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Overcoming Resistance to AC0010, a Third Generation of EGFR Inhibitor, by Targeting c-MET and BCL-2

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Abstract

AC0010 is a pyrrolopyrimidine-based irreversible inhibitor of epidermal growth factor receptor (EGFR), structurally distinct from previously reported pyrimidine-based irreversible EGFR inhibitors such as osimertinib and rociletinib. AC0010 selectively inhibits EGFR T790M mutation in both preclinical and clinical studies. However, AC0010 treatment eventually triggers drug resistance with unknown mechanism. To this end, we established two H1975 NSCLC-derived lines resistant to AC0010 after a series of drug exposure and selection in either nude-mice xenograft tumor (H1975-P) or cell culture (H1975-AVR) settings. Both lines obtained 100-fold resistance to AC0010 as compared to the parental lines. To elucidate underlying mechanism, we performed unbiased RNAseq-based profiling analysis and found that H1975-P cells had c-MET overexpression, whereas H1975-AVR cells had BCL-2 overexpression. AC0010 resistance was partially abrogated by targeting c-MET or BCL-2 using either pharmacological (small molecule inhibitors) and/or genetic (siRNA-based knockdown) approach, respectively. Our study shows that drug resistance to AC0010 can be developed *via* the different mechanism in a cell context–dependent manner and provides the proof-of-concept evidence for rational drug combinations to overcome resistance for maximal therapeutic efficacy.

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Introduction

Activation of epidermal growth factor receptor (EGFR) signaling, conferred by gene mutations or amplification, is tightly associated with the initiation, progression, and poor prognosis of non–small cell lung cancer (NSCLC) [1–3]. This discovery has led to the development of EGFR tyrosine kinase inhibitors (TKIs) as effective targeted therapies for patients with advanced disease [4]. Gefitinib and erlotinib are two first-generation EGFR TKIs, which showed impressive clinical efficacy for NSCLC patients with activating EGFR mutations [5–7]. Although the majority of patients with EGFR mutations benefited initially from these drugs, all patients ultimately had disease reoccurrence due to acquired drug resistance, most commonly *via* obtaining the T790M resistant mutation, as observed in 50% to 60% of resistant biopsies [8–10]. To overcome T790M-mediated resistance, second- and third-generation of EGFR TKIs have been developed [11–14]. However, second-generation of EGFR TKIs, when used as monotherapy, had demonstrated limited clinical benefits due to their poor therapeutic window derived from nonselectivity against both wild-type EGFR and EGFR T790M [15]. In contrast, the third generation of mutant-specific EGFR TKIs, which preferentially blocks both activating EGFR mutations and T790M, overcomes the selectivity issues and shows promising clinical outcomes [16]. For example, irreversible pyrimidine EGFR inhibitor osimertinib has demonstrated tumor responses in >60% of EGFR-

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mutant patients with T790M-mediated resistance [17,18]. AC0010, a novel pyrrolopyrimidine-based third-generation of EGFR TKI, has also demonstrated promising antitumor activity and a favorable safety profile in phase I/II clinical trial [19].

Similar to the earlier generation of EGFR inhibitors, drug resistance also developed for third-generation of EGFR TKIs [20]. For example, resistance to osimertinib arises after 9-13 months of therapy, by which acquired mutation C797S was detected in 40% of NSCLC patients [21]. Subsequently, resistance to the third generation of inhibitors, including osimertinib, CO-1686, and HM61713, was further investigated in both preclinical and clinical studies [22,23]. Mechanistically, in addition to the occurrence of acquired mutations in EGFR such as C797S and L718Q [24,25], other abnormalities in tumor cells were also identified that may contribute to resistance. The examples include a) amplification of cell surface receptors HER-2, MET [26], SRC [27], or ERBB2 [28]; b) constitutive activation of transducers downstream to EGFR [29]; c) activation of SFK/FAK and AKT [30,31]; and d) perturbation of the apoptotic machinery or phenotypic transformation [32]. Extensive studies to understand the mechanisms of acquired resistance against the third generation of EGFR TKIs with different chemical structures will certainly lead to better combinational strategies to benefit patients from EGFR-TKI–based targeted therapy. AC0010 is an oral and irreversible EGFR TKI with high selectivity against patients harboring EGFR-sensitive mutation and T790M-resistant mutation [19]. Compared with previous EGFR TKIs, AC0010 exhibited remarkably higher activity against EGFR with T790M than wild-type EGFR. Clinical studies indicated that AC0010 (over 350 mg/day) was highly effective in NSCLC patients with EGFR T790M mutation who experienced disease reoccurrence during prior therapies with gefitinib or erlotinib. The overall response rate of patients with EGFR T790M-positive mutation was about 50% at daily dose ≥350 mg, and Maximum Tolerated Dose (MTD) was not reached with daily dose up to 600 mg [33]. Thanks to the effectiveness of AC0010 in treatment of NSCLC patients with EGFR T790M mutation, AC0010 is currently in Phase II/III registration trial for those patients [34,35]. However, resistance to AC0010 was also found in clinical treatment, which can potentially restrict its therapeutic efficacy. It is important, therefore, to elucidate possible mechanism(s) for acquired resistance to AC0010 in advance, thus providing a rational strategy for the development of new combinational therapy for AC0010-resistant patients.

Here we reported the establishment of two H1975 NSCLC-derived lines resistant to AC0010 after a series of drug exposure in either cell culture (H1975-AVR) or nude-mice xenograft tumor setting (H1975-P). Both lines obtained 100-fold resistance to AC0010 as compared to the parental lines. Mechanistic investigation *via* RNAseq-based profiling study revealed that H1975-AVR cells had BCL-2 overexpression, whereas H1975-P cells had c-MET overexpression. Importantly, AC0010 resistance can be partially overcome by targeting either BCL-2 or c-MET *via* either pharmacological (small molecule inhibitors) or genetic (siRNA knockdown) approach, respectively. Our study, therefore, provides the proof-of-concept evidence for rational drug combinations to overcome AC0010 resistance for maximal therapeutic efficacy.

Materials and Methods

Chemicals

Gefitinib was purchased from Sciencechem (Jinan), afatinib was purchased from Langchem (Shanghai), crizotinib was purchased from PharmaBlock (Nanjing), and ABT-263 was purchased from Lollane (Shanghai). All other experimental compounds, including AC0010, Co-1686, and AZD9291, were synthesized in-house by ACEA Pharmaceutical Research, Hangzhou, Zhejiang, China. Stock solutions of all drugs were prepared in DMSO and stored at −80°C.

Cell Culture

Human NSCLC adenocarcinoma cell line NCI-H1975 was obtained from the American Type Culture Collection and maintained at 37°C with 5% CO_2 in the RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were routinely tested and verified to be free of mycoplasma contamination.

Resistant Cell Lines

AC0010-resistant H1975-P1 cells were derived *via in vivo* selection of NCI-H1975 cells injected into nude mice that developed resistance to AC0010. AC0010-resistant H1975-AVR1 cells were established *in vitro* by culturing parental H1975 cells in escalating doses of AC0010 (20 nM to 5 μM) for a prolonged period. For the development of drug resistance, culture media were replaced with fresh drug twice per week. DNA was extracted from H1975-P1 and H1975-AVR1 cells using the DNeasy Kit (Qiagen), and specific EGFR mutations were assessed using the therascreen EGFR RGQ PCR Kit (Qiagen).

To isolate single-cell clone, H1975-P1 or H1975-AVR1 cells were seeded into 96-well plates at a density of 0.5 cell/well. Wells containing only a single cell were expanded to form clones. DNA was extracted from each clone using the DNeasy Kit (Qiagen), and Sanger sequencing was performed on PCR products of exons 19, 20, and 21 of EGFR at the Sequencing Group of the Tsingke.

Cell Proliferation Assays

Cell proliferation was assayed by a cell viability reagent, WST-1, according to the manufacturer (Roche). Cells were seeded at 3000 cells per well onto 96-well plates and incubated for 24 hours, followed by compound treatment for 72 hours. Cell viability was then assayed by incubating cells with WST-1 reagent for 2-3 hours. Absorbance was measured at OD450/620 using the Beckman DTX880. The IC50 were determined using a nonlinear regression model with a sigmoidal dose response.

Colony-Forming Assay

H1975-P1-R1 and H1975-AVR1-R2 cells were cultured in RPMI culture medium. H1975-P1-R1 cells were seeded in 6-well plates at a density of 250 cells per well and then treated with AC0010 (2.0 μM), crizotinib (0.5 μM), or the combination. H1975-AVR1-R2 cells were seeded at a density of1000 cells per well in 6-well plates and then treated with AC0010 (1.8 μM), ABT-263 (0.065 μM), or the combination. The medium was changed every 3 days for a treatment period of 14 days to allow colony formation. The colonies were fixed and stained with Diff-Quick staining kit (SIEMENS).

RNA-seq Analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). A total of 5 μg of RNA per sample was used for RT reaction. Sequencing libraries were generated using NEBNextUltra RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina). After cluster generation, the

library preparations were sequenced on an Illumina Hiseq 2500 platform, and paired-end reads were generated. The expression level of each gene was measured as numbers of reads per kilobase of exon region in a gene per million mapped reads (RPKM). DESeq R package (1.18.0) was used to analyze differential expression of two conditions/groups. The *P* value was adjusted using *q* value [36]. FDR <0.05 and |log2Ratio| ≥ 1 found by DESeq were set as the threshold for significantly differential expression. The top Gene Ontology software was used to analyze differentially expressed genes (DEGs). Gene set enrichment analysis for KEGG pathways was performed to elucidate affected pathways.

qRT-PCR

The total RNA was isolated from H1975, H1975-P1-R1, and H1975-AVR1-R2 cells with RNeasy Mini kit (Qiagen), and cDNA was obtained by reverse transcription using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). The expression of c-MET, BCL-2, and CDH11 was measured using the TaqMan Gene Expression Assay (Hs01565584_m1, Hs00608023_m1, and Hs00901479_m1, Applied Biosystems). The qRT-PCR was performed in triplicate with TaqMan One-Step RT-PCR Master Mix (Applied Biosystems). Thermal cycler conditions are as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 95°C for 15 seconds followed by 60°C for 1 minute for 40 cycles. Control gene primer and probe pairs were purchased from Applied Biosystems (GAPDH, Hs02786624_g1). Relative expression of specific transcripts was determined by the following calculation: relative expression = 2^{-Ct}, where $Ct = (Ct_{target} - Ct_{control})_{drug\text{-}resistant\ cell} - (Ct_{target} - Ct_{control})_{vehicle\ cell}$.

siRNA and Transfection

The siRNA oligonucleotides targeting c-MET (ON-TARGET plus SMART pool) and negative control siRNA (ON-TARGET plus nontargeting pool) were purchased from Dharmacon. The cells were transfected with siRNA at a final concentration of 50 nM using Effectene (Qiagen, Carlsbad, CA) according to the manufacturer's instructions. The cells were harvested 48 hours posttransfection for protein extraction, followed by Western blotting or 72 hours for cell viability analysis.

Immunoblotting Analysis

Cells were seeded onto 6-well plates at the density of 1×10^6 cells per well. After 24 hours of culture in serum-containing media, cells were treated with test compound for 2 hours. Immunoblotting analysis was performed using the whole-cell extracts, and the blots were probed with following antibodies: phosphospecific EGFR (p-Y1068), total EGFR, phospho-AKT (Ser-473), total AKT, phospho-MET (Tyr1234/1235), phospho-ERK1/2 (p-T202/p-Y204), total ERK1/2 and BCL-2,BCL-XL,MCL-1,BIM (Cell Signaling Technology), and total c-MET and β-tubulin (Santa Cruz).

To perform immunoblotting analysis of xenograft tumors, the tumor tissues were diced into very small pieces using a clean razor blade and then transferred to microcentrifuge tubes containing RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. The tissues were homogenized, and the homogenized solutions were incubated on ice for 30 minutes followed by two rounds of centrifugation at 14,000 rpm for 30 minutes at 4 C. The supernatants were collected for immunoblotting analysis to probe p-EGFR, total EGFR, p-Akt, total Akt, p-Met, total Met, p-ERK1/2, and total ERK1/2. β-Tubulin was used as the loading control.

Flow Analysis

Apoptosis was determined by flow cytometry using a PE rabbit anti-active caspase-3 antibody (BD Biosciences). Briefly, cells were seeded at 1.8×10^6 cells per well onto 6-well plates and incubated for 24 hours, followed by compound treatment for 24 hours. After treatment, cells were collected by trypsinization, washed once with phosphate-buffered saline, then fixed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences) for 20 minutes at room temperature. Cells were washed with BD Perm/Wash buffer and subsequently stained with the PE rabbit anti-active caspase-3 antibody. Finally, cells were washed and resuspended in BD Perm/Wash buffer before analyzing by NovoCyte Flow cytometer (ACEA Biosciences).

Xenograft Models

All studies involving animals handing, care, and treatment reported here were conducted in Hangzhou ACEA Pharmaceutical Research Co., Ltd., and performed according to the guidelines and SOPs approved by Department of Science and Technology of Zhejiang Province, China.

The Nu/Nu nude mice were purchased from Beijing Vital River Laboratories of China. Six- to 8-week-old female mice were inoculated subcutaneously at the right flank with approximately 2.5×10^6 NCI-H1975-P1-R1 cells in 0.2 ml of medium for tumor development. The treatments were started when the tumor size reached approximately 200 mm^3. Animals were randomly divided into four groups (eight animals per group) including vehicle control [0.5% methylcellulose], crizotinib (12.5 mg/kg), AC0010 (100 mg/kg), or AC0010 plus crizotinib (12.5 mg/kg). The drugs were administrated orally once daily for continuous 14 days.

During the experimental period, mouse body weight and tumor volume were measured twice weekly. Tumor volumes were measured in two dimensions using a caliper, and the volume was expressed in mm^3 using the formula: $V = 0.5\ a \times b^2$, where *a* and *b* are the long and short diameters of the tumor, respectively. The tumor volume was then used for the calculation of tumor inhibitory rate (IR), which was an indication of antitumor effectiveness calculated by following equation: IR = (1 − relative mean tumor volume of treatment groups/relative mean tumor volume of control group) × 100%. Here relative mean tumor volume = mean tumor volume at the end of the experiment/mean tumor volume before dosing. SPSS 17.0 software (*t* test) was used for statistical analysis.

Results

Generation and Characterization of AC0010-Resistant Cells

To establish the resistance to third generation of EGFR inhibitor AC0010 for mechanistic study, we used H1975 NSCLC cells (harboring T790M/L858R double mutations) and generated two AC0010-resistant lines with two different approaches. The H1975-P1 cells were derived from *in vivo* passing of xenograft tumors in mice receiving AC0010 at 50 mg/kg for 17 days and continuous dosing at 500 mg/kg for another 98 days. The H1975-AVR1 cells were obtained by a series of *in vitro* culture exposure to AC0010 by dose escalation from 20 nM to 5 mM (Figure 1*A*). Cell proliferation assays showed that the IC50s against AC0010 were 2.8 μM and 1.6 μM, respectively, for H1975-P1 and H1975-AVR1 cells as compared to 14 nM for parental H1975 cells, indicating the successful establishment of AC0010-resistant lines (Figure 1*B*). The sensitivity of

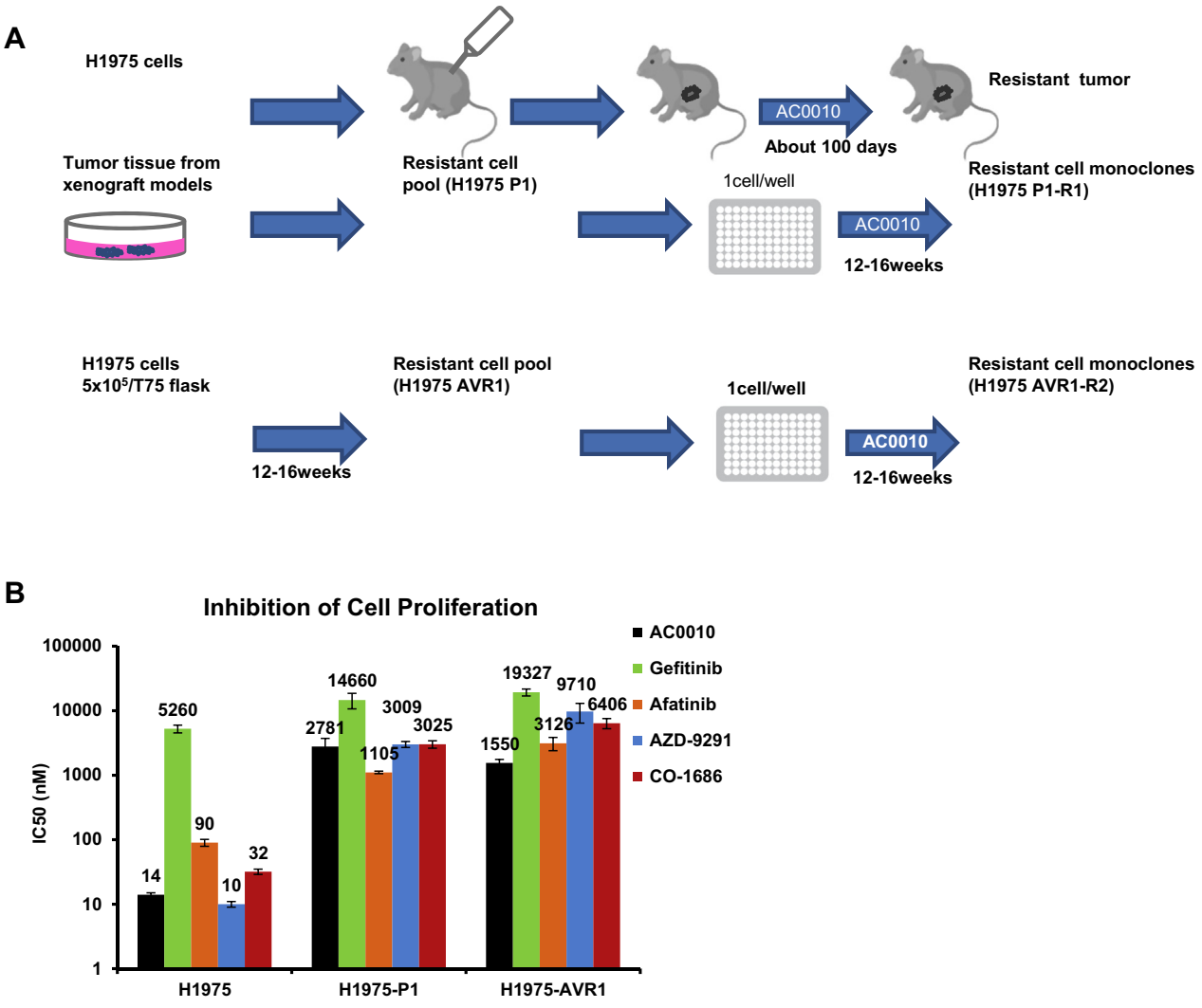


Figure 1. Establishment of lung cancer cell lines resistant to AC0010.(A) Schematic representation of generation of H1975-P1 and H1975-AVR1 cells resistant to EGFR inhibitor AC0010. (B) Sensitivity of H1975, H1975-P1, and H1975-AVR1 cells to various EGFR inhibitors. Two lines of resistant cells, along with parental H1975 cells, were treated with various concentrations of indicated EGFR inhibitors for 72 hours followed by MTT-based growth assay. Growth inhibition curves were generated and plotted. Shown are calculated IC_{50} values. The data are represented as mean ± SD of three independent experiments.

AC0010 resistant cells to the clinically relevant EGFR-TKIs gefitinib, afatinib, osimertinib, as well as CO-1686 was also examined. As expected, unlike parental H1975 cells which are only resistant to the first-generation EGFR TKI gefitinib, both H1975-P1 and H1975-AVR1 cells were resistant to all three generations of EGFR TKIs, including gefitinib (first); afatinib (second); and AC0010, osimertinib, and CO-1686 (third) (Figure 1*B*). Supplemental Figure 1 listed the chemical structures of several third-generation EGFR TKIs, including abivertinib (AC0010), osimertinib (AZD9291), rociletinib (CO-1686), olmutinib (HM61713), nazartinib (EGF816), lazertinib (YH25448), and naquotinib (ASP8273).

Sequencing analysis of EGFR exons 19-21 in H1975-P1-R1 or H1975-AVR1-R1 cell line, derived respectively from the single clone of H1975-P1 and H1975-AVR1 (Figure 1*A*), revealed that the T790M and L858R mutations found in parental H1975 cells were retained and no additional mutations were detected (Table S1), excluding the possible contribution of EGFR mutations within this region to drug resistance.

RNAseq Analysis Revealed Distinct Mechanisms Underlying the AC0010 Resistance

To understand the mechanism of resistance, we employed unbiased genome-wide RNAseq analysis of H1975-P1 and H1975AVR1 cells along with parental sensitive NCI-H1975 cells in an attempt to identify DEGs in the resistant cells. The genes with a two-fold change in RPKM and a false discovery rate–adjusted *P* value .05 in a pairwise analysis were considered as DEGs. Gene set enrichment analysis for KEGG pathways revealed top 20 statistics of pathway enrichment for H1975-P1 and H1975-AVR1, respectively (Supplemental Figures 2 and 3). Gene ontology–based functional categories of DEGs revealed that c-MET and MAPK signaling pathways showed high correlations with resistance in H1975-P1 cells, whereas BCL-2 and NF-κB pathways were correlated with that of H1975-AVR1 cells among many others (Figure 2, *A* and *B*, and Tables S2). A total of 109 or 146 genes were upregulated (Table S3), and 359 or 210 genes were downregulated by more than eight-fold in H1975-P1 and H1975-AVR1 cells, respectively. Large number of

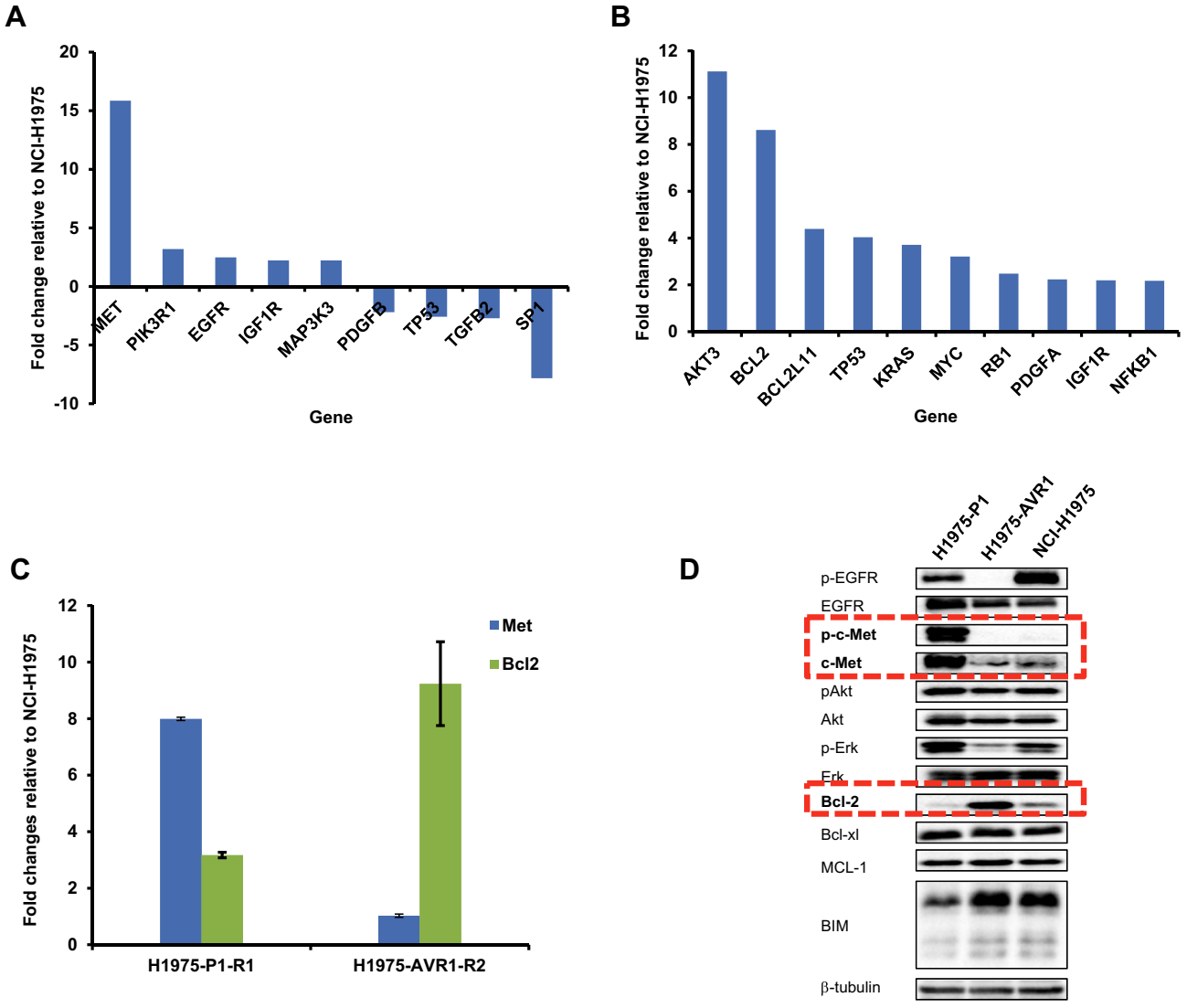


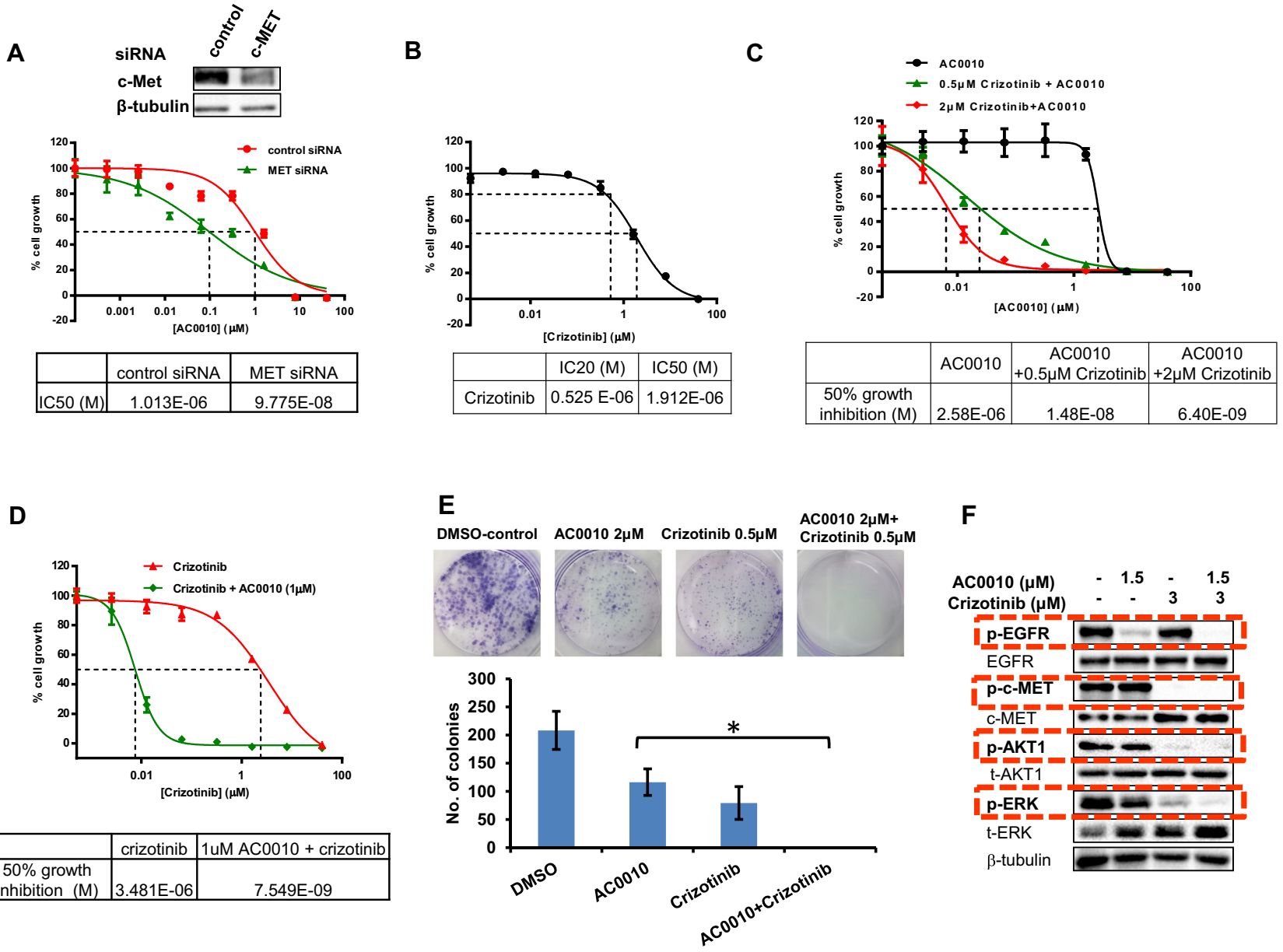
Figure 2. Elucidation of mechanisms of AC0010 resistance by RNAseq profiling.Total RNA was isolated and subjected to RNAseq profiling as detailed in the Materials and Methods section. Shown are genes in p53 and TGFβ pathways enriched in H1975-P1 cells (A), and apoptosis and NFκB pathways in H1975-AVR1 cells (B). Real-time PCR analysis to confirm increased expression of c-MET and BCL-2 in H1975-P1-R1 and H1975-AVR1-R2 cells, respectively (C). Western blot analysis of the levels of c-MET and BCL-2 in H1975-P1-R1 and H1975-AVR1-R2 cells as well as the phosphorylation status of c-MET, EGFR, and their downstream molecules (AKT1 and ERK) (D).

alterations in gene expression strongly suggests involvement of multiple signal pathways in conferring AC0010 resistance,

We next focused genes with following three criteria: a) highly differentially expressed, 2) known to be involved in drug resistance, and 2) have small molecule inhibitors currently in preclinical or clinical development. The c-MET and BCL-2 genes were chosen which had a 16-fold or 8.6-fold upregulation in H1975-P1 and H1975-AVR1 cells, respectively (Figure 2, *A* and *B*). We first used qRT-PCR to confirm their overexpression (Figure 2*C*). Western blotting analysis was also used to examine the levels of c-Met and BCL-2 in AC0010-resistant cells as well as the phosphorylation status of c-Met, EGFR, and their downstream molecules (AKT1 and ERK1/2) (Figure 2*D*). Development of AC0010 resistance led to a dramatic decrease of phosphorylated EGFR in both H1975-P1 and H1975-AVR1 cells as compared to NCI-H1975 cells (Figure 2*D*). Consistent with the c-MET overexpression at mRNA level, the c-Met protein level also significantly increased in H1975-P1 cells. The concurrent increase of the phosphorylated c-MET as well as downstream ERK phosphorylation further suggests that the activated c-MET pathway is likely associated with acquired AC0010 resistance of H1975-P1 cells. Likewise, a high level of BCL-2 protein was detected in H1975-AVR1 cells (Figure 2*D*). Finally, we determined the levels of a few additional BCL-2 family members and found that the levels of BCL-XL and MCL-1 maintained the same, whereas BIM level was higher in two resistant lines, likely due to the cellular compensatory response. Together, these data suggest the distinct mechanism underlying the AC0010 resistance acquired *via in vivo* and *in vitro* approaches, respectively.

Combination of AC0010 with Crizotinib Overcomes AC0010 Resistance in H1975 P1-R1 Cells

To investigate whether elevated c-MET is causally related to AC0010 resistance, we used two loss-of-function approaches to inactivate c-MET for AC0010 resensitization. Transfection of a



	control siRNA	MET siRNA
IC50 (M)	1.013E-06	9.775E-08

	IC20 (M)	IC50 (M)
Crizotinib	0.525 E-06	1.912E-06

	AC0010	AC0010 +0.5μM Crizotinib	AC0010 +2μM Crizotinib
50% growth inhibition (M)	2.58E-06	1.48E-08	6.40E-09

	crizotinib	1uM AC0010 + crizotinib
50% growth inhibition (M)	3.481E-06	7.549E-09

Figure 3. Overcoming AC0010 resistance by targeting c-MET in cell culture model.(A) H1975-P1-R1 cells were transfected with siRNA targeting c-MET, along with the control siRNA, and the sensitivity of transfected cells to AC0010 was determined by WST-1 assay. Western blot analysis was performed to show c-MET knockdown. (B) Growth curve of H1975-P1-R1 cells treated with various concentrations of crizotinib. (C) Growth curve of H1975-P1-R1 cells treated with various concentrations of AC0010 in combination with 2 μM crizotinib or 0.5 μM crizotinib (n = 3). (D) Growth curve of H1975-P1-R1 cells treated with 1 μM AC0010 in combination with various concentrations of crizotinib (n = 3). (E) Clonogenic survival assays of H1975-P1-R1 cells treated with AC0010, criztonib, or the combination with indicated concentrations (n = 3). (F) Western blot analysis of the phosphorylation status of c-MET, EGFR, and their downstream molecules (AKT1 and ERK) in H1975-P1-R1 cells treated with indicated drugs and concentrations.

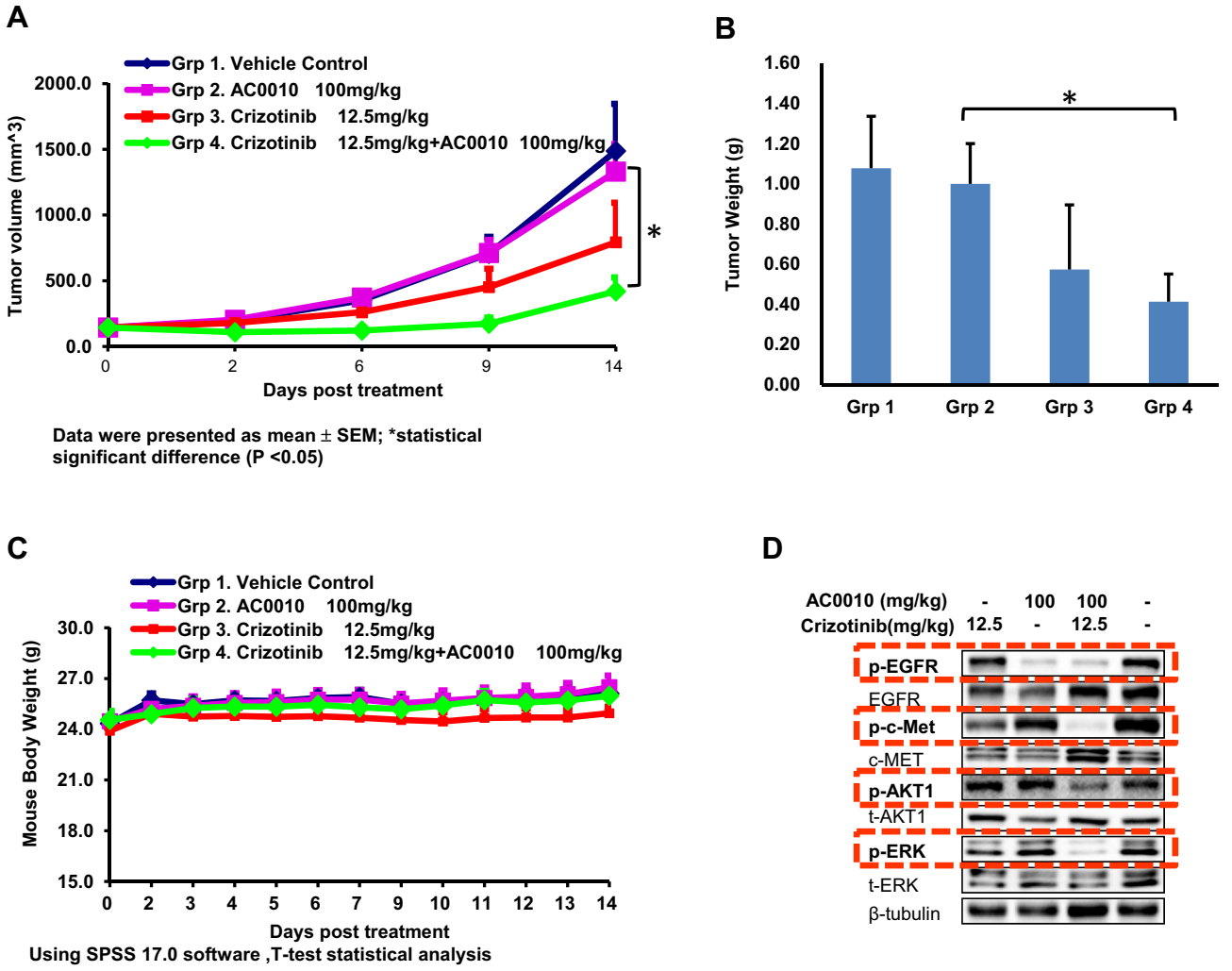
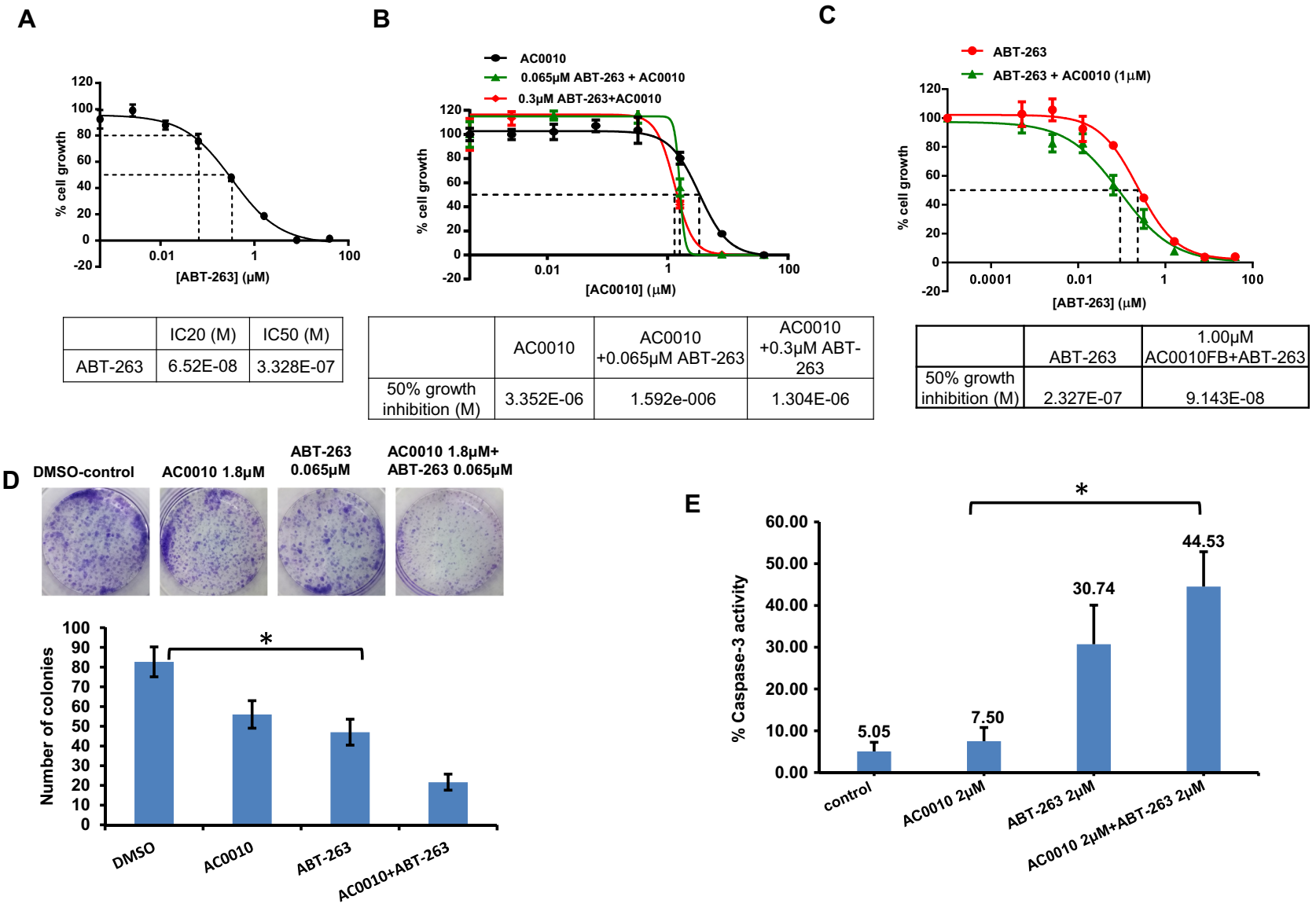


Figure 4. Overcoming AC0010 resistance by targeting c-MET in *in vivo* xenograft model. (A) Inhibition of H1975-P1-R1 tumor growth by AC0010 or crizotinib, alone or in combination, for a period of 14 days. Tumor growth curves are plotted as mean ± SEM (n = 8). (B) Tumor weights of each group were analyzed using SPSS 17.0 software; t test statistical analysis. (C) Mouse body weights of each group are plotted as mean ± SEM (n = 8). (D) H1975-P1-R1 tumor issues from mice treated with vehicle, AC0010, crizotinib, or AC0010 combined with crizotinib for 14 days were isolated and subjected to Western blotting analysis using indicated Abs.

siRNA oligonucleotide targeting c-MET into H1975-P1-R1 cells, a cell line derived from the single clone of H1975-P1 xenograft (Figure 1*A*), caused up to 60%-70% knockdown of c-MET level and resulted in a 10-fold increase in AC0010 sensitivity (IC50 reduced from 1013 nM to 98 nM) (Figure 3*A*). Likewise, crizotinib, a specific inhibitor of c-MET, inhibited H1975-P1-R1 cell proliferation in a dose-dependent manner (Figure 3*B*). We used the concentration of crizotinib that resulted in 20% (0.5 μM, IC_{20}) or 50% (2.0 μM, IC50) inhibition of cell proliferation for AC0010 sensitization. Indeed, while H1975-P1-R1 cells are very resistant to AC0010, combination with crizotinib at either concentration significantly sensitized cells to AC0010 (Figure 3*C*). Reciprocally, we fixed AC0010 concentration at 1.0 μM, measured cellular sensitivity to crizotinib, and found a significant sensitization with IC_{50} reduction from 3.4 μM to 0.0075 μM (Figure 3*D*). Finally, we measured overcome of drug resistance using clonogenic assay. The colony formation of H1975 P1-R1 cells was inhibited moderately with the treatment of AC0010 (2.0 μM) or crizotinib (0.5 μM), and their combination completely inhibited the colony formation (Figure 3*E*). Biochemically, while AC0010 alone (1.5 μM) had minimal, if any, effects on the phosphorylation of EGFR, c-MET, AKT1, and ERK, combination with crizotinib fully inhibited the phosphorylation of EGFR and c-MET, as well as the phosphorylation of their downstream AKT and ERK1/2. (Figure 3*F*), providing molecular basis for abrogation of AC0010 resistance. Collectively, these cell-based results supported a causal role of c-MET overexpression in conferring AC0010 resistance in H1975 P1 cells.

To further confirm the combinational effects *in vivo*, the efficacy of AC0010 in combination with crizotinib was evaluated in H1975-P1-R1 xenograft mouse model. Compared with vehicle control, oral administration of AC0010 alone at 100 mg/kg or crizotinib alone at 12.5 mg/kg caused minor or moderate suppression of tumor growth with tumor volume inhibitory rate at 11.7% and 48.1%, respectively. Combination of both significantly suppressed tumor growth with a tumor volume inhibitory rate of 73.5% (Figure 4, *A* and *B*). It is worth noting that, during the entire experimental period, no significant changes in animal body weights were observed among all the treatment groups (Figure 4*C*), suggesting that dosage used for each drug alone or in combination did not have apparent toxicity to



	IC20 (M)	IC50 (M)
ABT-263	6.52E-08	3.328E-07

	AC0010	AC0010 +0.065μM ABT-263	AC0010 +0.3μM ABT-263
50% growth inhibition (M)	3.352E-06	1.592e-006	1.304E-06

	ABT-263	1.00μM AC0010FB+ABT-263
50% growth inhibition (M)	2.327E-07	9.143E-08

Figure 5. (A) Growth curve of H1975-AVR1-R2 cells treated with various concentrations of BCL-2 inhibitor ABT-263. (B) Growth curve of H1975-AVR1-R2 cells treated with various concentrations of AC0010 in combination with 0.065 μM or 0.3 μM ABT-263 ($n = 3$). (C) Growth curve of H1975-AVR1-R2 cells treated with 1 μM AC0010 in combination with various concentrations of ABT-263 ($n = 3$). (D) Clonogenic survival assays of H1975-AVR1-R2 cells treated with AC0010, ABT-263, or the combination of AC0010 and ABT-263 ($n = 3$). (D) Apoptosis induction by AC0010 or ABT-263, alone or in combination, in H1975-AVR1-R2 cells. Apoptosis was determined by caspase-3 activation assay using flow cytometry ($n = 3$).

animals. To confirm that drugs indeed hit the corresponding targets, we performed Western blotting analysis in harvested tumor tissues and found that drug combination did inactivate EGFR, c-MET, AKT1, and ERK, which correlated with its maximal antitumor activity (Figure 4*D*). Taken together, both *in vitro* and *in vivo* models showed that c-MET overexpression is responsible for AC0010 resistance, which is resensitized by combination of a c-MET inhibitor or *via* siRNA targeting c-MET.

BCL-2 Inhibitor ABT-263 Overcomes Resistance of H1975-AVR1 to AC0010

Similarly, we next investigated whether BCL-2 overexpression (Figure 2*D*) is causally responsible for AC0010 resistance seen in H1975-AVR1 cells. We used Navitoclax/ABT263, a small molecule inhibitor of BCL-2, to treat H1975-AVR1-R2 cells, a single clone derived from H1975-AVR1 (Figure 1*A*), and generated an IC_{50} curve with IC_{20} and IC_{50} values of 65 nM and 333 nM, respectively (Figure 5*A*). We then combined ABT263 at either IC_{20} or IC_{50} concentration with various of doses of AC0010 and found that ABT263 can sensitize cells to AC0010 with a reduction of IC50 values from 2.3 μM to 1.2 μM or 0.97 μM, respectively (Figure 5*B*). Reciprocally, we fixed AC0010 concentration at 1.0 μM, measured cellular sensitivity to ABT263, and found a significant sensitization with IC_{50} reduction from 232 nM to 91 nM (Figure 5*C*).We also tested ABT263 sensitization using clonogenic assay and found that while treatment at IC_{20} value alone caused minimal to moderate suppression, the combination significantly inhibited colony formation which is statistically different (Figure 5*D*). Finally, we found that while AC0010 failed to induce apoptosis, ABT-263 alone can significantly induce it, which is further enhanced with drug combination in this AC0010-resistant line (Figure 5*E*). Taken together, it appears that BCL-2 overexpression is responsible at least in part for AC0010 resistance in H1975 AVR1 model derived from cell culture selection. Thus, combination of ABT-263 with third-generation EGFR inhibitors might be an effective strategy for the treatment of EGFR-T790M positive cancers with a decreased apoptotic response to EGFR inhibition.

Discussion

The major drawback for kinase-based targeted therapy is the development of drug resistance that abrogates the therapeutic efficacy. This is the case for the first generation of EGFR inhibitor. Investigation of underlying mechanism of drug resistance led to identification of acquired resistance through the T790M mutation [12]. The finding facilitated the discovery and development of the third-generation EGFR-TKIs that covalently bind to EGFR T790M, currently including irreversible pyrimidine inhibitors osimertinib [18], CO-1686 [14], and EGF-816 [37] and pyrrolopyrimidine-based inhibitor AC0010 [19]. Despite impressive initial outcomes with the third-generation EGFR-TKIs, new mutations and other mechanisms of resistance are also emerging. Preclinical studies and patient postprogression biopsies led to the identification of multiple resistance mechanisms to third-generation EGFR-TKIs, including insurgence of secondary mutations in the EGFR gene [38], activation of RAS pathway [39], HER2 [26], MET gene amplifications [40], and enhanced epithelial-to-mesenchymal transition [41]. Thus, thorough understanding of drug resistance mechanisms would provide rational strategy for effective drug combination to overcome acquired resistance.

Cell lines derived from resistance to targeted therapies have been a valuable tool for studying acquired resistance. In the present study, we successfully established two acquired resistant lines *via* continuous exposure of NCI-H1975 cells to AC0010 using *in vivo* nude mice xenograft and *in vitro* cell culture passaging, and confirmed their resistance to all three generations of EGFR-TKIs, including gefitinib, erlotinib, afatinib, rociletinib, osimertinib, and certainly AC0010 itself. Although a similar resistance to the treatment of AC0010 (IC_{50} values at 1-3 μM) was displayed by these two lines, distinct resistant mechanism was identified by RNAseq profiling. Specifically, both models revealed altered expression in many genes, but the pattern of expression is unique and distinctive between each other.

Our follow-up validation study mainly focused on druggable targets, namely, c-MET identified from *in vivo* model and BCL-2 from *in vitro* model; both are overexpressed, respectively, in resistant cells. Together with the finding that c-MET amplification is associated with the resistance to the treatment of osimertinib or CO-1686 [28], it appears that c-MET activation could be a common mechanism associated with the resistance to third-generation EGFR-TKIs. Furthermore, since EGFR and c-MET have been shown to act synergistically on the activation of downstream effectors such as AKT and ERK and ultimately regulate tumor cell proliferation, the c-MET pathway activation likely serves as a compensatory pathway in tumor cells for the loss of EGFR-driven signaling cascade. On the other hand, in cell culture–based resistant model, our study showed a causal role of BCL-2 overexpression in conferring the drug resistance. BCL-2 is a typical antiapoptotic survival protein whose overexpression would promote cancer progression and confer drug resistance to facilitate cancer reoccurrences [42,43]. We found that the combination with BCL-2 inhibitor navitoclax/ABT-263 overcomes the resistance of H1975-AVR1 cells to AC0010, consistent with a recent study by Hata et al. [44], reporting navitoclax/ABT-263 sensitization of EGFR T790M-negative drug-tolerant cells with diminished apoptotic response to third-generation EGFR inhibitors. Furthermore, BCL-2 inhibitor ABT-737 can significantly enhance killing of NSCLC cells by the first-generation EGFR inhibitor genfitinib and eradicated early TKI-resistant evaders [45,46].

It is worth noting that although C797S mutant was reported to be associated with AZD9291 resistance [47], we did not find this mutant in AC0010-resistant cells in either cell culture or nude mice selection settings (Table S1). In the first-in-human clinical study on AC0010, C797S mutant was not observed in a total of 16 patients with disease progression after AC0010 treatment [33]. Although how exactly resistant clones evolve during targeted therapy is unknown, the results from our study, along with the reports from other third-generation EGFR TKIs [25,26,28], would suggest that the resistance to targeted therapy may be determined in a context-dependent manner by complex factors, such as tumor heterogeneity, tumor microenvironment, and drug dosing.

Tumor resistance caused by activation of accessory pathways can be theoretically overcome by combination of the inhibitor of EGFR and involving molecules. Availability of small molecule inhibitors targeting c-MET and BCL-2 in clinical development provided us an excellent opportunity to test the causal relationship between their overexpression and AC0010 resistance. We demonstrated that the combination of AC0010 with crizotinib, a c-MET inhibitor, substantially inhibited the growth and survival of H1975-P1-R1 cells and fully inhibited the phosphorylation of EGFR and c-MET proteins and downstream molecules, AKT1 and ERK. The

combination effects were further confirmed *in vivo* using H1975-P1-R1 xenograft mouse model, showing synergistic inhibitory effects with 73.5% inhibitory rate at nontoxicity dose. For H1975-AVR1-R2 cells with elevated BCL-2 expression, treatment with navitoclax/ABT-263resulted in significant growth inhibition when acting alone, and its combination with AC0010 caused even more suppression of growth and survival by substantially inducing apoptosis. Thus, overexpression of c-MET and BCL-2 is causally responsible for acquired resistance to AC0010, respectively, for two established models. Similar combination approaches have been explored in several third-generation EGFR-TKIs. For example, concomitant treatment of osimertinib with selumetinib has been demonstrated to cause regression of osimertinib-resistant tumors in an EGFR-mutant/T790M transgenic model [40]. Therefore, the combination of EGFR TKI with inhibitors targeting resistant mechanism(s) would be an attractive strategy to overcome acquired resistance often seen during treatment of EGFR-mutant NSCLC. Our study provides a sound rationale for future investigation in the clinical setting.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.11.004.

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