BRIEF REPORT

# The Impact of On-Target Resistance Mediated by EGFR-T790M or EGFR-C797S on EGFR Exon 20 Insertion Mutation Active Tyrosine Kinase Inhibitors

Ikei S. Kobayashi, MD, PhD,[a] William Shaffer, MS,[a] Hollis Viray, MD,[a] Deepa Rangachari, MD,[a] Paul A. VanderLaan, MD, PhD,[b] Susumu S. Kobayashi, MD, PhD,[a,c] Daniel B. Costa, MD, PhD[a,*]

[a]*Division of Medical Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts*
[b]*Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts*
[c]*Division of Translational Genomics, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa, Japan*

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## ABSTRACT

**Introduction:** Mechanisms of resistance to EGFR exon 20 insertion mutation active inhibitors have not been extensively studied in either robust preclinical models or patient-derived rebiopsy specimens. We sought to characterize on-target resistance mutations identified in *EGFR* exon 20 insertion-mutated lung cancers treated with mobocertinib or poziotinib and evaluate whether these mutations would or would not have cross-resistance to next-generation inhibitors zipalertinib, furmonertinib, and sunvozertinib.

**Methods:** We identified mechanisms of resistance to EGFR exon 20 insertion mutation active inhibitors and then used preclinical models of EGFR exon 20 insertion mutations (A767_V769dupASV, D770_N771insSVD, V773_C774insH) plus common EGFR mutants to probe inhibitors in the absence/presence of EGFR-T790M or EGFR-C797S.

**Results:** Mobocertinib had a favorable therapeutic window in relation to EGFR wild type for EGFR exon 20 insertion mutants, but the addition of EGFR-T790M or EGFR-C797S negated the observed window. Zipalertinib had a favorable therapeutic window for cells driven by EGFR-A767_V769dupASV or EGFR-D770_N771insSVD in the presence or absence of EGFR-T790M. Furmonertinib and sunvozertinib had the most favorable therapeutic windows in the presence or absence of EGFR-T790M in all cells tested. *EGFR*-C797S in *cis* to all *EGFR* mutations evaluated generated dependent cells that were resistant to the covalent EGFR tyrosine kinase inhibitors mobocertinib, zipalertinib, furmonertinib, sunvozertinib, poziotinib, and osimertinib.

**Conclusions:** This report highlights that poziotinib and mobocertinib are susceptible to on-target resistance mediated by *EGFR*-T790M or -C797S in the background of the most prevalent EGFR exon 20 insertion mutations. Furmonertinib,

*Corresponding author.

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Address for correspondence: Daniel B. Costa, MD, PhD, Division of Medical Oncology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. E-mail: dbcosta@bidmc.harvard.edu

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sunvozertinib, and to a less extent zipalertinib can overcome EGFR-T790M compound mutants, whereas EGFR-C797S leads to covalent inhibitor cross-resistance—robust data that support the limitations of mobocertinib and should further spawn the development of next-generation covalent and reversible EGFR exon 20 insertion mutation active inhibitors with favorable therapeutic windows that are less vulnerable to on-target resistance.



*Keywords:* EGFR-T790M; EGFR-C797S; Mobocertinib; Zipalertinib; Furmonertinib; Sunvozertinib

## Introduction

Acquired resistance to targeted therapies is a common thread in oncogene-driven lung cancers. This phenomenon has been extensively studied in preclinical models and rebiopsy specimens of lung cancers driven by the most common EGFR mutations (exon 19 insertion/deletion or L858R), where it is established that a combination of on-target *EGFR* mutations, off-target activation of alternative oncogenic signaling, or cellular differentiation from epithelial to neuroendocrine phenotypes explains most cases with resistance to EGFR tyrosine kinase inhibitors (TKIs).[1] The major on-target resistance mutations have been identified for the approved reversible first-generation EGFR TKIs gefitinib and erlotinib (*EGFR*-T790M), the irreversible second-generation EGFR TKIs afatinib and dacomitinib (*EGFR*-T790M and *EGFR*-C797S), and the covalent mutation-selective third-generation EGFR TKI osimertinib (*EGFR*-C797S).[1,2]

Nevertheless, the heterogeneous group of EGFR exon 20 insertion mutations (approximately 10% of all EGFR mutants identified in lung adenocarcinomas) is usually insensitive to the aforementioned EGFR TKIs owing to their structure within the kinase domain of EGFR that precludes a therapeutic window to the drug motifs of gefitinib, erlotinib, afatinib, dacomitinib, and osimertinib.[3,4] Only recently have novel EGFR exon 20 insertion mutation active inhibitors reached clinical development, and this has led to the short-term regulatory approval (from 2021 to 2023) of the EGFR TKI mobocertinib.[5–15] The only other approved therapy is the dual EGFR-MET antibody amivantamab that has modest clinical activity as a single agent but more potential as an add-on therapy to traditional platinum-doublet chemotherapy.[5,16,17] Multiple more selective EGFR exon 20 insertion mutation active TKIs—such as zipalertinib, furmonertinib, and sunvozertinib—have entered later stage clinical trials seeking regulatory approval.[5] Mobocertinib achieved regulatory approval for patient care on the basis of initial phase 1 and phase 2 trials after failure of traditional therapies. The clinical results were modest at best, with the highest dose of mobocertinib 160 mg daily only achieving response rates below 30% with progression-free survival times below 8 months.[5] The data for poziotinib are even less robust.[5] In addition, mobocertinib has been reported to not be superior to traditional chemotherapy in the treatment-naive setting of advanced *EGFR* exon 20 insertion-mutated lung cancers (data not yet presented from ClinicalTrials.gov ID NCT04129502)—a result that has halted the clinical trial development of mobocertinib and will lead to removal of the regulatory approval of this EGFR TKI. Zipalertinib has undergone initial development with acceptable toxicities with seemingly more robust clinical activity than mobocertinib or poziotinib,[5,13] and an ongoing registration clinical trial (NCT05973773-REZILIENT3) is attempting to showcase the superiority of chemotherapy plus zipalertinib versus chemotherapy alone. Sunvozertinib is undergoing a similar path of clinical development but with seemingly even more robust clinical activity[5,14] and a registration phase 3 clinical trial (NCT05668988) that attempts to display superiority of sunvozertinib monotherapy versus platinum-doublet chemotherapy. Furmonertinib is already an approved EGFR TKI for more common *EGFR*-mutated lung cancers (those with *EGFR*-exon 19 insertion/deletion or -L858R) and is undergoing earlier stages of clinical development at high doses (160 mg or 240 mg daily instead of the approved 80 mg daily) for advanced *EGFR* exon 20 insertion-mutated lung cancer with promising clinical results[15] and a global registration trial (NCT05607550-FURVENT) of this EGFR TKI versus platinum-based chemotherapy.

Mechanisms of resistance to the aforementioned EGFR TKIs in the context of EGFR exon 20 insertion mutations have not been extensively studied in either robust preclinical models or in patient-derived rebiopsy specimens.[5–15] We sought to characterize on-target resistance mutations identified in *EGFR* exon 20 insertion-mutated lung cancer treated with mobocertinib or poziotinib and to establish whether these would or would have cross-resistance to the next-generation inhibitors zipalertinib, furmonertinib, or sunvozertinib with a goal of attempting to maximize precision oncology for this cohort of NSCLCs.

## Materials and Methods

### *Literature Review of Acquired Resistance to EGFR Inhibitors*

Genotype-inhibitor resistance data were obtained through a literature review of studies published in

**Table 1.** Mechanisms of Reported On-Target and Off-Target Resistance to EGFR Exon 20 Insertion Mutation Active TKIs

| EGFR Exon 20 Insertion Active TKI | On-Target Resistance (Clinical Samples) | On-Target Resistance (Preclinical Models) | Off-Target Resistance (Clinical Samples) |
|---|---|---|---|
| Mobocertinib | *EGFR*-T790M (prevalence unknown)<br>*EGFR*-C797S (prevalence unknown) | *EGFR*-T790M<br>*EGFR*-C797S | Not reported to date |
| Poziotinib | *EGFR*-T790M (13% prevalence) | *EGFR*-T790M<br>*EGFR*-C797S | *MET* amplification<br>*PIK3CA* mutation |
| Zipalertinib | Not reported to date | *EGFR*-C797S | Not reported to date |
| Sunvozertinib | Not reported to date | *EGFR*-C797S | Not reported to date |
| Furmonertinib | Not reported to date | *EGFR*-C797S | Not reported to date |

*Note*. The data were obtained from Vincent et al.,[9] Park et al.,[10] Elamin et al.,[11] Hamada et al.,[12] and the current report (Fig. 2).
TKI, tyrosine kinase inhibitor.

PubMed and other databases, including oncology meeting abstracts using the search field "EGFR exon 20 insertion" plus fields "mobocertinib," "poziotinib," "zipalertinib," "furmonertinib," and "sunvozertinib."

### Drugs

Osimertinib (LC Laboratories), mobocertinib (MedChemExpress), zipalertinib (MedChemExpress), and sunvozertinib (MedChemExpress) were dissolved in DMSO (Fisher Scientific) at 10 mM and stored at −80°C before dilutions. Furmonertinib (MedChemExpress) was mixed with DMSO at 5 mM immediately before each experiment.

### Cell Lines and Reagents

Ba/F3 murine cells were maintained as described previously[3,6–8] and interleukin-3 independent Ba/F3 cells were used for further experiments.[6–8] In the case of EGFR-WT–driven Ba/F3 cells, 10 ng/mL of EGF (PeproTech) was added to allow for interleukin-3 independence. All cells were grown at 37°C in a humidified atmosphere with 5% $CO_2$ and tested for absence of mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza) before the experiments (initiated within the initial one to five passages).

### EGFR-Mutant Constructs

The mutant *EGFR* constructs used in this study were A767_V769dupASV, D770_N771insSVD, V773_C774insH, delE746_A750, and L858R with or without *EGFR*-T790M or *EGFR*-C797S in *cis* to the aforementioned EGFR mutation. The resulting constructs were confirmed by nucleotide sequencing (Azenta Life Sciences).

### Cell Proliferation Assays

Cell viability was determined by CellTiter 96 aqueous one solution proliferation kit (Promega) for Ba/F3 cells, as previously described.[6–8] Inhibitory proliferation curves and the 50% inhibitory concentration ($IC_{50}$) were generated using GraphPad Prism 7 (GraphPad Software). Preclinical therapeutic window was calculated using logarithm of $IC_{50}$ of EGFR mutants compared with EGFR wild type (WT) with values below zero indicating sensitivity (favorable therapeutic window) and values above zero indicating resistance (unfavorable therapeutic window) to each EGFR TKI.

### Immunoblotting

Cells were treated with indicated EGFR TKIs for 6 hours at various concentrations. Cytoplasmic proteins were isolated through cell lysis for Western blotting, as detailed in prior reports.[3,6–8] Total EGFR (Santa Cruz Biotechnology), phospho-EGFR antibody (pY1068) (Cell Signaling), and $\beta$-actin (Cell Signaling) antibodies were used at 1:1000 dilution.

## Results

### On-Target Resistance EGFR Mutations Identified in Clinical Specimens

There are few reports of liquid biopsy or tissue rebiopsy in patients treated with mobocertinib or similar EGFR TKIs.[9–11] We were able to identify two studies that reported on liquid biopsy after mobocertinib resistance and one study evaluating on tissue rebiopsy after poziotinib resistance (Table 1). Both *EGFR*-T790M and *EGFR*-C797S have been reported (in unknown prevalence in larger cohorts) in mobocertinib-treated patients with lung cancer with *EGFR* exon 20 insertion-mutated lung cancer, whereas only *EGFR*-T790M was identified in poziotinib-treated cases (Table 1). To the best of our knowledge and from the available literature,[12] mechanisms of on-target or off-target resistance have not been reported from clinical cases that received the newer EGFR TKIs zipalertinib, furmonertinib, or sunvozertinib.

In view of these clinical findings, we decided to characterize in preclinical models on-target resistance mediated both by EGFR-T790M or EGFR-C797S in the

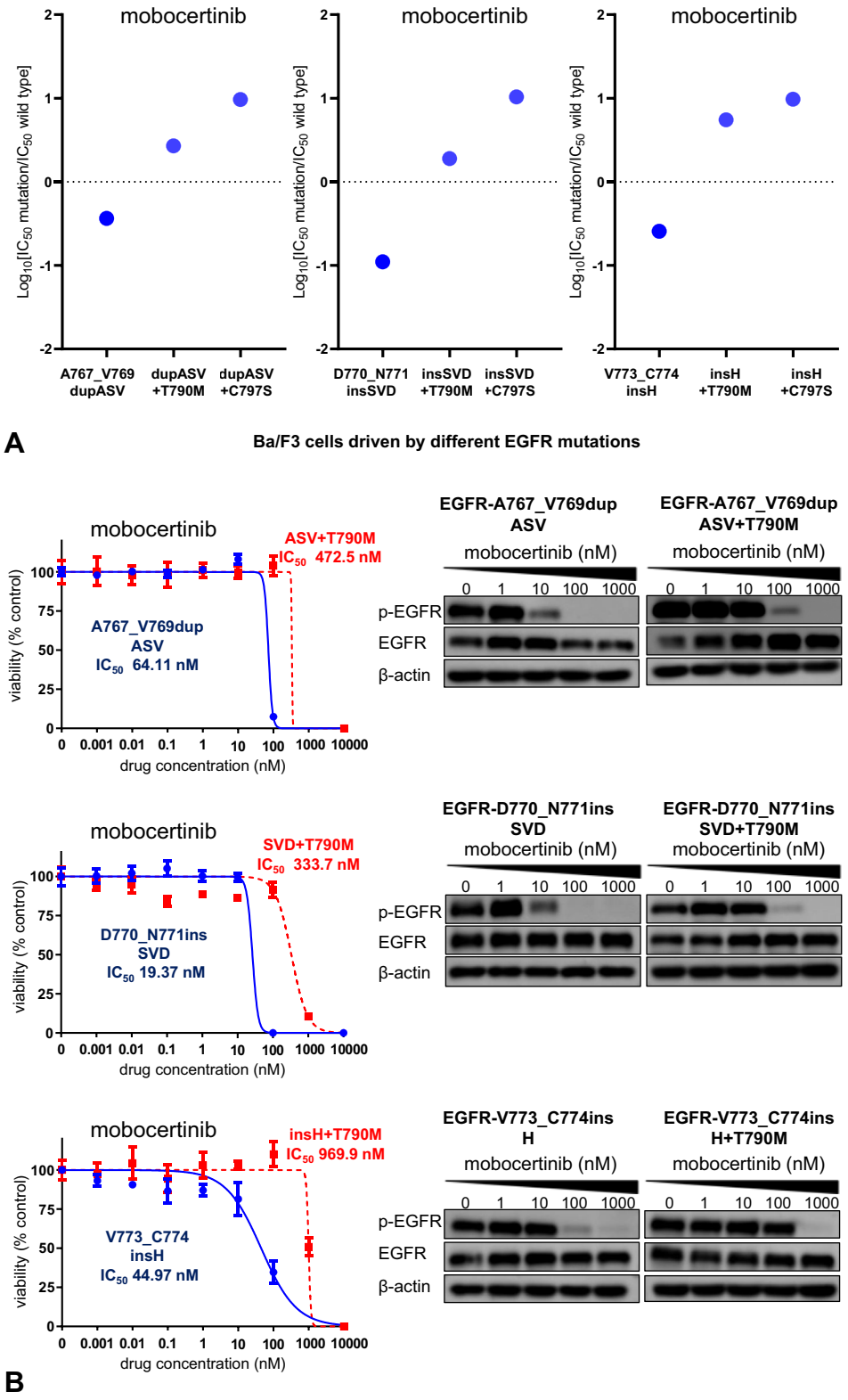


**Figure 1.** Characterization of EGFR-T790M as on-target resistance to mobocertinib. (*A*) Therapeutic window of mobocertinib in selected EGFR exon 20 insertion mutants (A767_V769dupASV, D770_N771insSVD, V773_C774insH) in the absence or presence of EGFR-T790M or -C797S. Cells were plated at a density of 10,000 cells per well (96-well plates) and grown over 3 days after treatment. Logarithm of the $IC_{50}$ of EGFR mutants compared with EGFR-WT is plotted with three separate experiments used to generate $IC_{50}$. Values below zero (0) indicate sensitivity, whereas values above 0 indicate resistance to EGFR TKIs. (*B*) Dose-response proliferation assays (percent viability) for cell lines harboring EGFR mutants (A767_V769dupASV, D770_N771insSVD, V773_C774insH) in the absence or presence of EGFR-T790M after exposure to increasing concentrations of

context of the most common clusters of representative EGFR exon 20 insertion mutants.

### *Preclinical Characterization of EGFR-T790M or EGFR-C797S as Modulators of Sensitivity to Different EGFR Exon 20 Insertion Mutation Active TKIs*

We generated compound mutations of *EGFR*-A767_V769dupASV, -D770_N771insSVD, and -V773_C774insH in cis to *EGFR*-T790M or *EGFR*-C797S and made Ba/F3 cells dependent on EGFR signaling to study different EGFR TKIs.

Mobocertinib had a favorable therapeutic window in relation to EGFR-WT for all EGFR exon 20 insertion mutants, but the addition of EGFR-T790M or EGFR-C797S negated that therapeutic window (Fig. 1*A*).

The dose-response proliferation assay curves highlighted a shift in sensitivity curves when each exon 20 insertion mutant had EGFR-T790M, with more than 6- to 20-fold increase in $IC_{50}$ for mobocertinib (Fig. 1*B*). The same pattern of EGFR-T790M–induced resistance to mobocertinib was noticeable at the protein level as delineated by the need of more than 10-fold higher dose of mobocertinib to achieve inhibition of EGFR autophosphorylation (Fig. 1*B*)—a measure of EGFR signaling. These results confirmed *EGFR*-T790M as a major on-target mechanism of resistance to mobocertinib in *EGFR* exon 20 insertion-mutated lung cancer.

We next evaluated the impact of compound EGFR-T790M mutants in other EGFR exon 20 insertion mutation active TKIs. Zipalertinib had a favorable therapeutic window in the presence or absence of EGFR-T790M for cells driven by EGFR-A767_V769dupASV and EGFR-D770_N771insSVD but not EGFR-V773_C774insH (Fig. 2*A*). Both furmonertinib and sunvozertinib had favorable therapeutic windows in the presence or absence of EGFR-T790M in all cells tested (Fig. 2*A*). The detailed dose-response curve of cells driven by EGFR-D770_N771insSVD against these EGFR inhibitors helps highlight how the presence of EGFR-T790M has minimal effects on inhibitory curves of furmonertinib and sunvozertinib, whereas zipalertinib is affected with an eightfold increase in $IC_{50}$ (Fig. 2*B*).

To place these results into context, we also evaluated zipalertinib, furmonertinib, sunvozertinib, mobocertinib, poziotinib, and osimertinib in more common TKI-sensitive EGFR mutants (EGFR-delE746_A750 and EGFR-L858R with or without EGFR-T790M), with all drugs—outside poziotinib—with favorable therapeutic windows in the presence of EGFR-T790M (Fig. 2*A*). Both osimertinib and poziotinib had unfavorable therapeutic windows in the context of EGFR exon 20 insertion mutations tested when combined with EGFR-T790M (Fig. 2*A* and *B*). These results highlight that the presence of EGFR-T790M generates different sensitivity/resistance patterns in the background of EGFR exon 20 insertion mutations when compared with the pan-EGFR TKI-sensitive classical EGFR mutants (exon 19 deletion and L858R).

The addition of EGFR-C797S to all EGFR mutants evaluated generated dependent cells that were resistant to (as measured by therapeutic window in relation to EGFR-WT) all the covalent EGFR TKIs tested (zipalertinib, furmonertinib, sunvozertinib, mobocertinib, poziotinib, and osimertinib) in this report (Fig. 2*A* and *B*), a result expected on the basis of the need for covalent bonding to EGFR amino acid position C797 for all these TKIs.[2,4]

## Discussion

To the best of our knowledge, the current report represents the largest preclinical report on the impact of on-target *EGFR* mutations in the efficacy and therapeutic window of EGFR exon 20 insertion mutation active inhibitors. Our data support that the first-generation of EGFR exon 20 insertion mutation active TKIs (such as poziotinib and mobocertinib) are susceptible to on-target resistance mediated by EGFR-T790M, and this type of compound mutation can be overcome by next-generation EGFR exon 20 insertion mutation active TKIs (such as zipalertinib, furmonertinib, sunvozertinib), whereas EGFR-C797S acts as an on-target mechanism of resistance to all the aforementioned TKIs that share a mechanism of covalent bond to amino acid position EGFR-C797.

These results expand on emerging reports from other groups[12] and may have implications for the clinical development of EGFR exon 20 insertion mutation active TKIs. Our preclinical data lend some insights into the shortcomings of mobocertinib and poziotinib. Both these first-generation EGFR exon 20 insertion mutation active TKIs have narrow therapeutic windows (highlighting the common dose-limiting skin and gastrointestinal adverse events) and are susceptible to on-target EGFR-T790M

mobocertinib. Three separate experiments were used to generate $IC_{50}$, and SDs are depicted in vertical bars. Western blotting of Ba/F3 cells driven by EGFR exon 20 insertion mutants in the absence or presence of EGFR-T790M. Cells were treated with mobocertinib for 6 hours at the indicated ascending concentrations. pEGFR at position 1068, total EGFR and $\beta$-actin (loading control) are exhibited. $IC_{50}$, 50% inhibitory concentration; pEGFR, phosphorylated EGFR; TKI, tyrosine kinase inhibitor; WT, wild type.

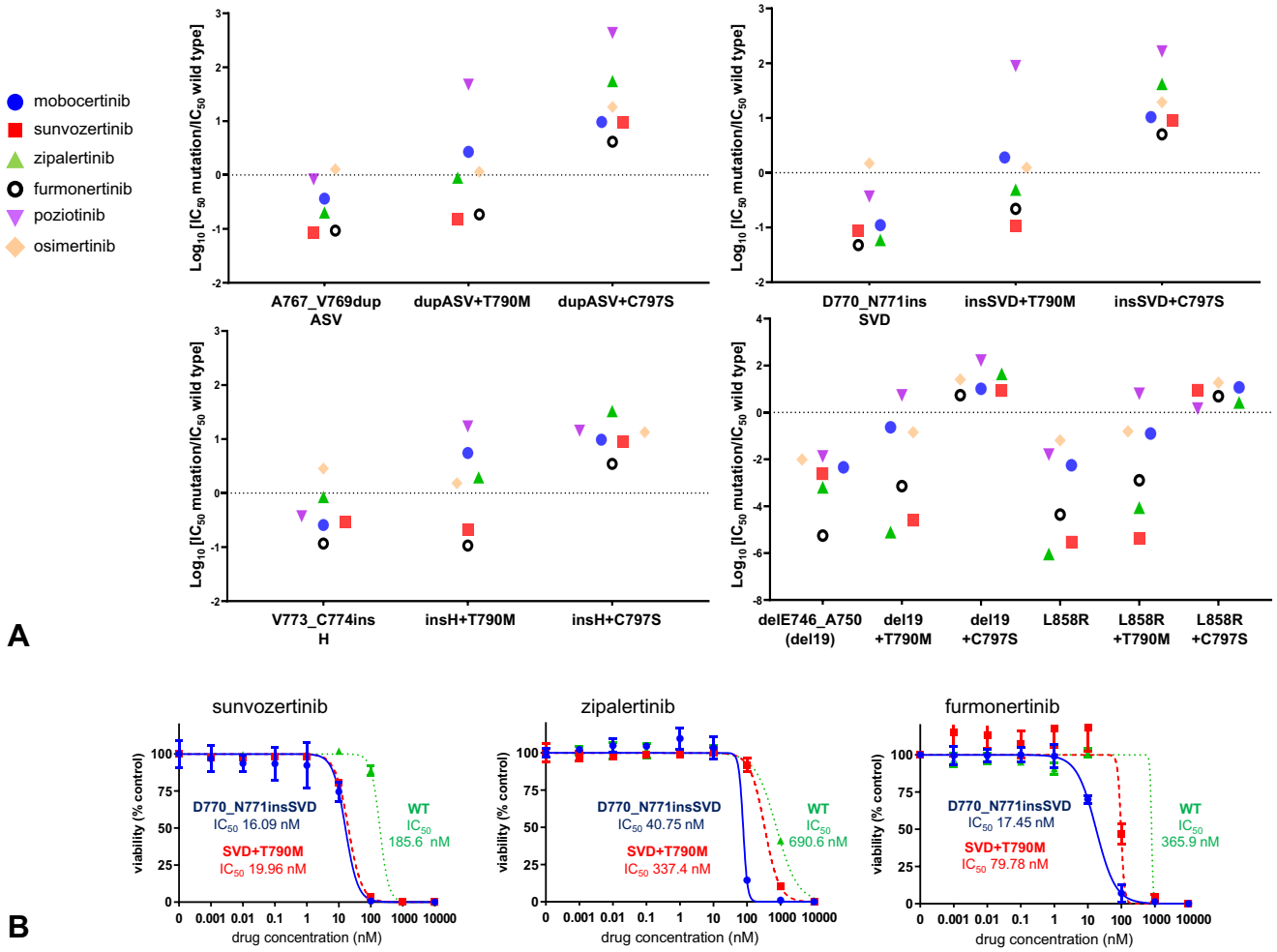


**Figure 2.** The impact of EGFR-T790M or EGFR-C797S in the sensitivity of different EGFR TKIs in the background of different types of EGFR mutants. (*A*) Therapeutic window of different EGFR TKIs to EGFR mutants. Cells were plated at a density of 10,000 cells per well (96-well plates) and grown over 3 days after treatment. $IC_{50}$ of EGFR mutants compared with EGFR-WT is plotted with three separate experiments used to generate $IC_{50}$. Values below zero (0) indicate sensitivity, whereas values above 0 indicate resistance to EGFR TKIs. The therapeutic window of Ba/F3 cells with EGFR mutants (A767_V769dupASV, D770_N771insSVD, V773_C774insH, delE746_A750, L858R in the presence or absence of EGFR-T790M or EGFR-C797S) were plotted for each EGFR TKI (osimertinib, poziotinib, mobocertinib, zipalertinib, furmonertinib, sunvozertinib). (*B*) Dose-response proliferation assays (percent viability) for cell lines harboring EGFR-D770_N771insSVD in the absence or presence of EGFR-T790M or EGFR-C797S after exposure to increasing concentrations of zipalertinib, furmonertinib, and sunvozertinib. Three separate experiments were used to generate $IC_{50}$, and SDs are depicted in vertical bars. $IC_{50}$, 50% inhibitory concentration; pEGFR, phosphorylated EGFR; TKI, tyrosine kinase inhibitor; WT, wild type.

resistance (highlighting the limited potency of the drugs).

The next-generation EGFR exon 20 insertion mutation active TKIs have better potential to achieving more meaningful clinical outcomes and obtaining durable regulatory approvals. Our data support that zipalertinib, furmonertinib, and sunvozertinib have improved therapeutic windows in relation to EGFR-WT in most tested models and are also EGFR TKIs with less susceptibility to on-target EGFR-T790M resistance. Specifically, furmonertinib or sunvozertinib was minimally affected by EGFR-T790M in all preclinical models used herein. These preclinical data dovetail with evolving clinical trial development for these drugs (as detailed in the Introduction[5,13–15]). The combined preclinical data from our report and others when added to the encouraging initial clinical responses plus the ongoing registration trials are supportive of a more robust pathway for future clinical utility of zipalertinib, furmonertinib, and sunvozertinib.[5,12–15] In the meantime, there will be a period without an EGFR TKI approved for *EGFR* exon 20 insertion-mutated lung cancer. The only other approved therapy is the dual EGFR-MET antibody amivantamab.[5] It is unknown from either preclinical models or clinical trials how to sequence amivantamab-based therapies with EGFR exon 20

insertion mutation active TKIs. As even next-generation EGFR exon 20 insertion mutation active TKIs (such as zipalertinib, furmonertinib, sunvozertinib) are susceptible to on-target EGFR-C797S resistance, there remains an unmet need to further develop EGFR exon 20 insertion mutation active TKIs with different mechanisms of EGFR kinase domain binding and other combinatory therapies for these recalcitrant lung cancers.

Limitations of our study include our focus on on-target resistance (that has an unknown prevalence in clinical cases treated with next-generation EGFR exon 20 insertion active TKIs), the lack of characterization of off-target mechanisms of resistance (albeit these have not been reported in clinical cases treated with mobocertinib, zipalertinib, furmonertinib, or sunvozertinib—as described in Table 1), and the lack of an in vivo preclinical model that would have allowed the analysis of the dual EGFR-MET antibody amivantamab in the context of EGFR-T790M or EGFR- C797S (it is expected that compound EGFR mutations will not affect amivantamab[5]). In addition, we were only able to obtain commercially available EGFR TKIs that have known covalent binding to EGFR-C797 and we did not have commercial access to test EGFR exon 20 insertion mutation active EGFR TKIs with a reversible (non-covalent) binding mode, such as the newly developed drug BAY 2927088. We also only focused on the first-, second-, and third-generation EGFR TKI-resistant cohort of EGFR exon 20 insertion mutations (represented herein by EGFR-A767_V769dupASV and EGFR-D770_N771insSVD but not EGFR-V773_C774insH mutants that our group and others have previously collated as the most common representatives for preclinical studies[2–8,12]) and purposely neglected to study the rare group of exon 20 insertions that have sensitivity to first-, second-, and third-generation EGFR TKIs—including EGFR-A763_Y764insFQEA that is pan-sensitive to EGFR TKIs and where we would not expect EGFR-T790M to be an on-target resistance to osimertinib or any of the EGFR exon 20 insertion active TKIs analyzed.[2–12]

In summary, our preclinical data characterize mechanisms of on-target resistance to EGFR TKIs mediated by EGFR-T790M or EGFR-C797S in the background of the most prevalent EGFR exon 20 insertion mutants. The agglomeration of results support zipalertinib, furmonertinib, and sunvozertinib as next-generation EGFR exon 20 insertion mutation active TKIs with superior therapeutic windows for clinical development than mobocertinib or poziotinib (both drugs have failed to gain sustained regulatory approval for clinical use) and as EGFR TKIs less susceptible to on-target EGFR-T790M resistance.

## CRediT Authorship Contribution Statement

**Daniel B. Costa:** Conceptualization, Resources, Data curation, Writing—original draft, Writing—review and editing, Visualization, Supervision, and Funding acquisition.

**Ikei S. Kobayashi:** Resources, Data curation, Writing—original draft, Writing—reviewed and editing, and Visualization.

**William Shaffer:** Resources, Data curation, Writing—reviewed and editing, and Visualization.

**Hollis Viray:** Resources, Data curation, and Writing—review and editing.

**Deepa Rangachari:** Resources, Data curation, and Writing—review and editing.

**Paul A. VanderLaan:** Resources, Data curation, and Writing—review and editing.

**Susumu S. Kobayashi:** Resources, Data curation, Writing—review and editing, Visualization, Supervision, and Funding acquisition.

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