

Original Article

Inhibition of Akt/NF- κ B/survivin pathway by embelin on castration-resistant prostate cancer cells

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Abstract: *Background:* Natural product embelin has been reported to exhibit anti-tumor activities in prostate cancer in preclinical models. While embelin was known to be a natural inhibitor of X-linked inhibitor of apoptosis (XIAP), whether other IAP family members also play a role in drug effects are incompletely understood. In the current study, the cell functions and IAP-related signaling pathways after embelin treatment were investigated in castration-resistant prostate cancer (CRPC) cells. *Materials and methods:* Cell viability was detected by MTT assay. Cell death was determined by trypan blue exclusion. Flow cytometry was applied to analyze cell cycle (propidium iodide, PI staining) and apoptosis (Annexin V/PI double staining). Caspase 3 activity was detected by fluorescence-based assay. Proteins were examined by Western blot. siRNA transfection was conducted using Lipofectamine 2000, and gene expression was tested by qPCR assay. *Results:* Embelin suppressed viability and cell number in CRPC cells, with IC₅₀ of 21.3 and 13.6 μ M in DU145 and PC-3, respectively. Embelin also increased trypan blue positive, Annexin V positive, G1 and sub-G1 cell populations. Further, embelin triggered caspase 3 activation and PARP cleavage, and inhibited XIAP, survivin, I κ B α , p-p65 and p-Akt. Survivin knockdown partially reduced drug-induced apoptosis. In the presence of PI3K inhibitor wortmanin and proteasome inhibitor MG132, embelin-mediated inhibition on cell proliferation and survivin expression was mitigated. *Conclusion:* Embelin inhibits proliferation, induces G1 arrest and triggers apoptosis in CRPC cells. Genetic and pharmacologic approaches identify Akt/NF- κ B/survivin signaling pathway as a possible target pathway for the drug. These new findings provide an alternative mechanism for embelin and suggest this natural medicine as a potential anti-tumor agent for CRPC.

Keywords: Embelin, apoptosis, Akt, NF- κ B, survivin, castration resistant prostate cancer

Introduction

Castration resistant prostate cancer (CRPC) remains a great challenge in the clinic since CRPC patients generally have more aggressive disease and worse quality of life. One typical feature for CRPC is its resistance to apoptosis, therefore CRPC is well recognized to be resistant to conventional therapies [1].

Inhibitor of apoptosis proteins (IAPs) are a group of anti-apoptotic proteins that are highly indicated in prostate cancer. IAP family includes XIAP, cIAP-1, cIAP-2, NIAP and survivin, each of which shares conservative protein domains while with different modifications [2]. Patient data have suggested that XIAP level is strongly associated with a reduced risk of tumor recurrence in prostate cancer, suggesting that it is a

novel prognosticator and a potential target for prostate cancer diagnosis and therapy [3]. Consistent with these findings, other researchers have shown that dysregulated XIAP contributes to the malignant behavior of prostate tumors [4]. In addition, survivin is shown to be correlated with Gleason score, T stage, lymph node metastasis and disease progression in prostate cancer patients [5].

In the apoptosis pathway, IAPs are important signaling components that negatively regulate apoptotic cascades. Specifically, XIAP effectively blocks caspases' activities including caspase 9 and caspase 3/7, therefore prevent cells from apoptosis [6]. By comparison, cIAP-1/2 can also bind to caspases, although the binding is not as strong as XIAP [7]. Additionally, the smallest protein in IAPs, survivin, functions to inhibit

caspsases' activation, thus negatively regulates apoptosis [8]. In terms of cell signaling network, IAPs themselves are regulated by multiple upstream kinases. One essential regulator is Akt kinase that controls expression of XIAP, c-IAPs and survivin, perhaps through NF- κ B [9, 10], since most of IAPs are NF- κ B target proteins [11, 12]. In addition, MAPK/ERK pathway can also modulate XIAP activity therefore protect stress-induced apoptosis [13].

Given the pivotal role of IAPs in cancer, inhibitors targeting IAPs have been actively studied for the sake of blocking IAPs' activities and resensitize tumor cells to apoptosis [14]. Particularly, small molecule inhibitors are widely investigated in various preclinical models [7, 15-17]. These inhibitors include oligopeptides mimicking N-terminal of Smac [7, 15], an endogenous IAP inhibitor released from mitochondria once triggered by apoptotic stimuli [18], and synthetic or semi-synthetic inhibitors [16, 17]. In prostate cancer, Dai et al. reports that a novel synthetic Smac-mimetic compound is able to enhance treatment outcomes of radiation both *in vitro* and *in vivo* [19]. In addition, a novel IAP inhibitor after screening is shown to effectively suppress prostate cancer cell proliferation, and induce apoptosis in multidrug-resistant cancer cells [20].

Embelin is a natural IAP inhibitor that has shown anti-tumor activities in various preclinical models of human malignancies, including cancers of lung [21], pancreas [22] and breast [23]. In preclinical prostate cancer models, embelin has shown effects on growth inhibition, cell cycle arrest and apoptosis induction, migration and invasion suppression *in vitro*, and tumor shrinkage *in vivo* [19, 24]. In addition, embelin is able to potentiate treatment response of prostate cancer cells to conventional therapies such as hormone therapy [25] and radiation therapy [19].

Mechanistically, although several signaling pathways have been elucidated for explaining embelin-mediated tumor suppressing effects [21, 26-28], embelin is originally demonstrated to directly block XIAP therefore exerting proapoptotic activity [24]. However, to date whether other IAP family members also play a role in drug efficacy are incompletely understood. In addition, treatment effects of this natural product in specifically CRPC cell models have not

been studied. In the current study, cell functions and expression of IAP members after embelin treatment were investigated in CRPC cells. Further, upstream signaling pathways that control identified IAP(s) are identified and validated using genetic and pharmacological methods.

Material and methods

Reagents

Embelin was purchased from Selleckchem (Houston, TX, USA). The powder was reconstituted in dimethyl sulfoxide (DMSO) with a stock concentration of 50 mM and stored at -20°C. All culture medium and supplemental reagents including trypsin and EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials (Hangzhou, China). Antibodies against poly (ADP-ribose) polymerase (PARP), cleaved PARP, XIAP, cIAP-1, cIAP-2, survivin, Tubulin and Actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Caspase 3, cleaved Caspase 3, I κ B α , p-p65 (S536), p65, p-Akt (S473) and Akt were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies were obtained from Boster Biological Technology (Wuhan, China). Wortmanin, MG132, protease inhibitors and other chemicals were from Sigma unless otherwise indicated.

Cell culture

CRPC cell lines PC-3, DU145 and C4-2B purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI-1640 (PC-3) or DMEM (DU145, C4-2B) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml), and incubated under 5% CO₂ at 37°C. The cells were passaged once every 3 days.

MTT assay

The cells were seeded into 96-well culture plates and incubated for 72 h after drug treatment. DMSO was used as an untreated control. The cells were assayed by adding 100 μ L/well of growth medium containing 1 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) for 4 h at 37°C in a CO₂ incubator. After the incubation, the MTT solu-

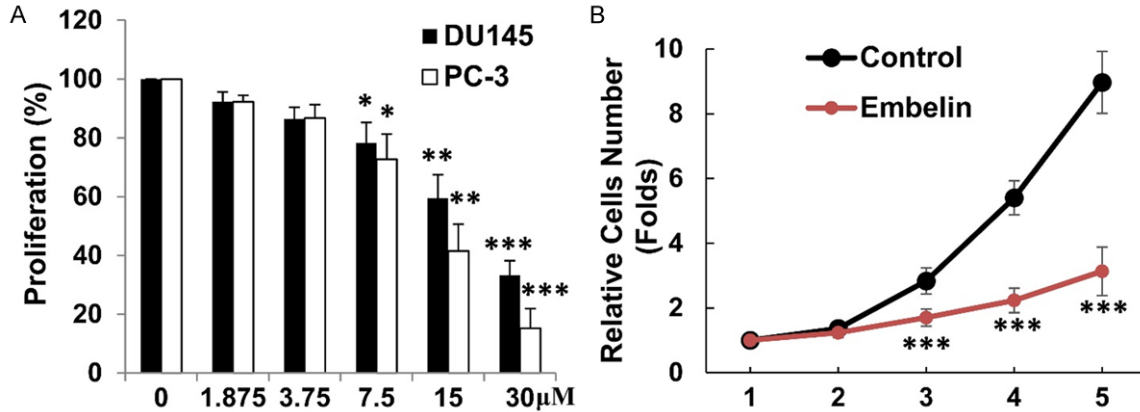


Figure 1. Effects of embelin on cell viability and proliferation in CRPC cells. A: DU145 and PC-3 cells were treated with embelin for 3 days. Cell viability was determined by MTT assay. Column, mean; Bars, SD ($n=3$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to DMSO control (t-test). B: C4-2B cells were treated with DMSO control or embelin (20 μ M). Every 24 hours, cells were trypsinized and mixed with trypan blue. Living cells exclusive from trypan blue were counted by hemocytometer. Relative cell number was calculated by dividing the counted number at each time by the seeding number on day 1. Bars, SD ($n=3$). ***, $P<0.001$ compared to Control in the same day (t-test).

tion was removed, and the cells were resuspended in 200 μ L of DMSO. The absorbance values were measured at 570 nm and were normalized to the corresponding control. The experiments were conducted in triplicate.

Cell proliferation and cell death assay

Cell proliferation was monitored by growth curve assay. Briefly, cells were seeded in a 24-well plate with equal density (10^4 /well) and treated with embelin. Viable cells were determined by trypan blue exclusion were counted at 24-h intervals over 4 days using a Coulter cell counter (Beckman). Cell death were characterized by trypan blue positive staining. Percentage of cell death was defined by the ratio of trypan blue positive cells with total cells.

Cell cycle

The cells were harvested and washed once with PBS. After centrifugation, the cells were fixed in 70% ice-cold ethanol overnight at -20° C. After a PBS wash, the cells were incubated in 500 μ L sample buffer containing 50 mg/L propidium iodide (PI) and 100 mg/L RNase for 30 min. The analysis of the apoptotic cells was performed on the flow cytometer (FACSCalibur, BD Biosciences). sub-G1 population was defined as hypodiploid populations in the cell cycle histogram. Data were analyzed using WinMDI 2.8 software (Purdue University Cytometry Laboratory).

Annexin V/PI staining

Flow cytometry was used to analyze cell apoptosis through Annexin V/PI staining and 2) sub-G1 analysis. Briefly, the cells were harvested and washed twice with ice-cold PBS. For 1), apoptotic cells that present phosphatidylserine on their outside surface were captured by Annexin V-FITC agent (KeyGen Biotech). Annexin V-FITC (1 μ l) was added to 500 μ l of Annexin V-FITC binding buffer, and the cells were incubated at room temperature for 30 min. After washing the cells with 1 \times binding buffer, 10 μ l propidium iodide (PI) was added to the binding buffer, and the cells were analyzed with a flow cytometer (FACS Calibur; BD Biosciences).

Western blot analysis

Whole cell lysates were extracted using the radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.5% SDS, 0.5% NP-40, 0.25% Sodium deoxycholate, 2 mM EDTA] and quantified with Bradford assay following the manufacturer's instructions (Bio-Rad, Hercules, CA). After addition of the sample loading buffer, protein samples were resolved on SDS-PAGE gels and subsequently transferred to nitrocellulose membranes (Bio-Rad). Each membrane was blocked and probed with desired primary antibody at 4° C overnight. The membrane was then washed three times using TBST (0.1% Tween 20) and incubated with HRP-conjugated secondary anti-

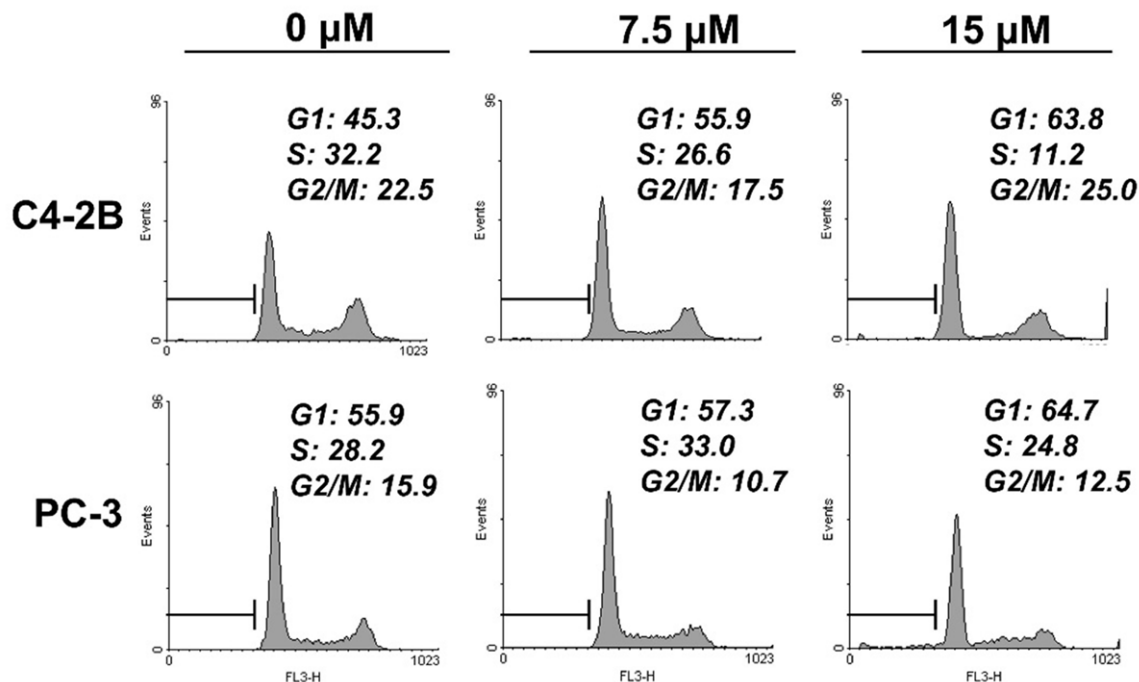


Figure 2. Effects of embelin on cell cycle distribution. C4-2B and PC-3 cells were treated by embelin (7.5 and 15 μ M) for 48 h. Control cells were treated by DMSO. Cells were harvested and stained with PI and processed for flow cytometry. Cell populations in each cell cycle phase were scored.

body at room temperature for 1 h. The immunoreactive protein was visualized using the chemiluminescent reagent ECL (Pierce, Rockford, IL).

Caspase 3 assay

Caspase 3 activation was determined using a fluorescence kit from Cayman Chemical (Ann Arbor, MI). Briefly, cells were dissolved in a lysis buffer (HEPES, pH 7.5, 50 mM; NaCl, 100 mM; EDTA, 1 mM; DTT, 5 mM; Triton X-100, 0.5%; Glycerol, 4%). Cell lysates were incubated with a fluorogenic substrate (Cayman Chemical) in a reaction buffer (containing 5 mM DTT) at 37°C for 2 h. Proteolytic release of the highly fluorescent product was monitored at λ_{ex} = 485 nm and λ_{em} = 535 nm using a microplate reader (Molecular Device, Sunnyvale, CA). Fluorescence signal increase was obtained by dividing the normalized activity of the treated sample with untreated control and expressed as "Fold" increase.

Transient transfection

Survivin siRNA and control scramble siRNA were purchased from GenePharma (Shanghai, China). siRNA oligonucleotides were transfected

into cells using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Transfected cells were immediately used for future experiments.

Quantitative RT-PCR analysis

Total RNA were extracted using Trizol (Invitrogen) following the manufacturer's instructions. Reverse transcription reaction with 1 μ g of total RNA in 100 μ l was done using TaqMan Reverse Transcription Kit (Applied BioSystems). For quantitative RT-PCR (qRT-PCR) reactions, SYBR Green-based reaction kit (Applied BioSystems) was used as an internal control. Q-PCR was performed with a Real-time PCR System (Eppendorf International, Germany). The sequences of the primers for *BIRC5*, *VEGF* and *ACTIN* were as follows: *BIRC5*, 5'-TGCCTGGCAGCCCTTTC-3' (forward) and 5'-CCTCCAAGAAGGGC-CAGTTC-3' (reverse); *VEGF*, 5'-ATACTCATGACCA-TCGCGCTAG-3' (forward) and 5'-AAAGAAGGC-CACAAAGCCAAAG-3' (reverse); *ACTIN*, 5'-CTCC-TCCTGAGCGCAAGTACTC-3' (forward) and 5'-TCCTGCTTGCTGATCCACATC-3' (reverse). The mRNA level of *BIRC5* and *VEGF* was normalized to the level of *ACTIN* and expressed as the relative expression (folds).

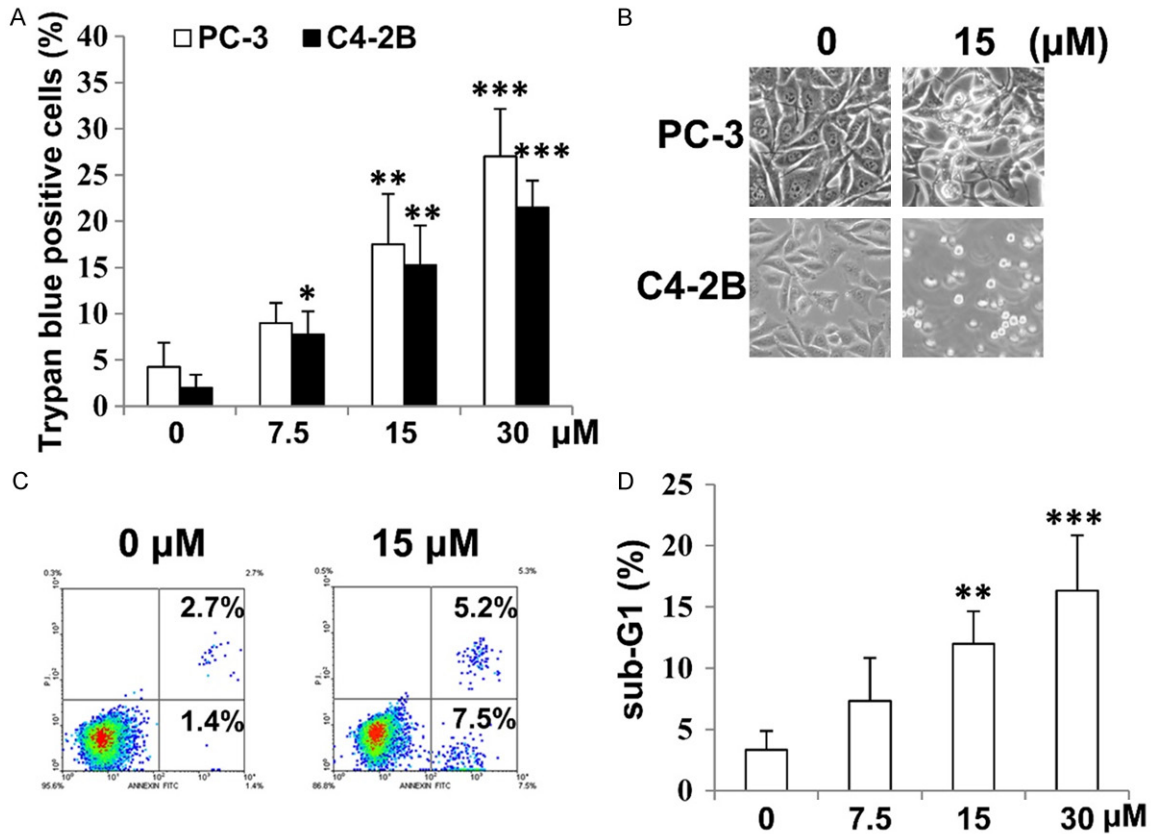


Figure 3. Embelin induces apoptosis in CRPC cells. A: Dead cells were scored as trypan blue positive populations in PC-3 and C4-2B. Cell death (%) was calculated by the ratio of dead cells with total cells. Columns, mean; bars, SD ($n=3$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to DMSO control (t-test). B: Cell morphology after embelin (15 μ M) treatment for 72 h. Original magnification, 200 \times . C: C4-2B cells were treated with embelin (15 μ M) for 24 h. Cells were subjected to Annexin V/PI staining followed by flow cytometry. Early (Annexin V+/PI+) and late (Annexin V+/PI-) apoptotic cells were scored. Representative images are shown. D: PC-3 cells were treated with varied concentrations of embelin for 72 h and presented to flow cytometry to detect sub-G1 populations. Columns, mean; bars, SD ($n=3$). **, $P<0.01$; ***, $P<0.001$ (t-test).

Statistical analysis

Experiments were conducted in triplicate. Data were analyzed using two-tailed Student's *t*-test. *P*-values were considered significant at <0.05 .

Results

Embelin inhibits CRPC cell proliferation

DU145 and PC-3 cells were treated with embelin (start from 30 μ M, 2-fold dilution) for 72 h and MTT assay was used to assess cell proliferation. As shown in **Figure 1A**, embelin inhibited proliferation in a dose-dependent manner, and the effects became significant at concentrations greater than 7.5 μ M. Using non-linear regression analysis, the IC_{50} was 21.3 μ M and 13.6 μ M for DU145 and PC-3, respectively. For another CRPC line C4-2B, cells were treated

with 20 μ M of embelin for up to 5 days, and cell number was scored every day. As expected, embelin significantly inhibited cell proliferation starting from day 3 (**Figure 1B**). These data suggests that embelin effectively suppresses CRPC cell proliferation.

Embelin induces cell cycle arrest in CRPC cells

C4-2B and PC-3 cells were treated with embelin (7.5, 15 μ M) for 48 h. Flow cytometry analysis indicated that embelin induced G1 arrest dose-dependently in both cells, although such effects were slightly weaker in PC-3 than in C4-2B cells (**Figure 2**).

Embelin triggers apoptosis in CRPC cells

PC-3 and C4-2B cells were exposed to embelin for 72 h. Trypan blue staining indicated that

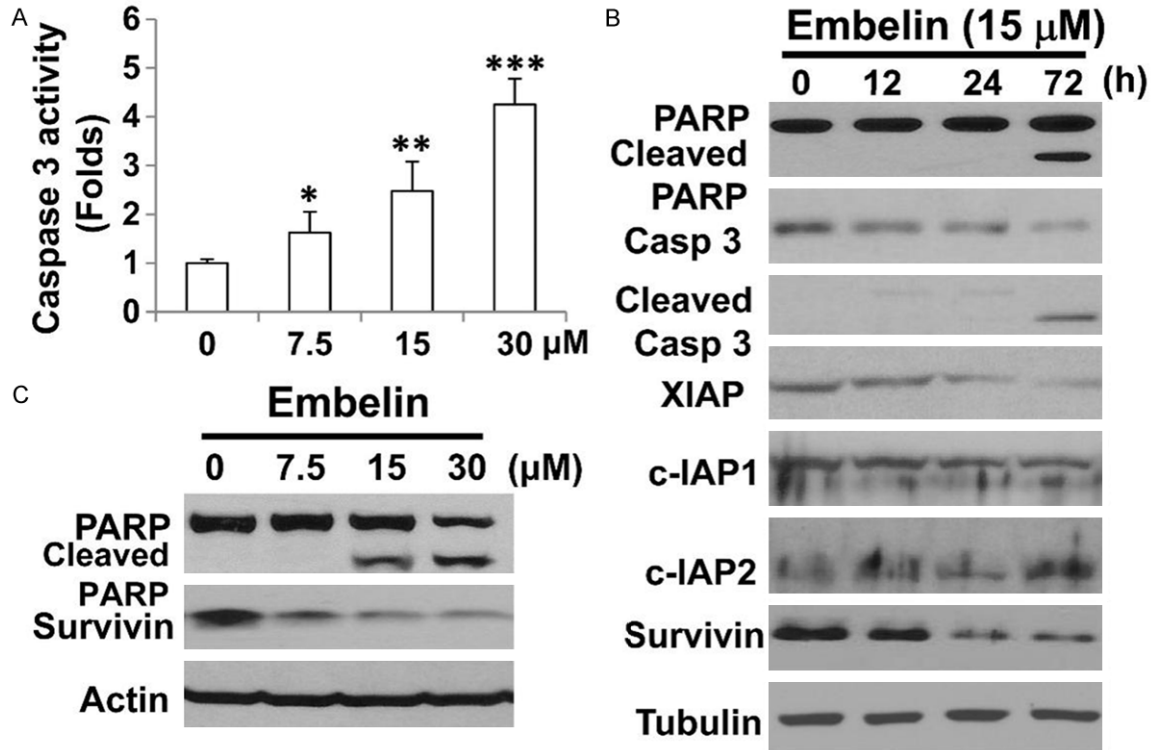


Figure 4. Molecular markers of apoptosis by embelin. A: PC-3 cells were treated with embelin for 72 h. Caspase 3 activity was detected by fluorescence assay. Fold of enzymatic activity was calculated by dividing the fluorescent signal in the treated sample by untreated control. Columns, mean; bars, SD ($n=3$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (t-test). B: PC-3 cells were treated with 15 μM of embelin for 12, 24 and 72 h. Caspase 3, PARP and IAP family proteins were analyzed by Western blot. Tubulin is a loading control. C: C4-2B cells were treated with varied concentrations of embelin for 72 h. PARP and survivin expression was analyzed by Western blot. Actin is a loading control.

embelin induced cell death in a dose-dependent manner (Figure 3A). At lower dose (7.5 μM), the induction was more significant in C4-2B than in PC-3, while at high dose (30 μM), embelin showed cell-killing effects in both cells (Figure 3A). These data were supported by morphological images that 72 h drug treatment resulted in clearly more floating cells (Figure 3B). To further detect whether embelin-induced cell death is apoptosis, after drug treatment (15 μM) for 48 h, C4-2B cells were subjected to Annexin V/PI double staining followed by flow cytometry. Representative diagrams in Figure 3C showed that drug treatment clearly increased Annexin V positive populations, indicating the enhancement of both early and late apoptosis. Similarly, sub-G1 test by flow cytometry after 72 h drug treatment showed that embelin enhanced hypodiploid cell population dose-dependently (Figure 3D). At the highest dose (30 μM), the sub-G1 population was ~5 times of that in the untreated control (Figure 3D).

These data suggest that embelin efficiently induces cell death and apoptosis in CRPC cells.

Embelin modulates apoptosis pathway

To determine the molecular signatures of apoptosis, PC-3 cells were treated with embelin with varied concentrations for 72 h and cell lysates were presented to detect activity of caspase 3, a typical hallmark located at the downstream of apoptotic cascades. Data in Figure 4A showed that consistent with sub-G1 change (Figure 3D), the drug dose-dependently facilitated caspase 3 activation, with the fold increase comparable to sub-G1 data (Figure 4A). Further, PC-3 cells were treated with 15 μM of embelin for 12, 24 and 72 h, and whole cell lysates were analyzed by Western blot to detect proteins involved in apoptotic pathways. Embelin clearly induced cleavage of caspase 3 and PARP (a typical substrate of caspase 3) at 72 h treatment (Figure 4B) that is in line with caspase 3

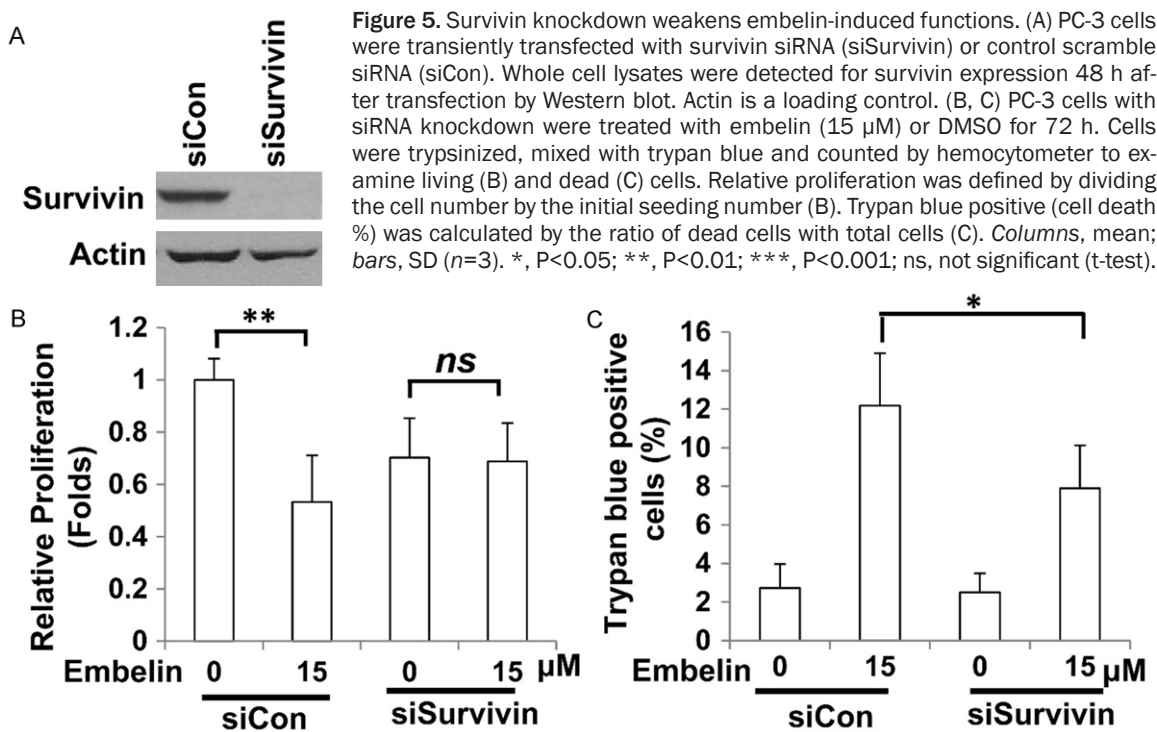


Figure 5. Survivin knockdown weakens embelen-induced functions. (A) PC-3 cells were transiently transfected with survivin siRNA (siSurvivin) or control scramble siRNA (siCon). Whole cell lysates were detected for survivin expression 48 h after transfection by Western blot. Actin is a loading control. (B, C) PC-3 cells with siRNA knockdown were treated with embelen (15 μM) or DMSO for 72 h. Cells were trypsinized, mixed with trypan blue and counted by hemocytometer to examine living (B) and dead (C) cells. Relative proliferation was defined by dividing the cell number by the initial seeding number (B). Trypan blue positive (cell death %) was calculated by the ratio of dead cells with total cells (C). Columns, mean; bars, SD (n=3). *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant (t-test).

functional activation (Figure 4A). Interestingly, when testing IAP family proteins, besides XIAP, embelen also inhibited survivin expression after 24 h treatment, while no inhibition was observed for cIAP-1 and cIAP-2 (Figure 4B). Similar data were seen in C4-2B cells that 72 h drug treatment resulted in clear PARP cleavage, as well as survivin inhibition (Figure 4C). These data suggested that embelen was able to trigger downstream effectors caspase 3/PARP breakdown, and more importantly, suppress anti-apoptotic protein survivin.

Survivin silencing partially attenuates embelen-induced cell functions

To determine whether survivin could be a possible target for embelen, genetic modifications were applied to silence survivin expression by siRNA transfection. By 24 h post-transfection, survivin was depleted in PC-3 cells introduced by survivin siRNA compared to the scramble control siRNA, indicating the efficient knockdown effects (Figure 5A). Further, knockdown cells were treated with 15 μM of embelen for 72 h to detect cell proliferation and cell death. While embelen significantly reduced proliferation in PC-3 cells with control siRNA, in survivin siRNA-transfected cells, such reduction by the drug was not significant (Figure 5B). Consistently, survivin siRNA modified cells showed sig-

nificantly less cell death than control cells after drug treatment (Figure 5C). These data suggested that blocking survivin is able to impair embelen-mediated anti-tumor functions.

Embelen suppresses Akt/NF-κB pathway

To further identify possible upstream pathways of survivin, Akt and NF-κB signaling molecules were detected, since they have been shown to be involved in regulating survivin expression [12]. Embelen treatment for 24 h resulted in clear reduction of IκBα, p-p65 (S536) and p-Akt (S473) in a dose-dependent manner (Figure 6A). To confirm whether survivin inhibition is a consequence of NF-κB pathway blockade, survivin gene *BIRC5* expression was tested using qPCR, since *BIRC5* is an NF-κB target gene [29]. Figure 6B showed that 24 h drug treatment at 15 μM significantly decreased *BIRC5* expression, which was comparable to the inhibition of the NF-κB target gene marker *VEGF*. These data clearly indicated that embelen is able to inhibit Akt/NF-κB pathway that leads to survivin gene reduction.

Inhibition of Akt/NF-κB pathway blunts embelen-induced cell growth inhibition and survivin reduction

To elucidate whether Akt/NF-κB pathway is necessary for embelen-mediated growth inhibi-

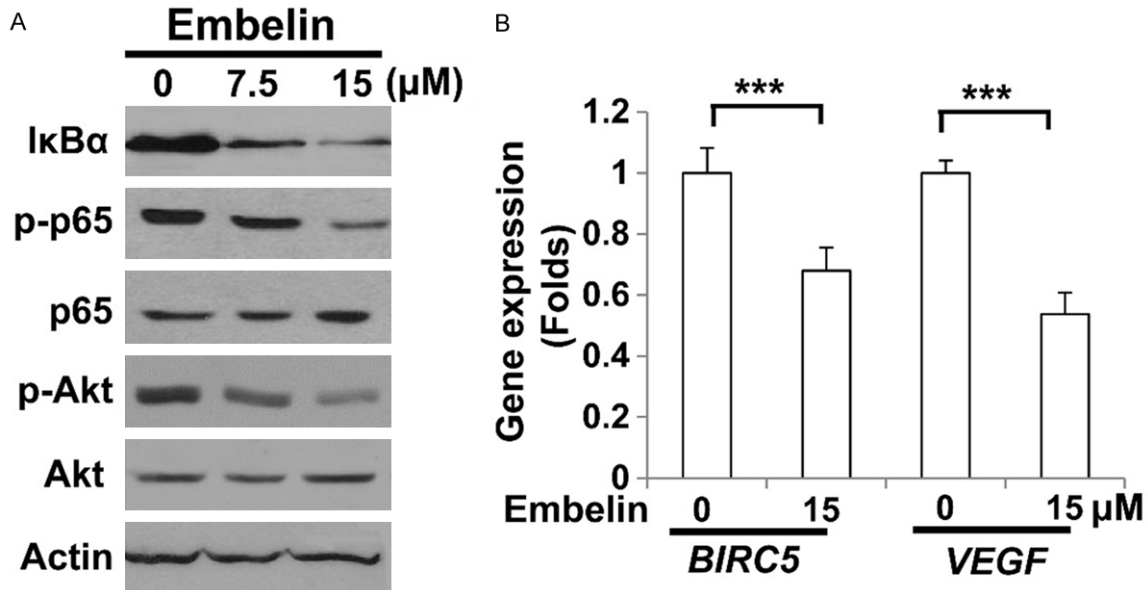


Figure 6. Effects of embelin on Akt/NF-κB pathway. A: PC-3 cells were treated with embelin (7.5, 15 μM) for 24 h and whole cell lysates were analyzed by Western blot. Actin is a loading control. B: PC-3 cells were treated with embelin (15 μM) for 24 h. Expression of *BIRC5* and *VEGF* were detected by qPCR. Columns, mean; bars, SD (n=3). ***, P<0.001 (t-test).

tion and survivin expression, small molecule kinase inhibitors were applied. These inhibitors included PI3K inhibitor wortmanin to block Akt activation, and proteasome inhibitor MG132 to block NF-κB activation. As expected, while embelin alone significantly inhibited cell proliferation, pre-treatment with either of these two inhibitors attenuated drug-induced inhibition (Figure 7A). At the molecular level, consistent with Figures 4B and 6A, embelin alone reduced survivin, p-p65 and p-Akt (Figure 7B), indicating the suppression of Akt/NF-κB/survivin pathway. However in the presence of wortmanin, although p-Akt and p-p65 were inhibited, the inhibitor failed to further reduce survivin (Figure 7B). Similar results were seen by MG-132, where the inhibitor alone was able to only inhibit p-p65, did not further decrease embelin-induced survivin reduction (Figure 7B). These data suggested that the Akt/NF-κB pathway is required for embelin-induced cell functions and survivin expression.

Discussion

CRPC cells are generally less sensitive to cytotoxic agents since pro-survival and anti-apoptotic pathways are commonly upregulated. Therefore, molecular agents designed to specifi-

cally target certain highlighted signaling pathways have been actively under investigation in CRPC. Interestingly, natural products are also intensively studied to treat CRPC in tissue culture and animal models since lead products have shown considerable effects by acting on different molecular targets [30-32].

Embelin has been reported as a natural product that shows potential anti-tumor efficacies for multiple cancer types including prostate cancer in preclinical models. Several cell signaling pathways have also been examined in the prostate cancer cells treated by embelin to highlight possible molecular targets [24, 33, 34]. Among those proteins, XIAP is perhaps the first target identified for this natural product therefore embelin is also named as a “natural IAP inhibitor” [24].

Nevertheless, in this study, we report for the first time that the Akt/NF-κB/survivin may be another important pathway for embelin-mediated effects in a group of CRPC cells. First, at cellular level, we show that embelin effectively inhibits cell proliferation in all 3 CRPC cell lines (Figure 1). Embelin also induces G1 cell cycle arrest (Figure 2) and cell death (Figure 3). Subsequent data demonstrate that such cell

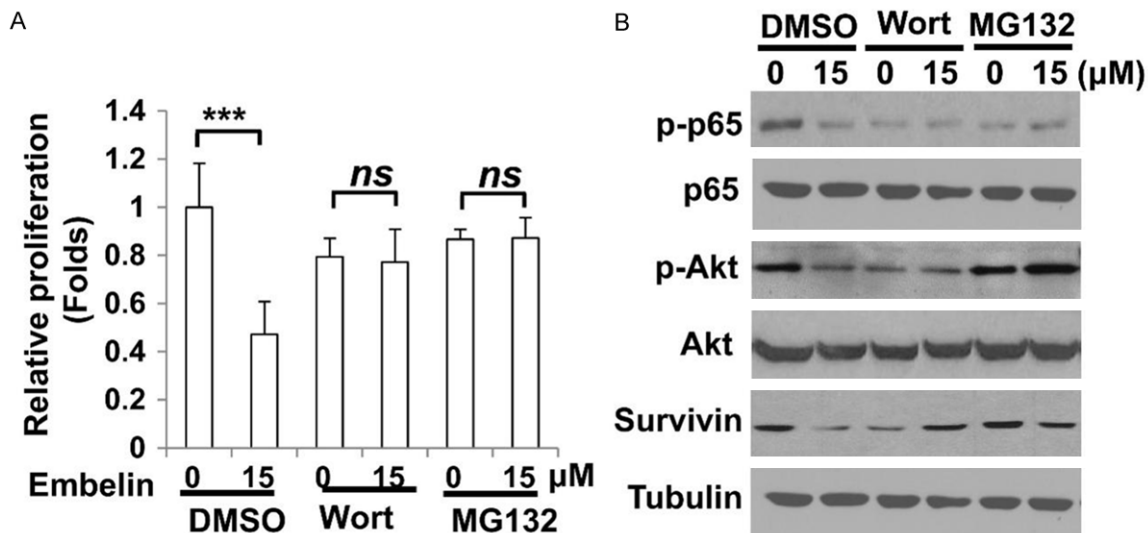


Figure 7. Inhibition of Akt/NF-κB pathway mitigates embelin-induced growth inhibition. PC-3 cells were pre-treated with wortmanin (100 nM) or MG132 (10 μM) for 1 h and then treated with embelin (15 μM) for 72 h (A) or 24 h (B). (A) Cells were trypsinized, mixed with trypan blue and counted by hemocytometer to examine living cells. Columns, mean; bars, SD ($n=3$). ***, $P<0.001$; ns, not significant (t-test). (B) Akt, NF-κB and survivin were analyzed by Western blot. Tubulin is a loading control.

death mode is apoptosis (Figure 3). At the molecular level, embelin inhibits survivin expression (Figure 4), as well as upstream Akt and NF-κB activity (Figure 6). Using siRNA and kinase inhibitors, we show that the Akt/NF-κB/survivin pathway is required for embelin-induced growth inhibition and apoptosis (Figures 5 and 7). Together, these data provide an alternative pharmacological mechanism for the anti-tumor activity of embelin.

Although embelin has been reported to exhibit functional inhibition in prostate cancer cells, to date there lacks specific report evaluating the drug efficacy in CRPC model. Our data are in line with previous studies that the IC_{50} range of embelin in CRPC cells (eg. PC-3) is 10~20 μM [25, 33], confirming the reproducibility of our experimental system. In addition, embelin is able to induce G1 arrest, which is consistent with several previous reports [35, 36], although other studies have different conclusions [19, 37]. Importantly, besides XIAP, we show that survivin is also involved as a molecular target for embelin, which is consistent with previous study [33, 38], suggesting XIAP may not be the only IAPs of the drug target. Further studies are designed to validate survivin as a key target of embelin in CRPC models. Identifying new signaling proteins is encouraging since it offers

possible new therapeutic strategies to interfere these pathways, therefore may hold value for future drug development particularly in CRPC.

NF-κB is a typical oncoprotein that is involved in multiple malignant functions such as survival and drug resistance. Most of IAPs are downstream targets of NF-κB including survivin, while Akt is one of the major upstream regulators of NF-κB [12]. In our study, we have identified Akt/NF-κB signaling as the upstream pathway that is responsible for survivin inhibition by embelin, since small molecule inhibitors targeting this pathway partially abolish embelin-induced cell functions and survivin reduction. It has been shown in the previous study that embelin is able to inhibit Akt phosphorylation and survivin in PC-3 cells [33]. However, the link between these two signaling molecules have not been established in that report. In our study, we connect survivin with Akt and NF-κB as upstream regulators for the first time, therefore clearly identify Akt/NF-κB/survivin as an alternative signaling pathway of embelin, at least in CRPC models. These new findings pave the way for highlighting one more possible molecular mechanism of embelin. It remains to be investigated, however, whether other key pro-survival pathways are also required for regulating survivin. For example, Zhang et al. re-

veals that c-Myc/RAF/MAPK pathway positively modulates survivin in the drug resistance of leukemia cells [39]. Alternatively, wnt/beta catenin is shown to control survivin expression at both transcriptional and translational level [40]. Elucidating other survivin-associated signaling pathways may aid to design possible combination therapy in the future by targeting both pathway components and survivin in order to yield maximum anti-tumor efficacies.

Taken together, our research data show that embelin is able to inhibit proliferation, induce cell cycle arrest and induce apoptosis in CRPC cells. More importantly, Akt/NF- κ B/survivin is a target pathway for embelin-mediated functions. These new findings provide another possible mechanism to explain the anti-tumor effects of embelin, and support embelin as a promising natural medicine for the treatment of CRPC.

Disclosure of conflict of interest

None.

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