國立交通大學

生物科技學院

分子醫學與生物工程研究所

碩士論文

探討 Securin 在 ACP-93 誘發細胞凋亡和抗腫瘤形成作用的角色

The role of securin in apoptosis and anti-tumorigenesis induced by ACP-93

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致謝

時間過得很快，歷經兩年的努力，我的碩士生涯將畫上句點。這篇論文，要感謝許多人的幫忙及支持才能順利完成。首先要謝謝我的指導教授趙瑞益老師，給我這個機會加入分子抗癌實驗室。在學業和研究上，老師不厭其煩的指導，讓我對細胞生物學和標靶抗癌等相關領域有深入的瞭解，學會研究上該具有的邏輯思考以及嚴謹的態度，並且啟發我面對困難和解決問題的能力；在生活上，老師也會適度的給予關心，為我們設想。

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Contents

中文摘要

Securin 又名腦下垂體腫瘤轉型基因，能促進癌細胞增生及腫瘤形成。ACP-93 是一種由 UK-1 衍生的新合成化合物。在本研究中我們探討 ACP-93 處理人類大腸癌細胞後，誘發細胞凋亡與抑制腫瘤形成的抗癌活性，以及 securin 可能扮演的調控角色。ACP-93 都會誘發 securin 功能正常與缺乏的 HCT116 人類大腸癌細胞之凋亡。以 4-32 μM ACP-93 處理 24 小時，誘發 caspase 3 和 caspase 8 的活化，以及造成 PARP 蛋白的分解。ACP-93 同時誘發 ATF-3 蛋白與磷酸化 H2AX 蛋白的表現。再者，ACP-93 誘發磷酸化 AKT 蛋白的增加。以 wortmannin 共同處理抑制 AKT 路徑後，會增加 ACP-93 所誘發的細胞毒性。更重要是 ACP-93 具有顯著抑制嚴重免疫缺失的老鼠與裸鼠中之異體移植人類大腸腫瘤的形成作用。此外，缺乏 securin 的 HCT116 大腸癌細胞在異體移植裸鼠中的腫瘤生長能力，比正常表現 securin 的 HCT116 癌細胞較差。由這些發現，ACP-93 具有誘發細胞凋亡和抗腫瘤的形成能力，而 securin 能促進腫瘤形成的作用。

Abstract

Securin, also known as pituitary-tumor transforming gene, promotes cancer cell proliferation and tumorigenesis. ACP-93 is novel synthetic compound that is derivative from UK-1. In this study, we investigated the anticancer abilities in apoptosis induction and tumor inhibition and the possible role of securin by ACP-93 in the human colon cancer. ACP-93 induced apoptosis in both the HCT116 securin-wild type and securin-null colon cancer cells. Treatment with ACP-93 (4-32 μM for 24 h) induced the activation of caspase 3 and caspase 8 and the protein cleavage of PARP in colon cancer cells. Meanwhile, ACP-93 induced the protein expression of ATF-3 and the phosphorylation of H2AX. Furthermore, ACP-93 increased the phosphorylation of AKT. The blockade of AKT pathway by co-treatment with wortmannin increased the ACP-93-induced cytotoxicity. More importantly, ACP-93 significantly inhibited the tumorigenesis of xenograft human colon tumors in severe combined immunodeficiency (SCID) and nude mice. Besides, the tumor growth ability in the HCT116 securin-null colon cancer cells was less than the securin-wild type HCT116 in xenograft nude mice. In summary, the findings indicate that ACP-93 induces apoptosis and anti-tumorigenesis in colon cancer, and securin can promote tumorigenesis.

Abbreviations

Apaf-1	apoptotic protease activating factor-1
APC	adenomatous polyposis coli
CREB	cyclic AMP response element-binding
DCF	dichlorofluorescein
DiOC6	3,3'-dihexyiloxadicarbocyanine iodide
DMSO	Dimethyl sulfoxide
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
HNPCC	hereditary nonpolyposis colorectal carcinoma
MMR	mismatch repair
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PARP	poly(ADPribose) polymerase
PBS	phosphate buffered saline
PI	propidium iodide
PTTG1	pituitary-tumor transforming gene-1
ROS	reactive oxygen species

SCID	severe combined immunodeficiency
TRAIL	TNF-related apoptosis-inducing ligand

1. Introduction

1.1. Colorectal cancer

Cancers are leading cause of mortality in the world. The statistical data demonstrated that the malignancy has been situated the first place of the causes of death in Taiwan [1]. Colorectal cancer is the second leading cause of cancer-related death in the Western world and its incidence is increasing in Asian countries [2]. At least 15% of cases are estimated to have a hereditary background, like familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal carcinoma (HNPCC) [3]. The genetic alterations of adenomatous polyposis coli (APC) were found in the majority of sporadic colorectal neoplasms [4, 5]. Hereditary nonpolyposis colorectal carcinoma also called “Lynch syndrome” that occured at an earlier age than in the general population [6]. Moreover, colon cancer has been found by germline mutations in DNA mismatch repair (MMR) genes, including *MLH1*, *MSH2*, and *MSH6* [6]. Moreover, high consumption of vegetables and fruits and the low sugar containing foods are likely to reduce risk of colon cancer. Besides, alcohol is another factor to increase risk [7].

1.2. Derivatives of benzimidazoles

The benzimidazole ring system is an important pharmacophore in medicinal chemistry and modern drug discovery. The benzimidazole derivatives are potent

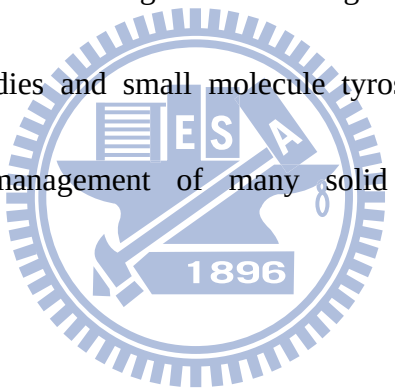
biologically active compounds and exhibit antiviral [8], antihypertension [9], against parasite properties [10] and anticancer activity [11]. The antitumor activity of the benzimidazole derivatives based on interference with the formation of microtubules [12] and as DNA topoisomerase I inhibitor [13]. For example, FB642 (Methyl-2-benzimidazole carbamate, carbendazim) is a systemic fungicide with antitumor activity both *in vivo* and *in vitro* [14-16]. It has been shown that FB642 can induce G_2/M arrest and apoptosis [14, 15]. Moreover, FB642 has undergone phase I clinical trials and is under further clinical development for treatment of cancer [16]. ABT-888 (2-[(R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide), currently in phase 2 trials [17, 18], is an orally bioavailable poly(ADP-Ribose) polymerase inhibitor with good penetration into the brain that potentiates DNA-damaging agents in preclinical tumor models [12].

1.3. Development of cancer therapy

The traditional therapeutic strategies involve radiotherapy, chemotherapy, immunotherapy, hormonal agent and anti-angiogenesis. There is no effective treatment for late stage and metastatic cancers of colorectal, prostate, pancreatic, breast, glioblastoma and melanoma cancers. Most of the currently available anti-cancer therapeutic strategies rely on the eradication of tumor cells [19]. Novel treatment

modalities are needed for these late stage patients because cytotoxic chemotherapy offers only palliation, usually accompanied with systemic toxicities and poor quality of life [20].

Novel technologies such as genomics and proteomics have increased the number of human genes known to be differentially expressed in normal and malignant tissues [21]. Advances in the understanding of cancer biology and specifically of cell signaling pathways have led to the identification of several potential molecular targets and to development of new agents directed against these targets. Targeted therapies, in the form of monoclonal antibodies and small molecule tyrosine kinase inhibitors have significantly altered the management of many solid tumors and hematologic malignancies [22].



1.4. Apoptosis

Apoptosis plays an important role in the regulation of cell number during development and tissue homeostasis [23]. The term was defined the morphologic features including cytoplasmic blebbing, chromatin condensation, cell shrinkage, nuclear fragmentation and cell rounding (loss of adhesion) [24]. The abnormal regulation of apoptosis resulting in increased or decreased activity is associated with a variety of clinical disorders including cancer, autoimmunity, neurodegenerative diseases,

hematopoietic disorders and infertility [25-27].

Apoptosis can be initiated through extrinsic or death receptor-link and intrinsic or mitochondria-dependent pathways [28]. The extrinsic pathway requires the binding of a ligand to death receptor on cell surface, such as TNF-α, Fas-ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), all of which can act as extracellular activators of apoptosis upon binding to their respective receptors [29, 30]. The intrinsic pathway is activated in response to intra-cellular stress, such as DNA damage, hypoxia, growth factor deprivation [19] and mediated by the mitochondrial release of cytochrome c [30]. Cytosolic cytochrome c induces the formation of the multisubunit apoptosome composed of apoptotic protease activating factor-1 (Apaf-1), procaspase 9 and either ATP or dATP [31, 32]. Next, caspase 3 activation is detected following the formation of the apoptosome [33]. All caspases possess an active-site cysteine and selectively cleave substrates after aspartic acid residues [33]. Poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation [34], was the first cellular protein to be identified as being specifically cleaved to the signature 89 kDa and 24 kDa fragment during apoptosis [35, 36]. PARP-1 is one of the prime target proteins for caspase 3 [37].

Intracellular reactive oxygen species (ROS) is considered to be a death signal in apoptosis [38, 39]. ROS induces disruption of the mitochondrial membrane potential

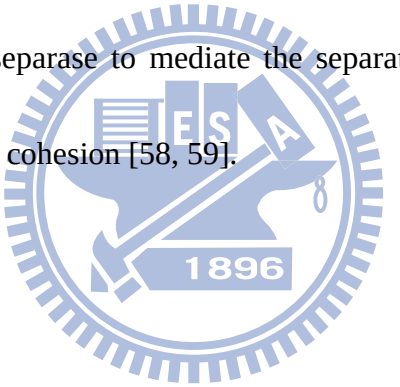
(MMP) and release of cytochrome c from mitochondria into the cytosol, where cytochrome c triggers caspase 9 activation and initiates caspases cascade which terminates cell to apoptosis [40, 41].

1.5. Securin and apoptosis

Securin, also known as the pituitary tumor-transforming gene-1 (PTTG1) was isolated from rat pituitary tumor cells in 1997 and identified as a pituitary-derived transforming gene [42]. It consists of a homologous family of proteins expressed in different species that includes Cut2 in fission yeast [43], Pds1 in budding yeast [44], Pim1 in *Drosophila* [45] and securin in human. PTTG1 is a multifunctional gene located at chromosome 5q33 and encoding 202 amino acid protein [46]. Securin participates in the maintenance of chromosome stability, cell-cycle progression, appropriate cell division [47], DNA repair [48], transactivation activity and apoptosis [49]. PTTG1 overexpression may cause both p53-dependent and p53-independent apoptosis [50]. In our previous study, expression of securin promotes cell apoptosis after radiation in colorectal cancer cell [51]. In contract, inhibition of securin expression increases apoptosis and chromosome instability following arsenite or cytochalasin B [52, 53]. Furthermore, securin depletion sensitizes human colon cancer cells to fisetin-induced apoptosis [54].

1.6. Securin and cell cycle

During most of the cell cycle, securin plays an important role in sister chromatid separation during anaphase [55]. Sister chromatid separation involves the proteolytic cleavage of cohesion proteins, a process that is mediated by separase, a cysteine protease [56]. In normal condition, securin prevents abnormal sister chromatid segregation by binding to the C-terminal domain of separase and inhibits its activity, maintains genomic stability [43, 57]. At the metaphase-anaphase transition, securin degradation ensues as a result of ubiquitination by anaphase-promoting complex (APC) with subsequent release of separase to mediate the separation of sister chromatid by cleavage of the chromosomal cohesion [58, 59].



1.7. Securin and cancer

Securin overexpression has been reported in a variety of endocrine-related tumors, especially pituitary [60], thyroid [61], breast [62], ovarian [63], and uterine tumors [64], as well as nonendocrine-related cancers involving the central nervous system [65], pulmonary system, and gastrointestinal system [66]. Induction of angiogenesis by PTTG was demonstrated the activation of proliferation, migration, and tube formation of human umbilical vein endothelial cells [67]. Overexpression of securin in mouse fibroblast cells results in cellular transformation and promoted tumor formation [42].

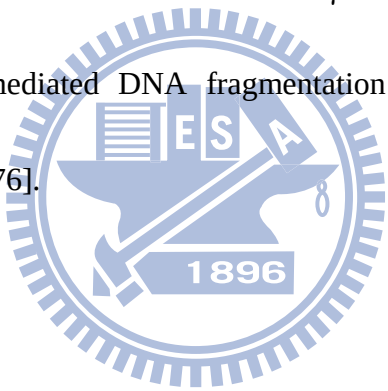
Securin may act as a transcription activator and involved in cellular transformation and tumorigenesis through activation of *c-myc* oncogene [68]. Securin has been identified that it is involved in tumorigenesis [69] and tumor invasiveness [49, 61, 66].

1.8. ATF3 and gamma-H2AX

ATF3 is a stress-inducible gene that encodes a member of the ATF/cyclic AMP response element-binding (CREB) family of transcription factors that contains a basic region/leucine zipper DNA-binding and binds to the cyclic AMP response element consensus sequence TGACGTCA [70]. In most cases, ATF-3 is induced by external stress signals such as ischemic injuries, mutagens, carcinogens, mitogenic cytokines, or endoplasmic reticulum stresses from abnormal protein processing [71]. ATF3 plays dichotomous roles in the cancer development [72]. It can either promote or suppress the cellular growth depending on the endogenous or exogenous texture of disease conditions. ATF-3 has been reported to affect cell death and cell cycle progression in cancer cells [73]. In tumorigenesis, ATF3 overexpression protected malignant MCF10CA1a human breast cancer cells from apoptosis and promoted their metastatic potential, associated with an up regulation of fibronectin-1, TWIST-1, and Slug transcripts, which are key regulators of cell-cell or cell-extracellular matrix interaction [72]. In contrast several studies have implicated ATF3 as a tumor suppressor, due to its

ability to induce apoptosis and cell cycle arrest. The loss of ATF3 function results in loss of tumor suppression [74].

H2AX is a histone H2A variant that plays essential role in the recruitment and accumulation of DNA repair proteins to sites of double-strand breaks (DSBs) damage [75]. In DSBs generation, the H2AX protein is phosphorylated (termed γ-H2AX) at serine residue 139 in the unique C-terminal motif SQEY within seconds and forms localized ‘‘foci” at DSBs sites [75]. Moreover, γ-H2AX is formed during apoptosis initiated by DNA damage. It has been found that γ-H2AX formation is a cellular response to endonuclease-mediated DNA fragmentation downstream from caspase activation during apoptosis [76].



1.9. AKT and survivin

The PI3K/AKT pathway regulates fundamental cellular functions, including cell growth and survival [77]. AKT mediates a variety of biological functions, including glucose uptake, protein synthesis, and inhibition of cell death [78]. AKT regulates cellular survival through phosphorylation of downstream substrates that indirectly or directly control the apoptotic machinery [79]. Survivin, a unique member of the inhibitor of apoptosis protein (IAP) family, plays an important role in regulating both apoptosis and cell division [80]. Survivin has been identified as a negative prognostic

factor in various cancer types and was implicated in resistance to apoptosis induction by anticancer agents [81]. Evidence for the up-regulation of survivin via the PI3K/AKT pathway was first shown in endothelial cells [82]. It has been reported that survivin expression is activated by the PI3K/AKT pathway, conferring cell survival and resistance to apoptosis in various malignant cells, including prostate [83], breast [84], and lung [85]. Several studies have demonstrated resistance of survivin-expressing cells to anticancer drug-induced apoptosis [86].

1.10. The purpose of the study

In this study, the anticancer abilities of ACP-93 on apoptosis and anti-tumorigenesis effects were investigated in the human colon carcinoma cells. Moreover, we investigated the role of securin in the tumorigenesis. Understanding the anticancer abilities and mechanisms of ACP-93 on apoptosis and anti-tumorigenesis in human colon cancer cells may contribute to potential colon cancer therapy.

2. Materials and Methods

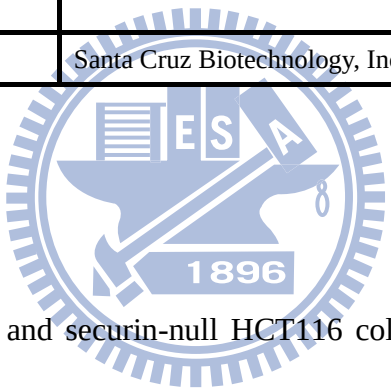
2.1. Chemicals and reagents

ACP-93 was synthesized and kindly provided by our collaborator Dr. Chinpiao Chen (National Dong Hwa University, Hualien, Taiwan). Wortmannin, propidium iodide and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). 3,3'-dihexyiloxadicarbocyanine iodide (DiOC6) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Calbiochem (San Diego, CA). Annexin V-FITC/PI kit was purchased from BioVision, Inc. (San Francisco, CA).

2.2. Antibodies

Anti-AKT and anti-phospho-AKT (ser473) were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Anti-ATF-3 (C-19), anti-bax (N-20), anti-bcl-2 (100), anti-actin (I-19) and anti-survivin (D-8) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-caspase 3 was purchased from BioVision, Inc. (San Francisco, CA). Anti-poly(ADP-ribose) polymerase (PARP) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-γ-H2AX and anti-caspase 8 were purchased from BD Bioscience. (Franklin Lakes, NJ).

Antibodies	Molecular Weight	Incorporation	Source	Antibody dilution
AKT	60 KDa	Cell Signaling Technology, Inc.	rabbit	1:1000
phospho-AKT (ser473)	60 KDa	Cell Signaling Technology, Inc.	rabbit	1:1000
ATF-3 (C-19)	21 KDa	Santa Cruz Biotechnology, Inc.	rabbit	1:500
Actin (I-19)	42 KDa	Santa Cruz Biotechnology, Inc.	mouse	1:5000
Bax (N-20)	21 KDa	Santa Cruz Biotechnology, Inc.	rabbit	1:1000
Bcl-2 (100)	29 KDa	Santa Cruz Biotechnology, Inc.	mouse	1:1000
Caspase 3	32 KDa	BioVision, Inc.	mouse	1:1000
Caspase 8	55 KDa	BD Bioscience.	mouse	1:2000
poly(ADP-ribose) polymerase (PARP)	116 KDa	Cell Signaling Technology, Inc.	rabbit	1:1000
phospho-histone H2AX (ser139)	13 KDa	BD Bioscience.	mouse	1:1000
Survivin (D-8)	16.5 KDa	Santa Cruz Biotechnology, Inc.	mouse	1:500



2.3. Cell culture

The securin-wild type and securin-null HCT116 colorectal carcinoma cell lines were kindly provided by Dr. B. Vogelstein of Johns Hopkins University (Baltimore, MD). The HCT116 cells were derived from an adult male colorectal carcinoma. The cells were maintained in McCoy's 5A medium. The complete medium was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/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 MTT 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 co-treated with or without PI3K/AKT inhibitor (wortmannin) and ACP-93 for 24 h in complete McCoy's 5A medium. Following treatment, the cells were washed with phosphate buffered saline (PBS) and then re-cultured in complete McCoy's 5A medium for 48 h. 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 surviving cell was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

2.5. Annexin V-PI assay

The level of apoptosis induced by ACP-93 was determined by Annexin V-propidium iodide (PI) analysis. The securin-wild type and securin-null HCT116 cells were treated with 0-32 μM ACP-93 for 24 h at 37℃. After treatment, the floating and adherent cells were collected and centrifuged at 1500 rpm for 5 min. Thereafter, the cell pellets were resuspended in 1× Annexin V binding buffer. Each sample was incubated

with fluorescein isothiocyanate (FITC)-conjugated-Annexin V and PI according to the manufacturer's instruction (BioVision, Mountain View, CA) for 5 min at room temperature. Finally, the samples were analyzed immediately using flow cytometer (FACS Calibur, BD Biosciences, Heidelberg, Germany). For each measurement, 10,000 cells were analyzed. Dot plots and histograms were analyzed by CellQuest software (BD Biosciences). Annexin V^-/PI^- cells were viable. The cells showed Annexin V^+/PI^- and Annexin V^+/PI^+, which indicated at early and late apoptosis, respectively.

2.6. Cell cycle analysis

The cell cycle progression after treatment with ACP-93 was measured by flow cytometry. The cells were plated at a density of 1×10^6 cells per 60-mm Petri dish in complete medium for 16-20 h, and then treated with 0-32 μM ACP-93 for 24 h at 37℃. At the end of treatment, the cells were collected and fixed with ice-cold 70% ethanol overnight at -20℃. Thereafter, the cell pellets were treated with 4 μg/ml PI solution containing 1% Triton X-100 and 50 μg/ml RNase for 30 min. To avoid cell aggregation, the cell solutions were filtrated through nylon membrane (Becton-Dickinson, San Jose, CA). Subsequently, the samples were analyzed by flow cytometry. For each measurement, 10,000 cells were analyzed for DNA content, and the percentage of cell cycle phases were quantified by ModFit LT software (Ver. 2.0, Becton-Dickinson).

2.7. Western blotting

At the end of treatment, the cells were lysed in the ice-cold whole cell extract buffer (pH 7.6) containing the protease inhibitors. The buffer containing 0.5 mM DTT, 0.2 mM EDTA, 20 mM HEPES, 2.5 mM $MgCl_2$, 75mM NaCl, 0.1 mM Na_3VO_4, 50 mM NaF, 0.1% Triton X-100, 1 mg/ml aprotinin, 0.5 mg/ml leupeptin, and 100 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride. The lysate was vibrated for 30 min at 4℃ and centrifuged at 10,000 rpm for 10 min. The protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL). The total cellular protein extracts were prepared. Equal amounts of proteins were subjected to electrophoresis using 10 to 12% sodium dodecyl sulfate-polyacrylamide gels and electrophoretic transfer of proteins onto polyvinylidene difluoride membranes. 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 sequentially hybridized with specific primary antibody and followed with a horseradish peroxidase-conjugated secondary antibody. Thereafter, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). To verify equal protein loading and transfer, actin was used as the protein loading control. The gel digitizing software, Un-Scan-It *gel* (Ver. 5.1, Silk

Scientific, Inc., Orem, UT), was used to analyze the intensity of bands on X-ray film.

2.8. ROS measurement

The ROS induction after treatment with ACP-93 was measured by flow cytometry. The cells were plated at a density of 7×10^5 cells per 60-mm Petri dish in complete medium for 16-20 h, then treated with 0-32 μM ACP-93 for 24 h at 37℃. After treatments, the cells were collected and resuspended in medium containing 5 μM 2',7'-dichlorodihydro-fluorescein diacetate (H_2DCF-DA). The compound is deacetylated by intracellular esterases converted to nonfluorescent dichlorodihydrofluorescein that reacts with H_2O_2 to form dichlorofluorescein (DCF), which is an oxidized green fluorescent compound. Cellular ROS content was measured by incubating the cells with stain solution for 30 min at room temperature in darkness. Finally, the collected cell pellets were resuspended in 1 ml ice-cold PBS and fluorescence was detected by flow cytometer (FACScan, Becton Dickinson, San Jose, CA). Data from 10,000 events per sample were collected and analyzed using the CellQuest software.

2.9. Xenografted human colon tumors in SCID and nude mice

Human colorectal carcinoma xenograft was developed in 4 week-old

CB17/Icr-Prkdcscid/Crl mice that were obtained from BioLASCO (BioLASCO Co., Ltd., Taipei, Taiwan). 5 week-old BALB/cAnN.Cg-*Foxn1nu*/CrlNarl mice were obtained from National Laboratory Animal Center (NLAC, Taiwan). After 1-2 weeks for environmental adaption, the mice were used for human colorectal cancer cell inoculation. For pre-treatment of ACP-93, the securin wild-type HCT116 cells were treated with or without 32 μM ACP-93 prior to inoculation into the flank of the severe combined immunodeficiency (SCID) mice (1×10^6 cells/mouse) by subcutaneous injection. The tumor size and the body weight of the mice were measured every four days. For post-treatment of ACP-93, solid HCT116 flank tumors were established by subcutaneous injection of 1×10^6 cells. After the tumors were visible, they received a 100 μl intra-tumoral injection of control vehicle (corn oil) or 30 mg/kg of ACP-93 once per four days during 12 days. The tumor size of the mice was measured by a digital caliper every four days and calculated by the following formula: (length) × $(\text{width})^2$ × 0.5. The tumors volumes were continuously measured until the mice were sacrificed.

2.10. Securin-wild type and securin-null colon tumor in xenograft nude mice

Human colorectal carcinoma xenograft was developed in 4 week-old CAnN.Cg-Foxn1nu/CrlBltw mice that were obtained from BioLASCO (BioLASCO Co., Ltd., Taipei, Taiwan). Solid securin-wild type and securin-null flank colon tumors were

established by subcutaneous injection of 2×10^6 cells. The tumor size and the body weight of the mice were recorded every four days until mice were sacrificed.

2.11. Statistical analysis

Each experiment was repeated at least three times. Data were analyzed using Student's *t* test or analysis of variance (a comparison of multiple groups), and a *p* value of < 0.05 was considered statistically significant in each experiment.

3. Results

3.1. ACP-93 induces apoptosis in both the securin-wild type and securin-null HCT116 cells

Fig. 1 shows the chemical structure of ACP-93. Previously, treatment with 2-32 μM ACP-93 for 24 h induced cytotoxicity in both the securin-wild type and securin-null HCT116 cells [87]. We have further assessed the apoptosis induction by ACP-93 in the securin-wild type and securin-null HCT116 cells in this study. The ACP-93 treated cells were analyzed by Annexin V-FITC-PI staining. The Annexin V^+/PI^- cells (early apoptosis) and Annexin V^+/PI^+ cells (late apoptosis) were increased following treatment with ACP-93 at 8 μM to 32 μM 24 h in both the securin-wild type and securin-null HCT116 cells (Fig. 2A). ACP-93 (32 μM for 24 h) significantly induced apoptosis level at average of 13.48% in the securin-wild type HCT116 cells and 11.82% in the securin-null HCT116 cells (Fig. 2B).

3.2. ACP-93 increases the protein levels of active caspase 3 and caspase 8 and the protein cleavage of PARP in the securin-wild type and securin-null HCT116 cells

To examine the apoptotic-regulated protein whether involve in ACP-93-induced apoptosis, caspases 3, caspase 8, PARP and bax proteins were analyzed by Western blot. The active form of caspase 3 (17 kDa and 12 kDa) protein was induced following treatment with 4-32 μM ACP-93 for 24 h in both the securin-wild type and securin-null

HCT116 cells (Fig. 3A). Also, the cleaved form of PARP (89 kDa) protein was elicited after treatment with 4-32 μM ACP-93 for 24 h. ACP-93 (32 μM for 24 h) significantly induced the active form of caspase 3 and the cleaved-PARP in the securin-wild type and securin-null HCT116 cells (Fig. 3B). However, the bax protein expression was not altered following ACP-93 treatment in the securin-wild type and securin-null HCT116 cells (Fig. 3A). To analyze the extrinsic apoptosis pathway whether regulated ACP-93-induced apoptosis, the active form of caspase 8 protein was analyzed by Western blot. As shown in fig. 4A, treatment with 4-32 μM ACP-93 for 24 h increased active caspase 8 protein levels. ACP-93 (32 μM for 24 h) significantly increased the active form of caspase 8 in both the securin-wild type and securin-null HCT116 cells (Fig. 4B). Actin was used as a loading control protein that was not altered by ACP-93.

3.3. ACP-93 induces γ-H2AX and ATF-3 proteins expression in the securin-wild type and securin-null HCT116 cells

To analyze the effect of ACP-93 on the apoptotic-regulated proteins expression, the phosphorylated H2AX and ATF-3 were analyzed to Western blot. As shown in fig. 5A, the protein expression of γ-H2AX was induced by ACP-93. Treatment with 32 μM ACP-93 for 24 h significantly induced protein expression in both the securin-wild type and securin-null HCT116 cells (Fig. 5B). Furthermore, the ATF-3 protein was also

induced following 4-32 μM ACP-93 for 24 h (Fig. 6A). ACP-93 significantly increased ATF-3 protein expression in both the securin-wild type and securin-null HCT116 cells (Fig. 6B). Actin was used as a loading control protein that was not altered by ACP-93.

3.4. ACP-93 induces ROS generation in the securin-wild type and securin-null HCT116 cells

The intercellular ROS level of the securin-wild type and securin-null HCT116 cells were determined by flow cytometry and staining with fluorescent dye H_2DCF-DA. The DCF fluorescence intensity was increased after 4-32 μM ACP-93 treatment in both the securin-wild type and securin-null HCT116 cells (Fig. 7).

3.5. ACP-93 inhibits cell proliferation and increases sub-G_1 fractions in the securin-wild type and securin-null HCT116 cells

The cell proliferation was inhibited by ACP-93 (32 μM) when the ACP-93-treated cells were re-cultured for 2-6 days (Fig. 8). To determine the possible involvement of ACP-93 in the regulation of cell cycle progression, the effect of ACP-93 on the securin-wild type and securin-null HCT116 cells were analyzed by flow cytometry (Fig. 9A). ACP-93 did not significantly alter the fractions of G_1, S and G_2/M phases in the securin-wild type and securin-null HCT116 cells. However, it markedly increased the fractions of sub-G_1 phase in both the securin-wild type and securin-null

HCT116 cells (Fig. 9B). Treatment with 32 μM ACP-93 for 24 h increased sub-G_1 fractions by about 7.7% and 9% in the securin-wild type and securin-null HCT116 cells, respectively.

3.6. ACP-93 induces anti-apoptotic protein expression in colon cancer cells

To investigate whether the anti-apoptotic proteins in regulating apoptosis by ACP-93, AKT, bcl-2 and survivin protein levels were analyzed by Western blot. Treatment with 4-32 μM ACP-93 for 24 h increased phospho-AKT (ser473), bcl-2 and survivin protein expression (Fig. 10). Total AKT protein expression did not alter by ACP-93. The protein levels of phospho-AKT (ser473), bcl-2 and survivin were increased at 4-24 h whereas decreased at 36 h by 32 μM ACP-93 treatment (Fig. 11). Actin was used as a loading control protein that did not alter by ACP-93. Besides, co-treatment with wortmannin significantly increased the ACP-93-induced cytotoxicity in the securin-wild type HCT116 cells (Fig. 12).

3.7. Securin promotes tumorigenesis of xenograft human colon tumors in nude mice

To study the role of securin in tumorigenesis, the securin-wild type and securin-null colon tumor xenograft nude mice were analyzed. The tumor developed in

four-week-old nude mice and each mouse received 2×10^6 cells by subcutaneously injection. The tumors of xenograft mice were growth to average 1995.5 mm^3 in the securin-wild type groups and 910.4 mm^3 in the securin-null groups after inoculation for 60 days (Fig. 13A). The solid securin-wild type and securin-null tumors were separated from mice (Fig. 13B).

3.8. ACP-93 inhibits tumorigenesis of xenograft human colon tumors in SCID and nude mice

To evaluate the anti-tumorigenesis effect of ACP-93, the colon tumor xenograft in SCID mice and nude mice were analyzed. In the SCID mice model, the tumors of xenograft mice were growth to 595 mm^3 in control groups and no visible tumors in ACP-93 pre-treatment xenograft mice until 40 days (Fig. 14A). Post-treatment of ACP-93 (30 mg/kg) in colon tumor xenograft SCID mice reveal inhibitory effect on tumorigenesis comparing to the control groups (Fig. 14B). The tumors of xenograft mice were growth to average 419 mm^3 in control groups and 296 mm^3 in ACP-93 treated groups after inoculation for 28 days. In addition, the nude mice model was investigated. Post-treatment of ACP-93 (30 mg/kg) in colon tumor xenograft nude mice reveal inhibitory effect on tumorigenesis comparing to the control groups (Fig. 15). The tumors of xenograft mice were growth to average 140 mm^3 in control groups and 47.8

mm^3 in ACP-93 treated groups after inoculation for 32 days.

4. Conclusion

In this study, we provide that ACP-93 induces apoptosis and anti-tumorigenesis in human colon cancer (Fig. 16). Securin plays important roles in anti-apoptosis and tumorigenesis; however, ACP-93 induced apoptosis in both of the securin-wild type and securin-null colon cancer cells. We suggest that ACP-93 induces apoptosis through a securin-independent pathway in human colon cancer cells. Furthermore, ACP-93 can simultaneously induce both apoptotic and anti-apoptotic signal proteins in regulating apoptosis and tumorigenesis.

5. Discussion

It has been shown that ACP-93 induced cytotoxicity in both the securin-wild type and securin-null HCT116 colon cancer cells [87]. In this study, we found that ACP-93 induced apoptosis in both the securin-wild type and securin-null HCT116 cells by using Annexin V-PI assays and the level of sub-G_1. ACP-93 induced the activation of caspase 3 and caspase 8 and the cleavage of PARP protein level in both the securin-wild type and securin-null HCT116 cells. Besides, ACP-93 displayed tumor inhibition on the xenografted human tumors in mice. Thus, we suggest that ACP-93 is a potential compound on the induction of apoptosis and the inhibition of tumorigenesis in human colon cancer cells.

Securin has been proposed as an oncogene that is expressed abundantly in most cancer cells [42, 60, 69]. It participates in the maintenance of chromosome stability, cell-cycle progression and appropriate cell division [47]. It also promotes cell proliferation and tumorigenesis [60, 69]. We found that the cell growth ability of the securin-null HCT116 cells were slower than the securin-wild type. Interestingly, the tumor growth ability of the securin-null colon tumor were slower than the securin-wild type in xenografted of nude mice. These findings indicate that securin plays an important role in tumorigenesis. Moreover, ACP-93 can display anti-tumorigenic effect in xenografted securin-wild type colon tumors of SCID and nude mice.

It has been reported that the presence of γ-H2AX is linked to caspase-controlled DNA fragmentation during apoptosis [76]. Also, γ-H2AX involves in the maintenance of genomic stability after treatment with DNA damaging agents [88]. ACP-93 elevated the phosphorylation of H2AX protein expression in human colon cancer cells. However, the γ-H2AX levels were similar between the securin-wild type and securin-null HCT116 cells following ACP-93 treatment. These results indicate that ACP-93 induces the phosphorylation of γ-H2AX through a securin-independent pathway but the activation of γ-H2AX may be regulated the ACP-93-induced apoptosis.

ATF-3 is a transcription factor that has been shown to dimerise with ATF-3 or other ATF/CREB proteins [72-74]. ATF-3 promotes apoptosis and reduces the metastatic potential in cancer development [74]. For example, ATF-3 overexpression results in inducing apoptosis of PC3 human prostate cancer cells [89] and reduced tumor size of subcutaneous HCT116 human colorectal cancer cells xenograft in nude mice [90]. The potential role of ATF-3 as a tumor suppressor is also supported by its defined role in transforming growth factor beta (TGF-β) signaling [91]. TGF-β is a potent tumor suppressor in epithelial cells, which signals via Smad3 activation to directly induce ATF-3 [74]. It is interested that ACP-93 induced ATF-3 protein expression in both the securin-wild type and securin-null HCT116 cells. If ATF-3 acts as a tumor suppressor, it may be responsible in the action of anti-tumorigenesis

following ACP-93 treatment. Nevertheless, the precise mechanism of ATF-3 in ACP-93induced tumorigenesis inhibition is required further investigation.

Recent direct evidence indicates that production of ROS is associated with the initiation of TRAIL-induced apoptosis [92]. It has been shown that exposure to H_2O_2 enhances FAS-induced caspase 8 activation [93]. ACP-93 induced ROS generation in both the securin-wild type and securin-null HCT116 cells that indicates through a securin-independent pathway. However, the correlation and mechanism of ROS generation and apoptosis by ACP-93 should be further studied.

Subsequently, ACP-93 elevated anti-apoptosis proteins, including phospho-AKT, bcl-2 and survivin protein expression. The activation of AKT prevents cell death by blocking apoptotic pathways [94]. AKT can directly regulate members of the bcl-2 super family and indirectly regulate apoptosis through the transcriptional factor that control apoptotic event [95]. Overexpressed survivin is associated with activation of the PI3K/AKT pathway [82]. The blockage of the PI3K/AKT pathway by exposure to wortmannin enhanced cytotoxicity in the ACP-93-treated cells. The results suggest that the activation of AKT pathway by ACP-93 may be a means by cancer cells to protect themselves from cell death.

Anticancer agents exert anticancer abilities by apoptosis induction and anti-tumorigenesis activity *in vitro* and *in vivo*. In this report, we provide that ACP-93

displays anticancer abilities in by apoptosis induction and tumor inhibition in colon cancer cellular and animal model.

6. Reference

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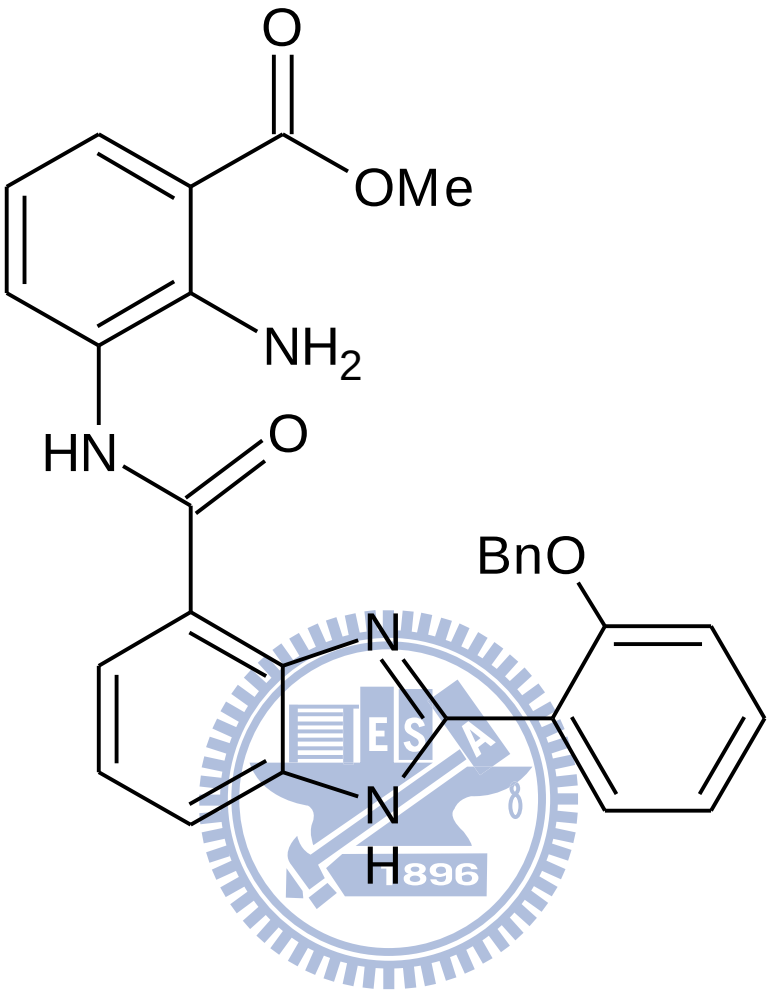


Fig. 1. The chemical structure of 2-amino-3-{[2-(2-benzyloxy-phenyl)-1H-benzoimidazole-4-carbonyl]-amino}-benzoic acid methyl ester (ACP-93).

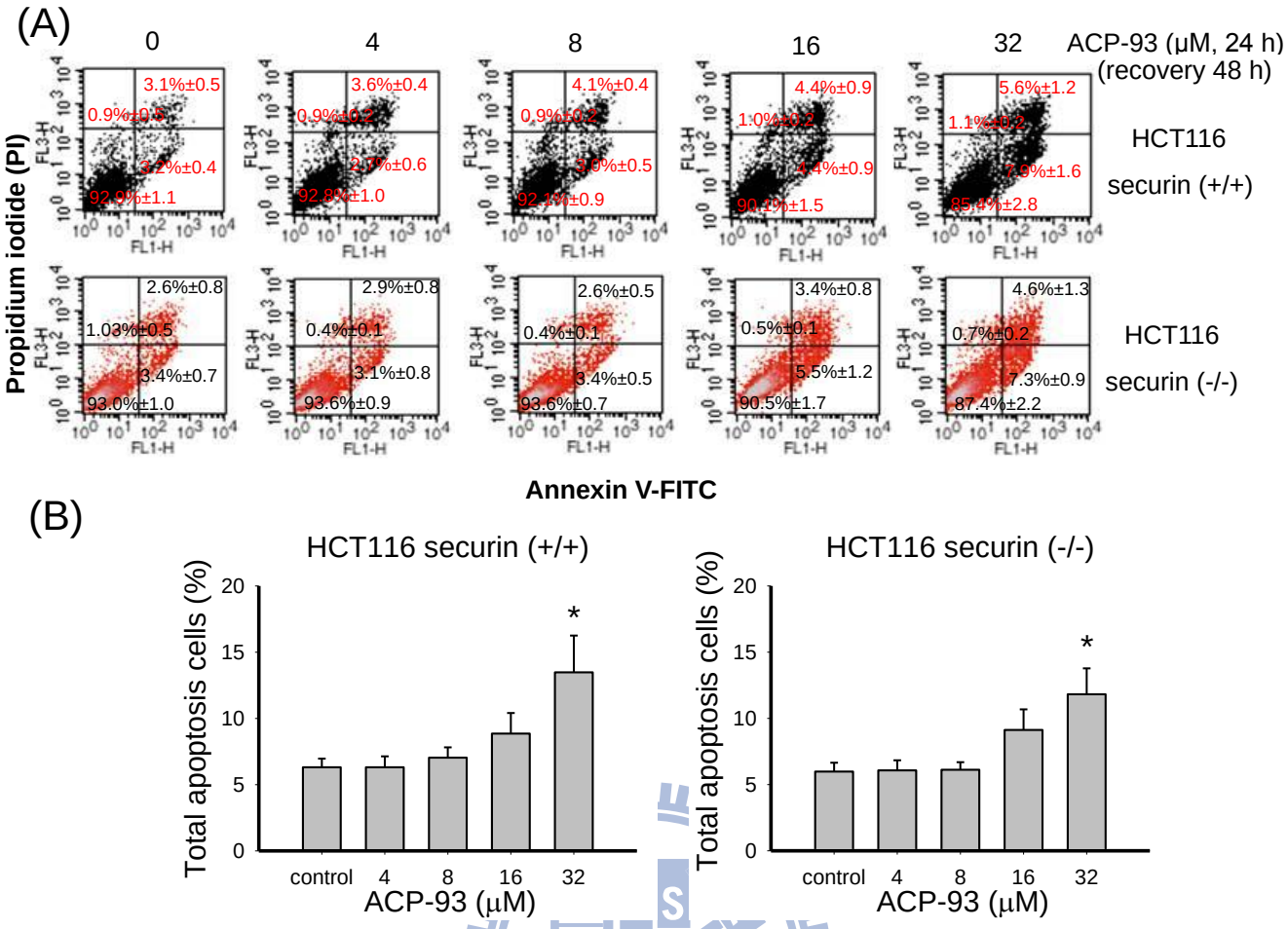


Fig. 2. Comparison of apoptosis between the securin-wild type and securin-null HCT116 cells after treatment with ACP-93. (A) The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the cells were re-cultured in fresh medium for 48 h. Apoptosis was determined by Annexin V-PI staining using flow cytometry analysis. The cell population of Annexin V^+/PI^- was indicated early apoptosis (lower right). The fraction of Annexin V^+/PI^+ was indicated late apoptosis (upper right). (B) The percentage of total apoptosis populations (combine early and late apoptosis) were quantified by CellQuest software. The results were obtained from four to seven experiments and the bar represents the mean ± S.E.M. $p < 0.05$ (*), indicates significant difference between control and ACP-93 treated samples.

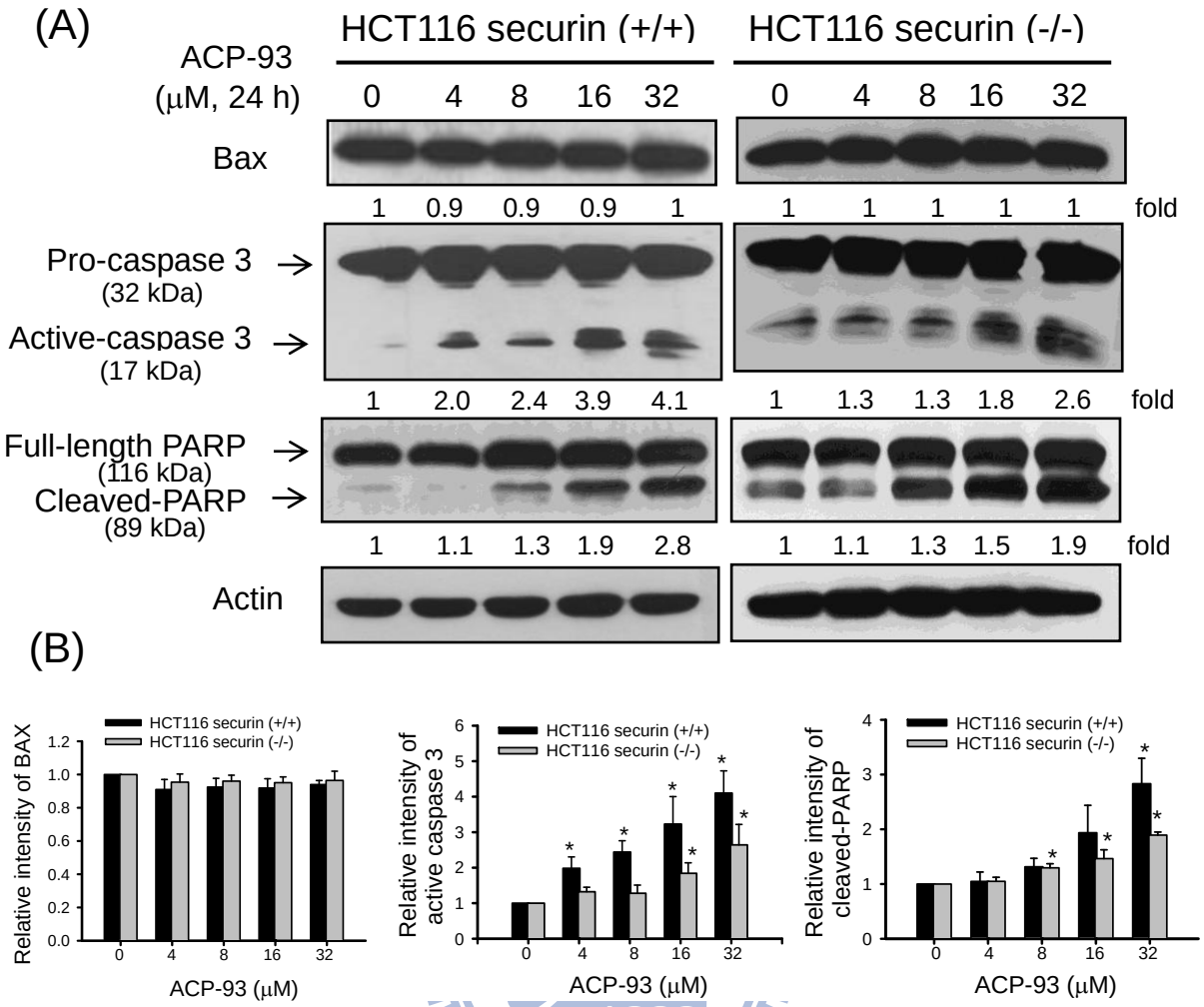


Fig. 3. Comparison of apoptosis-related protein expression between the securin-wild type and securin-null HCT116 cells after treatment with ACP-93. (A) The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the total protein extracts were subjected to Western blot analysis using anti-bax, anti-caspase 3, anti-PARP and anti-actin antibodies. Representative Western blot data are shown from one of three separate experiments with similar findings. (B) The relative protein intensity of bax, active caspase 3 and cleaved-PARP were from Western blot by semi-quantification. The results were obtained from three experiments and the bar represents the mean ± S.E.M. $p < 0.05$ (*), indicates significant difference between the control and ACP-93 treated samples.

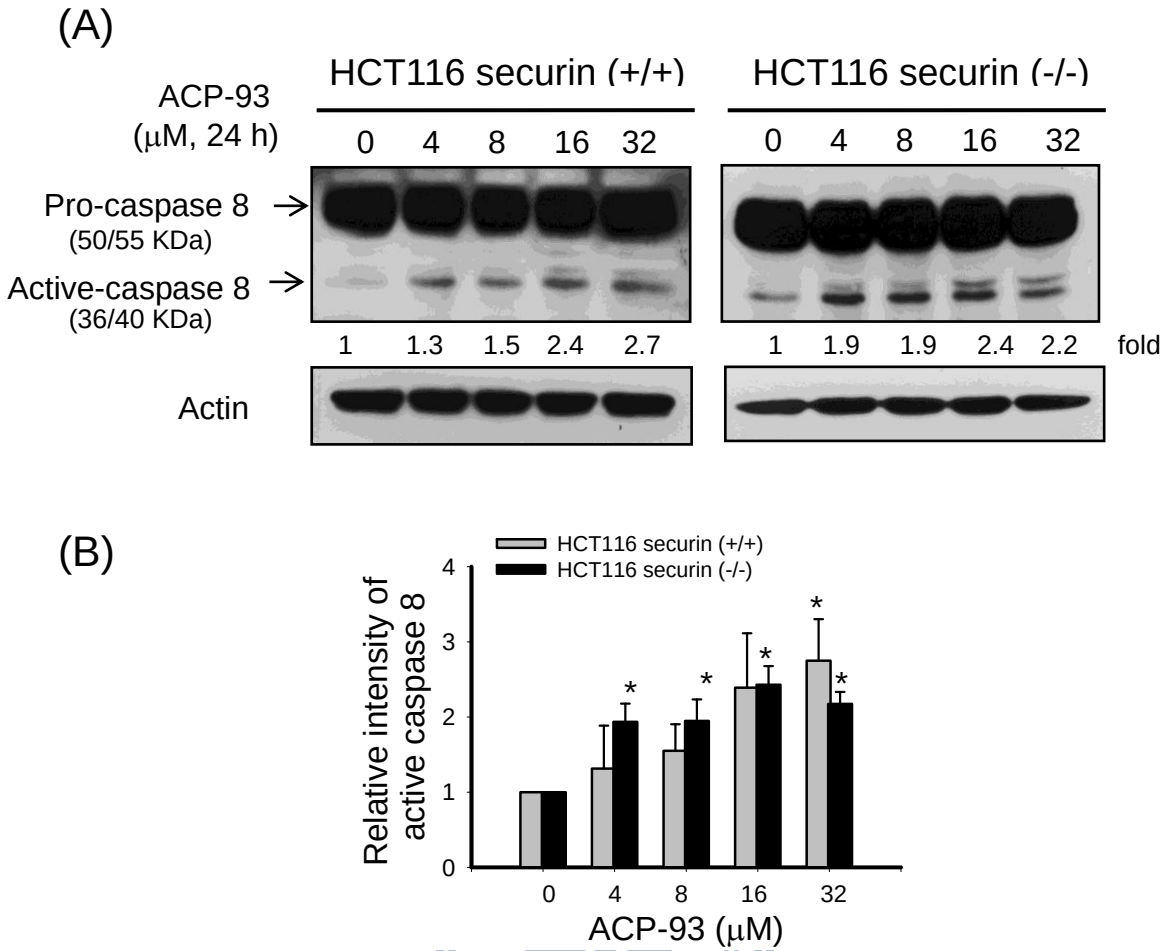


Fig. 4. Comparison of the caspases 8 activation between the securin-wild type and securin-null HCT116 cells after treatment with ACP-93. (A) The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the total protein extracts were subjected to Western blot analysis using anti-caspase 8 and anti-actin antibides. Representative Western blot data are shown from one of three separate experiments with similar findings. (B) The relative protein intensity of active caspase 8 was from Western blot by semi-quantification. The results were obtained from three experiments and the bar represents the mean ± S.E.M. $p < 0.05$ (*), indicates significants difference between the control and ACP-93 treated samples.

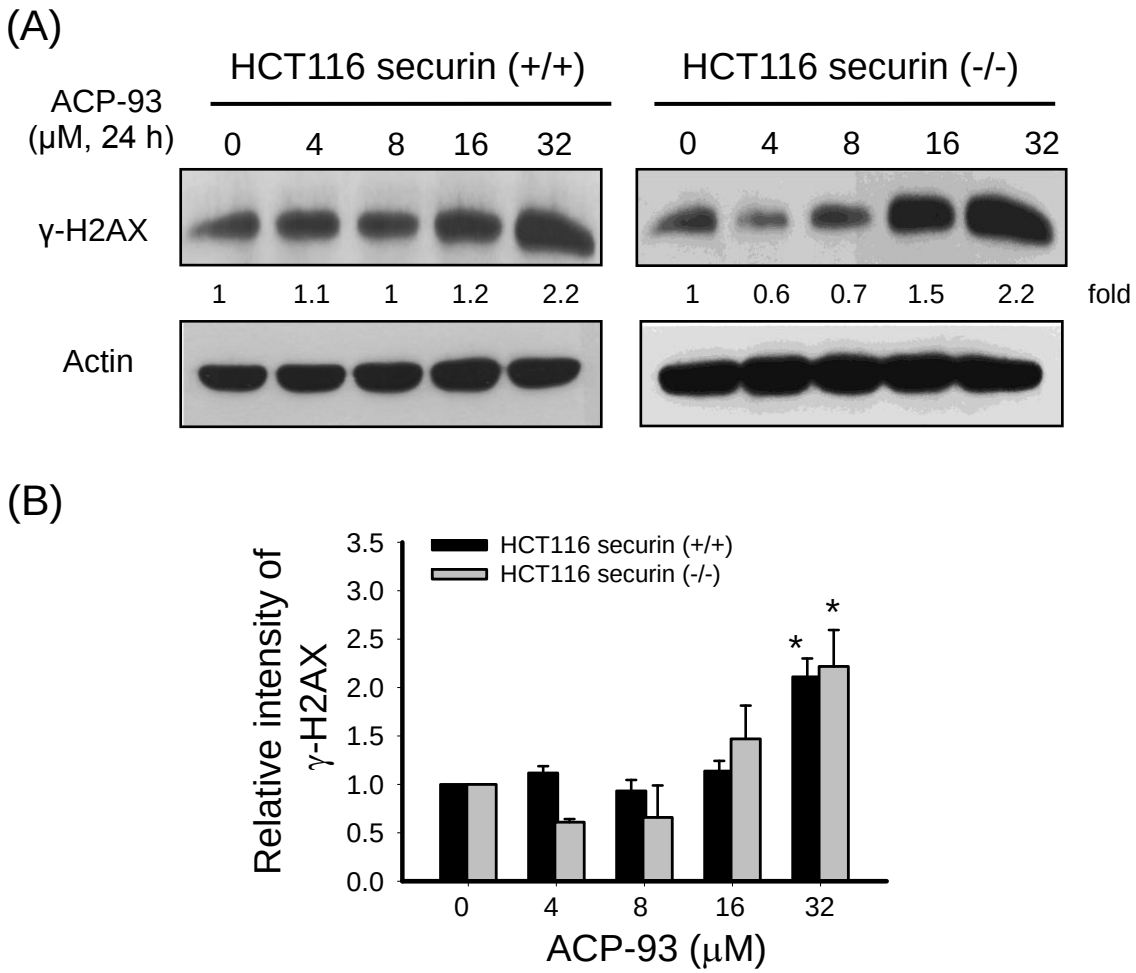


Fig. 5. Comparison of γ-H2AX protein expression between the securin-wild type and securin-null HCT116 cells after treatment with ACP-93. (A) The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the total protein extracts were subjected to Western blot analysis using anti-phosph-histone H2AX (ser139) and anti-actin antibodies. Representative Western blot data are shown from one of three separate experiments with similar findings. (B) The relative protein intensity of γ-H2AX was from Western blot by semi-quantification. The results were obtained from three experiments and the bar represents the mean ± S.E.M. $p < 0.05$ (*), indicates significant difference between the control and ACP-93 treated samples.

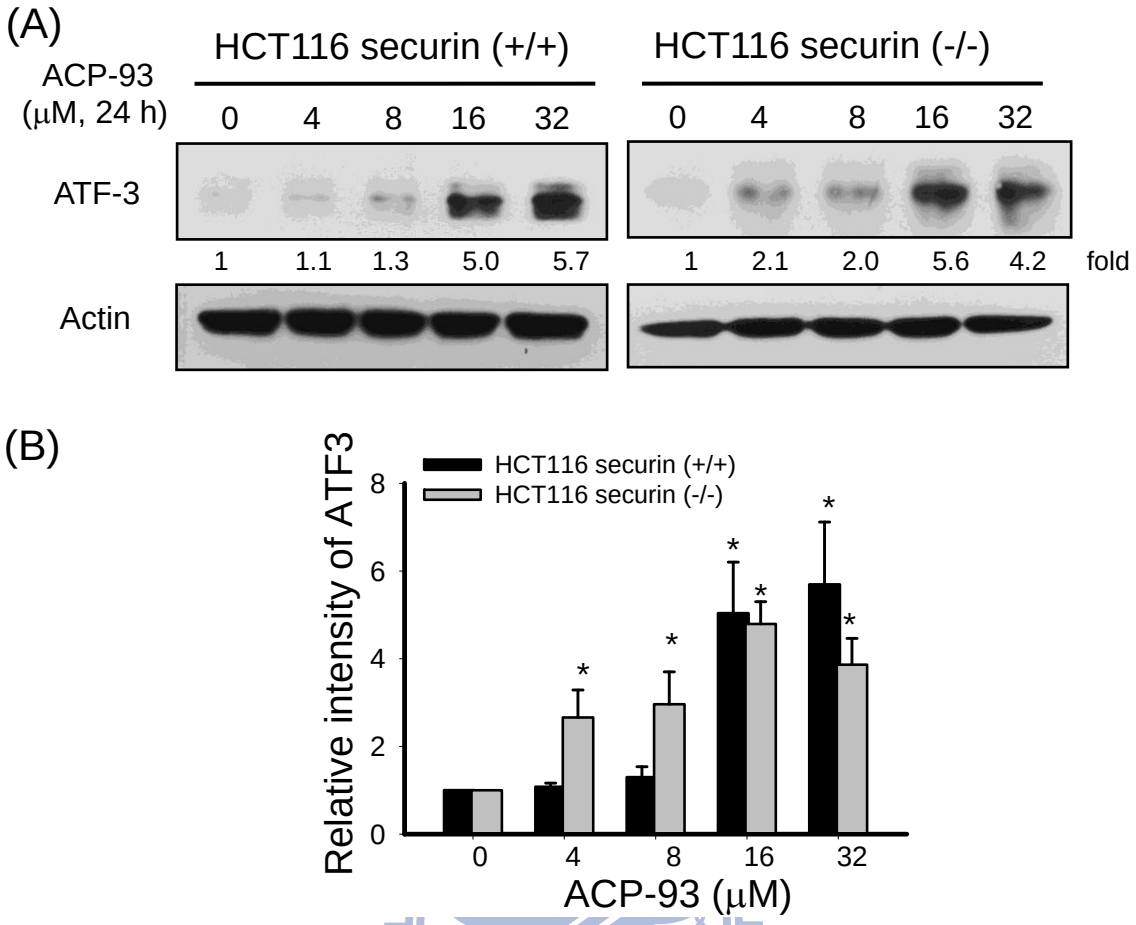


Fig. 6. Comparison of ATF-3 protein expression between the securin-wild type and securin-null HCT116 cells after treatment with ACP-93. (A) The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the total protein extracts were subjected to Western blot analysis using anti-ATF-3 and anti-actin antibodies. Representative Western blot data are shown from one of three separate experiments with similar findings. (B) The relative protein intensity of ATF-3 was from Western blot by semi-quantification. The results were obtained from three experiments and the bar represents the mean ± S.E.M. $p < 0.05$ (*), indicates significant difference between the control and ACP-93 treated samples.

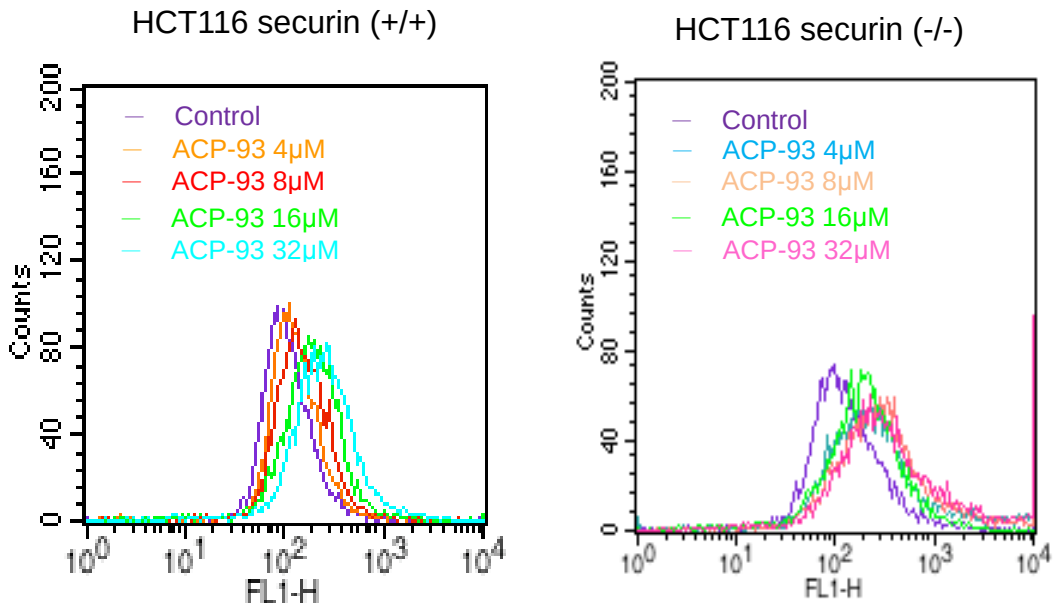


Fig. 7. Effect of ACP-93 on the ROS generation in the securin-wild type and securin-null HCT116 cells. The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the cells were incubated with 50 nM H_2DCF-DA and then analyzed by flow cytometer. Representative flow data are shown from one of four separate experiments with similar findings.

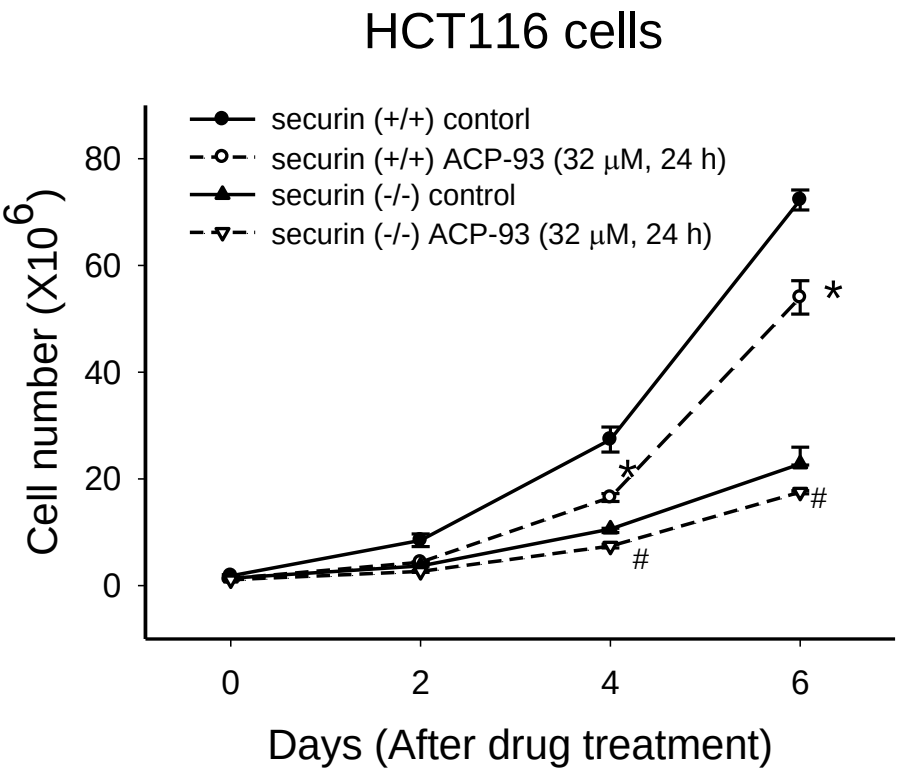


Fig. 8. Effects of ACP-93 on cell growth in the securin-wild type and securin-null HCT116 cells. The cells were plated at a density of 10^6 cells/p60 Petri dish for 20 h. Then the cells were treated with or without 32 μM ACP-93 for 24 h. After treatment, the cells were incubated for various times before they were counted by hemocytometer. The results were obtained from three experiments and the bar represents the mean ± S.E.M. $p < 0.05$ (*), indicates significant difference between control and ACP-93 treated securin-wild type samples. $p < 0.05$ (#), indicates significant difference between control and ACP-93 treated securin-null samples.

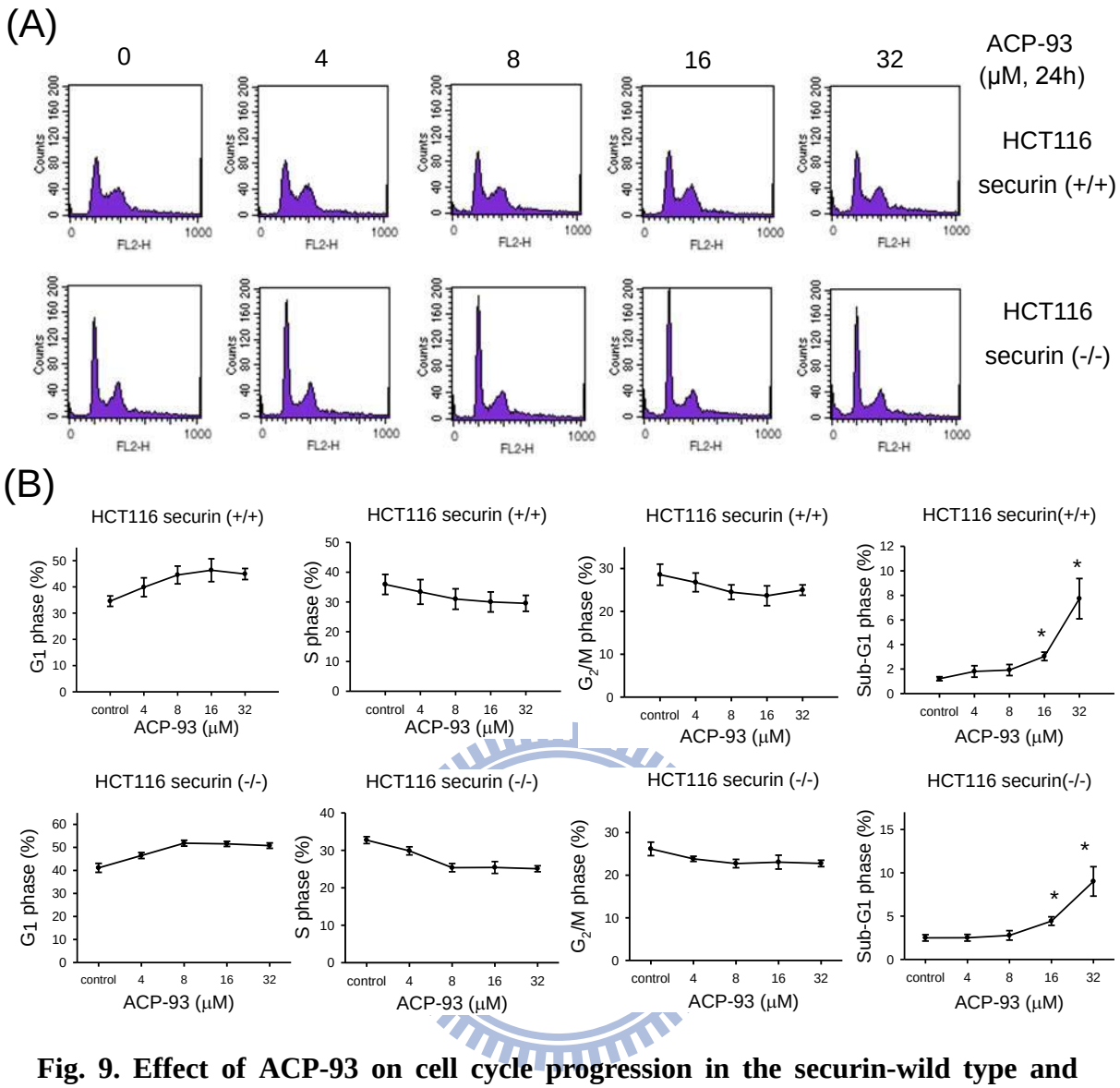


Fig. 9. Effect of ACP-93 on cell cycle progression in the securin-wild type and securin-null HCT116 cells. (A) The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the cells were trypsinized and then subjected to flow cytometry analysis. Representative flow data are shown from one of three to four separate experiments with similar findings. (B) The percentages of G_1, S, G_2/M and sub-G_1 fractions were quantified by ModFit LT software. The results were obtained from three to four experiments and the bar represents the mean ± S.E.M. $p < 0.05$ (*), indicates significant difference between control and ACP-93 treated samples.

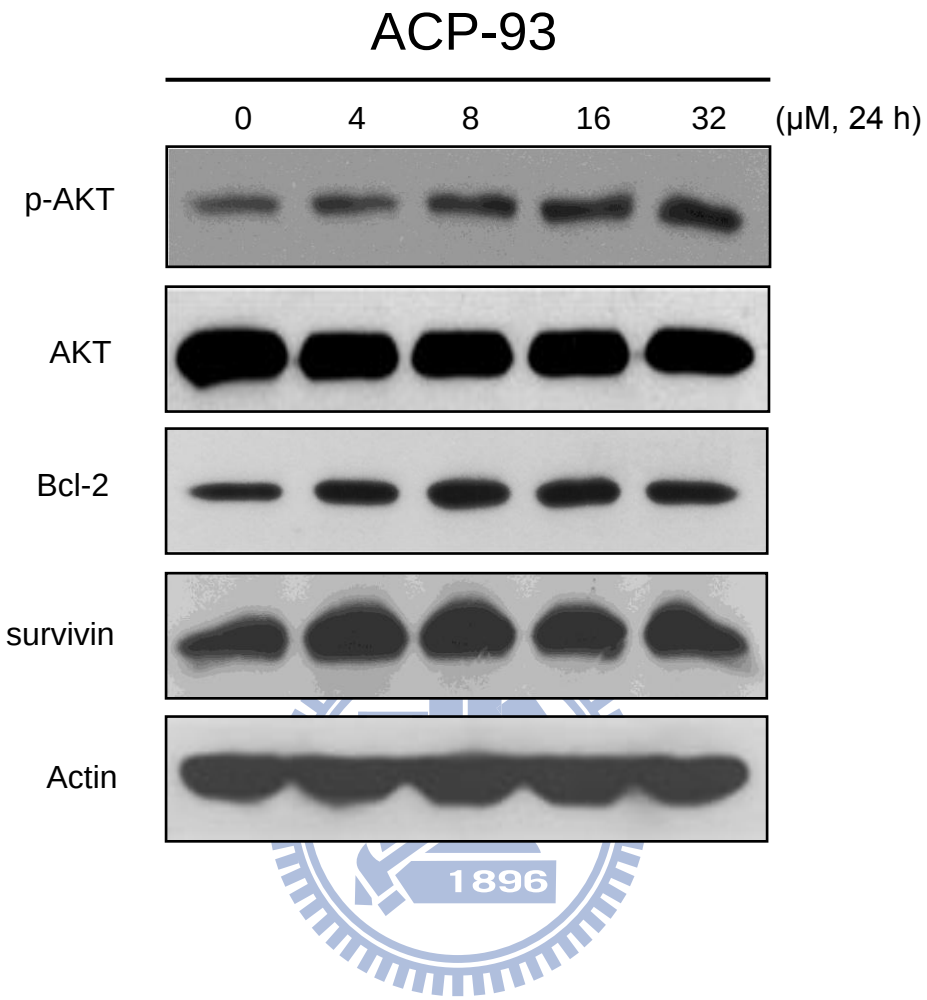


Fig. 10. Effect of ACP-93 on anti-apoptotic protein expression in the securin-wild type HCT116 cells. The cells were treated with 0-32 μM ACP-93 for 24 h. After treatment, the total protein extracts were subjected to Western blot analysis using anti-phospho-AKT (ser473), anti-AKT, anti-bcl-2, anti-survivin and anti-actin antibodies. Representative Western blot data are shown from one of three separate experiments with similar findings.

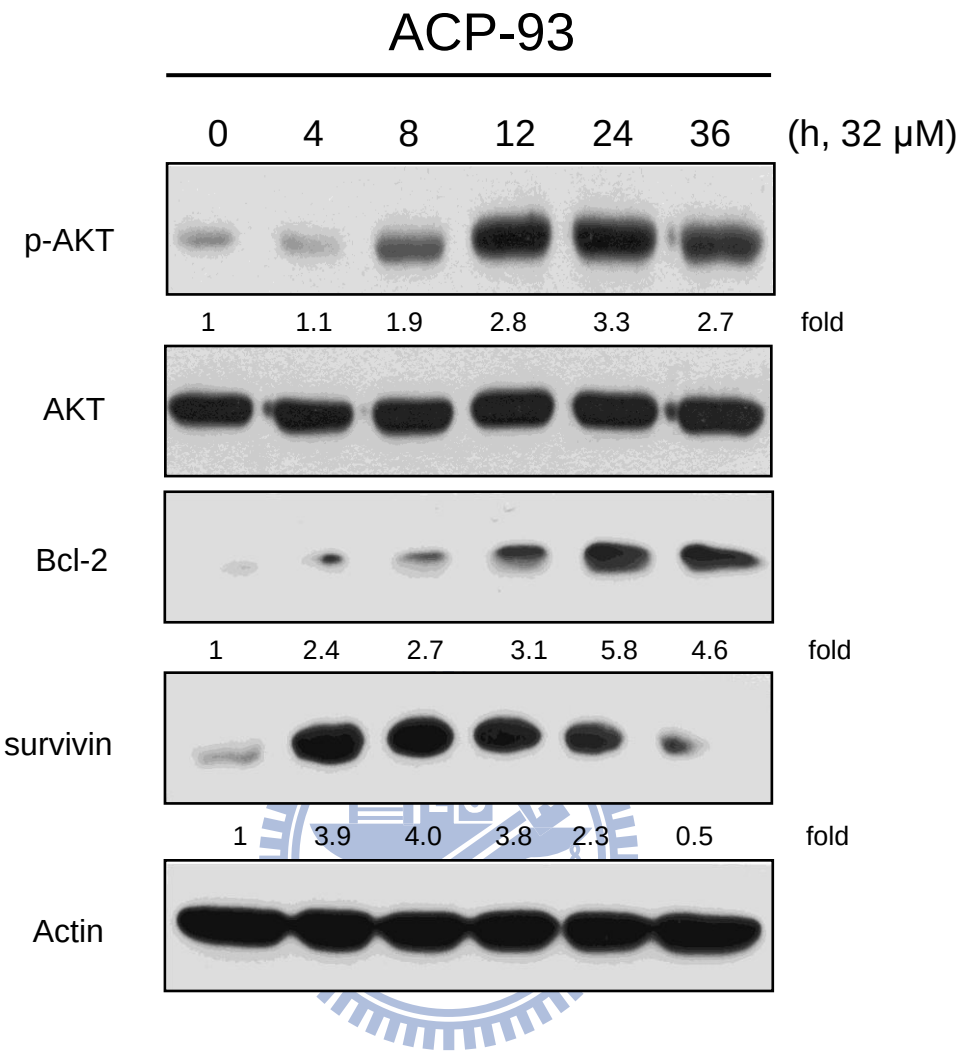


Fig. 11. Effect of ACP-93 on anti-apoptotic protein expression with various time treatment in the securin-wild type HCT116 cells. The cells were treated with 32 μM ACP-93 for 0-36 h. After treatment, the total protein extracts were subjected to Western blot analysis using anti-phospho-AKT (ser473), anti-AKT, anti-bcl-2, anti-survivin and anti-actin antibodies. Representative Western blot data are shown from one of three separate experiments with similar findings. The relative protein level of phospho-AKT, bcl-2 and survivin were from Western blot by semi-quantification.

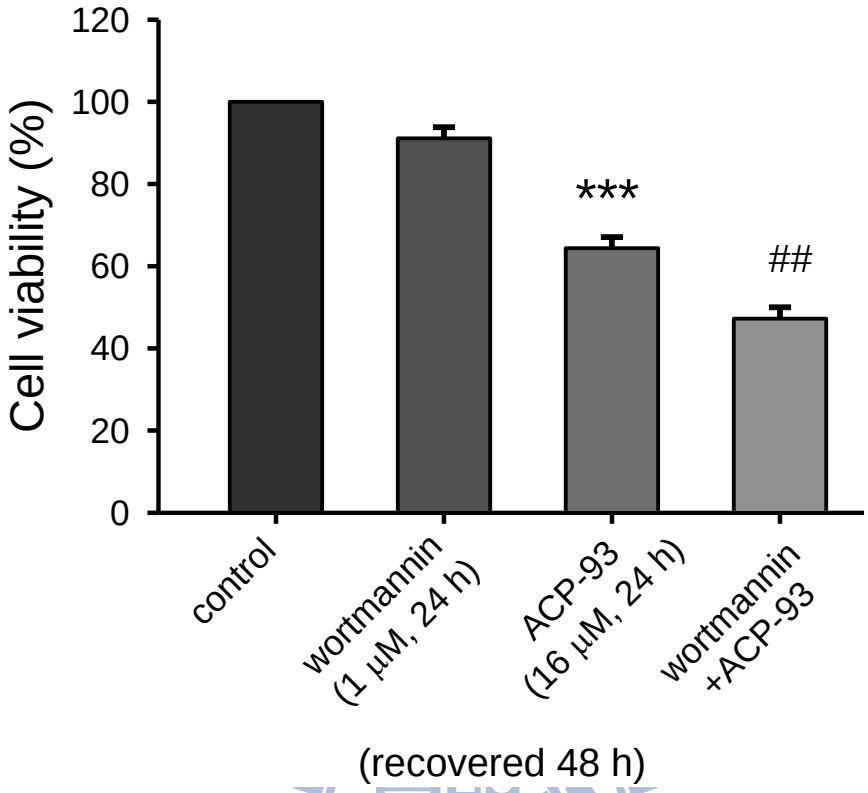
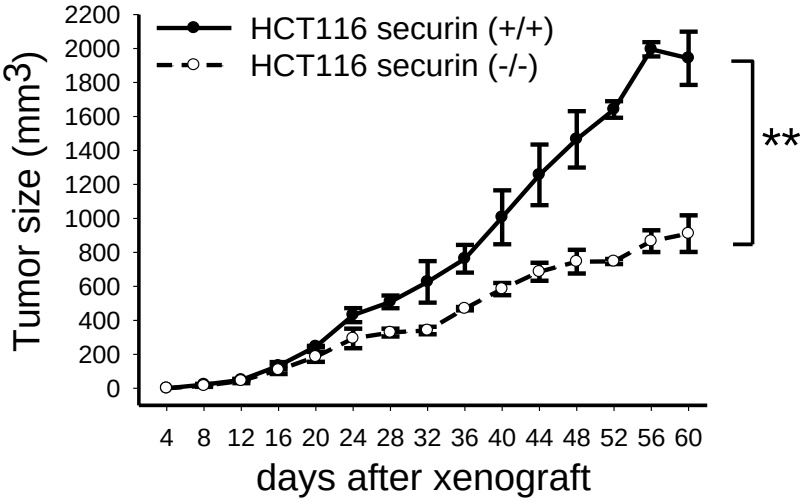


Fig. 12. Effect of a PI3K/AKT inhibitor (wortmannin) on the cell viability in the securin-wild type HCT116 cells by ACP-93 treatment. The cells were co-treated with 16 μM ACP-93 and 1 μM wortmannin for 24 h. At the end of treatment, the cells were washed with PBS and then re-cultured in fresh medium for 48 h. The cell viability was determined by MTT assay. The results were from six independent experiments and the bars represented mean ± S.E.M. $p < 0.001$ (***), indicates significant difference between control and ACP-93 treated samples. $p < 0.01$ (##), indicates significant difference between the ACP-93 treated samples and co-treatment with wortmannin samples.

(A)



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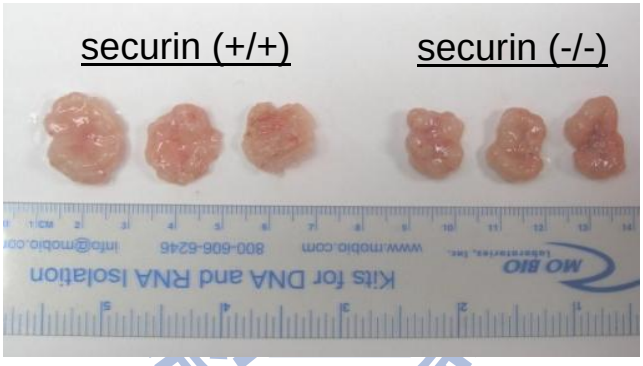

Fig. 13. Comparison of tumor formation between the securin-wild type and securin-null HCT116 in xenograft nude mice. (A) The securin-wild type and securin-null HCT116 cells were subcutaneously injected with 2×10^6 cells to the four-week-old nude mice. Each group contained three mice. The tumor volume was measured every four days during total 60 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 ACP-93 treated samples. (B) The visible tumors were separated from mice.

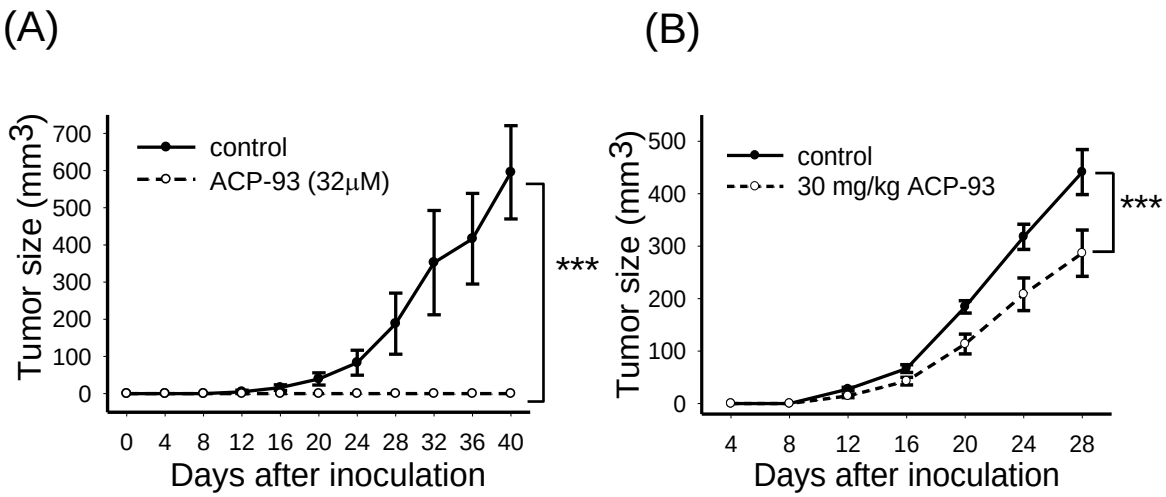

Fig. 14. Inhibition of tumorigenesis by ACP-93 in xenograft tumor of SCID mouse model. (A) HCT116 securin-wild type cells were pre-treated with or without 32 μM ACP-93 for 24 h. After treatment, the cells were subcutaneously injected with 1×10^6 cells to the four-week-old SCID mice. Each group contained five mice. The tumor volume was measured every four days during total 40 days. (B) The four-week-old SCID mice were subcutaneously injected with HCT116 1×10^6 cells. The mice bearing HCT116 xenografts were tumor injected with vehicle control (corn oil) or 30 mg/kg of ACP-93 once per four days from day 14 to day 22. Each group contained nine to ten mice. The tumor volume was measured every four days. The results were obtained from five to ten mice and the bar represents the mean ± S.E.M. $p < 0.001$ (***), indicates significant difference between the control and ACP-93 treated samples.

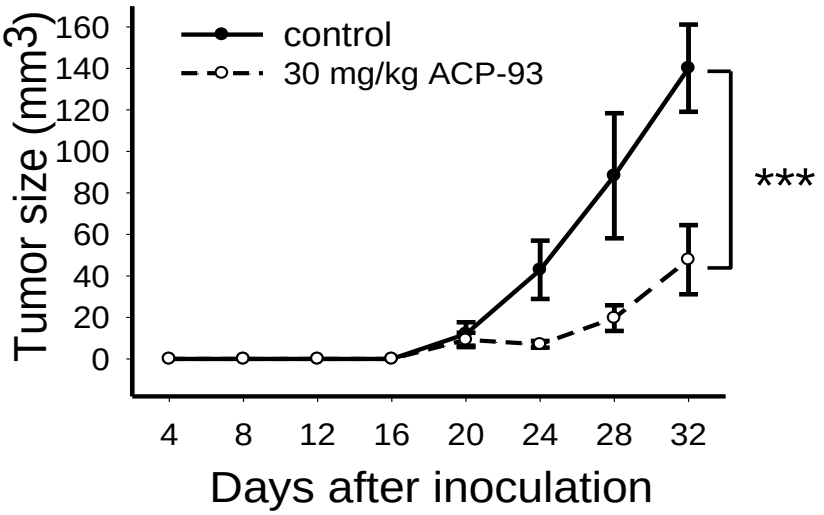

Fig. 15. Inhibition of tumorigenesis by ACP-93 in xenograft tumor of nude mouse model. The five-week-old nude mice were subcutaneously injected with HCT116 1 × 10^6 cells. The mice bearing HCT116 xenografts were tumor injected with vehicle control (corn oil) or 30 mg/kg of ACP-93 once per four days from day 24 to day 32. Each group contained three mice. The tumor volume was measured every four days. The results were obtained from three mice and the bar represents the mean ± S.E.M. $p < 0.001$ (***), indicates significant difference between the control and ACP-93 treated samples.

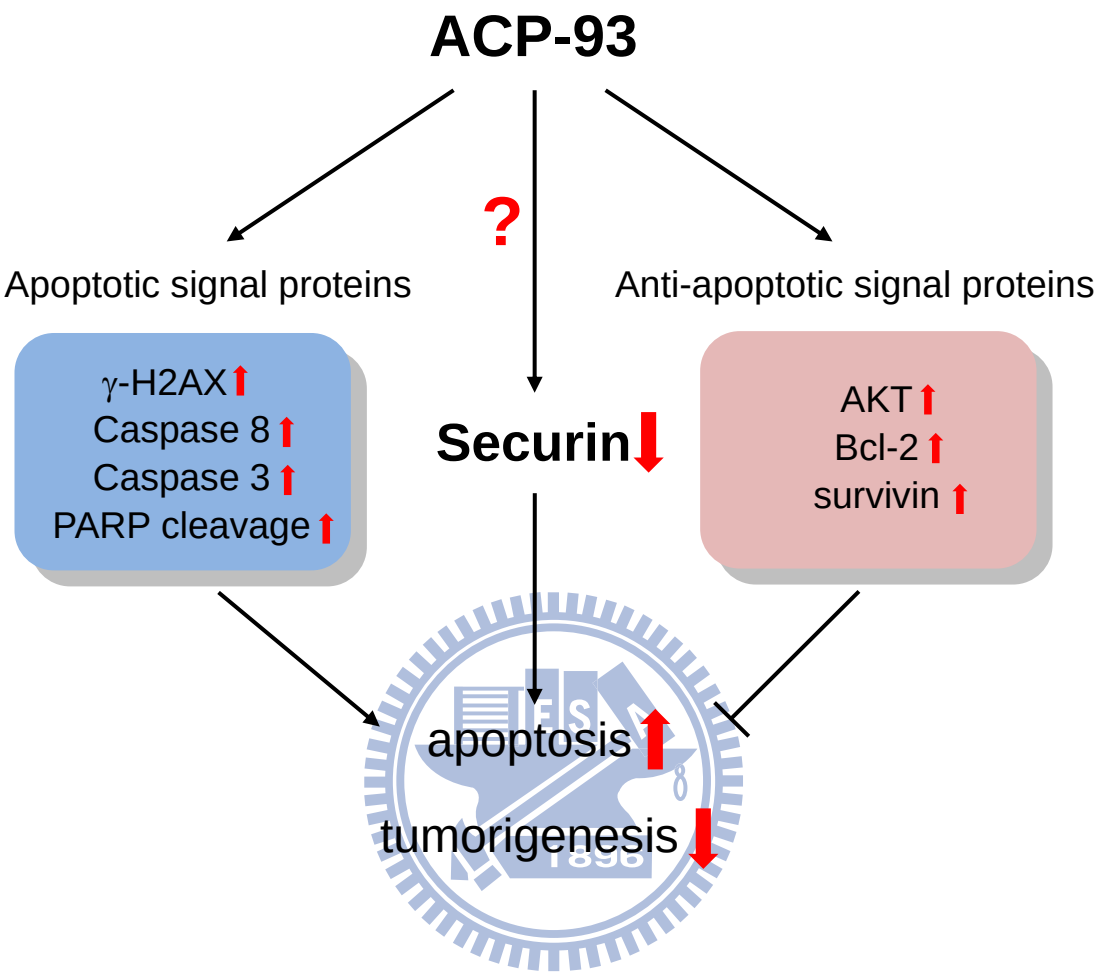

Fig. 16. Proposed model of apoptosis induction and anti-tumorigenesis by ACP-93.