Differential effect of psoralidin in enhancing apoptosis of colon cancer cells via nuclear factor-κB and B-cell lymphoma-2/B-cell lymphoma-2-associated X protein signaling pathways

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Received December 2, 2014; Accepted August 7, 2015

DOI: 10.3892/ol.2015.3861

Abstract. Worldwide, colon cancer is the third most common cancer in terms of incidence, following lung and breast cancer. Resistance to psoralidin frequently occurs following its use as an anticancer treatment. However, the mechanisms underlying the effects of psoralidin on colon cancer, remain to be elucidated. Hence, the present study investigated the anticancer effects and potential mechanism of action of psoralidin on SW480 human colon cancer cells. In the present study, an MTT assay was performed to measure the viability of SW480 cells. Additionally, an Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit, DAPI staining assay and caspase-3 colorimetric assay kits were used to analyze the cellular apoptosis of SW480 cells. The nuclear factor-κB (NF-κB) p65 activity and B-cell lymphoma-2 (Bcl-2)/Bcl-2-associated X protein (Bax) protein expression of SW480 cells was detected using NF-κB colorimetric assay kits and western blot analysis, respectively. Bcl-2 inhibitor ABT-737 was added to SW480 cells and the subsequent effects and mechanism of action of psoralidin on SW480 colon cancer cells was studied. In the present study, psoralidin reduced SW480 cell viability and enhanced the cellular apoptosis of SW480 cells in a dose-dependent manner. Caspase-3 activity of SW480 cells was increased following treatment with psoralidin. Additionally, psoralidin was able to reduce the NF-κB p65 activity of SW480 cells. Furthermore, psoralidin was able to reduce Bcl-2 protein expression and increase Bax protein expression in SW480 cells. Notably, Bcl-2 inhibitor was observed to enhance the effects of psoralidin on SW480 cells. The results of the present study suggest that psoralidin may be a candidate drug for the treatment of colon cancer by inhibition of the NF-κB and Bcl-2/Bax signaling pathways.

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Key words: nuclear factor-κB, B-cell lymphoma-2/B-cell lymphoma-2-associated X protein, colon cancer, psoralidin

Introduction

Colon cancer is a type of malignancy with high levels of incidence; worldwide, it is the third most commonly occurring malignancy in males, and the second most common in females (1). According to estimates from the International Agency for Research on Cancer, ~1.2 million novel cases of colon cancer were diagnosed in 2008, of which ~8% led to mortality (2). Colon cancer is more common in developed countries and regions, however due to economic development and the acceleration of urbanization, the diet and lifestyle of the population in developing countries have been altered (3). In recent years, the incidence and mortality of colon cancer has demonstrated an increasing trend in developing countries, which is higher than the world average (4).

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that is able to mediate inflammation and the immune response (5). Previous studies have revealed that NF-κB is associated with the occurrence and development of tumors, the inhibition of apoptosis and the induction of drug resistance (6,7). Identifying a natural inhibitor of NF-κB activity, and blocking the NF-κB signaling pathway which inhibits apoptosis, may be able to provide promising drug candidates for the treatment of malignant tumors (8). Yu *et al* (9) demonstrated that berberine was able to enhance the chemosensitivity of colon cancer cells to irinotecan via the suppression of NF-κB. Tanwar *et al* (10) concluded that etoricoxib reduced colon cancer development by inhibition of NF-κB.

The levels and interaction of B-cell lymphoma-2 (Bcl-2) family gene products are important for the regulation of apoptosis, during which the ratio of Bcl-2/Bcl-2-associated X protein (Bax) is critical (11). Investigating the expression of Bcl-2/Bax may be significant in the improvement of the study, diagnosis, treatment efficacy and prognosis assessment of tumors (12,13). Ko *et al* (14) demonstrated that soy soluble polysaccharide induced Bcl-2/Bax-mediated apoptosis of HCT-116 human colon cancer cells. Mao *et al* (15) reported that gastrin accelerated the action

of the cell apoptosis regulation complex Bcl-2/Bax in large intestine carcinoma. Zhao *et al* (16) revealed that β-sitosterol inhibited cell growth and induced apoptosis of human stomach cancer cells via a reduction of the Bcl-2/Bax ratio.

Current research has revealed that psoralidin contains a number of compounds, including coumarin, flavonoids and monoterpene phenols, which possess immunomodulatory, anti-inflammatory, antioxidant and anti-tumor effects (17). Furthermore, Hao *et al* (18) reported that psoralidin inhibited the proliferation of A549 human lung cancer cells through the generation of reactive oxygen species (ROS). Additionally, psoralidin has been observed to inhibit cell proliferation and induce apoptosis of androgen-independent prostate cancer cells through phosphatidylinositol 3-kinase-mediated Akt signaling (17). However, to the best of our knowledge, the mechanisms underlying the anticancer effects of psoralidin on colon cancer cells have not previously been studied. Therefore, in the present study, the mechanism of action of psoralidin was investigated in human colon cancer cells.

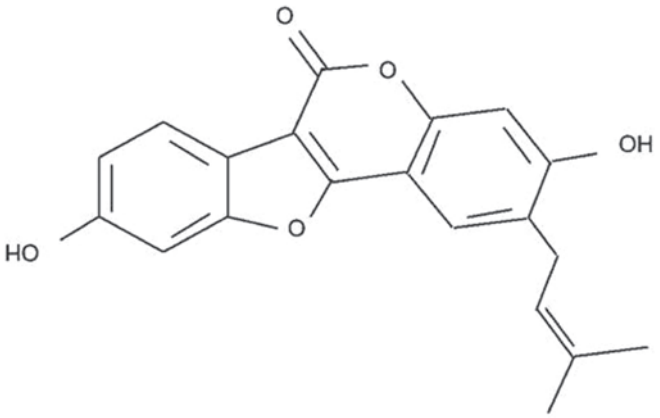

Figure 1. Chemical structure of psoralidin.

Materials and methods

Reagents and chemicals. The chemical structure of psoralidin (with purity ≥98%) is presented in Fig. 1. Psoralidin and DAPI reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium was obtained from KeyGen Biotechnology Co., Ltd. (Nanjing, China). Fetal bovine serum (FBS) was obtained from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). MTT was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit, caspase-3 colorimetric assay and NF-κB ELISA assay kits were purchased from Beyotime Institute of Biotechnology (Nanjing, China). ABT-737 was purchased from EMD Millipore (#HY-50907; Billerica, MA, USA).

Cell line and culture. The SW480 human colon cancer cell line was obtained from the Department of Oncology (Central Hospital of Jingzhou, Jingzhou, China). Cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37˚C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced every 2-3 days with fresh complete medium.

MTT assay. SW480 cells ($2.0x10^4$ cells/well) were cultured with psoralidin (0, 5, 10 and 20 μM) at 37˚C in a humidified atmosphere containing 5% CO_2 for 0, 24, 48 and 72 h in 96-well plates. SW480 cells were washed twice with phosphate-buffered saline (PBS; Sangon Biotech Co., Ltd.), prior to the addition of 10 μl MTT to each well. SW480 cells were incubated at 37˚C for 4 h. Subsequently, the culture medium was removed and 150 μl dimethyl sulfoxide (Invitrogen Life Technologies) was added to each well. SW480 cells were incubated for 20 min at room temperature with agitation. Cell viability of SW480 cells was determined by the MTT assay as described previously (19). Briefly, absorbance was measured at a wavelength of 570 nm using a microplate reader (2104-0010; PerkinElmer, Inc., Waltham, MA, USA). Cell viability was calculated as follows: Cell viability (%) = (mean absorbance of psoralidin treated groups/mean absorance of 0 μM psoralidin group) x 100.

Flow cytometric analysis of cellular apoptosis. SW480 cells ($2x10^6$ cells/well) were cultured with psoralidin (0, 5, 10 and 20 μM) (17) at 37˚C in a humidified atmosphere containing 5% CO_2 for 48 h in 6-well plates. SW480 cells were harvested and washed twice with cold PBS. Subsequently, SW480 cells ($1x10^6$ cells/ml) were resuspended in Annexin-V binding buffer. Annexin V-FITC (10 μl) was added and cells were incubated for 30 min in the dark at 4˚C. Subsequently, 5 μl PI was added to the cells and incubated for 10 min in the dark at room temperature. Cellular apoptosis of SW480 cells was immediately detected using flow cytometry (EPICS® ALTRA™; Beckman Coulter, Inc., Brea, CA, USA).

DAPI staining assay. SW480 cells ($2x10^6$ cells/well) were cultured with psoralidin (0, 5, 10 and 20 μM) at 37˚C in a humidified atmosphere containing 5% CO_2 for 0, 24, 48 and 72 h in 6-well plates. SW480 cells were washed twice with PBS. Subsequently, 0.5 ml 4% paraformaldehyde (Beijing DingGuo ChangSheng Biotechnology Co., Ltd., Beijing, China) was added to each well and fixed for 30 min at 4˚C. SW480 cells were washed twice with PBS, prior to the addition of sodium citrate (0.1%) containing 0.1% Triton X-100 and incubated for 5 min at 4˚C. Subsequently, DAPI was added to each well and incubated for 10-15 min at 4˚C in the dark. Apoptotic cells were excited by ultraviolet light. SW480 cells were observed and images were captured under a fluorescent microscope (Zeiss Axio Observer A1; Ziess AG, Oberkochen, Germany) at 340 nm.

Detection of caspase-3 activity. SW480 cells ($2x10^6$ cells/well) were cultured with psoralidin (0, 5, 10 and 20 μM) at 37˚C in a humidified atmosphere containing 5% CO_2 for 48 h in 6-well plates. Caspase-3 activity was detected by measuring fluorescence at a wavelength of 405 nm using a microplate reader (SN11693; Bio-Rad Laboratories, Inc., Hercules, CA, USA) with caspase-3 colorimetric assay kits.

Measurement of NF-κB activity. SW480 cells ($1x10^6$ cells/well) were cultured with psoralidin (0, 5, 10 and 20 μM) at 37˚C in a humidified atmosphere containing 5% CO_2 for 0, 24,

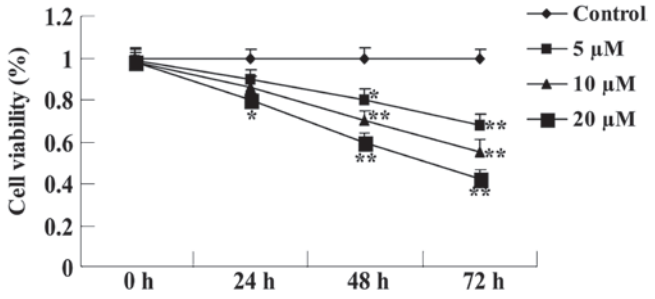

Figure 2. Psoralidin treatment reduces the viability of SW480 cells. Values are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 compared with the control group.

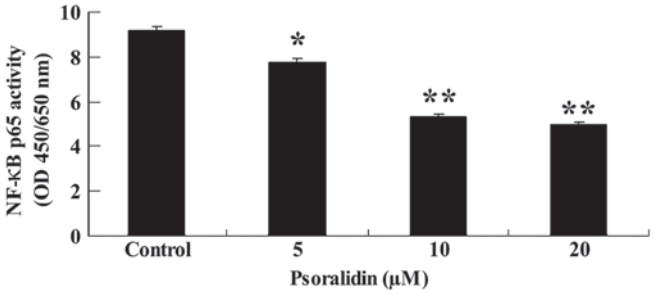

Figure 5. Psoralidin reduces the NF-κB activity of SW480 cells. Values are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 compared with control group. NF-κB, nuclear factor-κB; OD, optical density.

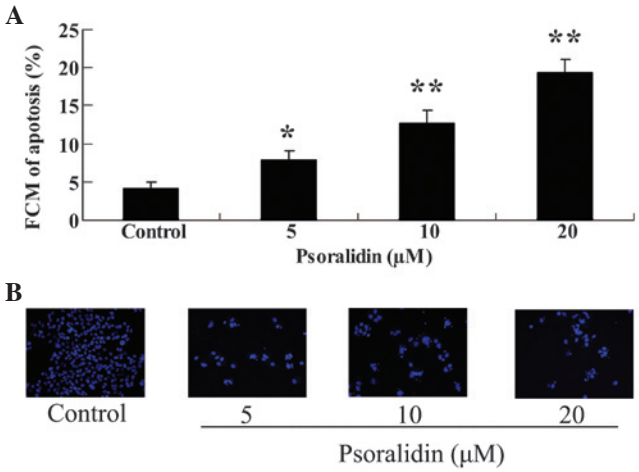

Figure 3. Psoralidin enhances cellular apoptosis of SW480 cells. Effect of psoralidin on the cellular apoptosis of SW480 cells examined by (A) flow cytometry and (B) DAPI staining assay (magnification, x200). Values are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 compared with control group. FCM, flow cytometry.

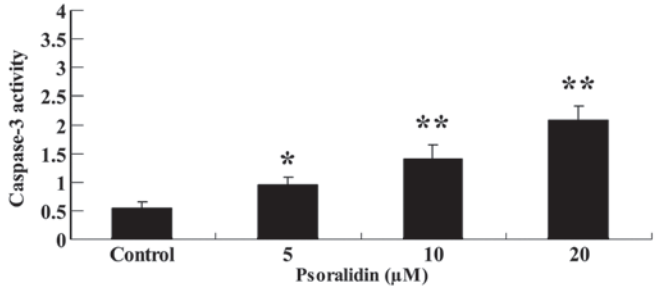

Figure 4. Psoralidin treatment enhances caspase-3 activity of SW480 cells. Values are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 compared with control group.

48 and 72 h in 6-well plates. Working solution (100 μl; 1% biotin labeling, 99% antibody) was added into every well and incubated at 37˚C for 1 h. Following incubation, the supernatant was dried and discarded, and 350 μl wash buffer was added to each well and washed for 1 min. Next, 3.90 μl tetramethylbenzidine substrate was added to each well, followed by incubation at 37˚C for 30 min in darkness. Then, 50 μl stop solution was added into every well. NF-κB activity was analyzed using ELISA assay kits (DRE20177; Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China) using a microplate reader (SN11693; Bio-Rad Laboratories, Inc.) at 450 nm, as described previously (20).

Western blot analysis. SW480 cells ($1x10^6$ cells/well) were cultured with psoralidin (0, 5, 10 and 20 μM) at 37˚C in a humidified atmosphere containing 5% CO_2 for 0, 24, 48 and 72 h in 6-well plates. SW480 cells were subsequently incubated with ice-cold lysis buffer (Beyotime Institute of Biotechnology) for 30 min on ice and then centrifuged at 12,000 x g for 10 min at 4˚C. The total protein concentration of soluble materials was determined using a bicinchoninic acid protein assay (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal quantities of protein were separated using 12% SDS-PAGE (Invitrogen Life Technologies) and then transferred onto a polyvinylidene difluoride membrane (0.22 mm; GE Healthcare Life Sciences, Piscataway, NJ, USA). Following blocking with Tris-buffered saline (Beyotime Institute of Biotechnology) containing 5% non-fat milk for 2 h, the membranes were incubated with monoclonal mouse anti-human Bcl-2 (1:1,500; cat. no. sc-7382; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), monoclonal mouse anti-human Bax (1:2,000; cat. no. sc-8432; Santa Cruz Biotechnology, Inc.) and monoclonal mouse anti-human β-actin (1:500; cat. no. sc-8432; Tiangen Biotech Co., Ltd., Beijing, China) overnight at 4˚C. The membrane was washed twice with Tris-buffered saline with Tween-20 (Sigma-Aldrich) for 2 h and then incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (1:1,000; cat. no. sc-358922; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Proteins were subsequently detected using enhanced chemiluminescence (DAB Horseradish Peroxidase Color Development Kit; Beyotime Institute of Biotechnology).

Effects of Bcl-2 inhibition. To further investigate the potential association between Bcl-2 inhibition and the effect of psoralidin on SW480 cells, SW480 cells were incubated with 1 μM Bcl-2 inhibitor (ABT-737; 99% purity; Sigma-Aldrich) and 10 μM psoralidin for 48 h at 37˚C in a humidified atmosphere containing 5% CO_2. The aforementioned MTT assay and western blotting protocols were then performed to determine cell viability and Bcl-2 protein expression levels, respectively.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Data were

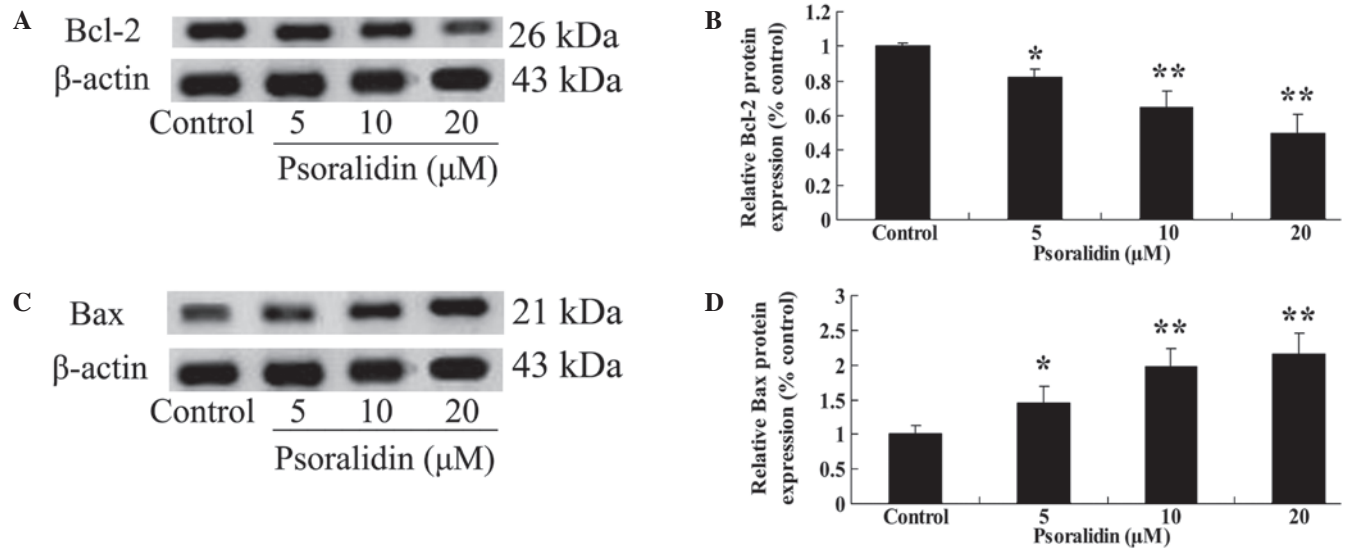

Figure 6. Psoralidin inhibits Bcl-2 and enhances Bax protein expression in SW480 cells. (A and B) Effect of psoralidin on Bcl-2 protein expression of SW480 cells by western blotting assay and statistical analysis of Bcl-2 protein expression of SW480 cells. (C and D) Effects of psoralidin on Bax protein expression of SW480 cells by western blotting assay and statistical analysis of Bax proteins expression of SW480 cells. Values are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 compared with control group. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

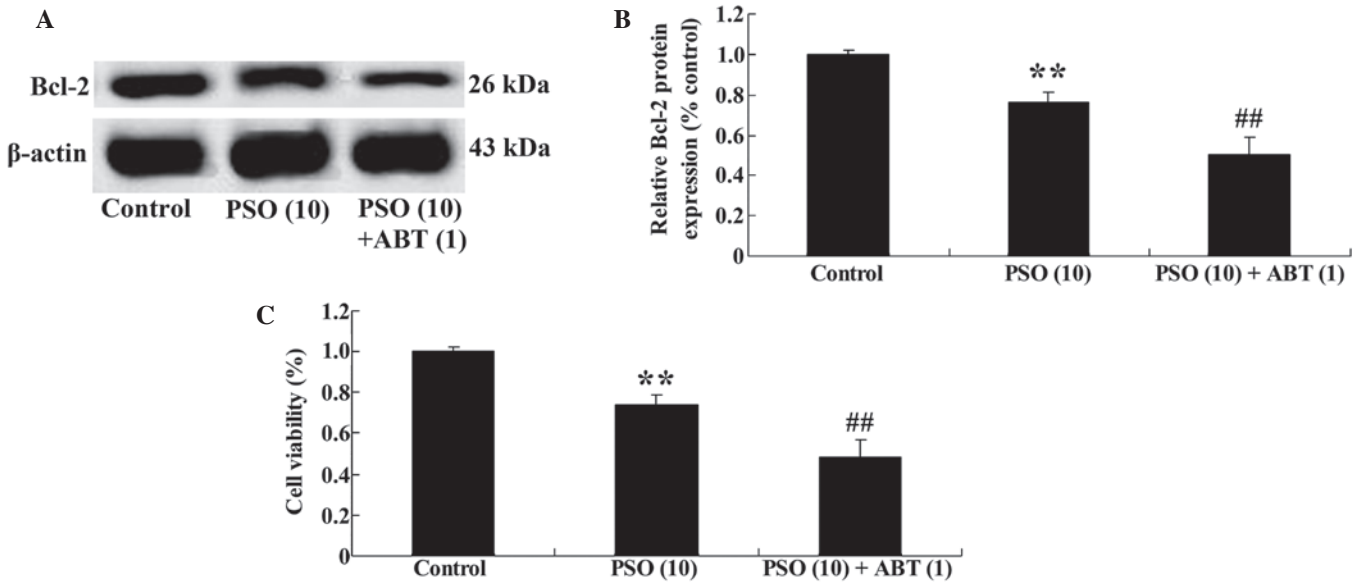

Figure 7. Bcl-2 inhibitor enhances the effect of psoralidin on SW480 cells. (A) Effect of Bcl-2 inhibitor on Bcl-2 protein expression of SW480 cells by western blotting assays; (B) statistical analysis of Bcl-2 protein expression of SW480 cells; and (C) Bcl-2 inhibitor enhances the effect of psoralidin on viability of SW480 cells. Values are expressed as the mean ± standard deviation. **P<0.01 compared with control group; $^{\#\#}$P<0.01 compared with PSO (10) group. Bcl-2, B-cell lymphoma-2; PSO, psoralidin; ABT, ABT-737.

analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Psoralidin treatment reduces the viability of SW480 cells. The anti-proliferative effect of psoralidin (0, 5, 10 and 20 μM) on the viability of SW480 cells was detected by MTT assay. As demonstrated in Fig. 2, psoralidin (5, 10 and 20 μM) treatment reduced the viability of SW480 cells in a dose- and time-dependent manner. This effect was particularly evident at a concentration of 20 μM, at which the proliferation of SW480 cells was significantly inhibited at 24, 48 and 72 h (P<0.01; Fig. 2). Additionally, treatment with 5 μM psoralidin for 72 h and 10 μM psoralidin for 48 and 72 h also significantly reduced SW480 cell viability (P<0.01; Fig. 2).

Psoralidin treatment promotes the apoptosis of SW480 cells. To determine whether apoptosis mediated the anti-proliferative effect of psoralidin (0, 5, 10 and 20 μM), apoptosis of SW480 cells was analyzed using flow cytometry and a DAPI staining assay. As demonstrated in Fig. 3A, psoralidin (0, 5, 10 and 20 μM) treatment promoted the apoptosis of SW480 cells in a dose-dependent manner. This effect of psoralidin was

particularly evident at concentrations of 10 and 20 μM, at which the apoptosis of SW480 cells at 48 h was significantly increased (P<0.01; Fig. 3A). In addition, treatment with 5 μM psoralidin for 72 h significantly enhanced the apoptosis of SW480 cells at 48 h (P<0.05; Fig. 3A). Notably, the results of the DAPI staining assay revealed that the apoptosis of SW480 cells was augmented in cells treated with psoralidin (5, 10 and 20 μM) at 48 h, compared with the control group (Fig. 3B).

Psoralidin treatment enhances caspase-3 activity of SW480 cells. To further investigate the anti-proliferative effect of psoralidin (0, 5, 10 and 20 μM) on the caspase-3 activity of SW480 cells, caspase-3 activity was measured using colorimetric assay kits. As demonstrated in Fig. 4, psoralidin (5, 10 and 20 μM) treatment increased the caspase-3 activity of SW480 cells in a dose-dependent manner. Following psoralidin (10 and 20 μM) treatment for 48 h, the caspase-3 activity of SW480 cells was significantly augmented (P<0.01), and following psoralidin (5 μM) treatment for 48 h, the caspase-3 activity of SW480 cells was also augmented (P<0.05; Fig. 4A and B).

Psoralidin treatment reduces the NF-κB activity of SW480 cells. The potential association between the anti-proliferative effect of psoralidin (0, 5, 10 and 20 μM) and NF-κB activity in SW480 cells was evaluated. NF-κB p65 activity was investigated using an ELISA assay kit. As demonstrated in Fig. 5, NF-κB p65 activity was reduced by psoralidin (5, 10 and 20 μM) treatment in a dose-dependent manner. NF-κB p65 activity was significantly decreased following psoralidin (10 and 20 μM) treatment for 48 h (P<0.01), and markedly decreased following psoralidin (5 μM) treatment for 48 h (P<0.05; Fig. 5).

Psoralidin treatment inhibits Bcl-2 and enhances Bax protein expression in SW480 cells. To determine whether there was an association between the anti-proliferative effect of psoralidin (0, 5, 10 and 20 μM) treatment on SW480 cells, and the Bcl-2/Bax signaling pathway, Bcl-2 and Bax protein expression in SW480 cells was detected by western blot analysis. As demonstrated in Fig. 6A and B, Bcl-2 protein expression was significantly inhibited by psoralidin (10 and 20 μM) treatment for 48 h (P<0.01), and markedly decreased following psoralidin (5 μM) treatment for 48 h (P<0.05; Fig. 6A and B). By contrast, Bax protein expression in SW480 cells was markedly promoted by psoralidin (5, 10 and 20 μM) treatment: Bax protein expression was significantly increased following psoralidin (10 and 20 μM) treatment for 48 h (P<0.01), and markedly increased following psoralidin (5 μM) treatment for 48 h (P<0.05; Fig. 6C and D).

Bcl-2 inhibitor is able to enhance the anti-proliferative effect of psoralidin on SW480 cells. To further investigate the potential association between Bcl-2 inhibition and the effect of psoralidin on SW480 cells, psoralidin-treated SW480 cells were incubated with a Bcl-2 inhibitor (ABT-737) for 48 h. ABT-737 inhibited Bcl-2 protein expression (Fig. 7A and B) and decreased the viability of SW480 cells (Fig. 7C), compared with the psoralidin (10 μM) treatment control group.

Discussion

Worldwide, colon cancer is the third most common cancer in terms of incidence, following lung and breast cancer (21). There are differences in the geographical distribution of colon cancer (22). In developed countries or regions, including Australia, New Zealand, Europe and North America, there is a higher prevalence of colon cancer, whereas in African and South Asian countries, incidence is reduced, and may be up to ten times lower (23). Colon cancer mortality rates are highest in Central and Eastern Europe, and lowest in Central Africa (24). In the present study, psoralidin treatment reduced the viability and promoted the apoptosis of SW480 cells in a dose-dependent manner. Furthermore, Hao *et al* (18) reported that psoralidin inhibited the proliferation of A549 cells by inducing ROS production. Szliszka *et al* (25) identified that psoralidin inhibited cell proliferation of prostate cancer cells by enhancement of tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis. Additionally in the present study, caspase-3 activity of SW480 cells was significantly enhanced following psoralidin treatment. Das *et al* (26) suggested that psoralidin may promote growth arrest in prostate cancer cells by activation of caspase-3 and caspase-9.

A number of studies have revealed that NF-κB is highly expressed within tumor cells, and is involved in cell proliferation, invasion, angiogenesis and inhibition of apoptosis (27). Activation of the NF-κB signal transduction pathway may weaken the antitumor effects of chemoradiation therapy (28). The Hut-78 cell line, which highly expresses NF-κB, is resistant to the anticancer drug Taxol; however, Jurket cells, which do not express NF-κB, are sensitive to Taxol. This suggests that NF-κB participates in regulation of the sensitivity of tumor cells to anticancer therapies (29). In the present study, it was observed that psoralidin treatment markedly reduced the NF-κB p65 activity of SW480 cells. Chiou *et al* (30) observed that psoralidin inhibited lipopolysaccharide-induced inducible nitric oxide synthase expression by mediating NF-κB signaling. Yang *et al* (17) reported that psoralidin regulated ionizing radiation-induced pulmonary inflammation by regulation of the phosphoinositide 3-kinase/Akt and NF-κB pathways.

Apoptosis is the primary mechanism for removal of unwanted cells during embryonic development and tissue repair, and disorders of the apoptotic mechanism are closely associated with tumorigenesis. Apoptosis is a process of active cell death that is controlled by genes (31). In particular, Bcl-2 family genes are significant for apoptosis and have been extensively studied. Bcl-2 family proteins may act on the apoptotic pathway and exert regulatory effect on apoptosis via various routes (32). The levels of and interaction between Bcl-2 and Bax gene products may also be the regulatory pathway center of Bcl-2 apoptosis, while the Bcl-2/Bax protein ratio is a significant switch in apoptosis regulation (33). It is known that psoralidin may decrease Bcl-2 protein expression and increase Bax protein expression in SW480 cells (34). Based on these findings, the present study demonstrated that Bcl-2 protein expression was markedly decreased following psoralidin treatment. It was previously revealed that psoralidin inhibited androgen-independent prostate cancer cells via Bcl-2/Bax signaling pathway inhibition (17). In the present study, it was

demonstrated that inhibition of Bcl-2 protein expression was capable of enhancing the anticancer effects of psoralidin on SW480 cells. Srinivasan *et al* (35) suggested that psoralidin inhibited cell proliferation and induced apoptosis of prostate cancer cells by mediation of NF-κB and Bcl-2 signaling pathways.

In conclusion, psoralidin decreased the expression levels of NF-κB and Bcl-2, increased the expression levels of Bax, promoted caspase-3 activity and subsequently induced apoptosis in human colon cancer SW480 cells. The results of the present study demonstrate the potential benefits of psoralidin in clinical practice.

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