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Baicalein induces cancer cell death and proliferation retardation by the inhibition of CDC2 kinase and survivin associated with opposite role of p38 mitogen-activated protein kinase and AKT

Jui-I Chao,[1] Wen-Chi Su,[1,2] and Huei-Fang Liu[1]

[1]Institute of Pharmacology and Toxicology, Tzu Chi University; [2]Department of Pharmacy, Tzu Chi General Hospital, Hualien, Taiwan

Abstract

The bioactive flavonoid baicalein has been shown to have *in vitro* growth-inhibitory activity in human cancer cells, although the mechanism of action is poorly understood. Baicalein (40–80 μmol/L for 24 h) more effectively induced cytotoxicity compared with other flavonoids (baicalin, catechin, genistein, quercetin, and rutin) in bladder cancer cells. Baicalein induced cell proliferation inhibition and apoptosis. The levels of cyclin B1 and phospho-CDC2 (Thr^{161}) were reduced, whereas the G_2-M phases were elevated by baicalein. Treatment of CDC2 kinase or CDC25 phosphatase inhibitors augments the baicalein-induced cytotoxicity. A variety of human bladder cancer cell lines expressed survivin proteins, which were located on the mitotic phases and regulated mitotic progression. Baicalein markedly reduced survivin protein expression. Transfection of a survivin small interfering RNA diminished the level of survivin proteins and increased the baicalein-mediated cell death. Overexpression of survivin enhanced cell proliferation and resisted the baicalein-induced cytotoxicity. Interestingly, baicalein induced the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and AKT. SB203580, a specific p38 MAPK inhibitor, attenuated proliferation inhibition and restored the protein levels of phospho-CDC2 (Thr^{161}) and survivin in the baicalein-exposed cells; conversely, blockade of AKT activation enhanced cytotoxicity and the reduction of phospho-CDC2 (Thr^{161}) and survivin proteins. As a whole, these findings provide that the opposite role of p38 MAPK and AKT regulates CDC2 kinase and survivin and the inhibition of CDC2-survivin pathway by baicalein contributes to apoptosis and proliferation retardation in cancer cells. [Mol Cancer Ther 2007;6(11):3039–48]

Introduction

Flavonoids, a group of polyphenolic compounds, are natural products in many fruits, vegetables, and all vascular plants (1, 2). Some kinds of flavonoids contain anticancer and chemopreventive activities (2–5). Baicalein, a bioactive flavonoid extracted from root of *Scutellaria baicalensis* or *Scutellaria radix*, exerts antitumor activity (6–8). It leads to cell cycle arrest and suppression of proliferation in cancer cells (6–8). Baicalein induces apoptosis of a variety of human cancer cell lines (8–12). However, the precise mechanism of apoptosis by baicalein is still ambiguous.

The balance between survival and apoptosis signal pathways controls the cancer pathogenesis. The p38 mitogen-activated protein kinase (MAPK) pathway has been associated with the induction of apoptosis in response to various cellular stresses (13–15). Anticancer drugs such as doxorubicin and paclitaxel induce the activation of p38 MAPK to mediate apoptosis (16). Moreover, p38 MAPK regulates apoptosis that is associated with the activation of caspases (14, 16). The phosphatidylinositol 3-kinase-AKT pathway regulates cell survival and apoptosis (17–19). The phosphorylation of AKT provides a survival signal to protect cells from apoptosis (19–21). It has been shown that AKT triggers cell survival through its ability to phosphorylate and to inactivate downstream targets such as bad (22) and caspase-9 (23).

The cell cycle arrest mediated by inappropriate activity of the cyclin-dependent protein kinases can trigger proliferation inhibition and apoptosis in cancer cells (24–26). The cyclin-dependent protein kinase 1 (CDC2) interacts with cyclin B1 that has been shown to play a critical role in the mitotic progression (26–28). Survivin is an inhibitor of apoptosis that is expressed in various human cancer cells but is undetectable in most normal adult cells (29, 30). Survivin displays both antiapoptosis and promotion of mitosis in cancer cells (26, 30–32). The stability of survivin resulted from the protein phosphorylation at Thr^{34} by the mitotic kinase complex CDC2/cyclin B1 (31, 33). Recently, it has been proposed as a new marker for bladder cancer detection (34–36). Urine detection of survivin is useful for the diagnosis and prognosis in bladder carcinomas (35, 36). It is correlated with decreased survival, unfavorable

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prognosis, and accelerated rates of recurrences in cancer therapy (37). Therefore, the blockade of CDC2-survivin pathway would induce apoptosis and cell cycle arrest in tumor cells, providing important strategy for cancer therapy.

In the present study, survivin is expressed in various human bladder cancer cell lines and reduced by baicalein. Roles of survivin were illustrated by using a small interfering RNA (siRNA) of survivin and a survivin overexpression vector (pCT-GFP-sur8) in the baicalein-exposed cells. Baicalein markedly inhibited the activation of CDC2/cyclin B1. Furthermore, p38 MAPK and AKT activated by baicalein exhibited the opposite role on the regulation of CDC2-survivin pathway and cancer cell death.

Materials and Methods

Flavonoids

Six types of polyphenolic compounds, including baicalein (a flavone), baicalin (a flavone glycoside), catechin (a flavan-3-OH), genistein (an isoflavone), quercetin (a flavonol), and rutin (a flavonol glycoside), were purchased from Sigma Chemical Co. All of flavonoids were dissolved in DMSO, and the concentration of DMSO was <0.8% in the control and drug-containing medium.

Reagents and Antibodies

2-(2-Mercaptoethanol)-3-methyl-1,4-naphthoquinone (Cpd 5) and 6-chloro-7-(2-morpholin-4-ylethylamino)quinoline-5,8-dione (NSC 663284) were kindly provided by Dr. C. Chen (National Dong-Hwa University, Hualien, Taiwan). Hoechst 33258, propidium iodide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical. Lipofectamine 2000 and BODIPY FL phallacidin were purchased from Invitrogen. Anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-AKT, anti-phospho-AKT (Ser^{473}), anti-phospho-CDC2 (Thr^{161}), SignalSilence survivin siRNA, SignalSilence control siRNA, anti-XIAP, and goat anti-rabbit IgG-horseradish peroxidase were purchased from Cell Signaling Technology, Inc. Anti-extracellular signal-regulated kinase-2 (C-14), anti-survivin (FL-142), anti-p38 (C-20), anti-BCL-2 (100), and the FITC-labeled goat anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. The Cy5-labeled goat anti-rabbit IgG was purchased from Amersham Pharmacia Biotech. Anti-CDC2 (Ab-1), anti-cyclin B1 (Ab-2), SB203580, alsterpaullone, and 1*L*-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate were purchased from Calbiochem.

Cell Culture

The bladder cancer cell lines were summarized in Supplementary Table S1.[3] TSGH8301 and BFTC905 cells were derived from bladder carcinomas of Chinese patients. RT4, T24, and HT1376 were derived from bladder carcinomas of Caucasian. TSGH8301, BFTC905, and RT4 were cultured in RPMI 1640 (Invitrogen). T24 and HT1376 were cultured in McCoy's 5A medium (Sigma Chemical) and DMEM (Invitrogen), respectively. The complete medium was additively supplemented with 10% fetal bovine serum.

Cytotoxicity Assay

The cells were plated in 96-well plates at a density of 1×10^4 per well in complete medium for 16 to 20 h. Then, the cells were treated with 0 to 80 μmol/L of baicalein for 24 h. After drug treatment, the cells were washed with PBS and recultured for 2 days. Thereafter, the medium was replaced, and the cells were incubated with 0.5 mg/mL MTT for 4 h. Finally, the cells were dissolved in DMSO, and the intensity of formazan was measured at 545 nm using a plate reader (OPTImax, Molecular Dynamics). The relative percentage of cell viability was calculated by dividing the absorbance of treatment (from the average of six-wells) by that of the control in each experiment.

Apoptosis Analysis

At the end of treatment, the cells were slightly washed with PBS and fixed with 4% paraformaldehyde solution for 1 h at 37°C. The nuclei were stained with 2.5 μg/mL Hoechst 33258 for 30 min. The number of apoptotic nuclei was counted by a hemocytometer under a fluorescence microscope. The morphology of apoptosis was confirmed by observation of the cell membrane blebbing and apoptotic bodies. At least 500 cells were examined for the calculation of apoptotic percentage in each treatment. The person counting the cells was blinded as to which treatments were being counted.

Cell Cycle Assay

Bladder cancer cells were plated at a density of 1×10^6 cells per 60-mm Petri dish in complete medium for 16 to 20 h. Thereafter, the cells were treated with 0 to 60 μmol/L of baicalein for 24 h. After drug treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at −20°C. The cell pellets were incubated with 4 μg/mL propidium iodide solution (containing 100 μg/mL RNase and 1% Triton X-100) for 30 min at 37°C. Then, the samples were analyzed by flow cytometer (FACScan, Becton Dickinson). The percentage of each cell cycle phases was analyzed by the ModFit LT software (Becton Dickinson).

Cell Number Assay

The cells were plated at a density of 7×10^5 per 100-mm Petri dish in complete medium for 16 to 20 h. Then, the cells were treated with 0 to 80 μmol/L of baicalein for 24 h. After drug treatment, the cells were washed twice with PBS and recultured in complete medium for various times before total cell number was counted by a hemocytometer.

Immunofluorescence Staining and Confocal Microscopy

The main procedure was according to our previous study (32). After treatment with or without baicalein, the cells were fixed in 4% paraformaldehyde solution in PBS for 1 h at 37°C. Briefly, the cells were incubated with rabbit anti-survivin (1:50) or rabbit anti-phospho-p38 (1:100) antibodies in PBS containing 10% fetal bovine serum overnight at 4°C. Thereafter, the cells were washed thrice

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

with 0.3% Triton X-100 in PBS. Subsequently, the cells were incubated with goat anti-rabbit Cy5 (1:100) or goat anti-mouse FITC (1:50) in PBS containing 10% fetal bovine serum for 2.5 h at 37°C. The nuclei, β-tubulin, and filamentous actin were stained with Hoechst 33258, the Cy3-labeled anti-β-tubulin, and BODIPY FL phallacidin, respectively. After staining, the samples were immediately examined under a Leica confocal laser-scanning microscope.

Western Blot Analysis

The total cellular protein extracts were prepared as described (32). Western blot analyses of cyclin B1, CDC2, phospho-CDC2, survivin, p38, phospho-p38, AKT,

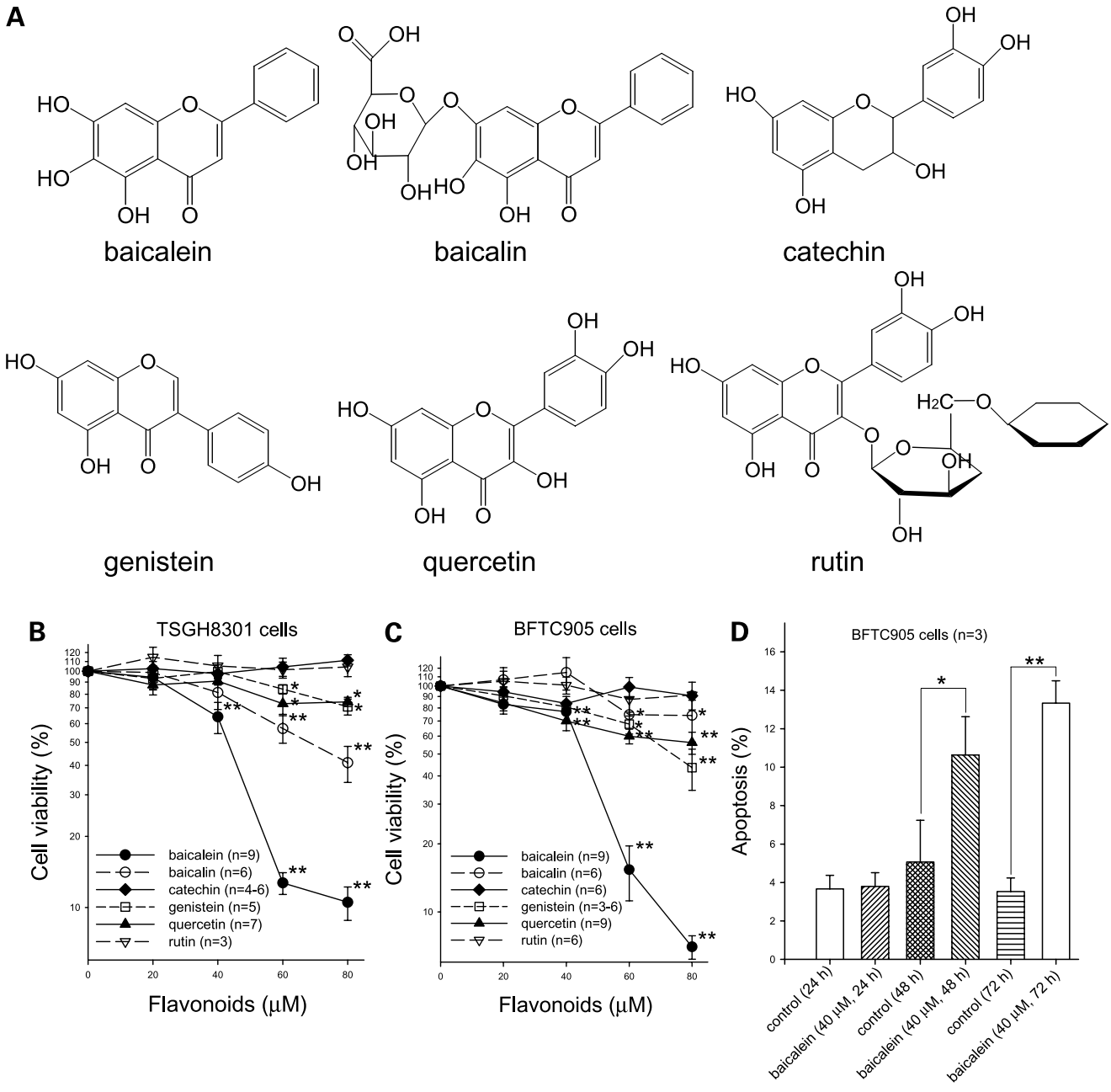

Figure 1. Effects of baicalein on the cytotoxicity and apoptosis in bladder cancer cells. **A,** chemical structures of flavonoids. **B** and **C,** TSGH8301 or BFTC905 cells were treated with 0 to 80 μmol/L baicalein, baicalin, catechin, genistein, quercetin, and rutin for 24 h. After drug treatment, the cells were recultured for 2 d. The cell viability was measured by MTT assay. *Points,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and flavonoid-treated samples. **D,** the cells were treated with or without 40 μmol/L baicalein for 24 to 72 h. The percentage of apoptosis was scored by the apoptotic nuclei under a fluorescence microscope. Results were obtained from three experiments. *Columns,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and baicalein-treated samples.

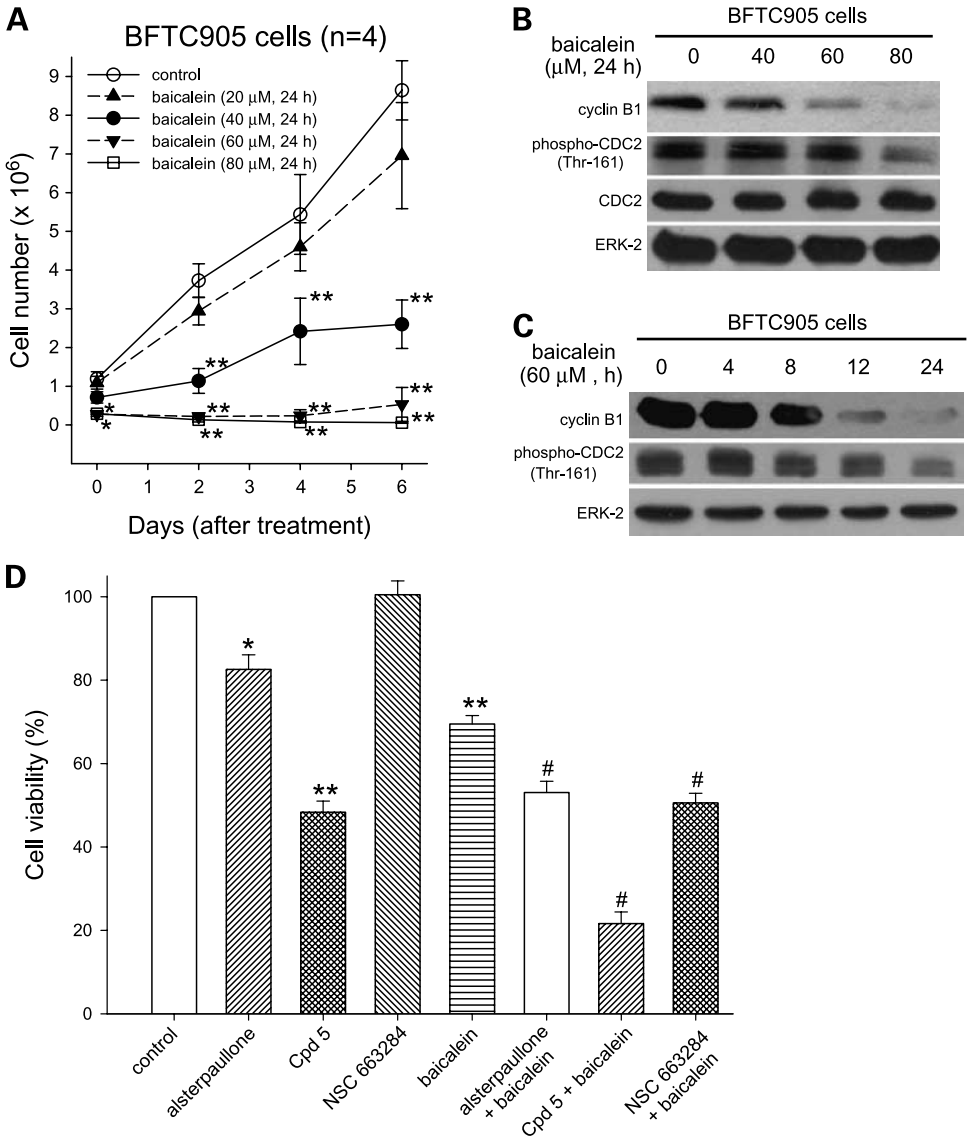

Figure 2. Effects of baicalein on the cell proliferation, cell cycle progression, and cyclin B1/CDC2 protein expression in bladder cancer cells. **A,** BFTC905 cells were treated with 0 to 80 μmol/L of baicalein for 24 h. After drug treatment, the cells were incubated for various times before they were counted by a hemocytometer. *Points,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and baicalein-treated samples. **B** and **C,** the cells were exposed to 0 to 60 μmol/L of baicalein for 24 h or 60 μmol/L baicalein for 0 to 24 h. The total protein extracts were prepared for Western blot analysis. Representative Western blot data were shown from one of three separate experiments with similar findings. *ERK-2,* extracellular signal-regulated kinase-2. **D,** BFTC905 cells were pretreated with or without alsterpaullone, Cpd 5, or NSC 663284 (5 μmol/L for 1 h) before exposure to 40 μmol/L baicalein for 24 h. The cell viability was measured by MTT assay. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and inhibitor- or baicalein-treated samples. #, $P < 0.05$, significant difference between inhibitor alone and baicalein plus inhibitor.

phospho-AKT, BCL-2, XIAP, and extracellular signal-regulated kinase-2 were done using specific antibodies. Briefly, proteins were separated on 10% to 12% SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were sequentially hybridized with primary antibody followed with a horseradish peroxidase–conjugated secondary antibody. Finally, the protein bands were visualized followed by detection with a chemiluminescence kit (Perkin-Elmer Life and Analytical Sciences). Scanned images were quantified using Un-Scan-It gel software (version 5.1; Silk Scientific, Inc.).

Transfection

A control siRNA and a survivin siRNA were used for transfection in BFTC905 cells. The cells were transfected with 40 nmol/L of control or survivin siRNA by using Lipofectamine 2000 reagent according to the manufacturer's recommendations. Moreover, we had constructed a survivin-expressed vector (pCT-GPF-sur8) and a control vector (pCT-GFP2) to examine the effect of survivin on cancer cells. BFTC905 cells were transfected with 5 μg/mL of control or survivin-expressed vectors. After transfection, the cells were subjected to cell number, cytotoxicity, or Western blot assays as described above.

Statistical Analysis

Data were analyzed by one-way or two-way ANOVA and further by post hoc tests using the statistical software of GraphPad Prism 4 (GraphPad Software). A P value of <0.05 was considered as statistically significant.

Results

Baicalein Elicits Higher Cytotoxicity Than Other Flavonoids in Bladder Cancer Cells

We examined two bladder cancer cell lines (TSGH8301 and BFTC905) on the cytotoxicity following treatment with

six flavonoids, including baicalein, baicalin, catechin, genistein, quercetin, and rutin. The chemical structures of various flavonoids were shown in Fig. 1A. The cell viability was reduced by treatment with 60 to 80 μmol/L of baicalein, baicalin, genistein, and quercetin in both TSGH8301 and BFTC905 cells; however, catechin and rutin did not significantly induce cytotoxicity in these cells (Fig. 1B and C). Baicalein was higher on the induction of bladder cancer cell death than other flavonoids. The IC_{50} value of 50% cell survived by baicalein was <50 μmol/L. However, the IC_{50} value of baicalein toward cultured human normal fibroblasts was >150 μmol/L (data not shown). Moreover, the percentage of apoptotic nuclei was increased following treatment with 40 μmol/L baicalein for 48 to 72 h in BFTC905 cells (Fig. 1D). Besides, baicalein (80 μmol/L for 24 h) increased the sub-G_1 phase (apoptotic fraction) by ~10% in BFTC905 cells (data not shown).

Baicalein Inhibits Cell Proliferation, Increases G_2-M Phases, and Blocks the Activation of CDC2/Cyclin B1

As shown in Fig. 2A, baicalein inhibited the cell proliferation via a concentration-dependent manner in BFTC905 cells. Higher concentrations of baicalein (60 and 80 μmol/L) almost completely blocked the cell proliferation ability (Fig. 2A). To characterize the effect of baicalein on cell cycle progression, the cells were treated with 0 to 80 μmol/L of baicalein for 24 h and then subjected to flow cytometry analyses. Baicalein (60–80 μmol/L for 24 h) significantly decreased the G_0-G_1 phases but increased the G_2-M phases in BFTC905 cells ($P < 0.05$). Treatment with 80 μmol/L baicalein for 24 h increased ~10% of the G_2-M phases than control. However, the S phase was not markedly altered by baicalein. Western blot analysis showed that baicalein decreased the protein levels of cyclin B1 and phospho-CDC2 (Thr^{161}) via a concentration- and time-dependent manner (Fig. 2B and C). However, total CDC2 proteins were not altered by baicalein (Fig. 2B). Extracellular signal-regulated kinase-2 protein was used as an internal control in this study that was not changed by baicalein. We have further examined the role of CDC2 activation after baicalein treatment by using a CDC2 kinase inhibitor (alsterpaullone) and the CDC25 phosphatase inhibitors (Cpd 5 and NSC 663284). Treatment with 5 μmol/L alsterpaullone or Cpd 5 for 1 h decreased the cell viability in BFTC905 cells (Fig. 2D). Moreover, pretreatment with alsterpaullone, Cpd 5, or NSC 663284 enhanced cytotoxicity when BFTC905 cells were exposed to 40 μmol/L baicalein for 24 h (Fig. 2D).

Baicalein Reduces Survivin Protein Expression in Human Bladder Cancer Cells

The human bladder cancer cell lines, including TSGH8301, BFTC905, RT4, T24, and HT1376, expressed survivin proteins (Fig. 3A). We have further determined the

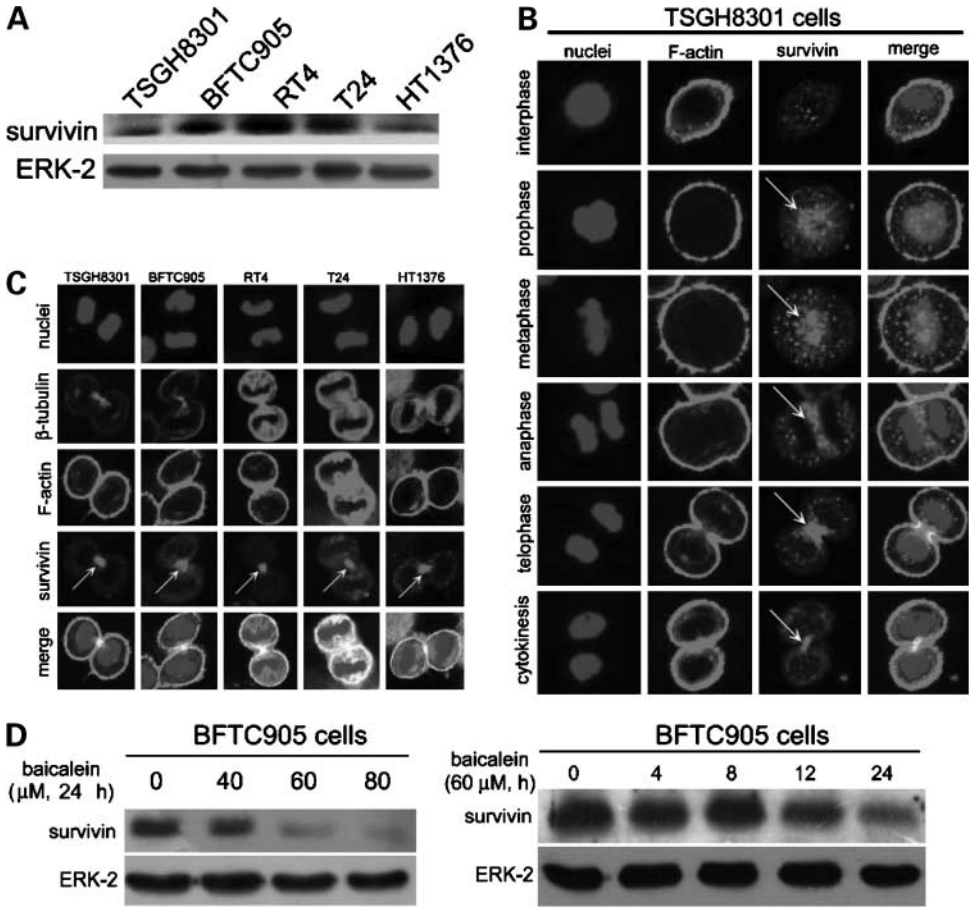

Figure 3. Effects of baicalein on the protein levels of survivin in bladder cancer cells. **A,** total protein extracts from bladder cancer cell lines were prepared for Western blot analysis using anti-survivin and anti-extracellular signal-regulated kinase-2 antibodies. **B** and **C,** the cells were incubated with rabbit anti-survivin antibody and then with goat anti-rabbit Cy5. The survivin protein displayed red fluorescence with goat anti-rabbit Cy5. The nuclei, filamentous actin (*F-actin*), and β-tubulin were stained with Hoechst 33258, BODIPY FL phallacidin, and the Cy3-labeled anti-β-tubulin, respectively. *Arrows,* location of survivin proteins. **D,** the cells were treated with or without baicalein. At the end of treatment, total protein extracts were prepared for Western blot analysis. Representative Western blot data were shown from one of three separate experiments with similar findings.

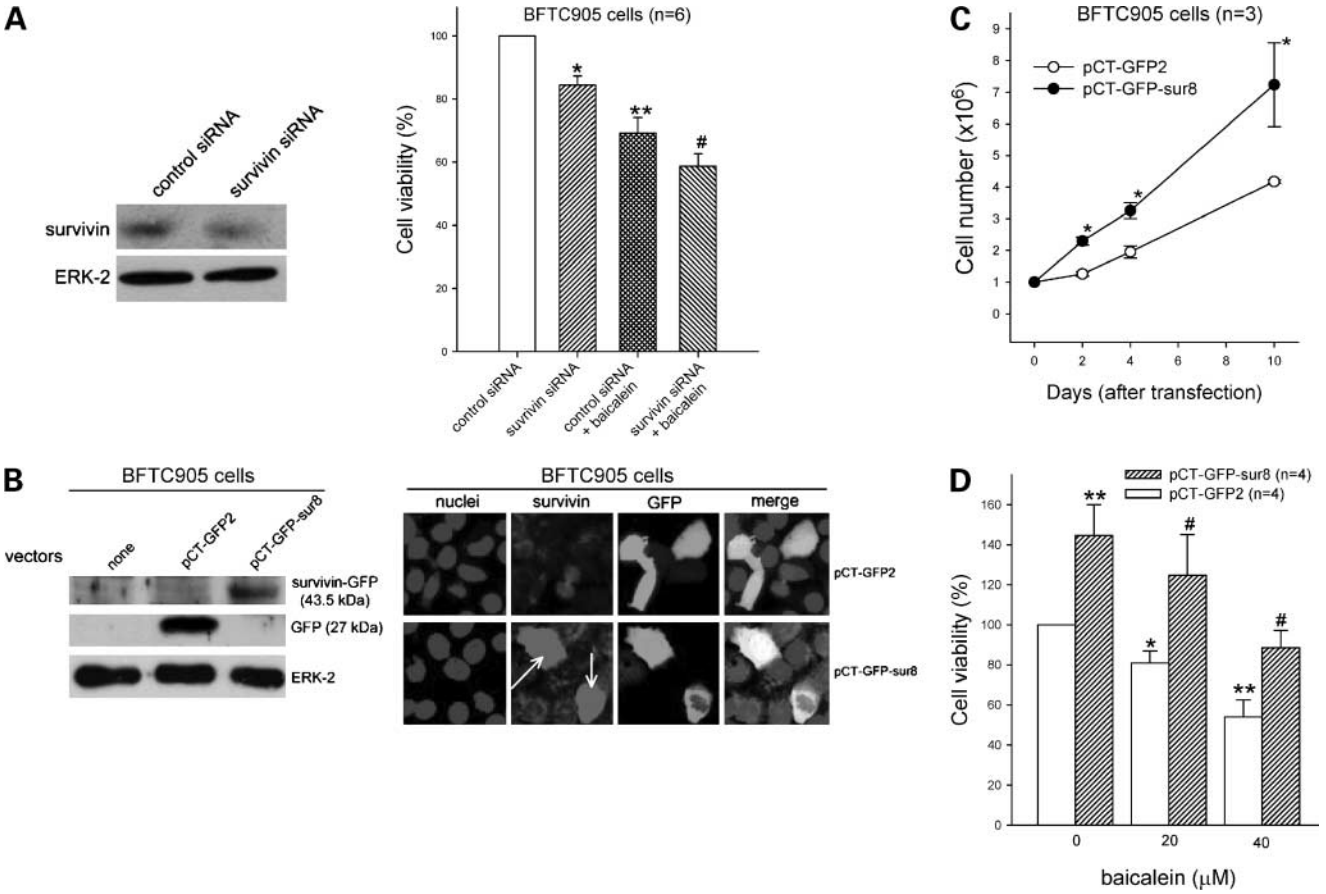

Figure 4. Effects of survivin on the baicalein-induced proliferation inhibition and cell death in bladder cancer cells. **A,** the control or survivin siRNA (40 nmol/L) was transfected to BFTC905 cells for 48 h. After transfection, the protein level of survivin was characterized by Western blot. Subsequently, the control or survivin siRNA-transfected cells were exposed to 40 μmol/L baicalein for 24 h. After treatment, the cell viability was measured by MTT assay. *Columns,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and survivin siRNA or baicalein-treated samples. #, $P < 0.05$, significant difference between control and survivin siRNA with baicalein treatment. **B,** the cells were transfected with 5 μg/mL pCT-GFP2 or pCT-GFP-sur8 vectors. The survivin-GFP and GFP proteins were characterized by Western blot using anti-GFP antibody. After transfection, the cells were subjected to immunofluorescence staining. The cells were incubated with rabbit anti-survivin antibody and then with goat anti-rabbit Cy5. The survivin protein displayed red fluorescence with goat anti-rabbit Cy5. The nuclei that were stained with Hoechst 33258 displayed the *blue color*. The GFP proteins displayed the *green color. Arrows,* survivin overexpressed cells. **C,** after transfection, the total cell numbers were counted. **D,** the control or pCT-GFP-sur8 – transfected cells were exposed to 0 to 40 μmol/L of baicalein for 24 h. The cell viability was measured by MTT assay. *Columns,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and baicalein-treated samples or between pCT-GFP2 and pCT-GFP-sur8 without baicalein treatment. #, $P < 0.05$, significant difference between pCT-GFP2 and pCT-GFP-sur8 with baicalein treatment.

location of survivin proteins in bladder cancer cells by using immunofluorescence staining and confocal microscopy. The intensity of red fluorescence (Cy5) indicated survivin proteins, which were expressed in mitotic phases in TSGH8301 cells (Fig. 3B, *arrows*). All of bladder cancer cell lines expressed survivin proteins, which concentrated on the midbodies during cytokinesis (Fig. 3C, *arrows*). The protein levels of survivin were decreased by baicalein in BFTC905 cells via a concentration- and time-dependent manner (Fig. 3D). Baicalein also inhibited the survivin protein expression in other bladder cancer cell lines (data not shown).

Existence of Survivin Increases the Cell Proliferation and Resists the Baicalein-Induced Cytotoxicity

To determine the role of survivin on the baicalein-induced cell death, a survivin siRNA and a survivin-expressed vector (pCT-GFP-sur8) were applied to examine the effect of baicalein on the cell viability and proliferation in bladder cancer cells. Transfection of survivin siRNA (40 nmol/L, 48 h) decreased ~30% of the survivin protein expression (Fig. 4A, *left*). Survivin siRNA or baicalein significantly inhibited cell survival in BFTC905 cells (Fig. 4A, *right*). Moreover, the cell death caused by baicalein (40 μmol/L for 24 h) was additively increased after transfection with 40 nmol/L survivin siRNA for 24 h (Fig. 4A, *right*). A survivin-expressed vector was called pCT-GFP-sur8, which produced a survivin-green fluorescent protein (GFP) fusion protein in cells. The survivin-GFP fusion proteins were characterized by using immunofluorescence staining and Western blot. Immunoblot analysis showed that transfection with pCT-GFP-sur8 vector expressed the survivin-GFP fusion protein (43.5 kDa) in BFTC905 cells (Fig. 4B, *left*).

The pCT-GFP2 vector expressed the GFP protein (27 kDa) in these cells. Moreover, transfection with pCT-GFP-sur8 vector produced the survivin-GFP fusion proteins in BFTC905 cells (Fig. 4B, *arrows*). The GFP proteins presented the green fluorescence (Fig. 4B). Overexpression of survivin by pCT-GFP-sur8 vector increased the cell proliferation (Fig. 4C) and the cell viability (Fig. 4D). Furthermore, BFTC905 cells transfected with pCT-GFP-sur8 vector were more resistant to cell death than control vector before treatment with baicalein (Fig. 4D).

Inhibition of the Phosphorylation of p38 MAPK Attenuates Cell Death and Restores the Protein Levels of Phospho-CDC2 and Survivin in the Baicalein-Treated Cells

The possible role of p38 MAPK in the baicalein-induced bladder cancer cell death was examined. Baicalein increased the protein levels of phospho-p38 MAPK via a time- and concentration-dependent manner in BFTC905 cells (Fig. 5A). The red fluorescence (Cy5) intensities exhibited by phospho-p38 proteins were elevated following exposure to 60 μmol/L baicalein for 24 h (Fig. 5B). The increased phospho-p38 proteins were concentrated on the nucleus (Fig. 5B, *arrow*). Pretreatment with a specific p38 MAPK inhibitor, SB203580, reduced the phospho-p38 proteins and recovered the protein levels of phospho-CDC2 (Thr^{161}) and survivin in the baicalein-exposed cells (Fig. 5C, *left*). The quantified data showed that SB203580 significantly restored the survivin proteins in the baicalein-exposed cells (Fig. 5C, *right*). SB203580 also significantly restored the phospho-CDC2 (Thr^{161}) proteins in the baicalein-treated cells ($P < 0.05$). However, BCL-2 and XIAP proteins were not altered by baicalein or SB203580

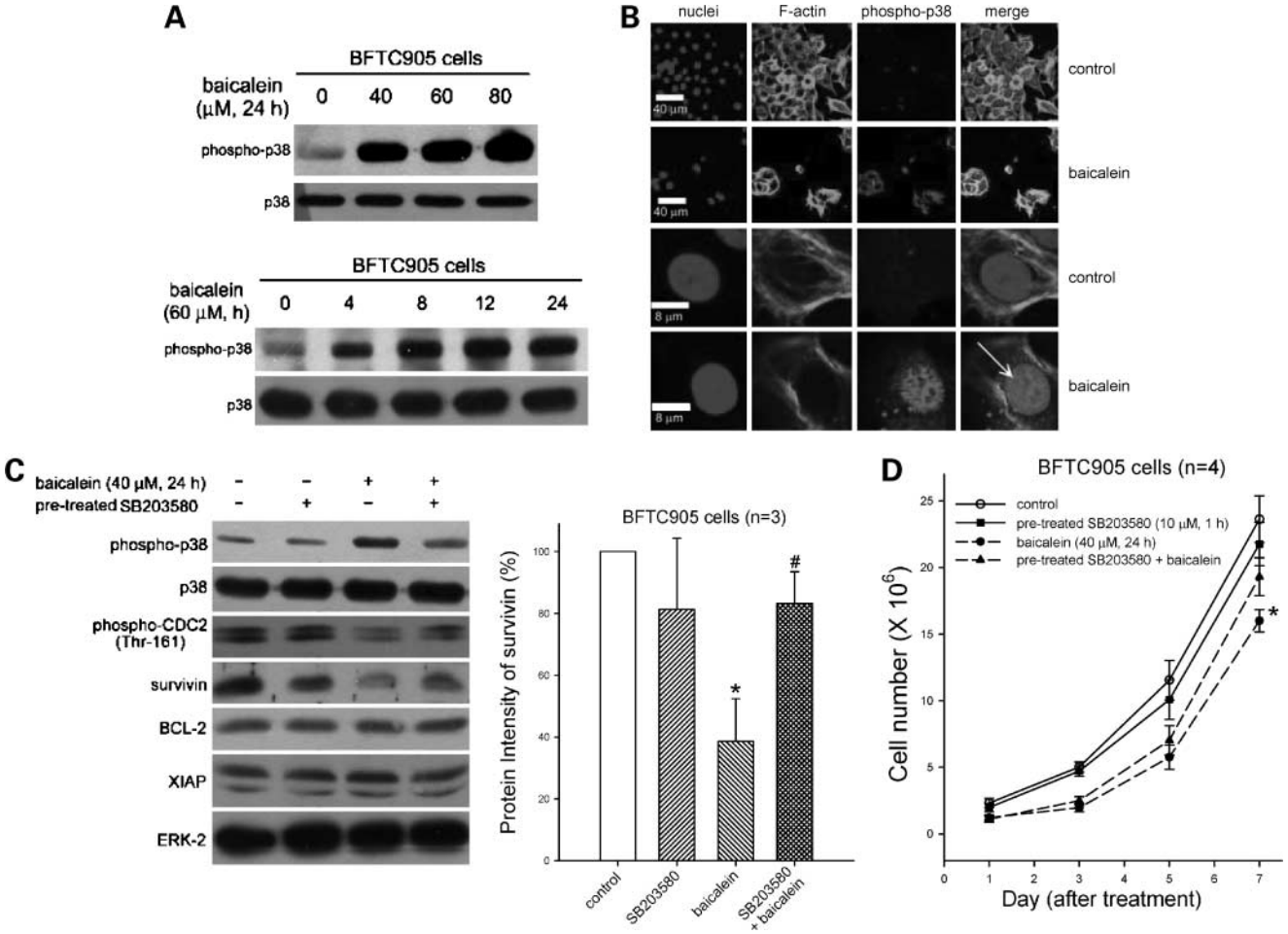

Figure 5. Effects of p38 MAPK on the survivin protein expression and the cell survival after treatment with baicalein in bladder cancer cells. **A,** BFTC905 cells were treated with or without baicalein. The protein levels of phospho-p38 and total p38 MAPK were analyzed by Western blot analysis. **B,** the cells were treated with or without 60 μmol/L baicalein for 24 h. The phospho-p38 proteins displayed red fluorescence. *Arrow,* phospho-p38 proteins located in the nucleus. **C,** the cells were pretreated with 5 μmol/L SB203580 for 1 h before exposure to 40 μmol/L baicalein for 24 h. Total protein extracts were analyzed by Western blot. Representative Western blot and immunofluorescence data were shown from one of three separate experiments with similar findings. The protein intensity of survivin was quantified from Western blots. *, $P < 0.05$, significant difference between control and baicalein-treated samples. #, $P < 0.05$, significant difference between baicalein alone and pretreatment with SB203580. **D,** BFTC905 cells were pretreated with 10 μmol/L SB203580 for 1 h before exposure to 40 μmol/L baicalein for 24 h. *Points,* mean; *bars,* SE. *, $P < 0.05$, significant difference between baicalein alone and pretreatment with SB203580.

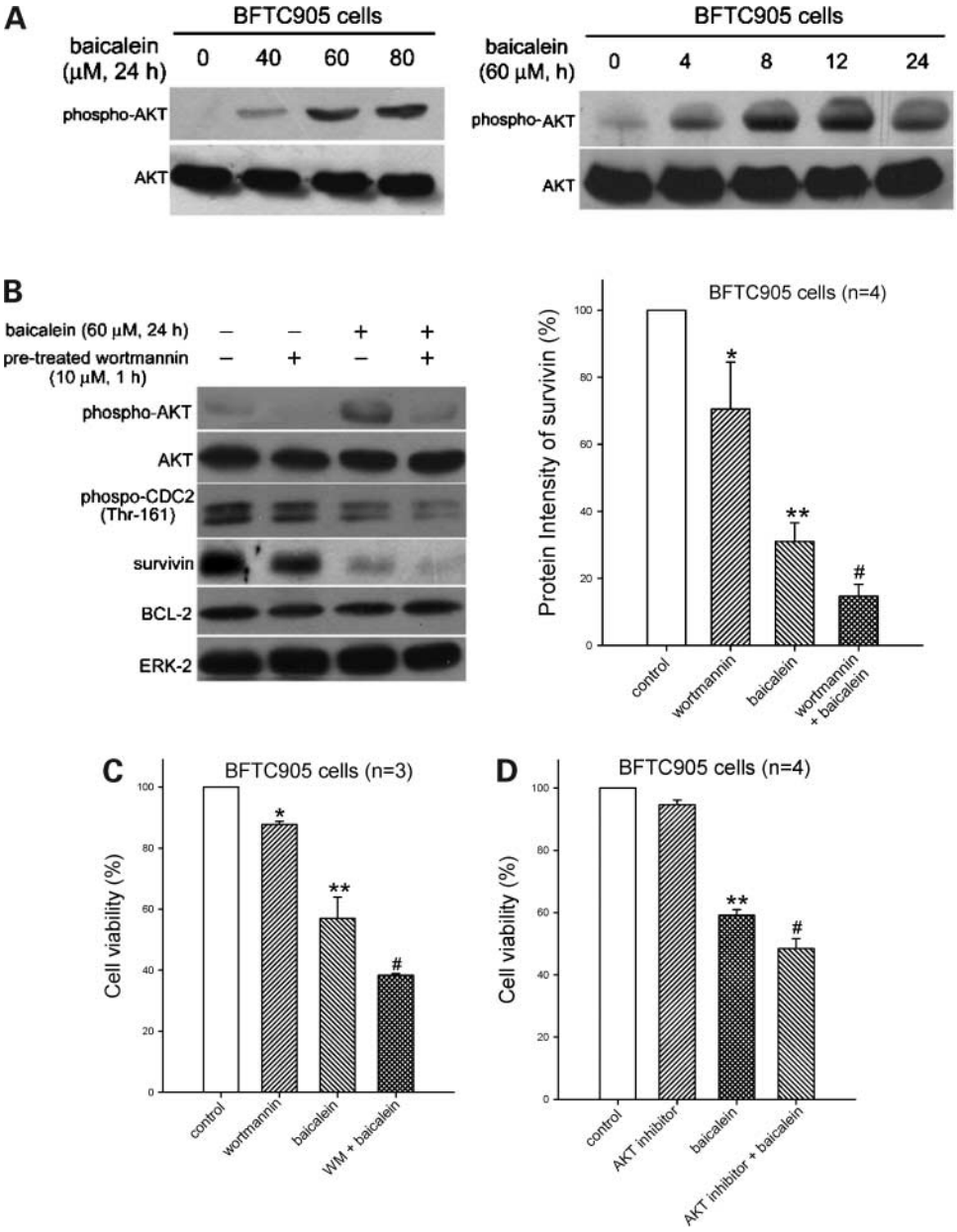

Figure 6. Effects of AKT on the survivin protein expression and the cell survival after treatment with baicalein in bladder cancer cells. **A,** BFTC905 cells were treated with or without baicalein. The protein levels of phospho-AKT and AKT were analyzed by Western blot. **B,** the cells were pretreated with 10 μmol/L wortmannin for 1 h before exposure to 40 μmol/L baicalein for 24 h. Total cell extracts were analyzed by Western blot. Representative Western blot data were shown from one of three to four experiments with similar findings. The protein intensity of survivin was quantified from Western blots. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and baicalein- or wortmannin-treated samples. #, $P < 0.05$, significant difference between baicalein alone and pretreatment with wortmannin. **C** and **D,** the cells were pretreated with 10 μmol/L wortmannin or 1*L*-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate for 1 h before exposure to 40 μmol/L baicalein for 24 h. After treatment, the cells were recultured for 2 d. The cell viability was measured by MTT assay. *Columns,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, significant difference between control and wortmannin- or baicalein-treated samples. #, $P < 0.05$, significant difference between baicalein alone and pretreatment with inhibitors.

(Fig. 5C). In addition, SB203580 attenuated the growth inhibition in the baicalein-exposed cells (Fig. 5D).

Inhibition of the Phosphorylation of AKT Increases the Cell Death and Enhances the Decreases of Phospho-CDC2 and Survivin Proteins in the Baicalein-Exposed Bladder Cancer Cells

Treatment with baicalein at 40 to 80 μmol/L for 24 h or 60 μmol/L for 4 to 24 h increased the phosphorylation of AKT in BFTC905 cells (Fig. 6A). Inhibition of the baicalein-induced AKT phosphorylation by wortmannin (a phosphatidylinositol 3-kinase inhibitor) enhanced the protein losses of phospho-CDC2 (Thr^{161}) and survivin (Fig. 6B). The quantified data showed that wortmannin significantly reduced the survivin protein expression and enhanced the baicalein-inhibited survivin expression (Fig. 6B). However, the BCL-2 protein level was not altered by baicalein or wortmannin (Fig. 6B). The cytotoxicity was promoted by pretreatment with wortmannin in the baicalein-exposed cells (Fig. 6C). Consistently, 1*L*-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (a specific AKT inhibitor) at 10 μmol/L treatment (38, 39) increased the baicalein-induced cell death (Fig. 6D).

Discussion

Baicalein more effectively induced cytotoxicity compared with other flavonoids (baicalin, catechin, genistein, quercetin, and rutin) in bladder cancer cells. Nevertheless, the

natural flavonoid baicalein displays low cytotoxicity to human normal cells. Previously, we reported that quercetin induced apoptosis but increased survivin protein expression (32). However, the protein level of survivin was not altered by genistein (26). Interestingly, the survivin protein expression was dramatically inhibited by baicalein in bladder cancer cells. Baicalein also blocked survivin expression in lung and breast cancer cells (data not shown). Transfection of survivin siRNA increased the baicalein-induced cytotoxicity; conversely, overexpression of survivin by a survivin-expressed vector enhanced cancer cell proliferation and resisted cell death from baicalein treatment. Accordingly, the blockage of survivin expression by baicalein mediates the apoptosis and proliferation inhibition in human cancer cells. Although this study provides the potential cancer therapy of baicalein by human cancerous cells *in vitro*, the human cancer therapeutics by baicalein or combination of the survivin gene knockdown need to be determined by *in vivo* model before clinical trials. Moreover, the possible pharmacokinetic and toxicologic barriers need further characterization.

The survivin activity and stability resulted from the phosphorylation of Thr^{34} by the mitotic kinase complex CDC2/cyclin B1 (31, 33). The activation of CDC2 is through the phosphorylation of Thr^{161} by CDC2-activating kinase and the dephosphorylation of Thr^{14} and Tyr^{15} by CDC25C phosphatase (40). Quercetin increases the survivin protein expression, which correlates with raising the protein levels of cyclin B1 and phospho-CDC2 (Thr^{161}; ref. 32). In contrast, baicalein reduced the cyclin B1 and phospho-CDC2 (Thr^{161}) proteins in bladder cancer cells. Cpd 5 and NSC 663284 have been shown to inhibit the CDC25 phosphatases, which cause the loss of CDC2 kinase activity (41, 42). Alsterpaullone is a CDC2 kinase inhibitor (43). Both CDC25 phosphatase and CDC2 kinase inhibitors enhanced the baicalein-induced cancer cell death. Indeed, it has been shown that baicalein can directly inhibit CDC2 kinase activity (44). Therefore, we suggest that the inhibition of CDC2/cyclin B1 by baicalein contributes to the reduction of survivin and the proliferation inhibition in cancer cells.

Interestingly, baicalein simultaneously induces the phosphorylation of p38 MAPK and AKT. Activation of p38 MAPK has been associated with the induction of apoptosis in response to various cellular stresses (15, 45, 46). In contrast, the activation of AKT provides a survival signal to protect cells from apoptosis (19–21). The phospho-CDC2 (Thr^{161}) and survivin proteins were restored by SB203580 (p38 MAPK inhibitor) in the baicalein-treated bladder cancer cells. It has been reported that the regulation of CDC25B phosphorylation by p38 MAPK is a critical event for initiating the G_2-M checkpoint after UV radiation (47). Accordingly, we suggest that the activation of p38 MAPK by baicalein inhibits the CDC2 kinase activity and the survivin expression for mediating the G_2-M arrest. Moreover, p38 MAPK can serve as a mediator of caspase-3–associated apoptosis (14, 48, 49). The loss of survivin expression disrupted antiapoptosis function and increased caspase-3 activity (30). SB203580 was effective in protecting bladder cancer cells from baicalein-mediated cell death. Thus, these findings suggest that p38 MAPK may be involved in the induction of apoptosis by inhibiting CDC2-survivin pathway in the baicalein-treated cancer cells.

The blockade of phosphatidylinositol 3-kinase-AKT pathway by phosphatidylinositol 3-kinase or AKT inhibitors enhanced the reduction of phospho-CDC2 (Thr^{161}) and survivin proteins and promoted the cytotoxicity in the baicalein-exposed cells. The inhibition of AKT pathway down-regulates survivin expression and enhances apoptosis in cancer cells (21). Recently, anticancer drug cisplatin activates AKT, which attenuates apoptosis by the up-regulation of survivin (50). Therefore, baicalein elicits the activation of AKT that may be from self-protection of cancer cells to resist cell death by which they defended the survivin level.

Taken together, we conclude that p38 MAPK and AKT display the opposite roles on the regulation of survivin expression in the baicalein-induced apoptosis. The blockade of CDC2-survivin pathway by baicalein mediates the induction of apoptosis and proliferation inhibition in human cancer cells. Baicalein may act as a potent inhibitor of CDC2-survivin for the chemoprevention and antitumorigenesis of cancers.

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