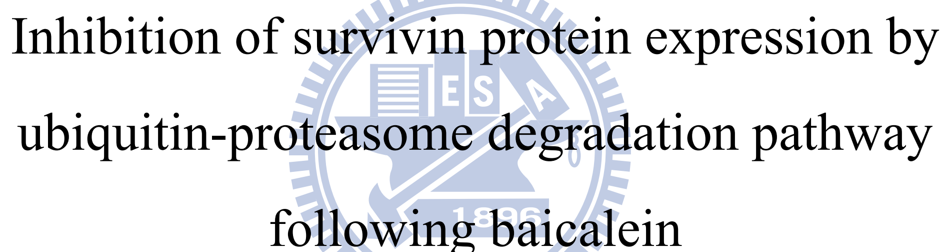
國立交通大學生物科技學系

碩士論文

黃芩素經由泛素-蛋白分解體降解路徑抑制 survivin 蛋白的表現



Inhibition of survivin protein expression by ubiquitin-proteasome degradation pathway following baicalein

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中華民國九十九年七月

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國立交通大學生物科技學系

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致謝

時間過的真快，在趙老師實驗室經歷了兩年的學習與訓練，到如今即將畢業了。學業上在老師的提攜以及學長姐的帶領下、同學間的教學相長，對於實驗研究有更進一步的了解與體認，並且增進了自己面對困難、解決問題的能力。在這段時間以來與實驗室夥伴們的學習相處下，我也學到更多待人處事的經驗，這是教科書上學不到的東西。在趙老師的指導下，使我學到做研究應有的邏輯觀念與學術素養，並且讓我有機會嘗試實驗室以往沒有的實驗技術和方法。實驗室的兩大支柱，惠芳學姊以及光凱學長，在我遭遇問題時挺身而出，指點迷津讓問題迎刃而解；並且在分生達人的啟蒙下，學習到的分生實驗與知識上讓我獲益良多。生技公司上班的和欽學長，讓我了解在業界所需具備的能力以及所應抱持的態度。在剛開始對實驗室不熟悉的階段，阿良跟真宜學姐從養細胞開始總是不厭其煩地一步一步帶領我走入研究的世界。在實驗室共患難、同進退的同學們，靜怡、繼慶跟勝壹兩年來的相處下，度過了許多美好的歡笑時光，也遭遇了許多困難與挫折。遇到困難時大家總是互相討論、幫助我解決問題以及面對當前的挑戰；遭遇挫折以及心情低落時，他們給我陪伴安慰以及背後的支持；閒暇之虞，出遊散心以及互相打嘴砲也成為我美好的回憶。實驗室的美女學妹團，婷婷、淳淳、阿簡和阿蓮，有了她們的加入使得原本枯燥乏味的實驗生活更顯多采多姿。我們的助理白雲大哥，成為我們實驗室的得力助手。還有昱耀、旻翰、Tammy 與玉梅有著年輕的活力，他們的認真的學習態度也是我效紡的對象。還有張家靖老師家與曾慶平老師家無數幫助過我的學長姐和同學們協助以及指導我解決實驗上許多的問題與困難。天天守候在外的大溝、耳洞跟皮皮，每天忠實陪伴著我們，在心情低落時，他們就像老朋友一樣安慰著你，陪你走到車棚為單調的生活帶來一絲樂趣。最後要歸功於我的家人，辛苦的從小供給我唸書到如今，他們是我最大的後盾，讓我沒有後顧之憂的完成碩士學位，並且感謝這些日子以來所有幫助過我的每一個人。

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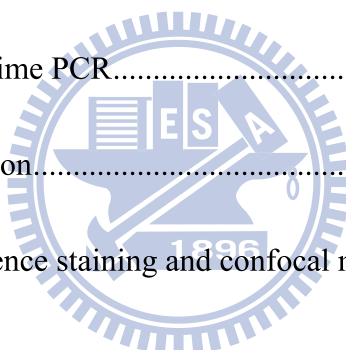

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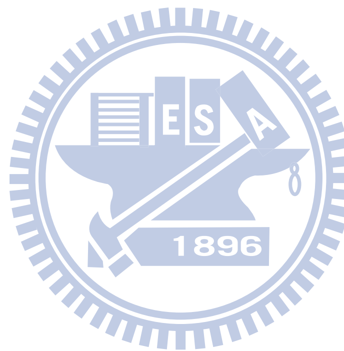

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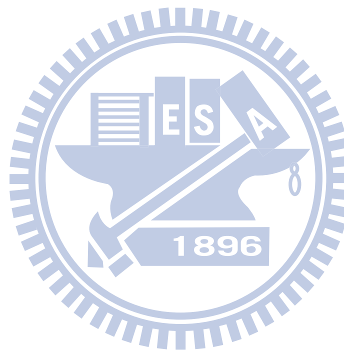


中文摘要

Survivin 是一種抗細胞凋亡的蛋白，具有促進癌細胞存活與增生的功能。黃芩素會誘發癌細胞生長停止及促進細胞凋亡的作用，然而黃芩素調控 survivin 蛋白表現的機制仍然不清楚。在本研究中，我們探討 BFTC905 人類膀胱癌細胞處理黃芩素後，survivin 的角色與調控機制。處理黃芩素明顯地抑制 BFTC905 細胞中 survivin 蛋白的表現。雖然如此，利用反轉錄聚合酶鏈反應與即時聚合酶鏈反應分析，發現黃芩素並不會影響 survivin mRNA 的表現量。利用蛋白質合成抑制劑 cycloheximide 處理後，會促進黃芩素減少 survivin 蛋白的表現量。相反地，處理一種蛋白分解體的抑制劑 MG132，能部分回復黃芩素所抑制的 survivin 蛋白表現，並且 MG132 會降低黃芩素所誘發的細胞凋亡。有趣地，利用免疫沉澱分析發現黃芩素會誘發 survivin 蛋白進行泛素化作用，泛素化的 survivin 蛋白被黃芩素大量誘發。此外，共同處理黃芩素與 oxaliplatin 會加強對 BFTC905 細胞的毒殺作用及抑制 survivin 蛋白的表現。綜合以上結果，我們推測黃芩素抑制人類膀胱癌細胞中 survivin 蛋白表現，是經由泛素-蛋白分解體的降解路徑。

Abstract

Survivn is an anti-apoptosis protein that plays the roles in promoting cancer cell survival and proliferation. Baicalein has been shown to induce growth inhibition and apoptosis in cancer cells; however, the regulation of survivin protein expression by baicalein remains unclear. In this study, we investigated the role and regulation of survivin following baicalein treatment in the BFTC905 human bladder cancer cells. Treatment with baicalein markedly inhibited survivin protein expression in BFTC905 cells. Nevertheless, the survivin mRNA level did not alter with baicalein treatment using reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. Treatment with a protein synthesis inhibitor (cycloheximide) increased the decrease of survivin protein level in the baicalein-treated cells. In contrast, a specific proteasome inhibitor (MG132) partially restored baicalein-inhibited survivin protein expression. Moreover, MG132 can reduce apoptosis induction by baicalein. Interestingly, baicalein induced the protein ubiquitination of survivin using immunoprecipitation assays. The ubiquitinated survivin proteins were increased by baicalein. Besides, co-treatment of baicalein and oxaliplatin enhanced the cytotoxicity and survivin protein inhibition in BFTC905 cells. As a consequence, we suggest that baicalein inhibits survivn protein expression through the ubiquitin-proteasome degradation pathway in the human bladder cancer cells.

1. Introduction

1.1. Flavonoids and anticancer activities

Flavonoids are a group of polyphenolic compounds that exist in plants, vegetables, and fruits (Havsteen, 1983). Flavonoids include flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (Manach et al., 2004; Scalbert and Williamson, 2000). Intake of flavonoids has been shown to reduce risk of cancer, inflammation and heart diseases (Havsteen, 2002; Middleton et al., 2000). Various types of flavonoids, such as luteolin, quercetin, kaempferol and catechin, display anticancer effects on growth inhibition and apoptosis (Brusselmans et al., 2005; Lee et al., 2005a; Psahoulia et al., 2007; Spencer et al., 2003; Yin et al., 2001). It has been shown that flavonoids can induce release of cytochrome c with activation of caspase-3 and caspase-9 to promote apoptosis (Michels et al., 2005; Wang et al., 1999). For example, epigallocatechin gallate (EGCG), the major catechin in tea, has been shown to induce apoptosis in various cancers, including leukemia (Hibasami et al., 1996), prostatic cancer (Brusselmans et al., 2003; Chung et al., 2001), gastric cancer (Horie et al., 2005), colon cancer (Chen et al., 2003), and lung cancer (Yang et al., 1998).

1.2. Apoptosis pathways

Apoptotic features include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, finally ending with the engulfment by macrophages or neighboring cells, thereby avoiding an inflammatory response in surrounding tissues (Savill and Fadok, 2000). Apoptosis can be separated two major pathways incliding extrinsic and intrinsic pathways. External apoptotic pathway is initiated by death ligands binding to their receptors such as CD-95/fas receptor and TNFα-receptor, which is followed by activation of initiator caspase-8 to induce the downstream apoptotic pathway (Walczak and Krammer, 2000). Intrinsic factors or intracellular stimuli such as DNA damage can mediate mitochondrial apoptotic pathway to initiate the release of cytochrome c and SMAC/DIABLO for apoptosis induction (Shi, 2002). Both extrinsic and intrinsic pathways lead to activation caspase-3 for apoptotic induction (Scaffidi et al., 1998; Shi, 2002). The inhibitor of caspase-activated deoxyribonuclease (CAD) can be cleaved by activated caspase-3 to release CAD (Enari et al., 1998; Sakahira et al., 1998). Then CAD enters the nucleus to degrade the chromosomal DNA and leading to DNA fragmentation and cell death (Enari et al., 1998). The NF-κB is a transcriptional factor which activates various anti-apoptotic genes expression, such as Bcl-2, Bcl-XL, and Mcl-1. These

antiapoptotic signals or proteins can promote cancer cell survival and tumorigenesis (Francois et al., 2005; Nakano et al., 2006; Suh and Rabson, 2004).

1.3. Baicalein and apoptosis

Baicalein is a bioactive flavonoid extracted from root of *Scutellaria baicalensis* or *Scutellaria radix* that contains anticancer activities (Bonham et al., 2005; Chao et al., 2007; Ma et al., 2005). It has been found that baicalein induce apoptosis in a variety of human cancer cells (Chao et al., 2007; Chen et al., 2000; Kuntz et al., 1999; Lee et al., 2005b; Ma et al., 2005; Pidgeon et al., 2002; Wang et al., 2009). Baicalein induces cancer cell death which is associated with regulating CDK1 kinase and survivin in bladder cancer cells (Chao et al., 2007). Moreover, baicalein induces apoptosis by decreasing Bcl-2 and increasing p53 and Bax human in lung cancer cells (Leung et al., 2007). In addition, NF-κB-regulated anti-apoptotic genes including Bcl-2 and Bcl-XL have been shown that they are suppressed by baicalein (Lee et al., 2005b; Pidgeon et al., 2002).

1.4. Baicalein and cell cycle progression

The regulation of cell cycle progression is regulated by cyclin dependent kinase (CDKs) and cyclins (Devault et al., 1991). CDKs participate cell cycle by binding

with cyclins and negatively regulated by CDK inhibitors (CDKIs) (Schwartz and Shah, 2005; Shapiro, 2006). Baicalein has been shown to reduce cyclin D proteins to mediate cell cycle arrest in breast, lung and prostate cancer cells (Lee et al., 2005b; Pidgeon et al., 2002). Furthermore, baicalein declines the protein expression of cyclin B1 and CDK1 protein levels in lung cancer cells (Lee et al., 2005b; Leung et al., 2007). Down-regulation of CDK1 and Cyclin B1 by baicalein is involved in the regulation of S phase progression (Leung et al., 2007). Baicalein suppresses CDK 2/4 expression and inhibits of the expression of p21 and p27 in prostate cancer cells (Pidgeon et al., 2002). Baicalein also involves G1 and G2 arrest in association with repression of CDK1, CDK2, cyclin D2 and cyclin A proteins, and with up-regulation of cyclin E, p15, p53 and p21 (Hsu et al., 2001). In addition, baicalein can induce G2/M arrest in leukemia cells (Roy et al., 2007). The levels of cyclin B1 and CDK1 are reduced for inducing G2/M arrest by baicalein in bladder cancer cells (Chao et al., 2007). Moreover, baicalein arrested S and G2/M phase in breast cancer cells (Wang et al., 2009). Accordingly, baicalein displays anticancer ability by mediating the blockage of cell cycle progression in various cancer cell types.

1.5. Baicalein and tumorigenesis inhibition

Baicalein has been shown to inhibit tumorgenesis in various mouse tumor model

(Bonham et al., 2005; Miocinovic et al., 2005). For example, baicalein displays the ability to attenuate tumor growth in pancreatic tumor mouse model (Tong et al., 2002). The antitumor effects of baicalein inhibits tumorigenesis in C3H/HeN mice implanted with murine bladder cancer cells (Ikemoto et al., 2004).

1.6. Survivin and cancer

The survivin gene is located on the human 17q25 chromosome that expresses a 16.5-kDa protein (Ambrosini et al., 1997). Survivin belongs to the smallest member of the IAP (inhibitor of apoptosis proteins) family (Deveraux et al., 1998), which contains a single 76-amino of the characteristic zinc finger baculovirus–inhibitor of apoptosis repeat (BIR) domain that is essential for the caspase-inhibitory function (Altieri, 2003; Ambrosini et al., 1997; Li et al., 1998). Survivin, it is homodimeric, arranged through hydrophobic surface of the BIR domain of each survivin monomer (Chantalat et al., 2000; Verdecia et al., 2000). Survivin, a unique inhibitor of apoptosis, expressed in embryonic and fetal organs in the developmental stages but undetectable in normal adult tissues (Ambrosini et al., 1997). Furthermore, survivin is selectively expressed in transformed cells and in most human cancers including lung, breast, pancreatic, and colon carcinomas, soft tissue sarcomas, brain tumors, melanoma, neuroblastoma, and hematologic malignancies (Adida et al., 1998;

Chakravarti et al., 2002; Grossman et al., 1999; Islam et al., 2000; Kappler et al., 2004; Kawasaki et al., 1998; Monzo et al., 1999; Satoh et al., 2001; Tanaka et al., 2000). Additionally, three isoforms of survivin have been found in human cells including survivin-2β, survivin-ΔEx3, and survivin-3β (Mahotka et al., 1999).

1.7. Survivin and cell division

Survivin is one of chromosome passenger proteins that regulates cell division (Ambrosini et al., 1997). The conserved mitotic complex of aurora-B, INCENP, borealin, and survivin is essential for chromosome movements during mitosis, proper spindle checkpoint surveillance, and execution of cytokinesis (Lens et al., 2006). Appendix 1 shows that survivin can control motosis progression (Mita et al., 2008). Chromosome passenger proteins are required to target the complex to kinetochores, correct misaligned chromosomes, properly form the central spindle and complete cytokinesis for equal and complete cell division (Gassmann et al., 2004; Honda et al., 2003; Wheatley et al., 2001). Survivin localizes to kinetochores at metaphase, transfers to the central spindle midzone at anaphase, and accumulates in midbodies at telophase (Vagnarelli and Earnshaw, 2004). Moreover, the phosphorylation of survivin on Thr^{34} by CDK1-cyclin B1 has been reported with increased survivin stability at metaphase to promote mitotic progression (O'Connor et al., 2000a).

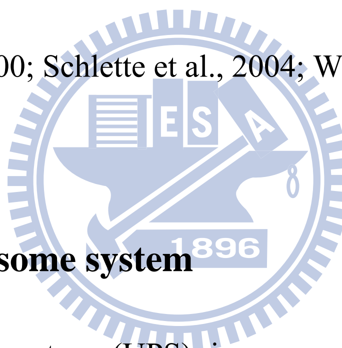
1.8. Survivin and apoptosis

Mammalian IAPs family including XIAP (X-linked IAP), c-IAP1, 2, NAIP (neuronal apoptosis-inhibiting protein), and survivin specifically inhibit the apoptosis (Miller, 1999; Salvesen and Duckett, 2002). XIAP, cIAPs, and NAIP have been shown to inhibit the caspase-3, -7, and -9 proteins by binding specific regions of caspases (Riedl and Shi, 2004). It has been shown that survivin inhibits caspase-3, -7, and -9 but not caspase-8 (Shin et al., 2001; Tamm et al., 1998). However, survivin also can mediate caspase-3-independent pathway (Banks et al., 2000). Smac/DIABLO is a pro-apoptotic protein and participates in the activation of caspase-9 (Srinivasula et al., 2000). Survivin has affinity with Smac/DIABLO to inhibit apoptosis by antagonizing the pro-apoptotic ability of Smac/DIABLO (Altieri, 2003).

1.9. Survivin and cancer therapy

Survivin displays both cell division and anti-apoptosis that can promote tumorigenesis. Survivin has been shown to promote angiogenesis (Blanc-Brude et al., 2003; Conway et al., 2003; Kawasaki et al., 2001; O'Connor et al., 2000b; Tran et al., 2002; Tran et al., 1999; Tu et al., 2003). Therefore, the blockage of survivin will

inhibit tumorigenesis and angiogenesis providing for cancer therapy. Antisense oligonucleotides, siRNA and dominant-negative mutants of survivin are successfully exploited to suppress survivin expression (Koul et al., 2006; Marusawa et al., 2003; Nakao et al., 2006; Zhang et al., 2001). Small-molecule antagonist suppresses survivin transcription by inhibiting promoter of survivin providing potential cancer therapy (Nakahara et al., 2007). Additionally, survivin has been predicted response to chemotherapy and radiotherapy in patients with bladder cancer (Als et al., 2007), breast cancer (Hinnis et al., 2007), multiple myeloma (Nakagawa et al., 2006), and lymphoma (Adida et al., 2000; Schlette et al., 2004; Watanuki-Miyauchi et al., 2005).



1.10. Ubiquitin-proteasome system

Ubiquitin-proteasome system (UPS) is an essential mechanism involved in cellular process such as degradation, cell cycle regulation, antigen processing, signal transduction and transcription (Boutillier et al., 1999; Nandi et al., 2006; Orlowski, 1999). One of UPS important function involves in ubiquitin-proteasome-dependent degradation of proteins (Ciechanover et al., 1980). The target protein is labeled with multiple ubiquitin moieties and degraded by the 26S proteasome (Ciechanover and Schwartz, 1998; Laney and Hochstrasser, 1999). The 76-amino-acid ubiquitin polypeptide (Ub) is participated to target proteins via reversible isopeptide linkages

between the ubiquitin and lysine side chains in the target proteins. Free Ub is recruited by the E1 (Ub-activating enzyme) and transferred to lysine residue of the E2 (Ub-conjugating enzyme) (Haas et al., 1982). E2 and substrate are bound by the E3 (Ub-protein ligases), which is responsible for substrate recognition. E3 involves that ubiquitin is transferred to a lysine amino groups of the target protein, then E2 and E3 are released (Hershko et al., 1986). The cyclic transfer of more Ub to the first Ub attached to the substrate is by E4 (ubiquitin-chain elongation factor)(Hoppe, 2005). Appendix 2 shows that ubiquitin-proteasome system (Donohue and Osna, 2003). Degradation of survivin occurs by the ubiquitin-proteasome pathway at the G1 phase, and it is stabilized when heat shock protein 90 (Altieri, 2004; Fortugno et al., 2003; Zhao et al., 2000). It has been shown that flavonoids such as kaempferol and quercetin enhance apoptosis by degradation of survivin in glioma cells (Siegelin et al., 2008; Siegelin et al., 2009). Furthermore, indomethacin reduced half-life of survivin and increased survivin ubiquitination (Chiou and Mandayam, 2007). In addition, chlamydocin involved survivin degradation by proteasome in ovarian cancer cells (De Schepper et al., 2003).

1.11. Survivin and bladder cancer

Bladder cancer is a universal malignancy of older adults with 14336 cases of

death in the year 2009 in the United States (Jemal et al., 2009). Survivin expression improves our prediction of cancer recurrence and survival in bladder cancer patients (Shariat et al., 2009). Survivin signaling pathways have been evaluated for survivin-targeted therapy in bladder cancer (Shariat et al., 2007). Blockage of survivin expression induces apoptosis and suppresses the growth of the tumor in bladder cancer cells (Fuessel et al., 2006; Ku et al.). Moreover, detection of survivin and its associated gene may provide an early biomarker of aggressive tumor behavior in the bladder cancers (Salz et al., 2005).

1.12. Oxaliplatin and cancer therapy

Oxaliplatin, a clinical anticancer drug, is a third-generation platinum compound that confers a different spectrum of activity compared with cisplatin (Hochster et al., 2003; Ramanathan et al., 2003). Like cisplatin, oxaliplatin acts as an alkylating agent on DNA, forming platinated intrastrand cross-linksbetween two adjacent guanine bases or two adjacent guanine–adenine bases that result in the blockage of replication and transcription (Fink et al., 1997). The combination has proven efficacy in 5-fluorouracil-resistant advanced disease and in previously untreated colorectal cancer (Andre et al., 1998; Maindrault-Goebel et al., 1999). Combination of oxaliplatin with a variety of anticancer drugs has been intensely evaluated for cancer

therapy in recent years. For example, combination of cyclooxygenase-2 inhibitors and oxaliplatin increases the growth inhibition and death in colon cancer cells (Lin et al., 2005). Moreover, oxaliplatin can inhibit survivin protein expression in cancer cells (Lin et al., 2005).

1.13. The purpose of this study

Our laboratory has provided that baicalein inhibited survivn protein expression in human bladder cancer cells (Chao et al., 2007). However, the regulation and mechanism of survivin expression after treatment with baicalein remains unclear. In this study, the regulation of survivin protein expression is investigated following baicalein treatment in the human bladder cancer cells. We provide that baicalein reduces survivin protein expression mediated by the ubiquitin-proteasome pathway. Moreover, the survivin protein expression is additionally inhibited by combination of baicalein and oxaliplatin. Understanding the mechanism by which survivin regulates baicalein-induced apoptosis may provide the identification of novel strategies for bladder cancer therapy.

2. Materials and methods

2.1. Reagents and Antibodies

Baicalein, oxaliplatin, Hoechst 33258, and 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO) Anti-survivin (FL-142 and D-8), goat anti-rabbit IgG horseradish peoxidase, goat anti-mouse IgG horseradish peoxidase, and the FITC (fluorescein isothiocyanate)-labeled goat anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ubiquitin (Apu2) and anti-actin (C4) antibodies were purchased from Millipore (Bedford, MA). MG132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) were purchased from Calbiochem (San Diego, CA). Baicalein was dissolved in DMSO, and the concentration of DMSO was < 1 % in the control and drug-containing medium.

2.2. Cell culture

The BFTC905 cell line was derived from human bladder papillary transitional cell carcinoma of a Chinese female patient. The cells were cultured in complete RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY, USA)

supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutamine (0.03 %, w/v), and cells were incubated at 37 °C and 5 % CO_2.

2.3. Cell viability assay

BFTC905 cells were plated in 96-well plates at a density of 1×10^4 cells/well for 16–20 h and then treated with various concentrations of baicalein for 24 h in RPMI-1640 medium. After the treatment, the cells were washed with PBS and were recultured in RPMI-1640 medium for 2 day. The cells were incubated with 0.5 mg/ml of MTT in RPMI-1640 medium for 4 h. The surviving cells converted MTT to formazan, which generates a blue-purple color when dissolved in dimethyl sulfoxide. The intensity of formazan was measured at 565 nm using a plate reader (VERSAmax, Molecular Dynamics Inc., CA) for enzyme-linked immunosorbent assays. The cell viability was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

2.4. Time-lapse of living cell image analysis

BFTC905 bladder cancer cells were plated at a density of 1.5×10^5 cells Petri 35-mm Petri dish in complete medium for 16 to 20 h. Then the cells were treated with

or without 60 μM baicalein by time-lapse observation under an optical phase contrast microscope with an incubator system (Olympus, Tokyo, Japan).

2.5. Annexin V/PI apoptosis assay

BFTC905 bladder cancer cells were plated at a density of 5×10^5 cells Petri 60-mm Petri dish in complete medium for 16 to 20 h. Thereafter, the cells were treated with or without 5 μM MG132 for 1 h, then were treated with or without 60 μM baicalein 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, and added 5 μl of Annexin-V-fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide (PI). Analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA).

2.6. Western blot

At 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 °C 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

in samples were subjected to electrophoresis of using 12 % sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes and the membranes were blocked overnight at 4 °C using blocking buffer (5 % non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05 % Tween 20 and 0.02 % sodium azide). Thereafter, the membrane were incubated for 2 h at 25 °C with specific primary antibodies followed by anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase conjugate secondary antibodies. The membranes were washed three times for 10 min with TBS containing 0.05 % Tween 20. The blot was incubated with enhanced chemiluminescence detection system (PerkinElmer Life Sciences) for 5 min and then exposed to X-ray film. To verify equal protein loading and transfer, actin was used as the protein loading control. The software of Un-Scan-It *gel* (Ver. 5.1, Silk Scientific, Inc.) was adopted for semi-quantification of the intensity in each band.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

BFTC905 cells were plated at a density of 2×10^6 cells per 60-mm Petri dish in culture medium. Total cellular RNA was purified by Trizol reagent (Invitrogen, California, USA) according to the manufacturer's protocol. RNA concentrations were

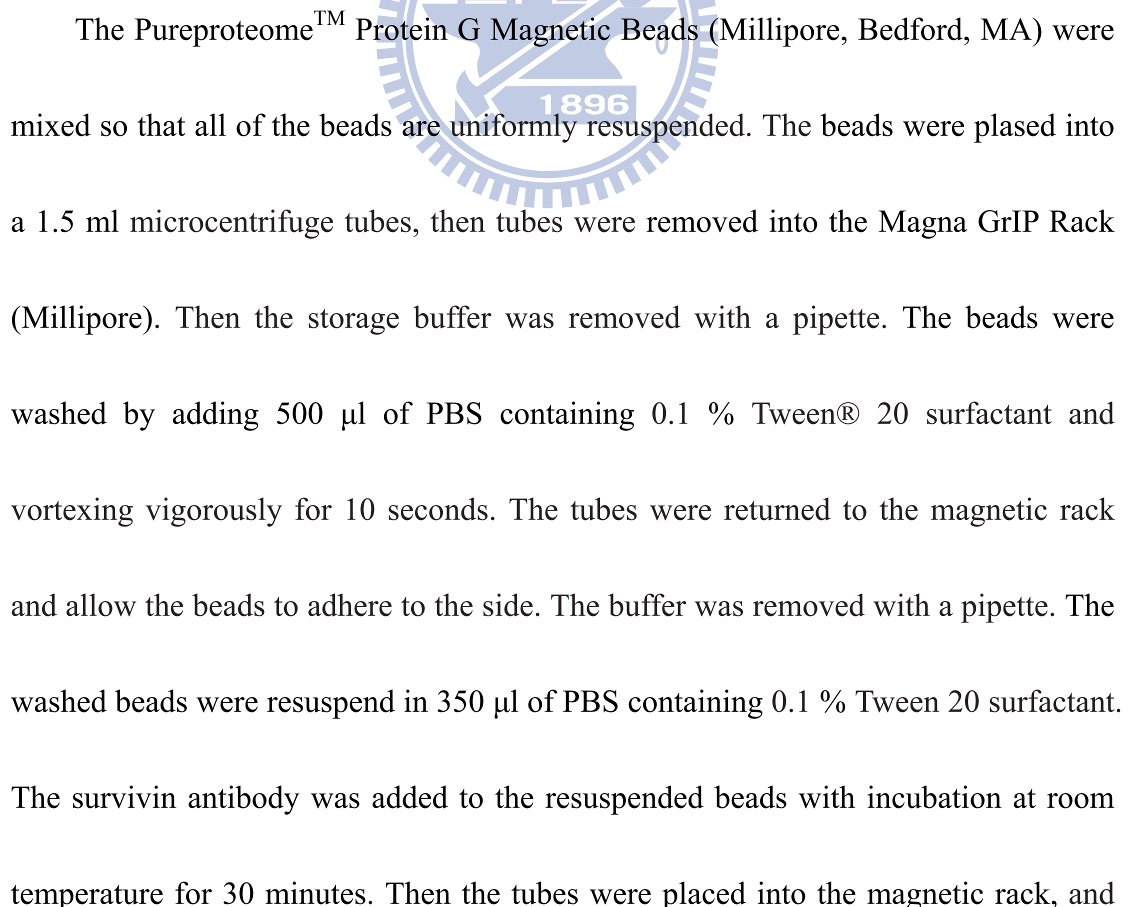
determined by a spectrophotometer (Eppendorf, Hamburg Germany). cDNAs were synthesized by SuperScript™ III reverse transcriptase (Invitrogen) with oligo-dT_{12-18} primer (Invitrogen). Each reverse transcript was amplified with GAPDH as an internal control. The following primer pairs were used for amplification: survivin, forward primer: 5'-GGCATGGGTGCCCCGACGTTG-3'and reverse primer: 5'-CAGAGGCCTCAATCCATGGCA-3'; GAPDH, forward primer: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse primer: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. RT-PCR was performed by a DNA thermal cycler, Mastercycler gradient (Eppendorf, Hamburg Germany), 56 °C for 30 s, and 72 °C for 40 s; and 72 °C for 5 min. The PCR products were visualized on 1.2 % agarose gels with ethidium bromide staining under UV transillumination with a digital camera system (DH27-S3, Medclub, Taoyuan, Taiwan).

2.8. Quantitative real-time PCR

Each real-time PCR was carried out in triplicate in a 25 μl volume using SYBR Green qPCR Master Mix (Fermentas Life Sciences,St. Leon-Rot, Germany) according to the manufacturer's protocol. Primers sequences were as follows: survivin 5'-ATTCGTCCGGTTGCGCTTTCC-3' and 5'-CACGGCGCACTTTCTTCGCAG-3'; β-Actin:

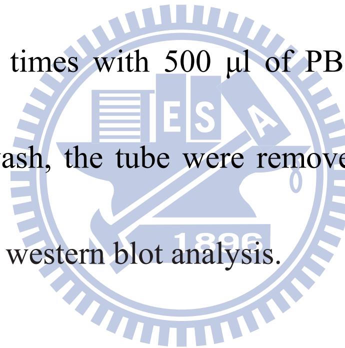
5’-GCGAGAAGATGACCCAGATC-3’ and 5’-GGATAGCAACGCCTGGATAG-3’. The PCR conditions were for 10 min at 95 °C for initial denaturation, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative gene expression quantifications were calculated according to the comparative Ct method using β-actin as an internal standard. The fold amplification of genes was respectively detected by calculating the $2^{-\Delta\Delta Ct}$ of the genes.

2.9. Immunoprecipitation



The Pureproteome™ Protein G Magnetic Beads (Millipore, Bedford, MA) were mixed so that all of the beads are uniformly resuspended. The beads were plased into a 1.5 ml microcentrifuge tubes, then tubes were removed into the Magna GrIP Rack (Millipore). Then the storage buffer was removed with a pipette. The beads were washed by adding 500 μl of PBS containing 0.1 % Tween® 20 surfactant and vortexing vigorously for 10 seconds. The tubes were returned to the magnetic rack and allow the beads to adhere to the side. The buffer was removed with a pipette. The washed beads were resuspend in 350 μl of PBS containing 0.1 % Tween 20 surfactant. The survivin antibody was added to the resuspended beads with incubation at room temperature for 30 minutes. Then the tubes were placed into the magnetic rack, and

then the buffer was removed with a pipette. The beads were washed 3 times with 500 μl of PBS containing 0.1 % Tween 20 surfactant. After the last wash, the tubes were removed from the rack and the cell lysates were added. According to the relative protein expression of survivin in control and baicalein-treated samples, the total protein lysates were adjusted in immunoprecipitation analysis for equal survivin protein expression of control and baicalein-treated sample. Then samples were immobilized survivin antibody at 2–8 °C with continuous mixing overnight. The tubes were placed into the magnetic rack, and then removed the sample with a pipette. The beads were washed 3 times with 500 μl of PBS containing 0.1 % Tween 20 surfactant. After the last wash, the tube were removed from the magnetic rack and added the sample buffer for western blot analysis.



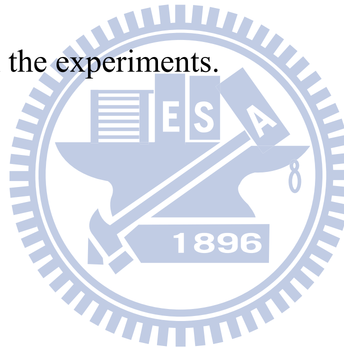
2.10. Immunofluorescence staining and confocal microscopy

To view the protein expression of survivin and ubiquitin after baicalein treatment, the cells were subjected to immunofluorescence staining and confocal microscopy. After fixation with 4 % paraformaldehyde solution, the cells were washed three times with PBS, and non-specific binding sites were blocked in PBS containing 10 % FBS and 0.3 % Triton X-100 for 1 h at 37 °C. Thereafter, the cells were separately incubated with mouse anti-survivin (1:200) antibody and rabbit anti-ubiquitin (1:400)

antibody in PBS containing 10 % FBS for 1 h at 37 °C, and washed three times with 0.3 % Triton X-100 in PBS. Then the cells were individually incubated with goat anti-mouse Cy3 (1:200) and anti-rabbit FITC in PBS containing 10 % FBS for 1 h at 37 °C. The nuclei were stained with Hoechst 33258. The samples were examined under a confocal microscope Fluoview 300 (Olympus, Tokyo, Japan).

2.11. Statistical analysis

Data were analyzed using Student's *t* test, and a p value of <0.05 was considered as statistically significant in the experiments.



3. Results

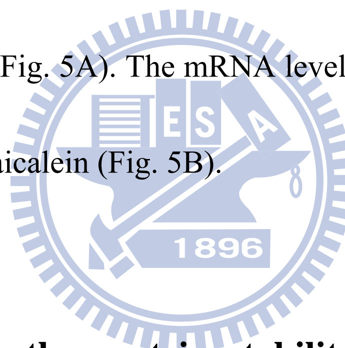
3.1. Baicalein induces cytotoxicity and proliferation inhibition in bladder cancer cells

To examine the cytotoxicity and proliferation following baicalein treatment in BFTC905 bladder cancer cells, the cells were analyzed by MTT assay. Treatment with 20–100 μM baicalein for 24 h significantly reduced the cell viability via a concentration-dependent manner in BFTC905 cells (Fig. 1). The value of IC50 (the concentration of 50 % inhibition of cell viability) was around 30 μM. Moreover, baicalein inhibited cell proliferation and induced cell death that can be observed by time-lapse living cell morphology observation alteration. The arrows show that baicalein induced the cell death at 24 h observation (Fig. 2). However, the untreated cells clearly displayed the increase of cell proliferation and cell number at 24 h observation (Fig. 2).

3.2. Baicalein inhibits survivin protein expression but not altered gene expression in bladder cancer cells

To study the effect of survivin protein expression by baicalein in BFTC905 bladder cancer cells, the baicalein-treated cells were analyzed by Western blot. The

protein levels of survivin were decreased by 20–80 μM baicalein for 24 h in BFTC905 cells (Fig. 3A and 3B). The quantified data also shows that baicalein significantly reduced survivin protein expression in BFTC905 cells (Fig. 3B). Moreover, we have further investigated the survivin expression on transcriptional levels by reverse transcription-PCR and real-time PCR. The qualities of total RNA extracts were presented by the contents of 28S rRNA and 18S rRNA (Fig. 4A). However, baicalein did not alter the survivin mRNA expression (Fig. 4B and 4C). The survivin mRNA expression in baicalein-treated cells was compared to the control for 24 h by real-time PCR (Fig. 5A). The mRNA level of survvin was not statistically altered by treatment with baicalein (Fig. 5B).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3.3. Baicalein reduces the protein stability of survivin in bladder cancer cells

To further determine the effect of baicalein on the protein stability and half-life of survivin proteins, a protein synthesis inhibitor (cycloheximide, CHX) was examined on the effect of survivin protein expression. Treatment with 10 μg/ml CHX for 24 h reduced around the half of total amount of survivin proteins (Fig. 6A). However, treatment with CHX and baicalein, the survivin protein levels were decreased more quickly than CHX alone at 6–24 h (Fig. 6B). Co-treatment of CHX

and baicalein almost completely blocked the survivin protein expression after 12 h period (Fig. 6B).

3.4. Proteasome inhibitor decreases baicalein-induced survivin protein degradation in bladder cancer cells

To investigate the role of proteasome on baicalein-induced down-regulation of survivin protein expression, MG132 (a proteasome inhibitor) was utilized in this study. Treatment with 60 μM baicalein for 24 h significantly reduced survivin protein expression (Fig. 7). Pre-treatment of BFTC905 cells with 5 μM MG132 potentially reversed survivin protein level in the baicalein-treated cells (Fig. 7). In annevin V/PI apoptosis assay, baicalein increased higher apoptosis level than untreated sample in BFTC905 cells (Fig. 8). Moreover, pre-treatment of MG132 potentially inhibited apoptosis in the baicalein-treated BFTC905 cells (Fig. 8).

3.5. Baicalein treatment increases ubiquitination of survivin

We have determined the effect of baicalein on the protein ubiquitination of survivin. As shown in Fig. 9, baicalein induced ubiquitinated survivin levels in BFTC905 cells. To further confirm the ubiquitination of survivin proteins, the baicalein-treated cells were subjected to immunofluorescence staining and confocal

microscopy. Baicalein reduced the intensity of red fluorescence (Cy3) of survivin proteins. However, baicalein induced high intensity of green fluorescence of ubiquitin proteins. The yellow color indicated that co-locolization of survivin and ubiquitin (Fig. 9, arrows).

3.6. Co-treatment of baicalein and oxaliplatin enhances the cytotoxicity and survivin protein inhibition in bladder cancer cells

As shown in Fig. 11, co-treatment with 10–50 μM baicalein and 1 μM oxaliplatin for 24 h enhanced the cytotoxicity in BFTC905 cells. To study the combination effect of baicalein and oxaliplatin on the survivin protein inhibition, the cells were co-treated with baicalein and oxaliplatin followed by Western blot analysis. Both of baicalein and oxaliplatin significantly reduced survivin protein expression (Fig. 12A and 12B). Co-treatment with baicalein and oxaliplatin for 24 h enhanced the decrease of the survivin protein expression (Fig. 12A and 12B).

4. Discussion

Various types of flavonoids display anticancer effects on growth inhibition and apoptosis (Brusselmans et al., 2005; Lee et al., 2005a; Psahoulia et al., 2007; Spencer et al., 2003; Yin et al., 2001). In this study, baicalein significantly inhibited cell viability in the human BFTC905 bladder cancer cells. We also found that baicalein induced growth inhibition and cell death by time-lapse observation in BFTC905 cells. Survivin has been demonstrated to inhibit apoptosis and to promote mitotic progression in cancer cells (Ambrosini et al., 1997; Li et al., 1998). Interestingly, baicalein significantly reduced survivin protein expression in BFTC905 cells. However, baicalein did not influence the survivin mRNA expression by RT-PCR and real-time PCR assays. As a consequence, we suggest that baicalein inhibits the survivin expression on the alteration of protein level but not gene expression.

Ubiquitin-proteasome pathway is an essential mechanism participating in cellular process. Proteasome degradation has also been shown to play an important role in regulation of apoptosis and cell proliferation by indomethacin (Chiou and Mandayam, 2007). Furthermore, kaempferol and quercetin enhanced apoptosis by degradation of survivin in glioma cells (Siegelin et al., 2008; Siegelin et al., 2009). It has been shown that survivin can be degraded via the ubiquitin-proteasome pathway

in a cell cycle-dependent manner (Zhao et al., 2000). We have shown that baicalein inhibited survivin protein expression (Chao et al., 2007); however, suppression of survivin on the post-translational level by baicalein has not been shown previously. We have further determined the half-life of survivin by baicalein. Baicalein enhanced the survivin protein degradation when the cells were co-treated with CHX. The data indicates that baicalein induces the survivin protein instability in bladder cancer cells. Furthermore, MG132 proteasome inhibitor prevented survivin protein degradation in the baicalein-treated cells. In addition, MG132 can reduce the baicalein-induced apoptosis. Baicalein also activated ubiquitination of survivin in bladder cancer cells. Accordingly, our findings suggest that baicalein inhibits survivin protein expression through the ubiquitin-proteasome pathway in human bladder cancer cells. We provide a model of baicalein-induced down-regulation of survivin as shown in Fig. 13.

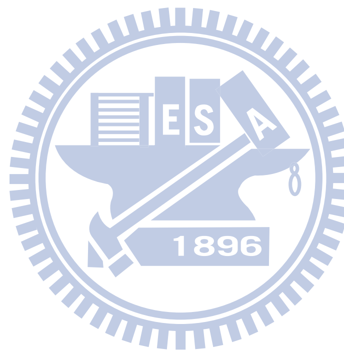
The stability of survivin resulted from the protein phosphorylation at Thr^{34} by the mitotic kinase complex CDK1/cyclin B1 (O'Connor et al., 2000a; Wall et al., 2003). Quercetin increases the survivin protein expression, which correlates with raising the protein levels of cyclin B1 and phospho-CDK1 (Kuo et al., 2004). The levels of cyclin B1 and CDK1 were reduced for inducing G2/M arrest by baicalein in bladder cancer cells (Chao et al., 2007). The role of CDK1/cyclin B1 on the regulation of survivin protein should be further investigated. It has been shown that

XAF1-XIAP complex enhanced degradation of survivin protein (Arora et al., 2007). Therefore, further investigations are required to determine the roles of XAF1-XIAP and CDK1/cyclinB1 on the regulation of survivin protein expression following baicalein in bladder cancer cells.

Combination of various anticancer agents may increase the efficiency of cancer therapy (Hochster et al., 2003; Ramanathan et al., 2003; Rathkopf et al., 2009). Previously, Oxaliplatin reduced survivin protein expression and induced apoptosis in cancer cells (Lin et al., 2005). In this study, we have further examined the anticancer effects of combination of baicalein and oxaliplatin on the cell viability and survivin expression in human bladder cancer cells. Both baicalein and oxaliplatin significantly induced cell death in BFTC905 cells. Interestingly, co-treatment of baicalein and oxaliplatin additively decreased the levels of survivin proteins and increased cytotoxicity in BFTC905 cells. Accordingly, the combination of baicalein and oxaliplatin may increase anticancer effects on survivin inhibition and cancer cell death, providing important strategy for cancer therapy.

5. Conclusion

We have summerized that the down-regulation of survivin by baicalein is mediated ubiquitin-proteasome degradation pathway in human bladder cancer cells (Fig. 13). Our findings indicate that the blockage of survivin by baicalein may provide the novel strategies for elevating the efficiency of cancer therapy in bladder cancer.



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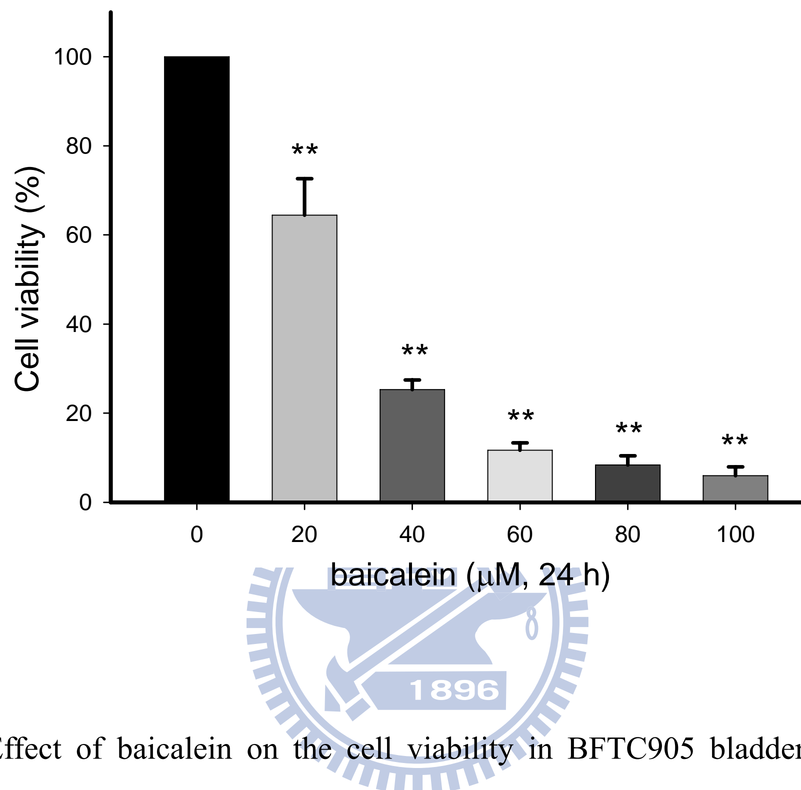


Fig. 1. Effect of baicalein on the cell viability in BFTC905 bladder cancer cells. BFTC905 cells were treated with 0–100 μM baicalein for 24 h. After drug treatment, the cells were recultured in fresh medium for 2 days. The cell viability was measured by MTT assay. Results were obtained from four independent experiments and the bar represents ± S.E. $p < 0.05$ (*) and $p < 0.01$ (**), indicate significance between untreated and baicalein-treated samples

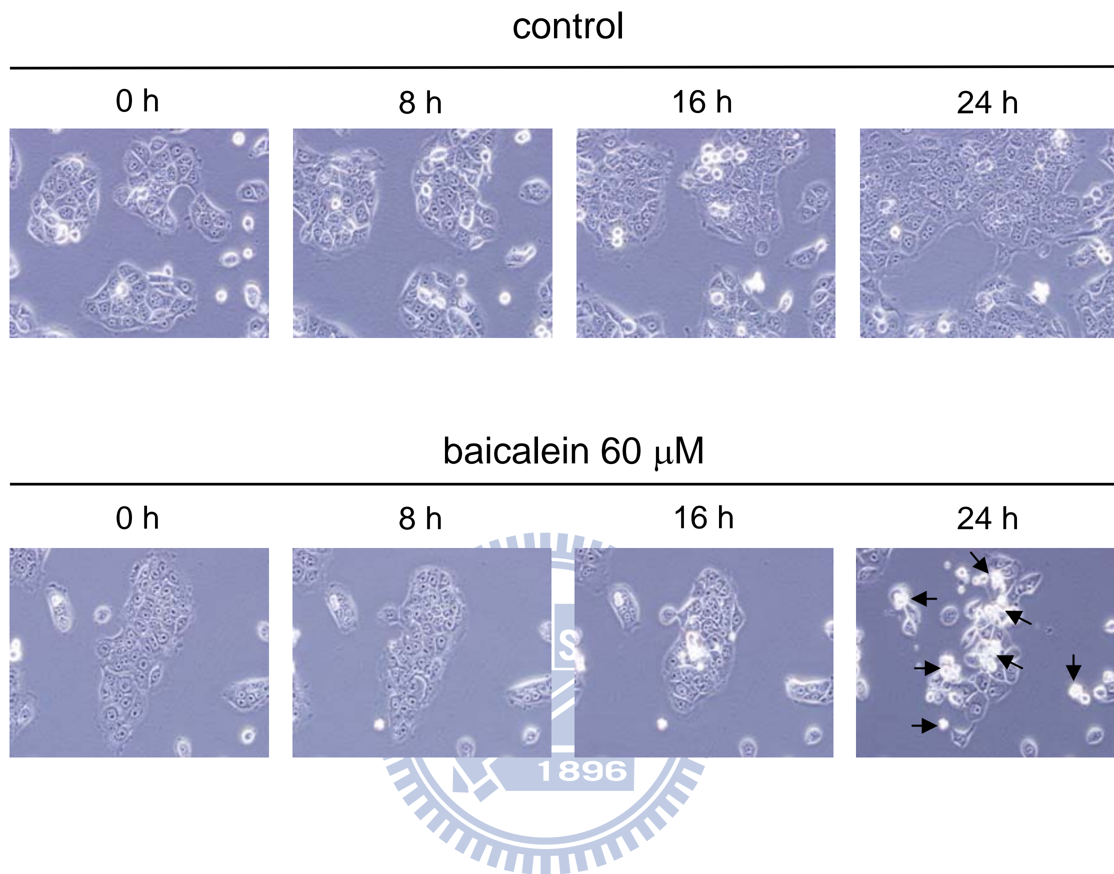


Fig. 2. Time-lapse observation of baicalein-induced cancer cell death. BFTC905 cells were treated with or without 60 μM baicalein by time-lapse observation from 0 to 24 h. The cell morphology of BFTC905 cells was observed under an optical phase contrast microscope with cell incubator system. The arrows indicate the cell death following baicalein treatment at 24 h observation.

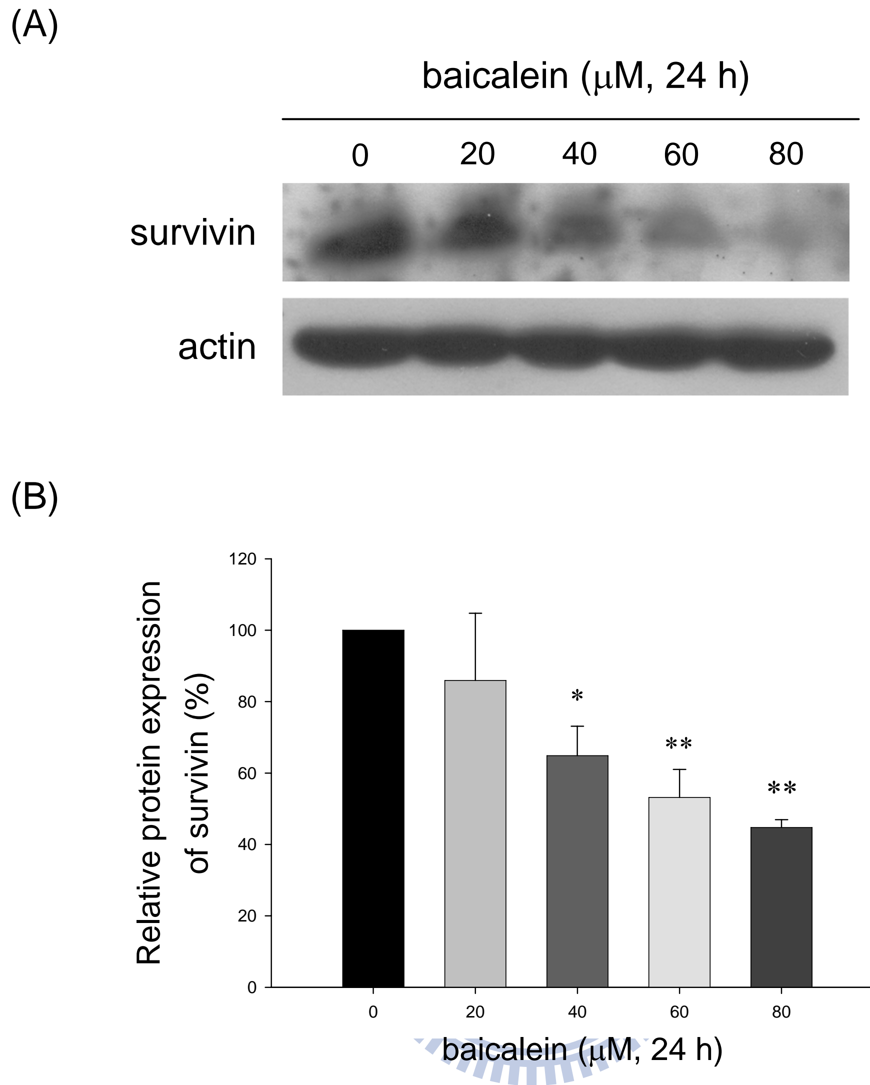


Fig. 3. Effect of baicalein on the protein levels of survivin in bladder cancer cells. (A) BFTC905 cells were treated with 0–80 μM baicalein for 24 h. Western blot data were shown from one of three experiments with similar findings. (B) The relative protein intensity of survivin was quantified from Western blots. Results were obtained from five independent experiments The bar represented the mean ± S.E. $p < 0.05$ (*) and $p < 0.01$ (**) indicate significance between control and baicalein-treated samples.

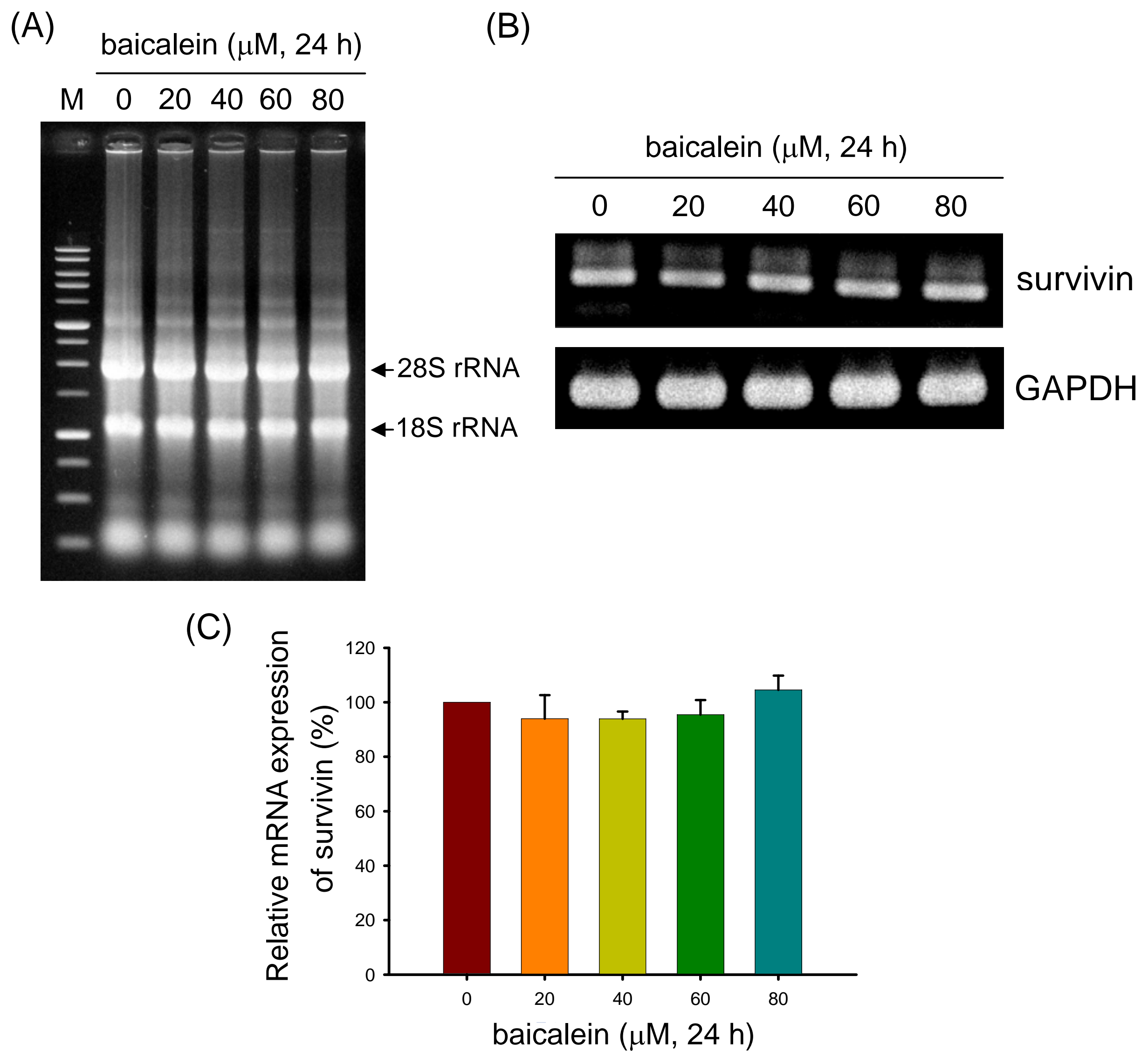


Fig. 4. Effect of baicalein on the survivin mRNA level in bladder cancer cells by RT-PCR analysis. (A) BFTC905 cells were treated with or without baicalein. At the end of treatment, total RNA from each sample was isolated according to the manufacturer's protocol. The bands of 28S and 18S rRNAs were indicated on the gel electrophoresis in each sample that was theoretically 2:1 when RNA integrity of the samples was reliable. (B) The survivin mRNA level was performed by RT-PCR, GAPDH was an internal control. (C) The bands of RT-PCR were quantified by UN-SCAN-IT. Results were from four independent experiments. Relative survivin mRNA expression was normalized to GAPDH and calibrated to levels in untreated samples.

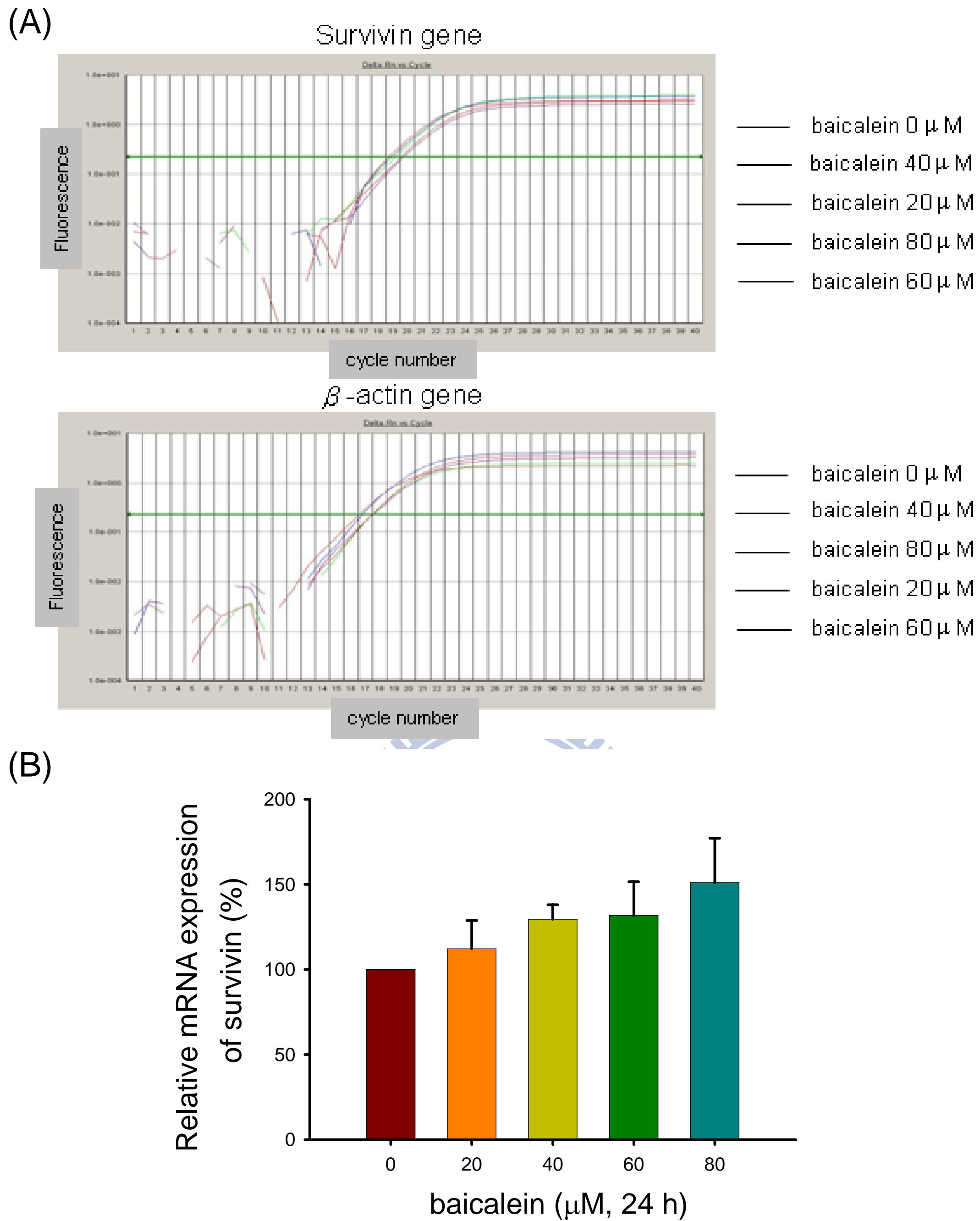


Fig. 5. Effect of baicalein on the survivin mRNA level in bladder cancer cells by real-time PCR. (A) BFTC905 cells were treated with 0–80 μM baicalein for 24 h. In the initial cycles of real-time PCR, there are little fluorescence signal. An increase in fluorescence above the baseline indicates the detection of accumulated target. The parameter CT is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. (B) Data were in triplicate from six independent experiments and were normalized to β-actin and calibrated to untreated samples.

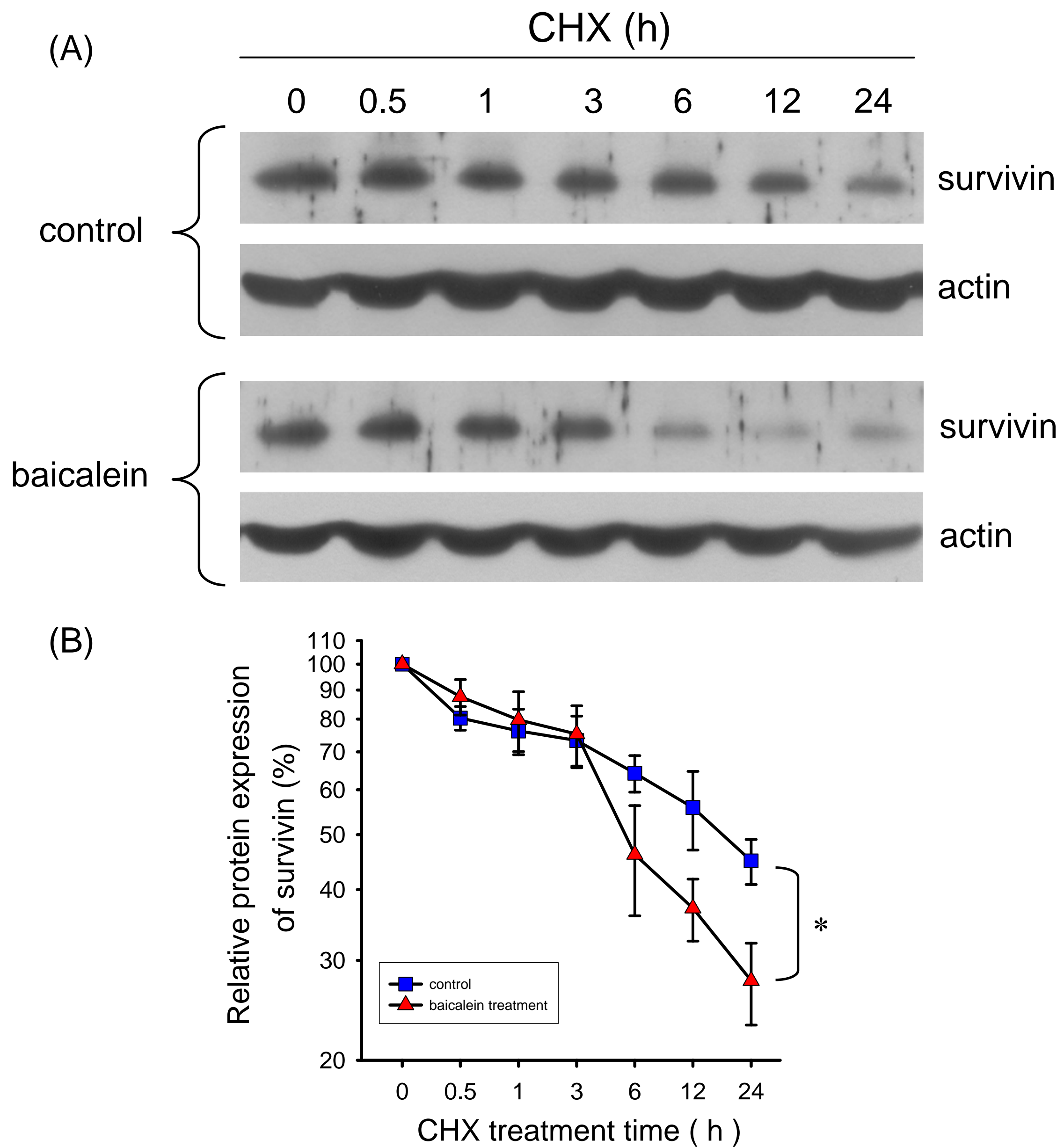
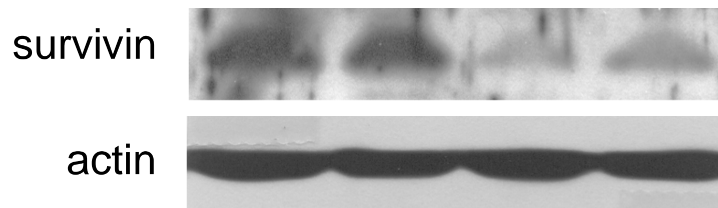


Fig. 6. Stability of survivin protein in the baicalein-treated in bladder cancer cells. (A) BFTC905 cells were pre-treated with or without 80 μM baicalein for 2 h, and then added with 10 μg/ml cycloheximide (CHX) for various time courses. The survivin protein levels were detected by Western blot. (B) The data were from three independent experiments. The Western blots were quantitated by UN-SCAN-IT and the protein expression of survivin was normalized to actin. The bar represents the mean ± S.E. $p < 0.05$ (*), indicates significance between control and baicalein treatment

(A)



(B)

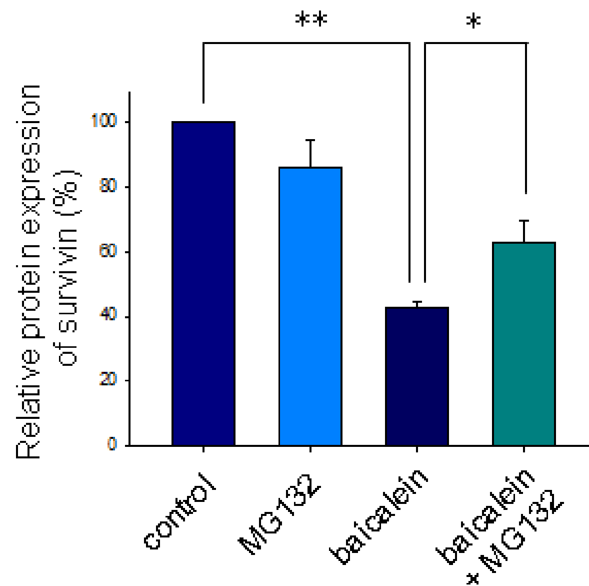


Fig. 7. Effect of MG132 (a proteasome inhibitor) on the baicalein-inhibited survivin protein expression in bladder cancer cells. (A) BFTF905 cells were in the presence or absence of 5 μM of MG132 for 1 h, then replaced MG132 for 60 μM baicalein for 24 h. Representative Western blot data were shown from one of five separate experiments with similar findings. (B) The protein intensity of survivin was quantified from Western blots. The bar represented the mean ± S.E. $p < 0.05$ (*) indicates significant difference between baicalein alone and pre-treatment with MG132 samples. $p < 0.01$ (**) indicates significant difference between control and baicalein-treated samples.

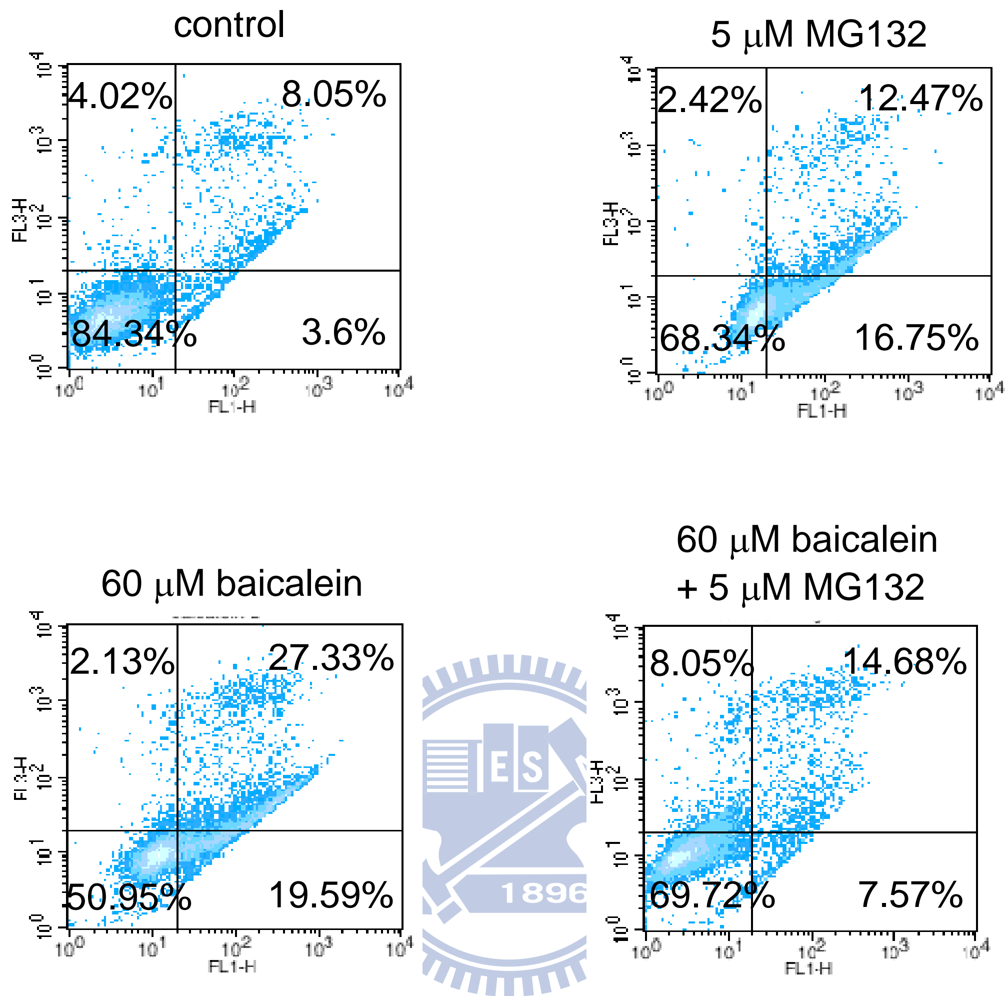


Fig. 8. Effect of MG132 on the apoptosis level in the baicalein-treated BFTC905 cells. The cells were pre-treated 5 μM MG132 for 1 h prior to treatment with 60 μM baicalein for 24 h. Apoptosis was determined by annexin V-PI staining using flow cytometry analysis. The cell population of annexin V(+)/PI(−) was indicated early apoptosis (lower, right). The fraction of annexin V(+)/PI(+) was indicated late apoptosis (upper, right). The population of apoptotic cells was averaged from two independent experiments.

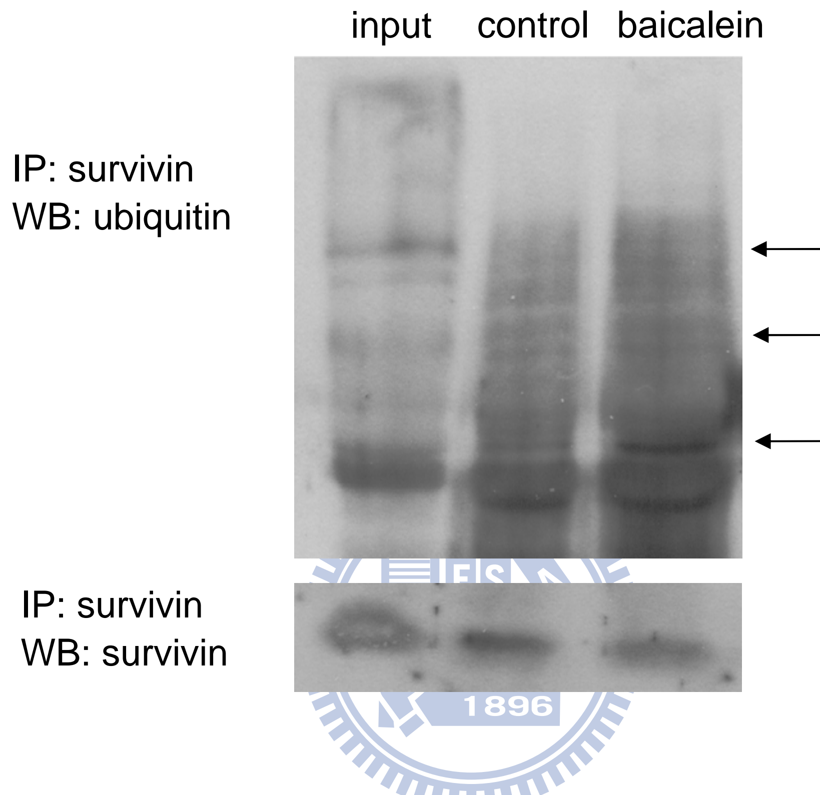


Fig. 9. Ubiquitination of survivin in baicalein-treated BFTC905 cells. The cells were treated with or without 80 μM baicalein for 24 h. Survivin proteins of BFTC905 cells were immunoprecipitated using monoclonal anti-survivin antibody. Ubiquitination was examined by Western blot analysis using polyclonal anti-ubiquitin antibody. The arrows indicate the bands of ubiquitination of survivin proteins.

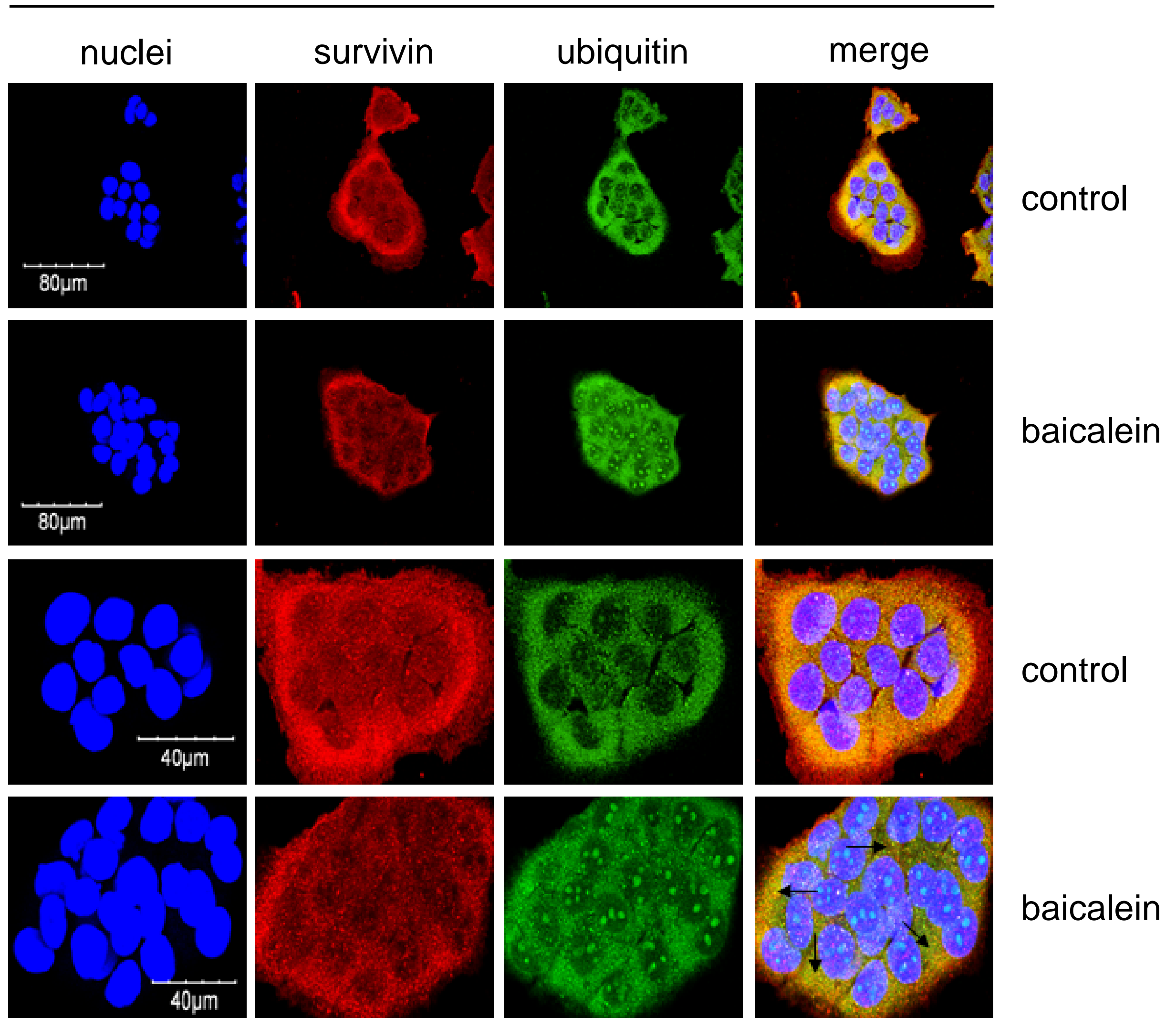


Fig. 10. Effects of baicalein on the co-expression of survivin and ubiquitin proteins in bladder cancer cells. BFTC905 cells were treated with or without 80 μM baicalein for 24 h. The cells were incubated with mouse anti-survivin and rabbit anti-ubiquitin antibody. Then the cells were incubated with goat anti-mouse Cy3 and goat anti-rabbit Hilyte 488. The survivin protein displayed red fluorescence with goat anti-mouse Cy3. The ubiquitin protein displayed green fluorescence with goat anti-rabbit Hilyte 488 and the nuclei were stained with Hoechst 33258.

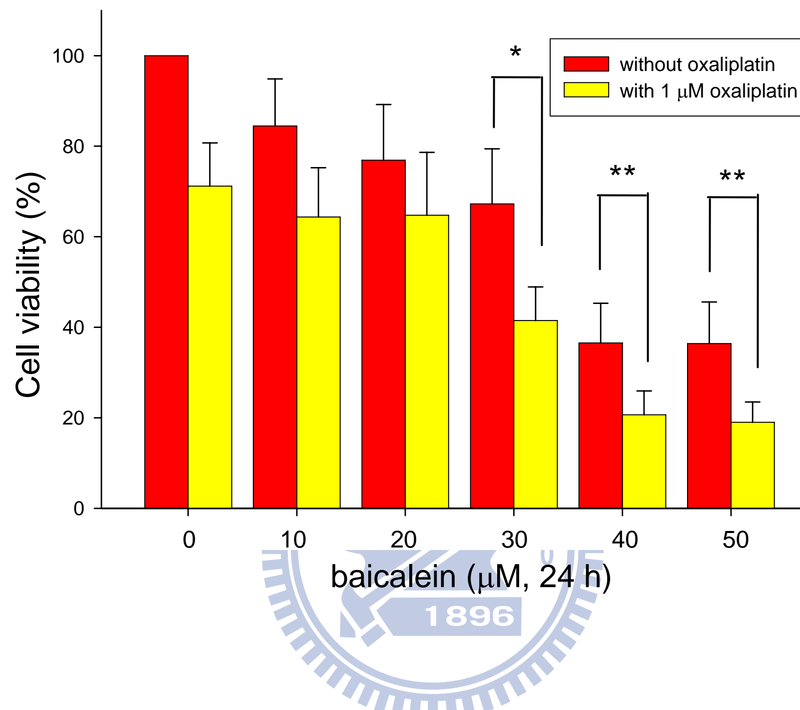


Fig. 11. Effect of co-treatment with baicalein and oxaliplatin on the cell viability in bladder cancer cells. BFTC905 cells were co-treated with 0–50 μM baicalein and 1 μM oxaliplatin for 24 h. Then the cell survival was measured by MTT assay. Results were obtained from five experiments and the bar represents ± S.E. $p < 0.05$ (*) and $p < 0.01$ (**), compared with the controls and co-treatment of baicalein and oxaliplatin.

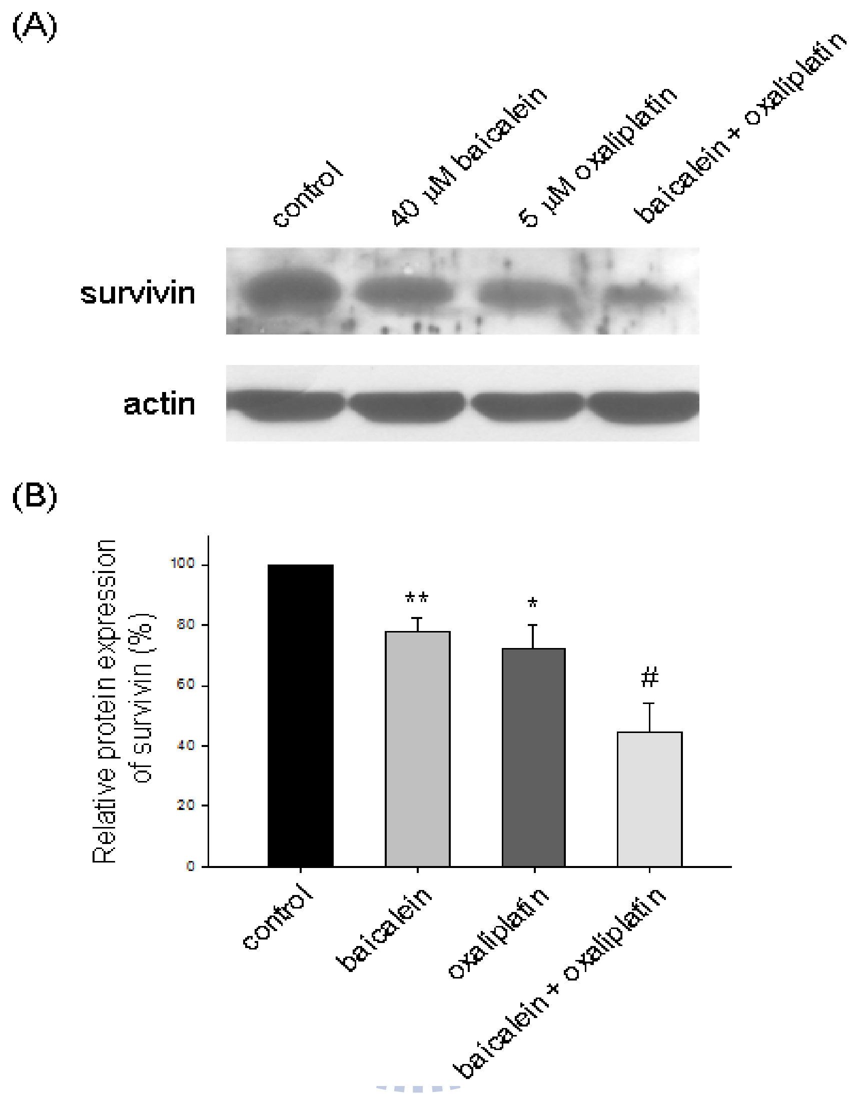


Fig. 12. Effects of baicalein and oxaliplatin on the protein level of survivin in bladder cancer cells. The cells were co-treated with 40 μM baicalein and 5 μM oxaliplatin for 24 h. The total protein extracts were subjected to Western blot analysis using anti-survivin and anti-actin antibodies. Representative Western blot data were shown from one of three separate experiments with similar findings. The bar represents the mean ± S.E. $p < 0.05$ (*) indicates significant difference between control and baicalein or oxaliplatin treated samples. $p < 0.05$ (#), indicates significant difference between the baicalein-treated sample and co-treatment of baicalein and oxaliplatin.

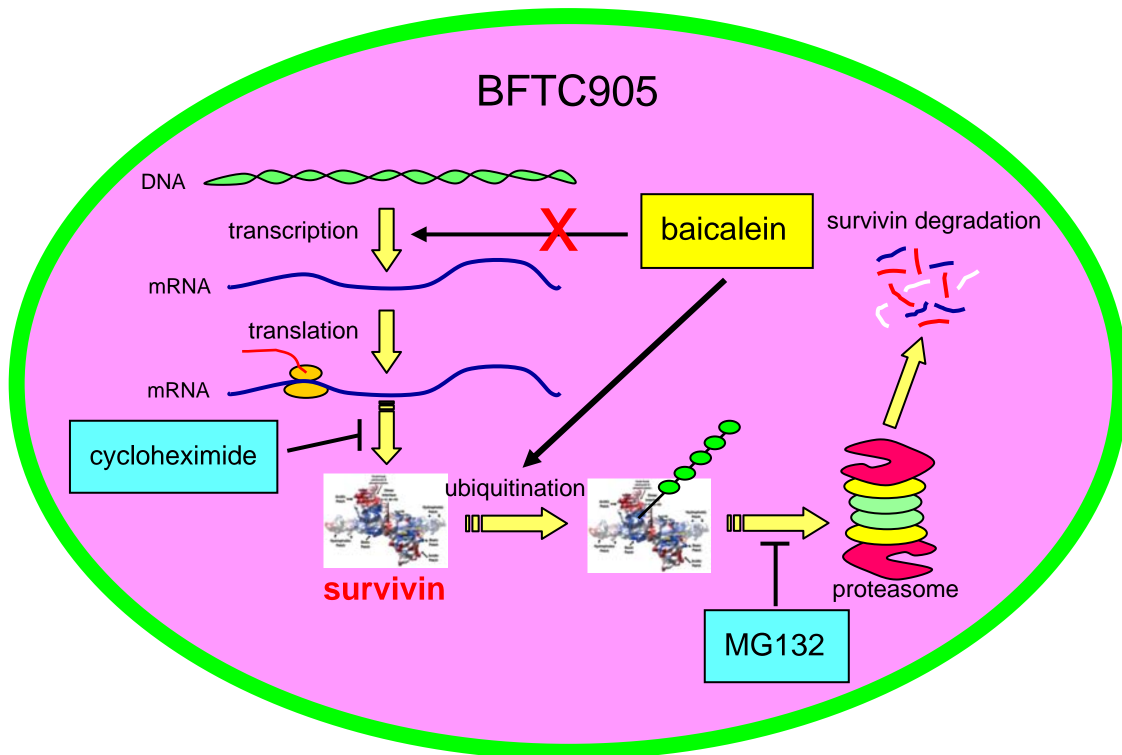
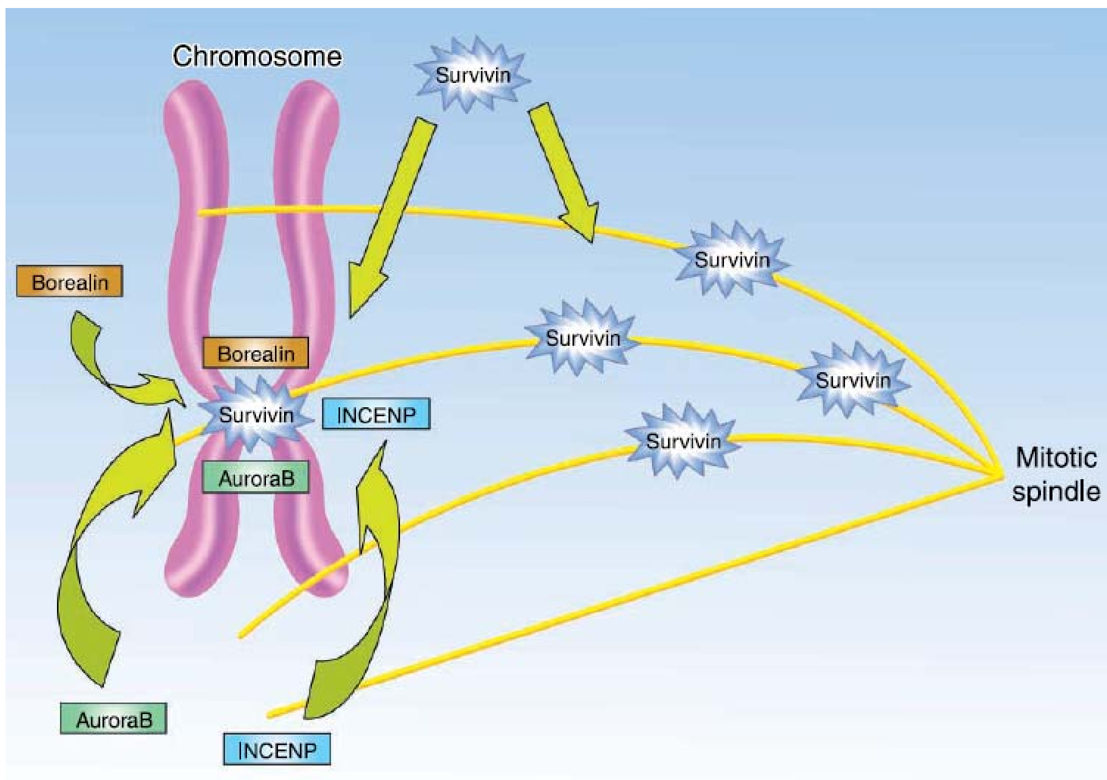
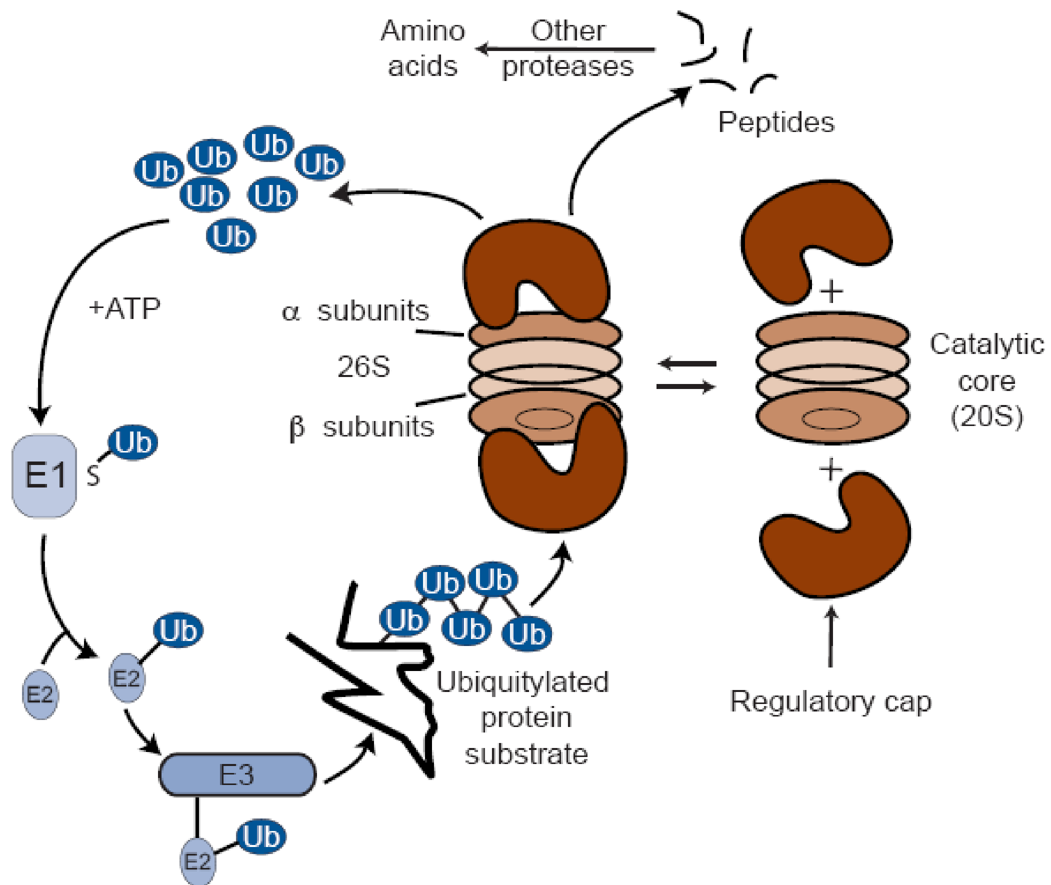


Fig. 13. Proposed model of survivin down-regulation by baicalein in human bladder cancer cells.

7. Appendixs



Appendix 1. Function of survivin in mitosis (From: Mita et al., 2008).



Appendix 2. Schematic representation of the ubiquitin–proteasome system (From: Donohue et al., 2002).