SFK/FAK Signaling Attenuates Osimertinib Efficacy in Both Drug-Sensitive and Drug-Resistant Models of EGFR-Mutant Lung Cancer

Eiki Ichihara¹, David Westover¹, Catherine B. Meador², Yingjun Yan¹, Joshua A. Bauer³,⁴,⁵, Pengcheng Lu⁶, Fei Ye⁶, Amanda Kulick⁷, Elisa de Stanchina⁷, Robert McEwen⁸, Marc Ladanyi⁹, Darren Cross⁸, William Pao¹,²,⁵, and Christine M. Lovly¹,²,⁵

Abstract

Mutant-selective EGFR tyrosine kinase inhibitors (TKI), such as osimertinib, are active agents for the treatment of EGFR-mutant lung cancer. Specifically, these agents can overcome the effects of the T790M mutation, which mediates resistance to first- and second-generation EGFR TKI, and recent clinical trials have documented their efficacy in patients with EGFR-mutant lung cancer. Despite promising results, therapeutic efficacy is limited by the development of acquired resistance. Here we report that Src family kinases (SFK) and focal adhesion kinase (FAK) sustain AKT and MAPK pathway signaling under continuous EGFR inhibition in osimertinib-sensitive cells. Inhibiting either the MAPK pathway or the AKT pathway enhanced the effects of osimertinib. Combined SFK/FAK inhibition exhibited the most potent effects on growth inhibition, induction of apoptosis, and delay of acquired resistance. SFK family member YES1 was amplified in osimertinib-resistant EGFR-mutant tumor cells, the effects of which were overcome by combined treatment with osimertinib and SFK inhibitors. In conclusion, our data suggest that the concomitant inhibition of both SFK/FAK and EGFR may be a promising therapeutic strategy for EGFR-mutant lung cancer. Cancer Res; 77(11) June 1, 2017 2990–3000. © 2017 AACR.

Introduction

EGFR tyrosine kinase inhibitors (TKI), such as erlotinib, gefitinib, and afatinib, are standard therapy in patients with advanced non–small cell lung cancer (NSCLC) harboring somatic EGFR mutations (1–3). However, acquired resistance to these agents invariably occurs, often within a year after initiation of therapy (4–7). Disease progression is mediated by the secondary EGFR "gatekeeper" mutation, T790M, in more than half of these cases (8–10).

Recently, mutant-selective third-generation EGFR TKIs, such as osimertinib (AZD9291) and rociletinib (CO-1686), have been developed with the goal of overcoming T790M-mediated resistance (11–14). In addition to increased potency against mutant EGFR, these TKIs also have less toxicity compared with first- or second-generation inhibitors because they "spare" wild-type EGFR. Recent clinical studies have demonstrated that osimertinib monotherapy induces a 61% response rate and durable benefit in patients with lung cancers harboring the EGFR T790M mutation (15), resulting in approval by the FDA for the treatment of patients with advanced EGFR T790M mutation–positive NSCLC. As with other EGFR TKIs, however, the magnitude and duration of response to osimertinib is variable and resistance inevitably develops, suggesting that targeting EGFR alone will not achieve long-term benefits. In addition, although most tumors initially decrease in size during EGFR TKI treatment, the tumors typically reach a steady state, implying that there may be mechanisms that make tumor cells tolerant to EGFR inhibitors, even in EGFR TKI–sensitive tumors.

We hypothesized that additional signaling pathways active in tumor cells may attenuate the effects of osimertinib, thereby limiting its full antitumor activity. We found that signaling downstream of EGFR through the AKT and MAPK pathways remained active even in the presence of osimertinib. Sustained signaling through these pathways under continuous EGFR inhibition appears to be, in part, regulated by Src family kinase (SFK) and focal adhesion kinase (FAK) signaling. Concomitant inhibition of EGFR, SFKs, and FAK most effectively enhanced osimertinib activity and suppressed the development of resistance. We also found that amplification of the SFK, YES1, caused resistance to osimertinib. These findings may lead to new therapeutic options for patients with EGFR-mutant lung cancer.
Materials and Methods

Kinome-wide siRNA library screen
PC-9/BRC1 cells were transfected with the GE Dharmacon Protein Kinase siRNA library (GU003505). The siRNA library included nine 96-well plates containing siRNAs targeting 714 kinases and kinase-related genes (4 individual siRNAs per gene) in a SMARTpool format. PC-9/BRC1 cells were reverse-transfected in 96-well plates (4,000 cells/well in 10% FBS) containing 5 pmol of siRNA/well using DharmaFECT4 (Dharmacon). Each plate also contained two wells with 5 pmol/well of nontargeting siRNA (siNT, Dharmacon). Twenty-four hours after transfection, cells were trypsinized and divided into replicate plates. After an additional 24-hour incubation, cells were treated with DMSO or osimertinib (5 nmol/L). After 72 hours of treatment, cells were incubated with Alamar Blue (Invitrogen) for 4 hours, and viability was measured by fluorescence readings using an EnSpire 2300 (544 nm excitation, 590 nm emission; PerkinElmer). The viability measurements were normalized to the siNT controls by taking the mean of the controls divided by each well's viability value.

The sensitizing index (SI) value was calculated for each siRNA to identify the gene targets that promote osimertinib sensitivity or resistance using the following formula, as previously described (16):

\[
SI = \frac{\text{Expected total viability effect - Observed combined effect}}{\text{Expected total viability effect}} = \frac{(Rc/Cc - Cd/Cc)}{Cd/Cc}.
\]

where \( R_c \) and \( C_c \) are the viability effect of siRNA without drug compared with siNT control, \( R_d \) and \( C_d \) are the viability effect of siRNA with drug compared with siNT control.

The Z-score was calculated as follows: Z-score = siRNA SI score – mean of all siRNAs in that plate)/SD of all siRNAs in that plate. This experiment was performed in duplicate. Any genes with a Z score > 2 and an SI ≥ 0.05 were considered significant.

Cell culture
PC-9, HCC827, NCI-H1975, NCI-H3225, and HCC4006 cell lines were purchased from the ATCC prior to 2009. The 11–18 cell line was generously provided by Koichi Hagiwara (Department of Respiratory Medicine, Jichi Medical University, Japan; ref. 17). PC-9/BRC1 were derived from PC-9 cells by chronic exposure to afatinib (18), HCC827, HCC4006, and 11–18 were cultured in RPMI1640 medium (Mediatech) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals) and penicillin (100 U/mL)/streptomycin (100 μg/mL; Mediatech). Cells were grown in a humidified incubator with 5% CO2 at 37°C. All cell lines are used for mycoplasma with the VenorGeM Mycoplasma Detection Kit (Sigma-Aldrich) approximately every 6 months. For experimental studies, the indicated cell lines were seeded at a passage number ranging from 5 to 25. For TKI-resistant cells, passage 1 was defined as the first passage following confirmation of a resistant phenotype. After 25 passages, cell lines were discarded to prevent genetic and phenotypic drift.

Derivation of osimertinib-resistant cell lines
To create resistant lines, parental cells were cultured with increasing concentrations of osimertinib, starting at the IC50 (19). At 80%–90% confluence, cells were trypsinized and divided into 2 tubes. Half were frozen and the rest were reseeded into a new dish at a 20%–30% higher dose of osimertinib. Fresh drug was added every 72–96 hours.

Inhibitors
Erlotinib was synthesized by the MSKCC Organic Synthesis Core. Osimertinib was either supplied by Astra Zeneca or purchased from Selleck Chemicals. All the other drugs were purchased from Selleck Chemicals.

Immunoblot and antibodies
The following antibodies were obtained from Cell Signaling Technology: EGFR (#2232), pEGFR Y1068 (#2234), HER3 (#4754), pHER3 Y1197 (#4561), pBRAF S445 (#2696), MEK (#9116), pMEK S217/222 (#9154), ERK (#9120), pERK T202/Y204 (#4102), PDK1 (#3062), pPDK1 S424 (#3061), AKT (#2272), pAKT S473 (#2927), pAKT T308 (#9275), ribosomal protein S6 (#2317), pS6 #240S244 (#3864), pFAK Y397 (#4756), SFKs (#2298), pSFKs Y416 (#2210), YES (#3201), Integrin Antibody Sampler Kit (#4749), PARP (#9542), E-cadherin (#3195), Vimentin (#3932), horseradish peroxidase (HRP)-conjugated anti-mouse (#7076), and HRP-conjugated anti-rabbit (#7074). The actin antibody (A2066) was purchased from Sigma-Aldrich. The RAF antibody (sc-55522) was purchased from Santa Cruz Biotechnology. For immunoblotting, cells were harvested, washed in PBS, and lysed in RIPA buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L sodium chloride, 5 mmol/L magnesium chloride, 1% Triton X-100; 0.5% sodium deoxycholate, 0.1% SDS, 40 mmol/L sodium fluoride, 1 mmol/L sodium oxalate, and complete protease inhibitors [a: 0.52. Body weight was assessed twice weekly. Mice were cared for in accordance with guidelines approved by the MSKCC Institutional Animal Care and Use Committee and Research Animal Resource Center.

siRNA experiments
siHER3, siSIRC, siYES1, and control oligos were used at a concentration of 10 nmol/L. Transfections were completed with DharmaFECT4 (Dharmacon) according to the manufacturer's protocol.

Expression constructs and transfections
Yes1 plasmid (#20665) was purchased from Addgene. The pWZL-Neo/Yes1 vector and the pSVS-G vector (Clontech) for production of the viral envelope were introduced into GP2-293 cells (80% confluence in a 10-cm dish) using Lipofectamine 2000 (Invitrogen). After 48 hours, viral particles released into the culture medium were concentrated by centrifugation at 15,000 × g for 3 hours at 4°C. The resulting pellet was
then suspended in fresh RPMI1640 medium and used to infect PC-9/BRc1 cells as previously described (20).

Vitronectin and fibronectin coating
Vitronectin and fibronectin (Sigma-Aldrich) were diluted in PBS and used to coat the culture surface with a minimal volume at 0.3 μg/cm² and 3 μg/cm², respectively. Plates were allowed to air dry for 1 hour at room temperature. Excess vitronectin or fibronectin was removed by aspiration.

Viability and proliferation assays
Cell viability was measured using the CellTiter Blue assay (Promega). Cells were seeded in 96-well plates at a density of 3,000 cells per well and drug treated on the following day. CellTiter Blue reagent was added 72 hours after drug addition, and fluorescence at 570 nm was measured on a Synergy MX microplate reader (Biotek). Cell proliferation was assayed by cell counting experiments. Cells were seeded in 6-well or 12-well plates on day 0, and treatment was started on day 1. Cell numbers were counted using a Coulter Counter (Beckman Coulter).

Statistical analysis
Difference in cell viability and comparison of xenograft tumor volumes was performed using Student t test in either R version 3.2.3, GraphPad Prism 5 (GraphPad Software), or GraphPad Prism 5 (GraphPad Software) version 5.01. Fisher exact test in R version 3.2.3 statistics package (R Core Team, 2015) or GraphPad Prism 5 (GraphPad Software). Fisher exact test in R version 3.2.3 statistics package was used to analyze whether different therapies influenced the likelihood of tumor recurrence in an in vivo model. A P value of ≤ 0.05 was considered significant for all analyses. Power analysis to determine appropriate sample size for in vivo work was done with the following parameters: α = 0.05, power = 0.8.

Results

Kinome-wide siRNA screen identifies rational targets for combination therapy with osimertinib
To identify kinases that attenuate the effects of osimertinib in EGFR-mutant lung cancer, we performed a kinome-wide siRNA screen using PC-9/BRc1 (EGFR exon19 deletion/T790M) cells (Supplementary Table S1; Supplementary Fig. S1A; ref. 18), and found 31 siRNAs that sensitized to osimertinib (Supplementary Table S2). Among the top 10 hits, we identified MAPK1 (encodes ERK2), BRAF, and PDK1 (encodes PDK1) as attenuating factors of osimertinib (Fig. 1A; Supplementary Table S2). In addition, PIK3CA and PIK3CB, both of which encode subunits of phosphoinositide 3-kinase (PI3K), were among the top 30 hits. PI3K and PDK1 are components of the AKT pathway, while BRAF and ERK2 are components of the MAPK pathway. These data suggest that signaling downstream of EGFR, through the AKT and MAPK pathways, remains active and may be independently contributing to proliferation/survival even in the setting of continuous EGFR inhibition.

On the basis of these data, we examined changes in the AKT and MAPK pathways after osimertinib treatment, focusing on the phosphorylation of key signaling kinases (Fig. 1B). EGFR phosphorylation was completely inhibited by osimertinib, while phosphorylation of AKT pathway components, including PDK1, AKT, and S6, was only partially blocked. Phosphorylation of BRAF, MEK, and ERK, components of the MAPK pathway, also persisted or became reactivated following osimertinib treatment.

Similar results were observed in other EGFR-mutant NSCLC cell lines (Fig. 1C; Supplementary Fig. S1B) and with other EGFR TKIs, including erlotinib and rociletinib (Supplementary Fig. S1C and S1D). These observations suggest that downstream signaling through the AKT and MAPK pathways is still partially active in the presence of continuous EGFR inhibition.

Next, we determined whether cotargeting EGFR and downstream pathways would result in a more complete inhibition of signaling and a greater effect on growth inhibition. Cotreatment of PC-9/BRc1 cells with osimertinib and either the pan-P38 inhibitor, BKM120, or the PDK1 inhibitor, GSK2334470, inhibited AKT phosphorylation (with no further effect on ERK phosphorylation) compared with osimertinib alone (Fig. 1D). Osimertinib plus BKM120 or GSK2334470 was also associated with greater inhibition of proliferation compared with osimertinib alone (Fig. 1E). We examined vertical inhibition of the MAPK pathway by cotreating PC-9/BRc1 cells with osimertinib and either a pan-RAF inhibitor, TAK632, or an allosteric MEK inhibitor, selumetinib. Neither TAK632 nor selumetinib could completely inhibit ERK phosphorylation at any time point (Supplementary Fig. S2A and S2B). However, combining either agent with osimertinib completely inhibited ERK phosphorylation (Fig. 1F) and more effectively blocked cell proliferation compared with any agent alone (Fig. 1G). These data suggest that addition of AKT or MAPK pathway inhibitors to EGFR-targeting therapies represent rational combination therapeutic strategies for EGFR-mutant lung cancer.

SF6s sustain MAPK pathway signaling in the presence of EGFR inhibition
We next explored potential mechanisms that could explain sustained signaling through the AKT and MAPK pathways in the presence of osimertinib. To exclude the possibility of insufficient drug levels, we examined the effects of higher doses of osimertinib. However, increased osimertinib concentrations showed no further inhibition of EGFR, AKT, or ERK phosphorylation and did not abrogate pathway reactivation at 96 hours (Fig. 2A). In addition, maximum loss of viability in PC-9/BRc1 was reached at 160 nmol/L osimertinib (Fig. 2B). These data suggest that sustained AKT and MAPK signaling is not due to insufficient EGFR inhibition.

We sought to better characterize the population of cells that remained viable after 96-hour exposure to osimertinib. To assess whether osimertinib inhibited cell growth less potently in the remaining population, we generated concentration–response curves using PC-9/BRc1 cells that were pretreated with either 100 nmol/L osimertinib or vehicle for 96 hours. We found no difference in EC50 for DMSO pretreated cells compared with osimertinib pretreated cells (3.46 vs. 3.44 nmol/L, P = 0.96; Supplementary Fig. S2C). Likewise, sequencing of EGFR exons 19, 20, and 21 after 96-hour osimertinib exposure did not reveal any new mutations within the EGFR kinase domain in any of five cell lines tested (data not shown). Taken together, these data suggest that osimertinib treatment is not rapidly selecting for clones with primary osimertinib resistance. Alternatively, to explore potential “bypass” signaling pathways, we profiled lysates from PC-9/BRc1 cells treated with osimertinib using a receptor tyrosine kinase array and found that phosphorylation of human epidermal growth factor receptor 3 (HER3) increased after drug treatment (Supplementary Fig. S3A and S3B). However, HER3 knockdown had no effect on AKT or ERK phosphorylation (Supplementary Fig. S3C), suggesting that HER3 does not act as a
“bypass” signal following osimertinib treatment in these cell models.

Next, we focused on a potential role for Src family kinases (SFK), as SFKs are known upstream regulators of the AKT and MAPK pathways (21). Interestingly, immunoblot analysis revealed increased phosphorylation of SFKs after osimertinib treatment (Figs. 2C and 3A). Treatment with PP2, a selective SFK inhibitor (22), or dasatinib, a multi-kinase inhibitor that targets SFKs, attenuated SFK activation in the presence of osimertinib (Fig. 2C, lanes 3 and 4 vs. lane 2). Notably, PP2 or dasatinib treatment also led to more profound inhibition of ERK phosphorylation compared with osimertinib alone (Fig. 2C, lanes 3 and 4 vs. lane 2), suggesting that activity of the MAPK pathway is sustained by SFKs in the absence of EGFR signaling. Furthermore, cotreatment of PC-9/BRC1 cells with PP2 or dasatinib enhanced growth-inhibitory effects compared with osimertinib monotherapy (Fig. 2D). Combining osimertinib with bosutinib or saracatinib (Fig. 2D), two other clinically relevant TKIs with anti-SFK activity (23, 24), also resulted in improved cell growth inhibition compared with osimertinib alone (Supplementary Fig. S4A–S4D).

Concomitant inhibition of FAK and SFKs is necessary to sustain MAPK and AKT pathway inhibition in the presence of EGFR blockade and to achieve greater inhibition of proliferation.

PP2, bosutinib, and saracatinib had minimal effect on AKT phosphorylation. Dasatinib, however, was better able to inhibit AKT phosphorylation (Fig. 2C, lane 3 vs. lane 4; Supplementary Fig. S4C). Moreover, dasatinib was consistently more efficacious than PP2, bosutinib, and saracatinib at increasing apoptosis (determined by expression of cleaved PARP) and inhibiting cell proliferation (Fig. 2C and D; Supplementary Fig. S4C and S4D). These data suggest that there are additional factors that attenuate the effects of osimertinib through incomplete AKT inhibition, and that these factors can be inhibited by dasatinib.

Focal adhesion kinase (FAK) is known to interact with SFKs. Notably, FAK activity can be inhibited by dasatinib, likely through an indirect mechanism (25). Analogous to SFKs, we also noticed that autophosphorylation of FAK increased after osimertinib treatment (Supplementary Fig. S5A). Therefore, we examined the expression of FAK and SFKs in multiple EGFR-mutant lung cancer cell lines by immunoblot (Fig. 3A; Supplementary Fig. S5B) and found that SFK and/or FAK phosphorylation was increased to varying extents after osimertinib treatment in all cell lines evaluated.

We next determined whether PF573228, a selective, ATP-competitive inhibitor of FAK (Supplementary Fig. S5C; ref. 26), could influence sensitivity of EGFR-mutant cells to osimertinib. PC-9/BRC1 cells were treated with osimertinib alone or in combination with PF573228 and/or PP2. Addition of PF573228 abrogated the osimertinib-induced increase in FAK phosphorylation (Fig. 3B, lane 3 vs. lane 2). Osimertinib combined with the SFK inhibitor, PP2, inhibited phosphorylation of SFKs and ERK (Fig. 3B, lane 4 vs. lane 2). Osimertinib/PF573228 and osimertinib/PP2 combinations both had modest effects on phosphorylated AKT or cleaved PARP. However, the triple combination of osimertinib, PF573228, and PP2 completely inhibited phosphorylation of FAK and SFKs, leading to complete inhibition of AKT, increased cleaved PARP (Fig. 3B, lane 5 vs. lanes 3 and 4), and enhanced growth-inhibitory effects (Fig. 3C). Similar results were observed for triple combinations that included bosutinib or saracatinib (Supplementary Fig. S5D). Osimertinib combined with dasatinib, which inhibits SFKs and can indirectly inhibit FAK activity (25, 27), showed effects similar to the osimertinib/FAK inhibitor/SFK inhibitor triple combination (Fig. 3B, lane 6 vs. lane 5; Supplementary Fig. S5E). These data suggest that activation of both SFKs and FAK plays a role in attenuating the effects of osimertinib, and that inhibiting both SFKs and FAK leads to enhanced growth-inhibitory effects in EGFR-mutant lung cancer cells.

The combinatorial effect of dasatinib with osimertinib was also examined in PC-9/BRC1 xenografts (Fig. 3D; Supplementary Fig. S6A and S6B). There was no significant body weight loss in either group (Supplementary Fig. S6A). Both osimertinib monotherapy and osimertinib combined with dasatinib inhibited tumor growth to almost undetectable levels (Supplementary Fig. S6B). Five weeks after treatment cessation, 3 of 7 mice in the osimertinib + dasatinib treatment arm showed no tumor growth (‘tumor cure’), while there were no tumor cures in the osimertinib monotherapy arm (Fig. 3D), although the difference in tumor regrowth cases between the two arms was not statistically significant ($P = 0.077$).

Given that integrins are reported to activate SFK/FAK signaling (28), we examined the expression of several integrins in the absence and presence of osimertinib treatment. Expression of integrins αvβ3, αvβ5, and αvß5 was increased after treatment with osimertinib (Fig. 3E). Moreover, after 24 hours of treatment with osimertinib plus SB273005, an antagonist of integrin αvβ3 and αvß5, we observed greater inhibition of AKT and ERK compared with osimertinib alone (Fig. 3F, lane 3 vs. lane 4). Next, as others had previously reported that cell–cell contacts influence signaling along the integrin–FAK pathway (29, 30) we investigated whether cells plated at higher density responded differently to osimertinib compared with cells plated at lower density. Interestingly, we observed an increase in SFK and FAK activity following osimertinib exposure that was more pronounced in the high density group (Supplementary Fig. S7A). These effects are consistent with the hypothesis that integrins are activating downstream signaling through SFK/FAK. To further investigate the necessity of integrins toward SFK/FAK–mediated AKT and MAPK signaling, we determined the effect of the integrin ligand, vitronectin, on integrin complexes αvβ3 and αvß5. In this experiment, PC-9/BRC1 cells were cultured in serum-free media as serum contains a large amount of extracellular matrix components, including vitronectin. Consistent with our hypothesis, cell culture dishes coated with vitronectin attenuated the effects of osimertinib, whereas fibronectin coating did not (Supplementary Fig. S7B). Collectively, these data suggest that integrins activate FAK/SFKs, thereby attenuating the effects of osimertinib.

On the basis of these findings, the potential mechanism whereby SFK/FAK signaling attenuates the effects of osimertinib in EGFR-mutant cells is shown schematically in Supplementary Fig. S7C. Under continuous inhibition of EGFR signaling by osimertinib, integrins can activate SFK/FAK, leading to sustained AKT and ERK phosphorylation. AKT or MAPK pathway inhibitors enhance the effects of osimertinib by directly inhibiting the respective downstream signaling, whereas dasatinib enhances the effects of osimertinib by inhibiting SFK/FAK, leading to simultaneous inhibition of both the AKT and MAPK pathways.
Figure 1.
Sustained AKT and MAPK pathway signaling following osimertinib treatment in EGFR-mutant lung cancer cells. A, PC-9/BRc1 cells were reverse transfected with siRNAs targeting 714 kinases and kinase-related genes (four individual siRNAs per gene) for 24 hours. Transfected cells were then treated with DMSO or osimertinib (5 nmol/L), and cell viability was measured following 72 hours of drug treatment. The average of SI of the four individual siRNAs was calculated for each gene using the formula described in the Materials and Methods. Dots represent the SI value of each siRNA. Positive SI value indicates a sensitizing effect. siRNAs are plotted in alphabetical order. (Continued on the following page.)
Osimertinib plus dasatinib combination therapy is superior to osimertinib in combination with either AKT or MAPK pathway inhibitors

To determine the optimal combination therapeutic strategy, we compared the growth-inhibitory effects of BKM120 (PI3K inhibitor), selumetinib (MEK inhibitor), or dasatinib (SFK inhibitor) with or without osimertinib in multiple EGFR-mutant lung cancer cell lines. Monotherapy with each drug inhibited tumor cell proliferation to varying degrees (Supplementary Fig. S8A). Addition of BKM120, selumetinib, or dasatinib enhanced the effects of osimertinib (Fig. 4A). However, the dasatinib–osimertinib combination was consistently the most effective at inhibiting tumor cell proliferation. We also determined the effects of the various combination therapies on apoptosis (Fig. 4B; Supplementary Fig. S8B). The selumetinib combination only modestly increase cleaved PARP in each of the cell lines evaluated, which is consistent with previously published data (31). In contrast, the greatest induction of cleaved PARP was consistently observed with the dasatinib combination. Moreover, the dasatinib combination was the most effective at delaying the emergence of resistant clones (Fig. 4C; Supplementary Fig. S8C and S8D).

Amplification of YES1, a SFK, mediates acquired resistance to osimertinib

Finally, we explored the role of SFK/FAK signaling in the setting of acquired resistance to osimertinib. For this purpose, we established five osimertinib (AZD9291) resistant cell lines (Supplementary Table S1) using a dose escalation method (18). Two of the five cell lines (H1975/9291 and SH450/9291) exhibited an epithelial-to-mesenchymal transition phenotype denoted by loss of E-cadherin and gain of vimentin expression (Supplementary Fig. S9A). In PC-9/BRC1/9291 cells, whole-exome sequencing revealed focal amplification (15.7 gene copy number) of YES1 on chromosome 18, which encodes the SFK, YES1. Immunoblot analysis showed considerably increased expression of YES1.
protein in PC-9/BRc1/9291 cells compared with the "parental" PC-9/BRc1 cells (Fig. 5A). Treatment of PC-9/BRc1/9291 cells with osimertinib alone inhibited EGFR phosphorylation, but did not inhibit downstream ERK, AKT or S6 phosphorylation (Fig. 5B). Combination therapy with osimertinib and the SFK inhibitors, PP2 or dasatinib, inhibited both EGFR and downstream effector signaling. Combination therapy also resulted in increased apoptosis as shown by cleavage of PARP. Importantly, pharmacologic or genetic inhibition of YES1 restored sensitivity to osimertinib in PC-9/BRc1/9291 cells (Fig. 5C and D). Moreover, ectopic expression of YES1 rendered the PC-9/BRc1 “parental” cells (from which the PC-9/BRc1/9291 cells were derived) less sensitive to osimertinib (Fig. 5E). Consistent with these results, dasatinib combined with osimertinib decreased the rate of tumor growth in a PC-9/BRc1/9291 xenograft model (Fig. 5F). Immunoblotting confirmed SFK phosphorylation was inhibited by the osimertinib/dasatinib combination (Supplementary Fig. S9B). There was no significant body weight loss in any group (Supplementary Fig. S9C).

**Discussion**

In this study, we sought to determine the most effective rational drug combination therapy to delay or prevent acquired resistance to osimertinib. Among the top hits in our kinome-wide siRNA screen were components of the MAPK and AKT pathways. Our
data reveal that signaling through these pathways is sustained or reactivated as early as 24–48 hours after addition of osimertinib, thereby attenuating the growth-inhibitory effects of this agent. Recent studies have also demonstrated that MAPK signaling induces acquired resistance to third-generation EGFR TKIs, including WZ4002 (32) and osimertinib (33). Consistent with our work herein, these studies demonstrated that the combination of WZ4002 with the MEK inhibitor, trametinib, showed more profound inhibition of tumor cell growth (31). In addition, we found that AKT pathway signaling is sustained in the presence of persistent EGFR inhibition, and that concomitant inhibition of EGFR and AKT pathway enhanced the effects of osimertinib. Interestingly, the efficacy was equivalent or better than that of EGFR plus MEK inhibition (Fig. 4A–C Supplementary Fig. S8A). In the previous studies, AKT pathway reactivation was reported to be a mechanism of acquired resistance to WZ4002 and trametinib combination therapy (31), suggesting that this pathway may play an important role in cancer cell survival under continuous EGFR inhibition.

Although the effects of simultaneous EGFR plus MAPK or AKT pathway inhibition are promising, it is unknown how these pathways are sustained or reactivated in the presence of continuous EGFR inhibition. A detailed mechanistic understanding of how and why EGFR-mutant cells have early downstream signaling reactivation is expected to result in improved therapeutic strategies. To this end, we found that phosphorylation of SFKs and FAK increased after osimertinib treatment. SFKs and FAK are known to interact with each other and mediate AKT and MAPK pathway signaling (21). Targeting SFKs with dasatinib enhanced the effects of osimertinib, resulting in both a more pronounced attenuation of effector signaling and a greater inhibition of cell growth. SFKs and FAK were also found to be activated through integrin signaling. Interestingly, integrin αβ3 has been associated with resistance to EGFR TKI treatment through forming integrin–KRAS complex (34), and integrin-targeted therapies are currently being tested in the clinic (35, 36).

Others have previously reported on the potential utility of SFK-targeting drugs in oncogene-driven NSCLC. Crystal and...
colleagues identified Src as a common mediator of ALK TKI resistance in cell cultures established from patients who had progressed on ALK TKI therapy (37). Likewise, Yoshida and colleagues reported that dasatinib could enhance the antitumor activity of irreversible EGFR TKIs, including the mutant-selective WZ4002, in cell lines with T790M-mediated resistance to erlotinib and gefitinib (38). In addition, previous work using models of other solid tumors, such as breast and thyroid cancer, have shown that dasatinib enhances the effects of targeted therapies via inhibition of SFK/FAK signaling (39, 40).
together, these studies support the conclusion that SFKs and FAK are common mediators of resistance to oncogene-targeting therapies across multiple tumor types. The combination of first-generation EGFR TKI plus dasatinib was evaluated in a phase II clinical trial in patients with EGFR-mutant lung cancer and acquired resistance to erlotinib or gefitinib (41). No radiographic responses were observed in the 12 patients who received combination therapy, two-thirds of whom were positive for T790M. However, we would not expect erlotinib to be sufficient to inhibit EGFR signaling in either T790M-induced acquired resistance or in non-T790M-bypass--induced acquired resistance. Considering that we observed no increase in apoptosis with dasatinib monotherapy (Fig. 4B), dasatinib appears to be most efficacious when coupled with potent EGFR inhibition. Interestingly, a phase I/II clinical trial of osimertinib plus dasatinib in EGFR-TKI-naive patients recently opened in the United States (NCT02954523).

Finally, we identified amplification of the SFK, YES1, as a potential mechanism of osimertinib resistance in vitro. Previous reports demonstrated that SFK signaling activation causes intrinsic or acquired resistance to EGFR TKIs in an in vitro model (42, 43). Furthermore, Src was reported to be a mechanism of intrinsic resistance to EGFR TKIs in EGFR-mutant NSCLC (43). Notably, YES1 amplification has been detected in patients with EGFR-mutant NSCLC at the time of EGFR TKI resistance (Ladanyi, and colleagues, manuscript in preparation). These data suggest that SFK signaling can attenuate the effects of EGFR TKIs in both EGFR TKI-sensitive and TKI-resistant tumor cells. Furthermore, these data suggest that multiple mechanisms may converge on SFK mediated signaling and resistance, for example, through upstream activation via integrins or genetic amplification events. This is analogous to MAPK pathway resistance, in which studies suggest multiple different events, for example, KRAS amplification, MAPK1 amplification, and BRAF mutation can all converge of activation of same pathway (33).

In conclusion, we found that inhibiting SFK/FAK, AKT or MAPK signaling enhanced the effects of osimertinib. Furthermore, osimertinib plus dasatinib was the most efficacious combination in several models of EGFR-mutant lung cancer, suggesting that this combination may produce more durable effects than osimertinib alone in a clinical setting. Overall, our studies provide mechanistic insights into early downstream signaling reactivation in the setting of continuous EGFR inhibition and suggest rational combination therapies for clinical use. Our results should prompt further investigation of third-generation EGFR TKIs and SFK/FAK inhibitors as combination therapy in patients whose tumors harbor EGFR mutations.

Disclosure of Potential Conflicts of Interest
R. McEwen has ownership interest (including patents) in Stocks. W. Pao is a global head of oncology discovery and translational area in Roche and has ownership interest (including patents) in Molecular MD. C.M. Lovly is a consultant/advisory board member of Ariad Pharmaceuticals, Clovis, Sequenom, Genoptix, Novartis, and NCCN. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: E. Ichihara, D. Westover, W. Pao, C.M. Lovly
Development of methodology: E. Ichihara, D. Westover, J.A. Bauer, C.M. Lovly
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Ichihara, D. Westover, C.B. Meador, Y. Yan, J.A. Bauer, A. Kulick, E. de Stanchina, M. Ladanyi, C.M. Lovly
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Ichihara, D. Westover, Y. Yan, J.A. Bauer, P. Lu, F. Ye, R. McEwen, D. Cross, W. Pao, C.M. Lovly
Writing, review, and/or revision of the manuscript: E. Ichihara, D. Westover, C.B. Meador, J.A. Bauer, F. Ye, M. Ladanyi, D. Cross, W. Pao, C.M. Lovly
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Ichihara, Y. Yan, W. Pao, C.M. Lovly
Study supervision: W. Pao, C.M. Lovly

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Eiki Ichihara, David Westover, Catherine B. Meador, et al.


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