Apoptotic Signaling in Bufalin- and Cinobufagin-Treated Androgen-Dependent and Androgen-Independent Human Prostate Cancer Cells

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Running Title: BF & CB on Apoptotic Signaling in Prostate Cancer Cells

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ABSTRACT

Prostate cancer is one of the most common malignant tumors among men in the United States. The mortality in Asia is also increased. Bufalin and cinobufagin are extracts of toxic glands from toads, and are traditionally used as treatments of heart failure. Our previous studies indicated that digitalis-like bufalin and cinobufagin exerted antiproliferative effects on prostate cancer cells. Moreover, calcium played an important role in digitalis-induced apoptosis in prostate cancer cells. In the present study, we examined the apoptosis-related transduction pathway induced by bufalin and cinobufagin on androgen-dependent and -independent prostate cancer cell lines. By Western blot, the protein expressions of active caspase 3 were elevated in LNCaP, DU145, and PC3 cells after combination of bufalin or cinobufagin with EGTA. The expressions of Fas, the death receptor on cell membrane, were increased in three types of human prostate cancer cell lines after treatment of bufalin or cinobufagin. After separation of nucleus and mitochondrial fractions, the expressions of Bax were down-regulated in nucleus and elevated in mitochondrial fractions after treatment with bufalin or cinobufagin for 12 h in LNCaP and DU145 cells and for 24 h in PC3 cells. The protein expressions of cytosolic cytochrome c were also increased after treatment of bufalin or cinobufagin in three types of cell lines. These results suggested that the extracellular calcium influx might not contribute to the apoptosis induced by bufalin or cinobufagin. However, Fas expression could be involved in the increase of mitochondrial Bax and cytosolic cytochrome c induced by bufalin and cinobufagin in human prostate cancer cells.
INTRODUCTION

Prostate cancer has high incidence, and is one of the most common cause of cancer death of men in the United States (1). In addition, the incidence and mortality of prostate cancer also increase in Asia countries for the past decade. Traditionally, radiation therapy and surgery are curative treatments for prostate cancer in situ. Androgen ablation is the mainstay treatment for metastatic prostate cancer; however, it results in the recurrence of androgen-independent cancer cells in 80% of patients, which has the median survival for six to nine months (2). There are still no effective chemotherapeutic drugs for hormone-refractory prostate cancer. Therefore, it is important to find some novel and effectual treatments for hormone-insensitive prostate cancer.

Bufalin, one of the prominent components of Chan-Su extracts from the venom of *Bufo bufo gargarizan*, is reported as a Na\(^+\)-K\(^+\)-ATPase inhibitor which results in an elevation of intracellular calcium concentration (3). On the basis of this mechanism, bufalin may increase vasoconstriction, and be used as a treatment for heart failure in Chinese medicine. Disruption of intracellular calcium homeostasis induces apoptosis in diverse cell types (4). Our previous results have illustrated that bufalin, with digitalis-like activity, is able to induce the increase of intracellular calcium and cell apoptosis in prostate cancer cells, but the detail mechanism remains unclear (5). On the other hand, bufalin is also known as a topoisomerase II inhibitor. Topoisomerase II is a nuclear enzyme that relaxes supercoiled DNA at the time of DNA replication. Topoisomerase II inhibitors, such as etoposide and adriamycin, maintain the complex of topoisomerase II and the 5'-cleaved ends of the DNA resulting in protein-linked DNA double strand breaks (6). According to the mechanism described above, bufalin may induce leukemia cell differentiation (7) and apoptosis (8). Furthermore, inhibition of solid tumor growth, endothelial cell proliferation, and angiogenesis caused by bufalin in vitro have also been reported (9). Since inhibitors of topoisomerase and Na\(^+\)-K\(^+\)-ATPase have been demonstrated to induce apoptosis in some cancer cell lines, it is interesting to investigate the effects of bufalin and cinobufagin, another major component of Chan-Su, in androgen-dependent, LNCaP, and androgen-independent, DU145 and PC3, prostate cancer cell lines.

Inducing cell apoptosis has been the target mechanism for chemotherapeutic drugs to treat a
variety of cancer. Caspase cascade is a well known key pathway in the apoptotic signal transduction. Caspases are normally present in the cell as zymogens (procaspases), and could be divided into two types of subfamilies: upstream initiator caspases (caspase 8 and 9), which are involved in regulatory events, and downstream effector caspases (caspase 3, 6, and 7), which are directly response for the change of cell morphological events (10). There are two major apoptotic pathways on the upstream of caspase cascade to date, which are the intrinsic pathway (the mitochondria) and the extrinsic pathway (the cell surface receptors) (11). Since bufalin is an inhibitor of topoisomerase II, it may induce DNA damage and activate mitochondrial pathway to regulate cell apoptosis. Bcl-2 family may be divided into two functional subfamilies such as pro-apoptotic proteins (Bax and Bid) and anti-apoptotic proteins (Bcl-2 and Bcl-xL). Bcl-2 family members translocate to mitochondria and mediate the membrane potential to induce cytochrome c release. The cytosolic cytochrome c is further involved in the signal transduction of caspase activation and finally causes cell apoptosis.

The major objectives of the present study were to explore the mechanism of antiproliferative effects occasioned by both bufadienolides depended on the measurement of protein expressions of caspases as well as the upstream molecules, Fas, Bax and cytochrome c. Such investigations may, in part, illustrate the antitumor effects of bufalin and cinobufagin in prostate cancer, and help to provide a new treatment for cancer patients.
MATERIALS AND METHODS

Cells and Culture Conditions.

Androgen dependent human prostate carcinoma cell line LNCaP was purchased from Culture Collection and Research Center (CCRC) of Food Industry Research and Development Institute (FIRDI), Taiwan, ROC. Androgen independent prostate cancer cell lines DU145 and PC3 were provided by Dr. C. R. Jan (Department of Medical Education and Research, Veterans General Hospital-Kaohsiung, Kaohsiung, Taiwan, ROC). Cell lines were maintained in RPMI 1640 (Gibco Laboratories, Buffalo, Grand Island, NY, USA)(LNCaP) or in Dulbecco’s Modified Eagle’s Media (DMEM, Gibco Laboratories, Buffalo, Grand Island, NY, USA)(DU145 and PC3) with 50 IU/ml potassium penicillin G (Sigma, St. Louis, MO, USA), 50 IU/ml streptomycin sulfate (Sigma, St. Louis, MO, USA) and 10% fetal calf serum (FCS, PAA, Pasching, Austria) as standard media in an atmosphere of 5 % CO$_2$ at 37°C.

Immunoblotting Assessment.

After culture under the treatment of bufalin or cinobufagin at certain time points, cells were harvested and washed twice by ice-cold PBS. Cells were lysed in RIPA buffer (50 mM Tri-HCl, pH 7.4, 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na-orthovanadate, 1 mM NaF) for 30 min on ice. The lysate was centrifuged at 10,000×g in 4°C for 15 min, and the supernatant was collected. Equal amount of cell extract proteins (50-100 μg) was subjected to 15% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH, USA). Membranes were incubated in blocking solution (5% dry milk in TBST containing 20 mM Tris-HCl, 135 mM NaCl, 0.1 % Tween 20, pH 7.6) followed by incubation with primary antibody overnight. The following primary antibodies were used as 1 μg/ml: caspase 3 from Imgenex (San Diego, CA, USA); or β-actin from Sigma (St. Louis, MO, USA). After washing for three times by TBST, the blot was incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:20,000, Promega Corporation, Madison, WI, USA), and proteins were visualized using
enhanced chemiluminescence detection (ECL, Western blotting reagents, Amersham International, Bucks, UK).

**Isolation of Cytosolic and Mitochondrial Fractions.**

Release of cytochrome c from mitochondria and translocation of Bax to mitochondria were measured by immunoblotting as described previously (12). Briefly, drug treated cancer cells were collected and washed twice by ice-cold PBS. The cell pellet was resuspended in 1 ml of extraction buffer (20 mM K⁺-Hepes, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂ · 6H₂O, 0.1 mM EDTA, 1 mM EGTA, and protease inhibitors). Cells were lysed by 40 passages through a 26-gauge needle, and homogenates were centrifuged at 1000 x g for 5 min. The supernatant was again centrifuged at 10,000 x g for 15 min following the separation of supernatant (cytosolic fraction) from pellet (mitochondrial fraction). Cytosolic fraction was then concentrated to 50-100 μl using centrifugal concentration device for 10-kDa molecular mass (microcon YM-10, Millipore Co., Bedford, MA, USA) according to the manufacturer’s instructions. The resulting mitochondrial pellets were resuspended in 50 μl of cell lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluride, and protease inhibitor mixture). These fractions were separated on 15% SDS-PAGE with an equal amount of protein loaded onto each lane as determined by Bradford assay reagent. Cytochrome c and Bax were detected by mouse monoclonal antibody at dilution of 1:400 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Each experimental condition was repeated by three times.

**Isolation of Nucleus Fraction.**

The expression of Bax in nucleus was examined by Western blot. After treatment for specific time point, cells were harvested and washed twice by saline. Cells then incubated in hypotonic buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂ · 6H₂O, 1 μl/ml PMSF, 1μl/ml aprotinin, pH 7.9) for 10 min on ice. After incubation, buffer B (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂ · 6H₂O, 2.5% NP-40, pH 7.9) was further added in cell-suspended buffer A. After centrifugation at 1000 x g for 10 min, the supernatant was removed, and the nucleus pellet was homogenized in buffer C (20 mM
HEPES, 0.45 M NaCl, 1 mM EDTA, pH 7.9) for 20 min on ice. The homogenate was centrifuged at 10,000 x g for 15 min. Equal amount of nucleus extracts were subjected to 15% SDS for detecting the expression of Bax in nucleus.

**Cell Surface Fas Analysis.**

The expression of Fas was analyzed using flow cytometry. Cells were exposed to medium with or without $10^{-5}$ M of bufalin or cinobufagin for 24 h (LNCaP and DU145) or 36 h (PC3). Harvested cells were washed twice by 1mM EDTA PBS. Cells were then washed twice by flow cytometric buffer (PBS containing 1% FCS and 0.01% sodium azide). Resuspended cells incubated with mouse anti-human Fas monoclonal antibody (1 ug/ml, BD Transduction Laboratories, Franklin Lakes, NJ, USA) or with anti-mouse IgG as negative control at 4°C for 2 h. After washing twice, cells were further incubated with FITC-conjugated rat anti-mouse IgG (10 ug/ml, eBioscience, San Diego, CA, USA) in the dark at 4°C for 2 h. After washing twice again, cells were analyzed by flow cytometry (13).

**Statistics.**

All values are given as the mean ± standard error of the mean (SEM). In some cases, Student’s $t$ test was used. In others, means were tested for homogeneity by one-way analysis of variance (ANOVA), and the difference between specific means was tested for significance by Duncan’s multiple-range test (14). The difference between two means was considered statistically significant when $P<0.05$. 
RESULTS

Induction of Bufalin and Cinobufagin on Bax Translocation and Cytochrome c Release.

Bufalin and cinobufagin inhibit the proliferation of prostate cancer via caspase activations (5). Alterations of mitochondria leading to the release of cytochrome c are thought to mediate caspase activation in many models of cell death programs. The role of mitochondria is crucial participants in cell apoptosis by targeting Bcl-2 family members to mitochondrial outer membrane (11). We used Western blotting to analyze the protein levels of nucleus and mitochondrial Bax, a proapoptotic molecule in Bcl-2 family, and cytosolic cytochrome c. Consistent with this mechanism, treatment of bufalin resulted in a reduction of Bax in nucleus fraction, and in an elevation in mitochondrial fraction in three types of prostate cancer cell lines (Fig. 1). The release of cytochrome c into cytosolic fraction was also found in three prostate cancer cell lines after bufalin or cinobufagin treatment (Fig. 2). Significant results were found at time points 12 h for LNCaP cells (Fig. 1A, 2A) and DU145 cells (Fig. 1B, 2B); and at 24 h for PC3 cells (Fig. 1C, 2C). The increase of cytochrome c release and Bax translocation is in a dose-dependent manner.

p53 might be involved in Bufalin- and Cinobufagin-Induced Apoptosis in Prostate cancer cells with wild p53.

p53 mediates cell apoptosis-stimulated by many chemotherapeutic drugs. To examine whether p53 manipulates cell apoptosis after treatment of bufalin and cinobufagin, protein expression of p53 in whole cell lysate was analyzed by Western blot in three prostate cancer cells. Figure 3 shows that the expression of p53 is elevated by the treatment of bufalin or cinobufagin in LNCaP cells with wild type p53. These were time- and dose-dependent effects. There was no increase of p53 expression in DU145 or PC3 cells which had mutated or null p53 (data not shown).

Fas-Mediated Apoptotic Signals can be Executed by Bufalin and Cinobufagin in p53-Wild or –Mutated Prostate Cancer Cells.

Fas, a death receptor on cell surface, is activated by death stimuli to induce cell apoptosis.
Previous results show that p53 may be involved in the signal transduction of cell apoptosis induced by bufalin or cinobufagin in LNCaP cells which have wild type p53. To investigate whether Fas mediates cell apoptosis induced by bufalin or cinobufagin in prostate cancer cells with mutated or null p53, flow cytometry was used to examine the expression of Fas on the cell surface. In figure 4, 5, and 6, the percentage of Fas expression on the cell surface was elevated after treatment for 24 h (LNCaP and DU145) or 36 h (PC3). The increase amount of Fas was more significant on DU145 (from 30% in control to about 50% in treated group) and PC3 (from 6% to about 20%) cells than on LNCaP (from 20% to about 27%) cells.

**Extracellular Calcium might not be the Main Factor of Bufalin and Cinobufagin-induced Apoptosis in Prostate Cancer Cells.**

Lin, et al. (15) suggests that cell apoptosis induced by digoxin may inhibit by reducing the extracellular calcium after EGTA treatment. Bufalin is a digitalis-like molecule. To examine whether extracellular calcium manipulates the bufalin- and cinobufagin-induced apoptosis, the expression of active caspase 3 was analyzed by Western blot after co-treatment of bufalin or cinobufagin with EGTA. Figure 7 shows that EGTA alone couldn’t activate caspase 3 (lane 2) after treatment for 12 h (LNCaP, 7A and DU145, 7B) or 24 h (PC3). Bufalin and cinobufagin elevated the expression of active caspase 3 (lane 3, 4), but after co-treatment of bufalin or cinobufagin with EGTA, the expressions of active caspase 3 were still increased (lane 5, 6) in comparison with vehicle group (lane 1).
DISCUSSION

We had demonstrated that (a) mitochondrial pathway was involved in the apoptosis induced by both bufadienolides via releasing cytochrome \( c \) from mitochondria to cytosol, and the proapoptotic Bcl-2 family member, Bax, manipulates these phenomena, (b) both bufadienolides induced elevated the expression of p53 in LNCaP cells which has wild type p53, (c) the expressions of Fas on the cell surface were increased more significant in DU145 and PC3 cells than in LNCaP cells after treatment of bufalin or cinobufagin, and (d) EGTA couldn’t block the expression of active caspase 3 stimulated by both bufadienolides.

Prostate cancer is a worldwide malignant and age-related cancer in men. The nature history of prostate cancer follows a formation of progression from localized disease with androgen-dependent characters to a more accelerated, invasive, and metastatic disease which often connects with gain of androgen-refractory capacity (16). Men with metastatic prostate cancer are prospected to survive for six to nine months by means. Therefore, it is essential to develop an efficacious therapy targeting to the characters of prostate cancer and to eliminate the ravage cause by prostate cancer in men.

Bufalin is a digitalis-like molecule of animal source. The capability of bufalin to inhibit Na\(^+\)-K\(^+\)-ATPase is greater than that of ouabain which is the most effective molecule among digitalis in inducing the antiproliferative effects on prostate cancer cells (17, 18). It is known that prostate cancer cells express the Na\(^+\)-K\(^+\)-ATPase, which plays as a target for digitalis-like drugs (19). As a Na\(^+\)-K\(^+\)-ATPase inhibitor, bufalin brings about the increase of intracellular calcium in prostate cancer cells. Role of intracellular calcium has been studied in prostate cells. Androgen ablation leads to apoptosis associated with calcium influx and calcium-regulated endonuclease activation in normal prostate cells (20). Besides, thapsigargin, a calcium ionophore, directly stimulates an increase of intracellular calcium, which triggers apoptosis in androgen-dependent and androgen-refractory prostate cancer cells (21). In addition, bufalin also functions as an inhibitor of topoisomerase II. Topoisomerase II is the target of several antitumor drugs, such as etoposide, adriamycin, genistein, and ICRF-193 (22). By blocking the enzyme function of topoisomerase II, DNA double strand breaks remain, and DNA damage occurs. Cells are able to recognize such DNA damage and to
eliminate the injured cells by apoptosis.

Based on either increase intracellular calcium or stimulating DNA damage, bufalin may cause cell apoptosis. In the present study, bufalin and cinobufagin were employed to investigate the antiproliferative effects and apoptotic signaling induced by both bufadienolides in androgen-dependent and -independent prostate cancer cells. It has been reported that DNA damage might induce Bax translocation to mitochondria, in turn, resulted in release of cytochrome c (6, 23). Our results showed that bufalin stimulated Bax translocation from nucleus to mitochondria (Fig. 1). Furthermore, cytosolic cytochrome c was also elevated by the treatment of bufalin and cinobufagin (Fig. 2). These results indicated that mitochondrial pathway was involved in the signal transduction of bufalin- and cinobufagin-induced apoptosis.

DNA damage caused by topoisomerase II inhibitor might induce the phosphorylation of p53. The active form of p53 prompted Bax translocation to mitochondria and cytochrome c release to cytosol (6, 23). Figure 3 shows that the expression of p53 was increased in LNCaP cells after treatment of bufalin and cinobufagin. The mechanism described above might be the signal transduction caused by bufalin and cinobufagin in LNCaP cells which had wild type p53.

In DU145 and PC3 cells, with the mutant and null p53, c-Jun NH2-terminal kinas (JNK)-dependent Fas activated apoptosis may replace the p53-dependent apoptotic pathway (13, 21). In Fas-manipulated apoptotic pathway, activated caspase 8 can directly activate the downstream caspase 3, and it can also cross talk with mitochondrial pathway through cleavage of Bid, a Bcl-2 family protein. Truncated Bid (tBid) translocates to mitochondria, inducing cytochrome c release, which sequentially activates caspase 9 and 3 (24). Our results also indicated that Fas mediated the bufalin- and cinobufagin-induced apoptosis in prostate cancer cells (Fig. 4, 5, and 6). The elevations of Fas were found more significant in androgen-independent prostate cancer cells which had mutant p53. Androgen-dependent LNCaP cells have wild type p53, thus the death stimuli from both bufadienolides may go through either p53-mediated or Fas-mediated apoptotic pathways. This might explain the reason of less expression of Fas on the cell surface of LNCaP cells.

Digitalis, a Na\(^+\)-K\(^+\)-ATPase inhibitor, induces cell apoptosis in androgen-dependent and -independent prostate cancer cell lines (5, 15). Our previous results indicate that extracellular
calcium may mediate the digitalis-induced apoptosis in prostate cancer cells (15). Caspase activation is involved in the apoptotic signal transduction induced by digitalis or bufalin (5, 15). Figure 7 suggested that eliminating the extracellular calcium couldn’t block the apoptosis induced by both bufadienolides. Although bufalin is one of the digitalis-like molecules, it may have some other mechanism to induce cell apoptosis, such as being an inhibitor of topoisomerase II.

These results indicated that bufalin and cinobufagin induced cell apoptosis in androgen-dependent prostate cancer cells which had wild type p53 through p53 or Fas, or in androgen-independent prostate cancer cells which had mutant p53 through Fas. Bax translocation and cytochrome c release were involved in p53- or Fas-mediated apoptotic pathway after treatment of bufalin or cinobufagin.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1 Effects of bufalin on the protein expression of Bax translocation in LNCaP, DU145, and PC3 cell lines. Cells were incubated with bufalin for 12 h (LNCaP and DU145) or 24 h (PC3). Cell lysates were separated in nucleus and mitochondrial fractions and analyzed by 15% SDS-PAGE. A, LNCaP cells; B, DU145 cells, C, PC3 cells. Each lane was loaded with 100 ug protein. Similar results were obtained in three other experiments.

Fig. 2 Effects of bufalin or cinobufagin on the protein expressions of cytosolic cytochrome c in LNCaP, DU145, and PC3 cell lines. LNCaP (A) and DU145 (B) cells were incubated with bufalin or cinobufagin for 12 h, whereas, PC3 (C) cells were treated for 24 h. Cell lysates were separated to obtain the cytosolic fraction. Samples were sized in 15% SDS-PAGE and analyzed by Western blotting. Each lane was loaded with 100 μg protein of samples. Similar results were obtained in three other experiments.

Fig. 3 Effects of bufalin or cinobufagin on the protein expression of p53 in LNCaP cells. Cells were incubated with bufalin or cinobufagin for 12 h or 24 h. Cell lysates were sized in 10% SDS-PAGE and analyzed by Western blotting. Each lane was loaded with 100 μg protein of samples. Similar results were obtained in three other experiments.

Fig. 4 Effects of bufalin or cinobufagin on the Fas expression on the cell surface of LNCaP cells. Cells were incubated with bufalin or cinobufagin for 24 h. Cells were washed and incubated with mouse anti-human Fas monoclonal antibody or with anti-mouse IgG as negative control at 4°C for 2 h. After washing twice, cells were further incubated with FITC-conjugated rat anti-mouse IgG and analyzed by flow cytometry. Similar results were obtained in three other experiments.

Fig. 5 Effects of bufalin or cinobufagin on the Fas expression on the cell surface of DU145 cells.
Cells were incubated with bufalin or cinobufagin for 24 h. Cells were washed and incubated with mouse anti-human Fas monoclonal antibody or with anti-mouse IgG as negative control at 4°C for 2 h. After washing twice, cells were further incubated with FITC-conjugated rat anti-mouse IgG and analyzed by flow cytometry. Similar results were obtained in three other experiments.

Fig. 6 Effects of bufalin or cinobufagin on the Fas expression on the cell surface of PC3 cells. Cells were incubated with bufalin or cinobufagin for 36 h. Cells were washed and incubated with mouse anti-human Fas monoclonal antibody or with anti-mouse IgG as negative control at 4°C for 2 h. After washing twice, cells were further incubated with FITC-conjugated rat anti-mouse IgG and analyzed by flow cytometry. Similar results were obtained in three other experiments.

Fig. 7 Effects of co-treatment of bufalin or cinobufagin with EGTA on the protein expression of caspase 3 in LNCaP, DU145 and PC3 cells. Cells were incubated with bufalin or cinobufagin for 24 h (LNCaP and DU145) or 48 h (PC3). Cell lysates were sized in 15% SDS-PAGE and analyzed by Western blotting. Each lane was loaded with 100 μg protein of samples. Lane 1: vehicle; lane 2: EGTA 1 mM; lane 3: bufalin, 5x10⁻⁶ M; lane 4: cinobufagin 5x10⁻⁶ M, lane 5: bufalin+EGTA; lane 6: cinobufagin+EGTA. Similar results were obtained in three other experiments.
Figure 1

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- 45 kDa β-Actin
- 20 kDa Bax
Figure 2

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45 kDa β-Actin
14 kDa Cytochrome c
### Figure 3

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**Notes:**
- 53 kDa
- 45 kDa
Figure 4

Cell type
Incubation time
Anti-Fas antibody

LNCaP
24 h

Vehicle

Bufalin
(10⁻⁵ M)

Cinobufagin
(10⁻⁵ M)

cell number

Log FITC

4.74%

21.87%

31.36%

26.28%
Figure 5

Cell type  
Incubation time  
Anti-Fas antibody  
Vehicle  
Bufalin (10^{-5} M)  
Cinobufagin (10^{-5} M)  
DU145  
24 h  

![Graph showing cell number vs. Log FITC for different conditions and treatments.](image)
Figure 6

Cell type
Incubation time
Anti-Fas antibody

PC3
36 h

--
3.39%

+ 6.61%

Vehicle

Bufalln
(10^{-8} M)

25.21%

Cinobufagin
(10^{-5} M)

16.09%
Figure 7

A: LNCaP

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C: PC3

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</table>

EGTA 1 mM   -   +   -   -   +   +
Bufalin 5X10⁻⁶ M   -   -   +   -   +   -
Cinobufagin 5X10⁻⁶ M   -   -   -   +   -   +