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Inhibition of cell proliferation and migration by oxidative stress from ascorbate-driven juglone redox cycling in human bladder-derived T24 cells

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ABSTRACT

The effects of juglone on T24 cells were assessed in the presence and absence of ascorbate. The EC_{50} value for juglone at 24 h decreased from 28.5 μM to 6.3 μM in the presence of ascorbate. In juglone-treated cells, ascorbate increased ROS formation (4-fold) and depleted GSH (65%). *N*-acetylcysteine or catalase restricted the juglone/ascorbate-mediated effects, highlighting the role of oxidative stress in juglone cytotoxicity. Juglone alone or associated with ascorbate did not cause caspase-3 activation or PARP cleavage, suggesting necrosis-like cell death. DNA damage and the mild ER stress caused by juglone were both enhanced by ascorbate. In cells treated with juglone (1–5 μM), a concentration-dependent decrease in cell proliferation was observed. Ascorbate did not impair cell proliferation but its association with juglone led to a clonogenic death state. The motility of ascorbate-treated cells was not affected. Juglone slightly restricted motility, but cells lost their ability to migrate most noticeably when treated with juglone plus ascorbate. We postulate that juglone kills cells by a necrosis-like mechanism inhibiting cell proliferation and the motility of T24 cells. These effects are enhanced in the presence of ascorbate.

1. Introduction

Juglone (5-hydroxy-1,4-naphtoquinone) is a pigment found in the roots, wood, bark, leaves and nuts of *Juglans* sp. (Juglandaceae) walnut trees [1]. In its natural environment, juglone has a critical ecological function, acting as an allelopathic compound, very effective in the plant competition fight [2]. Several studies have shown potential anticancer activity of juglone against a representative panel of human tumor cell lines [3–5]. Moreover, juglone had *in vivo* anticancer and radiosensitizing activities against an experimental chemo/radio resistant melanoma, B16F1, in C57BL/6J mice [6]. When considering the type of cell death induced by juglone, the final outcome seems to be dependent on the exposed cell lines, which may vary in terms of sensitivity: Juglone triggered apoptosis in SGC-7901 gastric cancer cells [7], HL-60 leukemia cells [8] and A549 adenocarcinomic alveolar basal epithelial cells [9]; however, necrosis was observed in B16F1 melanoma cells exposed to juglone [1].

In the search for new redox active chemotherapeutic agents, we decided to explore a particular vulnerability of cancer cells by impairing their redox status. Indeed, as a result of metabolic alterations and the activity of oncogenes, cancer cells often exhibit high levels of reactive oxygen species (ROS), which can stimulate cell proliferation and promote genetic instability [10]. In addition, cancer cells have low antioxidant defense activity [11]. These biochemical differences between normal and cancer cells represent a specific vulnerability that can be selectively targeted for cancer therapy. In this context, previous studies have shown that redox active quinones associated with pharmacological doses of ascorbate generate redox cycling that potentiates, both *in vitro* and *in vivo*, the anti-tumor effects of quinone [12–15]. It should be noted that because of an over-expression of GLUT1 glucose transporters, cancer cells may increase the uptake of vitamin C and accumulate ascorbate [16,17].

Because the cytotoxicity of juglone has been associated with the ability to induce oxidative stress [18], we hypothesized that ascorbate may enhance the redox cycling of this quinone-bearing

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molecule. The aim of this study was, therefore, to investigate the biological effects of juglone on human bladder-derived tumor T24 cells in the absence and presence of ascorbate. We investigated the cytotoxic profile, the induction of oxidative stress (ROS formation and GSH levels), and the type of cancer cell death (apoptosis or necrosis). Additional markers of cellular stress, such as oxidative DNA lesions and endoplasmic reticulum (ER) stress, were also assessed by measuring the levels of phosphorylated histone H2AX (γH2AX) and the eukaryotic initiation factor 2 (eIF2α), respectively. Finally, the effects of juglone alone or combined with ascorbate were evaluated on some key malignant-acquired capabilities, namely increased cell proliferation and motility.

2. Material and methods

2.1. Chemicals and antibodies

Juglone was synthesized by green chemistry, following the photochemical procedure described by Oelgemöller et al. [19]. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Gibco (USA). Sodium ascorbate, *N*-acetylcysteine (NAC), catalase (CAT), DMSO, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 5-sulfosalicylic acid (SSA), NADPH, bovine serum albumin (BSA), and protease inhibitor cocktail were purchased from Sigma–Aldrich (USA). The 7-amino-4-trifluoromethylcoumarin conjugated (Ac-DEVD-AFC) was from Enzo Life Sciences (USA). The phosphatase inhibitor cocktail was from Calbiochem (Merck4Biosciences). Rabbit polyclonal antibody against γH2AX was from Upstate (USA), and rabbit monoclonal antibody against phospho-eIF2α (Ser51) was from Cell Signaling Technology (USA). Mouse monoclonal antibody against eIf2α was from Abcam (UK) and rabbit polyclonal antibody against poly (ADP-ribose) polymerase (PARP) was 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-LICR-Brussels). They were cultured at 37 °C under 5% CO_2 atmosphere with 95% air humidity. DMEM was used, supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL).

2.3. Cell viability

Cytotoxicity was measured using the MTT assay [20]. Briefly, 10^4 cells/well were plated onto 96-well plates and, after confluence, were exposed to juglone (0–40 μM) in the absence or in the presence of ascorbate (1 mM) for up to 24 h. 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. Cell morphology was evaluated using a microscope and a photography unit with the Motics Image Plus 2.0 software (Ted Pella Inc., Redding, USA).

2.4. Oxidative stress markers

Intracellular ROS were evaluated as reported by Glorieux et al. [21]. Cells (15.000) were loaded with 10 μM DCFH-DA in HBSS at 37 °C and incubated for 30 min. Excess DCFH-DA was removed by washing with fresh HBSS. Cells were then incubated for 2 h with the test compounds, washed twice more with HBSS, and then 100 μL of HBSS/well was added. The intensity of fluorescence was measured with a microplate reader (Victor™ X2, PerkinElmer) at 485 nm for excitation and 530 nm for emission. Changes in ROS levels were determined by calculating $\Delta F = (F_t - F_c)/F_c$, where F_t represents the fluorescence read at each time point and F_c the control fluorescence.

Intracellular GSH was estimated according to Griffith [22]. Treated cells were washed with cold PBS and acidified with 5% SSA. The samples were submitted to two freeze–thaw cycles and centrifuged at 4 °C (10.000 *g*/10 min). Ten microliters of the supernatant were then placed in a reaction mixture containing 0.2 U/mL of glutathione reductase, 50 mg/mL of DTNB and 1 mM of EDTA at pH 7. The reaction was initiated by adding 50 mM NADPH and changes in absorbance were recorded at 412 nm. Results were expressed as nmol of GSH and normalized to the protein content using the method of Lowry et al. [23].

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) from whole cellular homogenates 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 380 nm for excitation and 505 nm for emission (Perkin Elmer, Waltham, USA). Results are expressed as Units/mg protein, as originally described by Nicholson et al. [24]. Sanguinarine 5 μM, a flavonoid known to induce apoptosis [25], was used as a positive control.

2.7. Colony formation assay

The potential to induce clonogenic death was evaluated according to Franken et al. [26]. Cells (500) were treated for 2 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 by crystal violet and colonies with more than 50 cells were counted.

2.8. Wound healing assay

Cell culture plates with inserts (Ibidi™) were used according to the manufacturer's protocol. In each plate-set, the inserts formed two small chambers, which were filled with 3.5×10^4 cells and incubated overnight. The inserts were then removed and fresh serum-free medium was provided containing the respective treatments. Images were captured at regular time intervals using a

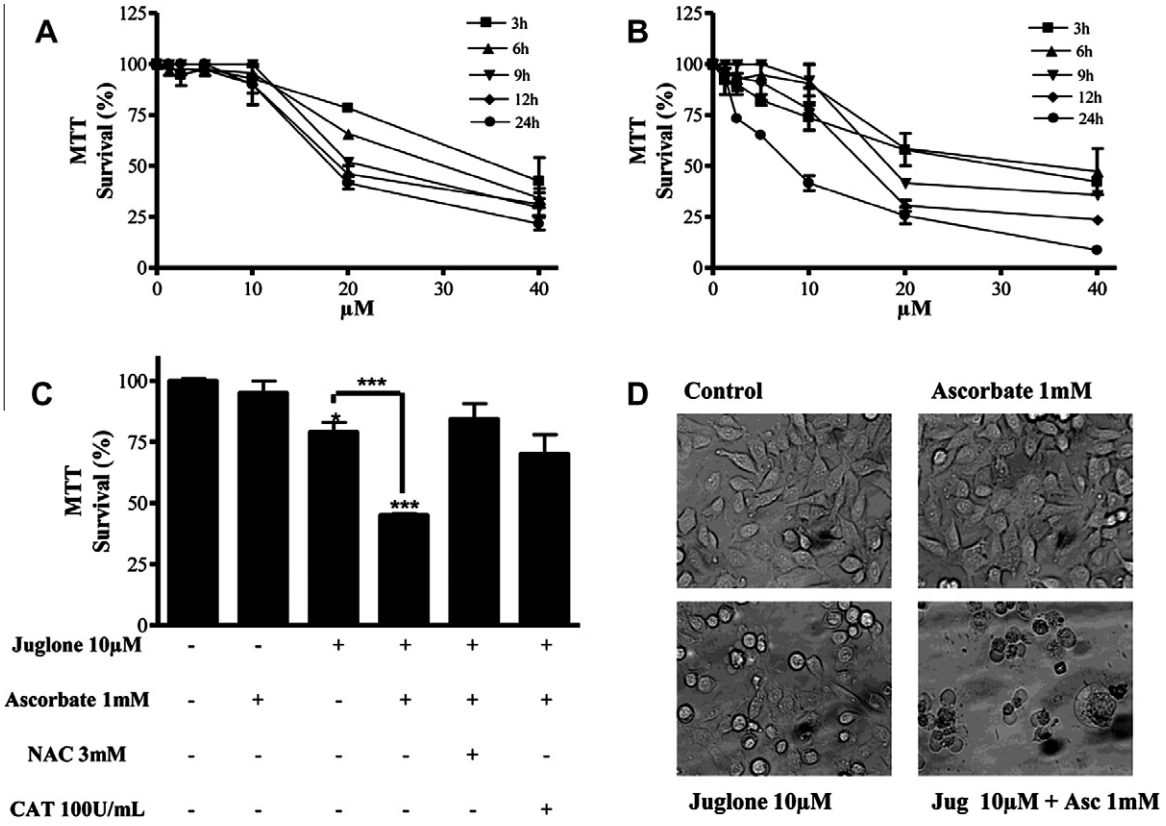

Fig. 1. T24 cells were incubated for 3, 6, 9, 12 or 24 h with juglone alone at different concentrations ranging from 0 to 40 μM (A) or associated with 1 mM ascorbate (B). In addition, cells were incubated for 24 h as indicated and supplemented with *N*-acetylcysteine (NAC) at 3 mM or catalase (CAT) at 100 U/mL (C). Morphology of T24 cells incubated for 24 h with 1 mM ascorbate or juglone (10 μM) alone or associated with 1 mM ascorbate (D). (*) and (***) denote statistical difference at $p < 0.01$ and $p < 0.001$ compared to control or between indicated treatments.

microscope coupled to a photography unit equipped with Motics Image Plus 2.0 software (Ted Pella Inc., Redding, USA).

2.9. Data analysis

Data were analyzed with the ANOVA test followed by the Bonferroni test *post hoc*. The comparisons and the EC_{50} values were performed using GraphPad Prism software (San Diego, USA). Values of $p < 0.05$ were considered to be statistically significant.

3. Results

The cytotoxicity of juglone on T24 cells is shown in Fig. 1. Cells treated with juglone showed a concentration-dependent decreased capacity to reduce MTT (Fig. 1A). A stronger cytotoxic effect was observed when juglone was associated with 1 mM ascorbate (Fig. 1B). The EC_{50} value at 24 h for juglone decreased from 28.5 μM to 6.3 μM when juglone was administered with ascorbate. Antioxidants, e.g., NAC and CAT, protected against the oxidative stress induced by the association of juglone plus ascorbate (Fig. 1C). Fig. 1 also shows that the cytotoxic effect of juglone plus ascorbate was higher than the cytotoxicity of either product administered separately. Accordingly, the morphology of cells treated with juglone alone was different from ascorbate and untreated control cells and clearly distinguishable from juglone/ascorbate-treated cells (Fig. 1D).

Fig. 2 shows the effects of juglone alone or with ascorbate on several oxidative markers and on the integrity of the PARP protein. Fig. 2A shows that the addition of ascorbate to juglone-treated cells enhanced by about 4-fold the production of intracellular ROS, whereas the antioxidants, NAC or CAT, restricted ROS formation. Fig. 2B shows that juglone plus ascorbate caused GSH depletion (65%), but partial recovery was seen in the presence of NAC or CAT. Juglone caused additional cellular injury as shown by the increase in the phosphorylated protein band of γ-H2AX (DNA damage), and the appearance of the phosphorylated protein band corresponding to eIF2α, suggesting mild ER stress (Fig. 2C). Both processes were strongly enhanced by the presence of ascorbate.

The demise of T24 cells caused by juglone was examined using two assays reflecting activation of caspase-3, namely DEVDase activity and PARP cleavage. The cleavage of PARP protein from a molecular size of 116 kD, corresponding to the whole protein, to the cleaved fragment of about 89 kD was only observed in sanguinarine-treated cells, and not in juglone-treated cells with or without ascorbate (Fig. 2D). Compared to the control untreated cells with DEVDase activity of 2.0 ± 1.0 U/mg, sanguinarine (5 μM) triggered caspase-3 activation with activity reaching 95 ± 8 U/mg after 4 h of incubation. Juglone, alone or associated with ascorbate, was unable to induce caspase-3 activity, with values non-distinguishable from controls at about 2–3 U/mg even after 24 h of incubation (data not shown). These data rule out a putative involvement of a caspase-3 dependent mechanism leading to cell death, and suggests a mechanism of cell death closer to necrosis.

To further characterize the effects of juglone on T24 cells, we explored whether cellular proliferation and cell migration were impaired by juglone alone or in association with ascorbate. Fig. 3 shows that in cells treated just with juglone (1–5 μM), there was a concentration-dependent decrease in cell proliferation. Ascorbate did not impair the capacity of T24 cells to proliferate, but its

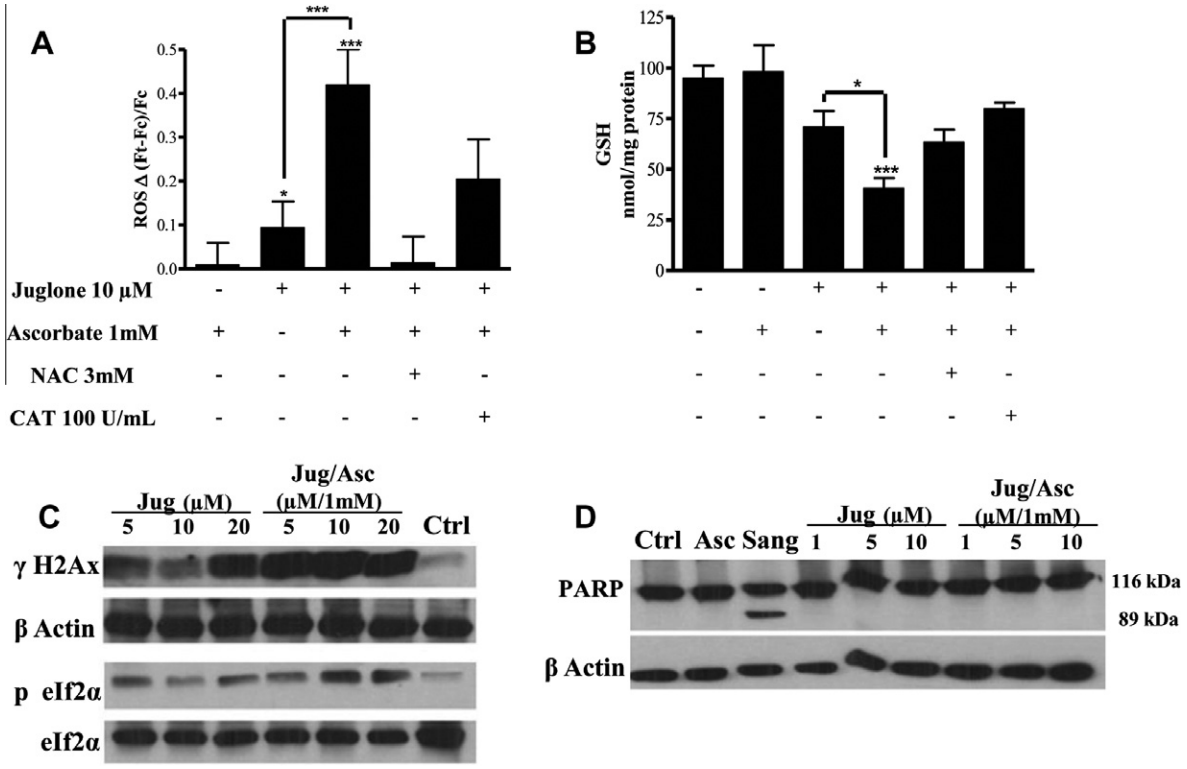

Fig. 2. T24 cells were incubated under different conditions as indicated in the figure after 2 h of treatment for ROS formation (A) and after 3 h for GSH (B). Immunoblots were performed as reported in the Materials and Methods in whole cell homogenates after 4 h treatments of juglone alone (1–20 μM) and in the presence of 1 mM ascorbate (C); or juglone alone (1–10 μM) and in the presence of 1 mM ascorbate (D). Sanguinarine 5 μM (Sang) was used as positive control of caspase-3 activation. Results are means from three separate experiments. (*) and (***) denote statistical difference at $p < 0.01$ and $p < 0.001$compared to control or between indicated treatments.

association with juglone, even at doses as low as 1 μM, led to a clonogenic death state. The addition of NAC or CAT restored the cellular capacity to proliferate, suggesting that juglone and ascorbate induce clonogenic death likely by an oxidative mechanism. The number of colonies was estimated from plates of stained cells (Supplementary data).

Fig. 4 shows that migration of control, untreated T24 cells was detectable after 12 h of incubation. From this point, migration was consistent, resulting in closure of the layer within 24 h. In ascorbate-treated cells there were no major differences compared to the control conditions. A partial restriction of cell motility was observed with juglone alone between 12 and 20 h. T24 cells notably lost their motility when treated with the association of juglone plus ascorbate.

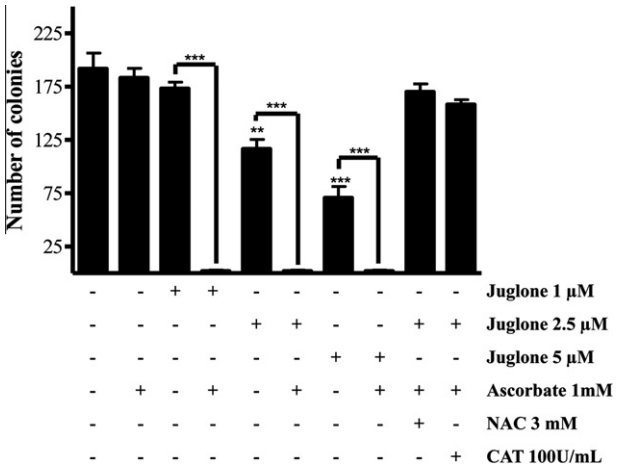

Fig. 3. T24 cells were incubated for 2 h under different conditions as indicated in the figure. After 10 days, colonies were stained and counted. Results are means from three separate experiments. (**) and (***) denote statistical differences at $p < 0.01$ and $p < 0.001$ compared to untreated control cells or between the indicated treatments.

4. Discussion

There is considerable interest in the antitumor effects of some quinoid compounds [18,27,28]. Over the last few decades, considerable effort has been put into understanding the mechanisms underlying the potential of the antitumor naphthoquinones. Two main features are generally accepted as being critical for the cytotoxicity of these agents: (a) the ability to generate ROS and (b) their electrophilicity enabling them to form adducts with some cell constituents. By evaluating the electrophilicity of 1.4-naphtoquinone and some of its hydroxylated derivatives, juglone has been classified as a molecule endowed with low electrophilicity [18]; hence, its cytotoxicity is expected to be due to an induction of cellular ROS generation. Our findings corroborate these predictions. First, we showed increased levels of ROS in T24 cells exposed to juglone alone or in association with ascorbate. Second, ROS generation was accompanied by consumption of intracellular GSH. Third, addition of the antioxidants, NAC and CAT, strongly prevented the cytotoxic effects of juglone-ascorbate. Taken together, these data reinforce the major role of oxidative stress in juglone cytotoxicity.

The mechanisms triggered by the juglone-ascorbate association leading to cell death were also explored. Attacks on DNA can cause oxidative damage able to trigger either cell death or senescence (clonogenic death) [29,30]. In this context, the phosphorylation of histone variant H2AX at serine 139 has been widely used as a sensitive marker of DNA damage [30]. Our results are in agreement

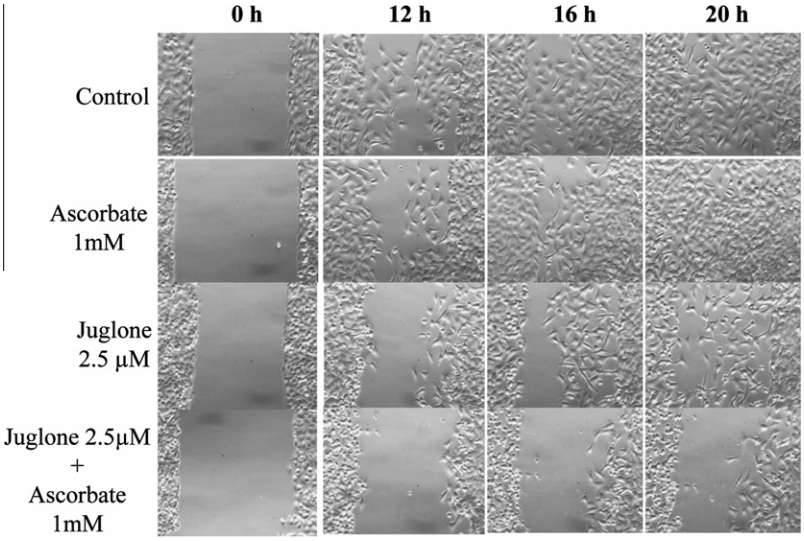

Fig. 4. Migration of T24 cells incubated for different times and under different experimental conditions.

with those published by Paulsen and Ljungma [31] who reported that juglone induced rapid phosphorylation of H2AX in all phases of the cell cycle. On the other hand, oxidative toxicity is also involved in leading vulnerable cells to death by a mechanism related to ER stress [32]. It is tempting to expect that oxidative stress via DNA damage and early cell senescence may lead to the accumulation of misfolded proteins and activate an unfolded protein response. As reported, mild ER stress was also observed in cells exposed to juglone alone or associated with ascorbate, as shown by the appearance of the phosphorylated protein band corresponding to eIF2α.

Previous reports indicate that juglone can induce apoptosis in some cell lines [7–9], whereas necrosis was caused by some concentrations in other types of cell [1,33]. Results of the lactate dehydrogenase release assay obtained by Aithal et al. [1] showed that B16F1 cells treated with juglone presented membrane burst, something that the authors proposed was related to the cytotoxicity of juglone. On this basis and from our results it seems that the type of cell demise depends on the cell type, its sensitivity, and the concentration of juglone. In T24 cells, markers of caspase-3 activation (DVEDase activity and PARP cleavage) and the morphologic changes induced by juglone alone or in association with ascorbate over a large range of concentrations, suggest induction of necrosis-like death.

A concentration-dependent inhibition of proliferation of T24 cells was induced by the juglone-ascorbate association resulting in a clonogenic death state, i.e., a total loss of cellular ability to proliferate. Moreover, in juglone-ascorbate treated T24 cells, motility was markedly reduced compared to the control untreated cells. Movement of cancer cells into tissue surrounding the tumor and the vasculature are the first steps in the spread of metastatic cancers [34]. In this context, the study of the molecular bases of invasion has been focused on the detection of intracellular targets, such as the cell surface and extracellular proteins that mediate sensing, adhesion, and proteolysis, as well as the signal transduction pathways that regulate invasion, angiogenesis and tumor cell proliferation [35]. We postulate that juglone may impair both tumor cell proliferation and tumor cell motility via a redox mechanism and that these effects are enhanced in the presence of ascorbate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.150.

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