Customized PEG Linkers Improve Tumor Delivery of RNA Antagonist Oligonucleotides

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Introduction

Locked Nucleic Acid (LNA) represents third generation antisense technology with high target mRNA binding affinity and excellent tissue stability. Nanomolar to sub-nanomolar concentrations of LNA oligonucleotide (LNA-ON) can effectively inhibit target mRNA, and consequently diminish protein expression, in human cancer cells in the presence of transfection reagents (e.g. lipofectamine). LNA has emerged as a promising new therapeutic platform for many human diseases including cancers. Nevertheless, systemic delivery of LNA-ONs may be further improved if more favorable pharmacokinetic profile, specific tumor targeting and improved tumor cell penetration were possible. We describe here the utility of Customized PEG linkers incorporating cell penetrating peptides (CPP) or folate as targeting agent via releasable linkers. These constructs enhance cellular uptake of LNA-ONs resulting in potent down-modulation of target mRNA in human tumor cells and improved tumor delivery of LNA-ONs in mice.

Customized PEG-LNA conjugates

LNA 1 : anti-Survivin LNA
LNA 2 : anti-erbB3-LNA
TAT = CYGRKKRRQRRR-NH2

FAM = 6-carboxy-fluorescein
Tamra = Tetramethyl-6-Carboxy Rhodamine

TAT Conjugates

Table 1: PEG-LNA conjugates using TAT

MW of PEG	Conjugates
20 k Da	TAMRA-TAT-PEG-LNA2-FAM
10 kDa, 20 kDa, 40 kDa	TAT-PEG-LNA1

Folate Conjugates

Table 2: PEG-LNA conjugates using folate

MW of PEG	Conjugates
5 kDa, 40 kDa	Folate-PEG-LNA2-FAM
5 kDa, 40 kDa	Folate-PEG-LNA2

In vitro cellular uptake studies of CPP- PEG-LNA conjugates

Objective: To evaluate the cellular internalization of PEG-LNA conjugates using TAT peptide

Fluorescence Microscopy
Tamra-TAT-PEG-LNA-FAM

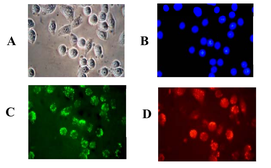


Fig 1: Fluorescence microscopy: 1μM Tamra-TAT-PEG-LNA2-FAM in 15PC3 cells. **A.** Light **B.** Blue-fluorescent-Hoechst 33342 dye **C.** Green-fluorescent-FAM **D**. Red-fluorescent-Tamra

Confocal Microscopy
Tamra-TAT-PEG-LNA-FAM

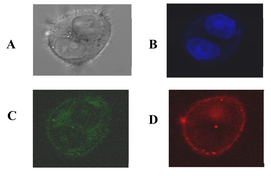


Fig 2: Confocal Microscopy: 500 nM Tamra-TAT-PEG-LNA2-FAM in KB cells **A.** Light **B**.Blue-Fluorescent-Hoechst Dye **C**.Green-fluorescent-FAM **D**. Red-Fluorescent Tamra

Procedure: 15PC3 or KB cells were plated overnight at 37 ºC. Next day compounds were incubated with cells for 24 hrs at 37 ºC. Cells were washed and the samples were analyzed by fluorescence and confocal microscopy.

Conclusion: Both fluorescence and confocal microscopy show improved cellular uptake of LNA using TAT.

In vitro cellular uptake studies of Folate-PEG-LNA conjugates in KB cells

Objective: To evaluate the cellular internalization of PEG-LNA conjugates using folate and the specificity of binding to the folate receptor

Fluorescence microscopy of Folate –PEG-LNA-FAM

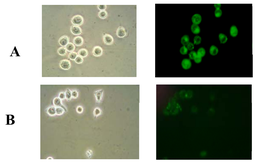


Fig 5: Fluorescence microscopy: A. 100 nM Folate-PEG-LNA2-FAM in KB cells. **B.** Blocking by 1 μM of cold folate.

Confocal microscopy study Folate-PEG-LNA-FAM

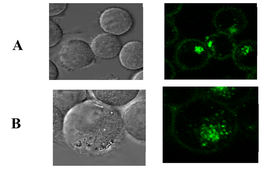


Fig 6: Confocal microscopy: 100 nM Folate-PEG-LNA2-FAM in KB cells **A.** 5k PEG **B**. 40k PEG

Procedure: KB cells were plated overnight at 37 ºC. Next day compounds were incubated with cells for 24 hrs at 37 ºC. Cells were washed and the samples were analyzed by fluorescence and confocal microscopy.

FACS analysis of folate receptor binding Folate-PEG-LNA-FAM

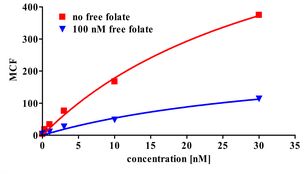


Fig 7: Binding to Folate receptor Folate-5KPEG-LNA2-FAM binds specifically to the folate receptors in KB cells

Procedure: KB cells were exposed for 4 hours at 37 ºC to increasing concentrations of Folate-PEG-LNA2-FAM, with or without 100 nM free folate. Cells were washed and analyzed by FACS for binding to the FAM-labeled PEG-LNA.

Conclusion: Both fluorescence and confocal microscopy show improved cellular uptake of LNA using folate. The specific binding of Folate-PEG-LNA-FAM to the folate receptor and subsequent internalization in KB cells can be blocked by the addition of free folate as indicated by both FACS analysis and fluorescence microscopy.

In vitro efficacy study

Objective: To evaluate the *in vitro* mRNA down-modulation of PEG-LNA conjugates without transfection

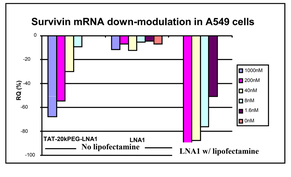


Fig 3: qRT-PCR analysis

Procedure: A549 cells were transfected by native and PEGylated LNA. The cells were harvested and analyzed by qRT-PCR for survivin mRNA down-modulation

Conclusion: PEG-LNA conjugates have demonstrated potent dose-dependent mRNA down-modulation by qRT-PCR analysis without the use of lipofectamine.

In vivo bio-distribution study

Objective: To evaluate the tumor accumulation of PEG-LNA conjugates in mice

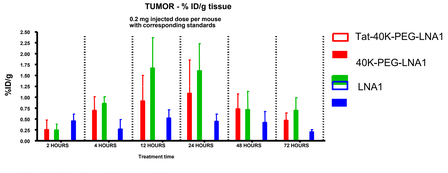


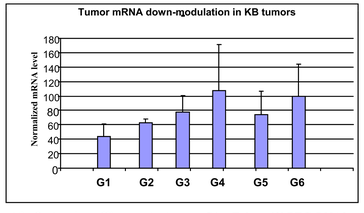
Fig 4: Bio-distribution study

Procedure: A549 tumor bearing athymic nude Balb/c mice were injected with a single dose of 10 mg/kg naked LNA or LNA conjugates. At various time points, mice were sacrificed and LNA concentration in tumors was quantified using an ELISA hybridization assay.

Conclusion: Customized PEG linkers with 40 kDa PEG enhance tumor accumulation.

In vivo efficacy studies of Folate-PEG-LNA conjugates

Objective: To evaluate the tumor and liver mRNA down-modulation in mice using Folate-PEG-LNA conjugates



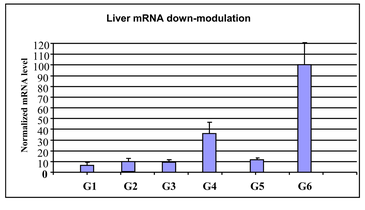


Fig 8: *In vivo* efficacy study: **G1 = Folate-40kPEG-LNA2 ; G2 = 40kPEG-LNA2; G3 = Folate-5kPEG-LNA2; G4 = 5kPEG-LNA2 ; G5 = LNA2 G6 = Saline**

Procedure: Athymic nude Balb/c mice with KB tumors were injected with a dose of 35 mg/kg of naked LNA or LNA conjugates q3dx4 for 12 days. Tumor and liver samples were isolated and analyzed by qRT-PCR for mRNA down-regulation.

Conclusion: Folate-PEG-LNA conjugates demonstrated potent tumor and liver erbB3 mRNA down-modulation in mice compared to naked erbB3 LNA.

Conclusions

A series of PEG-LNA conjugates have been successfully made using Customized Linkers incorporating CPPs and folate to study the *in vitro* and *in vivo* activities of LNA oligonucleotides. The studies have demonstrated:

- **PEG-LNA conjugates with cell penetrating peptide (TAT) have shown potent dose-dependent and specific target mRNA down-modulation in vitro without the use of lipofectamine.**
- **PEGylation increases tumor accumulation of LNA-ONs**
- **Folic acid PEG-conjugates enhance intracellular uptake of LNA-ONs**
- **Folic acid PEG-conjugates enhance mRNA down-modulation in mice compared to naked LNA-ONs**

The improved delivery and efficacy of LNA-ONs in tumor bearing mice is attributed to both the enhanced permeation and retention effect (EPR) and targeted delivery. Therefore, customized PEG linkers may provide a novel approach for more efficient in vivo delivery of oligonucleotides including LNA-ONs and siRNAs.

References

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