

# Antitumor activity of tarloxotinib, a hypoxia-activated EGFR TKI, in patient-derived lung cancer cell lines harboring EGFR exon 20 insertions

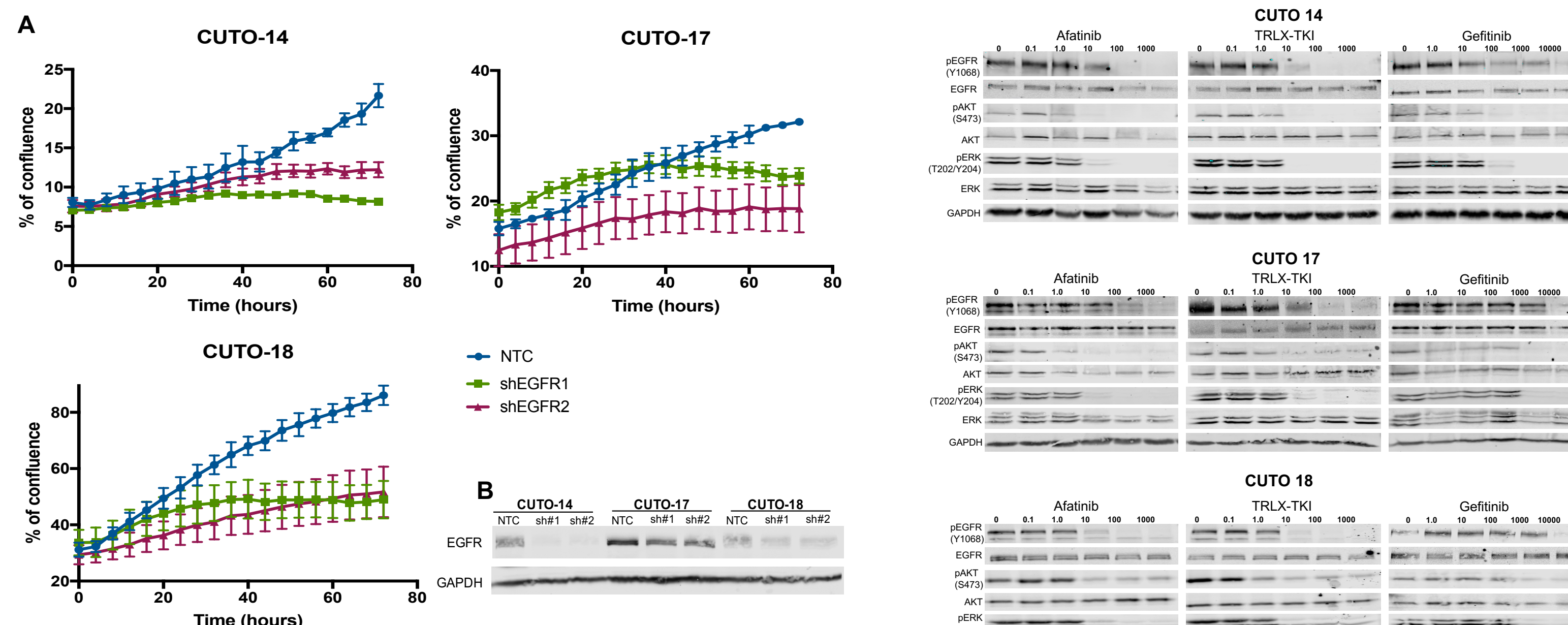
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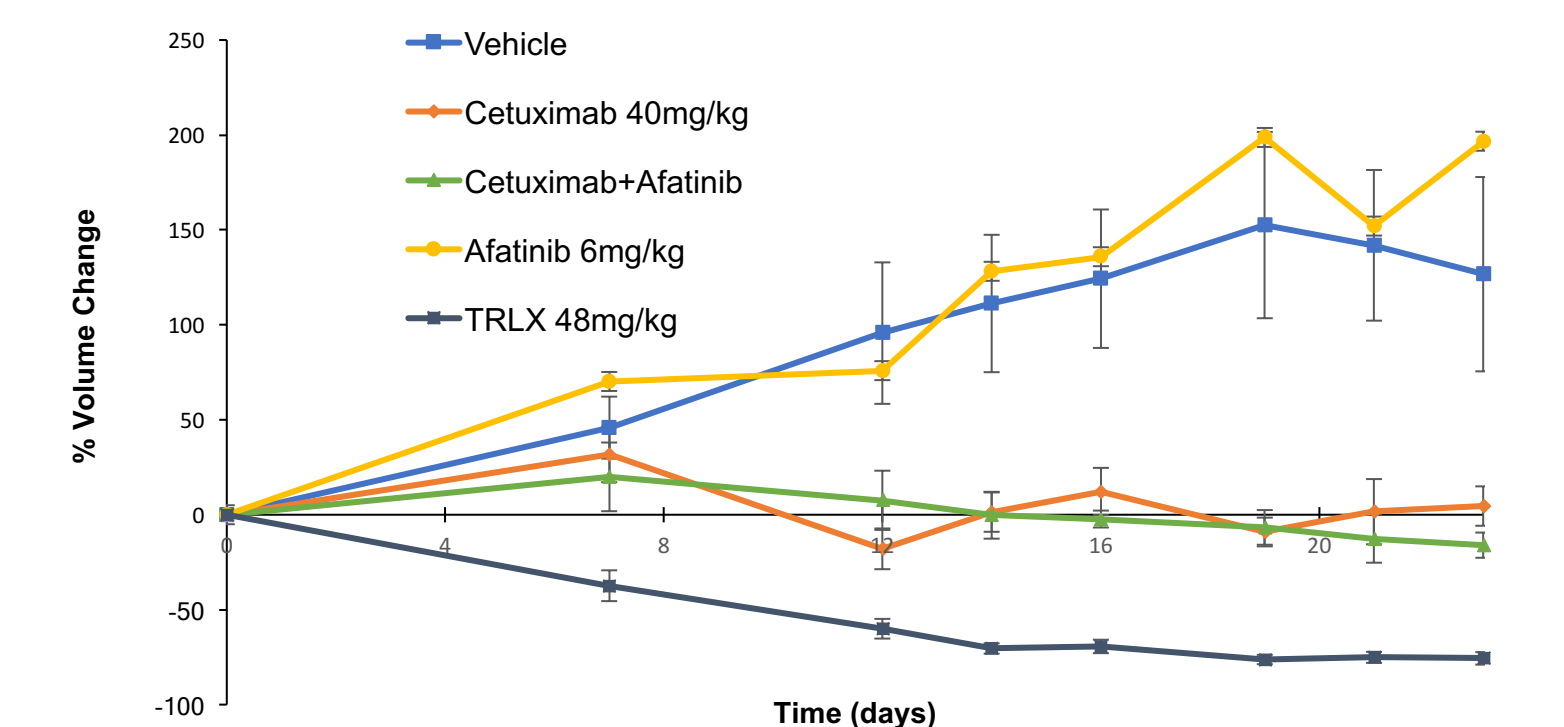


## Introduction

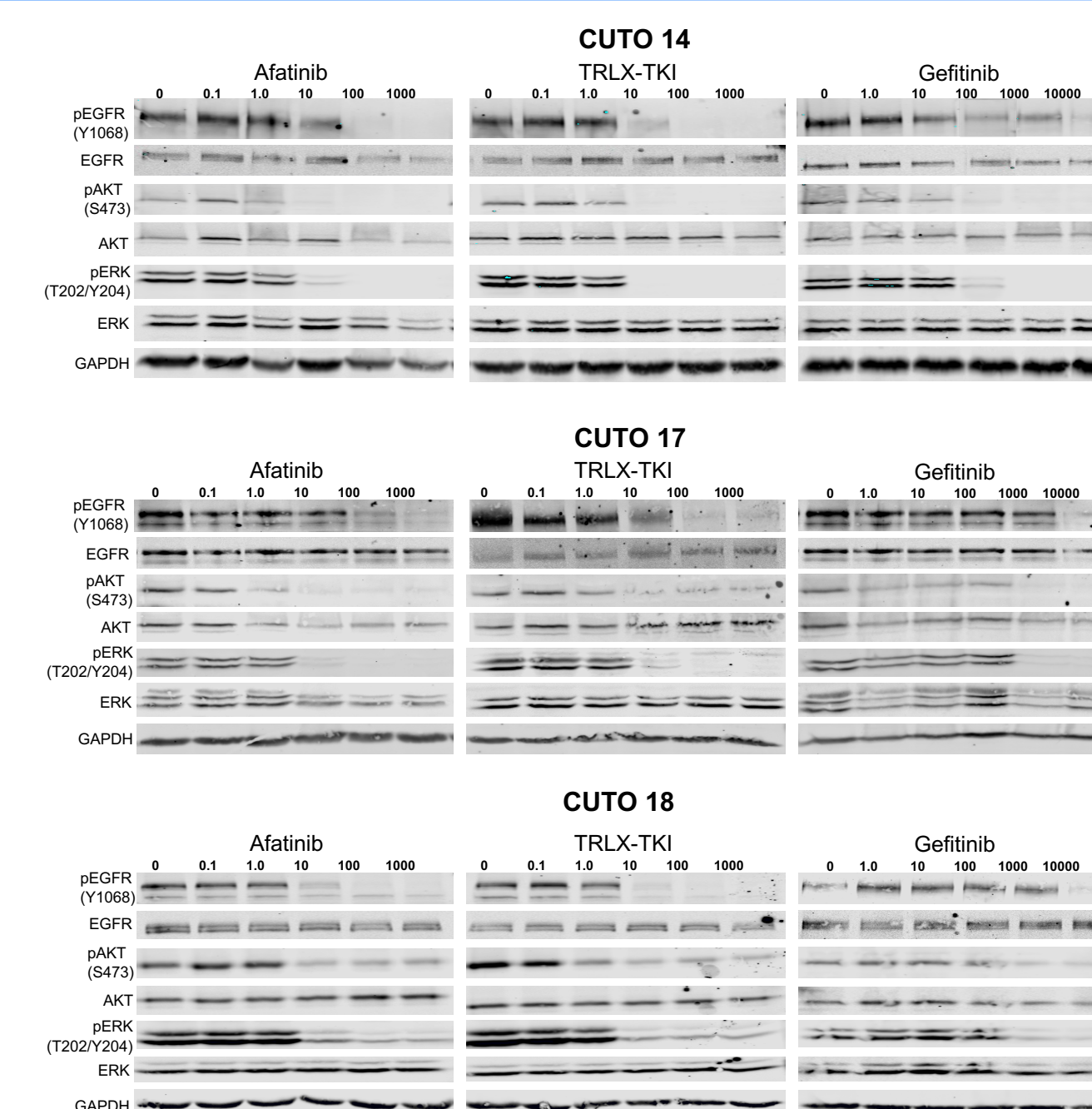
- NSCLC is the leading cause of cancer-related deaths<sup>1</sup>
- 15-50% of NSCLC is caused by mutations in the epidermal growth factor receptor (*EGFR*) gene.
- 90% of mutations in *EGFR* cluster in exon 19 and exon 21, and most of these NSCLC patients have radiographic response to FDA-approved EGFR TKIs such as erlotinib, gefitinib, and afatinib<sup>2,3</sup>
- Exon 20 in-frame insertion mutations in *EGFR* have been identified in 4-10% of NSCLC patients with *EGFR* mutations and are associated with lack of response to approved EGFR TKIs<sup>2,4</sup>
- In vitro*, ~100-fold higher concentrations of EGFR TKIs required for growth inhibition and these doses are not clinically achievable<sup>5,6</sup>
- Our understanding of exon20 insertions is limited by the lack of representative preclinical models. Our laboratory is the first to isolate and propagate several patient-derived exon 20 mutant cell lines which will give insight to the biology of the NSCLC tumors that harbor these *EGFR* mutations
- Solid tumors are often hypoxic, which can lead to resistance to radiotherapy and chemotherapy. To transform this poor risk feature into a vulnerability, tarloxotinib was designed as a prodrug that is reduced under hypoxic conditions to release an irreversible EGFR/HER2 inhibitor (TRLX-TKI).
- Tarloxotinib may increase the therapeutic ratio over conventional anti-EGFR therapy by inhibiting EGFR with greater dose-intensity in hypoxic tumors.
- In this study, we evaluate the effect of tarloxotinib on patient derived cell lines harboring *EGFR* exon 20 insertion mutations.



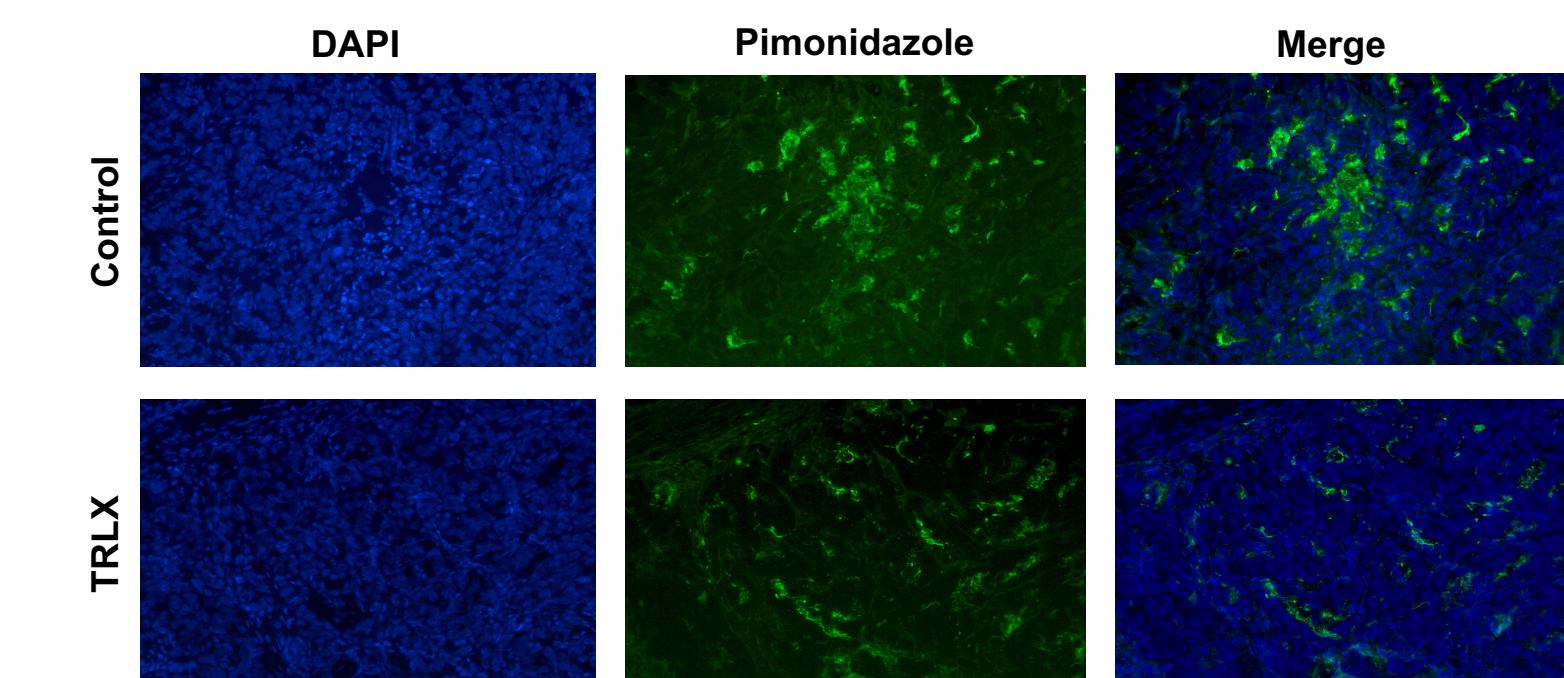
**Figure 2. Patient derived EGFR exon 20 insertion mutations cell lines are dependent on EGFR for proliferation.** A) Cell lines were transfected with one of two distinct shEGFR (shEGFR1 or shEGFR2) or with non targeted shRNA (NTC); proliferation was monitored by IncuCyte live-cell imaging system continuously for 80 h. Cell growth is expressed as an increase in percentage of confluence. B) Immunoblot of lysates collected at 72h post-transduction demonstrating reduction in EGFR protein levels.



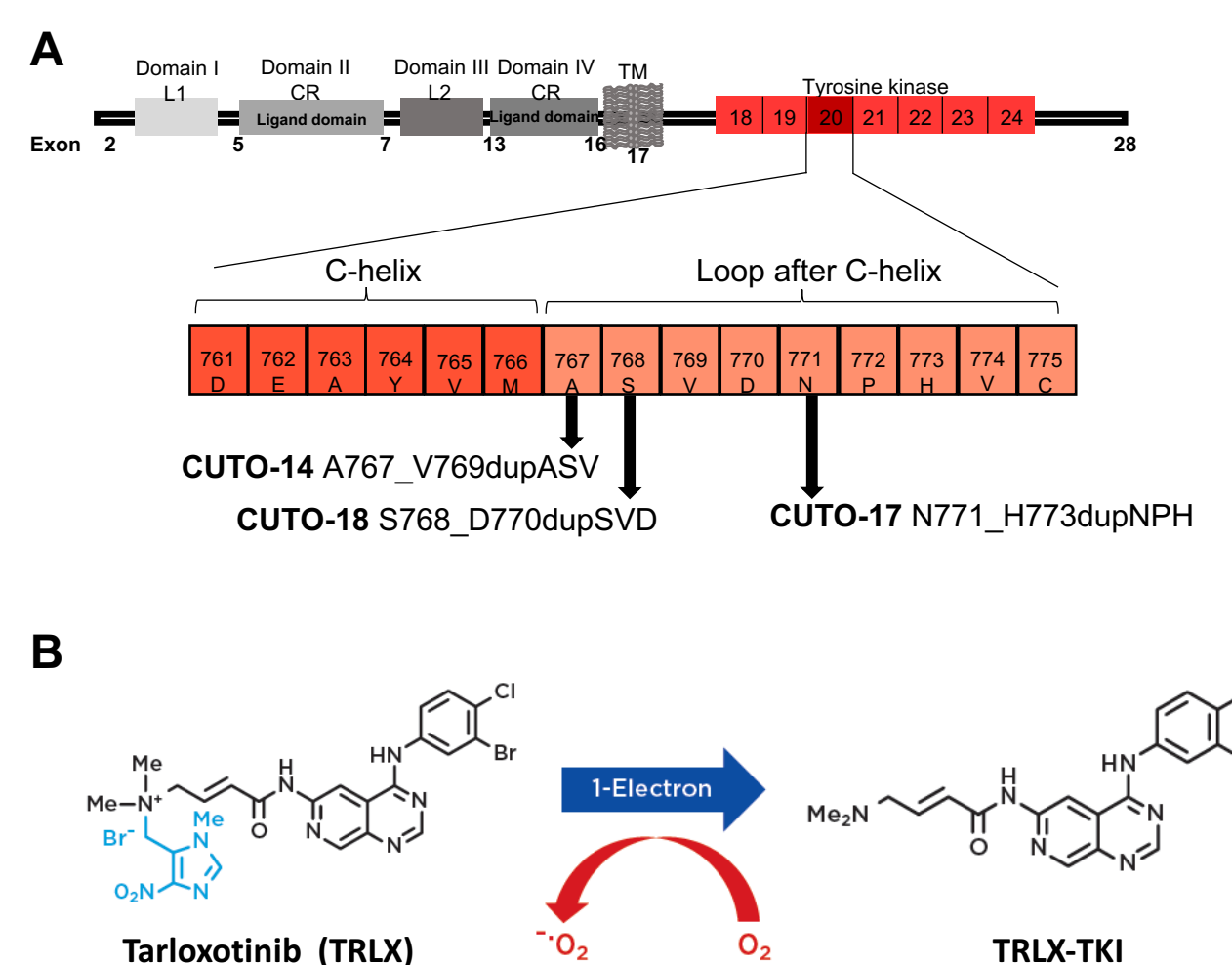
**Figure 5. Tarloxotinib inhibits tumor growth of an EGFR exon 20 insertion mutation xenograft model.** Percent changes from baseline tumor volume in nude mice injected subcutaneously with CUTO-14 cells and treated with vehicle, cetuximab (40mg/kg, two times a week, IP), afatinib (6mg/kg, daily, PO), combination of cetuximab and afatinib, or tarloxotinib (48mg/kg, once a week, IP).



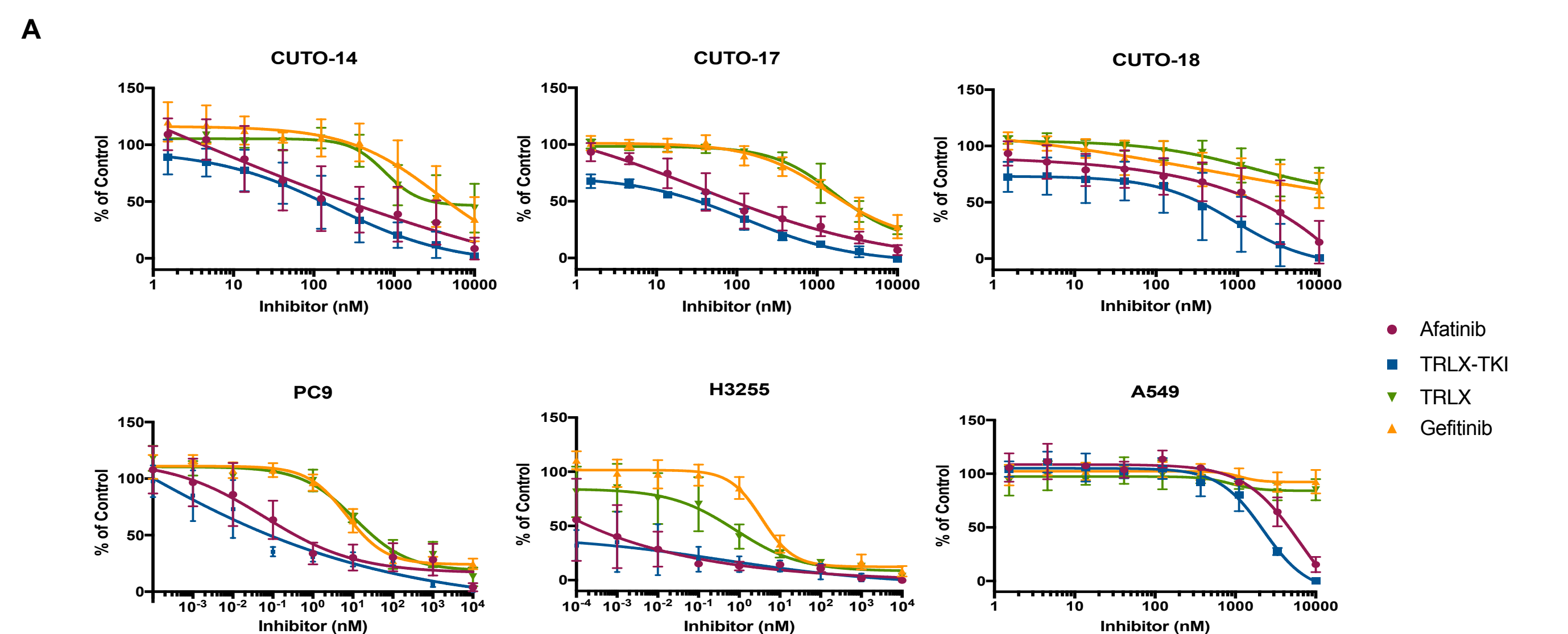
**Figure 4. Tarloxotinib-TKI inhibits EGFR phosphorylation and signaling in EGFR exon 20 insertion mutation cell lines.** Cells were treated with the indicated doses of afatinib, gefitinib, and TRLX-TKI (active drug) for 2 hours, lysed and analyzed by immunoblot. Experiments were done by triplicate.



**Figure 6. Hypoxia in CUTO-14 xenograft tumors.** Mice bearing subcutaneous tumors were treated with vehicle (control) or tarloxotinib (TRLX, 48mg/kg). Pimonidazole (60mg/kg) was administered 60 min before sacrifice and 6 hr after the last dose of TRLX from experiment in figure 5. Excised tumors were formalin-fixed, paraffin-embedded and stained for pimonidazole (green) and dapi (blue).



**Figure 1. A)** Schematic of EGFR structure indicating the localization of exon 20 insertion mutations identified in our patient derived cell lines (CUTO-14, CUTO-17, and CUTO-18). **B)** Representation of tarloxotinib conversion to its irreversible EGFR/HER2 inhibitor. Attachment of a hypoxia trigger to tarloxotinib significantly reduces the potency of the prodrug, allowing for administration at higher relative concentrations than the cognate TKI (TRLX-TKI).



**Figure 3. Tarloxotinib-TKI (TRLX-TKI) inhibits proliferation of patient derived cell lines harboring EGFR exon 20 insertion mutations.** A) Dose response curves of cell viability of EGFR exon 20 insertion mutation patient derived cell lines (upper panel), mutated EGFR (PC9, H3255) and wild type EGFR (A549) (lower panel). Cells were treated with afatinib, gefitinib, tarloxotinib (pro-drug, TRLX) and Tarloxitinib-TKI (active drug, TRLX-TKI) for 72 hours and measured by MTS. Experiments were done in triplicate; mean and  $\pm$ SEM is plotted. B) IC<sub>50</sub> values of the proliferation experiments.

**B**

IC <sub>50</sub> (nM)	CUTO-14	CUTO-17	CUTO-18	PC9	H3255	A549
Afatinib	203	89	709	<1nM	<1nM	4545
TRLX-TKI	208	33	345	<1nM	<1nM	2098
TRLX	5508	2108	124165	18	3.54	>10000
Gefitinib	3681	2213	>10000	30	0.80	>10000

## Conclusions

- We generated three patient-derived cell lines that harbor unique *EGFR* exon 20 insertion mutations.
- These cell lines depend on EGFR signaling for their proliferation and are resistant to approved EGFR inhibitors compared to sensitizing *EGFR* mutations in exon 19 and 21.
- Tarloxotinib-TKI (TRLX-TKI, active drug) inhibits proliferation *in vitro* of EGFR exon 20 insertion mutation cell lines
- Tarloxitinib (TRLX, pro-drug) has 30-70 fold lower activity *in vitro* under normoxic conditions (depending on the cell line).
- TRLX-TKI inhibits EGFR downstream signaling *in vitro*.
- TRLX significantly reduces tumor volume in an EGFR exon 20 insertion mutation xenograft model (CUTO-14).

## References

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**Funding:** Developmental Therapeutics Program, University of Colorado Cancer Center (NIH/NCI P30CA046934).