In vitro and *in vivo* characterization of two novel β-catenin RNA antagonists, EZN-3889 and EZN-3892

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Abstract #601

INTRODUCTION

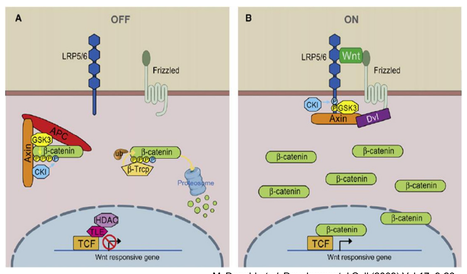
β-catenin is a central signaling molecule within the Wnt pathway. Nuclear β-catenin functions as a transcripional regulator. In the absence of Wnt ligands, β-catenin is bound by Axin and APC allowing phosphorylation of β-catenin by GSK-3 and CK1. This event labels β-catenin for proteosomal degradation initiated by β-TrCP. In the presence of Wnt ligands, Axin is sequested to the plasma membrane resulting in stabilized β-catenin which accumulates and translocates to the nucleus. Nuclear β-catenin interacts with the TCF/LEF family of DNA bound proteins and regulates target gene expression. Activation of the Wnt pathway results in the regulation of many target genes important in cancer development.

Activating events throughout the Wnt pathway are common in human cancers. This is well characterized in colon where ~90% of cancers have APC mutations. Wildtype APC colon cancers frequently have β-catenin or Axin mutations; underpinning the importance of this pathway in cancer development. β-catenin mutations are also found in many other human malignancies and as such, inhibition of β-catenin is likely to have therapeutic effects in many cancers.

We report here the identification and characterization of two β-catenin LNA-based mRNA-antagonists, EZN-3889 and EZN-3892. Both LNAs are able to potently inhibit β-catenin mRNA and protein *in vitro* and inhibit the growth of numerous cancer cell lines. Inhibition of β-catenin using these LNAs results in the inhibition of spheroid formation in the SW480 cell line. Both LNAs are well tolerated *in vivo* with >90% knockdown of β-catenin mRNA observed in mouse liver. Excitingly, treatment of mice bearing Colon-EZN tumors intravenously with EZN-3892 results in significant inhibition of tumor growth. A wide therapeutic window exits for EZN-3892 making the therapeutic development of these LNAs highly desirable. Further work characterizing these β-catenin mRNA antagonists is ongoing.

Targeting the Wnt pathway and β-catenin

The Wnt pathway as a therapeutic target for cancer treatment

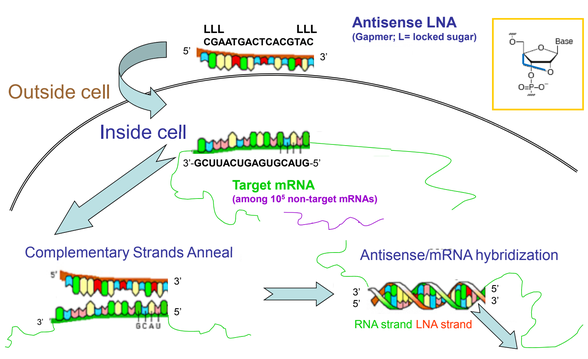


McDonald *et al*, Developmental Cell (2009) Vol 17; 9-26.

β-catenin:

- Transcription factor controlling cell survival, growth, cell cycle, cancer stem cells, metastasis
- Activated in multiple cancers via many mechanisms (e.g. APC, β-catenin, Axin mutations)

LNA-based RNA antagonism



Favorable physical properties of LNA-based oligonucleotides include

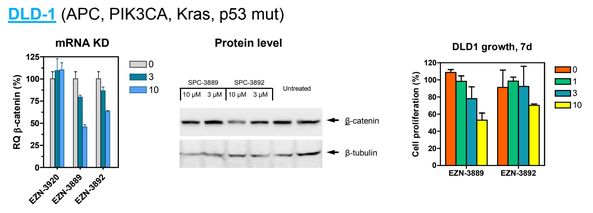
- Highest affinity for mRNA target compared to other anti-sense technology
- Single stranded molecule and relatively short sequence (12-16mer) needed for effective targeting
- Highly stable in vitro and in vivo

Targeted inhibition of β-catenin in "gymnotic culture"

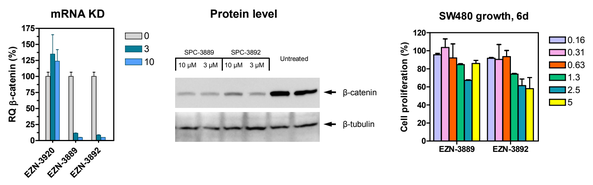
Figure 1. *EZN-3889- and EZN-3892-mediate inhibition of β-catenin in DLD-1, SW480, and 15PC3 cells without transfection.*

Cells were treated with 1-10 μM of EZN-3889 or EZN-3892 for 6-7 days. Specific down-regulation of target mRNA by RT-qPCR and down modulation of β-catenin at the protein level by Western analysis were observed in all three cell lines. However, the treatments effects on cell growth as assessed by MTT or CellTiterGlo assay were moderate. Two out of three cell lines (DLD-1 and SW480) have APC mutations which should render β-catenin active in the cell and drive proliferation. It is interesting that the inhibition of proliferation in these lines is moderate and comparable to a line that does not have activating mutations in the β-catenin degradome (15PC3). 15PC3 however, is PTEN deficient which could activate β-catenin indirectly by activating Akt and inactivating GSK-3.

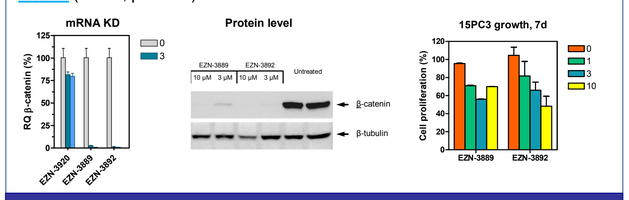
DLD-1 (APC, PIK3CA, Kras, p53 mut)



SW480 (APC, Kras, p53 mut)



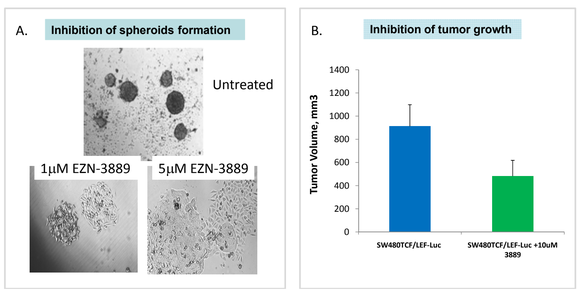
15PC3 (PTEN, p53 mut)



EZN-3889 and 3892 inhibits spheroid formation of SW480 cells in culture

Figure 2. *Without lipofection, EZN-3889 and 3892 (not shown) inhibited the formation of SW480 spheroids and growth of the spheroids in mice*

Cells were incubated EZN-3889 for 6 days at 1 and 5 μM after which an absence of spheroidal growth in observed (A). The SW480 cells treated with 10 μM EZN-3889 were injected into nude mice and had a reduced tumor growth (B).

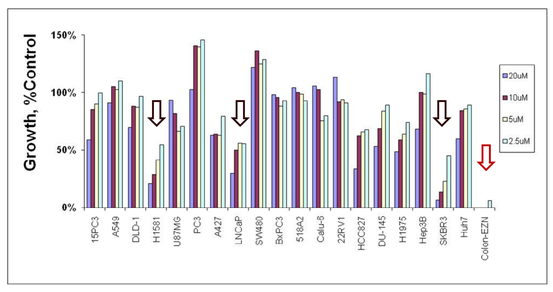


This observation could be indicative of an inhibition of the E-cadherin associated cytoplasmic β-catenin or may suggest an impact on the cancer stem cell population within the SW480 cell line.

Screening of cell lines sensitive to β-catenin LNA in gymnotic culture

Figure 3. *Identification of tumor cell lines sensitive to EZN-3889-mediated growth inhibition without lipofection a using long-term culture assay.*

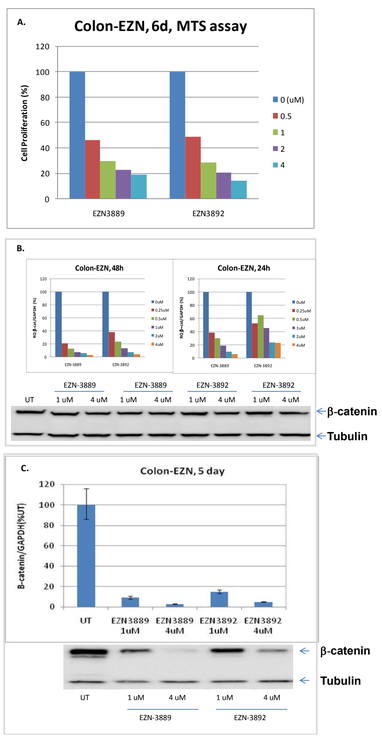
A panel of human tumor cell lines were treated with up to 20 μM of EZN-3889 for 7 days. Cell number was determined by MTS assay. Arrows indicate sensitive cell lines and potential xenograft models for compound evaluation *in vivo*.



Identification of Colon-EZN cell line that is highly sensitive to β-catenin knock down

Figure 5. *EZN-3889 and EZN8992 are able to potently inhibit proliferation of the APC mutant Colon-EZN cell line by knocking down β-catenin mRNA and protein.*

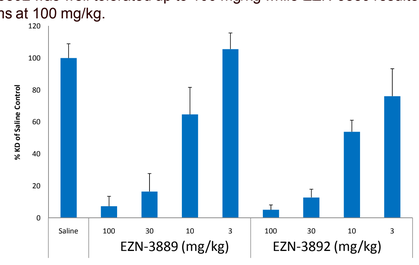
Colon-EZN cells were treated with a dose titration of β-catenin LNA up to 4 μM in culture and cell number was assessed 6 days after plating by MTS assay (A). The ability of EZN-3889 and EZN-3892 to knockdown β-catenin mRNA and protein was assessed 24 and 48 h post LNA addition. While mRNA is significantly inhibited at both of these time points protein levels remain unchanged (B). Five days after LNA addition to Colon-EZN cells in culture a concomitant inhibition of β-catenin mRNA and protein is observed (C).



Knock down of target mRNA *in vivo*

Figure 6. *Evaluation of EZN-3889 and EZN-3892 mediated knockdown of β-catanin mRNA in vivo without delivery agent*

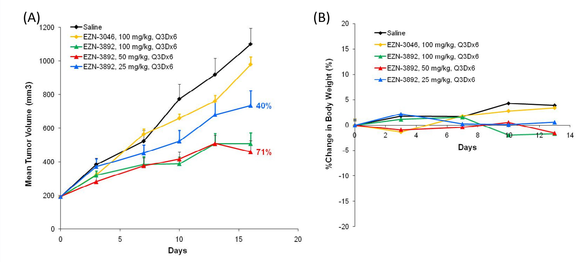
EZN-3889 or EZN-3892 were designed to target both human and mouse β-catenin. The compounds can thus be easily evaluated in mice for targeting *in vivo*. Nude mice were treated with 100, 30, 10 or 3 mg/kg of EZN-3889 or EZN-3892 prepared in saline, Q3Dx3, IV. Twenty four hours following the third dose, mice were euthanized and liver collected. The β-catenin mRNA levels in mouse liver were determined by RT-qPCR. Treating the mice with EZN-3889 and EZN-3892 resulted in approximately 90% reduction of β-catenin mRNA in mouse liver. EZN3892 was well tolerated up to 100 mg/kg while EZN-3889 resulted in ¼ animal deaths at 100 mg/kg.



EZN-3892 is efficacious in Colon-EZN tumor xenografts grown in nude mice

Figure 7. *EZN-3892 inhibits the growth of Colon-EZN xenografts.*

Colon-EZN cells were injected in nude mice, animals were dosed intravenously with either vehicle (Saline) or EZN-3892 at a Q3Dx6 schedule. Dose responsive tumor growth inhibition is observed with 25 and 50 mg/kg of EZN-3892 resulting in 40 and 71% tumor growth inhibition, respectively. Increasing the dose to 100 mg/kg did not increase tumor growth inhibition (A). EZN-3892 was well tolerated with minimal body weight loss observed (B).



CONCLUSIONS

1. The β-catenin antagonists potently and specifically inhibit β-catenin mRNA and protein without transfecton. Inhibition of β-catenin levels in these cell lines impacts cell proliferation.
2. Identification of the Colon-EZN cell line as particularly sensitive to β-catenin knockdown. Inhibition of β-catenin in Colon-EZN cells leads to potent inhibition of cell growth (EC_{50}~80nM) without transfection.
3. The β-catenin LNA antagonists have ability to knockdown β-catenin mRNA levels in mouse liver at safe and tolerated doses.
4. EZN-3892 significantly inhibits the growth of Colon-EZN cells when grown as xenografts in immuno-compromised mice. EZN-3892 is well tolerated at the schedule tested.
5. Further studies ongoing to identify more cell lines sensitive to β-catenin inhibition without transfection and to understand the activity of these LNA antagonists in more mouse models of cancer.

ACKNOWLEDGEMENTS

The design and discovery of EZN-3889 and EZN-3892 has been done in collaboration with Santaris Pharma A/S, Denmark. EZN-3889 and EZN-3892 are being developed by Enzon under a license with Santaris.

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