Original Article

The Inhibition Effect of 2-Methoxy-1,4-naphthoquinone on Human Hepatoma Cell Lines: A *in vitro* and *in vivo* Studies

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ABSTRACT

Background and Aim: To study the antitumor activity of 2-Methoxy-1,4-naphthoquinone (MNQ) against hepatocellular carcinoma (HCC) cells, in an attempt to fill the knowledge gap of anti-liver cancer with MNQ. **Materials and Methods:** Cell viability in the presence of MNQ was assessed by MTT and effect of MNQ on HepG2 cell cycle by flow cytometry. To analyze apoptosis and its molecular mechanisms, we used a combination of Hoechst 33342 staining, annexin V binding, Rhodamine 123 staining, Real-time quantitative PCR (qPCR), western blotting and confocal microscopy. On the other hand, *in vivo* tumor growth was measured by a xenograft tumor nude mice model. Finally, the hematoxylin and eosin staining were used to observe the pathological changes of tumor tissue. **Results:** The results indicate that MNQ induced apoptosis in HCC cell lines, as demonstrated by a significant increasing of mitochondrial membrane potential and G0/G1 cell cycle arrest in HepG2 cells. Furthermore, MNQ significantly up-regulated BAD and down-regulated the expression of anti-apoptotic factors NF-κB and Bcl-2 at protein and transcription levels. Finally, *in vivo* studies revealed that MNQ significantly inhibited tumor growth. **Conclusion:** MNQ suppress the proliferation and induces apoptosis of HCC cells through mitochondrial and Rel/NF-κB signal pathways.

Keywords: 2-Methoxy-1,4-naphthoquinone, Hepatocellular carcinoma, Apoptosis, Cell cycle, Hematoxylin and Eosin, Antitumor mechanisms.

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INTRODUCTION

Liver cancer is highly prevalent worldwide, but its incidence rate is increasing in most developed countries. Primary liver cancer is the third leading cause of cancer-related death in the world.[1] Hepatocellular carcinoma accounts for more than 80% of the total number of liver cancer, and drug resistance often occurs in the treatment of HCC with chemotherapy drugs.[2] In addition, the use of chemotherapy drugs often brings huge side effects, such as bone marrow suppression, immunosuppression and gastrointestinal toxicity.[3] However, natural Chinese medicine monomers have the advantages of no damage to the gastrointestinal tract, small liver and kidney stimulation, safety, and few side effects.[4] In recent years, a variety of new anticancer drugs are derived from natural plant products. They can effectively inhibit tumor growth but have relatively low toxicity.[5]

2-Methoxy-1,4-naphthoquinone (MNQ) is a naturally occurring plant chemicals isolated from the genus *Impatiens*, which includes *Impatiens balsamina* L, *Impatiens bicolor* and *Impatiens glandulifera* Royl.[6-7] *Swertia calycina* (Gentianaceae) was also found to contain this compound.[8] Several bioactivities have already been demonstrated by MNQ including antipruritic,[9] anti-inflammatory,

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antiallergic,[10] and anticancer,[11-12] activities. Antimicrobial activity against fungal and bacterial pathogens has also been reported.[13-14]

In recent years, the anticancer activities and medicinal value of MNQ have become a new focal point. Much work has been conducted in several representative cancer cell lines including MKN45, MDA-MB-231 and HepG2 cells.[15-17] All the same, systematic study of MNQ's inhibit the growth of human hepatoma cell lines both *in vitro* and *in vivo* has not been documented. Therefore, this study aims to explore the effect of MNQ on hepatocellular carcinoma and its mechanism. In this study, we confirmed the antitumor activity of MNQ on HCC *in vitro* and *in vivo*. MNQ can reduce the expression of Bcl-2 and NF-κB protein in HepG2 cells, inhibit cell proliferation and promote apoptosis.

MATERIALS AND METHODS

Chemicals and Reagents

2-Methoxy-1,4-naphthoquinone (purity, ≥98%; molecular weight, 188.18; formula, $C_{11}H_8O_3$), MTT and PI was obtained from Sigma-Aldrich (St. Louis, MO, USA). The solubility of purchased MTT and PI was >50 mg/ml in DMSO. The Membrane Potential Detection Kit was purchased from Cell Technology. The following reagents were obtained from Beyotime: RNase, Immunol Staining kit, Immunol Staining Secondary Antibody and materials for western blot analysis. SYBR PCR Mix and the RT-PCR kit were purchased from Takara (Dalian, China). Antibodies for human reactive anti-Bcl-2, BAD, β-actin and NF-κB and the secondary antibody were purchased from Cell Signaling Technology (CST, USA).

Cell Culture

The three human hepatoma cell lines used for these studies (HepG2, Bel-7402 and HuH-7) were a generous gift from the School of Pharmaceutical Sciences, Sun Yat-sen University. HepG2 and Huh-7 cells were maintained in DMEM medium supplemented with high amounts of glucose (GIBCO, UK) and 10% fetal bovine serum (FBS) in a humidified incubator at 37°C and 5% CO_2 (Sanyong, Japan). Bel-7402 cells were cultured in RPMI 1640 medium (GIBCO, UK) with 10% FBS (Hyclone, Logan, UT, USA).

Cell Viability Assay

Cell viability was measured using MTT assay. Bel-7402, HepG2 and HUH-7 cells were inoculated in 96 well plates with 1×10^4 cells per well. Treated with different concentrations of MNQ (1.7188, 3.4375, 6.875, 13.75, 27.5 and 55 μM) for 24, 48 and 72 hr. 5-fluorouracil (5-Fu, 30μM) was used as a positive control. After the specified time, 10 μL MTT (5 mg/mL) was added to each well, and the culture was continued for 4 hr. After centrifugation at 1000 r/min for 5 min, 100 μL DMSO was added to each empty hole and shaken at a low speed for 10 min on the shaker. Absorbance was measured on a microplate reader (AMR-100, Allsheng, China) at 490 nm.

Hoechst 33342 Staining

HepG2 cells were seeded on glass coverslips and treated with MNQ (2.5, 5, 10 μM) at different concentrations for 48 hr. The cells were washed with PBS, resuspended in Hoechst 33342 solution (5 μg/mL), and cultured at 37°C for 30 min. After two washes with PBS, the glass coverslips were placed on the microslide, which was overlaid with 50 μl of 50% glycerol. Then the stained cells were observed under fluorescence microscope. Using this staining method, live cells had bright blue intact nuclei, chromatin condensation or breakage in apoptotic cells.

Annexin V Binding

HepG2 cells were treated with different concentrations of MNQ (0, 2.5, 5, 10 μM) for 72 hr. Washed twice with cold PBS, harvested cells (1×10^5 cells/ml) and reselected in 1x binding buffer. Next, 5μl FITC annexin V and 5μl propidium iodide (PI) (Beyotime, China) were added and incubated in dark for 10 min at room temperature. Different subsets of apoptotic cells were determined by flow cytometry (BD FACS Calibur, USA).

Rhodamine 123 Staining

As an important indicator of mitochondrial disorder, mitochondrial membrane potential can be detected by Rhodamine 123 (Rh123).[18] HepG2 cells were treated with different concentrations of MNQ (1, 2, 4 μM) for 48hr. The cultured cells (1×10^5 cells/ml) were collected and resuspended in 1 ml medium. Rh123 dye solution was added and incubated at 37°C for 30 min, followed by flow cytometry analysis (BD FACS Calibur, USA).

Cell Cycle Analysis

After 36 hr starvation, cells were treated with different concentrations of MNQ (2.5, 5, 10 μM) for 48hr. Cells were collected and fixed with 70% ethanol at -20°C for 24 hr, then incubated with PI and RNase for 30 min. Cycle analysis was performed using flow cytometry (BD FACS Calibur, USA).

RT-qPCR Analysis

HepG2 cells were seeded on 6-well plates and incubated with MNQ (2.5, 5, 10 μM) at different concentrations for 48 hr. Total RNA extraction using Trizol. According

to manufacturer ' s instructions, RNA was reverse transcribed into cDNA using Prime Script TM RT kit. Expression levels of NF-κB, BAD and Bcl-2 were determined by quantitative PCR (qPCR) using SYBR PCR Mix (TaKaRa, Japan) in a Mini Option Real-Time PCR detection system (Bio-Rad). The primers used in the experiment are as follows. NF-κB primers were: 5'-AGGAGAGGATGAAGGAGTTGTG-3' (forward) and5'-CCAGAGTAGCCCAGTTTTTGTC-3'(reverse); Bcl-2 primers were: 5'-GTTTGATTTCTCCTGGCT GTCTC-3' (forward) and 5'-GAACCTTTTGCATAT TTGTTTGG-3' (reverse); BAD primers were: 5'-ATGTTCCAGATCCCAGAGTTTG-3' (forward) and 5'-GGTAGGAGCTGTGGCGACT-3' (reverse); β-actin primers were: 5'-TGACGTGGACATCCGCA AAG-3' (forward) and 5'-CTGGAAGGTGGACAG CGAGG-3' (reverse).

Western Blot Analysis

RIPA buffer (Beyotime, China) was used to lyse cells to obtain total protein, and the protein concentration was determined by BCA method. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore, Burlington, USA). After blocking with 5% skim milk powder, the primary antibody was incubated overnight at 4°C. The next day, the second antibody was incubated at room temperature for 2 hr. Protein bands were detected by enhanced chemiluminescence. We used two methods to detect protein expression. NF-κB expression was analyzed by western blot analysis using the wet transfer method, but for the smaller proteins Bcl-2 and BAD, the semi-dry transfer method was used for greater accuracy of detection.

Confocal Fluorescence Microscopy

HepG2 cells (2×10^4 cells/well) were seeded in 48-well plates. Subsequently, MNQ (1.25, 2.5, 5, 10 μM) was treated with different concentrations for 48 hr. Wash with PBS, collect cells and fix them with Immunol Staining Fix Solution (Beyotime, China) for 15 min. Then the buffer was Immunol Staining Blocking (Beyotime, China) for 1 hr at room temperature. Cells were incubated overnight with primary antibodies at 4°C. The next day, incubated with the corresponding second antibody. Finally, the cells were incubated with DAPI for 3 min at room temperature and observed by confocal laser scanning microscopy (Fluoview FV1000, Olympus, Japan). Data were processed by ImageJ software.

In vivo Studies

Five-week-old BALB/c nude mice (n = 15) were purchased from Guangdong Medical Laboratory Animal Center. After 3 days of adaptation, 0.2 mL HepG2 cells (7×10^6 cells/mL) were subcutaneously injected into the right armpit area of mice. The subsequently formed tumors were allowed to reach a diameter of 3 to 4 mm. Subsequently, the mice were randomly divided into three groups: The control group was administered saline (0.2ml/d, ip); MNQ (10 mg/kg/d, ip) was administered to the medication group; the positive group was administered 5-FU(15 mg/kg/d, ip). Measure tumor volume and weight every three days. The tumor volume is calculated as follows: (width^2 × length)/2. After 21 days of administration, the mice were euthanized and the xenografts were dissected. All animal experiments were approved by the Ethics Committee of the Guangdong Pharmaceutical University.

Hematoxylin and eosin (HE) staining assay

Tumor tissue was fixed with paraformaldehyde for 48 hr, then embedded in paraffin, cut into 4-5 μM sections. After xylene dewaxing and ethanol hydration, stained with hematoxylin and eosin. The morphology of HE-stained tumor tissue was observed under an optical microscope (Nikon Eclipse E100, Japan).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 26.0 (IBM, Armonk, USA). All experimental results were expressed as the mean ± standard deviation (SD) of triplicate samples. One-way analysis of variance and Duncan's multiple comparison test were used to determine the differences between the average values of different groups. p value < 0.05 was considered significant.

RESULTS

MNQ inhibits the proliferation of human hepatocellular carcinoma cells

The effects of MNQ on HepG2, Bel-7402 and HUH-7 cell viability were determined by an MTT assay. 5-FU (30 μM) as positive control group. As shown in Figure 1, MNQ could significantly suppress the proliferation of HepG2, HUH-7 and Bel-7402 cells in a time and dose-dependent manner. The IC_{50} values of the human hepatoma cells exposed to 1.72-55 μM MNQ for 48 hr ranged from 2.29-14.29 μM. At the dose of 27.5 μM, the inhibitory effect of MNQ on three HCC cells was similar to that of the positive drug 5 - FU. We selected HepG2 cells for further study, because MNQ had the strongest inhibitory effect on HepG2 cells at high doses.

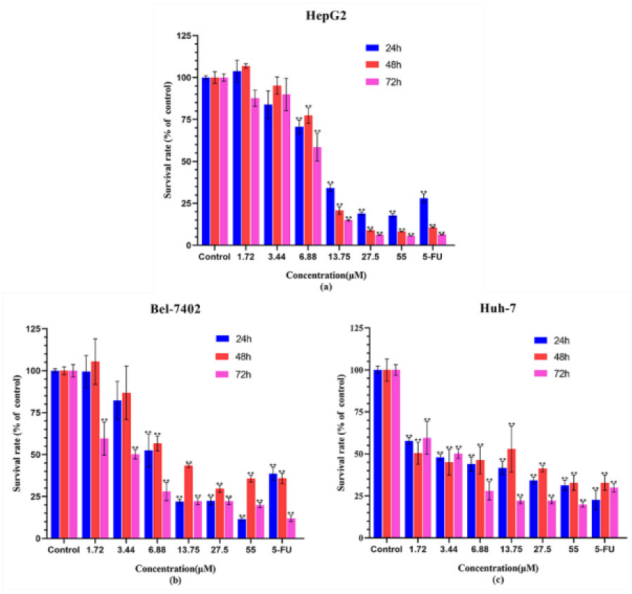

Figure 1: Anti-proliferative activity of MNQ on HepG2 (a), Bel-7402 (b) and HUH-7 (c) cells was detected by MTT assay 24, 48, and 72 hr after treatment.

Data are shown as mean±SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control.

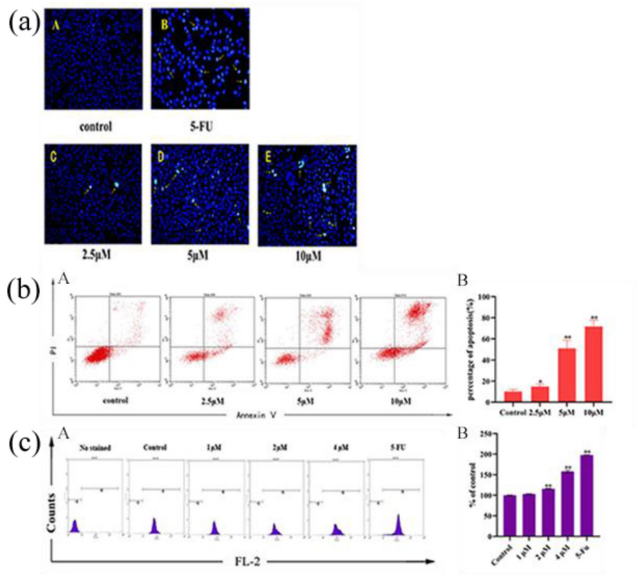

Figure 2: Effect of MNQ on apoptosis of HepG2 cells.

(a). The effect of MNQ on the morphology of HepG2 cells was assayed by Hoechst 33342 staining (magnification 400×). The yellow arrows indicate the cells undergoing apoptosis. (b). Assessment of apoptosis induced by MNQ in HepG2 cells using flow cytometry with annexin V-FITC/PI staining. The results are expressed as percent apoptotic cells. (c). Distribution map of the mitochondrial membrane potentials after treatment with MNQ for 48 hr. The fluorescence emission was measured by flow cytometry. Data are shown as mean±SD. *$p < 0.05$, **$p < 0.01$ vs control.

Apoptosis induced by MNQ

In order to further explore the molecular mechanism of MNQ against proliferation, we analyzed the ability of MNQ to induce apoptosis in HepG2 cells. As shown in Figure 2 (a), MNQ treatment of HepG2 cells for 48 hr, chromatin condensation, volume reduction and nuclear fragmentation, which is a typical apoptotic morphology. 5-FU group also showed obvious apoptosis characteristics. However, the cells in the control group grew well and stained weakly blue. Flow cytometry was used to further prove the apoptosis effect of MNQ on HepG2 cells. Compared with the control group, MNQ significantly increased the number of early and late apoptosis in HepG2 cells. The apoptosis rates after 48 hr treatment with different doses of MNQ (0, 2.5, 5, 10 μM) were 10.14 ± 2.00%, 14.89 ± 2.12%, 51.07 ± 7.59% and 71.73 ± 6.01%, respectively (Figure 2(b)). The effect of MNQ on MMP in HepG2 cells was investigated by Rhodamine 123 staining. As shown in Figure 2 (c), the average fluorescence intensity of HepG2 cells treated with different doses of MNQ (0, 1, 2, 4 μM) were 100 ± 0.76%, 103.13 ± 0.48%, 116.11 ± 0.64% and 158.04 ± 2.89%, respectively. Therefore, MNQ treatment resulted in significant depolarization of MMP in HepG2 cells, suggesting that mitochondrial function was impaired and apoptosis occurred.

Effect of MNQ on cell cycle progression

Next, we explored the effect of MNQ on the cell cycle progression of HepG2. After 48 hr of MNQ treatment, the percentage of cells in G0/G1 phase increased (0-10 μM MNQ: 66.68 ± 1.68% to 80.35 ± 1.13%, respectively; $P < 0.01$). In addition, the percentage of cells in S phase decreased (0-10 μM MNQ: 11.11 ± 0.35% to 7.26 ± 0.06%, respectively; $p < 0.01$). Our results showed that MNQ could arrests HepG2 cells in G0 / G1 phase. Because our experiment adopts PI single staining, the sub-G1 events of a large number of late apoptotic cells were not obvious. Flow cytometry results showed that the percentage of cell cycle in each stage of HepG2 cells was significantly changed after MNQ treatment.

Effect of MNQ on NF-κB, Bcl-2 and BAD gene and protein expression

After MNQ treatment for 48 hr, the expression levels of NF-κB, Bcl-2 and BAD genes were analyzed by RT-qPCR using the 2 (-DeltaDeltaC (T)) method.[19] The results in Table 1 show that the Bcl-2 and NF-κB expression levels decreased to 0.19±0.04, 0.18±0.03 and 0.12±0.01 and 0.78±0.06, 0.44±0.08 and 0.14±0.02, respectively, and BAD expression increased to 1.18±0.25, 1.31±0.13 and 1.42±0.27 compared to the control group (2.5, 5 and 10 μM MNQ). Most eukaryotic protein expression is controlled at the level of gene transcription; therefore, changes in gene expression levels are likely to affect protein expression levels. These changes affect

Table 1: Quantitative analysis of NF-κB, Bcl-2 and BAD expression in HepG 2 cells treated with MNQ for 48 hr by real time RT-PCR.

Concentration(μM) NF-κB		F value ($2^{-\Delta\Delta Ct}$)		
		Bcl- 2	BAD	
Control	0μM	1.00±0.12	1.00±0.20	1.00±0.23
MNQ	2.5μM	0.78±0.06*	0.19±0.04***	1.18±0.25
	5μM	0.44±0.08**	0.18±0.03***	1.31±0.13*
	10μM	0.14±0.02***	0.12±0.01***	1.42±0.27*

Note: F Valule indicates the fold change in gene mRNA expression. Data are the mean ± SD of at least three independent experiments. The difference was significant for *p<0.05 versus the control. The difference was markedly significant for **p<0.01 and ***p<0.001 versus the control.

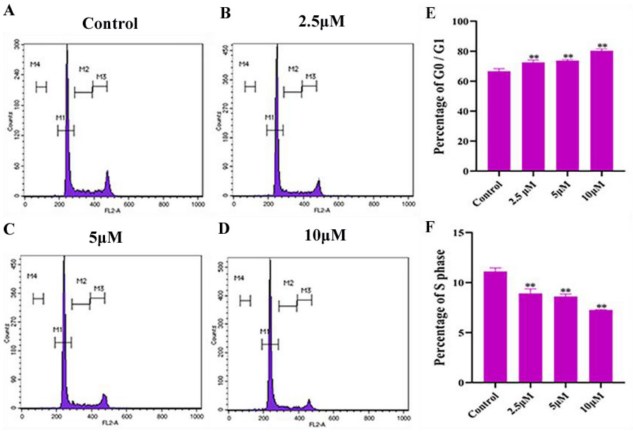

Figure 3: Cell cycle analysis of HepG2 cells after MNQ treatment.

The percentage of non-apoptotic cells within each cell cycle was determined by flow cytometry. Data are shown as mean±SD. *p < 0.05, **p < 0.01 vs control.

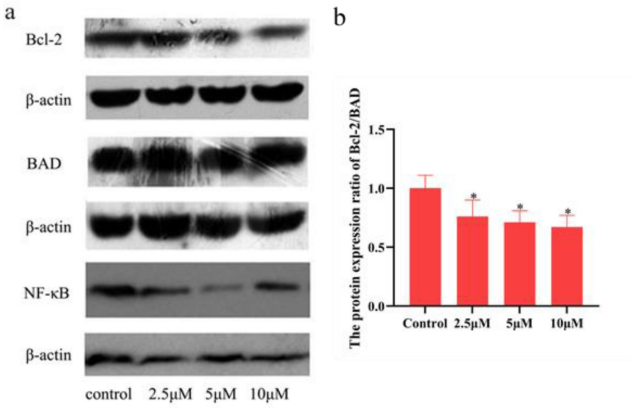

Figure 4: Effect of MNQ on the expression of Bcl-2, BAD and NF-κB in HepG2 cells.

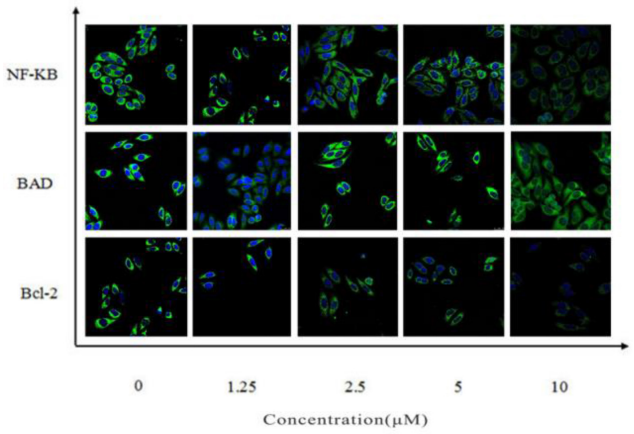

Figure 5: Confocal microscopy images of the expression levels of NF-κB, BAD and Bcl-2 after treatment with the indicated concentrations of MNQ for 48 hr.

The cell nuclei were stained with propidium iodide (blue). The proteins were detected with a combination of primary antibodies and secondary antibodies conjugated with immunofluorescent tags (green). All photos were taken with a 63× oil immersion lens.

the proteins' biological function and the intracellular signaling pathways to which they belong, which can alter tumor cell behavior.[20-21]

To further investigate the apoptotic pathways induced by MNQ in HepG2 cells, we investigated the protein changes of Bcl-2, BAD and NF-κB. After treatment with MNQ, NF-κB and Bcl-2 expression in cells was significantly decreased compared with control group for (2.5 μM, 5 μM and 10μM MNQ, respectively) (Figure 4a). The increased expression of BAD protein was also observed, and the Bcl-2/BAD ratio was less than 1. It is well known that a decreased ratio of Bcl-2/BAD results in the deletion of MMP and cytochrome c release and may subsequently trigger apoptosis.[22-23] Interestingly, our data coordinated well with results from previous studies, which illustrate that MNQ has a positive role in inducing apoptosis. We also examined the ability of MNQ to initiate apoptosis using an immunofluorescence staining assay. HepG2 cells were treated with MNQ for 48 hr. Confocal fluorescence microscopy was then used to detect the expression levels of the above- mentioned apoptosis-related proteins. Consistent with the above results, the expression of Bcl-2 and NF-κB was significantly decreased, while the expression of BAD was significantly increased (Figure 5).

MNQ inhibits tumor growth *in vivo*

Study on the anti-tumor effect of MNQ *in vivo* by xenograft model. Figure 6A shows the size of tumors treated with different drugs. Tumor size and volume increased apparently in nude mice treated with saline, but decreased significantly in nude mice treated with MNQ or 5-FU. Together, these results are indicative of the strong anti-hepatocellular carcinoma potential of both MNQ and 5-Fu. We next removed the tumors, sectioned them (5 μm thick) and processed them for HE staining to histopathological diagnosis. Figure 6B shows that in the cells of the normal tissues, nuclei were intact, nuclear membranes and the nucleolus appeared as a full

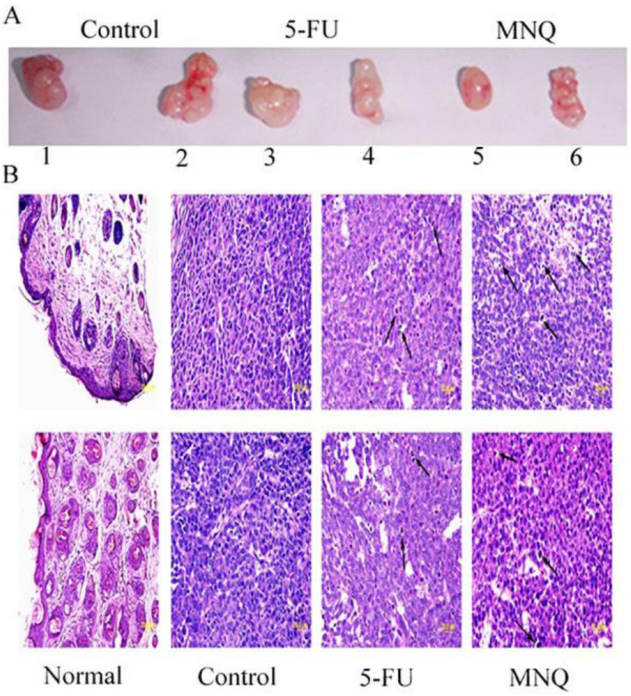

Figure 6: Effects of MNQ on xenograft tumors in nude mice.
A. Photos of tumors from tumor-burdened nude mice after sacrifice. 1, 2: tumor tissue of nude mice in the control group; 3, 4: tumor tissue of nude mice in the 5-Fu group; 5, 6: tumor tissue of nude mice in the MNQ group. B. Pathological changes of the tumor tissues 21 days after treatment (HE staining, original magnification: 400 ×). Normal: untreated left axillary subcutaneous tissue from transplantation tumors in nude mice. 1, 2: tumor histology from nude mice in the control group; 3, 4: tumor histology from nude mice in the 5-Fu group; 5, 6: tumor histology from nude mice in the MNQ group. Note: The arrows represent the positon of apoptotic cells.

circle and were centrally located in the cell, cells were arranged in an orderly manner and interstitial cells were abundant. As for the tumor tissues, the morphological characteristics were obvious; cells were tightly packed and stromal cells were rare. Nuclear volume of tumor cells increased in saline control group, as well as higher nucleus/cytoplasm ratios, and the number of interstitial cells had decreased significantly. The above results showed that the cells in saline control group grew vigorously. However, MNQ and 5-Fu groups showed increased interstitial cells, nuclear fragmentation, nuclear condensation and chromatin condensation. Apoptotic cells were observed in HE staining specimens of 5-Fu and MNQ groups.

DISCUSSION

In an attempt to search for new and better phytochemicals against hepatocellular carcinoma, we investigated the activity of MNQ, which has gained prominence as a possible anticancer agent in recent years.[24-26] *In vitro* experiments performed in this study demonstrated that MNQ exerts a significant cytotoxic effect in human hepatoma cell lines. This inhibitory effect was further verified *in vivo*. These results indicate that this compound might have potential as a treatment for hepatocellular carcinoma.

Inhibition of cancer growth has been a continuous focus in the world of cancer treatments. The suppression of cancer cell proliferation and induction of cell necrosis are two of the most significant means by which to defeat tumor growth.[27] In concurrence with an earlier report that indicated that MNQ was able to greatly inhibit growth of HepG2 cells,[28] our results, obtained by MTT assays, indicated that MNQ induced a concentration- and time-dependent cytotoxic effect in three types of human hepatoma cells (Figure 1). Apoptosis refers to the automatic and orderly death of cells in order to maintain the stability of the internal environment.[29] The process of apoptosis has obvious morphological changes, such as cell volume reduction, nuclear fragmentation and DNA degradation.[30] In order to explore the role of apoptosis in the anti-cancer effect of MNQ, Hoechst 33342 staining and annexin V-FITC/PI double staining were used to detect the apoptosis of HepG2 cells after MNQ treatment. Hoechst 33342 staining showed that the cells showed obvious signs of apoptosis (Figure 2(a)). Data from flow cytometry analysis revealed that MNQ significantly promote apoptosis in HepG2 cells. Even a concentration as low as 5 µM was capable of inducing apoptosis in 51.07% of cells (Figure 2(b)). The data from the MTT and apoptosis assays point to the great potential of MNQ in inducing apoptosis. To pinpoint the molecular factors underlying the strong anti-proliferation and anti-apoptotic abilities of MNQ, MMP and cell cycle distributions were assessed. Cell cycle arrest inhibits cell growth and promotes apoptosis.[31-32] After MNQ treatment of HepG2 cells, MMP loss (Figure 2(c)) and G0 / G1 phase block were observed (Figure 3).

The Bcl2 protein family plays an important role in the survival and destruction of various cancers.[33-35] The delicate balance between BAD and Bcl-2 proteins mediates mitochondrial-induced apoptosis.[36-37] In a previous study, Beg AA *et al.*[38] found that mice lacking RelA subunit of NF-κB may cause a massive degeneration of the liver by programmed cell death or apoptosis. NF-κB often inhibits apoptosis of tumor cells by activating a variety of anti-apoptotic factors.[39-40] The Rel/NF-κB signaling pathway regulates anti-apoptotic genes, blocking NF-κB can lead to tumor cells stop proliferation and increase apoptosis susceptibility.[41-44] Based on the above findings, we analyzed a few of the vital genes in this process including NF-κB, BAD and Bcl-2. MNQ was found to downregulate Bcl-2 and NF-κB and upregulate BAD expression as determined

by RT-qPCR (Table 1). The results from western blot analysis and confocal fluorescence microscopy were in line with the results obtained by RT-qPCR (Figure 4, Figure 5), which reflects a decreasing Bcl-2/BAD protein ratio. A balance between the upregulation and downregulation of the expression in Bcl-2 family members determines whether cells will undergo apoptosis or survive. Our current results show that MNQ downregulated the expression of the Bcl-2 family proteins as well as NF-κB and upregulated the expression of the BAD. This regulation resulted in a reduced Bcl-2/BAD expression ratio. The above results showed that MNQ induced apoptosis in HepG2 cells is dependent on the reciprocal regulation of Bcl-2 and BAD and the transcriptional block elicited by the Rel/NF-κB signaling pathway.

To ascertain whether the observed inhibitory effect of MNQ on human hepatoma cell lines was also effective in animal models, further experiments were used to measure the anti-hepatocellular carcinoma activities of MNQ in BALB/c nude mice. HE staining was performed to verify that the tumor-bearing naked mouse model was successfully established. The results were exciting because MNQ had similar anticancer effects to 5-Fu (Figure 6A). Tumor biopsies from each group were stained by HE and obvious degeneration of the tumor cells was observed in the MNQ group (Figure 6B). To analyses the results of HE staining, we found that the existence of apoptotic bodies, which may closely related to the decrement tumor volumes after the drug was administered. Thus the tumor suppression effect of MNQ *in vivo* has reached an agreement with that *in vitro*. These results suggest that we can obtain a more effective monomeric compound for subsequent research by modification of the chemical structure of the naphthalene quinones, thus forming a good foundation for the development of high-efficiency antitumor drugs.

CONCLUSION

In conclusion, our data verify that MNQ exerts a promising anti-proliferation activity against human hepatoma cells *in vitro*. The variety of techniques used in this study present a more systematic and complete analysis of the effects of MNQ on cancer cells than in previous studies. Furthermore, existing literature regarding MNQ research mainly concentrated on its extraction process and on *in vitro* activity studies; our research is the first attempt to use HepG2 cells and male BALB/c naked mice for an *in vivo* study of MNQ's antitumor activity. Thus, our study provides a new experimental basis for future pharmaceutical research and clinical applications of MNQ in hepatocellular carcinoma treatments.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS

MNQ: 2-Methoxy-1,4-naphthoquinone; **qPCR:** Real-time Quantitative polymerase chain reaction; **MTT:** 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl Tetrazolium Bromid; **HCC:** Hepatocellular carcinoma; **MMP:** Mitochondrial membrane potential; **DMSO:** Dimethyl sulfoxide; **DMEM:** Dulbecco's Modified Eagle's medium; **RPMI:** Roswell Park Memorial Institute; **PI:** Propidium iodide; **Rh123:** Rhodamine 123; **HE staining:** Hematoxylin and eosin staining.

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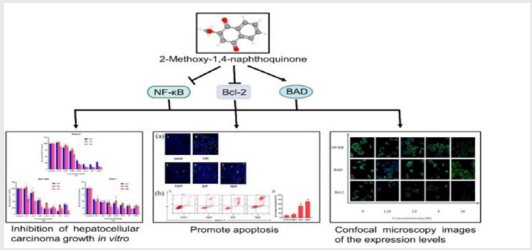
PICTORIAL ABSTRACT



SUMMARY

MNQ inhibits the growth of human hepatocellular carcinoma cell line and induces apoptosis. Animal experiments also show that MNQ can promote the regression of liver cancer.

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