Novel Releasable PEG-Conjugates of LNA Antisense Oligonucleotides as Potent mRNA Antagonists to Cancer Targets

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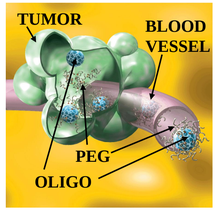
Introduction

Locked Nucleic Acid (LNA) antisense oligonucleotides (ASO), which can down-modulate mRNA expression in single digit nanomolar to high picomolar range have emerged as promising therapeutic agents for cancer treatment[1]. Beyond that LNA ASOs have been shown to down-modulate target without any delivery agent *in vitro* and *in vivo*, resulting in tumor growth inhibition[2]. To take advantage of accumulation into solid tumors *via* enhanced permeability retention[3] (EPR), conjugation of single stranded LNA ASO to branched polyethylene glycol (PEG) with a releasable linker was developed. Here we report an efficient conjugation method in multiple gram quantities and high purity without the need for an additional purification step after conjugation to minimize side products and maximize yield. Purity and loading density of LNA ASO in the PEG-conjugates was confirmed by SEC-MALS and MALDI-MS spectroscopy. In addition, melting temperature (T_m) studies have shown conjugation of releasable branched PEG linker to the 5'-end of LNA ASO did not perturb ability of hybridization to the complementary RNA *in vitro*. PEG-conjugates of LNA ASO showed improved pharmaceutical properties such as prolonged plasma half-life and stability[4] of PEG LNA conjugate. PEG-conjugates of LNA ASO resulted in comparable potency in down-modulation of target mRNA in mouse liver when compared to the unmodified LNA ASO. In addition, PEG-conjugates of LNA ASO targeting important cancer targets such as human HER3 showed better tumor growth inhibition in xenograft mouse models when compared to the equal dose of unmodified LNA ASO. The further assessment of releasable PEG linkers for oligonucleotide therapeutics development and other[5] agents such as small molecule drugs, antibody drug conjugates (ADC), peptides and proteins is warranted.

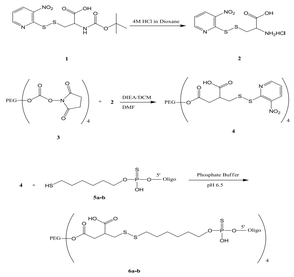
Hypothesis

- PEGylation will prolong circulation time of LNA oligonucleotides in plasma.
- PEGylation will improve tumor targeting of LNA oligonucleotides *via* enhanced permeability and retention (EPR).
- Prolonged circulation time in plasma and tumor uptake *via* EPR may translate to better therapeutic efficacy.

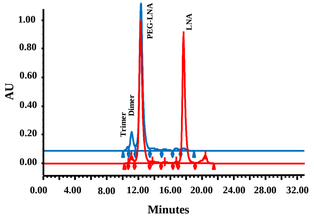


PEGylation Reaction Scheme & SEC-MALS Analysis

LNA ASO PEGylation Reaction Scheme



SEC-MALS Results of Reaction and Purified Products



- PEGylation Reaction Mixture
- Product after UF/DF Purification

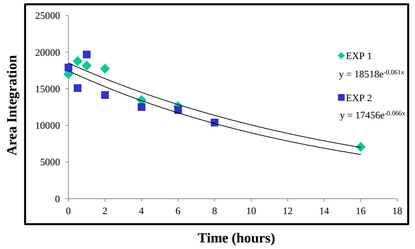
LNA Oligo PEGylation Reaction Yields & PEG-LNA ASO Stability in Rat Plasma

LNA Oligonucleotide PEGylation Reaction Yields

LNA-Oligonucleo-tides	Pegylation Reaction Scale (by UV at 260 nm)	Crude Product Analysis by HPLC (Area %)	Final Analysis		Yield[a]
			HPLC Purity (%)	Purity by SEC-MALS	
PEG-EZN-3920	3.43 g (657 µmol)	58.3%	99.9%	86% pure with 12% dimer	56.9%
PEG-EZN-3892	4.02 g (767 µmol)	59.1%	99.9%	86% pure with 11% dimer	57.0%

Note: a) Yield was based on UV at 260 nm of free LNA oligonucleotide

Two Repeating Kinetics Experiments of PEG-EZN3920 LNA Stability in Rat Plasma



HPLC based assay was used to analyze the PEG-EZN3920 conjugate stability, and plot the area integration of PEG-EZN3920 LNA over time. The calculated average t ½ or half life of PEG-LNA in rat plasma is ~ 10.9 hrs

Results:

- PEG-LNA conjugates were purified by UF-DF without additional preparative HPLC to maximize reaction yield
- In rat plasma 50% of free EZN3920 ASO is released from PEG-EZN3920 conjugate in ~10.9 hours

Melting Temperature (T_m) of EZN3920 and PEG-EZN3920 LNA-Oligonucleotide

Objective: Comparison of Unmodified and PEG-LNA ASO for *in vitro* Hybridization to RNA Complement

Figure A. Normalized temperature dependent UV dissociation profiles for the PEG-EZN-3920-RNA duplex monitored as a function of concentration

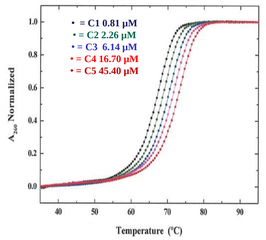


Figure B: van't Hoff analysis of concentration-dependent EZN-3920-RNA duplex & PEG-EZN-3920-RNA duplex dissociation

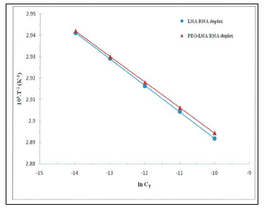


Table Comparison of concentration-dependent transition temperatures (T_m) for the EZN3920-RNA and PEG-EZN3920-RNA duplexes[1]

Conc.$_{Duplex}$ (µM)	T_m of EZN3920-RNA Duplex (°C)	T_m of PEG-EZN-3920-RNA Duplex (°C)
0.42	67.0	66.9
1.13	68.4	68.3
3.07	69.9	69.7
8.35	71.3	71.1
22.70	72.8	72.5

[1] The standard deviation for T_m is within ± 0.5 °C

Procedure:

A. Normalized temperature-dependent UV dissociation profiles for the EZN320 monitored as a function of concentration.
B. van't Hoff analysis of concentration-dependent dissociation of EZN3920/RNA and PEG-LNA/RNA duplexes.
C. Comparison of concentration-dependent transition temperatures (T_m) for the EZN3920/RNA and PEG-EZN3920/ RNA duplexes

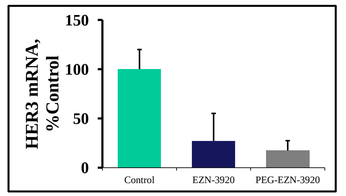
Results:

- Analysis of the temperature dependent dissociation profiles for duplexes of unmodified EZN3920 and PEG-EZN3920 with complimentary RNA reveals comparable thermal stabilities (based on above Table, T_m = 69.9 ± 0.1 ^{0}C at C_{duplex} = 3.07 µM)
- Unmodified and PEGylated LNA and complimentary RNA duplexes showed comparable van't Hoff dissociation enthalpies (ΔH_{vH} = 163.4 ± 3.0 K.Cal.mol^{-1}) and van't Hoff thermodynamic stabilities (ΔG_{vH} = 27.6 ± 3.0 K.Cal.mol^{-1})

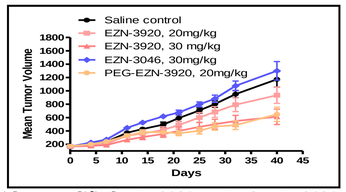
HER3-mRNA Knockdown by EZN3920 &PEG-EZN3920 ASO in Xenografts

Objective: Comparative *in vivo* target mRNA Knockdown by Unmodified EZN3920 & PEG-EZN3920

A. HER3 mRNA Knockdown in Liver



B. Tumor Growth Inhibition (TGI) in HCC827 Xenograft Model



Procedure: A. Mice were injected i.v. (q3d x 4) with unmodified EZN3920 or PEG-EZN3920 (10 mg/kg). Liver samples were collected 24 hrs after the last dose and analyzed by qRT-PCR for HER3 mRNA knockdown. B. TGI was performed in HCC827 tumor xenograft model. Tumor-bearing mice were injected i.v. (q3d X 8) with unmodified EZN3920 or PEG-EZN3920 with the above indicated doses.

Results: PEG-EZN3920 resulted in 92% mRNA knockdown in liver and 83% in HCC827 tumor model whereas unmodified EZN3920 showed 88% knockdown in liver and 73% in HCC827. PEG-EZN3920 at 20 mg/kg showed comparable anti-tumor activity to 30 mg/kg of unmodified or unmodified EZN3920

Conclusions

This study has demonstrated:

- PEG-LNA-antisense oligonucleotide conjugates with releasable linkers were synthesized efficiently in high yields without chromatographic purification.
- PEGylation of EZN3920 LNA oligonucleotide at 5'-end does not perturb hybridization to the RNA complement.
- PEG-EZN3920 showed prolonged circulation time and stability in plasma
- PEG-EZN3920 LNA oligonucleotide conjugate shows comparable mRNA target down modulation in the tumor to unmodified LNA oligonucleotide.
- PEG-EZN3920 LNA oligonucleotide at 20 mg/kg dose shows comparable tumor growth inhibition to unmodified EZN3920 LNA oligonucleotide at 30 mg/kg in HCC827 tumor xenograft model.

The *in vivo* effects observed with PEG-LNA oligonucleotide conjugates may be due to EPR effect within the tumor, which has previously been observed with other[5] PEGylated molecules.

References

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