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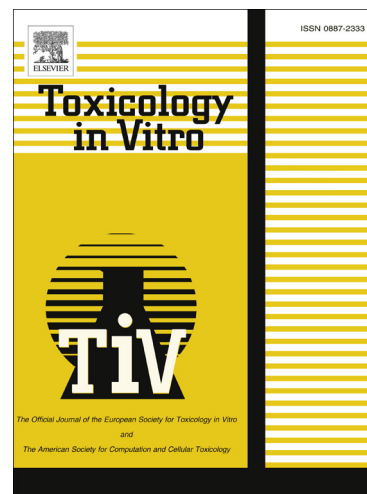
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1 **Novel *o*-naphthoquinones induce apoptosis of EL-4 T lymphoma cells through the**  
2 **increase of reactive oxygen species**

3  
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**1 ABSTRACT**

2

3 Novel  $\beta$ -lapachone analogs 2-phenyl-3,4-dihydro-2H-benzo[h]chromene-5,6-dione  
4 (NQ1), 2-p-tolyl-3,4-dihydro-2H-benzo[h]chromene-5,6-dione (NQ3) and 2-methyl-2-  
5 phenyl-3,4-dihydro-2H-benzo[h]chromene-5,6-dione (NQ7), which have trypanocidal  
6 activity, were assayed for cytotoxic effects on murine EL-4 T lymphoma cells. The NQs  
7 inhibited the proliferation of EL-4 cells at concentrations above 1  $\mu$ M. Nuclear staining  
8 of the EL-4 cells revealed chromatin condensation and a nuclear morphology  
9 compatible with the induction of apoptosis. Flow cytometry assays with annexin V-  
10 FITC and propidium iodide confirmed the cell death by apoptosis. Using electron  
11 paramagnetic resonance (EPR), a semiquinone radical was detected in EL-4 cells treated  
12 with NQs. In addition, a decrease in the GSH level in parallel with reactive oxygen  
13 species (ROS) production was observed. Preincubation with n-acetyl-l-cysteine (NAC)  
14 was able to reverse the inhibitory effects of the NQs on cell proliferation, indicating that  
15 ROS generation is involved in NQ-induced apoptosis. In addition, the NQs induced a  
16 decrease in the mitochondrial membrane potential and increased the proteolytic  
17 activation of caspases 9 and 3 and the cleavage of Poly (ADP-Ribose) Polymerase  
18 (PARP). In conclusion, these results indicate that redox cycling is induced by the NQs  
19 in the EL-4 cell line, with the generation of ROS and other free radicals that could  
20 inhibit cellular proliferation as a result of the induction of the intrinsic apoptosis  
21 pathway.

22

23 *Keywords* : *o*-naphthoquinones; oxidative stress; apoptosis.

24

**25 Abbreviations list**

26 NQ1: 2-phenyl-3,4-dihydro-2H-benzo[h]chromene-5,6-dione; NQ3: 2-p-tolyl-3,4-  
27 dihydro-2H-benzo[h]chromene-5,6-dione; NQ7: 2-methyl-2-phenyl-3,4-dihydro-2H-  
28 benzo[h]chromene-5,6-dione; DMFA: *N,N*-dimethyl-formamide; AO: acridine orange;  
29 EB: ethidium bromide; PI: propidium iodide; EPR: electron paramagnetic resonance;  
30 ROS: reactive oxygen species; H2DCF-DA: 2,7-dichlorodihydrofluorescein diacetate;  
31 DTNB: 5,5'-dithiobis-2-nitrobenzoic acid; TCA: trichloroacetic acid; TNB: 2-nitro-5-  
32 thiobenzoic acid; NAC: n-acetyl-l-cysteine; Rho-123: rhodamine-123; PARP: Poly  
33 (ADP-Ribose) Polymerase; PTP: permeability transition pore; MMP: mitochondrial  
34 membrane potential.

## 1 1. INTRODUCTION

2  
3 Quinones are considered privileged structures in medicinal chemistry because of their  
4 biological activities and structural properties (Bolton et al., 2000; Costantino and  
5 Barlocco, 2006). Indeed, several quinones, such as doxorubicin, mitomycin, and  
6 mitoxantrone, are medicines that are still used clinically for the treatment of solid  
7 cancers (Edmonson et al., 1993). Among quinones, naphthoquinones are especially  
8 important due to their biological activities and structural properties (Costantino and  
9 Barlocco, 2006). The biological profiles of these molecules are based on their *ortho*- or  
10 *para*-quinoid moiety. This group generally accepts one and/or two electrons to form the  
11 corresponding radical species, and the driving force behind this redox property is the  
12 formation of a fully aromatic system (Asche, 2005; Hillard et al., 2008). In biological  
13 systems, naphthoquinones are reduced to semiquinone radicals through two different  
14 pathways (de Witte et al., 2004; Elingold et al., 2009; Fernandez Villamil et al., 2004;  
15 Thor et al., 1982). The one-electron reduction and direct formation of semiquinone is  
16 catalyzed primarily by microsomal NADPH-cytochrome P450 reductase. The two-  
17 electron reduction is catalyzed by the cytosolic flavoprotein DT-diaphorase (NADPH  
18 (quinone acceptor) oxidoreductase NQO1) and generates hydroquinone, which can be  
19 reoxidized and indirectly form the semiquinone (Cadenas, 1995; dos Santos et al., 2004).  
20 This radical is capable of transferring electrons to molecular oxygen, thereby returning  
21 to its original quinoidal structure and generating a superoxide anion radical ( $O_2^{\bullet-}$ ).  
22 Superoxide dismutases to hydrogen peroxide ( $H_2O_2$ ), and then the hydroxyl radical  
23 ( $OH^{\bullet}$ ) can be formed (Boveris et al., 1978a; Molina Portela et al., 1996; Molina Portela  
24 and Stoppani, 1996). These highly reactive oxygen species (ROS) can cause damage to  
25 DNA, lipids, and proteins (Brunmark and Cadenas, 1989; Valko et al., 2007). A great  
26 number of physiological functions are controlled by redox-responsive pathways (Droge,  
27 2002). It has been reported that the induction of apoptosis via the mitochondrial  
28 pathway can be mediated by an increase in the ROS level (Hampton et al., 1998;  
29 Kroemer et al., 2007; Seshadri et al., 2011; Xu et al., 2012; Xu et al., 2010).

30  
31 Among naphthoquinones, *o*-naphthoquinones are interesting because of their electronic  
32 structure, which makes them more active than *p*-naphthoquinones against trypanosomatids  
33 and human cancer cell lines (Boveris et al., 1978a; Kongkathip et al., 2003; Lopes et al.,  
34 1978; Molina Portela et al., 1996). The natural *o*-naphthoquinone  $\beta$ -lapachone has been

1 intensely investigated not only due to its trypanocidal activity but also for clinical use in  
2 cancer therapy (Goijman and Stoppani, 1985; Pardee et al., 2002). The studies on  $\beta$ -  
3 lapachone focused on cancer chemotherapy pointed to topoisomerases I and II as its  
4 biochemical targets in the induction of apoptosis (Li et al., 1995). The cytotoxicity of  $\beta$ -  
5 lapachone prompted the synthesis of a number of *o*-naphthoquinones to establish the  
6 structural requirements for optimal therapeutic use. Ferreira et al. (2011) synthesized  
7 new derivatives from  $\beta$ -lapachone. Among these compounds are 2-phenyl-3,4-dihydro-  
8 2*H*-benzo[*h*]chromene-5,6-dione (NQ1), 2-*p*-tolyl-3,4-dihydro-2*H*-benzo[*h*]chromene-  
9 5,6-dione (NQ3) and 2-methyl-2-phenyl-3,4-dihydro-2*H*-benzo[*h*]chromene-5,6-dione  
10 (NQ7) (Figure 1), which were only investigated against bloodstream trypomastigotes of  
11 *Trypanosoma cruzi*, the etiologic agent of Chagas disease, and proved to be more active  
12 than  $\beta$ -lapachone (Ferreira et al., 2011).

13

14 In the present study, we investigated the cytotoxic effects of NQs on EL-4 murine  
15 lymphoma cells and analyzed the mechanisms of action involved. To better understand  
16 the mechanism underlying the cytotoxicity of these compounds, studies involving the  
17 analysis of changes in nuclear morphology, the permeability of membranes,  
18 phosphatidyl serine externalization, semiquinone formation, ROS generation and  
19 mitochondrial membrane potential were performed. The activation of the proteolytic  
20 cascade of enzymes involved in apoptosis, such as caspase 9 and caspase 3, and the  
21 inactivation by cleavage of poly(ADP-ribose) polymerase (PARP) were investigated to  
22 analyze the intracellular signals involved. Our results indicate that the production of  
23 ROS and the semiquinone radical formation induced by NQ redox cycling may induce  
24 mitochondrial (intrinsic) pathway-dependent apoptosis involving the proteolytic  
25 activation of caspases 9 and 3 and PARP cleavage.

26

27

## 28 **2. MATERIALS AND METHODS**

29

### 30 **2.1. Chemicals**

31

32 The NQs used in this study were synthesized as previously described (Ferreira et al.,  
33 2011). The  $\beta$ -lapachone analogs were synthesized by the Knoevenagel condensation of  
34 2-hydroxy-1,4-naphthoquinone with paraformaldehyde or arylaldehydes, followed by a

1 hetero-Diels-Alder reaction with substituted styrenes in aqueous ethanol media,  
2 generating different  $\beta$ -lapachone analogs. The structures of the synthesized compounds  
3 were confirmed using spectroscopic techniques, such as  $^1\text{H}$  and  $^{13}\text{C}$  NMR, infrared  
4 spectroscopy, and HRMS (ESI), and our data are consistent with those reported  
5 previously.

6 The chemical structures of NQs are shown in Figure 1. These compounds were provided  
7 as lyophilized powders and were diluted in *N,N*-dimethyl-formamide (DMFA). Controls  
8 received the same volume of solvent, whose concentration never exceeded 0.5% (v/v).  
9 RPMI 1640 cell culture medium, fetal calf serum, glutamine, penicillin and  
10 streptomycin were purchased from Gibco (USA). [ $^3\text{H}$ ]-Thymidine was purchased from  
11 New England Nuclear, Perkin Elmer Inc, Boston, MA, USA. HCl, NaCl,  $\text{CaCl}_2$ , NaOH  
12 and KCl were provided by Merck Química Argentina S.A. (Buenos Aires, Argentina).  
13 *N*-Acetylcysteine (NAC), trypan blue, fetal calf serum (FCS), fetal bovine serum (FBS),  
14 phosphate-buffered saline (PBS), Hoechst 33258, acridine orange (AO), ethidium  
15 bromide (EB), DMFA, HEPES, annexin V-FITC, propidium iodide (PI),  $\text{KH}_2\text{PO}_4$ ,  
16  $\text{K}_2\text{HPO}_4$ , NADPH, 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA),  
17 trichloroacetic acid (TCA), GSH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB),  
18 glutathione reductase, 4-vinylpyridine, rhodamine 123 (Rho-123), Trizma base,  
19 [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), NP-40, NaF,  $\text{Na}_3\text{VO}_4$ , sodium  
20 deoxycholate, phenylmethylsulfonyl fluoride, aprotinin, pepstatin, leupeptin, SDS,  
21 glycerol, bromophenol blue, 2-mercaptoethanol, acrylamide,  $\text{N}'$ , $\text{N}'$ -methylene  
22 bisacrylamide and Tween were acquired from Sigma Chemical Co. (St. Louis, Mo,  
23 USA). Rabbit anti-active caspase 9 and rabbit anti-active caspase 3 antibodies were  
24 purchased from Abcam<sup>R</sup> (USA). Mouse anti-cleaved PARP was acquired from BD  
25 Biosciences (San Jose, CA, USA). The rabbit anti-actin antibody was purchased from  
26 Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Anti-rabbit or anti-mouse  
27 secondary antibodies coupled to horseradish peroxidase were acquired from Signaling  
28 Technology, Inc. (Beverly, MA, USA). All other chemicals used in this study were of  
29 analytical grade.

30

## 31 **2.2. Cell suspensions and culture conditions**

32

33 EL-4 mouse T lymphoma cells (American Type Culture Collection, ATCC, Manassas,  
34 VA, USA) were cultured at an optimal concentration of  $1-5 \times 10^5$  cells/ml in RPMI 1640

1 medium supplemented with 10% FCS, 2 mM glutamine, 100 g/ml of penicillin and 150  
2 g/ml of streptomycin. The culture medium was changed every day after the cells had  
3 reached the exponential growth phase. The cells were kept in T-25 culture flasks  
4 (Corning, NY) at 37 °C with a 5% CO<sub>2</sub> atmosphere.

### 6 **2.3. Proliferation assays and viability studies**

7  
8 EL-4 cells were cultured for 24 h in the absence (control) or presence of increasing  
9 concentrations of the NQs, and proliferation was evaluated in 96-well microplates  
10 (Nunc™) using a pulse of [<sup>3</sup>H]thymidine ([<sup>3</sup>H]-TdR, 20 Ci/mmol) 16 h before the end of  
11 the incubation period. At the end of culture incubation period, the cells were harvested,  
12 and the amount of [<sup>3</sup>H]-TdR incorporated to the DNA was quantified using a liquid  
13 scintillation counter (Barreiro Arcos et al., 2003). In the experiments involving NAC  
14 treatment, EL-4 cells were incubated with NAC for 18 h prior to culturing the cells  
15 under the conditions described previously (Donadelli et al., 2006). The results were  
16 expressed as the dpm values for the NQ-treated cells minus the dpm values for the  
17 control (cells treated with vehicle). Cytotoxic activity expressed by IC<sub>50</sub>±SE (M) of  
18 compounds for EL-4 cell line was obtained by nonlinear regression from three  
19 independent experiments. Cell viability was determined by trypan blue exclusion.  
20 Viability is expressed as the percentage of living cells and was calculated as follows:  
21  $[\text{number of viable cells} / \text{number of total cells}] \times 100$ .

### 23 **2.4. Chromatin condensation assay with Hoechst 33258 staining**

24  
25 T lymphoma cells were cultured in RPMI 1640 medium supplemented with FBS in the  
26 absence or presence of NQs for the indicated times, and then the nuclear morphology of  
27 the cells was examined. Briefly, the cells were washed and resuspended in PBS at a  
28 concentration of  $1 \times 10^6$  cells/ml, plated onto slides and fixed with ethanol. A nuclear  
29 staining solution containing 0.01 mg/ml Hoechst 33258 was added for 10 min. The cells  
30 were washed three times with PBS, and the nuclear morphology was then examined  
31 using a fluorescence microscope (Nikon Diaphou; Nikon Inc., Melville, NY).

### 33 **2.5. Acridine orange staining**

1

2 The presence of apoptotic morphology was analyzed by double staining with acridine  
3 orange and ethidium bromide (AO/EB). EL-4 cells were plated at a density of  
4  $1 \times 10^6$  cells/ml and were incubated with NQs at  $5 \mu\text{M}$  for different times. Then, the cells  
5 were washed twice and resuspended in 300  $\mu\text{l}$  of PBS. Ten microliters of a fluorescent  
6 dye mixture containing AO and EB (both  $100 \mu\text{g/ml}$ ) were added to the cells for 10 min.  
7 Freshly stained cell suspensions were dropped onto glass slides and covered by  
8 coverslips. The slides were observed under a UV-fluorescence microscope (Nikon  
9 Diaphou; Nikon Inc., Melville, NY) at a magnification of 1000x (Coligan, 1995).

10

### 11 ***2.6. Quantification of apoptosis by flow cytometry***

12

13 EL-4 cells were incubated in the absence or presence of NQs ( $5 \mu\text{M}$ ) for different times.  
14 Then,  $1 \times 10^6$  cells were washed once with PBS, resuspended in 0.5 ml of staining buffer  
15 (10 mM HEPES/NaOH, pH 7.5; 0.14 M NaCl; 2.5 mM  $\text{CaCl}_2$ ) and then incubated for  
16 15 min in the dark with  $5 \mu\text{l}$  of annexin V-FITC (1 mg/ml) and  $10 \mu\text{l}$  of propidium  
17 iodide (PI, 1 mg/ml) (Vermes et al., 1995). The labeled cells were analyzed by flow  
18 cytometry (FACSCalibur, Becton Dickinson Biosciences), and the fluorescence  
19 intensities at 580 nm (PI) and 520 nm (annexin V-FITC) were quantified. The data were  
20 analyzed with WinMDI 2.8 and were expressed as the percentage of cells in each  
21 subpopulation (unstained, stained with PI, stained with FITC or stained with both) with  
22 respect to the total number of cells analyzed (Walsh et al., 1998).

23

### 24 ***2.7. Analysis of free radical semiquinones***

25

26 Semiquinone radicals were detected by electron paramagnetic resonance (EPR) at room  
27 temperature under anaerobic conditions as previously described (Elingold et al., 2009).  
28 Briefly, EL-4 cells ( $2 \times 10^6$ ) were incubated with NQs ( $5 \text{ mM}$ ) in potassium phosphate  
29 buffer (0.1 M, pH 7.4) and NADPH (20 mM) in a final volume of 0.15 ml. The time  
30 elapsed between the preparation of the reaction mixture and the recording of the EPR  
31 spectra was approximately 5 min, and this period was considered as the incubation time.  
32 EPR measurements were performed on a Bruker BioSpin EMX Plus spectrometer  
33 (Bruker, Karlsruhe, Germany). The instrumental conditions were as follows: microwave



1 power, 20 mW; microwave frequency, 9.75 GHz; modulation amplitude, 1 G;  
2 modulation frequency, 50 kHz; time constant, 164 ms; and centerfield, 3490 G.

### 3 4 **2.8. Reactive oxygen species (ROS) measurement**

5  
6 The intracellular level of reactive oxygen species (ROS) was determined using the  
7 fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) (LeBel et al.,  
8 1992). EL-4 cells were cultured in the absence or presence of the NQs (5 M). After the  
9 appropriate culture duration,  $1 \times 10^6$  cells were washed and resuspended in 1 ml of PBS.  
10 Cells were incubated with 10  $\mu$ M DCF-DA for 20 min at 37°C, and the fluorescence  
11 was quantified using a FACSCalibur flow cytometer (Becton Dickinson Biosciences) at  
12 480 nm. In the experiments involving NAC treatment, EL-4 cells were incubated with  
13 NAC (12.5 mM) for 18 h prior to culturing the cells under the conditions described  
14 previously. The data were analyzed using WinMDI 2.8.

### 15 16 **2.9 Determination of the intracellular glutathione levels**

17  
18 The glutathione levels were determined using the method of Griffith (Griffith, 1980).  
19 Briefly, EL-4 cells were cultured in the absence or presence of NQs for different times.  
20 Then, cells were washed, resuspended in PBS and pelleted at 1000 x g for 10 min. The  
21 cell pellets were resuspended in 200  $\mu$ l of TCA (0.5%) and frozen at -20°C. Then, the  
22 pellets were thawed and centrifuged to remove the cellular debris. The supernatants  
23 were used to quantify the total glutathione and GSSG levels. The total glutathione level  
24 was determined using an enzyme recycling procedure, in which the GSH is oxidized by  
25 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of  
26 glutathione reductase. The formation of 2-nitro-5-thiobenzoic acid (TNB) was  
27 monitored at 412 nm. In the experiments involving NAC treatment, EL-4 cells were  
28 incubated with NAC for 18 h prior to culturing the cells under the conditions described  
29 previously. The total glutathione values of the sample were extrapolated to a standard  
30 curve of known concentrations of GSH. For GSSG quantification, samples were  
31 preincubated with 4-vinylpyridine. The level of reduced glutathione was determined by  
32 the formula  $GSH = [\text{total glutathione} - GSSG]$ .

### 33 34 **2.10 Determination of the mitochondrial membrane potential**

1  
2 EL-4 cells were cultured in the absence or presence of NQs for 3.5 h, and aliquots of the  
3 cell cultures ( $1 \times 10^6$  cells/ml) were incubated with  $1 \mu\text{M}$  rhodamine 123 (Rho-123) for  
4 30 min. The cells were then centrifuged and resuspended in PBS. Changes in the  
5 mitochondrial membrane potential were detected by flow cytometry using a  
6 FACSCalibur flow cytometer.

7

### 8 **2.11 Immunoblot analysis**

9

10 EL-4 cells were cultured in the absence or presence of NQs ( $5 \mu\text{M}$ ) for different times.  
11 Then, the cells were lysed for 30 min at  $4^\circ\text{C}$  in lysis buffer (50 mmol/L Tris-HCl, 150  
12 mmol/L NaCl, 1 mmol/L EGTA, 1% NP-40, 1 mmol/L NaF, 1 mmol/L  $\text{Na}_3\text{VO}_4$ , 0.25%  
13 sodium deoxycholate,  $1 \mu\text{mol/L}$  phenylmethylsulfonyl fluoride,  $10 \mu\text{g/mL}$  aprotinin,  $10$   
14  $\mu\text{mol/L}$  pepstatin and  $10 \mu\text{mol/L}$  leupeptin). After centrifugation ( $14,000 \text{ g}$ , 15 min,  
15  $4^\circ\text{C}$ ), the whole-cell protein extracts ( $50 \mu\text{g}$ ) were mixed with SDS sample buffer (2%  
16 SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.2% bromophenol blue and 1%  
17 (v/v) 2-mercaptoethanol). Equal amounts of protein were separated by SDS-PAGE on  
18 10% polyacrylamide gels and transferred to PVDF membranes. Nonspecific binding  
19 sites on the nitrocellulose membranes were blocked with blocking buffer (5% nonfat  
20 dried milk containing 0.1% Tween 20 in 100 mM Tris-HCl, pH 7.5, and 0.9% NaCl)  
21 for 1 h. Then, the PVDF membranes were incubated overnight with rabbit anti-active  
22 caspase 9, rabbit anti-active caspase 3, mouse anti-cleaved PARP or rabbit anti- $\beta$ -actin  
23 antibodies, all of which were used at a dilution of 1:1000. After being washed three  
24 times for 10 min with PBS-Tween, the membranes were subsequently exposed to  
25 anti-rabbit or anti-mouse (1:2500) antibodies coupled to horseradish peroxidase for 1 h.  
26 After the membranes were washed three times for 10 min with PBS-Tween and once  
27 with PBS, an enhanced chemiluminescent system (GE Healthcare Bio-Sciences) was  
28 used for detection. The amount of protein loaded in each well was determined using a  
29 rabbit anti- $\beta$ -actin antibody (1:1000). Densitometry analysis of the bands was performed  
30 using Image J software (version 5.1, Silk Scientific Corporation, NIH, Bethesda, MA,  
31 USA). The densitometric intensities of the analyzed protein were normalized to those of  
32 the corresponding bands for  $\beta$ -actin.

33

### 1 **2.13. Statistical analysis**

2  
3 The effect of the NQs was calculated by using the corresponding results for the sample  
4 containing DMFA as the control value. Data were expressed as mean  $\pm$  SE of the  
5 number of independent experiments indicated in the figure legends, each one  
6 performed in triplicate. Analysis of variance (ANOVA) was used to assess the statistical  
7 significance of the differences followed by either Dunnett or Tukey post-test. A value of  
8  $p < 0.05$  was considered statistically significant. The data were analyzed with the  
9 GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

## 11 **3. RESULTS**

### 13 **3.1 Effects of NQs on cell proliferation.**

14  
15 The evaluation of the proliferative activity of cells growing in the presence of different  
16 drugs is widely used to investigate the mechanisms of cytotoxicity. To determine the  
17 effect of the NQs on the proliferation of EL-4 cells, the amount of [ $^3\text{H}$ ]-TdR  
18 incorporated into DNA was quantified. Figure 2A shows the proliferation curves of EL-  
19 4 cells treated with increasing concentrations of NQs for 24 h. A significant inhibitory  
20 effect was observed at concentrations as low as 1  $\mu\text{M}$ , and this effect was concentration  
21 dependent. Moreover, it was also demonstrated using the trypan blue exclusion method  
22 that NQs (5  $\mu\text{M}$ ) were able to induce a decrease in cell viability in a time-dependent  
23 manner, reaching maximum values at 7 h of incubation; this decrease in viability led to  
24 an inhibition of EL-4 cell proliferation (Figure 2B). In terms of cytotoxicity, NQ1  
25 ( $\text{IC}_{50}=0.7\pm 0.2 \mu\text{M}$ ), NQ3 ( $\text{IC}_{50}=1.1\pm 0.1 \mu\text{M}$ ) and NQ7 ( $\text{IC}_{50}=1.0\pm 0.2 \mu\text{M}$ ) resulted  
26 significantly more active against EL-4 cell line than  $\beta$ -lapachone ( $\text{IC}_{50}=2.0\pm 0.2 \mu\text{M}$ ),  
27 the naphthoquinoidal precursor.

### 29 **3.2 Effects of NQs on cell death.**

30  
31 To study the mechanisms by which NQs induce cell death, we determined whether the  
32 growth-inhibitory effect of NQs was related to the induction of apoptosis and/or  
33 necrosis. To this end, control EL-4 cells and NQ-treated cells (5  $\mu\text{M}$ ) were labeled using

1 AO/EB double staining, and the percentages of viable, apoptotic and necrotic cells were  
2 determined by fluorescence microscopy. As shown in Figure 3A, control cells had a  
3 normal morphology and green fluorescence, indicating that the cells were viable. The  
4 nuclei of the cells were all similar sizes, regularly shaped and evenly stained. Treatment  
5 with NQs (5  $\mu$ M) for 3.5 h increased the number of cells with morphological changes  
6 and enhanced the fluorescence. In the groups of cells treated for 7 h, the level of orange  
7 cell fluorescence was significantly enhanced, and the chromatin was condensed,  
8 indicating that these cells were late apoptotic cells.

9  
10 To further determine if the cell death mechanism was apoptosis or necrosis, control EL-  
11 4 cells and cells treated with NQs (5  $\mu$ M) for 7 h were labeled with Hoechst 33258 dye,  
12 and the nuclear morphology was observed by fluorescence microscopy. Control EL-4  
13 cells had a normal nuclear morphology, similar sizes and regular shapes. However, the  
14 NQ-treated cells had a nuclear morphology compatible with apoptosis, with the  
15 reduction of the nuclear and cytoplasmic volumes and chromatin condensation (Figure  
16 3B).

17  
18 The populations of apoptotic (early or late), necrotic and viable cells were quantified  
19 using the annexin V-FITC/PI double staining method. As shown in Figure 3C, treatment  
20 with NQs (5  $\mu$ M) decreased cell viability and increased the percentage of cells in the  
21 early and late stages of apoptosis in a time-dependent manner. NQ treatment did not  
22 induce a significant increase in the percentage of necrotic cells (annexin V-/ IP+). These  
23 results are in agreement with the inhibition of cell proliferation observed in the presence  
24 of NQs.

### 25 26 ***3.3 Effects of NQs on semiquinone radical generation.***

27  
28 The NADPH-dependent reduction of NQs in EL-4 cells was confirmed by EPR,  
29 indicating that the *o*-seminalphthoquinone radical was formed (Figure 4). At least five  
30 lines (a quintuplet) would be expected for these spectra due to the coupling of the free  
31 electron with the four aromatic protons C7-C10 in the naphthalene ring. Under our  
32 experimental conditions, the quintuplet was clearly observed for NQ7 but was poorly  
33 resolved for the other drugs. The apparent splitting constants varied between 1.45 and  
34 1.60 gauss, in agreement with the obtained values for other *o*-seminalphthoquinones

1 (Kumar et al., 2006). The omission of the NQ or NADPH prevented the appearance of  
2 the semiquinone signals. In the absence of cells, there were small signals corresponding  
3 to chemical (non-enzymatic) reduction.

#### 4 5 **3.4 Effects of NQs on ROS production.**

6  
7 The oxidative stress associated with the autoxidation of the semiquinone free radical,  
8 which produces the superoxide anion, hydrogen peroxide, and other ROS, has been  
9 shown to be related to naphthoquinone's cytotoxicity. To probe the role of ROS  
10 generation and increasing oxidative stress as a mechanism associated with NQ toxicity  
11 toward EL-4 cells, we measured the intracellular ROS level using the fluorescent probe  
12 2,7 dichlorofluorescein diacetate (DCFDA). Figures 5A and 5B show a significant  
13 increase in ROS accumulation after a 10 min incubation with NQs (5  $\mu$ M). The highest  
14 ROS level was observed after 30 min of incubation. After a longer period of incubation,  
15 the ROS level was lower (data not shown).

16  
17 To further explore the role of oxidative stress in NQ-mediated toxicity, ROS were  
18 quantified using the natural antioxidant N-acetyl cysteine (NAC), the most bioavailable  
19 precursor of glutathione (Gross et al., 1993). After 18 h incubation with NAC (12.5  
20 mM) a reversal of NQs-induced ROS increasing was observed (Control: 199.18 $\pm$ 9.39;  
21 NQ1: 201.25 $\pm$ 10.51; NQ3: 205.36 $\pm$ 9.52; NQ7: 210.51 $\pm$ 11.32 MFI) (Supplemental  
22 Figure 1).

#### 23 24 **3.5 Effects of NQs on the cellular redox state.**

25  
26 The increased generation of ROS by NQs likely contributes to oxidative stress, which  
27 may ultimately lead to the observed cytotoxicity. The reduced/oxidized glutathione ratio  
28 (GSH/GSSG) is used to evaluate the oxidative stress status in biological systems (Gago-  
29 Martinez et al., 2004). As seen in Table 1, NQs (5  $\mu$ M) caused a statistically significant  
30 decrease in the GSH/GSSG ratio after a 30 min incubation due to a reduction in the  
31 GSH level and an increase in the GSSG level. The pre-incubation of the cells with NAC  
32 (12.5 mM) for 18 h before treatment with the NQs (5  $\mu$ M) for 30 min, restored the  
33 GSH/GSSG ratio to the respective control value.

34

1 NAC effect was also studied on EL-4 cell proliferation, analyzed by [<sup>3</sup>H]-TdR  
2 incorporation into DNA. The pre-incubation of the cells with NAC (12.5 or 15 mM) for  
3 18 h before treatment with the NQs (2.5 or 5 μM) for 24 h reversed the growth-  
4 inhibitory effect of the NQs. This effect was observed in a NAC and NQ concentration-  
5 dependent manner (Figure 6). It is worth mentioning that the NAC concentrations used  
6 in this experiment had no effects on the proliferation of control cells.

### 8 ***3.6. Effects of NQs on the mitochondrial membrane potential.***

9  
10 To better understand the mechanisms underlying the toxicity of NQs, their ability to  
11 interfere with mitochondria was assessed using the fluorescent dye rhodamine 123. A  
12 significantly decrease in the MMP was observed in EL-4 cells treated with NQs (5 μM).  
13 The lowest MMP values were observed after 3.5 h of incubation (Figure 7). This  
14 decrease in the mitochondrial membrane potential could occur due to the opening of the  
15 mitochondrial permeability transition pore, which allows the release of proteins such as  
16 cytochrome c that trigger the apoptotic pathway (Kroemer et al., 2007).

### 18 ***3.7 Effects of NQs on the proteolytic activation of caspases and PARP cleavage***

19  
20 The involvement of the mitochondrial apoptotic pathway was investigated by Western  
21 blotting. As shown in Figure 8, the incubation of EL-4 cells with NQs (5 μM) induced  
22 the proteolytic activation of caspases 3 and 9 and increased the level of the cleaved form  
23 of PARP in a time-dependent manner. As expected, the increase in the level of the  
24 active form of caspase 3 occurred after the proteolytic activation of caspase 9 and prior  
25 to the cleavage of PARP, showing that these events are sequential.

## 27 **4. DISCUSSION**

28 β-Lapachone, an *o*-naphthoquinone, proved to be an effective cytostatic agent against  
29 different tumor cells, such as murine leukemia, melanoma, and hepatoma cells and  
30 human leukemia, colon carcinoma, lymphoma, and glioma cells, as well as epidermoid  
31 laryngeal, ovarian, breast, lung and prostate cancer cells (Dolan et al., 1998; Frydman et  
32 al., 1997; Li et al., 1993; Li et al., 1995; Li et al., 1999b; Pardee et al., 2002; Planchon

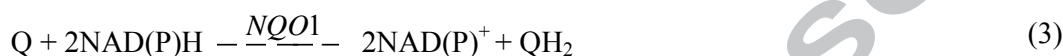
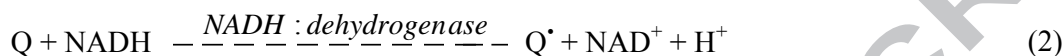
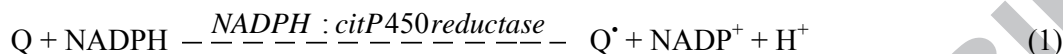
1 et al., 1999; Samali et al., 1999). Based on these effects, the clinical use of  $\beta$ -lapachone  
2 has been suggested (Pardee et al., 2002), and the synthesis of a number new of *o*-  
3 naphthoquinones, such as NQs, has been pursued (Ferreira et al., 2011). The data  
4 presented in this paper demonstrate that NQs induce the intrinsic apoptotic pathway in a  
5 manner associated with a significant ROS increase in the EL-4 cell line.

6 Our results show that NQs decrease cell proliferation in a concentration-dependent  
7 manner. Moreover, NQs decreased cell viability in a time-dependent manner at the same  
8 concentration that interfered with cell proliferation, demonstrating that at least part of  
9 the observed effect on proliferation was actually due to the compounds' ability to  
10 induce cell death. The data show that the type of cell death induced by treatment with *o*-  
11 naphthoquinones depends on the target cell type, time, and drug dose (Li et al., 1999b;  
12 Pardee et al., 2002). The results of the present study confirm that NQs (5  $\mu$ M) can  
13 induce apoptosis in the EL-4 cell line. This finding was supported by three methods:  
14 Hoechst 33258 staining, double staining with AO/EB and double staining with annexin  
15 V-FITC and PI. We observed apoptotic features such as chromatin condensation and  
16 alterations in the structure, size, and shape of the nucleus that are characteristic of  
17 apoptosis. A time-course analysis of the cellular biochemistry showed that the  
18 population of apoptotic cells analyzed by double staining formed predominantly after a  
19 short incubation period (3.5 h) with NQs. After prolonged incubation, a higher  
20 percentage of late apoptotic cells was noted.

21  
22  
23 The most prominent chemical feature of quinones (Qs) is their ability to undergo redox  
24 cycling to generate ROS, and thus, damage to tumor cell results from the ability of Qs  
25 to undergo enzymatic reduction to the semiquinone radical (Q $\cdot$ ) (reactions 1, 2 and 3)  
26 (Inbaraj and Chignell, 2004; Kalyanaraman et al., 1991; Seshadri et al., 2011). Under  
27 aerobic conditions, the semiquinone radical participates in redox cycling to generate the  
28 superoxide anion radical O $_2^{\cdot-}$  and H $_2$ O $_2$  (reactions 4-7). Increased ROS may cause  
29 damage to DNA, lipids, and proteins, leading to apoptosis (Boveris et al., 1978b;  
30 Halliwell and Aruoma, 1991; Valko et al., 2007). We demonstrated that NQs (5 mM)  
31 generated semiquinone radicals in EL-4 cells. The detection of semiquinone radicals  
32 formed by enzymatic reduction requires very high concentrations of the quinone due to  
33 the relatively slow rates of formation of the semiquinone by the enzyme and their much  
34 faster rates of decay (reaction 8) (Butler et al., 1987; Cadenas, 1995). The redox

1 activation of these NQs by intracellular reductases in EL-4 cells and the intracellular  
 2 localization of the semiquinone and the derived oxygen radicals do not rule out plasma  
 3 membrane effects originating from this process (Kumar et al., 2002).

4  
 5  
 6



7  
 8 Our study confirmed that NQs produce ROS in the EL-4 cell line. It was observed that  
 9 ROS production measured by the DCFDA method is a time-dependent effect, reaching  
 10 maximum values at 30 min of incubation. At longer times, we observed a decrease in  
 11 the intracellular content of ROS (data not shown). This decrease can be attributed to the  
 12 termination reactions that involve free radicals, as well as to the dissemination of these  
 13 species to the extracellular space, making them undetectable by flow cytometry. In close  
 14 agreement with our results, several studies using other *o*-naphthoquinones, such as  $\beta$ -  
 15 lapachone-based 1,2,3-triazoles, suggest that the induction of apoptosis is associated  
 16 with ROS production in different human cell lines from leukemias, melanomas, colon  
 17 tissue and central nervous system tissue (da Silva et al., 2011). In addition, ROS  
 18 formation and apoptosis have also been observed in response to treatment with 1,2-  
 19 naphthoquinone in human MCF-7 breast cancer cells (Lin et al., 2007), with 7-hydroxy-  
 20  $\beta$ -lapachone in human cell lines from breast, cervix, lung and colon (Rios-Luci et al.,



1 2012) and with 3'-nitro-3-phenylamino nor- $\beta$ -lapachone against HL-60 cell line (Araujo  
2 et al., 2012).

3

4 Oxidized glutathione (GSSG) accumulates in cells under conditions of oxidative stress,  
5 and the GSH/GSSG ratio is a good indicator of this process (Nogueira et al., 2004).  
6 Numerous studies demonstrate that there is a close correlation between the increase in  
7 ROS production and the decrease in the intracellular GSH levels (Armstrong et al.,  
8 2002). A decrease in the GSH/GSSG ratio with NQ treatment is indicative of oxidative  
9 stress in treated EL-4 cells.

10 N-Acetyl-cysteine (NAC) protects cells by promoting GSH synthesis and scavenging  
11 ROS (Zafarullah et al., 2003). The addition of this precursor to the cell cultures resulted  
12 in a reversal of NQs-induced ROS increasing, GSH/GSSG ratio decrease and cell  
13 proliferation inhibition, confirming that the mechanism of action of these drugs is based  
14 on the generation of ROS. It should be noted that it is essential to pre-incubate EL-4  
15 cells with NAC to observe significant differences in the proliferative response with  
16 respect to cells treated with NQs alone. This behavior may be related to the time  
17 required for the synthesis of GSH from NAC or to the fact that the cells previously  
18 required a higher concentration of GSH to compensate for the increase in ROS  
19 production caused by the action of NQs.

20

21 The alteration of the GSH/GSSG redox couple induced by ROS governs the cell death  
22 pathways by inducing the opening of the mitochondrial permeability transition pore  
23 (PTP), leading to the collapse of the mitochondrial membrane potential (MMP) and the  
24 release of proapoptotic factors (Coppola and Ghibelli, 2000; Hampton et al., 1998;  
25 Kroemer et al., 2007). Several studies have indicated that mitochondrial dysfunction  
26 and cellular energy depletion play a major role in the mechanism of cell killing by  
27 quinones with differing structures and chemical reactivities (Pritsos and Vimalachandra,  
28 1995; Tudor et al., 2003). In accordance with these studies, a decrease in the MMP was  
29 observed in the NQ-treated EL-4 cells. These mitochondrial alterations then induce the  
30 downstream apoptotic events such as cytochrome c release and apoptosome complex  
31 formation involving Apaf-1 and procaspase 9, resulting in caspase 9 activation. Our  
32 results show that NQ treatment induces the proteolytic cleavage of procaspase 9 in EL-4  
33 cells, generating a 37 kDa fragment that corresponds to the active form of the enzyme.  
34 Procaspase 3 is a target of caspase 9, whose action releases an 18 kDa fragment with

1 catalytic activity. In this context, our results also suggest that apoptosis induced by NQs  
2 is dependent on caspase 3, a conclusion that is supported by the evidence that the DNA  
3 repair enzyme PARP-1 is cleaved. We found a time-dependent increase in the cleavage  
4 of PARP that was downstream of caspase 9 and caspase 3, indicative of a cascade of  
5 sequential events. These results indicate that the NQs induce apoptosis through the  
6 activation of the intrinsic or mitochondrial pathway, as has been demonstrated for  $\beta$ -  
7 lapachone in human cancer cell lines derived from prostate (Weller et al., 1997),  
8 malignant glioma (Planchon et al., 1995), leukemia (Woo et al., 2006), colon  
9 (Wuerzberger et al., 1998) (Choi et al., 2003), breast (Li et al., 1999a), ovarian (Lai et  
10 al., 1998), hepatoma (Li et al., 2000), and pancreatic tumors (Ough et al., 2005) and for  
11 other *o*-naphthoquinones at concentrations in the range of 1-10  $\mu$ M (IC<sub>50</sub>) (D'Anneo et  
12 al., 2010; Tudor et al., 2003). However, we can not exclude the possibility that the  
13 extrinsic apoptotic pathway also contribute to NQs-induced apoptosis in EL-4 cells.

14

15 It is worth mentioning that no significant difference was found between NQs in any of  
16 the analyzed parameters in the EL-4 cell line. This result can be easily explained by the  
17 structural characteristics of the studied naphthoquinones. They do not differ in terms of  
18 their electronic, steric and lipophilic properties, with the only structural difference being  
19 in the position of a methyl group or the absence thereof. However, NQs were more  
20 active than  $\beta$ -lapachone against EL-4 cell line. Thus, these new derivatives present a  
21 promising profile for further experimental investigations.

22

23 Finally, our results indicate that novel NQs induce redox cycling through the production  
24 of ROS in EL-4 cells. This mechanism could be responsible for the inhibition of cell  
25 proliferation and for the cell death by apoptosis. The evaluation of the effectiveness of  
26 drugs with cytotoxic effects against tumor lines and the analysis of the mechanisms of  
27 action involved could be helpful in the treatment of cancer. In addition, the inhibitory  
28 effects of NQs in *in vivo* tumor models in mice may constitute an important part of the  
29 study of potential antitumoral drugs.

30

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ACCEPTED MANUSCRIPT

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2 **Figure captions**  
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5 **Figure 1: Chemical structure of NQs used in this study.** NQ1: 2-phenyl-3,4-dihydro-  
6 2*H*-benzo[*h*]chromene-5,6-dione, NQ3: 2-*p*-tolyl-3,4-dihydro-2*H*-benzo[*h*]chromene-  
7 5,6-dione, NQ7: 2-methyl-2-phenyl-3,4-dihydro-2*H*-benzo[*h*]chromene-5,6-dione.  
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10 **Figure 2: Effects of NQs on EL-4 cells proliferation and viability.** EL-4 cells were  
11 cultured for 24 h in the absence or presence of increasing concentrations of NQs (●  
12 NQ1, □ NQ3 and ▲ NQ7). Cell proliferation was determined by [<sup>3</sup>H]-TdR  
13 incorporation (A). Values [desintegrations per minute (dpm)] are means ± SE of 4  
14 independent experiments performed in triplicate. After incubation in the absence  
15 (control) or presence of NQs (5 μM) for the indicated times, the percentage of viable  
16 cells was quantified by trypan blue exclusion dye (B). Values are the means ± SE of 4  
17 independent experiments performed in duplicate. \*Significantly different from the  
18 corresponding control value (p < 0.05).  
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21 **Figure 3: Action of NQs on cell death induction in EL-4 line.** (A) EL-4 cells were cultured  
22 for 3.5 and 7 h in the absence (control) or presence of NQs (5 μM) and then labeled with  
23 acridine orange and ethidium bromide (both 100 μg/ml). Viable cells (stained in green) and  
24 apoptotic cells (stained in orange) were observed by fluorescent microscopy (1000x). Results  
25 are representative of 4 independent experiments. (B) EL-4 cells were incubated for 7 h in the  
26 absence (control) or presence of NQs (5 μM) and then stained with Hoechst 33258 (0.5  
27 μg/ml) dye. Nuclear morphology was observed by fluorescent microscopy (1000x). Results  
28 showed are representative of 3 independent experiments. (C) EL-4 cells were cultured for 3.5  
29 and 7 h in the absence (control) or presence of NQs (5 μM) and then labeled with propidium  
30 iodide (PI) and annexin V-FITC (annexin). Percentage of viable cells (PI-/annexin-), in early  
31 apoptosis (PI-/annexin+), delayed apoptosis (PI+/annexin+) or necrosis (PI+/annexin-) was  
32 quantified by flow cytometry. Percentages of cells corresponding to each quadrant are  
33 indicated in parentheses. Dot blot graphs showed are representative of 3 independent  
34 experiments. (D) Percentages of cells corresponding to each stage are shown. Results are  
35 expressed as mean ± SE from 3 independent experiments performed in triplicate.  
36 \*Significantly different from corresponding control value (p < 0.05).  
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39 **Figure 4: Electron Paramagnetic Resonance (EPR) spectrum of *o*-semi-**  
40 **naphthoquinones.** EL-4 cells were incubated with NQs (5 mM) and NADPH (20 mM)  
41 under anaerobic conditions. *O*-seminaphthoquinones signals were obtained following  
42 the procedure described in materials and methods sections. Spectrums (A), (B) and (C)  
43 correspond to NQ1, NQ3 and NQ7, respectively. Spectrums (D) to (F) are  
44 representative of EL-4 cells incubated with NQs in absence of NADPH (D); EL-4 cells  
45 incubated with NADPH in absence of NQs (E), and NQs incubated with NADPH in  
46 absence of cells (F). The arrow indicates a small signal corresponding to the chemical,  
47 non-enzymatic reduction. Spectrum are representative of 3 independent experiments.  
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1 **Figure 5: Effect of NQs on the generation of reactive oxygen species (ROS) in the**  
2 **EL-4 cells.** EL-4 cells were cultured in the absence (control) or presence of NQs (5  $\mu$ M)  
3 for the indicated times and then labeled with H2DCF-DA to quantify the ROS  
4 production by flow cytometry (A). The mean fluorescence intensity (MFI) of each  
5 treatment is indicated in parenthesis. The histograms showed are representative of 3  
6 independent experiments. (B) The quantification of ROS production in function of the  
7 time of treatment with NQs is shown in the bar graph. Results are expressed as mean  $\pm$   
8 SE from 3 independent experiments performed in triplicate. \*Differ significantly respect  
9 to control (p 0.05).

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12 **Figure 6: Effect of NAC on the proliferation of EL-4 cell cultured with NQs.**  
13 Quantification of EL-4 cells proliferation preincubated for 18 h with different  
14 concentrations of NAC and then cultured for 24 h in the presence of 2.5  $\mu$ M or 5  $\mu$ M of  
15 NQ1 (A), NQ3 (B) or NQ7 (C). Bars corresponding to control indicate basal values of  
16 cell proliferation in the absence (white bars) or the presence (black bars) of NAC and  
17 without NQs. A dose-response effect of NAC on NQs inhibitory actions is depicted for  
18 the two concentrations of NQs (light and dark grey bars). Results are expressed as mean  
19  $\pm$  SE from 3 independent experiments performed in triplicate. \*Differ significantly from  
20 the control value (p<0.05).

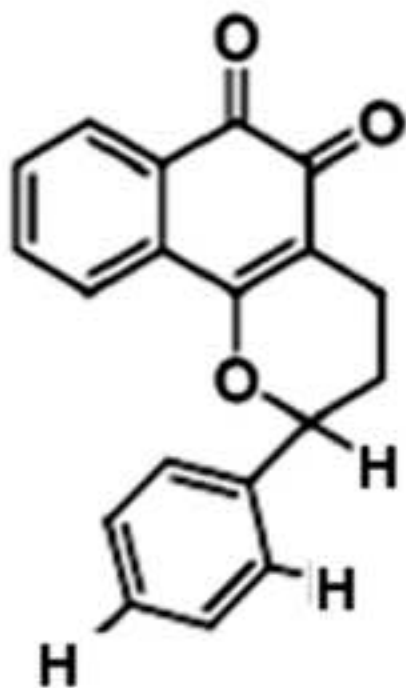
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22 **Figure 7: Effect of NQs on mitochondrial membrane potencial.** EL-4 cells were  
23 cultured for 3.5 h in the absence (control) or presence of NQs (5 M) and then labeled with  
24 rhodamine-123 (1  $\mu$ M). Changes in mitochondrial membrane potential were detected by  
25 flow cytometry. The histograms shown are representative of 3 independent experiments  
26 performed in triplicate. The bar graph indicates the mean fluorescence intensity (MFI) of  
27 rhodamine-123 retained inside the mitochondria. Results are expressed as mean  $\pm$  SE  
28 from 3 independent experiments performed in triplicate. \*Differ significantly respect to  
29 control (p<0.05).

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31 **Figure 8: Caspases proteolytic activation and Poly (ADP-Ribose) Polymerase**  
32 **(PARP) cleavage mediated by NQs.** EL-4 cells were cultured in the absence (control,  
33 C) or presence of NQs (5  $\mu$ M) for the indicated times. Proteolytic activation of caspases  
34 9 and 3 and PARP cleavage were analyzed by western blot. Specific antibodies showed  
35 bands for active caspase 9 (37 kDa), active caspase 3 (17 kDa), cleaved PARP (89 kDa)  
36 and  $\beta$ -actin (43 kDa). Densitometry analyses of the results are shown in the bar graphs.  
37 Values are the means  $\pm$  SE of 3 independent experiments.  $\beta$ -actin bands were used as  
38 protein load control. \*Differ significantly respect to control (p<0.05).

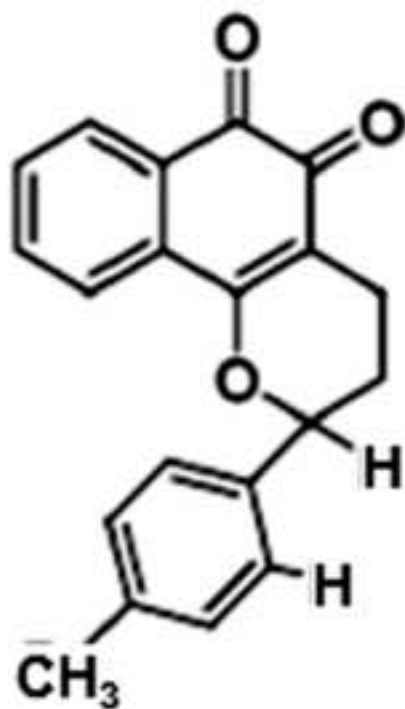
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40 **Supplemental Figure 1: Effect of NAC on the generation of reactive oxygen species**  
41 **(ROS) mediated by NQs in the EL-4 cells.** EL-4 cells were cultured in the absence  
42 (control) or presence of NQs (5  $\mu$ M) for 30 min and then labeled with H2DCF-DA to  
43 quantify the ROS production by flow cytometry. NAC (12.5 mM) was added 18 h  
44 before compounds exposure. (A). The mean fluorescence intensity (MFI) of each  
45 treatment is indicated in parenthesis. The histograms showed are representative of 3  
46 independent experiments. (B) The quantification of ROS production in absence or  
47 presence of NAC pre-treatment is shown in the bar graph. Results are expressed as  
48 mean  $\pm$  SE from 3 independent experiments performed in triplicate. \*Differ  
49 significantly respect to control (p 0.05).

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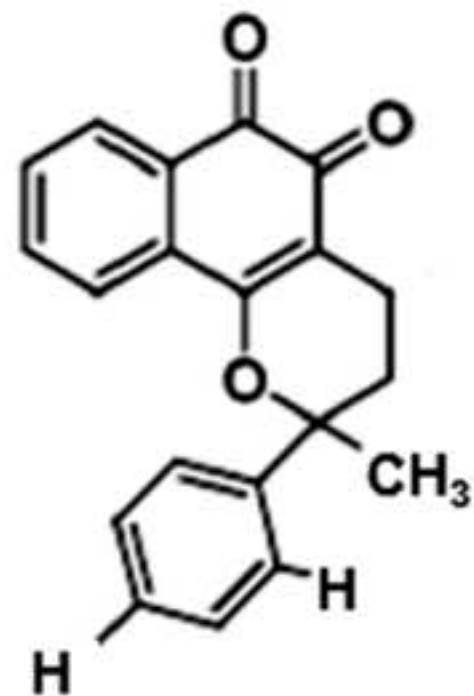
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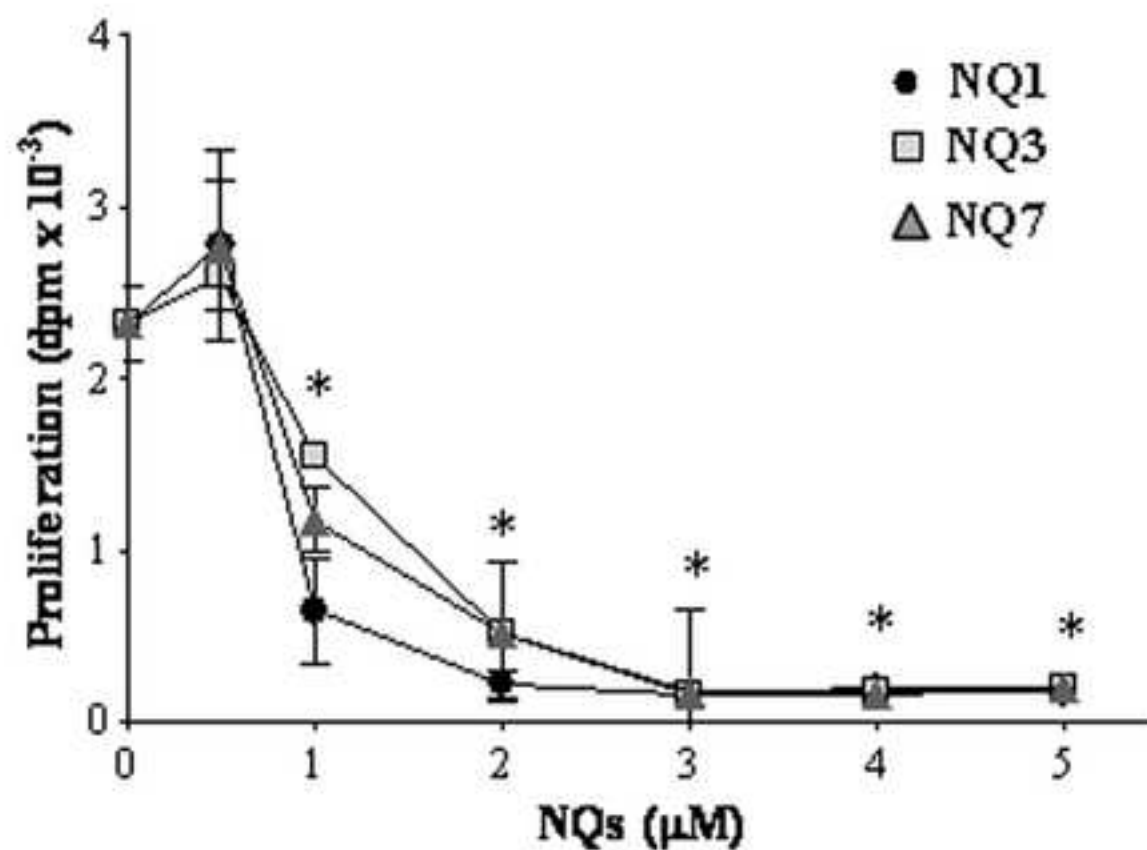
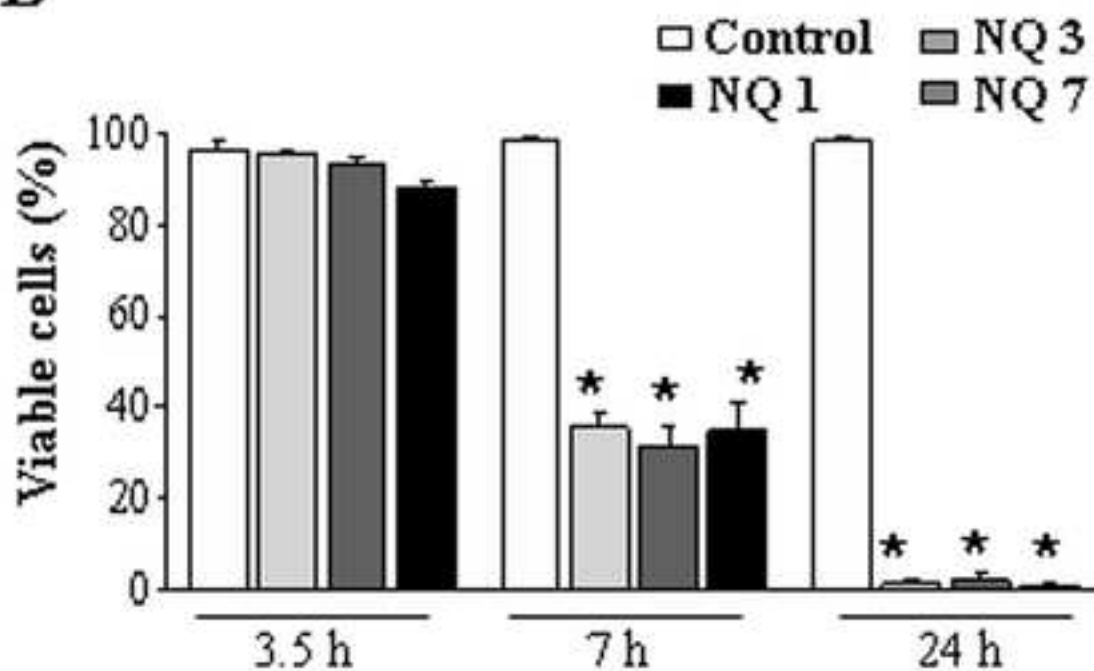


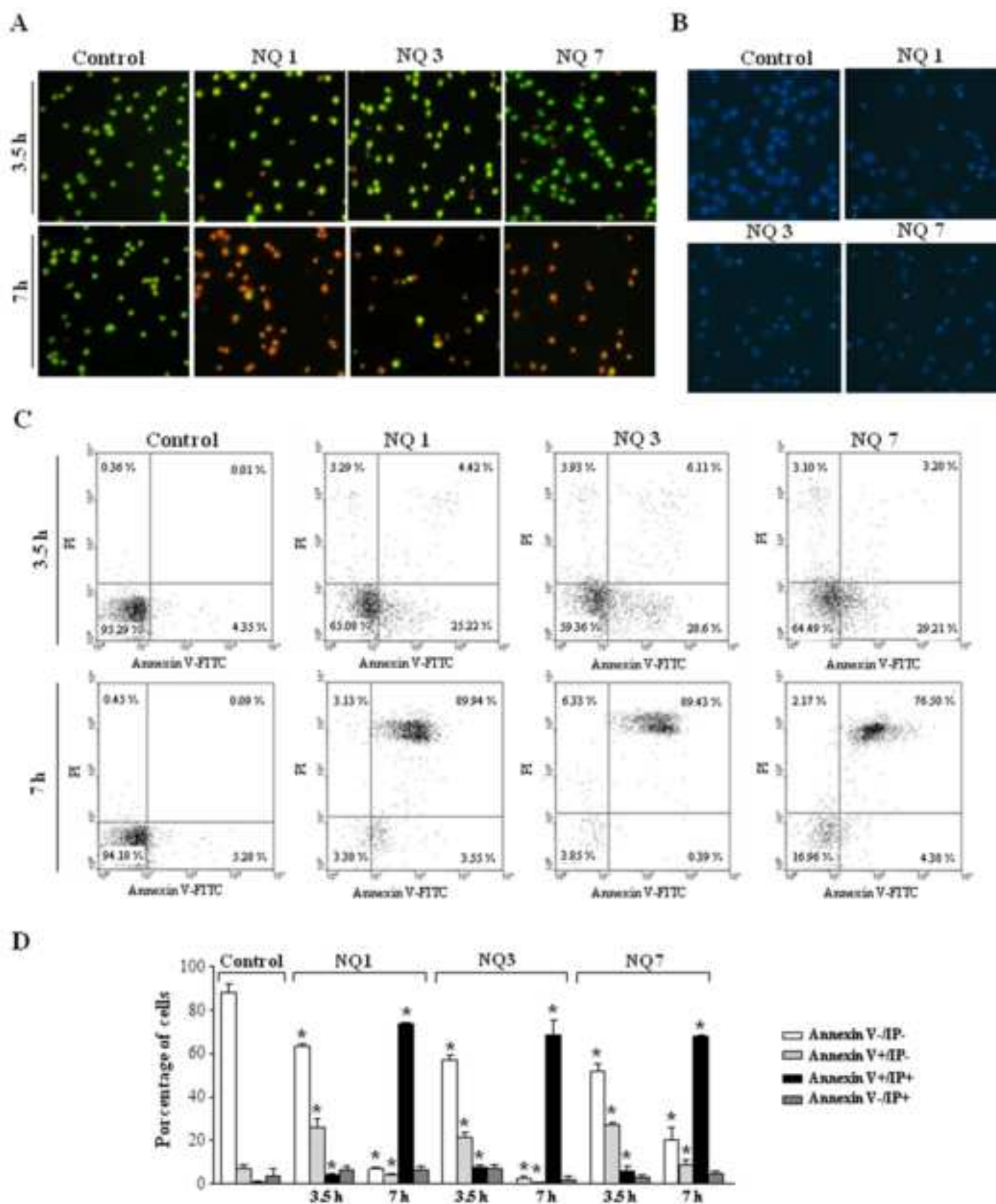
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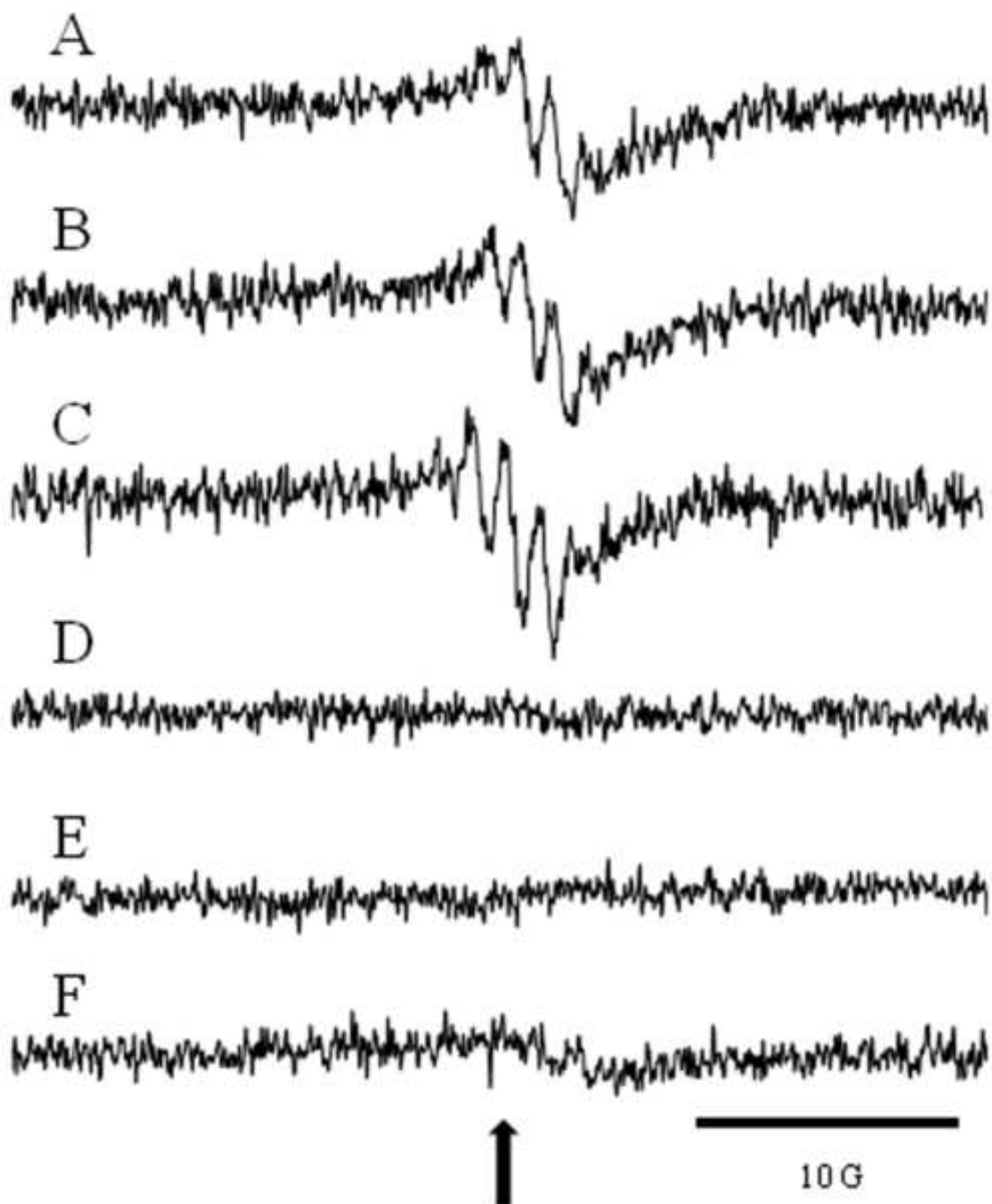


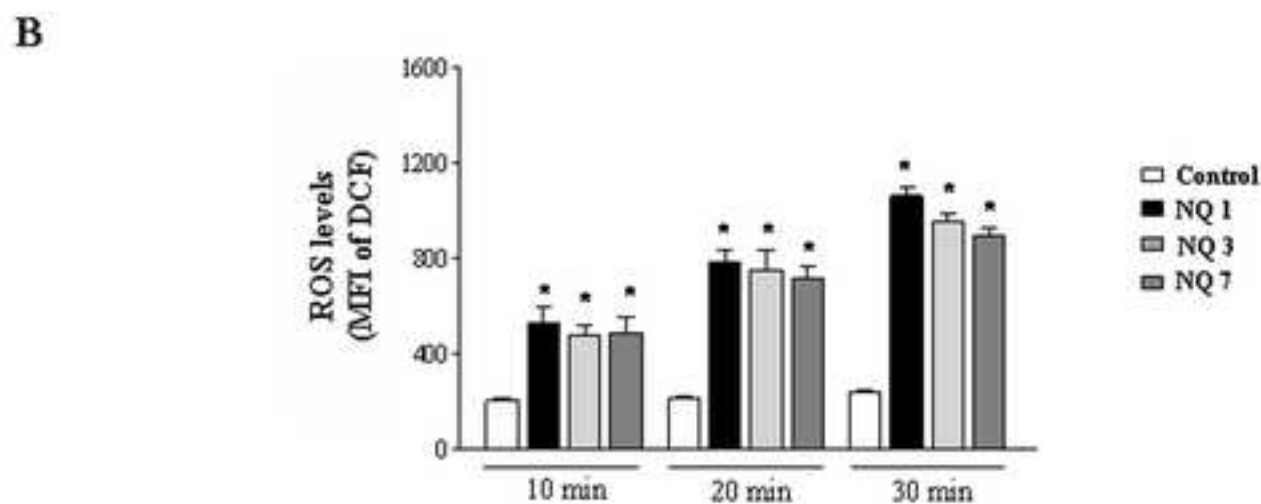
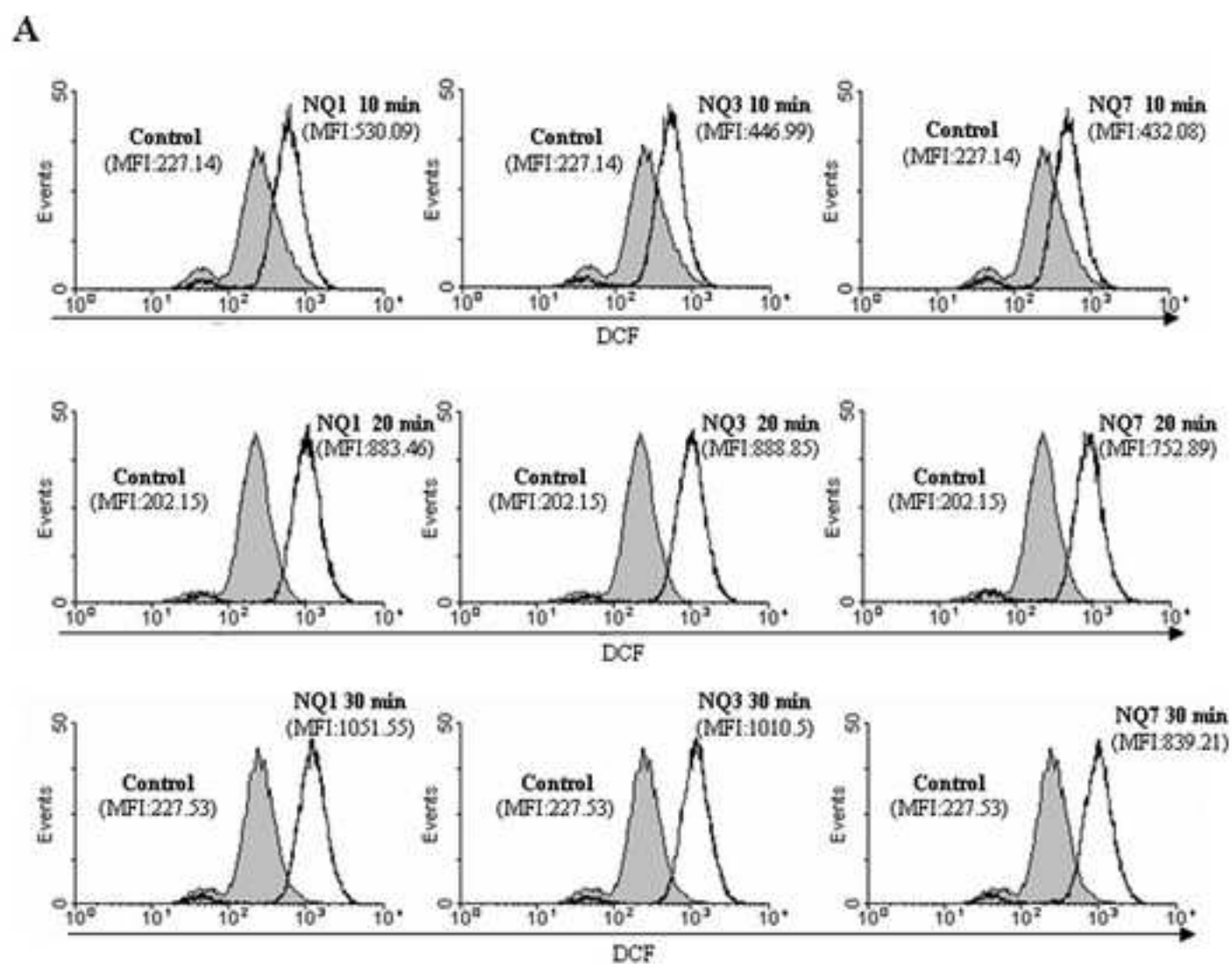
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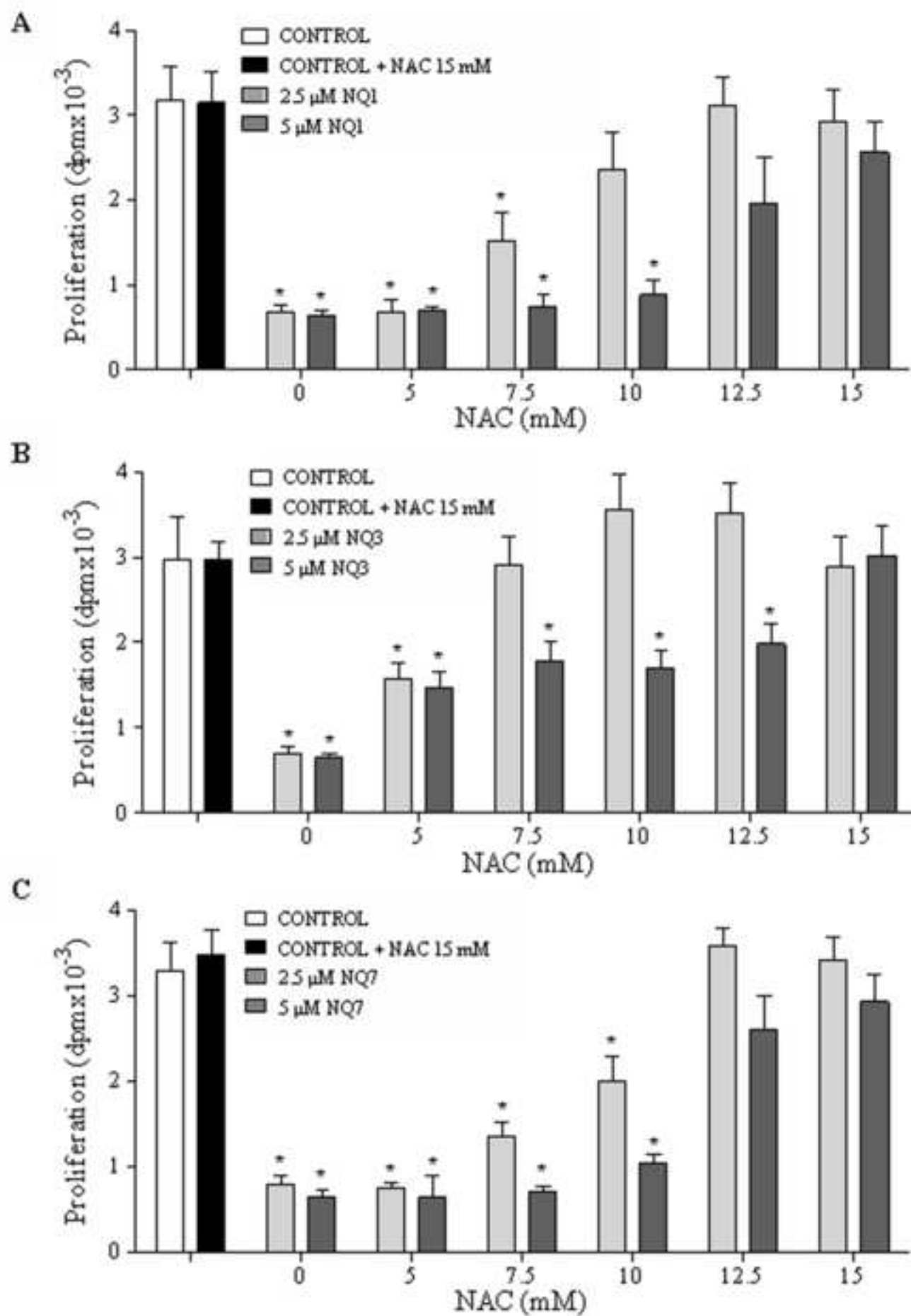


**A****B**

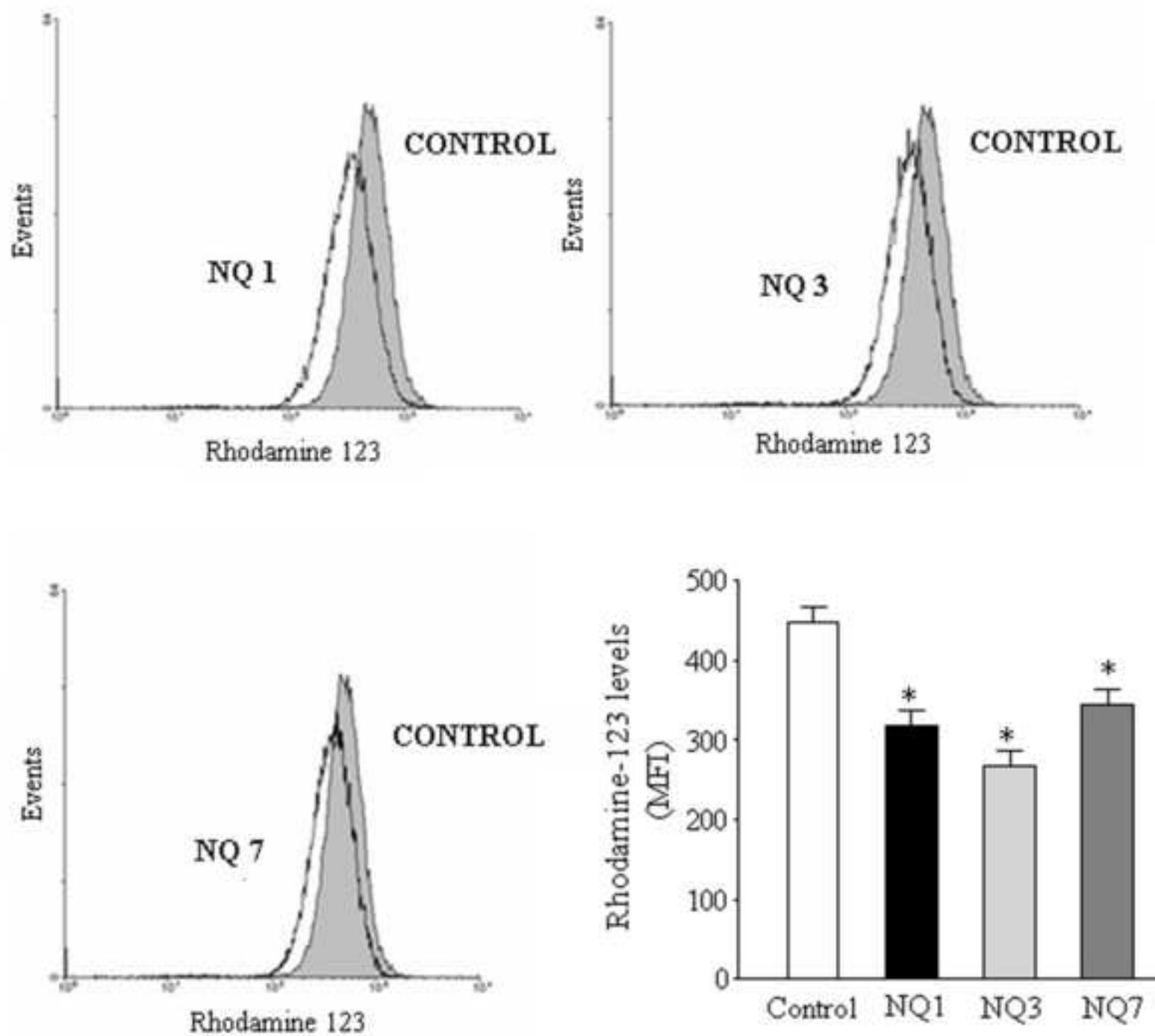


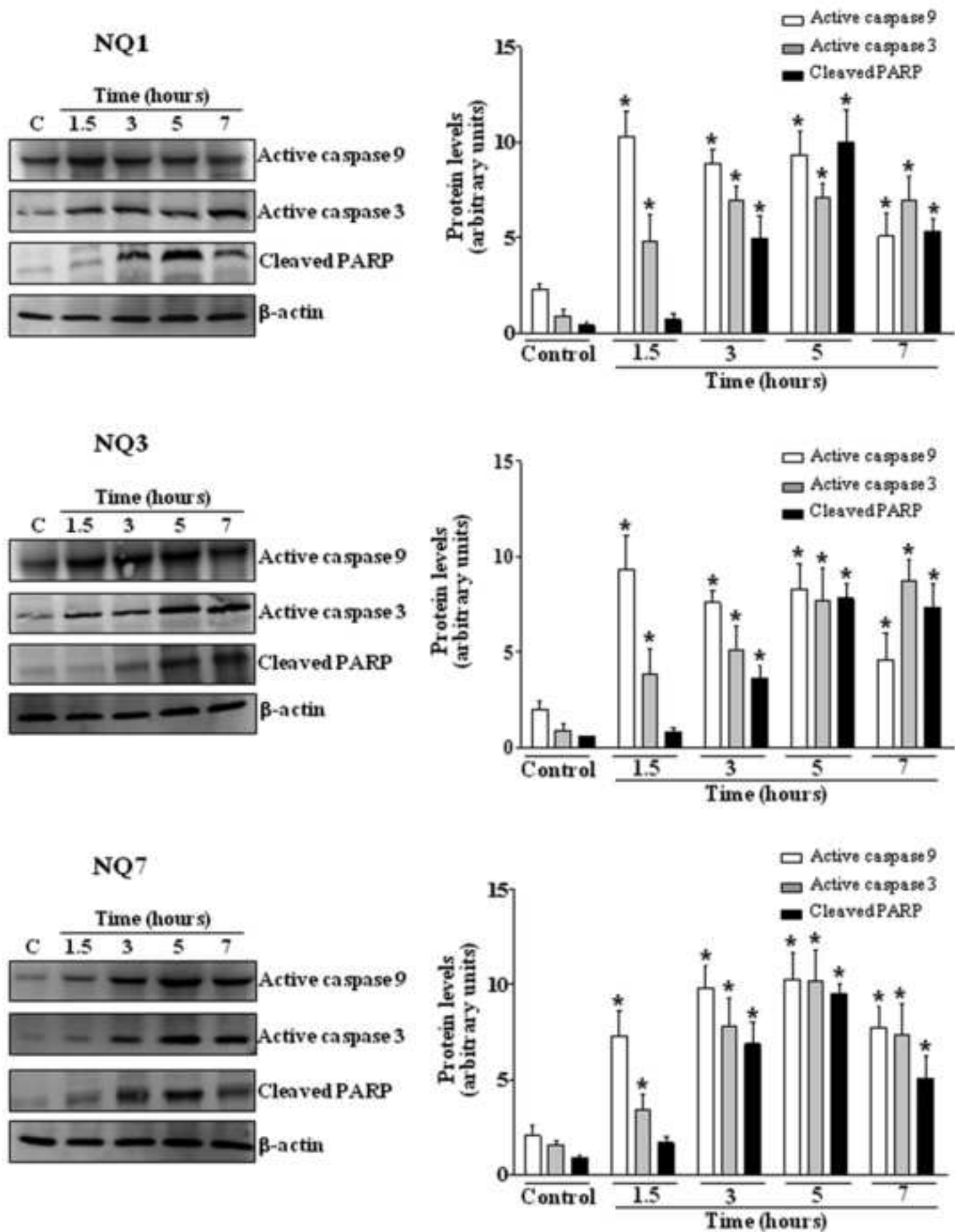












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**Table 1**

Effect of NQs in glutathione redox state

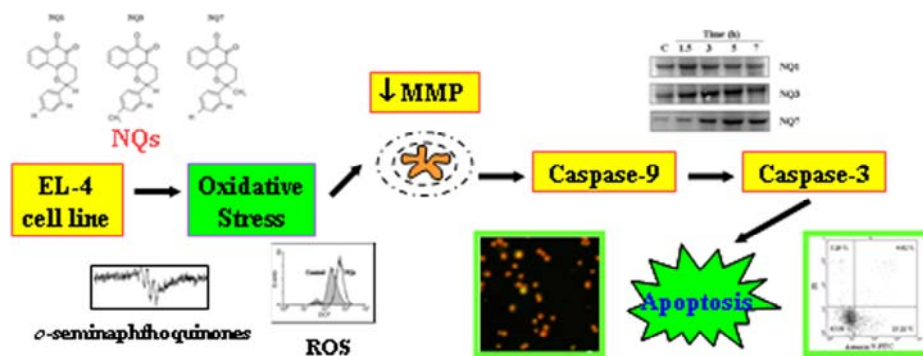
Treatment	Total glutathione pmol/1x10 <sup>6</sup> cells	GSH	GSSG	GSH/GSSG
Control	20.57±0.85	15.95±1.2	4.62±0.35	3.45±0.15
NQ1	19.14±0.89	9.57±1.66*	9.57±0.77*	1.00±0.25*
NQ3	17.27±0.95	8.69±1.78*	8.58±0.83*	1.01±0.30*
NQ7	16.83±0.94	6.93±1.61*	9.90±0.67*	0.70±0.30*
Control+NAC	25.28±1.00*	22.23±1.36*	3.05±0.36	7.29±0.18*
NQ1+NAC	25.50±1.02*	22.38±1.28*	3.12±0.26	7.17±0.14*
NQ3+NAC	25.91±0.98*	22.66±1.31*	3.25±0.33	6.97±0.16*
NQ7+NAC	26.14±1.01*	22.61±1.30*	3.53±0.29	6.40±0.14*

EL-4 cells were cultured for 30 min in the absence (control) or the presence of NQs (5  $\mu$ M). For NAC treatment, EL-4 cells were incubated with NAC (12.5 mM) for 18 h prior to incubation with NQs. GSH and GSSG content was quantified according to the procedures described in materials and methods. Each value represents the mean  $\pm$  SE of three independent experiments, each in triplicate. \*p<0.05 compared to the respective control.

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Graphical abstract



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#### **HIGHLIGHTS**

Novel  $\beta$ -lapachone analogs (NQs) cause cytotoxicity in EL-4 lymphoma cells.  
NQs (5  $\mu$ M) can induce apoptosis in the EL-4 cell line.  
ROS generation and oxidative stress are involved in NQ-induced apoptosis.  
NQs induce apoptosis through the activation of the intrinsic pathway.