Berberine exhibits antitumor effects by activating autophagy and apoptosis in anaplastic thyroid carcinoma cells by regulating ROS and the PI3K/AKT/mTOR signaling pathways

Xiang-Zhe Shi
Sheng Zhao
Yan Wang
Meng-Yao Wang
Chen Xiong
Su-Wen Su
Yan-Zhao Wu (✉ wyz315@163.com)

Research Article

Keywords: anaplastic thyroid carcinoma, berberine, autophagy, apoptosis, PI3K/AKT/mTOR, ROS, doxorubicin, synergism

Posted Date: January 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2421091/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License.

Read Full License
Abstract

Anaplastic thyroid carcinoma (ATC) is the most lethal thyroid carcinoma. Doxorubicin (DOX) is the only drug approved for anaplastic thyroid cancer treatment, but its clinical use is restricted due to irreversible tissue toxicity. Berberine (BER), an isoquinoline alkaloid extracted from Coptidis Rhizoma, has been proposed to have antitumor activity in many cancers. However, the underlying mechanisms by which BER regulates apoptosis and autophagy in ATC remain unclear. Thus, the present study aimed to assess the therapeutic effect of BER in CAL-62 and BHT-101 cells as well as the underlying mechanisms. In addition, we assessed the antitumor effects of a combination of BER and DOX in ATC cells. The present results showed that BER significantly inhibited cell growth and induced apoptosis in ATC cells. BER treatment also significantly upregulated the expression of LC3B-II and increased the number of GFP-LC3 puncta in ATC cells, suggesting that BER induced a high level of autophagy. Inhibition of autophagy by 3-methyladenine (3-MA) suppressed BER-induced autophagic cell death, which confirmed the anticancer role of autophagy induced by BER. Moreover, BER induced the generation of reactive oxygen species (ROS). N-acetylcysteine (NAC), a potential ROS scavenger, substantially suppressed the expression of autophagy-regulated proteins and apoptosis proteins induced by BER. Moreover, BER and DOX cooperated to promote apoptosis and autophagy in ATC cells. Mechanistically, we demonstrated that BER regulated the autophagy and apoptosis of human ATC cells through the PI3K/AKT/mTOR pathways. Taken together, the present findings indicated that BER induces apoptosis and autophagic cell death by activating ROS and regulating the PI3K/AKT/mTOR signaling pathway.

Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most lethal aggressive malignancies and is characterized by high levels of rapid proliferation, extrathyroidal invasion, and distant metastasis. Unfortunately, unlike differentiated thyroid carcinoma, most ATC patients are resistant to conventional treatment, including surgery, radiotherapy, chemotherapy (multimodal therapy), and immunotherapy (Chen et al., 2017; Deeken-Draisey et al., 2018). Due to a lack of effective treatment measures, ATC is the major cause of all thyroid carcinoma-related deaths with a median survival time of 3 to 9 months, and the 1-year overall survival rate of ATC patients after diagnosis is only 20% (Kim et al., 1983; Ranganath et al., 2015).

In recent years, natural compounds from traditional Chinese medicinal plants have received increasing attention due to their potential antitumor activities and few side effects (Liu et al., 2019; Qi et al., 2015). Berberine (BER) is a type of isoquinoline alkaloid. As the main component of Coptis chinensis, BER has been widely used in the clinical treatment of gastroenteritis and other intestinal infectious diseases with almost no toxic side effects. Modern studies have also found that BER has many drug properties, such as antitumor and cardioprotective effects (Tan et al., 2016; Tabeshpour et al., 2017; Zou et al., 2017; Gong et al., 2020; Li et al., 2017). Although studies have shown that BER inhibits a variety of tumor cells, only a few studies included thyroid cancer. Through a literature search, we found that BER inhibits the
proliferation of thyroid cancer cells in a dose-time-dependent manner, while the cytotoxicity of normal thyroid cells to BER is relatively low (Li et al., 2017b; Park et al., 2012).

Apoptosis is a process of programmed cell death. Unlike apoptosis, autophagy is normally a cell-survival process that maintains cellular homeostasis by removing misfolded proteins and damaged organelles, thereby providing additional nutrients and energy to the cell (Levy et al., 2017). In cancer therapy, autophagy behaves as a double-edged sword because it has a cytoprotective effect in some situations but is an alternative cell death pathway (namely, type II PCD) in other situations (Singh et al., 2018). Thus, autophagy-dependent cell death provides molecular mechanisms and implications for cancer therapy. Growing evidence has demonstrated that BER, as a potent natural autophagic modulator, induces or inhibit autophagy in a variety of cancer cells (Zhang et al., 2020; Liu et al., 2020b; Wang et al., 2020). Thus, further research is needed to determine whether BER regulates the growth of ATC cells by modulating autophagy.

Many studies have shown that the phosphatidylinositide-3 kinase (PI3K)/protein kinase B (AKT) signaling pathway activates mammalian rapamycin target protein (mTOR), thus regulating autophagy (Wang et al., 2019; Fan et al., 2016). More importantly, the PI3K/AKT/mTOR signaling pathway plays critical roles in the development and progression of various human cancers, including ATC (Saji et al., 2010; Petrulea et al., 2015). However, it is unclear whether PI3K/AKT/mTOR signaling mediates BER-mediated autophagy in ATCs.

DOX is the only drug approved by the Food and Drug Administration (FDA) for ATC treatment based on the guidelines (Haddad et al., 2015). However, the drug resistance of tumors to DOX and the toxicity of DOX itself, especially cardiac toxicity, are the most prominent and serious consequences that reduce the effect of chemotherapy, which depends on the dose. Previous studies have shown that BER combined with DOX does not interfere with the antitumor effect of DOX but significantly promotes the sensitivity and antitumor effect of DOX on a variety of human cancer cells (Mittal et al., 2014; Tong et al., 2012). Moreover, our recent studies have reported that BER alleviates DOX-induced cardiotoxicity in vitro and in vivo (Xiong et al., 2018; Wu et al., 2019). However, it has not been reported whether BER enhances the anti-ATC effects of DOX, and the underlying molecular mechanisms of BER have not yet been elucidated.

The aims of the present study were to investigate the anticancer effects of BER on ATC cancer cell lines in vitro. We also analyzed the level of autophagy and apoptosis after BER treatment to provide crucial insight into the application of BER in ATC treatment. Additionally, we aimed to further investigate the effects of BER on the sensitivity of ATC cells to DOX.

Materials And Methods

*Cell culture and chemicals.* The CAL-62 and BHT-101 human ATC cell lines were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and DMEM (Gibco, USA), respectively, with 10% fetal bovine serum (ExCell Bio,
China) and 1% penicillin/streptomycin (BI, Israel). All cells were incubated at 37°C with 5% CO₂. BER (Solarbio, China) was dissolved in water and diluted with RPMI 1640 or DMEM to achieve the desired concentration. Bafilomycin A1 (Baf-A1), rapamycin (RA), SC79, wortmannin (WOR), MK-2206, 3-methyladenine (3-MA), and N-acetylcysteine (NAC) were purchased from MCE (NJ, USA).

**Cell survival assay.** A CCK-8 kit (Roche Diagnosis, Indianapolis, IN, USA) was used to measure cell viability. ATC cells were plated in 96-well plates at a density of 4×10³ cells per well 24 h before treatment. ATC cells were then treated with various concentrations of BER for 24, 48, and 72 h. At each time point, 10 µL of CCK-8 reagent was added into each well for an additional 2 h at 37°C, and a microplate reader (NanoDrop, USA) was utilized to detect the absorbance at 450 nm. The cell survival rate is presented as the absorbance relative to that of controls.

**Flow cytometry assay.** The flow cytometric apoptosis assays were performed according to the manufacturer’s instructions. In brief, the Annexin V-FITC Apoptosis Detection kit (BD Pharmingen, USA) was used to detect cell apoptosis. Cells were treated with various concentrations of BER for 72 h. Both attached and floating cells were harvested, washed twice with ice-cold PBS, and suspended in 300 µl of binding buffer. Cells were stained with 5 µl of Annexin V-FITC and incubated at room temperature for 15 min in the dark, and then they were then stained with 5 µl of propidium iodide (PI) and incubated at room temperature for 5 min in the dark. Finally, 200 µl 1× binding buffer was added to each sample, and apoptosis was analyzed by flow cytometry (Agilent Novocyt, USA).

**Colony Formation Assay.** A colony-forming assay was performed to determine clonogenic cell death. Monolayer culture was performed to measure colony formation. ATC cells (700/well) were seeded into 6-well plates and treated with various doses of BER, and the medium was refreshed every 72 h. After 15 days of culture for colony formation, cells were fixed with 4% paraformaldehyde for 10 min and then washed with PBS, and the colonies were stained with a 1% crystal violet solution for 15 min. Each assay was performed in triplicate, and the surviving fraction was determined.

**Western blot analysis.** After treatment with BER for 72 h, cells were harvested and lysed with RIPA buffer (Solarbio, China) containing PMSF (Solarbio, China), and the protein concentration was quantified using BCA assays (Lianke Biotech). The proteins were electrophoresed using 8%-12% SDS–PAGE gels and then transferred to PVDF membranes (Millipore). The membranes were then blocked in 5% nonfat milk for 1.5 h and incubated at 4°C overnight with the following primary antibodies: p62 (MBL, 1:1000), LC3B (GeneTex, 1:1000), cleaved caspase3 (Abcam, 1:500), cleaved PARP1 (Abcam, 1:1000), PI3K (Abcam, 1:1000), p-PI3K (Abcam, 1:1000), AKT (Abcam, 1:10000), p-AKT (Abcam, 1:1000), mTOR (Abcam, 1:1000), p-mTOR (Abcam, 1:1000) and GAPDH (ABclonal, 1:6000). The membranes were further incubated with the corresponding secondary antibodies (Rockland, USA) for 2 h at RT. The protein bands were detected using the Odyssey system.

**Quantification of GFP-LC3 puncta.** ATC cells were transfected with a pCDNA2 GFP-LC3 plasmid using Lipofectamine 3000 (Invitrogen, USA) in serum- and antibiotic-free medium for 12 h followed by
treatment with BER for 12 h. The accumulation of GFP-LC3 was examined by fluorescence microscopy (Olympus, Japan).

**Measurement of cytosolic ROS.** An ROS Assay Kit (Beyotime, Shanghai, China) was used to determine ROS levels using 2',7'-dichlorofluorescein diacetate (DCFH-DA) by flow cytometry analysis. ATC cells were treated with BER and the ROS inhibitor, NAC, for 24 h at 37°C, and they were then incubated in RPMI-1640 medium containing 5 µmol/ml DCHF-DA for 30 min at room temperature. The fluorescence intensity was determined using a flow cytometer (Agilent Novocyte, USA), and the data were analyzed using FlowJo software.

**Statistical analysis.** The data analysis was performed at least three times using GraphPad Prism 8.0, and the results are expressed as the mean ± SD. Student's t tests were performed to evaluate the significant differences between the control and treated groups, and \( P \) values < 0.05 were considered statistically significant.

**Results**

**BER inhibits the viability and proliferation of ATC cells**

To investigate the effects of BER on the proliferation of ATC cells, the viability of two anaplastic thyroid carcinoma cell lines (CAL-62 and BHT-101) was evaluated. We first evaluated the cytotoxicity of BER at different concentrations (10, 20, 40, 80, 160 and 200 µM) after 24, 48 and 72 h of treatment by the CCK-8 assay. The results showed that BER significantly inhibited the viability of both ATC cell lines in a time- and dose-dependent manner (Fig. 1A). The half maximal inhibitory concentration (IC\(_{50}\)) of BER in CAL-62 cells was 124.60, 40.18 and 30.39 µM at 24, 48 and 72 h, respectively. Similarly, the IC\(_{50}\) values of BER in BHT-101 cells treated for 24, 48 and 72 h were 70.29, 38.44 and 22.92 µM, respectively. The strongest effect of BER on the inhibition of cell growth was observed 72 h after treatment. According to the CCK-8 assay results of the examined cell lines, BHT-101 was the most sensitive cell line to BER. Hence, we subsequently used 80 and 40 µM BER for 72 h to treat CAL-62 and BHT-101 cells. Furthermore, the number of colonies in the BER-treated cells was significantly decreased with increasing BER concentrations (Fig. 1B). Taken together, these data suggested that BER inhibits cell growth and proliferation in CAL-62 and BHT-101 cells.

**BER induces apoptosis in ATC cells**

To determine whether the inhibitory proliferative effect of BER in CAL-62 and BHT-101 cells was associated with apoptosis, the expression levels of cleaved caspase 3 and cleaved PARP1, two apoptosis protein biomarkers (Cohen, 1997), in berberine-treated ATC cells were examined. Figure 2A shows that BER significantly increased the protein expression of cleaved caspase 3 and cleaved PARP1 in both ATC cell lines in a dose-dependent manner. Furthermore, CAL-62 and BHT-101 cells were exposed to various concentrations of BER for 72 h and analyzed by flow cytometry (Fig. 2B). Flow cytometry analysis indicated that the apoptosis rates induced by BER treatment at 20, 40 and 80 µM were 46.32 ± 1.96%,
56.17 ± 2.57% and 66.27 ± 1.09%, respectively, in CAL-62 cells. In addition, the apoptosis rates induced by BER treatment at 10, 20 and 40 µM in BHT-101 cells were 4.02 ± 0.41%, 8.90 ± 0.32% and 7.55 ± 0.91%, respectively (Fig. 2C). These results indicated that BER triggered dose-dependent apoptosis in ATC cell lines. Compared to the control group, the 80 and 40 µM BER treatment groups induced higher levels of apoptosis in CAL-62 (P < 0.01) and BHT-101 (P < 0.001) cells, respectively.

**BER induces autophagy in ATC cells**

Figure 1A and 2A show that the apoptosis rate did not match the inhibition rate of BER on ATC cell viability, which suggested that BER-induced cell death may involve other mechanisms. BER exerts anticancer activity in various cancer types by inducing autophagy (Mohammadinejad et al., 2019). To explore whether BER triggers autophagy in ATC cells, we first detected the protein expression of autophagy-related factors, including LC3 and p62, in CAL-62 and BHT-101 cells. Because the conversion of LC3-I to LC3-II is commonly used to evaluate autophagy (Klionsky et al., 2021), we evaluated the conversion of LC3-I to LC3-II. After BER treatment for 72 h, the LC3- /LC3- ratio (LC3B) was significantly increased, and the levels of p62, a substrate of autophagosome degradation (Pankiv et al., 2007), were also increased (Fig. 3A). To further confirm that BER causes autophagy in ATC cells, an GFP-LC3 puncta autophagy assay was used to monitor autophagy through direct fluorescence microscopy. In CAL-62 and BHT101 cells treated with 80 and 40 µM BER, respectively, GFP-LC3 puncta were significantly increased compared to untreated cells (Fig. 3B), which demonstrated that BER induced autophagy in ATC cells.

Because the accumulation of LC3-II may be attributed to an increase in autophagosome formation or a decrease in lysosomal fusion and degradation, cells were incubated with bafilomycin A1 (BafA1), an inhibitor of the autophagosome, to block autophagic flux. The results showed that BafA1 (2 µM) treatment elicited a significant increase in the LC3- /LC3- ratio (LC3B) and p62 expression in ATC cells treated with BER (Fig. 3C), which excluded the possibility of lysosomal dysfunction causing LC3-II accumulation. These data revealed that BER treatment induces autophagy in ATC cells.

Because the above data indicated that BER promotes both apoptosis and autophagy in ATC cells, we further investigated the role of BER-induced autophagy in cell death by pretreating cells the 3-methyladenine (3-MA; 2 mM) autophagy inhibitor for 2 h followed by BER treatment for 72 h. We then calculated the cell viability of BER-treated ATC cells with or without pretreatment with 3-MA. When ATC cells were cotreated with BER and 3-MA, the BER-induced accumulation of LC3B was significantly blocked, and the expression of c-PARP1 was significantly decreased (Fig. 3D). Flow cytometry analysis was utilized to further explore the combination of BER and 3-MA. Compared to the BER group, cotreatment with BER and 3-MA efficiently reduced cell apoptosis (Fig. 3E and F). In addition, ATC cells pretreated with 3-MA showed significantly enhanced viability compared to BER-treated ATC cells (Fig. 3G). These results indicated that the antiproliferative and proapoptotic effects of BER on ATC cells were decreased under cotreatment with BER and 3-MA. Thus, these findings suggested that autophagy may play a partial but significant role in BER-induced cell growth inhibition.
BER induces cytotoxicity via the PI3K/AKT/mTOR signaling pathway

As the PI3K/AKT/mTOR pathway has been demonstrated to be related to cancer progression and cell autophagy (Wang et al., 2019; Fan et al., 2016), we investigated whether the PI3K/AKT/mTOR signaling pathway is involved in the effects of BER in ATC. Compared to control cells, western blot analysis demonstrated that the ratios of p-PI3K/PI3K and p-mTOR/mTOR were significantly lower in both types of cancer cells treated with BER, while the ratios of p-AKT/AKT were increased in CAL-62 and BHT-101 cells treated with BER (Fig. 4A). Interestingly, although BER blocked mTOR activation, it simultaneously activated AKT. These results suggested that the BER-induced mTOR inhibition was not dependent on its effect on AKT but that BER-induced AKT activation may be due to mTOR inhibition. Thus, the AKT/mTOR pathway, as well as its feedback loop, are more likely involved in these processes (Wan et al., 2007). These results indicated that the mechanism by which BER inhibits the proliferation of ATC cells may involve modulation of the PI3K/AKT/mTOR signaling pathway.

To further confirm whether the PI3K/AKT/mTOR pathway participates in BER-triggered cytotoxicity in anaplastic thyroid cancer cells, we tested whether cotreatment with wortmannin (WOR) (a PI3K inhibitor), MK-2206 (an AKT inhibitor), and rapamycin (RA) (a mTOR inhibitor) enhances the BER-induced reduction in cellular growth. The CCK8 assay demonstrated that although BER inhibited the viability of ATC cells, RA, WOR, and MK-2206 pretreatment increased the inhibitory effect of BER on ATC cell viability (Fig. 4B). Therefore, these data demonstrated that BER suppresses ATC cell growth by regulating the PI3K/AKT/mTOR signaling pathway.

BER mediates autophagy and apoptosis by regulating the PI3K/Akt/mTOR signaling pathway

We next investigated whether the PI3K/AKT/mTOR pathway participates in BER-induced apoptosis and autophagy in ATC cells. Cells were treated with BER for 72 h with or without pretreatment with the WOR (PI3K inhibitor), SC79 (AKT activator) and RA (mTOR inhibitor) for 30 min. Western blot analysis showed that the expression of cleaved PARP and the LC3-/LC3+ ratio (LC3B) were increased in cells treated with both WOR and BER compared to BER-treated cells (Fig. 5A). Flow cytometry analysis showed that ATC cells treated with BER and WOR had a higher apoptotic rate than those treated with BER alone (Fig. 5B). Moreover, the apoptosis and autophagy-related protein levels were similarly increased after BER treatment alone and cotreatment with RA, an inhibitor of mTOR (Fig. 5E). Moreover, flow cytometry analysis indicated that the apoptotic rates of cells cotreated with BER and RA were greater than those in cells treated with BER alone (Fig. 5F). Additionally, western blot analysis demonstrated that pretreatment of ATC cells with SC79 significantly increased the BER-induced p-AKT/AKT ratio but reduced the LC3II/I ratio and cleaved PARP1 expression (Fig. 5C). Flow cytometry analysis showed that cells treated with BER and SC79 had a lower apoptotic rate than cells treated with BER alone (Fig. 5D). Hence, activation of AKT via SC79 treatment may restore the autophagy and apoptosis induced by BER. Taken together, these
findings indicated that the PI3K/AKT/mTOR signaling pathway may have a vital role in the regulation of BER-induced autophagy and apoptosis.

**BER induces ATC cell autophagy and apoptosis via ROS accumulation**

Previous studies have demonstrated that ROS are a major factor in BER-induced apoptosis (Fang et al., 2021; Kim et al., 2021). Excessive ROS production induces tumor cell autophagy and apoptosis (Liu et al., 2020; Wen et al., 2019). To further investigate the mechanism by which BER promotes autophagy and apoptotic responses in ATC cells, the DCHF-DA oxidation-activated fluorescent dye was used to detect intracellular ROS via flow cytometry analysis. BER treatment significantly increased ROS levels, whereas pretreatment with the N-acetylcysteine (NAC) ROS inhibitor significantly decreased ROS levels, which suggested that BER stimulated ROS generation in ATC cells (Fig. 6A). Furthermore, pretreatment with NAC significantly reversed the BER-induced decrease in cell viability in ATC cells (Fig. 6B). To further investigate whether BER-induced ROS triggers autophagy and apoptosis, the LC3- /LC3- ratio (LC3B) and cleaved PARP1 were detected by western blot analysis, which demonstrated that NAC inhibited BER-induced autophagy and apoptosis (Fig. 6C). These results demonstrated that increased ROS production is one of the upstream events contributing to BER-induced activation of autophagy and apoptosis. Furthermore, the effects of NAC and BER on apoptosis were detected by flow cytometry analysis. Figure 6D shows that NAC alone did not affect the apoptosis rate of ATC cells but that NAC reduced the apoptosis rate of BER-treated ATC cells ($P < 0.01$ or $P < 0.001$). Collectively, these data indicated that BER induces cellular ROS accumulation, thereby promoting apoptosis and autophagic cell death.

**BER promotes the sensitivity of ATC cells to DOX**

DOX has shown encouraging clinical activity as a chemotherapeutic for ATC (Haddad et al., 2015). Because BER has antitumor activities toward ATC cells, we next tested the effect of a combination of BER and DOX on the proliferation of ATC cells. We first treated CAL-62 cells with increasing concentrations of DOX for different time points (48–72 h), and cell viability was measured and quantified by the CCK-8 assay. As shown in Fig. 7A, DOX inhibited cell growth in a dose- and time-dependent manner. The IC$_{50}$ values of DOX were 0.298 and 0.098 µM at 48 and 72 h in CAL-62 cells, respectively. To investigate the combinatorial anticancer effects of BER and DOX, we treated CAL-62 cells with DOX at concentrations of 0.2, 0.5 and 1 µM combined with 40 µM BER. Compared to 0.5 and 1 µM DOX alone, the combination of DOX and BER did not induce a reduction in cell viability. When the concentration of DOX was reduced to 0.2 µM, the cell viability was significantly reduced by adding BER (Fig. 7B), which indicated that BER reduced the dosage of low concentrations of DOX. These findings suggested that BER enhanced the sensitivity of ATC cells to DOX treatment. Furthermore, Chou-Talalay analysis (Chou, 1984) was used to calculate the combination index (CI) of BER and DOX for ATC cells. CI values less than one are defined as synergism, while a CI greater than one is defined as antagonism. As shown in Table 1, BER combined with DOX exhibited a synergistic inhibitory effect in CAL-62 cells with CI values of 0.80–0.97 (CI < 1). Thus, we selected BER (40 µM) and DOX (0.2 µM) as the final experimental concentrations for further analysis. The above results revealed that BER may improve the sensitivity of ATC cells to DOX treatment.
Table 1
The combination index (CI) of BER and DOX was calculated for the CAL-62 cell.

<table>
<thead>
<tr>
<th>DOX(µM)</th>
<th>BER(µM)</th>
<th>Combination index (CI)</th>
<th>Cell viability(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>10</td>
<td>6.92928</td>
<td>78.83%</td>
</tr>
<tr>
<td>0.1</td>
<td>20</td>
<td>2.08118</td>
<td>48.37%</td>
</tr>
<tr>
<td>0.2</td>
<td>40</td>
<td>0.97185</td>
<td>23.97%</td>
</tr>
<tr>
<td>0.4</td>
<td>80</td>
<td>0.80753</td>
<td>13.63%</td>
</tr>
</tbody>
</table>

Because BER induces autophagic death in ATC cells, we investigated whether cotreatment with DOX and BER enhances autophagy induction. As shown in Fig. 7C, both BER and DOX alone increased the relative expression of the LC3B and p62 autophagy-promoting proteins in CAL-62 cells compared to the control ($P < 0.05$ or $P < 0.01$). Interestingly, BER and DOX combination therapy had higher expression levels of LC3B and p62 compared to either treatment alone ($P < 0.05$ or $P < 0.01$). Furthermore, the apoptotic effect of the combination of BER and DOX was further explored by flow cytometry analysis. The results showed that a combination of BER and DOX enhanced ATC cell apoptosis compared to DOX monotherapy (Fig. 7D). Collectively, these results provided additional evidence that the combination of BER with DOX promotes apoptosis and autophagy activation in human ATC cells.

Discussion

BER, the main medicinal component of Coptis chinensis, is a natural plant product with many applications. BER is accepted as a promising anticancer agent in different types of tumors through multiple mechanisms (Liu et al., 2019b). Recently, Gao et al. reported that BER enhances the sensitivity of breast cancer cells to different conventional chemotherapeutic drugs (Gao et al., 2019). Pretreatment with BER is effective in promoting the antitumor effects of 5-fluorouracil and cisplatin in laryngeal cancer cells (Palmieri et al., 2018). Importantly, BER has attracted much attention because it has low toxicity in relatively high doses and exhibits significant therapeutic activities without major adverse effects (Kumar et al., 2015). However, the efficacy of BER against ATC has rarely been reported, and the potential molecular mechanism remains unknown. The present study showed that BER induced a decrease in cell viability, apoptosis, and autophagy in CAL-62 and BHT-101 cells. Furthermore, combining DOX with BER generated a synergistic anticancer effect in ATC cells.

Autophagy is commonly considered to play a dual role in tumor suppression and cancer promotion, which may provide novel opportunities for anticancer drug development (Singh et al., 2018). BER, as a potential autophagy modulator, may trigger or inhibit autophagy and exert its anticancer effects (Mohammadinejad et al., 2019). For instance, BER induces autophagic cell death in breast cancer cells while inhibiting autophagy in lung cancer cells, suggesting the cytoprotective role of autophagy (Wang et al., 2016; Meng et al., 2017). Further research is consequently needed to determine whether BER exhibits anticancer effects through modulation of autophagy in ATC cells. As previously reported, the presence of
LC3-II has been considered an indicator of autophagosome formation (Mohammadinejad et al., 2019). In the present study, we found that BER triggered autophagy and promoted the conversion of LC3-I to LC3-II. Interestingly, the level of p62 also increased, which is related to the degradation of autophagy. As a stress protein, p62 is also affected by oxidative stress and toxic substances. When these conditions act on cells, p62 also increases, which may be accompanied by an increase in autophagy (Moscat et al., 2016). BER also promoted an increase in the LC3-II green fluorescent puncta number in the cytoplasm. In the presented study, we used 3-methyladenine (3-MA) and bafilomycin A1 (BafA1) to understand the role of BER-induced autophagy. When cells were cotreated with BER and BafA1, the BER-induced LC3-II and p62 accumulation was greater than that of cells treated with BER alone. Thus, these findings indicated that autophagy occurred in BER-treated ATC cancer cells. To further investigate the role of autophagy in BER-induced apoptosis of ATC cells, we used 3-MA, a classical inhibitor of autophagy. The combination of BER with 3-MA significantly attenuated the production of LC3-II and cleaved PARP1 compared to treatment with BER alone, which partly indicated that the BER-regulated autophagy may be a form of cell death and not a protective mechanism. These results suggested that BER represses human ATC cell growth by inducing cytostatic autophagy.

Autophagy and apoptosis are two distinct processes that maintain homeostasis, both of which are associated with the death of tumor cells. The present results indicated that the protein levels of cleaved caspase 3 and cleaved PARP1 were significantly upregulated by BER in a concentration-dependent manner. In addition, the inhibition of ATC cell proliferation was decreased after combined treatment with BER and 3-MA compared to BER treatment alone. Rapamycin (RA), an autophagy inducer that targets mTOR (Pan et al., 2009), significantly reversed the BER-reduced cell viability. Moreover, flow cytometry analysis suggested that RA pretreatment enhanced BER-induced apoptosis, while 3-MA reduced BER-induced cell death in ATC cells. These findings indicated that BER-induced apoptosis is reduced or increased after the inhibition or promotion of autophagy by pharmaceutical tools of autophagy. The present findings also revealed that BER induces apoptosis and autophagy in ATC cells. Therefore, the relationship between apoptosis and autophagy may be mutually regulated with treatment to achieve anticancer effects. Additionally, both autophagy and apoptosis may be triggered by common upstream signals, resulting in the activation of combined autophagy and apoptosis.

Because the PI3K/AKT signaling pathway plays a fundamental role in thyroid carcinogenesis and progression, it is a potential therapeutic target in ATC treatment (Saji et al., 2010; Xing et al., 2010). Previous studies have demonstrated that inactivation of the PI3K/AKT/mTOR signaling pathway is involved in autophagy induction (Wang et al., 2019; Fan et al., 2016). Thus, the PI3K/AKT/mTOR pathway may be associated with both apoptosis and autophagy, and it may play a crucial role in BER-induced apoptosis and autophagy in ATC cells. Here, western lot analysis indicated that BER significantly blocked the activation of the PI3K and mTOR proteins as evidenced by the decreased phosphorylation of these proteins but increased phosphorylation of AKT. Interestingly, our results were consistent with previous studies (Hyun et al., 2010; Eo et al., 2014) demonstrating that BER treatment activates the AKT signaling pathways in chondrosarcoma and HepG2 cells, resulting in apoptosis. Furthermore, blocking the PI3K/AKT/mTOR pathway by WOR, MK2206, and RA enhanced the BER-induced cell growth inhibition.
Therefore, we further confirmed that BER represses ATC cell growth through the PI3K/AKT/mTOR pathway. Importantly, in the present study, we simultaneously investigated the roles of PI3K/AKT/mTOR signaling in BER-induced autophagy and apoptosis, and we found that inhibition of PI3K and mTOR significantly increased BER-induced cytostatic autophagy and apoptosis, leading to significant inhibition of cell proliferation, while SC79 (an AKT activator) elicited the opposite effect. These data indicated that PI3K/AKT/mTOR signaling is involved in BER-induced autophagy and apoptosis.

Because the ability of BER to produce free radicals has been reported in various cancer cell lines (Fang et al., 2021; Kim et al., 2021), we investigated the ability of BER to alter intracellular redox potential in ATC cells. In the present study, BER induced a significant increase in ROS generation, which was clearly inhibited by an ROS scavenger (NAC). In addition, a decreased level of ROS through pretreatment with NAC reversed the BER-induced cell viability decrease, apoptosis, and autophagy. These findings demonstrated that BER induces the generation of ROS, which contributes to cell death via apoptosis and/or autophagy.

DOX has been approved by the American Thyroid Association (ATA) guidelines as a conventional single agent in ATC treatment (Smallridge et al., 2012). However, the antitumor activities of combining DOX with BER in ATC are unknown. Thus, we tested the synergistic anticancer effects of DOX and BER in combination on ATC cancer cell lines. The combination therapy of DOX and BER significantly inhibited ATC cell proliferation and induced cell apoptosis compared to DOX or BER monotherapy. In addition, BER combined with DOX exhibited a synergistic inhibitory effect in ATC cells with CI values less than one, indicating that these two compounds in combination markedly exert synergistic growth inhibitory effects on ATC cells. Furthermore, concomitant treatment with DOX and BER enhanced cell autophagy by enhancing the expression of p62 and LC3-II. These data demonstrated that cotreatment with DOX and BER markedly exerts a synergistic growth inhibitory effect on ATC cells by triggering apoptosis and autophagic cell death. Therefore, cotreatment with DOX and BER significantly inhibits cell growth and may be a promising strategy for ATC chemoprevention.

In conclusion, the present results demonstrated that BER exerts antitumor effects in ATC by inhibiting proliferation, promoting apoptosis, and inducing autophagy through the PI3K/AKT/mTOR signaling pathway. In this process, ROS act as a potential target of BER-mediated induction of autophagy and apoptosis. Furthermore, we demonstrated that cotreatment with DOX and BER exhibits synergistic chemopreventive effects by inducing apoptosis and autophagic cell death. Taken together, these results suggested that cotreatment with DOX and BER may be a potential therapeutic approach for ATC. Our findings may shed light on the underlying mechanisms of the anti-ATC effects of BER (Fig. 8), which may serve as a promising drug for treating ATC.

**Abbreviations**

ATC, anaplastic thyroid carcinoma; DOX, doxorubicin; BER, berberine; ROS, reactive oxygen species
Declarations

Acknowledgements

Not applicable.

Funding

This study was supported by the Natural Science Foundation of Hebei Province (H2020206273 and H2021206012).

Availability of data and materials

This article had included all data.

Authors’ contributions

YZW, CX designed this article. XZS, SZ, YW and MYW carry out experiments and analyzed data. CX wrote the manuscript. SWS and YZW helped to revise the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

There is no conflict of interest in this manuscript.

References


**Figures**
Figure 1

BER inhibits the viability and proliferation of ATC cells. (A) The CAL-62 and BHT-101 ATC cell lines were incubated with increasing doses of BER (0-200 µM) for 24, 48, and 72 h to analyze cell proliferation using the CCK-8 assay. (B) BER inhibited colony formation of ATC cell lines. Representative images of CAL-62 and BHT-101 cell contact-dependent colony formation. Each experiment is representative of three
independent experiments. The data are presented as the mean ± SD of three separate experiments; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. 0 µM. BER, berberine; IC$_{50}$, half maximal inhibitory concentration.

**Figure 2**

BER induces apoptosis in ATC cells. (A) The expression of apoptosis-related proteins was evaluated by western blot analysis. CAL-62 and BTH-101 cells were treated with a series of concentrations of BER for
72 h, and western blot analysis was used to evaluate the levels of apoptosis pathway-related proteins using anti-PARP1 and anti-caspase-3 antibodies. (B) AV/PI flow cytometric analysis was used to detect the effect of BER on the apoptosis of ATC cells. To determine the percentage of early and late apoptotic populations, the cells were dual stained with dUTP-FITC and PI and detected by flow cytometric analysis. (C) The cell apoptosis rate was calculated from the flow cytometry results. Data are presented as the mean ± SD. All data are representative of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. 0 µM.
Figure 3

BER-induced autophagy contributes to cell death in ATC cells. (A) The expression of autophagy-related proteins was evaluated in CAL-62 and BHT-101 cells after treatment with the indicated concentrations of BER for 72 h by western blot analysis. GAPDH was used as a control. Representative column diagrams showing the results of relative protein expression. (B) CAL-62 and BHT-101 cells were transiently transfected with GFP-LC3 expression plasmids for 12 h followed by treatment with 80 or 40 μM BER for 72 h. Scale bar, 100 μm. (C) ATC cells were pretreated with or without 2 μM bafilomycin A1 (BafA1, an autophagic flux inhibitor) for 2 h followed by treatment with or without BER for 72 h. Cells were then harvested for western blot analysis to examine the LC3- /LC3- ratio (LC3B) and p62 levels. GAPDH was used as a control. Representative column diagrams showing the results of relative protein expression. (D) The cells were cultured with BER for 72 h in the absence or presence of 2 mM 3-MA, and western blot analysis of the LC3- /LC3- ratio (LC3B) and c-PARP1 was performed. (E) Cells were treated as in D, and apoptosis was detected by flow cytometry. (F) The cell apoptosis rate was calculated from the flow cytometry results. (G) Cells were treated as in D, and the inhibitory rate was measured by CCK-8 assays. Data are expressed as the mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the BER group.
Figure 4

BER inhibits the PI3K/AKT/mTOR pathway. (A) The phosphorylation levels of PI3K, AKT, and mTOR in ATC cells treated with or without BER for 72 h were detected by western blot analysis. Quantification of the relative gray value of bands compared to GAPDH. (B) Effects of the PI3K/AKT/mTOR signaling pathways on BER-induced inhibition of ATC cell viability. CAL-62 and BHT-101 cells were pretreated with 1 μM wortmannin (WOR, a PI3K inhibitor), 0.5 μM MK2206 (an AKT inhibitor), or 0.2 μM rapamycin (RA, an
mTOR inhibitor) for 2 h prior to 80 or 40 μM BBR treatment for 72 h, and cell viability was detected using CCK-8 assays. Data are expressed as the mean ± SD from three independent experiments. *P<0.05, **P<0.01, and ***P<0.001 vs. the control group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the BER group.

Figure 5
BER mediates autophagy and apoptosis by regulating the PI3K/AKT/mTOR signaling pathway. CAL-62 and BHT-101 cells were pretreated with 1 μM WOR (a PI3K inhibitor), 4 μg/ml SC79 (an AKT activator), and 0.2 μM RA (a mTOR inhibitor) for 2 h prior to 80 or 40 μM BBR treatment for 72 h. (A) Cells were treated with or without BER in combination with or without 1 μM WOR, and the levels of the apoptosis-related protein, cleaved PARP1, and the autophagy-related protein, LC3B, were determined by western blot analysis. (B) Cells were treated as in A, and apoptosis was detected using flow cytometry. (C) Cells were treated with or without BER in combination with or without 4 μg/ml SC79, and the levels of the apoptosis-related protein, cleaved PARP1, and the autophagy-related protein, LC3B, were determined by western blot analysis. (D) Cells were treated as in C, and apoptosis was detected using flow cytometry. (E) Cells were treated with or without BER in combination with or without 0.2 μM RA, and the levels of the apoptosis-related protein, cleaved PARP1, and the autophagy-related protein, LC3B, were determined by western blot analysis. (F) Cells were treated as in E, and apoptosis was detected using flow cytometry analysis. Data are expressed as the mean ± SD of triplicate experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the BER group.
Figure 6

BER induces ATC cell autophagy and apoptosis via ROS accumulation. (A) After being treated with 80 or 40 µM BER and/or 2 mM NAC (an ROS inhibitor) for 72 h, CAL-62 and BHT-101 cells were stained with the DCHF-DA oxidation-activated fluorescent dye and analyzed using flow cytometry to detect intracellular ROS. (B) Cell viability was evaluated by the CCK-8 assay. (C) ATC cells were treated as in A, and western blot analysis was used to measure the phosphorylation levels of AKT, the protein levels of
cleaved PARP1 (an apoptosis-related protein), and the protein levels of LC3B (an autophagy-related protein). (D) Cells were treated as in A, and apoptosis was detected using flow cytometry. Data are expressed as the mean ± SD of triplicate experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the BER group.

Figure 7
Effects of BER, DOX, and their combination on the growth of ATC cell lines. (A) CAL-62 cells were treated with different concentrations of DOX for 48 or 72 h. Cell viability was detected using a CCK-8 assay. ns indicates no significant difference. (B) CAL-62 cells were treated with different concentrations of DOX (0.2, 0.5, and 1 μM) in the presence or absence of BER (40 μM) for 72 h. ns indicates no significant difference. The results for (C) CAL-62 cells were analyzed using the CalcuSyn program (Biosoft) based on the Chou-Talalay method. The combination index (CI) of BER and DOX was calculated for CAL-62 cells. (D) The expression of the autophagy-related proteins, LC3 and p62, was evaluated by western blot analysis. (E) Flow cytometry was employed to evaluate cell apoptosis. Data are expressed as the mean ± SD of triplicate experiments. *P < 0.05 and **P < 0.01 vs. the control group; #P < 0.05 and ##P < 0.01 vs. the BER group or DOX group. CI < 1 indicates synergy, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism. BBR indicates berberine.

Figure 8

Schematic diagram illustrating the potential pathway associated with BER-induced apoptosis and autophagy. BER promotes the downregulation of PI3K/AKT/mTOR signaling and the upregulation of ROS-mediated cell dysfunction, ultimately resulting in autophagic cell death.