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γ Secretase Inhibitor BMS-708163 Reverses Resistance to EGFR Inhibitor via the PI3K/Akt Pathway in Lung Cancer

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ABSTRACT

Lung adenocarcinoma cells harboring epidermal growth factor receptor (EGFR) mutations are sensitive to EGFR tyrosine kinase inhibitor (TKI). Acquired resistance to EGFR TKI develops after prolonged treatment. The aim of this study was to investigate the effect of the novel γ secretase inhibitor BMS-708163 on acquired resistance to the EGFR TKI gefitinib. We did not observe known mechanisms of acquired resistance to EGFR TKI, including the EGFR T790M mutation and MET gene amplification in the gefitinib-resistant PC9/AB2 cells. BMS-708163 inhibited PI3K/Akt expression and sensitized PC9/AB2 cells to gefitinib-induced cytotoxicity. In contrast, BMS-708163 had no significant effect on gefitinib sensitivity in PC9 parental cells. Combined treatment with BMS-708163 and gefitinib induced high levels of apoptosis. Our in vivo studies showed that combined treatment of gefitinib and BMS-708163 inhibited the growth of PC9/AB2 xenografts. In conclusion, our data show that combined treatment of gefitinib and γ secretase inhibitors may be useful for treating lung adenocarcinomas harboring EGFR mutations with acquired gefitinib resistance. J. Cell. Biochem. 116: 1019–1027, 2015.

KEY WORDS: γ SECRETASE INHIBITOR; GEFITINIB; DRUG RESISTANCE; LUNG CANCER

Lung cancers with mutations in the epidermal growth factor receptor (EGFR) gene are a well-described molecular subgroup of lung adenocarcinomas. This subgroup is characterized by high prevalence in females, never-smokers, and Asians, and sensitivity to EGFR tyrosine kinase inhibitor (TKI) gefitinib or erlotinib [Jackman et al., 2009]. However, disease progression develops in most lung cancer patients with EGFR mutations after a median of 10–14 months of treatment with EGFR TKI. Potential mechanisms associated with acquired resistance to gefitinib include acquisition of the T790M mutation in exon 20 of the EGFR, MET amplification, and hepatocyte growth factor (HGF) overexpression [Godin-Heymann et al., 2007; Yano et al., 2008; Mcdermott et al., 2010].

The Notch signaling pathway is a conserved ligand–receptor signaling pathway that plays critical mechanistic roles in cell proliferation, survival, apoptosis, and differentiation which affects the development and function of many organs [Miele, 2006]. Recently, Notch is found to be involved in not only chemotherapy drug-resistance [Bao et al., 2011; McAuliffe et al., 2012; Liu et al., 2013] but also EGFR TKI resistance [Xie et al., 2013]. Blockade of an upregulated Notch signaling pathway can be achieved by inhibiting the formation of the main force of Notch activity, the Notch intracellular domain (NICD) [Takebe et al., 2014]. Thus, a pharmacological approach using γ secretase inhibitor to prevent the final cleavage step of the precursor form of Notch to decrease the levels of NICD could be a novel therapeutic approach for the treatment of lung cancer by overcoming drug-resistance of cancer cells.

The aim of this study was to investigate the effect of the novel γ secretase inhibitor BMS-708163 on acquired resistance to EGFR TKI. Our results show that BMS-708163 reverses sensitivity to gefitinib in

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PC9/AB2 cells in vitro and in vivo. BMS-708163 induces apoptosis and enhances cell cycle arrest at the G1 phase. We also demonstrate that EGFR TKI acquired resistance in PC9/AB2 cells is mainly driven by Notch1-mediated activation of PI3K/Akt. Our findings suggest that inhibition of γ secretase may represent a novel approach to treating lung cancer.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

The PC9 cell line harboring the EGFR exon 19 deletion (Del E746–A750) was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). NCI-H820 cell line harboring the E746–E749 deletion mutation and the T790M mutation as well as MET amplification was purchased from the American Type Culture Collection (Manassas, VA). A PC9/AB2 cell line with acquired resistance to gefitinib was kindly provided by Shanghai Pulmonary Hospital [Ju et al., 2010]. To derive the PC9/AB2 cells, PC9 cells were cultured in medium containing 0.2 μM gefitinib for 7 days, washed, and cultured in drug-free medium for 14 days. The cells were then seeded into medium containing 0.5 μM of gefitinib in 96-well plates for subcloning. After 28 days, the colonies were harvested. The subcloned cells showed a more than 100-fold increase in resistance to gefitinib-induced growth inhibition, as determined by cytotoxicity assay. The gefitinib-resistance phenotype has been previously shown to be stable for over 6 months under drug-free conditions [Koizumi et al., 2005]. All the cell lines were characterized by mycoplasma detection, DNA-Fingerprinting, isozyme detection and cell viability analysis. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 2 mmol/L L-glutamine. Gefitinib (N-(3-chloro-4-fluoro-phenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy) quinazolin-4-amine) was purchased from Tocris Bioscience (Bristol, England). BIBW2992 and MRK003 were kindly provided by Boehringer Ingelheim and Merk Co., respectively. BMS-708163 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3D GROWTH ASSAY

The well of a chamber slide was initially coated with 100% Matrigel (BD Biosciences, San Jose, CA) and allowed to solidify, forming a gelled bed of basement membrane approximately 1 mm thick [Guix et al., 2008]. The cells were seeded into the chamber as a single-cell suspension. DMSO, gefitinib (1μM), MRK003 (10 mM), BMS-708163 (10 μM), or a combination of these drugs were added into the medium 12 h after cell seeding. The medium and drugs were replenished every 2 days for 20 days. The cells proliferated and formed clusters after 5 days in 3D culture, and subsequently formed colonies. Photographs were taken after culturing the cells for 20 days.

APOPTOSIS ANALYSIS

The cells were seeded at densities of 1.0×10^5 cells/plate in 10 cm^2 plates for fluorescence-activated cell sorter (FACS) analysis. Twenty-four hours after plating, the cell culture medium was replaced with 15 mL DMEM that contained 0.1% FBS with gefitinib (1 μM), BMS-708163 (10 μM), or a combination of gefitinib and BMS-708163 for 6 h. The cells were incubated for another 48 h, harvested, and collected. Cell apoptosis was detected using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). The analysis was performed on a FACSCalibur flow cytometer with CellQuest Software (Becton Dickinson) [Xie et al., 2013; Fofaria et al., 2014].

CELL CYCLE ANALYSIS

The cells were trypsinized, washed with 1 x PBS and fixed with 70% ethanol overnight at 4°C. The cells were then spun down, washed twice and incubated with 20 μg/mL propidium iodide and 10 μg/mL RNAse for 30 min, and washed and the DNA content was analyzed by flow cytometry [Ravi et al., 2014].

SIRNA TRANSFECTION

The PI3K siRNA (Cat No. sc-39125) and the control siRNA (Cat No. sc-44236) were purchased from Santa Cruz Biotechnology. PC9/AB2 cells were grown to 60% confluence, and PI3K and control siRNAs were transfected using the Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Briefly, Lipofectamine was incubated with serum-free medium for 10 min, mixed with siRNA (80 nM), and incubated for 20 min at room temperature before the mixture was diluted with medium and added to the PC9/AB2 cells. After culturing for 30 h, the cells were washed, and then resuspended in fresh culture media in the presence of DMSO or BMS-708163 (10 μM) for an additional 48 h.

CYTOTOXICITY ASSAY

The cell viability was assessed using a tetrazolium salt (WST-8)–based colorimetric assay from the Cell Counting Kit 8 (CCK-8; Dojindo Molecular Technology, Japan). The cells were seeded into 96-well plates at an initial density of 5×10^3 cells/well and cultured for 24 h, after which the cells were cultured with DMSO, increased concentrations of gefitinib or BMS708163, BIBW2992, or the combination of BMS708163 and BIBW2992 for an additional 48 h. The A450 was measured in a microplate reader after 10 μL of CCK-8 solution was added and incubated for 1 h. The percentage of growth is shown relative to untreated controls.

WESTERN BLOT ANALYSIS

Total protein was isolated from the cells using Cell Extraction Buffer (Biosource, Camarillo, CA) supplemented with protease and phosphatase inhibitors. The lysates were precleared using centrifugation, and the protein concentrations were measured using the bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, IL). The primary antibodies used in this study included: active caspase 3, PARP, Ki-67, PI3K, Akt, mTOR, pEGFR, EGFR (Cell Signaling Technology, Boston, MA), Notch1 (Abcam Corporation, Burlingame, CA), and HES1 (Santa Cruz Biotechnology). Equal protein loading was confirmed by probing for β-actin (Santa Cruz Biotechnology) expression. Appropriate secondary antibodies conjugated to horseradish peroxidase were used, including anti-mouse or anti-rabbit IgG (GE-Healthcare, Chiltern, England). The proteins were visualized by Amersham enhanced chemiluminescence (GE-Healthcare).

TUMOR XENOGRAFTS IN NUDE MICE

All in vivo experiments were approved by the Institutional Review Board of the First Affiliated Hospital of Guangzhou Medical University and performed according to the Guidance of Institutional Animal Care and Use Committee of Guangzhou Medical University. Four- to six-week-old female Balb/c athymic (nu + /nu +) mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (China). The mice were anesthetized with ether. The mice were acclimatized for one week before being injected with 1.5×10^6 PC9/AB2 cells that had been resuspended in 200 μL of matrigel. When established tumors of approximately 150–300 mm^3 in diameter were detected, the mice were randomly divided into groups and fed orally by gavage with either vehicle (1% methylcellulose, 0.2% Tween 80 in sterilized water), gefitinib (3 mg/kg diluted in vehicle), BMS-708163 (10 mg/kg diluted in vehicle), or a combination of gefitinib (3 mg/kg) and BMS-708163 (10 mg/kg) for 5 days/week. Each treatment group consisted of eight mice. The tumor volume were measured and calculated every five days using the following formula: $\pi/6 \times$ (larger diameter) $\times$ (smaller diameter)^2. After 30 days, mice were killed by cervical dislocation.

IMMUNOHISTOCHEMISTRY (IHC) ASSAY

Caspase 3 (Cat No. Ab 32351) and Ki-67 (Cat No. Ab 15580) antibodies were purchased from Abcam Corporation and used for immunohistochemistry assay of the tumor tissue resected from the subcutaneous xenograft model. After dewaxing in xylene and rehydrating stepwise in an ethanol gradient, the sections were subjected to heat-induced antigen retrieval. Endogenous peroxidase activity and nonspecific binding were blocked with 3% H_2O_2 and nonimmune sera, respectively. The sections were then incubated with primary antibodies overnight at 4°C. The following day, the primary antibody was detected using a Streptavidin-Biotin kit (Maixin Biotechnology, Fuzhou, China) according to the manufacturer's instructions. The immunolabeled sections were visualized with 3,3′-diaminobenzidine and counterstained with hematoxylin. The sections were counterstained with hematoxylin, dehydrated, and mounted. Sections were stained in parallel without primary antibody to provide a negative control. Cytoplasmic staining of caspase 3 or nuclear staining of Ki-67 in tumor cells was considered to be positive [Xie et al., 2012].

DNA SEQUENCING

Genomic DNA was extracted from PC9/AB2 cells using a QIAmp DNA Mini Kit (Qiagen, Shanghai, China). The EGFR exon 20 was amplified and sequenced as previously described [Lynch et al., 2004]. Sense and antisense sequences were obtained from the products of the amplification reactions. The reference cDNA used to determine the nucleotide positions was GeneBank NM_201283. The PCR sequencing reaction was performed at 96 °C for 1 min, followed by 25 cycles at 96 °C for 30 s, 50°C for 15 s, and 60 °C for 4 min. The PCR products were subcloned into the pGEM-T easy cloning vector (Invitrogen) and sequenced. The sequencing was conducted using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). NCI-H820 cell line harboring the EGFR T790M mutation was used as positive control.

FISH ANALYSIS FOR MET AMPLIFICATION

Slides were prepared from fixed NCI-H820 and PC9/AB2 cell pellets available in the laboratory. The slides were subjected to a dual-color FISH assay using a mixture of the CEP7 SG probe (Abbott Molecular, Des Plaines, IL) and a homebrew MET probe prepared with the human DNA insert from the BAC clone RP11–95I20. A MET/CEP ratio was established based on counting at least 200 cells. Samples with a MET: CEP7 ratio greater than 2 were considered to have MET amplification (low amplification ≤ 3, high amplification > 3) [Yu et al., 2013]. The NCI-H820 cell line was used as positive control for MET amplification [Bean et al., 2007]. Images were captured using a CCD camera and merged using dedicated software (CytoVision, Applied Imaging, San Jose, CA).

STATISTICAL ANALYSIS

The Student's *t* test was used to evaluate the statistical significance of the results. All *P* values represent two-sided tests of statistical significance. All analyses were performed with the SPSS 16.0 software package. $P < 0.05$ was considered to be statistically significant.

RESULTS

GEFITINIB ACQUIRED RESISTANT PC9/AB2 CELLS REMAIN SENSITIVE TO BMS-708163

PC9/AB2 cells were generated by culturing PC9 cells in increasing concentrations of gefitinib, according to previously described methods [Xie et al., 2013]. Notably, BIBW2992 was cytotoxic to gefitinib-resistant PC9/AB2 cells (Fig. 1A). To exclude common mechanisms of acquired resistance, we performed DNA sequencing and FISH assay. The EGFR exon 20 wild-type DNA sequence was GAAGCCTACGTGATGGCCAGCGTGGACAACCCCCACGTGTGC CGC CTGCTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGC TCATGCCCTT CGGCTGCCTCCTGGACTATGTCCGGGAACACAAA-GACAATATTGGCTCCCAGTACCTGCTCAACTGGTGTGTGCAGATCG CAAAG (189 bp; Fig. S1, upper panel). The product of exon 20 amplification was subcloned. The positve control (NCI-H820) cells exhibited a novel C-to-T (antisense G-to-A) base-pair change. However, PC9/AB2 cells showed no T790M mutation (Fig. S1, lower panel). The MET gene status in the two cell lines were analyzed. The mean MET copy number was seven in the control NCI-H820 cells , while no MET amplification was observed in the PC9/AB2 cells (on average, there was one copy of MET (red signal) per cell) (Fig. 1B). The effect of BMS-708163 on gefitinib sensitivity in PC9/AB2 cells was then investigated. A low dose of BMS-708163 (5 μM) had no significant effect on the gefitinib sensitivity of PC9/AB2 cells. However, a higher dose of BMS-708163 ($\geq$20 μM) reversed sensitivity to gefitinib in PC9/AB2 cells (Fig. 1C). Additionally, BMS-708163 had no significant effect on gefitinib sensitivity in PC9 parental cells.

Similarly, cytotoxicity assay showed that higher concentration of BMS-708163 ($\geq$20 μM) in combination with BIBW2992 had synergistical cytotoxic effect on PC9/AB2 cells compared with BIBW2992 alone (Fig. 1D). The 3D growth assay revealed that the PC9/AB2 cells formed less and smaller colonies in the presence of γ

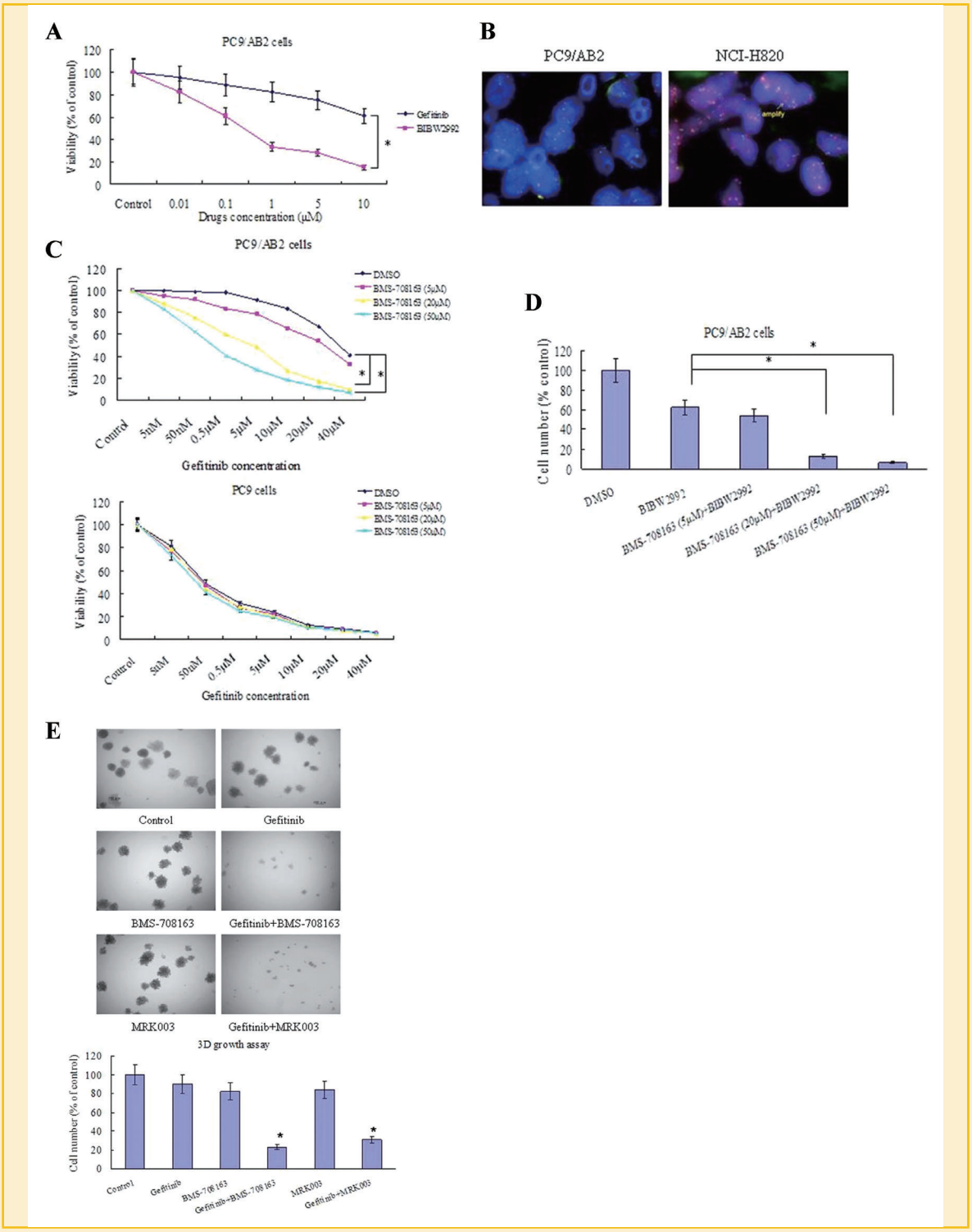

Fig. 1. Effect of BMS-708163 on PC9/AB2 cell sensitivity to gefitinib. A: PC9/AB2 cells were treated with increasing concentrations of gefitinib or BIBW2992 for 48 h. Afterwards, the viability was measured using a CCK-8 kit. The bars indicate the means ± standard deviation (SD) from three independent experiments. * indicates a significant difference ($P < 0.05$). B: Hybridization of a MET/CEP7 probe set with PC9/AB2 and NCI-H820 cells. On average, only one MET signal (red) per cell was observed in PC9/AB2 cells, while seven MET signals were detected in the control NCI-H820 cell nuclei. C: PC9/AB2 and PC9 cells were treated with different concentrations of BMS-708163 or DMSO. The viability was measured using a CCK-8 kit. * indicates a significant difference ($P < 0.05$). D: The cells were treated with different concentrations of BMS-708163, BIBW2992, or both, as indicated, for 48 h. The cell viability was measured with a CCK-8 kit and is presented as a percentage of untreated cells. * indicates a significant difference ($P < 0.05$). E: PC9/AB2 cells were grown in matrigel with DMSO, gefitinib (1 μM), MRK003 (10 mM), BMS-708163 (10 μM), or a combination of these drugs. Images of the colonies were taken after 20 days. The cells were then harvested and quantified. *$P < 0.05$ compared to the control.

secretase inhibitor BMS-708163 (10 μM) or MRK003 (10 mM) alone than the cells treated with gefitinib (1 μM), although there was no significant difference between these three groups. However, when BMS-708163 or MRK003 was combined with gefitinib, the colony growth of PC9/AB2 cells was significantly attenuated (Fig. 1E).

INDUCTION OF APOPTOSIS BY BMS-708163 IN PC9/AB2 CELLS

PC9/AB2 cells were treated with gefitinib (1μM), BMS-708163 (10 μM), or a combination of gefitinib and BMS-708163 for 12 h. Western blot analysis showed that treatment with BMS-708163 increased the expression of active caspase 3 and PARP and reduced the expression of Ki-67 in PC9/AB2 cells compared to cells treated with gefitinib alone. Moreover, combined treatment with BMS-708163 and gefitinib increased the expression of active caspase 3 and PARP compared to treatment with BMS-708163 alone (Fig. 2A). Flow cytometry analysis with Annexin V further revealed that treatment with BMS-708163 plus gefitinib greatly increased apoptosis of PC9/AB2 cells compared to treatment with gefitinib or BMS-708163 alone. These results indicate that exposure to BMS-708163 in combination with gefitinib is sufficient for the induction of apoptosis in PC9/AB2 cells (Fig. 2B). To identify the mechanism behind the BMS-708163-mediatd inhibition of PC9/AB2 cell growth, we examined the effect of BMS-708163 on cell cycle arrest. The cells were treated with various concentrations of BMS-708163 and analyzed by flow cytometry. Our results showed a concentration-dependent effects of BMS-708163 on the number of PC9/AB2 cells in the G1 phase. A high dose of BMS-708163 (≥20 μM) induced a significant level of G1 phase arrest in PC9/AB2 cells (Fig. 2C). The PC9/AB2 cells were then pretreated with gefitinib (1μM), BMS-708163 (10 μM), or a combination of gefitinib and BMS-708163. We observed a significant increase in G1 phase arrest in the cells treated with BMS-708163 alone or with a combination of gefitinib and BMS-708163 when compared to the control ($P < 0.05$). Additionally, treatment with gefitinib plus BMS-708163 slightly enhanced the accumulation of PC9/AB2 cells in the G1 phase compared to treatment with BMS-708163 alone; however, there was no significant difference between these two groups. There was no significant difference in the number of cells in G1 phase arrest when treated with gefitinib or the control (Fig. 2D). These results suggest that BMS-708163 induces apoptosis and enhances cell cycle arrest at the G1 phase in PC9/AB2 cells.

ACQUIRED RESISTANCE TO EGFR TKI IN PC9/AB2 CELLS IS MAINLY DRIVEN BY NOTCH1-MEDIATED ACTIVATION OF PI3K/AKT

To further investigate the underlying mechanism of acquired resistance to EGFR TKI in PC9/AB2 cells, we analyzed the expression

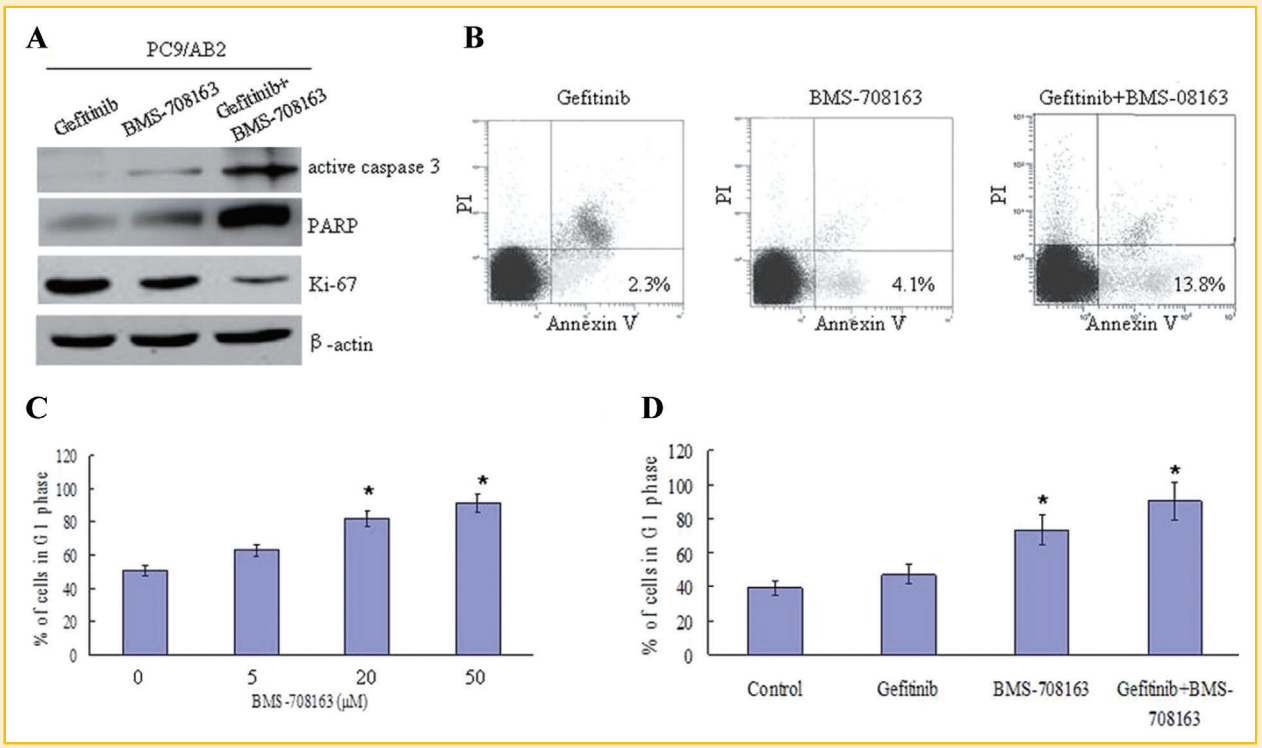

Fig. 2. Induction of apoptosis by BMS-708163 in PC9/AB2 cells. A: PC9/AB2 cells were treated with gefitinib (1μM), BMS-708163 (10 μM), or a combination of gefitinib and BMS-708163 for 12 h. Western blot analysis was performed to detect the expressions of active caspase 3, PARP and Ki-67. β-actin was used as the loading control. B: PC9/AB2 cells were incubated with gefitinib (1μM), BMS-708163 (10 μM), or a combination of gefitinib and BMS-708163 for 6 h, and then washed twice with PBS. The resulting cultures were incubated in fresh medium for 48 h. The number of apoptotic cells were determined using an Annexin V assay kit according to the manufactory protocol. C: The concentration-dependent effect of BMS-708163 on the number of PC9/AB2 cells in the G1 phase. *$P < 0.05$ compared to the control (DMSO). D: PC9/AB2 cells were pretreated with control, gefitinib (1 μM), BMS-708163 (10 μM), or a combination of gefitinib and BMS-708163 for 48 h, stained with propidium iodide and analyzed for cell cycle arrest by flow cytometry. *$P < 0.05$ compared to the control.

of Notch1/HES1 and its downstream genes in PC9/AB2 and PC9 parental cells treated with the γ secretase inhibitor BMS-708163. As shown in Fig. 3A, Notch1 was only overexpressed in PC9/AB2 cells. BMS-708163 treatment effectively downregulated the expression of Notch1, HES1, PI3K and Akt in PC9/AB2 cells. However, we did not observe any reduction in mTOR expression in PC9/AB2 cells after exposure to 10 μM BMS-708163. Meanwhile, BMS-708163 treatment had no effect on the expressions of Notch1/HES1 and PI3K/Akt in the PC9 parental cells (Fig. 3A). These results suggest that acquired resistance to gefitinib is mainly driven by Notch-mediated activation of PI3K instead of mTOR and that Notch inhibition is essential to restore sensitivity to gefitinib. In addition, the western blot assay revealed that EGFR phosphorylation was mostly inhibited when PC9/AB2 cells were treated with gefitinib in combination with either BMS-708163 or the PI3K inhibitor LY294002. Of note, triple combination treatment (with BMS-708163, LY294002, and gefitinib) resulted in increased inhibition of pEGFR expression compared to treatment with gefitinib plus BMS-708163. However, treatment with the mTOR inhibitor rapamycin (100 μM) did not affect the expression of pEGFR (Fig. 3B). Our results showed that treatment with BMS-708163 greatly decreased the expression of PI3K in PC9/AB2 cells, indicating that PI3K is a downstream target of Notch. We next transfected PC9/AB2 cells with PI3K siRNA (siPI3K). The cytotoxicity assay showed that transfection with siPI3K greatly increased PC9/AB2 cell sensitivity to gefitinib. Adding BMS-708163 greatly enhanced the inhibitory effect on PC9/AB2 cells transfected with siPI3K (Fig. 3C).

PC9/AB2 XENOGRAFT LUNG CANCER FORMATION IS INHIBITED BY COMBINED BLOCKADE OF EGFR AND NOTCH

We hypothesized that the addition of γ secretase inhibitor to gefitinib would prevent or delay development of acquired resistance

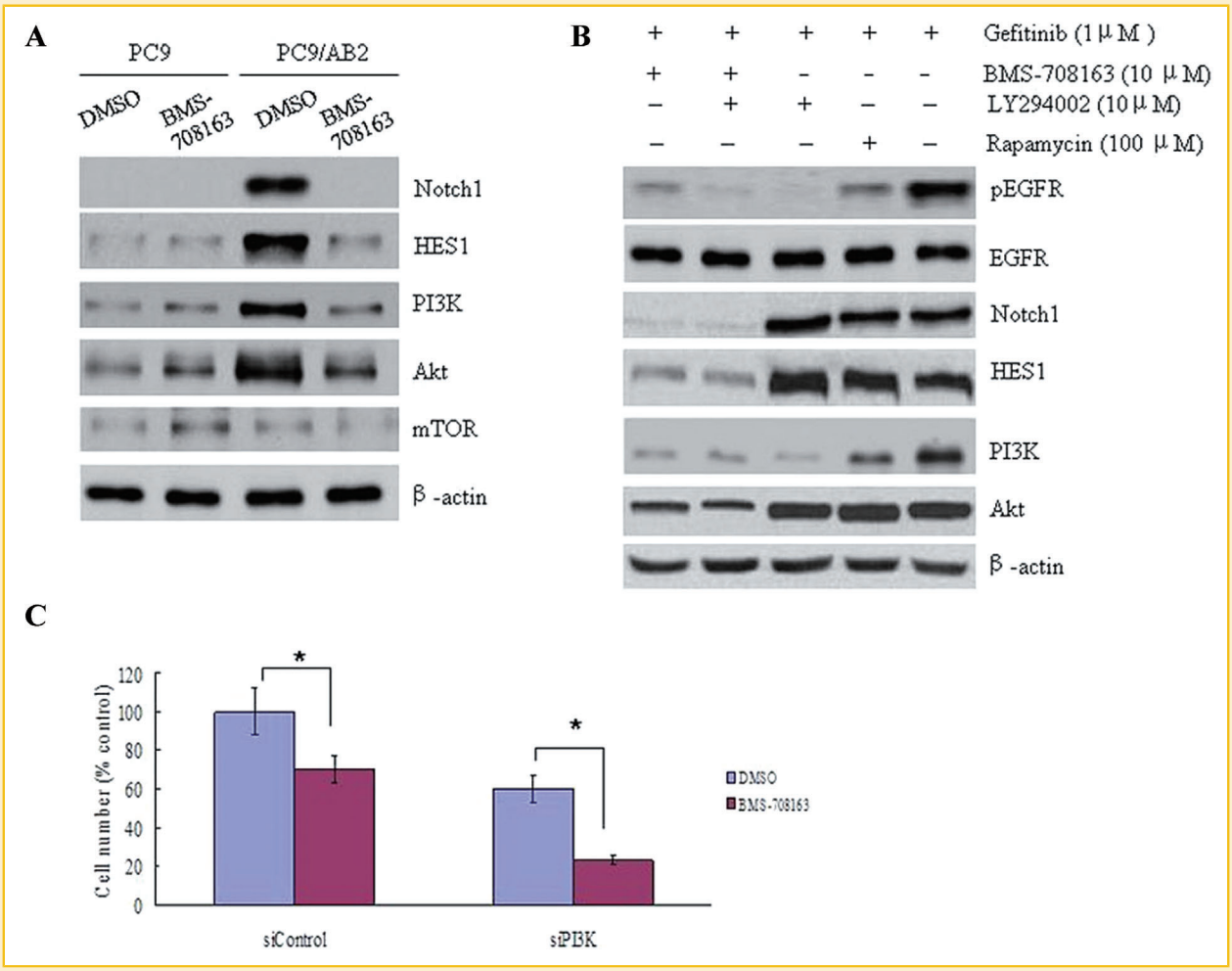

Fig. 3. Acquired resistance to EGFR TKI in PC9/AB2 cells is mainly driven by Notch1-mediated activation of the PI3K/Akt pathway. A: PC9 and PC9/AB2 cells were treated with DMSO or BMS-708163 (10 μM) for 12 h. Western blot analysis was performed to detect the expression of Notch1, HES1, PI3K, Akt and mTOR. β-actin was used as the loading control. B: PC9/AB2 cells were treated with gefitinib (1 μM), BMS-708163 (10 μM), LY294002 (10 μM), rapamycin (100 μM) or a combination as indicated for 12 h. Western blot analysis was performed to detect the expression of p-EGFR, EGFR, Notch1, HES1, PI3K, and Akt. β-actin was used as the loading control. C: PC9/AB2 cells were transfected with PI3K siRNA (siPI3K) or control siRNA (siConrol). The cells were treated with BMS-708163 (10 μM) or DMSO for 48 h. Cell viability was measured with a CCK-8 kit and the result are presented as a percentage of untreated cells. * indicates a significant difference ($P < 0.05$).

to gefitinib in a PC9/AB2 xenograft lung cancer models. Nude mice bearing PC9/AB2 xenografts were randomized to control, gefitinib, BMS-708163 or the combination (both drugs) therapy groups for 30 days. BMS-708163 monotherapy had a minor inhibitory effect on PC9/AB2 tumor growth compared with gefitinib alone. However, we observed a significant inhibition of tumor growth in the PC9/AB2 xenograft lung cancer model in response to combined treatment with BMS-708163 and gefitinib (Fig. 4A). BMS-708163 monotherapy resulted in a slight increase in caspase 3 expression as well as a mild decreased in Ki-67 expression in vivo (Fig. 4B). A marked increase in caspase 3 expression and a reduction in Ki-67 staining were observed in the xenograft lung cancer samples treated with BMS-708163 plus gefitinib, which was consistent with the enhanced apoptotic activity as measured by western blot (Fig. 2A) and with the Annexin V assay kit (Fig. 2B), respectively. These results indicate that the combined inhibition of Notch and EGFR abrogates the acquired resistance to gefitinib.

DISCUSSION

Most advanced non-small cell lung cancers (NSCLC) with activating EGFR mutations (exon 19 deletions or L858R) initially respond to the EGFR TKI gefitinib or erlotinib. However, most tumors eventually develop acquired resistance to EGFR TKI. Current research is focusing on identifing other mechanisms that account for the approximately 30–40% of TKI-resistant EGFR mutant tumors that do not carry secondary resistance mutations or MET amplification [Nguyen et al., 2009; Kosaka et al., 2011]. We were the first to show that acquired resistance to gefitinib in PC9/AB2 cells is associated with Notch1 overexpression [Xie et al., 2013]. In this study, weshow that neither MET amplification nor EGFR T790M account for acquired resistanc to gefitinib in PC9/AB2 cells. The goal of this study was to determine whether the novel γ secretase inhibitor BMS-708163 can overcome the acquired resistance to EGFR TKI.

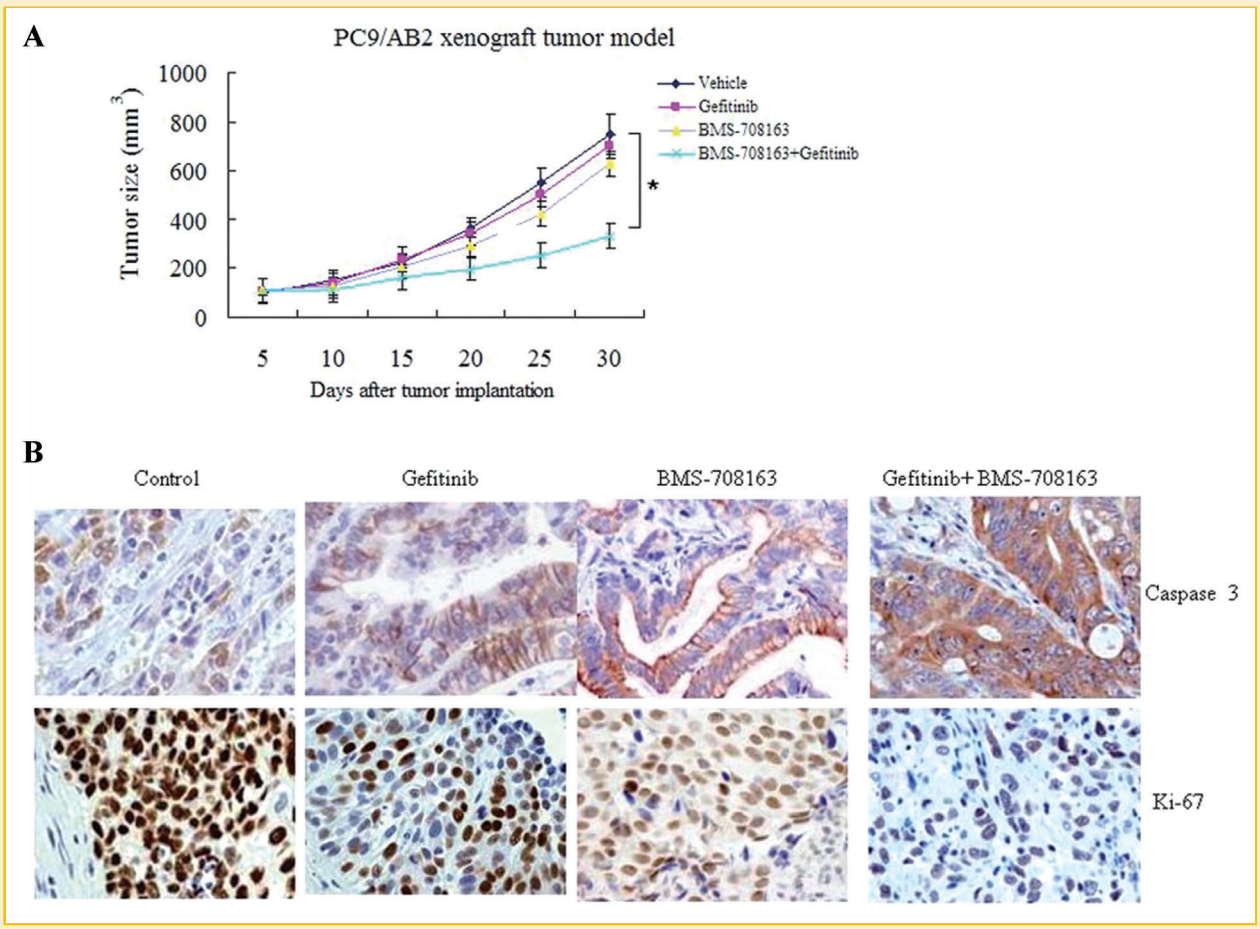

Fig. 4. PC9/AB2 xenograft lung cancer development is inhibited by combined blockade of EGFR and Notch. A: Lung cancer cells (1.5×10^6) resuspended in 200 μL of matrigel (BD Biosciences, USA) were subcutaneously injected into the right flank of nude mice. Mice bearing tumors of 150 to 300 mm^3 in size were then randomly divided into groups that received either vehicle (1% methylcellulose, 0.2% Tween 80 in sterilized water), gefitinib (3 mg/kg diluted in vehicle), BMS-708163 (10 mg/kg diluted in vehicle), or a combination of gefitinib (3 mg/kg) and BMS-708163 (10 mg/kg) for 5 days/week. The tumor volumes were measured every five days. Each treatment group consisted of eight mice. The data shown represent the average (± SD). *$P < 0.05$ when compared to the control. B: The tumors were resected from the sacrificed nude mice and fixed in 10% neutral buffered formalin. Immunohistochemistry assays were conducted for caspase 3 and Ki-67. Photomicrographs were taken at 400× magnification.

The Notch signaling pathway plays critical mechanistic role in tumor cell proliferation, the epithelial-mesenchymal transition, chemoresistance, and tumorigenesis. Emerging evidence suggests that the Notch signaling pathway is one of the most important signaling pathways in drug-resistant tumor cells. Pharmacological inhibition of Notch signaling may enhance the efficacy of cancer therapy. Moreover, downregulation of the Notch pathway could induce drug sensitivity, leading to increased inhibition of cancer cell growth, invasion, and metastasis [Wang et al., 2010; Capaccione and Pine, 2013]. Because Notch signaling is activated via the enzymatic activity of γ secretase, inhibition of γ secretase is becoming an exciting new area of drug development for the targeted therapy of many human cancers. The Notch pathway has tremendous potential as a new target in cancer therapy. Several Notch inhibitors have been investigated, and while the γ secretase inhibitor is less specific, it has the potential advantage of pan-Notch inhibition [Espinoza et al., 2013].

The novel, orally available, small molecule, BMS-708163 was identified as a noncompetitive γ secretase inhibitor. In this study we show that BMS-708163 reverses sensitivity to the first generation EGFR TKI gefitinib in PC9/AB2 cells. Treatment with BMS-708163 in combination with BIBW2992 had a synergistical cytotoxic effect on PC9/AB2 cells. We also show that another γ secretase inhibitor (MRK003) had a similar inhibitory effect on PC9/AB2 colony growth in a 3D growth assay. Although studies have elaborated the apoptotic mechanism that occurs during γ secretase inhibitor-mediated cell death, it remains unclear how γ secretase inhibitors induce apoptosis. Osipo et al. found that a γ secretase inhibitor alone was sufficient to increase apoptosis in trastuzumab-resistant BT474 cells by 20%, which increased to 30% when combined with trastuzumab [Osipo et al., 2008]. We showed that BMS-708163 enhanced sensitivity to gefitinib as well as BIBW2992 by promoting apoptosis through activation of a caspase pathway in vitro. Moreover, a marked increase in caspase 3 staining accompanied by a reduction in Ki-67 staining was observed in the xenograft lung cancer samples treated with BMS-708163 plus gefitinib. This is in agreement with Curry et al., who showed that a γ secretase inhibitor indirectly activated caspase 3 in murine cells [Curry et al., 2005].

Inhibition of apoptosis in Notch-transformed tumors and resistance to chemotherapy appear to be dependent on the PI3K/Akt pathway [Meurette et al., 2009]. However, the role of crosstalk between Notch and the PI3K/Akt pathway in acquired resistance to EGFR TKI remains unclear. While further studies are needed to elucidate the relationship between Notch and the PI3K/Akt pathway, previous data indicate that Notch does not directly affect PI3K or Akt [Dang, 2012]. Xiao et al. found that inhibition of the Jagged/Notch pathway inhibited retinoblastoma cell proliferation by suppressing the PI3K/Akt pathway [Xiao et al., 2014]. Similarly, our study suggests that acquired resistance to gefitinib is mainly driven by Notch1-mediated activation of the PI3K/Akt pathway, instead of mTOR pathway. Thus, BMS-708163-mediated inhibition of Notch1 induces apoptosis in gefitinib acquired resistant PC9/AB2 cells in a PI3K/Akt pathway-dependent manner. Fei et al. showed that mTOR was highly activated in resistant lung cancer cells and that mTOR inhibition is likely a good strategy for treating patients with EGFR mutant NSCLC who develop EGFR TKI resistance [Fei et al., 2013]. However, in our study, we observed no significant difference in mTOR expression between PC9 parental cells and PC9/AB2 cells. The addition of mTOR did not affect the sensitivity of TKI- resistant cells to gefitinib, indicating that mTOR is not associated with TKI resistance in PC9/AB2 cells.

In conclusion, the γ secretase inhibitor BMS-708163 appears to be a novel strategy for the reversal of acquired resistance to EGFR TKI during lung cancer treatment. Further prospective clinical investigation is thus needed.

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