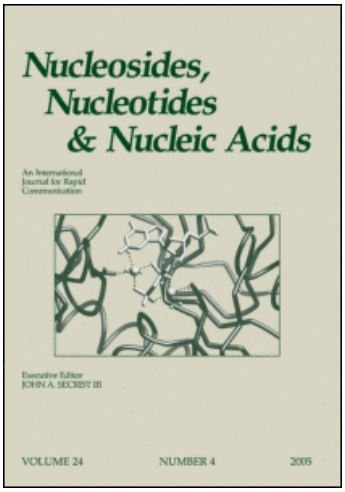

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DOWN-MODULATION OF SURVIVIN EXPRESSION AND INHIBITION OF TUMOR GROWTH IN VIVO BY EZN-3042, A LOCKED NUCLEIC ACID ANTISENSE OLIGONUCLEOTIDE

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□ *Survivin plays an important role in preventing apoptosis and permitting mitosis, and is highly expressed in various human cancers. EZN-3042 is a locked nucleic acid antisense oligonucleotide (LNA-AsODN) against survivin. We report the effects of EZN-3042 in animal models. In a chemical-induced liver regeneration model, treatment with a mouse homolog of EZN-3042 resulted in 80% down-modulation of survivin mRNA. In A549 and Calu-6 lung xenograft models, treatment with EZN-3042 single agent induced 60% down-modulation of survivin mRNA in tumors and 37–45% tumor growth inhibition (TGI). In Calu-6 model, when EZN-3042 was combined with paclitaxel, an 83% TGI was obtained. EZN-3042 is currently being evaluated in a Phase 1 clinical trial as a single agent and in combination with docetaxel.*

Keywords Survivin; locked nucleic acid; antisense; liver regeneration; oligonucleotide; mRNA

1. INTRODUCTION

Survivin, a member of the family of inhibitors of apoptosis proteins (IAPs), has become a compelling molecular target in cancer research since its discovery more than 10 years ago. The mechanistic role of survivin in cancer cell division and survival appears to place the target in critical networks required for cancer cell survival.[1–3] In particular, as an IAP, survivin inhibits cell death mediated by apoptosis. In addition, the protein is up-regulated

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All authors are full-time employees of Enzon Pharmaceuticals and own company's stock options and/or units.

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during cell division, is associated with centrosomes and mitotic spindle microtubules, and controls chromosome spindle-checkpoint assembly, thereby insuring proper cell division.

Survivin is an attractive target for down-regulation in cancer therapy, as it is overexpressed compared with normal tissues in a variety of cancers, including those derived from prostate,[4] lung,[5] breast,[6] colon,[7] stomach,[8,9] pancreas,[10] liver,[11] uterus,[12] and ovary.[13,14] Further, survivin is undetectable in most adult tissue[15] (exceptions are CD34+ stem cells, placenta, basal cells of the colonic epithelium, and thymus).[16–19] Overexpression of survivin is correlated with advanced cancer, reduced survival, resistance to therapy and accelerated recurrences.[4–7,20–24] In preclinical models, inhibition of survivin expression (e.g., by antisense methodology) or inhibition of survivin function (e.g., by in vitro inhibition of cyclin-dependent kinase 1 [CDK1]-mediated phosphorylation of the protein) leads to cell death.[25–28] This is particularly apparent when CDK1 inhibition is combined with paclitaxel therapy.[25]

Like many targets in oncology, survivin is devoid of enzymatic activity and functions by protein-protein interaction within the cell. While indirect inhibitors using small molecules have been pursued, direct inhibitors using RNA antagonism may be the only effective way to specifically target survivin. While many molecules have been claimed to inhibit the expression or function of survivin, most small-molecule antagonists indirectly affect survivin.[29,30] Direct inhibitors of survivin include gene-therapy approaches and antisense oligonucleotides (AsODNs). Currently, one AsODN (LY2181308[31]) and one small-molecule inhibitor (YM155[29,32,33]) are being evaluated in early-stage clinical programs in patients with cancer.

Antisense technology has significantly advanced in the last decade. The first and second generations of AsODNs, which have evolved over the past 20 or more years, were made of DNA phosphorothioates coupled with other chemical modifications known as 2′-methoxy modifications of the ribose sugar or 2′-monomethoxyethyl (MOE) modification (such as that used in LY2181308). The thiophosphate group of the phosphorothioate backbone not only increases stability towards nucleases but also improves the pharmacokinetic properties of the oligonucleotide. The latest generation of AsODNs contain an engineered O2′- to C4′-linkage within the ribose sugar.[34,35] This stabilizes, or "locks" the ribose in the 3′-endo structural conformation that is favored for RNA binding; hence, the name "locked nucleic acid (LNA)." LNAs have an exceptionally high binding affinity to RNA compared with conventional DNA oligonucleotides and 2′-MOE-based oligonucleotides. This translates into low single-digit nanomolar or high picomolar IC_{50} values for mRNA down-modulation that have been achieved in cell culture for several LNA-AsODNs.[36,37] Therefore, LNA-AsODNs have potencies similar to those of siRNAs but without the inherent instability of siRNAs.[38] In addition, the

LNA modification substantially improves nuclease resistance and binding affinity which permits reduction in oligonucleotide length.

EZN-3042 is a survivin mRNA antagonist that uses LNA technology to specifically down-modulate survivin mRNA and protein.[37] The compound is designed as a 16-mer LNA gapmer targeting the region comprising the stop codon of the open reading frame in exon 4 of the survivin transcript.[37] Previously, it was shown in transfected cells that EZN-3042 could potently and specifically down-modulate survivin mRNA (IC_{50} in low nM range) and elicit downstream effects on apoptosis and the cell cycle in 15PC3 prostate cancer cells.[37] EZN-3042 was substantially more potent in its ability to inhibit tumor cell growth and to induce apoptosis and cell-cycle arrest compared to LY2181308.[37] In vivo, EZN-3042 also demonstrated activity in a prostate xenograft model when used in combination with paclitaxel.[37]

Here, we report that systemic administration of EZN-3042 induced down-modulation of survivin was associated with the growth inhibition of lung tumors implanted into nude mice. EZN-3042 also enhanced the antitumor effects of the small-molecule drug, paclitaxel. Further, we show that EZN-3042 potently and specifically down-modulated survivin mRNA in a liver regeneration model.

2. MATERIALS AND METHODS

2.1. Cell Lines and Animals

Lung cancer cell lines (Calu-6 and A549) were obtained from American Type Culture Collection (Manassas, VA, USA) and were grown in minimum essential medium (Eagle) or Ham's F12K medium, respectively, supplemented with 10% fetal bovine serum at 37°C.

Seven- to 8-week-old female BALB/c homozygous and 4- to 5-week-old female athymic nude mice were purchased from Harlan (Indianapolis, IN, USA). All animal studies were approved by the University of Medicine and Dentistry New Jersey Institutional Animal Care and Use Committee.

2.2. LNA Oligonucletides Synthesis

Oligonucleotides were synthesized using the phosphoramidite approach on an AKTA Oligopilot RNA/DNA synthesizer at 1 to 8 millimol scale. Detritylation was performed with 3% dichloroacetic acid (DCA) in toluene (v/v). Coupling step was carried with 0.3M solution of -5-(benzylthio)-1H-tetrazole in dry acetonitrile for 3-minutes and 10-minutes recycle time for DNA and LNA, respectively. Sulfurization step was carried out using 0.2 M phenylacetyl disulfide (PADS) in 1:1 acetonitrile (ACN)/3-picoline solution for 3 minutes on the solid support. After final detritylation at 5′-end, solid

support was treated with 20% diethylamine (DEA) in dry acetonitrile for 15 to 30 minutes, followed by base deprotection in concentrated aqueous ammonia for 16 hours at 55°C. The crude oligonucleotides were purified by ion exchange chromatography on a strong anion exchange resin (Source 30 Q). Pure fractions containing full-length product were pooled, pH adjusted, desalted by reversed-phase HPLC and freeze dried to give the final product. The LNA/DNA gapmer oligonucleotides were characterized by ion-pair high performance liquid chromatography and mass-spectrometry (reversed-phase and electro spray-ionization mass spectrometry) and quantified by UV spectrometry at 260 nm prior to use.

EZN-3042 (5′-CTCA atc cat gg CAG c-3′), where upper case stands for LNA and lower case stands for DNA) is a fully phosphorothioated 16-nt LNA/DNA gapmer antisense oligonucleotide. EZN-3042 is complete homologous to human and is designed to target the stop-codon of the open reading frame in exon 4 of the survivin mRNA transcript. EZN-3046 (5′-CGCA gat tag aa ACC t -3′) is a scrambled control LNA/DNA gapmer fully phosphorothioated oligonucleotide with four mismatches in the LNA region and a complete mismatched gap DNA in the middle of the sequence. EZN-3836 (5′-CTCA gca tta gg CAG c-3′) is an anti-survivin LNA oligonucleotide homologous to mouse with six mismatches compared to the EZN-3042 oligonucleotide.

2.3. In Vitro Studies

2.3.1. Transfection

Cells were seeded 24 hours before transfection in either 12- or 96-well plates (Nunc, Rochester, NY, USA) at a density of 100,000 cells (for mRNA and protein analysis) or 10,000 cells (for proliferation assay), respectively. After 24 hours, cells were washed once with PBS and then incubated with various concentration of either EZN-3042 or scrambled control oligonucleotide, EZN-3046, along with 10 μg/mL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in OPTI-MEM medium (GIBCO, Carlsbad, CA, USA). After 6-hours incubation, the transfection mixture was replaced by culture medium, and in vitro assays were performed as described below.

2.3.2. In Vitro Assays

For RT-PCR analysis, after 24-hours transfection, cells in 12-well plates were lysed in lysis/binding solution, and total cellular RNA was extracted using the Ambion RNAqueous kit and converted to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Survivin mRNA was measured in quartiles by relative quantification real-time PCR (qRT) using TaqMan gene expression assay Hs00153353m1 (reporter sequence CAAGAACAAAATTGCAAAGGAAACC) and reference gene GAPDH (4326317E, Applied Biosystems) in 9700HT Fast Real Time PCR System (Applied Biosystems) and analyzed by RQ Manager Software.

For protein analysis, after 48-hour transfection, cells in 12-well plates were lysed in SDS-PAGE sample buffer. 50 μg of total protein of each sample was electrophoresed on 4–20% SDS-PAGE gels at 100 volts for 1 hour and blotted on nitrocellulose membrane in an Electroblotter at 100 mA for 1 hour. The blotted membrane was incubated with 0.1 μg/mL anti-survivin monoclonal antibody (ab-8228, Abcam) and anti-alpha-tubulin antibody (sc-2302; Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C for 16 hours and subsequently in 0.1 μg/mL horseradish peroxidase-labeled anti-mouse IgG antibody at 23°C for 1 hour. After developing in ECL detection reagent (GE Healthcare, Piscataway, NJ, USA), the membrane was read by FUJIFILM LAS 3000 (FUJIFILM, Edison, NJ, USA).

For measuring antiproliferative activity, after 72-hour transfection, cells in 96-well plates were washed once. Subsequently, cell Titer 96 Aqueous One Solution (MTS) reagent (Promega) was added, and the resulting color was analyzed at 490 nm using a Spectramax 340PC reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. In Vivo Studies

2.4.1. Maximum Tolerated Dose (MTD) in Nude Animals

To support xenograft therapeutic efficacy studies, the MTD of EZN-3042 was determined in nude mice. Mice were injected with EZN-3042 i.p. at various dose levels every other day. Mice were monitored daily for mortality and signs of distress (such as lethargy, loss of gait and loss of body weight). Mice were sacrificed when body weight loss was >20% of the pretreatment body weight.

2.4.2. Development of Liver Regeneration Model

Naïve BALB/c mice were injected i.p. with carbon tetrachloride (CCl_4) (1 mL/kg CCl_4 dissolved in olive oil [1:1 volume/volume {v/v}]). At various timepoints, (24, 48, and 96 hours) post-injection of CCl_4, mice were sacrificed and liver was harvested; 5−10 mg of each liver sample was transferred individually into 1 mL lysis/binding solution in 1.5 mL microtubes and minced with microtube pestles. The total cellular RNA extraction, cDNA synthesis and relative qRT-PCR were performed as described above. The mouse survivin mRNA expression was determined by TaqMan gene expression assay Mm00599749_m1 (reporter sequence CACCCGGAAATACACGCTCC-CTCCA) and reference gene mouse GAPDH (4352339E, Applied Biosystems).

2.4.3. In Vivo Efficacy in Liver Regeneration Model

Mice were treated with EZN-3836 (murine homolog of EZN-3042) either twice daily or once daily at two dose levels (50 or 40 mg/kg) for 5 days. The

dosing was initiated 2 days before CCl_4 injection. On Day 3, mice were injected i.p. with 1 mL/kg CCl_4 dissolved in olive oil (1:1 v/v) 30 minutes after the morning treatment. On Day 5 (second day after CCl_4 treatment), 10 mice from each group were sacrificed. Livers were harvested and homogenized, and survivin mRNA levels were analyzed via qRT-PCR.

2.4.4. In Vivo Therapeutic Efficacy in Xenografts

Subcutaneous (s.c.) tumor xenograft models were established by injecting either 1×10^6 Calu-6 or A549 cells/mouse into the right axillary flank of 4- to 5-week-old female nude mice. When tumors reached an average volume of 75 to 200 mm^3, mice received treatment with EZN-3042 or scrambled (control) oligonucleotide, EZN-3046. Treatment generally was administered at the MTD or a dose lower than the MTD. Compounds were given either by i.p. route or via continuous infusion using osmotic pumps. The mice were monitored for tumor sizes and body weights. The animals were euthanized when individual tumor volumes reached >1,650 mm^3. In some studies, a cohort was sacrificed after receiving four to five doses, and tumors were harvested to determine mRNA down-modulation via qPCR.

2.5. Data Analysis

For in vitro mRNA down-modulation studies, the survivin mRNA level was normalized to GAPDH mRNA levels and plotted relative to an average value of the control (saline-treated) samples from the same transfection, which was set to 100%. For in vitro cytotoxicity studies, dose-response curves were generated from the mean of triplicate determinations, and IC_{50} values were obtained using the GraphPad Prism software (Advanced Graphics Software, Encinitas, CA, USA). For efficacy studies, percentage tumor growth inhibition (% TGI) was calculated using the formula $([C - T]/C) \times 100$, where C = mean tumor volume of the control group at a specified time, and T = mean tumor volume of the treatment group at the same time. Differences between treatments were compared using ANOVA with statistical significance $p < 0.05$.

3. RESULTS

3.1. In Vitro mRNA and Protein Down-Modulation

EZN-3042 was a potent inhibitor of survivin mRNA. The estimated IC_{50} values for survivin mRNA down-modulation were approximately <1 nM at 24 hours after transfection in Calu-6 and A549 cells (Figures 1A and 1B). Control oligonucleotide (EZN-3046) with no complementarity to the survivin transcript did not down-modulate survivin mRNA even at the highest

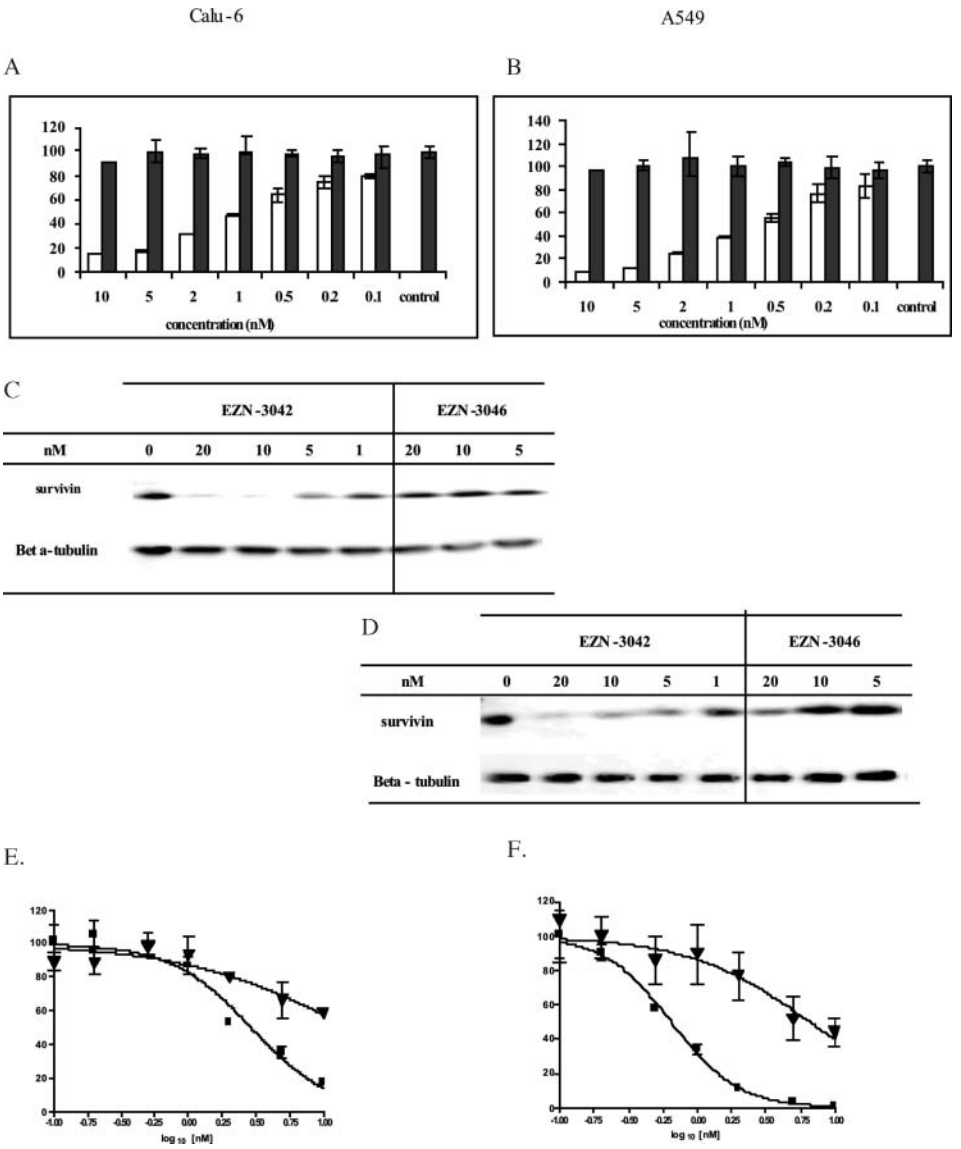

FIGURE 1 A and B) mRNA down-modulation by EZN-3042 in Calu-6 (A) or A549 (B) cells. Serial dilutions of EZN-3042 or control (scrambled) LNA-oligonucleotide, EZN-3046, were transfected via lipofectamine 2000 into cells. Survivin mRNA levels upon treatment with LNA-oligonucleotides were analyzed by qPCR at 24 hours after transfection using GAPDH as the housekeeping control gene. Y-axis (%RQ: relative quantification) represents percentage of mRNA downregulation compared to controls (mock transfected) ± S.D. ($n = 4$). C and D) Protein down-modulation by EZN-3042 in Calu-6 (C) or A549 (D) cells. EZN-3042 or EZN-3046 were transfected into cells, and survivin protein levels were monitored at 48 hours by Western blot analysis. Beta-tubulin was used as a protein-loading control. E and F) Antiproliferative activity of EZN-3042 in Calu-6 (E) or A549 (F) cells. Serial dilutions of EZN-3042 (■) or control LNA-oligonucleotide, EZN-3046 (▲), were transfected via lipofectamine 2000 into cells. After 72 hours, MTS dye was added, and the resulting color was analyzed at 490 nm. The inhibition of growth rate change in OD was plotted against the oligonucleotide concentration relative to saline control, which was set to 100%. Y-axis (% survivival) represents mean ± S.D. ($n = 3$) from one representative experiment.

concentration tested (10 nM). Therefore, EZN-3042-dependent survivin reduction was sequence specific. In addition, survivin protein expression was decreased dose dependently in both Calu-6 (Figure 1C) and A549 cells (Figure 1D) transfected with 20, 10, 5, and 1 nM of EZN-3042, compared to scrambled LNA control, EZN-3046, and to PBS control. The potent mRNA and protein down-modulation of survivin by EZN-3042 translated into strong antiproliferative activity. After 72 hours, the IC_{50} of EZN-3042 in Calu-6 and A549 cells were 2.9 ± 0.28 nM and 0.65 ± 0.03 nM, respectively (Figures 1E and 1F). For the control oligonucleotide, EZN-3046, the antiproliferation effects were seen at high concentrations but, even at the highest concentration tested (10 nM), 60% and 40% cells were still viable in Calu-6 and A549 cells, respectively.

3.2. In Vivo Efficacy in Liver Regeneration Model

Liver injury was induced by treatment with i.p. CCl_4 injection (1 mL/kg CCl_4 dissolved in olive oil [1:1 volume/volume {v/v}]). Under this treatment, expression of survivin mRNA was greatly elevated (34-fold) during the subsequent liver tissue repair (2 days after CCl_4) injection (Figure 2A). The liver regeneration model was used to test if LNA-AsODN targeting survivin could diminish the resulting spike in survivin levels in the regenerating liver tissue. Because EZN-3042 (the LNA-AsODN against human survivin) has six mismatches against mouse survivin, a mouse homolog with a perfect match to the same base positions in the mouse gene (EZN-3836) was made and used for this study. Treatment with EZN-3836 at 50 mg/kg or 40 mg/kg twice daily for 9 doses resulted in ~80% or 60% decrease of survivin mRNA (Figure 2B). Even when given at 40 mg/kg daily for a total of 4 doses, EZN-3836 treatment resulted in a 33% decrease in survivin mRNA. The effect was specific, as the survivin mRNA levels for the control oligonucleotide (EZN-3046) were not reduced significantly compared to the levels for the untreated animals. Furthermore, the specificity also was demonstrated since no down-modulation was achieved by treatment with EZN-3042 (six mismatches to murine survivin; data not shown).

3.3. Determination of MTD of EZN-3042 in Nude Animals

To support xenograft therapeutic efficacy studies, the MTD of EZN-3042 was determined in nude mice. The mice were given multiple i.p. injections of EZN-3042 (q2d × 15) at 10, 25, 50, 75, or 100 mg/kg dose levels. In all dose groups, there was no significant reduction in mean body weight of animals, and no deaths were recorded. Therefore, the MTD of EZN-3042 when given q2d × 15 in nude mice was defined as >100 mg/kg.

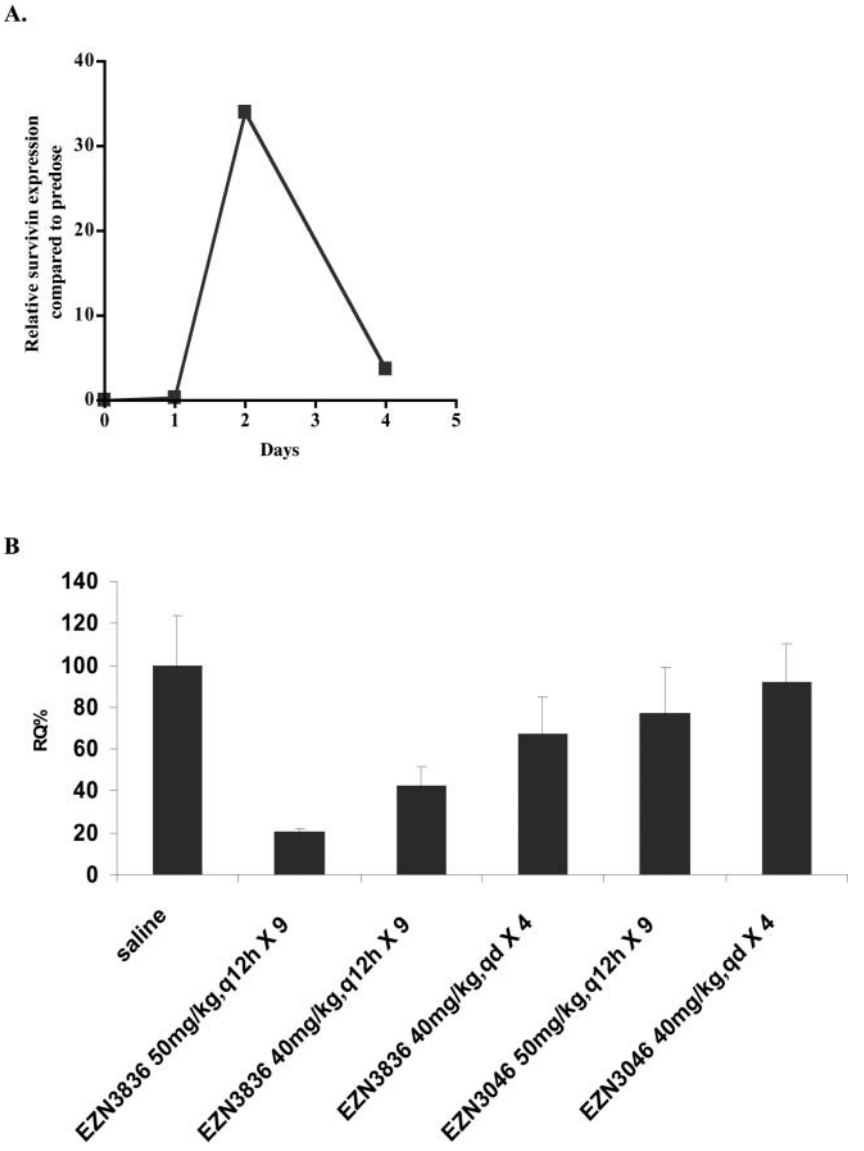

FIGURE 2 A) Liver regeneration mediates a rapid and significant increase in survivin in the liver. Mice were injected i.p. with carbon tetrachloride (CCl4) (1 mL/kg CCl4 dissolved in olive oil [1:1 volume/volume {v/v}]). At various timepoints, (24, 48, and 96 hours) post-injection of CCl4, mice were sacrificed, livers harvested and qRT-PCR performed to determine survivin mRNA expression. Results are expressed as relative survivin mRNA compared to predose ($n = 3$). B) mRNA down-modulation of survivin in liver regeneration model. Mice were treated with EZN-3836 (murine homolog of EZN-3042) at doses and schedules indicated for 5 or 6 days. As a control, mice also received control oligonucleotide, EZN-3046. On Day 3, mice received i.p. 1 mL/kg CCl_4 dissolved in olive oil (1:1 v/v) to induce liver damage. On Day 5 (second day after CCl_4 treatment), 10 mice from each group were sacrificed. Livers were harvested and homogenized, and survivin mRNA levels were analyzed via qRT-PCR. Survivin mRNA levels on treatment with LNA-oligonucleotides were analyzed by qPCR using GAPDH as the housekeeping control gene. Y-axis (%RQ: relative quantification) represents percentage of mRNA downregulation compared to controls (saline treated group) $\pm$ S.D. ($n = 10$).

3.4. Therapeutic Efficacy in Xenograft Models

Single agent in vivo therapeutic efficacy of EZN-3042 was evaluated in xenograft models of human lung cancer (Calu-6 and A549). In the Calu-6 xenograft model, treatment with 100 mg/kg EZN-3042 q2d × 12 was well tolerated by mice and resulted in 37% TGI at end of the study. The tumor volumes were significantly different from controls for the duration of the study ($p < 0.01$ to 0.0005) (Figure 3A). In the A549 xenograft model, EZN-3042 demonstrated significantly better therapeutic efficacy than EZN-3046 (scrambled LNA-AsODN) and saline. EZN-3042 at 100 mg/kg (q2d × 13, i.p.) resulted in a TGI of 42% (Figure 3B). Tumors treated with EZN-3042 100 mg/kg were significantly smaller than controls on Day 27 (last day of the study) ($p < 0.05$). This TGI was specific, as the tumors treated with control LNA-AsODN, EZN-3046, had no effect on growth inhibition. When EZN-3042 was given via a continuous infusion for 7 days, a TGI of 45% was obtained (Figure 3B). Additionally, EZN-3042 treatment as a continuous infusion also resulted in 60% down-modulation of survivin mRNA compared to control (Figure 3C).

Inhibition of survivin could potentiate cancer chemotherapeutic agents that activate apoptotic pathways and/or induce cell-cycle arrest, which is consistent with studies that have shown down-modulation of survivin sensitizes cancer cells to chemotherapeutic agents. Since paclitaxel induces apoptosis and G2/M cell-cycle arrest, the effect of EZN-3042 in combination with paclitaxel was examined in the Calu-6 lung xenograft model. When tumors reached an average volume of approximately 100 mm^3, mice began a dose regimen of paclitaxel 17 mg/kg q4d × 3 (starting on Day 1). Groups of animals also were given EZN-3042 100 mg/kg q4d × 3 (starting on Day 2) alone or in combination with paclitaxel. Paclitaxel as a single agent had 57% TGI. EZN-3042 as single agent had 13% TGI, which was less than observed above due to reduced dosing frequency. This was required for optimizing the combination effects with paclitaxel administration. When EZN-3042 was combined with paclitaxel, an 83% TGI was obtained (Figure 4).

4. DISCUSSION

In this article, we describe an LNA-AsODN, EZN-3042, that specifically and potently down-modulated survivin mRNA expression and yielded anticancer effects in A549 and Calu-6 lung cancer models in vitro and in vivo. In vitro, although, control oligonucleotide with no complementarity to the survivin transcript did not down-modulate survivin mRNA, antiproliferation effects were seen at high concentrations. It is possible that nonspecific antiproliferative activity observed at high doses was exacerbated in the presence of lipofectamine or at high concentrations off-target gene downregulation and toxicities occur. However, growth inhibitory and downregulation effects

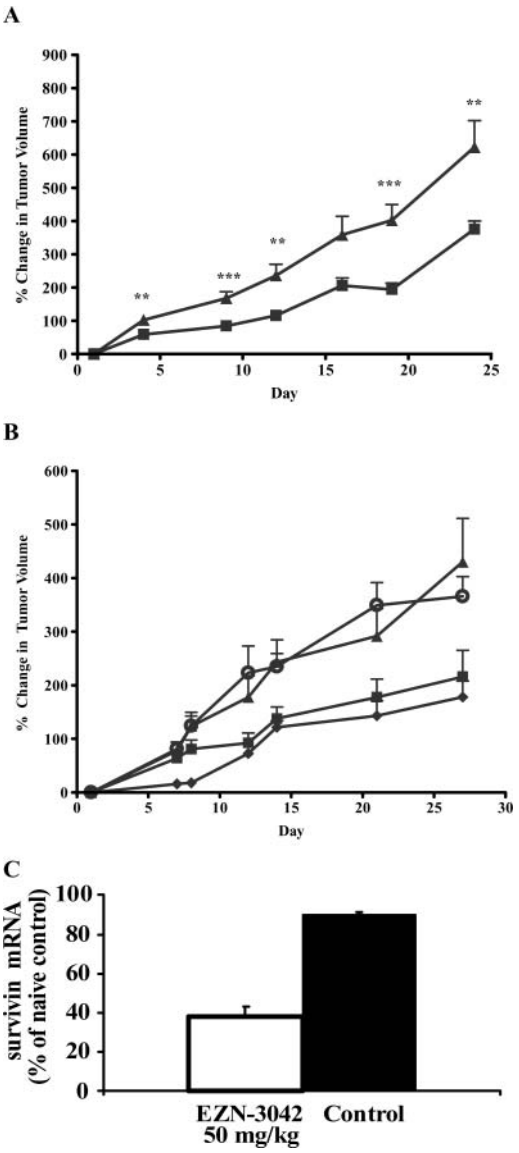

FIGURE 3 Antitumor efficacy of EZN-3042 in xenograft models of lung cancer (A) Calu-6 and (B) A549. Treatment was initiated in xenografts when mean tumor volume reached ~120 mm^3 (A549) or ~270 mm^3 (Calu-6). Mice were treated with either saline (▲), EZN-3042 (■) (100 mg/kg, q2d × 12–13 given i.p.), EZN-3042 (♦) (50 mg/kg, given as continuous s.c. infusion for 7 days) or control oligonucleotide, EZN-3046 (○) (100 mg/kg, q2d × 13 given i.p.). Body weights and tumor volumes were monitored twice weekly during the study. Data represent mean percentage of change in tumor volume ± standard deviation ($n = 10$). $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.0005$. C) mRNA down-modulation of survivin in A549 tumor samples. Mice injected with EZN-3042 50 mg/kg as a continuous s.c. infusion for 7 days using an Alzet pump. Twenty-four hours following pump removal, five mice were euthanized. Tumor samples from each mouse were collected, cut into 1- to 5-mm^3 pieces, placed in RNAlater solution, and analyzed later by qRT-PCR.

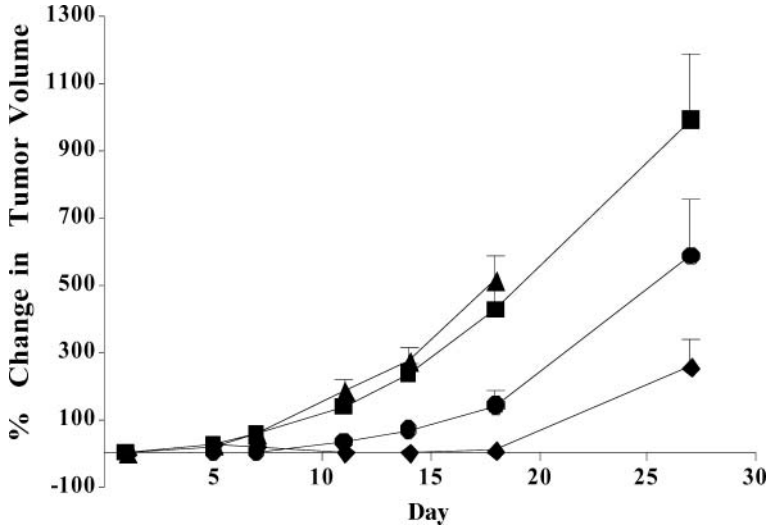

FIGURE 4 Antitumor efficacy of EZN-3042 in combination with paclitaxel in Calu-6 lung xenograft model. Mice were treated with either saline (▲), EZN-3042 (■) (100 mg/kg, q4d × 3), paclitaxel (●) (17 mg/kg q4d × 3), or the combination of paclitaxel and EZN-3042 (◆). Paclitaxel treatment preceded EZN-3042 treatment. Body weights and tumor volumes were monitored twice weekly during the study. Data represent mean percentage change in tumor volume ± standard deviation ($n = 10$).

of EZN-3042 in vivo in both liver model and xenogrfat models were specific. We used two approaches in vivo to evaluate the efficacy of EZN-3042.

In the first approach to investigate the capacity of EZN-3042 to exhibit anti-survivin down-modulation in vivo, a liver regeneration model for survivin expression was adapted from the method of Deguchi et al.[39] This model was chosen since AsODNs preferentially distribute and accumulate in the liver. Here, we showed that survivin mRNA was significantly elevated during liver regeneration and that a mouse homolog of EZN-3042 potently and specifically down-modulated survivin expression 2 days after induction of liver regeneration.

In an alternate approach using traditional lung xenograft models, we showed that EZN-3042 treatment resulted in TGI that was associated with survivin mRNA down-modulation in treated tumors. The TGI effects were significantly improved when the drug was combined with paclitaxel. Several in vitro approaches using AsODNs or other agents targeting survivin have shown that down-modulation of survivin sensitizes cancer cell lines to chemotherapeutic agents like paclitaxel, etoposide, topotecan, cisplatin and doxorubicin, as well as radiation therapy.[40–45] The effect of paclitaxel probably depends on the release of SMAC/DIABLO, which induces apoptosis by inhibiting X-linked inhibitor of apoptosis (XIAP).[46] Survivin can potentially interact with SMAC/DIABLO and thus abrogates its inhibitory effect on XIAP. Survivin also plays a major role in docetaxel-induced apoptosis in vitro. In prostate cancer cells treated with docetaxel, apoptosis is

induced by the binding of survivin to SMAC/DIABLO and to the mitotic spindle, which induces G2-M arrest.[47] Resistance to docetaxel is associated with increased levels of survival,[47] and chemoresponse to docetaxel is associated with expression of survivin splice variants.[48] In vitro, docetaxel resistance was reversed in gastric cancer cells when mRNA expression of survivin was down-modulated by gambogic acid, a survivin inhibitor.[49] In addition to chemotherapeutics, sensitization to radiation therapy by inhibiting survivin expression with a ribozyme or an AsODN has been demonstrated in melanoma cells[50] and lung cancer cells,[51] respectively.

Besides these two approaches, a more appropriate strategy would be to evaluate the efficacy of EZN-3042 in orthotopic animal models. Since LNA-AsODNs reside for long duration in the liver, kidney and skin, the utility of EZN-3042 in orthotopic tumor models of these organs should be explored further.

Hansen et al., have previously suggested that EZN-3042 (or SPC-3042) may also downregualte Bcl-2 mRNA and protein levels.[37] The authors could not define the mechanism behind this phenomenon but suggested that evidently it is related to the specific sequence of SPC3042. The authors also concluded that as a link between transcription control of survivin and Bcl-2 is not evident, the observed effect on the Bcl-2 transcript is possibly not a direct result of survivin inhibition. In the current work, we did not evaluate the effects of EZN-3042 on Bcl-2. However, the efficacy of EZN-3042 could likely be due to multiple mechanisms of actions and further studies should explore these mechanisms.

Currently only a few therapeutic agents selectively and effectively inhibit the synthesis of survivin. An antisense molecule that specifically inhibits survivin mRNA expression (LY2181308) has recently completed Phase I trials. Although clinical activity was limited to stable disease, reduction in survivin expression in tumors after dosing was documented.[31] Two other small-molecule transcriptional repressors of survivin (YM155 and EM-1421) have been described. Preliminary activity of YM155, has been noted in Phase 1 trials in prostate cancer and large-cell lymphoma[29,32] and in a Phase 2 trial in patients with non-small-cell lung cancer.[33]

The doses of EZN-3042 used for the animal efficacy study are approximately 50–100 mg/kg when the compound is given every other day (in xenograft models) or daily and twice daily up to a 5-day cycle (liver knockdown studies). Since certain phosphorothioate-containing LNA oligonucleotides have been reported to induce inflammation or liver toxicity in mice,[52] gross observation for signs of toxicity were studied. No lethality, significant weight loss, or gross pathological changes in the liver were observed in the efficacy experiments. Therefore, further examination of liver toxicity as assessed by changes in alkaline phosphatase, alanine aminotransferase, or histological studies was not done in these studies, but merits attention in the future.

Based on the data presented in this manuscript, a Phase 1, open-label, non-randomized, dose-escalation single-agent and combination with docetaxel study to determine the MTD, safety and pharmacologic profile of EZN-3042, is ongoing in patients with advanced solid tumors or lymphoma. In conclusion, EZN-3042 is a potent and selective LNA-AsODN against survivin that has tumor growth inhibitory effects both as single agent and in combination with paclitaxel in animal models.

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