Baicalaína provoca autofagia e não apoptose em células de várias neoplasias humanas

**Baicalein induces autophagic cell death through AMPK/ULK1 activation and downregulation of mTORC1 complex components in human cancer cells**

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**Abstract**

Baicalein, a flavonoid and aglycon hydrolyzed from baicalin, has anticancer properties in several human carcinomas, but its molecular mechanisms of action remain unclear. Here, we show that baicalein leads to human cancer cell death by inducing autophagy rather than apoptosis, because cell death induced by baicalein was completely reversed by suppressing the expression levels of key molecules in autophagy such as Beclin 1, vacuolar protein sorting 34 (Vps34), autophagy-related (Atg)5 and Atg7, but not by pan-caspase inhibitor. Our data revealed that baicalein significantly increased the number of green fluorescence protein–cytosol-associated protein light chain 3 (GFP–LC3)–containing puncta and LC3B–II expression levels, which were further enhanced by chloroquine treatment. Furthermore, a luciferase-based reporter assay showed that the ratio of RLuc–LC3wt/RLuc–LC3G120A was greatly reduced. The data suggested that baicalein induced not only autophagosome formation, but also autophagic flux. Experiments using short interfering RNAs and pharmacological inhibitors revealed that Beclin 1, Vps34, Atg5, Atg7 and UNC-51 (*Caenorhabditis elegans*)-like kinase 1 (ULK1) play pivotal roles in mediating baicalein-induced autophagy. Moreover, baicalein activated AMP-activated protein kinase (AMPK)α, leading to ULK1 activation through phosphorylation at Ser555, whereas both protein and mRNA levels of mammalian target of rapamycin (mTOR) and Raptor, upstream inhibitors of ULK1 and autophagy, were markedly downregulated by baicalein. Our data suggest that the anticancer effects of baicalein are mainly due to autophagic cell death through activation of the AMPK/ULK1 pathway and inhibition of mTOR/Raptor complex 1 expression. These results provide new mechanistic insights into the anticancer functions of autophagy inducers, such as baicalein, which may be used as potential therapeutics for cancer treatment.

**Introduction**

Autophagy is a highly conserved evolutionary process in which portions of the cytosol and organelles are sequestered into a double-membrane vesicle, the autophagosome, and delivered to the lysosome for breakdown and eventual recycling of the resulting
macromolecules to maintain homeostasis [1-3]. The formation of autophagosomes is initiated by class III phosphoinositide 3-kinase (PtdIns3K), vacuolar protein sorting 34 (Vps34) and Beclin 1, and the initiation is controlled by the UNC-51 (C. elegans)-like kinase 1 complex (ULK1; human homologue of ATG1), including autophagy-related 13 (Atg13) and focal adhesion kinase family interacting protein of 200 kDa (FIP200; Atg17), which acts downstream of the mammalian target of rapamycin (mTOR) [4, 5]. mTOR is a central negative regulator of autophagy and the regulatory-associated protein of mTOR (Raptor)-containing mTORC1 complex is known to interrupt autophagosome formation [4]. Formation of PtdIns3P by the Beclin 1/Vps34 complex is essential for the recruitment of other Atg proteins, which are major building blocks of autophagosomes [5]. The initiation phase proceeds with formation of the Atg5–Atg12–Atg16 complex, which promotes the recruitment and conversion of cytosol-associated protein light chain kinase (LC3-I) to a phosphatidylethanolamine-conjugated form (LC3-II); this is then incorporated into the membrane through Atg7- and Atg3-dependent activation. The Atg proteins are recycled to the cytosol after the completion of autophagosome formation, whereas LC3-II remains in mature autophagosomes until fusion with lysosomes is completed. Therefore, LC3-II is commonly used to monitor autophagy.

Autophagy is considered to be a double-edged sword in cancer development, progression and treatment response, because it can protect cancer cells from metabolic stress through lysosomal degradation and recycling of proteins and organelles, while also acting as an alternative route of programmed cell death [2] by sharing common components, stimuli and signaling pathways with apoptosis. Appropriate modification of autophagy, such as inhibiting cytoprotective autophagy or promoting autophagic cell death, is considered important for the development of cancer therapeutic strategies.

Baicalein (3,4,5-trihydroxyfurane), a flavonoid with well-established anti-inflammatory and antioxidant properties, is extracted from the root of Scutellaria baicalensis [6-11]. Furthermore, baicalein has an inhibitory effect on several cancers including colorectal, lung, breast and prostate carcinomas. The antitumor activity of baicalein is mediated by modulating cyclin-dependent kinases (cdk) to induce cell-cycle arrest, and by inducing cytochrome c release from mitochondria and activation of caspase 3, caspase 7 and poly(ADP-ribose) polymerase (PARP) through the downregulation of myeloid leukemia 1 (Mcl-1). However, whether or not the anticancer properties of baicalein are mediated by autophagy has not been investigated.

Here, we report a new mechanism by which baicalein suppresses the growth of human cancer cells, providing evidence that baicalein induces autophagic cell death in human breast and prostate cancer cells through AMP-activated protein kinase (AMPK)–ULK1 activation by modulating the expression level of anti-autophagic molecules including mTOR and Raptor.

Results

**Baicalein reduces mitochondrial membrane potential and induces cell-cycle arrest leading to human prostate and breast cancer cell death**

Baicalein is one of main flavonoids extracted from the root of *S. baicalensis* and is an aglycon, which is hydrolyzed from baicalin by intestinal microflora and has greater
potency with less toxicity in inhibiting inflammation, hypertension, hepatitis and allergies, as well as cancer. There is accumulating evidence demonstrating the antitumor activities of baicalein, but its modes of action are mainly attributed to apoptosis and cell-cycle arrest, and remain unclear.

In order to investigate the anticancer mode of action of baicalein, we first tested its biological effects by measuring cell proliferation and cell-cycle distribution in human prostate (PC-3 and DU145) and breast (MDA-MB-231) cancer cells. Cells were cultured in the absence or presence of baicalein, and attached cells were stained with crystal violet after 4 days. As shown in Fig. 1A, treatment with 20 μg·mL$^{-1}$ baicalein inhibited the growth of > 90% of PC-3 and MDA-MB-231 cells, and even the lowest dose of baicalein tested (2.5 μg·mL$^{-1}$) induced cell death in > 60% of cells (data not shown). DU145 cells were more sensitive to baicalein and their proliferation was almost completely inhibited at a dose of 2.5 μg·mL$^{-1}$. We also observed similar results in H460 human lung cancer cells, HepG2 hepatoma cells and MiaPaCa pancreatic cancer cells (data not shown). The sub-G$_0$ cell population increased ~10-fold, from 2.98% in the control to 35% after 24 h treatment with baicalein, suggesting that baicalein-mediated growth suppression in PC-3 cells may be due to the induction of cell-cycle arrest and apoptosis (Fig. 1B). We confirmed these results by measuring the expression levels of cell-cycle regulatory proteins. Cell-cycle regulators, such as cell division cycle protein 2, cdk2, cdk4 and cyclin D1, were significantly downregulated, as reflected by the phosphorylation status of the retinoblastoma protein (pRb). Furthermore, there was a slight increase in p21 levels (Fig. 1C). Taken together, our data suggest that baicalein might suppress the growth of different human cancer cells by inducing cell-cycle arrest, indicating its chemotherapeutic potential against human cancers.
Baicalein induces a collapse in mitochondrial membrane potential and cell-cycle arrest. (A) Human cancer cell lines were treated with baicalein at the indicated concentrations for up to 4 days. After 4 days, cells were stained with crystal violet to evaluate the cell viability. (B, C) PC-3 cells were cultured for 24 h in the presence or absence of 10 μg·mL⁻¹ baicalein and DNA content was analyzed by using propidium iodide.

Figure 1.

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staining and fluorescence-activated cell sorting. The percentage of cells with a hypodiploid DNA content (sub-G1) and cells in G1, G2/M and S phases were indicated and plotted. Separately, cell lysates were analyzed by western blot. (D) PC-3 cells were treated with baicalein (5 μg·mL−1 for 6–24 h) or carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 25 μm for 1 h) and mitochondrial membrane potential was determined by using JC-1 staining and flow cytometry. Data shown are the average of triplicate determinations ± SD and are representative of two or three independent experiments (A–D).

Loss of mitochondrial membrane potential (Δψmt) is an indicator for the initiation of cellular apoptosis or oxidative/metabolic stress. Because our data indicated an antiproliferative effect of baicalein in cancer cells, we determined whether baicalein treatment induced changes in Δψmt. PC-3 cells cultured in the absence or presence of 5 μg·mL−1 baicalein for 24 h were stained with JC-1 reagent, which emits an orange–red fluorescence when mitochondria are intact. As shown in Fig. 1D, left, baicalein triggered an increase in the mitochondrial depolarization in PC-3 cells, leading to a dramatic reduction in JC-1 J-aggregate formation and increased green monomer fluorescence indicative of cellular stress. The proton gradient uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone was used as a positive control to dissipate the electrochemical potential across the inner mitochondrial membrane. PC-3 cells stained with JC-1 dye following treatment with 5 μg·mL−1 baicalein for the indicated time were analyzed by flow cytometry as well. Healthy cells initially detected in the R2 region accumulated in the R3 region (which contained apoptotic cells or cells depolarized under stress) in a time-dependent manner. Our data showed an increase in the R3 cell population from 7.77% in control cells to 36.38 and 92.38%, 6 and 18 h after baicalein treatment, respectively (Fig. 1D, right). This increase corresponded to a loss of orange–red fluorescence and an increased number of green fluorescent cells, as determined by fluorescence microscopy.

**Baicalein may induce prostate cancer cell death through a caspase-independent/alternative mechanism**

In order to test whether the loss of Δψmt (Fig. 1D) leads to cell apoptosis, we detected the levels of anti- or proapoptotic proteins, cleavage of caspase 3 and PARP following baicalein treatment in PC-3 cells. As shown in Fig. 2A, baicalein treatment for 24 h increased PARP cleavage and the active form of caspase 3, although to a lesser extent. Bcl-2 and Bcl-xL protein levels remained unchanged, whereas Bim, a proapoptotic BH3-only protein known to antagonize anti-apoptotic members of the Bcl-2 family, was noticeably induced. However, caspase 3 activation was not as significant as expected, and we were only able to detect cleavage when the film was exposed for > 20 min. A time-course experiment with baicalein revealed that it took 12 h for baicalein to show even trace amounts of active caspase 3 (Fig. 2A, far left, and Fig. 2B). Moreover, PARP cleavage by baicalein was not even blocked by Z-VAD-FMK, a pan-caspase inhibitor (Fig. 2A, right). Because apoptosis inducers, such as transforming growth factor β1 and dithiothreitol have been reported to induce PARP cleavage in a caspase-independent manner [12], we concluded that that baicalein-induced PARP cleavage might not be associated with caspase activation. We further investigated the effect of baicalein on caspase 3 activation by comparing various apoptotic reagents such as doxorubicin (2 or 5 μm), staurosporin (25 or 50 nm), cisplatin (20 or 40 μm) and etoposide (5 μm) (Fig. 2C). Even though baicalein (2 or 5 μg·mL−1 equivalent to 9.3 or 18.6 μm)
increased the cleaved form of caspase 3, it seems weaker than the other drugs tested (Fig. 2C). Moreover, cleavage of caspase 3 induced by doxorubicin, cisplatin or etoposide was completely blocked by Z-VAD-FMK, whereas baicalein-mediated caspase 3 cleavage remained intact, although to a lesser extent. We further measured caspase activity using fluorescent-labeled inhibitor of caspase (FLICA) probes, which covalently bind active caspases, after treatment with 5 or 10 μg·mL\(^{-1}\) of baicalein for 24 h, or 40 μm of cisplatin as a positive control in PC-3 cells. Caspase-negative cells occurred in the lower log fluorescence output decades (labeled as M1) of the FL1H (X-axis), whereas caspase-positive cells appeared as a separate peak on the right of the histogram (labeled M2) in Fig. 2D. Cisplatin, a positive control, significantly increased the activity of caspases from 4.2% in untreated cells to 81.8% in cisplatin-treated cells, and such induction was substantially suppressed to 29.6% by Z-VAD-FMK. By contrast, caspase activity by baicalein only increased to 5.3 and 6.3% at doses of 5 and 10 μg·mL\(^{-1}\), respectively, compared with 4.2% in control cells treated with vehicle alone (Fig. 2D), even though we were able to observe significant cell death (Figs 1A,B and 2E). Furthermore, Z-VAD-FMK treatment was able to block cell death induced by etoposide, but failed to reverse growth inhibition mediated by baicalein (Fig. 2E). Cells stimulated by baicalein were stained with PtdIns and FLICA, and analyzed by flow cytometry; no significant cell death was observed at 6 h of baicalein addition. However, 51.15% of cells were PtdIns-positive (upper left) and 6.96% were PtdIns- and FLICA-positive (upper right) after 24 h of stimulation with baicalein, showing 70% cell death at 24 h (Fig. 2F). We usually observed cell death by baicalein at least 12 h after treatment (data not shown). In addition, baicalein-mediated inhibition of cell growth was not counteracted by Z-VAD-FMK even after 48 or 72 h (data not shown). The data suggested that apoptosis did not seem to be the main mechanism of baicalein-mediated cell death, indicating that an alternative mechanism, such as autophagy, may be involved.
Figure 2.
Baicalein induces prostate cancer cell death through a caspase-independent mechanism. (A) PC-3 cells were treated with 10 μg·mL⁻¹ of baicalein for 24 h in the absence or presence of 20 μm of Z-VAD-FMK. Thirty to 50 μg of protein was analyzed by western blotting to measure the protein level of poly(ADP-ribose) polymerase (PARP), cleaved caspase 3, Bcl-xL, B-cell lymphoma 2 (Bcl-2) and Bim. (B) Cells were incubated with 5 μg·mL⁻¹ baicalein for the indicated time and protein samples were subjected to western blot analysis to measure active form of caspase 3. (C) The levels of cleaved caspase 3 in PC-3 cells were compared after 24 h treatment with various cytotoxic reagents such as doxorubicin (2 or 5 μm), cisplatin (20 or 40 μm), Baicalein (2 or 5 μg·mL⁻¹; 9.3 or 18.6 μm), etoposide (5 μm) and staurosporine (25 or 50 nm) in the absence or presence of 20 μm Z-VAD-FMK. (D) Active caspses bound to FLICA were analyzed by flow cytometry. Cisplatin was used as a positive control for caspase activation. (E) PC-3 cells were incubated with baicalein (5 μg·mL⁻¹) or etoposide (5 μm) in the absence or presence of Z-VAD-FMK for 48 h and cell growth was measured by using crystal violet staining. (F) Separately, PC-3 cells were harvested 6 or 24 h after baicalein (5 μg·mL⁻¹) treatment, stained with both PtdIns and FLICA, and analyzed by flow cytometry. Results are representative of 2–3 independent experiments (A–F).

### Baicalein anticancer properties are mediated through the induction of autophagy in human cancer cells

Autophagy is a normal catabolic process to degrade dysfunctional proteins and damaged organelles and maintain cellular homeostasis. In order to test whether baicalein induced autophagic cell death in human prostate and breast cancer cells, we first examined the protein level of the lipidated form of LC3B (LC3B-II), a mammalian homologue of Atg8 localized in autophagosomes. Human PC-3 and DU145 prostate cancer cells were treated with different doses of baicalein ranging from 0.5 to 10 μg·mL⁻¹ for 24 h and LC3B-II protein expression was determined using western blot analysis. As shown in Fig. 3A, LC3B-II expression was slightly increased after treatment with 2.5 μg·mL⁻¹ baicalein and increased further in a dose-dependent manner in both cell lines. Moreover, the induction of LC3B-II expression was inversely correlated with the cyclin D3 expression level, indicating cell death (Fig. 3A). We also performed time-course experiments for baicalein treatment. The LC3B-II protein level was substantially upregulated as early as 9 h after addition of baicalein and further increased up to 24 h, whereas cyclin D2 and D3 levels decreased (Fig. 3B). Because an association of p62 with LC3 is known to facilitate the autophagy-mediated degradation of p62 associated with protein aggregates, the effect of baicalein treatment on the p62 protein level was measured; there were no significant changes in p62 levels observed in PC-3, DU145 and MDA-MB-231 cells (data not shown). Even though the inverse correlation between LC3B and p62 levels in autophagy is well established, whether it is a universal phenomenon remains controversial [13-15]. Moreover, silencing of p62 in PC-3 cells did not affect baicalein-induced autophagy, suggesting that it may be independent of p62 (data not shown). Taken together, our data indicated that baicalein could induce autophagy in both a dose- and time-dependent manner in human cancer cells.
Figure 3.
Baicalein induces autophagic flux as well as autophagosome formation in human cancer cells. (A–D) Cell lysates were prepared from cells treated with baicalein at doses ranging from 0.5 to 10 μg·mL⁻¹ for the indicated times. (C, D) Cells were incubated with 3-MA for 1 h prior to baicalein addition. Protein samples were analyzed by western blot to determine the expression levels of cytosol-associated protein light chain 3B-II (LC3B-II), cyclin D2 and D3. (E) Cells were transiently transfected with 2 μg of control vector or green fluorescent protein–cytosol-associated protein light chain 3 (GFP–LC3) followed by baicalein stimulation for 24 h. Samples were subjected to confocal microscopy. (F) Cells were transduced with BacMam LC3B–FP (wild type; wt) or BacMam LC3B–G120A–FP (mutant) and treated with baicalein or chloroquine (CQ). Cells were then washed, fixed and analyzed by confocal microscopy to determine LC3 puncta formation (E, F). (G) After 24 h of baicalein treatment, cells were stained with Cyto-ID Green Detection Reagent and analyzed by flow cytometry (the black and green lines represent control and baicalein-treated cells, respectively). (H) Cells were transiently transfected with 1 μg of RLuc–LC3-wt or RLuc–LC3-G120A-mutant and incubated with baicalein for 24 h. Samples were analyzed by using a luciferase assay and autophagic flux was determined by calculating the RLuc–LC3-wt/RLuc–LC3-G120A ratio (P < 0.0001 vs. control). (I) Cells were stimulated with the indicted doses baicalein for 24 h in the absence or presence of 50 μM CQ. CQ was added 1 h prior to Baicalein for CQ (pre). For CQ (1 h) or CQ (5 h), cells were incubated with baicalein and CQ was added 1 or 5 h before harvesting samples. Thirty to 50 μg of protein was analyzed to determine the level of LC3B-II expression. Data shown are the average of triplicate determinations ± SD and are representative of two or three independent experiments (A–I).

To confirm that baicalein induces autophagy, cells were incubated with the autophagy inhibitor 3-methyladenine (3-MA) followed by treatment with 5 μg·mL⁻¹ baicalein for 24 h. As shown in Fig. 3C, pretreatment of MDA-MB-231 with 10, 20 and 30 mm 3-MA for 1 h inhibited the baicalein-mediated induction of LC3B-II, and a similar effect was also observed in PC-3 cells (Fig. 3D), suggesting that baicalein regulates autophagy. Interestingly, the ability of 3-MA to block baicalein-induced autophagy was greater at 10 mm than at 20 and 30 mm (Fig. 3C), and this may be explained by the observation that higher doses of 3-MA trigger cell death and by a dual role of 3-MA in inhibiting autophagy and promoting autophagic flux reported [16].

Baicalein induces autophagic flux as well as the formation of autophagosomes

A glycine residue of LC3-I is conjugated with phosphatidylethanolamine to produce LC3-II, which is then localized to autophagosomes. Therefore, LC3-II can be visualized as punctate structures primarily representing autophagosomes, whereas LC3-I remains diffused in the cytosol. In order to further examine baicalein-induced autophagy, PC-3 cells transfected with green fluorescence protein (GFP)–LC3 were cultured in the absence or presence of 5 μg mL⁻¹ baicalein for 24 h and analyzed using confocal microscopy. The number of GFP–LC3-containing puncta was greatly increased in cells treated with baicalein, whereas GFP–LC3 in control cells was mainly cytosolic (Fig. 3E). We further investigated baicalein-induced autophagy using BacMam LC3B–
FP, a baculovirus expressing LC3B–fluorescent protein, to allow for high transduction efficiency. PC-3 cells were transduced with either BacMam LC3B–FP or BacMam LC3B–G120A–FP, followed by treatment with either 5 μg·mL⁻¹ baikalein or 50 μm chloroquine (CQ) for 24 h. BacMam LC3B–G120A–FP was used as a negative control for autophagic puncta formation because the G120A mutation protects LC3B from cleavage and subsequent phosphatidylethanolamine-lipidation during autophagy. CQ was used as a positive control because it can inhibit autophagy by blocking lysosomal fusion with autophagosomes, resulting in abrogated autophagic flux and accumulation of LC3-II puncta. As shown in Fig. 3F, cells transduced with LC3B–FP had a significant number of LC3B–FP puncta 24 h after baikalein addition, and similar puncta formation was observed in CQ-treated cells. By contrast, LC3B–G120A–FP did not accumulate in puncta in cells stimulated with baikalein and had a diffuse cytosolic distribution. Separately, cells were stained with Cyto-ID Green Detection Reagent and analyzed by flow cytometry after 24 h of baikalein treatment. Compared with control cells (black solid line), the histogram representing baikalein-treated cells was shifted to the right (Fig. 3G, green line), suggesting that baikalein induced autophagic vacuole formation in PC-3 cells.

GFP–LC3 puncta formation generally indicates the level of autophagic activity and represents either induced autophagosome generation or inhibition of autophagosome lysosomal fusion. Therefore, we tested the effect of baikalein on autophagic flux using a luciferase-based assay. Cells were transfected with either RLuc–LC3–wild type (wt) or RLuc–LC3–G120A (mutant), followed by baikalein treatment for 24 h, and the level of autophagic flux was calculated as the ratio of LC3–wt/LC3–G120A expression. Consistent with the data shown in Fig. 3G, baikalein treatment increased not only autophagosome generation, but also autophagic flux, as indicated by the decreased LC3–wt/LC3–G120A ratio (Fig. 3H). We confirmed this by incubating cells with CQ and analyzing the change in LC3B-II level. CQ was either pretreated 2 h prior to baikalein or cells were incubated in the presence or absence of baikalein followed by CQ for 1 or 5 h before harvested. As shown in Fig. 3J, baikalein-induced LC3B-II expression was further increased by 50 μm CQ in both PC-3 and MDA-MB-231 cells. Taken together, our data indicated that baikalein induced autophagic flux as well as the formation of autophagosomes.

**Interrupting the nucleation process suppresses baikalein-mediated autophagy**

We next investigated signaling pathways that might be involved in baikalein-induced autophagy. Cells were treated with various signaling pathway inhibitors including LY294002 (PtdIns3K), SB202190 (p38 mitogen-activated protein kinase), PD98058 (extracellular signal-regulated kinase) and SP600125 (c-jun N-terminal kinase) followed by the addition of baikalein. Among inhibitors tested, LY294002 suppressed LC3B-II levels induced by baikalein to a great extent in both PC-3 and MDA-MB-231 cells (Fig. 4A). Such inhibition by LY294002 was dose-dependent and occurred at concentrations as low as 1 μm of LY294002 (data not shown). Because LY294002 inhibits class III as well as class I PtdIns3K, and class III PtdIns3K/Vps34 along with Beclin 1 is required in the membrane nucleation process of autophagy, we addressed the role Vps34 and Beclin 1 in baikalein-induced autophagy by using RNA interference. First, PC-3 and MDA-MB-231 cells were transfected with small interfering RNAs (siRNAs) targeting Vps34 and Beclin 1, followed by treatment with baikalein for 24 h.
Data showed that Beclin 1 or Vps34 silencing markedly suppressed the LC3B-II level induced by baicalein (Fig. 4B), suggesting that nucleation factors, including Beclin 1 and class III PtdIns3K/Vps34, may play a key role in baicalein-mediated autophagy. We next examined the role of Atg5, Atg7 and ULK1 proteins in baicalein-induced autophagy. As shown in Fig. 4C, silencing of ULK1, Atg5 or Atg7 substantially suppressed the ability of baicalein to induce autophagy in PC-3 cells. Although we were not able to see such an effect for the individual knockdown of ULK1, Atg5 or Atg7 in MDA-MB-231 cells (data not shown), the concomitant downregulation of those proteins significantly inhibited baicalein-induced autophagy (Fig. 4C, right). Separately, we examined whether silencing the genes tested above could reverse the cell growth inhibited by baicalein. PC-3 cells were transfected with control siRNA, or siRNAs against Vps34, ULK1, Atg5, Atg7 and Beclin, followed by treatment with 5 μg·mL⁻¹ baicalein for 48 h (Fig. 4D). Cells stained with crystal violet were subjected to spectrophotometry at 550 nm. The cell growth was suppressed ~ 80% by baicalein in cells transfected with control siRNA, whereas baicalein failed to induce cell death when Vps34, ULK1, Atg5, Atg7 or Beclin 1 was silenced, suggesting that autophagic cell death might be the key mechanism for the anticancer properties of baicalein.
Figure 4.
Activation of AMPK/ULK1 mediates, and interruption of the nucleation process suppresses baicalein-induced autophagy. (A, E, F) Cells were preincubated with or without various signaling inhibitors such as LY294002, SB202190, PD98058 and SP600125 for 1 h prior to baicalein treatment for 24 h. (B, C) Cells were transfected with 40 nm of siRNAs against the indicated genes and cultured with or without baicalein for 24 h. (A–C, E, F) Protein expression levels were determined by using specific antibodies. (D) Separately, cells transfected with 40 nm siRNA indicated were further cultured in the absence or presence of baicalein for 48 h, and subjected to crystal violet staining and spectrophotometry at 550 nm (*P < 0.0001 vs. control, #P < 0.0001 vs. baicalein only treatment). Data shown are the average of triplicate determinations ± SD and representative of two or three independent experiments (A–F).

**Baicalein induces autophagy by activating AMPK/ULK1 and negatively regulating mTOR/Raptor expression**

ULK1 is a serine/threonine kinase, which acts downstream of the autophagy inhibitor mTOR and forms a large complex with Atg13 and FIP200 to initiate autophagy [17]. mTOR interacts with ULK1 through Raptor, resulting in ULK1 inhibition through phosphorylation at Ser757, whereas Ser555 phosphorylation by AMPK activates ULK1 under nutrient-deprived conditions, thereby initiating autophagy by reversing the inhibitory effect of mTOR [18]. Because si-ULK1 blunted the ability of baicalein to induce cell death (Fig. 4D), as well as LC3B-II level (Fig. 4C), we studied whether baicalein could regulate ULK1 activity through AMPK to induce autophagy. As shown in Fig. 4E, the induction of LC3B-II level by baicalein was accompanied by increased ULK1 Ser555 and decreased Ser757 phosphorylation levels, whereas the total ULK1 level remained unchanged. Moreover, AMPK was significantly phosphoactivated by baicalein at Thr172, and such phosphorylation was noticeably suppressed by blocking class III PtdIns3K activity with LY293002 (Fig. 4E,F). Taken together, our findings suggested that baicalein activated AMPK, thereby activating ULK1 and reducing the inhibitory effect of mTOR, leading to cancer cell death through autophagy.

We next investigated the possible mechanism through which baicalein blocks the ability of mTOR to inhibit autophagy. First, the expression levels of mTOR and Raptor, key components of mTORC1, were determined using western blot analysis. Treatment with 5–10 μg·mL⁻¹ baicalein for 24 h markedly decreased the protein levels of mTOR and Raptor in PC-3 (Fig. 5A). Moreover, conventional RT-PCR analysis revealed that the baicalein-mediated downregulation of mTOR and raptor proteins was due to the reduced level of mRNA expression (Fig. 5B). The data were further confirmed using real-time PCR (Fig. 5C,D), and downregulation of their message levels occurred as early as within 3 or 6 h of baicalein addition, suggesting a transcriptional mechanism. In summary, our data suggested that baicalein is a novel autophagy inducer, which functions by activating the AMPK/ULK1 pathway and negatively regulating mTOR complex. Furthermore, this study provides new insights into the modes of action of baicalein (Fig. 5F), indicating that baicalein has potential as a therapeutic agent for cancer treatment.
Figure 5.

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Suppression by baicalein of mTOR/Raptor expression may lead cancer cells to the autophagic cell death. (A) Protein levels of mTOR and Raptor were determined following baicalein treatment. (B, C) Total RNA was isolated from PC-3 and MDA-MB-231 cells stimulated with baicalein treatment for the indicated time, and subjected to conventional PCR (B) or quantitative real time PCR (C) to determined expression levels of mTOR and raptor. Data shown are the average of triplicate determinations ± SD and representative of two or three independent experiments (A–D). (F) The diverse mechanism of action of baicalein in autophagy induction.
Discussion

Baicalein, the major bioactive flavone extracted from *S. baicalensis*, is more potent than baicalin, which is hydrolyzed by microflora to baicalein. This is further supported by a recent publication showing that pretreatment of baicalin with glycoside hydrolase substantially enhanced its antiproliferative activity in human colorectal and breast cancer cells [19].

To date, studies on the anticancer properties of baicalein have mainly focused on apoptosis, and several mechanisms of action including cdk modulation, cytochrome c release and the activation of caspase 3, caspase 7 and PARP through myeloid leukemia 1 downregulation, have been reported [20-23]. Here, we report new insights into baicalein anticancer properties, providing strong evidence that autophagy, rather than apoptosis, is the key mechanism through which baicalein induces PC-3 and MDA-MB-231 cell death.

We found that baicalein stimulated ULK1 phosphorylation at Ser555. Several studies have shown that the ULK1 complex containing ULK1, FIP200 and Atg13 is the first responder in the canonical autophagy pathway. The serine/threonine kinase ULK1 is phosphorylated at different sites by multiple kinases, including AMPK, mTOR and protein kinase B, thereby acting as a key node in autophagy regulation [24]. The energy sensors, mTOR and protein kinase B, phosphorylate ULK1 on Ser757 [18, 25, 26] and Ser774, respectively, thereby inhibiting ULK1-mediated autophagy, whereas AMPK stimulates autophagy by phosphorylating ULK1 on Ser555, which is the main site for AMPK-dependent 14-3-3 binding [24].

AMPK acts as a cellular energy sensor that is activated by metabolic stresses such as hypoxia, exercise and starvation. Once activated, AMPK stimulates energy production and limits energy use to ensure cellular survival. AMPK activators, such as 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside or metformin have emerged as potential therapeutics for the treatment of numerous human diseases including obesity, diabetes and cancer. Accumulating evidence suggests that phosphoactivation of AMPK suppresses the proliferation of cancer cells *in vivo* and *in vitro*. Our study revealed that baicalein treatment led to AMPK phosphorylation on Thr172, thereby transmitting cellular signals in the autophagy interaction network, indicating that baicalein might be a prospective therapeutic agent for human cancers.

In its active state, the mTORC1 complex-containing Raptor is associated with the ULK1 complex and blocks autophagy and ULK1 kinase activity by phosphorylating ULK1 Ser757 and Atg13 [4, 17, 27]. Therefore, dephosphorylation of ULK1 Ser757 or AMPK-induced phosphorylation of ULK1 Ser555 leads to dissociation of the ULK1 complex from mTORC1, thereby stimulating the proautophagic interaction between ULK1 and AMPK [26]. In this study, we observed a significant increase in the levels of ULK1 and AMPK phosphorylation at Ser555 and Thr172, respectively, and reduced inhibitory phosphorylation on Ser757 of ULK1 in response to baicalein. Consistent with these results, silencing of ULK1 using siRNA markedly suppressed baicalein-mediated LC3B-II induction in PC-3 cells (Fig. 4C). Moreover, we unexpectedly found that the protein levels of mTOR and Raptor were substantially reduced by baicalein treatment and that the reduction reflected the message levels of these two major components of mTORC1. This might explain the diminished ability of the mTORC1 complex to inhibit
autophagy by dissociating the proautophagic ULK1 complex from Raptor. Our findings indicate a model in which baikalein induces autophagy by activating the AMPK–ULK1–mTORC1 axis. Interestingly, baikalein-induced AMPK activation was suppressed by LY293002, an inhibitor of class I and class III PtdIns3K. How this inhibitor acts upstream of AMPK in response to baikalein needs to be studied further.

Beclin 1–PtdIns3K class III complexes are initial responders in the autophagy pathway and are essential for expanding the phagophore membranes and autophagosome formation [5] by producing PtIns3P, a signaling lipid for recruiting autophagy effectors. Moreover, Atg5 and Atg7 are critical genes for macroautophagy, given the evidence that mice lacking these genes have impaired constitutive and starvation-induced autophagy [28]. In this study, siRNAs targeting Vps34, Beclin 1, Atg5 and Atg7 were able to block the ability of baikalein to induce autophagy. This suggests that baikalein most likely works at the initiation and nucleation, rather than the elongation or maturation, steps of the autophagosome formation process.

In conclusion, our data provide evidence that baikalein is an autophagy inducer with anticancer function, and the novel mechanisms of action include upregulation of AMPK/ULK1 and downregulation of mTOR/Raptor expression, thereby leading to ULK1 dissociation from the mTORC1 complex, an autophagy inhibitor. Thus, autophagy activation by the bioactive flavone baikalein may have potential as an effective therapeutic strategy, chemoprevention and targeted therapy, or as a chemotherapeutic adjuvant for cancer treatment.

Materials and methods

Materials

The sources for the following reagents were: baikalein (#465119) and 3-MA (#M9281), Sigma-Aldrich (St. Louis, MO, USA); CQ (#193919), MP Biochemicals (Santa Ana, CA, USA); LY294002 (#440202), Calbiochem (La Jolla, CA, USA); bafilomycin-A1 (#BML-CM110-0100), Enzo Lifesciences (Farmingdale, NY, USA); anti-LC3 (#3868P), anti-(Beclin 1) (#3495P), anti-p62 (#5114S), anti-Atg5 (#8540), anti-Atg7 (#8558S), anti-(ULK1) (#4773), anti-(p-ULK1) (S555; #5869P), anti-(p-ULK1) (S757; #6888), anti-AMPK (#5832P), anti-(p-AMPK) (T172; #2535P), anti-PARP (#9542S), anti-(cyclin D3) (#2936S), anti-(cyclin D1) (#2926S), anti-(p-Rb) (S807/S811; #9309P), anti-(PtdIns3K class III) (#3359P), anti-Bcl2 (#2870P) and anti-(Bcl-XL) (#2764P), Cell Signaling (Danvers, MA, USA); anti-(caspase 3) (SC-7148) and anti-(cyclin D2) (SC-593), Santa Cruz, Santa Cruz, CA, USA; anti-(β-actin) (A5441), Sigma Aldrich (St. Louis, MO, USA); and characterized fetal bovine serum, HyClone (Logan, UT, USA).

Cell culture

PC-3, MDA-MB-231 and DU145 human cancer cell lines purchased from Korean Cell Line Bank (KCLB, Seoul, Korea) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum at 37 °C in a 95% air/5% CO₂ environment and passaged every 3–4 days (at subconfluence). All experiments were performed under low serum conditions (DMEM containing 1% fetal bovine serum and 15 mm HEPES), unless otherwise specified.
Western blot analysis

Samples were analyzed by immunoblotting as described previously [29, 30]. Cells were plated at a density of $2 \times 10^5$ cells·well$^{-1}$ in six-well plates containing 2 mL·well$^{-1}$ of DMEM supplemented with 1% fetal bovine serum and 15 mm HEPES and then treated with baicalein at doses ranging from 0 to 10 $\mu$g·mL$^{-1}$ for the indicated times. After treatment, cells were washed with 1 × PBS and lysed at 4 °C with ice-cold RIPA buffer (PBS, 1% Nonidet P-40, 0.1% SDS and 0.5% sodium deoxycholate) containing 1 mm sodium orthovanadate, complete Mini-EDTA-free Protease Inhibitor Mixture and 1 mm phenylmethylsulfonyl fluoride. Lysates were centrifuged at 16 700 g for 20 min at 4 °C and the protein content of the supernatants was quantified using the BCA protein assay [23225; Pierce (Thermo Scientific), Waltham, MA, USA]. Thirty to 50 $\mu$g of protein from whole-cell lysates was analyzed by using western blot analysis. Lysates were boiled for 5 min in SDS/PAGE loading buffer containing 5% 2-mercaptoethanol, electrophoresed through 8 or 10% Tris/glycine gels, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline (10 mm Tris/HCl, pH 8.0 and 150 mm NaCl) containing 5% non-fat dry milk and incubated with the indicated primary antibodies and the appropriate horseradish peroxidase conjugated secondary antibody (1 : 5000; Jackson Immunoresearch Laboratory, West Grove, PA, USA). The Super Signal Chemiluminescence Substrate System (Pierce) was used to visualize protein bands.

Luciferase assay

RLuc–LC3-wt and RLuc–LC3-G120A-mutant constructs [31] were kindly provided by Dr. Marja Jäättelä (Danish Cancer Society Research Center, Denmark). Cells were transfected by using Lipofectamine Plus transfection reagent (15388-100; Invitrogen, Carlsbad, CA, USA) as described previously [32]. PC-3 cells were plated at a density of $2 \times 10^5$ cells·well$^{-1}$ in DMEM supplemented with 1% fetal bovine serum and 15 mm HEPES and transiently transfected for 3 h in serum-free medium with 2 $\mu$g of GFP-LC3/well. After 3 h, the medium was replaced with DMEM containing 1% fetal bovine serum and 15 mm HEPES. Cells were then cultured in the absence or presence of baicalein for additional 24 h before lysis or analysis.

GFP–LC3 transfection and confocal microscopy

PC-3 cells were plated on cover glass-bottom dishes and transfected with GFP-tagged LC3 using Lipofectamine 2000 reagent according to the manufacturer's protocol (11668-019; Invitrogen) followed by treatment with baicalein or control vehicle. Cells were then washed with 1X PBS and fixed with 4% formaldehyde/PBS solution. LC3 puncta formation was analyzed by imaging with a confocal microscope.

RNA interference

RNA interference was performed by using siRNA directed towards specific genes. Cells were transfected with siRNAs by using Lipofectamine RNAmax (1122868; Invitrogen) in serum-free medium and cultured for 6 h. Cells were supplemented with serum and treated with the indicated reagents. Cells were harvested and lysates were prepared 48 h after siRNA transfection or 24 h after drug treatment.
Real-time PCR

Total RNA from PC-3 and MDA-MB-231 cells treated with indicated compounds was extracted using Trizol (FATRR001; Favorgen, Changjih, Pingtung, Taiwan) according to manufacturer's instructions. One microgram of RNA was used to synthesize cDNA, which was then subjected to qPCR (Illumina, San Diego, CA, USA) using the following primers. GAPDH (forward 5′-GATCATCAGCAATGCCTCCT-3′ and reverse 5′-TGTGGTATGAGTCTCCA-3′), mTOR (forward 5′-CTGGGACTCAAATGTGTGCAGTTC-3′ and reverse 5′-GAACAATAGGGTGAATGATCCGGG-3′), and Raptor (forward 5′-TCGTCAGTCTCCAAGCAG-3′ and reverse 5′-GGGTGATTTGGGTTGATTGC-3′).

Crystal violet staining and cell viability assay

Cells were plated at a density of 1 × 10^5 cells·well⁻¹ in 12-well plates or at 2 × 10^5 cells·well⁻¹ in six-well plates containing 1 or 2 mL DMEM supplemented with 1% fetal bovine serum and 15 mm HEPES per well, respectively, and incubated overnight to allow attachment. Baicalein or control vehicle was added and cells were cultured for the indicated times. Cells were then fixed with 2% formalin/PBS and incubated with 0.2 mg·mL⁻¹ of crystal violet solution to stain the nuclei. After washing twice with 1 X PBS, the crystal violet dye was eluted by adding 1% Triton X-100/PBS and the absorbance at 550 nm was determined.

Cell-cycle analysis and detection of autophagy and *in vitro* caspase activity by flow cytometry

For cell-cycle analysis, cells (1.5 × 10^6) were detached from the plates, washed once in 1 X PBS, fixed in 90% methanol and then treated with 0.1 mg·mL⁻¹ ribonuclease A. Then, cells were incubated with 50 μg·mL⁻¹ of propidium iodide (Molecular Probes, Waltham, MA, USA) and analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Propidium iodide incorporation in cells was analyzed at excitation and emission wavelengths of 488 and 585 nm, respectively. Sub-G₁ cells with a DNA content < 2n were considered apoptotic.

*In vitro* caspase activity by flow cytometry

*In vitro* caspase detection was performed using a FAM–FLICA™ kit (#637; Immunoochemistry Technology, Bloomington, MN, USA), according to the manufacturer's instructions. Cells were treated with the reagents indicated and 30× FLICA solution was added to the cell suspension at a rate of 1 : 30. After incubation at 37 °C for 1 h in the dark, cells were washed with 2 mL of 1× apoptosis wash buffer followed by centrifugation at 400 g for 5 min at room temperature. The cell pellet was again washed with 1 mL of apoptosis wash buffer and resuspended in apoptosis wash buffer; cells were further stained either with control vesicle or propidium iodide for 1 h on ice. Cells were then washed to remove excess PtdIns from the media and fixative was added at a rate of 1 : 10. After incubation for 15 min at room temperature in the dark, samples were subjected to flow cytometry analysis, measuring FAM on the FL1 channel and red fluorescence (PtdIns) on the FL2 channel.
Detection of autophagy by flow cytometry

Autophagy was measured using a Cyto-ID Autophagy Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA), according to the manufacturer's instructions. After treatment with or without baicalein for 24 h, cells were trypsinized and centrifuged at 80 g for 5 min to pellet. Cells were then washed with 1× assay buffer and collected by centrifugation. Two hundred and fifty microliters of the diluted Cyto-ID Green stain solution was added to cells resuspended in 250 μL of indicator-free cell culture medium containing 5% fetal bovine serum, followed by incubation for 30 min at room temperature in the dark. After incubation, cells were washed with 1× assay buffer and resuspended in 500 μL of fresh 1× assay buffer before being subjected to flow cytometry analysis.

Statistical analysis

Statistical significance was calculated with one-way analysis of variance (ANOVA; Tukey's post hoc analysis), and accepted at the level of $P < 0.05$.

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