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Induction of Apoptosis in Human DU145 Prostate Cancer Cells by KHC-4 Treatment

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Abstract

Prostate cancer (CaP) is one of the most prevalent cancers worldwide and the incidence and mortality rates have been rapidly increasing in recent years in Taiwan. Therefore, it is important to development anti-cancer therapy. In this study, KHC-4 was identified from 2-phenyl-4-quionolone derivatives in human prostate cancer cells and as a potential antitumor agent. In this study, we have identified KHC-4 induced apoptosis effects in castration-resistant prostate cancer DU145 cells, and the IC_{50} value of KHC-4 was 0.1 μM. KHC-4 suppressed the survival signaling p-PI3K and p-Akt and protein levels of Bcl-2 and Bcl-xL, upregulated Bax, cytochrome *c* and caspase 8/9 and induced apoptosis by mitochondrial-dependent pathway. In JC-1 assay monitored the loss of membrane potential in KHC-4 treatments. TUNEL assay results showed DNA fragmentation in KHC-4 induced apoptosis. We concluded that KHC-4 exerted anti-tumor effects in DU145 cells by induction of apoptosis.

Key Words: anti-tumor effects, apoptosis, 2'-fluoro-6-morpholinyl-2-phenyl-4-quinolone, prostate cancer

Introduction

Prostate cancer (CaP) is one of the most prevalent cancers worldwide, and is the second leading cause of cancer-related mortality in male in the United States (2). Recently, CaP has become more common in Asia, where it is ranked as one of the top 10 most common cancers in Taiwan (14, 15). There are several treatments for localized CaP, such as by surgery and radiation. Advanced CaP could be treated by androgen ablation therapy. However, in the advanced stage, CaP develops castration-resistance (7). Therefore, it is necessary to develop alternative agents for the treatment of castration-resistant CaP.

Microtubules are composed of the α- and β-tubulin heterodimers and are involved in many essential cellular functions. Microtubules are important cytoskeletal components involved in the regulation of mitotic events, including cell shape determination, cell motility, vesicle motility and cell division (12).

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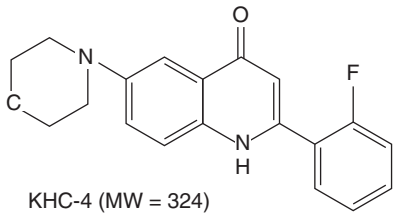

Fig. 1. The chemical structure and molecular weight of KHC-4 (2'-fluoro-6-morpholinyl-2-phenyl-4-quinolone).

Disruption of microtubules can induce cell-cycle arrest in the M phase, consequently triggering signals for apoptosis (20). Microtubule-targeted drugs, including polymerizing agents (paclitaxel and docetaxel) and depolymerizing drugs (vincristine), are widely used as anticancer drugs (17, 19, 21).

Cancer chemotherapeutic agents exert their effects through the disturbance of the microtubule dynamics, leading to dysregulation of mitotic spindles and the causative mitotic arrest in cancer cells (1). Several studies have reported that the 2-phenyl-4-quinolones and related quinolone derivatives display effects against a broad spectrum of human cancer cell lines (5, 9-11, 22). Moreover, the anticancer effect in CaP cell has been identified (1). Notably, 2'-fluoro-6-morpholinyl-2-phenyl-4-quinolone (KHC-4) (the structure is presented in Fig. 1) has been identified from 2-phenyl-4-quinolone derivatives to have anti-CaP effects (7). In this study, we investigated the anticancer mechanisms of KHC-4 in DU145 cells. Our results indicated that apoptosis was induced and caused the anticancer effects of KHC-4 in DU145 cells.

Materials and Methods

Materials

KHC-4 was kindly provided by Sheng-Chu Kuo, Graduate Institute of Pharmaceutical Chemistry, School of Medicine, China Medical University, Taichung, Taiwan, ROC. Antibodies used in these experiments were all obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)

CaP Cell Line Culture

Human CaP cell lines, DU145, were kindly provided by Dr. Paulus Shyi-Gang Wang, Department of Physiology, School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC. Cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, 1.5 g/l sodium bicarbonate and 1 mM sodium pyruvate in humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell Viability

To determine the effects of KHC-4 on cell viability, DU145 cells were treated with KHC-4 for 48 h and cell viability was estimated using a colorimetric assay based on tetrazolium dye (MTT) conversion into a blue formazan product at 570 nm using an enzyme-linked immunosorbent assay plate reader. The cell viability (cell number) of DU145 cells was determined according to the absorbance corrected to a background test.

Western Blot

After the indicated exposure time of cells to drug treatment, DU145 cells were washed twice with PBS and resuspended in lysis buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 1.0 mM EDTA, pH 7.5, 10% glycerol, 1 mM BME, 1% IGEPAL-630 and proteinase inhibitor cocktail obtained from Roche, Mannheim, Germany). After incubation for 30 min on ice, the supernatant was collected by centrifugation at 12,000 × *g* for 20 min at 4°C, and the protein concentration was determined by the Lowery method. For Western blot analysis, 40 μg protein was separated by electrophoresis in 12% gradient SDS–PAGE and transferred onto nitrocellulose membranes. Non-specific protein binding was blocked in blocking buffer at room temperature for 1 h (5% milk, 20 mM Tris-HCl, pH7.6, 150 mM NaCl and 0.1% Tween 20). The membranes were blotted with the indicated antibodies and incubated at 4°C overnight. Densitometric analysis of the immunoblots was performed using the AlphaImager 2200 digital imaging system (Digital Imaging System, San Diego, CA, USA). Experiments were performed in triplicates.

JC-1 Stain

DU145 cells were treated with the indicated doses of KHC-4 for 6 h and JC-1 staining was performed using the JC-1 Mitochondrial Membrane Potential Assay Kit (CS0390, Sigma, St. Louis, MO, USA). Briefly, cells were washed twice with PBS and incubated with the JC-1 stain solution per well for 20 min at 37°C in a humidified atmosphere containing 5% CO_2. JC-1 stain solution was removed and the cells were washed with a wash buffer. The fluorescence was visualized using a fluorescence microscope coupled with an image analysis system.

TUNEL Assay

The apoptosis was assessed by the TUNEL assay.

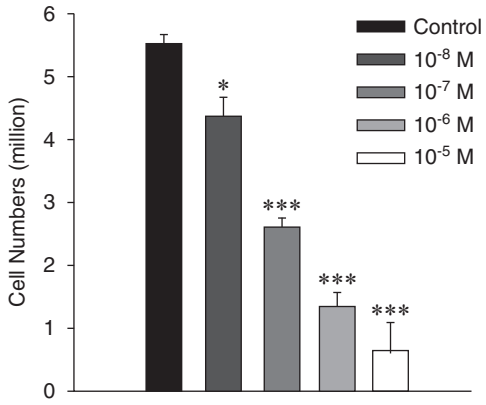

Fig. 2. Cell survival effects of KHC-4 in DU145 CaP cell line. Cell survival was measured by MTT assay after 48 h of KHC-4 treatment at different concentrations (0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M). Each value represents mean ± SE. *$P < 0.05$; ***$P < 0.001$.

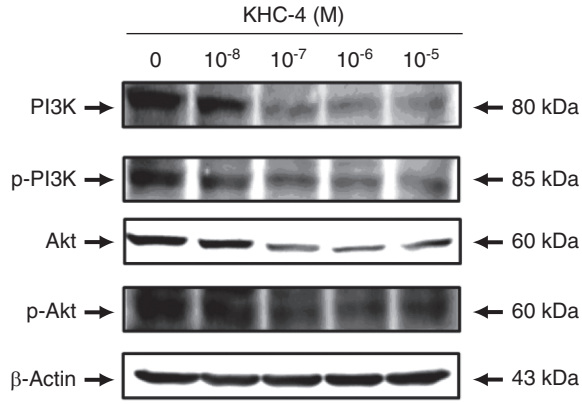

Fig. 3. Effects of KHC-4 on the survival pathway proteins. DU145 cells plated onto 6-well chamber dish were treated with the indicated concentration of KHC-4, and cells were then harvested and lysed for the detection of PI3K, p-PI3K, Akt, p-Akt and β-actin protein expression.

After KHC-4 treatment, DU145 cells grown in 24-well plates were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed with PBS and permeabilized with 0.1% Triton X-100 for 5 min at 4°C. After washing, the samples were incubated with the TUNEL reaction (TdT-mediated digoxigenin-dUTP nick-end labeling) kit for 1 h at 37°C. This was followed by washes with PBS and DAPI was added for 5 min. TUNEL-positive cells were identified with an Olympus ckx41 fluorescence microscope (Culture Microscopes, Olympus, Center Valley, PA, USA) using an excitation wavelength in the range of 450-500 nm and a detection wavelength in the range of 515-565 nm (green).

Statistics

Each experiment was duplicated at least three times. All values were presented as the mean ± standard error of mean (SEM), and statistical comparisons were made using the Student's *t*-test. The Statistically significant difference between two means was defined at $P < 0.05$.

Results

Effects of KHC-4 on Cell Viability of DU145 Cells

The cell viability of DU145 cells treated with various concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) of KHC-4 for 48 h was presented as cell numbers (Fig. 2). The results showed that decreases in DU145 cell viability were time- and dose-dependent with an IC_{50} of 10^{-7} M. The results showed that treatment with KHC-4 higher than 10^{-7} M significantly decreased the viability of DU145 cells after 48 h treatment. In the analysis of proteins of the PI3K-Akt cell survival pathway (Fig. 3), down-regulation of the proteins was shown, correlating with cell survival inhibition by KHC-4 treatment at a starting concentration of 10^{-7} M (13).

KHC-4 Disrupts Mitochondrial Membrane Potential and Suppressed Related Proteins in DU145 Cells

Western blot analysis showed that KHC-4 down-regulated the Bcl-2 and Bcl-xL protein levels accompanied by an increase in Bax expression in KHC-4-treated cells (Fig. 4). However, KHC-4 had little effects on the pro-apoptotic membrane protein, Bak (Fig. 4).

Effects of KHC-4 on the Activation of Caspases

Western blot analysis was performed in the determination of caspase proteins expression levels in KHC-4-induced apoptosis to in DU145 cells. Here, pro-caspase 8 (55 kDa) was cleaved into its active form and the increase in active fragment of caspase 8 (22 kDa) was detected after KHC-4 treatment (Fig. 5A). Pro-caspase 9 (46 kDa) was cleaved into its active form after KHC-4 treatment and the active fragment of caspase 9 (35 kDa) was detected at 48 h compared with the control (Fig. 5B).

JC-1 and TUNEL Stain Assays

Furthermore, JC-1 staining showed decreased levels of mitochondria membrane potential ($\Delta\Psi m$) after KHC-4 treatment (Fig. 6A). Mitochondrial integrity or the loss of $\Delta\Psi m$ has been linked to the initiation

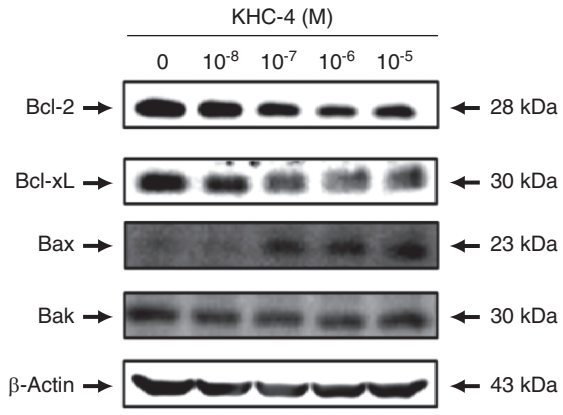

Fig. 4. Effects of KHC-4 on the mitochondria-dependent apoptotic proteins. DU145 cells plated onto 6-well chamber dish were treated with the indicated concentration of KHC-4, and cells were then harvested and lysed for the detection of Bcl-2, Bcl-xL, Bax, Bak and β-actin protein expression.

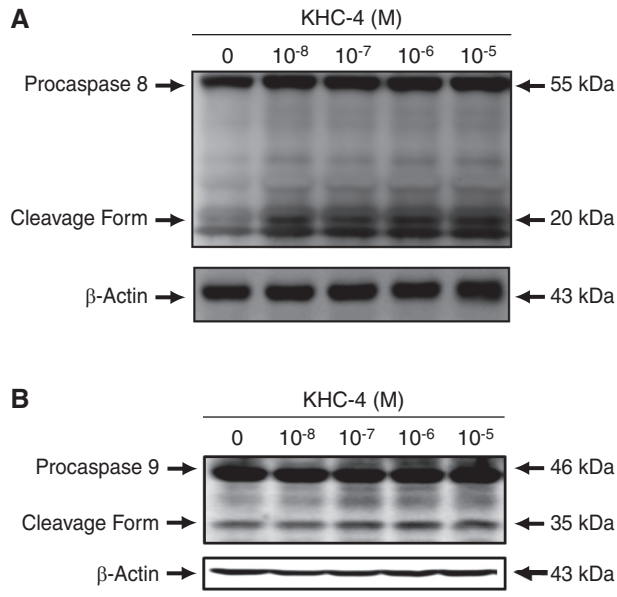

Fig. 5. Effects of KHC-4 on caspase activation and apoptotic effects in DU145 cells. DU145 cells plated onto 6-well chamber dish were treated with indicated concentration of KHC-4 for 48 h, and cells were then harvested and lysed for the detection of (A) caspase 8 (B) caspase 9 expression.

and activation of some apoptotic cascades. Apoptotic cells were easily found under fluorescence microscope using the TUNEL assay. Results showed increasing DNA fragmentation following KHC-4 addition at concentrations from 10^{-8} to 10^{-5} M (Fig. 6B).

Discussion

In previous studies, some anti-microtubule agents used have complex structures and pharmaceutical synthesis was difficult. On the contrary, 2-phenyl-4-quinolone is a 4-quinolone analogue has a simple structure and conforms to the criteria of pharmacological properties. In previous studies, 2-phenyl-4-quinolone displayed anti-mitotic and anti-tumor effects by inhibiting tubulin polymerization in several cancer types, including hormone-resistant CaP cells (1). CHM-1, a synthetic 6,7-substituted 2-phenly-4-quinolone, was identified as anti-tumor agent in HA22T, Hep3B and HepG2 hepatoma cells (20). In this study, KHC-4, a novel synthetic quinolone was shown to exhibit significant anti-proliferation and anti-migration effects against human CaP cells, DU145. Mitochondria-mediated signaling pathways were shown to play a major role in cell apoptosis. Members of the Bcl-2 family of proteins are important regulators of mitochondrial functions with individual members exerting anti-apoptotic (*e.g.* Bcl-2, Bcl-xl) or inducing proapoptotic effects (*e.g.* Bax, Bad) (4, 8, 16, 18). Our results indicated that KHC-4 upregulated proapoptotic Bax levels and downregulated levels of antiapoptotic Bcl-2 and Bcl-xl, concomitantlywith a loss of $\Delta\Psi m$ (6).

In MTT assay, the cell viability was confirmed by the measurement of cellular metabolic activity *via* NADPH-dependent cellular oxidoreductase. The loss of $\Delta\Psi m$ can also cause the MTT reduction failure. Thus, KHC-4 (10^{-4} M) treatment caused the death of most DU145 CaP cells through cellular metabolic activity inhibition (Fig. 2).

On the other hand, KHC-4 treatments induced apoptosis in DU145 CaP cells resulted in the cleavage of caspase proteins from pro-form to an activated cleavage-form. Caspase protein activation subsequently caused DNA damages as aDU145 cell death mainly through $\Delta\Psi m$ loss leading to cell autophagic effects and also through caspase protein cleavage.

Caspases 8 and 9 are known to promote apoptosis in response to mitochondrial and death-inducing signals from cell surface receptors (3). Results showed that KHC-4 induced cleavage of pro-caspase 8 and 9 and the TUNEL assay results also indicated early DNA degradation phenomenon. These results verified that KHC-4 induced DU145 cell apoptosis through mitochondria-dependent events and extrinsic death receptor pathways.

Taken together, our results showed that KHC-4 displayed pro-apoptotic effects in human DU145 CaP cells. Consequently, the participation of Bcl-2 family members, including the decrease of Bcl-2 and Bcl-XL, regulated the apoptotic signaling pathway induced by KHC-4.

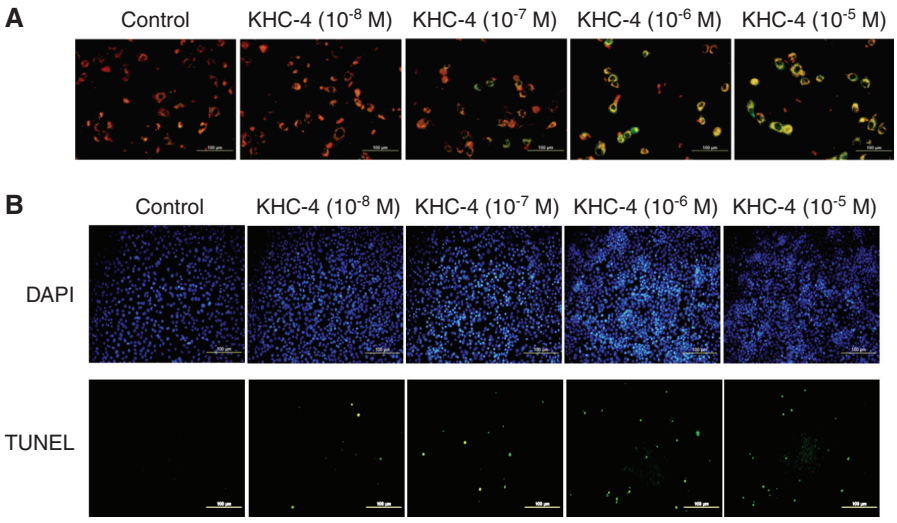

Fig. 6. Images of KHC-4-induced mitochondrial membrane instability and apoptosis in DU145 cells. (A) The DU145 cells were treated with indicated concentration of KHC-4 for 6 h and then treated by JC-1 assay and imaged by microscopy. The JC-1 (yellow) shows the mitochondrial membrane instability in the KHC-4 treated cells. (B) The DU145 cells were treated with indicated concentration of KHC-4 for 48 h and were then subject to TUNEL assay and imaged by microscopy. The TUNEL spots (green) indicate the DNA fragments in the nuclei of the cell in comparison with intact nuclei stained by DAPI (blue).

Acknowledgments

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