An antisense molecule to HER3 sustains growth inhibitory effects in gefitinib resistant cells that are independent of MET overexpression

Zhengxing Qu, Yaming Wu, Yixian Zhang, Victoria Shi, Peifang Zhu, Maoliang Wang, Steven Kim, Patricia Kraft, Lee M. Greenberger, Ivan D. Horak.

Enzon Pharmaceuticals, Inc., Piscataway, NJ 08854, USA

timothy.qu@enzon.com

Abstract #307

INTRODUCTION

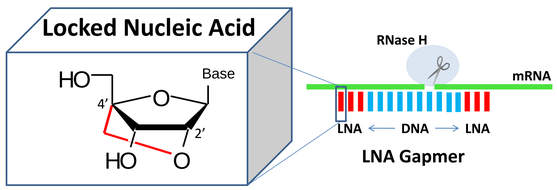
Treatments with selective tyrosine kinase inhibitors, e.g. gefitinib and erlotinib, in advanced NSCLC patients harboring EGFR mutations have given promising results. However, after initial response to the treatment, acquired drug-resistance cancers eventually develop, due to additional mutations in EGFR or other mechanisms independent of EGFR. Although HER3 is not typically amplified or overexpressed in many tumor cell lines like EGFR or HER2, it is emerging as a critical family member since 1) it is a key link to the PI3K/AKT signaling pathway for HER family members, 2) it can heterodimerize with EGFR and HER2, and 3) it can be activated via autocrine signaling by binding its cognate ligand, heregulin, or other receptor tyrosine kinases (e.g. MET). These features help explain why increased activation of HER3 can mediate resistance to EGFR and HER2 inhibitors such as gefitinib, erlotinib, lapatanib, or Herceptin.

We have been attempting to understand the basis of unusual sensitivity of the lung carcinoma cell line, HCC827 to gefitinib (IC_{50} ~10 nM) and acquired resistance mechanisms after the cells were chronically exposed to increasing concentrations of gefitinib in vitro. We have focused on inhibition of HER3 by using LNA-antisense technology. We report here the biological activities of a HER3 mRNA antagonist, EZN-3920, against HCC827 parental and the gefitinib-resistant cells.

METHODS

- Gene targeting and cell growth assays. The ability of EZN-3920 to down modulate its direct target HER3 mRNA, protein, and to inhibit cell growth was evaluated by qRT-PCR, immunoblotting, and MTT, respectively. Cell treatment was conducted by addition of EZN-3920 cell into the culture medium at designed concentrations without transfection reagent.
- Genomic DNA analysis. EGFR copy number in HCC728 and GR clones were determined by qPCR using TaqMan gene copy number assay. Purified genomic DNA was used as templates.
- Drug combination study. The gefitinib-resistant clone and parent HCC827 were treated with varying concentrations of EZN-3920 (0-4 uM) or EZN-4150 (0-20 uM) alone, or in combination (a ratio of EZN-3920 and EZN-4150 1:5), for 6 days. Cell proliferation was determined by MTT assay. A formal quantitative analysis of the data was used to calculate the combination index (CI) based on a multiple drug-effect equation (Reynolds 2005 Mol Med).

LNA TECHNOLOGY & ACTION



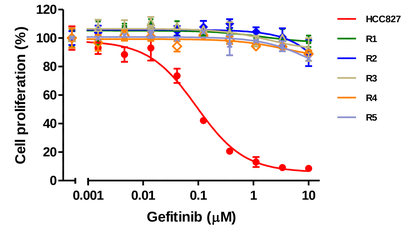
- 3rd generation LNA-based antisense technology
- Very high mRNA affinity
- Excellent plasma stability
- Long tissue residence time (days)
- Short sequence (14-16-mer)
- IV administration without delivery vehicle (unlike siRNA)

REFERENCES

- Engelman JA, *et al.* MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007; 316:1039-1043
- Amann J. *et al.* Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. Cancer Res. 2005; 65:226-35

Gefitinib-resistant HCC827 cells independent MET overexpression

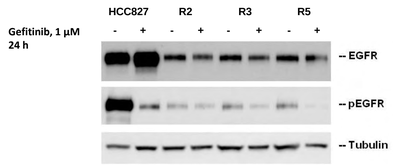
Figure 1. Sensitivities of HCC827 and the stable gefitinib-resistant clones to gefitinib treatment *in vitro*



R1, 2, 3, 4, and R5 are subclones of the lung cancer cell line HCC827 (ATCC CRL-2868) that had been chronically adapted to grow in culture medium containing increasing concentrations of gefitinib, up to 250 nM. While the parent HCC827 is amongest the most sensitive cell lines to gefitinib, these clones are highly resistant to gefinitib (IC_{50} >10 μM). These cells had been propagated in medium without gefitinib for over 30 passages and retained the drug-resistant property.

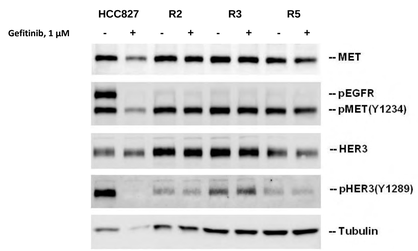
Figure 2. Western blotting analyses of EGFR, MET and HER3 protein levels in HCC827 and the gefitinib-resistant clones. The gefitinb-resistant clones are distinct from that reported to be driven by HER3 hyperactivation associated with MET amplification (Engelman et al. Science 2007).

A



- ❑ Lower levels of EGFR expression are presence in the resistant cells
- ❑ High level pEGFR in parent cells, but not in the resistant clones

B



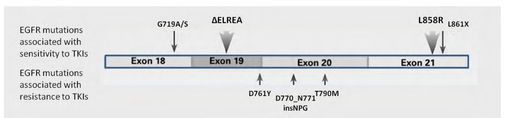
- ❑ Comparable MET protein levels in HCC827 and the resistant clones
- ❑ Persistent pHER3(Y1289) in the resistant cells after gefitinib treatment

Table 1. RT-PCR and sequencing of EGFR cDNA, exon 19-28

Cell line	EGFR (%)*	$EGFR^{\Delta 746\text{-}750}$ (%)*
HCC827	ND	100
R1	66.2±0.5	33.8±0.5
R2	64.5±1.7	35.5±1.7
R3	81.0±0.1	19.0±0.1
R4	68.2±1.3	31.8±1.3
R5	76.2±0.4	23.5±0.4

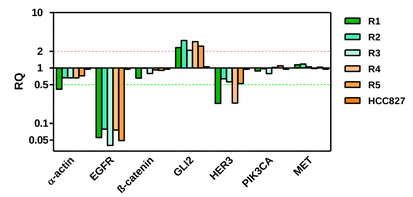
* Estimated based on average signal intensities of sequencing chromatogram.

The intracellular portion of EGFR (exon19-28) in the gefitinib-resistant clones, R1-R5, were sequenced. None of the mutations known to associate with TKI-resistance were found. While only mRNA encoding $EGFR^{\Delta 746\text{-}750}$ was found in HCC827 cells (J. Amann et al. 2006), both $EGFR^{\Delta 746\text{-}750}$ and wild-type EGFR mRNA were found in the resistant cells and the wild-type EGFR was dominant.



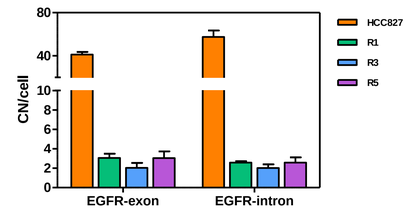
Gefitinib-resistance resulted from loss of EGFR amplification

Figure 3. Gene expression profiling confirmed that HCC827 gefitinib-resistant clones have low EGFR expression and no MET amplification



Gene expression analysis confirmed a 20-fold lower EGFR mRNA level, but a comparable MET mRNA level in the gefitinib-resistant clones relative to those in the parent HCC827 cell. Higher and lower GLI2 and HER3 mRNA levels were observed in the resistant clones.

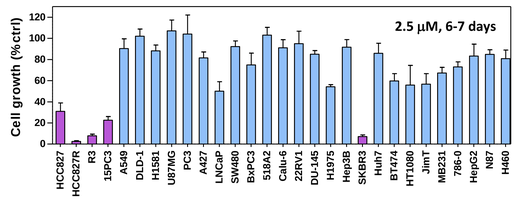
Figure 4. Determination of EGFR gene copy number in HCC827 and the Gefitinib resistant clones



Genomic DNA was isolated and prepared from the cell at exponential growth. Gene copy number was determined using the TaqMan gene copy number kit. Primer pairs specifically amplify a fragment of EGFR intron 2 (EGFR-intro) and exon 3 (EGFR-exon) were used.

Gefitinib-resistant cells remain sensitive to EZN-3920 induced growth inhibition

Figure 5. Gefitinib-resistant HCC827 cell lines maintain the sensitivity to the HER3 antagonizing LNA oligonucleotide, EZN-3920



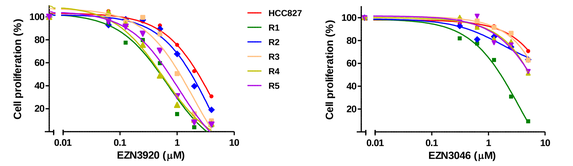
Cell lines were treated with 2.5 μM EZN-3920 for 6 or 7 days. Cell proliferation was determined by MTS assay. The results are from 5 independent assay plates, triplicate each. Average values and standard deviations are shown. HCC827R is the resistant cell pool, from which, R1-R5 clones were isolated.

CONCLUSIONS

- ❑ Diversed mechanisms exist for cells become resistant to selective TKI agents. Gefitinib-resistance could be the result of tumor cells with loss of EGFR amplification and expression of wild-type EGFR escaping from gefitinib treatment.
- ❑ Gefitinib hypersensitivity may indicate that cells are dependent on HER3 and will be inhibited by HER3 antisense molecules
- ❑ Pharmacological manipulation to down-regulate HER3 by EZN-3920 could prove to be a translational approach to controlling HER3-mediated tumor growth in cancer patients
- ❑ Down-regulation of both HER3 and PIK3CA with antisense LNA combination could be an effective approach to treat certain type of drug-resistant tumors

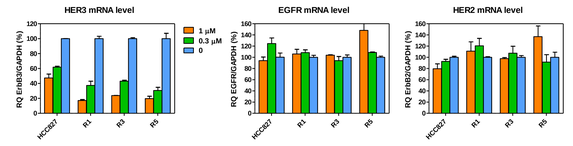
Targeted effects of to EZN-3920 in the gefitinib-resistant cells

Figure 6. Gefitinib resistant HCC827 cell lines were equally or more sensitive to the treatment of EZN-3920



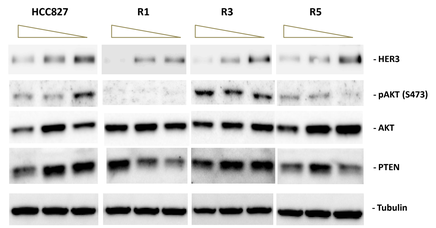
The gefitinib-resistant clones and parent HCC827 were treated with varying concentrations of EZN-3920 or a control oligonucleotide (EZN-3046) for 6 days. Cell proliferation was determined by MTT assay. Shown are representative data of multiple independent assays. R1, R3, R4, and R5 were consistently more sensitive than the parent HCC827 to EZN-3920, but not to the control LNA compound, EZN-3046.

Figure 7. Specificity of down-modulation of HER3 mRNA by EZN-3920 in HCC827 cells and the gefitinib-resistant clones



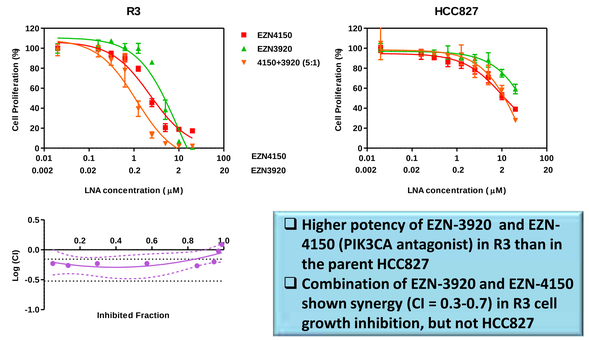
HCC827 and the gefitinib-resistant cells were treated with 1, 0.3 or 0 μM of EZN-3920 for 3 days. HER3, EGFR and HER2 mRNA levels were determined by real-time PCR using TaqMan gene expression assays.

Figure 8. Down-modulation of HER3 protein by EZN-3920 in HCC827 cells. Cells were treated with 1, 0.3 or 0 μM of EZN-3920 for 3 days.



- ❑ Distinct expression profiles observed between the resistant clones
- ❑ Down-regulation of HER3 in HCC827 and gefitinib-resistant cells
- ❑ Persistent pAKT levels in the resistant cells, R3 and R5

Figure 9. The synergetic growth inhibitory effect of HER3 and PIK3CA LNA in gefitinib-resistant cells (see Methods for experiment details)



- ❑ Higher potency of EZN-3920 and EZN-4150 (PIK3CA antagonist) in R3 than in the parent HCC827
- ❑ Combination of EZN-3920 and EZN-4150 shown synergy (CI = 0.3-0.7) in R3 cell growth inhibition, but not HCC827

ACKNOWLEDGEMENTS

- EZN-3920 and EZN-4150 are developed by Enzon Pharmaceuticals, Inc. under a license from Santaris Pharma A/S, Denmark.