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Original Article

F68-*cis*-curcumin inhibits proliferation of DU-145 hormone-independent prostate cancer cells via downregulating PI3K/AKT signaling pathways

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Abstract: Objective: The current study investigated the therapeutic potential of a new flexible acid-responsive micelle formulation for prostate cancer therapy, produced by the covalent conjugation of Cur to the hydrophilic terminals of Pluronic F68 chains through *cis*-aconitic anhydride linkers (F68-*cis*-Cur). Methods: Cell viability assays were conducted using 2,000 cells. They were seeded into 96-well plates and cultured overnight. Cytotoxicity levels of F68-*cis*-Cur were demonstrated in DU145 cells using MTT assays. Absorbance values were measured at 570 nm using a microplate reader. Apoptotic rates of DU145 cells were detected using flow cytometry. Stripe grayscale values of the membranes were measured using the Image lab version. Results: Results of the current study demonstrated that F68-*cis*-Cur micelles notably suppressed proliferation of DU145 prostate cancer cells, in a dose- and time-dependent manner. This growth inhibition was accompanied by G2-phase cell cycle arrest and more prominent induction of apoptosis, compared with free Cur. This formulation also downregulated the phosphorylation of AKT, leading to cellular apoptosis by inhibiting B cell lymphoma-2 expression and upregulated expression of Bax and cleaved poly (ADP) ribose polymerase. Conclusion: F68-*cis*-Cur particles exhibit promising antineoplastic activity. Thus, they may be valuable in the treatment of prostate cancer.

Keywords: Polymer-drug conjugate, curcumin, Pluronic F68, prostate cancer

Introduction

Prostate cancer (PCa) is one of the most frequently diagnosed adult malignancies in developed countries [1]. Androgen deprivation therapy (ADT) is the principal treatment for primary PCa [2]. However, resistance to ADT often develops. Thus, the cancer acquires a castration-resistant phenotype, for which there are no effective therapy methods [3]. Therefore, it is necessary to identify novel agents with better curative efficacy and lower toxicity levels for treatment of castration resistant PCa. Conventional chemotherapy leads to off-target effects. Resistance develops against anticancer drugs. Healthy organ toxicity often ensues [4]. Epidemiological studies have demonstrated that risks and morbidity rates of multiple types of cancer may be reduced by using natural polyphenol compounds in the daily diet [5]. Antitumor natural compounds may be highly valuable for treatment of PCa, characterized by high morbidity and long latency [6]. Moreover, natural polyphenol compounds may be valuable as adjuvant therapy following surgery, chemotherapy, and/or radiation therapy [7].

Of the various natural antitumor agents, curcumin (Cur) is a polyphenolic compound. It has exhibited extensive anticancer efficacy in PCa [8]. Recent studies have reported that Cur plays a key role in cell growth, migration, inva-

sion, apoptosis, and cell cycle regulation by modulating multiple signaling pathways [9, 10]. Over 40 clinical trials are currently investigating the therapeutic efficacy of Cur in several types of cancer. In view of these data, the current study aimed to investigate the potential of Cur, formulated as pills or gel capsules, in either delaying onset or inhibiting the process of carcinogenesis. Despite promising antitumor properties, the therapeutic efficacy of Cur has been limited due to poor aqueous solubility (< 1 μg/mL), rapid degradation in the physiological environment [11], and low bioavailability [12]. Delivery of Cur via nanocarriers is a promising protocol designed to overcome these shortcomings. Cur has been conjugated to various polymers, including methoxy poly (ethyleneglycol)-poly (lactic acid) (mPEG-PLA) [13], mPEG-PLA-Tris [14], poly (D, L-lactic-co-glycolic acid) [15], alginate [16], cholesteryl-hyaluronic acid [17], and Pluronic F68 [18]. One of these strategies, including Cur covalently conjugated to Pluronic F68 block through *cis*-aconitic anhydride linkage micelles, generated via the self-assembly of F68 with Cur covalently conjugated to the polymer chains, has been reported as a potential reagent increasing intracellular drug delivery in tumor therapy.

The current study generated a flexible acid-responsive micelle formulation by covalently conjugating Cur to the hydrophilic terminals of Pluronic F68 chains via *cis*-aconitic anhydride linkers (F68-*cis*-Cur), aiming to enhance intracellular drug release. Furthermore, cytotoxicity and antitumor efficacy levels of this formulation were evaluated.

Materials and methods

Materials

All reagents, solvents, and chemicals were acquired from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). F68-*cis*-Cur (FCC) conjugate, produced by the covalent conjugation of Cur to the hydrophilic terminals of Pluronic F68 chains through cis-aconitic anhydride linkers, was prepared based on a previously published project using the nanoprecipitation method [19]. F68-*cis* (F68-Cis), an empty vector free of Cur, was produced by the covalent conjugation of the hydrophilic terminals of Pluronic F68 chains and cis-aconitic anhydride linkers. It was applied as a control in all experiments.

Cell culturing

DU-145 human PCa cell lines, which exhibit androgen-independent (AI) characteristics, were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA) with 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C under a 5% CO_2 atmosphere. Cell viability assays used 2,000 cells. They were seeded into 96-well plates and cultured overnight. Colony formation assays, using 0.6×10^3 per well, were seeded into six-well plates and incubated for 24 hours. The cells were grouping according to the experiment. The current study was approved by the Ethics Committee of Nanfang Hospital of Southern Medical University.

Cell viability assays

Cytotoxicity levels of F68-*cis*-Cur were demonstrated in DU145 cells using MTT assays. Briefly, 2000 cells were seeded into 96-well plates and cultured overnight. They were incubated with different concentrations of F68-*cis*-Cur for the indicated times with added MTT (20 μl; 5 mg/mL) at 37°C for 2 hours. Subsequently, 150 μl dimethyl sulfoxide was used to dissolve the precipitates. Absorbance values were measured at 570 nm using a microplate reader. Three duplicate wells were created for this experiment.

Colony formation assays

Determining the effects of F68-*cis*-Cur on clonogenic potential, DU145 cells (0.6×10^3 per well) were seeded into six-well plates and incubated for 24 hours. This was followed by the addition of Cur or F68-*cis*-Cur (10 μM) for 2 weeks. The number of colonies was calculated after staining with Giemsa.

Cell cycle analysis

Effects of F68-*cis*-Cur, followed by the addition of Cur (0, 5, 10 μM) or F68-cis-Cur (0, 5, 10 μM), on cell cycle were determined by flow cytometry. Briefly, DU145 cells were harvested and fixed in 70% ice-cold ethanol overnight at 4°C. The cells were washed in ice-cold PBS, then exposed to 125 U/mL RNaseA (Sigma) for

30 minutes at 37°C. Subsequently, the cells were stained with 400 μl propidium iodide (KeyGen, Nanjing, China) for 30 minutes. Cell cycle analysis was conducted using a FACScan flow cytometry analyzer (FACSCalibur, Becton Dickinson). Each experiment was repeated three times.

Cellular apoptosis analysis

Effects of F68-*cis*-Cur, followed by the addition of Cur (0, 5, 10 μM) or F68-cis-Cur (0, 5, 10 μM), on cellular apoptosis were examined with Annexin V-FITC and propidium iodide (PI), according to manufacturer instructions (KeyGen). Apoptotic rates of DU145 cells were detected by flow cytometry (FACSCalibur, BD Biosciences).

Western blotting

Regarding Western blotting, 1×10^6 DU145 cells were seeded in a 10-cm culture dish and exposed to 5-10 μM Cur or F68-*cis*-Cur for 24 hours. Total protein lysates were extracted using radioimmunoprecipitation assay (RIPA) buffer. Cell lysates were then lysed with 2X SDS lysis buffer (Boster Biological Technology, Ltd., Wuhan, China). Equal amounts of protein were loaded and separated by SDS polyacrylamide gel electrophoresis. They were electro-transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked with 5% non-fat milk or BSA in Tris-buffered saline containing 0.05% Tween-20 for 1 hour. The membranes were immunoblotted with primary antibodies against Bax (Acbam, #07856), Bcl-xL (Acbam, #83145), poly (ADP-ribose) polymerase (PARP, #9532), cleaved-PARP1 (#9505), and caspase-3 (#9662) (1:1,000, Cell Signaling Technology, Inc., Danvers, MA, USA). AKT (CST, #2938), p-AKT (CST, #4060), phosphatidylinositol-3 kinase (PI3K), (CST, #4249), and β-actin, for protein internal control, were purchased from Sigma, #A2228. Following incubation with the primary antibodies at 4°C overnight, the membranes were washed. They were then probed with the appropriate secondary antibodies (Boster Biological Technology, Ltd.) for 1 hour at room temperature. All immunoreactive bands were detected with an enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Rockford, IL, USA), in accordance with manufacturer instructions. β-actin was applied as the loading control protein. Stripe grayscale values of the membranes were measured by Image lab version 5.2.1 build 11. The internal control was β-actin.

Statistical analysis

Statistical analyses were conducted using SPSS software (SPSS Standard version 20.0; IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard deviation (SD). Student's t-tests or one-way ANOVA were performed to compare differences between two independent samples. *P*-values < 0.05 indicate statistically significant differences. All experiments were repeated three times.

Results

F68-cis-Cur is cytotoxic to DU145 cells

Effects of F68-*cis*-Cur (**Figure 1A**) on cell viability were assessed in DU145 PCa cells. As expected, F68-*cis*-Cur markedly suppressed cell proliferation in dose- and time-dependent manners (**Figure 1B**, **1C**). Consistently, colony formation in the DU145 cells treated with F68-*cis*-Cur was significantly lower than that in the control group ($P < 0.05$, $n = 3$) (**Figure 1D**, **1E**). Taken together, current findings indicate that F68-*cis*-Cur was effective in inhibiting the growth of DU145 cells via induction of cell death.

F68-cis-Cur induces G2 cell cycle arrest in DU145 cells

Identifying whether the suppression of proliferation upon treatment with F68-*cis*-Cur may be implicated in cell cycle profile alterations, flow cytometry analysis was performed, aiming to quantify cellular DNA content. Cell percentages in DU145 cells treated with F68-*cis*-Cur were significantly lower than those in the control group ($P < 0.05$) (**Figure 2A**, **2B**). Results demonstrated that F68-*cis*-Cur caused cell cycle arrest in the G2 phase, partly explaining the suppressed cell proliferation.

F68-cis-Cur treatment results in intrinsic apoptosis of DU145 cells

The current study further examined the involvement of F68-*cis*-Cur on cellular apoptosis via flow cytometry. As presented in **Figure 3A**, **3B**, apoptosis rates in DU145 cells treated with

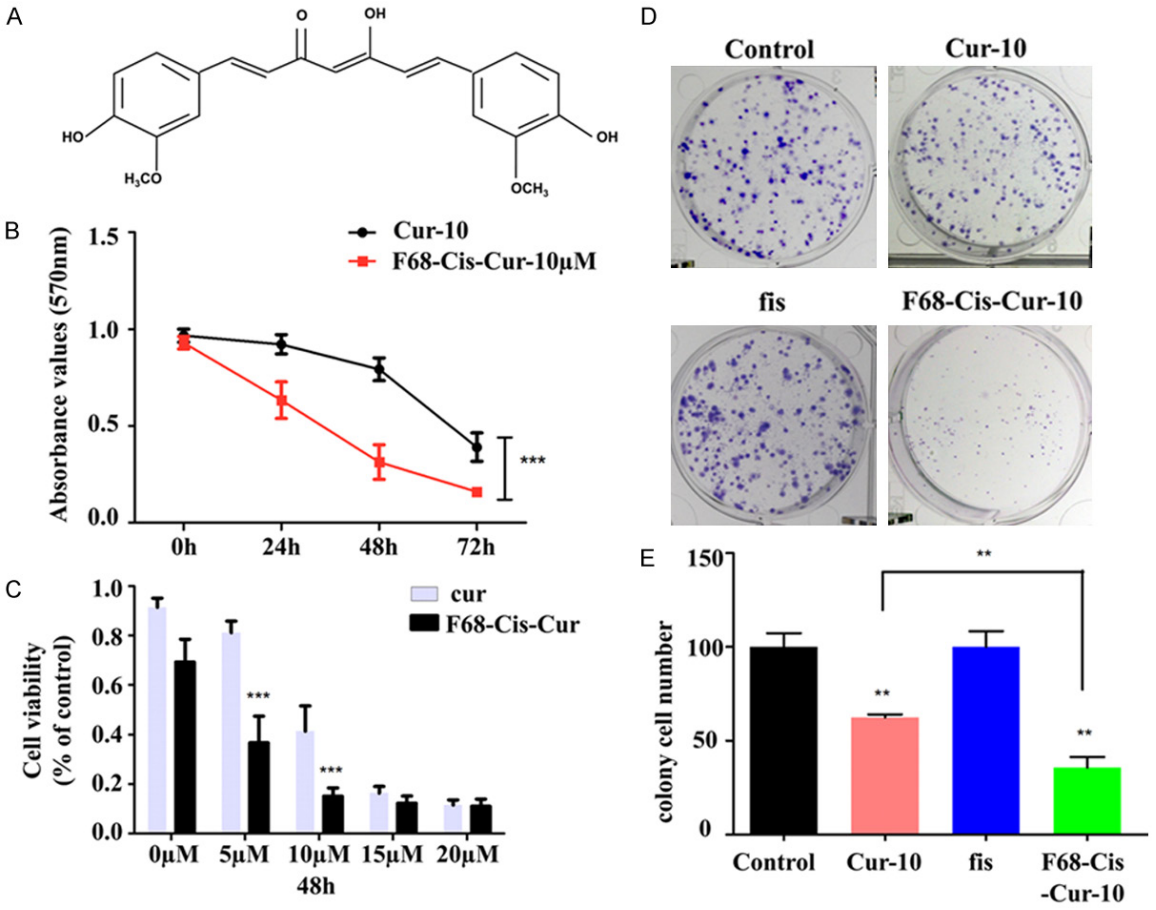

Figure 1. Effects of F68-*cis*-Cur on DU145 cell viability and cell colony formation ability. A. Chemical structure of curcumin (Cur); B. Kinetics of cell viability in DU145 cells treated with F68-*cis*-Cur (10 *μmol/l*) Cur-10 mean curcumin10 *μmol/l*. F68-cis-10 mean Cur to the hydrophilic terminals of Pluronic F68 chains through cis-aconitic anhydride linkers 10 *μmol/l*; C. Dosage effects of F68-*cis*-Cur on the viability of DU145 cells. DU145 cells were treated with F68-*cis*-Cur for the indicated times. Cur-10 mean curcumin 10 μmol/l. F68-cis-10 mean Cur to the hydrophilic terminals of Pluronic F68 chains through cis-aconitic anhydride linkers 10 μmol/l. D and E. The colony formation ability of the tumor cells was calculated. Data represents the mean ± standard error of the mean ($^{*}P < 0.05$, n = 3) as the percentage of viable cells normalized to the percentage of viable cells in dimethyl sulfoxide (DMSO)-treated (control) cells. Cur-10 means curcumin 10 μmol/l. F68-cis-10 means Cur to the hydrophilic terminals of Pluronic F68 chains through cis-aconitic anhydride linkers 10 μmol/l. Fis mean F68-cis. Data are presented as the mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the respective DMSO-treated (control) cells. All experiments were replicated three times.

F68-*cis*-Cur were significantly increased, compared with those of the control groups.

It has been demonstrated that caspases, Bcl-2, BclxL, and PARP, are closely associated with cellular apoptosis in PCa [20, 21]. Therefore, the current study measured activation of Bcl-2/caspase pathways. As expected, F68-*cis*-Cur upregulated expression of Bax but inhibited the activation of Bcl-2 in DU145 cells (**Figure 3A**). Results suggest that F68-*cis*-Cur induced apoptosis of DU145 cells via inhibition of the key anti-apoptotic protein Bcl-2 and induction of PARP cleavage.

F68-cis-Cur inhibited PI3K/AKT signaling in DU145 cells

Canonical PI3K/AKT signaling has been found to play a key role in cancer cell proliferation [21]. F68-cis-Curcumin suppresses cell growth in hormone independent prostate cancer DU-145 cells by downregulating PI3K/AKT signaling (**Figure 5**). It was observed that the DU145 cells were significantly decreased after being treated with 5 or 10 μM F68-*cis*-Cur for 24 hours (**Figure 3A**). Western blot analysis demonstrated that F68-*cis*-Cur also significantly decreased expression of p-AKT in the DU145

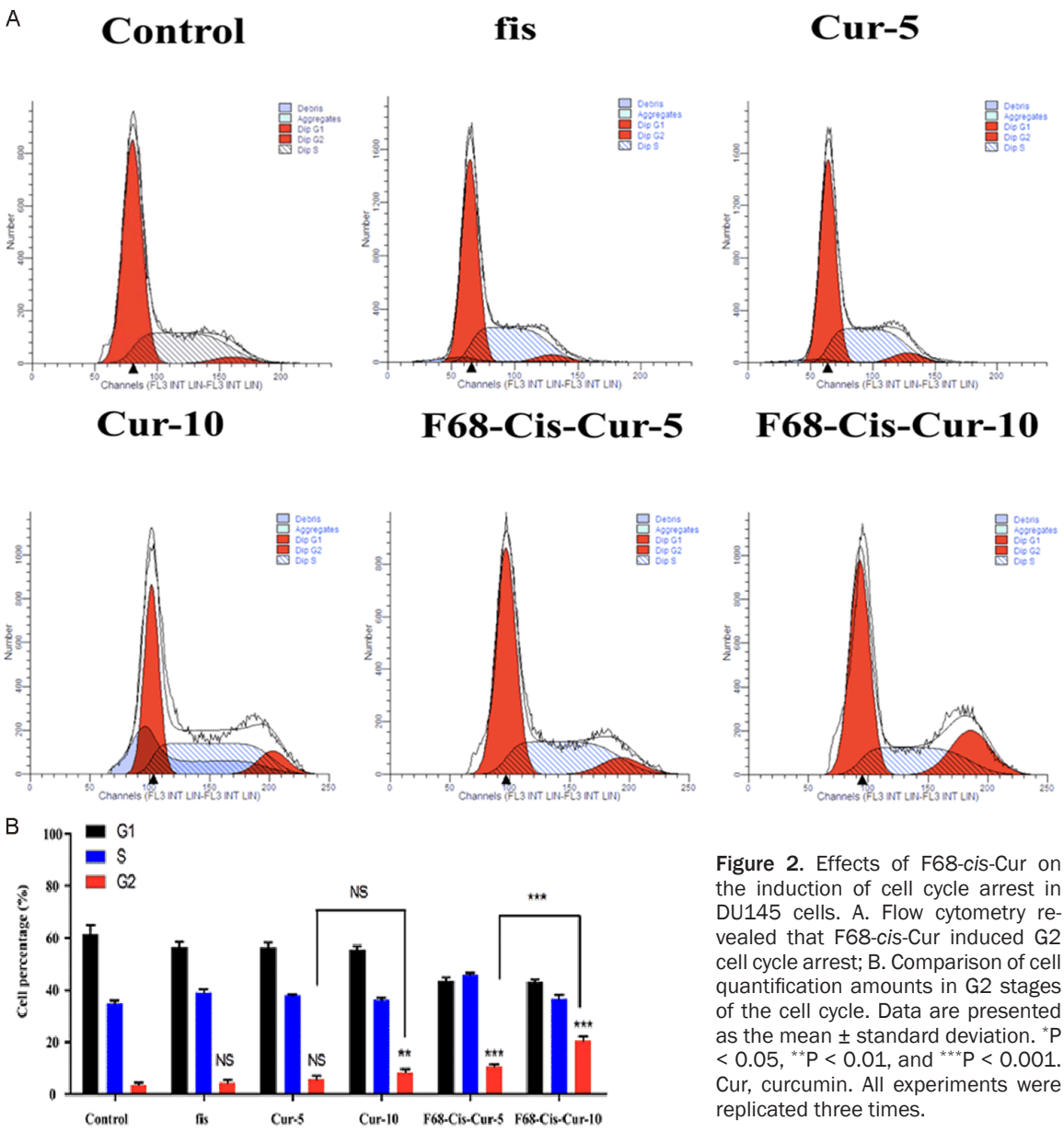

Figure 2. Effects of F68-*cis*-Cur on the induction of cell cycle arrest in DU145 cells. A. Flow cytometry revealed that F68-*cis*-Cur induced G2 cell cycle arrest; B. Comparison of cell quantification amounts in G2 stages of the cell cycle. Data are presented as the mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Cur, curcumin. All experiments were replicated three times.

cells treated with F68-*cis*-Cur, compared with the control group ($P < 0.05$, **Figure 4A-C**). Present data indicates that F68-*cis*-Cur may interfere with the proliferation of DU145 cells through inhibition of PI3K/AKT signaling pathways.

Discussion

Cur has recently attracted attention due to the antiproliferative effects it provides on various cancer cell lines [20, 22, 23]. Although studies have proven that Cur can inhibit cell growth and induce apoptosis in various malignancies, such as hepatocellular carcinoma, PCa [24], and thyroid carcinoma [25], markedly limited intracellular drug release has restricted its clinical application. Thus, the current study introduced a flexible acid-responsive micelle program by covalently conjugating Cur to the hydrophilic terminals of Pluronic F68 chains through *cis*-aconitic anhydride linkers (F68-*cis*-Cur), aiming to improve drug release. Thus, the current study investigated the effectiveness and mechanisms of action of F68-*cis*-Cur in PCa treatment *in vitro*.

Results demonstrated that Cur micelles (F68-*cis*-Cur) exhibited enhanced cytotoxicity and

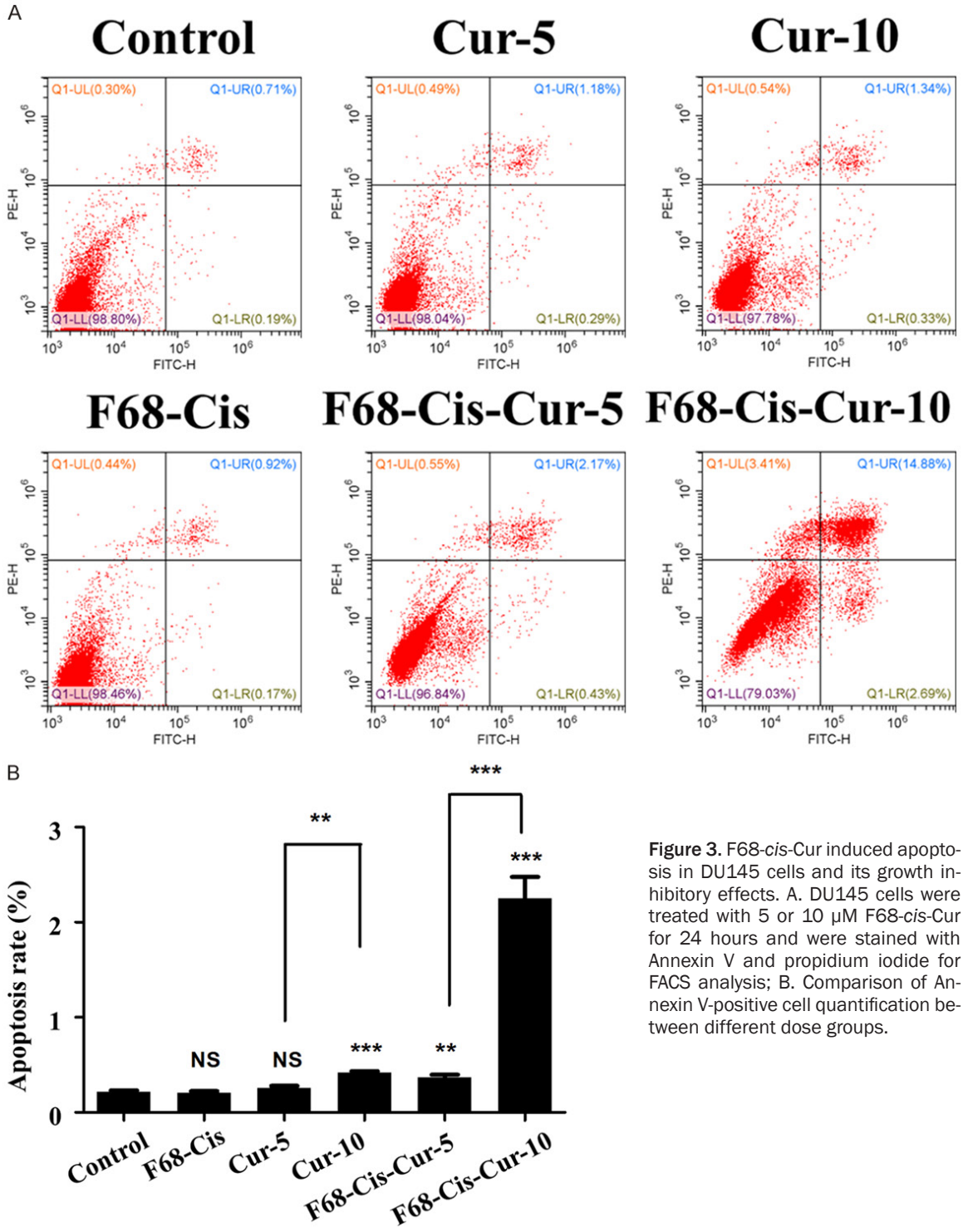

Figure 3. F68-*cis*-Cur induced apoptosis in DU145 cells and its growth inhibitory effects. A. DU145 cells were treated with 5 or 10 μM F68-*cis*-Cur for 24 hours and were stained with Annexin V and propidium iodide for FACS analysis; B. Comparison of Annexin V-positive cell quantification between different dose groups.

induced apoptosis of PCa cells *in vitro* more prominently, compared with free Cur. Moreover, it was demonstrated that Cur micelles exert antiproliferative effects on PCa cells by inhibiting intrinsic apoptotic signaling and enhancing PI3K/AKT signaling pathways.

The intrinsic apoptotic signaling pathway has been shown to be involved in several types of malignancies, including PCa. Dysregulation of cellular apoptosis and/or the cell cycle results in the unlimited proliferation of cancer cells [26]. Results of the present study demonstrat-

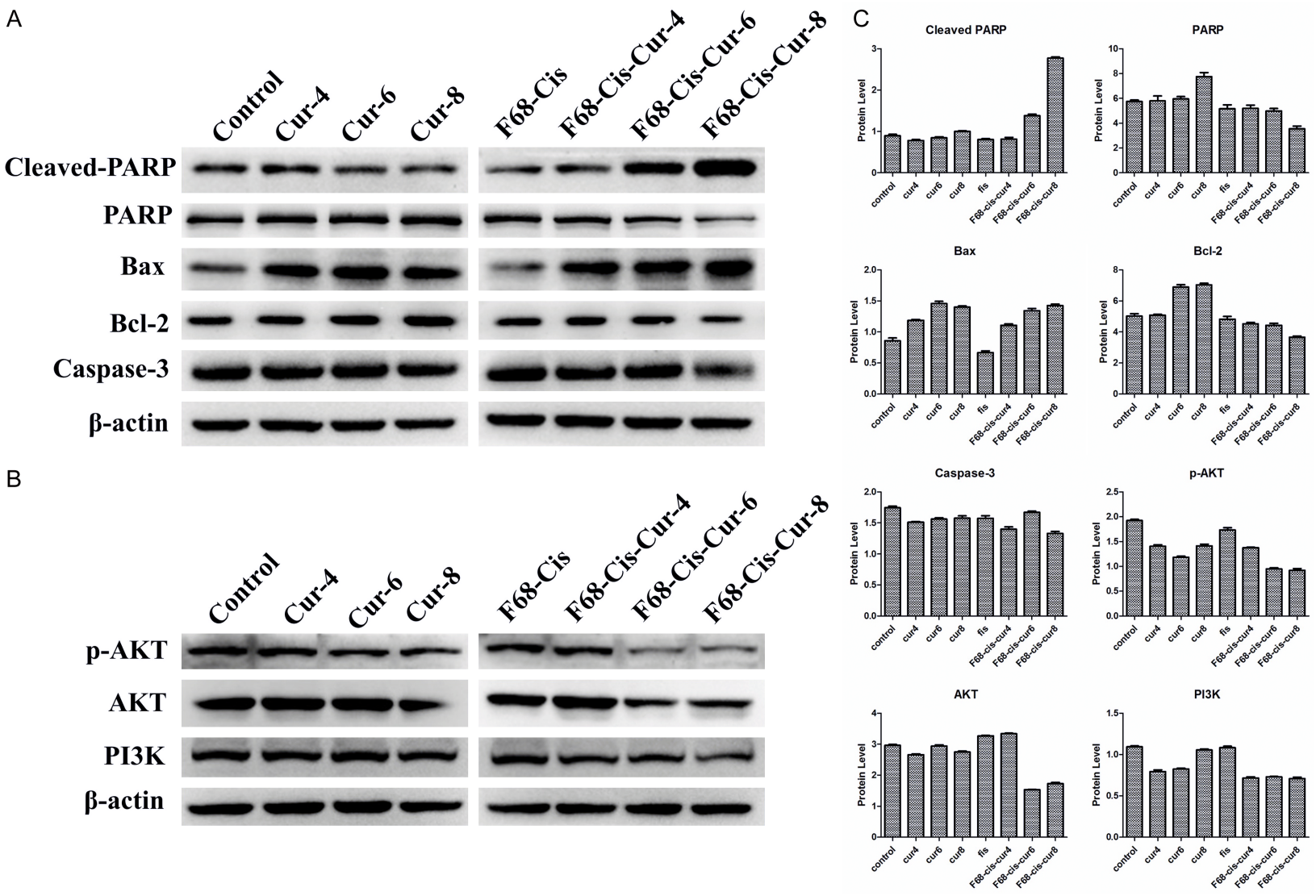

Figure 4. F68-*cis*-Cur suppressed activation of PI3K/AKT signaling pathways. (A and B) Expression of poly(ADP) ribose polymerase (PARP), cleaved PARP, Bax, B cell lymphoma-2, caspase-3, and components of the PI3K/AKT signaling pathways were analyzed by Western blotting after a similar F68-*cis*-Cur treatment procedure. β-actin served as the loading control; (C) Quantification of protein levels shown in (C) normalized to β-actin. Cur, curcumin; PI3K, phosphatidylinositol-3 kinase. All experiments were replicated three times.

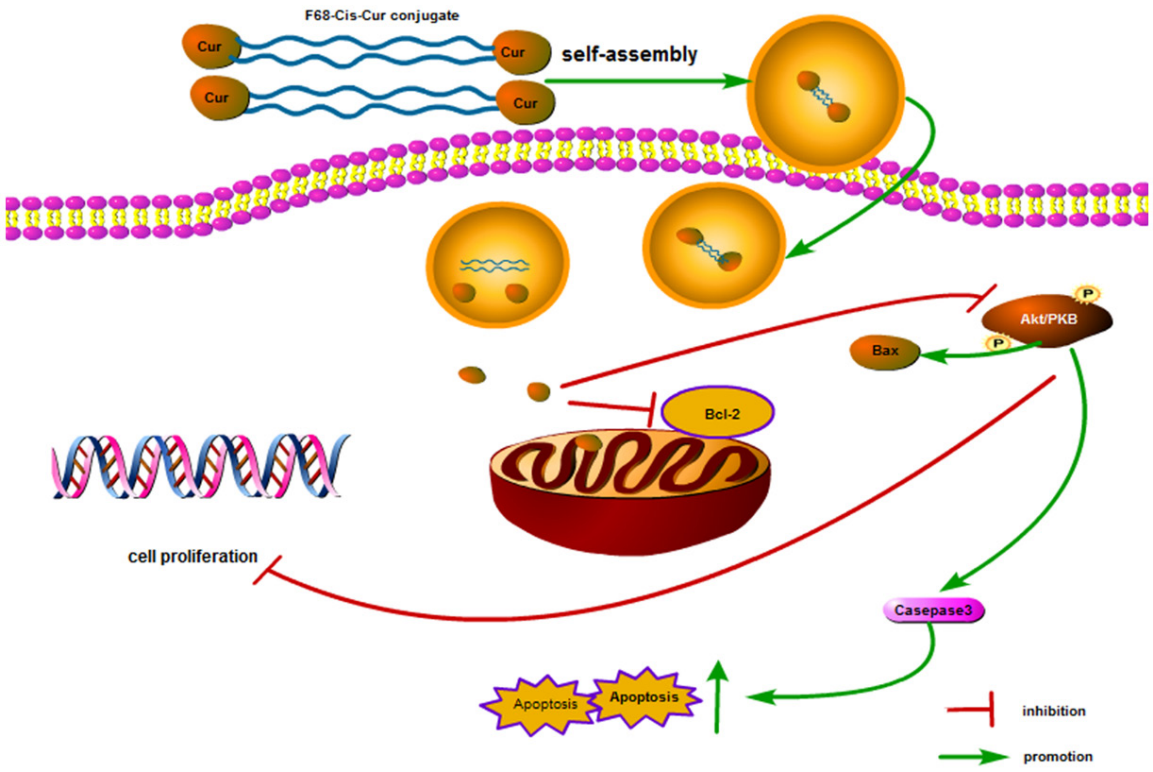

Figure 5. Schematic model for the roles of F68-Cis-Cur. F68-*cis*-Curcumin suppresses cell growth in hormone independent prostate cancer DU-145 cells by down regulating PI3K/AKT signaling.

ed that F68-*cis*-Cur inhibited the proliferation of DU145 cells, partly by contributing to the aberrant expression of intrinsic apoptotic pathways, as well as downstream signaling molecules, including inhibition of Bcl-2. Moreover, it enhanced expression of pro-apoptotic proteins Bax and cleaved PARP. In addition, results revealed that F68-*cis*-Cur induced G2/M cell cycle arrest in PCa cells. Present results are consistent with similar observations regarding Cur analog WZ35 in PCa [27]. Those results demonstrated that F68-*cis*-Cur was involved in the intrinsic apoptotic signaling pathway, which participates in cellular apoptosis-regulated gene expression. It plays an important role in the promotion of cellular apoptosis in PCa.

It has been reported that Cur mediates AKT signaling in Pca [28, 29]. Therefore, further reducing the dose of Cur to levels sufficient to suppress AKT phosphorylation, without changing its function, may prove beneficial in tumor treatment [30, 31]. Chaudhary et al. [32] reported that Cur decreases protein kinase B (PKB/AKT) activation and restrains the complete activation of AKT, in a time-dependent manner. The present study also showed that PI3K/AKT signaling was aberrantly decreased following exposure to F68-*cis*-Cur in DU-145 cells. Therefore, it was hypothesized that F68-*cis*-Cur is involved in the regulation of PI3K/AKT pathways, one of the major drivers of cell proliferation and survival in PCa.

In conclusion, present findings provide evidence that F68-*cis*-Cur conjugates exerts potent anticancer effects on androgen-resistant PCa cells *in vitro*. Present results may be the basis of a novel strategy for Cur-based antineoplastic drug design. Results also suggest that F68-*cis* may be a promising candidate for androgen-resistant PCa treatment. However, further basic and clinical studies are required to verify the potential of F68-*cis*-Cur conjugates as a targeted therapy method for PCa.

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Disclosure of conflict of interest

None.

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