Activity of tarloxotinib-E in cells with EGFR exon-20 insertion mutations and mechanisms of acquired resistance

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Abstract
Background: Approximately 10% of non-small cell lung cancers (NSCLCs) that harbor epidermal growth factor receptor (EGFR) gene mutations have in-frame insertions in exon 20 of the EGFR gene. These tumors do not usually respond to currently available EGFR-tyrosine kinase inhibitors (TKIs). Tarloxotinib is a novel hypoxia-activated prodrug that releases a potent, irreversible pan-ERBB TKI (tarloxotinib-E) under solid tumor hypoxia.

Methods: We examined the efficacy of tarloxotinib-E against several types of Ba/F3 cells with introduced EGFR exon 20 mutations (EGFR A763insFQEA, V769insASV, D770insSVD, H773insH and H773insNPH mutations). We assayed growth inhibition for tarloxotinib (prodrug), tarloxotinib-E (active form), poziotinib, afatinib, and osimertinib in Ba/F3 cells with each EGFR exon 20 mutation. We also explored acquired resistance mechanisms to tarloxotinib-E by establishing cells with resistance to tarloxotinib-E via chronic drug exposure after N-ethyl-N-nitrosourea mutagenesis treatment.

Results: Among all tested Ba/F3 cell lines, IC50 was ≥72.1 times higher for tarloxotinib than for tarloxotinib-E, which implies a wide therapeutic window with this prodrug strategy. Tarloxotinib-E was efficacious against all tested Ba/F3 cells except for H773insH, which was less sensitive to all tested EGFR-TKIs. As acquired resistance mechanisms to tarloxotinib-E, we identified either T790M or C797S secondary mutations, depending on the original EGFR exon 20 mutation.

Conclusions: These findings indicate that tarloxotinib-E could be effective for NSCLC with EGFR exon 20 mutations. Our results also show that T790M or C797S mutations can confer acquired resistance to tarloxotinib-E, and suggest that resistance mechanisms are influenced by the baseline EGFR exon 20 mutations.

KEYWORDS
EGFR mutation, lung cancer, molecular targeted therapy, acquired resistance, secondary mutations

INTRODUCTION
Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) monotherapy is a standard of care for patients with recurrent or metastatic non-small cell lung cancers (NSCLC) that harbor EGFR mutations, such as exon 19 deletion and L858R point mutation.1 However, approximately 10%–12% of EGFR-mutated NSCLC tumors have an in-frame insertion within exon 20 of EGFR.2 These tumors currently have no effective or approved targeted therapies. For example, response rates of first generation (1G) TKI erlotinib, 2G-TKI afatinib, or 3G-TKI osimertinib in patients with EGFR exon 20 mutations were reportedly 6%–11%.3–5 An irreversible, repurposed 2G EGFR-TKI,
poziotinib, reportedly has therapeutic potential for these tumors. However, because of its potent inhibition of wild-type EGFR, severe skin and gastrointestinal adverse effects have been reported in clinical trials.

Tarloxotinib is a hypoxia-activated prodrug that is designed to release a potent and irreversible pan-ERBB TKI (tarloxotinib-E) under pathophysiological hypoxia (<0.1% O₂), which is often present in solid tumors. Briefly, one-electron reduction of tarloxotinib under oxygen-deficient conditions leads to loss of the trigger moiety, releasing the neutral, diffusible “warhead” tarloxotinib-E. Mechanistic studies for this conversion show that tarloxotinib is cell-excluded because of the positive charge of the quaternary ammonium cation to which the 4-nitroimidazole trigger is excluded. Anoxic conditions deplete cell-associated tarloxotinib with a concomitant increase in the cell-associated tarloxotinib-E concentrations, which inhibit proliferation in various EGFR-expressing cell lines. The geometry of tarloxotinib-E release was spatially coordinated in vivo, with hypoxic regions in tumors from mice with SiHa tumor xenografts. In looking for molecules with important roles in hypoxic regions in tumors from mice with SiHa tumor xenografts, tarloxotinib-E release was spatially coordinated in vivo, with hypoxic regions in tumors from mice with SiHa tumor xenografts. In looking for molecules with important roles in hypoxic regions in tumors from mice with SiHa tumor xenografts.

METHODS

Cell culture, reagents, and establishment of Ba/F3 cells with EGFRExon20

The interleukin-3-dependent murine pro-B-cell line Ba/F3 was provided by Riken Bio Resource Center. Cells were cultured in RPMI 1640 medium (Wako) with 10% FBS (Sigma-Aldrich). EGFR-TKIs (afatinib, poziotinib, and osimertinib) were purchased from Selleck Chemicals. Tarloxotinib and tarloxotinib-E were provided by Rain Therapeutics, Inc. Mycoplasma contamination was checked routinely using the TaKaRa PCR Mycoplasma Detection Set (Takara).

Each EGFR exon 20 mutation was introduced into the Ba/F3 cells by retroviral vectors as previously described. We generated A763insFQEA, D770insSVD, and H773insNPH mutations from full-length complementary DNA that encoded the wild-type EGFR gene (Addgene), using the Prime STAR Mutagenesis Basal Kit (Takara) with specific primers (Table S1). Full-length complementary DNA that encoded V769insASV and H773insH was purchased from Addgene. We also used Ba/F3 cells with secondary mutations, plus either EGFR exon 19 deletion or L858R point mutation, which our previous study showed to be potential factors in resistance to frontline osimertinib.

Growth inhibition assay

We seeded 2000 transfected Ba/F3 cells into each well of 96-well plates, which were grown in RPMI 1640 medium supplemented with 10% FBS for 24 h. DMSO or an EGFR-TKI at the indicated concentration was then added, and the cells were cultured for a further 72 h. We used colorimetric assays to estimate the growth inhibition of each drug using the Cell Counting Kit-8 reagent (Dojindo Laboratories), following the manufacturer’s protocol. Each experiment was performed in triplicate.

Establishment of tarloxotinib-E- or poziotinib-resistant cells

Cells with acquired resistance against tarloxotinib-E or poziotinib were established using the ENU (Sigma-Aldrich) mutagenesis technique as previously described. ENU exposure was performed at the concentration of 100 μg/ml for 24 h. We seeded 5 × 10⁶ cells into 24 wells of 96-well plates in the presence of tarloxotinib-E (200 nM) or poziotinib (200 nM). These plates were incubated for two weeks with medium changes twice weekly; surviving clones were isolated. After establishing resistant cells, total DNA was extracted, and we explored secondary EGFR mutations.

| Table 1 FREQUENCIES OF EGFR EXON 20 INSERTIONS IN COSMIC DATABASE (SEARCHED ON JUNE 24, 2019) |
|-----------------|-----------------|-----------------|
| 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 |
RESULTS

Efficacy of tarloxotinib-E against various EGFR exon 20 mutations

Efficacies of afatinib, poziotinib, osimertinib, tarloxotinib, and tarloxotinib-E were evaluated using cell growth inhibition assays (Figure 1, Table 2). Among all tested Ba/F3 cell lines, IC_{50} was ≥72.1 times higher for tarloxotinib than for tarloxotinib-E, which implies a wide therapeutic window with this prodrug strategy. Using the cutoff value of 10 nM (50 nM for osimertinib because of its higher clinically achievable concentration), Ba/F3 cells with A763insFQEA showed sensitivity to all tested EGFR-TKIs. Ba/F3 cells with V769insASV, H770insSVD, or H773insNPH were sensitive to tarloxotinib-E and poziotinib, but not to afatinib or osimertinib. None of the tested EGFR-TKIs showed strong efficacy against Ba/F3 cells with H773insH.

Efficacy of tarloxotinib-E against potentially osimertinib-resistant cells

We also evaluated the efficacy of tarloxotinib and tarloxotinib-E in Ba/F3 cells with potential resistance-conferring mutations that may arise after front-line osimertinib treatment.13 In our previous study, we established Ba/F3 cells with EGFR exon 19 deletions together with one of L718Q, L718V, L792F, L792H, or C797S mutations; and Ba/F3 cells with L858R point mutation together with one of L718Q, L718V, L792F, L792H, C797G, or C797S mutations. Tarloxotinib-E showed quite low IC_{50} values against these Ba/F3 cells, except for those with L858R plus C797S (Table 3). However, tarloxotinib-E was ineffective against triple mutations, such as EGFR exon 19 deletion or L858R mutation plus T790M plus C797S, which could arise as an acquired resistance mechanism to osimertinib in a second-line setting after 1G or 2G EGFR-TKI therapy.

Secondary resistance mutations after tarloxotinib-E treatment

We also explored acquired resistance mechanisms to tarloxotinib-E in Ba/F3 cells with EGFR exon 20 mutations. After chronic exposure to tarloxotinib-E, we acquired 62 tarloxotinib-E-resistant clones. We also established 57 poziotinib-resistant clones, to confirm results after ending the tarloxotinib-E experiments. The A763insFQEA cells were highly sensitive to tarloxotinib-E and only one resistant clone (with acquired C797S) was obtained. In Ba/F3 cells with V769insASV, all 14 tarloxotinib-E-resistant clones and all 15 poziotinib-resistant clones developed a T790M mutation. In Ba/F3 cells with D770insSVD, all 24 tarloxotinib-E-resistant clones and 20 poziotinib-resistant clones developed a C797S mutation. In Ba/F3 cells with H773insNPH, 22 of 23 tarloxotinib-E-resistant clones and 21 of 22 poziotinib-resistant clones developed a T790M mutation; the other clones harbored a C797S mutation. The growth inhibitory assay showed that the IC_{50} values of these resistant cells were >100 times higher than parental cells, and these cells were also insensitive to 2G-TKI afatinib and 3G-TKI
osimertinib (Table 4). We introduced a C797S secondary mutation into Ba/F3 cells with V769insASV, which developed a T790M mutation through chronic drug exposure. We also introduced a T790M secondary mutation into Ba/F3 cells with D770insSVD, which developed a C797S mutation through chronic drug exposure. These cells were also insensitive to tarloxotinib-E or poziotinib (Table 4).

### DISCUSSION

Development of effective therapy for NSCLC with EGFR exon 20 mutation is an unmet clinical need. Some rare EGFR exon 20 mutations are sensitive to currently available EGFR-TKIs; e.g., A763insFQEA was sensitive to erlotinib16 and D770delinsGY showed partial response to an irreversible EGFR-TKI, dacomitinib.17 However, currently available EGFR-TKIs usually cannot inhibit EGFR exon 20 mutations. Many EGFR exon 20 mutation variants are reported in the COSMIC database. In this study, we established Ba/F3 cells with five most common EGFR exon 20 mutations, including the aforementioned A763insFQEA mutation, which was sensitive to erlotinib. These five variants accounted for 72.2% of all recurrent EGFR exon 20 mutations in the COSMIC database (Table 1).

As expected, A763insFQEA was sensitive to all tested EGFR-TKIs including tarloxotinib-E and poziotinib. Our results also showed that V769insASV, D770insSVD, and H773insNPH were sensitive to both tarloxotinib-E and poziotinib. These results suggest that tarloxotinib-E and...
poziotinib may be effective against many EGFR exon 20 mutations. Therefore, if conversion from tarloxtinib to tarloxtinib-E in tumor tissues of human subjects is confirmed, tarloxtinib might be used to treat lung cancers with EGFR exon 20 mutations. However, we found that the fourth most common EGFR exon 20 mutation, H773insH, did not respond well to either tarloxtinib-E or poziotinib. We also showed that tarloxtinib-E and poziotinib were not active against so-called triple mutations, such as exon 19 deletion or L858R plus T790M plus C797S mutations, which can arise after 1G/2G EGFR-TKI and osimertinib treatment failures. As tarloxtinib-E and poziotinib are quite potent irreversible EGFR-TKIs, another strategy, such as combination of an allosteric inhibitor and a quinazoline-based EGFR-TKI, will be needed to inhibit these resistant EGFR mutants. Notably, however, tarloxtinib-E showed quite low IC50 values against Ba/F3 cells with secondary mutations that may confer acquired resistance to front-line osimertinib.13 Osimertinib was approved as a first-line treatment for lung cancer with EGFR mutations in 2018, and therefore tarloxtinib may be a suitable second-line EGFR-TKI for lung cancers with exon 19 deletions or with L858R point mutation.

In analyzing the potential resistance mechanisms to tarloxtinib-E and poziotinib, we found that the acquisition of a T790M or C797S mutation to an EGFR exon 20 mutation was enough to confer resistance to these drugs (Table 4). Additionally, we observed some preference for specific secondary mutation in each type of EGFR exon 20 mutation (T790M for V769insASV and H773insNPH, and C797S for D770insSVD) in the drug exposure experiments. Although the mechanism for these preferences is unclear, the aforementioned study17 also observed that only T790M developed after chronic dacomitinib exposure in D770delinsGY Ba/F3 cells, whereas Ba/F3 cells that contained either T790M or C797S together with D770delinsGY were resistant to dacomitinib and afatinib. Similarly, EGFR with an exon 19 deletion—but not EGFR L858R—reportedly developed resistance to the 3G inhibitor osimertinib via L724S, which further supports the notion that resistance mutations are influenced by baseline mutations.19

In conclusion, our results indicate that tarloxtinib-E could be effective for most NSCLCs with EGFR exon 20 mutations, or those for which front-line osimertinib treatment is no longer effective. However, continuous efforts to develop novel EGFR inhibitors are necessary, as some EGFR mutants, such as D770insH or EGFR exon 20 mutation plus T790M or C797S, are refractory to tarloxtinib-E and poziotinib in vitro.

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**CONFLICT OF INTEREST**

Dr Suda has received honoraria from Boehringer Ingelheim, has been a consultant/advisory board member for AstraZeneca, and has received research funding (through Kindai University Faculty of Medicine) from Rain Therapeutics and Boehringer Ingelheim. Mr Vellanki is currently an employee of Rain Therapeutics and owns stock in Rain Therapeutics. Dr Tirunagaru is currently an employee of Rain Therapeutics and owns stock in Rain Therapeutics. Dr Doebele is currently an employee of Rain Therapeutics and owns stock in Rain Therapeutics. Dr Doebele has received personal fees from Genentech/Roche, Ignyta, Blueprint Medicines, Green Peptide, AstraZeneca, Anchiano, Takeda/Millenium, and Bayer outside the submitted work; in addition, Dr Doebele has a patent for U.S. Provisional Patent Application No. 62/712531 pending and licensed to Rain Therapeutics; and The University of Colorado has received licensing fees from Foundation Medicine, Ignyta, Scorpion Therapeutics, Voronoi, Pearl River, Black Diamond Therapeutics, and Genentech for biological materials derived in Dr Doebele’s laboratory. Dr Mitsudomi has received honoraria from AstraZeneca, Boehringer Ingelheim, Chugai, and Pfizer, has been a consultant/advisory board member for AstraZeneca, Chugai, and Boehringer Ingelheim, and has received research funding (through Kindai University Faculty of Medicine) from AstraZeneca, Boehringer Ingelheim, and Chugai. The other authors declare no conflicts of interest.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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