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# Antiproliferative effects of phenylaminonaphthoquinones are increased by ascorbate and associated with the appearance of a senescent phenotype in human bladder cancer cells

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ABSTRACT

Quinone-containing molecules have been developed against cancer mainly for their redox cycling ability leading to reactive oxygen species (ROS) formation. We have previously shown that donor-acceptor phenylaminonaphthoquinones are biologically active against a panel of cancer cells. In this report, we explored the mechanisms involved in cancer cell growth inhibition caused by two phenylaminonaphthoquinones, namely **Q7** and **Q9**, with or without ascorbate (ASC). The results show that **Q7** and **Q9** are both redox cyclers able to form ROS, which strongly inhibit the proliferation of T24 cells. **Q9** was a better redox cycler than **Q7** because of marked stabilization of the semiquinone radical species arising from its reduction by ascorbate. Indeed, ASC dramatically enhances the inhibitory effect of **Q9** on cell proliferation. **Q9** plus ASC impairs the cell cycle, causing a decrease in the number of cells in the G2/M phase without involving other cell cycle regulating key proteins. Moreover, **Q9** plus ASC influences the MAPK signaling pathways, provoking the appearance of a senescent cancer cell phenotype and ultimately leading to necrotic-like cell death. Because cellular senescence limits the replicative capacity of cells, our results suggest that induction of senescence may be exploited as a basis for new approaches to cancer therapy.


## 1. Introduction

Cancer is a major health problem affecting humans. Nearly 15 million new cases are diagnosed globally every year [1], but the use of conventional cancer chemotherapy is limited because of its toxic side effects [2].

As part of our ongoing studies concerning the preparation of potential biologically active compounds [3–5], we were interested in the synthesis of diversely substituted donor–acceptor phenylaminonaphthoquinones. Briefly, using an MTT-based screening assay, we have shown that these compounds are biologically active against a panel of cancer cells. Among this series, two quinones (namely **Q7** and **Q9**) markedly impaired the cellular ability to reduce MTT in three different cancer cell lines, while having a low cytotoxicity on healthy fibroblasts [4]. Previous data also have shown that the association of ascorbate (ASC) with some quinone-containing compounds potentiates their antitumor activity [3,6,7].

Because ascorbate enhances the redox cycling of menadione (a 1,4-naphthoquinone) leading to activation of the p38 MAPK pathway in breast MCF-7 cancer cells [8], we hypothesized about a potential involvement of this pathway in the mechanisms by which phenylaminonaphthoquinones cause cell growth arrest. On the other hand, the enzymatic cascades of ERK1/2 and p38 MAPK have been reported to be involved in premature senescence [9,10]. Cellular senescence is a growth-arrest program that limits the lifespan of mammalian cells and prevents unlimited cell proliferation [11]. Interestingly, when apoptosis fails to induce cancer cell death, the induction of cellular senescence is attractive because of its relationship with tumor suppression.

The aim of this paper was, therefore, to explore the effects of phenylaminonaphthoquinones, alone or associated with ascorbate (ASC), on cancer cell growth and cellular senescence. We describe the antiproliferative effects and the senescent phenotype induced by the selected quinones, namely **Q7** [2-(4-hydroxyanilino)-1,4-

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naphthoquinone] and **Q9** [2-(4-methoxyanilino)-1,4-naphthoquinone], in T24 bladder carcinoma cells. We also report their effects on MAPK signaling pathways and on cancer cell survival.

## 2. Material and methods

### 2.1. Chemicals and antibodies

Quinones **Q7** [2-(4-hydroxyanilino)-1,4-naphthoquinone] and **Q9** [2-(4-methoxyanilino)-1,4-naphthoquinone] were synthesized by amination of 1,4-naphthoquinone with the respective arylamines, under aerobic conditions, using $CeCl_3 \cdot 7H_2O$ as the Lewis acid catalyst as previously described [4]. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Gibco (USA). Sodium ascorbate, bovine serum albumin (BSA), nocodazole and the protease inhibitor cocktail were purchased from Sigma–Aldrich (USA). MAPK inhibitors PD98054, SB202190, and SP600125 were from Calbiochem (Millipore). The 7-amino-4-trifluoromethylcoumarin conjugated (Ac-DEVD-AFC) was from Enzo Life Sciences (USA). The phosphatase inhibitor cocktail was from Calbiochem (Merck Biosciences). JNK, p38 and ERK1/2 antibodies were purchased from Cell Signaling Technology (USA). Survivin, p53, p27, p21 and rabbit polyclonal antibody against poly (ADP-ribose) polymerase (PARP) were from Santa Cruz Biotechnology, Inc. (USA). Secondary antibodies were from Dako (Denmark) and Chemicon (Millipore, USA). All other chemicals were made from ACS grade reagents.

### 2.2. Cell culture

The human bladder carcinoma T24 cells were a gift from Dr. F. Brasseur (Ludwig Institute for Cancer Research-Brussels). They were cultured as previously described [6].

### 2.3. Cell viability

Cytotoxicity was measured using MTT [12] and trypan blue [13] assays. For the MTT assay, $10^4$ cells/well were plated onto 96-well plates and, after confluence, cells were treated for 24 h with the respective treatments. Cells were then washed twice with PBS and incubated for 2 h with MTT (0.5 mg/mL). Formazan crystals were solubilized by adding DMSO (100 μL/well) and the colored solutions were read at 550 nm. To verify the involvement of MAPK in the cytotoxicity, cells were pretreated for 2 h with the following MAPK inhibitors: (a) PD98054 (10 μM), (b) SB202190 (10 μM), (c) SP600125 (20 μM).

The Trypan blue assay was performed after a 24 h-exposure of T24 cells to quinones (10 μM), alone or with ASC (1 mM). After staining, cells were counted by a TC10 automated cell counter (Bio-Rad, USA), and the percentage cell viability was determined.

### 2.4. Cell cycle analysis

Cells were plated into 6-well plates ($50–100 \times 10^3$) and synchronized with nocodazole (30 ng/ml) for 14 h. Cells were treated for up to 72 h with the respective treatments. Cells were then washed and the cell pellet was resuspended in ice-cold 80% ethanol and kept at −20 °C overnight. Finally, cells were washed again with PBS and incubated for 20 min in a saponin-based permeabilization solution (BSA 1%, ribonuclease A 0.2 mg/ml and propidium iodide 50 μg/ml). Flow cytometry was performed using an LSRFortessa (BD Biosciences) FACS data were analyzed using the FlowJo software (Treestar).

### 2.5. Immunoblotting assays

After treatments, cells were washed with PBS and lysed in RIPA buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate and 1 mM phenylmethylsulfonyl fluoride) supplemented with 1% protease inhibitor and 3% phosphatase inhibitor cocktails. After denaturation in Laemmli buffer (60 mM Tris–Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), equal amounts of protein (30 μg) were subjected to SDS–PAGE electrophoresis followed by electroblot to nitrocellulose membranes. After blocking and washing, the membranes were incubated overnight with the primary antibodies, washed again and further incubated with the secondary antibodies. Immunodetection was performed using the enhanced chemiluminescence (ECL) detection kit (Amersham, UK) for HRP-coupled secondary antibodies. β-Actin served as a loading control.

### 2.6. Caspase-3 activity

After treatments, cells were washed twice with PBS, lysed, centrifuged, and the supernatants were incubated with the fluorogenic caspase-3 substrate, Ac-DEVD-AFC. Fluorochrome release after peptide cleavage was determined kinetically at room temperature using a Victor X2 spectrophotometer at $\lambda = 380$ nm for excitation and $\lambda = 505$ nm for emission (Perkin Elmer, Waltham, USA). Results are expressed as Units/mg protein, as originally described by Nicholson et al. [14]. Sanguinarine (5 μM), a flavonoid known to induce apoptosis [15], was used as a positive control.

### 2.7. Colony formation assay

The potential to induce clonogenic death was evaluated according to Franken et al. [16]. Cells (500/well) were treated for 24 h with the respective treatments. They were then washed twice with warm PBS and fresh medium was added. After 10–12 days, cells were stained with crystal violet and colonies with more than 50 cells were counted.

### 2.8. Electron paramagnetic resonance (EPR)

Phenylaminonaphthoquinones and ASC were solubilized at 10–100 μM and 1 mM, respectively. EPR spectra were obtained at X-band (9.785 GHz) on a Bruker EMX spectrometer equipped with a variable temperature controller, BVT-3000, using 5 mW microwave power, a modulation frequency of 100 kHz and a modulation amplitude of 0.63 G. The recording of EPR spectra was started 1 min after inserting a sample into the cavity. The scan time was 20.9 s. All spectra were collected at 37 °C as previously reported [7].

### 2.9. Senescence assays

Cells were exposed for 24 h to **Q7** (10 μM) or **Q9** (4 μM), alone or with 1 mM ASC. Induction of senescence was assessed by measuring SA-β-galactosidase (β-Gal) activity using the senescence cells histochemical staining kit from Sigma–Aldrich (USA) and according to the procedures described by the manufacturer. Cell staining and morphology were assessed by microscopy. Images were captured by the Motics Image Plus 2.0 software (Ted Pella Inc., Redding, USA). We used an arbitrary quantitative analysis to record the number of β-Gal positive stained cells in each experimental condition. Briefly, β-Gal positive cells were counted by analyzing approximately 30 cells for each magnified field (X) and 10 fields were taken into account. Data were recorded as percentage of senescent cells.