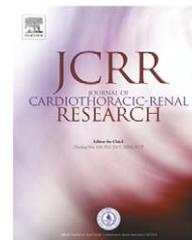




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DMNQ S-53 induces apoptosis and inhibits the growth of Lewis lung carcinoma cells in vitro and in vivo

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KEYWORDS

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Summary *Background:* 6-(1-Proproxyiminomethyl)-5,8-dimethoxy-1,4-naphthoquinone S-53 (DMNQ S-53) was synthesized to develop a novel anti-tumor agent against lung cancer. *Methods:* Cytotoxicity assay, DNA fragmentation assay, cell cycle analysis, mitochondrial potential measurement and Western blotting were employed in vitro and also Lewis lung carcinoma (LLC) animal model was used for evaluating the anti-tumor of DMNQ S53 in vivo. *Results and conclusions:* DMNQ S-53 exerted cytotoxicity against LLC cells with IC₅₀ of ~5 μM. DMNQ S-53 also increased the sub G1 cell population stained by propidium iodide (PI) as well as ladder-like DNA fragmentation in a concentration dependent manner. Western blot analysis revealed that DMNQ S-53 induced apoptosis was associated with the activation of caspase-9 and -3, cleavage of poly (ADP-ribose) polymerase (PARP) and the increased ratio of Bax to Bcl-2 expression in LLC cells in a concentration dependent manner. In addition, DMNQ S-53 reduced mitochondrial potential suggesting the involvement of the mitochondrial intrinsic pathway. Furthermore, intraperitoneally injection of DMNQ S-53 resulted in the inhibition of the tumor volume/weight of LLC cells inoculated on the flank of C57BL6 mice up to ~50%. Taken together, these results strongly indicate that DMNQ S-53 may inhibit LLC tumor growth in vitro and in vivo via apoptosis induction through the mitochondria-mediated caspase activation pathway.

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Introduction

Many pharmaceuticals with anti-tumor activity, including heyneanol [1], cantharidin [2], bufalin [3] and emodin [4] have been isolated from natural products and used for the treatment of cancer. The current problems of cancer treatment are not only how to effectively increase anti-cancer activity but also how to reduce side effects. Shikonin, a red naphthoquinone pigment isolated from the ground rhizome of *Lithospermum erythrorhizon* SIEB., was known to have various biological activities such as anti-tumor [5], anti-bacterial [6], anti-inflammatory [19] and wound healing effects [7]. However, shikonin has limited solubility and induces adverse side effects. For instance, the exposure of hepatocytes to naphthazarin, the structural skeleton of shikonin, caused a marked decrease of intracellular glutathione and increased cell death.

To overcome these weak points, many compounds, containing naphthazarin structure, were synthesized [8,9] including 6-(1-propoxyiminomethyl)-5,8-dimethoxy-1,4-naphthoquinone S-53 (DMNQ S-53). A related analog, 2-hyim-DMNQ S-33 has been shown to prolong the life span of mice bearing sarcoma 180 tumor with T/C of 239% [10]. However, there has been no report of biological studies with DMNQ S-53. Thus, in the present study, we examined the apoptotic and anti-tumor activities of DMNQ S-53, in vitro and in vivo using the Lewis lung carcinoma (LLC) model.

Materials and methods

Drug and chemical reagents

DMNQ S-53 was synthesized at College of Pharmacy, Chungnam University, Taejon, Korea (Fig. 1). Eagle's Minimal Essential Medium (EMEM; GIBCO BRL, Life Technologies Inc., NY) and antibiotics and antimycotics were purchased from GIBCO (Grand island, NY). Fetal bovine serum (FBS) was from JRH (Lenexa, KS). HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), sodium bicarbonate, XTT (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide), propidium iodide (PI), RNase A, Proteinase K, bovine serum albumin (BSA), *N*-methyl-dibenzo-pyrazine methyl sulfate (PMS) and poly-L-lysine were obtained from Sigma (St. Louis, MO). Tetramethylrhodamine ethyl ester (TMRE) was purchased from Fluka (St. Gallen, Switzerland). Protease inhibitor cocktail from Boehringer Mannheim (Indianapolis, IN) and

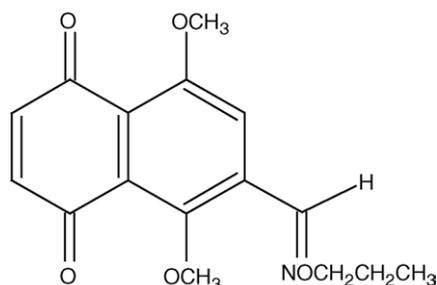


Figure 1 Chemical structure of 6-(1-propoxyiminio)-5,8-dimethoxy-1,4-naphthoquinone S-53.

DC protein assay kit II were obtained from Bio-Rad (Hercules, CA). Rabbit anti-human/mouse PARP (9542), rabbit anti-human/mouse cleaved caspase-3 (9661) and rabbit anti-mouse caspase-9 (9504) rabbit anti-human/mouse Bax (2772) antibodies were from Cell Signaling Technology (Beverly, MA). Mouse anti-human/mouse Bcl-2 (sc-509) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human/mouse β -actin (A5316) was from Sigma. Goat anti-mouse IgG HRP conjugated secondary antibody and goat anti-rabbit IgG HRP conjugated secondary antibody were from Zymed (San Francisco, CA). 4 \times NuPAGE LDS sample buffer, 4–12% NuPAGE Bis–Tris gels and NuPAGE MES SDS running buffer were from Invitrogen (Carlsbad, CA). Hybond ECL transfer membrane and enzyme chemiluminescent substrate (ECL) Western blotting detection kit were from Amercham Pharmacia (Arlington Heights, IL). X ray films were from Agfa-Gevaert (CP-BU, N.V., Belgium).

Cell culture

Lewis lung carcinoma (LLC) cells, originated spontaneously from mouse lung, were kindly provided by Dr. Ikuo. Saiki (Toyama Medical and Pharmaceutical University, Toyama, Japan). They were maintained in EMEM supplemented with 10% heat-inactivated FBS, 100 units/ml antibiotics and antimycotics and 2.2 g/l sodium bicarbonate and incubated in a humidified atmosphere of 5% CO₂ at 37°C. LLC cells were harvested by incubating in 1 mM EDTA for 2 min. The cells are seeded for 24 h then used for the experiments.

Cytotoxicity assay

The cytotoxicity of DMNQ S-53 was measured by XTT colorimetric assay. DMNQ S-53 was dissolved with dimethyl sulfoxide (DMSO). The cells were seeded onto 96-well microplates at a density of 1×10^4 cells per well in 100 μ l of EMEM. After incubation at 37°C in a humidified incubator for 24 h, the cells were treated with various concentrations of DMNQ S-53 in serum-free EMEM for 24 h. After incubation, a 50 μ l of XTT (1 mg/ml in PBS) mixture containing PMS (1.53 mg/ml in PBS) at the ratio of 100:1 was added to each well of the plate. The cells were incubated at 37°C for 2 h and the optical density was measured using microplate reader (Molecular Devices Co.) at 450 nm. Cell viability was calculated as a percentage of viable cells in DMNQ S-53 treated group versus vehicle-treated control by following equation. Cell viability (%) = [OD (DMNQ S-53) – OD (Blank)] / [OD (Control) – OD (Blank)] \times 100.

Cell cycle analysis

After treatment with or without DMNQ S-53 for 24 h, the cells were collected and washed twice with ice-cold PBS. Cell pellets were fixed in 70% cold ethanol overnight at –20°C. Fixed cells were centrifuged, washed and resuspended in 100 μ l of PBS containing 10 μ l of RNase A (10 mg/ml) and incubated for 1 h at 37°C. The cells were stained by adding 900 μ l of PI (50 μ g/ml) for 30 min at room temperature in the dark. After filtering with nylon mesh (40 μ m), DNA contents of stained cells were analyzed using FACS Vantage SE and CellQuest program (Becton Dickinson, Heidelberg, Germany).

DNA fragmentation assay

The cells treated with or without DMNQ S-53 for 24 h, were collected and washed twice with PBS. The pellet was lysed in 450 μ l of lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 0.2% Triton X-100) and incubated for 10 min on ice. The lysates were centrifuged for 15 min at $13,000 \times g$ and the supernatants were incubated at 50 °C overnight with proteinase K (2 mg/ml, Sigma). DNA was precipitated with 100% ethanol and 3 M sodium acetate for 2 h at -70 °C. DNA was pelleted at $12,000 \times g$ for 15 min and washed twice with 70% ethanol. DNA was dissolved in distilled water containing 1 mg/ml RNase A, incubated for 30 min at 37 °C, and analyzed on 1.5% agarose gels.

Western blotting

The cells treated with or without DMNQ S-53 were collected and washed twice with cold PBS. Cell pellets were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na_3VO_4 , 1 mM NaF, protease inhibitor cocktail) for 20 min on ice. Cell lysates were centrifuged at $14,000 \times g$ for 20 min at 4 °C. Protein concentrations were determined using a Bio-Rad DC protein assay kit II. The lysates containing 20 μ g of protein were mixed with 4 \times NuPAGE LDS sample buffer and boiled at 100 °C for 5 min. The protein was separated through NuPAGETM Bis-Tris gels with 1 \times NuPAGE MES SDS running buffer. Gels were electrotransferred onto a Hybond ECL transfer membrane with transfer buffer (25 mM Tris, 250 mM glycine, 20% methanol) at 300 mA for 90 min. To block the nonspecific binding site, membranes were treated with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) for 90 min at room temperature. The membranes were incubated with primary antibody against Bcl-2 (1:200), Bax (1:1000), cleaved caspase-3 (1:1000), Procaspase-9 (1:1000), PARP (1:1000) and β -actin (1:3000) in TBST containing 3% nonfat dry milk at 4 °C overnight. The membranes were washed with TBST three times for 30 min at room temperature and incubated with goat anti-rabbit IgG HRP conjugated secondary antibody (1:3000) in TBST that contained 3% nonfat dry milk for 90 min at room temperature. After incubation with secondary antibody, the membranes were washed with TBST three times for 30 min at room temperature. The proteins were developed using an ECL Western blotting detection kit and exposed to X-ray films.

Measurement of mitochondrial membrane potential

LLC cells (1×10^6 /ml) were treated with various concentrations (2, 5, 10 μ M) of DMNQ S-53 for 24 h. The cells were washed with cold PBS and stained by adding 150 nM the fluorescent potential dependent indicator, tetramethylrhodamine ethyl ester (TMRE) for 30 min at 37 °C. Then mitochondrial membrane potential was detected by flow cytometry (FACS Vantage SE, Becton Dickinson, Heidelberg, Germany) at 582 nm.

Tumor growth in LLC-bearing mice

Female C57BL/6 mice, five weeks old, were purchased from Daehan Biolink Co., LTD (Chungbuk, Korea) and were given

food and water ad libitum. Mice were housed in a room maintained at 25 ± 1 °C with 55% relative humidity. LLC cells (5×10^5 /100 μ l PBS) were subcutaneously implanted on the right flank of C57BL6 mice. DMNQ S-53 was dissolved in PBS containing 1% DMSO. Five days later, mice were given a daily i.p. injection of DMNQ S-53 at 0.5 mg/kg/day. Tumor volumes were measured every four days with a caliper, and calculated according to the formula $[(\text{length} \times \text{width}^2)/2]$, where length represents the largest tumor diameter and width. All mice were sacrificed 21 days after inoculation of LLC cells and tumors were removed and weighed.

Statistical analysis

All values were expressed as mean \pm S.D. Statistical significance was compared between two groups by Student's *t*-test.

Results

DMNQ S-53 exerted cytotoxicity against LLC cells

To examine the cytotoxic effect of DMNQ S-53 on LLC cells, the cells were treated with various concentrations of DMNQ S-53 for 24 h. The viable cell number was evaluated by XTT assay. Treatment with DMNQ S-53 effectively inhibited the growth of LLC cells in a concentration-dependent manner. The IC_{50} of DMNQ S-53 was approximately 5 μ M (Fig. 2).

DMNQ S-53 increased the sub-G₁ cell population and induced DNA fragmentation in LLC cells

To determine whether DMNQ S-53 can induce apoptosis that may account for the observed effect on cell viability, a number of apoptosis assays were performed. First, we carried out cell cycle analysis to detect sub-G₁ apoptotic population. Cells were treated with various concentrations of DMNQ S-53 for 24 h, and the DNA contents were analyzed by flow cytometry after PI staining. As shown in Fig. 3, DMNQ S-53 significantly increased sub G₁ cell population to 5.27, 11.39 and 63.94% at 2, 5 and 10 μ M, respectively.

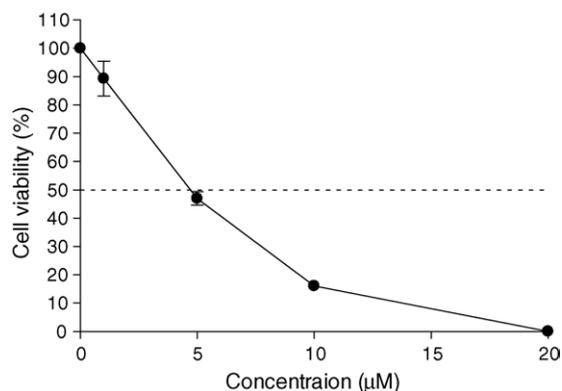


Figure 2 Effect of DMNQ S-53 on the cytotoxicity against LLC cells. The cells were treated with various concentrations of DMNQ S-53 for 24 h and cell viability was determined by XTT assay. All data were expressed as mean \pm S.D. of triplicates.

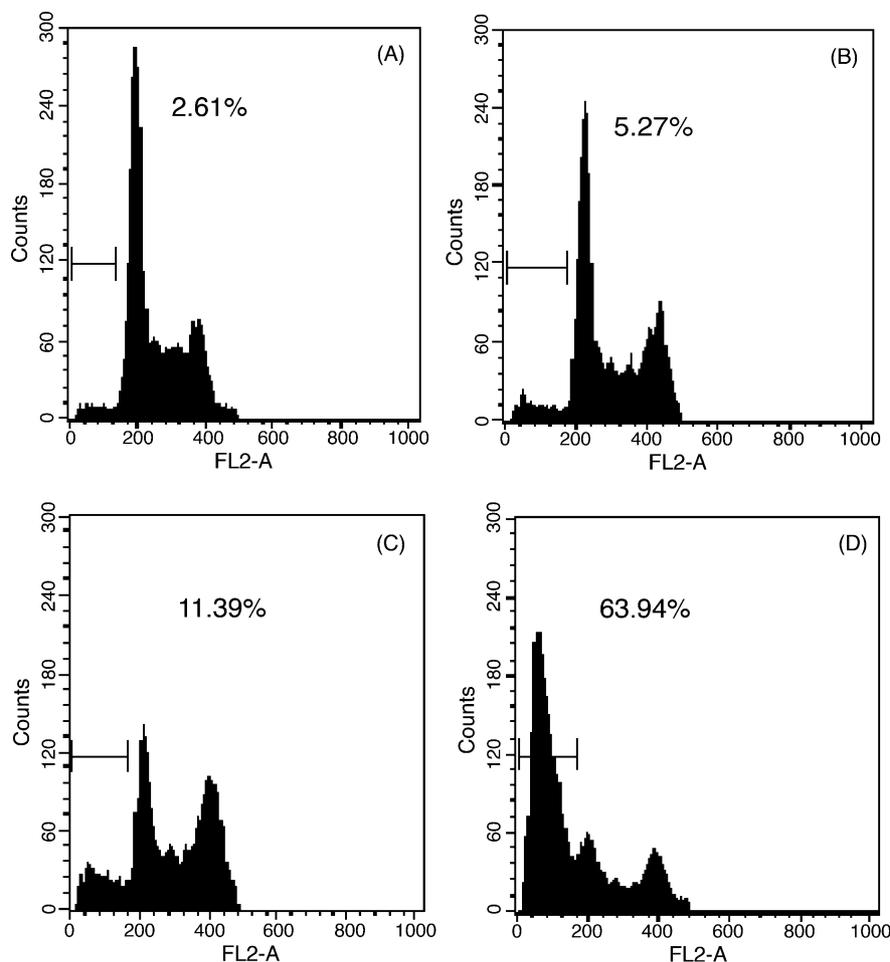


Figure 3 Exposure to DMNQ S-53 increased sub-G₁ cell population by cell cycle analysis. Cell cycle analysis was performed in LLC cells exposed to various concentrations of DMNQ S-53 for 24 h. A, Control; B, 2 μ M; C, 5 μ M; D, 10 μ M. DNA contents were analyzed using CellQuest Software with a flow cytometry system.

Next, we confirmed the apoptotic activity of DMNQ S-53 by DNA fragmentation assay as shown in Fig. 4. A ladder pattern of internucleosomal cleavage was observed in LLC cells exposed to DMNQ S-53 in a concentration dependent manner.

DMNQ S-53 regulated apoptosis related proteins in LLC cells

To investigate the molecular mediators of apoptosis induced by DMNQ S-53, the expression levels of Bax, Bcl-2, caspase-3, -9 and PARP were examined by Western blotting. As shown in Fig. 5A, the expression of procaspase-9, an initiator caspase, was decreased. The active form of caspase-3, an effector caspase, was increased in a concentration-dependent manner in DMNQ S-53 treated LLC cells (Fig. 5A). The caspase-3 substrate PARP was cleaved following treatment with DMNQ S-53 (2, 5 and 10 μ M) for 24 h. The expression of pro-apoptotic protein Bax was not changed, while that of Bcl-2, an anti-apoptotic protein resident on mitochondria membrane, was down-regulated

in a concentration dependent manner as shown in Fig. 5B. The ratio of Bax to Bcl-2 was increased in DMNQ S-53 treated LLC cells. These data collectively support that at least one pathway of caspase activation induced by S-53 treatment involved mitochondria-caspase-9 pathway.

DMNQ S-53 reduced mitochondrial membrane potential in LLC cells

Mitochondrial membrane potential in the cells with various concentrations of DMNQ S-53 for 24 h was analyzed by flow cytometry after TMRE staining. As shown in Fig. 6, DMNQ S-53 reduced fluorescence intensity reflecting mitochondrial membrane potential in a concentration dependent manner.

DMNQ S-53 inhibited the growth of LLC cells inoculated on the flank of mice

To evaluate the anti-tumor activity of DMNQ S-53 in vivo, C57/BL6 mice were subcutaneously inoculated with LLC cells and were given daily i.p. injection of DMNQ S-53 in PBS con-

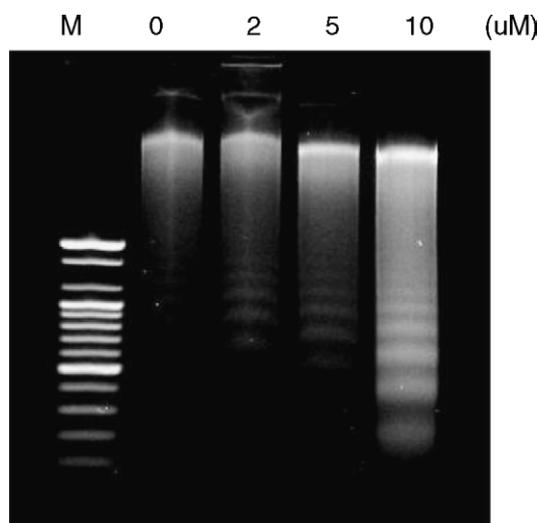


Figure 4 Exposure to DMNQ S-53 induced DNA fragmentation in LLC cells. LLC cells (1×10^6 /ml) were treated with various concentrations (2, 5, 10 μ M) of DMNQ S-53 for 24 h. Fragmented DNA was separated by 1.5% agarose gel electrophoresis and visualized under UV light. Lane 1, 100bp Marker; lane 2, Control; lane 3, 2 μ M; lane 4, 5 μ M; lane 5, 10 μ M of DMNQ S-53.

taining 1% DMSO every day. The tumor size was measured every four days for 21 days. As shown in Fig. 7, treatment of DMNQ S-53 effectively inhibited the growth of LLC cells control group in a time dependent manner at a dose of

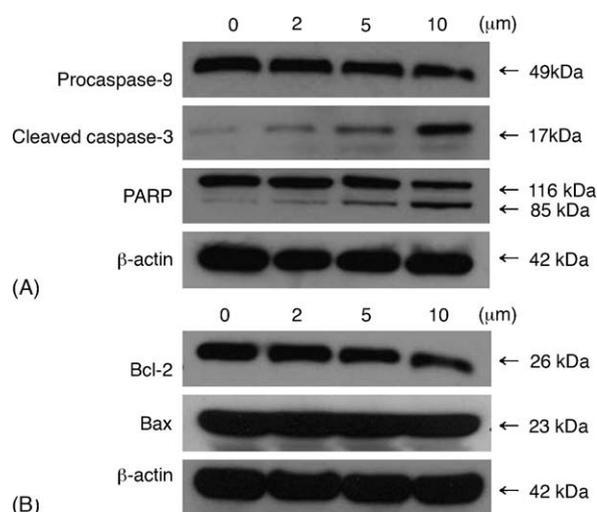


Figure 5 Effect of DMNQ S-53 treatment on selected caspases (A) and mitochondria apoptosis-related proteins (B) in LLC cells. Cells were treated with various concentrations of DMNQ S-53 for 24 h. The expression of procaspase-9, cleaved caspase-3 and cleaved PARP as well as Bcl-2 and Bax (B) were evaluated by Western blotting.

0.5 mg/kg. The tumor weight at termination of experiment was inhibited to ~50% of untreated control by DMNQ S-53 as shown in Fig. 8. It is noteworthy that DMNQ S-53 did not show any side effects such as weight loss, hair loss,

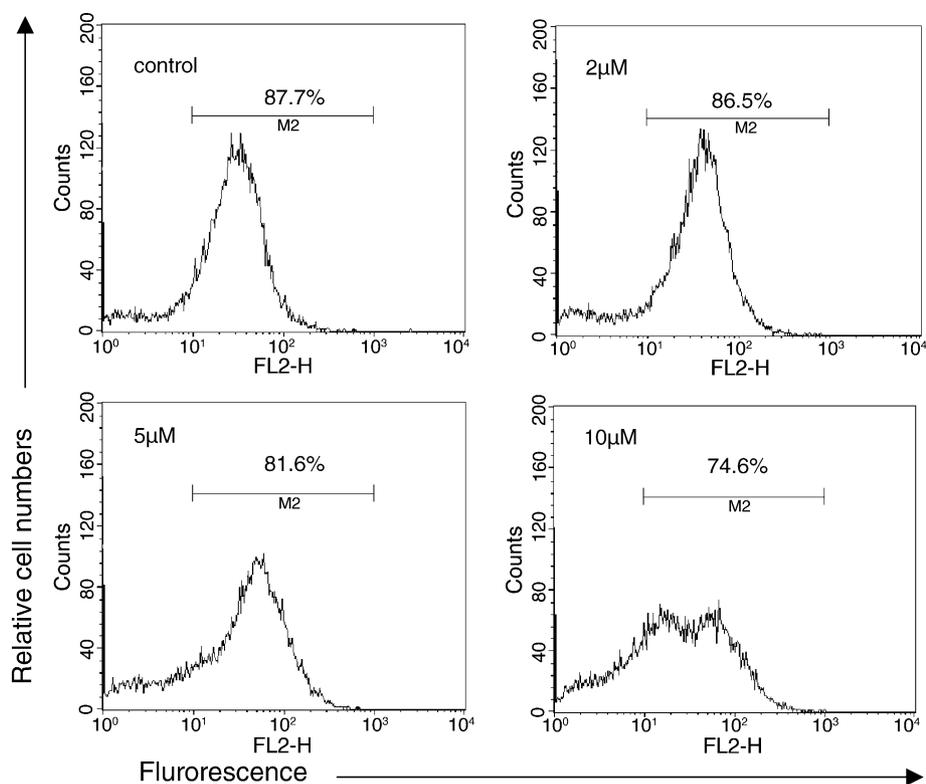


Figure 6 Effect of DMNQ S-53 on mitochondrial membrane potential in LLC cells. LLC cells (1×10^6 /ml) were treated with various concentrations (2, 5, 10 μ M) of DMNQ S-53 for 24 h, then stained with tetramethylrhodamine ethyl ester (TMRE) for 30 min at 37 $^{\circ}$ C. Change of mitochondrial membrane potential was detected by flow cytometry (FACS Vantage SE, Becton Dickinson, Heidelberg, Germany) at 582 nm.

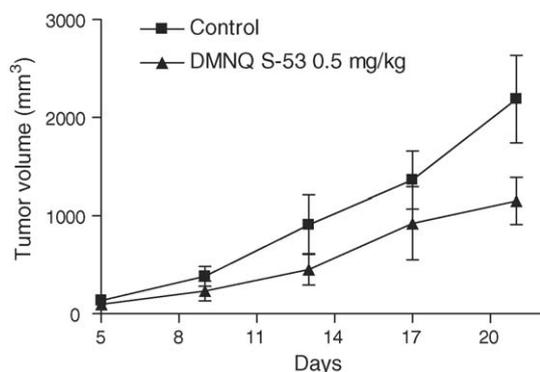


Figure 7 Effect of DMNQ S-53 on the growth of LLC cells inoculated on the flank of C57BL6 mice. LLC ($5 \times 10^5/100 \mu\text{l}$ PBS) were subcutaneously injected into the right flank of mice. Five days later, mice were given daily intraperitoneally injection of DMNQ S-53 every day for 16 days. Tumor volumes were measured every four days with a caliper as described in Materials and methods. All data were expressed as mean \pm S.D. $n = 10$.

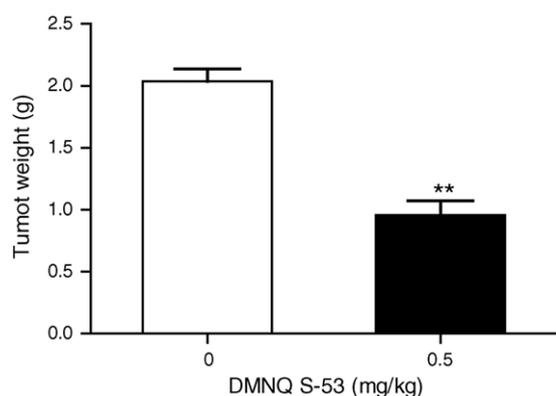


Figure 8 DMNQ S-53 treatment reduced tumor weight in LLC-bearing mice. All mice were sacrificed 21 days after LLC cells inoculation and the tumors were removed and weighed. All data were expressed as mean \pm S.D. ($n = 5$). The statistically significant differences between control and DMNQ S-53 treated groups were calculated by Student's *t*-test (** $p < 0.01$).

mortality and lethargy during this in vivo study (data not shown).

Discussion

Apoptosis is a strictly controlled mechanism of cell suicide triggered by certain internal or external signals [11,12]. Because many chemotherapeutic drugs have been shown to induce apoptosis in malignant cells, apoptosis has currently been a target for developing anti-tumor drugs [13–15].

In the present study, the apoptotic mechanism of DMNQ S-53, a new shikonin derivative and its in vivo anti-tumor efficacy were, evaluated in LLC cells. DMNQ S-53 significantly inhibited the viability of LLC cells with IC_{50} of $\sim 5 \mu\text{M}$ by XTT assay.

The extent of apoptosis as defined by the percentage of cells in the sub-G1 fraction of the cell cycle [15] was increased by DMNQ S-53 to over 60% at $10 \mu\text{M}$ concentration.

DNA fragmentation assay was confirmed that DMNQ S53 exerted cytotoxicity against LLC cells through apoptosis by DNA laddering, a characteristic feature of apoptosis in LLC cells. These data strongly demonstrate that DMNQ S-53 selectively induce apoptotic cell death of LLC cells. However, whether necrosis was also induced was not specifically investigated.

Caspases are well known to mediate apoptosis induced by many apoptotic stimuli. One pathway of caspase activation is initiated by the mitochondrial cytochrome *c* release, which then binds to apoptotic protease activation factor (Apaf)-1 and subsequently activates caspase-3 via proteolytic processing [16]. Activated caspase-3 cleaves a number of substrates including the DNA repair associated nuclear enzyme poly (ADP-ribose) polymerase (PARP) to form a 89 kDa fragment [17] that has become a hallmark for apoptosis. A second pathway is the activation of caspase-8 by binding of death ligands to cell membrane death receptors. Activated caspase-8 also cleaves caspase-3, which mediates many of the apoptotic execution events. Furthermore, activated caspase-8 cleaves cytosolic BID, a BH3 domain-containing pro-apoptotic Bcl-2 family member. Truncated Bid translocates into the mitochondria, resulting a cross-talk with the mitochondrial pathway by increasing cytochrome *c* release and the activation of caspase-9 [18]. In our study, DMNQ S-53 induced apoptosis was associated with activation of caspase-3 and 9 and cleavage of Poly (ADP-ribose) polymerase (PARP) in a concentration dependent fashion.

Consistent with a mitochondrial involvement in apoptosis signaling, DMNQ S-53 decreased mitochondrial membrane potential and effectively increased the ratio of Bax pro-apoptotic protein to Bcl-2 anti-apoptotic protein in LLC cells.

Furthermore, our animal experiment showed that i.p. administration of DMNQ S-53 at 0.5 mg/kg effectively suppressed the tumor volume/weight by 50% without signs of toxicity as indicated by the lack of effect on body weight gain. Such an experiment indicates that S-53 possesses selectivity against tumor cell growth/survival and holds good potential for improved clinical efficacy with fewer side effects.

In conclusion, DMNQ S-53 induced apoptosis in LLC cells that was associated with the cleavage of PARP, activation of caspases-9, and 3. The apoptosis effect may in part account for its efficacy to inhibit the growth of LLC tumor cells in vivo. These findings collectively suggest that DMNQ S-53 may be an anti-tumor drug candidate with improved efficacy and tolerance profile in comparison with shikonin.

Acknowledgements

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