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AZD3759 enhances radiation effects in non-small-cell lung cancer by a synergistic blockade of epidermal growth factor receptor and Janus kinase-1

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ABSTRACT

AZD3759 is a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) on the basis of gefitinib and has been proven to enter the central nervous system. Although the promising antitumor effects of AZD3759 on non-small cell lung cancer (NSCLC) have been demonstrated in clinical trials, the regulatory effects of this inhibitor on the antitumor efficacy of radiation (RA) are unclear. The present study aimed to compare the effects of AZD3759 and osimertinib on RA efficacy in NSCLC and explore the potential mechanism of action of AZD3759. We found that the survival in RA-treated NSCLC cells was significantly decreased by treatment with 500 nM AZD3759 and osimertinib at the RA dosage of 8 Gy. The apoptotic rate, cell cycle arrest, and DNA damage in RA-treated NSCLC cells and brain metastasis in RA-treated xenograft nude mice were significantly enhanced by the co-administration of AZD3759 and osimertinib, respectively. In addition, AZD3759 showed a significantly stronger efficacy than osimertinib did. Mechanistically, the receptor tyrosine kinase signaling antibody array revealed that Janus kinase-1 (JAK1) was specifically inhibited by AZD3759, but not by osimertinib. The effects of AZD3759 on RA efficacy in PC-9 cells and in a brain metastasis animal model were significantly abolished by the overexpression of JAK1. Collectively, our results suggested that AZD3759 promoted RA antitumor effects in NSCLC by synergistic blockade of EGFR and JAK1.

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NSCLC; EGFR mutant; JAK1

Introduction

Metastatic encephaloma is a common type of intracranial tumor, and the morbidity of this disease is approximately 10-fold higher than that of intracranial primary tumors. Approximately half of the metastatic encephaloma cases originate from lung carcinoma, of which 85% are diagnosed as non-small cell lung cancer (NSCLC) [1]. In the progression of NSCLC, brain metastasis occurs in 30–50% of NSCLC patients [2]. It has been reported that a higher incidence rate of brain metastasis is observed in the presence of epidermal growth factor receptor (EGFR) mutations [3]. The survival rate and quality of life in patients with NSCLC are significantly impacted by brain metastasis. Effective methods to control the brain metastasis of NSCLC are urgently required in the clinic [4,5].

The blood-brain barrier (BBB) significantly limits the application of chemotherapy drugs

in the treatment of brain metastasis of NSCLC, which is mainly treated by the local interventions, including radiotherapy (RA) and surgery [6]. It was recently reported that in NSCLC patients with brain metastases and certain EGFR mutations, a relatively high intracranial remission rate and prolonged survival can be achieved by treatment with small molecules EGFR tyrosine kinase inhibitors (EGFR-TKIs) [7,8]. In a phase II clinical trial in South Korea, in NSCLC patients with brain metastases and EGFR-TKI sensitive mutations, an overall response rate of approximately 70–89% and overall survival (OS) of 15.9–21.9 months were achieved by treatment with erbitinib monotherapy [8]. Therefore, EGFR-TKIs are believed to be promising agents for the treatment of NSCLC patients with brain metastases and EGFR-TKI sensitive mutations.

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In recent years, more attention has been paid to the relationship between EGFR and the development of lung carcinoma, and EGFR-TKIs targeting EGFR have been widely applied for the treatment of advanced lung carcinoma [9]. Osimertinib is the 3rd generation of EGFR-TKIs and the only agent used for the treatment of NSCLC patients with EGFR T790M positive mutations [10]. AZD3759 is a reformative novel EGFR-TKI based on gefitinib and has been shown to enter the central nervous system (CNS) by passing through the BBB [11]. The BBB penetrability of AZD3759 has been reported to be significantly higher than those of erbitinib and gefitinib. The growth of intracranial tumors in the mouse model of brain metastasis can be dramatically suppressed by AZD3759 monotherapy, accompanied by prolonged survival in mice [12]. In addition, higher concentrations of AZD3759 in the CNS and promising anti-tumor efficacy have been observed in clinical trials of AZD3759 [13]. However, the mechanism of AZD3759 or its effect on antitumor efficacy of RA are unclear. We hypothesized that AZD3759 promoted the inhibitory effects of RA on the brain metastasis of NSCLC cells by a synergistic blockade of EGFR and Janus kinase 1 (JAK1). In the present study, we aimed to investigate the mechanism of action of AZD3759 and to compare its antitumor efficacy with that of osimertinib in order to provide more non-clinical evidence for the application of AZD3759 for the treatment of NSCLC patients with brain metastases and EGFR-TKI sensitive mutations in the clinic.

Methods and materials

Cell lines and treatments

The EGFR-mutated NSCLC cell lines PC-9 and H3255 were purchased from ATCC (Manassas, Virginia, USA), and luciferase labeled PC-9 (PC-9-LUC) cells were prepared and obtained from Cloud-Clone Corp (Wuhan, China). Cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin, the condition of which was 5% CO₂ and 37°C.

Reagents

Osimertinib and AZD3759 were obtained from AbMole (Texas, USA). In the *in vitro* experiments, osimertinib and AZD3759 stock solutions were prepared in DMSO, which was diluted in serum-free RPMI-1640 medium before dosing. In the *in vivo* experiments, a vehicle (0.5% methylcellulose [w/v] and 0.4% Tween 80 [v/v] in sterile water) was used to dissolve osimertinib and AZD3759 for oral administration to xenograft nude mice.

Clonogenic assay[14]

PC-9 cells, H3255 cells, and PC-9 cells transfected with siRNA or siNC were seeded in 6-well plates and pre-treated with osimertinib or AZD3759 for 1 h, followed by different doses of RA (0, 1, 2, 4, 6, and 8 Gy) on day 1. On days 2 and 3, the cells were incubated with the same concentrations of osimertinib or AZD3759. After incubation for 7 days, the cells were stained with 0.5% crystal violet (Sigma, Missouri, USA) in 10% methanol for 30 min, followed by counting of colonies with more than 50 cells. The surviving cell fraction was calculated at each concentration by dividing the total number of colonies after irRA by the number of colonies without irRA.

In vitro experiments

Five groups of cells treated with different strategies were created for the *in vitro* experiments: RA, AZD3759, AZD3759 + RA, osimertinib, and osimertinib + RA groups. Both PC-9 and H3255 cells were used for the *in vitro* assays. In the RA group, cells were irradiated (2.5 Gy). In the AZD3759 group, the cells were treated with 500 nM AZD3759. In the AZD3759 + RA group, cells were irradiated (2.5 Gy) following pretreatment with 500 nM AZD3759 for 30 min. In the osimertinib group, the cells were treated with 500 nM osimertinib. In the osimertinib + RA group, cells were irradiated (2.5 Gy) following pretreatment with 500 nM osimertinib for 30 min.

Flow cytometry for the analysis of apoptosis[15]

In brief, cells were seeded on 6-well plates at a density of 2×10^5 cells/well and resuspended in 500 μ L of annexin V binding buffer containing 5 μ L of PI and 5 μ L of annexin V-FITC, followed by detection of the samples by flow cytometry (BD, New Jersey, USA) to determine the apoptotic rate in each group.

Flow cytometry for the analysis of cell cycle[16]

After centrifugation at $750 \times g$ for 5 min and three washes with PBS buffer, cells were counted and fixed in 70% ice-cold ethanol overnight at 4°C, followed by centrifugation at 1200 g for 5 min. Cells were then resuspended and washed with PBS buffer, followed by staining with 50 μ g/mL PI and 50 μ g/mL RNase at 37°C for 30 min. Finally, cell cycle analysis was performed using flow cytometry (BD, New Jersey, USA).

Western blotting assay[17]

The lysis buffer (Cell Signaling Technology, California, USA) was used for the extraction of total proteins from the cells or tissues, followed by quantification using a BCA kit (Shanghai Ze Ye Biotechnology Co., Ltd, Shanghai, China). After loading approximately 30 μ g protein, 12% SDS-PAGE was used to separate the proteins, which were then transferred to a PVDF membrane (Cell Signaling Technology, California, USA). Then, the protein-loaded PVDF membrane was mixed with 5% skim milk to block the nonspecific binding proteins, followed by incubation in a solution containing the primary antibody against cleaved caspase 3 (1:800, Abcam, Cambridge, UK), total caspase 3 (1:800, Abcam, Cambridge, UK), cleaved PARP (1:800, Abcam, Cambridge, UK), total PARP (1:800, Abcam, Cambridge, UK), JAK1 (1:800, Abcam, Cambridge, UK), and GADPH (1:800, Abcam, Cambridge, UK). The membrane was subsequently incubated with a secondary antibody (Abcam, Cambridge, UK). Finally, the bands were visualized with ECL kits (Cell Signaling Technology, California, USA), followed by quantification of the relative expression levels of target proteins using ImageJ software.

DNA damage assay[18]

DNA damage in PC-9 cells, H3255 cells, or JAK1 knockdown PC-9 cells was determined using immunofluorescence with γ -H2AX. After different treatments, cells were fixed using 4% paraformaldehyde, permeabilized, and incubated with 5% goat serum dissolved in 0.2% Triton X-100 PBS buffer for blocking. Then, the samples were incubated with the primary antibody against γ -H2AX (1:200, Abcam, Cambridge, UK), followed by incubation with the fluorescently labeled secondary antibody (Aviva Systems Biology, California, USA), and nuclei were counterstained with Vectashield DAPI mounting medium (Vector Labs, CA, USA). Finally, images were acquired using confocal microscopy (Keyence, Tokyo, Japan).

Receptor tyrosine kinase signaling antibody array[19]

The PathScan RTK Signaling Antibody Array Kit (CST, Boston, USA) which contained 39 antibodies was used to evaluate the effects of osimertinib and AZD3759 on the phosphorylated forms of receptor tyrosine kinases (RTKs) or key signaling proteins. PC-9 cells were treated with 500 nM osimertinib or AZD3759 for 30 min, followed by 10 Gy of RA and 24 hour incubation. Subsequently, the cells were collected and RTK array analysis was performed according to the manufacturer's instructions. LumiGLO and Peroxide reagent (CST, Boston, USA) were used to develop the membrane, and the expression ratio of RTKs was densitometrically quantified using ImageProPlus software. For comparison among different stimulation conditions, sets were normalized to allow equal intensities of the positive controls.

Overexpression of JAK1[20]

Overexpression of JAK1 in PC-9 cells was conducted by transfection with lentivirus loaded with pcDNA3.1-JAK1 (Genscript, Nanjing, China), which was transfected together with Lipofectamine 2000 (Invitrogen, California, USA) for 48 h. pcDNA3.1-NC was used as the negative control. Efficacy was confirmed using Western blotting.

Luciferin activity measurements in PC-9-LUC cells [21]

PC-9-LUC cells at the logarithmic phase were seeded in 96-well plates with cell numbers of 4×10^4 , 2×10^4 , 1×10^4 , 5000, 2500, 1250, 625, 312, and 156, respectively, with blank medium as the negative control. After incubation for 24 h, the substrate of luciferase was added at a concentration of 150 $\mu\text{g/mL}$, followed by immediate detection using the IVIS spectrum system (PerkinElmer, Massachusetts, USA).

CCK-8 cell viability assay [22]

In brief, 10 μL CCK-8 solution was added to the cells, followed by incubation at 37°C for 2 h, followed by measuring the absorbance at 450 nm using a microplate reader (J&H technology Co., Ltd, Jiangsu, China) to determine cell viability.

The establishment of brain metastasis of NSCLC [23]

Approximately 7-8-anesthesiaweek BALB/C nude mice were purchased from Charles River Laboratories (Beijing, China) and were adapted in our laboratory for 2 weeks. After anesthesia by intraperitoneal injection with 5% chloral hydrate, mice were fixed on the platform and approximately 100 μL of cell suspension was injected at the midpoint of the supra sternal notch to the left of the xiphoid process. The tumor cells were injected into the ventriculus sinister using an intravenous bolus. Brain metastasis was monitored by detecting fluorescence intensity.

In vivo grouping for the pharmacological experiments

When the intracranial fluorescent signal in animals was higher than 5×10^6 photons/s, nude mice were treated with different strategies. Six groups were generated: Control (nude mice inoculated with PC-9-LUC cells were administered vehicle), RA (nude mice planted with PC-9-LUC cells were treated with RA, 30 Gy/10 daily fraction, five fractions per week for 3 weeks), AZD3759 (nude mice planted with PC-9-LUC cells were

administered 15 mg/kg/day AZD3759 [24] orally for 3 weeks), AZD3759 + RA (nude mice inoculated with PC-9-LUC cells were administered 15 mg/kg/day AZD3759 concurrently with RA), osimertinib (nude mice planted with PC-9-LUC cells were administered 25 mg/kg/day osimertinib [25] orally for 3 weeks), and Osimertinib + RA (nude mice planted with PC-9-LUC cells were administered 15 mg/kg/day osimertinib concurrently with RA). RA treatment was performed at room temperature at a dose rate of 1.6 Gy/min using an XCELL 160 X-ray system (Kubtec, Stratford, CT, USA). AZD3759 or osimertinib was administered one hour prior to the RA. Tumor growth was monitored by measuring bioluminescence signals at 1 week and 3 weeks after inoculation. Tumor volumes were calculated at the end of the experiment.

In vivo grouping to verify AZD3759 mechanism of action

In this experiment, three groups were created: RA (nude mice inoculated with PC-9-LUC cells were treated with RA alone), AZD3759 + RA (nude mice inoculated with PC-9-LUC cells were administered 15 mg/kg/day AZD3759 concurrently with RA), and AZD3759 + RA+JAK1 (nude mice inoculated with JAK1-overexpressed PC-9-LUC cells were administered 15 mg/kg/day AZD3759 concurrently with RA). RA treatment was performed at room temperature at a dose rate of 1.6 Gy/min using an XCELL 160 X-ray system (Kubtec, Stratford, CT, USA). AZD3759 was administered one hour prior to the RA. Tumor growth was monitored by measuring bioluminescence signals at 1 week and 3 weeks after planting. Tumor volumes were calculated at the end of the experiment.

Statistical analysis

The data in the present study are presented as mean \pm SD and analyzed using GraphPad software. The difference between two groups was analyzed using the t-test, and the difference among groups was analyzed using the one-way ANOVA method. $P < 0.05$ indicated a significant difference.

Ethics statements

We declare that all animal experiments involved in this manuscript were authorized by the ethical committee of Affiliated Hangzhou Cancer Hospital Zhejiang University School of Medicine and carried out according to the guidelines for care and use of laboratory animals, as well as to the principles of laboratory animal care and protection.

Results

The sensitivity of NSCLC cells to RA was significantly enhanced by AZD3759

To investigate the impact of AZD3759 on the sensitivity of NSCLC cells to RA and to compare the efficacy of AZD3759 with that of osimertinib, a clonogenic assay in PC-9 and H3255 NSCLC cells following treatment with AZD3759 or osimertinib once a day for 3 consecutive days and irRA on the first day was performed. As shown in Figure 1(a,b), a dose-dependent decline in clonogenic survival in both PC-9 and H3255 cells was observed in the osimertinib group, while similar effects were also observed in the AZD3759 group

(Figure 1(c,d)). However, when the concentration reached 400 nM or 500 nM and the dosage of RA reached 6 or 8 Gy, compared to the control, a more significant decrease in clonogenic survival was observed in the AZD3759 group than in the osimertinib group (* $p < 0.05$, control; ** $p < 0.01$, vs. control), indicating that AZD3759 had significantly stronger effect on the sensitivity of NSCLC cells to RA than osimertinib did.

The RA-induced apoptosis is enhanced by AZD3759

To explore and compare the effects of AZD3759 and osimertinib on the RA-induced apoptosis in NSCLC cells, flow cytometry was used to determine the apoptotic rate and cell cycle, and the expression level of apoptotic proteins was evaluated. As shown in Figure 2(a), in PC-9 cells, compared to the RA group, the apoptotic rate was significantly elevated from 17.43% to 21.64% and 20.05% after co-treatment with AZD3759 and osimertinib, respectively (** $p < 0.01$ vs. RA). In addition, compared to the AZD3759 group, the apoptotic rate was dramatically decreased from 13.10% to 10.34% in the osimertinib group (# $p < 0.05$, vs. AZD3759). Similar results were observed in H3255 cells, except that

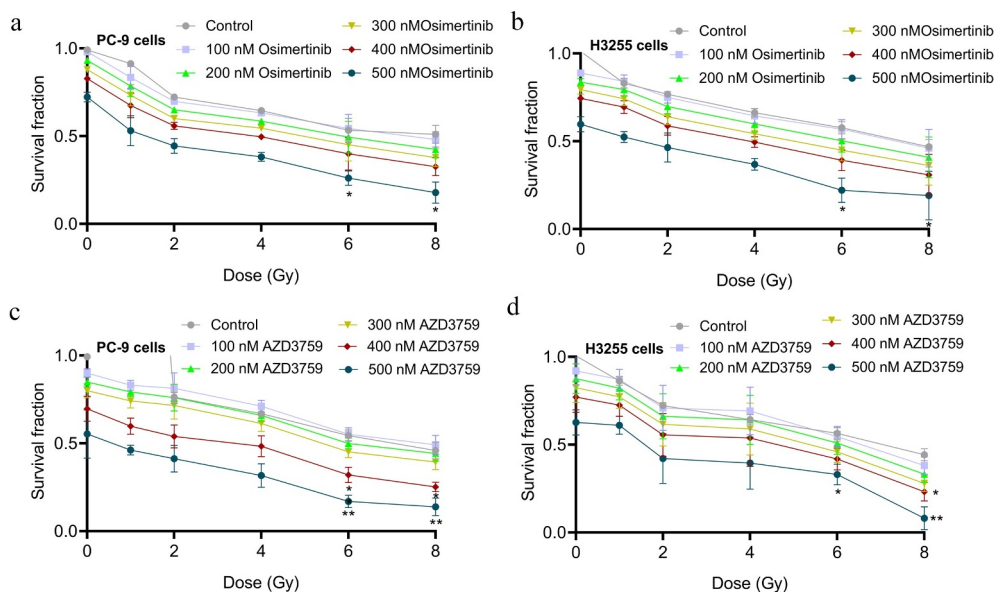


Figure 1. Effects of AZD3759 and osimertinib on the sensitivity of NSCLC cells to RA are evaluated using the clonogenic assay. (a) Survival fraction is determined after PC-9 cells are treated with different concentrations of osimertinib. (b) Survival fraction is determined after H3255 cells are treated with different concentrations of osimertinib. (c) Survival fraction is determined after PC-9 cells are treated with different concentrations of AZD3759. (d) The survival fraction was determined after H3255 cells were treated with different concentrations of AZD3759 (* $p < 0.05$ vs. control, ** $p < 0.01$ vs. control). NSCLC, non-small cell lung cancer; RA, radiation.

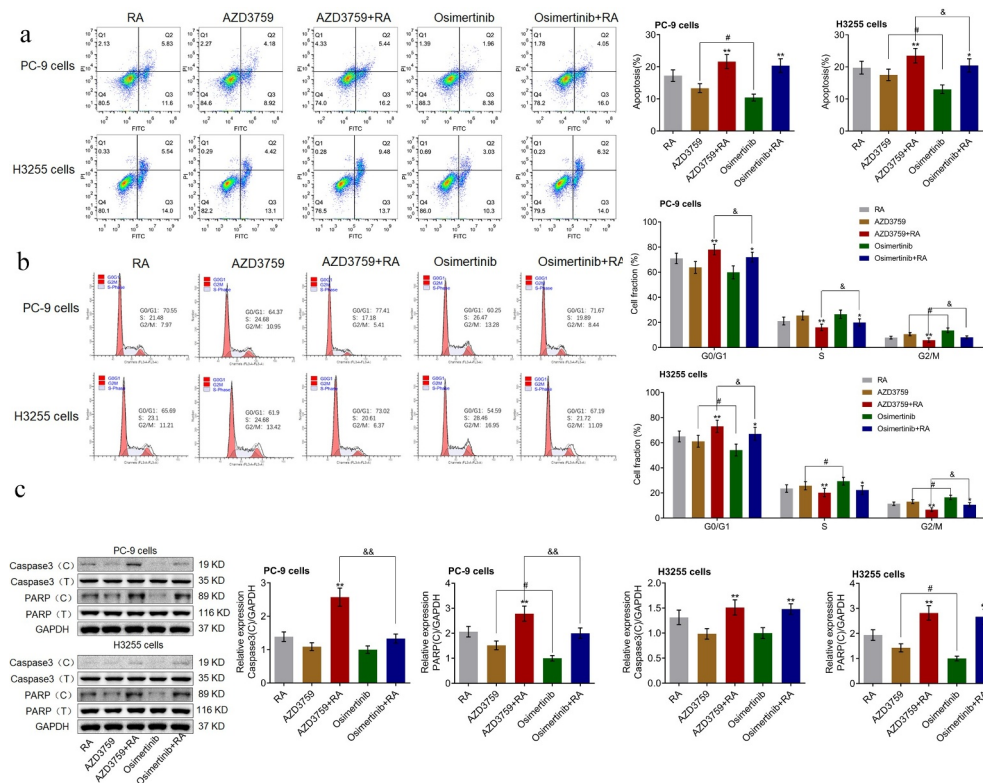


Figure 2. AZD3759 promoted the RA-induced apoptosis in NSCLC cells. (a) The apoptotic rate in PC-9 cells and H3255 cells treated with different strategies is evaluated using flow cytometry. (b) The cell cycle of PC-9 cells and H3255 cells treated with different strategies is evaluated using flow cytometry. (c) The expression level of caspase 3, caspase 3 (C), PARP, and PARP (C) is determined using Western blotting assay (* $p < 0.05$ vs. RA, ** $p < 0.01$ vs. RA, & $p < 0.05$ vs. AZD3759 + RA, # $p < 0.05$ vs. AZD3759). NSCLC, non-small cell lung cancer; RA, radiation; caspase 3 (C), cleaved caspase 3; PARP (C), cleaved PARP.

a significant difference in apoptotic rate was observed between the AZD3759+ RA and the osimertinib + RA group (& $p < 0.05$, vs. AZD3759+ RA). These data indicated that AZD3759 significantly enhanced RA-induced apoptosis in NSCLC cells, while the effect of AZD3759 was significantly stronger than that of osimertinib in H3255 cells.

We further evaluated the effects of AZD3759 and osimertinib on RA-induced cell cycle arrest using flow cytometry. As shown in Figure 2(b), in PC-9 cells, compared to RA, the proportion of G0/G1 was significantly increased from 70.55% to 77.41% by co-treatment with AZD3759 (** $p < 0.01$ vs. RA) and slightly elevated from 70.55% to 71.67% after co-treatment with osimertinib. A significant difference in the proportion of G0/G1 was observed between the AZD3759 + RA and the osimertinib + RA group (& $p < 0.05$, vs. AZD3759 + RA). In addition, compared to AZD3759, the proportion of G0/G1 was significantly declined from 64.37% to 60.25% in the osimertinib group (# $p < 0.05$, vs. AZD3759). Similar results were observed in the

H3255 cells. These data indicated that AZD3759 significantly enhanced RA-induced cell cycle arrest in NSCLC cells, the effect of which was significantly stronger than that of osimertinib.

Lastly, the effects of AZD3759 on RA-induced apoptosis were confirmed by detecting the expression of apoptotic proteins. As shown in Figure 2(c), in PC-9 cells, compared to RA, cleaved caspase 3 (caspase 3 (C)) and cleaved PARP (PARP (C)) were significantly upregulated by the co-administration of AZD3759 (** $p < 0.01$ vs. RA). Compared to the AZD3759 group, a significant decrease in the expression of PARP (C) was observed in the osimertinib group (# $p < 0.05$, AZD3759). A significant difference in the expression levels of caspase 3 (C) and PARP (C) was observed between the AZD3759+ RA and the osimertinib + RA group (&& $p < 0.01$ vs. AZD3759+ RA). In H3255 cells, caspase 3 (C) and PARP (C) were significantly upregulated by the co-administration of AZD3759 and osimertinib

(** $p < 0.01$ vs. RA). Compared to the AZD3759 group, a significant decrease in the expression of PPAR (C) was observed in the osimertinib group ($\#p < 0.05$, AZD3759).

RA-induced DNA damage is enhanced by AZD3759

DNA damage is one of the anti-tumor mechanisms of RA. The effects of AZD3759 and osimertinib on RA-induced DNA damage were investigated. As shown in Figure 3, in both PC-9 cells and H3255 cells, γ -H2AX immunofluorescence was rarely observed in the AZD3759 and osimertinib monotherapy groups. Compared to RA, the fluorescence intensity was significantly elevated by co-treatment with AZD3759 and osimertinib (* $p < 0.05$ vs. RA, ** $p < 0.01$ vs. RA). Compared to the AZD3759+ RA group, the fluorescence intensity was dramatically decreased in the osimertinib + RA group (&& $p < 0.01$ vs. AZD3759 + RA). These data

revealed that AZD3759 enhanced RA-induced DNA damage in NSCLC cells, with significantly higher efficacy than that observed with osimertinib.

Fluorescence intensity is positively correlated with the number of PC-9 LUC cells

To ensure that the fluorescence intensity could be used as a representative for the proliferation of PC-9 LUC cells in in vivo experiments, the correlation between the fluorescence intensity and the number of PC-9 LUC cells was determined. Only one NSCLC cell line (PC-9-WT) was used in subsequent in vivo experiments and verification experiments. First, as shown in Figure 4(a), compared to the wild type PC-9 cells, luciferin activity was significantly elevated in the PC-9 LUC group (** $p < 0.01$ vs. PC-9-WT). A linear correlation between the number of photons and the number of cells was observed (Figure 4(b)), with an R^2 value of 0.9726. In addition, no significant

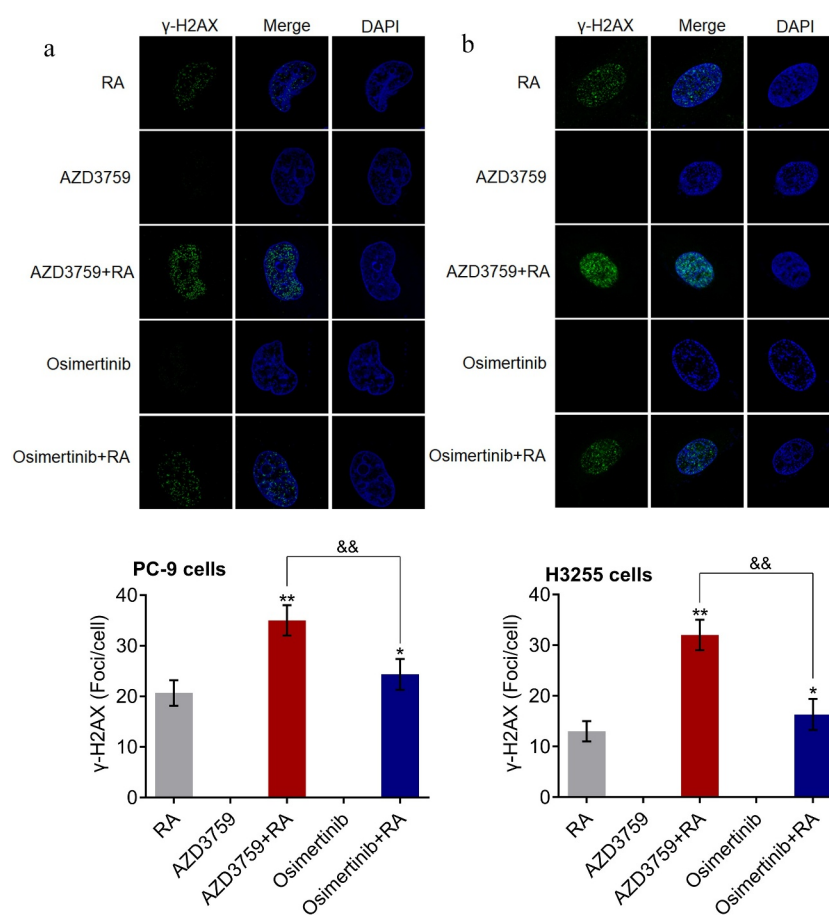


Figure 3. AZD3759 enhances the RA-induced DNA damage in NSCLC cells. DNA damage in PC-9 cells (a) and H3255 cells (b) is evaluated using the γ -H2AX immunofluorescence assay (* $p < 0.05$ vs. RA, ** $p < 0.01$ vs. RA, && $p < 0.01$ vs. AZD3759 + RA). NSCLC, non-small cell lung cancer; RA, radiation.

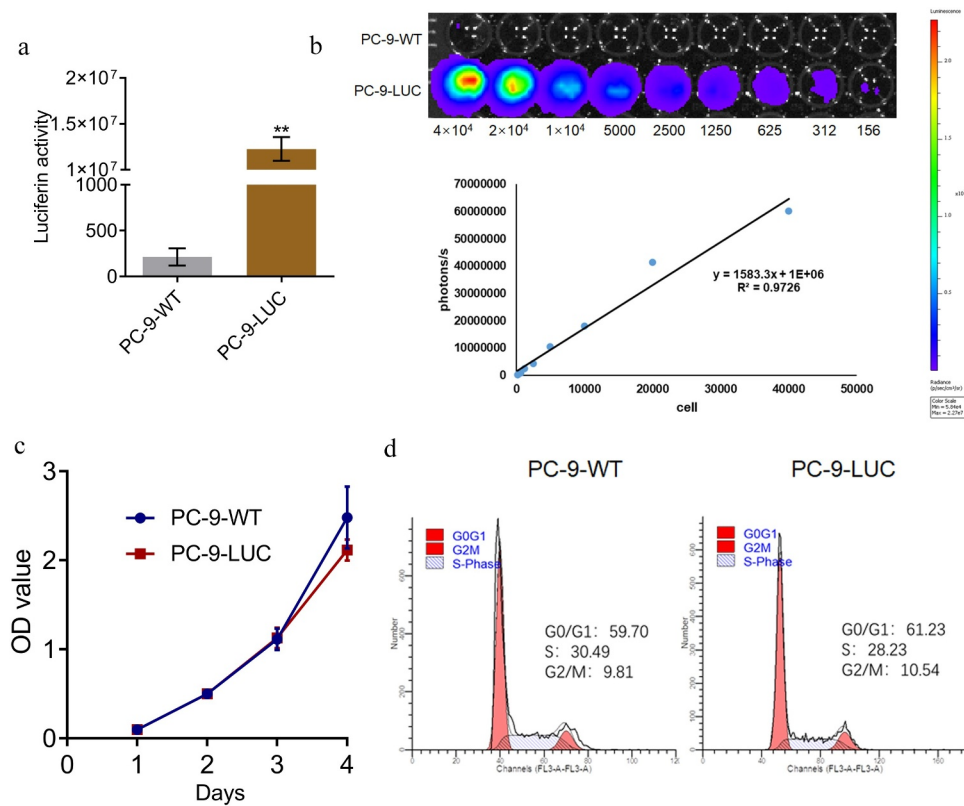


Figure 4. Fluorescence intensity is positively correlated with the number of PC-9 LUC cells. (a) Luciferin activity in PC-9-WT and PC-9-LUC cells is determined using the IVIS spectrum system (** $p < 0.01$ vs. PC-9-WT). (b) Photons of different number of PC-9-LUC cells are detected using the IVIS spectrum system. (c) The OD value of PC-9-WT and PC-9-LUC cells is measured using CCK-8 assay. (d) Cell cycle in PC-9-WT and PC-9-LUC cells is determined using flow cytometry assay. OD, optical density; WT, wild type.

difference was observed in the optical density values detected by CCK-8 assay and in the G0/G1 proportion detected by flow cytometry between the PC-9-WT and PC-9 LUC cells, indicating that the proliferation and cell cycle of PC-9 cells were not affected by the transfection of luciferase.

Inhibitory effect of RA on the brain metastasis of PC-9 cells is enhanced by AZD3759

To explore the effect of AZD3759 and osimertinib on brain metastasis in RA-treated xenograft nude mice, PC-9-LUC cells were inoculated and this was followed by treatment with AZD3759 and osimertinib, respectively. As shown in Figure 5(a-b), no significant difference was observed 1 week post treatment among all groups. On the third week post-treatment, compared to the control, the average signal was significantly decreased in the RA, AZD3759, and osimertinib groups (* $p < 0.05$, ** $p < 0.01$, vs. control). Compared to the RA group, the average signal was dramatically reduced by the co-

administration of AZD3759 and osimertinib, respectively (## $p < 0.01$ vs. RA), while a significant difference was observed between the AZD3759 + RA and osimertinib + RA groups (& $p < 0.05$ vs. AZD3759 + RA). At the end of the experiment, the animals were sacrificed, and the volume of the brain metastatic tumor was measured. As shown in Figure 5(c), the tumor volume was greatly suppressed in the RA, AZD3759, and osimertinib treatments (** $p < 0.01$, vs. control). Compared to RA, the tumor volume was dramatically inhibited by the co-administration of AZD3759 or osimertinib (## $p < 0.01$ vs. RA). In addition, compared to the AZD3759 + RA group, a significantly larger tumor volume was observed in the AZD3759 + osimertinib group ($p < 0.05$ vs. AZD3759 + RA). These data indicated that AZD3759 enhanced the inhibitory effect of RA on the brain metastasis of PC-9 cells, and this effect was significantly stronger than that of osimertinib.

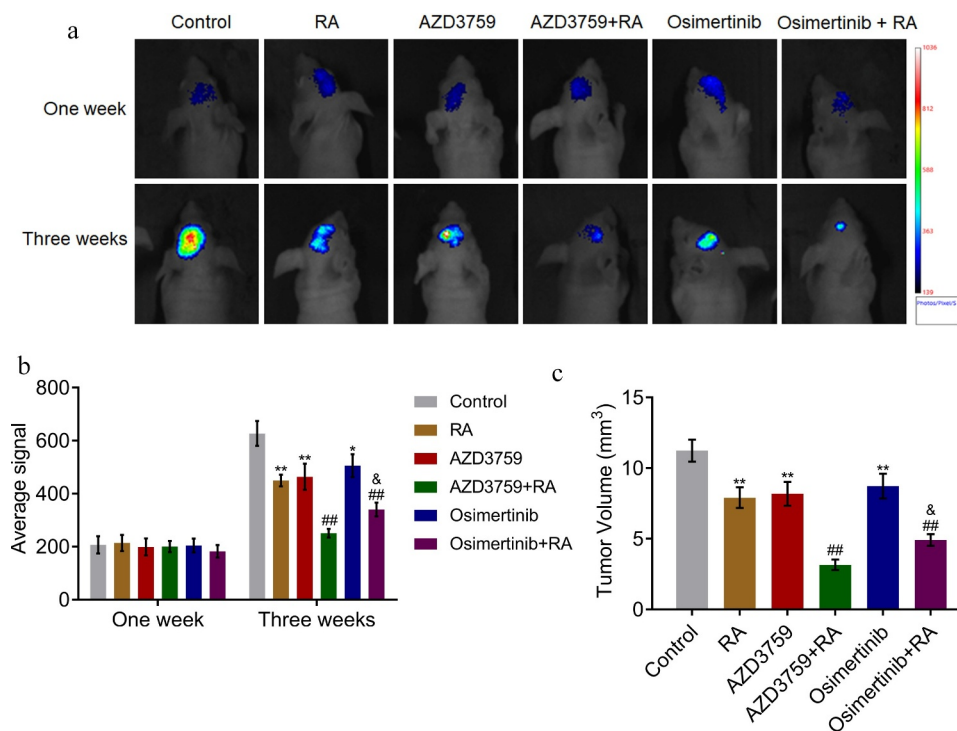


Figure 5. AZD3759 promotes the inhibitory effect of RA on the brain metastasis by PC-9 cells in xenograft mice. (a) Live imaging of the animals in each group is performed 1 week and 3 weeks post treatments. (b) Average signal levels are quantified using the IVIS spectrum system (* $p < 0.05$ vs. control, ** $p < 0.01$ vs. control, ## $p < 0.01$ vs. RA, & $p < 0.05$ vs. AZD3759 + RA). (c) Tumor volumes are calculated at the end of the experiments (** $p < 0.01$ vs. control, ## $p < 0.01$ vs. RA, & $p < 0.05$ vs. AZD3759 + RA). RA, radiation.

AZD3759 enhances the anti-tumor efficacy of RA by inhibiting both EGFR and JAK1

To explore the mechanistic basis underlying higher anti-tumor efficacy of AZD3759 compared to that of osimertinib, an RTK signaling antibody array analysis was conducted. As shown in Figure 6(a), compared to RA, EGFR was down-regulated by both AZD3759 and osimertinib. However, the reduced expression of JAK1 was only observed in the AZD3759 + RA group, which was not observed in the osimertinib + RA group. We suspected that the inhibitory effect of JAK1 might be responsible for the enhanced anti-tumor properties of AZD3759 compared to those of osimertinib. Therefore, JAK1-overexpressed PC-9 cells were established, which was confirmed by Western blotting (Figure 6(b)). The clonogenic assay (Figure 6(c)) revealed that, compared to the control, JAK1-overexpressed PC-9 cells had significantly elevated survival fractions at all concentrations of AZD3759. As shown in Figure 6(d), compared to RA, the number of γ -H2AX foci per cell was significantly increased by the co-administration of AZD3759, which was

dramatically abolished by the overexpression of JAK1 (** $p < 0.01$ vs. RA, ## $p < 0.01$ vs. AZD3759 + RA).

We further performed a mechanism verification experiment (grouping and methods are presented in the method section). As shown in Figure 7, three weeks post-treatment, compared to RA, the average signal and tumor volume were significantly suppressed by the co-administration of AZD3759, which was significantly reversed by the overexpression of JAK1 (** $p < 0.01$ vs. RA, ## $p < 0.01$ vs. AZD3759 + RA). These results indicated that the facilitating effects of AZD3759 on the anti-metastasis property of RA were closely related to the inhibition of JAK1.

Discussion

We hypothesized that AZD3759 promoted the inhibitory effects of RA on the brain metastases by NSCLC cells by a synergistic blockade of EGFR and JAK1. The present study compared the anti-tumor efficacy of osimertinib and AZD3759 and investigated the mechanism of action of AZD3759.

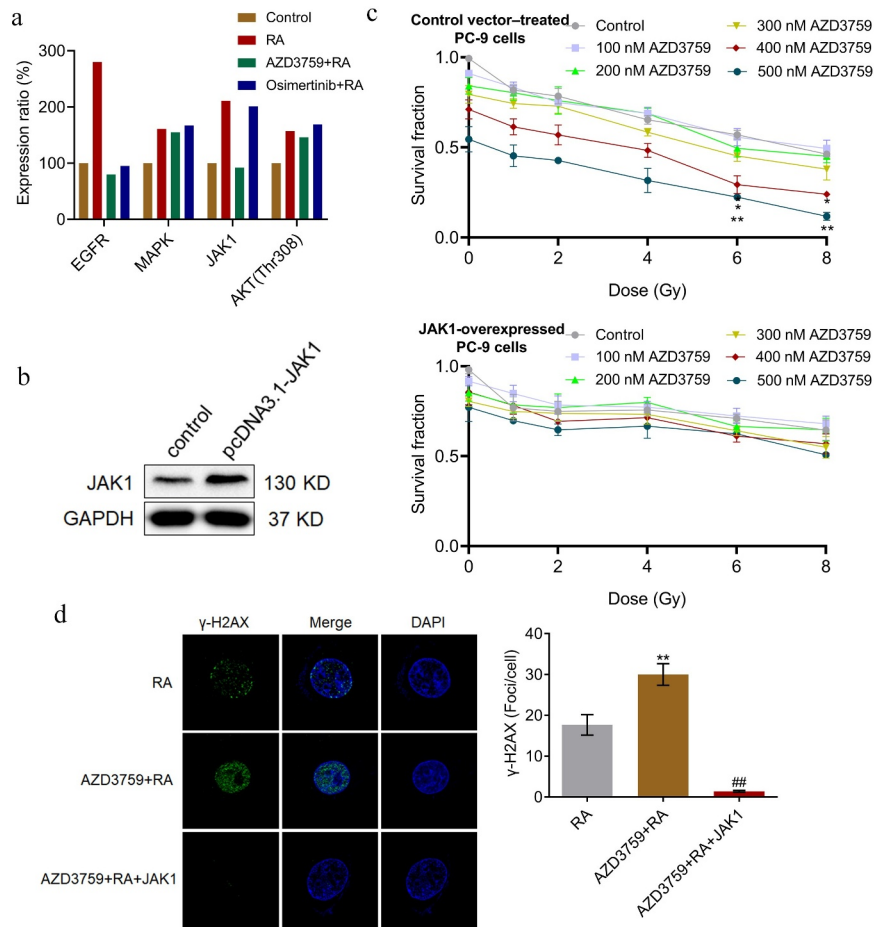


Figure 6. JAK1 overexpression abolishes effects of AZD3759 on clone formation and DNA damage in RA-treated PC-9 cells. (a) Effects of AZD3759 and osimertinib on RTK pathway activity are evaluated using the RTK signaling antibody array. (b) Transfection efficacy is confirmed using Western blotting assay. (c) Survival fraction is determined after PC-9 cells or JAK1-overexpressing PC-9 cells are treated with different concentrations of AZD3759 (* $p < 0.05$ vs. control, ** $p < 0.01$ vs. control). (d) DNA damage is evaluated using the γ -H2AX immunofluorescence assay (** $p < 0.01$ vs. RA, ### $p < 0.01$ vs. AZD3759 + RA). JAK, Janus kinase 1; RA, radiation; RTK, receptor tyrosine kinase.

We first explored the effects of AZD3759 on the sensitivity of NSCLC cells to RA, and then checked the impact of AZD3759 on RA-induced apoptosis in NSCLC cells. The facilitating effects of AZD3759 on RA-induced DNA damage in NSCLC cells were explored. For the in vivo experiments, the regulatory effect of AZD3759 on the inhibitory effect of RA on the brain metastasis of PC-9 cells was determined using a xenograft model. Finally, the involvement of JAK1 in the antitumor mechanism of AZD3759 was investigated and verified.

The ideal clinical output is not achieved by the treatment of NSCLC brain metastasis with RA therapy alone. The median survival time for whole brain radiotherapy (WBRT) is currently

approximately 3–6 months and the objective response rate (ORR) for WBRT is 50–70%, with a one-year survival rate of 10–15% [26]. EGFR-TKIs are currently widely applied as adjuvant therapy combined with RA therapy for the treatment of NSCLC patients with brain metastasis. Welsh [27] recruited 40 NSCLC patients with brain metastasis, and EGFR mutations were observed in 9 patients, with 9 EGFR-naïve patients. After treating the patients with RA combined with EGFR-TKIs, compared to EGFR-naïve patients (9.3 months), significantly higher OS (19.1 months) was observed in EGFR mutant patients. Osimertinib is a third-generation EGFR-TKI that has been proven to achieve clinical progression during the treatment of brain metastatic NSCLC patients [25].

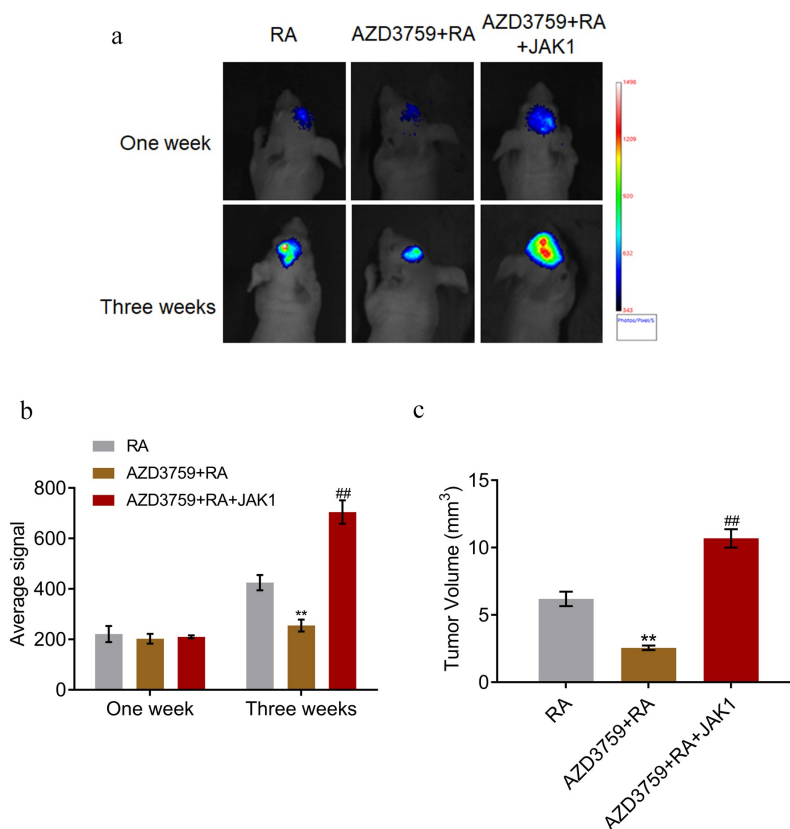


Figure 7. JAK1 overexpression abolishes effects of AZD3759 on the brain metastasis by PC-9 cells in RA-treated nude mice. (a) Live imaging of the animals in each group is performed 1 week and 3 weeks post treatments. (b) Average signal levels are quantified using the IVIS spectrum system (** $p < 0.01$ vs. RA, ## $p < 0.01$ vs. AZD3759 + RA). (c) Tumor volumes are calculated at the end of the experiments (** $p < 0.01$ vs. RA, ## $p < 0.01$ vs. AZD3759 + RA).

AZD3759, also known as Zorifertinib, is a type of EGFR-TKI specially designed to treat brain metastatic NSCLC patients because of its ability to penetrate the BBB, which shows significant affinity for L858R mutant NSCLC. Several *in vitro* studies have revealed the promising antitumor efficacy of AZD3759. Chao reported that AZD3759 induced apoptosis in hepatoma cells by activating a p53-SMAD4 positive feedback loop [28]. Li claimed that the inhibitory effects of RA on brain metastasis from EGFR-mutant NSCLC cells were significantly enhanced by AZD3759 [24]. Our previous research revealed the antitumor effects of AZD3759 on glioma cells [29]. Yang [12] reported the results of phase I clinical trials on the treatment of brain metastatic NSCLC patients with AZD3759 in 2015, which showed high permeability across the BBB (29.5×10^{-6} cm/s). Recently, a phase 1, open-label, dose-escalation, and dose-expansion study of AZD3759 showed promising efficacy of AZD3759 in EGFR-mutated NSCLC patients with CNS metastases [13]. In the EGFR-mutant brain

metastatic animal model of NSCLC, a significant decrease in tumor volume was observed in AZD3759 treated mice [12]. In the present study, we compared the effects of AZD3759 and osimertinib in RA-treated NSCLC cells and a xenograft brain metastatic animal model. The results indicated that both AZD3759 and osimertinib significantly increased the effects of RA-induced reduction in cell survival in the clonogenic assay, promoted apoptosis and DNA damage, and suppressed brain metastasis in xenograft nude mice. These observations were consistent with the reports that reported inhibitory effects of AZD3759 or osimertinib against the brain metastasis of EGFR mutated NSCLC [24,30]. In addition, effects of AZD3759 on the anti-tumor function of RA were significantly stronger than those of osimertinib, which was verified in both *in vitro* and *in vivo* experiments.

In the present study, to explore the potential mechanism underlying the stronger efficacy of AZD3759 compared to osimertinib, we performed

the RTK signaling antibody array analysis in PC-9 cells after co-treatment with AZD3759 and RA or osimertinib and RA. JAK1 was found to be the specific RTK suppressed by AZD3759 compared to osimertinib. JAK belongs to the non-receptor tyrosine kinase family with four family members, including JAK1, JAK2, JAK3, and TYK2, which can activate STAT proteins under the stimulation of multiple cytokines and growth factors [31–33]. The JAK1/STAT pathway is strictly regulated in normal cells. However, in tumor cells, this pathway can be persistently activated upstream of the JAK family protein members [34]. Among all JAK1/STAT pathways, the JAK1/STAT3 pathway is reported to play an important role in the proliferation, apoptosis, migration, and invasion of tumor cells [35–37]. In the present study, to verify that AZD3759 exerted anti-tumor properties not only by inhibiting EGFR signaling, but also by suppressing JAK1 signaling, we established JAK1-overexpressed PC-9 cells. The *in vitro* verification experiments indicated that the effects of AZD3759 on clone formation and DNA damage in RA-treated PC-9 cells were significantly abolished by the overexpression of JAK1. *In vivo* verification experiments revealed that the effects of AZD3759 on the brain metastasis by PC-9 cells in RA-treated nude mice were also dramatically abolished by the overexpression of JAK1. We concluded that the inhibitory effect of JAK1 was responsible for the stronger anti-tumor properties of AZD3759 compared to those of osimertinib. However, this conclusion will be verified in our future work. For example, parallel comparisons of apoptosis and DNA damage in RA-treated JAK1 overexpressed PC-9 cells incubated with AZD3759 and osimertinib will be conducted, as well as a parallel comparison of their anti-tumor efficacies *in vivo*. In addition, the effects of AZD3759 on the activity of STAT3 will be further investigated to confirm the inhibitory effects of AZD3759 on the JAK1/STAT3 pathway.

Conclusions

Our data revealed that AZD3759 enhanced RA effects in NSCLC cells by a synergistic blockade of EGFR and JAK1, which provides the

fundamental basis for the combination of AZD3759 and RA for the treatment of clinical NSCLC.

Highlights

- AZD3759 promoted the inhibitory effects of RA on NSCLC;
- AZD3759 showed superior effects on RA-treated NSCLC than Osimertinib;
- AZD3759 showed a synergistic blockade of EGFR and JAK1.

Consent for publication

The manuscript is agreed for publication by the authors.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors' contributions

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 Acquisition of data: Qingqing Yu
 Analysis and interpretation of data: Ke Zhang, Wei Yin
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 Obtaining funding: Ruing Zhao
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 Revision of manuscript for important intellectual content: Shenglin Ma, Qinghua Deng

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