Large Molecule Therapeutics

Downregulation of HER3 by a Novel Antisense Oligonucleotide, EZN-3920, Improves the Antitumor Activity of EGFR and HER2 Tyrosine Kinase Inhibitors in Animal Models

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Abstract

Among the four human EGF receptor (HER) family members (EGFR, HER2, HER3, HER4), HER3 is of particular interest as it interacts with HER2 and EGFR via heterodimerization and is a key link to the phosphoinositide 3-kinase (PI3K)/AKT signal transduction axis. Recent studies indicate that HER3 plays a critical role in mediating resistance to agents that target EGFR or HER2. As HER3 lacks significant kinase activity and cannot be inhibited by tyrosine kinase inhibitors, neutralizing antibodies and alternative inhibitors of HER3 have been sought as cancer therapeutics. We describe here a locked nucleic acid (LNA)-based HER3 antisense oligonucleotide, EZN-3920, that specifically downmodulated the expression of HER3, which was associated with growth inhibition. EZN-3920 effectively downmodulated HER3 expression, HER3-driven PI3K/AKT signaling pathway, and growth in tumors derived from BT474M1 breast and HCC827 lung carcinoma cell lines, which overexpress HER2 and EGFR, respectively. Furthermore, when EZN-3920 was coadministered with gefitinib or lapatinib in xenograft tumor models, enhanced antitumor activity compared with the effect of monotherapy was found. The effect was associated with a blockade of induced HER3 mRNA expression caused by lapatinib or gefitinib treatment. Finally, EZN-3920 sustained its antiproliferative effect in trastuzumab-resistant cells and three independently derived gefitinib-resistant cells. Our findings show that downmodulation of HER3 by EZN-3920 leads to the suppression of tumor growth in vitro and in vivo, suggesting that HER3 can be an effective target for the treatment of various cancers that have been activated by HER3 alone or where HER3 activation is associated with EGFR or HER2 expression.

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Introduction

Members of the human EGF receptor (HER) family of receptor tyrosine kinases (RTK) designated as HER1 (EGFR), HER2 (ErbB2 or p185HER2/neu), HER3, and HER4 underlie the pathogenesis of many types of human cancer (1–4). Extracellular ligand binding initiates signaling by causing specific homodimeric or heterodimeric receptor formation and activation of the cytoplasmic kinase domain that phosphorylates tyrosines in the tail region of each receptor. The phosphorylated HER members become a docking station for various proteins and thereby activate at least 3 major intracellular signaling cascades (RAS/ERK, AKT/PI3K/PTEN, and JAK/STAT), which ultimately regulate cell growth, differentiation, migration, and survival.

Hyperactivation of EGFR or HER2 are already known to mediate tumor growth. Consistent with this, small molecules and antibodies that bind to ErbB1/EGFR (gefitinib, erlotinib, erbitux) and/or HER2 (e.g., trastuzumab, lapatinib) have already proven anti-cancer activity. However, in the past few years, new data have revealed that HER3 plays a vital role in cancer growth and progression (5–8). HER3 is overexpressed in various cancers, including breast, colon, gastric, prostate, lung, hepatocellular, head and neck cancers, and melanoma, and is implicated in tumorigenesis and prognosis (7–11). It is now clear that heregulin binding to HER3 or HER3 dimerization with EGFR or HER2 leads to hyperphosphorylation of cytoplasmic tail of HER3, which in turn results in AKT signaling (12–14). Beyond this, HER3 can be hyperphosphorylated in patients with gefitinib-resistant cancer (4) as well as lapatinib- or trastuzumab-resistant tumor cell lines via receptor tyrosine kinases (15, 16). The mRNA

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encoding HER3 is also increased in response to treatment of cells with HER1 inhibitor (gefitinib), HER1/2 inhibitors (lapatinib), AKT inhibitors, phosphoinositide 3-kinase (PI3K) inhibitors, or anti-androgens (15–20).

Experimental downmodulation of HER3 using genetic or pharmacologic methods supports the notion that HER3 is an important driver in cancer growth. Consistent with this, reduced HER3 expression by conditional knockout or LNA-based antisense molecule, designated as EZN-3920, in a polyoma-T model of breast cancer inhibits PI3K activation and tumor formation in this genetically engineered mouse model (21). In addition, siRNA and short hairpin RNA (shRNA)-directed downmodulation of HER3 resulted in tumor growth inhibition in lung adenocarcinoma (22) and decreased disease progression as well as prolonged survival in xenograft mouse models of ovarian cancer (13) or of HER2-overexpressing breast cancer (5). Furthermore, a recent study showed the sensitivity of a subset of head and neck cancers to HER2 kinase inhibitor is dependent on the co-expression of HER3 and its ligand neuregulin-1 (14). In vitro, targeting HER3 using siRNAs and antisense deoxyoligonucleotides (AS-ODNs) potently inhibits proliferation and promotes apoptosis in cells sensitive and insensitive to EGFR and HER2 inhibitors (5, 13, 17, 22, 23). Finally, a monoclonal antibody to HER3 inhibited tumor growth, especially in cell lines that secrete heregulin, a ligand for HER3 (12).

In this study, we have further explored the use of the HER3 antisense oligonucleotide EZN-3920, which is an LNA-ASO. LNA-ASOs have shown very high binding affinity to mRNA, excellent potency for target mRNA downmodulation, improved resistance to nuclease digestion, and excellent stability in plasma and tissues in preclinical studies (24). Furthermore, LNA-ASOs simply prepared in saline are highly effective in vitro and in vivo (25–29). It is shown here that EZN-3920 suppressed tumor growth both in vitro and in vivo in tumors that co-express HER1 or HER2 or develop resistance to HER1/2 inhibitors. The evidence suggests that the efficacy of EZN-3920 alone or in combination with other anti-HER drugs such as lapatinib, trastuzumab, or PI3K inhibitors may overcome emerging resistance to agents that target the HER/PI3K/AKT axis.

Materials and Methods

Cell culture

Cell culture reagents were purchased from Invitrogen unless otherwise mentioned. All cell lines unless otherwise motioned were obtained from American Type Culture Collection (ATCC) and were maintained according to ATCC’s recommendations in a medium supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO2 and 95% air. HCCS27gr6 cancer cells were obtained from Dr. Jeffrey Engelman’s laboratory (Department of Medicine, Harvard Medical School, Charlestown, MA) grown in RPMI-1640 plus 10% FBS. Both PC9 and PC9GR cancer cells were obtained from Pasi A. Jänne’s laboratory (Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA) and grown in RPMI-1640 containing 10% FBS. BT474M1 breast cancer cells were obtained from California Pacific Medical Center and maintained in DMEM/F12 (1:1) containing 10% FBS. The 15PC3 human prostate cancer cell line was provided by Dr. F. Bass (Neurointuigen Laboratory, Academic Medical Center, Amsterdam, the Netherlands) and maintained in Dulbecco’s Modified Eagles’ Media (DMEM) plus 10% FBS. Authentication of used cell lines was not done by the authors.

Synthesis of oligonucleotide

Oligonucleotides were synthesized as described previously (26). EZN-3920 (5'-TAGctgctacctCTC-3') is a fully phosphorothioated oligonucleotide. Capital letters denote LNA monomers, and lowercase letters denote DNA monomers. 5'-Cy5.5-labeled EZN-3920 and a scrambled oligonucleotide, designated as EZN-3046 (5'-CCGagtagaAAcct-3') and abbreviated as EZN-SCR, also were made according to the method described previously (29).

mRNA downmodulation in human xenografts

Twenty-four hours post-last dose, tumors were harvested and cut into 3- to 5-mm3 pieces. Five to 10 mg tumor sample was transferred into 1 mL lysis/binding solution in 1.5 mL Lysing Matrix D tubes and homogenized in Fastprep (MP BIO). RNA purification, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) were carried out as previously described (26). Targets and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were quantified using TaqMan Assay Kits (Applied Biosystems) according to the manufacturer’s instructions. The following probes were used for qRT-PCR: Hs00176538_m1 for HER3, 4326317E for GAPDH, Hs00193306_m1 for EGFR1, and Hs00170433_m1 for HER2.

Western blot analysis

Western blot analysis was conducted as previously described (29). The antibodies used are as follows: HER3 (sc-285), EGFR (sc-03), Akt (5G3, #2966), p-Akt (ser473, #4791), p-p53 (Ser15, #9284), and p53 (1C12, #2524). All antibodies were derived from Cell Signaling Technology, Inc. α-Tubulin (B-5-1-2, T5168) and anti-GAPDH-peroxidase (G9295) were purchased from Sigma. Anti-Rabbit IgG (H + L) HRP conjugate (W4011) and anti-mouse IgG (H + L) HRP conjugate (W4021) were purchased from Promega. GAPDH and α-tubulin served as the loading controls.

Localization of Cy5.5-labeled EZN-3920 in BT474M1 tumors

Once the BT474M1 tumors reached an average volume of 200 mm3, tumor-bearing mice were injected i.v. with 40 mg/kg unlabeled or Cy5.5-labeled EZN-3920 on day 0 (first day of dosing) and day 4. The tumors were excised 24 hours later and imaged whole by the
Xenogen Lumina as described previously (29). Following imaging, tumors were cryopreserved with 4% paraformaldehyde, sectioned, and imaged with fluorescent microscopy.

**Determination of the concentration of EZN-3920 in tumors**

An LC/MS/MS system (29), 4000 QTrap mass spectrometer (AB Sciex) interfaced with an AcQuity UPLC (Waters), was used to determine EZN-3920 concentration in tumor tissues. Briefly, analyte EZN-3920 and an EZN-4176 (internal standard) were eluted on Clarity 3u Oligo-RP column (50 × 2.00 mm², Phenomenex) with a linear gradient of mobile phase A/B from 80/20 to 50/50 in 8 minutes, and mobile phase A consisting of 0.4 mol/L hexafluoro-2-propyl (HFIP) and 16.3 m mol/L triethyl amine (TEA) in 5% methanol aqueous solution and mobile phase B consisting of 0.4 mol/L HFIP and 16.3 m mol/L TEA in 60% methanol. The flow rate was 0.15 mL/min. Column temperature was set at 65°C. The turbo IonSpray source was operated with negative mode at 40°C. The ion transitions of m/z = 651.5 (precursor ion) to m/z = 95 (product ion) for EZN-3920 and m/z = 751.8 (precursor ion) to m/z = 95 (product ion) for EZN-3920 were monitored, and declustering potential and collision energy were optimized to DP = −40 V and CE = −100 V (EZN-3920), and DP = −60 V and CE = −100V (EZN-4176).

**Results**

**Selection of EZN-3920 and models for in vitro studies**

EZN-3920 was designed to bind with 100% complementarity to HER3 mRNA and initiate RNase H–mediated degradation of mRNA. An initial exploration of the activity of EZN-3920 was one in an in vitro cell–based assay where EZN-3920 was added to cells in the presence of transfection agent. It downmodulated HER3 mRNA in 15PC3 and HuH-7 cells with an IC50 of 0.24 and 0.36 nmol/L, respectively (Supplementary Table S1). EZN-3920 also showed potent target and growth inhibition in a panel of cancer cell lines (Supplementary Table S2). In 15PC3 prostate cancer cells, HER3 and p-AKT levels were decreased (IC50 < 5 nmol/L; Supplementary Fig. S1A) and concomitantly caspase-3/7 activities increased (Supplementary Fig. S1B) in response to the treatment of EZN-3920. The data were consistent with the function of HER3 in cancer cells. Recently, we also showed that EZN-3920 was effective in downmodulating mRNA and HER3 protein in the absence of lipofection (30). The effect was specific as EZN-3920 did not downmodulate EGFR or HER2 (see below). Beyond that, target inhibition in tumors derived from 15PC3 cells after i.v. administration of EZN-3920 was detected (30). In addition, the compound downmodulated HER3 and inhibited tumor growth in a mouse model of mammary carcinoma driven by the polyomavirus middle T oncogene (21). On the basis of this initial favorable activity, we decided to extend our efficacy studies in additional models in which HER3 plays a key role in tumor development.

To find suitable models for in vivo studies, we screened multiple cell lines that may depend on HER3 for growth (4, 5, 13, 31–33) and tested the effect of EZN-3920 on the growth of these lines. As transfection is highly artificial as recently reported (34) and does not represent the context of our in vivo experiments in which no transfection systems were used, the remainder of the in vitro studies were conducted without lipofection. Table 1 shows that HER3 was highly expressed in BT474M1, SKBR3, and HCC827 when compared with OVCA5 and A549. EZN-3920 showed potent growth inhibition in BT474M1, SKBR3, and HCC827 cells (Table 1) but far less or no activity in OVCA5 or A549 cells. This observation allowed us to focus on the characterization of the effect of EZN-3920 in BT474M1 and HCC827 models as, in our hands, SKBR3 is not tumorigenic in xenograft models (data not shown), and the potency of EZN-3920 in OVCA5 and A549 was low. Beyond this, HER2 and EGFR are drivers of cell growth in BT474M1 and HCC827, respectively, as the BT474M1 cell line overexpresses HER2 and HER3 knockdown resulted in tumor regression (5), whereas the HCC827 cell line overexpresses EGFR and is inhibited by gefitinib (35). Figure 1A shows that EZN-3920 effectively and specifically downmodulated HER3 mRNA but not other family members, such as EGFR and HER2, in a dose-dependent manner in HCC827 cells. The specificity was expected as EZN-3920 has 4 and 3 base mismatches with the complementary region of EGFR and HER2, respectively (Supplementary Table S1) and such mismatches typically can result in an inability for downmodulating mRNA (26, 36). This effect was not due to off-target effect of the oligonucleotide backbone as the control oligonucleotide EZN-SCR failed to downmodulate HER3 mRNA in 15PC3 (Supplementary Fig. S2) or HCC827 cells (Fig. 1). In BT474M1 cells, EZN-3920 also showed specific downmodulation of the intended target HER3 but not EGFR nor HER2 (Fig. 1B). To make sure that mRNA downmodulation was associated with reduction of protein expression, we conducted Western blot analysis on the

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Cell line</th>
<th>HER3 expression</th>
<th>IC50b μmol/L</th>
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<tbody>
<tr>
<td>Breast</td>
<td>BT474M1</td>
<td>++</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Breast</td>
<td>SKBR3</td>
<td>+++</td>
<td>0.5 ± 0.003</td>
</tr>
<tr>
<td>NSCLC</td>
<td>HCC827</td>
<td>++</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>Ovarian</td>
<td>OVCA5</td>
<td>+</td>
<td>5 ± 0.71</td>
</tr>
<tr>
<td>NSCLC</td>
<td>A549</td>
<td>+/-</td>
<td>&gt;10</td>
</tr>
</tbody>
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aRelative HER3 mRNA level measured by qRT-PCR: +/−, Ct ≥ 35; +, 30 < Ct < 35; ++, 25 < Ct < 30; ++++, Ct < 25.
bCell growth (6 days) was measured by MTS assay as described in Materials and Methods.
lysates obtained from both cell lines after they were treated with EZN-3920 or EZN-SCR. In both cell lines, treatment with EZN-3920 resulted in dose-dependent downmodulation of HER3 protein and phospho (p)-HER3 levels (Fig. 1C). The reduced expression level of HER3 was associated with deactivation of AKT pathway by EZN-3920 in a dose-dependent manner. The effects were specific as AKT or tubulin levels were unchanged. The downmodulation of HER3 had no effect on HER2 or p-HER2, suggesting the effect of EZN-3920 was specific and the observed decrease in HER3 protein level was sufficient to impact on the important downstream signaling such as p-AKT.

Antitumor activity in xenograft mode

In vivo efficacy of EZN-3920 was first evaluated in the HCC827 lung cancer model. EZN-3920 administered at 30 and 40 mg/kg given twice a week for 4 weeks inhibited the growth of HCC827 tumors by approximately 60% and 80%, respectively, on day 28, whereas EZN-SCR did not inhibit tumor growth (Fig. 2A). To relate tumor growth inhibition to HER3 downmodulation, the effect of EZN-3920 on HER3 was tested in a separate short-term study. After 5 doses of EZN-3920 administered at 30 and 40 mg/kg, 40% and 60% of the human HER3 mRNA in tumors was downmodulated, respectively, based on qRT-PCR analysis (Fig. 2B). Furthermore, Western blot analysis of each individual tumors revealed that HER3, p-HER3,
p-AKT, p-S6K, and cyclin D levels were significantly inhibited (Fig. 2C). The Western blot analysis was further supported by immunohistochemical (IHC) data (Fig. 2D), which showed that the membrane staining of HER3 or p-HER3 was decreased in response to the treatment of EZN-3920. Similar to what was observed in vitro, p-AKT level in both cytoplasm and nucleus of the tumors was reduced (Fig. 2D). The inhibition of HER3 consequently promoted apoptosis (34) as revealed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Fig. 2E), confirming the importance of HER3 in tumor growth and survival.

The efficacy of EZN-3920 was explored in a second xenograft model. In this case, tumors derived from BT474M1 breast cancer cells were used as downregulation of HER3 in this model has been shown to inhibit tumor growth (5). Similar to the HCC827 model, the growth of BT474M1 was also significantly inhibited by EZN-3920 in a dose-dependent manner (Fig. 3A). The observed effect was specific as EZN-SCR was not effective in inhibiting tumor growth. Furthermore, IHC analysis revealed that the growth was associated with the inhibition of the target (Fig. 3B).

Accumulation of EZN-3920 and target downmodulation in tumors

To ascertain that the antitumor effect was associated with the presence of EZN-3920 in the tumors, we evaluated the presence and duration of EZN-3920 in established BT474M1 tumors using LC/MS/MS method (29). Figure 4A shows that the concentration of EZN-3920 in the tumors reached 4 and 8 μmol/L 4 hours after a single dose of EZN-3920 and multiple doses of EZN-3920 (q3dx3), respectively. The concentration of EZN-3920 in
the tumor remained unchanged during the 24-hour period and decreased slightly after 48 hours. These concentrations matched or exceeded the level of EZN-3920 needed to inhibit cell growth in vitro (Fig. 1). We next asked if the compound was, in fact, localized inside the cells. To locate the compound inside the cells, we generated a Cy5.5-labeled EZN-3920 and determined that it was active as shown by HER3 mRNA downmodulation in the liver of mice (Supplementary Fig. S3). Tumor-bearing mice were then injected with 40 mg/kg of Cy5.5-labeled EZN-3920 at 0 and 4 hours. After 24 hours, animals were sacrificed, and tumors were analyzed by fluorescence imaging.

As reported previously (29), a high-intensity signal in the tumor was observed 24 hours posttreatment, indicating localization of Cy5.5-EZN-3920 in tumors (Fig. 4B, bottom). To confirm the observation, we next carried out sectioning of the tumor and microscopically examined the tumor. Figure 4C shows that the compound, Cy5.5-EZN-3920 was present in tumor. Furthermore, the compound was primarily localized inside the cells as it was located around the nuclei [detected by 4′,6-diamidino-2-phenylindole DAPI staining], providing additional evidence to support previously shown target inhibition data.

**EZN-3920 potentiates the effect of tyrosine kinase inhibitor in tumor growth inhibition**

Lapatinib, a small-molecule dual inhibitor of HER2 and EGFR tyrosine kinase activity, is currently used in the treatment of HER2-positive breast cancer. Interestingly, it was shown recently that inhibiting lapatinib causes increases in HER3 mRNA as well as HER3 protein and p-HER3 levels through a negative feedback loop that involves the PI3K/AKT pathway (15–20). This effect ultimately results in resistance to lapatinib. Consistent with...
HER3 mRNA Antagonist

this, a combination of lapatinib with a neutralizing HER3 antibody AMG-888 showed better efficacy than either agent alone (15), and a combination of PI3K inhibitor BEZ235 with trastuzumab also resulted in a greater antitumor effect (16). Therefore, we reasoned that treatment of cancer cells with lapatinib in the presence of EZN-3920 should also yield improved efficacy over a single agent. We thus used BT474M1 as a model system. In BT474M1 cells, lapatinib administered at a clinically achievable level (1 μmol/L; ref. 37) significantly induced HER3 mRNA (Fig. 5A) and HER3 protein levels (Fig. 5B). However, lapatinib-induced HER3 expression was effectively reduced by EZN-3920 in a dose-dependent manner (Fig. 5A and B), but HER3 mRNA expression was not reduced by the scrambled oligonucleotide control. Consequently, the combination of both agents exerted much greater growth inhibition than either agent alone (Fig. 5C). The combinatorial effect appeared to be synergistic as a noneffective dose (5 μmol/L) of EZN-3920 with a moderately effective (40% growth inhibition with EZN-5CR) dose of lapatinib resulted in 60% growth inhibition (Fig. 5C). We next tested the effect of this combination in a tumor xenograft model. Tumor-bearing mice were treated with lapatinib [100 mg/kg, intraperitoneally (i.p.)] in combination with a suboptimal dose of EZN-3920 (30 mg/kg). Lapatinib or EZN-3920 alone showed moderate antitumor effect. However, when combined, the tumor growth was completely inhibited (Fig. 5D). Structure of gefitinib and lapatinib are shown in Fig. 5G and H, respectively.

To further support a combination approach, we conducted a separate study using an EGFR small-molecule inhibitor, gefitinib, which also induces HER3 (19). Similarly, while EZN-3920 or gefitinib exerted significant antitumor activities, the combination resulted in the regression of HCC827 tumor xenograft (Fig. 5E). Analysis of tumor samples (Supplementary Fig. S4) shows that there was a rapid increase in HER3 expression in tumors after mice was treated with gefitinib for only 12 hours. However, this induction was prevented by EZN-3920 treatment (Supplementary Fig. S4). Moreover, as heterodimerization of HER2 with HER3 activates PI3K/AKT pathway, we next tested the combination of EZN-3920 with a PI3KCA (PI3K constitutively active)-specific antisense molecule EZN-4150 (30). The combination of these 2 LNAs showed greater antitumor activity than either agent administered alone (Fig. 5F).

**Antiproliferation activity of EZN-3920 in gefitinib- or trastuzumab-resistant cells**

To explore the potential use of EZN-3920 in drug-resistant lung cancer, we next generated gefitinib-resistant HCC827 through chronic exposure of cells with increasing amount of gefitinib over a 4-month period of time. When the concentration reached 1 μmol/L, the cells were cloned and tested for their responses to gefitinib treatment. Figure 6A (top) shows that all clones were highly resistant to the treatment of gefitinib, up to 10 μmol/L, whereas the IC50 for gefitinib in the parental cells was approximately 10 nmol/L. The results were consistent with the published data (4). Interestingly, the clones showed enhanced sensitivity to EZN-3920 (Fig. 6A, bottom). The results are encouraging as it suggests that EZN-3920 is capable of overcoming resistance induced by small-molecule inhibitors. To confirm the results, we obtained additional gefitinib-resistant cells and tested the effect of EZN-3920 in these cells. Resistance to gefitinib in HCC827GR and PC9GR and were first validated (top of Fig. 6B and C). Interestingly, sensitivity to EZN-3920 was retained in both HCC827GR and PC9GR (bottom of Fig. 6B and C). Beyond that, we also generated trastuzumab-resistant BT474M1 (Fig. 6D, top) after chronic exposure of the cells to increasing concentration of trastuzumab. Figure 6D (bottom) shows that the trastuzumab clone was sensitive to the treatment of EZN-3920. Thus, all the resistant lines remained sensitive to EZN-3920 treatment, suggesting that HER3 plays a centric role in the resistance of different modalities. This growth inhibition in the resistant cells was not simply associated with general cytotoxicity but target inhibition based on mRNA assessment (Supplementary Fig. S5).

**Discussion**

Unlike most members of the HER family, HER3 has a potent ability to activate downstream PI3K and Akt pathway once it dimerizes with HER2, mediate resistance to HER1 or HER2-targeted therapeutics, and play a critical role in cancer growth. Unlike HER1 or HER2, HER3 does not appear to have appreciable kinase activity (38–41), so ATP-mimetic small-molecule inhibitors may not be able to inhibit HER3-mediated tumor growth. On the other hand, inhibition of HER3 activity can also be achieved by antibodies that block ligand binding to HER3 (12). However, as the cytoplasmic tail of HER3 can be phosphorylated and thereby hyperactivated by other growth factors (4), HER3 antibodies may not be effective in all patients, and resistance to these agents is highly likely, as the phosphorylated tail of HER3 still engages the critical PI3K pathway. Beyond this, isoforms of HER2 that are devoid of the extracellular domain and expressed in many breast cancers have been documented (42), and thus may prohibit the use of antibodies that target HER2 or HER2/3 heterodimers. Therefore, an RNA antagonist to HER3 offers a unique solution to controlling HER3-mediated tumor growth. We show here that EZN-3920 is a specific inhibitor of HER3, as it downmodulated HER3 mRNA, protein, and consequently p-AKT levels (Figs. 1C and 2C). Furthermore, we showed that concomitant with HER3 downmodulation, the basal level of p-AKT as well as neuregulin-1-induced p-AKT could be suppressed by EZN-3920 in FaDu head and neck cancer cells (Supplementary Fig. S6). Consistent with the inhibition of p-AKT, p-S6K was also inhibited in neuregulin-1–treated cells. More interestingly, we also noticed the reduction p-MAPK from cells treated with or without neuregulin-1 (Supplementary Fig. S6), suggesting that inhibition of
Figure 5. EZN-3920 potentiated the effect of lapatinib, gefitinib, or PDKCA inhibitor. A, EZN-3920 inhibited lapatinib-induced HER3 mRNA in BT474M1 cancer cells as determined by qRT-PCR. *, P < 0.05 compared with untreated group. B, EZN-3920 inhibited lapatinib-induced HER3 protein expression in BT474M1 cells.
HER3 has the advantage to block both PI3K/AKT and ERK pathways. Recently, it has been shown that the treatment of lapatinib resulted in an increase in the p-HER3 level via mRNA induction due to compensatory pathways (15–20). Consistent with this, HER3 mRNA is also induced by AKT (20) or PI3K inhibitor through derepression of FoxO3a transcription factor (15–16). This enhanced p-HER3 level

Figure 6. Gefitinib- or trastuzumab-resistant cells remained sensitive to EZN-3920. Cells were treated with different concentrations of EZN-3920 or EZN-SCR control oligonucleotide for 6 days. Cell proliferation was determined by MTT assay. A, effect of EZN-3920 on HCC827 and gefitinib-resistant HCC827 cells. B, effect of EZN-3920 on HCC827 and gefitinib-resistant HCC827GR cells. C, effect of EZN-3920 on PC9 and gefitinib-resistant PC9 cells. D, effect of EZN-3920 on BT474M1 and trastuzumab-resistant BT474M1 cells. All data are mean ± SD.
underscores the importance of HER3 in cell survival. It is therefore logical to consider the combination of HER3 antagonist such as EZN-3920 with lapatinib. Indeed, when we combine the 2, greater antitumor activity was observed than either agent alone. Our data are in agreement with the data published by Garrett and colleagues (15), which showed that a neutralizing HER3 antibody AMG sensitized HER-positive BT474 breast cancer cells to lapatinib both in vitro and in vivo. We observed that the combination was not just unique to lapatinib. When we combined EZN-3920 with gefitinib or a specific PI3KCA antisense molecule EZN-4150 in HCC827 non–small cell lung cancer (NSCLC) xenograft model, greater efficacy was achieved than either agent alone. It is worth noting that the benefit of combining agents to target multiple members of the family was also recently shown by a two-in-one antibody against HER3 and EGFR (43). In our case, the improvement in efficacy is clearly an on-target effect as pretreatment of tumorbearing mice with EZN-3920 suppressed a 3-fold induction of HER3 mRNA by gefitinib (Supplementary Fig. S5). The combination of EZN-3920 with tyrosine kinase inhibitors or inhibitors of the PI3K/AKT pathway may thus provide a novel and much more effective approach for treating various cancers.

An inducible HER3 level plays an important role in gefitinib resistance (4, 17). Logically, inhibition of HER3 should overcome the resistance. To test this hypothesis, the activity of EZN-3920 in gefitinib-resistant cells was examined in vitro. Gefitinib-resistant cells (HCC827R) were generated by selecting HCC827 lung carcinoma cells for resistance to the agent in vitro. HCC827R showed significant reduction in p-EGFR and EGFR levels (44), but unlike other HCC827 selected for resistance to gefitinib (4), this particular cell line does not amplify MET. EZN-3920 had better potency in HCC827R when compared with its parental cells, suggesting that HER3 still plays a role in the survival of HCC827R. We subsequently showed that cell lines with entirely different resistance mechanisms such as PC9GR (T790M mutation; ref. 45) and HCC827GR (MET amplification; ref. 4) were still sensitive to EZN-3920 treatment. Furthermore, EZN-3920 showed equal if not more potent activity in a trastuzumab-resistant cell line, suggesting the ability of EZN-3920 to overcome resistance mediated through different mechanisms.

Collectively, these data suggest that downregulation of HER3 by the antisense molecule EZN-3920 offers a unique opportunity to confirm HER3-driven cancers. The reported in vitro and in vivo activities are encouraging and supportive of further development of this compound. At the moment, several anti-HER3 antibodies including AMG-888 and MEHD7945A are being evaluated in clinical trials with such compounds with EZN-3920. It will be extremely important to develop a biomarker strategy to increase success rates of clinical trials and maximize the benefit to patients. Some preclinical data have been generated to suggest that the expression of ligand neu- regulin-1 is associated with the activation of HER3 in ovarian (13) and head and neck cancer (14). Additional preclinical and clinical data as well as data integration will help to provide a predictive biomarker for HER3-targeted therapy.

Disclosure of Potential Conflicts of Interest
L.M. Greenberger is employed (other than primary affiliation; e.g., consulting) in Bristol-Myers Squibb as Exec Director, Strategic Transactions and has ownership interest (including patents) in Enzon. Z. Qu is employed (other than primary affiliation; e.g., consulting) in Enzon Pharmaceuticals, Inc. as a scientist. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: Y. Wu, Y. Zhang, Z. Qu, V. Shi, I.D. Horak, L.M. Greenberger
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References


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