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ORIGINAL ARTICLE

Combination of MEK Inhibitor and the JAK2-STAT3 Pathway Inhibition for the Therapy of Colon Cancer

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Abstract

The study aimed to investigate the reason of HCT116 cell resistance to MEK inhibitor, and the combination treatment effects of MEK inhibitor AZD6244 and JAK2/STAT3 inhibitor AG490 on colon cancer in vitro and in vivo, including cell viability, apoptosis, and explore the partial mechanisms focused on AZD6244 promoted the activation of JAK2-STAT3 pathways. In vitro, we examined the HCT116 cell viability by CCK8, cell apoptosis by flow cytometry; Western blot measured p-ERK, p-JAK2, p-STAT3 and STAT3 expression. In vivo, nude mice were subcutaneously injected by HCT116 cells. The tumor volume and weight were detected. HCT116 cell resistance to MEK inhibitor AZD6244, which inhibited the activation of ERK and promoted the activation of JAK2-STAT3 signaling. The combination treatment of AZD6244 and AG490 significantly inhibited cell viability and induced cell apoptosis, and completely inhibited the activation of ERK and JAK2-STAT3 signaling. Combination treatment of AZD6244 and AG490 had a stronger effect than that of AZD6244 as a monotherapy in vitro and in vivo. The treatment of AZD6244 on K-Ras mutations HCT116 cells promoted the activation of JAK2/STAT3 signaling. JAK2/STAT3 inhibitor AG490 synergistically increases effects of AZD6244 on colon cancer in vitro and in vivo. Collectively, these results provide a rationale for combining inhibitors of the JAK/STAT pathway and MEK inhibitors to reduce the potential impact of drug resistance.

Keywords MEK inhibitors · Colon cancer · JAK2 · STAT3

Introduction

Colorectal cancer (CRC) is the third most frequent cancer in men, after lung and prostate cancer, and is the second most frequent cancer in women after breast cancer [1]. It is also the third cause of death in men and women separately and is the second most frequent cause of death considering both genders. Despite growing understanding of oncogenesis and successful identification of proto-oncogenes and tumor suppressor genes involved in the tumorigenesis of CRC, the biological and molecular mechanisms in CRC are poorly understood [2]. Surgery represents the mainstay of treatment in early cases but often patients are primarily diagnosed with an advanced stage of disease, and sometimes also distant metastases are present.

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Ras proteins play a direct causal role in human cancers. Oncogenic mutant Ras proteins are highly prevalent in multiple human tumors [3]. Moreover, cancers with a high prevalence of K-Ras mutations, such as pancreatic carcinomas, colorectal cancers, and lung cancers are difficult to treat. Clinical outcomes are poor even with aggressive and toxic medical interventions [4, 5]. Oncogenic K-Ras mutations occur in 45% of colorectal carcinomas, and the resistance to MEK inhibitors are associated with these mutations [6]. Suppressing K-Ras mutants has become a promising concept for new therapies. However, K-Ras mutant has been proven highly tricky to the drug, and no small molecular K-Ras mutant inhibitors are available for clinical trials yet [7]. With the failure of directly inhibiting K-Ras mutant, inhibiting downstream effectors of K-Ras appears a promising alternative [8]. K-Ras signals via downstream effectors such as MAPK, PI3K/AKT and STAT3 signaling cascade [9]. It has been shown MAPK signaling plays a more critical role in tumor maintenance than PI3K signaling in K-Ras mutant pancreatic and lung tumors. Drug development efforts have mostly focused on components of the classical Ras-activated MAPK pathway. As part of this path, MEK1/2, a dual-specific kinase

required for activation of ERK1/2, plays crucial roles in tumorigenesis, cell proliferation and inhibition of apoptosis. Therefore, MEK1/2 inhibition is an attractive therapeutic strategy in some cancers [10]. Inhibiting the downstream effector MEK1/2 has proven to be effective in preclinical studies. Several MEK inhibitors have been developed and are under investigation in clinical studies in colon cancer [11, 12]. MEK inhibitors have the potential to inhibit tumors dependent on MAPK signaling pathway. Despite the short-term effectiveness of MEK inhibitors in treating various cancers in preclinical and clinical settings, some cancer resistant to these inhibitors in long-term studies [13, 14]. However, the underlying mechanism is not very clear. Elucidating the mechanism of cancer cell resistance to MEK inhibitors is critical for the development of more effective therapies.

Some results have shown that the MEK inhibition in K-Ras mutant pancreatic cancer unexpectedly induced STAT3 phosphorylation/activation.STAT3 is an oncogene, which is constitutively activated in multiple types of human cancers and contributes to cancer progression [15].

In the present study, we assess the effects of MEK inhibitors AZD6244 on the JAK2-STAT3 pathway in Colorectal cancer cell line HCT116 and further discuss the effects of the combination of MEK inhibitor and the JAK2-STAT3 pathway inhibition for the therapy of colon cancer in vitro and in vivo. This study provides an experimental basis for Elucidating the mechanism of cancer cell resistance to MEK inhibitors, which is critical for the development of more effective therapies.

Materials and Methods

Reagents

AG490, JAK2-STAT3 pathway inhibitor, was purchased from Sigma (USA), was dissolved in ethanol and added into the culture medium at the indicated concentration. AZD6244 was obtained from Selleck Chemicals (USA), dissolved in DMSO at the appropriate concentration; Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from Gibco Co., Ltd. (USA). CCK kit from Dojindo Molecular Technologies, Inc., (Japan). The Annexin V-FITC and PI-PE apoptosis kit were purchased from Becton, Dickinson, and Company (USA). The primary antibody p-JAK2, p-STAT3, STAT3, p-ERK, and β-actin were purchased from Santa Cruz Co., Ltd. (USA); enhanced chemiluminescence reagent was obtained from Pierce Biotechnology Inc. (USA).

Cell Culture

The HCT116 human colon cancer cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences, (China). Cells were maintained in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

Administration and Cell Viability Assay

HCT116 cells were treated with AZD6244 at different concentration (5, 10, 20 μM) and a different time (24 h, 48 h, 72 h). Each group HCT116 cells (10^4/well) were plated in 96-well plates, six replicates, and then incubated at 37 °C with 5% CO_2 for 48 h. 4 h before the end of the incubation, 20 μl of CCK-8 was added to each well. The absorbance was measured by Multiscan Spectrum (BioTek Co., Ltd., USA). The results were described as the average of 6 counts.

According to the results of cell viability, we choose the appropriate time (24 h), and concentration of single-agent or combination of MEK inhibitor AZD6244 (5 μM) and the JAK2-STAT3 pathway inhibition AG490 (50 μM) was added into the HCT116 cells.

In vivo tumor experiments, AZD6244 were resuspended in water supplemented with 0.5% hypromellose and 0.1% Tween 80 and administered by oral gavage twice daily as described previously [16].

Flow Cytometric Assay

Each group of cells (5×10^5cells) was transferred to a tube to which annexin V (5 μL) and propidium iodide (5 μL) was added. The cells were then allowed to incubate at room temperature for 15 min and analyzed using FC500 flow cytometer (Beckman Coulter). Data were analyzed with FCS Express 4. P1, dead cells; P2, dead/late apoptotic cells; P3, normal cells; P4, early apoptotic cells.

In Vivo Tumor Experiments

To confirm the effects of a combination of MEK inhibitor AZD6244 (25 mg/kg) and the JAK2-STAT3 pathway inhibition AG490 (5 mg/kg) on the HCT116 induced tumor in vivo, we subcutaneously injected 4×10^5 HCT116 cells into the nude mice (3–4 weeks old, $n = 8$). After seven days of tumor inoculation, mice have treated with AZD6244 (oral gavage twice daily) or AG490 (injection intraperitoneally every day) treatment for two weeks.

Tumour growth was determined by measuring the tumor volume, which was assessed every four days, for seven times. The tumor volume was calculated according to the formula (volume = 1/2 length × width^2). At the end of the experiment (at day 28), the tumor was removed and weighed.

Western Blot Analysis

Each group of cells was washed with PBS three times, then lysed in cell lysis buffer with one mM PMSF, ten mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, followed by centrifugation (12,000 rpm) for 10 min and the supernatant was obtained. A total of 20 μg of protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (PVDF membrane, Millipore, USA). The PVDF membranes were incubated with primary antibodies of p-JAK2, p-STAT3, STAT3, p-ERK (1:1000) and mouse monoclonal anti-β-actin (1:5000) at 4 °C overnight. Then the membranes were incubated with secondary antibodies conjugated with HRP, and detection was achieved by measuring the chemiluminescence of the blotting agent after exposure of the filters on films. Finally, the densities of the bands were quantified with a computerized densitometer (ImageJ Launcher, Broken Symmetry Software). Equivalent protein loading and transfer efficiency were verified by staining for β-actin.

Statistical Analysis

Data are expressed as the mean and standard deviation (SD). The analysis of variance (ANOVA) and Student's t test was used to determine significant differences between groups. Calculations were performed using the SPSS version 11.5 statistical package. Values of $P < 0.05$ were considered significant.

Results

The Inhibited Effect of AZD6244 on the HCT116 Cell Viability Weakened, Meanwhile AZD6244 Promoted STAT3 Activation

To evaluate the effect of MEK inhibitors AZD6244 on HCT116 cell viability, we first treated HCT116 cells with different concentrations of AZD6244 (5, 10, 20 μM) for 24 h, 48 h, and 72 h. At a high concentration (5, 10 μM), AZD6244 treatment did not significantly inhibit cell viability at 24, 48, 72 h, while AZD6244 (20 μM) can inhibit HCT116 viability until 48 h (Fig. 1a).

To probe the resistant mechanism of the HCT116 cell to AZD6244 treatment, we detected the expression and activation of the related protein. AZD6244 (5, 10 μM) can significantly decrease the activation of ERK, at the same time AZD6244 promoted STAT3 activation, which may result in invalid of AZD6244 (5, 10 μM) on cell viability. AZD6244 did not affect the expression of STAT3 (Fig. 1b).

MEK Inhibitors AZD6244 Promoted JAK2-STAT3 Pathway Activation and the Combination of AZD6244 and JAK2-STAT3 Inhibitor AG490 Enhanced the Inhibited Effected on the HCT116 Cell Viability

Compared to control groups, AZD6244 (5 μM) alone and AG490 (50 μM) alone had no effect on cell viability. Whereas the combination of AZD6244 (5 μM) and AG490 (50 μM) significantly inhibited the cell viability compared with AZD6244 and AG490 respectively (Fig. 2a).

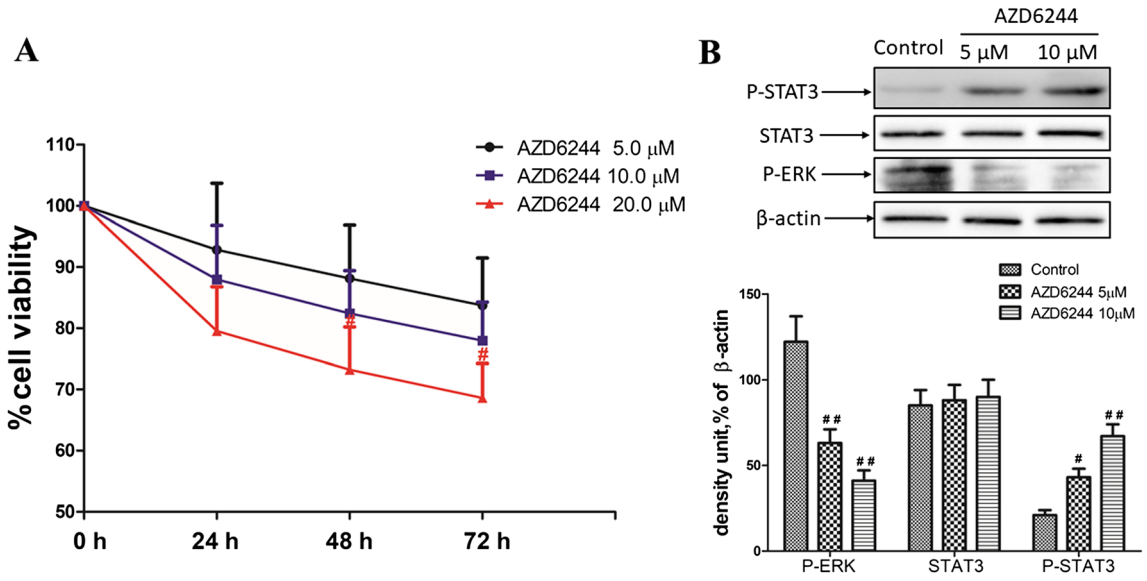

Fig. 1 The effect of MEK inhibitors AZD6244 on HCT116 cell viability and related proteins. **a**: The effect of MEK inhibitors AZD6244 on HCT116 cell viability. Data are expressed as mean ± SD with 6 replicates for each group. **b**: The effect of AZD6244 on p-ERK and p-STAT3 by Western blot. β-actin was measured as an internal control. **c**: Densitometric analysis from the above immunoblots is shown as a bar chart. The results are representative of three independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus control

We further observed the effect of combination AZD6244 and AG490 on the related protein. AZD6244 (5 μM) alone can inhibit the activation of ERK as expected, and upregulated the expression of p-JAK2, p-STAT3 as well. Whereas the combination of AZD6244 (5 μM) and AG490 (40 μM) significantly inhibited not only p-ERK but also p-JAK2, p-STAT3 (Fig. 2b). The results showed that AZD6244 inhibited the activation of ERK, at the same time promoted the activation of JAK2-STAT3. AG490 as the inhibitor of the JAK2-STAT3 pathway, the combination of AZD6244 and AG490 can completely block the activation of two paths.

Combination Treatment of AZD6244 and AG490 Promoted HCT116 Cell Apoptosis

P2 (dead/late apoptotic cells) + P4 (early apoptotic cells) represented the percent of apoptosis cells. Compared to control groups, AZD6244 (5 μM) alone and AG490 (50 μM) alone had no effect on cell apoptosis. Whereas the combination of AZD6244 (5 μM) and AG490 (50 μM) significantly inhibited the cell viability compared with AZD6244 and AG490 respectively. These findings suggested that AG490 synergistically increased the effects of AZD6244 on HCT116 cell apoptosis (Fig. 3).

The Effects of Combination Treatment of AZD6244 and AG490 on Nude Mice In Vivo

To observe the effects of a combination treatment of AZD6244 and AG490 in vivo, we detected tumor volume and tumor weight in the HCT116 colon cancer cells in nude mice. As shown in Fig. 4, HCT116 cells formed large tumors in nude mice from day 16. AZD6244 (25 mg/kg) did not significantly inhibit tumor proliferation compared to the control group (no treatment) in vivo, including tumor volume and tumor weight. Combination treatment of AZD6244 and AG490 had a stronger inhibitory effect than that of the AZD6244 alone.

Discussion

Members of the Ras protein subfamily (H-, K- and N-Ras) function as molecular switches in cellular signal transduction. The Ras/MAPK pathway is the best characterized of the mammalian MAPK signal transduction networks, consisting of the Ras proteins, a family of small G-coupled molecules, the Raf kinases (MAP3K), the MAP2K kinases (MEK1 and MEK2) and the pathway distill kinases ERK1 and ERK2 [17].

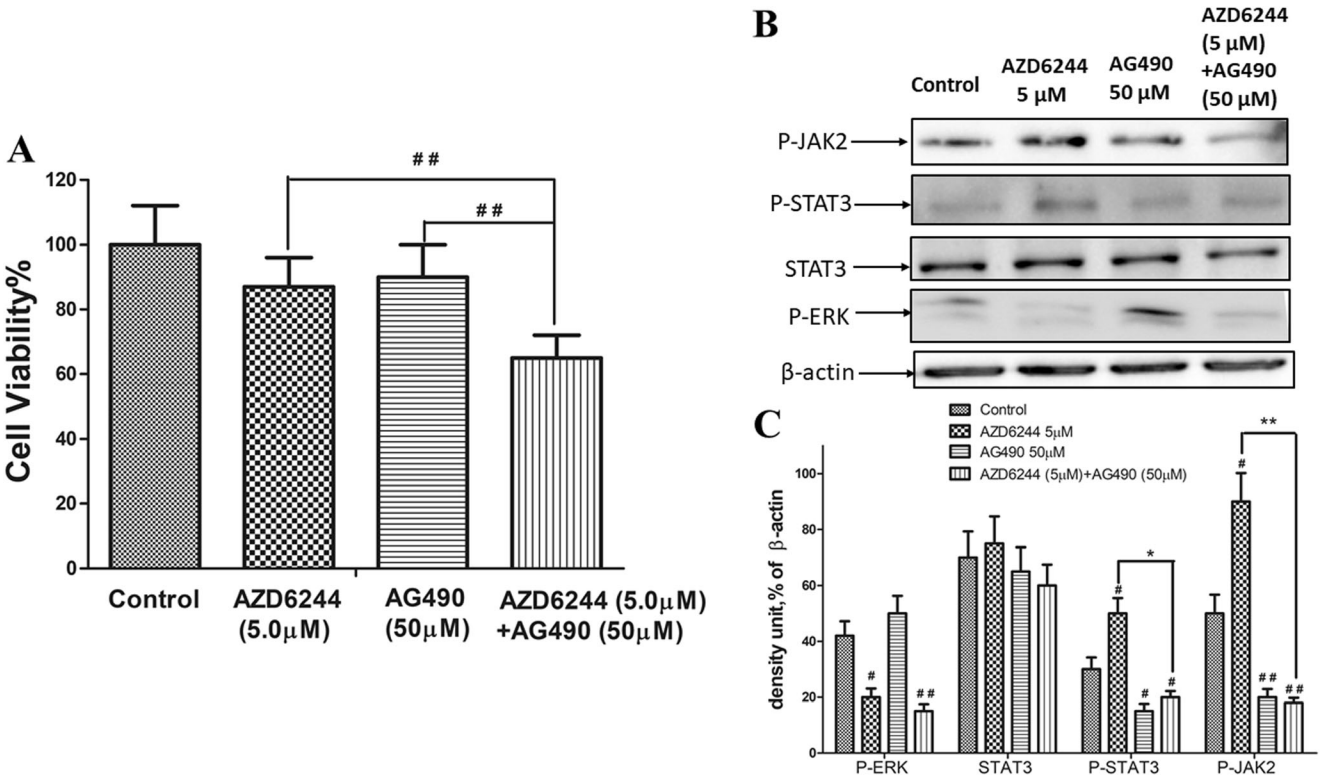

Fig. 2 The effect of combination AZD6244 and AG490 on HCT116 cell viability and the activation of ERK and JAK2-STAT3 pathway. **a**: The effect of combination AZD6244 and AG490 on HCT116 cell viability. Data are expressed as mean ± SD with 6 replicates for each group. **b**: The effect of combination AZD6244 and AG490 on p-ERK, p-JAK2 and p-STAT3 by Western blot. β-actin was measured as an internal control. **c**: Densitometric analysis from the above immunoblots is shown as a bar chart. The results are representative of three independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus control; $^{*}P<0.05$, $^{**}P<0.01$ versus AZD6244 group

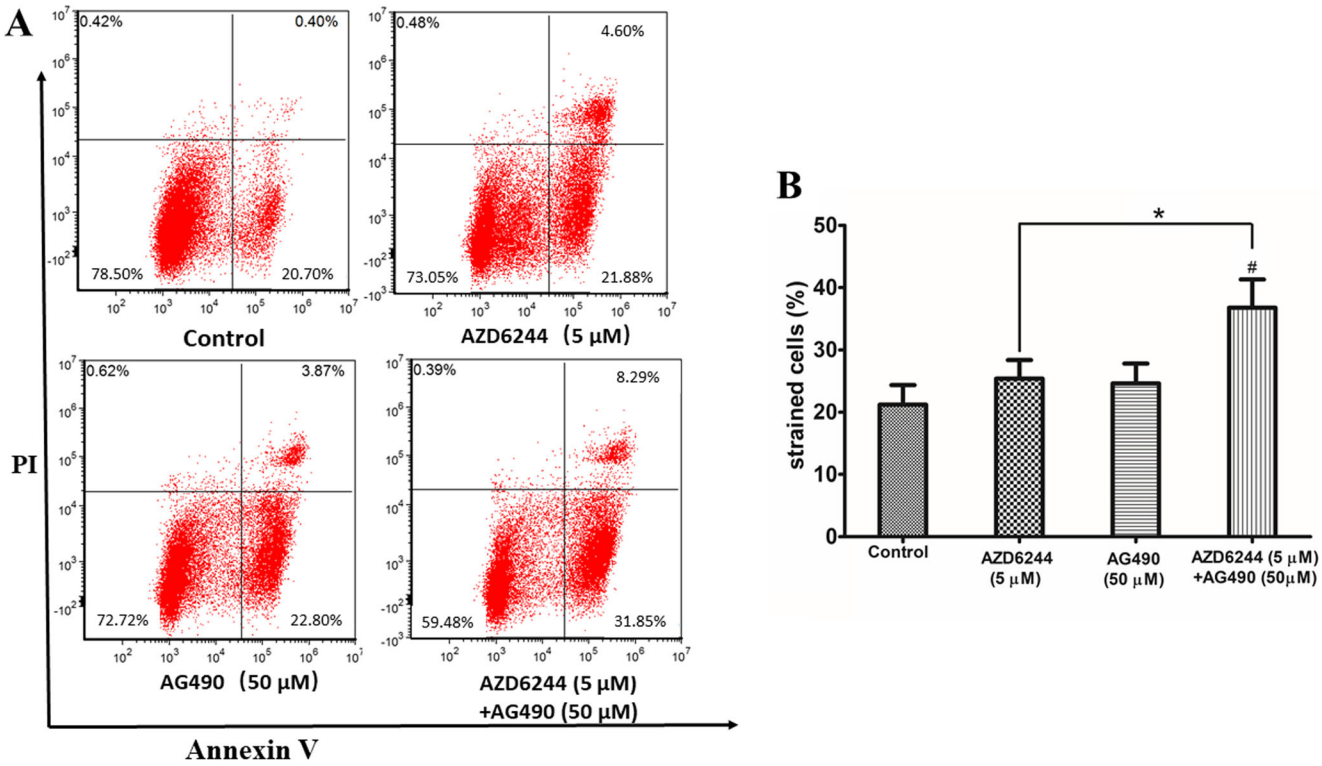

Fig. 3 Combination treatment of AZD6244 and AG490 promoted MCF-7 cell apoptosis. HCT116 cells were treated by each treatment approach. These cells were harvested, then stained with antibodies annexin V and propidium iodide and analyzed by flow cytometry. **a**: Dot plot stained with annexin V and propidium iodide. **b**: Histogram presentation of apoptotic cell percentages. Data are shown as the means ±SD from 3 replicate experiments. $^{\#}P<0.05$ versus control; $^{*}P<0.05$ versus AZD6244 group. P1, dead cells; P2, dead/late apoptotic cells; P3, normal cells; P4, early apoptotic cells

Approximately 30% of malignancies contain activating mutations in a Ras proto-oncogene, with pancreatic (90%), colon (50%) and thyroid (50%) carcinomas demonstrating the highest prevalence. Nearly 50% of colon cancers harbor activating mutations in Kras, whereas Nras mutations occur in a smaller percentage (~5%), but rarely H-Ras, and seem to arise at a later stage in the development of malignancy [18]. The basis for this difference remains unclear.

The Ras/MAPK network is frequently deregulated in malignancy and contributes to many of the hallmarks of oncogenesis, including abnormal cellular proliferation, impaired apoptosis, enhanced angiogenesis, metastasis and the development of drug resistance. MEK1 and MEK2 are ideal targets; not only do they play a key role in tumor development and progression [19], they have narrow substrate specificities and distinctive structural characteristics. Numerous potent, selective allosteric MEK inhibitors have been developed and have undergone clinical evaluation of their ability to inhibit tumor growth [20]. Preclinical studies showed efficient inhibition of phosphorylation of ERK1 and ERK2, which correlates with potent growth inhibition in cancer cell lines with mutant RAS with elevated phosphorylation of MEK1 and MEK2 [21]. However, most MEK inhibitors have demonstrated limited clinical efficacy as single-agent therapies [19, 20].

AZD6244 is a potent MEK inhibitor that has demonstrated significant tumor suppressive activity in some preclinical solid tumor models [22, 23]. Whereas K-Ras mutated tumors did not show ideal effect. Our results showed that at a high concentration, AZD6244 treatment did not significantly inhibit cell viability though it inhibited ERK activation. AZD6244 (20 μM). As a result of these studies, there has not been an advocacy for AZD6244 as a monotherapy.

K-Ras signals via downstream effectors such as MAPK, PI3K/AKT and STAT3 signaling cascade [9, 24]. Abnormalities of the JAK/STAT pathway are involved in the pathogenesis of several cancers [25, 26]. In the present study, we found that AZD6244 significantly inhibited MEK downstream effectors ERK activation, at the same time promoted STAT3 activation, which may be the reason for resistance to MEK inhibitor.

Therefore, we used JAK2/STAT3 inhibitor AG490 to observe the effect of combination with AZD6244. AZD6244 alone and AG490 alone did not affect cell viability, whereas the combination of AZD6244 and AG490 significantly inhibited the cell viability compared with AZD6244 and AG490 respectively. In the apoptosis experiments, we had the similar results. We further detected the related protein, and the results showed that the combination of AZD6244 and AG490 significantly inhibited ERK and STAT3

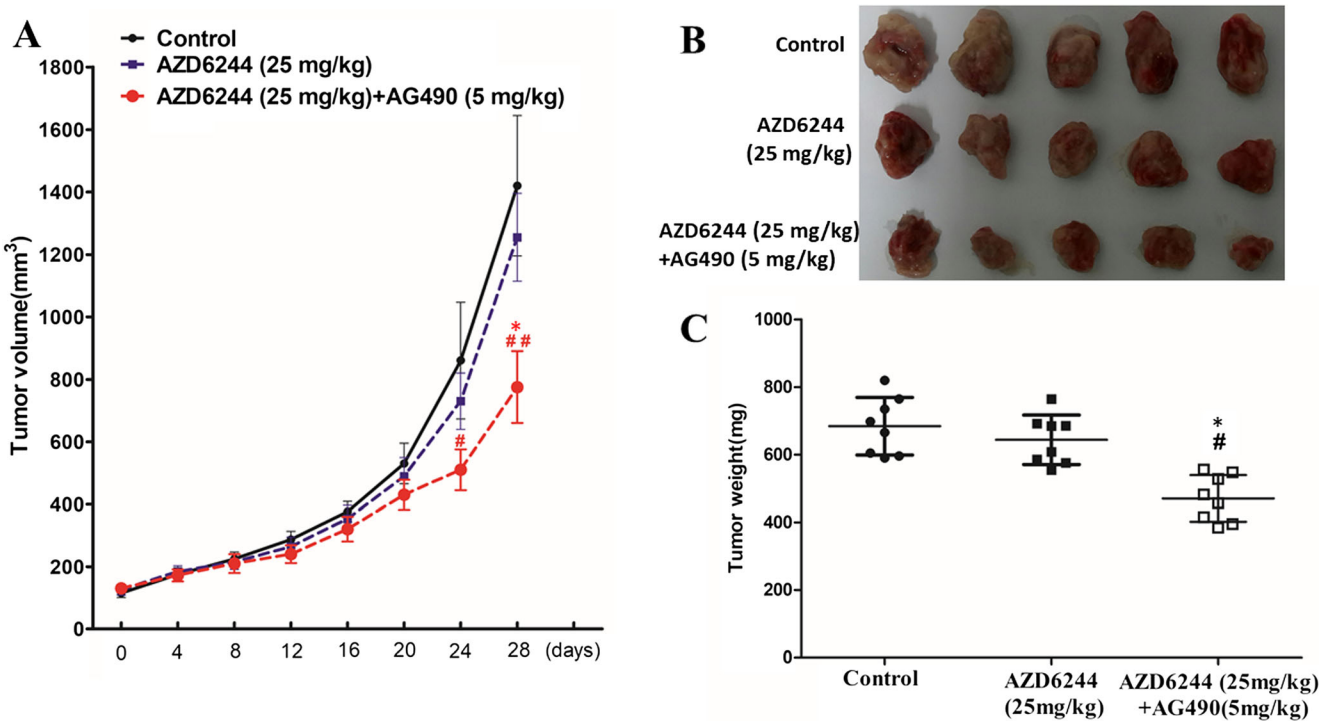

Fig. 4 The effects of combination treatment of AZD6244 and AG490 in vivo. **a**: The effects of combination treatment of AZD6244 and AG490 on the tumor volume were measured. HCT116 cells were transplanted subcutaneously into nude mice. Tumour growth was monitored every 4 days. Data are expressed as mean ± SD with 8 mice for each group. **b**: Representative tumor masses of each group. **c**: The tumor weight of combination treatment of AZD6244 and AG490 were measured. Data are shown as the means±SD from 8 animals. $^{\#}P<0.05$ versus control; $^{*}P<0.05$ versus AZD6244 group

activation, which ultimately inhibited cell viability and induced apoptosis.

HCT116 cells carry K-Ras mutations, the resistance of the cell lines to AZD6244 coincides with activation of JAK2/STAT3 following MEK inhibition. AZD6244 combined with JAK2/STAT3 inhibition synergistically induced apoptosis in the cells. In vivo, the combination treatment of AZD6244 and AG490 obviously inhibited tumor volume and tumor weight in the nude mice caused by the HCT116 cell. These results suggest that AG490 synergistically increases effects of AZD6244 on breast cancer in vitro and in vivo. This effect can be explained that loss of negative feedback regulation in the MAPK pathway after MEK inhibition could be a significant cause for the lack of clinical efficacy [19, 20]. That was to say that MEK inhibitors reduce the activity of ERK and then relieve the feedback inhibition of RAF, resulting in enhancement of RAF kinase and activation of JAK2/STAT3 [27]. However, no essential studies on the exact molecular mechanisms of the effects.

Conclusion

The current study demonstrated that the treatment of AZD6244 on K-Ras mutations HCT116 cells promoted the activation of JAK2/STAT3 signaling. The combination treatment of MEK inhibitors AZD6244 and JAK2/STAT3 inhibitor AG490 inhibited cell viability and induced apoptosis compared to AZD6244 as a monotherapy. AG490 synergistically increases effects of AZD6244 on colon cancer in vitro and in vivo. Collectively, these results provide a rationale for combining inhibitors of the JAK/STAT pathway and MEK inhibitors to reduce the potential impact of drug resistance.

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Author's Contribution Yanan zhu contributed to the design of the study, served as the study coordinator, and helped to review the manuscript. Jianying Jin designed the study, performed experiments, collected data and wrote the manuscript. Qunyi Guo, Jingjing Xie, Dan Jin helped perform experiments and interpret data. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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