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Growth Inhibitory Effects of Vitamin K2 (Menaquinones- MK4) on Bladder Carcinoma and Prostate Carcinoma Cells Via Two Possible Pathways of Apoptosis Implicating PUMA, BAX and Cleaved-PARP

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Conflicts of Interest
There are no conflicts to declare.

ABSTRACT

Ubiad1 is a tumor suppressor gene ubiquitously expressed in normal human tissues and its protein harbors a prenyltransferase functional domain conserved throughout the evolution. The ability of human UBIAD1 enzyme to synthesize menaquinone-4 (MK-4) rescues mitochondrial dysfunction in Drosophila and effectively initiates apoptosis in various types of tumors through a caspase-dependent pathway. The objective is to study menaquinone-4-induced apoptosis signaling pathway and to investigate its anti-tumor activity in human prostate (PC-3, LNCaP) and bladder (T24, J82) tumor cell lines, an approach that has not been undertaken yet. Cell viability of cancerous cells pre-treated with MK-4 showed a significant decrease in dose- and time-dependent manner. Moreover, flow cytometry detected apoptotic cells after treatment with the vitamin. Taken together, these observations suggest that MK-4 could inhibit cell growth by promoting apoptosis in prostate and bladder carcinoma cells via different-mediated signal pathways, making it a potential therapeutic molecule for the prevention and the cure of cancers.

Keywords: MK-4, BLADDER CANCER, PROSTATE CANCER, APOPTOSIS, ANTICANCER THERAPY

Introduction

Menaquinone 4 biosynthesis

K vitamins refer to essential lipid-soluble vitamins found in food and dietary supplements. The human body requires vitamin K to activate the synthesis of other factors. Vitamin K2 (VK2) also known as menaquinone

is one of the three forms of vitamin K, the other two being vitamin K1 (VK1, phylloquinone) and K3 (VK3, menadione). VK2 is naturally found in fatty meat, dairy products, and fermented food (Nakagawa et al. 2010).

Menaquinone-4 (MK-4), the most common variant of vitamin K2, is a short chain menaquinone distributed in different animal tissues and found in the brain, kidneys and pancreas at high concentrations (M. J. Shearer and Newman 2014). And it was in 2010 that Nakagawa and his colleagues defined a novel function to UBIAD1 as a menaquinone-4 (MK-4, vitamin K2) biosynthetic enzyme, a predominant form in humans (Nakagawa et al. 2010). Vitamin K2 derives from the conversion of dietary phylloquinone (K1) into menadione (K3). The latter being an intermediate variant prenylated by geranylgeranyl pyrophosphate enzyme (GGPP) to produce the final component K2 (reaction catalyzed by UBIAD1 protein) (Hirota et al. 2013; Nakagawa et al. 2010; Martin J. Shearer and Newman 2008). In *Escherichia coli* bacteria, *menA* gene acts as the human UBIAD1 and confers the prenyltransferase activity from menaquinones (Suvarna et al. 1998; Cheng and Li 2014).

A number of studies have associated K vitamins to exceptional health benefits such as corneal health and visual acuity (Nickerson et al. 2013). Importantly, vitamin K2 and K3 were found to generate oxidative stress and inhibit multiple cell cells, primarily by inducing cell death, necrosis and cell cycle arrest of as they are both redox-cycling and alkylating quinones (Yokoyama et al. 2008; Shibayama-Imazu, Aiuchi, and Nakaya 2008; Jamison et al. 2004; Jamison et al. 2010; Gilloteaux et al. 2010). These findings were corroborated with those found in colon (Kawakita et al. 2009), pancreatic cancer cell lines (Showalter et al. 2010), hepatocellular carcinoma Smmc-7721 cells (Li et al. 2010) and RCC (renal cell carcinoma) cell lines (Fredericks et al. 2013).

Although the growth inhibition induced by VK3 showed a high potency, the vitamin is considered as highly toxic. In the other hand, VK2 acquires a very limited toxicity without any evident side effects. However, VK1 remains the one providing the weakest function among the three forms (Karasawa et al. 2013).

The purpose of this study is to investigate the effect of the product of UBIAD1 expression; the vitamin K2 on prostate cancer cells and bladder cancer cells in order to clarify the function of this vitamin in tumorigenesis, a discovery that might provide therapeutic resolutions and may assist with the development of future treatments for cancer.

Materials and methods

Cells and cell lines

The human bladder cancer cell lines (T24, J82), human prostate cancer cell lines (PC-3 and LNCaP) and human hepatic normal cell lines (L-02) were obtained from the American Type Culture Collection (Manassas, VA, USA). The two types of bladder carcinoma cell lines were both seeded and cultured in Minimum Essential Medium Eagle (MEM) containing 10% Foetal Bovine Serum (FBS) (Gibco, USA). Prostate cancer cells (PC-

3) were cultured in Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 medium mixture (1:1). LNCaP cells were maintained in Roswell Park Memorial Institute-1640 (RPMI 1640; Sigma Aldrich, China) medium supplemented with 10% FBS (v/v). L02 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (v/v). All incubations were performed at 37°C in a cell culture humidified incubator with 5% CO_2.

Drug preparation, antibodies and reagents

Menaquinone 4 (vitamin K2) was purchased from Sigma (USA) and totally dissolved in 99.9% ethanol at stock solutions of 50mM then diluted in culture medium MEM, DMEM:F12=1:1, RPMI 1640 and DMEM depending on the type of cell lines used (T24, J82, PC-3, LNCaP and HEK293 cells, respectively) to prepare the five working concentrations (5µM, 10µM, 25µM, 50µM and 100µM) for a final volume of 100mL (9:1). Ethanol was added to cultures at 0.1% (v/v) as a solvent control. Photodegradation of the vitamin was prevented by preparing vitamin solutions in a darkened lamina flow hood.

CellTiter 96® AQeous One Solution Cell Proliferation Assay was provided from Promega (USA). MTS tetrazolium compound (MTT) used in MTT assay was purchased from Sigma Chemical Co. (St. Louis, MO). Reagents necessary for apoptosis, we cite ANNEXIN V-FITC (Fluorescein Isothiocyanate)/PI (Propidium Assay Kit were from Zomanbio (PRC).

Primary antibodies against PUMA, BAX, cleaved-PARP and PARP were purchased from Protein tech (USA). Primary antibodies detecting actin were purchased from Cell Signaling Technology (USA). Horseradish Peroxidase (HRP)-conjugated secondary antibodies were synthesized by Abgent (USA) and anti-mouse and anti-rabbit antibodies were from Abcam (USA).

Treatment with Menaquinone-4 under dose- and time-dependent conditions

Each type of bladder carcinoma cells and prostate carcinoma cells was firstly plated and cultured overnight on distinct 6-well plates (diameter of 5 $\times 10^5$) at 37°C, 5% CO_2. After being washed with 1X PBS buffer solution, cells were trypsinized with 0,25% Trypsin/EDTA (Gibco, USA) and incubated with different concentrations of MK-4 (5µM, 10µM, 25µM, 50µM and 100µM) for 24 hours or 48 hours at 37°C, 5% CO_2 to analyze dose-response and time-response of cells. The different concentrations of MK-4 (5µM, 10µM, 25µM, 50µM and 100µM) were diluted in the appropriate media (MEM, DMEM:F12=1:1, RPMI 1640 or DMEM depending on the type of cell lines used T24, J82, PC-3, LN-CaP and HEK293 cells, respectively) as mentioned in the previous part. Cells treated with medium only (without MK-4) served as negative control samples.

MTT assay: Cell survival

Each of T24, J82, PC-3, LNCaP human cancer cell lines were seeded in 96-well cells cluster (diameter of 1×10^5) at an approximate concentration of 1×10^5 cells per well and incubated overnight at 37°C in 5% CO_2 incubator. After two days, each type of cell lines was exposed respectively with MEM, DMEM:F12=1:1 or RPMI 1640-containing varying concentrations of MK-4 (5 µM, 10µM, 25 µM, 50 µM and 100 µM) for a period of 24 hours or 48 hours (dose- and time-dependent analysis). Cells treated with medium only served as a negative control group.

After 24 and 48 hours of MK-4 treatment, cellTiter 96® AQeous One Solution Cell Proliferation Assay with MTS tetrazolium compound were introduced to the cells. Cells were then maintained for 3 hours in 5% CO_2 at 37°C. After the incubation, the cells were ready to be tested for cell viability resorting to MTT assay.

Cell cycle analysis

Equal numbers of T24, J82, PC-3 and LNCaP cells were cultured in different 6-well plates and treated by varying doses of MK-4 (5µM, 25µM, 50µM and 100µM) for 24 hours and 48 hours (dose- and time-dependent analysis), respectively. Cells treated with medium only served as a negative control group. The cells were maintained in the humidified 5% CO_2 incubator at 37°C for 24 or 48 hours. Following the required period of menaquinone 4 time-dependent manner, cells were harvested, washed with 1× PBS and resuspended by trypsin/EDTA to be spin down at 2000rpm for 10 minutes. Pellets were then rewashed with 1× PBS and centrifuged under the same conditions. Next, cells were fixed with 500µl ice-cold 70% ethanol and incubated overnight at 4°C. After a succession of washings by 1× PBS, samples were resuspended in 25 µl of Propidium Iodide (1mg/ml) staining solution and 10 µl of RNase A (10 mg/µl) (Sigma, USA) and incubated for 30 min at 37°C, 5% CO_2, kept away from the light. Cell cycle was analyzed by flow cytometry (Beckman coulter FC500).

Apoptosis analysis

Bladder carcinoma T24, J82 cells and prostate carcinoma PC-3 and LNCaP cells were cultured in 6-well plates and treated by four varying doses of MK-4 (5 µM, 25 µM, 50 µM and 100 µM) for 24 hours and 48 hours (dose- and time-dependent analysis), respectively. Cells treated with medium only served as a negative control group. The cells were maintained in the humidified 5% CO_2 incubator at 37°C for 24 or 48 hours. Following the required period of menaquinone 4 time-dependent manner, cells were harvested, washed with 1× PBS and resuspended by trypsin/EDTA to be spin down at 1500rpm for 5-10 minutes. Pellets were then rewashed with 1× PBS and centrifuged under the same conditions. Next, cells were incubated with 500 µl of binding buffer 1× (1 dose of 10× annexin V Binding Buffer to 9 doses of distilled water), 5 µl of annexin and 10 µl of PI Apoptosis Assay Kit at room temperature for 15 minutes in the dark, following the manufacturer's

instructions. Unstained cells (not treated by MK-4), Cells stained with annexin V-FITC (no PI) and cells stained with PI (no Annexin V-FITC) were controls used to set up compensation and quadrants of flow cytometry. After a final wash in 1× PBS, bladder carcinoma and prostate carcinoma cells were immediately analyzed by flow cytometry (Beckman coulter FC500). Samples were excited by the light wavelength of 488 nm and the emitted green fluorescence of Annexin (FL1) and red fluorescence of PI (FL2) were measured using 525 nm and 575 nm barrier filters, respectively. Data were analyzed by flow cytometer software and plotted for annexin V-FITC and PI. Live, early apoptotic and late apoptotic/necrotic cells were represented as annexin V-FITC$^-$/PI$^-$, annexin V-FITC$^+$/PI$^-$and annexin V-FITC$^+$/PI$^+$, respectively. Each experiment was repeated more than five times.

Protein extraction and western blotting analysis

To determine the expression of associated proteins, western blot was performed. T24, J82 human bladder tumor cells and PC-3, LNCaP prostate tumor cells were harvested after treatment with MK-4 (25 μM, 50 μM, and 100 μM) for 24 h or with a constant dose of 100 μM for a time-dependent manner (12, 24 or 48hrs). The total proteins were lysed and extracted on ice using Radio Immune Precipitation (RIPA) buffer (Beyotime, China) supplemented with phosphatase inhibitor cocktail tablet (dilution of 100:1) purchased from Google Biology (Wuhan, PRC). Protein concentration was determined using enhanced BCA protein assay kit. The extracted protein samples were mixed with protein loading buffer (5:1) and boiled for 8 minutes. Protein samples were separated by sodium lauryl sulfate (SDS)-polyacrylamide gel (PAGE) (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (from Millipore Immobion, USA). Next, the membranes were blocked with 5% fat-free milk dissolved in Tris-Buffered Saline (TBS) containing Tween-20 (TBST buffer) for 2 hours at room temperature and then probed with primary antibodies. The preparation was first incubated overnight at 4°C then with HRP-conjugated antibodies and analyzed by immunoblotting. The immunoblotting consists of detecting protein expression signals by enhanced chemiluminescence detection solution (WesternBright ECL, Advansta, USA). The intensity of target proteins was quantified by Quantity One software (BioRad, USA).

Results

1. **Antitumor activity of menaquinone-4 (Repression of cell viability)**

As shown in Fig 1, bladder carcinoma T24 and J82 cell viability expressed both dose- and time-dependent relationships. Interestingly, the cell viability of both T24 and J82 cells greatly decreased after 48 h of MK-4 treatment with 5µM, 10µM, 25µM, 50µM and 100µM concentrations. Untreated cells were medium MK-4-free and were used as controls.

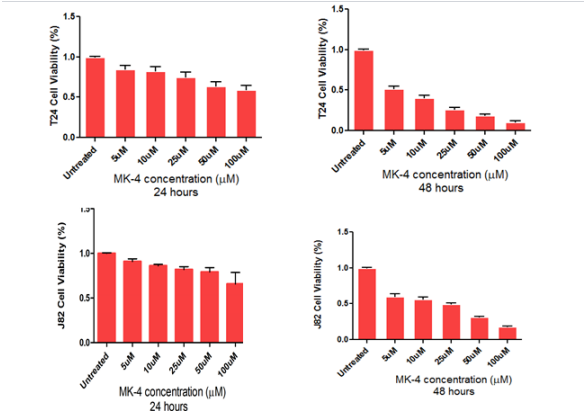


Figure 1. MK-4 inhibits cell proliferation of T24 and J82 cells in vitro in a dose- and time-dependent manner. T24 and J82 cells were treated by varied concentrations of MK-4 (dose-dependent) following an exposure of 24 h or 48 h (time-dependent) and were tested by MTT assay. Bars represent the corresponding standard deviations. The viability of bladder carcinoma T24 and J82 cells were significantly affected by all MK-4 concentrations, mainly after 48 h of exposure. Cell viability was made relative to untreated control cells (100%). Data are expressed as mean ± SD from three independent experiments.

In Fig 2, cell growth was inhibited in prostate carcinoma PC-3 and LNCaP dose- and time-dependently. It started to significantly decrease as MK-4 doses were higher (25μM, 50μM and 100μM) during 24 h of MK-4 treatment concentrations. Interestingly, the cell viability of PC-3 and LNCaP cells remarkably decreased after 48 h of MK-4 treatment with 5μM, 10μM, 25μM, 50μM and 100μM concentrations.

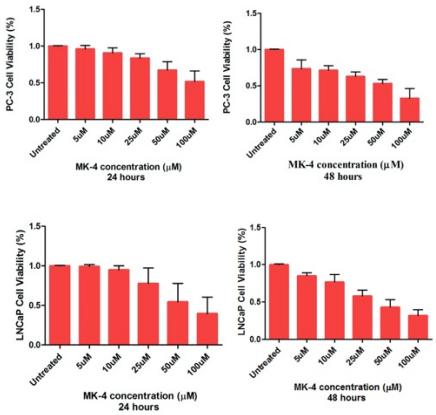


Figure 2. MK-4 inhibits cell proliferation of PC-3 and LNCaP cells in vitro in a dose- and time-dependent manner. Antitumor activity and dose– and time–response cell viability was measured by MTT assay following a 24 h or 48 h (time-dependent) exposure to VK2 (dose-dependent; 5μM, 10 μM, 25μM, 50μM and 100μM). Bars represent the corresponding standard deviations. The viability of human prostate carcinoma PC-3 and LNCaP cells were significantly decreased when treated with 25μM, 50μM and 100μM of MK-4 concentrations after 24h. Under the same time of exposure, PC-3 and LNCaP cells viability were not significantly affected by 5μM, 10μM of MK-4, when compared to that of untreated control cells. The

considerable decrease in cell viability was observed after 48 h of exposure with approximately all MK-4 doses for both prostate cell lines. Cell viability was made relative to untreated control cells (100%). Data are expressed as mean ± SD from three independent experiments.

In the purpose to reassert the anti-growth function of MK-4 in cell viability, it was necessary to examine the effect of this vitamin (5µM, 10µM, 25µM, 50µM and 100µM), respectively, for 24 h and 48 h treatment on normal cells. Therefore, L-02 normal hepatocytes cells were selected as a negative control and were assessed with MTT assay. As expected, no growth inhibition was induced by MK-4 dose- and time-dependent in L-02 normal hepatocytes cells (Fig 3). These results confirm that MK-4 does not induce its growth inhibitory function on normal control cells.

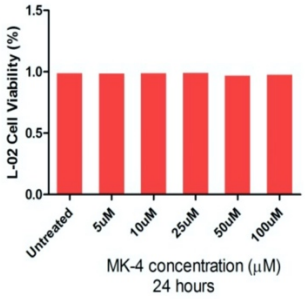


Figure 3. MK-4 does not inhibit cell proliferation in L-02 normal hepatocyte cells *in vitro* in a dose-dependent manner. As a control group, *L-02* cells were treated by varied concentrations of MK-4 (dose-dependent) for 24h and were tested by MTT assay. The viability of normal hepatocyte *L-02* cells was not affected by MK-4 treatment during 24 h of exposure. Cell viability was made relative to untreated control cells (100%). Data are expressed as mean ± SD from three independent experiments.

2. **Menaquinone-4 effects on the tumor cell populations: the vitamin contributes in cell cycle arrest of human carcinoma cells**

Treatment of T24 cells with various concentrations of MK-4 for 24h and 48h caused a significant cell cycle arrest in G1/M phase compared to that of control cells (Fig 4A). Moreover, T24 cells also showed the subG1 phase, one of the apoptotic hallmarks, when incubated with 50µM and 100µM of MK-4 for 24h. When treated for 48 h, T24 cells exhibited the subG1 phase earlier with 25µM, 50 µM and 100µM of MK-4 (Fig 4A). Treatment by MK-4 for 48h induced J82 cells to undergo a cell cycle arrest starting from the dose of 10µM and subG1 phase was induced under the same concentration of MK-4 (Fig 4B). Continuous MK-4 treatment of PC-3 and LNCaP cells for 24h and 48h resulted in a cell cycle arrest in G1/M phase compared to that of control cells (Fig 5A and Fig 5B). SubG1 was significantly important after exposing prostate carcinoma cell lines to 25 µM, 50µM and 100µM doses of MK-4 for 48h (Fig 5A and Fig 5B).

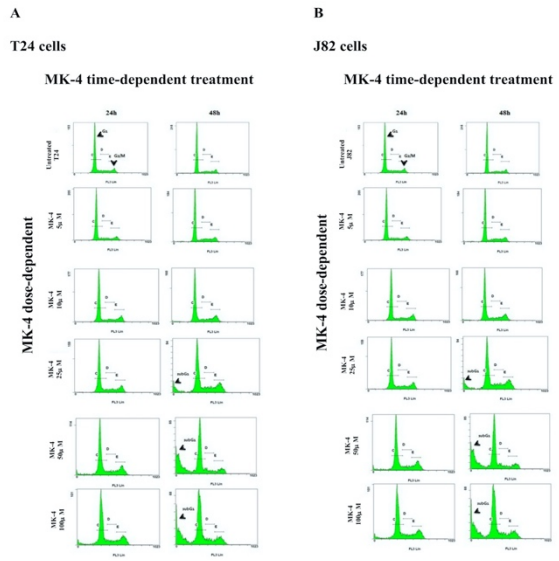


Figure 4. Cell cycle arrest induced by MK-4 in T24 and J82 human bladder carcinoma cell lines under dose- and time-dependent conditions. Cells were transfected with MK-4 at 5μM, 10μM, 25μM, 25μM, 50μM, and 100μM for 24 or 48 hours and then subject to flow cytometric analysis. (A) Flow cytometric plots for cell cycle distribution of T24 cells at different concentrations of MK-4 exposed for 24h (on the left) and 48h (on the right). (B) Flow cytometric plots for cell cycle distribution of J82 cells at different concentrations of MK-4 exposed for 24h and 48h.

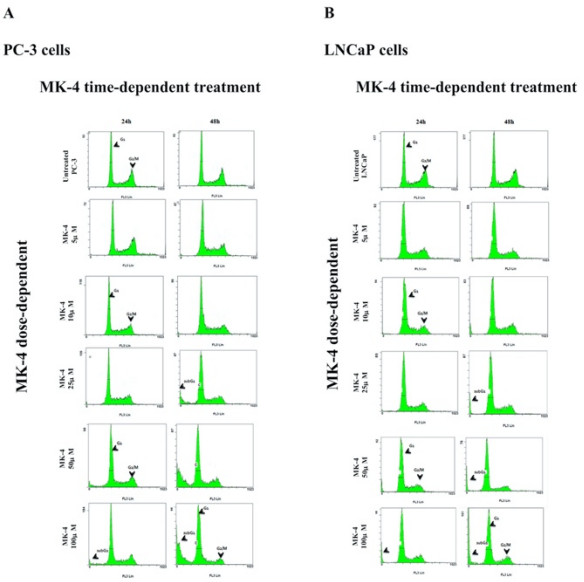


Figure 5. MK-4 induced cell cycle arrest in PC-3 and LNCaP human prostate carcinoma cell lines under dose- and time-dependent conditions. To examine the effects of MK-4 on prostate cancer cells, PC-3 and LNCaP were incubated with various doses of MK-4 (5μM, 10μM, 25μM, 25μM, 50μM and 100μM) for different periods of time (24 or 48h). (A) Flow cytometric plots for cell cycle distribution of PC-3 cells at different concentrations of MK-4 exposed for 24h (on the left) or 48h (on the right). (B) Flow cytometric plots for cell cycle distribution of LNCaP cells at different concentrations of MK-4 exposed for 24h (on the left) or 48h (on the right). The cell cycle arrest in human prostate carcinoma cell lines following the vitamin treatment

is observed, however it is more significant after an exposure of 48h for cells. The effect of MK-4 in cells is weaker following the exposure of 24h, inducing a significant cell cycle arrest after 50µM administration in PC-3 and LNCaP cells.

3. Menaquinone-4 induced apoptosis in human carcinoma cells

Significant differences were observed between the control and the MK-4-treatment in both cell lines. In Fig 6. A1, significant increase of late apoptosis was indicated in MK-4-treated T24 cells with 50 μM (1% of early apoptotic cells and 38,5% of late apoptotic cells) and with 100 μM (4.5% of early apoptosis and 84.3%% of late apoptosis) for an incubation of 24 h (Fig 6. A1). As the concentrations increased, the apoptosis rate was significantly higher indicating 95.3% of late apoptotic cells when the incubation was of 50 µM during 48 h (Fig 6. A1). T24 cells were 99.6% damaged when treated with 100 µM of MK-4 under same time conditions. Taken together, these results clearly demonstrate that MK-4 induces a dose-dependent and a time-dependent apoptosis in T24 cells supporting the data collected by flow cytometry. Comparison of late apoptosis of T24 cells at different time-exposures is shown in percentage in figure 6. B2. Similarly, death mechanism of T24 cells under vitamin treatment for 48 hours is shown (fig 6. B2).

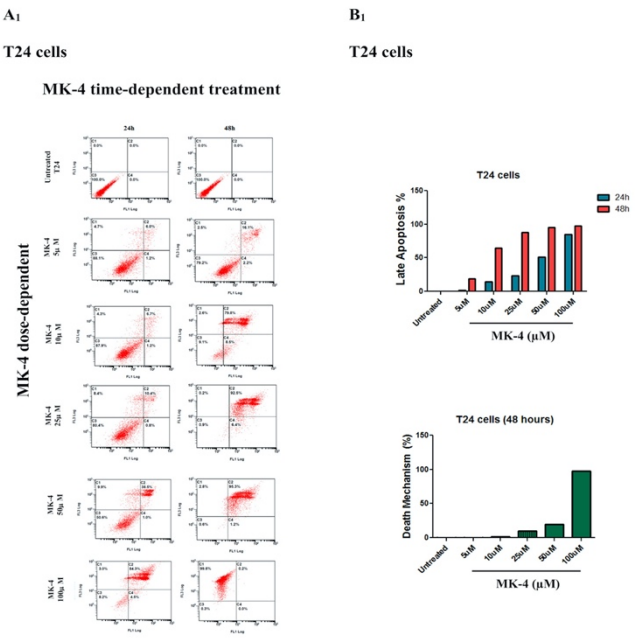


Figure 6. Apoptosis induced in T24 bladder carcinoma cell lines by MK-4 dose- and time-dependent manner. (A_1) A representative result of flow cytometry for a quantitative evaluation of cell apoptosis and necrosis of T24 cells stained with Annexin V-FITC/PI after non-treatment (control) or treatment with MK-4 at concentration of 5μM, 10μM, 25 μM, 50 μM and 100 μM for 24h or 48h (Annexin V^-/PI^- represents viable cells, Annexin V^+/PI^- represents early apoptotic cells and Annexin V^+/PI^+ represents late apoptosis or necrotic cells). (B_1) Late apoptosis and damaged cells (necrotic cells) rate for T24 cells treated with 5μM, 10μM, 25 μM, 50 μM and 100 μM of MK-4 for duration of 24h or 48h. Experiments were repeated four times and the

percentage of late apoptotic cells (mean ± SD) as well as the death mechanism rate for each treatment group is shown. C_1: necrotic cells; C_2: late apoptotic cells; C_3: healthy cells; C_4: early apoptotic cells.

Exposure of 48 h with 100μM of the vitamin indicated that 60% of J82 cells undergo only an early apoptosis (Fig 7. A2). Comparison of late apoptosis of J82 cells at different time-exposures is shown in percentage in figure 7. B2. Similarly, the death mechanism of J82 cells under vitamin treatment for 48 hours is shown (fig 6. B2).

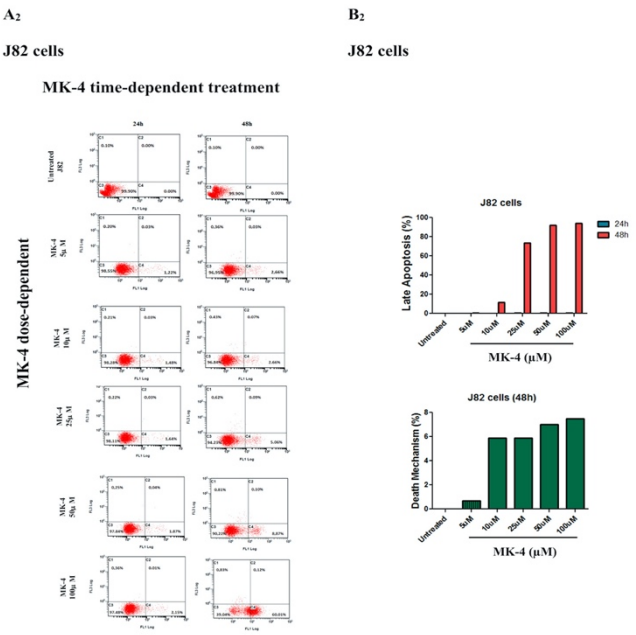
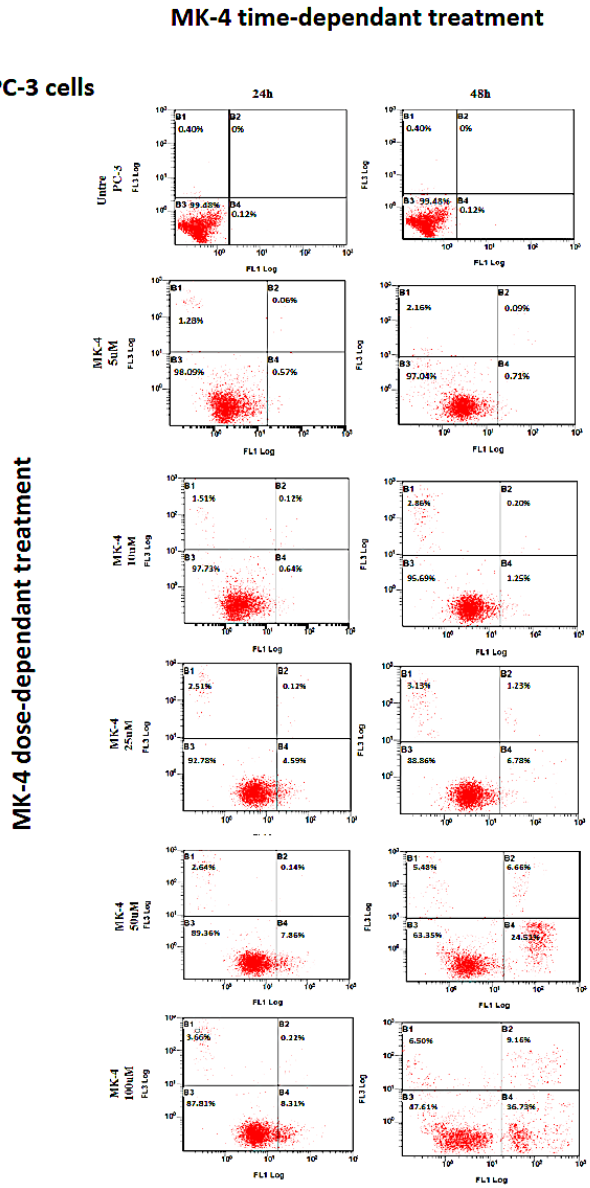


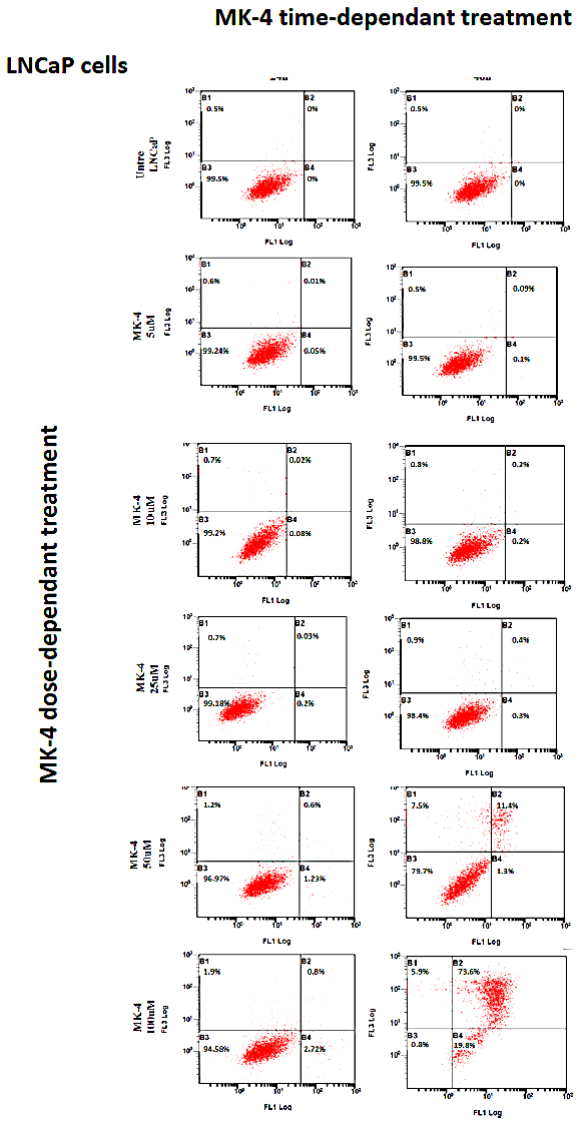
Figure 7. Apoptosis induced in J82 bladder carcinoma cell lines by MK-4 dose- and time-dependent manner. (A_2) A representative result of flow cytometry for a quantitative evaluation of cell apoptosis and necrosis of J82 cells stained with Annexin V-FITC/PI after non-treatment (control) or treatment with various concentrations of MK-4 (5μM, 10μM, 25 μM, 50 μM and 100 μM) for 24h or 48h (Annexin V^-/PI^- represents viable cells, Annexin V^+/PI^- represents early apoptotic cells and Annexin V^+/PI^+ represents late apoptosis or necrotic cells). (B_2) the bar graphs show the percentage of J82 late apoptotic cells and damaged cells after treatment with 5μM, 10μM, 25 μM, 50 μM and 100 μM of MK-4 for 24h or 48h. Data for each treatment group are mean ± SD of four independent experiments. C_1: necrotic cells; C_2: late apoptotic cells; C_3: healthy cells; C_4: early apoptotic cells.

Both prostate tumor cells incubated with the vitamin for 24h did not significantly undergo apoptosis (Fig S1). Late apoptotic cells counted for 6.66% and 9.16% after incubation with 50 μM and 100 μM for 48 h. Taken together, these results clearly demonstrate that MK-4 induces a dose-dependent and a time-dependent apoptosis in PC-3 cells that is not as significant as induced in T24 bladder tumor cells.



Supplementary fig. 1 Apoptosis induced in PC-3 prostate carcinoma cell lines following MK-4 dose- and time-dependent manner. A representative result of flow cytometry for a quantitative evaluation of cell apoptosis and necrosis of PC-3 cells stained with Annexin V-FITC/PI after non-treatment (control) or treatment with various concentrations of MK-4 (5μM, 10μM, 25 μM, 50 μM and 100 μM) for 24h or 48h (Annexin V^-/PI^- represents viable cells, Annexin V^+/PI^- represents early apoptotic cells and Annexin V^+/PI^+ represents late apoptosis or necrotic cells). B_1: necrotic cells; B_2: late apoptotic cells; B_3: healthy cells; B_4: early apoptotic cells.

Exposure for 48 h with 50μM and 100μM of the vitamin allowed a significant amount of LNCaP cells to suddenly undergo a late apoptosis with 11.4% and 73.60% (*vs* 1.23% and 2.72% of early apoptosis), respectively (Fig S2). This late apoptosis result marks an important difference with that undergone by PC-3 cells under the same conditions.

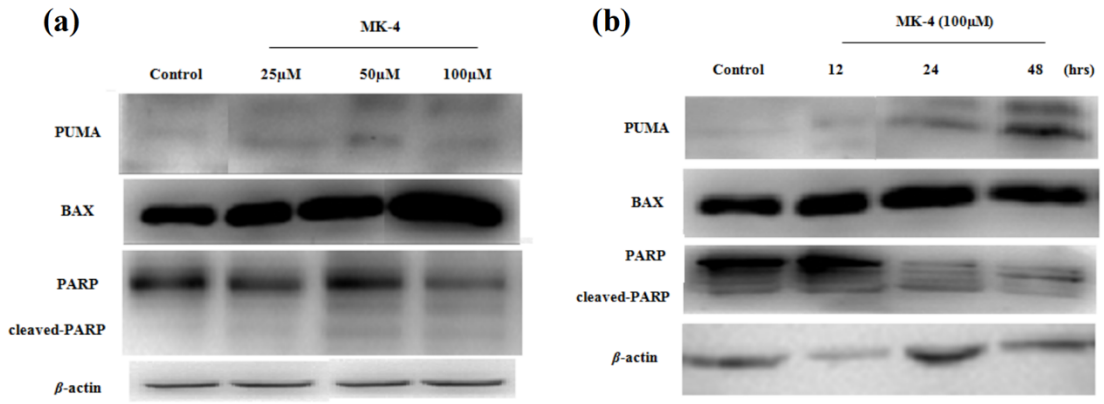


Supplementary fig. 2 Apoptosis induced in LNCaP prostate carcinoma cell lines following MK-4 dose- and time-dependent manner. A representative result of flow cytometry for a quantitative evaluation of cell apoptosis and necrosis of LNCaP cells stained with Annexin V-FITC/PI after non-treatment (control) or treatment with various concentrations of MK-4 (5μM, 10μM, 25 μM, 50 μM and 100 μM) for 24h or 48h (Annexin V^-/PI^- represents viable cells, Annexin V^+/PI^- represents early apoptotic cells and Annexin V^+/PI^+ represents late apoptosis or necrotic cells). B_1: necrotic cells; B_2: late apoptotic cells; B_3: healthy cells; B_4: early apoptotic cells.

4. Effects of menaquinone-4 on expression of apoptosis-related proteins

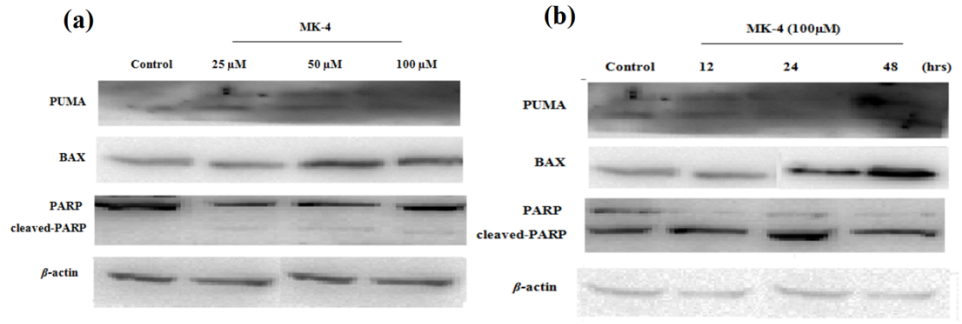
As indicated in the Figure S3a, VK2 (MK-4) potently increased the expression of the pro-apoptotic PUMA, BAX and cleaved-PARP proteins in a concentration-dependent manner (25μM, 50μM, and 100μM) after 24hrs of exposure with MK-4, compared with control cells. Following a constant concentration of MK-4 (100μM) treatment, levels of PUMA and cleaved-PARP were

significantly high in a time-dependent manner (12, 24 or 48hrs). Meanwhile, levels of BAX protein did not show a significant change for 48hrs (Fig S3b).



Supplementary fig. 3 Western blot analysis of PUMA, BAX, PARP and cleaved-PARP expression levels in T24 bladder tumor cells after treatment with MK-4. (a) Representative results of western blotting analysis of PUMA, BAX, PARP and cleaved-PARP expression levels in T24 cells exposed to three various doses of MK-4 (25μM, 50μM and 100μM) for 24 hrs. (b) Representative results of western blotting analysis of the same proteins expression levels in T24 treated cells with 100μM of MK-4 in time-dependent conditions (12, 24 or 48hrs). β-actin served as loading control. Molecular weight of the analyzed proteins is as follows: PUMA (21kDa), BAX (21kDa), PARP (116kDa), cleaved-PARP (89kDa), β-actin (42kDa).

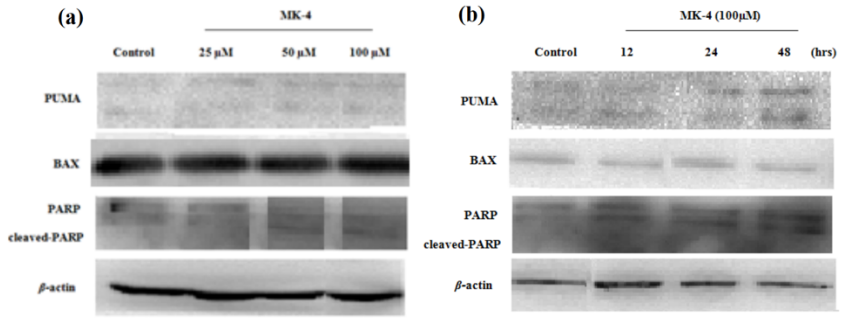
Although BAX protein expression is also induced with 50μM and 100μM doses of the vitamin, its level is not as significant as the two previous pro-apoptotic proteins (Fig S4a).



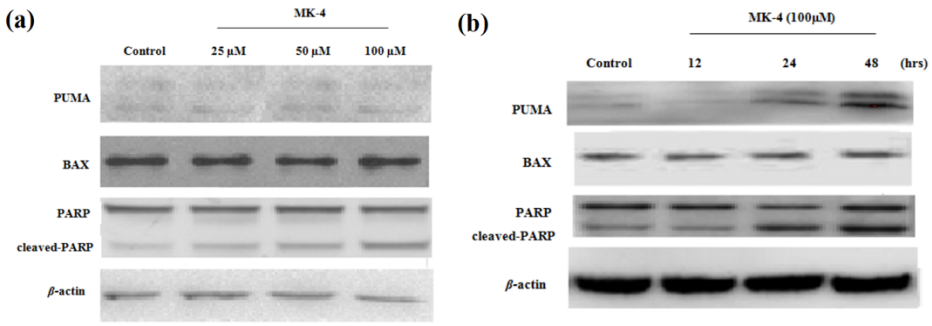
Supplementary fig. 4 Immunoblotting of PUMA, BAX, PARP and cleaved-PARP expression levels in J82 bladder tumor cells in a dose- and time-dependent manner. (a) Representative results of western blotting analysis of PUMA, BAX, PARP and cleaved-PARP expression levels in J82 cells treated with MK-4 (25μM, 50μM and 100μM) for 24 hrs. (b) Representative results of western blotting analysis of the same proteins expression levels in J82 treated cells with 100μM of MK-4 in time-dependent conditions (12, 24 or 48hrs). β-actin served as loading control. Molecular weight of the analyzed proteins is as follows: PUMA (21kDa), BAX (21kDa), PARP (116kDa), cleaved-PARP (89kDa), β-actin (42kDa).

Treatment of vitamin K_2 upregulates the expression of PUMA and cleaved-PARP proteins but does not increase the expression of the proapoptotic protein Bax in LNCaP cells (Fig 5S). In both experiments, vitamin

K_2 treatment to LNCaP cells did not induce any significant change in the expression of BAX, whereas the expression of PUMA and cleaved-PARP was increased, especially after treatment for 48hrs (Fig S6a and Fig S6b).



Supplementary fig. 5 Western blot analysis of PUMA, BAX, PARP and cleaved-PARP expression in PC-3 prostate tumor cells in a dose- and time-dependent manner. Immunoblotting was determined by using the corresponding antibodies as detailed in Materials and Methods. (a) Representative blots of PUMA, BAX, PARP and cleaved-PARP expression levels in PC-3 cells treated with MK-4 (25μM, 50μM and 100μM) for 24 hrs. (b) Representative results of western blotting analysis of the same proteins expression levels in PC-3 cells treated with MK-4 (100μM) for 12, 24 or 48hrs (time-dependent conditions). β-actin served as loading control. Molecular weight of the analyzed proteins is as follows: PUMA (21kDa), BAX (21kDa), PARP (116kDa), cleaved-PARP (89kDa), β-actin (42kDa).



Supplementary fig. 6 Western blot analysis of PUMA, BAX, PARP and cleaved-PARP expression in LNCaP prostate tumor cells in a dose- and time-dependent manner. Immunoblotting was determined by using the corresponding antibodies as detailed in Materials and Methods. (a) Representative blots of PUMA, BAX, PARP and cleaved-PARP expression levels in LNCaP cells treated with MK-4 (25μM, 50μM and 100μM) for 24 hrs. (b) Representative results of western blotting analysis of the same proteins expression levels in LNCaP cells treated with MK-4 (100μM) for 12, 24 or 48hrs (time-dependent conditions). β-actin served as loading control. Molecular weight of the analyzed proteins is as follows: PUMA (21kDa), BAX (21kDa), PARP (116kDa), cleaved-PARP (89kDa), β-actin (42kDa).

Discussion

The identification of MK-4-dependent suppression of cancer cell growth and its direct target genes is critical for understanding the biological significance of the vitamins in prostate/bladder cancer development, progression and metastasis. It has been reported that treatment with MK-4 might regulate the expression of a number of important genes that play a role in cell proliferation, cellular division, differentiation and apoptosis in prostate and bladder cancer. It is suggested that these regulated genes might be cancer-related genes and therefore, this vitamin produced by UBIAD1 could be a new mechanism for regulating gene expression in human tumors. Vitamin K compounds contain a quinone group in their structure similar to some widely used antitumor agents such as Adriamycin and daunorubicin. The quinone group is involved in cell growth inhibition and anti-tumor activity through the generation of free radicals and active oxygen species (Dorr 1996). In the present study, a series of experimental procedures of MK-4 have been conducted in human bladder and prostate carcinoma cell lines by exposure at various doses for 24 and 48hrs. Our data demonstrated that MK-4 markedly reduced prostate (PC-3 and LNCaP) and bladder (T24 and J82) cell viability and cell cycle progression in both dose- and time-dependent manner.

To explore the antigrowth-inducing effect of MK-4, bladder and prostate tumor cell lines were treated with the vitamin (5µM, 10µM, 25µM, 50µM and 100µM) for 24 or 48hrs. The results indicated that MK-4 exerts an anti-proliferative action in a dose- and time-dependent on T24 and J82 (bladder) and PC-3 and LNCaP (prostate) cancer cell lines. These findings are in accordance with prior studies that showed same effects of the vitamin in a variety of cancer cells including leukemia, lung cancer, human ovarian cancer cells, lung carcinomas and hepatocellular carcinoma (Miyazawa et al. 2001; Yokoyama et al. 2005; Sibayama-Imazu et al. 2008; Yoshida et al. 2003; Otsuka et al. 2004).

Furthermore, it was necessary to determine whether apoptosis contributed to the anti-growth activity of the menaquinone. Flow cytometry indicated that treatment with MK-4 induced a concentration-dependent augmentation (with 50µM and 100µM) as well as a time-dependent increase (a great significance for 48hrs exposure) of apoptotic cells, particularly for bladder cancer cells.

In order to broaden our understanding to the investigation in the mechanisms involved in MK-4-induced apoptosis in the human prostate and bladder carcinoma cells, essential proteins-mediated apoptosis was examined. In this current study, a significant upregulation of PUMA, Bax and cleaved-PARP proteins was observed in both bladder and prostate cell lines, especially with MK-4 at 50μM and 100μM (dose-dependent) treated for 24hrs and 48hrs (time-dependent). Therefore, MK-4 acquires dose- and time-dependent apoptotic-induced effects via the activation of key proteins (PUMA, Bax and cleaved-PARP) that eventually mediate apoptotic cell death in bladder and prostate cancer cells. These results observed in bladder) and prostate cancer cells are significantly consistent with previous reports using MK-4 in various human cancer cells (Karasawa

et al. 2013; Wei, Wang, and Carr 2010; Showalter et al. 2010; Sada et al. 2010; Li et al. 2010; Kawakita et al. 2009; Yokoyama et al. 2008; Shibayama-Imazu, Aiuchi, and Nakaya 2008; Yokoyama et al. 2005).

In the contrary of bladder cancer cells, Bax was not highly expressed in prostate cancer cells even with larger doses of MK-4 exposure (25µM, 50µM, 100µM) for 24 and 48hrs. These observations suggest that apoptosis induced by menaquinone might not be mediated by the intrinsic pathway in prostate cells. The intrinsic pathway (mitochondrion-mediated cell death) is mainly executed by the mitochondrial protein Bax that initiates the release of cytochrome c into the cytosol to form apoptosomes, and the activation of downstream caspase-9 which in turn activates caspase-3 (Green and Kroemer 2004). The extrinsic pathway is a death receptor-induced pathway initiated by the ligation of death receptors which activates p53 phosphorylation and contributes to caspase-8 expression followed by caspase-3 (Cheung, Arora, and Korneluk 2006). The induction of apoptosis after pre-treatment with VK2 in Smmc-7721 HCC cells was found independent of the intrinsic apoptotic pathway and primarily associated with p53 activation (15). This independent cell apoptosis induced by MK-4 in cancer cells is still hypothetical as it can be contradictory from a cancer cell type to another. In bladder cells, MK-4 binds to the pro-apoptotic protein Bax that activates the acidic phospholipid cardiolipin (reactive oxygen species generation-dependent) (Green and Kroemer 2004) which in turn induces the release of cytochrome c from the mitochondria and subsequently results in activating caspase-3. Conversely, the vitamin may stimulate the extrinsic apoptosis pathway in prostate cells by activating caspase-8 prior the induction of caspase-3. The intermediate factors involved in the mechanism of menaquinone-dependent activation of caspase-3 are yet to be confirmed. These mediators include c-Jun N-terminal kinase (JNK), extracellular-signal-related kinase (ERK), mitogen-activated protein kinase (MAPK) and p53 pathways and require further investigation. In addition, it is important to further investigate the expression level of caspase-8 in prostate cancer cells to determine whether the extrinsic pathway also initiates the mechanism of vitamin-dependent induction of cell death in prostate cells.

Moreover, a future study investigating the down-regulation of caspase-9 in prostate cells should be conducted with the aim to confirm that the antitumor effect of MK-4 generates an apoptosis totally not mediated by the intrinsic pathway (mitochondrial pathway) in this type of cancer cells.

Conclusion

MK-4 activity is capable of restricting the growth of cancer cell lines in *vitro* by promoting cell-cycle arrest and apoptosis via a specific signalling pathway. VK2 (menaquinone) seems to be a promising and safe chemotherapeutic candidate without prominent adverse drug effects that might be an option of using it as a therapeutic medicine (alone or combined to other chemotherapy agents) for the prevention and/or treatment of patients with bladder and prostate cancer. The combination of MK-4 with other antitumor agents should be also considered in future animal studies and are expected to synergistically induce better effects in the restriction/suppression of tumor cell growth.

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This paper is dedicated to the memory of Professor Hong Ling who has suddenly passed away on February 8th, 2020 amid coronavirus epidemic (COVID-19) in China. A sincere gratitude to him as he was my former Supervisor, for the support he has given me to achieve this research. I particularly thank Dr. Fengsen Duan for assisting me and guiding me all along my project. Thank you to Chinese Scholarship Council (CSC) for covering our studies and to Huazhong University of Science and Technology for providing us technical assistance to produce and complete this research during my PH.D. research. I am also thankful to the school of Life Sciences and Technology for making the platform of instruments available for our data analyses as well as their technical assistance.

Statistical analyses

Results are shown as the mean ± standard deviation (SD). Statistical data were compared using the Student's *t*-test and $p < 0.05$ as statistically significant. All experiments have been repeated at least four times.

Compliance with ethical standards

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Authors' contributions

Professor Ling Hong and Samira Zohra Midoun came up with the research project and contributed to the conception of the experimental research design. Samira Zohra Midoun performed the experiments and analyzed the data. Yongle Chen, Yang Hu and Yanshun Li performed cell culture experiments. Samira Zohra Midoun wrote the manuscript. Professor Ling Hong was responsible for the overvall supervision. All authors reviewed the manuscript.

Data availability

The relevant data are all available in this research paper as well as in the part of additional materials.

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