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Growth inhibitory effects of 3′-nitro-3-phenylamino nor-beta-lapachone against HL-60: A redox-dependent mechanism

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ABSTRACT

In this study, the cytotoxicity, genotoxicity and early ROS generation of 2,2-dimethyl-(3*H*)-3-(*N*-3′-nitrophenylamino)naphtho[1,2-*b*]furan-4,5-dione ($QPhNO_2$) were investigated and compared with those of its precursor, nor-beta-lapachone (nor-beta), with the main goal of proposing a mechanism of antitumor action. The results were correlated with those obtained from electrochemical experiments held in protic (acetate buffer pH 4.5) and aprotic (DMF/$TBABF_4$) media in the presence and absence of oxygen and with those from dsDNA biosensors and ssDNA in solution, which provided evidence of a positive interaction with DNA in the case of $QPhNO_2$. $QPhNO_2$ caused DNA fragmentation and mitochondrial depolarization and induced apoptosis/necrosis in HL-60 cells. Pre-treatment with *N*-acetyl-L-cysteine partially abolished the observed effects related to the $QPhNO_2$ treatment, including those involving apoptosis induction, indicating a partially redox-dependent mechanism. These findings point to the potential use of the combination of pharmacology and electrochemistry in medicinal chemistry.

1. Introduction

Cancer is the second leading cause of death worldwide. Although cancer is often referred to as a single disease, it actually consists of more than 100 different conditions. These diseases are characterized by uncontrolled cell growth and the spread of abnormal cells (Hanahan and Weinberg, 2000).

Drugs containing a quinone moiety, such as anthracyclines and mitoxantrones show excellent anticancer activity (Foye, 1995), which justifies the numerous studies in the literature on the synthesis and evaluation of either natural quinones or their analogues as potential antitumor agents (da Silva Júnior et al., 2007, 2009, 2010; Montenegro et al., 2010). Two major mechanisms of quinone cytotoxicity have been proposed: stimulation of oxidative stress and alkylation of cellular nucleophiles, which encompass a large range of biomolecules (Asche, 2005; de Abreu et al., 2002a; Hillard et al., 2008), such as DNA and some enzymes, e.g., topoisomerase and protein tyrosine phosphatases (Bova et al., 2004).

One of the most widely studied quinones is beta-lapachone, a natural compound found in the lapacho tree that can be synthesized easily from lapachol by acid cyclization. Beta-lapachone and its related compound nor-beta-lapachone (nor-beta, Fig. 1) are cytotoxic to many human cancer cell lines at concentrations in the IC_{50} range of 1–10 μM (da Silva Júnior et al., 2007). In a previous study, our group demonstrated that the structural modifications of nor-beta can enhance its anticancer activity against cancer cell lines (da Silva Júnior et al., 2007, 2009, 2010) and that 2,2-dimethyl-(3*H*)-3-(*N*-3′-nitrophenylamino)naphtho[1,2-*b*]furan-4,5-dione ($QPhNO_2$, Fig. 1) is one of the most active substances, with an IC_{50} below 2 μM (da Silva Júnior et al., 2007).

Thus, the aim of the present work was to evaluate the mechanism of action involved in $QPhNO_2$ cytotoxicity and genotoxicity in the leukemia cell line HL-60 compared with its precursor

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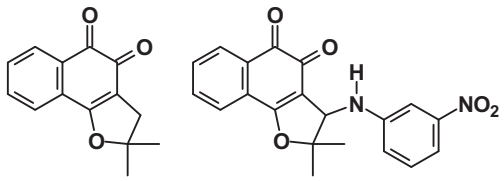

Fig. 1. Chemical structures of nor-beta-lapachone (nor-beta) and 2,2-dimethyl-(3*H*)-3-(*N*-3′-nitrophenylamino)naphtho[1,2-*b*]furan-4,5-dione ($QPhNO_2$), antitumor compounds.

quinone nor-beta. Electrochemical experiments were performed and contributed to the elucidation of the mechanism of action. This approach is particularly suitable for states of the disease associated with oxidative stress of cells, as in cancer (de Souza et al., 2010; Hileman et al., 2004). Additionally, this electrochemical approach can provide an understanding of the interaction between anticancer drugs and DNA, yielding information on the extent of DNA damage (de Abreu et al., 2008; Oliveira-Brett et al., 2002; Rauf et al., 2005).

2. Experimental

2.1. Materials

2,2-Dimethyl-(3*H*)-3-(*N*-3′-nitrophenylamino)naphtho[1,2-*b*]furan-4,5-dione ($QPhNO_2$, $C_{20}H_{16}O_5N_2$, molecular mass 364.35 g/mol) was prepared as described previously (da Silva Júnior et al., 2007). Stock solutions for pharmacological assays were prepared by dissolving $QPhNO_2$ and nor-beta in 0.1% DMSO immediately prior to use. Doxorubicin hydrochloride (adriamycin, CAS No. 25316-40-9) (Dox) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). RPMI 1640 growth medium supplemented with 2% glutamine, fetal bovine serum, streptomycin and penicillin was purchased from Gibco® (Invitrogen, Carlsbad, CA, USA). Calf thymus dsDNA (sodium salt, type I) was purchased from Sigma (St. Louis, MO, USA). Aqueous acetate buffer solutions (0.1 M, pH 4.5), which were used in the electrochemical experiments involving DNA, were prepared from analytical grade reagents and purified water (conductivity < 0.1 μS/cm) obtained from a Millipore (Milford, MA, USA) Milli-Q system. Dimethylformamide (DMF) and tetrabutylammonium tetrafluoroborate ($TBABF_4$) were used in the electrochemical experiments (aprotic medium) and prepared from analytical grade reagents supplied by Sigma Aldrich.

2.2. HL-60 cell culture and MTT assay

HL-60 cells (human promyelocytic leukemia line) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C in a 5% CO_2 atmosphere. The cytotoxicity of compounds (0.009–5 μg/mL) was evaluated using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay (Mosmann, 1983) after 24 h of incubation. Doxorubicin was used as a positive control. In a second set of experiments, *N*-acetyl-L-cysteine (NAC, 5 mM) was pre-incubated with the cells for 1 h before drug addition, and after 24 h, cytotoxicity was measured, as previously described.

2.3. Analysis of mechanisms involved in the cytotoxic activity

Additional experiments were performed to elucidate the mechanisms involved in the cytotoxic action of nor-beta and $QPhNO_2$ using HL-60 cells (3×10^5 cells mL^{-1}) after drug exposure for 24 h. Compounds were dissolved in DMSO to make a 1 mg mL^{-1} stock solution and added to the cell culture to obtain a final concentration of 0.5, 1.0 or 2.0 μM $QPhNO_2$, based on its IC_{50} value, or 1.0 or 2.0 μM nor-beta. Doxorubicin (0.5 μM) was used as a positive control.

2.3.1. Measurement of the generation of reactive oxygen species

After the quinone treatment, cells were loaded with 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA) (20 μM) and incubated at 37 °C for 30 min in the dark, as proposed by Lebel et al. (1992). Doxorubicin and beta-lapachone were used as positive controls. The experiments were repeated in the presence of NAC (5 mM) pre-incubated with the cells for 1 h before drug addition. The cells were then harvested, washed, resuspended in PBS and analyzed immediately by flow cytometry at excitation and emission wavelengths of 490 and 530 nm, respectively.

2.3.2. Flow cytometry analysis

All experimental procedures adopted in the flow cytometry analysis essentially followed the methodology described by Montenegro and coworkers (2010). The evaluated parameters included cell membrane integrity, internucleosomal DNA fragmentation, cell cycle, mitochondrial depolarization, phosphatidylserine (PS) externalization and caspase 3/7 activation. For all the tested compounds, five thousand events were evaluated per experiment, and cellular debris was omitted from the analysis. HL-60 cell fluorescence was then determined by flow cytometry in a Guava EasyCyte Mine® using Guava Express Plus software. Internucleosomal DNA fragmentation and the cell cycle were analyzed by ModFit LT for Win32 version 3.1. The experiments were performed in triplicate.

To verify the participation of ROS in the quinone activity, NAC (5 mM) was pre-incubated with the cells for 1 h prior to drug addition, and after 24 h, cell membrane integrity, internucleosomal DNA fragmentation and phosphatidylserine (PS) externalization were measured, as previously described.

2.4. DNA gel electrophoresis

During the apoptotic process, DNA is cleaved in a distinctive way at internucleosomal sites by a specific caspase-activated endonuclease, thus yielding fragments in multiples of 200 bp, which appear as a characteristic "ladder" when DNA is separated by gel electrophoresis (Enari et al., 1998). Fragmented DNA was isolated as described by Ausubel et al. (1990), using DNAzol® Reagent (Gibco® – Invitrogen, Carlsbad, CA, USA) after 24 h of incubation. Electrophoresis was performed in a 1.5% agarose gel.

2.5. Comet assay in HL-60 cells

The alkaline comet assay was performed as described by Singh et al. (1988) with minor modifications. Briefly, HL-60 cells were incubated for 3 or 24 h with five concentrations of $QPhNO_2$ (0.5, 1.0, 2.0, 5.0 or 10 μM) and with nor-beta at 2.0 or 10 μM. Then, the cells were processed and dissolved in 0.75% low melting point agarose and immediately spread onto a glass microscope slide precoated with a layer of 1% normal melting point agarose. The slides were further incubated in ice-cold lysis solution (pH 10.0) at 4 °C for at least 1 h. After the lysis procedure, the slides were placed in a horizontal electrophoresis unit filled with enough fresh buffer (300 mM NaOH and 1 mM EDTA, pH ~13.0) to cover the slides for 20 min at 4 °C. Electrophoresis was conducted for 20 min at 25 V (300 mA). The slides were then neutralized (0.4 M Tris, pH 7.5) and fixed with ethanol 100%. After the staining step with ethidium bromide, the gels were dried at room temperature overnight, and 50 cells from each of two replicate slides were selected and analyzed for each concentration of test substance. These cells were

scored visually into five classes according to tail length: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1–2× the diameter of the head; (4) class 3: with a tail longer than 2× the diameter of the head; and (5) class 4: comets with no heads. The damage index is based on the length of migration and the amount of DNA in the tail and is considered a sensitive measure of DNA. The damage index thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). The vehicle was used as a negative control, and doxorubicin (0.5 μM) was used as a positive control.

2.6. Electrochemical approaches

Electrochemical experiments, including cyclic voltammetry (CV) and differential pulse voltammetry (DPV), were performed using an Autolab (Echo-Chemie, Utrecht, Netherlands) PGSTAT 20 or PGSTAT-30. The working electrode was a BAS (Bioanalytical Systems, West. Lafayette, IN, USA) 3-mm diameter GC electrode, the counter electrode was a platinum coil, and the reference electrode was AgAgCl, Cl^- (0.1 M); all of the electrodes contained in a single-compartment electrochemical cell with a 10-mL capacity. In CV experiments, a scan rate of 0.100 V s^{-1} was chosen for comparison and for figures. It was only necessary to degas the cell with a nitrogen flux for reduction studies.

CV experiments were performed with $QPhNO_2$ and nor-beta in aprotic media (DMF + 0.1 M $TBABF_4$) on a glassy carbon electrode in the absence and presence of oxygen to investigate their electrochemical reduction mechanisms and possible oxygen interaction with the electrochemically generated radical anions, at *E*pIc (from $QPhNO_2$ and nor-beta). The parameters analyzed were the observed anodic shift in the potential of the first reduction wave (*E*pIc) and the current increase on the same peak (*I*pIc). Each compound was added to the supporting electrolyte, and the solution was degassed with N_2, with a subsequent CV run. Oxygen was then bubbled into the cell, and its concentration was monitored with an oxymeter (DM-4 Digimed). Cyclic voltammograms were recorded at different oxygen concentrations.

For reduction and oxidation studies in protic media, the CV and DPV of 0.1 and 1 mM solutions of $QPhNO_2$ and nor-beta (previously dissolved in 1 mL ethanol) in acetate buffer (0.1 M, pH 4.5) were performed using a bare GC electrode. For the DPV measurements, the optimized differential pulse voltammetry parameters were as follows: pulse amplitude (Δ*E*sw) of 50 mV, pulse width of 70 ms and scan rate of 5 mV s^{-1} [using a step potential (Δ*E*s) of 0.002 V]. This supporting electrolyte was used for all of the experiments involving DNA. All experiments were performed at room temperature (25 ± 1 °C).

2.7. Preparation of the dsDNA-GC biosensor

The electrochemical procedure for the investigation of the $QPhNO_2$-dsDNA interaction involved three steps: preparation of the electrode surface, immobilization of dsDNA gel and voltammetric transduction, as previously described (De Abreu et al., 2008; Diculescu et al., 2005). For each series of experiments, an identical dsDNA-GC electrode was prepared as a reference blank to serve as a control. This electrode was not treated with substrate but received the same pre- and post-treatments as the test electrode. The procedure produced a thick-layer dsDNA-modified electrode.

2.8. Interaction of dsDNA-GC biosensor with $QPhNO_2$

Two different protocols were used to examine the interaction of $QPhNO_2$ with the biosensor. In the first method, the dsDNA-GC surface was dried under a stream of nitrogen, after which the electrode was coated with 20 μL of a solution of $QPhNO_2$ in ethanol P.A., allowed to rest for 5 min and then dried again under a stream of nitrogen until the gel was completely dry. After this step, 5 mL of acetate buffer was added to the cell, and DPV experiments were conducted. In the second method, the biosensor was immersed in a solution of $QPhNO_2$ (5, 10 or 20 μM) for 15 min, after which electrochemical measurements were taken immediately. The same procedure was also applied to the biosensor immersed only in acetate buffer.

2.9. Interaction of ssDNA with $QPhNO_2$

Single-stranded DNA (ssDNA) was prepared by dissolving 3.0 mg of dsDNA in 1.0 mL of chloridric acid (1 M) and heating for 1 h until complete dissolution. This treatment was followed by neutralizing the solution with 1.0 mL of sodium hydroxide (1 M) and adding 9 mL of acetate buffer (Diculescu et al., 2005).

Freshly prepared ssDNA solution was added to the cell, and single-scan DPV experiments were conducted in the range of 0 to +1.4 V *vs.* AgAgCl, Cl^- (0.1 M). Two peaks corresponding to the oxidation of the guanine and adenine bases appeared at potentials of +0.815 V and +1.131 V, respectively. After the first run, the electrode was washed, polished and returned to the ssDNA solution. After cleaning the surface, the GC electrode was inserted into a solution containing $QPhNO_2$ (at different concentrations of 5–46 μM) and ssDNA, and the DPV experiment was repeated. A clean GC electrode was also employed in the DPV experiments involving a 20 μM solution of $QPhNO_2$ alone, and the current of peak Ia was used for comparison.

2.10. Statistical analysis

The IC_{50} values for the MTT assay were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA) from 3 to 4 independent experiments performed in triplicate.

The data are presented as the means ± S.D. from three independent experiments. Differences between experimental groups were compared by one-way ANOVA followed by Newman–Kells test for multiple comparison ($p < 0.05$), whereas Student's *t* tests were used to compare data obtained in the absence or presence of NAC ($p < 0.05$).

3. Results and discussion

3.1. Cytotoxicity against the leukemia cell line HL-60

The inhibitory effects of nor-beta and its nitrophenylamine derivative $QPhNO_2$ were initially determined on the growth of HL-60 cells. The HL-60 cell line could be considered a suitable model to study compounds derived from beta-lapachone because

Table 1
Growth inhibitory activity expressed by IC_{50} (μM) of compounds for HL-60 cell line in the absence or in the presence of *N*-acetylcysteine (NAC, 5 mM) after 24 h of incubation. NAC was pre-incubated with the cells 1 h before the tested compounds.

Compounds	IC_{50} (μM)[a]	
	Without NAC	+NAC (5 mM)
Nor-beta	2.01 ± 0.17	2.72 ± 0.08*
$QPhNO_2$	0.32 ± 0.04	1.03 ± 0.10*
Doxorubicin	0.20 ± 0.002	0.31 ± 0.05

[a]Data are presented as mean IC_{50} values ± standard error of the mean from 3 to 4 independent experiments. Doxorubicin was used as a positive control.
* $p < 0.05$, comparing values in the absence or in the presence of NAC by Student *t* test.

the cytotoxic effects and apoptosis-inducing properties of this compound have already been demonstrated using this cell line (Planchon et al., 1995, 1999).

As shown in Table 1, both $QPhNO_2$ and nor-beta exhibited a strong inhibitory effect on HL-60 cell proliferation after 24 h of incubation, with IC_{50} values of 0.32 and 2.01 μM, respectively, while doxorubicin showed an IC_{50} value of 0.22 μM (Table 1). These findings are in agreement with a previous publication showing the effects of these compounds in HL-60 cells but only after a longer incubation period of 72 h (da Silva Júnior et al., 2007). The introduction of the *meta*-nitrophenylamine group in C-3 of nor-beta, leading to $QPhNO_2$, could increase its growth inhibitory effects toward HL-60 cells, regardless of the incubation period. It is interesting to note that while $QPhNO_2$ presents a higher IC_{50} value (0.91 μM) after 72 h incubation, the other quinones, nor-beta and doxorubicin, presented a clear time-dependency, increasing their activity after 72 h.

Quinones are redox active molecules that form semiquinones and hydroquinones that can redox cycle in the presence of oxygen, leading to the formation of reactive oxygen species (ROS) (Asche, 2005; de Abreu et al., 2002a; Hillard et al., 2008; Ferreira et al., 2009). In fact, it is postulated that ROS generation and the alkylation of cellular nucleophiles, including DNA and enzymes with a –SH group, account for the mechanism of cytotoxic action of drugs containing a quinone moiety (Asche, 2005; de Abreu et al., 2002a; Hillard et al., 2008; Ferreira et al., 2009).

In view of this fact, we measured the cytotoxic effect of $QPhNO_2$ in the presence of NAC, an antioxidant that acts as a ROS scavenger (Zafarullaha et al., 2003). The IC_{50} value for $QPhNO_2$ increased from 0.32 to 1.03 μM and that for nor-beta increased from 2.01 to 2.72 μM (Table 1, column 3). While these data suggest the participation of ROS in $QPhNO_2$ cytotoxic effects, they do not exclude other direct targets. Doxorubicin effects, in contrast, were not affected by NAC treatment (Table 1). ROS production was also evaluated in HL-60 cells by flow cytometry using the oxidation-sensitive fluorescent dye H_2-DCF-DA after 1 h of incubation. $QPhNO_2$ and nor-beta stimulated ROS generation, while doxorubicin was inactive (Fig. 2). ROS generation was higher in the first hour than after 3 h of incubation (data not shown). The pre-incubation with NAC protected the cells from oxidative stress, reducing intracellular ROS generation.

Cell death can be classified according to its morphological appearance, which may be apoptotic, necrotic, autophagic or associated with mitosis catastrophe (Melino, 2001; Okada and Mak, 2004). Cells undergoing apoptosis show typical well-defined morphological changes, including plasma membrane blebbing, chromatin condensation with margination of chromatin to the nuclear membrane, karyorrhexis (nuclear fragmentation), and

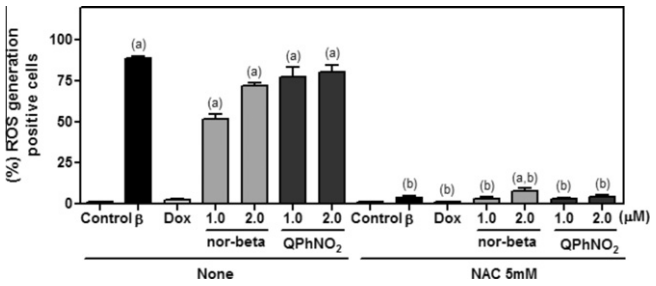

Fig. 2. Intracellular reactive oxygen species generation caused by $QPhNO_2$ and nor-beta in HL-60 cells, after 1 h incubation. The negative control (Control) was the vehicle used for diluting the tested substance. Doxorubicin and beta-lapachone (β) were used as positive controls. Data are presented as mean values ± S.E.M., from three independent experiments performed in triplicate. (a) $p < 0.05$ compared to control by ANOVA, followed by Newman–Keuls Multiple Comparison Test. (b) $p < 0.05$ comparison for each drug in the presence and in the absence of *N*-acetyl-L-cysteine (NAC, 5 mM) by Student *t* test.

formation of apoptotic bodies (Kerr et al., 1972), as already described. Cells treated with $QPhNO_2$ displayed those features, suggesting that this compound induces apoptosis in HL-60 leukemia cells (data not shown).

Considering the observed morphological features, we conducted a flow cytometry analysis of several cellular and biochemical events to assess the mechanisms involved in cell death induced by the tested compounds. In the initial stages of apoptosis, the cell shrinks while the membrane remains intact; however, necrotic cell swelling occurs as a result of the early failure of membrane integrity (Darzynkiewicz et al., 1992; Ziegler and Groscurth, 2004). Nor-beta and $QPhNO_2$ reduced the density of HL-60 cells in a concentration-dependent manner (Fig. 3A). Additionally, both compounds induced internucleosomal DNA fragmentation (Fig. 3C), whereas membrane disruption was only observed in the presence of $QPhNO_2$ at 1 and 2 μM (Fig. 3B).

Apoptosis was confirmed by phosphatidylserine (PS) externalization, caspase 3 and 7 activation and DNA laddering (Figs. 4 and 5). $QPhNO_2$ was again shown to be more active than its prototype nor-beta. Necrosis was also observed in $QPhNO_2$-treated cells (1 and 2 μM), which is compatible with the previously observed loss of membrane integrity. However, it is not possible to state whether necrotic cells corresponds to a secondary necrosis that occurs later in the apoptotic process.

Caspases are essential molecules in apoptosis. Among them, caspase 3 is the death promoter protease that can be activated either by a dependent or independent mitochondrial cytochrome *c* release and caspase 9 function. Additionally, caspase 3 is essential for some hallmarks of apoptosis, such as chromatin condensation and formation of apoptotic bodies. Several authors have reported that beta-lapachone induces apoptosis in cancer cell lines at 5 μM (Gupta et al., 2002; Planchon et al., 1995). Therefore, for the first time, we report that both compounds induce apoptosis, as observed by phosphatidylserine externalization, caspase 3 and 7 activation and DNA fragmentation.

ROS have been recognized as key molecules, which can selectively modify proteins and thus regulate cellular signaling, including apoptosis. A variety of anticancer agents induce apoptosis through the generation of ROS (Eskes et al., 2000; Mizutani et al., 2002). ROS generation is also known to contribute to mitochondrial damage, in which pro-apoptotic proteins from the cytosol are translocated and integrated into the outer mitochondrial membrane, leading to the formation of pores that release cytochrome *c*; the cytochrome *c* then binds to APAF-1 and caspase 9, forming a complex called the apoptosome, which leads to activation of caspase 3 (Eskes et al., 2000; Li et al., 1997). In this context, the generation of ROS should present a role in the initiation of the apoptotic process induced by $QPhNO_2$. It is important to note that doxorubicin is a poor pro-oxidant when compared with $QPhNO_2$ and nor-beta, suggesting a different mechanism of action for this molecule.

To evaluate the role of ROS in the apoptosis-inducing properties of the tested compounds, the cells were pre-treated with NAC at 5 mM. The $QPhNO_2$ effects on cell number (Fig. 3A), DNA fragmentation (Fig. 3C), membrane integrity (Fig. 3B) and phosphatidylserine externalization (Fig. 4) at a concentration of 0.5 μM were inhibited after pre-treatment with NAC (Figs. 3 and 4), whereas at 1 and 2 μM, $QPhNO_2$ effects remained unaltered. These findings reinforce the importance of oxidative stress in the apoptosis process triggered by this molecule. However, they strongly suggest additional targets because while NAC abolished ROS generation induced by $QPhNO_2$ at 1 and 2 μM (Fig. 2), it did not inhibit the appearance of apoptotic features at the equal concentrations (Figs. 3 and 4).

DNA is also recognized as a target of quinones. The cytotoxic effects of doxorubicin are generally related to its ability to damage

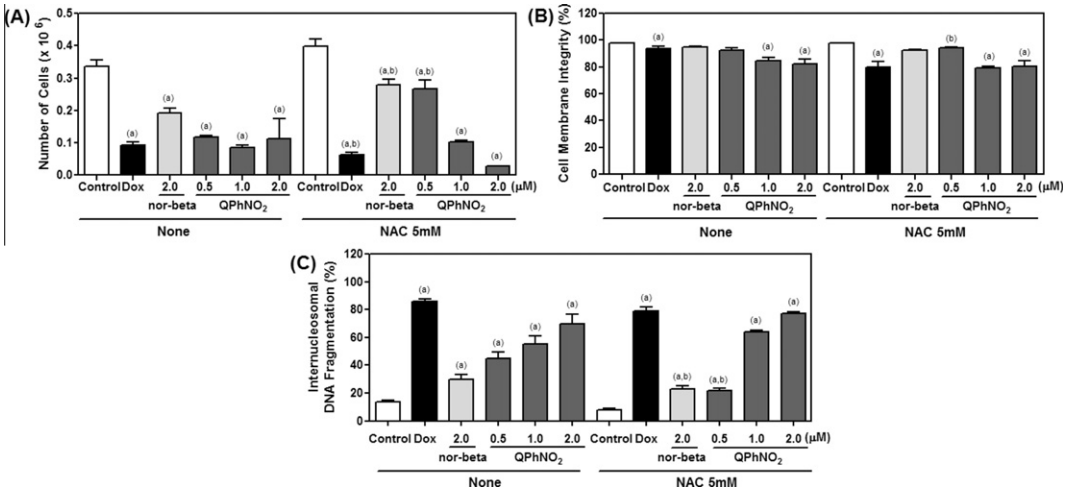

Fig. 3. Effect of $QPhNO_2$ and nor-beta in HL-60 cell density of cells (A) and cell membrane integrity (B) using propidium iodide; internucleosomal DNA fragmentation using propidium iodide, Triton X-100 and citrate (C) determined by flow cytometry after 24 h incubation. Doxorubicin (Dox, 0.5 μM) was used as a positive control. The negative control (Control) was the vehicle used for diluting the tested substance. Five thousand events were analyzed in each experiment. Data are presented as mean values ± S.E.M. from three independent experiments performed in triplicate. (a) $p < 0.05$ compared to control by ANOVA, followed by Newman–Keuls Multiple Comparison Test. (b) $p < 0.05$ comparison for each drug in the presence and in the absence of *N*-acetyl-L-cysteine (NAC, 5 mM) by Student *t* test.

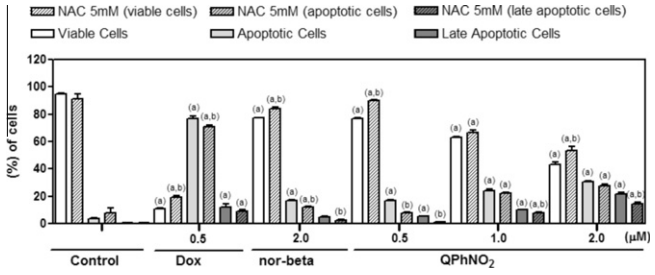

Fig. 4. $QPhNO_2$ (0.5, 1 and 2 μM) and nor-beta (2 μM) induces phosphatidylserine (PS) externalization. PS externalization was determined by flow cytometry using Annexin V–PE and 7-AAD after 24 h of incubation in the absence and presence of *N*-acetyl-L-cysteine (5 mM). The negative control (Control) was the vehicle used to dilute the tested substance. Doxorubicin (Dox, 0.5 μM) was used as a positive control. The percentages of early and late apoptotic and viable cells are indicated. Data are presented as mean values ± S.E.M. from three independent experiments performed in triplicate. Five thousand events were analyzed in each experiment. (a) $p < 0.05$ compared to control by ANOVA, followed by Newman–Keuls Multiple Comparison Test. (b) $p < 0.05$ comparison for each drug in the presence and in the absence of *N*-acetyl-L-cysteine (NAC, 5 mM) by Student *t* test.

cancer cell DNA, which is a consequence of its interaction and inhibition of DNA topoisomerase II, its induction of double-stranded DNA breaks, and its direct intercalation into DNA, modifying helical torsion (Ozben, 2007).

The comet assay is a sensitive and relatively simple technique to quantify DNA damage in individual cells (Singh et al., 1988). HL-60 cells treated with nor-beta did not display any DNA damage in the tested time frame and dosage. $QPhNO_2$ showed a different profile, with concentration-dependent damage and frequency of damage after 3 and 24 h of treatment, especially at higher concentrations (10 μM) (Fig. 6). Doxorubicin, as expected, was a very strong genotoxic compound, increasing the DNA damage index and frequency at 0.5 μM (Fig. 6). Thus, $QPhNO_2$ directly interacts with DNA but at higher concentrations than those necessary to induce apoptosis.

3.2. Electrochemical experiments

Electrochemical methods (analytical and preparative) and electrochemical (thermodynamic and kinetic) parameters have been shown to be extremely useful in biomedical chemistry with respect to the mechanisms of biological electron-transfer processes. The high versatility of electrochemical methodologies allows the mimicking of a large spectrum of biological environments (Hillard et al., 2008). With this in mind, electrochemical proof of the pro-oxidant activity of $QPhNO_2$ and nor-beta was assayed using cyclic voltammetry in the presence of oxygen in aprotic media, which provided a good model of the membrane environment in which peroxidation processes take place (Ossowski et al., 2000). The electrochemistry of both quinones in aprotic media on GCE and mercury has already been reported (de Souza et al., 2010; Hernández et al., 2008). A detailed study of the influence of oxygen concentration on *E*pIc and *I*pIc of both quinones was performed, as described previously for lapachol and isolapachol (Goulart et al., 2003, 2004). The addition of O_2 to the system caused remarkable changes to the position of the first reduction peak potential (*E*pIc) as well as to the shape of the other waves of $QPhNO_2$ (Fig. 7A). The peak of oxygen reduction (EpO_2) in this medium occurred at −0.894 V. These effects include a) an increase in the height and anodic shift of the first cathodic wave (Ic) (inset, Fig. 7A, which is related to the generation of the semiquinone, and b) a disappearance of the corresponding anodic wave (Ia) (Fig. 7A).

The position of the $QPhNO_2$ first reduction peak changed and shifted toward more positive potentials (Δ = 0.032 V) with oxygen concentration (Fig. 7A). The larger shift (Δ *ca.* 0.048 V) occurred at an oxygen/$QPhNO_2$ concentration ratio of 0.093. The reduction current increased by 28% at oxygen concentrations as low as 0.096 mM and reached its maximum, with a 162% increase, in $[O_2]$ = 0.806 mM (Fig. 7A, inset).

Data obtained from the addition of oxygen at different concentrations (Fig. 7B) indicate that the apparent association constant between the electrogenerated semiquinone (from $QPhNO_2$) and O_2 from the graph Ip_{R1}/Ip_{O1} vs. kapp$[O_2]RT/nFv$ is 0.72 s^{-1}, considering that the maximum solubility of oxygen in DMF is 1.85 mM at 25 °C (de Abreu et al., 2007). In similar experiments and conditions, the apparent association constant for nor-beta is 0.55 s^{-1} (Fig. 7C), which is lower than that of $QPhNO_2$.

In this study, using electrochemical methods, we have demonstrated that the anion radicals of both quinones [nor-beta$Q^{\cdot -}$] and [$Q^{\cdot -}$]-$PhNO_2$ interact with O_2 according to an EC mechanism, which yields the original quinone and peroxyl radicals (Goulart et al., 2003, 2004). These facts support the possible intermediacy of ROS in the molecular mechanism of action of $QPhNO_2$.

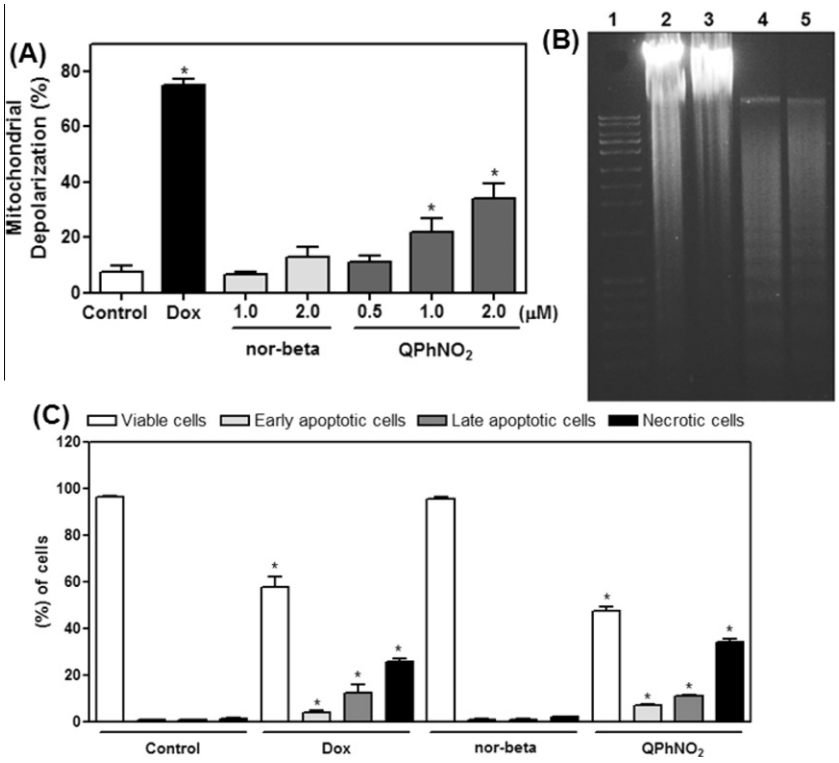

Fig. 5. Apoptosis-inducing effects of $QPhNO_2$ and nor-beta at 2.0 μM in HL-60 cells. (A) Mitochondrial depolarization using rhodamine 123 (B) Caspases 3/7 activity was also determined by flow cytometry using FAM and 7-AAD. The negative control (Control) was the vehicle used for diluting the tested substances. Doxorubicin (Dox, 0.5 μM) was used as a positive control. Data are presented as mean values ± S.E.M. from three independent experiments performed in triplicate. Three thousand events were analyzed in each experiment. $^{*}p < 0.05$ compared to control by ANOVA followed by Newman–Keuls Multiple Comparison Test. (C) DNA laddering analysis of HL-60 cells that had been treated with nor-beta at 2 μM (line 3), Dox at 0.5 μM (line 5) and $QPhNO_2$ at 2 μM (line 4) after 24 h incubation. Negative control cells (line 2) were treated with vehicle alone. Line 1: Marker 1 bp.

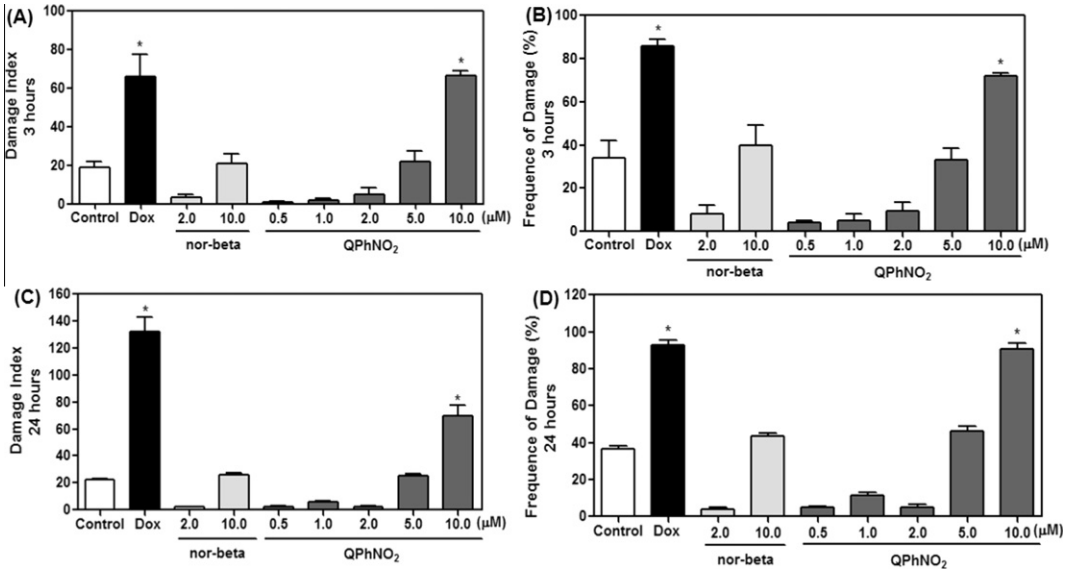

Fig. 6. Effect of $QPhNO_2$ (0.5 up to 10 μM), nor-beta (2.0 and 10 μM) and doxorubicin (Dox) on DNA damage index (3 h, A and 24 h, C) and on DNA damage frequency (3 h, B and 24 h, D) in comet assay. HL-60 cells were exposed for 3 h and 24 h. Bars represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$, ANOVA followed by Student–Newman–Keuls test.

Because DNA is also a possible target for the action of quinones, electrochemical studies in protic medium could provide valuable information. CV and DPV of 0.1 and 1 mM solutions of $QPhNO_2$ were performed. As shown in Fig. 8A, $QPhNO_2$ demonstrated a behavior represented by two reduction peaks (EpIc = −0.256 V and EpIIc = –0.826 V) and the corresponding oxidation peaks (EpIa = –0.098 V, EpIIa = +0.072 V). In comparison with the CV of nor-beta (da Silva Júnior et al., 2009) and beta-lapachone (de Abreu et al., 2002b) and considering the facility of quinone reduction, it is suggested that the first reduction peak observed (Ic) for $QPhNO_2$ (Fig. 8A) is related to the reduction of quinone by $2e^{-}/2H^{+}$ capture, whereas the second stage of reduction (IIc) is related to the

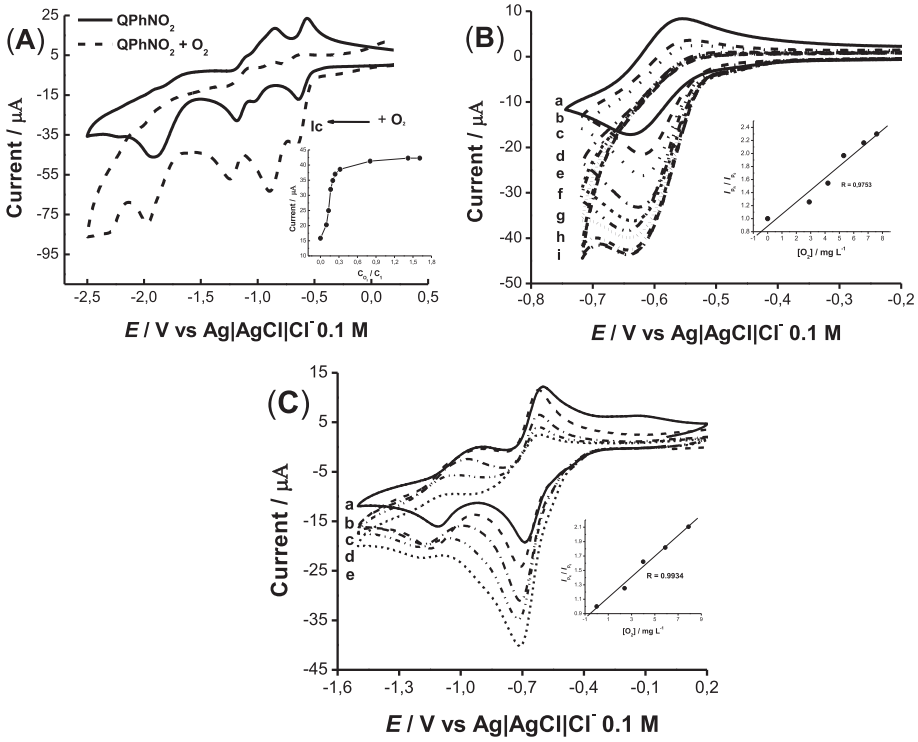

Fig. 7. Cyclic voltammograms (CV) on glassy carbon electrode (3 mm diameter), in DMF + $TBABF_4$, 0.1 M, *t* 0.100 V s^{-1}. (A) CV of $QPhNO_2$ (c_{QPhNO2} = 1 mM), in the absence and presence of oxygen at 0.100 V s^{-1}. (Inset) Effect of oxygen concentration on the peak current of the first wave of reduction of $QPhNO_2$ (I_{p1c}). (B) CV of $QPhNO_2$ (c_{QPhNO2} = 1 mM), in the presence of different oxygen concentrations: (a) 0, (b) 0.096, (c) 0.131, (d) 0.165, (e) 0.200, (f) 0.237, (g) 0.318, (h) 0.806, (i) 1.42 mM. Inserted curve I_{p1c}/I_{p1a} depending on the concentration of oxygen. (C) CV of nor-beta ($c_{nor\text{-}beta}$ = 1 mM). Same conditions, different oxygen concentrations: (a) 0, (b) 0.075, (c) 0.125, (d) 0.184 and (e) 0.246 mM.

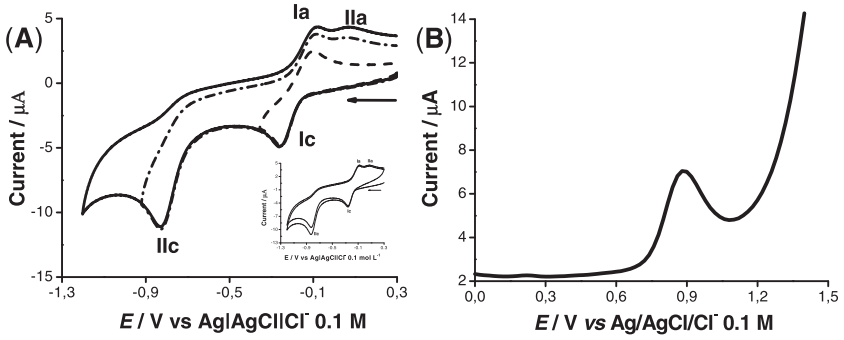

Fig. 8. CV of $QPhNO_2$ (c_{QPhNO2} = 0.1 mM), on glassy carbon electrode (3 mm diameter), in acetate buffer (0.1 M) + EtOH (50%) (pH 4.5), at 0.100 V s^{-1}. (A) Cathodic run, from −0.3 V up to −1.2 V. (B) Differential pulse voltammogram (DPV) of $QPhNO_2$ (c_{QPhNO2} = 0.1 mM), anodic run, from 0 to +1.5 V measured with a pulse amplitude of 50 mV, a pulse width of 70 ms and scan rate of 5 mV s^{-1}.

irreversible reduction of the nitro group in one step with the entrance of $4e^-/4H^+$ (Cavalcanti et al., 2004; Goulart et al., 2007). The electrogenerated hydroxylamine (oxidation peak IIa) was also shown to be unstable, whereas the expected electrochemical system ArNHOH ⇆ ArNO + $2H^+$ + $2e^-$ was not visible (second cycle, inset, Fig. 8A).

The oxidation behavior of $QPhNO_2$ (Fig. 8B) was represented by one irreversible and diffusion-controlled process (*E*pIIIa shifted with scan rate): in the DPV, peak IIIa was located at +0.884 V and was likely related to the oxidation of the aromatic amino group in the molecule, whereas nor-beta did not show oxidation peaks (data not shown).

The interaction between $QPhNO_2$ and dsDNA was analyzed using thick-film dsDNA-biosensors (Fig. 9); undesirable binding of drug molecules to the electrode surface was avoided by completely covering the electrode surface with dsDNA (de Abreu et al., 2008).

Despite the presence of the oxidation wave of $QPhNO_2$ at a potential very close (*E*pIIIa = + 0.884 V) to that of guanosine (approximately +0.80 V *vs.* Ag/AgCl), a DNA-constituent base, it was possible to observe a positive interaction, which was indicated by the presence of the waves of guanosine and adenosine, using a very low concentration of $QPhNO_2$ (Fig. 9A and B) due to solubility problems.

The positive interaction between $QPhNO_2$ and ssDNA in solution is depicted in Fig. 10. Signals associated with the oxidation of the guanine (G) and adenine (A) bases (+0.83 V and +1.16 V vs. Ag/AgCl 0.1 M, respectively) in ssDNA were very intense. However, in the presence of $QPhNO_2$, the current intensity of the oxidation peaks decreased in a concentration-dependent manner until the

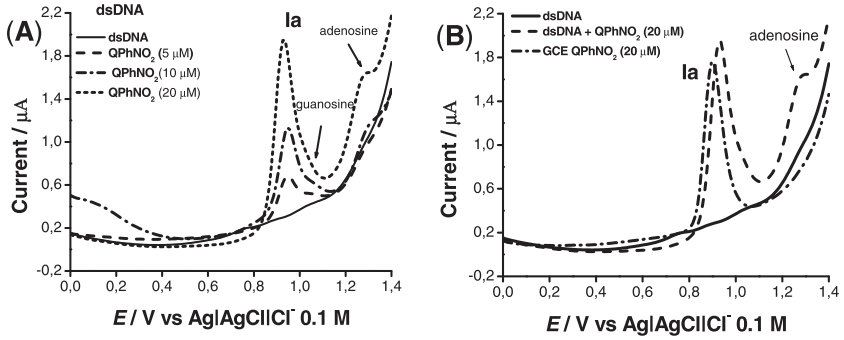

Fig. 9. Aqueous acetate buffer 0.2 M; pH 4.5. (A) DPV recorded following 15 min of exposure of a dsDNA biosensor to different concentrations of $QPhNO_2$; (B) DPV of $QPhNO_2$, c_{QPhNO2} = 20 μM, on glassy carbon electrode. Peak Ia is related to the oxidation of $QPhNO_2$ and the peaks associated with the oxidation of guanosine and adenosine are marked with arrows.

signals leveled out (Fig. 10B and C). Increasing the concentration was precluded by dissolution problems, with the precipitation of $QPhNO_2$. The oxidation potentials of guanine and adenine were kept nearly constant.

A different behavior (currents related to the oxidation of guanine and adenine remained practically unaltered at concentrations up to 200 μM) was reported for nor-beta (Cavalcanti et al., 2011) in studies with ssDNA, and these results suggest that those compounds do not damage DNA directly (Brett et al., 2002; de Abreu et al., 2002a,b).

The positive correlation between the electrochemical and pharmacological experiments can be expanded using already reported data obtained from doxorubicin analogs. The interaction of dsDNA (calf thymus) and daunorubicin in solution and on the electrode surface was previously studied using cyclic voltammetry and particularly by constant-current chronopotentiometric stripping analysis using carbon paste electrodes, which revealed the intercalation of this drug between the base pairs in dsDNA (Wang et al., 1998). Adriamycin, a cancerostatic anthracycline antibiotic, causes considerable tumor cell death, together with the induction of breaks in single- and double-stranded DNA. The interaction of adriamycin with DNA was also investigated using an electrochemical DNA-biosensor. Its intercalation in DNA disrupts the double helix, and the detection of guanine and 8-oxoguanine could mimic a possible mechanism for the *in vivo* adriamycin drug action (Piedade et al., 2002). Recently, Cavalcanti and coworkers reported the positive interaction between doxorubicin and ssDNA (Cavalcanti et al., 2011).

As previously shown with respect to DNA, electrochemical studies indirectly allow evidencing the generation of ROS. Experiments performed in the presence of oxygen showed that $QPhNO_2$ reacts faster with oxygen than nor-beta and provokes a greater release of ROS. These findings corroborate with those obtained by flow cytometry. The underlying rationale for this fact may be the structure of the reduced nitroquinone (de Souza et al., 2010; Hernández et al., 2008). The redox-cycling of quinones may be initiated by either a one- or two-electron reduction. The one-electron reduction of quinones is catalyzed by NADPH–cytochrome P450

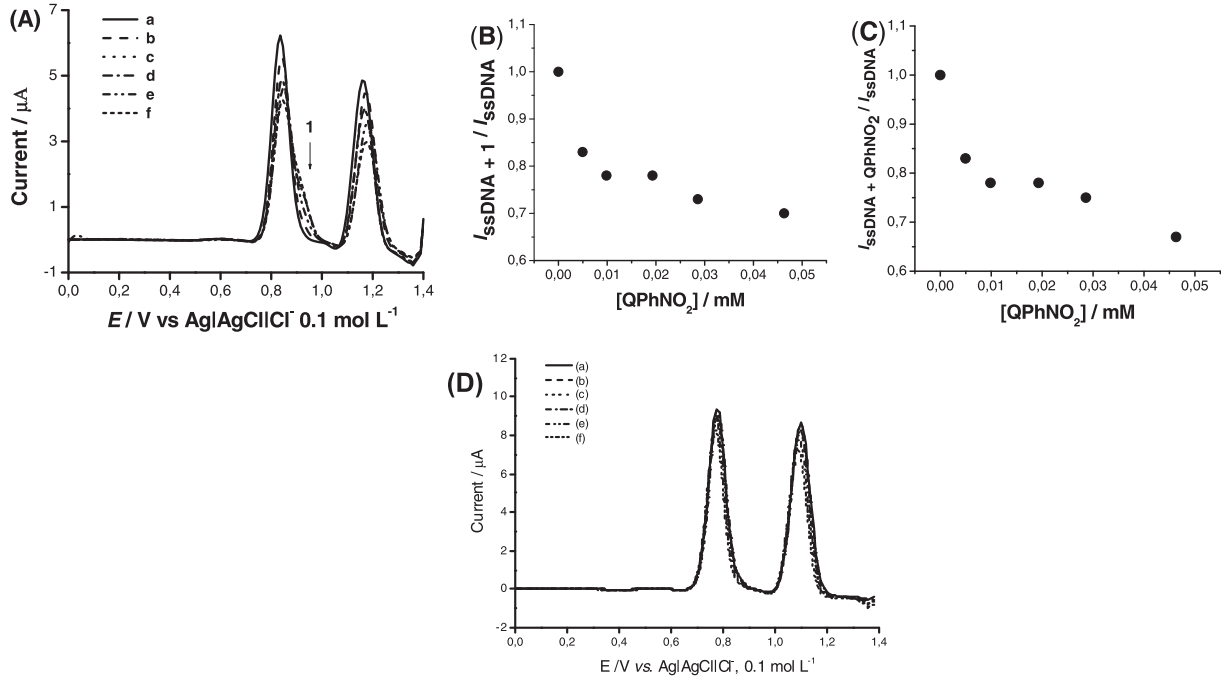

Fig. 10. Differential pulse voltammograms (DPV), aqueous ethanol (27%), acetate buffer (0.1 mol L^{-1}), pH 4.5. Glassy carbon electrode vs. Ag|AgCl|Cl^- (0.1 M), v = 0.010 V s^{-1}. (A) Effects on ssDNA of increased amounts of $QPhNO_2$, both in solution: (a) 0, (b) 0.005, (c) 0.010, (d) 0.020, (e) 0.030, (f) 0.046 mM. (B and C) Graphs of peak current of oxidation of ssDNA, depending on the concentration of $QPhNO_2$. (D) Effects on ssDNA of increased amounts of nor-beta: 0.010 (b), 0.020 (c), 0.060 (d), 0.100 (e) and 0.200 mM (f) and (a) only ssDNA solution.

reductase, generating unstable semiquinones. The semiquinones transfer electrons to molecular oxygen and return to their original quinoidal formation, thus generating a superoxide anion radical ($O_2^{\cdot -}$). Superoxide can dismutate into hydrogen peroxide (H_2O_2) by a SOD-catalyzed reaction, and a hydroxyl radical ($HO^{\cdot}$) would be subsequently formed by the iron-catalyzed reduction of peroxide by a Fenton reaction (Hillard et al., 2008). All of these highly reactive ROS may react directly with DNA or other cellular macromolecules, such as lipids and proteins, leading to cell damage.

4. Conclusions

In conclusion, $QPhNO_2$ cytotoxicity is based on apoptosis, which is partially caused by ROS release, and DNA is also a target for this nitroquinone. This study illustrates how electrochemistry, biochemistry and pharmacology can be integrated to elucidate biological mechanisms of action.

Conflict of interest

Authors declare no conflict of interest.

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