Heterogeneous Mechanisms of Primary and Acquired Resistance to Third-Generation EGFR Inhibitors


Abstract

Purpose: To identify novel mechanisms of resistance to third-generation EGFR inhibitors in patients with lung adenocarcinoma that progressed under therapy with either AZD9291 or rociletinib (CO-1686).

Experimental Design: We analyzed tumor biopsies from seven patients obtained before, during, and/or after treatment with AZD9291 or rociletinib (CO-1686). Targeted sequencing and FISH analyses were performed, and the relevance of candidate genes was functionally assessed in vitro models.

Results: We found recurrent amplification of either MET or ERBB2 in tumors that were resistant or developed resistance to third-generation EGFR inhibitors and show that ERBB2 and MET activation can confer resistance to these compounds. Furthermore, we identified a KRAS mutation in a patient with acquired resistance to AZD9291 as a potential driver of acquired resistance. Finally, we show that dual inhibition of EGFR/MEK might be a viable strategy to overcome resistance in EGFR-mutant cells expressing mutant KRAS.

Conclusions: Our data suggest that heterogeneous mechanisms of resistance can drive primary and acquired resistance to third-generation EGFR inhibitors and provide a rationale for potential combination strategies. Clin Cancer Res; 22(19); 4837–47.

Introduction

In EGFR-mutant lung adenocarcinoma, targeted therapy with EGFR tyrosine kinase inhibitors (TKI) performs substantially better than standard chemotherapy in terms of response rate (RR), progression-free survival (PFS), and tolerability (1). Unfortunately, all patients will ultimately experience relapse with a median PFS of 7 to 16 months.

The major cause of resistance is the EGFR mutation T790M, at the gatekeeper site (50%–60%; refs. 2–4). A third generation of covalent EGFR TKIs that specifically target EGFR T790M as well as the activating EGFR mutations (e.g., L858R or exon 19 deletion),
Transitional Relevance

Although the majority of EGFR<sup>T790M</sup>-mutant adenocarcinomas of the lung respond well to third-generation EGFR inhibitors, resistance to these inhibitors remains a major challenge in the field. We uncover ERBB2 and MET activation as potential bypass-track mechanism to third-generation EGFR inhibitors and suggest that treatment with third-generation EGFR inhibitors may select for EGFR-mutant subclones that tolerate cooccurring mutations in the MAPK pathway. Together with previous preclinical studies, our data support the notion that activation of MAPK signaling might play a role in resistance to these drugs. Our findings might be of broad interest to basic cancer scientists and medical oncologists alike, as they provide insight related to the biology of EGFR-mutant adenocarcinomas and have immediate implications for genetically stratified treatment of these patients.

Patients and Methods

Study population

Whenever possible, biopsies were obtained before treatment and after radiographic progression under TKI therapy (with the exception of P2 and P3), at the times and with the methods described in Table 1. Standard histopathology was performed to confirm the histologic subtype. Patients were treated in trials NCT01802632 and NCT01526928. The Institutional Review Board (IRB) and the responsible ethics committee approved FISH analyses

FISH for MET and ERBB2 was performed on formalin-fixed paraffin-embedded tissue using labeled dual-color probes. Sections of 1.5 µm were cut and hybridized with labeled probes for MET or ERBB2 and the respective centromeric reference probe (ZytoLight Spec MET/CEN7 probes; ZytoLight Spec ERBB2/CEN17 probes by ZytoVision). Slides were reviewed at ×630 and scored according to appropriate guidelines (17). For ERBB2, the gastric cancer scoring system was used because no lung-specific recommendations are available (18).

DNA extraction and sequencing

Total DNA was obtained from formalin-fixed paraffin-embedded tumor tissue. DNA from sections was extracted using the Puregene Extraction Kit (Qiagen) according to the manufacturer’s instructions. DNA was eluted in 1× TE buffer (Qiagen), diluted to a working concentration of 150 ng/μL and stored at −80°C.

Targeted sequencing

Targeted sequencing analysis was performed as described previously (19) using a custom-made lung cancer panel consisting of 102 amplicons for the detection of hotspot mutations in 14 lung cancer–related genes. Isolated DNA (up to 50 ng) was amplified with two customized Ion AmpliSeq Primer Pools. Library products were quantified using Qubit 2.0 Fluorometer (Qubit ds DNA HS Kit; Life Technologies), diluted, and pooled in equal amounts. Six to 8 pmol/L was spiked with 5% PhiX DNA (Illumina) and sequenced with the MiSeq reagent Kit V2 (300-cycles; Illumina). Data were exported as FASTQ files and analyzed using the in-house pipeline.

Generation of stably transduced cell lines

H1975 (CRL-5908) and HCC827 (CRL-2868) cells were obtained from the ATCC. PC9, PC9GR and HCC827GR cells were kindly provided by Dr. Hongbin Ji (Shanghai Institutes for Biological Sciences, China), Dr. Passi Jänne (Broad Institute, Cambridge, MA), and Dr. Jeffrey Engelman (Massachusetts General Hospital, Boston, MA), respectively. Cells were cultured with RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO₂.

Generation of cells harboring pBabe-puro empty vector, EGFR<sup>T790M</sup>, ERBB2<sup>W1241X</sup>, KRAS<sup>G12V</sup>, or KRAS<sup>G12S</sup> was done by stable transduction as described previously (20) using a pBabe-puro retroviral vector (Addgene). Cells expressing the corresponding mutants were generated by retroviral transduction and subsequent puromycin selection. The expression of the mutant was verified by RT-PCR and further Sanger sequencing and by Western blot analysis.

cDNA transcription

RNA was isolated from PC9<sup>KRAS-G12S</sup> and HCC827<sup>KRAS-G12S</sup> cells using TRIzol reagent (Invitrogen) and cleaned up using the RNeasy MinElute Cleanup Kit (Qiagen) following the manufacturers’ protocols. Finally, 1 µg of RNA was transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, #18064).

Reagents

AZD9291, rociletinib, afatinib, crizotinib, trametinib, and selumetinib were synthesized according to published methods. For cell culture experiments, each compound was dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored as 10 mmol/L stocks at −80°C. HGF (R&D Systems) was resuspended in PBS +0.1% BSA and aliquots stored at −20°C.

Cell viability assays

Cells were plated into 96-well culture plates in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin, at a density of 1,500 cells per well, cultured overnight, and treated the following day. Cell viability was determined after 4 to 6 days by measuring cellular ATP content (CellTiter-Glo, Promega) as described previously (20). GI<sub>50</sub> values were calculated by plotting...
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**Abbreviations:** BEVA, bevacizumab; CARBO, carboplatin; CS, cisplatin; DOCE, docetaxel; GEMCI, gemcitabine; Het, heterogeneity; OP, surgery; OS, overall survival; PACLI, paclitaxel; PEM, pemetrexed; RTX, radiotherapy; TRO, trofosfamide; VINO, vinorelbine.

*Still alive.*

*Obtained during therapy.*

*Treatment ongoing.*
luminescence intensity against drug concentration in nonlinear curves using GraphPad Prism (GraphPad). Synergy scores were calculated as described previously (21). For long-term viability assays, the reported “Cell number change (%)” was determined as: 100 × (Value/Baseline)/Baseline. Each point was performed with three replicates. Unless otherwise noted, three independent experiments were performed, and a representative result is presented. Error bars are mean ± SD.

Crystal violet staining
Cells were plated at a density of 1 million cells per well and incubated overnight. Cells were treated with AZD9291 the next day and then again 72 hours after. After 6 days of drug exposure, cells were fixed with 1% paraformaldehyde. For staining, a solution of 0.1% crystal violet was added and incubated for 20 minutes. Plates were inverted and dried overnight, and images were taken the next day.

Western blot analyses
Cells lysates were blotted as described previously (20). The following antibodies were used: phospho-EGFR (Y1068), total EGFR, phospho-ERBB2 (Y1248), total ERBB2, phospho-MET (Y1234/Y1235), total MET, phospho-AKT (Ser473), total AKT, phospho-ERK (T202/Y204), total ERK, actin (Cell Signaling Technology), and conjugated antibodies to rabbit and mouse (Millipore).

Statistical analyses
Statistical analyses were performed using GraphPad Prism 5.0. We used the Student t test (unpaired, two-sided). A P value of <0.05 was used as a threshold considered to call statistical significance. PFS was assessed from start of the respective therapy until progression or death. Survival analyses were performed using Kaplan–Meier statistics, providing median and 95% confidence interval (CI). For response evaluation, the Response Evaluation Criteria in Solid Tumors (RECIST V1.1; ref. 22) were used.

Results
Mechanisms of primary and acquired resistance to third-generation EGFR inhibitors
We obtained specimens from seven patients treated with third-generation EGFR inhibitors AZD9291 (n = 5) or rociletinib (n = 2), within two clinical trials (clinicaltrials.gov, NCT01802632 and NCT02147990) after acquired resistance to first- or second-generation EGFR kinase inhibitors (Fig. 1). Positive resistance to first- or second-generation EGFR kinase inhibitors. The PFS for third-generation EGFR TKI therapy ranged from 1.2 to 19.4 months (median 4.2 months; 95% CI, 3.7–4.7 months, Fig. 1A). The best reduction of the sum of the largest tumor diameters under treatment, according to RECIST V1.1 (22), ranged between 0.1% and 79.2%, with 3 patients presenting growth of non-target lesions (P1, P3, and P5, Fig. 1B).

The pattern of response varied across these patients: two patients (P1 and P3) experienced primary resistance to rociletinib (defined as progression on the first restaging scan), whereas two patients had stable disease (P2 and P4) under treatment with AZD9291. Finally, three cases (P5, P6, and P7) presented with partial responses to treatment with AZD9291, with reductions in diameters of more than 50% (Fig. 1A and B). For these patients, unfortunately, disease recurred after 16.5, 19.4, and 7 months, respectively.

To characterize the molecular mechanisms that may be linked with such heterogeneous response patterns, we performed FISH for known EGFR resistance markers (ERBB2 and MET) and targeted next-generation sequencing on tumor tissue from these patients (see Patients and Methods). FISH analyses of a massively progressive pleural effusion (non-target lesion) collected after 3 weeks of treatment initiation from P1 (PD, 625 mg rociletinib twice daily) and a lung biopsy sample collected before treatment from P2 (SD, 180 mg AZD9291 daily), revealed ERBB2 amplification (7.3 and 3.7 gene copies, respectively; Fig. 1C).

Because ERBB2 amplification has been shown to confer resistance to first-generation EGFR TKIs (23), we hypothesized that amplified ERBB2 might substitute for EGFR signaling in this context and explain the lack of response to of AZD9291 and rociletinib in P1 and P2. To test this hypothesis, we evaluated the impact of ERBB2 overexpression on the sensitivity to AZD9291 and rociletinib in PC9GR cells (EGFR<sup>T790M</sup>). Despite a relatively low overexpression of ERBB2 in PC9GR cells, we observed decreased sensitivity to both drugs at nanomolar concentrations (Fig. 1D and Supplementary Fig. S1A). Thus, our results indicate that ERBB2 activation may contribute to resistance against rociletinib and AZD9291.

The tumor biopsy collected before treatment of P3 (PD, 625 mg rociletinib twice daily) showed high-level amplification of MET (MET/CEN7 ratio 2.10, 11.42 MET copies; Fig. 2A). Unfortunately, no baseline comparative sample (before erlotinib treatment) was available for this patient. In the case of P4 (SD, 80 mg AZD9291 daily), an initial tumor reduction (20.8%) was followed by an increase in the tumor volume after 4.1 months of treatment (Fig. 2B). In this patient too, the biopsy taken after the initial response exhibited high-level amplification of MET (MET/CEN7 ratio 5.35, 11.42 MET copies) that was not observed before therapy with AZD9291 (Fig. 2C). P5 (PR, 80 mg AZD9291 daily) had an initial tumor response of 79% and presented a new lesion in the liver that harbored a high-level MET amplification (MET/CEN7 ratios 1.88, 21.92 MET copies, Fig. 2D, left) and ERBB2 amplification (4.25 gene copies, Fig. 2D, right). Of note, targeted sequencing of these samples did not show additional clinically relevant mutations (Table 1).

To functionally test whether MET amplification might reduce the sensitivity to AZD9291 or rociletinib, we stably overexpressed E<sup>GRF<sub>T790M</sub></sup>–mutant constructs in HCC827 cells (MET wild-type) and their gefitinib-resistant derivative, HCC827GR cells (MET amplified; Supplementary Fig. S1B). In line with previous reports (24), the presence of MET amplification decreased the sensitivity to both third-generation EGFR inhibitors (Fig. 2E) and led to sustained phosphorylation of ERK and AKT (Fig. 2F). Furthermore, concomitant inhibition with either AZD9291 or rociletinib and the MET inhibitor, crizotinib, resulted in pronounced cytotoxicity and reduced AKT and ERK activation in HCC827GR/E<sup>GRF<sub>T790M</sub></sup> cells (Fig. 2E and C). These results were confirmed in E<sup>GRF<sub>T790M</sub></sup>-expressing cells (H1975 and PC9GR) where MET was activated through addition of exogenous HGF (Supplementary Fig. S1C–S1F).

Thus, our clinical observations are in line with previous reports where both ERBB2 and MET amplification has been found to be associated with loss of efficacy of third-generation EGFR inhibitors in E<sup>GRF<sub>T790M</sub></sup>-mutant tumors (25). More importantly, we provide functional evidence that activation of ERBB2 and MET signaling may induce resistance to this new class of EGFR inhibitors.
Finally, patient 6 (P6) presented with a tumor response of 72.6% followed by emergence of new liver metastases 19.4 months after start of therapy. The liver biopsy revealed the retention of the initial \textit{EGFR} exon19 deletion (allelic fraction, AF: 42.6%, Supplementary Fig. S2A) and the \textit{EGFR} T790M mutation (AF: 27.6%) together with the presence of a secondary \textit{EGFR} C797S mutation (Fig. 2H) that was not present in the pretreatment biopsy (Supplementary Fig. S2B). Of note, the liver biopsy obtained at relapse also harbored an intermediate-level \textit{MET} amplification (MET/CEN7 ratios 1.14, 5.67 \textit{MET} copies, Fig. 2I). The \textit{EGFR} C797S mutation abrogates the irreversible binding of third-generation EGFR inhibitors and has been described to confer resistance to AZD9291 thus providing a rational for the observed resistance phenotype in P6 (10, 26, 27).

**KRAS mutation and resistance to third-generation EGFR inhibition**

In our cohort, a fourth patient (P7) relapsed after having initially responded to treatment with AZD9291. This patient received different lines of treatment before developing resistance through \textit{EGFR}T790M under combination therapy with afatinib and cetuximab. After enrollment into the AURA trial (NCT01802632) the tumor responded to AZD9291 treatment (160 mg/d) with a PR (best response: 54.3%, Fig. 3A) for 8 months. However, the fifth follow-up revealed the appearance of a new lesion in a fast growing cervical lymph node. Targeted sequencing of the specimens obtained before and after treatment with AZD9291 (both from the cervical lymph nodes) showed a slight decrease in the fraction of reads containing the original activating \textit{EGFR} mutation (43% before treatment with AZD9291 vs. 32% after treatment, Supplementary Fig. S2C) and the disappearance of \textit{EGFR}T790M (25% before treatment with AZD9291, Supplementary Fig. S2D). Finally, in two independent sequencing runs we found a novel \textit{KRAS}G12S mutation (38% of the reads), which had not been detected before therapy with AZD9291 (Fig. 3B). In general, the disproportionate levels of C>T/G>A changes that occur as a consequence of formalin fixation are more apparent at low allelic fractions (1%–10%; ref. 28). In the relapse sample of

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**Figure 1.**

Primary and acquired resistance to third-generation EGFR inhibitors. A, duration of treatment of patients that underwent therapy with AZD9291 (blue) or rociletinib (green). PD, progressive disease; SD, stable disease; PR, partial response. MET and/or \textit{ERBB2} amplification were detected before treatment (gray) or at relapse (black) under AZD9291 or rociletinib. B, waterfall plots showing best percentage change in the size of target or non-target (‘’) lesions. C, FISH analyses with probes for chromosomal loci containing \textit{ERBB2} (P1 and P2, green signal) and the respective centromeric reference probe (red signal). D, viability assays of PC9GR\textsuperscript{ERBB2} and PC9GR\textsuperscript{ERBB2} cells indicating the change in cell number after 6 days of treatment with DMSO or increasing concentrations of either AZD9291 or rociletinib, in comparison with the number of cells at the initiation of drug exposure. **, P < 0.01; ***, P < 0.00.
Figure 2.
Activation of MET confers resistance to third-generation EGFR inhibitors. **A,** FISH analyses with probes for chromosomal loci containing MET (P3, green signal) and the respective centromeric reference probe (red signal). **B,** tumor response analysis for P4 using the quantitative imaging tool mint Lesion. Here, the volumes of the target lesions are shown and added up, according to RECIST. This representation shows how the different lesions respond individually as well as the sum of the changes in the target lesions. Follow-up scans (*) were performed every 8 weeks. **C,** FISH analyses assessing the presence of MET amplification in the liver lesion collected before AZD9291 and after relapse. (Continued on the following page.)
and phosphorylation of the MET, EGFR, AKT, and ERK, as well as expression of BIM and actin was monitored by immunoblotting.

Similar results were observed with the first-generation EGFR inhibitors. The role of ERBB2 activation in resistance has been previously reported for KRAS (10) but was not observed in the lymph node biopsy (Supplementary Fig. S2F). Moreover, the KRASG12S mutation was not detected in the plasma of this patient. These observations suggest that heterogeneous mechanisms of acquired resistance might be operant in this patient.

To investigate whether expression of an activated KRAS allele might contribute to resistance to third-generation EGFR inhibitors we stably transduced EGFR-mutant PC9 cells with the KRASG12S-mutant. PC9 cells that expressed the KRASG12S-mutant were selected under treatment with puromycin for at least 2 weeks. The resulting PC9KRAS-G12S cells expressed both the original EGFR exon19 deletion and the KRASG12S mutation (Supplementary Fig. S3A). Concomitant expression of both mutants was also observed in a PC9KRAS-G12S clone obtained by serial dilution (Supplementary Fig. S3B).

When compared with the KRAS wild-type transduced cells (PC9KRAS-wt), PC9KRAS-G12S were less sensitive to AZD9291 (Fig. 3C and D). Furthermore, introduction of KRASG12S resulted in increased KRAS expression and sustained ERK phosphorylation under treatment with AZD9291 (Fig. 3E). Similar results were observed in HCC827KRAS-G12S cells (Supplementary Fig. S3C and S3D) where the introduction of the KRAS mutation also resulted in decreased sensitivity to rociletinib (Supplementary Fig. S3C). Confirming a role of MAPK signaling in inducing resistance in PC9KRAS-G12S cells, combined treatment with AZD9291 and the MEK inhibitor, trametinib, was highly synergistic ( synergy score, 5.48; ref. 21; P value of 4.14E–08; Fig. 3F and Supplementary Fig. S4A). Consistent with these results, AZD9291 failed to fully inhibit downstream MAPK signaling except in the presence of trametinib (Fig. 3G). Similar results were observed with the combination of AZD9291 and selumetinib (Fig. 3F and Supplementary Fig. S4B). Finally, concomitant EGFR and MEK inhibition was also synergistic in HCC827KRAS-G12S cells (Supplementary Fig. S4C and S4D). These findings demonstrate that mutant KRAS may induce acquired resistance to third-generation EGFR inhibitors.

**Discussion**

Here, we provide clinical and functional evidence for the role of ERBB2 and MET amplifications as well as KRAS mutations as possible mechanisms of resistance to third-generation EGFR inhibitors. The role of ERBB2 amplification in resistance has been previously reported for first-generation EGFR inhibitors (23). The activity of AZD9291 against ERBB2 is known to be limited and despite the presence of a metabolite AZS104 that shows inhibition of ERBB2 it is conceivable that ERBB2 signaling may reduce the overall activity of third-generation inhibitors in patients (6).

Because the potency against ERBB2 was greater with the AZ5104 metabolite than with AZD9291, it would be interesting to determine the metabolites levels in the plasma of patients to predict the emergence of ERBB2 as a potential bypass mechanism of resistance to AZD9291. Our functional **in vitro** results suggest that the activity of both AZD9291 and rociletinib may decrease through specific activation of ERBB2 signaling. These observations go in line with the studies reporting ERBB2 as a mechanism of resistance to first-generation EGFR inhibitors (23). The use of patient-derived **EGFR rm** mutant cells may help defining the activity of these inhibitors on ERBB2 and characterizing the molecular context of the role of ERBB2 as a mechanism of resistance to third-generation EGFR inhibitors.

The role of MET amplification in acquired resistance to first-generation EGFR inhibitors has been studied extensively (25–31). According to our results MET activation may similarly play a role in resistance to third-generation EGFR inhibitors as MET is not a relevant off-target for these drugs. Our observations also confirm previous reports on the efficacy of the combination of rociletinib with crizotinib in these tumors (32). Combination therapies have proven successful in the setting of resistance to first-generation EGFR inhibitors (33) and early-phase clinical trials are evaluating the combination of rociletinib with crizotinib (32), or AZD9291 with the MET inhibitor, savolitinib, in **EGFR rm** lung cancer (34). It might therefore be important to determine the presence of these alterations before therapy with any EGFR inhibitor.

Interestingly, we identify an activating KRASG12S mutation in a tumor rebiopsy obtained at the time of acquired resistance to AZD9291. This mutation is of particular interest because EGFR and KRAS mutations typically occur in a mutually exclusive fashion in lung cancer (16, 35). Indeed, a recent report evidenced that the expression of inducible KRASG12S constructs in EGFR-mutant PC9 cells results in decreased cell viability after 7 days of selection with doxycycline (16). Anecdotal reports show evidence of co-occurrence of KRAS and EGFR mutations in lung cancer patients (36, 37). More recently Hata and colleagues (14) described two distinct evolutionary pathways for the development of resistance to EGFR inhibition. In this study, cell lines that developed resistance at late stages were enriched for NRAS and KRAS mutations suggesting that under prolonged selection a distinct population of EGFR-mutant cells may well tolerate the presence of oncogenic MAPK pathway activation. In our functional experiments, decreased cell viability was observed in cells expressing both EGFR and KRAS mutants; however, this phenotype was overcome after about 10 passages under antibiotic selection. In line with our results, in the study performed by Unni and colleagues (16), the induction of mutant KRAS rescued PC9 cells from the lethal effects of erlotinib, thus implying that the toxicity of coexpression of mutant KRAS and EGFR depend on the kinase activity of mutant EGFR. We speculate that EGFR inhibition through AZD9291 may functionally deplete oncogenic EGFR.
Figure 3.
Acquired resistance to AZD9291 mediated by acquired KRAS<sup>G12S</sup> mutation. A, tumor response analysis for PS using the quantitative imaging tool mint Lesion showing the volumes of the target lesions. This representation shows how the different lesions respond individually as well as the sum of the changes in the target lesions. Follow-up scans (‘†’) were performed every 8 weeks. B, Integrative Genomics Viewer (IGV)-based visualization of reads of targeted NGS that identify the wild type KRAS (baseline, top) and an acquired C→T mutation in 38% of reads, encoding a KRAS<sup>G12S</sup> mutation (relapse, bottom). C, long-term viability assay of PC9<sup>KRAS<sup>wt</sup></sup> and PC9<sup>KRAS-G12S</sup> cells treated with AZD9291 at the indicated concentrations for 6 days. Cell viability is presented as the percentage of change in cell number (compared with baseline). D, long-term proliferation assay of PC9<sup>KRAS<sup>wt</sup></sup> and PC9<sup>KRAS-G12S</sup> cells exposed to increasing concentrations of AZD9291 for 6 days. Cells were stained using crystal violet; **, P < 0.01; ***, P < 0.001. E, cellular signaling of PC9<sup>KRAS<sup>wt</sup></sup> and PC9<sup>KRAS-G12S</sup> cells treated with increasing concentrations of AZD9291 for 24 hours. Whole-cell lysates were analyzed by immunoblotting. F, long-term viability assay of PC9<sup>KRAS-G12S</sup> cells demonstrating the change in cell number after treatment with AZD9291 (100 nmol/L) alone or in combination with either trametinib (1 µmol/L) or selumetinib (1 µmol/L), in comparison with the cell number before drug exposure. Cell viability was determined after 6 days of treatment. G, PC9<sup>KRAS-G12S</sup> cells were treated with AZD9291 (100 nmol/L) alone or in combination with either trametinib (1 µmol/L) for 24 hours and lysed. Protein expression and phosphorylation levels of EGFR, AKT, ERK and the expression of KRAS, BIM, and actin were monitored by immunoblotting.


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signaling to a degree that would allow the emergence of cells that harbor EGFR and KRAS mutations. Unfortunately, our analyses do not permit distinguishing the individual clones that harbor each individual lesion.

RAS activation is an alternative bypass pathway of resistance in lung adenocarcinomas. Reactivation of the MAPK pathway via either KRAS copy number gain or decreased expression of the MAPK phosphatase DUSP6 was associated with resistance to ALK inhibitors to KRAS mutations in patients with EML4-ALK–rearranged lung adenocarcinoma (38). Furthermore, activation of members of the RAS family was shown to confer resistance to ROS1 inhibitors (39). In EGFR-mutant lung cancers, resistance to EGFR TKIs may be associated with increased dependency on RAS-MAPK signaling, including ERK activation, loss of NRAS, and CRKL amplification (13, 40–42). Amplification of MAPK1 was reported as a resistance mechanism of WZ4002 and has been observed in AZD9291 (12). Moreover, NRAS mutations as well as copy-number gain of KRAS and NRAS (12, 13) were recently identified as mechanisms of resistance to AZD9291 in preclinical models. Our data therefore add to these findings and provide clinical evidence for a possible role of MAPK pathway activation in the context of acquired resistance to third-generation EGFR inhibitors.

Our findings also highlight the role of dual EGFR/MEK inhibition in cells that express EGFR and KRAS mutations and are in line with previous reports that identified that this combination may be effective in cells that display EGFR-TKI resistance through activation of MAPK signaling (13). The combination of AZD9291 and selumetinib is currently being evaluated in a phase I trial (NCCT21434466), and may thus help to further delineate the role of MAPK signaling in modulating the response to third-generation EGFR inhibitors.

Of note, a plasma sample collected from P7 one month after the cervical lymph node had been obtained for these studies, revealed the presence of a EGFR C797S mutation (AF: 1.3%), as well as EGFR T790M (AF: 0.7%), yet no KRAS mutation (Subject 2; ref. 10). Neither the EGFR C797S nor the EGFR T790M mutations were detected in the lymph node lesion after progression (Supplementary Fig. S2D and S2F), thus suggesting that the KRAS mutation might not necessarily be the unique driver of resistance in this patient and likely reflecting heterogeneity in the acquisition of resistance to third-generation EGFR inhibitors. Our data provide further evidence multiple clones with unique resistance mechanisms may give rise to the overall resistance observed in patients treated with third-generation TKIs (43).

The analysis of a larger cohort of patients would be key to confirm our observations and perform a more comprehensive profile of primary and/or acquired resistance to AZD9291 and rociletinib. Moreover, a longitudinal analysis of the emergence of multiple mechanisms of resistance using liquid biopsies could provide evidence of the prevalence of each mechanism and help defining their individual role in resistance to third-generation EGFR inhibitors.

Disclosure of Potential Conflicts of Interest

M. Scheller is a consultant/advisory board member for Boehringer Ingelheim, and Novartis. C.M. Lovly reports receiving speakers bureau honoraria from Abbott Molecular, and Qiagen; and is a consultant/advisory board member for Ariad, Clovis, Genentech, Novartis, Pfizer, and Sequenom. S. Merkelbach-Bruse reports receiving speakers bureau honoraria from AstraZeneca; and is a consultant/advisory board member for AstraZeneca, and Roche Pharma. Johannes M Heuckmann holds ownership interest (including patents) in NEO New Oncology. J.C. Heukamp is an employee of NEO New Oncology. W. Pao holds ownership interest (including patents) in MolecularMD. Martin Peifer is a consultant/advisory board member for NEO New Oncology GmbH. R. Büttner reports receiving speakers bureau honoraria from, and is a consultant/advisory board member for AstaZeneca, Boehringer-Ingelheim, Bristol-Myers Squibb, Lilly, Merck-Serono, MSD, Pfizer, Qiagen, and Roche. J. Wolf reports receiving speakers bureau honoraria from AstraZeneca, Clovis, and Novartis. R.K. Thomas reports receiving speakers bureau honoraria from AstraZeneca, Bayer, Boehringer Ingelheim, Clovis, Daiichi-Sankyo, Johnson & Johnson, Lilly, Merck, MSD, Parna, Roche, and Sanoft-Aventis; and is a consultant/advisory board member for New Oncology AG. No potential conflicts of interest were disclosed by the other authors.

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Heterogeneous Mechanisms of Primary and Acquired Resistance to Third-Generation EGFR Inhibitors

Sandra Ortiz-Cuaran, Matthias Scheffler, Dennis Plenker, et al.


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