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Original Article

Concurrent inhibition of ErbB family and MEK/ERK kinases to suppress non-small cell lung cancer proliferation

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Abstract: Lung cancer ranks as the most common cancer and leading cause of cancer-related deaths worldwide. Of all lung cancer types, non-small cell lung cancer (NSCLC) accounts for 85 percent of all cases. The high mortality of NSCLC occurs mainly because of poor prognosis in patients with recurrent and metastatic cancer. Cisplatin-containing chemotherapy is the first option to treat recurrent and metastatic NSCLC. Additionally, targeted therapy plays an important role to prolong life in patients. Currently, EGFR inhibitors are the most important targeted anti-cancer drugs for patients with EGFR mutations in the clinical setting. Another important kinase inhibitor for targeted therapy is the MEK inhibitor, Trametinib, which is often used for patients with BRAF mutation or MEK/ERK activation in the tumors. In this study, we determined whether a combination of the pan-ErbB kinase inhibitor, Afatinib, and MEK inhibitor, Trametinib, could more effectively inhibit NSCLC cell proliferation when compared to either single treatment. We found that Afatinib inhibited phosphorylation of EGFR, HER2, HER3, and HER4, as well as Akt, whereas it elevated ERK phosphorylation. Conversely, Trametinib treatment led to ERK inhibition, but induced Akt phosphorylation. However, the combination of Afatinib and Trametinib inhibited all of the above-mentioned signaling pathways and synergistically suppressed cell proliferation. Our data indicate that co-targeting of ErbB family and MEK/ERK pathways through a combination of Afatinib and Trametinib could be a potential effective strategy to treat NSCLC.

Keywords: Non-small cell lung cancer, Afatinib, MEK inhibitor, PI3K, Akt

Introduction

Lung cancer is the one of the most common cancers in the United States and the leading cause of cancer-related deaths worldwide [1, 2]. Lung cancers consist of several types based on the microscopic appearance of the cancer cells, which include small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2, 3]. NSCLC comprises about 85-90% of all lung cancer cases and accounts for the major cause of lung cancer deaths. The main treatment options for NSCLC include surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy. Surgery and/or radiation therapy achieve positive outcomes for patients with early stage tumors (Stages I and II). For patients with locally advanced disease (Stage III), chemotherapy combined with surgery and radiation therapy appears to be the best option. For metastatic or recurrent disease, chemotherapy, targeted therapy, or a combination would be the primary choice [4-7].

Currently, the major targeted therapy for NSCLC is to target epidermal growth factor receptor (EGFR) because many NSCLC cells have EGFR amplifications or mutations that play crucial roles in advanced NSCLC [8-10]. These drugs include Gefitinib and Erlotinib, which target EGFR, and Afatinib and Dacomitinib, which target EGFR, HER2, and HER4 [11-15]. It is expected that Afatinib or Dacomitinib may more effectively suppress NSCLC cell growth because it can target more ErbB family members. However, clinical results demonstrate that while EGFR

inhibitors can shrink tumors for several months, the disease will eventually progress [6, 11-14, 16-18]. Thus, we must define the mechanisms by which NSCLC cells develop resistance to EGFR inhibitor treatment.

Some NSCLC cells have changes in the *BRAF* gene, which leads to activation of the MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway) to regulate NSCLC growth. Recently, multiple MEK inhibitors have been reported to inhibit NSCLC *in vitro* and *in vivo* [19-21]. An important MEK inhibitor, Trametinib, has recently acquired FDA approval for use in NSCLC therapy [9, 10, 15, 22-24]. It would be interesting to test whether Trametinib alone, or in combination with another targeted drug, could suppress NSCLC even for patients without *BRAF* changes.

In this study, we set out to investigate the molecular links between EGFR and its downstream targets, such as the PI3K/Akt, mTOR/S6K/S6, and MEK/ERK pathways following treatment with EGFR or MEK inhibitors. Specifically, we wanted to determine whether a combination of the EGFR inhibitor, Afatinib, with the MEK inhibitor, could more effectively suppress NSCLC growth when both were used for NSCLC therapy. We found that Afatinib inhibited all ErbB family members, including EGFR, HER2, HER3, and HER4, as well as Akt, whereas it elevated ERK phosphorylation levels. Conversely, Trametinib inhibited ERK but led to induction of Akt phosphorylation in multiple NSCLC cell lines. A combination of Afatinib and Trametinib inhibited all the above signaling pathways and led to increased inhibition of cell proliferation. These results suggest that the combination of Afatinib and Trametinib could be an effective strategy to treat NSCLC.

Materials and methods

Cell lines

NSCLC cell lines A549 and NCI-H522 were generous gifts from Dr. Feng Jiang (Department of Pathology, University of Maryland, Baltimore, MD) and Drs. Yuping Mei and Li Mao (Department of Oncology and Diagnostic Sciences, University of Maryland School of Dentistry, Baltimore, MD), respectively. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and NCI-H522 in RPMI-1640, and both were supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/mL penicillin and streptomycin (Gibco).

Reagents and antibodies

Protease and phosphatase inhibitors were purchased from Roche. Afatinib and Trametinib were purchased from Selleckchem. Antibodies against phospho-EGFR-Y1068 (CST-3733), phospho-HER2-Y1248 (CST-2247), HER2 (CST-4290), phospho-HER3-Y1289 (CST-2842), HER3 (CST-12708), phospho-Akt-S473 (CST-4508), Akt (CST-2938), phospho-S6K-T389 (CST-9206), S6K (CST-9202), phospho-S6 (CST-2211), S6 (CST-2317), phospho-ERK-T202/Y204 (CST-4370), ERK (CST-4348), cleaved caspase 3 (CST-9664 and CST-9662), and GAPDH (CST-5174) were purchased from Cell Signaling. Anti-EGFR (SC-03) was purchased from Santa Cruz Biotechnology.

Cell lysis and western blot analysis

Cells were lysed and Western blot analysis was performed as described previously [25].

Cell proliferation assays

Sulforhodamine B (SRB) cytotoxicity assays were performed to test the effects on cell growth following treatment with Afatinib, Trametinib, or a combination, according to previously published protocols [26].

Measuring apoptosis with annexin V/propidium iodide staining

Cells were trypsinized, washed with PBS and Annexin V binding buffer, and re-suspended in 1 mL Annexin V binding buffer. Cells were then stained with 0.5 μL of Annexin V and 0.7 μL of propidium iodide (PI) for 15 minutes at room temperature. Stained cells were then analyzed by flow cytometry on the BD FACSCanto II™ Cell Analyzer (BD Biosciences). The data were analyzed using FCS Express 6 software. Experiments were performed twice in triplicate and statistical analysis was performed.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed using GraphPad

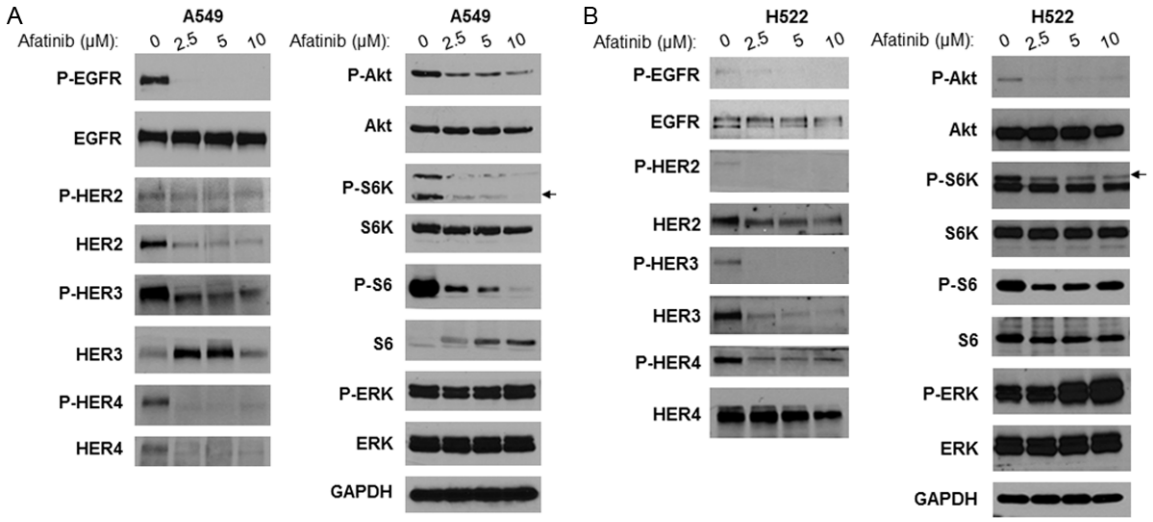

Figure 1. Inhibition of ErbB family members including EGFR, HER2, HER3, and HER4, as well as the Akt/mTOR signaling pathway, and induction of the ERK pathway by Afatinib in NSCLC cells. (A and B) Cell lysates from A549 cells (A) and H522 cells (B) treated with increasing doses of Afatinib for 24 hours were prepared and phosphorylation and total levels of EGFR, HER2, HER3, and HER4 (left panel), and Akt, S6K, S6, and ERK (right panel), as well as GAPDH expression, were detected by Western blot.

Prism, version 7.04 (GraphPad Software, Inc.). *P* values < 0.05 were considered statistically significant (**P* < 0.05; ***P* < 0.01; ****P* < 0.005).

Results

Afatinib inhibits ErbB family members, but induces the MEK/ERK pathway in NSCLC cells

Previous studies have shown that Afatinib inhibited EGFR, HER2, and HER4 [27-31]. We treated NSCLC cell line A549 with increasing doses of Afatinib for 24 hours and measured its effects on the phosphorylation of EGFR, HER2, HER3, and HER4. We found that Afatinib treatment dramatically inhibited phosphorylation of EGFR, HER2, HER3, and HER4 (**Figure 1A**, left panel). Consistent with this inhibition, phosphorylation of Akt, S6K, and S6 was also significantly inhibited, which indicated that Afatinib could inhibit the Akt/mTOR/S6K/S6 signaling pathway (**Figure 1A**, right panel). Interestingly, ERK phosphorylation was not inhibited at doses of 2.5 or 5.0 μM, but was induced at 10 μM (**Figure 1A**, right panel). In order to confirm our results in A549 cells, we also treated NCI-H522 cells, another NSCLC cell line, with similar doses of Afatinib for 24 hours and tested the effects on the same signaling pathways. Consistently, Afatinib inhibited phosphorylation of EGFR, HER2, HER3 and HER4 (**Figure 1B**, left panel), as well as Akt, S6K, and S6, but elevated ERK phosphorylation in a dose-dependent manner (**Figure 1B**, right panel). Our data indicated that Afatinib inhibited ErbB family members, and subsequently inhibited the Akt/mTOR pathway, whereas it induced the MEK/ERK pathway in NSCLC cells.

MEK inhibitors inhibit ERK but elevate the PI3K/Akt/mTOR cascade

Trametinib is a MEK inhibitor that recently gained FDA approval to treat NSCLC patients with RAS/BRAF mutations or amplifications [32-36]. We treated A549 cells with increasing doses of Trametinib and determined its effect on Akt and ERK phosphorylation. Trametinib led to dose-dependent inhibition of ERK phosphorylation, whereas it induced Akt phosphorylation (**Figure 2A**). In addition, Trametinib partially inhibited phosphorylation of S6K and S6, two downstream targets of mTOR (**Figure 2A**). To confirm our results, we also determined the effects of other MEK/ERK inhibitor treatments on phosphorylation of ERK and Akt, as well as the mTOR pathway. PD0325901 is a novel MEK inhibitor that is currently under clinical trials for use in treatment of multiple cancers [37-40]. Consistent with the Trametinib treatment data, PD0325901 inhibited ERK activation, but led to elevated Akt phosphorylation. Likewise, the phosphorylation of S6K and S6 was also inhib-

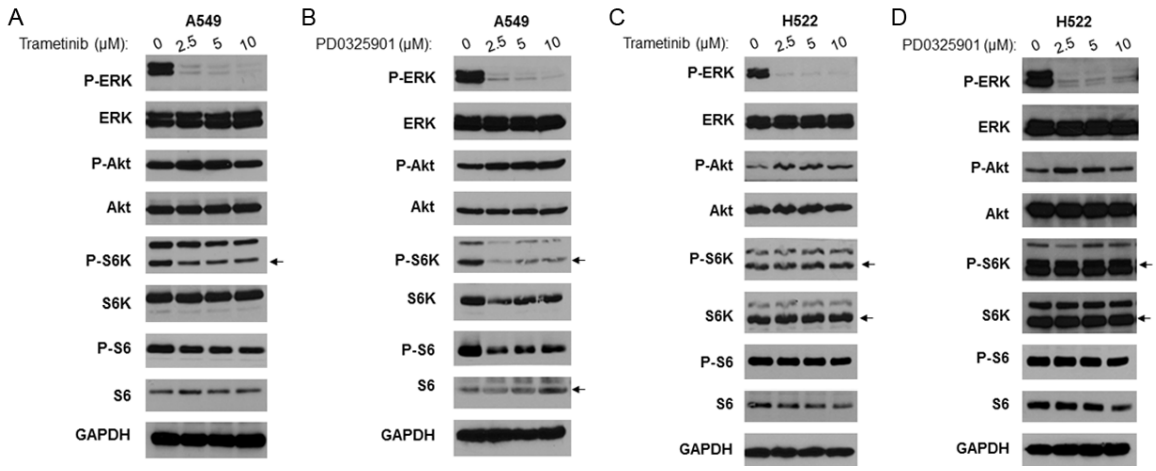

Figure 2. Inhibition of ERK phosphorylation and induction of Akt phosphorylation by MEK inhibitors, Trametinib or PD0325901 in NSCLC cells. (A and B) A549 cells were treated with different doses of Trametinib (A) or PD0325901 (B) for 24 hours, lysed, and phosphorylation and total levels of ERK, Akt, S6k, and S6, as well as GAPDH expression, were detected by Western blot. (C and D) H522 cells were treated with different doses of Trametinib (C) or PD0325901 (D) for 24 hours and proteins as described for (A and B) were detected by Western blot.

ited by PD0325901 treatment (**Figure 2B**). Next, we determined the effects of Trametinib or PD0325901 on the phosphorylation of ERK, Akt, S6K, and S6 in NCI-H522 cells. Interestingly, Trametinib (**Figure 2C**) and PD0325901 (**Figure 2D**) inhibited ERK phosphorylation and induced Akt phosphorylation but had no significant effects on S6K or S6 phosphorylation. In summary, MEK inhibitors blocked the MEK/ERK pathway and induced Akt phosphorylation, whereas they differentially affected mTOR/S6K/S6 pathways.

Inhibition of both PI3K/Akt and MEK/ERK pathways following combination of Afatinib and MEK inhibitors

We next determined whether a combination of Afatinib and MEK inhibitor could more effectively block phosphorylation of Akt, ERK, S6K, and S6. Consistent with the results in **Figure 1**, treatment of A549 cells with 5 μM Afatinib blocked Akt, S6K, and S6 phosphorylation, but had no effect on ERK phosphorylation (**Figure 3A**, lane 2 versus lane 1). In addition, Trametinib treatment completely blocked ERK phosphorylation and partially blocked S6K and S6 phosphorylation, which were accompanied by induction of Akt phosphorylation (**Figure 3A**, lane 3 versus lane 2). However, a combination of Afatinib and Trametinib blocked phosphorylation of Akt, ERK, S6K, and S6 (**Figure 3A**). Similarly, a combination of Afatinib with PD0325901 also blocked this phosphorylation in A549 cells (**Figure 3B**). Moreover, a combination of Afatinib with Trametinib (**Figure 3C**) or PD0325901 (**Figure 3D**) significantly blocked phosphorylation of Akt, ERK, S6K, and S6 in H522 cells when compared to either single inhibitor treatment regardless of the earlier results that MEK inhibitors (both Trametinib and PD0325901) induced S6K and S6 phosphorylation.

Afatinib and Trametinib synergistically inhibits cell proliferation

We next determined whether Afatinib and Trametinib could cooperate to inhibit cell proliferation. A549 cells were treated with vehicle control, or increasing doses of Afatinib or Trametinib, alone or combined, for 72 hours, after which we measured cell proliferation. Afatinib or Trametinib treatment alone led to inhibition of cell proliferation in a dose-dependent manner, whereas the combination increased inhibition of cell proliferation compared to either single treatment (**Figure 4A**). Furthermore, we found that the Afatinib and Trametinib combination increased inhibition of cell proliferation compared to the single treatments in H522 cells (**Figure 4B**). Furthermore, we utilized the CalcuSyn 2.0 software to calculate the combination index value (CI) according to the Chou-Talalay method [41]. A CI value greater than 1 is defined as antagonism, equal

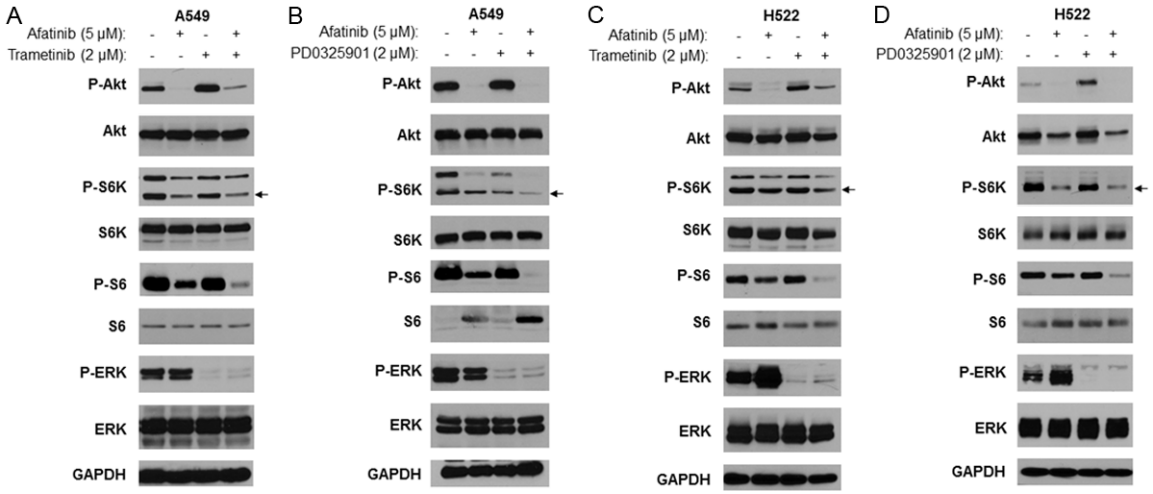

Figure 3. Inhibition of Akt/S6K/S6 and ERK pathways by a combination Afatinib with MEK inhibitors in NSCLC cells. (A and B) A549 (A) or H522 (B) cells were treated with DMSO control, Afatinib, Trametinib, or a combination for 24 hours and phosphorylation and total levels of Akt, S6k, S6, and ERK, as well as GAPDH expression were detected by Western blot. (C and D) A549 (C) or H522 (D) cells were treated with DMSO control, Afatinib, PD0325901, or a combination for 24 hours and the indicated proteins were analyzed by Western blot.

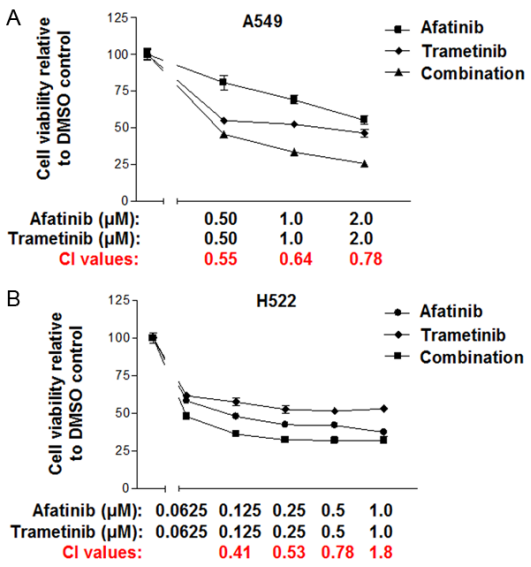

Figure 4. Afatinib and Trametinib synergistically suppress cell proliferation in NSCLC cells. (A and B) A549 (A) or H522 (B) cells were treated with different doses of Afatinib, Trametinib, or a combination for 72 hours and cell proliferation was determined by MTS assay. The experiments were performed in triplicate, and the combination index values (CI values) were determined according to the Chou-Talalay method.

to 1 as additive, and less than 1 as synergy. The CI values in combination treatments were less than 1 (**Figure 4A** and **4B**), which demonstrated that Afatinib and Trametinib synergistically inhibit cell proliferation.

Afatinib and Trametinib cooperate to induce apoptosis and cell death in NSCLC cells

To determine whether a combination of Afatinib and Trametinib could induce an increase in apoptosis compared to either single treatment, we first tested their effects on caspase-3 cleavage. Treatment with Afatinib (5 μM) alone led to no caspase-3 cleavage, while treatment with 2 μM Trametinib caused caspase-3 cleavage; however, the combination of Afatinib and Trametinib induced significant caspase-3 cleavage after 48 hours in A549 cells (**Figure 5A**). Similar results were found in H522 cells (data not shown). Apoptosis measurement by Annexin V showed that Afatinib or Trametinib treatment alone increased apoptosis and cell death compared to vehicle control treatment, whereas the combination significantly increased apoptosis and cell death in A549 cells (**Figure 5B** and **5C**). Similarly, Afatinib and Trametinib cooperated to induce apoptosis and cell death in H522 cells (data not shown). Our data indicate that a combination of Afatinib and MEK inhibitors synergistically induced apoptosis and cell death in NSCLC cells.

Afatinib and Trametinib inhibit cell cycle G1/S transition in NSCLC cells

We further determined the effects of Afatinib, Trametinib, or their combination on the cell cycle in H522 cells. Treatment with only Afatinib

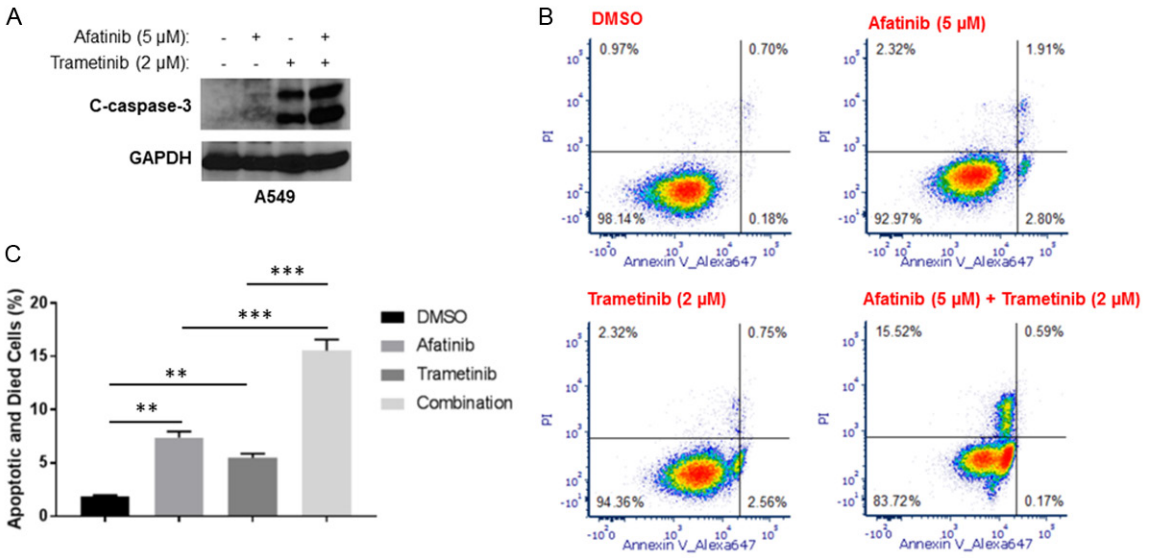

Figure 5. Afatinib and Trametinib cooperate to induce apoptosis in NSCLC cells. (A) A549 cells were treated with vehicle control, Afatinib, Trametinib, or a combination for 48 hours, lysed, and expression of cleaved caspase-3 was detected by Western blot. (B) A549 cells were treated with vehicle control, Afatinib, Trametinib, or a combination for 48 hours. Cell apoptosis was measured by Annexin V. (C) Experiments in (B) were performed twice in triplicate, early and late stage apoptotic and dead cells were counted, and results were presented as mean ± SD. Student's t-test using GraphPad Software were performed for statistical analysis. *P* values less than 0.05 were considered statistically significant.

(0.5 μM) or Trametinib (0.5 μM) led to significant G1-cycle arrest, while the combination increased G1-cycle arrest in comparison to either single treatment (**Figure 6A** and **6B**). Similar results were found in A549 cells (data not shown). Our data indicated that inhibition of cell proliferation by Afatinib, Trametinib, or a combination involved G1-cycle arrest.

Discussion

In this study, we showed that the ErbB family inhibitor Afatinib inhibited the phosphorylation of EGFR, HER2, HER3, and HER4, as well as Akt, yet still induced ERK phosphorylation. MEK/ERK inhibition by MEK inhibitors such as Trametinib or PD0325901 inhibited phosphorylation of ERK, but up-regulated Akt. A combination of Afatinib and MEK inhibitor blocked all ErbB family members, as well as Akt and ERK, which resulted in significant inhibition of cell proliferation, apoptosis induction, and cell cycle arrest. These data demonstrated that co-targeting ErbB family and MEK/ERK pathways through a combination of Afatinib and Trametinib could more effectively treat NSCLC.

Previous studies have also demonstrated that inhibition of the PI3K/Akt pathway by PI3K inhibitors caused reactivation of the MER/ERK pathway through feedback mechanisms [42-47]. In this study, our data indicate that Afatinib inhibits the Akt/mTOR pathway consistent with PI3K inhibitor treatment. However, it remains unclear whether Afatinib induces ERK activation through PI3K inhibition in NSCLC cells.

Our data are consistent with results by multiple groups who demonstrated that inhibition of MEK/ERK by MEK inhibitors caused up-regulation of the PI3K/Akt pathway, and a combination of MEK and PI3K inhibitors synergistically inhibited cell proliferation in multiple cancers, including NSCLC [44-48]. An important study by Hutchinson, *et. al.*, reported that MEK inhibition by Trametinib up-regulated ErbB family and Akt activities, which, in turn, regulated MEK inhibitor sensitivity in a subset of driver-negative melanoma [48]. It is not clear whether Trametinib activation of Akt occurs through ErbB family activation in NSCLC. In addition, since there are currently no FDA-approved PI3K inhibitors for use in NSCLC treatment, one would expect that Afatinib, in combination with MEK inhibitor Trametinib, could more effectively suppress NSCLC through ErbB family and PI3K inhibition.

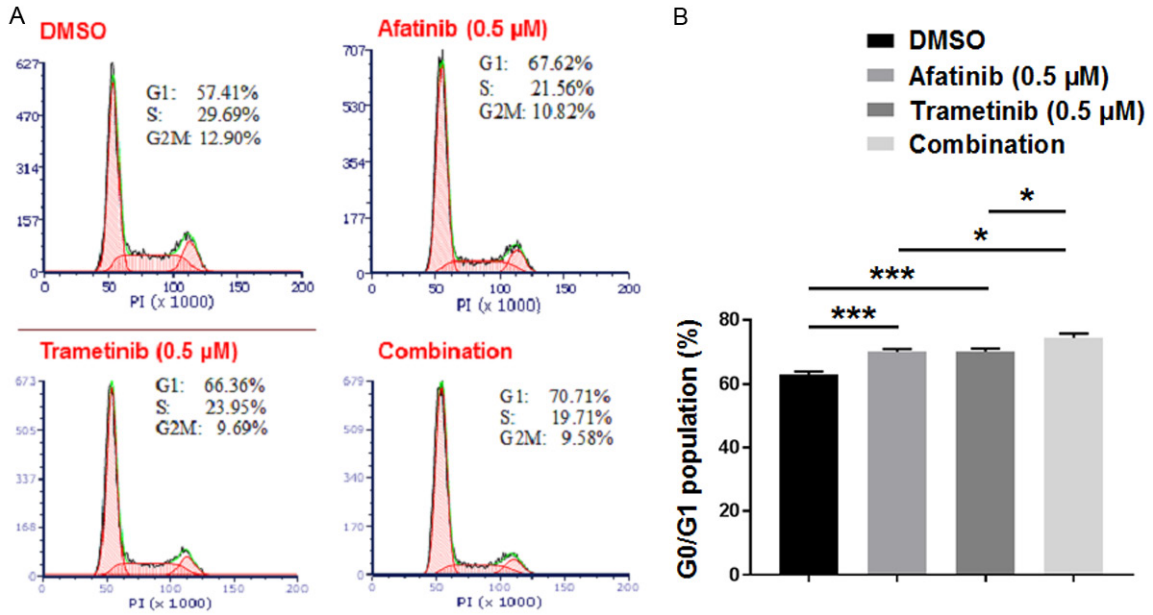

Figure 6. Afatinib and Trametinib led to inhibition of cell cycle G1/S transition in NSCLC cells. (A) H522 cells were treated with vehicle control, Afatinib, Trametinib or a combination for 48 hours and cell cycle distribution was assessed by flow cytometry. (B) Experiments in (A) were performed twice in triplicate, cell cycle distribution in G1, S, and G2/M were counted. Results were presented as mean ± SD. Student's t-test using GraphPad Software were performed for statistical analysis. *P* values less than 0.05 were considered statistically significant (*P<0.05; **P<0.01, and ***P<0.005).

Interestingly, we found that although MEK inhibitors blocked the MEK/ERK pathway and induced Akt phosphorylation, they differentially affected mTOR/S6K/S6 pathways in a cell type-specific manner (**Figure 3**). It has been shown that Akt activated mTOR through TSC2 phosphorylation and inhibition [49-52]. Additionally, activation of MEK/ERK pathway also led to up-regulation of the mTOR/S6K/S6 pathway through ERK phosphorylation and TSC2 inhibition [45, 53, 54]. The differences of the effects of MEK inhibitors on mTOR activation in A549 and H522 cells could be due to the fact that Akt or ERK signaling differentially controls mTOR activity in a cell type-specific manner. It would be interesting to determine more detailed mechanisms for this phenomenon.

Afatinib is usually used to treat NSCLC patients with EGFR amplification of mutations, whereas MEK inhibitors are used to treat patients whose tumors have BRAF signaling mutations and/or abnormal activation [6, 11-14, 16-21]. It is not clear whether Afatinib will induce the MEK/ERK pathway or whether MEK inhibitors will induce elevated Akt activity in these NSCLC tumors. It is also important to determine whether the combination of Afatinib with Trametinib could more effectively suppress growth in tumors with EGFR or BRAF mutations.

Afatinib is most often used to treat NSCLC patients with EGFR amplification of mutations, whereas MEK inhibitors primarily treat patients whose tumors have BRAF signaling mutations and/or abnormal activation of MEK/ERK [6, 11-14, 16-21]. A549 cells have wild-type EGFR and KRAS mutations, whereas H522 cells have both wild-type EGFR and KRAS [55]. Therefore, Afatinib induction of the MEK/ERK signaling pathway could be KRAS-independent. It is not clear whether Afatinib will induce the MEK/ERK pathway or whether MEK inhibitors will induce elevated Akt activity in NSCLC tumors with different genetic backgrounds. An important study by Shi, *et. al.,* showed that modulation of MEK/ERK-dependent Bim and Mcl-1 degradation by MEK inhibitor Selumetinib is crucial to regulate EGFR-mutant NSCLC cell sensitivity and resistance to the third generation EGFR inhibitor, AZD9291 [56]. It is also important to determine whether the combination of Afatinib and Trametinib could more effectively suppress growth in tumors with EGFR or BRAF mutations.

It should be noted that a very recent study by Yee, PS, *et. al.,* showed that Afatinib and Trametinib synergistically inhibited Head and Neck Squamous Cell Carcinoma (HNSCC) growth in pre-clinical models of oral squamous cell carcinoma [57]. These findings are very similar to our results. In addition, we recently also found that a combination of Afatinib and PD0325901 dramatically inhibited cisplatin-resistant HNSCC proliferation [58]. Therefore, co-targeting of ErbB family and MER/ERK pathways could be effective to inhibit cancer proliferation and survival in multiple cancer types.

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Disclosure of conflict of interest

None.

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