Marked sensitivity to mRNA targeting by LNA antisense in tumor cells without a delivery system: Lessons learned

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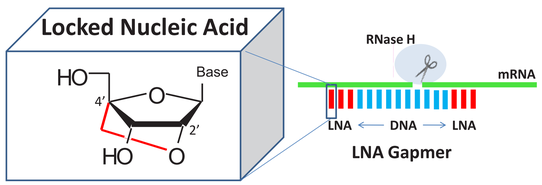
INTRODUCTION

Unlike siRNAs, single-stranded locked nucleic acid-based antisense oligonucleotides (LNA-ONs) have shown the ability to down regulate mRNAs *in vitro* and *in vivo* without any delivery systems such as transfection reagents or liposomes. Hence, LNA-ONs may have significant advantages as a therapeutic compared to siRNAs. Investigation of LNA-ONs that target HIF-1α, survivin, or androgen receptor in cancer patients is ongoing. We assess here 1) down-regulation efficiency using LNA-ONs against PIK3CA, β-catenin, and HER3 in multiple cell lines and in cells prepared directly from patient tumors, 2) the correlation of target down-regulation and growth inhibition *in vitro* with tumor growth inhibition *in vivo*, 3) the correlation of intratumoral LNA-ON concentration with target down-regulation *in vivo* in xenograft models, and 4) genes that are differentially expressed in LNA-ONs resistant cells as compared to permissive cells.

METHODS

LNA-ONs were either added to tissue culture media (i.e. no transfection) *in vitro* or prepared in saline and given IV *in vivo*. Endpoints were measured by qRT-PCR, MTT, Western Blot analysis, and tumor size. Gene Expression Profiling was performed by Asuragen, Inc. Concentration of LNA-ONs in tissues was measured by LC/MS/MS. Primary tumors samples obtained and treated by Precision Therapeutics Inc (PTI) and down-regulation analysis carried out by Enzon Pharmaceuticals.

WHY USE LNA TECHNOLOGY?



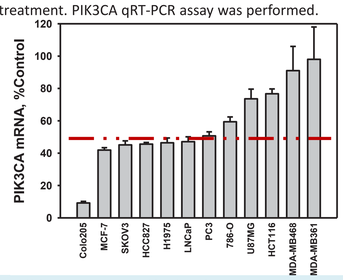
- 3rd generation LNA-based antisense technology results in:
  - Improved T_m (+2 to +8^0 per LNA monomer)
  - Excellent plasma stability ($T_{1/2}$ = 15 hrs)
  - Long tissue residence time (Liver $T_{1/2}$ = weeks)
  - Short sequence (14-16-mer)
  - IV administration without delivery vehicle

LNA-ONs USED IN THE STUDIES

Compounds	Target
EZN-3920	HER3
EZN-3892	β-catenin
EZN-3889	β-catenin
EZN-4150	PI3KCA
EZN-4176	Androgen Receptor
EZN-2968	HIF-1α
EZN-3046 scrambled control	None

Down-regulation of mRNA by LNA-ON in cell lines

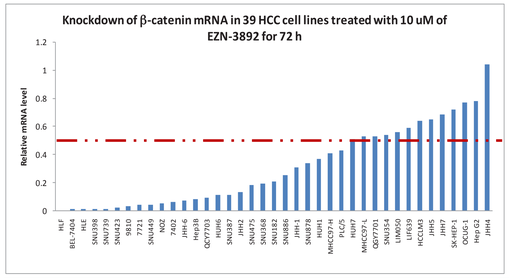
Fig. 1. Effect of LNA-ONs without lipofection on mRNA in human cancer cell lines. Cells were treated with EZN-4150 at 5 μM. Cells were harvested 2 days after treatment. PIK3CA qRT-PCR assay was performed.



58% of the cell line show > 50% PIK3CA mRNA down-regulation without lipofection. Similar data were obtained with EZN-3920 HER3 LNA-ON (Zhang et al, 2011 Gene Therapy. 18:326)

Down-regulation mRNA by LNA-ON in hepatocellular cell lines

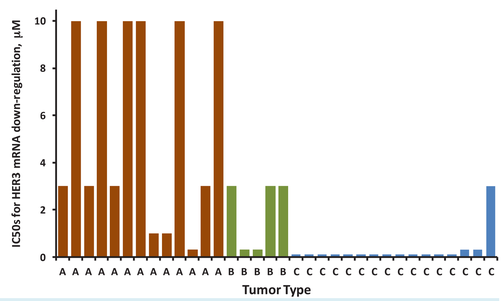
Fig. 2. Effect of LNA-ONs without lipofection on mRNA. Multiple human hepatocellular carcinoma cell (HCC) lines were treated with EZN-3892 at 10 μM. Cells were harvested 3 days after treatment. β-catenin qRT-PCR assay was performed.



β-catenin mRNA down-regulation > 50% was observed in 69% of HCC cell lines. Similar data (not shown) were obtained with EZN-2968 HIF-1α LNA-ON

Down-regulation of mRNA in primary tumor cells

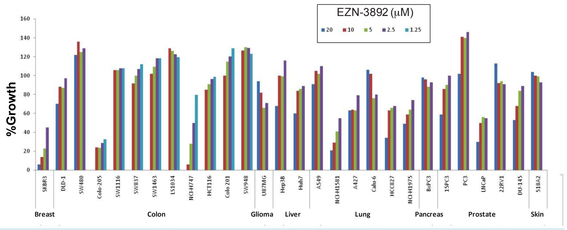
Fig. 3. Down-regulation of mRNA in primary tumor cells by LNA-ON. Primary tumor cells from 3 tumor types were prepared from cancer patients according to Precision Therapeutics Inc's procedure, plated, and treated with EZN-3920 without lipofection at various concentrations. Cells were harvested 2 days after treatment. qRT-PCR assay was performed.



•Potency in target down-regulation varied among tumor samples
•Tumor type C demonstrated superior permissiveness to LNA-ON

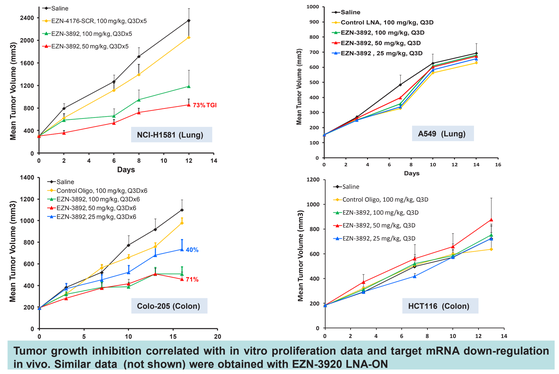
Correlation *between in vitro* and *in vivo* growth inhibition

Figure 4. Inhibition of solid tumor cell lines growth by LNA-ON without lipofection. Cells from various tumor histologies were treated by adding EZN-3892 to cell cultures at doses up to 20 μM without lipofection for 7 days prior to MTT assay.



•SKBR3, Colo-205, NCI-H747 and NCI-H1581 cells were more sensitive to EZN-3892 than others (EC50 ~ 250 nM-8 μM) in growth assay
• β-catenin mRNA was down-regulated efficiently (EC50~ 200 nM to 3 μM) in these cell lines
•Colo-205 and NCI-H1581 were chosen for in vivo efficacy test because they grow well on the flank of mice; HCT116 and A549 were also chosen for in vivo efficacy test because potency in growth inhibition and target down-regulation were poor (EC50 > 20 μM)

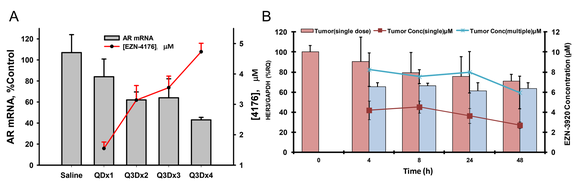
Fig. 5. Effect of LNA-ON on tumor growth in mice. NCI-H1581, Colo-205, A549, and HCT116 tumor bearing mice were dosed IV with EZN-3892 or control LNA simply dissolved in saline with indicated doses and schedules.



Tumor growth inhibition correlated with in vitro proliferation data and target mRNA down-regulation in vivo. Similar data (not shown) were obtained with EZN-3920 LNA-ON

Intratumoral PK/PD relationship

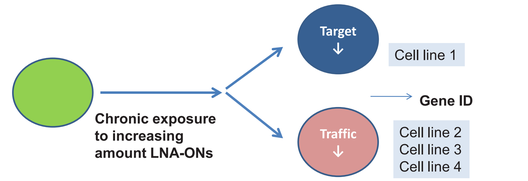
Fig. 6. Intratumoral PK/PD correlation. (A) Castration-resistant C4-2b tumor-bearing mice were treated with 40 mg/kg EZN-4176 at indicated dosing schedule. Twenty four hours after the last dose, mice were sacrificed and tumors harvested. AR mRNA level (grey bars) and EZN-4176 concentration in the tumors (line) were measured. (B) NSCLC HCC827 tumor-bearing mice were treated with 30 mg/kg EZN-3920 either single dose or multiple doses (q3dx3). At indicated times after the last dose, mice were sacrificed and analyzed for HER3 mRNA level and EZN-3920 concentration. All data are mean ± SEM (n = 5).



• mRNA antagonist concentration increases in tumor with repeated dosing
• Increasing concentration of mRNA antagonist in the tumor is associated with increased down-regulation of target mRNA

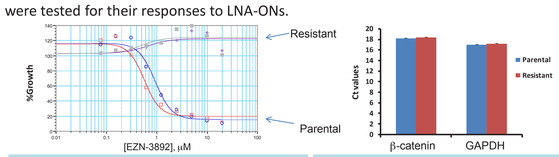
Generating cells resistant to LNA-ON

Fig. 7. Generation of LNA-ON-resistant cell lines.



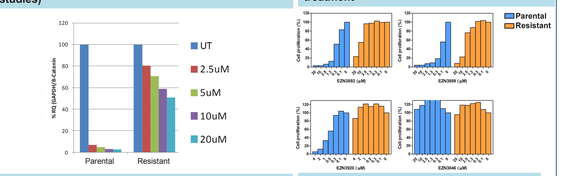
Two types of resistant mechanisms were identified:
Type 1: Level of target protein reduced compared to parent cell line (cell line 1)
Type 2: Level of target protein remained the same; down-regulation efficiency of mRNA by LNA-ON reduced (cell lines 2-3)

Fig. 8. Characterization of type 2 resistant cell lines. Parental and resistant cells were tested for their responses to LNA-ONs.



1. Resistant cells do not demonstrate growth inhibition compared to parental (two independent studies)

3. No change in β-catenin or GAPDH mRNA in resistant cells in response to EZN-3892 treatment

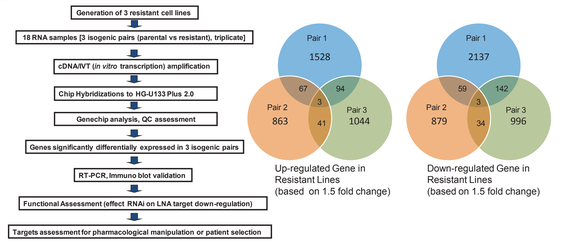


2. Resistance is associated with reduced KD of target

4. Resistant cells are cross resistant to multiple LNA-ONs

Genomic analysis of resistant cell lines

Fig. 8. Genomic analysis of the mechanism of resistance.



•Preliminary results suggest that genes are differentially expressed in resistant cells
•Validation of these differentially expressed genes and understanding of their roles in target down-regulation may assist in selection of tumors and patients who may benefit the most from LNA-ON therapy

CONCLUSIONS

- **LNA-based oligonucleotides (LNA-ONs) result in down-regulation of target in vitro and *in vivo* in many cancer cell lines including primary tumor cells without the need for transfection**
- ***In vitro* activities as assessed by potency in growth inhibition and target down-regulation may predict *in vivo* anti-tumor response**
- **LNA-ONs administered IV achieve tumor concentration required to effectively down regulate target in vitro** (Zhang et al, 2011 Gene Therapy. 18:326)
- **Cells resistant to LNA-ONs have been generated**
- **Understanding the mechanism of resistance to LNA-ON may assist in the selection of tumors and patients for therapy with LNA-ON**

LNA-ONs are being developed by Enzon under a license with Santaris Pharma A/S.

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