AKT serine/threonine protein kinase modulates baicalin-triggered autophagy in human bladder cancer T24 cells

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Abstract. Baicalin is one of the major compounds in the traditional Chinese medicinal herb from *Scutellaria baicalensis* Georgi. We investigated the molecular mechanisms of cell autophagy induced by baicalin in human bladder cancer T24 cells. Baicalin inhibited cell survival as shown by MTT assay and increased cell death by trypan blue exclusion assay in a concentration-dependent manner. Baicalin did not induce apoptotic cell death in T24 cells by TUNEL and caspase-3 activity assay. Baicalin induced the acidic vesicular organelle cell autophagy marker, manifested by acridine orange (AO) and monodansylcadaverine (MDC) staining and cleavage of microtubule-associated protein 1 light chain 3 (LC3). The protein expression levels of the Atg 5, Atg 7, Atg 12, Beclin-1 and LC3-II were upregulated in T24 cells after baicalin treatment. Inhibition of autophagy by 3-methyl-adenine (an inhibitor of class III phosphatidylinositol-3 kinase; 3-MA) reduced the cleavage of LC3 in T24 cells after baicalin treatment. Furthermore, protein expression levels of phospho-AKT (Ser473) and enzyme activity of AKT were downregulated in T24 cells after baicalin treatment. In conclusion, baicalin triggered cell autophagy through the AKT signaling pathway in T24 cells.

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Introduction

The morphological processes leading to cell death include apoptosis, necrosis and autophagy (1-3). Autophagy (or called self-eating) is a process maintaining cellular homeostasis (4,5). When the cells undergo cellular damage, autophagy is required for the promotion of cellular survival (4,6). Autophagy involves the autophagosome formation (a double-membrane structure), which fuses with a lysosome to form an autophagolysosome, finally resulting in degradation of the captured proteins or organelles by lysosomal enzymes (7,8). Several reports have shown that autophagy-related (Atg) proteins and microtubule-associated protein 1 light chain 3 (LC3) are major proteins involved in autophagy processes (9-11). Induction of Atg and LC3 protein levels has been linked with altering a variety of cellular signaling pathways, such as adenosine monophosphate-activated protein kinase (AMPK) (12-14), mitogen-activated protein kinase (MAPK) (15,16) and PI3K/Akt pathways (17,18). Previous studies indicated that suppression of PI3K/Akt is involved in regulating autophagy formation (19-21).

Chinese herbs are used for treatment of diseases in Taiwan and in China for a long time (22,23). Baicalin is one of the major flavonoids (molecular formula: C21H18O11; Fig. 1) in the traditional Chinese medicinal herb 'Huang qin' (*Scutellaria baicalensis* Georgi) (24,25). The baicalin exhibits many different pharmacological actions such as anti-oxidant (26), photo-protective (27), neural protective (28,29), anti-depressant (30), anti-inflammatory (31,32), anti-viral (33,34), anti-hepatotoxicity (35,36) and anticancer effects (37-39). Baicalin induces CA46 Burkitt lymphoma cell apoptosis through inhibiting the PI3K/Akt kinase activity (40). Baicalin

induces apoptosis in SW620 colorectal cancer cells *in vitro* and anticancer activity in HCT-116 cells *in vivo* (41,42), and Zheng *et al* demonstrated that baicalin induces apoptosis in leukemia HL-60/ADR cells through inhibiting the PI3K/Akt kinase (43). Our previous study demonstrated that baicalin induced apoptosis in leukemia HL-60 cells through ER stress and mitochondrial-dependent pathways (44). Recently, Zhang *et al* pointed out that baicalin induces autophagy in human hepatocellular carcinoma SMMC-7721 cells (45); however, there is no evidence to show the effects of baicalin on the induction of autophagy in human bladder cancer T24 cells. In the present study, we investigated the pharmacological effects of the baicalin on inhibition of cell growth and induction of cell autophagy in T24 cells. Our results indicated that baicalin might contribute to cell autophagy via the Akt pathway in T24 cells.

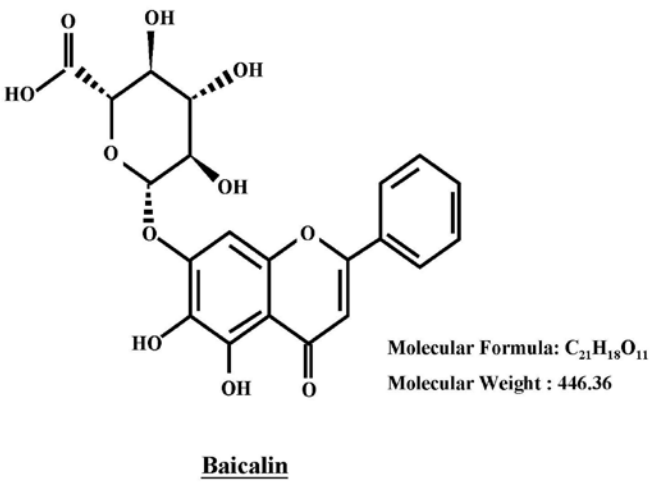

Figure 1. The chemical structure of baicalin.

Materials and methods

Chemicals and reagents. Acridine orange (AO), Baicalin, 3-methyladenine (3-MA), monodansylcadaverine (MDC) and tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). AKT kinase assay kit was obtained from Cell Signaling Technology (Danvers, MA, USA). Tdt-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay kit was purchased from Roche Diagnostics (GmBH, Mannheim, Germany). Caspase-3 activity assay kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA). The primary antibodies against Atg 5, Atg 7 and Atg 12, Beclin, LC3-II, AKT and phospho-AKT (Ser473) were purchased from Cell Signaling Technology. Antibody against β-actin was obtained from Sigma Chemical Co. All peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) detection kit was obtained from Pierce Chemical (Rockford, IL, USA).

Cell culture. The T24 human bladder cancer cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were grown in McCoy's 5a medium fortified with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin and incubated at 37˚C under a humidified 5% CO_2 atmosphere (46).

Cell viability and morphology. The cell viability was assessed by the MTT assay. Briefly, the T24 cells were cultured in a 96-well plate at the density of $1x10^4$ cells/per well and were incubated with 0, 50, 100, 150 and 200 μM of baicalin for 24 h. For the study of autophagy inhibition, cells were pre-treated with 3-MA (10 mM) for 1 h before the 24-h treatment of baicalin with indicated concentrations. At the end of baicalin treatment, culture medium containing MTT (0.5 mg/ml) was added to each well after washing the cells. The cells were then incubated at 37˚C for 4 h and the supernatant was removed. The formed blue formazan crystals in viable T24 cells were dissolved with isopropanol/0.04 N HCl, followed by measurement of the absorbance of each well at 570 nm with the ELISA reader with a reference wavelength of 620 nm. All experiments were performed in triplicate. The cell viability of each treatment was expressed as percentage of the control. The morphological examination of autophagic vacuoles in baicalin-treated cells was determined under a phase-contrast microscope (18).

Trypan blue exclusion assay for cell death. Trypan blue exclusion assay was used to evaluate cell death induced by baicalin treatment. T24 cells in a 24-well plate ($2.5x10^5$ cells/per well) were incubated with 0, 50, 100, 150 and 200 μM of baicalin. After 24 h, cells were stained with 0.25% trypan blue solution and the numbers of dead cells were determined by Countess Automated Cell Counter (Invitrogen/Life Technologies) (18).

TUNEL staining. TUNEL staining was performed to detect apoptotic cells according to the manufacturer's protocol (*in situ* cell death detection kit; Roche Diagnostics). T24 cells in a 24-well plate ($2.5x10^5$ cells/per well) were exposed to 0, 50, 100, 150 and 200 μM of baicalin for 24 h. At the end of the incubation, cells were collected, fixed with 70% ethanol and washed twice with ice-cold PSB. After incubated in the dark for 30 min at 37˚C in 100 μl of TdT-containing solution, the T24 samples were washed once before flow cytometry analysis of the TUNEL-positive cells using a FACSCalibur (Becton-Dickinson). The median fluorescence intensity was quantified by CellQuest software (18).

Caspase-3 activity assays. The caspase-3 activity assay was performed according to the manufacturer's instructions (Caspase Colorimetric Kit; R&D Systems Inc.). Briefly, after a 24-h incubation with 0, 50, 100, 150 and 200 μM of baicalin, T24 cells (~$1x10^7$/75-T flask) were harvested. The collected cells were then lysed in the lysis buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT], followed by centrifugation to collect total proteins in the supernatant. The cell lysate containing 50 μg proteins were then incubated for 1 h at 37˚C with caspase-3 specific substrate (Ac-DEVD-pNA) in the reaction buffer. The caspase-3 activity was determined by measuring O_{D405} of the released pNA (18).

Detection of acidic vesicular organelles (AVO) with acridine orange (AO) and acidic autophagic vacuoles with monodansylcadaverine (MDC). T24 cells were seeded on sterile coverslips in tissue culture plates with a density of $5x10^4$ cells/per coverslip. After 0 or 200 μM of baicalin treatment for 24 h, cells were stained with either acridine orange (AO) or 0.1 mM monodansylcadaverine (MDC) at 37˚C for 10 min. After three washes with PBS, cells were immediately visualized by fluorescence microscopy (Nikon, Melville, NY, USA) for the detection of acidic vesicular organelles and MDC-positive autophagic vacuoles (18,47).

Western blot analysis. T24 cells ($1x10^7$/75-T flask) were treated with 0, 50, 100, 150 and 200 μM of baicalin for 24 h, then harvested, lysed and the total proteins were collected by SDS sample buffer. In brief, ~30 μg of protein from each treatment was resolved on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a nitrocellulose membrane. The transferred membranes were blocked in 5% non-fat dry milk in 20 mM Tris-buffered saline/0.05% Tween-20 for 1 h at room temperature followed by incubation with primary antibodies against indicated autophagic-associated proteins or AKT and autophagy pathway-related proteins at 4˚C overnight. At the end of incubation, membranes were washed with Tris-buffered saline/Tween-20 and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). The blots were developed by a chemiluminescence kit (Millipore, Bedford, MA, USA), followed by X-ray film exposure. Each membrane was stripped and reprobed with anti-β-actin antibody to ensure equal protein loading during the experiment (18).

In vitro AKT kinase assay. In brief, T24 cells ($1x10^7$/75-T flask) were treated with 0, 50, 100, 150 and 200 μM of baicalin for 6 h. Cells were lysed in ice-cold lysis buffer provided by the kit. The 200 μg of protein from each time-point of treatment was immuno-precipitated with 2 μg of anti-AKT antibody overnight. Immuno-precipitates were extensively washed and then incubated with 1 μg of GSK-3 α/β fusion protein substrate in 50 μl of kinase buffer for 30 min at 30˚C. Reactions were stopped by SDS loading buffer and samples were separated on 12% SDS-PAGE. The phospho-GSK-3 α/β (Ser219) was detected by immunoblotting (18).

Statistical analysis. All the statistical results are presented as the mean ± SEM for the indicated numbers of separate experiments. Statistical analyses of data were done using one-way ANOVA followed by Student's t-test and $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ were considered statistically significant (18).

Results

Baicalin decreased the viability of T24 human bladder cancer cells. The human bladder cancer cells T24 were treated with baicalin (0, 50, 100, 150 and 200 μM) for 24 h. Results from the MTT assay showed that even though 50 μM of baicalin did not reduce cell viability, increased concentrations of baicalin treatment (100, 150 and 200 μM) significantly led to decrease of cell viability in T24 cells in a concentration-dependent manner (Fig. 2A). Fig. 2B showed that baicalin increased the number of cell death at 100, 150 and 200 μM in a concentration-dependent manner using the trypan blue exclusion assay.

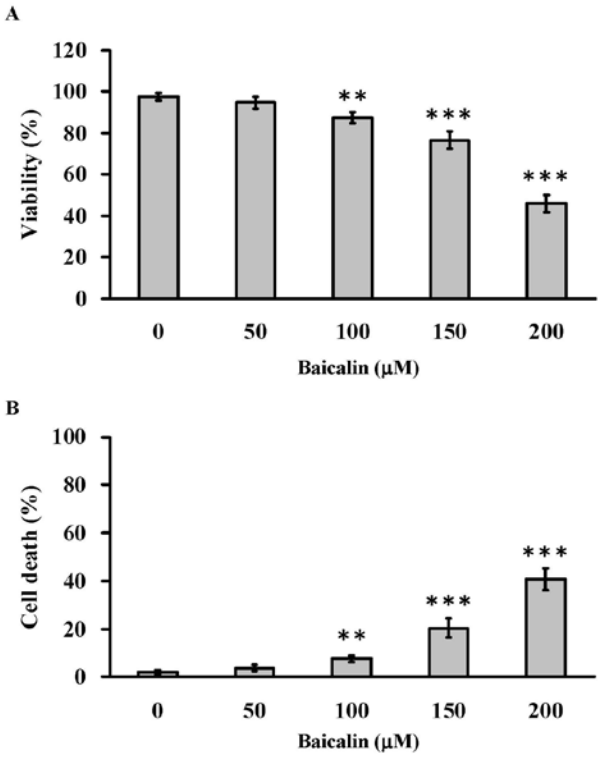

Figure 2. Effects of baicalin on cell viability and cell death in human bladder cancer T24 cells. (A) T24 cells were treated with various concentrations of baicalin (0, 50, 100, 150 and 200 μM) for 24 h. The viability of cells was determined by MTT assay. (B) The percentage of cell death was determined by the trypan blue exclusion assay. Data are expressed as the mean ± SEM of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$, significant compared with the control (0 μM) group.

Baicalin induces caspase-independent cell death in T24 cells. To verify whether baicalin induced apoptosis in T24 cells, cells were treated with baicalin (0, 50, 100, 150 and 200 μM) for 24 h before subjected to TUNEL staining. Fig. 3A indicated that the percentages of TUNEL-positive cells in baicalin-treated groups were <5%. In addition, to further examine whether the cell death caused by baicalin treatment was mediated through caspase-3 activation, protein samples collected from T24 cells after baicalin (0, 50, 100, 150 and 200 μM) treatment were analyzed for caspase-3 activity. The caspase-3 activity assay showed no changes in baicalin-treated cells regardless of the baicalin concentrations (Fig. 3B). Our results demonstrated that apoptosis and the activation of caspase-3 were not involved in baicalin-induced cell death.

Baicalin induces cell autophagy in T24 cells. We further investigated whether the cell death caused by baicalin treatment was mediated by autophagy. T24 cells were treated with baicalin (0, 50, 100, 150 and 200 μM) for 24 h and the formation of autophagic vacuoles was examined under a phase contrast microscope. As shown in Fig. 4A, 100 and 200 μM of baicalin treatment induced the formation of autophagic vacuoles, while baicalin at control and 50 μM induced hardly

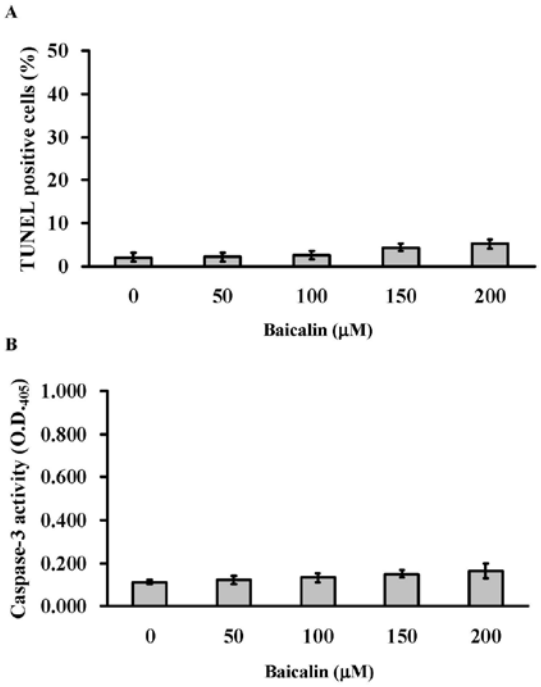

Figure 3. Effects of baicalin on cell apoptosis and caspase-3 activity in human bladder cancer T24 cells. (A) T24 cells were treated with various concentrations of baicalin (0, 50, 100, 150 and 200 μM) for 24 h. TUNEL staining was applied to determine the percentage of apoptotic cells (B) Analysis of caspase-3 activities in baicalin-treated T24 cells. After 24-h exposure to different doses of baicalin, the T24 cell lysate was pre-treated with z-DEVE-fmk (the specific inhibitor of caspase-3) for 1 h followed by measuring the caspase-3 activities. The OD_{405} of the released pNA was measured as the indication of caspase-3 activities. Data are presented as the mean ± SEM from three independent experiments.

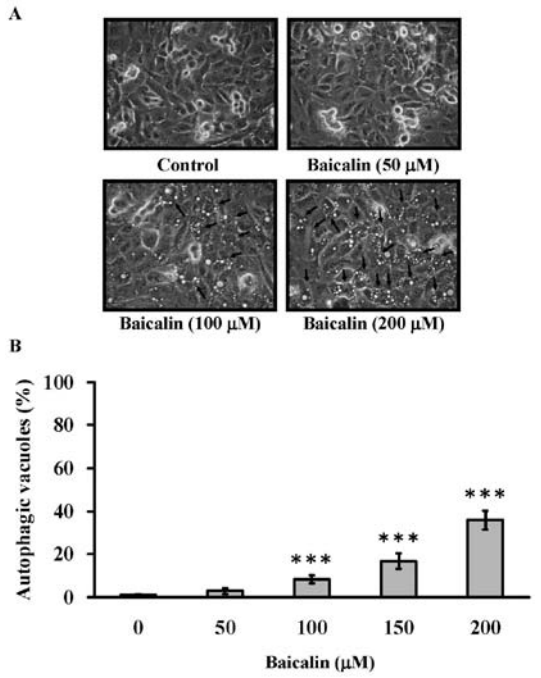

Figure 4. Baicalin induces cell autophagy in human bladder cancer T24 cells. (A) T24 cells were treated with baicalin (0, 50, 100 and 200 μM) for 24 h and were examined for the formation of autophagic vacuoles by a phase contrast microscope (magnification, x400). (B) The percentage of autophagic vacuoles from each treatment were quantified and presented as the mean ± SEM. Data were collected from three independent experiments. ***p<0.001, significant compared with the control (0 μM) group.

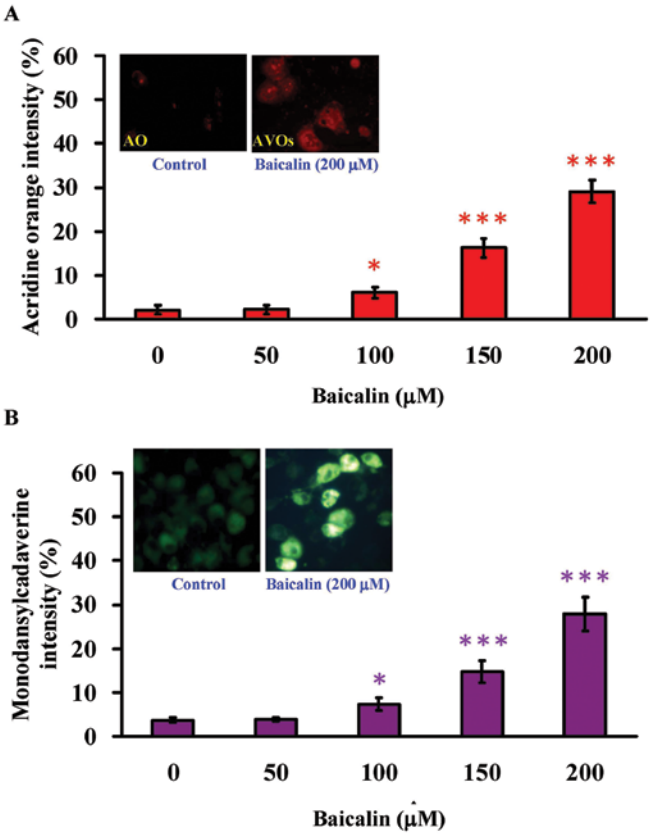

Figure 5. Baicalin induces autophagy markers in human bladder cancer T24 cells. (A) After treatment with various concentrations of baicalin (0, 50, 100, 150 and 200 μM) for 24 h, the cytosolic acidic vesicular organelles (AVOs) were stained by acridine orange (AO) staining. The representative images of AO staining in the presence and absence of baicalin (200 μM) in the upper panel; and quantitative data of AO intensity are shown. (B) Autophagic vacuoles in baicalin-treated T24 cells were stained by fluorescent probe monodansylcadaverine (MDC) in the upper panel and MDC intensities at each treatment are shown. *p<0.05, ***p<0.001, significant compared with the control (0 μM) group.

any formation of autophagic vacuoles. In addition, the amount of autophagic vacuole formation was significantly elevated in a concentration-dependent manner in higher baicalin concentration groups (≥100 μM) (Fig. 4B). Especially, upon the challenge of 200 μM baicalin for 24 h, ~40% of cells manifested autophagic vacuoles.

One of the hallmarks of autophagic cell death is the cytosolic acidic vesicular organelles (AVO) (48). Through the staining of acridine orange (AO), a lysotropic dye that emits bright red fluorescence inside the low pH acidic vesicles, AVO were noticeably observed in the cytoplasm of baicalin-treated T24 cells (200 μM of baicalin) when compared to the control group by the fluorescence microscopy. In addition, as the concentration of baicalin increased, the measured AO intensity became stronger (Fig. 5A). Furthermore, we confirmed the autophagic cell death caused by baicalin treatment using monodansylcadaverine (MDC) staining. MDC is another widely used fluorescent marker that preferentially accumulates in autophagic vacuoles. As shown in Fig. 5B, T24 cells treated 200 μM of baicalin for 24 h clearly showed autophagic vacuoles, while very few autophagic vacuoles were observed in the control group. Again, the MDC intensity increased as

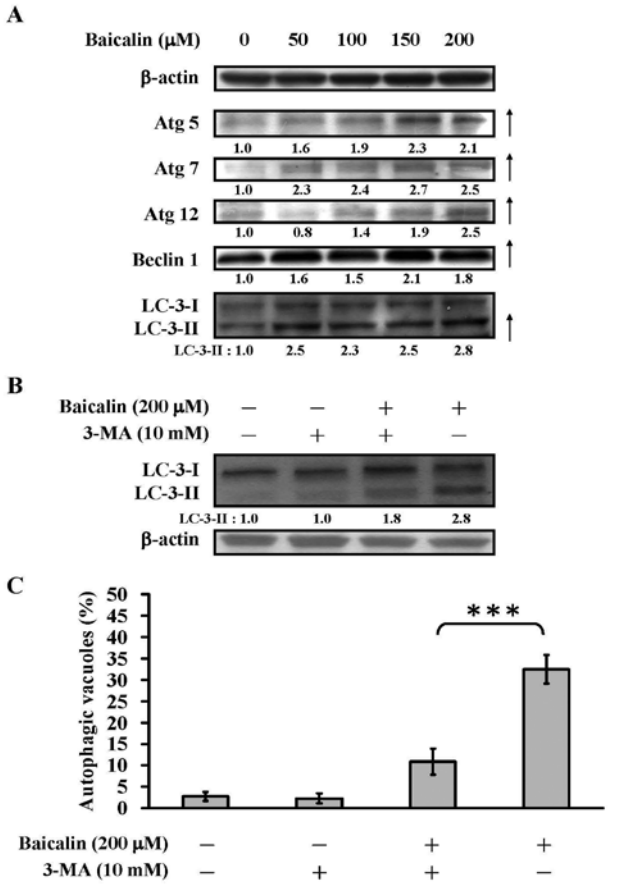

Figure 6. Baicalin increased the expression of autophage-associated proteins in human bladder cancer T24 cells. (A) T24 cells were treated with different concentrations (0, 50, 100, 150 and 200 μM) of baicalin as indicated for 24 h. The protein levels of Atg 5, Atg 7 and Atg 12, Beclin 1 and LC-3 were examined by western blot analysis. The numbers below each line of the protein bands indicate increasing folds of that protein level when compared to the control group (defined as 1.0). The protein level of β-actin was used as the internal standard. (B) Prior to baicalin treatment (200 μM), cells were pre-treated with/without 3-MA (10 mM) followed by western blot analysis of protein level of LC-3. (C) The percentage of autophagic vacuoles was measured after the exposure of T24 cells to baicalin and 3-MA interventions as indicated. Data are presented as the mean ± SEM from three independent experiments. ***p<0.001, significant compared with the control (0 μM) group.

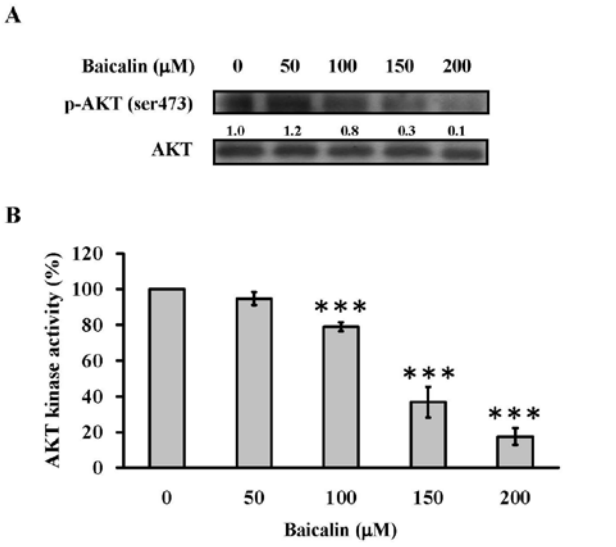

Figure 7. Baicalin inhibits AKT protein level and kinase activity in T24 human bladder cancer cells. (A) T24 cells were treated with different concentrations (0, 50, 100, 150 and 200 μM) of baicalin as indicated for 12 h. The protein levels of AKT and phosphor-AKT (ser 473) were examined by western blot analysis. (B) AKT kinase activity was measured by an *in vitro* kinase activity assay. Data are presented as the mean ± SEM from three independent experiments. ***p<0.001, significant compared with the control (0 μM) group.

the baicalin concentration increased. Our results indicated that autophagy was the mechanism underlying baicalin-induced cell death.

Baicalin regulates the autophagy-associated protein levels in T24 cells. It has been shown that the autophagic cell death is associated with the elevations of autophagosome formation protein levels. Those proteins includes light-chain-3 (LC-3), Atg complex (Atg 5, Atg 7 and Atg 12) and Beclin-1 (18,49). As examined by western blot analysis, baicalin increased the protein expression of Atg 5, Atg 7 and Atg 12, Beclin-1 and LC-3 II (Fig. 6A). For example, when compared with the control group, the respective protein levels of Atg 5, Atg 7 and Atg 12, Beclin-1 and LC-3 II were 2.1-, 2.5-, 2.5-, 1.8- and 2.8-fold higher after treated with 200 μM of baicalin for 24 h.

Among the afore-mentioned proteins, the microtubule-associated protein light-chain 3 (LC-3) is a reliable autophagic membrane marker for the detection of early autophagosome formation (50,51). The conversion of LC-3I to LC-3II is indicative of autophagic activity. We next examined whether 3-methyladenine (3-MA), a commonly used reagent that inhibits autophagy by blocking autophagosome formation via the inhibition of type III phosphatidylinositol 3-kinases (PI-3K), could attenuate the elevated LC-3 II expression induced by baicalin. As shown in Fig. 6B, 200 μM of baicalin treatment upregulated the LC-3 II protein levels to 2.8-fold when compared to the control group. Nevertheless, 3-MA pretreatment (10 mM) decreased the LC-3II expression level to 1.8-fold in the presence of 200 μM of baicalin. The quantitative data from the numbers of autophagic vacuoles also indicated that the baicalin-induced autophagic vacuoles formation was sharply diminished upon 3-MA pretreatment (Fig. 6C). The experimental results (Fig. 6) indicated that baicalin induced autophagic cell death through upregulation of proteins associated with autophagosome formation and it could be attenuated by 3-MA.

Baicalin blocks the AKT signaling in T24 cells. The AKT activity has been demonstrated to contribute to autophagic cell death (6,40). We next performed western blot analysis and AKT kinase activity assay to investigate whether the AKT signaling was involved in the baicalin-induced autophagic cell death in T24 cells. The present study showed that baicalin decreased the phosphor-AKT (Ser473) protein levels in T24 cells in a concentration-dependent manner (Fig. 7A). In addition, baicalin inhibited AKT kinase activity and the inhibition was concentration-dependent (Fig. 7B). Our data implied that baicalin induced cell autophagy in T24 cells through blocking the AKT signaling.

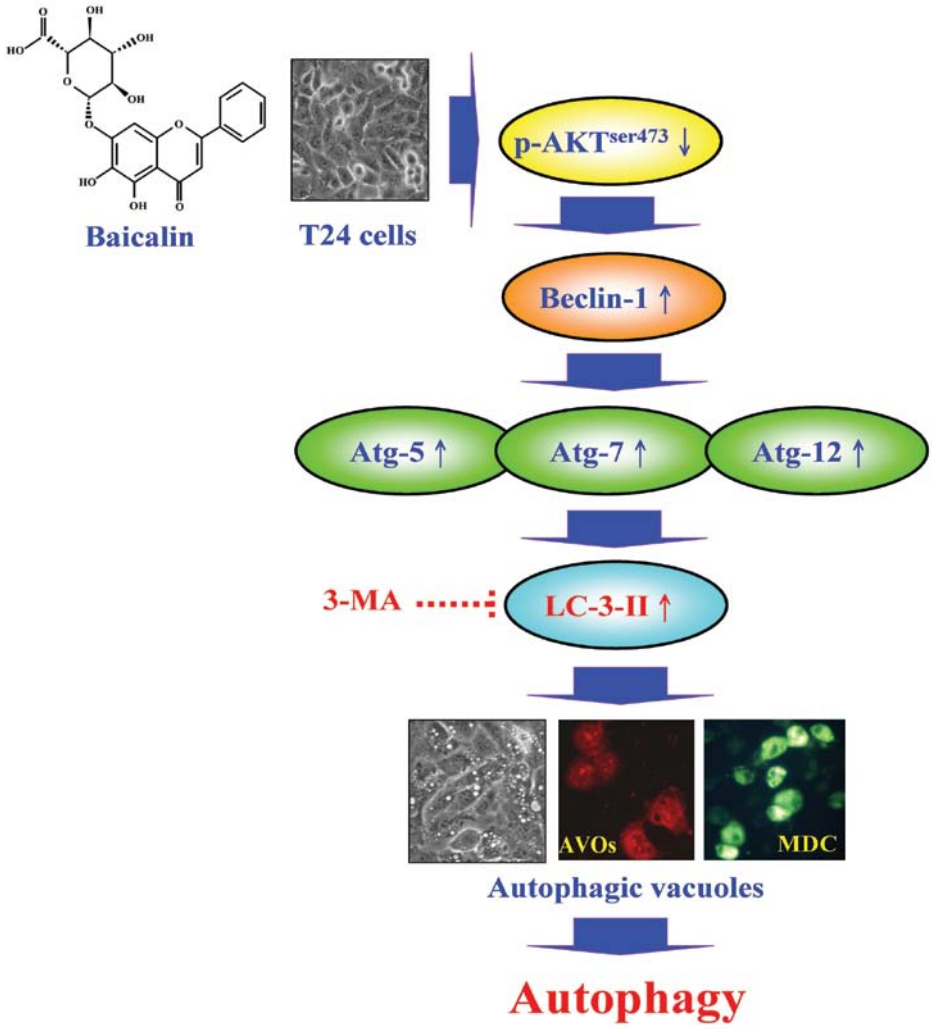

Figure 8. The proposed model of baicalin induced cell autophagy in T24 human bladder cancer cells.

Discussion

Previous studies have showed that *Scutellaria baicalensis* Gerogi containing over 30 different kinds of flavonoids (24,52,53), including baicalin, baicalein, oroxylin A and wogonin (53-55). It was reported that ethanol extracts of *Scutellaria baicalensis* Gerogi prevent oxidative damage (56,57) and has anti-inflammation (58,59) and anti-angiogenesis effects (60). In addition, *Scutellaria baicalensis* Gerogi extract triggers G_2/M arrest and caspase-dependent apoptosis by modulating ERK pathway in HSC-T6 cells (58). In the present study, we focused on the baicalin from *Scutellaria baicalensis* Gerogi for their anticancer effect on human bladder cancer T24 cells. Baicalin is a natural flavonoids compound with anticancer activity and low toxicity against normal cells (61,62). Previous reports showed that baicalin exerted anti-proliferative ability and induced apoptotic effects in many cancer cell lines (CA46, SW620, HCT-116, HL-60/ADR and HL-60) (40,41,43,44,63,64). In this study, we investigated the anticancer effects of baicalin on T24 human bladder cancer cells *in vitro*. Our results showed that baicalin exerted a significant anti-proliferative effect on T24 cells (Fig. 2A). Baicalin is a new anticancer agent and has apoptotic effect on T24 cells. However, the apoptotic TUNEL-positive cells and caspase-3 activity did not change in baicalin-treated T24 cells (Fig. 3). Data suggested that there may be another mechanism involved in baicalin-induced cell death in T24 cells.

Many studies have suggested the autophagy has a cancer suppressor role (65). Several traditional Chinese medicines such as arsenic trioxide (AS_2O_3) (66), berberine (67), bufalin (18) and kaempferol (68) have been demonstrated to induce autophagy and to exert anticancer activity in cancer cells. Intriguingly, baicalin-induced autophagy in T24 cells was demonstrated by autophagic vesicle formation (Fig. 4). Baicalin induced autophagy generation shown by larger bright-red AO-stained vacuoles (Fig. 5A) and induction of the LC3 cleavage (Fig. 6A). In contrast, protein levels of the LC3-II, Beclin-1, Atg 5, Atg 7 and Atg 12 were upregulated in T24 cells after baicalin treatment (Fig. 6A). When T24 cells were pre-treated with 3-MA followed by treatment with baicalin, LC3 protein cleavage (Fig. 6B) and autophagic vesicle formation (Fig. 6C) were significantly decreased compared with the baicalin alone treatment group. Our results demonstrated that baicalin-induced cell death possibly involved autophagy, and is the first detailed evidence that baicalin induced autophagy in T24 cells. Our

findings are in agreement with previous studies that baicalin induced autophagy in SMMC-7721 cells (45).

Akt serine/threonine kinase [also called protein kinase B (PKB)] is one of the most regularly activated protein kinases in human bladder cancer (69-71). Activation of Akt is associated with anti-apoptosis, cell proliferation and cellular energy metabolism (72). The AKT pathway is frequently activated in human bladder cancer cells. Askham *et al* demonstrated that the AKT1 G49A (E17K) mutation led to constitutive AKT1 activation and was found in 4.8% bladder cancer cell lines and 2.7% bladder tumors (73). Regulating the Akt pathway is potentially essential for developing therapeutic inhibitors in human bladder cancer. Dickstein *et al* demonstrated that the AKT inhibitor AZ7328 has synergistic effect on inducing apoptosis with autophagy inhibitors in human bladder cancer cells (74). Wu *et al* demonstrated that PI-3 kinase inhibitor LY294002 inhibits cell proliferation and sensitizes doxorubicin in human bladder cancer cells (75). Our study demonstrated baicalin induced autophagy accompanied with downregulation of phospho-AKT (Ser473) protein level (Fig. 7A) and Akt kinase activity (Fig. 7B). Previous studies demonstrated that baicalin induced apoptotic cell death through inhibiting the AKT signaling pathway in CA46 Burkitt lymphoma and leukemia HL-60/ADR cells (40,76). In the present study, the result showed that the AKT pathway is associated with the induction of autophagy in baicalin-treated T24 cells.

The molecular mechanisms underlying the inhibitory effect of baicalin on T24 cell proliferation are summarized in Fig. 8. In conclusion, baicalin induces autophagy through the Akt signaling pathway in T24 human bladder cancer cells. Our findings imply that baicalin may be used as a novel anticancer drug candidate for the treatment of human bladder cancer.

Acknowledgements

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