

Andrographolide attenuates bupivacaine-induced cytotoxicity in SH-SY5Y cells through preserving Akt/mTOR activity

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Background: Bupivacaine (Bup) is the most commonly used local anesthetic. However, Bup induces cytotoxicity, especially in older patients. Recent reports have indicated that andrographolide (Andro) exhibits protective effects on human neurons. Nevertheless, whether Andro can inhibit Bup-induced cytotoxicity remains unclear. As such, we investigated the effect of Andro on Bup-induced cytotoxicity of SH-SY5Y cells in the present study.

Methods: Western blotting was used to examine expression of Bax, Bcl2, active caspase 3, p-Akt, and p-mTOR in SH-SY5Y cells. In addition, ELISA was used to detect levels of total glutathione and reactive oxygen species in cells.

Results: We found that Andro attenuated Bup-induced cytotoxicity of SH-SY5Y cells. In addition, Andro inhibited Bup-induced apoptosis via downregulating the expression of Bax and active caspase 3 and upregulating the proteins Bcl2, p-Akt, and p-mTOR in SH-SY5Y cells. Moreover, Andro alleviated Bup-induced oxidative damage in SH-SY5Y cells via downregulating the level of reactive oxygen species and upregulating of the level of total glutathione. More significantly, inhibition of Akt abolished the protective effect of Andro in Bup-treated SH-SY5Y cells.

Conclusion: Our findings indicated that Andro played a neuroprotective role via preserving Akt/mTOR activity and increasing antioxidative status in Bup-treated SH-SY5Y cells. Therefore, Andro may be a potential agent for the treatment of human cytotoxicity induced by Bup.

Keywords: andrographolide, bupivacaine, apoptosis, Akt, cytotoxicity

Introduction

Local anesthetics have been found to induce brain neural injury in human patients.¹ Local anesthetics can induce permanent injury in young patients, and even affect neurobehavioral outcomes.² Bupivacaine (Bup) is a common local anesthetic used for postoperative pain relief.³ A recent study indicated that 5% Bup can induce histopathological abnormalities in a rat model.⁴ Meanwhile, injection of Bup can also lead to serious sciatic nerve damage in rats.⁵ Even with a normal or lower dose, Bup can induce neurotoxicity in cells.⁶ As such, it is urgent that we develop novel effective methods for the treatment of local anesthetic neurotoxicity.

Andrographolide (Andro) is a natural diterpenoid extracted from the traditional Chinese herbal medicine *Andrographis paniculate*.⁷ Andro has been revealed to exhibit a variety of biological activities, including antitumor, anti-inflammatory, antiviral, and

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antioxidation actions.^{8–12} A previous study indicated that Andro can stimulate neurogenesis in the adult hippocampus.¹³ Liang et al found that Andro may exhibit neuroprotective effects in nervous system diseases.¹⁴ Meanwhile, a recent study showed that Andro exerted strong neuroprotective effects in a mouse model of Parkinson's disease.¹⁵ However, it remains unclear whether Andro provides neuroprotection against Bup. In addition, the Akt-signaling pathway participates in regulating cell growth, survival, and death.¹⁶ Studies have indicated Bup-induced apoptosis in neural injury via inactivation of the Akt pathway.^{17,18}

Therefore, our main purpose was to investigate the effect of Andro on Bup-induced neurotoxicity in SH-SY5Y cells. In this study, an in vitro model of Bup-induced cytotoxicity was first established. Then, mechanisms by which Andro regulates Bup-induced injury in SH-SY5Y cells were evaluated.

Methods

Cell cultures

The human neuroblastoma cell line SH-SY5Y was purchased from the American Type Culture Collection (Rockville, MD, USA). SH-SY5Y cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific) and 100 U/mL penicillin–streptomycin at 37°C in a humidified 5% CO₂ incubator. Cell-culture medium was changed daily. Andro (365645) and Bup (B5274) were obtained from Sigma-Aldrich (St Louis, MO, USA). AZD5363 was purchased from MedChem Express (Monmouth Junction, NJ, USA).

CCK8 assay

A CCK8 assay kit (Beyotime, Haimen, China) was used to determine cell viability. SH-SY5Y cells were seeded into a 96-well plate at a density of 5×10^3 cells/well overnight. When cell confluence had reached about 80%, SH-SY5Y cells were incubated with Bup, Andro, or AZD5363. After that, 10 μ L CCK8 solution was added to each well and cultured at 37°C for another 3 hours. OD values were measured using a microplate reader (Thermo Fisher Scientific) at 450 nm.

Immunofluorescence assay

SH-SY5Y cells were seeded into 24-well plates at a density of 4×10^5 cells/well overnight. For treatment 1 (Figure 2A), SH-SY5Y cells were incubated with Andro (0 or 200 μ M) for 12 hours. Then, the culture medium was

changed and cells incubated with Bup (0 or 400 μ M) for another 48 hours at 37°C. After that, cells were prefixed in 4% paraformaldehyde at room temperature for 20 minutes and fixed in cold methanol for 10 minutes at –20°C. Later, cells were incubated with primary antibodies for anti-Ki67 (1:1,000; Abcam, Cambridge, UK) and DAPI (1:1,000; Abcam) at 4°C overnight.

For treatment 2 (Figure 5A), SH-SY5Y cells were incubated with Andro (0 or 200 μ M) for 12 hours. Then, the culture medium was changed and cells incubated with 10 nM AZD5363 for 1 hour. Later, the culture medium was changed and cells incubated with Bup (0 or 400 μ M) for another 48 hours. Next, cells were incubated with primary antibodies for anti-p-Akt (1:1,000; Abcam) and DAPI (1:1,000) at 4°C overnight. After that, cells were incubated with goat antirabbit IgG secondary antibodies (1:5,000; Abcam) at 37°C for 1 hour. Cells were visualized with fluorescence microscopy (Olympus, Tokyo, Japan).

Flow-cytometry analysis of cell apoptosis

SH-SY5Y cells were seeded on six-well plates at a density of 5×10^5 cells/well overnight. SH-SY5Y cells were incubated with Bup, Andro, or AZD5363. After that, collected cells were washed with cold PBS and resuspended in 500 μ L binding buffer at 4°C. Staining with annexin V–FITC–PI (Thermo Fisher Scientific) was used to detect cell apoptosis according to the manufacturer's specifications. Results were measured with flow cytometry (BD Bioscience, San Jose, CA, USA).

Western blot analysis

Total cellular protein was quantified with a BCA protein assay kit (Beyotime). An equal amount of protein samples (40 μ g/lane) was separated by polyacrylamide-gel electrophoresis. Then, proteins were transferred to polyvinylidene fluoride membranes (Thermo Fisher Scientific). After blocking with 5% nonfat milk for 50 minutes, membranes were incubated with primary antibodies overnight at 4°C. After that, membranes were incubated with secondary antibody for 1 hour at room temperature. Primary antibodies used were anti-Bax (1:1,000), anti-Bcl2 (1:1,000), anti-active caspase 3 (1:1,000), anti-p-Akt (1:1,000), anti-p-mTOR (1:1,000), and anti- β -actin (1:1000). The secondary antibody was goat antirabbit IgG (1:5,000). All these antibodies were provided by Abcam. Finally, images protein bands were captured with an electrochemiluminescence reagent (Santa Cruz Biotechnology). The density of blots for targets was normalized to β -actin.

Glutathione assay

ELISA kits (Beyotime) were used to detect levels of intracellular reduced glutathione (GSH) and oxidized GSH (GSSG). Absorbance was read at 450 nm by a microplate reader (Thermo Fisher Scientific). The level of total GSH was the sum of GSH and GSSG.

Intracellular ROS assay

A reactive oxygen species (ROS) assay kit (Beyotime) was used to detect the level of intracellular ROS. Intracellular ROS level was also detected with a microplate reader (Thermo Fisher Scientific).

Statistical analysis

Each group was subjected to least three independent experiments, and all data are expressed as means \pm SD. Student's *t*-test was used to analyze comparison between two groups. Comparisons among multiple groups were made with one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

Effects of Bup or andro on growth of SH-SY5Y cells

The chemical structure of Andro is indicated in Figure 1A. CCK8 assays were used to investigate the effects of Bup or Andro on the growth of SH-SY5Y cells. As shown in Figure 1B, Bup significantly inhibited the viability of SH-SY5Y cells in a dose-dependent manner. Since 400 μ M Bup induced about 50% growth inhibition, 400 μ M Bup was utilized in the following experiments. Meanwhile, Andro had a very limited effect on cell proliferation when the concentration reached 200 μ M (Figure 1C).

However, 400 μ M Andro had significant cytotoxicity. Therefore, 200 μ M Andro was utilized in the following experiments.

Andro alleviated Bup-induced cytotoxicity in SH-SY5Y cells via inhibition of apoptosis

As indicated in Figure 2A, the cytotoxicity of 400 μ M Bup on SH-SY5Y cells was significantly alleviated by Andro (100 or 200 μ M) treatment. Next, a Ki67 immunofluorescence assay was used to determine cell viability as well. The results showed Bup markedly inhibited cell proliferation, which was notably reversed by 200 μ M Andro (Figure 2, B and C). In addition, flow cytometry was applied to explore further the apoptosis in SH-SY5Y cells. The rate of early and late apoptotic stage in Bup-treated SH-SY5Y cells was about 10% and 20.00%, respectively. However, the rate of early- and late-apoptosis stages in Bup-treated SH-SY5Y cells was markedly decreased to about 4% and 10% in the presence of Andro (Figure 2, D and E), respectively. These results indicated that Andro significantly decreased Bup-induced apoptosis in SH-SY5Y cells. As such, Andro inhibited Bup-induced cytotoxicity via inhibition of apoptosis in SH-SY5Y cells.

Andro attenuated Bup-induced cytotoxicity in SH-SY5Y cells via increasing antioxidative status

Oxidative status is an important cell characteristic included in the response to cellular environment transformation. Generation of ROS and GSH mediates apoptosis in neuroblastoma SH-SY5Y cells.^{19,20} As shown in Figure 3, A and B, Bup

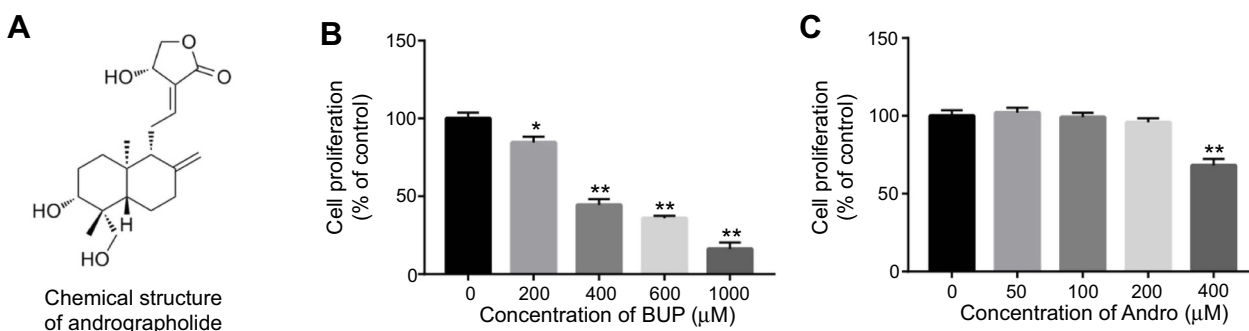


Figure 1 Inhibitory effects of Bup or Andro on SH-SY5Y cell growth.

Notes: (A) Chemical structure of Andro. (B) SH-SY5Y cells were exposed to Bup (0, 200, 400, 600, or 1,000 μ M) for 48 hours. Then, cell viability was determined using CCK8 assay. (C) SH-SY5Y cells were exposed to Andro (0, 50, 100, 200, or 400 μ M) for 12 hours. Then, cell viability was determined using CCK8 assay. * $P < 0.05$; ** $P < 0.01$.

Abbreviations: Bup, bupivacaine; Andro, andrographolide.

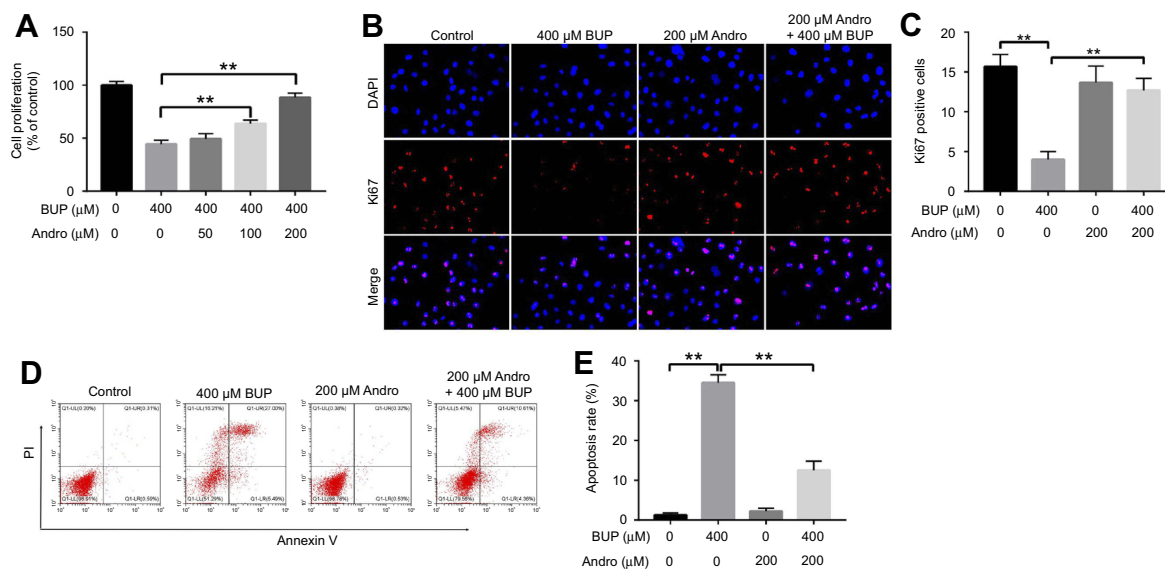


Figure 2 Andro alleviated Bup-induced cytotoxicity via inhibition of apoptosis in SH-SY5Y cells.

Notes: (A) SH-SY5Y cells were incubated with Andro (0, 50, 100 or 200 μM) for 12 hours. Then, the culture medium was changed and cells incubated with Bup (0 or 400 μM) for another 48 hours. Cell viability was detected with CCK8 assay. (B) SH-SY5Y cells were incubated with Andro (0 or 200 μM) for 12 hours. Then, the culture medium was changed and cells incubated with Bup (0 or 400 μM) for another 48 hours. Proliferation of SH-SY5Y cells was determined using Ki67 staining. Representative fluorescence images of Ki67 and DAPI staining (magnification 200×). (C) Ki67 positive cells in each group was counted. (D) Cell apoptosis in SH-SY5Y cells were detected by annexin V-PI staining. (E) The cell-apoptosis rate in each group was calculated. ** $P < 0.01$.

Abbreviations: Bup, bupivacaine; Andro, andrographolide.

significantly increased the level of ROS and decreased the level of total GSH in SH-SY5Y cells compared with the control group. However, levels of ROS and GSH in SH-SY5Y cells recovered to normal status in the presence of Andro compared with the Bup-treated group (Figure 3, A and B). These data suggested that Andro attenuated Bup-induced cytotoxicity via increasing antioxidative status in SH-SY5Y cells.

Andro inhibited Bup-induced cytotoxicity in SH-SY5Y cells via activating p-Akt and p-mTOR

To explore further the effects of Andro on Bup-induced apoptosis in SH-SY5Y cells, Western blotting was performed. It has been reported that Bcl2 and caspase 3 are proapoptotic proteins, while Bax is an antiapoptotic protein.²¹ In addition, the Akt-mTOR pathway plays an important role in cell survival and death.²² As indicated in Figure 4, Bup significantly increased Bax and active caspase 3 expression and decreased levels of Bcl2, p-Akt, and p-mTOR in cells compared with the control group. However, these effects were markedly reversed in the presence of Andro (Figure 4). Therefore, these data suggested that Andro attenuated Bup-induced cytotoxicity in SH-SY5Y cells via activating the Akt-mTOR pathway.

Akt inhibitor abrogated the protective effect of Andro in Bup-treated SH-SY5Y cells

To investigate further whether Andro attenuated Bup-induced cytotoxicity via the Akt pathway, the Akt-selective inhibitor AZD5363 was used. As indicated in CCK8 assays, the protective effect of Andro in SH-SY5Y cells against Bup was abrogated by AZD5363 treatment; however, AZD5363 notably increased Bup-induced cytotoxicity (Figure 5A). Meanwhile, the antiapoptotic effect of Andro in Bup-treated SH-SY5Y cells was also reversed by AZD5363 treatment (Figure 5, B and C). These data suggested that the Akt inhibitor abrogated the protective effect of Andro in Bup-treated SH-SY5Y cells.

In addition, AZD5363 abolished the upregulation of p-Akt and p-mTOR in cells, even in the presence of Andro (Figure 6, A-C). Meanwhile, the expression of active caspase 3 in cells was significantly increased with treatment by AZD5363. Similarly, immunofluorescence assays indicated the expression of p-Akt in SH-SY5Y cells was significantly inhibited by AZD5363, even in the presence of Andro (Figure 6, D and E). All these data confirmed that Andro inhibited Bup-induced cytotoxicity of SH-SY5Y via preserving the Akt-mTOR pathway, which was abrogated by AZD5363.

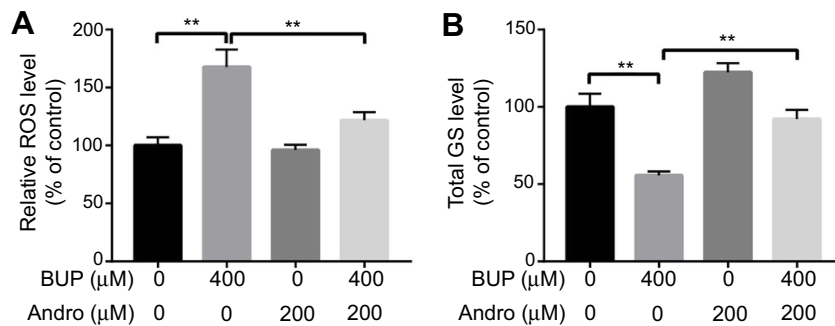


Figure 3 Andro attenuated Bup-induced cytotoxicity via increasing anti-oxidative status in SH-SY5Y cells.

Notes: SH-SY5Y cells were incubated with Andro (0 or 200 μM) for 12 hours. Then, the culture medium was changed and cells incubated with BUP (0 or 400 μM) for another 48 hours. Production of ROS and total GSH SH-SY5Y cells were detected with ELISA kits. ** $P < 0.01$.

Abbreviations: Bup, bupivacaine; Andro, andrographolide; ROS, reactive oxygen species; GSH, glutathione.

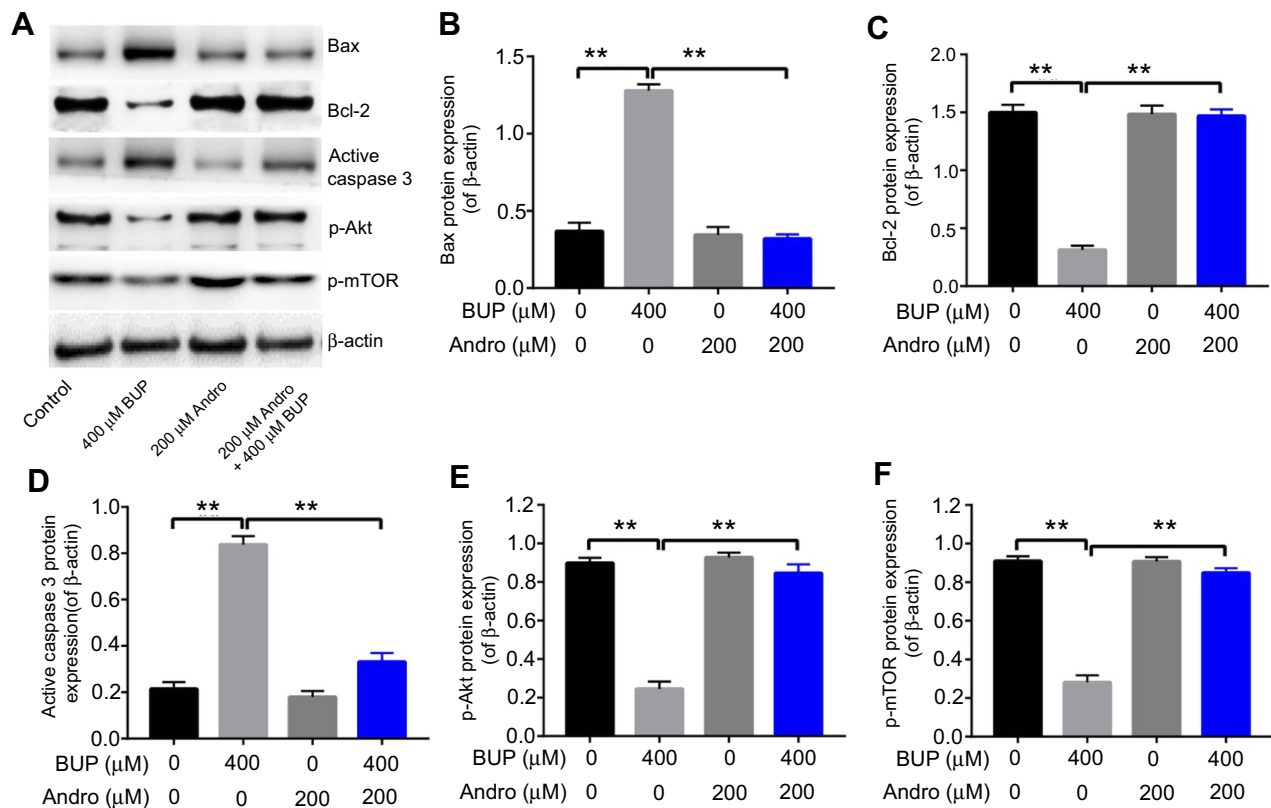


Figure 4 Andro attenuated Bup-induced cytotoxicity via activating p-Akt and p-mTOR in SH-SY5Y cells.

Notes: SH-SY5Y cells were incubated with Andro (0, 50, 100, or 200 μM) for 12 hours. Then, the culture medium was changed and cells incubated with Bup (0 or 400 μM) for another 48 hours. (A) Expression of Bax, Bcl2, active caspase 3, p-Akt, and p-mTOR in Bup-induced SH-SY5Y cells was analyzed by Western blotting. (B) Relative Bax expression was quantified by normalizing to β-actin. (C) Relative Bcl2 expression was quantified by normalizing to β-actin. (D) Relative active caspase 3 expression was quantified by normalizing to β-actin. (E) Relative p-Akt expression was quantified by normalizing to β-actin. (F) Relative p-mTOR expression was quantified by normalizing to β-actin. ** $P < 0.01$.

Abbreviations: Bup, bupivacaine; Andro, andrographolide.

Discussion

In the present study, we found that Andro protected SH-SY5Y cells from Bup-induced cytotoxicity. Pretreatment with 200 μM Andro significantly suppressed Bup-induced cytotoxicity, apoptosis, and ROS accumulation. A study has

shown that Bup induced neurotoxicity in neuroblastoma Neuro2a cells via activating apoptosis.²³ Wen et al showed that Bup induced neural injury in SH-SY5Y cells,²⁴ consistent with the results of this study. In addition, we found that 200 μM Andro had no cytotoxicity on SH-SY5Y cell

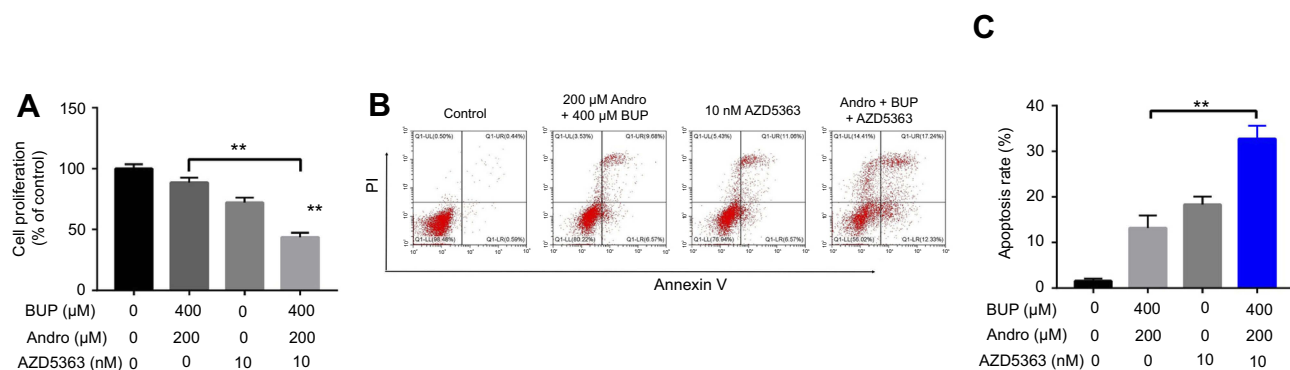


Figure 5 An Akt inhibitor abrogated the protective effect of Andro in Bup-treated SH-SY5Y cells.

Notes: SH-SY5Y cells were incubated with Andro (0 or 200 μM) for 12 hours. Then, the culture medium was changed and cells incubated with 10 nM AZD5363 for 1 hour. Later, the culture medium was changed and cells incubated with Bup (0 or 400 μM) for another 48 hours. **(A)** Proliferation of SH-SY5Y cells was determined using CCK8 assay. **(B)** Apoptotic cells were detected with annexin V-PI staining. **(C)** Cell-apoptosis rates were calculated. $**P < 0.01$.

Abbreviations: Bup, bupivacaine; Andro, andrographolide.

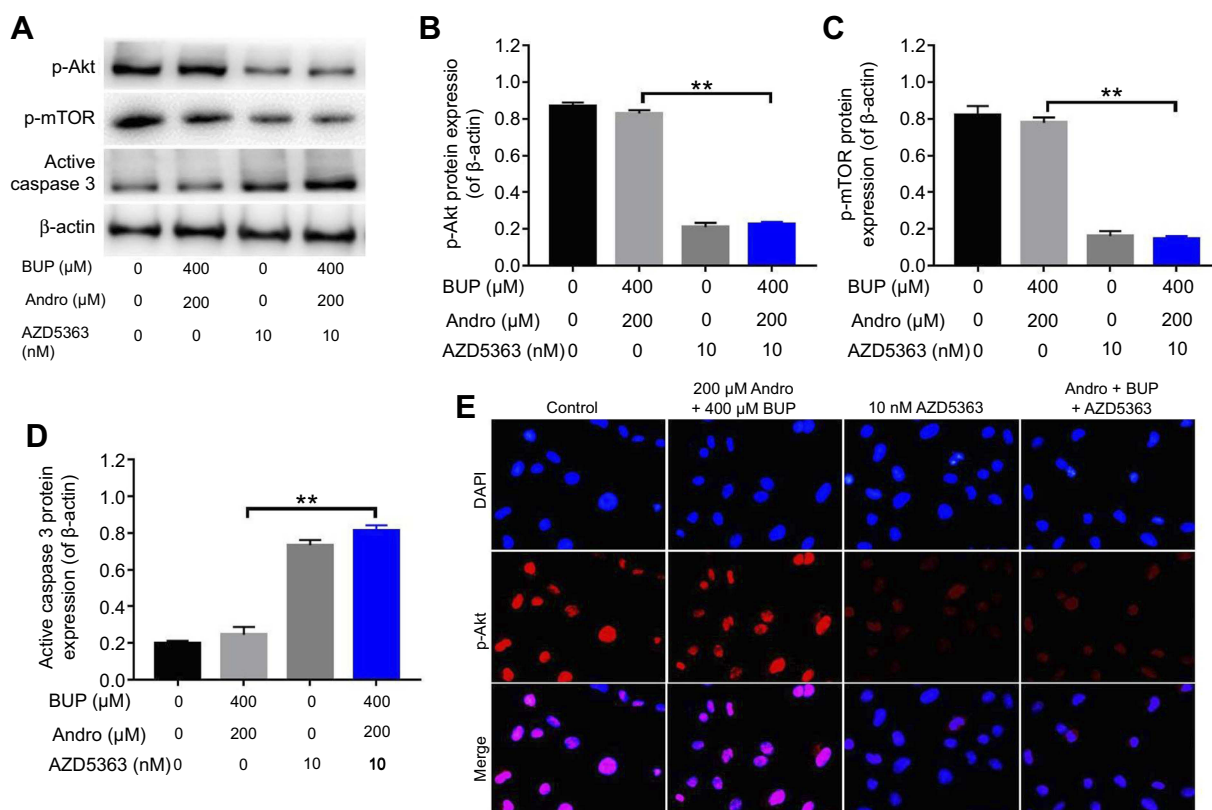


Figure 6 An Akt inhibitor abrogated the protective effect of Andro in Bup-treated SH-SY5Y cells via inhibition of the Akt-mTOR pathway.

Notes: SH-SY5Y cells were incubated with Andro (0 or 200 μM) for 12 hours. Then, the culture medium was changed and cells incubated with 10 nM AZD5363 for 1 hour. Later, the culture medium was changed and cells incubated with Bup (0 or 400 μM) for another 48 hours. **(A)** Expression of p-Akt, p-mTOR, and active caspase 3 in SH-SY5Y cells was analyzed by Western blotting. **(B)** Relative p-Akt expression was quantified by normalizing to β -actin. **(C)** Relative p-mTOR expression was quantified by normalizing to β -actin. **(D)** Relative active caspase 3 expression was quantified by normalizing to β -actin. **(E)** Representative fluorescence images of p-Akt and DAPI (magnification 400 \times). $**P < 0.01$.

Abbreviations: Bup, bupivacaine; Andro, andrographolide.

proliferation. Consistently with previous research, ginkgolide B had no effect on cell proliferation, while pretreatment with ginkgolide B protected SH-SY5Y cells from Bup-induced neurotoxicity.²⁵ Furthermore, Chen et al indicated

that paeoniflorin attenuated Bup-induced neurotoxicity via downregulation of Bax and caspase 3 levels in SH-SY5Y cells.²¹ Harato et al also showed that Bup induced apoptosis in mouse neuro2a cells via increasing the expression of

caspace 3 and inducing ROS generation.²⁶ In this study, Andro reversed Bup-induced cytotoxicity in SH-SY5Y cells via downregulation of the level of proapoptotic Bax and caspase 3 proteins and upregulation of the level of the antiapoptotic Bcl2 protein. Our results are consistent with previous reports that Andro can reverse Bup-caused neurotoxicity via inhibiting apoptosis in SH-SY5Y cells.

An important element of cellular survival is oxidoreductive homeostasis. In neuronal cells, the course of cell apoptosis is associated with ROS and GSH generation.^{27,28} Park et al found that Bup-induced apoptosis was associated with the production of ROS.²⁹ Lu et al showed Bup-induced apoptosis in SH-SY5Y cells via induction of ROS production and activation of the AMPK pathway.³⁰ However, inhibition of GSH can induce apoptosis. Our findings are consistent with previous reports that Bup increased ROS generation and decreased total GSH level in SH-SY5Y cells. Moreover, Andro is considered an antioxidant,³¹ and we demonstrated the effects of Andro on ROS and total GSH content in Bup-treated SH-SY5Y cells. We found that ROS content was reduced and the level of total GSH increased with Andro treatment. Supporting our finding, Andro-attenuated hypoxia-induced the production of ROS.³² As such, Andro recover ROS and GSH to normal levels in Bup-treated SH-SY5Y cells and played an important role in maintaining oxidoreductive homeostasis.

Akt, a serine/threonine-specific protein kinase, can modulate cell death and survival in multiple cellular processes.³³ The primary direct downstream effector protein of Akt is mTOR.³³ mTOR is a main nodal point of regulation for ribosome biogenesis and protein translation.³³ The Akt-signaling pathway has been found to be involved in Bup-induced neural injury.³⁴ In this study, we observed that Bup markedly decreased the expression of p-Akt and p-mTOR. This is consistent with Zhao et al's report that Bup induced neurotoxicity in SH-SY5Y cells via inhibiting the PI3K–Akt pathway.³⁴ Importantly, we found that Andro attenuated Bup-induced cytotoxicity via preserving Akt/mTOR activity in SH-SY5Y cells. Chen et al also found that Andro inhibited endothelial cell apoptosis through activation of the PI3K–Akt signaling pathway.³⁵ Therefore, it is presumed that Andro rescued Bup-induced cytotoxicity via preserving Akt/mTOR activity in SH-SY5Y cells. To verify these data, the Akt-selective inhibitor AZD5363 was used in this study. AZD5363 abrogated the protective effect of Andro in Bup-treated SH-SY5Y cells. These results suggested that Andro exerted its

neuroprotective effects through preserving the Akt–mTOR signaling pathway. However, a limitation of the current study was the lack of animal experiments, which hindered the clinical use of Andro.

Conclusion

In summary, this study indicated that Andro alleviated Bup-induced cytotoxicity in SH-SY5Y cells via inhibition of apoptosis and ROS generation and activation of the Akt–mTOR signaling pathway. Therefore, Andro may be a potential agent for the treatment of human cytotoxicity induced by Bup.

Disclosure

The authors report no conflicts of interest in this work.

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