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Opposite Expression of Securin and γ-H2AX Regulates Baicalein-Induced Cancer Cell Death

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ABSTRACT

Securin and γ-H2AX have been shown to regulate cell survival and genomic stability. However, it is still unknown how the expression and regulation of these proteins is altered following treatment with baicalein, a natural flavonoid extracted from the *Scutellaria baicalensis* root. In the present study, we investigate the possible roles of securin and γ-H2AX in baicalein-induced cancer cell death. Baicalein reduced cell viability in a variety of human cancer cell lines, including bladder, cervical, colon, and lung cancer cells. Interestingly, baicalein treatment (40–80 μM for 24 h) markedly inhibited securin expression, while the levels of γ-H2AX were elevated. Abnormal spindle formation and chromosomal segregation were induced by baicalein. Furthermore, wild type HCT116 cancer cells had a higher incidence of cytotoxicity and apoptosis than securin-null HCT116 cells following treatment with baicalein. In contrast, baicalein increased the levels of γ-H2AX to a similar extent in both cell types. Transfection with H2AX siRNA further increased baicalein-induced cell death. Additionally, blockade of the AKT pathway by treatment with wortmannin or AKT shRNA lowered the levels of γ-H2AX and enhanced cytotoxicity in baicalein-treated cells. Taken together, our findings suggest that the opposing effects of baicalein on securin and γ-H2AX levels may be involved in the regulation of cell viability and genomic stability by this compound. J. Cell. Biochem. 111: 274–283, 2010.

KEY WORDS: BAICALEIN; SECURIN; Γ-H2AX; AKT; CELL VIABILITY; APOPTOSIS

Baicalein, a bioactive natural flavonoid extracted from the roots of *S. baicalensis* and *Scutellaria radix* exerts anticancer activity [Bonham et al., 2005; Ma et al., 2005; Miocinovic et al., 2005]. Flavonoids are a group of polyphenolic compounds that exist in many fruits, vegetables, and all vascular plants [Havsteen, 1983; Di Carlo et al., 1999]. Some flavonoids contain anticancer and chemopreventive activities [Plaumann et al., 1996; Di Carlo et al., 1999; Kobayashi et al., 2002; Naasani et al., 2003]. It has been shown that baicalein induces apoptosis in a variety of human cancer cells [Kuntz et al., 1999; Chen et al., 2000; Pidgeon et al., 2002; Lee et al., 2005; Ma et al., 2005]. Baicalein induces higher bladder cancer cell death than other flavonoids, including baicalin, catechin, genistein, quercetin, and rutin [Chao et al., 2007]. In addition to inducting apoptosis, baicalein leads to growth inhibition in cancer cells [Bonham et al., 2005; Ma et al., 2005; Miocinovic et al., 2005]. Baicalein also enacts anticancer activity by inhibiting platelet-type 12-lipoxygenase (12-LOX), which has been shown to regulate growth, metastasis, and angiogenesis in prostate cancer [Pidgeon et al., 2002].

Securin is also called the pituitary-tumor transforming gene (PTTG) [Pei and Melmed, 1997; Dominguez et al., 1998; Heaney et al., 1999; Saez et al., 1999; Zou et al., 1999]. During normal cell division, securin acts as an anaphase inhibitory protein in order to prevent abnormal chromosomal segregation and maintain genomic stability [Yamamoto et al., 1996; Jallepalli et al., 2001; Stemmann et al., 2001; Chao et al., 2006]. Securin is overexpressed in various human cancer cells [Dominguez et al., 1998; Heaney et al., 1999; Saez et al., 1999; Zou et al., 1999]. Furthermore, it promotes cancer cell proliferation and tumorigenesis [Zhang et al., 1999; Zou et al., 1999; Hamid et al., 2005]. However, overexpression of securin induces apoptosis [Yu et al., 2000; Hamid and Kakar, 2004], aneuploidy [Christopoulou et al., 2003], and genomic instability

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[Kim et al., 2005, 2007]. Thus, securin can play different roles in the regulation of cell survival, apoptosis, and genomic stability under various cellular conditions or treatments.

The efficacy of anticancer drugs during cancer therapy can be influenced by the induction of apoptotic or survival signaling pathways. The phosphatidylinositol 3-kinase (PI3K)-AKT pathway has been shown to mediate cell survival [Kulik et al., 1997; Downward, 1998; Cross et al., 2000]. The activation of AKT prevents cell death by the blocking apoptotic pathways [Downward, 1998; Martelli et al., 2003; Kim et al., 2004]. It has been shown that the phosphorylation of AKT stimulates cell survival by leading to the phosphorylation and inactivation of downstream apoptotic proteins, such as the caspases and Bad [Datta et al., 1997; Cardone et al., 1998]. H2AX is a variant of the histone H2A that maintains genomic stability [Paull et al., 2000; Redon et al., 2002]. The phosphorylation of H2AX at serine-139, which creates the γ-H2AX protein, can be activated by DNA damaging agents [Paull et al., 2000; Redon et al., 2002]. γ-H2AX also has a role in the modulation of caspase-mediated apoptosis [Rogakou et al., 2000].

In this study, the role of securin and γ-H2AX in the regulation of baicalein-induced cancer cell death was investigated. Interestingly, the protein level of securin was markedly reduced by baicalein in human cancer cells. Wild type cancer cells were more susceptible to cell death than securin-null cells following treatment with baicalein. In contrast, baicalein elevated the protein levels of phosphorylated AKT and γ-H2AX. Blocking the AKT and γ-H2AX pathways increased baicalein-induced cytotoxicity. Herein, we communicate the novel insight that the presence of securin and the activation of AKT and γ-H2AX may underlie the regulation of cell viability by baicalein.

MATERIALS AND METHODS

CHEMICALS AND ANTIBODIES

Baicalein, wortmannin, Hoechst 33258, and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA). Anti-AKT (#9272) and anti-phospho-AKT (serine-473) (#9271) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-ERK-2 (C-14), anti-GFP (FL), goat anti-rabbit IgG horseradish peoxidase, goat anti-mouse IgG horseradish peoxidase, and the FITC (fluorescein isothiocyanate)-labeled goat anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-securin antibody was purchased from Abcam (Cambridge, MA). The γ-H2AX antibody was purchased from Upstate (Temecula, CA).

CELL CULTURE

BFTC905 cells were derived from the human bladder carcinoma. A549 cell line was derived from the lung adenocarcinoma. RKO was a colon carcinoma cell line. HeLa was a cervical cancer cell line. The securin-wild type and securin-null HCT116 colorectal cancer cell lines were kindly provided by Dr. B. Vogelstein of the Johns Hopkins University. BFTC905 and A549 cells were cultured in RPMI-1640 medium (Invitrogen). RKO and HeLa cells were maintained in DMEM medium (Invitrogen). The securin-wild type and securin-null HCT116 cells were maintained in McCoy's 5A medium (Sigma Chemical). The complete medium was supplemented with 10% fetal bovine serum (FBS) and contained 100 units/ml penicillin and 100 μg/ml streptomycin. These cells were cultured at 37°C and 5% CO_2 in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

CELL VIABILITY ASSAY

To evaluate the cytotoxicity of baicalein in BFTC905, A549, RKO, HeLa and HCT116 cell lines, the cells were plated in 96-well plates at a density of 1×10^4 cells/well in complete medium for 16–20 h. Thereafter, the cells were treated with 10–80 μM of baicalein for 24 h. At the end of treatment, the cells were washed with phosphate-buffered saline (PBS), and re-cultured for 2 days. Finally, the cells were incubated with 0.5 mg/ml of MTT for 4 h and then dissolved in DMSO. The intensity of formazan was measured at 565 nm using a plate reader (Molecular Dynamics, OPTImax). The relative percentage of cell viability was calculated by dividing the absorbance of treatment by that of the control in each experiment.

ANNEXIN V-PI STAINING

The level of apoptosis induced by baicalein was determined by annexin V-propidium iodide (PI) analysis. The cells showed Annexin V^+/PI^- and Annexin V^+/PI^+, which indicated at early and late apoptosis, respectively. The annexin V-PI staining was used to examine the cells by incubated with fluorescein isothiocyanate (FITC)-conjugated-Annexin V and PI according to the manufacturer's instruction (BioVision, Mountain View, CA). After treatment with baicalein, the cell number of one million cells was collected by centrifugation at 200*g* for 5 min. Thereafter, the cells were incubated with Annexin V-PI labeling solution at 25°C for 5 min. Finally, the samples were immediately analyzed by flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA). The percentage of Annexin V-PI staining cells was quantified from a minimum of 10,000 cells by CellQuest software (BD Biosciences).

IMMUNOFLUORESCENCE STAINING AND CONFOCAL MICROSCOPY

To examine the location and expression of securin and γ-H2AX proteins, the cells were subjected to immunofluorescence staining and confocal microscopy as described previously [Chao and Liu, 2006]. At the end of treatment, the cells were fixed with 4% paraformaldehyde solution, and then washed three times with PBS. The non-specific binding sites were blocked in PBS containing 10% FBS and 0.3% Triton X-100 for 1 h. Thereafter, the cells were incubated with mouse anti-securin (1:50) or anti-γ-H2AX (1:100) antibodies in PBS containing 10% FBS overnight at 4°C, and washed three times with 0.3% Triton X-100 in PBS. Then the cells were incubated with goat anti-mouse FITC (1:50) in PBS for 2.5 h at 37°C. The nuclei (chromosomes) and β-tubulin were stained with Hoechst 33258 and the Cy3-labeled anti-β-tubulin, respectively. Finally, the cells were observed under a laser scanning confocal microscope (Leica, Wetzlar, Germany).

WESTERN BLOT

Total cellular protein extracts were prepared as described [Chao and Liu, 2006]. Western blot analyses of securin, phospho-AKT, AKT, γ-H2AX, ERK-2, and GFP were performed using specific antibodies. Briefly, the proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel, and electrophoretic transfer of proteins onto polyvinylidene difluoride membranes. The membranes were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated secondary antibody. Thereafter, the protein bands were visualized followed by detection with a chemiluminescence kit (PerkinElmer Life and Analytical Sciences, Boston, MA).

TRANSFECTION

BFTC905 cells (2×10^6 cells/60-mm Petri dish) were transfected with control siRNA, H2AX siRNA, control shRNA pSuper-EGFP vector, and AKT shRNA pSuper-EGFP vector by using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). A control siRNA (5′-GUCAACGGAUUUGGUCGA-3′) and four H2AX siRNAs (5′-GGGACGAAGCACUUGGUAA-3′; 5′-CGACUAGAACCUUAGGCAU-3; 5′-GGAAA GAGCUGAGCCGCUU-3′; 5′-GAACUGGAAUUCUGCAGCU-3′ (Dharmacon, Lafayette, CO) were employed for transfection in BFTC905 cells. The cells were transfected with 50–100 nM of control or H2AX siRNAs by using Lipofectamine™ 2000 reagent according to the manufacturer's recommendations. In addition, BFTC905 cells were transfected with 10–50 μg control or AKT shRNA pSuper-EGFP vectors. After transfection with siRNAs or vectors, the cells were subjected to cytotoxicity or Western blot assays as described above.

STATISTICAL ANALYSIS

Data were analyzed by one-way or two-way analysis of variance (ANOVA), and further post-hoc tests using the statistic software of GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). Each experiment was repeated at least 3 times. A P-value of <0.05 was considered as statistically significant.

RESULTS

BAICALEIN REDUCES CELL VIABILITY IN HUMAN CANCER CELLS

A variety of human cancer cells, including colorectal (RKO), bladder (BFTC905), cervical (HeLa), and lung (A549) cancer cells, were exposed to baicalein and analyzed by cytotoxic MTT assays. Baicalein (20–80 μM for 24 h) decreased cell viability in a concentration-dependent manner in these cancer cells (Fig. 1A). Moreover, treatment with 80 μM of baicalein for 24 h significantly increased cytotoxicity in all cell types. However, BFTC905 cells had relatively higher levels of cell death than RKO, HeLa or A549 cells following baicalein treatment. We further analyzed baicalein-induced apoptosis by Annexin V-PI staining assays. As shown in Figure 1B, treatment with 60 μM of baicalein for 24 h increased the number of Annexin V^+/PI^- cells (cells undergoing early apoptosis) by an average of 10.1% and the number of Annexin V^+/PI^+ cells (cells undergoing late apoptosis) by an average of 19.4% in BFTC905 cells.

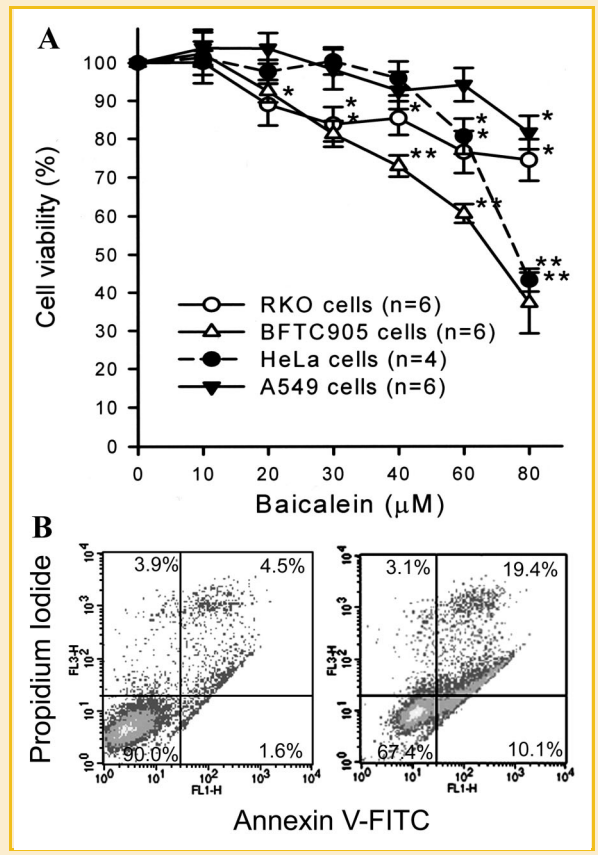


Fig. 1. Effect of baicalein on cell viability in various human cancer cells. A: RKO, BFTC905, HeLa, and A549 cancer cells were separately treated with 0–80 μM of baicalein for 24 h. After treatment, cells were re-cultured for 2 days. Cell viability was measured by MTT assay. The results were obtained from four to six experiments. Bars represent mean ± SE. $^*P<0.05$ and $^{**}P<0.01$ indicate a significant difference between control and baicalein-treated samples. B: BFTC905 cells were treated with or without 60 μM of baicalein for 24 h. Apoptosis was determined by Annexin V-PI staining using flow cytometry analysis. Cells staining with Annexin V^+/PI^- are those undergoing early apoptosis (lower right), whereas Annexin V^+/PI^+ stained cells are undergoing late apoptosis (upper right). Populations of apoptotic cells were quantified using CellQuest software. Data are shown from one of three independent experiments with similar findings.

SECURIN-EXPRESSING HCT116 CELLS DISPLAY HIGHER CELL DEATH THAN SECURIN-NULL CELLS FOLLOWING BAICALEIN TREATMENT

To examine the role of securin in baicalein-induced cytotoxicity, securin-expressing, wild type HCT116 colorectal cancer cells and their securin-null counterparts were subjected to MTT assays. As shown in Figure 2, baicalein reduced cell viability in both wild type and securin-null HCT116 cells. We found that wild type HCT116 cells exhibited a greater susceptibility to cell death than securin-null HCT116 cells when exposed to 40–80 μM of baicalein (Fig. 2A). Furthermore, treatment with 60 μM of baicalein for 24 h increased the levels of Annexin V^+/PI^- and Annexin V^+/PI^+ cells in both wild type and securin-null HCT116 cells (Fig. 2B). However, wild type cells displayed a relatively higher incidence of apoptosis than

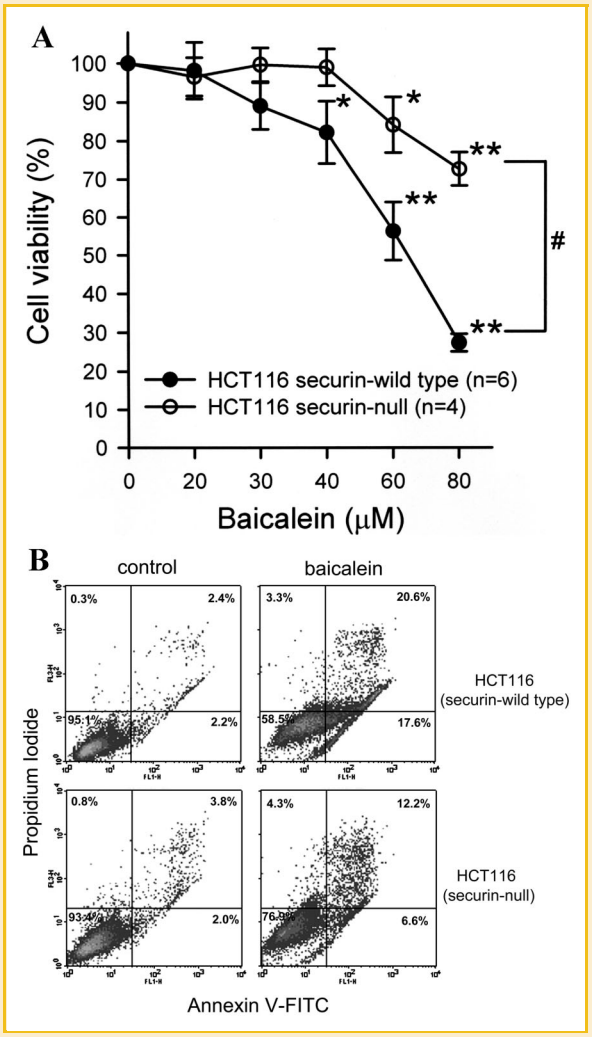


Fig. 2. Comparison of cytotoxicity and apoptosis after treatment with baicalein between wild type and securin-null HCT116 colorectal cancer cells. A: Cells were treated with or without baicalein for 24 h. After treatment, cells were re-cultured for 2 days. Cell viability was measured by MTT assay. The results were obtained from four to six experiments and bars represent the mean ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference between the control and baicalein-treated samples in wild type or securin-null HCT116 cells. $^{\#}P < 0.05$ indicates a significant difference between wild type and securin-null HCT116 cells in the context of baicalein treatment. B: Wild type and securin-null cells were treated with or without 60 μM of baicalein for 24 h. Apoptosis was determined by Annexin V-PI staining using flow cytometry analysis. The population of Annexin V^+/PI^- cells represents cells undergoing early apoptosis (lower right), whereas the fraction of Annexin V^+/PI^+ cells are those undergoing late apoptosis (upper right). Populations of apoptotic cells were quantified using CellQuest software. Data are shown from one of three separate experiments with similar findings.

securin-null cells following exposure to baicalein (approximately 20% more apoptotic cells).

BAICALEIN INHIBITS SECURIN EXPRESSION IN CANCER CELLS

We examined the protein expression and localization of securin in BFTC905 cells by immunofluorescence staining and confocal microscopy. Red fluorescence indicates the location of microtubules (cytoskeleton) that were stained with Cy3-labeled anti-β-tubulin antibody, and blue fluorescence shows the location of nuclei or chromosomes that were stained with the Hoechst 33258 dye (Fig. 3A,C). Green fluorescence indicates the location of securin, which is highly expressed during prophase and metaphase in BFTC905 cells (Fig. 3A, arrows). Treatment with 20–80 μM of baicalein for 24 h reduced the levels of securin protein in a concentration-dependent manner in BFTC905 cells (Fig. 3B). Protein levels of ERK2 were used as an internal control [Kuo et al., 2004; Chao et al., 2007], as these levels were not affected by baicalein treatment. Baicalein also caused irregular anaphase progression, abnormal spindles (stars), and aberrant chromosomal segregation (arrows) in these cells (Fig. 3C).

BLOCKING AKT SIGNALING BY TREATMENT WITH WORTMANNIN OR shRNA TARGETING AKT ENHANCES BAICALEIN-INDUCED CANCER CELL DEATH

Treatment with 20–80 μM of baicalein for 24 h increased the phosphorylation of AKT in a concentration-dependent manner in BFTC905 cells (Fig. 4A). Wortmannin (WB), a PI3K-AKT pathway inhibitor, enhanced baicalein-induced cancer cell death (Fig. 4B). To further examine the role of AKT in the regulation of baicalein-induced cytotoxicity, an AKT shRNA was expressed from the pSuper-EGFP vector. Both the empty vector and vector encoding AKT shRNA expressed green fluorescent protein (GFP) in BFTC905 cells (Fig. 4C, lower bands). Transfection with 10 μg of AKT shRNA reduced the protein levels of AKT (Fig. 4C). Following transfection with AKT shRNA, BFTC905 cells exhibited increased sensitivity to baicalein-induced cell death (Fig. 4D).

BAICALEIN ELEVATES γ-H2AX EXPRESSION IN HUMAN CANCER CELLS

The effect of baicalein on the protein expression and localization of γ-H2AX were analyzed in BFTC905 cells by Western blot and confocal microscopy, respectively. Treatment with 40–80 μM of baicalein increased γ-H2AX protein expression in a concentration-dependent manner (Fig. 5A). Baicalein treatment (60-μM for 4–24 h) also increased γ-H2AX protein levels in a time-dependent manner (Fig. 5B). In confocal imaging, green fluorescence indicates the location of γ-H2AX. The protein was present in the nucleus of BFTC905 cells treated with 60 μM of baicalein for 24 h (Fig. 5C, arrows). Transfection with 100 nM of H2AX siRNA for 24 h increased cell death in BFTC905 cells by ~20% (Fig. 5D). Moreover, transfection with H2AX siRNA further increased baicalein-induced cancer cell death.

BAICALEIN ACTIVATION OF AKT AND γ-H2AX DOES NOT CORRELATE WITH SECURIN INHIBITION

Baicalein inhibits securin expression in wild type HCT116 cells but not in securin-null cells (Fig. 6). Treatment with 20–80 μM of baicalein for 24 h elevated the levels of phosphorylated AKT and γ-H2AX in both wild type and securin-null HCT116 cells to a similar degree. Total levels of AKT and ERK2 were not altered by baicalein. Baicalein also increased the levels of phosphorylated AKT and γ-H2AX in BFTC905 cells (Fig. 7). Additionally, we found that treatment with the AKT pathway inhibitor, wortmannin, decreased

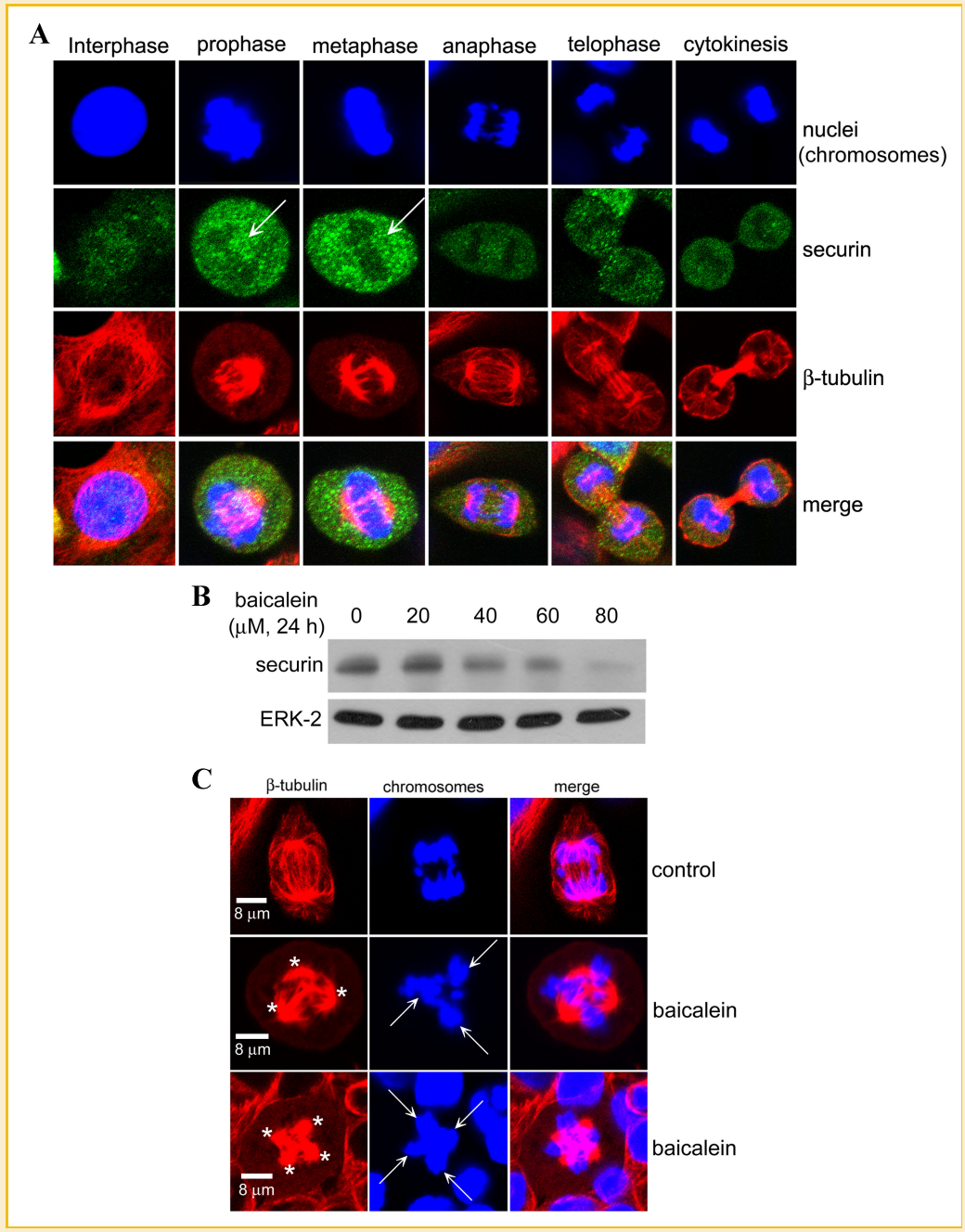


Fig. 3. Effect of baicalein on securin protein expression in BFTC905 cancer cells. A: β-tubulin was stained with Cy3-labeled mouse anti-β-tubulin antibody that has red fluorescence. Blue fluorescence indicates nuclei or chromosomes following staining with Hoechst 33258. Green fluorescence indicates the location of securin. Securin was highly expressed during prophase and metaphase in BFTC905 cells (arrows). B: BFTC905 cells were treated with 0–80 μM of baicalein for 24 h. At the end of treatment, total protein extracts were prepared for Western blot analysis. Representative Western blot data are shown from one of three separate experiments with similar findings. C: Cells were treated with or without 60 μM of baicalein for 24 h. β-Tubulin and nuclei were stained with Cy3-labeled mouse anti-β-tubulin antibody and Hoechst 33258, respectively. Stars indicate abnormal spindles. Arrows indicate abnormal chromosomal segregation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the levels of phosphorylated AKT and γ-H2AX in these baicalein-treated cells. In contrast, securin expression was not significantly altered by wortmannin in baicalein-exposed BFTC905 cells. Furthermore, transfection of BFTC905 cells with 50–100 nM of H2AX siRNA for 48 h lowered γ-H2AX expression but did not alter the levels of phospho-AKT, total AKT, or securin (Fig. 8A). We have further demonstrated that the expression of AKT and securin were not altered when baicalein-treated cells were transfected with H2AX siRNA (Fig. 8B).

DISCUSSION

Baicalein is a bioactive flavonoid extracted from the roots of *Scutellaria baicalensis* and *S. radix* that has been evaluated for use

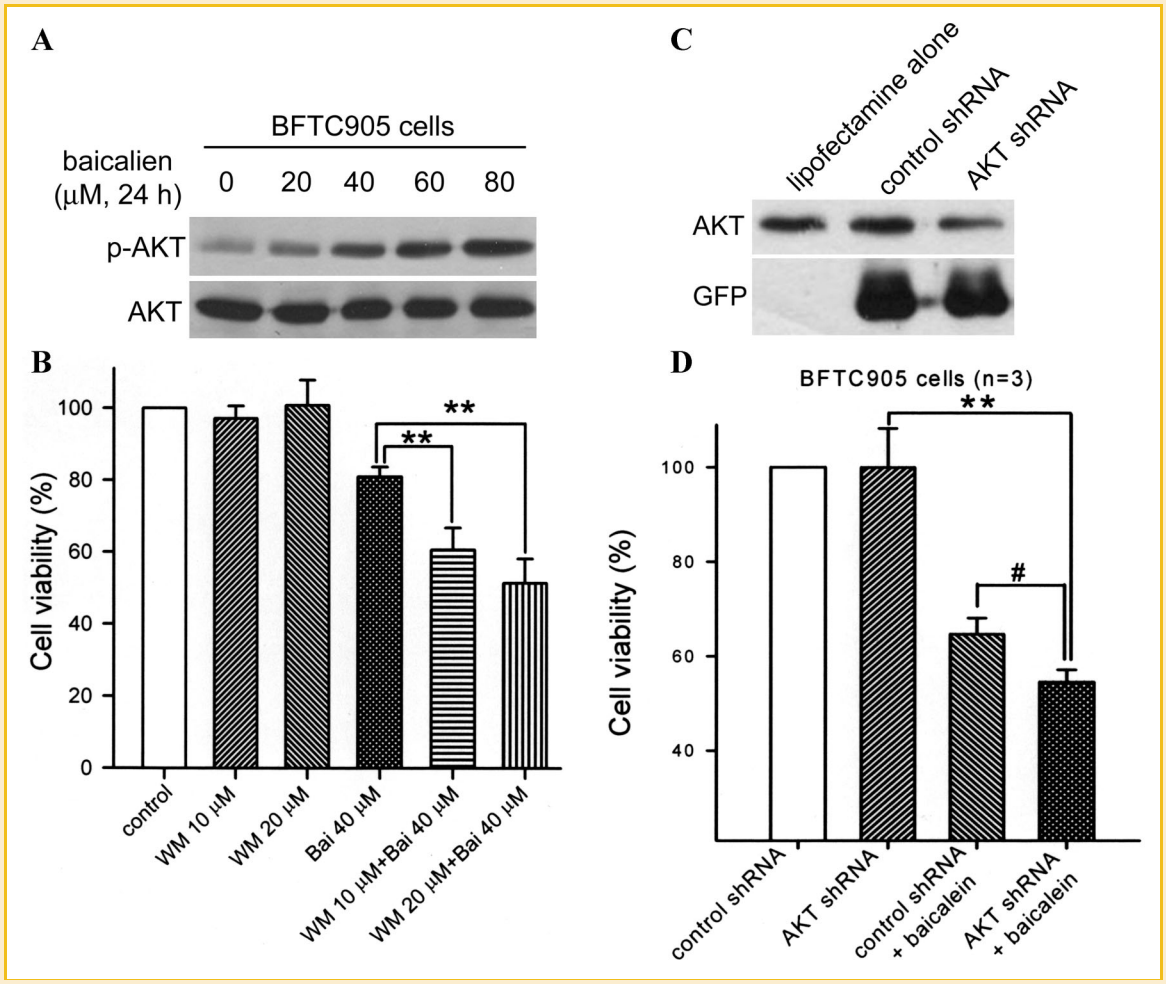
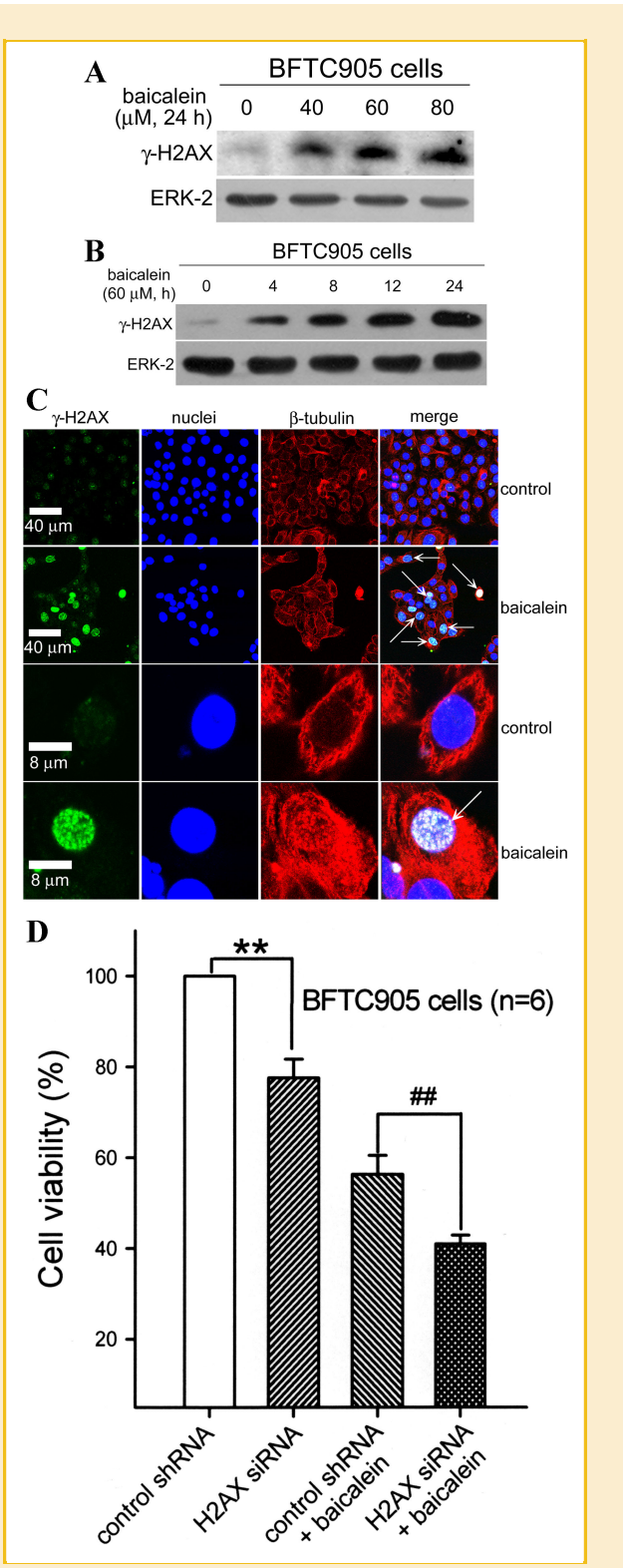


Fig. 4. Effect of wortmannin and AKT shRNA on cell viability in baicalein-treated BFTC905 cells. A: After baicalein treatment, total protein extracts were prepared for Western blot analysis using specific anti-phospho-AKT and anti-AKT antibodies. Representative Western blot data are shown from one of three experiments with similar findings. B: BFTC905 cells were pre-treated with 10 or 20 μM of wortmannin for 1 h before exposure to 40 μM of baicalein for 24 h. Cell viability was measured by MTT assay. The results were obtained from eight experiments. Bars represent the mean ± SE. $^{**}P < 0.01$ indicates a significant difference between control and wortmannin-treated cells in the context of baicalein treatment. C: BFTC905 cells were transfected with 10 μg of empty pSuper-EGFP vector or with pSuper-EGFP encoding AKT shRNA for 48 h. The levels of AKT and GFP were determined by Western blots using specific anti-AKT and anti-GFP antibodies, respectively. D: Cells were transfected with 50 μg of empty pSuper-EGFP vector or with pSuper-EGFP encoding AKT shRNA for 24 h before exposure to 40 μM of baicalein for 24 h. At the end of treatment, cell viability was measured by MTT assay. The results were obtained from three experiments. Bars represent the mean ± SE. $^{**}P < 0.01$ indicates a significant difference between control and baicalein-treated samples. $^{\#}P < 0.05$ indicates a significant difference between control and AKT shRNA-treated cells in the context of baicalein treatment.

in cancer therapy. In this report, we demonstrate that baicalein increases cytotoxicity in a variety of human cancer cells, including bladder, colorectal, cervix, and lung cells. Human bladder cancer cells showed a relatively greater level of cytotoxicity in response to baicalein treatment. Moreover, baicalein is more effective at inducing cytotoxicity in human bladder cancer cells than other flavonoids, including baicalin, catechin, genistein, quercetin and rutin [Chao et al., 2007]. Accordingly, baicalein is a potential natural flavonoid that may be developed for bladder cancer prevention and therapy.

The inhibition of survival pathways or the induction of apoptosis pathways by anticancer agents induces the inhibition of cancer cell proliferation, a phenomenon that may be exploited for cancer therapy. The securin protein is also referred to as vSecurin or PTTG [Pei and Melmed, 1997; Dominguez et al., 1998; Saez et al., 1999; Zou et al., 1999]. Securin is overexpressed in a variety of human cancer cells [Dominguez et al., 1998; Heaney et al., 1999; Saez et al., 1999; Zou et al., 1999]. This protein mediates cancer cell proliferation and tumorigenesis [Kakar and Jennes, 1999; Zhang et al., 1999]. Securin can interact with p53 to block its transcriptional activity, which prevents apoptosis of human lung cancer cells [Bernal et al., 2002]. In contrast, the overexpression of securin triggers apoptosis [Yu et al., 2000]. It has been shown that when securin mediates apoptosis, it does so by inducing p53 expression [Hamid and Kakar, 2004]. Therefore, securin can play varying roles in cell survival and apoptosis. We have found that baicalein markedly attenuates securin expression in human bladder and colorectal cancer cells. Interestingly, wild type cancer cells exhibited higher cytotoxicity than securin-null cancer cells following baicalein treatment. These findings indicate that the

presence of securin may increase baicalein-induced cancer cell death. Accordingly, we propose that securin may interact with p53, and baicalein-induced apoptosis may be mediated by p53-dependent downstream gene expression. However, the precise role of p53 activation in the regulation of apoptosis by baicalein should be further investigated.



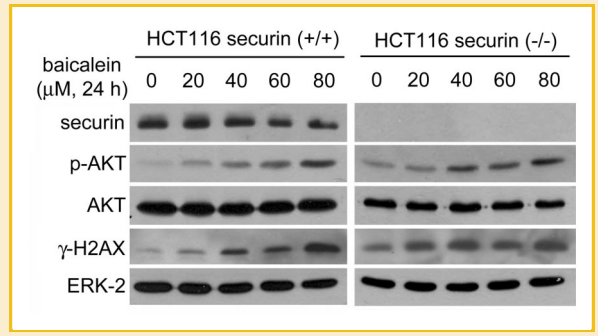


Fig. 6. Comparison of the expression of securin, AKT, and γ-H2AX between wild type and securin-null HCT116 cells after treatment with baicalein. Cells were treated with 0–80 μM of baicalein for 24 h. At the end of treatment, total protein extracts were prepared for Western blots with anti-securin, anti-phospho-AKT, anti-AKT, anti-γ-H2AX, and anti-ERK2 antibodies. Representative Western blot data are shown from one of three separate experiments with similar findings.

During normal cell division, securin prevents abnormal sister chromatid separation during mitotic progression by binding with separase [Jallepalli et al., 2001; Nasmyth, 2001; Stemmann et al., 2001]. At the metaphase-anaphase transition, securin is degraded, allowing the release of separase, which mediates the separation of sister chromatids by cleaving chromosomal cohesin [Nasmyth, 2001; Stemmann et al., 2001]. Securin has been shown to prevent aberrant chromosomal segregation when cellular DNA or spindles are damaged [Funabiki et al., 1996; Yamamoto et al., 1996; Chao et al., 2006]. Chromosomal aberrations were found in securin knockout mice [Wang et al., 2001; Chesnokova et al., 2005]. We found that securin was highly expressed in BFTC905 cancer cells during prophase and metaphase. Baicalein markedly reduced securin levels and induced abnormal spindle formation and chromosomal segregation. Chromosomal aberration is associated with the progression of carcinogenesis [Lengauer et al., 1997; Lengauer et al., 1998]. However, if the chromosomal damage is beyond repair, the cells will enter into apoptosis. It has been reported that securin is required for genomic stability during mitosis

Fig. 5. Effect of baicalein on γ-H2AX phosphorylation in BFTC905 cells. A: Cells were treated with 0–80 μM of baicalein for 24 h. B: BFTC905 cells were treated with 60 μM of baicalein for 0–24 h. At the end of treatment, total protein extracts were prepared for Western blot analysis. Representative Western blot data are shown from one of three separate experiments with similar findings. C: Cells were treated with or without 60 μM of baicalein for 24 h. γ-H2AX is indicated by green fluorescence from staining with goat anti-mouse antibody conjugated to FITC. β-Tubulin and nuclei were stained with Cy3-labeled mouse anti-β-tubulin antibody and Hoechst 33258, respectively. Arrows indicate γ-H2AX in the nucleus. D: Cells were transfected with 100 nM of control or H2AX siRNA for 24 h before exposure to 40 μM of baicalein for 24 h. Cell viability was measured by MTT assay. The results were obtained from 6 separate experiments. Bars represent the mean ± SE. $^{**}P < 0.01$ indicates a significant difference between control and H2AX siRNA-treated samples. $^{\#\#}P < 0.05$ indicates a significant difference between control and H2AX siRNA-treated cells in the context of baicalein treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

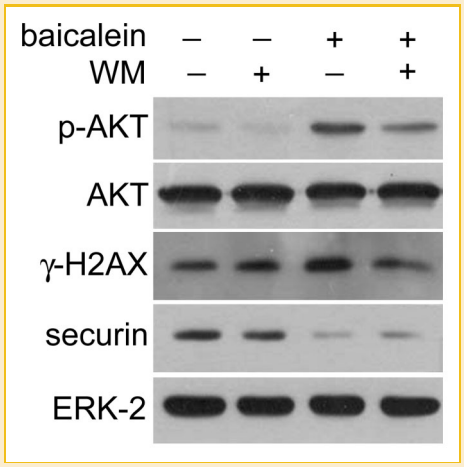


Fig. 7. Effect of wortmannin on the levels of securin, AKT, and γ-H2AX in baicalein-treated cells. BFTC905 cells were pre-treated with or without 10 μM of wortmannin for 24 h before exposure to 60 μM of baicalein for 24 h. The levels of phospho-AKT, AKT, γ-H2AX, securin, and ERK2 were analyzed by Western blot. Representative Western blot data are shown from one of three separate experiments with similar findings.

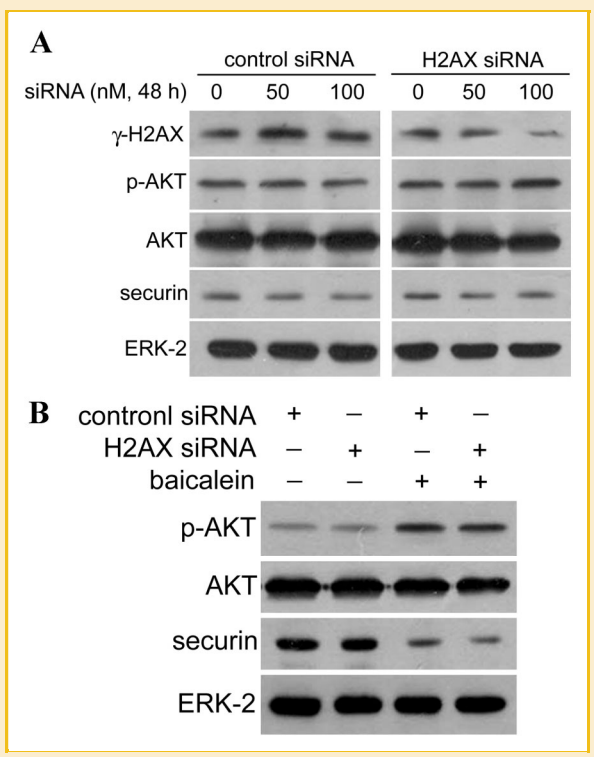


Fig. 8. Effect of H2AX siRNA on the levels of securin, AKT, and γ-H2AX in baicalein-treated cells. A: BFTC905 cells were transfected with 50–100 nM of control or H2AX siRNA for 48 h. B: Cells were transfected with 100 nM of control siRNA or H2AX siRNA for 24 h and then exposed to 40 μM of baicalein for 24 h. The levels of phospho-AKT, AKT, γ-H2AX, securin, and ERK2 were analyzed by Western blot. Data are shown from one of three separate experiments with similar findings.

[Yanagida, 2000; Jallepalli et al., 2001]. Securin also regulates DNA repair when cells are exposed to UV and X-ray irradiation [Nagao et al., 2004]. Therefore, we suggest that baicalein can block securin expression, thus inducing abnormal mitotic progression and genomic instability in cancer cells.

It was furthermore demonstrated that baicalein elevates the phosphorylation of γ-H2AX and AKT. The activation of AKT is a survival signal to protect cells from apoptosis [Downward, 1998; Martelli et al., 2003; Kim et al., 2004]. It has been proposed that the presence of γ-H2AX is linked to caspase-controlled DNA fragmentation during apoptosis [Rogakou et al., 2000]. However, γ-H2AX is also involved in the maintenance of genomic stability after treatment with DNA damaging agents [Paull et al., 2000; Redon et al., 2002]. The blockage of the PI3K-AKT pathway by exposure to wortmannin or AKT shRNA enhanced cytotoxicity in baicalein-treated cells. Moreover, decreasing γ-H2AX expression by transfection with H2AX siRNA increased baicalein-induced cytotoxicity. These results indicate that the activation of AKT and γ-H2AX by baicalein may be a means by which cancer cells protect themselves from cell death. The phosphorylation of AKT and γ-H2AX was elevated to a similar extent in both wild type and securin-null HCT116 cancer cells following baicalein treatment. Therefore, we suggest that baicalein induces the phosphorylation of AKT and γ-H2AX through a securin-independent pathway.

In summary, we demonstrate that the expression of securin in cancer cells can modulate baicalein-induced cytotoxicity and apoptosis. Blocking the AKT and γ-H2AX pathways increases baicalein-induced cancer cell death. Understanding the mechanisms by which securin, AKT, and γ-H2AX regulate baicalein-induced cancer cell death may allow the identification of novel strategies for cancer therapy.

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