T790M or C797S confers acquired resistance to tarloxotinib and poziotinib in EGFR exon 20 insertion-driven lung cancer models *in vitro*

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Introduction

Unmet needs in EGFR mutated lung cancer treatment

- Approximately 10-12% of all EGFR mutated lung cancers[1] harbor EGFR exon 20 insertion mutation and represent a unique subset of patients for whom there are currently no effective or approved targeted therapies. First generation (1G) EGFR-TKI, gefitinib and erlotinib, 2G EGFR-TKI afatinib, and 3G EGFR-TKI osimertinib showed low response rates of 6 -11%. [2-4]
- Recent evidence suggests the therapeutic potential of the novel hypoxia-activated prodrug, tarloxotinib[5], a potent pan-ErbB inhibitor, or the repurposed 3rd generation inhibitor, poziotinib, in these tumors. In our previous study, we have shown that tarloxotinib-E, the activated form of tarloxotinib, effectively inhibited proliferation of Ba/F$_3$ cells which express one of four different EGFR exon 20 insertion mutations[6]

Tarloxotinib: A hypoxia-activated irreversible pan-ErbB inhibitor

- Tarloxotinib is a hypoxia-activated prodrug (HAP) that releases a potent irreversible pan-ErbB TKI (tarloxotinib-E) under pathophysiological hypoxia present in solid tumors.
- STEAP$_4$ (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.[7]
- 80% (15/18) of NSCLC tumors were demonstrated to be hypoxic by [18F]HX$_4$ imaging. [8]
- Tarloxotinib was designed to increase therapeutic ratio over conventional EGFR-TKI therapy, thus inhibiting mutant forms of EGFR (and HER2) with increased dose-intensity in hypoxic 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 approved EGFR TKIs.
- Tarloxotinib is currently in phase 2 clinical trial for EGFR and HER2 exon 20 insertion mutations.

Figure 1. Tarloxotinib conversion to its irreversible pan-ErbB inhibitor.

- Tarloxotinib (prodrug) conversion to irreversible pan-ErbB inhibitor (active drug).
  - Step 1: Tarloxotinib (prodrug) receives an electron from STEAP$_4$ reductase.
  - Step 2: In the absence of oxygen, radical anion intermediate fragments to form tarloxotinib-E (active drug). When oxygen is present, O2 scavenges electron, and prodrug is retained.
- Addition of a hypoxia trigger (red) to tarloxotinib-E significantly reduces the potency of the prodrug, allowing for administration of a higher relative dose.

References

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Purpose of the study

- To investigate secondary EGFR mutations that cause acquisition of resistance to tarloxotinib-E in lung cancers with EGFR exon 20 insertion mutation.

Materials and Methods

Establishment of Ba/F$_3$ cells expressing mutant forms of EGFR

- The murine pro-B cell line Ba/F$_3$ (RCB0805) was obtained from RIKEN Bio Resource Center (Tsukuba, Japan). Ba/F$_3$ cells that express one of EGFR exon 20 insertions (Table 1) were established in our previous study. In this study, we established Ba/F$_3$ cells that express one of EGFR exon20 insertions with a concurrent secondary *EGFR* mutation in the same allele. Detailed methods for the establishment of Ba/F$_3$ cells were described in our previous study. [9]

Cell growth inhibition assay

- Cell growth inhibition assay for one of the following TKIs, tarloxotinib-E (activated form), tarloxotinib (pro-drug before activation), afatinib, poziotinib, and osimertinib, were preformed as previously described. [7] Briefly, 2000 cells were seeded in each well of 96-well plates. Twenty-four hours later, DMSO or a TKI at indicated drug concentration were added, and the cells were cultured for additional 72 hours. We used a colorimetric assay to estimate the growth inhibition of each drug using the Cell Counting Kit-8 reagent (Dojindo Laboratories, Kumamoto, Japan). Each experiment was performed in triplicate.

ENU mutagenesis assay

- ENU acquired resistant cells against tarloxotinib-E or poziotinib were established using ENU (Sigma Aldrich) mutagenesis technique. ENU exposure was performed at the concentration of 100 ug/ml for 24h. Fifty-thousand cells were seeded into 24 wells of 96-well plates in the presence of tarloxotinib-E (200 nM) or poziotinib (200nM). These plates were incubated for 2 weeks with medium changes twice weekly, and surviving clones were isolated. The secondary EGFR mutations were explored by direct sequencing.

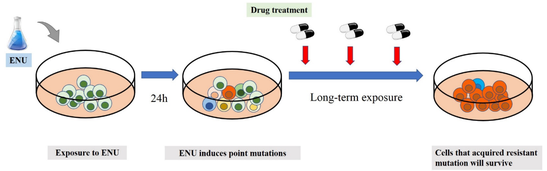

Figure 3. Brief protocol of ENU mutagenesis and establishment of drug-resistant Ba/F$_3$ clones

Results

EGFR exon 20 insertions evaluated in this study

- Using the COSMIC database, we searched for EGFR exon 20 insertion mutations which have been reported in two or more lung cancer patients. We selected the top 5 mutation subtypes for this study, which covered 72.2% of all reported EGFR exon 20 insertions in lung cancers.

Frequency of mutation subtypes		Numbers of reports	(%)
1	V769insASV	89	29.5
2	D770insSVD	61	20.2
3	H773insNPH	34	11.2
4	H773insH	18	6.0
5	A763insFQEA	16	5.3
	Others	84	27.8

Table 1. Frequencies of EGFR exon 20 insertions in COSMIC database

Tarloxotinib-E potently inhibits EGFR exon 20 insertion mutations *in vitro*

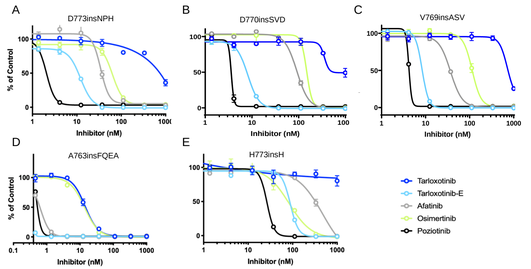

Figure 2. Growth inhibition of Ba/F$_3$ cell lines expressing various EGFR exon 20 insertion mutations

IC$_{50}$ (nM)	Afatinib	Poziotinib	Osimertinib	Tarloxotinib	Tarloxotinib-E
A763insFQEA	0.7	0.7	14.6	15.2	<0.5
V769insASV	35.5	4.8	118.4	675.9	7.6
D770insSVD	86.0	2.7	184.7	990.1	7.3
H773insNPH	35.8	2.2	61.9	714.0	9.9
H773insH	325	22.8	77.7	>1000	73.1

Partially updated based on repeated confirmation analyses

Table 2. Cellular potency in Ba/F$_3$ cells expressing various EGFR exon 20 insertion mutations

Tarloxotinib-E treatment leads to secondary resistant mutations in Ba/F$_3$ cells expressing EGFR exon 20 insertion mutations *in vitro*

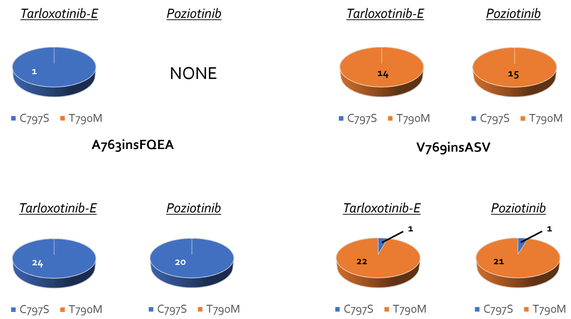

Figure 4. Pattern of secondary mutations to drug treatment in Ba/F$_3$ cell lines expressing EGFR exon 20 insertion mutations

Type of EGFR mutation		Afatinib	Poziotinib	Osimertinib	Tarloxotinib	Tarloxotinib-E
A763insFQEA	+T790M*	17.4	13.4	30.0	547.6	5.6
	+C797S	45.2	17.7	959.2	>1000	186.8
V769insASV	+T790M	415.1	263.4	55.1	>1000	104.3
	+C797S*	985.2	619.7	>1000	>1000	>1000
D770insSVD	+T790M*	741.1	214.6	54.1	>1000	471.7
	+C797S	696.3	409.8	>1000	>1000	507.9
H773insNPH	+T790M	>1000	534.9	107.3	>1000	496.6
	+C797S	>1000	>1000	>1000	>1000	>1000

*These cells were established by transfection of mutant EGFR. Others were established via chronic drug exposure after ENU mutagenesis.

Table 3. IC$_{50}$ values of EGFR-TKIs in Ba/F$_3$ cells with exon 20 insertion plus a secondary mutation

Conclusions

- Tarloxotinib-E (active drug) demonstrates potent *in vitro* activity against Ba/F$_3$ cells with EGFR exon 20 insertions except for H773insH. Ba/F$_3$ cells with H773insH were insensitive to all tested EGFR-TKIs.
- ENU mutagenesis followed by chronic drug exposure resulted in the development of acquired resistant cells with secondary EGFR mutation to tarloxotinib-E.
- T790M and C797S were identified as the major resistance mechanisms to tarloxotinib-E treatment in Ba/F$_3$ cells with EGFR exon 20 insertion mutation.
- Loss of potency was observed for secondary resistance mutations for tarloxotinib-E.
- Interestingly, secondary mutations showed preference based on the type of the original exon 20 insertion.
- This secondary mutation preference pattern was the same for tarloxotinib-E and poziotinib.
- Secondary resistance mutations appear to be based on the original EGFR exon20 insertion mutation and not drug specific.

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