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# 2-Methoxy-1,4-naphthoquinone (MNQ) induces apoptosis of A549 lung adenocarcinoma cells via oxidation-triggered JNK and p38 MAPK signaling pathways

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ABSTRACT

*Aim:* The compound 2-methoxy-1,4-naphthoquinone (MNQ) was previously shown to be cytotoxic against several cancer cell lines, but its mode of action is poorly understood. In this study, we aimed to explore the molecular mechanism of MNQ-induced cytotoxicity of A549 lung adenocarcinoma cells.
*Main methods:* The growth inhibition potential of MNQ was analyzed using sulforhodamine B assay, flow cytometry cell cycle analysis and Annexin V apoptosis assay. Oxidative stress was determined using 2′,7′-dichlorofluorescein diacetate to measure intracellular reactive oxygen species level and comet assay to measure DNA damage. Western blotting was performed to study the activation of mitogen-activated protein kinase signaling pathways.
*Key findings:* MNQ induced apoptosis of A549 cells independent of cell cycle arrest, and is mediated by the JNK and p38 MAPK signaling pathways. Further analysis demonstrated that these signaling pathways were stimulated by oxidative DNA damage caused by increased ROS generation in MNQ-treated A549 cells.
*Significance:* This study is the first to provide an insight into the molecular mechanism of MNQ-induced cytotoxicity of a lung cancer cell, which demonstrates the potential of MNQ as a potential chemotherapeutic drug for lung cancer treatment.



## 1. Introduction

Lung cancer causes the highest cancer-related mortality globally [1], with an estimate of 1.59 million deaths reported in the year 2012 [2]. This disease can be generally categorized as either non-small cell lung carcinoma (NSCLC), which comprises 85% of the total lung cancer cases reported, or small cell lung carcinoma (SCLC) which accounts for the remaining 15% [3]. Despite the various treatments available for lung cancer management, the survival rates reported are usually low [4,5,6]. In addition, the current chemotherapeutic regimens are compromised with detrimental side effects [7]. Therefore, this warrants the need for more efficient therapeutic approaches.

Quinones are a class of organic compounds in which many of its members were observed to possess anticancer properties by modulating intracellular processes to trigger the cell death mechanisms. These processes include the MAPK signaling pathways, which are deregulated in different types of cancers to promote cell survival and proliferation while evading growth suppression and cell death [8,9,10,11,12]. However, certain anticancer quinones have been observed to overcome this aberration by stimulating the MAPKs to activate the apoptotic pathways [13,14,15,16].

In addition, several quinones were also observed to promote cancer cell death in a reactive oxygen species (ROS)-dependent mechanism [17,18,19,20]. The redox systems in most cancer cells were observed to be aberrant, with higher levels of ROS higher compared to normal cells [21]. This higher but non-lethal level of ROS is believed to play an important role in carcinogenesis as they serve as second messengers to alter the signaling pathways, thus promoting proliferation and survival of cancer cells [22,23]. Nevertheless, it is also known that high concentrations of ROS will completely overwhelm the antioxidant defense mechanism, causing extreme oxidative damage to cellular components and results in cell death [24,25]. In fact, several chemotherapeutic drugs function by inducing ROS generation in cancer cells in order to eliminate them [26,27].

The compound 2-methoxy-1,4-naphthoquinone (MNQ) (Fig. 1) is a quinone extracted from garden balsam (Impatiens balsamina). This compound has been observed to exert an anticancer effect against several cancer cell lines, including HepG2 hepatocarcinoma cells, MDA-MB-231 breast cancer cells, and MKN45 gastric adenocarcinoma cells [28,29,30]. Nevertheless, its mechanism of action is not fully understood. In this study, we investigated the growth inhibitory potential of MNQ on A549

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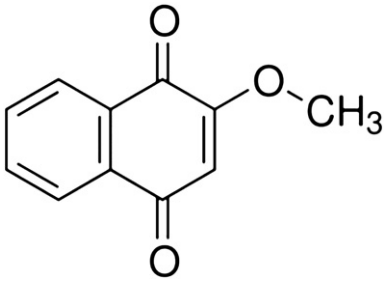

**Fig. 1.** Chemical structure of 2-methoxy-1,4-naphthoquinone (MNQ).

lung adenocarcinoma cells and elucidated the molecular mechanism of MNQ in inducing A549 lung cancer cell death in regard to MAPK signaling and redox balance.

## 2. Materials and methods

### 2.1. Chemicals and reagents

2-Methoxy-1,4-naphthoquinone (MNQ), RPMI 1640, RNase, propidium iodide (PI), N-acetylcysteine (NAC) and dichlorofluorescein diacetate (DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), JNK inhibitor SP600125 and p38 inhibitor SB203580 were purchased from Calbiochem (San Diego, CA, USA). Primary antibodies against ERK, JNK, p38, and β-actin, as well as HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while primary antibodies against phospho-ERK, phospho-JNK and phospho-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Fetal bovine serum (FBS) was purchased from JR Scientific (Woodland, CA, USA). Annexin V-FITC was purchased from BD Biosciences (San Jose, CA, USA).

### 2.2. Cell lines and cell culture

A549 lung adenocarcinoma epithelial cell line was purchased from American Type Cell Culture Collection (Manassas, USA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were grown as monolayers at 37 °C and 5% $CO_2$.

### 2.3. Sulforhodamine B (SRB) growth inhibition assay

The growth inhibitory potential of MNQ was measured using In Vitro Toxicology Assay Kit Sulforhodamine B-Based (Sigma-Aldrich, St. Louis, MO). Briefly, $5 \times 10^3$ A549 cells were seeded in a 96-well cell culture dish and treated with different concentrations of MNQ (0, 0.3125, 0.625, 1.25, 2.5, 5.0, 10, and 20 μM) for 48 h, or 10 μM MNQ for 24, 48 and 72 h. The cells were then fixed with TCA and washed and stained with sulforhodamine B (SRB) solution. After washing off unincorporated SRB dye with 1% acetic acid, the protein-bound dye was solubilized with 10 mM Tris and the absorbance was read at 490 nm using the iMark Microplate Reader (Bio-Rad, Hercules, CA).

### 2.4. Cell cycle analysis

A549 cells were seeded in a 6-well dish and treated with 10 μM MNQ for 6, 12, 24, and 48 h. The cells were subsequently harvested and fixed with ethanol. The fixed cells were then washed with PBS and resuspended in 20 μg/ml RNase (Sigma-Aldrich, St. Louis, MO) with 5 μg/ml PI and incubated in the dark for 30 min. The samples were then analyzed using an Accuri C6 (BD Biosciences, San Jose, CA) flow cytometer. The data obtained were analyzed using the ModFit LT version 4 software (Verity Software House).

### 2.5. Annexin V apoptosis assay

A549 cells were seeded into a 6-well dish and treated with 10 μM MNQ for 3, 6, 9, and 12 h. The cells were harvested, washed with PBS and resuspended in 1× binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$) containing Annexin V-FITC and propidium iodide. The cells were then incubated in the dark with Annexin V-FITC and propidium iodide (PI) before being analyzed on the Accuri C6 flow cytometer.

### 2.6. SDS-PAGE and Western blot

A549 proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL) at 4 °C with shaking for 30 min. The lysates were then collected and centrifuged at 14,000 × *g* for 10 min at 4 °C to collect the supernatant. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) before equivalent amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting protein bands were transferred onto a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The PVDF membrane was then blocked with 1% BSA and incubated with the desired primary antibody. The membrane was then incubated with the appropriate HRP-conjugated secondary antibody before visualizing by enhanced chemiluminescence (ECL) using Amersham ECL Select (GE Healthcare, Buckinghamshire, UK) on the BioSpectrum 600 Imaging System (UVP, Upland, CA).

### 2.7. Reactive oxygen species (ROS) measurement assay

A549 cells were seeded into a black 96-well dish and incubated with 2′,7′-dichlorofluorescein diacetate (DCFDA) for 60 min. The DCFDA solution was washed off and the cells were incubated with media without MNQ or with MNQ at concentrations of 10 or 20 μM. The black 96-well dish was placed in the FLUOstar OPTIMA fluorescence microplate reader (BMG LABTECH, Ortenberg, Germany) with excitation and emission filters set at 495 nm and 519 nm, respectively. The fluorescence signals were collected every 30 min for 3 h.

### 2.8. Single cell gel electrophoresis (comet assay)

A549 cells were seeded into 60 mm dishes and treated with 10 μM MNQ for 1 h. The cells were then trypsinized and washed with cold PBS. Comet assay was then performed using the OxiSelect Comet Assay Kit (Cell Biolabs, SD) according to the manufacturer's instruction. Briefly, harvested individual cells were mixed with molten low melt agarose and applied on the OxiSelect Comet Slide. The embedded cells were then lysed using the Lysis Buffer and treated with Alkaline Solution to relax and denature the DNA. Subsequently, electrophoresis of the samples was carried out under alkaline condition at 1 V/cm and 300 mA for 30 min. After electrophoresis, the samples were stained with Vista Green fluorescence dye for 15 min and viewed using the Eclipse Ti-E fluorescence inverted microscope (Nikon Instruments, Japan). Analysis was done using CASP comet assay software (CaspLab) and results were expressed as Tail Moment (Tail DNA% × Tail Length).

### 2.9. Pathway inhibition and ROS scavenging

For inhibition of JNK and p38 MAPK activations as well as ROS scavenging, A549 cells were pre-treated with 20 μM specific chemical inhibitors (SP600125 or SB203580) for 1 h or 5 mM ROS scavenger NAC for 30 min before treatment with MNQ for indicated periods.