:7.4 and consequent hydration of a lipid film (solvent evaporation-lipid film hydration method). The lipid film was composed of DOPE:CHEMS:PEG2000-DSPE:Maleimide-PEG2000-DSPE (6:4:0.3:0.3). The prepared lipoplexes with a total lipid concentration of 10 mM and RNA concentration of 1 uM were extruded repeatedly through polycarbonate membranes with a final pore size of 100 nm by a high-pressure extruder.

A solution of 0.5 M Hepes, 0.5 M Hydroxylamine, 0.25 mM EDTA at pH:7 was added to N-succinimidyl S-acetyltioacetate (SATA) modified Anginex at volume ratio of 1/10 and incubated for 45 minutes at room temperature to deprotect the SATA groups. Unprotected peptides were then added to lipoplexes at a concentration of 10 ug peptide per 1 umol phospholipid and left at 4°C overnight. Uncoupled peptides were separated by ultracentrifuge for 60 minutes at 200,000XG (Brandwijk et al., 2007).

Hydrodynamic diameter of lipoplexes was measured by dynamic light scattering (DLS) on an ALV CGS-3 system (Malvern Instruments Ltd., Worcestershire, UK) and Nanosight LM-10SH (Nanosight Ltd. Wiltshire, UK). ζ- potential was determined by Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, UK).

Complex stability was evaluated by gel retardation and encapsulation efficiency was determined by Ribogreen assay after treatment of samples with Triton X-100 5% and dextran sodium sulfate (DSS)1%.

Cytotoxicity was studied by Lactate Dehydrogenase Activity Assay (LDH) on HUVEC. Cell binding and uptake were investigated by flow cytometry after treatment of HUVEC with AxL containing Alexa488-labeled siRNA. Gene knock-down was determined by measuring expression of VEGFR2 in HUVEC using Western blot.

RESULTS AND DISCUSSION

Monodisperse AxL with an average size of 126 nm and a net negative charge were prepared and were shown to protect the encapsulated siRNA when treated with Triton X-100 5% and dextran sodium sulfate (DSS) 1% as demonstrated in Fig. 1.

Figure 1. Gel electrophoresis of AxL before and after treatment with Triton X-100 and DSS to evaluate complexation stability (1: Marker, 2: Naked siVEGFR2, 3: Empty AxL, 4: siVEGFR2-AxL, 5: siVEGFR2-AxL+Triton X-100 5%+DSS1%)
AxL caused lower toxicity in HUVEC’s compared to cationic DOTAP liposomes and Lipofectamine2000 as measured by cytosolic LDH release (Fig. 2).

Uptake (i.e. binding and internalization) of AxL to HUVEC was noticeably higher in comparison to formulations prepared with a non-binding control peptide (RAD). Binding was approximately half of that of Lipofectamine2000 which could be related to the cationic nature of Lipofectamine2000 resulting in very high binding to cells (Fig. 3). Cell uptake, however, was comparable between AxL and Lipofectamine2000 and improved over time (Fig. 4). The higher fluorescence intensity observed in the uptake study compared to the binding experiment could be attributed to the dequenching of complexed Alexa488-labeled siVEGFR2.

Biological activity of delivered siVEGFR2 was evaluated 48hr after treatment with AxL. Expression of VEGFR2 was reduced by 61% when HUVEC were treated with AxL (Fig. 5).

CONCLUSION

In this study, Anginex lipoplexes encapsulating siRNA with an average size of 126 nm and a net negative charge were prepared. These complexes were stable when incubated with lipid bilayer-disruptive and anionic agents (Triton X-100 and dextran sodium sulfate) and showed low toxicity. Binding of AxL to HUVEC was lower than Lipofectamine2000. Efficient Anginex-specific cellular uptake occurred over time and transfections resulted in up to 20% gene knock-down compared to empty AxL. Surprisingly, the lipoplex we developed showed an intrinsic downregulation of VEGFR2 irrespective of RNA-payload or targeting ligand. In contrast to Lipofectamine2000, where VEGFR2 downregulation was RNA-sequence dependent. We are currently exploring the underlying mechanism behind this phenomenon. Moreover, empty lipoplexes showed silencing of VEGFR2 expression. The reason could be that binding of galectin-1 to neuropilin-1 can trigger the down-regulation of VEGFR2-mediated signaling pathway (Hsieh et al., 2008). Taken together, Anginex lipoplexes can be considered as a promising candidate for anti-angiogenic therapy with small ncRNAs through siRNA delivery and Anginex activity. Therefore, the study will proceed with in vivo experiments to investigate tissue distribution and efficacy.

REFERENCES


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