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Pazopanib restricts small cell lung cancer proliferation via reactive oxygen species-mediated endoplasmic reticulum stress

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Abstract

Background: Pazopanib is an approved multitarget anticancer agent for soft tissue sarcoma (STS) and renal cell carcinoma (RCC), which is also under clinical investigation for other malignancies, including small cell lung cancer (SCLC). However, the potential anti-SCLC mechanisms of pazopanib remain unclear.

Methods: Cell viability was evaluated by CCK-8, apoptotic cell detection was conducted using annexin V/PI staining followed by flow cytometry, and Western blot analysis was used to detect the apoptotic-related molecules and ER-stress pathway effectors. The intracellular reactive oxygen species (ROS) level was determined by DCFH-HA staining followed by flow cytometry. An NCI-H446 xenograft model was established to evaluate pazopanib on tumor suppression in vivo. Immunohistochemistry (IHC) was used to assess the proliferative activity of xenograft in NCI-H446 cell-bearing NOD-SCID mice.

Results: Pazopanib dose- and time-dependently inhibited SCLC cell proliferation induced significant apoptosis in SCLC cell lines, increased cleaved-caspase3 and Bax, and decreased Bcl-2. Moreover, the PERK-related ER-stress pathway was potently activated by pazopanib treatment, inhibiting ER-stress by salubrinal significantly reversing pazopanib-mediated apoptosis in SCLC cell lines. Furthermore, pazopanib-induced intracellular ROS levels increased, while inhibiting ROS by NAC significantly reversed pazopanib-induced apoptosis in SCLC cells. In addition, pazopanib significantly suppressed NCI-H446 xenograft growth and decreased Ki67 positive cells in the tumor.

Conclusion: Our findings indicate that pazopanib induces SCLC cell apoptosis through the ER-stress process via upregulation of ROS levels. Further investigation of relevant biomarkers to accurately select patients for benefit from pazopanib should be further investigated.

KEYWORDS
apoptosis, endoplasmic reticulum stress (ER-stress), Pazopanib, reactive oxygen species (ROS), small cell lung cancer (SCLC)

INTRODUCTION

Lung cancer is a malignant disease that remains a serious public health problem with high mortality and morbidity rates worldwide, especially in China, where lung cancer has replaced liver cancer as the number one cause of death among all malignancies since 2008.[1] Although small cell lung cancer (SCLC) accounts for approximately only 15% of all types of lung cancer, the patient prognosis is discouraged with a 5-year survival rate of less than 5% and an average overall survival period of only 2–4 months for those without any active therapy.[2] SCLC can be classified into limited and extensive stages based on the veterans affairs lung study group staging criteria. Regrettably, the extensive stage accounts for approximately one-third of newly diagnosed cases, which means treatment options for SCLC are limited (primarily chemotherapy), and therapy

effects are poor. Hence, novel treatment strategies for SCLC urgently need to be investigated.

Pazopanib (GW786034) is one of the new multitargeted receptor tyrosine kinase inhibitors (TKIs), approved for the treatment of patients with advanced renal cell carcinoma.[3] The potential targets of pazopanib include VEGFR1, VEGFR2, VEGFR3, PDGFR, FGFR, c-Kit, and c-Fms/CSF1R.[4,5] In addition, the clinical investigation of pazopanib on soft-tissue sarcoma, gastrointestinal stromal tumors, platinum-resistant/refractory epithelial ovarian cancer, and other malignant tumors is under development.[6–8] For SCLC, pazopanib has shown significant anticancer effects in a preclinical model and clinical trials; for example, second-line treatment with pazopanib in platinum-sensitive SCLC is well tolerated and resulted in promising objective responses and disease control.[9] In addition, pazopanib maintenance after first-line etoposide and platinum chemotherapy significantly prolonged progression-free survival (PFS) in patients with extensive disease-SCLC.[10] However, few studies have demonstrated the potential mechanisms of action of pazopanib on SCLC.

As a central subcellular organelle, the endoplasmic reticulum (ER) is where secreted and transmembrane proteins are synthesized, folded, and modified.[11] Although this process is tightly regulated, various internal and external factors can disrupt this protein folding capacity and trigger an ER stress state characterized by a buildup of misfolded or unfolded proteins, named the unfolded protein response (UPR).[12] Genetic, transcriptional, and metabolic disorders in tumor cells will result in sustained ER stress and eventually affect their function, destiny, and survival.[13] The UPR is initiated by three resident transmembrane proteins that function as sensors of protein-folding stress: inositol-requiring protein 1α (IRE1α),[14] PRKR-like ER kinase (PERK,) and activating transcription factor 6 (ATF6).[15] Under ER stress conditions, molecular chaperone-binding immunoglobulins (BiP) are titrated away from the sensor due to higher affinity binding to misfolded protein resulting in activation of the ER stress sensor and its downstream signals.[16] Among the above three pathways, PERK-dependent phosphorylation of eIF2α reduces protein synthesis by generally inhibiting 5′ cap-dependent translation while selectively increasing the cap-independent translation of ATF4, which subsequently activates the transcription of factor C/EBP homologous protein (CHOP)-mediated apoptosis. More critically, this process is independent of other pathways and can be initiated by enriched reactive oxygen species levels level.[17] The ER stress is a typical biological process that a series of anticancer agents utilize to activate apoptosis.[18] Hence, we suspected whether pazopanib induces ER stress-mediated anticancer effects on SCLC cells.

In the present study, we illustrate that pazopanib can induce SCLC cell apoptosis through PERK-dependent ER stress via upregulation of ROS levels. Our findings may provide clues to the problematic situation of SCLC management.

METHODS

Chemical and biochemical reagents

Pazopanib HCl (GW786034 HCl), salubrinal, and *N*-acetylcysteine were purchased from Selleckchem. Cell counting kit-8 (CCK-8) was purchased from Beyotime. Dimethyl sulfoxide (DMSO), hydroxypropylmethylcellulose, and Tween-80 were obtained from Sigma-Aldrich. The RPMI-1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Biological Industries. All other reagents were purchased from Sigma-Aldrich.

Cell lines and cell culture

The human SCLC cell lines NCI-H446 and NCI-H82 were obtained from the National Collection of Authenticated Cell Cultures. All cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. All cells were identified as free of contamination.

Cell proliferation assay

A cell viability test was performed by CCK-8 kit to evaluate the proliferation of SCLC cells under pazopanib treatment, as previously described.[19] In brief, 5×10^3 cells/well were cultured in 96-well plates in 180 μl of complete medium. Different concentrations of pazopanib in 20 μl of complete medium were added to each well (range 0–30 μM). After incubation for 24, 48, and 72 h, the plates were centrifuged, and the medium was discarded. The cells were subsequently incubated with a complete medium (100 μl) containing CCK-8 (10 μl) for 4 h at 37°C. An iMark multiplate reader (Bio-Rad) was used to obtain the OD (under 450 nm) value of each well. The relative cell viability was calculated using the following equation: Cell viability $= (OD_{\text{repeseting group}} - OD_{\text{blank}})/(OD_{\text{control group}} - OD_{\text{blank}}) \times 100\%$. The median inhibitory concentration (IC_{50}) was calculated using Graphpad 9.00 software and the "nonlinear regression (curve fit)" model.

Apoptotic cell detection

Apoptotic cells were evaluated following pazopanib treatment via annexin V/PI apoptosis detection kit from Beyotime. The NCI-H446 or NCI-H82 cells were treated with different concentrations of pazopanib. After 24 h, the staining buffer (200 μl) was used to resuspend the cells at a density of 1×10^5. The annexin V and PI (5 μl, respectively) were mixed in the dark for 30 min at room temperature. Subsequently, a BD Accuri C6 (BD Biosciences) flow cytometer was used to detect the apoptotic cells. FlowJo 10.0 software (BD Biosciences) was used to analyze the data.

ROS detection

The DCFH-DA diacetate (Beyotime) was used as a probe to detect ROS alteration in SCLC cells following pazopanib treatment. In brief, after treatment with pazopanib, the NCI-H446, and NCI-H82 cells were cultured with DCFH-DA (10 μM) in the dark at 37°C for 20 min. The cells were then washed and resuspended in PBS. Subsequently, the cells were analyzed with a BD Accuri C6 (BD Biosciences) flow cytometer. The data were analyzed using FlowJo 10.0 software (BD Biosciences) for flow cytometry.

Western blot

The key molecules involved in ER stress processes were evaluated by Western blot. Specifically, following treatment with pazopanib, the NCI-H446 and NCI-H82 cells were lysed by RIPA buffer on ice and centrifuged at 12000 g for 15 min to obtain the total cell protein lysates. The total protein concentrations were analyzed using a BCA protein quantification kit (Beyotime). Each sample's total protein (20 μg) was subjected to SDS-PAGE and transferred onto a 0.2 μm polyvinylidene difluoride membrane (Merk Millipore). The membranes were incubated with nonfat milk (5%) to block the potential nonspecific proteins and subsequently incubated with primary antibodies (cleaved caspase-3, PARP, BAX, Bcl-2, p-eIf2α, eIf2α, PERK, BiP, ATF2, CHOP, and β-actin) at 4°C for 8 h. Then, TBST buffer was used to wash the membrane five times, and HRP-conjugated secondary antibodies were incubated for 2 h at room temperature. After washing with TBST buffer five times, the membranes were incubated with ECL and visualized by Gel Doc XR+ (Bio-Rad). Image J software was used to analyze the relative intensity of each band, and GraphPad software was used for the statistical analysis.

Xenograft model

The animal study protocol was approved by Tianjin Medical University Animal Experimental Ethics Committee. Specifically, 6-week-old NOD/SCID female mice were used as an animal model, allowed free access to food and water, and maintained under specific pathogen-free (SPF) feeding conditions. A total of 100 μl PBS containing NCI-H446 cells (2×10^7) were injected subcutaneously into the left hindlimbs of the mice. The mice were randomly divided into three groups ($n = 6$): the control group treated with 0.5% hydroxypropylmethylcellulose and 0.1% Tween-80 in water, daily treatment by oral gavage; the pazopanib group treated with pazopanib (30 mg/kg, suspended in 0.5% hydroxypropylmethyl cellulose and 0.1% Tween-80 in water, daily treatment by oral gavage); and the pazopanib + NAC group, treated with pazopanib with NAC (50 mg/kg, suspended in 0.5% hydroxypropylmethyl cellulose and 0.1% Tween-80 in water, daily treatment by oral gavage). The treatment process was sustained for about 2 weeks (13 days), and daily tumor volumes were monitored.

Immunohistochemistry (IHC)

Tumor xenografts were separated from the mice and subsequently fixed in 4% paraformaldehyde. The tumors were then washed and dehydrated using graded ethanol before being embedded in paraffin and consecutively sectioned at a thickness of 4 μm. EDTA method was used to complete antigen retrieval. After blocking with goat serum, the slides were incubated with the anti-Ki67 antibody at room temperature for 1 h. Subsequently, secondary antibodies and DAB were incubated to visualize the positive staining. IHC score was evaluated as previously described.[20]

Statistical analysis

The data were analyzed using Graphpad 8.00 software. Data are expressed as mean ± SD, and three independent experiments were conducted. Unpaired student's *t*-test was used to carry out the significance of comparing both groups. Multigroup comparisons of the means were carried out by one-way analysis of variance (ANOVA) test Student–Newman–Keuls post hoc analysis for comparison of groups ≤3. Post hoc Tukey's test was conducted for groups ≥3. Statistical significance for all tests was set when p was <0.05 (*).

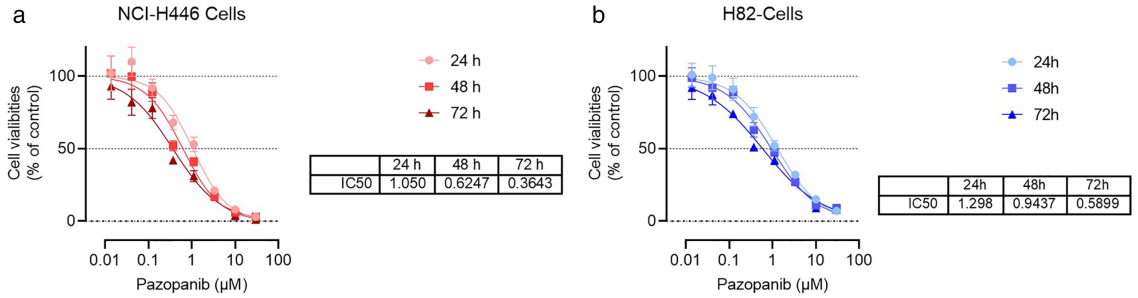
RESULTS

Pazopanib significantly inhibits SCLC cell proliferation

First, we used the CCK-8 assay to determine the antiproliferative effects of pazopanib on SCLC cell lines NCI-H446 and NCI-H82. In this study, different pazopanib concentrations were applied (range 0–30 μM) and incubated with NCI-H446 or NCI-H82 cells for 24, 48, and 72 h, respectively. As illustrated in Figure 1a, pazopanib significantly decreased NCI-H446 cell proliferation in a dose- and time-dependent manner, with IC_{50} (24 h) value at 1.05 μM. Moreover, as shown in Figure 1b, pazopanib could also induce potent cell death in NCI-H82 cells in a dose- and time-dependent manner. The IC_{50} on 24 h incubation of pazopanib for NCI-H82 cells was 1.298 μM, similar to that for NCI-H446 cells, indicating that pazopanib significantly antagonized SCLC cell proliferation.

Pazopanib induces significant apoptosis in SCLC cells

To further determine the mechanism of action on pazopanib-induced SCLC cell proliferative inhibition, the apoptotic cells of NCI-H446 and NCI-H82 after pazopanib



	24 h	48 h	72 h
IC50	1.050	0.6247	0.3643

	24h	48h	72h
IC50	1.298	0.9437	0.5899

FIGURE 1 Antiproliferative effects of pazopanib on small cell lung cancer (SCLC) cell lines. The concentration-viability curve of pazopanib on SCLC cell lines (a) NCI-H446 and (b) NCI-H82 were detected by cell counting kit-8 (CCK-8) assays for 24 h, 48 h, and 72 h. The relative IC50 values of pazopanib at different time points are illustrated in the sheet. Data are expressed as mean ± SD in at least three independent experiments

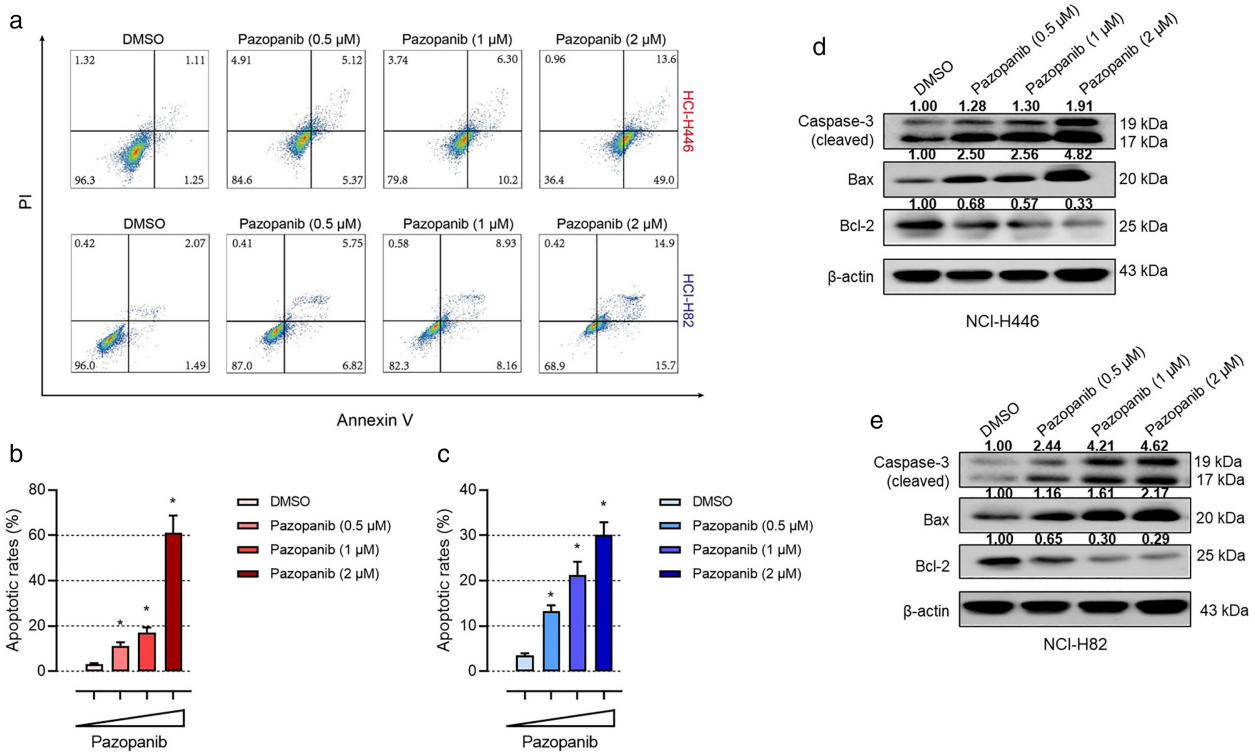


FIGURE 2 Pazopanib induces apoptosis in small cell lung cancer (SCLC) cell lines. (a) Dot-blots detected by flow cytometry indicated the apoptotic cells in NCI-H446 and NCI-H82 cells following pazopanib (0.5, 1, and 2 μM) treatment for 24 h. The apoptotic cells in (b) NCI-H446 and (c) NCI-H82 were calculated by adding the cell rates from annexin V+ and annexin V+/PI+ quadrant from the FlowJo software. The cleaved-caspase3, Bax, and Bcl-2 in (d) NCI-H446 and (e) NCI-H82 were detected by Western blot analysis following pazopanib treatment (0.5, 1, and 2 μM) treatment for 24 h. Data are expressed as mean ± SD in at least three independent experiments. The numerical values illustrated on the bands indicated the relative intensity (% of loading control). Statistical significance was recognized when $p < 0.05$ (*)

treatment were evaluated by flow cytometry via annexin V/PI staining. As shown in Figure 2a–2C, pazopanib dose-dependently induced both NCI-H446 and NCI-H82 apoptosis. The apoptosis-related molecules were detected by Western blot. As illustrated in Figure 2d and e, enhanced cleaved-caspase3 was observed in both NCI-H446 and NCI-H82 cells after pazopanib treatment. Moreover, Bax was significantly upregulated while Bcl-2 was potently decreased after pazopanib treatment in both NCI-H446 and NCI-H82 dose-dependently. This result indicated that pazopanib inhibited SCLC cell proliferation in an apoptosis-dependent manner.

Pazopanib-mediated SCLC cell apoptosis is an ER stress-dependent pathway

To further evaluate the potential mechanism of action on pazopanib-induced SCLC cell apoptosis, the critical molecules in ER stress were determined as ER-stress is one of the significant regulation biological processes in cancer cells. As illustrated in Figure 3a and b, the ER-stress key molecules PERK, BiP, p-eIF2α, ATF4, and CHOP were significantly dose-dependently increased after pazopanib treatment of both NCI-H446 and NCI-H82 cells, indicating that the induction of

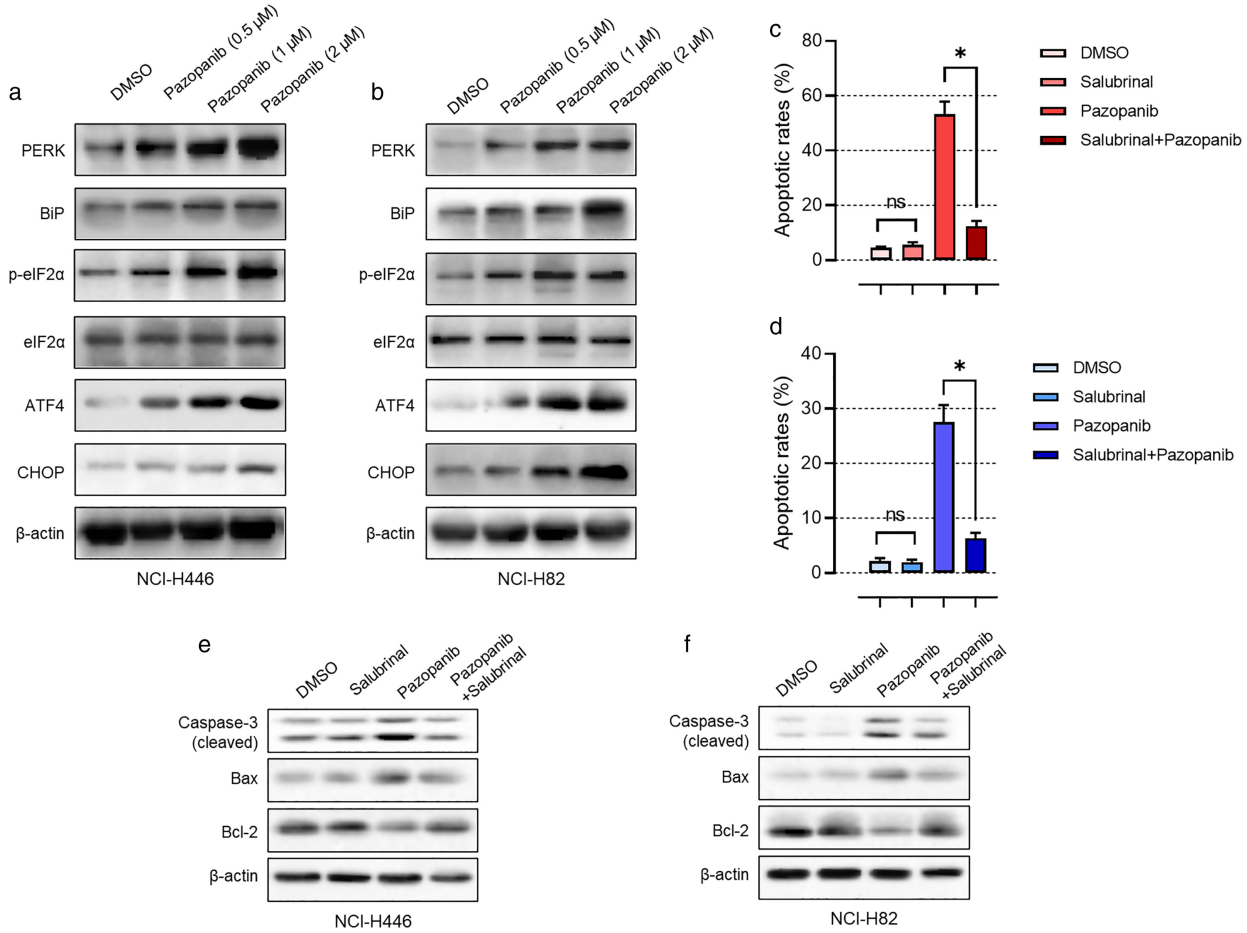


FIGURE 3 Pazopanib activates PERK-mediated ER stress. Western blot showed the PERK-mediated ER stress key molecule expression in both (a) NCI-H446 and (b) NCI-H82 cells following pazopanib (0.5, 1, and 2 μM) treatment for 24 h. Salubrinal was used as a dephosphorylation inhibitor of eIF2α, and the apoptotic cells (c) NCI-H446 and (d) NCI-H82 in single- and combination-used of salubrinal and pazopanib were evaluated by flow cytometry. The cleaved-caspase3, Bax, and Bcl-2 in (e) NCI-H446 and (f) NCI-H82 after salubrinal intervention were determined by Western blot. The numerical values illustrated on the bands indicated the relative intensity (% of loading control). Data are expressed as mean ± SD in at least three independent experiments. Statistical significance was recognized when $p < 0.05$ (*)

ER-stress may be responsible for pazopanib-induced SCLC cell death. Subsequently, the eIF2α dephosphorylation inhibitor salubrinal was coadministered with pazopanib to validate this hypothesis. As illustrated in Figure 3c and d, the combination use of salubrinal with pazopanib potently inhibits the pazopanib-induced apoptosis in both NCI-H446 and NCI-H82 cells. In addition, the apoptosis-related molecules, including caspase3, Bax, and Bcl-2, were all altered by coadministration of pazopanib with salubrinal (Figure 3e and f). However, single-use of salubrinal did not significantly change the apoptotic cells or apoptosis molecules in SCLC cells. This result indicated that the activation of ER-stress pathways is responsible for pazopanib-induced SCLC cell apoptosis.

ROS is responsible for pazopanib-induced SCLC cell apoptosis

ROS levels in both NCI-H446 and NCI-H82 cells were detected after pazopanib treatment. As shown in Figure 4a-4c, the flow cytometry results illustrated that pazopanib could significantly increase the intracellular ROS level in both NCI-H446 and NCI-H82 cells in a dose-dependent manner. The highest ROS positive cell rates were nearly 60% at 2 μM of pazopanib. Moreover, the intracellular ROS inhibitor NAC was coadministered with pazopanib, and annexin V/PI flow cytometry was used to detect apoptotic cells. As illustrated in Figure 4d and e, although single use NAC did not significantly alter the apoptotic cells in NCI-H446 or NCI-H82 cells, coadministration of pazopanib with NAC potently reversed the pazopanib-induced apoptosis in both NCI-H446 or NCI-H82 cells. This result indicated that the increased ROS level is responsible for pazopanib-induced SCLC cell apoptosis, which might be related to the activation of ER stress.

Pazopanib significantly suppresses xenografts, reversed by NAC

To further evaluate the anticancer effects of pazopanib and the mechanisms of action on inhibition of SCLC proliferation, an NCI-H446 cell xenograft NOD-SCID mouse model

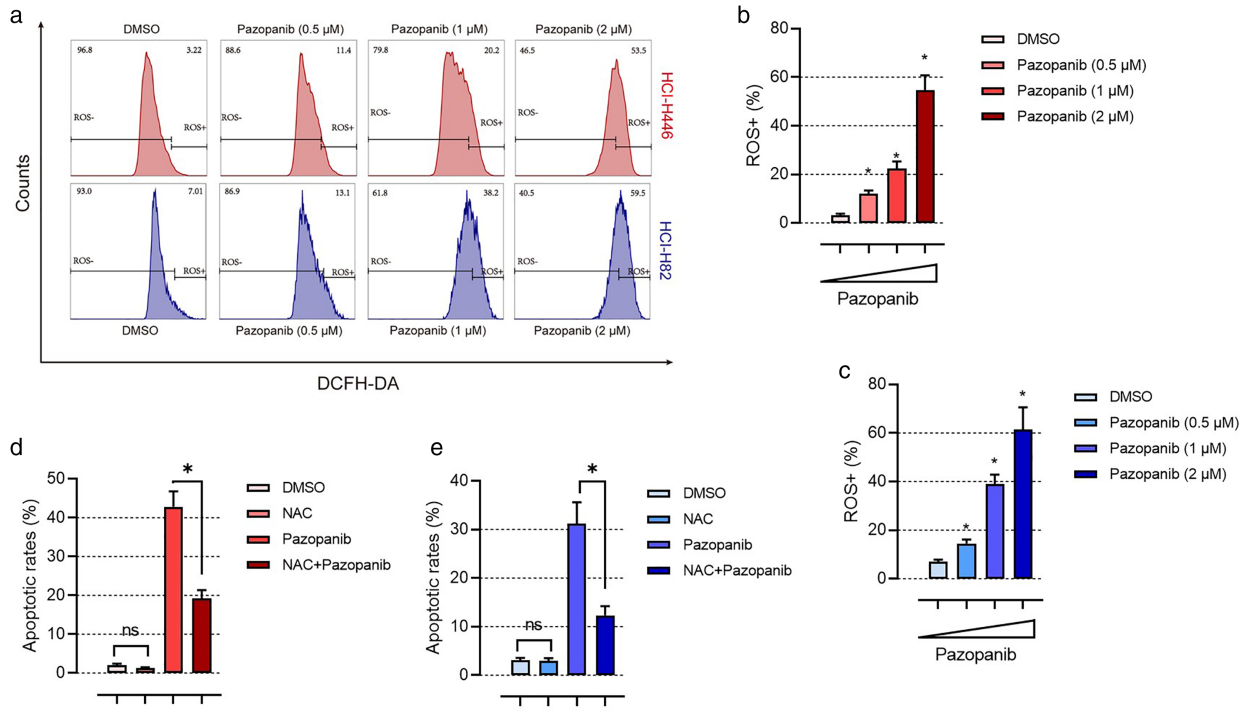


FIGURE 4 Pazopanib induces small cell lung cancer (SCLC) cell apoptosis through increased ROS levels. (a) The intracellular ROS level in NCI-H446 and NCI-H82 cell treatment with pazopanib (0.5, 1, and 2 μM) for 24 h was determined by DCFH-DA staining followed by flow cytometry. The statistical ROS positive cells in (b) NCI-H446 and (c) NCI-H82 were analyzed according to the data in (a) by FlowJo software. The NAC was used as a ROS inhibitor, and the apoptotic cells (d) NCI-H446 and (e) NCI-H82 in single- and combination-use NAC and pazopanib were evaluated by flow cytometry. Data are expressed as mean ± SD in at least three independent experiments. Statistical significance was recognized when $p < 0.05$ (*)

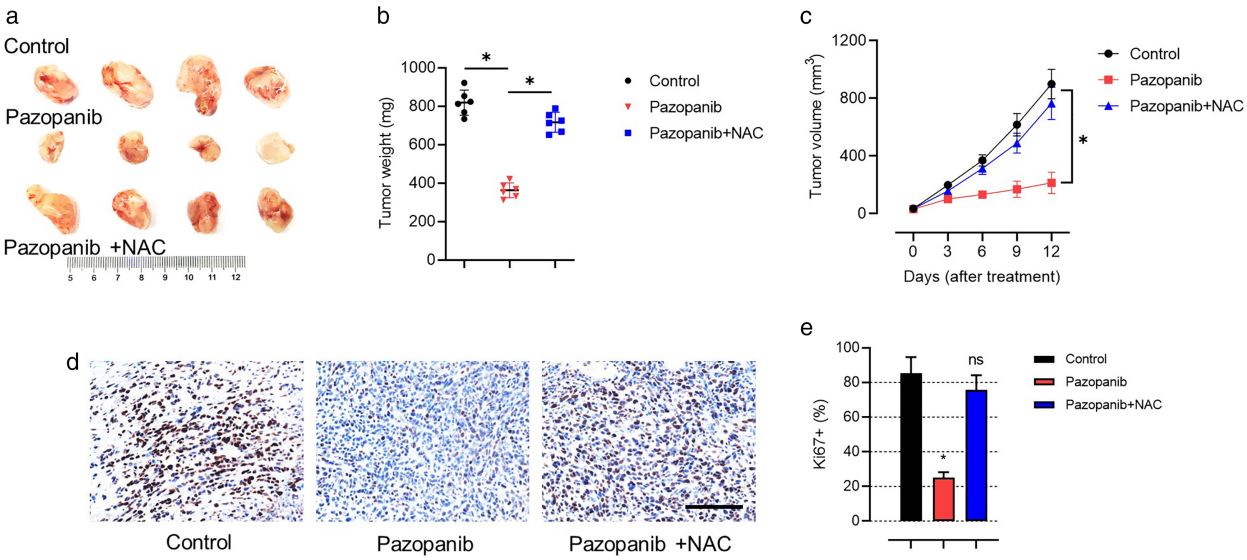


FIGURE 5 Pazopanib suppresses small cell lung cancer (SCLC) xenograft growth in vivo. (a) Images of NCI-H446 cells xenografts at the end of the treatment (pazopanib or pazopanib coadministration with NAC) of NOD-SCID mice ($n = 6$). (b) The tumor weight at the end of the treatment with pazopanib or pazopanib coadministration with NAC. (c) The tumor volume monitor data throughout the treatment processes. (d) Ki67 positive cells in tumor tissues by IHC detection. (e) Ki67 positive cells of (f) analysis by image J software. Data are expressed as mean ± SD in at least three independent experiments. Statistical significance was recognized when $p < 0.05$ (*)

was established, who were treated with single pazopanib or in combination with NAC. As illustrated in Figure 5a and b, administration of pazopanib significantly suppressed the growth of NCI-H446 xenograft. In addition, the dynamic tumor growth curve shown in Figure 5c indicated similar results. Interestingly, coadministration of pazopanib with

NAC significantly reversed the suppression of NCI-H446 xenograft growth. The IHC results for Ki67 detection shown in Figure 5d and e also indicated that pazopanib significantly decreased the proliferative activity in NCI-H446 xenograft, which can be reversed by NAC. These results further confirmed the abovementioned hypothesis that ROS is involved in pazopanib-induced inhibitory effects of SCLC cells.

DISCUSSION

Small cell lung cancer is the most malignant subtype of all lung cancers, and patients are often diagnosed with early distant metastasis and primary resistance to multiple antineoplastic agents.[21] The current treatment strategy for small cell lung cancer is mainly chemotherapy, but the drawbacks, such as low remission rate and high side effects, have limited its further clinical application.[22] Some targeted drugs, such as antiangiogenic drugs, are still in the clinical evaluation stage. Some have complicated mechanisms of action, and their production of antitumor activity may not be achieved through their initially designed targets.[23] Therefore, the evaluation of novel mechanisms of antitumor drugs that have entered clinical trials is essential for the treatment of small cell lung cancer and many other malignancies.

Pazopanib is an oral multitargeted tyrosine kinase inhibitor (TKI) that delivers antineoplastic effects by suppressing selective inhibition of vascular endothelial growth factor receptor (VEGFR)-mediated angiogenesis and by directly blocking growth-promoting receptor tyrosine kinases (RTKs), including platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs), and KIT.[24] Pazopanib is currently recognized as an attractive therapy alternative for soft tissue sarcoma (STS) and renal cell carcinoma (RCC).[25] Nevertheless, despite many clinical trials assessing its therapeutic efficacy in many other cancer types, the results have been less than satisfactory. For example, a phase III trial evaluated pazopanib as maintenance therapy after standard first-line platinum-based chemotherapy in patients with advanced non-small cell lung cancer (NSCLC). Compared with placebo, pazopanib failed to improve OS and PFS after platinum-based therapy in patients with NSCLC.[26] However, in another phase II study (KCSG-LU12-07) clinical trial for SCLC, pazopanib maintenance significantly prolonged PFS in patients with ED-SCLC,[10] suggesting that pazopanib might be a good candidate for SCLC treatment. Nevertheless, the potential mechanisms of action of pazopanib on SCLC cells remain to be determined.

Although pazopanib has been investigated as an antiangiogenic agent which can selectively inhibit VEGFR, in the present study, our main finding was that pazopanib could inhibit SCLC cell proliferation via ROS-mediated activation of ER-stress, which ultimately induces apoptosis, rather than blocking angiogenesis. Pazopanib has previously been documented to upregulate the proapoptotic molecule Bax in colorectal cancer cells.[27] Our results found that pazopanib significantly upregulated Bax while it downregulated Bcl-2, an antiapoptotic protein. Hence, it is confirmed that pazopanib induced direct apoptosis in SCLC cells. ROS accumulation is mainly responsible for higher oxidative stress levels in cancer cells than in normal cells, and compels cancer cells with a balanced upregulation antioxidant approach.[28] Interestingly, excessive levels of ROS synergistically induce cytotoxicity via the regulation of DNA damage, autophagy, metabolism, migration, and ER stress.[29,30] ROS-mediated ER stress is a critical approach in which a series of anticancer agents are utilized to induce apoptotic cell death in cancer cells.[31] Hence, we first evaluated whether ER-stress molecules were activated by pazopanib treatment in SCLC cells. Three effective apparatus are responsible for ER-stress, in which the PERK is independent of others related to a high level of ROS. Our results showed that pazopanib significantly increased PERK expression in SCLC cells, followed by BiP and p-eIF2α upregulation, ultimately activating transcriptional factor CHOP expression. To validate the apoptotic effects mediated by pazopanib induced by ER-stress, the eIF2α dephosphorylation inhibitor salubrinal[32] was coadministered with pazopanib, followed by apoptotic detection. The result showed that salubrinal reversed pazopanib-induced SCLC cell apoptosis, suggesting that ER-stress is involved in pazopanib-induced apoptosis in SCLC cells.

Predictably, after treatment with pazopanib, the intracellular ROS level in SCLC cells was significantly increased. However, whether the increased ROS is related to ER-stress-induced apoptosis after pazopanib treatment is unknown. Hence, we intervened with the pazopanib by ROS inhibitor, NAC. The result indicated that NAC could potently reverse pazopanib-induced SCLC cell apoptosis, suggesting that pazopanib-induced SCLC cell apoptosis is ROS-ER stress-dependent.

To further validate the anti-SCLC effects of pazopanib and the potential mechanisms of action, an NCI-H446 xenograft model was established using NOD-SCID mice. Pazopanib significantly suppressed the growth of xenograft, weakening the proliferation activity in tumor cells (Ki67), while NAC can reverse such effects, which further verified the findings in vitro.

In conclusion, the present study indicated that pazopanib showed anti-SCLC effects in vitro and in vivo. In addition, ROS-mediated ER stress is responsible for pazopanib-induced apoptosis in SCLC. However, further investigation of relevant biomarkers to accurately select patients who might benefit from pazopanib should be further investigated.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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