Novel PEG-Nanoparticles Enhance Delivery of RNA Antagonist Oligonucleotides in Tumor Cells and in Mice

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Introduction

It has been increasingly attractive to target specific genes associated with human diseases. Oligonucleotide based molecules including locked nucleic acid and siRNA have the potential to prevent the unwanted genes from expression and may represent the next generation of new therapeutics. Locked nucleic acid oligonucleotides (LNA-ONs) can down modulate mRNA expression in transfected human cancer cells with very high potency (IC50 ~ 0.5 – 5.0 nM). Nonetheless, LNA-ONs could still benefit from further enhancement in cellular uptake and target tissue accumulation via effective delivery strategies. We have disclosed before that PEGylation of oligonucloetides using Customized Releasable Linkers could improve the pharmacokinetic properties and tumor accumulation of antisense molecules. [1] In addition, lipid-based nanoparticle strategies are among other major non-viral gene methods to deliver oligonucleotides *in vivo* to improve their pharmacokinetic profile, cell penetration, and specific tumor targeting. [2]

Recently, coated cationic lipid (CCL) [3] and stable nucleic acid-lipid particle (SNALP) [4] formulations were reported to achieve nanoparticles with small sizes, high nucleic acid encapsulation, good serum stability, and long circulation time. However, they didn't show significantly improved *in vivo* activities especially in organs other than liver as compared to the use of the naked oligonucleotides. Further improvement in the specific tumor targeting and tumor cell penetration will practically increase the possibility of using novel oligonucleotide based molecules to treat broad spectrum of human diseases. We describe here the utility of novel nanoparticle delivery systems that enhance the cellular uptake of LNA-ONs and result in potent down-modulation of target mRNA in human tumor cells without transfection and improve the cellular delivery of LNA-ONs in tumor-bearing mice.

Nanoparticles in study

Name	LNA	Size (nm)	Zeta potential (mV)	Poly dispersity	LNA conc. (mg/ml)
NP-1	LNA	79.9	+24	0.125	1.6
NP-2	Scrambled LNA	84.6	+21	0.092	1.57
NP-3	LNA-FAM	76.1	+22	0.216	0.134
NP-4*	LNA-FAM	85.6	+22	0.073	1.75
NP-5	None	77.9	+38	0.243	0

Table 1. Nanoparticle formulations used *in vitro* and in vivo studies

Nanoparticle composition:
C16-PEG:DSPE-PEG:Lipid 1: Cholesterol:DOPE = 1:1:18:20:60;
Lipid 1 = novel cationic lipid; FAM = 6-Carboxy Fluorescein; NP-4*: lipid labeled with Rhodamine

Stability

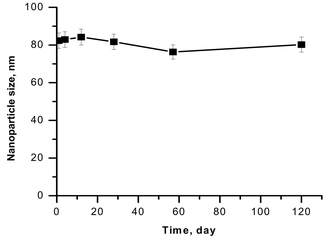
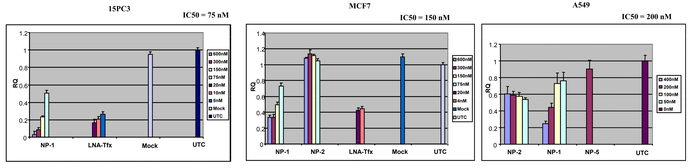


Fig 1: NP-1 stability in PBS at 4 °C.

Excellent stability in saline

In Vitro efficacy studies – mRNA down-modulation

Objective: To evaluate the *in vitro* mRNA down-modulation of nanoparticle in different cell lines without transfection



LNA-TFx = LNA with lipofectamine; Mock = Lipofectamine reagent only ; UTC = untreated control

Fig 2: qRT-PCR analysis: Target mRNA down-modulation by lead nanoparticle NP-1in different cells lines: Potency for mRNA down modulation: 15PC3 > MCF7 = A431 = N87 > A549 > DU145 = KB

Procedure: Cells were treated overnight with nanoparticles and harvested and analyzed by qRT-PCR. Samples of untreated served as reference point, and LNA with lipofectamine transfection as positive control.

Conclusion: NP-1 has demonstrated potent dose-dependent and target specific mRNA down-modulation by qRT-PCR analysis in multiple cell lines.

Protein down-modulation

Objective: To evaluate the *in vitro* protein down-modulation of nanoparticle in A431 cell lines without transfection

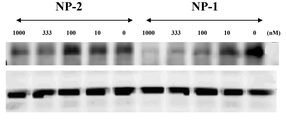


Fig 3: Western Blot analysis: Target protein down-modulation by lead formulation NP-1 and scrambled LNA formulation NP-2

Procedure: After being transfected with LNA nanoparticles for 24 h, A431 cells were harvested and lysed in SDS-PAGE buffer. 20 ug of total protein of each sample was electrophoresed on a 4-10% PAGE gel and transferred to nitrocellulose membrane using a semi-dry electroblotter. The western analysis was performed with enhanced chemiluminescence using monoclonal anti-target antibody and horseradish hyperoxidase-labeled anti-mouse IgG secondary antibody.

Conclusion: NP-1 was potent in protein knock-down.

In Vitro cellular uptake studies

Objective: To evaluate the cellular trafficking of nanoparticles in human cancer cells

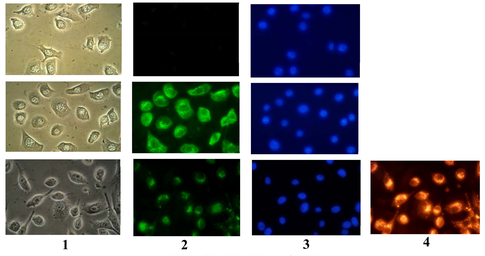


Fig 4: Fluorescence microscopy: (A) LNA-ON (B) NP-3 (C) NP-4
(1) Light **(2)** Green-FAM **(3)** Blue-Hoechst-Nuclear staining Dye **(4)** Red Dye Rhodamine
Procedure: 15PC3 cells were incubated with nanoparticles for 24 h at 37ºC. Cells were washed and the samples were analyzed by fluorescence microscopy.

Conclusion: NP-3 and NP-4 can penetrate cancer cell membrane and accumulate in the cytoplasm and nucleus.

Intratumoral administration (*In vivo*)

Objective: To evaluate the tumor mRNA down-modulation by intratumoral administration of nanoparticles in mice

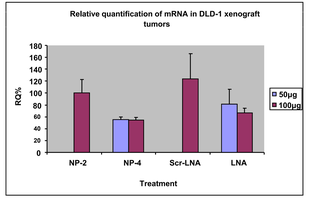


Fig 5: *In vivo* efficacy study: mRNA down-modulation by nanoparticles administered through intratumoral injection

Procedure: LNA-ONs were injected q3dx2 directly into DLD-1 tumors established in athymic nude mice either in naked form or in nanoparticles. Electroporation was applied after injection of naked LNA-ONs. Tumor samples were harvested 18 h after the last injection and analyzed by qRT-PCR for mRNA down-regulation

Conclusion: NP-4 showed mRNA down-modulation in tumor.

Intravenous administration (*In vivo*)

Objective: To evaluate the tumor and liver mRNA down-modulation by intravenous administration of nanoparticles in mice

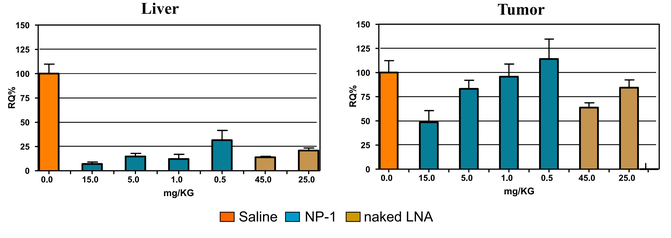


Fig 6: *In vivo* efficacy study: mRNA down-modulation by lead nanoparticle NP-1

Procedure: Athymic nude mice with 15PC3 tumors were injected with naked LNA or nanoparticles q3dx4 for 12 days. Tumor and liver samples were harvested and analyzed by qRT-PCR for mRNA down-regulation.

Conclusion: NP-1 showed dose-dependent mRNA down-modulation in tumor and liver at lower doses than naked LNA after intravenous administration in mice.

Conclusions

- **Novel nanoparticles using Enzon's Customized lipid have been identified**
- **The novel nanoparticles were stable in buffer and smaller than *100* nm with narrow size distribution**
- **Nanoparticles of LNA-ON allow efficient cellular uptake of LNA-ON as well as dose-dependent down-modulation of target mRNA and protein in multiple human cancer cell lines without transfection**
- **Intra-tumoral injected nanoparticles knocked down specific mRNA in tumor cells more efficiently than naked LNA with electroporation**
- **When given intravenously, the nanoparticles of LNA-ON was > 30-fold and > 3-fold more effective than naked LNA at silencing mRNA in liver and tumor, respectively**

These data show that novel nanoparticles provide a promising approach for more efficient in vivo delivery of oligonucleotides including LNA-ONs and siRNAs.

References

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