

ENABLING TECHNOLOGIES

Down-modulation of cancer targets using locked nucleic acid (LNA)-based antisense oligonucleotides without transfection

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Usually, small interfering RNAs and most antisense molecules need mechanical or chemical delivery methods to down-modulate the targeted mRNA. However, these delivery approaches complicate the interpretations of biological consequences. We show that locked nucleic acid (LNA)-based antisense oligonucleotides (LNA-ONs) readily down-modulate genes of interest in multiple cell lines without any delivery means. The down-modulation of genes was quick, robust, long-lasting and specific followed by potent down-modulation of protein. The efficiency of the effect varied among the 30 tumor cell lines investigated. The most robust effects were found in those cells where nuclear localization of the LNA-ON was clearly observed. Importantly, without using any delivery agent, we demonstrated that HER3 mRNA and protein could be efficiently down-modulated in cells and a tumor xenograft model. These data provide a simple and efficient approach to identify potential drug targets and animal models. Further elucidation of the mechanism of cellular uptake and trafficking of LNA-ONs may enhance not only the therapeutic values of this platform but also antisense molecules in general.

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INTRODUCTION

In recent years, antisense and small interfering RNA (siRNA) have become routine and useful tools to down-modulate genes to identify a drug's target. However, both approaches are limited because of the instability or lack of efficient cellular uptake of these oligonucleotides (ONs). The earlier generation antisense molecules such as phosphorothioate ONs have relatively low potency and short stability, and general toxicities are associated with the backbone of the molecules.^{1–5} Although siRNA technology has revolutionized the biological research, it also faces challenges such as stability, immune activation and off-target effects.^{6–10} Beyond this, ON-based molecules do not penetrate the plasma membrane efficiently. Therefore, membrane-permeabilizing agents are required to efficiently get ONs into the cells. False-positive results have been common in preclinical studies because of the use of delivery methods such as lipofection, virus and electroporation.^{6–12} Additionally, the use of delivery methods has made down-modulation relatively laborious and results more inconsistent because many factors such as cell density, amount of transfection reagent used, viral titer and duration of treatment can affect the down-modulation efficiency. Therefore, stringent validation is required before any target is considered relevant to the cancer therapy. Moreover, without a delivery agent, phosphorothioate antisense and siRNA molecules are not readily applicable to *in vivo* target modulations outside the liver.

Locked nucleic acid (LNA)-ONs are a new generation of antisense ONs.^{13–17} Unlike previous ON designs, the ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon nucleoside.^{17–20} This modification provides ONs with an exceptionally high binding affinity for mRNA compared with

conventional DNA ONs. Additionally, LNA-ONs are resistant to nucleases and therefore provide exceptional stability in plasma and cell culture medium.^{13–17} In the presence of transfection reagents, LNA-ONs have low single-digit nanomolar or high picomolar IC₅₀ values for mRNA down-modulation in cell culture.^{21–22} As LNA-ONs are not only stable but also highly potent under transfection condition, along with others,²³ we have been able to examine the effects of LNA-ONs on mRNA down-modulation without transfection using an *in vitro* system. We confirmed that single addition of LNA-ONs to cell-culture media (without any transfection reagent or termed as *gymnosis*²³) caused specific down-modulation of target genes in multiple cells. Time- and dose-dependent studies revealed that the down-modulation of mRNA and protein was robust, efficient and sustained. Using this approach, we evaluated several potential cancer targets. Additionally, we screened over 30 cell lines to investigate the consequence of the gene inhibition and found very diverse responses in terms of growth among the cell lines tested. Potent down-modulation of target mRNA appeared to correlate with nuclear localization of the antisense ONs. In sum, in certain cell lines, LNA-ONs represent an attractive option for rapid, simple and specific determination of gene function and drug target validation.

RESULTS

Simple and efficient down-modulation of target genes

We have previously identified LNA-ONs that down-regulated HER3, β -catenin, PIK3CA (phosphatidylinositol 3-kinase P110 α) and heat shock protein 27 (HSP27) mRNA in cells transfected with lipofectamine. The IC₅₀ values for mRNA down-modulation were typically in

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the 1–5 μM ranges. Recent studies without lipofectamine were conducted. LNA–ONs targeting HER3, β -catenin, PIK3CA or HSP27 were simply added to 15PC3 prostate cancer cells. In the absence of lipofection, all LNA–ONs potently down-modulated their respective targets, with IC_{50} values ranging from 300 to 600 nM (Figure 1). The effect was specific because HER3 mRNA was only down-modulated by EZN-3920 (anti-HER3) but not by EZN-3889 (anti- β -catenin) (Figure 1a). The β -catenin mRNA was only down-modulated by EZN-3889 but not by EZN-3920 (Figure 1b). The same results were obtained with antisense molecules to PIK3CA (EZN-4150) and HSP27 (EZN-4516) (Figures 1c and d).

To understand the effect of treatment time on mRNA levels, we also evaluated target gene down-modulation at different time points using three different cell lines: 15PC3, PC3 prostate cancer and SW480 colorectal cancer cells. Time-course studies in 15PC3 and PC3 cells (Figures 2a and c) revealed a specific and rapid down-modulation of target mRNA, which reached maximum around 24 h. EZN-3920-mediated target mRNA down-modulation in 15PC3 cells can be detected by real-time quantitative PCR as early as 8 h (Supplementary Figure S1). Prolonged incubation over 5 days did not seem to yield additional down-modulation (Figures 2a and c). Interestingly, similar to what was reported,²³ the down-modulation was maintained during the entire study with just one treatment, suggesting the sustained action of LNA–ONs may be due to their superior affinity and stability. The kinetics of down-modulation appeared to be cell-type dependent because down-modulation in SW480 was progressively more efficient over time (Figure 2d) with EZN-3889 against β -catenin. The effect was specific because a HER3 antisense (EZN-3920) failed to down-modulate β -catenin but HER3 (Supplementary Figure S2). To understand how common this observation is, we surveyed about 30 cell lines for their ability to down-modulate genes. Supplementary Table S1 summarizes the down-modulation results based on >50% mRNA reduction of at least one target gene among the four tested LNA–ONs at 5 μM . The results showed a diverse response to the treatment of

LNA–ONs, suggesting that down-modulation efficiency can vary significantly among cell lines.

Correlation between effective down-modulation and nuclear localization of LNA–ONs

The diverse response of cell lines in terms of mRNA down-modulation could be related to multiple factors such as uptake efficiency, endosomal entrapment and release, and ribonuclease H (RNase H) activity. To understand the mechanism underlying the diverse response, we labeled HER3 antagonist EZN-3920 with FAM (3-Oxo-3',6'-dihydroxy-1,3-dihydrospiro[isobenzofuran-1,9'-[9H]xanthene]-6-carboxamido-hexyl-1-O-phosphate) and examined the cellular uptake and localization of the ON. Among six cell lines with different sensitivities to LNA–ON treatment, there was no significant difference in the kinetics of FAM-EZN-3920 uptake (Supplementary Figure S3). However, by examining 15PC3 and A549 cells with fluorescence microscopy, we observed that efficient target down-modulation appeared to correlate with nuclear localization of FAM-EZN-3920. The compound was accumulated in the cytoplasm in the perinuclear space in both cell lines (Figures 3a and b). In 15PC3 cells, the majority of the EZN-3920 was located in the cytoplasm in the early time points such as 2 or 6 h (Figure 3a). There appeared to be a slight increase in nuclear localization of the FAM-labeled compound by comparing the images of 6 h with those of 2 h. The nuclear staining became quite evident at 24 h. We noticed that this translocation occurred prominently in 15PC3 cells but minimally in A549 cells (Figure 3b, top panel). Interestingly, the nuclear localization of FAM-EZN-3920 correlated with the ability of EZN-3920 to down-modulate its target (Figure 3c) and growth as well (Figures 5a and b) in these cells. To validate this observation, we studied other cell types such as HCC827 and DLD-1. HER3 mRNA was efficiently down-modulated by EZN-3920 in HCC827 cells but not in DLD-1 cells. The down-modulation efficiency clearly matched the results of nuclear localization of FAM-EZN-3920 (Supplementary Figure S4). To ensure that the

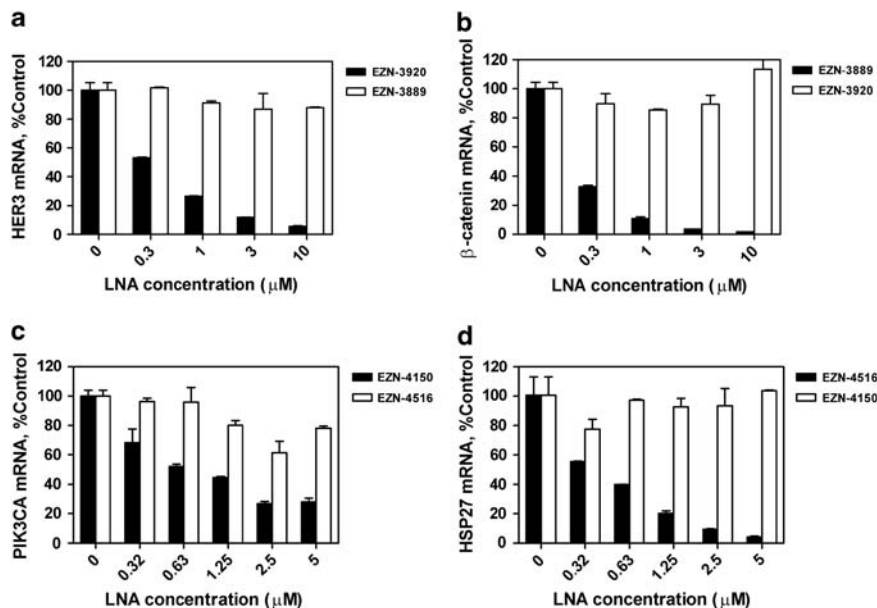


Figure 1 Down-modulation of target mRNA with LNA–ONs in the absence of lipofection in 15PC3 cells. LNA–ONs were added to tissue culture media at the indicated concentrations and incubated with 15PC3 cells (plated at approximately 2000 cm^{-2}). Down-modulation of HER3 (a), β -catenin (b), PIK3CA (c) and HSP27 (d) mRNA after treatment for 3 (a, b) or 6 days (c, d) were determined by quantitative real-time PCR with TaqMan gene expression assay. LNA–ONs designed to specifically targets are HER3 (EZN-3920), HSP27 (EZN-4516), PIK3CA (EZN-4150) or β -catenin (EZN-3889). Data are shown as means \pm s.e. ($n=3$).

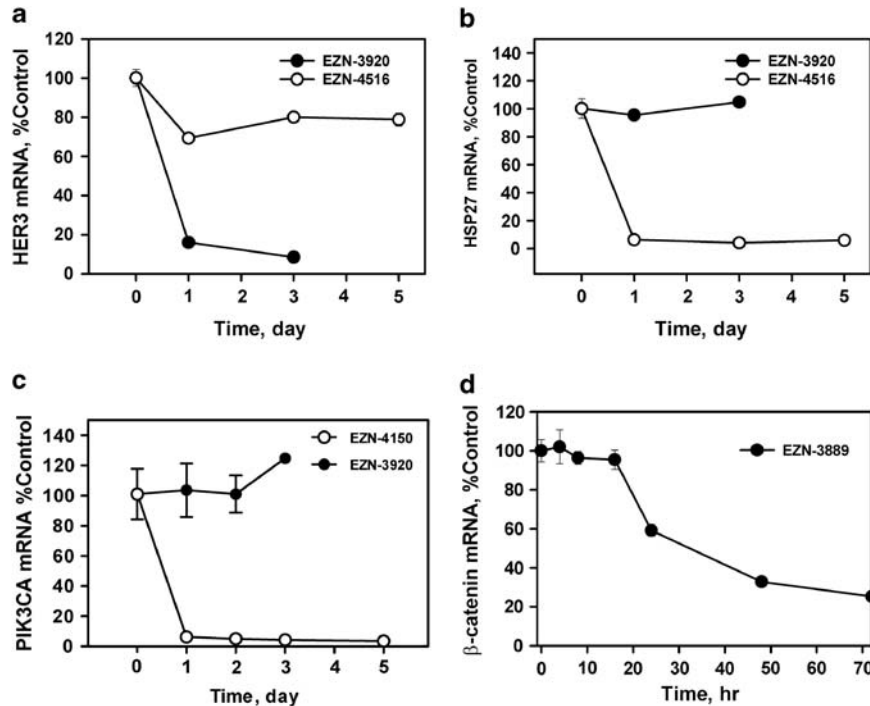


Figure 2 Kinetics of target mRNA down-modulation with naked LNA-ONs. (a, b) 15PC3 was plated at approximately $2,000\text{ cm}^{-2}$ in six-well plates and cultured for 5 days. At different time points, $5\text{ }\mu\text{M}$ of either EZN-3920 or EZN-4516 were added to designated wells to start the treatment. At the end of day 5, the cells were harvested and total RNA extracted for determining the time course of HER3 (a) or HSP27 (b) mRNA levels. (c) PC3 cells were similarly treated with $5\text{ }\mu\text{M}$ of either EZN-4150 or EZN-3920 and measured for PIK3CA mRNA levels. (d) SW480 were treated with $5\text{ }\mu\text{M}$ of EZN-3889 and measured for β -catenin mRNA levels at different time points. Data are shown as means \pm s.e. ($n=3$). The specificity of EZN-3889-mediated down-modulation of β -catenin mRNA in SW480 is shown in Supplementary Figure S2.

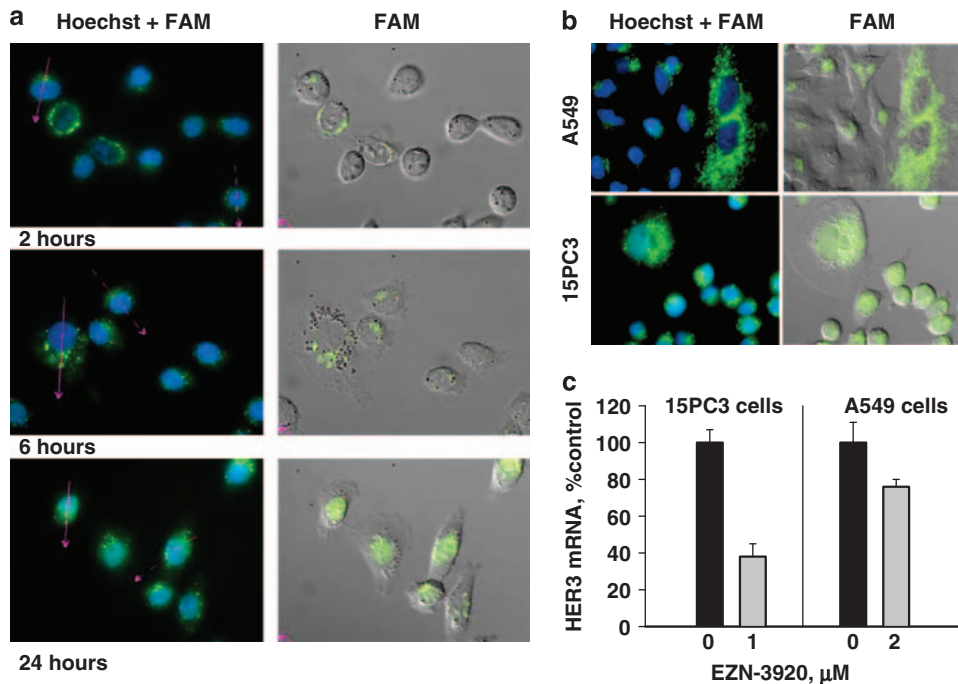


Figure 3 Nuclear localization of LNA-ONs correlated with mRNA down-modulation. (a) 15PC3 cells were treated with FAM-labeled EZN-3920 for the designated time and examined using fluorescence microscopy for FAM and Hoechst signals. Cells with apparent nuclear staining of FAM-EZN-3920 are indicated with arrows. (b) Comparison of FAM and Hoechst signals detected in 15PC3 and A549 cells after cells were incubated in the media containing $2\text{ }\mu\text{M}$ FAM-labeled EZN-3920 for 24 h. (c) 15PC3 and A549 were treated with 1 and $2\text{ }\mu\text{M}$ EZN-3920, respectively. HER3 mRNA levels were examined after 24 h. Error bars indicate s.d. ($n=3$).

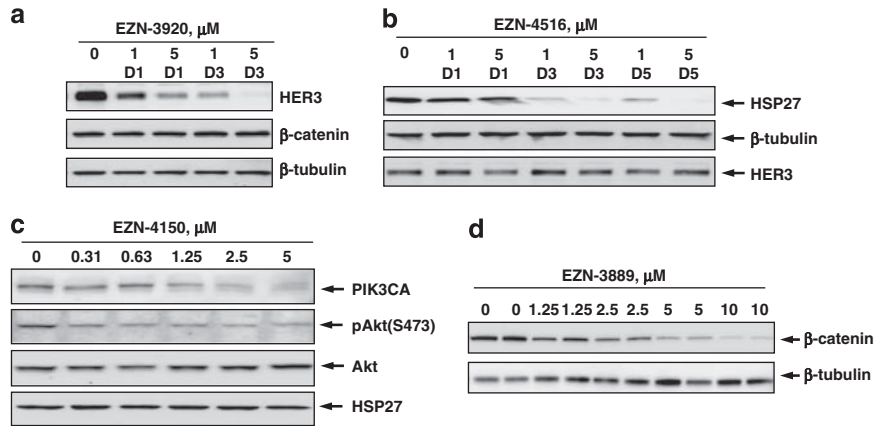


Figure 4 Efficient protein down-modulation by LNA-ONs. (a) 15PC3 cells were treated with 1 or 5 μM EZN-3920 and analyzed for HER3 protein levels by immunoblotting on day 1 or day 5. β -catenin and tubulin were also analyzed for specificity. (b) 15PC3 cells were treated with indicated concentrations of EZN-4516. HSP27 protein levels were analyzed on day 1, 3 or 5. HER3 and β -tubulin proteins were also probed to indicate specificity. (c) 15PC3 cells were treated with indicated concentrations of EZN-4150 for 6 days. PIK3CA, p-AKT, AKT and HSP27 proteins were analyzed by immunoblotting. (d) SW480 cells were treated with indicated concentrations of EZN-3889 (duplicates) for 6 days and analyzed for β -catenin or β -tubulin protein levels by immunoblotting.

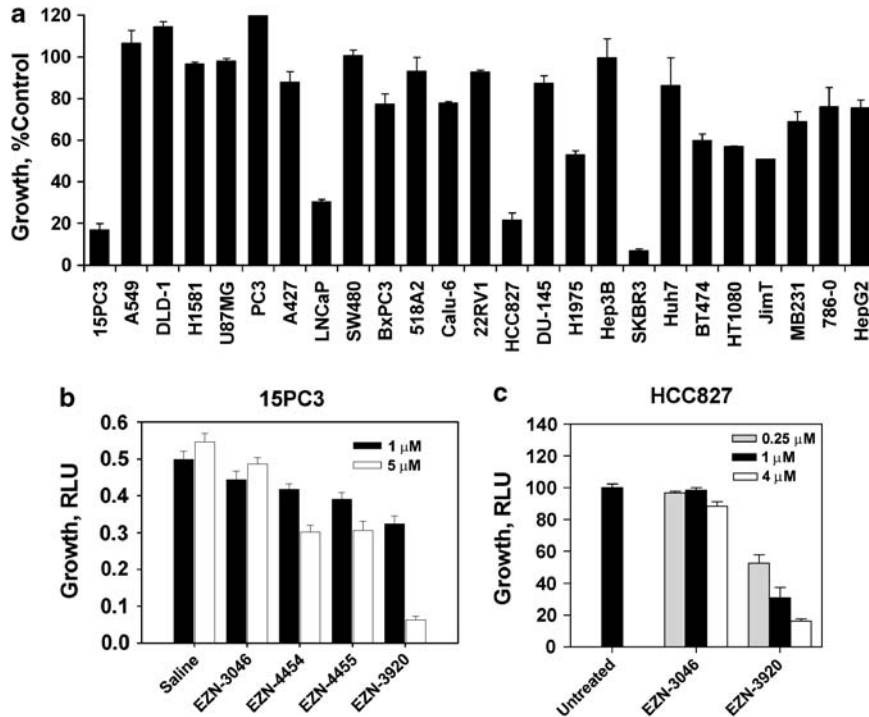


Figure 5 Effect of LNA-ONs on the growth of cancer cells. (a) Multiple cell lines were plated at approximately 1000 cm^{-2} and treated with 2.5 μM EZN-3920. MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed on day 7 to show the effect of compound on the growth of cells. Data are means \pm s.e. ($n=8$); (b, c) 15PC3 or HCC827 cells were treated with 0.25–5 μM EZN-3920 or indicated control LNA-ONs for 6–8 days. The control ONs were a scrambled ON (EZN-3046) and three base pair mismatched compared with EZN-3920 (EZN-4454, 4455). Data are means \pm s.e. ($n=3$).

nuclear signal was not simply because of degraded product of FAM-EZN-3920 that might have freely travelled into the nucleus, we monitored the intact FAM-EZN-3920 level in 15PC3 culture. Our data show that there was no significant degradation of FAM-EZN-3920 during the study (Supplementary Figure S5). Therefore, the signal detected in the nucleus was indeed from intact FAM-EZN-3920.

Potent down-modulation of protein in cells

We next asked if potent mRNA down-modulation would result in protein inhibition. We examined all four targets, HER3, HSP27,

PIK3CA and β -catenin, using LNA-ONs designated. All four LNA-ONs resulted in potent down-modulation of their corresponding target protein in cells with only one-time addition of LNA-ONs to the media (Figures 4a and d). The reduction of protein levels was robust, and in some cases, >90% inhibition of protein expression was achieved (Figures 4a, b and d). Interestingly, the protein down-modulation of HER3 occurred very quickly and in a dose-dependent manner, suggesting a relatively short half-life of HER3 (Figure 4a). Treatment of 5 μM EZN-3920 led to >50% protein down-modulation within 24 h and an even more pronounced effect (>95%) by day 3.

Additionally, the down-modulation of protein was specific. For example, HER3 LNA-ONs potently down-modulated HER3 but not β -catenin (Figure 4a), while a β -catenin-specific LNA-ON down-modulated β -catenin (Figure 4d) but not HER3 (Supplementary Figure S6). Similarly, EZN-4516 treatment resulted in >90% reduced HSP27 protein by day 3 but had no effect on HER3 level (Figure 4b). Interestingly, treatment with a specific PIK3CA LNA-ON resulted in dose-dependent down-modulation of PIK3CA protein. *P*-AKT level also was reduced as expected because it is controlled by PIK3CA (Figure 4c). However, a dose-dependent effect was not evident, suggesting that *P*-AKT may be controlled by multiple upstream signaling pathways. To rule out the possibility that LNA-ONs inhibited protein synthesis through steric blockage of initiation complex, we tested EZN-3920 in an *in vitro* transcription and translation assay. The newly synthesized lumio fusion protein comprised target sequence of EZN-3920 and was readily detected real-time by fluorometer. EZN-3920 and a non-targeting control, EZN-4150, failed to block the synthesis of their respective proteins (Supplementary Figure S7) at concentration as high as 500 nM, consistent with the hypothesis that the down-modulation of protein expression observed in Figure 4 was due to RNase H-dependent degradation of target mRNA.

Inhibition of cell proliferation

Having demonstrated efficient mRNA and protein down-modulation, we decided to test the effect of LNA-ONs on the growth of a battery of cells. The results may shed some light on the importance of particular target in cancer cells and guide us in selecting potential tumor models for *in vivo* testing. For proof-of-principle, we tested HER3 LNA-ON in growth assay. To our surprise, among 30 cell lines tested, only a few

lines showed sensitivity toward HER3. All the sensitive cell lines contain relatively high HER3 expression, suggesting HER3 may have a major role in the growth of these cell lines. To show that the growth inhibition by EZN-3920 treatment was specific, we also tested multiple control LNA-ONs (Figure 5b), which failed to show significant growth inhibition in 15PC3. Similarly, in another sensitive cell line (HCC827), growth inhibition was only observed with the treatment of EZN-3920 but not a control ON, EZN-3046 (scrambled sequence of survivin antisense EZN-3042), indicating that the inhibition was specific (Figure 5c).

In vivo down-modulation HER3 mRNA and protein

Treatment of LNA-ONs in the absence of transfection agent resulted in efficient target inhibition in cell culture. This helps explain why LNA-ONs given in saline can down-modulate target mRNA in liver, which has been shown using an anti-HIF-1 α LNA-ON.²¹ To further validate the utility of this approach *in vivo*, we tested our LNA-ONs in a 15PC3 xenograft model. EZN-3920 dissolved in saline was intravenously injected into nude mice-bearing tumor at different doses and schedules (qd and q3d). Saline and EZN-3046 were used as controls. When the studies were terminated, tumor samples were collected for examination of mRNA and protein levels. In these short-term studies, we observed no apparent tumor growth inhibition using these dosing schedules. However, after 5 daily intravenous administration (qd \times 5), EZN-3920 (3–80 mg kg⁻¹) down-modulated HER3 mRNA level in the 15PC3 tumors in a dose-dependent manner compared with the saline control (Figure 6a). This effect appeared to be specific because EZN-3920 did not affect the expression level of HIF-1 α and EZN-3046 did not inhibit the HER3 mRNA in tumors even at 80 mg kg⁻¹ (Figure 6b)

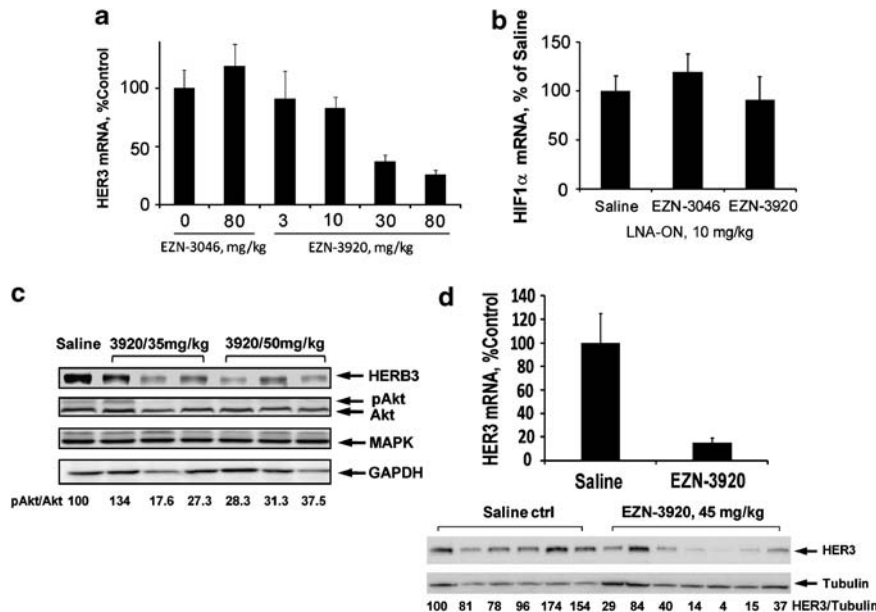


Figure 6 *In vivo* mRNA and protein down-modulation. (a, b) Nude mice-bearing 15PC3 tumors were injected with indicated amount of EZN-3920 or EZN-3046 control (iv, qd \times 5). Tumors were harvested and analyzed for HER3 mRNA on day 6. mRNA down-modulation of HER3 or HIF-1 α in 15PC3 tumors were determined by RT-quantitative PCR (qPCR) with TaqMan gene expression assays. Data are means \pm s.e. ($n=8$) (c) Nude mice-bearing 15PC3 tumors were injected with indicated amount of EZN-3920 (iv, q3d \times 6). Two days after the last dosing, the tumor tissues were harvested. Protein levels of HER3, p-AKT, AKT, mitogen-activated protein kinase (MAPK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in tumor samples were analyzed with western blot analysis. Data from individual animals are shown. Relative quantitation of pAkt and Akt levels (indicated at the bottom) were carried out by using Multi Guage Image Analysis software (Fujifilm Life Science, Minato-ku, Tokyo, Japan). (d) Nude mice-bearing 15PC3 tumors were injected with indicated amount of EZN-3920 (iv, q3d \times 6). Two days after the last dosing, the liver tissues were harvested. The top panel shows the relative mRNA level of HER3 determined by RT-qPCR. Protein levels of HER3 and tubulin were analyzed with western blot analysis (lower panel). Data from individual animals are shown. Quantitation of relative HER3 levels was carried out by using the Multi Guage Image Analysis software. The numbers denote the % of normalized ratios of HER3 over tubulin.

while similar dose of EZN-3920 down-modulated about 90% of HER3 mRNA in the liver (data not shown). The target down-modulation in tumors by EZN-3920 was confirmed by HER3 protein examination using western blot (Figure 6c). These results suggest that EZN-3920 can be systematically delivered to the tumors and can specifically inhibit the target expression without any delivery agent. Inhibition of HER3 also resulted in the reduction of *P*-AKT level but no apparent changes in mitogen-activated protein kinase, confirming the role of HER3 in the activation of phosphatidylinositol 3 kinase/AKT pathway (Figure 6c). To confirm *in vivo* activity of EZN-3920, we also examined the level of HER3 in liver harvested from the EZN-3920-treated mice in a separate study. As expected, EZN-3920 significantly down-modulated HER3 mRNA and protein level (Figure 6d). It is also worth mentioning that consistent with *in vitro* studies, systemic administration of EZN-3920 had a long-lasting effect on target mRNA down-modulation. By real-time quantitative PCR assay, we estimated that a single intravenous injection of EZN-3920 could down-modulate HER3 expression in the tumor and mouse liver for at least 4 days (Supplementary Figure S8). In summary, our data validated the approach used in cell-based assays and suggest that the cellular data were useful in predicting *in vivo* target down-modulation.

DISCUSSION

As ONs do not effectively penetrate cells well, antisense molecules and siRNAs use transfection conditions in tissue culture and various delivery technologies in animals to enhance biological activities *in vivo*.²⁴ However, transfection reagents are known to substantially alter cellular function and thereby significantly complicate the interpretation of the results.²⁵ On the other hand, while much progress has been made in delivery of siRNAs in animals using various methods, their utilities for cancer treatment have yet to be proven.

We have been developing LNA-ONs to several key cancer targets including survivin, HIF-1 α , androgen receptor, Gli2, β -catenin, HER3, PIK3CA and HSP27. LNA-ONs were chosen because these molecules are highly stable in plasma, have extraordinary high affinity to mRNA and potently silence mRNA in cells under transfection conditions (IC₅₀ in the high picomolar–low nanomolar range). The major finding of this work has confirmed²³ and extended the observation that LNA-ONs, when used in the high nanomolar–low micromolar range, readily down-modulated gene and protein of interests in multiple cell lines without any delivery vehicles.²³ Importantly, we also show that the down-modulation efficiency did not diminish but improved over time without further addition of LNA-ONs (Figure 2). Using this approach, we have been able to down-modulate multiple targets in > 30 cell lines. Although earlier generation of antisense such as phosphorothioate ONs can down-modulate target in cells without delivery vehicles, high micromolar concentrations and repeated treatments are usually required to achieve significant target inhibition.^{26–27} Beyond this, phosphorothioate ONs and siRNA molecules are rapidly degraded in tissue culture media or plasma. Furthermore, this study, which was performed in 96-well plates, also demonstrated that LNA-ONs are amenable for high-throughput format to screen cell lines or to assess the therapeutic values of LNA-ONs as single agent or in combination with other anticancer drugs. Therefore, LNA-ONs represent an attractive option for rapid, simple and specific determination of gene function, target validation and therapeutic applications.

LNA-ONs also can facilitate the identification of drug-sensitive tumors for further *in vivo* evaluation. For example, HER3 is an important factor in tumor growth and survival.^{28–29} We show that the growth of multiple HER3-positive lines was reduced with the

treatment of HER3 LNA-ON, EZN-3920, but not the control ONs (Figures 5a–c), supporting that HER3 is a valid oncology target in these cell types. Of course, more definitive validations such as rescue experiments are needed to confirm our findings. Most importantly, when tumor-bearing mice were given EZN-3920 prepared in saline, HER3 mRNA and proteins in tumors were specifically inhibited (Figure 6), demonstrating the ease of use LNA-ONs target inhibition *in vivo*. Biological consequences of this inhibition are being investigated.

LNA-based therapeutics may circumvent the issues associated with delivery vehicles such as toxicity and false-positive results, due in part to improved properties such as stability, affinity and resistance to nuclease digestion. Our data (Figures 6a and c) already show that EZN-3920 down-modulated HER3 mRNA and protein level in tumors without any delivery formulation. However, one drawback of this approach is that certain tumor cell line grown *in vitro* may be preferentially sensitive to LNA-ONs (Supplementary Table). This may be true in tumor-bearing animals as well.

Interestingly, in contrast to a recent finding,²³ we utilized a FAM-labeled EZN-3920 to demonstrate a strong correlation between nuclear localization of ONs and efficiency of down-modulation (Figure 3 and Supplementary Figure S4). Degradation of mRNA by LNA-ONs is primarily mediated by RNase H, which is present in both the cytoplasm and the nucleus.³⁰ The association of efficient mRNA down-modulation with nuclear localization of the LNA ON is consistent with the RNase H located in the nucleus. The mechanisms underlying the nuclear localization of LNA ONs in these sensitive cells are unclear. As ONs can readily shuttle between the cytoplasm and the nucleus,³¹ our data thus suggest the involvement of specific surface proteins in transporting ONs into cells. It also is possible that ONs can escape from restricted compartments, possibly endosomes, via specific yet unidentified certain channels in these specific cell types, allowing effective RNase H-mediated mRNA degradation. This conclusion is further supported by our finding that EZN-3920 was not able to inhibit translation via steric blocking (Supplementary Figure S7). Future studies of sensitive and resistant cell lines may offer an opportunity to identify the underlying mechanisms that promote ON trafficking into the nucleus. This knowledge may facilitate selection of patients with higher probability to benefit from these therapeutic strategies. Moreover, the identification of reagents that can convert insensitive cells to sensitive cells, possibly by enhancing LNA-ONs entry into cells or nucleus, may offer great value for RNA antagonist-based targeted cancer therapy.

MATERIALS AND METHODS

Cell culture

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise mentioned. All cell lines were obtained from ATCC (Manassas, VA, USA) and were maintained according to the ATCC's instruction in medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The 15PC3 human prostate cancer cell line was kindly provided by Dr F Bass (Neurozintuigen Laboratory, Academic Medical Center, Amsterdam, The Netherlands), grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, 2 mM glutamax I and 25 μ g ml⁻¹ gentamicin, at 37 °C in 5% CO₂. The HUH-7 liver cancer cell line was provided by Santaris Pharma (Horsholm, Denmark) and cultured in the above growth medium and supplements plus 1 \times Non Essential Amino Acid (Sigma, St Louis, MO, USA).

Oligonucleotide

All ONs (except EZN-3046) used are 16-nucleotide long gapmers consisting of locked nucleic acid (LNA, minor letters) flanks and a DNA (capital letters) gap.

Table 1 LNA-ONs used in the study

Compounds	Sequences	Targets
EZN-3920	5'-TAGcctgtcacttCTC-3'	HER3
EZN-4454 (control for EZN-3920)	5'-TAGccttgacctCTC-3'	None
EZN-4455 (control for EZN-3920)	5'-TAGcctgtcccatCTC-3'	None
EZN-3046 scrambled control	5'-CGCAGattagaaACCT-3'	None
EZN-3889	5'-CCAtctgtgatCAT-3'	β -catenin
EZN-4516	5'-GGCacagccagtGCG-3'	HSP27
EZN-4150	5'-AGCattcattccACC-3'	PIK3CA

Abbreviations: LNA, locked nucleic acid; ON, oligonucleotide. All ONs except EZN-3889 correspond to complementary coding region of their respective mRNA sequence. All ONs are 100% match to their mouse mRNA. The ONs have fully thiolated backbone. The small caps in the sequences indicate DNA while caps indicate LNA. The quantification of the ONs was performed by measuring OD260 nm value on an UV NanoDrop spectrometry ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Table 1 shows the sequence and corresponding target of each LNA-ON used in the study.

Solid-phase ON synthesis, purification and analysis of LNA/DNA gapper ONs

ONs were synthesized using the phosphoramidite approach on an OligoPilot automated synthesizer (GE Healthcare, Piscataway, NJ, USA) as described previously.²¹ Similarly, 5'-fluorescein-labeled EZN-3920 LNA/DNA gapper was synthesized by 5'-fluorescein phosphoramidite (6-FAM) using the solid-phase DNA synthesis approach described above. However, after cleavage and deprotection in ammonium hydroxide, 5'-fluorescein-labeled EZN-3920 was purified by reversed-phase instead of ion-exchange chromatography. After purification and subsequent desalting by reversed-phase chromatography, 5'-fluorescein-labeled EZN-3920 ON was freeze dried.

Total RNA purification and quantitative real-time PC

The methodologies were described previously.²¹ The TaqMan Gene Expression kits used in the studies include: Hs00356629_g1 (HSP27), Hs00170025_m1 (β -catenin), Hs00176538_m1 (HER3) and Hs00180679_m1 (PIK3CA). In most assays, the relative levels of gene expression were determined by using standard curves and normalized to the endogenous glyceraldehyde 3-phosphate dehydrogenase.

Western blot analysis

The mammalian protein extraction reagent and tissue protein extraction reagent (Thermo Scientific, Rockford, IL, USA) were used for total protein extraction from tumor cell and tissue samples, respectively. Total protein concentrations were measured by Bradford assay (Bio-Rad, Hercules, CA, USA). For western blot analysis, 50–100 μ g of lysates was size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad) and transferred onto polyvinylidene fluoride membranes (Bio-Rad). The blots were subsequently incubated with blocking buffer, primary antibody and second antibody (Promega, Madison, WI, USA) and visualized using enhanced chemiluminescence ECL detection kit (Thermo Scientific). The human antibodies used are as follows: HER3 (sc-285), EGFR (sc-03), Akt (5G3, #2966), p-Akt (ser473, #4051), p-HER3 (Tyr1289, #4791), p-p53 (Ser15, #9284), p-53 (1C12, #2524), were from Cell Signaling Technology, Inc. (Denver, MA, USA) α -tubulin (B-5-1-2, T5168) and anti-glyceraldehyde 3-phosphate dehydrogenase-peroxidase (G9295) were from Sigma. Anti-rabbit immunoglobulin G (H+L) horseradish peroxidase conjugate (W4011) and anti-mouse immunoglobulin G (H+L) horseradish peroxidase conjugate (W4021) were from Promega. Glyceraldehyde 3-phosphate dehydrogenase and α -tubulin served as the loading controls.

Localization of FAM-EZN-3920

Cells (15 000 cells per well in phenol-red free RPMI, containing 10% heat inactivated-fetal bovine serum), were incubated for 48 h at 37 °C (5% humidified CO₂) in two-well coverglass slides (Lab-Tek II Chambered #1.5. Borosilicate Coverslip, VWR International, West Chester, PA, USA). The

medium was refreshed, the FAM-EZN-3920 added to give a final concentration of 5 μ M and incubation continued for 2, 6 or 24 h, as indicated. The nuclear stain, Hoechst 33342 (Sigma Aldrich, St Louis, MD, USA; 2 μ g ml⁻¹), was added to each chamber for 20 min at 37 °C, followed by four washes with warmed Dulbecco's phosphate-buffered saline (containing 0.01% bovine serum albumin, 20 mM HEPES at pH 7.4) to removed all free fluorescent compound. The cells were gently overlaid with fresh, warmed Dulbecco's phosphate-buffered saline (containing 0.01% bovine serum albumin, 20 mM HEPES at pH 7.4) and maintained at 37 °C on a warming microscope stage during image acquisition. Fluorescent or differential interference contrast (DIC) images were acquired within 15 min with a 60 \times oil immersion objective on a Nikon Inverted Eclipse TE2000 microscope (Nikon Instruments Inc., Melville, NY, USA) at 2-, 4- or 8-s fixed exposure times and analyzed for qualitative comparison using Nikon NIS-Elements AR3.0 software, Nikon Instruments Inc. (following fluorescent background compensation and adjustment to identical grayscale ranges at each exposure time).

Cell proliferation assay

Effect of EZN-3920 treatment without lipofectamine on cell growth was evaluated in a panel of tumor cell lines. The cells were plated at low density (500–1000 cells cm⁻²). Appropriate dilutions of EZN-3920 in media were added and cells were cultured for 6–8 days. Cell proliferation was examined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using the Celltiter 96 Aqueous One solution cell proliferation assay kit (Promega) following the manufacturer's instructions. Briefly, 20 μ l of the MTS tetrazolium compound solution was added to the culture in a 96-well plate and incubated for 1–3 h at 37 °C in 5% CO₂ depending on cell density. The 490 nm absorbance values were measured in a Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Tumor xenograft model and ON treatment

For tumor model development, 15PC3 human prostate cancer cells were subcutaneously injected into the right axillary flank of 5- to 6-week-old athymic nu/nu female mice (Harlan Laboratories, Indianapolis, IN, USA). When tumors reached an average volume of 70–100 mm³, the models were used for the ON evaluation. The ONs were injected intravenously at the specified doses and schedules. The ONs were dissolved in saline, and the concentration was determined by measuring optical density 260 nm values. Saline or EZN-3046 served as the control. When the study was terminated, the tumor and liver samples were collected for mRNA and protein analyses. Animal experiments were conducted in the animal facility of UMDNJ (University of Medicine and Dentistry, NJ, USA) in accordance with the current guidelines for animal welfare. All *in vivo* studies were performed according to UMDNJ IACUC protocols.

CONFLICT OF INTEREST

All authors are employees or former employees of Enzon Pharmaceutical Inc.

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