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Benzyldihydroxyoctenone, a novel anticancer agent, induces apoptosis *via* mitochondrial-mediated pathway in androgen-sensitive LNCaP prostate cancer cells

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ABSTRACT

This study was aimed to evaluate detailed mechanisms on the apoptotic induction of benzyldihydroxyoctenone, a novel compound isolated from *Streptomyces* sp. KACC91015, in androgen-sensitive LNCaP prostate cancer cells. Benzyldihydroxyoctenone, designated as F3-2-5 in the current study, caused accumulation of apoptotic sub-G_1 phase in the flow cytometric analysis using propidium iodide staining. Moreover, the typical apoptotic DNA fragmentation of the LNCaP cells treated with 30 μM of F3-2-5 was confirmed using the TUNEL assay. This apoptotic induction of F3-2-5 in the LNCaP cells was associated with the cytochrome *c* release from mitochondria to cytosol, and the activation of procaspase-8, -9, and -3, as well as the specific proteolytic cleavage of poly(ADP-ribose) polymerase (PARP). In addition, F3-2-5 treatment caused the down-regulation of the antiapoptotic protein, such as Bcl-2 and Bcl-X_L, but the proapoptotic protein, such as Bax, was not influenced. To investigate whether apoptotic induction by F3-2-5 is also due to the down-regulation of androgen receptor (AR), Western blot analysis and quantitative RT-PCR were conducted in F3-2-5-treated LNCaP prostate cancer cells. We found that F3-2-5 significantly inhibited the expression levels of AR and prostate-specific antigen (PSA) proteins in a time-dependent manner, as well as F3-2-5 abrogated the up-regulation of *AR* and *PSA* genes with and without DHT. Therefore, F3-2-5 has been shown to be an androgen antagonist, suggesting that F3-2-5 could be a potent agent for the treatment of both androgen-dependent and hormone-refractory prostate cancer.

Apoptosis has been characterized as a fundamental cellular event to maintain the physiological balance and homeostasis of the organism. The relationship between dysregulation of apoptosis and cancer formation has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression and metastasis involve the alteration of normal apoptotic pathways.[1]

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of the death receptors located on the cell membrane, such as Fas and TNFR1. In contrast, the intrinsic pathway is initiated through the release of signal factors, such as cytochrome *c*, by mitochondria.[2] Recently, considerable attention has been devoted to the sequence of events referred to as apoptotic cell death and the role of this process in mediation of the lethal effects of the diverse chemotherapeutic agents. Accordingly, many chemotherapeutic agents reportedly exert their antitumor effects by induction of apoptosis in various cancer cells.[3–7]

Prostate cancer is the most frequently diagnosed cancer and is the leading cause of cancer death in men in the US with an estimated 234,460 new cases and 27,350 deaths in 2006.[8] Despite the initial efficacy of androgen deprivation therapy, most patients with advanced prostate cancer eventually develop resistance to this therapy and progress to hormone-refractory prostate cancer (HRPC), for which there is no curative therapy.[9] Therefore, novel targeted therapeutic approaches must be developed for the treatment of HRPC.[10]

Androgen, such as testosterone and DHT, exerts its biological effects by binding to androgen receptor (AR) and activating AR transcriptional activity. AR is a member of the steroid receptor superfamily and is a nuclear transcription factor. Upon binding to AR, androgen activates AR, which, in turn, interacts with androgen response elements (ARE) in the promoter of target genes including prostate-specific antigen (PSA), regulating the transcription of target genes. PSA is a clinically important marker used to monitor diagnosis, treatment response, prognosis, and progression in patients with prostate cancer.[11]

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Studies on the progression of prostate cancer have indicated that an increase in AR at mRNA and protein level is necessary to convert prostate cancer from a hormone-sensitive to a hormone-refractory stage,[12] and that overexpressed AR linked to p21 silencing may be responsible for androgen independence and resistance to apoptosis.[13] Therefore, AR is a key target for the treatment of both early stage prostate cancer and HRPC, and the inactivation of AR expression should be an important approach for the successful treatment of HRPC.

Previously, the current authors reported on the isolation of F3-2-5 from a culture broth of *Streptomyces* sp. KACC91015 (Fig. 1), and F3-2-5 treatment induced the cell cycle arrest in G_1 phase and apoptosis in HeLa cells. However, F3-2-5 showed no antiproliferative effect on normal lymphocytes and normal fibroblasts used as controls.[14] As our continuous studies, the current paper presents the underlying apoptotic mechanism of F3-2-5 in androgen-sensitive LNCaP prostate cancer cells.

To quantify the degree of apoptosis, we analyzed the amount of apoptotic sub-G_1 DNA by flow cytometric analysis of fixed nuclei using PI staining. As shown in Figure 2A, the addition of F3-2-5 to LNCaP cells resulted in progressively increased accumulation of the sub-G_1 phase in a time-dependent manner. Owing that PI staining may yield false positive data, the TUNEL assay was employed as a more discriminating measure. Consistent with the results of PI staining, a time-dependent apoptotic DNA fragmentation also was observed in LNCaP cells (Fig. 2B). Taken together, these findings provide evidence that the inhibitory effect of F3-2-5 on LNCaP cell growth may be due to its apoptogenic properties.

Several apoptosis-associated genes or proteins have been shown to play critical roles in regulating apoptosis. These include caspases, Bcl-2 family members, cytochrome *c*, and PARP. To determine whether these proteins are involved in the mediation of F3-2-5-induced cell death in LNCaP cells, we examined caspases activation and cleavage of PARP by Western blotting.

As shown in Figure 3A, the involvement of caspase-3 activation is further supported by immunoblotting analysis in which F3-2-5 evidently induced proteolytic cleavage of procaspase-3 into its active form, 11 and 17 kDa fragments. In addition, the expression of procaspase-8 and -9 were gradually decreased, and the time-dependent cleavage of procaspase-8 was also revealed in F3-2-5-treated LNCaP cells. PARP, a key substrate of caspase-3, is a nuclear enzyme which is involved in DNA repair, and it has been demonstrated that the 116 kDa PARP is cleaved into a 85 kDa fragment.[15] Figure 3A shows that PARP is cleaved into a 85 kDa fragment after the treatment of F3-2-5.

Because a major mechanism of caspase-9 activation was through cytochrome *c* released from mitochondria, we examined the effect of F3-2-5 on cytosolic cytochrome *c* levels. As shown in Figure 3B, cytosolic cytochrome *c* was gradually increased, indicating release from mitochondria into the cytosol by F3-2-5.

Many lines of evidence demonstrate that Bcl-2-related proteins play an important role in either inhibition or promotion of apoptosis.[16] Our results from Western blot analysis showed that a significant decrease in Bcl-2 protein level after 48 h of F3-2-5 treatment, and a decrease in Bcl- X_L protein level was observed after 24 h of treatment. Furthermore, there was no change in the level of Bax

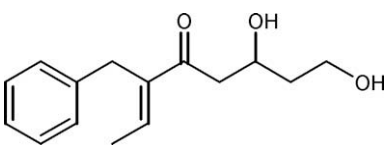

Figure 1. The chemical structure and nomenclature of F3-2-5, (*E*)-3-benzyl-6,8-dihydroxyoct-2-en-4-one.

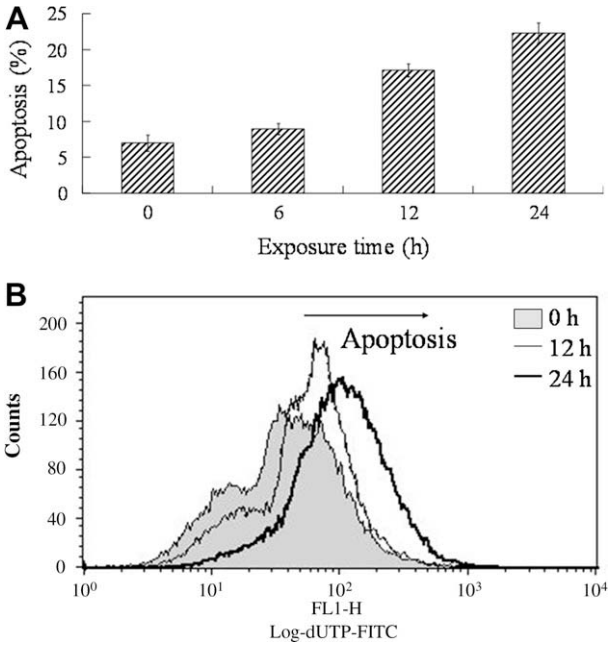

Figure 2. F3-2-5-induced apoptosis in LNCaP cells. (A) The LNCaP cells were treated with 30 μM of F3-2-5 for various times, stained with PI, and their DNA content analyzed by flow cytometry with Cell Quest software. A total of 10,000 nuclei were analyzed for each sample. Data are means ± SD of three separate experiments. (B) Apoptotic DNA fragmentation by F3-2-5 in LNCaP cells, as determined by TUNEL assay. The cells were treated with 30 μM of F3-2-5 for indicated times, then fixed, permeabilized, stained with the fluorescent TUNEL reagent and analyzed by flow cytometry.

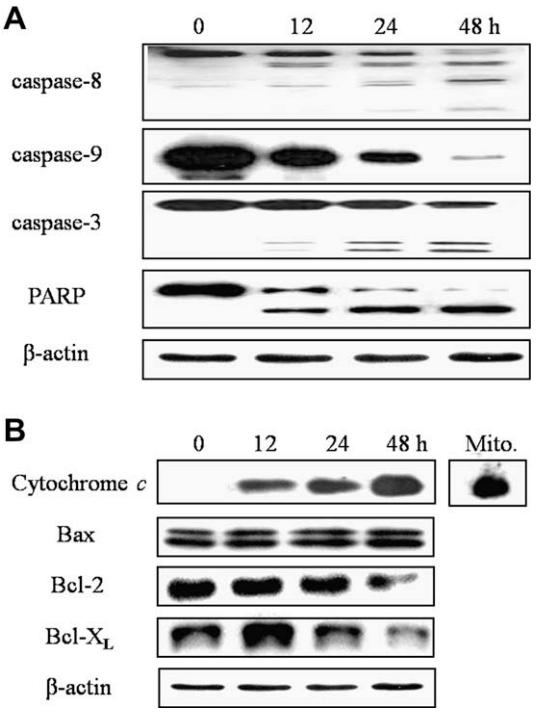

Figure 3. Effect of F3-2-5 on apoptosis-related proteins. Total cell lysates from cells treated for various times with 30 μM of F3-2-5 were separated electrophoretically on 8–14% polyacrylamide gels and immunoblotted with an antibody against each protein, and β-actin as an internal control. (A) Effect of F3-2-5 on activation of caspase-8, -9, and -3, and on cleavage of PARP. (B) Effect of F3-2-5 on cytochrome *c* release and expression Bcl-2 family proteins, Bax, Bcl-2, and Bcl-X_L. Mito.: mitochondrial fractions.

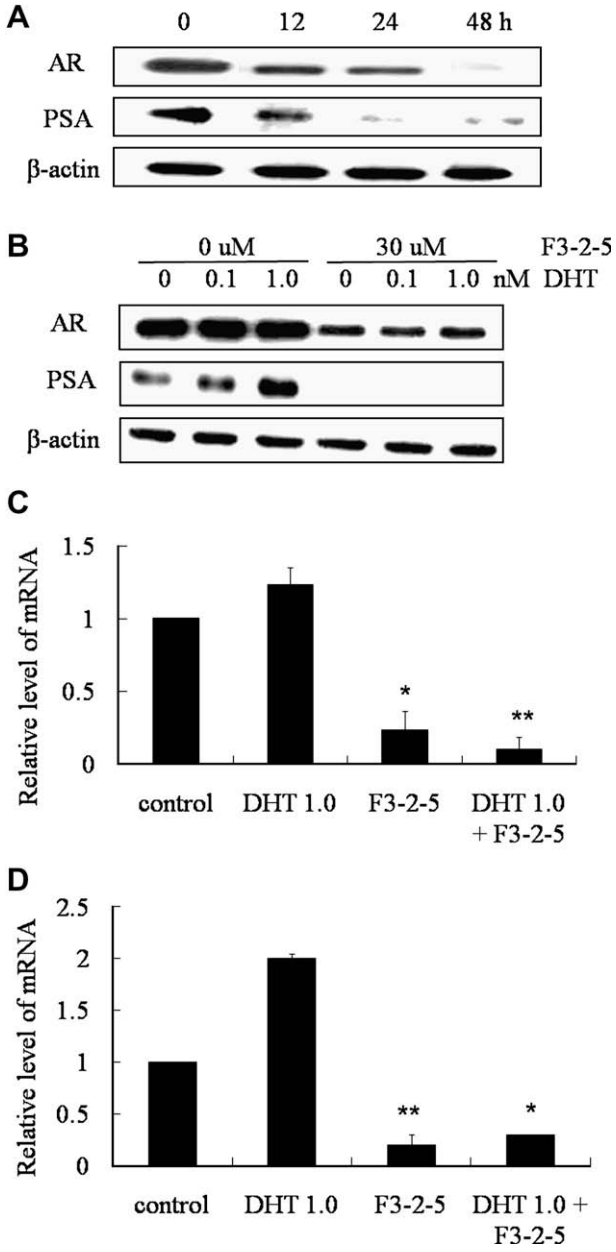

Figure 4. (A) F3-2-5 significantly inhibited the expression of AR and PSA at protein levels and the secretion of PSA in LNCaP cells. F3-2-5 inhibited the expression of AR and PSA protein in a time-dependent manner. (B) Western blot analysis showed that 30 μM of F3-2-5 abrogated the induction of AR and PSA expression induced by 0.1 and 1 nM of DHT treatment for 2 h in LNCaP cells. The quantitative RT-PCR demonstrated that F3-2-5 significantly down-regulated the transcription of *AR* (C) and *PSA* gene (D) in LNCaP cells with and without DHT (1.0 nM). $^{*}p < 0.05$, $^{**}p < 0.01$.

protein after F3-2-5 treatment (Fig. 3B). These results suggest that F3-2-5-induced apoptosis may be mediated through the down-regulation of Bcl-2 and Bcl-X_L antiapoptotic proteins, and increase of the Bax/Bcl-2 ratio in LNCaP cells.

To investigate whether induction of apoptosis by F3-2-5 is due to the down-regulation of *AR* gene, Western blot analysis and quantitative RT-PCR were conducted in F3-2-5-treated LNCaP prostate cancer cells.[17,18] As shown in Figure 4, we found that F3-2-5 significantly inhibited the expression levels of AR and PSA proteins in a time-dependent manner, as well as F3-2-5 abrogated the up-regulation of *AR* and *PSA* genes in LNCaP cells with and without DHT. Therefore, F3-2-5 has been shown to be an androgen antagonist, suggesting that F3-2-5 could be a potent agent for the treatment of both androgen-dependent and hormone-refractory prostate cancer.

In conclusion, a novel benzyldihydroxyoctenone derivative (F3-2-5) exhibits an antiproliferative effect by induction of apoptosis that is associated with increase of Bax/Bcl-2 ratio, cytochrome *c* translocation, activation of caspase-3, -9, and -8, and cleavage of PARP in LNCaP cells. Furthermore, F3-2-5 significantly inhibited the expression levels of AR and PSA proteins in LNCaP prostate cancer cells in a time-dependent manner. In addition, F3-2-5 down-regulated the transcription of *AR* and *PSA* genes, suggesting that F3-2-5 could be a potent agent for the treatment of both androgen-dependent and hormone-refractory prostate cancer.

Acknowledgments

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17. *Western blot analysis.* The LNCaP cells were cultured in RPMI 1640 with 10% FBS for 48 h. Cells were then treated with 30 μM of F3-2-5 in 100% ethanol for 24 h followed by treatment with and without DHT (0.1 and 1 nM) for 2 h. After treatment, cells were lysed and protein concentrations were then measured using bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The proteins were subjected to SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with various primary antibodies, and subsequently incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).
18. The quantitative RT-PCR: The LNCaP cells were treated as described above.[17] Total RNA was extracted from the cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (2 μg) from each sample was subjected to reverse transcription using the SuperScript First-Strand cDNA Synthesis Kit (Invitrogen, USA) and the cDNAs were subjected to real-time PCR analysis for AR and PSA expression. Real-time PCR reactions were carried out in iCycler iQ (Bio-Rad, USA). The specific primers for *AR* were as follows: 5′-GTGGACGACCAGATGGCTGT-3′ and 5′-GAAGAGTAGCAGTGCTTTCATGC-3′. The primers for *PSA* were as follows: 5′-TTGTCTTCCTC ACCCTGTCC-3′ and 5′-CAT CAGGAACAAAAG CGTGA-3′. The primers for *GADPH* were as follows: 5′-GTGG GGCGCCCCAGGCACCAGGGC-3′ and 5′-CTCCTTAATCTCACGCACGATTTC-3′. PCR amplification efficiency and linearity for each gene including targeted and control genes were tested. The relative quantization was calculated using the comparative threshold cycle method.[19] The house-keeping gene *GAPDH* was used to confirm the homogeneity of the DNA products.
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