Combined EGFR/MEK Inhibition Prevents the Emergence of Resistance in EGFR-Mutant Lung Cancer

INTRODUCTION

Tyrosine kinase inhibitors (TKI) such as erlotinib or gefitinib that target EGFR are the standard of care for patients with EGFR-mutant non–small cell lung cancer (NSCLC; refs. 1, 2). However, TKI therapies are not curative: Most patients with EGFR-mutant NSCLC treated with first- or second-generation EGFR TKIs, such as gefitinib, erlotinib, and afatinib, develop resistance within 9 to 14 months (1, 2). Extensive research has shown that a variety of mechanisms can lead to acquired drug resistance. The most common mechanism (detected in 60% of patients) is a secondary mutation in EGFR itself (EGFR<sup>T790M</sup>; refs. 3–5). In addition, activation of other kinases (MET, IGF1R, BRAF, HER2, AXL, or FGFR) or alterations in downstream pathway components (MAPK1, NF1, PIK3CA activation, PTEN loss, or NF1 loss) that bypass the requirement for EGFR to maintain activation of downstream ERK1/2 and AKT pathways can lead to acquired resistance (6–16).

A recent breakthrough in the treatment of EGFR<sup>T790M</sup>-mutant cancers occurred with the development of mutant-selective pyrimidine-based EGFR TKIs, which include the inhibitors WZ4002, CO-1686, AZD9291, and HM61713 (17–19). CO-1686, AZD9291, and HM61713 are in phase I–III clinical trials and have demonstrated responses in >50% of patients with tumors harboring the EGFR<sup>T790M</sup> mutation (20–22). In addition, their reduced affinity for wild-type EGFR provokes less toxicity than other TKIs (20–22). However, it is fully anticipated that resistance will also occur to this class of EGFR inhibitors. In previous studies, we and others investigated resistance to WZ4002 and found evidence of genetic alterations leading to the EGFR-independent activation of ERK1/2 (13, 23).

Prior studies have focused on a sequential approach of treatment with EGFR TKIs by first identifying mechanisms of resistance to single-agent EGFR inhibitors and then developing strategies to overcome individual resistance mechanisms. Given the wide variety of identified resistance mechanisms, it is impractical to implement this approach clinically. An alternative strategy is to identify a combination treatment approach that prevents the occurrence of more than one resistance mechanism. This approach could have broad utility as an initial therapeutic treatment or at the time of EGFR<sup>T790M</sup> development. Here, we investigate the effectiveness of cotargeting EGFR and MEK using in vitro and in vivo models of EGFR inhibitor–sensitive and –resistant cancers. We demonstrate that this combination is more effective than single-agent WZ4002 at treating cancers with EGFR<sup>T790M</sup> and can also prevent the emergence of both T790M- and non–T790M-mediated drug resistance. These findings have significant therapeutic implications for patients with EGFR-mutant NSCLC.
RESULTS

ERK1/2 Reactivation Occurs after TKI Treatment

Preclinical and clinical studies suggest that mutant-selective EGFR inhibitors are a promising treatment option for T790M-positive tumors (17–22). However, it is unclear whether these agents are best used clinically as first-line treatment in patients with EGFR-mutant lung cancer or following the development of acquired resistance to current EGFR inhibitors. To determine whether resistance is prevented or delayed by initial treatment with mutant-selective EGFR inhibitors, we assessed the emergence of acquired resistance to gefitinib or to the mutant-selective EGFR inhibitor tool compound WZ4002 treatment in PC9 cells, which develop T790M in response to gefitinib treatment (24, 25). Low-confluent cells were treated in a 96-well plate format, and colonies of 50% confluence were scored weekly (7, 13). We found that both WZ4002 and gefitinib-resistant colonies begin to appear within 3 to 6 weeks, indicating that the emergence of resistance is not delayed by WZ4002 treatment in this cell line (Fig. 1A). Although ERK1/2 and AKT were initially inhibited to similar degrees, ERK1/2 reactivation was observed within just days following continuous exposure to treatment with gefitinib or WZ4002 (Fig. 1B). Next, we assessed whether the addition of a MEK inhibitor to WZ4002 was able to maintain ERK inhibition. The MEK inhibitor trametinib effectively inhibits ERK phosphorylation at 30 nmol/L in several EGFR-mutant cell lines but has little effect on cell viability (Supplementary Fig. S1A and S1B). AKT activation is increased by MEK inhibition in some cell lines (e.g., HCC4006, HCC2279), which has been previously reported to be caused by hyperactivation of HER3 (26). TKI-naive EGFR-mutant cell lines (PC9, HCC4006, HCC2279, and HCC2935) treated with WZ4002 plus trametinib (W+T) presented with less ERK1/2 reactivation compared with treatment with WZ4002 alone (Fig. 1C). Consistent with prolonged inhibition of ERK1/2 phosphorylation, the W+T combination also significantly sustained inhibition of ERK1/2-dependent gene transcription at 24 hours as compared with WZ4002 alone (Supplementary Fig. S1C and S1D; refs. 13, 27).

Prevention versus Treatment of Acquired Resistance

Given that genomic alterations resulting in ERK reactivation also develop following several months of drug exposure and mediate WZ4002 resistance, we sought to determine whether trametinib-mediated ERK1/2 inhibition enhances WZ4002-targeted toxicity and prevents the emergence of drug resistance (13). In order to evaluate the effect of W+T on cell viability, we assessed the effect of W+T treatment in TKI-naive cell lines (PC9, HCC4006, HCC2279, and HCC2935) at 72 hours (Fig. 2A). Overall, the addition of 30 nmol/L trametinib to WZ4002 treatment had a minor impact on cellular viability. Although the combination was not synergistic in these short-term viability assays, W+T treatment was very effective in preventing the emergence of resistance in TKI-naive cell lines; inhibition of growth in response to 100 nmol/L WZ4002 was significantly improved by the addition of 30 nmol/L trametinib as measured by the percentage of colony-positive wells at 6 weeks (Fig. 2B). It is important to note that these cell lines have diverse yet predictable mechanisms of acquired resistance: PC9 cells are reported to display T790M mutation or IGF1R activation, HCC4006 cells may undergo epithelial–mesenchymal transition, HCC287 cells amplify MET, and HCC2279 cells are resistant to EGFR TKIs due to a reduced ability to induce apoptosis through BIM upregulation (8, 24, 25, 28). Interestingly, the ability of W+T treatment to prevent the emergence of drug resistance was not influenced by these diverse mechanisms.

We next determined whether W+T is effective in delaying the emergence of acquired resistance in cell lines harboring an established T790M mutation, including HCC827 EPR, PC9 GR4, PC9 DR1, and H1975 cells in Fig. 2C (for a
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WZ4002 treatment reduced the appearance of colonies in all cell lines (Fig. 2E). The addition of trametinib to reduce the incidence of resistance was assessed at 6 weeks combination treatment. Next, the ability of WZ4002 + HGF pathway (PC9 PFR3 and H1650) was not affected by combination treatment. However, when cell lines harbored established EGFR TKI resistance mechanisms other than the T790M mutation, WZ4002 + trametinib, or a combination thereof, and the percentage of wells at 50% or greater confluence was assessed at 6 weeks (n = biologic triplicates, 1 plate (60 wells) each). Graph is representative of two or three independent experiments per cell line. F, PC9 cells were treated with DMSO control (media), 100 nmol/L IGF1R inhibitor BMS754807 (BMS), or WZ4002 combinations weekly (top). HCC287 cells were treated with DMSO (media), 100 nmol/L WZ4002, 30 nmol/L trametinib, 100 nmol/L crizotinib (criz), or WZ4002 combinations weekly (bottom). Positive wells (n = three biologic replicates, 1 plate each) were assessed weekly and graphed mean ± SD. Each experiment was repeated two times.

Figure 2. Acquired resistance is prevented by W+T combination treatment in TKI-naïve and T790M+ cell lines. A, cell lines were treated with increasing doses of WZ4002 alone or in combination with 30 nmol/L trametinib (tram), and viability was assessed by MTS (n = 3 independent experiments, mean ± SD). TKI-naïve cell lines (B), T790M+ resistant cell lines (C), and cell lines harboring non-T790M mechanisms of TKI resistance (D) were treated weekly with 100 nmol/L WZ4002, 30 nmol/L trametinib (tram), or a combination thereof for 6 weeks. Arrows, wells did not achieve 50% confluence by 6 weeks. Data is representative of 2 to 3 independent experiments (180 wells per condition). E, cell lines were treated with DMSO (media), 100 nmol/L WZ4002, 30 nmol/L trametinib, or a combination thereof and to reduce the incidence of W+T-resistant colonies. Similar to TKI-naïve cell lines, the W+T combination was able to significantly delay the emergence of acquired resistance and to reduce the incidence of W+T-resistant colonies. However, when cell lines harbored established EGFR TKI resistance mechanisms other than the T790M mutation, the combination was less effective (Fig. 2D). Combination treatment was able to slightly delay the emergence of resistance but did not affect the overall incidence of acquired resistance in cell lines with preexisting MET amplification (HCC827 GR6 and DFC181) or activation (HCC827 + HGF) as well as MAPK1 amplification (PC9 PFR3) and H1650). Growth of cell lines with EGFR-independent activation of the AKT pathway (PC9 PFR3 and H1650) was not affected by combination treatment. Next, the ability of W+T treatment to reduce the incidence of resistance was assessed at 6 weeks for all cell lines (Fig. 2E). The addition of trametinib to WZ4002 treatment reduced the appearance of colonies in TKI-naive and T790M+ cell lines (9 cell lines; 0/9 reached 100% positive wells at 6 weeks and are considered long-term “W+T sensitive”); the majority of cell lines with established non-T790M mechanisms of resistance exhibited 100% positive wells at 6 weeks (5/6 cell lines, hereafter defined as “W+T resistant”). Overall, W+T treatment was significantly more effective in TKI-naïve or T790M+ cells but had little effect in most cell lines with non-T790M TKI resistance mechanisms (P = 0.0020, Fisher exact test).

As W+T combination treatment prevents the emergence of acquired resistance in EGFR TKI-naïve cell lines known to develop a variety of TKI resistance mechanisms, we hypothesized that W+T treatment may be superior to combination treatments designed to target just one resistance mechanism predicted to arise in a given cell line. PC9 cells are known to develop EGFR (T790M) or IGF1R activation as mechanisms of TKI resistance, whereas HCC827 cells develop MET amplification (6, 7, 24, 25). To prevent...
MET amplification in HCC827 cells, the clinically used MET inhibitor crizotinib was combined with WZ4002, whereas PC9 cells were treated with the IGF1R inhibitor BMS754807 and WZ4002 (Fig. 2F). In both cases, the targeted combination was able to delay the growth of colonies but not to the extent of W\textsuperscript{+}T treatment. However, WZ4002 plus crizotinib was equivalent or superior to W\textsuperscript{+}T when treating HCC827 cells with existing TKI resistance, including those with MET amplification or aberrant HGF signaling (Supplementary Fig. S2).

**W\textsuperscript{+}T Treatment Effectively Inhibits S6 Activation in Sensitive Cell Lines and Enhances Apoptosis**

To identify predictive biomarkers associated with long-term W\textsuperscript{+}T sensitivity, we assessed the ability of W\textsuperscript{+}T treatment to inhibit EGFR-dependent signaling cascades, including EGFR, ERK1/2, AKT, and S6 phosphorylation in the HCC827 TKI-naive and matching TKI-resistant cell lines (Fig. 3A). In cell lines that are sensitive to long-term W\textsuperscript{+}T treatment (e.g., HCC827 and HCC827 EPR cell lines), EGFR and downstream pathways are inhibited by both WZ4002 and the W\textsuperscript{+}T combination treatment. In cell lines that develop resistance to long-term W\textsuperscript{+}T treatment (e.g., HCC827 GR6 and HCC827 +HGF), EGFR and ERK1/2 are efficiently inhibited by W\textsuperscript{+}T combination treatment, but AKT and S6 inhibition was impaired. EGFR, ERK1/2, AKT, and S6 phosphorylation was assessed in the complete panel of cell lines shown in Fig. 2E (Supplementary Fig. S3).

In the majority of W\textsuperscript{+}T-sensitive cell lines, WZ4002 alone or W\textsuperscript{+}T treatment reduced the activity of EGFR-dependent pathways. W\textsuperscript{+}T-resistant cell lines treated with W\textsuperscript{+}T exhibited reduced EGFR and ERK1/2 activation, but S6 activation was unchanged. An exception was seen with the H1975 cell line, wherein WZ4002 or W\textsuperscript{+}T treatment did not inhibit S6 activation despite a W\textsuperscript{+}T-mediated reduction in the emergence of drug resistance at 6 weeks (as shown in Fig. 2C). However, this cell line generates a high frequency of W\textsuperscript{+}T-resistant clones compared with other TKI-naive or T790M-positive cell lines.

**Figure 3.** Effect of W\textsuperscript{+}T treatment on signaling and apoptosis. A, cell lines were treated with DMSO (−), 100 nmol/L WZ4002 (W), 30 nmol/L trametinib (T), or the combination (C) for 8 hours, and phosphorylation (p) of EGFR, AKT, ERK1/2, and S6 was assessed. Hsp90 was assessed as a loading control. Long-term sensitivity or resistance of each cell line to W\textsuperscript{+}T treatment is indicated below the figure. Image is representative of three independent experiments. B, TKI-sensitive and TKI-resistant cell lines were treated with 100 nmol/L WZ4002 (W), 30 nmol/L trametinib (T), or a combination thereof (C) for 24 hours, and BIM levels were assessed. Hsp90 was used as a loading control. Image is representative of three independent experiments. C, cell lines were treated with DMSO control (media), 100 nmol/L WZ4002, 30 nmol/L trametinib (tram), or a combination thereof for 72 hours. Caspase 3 activity of lysates was measured, and fold change was calculated relative to DMSO control (n = 3 to 4 independent experiments, mean ± SD). Significance was calculated by one-way ANOVA (P < 0.05).
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AUGER article

EGFR

L858R/T790M NSCLC. This model was previously demonstrated in a genetically engineered mouse model (GEMM) of tumor outgrowth for at least 14 weeks, no tumor cures were observed in tumors from mice treated with W+T compared with individual treatments, as analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Fig. SC and D; Supplementary Fig. SSB). Treatment differences between WZ4002 and W+T conditions became more apparent after 2 weeks; tumors treated with WZ4002 or trametinib began to rebound, whereas W+T-treated tumors were inhibited beyond 20 weeks (Fig. SE and Supplementary Fig. S5C and S5D). Trametinib alone was unable to prevent tumor growth, and the addition of WZ4002 to trametinib at the time of tumor regrowth caused a transient reduction in tumor volume. Overall, W+T treatment was able to significantly extend the survival of mice beyond WZ4002 alone (Fig. SF, P = 0.0097; log-rank test).

Mechanisms of Acquired Resistance to EGFR/MEK Inhibition

To study W+T–acquired resistance, we continued W+T treatment of the EGFR<sup>L858R/T790M</sup> GEMMs until tumor outgrowth was observed (Supplementary Fig. S6A and S6B). These tumors were harvested and the EGFR signaling pathway was assessed by IHC to identify any reactivated components. Phosphorylation of EGFR and ERK1/2 was assessed, and S6 and 4EBP1 were also evaluated as a readout of the mTOR pathway (Fig. 6A and Supplementary Fig. S6C). Notably, we found an unusual degree of heterogeneity between adjacent tumor nodules in each mouse, and were able to identify tumor nodules with significant phosphorylation residing next to nodules in which phosphorylation was not detected for EGFR, ERK1/2, S6, and 4EBP1 (Fig. 6A). However, EGFR and ERK1/2 reactivation by the W+T combination was maintained in the majority of resistant nodules, whereas S6 was frequently reactivated, occurring in 15 of 27 (67%) tumor nodules examined (Fig. 6B). Reactivation of both AKT and S6 was also detected by Western blot of tumor lysates (Supplementary Fig. S6D). However, no genetic lesions known to increase mTOR activation (e.g., PTEN, AKTI, TSC1, TSC2, p53a, PISKCA) were identified in these resistant tumors (data not shown). To confirm the impact of mTOR signaling on W+T-resistant tumors, we administered the TORC1/2 inhibitor Torin2 with the W+T combination in two mice exhibiting W+T resistance and assessed changes in tumor volume for 4 weeks (Fig. 6C; ref. 33). Torin2 has minimal activity by itself (data not shown). In both cases, the addition of Torin2 to W+T treatment resulted in tumor shrinkage (Fig. 6D).

To determine if mTOR activation was also observed after W+T resistance was generated in vitro, PC9 cells were seeded in 96-well plates at low confluence and treated weekly with W+T until resistant clones emerged. These drug-tolerant populations were then treated with W+T for 8 hours, and EGFR, AKT, ERK1/2, and S6 inhibition was assessed (Fig. 6E).
Although W+T could still inhibit EGFR and ERK1/2 phosphorylation, AKT and S6 phosphorylation was unaffected by treatment. Torin2 treatment alone was able to inhibit S6 phosphorylation and decrease the viability of both the parental and W+T drug-tolerant populations (Supplementary Fig. S7A and S7B). Furthermore, the addition of Torin2 to W+T combination treatment was able to restore sensitivity of the PC9 W+T-resistant cells almost to that of the parental PC9 levels (Fig. 6F; Supplementary Fig. S7C)

**DISCUSSION**

The development of acquired drug resistance limits the long-term clinical success of EGFR inhibitors in lung cancer. The identification and clinical development of mutant-selective EGFR inhibitors provide the opportunity to both treat patients with EGFR<sup>T790M</sup>-mediated drug resistance and to develop combination treatment approaches that previously were not possible due to combined toxicity of EGFR inhibitors and agents targeting signal transduction pathways.

Here, we propose that cotargeting EGFR and MEK is a more effective combination strategy than just targeting EGFR alone. There are two potential uses for this approach. The addition of a MEK inhibitor can enhance the efficacy of WZ4002 in EGFR<sup>T790M</sup>-containing models. Through the use of in vitro models, xenograft models, and EGFR<sup>L858R/T790M</sup> GEMMs, we demonstrate that the W+T combination is significantly better at delaying the onset of resistance than WZ4002 alone, and cures some xenograft models (Figs. 2, 4,
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**Figure 5.** Cotargeting EGFR and MEK prolongs effective treatment duration in EGFR*L858R/T790M* genetically engineered mice. **A,** tumor volume after 2 weeks of vehicle, WZ4002, trametinib (tram), or a combination thereof. Significance was assessed by two-tailed t test, *P = 0.0041*. A subset of single-agent WZ4002-treated mice is published in a previous report (13). **B,** phosphorylation (p) of EGFR, AKT, ERK1/2, S6, and 4EBP1 was assessed by IHC after two doses of vehicle, trametinib, WZ4002, or combination. H&E, hematoxylin and eosin. **C,** analysis of apoptosis by TUNEL staining after two doses of WZ4002, trametinib, or a combination thereof. **D,** quantification of TUNEL-positive cells. Significance was assessed by one-way ANOVA, *P < 0.001*. **E,** tumor volume after W+T treatment. Upon tumor growth, mice treated with trametinib (purple line) were treated with a combination of WZ4002 and trametinib (green line). A subset of single-agent WZ4002-treated mice was previously reported (ref. 13; *n* = 5, mean ± SEM). **F,** survival of W+T–treated mice compared with WZ4002 alone. Significance was determined by the log-rank test, *P = 0.0097*. One mouse in the combination treatment group was euthanized at 12.3 weeks for a leg problem unrelated to treatment. A necropsy was performed, and no tumor was observed (data not shown).

A second clinical use for this combination is the prevention of drug resistance, which has broad clinical applicability, as our studies demonstrate that the W+T combination prevents both T790M-dependent and T790M-independent drug resistance (Fig. 2). Compared with WZ4002 alone, this combination was able to cure a fraction of tumors in the xenograft models. Although this represents an improvement over the single agent, these data suggest that additional strategies are still needed and provide a starting point for the development of effective combination strategies using EGFR TKIs. In fact, this approach is better than a preventative treatment designed for the specific resistance mechanism known to arise as a result of EGFR inhibition (e.g., MET/EGFR inhibition in HCC827 cells that develop MET amplification in response to EGFR inhibitors).

In contrast, the W+T combination is relatively ineffective in models with established non–T790M-acquired resistance, likely because compensatory pathways allow for proliferation even in the presence of combined EGFR/MEK inhibition (Fig. 2D). Our studies suggest that one such pathway is the mTOR pathway, as W+T is unable to downregulate S6 phosphorylation in the majority of resistant cell lines, in contrast with sensitive cell lines (Fig. 3A). In addition, acquired resistance to W+T treatment in EGFR*L858R/T790M* GEMMs and PC9 cells is associated with S6 reactivation (Fig. 6). Further support for the significance of the mTOR pathway in resistance to W+T treatment is seen by the ability of the mTOR inhibitor Torin2 to resensitize cells to W+T treatment both in vitro and in vivo (Fig. 6). The mTOR pathway is a convergence point for many signaling pathways, including EGFR and other...
receptor tyrosine kinases, and additionally can be affected by mutation or loss of tumor suppressors, such as PTEN, TSC1, or NF2 (34). As mTOR and downstream S6 activation can occur in response to a diverse array of survival signals, S6 may have utility as a general biomarker for optimal TKI response. S6 has also been reported as a putative biomarker for response to RAF or MEK inhibitors in melanoma and for afatinib plus cetuximab combination treatment in NSCLC (35, 36).

The enhanced efficacy of the W+T combination compared with WZ4002 alone is likely due to at least two mechanisms. The addition of a MEK inhibitor provides more prolonged and effective pathway inhibition than WZ4002 alone (Fig. 1C) and hence may lead to a delay in the onset of resistance. Genetic alterations leading to ERK reactivation have also been identified in preclinical models of single-agent EGFR TKI resistance (13). In addition, reactivation of ERK1/2 signaling has also been observed in preclinical models of drug resistance to AZD9291, suggesting that this observation is not WZ4002 specific (23). A second mechanism contributing to the enhanced efficacy of W+T that may be a result of enhanced pathway inhibition is the increase in apoptosis observed both in vitro (Fig. 3C) and in vivo (Fig. 5C and D). It is possible that in the presence of this increased apoptotic effect, it is more difficult for a resistant clone to emerge, especially when cell proliferation may be compromised because of MEK inhibition. In accordance with this hypothesis, many of the observations demonstrating the enhanced efficacy of the W+T combination are evident only in long-term in vitro and in vivo studies. The relative contributions of these two mechanisms, more effective pathway inhibition and enhanced apoptosis, to the improved in vitro and in vivo efficacy observed with the WZ4002 and trametinib combination remain to be determined.

Figure 6. Heterogeneous resistance mechanisms develop in response to W+T combination treatment in the EGFR<sup>L858R/T790M</sup> genetically engineered mice. A, phosphorylation (p) of EGFR, ERK1/2, S6, and 4EBP1 was assessed by immunohistochemistry, and instances of positive nodules adjacent to negative nodules (highlighted with dotted lines) were observed. B, total number of tumor nodules IHC positive (red) or negative (black) for EGFR, ERK, S6, and 4EBP1 phosphorylation. Percentage of positive cases is listed above each bar (n = 27). C, tumor volume of W+T-resistant tumors after treatment with a combination of WZ4002, trametinib, and Torin2. Yellow T indicates tumor mass. D, quantification of tumor volume for each mouse after initiation of W+T plus Torin2 combination treatment. E, PC9-derived cell lines tolerant to W+T (WT1, 3, and 4) were treated with 100 nmol/L WZ4002 + 30 nmol/L trametinib, and phosphorylation of EGFR, AKT, ERK1/2, and S6 was assessed. Image is representative of two independent experiments. F, viability of W+T-tolerant cell lines was assessed after the addition of 10 nmol/L Torin2 to W+T cotreatment at 72 hours (n = 3 independent experiments, mean ± SD).
The success of W+T in preventing and treating drug resistance suggests that this combination may be of use clinically and more effective than single-agent therapy with mutant-selective inhibitors, such as AZD9291 or CO-1686.

Our study provides guidance as to where this combination should be evaluated, specifically either as initial therapy in EGFR TKI-naïve patients or in those who have developed EGFR T790M-mediated drug resistance. However, clinical trials will be required to confirm superior efficacy and tolerability in lung cancer patients. An ongoing clinical trial is evaluating the combination of AZD9291 and the MEK inhibitor selumetinib (NCT02143466), and findings from this study will further inform the best strategy to prevent or treat drug resistance in EGFR-mutant lung cancer patients. These studies will provide a starting point toward improving EGFR TKI combination strategies.

METHODS

Cell Lines and Reagents

The NSCLC cell lines PC9, HCC827, HCC4006, HCC2935, H1975 (subclone 2), DFCIC81, H1650, and drug-resistant counterparts were grown in RPMI-1640 (Gibco), 10% FBS, and 1% penicillin/streptomycin (Gibco). The PC9 cells were obtained from Dr. Nishio (Kinki University, Osaka, Japan) in 2005. The HCC827 cells were obtained from Dr. Adi Gazdar (UT Southwestern, Dallas, TX) in 2004. The HCC827 EPR cell line was generated by the laboratory of Tetsuya Mitsudomi (Kinki University, Osaka, Japan) in 2011, and the DFCIC81 cell line was generated in the Jänne laboratory in 2011. HCC827 + HGF cells were created between 2006 and 2013 by transduction of JPI698-HGF into HCC827 cells, followed by 5 days of 25 μg/mL bacitracin selection. PC9 W–T–tolerant cell lines were created between 2006 and 2014 by plating 350 cells per well in 96-well format and treating with 100 nmol/L WZ4002 + 30 nmol/L trametinib weekly. Cell line identity was confirmed by fingerprinting (Promega Powerplex 16 System) for the following cell lines: HCC4006, PC9, PC9 PFR3, PC9 GR4, PC9 DR1, PC9 WZR12, PC9 WTR4, HCC827, HCC827 EPR, HCC827 GR6, H1975 subclone 2, HCC2935, HCC2279, and H1650. Fingerprinting was performed in September 2014 at the Genomic Core Laboratory, Michigan State University.

Generation of Mouse Cohorts and Treatment

All care and treatment of experimental animals was in accordance with Harvard Medical School/Dana-Farber Cancer Institute (DFCI) Institutional Animal Care and Use Committee guidelines. All mice were housed in a pathogen-free environment at the DFCI animal facility and handled in strict accordance with Good Animal Practice as defined by the Office of Laboratory Animal Welfare.

Mice were purchased from Charles River Laboratories International Inc. (Nu/Nu mice) for the following cell lines: HCC4006, PC9, PC9 PFR3, PC9 GR4, PC9 DR1, PC9 WZR12, PC9 WTR4, HCC827, HCC827 EPR, HCC827 GR6, H1975 subclone 2, HCC2935, HCC2279, and H1650. Fingerprinting was performed in September 2014 at the Genomic Core Laboratory, Michigan State University. WZ4002 was dissolved in 10% NMP (10% 1-methyl-2-pyrrolidinone: 90% PEG-300), and trametinib was dissolved in 0.5% hydroxypropyl methylcellulose with 0.2% Tween 80. WZ4002 was dosed as 50 mg/kg daily orally, trametinib was given as 2.5 mg/kg for 5 consecutive days followed by three times weekly orally; the combination dosing schedule and dosage were the same as the single reagents; vehicle control mice were given 10% NMP daily. Torin2 was dissolved in captisol (1:80) followed by dilution in water to 4 mg/mL and was administered at 40 mg/kg daily. Mice treated with Torin2 plus W+T were initially treated with WZ4002, followed by W+T until the development of resistance, and then Torin2 plus W+T.

Doxycycline-inducible EGFRIGFR(III)790M:copA741TL (TIL) transgenic mice were generated as previously described (17, 39). Mice were fed a doxycycline diet at 6 weeks of age to induce expression of mutant EGFR. Mice were evaluated by MRI after 12 to 16 weeks of doxycycline diet to quantify lung tumor burden before being assigned to various treatment study cohorts. All the treatment mice had an equal amount of initial tumor burden. MRI evaluation was repeated every 2 weeks during the treatment.

Histology and Immunohistochemistry

Mice were sacrificed with CO2, and half-dissected tumors were snap-frozen in liquid nitrogen for preparation of protein lysates or fixed in 10% neutral-buffered formalin overnight at room temperature, transferred to 70% ethanol, embedded in paraffin, and sectioned at 5 μm for IHC staining. Hematoxylin and eosin (H&E) stains were performed according to the antibody manufacturers’ recommendations. The following antibodies were purchased from Cell Signaling: anti-phospho-AKT (Ser473, 4060; 1:2,000), total AKT (9272; 1:3,000), phospho-EGFR (Tyr 1068; 3777; 1:1,000), total EGFR (2232; 1:1,000), phospho-ERK1/2 (Thr 202/204; 9101; 1:3,000), total ERK1/2 (9102; 1:3,000), pS6 (Ser240/244; 5364; 1:2,000), S6 (2217; 1:3,000), and BIM (2933; 1:2,000). HSP90 was obtained from Santa Cruz Biotechnology (sc-7947; 1:3,000).

Caspase 3 Activity

Cells were plated 0.6 × 106 per 10-cm plate and treated with the indicated drugs the following day. Cells were washed with PBS and resuspended in lysin buffer (CaspASE colorimetric assay; Promega). Nonadherent cells were collected from the media by centrifugation, washed in PBS, and added to the lysate. Complete lysis was carried out by rapid freeze–thaw cycles. The assay was prepared as suggested by the manufacturer protocol using 30 μg of lysate per well and read at 405 nm after an overnight incubation at 37°C.

Cell Growth and Proliferation Assays

MTS assays were performed using previously described methods (38). To measure the emergence of acquired resistance, cell lines were plated in triplicate 96-well plates (350 cells/well), with the exception of DFCIC81 (700 cells/well). Cells were treated with the indicated drugs the following day, and then treatments were exchanged every 7 to 9 days thereafter. Positive wells were scored as greater than 50% confluent and assessed weekly.

Antibodies and Western Blotting

Cells were plated at 0.6 × 106 cells per well in 10-cm plates for assessment of ERK1/2 reactivation (Fig. 1A). Cells were washed twice with ice-cold PBS and lysed in RIPA T-X100 buffer (Boston Bioproducts). To assess ERK1/2 reactivation (Fig. 1B), 0.2 × 106 cells were plated in 6-well plates and treated with the indicated concentration of drugs. Cells were washed once with ice-cold PBS and lysed in RIPA T-X100. To assess BIM levels, 0.6 × 106 cells were plated in 10-cm plates, treated with the indicated drugs for 24 hours, and lysed with CaspACE 3 lysin buffer (Promega). Immunoblotting was performed according to the antibody manufacturers’ recommendations. The following antibodies were purchased from Cell Signaling: anti-phospho-EGFR (Tyr 1068; 3777; 1:1,000), total EGFR (2232; 1:1,000), phospho-ERK1/2 (Thr 202/204; 9101; 1:3,000), total ERK1/2 (9102; 1:3,000), pS6 (Ser240/244; 5364; 1:2,000), S6 (2217; 1:3,000), and BIM (2933; 1:2,000). HSP90 was obtained from Santa Cruz Biotechnology (sc-7947; 1:3,000).

Histology and Immunohistochemistry

Mice were sacrificed with CO2, and half-dissected tumors were snap-frozen in liquid nitrogen for preparation of protein lysates or fixed in 10% neutral-buffered formalin overnight at room temperature, transferred to 70% ethanol, embedded in paraffin, and sectioned at 5 μm for IHC staining. Hematoxylin and eosin (H&E) stains were performed according to the antibody manufacturers’ recommendations. The following antibodies were purchased from Cell Signaling: anti-phospho-AKT (Ser473, 4060; 1:2,000), total AKT (9272; 1:3,000), phospho-EGFR (Tyr 1068; 3777; 1:1,000), total EGFR (2232; 1:1,000), phospho-ERK1/2 (Thr 202/204; 9101; 1:3,000), total ERK1/2 (9102; 1:3,000), pS6 (Ser240/244; 5364; 1:2,000), S6 (2217; 1:3,000), and BIM (2933; 1:2,000). HSP90 was obtained from Santa Cruz Biotechnology (sc-7947; 1:3,000).
performed in the Department of Pathology in Brigham and Women’s Hospital; IHC for phospho-EGFR, AKT, ERK1/2, S6, and 4EBP1 was done using previously described methods (17).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software. MTS assays are represented as the mean three independent experiments ± SD. In vivo experiments are represented as the mean ± SEM. Significance of caspase 3 activity and TUNEL staining was assessed by ordinary one-way ANOVA. Significance of caspase 3 induction was assessed by the Fisher exact test. Survival analysis of W24002 versus W1T–treated EGFR LT mice was assessed by the log-rank test.

Disclosure of Potential Conflicts of Interest

N. Rosen reports receiving commercial research grants from Chugai and Wellspring and is a consultant/advisory board member for AstraZeneca, Chugai, Eli Lilly, and Takeda-Millennium. N.S. Gray has ownership interest (including patents) in Gatekeeper Pharmaceuticals; is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceuticals, Clovis Oncology, Genentech, Merrimack, Pfizer, and Sanofi, and has provided expert testimony for Lab Corp. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Tetsuya Mitsudomi for providing the HCC827 EPR cell line and Magda Bahcall for helpful discussions and manuscript preparation.

Grant Support

This study was supported by grants from the NIH RO1CA114465 (to P.A. Jänne), RO1CA135257 (to P.A. Jänne), and PO1CA154303 (to P.A. Jänne, N.S. Gray, and K.-K. Wong).

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Received January 13, 2015; revised May 26, 2015; accepted May 29, 2015; published OnlineFirst June 2, 2015.

REFERENCES

8. Tricker et al.


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