Pharmacological and Structural Characterizations of Naquotinib, a Novel Third-Generation EGFR Tyrosine Kinase Inhibitor, in EGFR-Mutated Non-Small Cell Lung Cancer

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Abstract

Multiple epidermal factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKI) have been developed to effectively inhibit EGFR-derived signals in non–small cell lung cancer (NSCLC). In this study, we assessed the efficacy of EGFR-TKIs, including a novel third-generation inhibitor naquotinib (ASP8273), in clinically relevant EGFR mutations, including L858R, exon 19 deletion, L858R+T790M, exon 19 deletion+T790M with or without a C797S mutation, and several exon 20 insertion mutations. Using structural analyses, we also elucidated the mechanism of activation and sensitivity/resistance to EGFR-TKIs in EGFR exon 20 insertion mutations. The efficacy of naquotinib in cells with L858R, exon 19 deletion and exon 19 deletion+T790M was comparable with that of osimertinib. Interestingly, naquotinib was more potent than osimertinib for L858R+T790M. Additionally, naquotinib and osimertinib had comparable efficacy and a wide therapeutic window for cells with EGFR exon 20 insertions. Structural modeling partly elucidated the mechanism of activation and sensitivity/resistance to EGFR-TKIs in two EGFR exon 20 insertion mutants, A767_V769dupASV and Y764_V765insHH. In summary, we have characterized the efficacy of EGFR-TKIs for NSCLC using in vitro and structural analyses and suggested the mechanism of activation and resistance to EGFR-TKIs of EGFR exon 20 insertion mutations. Our findings should guide the selection of appropriate EGFR-TKIs for the treatment of NSCLC with EGFR mutations and help clarify the biology of EGFR exon 20 insertion mutations. Mol Cancer Ther; 17(4): 740–50. ©2018 AACR.

Introduction

Lung cancers with mutation in the epidermal growth factor receptor (EGFR) account for a significant subgroup of non–small cell lung cancers (NSCLC; refs. 1, 2). Somatic mutations of the tyrosine kinase domain of EGFR, such as in-frame deletions around the LREA motif in exon 19 (exon 19 deletions) and the L858R point mutation in exon 21, are classic mutations, accounting for approximately 80% to 90% of EGFR mutations in NSCLC (3, 4). These mutations destabilize the inactive conformation of EGFR and induce its constitutive activation (5–7). Activated EGFR transduces downstream signals, including the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/protein kinase B (AKT) pathways (8, 9). Studies demonstrating the efficacy of first-generation EGFR tyrosine kinase inhibitors (EGFR-TKI) such as gefitinib and erlotinib against NSCLC, such as alevatinib against NSCLC harboring the aforementioned mutations had significantly changed the treatment strategy for NSCLC (10–15). In addition to first-generation drugs, the efficacy of second-generation EGFR-TKIs, such as afatinib, in patients with NSCLC bearing these mutations has been demonstrated (16). However, NSCLC was shown to acquire resistance to first- and second-generation EGFR-TKIs in approximately 1 year. EGFR T790M mutation is found in approximately 50% of NSCLC resistant to first- and second-generation EGFR-TKIs (17, 18). To overcome the EGFR T790M-mediated resistance, third-generation EGFR-TKIs have been developed (19–22). Third-generation EGFR-TKIs bind irreversibly to the ATP binding pocket of EGFR by covalently binding to Cys797, thereby blocking EGFR-mediated signals. Osimertinib is an FDA-approved third-generation EGFR-TKI that has been shown to significantly improve the prognosis of patients with NSCLC harboring the EGFR T790M mutation in multiple clinical trials (23, 24). Naquotinib, previously known as ASP8273, is a novel third-generation EGFR-TKI (25), and a preclinical study has reported its efficacy for cell lines harboring EGFR mutations with or without a T790M mutation (26). However, naquotinib has not been fully characterized.
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and directly compared with other EGFR-TKIs including osimertinib. In this study, we compared the efficacy of naquotinib with that of other EGFR-TKIs in several clinically relevant NSCLC cell lines harboring EGFR mutations.

In addition, we performed structural evaluations of several EGFR exon 20 insertion mutants. It is noteworthy to mention that exon 20 insertions are the third major subgroup of EGFR mutations, accounting for approximately 4% to 10% of all EGFR mutations (27, 28). EGFR exon 20 insertions have been associated with resistance to first- and second-generation EGFR-TKIs. One exception is the A763_Y764insFQEA mutation, which we previously reported as a sensitizing mutation to first-generation EGFR-TKIs (29). We have also reported the potential efficacy of osimertinib against tumors with exon 20 insertions in a previous study (30). However, findings that clarify the mechanism of activation and resistance of NSCLC with exon 20 insertions to first- and second-generation EGFR-TKIs are limited, and therefore the mechanism was addressed in this study. Our findings should guide the selection of appropriate EGFR-TKIs for the treatment of NSCLC with EGFR mutations and help clarify the biology of EGFR exon 20 insertion mutations.

Materials and Methods

Cell lines

Five human NSCLC cell lines were used: PC-9 [EGFR exon 19 deletion (delE746-A750)]; H3255 (EGFR L858R); PC-9ER [EGFR exon 19 deletion (delE746-A750) + T790M]; BID007 [EGFR exon 20 insertion (A763_Y764insFQEA)]; and H1975 (EGFR L858R + T790M). PC-9, H3255, and BID007 cells were a kind gift from Dr. Susumu Kobayashi (Beth Israel Deaconess Medical Center, Boston, MA). H1975 cells were purchased from the ATCC in April 2017. PC-9ER cells developed resistance to erlotinib after chronic exposure to the drug and the acquisition of a T790M mutation. Cell authentication for PC-9 and H3255 was performed by the authors in June 2015 using genetic profiling of polymorphic short tandem repeat (STR) loci (Tokara).

Ba/F3 stable cell lines

IL3-dependent murine pro-B cell line Ba/F3 stably expressing the wild-type or mutated EGFR was created as previously described (29). Ba/F3 cells harboring EGFR mutations were maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum at 37°C and 5% CO2 incubator in the presence of epidermal growth factor (EGF; 10 ng/mL). The Ba/F3 cells harboring EGFR-TKIs were maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. Ba/F3 cells expressing wild-type EGFR were maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator; the presence of epidermal growth factor (EGF; 10 ng/mL). The EGFR mutations examined in this study include the delE747_P753insS (exon 19 del), L858R, delE747_P753insS + T790M (exon 19 del + T790M), L858R + T790M, delE747_P753insS + C797S (exon 19del + T790M + C797S), L858R + T790M + C797S, A763_Y764insFQEA, Y764_V765insHH, A767_V769dupASV, and D770_N771insNPG.

Reagents

Erlotinib and afatinib were purchased from LC Laboratories. Osimertinib (AZD9291) was purchased from Selleck Chemicals. Naquotinib was provided by Astellas Pharma. These compounds were dissolved in dimethyl sulfoxide (DMSO). Total EGFR antibody (#4267), total AKT antibody (#9272), phospho-AKT (S473) antibody (D9E; #4060), total p44/42 MAPK antibody (#9102S), and phospho-p44/42 MAPK (T202/204) antibody (#9101S) were purchased from Cell Signaling Technology. Phospho-EGFR (Y1068) antibody (44788G) was purchased from Invitrogen/Life Technologies, and actin antibody was purchased from Sigma-Aldrich.

Kinase inhibitory profiling

The inhibitory effect of naquotinib at the concentration of 1 and 10 nmol/L against 102 human tyrosine kinases and 28 human serine/threonine kinases was investigated by Mobility Shift Assay at Carna Biosciences, Inc. Naquotinib was incubated with each kinase for 30 minutes at room temperature. After incubation, ATP and substrate mixture was added to start enzymatic reaction. The reaction mixture was applied to LabChip 3000 (PerkinElmer), and the product and substrate peptide peaks were separated and quantitated.

Cell proliferation assay

The lung cancer cells or Ba/F3 cells were plated in a 96-well plate with or without EGFR-TKI. Control cells were treated with an equivalent concentration of DMSO. At 72 hours after treatment, cell proliferation assay was performed by adding 20 μL CellTiter 96 Aqueous One Solution Reagent (Promega) to each well. The absorbance at 490 nm was read using a multiple reader. Data are expressed as the percentage of growth relative to that of the controls. All experiments were performed at least three times.

Immunoblotting analysis

Cells were treated with EGFR-TKI at the indicated concentration for 4 hours. Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology). Equal amounts of protein (20 μg) were loaded per lane on sodium dodecyl sulfate-polyacrylamide gels, and separated proteins were transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk (Becton Dickinson), the membranes were incubated overnight with primary antibodies at 4°C and then with secondary antibodies for 30 minutes. To detect the proteins, the membranes were incubated with agitation in LumiGLO reagent and peroxide (Cell Signaling Technology) and then exposed to an X-ray film.

Apoptosis assay

Ba/F3 cells harboring EGFR exon 19del+T790M or EGFR L858R+T790M were maintained in 6-well plates. The cells were treated with different EGFR-TKIs at the indicated concentrations for 48 hours. Control cells were treated with an equivalent concentration of DMSO. We analyzed cell apoptosis using the Annexin V Apoptosis Detection Kit APC (eBioscience) according to the manufacturer’s protocol. The proportion of apoptotic cells was evaluated by flow cytometry using the Gallios flow cytometer system (Beckman Coulter).

Y764_V765insHH and A767_V769dupASV model structures

The crystal structure of EGFR T790M in a complex with naquotinib was used as the template model. Briefly, the EGFR kinase domain harboring the T790M mutation (residues 695–1022) was expressed in insect cells and purified accordingly. The purified EGFR T790M protein was crystallized using the sitting drop vapor diffusion method. The PDB accession number for the EGFR T790M crystal structure in a complex with naquotinib is 5Y9T. The Y764_V765insHH and A767_V769dupASV sequences were published in the Protein Data Bank (PDB) under the accession number 5Y9T.
aligned using the Structure Prediction Wizard. HH or ASV residues were inserted to conserve the Glu762 residue, which is indispensable for the kinase activity, resulting in a C-terminus shift.

The crystal structure of the EGFR T790M complex was superimposed with the structural model of Y764_V765insHH or A767_V769dupASV with three PDB coordinates (PDBID: 4ZAU, 4G5P, and 4HJO) using the Molecular Operating Environment (MOE; Chemical Computing Group), and images were generated using the MOE tool.

**Statistical analysis**

Statistical analysis was performed using the GraphPad Prism software, version 4.0 (GraphPad Software). The 50% inhibitory concentration (IC50) values were calculated using the GraphPad Prism software.

**Results**

**Efficacy of naquotinib and other EGFR-TKIs for human lung cancer–derived cell lines**

To evaluate the efficacy of EGFR-TKIs including naquotinib for human lung cancer–derived cell lines harboring EGFR mutations, the cells were subjected to MTS cell proliferation assays in the presence or absence of EGFR-TKIs. We observed a clear sensitivity and resistance pattern; in lung cancer cell lines harboring the IC50 values are shown in Supplementary Table S1. For lung cancer and L858R mutations, all EGFR-TKIs evaluated were able to inhibit cell proliferation. For cells bearing the classical EGFR mutations, all EGFR-TKIs showed a wide therapeutic window with a selectivity index (SI) value of approximately 2, indicating that the IC50 values for the mutants were approximately 100-fold lower than those of the wild-type (Supplementary Fig. S2). For cells with exon 19del + T790M or L858R + T790M, osimertinib and naquotinib, but not afatinib, demonstrated low SI values. For cells with exon 19del + T790M + C797S or L858R + T790M + C797S, none of the EGFR-TKIs showed low SI values. These data are consistent with the findings from human lung cancer–derived cell lines described above.

To confirm that the inhibition of Ba/F3 cell proliferation was mediated through the inhibition of EGFR and its downstream signaling, we performed an immunoblot analysis. The inhibition pattern observed with the immunoblot analysis was generally consistent with the results of the MTS cell proliferation assay (Supplementary Fig. S3). For Ba/F3 cells transduced with mutant alleles for different EGFR-TKIs bearing other exon 20 insertion mutations, the IC50 values were 74 and 9 nmol/L for osimertinib and naquotinib, respectively. In addition, apoptosis was more profound in naquotinib-treated cells than in osimertinib-treated cells (Supplementary Fig. S1).

In wild-type cells, afatinib demonstrated the strongest inhibition of cell growth. To determine the therapeutic window of the EGFR-TKIs, we used a previously established model in which we calculated the IC50 ratios of Ba/F3 cells transduced with mutant and wild-type EGFR. For cells bearing the classical EGFR mutations, all EGFR-TKIs showed a wide therapeutic window with a selectivity index (SI) value of approximately 2, indicating that the IC50 values for the mutants were approximately 100-fold lower than those of the wild-type (Supplementary Fig. S2). For cells with exon 19del + T790M or L858R + T790M, osimertinib and naquotinib, but not afatinib, demonstrated low SI values. For cells with exon 19del + T790M + C797S or L858R + T790M + C797S, none of the EGFR-TKIs showed low SI values. These data are consistent with the findings from human lung cancer–derived cell lines described above.

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**Efficacy of EGFR-TKIs for EGFR exon 20 insertion mutations**

EGFR exon 20 insertions have been reported in tumors resistant to first- and second-generation EGFR-TKIs. An exception to this is the A763_Y764insFQEA mutation, which we previously reported as a mutation that retains sensitivity to first- and second-generation EGFR-TKIs. We have previously reported the therapeutic window and potential efficacy of osimertinib for resistant cells with EGFR exon 20 insertion mutations (29). To evaluate the efficacy of EGFR-TKIs including naquotinib, we performed MTS cell proliferation assays for Ba/F3 cells harboring different EGFR exon 20 insertion mutations. The EGFR exon 20 insertions evaluated in this study are A763_Y764insFQEA (FQEA), Y764_V765insHH (HH), A767_V769dupASV (ASV), and D770_N771insNPG (NPG). For Ba/F3 cells bearing the A763_Y764insFQEA mutation, all EGFR-TKIs were able to inhibit the cell proliferation. For Ba/F3 cells with a C797S mutation (exon 19del + T790M + C797S or L858R + T790M + C797S; ref. 31), none of the EGFR-TKIs inhibited the cell proliferation. Of note, for cells with the L858R + T790M mutation, the IC50 value of naquotinib was slightly lower than that of osimertinib. The calculated IC50 values were 74 and 9 nmol/L for osimertinib and naquotinib, respectively. In addition, apoptosis was more profound in naquotinib-treated cells than in osimertinib-treated cells (Supplementary Fig. S1).

In wild-type cells, afatinib demonstrated the strongest inhibition of cell growth. To determine the therapeutic window of the EGFR-TKIs, we used a previously established model in which we calculated the IC50 ratios of Ba/F3 cells transduced with mutant and wild-type EGFR. For cells bearing the classical EGFR mutations, all EGFR-TKIs showed a wide therapeutic window with a selectivity index (SI) value of approximately 2, indicating that the IC50 values for the mutants were approximately 100-fold lower than those of the wild-type (Supplementary Fig. S2). For cells with exon 19del + T790M or L858R + T790M, osimertinib and naquotinib, but not afatinib, demonstrated low SI values. For cells with exon 19del + T790M + C797S or L858R + T790M + C797S, none of the EGFR-TKIs showed low SI values. These data are consistent with the findings from human lung cancer–derived cell lines described above.

To confirm that the inhibition of Ba/F3 cell proliferation was mediated by the inhibition of EGFR and its downstream signaling, we performed an immunoblot analysis. The inhibition pattern observed with the immunoblot analysis was generally consistent with the results of the MTS cell proliferation assay (Supplementary Fig. S3). For Ba/F3 cells transduced with mutant alleles for different EGFR-TKIs bearing other exon 20 insertion mutations, the IC50 values were 74 and 9 nmol/L for osimertinib and naquotinib, respectively. In addition, apoptosis was more profound in naquotinib-treated cells than in osimertinib-treated cells (Supplementary Fig. S1).
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Figure 1.

Sensitivity of lung cancer cell lines to EGFR-TKIs. A, MTS assays of PC-9, H3255, PC-9ER, and H1975 cells exposed to different EGFR-TKIs. Data represent the mean ± standard deviation. B, PC-9, H3255, PC-9ER, and H1975 cells were treated with different EGFR-TKIs at the indicated concentrations for 4 hours prior to immunoblotting for phosphorylated (p) and nonphosphorylated forms of EGFR, AKT, and ERK. Actin was used as a loading control.
proliferation (Fig. 3A). The calculated IC₅₀ values are shown in Supplementary Table S2.

For cells with the A763_Y764insFQEA mutation, all EGFR-TKIs showed a wide therapeutic window with an SI value of approximately 2, indicating that the IC₅₀ values for the mutants were approximately 100-fold lower than those for the wild-type (Supplementary Fig. S4). For cells harboring other EGFR exon 20 insertion mutations, osimertinib and naquotinib showed a wide therapeutic window, but not for Y764_V765insHH. In an immunoblot analysis, all EGFR-TKIs evaluated showed limited efficacy for Ba/F3 cells with a Y764_V765insHH mutation (Supplementary Fig. S5). A similar sensitivity pattern was also observed in a human lung cancer-derived cell line, BID007, which also harbors the EGFR A763_Y764insFQEA mutation (Fig. 3B; Supplementary Table S1). Immunoblotting for the BID007 cells also showed a similar inhibition pattern of EGFR and its downstream signals, which was consistent with the findings of the MTS cell proliferation assay (Fig. 3C). These results indicate a comparable efficacy for naquotinib and osimertinib in several EGFR exon 20 insertion mutants.

Structural characterization of EGFR exon 20 insertion mutations

The findings described above indicate a clear efficacy difference for EGFR-TKIs among cells bearing different EGFR mutations, especially in cells with EGFR exon 20 insertion mutations. Previously, data on the mechanism of activation and drug sensitivity of EGFR exon 20 insertion mutants based on structural analyses had been limited. In this study, we performed a structural modeling of EGFR bearing different exon 20 insertion mutations. Among the four EGFR exon 20 insertion mutations, D770_N771insNPG and A763_Y764insAA mutation and D770_N771insNPG and A763_Y764insAA have been evaluated extensively in a previous study (29). In this study, we focused on the A767_V769dupASV mutation, one of the most common EGFR exon 20 insertion mutations, and the Y764_V765insHH mutation, the most resistant mutation examined in this study. Both models assumed that the catalytically indispensable Glu762 retains its position and that the inserted residues shift toward the C-terminus. We generated a model for EGFR A767_V769dupASV to structurally analyze its mechanism of activation and difference in EGFR-TKIs sensitivity. The model showed that the ASV insertion forms a residual loop at the end of the C-helix. This insertion does not directly affect EGFR-TKIs upon inhibitor superimposition (Supplementary Fig. S6). Because the insertion site of A766_V769dupASV is only one residue different from that of D770_N771insNPG, their mechanism of activation and difference in inhibitor sensitivity may be similar. As observed in the crystal structure of EGFR D770_N771insNPG, the inserted residues form a tight turn at the end of the C-helix, which may shift the kinase activity equilibrium to an upregulated state (29). This model suggested that the A767_V769dupASV and D770_N771insNPG mutations have a similar conformational effect.

To thoroughly analyze the inhibitor binding effect of the Y764_V765insHH insertion, we generated a structural model of EGFR Y764_V765insHH. The structural model with C-terminal insertion showed that the inserted histidine residues displace Val765 and Met766 (Fig. 4A). By superimposing EGFR-TKIs with this model structure, we observed that the second histidine insertion in the Val765 position would lose its hydrophobicity toward Leu858 and thus keeping it flipped in its active conformation (32).

Biological validation of the EGFR Y764_V765insHH structure

In order to validate the structural models above, we performed further in vitro analyses. First, to investigate whether the effect of the insertion (i.e., the shift toward C-terminus) alone is sufficient to upregulate the kinase activity, we constructed an EGFR M766_A767insAA mutant. We did not construct a Y764_V765insHH mutant because this mutation may alter the structure of the EGFR kinase pocket, especially the Met766. To investigate whether this mutation activates EGFR, we transduced it to Ba/F3 cells. As expected, Ba/F3 cells transduced with wild-type EGFR failed to grow in the absence of EGF. However, interestingly, Ba/F3 cells transduced with D770_N771insASV grew even in the absence of EGF at a rate comparable to cells with wild-type receptor with EGF (Fig. 5A). These results demonstrated that the effect of the insertion alone was sufficient to upregulate the EGFR kinase activity.

Next, to confirm the structural finding that the replacement of Y765 and M766 to H765 and H766, respectively (Y765H+M766H) may result in EGFR-TKI insensitivity, especially to erlotinib and afatinib, we constructed EGFR bearing the L858R+V765H+M766H mutation. We transduced this mutation to Ba/F3 cells and performed an MTS cell proliferation assay in the presence of EGFR-TKIs. As predicted by the structural analysis, the L858R+V765H+M766H mutant showed remarkable insensitivity toward erlotinib and afatinib (Fig. 5B; Supplementary Table S3). These data supported the findings that replacement of Y765 and M766 to H765 and H766 (Y765H+M766H) induced insensitivity to EGFR-TKIs. In summary, we have demonstrated that the insertion of histidine residues in the position of Val765 and Met766 in Y764_V765insHH upregulated the EGFR kinase activity, and that it caused a steric insensitivity to the particular EGFR-TKIs.

Discussion

In this study, we have characterized the efficacy of multiple EGFR-TKIs including a novel third-generation EGFR-TKI, naquotinib, for clinically relevant EGFR mutations. The inhibitory effects of naquotinib on various kinases are shown in Supplementary Table S4. Naquotinib exhibited mutation-selective inhibitory effects for EGFR. Other than mutated EGFR, naquotinib inhibited several kinases, including BTK, JAK3, and TKX. A difference in the inhibitory profile between naquotinib and osimertinib (20) was observed, which may affect efficacy or safety.
Figure 2.
Sensitivity of Ba/F3 cells expressing wild-type (WT) or mutant EGFR to EGFR-TKIs. MTS assays were conducted for Ba/F3 cells expressing the indicated EGFR genotypes. Data represent the mean ± standard deviation.
Figure 3.
Effect of EGFR-TKIs on Ba/F3 or lung cancer cells harboring EGFR exon 20 insertion mutations. A, Sensitivity of Ba/F3 cells expressing EGFR exon 20 insertion mutations to EGFR-TKIs. MTS assays were conducted for Ba/F3 cells expressing the indicated EGFR genotypes. Data represent the mean ± standard deviation. B, The sensitivity of BID007 cells expressing EGFR exon 20 insertion mutation, A763_Y764insFQEA, to EGFR-TKIs. MTS assays were conducted in BID007 cells. Data points represent the mean ± standard deviation. C, BID007 cells were treated with the indicated concentrations of EGFR-TKIs for 4 hours prior to immunoblotting for the phosphorylated (p) and nonphosphorylated forms of EGFR, AKT, and ERK. Actin was used as a loading control.
of these inhibitors in lung cancer patients. In our cell-based assays, the efficacy of naquotinib and osimertinib was generally comparable. For most of the EGFR mutations, except for C797S-positive mutations and Y764_V765insHH, naquotinib demonstrated low IC50 values and a wide therapeutic window. The therapeutic window for these EGFR-TKIs is an important determinant of their potential toxicity in human. To quantify this, we used an in vitro model, which we described in a previous study (30), to determine the ratio of IC50 values in Ba/F3 cells transduced with mutant or wild-type EGFR.

For EGFR_L858R+T790M, naquotinib showed lower IC50 values and a wider therapeutic window than osimertinib. However, whether the difference observed in the in vitro study is clinically relevant remains unclear. Further characterizations of naquotinib through additional in vitro and in vivo studies are required.

Structural analysis can offer insight into the mechanism of protein activation and relationship between protein structure and drug sensitivity. Structural analyses for EGFR with L858R or G719S mutations have been performed in numerous studies (5, 7, 33). Both L858R and G719S mutations lock the EGFR kinase domain in a constitutively active conformation by destabilizing the inactive conformation. However, for EGFR with exon 20 insertion mutations that displayed resistance to first- and second-generation EGFR-TKIs, the structural basis for resistance is poorly understood. In a previous study, we have clarified the mechanism of activation and sensitivity for EGFR_D770_N771insNPG and A763_Y764insFQEA using crystal structure and structural modeling, respectively (29). For the D770_N771insNPG mutation, the crystal structure revealed an unaltered ATP binding pocket, and the inserted residues form a wedge at the end of the C-helix, a key structural component for activation (34). For the A763_Y764insFQEA mutation, structural modeling revealed that the inserted residues shift the register of the C-helix in the N-terminal direction, altering the structure in the region that is also affected by the L858R mutation. In this study, we have partially elucidated the mechanism of activation and sensitivity of two EGFR exon 20 insertion mutations associated with resistance to first- and second-generation EGFR-TKIs, i.e., the A767_V769dupASV and Y764_V765insHH mutations. For both mutations, the models assumed that the catalytically
indispensable Glu762 retains its position and that the inserted residues shift toward the C-terminus. This assumption was supported by results of the in vitro experiments. Interestingly, a shift toward the C-terminus by two amino acids activated the EGFR, as demonstrated by the ligand-independent proliferation of Ba/F3 cells transduced with EGFR M766_A767insAA. Although different insertions may yield slight differences in the structure, insertion of several residues in this EGFR region (i.e., near the end of the C-helix) should have a similar structural effect.

In addition, we have proposed a mechanism for resistance associated with EGFR Y764_V765insHH mutation, one of the most resistant mutations. We demonstrated that in
Y764, Y765insHH, histidine residues inserted in the Val765 and Met766 positions upregulated the EGFR kinase activity and caused steric insensitivity to the particular EGFR-TKIs. These findings shed light on the biology of EGFR exon 20 insertion mutations.

In summary, we have characterized the efficacy of different EGFR-TKIs including naqutinib, a novel third-generation EGFR-TKI, using in vitro and structural analyses. Results of this study also partially clarified the mechanism of activation and resistance of EGFR exon 20 insertion mutations to EGFR-TKIs. Our findings should help to determine the appropriate EGFR-TKIs for the treatment of NSCLC with EGFR mutations and shed light on the biology of specific subsets of EGFR mutations.

Disclosure of Potential Conflicts of Interest

K. Soejima reports receiving a commercial research grant from Taiho and has received speakers bureau honoraria from AstraZeneca, Chugai, Ono, MSD and Eli Lilly. No potential conflicts of interest were disclosed by the others.

Authors’ Contributions

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References


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