Chemico-Biological Interactions 220 (2014) 41–50

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

# The depletion of securin enhances butein-induced apoptosis and tumor inhibition in human colorectal cancer

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ARTICLE INFO

*Article history:*
Received 13 October 2013
Received in revised form 8 January 2014
Accepted 4 June 2014
Available online 12 June 2014

*Keywords:*
Butein
Securin
Mitosis
Apoptosis
Colorectal tumor inhibition

ABSTRACT

Butein (3,4,2′,4′-tetrahydroxychalcone) is a promising natural polyphenolic compound that shows the growth inhibitory activity in human cancer cells; however, the precise mechanism is still unclear. Securin plays pivotal role in cancer cell proliferation and tumorigenesis. Here, we report the presence of securin that could modulate apoptosis and tumor growth ability in the butein-treated human colorectal cancer. Butein induced caspase-3 activation and PARP protein cleavage for apoptosis induction in human colorectal cancer cells. Interestingly, butein reduced the securin protein levels but conversely increased the phospho-histone H3 proteins, mitotic arrest and abnormal chromosomes segregation in cancer cells. The securin-null colorectal cancer cells were more sensitive on the reduction of cell viability than the securin-wild type cancer cells following butein treatment. The loss of securin in human colorectal cancer cells decreased tumor growth ability in nude mice. Moreover, butein reduced the tumor size of xenografted human colorectal tumors of nude mice. Taken together, this study demonstrates for the first time that the depletion of securin mediates the butein-induced apoptosis and colorectal tumor inhibition.



## 1. Introduction

Colorectal cancer is one of the leading cause and death in the world [1,2]. Development of novel anticancer agents for colorectal cancer prevention or therapy is still highly desired. Chalcone belongs to the polyphenol flavonoid family that exerted a variety of biological activities, including anticancer ability. Butein (3,4,2′,4′-tetrahydroxychalcon) is a chalcone flavonoid. Butein has been shown to display anticancer activities against various human cancer cells, including colorectal carcinoma [3], leukemia [4], melanoma [5], breast carcinoma [6], hepatoma [7], and malignant pleural mesothelioma [8]. In addition to anticancer ability, butein is an anti-adipogenic compound [9]. Butein can induce cell death or growth inhibition in cancer cells [3–8]. Butein-induced apoptosis is associated with the activation of caspases [4,5]. Moreover, butein could block signal molecules such as tyrosine kinases of the survival pathways [10,11]. Nonetheless, the precise mechanism of butein in apoptosis and tumor inhibition in colorectal cancer remains unclear.

Securin is also called as the pituitary-tumor transforming gene (PTTG) [12–17]. In general condition, securin plays a vital role to prevent abnormal chromosome segregation and to maintain genomic stability [18–20]. It may participate in DNA repair and prevention of aberrant chromosomal segregation when cellular DNA or spindles are damaged [20–23]. However, securin was highly expressed in a variety of human cancers [13–17,24,25] and promoted cancer cell proliferation and tumorigenesis [13,26,27]. The protein levels of securin have been correlated with tumor invasiveness and metastasis [28].

The cell cycle arrest mediated by inappropriate activity of the cyclin-dependent protein kinases (CDKs) can trigger the proliferation inhibition and apoptosis of cancer cells [29,30]. CDKs have been developed as a new target for cancer therapeutics [31,32]. Entry into mitosis is controlled by cyclin dependent kinase 1 (CDK1), also referred to as CDC2 [30,33–35]. The interaction of CDC2 and cyclin B1 plays a critical role in mitotic progression [30,33–35]. The phosphorylation of CDC2 on Thr161 by

*Abbreviations:* PTTG, pituitary-tumor transforming gene; CDKs, cyclin-dependent protein kinases; CDK1, cyclin dependent kinase 1; FBS, fetal bovine serum; MTT, 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; FITC, fluorescein isothiocyanate; PARP, poly(ADP-ribose) polymerase.

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http://dx.doi.org/10.1016/j.cbi.2014.06.006
0009-2797/

CDK-activating kinase (CAK) is required for CDC2 activity [36]; in contrast, the phosphorylated sites of CDC2 on Tyr15 by Wee1 [37–39] and Thr14 by Myt1 [40] inhibit CDC2 activity. Furthermore, CDC25C, a phosphatase, can dephosphorylate Tyr15 and Thr14 to activate CDC2 [41].

Securin expression has been used as a new marker of malignant colorectal carcinoma and may represent a therapeutic target [16]. The regulation of securin following treatment with butein in cancers was previously undetermined. In this study, the depletion of securin enhanced the butein-induced apoptosis and colorectal tumor inhibition. The blockade of securin by butein may provide important insight for colorectal cancer prevention and therapeutics.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

Butein, Hoechst 33258, Cy3-labeled mouse anti-β-tubulin, propidium iodide (PI), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). Butein was dissolved in DMSO. The concentration of DMSO was used <1% in control and the butein-containing medium. Anti-CDC2, anti-phospho-CDC2 (Tyr15), anti-phospho-CDC2 (Thr14), anti-phospho-CDC2 (Thr161), anti-phospho-histone H3 (Ser10), anti-poly (ADP-ribose) polymerase (PARP) and anti-phospho-p53 (Ser15) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-caspase-3 antibody was purchased from BioVision (BioVision, Inc., USA). Anti-cyclin B1 (Ab-2) antibody was purchased from Oncogene Sciences (Cambridge, MA). Anti-p53 (DO-1), anti-actin (I-19), goat anti-rabbit IgG horseradish peoxidase, and goat anti-mouse IgG horseradish peoxidase antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

### 2.2. Cell lines and cell culture

RKO was a human colorectal carcinoma cell line that expressed the wild-type p53 [42]. SW480 cells contained the double mutations of p53 gene, a G-to-A mutation in codon 273 and a C-to-T mutation in codon 309, that was established from the colorectal adenocarcinoma of a 50-year-old white male [43,44]. The wild type, securin-null, and p53-null HCT116 human colorectal carcinoma cell lines were kindly provided by Dr. B. Vogelstein of Johns Hopkins University (Baltimore, MD). RKO and SW480 cells were maintained in DMEM medium (Gibco, Life Technologies, Grand Island, NY). The HCT116 human colorectal carcinoma cells were cultured in complete McCoy's 5A medium (Sigma Chemical). These complete media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutamine. The cells were cultured at 37 °C and 5% $CO_2$ in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

### 2.3. Cytotoxicity assay

The cells were plated in 96-well plates at a density of $1 \times 10^4$ cells/well for 16–20 h. Thereafter, the cells were treated with various concentrations of butein for 24 h, and then the cells were washed with phosphate-buffered saline (PBS) and were replaced fresh complete McCoy's 5A medium for cultured 2 days. Subsequently, the cells were incubated with 0.5 mg/ml of MTT in fresh complete McCoy's 5A medium for 4 h. The surviving cells converted MTT to formazan by forming a blue–purple color when dissolved in dimethyl sulfoxide. The intensity of formazan was measured at 565 nm using a microplate reader (VERSAmax, Molecular Devices Inc., CA). The relative percentage of cell viability was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

### 2.4. Time-lapse observation of cell morphological alteration

HCT116 colorectal cancer cells were plated at a density of $2 \times 10^5$ cells per 35-mm Petri dish in complete medium for 16–20 h. Then the cells were treated with or without 40 μM butein. The cellular morphological alteration was recorded by time-lapse observation under an optical phase contrast microscope with an incubator system (OLYMPUS IX71, Japan). The pictures were edited by DP manager software (Ver. 3.3.1, OLYMPUS).

### 2.5. Cell cycle analysis

The cells were plated at a density of $1 \times 10^6$ cells per 60-mm Petri dish in complete medium for 16–20 h. At the end of treatment with butein, the cells were collected and fixed with ice-cold 70% ethanol overnight at −20 °C. After centrifugation, the cell pellets were treated with 4 μg/ml PI solution containing 1% Triton X-100 and 100 μg/ml RNase at 37 °C for 30 min. After re-centrifugation, the cell pellets were resuspended in 1 ml ice-cold PBS. To avoid cell aggregation, the cell solutions were filtrated through nylon mesh membrane. Finally, the samples were analyzed by a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA). A minimum of ten thousand cells was analyzed for DNA content, and the percentage of cell cycle phases was quantified by ModFit LT software (Ver. 2.0, BD Biosciences).

### 2.6. Annexin V and PI assays

The cells were plated at a density of $7 \times 10^5$ cells per 60-mm Petri dish in complete medium for 16–20 h. Thereafter, the cells were treated with or without 10–40 μM butein for 24 h. Apoptotic cells was performed using an Annexin-V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Then cells were collected and resuspended in 500 μl of binding buffer, added 5 μl of Annexin-V-FITC and 5 μl of PI. Finally, the samples were analyzed by flow cytometer using CellQuest software (BD Biosciences). The cells showed Annexin V(+)/PI(−) and Annexin V(+)/PI(+), which indicated at early and late apoptosis, respectively.

### 2.7. Western blot

At the end of treatment, the cells were lysed in the ice-cold whole cell extract buffer containing the protease inhibitors. The lysate was vibrated for 30 min at 4 °C and centrifuged at 10,000 rpm for 10 min. Protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of proteins were subjected to electrophoresis using 12% sodium dodecyl sulfate–polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes and then the membranes were blocked overnight at 4 °C using blocking buffer (5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20 and 0.02% sodium azide). Thereafter, the membranes were incubated for 2 h at 25 °C with specific primary antibody followed by anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase conjugated secondary antibodies. Then the membranes were washed three times for 10 min with washing solution. Finally, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). A gel-digitizing software, Un-Scan-It gel (ver. 5.1; Silk Scientific, Inc.), was used to analyze the intensity of bands on X-ray film by semi-quantification.

### 2.8. Immunofluorescence staining and confocal microscopy

The cells were cultured on coverslips, which were kept in 35-mm Petri dish at a density of $5 \times 10^5$ per well for 16–20 h. After treatment with or without butein, the cells were washed with PBS, and then fixed with 4% paraformaldehyde solution overnight at 4 °C. After fixation, the cells were washed three times with PBS, and non-specific binding sites were blocked in PBS containing 10% FBS and 0.3% Triton X-100 for 1 h at 37 °C. Thereafter, the cells were incubated with rabbit anti-phospho-histone H3 (1:100) antibody 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 anti-rabbit IgG-Hylite 488 (1:100) in PBS containing 10% FBS for 1 h at 37 °C, and washed three times with 0.3% Triton X-100 in PBS. In addition, the samples incubated with mouse anti-securin (1:100) antibody 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 anti-mouse IgG-Cy3 (1:100) in PBS containing 10% FBS for 1 h at 37 °C. The β-tubulin and nuclei were stained with the Cy3-labeled anti-β-tubulin and Hoechst 33258, respectively. After staining, the samples were immediately examined under Multiphoton Confocal Microscope System (TCS-SP5-X AOBS, Leica, Germany).

### 2.9. Mitotic index analysis

The cells were cultured on coverslips in a 35-mm Petri dish at a density of $5 \times 10^5$ for 16–20 h. After treatment with or without 40 μM butein for 24 h, the cells were carefully and gently washed with PBS (pH 7.4) and then fixed with 4% paraformaldehyde solution in PBS for one hour at 37 °C. The cells were incubated with rabbit anti-phosphorylated histone H3 (Ser10) antibody. Then the cells were incubated with goat anti-rabbit IgG-Hylite 488. The β-tubulin was stained with the Cy3-labeled mouse anti-β-tubulin (1:50) for 30 min at 37 °C. Finally, the nuclei were stained with 2.5 μg/ml Hoechst 33258 for 30 min. Mitotic index indicated the percentage of mitotic cell number/total counted cells that was counted under a fluorescence microscope in each treatment. Prophase, metaphase, anaphase, and telophase in the mitotic phases were separately counted under a fluorescence microscope.

### 2.10. Analysis of phospho-histone H3 by flow cytometer

The HCT116 colorectal cells were treated with or without butein. Thereafter, the cells were harvested and fixed with 75% alcohol at −20 °C for overnight. The samples were collected and incubated with 10% bovine serum albumin in PBS at 4 °C for 1 h. After inculcation, the samples were further incubated with rabbit anti-phospho-histone H3 antibody (1:100), and then incubated with anti-rabbit IgG-Hylite 488 (1:100) at 4 °C for 2 h. At the end of incubation, the cells were resuspended in 1 × PBS and immediately analyzed by a flow cytometer (FACS Calibur, BD Biosciences). The fluorescence intensities of phospho-histone H3 from Hylite 488 were analyzed and quantified using CellQuest software (BD Biosciences).

### 2.11. Xenografted human colorectal tumors in nude mice

BALB/cAnN.Cg-*Foxn1nu*/CrlNarl mice (3-week-old male) were obtained from BioLASCO (BioLASCO Co., Ltd., Taipei, Taiwan). After 1 week for environmental adaption, the mice were inoculated with human colorectal cancer cells. The wild type and securin-null HCT116 colorectal cancer cells were subcutaneously injected with $2 \times 10^6$ cells to the four-week-old nude mice. Each group contained three mice. The xenograft tumor volume in the mice was calculated by the following formula: $(\text{length}) \times (\text{width})^2 \times 0.5$ using a digital caliper. The tumor volume was measured every four days during total 60 days. To further investigate the anti-tumor ability by treatment with butein, the HCT116 colorectal cancer cells were subcutaneously injected with $2 \times 10^6$ cells to the four-week-old nude mice. Each group contained three mice. After 10 days inoculation, the mice bearing colorectal xenograft tumors were injected with vehicle control (corn oil) or 40 mg/kg of butein. After butein treatment, the tumor volume was measured every four days during total 28 days. The visible tumors were harvested from scarified mice. The above animal studies had been permitted by the Committee of Animal Study in National Chiao Tung University. Moreover, the animal care and sacrifice were according to international animal care guidelines.

### 2.12. Statistical analysis

Each experiment was repeated at least three times. Data from the population of cells treated with different conditions were analyzed using paired Student's *t*-test. In a comparison of multiple groups, data were analyzed by one-way or two-way analysis of variance (ANOVA), and further post Tukey's tests using the statistic software of GraphPad Prism 5 (GraphPad software, Inc. San Diego, CA). A $p$ value of $<0.05$ was considered as statistically significant in each experiment.

## 3. Results

### 3.1. The depletion of securin enhances butein-induced cell death and apoptosis in human colorectal cancer cells

To examine the role of securin in the cell survival of butein-treated cells, the cell viability and apoptosis assays were determined between the HCT116 wild type (WT) and securin-null colorectal cancer cells by treatment with butein. Treatment with butein (10–40 μM for 24 h) significantly reduced the cell viability via a concentration-dependent manner in both the securin-wild type and securin-null HCT116 cells (Fig. 1A). However, the HCT116 securin-null cancer cells were more sensitive on the reduction of cell viability than the securin-wild type cells following butein treatment (Fig. 1A). The values of $IC_{50}$ (the concentration of 50% inhibition of cell viability) were around 26.6 μM and 18.2 μM in the securin-wild type and securin-null HCT116 cells, respectively. The apoptosis was analyzed by Annexin V-FITC and PI staining. The basal level of apoptosis in the securin-null HCT116 cells was relative higher than the wild type cells. Treatment with butein (10–40 μM for 24 h) increased the Annexin V(+)/PI(−) cells (early apoptosis) and Annexin V(+)/PI(+) cells (late apoptosis) in both the securin-wild type and securin-null HCT116 cells (Fig. 1B). After calculating total apoptotic population, the securin-null cells were displayed higher levels of apoptosis than the wild type cells by treatment with butein at the same concentrations (20–40 μM) (Fig. 1C and D).

### 3.2. Butein induces the protein levels of active caspase-3 and PARP cleavage in human colorectal cancer cells

The effect of butein on the cellular morphological alteration was recorded by time-lapse live cell observation. The untreated cells were observed the cellular proliferation and became confluent in culture dish after 48 h (Fig. 2A). In contrast, butein induced the abnormal round-up morphology and blocked the cellular proliferation (Fig. 2A). These cells were undergoing cell death by treatment with butein (Fig. 2A, stars). To examine the apoptotic pathway after treatment with butein, the protein levels of activated caspase-3 and PARP cleavage were analyzed by Western blot. The

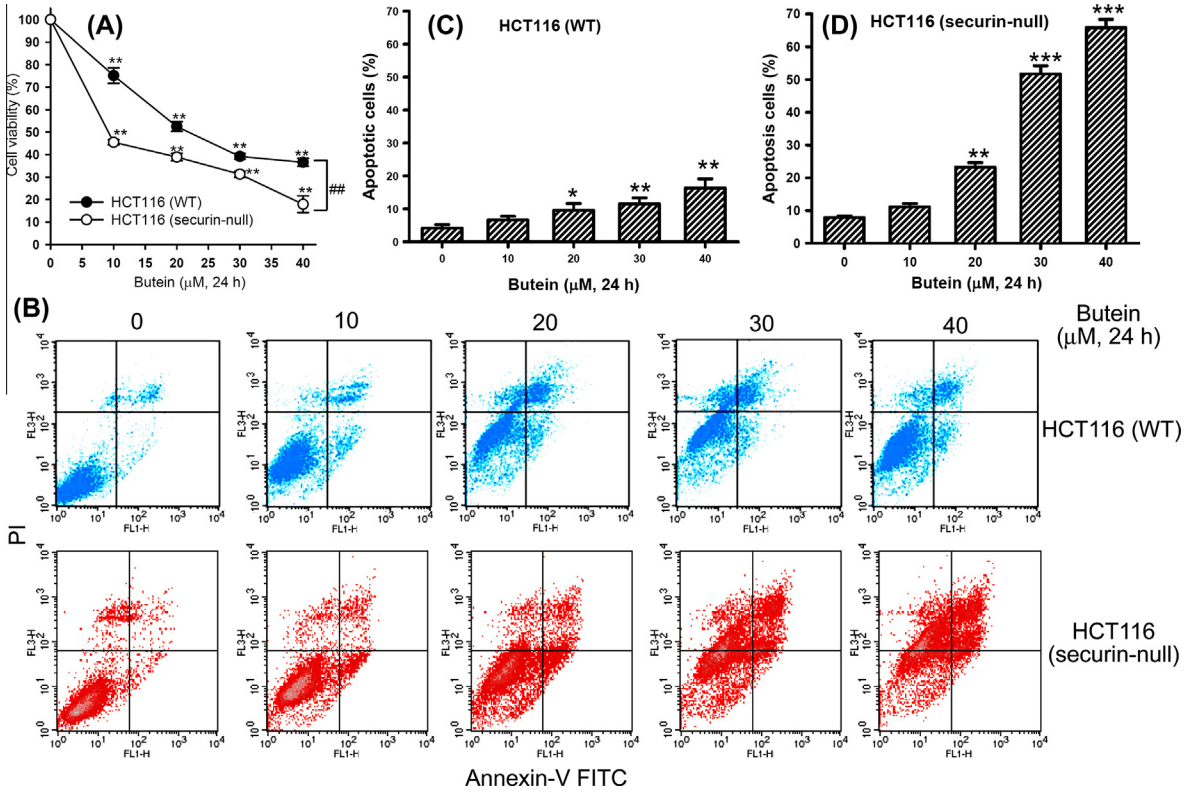


**Fig. 1.** The loss of securin increases the reduction of cell viability by treatment with butein in human colorectal cancer cells. (A) The HCT116 wild type (WT) and securin-null colorectal cancer cells were treated with or without 10–40 μM butein for 24 h. At the end of treatment, the cells were re-cultured in fresh medium for 2 days. Then the cell viability was measured by MTT assay. Results were obtained from four experiments and the bar represents the mean ± S.E.M. $^{**}p < 0.01$ indicates significance between control and butein-treated samples. $^{\#\#}p < 0.01$ indicates significant difference between the HCT116 (WT) and securin-null cancer cells by butein treatment at the same concentrations. (B) The levels of apoptosis were measured by Annexin-FITC and PI 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). (C) HCT116 WT cells and (D) HCT116 securin-null cells in the total population of apoptotic cells including early and late apoptosis were quantified by CellQuest software in flow cytometer. Results were obtained from four experiments and the bar represents the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant difference between control and butein treated samples.

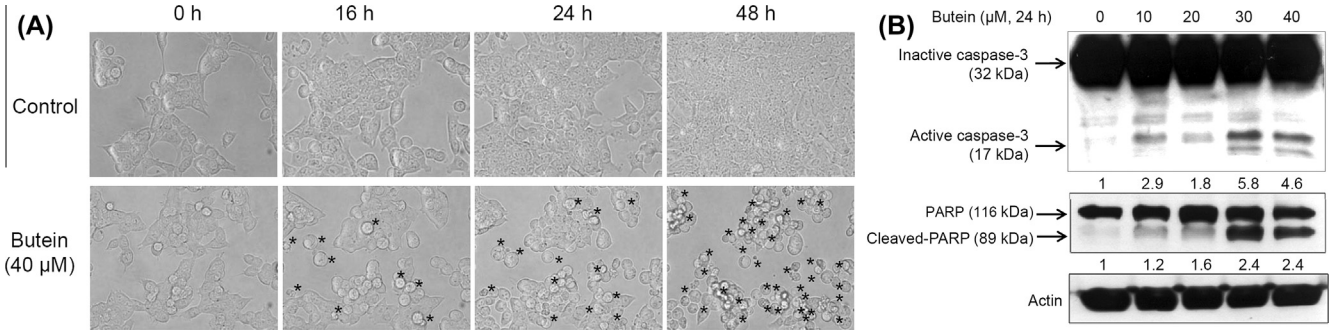


**Fig. 2.** Butein induces caspase-3 activation and PARP protein cleavage for apoptosis in human colorectal cancer cells. (A) The time-lapse observation of cellular morphology following treatment with or without butein was recorded under an optical phase contrast microscope with cell incubator system. HCT116 cells were treated with or without butein (40 μM for 0–48 h). The stars indicate the round-up morphology and are undergoing cell death following butein treatment. (B) HCT116 cells were treated with or without 10–40 μM butein for 24 h. The protein levels of active caspase-3 (17 kDa) and cleaved PARP (89 kDa) were analyzed by Western blot. The representative Western blot data were shown from one of three separate experiments with similar findings. Actin was a loading control. The relative intensity of proteins (fold) was from Western blots by semi-quantification.

active forms of caspase-3 (12 kDa and 17 kDa) and the cleaved form proteins of PARP (89 kDa) were induced by treatment with butein (10–40 μM for 24 h) (Fig. 2B).

### 3.3. Butein induces mitotic arrest and modulates CDC2 phosphorylation in human colorectal cancer cells

To investigate the role of securin in the butein-induced cell cycle arrest, the securin-wild type and securin-null HCT116 cells were analyzed by flow cytometry. Butein increased the G2/M fractions but decreased the G1 fractions in both the securin-wild type and securin-null HCT116 cells (Fig. 3A and B). Moreover, the securin-null cancer cells were higher increase of G2/M fractions than the securin-wild type cancer cells by treatment with butein at 20–30 μM (Fig. 3A and B). To determine whether butein induced G2 or M arrest, we further analyzed the mitotic index. Butein markedly increased the mitotic index in these cells (Fig. 4A). The mitotic stages including prophase (with condensed DNA but no

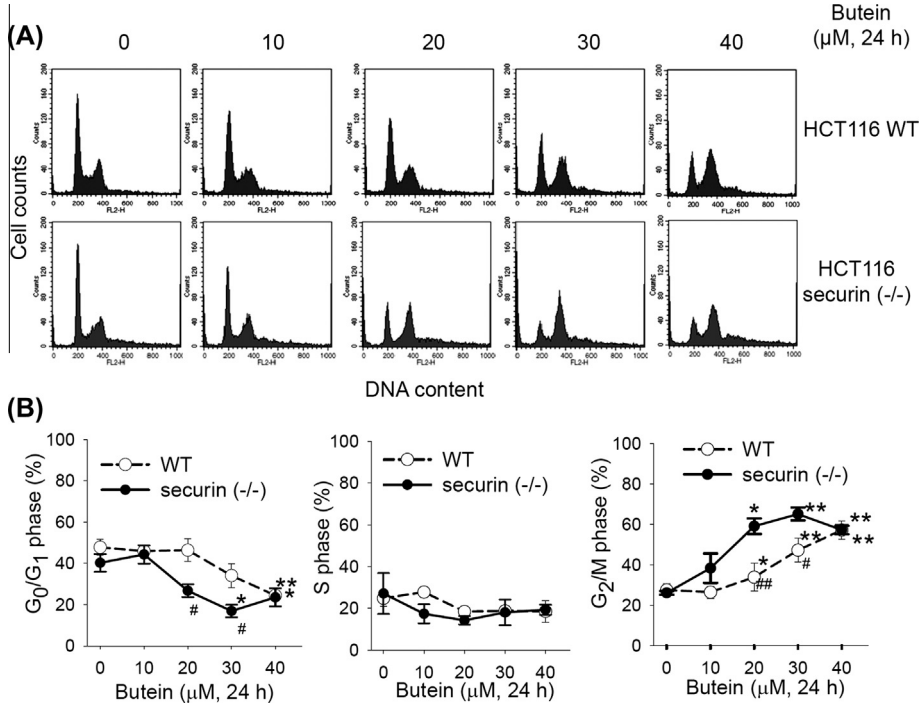


**Fig. 3.** The loss of securin increases the butein-induced G2/M fractions but reduced G1 fractions in human colorectal cancer cells. (A) The HCT116 (WT) and HCT116 securin-null colorectal cancer cells were treated with or without 10–40 μM butein for 24 h. At the end of treatment, the cells were trypsinized and then subjected to flow cytometry analysis. The representative flow data were shown from one of five separate experiments with similar findings. (B) The percentages of G1, S and G2/M fractions were quantified by ModFit LT software. Results were obtained from five experiments and the bar represents the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant difference between control and butein treated samples. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ indicate significant difference between the HCT116 (WT) and HCT116 securin-null cancer cells by butein treatment at the same concentrations.

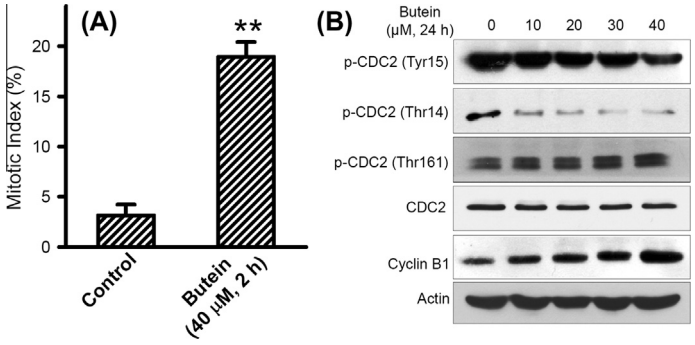


**Fig. 4.** Butein increases the mitotic index and modulates the protein levels of phospho-CDC2 and cyclin B1 in human colorectal cancer cells. (A) HCT116 cells were treated with or without 40 μM butein for 24 h. The β-tubulin and nuclei of cells were stained with the Cy3-labeled mouse anti- β-tubulin and Hoechst 33258, respectively. Mitotic index (the percentage of mitotic cell number/total cell number) was counted under a fluorescence microscope. Results were obtained from three experiments and the bar represents the mean ± S.E.M. $^{**}p < 0.01$ indicates significant difference between control and butein treated samples. (B) The cells were treated with or without 10–40 μM butein for 24 h. The total protein extracts were prepared for immunoblot analysis using specific anti-CDC2, anti-phospho-CDC2 (Tyr-15), anti-phospho-CDC2 (Thr-14), and anti-phospho-CDC2 (Thr-161), and anti-cyclin B1 antibodies. Actin was a loading control. The representative Western blot data were shown from one of three separate experiments with similar findings.

spindle), metaphase (with a spindle but no visible separation of sister chromatids), anaphase (with a spindle and clearly separated sister chromatids), and telophase (with a spindle and clearly separated cells) were counted under fluorescence microscope. The percentages of prophase, metaphase, anaphase and telophase were 51.9%, 45.8%, 0.7%, and 1.5%, respectively. Furthermore, butein decreased the protein levels of phospho-CDC2 (Thr-14) and phospho-CDC2 (Tyr-15) but conversely increased the protein levels of phospho-CDC2 (Thr-161) and cyclin B1 (Fig. 4B). The total CDC2 protein levels were not altered in the butein-treated cells.

### *3.4. Butein induces phosphorylated histone H3 (Ser10) and abnormal chromosomes segregation*

To investigate the effect of butein-induced mitotic arrest, the butein-treated cells were measured by a specific mitotic marker, phospho-histone H3 (Ser10). Butein markedly increased the protein levels of phospho-histone H3 via a concentration-dependent manner in colorectal cancer cells (Fig. 5A). To further examine the cellular morphologic alteration by butein, the cells were treated with or without butein (30 μM for 24 h) and subjected to

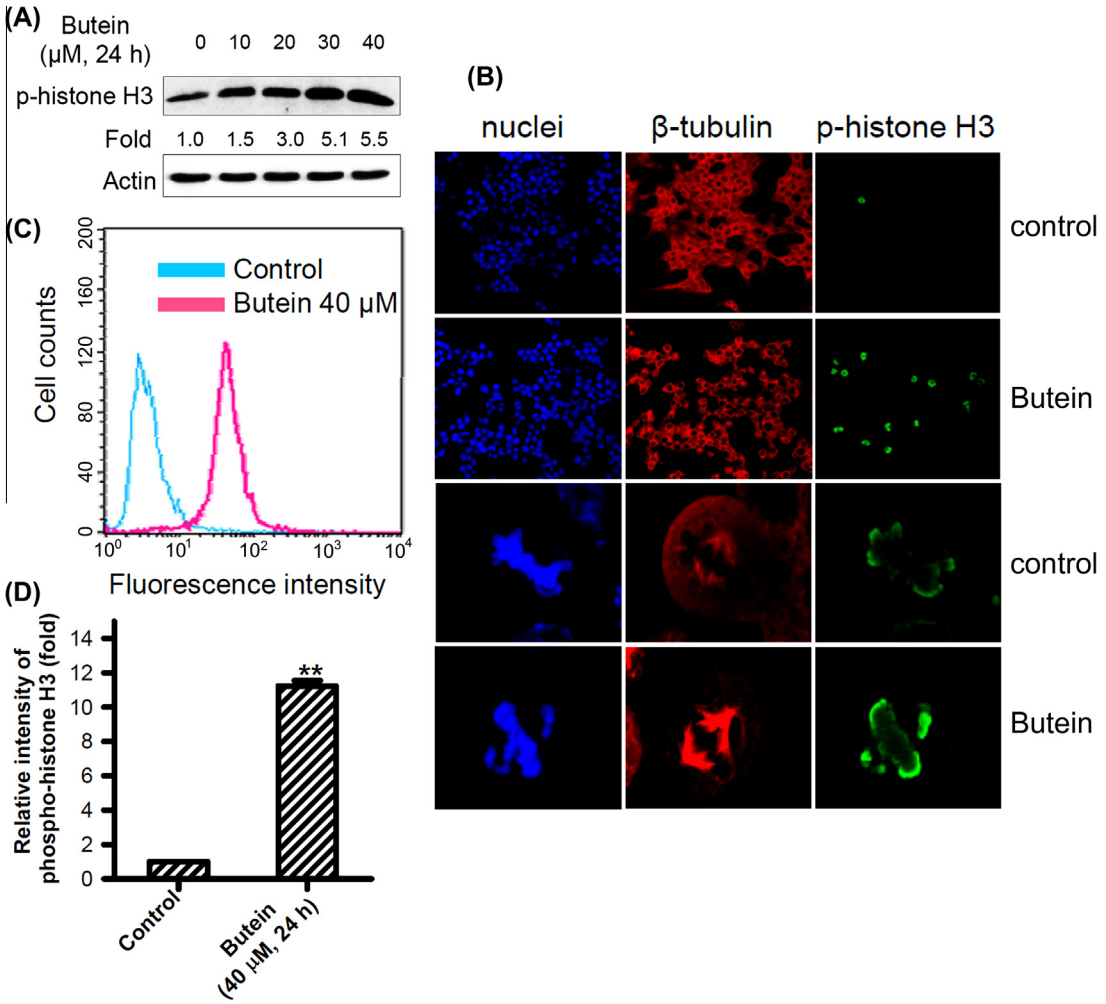


**Fig. 5.** Butein increases phospho-histone H3 and abnormal chromosome separation in human colorectal cancer cells. (A) HCT116 cells were treated with or without 10–40 μM butein for 24 h in HCT116 cells. The total protein extracts were prepared for immunoblot analysis using anti-phospho-histone H3 and anti-actin antibodies. Actin was a loading control. The representative Western blot data were shown from one of three separate experiments with similar findings. The relative intensity of proteins (fold) after butein treatment was from Western blots by semi-quantification. (B) The cells were treated with or without 30 μM butein for 24 h. At the end of treatment, the cells were incubated with rabbit anti-phospho-histone H3 (Ser10) and then incubated with goat anti-rabbit IgG-Hylite 488. The phospho-histone H3 (Ser10) proteins displayed green fluorescence with goat anti-rabbit IgG-Hylite 488. The β-tubulin and nuclei were stained with the Cy3-labeled mouse anti-β-tubulin and Hoechst 33258, respectively. (C) The untreated or butein-treated cells were incubated with rabbit anti-phospho-histone H3 (Ser10) antibody. Then the cells were incubated with goat anti-rabbit IgG-Hylite 488. Fluorescence intensity of phospho-histone H3 was detected in whole cell population by flow cytometer. X-axis indicates the fluorescence intensity of Hylite 488. Y-axis indicates the cell counts. (D) The fluorescence intensities were quantified from a minimum of 10,000 cells by CellQuest software of flow cytometer. Results were obtained from three experiments and the bar represents the mean ± S.E.M. $^{**}p < 0.01$ indicates significant difference between control and butein treated samples.

immunofluorescence staining and confocal microscopy. The green fluorescence intensity exhibited by phospho-histone H3. The red fluorescence intensity exhibited by the β-tubulin proteins of cytoskeleton. The blue color was represented the location of nuclei or chromosomes by staining with Hoechst 33258. Butein increased the green fluorescence intensities of phospho-histone H3 in the mitotic cells and induced abnormal chromosome segregation in these cells (Fig. 5B). In addition, we confirmed that butein increased the fluorescence intensity of phospho-histone H3 using flow cytometer analysis (Fig. 5C). Treatment with 40 μM butein increased the fluorescence intensity of phospho-histone H3 proteins to 10-folds by comparison with untreated cells (Fig. 5D).

### 3.5. Butein inhibits securin protein expression in human colorectal cancer cells

Fig. 6A shows that butein markedly decreased the securin protein levels in colorectal cancer cells. Actin was used as a loading control protein that was not altered by butein. After quantification, butein significantly reduced the securin protein levels in a concentration-dependent manner (Fig. 6B). Besides, the fluorescence intensities of securin proteins were decreased by treatment with butein (30 μM for 24 h) in these cells (Fig. 6C).

### 3.6. The depletion of securin enhances the reduction of colorectal tumor size in the xenografted nude mice following butein treatment

To investigate the role of securin in tumor growth ability, the securin-wild type and securin-null HCT116 cells were subcutaneously injected into nude mice, and compared the tumor size in the xenografted nude mice. The wild type colorectal cancer cells were relative higher growth ability than the securin-null cancer cells in the xenografted tumors of nude mice (Fig. 7A). The visible human colorectal tumors from scarified mice showed that the loss of securin reduced tumor size (Fig. 7B). To verify the anti-tumor ability of butein, the nude mice-bearing colorectal xenograft tumors were treated with vehicle control (corn oil) or 40 mg/kg of butein. After butein treatment, the tumor volume was measured every four days during total 28 days. Fig. 8A shows that the tumor size was significantly decreased in the butein-treated groups by comparison with

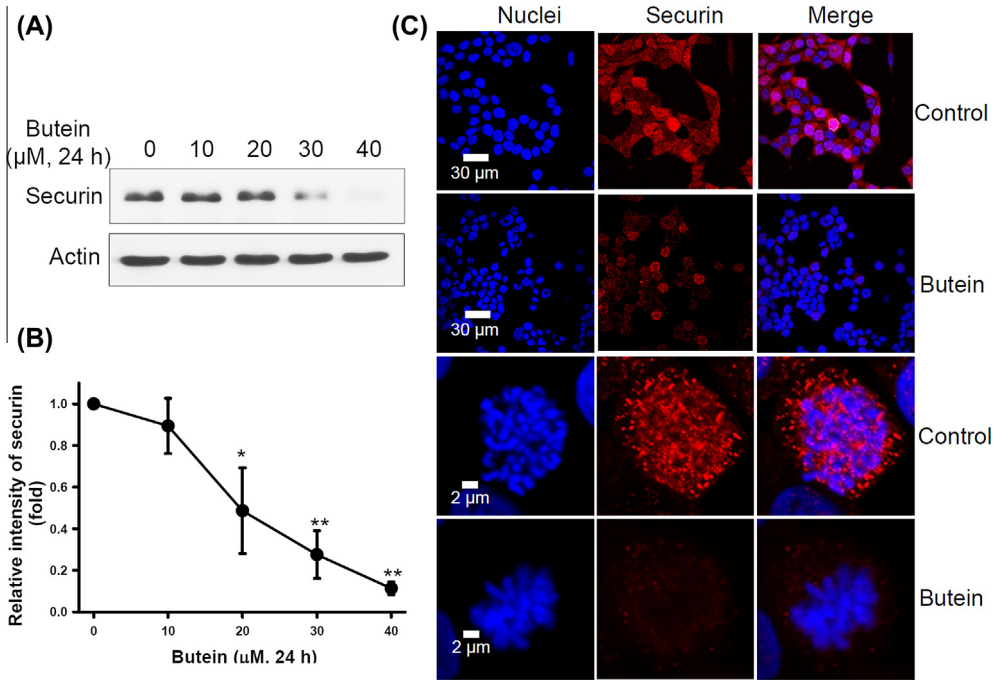


**Fig. 6.** Butein inhibits the protein levels of securin in human colorectal cancer cells. (A) HCT116 cells were treated with or without 10–40 μM butein for 24 h. The total protein extracts were prepared for Western blot analysis using specific anti-securin and anti-actin antibodies. Actin was a loading control. (B) The relative intensity of securin from Western blot was analyzed by semi-quantification. Results were obtained from three experiments and the bar represents the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant difference between control and butein treated samples. (C) The cells were treated with or without 30 μM butein for 24 h. At the end of treatment, the cells were incubated with mouse anti-securin and then incubated with goat anti-mouse Cy3. The securin proteins displayed red fluorescence with goat anti-mouse Cy3. The nuclei were stained with Hoechst 33258. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

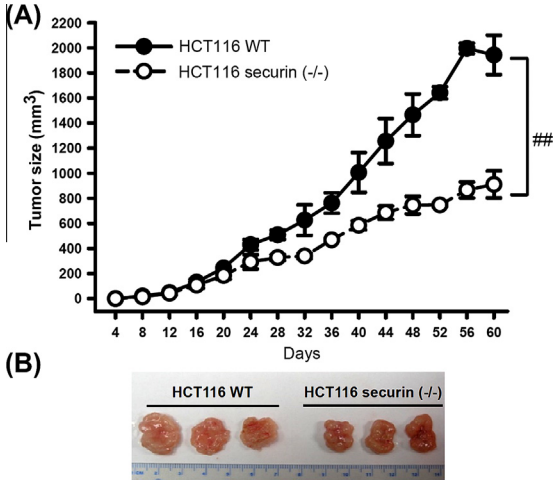


**Fig. 7.** The tumor growth ability between the wild type and securin-null colorectal cells in the xenografted nude mice. (A) The securin-wild type and securin-null HCT116 cells were subcutaneously injected with $2 \times 10^6$ cells to the four-week-old nude mice. The tumor volume was measured every four days until day 60. The results were obtained from three mice in each group and the bar represents the mean ± S.E.M. $^{\#\#}p < 0.01$ indicates significant difference between the securin-wild type and securin-null HCT116 colorectal tumors. (B) The visible colorectal tumors were harvested from scarified mice.

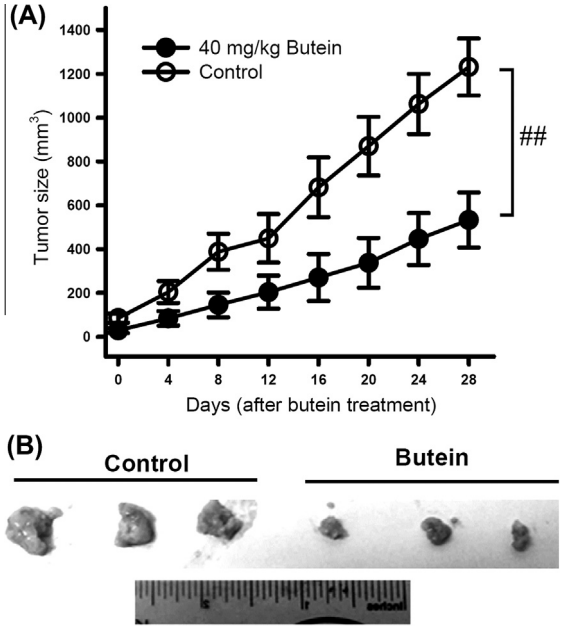


**Fig. 8.** Butein reduces tumor size in the xenografted human colorectal tumors of nude mice. (A) HCT116 colorectal cancer cells were subcutaneously injected with $2 \times 10^6$ cells to the four-week-old nude mice. Each group contained three mice. After 10 days inoculation, the mice bearing colorectal xenograft tumors were injected with vehicle control (corn oil) or 40 mg/kg of butein. After butein treatment, the tumor volume was measured every four days during total 28 days. The results were obtained from three mice in each group and the bar represents the mean ± S.E.M. $^{\#\#}p < 0.01$ indicates significant difference between the control and butein treated samples. (B) The visible colorectal tumors were harvested from scarified mice.

the control groups. The average tumor size was ~1200 $mm^3$ in the control groups after inoculation at 38 days (Fig. 8A). However, treatment with butein reduced to average tumor size ~550 $mm^3$. The visible tumors also showed the reduction of tumor size in the butein-treated groups (Fig. 8B).

## 4. Discussion

Colorectal cancer is one of the leading cause and death in the world [1,2]. Development of novel strategies and anticancer drugs for colorectal cancer therapy is highly required. Securin is highly expressed in human colorectal cancer cells; moreover it has been proposed as a new marker and therapeutic target for colorectal carcinoma [16]. Butein is promising natural chalcone flavonoid. It has been shown that butein displays anticancer abilities against varying human cancer cells [3–8]. In this study, the anticancer activities of butein were demonstrated in colorectal cancer model *in vitro* and *in vivo*. The depletion of securin enhanced the butein-induced apoptosis and tumor inhibition in human colorectal cancer. We show the novel insight of butein, which is a potent securin and mitotic inhibitor that has ability to reduce colorectal tumor size.

The induction of cell death pathways or the blockage of survival signal pathways may contribute the efficacy of anticancer drugs for cancer therapy. Butein has reported to block anti-apoptotic signal molecules such as tyrosine kinases in the survival pathways of cancers [10,11]. Securin has been shown to promote cancer cell survival [13,26,27]. It was highly expressed in various human cancers [13–17,24,25]. Furthermore, the protein levels of securin have been correlated with tumor invasiveness and metastasis [28]. Interestingly, we found that butein markedly reduced the securin protein levels in colorectal cancer cells. The depletion of securin enhanced the butein-induced cell death and apoptosis in these cells. Moreover, butein induced apoptosis related to the activation of caspase-3 and PARP protein cleavage in colorectal cancer cells. Indeed, butein has been reported to induce apoptosis through the activation of caspases [4,5]. Activation of caspases can cleave specific cellular substrates such as poly (ADP-ribose) polymerase (PARP) during apoptosis [45,46]. Accordingly, these findings suggest that the inhibition of securin by butein may mediate the caspase-dependent pathway for apoptosis induction.

Subsequently, we show that butein is a mitotic inhibitor in colorectal cancer cells. Previously, butein has shown to induce G2/M phase arrest in human hepatoma cancer cells [7]. According to cellular morphological observation and mitotic index analyses, the butein-treated colorectal cancer cells were arrested in prophase and metaphase stages. The phosphorylation of Thr161 of CDC2 by CAK is required for CDC2 activity [36], whereas phosphorylation of Tyr15 [37–39] and Thr14 [40] inhibits CDC2 activity. We found that butein increased the protein levels of phosphorylated CDC2 (Thr161) but reduced the protein levels of phosphorylation sites at Tyr15 and Thr14. The protein levels of cyclin B1 were also elevated by treatment with butein. The cell cycle arrest by inappropriate activity of CDKs could induce proliferation inhibition and apoptosis in cancer cells [29,30]. It has been shown that down-regulation of securin in cancer cells contributes to the suppressive effects of CDK inhibitor [47]. Accordingly, these findings suggest that butein can modulate the mitosis-regulated protein levels, including phosphorylated CDC2 and cyclin B1, to induce the blockade of mitotic exit and subsequently induces apoptosis.

The phosphorylation of histone H3 is critical for proper chromosome condensation and segregation in mitotic cells [48]. The phosphorylation of histone H3 (Ser10) has been used as a mitotic marker [49]. Interestingly, butein markedly increased the levels of phospho-histone H3 (Ser10); at the same time, it induced abnormal chromosomes segregation in these cells. Besides, we found that the loss of securin increased the butein-induced mitotic aberration. Securin is required for genomic stability during mitosis. Securin controls sister chromatid separation and progression from metaphase to anaphase, and its defects can result in chromosomal instability [18–20]. Securin is also involved in DNA repair and maintains chromosomal stability when cellular DNA or spindles are damaged [20–23]. Mitotic catastrophe is characterized by missegregation of chromosomes, leading to an aberrant mitosis or imperfect cell division [50,51]. Anticancer agents have been shown to induce mitotic catastrophe for cell death [52,53]. Thus, butein induces the depletion of securin that may induce abnormal chromosomes separation and mitotic catastrophe in human colorectal cancer cells. p53 is an important target for cancer treatment [54,55]. Previously, we found that p53 may modulate the oxaliplatin-induced cytotoxicity in human colorectal cancer cells [56]. Nonetheless, the inhibition of securin expression induced by arsenite increases the chromosomal instability and apoptosis via a p53-independent pathway [20]. We also examined the role of p53 by treatment with butein (please see the Supporting information). Butein reduced the cell viability in both the p53-wild type and p53-null HCT116 colorectal cancer cells (Fig. S1A). The p53-null HCT116 cells were more susceptible to increase ~20% cell death than the p53-wild type HCT116 cells following butein treatment. Consistently, the p53-mutational SW480 colorectal cancer cells were more susceptible to increase cell death than the p53-wild type RKO cells after treatment with butein (Fig. S1B). These findings indicate that butein still displays anticancer ability in the p53-mutational colorectal cancer cells. Furthermore, we found that butein increased the phosphorylation of p53 at Ser15 in both the p53-wild type HCT116 and RKO cancer cells but not alter the total p53 protein levels (Fig. S2). The phosphorylation of p53 at Ser15 plays an important role for p53 activation and stabilization [57–59]. It has been shown that the phosphorylation of p53 at Ser15 may transmit a survival signal and modulate apoptosis in response to several stimuli such as quercetin [60] and nitric oxide [61]. We suggest that activation of p53 may modulate the cell death in the butein-treated human colorectal cancer cells.

In summary, butein is a potent securin and mitotic inhibitor, which may induce abnormal CDC2 phosphorylation, mitotic arrest, apoptosis, and tumor inhibition in the human colorectal cancer. Understanding the mechanism by which securin regulates the butein-induced cell death and tumor inhibition may contribute to novel therapeutic strategies in colorectal cancer.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

## Transparency Document

The Transparency document associated with this article can be found in the online version.

## Acknowledgements

We thank Dr. B. Vogelstein of the Johns Hopkins University for providing the human securin-null and p53-null HCT116 colorectal cancer cell lines. This work was supported by the grants from NSC 100-2627-B-009-007, NSC 101-2627-B-009-006 and NSC 102-2627-B-009-006 of National Science Council, Taiwan. The authors also thank the core facilities of Multiphoton and Confocal Microscope System (MCMS) and IVIS in National Chiao Tung University.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2014.06.006.

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