Utility of Customized PEG Linkers for the delivery of oligonucleotides without the use of transfection agents

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ABSTRACT

RNA antagonists using locked nucleic acid (LNA) antisense oligonucleotides are among the most promising therapeutics to treat cancer. Unlike the other antisense oligonucleotides, LNA-based oligonucleotides have high target binding affinity and long tissue stability. LNA can inhibit the mRNA expression at nanomolar and sub-nanomolar concentrations in the presence of lipofectamine. However, systemic delivery of LNA is still a challenge. Using our Customized PEG linkers we achieved in vitro mRNA down-modulation in the absence of transfection agents in a dose dependent manner. By attaching targeting moieties to these PEG linkers, cell specific binding and internalization were observed in fluorescence microscopy studies. Preclinical xenograft models were used to study the bio-distribution and tumor accumulation of PEG-LNA conjugates.

Keywords: Locked Nucleic acid (LNA), antisense oligonucleotide (AS-ODN), poly(ethylene glycol) (PEG), Customized PEG linker, mRNA down-modulation

1 INTRODUCTION

Antisense oligodeoxynucleotides (AS-ODN) can efficiently knock down the expression of target genes that are involved in the development of many human diseases, including cancers in a sequence-specific manner. AS-ODN is one of the most promising therapeutic macromolecules. Lock nucleic acid or LNA is the third generation of antisense technology with exceptionally high binding affinity and stability. As a result, LNA can down modulate mRNA in vitro with efficiency similar to siRNA technology.

There are several major in vivo delivery hurdles to overcome when using antisense oligos as therapeutic agents. AS-ODN is distributed and eliminated rapidly after IV injection. In addition, the polyanionic character of phosphorothioate oligonucleotides impairs its ability to penetrate cellular membranes. Carrier systems such as cationic lipids have been designed to shield the negative charge and enhance oligonucleotide delivery and cellular uptake, but to date these have not proven to be clinically useful. It is obvious that there are at least two major factors limit the use of AS-ODN oligonucleotides as therapeutic agents: short circulation half-life and their inadequate ability to cross cell membranes.

While PEGylation can generally improve the pharmacokinetic profiles of oligonucleotides [1, 2], poor cellular uptake due to their poly-anionic nature still remains as the major hurdle. To address this challenge, new Customized PEG linkers with positive PEG backbone are under development. The idea is to incorporate sufficient positive charges into the backbone of PEG for the conjugation with oligonucleotide. The overall appearance of such PEG-oligo conjugate will be neutral or even slightly positive for better cell membrane binding. Cell penetrating peptides (CPP) are a class of positively charged peptides which can enhance the cellular uptake [3]. When conjugated to CPP, the molecule which has difficulty to cross the cell membrane by itself can now have better ability to get inside the cells [4]. Our preliminary data showed that when CPPs were conjugated to PEG-LNA, we could down modulate mRNA in human cancer cells without the help of transfection agent. To further increase the in vivo efficacy, active binding functions have also been incorporated into these novel conjugates. There are many targeting agents which have been used for drug delivery, including folic acid [5], peptide [6], and antibodies [7]. When we incorporated these targeting moieties into our PEG-LNA conjugates, cell specific binding and internalization were observed in fluorescence microscopy studies. Furthermore, in order to find the relationship of tumor accumulation and the size of PEG, a series of PEG-LNA conjugates were prepared with different PEG sizes for bio-distribution studies in mice.

2 EXPERIMENTAL

2.1. Preparation of the PEG-LNA conjugates

LNA oligonucleotides with C6-NH_2 or C6-SH linker were conjugated to active PEG linkers followed by the addition of targeting agent or CPP. The products were purified by ion-exchange column chromatography.

2.2. Fluorescence Microscopy studies

Human lung cancer A549 cells were seeded to 4 well slides (30K / 500 µL/ well), incubated overnight at 37 ºC. The medium was then aspirated and 135 µL of 10% FBS growth medium was added. The cells were then treated with 15 µL solution of PEG-LNA compound (1 µM) and incubated for 24 hr at 37 ºC. The slides were washed three

times with 1% BSA PBS and the samples were analyzed by fluorescence microscopy.

2.3. In vitro efficacy study

A549cells were seeded at 2.5 x 105/12-well and incubated overnight at 37 °C. The cells were washed once with Opti-MEM, then the PEG-LNA compounds were added with or without lipofectamine, and incubated for 24 hours. Total RNA was prepared using RNAqueous Kit (Ambion) following the manufacturer's instructions. The RNA concentrations were determined by OD at 260 nm, and 2.0 µg of total RNA were used for cDNA synthesis. Real-time PCR was conducted on ABI 7300 instrument. 100 ng of each sample was analyzed by Multiplex Tagman gene expression assays using human survivin gene set (Hs 00153353 m1) and GAPDH as house keeping gene.

2.4. Bio-distribution study

Animals were injected with A549 cells and tumors were grown to ~100 mm^3. PEG-LNA compounds and naked LNA were injected at 10 mg/kg dosing at q3d x4. After completion of dosing, the animals were sacrificed and tumor samples were analyzed using RNA hybridization assay.

3 RESULTS

3.1 Florescence microscopy studies

Dye labeled PEG-LNA conjugates were prepared using FAM labeled LNA. Two different targeting moieties RGD peptide and folic acid were used to study the cell specific cellular uptake by fluorescence microscopy (Table 1).

Table 1. PEG-conjugates for in vitro studies

PEG size	Compound Name
20 kDa	RGD-PEG-LNA-FAM
5 kDa	folate-PEG-LNA-FAM
20 kDa	TAT-PEG-LNA

U87 cells which over-expressing RGD receptors were treated with 100 nM of RGD-PEG-LNA-FAM. Internalization of PEG-LNA conjugate was clearly observed after 24 h of incubation without any transfection agents (Fig 1). Meantime, naked LNA by itself did not show any uptake under the same conditions. Competitive binding studies were performed by incubating the cells with cold RGD peptide and followed by treating with PEG-LNA compounds. Inhibition of binding was observed.

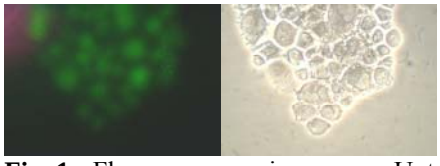

Fig 1: Fluorescence microscopy: Uptake of RGD-PEG-LNA-FAM (100 nM) in U87 cells at 24 h, 37 °C.

Similar binding and internalization effects were obtained when folate receptors over-expressing KB cells were treated with folate-PEG-LNA-FAM conjugate (Fig 2). Again, binding was inhibited completely by the addition of free folic acid to the medium prior to the addition of PEG-LNA compound.

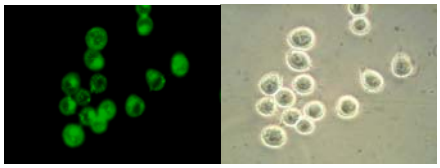

Fig 2: Fluorescence microscopy: 100 nM Folate-PEG-LNA-FAM in KB cells at 24 h, 37 °C.

3.2. In vitro efficacy study

We then studied the in vitro efficacy of TAT-PEG-LNA construct in A549 cell line using anti-survivin LNA. The RT-PCR results showed that TAT-PEG-LNA conjugate could down regulate survivin mRNA in a dose-dependent manner with an IC_{50} of ~100 nM without any transfection agents (Fig.3).

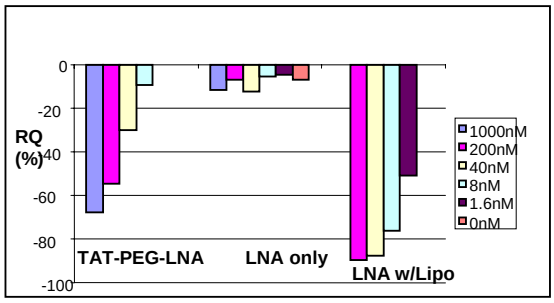

Fig 3: Survivin mRNA down-modulation in A549 cells

3.3. Bio-distribution study

We prepared PEG-LNA conjugates with two different PEG sizes, 40 kDa and 10 kDa, to study the LNA bio-distribution in mice (Table 2).

Table 2. PEG-LNA conjugates for bio-distribution studies

PEG size	Compound Name
40 kDa & 10 kDa	TAT-PEG-LNA
40 kDa & 10 kDa	PEG-LNA

The 40 kDa PEG-LNA conjugates showed higher tumor accumulation compared to naked LNA or 10k PEG-LNA conjugates. At 12 hours after dosing, about 1.75% of total injected dose of 40 kDa PEG-LNA conjugate was found in the solid tumor (Fig. 4)

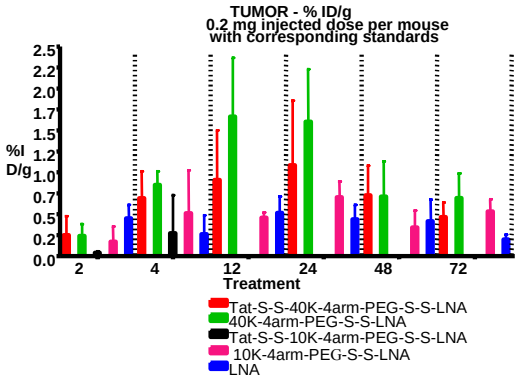

Fig. 4 Bio-distribution study of PEG-LNA

4 DISCUSSION

A series of PEG-LNA conjugates have been successfully prepared using Customized PEG Linkers to study the in vitro activities of LNA oligonucleotides. PEG-LNA conjugates targeting survivin have demonstrated potent dose-dependent and sequence-specific mRNA down modulation by RT-PCR study in the absence of lipofectamine. Cell penetrating peptide TAT has demonstrated capability to transfect cells and induced internalization of PEG-LNA conjugate inside the cancer cells.

By using RGD peptide and Folic acid as targeting agents in the PEG-LNA constructs, we also demonstrated cell specific binding and cellular uptake by fluorescence microscopy studies. U87 cell are known to express high levels of integrins and the RGD-PEG-LNA compound showed specific binding and internalization to these cells. Folate receptor is over expressed in many cancerous cell lines. We chose folic acid as another targeting moiety to incorporate into our PEG-LNA constructs. We observed very strong binding and uptake when folate-PEG-LNA-FAM was incubated with KB cells which highly express folate receptors on their surfaces.

One of the goals of our study was to proportionally increase the tumor accumulation of LNA in solid tumor. We screened PEG-LNA constructs with different molecular weight of PEG in tumor bearing mice to study the impact of PEG size in bio-distribution. Results from our experiment indicated that compounds with higher PEG size have better tumor accumulation compared to lower molecular weight PEG-LNA conjugates or free LNA. This is consistent with the previous reports on the EPR effect of high molecular weight PEG [8].

Currently, the leading PEG-LNA compounds are being screened in preclinical xenograft models to assess their capability to down modulate target mRNA in tumors.

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