

Inhibition of Anlotinib-induced autophagy enhances pyroptosis and increases osteosarcoma sensitivity to anlotinib

Jia-Lin He

Affiliated Hospital of Qingdao University

Xiang Li

Affiliated Hospital of Qingdao University

Zi-Shuo Xi

Affiliated Hospital of Qingdao University

Jie Wang

Affiliated Hospital of Qingdao University

Shuo Wang

Ocean University of China

Ming-Yang Wang

Affiliated Hospital of Qingdao University

Zhong-Yi Chen

Affiliated Hospital of Qingdao University

Lin Quan

Affiliated Hospital of Qingdao University

Bin Yue

Affiliated Hospital of Qingdao University

Bingxin Zheng

bonetumor@qdu.edu.cn

Affiliated Hospital of Qingdao University

Article

Keywords: Osteosarcoma, Anlotinib, pyroptosis, autophagy, ROS

Posted Date: September 30th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10964447/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Background

Osteosarcoma (OS) is the most common primary malignant bone tumor, and therapeutic resistance remains a major challenge in improving patient outcomes. Although anlotinib exhibits promising antitumor activity against osteosarcoma, its efficacy may be limited by protective autophagy. This study aimed to investigate the roles of apoptosis, pyroptosis, and autophagy in the antitumor activity of anlotinib and to determine whether inhibition of autophagy could enhance its therapeutic efficacy.

Methods

The effects of anlotinib on osteosarcoma cells were evaluated in 143B and KHOS cell lines using CCK-8, colony formation, EdU incorporation, flow cytometry, TUNEL staining, western blotting, immunofluorescence, ELISA, transmission electron microscopy, ROS detection, and JC-1 assays. Autophagy was pharmacologically modulated using rapamycin and chloroquine to investigate its regulatory role in anlotinib-induced pyroptosis, oxidative stress, and mitochondrial dysfunction. The therapeutic efficacy of combined anlotinib and chloroquine treatment was further validated in a mouse xenograft model.

Results

Anlotinib significantly inhibited osteosarcoma cell proliferation and induced apoptosis in a dose-dependent manner. It also activated NLRP3/GSDMD-mediated pyroptosis while simultaneously inducing autophagy, accompanied by increased ROS production and mitochondrial dysfunction. Pharmacological activation of autophagy attenuated anlotinib-induced pyroptosis, oxidative stress, and mitochondrial damage, whereas autophagy inhibition by chloroquine markedly enhanced these effects. In vivo, combined treatment with anlotinib and chloroquine significantly suppressed tumor growth compared with anlotinib monotherapy and further increased the expression of pyroptosis-related proteins.

Conclusions

This study identifies protective autophagy as a critical mechanism limiting the antitumor efficacy of anlotinib in osteosarcoma. Pharmacological inhibition of autophagy enhances anlotinib-induced pyroptosis and improves its antitumor activity, providing preclinical evidence that targeting the autophagy–pyroptosis axis may represent a promising therapeutic strategy for overcoming resistance and improving osteosarcoma treatment.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor, characterized by its high malignancy and propensity for local invasion, recurrence, and metastasis, which significantly jeopardizes patient survival[1, 2]. The introduction of chemotherapy in the treatment of OS has increased the long-

term survival rate from 20% to 65%-70%[3]. However, approximately one-third of patients still experience poor outcomes. Furthermore, chemotherapy, while effective in killing tumor cells, also causes significant damage to healthy tissues. Therefore, a key goal in OS treatment is to specifically target tumor cells without harming normal tissues[4]. With advancements in medicine, targeted therapies have emerged as a promising approach, offering improved long-term survival for patients. Unlike traditional radiotherapy and chemotherapy, targeted therapies can accurately target key molecules involved in the development and progression of the disease, thereby enabling precise treatment while minimizing damage to normal tissues.

Angiogenesis plays a crucial role in the proliferation, migration, and invasion of malignant tumors[5]. Previous studies have shown that the growth and metastasis of osteosarcoma are closely associated with angiogenesis[6]. Anlotinib, a multi-target tyrosine kinase inhibitor, exerts its anti-tumor effects by targeting key receptors involved in tumor growth, angiogenesis, and metastasis, including vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), and c-kit[7]. It effectively reduces tumor blood supply, inhibits the formation of new blood vessels, and interferes with tumor cell survival and migration. Anlotinib has demonstrated significant anti-tumor activity in various cancers, including non-small cell lung cancer (NSCLC), soft tissue sarcomas (STS), and osteosarcoma[5, 7, 8]. Research has shown that Anlotinib significantly improves the overall survival of patients with advanced NSCLC, with a median survival increase of 3.3 months compared to the placebo group [8]. Despite its promising anti-tumor potential, further strategies are required to enhance its efficacy in cancer treatment.

Autophagy is a crucial self-degradation process that clears misfolded or aggregated proteins and damaged organelles (such as mitochondria, endoplasmic reticulum, and peroxisomes). By degrading and recycling intracellular substrates, autophagy effectively responds to cellular stress induced by factors like nutrient deprivation, hypoxia, and drugs [9–11]. In the early stages of carcinogenesis, autophagy aids in the removal of abnormal cells and prevents cancer development. However, during tumor progression, cancer cells exploit enhanced autophagy to maintain survival, promote growth, metastasis, and evade cell death, ultimately contributing to drug resistance[9, 12]. Studies have established a link between autophagy and tumor resistance to chemotherapy. For instance, in ovarian cancer, resistance to paclitaxel is induced by TXNDC17-mediated autophagy [13], and ovarian cancer also develops resistance to cisplatin through autophagy [14]. Additionally, research has shown that Anlotinib can induce protective autophagy, enabling tumor cells to evade the cytotoxic effects of anti-cancer drugs. For example, Anlotinib induces apoptosis and protective autophagy in human lung cancer cell lines, and inhibiting autophagy enhances Anlotinib's cytotoxicity[15]. However, research on the impact of autophagy on the anti-tumor effects of Anlotinib in osteosarcoma is still limited.

Pyroptosis is a form of lytic and inflammatory programmed cell death, and studies have shown that pyroptosis can improve the immunosuppressive tumor microenvironment, potentially serving as a strategy to enhance osteosarcoma treatment[16]. In recent years, there has been increasing attention on the interaction between autophagy and pyroptosis in various physiological and pathological processes.

Liraglutide has been shown to inhibit NLRP3 inflammasome activation and pyroptotic cell death by enhancing mitochondrial autophagy[17]. The neuroprotective effects of autophagy activation are mediated through the suppression of pyroptosis[18]. In light of these findings, this study aims to explore potential mechanisms to enhance the anti-tumor efficacy of Anlotinib in osteosarcoma treatment. We hypothesize that inhibiting Anlotinib-induced autophagy may increase pyroptosis, thereby enhancing its therapeutic effect on osteosarcoma.

Materials and methods

Cells and Reagents

The osteosarcoma cell lines 143B and KHOS used in this study were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured at 37°C in a 5% CO₂ atmosphere. The culture medium was supplemented with 10% fetal bovine serum (provided by Kordsa Creatures, Jiangsu, China). The cell culture media used include DMEM (Solarbio, Beijing, China) and RPMI 1640 (Solarbio, Beijing, China). The following antibodies were used in this study: Rabbit anti-NLRP3 antibody (DF7438) (Affinity, Jiangsu, China), Rabbit anti-GSDMD antibody (20770-1-AP) (Proteintech, Wuhan, China), Rabbit anti-GSDMD-N antibody (ab215203) (Abcam, UK), Rabbit anti-activated IL-1 β antibody (AF4006) (Affinity, Jiangsu, China), Rabbit anti-P62 antibody (Bioss, Beijing, China), Rabbit anti-GAPDH antibody (AB0037) (ShareBio, Shanghai, China), Rabbit anti-Beclin1 antibody (ab210498) (Abcam, UK), and Rabbit anti-LC3 antibody (14600-1-AP) (Proteintech, Wuhan, China).

CCK-8 Assay

After cells had adhered, they were treated with different concentrations of Anlotinib. Cell viability was evaluated using the CCK-8 assay kit (ShareBio, Shanghai, China). After incubation for 1 hour at 37°C with 5% CO₂, the OD at 450 nm was measured using a microplate reader. The procedure was repeated every 12 hours to monitor cell viability.

IC50 Determination

The IC₅₀ of Anlotinib was determined using the Cell Counting Kit-8 (CCK-8) assay (ShareBio, Shanghai, China). Cells were seeded in 96-well plates at a density of 5,000 cells per well and incubated overnight at 37°C in a 5% CO₂ humidified atmosphere. The following day, cells were treated with various concentrations of Anlotinib for 24 hours. After treatment, CCK-8 solution was added to each well, and the plates were incubated at 37°C for 1–2 hours. The optical density (OD) at 450 nm was measured using a microplate reader. The IC₅₀ value was defined as the drug concentration required to reduce cell viability by 50%. Data were fitted to a nonlinear regression curve using GraphPad Prism software.

Colony Formation Assay

Cells (5×10^3 per well) were seeded in 6-well plates and incubated with varying concentrations of Anlotinib for 14 days at 37°C. After fixation with 4% paraformaldehyde (PFA) at room temperature for 30 minutes, colonies were stained with 0.1% crystal violet for 30 minutes. Colonies were visualized and counted.

EdU Assay

Cell proliferation was assessed using the 488 Click-iT EdU Imaging Kit (ShareBio, Beijing, China). Following the manufacturer's instructions, EdU-labeled cells were processed and analyzed according to the kit's protocol. Proliferation was observed and photographed using a Nikon fluorescence microscope (Nikon, Shanghai, China).

Calcein-AM/PI Cell Viability and Cytotoxicity Assay

Cell viability and cytotoxicity were assessed using the Calcein-AM/PI Cell Viability/Cytotoxicity Assay Kit (Beyotime, Shanghai, China). Osteosarcoma cells were seeded in 96-well plates and treated with varying concentrations of Anlotinib. After treatment, the cells were stained with Calcein-AM and PI, where Calcein-AM stains live cells green and PI stains dead cells red. Fluorescence intensity of green and red signals was measured using a fluorescence microscope or microplate reader to calculate cell viability and cytotoxicity, thereby evaluating the therapeutic effect of the drug.

TUNEL Assay

Cell apoptosis was evaluated using the 488 TUNEL Apoptosis Assay Kit (ShareBio, Beijing, China). Cells were treated according to the kit instructions, and apoptosis was observed and imaged using a Nikon fluorescence microscope (Nikon, Shanghai, China).

Flow Cytometry Analysis

Flow cytometry was employed to assess cell apoptosis and viability. After treatment, cells were stained with Annexin V-FITC and PI (Yeasen, Shanghai, China) to distinguish early apoptotic cells and late apoptotic/necrotic cells, respectively. The stained cells were analyzed using a flow cytometer, and the proportions of live cells, early apoptotic cells, and late apoptotic/necrotic cells were calculated based on fluorescence intensity.

Western Blot Analysis

Proteins from cells or tissues were extracted using RIPA lysis buffer (Beyotime, China). Total protein concentration was determined using a BCA assay kit (PC0022, Solarbio, Beijing, China). Equal amounts of protein (20–40 µg per well) were separated by SDS-PAGE and transferred to PVDF membranes.

Membranes were blocked for 1 hour at room temperature with 5% non-fat milk in TBS-T buffer, followed by overnight incubation at 4°C with primary antibodies. After washing, membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 hour. Protein bands were visualized using ECL substrate (SB-WB012, ShareBio, Shanghai, China) and detected using the Alliance Q9 Advanced Multipurpose Chemiluminescent Imaging System (UVITEC, UK).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde and treated with 0.1% Triton X-100 at room temperature to enhance membrane permeability. The samples were blocked with BSA blocking solution and incubated overnight at 4°C with primary antibodies against LC3B and NLRP3. Secondary antibodies were then applied at room temperature. Images were captured using a confocal microscope (Nikon).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells treated with Anlotinib were analyzed for IL-1 β and IL-18 levels using commercially available ELISA kits. Absorbance was measured at 450 nm using a microplate reader, and the antigen/antibody concentration was calculated using the standard curve.

Electron Microscopy

Cells treated with Anlotinib and untreated cells were collected by scraping from the culture plate, followed by centrifugation to remove the supernatant. Cells were fixed overnight at 4°C with 2.5% glutaraldehyde. Ultrathin sections (100 nm) were prepared and stained with uranyl acetate and lead citrate. Samples were examined using a transmission electron microscope (H-600; Hitachi, Tokyo, Japan).

Detection of Intracellular ROS Production

Intracellular reactive oxygen species (ROS) levels were measured using a ROS assay kit (Solarbio, Beijing, China) and observed via fluorescence microscopy.

Mitochondrial Membrane Potential (MMP) Evaluation

Changes in mitochondrial membrane potential were assessed using a mitochondrial membrane potential detection kit (JC-1) (Solarbio, Beijing, China). Fluorescence was observed, with a shift from red to green fluorescence indicating a decrease in membrane potential.

Immunohistochemistry

Paraffin-embedded tissue sections were first dewaxed and hydrated, followed by antigen retrieval by heating in a citrate buffer (pH 6.0) at 95–99°C. After antigen retrieval, sections were washed with distilled water and PBS, then treated with 3% hydrogen peroxide (H₂O₂) to block endogenous peroxidase activity. Subsequently, sections were incubated with 5% goat serum at room temperature to block non-specific binding. After blocking, sections were incubated overnight at 4°C with a 1:50 dilution of the primary antibody. The following steps included washing with PBS, incubation with secondary antibody,

and color development using HRP substrate solution. After staining, sections were counterstained with hematoxylin, dehydrated, and mounted. The immunohistochemistry procedure for animal tissues was similar, with modifications in antibody types and dilution ratios.

Xenograft Tumor Model

Female BALB/c nude mice (4–6 weeks old, weighing 16–20 g) were purchased from Beijing Huafukang Biotechnology Co., Ltd. (License No.: SCXK (Jing) 2024-0003), Beijing, China. The whole transportation process was performed in full compliance with the transportation specifications for SPF-grade laboratory animals. The mice were housed under specific pathogen-free (SPF) conditions in the Laboratory Animal Platform of the Biomedical Center at Qingdao University, with a controlled temperature ($22 \pm 2^\circ\text{C}$), relative humidity ($50 \pm 10\%$), and a 12-hour light/dark cycle. All animals had free access to sterilized food and water. All animal manipulations were conducted after one week of adaptive rearing. All experimental procedures were performed in accordance with the institutional guidelines for the care and use of laboratory animals and were approved by the Laboratory Animal Welfare Ethics Committee of Qingdao University (approval number: No.202501BALB/C-nude66202502185; approval date: March 6, 2025).

At the end of the experiment, mice were first anesthetized by inhalation of isoflurane (3–5% v/v in oxygen, flow rate 1 L/min) using a small animal anesthesia induction chamber (RWD Life Science Co., Ltd., Shenzhen, China). Once the righting reflex was lost, animals were transferred to a standardized CO₂ euthanasia chamber. CO₂ was delivered at a flow rate of 30% of the chamber volume per minute, and animals were maintained in the CO₂ atmosphere for at least 2 minutes after respiratory arrest to ensure death. Death was confirmed by the absence of heartbeat and respiratory movements. This euthanasia protocol is consistent with the AVMA Guidelines for the Euthanasia of Animals (2020 Edition). No additional chemical agents were administered for euthanasia.

Results

Anlotinib Inhibits Osteosarcoma Cell Proliferation

To investigate the anti-proliferative effects of Anlotinib on osteosarcoma cells, cell viability was first assessed in the 143B and KHOS cell lines following treatment with varying concentrations of Anlotinib for different time periods. Anlotinib treatment resulted in a dose- and time-dependent inhibition of cell viability in both 143B and KHOS cells (Fig. 1A and 1B). The half-maximal inhibitory concentration (IC₅₀) values for Anlotinib were determined to be 12.32 μM for 143B cells and 15.92 μM for KHOS cells (Fig. 1C and 1D). These results suggest that Anlotinib effectively inhibits osteosarcoma cell proliferation in a concentration-dependent manner. To further evaluate the long-term effects of Anlotinib on cell growth, colony formation assays were conducted. As shown in Fig. 1E and 1F, Anlotinib significantly reduced the number of colonies formed by both 143B and KHOS cells, indicating its potent anti-proliferative effect. EdU incorporation assays were performed to assess the impact of Anlotinib on DNA synthesis. Treatment with Anlotinib significantly reduced the number of EdU-positive cells in both 143B and KHOS

cell lines (Fig. 1G-I), indicating that Anlotinib effectively inhibits DNA synthesis and cell proliferation. These findings collectively demonstrate that Anlotinib exerts a strong anti-proliferative effect on osteosarcoma cells by inhibiting DNA synthesis and colony formation.

Anlotinib Induces Apoptosis in Osteosarcoma Cells

To assess the apoptotic effect of Anlotinib on osteosarcoma cells, 143B and KHOS cell lines were treated with Anlotinib at IC₅₀ and 2×IC₅₀ concentrations, and PI/AM staining was performed. Treatment with Anlotinib significantly increased the proportion of PI-positive cells in both 143B and KHOS cells, indicating a marked increase in cell death (Fig. 2A and 2B). Subsequently, TUNEL staining was conducted to detect apoptotic cells. As shown in Fig. 2C and 2D, Anlotinib treatment significantly increased the number of apoptotic cells in both 143B and KHOS cell lines, further confirming its pro-apoptotic effect. In addition, flow cytometry analysis was performed to quantify the proportions of early and late apoptotic cells. Anlotinib treatment resulted in a significant increase in both early and late apoptotic cell populations in 143B and KHOS cells at IC₅₀ and 2×IC₅₀ concentrations (Fig. 2E–H). These results provide compelling evidence that Anlotinib induces apoptosis in osteosarcoma cells in a concentration-dependent manner.

Anlotinib Induces Pyroptosis and Autophagy in Osteosarcoma Cells

To investigate the effects of Anlotinib on pyroptosis and autophagy, we first assessed the expression of pyroptosis-related proteins. Western blot and immunofluorescence analyses revealed that Anlotinib significantly upregulated the expression of NLRP3, GSDMD-N, and IL-1 β in both 143B and KHOS cells (Fig. 3A and 3B), with enhanced fluorescence intensity observed in immunofluorescence staining. ELISA results further confirmed that the secretion of IL-1 β and IL-18 was significantly increased following Anlotinib treatment (Fig. 3C), indicating the activation of pyroptosis. Next, we examined the impact of Anlotinib on autophagy-related proteins. Western blot analysis showed a significant increase in the expression of Beclin-1 and LC3-II, while P62 expression was decreased (Fig. 3D). Immunofluorescence staining further confirmed the upregulation of LC3 expression, supporting the activation of autophagy (Fig. 3E). Transmission electron microscopy (TEM) analysis revealed a significant increase in the number of autophagosomes in Anlotinib-treated cells, suggesting enhanced autophagy (Fig. 3F). To evaluate the effect of Anlotinib on oxidative stress, ROS levels were measured using a ROS assay. Anlotinib treatment significantly increased ROS levels in both 143B and KHOS cells, with enhanced fluorescence intensity observed (Fig. 3G). Finally, JC-1 staining was performed to assess mitochondrial membrane potential. Anlotinib treatment resulted in a significant decrease in mitochondrial membrane potential, as indicated by a reduction in red fluorescence (aggregated JC-1) and an increase in green fluorescence (monomeric JC-1), suggesting mitochondrial damage (Fig. 3H). Together, these results provide compelling evidence that Anlotinib induces both pyroptosis and autophagy in osteosarcoma cells, contributing to cellular stress and damage.

Impact of Autophagy Regulation on Anlotinib-Induced Pyroptosis, Mitochondrial Damage, and Oxidative Stress

To explore the role of autophagy in Anlotinib-induced pyroptosis, mitochondrial damage, and oxidative stress, we first investigated the effect of autophagy modulation on the expression of pyroptosis-related proteins. Western blot and immunofluorescence analyses showed that the autophagy activator rapamycin (RAPA) significantly reduced the expression of NLRP3, GSDMD, and GSDMD-N induced by Anlotinib (Fig. 4A and 4C), whereas the autophagy inhibitor chloroquine (CQ) enhanced their expression (Fig. 4B and 4D). Statistical analysis confirmed that RAPA significantly inhibited NLRP3 expression, while CQ significantly enhanced it. Next, we assessed mitochondrial membrane potential and ROS levels to evaluate the impact of autophagy regulation on mitochondrial function. JC-1 staining and ROS assays revealed that RAPA attenuated the decline in mitochondrial membrane potential and the increase in ROS levels induced by Anlotinib (Fig. 4E and 4G). In contrast, CQ further exacerbated these effects (Fig. 4F and 4H). Statistical analysis indicated that autophagy plays a critical role in regulating cellular stress responses induced by Anlotinib. These findings underscore the pivotal role of autophagy in modulating Anlotinib-induced pyroptosis, mitochondrial damage, and oxidative stress in osteosarcoma cells.

Autophagy Inhibitor CQ Enhances the Anti-Tumor Effect of Anlotinib

To evaluate the impact of autophagy inhibition on Anlotinib's anti-tumor effects, we utilized a mouse xenograft model. As shown in Fig. 5A-C, treatment with Anlotinib alone significantly inhibited tumor growth, while combining Anlotinib with the autophagy inhibitor chloroquine (CQ) further enhanced the anti-tumor effects. Both tumor weight (Fig. 5B) and volume (Fig. 5C) were significantly reduced in the combination treatment group compared to Anlotinib alone. Further analysis via immunohistochemistry (IHC) and Western blotting revealed that Anlotinib treatment increased the expression of NLRP3 and GSDMD-N (Fig. 5D and 5E). However, when combined with CQ, the expression of these proteins was significantly elevated, suggesting that cell pyroptosis was further activated by the combination treatment. These findings indicate that autophagy inhibition by CQ potentiates the anti-tumor efficacy of Anlotinib, likely through enhanced activation of pyroptosis.

Discussion

Anlotinib is a multi-targeted tyrosine kinase inhibitor (TKI) that primarily targets receptor tyrosine kinases (RTKs), which are involved in various critical physiological processes such as cell proliferation, apoptosis, and growth. Additionally, RTKs are implicated in the pathogenesis of several diseases, including cancer, metabolic and autoimmune disorders, infectious diseases, and neurodegenerative conditions[7]. Anlotinib has been shown to inhibit angiogenesis and tumor cell signaling, demonstrating significant anti-tumor activity in various malignancies[19].

This study further investigates the mechanisms underlying the therapeutic effects of Anlotinib in osteosarcoma. Our findings indicate that Anlotinib not only effectively inhibits osteosarcoma cell proliferation and growth but also enhances its anti-tumor efficacy by inducing apoptosis and pyroptosis. However, autophagy appears to play a protective role in this context, aiding tumor cells in evading the cytotoxic effects of Anlotinib, thereby potentially undermining the overall therapeutic outcomes.

Our study confirms that Anlotinib treatment significantly inhibits the proliferation of osteosarcoma cells, while also notably increasing the proportion of cell death. Previous studies have demonstrated that Anlotinib exerts its anti-tumor effects through the induction of apoptosis. For example, in breast cancer and hepatocellular carcinoma, Anlotinib has been shown to activate apoptotic pathways[20, 21]. Consistent with these findings, our flow cytometry results indicate that Anlotinib treatment significantly increases the proportions of early apoptosis, late apoptosis, and necrotic cells in osteosarcoma cell lines, further supporting the ability of Anlotinib to induce apoptosis and inhibit cell survival.

To further investigate the specific form of cell death, we focused on verifying the role of pyroptosis, a form of programmed cell death. Pyroptosis is a pro-inflammatory form of programmed cell death mediated by Gasdermin family proteins, which occurs via classical (caspase-1 dependent) and non-classical (caspase-4/5/11 dependent) pathways. Notably, inflammasome-mediated pyroptosis is classified under the classical caspase-1-dependent pathway[22]. In this pathway, NLRP3, a pattern recognition receptor (PRR), is one of the most common inflammasome sensors. NLRP3 can be activated by various endogenous and exogenous stimuli, including viruses, bacterial toxins, reactive oxygen species (ROS), endogenous damage signals, and certain drugs[22]. Upon activation, NLRP3 inflammasomes recruit and cleave pro-caspase-1, resulting in the production of active caspase-1. Active caspase-1 then cleaves Gasdermin D (GSDMD), releasing its N-terminal fragment (GSDMD-NT), which forms non-selective pores in the cell membrane. Additionally, caspase-1 cleaves and activates the precursors of IL-1 β and IL-18, converting them into their active forms, which are subsequently released into the extracellular space through the membrane pores formed by GSDMD. This process is accompanied by cell swelling, rupture, and the release of numerous inflammatory cytokines, leading to intense local inflammation and immune responses[23, 24]. Thus, due to its unique inflammatory characteristics, pyroptosis has attracted widespread attention.

According to a recent study by Chen Wang et al, Anlotinib has been shown to induce the release of pyroptosis-related proteins and inflammatory cytokines in bladder cancer cells, thereby promoting anti-tumor immune responses[25]. This finding aligns closely with the classical activation mechanism of the NLRP3 inflammasome, suggesting that Anlotinib may induce tumor cell pyroptosis through triggering inflammasome signaling pathways. Based on this premise, we designed experiments to determine whether Anlotinib not only induces apoptosis but also triggers pyroptosis. Our experimental results demonstrate that Anlotinib treatment significantly increased the levels of pyroptosis-related proteins (e.g., NLRP3, GSDMD-N) and inflammatory cytokines (e.g., IL-1 β , IL-18), indicating that Anlotinib induces pyroptosis in osteosarcoma cells.

Therefore, Anlotinib not only inhibits tumor growth by inducing apoptosis but also enhances its anti-tumor efficacy by inducing pyroptosis, further reinforcing its potential as an effective therapeutic agent for osteosarcoma.

As previously discussed, numerous studies have shown that the NLRP3 inflammasome can sense reactive oxygen species (ROS). NLRP3 may directly sense ROS generated by normal or dysfunctional mitochondria or indirectly sense ROS produced by other activating factors [26, 27]. Wang et al. observed that ROS generation and mitochondrial membrane potential (MMP) collapse are closely associated with the activation of the NLRP3 inflammasome. Loss of mitochondrial membrane potential can lead to ROS accumulation, which, in turn, promotes the activation of the NLRP3 inflammasome and initiates pyroptosis [28].

However, ROS are not only closely related to pyroptosis but also play a crucial role in autophagy. Autophagy has a complex dual role in cancer. In the early stages of cancer, autophagy maintains cellular homeostasis and suppresses tumor initiation. Once the tumor is formed and progresses to later stages, autophagy becomes a critical support mechanism for tumor cell survival and development. At this point, tumor cells rely on autophagy to maintain their high metabolic rate and evade detrimental factors that could induce cell death. Therefore, autophagy becomes an essential factor in tumor maintenance, progression, and drug resistance[9, 12, 29]. ROS accumulation disrupts cellular homeostasis, leading to oxidative stress and mitochondrial dysfunction, and induces autophagy. Oxidative stress plays a facilitating role in autophagy formation, and conversely, autophagy reduces oxidative damage by engulfing and degrading oxidative materials, thereby protecting cells from further damage[30].

As a key signaling molecule in oxidative stress, ROS plays a pivotal role in the tumor microenvironment (TME), coordinating the complex interactions between immune responses and modes of cell death, such as autophagy and apoptosis[31]. Building upon existing research, Anlotinib, a multi-targeted tyrosine kinase inhibitor, has been shown to significantly influence ROS and autophagy processes in various cancer cells. For instance, Anlotinib dose-dependently increases ROS levels and activates autophagy, thereby inducing ferroptosis and enhancing its anti-tumor effects in thyroid cancer cells. Further autophagy inhibition potentiates these effects, indicating a synergistic role of ROS and autophagy in modulating Anlotinib's therapeutic efficacy[32]. Yang et al. found that Anlotinib induces apoptosis in human pancreatic cancer cells by accumulating ROS[33]. Additionally, Anlotinib dose- and time-dependently induces autophagy in human lung cancer cells [15], and promotes apoptosis in breast cancer cells via autophagy induction[21]. In glioblastoma cells, Anlotinib induces autophagy by increasing the levels of Beclin-1 and microtubule-associated protein 1 light chain 3B (LC3B)[34].

Consistent with these studies, our experimental results demonstrate that Anlotinib treatment significantly increases ROS levels in osteosarcoma cell lines, while mitochondrial membrane potential changes and mitochondrial damage are exacerbated. Additionally, the level of autophagy in the treated cells is also elevated. These findings are in agreement with existing literature and suggest that Anlotinib enhances both oxidative stress and autophagy in osteosarcoma cells.

According to existing research, combining anticancer drugs with autophagy inhibition can improve anti-tumor effects[35, 36], Autophagy plays a crucial role in the development of drug resistance in cancer therapy. Studies have shown that elevated autophagy levels are associated with resistance to BRAF inhibitors, such as vemurafenib, in melanoma. Interestingly, autophagy inhibition has been shown to effectively reverse this resistance[37]. These findings suggest that modulating autophagy could provide a novel strategy to overcome drug resistance during tumor treatment.

To further explore the effects and mechanisms of Anlotinib in osteosarcoma, this study integrates current literature and experimental data, focusing on the interplay between Anlotinib, autophagy, and pyroptosis. Autophagy and pyroptosis are two important forms of programmed cell death, and both are closely involved in cellular responses, immune regulation, and disease progression. Previous studies have shown that autophagy inhibits pyroptosis by suppressing ROS accumulation and mtDNA release[38, 39]. These studies provide a theoretical foundation for understanding the interaction between autophagy and pyroptosis.

In this study, we used a combination of autophagy inhibitors and activators with Anlotinib treatment to investigate the role of autophagy in Anlotinib therapy and its regulatory effects on pyroptosis. Our results demonstrate that under autophagy inhibition, ROS levels significantly increased in osteosarcoma cells, accompanied by exacerbated mitochondrial damage. Western blot (WB) and immunofluorescence staining further revealed a significant increase in pyroptosis levels. These findings suggest that autophagy inhibition leads to ROS accumulation and further mitochondrial dysfunction, thereby promoting the occurrence of pyroptosis. Conversely, when autophagy was activated, ROS levels significantly decreased, the number of damaged mitochondria was reduced, and pyroptosis was suppressed. This suggests that autophagy plays a critical protective role during Anlotinib treatment in osteosarcoma, likely by regulating ROS levels and mitochondrial membrane potential changes to inhibit pyroptosis.

For the first time, we demonstrated that inhibiting Anlotinib-induced autophagy increases pyroptosis, thereby enhancing Anlotinib's anti-tumor efficacy and alleviating drug resistance. These findings further emphasize the important role of autophagy in Anlotinib treatment for osteosarcoma. Autophagy not only protects tumor cells from premature death by regulating oxidative stress and mitochondrial function but may also delay tumor cell death by reducing the onset of pyroptosis during drug treatment. Notably, inhibiting autophagy may promote pyroptosis and enhance the anti-tumor effect, providing new directions for developing combination therapies.

Therefore, regulating autophagy levels could serve as a potential strategy to improve Anlotinib efficacy and overcome resistance. In this study, we extensively explored the anti-tumor effects of Anlotinib in osteosarcoma, particularly focusing on the interplay between autophagy and pyroptosis. Despite our study providing important experimental data to elucidate the therapeutic mechanisms of Anlotinib, there are several limitations. First, we have preliminarily shown that autophagy regulates ROS levels and mitochondrial function to inhibit pyroptosis. However, the role of ROS in cells is complex and diverse, and

its exact function in the interaction between autophagy and pyroptosis remains unclear. Future studies should further investigate the precise role of ROS in regulating the interplay between autophagy and pyroptosis. Additionally, although our experiments suggest that Anlotinib enhances osteosarcoma cell death, translating these findings into clinical practice remains challenging. Overcoming these limitations will pave the way for future research to provide new theoretical insights and practical directions for enhancing Anlotinib's anti-tumor efficacy, overcoming resistance, and developing more effective therapeutic strategies.

In conclusion, this study unveils a dual mechanism by which anlotinib exerts its antitumor effects in osteosarcoma. On one hand, anlotinib induces tumor cell death by activating apoptosis and pyroptosis. On the other hand, we provide the first evidence that modulating autophagy in osteosarcoma can enhance the antitumor efficacy of anlotinib and overcome drug resistance, offering new insights for the development of combination therapeutic strategies.

Conclusion

In conclusion, this study highlights the critical regulatory roles of autophagy and pyroptosis in the antitumor effects of anlotinib in osteosarcoma. Inhibition of anlotinib-induced autophagy significantly enhances pyroptosis, thereby improving its antitumor activity. These findings provide a potential new therapeutic target for osteosarcoma treatment. However, further investigations are needed to elucidate the molecular mechanisms underlying the regulation of autophagy and pyroptosis by anlotinib, as well as to evaluate the clinical applicability of this strategy. Through in-depth studies, we believe that the combination of anlotinib and autophagy inhibitors holds promise for delivering greater therapeutic benefits to osteosarcoma patients.

Declarations

Ethics approval and consent to participate

All animal experiments were performed in accordance with the institutional guidelines for the care and use of laboratory animals and were approved by the Ethics Committee of Qingdao University (approval number: No.202501BALB/C-nude66202502185).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Shandong Provincial Natural Science Foundation (ZR2021QH038), Shandong Province Higher Education Institutions Youth Innovation Team Development Plan (2024KJJ046), and Qingdao Outstanding Health Professional Development Fund.

Author Contribution

Jia-Lin He and Xiang Li performed the majority of the experiments, analyzed the data, and drafted the manuscript. Zi-Shuo Xi, Jie Wang, and Shuo Wang performed part of the experiments. Ming-Yang Wang, Zheng-Yi Chen, and Lin Quan participated in manuscript preparation and revision. Bin Yue and Bingxin Zheng conceived and designed the study, supervised the project, revised the manuscript, and acquired funding. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Data Availability

The data sets analyzed within the current study are available from the corresponding author on reasonable request.

References

1. Jiang, H. et al. Establishment of a prognostic risk model for osteosarcoma and mechanistic investigation. *Front. Pharmacol.* **15**, 1399625 (2024).
2. Chen, C. et al. Immunotherapy for osteosarcoma: Fundamental mechanism, rationale, and recent breakthroughs. *Cancer Lett.* **500**, 1–10 (2021).
3. Isakoff, M. S., Bielack, S. S., Meltzer, P. & Gorlick, R. Osteosarcoma: Current Treatment and a Collaborative Pathway to Success. *J. Clin. Oncol.* **33** (27), 3029–3035 (2015).
4. Xie, D. et al. Targeted Delivery of Chemotherapeutic Agents for Osteosarcoma Treatment. *Front. Oncol.* **12**, 843345 (2022).
5. Wang, G. et al. Anlotinib, a novel small molecular tyrosine kinase inhibitor, suppresses growth and metastasis via dual blockade of VEGFR2 and MET in osteosarcoma. *Int. J. Cancer.* **145** (4), 979–993 (2019).
6. Wang, L. H. et al. CTGF promotes osteosarcoma angiogenesis by regulating miR-543/angiopoietin 2 signaling. *Cancer Lett.* **391**, 28–37 (2017).

7. Shen, G. et al. Anlotinib: a novel multi-targeting tyrosine kinase inhibitor in clinical development. *J. Hematol. Oncol.* **11** (1), 120 (2018).
8. Han, B. et al. Effect of Anlotinib as a Third-Line or Further Treatment on Overall Survival of Patients With Advanced Non-Small Cell Lung Cancer: The ALTER 0303 Phase 3 Randomized Clinical Trial. *JAMA Oncol.* **4** (11), 1569–1575 (2018).
9. Zamame Ramirez, J. A., Romagnoli, G. G. & Kaneno, R. Inhibiting autophagy to prevent drug resistance and improve anti-tumor therapy. *Life Sci.* **265**, 118745 (2021).
10. Glick, D., Barth, S. & Macleod, K. F. Autophagy: cellular and molecular mechanisms. *J. Pathol.* **221** (1), 3–12 (2010).
11. Parzych, K. R. & Klionsky, D. J. An overview of autophagy: morphology, mechanism, and regulation. *Antioxid. Redox. Signal.* **20** (3), 460–473 (2014).
12. Mizushima, N. & Komatsu, M. Autophagy: renovation of cells and tissues. *Cell* **147** (4), 728–741 (2011).
13. Zhang, S. F. et al. TXNDC17 promotes paclitaxel resistance via inducing autophagy in ovarian cancer. *Autophagy* **11** (2), 225–238 (2015).
14. Zhou, F. et al. Down-regulation of OGT promotes cisplatin resistance by inducing autophagy in ovarian cancer. *Theranostics* **8** (19), 5200–5212 (2018).
15. Liang, L. et al. Autophagy inhibition potentiates the anti-angiogenic property of multikinase inhibitor anlotinib through JAK2/STAT3/VEGFA signaling in non-small cell lung cancer cells. *J. Exp. Clin. Cancer Res.* **38** (1), 71 (2019).
16. Jin, J. et al. Mitochondria-Targeting Polymer Micelle of Dichloroacetate Induced Pyroptosis to Enhance Osteosarcoma Immunotherapy. *ACS nano.* **16** (7), 10327–10340 (2022).
17. Yu, X. et al. Liraglutide ameliorates non-alcoholic steatohepatitis by inhibiting NLRP3 inflammasome and pyroptosis activation via mitophagy. *Eur. J. Pharmacol.* **864**, 172715 (2019).
18. Gao, C. et al. Autophagy Activation Represses Pyroptosis through the IL-13 and JAK1/STAT1 Pathways in a Mouse Model of Moderate Traumatic Brain Injury. *ACS Chem. Neurosci.* **11** (24), 4231–4239 (2020).
19. Sun, Y. et al. Safety, pharmacokinetics, and antitumor properties of anlotinib, an oral multi-target tyrosine kinase inhibitor, in patients with advanced refractory solid tumors. *J. Hematol. Oncol.* **9** (1), 105 (2016).
20. He, C., Wu, T. & Hao, Y. Anlotinib induces hepatocellular carcinoma apoptosis and inhibits proliferation via Erk and Akt pathway. *Biochem. Biophys. Res. Commun.* **503** (4), 3093–3099 (2018).
21. Chen, S. et al. Anti-cancer Drug Anlotinib Promotes Autophagy and Apoptosis in Breast Cancer, *Frontiers in bioscience. (Landmark edition).* **27** (4), 125 (2022).
22. Bibo-Verdugo, B., Snipas, S. J., Kolt, S., Poreba, M. & Salvesen, G. S. Extended subsite profiling of the pyroptosis effector protein gasdermin D reveals a region recognized by inflammatory caspase-11. *J. Biol. Chem.* **295** (32), 11292–11302 (2020).

23. Liu, Z. et al. Caspase-1 Engages Full-Length Gasdermin D through Two Distinct Interfaces That Mediate Caspase Recruitment and Substrate Cleavage. *Immunity* **53** (1), 106–114e5 (2020).
24. Swanson, K. V., Deng, M. & Ting, J. P. The NLRP3 inflammasome: molecular activation and regulation to therapeutics, *Nature reviews. Immunology* **19** (8), 477–489 (2019).
25. Wang, C. et al. Anlotinib Enhances the Therapeutic Effect of Bladder Cancer with GSDMB Expression: Analyzed from TCGA Bladder Cancer Database & Mouse Bladder Cancer Cell Line. *Pharmgenomics Pers. Med.* **16**, 219–228 (2023).
26. Dominic, A., Le, N. T. & Takahashi, M. Loop Between NLRP3 Inflammasome and Reactive Oxygen Species. *Antioxid. Redox. Signal.* **36** (10–12), 784–796 (2022).
27. Minutoli, L. et al. ROS-Mediated NLRP3 Inflammasome Activation in Brain, Heart, Kidney, and Testis Ischemia/Reperfusion Injury, *Oxid Med Cell Longev* (2016) 2183026. (2016).
28. Wang, Y. et al. Mitochondrial ROS promote macrophage pyroptosis by inducing GSDMD oxidation. *J. Mol. Cell Biol.* **11** (12), 1069–1082 (2019).
29. Debnath, J., Gammoh, N. & Ryan, K. M. Autophagy and autophagy-related pathways in cancer, *Nature reviews. Mol. cell. biology.* **24** (8), 560–575 (2023).
30. Li, L., Tan, J., Miao, Y., Lei, P. & Zhang, Q. ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. *Cell. Mol. Neurobiol.* **35** (5), 615–621 (2015).
31. Ren, Y. et al. Multifaceted role of redox pattern in the tumor immune microenvironment regarding autophagy and apoptosis. *Mol. Cancer.* **22** (1), 130 (2023).
32. Wu, J. et al. Autophagic blockade potentiates anlotinib-mediated ferroptosis in anaplastic thyroid cancer. *Endocr. Relat. Cancer* **30**(9) (2023).
33. Yang, L. et al. Reactive oxygen species mediate anlotinib-induced apoptosis via activation of endoplasmic reticulum stress in pancreatic cancer. *Cell. Death Dis.* **11** (9), 766 (2020).
34. Xu, P., Wang, H., Pan, H., Chen, J. & Deng, C. Anlotinib combined with temozolomide suppresses glioblastoma growth via mediation of JAK2/STAT3 signaling pathway. *Cancer Chemother. Pharmacol.* **89** (2), 183–196 (2022).
35. Amaravadi, R., Kimmelman, A. C. & White, E. Recent insights into the function of autophagy in cancer. *Genes Dev.* **30** (17), 1913–1930 (2016).
36. Levy, J. M. & Thorburn, A. Targeting autophagy during cancer therapy to improve clinical outcomes. *Pharmacol. Ther.* **131** (1), 130–141 (2011).
37. Ma, X. H. et al. Targeting ER stress-induced autophagy overcomes BRAF inhibitor resistance in melanoma. *J. Clin. Investig.* **124** (3), 1406–1417 (2014).
38. Zhuo, L. et al. Rapamycin Inhibited Pyroptosis and Reduced the Release of IL-1 β and IL-18 in the Septic Response, *Biomed Res Int* (2020) 5960375. (2020).
39. Liu, Z. et al. XBP1 deficiency promotes hepatocyte pyroptosis by impairing mitophagy to activate mtDNA-cGAS-STING signaling in macrophages during acute liver injury. *Redox Biol.* **52**, 102305 (2022).

Figures

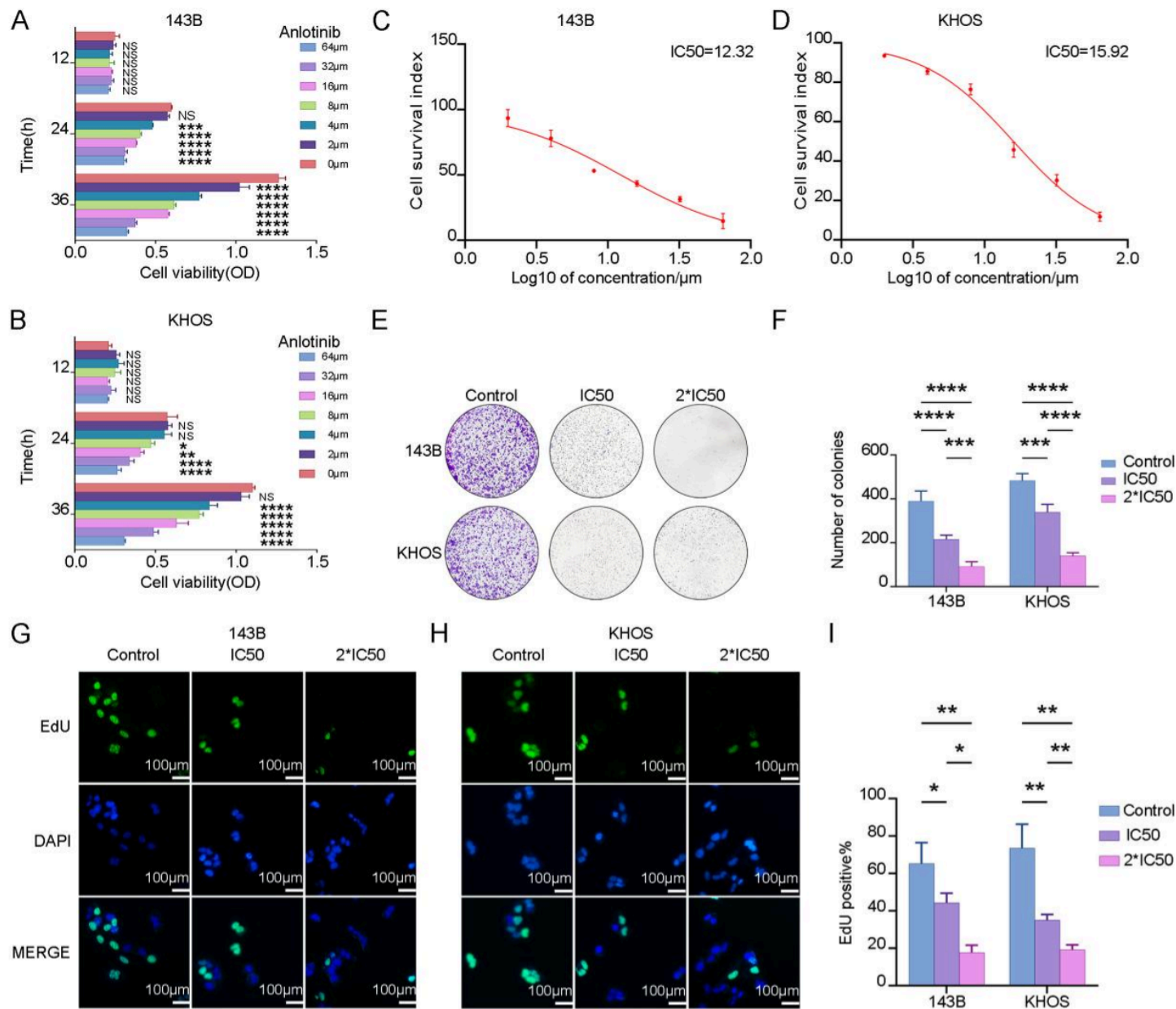


Figure 1

Antiproliferative effects of anlotinib on osteosarcoma. (A, B) Anlotinib inhibits the viability of 143B (A) and KHOS (B) cells in a dose- and time-dependent manner. (C, D) The IC_{50} values of anlotinib were calculated as 12.32 μ M for 143B cells and 15.92 μ M for KHOS cells. (E, F) Clonogenic assays demonstrated that anlotinib significantly reduced the number of cell colonies. (G–I) EdU incorporation assays revealed that anlotinib inhibits DNA synthesis, as evidenced by a significant reduction in EdU-positive cells. Scale bar = 100 μ m. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

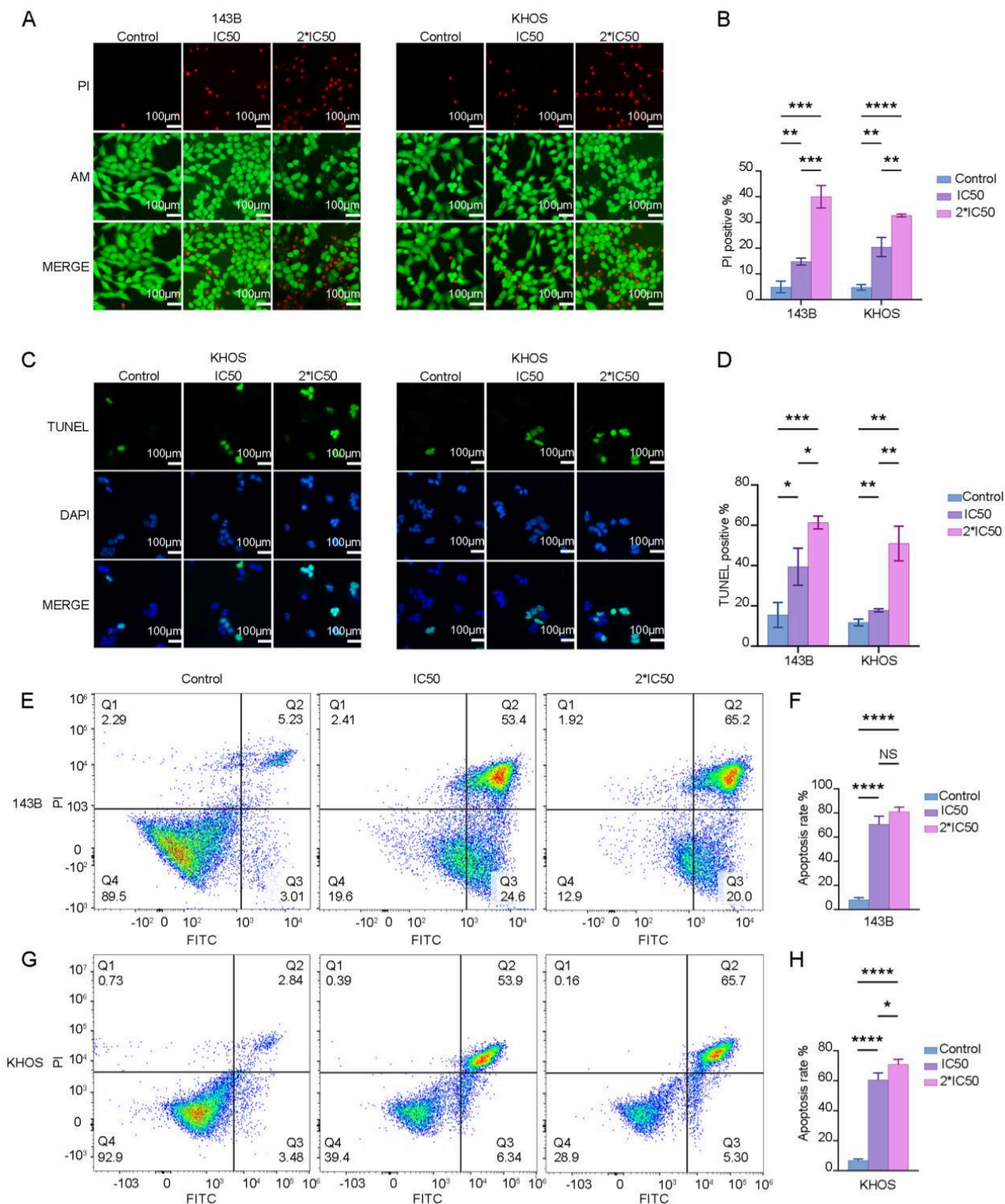


Figure 2

Anlotinib induces apoptosis in osteosarcoma cells. (A, B) PI/AM staining demonstrates that anlotinib (at IC50 and 2×IC50 concentrations) significantly increases the proportion of PI-positive cells in 143B and KHOS cells. Scale bar = 100 μm. (C, D) TUNEL staining results show an increase in the number of apoptotic cells in 143B and KHOS cells following anlotinib treatment. Scale bar = 100 μm. (E–H) Flow cytometry analysis reveals that anlotinib induces both early and late apoptosis in 143B and KHOS cells,

with significant differences between treated (IC50 and 2×IC50) and control groups. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

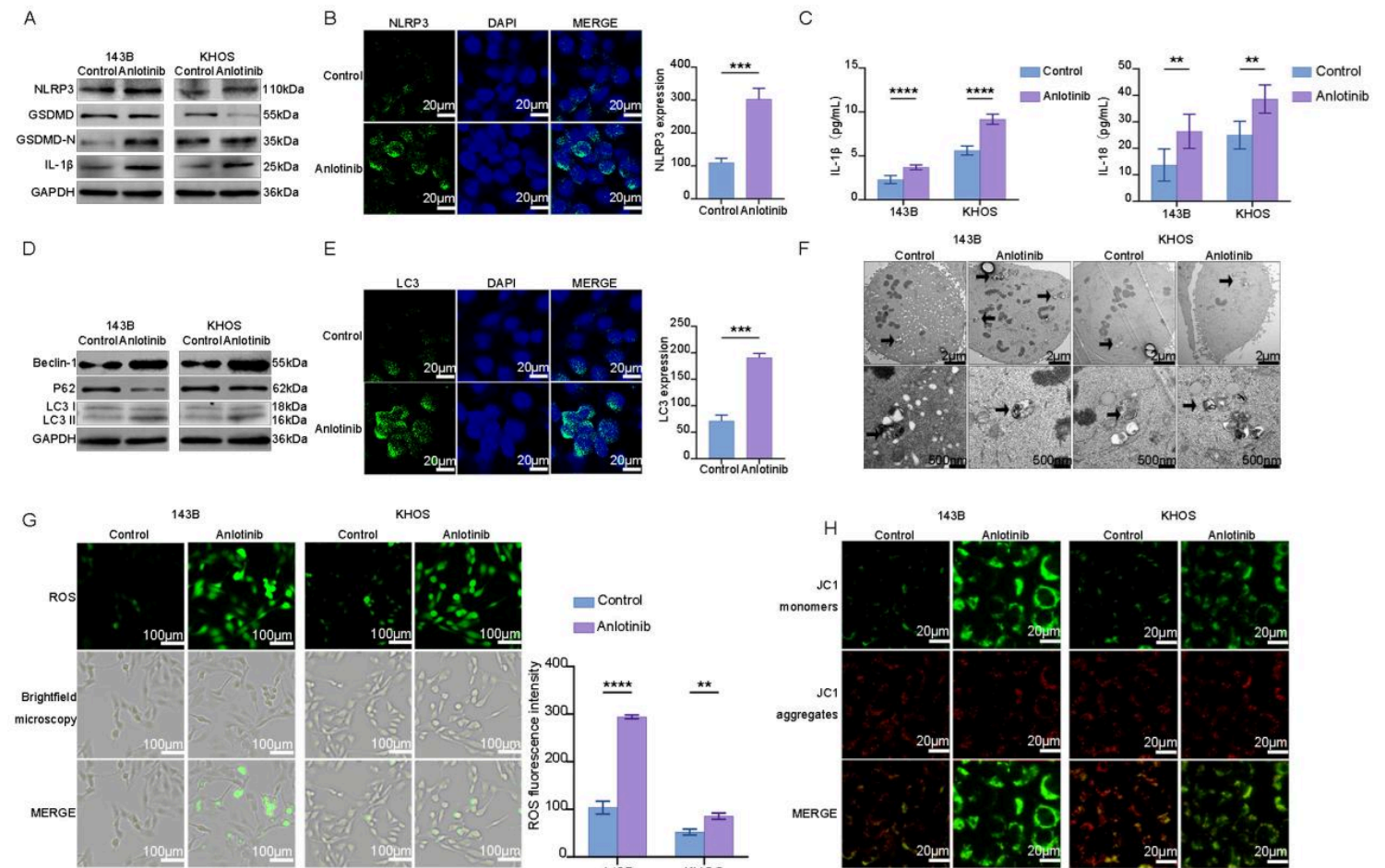


Figure 3

Anlotinib induces pyroptosis and autophagy in osteosarcoma cells. (A, B) Western blot and immunofluorescence analyses show that anlotinib significantly upregulates the expression of NLRP3, GSDMD-N, and IL-1 β (A, B). Scale bar = 20 μ m. (C) ELISA results demonstrate a significant increase in the secretion of IL-1 β and IL-18 following anlotinib treatment. (D) The expression of autophagy-related proteins Beclin-1 and LC3-II is significantly upregulated, while P62 expression is downregulated. (E) Immunofluorescence staining shows an increase in LC3 expression. (F) Transmission electron microscopy reveals a significant increase in autophagosome structures. Scale bar = 2 μ m. (G) ROS detection shows that anlotinib significantly increases ROS levels in 143B and KHOS cells, with enhanced fluorescence intensity. Scale bar = 100 μ m. (H) JC-1 staining indicates a decrease in mitochondrial membrane potential in the anlotinib-treated group, with reduced red polymer fluorescence and increased green monomer fluorescence, suggesting significant mitochondrial damage. Scale bar = 20 μ m. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

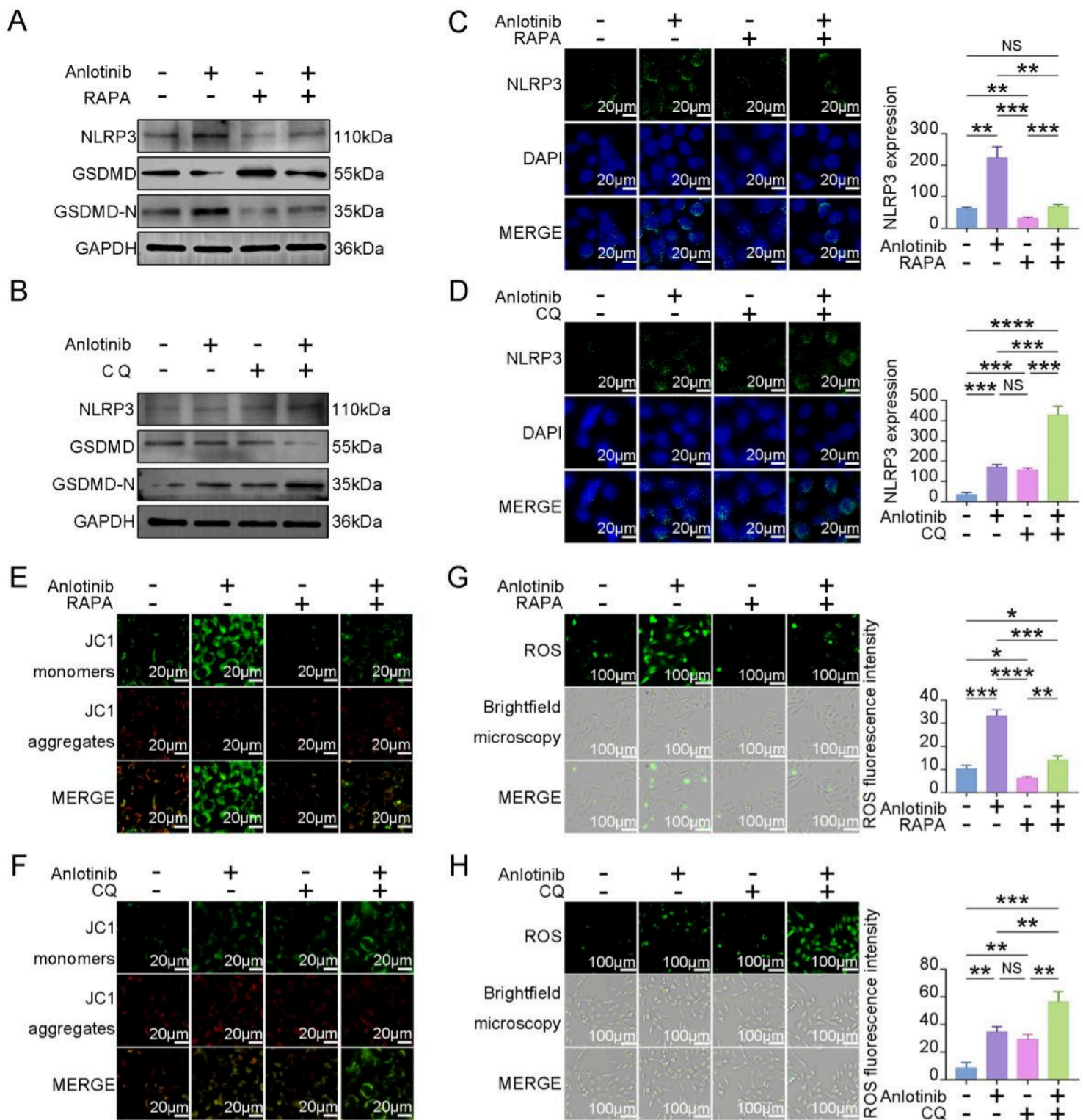


Figure 4

The effect of autophagy regulation on anlotinib-induced pyroptosis, mitochondrial damage, and oxidative stress. (A–D) Western blot and immunofluorescence analyses show that the autophagy activator rapamycin (RAPA) significantly reduces the expression of NLRP3, GSDMD, and GSDMD-N induced by anlotinib (A, C), while the autophagy inhibitor chloroquine (CQ) enhances their expression (B, D). Statistical analysis indicates that RAPA significantly inhibits NLRP3 expression, whereas CQ significantly

enhances its expression. (E–H) JC-1 staining and ROS detection reveal that RAPA attenuates the anlotinib-induced decrease in mitochondrial membrane potential and the increase in ROS levels (E, G), while CQ further exacerbates these effects (F, H). Statistical analysis suggests that autophagy plays a critical regulatory role in the cellular stress response induced by anlotinib. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

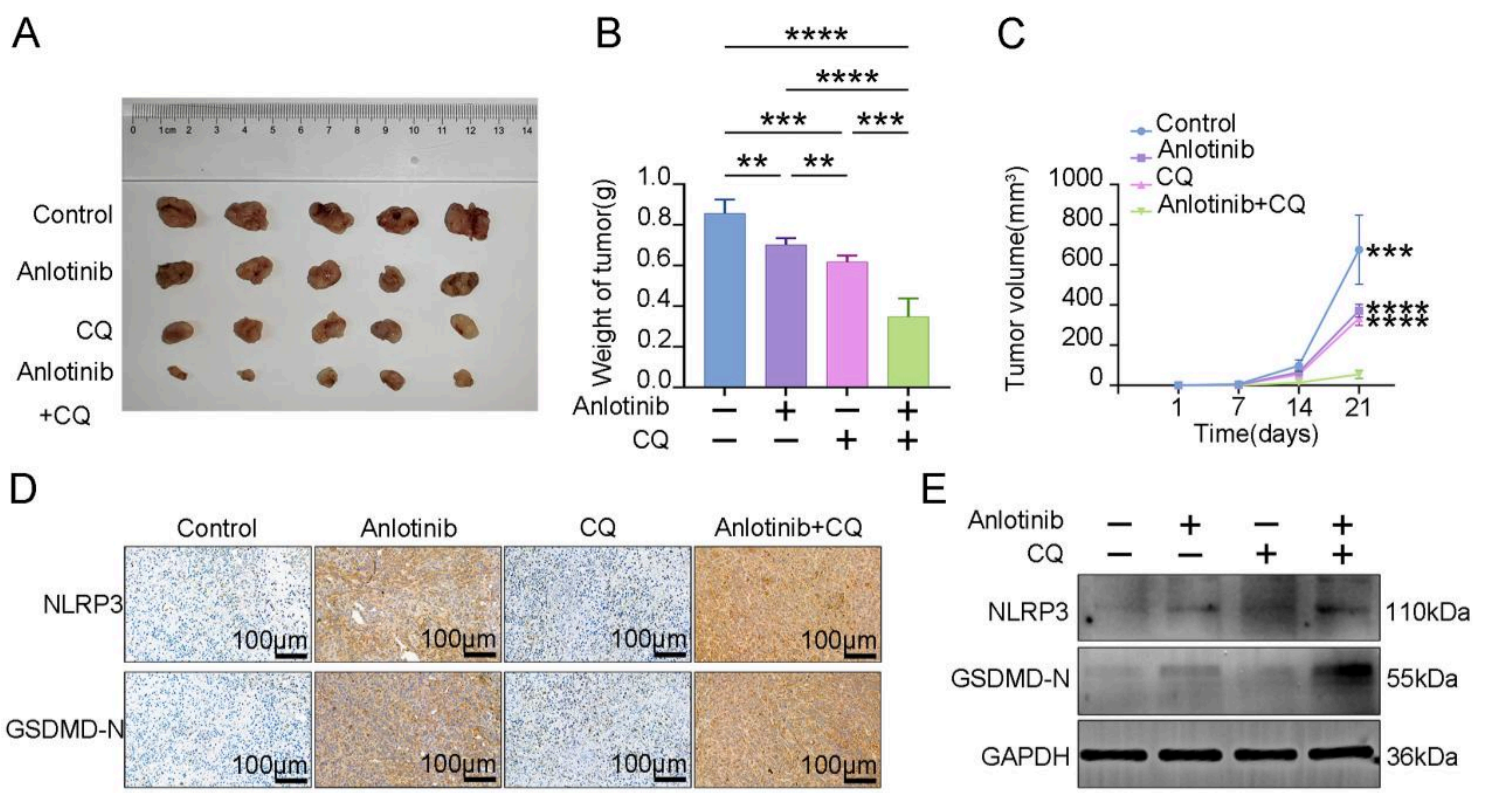


Figure 5

The autophagy inhibitor CQ enhances the antitumor effects of anlotinib. (A–C) Mouse xenograft model results show that anlotinib alone significantly inhibits tumor growth, while the combination of the autophagy inhibitor CQ and anlotinib further enhances the antitumor effect. Both tumor weight (B) and volume (C) are significantly reduced. (D, E) IHC and Western blot analyses show that anlotinib treatment increases the expression of NLRP3 and GSDMD-N, while the combination with CQ significantly further enhances the expression of these proteins, indicating further activation of pyroptosis. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GraphicalAbstract.docx](#)
- [supplementarymaterial.pdf](#)