STEAP4 ISH Diagnostics for Tarloxotinib Activation in EGFR/HER2 Mutant Cancers

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Hui Yu[1], Shevan Silva[2], Adriana Estrada-Bernal[1], Christopher J. Rivard[1], Maria Abbattista[2], Jeff Smaill[2], Chris Guise[2], Chris Squire[3], Robert C. Doebele[1], Shivaun Lueke Pickard[1], Kenichi Suda[4], Paul Mitchell[5], Adam V. Patterson[2], Avanish Vellanki[6], Vijaya G. Tirunagaru[6] and Fred R. Hirsch[7]

[1] University of Colorado, Division of Medical Oncology, Aurora, CO, [2] Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand, [3] School of Biological Sciences, The University of Auckland, Auckland, New Zealand, [4] Division of Thoracic Surgery, Department of Surgery, Kindai University Faculty of Medicine, Osaka-Sayama, Japan, [5] Olivia Newton-John Cancer, Wellness and Research Centre, Heidelberg, Australia, [6] Rain Therapeutics, Inc., Newark, CA, [7] Icahn School of Medicine at Mount Sinai, NY.

Introduction

- Tarloxotinib is a hypoxia-activated prodrug (HAP) that releases tarloxotinib-E, a potent irreversible pan-ErbB TKI under pathophysiological hypoxia present in solid tumors
- Tumor selective release of tarloxotinib-E increases dose intensity and significantly enhances the tolerability due to reduced WT EGFR-mediated side effects compared to marketed EGFR TKIs
- Tarloxotinib-E is a potent inhibitor against a range of EGFR and HER2 mutations including exon20 insertion mutations[1]
- STEAP4 (Six-Transmembrane Epithelial Antigen of Prostate 4) was identified as the one-electron reductase that is a major contributor to the conversion of tarloxotinib to tarloxotinib-E in hypoxic tumors[2]
- STEAP4 is a FAD and β-type heme-containing transmembrane metalloreductase that functions to reduce extracellular $Fe3+$ and $Cu2+$ in an NADPH-dependent manner
- STEAP4 knockout by CRISPR/Cas9 in SiHa cells led to a 92% decrease in anoxic tarloxotinib metabolism in vitro. Tumors derived from the STEAP4 knockout clone had a 89% decrease in effector/prodrug ratio relative to parental SiHa tumors[2]
- Conversely, STEAP4 overexpression in C33A cells resulted in a significant increase in tarloxotinib metabolism[2]

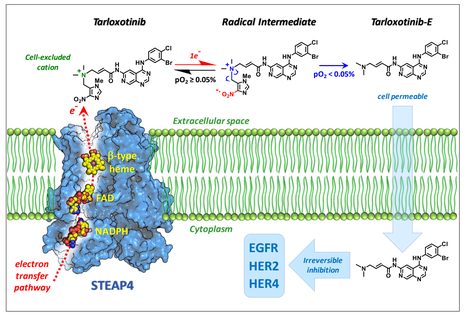


Figure 1. Proposed mechanism of tarloxotinib activation by STEAP4[1]

- Tarloxotinib conversion to its irreversible EGFR/HER2/HER4 inhibitor. Addition of a hypoxia trigger to tarloxotinib-E significantly reduces the potency of the prodrug, allowing for higher dose intensity
- Step 1: Tarloxotinib (prodrug) receives an electron from STEAP4
- Step 2: In the absence of oxygen, radical anion intermediate fragments to form tarloxotinib-E (active drug)

Note: When oxygen present, O_2 scavenges electron, and prodrug is retained

Methods

***STEAP4 in situ* hybridization (ISH).** Slides were stained on a Leica Bond RX autostainer using the RNAScope 2.5 LSx Brown Reagent kit (#322700) with the RNAscope 2.5 LSx STEAP4 probe (#542158). The RNAscope 2.5 LSx PPIB probe (#313908) was used as a positive control. For mRNA ISH detection, the protocol used was the RNAscope 2.5 LSx DAB ISH with an additional fifteen minute exposure with the AMP 5 DAB step. For heat pretreatment, the protocol used was the RNAscope 2.5 LSx at 95 degrees centigrade and exposed to epitope retrieval 2 (high pH). The enzyme protease was used for pretreatment using Leica protocol RNAscope 2.5 LSx Enzyme. Hybridization was extended by twenty-five minutes at 42 degrees centigrade, using Leica protocol RNAscope 2.5 LSx Hybridization. Following staining, slides were cleared and dehydrated on an automated Tissue-Tek Prisma platform and cover slipped using a Tissue-Tek Film cover slipper.

Increased tarloxotinib metabolism in *STEAP4* expressing xenografts

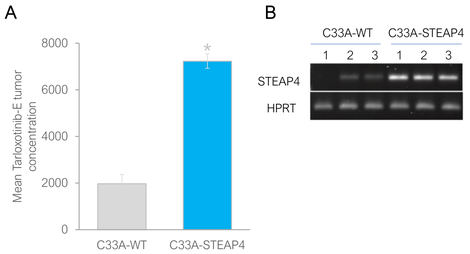


Figure 2. A. Tarloxotinib metabolism in parental and STEAP4 overexpressing C33A xenografts grown in NIH-III mice, * P <0.001. **B.** Tumors were harvested 3 hours following a single dose (48 mg/kg, i.p.) of tarloxotinib. *STEAP4* expression in xenografts by qPCR.

- Tarloxotinib metabolism was measured in an isogenic pair of parental (WT) and STEAP4 overexpressing C33A xenografts
- Significant increases in tarloxotinib metabolism in STEAP4 overexpressing xenografts relative to parental xenografts

Development and validation of *STEAP4* ISH

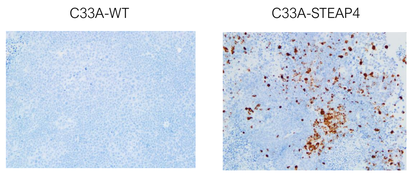
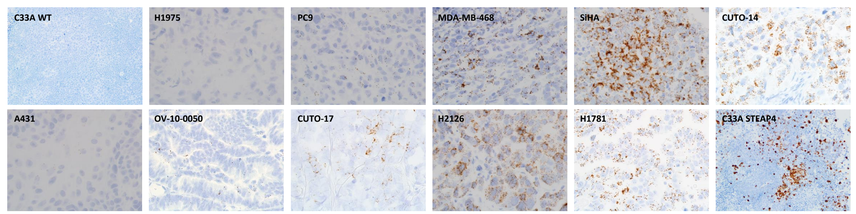


Figure 3. STEAP4 ISH staining in C33A-WT and C33A-STEAP4 overexpressing cells.

Score	ISH Scoring criteria
0	No staining or <1 dot/10 cells
1	1-3 dots/cell
2	4-9 dots/cell. None or very few dot clusters
3	10-15 dots/cell and <10% dots are in clusters
4	>15 dots/cell and >10% dots are in clusters

- *STEAP4* ISH assay (RNAScope) developed using human *STEAP4* RNAScope probes from ACDBio
- C33A cells have low endogenous *STEAP4* expression
- *STEAP4* overexpression in C33A cells detected by the ISH assay

STEAP4 ISH staining in human tumor xenografts



B

Xenograft	Dose (mg/kg)	Tumor Regression	Tumor Growth inhibition
H1975	70	✔	
A431	30	✔	
PC9	7.5	✔	
OV-10-0050	26	✔	
CUTO-17	48		✔
CUTO-14	48	✔	
H1781	48		✔

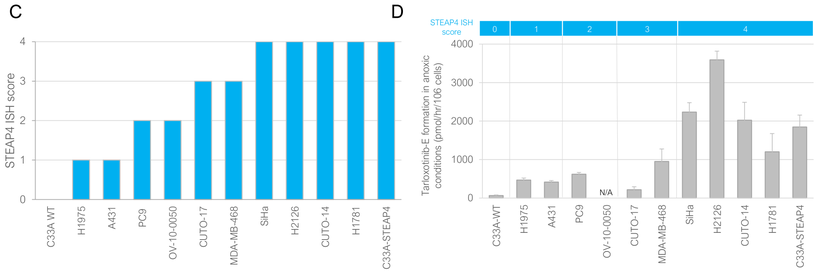


Figure 4. A. STEAP4 ISH staining in EGFR/HER2 mutant and *CLU-NRG1* xenograft tumor sections. **B.** *In vivo* anti-tumor activity of tarloxotinib in various xenograft models. **C.** *STEAP4* ISH scoring of various FFPE xenograft tumor sections with RNAScope ISH scoring guidelines. **D.** Tarloxotinib metabolism under anoxic conditions determined by mass spectrometry in various cell lines *in vitro*.

EGFR mutation positive tumors demonstrate *STEAP4* expression in primary and metastatic sites

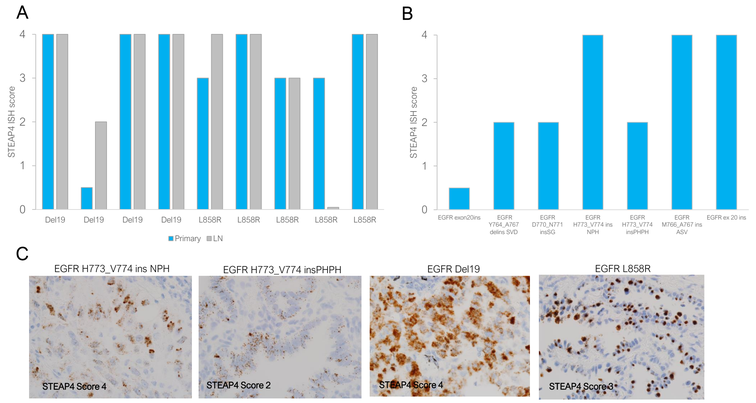


Figure 5. A. *STEAP4* ISH staining and scoring of EGFR mutant (del 19 and L858R) lung cancer patient tumor FFPE sections from the primary tumor and lymph node (LN) metastasis. **B.** *STEAP4* ISH staining and scoring of EGFR exon 20 insertion mutant lung cancer patient tumor FFPE sections. **C.** *STEAP4* ISH staining of FFPE sections from EGFR mutant lung cancer patients.

STEAP4 mRNA levels increased in EGFR mutant NSCLC samples

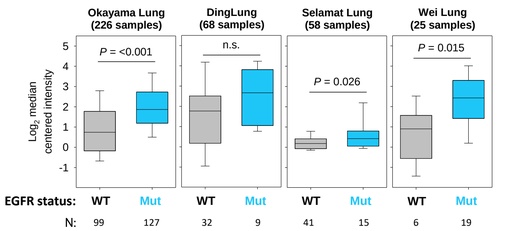


Figure 6. ONCOMINE analysis of *STEAP4* expression in four cohorts of lung cancer datasets based on EGFR mutation status (WT = wildtype EGFR, Mut = mutant EGFR).

- Analysis of *STEAP4* expression in lung adenocarcinoma tumor samples across four independent datasets suggests an EGFR-mutant genotype may be associated with increased *STEAP4* expression
- The genomic proximity of *EGFR* (7p12) and *STEAP4* (7q21) suggests the potential for increased copy number of *STEAP4* due to chromosome 7 polysomy, a frequent co-occurrence in EGFR mutant NSCLC

Conclusions

- ISH assay for human STEAP4 was developed using RNAScope technology and validated using an isogenic pair of parental and STEAP4 overexpression C33A xenografts
- *STEAP4* ISH staining *in situ* across a panel of xenografts relates well with tarloxotinib metabolism data *in vitro* in matched cell lines
- High *STEAP4* expression was observed in EGFR mutant (del19, L858R and exon 20 insertion) lung cancer FFPE samples and 100% of the samples were positive
- Good correlation of *STEAP4* ISH staining was observed in primary tumor and lymph node metastasis in EGFR mutant lung cancer FFPE samples
- Expression data (ONCOMINE) supports observation of elevated *STEAP4* mRNA in EGFR mutant NCSLC
- STEAP4 may represent a biomarker for tarloxotinib conversion to tarloxotinib-E
- *STEAP4* transcript abundance will be analyzed by ISH in the ongoing tarloxotinib clinical trial (RAIN-701, NCT03805841)

References

1. Suda et al., Potent in vitro activity of Tarloxotinib for EGFR C797S and other mutations refractory to current EGFR tyrosine kinase inhibitors. AACR 2019.
2. Silva et al., The hypoxia-activated EGFR/HER2 inhibitor Tarloxotinib is activated by the plasma membrane reductase STEAP4. ENA 2018.

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