國立交通大學

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碩士論文

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Contents

摘要

Securin 是一種調控有絲分裂與細胞凋亡的重要蛋白。紫鉚花素是一種具有抑制癌細胞增生作用的多酚類化合物，然而，securin 在紫鉚花素所誘導的有絲分裂停止與細胞凋亡的調控作用仍不清楚。本篇研究的目的，主要探討紫鉚花素誘發人類大腸癌細胞的有絲分裂停止與細胞凋亡，以及 securin 的功能。處理紫鉚花素後會顯著造成 HCT116 大腸癌細胞的有絲分裂停止與細胞凋亡，此外，紫鉚花素增加磷酸化組蛋白-3 表現量與有絲分裂指數，紫鉚花素誘發大量位在染色體的磷酸化組蛋白-3，並停在有絲分裂的中期。再者，在缺少 securin 基因的大腸癌細胞比 securin 功能正常的 HCT116 大腸癌細胞，對紫鉚花素細胞毒性更為敏感。並且紫鉚花素會大量地抑制 securin 功能正常的 HCT116 癌細胞中 securin 蛋白的表現。進一步研究發現，紫鉚花素會透過活化 caspase-3 引發細胞凋亡。此外，紫鉚花素增加磷酸化 p53 蛋白，然而在缺少 p53 基因的 HCT116 大腸癌細胞對紫鉚花素所誘發的細胞死亡比 p53 正常功能的細胞較為敏感。綜合以上結果，我們推測 securin 與 p53 參與調控紫鉚花素所造成的有絲分裂期停止及細胞凋亡。

ABSTRACT

Securin has been shown to play important roles in controlling apoptosis and mitosis. Butein is a natural polyphenolic compound, which has been shown growth inhibitory activity in human cancer cells. However, the regulation of securin on butein-induced mitotic arrest and apoptosis is poorly understood. In this study, we investigated the role of securin on regulating mitotic arrest and apoptosis after treatment with butein in the human colon cancer cells. Butein markedly induced the mitotic arrest and apoptosis in HCT116 colon cancer cells. Additionally, butein increased the levels of phospho-histone H3 and mitotic index. The phosphorylated histone H3 proteins were located in chromosomes of metaphase following treatment butein. Moreover, the securin-null colon cancer cells were more sensitive than the securin-wild type cancer cells to cytotoxicity by butein. The securin protein expression was markedly reduced by butein in the HCT116 securin-wild type cancer cells. Furthermore, butein induced caspase-3 activation for apoptosis. Besides, butein increased the phospho-p53 (Ser15) levels. However, the p53-null HCT116 cancer cells were more sensitive on cell death than the p53-wild type HCT116 cancer cells following butein treatment. Taken together, we suggest that butein induces mitotic arrest and apoptosis, which may regulate by securin and p53.

ABBREVIATIONS

AIF: apoptosis inducing factor

CAK: cyclin dependent kinase-activating kinase

CDC2: cell division control protein 2

CDC25C: cell division cycle 25 homolog C

CDKs: cyclin dependent kinases

CDKIs: cyclin dependent kinase inhibitors

DMSO: dimethyl sulfoxide

EGFR: epidermal growth factor receptor

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

IAP2: inhibitor of apoptosis protein 2

MTT: 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide

PARP: poly (ADP-ribose) polymerase

PBS: phosphate-buffered saline

PI: propidium iodide

PTTG1: pituitary tumor-transforming gene 1

ROS: reactive oxygen species

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TNF: tumor necrosis factor

MMP-9: matrix metallopeptidase 9

1. Introduction

1.1. Colorectal cancer

Colorectal cancer is one of the leading causes of cancer mortality in the world. Epidemiological studies have linked increased risk of colorectal cancer with a diet high in red meat and animal fat, low-fiber diet, and low overall intake of fruits and vegetables [1]. Colorectal cancer comes in many forms, including adenocarcinoma, leiomyosarcoma, lymphoma, melanoma, and neuroendocrine tumors. Adenocarcinoma is the most common type of colorectal cancer [2]. In the past 10 years, an unprecedented advance in systemic therapy for colorectal cancer has dramatically improved outcome for patients with metastatic disease [3]. Until the mid-1990s, the only approved agent for colorectal cancer was 5-fluorouracil [4]. New agents that became available in the past 10 years include cytotoxic agents such as irinotecan and oxaliplatin, oral fluoropyrimidines, and biologic agents such as bevacizumab, cetuximab, and panitumuma [5]. Development of novel anticancer drugs for colon cancer therapy is highly desired.

1.2. Anticancer activities of chalcones

Chalcones, a group of aromatic enones, forms the central core of a variety of important biological compounds, which belong to the flavonoid family and are often responsible for the yellow pigmentation in plants [6]. These phenolic compounds all

bear a 1,3-diphenyl-2-en-1-one framework. Chalcones have a variety of biological activities, including antifungal [7], antibacterial, antiprotozoal [8, 9], antimutagenic, antitumorogenic [10] and anti-inflammatory properties [8, 11, 12]. Various of chalcones, such as flavokawain B [13-15], isoliquiritigenin [16], and isobavachalcone [17, 18] have been shown to induce apoptosis in different types of cancer cells. These triterpenoids have a common target, Bcl-2 protein, which can induce apoptosis in cancer cells [6, 19]. Chalcones also have potential to block the NF-κB activation and inhibit proliferation, invasion, metastasis and angiogenesis [20-24].

1.3. Butein and its anticancer activities

Butein (3,4,2′,4′-tetrahydroxychalcon), a plant polyphenol flavonoid extracted from the stembark of cashews and *Rhus verniciflua* stokes, has traditionally been used for the treatment of pain, parasitic, and thrombotic diseases [25]. Butein has anticancer activities against cancers, including leukemia [26], melanoma [27], breast carcinoma [28, 29], colon carcinoma [30, 31], osteosarcoma [32], and hepatic stellate cells [33]. Butein can induce apoptosis in different types of cancer cells [23, 34, 35]. It has been shown that the apoptotic effect of butein is due to its inhibition of the expression of such NF-κB-regulated gene products as IAP2, Bcl-2, and Bcl-xL [23]. Also, butein can increase caspase-3 activity and expression of death receptor DR5 [36]. Moreover, butein induced cell cycle arrest and apoptosis in human hepatoma

cancer cells [34]. Butein also down-regulated MMP-9 in human leukemia cells [23]. In addition, butein is a tyrosine kinase inhibiter to cause inhibition of EGF-induced tyrosine phosphorylation of EGFR in cancer cells [35].

1.4. Apoptosis

Characteristic apoptotic features include membrane blebbing, cell shrinkage, chromatin condensation, and formation of a DNA ladder with multiple fragments caused by internucleosomal DNA cleavage [37]. There are two major apoptosis pathways, including intrinsic (also called ‘mitochondrial’ or ‘Bcl-2-regulated’) and extrinsic (also called ‘death receptor’) apoptosis signaling, in cells responsive to apoptotic stimuli [38, 39]. The extrinsic pathway is initiated by binding of the transmembrane death receptors such as Fas, tumor necrosis factor (TNF) receptor, DR3, DR4, or DR5 with their specific ligands, which is followed by activation of initiator caspase-8 to induce apoptosis [40]. The intrinsic pathway is activated by intrinsic death stimuli such as reactive oxygen species (ROS), DNA-damaging reagents, resulting in the release of cytochrome-c and the activation of caspase-9 which in turn activates caspase-3 [41]. Both extrinsic and intrinsic pathways lead to activation caspase-3 for apoptotic induction [42, 43]. Activation of caspases can cleave specific cellular substrates, including cytoplasmic structural proteins such as actin and nuclear proteins such as poly (ADP-ribose) polymerase (PARP) for

inducing cell death [19, 44, 45]. Failure of apoptosis regulation results in pathological conditions including cancer development [46].

1.5. Cell cycle progression

Cell cycle progression is an important biological event to control normal cells, which almost universally becomes aberrant or deregulated in transformed and neoplastic cells. The cell cycle consists of four orderly and tightly regulated phases, including G_1, S, G_2 and M [47-49]. The regulation of cell cycle progression is regulated by cyclin dependent kinases (CDKs) and cyclins [50]. CDKs participate in cell cycle by binding with cyclins and negatively regulated by CDK inhibitors (CDKIs) [48]. In order to regulate cell cycle progression, different checkpoints are set at various stages of the cell cycle [51]. Entry into mitosis is controlled by CDC2, also known as CDK1 (Cyclin Dependent Kinase 1), which is regulated by the cell cycle-dependent synthesis and degradation of cyclin B1, which accumulates during G_2/M and disappears at the end of mitosis. CDC2 is also regulated by phosphorylation at different three sites [52]. Phosphorylation of threonine 161 by CDK-activating kinase (CAK) is required for CDC2 activity [53], whereas phosphorylation of tyrosine 15 by Wee1 [51, 54, 55] and threonine 14 by Myt1 [56] inhibits CDC2 activity. At the onset of mitosis, the phosphatase CDC25C dephosphorylates tyrosine 15 and threonine 14 to activate CDC2 [57].

1.6. Securin and apoptosis

Securin consists of a homologous family of proteins expressed in different species [58-61]. Securin participates in DNA repair after radiation [62]. Securin overexpression has been reported in a variety of endocrine-related tumors [63-66] and nonendocrine-related cancers [67-69]. It has been shown that securin can promote the cell proliferation and tumorigenesis [70, 71]. Securin levels correlate with tumor invasiveness, and it has been identified as a key signature gene associated with tumor metastasis [72]. It has been reported that overexpression of securin induces apoptosis [70, 73], aneuploidy [74], genomic instability [75, 76], angiogenesis [77, 78], and senescence [79].

1.7. Securin and cell division

Securin has a well-established role in binding and inhibiting separase, an enzyme that cleaves the chromosomal cohesion, and thereby ensuring the appropriate timing of sister chromatid separation to prevent abnormal sister chromatid separation in the mitosis progression [14, 80-84]. Securin can prevent aberrant chromosomal segregation when cellular DNA or spindles are damaged [59, 60, 85]. Securin accumulates during G_2 and prophase and is destroyed at the onset of anaphase [14, 86].

1.8. Purpose of this study

The regulation of securin in the butein-induced mitotic arrest and apoptosis was still unclear. In this study, the anticancer abilities of butein on mitotic arrest and apoptosis are investigated in the human colon cancer cells. Understanding the mechanism by which securin regulates butein-induced mitotic arrest and apoptosis may provide the identification of novel strategies for colon cancer therapy.

2. Materials and methods

2.1. Chemicals and reagents

Butein (B-178), Hoechst 33258, Cy3-labeled mouse anti-β-tubulin (c-4585), propidium iodide, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and the Cy3-labeled mouse anti-β-tubulin were purchased from Sigma Chemical (St. Louis, MO). Butein was dissolved in DMSO, and the concentration of DMSO was < 1 % in the control and drug-containing medium.

2.2. Antibodies

Anti-CDC2, anti-phospho-CDC2 (Tyr15), anti-phospho-CDC2 (Thr14), anti-phospho-CDC2 (Thr161), anti-phospho-histone H3 (Ser10), anti-phospho-p53 (Ser15), and anti-poly (ADP-ribose) polymerase (PARP) was 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-actin (I-19) antibody, goat anti-rabbit IgG horseradish peoxidase, and goat anti-mouse IgG horseradish peoxidase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Antibody	Source	Molecular Weight	Brand	Catalog
Actin	mouse	42	Santa Cruz	sc-1616
CDC2	rabbit	34	Oncogene	PC-25
Cyclin B1	mouse	60	Oncogene	CC03
Caspase-3	mouse	32	Biovision	3004-100
PARP	rabbit	17	Cell Signaling Tech	#9542
Phospho-CDC2 (Thr161)	rabbit	34	Cell Signaling Tech	#9114
Phospho-CDC2 (Tyr15)	rabbit	34	Cell Signaling Tech	#4539
Phospho-CDC2 (Thr14)	rabbit	34	Cell Signaling Tech	#2543
Phospho-p53 (Ser15)	rabbit	53	Cell Signaling Tech	#9284
Phospho-histone H3 (Ser10)	rabbit	17	Cell Signaling Tech	#9701
Securin	mouse	22	Abcam	ab3305
Anti-rabbit IgG-HRP	goat	secondary antibody	Santa Cruz	sc-2004
Anti-mouse IgG-HRP	goat	secondary antibody	Santa Cruz	sc-2005
Anti-rabbit IgG-Hylite 488	goat	secondary antibody	Jackson	115-485-003

2.3. Cell culture

The wild type, securin (-/-), and p53 (-/-) HCT116 colorectal carcinoma cell lines were kindly provided by Dr. B. Vogelstein of Johns Hopkins University (Baltimore, MD). These cell lines were cultured in complete McCoy's 5A medium (Sigma Chemical) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and sodium bicarbonate. These cells were maintained at 37℃ and 5% CO_2 in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

2.4. Cytotoxicity assay

The cells were plated in 96-well plates at a density of 1×10^4 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.

2.5. Time-lapse observation of cell death

HCT116 colon cancer cells were plated at a density of 2×10^5 cells per 35-mm Petri dish in complete medium for 16-20 h. Then the cells were treated with or without 40 μM butein 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)

2.6. Cell cycle analysis

The cell cycle progression after treatment with butein was measured by flow cytometer. The cells were plated at a density of 1×10^6 cells per 60-mm Petri dish in complete medium for 16-20 h. After treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at -20 ℃. 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 ℃ for 30 min. After re-centrifugation, the cells resuspended in 1 ml ice-cold PBS. To avoid cell aggregation, the cell solutions were filtrated through nylon mesh membrane. Subsequently, the samples were analyzed by CellQuest software in flow cytometer (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, Becton-Dickinson).

2.7. Annexin V and PI assays

The cells were plated at a density of 7×10^5 cells per 60-mm Petri dish in complete medium for 16-20 h. Thereafter, the cells were treated with 0-40 μM butein for 24 h. Apoptotic cells was performed using an annexin-V-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-fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide (PI). Finally, the samples were analyzed by flow cytometer using CellQuest software (FACScan, Becton–Dickinson, San Jose, CA). The cells showed annexin V(+)/PI(−) and annexin V(+)/PI(+), which indicated at early and late apoptosis, respectively.

2.8. Western blot

After the end of drug 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 ℃ and centrifuged at 10,000 rpm for 10 minutes. 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. To verify equal protein loading and transfer, proteins were then transferred to polyvinylidene difluoride membranes and the membranes were blocked overnight at 4 ℃ 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). The membranes were then incubated for 2 h at 25℃ with specific primary antibody followed by anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase conjugated secondary antibodies. 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.

2.9. Immunofluorescence staining and confocal microscopy

The cells were cultured on coverslips, which were kept in 35-mm Petri dish at a density of 5×10^5 per well for 16-20 h. After treatment with or without 40 μM butein for 24 h, the cells were washed with PBS. Then fixation with 4% paraformaldehyde solution overnight at 4 ℃, 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 ℃. Thereafter, the cells were separately incubated with rabbit anti-phospho-histone H3 (1:100) antibody in PBS containing 10 % FBS overnight at 4℃, 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 ℃, and washed three times with 0.3 % Triton X-100 in PBS. The samples incubated with mouse anti-securin (1:100) antibody in PBS containing 10 % FBS overnight at 4℃, 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 ℃. 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 Olympus confocal microscope (Olympus, Tokyo, Japan).

2.11. Mitotic index analysis

The cells were cultured on coverslips in a 35-mm Petri dish at a density of 5 × 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 ℃. 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 ℃. 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. The prophase, metaphase, anaphases, and telophase in total mitotic phases were counted under fluorescence microscope.

2.12. Analysis of phospho-histone H3 by flow cytometer

After treatment with butein for 24 h, HCT116 cells were harvested and fixation with 75% alcohol in -20℃ overnight. Subsequently, the samples were incubated with 10 % bovine serum albumin in PBS for 1 hour at 4 ℃. After that, the samples were incubated with rabbit anti-phospho-histone H3 antibody (1:100), and following incubated with anti-rabbit IgG-Hylite 488 (1:100) for 2 hours at 4 ℃ in dark. 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 were quantified the green fluorescence using CellQuest software (BD Biosciences).

2.13. 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. Butein decreases the cell viability and increases the G_2/M fractions in human colon cancer cells

Fig. 1 shows the chemical structure of butein. To examine the cytotoxicity and proliferation following butein treatment in HCT116 colon cancer cells, the cells were analyzed by MTT assays. Treatment with 10-40 μM butein for 24 h significantly reduced the cell viability via a concentration-dependent manner in both the securin (+/+) and securin (-/-) HCT116 cells (Fig. 2). The values of IC_{50} (the concentration of 50 % inhibition of cell viability) were calculated according to Appendix 1. The values of IC_{50} were around 26.6 μM and 18.2 μM in the securin (+/+) and securin (-/-) HCT116 cells, respectively. The securin (-/-) cancer cells were more sensitive than the securin (+/+) cancer cells to cytotoxicity by butein (Fig. 2).

To investigate the effect of butein on the cell cycle progression and the role of securin on the cell cycle progression, the securin (+/+) and securin (-/-) HCT116 cells were treated with butein and compared by flow cytometry. Comparing with untreated and butein-treated samples, butein decreased the G_1/G_0 fractions and dramatically increased the G_2/M fractions in both the securin (+/+) and securin (-/-) HCT116 cells (Fig. 3A and 3B). Moreover, the securin (-/-) cancer cells were higher increase of G_2/M fractions than the securin (+/+) cancer cells after treatment with butein at 20-30

μM.

3.2. Butein inhibits securin protein expression in human colon cancer cells

To investigate the protein expression of securin in the butein-induced cancer cell death, the cells were analyzed by Western blot. The securin protein expression was reduced by butein in securin (+/+) HCT116 cells (Fig. 4A). Actin was used as a loading control protein. Comparing with untreated samples, butein significantly decreased the expression of securin in a concentration-dependent manner in the securin (+/+) HCT116 cells (Fig. 4B).

We have further examined the effect of butein on the securin protein expression by immunofluorescence staining and confocal microscopy. As shown in Fig. 5, the red fluorescence intensity (Cy 3) exhibited by securin and the blue color was represented the location of nuclei by staining with Hoechst 33258. The fluorescence intensities of securin proteins were decreased after treatment with 30 μM butein for 24 h (Fig. 5).

3.3. Butein induces histone H3 phosphorylation, mitotic arrest and aberrant chromosome separation

To determine whether G_2 or M phases induced by butein, the cells were treated butein and analyzed the protein levels of phospho-histone H3, a mitotic marker protein by Western blot and immunofluorescence staining. Comparing with untreated samples, butein significantly increased the expression of phospho-histone H3 in a

concentration-dependent manner in the securin (+/+) HCT116 cells (Fig. 6). However, butein did not alter the phospho-histone H3 protein level in the securin (-/-) HCT116 cells (Fig. 6). To examine butein on the cell morphology and cellular location of phospho-histone H3 proteins in HCT116 cells, the cells were treated with or without butein (30 μM for 24 h) and subjected to immunofluorescence staining and confocal microscopy. The green fluorescence intensity exhibited by phospho-histone H3, the red fluorescence intensity exhibited by β-tubulin proteins of the cytoskeleton, and the blue color was represented the location of nuclei by staining with Hoechst 33258. Treatment with butein increased the accumulation of phospho-histone H3 in mitotic cells (Fig. 7). Moreover, butein induced abnormal chromosome segregation in the mitotic cells (Fig. 7). In addition, the fluorescence intensity of phospho-histone H3 proteins was increased by butein by indirect phospho-histone H3 immunofluorescence staining and flow cytometer (Fig. 8A). The fluorescence intensity of phospho-histone H3 proteins was elevated to 10-folds by 40 μM butein treatment comparing to untreated HCT116 cells (Fig. 8B).

Moreover, we have analyzed the mitotic index after treatment with butein. Butein markedly increased the mitotic cell number (Fig. 9). The stages of mitotic cells after treatment with butein in prometaphase (with condensed DNA but no 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. Besides, butein decreased the protein levels phospho-CDC2 (Thr-14) and slightly reduced phospho-CDC2 (Tyr-15) in colon cancer cells (Fig. 10). In contrast, butein increased the protein levels phospho-CDC2 (Thr-161) and cyclin B1 (Fig. 10). However, the total CDC2 protein level was not altered in the butein-treated cells.

3.4. Butein induces apoptosis in human colon cancer cells

The effect of butein on the induction of cell death was observed by time-lapse living cell morphology. The arrows indicate that butein induced the cell death morphology and reduced cell number (Fig. 11). However, the untreated cells clearly displayed the increase of cell proliferation and cell number after 48 h observation (Fig. 11).

We further assessed apoptosis from the cells that had been exposed to butein by annexin V and PI staining analysis. The control cells were not significantly stained with fluorochromes; however, the annexin V (+) apoptosis cells were increased by treatment with 40 μM butein for 24 h in HCT116 cells (Fig. 12A). After quantification of apoptotic cell number, butein induced apoptosis via a concentration-dependent

manner in HCT116 cells (Fig. 12B).

3.5. Butein increases the protein levels of active caspase-3 and cleavage of PARP in colon cancer cells

To examine the induction of apoptosis pathway following butein treatment, HCT116 cells were analyzed the protein levels of the caspase-3 activation and PARP cleavage using Western blot. The active forms of caspase-3 (12 kDa and 17 kDa) were induced following treatment with butein (Fig. 13). Moreover, the cleaved form proteins of PARP (89 kDa) were increased by exposure to butein in HCT116 cells.

3.6. Butein induces phosphorylation of p53 (Ser15) and existence of p53 reduces the butein-induced cell death

Treatment with 10-40 μM butein for 24 h significantly reduced the cell viability via a concentration-dependent manner in both the p53 (+/+) and p53 (-/-) HCT116 cells (Fig. 14). The values of IC_{50} were around 26.6 μM and 18.1 μM in the p53 (+/+) and p53 (-/-) HCT116 cells, respectively. We had examined the effect of butein on the p53 protein expression in HCT116 cancer cells. Butein did not alter p53 protein level but increased phospho-p53 (Ser15) proteins in HCT116 cells (Fig. 15A). The semi-quantified data showed that butein (30-40 μM for 24 h) significantly elevated the protein levels of phospho-p53 (Ser15) (Fig. 15B).

4. Conclusion

We provide a model that the butein induces mitotic arrest and apoptosis in human colon cancer cells (Fig. 16). Butein induces mitotic arrest and apoptosis that may be regulated by the inhibition of securin and the activation of p53. Understanding the mechanisms by which securin and p53 regulates mitotic progression and apoptosis following butein may contribute to novel therapeutic strategies in colon cancers.

5. Discussion

Various types of chalcones display anticancer effects on growth inhibition and apoptosis [11, 87-89]. In this study, we provide for the first time that butein induces mitotic arrest and apoptosis in human colon cancer cells. The cell cycle arrest at G_2/M phase has been suggested to mediate the antiproliferative activities of butein [34]; however, butein induced G_2 or mitotic arrest still unclear. Our results further provide that butein is a potent agent to induce mitotic arrest in cancer cells.

The phosphorylation of histone H3 is known to be involved in cell division [90]. Phosphorylation of histone H3 is critical for proper chromosome condensation and segregation in mitotic cells [91]. We found that butein significantly increased the levels of phospho-histone H3, which located in chromosomes in colon cancer cells. It is indicated that butein can induce mitotic cells. According to confocal microscope observation and mitotic index analysis, the butein-treated HCT116 colon cancer cells arrested in prophase and metaphase stages. Thus, we suggest that butein blocks mitotic arrest at the metaphase/anaphase transition.

Securin controls sister chromatid separation, progression from metaphase to anaphase; its defects can result in chromosomal instability [81, 92, 93]. It has been reported that securin is required for genomic stability during mitosis [60, 81]. Moreover, securin regulates DNA repair following UV and X-ray damages [62]. Thus,

the inhibition of securin would block the cell survival and proliferation in tumor cells, providing important strategy in cancer therapy. Treatment with anticancer agents including ultraviolet, doxorubicin, and bleomycin decreased the securin expression in cancer cells [94, 95]. We found that butein markedly reduced securin protein expression and abnormal chromosomal segregation in colon cancer cells. We suggest the down-regulation of securin may mediate genomic instability and abnormal chromosome separation in human colon cancer cells. Interestingly, comparing with the securin (+/+) and securin (-/-) HCT116 cells by butein treatment, we found that the securin (-/-) cells were higher on the increase of G_2/M fractions than the securin (+/+) cancer cells; however, butein did not alter the phospho-histone H3 protein level in the securin (-/-) HCT116 cells. We suggest that butein may block securin expression to induce abnormal mitotic progression and inhibiting cell proliferation. Nevertheless, whether butein induces G_2 phase arrest in the securin (-/-) HCT116 cells need to be further investigated.

Activation of CDC2/cyclin B1 complex is a well-known mechanism to regulate mitotic progression. The activation of CDC2/cyclin B1 complex promotes mitotic progression, including chromosome condensation, nuclear envelope breakdown, and spindle pole assembly. Phosphorylation of Thr61 by CAK is required for CDC2 activity [53], whereas phosphorylation of Tyr5 by Wee1 [51, 54, 55] and Thr14 by

Myt1 [56] inhibits CDC2 activity. At the onset of mitosis, the phosphatase CDC25C dephosphorylates Tyr15 and Thr14 to activate CDC2 [57]. Cyclin B1 is degraded to allow mitotic exit and cytokinesis [96, 97]. We found that butein increased the activation of CDC2 on Thr161 phosphorylation and reduced inhibitory phosphorylation at Thr14. The protein level of cyclin B1 was also increased by butein treatment. The alterations in CDC2 phosphorylation and cyclin B1 by butein may maintain spindle checkpoint activation and further inhibits exit from mitosis.

If the exit out of G_2 into mitotic phase occurs in the setting of coexistent DNA damage, the tumor cells undergo apoptosis and induction of a form of cell death called “mitotic catastrophe” [98, 99]. Mitotic catastrophe is characterized by missegregation of chromosomes, leading to an aberrant mitosis or imperfect cell division [100]. It has been suggested that mitotic catastrophe is accompanied by chromatin condensation and mitochondrial release of proapoptotic proteins such as cytochrome c and AIF [101]. We found that butein induced apoptosis related to the activation of caspase-3 and cleavage of PARP. Accordingly, we suggest that butein causes mitotic catastrophe and subsequently induces caspase-dependent pathway for cancer cell death.

The tumor suppressor p53 has been proposed as an important target for cancer treatment [102, 103]. The diverse phosphorylation sites of p53 have been indicated to

play important roles in the regulation of many cellular responses [104-106]. The phosphorylation of p53 at Ser15 is an important target for p53 activation [107] and stabilization [104, 108]. Moreover, the phosphorylation of p53 at Ser15 may transmit a survival signal and may suppress apoptosis in response to several stimuli [109]. We found that butein increased the phosphorylation of p53 at Ser15 in HCT116 colon cancer cells. Butein induced the cytotoxicity in both the p53 (+/+) and p53 (-/-) colon cancer cells. However, the p53 (-/-) cells were more susceptible to cell death than the p53 (+/+) cells in the increase about 21% following butein. These findings suggest that butein still displays anticancer ability in the p53-mutational or -nonfunctional cancer cells.

6. References

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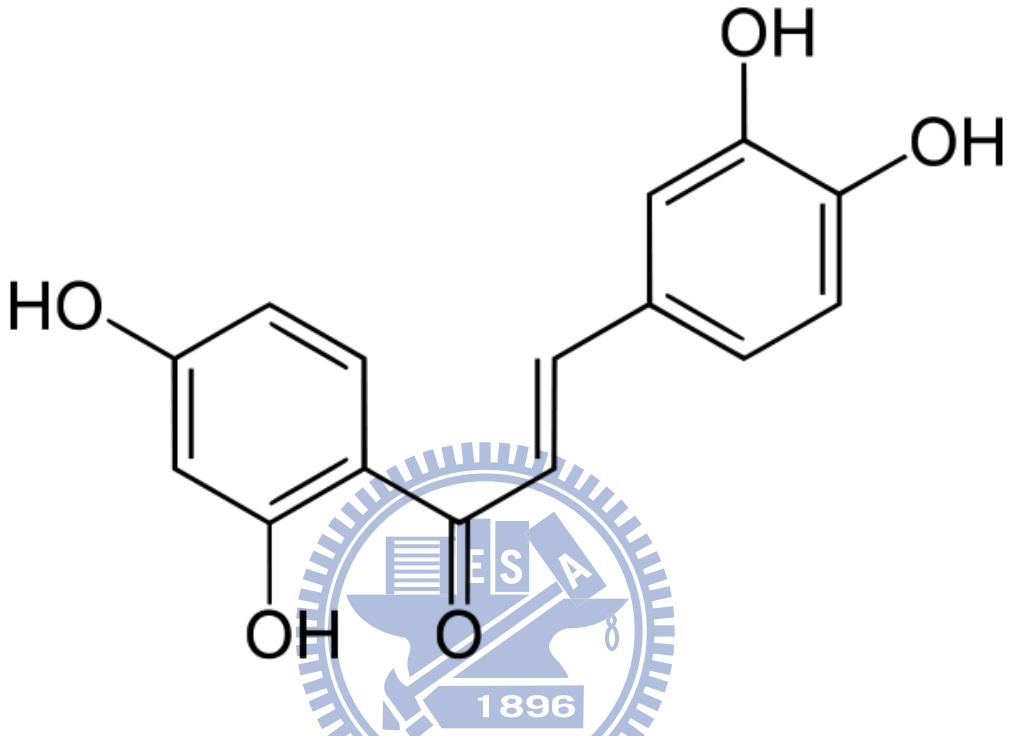


Fig. 1. Chemical structure of 3, 4, 2', 4'-tetrahydroxychalcone (butein)

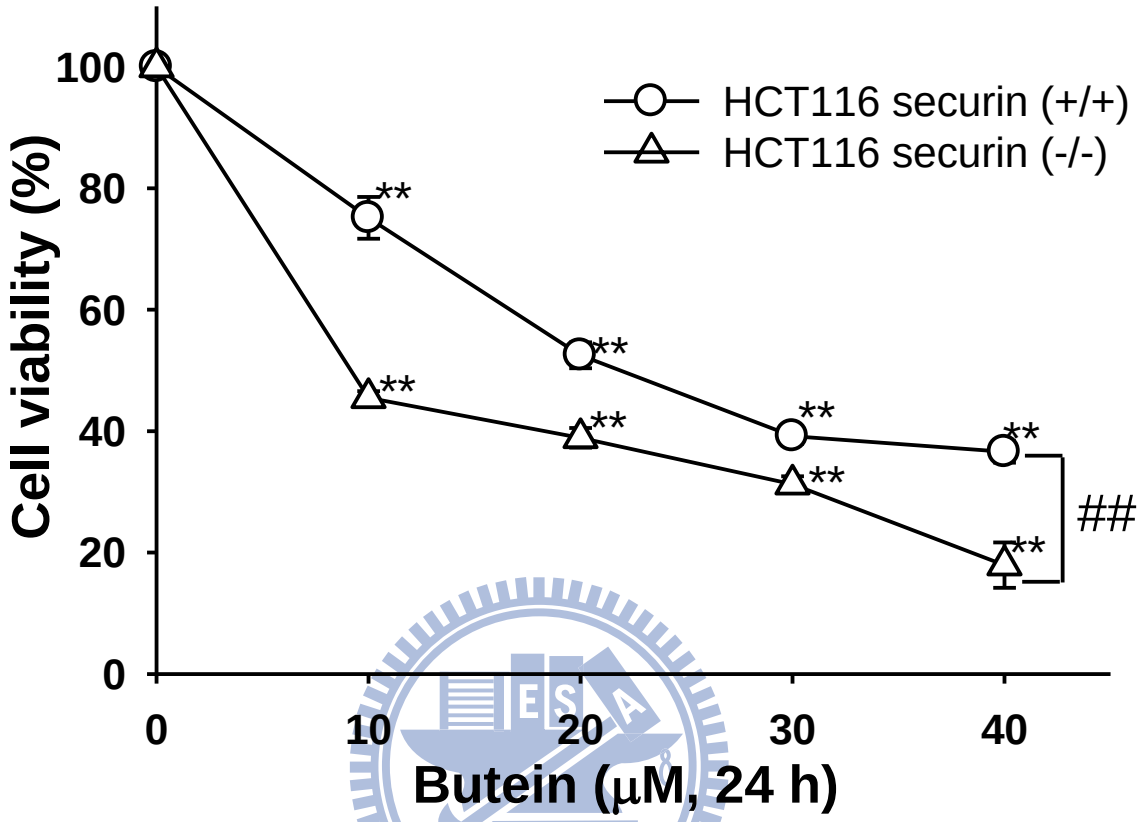


Fig. 2. Comparison of cell viability between the HCT116 securin (+/+) and securin (-/-) cells by butein. The cells were treated with 0-40 μM butein for 24 h. After drug treatment, the cells were recultured in fresh medium for 2 days. The cell viability was measured by MTT assay. Results were obtained 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 securin (+/+) and securin (-/-) HCT116 cancer cells by butein treatment at the same concentration.

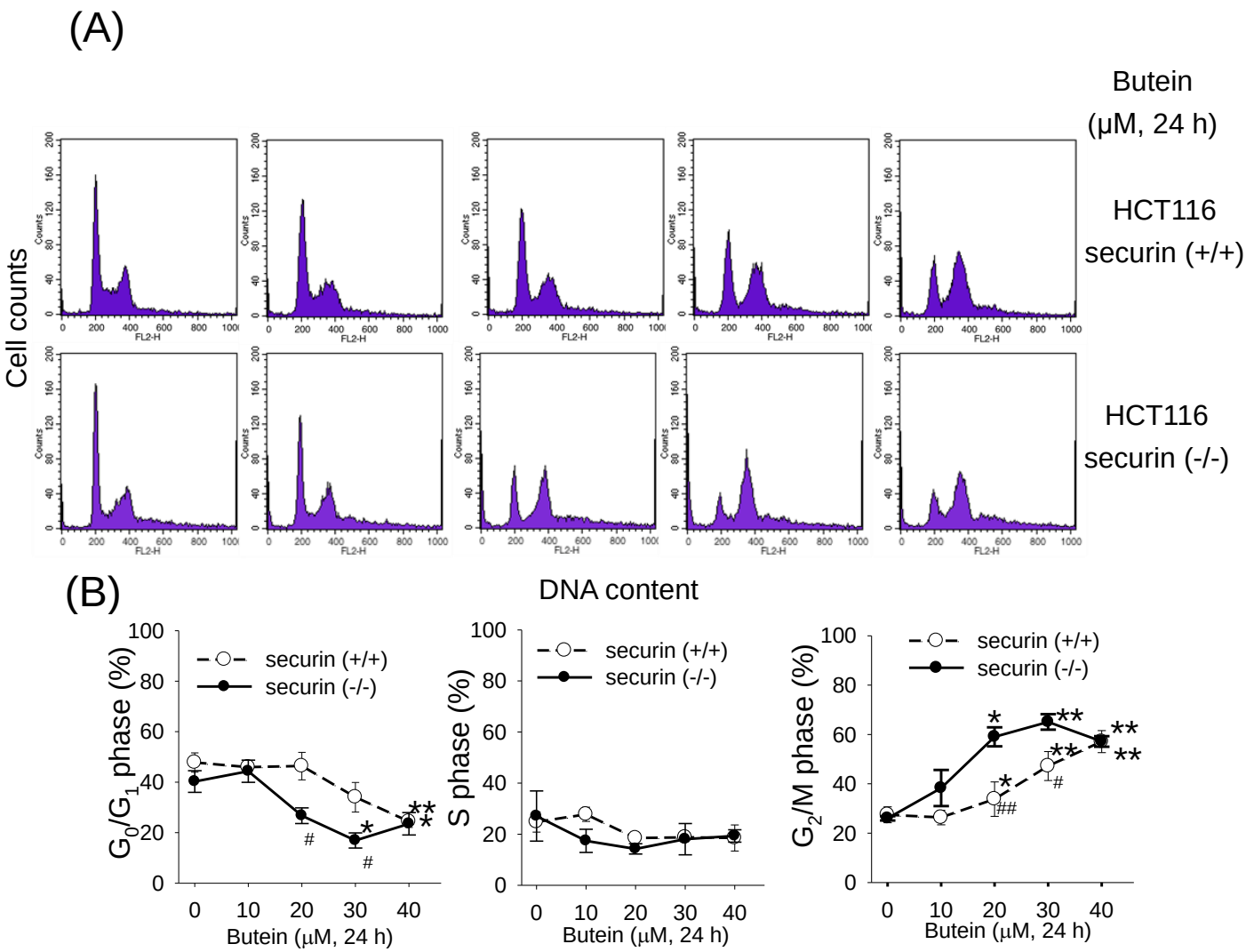


Fig. 3. The effect of butein on cell cycle progression between the HCT116 securin (+/+) and securin (-/-) cells. (A) The cells were treated with 0-40 μM butein for 24 h. The cells were trypsinized and then subjected to flow cytometry analysis. The representative flow data were shown from one of five separate experiments with similar findings. (B) The percentages of G_0/G_1, S and G_2/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 $^{\#\#}p < 0.01$ indicates significant difference between the securin (+/+) and securin (-/-) HCT116 cancer cells by butein treatment at the same concentration.

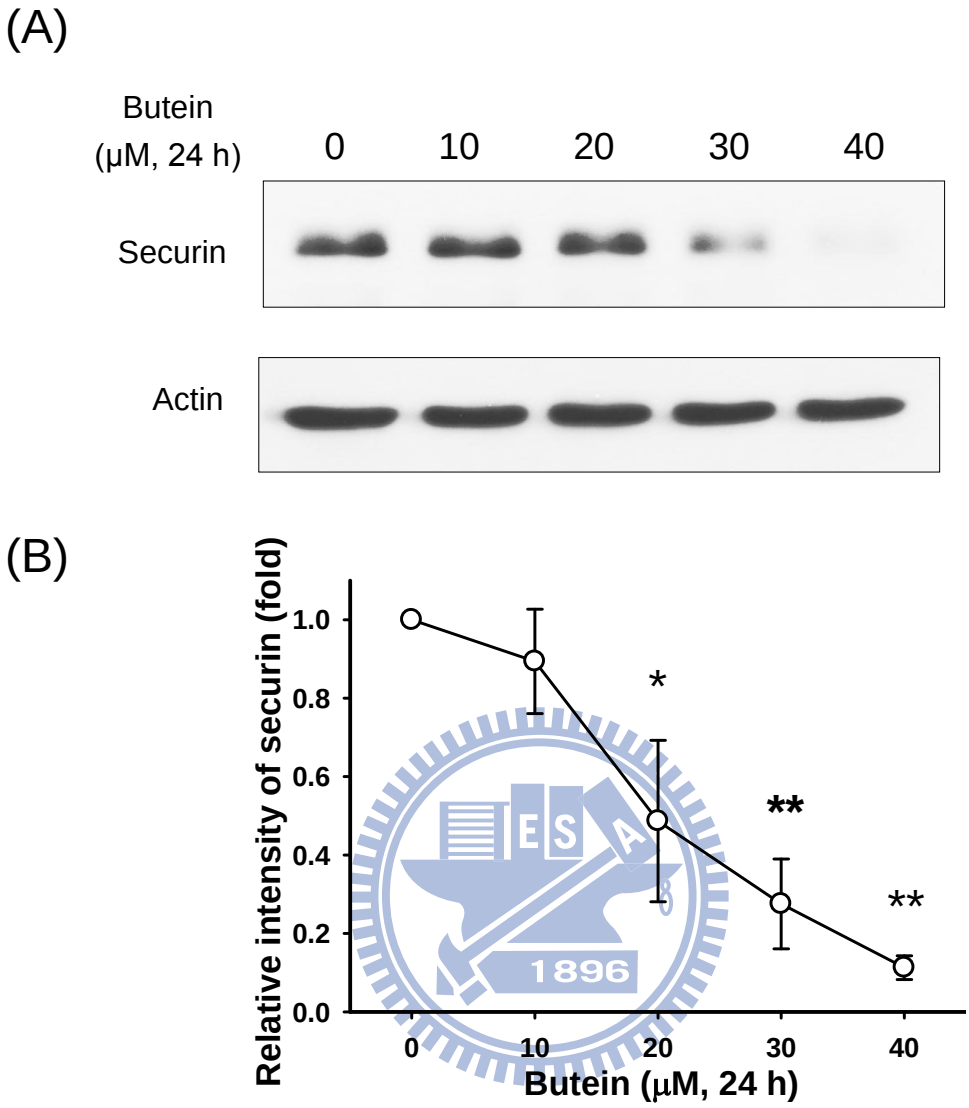


Fig. 4. The effect of butein on the protein expression of securin in the HCT116 securin (+/+) cells. (A) The cells were treated with 0-40 μM butein for 24 h. The total protein extracts were prepared for immunoblot analysis using anti-securin and anti-actin antibodies. Actin was a loading control. (B) The relative intensity of securin was from Western blot 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.

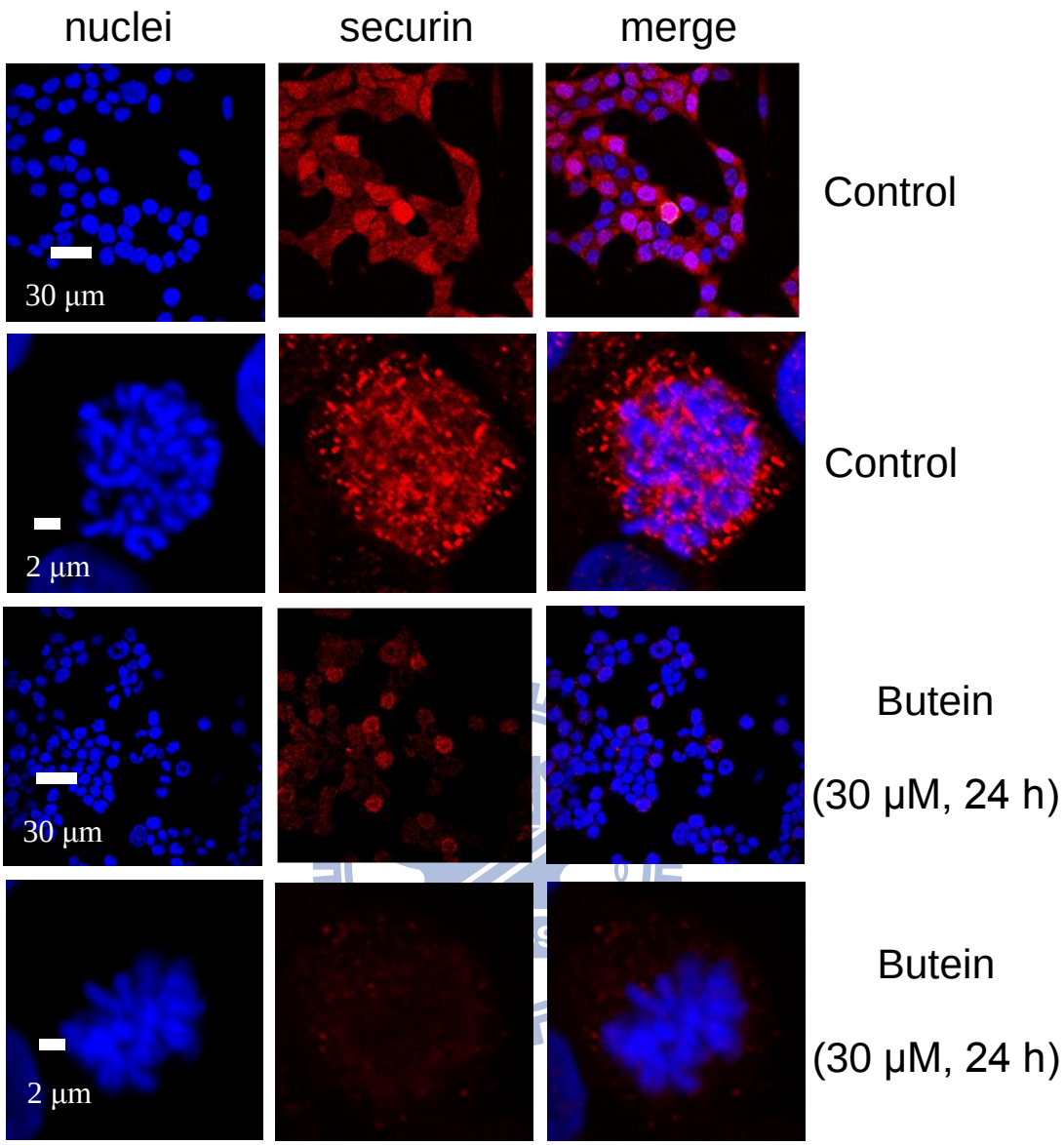


Fig. 5. The protein expression and location of securin after treatment with butein in the HCT116 securin (+/+) cells. The cells were treated with or without 30 μM butein for 24 h. At the end of treatment, the cells were incubated with mouse anti-securin and then incubated with goat anti-mouse Cy3. The securin proteins displayed red fluorescence with goat anti-mouse Cy3. The nuclei were stained with Hoechst 33258.

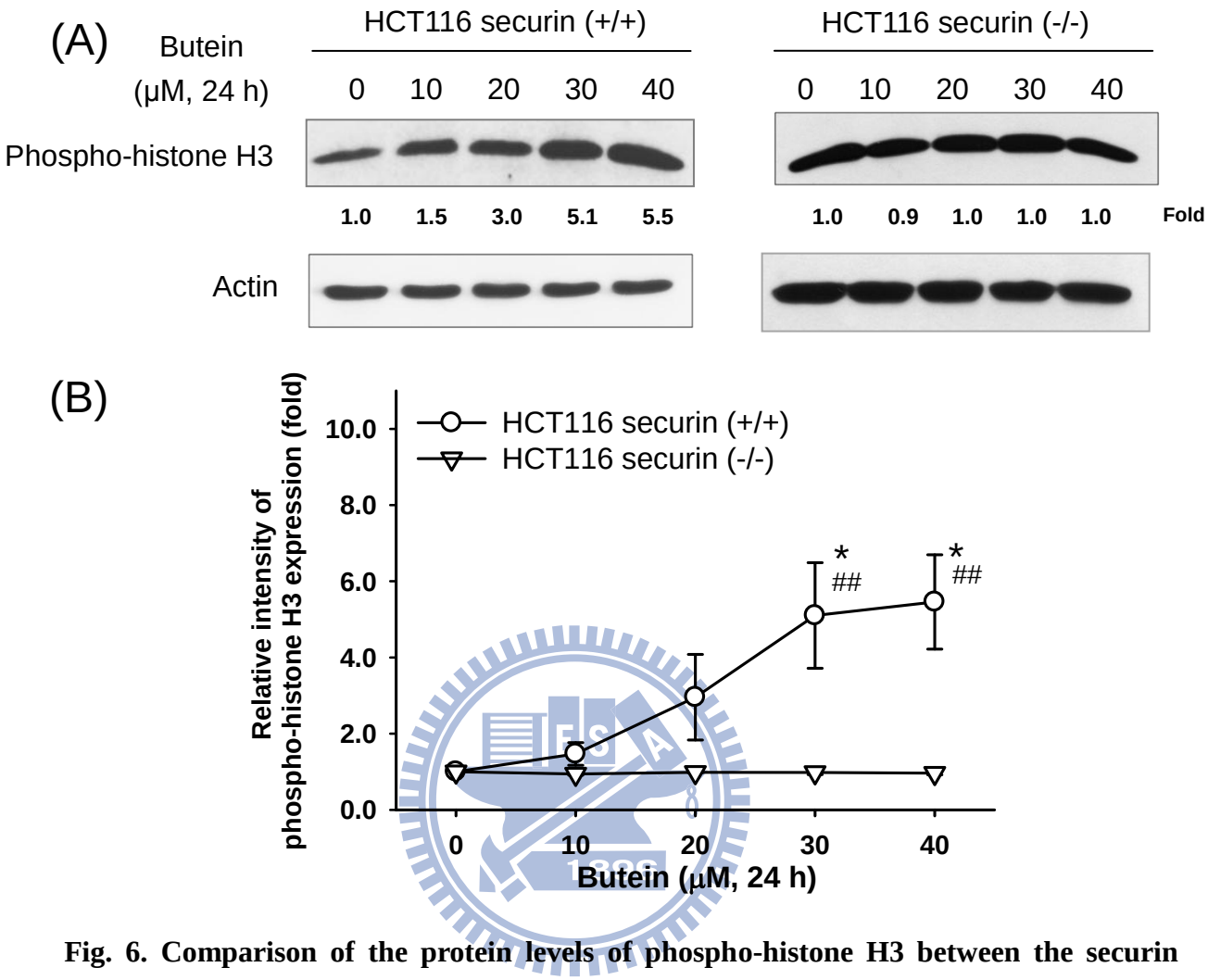


Fig. 6. Comparison of the protein levels of phospho-histone H3 between the securin (+/+) and securin (-/-) HCT116 cells following butein treatment. (A) The cells were treated with 0-40 μM butein for 24 h. The total protein extracts were prepared for immunoblot analysis using anti-phospho-histone H3 and anti-actin antibodies. Actin was a loading control. (B) The relative intensity of phospho-histone H3 was from Western blot by semi-quantification. Results were obtained from three experiments and the bar represents the mean ± S.E.M. *$p < 0.05$ indicates significant difference between control and butein treated samples. $^{\#\#}p < 0.01$ indicates significant difference between the securin (+/+) and securin (-/-) HCT116 cells by the butein treatment at the same concentration.

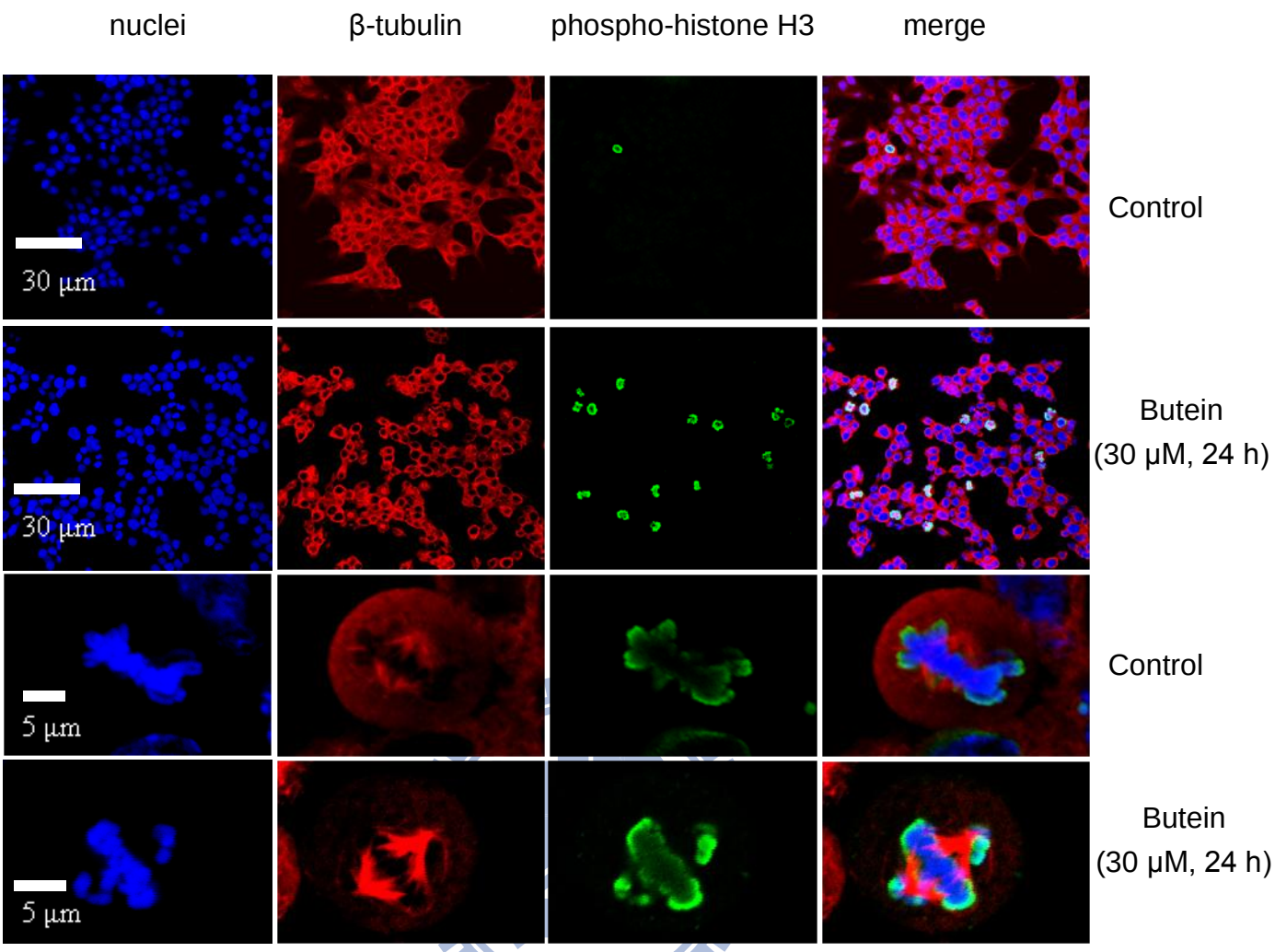


Fig. 7. Butein increases phospho-histone H3 and abnormal chromosome separation in the HCT116 securin (+/+) cells. The cells were treated with or without 30 μM butein for 24 h. At the end of treatment, the cells were incubated with rabbit anti-phospho-histone H3 (Ser10) and then incubated with goat anti-rabbit 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.

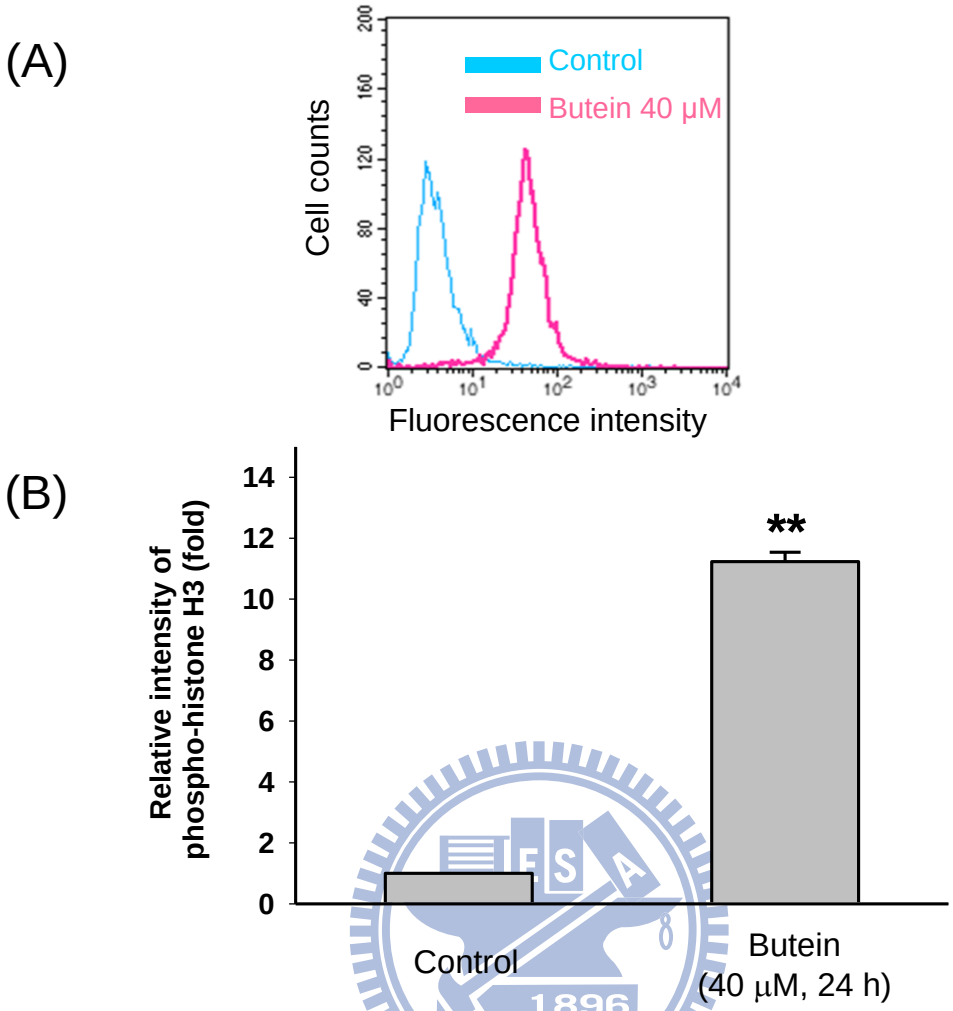


Fig. 8. The effect of butein on the protein level of phospho-histone H3 in the HCT116 securin (+/+) cells by flow cytometer. (A) The cells were treated without or with 40 μM butein for 24 h. The cells were incubated with rabbit anti-phospho-histone H3 (Ser10) antibody. Then the cells were incubated with 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. (B) 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.

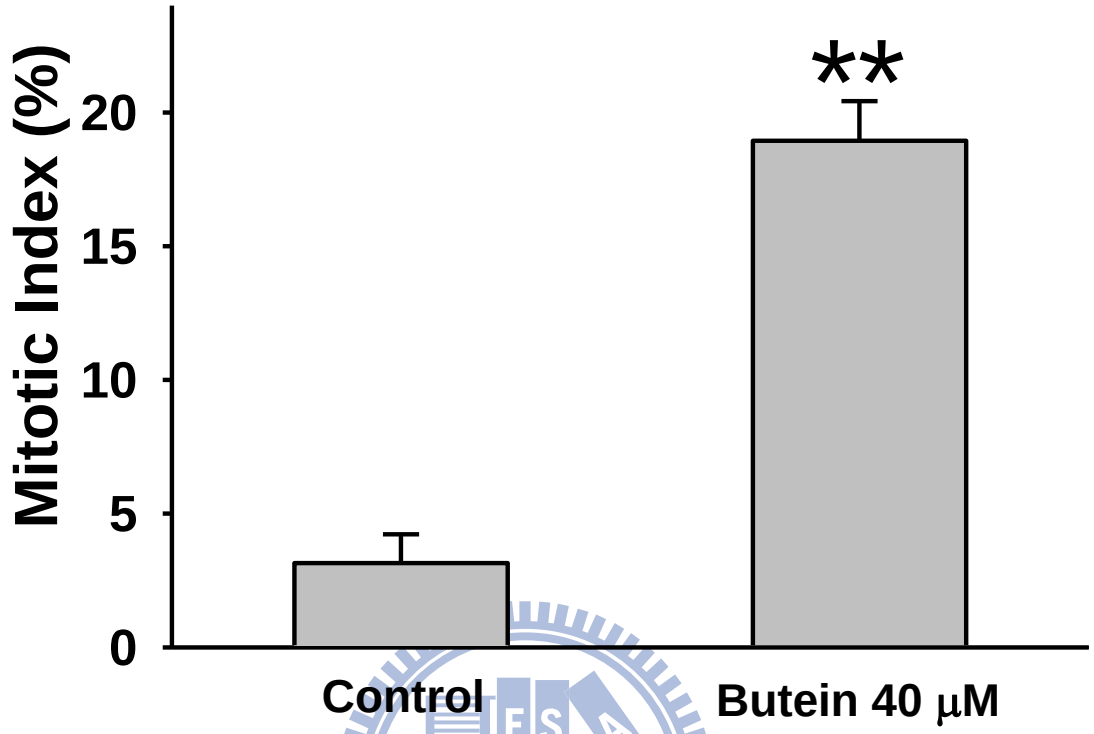


Fig. 9. The effect of butein on mitotic index in the HCT116 securin (+/+) cells. The cells were treated with or without 40 μM butein for 24 h. Mitotic index (the percentage of mitotic cell number/total cell number) was counted under a fluorescence microscope. The β-tubulin and nuclei of cells were stained with the Cy3-labeled mouse anti-β-tubulin and Hoechst 33258, respectively. 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.

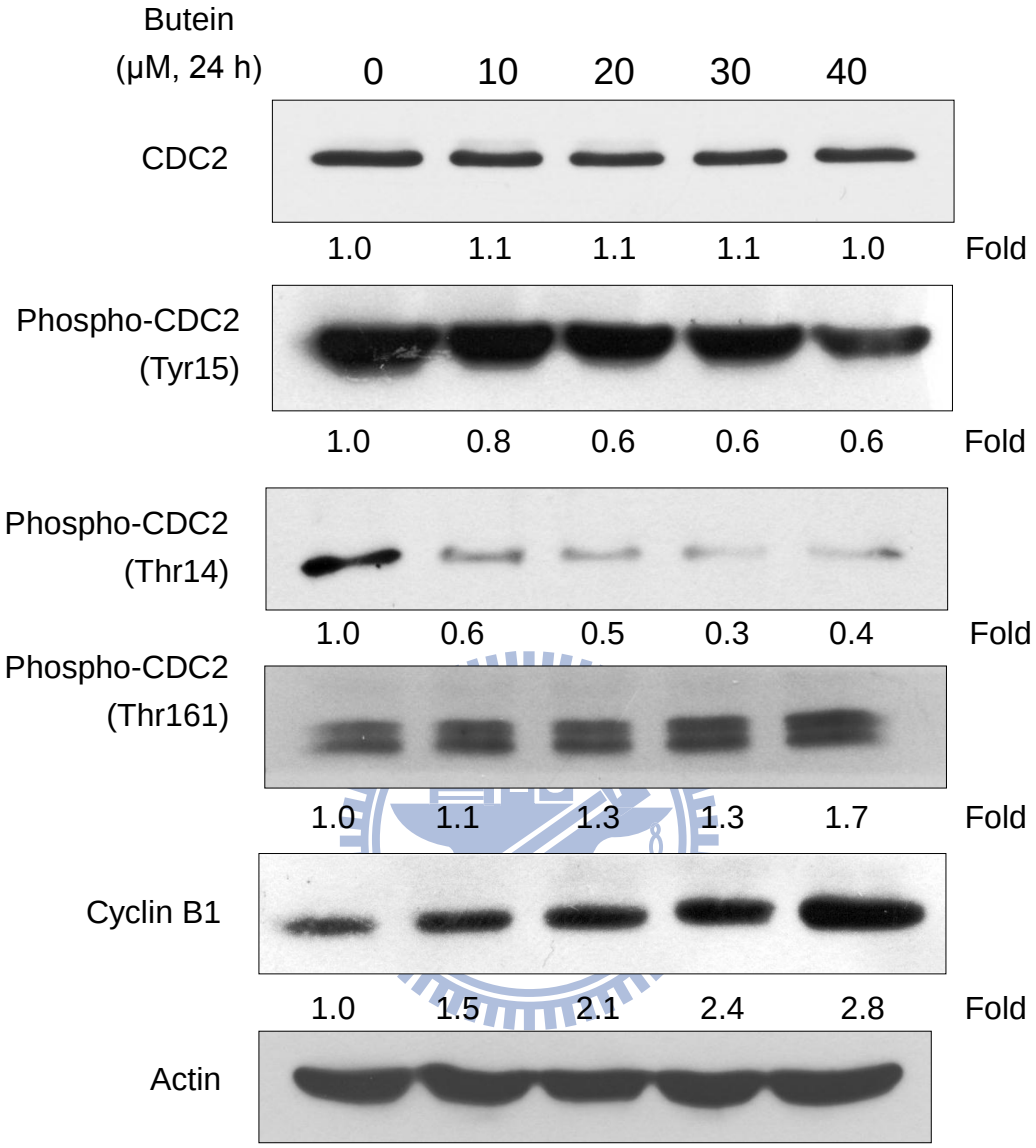


Fig. 10. The effect of butein on protein expression of phospho-CDC2, CDC2 and cyclin B1 in the HCT116 securin (+/+) cells. The cells were treated with 0–40 μM Butein for 24 h. The total protein extracts were prepared for immunoblot analysis using specific anti-CDC2, anti-phospho-CDC2 (Tyr-15), anti-phospho-CDC2 (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.

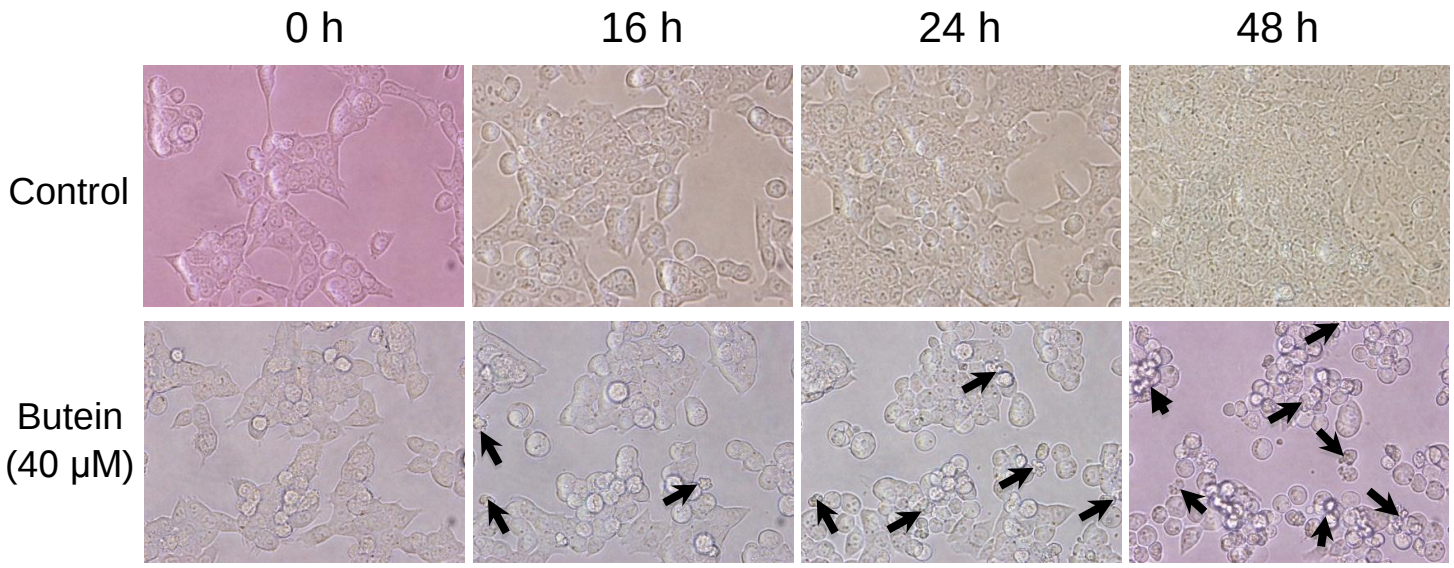


Fig. 11. Time-lapse observation in the butein-induced cancer cell death of the securin (+/+) HCT116 cells. The cells were treated with or without 40 μM butein by time-lapse observation from 0 to 48 h. The morphology of cells was observed under an optical phase contrast microscope with cell incubator system. The arrows indicate the cell death following butein treatment.

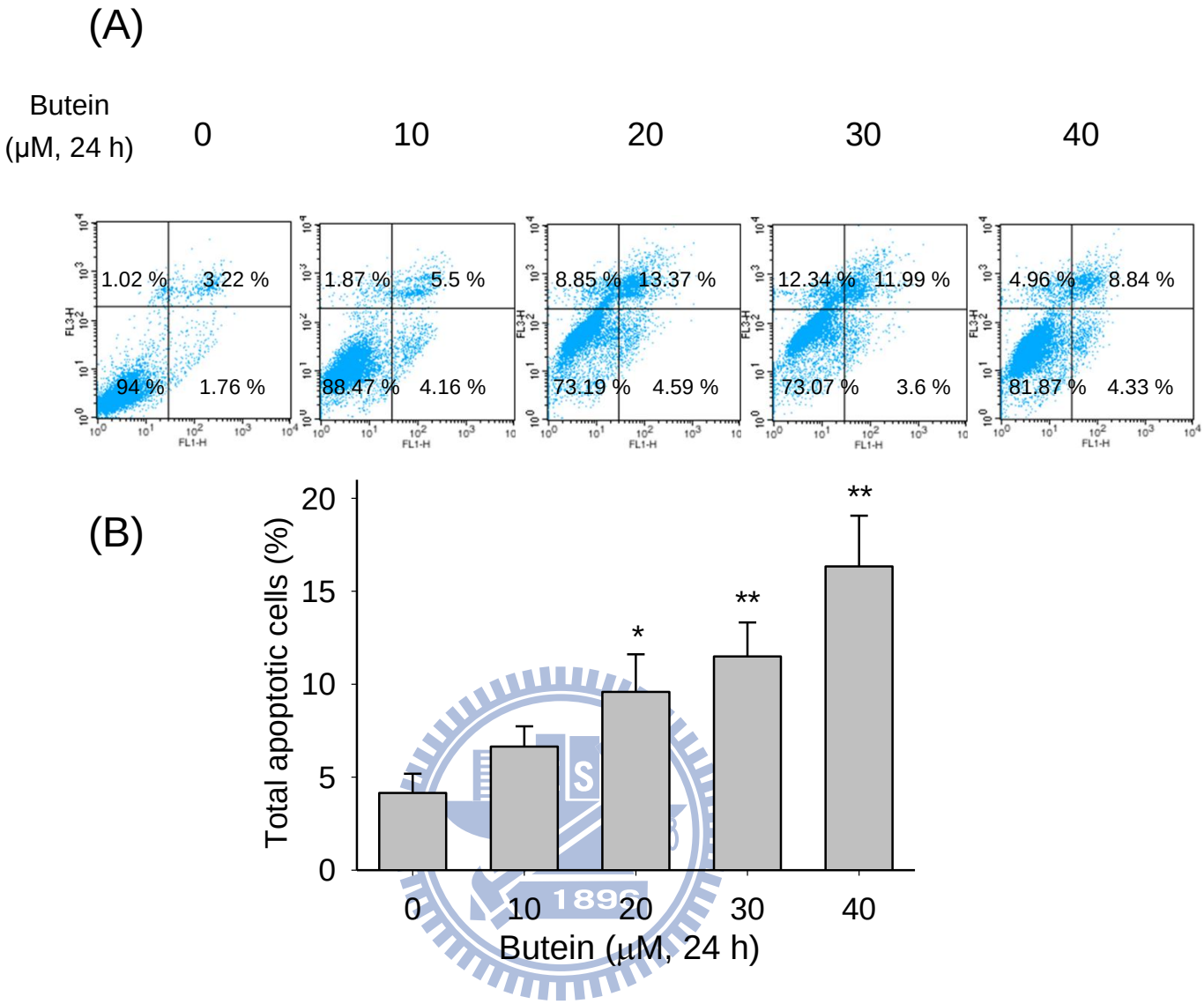


Fig. 12. The effect of butein on apoptosis in the HCT116 securin (+/+) cells. (A) The cells were treated with 0-40 μM butein for 24 h. The apoptosis was measured by Annexin and PI assay. The population of Annexin V+/PI- cells represents cells undergoing early apoptosis (lower right), whereas the fraction of Annexin V+/PI+ cells are those undergoing late apoptosis (upper right). (B) Populations of total 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. *$p < 0.05$ and **$p < 0.01$ indicate significant difference between control and butein treated samples.

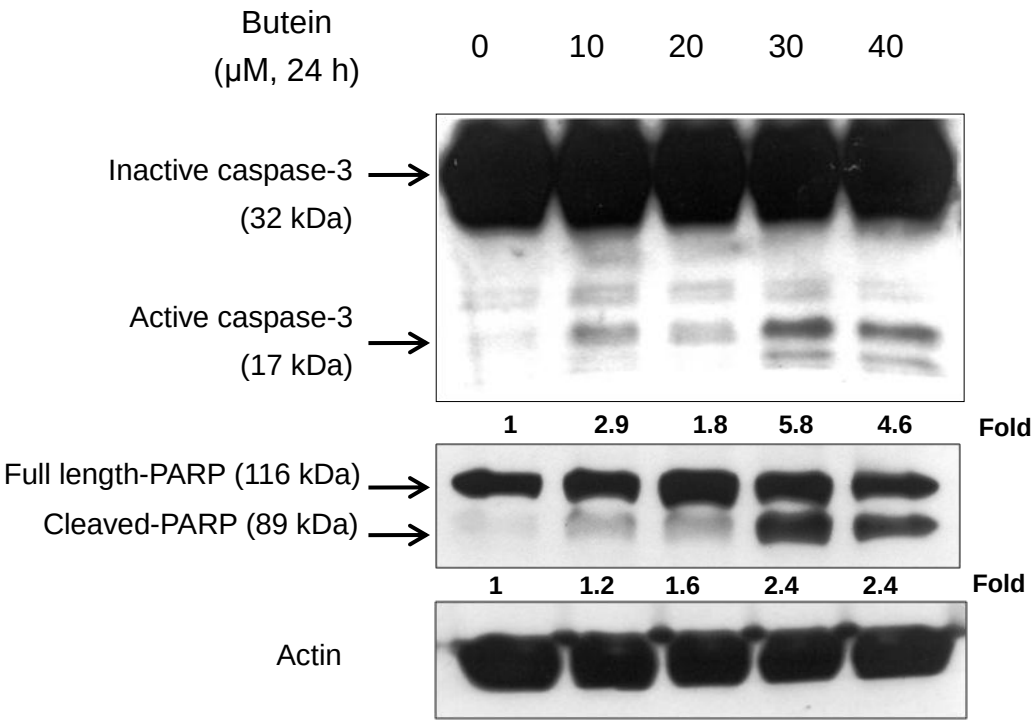


Fig. 13. The effect of butein on the activation of caspase-3 and the cleavage of PARP in the HCT116 securin (+/+) cells. The cells were treated with 0-40 μM butein for 24 h. The protein levels of active caspase-3 (17 kDa) and cleaved-PARP (89 kDa) were analyzed by Western blot. The representative Western blot data were shown from one of three separate experiments with similar findings. Actin was a loading control.

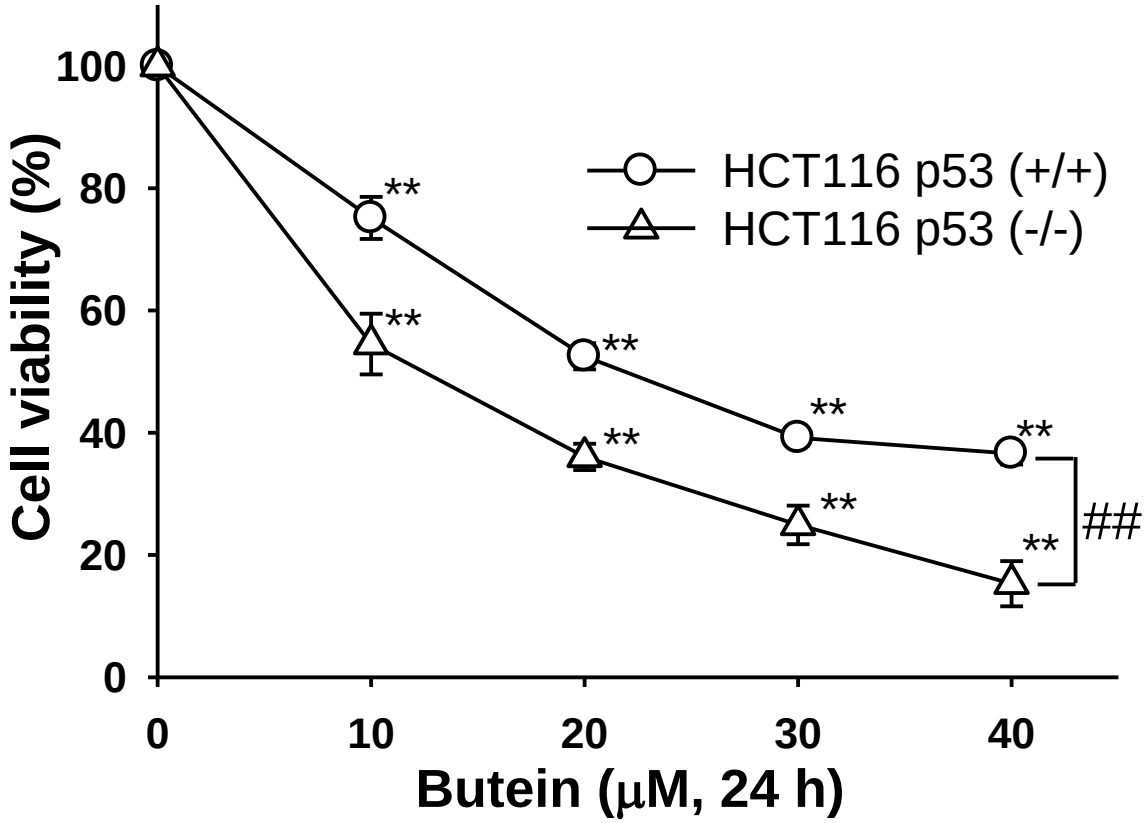


Fig. 14. Comparison of cell viability between the HCT116 p53 (+/+) and p53 (-/-) cells following butein treatment. The cells were treated with 0–40 μM butein for 24 h. After drug treatment, the cells were recultured in fresh medium for 2 days. The cell viability was measured by MTT assay. Results were obtained from four experiments and the bar represents the mean ± S.E.M. *$p < 0.05$ and **$p < 0.01$ indicate significance between control and butein-treated samples. $^{\#\#}p < 0.01$ indicates significant difference between the p53 (+/+) and p53 (-/-) HCT116 cancer cells by butein treatment at the same concentration.

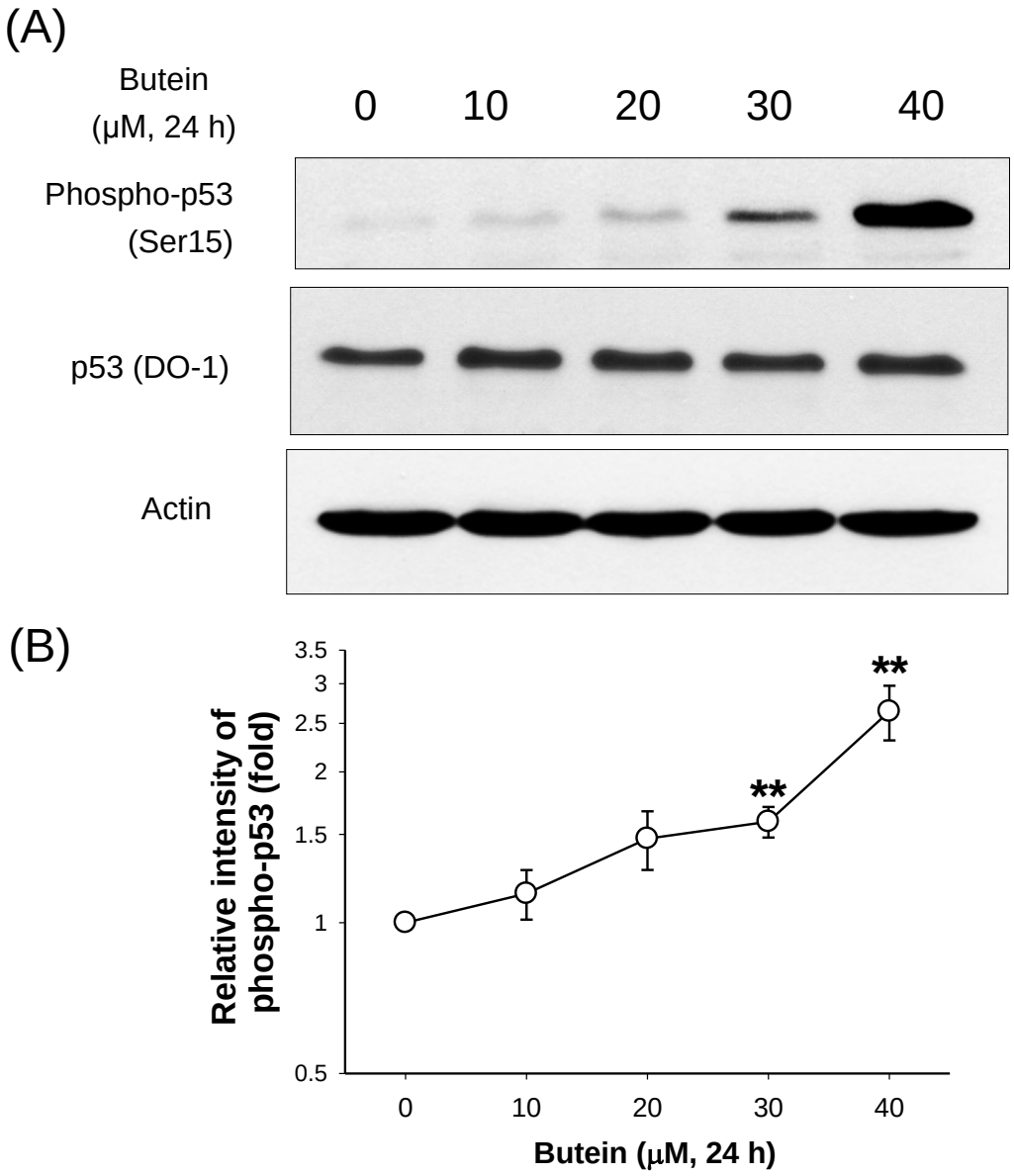


Fig. 15. The effect of butein on the protein expression of p53 and phospho-p53 (Ser15) in the HCT116 securin (+/+) cells. (A) The cells were treated with 0–40 μM butein for 24 h. The total protein extracts were prepared for immunoblot analysis using anti-p53, anti-phospho-p53 (Ser15) and anti-actin antibodies. Actin was a loading control. (B) The relative intensity of phospho-p53 (Ser15) was from Western blot by semi-quantification. 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.

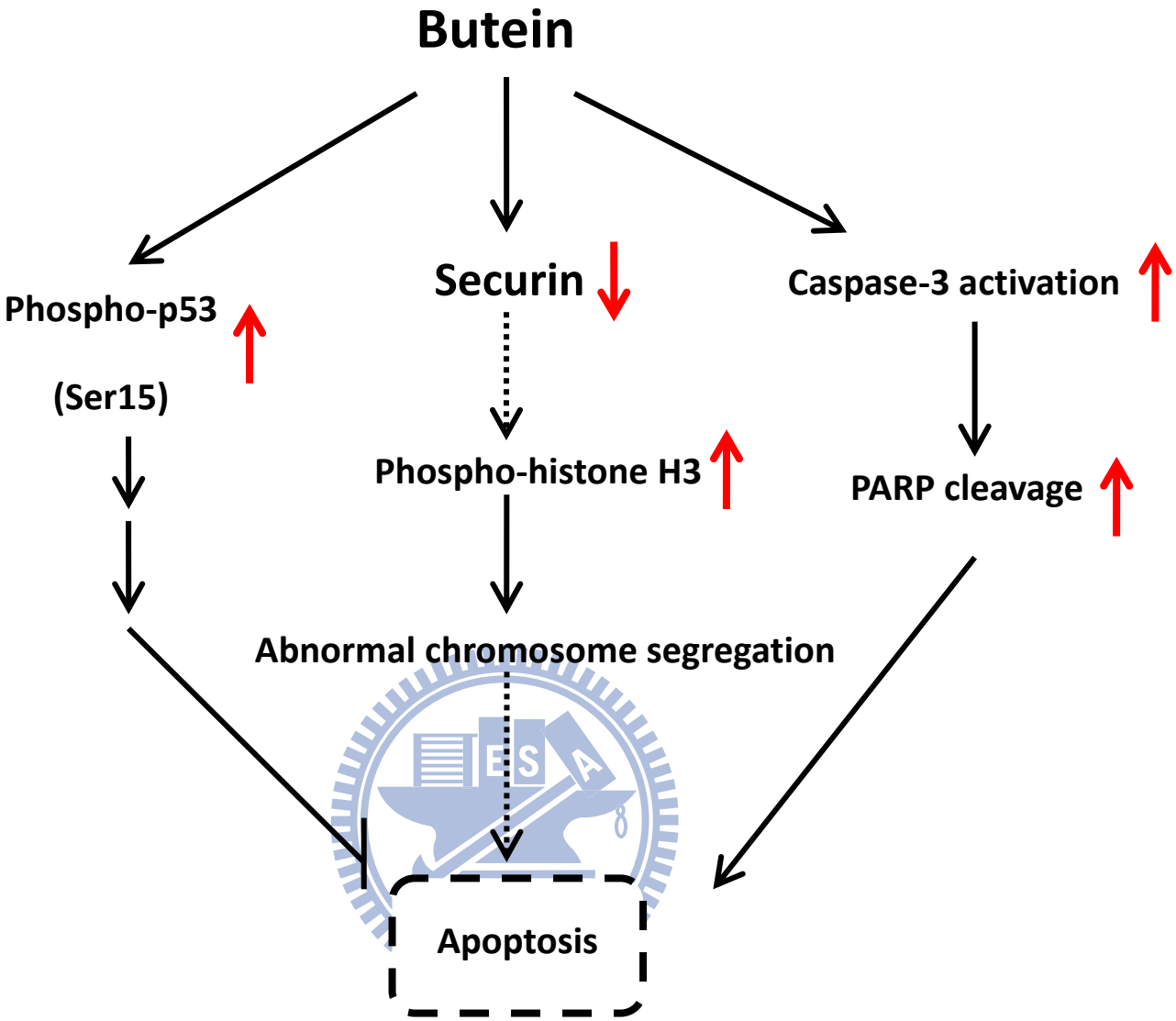
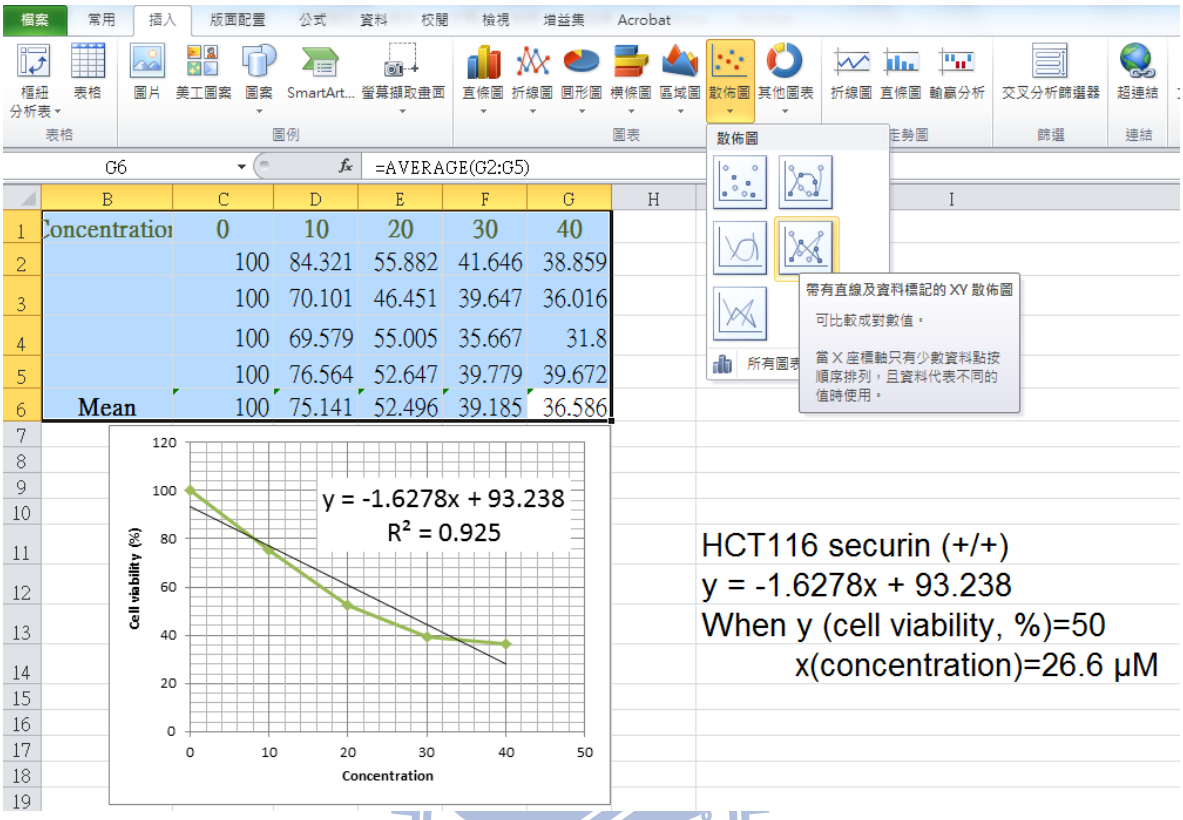


Fig. 16. Proposed model of apoptosis induction and mitotic arrest induced by butein



Appendix 1. Calculation of IC_{50} value of butein in the HCT116 securin (+/+) and securin (-/-) cells. The mean percentage of cell viability by butein in different concentrations (10, 20, 30, 40 μM) from MTT assays was calculated by Excel software. The equation (y=ax+b) and linear regression were analyzed by Excel software. The y value (viability, %) is indicated as 50. The x value shows the IC_{50} value. After calculation, the IC_{50} values were 26.6 μM and 18.2 μM in the HCT116 securin (+/+) and securin (-/-) cells, respectively.