$K_9(C_4H_4FN_2O_2)_2Nd(PW_{11}O_{39})_2 \cdot 25H_2O$ induces apoptosis in human lung cancer A549 cells

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Abstract. Lung cancer is the leading cause of cancer-associated mortality worldwide. The present study investigated the effects of $K_9(C_4H_4FN_2O_2)_2Nd(PW_{11}O_{39})_2 \cdot 25H_2O$ (FNdPW), a chemically synthesized polyoxometalate that contains rare earth elements, on lung cancer growth, and explored the mechanism underlying its actions. The effects of FNdPW on the cell viability and apoptosis of human lung cancer A549 cells were measured using MTT assay, acridine orange/ethidium bromide staining and electron microscopy. The expression of apoptosis-related proteins, including B-cell lymphoma (Bcl)-2-associated death promoter (Bad), phosphorylated (p)-Bad, X-linked inhibitor of apoptosis (XIAP), apoptosis-inducing factor (AIF), Bcl-2-associated X protein (Bax) and Bcl-2, was determined by western blotting. Caspase-3 activity was measured using a caspase-3 activity kit. After 72 h of incubation, FNdPW reduced cell viability and induced apoptosis in A549 cells in a concentration- and time-dependent manner. FNdPW upregulated the pro-apoptotic Bad and Bax proteins, and downregulated the anti-apoptotic p-Bad, Bcl-2 and XIAP proteins. Furthermore, FNdPW also enhanced caspase-3 activity and increased the protein level of AIF in A549 cells, which was independent of the caspase-3 pathway. These events were associated with the regulation exerted by FNdPW on multiple targets involved in A549 cell proliferation. Therefore, FNdPW may be a novel drug for the treatment of lung cancer.

Introduction

Lung cancer is currently the leading cause of cancer-associated mortality worldwide, with an estimated 87,750 mortalities in males and 72,590 in females in 2012 (1). According to its histopathological classification, lung cancer can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC), which is the most common histological type of lung cancer. The incidence of NSCLC has increased annually; it is a heterogeneous disease that is difficult to treat, and remains the leading cause of cancer-associated mortality worldwide (2). In recent years, despite recent advances in surgery, irradiation, chemotherapy and targeted therapy, the 5-year survival rate of patients with advanced stage NSCLC has remained markedly low (3). Traditional chemotherapy has been widely used in the treatment of NSCLC, but severe side effects and drug resistance have limited its use in the clinic. Therefore, alternative therapeutic drugs that can effectively treat lung cancer have garnered recent attention (4,5).

Since 1970, polyoxometalates have been widely used in analytical chemistry, medicine, catalysis and material science (6), and a large number of them are potential antitumor, antiviral and antibacterial drugs (7,8). The biological effects of rare earth elements have attracted much attention (9-11), since they exhibit inhibitory effects on tumor cells such as B16 mouse melanoma cells, HeLa human cervical cancer cells, HepG2 human liver carcinoma cells and PAMC82 human gastric cells (12). Rare earth elements can inhibit tumor growth; thus, it is plausible that the introduction of rare earth elements into the structure of polyoxometalates may enhance their antitumor activities. $K_9(C_4H_4FN_2O_2)_2Nd(PW_{11}O_{39})_2 \cdot 25H_2O$ (FNdPW) is a chemically synthesized drug that contains synthesized rare earth elements, which have a low cytotoxicity (8). However, the physiological and pathophysiological roles of FNdPW in A549 cells and its underlying mechanisms of action remain to be determined. In the present study, it was demonstrated that FNdPW significantly decreased the viability of human A549 lung cancer cells and induced apoptosis, with significant activation of caspase 3-dependent and caspase 3-independent signaling pathways.

Materials and methods

Reagents. Antibodies against B-cell lymphoma (Bcl)-2 (D160117) and Bcl-2-associated X protein (Bax) (D120073) were purchased from Sangong Biotech Co., Ltd. (Shanghai, China). Antibodies against Bcl-2-associated death promoter (Bad) (9293), phosphorylated (p)-Bad (5284),

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apoptosis-inducing factor (AIF) (4642) and X-linked inhibitor of apoptosis protein (XIAP) (14334) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Other chemicals were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

A549 cell culture and drug chemical synthesis. A549, a human lung cancer cell line, was provided by Harbin Medical University (Harbin, China). Cells were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an incubator containing humidified air with 5% (v/v) CO_2. FNdPW was purchased from the Department of Inorganic Chemistry of Harbin Medical University.

Cell viability assay. Cells were seeded in 96-well plates at a density of $8x10^3$ cells/well 24 h prior to treatment. Cells were treated with FNdPW ($3.17x10^{-7}$, $6.69x10^{-7}$, $3.02x10^{-6}$, $5.87x10^{-6}$ or $8.52x10^{-6}$ mol/l). After 72 h, 15 μl (5 mg/ml) MTT (Sigma-Aldrich; Merck Millipore) was added to each well and incubated at 37˚C for 4 h. Then, the MTT solution was removed, and 150 μl dimethyl sulfoxide was added to dissolve the crystals. Then, the mixture was agitated for 10 min to fully dissolve the crystals. A microplate reader (Tecan Group Ltd., Männedorf, Switzerland) was used to measure the absorbance at a wavelength of 570 nm. Cell viability was expressed as the percentage change in the absorbance values of the treated group compared with the control group.

Electron microscopy (EM). A549 lung cancer cells were cultured in 60-mm plates, collected in PBS and fixed with 2% (v/v) paraformaldehyde containing 2.5% (w/v) glutaraldehyde (Paesel & Lorei GmbH & Co. KG, Duisburg, Germany) buffered with Hank's modified salt solution at 4˚C for 4 h. The cells were further fixed in 1% (w/v) osmium tetroxide solution buffered with 0.1 M cacodylate (pH 7.2) at 4˚C for 2 h. Subsequently, cells were scraped off the plastic and dehydrated in ethanol. Dehydration was completed in propylene oxide. The specimens were embedded in Araldite (SERVA Electrophoresis GmbH, Heidelberg, Germany). Ultrathin sections were produced on an FCR Reichert Ultracut ultramicrotome (Leica Microsystems, Inc., Buffalo Grove, IL, USA), mounted on pioloform-coated copper grids and contrasted with lead citrate. Specimens were analyzed and documented by EM (10A; Zeiss GmbH, Jena, Germany).

Acridine orange (AO)/ethidium bromide (EB). A549 cells in exponential growth phase were cultivated on sterile coverslips for 24 h and subsequently treated with $5.87x10^{-6}$ mol/l FNdPW for 72 h. The cells were washed twice with PBS and then mixed with 1 ml dye mixture containing 100 mg/ml AO and 100 mg/ml EB in PBS (13). Cellular morphological changes were examined using fluorescence microscopy (magnification, x200). The percentage of apoptotic cells was calculated with the following formula: Apoptotic rate (%) = number of apoptotic cells/number of total cells (14,15).

Western blot analysis. Total protein samples were extracted from A549 cells. Cells cultured on 25-mm dishes or 6-well plates were lysed in a lysis buffer (P0013; Beyotime Institute of Biotechnology, Haimen, China) containing protease and phosphatase inhibitors. After centrifugation at 13,500 x *g* for 15 min at 4˚C, cell lysates were collected. Protein concentration was assessed using the bicinchoninic acid assay. Aliquots of protein were then mixed with Laemmli sample buffer and boiled at 100˚C for 5 min. Samples (60 μg protein) were resolved on 10-12% SDS-PAGE, followed by transfer to nitrocellulose membranes. For visualization, blots were probed with antibodies against Bcl-2 (1:1,000 dilution), Bax (1:1,000 dilution), Bad (1:500 dilution), p-Bad (1:500 dilution), XIAP (1:500 dilution), AIF (1:500 dilution) and GAPDH (1:1,000 dilution) at room temperature (21-23˚C) for 1 h. Signals were detected using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution; Cell Signaling Technology, Inc.) for 1 h at room temperature. The obtained digital images of the western blots were used for densitometry determinations with Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). Western blot bands were quantified using Odyssey version 3.0 software (LI-COR Biosciences, Lincoln, NE, USA) by measuring the band intensity (area x absorbance) for each group and normalizing the value to that of the GAPDH band, which served as an internal control.

Caspase-3 activity assay. Caspase-3 activity was analyzed using a caspase-3 activity assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol, using the substrate peptides acetyl (Ac)-DEVD-p-nitroanilide (pNA), Ac-IETD-pNA and Ac-LEHD-pNA (Beyotime Institute of Biotechnology). Briefly, the supernatant of the cell lysates was mixed with buffer containing the above substrate peptides to allow the binding of caspase-3 to pNA. The release of pNA was quantified by determining the absorbance with an ELISA reader at 405 nm. The caspase activities were expressed as a percentage over the control.

Data analysis. Data are shown as means ± standard deviation of 3-6 independent experiments, and were evaluated using an unpaired Student's *t*-test. Statistical analysis was conducted with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

FNdPW suppresses the viability of A549 cells. The antiproliferative effect of FNdPW on A549 cells was examined by exposing the cells to different concentrations ($3.17x10^{-7}$, $6.69x10^{-7}$, $3.02x10^{-6}$, $5.87x10^{-6}$ or $8.52x10^{-6}$ mol/l) of FNdPW for 72 h. Cell growth was inhibited in a dose- and time-dependent manner (Fig. 1). In the presence of $5.87x10^{-6}$ mol/l FNdPW, A549 cells exhibited ~50% inhibition of proliferation after treatment for 72 h. Thus, this concentration and treatment time were used in subsequent experiments.

FNdPW induces apoptosis in A549 cells. Cell viability is a balance between proliferation and apoptosis (16). To investigate whether FNdPW regulates apoptosis, AO/EB staining and EM were used to detect the number of apoptotic cells. The results from our fluorescence microscopy analysis are shown

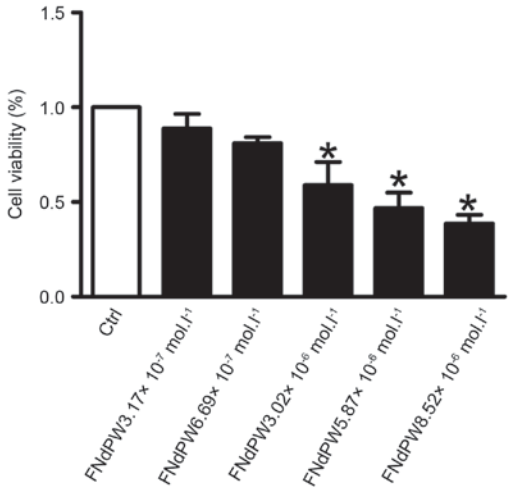

Figure 1. FNdPW inhibits the survival of A549 cells. A549 cells were treated with FNdPW at concentrations of 3.17x10-7, 6.69x10-7, 3.02x10-6, 5.87x10-6 or 8.52x10-6 mol/l for 72 h. Relative cell viability was determined by MTT assay. *P<0.05 compared with Ctrl (n=6 independent experiments for each condition). Ctrl, control; FNdPW, $K_9(C_4H_4FN_2O_2)_2Nd(PW_{11}O_{39})_2{\cdot}25H_2O$.

in Fig. 2A. Three types of cells were recognized under the fluorescence microscope: Living cells (green), apoptotic cells (yellow) and necrotic cells (red). FNdPW induced the presence of a substantial number of apoptotic cells (P=0.0365). Under EM, cells with FNdPW exhibited robust changes in their microstructure, including cell surface microvilli reduction, nuclear chromatin condensation, invagination and membrane blistering (Fig. 2B).

FNdPW activates pro-apoptotic signaling pathways. To explore the mechanisms by which FNdPW induces apoptosis in A549 cells, the downstream proteins of the FNdPW apoptotic pathway, including Bax, Bcl-2, Bad, p-Bad and XIAP, were determined. Fig. 3 demonstrates that FNdPW upregulated Bad, Bax and AIF, and downregulated p-Bad, Bcl-2 and XIAP expression (Fig. 3A, B and D). In addition, to determine the potential toxic effects of FNdPW, the cellular distributions of AIF were detected by western blotting. As shown in Fig. 3E, FNdPW increased the protein level of AIF in the nucleus, which is independent of the caspase-3 (Fig. 3C) pathway.

Discussion

Our present understanding of the precise mechanisms underlying the use of FNdPW for treating human cancer is still incomplete (17). In our study, it was demonstrated that FNdPW, a polyoxometalate that includes rare earth elements, has anti-proliferative effects on A549 cells by causing apoptosis. In addition, it was identified that activation of both caspase-3-dependent and caspase-3-independent pathways are mechanisms underlying FNdPW-induced apoptosis, which led to the suppression of liver cancer cell growth. Therefore, the current study provides new insights into the pathophysiological role of FNdPW in lung cancer diseases.

Lung cancer is the most common malignant cancer worldwide, and is associated with high fatality rates in humans. The use of chemotherapy for the clinical management of lung cancer causes notable side effects (18,19); therefore, new effective drugs to treat lung cancer are required. Previous studies have shown that polyoxometalates exert their antitumor properties by regulating cell invasion, proliferation and migration in a variety of malignancies such as breast, kidney, lung, ovary, pancreas and prostate cancer (17). However, FNdPW has not been reported to induce apoptosis in human lung cancer cells. It appears that the ability of FNdPW to inhibit cell proliferation may depend upon the cell type; different cell types have different predominant apoptotic signaling pathways. Apoptosis (programmed cell death), is an important homeostatic mechanism balancing cell division and cell death, while maintaining the appropriate cell number in the body (20). Therefore, the identification of novel drugs that trigger the apoptosis of tumor cells has become an attractive strategy in anticancer drug research (21). The Bcl-2 family members are essential intracellular players in the apoptotic machinery (22). Several studies have reported that Bax, Bcl-2 and caspase-3 are key molecules that cause apoptosis in lung cancer cells (23). Combination treatment with triptolide and hydroxycamptothecin synergistically enhances apoptosis by increasing the ratio of Bax/Bcl-2 in A549 cells (24). Formononetin-induced apoptosis was accompanied by upregulation of Bax and downregulation of Bcl-2, with the consequent dysfunction of mitochondria and activation of caspase-3 in A549 cells (25). *Cactus pear* extracts increased caspase3 and Bax protein levels, and decreased the Bcl-2 protein level in the lung cancer cell line A549 (26). *Cochinchina momordica* seed induces lung cancer cell apoptosis by activating caspase-3 via upregulation of Bax and downregulation of Bcl-2 (27). In the present study, it was observed that the expression of Bcl-2 protein in A549 cells was significantly lower than that in the control group (P=0.0396), whereas the expression of Bax protein was significantly higher than that in the control group (P=0.0236). The ratio of Bcl-2/Bax significantly decreased upon treatment with FNdPW, indicating that FNdPW induced apoptosis in A549 cells. In addition, FNdPW treatment significantly increased the levels of Bax and decreased the levels of Bcl-2. These data indicated that the caspase-3-dependent apoptotic signaling pathway serves a critical role in the anticancer activity of FNdPW.

Bad, a member of the Bcl-2 family, normally binds to the Bcl-2/Bcl-extra large (xL) complex and triggers apoptosis. However, p-Bad dissociates from this complex, resulting in the release of Bcl-2/Bcl-xL and the suppression of apoptosis (28). XIAP interacts with caspase-3 to block its full activation, substrate cleavage and cell death (29). To fully understand how FNdPW increases the ratio of Bax over Bcl-2 and activates caspase-3, the present study examined the levels of Bad, p-Bad and XIAP proteins, and noticed that FNdPW treatment significantly increased the levels of Bad and decreased the levels of p-Bad and XIAP .These data indicated that caspase-3-dependent apoptotic signaling serves a critical role in the anticancer activity of FNdPW.

The caspase family is important in apoptosis, with the cysteine aspartic acid protease caspase-3 being a key molecule that transmits apoptotic signals in multiple signaling pathways (30). The present study demonstrated that caspase-3 activity expression in cells increased following treatment with FNdPW. Although caspases are important mediators of

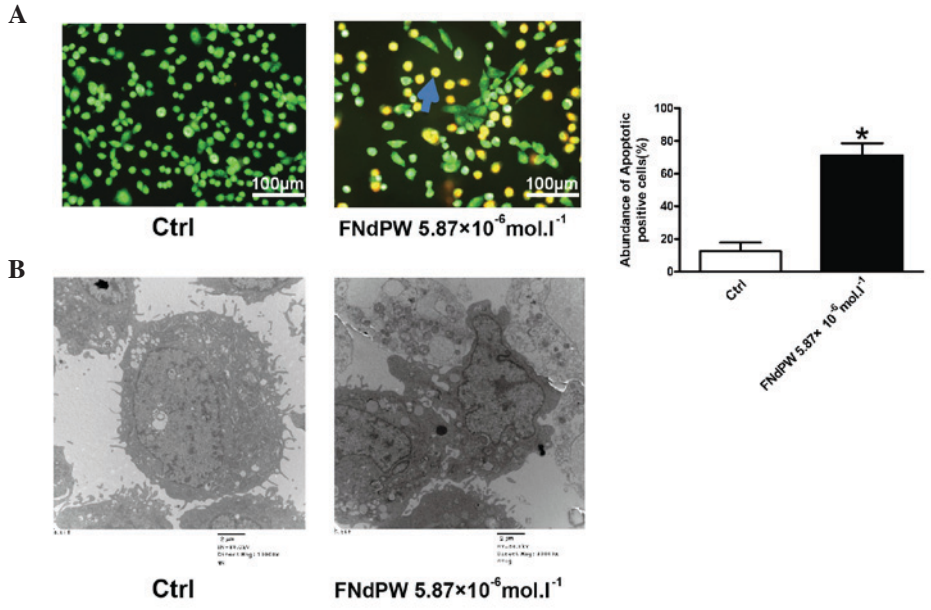

Figure 2. FNdPW induces apoptosis in A549 cells. (A) AO/EB staining was used to detect changes in the nucleus. Statistical bar graph of apoptotic cells by AO/EB staining. Blue arrow indicates apoptotic cells. (B) Transmission electron microscopy was employed to estimate micro-morphological changes (magnification, x10,000). *P<0.05 compared with Ctrl (n=3 independent experiments for each group). Ctrl, control; FNdPW, $K_9(C_4H_4FN_2O_2)_2Nd(PW_{11}O_{39})_2{\cdot}25H_2O$; AO, acridine orange; EB, ethidium bromide.

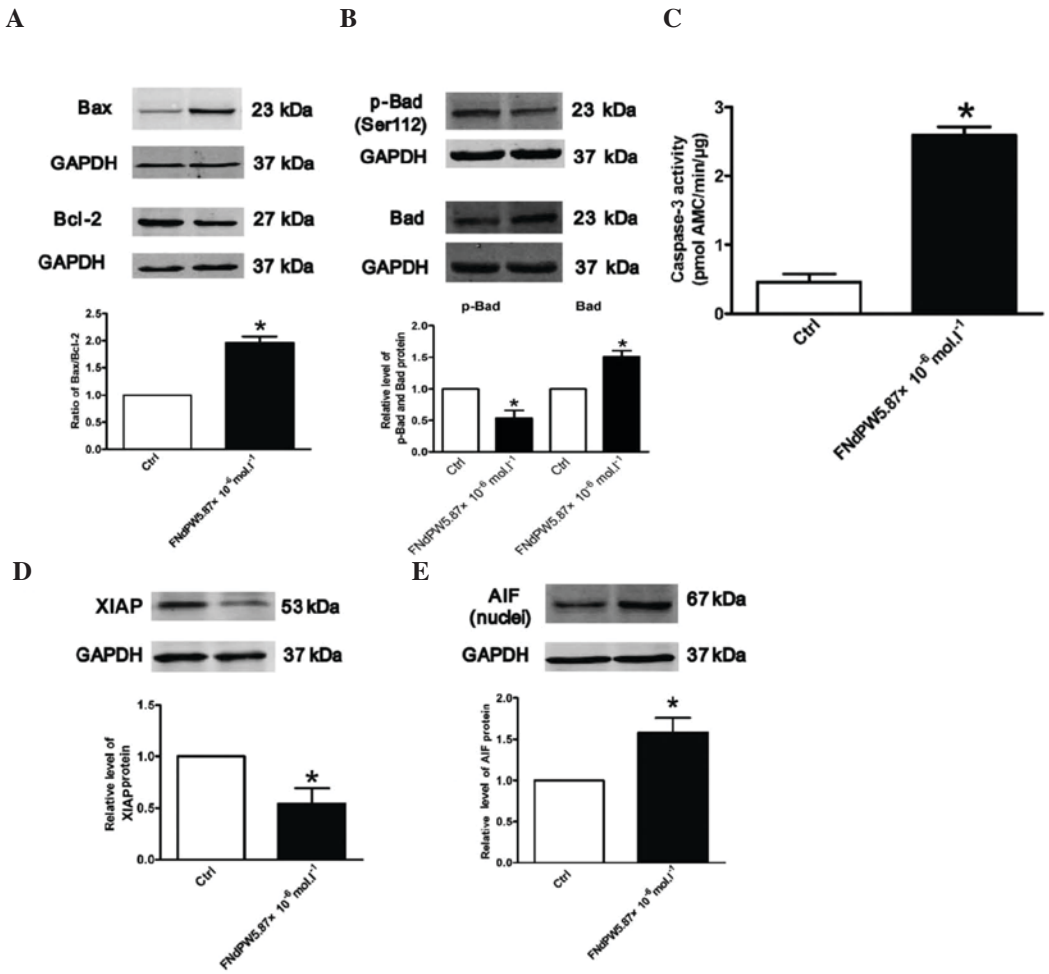

Figure 3. FNdPW alters p-Bad, Bad, Bax, Bcl-2, XIAP and AIF expression, and promotes caspase-3 activation. Western blotting was used to detect (A) Bax, Bcl-2, (B) p-Bad, Bad, (D) XIAP and (E) AIF expression in A549 cells treated with FNdPW. Relative expression of Bax, Bcl-2, p-Bad, Bad, XIAP and AIF was normalized to GAPDH (n=3 independent experiments per each group). (C) Activation of caspase-3 by FNdPW. Data are averaged from five independent experiments for each group. *P<0.05 compared with Ctrl. Bcl, B-cell lymphoma; Bax, Bcl-2-associated X protein; Bad, Bcl-2-associated death promoter; p, phosphorylated; AIF, apoptosis-inducing factor; XIAP, X-linked inhibitor of apoptosis protein; Ctrl, control; FNdPW, $K_9(C_4H_4FN_2O_2)_2Nd(PW_{11}O_{39})_2{\cdot}25H_2O$.

apoptosis, there is accumulating evidence for the existence of caspase-independent mechanisms of cell death (28,29). AIF is a putative caspase-independent effector of cell death that has recently been cloned and characterized. It is a mitochondrial intermembrane flavoprotein that is released from mitochondria and translocates to the nucleus in response to specific death signals (31,32). The present study observed that FNdPW treatment resulted in the translocation of AIF from mitochondria to the nucleus, suggesting that the caspase-3-independent signaling pathway is involved in the apoptosis induced by FNdPW.

In conclusion, the present study demonstrated for the first time that FNdPW markedly inhibits cell proliferation by inducing apoptosis in A549 cells. This event is likely to be associated with multiple targets involved in A549 cell proliferation which are regulated by FNdPW. FNdPW increased the expression of Bad and decreased the level of p-Bad, which subsequently mediated an increase in the ratio of Bax over Bcl-2, thus leading to the activation of caspase-3. Our findings suggest that FNdPW is a potential drug for the clinical treatment of lung cancer, provided that FNdPW can be applied to the right types of lung cancer in the adequate cellular context.

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