Inhibiting ROS-NF-κB-dependent autophagy enhanced brazin-induced apoptosis in head and neck squamous cell carcinoma

Zhi-Jing He, Fei-Ya Zhu, Shi-Sheng Li, Liang Zhong, Hong-Yu Tan, Kai Wang.

Department of Oral and Maxillofacial Surgery, The Second Xiangya Hospital of Central South University, Changsha, Hunan, PR China

Department of Otorhinolaryngology Head and Neck Surgery, The Second Xiangya Hospital of Central South University, Changsha, Hunan, PR China

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Abstract

Autophagy modulation has been considered a potential therapeutic strategy for head and neck squamous cell carcinoma (HNSCC). A previous study confirmed that brazin might possess significant anti-carcinogenic activity. However, whether brazin induces autophagy and its roles in cell death in HNSCC are still unclear. In this study, we have shown that brazin induced significant apoptosis in the Cal27 HNSCC cell line but not in oral keratinocyte cell line (OKC). In addition to showing apoptosis induction, we demonstrated the brazin-induced autophagic response in the Cal27 cells, as evidenced by the formation of GFP-LC3 puncta, and also showed the upregulation of LC3-II and Beclin-1. Moreover, pharmacologically or genetically blocking autophagy enhanced the brazin-induced apoptosis, indicating the cytoprotective role of autophagy in brazin-treated Cal27 cells. Moreover, brazin activated nuclear factor kappa B (NF-κB) nuclear translocation and increased NF-κB p65 reporter activity, which contributed to the upregulation of autophagy-related genes, including LC3-II and Beclin-1. Importantly, we found that brazin triggered reactive oxygen species (ROS) generation in Cal27 cells. Furthermore, N-acetyl-cysteine (NAC), a ROS scavenger, abrogated the effects of brazin on the NF-κB p65-dependent autophagy. Taken together, our results demonstrated that brazin increased the NF-κB p65-dependent autophagy through the promotion of ROS signalling pathways in HNSCC. These data also suggest that a strategy of blocking ROS-NF-κB p65-dependent autophagy to enhance the activity of brazin warrants further attention for the treatment of HNSCC.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a malignancy found worldwide with an aggressive process and dismal prognosis, and HNSCC is also highly correlated with tobacco and alcohol abuse, betel quid chewing and human papilloma virus (HPV) infection (Murphy et al., 2016). Although surgical resection is traditionally the initial treatment for early-stage cancer, the addition of chemotherapy has shown a notable trend of increasing overall survival and progression-free survival in the advanced and metastatic stages of cancer for which cisplatin and paclitaxel are most widely used (Aminuddin and Ng, 2016). Despite recent advances in chemotherapy for gastric cancer, the clinical effect remains unsatisfied. Therefore, novel drugs or adjuvants with high efficacy and low toxicity are badly needed.

Brazilin, a purified natural product from sappan wood (Caesalpinia sappan L), is suggested to possess anti-inflammatory, anti-proliferative, and anti-oxidant activities when studied in various animal model systems and cell cultures (Nirmal et al., 2015). This compound has recently attracted considerable attention because of accumulating data demonstrating its strong inhibitory effect on bladder carcinoma, multiple myeloma, and glioblastoma (Kim et al., 2012). Several studies have revealed that brazin causes cell cycle arrest and triggers apoptosis in some human carcinoma cells (Kim et al., 2012; Lee et al., 2013). However, the potential anticancer properties of brazin against HNSCC cells are not well known.

Autophagy is a homeostatic, catabolic degradation process whereby cellular proteins and organelles are engulfed by autophagosomes, digested in lysosomes, and recycled to sustain cellular metabolism (Yang et al., 2011). Autophagy can function as a cellular housekeeper by removing damaged organelles and recycling macromolecules; therefore, autophagy can protect cancer cells, particularly during malignant transformation and carcinogenesis. A growing body of evidence indicates that autophagy can help human
cancer cells survive by conferring apoptosis resistance, and inhibition of autophagy causes caspase-dependent apoptotic cell death, especially in the presence of other therapies (Button et al., 2016; Chen et al., 2016a; Dou et al., 2016; Yeo et al., 2016). Understanding the interplay between apoptosis and autophagy in tumours is crucial to identify new targets for cancer therapy and improve the therapeutic efficiency. Thus, it is necessary to determine whether increased autophagic activity is observed in HNSCC cells in response to brazilin, and this effect has been hypothesized to significantly reduce treatment efficacy.

NF-κB p65 is a dimeric transcription factor whose activity is controlled at the subcellular level, and it comprises a family of inducible transcription factors (Huang et al., 2016). Recently, NF-κB p65 activation has been shown to be involved in multiple aspects of oncogenesis, including inflammatory responses, cellular differentiation, proliferation, and survival, in almost all multicellular organisms (Lu and Stark, 2015; Zhou et al., 2015; Espinosa et al., 2015). NF-κB p65 is dysregulated in many forms of cancer. NF-κB p65 exerts different, even contradictory effects, depending on the cell type or the variety of stress. Recent studies have suggested a correlation between autophagy and NF-κB p65 pathway in human cancer (Vequaud et al., 2015; Shaw et al., 2008; Ling et al., 2012; Wang et al., 2011). Importantly, under stressful conditions, such as treatment using chemotherapy, an intricate interplay between the homeostatic pathways for NF-κB p65 and autophagy and the apoptotic executive process may take place in cancer cells that will ultimately whether their fate is cell death or survival.

In this study, our results indicate that brazilin induces obvious apoptosis in HNSCC. Furthermore, apoptosis is not the sole consequence of brazilin deprivation because brazilin treatment rapidly activates an autophagic process. Moreover, brazilin activated nuclear factor kappa B (NF-κB p65) nuclear translocation and increased NF-κB p65 reporter activity, which contributed to the upregulation of autophagy-related genes, including LC3-II and Beclin-1. Pharmacological or genetic inhibition of NF-κB p65-dependent autophagy sensitized brazilin-induced apoptosis in HNSCC cells. A link between brazilin-induced NF-κB p65-dependent autophagy and ROS production was also observed. These findings indicate that autophagy provides a cytoprotective mechanism in brazilin-treated HNSCC cells, and inhibition of autophagy may improve the therapeutic efficacy of brazilin in the treatment of HNSCC.

2. Materials and methods

2.1. Drugs and reagents

Brazilin, Bafilomycin A1, DCFH-DA, and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO), and Lipofectamine 2000 was bought from Invitrogen (Carlsbad, CA). Stock solutions were prepared in dimethyl sulfoxide (DMSO), stored at −20 °C, and diluted in fresh medium for each experiment. The final concentration of DMSO did not exceed 0.5% in any of the experiments to prevent cell toxicity.

2.2. Cell lines

Human HNSCC Cal27 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco) in a humidified incubator at 37 °C with 5% CO2. 2–3 passive primary cultured oral keratinocyte cell line (OKC) was used for a normal control. Cells were passed by dissociation with 0.25% trypsin-EDTA solution (Gibco) for 1–2 min after having grown to 80–90% confluency.

2.3. Nuclear and cytoplasmic protein extraction

After stimulation, the cytoplasmic and nuclear proteins of Cal27 cells were extracted using Nuclear and Cytoplasmic Extraction Reagents obtained from Thermo Fisher strictly according to the manufacturer’s instructions.

2.4. Luciferase reporter assays

To detect the activation of NF-κB, Cal27 cells were transfected with plasmids encoding NF-κB-responsive firefly luciferase and Renilla luciferase using Lipofectamine 2000. After transfection for 48 h, the cells were washed with phosphate-buffered saline, and exposed to different doses brazilin of for 24 h, and then assayed for luciferase activity using the dual-luciferase reporter assay kit (Promega Corporation, WI, USA) according to the manufacturer’s instructions (Yang et al., 2016).

2.5. Western blot analysis

Cal27 cells were treated with the indicated concentrations of brazilin and pretreated with or without Bafilomycin A1 for 48 h. Then, the cells were harvested and washed with cold phosphate-buffered saline (PBS). The proteins were extracted with RIPA Cell Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China) and kept on ice for at least 30 min. The lysates were centrifuged at 12,000 g at 4 °C for 10 min and then the supernatant was transferred to a fresh tube. After the protein concentration was measured using the bicinchoninic acid (BCA) method, an equal quantity of total protein per lane was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk and powder in 0.05% Tris-buffered saline and Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4 °C with specialized antibodies. After overnight incubation, the membranes were washed three times and then incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. Then, blots were developed using a West Pico enhanced chemiluminescence detection kit (Thermo). Antibodies against NF-κB p65, LC3, and PARP were purchased from Cell Signalling (Beverly, MA, USA); antibodies against Beclin-1, Bcl-2, p62 and Bax were purchased from

Fig. 1. Brazilin induces apoptosis in HNSCC cells. (A) Cal27 cells were treated with 5 μM, 10 μM, and 20 μM brazilin for 24 h and stained with Annexin V/PI and then analysed by flow cytometry. The percentages of Annexin V-positive cells are presented in bar charts; (B) Clonogenic assay. Cal27 cells were plated at low density, treated with different concentrations of brazilin and cultured for 7 days. Representative pictures (left panel) and a summary graph (right panel) are shown; (C) Cal27 cells were treated with different concentrations of brazilin for 24 h, and then western blot analysis was performed to assess the expression level of Bcl-2, Bax and cleaved-PARP (Cl-PARP); GAPDH served as a loading control; (D) Cal27 cells were treated with 10 μM brazilin for 6, 12 and 24 h and then western blot analysis was performed to assess the expression level of Bcl-2, Bax and Cl-PARP; GAPDH served as a loading control; (E) Caspase-3 activity of Cal27 cells after 24 h of brazilin treatment at the indicated concentrations. The percentages of caspase-3 activity are presented in bar charts; (F) Cal27 cells were treated with 10 μM brazilin for 6, 12 h and 24 h and then the caspase-3 activity was determined. The percentages of caspase-3 activity are presented in bar charts; the data are presented as the means ± SEM. One-way ANOVA followed by Dunnett analysis was performed using GraphPad Prism 5. *P < 0.05, **P < 0.01 versus the control group (n = 6).
Santa Cruz (Santa Cruz, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and foetal bovine serum were obtained from Gibco (Life Technologies Gibco/BRL, New York, NY, USA). NF-kB p65 and Beclin1 siRNAs were purchased from Shanghai GenePharma (Shanghai, China). Proteins were visualized using HRP-conjugated anti-rabbit or anti-mouse IgG and DAB as the HRP substrate (Piet et al., 2013).

2.6. Clonogenic assay

For the clonogenic assay, 1 × 10³ Cal27 cells were plated in 35-mm culture dishes in complete DMEM. Cells were grown under these conditions for 7–10 days, and adherent separated clones were counted.

2.7. Apoptosis assay

An Annexin V-FITC/PI Detection Kit (BD Biosciences, San Diego, CA, USA) was used for the determination of cell apoptosis. Cal27 cells were exposed to brazilin with or without the autophagy inhibitor Bafilomycin A1 for 48 h, then harvested and washed twice with cold PBS, and re-suspended in binding buffer at a concentration of 1 × 10⁶ cells/mL. Subsequently, according to the manufacturer’s instructions, the cells were stained with Annexin V-FITC and PI for 15 min at 37 °C. Then, the cells were analysed immediately using a FACS Calibur flow cytometer (Becton-Dickinson, CA, USA). The percentages of Annexin V-positive cells are presented in bar charts. Caspase-3 activity of OKC cells after 24 h of brazilin treatment at the indicated concentrations. The means ± SEM. One-way ANOVA followed by Dunnett analysis was performed using GraphPad Prism 5. *P < 0.05, **P < 0.01 versus the control group (n = 6).

Fig. 2. Brazilin did not cause apoptosis in oral keratinocyte cell line (OKC) (A) OKC cells were treated with 5 μM, 10 μM, and 20 μM brazilin for 24 h and stained with Annexin V/PI, and then analysed by flow cytometry. The percentages of Annexin V-positive cells are presented in bar charts; (B) Caspase-3 activity of OKC cells after 24 h of brazilin treatment at the indicated concentrations. The percentages of caspase-3 activity are presented in bar charts; (C) OKC cells were treated with different concentrations of brazilin for 24 h, and then western blot analysis was performed to assess the expression level of Bcl-2, Bax and cleaved-PARP (Cl-PARP); GAPDH served as a loading control; the data are presented as the means ± SEM. One-way ANOVA followed by Dunnett analysis was performed using GraphPad Prism 5. *P < 0.05, **P < 0.01 versus the control group (n = 6).
2.8. LC3 immunofluorescence staining

Cal27 cells stably expressing GFP-LC3 were obtained by transfecting the cells with the GFP-LC3 plasmid and selecting with G418. Transfection using Lipofectamine 2000 Reagent was carried out according to the manufacturer’s protocol. After transfection, the cells were washed twice with phosphate-buffered saline (PBS), and fresh DMEM was added for further incubation. The images were photographed using a fluorescence microscope (Leica, Brunswick, Germany) (Pi et al., 2015).

2.9. Measurement of Reactive Oxygen Species (ROS)

Accumulated cells were treated with brazilin in the presence or absence of NAC for 2 h and then loaded with 25 mM DCF-DA. After incubation for 30 min at 37 °C in a 5% CO₂ incubator, cells were washed twice with HBSS solution, suspended in complete media and then examined under a fluorescence microscope to detect the intracellular accumulation of ROS.

2.10. Gene knockdown using siRNA

The siRNAs for Beclin1 and NF-κB p65 and the control siRNA...
were all purchased from Shanghai GenePharma. Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were incubated for 48 h before further treatment (Li et al., 2016a).

2.11. RT-PCR

Total RNA was extracted using Trizol reagent, and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen).
qRT-PCR and data collection were performed with an ABI PRISM 7900HT sequence detection system.

2.12. Statistical analysis

One-way ANOVA, followed by Tukey’s post hoc test, was used to determine the statistical differences between treatment groups. \( P < 0.05 \) was considered statistically significant; all tests were two-sided, and no corrections were applied for multiple significance testing. All the experiments were repeated at least three times. Drug and biomarker distributions are represented as the mean values ± s.e.m. * and ** indicate \( P < 0.05 \) and \( P < 0.01 \), respectively.

3. Results

3.1. Brazilin induces apoptosis in HNSCC cells

We applied flow cytometry following Annexin V-FITC and propidium iodide (PI) dual labelling to detect the effects of brazilin treatment on apoptotic induction in Cal27 cells. Cultured Cal27 cells were treated with brazilin at increasing concentrations. As shown in Fig. 1A, cells were treated with 5, 10 and 20 \( \mu \)M brazilin for 24 h or 10 \( \mu \)M brazilin for 6, 12, and 24 h, and we found that brazilin induced cell apoptosis in a dose- and time-dependent manner. Consistently, the results of the clonogenic assay demonstrated the dose- and time-dependent effects (Fig. 1B). Both cleaved PARP and the ratio of Bax to Bcl-2 proteins, which are all key indicators of apoptosis, increased in Cal27 cells that were treated with different concentrations of brazilin for 24 h or with 10 \( \mu \)M brazilin for the indicated time periods (Fig. 1C and D). We also found that treatment with brazilin increased caspase-3 activity in a dose- and time-dependent manner (Fig. 1E and F). It was worth to note that brazilin, at the same concentrations, did not cause apoptosis in oral keratinocyte cell line (OKC) (Fig. 2A, B and C). Together, these results demonstrate that apoptosis was involved in the response of Cal27 cells to brazilin treatment.

3.2. Brazilin induces autophagy in HNSCC cells

Several studies have suggested that autophagy may act as a protective mechanism in tumour cells and that autophagy induction is a common event in cancer cells in response to various chemotherapeutic treatments (Song et al., 2015; Fan et al., 2015; Chen et al., 2016b). Thus, we examined whether brazilin induced...
autophagy through the membrane-bound, autophagosome-associated form of microtubule-associated protein 1 light chain 3 (LC3-II) to examine its potential as a marker to monitor the effect of brazilin on autophagy induction (Pi et al., 2015). First, we constructed a Cal27 cell line stably expressing the GFP-LC3 fusion gene and used a fluorescence microscope to detect GFP-LC3 puncta dots. As shown in Fig. 3A, after treatment with different concentrations of brazilin for 24 h, Cal27 cells displayed more green fluorescence than that in the negative controls. In agreement with the results of the formation of GFP-LC3-labelled vacuoles examined by fluorescence microscopy, conversion of the cytoplasmic form of LC3 (LC3-I, 18 kDa) to the pre-autophagosomal and autophagosomal membrane-bound form of LC3 (LC3-II, 16 kDa) was biochemically demonstrated by western blot and shown to have a dose- and time-dependent effect (Figs. 3B and 2C). Moreover, induction of autophagy was identified by two well-established measurements of autophagy, that is, enhancement of Beclin-1 and degradation of p62 (Fig. 3B and C). Taken together, our results indicate that brazilin treatment induced autophagic flux in the HNSCC cells.

3.3. Inhibition of brazilin-induced autophagy sensitized Cal27 cells to brazilin-induced apoptotic cell death

Autophagy is considered as a double-edged sword (Buffen et al., 2014; Li et al., 2016b; White and DiPaola, 2009). It can prolong the survival of cancer cells and enhance the resistance to apoptosis, and paradoxically, defective autophagy has been linked to increased tumorigenesis. Given that manipulation of autophagy may improve the efficacy of anticancer therapeutics, we were eager to determine whether the brazilin-elicited autophagy in Cal27 favoured cell survival or cell death. We used several approaches to investigate this question. Since Beclin1 is an essential protein for autophagy activation, Beclin1 deficiency can significantly interrupt autophagy (Zhang et al., 2016; Takahashi et al., 2009). Therefore, to determine the precise role of autophagy in brazilin-treated Cal27 cells, autophagy inhibition by Beclin1 siRNA transfection was then performed. Cal27 cells transfected with Beclin-1 siRNA showed a reduced level of LC3-II accumulation after brazilin treatment compared with that for a scrambled siRNA control, indicating the...
involvement of Beclin-1 in brazilin-mediated autophagy in Cal27 cells (Fig. 4A). To test whether autophagy acts as a cytoprotective mechanism in our system, we inhibited autophagy in Cal27 cells using Bafilomycin A1 (Baf A1), which is an inhibitor of the lysosomal V-ATPase and which causes an accumulation of autophagosomes due to a defect in the fusion between autophagosomes and lysosomes (Pi et al., 2013). We observed that the presence of 10 nM Baf A1 significantly increased the level of LC3II and cleaved PARP in Cal27 cells treated with 10 mM brazilin (Fig. 4B). In agreement with the data derived from pharmacological inhibitors, knockdown of Beclin-1 by siRNA increased the level of cleaved PARP, as assayed by western blot analysis (Fig. 4C). Moreover, brazilin remarkably increased the caspase-3 activity after inhibited autophagy flux (Fig. 4D and E). These results reveal that inhibition of brazilin-induced autophagy sensitized Cal27 cells to brazilin-induced apoptotic cell death.

3.4. Brazilin induced autophagy by activating NF-κB p65 pathways in HNSCC cells

Nuclear factor kappa B (NF-κB p65) is a family of dimeric transcription factors central to coordinating inflammatory responses and cellular differentiation, proliferation, and survival in almost all multicellular organisms (Lu and Stark, 2015; Zhou et al., 2015; Espinosa et al., 2015). Recent studies have suggested a correlation between autophagy and the NF-κB p65 pathway in human cancer (Vequaud et al., 2015; Shaw et al., 2008; Ling et al., 2012; Wang et al., 2011). Based on these results, we investigated whether NF-κB p65 is involved in the brazilin action in Cal27 cells. Activation of NF-κB was determined through the detection of its translocation into cell nuclei from its initial location in the cytoplasm where it exists in an inactive form. Western blotting of the NF-κB p65 protein in the nuclear and cytosolic fractions of Cal27 cells indicated...
that brazilin treatment led to an enhancement of nuclear NF-κB and a reduction of cytosolic NF-κB (Fig. 5A). The relative level of NF-κB p65 in the nuclear and cytoplasmic fractions was compared with those of GAPDH and histone 3, respectively and quantified by image analysis. To explore whether the NF-κB p65 pathway is involved in brazilin-induced apoptosis in HNSCC cells, Cal27 cells were transfected with an NF-κB-luciferase expression vector prior to brazilin stimulation, and the transcription factor activity of NF-κB p65 was assessed with a luciferase reporter gene assay. As expected, brazilin greatly increased the NF-κB p65 luciferase activity (Fig. 5B). What’s more, by using RT-PCR, we found that the protein levels of LC3 and Beclin-1 were also increased (Fig. 5C and D). These results suggested that brazilin induced autophagy by activating the NF-κB p65 pathway in HNSCC cells.

3.5. NF-κB p65 mediates brazilin-induced autophagy in HNSCC cells

To further determine whether NF-κB p65 could mediate brazilin-induced autophagy, NF-κB p65 was inhibited with NF-κB p65-specific siRNA prior to brazilin treatment in Cal27 cells. As shown in Fig. 6A, si-NF-κB p65 abrogated the brazilin-induced NF-κB p65-luciferase activity. Notably, inhibition of NF-κB p65 activity decreased the levels of brazilin-induced autophagy-related genes, including LC3 and Beclin-1 (Fig. 6B and C). Additionally, the protein levels of LC3 II/LC3 I and Beclin-1 were also suppressed (Fig. 6D). These results suggested that brazilin induced autophagy by activating the NF-κB p65 pathway in Cal27 cells.

3.6. ROS is an upstream signalling molecule that activates the NF-κB p65-dependent autophagy pathway

Accumulating evidence indicates that intracellular ROS can induce apoptosis and autophagy in cancer cells (Qin et al., 2015; Kaminsky and Zhivotovsky, 2014; Radogna et al., 2016). Thus, we examined whether brazilin-induced apoptosis and autophagy involves the generation of ROS in Cal27 cells. Brazilin significantly induced ROS generation in a dose- and time-dependent manner (Fig. 7A and B), and the ROS scavenger N-acetyl-L-cysteine (NAC) significantly decreased the intracellular ROS generation of the cells (Fig. 7C). In addition, pretreatment with NAC significantly inhibited the activation of NF-κB p65 in brazilin-treated cells, indicating that ROS is an upstream signalling molecule that activates the NF-κB p65 pathway (Fig. 7D and E). NAC also significantly suppressed the protein expression of Beclin-1 and LC3-II and increased the expression of p62, suggesting that autophagy was inhibited by NAC administration (Fig. 8A). Furthermore, incubation of cells with NAC for 1 h prior to treatment with brazilin increased the caspase-3 activity (Fig. 8B). Taken together, these results showed that ROS is involved in brazilin-induced autophagy in Cal27 cells and that the brazilin-promoted NF-κB p65 signalling pathway is partly dependent on ROS generation.

4. Discussion

HNSCC is the most frequent type of head and neck cancer and it usually arises from the mucosal surfaces of several organs, including the nasal cavity, paranasal sinuses, oral cavity, tongue, pharynx, and larynx (Aminuddin and Ng, 2016). Globally, it is the sixth most common type of cancer, and it has low survival rates due to late stage of diagnosis and the high rates of recurrence. Chemotherapy is an effective weapon in the battle against HNSCC, but numerous cancer patients either are not sensitive to chemotherapy or develop drug resistance to current chemotherapy regimens (AlQathama and Prieto, 2015). Therefore, the discovery of an effective chemotherapy mechanism that enhances tumour sensitivity to chemotherapeutics is necessary and urgent.
Brazilin is a naturally occurring red pigment that is oxidized by air and light. Brazilin is a promising chemopreventive agent since it is generally non-toxic and interferes with the process of carcinogenesis. Several synthetic types of brazilin analogues have been demonstrated to induce apoptosis in a number of human cancer cell lines (Lee et al., 2013). In our study, cleaved PARP, the ratio of Bax to Bcl-2 proteins and the caspase-3 activity increased in Cal27 cells after treated with brazilin, but at the same concentrations brazilin did not cause apoptosis in oral keratinocyte cell line (OKC), indicating that brazilin has potential for use as a drug for HNSCC treatment and might be a tumour-specific agent. However, the results of those basic research studies that utilized brazilin were unsatisfactory due to the problem of drug resistance.

Autophagy is a lysosome-dependent mechanism by which dysfunctional or damaged intracellular organelles are broken down and recycled through the lysosomes (Sun et al., 2015). As a putative adaptive catabolic process, autophagy plays an important role in many human cancers (Fan et al., 2015; Li et al., 2016b; Copetti et al., 2009). Inhibition of autophagy in these conditions can lead to increased HNSCC cell death. Fan TF et al. found that suppressing autophagy can enhance the efficacy of STAT3 signalling blockade in HNSCC cells (Fan et al., 2016). Chang et al. reported that inhibition of autophagy in HNSCC cells increased bortezomib-induced apoptosis in HNSCC by reducing autophagy (Chang and Wang, 2016). Consistent with these findings, our experiments showed that pharmacological or genetic inhibition of autophagy leads to increased brazilin-induced apoptosis, indicating that brazilin-mediated autophagy is a pro-survival mechanism rather than a pro-death mechanism. We also examined the potential mechanisms of brazilin-induced autophagy. The nuclear factor kappa B (NF-κB p65) family has been shown to regulate autophagy in various human cancers, and it is well known that NF-κB p65 plays key roles in biological activities, including autophagy, apoptosis, inflammation, survival and metabolic disorders (Vequaud et al., 2015; Shaw et al., 2008; Ling et al., 2012; Wang et al., 2011). Activation of NF-κB p65 was evaluated by detecting its translocation into cell nuclei from its initial location in the cytoplasm, where it exists in an inactive form (Bak et al., 2016), and nuclear translocation of NF-κB p65 stimulates autophagy in various cell types via the increased expression of numerous autophagy-related genes, including proteins in the autophagy core machinery (Copetti et al., 2009). In our research, brazilin treatment activated NF-κB p65 nuclear translocation and increased NF-κB p65 reporter activity, which contributed to the upregulation of autophagy-related genes, including LC3-II and Beclin-1. Moreover, downregulation of NF-κB p65 by siRNA can suppress brazilin-induced autophagy, indicating that the NF-κB p65 signalling pathway was involved in the brazilin-induced autophagy in the HNSCC cells. Interestingly, a previous study reported that the anti-inflammatory properties of brazilin are closely associated with its ability to inhibit the activities of NF-κB p65 (Jia et al., 2016; Lee et al., 2012; Jayakumar et al., 2014). The apparently contradictory data suggest that activation or inhibition of NF-κB p65 by brazilin depends on the cell type and disease model. However, it remains unknown which mechanism mediates the effect of brazilin on the enhancement of NF-κB p65 activity. Recent evidence has linked the accumulation of ROS to NF-κB p65 activation in the invasion and migration of cancer (Cao et al., 2016; Liu et al., 2016). Following brazilin exposure, we have found that brazilin induces ROS in a time- and dose-dependent fashion, an effect that was abolished by pretreatment of cells with NAC. Furthermore, the incubation of HNSCC cells with NAC for 1 h prior to treatment with brazilin revealed that the inhibition of ROS generation abrogated the effect of brazilin on the NF-κB p65 pathway. Based on these results, we suggest that ROS is an important cellular mediator that triggers the NF-κB p65-dependent pathway after the administration of brazilin in HNSCC.

Collectively, our results lead us to report that brazilin induces obvious apoptosis in HNSCC cells. Moreover, apoptosis is not the sole consequence of brazilin deprivation; brazilin treatment rapidly activates an autophagic process. A link between brazilin-induced autophagy and ROS production was also observed. Pharmacological or genetic inhibition of NF-κB p65-dependent autophagy sensitized HNSCC cells to brazilin-induced apoptosis. These findings indicate that autophagy provides a cytoprotective mechanism in HNSCC cells treated with brazilin, and inhibition of autophagy may improve the therapeutic efficacy of brazilin for the treatment of HNSCC.

Disclosure of conflict of interest

None.

Author contributions

Zhi-ting He, Kai Wang conceived and designed the experiments; Zhi-ting He, Fei-ya Zhu and Shi-Sheng Li performed the experiments; Liang Zhong, Hong-yu Tan analysed the data; Zhi-ting He drafted the manuscript; Kai Wang critically revised and approved the manuscript.

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