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Mechanism of action of novel naphthofuranquinones on rat liver microsomal peroxidation

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ABSTRACT

In order to elucidate the effect on mammal systems of new derivatives from 2-hydroxy-3-allyl-naphthoquinone, α-iodinated naphthofuranquinone (NPPN-3223), β-iodinated naphthofuranquinone (NPPN-3222) and β-methyl naphthofuranquinone (NPPN-3226) synthesized as possible trypanocidal agents, their effect on rat liver microsomal lipid peroxidation was investigated. They (a) inhibited NADPH-dependent, iron-catalyzed microsomal rat liver lipid peroxidation; (b) did not inhibit the *tert*-butyl hydroperoxide-dependent lipid peroxidation; (c) did not inhibit ascorbate–lipid peroxidation with the exception of NPPN-3226 which did inhibit it; (d) stimulated NADPH oxidation and microsomal oxygen uptake; (e) increased superoxide anion formation by NADPH-supplemented microsomes and (f) stimulated ascorbate oxidation. The three drugs were reduced to their seminaphthofuranquinone radical by the liver NADPH–P450 reductase system, as detected by ESR measurements. These results support the hypothesis that naphthofuranquinones reduction by microsomal NADPH–P450 reductase and semiquinone oxidation by molecular oxygen diverts electrons, preventing microsomal lipid peroxidation. In addition, hydroquinones and/or semiquinones formed by naphthofuranquinones reduction would be capable of lipid peroxidation inhibition and on interacting with the lipid peroxide radicals can lead to an antioxidant effect as we suggested for NPPN-3226 in close agreement to the inhibition of ascorbate–lipid peroxidation. Due to the properties of these molecules and their incoming structure developments, naphthofuranquinones would be considered as potentially promising therapeutic agents, mainly against Chagas disease.

1. Introduction

Quinones are widely distributed in nature and make up an important group of substrates for flavoenzymes. Their fundamental feature is the ease of reduction and, therefore, the ability to act as an oxidizing or dehydrogenating agent, being this redox property the driving force in the formation of a fully aromatic system [1]. Naphthoquinones are considered privileged structures in medicinal chemistry on the basis of their biological activities and structural properties [2]. Lipophilic *o*-naphthoquinones possess antifungal, antibacterial, trypanocidal and cytostatic effects. Among these quinones, β-lapachone (3,4-dihydro-2,2-dimethyl-2-*H*-naptho[1,2*b*] pyran5,6-dione) isolated from the lapacho tree (*Tabebuia avellanedae*) has proved to be an effective cytostatic agent to a variety of human cancers lineages [3–7]. This naphthoquinone is under investigation, especially for the treatment of specific cancers with elevated NAD(P)H quinone oxidoreductase 1(NQO1) levels, such as breast, non-small cell lung, pancreatic, colon, and prostate cancers [8–10], and it is currently in phase II clinical trials for the treatment of pancreatic cancer [8–11]. β-Lapachone induces a novel caspase and p53-independent cell death

Abbreviations: SOD, superoxide dismutase; ROS, reactive oxygen species; DTD, DT-diaphorase; DMFA, *N*,*N*-dimethyl formamide; EDTA, ethylenediamene tetraacetic acid; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; *t*-BuOOH, *tert*-butyl hydroperoxide; TBA, thiobarbituric acid; MDA, malondialdehyde; DPPH, 2,2-diphenyl-1-picryl-hydrazyl; DETAPAC, diethylenetriaminepentaacetic acid; BSA, serum bovine albumin; TEMPOL, 2,2,6,6-tetramethyl piperidine-N-oxyl; NFQs, naphthofuranquinones molecules whose chemical formulae are described in text and in Fig. 1.

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in human cancer cells lineages overexpressing NQO1 that substantially enhances the toxicity of β-lapachone, reducing the quinone to an unstable hydroquinone that rapidly undergoes a two-step oxidation back to the parent compound, perpetuating a futile redox cycle [12,13]. The redox-cycling of quinones may be initiated by either one- or two-electron reduction. The one-electron reduction of quinones is catalyzed by NADPH–cytochrome P450 reductase generating unstable semiquinones [14]. They transfer electrons to molecular oxygen and return to their original quinoidal formation, thus generating superoxide anion radical ($^{\bullet}O_2^-$). Superoxide can dismutate to hydrogen peroxide (H_2O_2), by a SOD-catalyzed reaction, and then, by the Fenton reaction, hydroxyl radical ($^{\bullet}HO$) would be formed by the iron-catalyzed reduction of peroxide [15–17]. All these highly ROS may react directly with DNA or other cellular macromolecules, such as lipids and proteins, leading to cell damage. Unlike most other cellular reductases, two-electron reduction of quinone can also be catalyzed by cytosolic and mitochondrial DTD directly to the hydroquinone [18–20].

Although there are several reports on the biological activity of naphthopyranquinones, such as β-lapachone, data in the literature about naphthofuranic analogues are scarce. To find more potent naphthoquinones, Silva et al. [21] synthesized new derivatives from 2-hydroxy-3-allyl-naphthoquinone. Among these compounds stand α-iodinated naphthofuranquinone (NPPN-3223), β-iodinated naphthofuranquinone (NPPN-3222) and β-methyl naphthofuranquinone (NPPN-3226) (Fig. 1) that proved to be active against trypomastigotes of *Trypanosoma cruzi*, the etiological agent of Chagas disease [22].

In the present study, we examined the effect of these three NFQs on microsomal rat liver lipid peroxidation, on the production of ROS and on semiquinone formation. This report shows that the production of reactive oxygen species by NFQs redox-cycling may involve reaction sequences in which the semiquinone is an intermediary. The antioxidant property of NPPN-3226 is also proposed.

2. Materials and methods

2.1. Chemicals

The NFQs were synthesized as previously described [21]. These compounds were provided as a lyophilized powder and diluted in dimethylformamide (DMFA). Controls received the same volume of solvent, whose concentration never exceeded 0.5% (v/v). HCl, NaOH and KCl were provided by Merck Química Argentina S.A. (Buenos Aires, Argentina). Ethylenediamine tetraacetic acid (EDTA), tris (hydroxymethyl) aminomethane, thiobarbituric acid (TBA), Na_2HPO_4, KH_2PO_4, $MgCl_2$, $FeCl_3$, $FeSO_4$, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), $NADP^+$, ADP, NADPH, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), sodium ascorbate, diethylenetriaminepentaacetic acid (DETAPAC), *tert*-butyl hydroperoxide (*t*-BuOOH), cytochrome *c* partially acetylated, superoxide dismutase (SOD), catalase and serum bovine albumin (BSA) were acquired from Sigma Chemical Co. (St. Louis, MO, USA), *N*,*N*-dimethyl-formamide (DMFA) and methanol were provided by Riedel de Haen AG (Seeize, Germany). All the other chemicals used in this study were of analytical grade.

2.2. Microsomal preparations

Microsomes were obtained from the livers of 20-h fasted, male Wistar rats (240–280 g) fed a Purine-like rat chow. The liver was removed and placed quickly in an ice-bath [23,24]. Briefly, the liver tissue was homogenized in 50 mM Tris–HCl, 150 mM KCl (pH 7.4) (4 ml buffer/g liver). Centrifugations at 600 × *g* for 10 min and then at 10,800 × *g* for 15 min were performed to eliminate nuclear and mitochondrial fractions, respectively. The microsomal fraction was obtained by centrifugation of the supernatant for 60 min at 105,000 × *g*. Microsomes were washed with 150 mM KCl and centrifuged for 1 h at 105,000 × *g* twice, and either used immediately or stored in liquid nitrogen for 3 months. All procedures were performed at 4 °C. No superoxide dismutase or catalase activities were found in the microsomal suspension (data not shown).

2.3. Assay of lipid peroxidation

For the assay of NADPH-dependent lipid peroxidation, the incubation mixture consisted of liver microsomes (1.5 mg protein/ml), the NADPH-generating system (0.55 mM $NADP^+$, 5.5 mM G6P, 1.4 units/ml G6PD, 5.5 mM $MgCl_2$), 1.7 mM ADP, 0.1 mM $FeCl_3$, 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.4 [25]. The $FeCl_3$–ADP mixture was prepared immediately before use. After equilibration at 37 °C, the reaction was initiated by adding G6PD. For the ascorbate–iron-induced lipid peroxidation, the incubation mixture consisted of microsomes (1.5 mg protein/ml, preheated in a water bath at 100 °C for 8 min), 2.0 mM ADP, 67 μM $FeCl_3$, 0.5 mM sodium ascorbate, 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.4. The reaction was started by adding ascorbate, which was prepared immediately before use and kept under a stream of nitrogen. For the *t*-BuOOH-Fe induced peroxidation [26], the incubation mixture consisted of 0.11 mM EDTA, 0.10 mM $FeSO_4$, 2.6 mM *t*-BuOOH, microsomes (1.5 mg protein/ml) and phosphate buffer as with the NADPH-generating system. EDTA and $FeSO_4$ were added as an EDTA–$FeSO_4$ complex, which was prepared immediately before use and kept under a stream of nitrogen; then, the reaction was started by adding *t*-BuOOH. The final volume of the incubation mixture was always 0.75 ml. Incubation time was 60, 90 or 20 min with NADPH-dependent, ascorbate or *t*-BuOOH–lipid peroxidation system, respectively. The NFQs were added dissolved in DMFA. Controls received the same of solvent whose concentration never exceeded 1% (v/v); DMFA failed to affect the rate of lipid peroxidation. Incubations were performed in a New Brunswick gyratory shaker at 90 cycles/min at 37 °C under air. Other experimental conditions are described in Section 3. The MDA content of these samples was measured by the TBA method [27]. Absorbance was measured spectrophotometrically at 535 nm in a DU-800 Beckman Coulter spectrophotometer and the amount of MDA was calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.4. Antiradical activity determination

Scavenging of free radical was tested in a methanolic solution of DPPH [28]. DPPH is a stable free radical and accepts an electron or hydrogen to become a stable diamagnetic molecule [29]. The degree of decoloration of the solution indicates the scavenging efficiency of the added NFQ. Aliquots of 0.5 ml NFQ in methanol were added to 1 ml DPPH solution (10 mg/l). After 15 min, the reductive capability of DPPH radicals was measured by the decrease in absorbance (Abs) at 517 nm determined in a DU-800 Beckman Coulter spectrophotometer. A reference sample was prepared with 1 ml of methanol. Antiradical activity was calculated as a percentage of DPPH decoloration using the following equation:

$$\text{Antiradical activity} = 100 \times \left(\frac{1 - \text{Abs sample}}{\text{Abs reference}}\right)$$

2.5. Assay of NADPH-oxidase activity

The NFQs were assayed as substrates for the microsomal NADPH–cytochrome P450 reductase by measuring the rate of NADPH disappearance in the presence of microsomes at 30 °C. The reaction mixture consisted of microsomes (0.5 mg protein/ml),

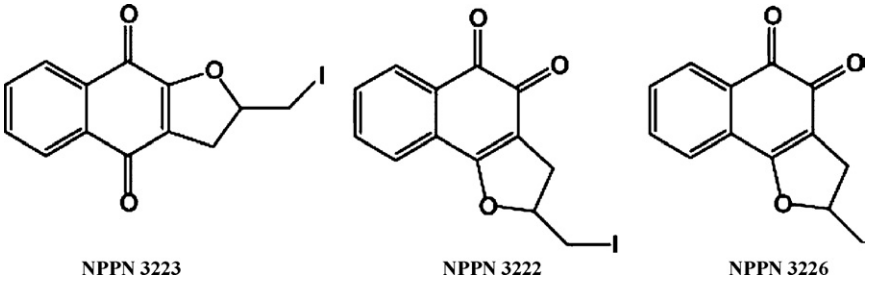

Fig. 1. Naphthofuranquinones structures: 2-iodomethyl-2,3-dihydro-naphtol[2,3-b]furan-4,9-dione (NPPN-3223); 2-iodomethyl-2,3-dihydro-naphto[1,2-b]furan-4,9dione (NPPN-3222); 2-methyl-2,3-dihydro-naphtol [1,2-b]furan-4,9-dione (NPPN-3226).

0.25 mM NADPH, 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.4. The reaction was started by the addition of the NFQs. The rate of NADPH oxidation was measured for 5 min at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) in a DU-800 Beckman Coulter spectrophotometer.

2.6. Oxygen uptake measurements

Oxygen uptake was measured polarographically, with a model 5/6 Oxygraph (Gilson Medical Electronics, Middleton, WI, USA) fitted with a Clark oxygen electrode, and thermostatized at 30 °C. Oxygen uptake in microsomal reactions was conducted in a 1.8 ml closed and magnetically stirred glass chamber containing microsomes (1.5 mg of protein/ml) suspended in 130 mM KCl and 23 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.4, and NFQ or DMFA.

The NFQs effect on ascorbate oxidation was measured polarographically as described above. The reaction mixture contained 5.5 mM sodium ascorbate, 1.0 mM DETAPAC, 100 mM Tris–HCl buffer, pH 7.5, and NFQ or DMFA [30].

2.7. Determination of superoxide anion generation

Production of $^{\bullet}O_2^-$ was measured using the cytochrome *c* partially acetylated method [31,32]. The reaction mixture contained microsomes (0.26 mg protein/ml), 15 μM cytochrome *c* partially acetylated, 0.16 mM NADPH and buffer (130 mM KCl, 23 mM KH_2PO_4–Na_2HPO_4, pH 7.4). Cytochrome *c* reduction was determined at 550 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$) in a DU-800 Beckman Coulter spectrophotometer at 30 °C. The rate of cytochrome *c* reduction sensitive to addition of 100 U/ml SOD was considered rate of $^{\bullet}O_2^-$ production.

2.8. ESR measurements

Semiquinone radicals were generated through the reduction of NFQs by the liver microsomal NADPH–P450 reductase system. The reaction mixtures for ESR spectroscopy contained, in a final volume of 2 ml, 5 mM each NFQ, 10 mM NADPH, NADPH generator system as described above (item 2.3), and liver microsomes. In order to obtain anaerobic conditions for the ESR measurements, the assay media in the absence of NFQ was bubbled with a stream of nitrogen for 5 min. At the same time, the NFQ solution was bubbled with nitrogen, as described above. A sample of the deoxygenated, concentrated NFQ solution was added to the deoxygenated assay medium under anaerobic conditions. Lastly, the reaction medium was transferred to the spectrometer cell previously flushed with nitrogen and the spectrum was recorded. The time elapsed from the reaction mixture preparation to ESR spectra recording was about 5 min, a period considered as the incubation time. ESR measurements were performed at room temperature under anaerobic conditions using a Bruker ECS 106 ESR spectrometer (Bruker, Karlsruhe, Germany) equipped with an ER 4102ST cavity. General instrumental conditions were: microwave power, 20 mW; microwave frequency, 9.75 GHz; modulation amplitude, 1 G; modulation frequency, 50 kHz; time constant, 164 ms. The field was centered at 3490 G. Radical concentrations were determined by double integration of spectra using 2,2,6,6-tetramethyl piperidine-N-oxyl (TEMPOL) as standard.

2.9. Statistical analysis

The effect of the NFQs was calculated by taking the result in the sample containing DMFA as the control value. GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA) was used to calculate the standard errors of independent experiments involving duplicate analyses for each sample condition. Statistical analysis was done with one-way analysis of variance (ANOVA) test for repeated measurements and either Dunnett or Tukey post-test. Significance was accepted at $p < 0.05$.

3. Results

3.1. Effects of the naphthofuranquinones on NADPH-dependent lipid peroxidation

In microsomes, NADPH-cytochrome P450 reductase is involved in NADPH-dependent enzymatic lipid peroxidation [25,33]. Lipid peroxidation, which can be measured by the TBA method, occurs when rat liver microsomes are incubated in the presence of Fe^{3+}–ADP/NADPH. Table 1 shows the effect of the NFQs on MDA production by liver microsomes. The results show that these compounds inhibited lipid peroxidation in a concentration dependent manner. Maximal inhibition values ranged between 88 and 94% at 1 μM, being NPPN-3226 a more effective inhibitor when compared to NPPN-3222 and NPPN-3223.

Table 1
Effect of naphthofuranquinones on lipid peroxidation by liver microsomes incubated with the NADPH-generating system.

NPPN (μm)	MDA-equivalents (nmol/mg protein)		
	3222	3223	3226
0.001	17.69 ± 1.36	16.60 ± 0.63	15.58 ± 0.99
0.010	16.36 ± 1.46	15.53 ± 0.87	15.21 ± 1.08
0.025	15.18 ± 1.40	14.62 ± 0.59	11.91 ± 2.07** (37)
0.050	14.90 ± 0.93	14.34 ± 2.44	9.51 ± 1.60** (50)
0.100	9.38 ± 1.65** (51)	10.41 ± 2.61** (45)	5.78 ± 1.19** (69)
1.000	1.15 ± 0.22** (94)	2.30 ± 0.14** (88)	1.59 ± 0.03** (92)

Microsomes were incubated with reaction mixture containing NADPH-generating system, as described in Section 2. DMFA 19.00 ± 2.00 nmol MDA/mg protein. Values are mean ± SEM of at least three duplicate, independent measurements. Percent inhibition of lipid peroxidation is given in parentheses.

** Analysis of variance: $p < 0.01$ (NPPN against control sample).

Table 2
Effect of naphthofuranquinones on lipid peroxidation by liver microsomes incubated with ascorbate or *t*-BuOOH.

NFQ(μM)	Ascorbate–lipoperoxidation	*t*-BuOOH–lipoperoxidation	
	MDA-equivalents (nmol/mg protein)	MDA-equivalents (nmol/mg protein)	
		−NADPH	+NADPH
None	38.9 ± 1.89	4.61 ± 0.31	5.4 ± 0.39
NPPN-3222			
0.01	33.7 ± 3.45	4.8 ± 0.12	5.7 ± 0.34
0.1	32.6 ± 2.60	4.6 ± 0.03	4.5 ± 0.25** (17)
1	33.2 ± 2.36	4.6 ± 0.03	3.4 ± 0.19** (37)
NPPN-3223			
0.01	38.1 ± 1.43	5.1 ± 0.35	5.4 ± 0.32
0.1	35.7 ± 1.32	4.7 ± 0.05	5.0 ± 0.18
1	33.9 ± 2.05	4.6 ± 0.11	3.6 ± 0.28** (33)
NPPN-3226			
0.01	26.3 ± 2.58** (32)	4.6 ± 0.03	5.6 ± 0.22
0.1	26.3 ± 2.95** (32)	4.7 ± 0.07	4.8 ± 0.23
1	24.8 ± 2.87** (36)	4.7 ± 0.04	3.5 ± 0.15** (35)

Microsomes were incubated with reaction mixture containing lipid peroxidation inducers, as described in Section 2. Values are mean ± SEM of at least three duplicate, independent measurements. Percent inhibition of lipid peroxidation is given in parentheses.
** Analysis of variance: $p < 0.01$ (NPPN against control sample).

3.2. Ascorbate–iron and t-BuOOH-dependent lipid peroxidation

In the ascorbate–iron system, the lipid peroxidation was initiated by ascorbate and previously inactivated microsomal enzymes (non-enzymatic lipid peroxidation) [34]. The results in Table 2 show that ascorbate-dependent lipid peroxidation was inhibited only in the presence of NPPN-3226 while no effect was observed in the presence of both NPPN-3222 and NPPN-3223. In the *t*-BuOOH-initiated lipid peroxidation, the homolytic cleavage of the *t*-BuOOH bond by microsomal cytochrome P450 generates the terbutyl radical which undergoes β-scission or initiates lipid peroxidation by abstracting a hydrogen atom from a lipid, as previously proposed by Weiss and Estabrook [26] for cumene hydroperoxide-dependent lipid peroxidation. Table 2 shows that the NFQs inhibited *t*-BuOOH–lipid peroxidation after the addition of NADPH, but on omission of NADPH no inhibition was observed.

3.3. Naphthofuranquinone redox-cycling in the presence of ascorbate

Quinones are reduced by ascorbate to semiquinone [35], and one-electron transfer from the semiquinone to dioxygen yields the superoxide anion radical [36]. In order to compare the capability of the assayed NFQs for redox-cycling, their action on ascorbate oxidation was measured. The three NFQs increase oxygen uptake in a concentration dependent manner (Fig. 2) indicating that they were reduced by ascorbate. Furthermore, NPPN-3222 and NPPN-3223 showed to be more effective as compared with NPPN-3226 ($p < 0.001$ at 25 μM).

3.4. Effects of naphthofuranquinones on superoxide anion production and oxygen uptake by liver microsomes

The antioxidant property of a quinone may support a relation to its activity as a substrate for microsomal "quinone-reductase" or, in other words, to the possibility of quinone redox-cycling, which can be demonstrated by $^{\bullet}O_2^-$ production (Fig. 3). Both NPPN-3222 and NPPN-3223 were more effective $^{\bullet}O_2^-$ generators than NPPN-3226. Oxygen uptake constituted an indicator of free radical production [37]. In close agreement with the NFQs effect on $^{\bullet}O_2^-$ production,

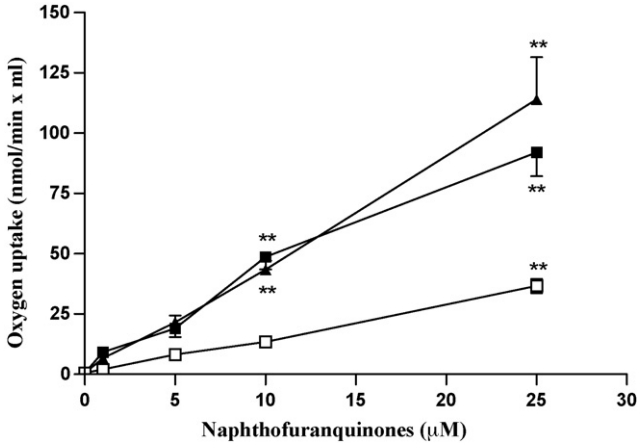

Fig. 2. Effect of naphthofuranquinones on oxygen uptake by reduction with ascorbate. 3222 (■); 3223 (▲); 3226 (□). Experimental conditions are described in Section 2. Values are mean ± SEM of at least five duplicate, independent measurements, and were compared to the DMFA-containing sample.**$p < 0.01$. NPPN-3222 10 μM *vs* 3226 10 μM, $p < 0.01$; 3222 25 μM *vs* 3226 25 μM, $p < 0.001$; 3223 25 μM *vs* 3226 25 μM, $p < 0.001$.

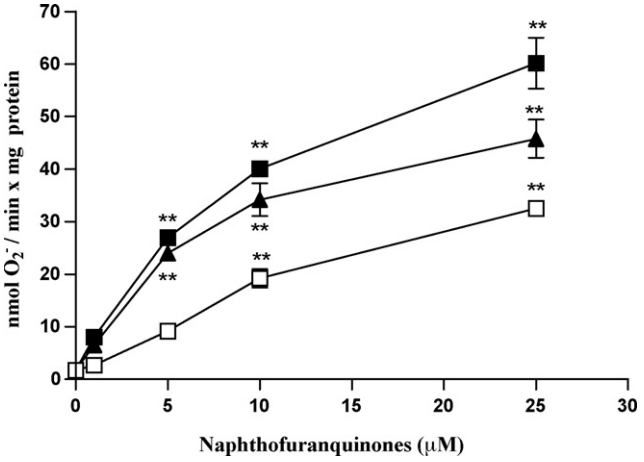

Fig. 3. Effect of naphthofuranquinones on superoxide anion production. 3222 (■); 3223 (▲); 3226 (□). Experimental conditions are described in Section 2. Values are mean ± SEM of at least three duplicate, independent measurements, and were compared to the DMFA-containing sample. **$p < 0.01$.

the three compounds increased oxygen uptake in a concentration dependent manner (Fig. 4). Similar results were obtained by measuring microsomal NADPH-oxidase activity (Table 3).

3.5. Semiquinone radical generation

The NADPH-dependent NFQ reduction through redox-cycling was confirmed by ESR, which demonstrated the seminaphthofuranquinone radical formation (Fig. 5). Omission of the quinone or reductant system prevented the appearance of the semiquinone

Table 3
Effect of naphthofuranquinones on NADPH oxidation.

NFQ (μM)	NPPN-3222	NPPN-3223	NPPN-3226
0.1	1.59 ± 0.52	1.17 ± 0.23	2.33 ± 0.71
1	10.55 ± 0.84**	8.85 ± 2.26**	7.79 ± 1.17**
10	18.73 ± 1.59**	15.32 ± 3.22**	16.20 ± 1.78**

Experimental conditions are described in Section 2. Values are mean ± SEM of at least three duplicate, independent measurements, and were compared to the DMFA-containing sample.
** $p < 0.01$.

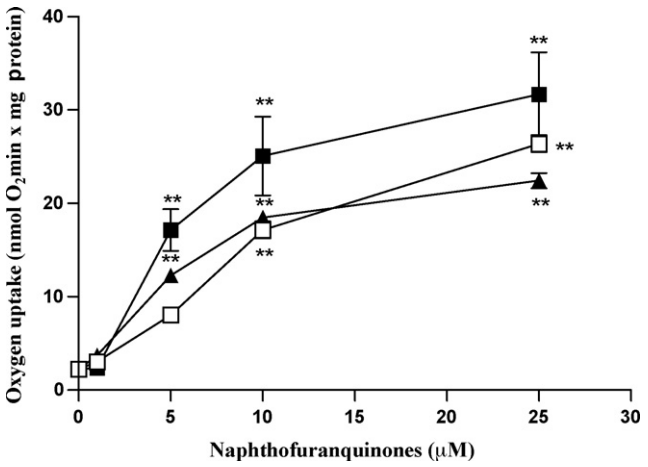

Fig. 4. Effect of naphthofuranquinones on microsomal oxygen uptake. 3222 (■); 3223 (▲); 3226 (□). Experimental conditions are described in Section 2. Values are mean ± SEM of at least three duplicate, independent measurements, and were compared to the DMFA-containing sample. $^{**}p < 0.01$.

signals (data not shown). The spectrum, quintuplet signals, show major splitting attributable to four aromatic protons and is consistent with four equivalent coupling sites at protons C7–C10 at the naphthalene ring. Hyperfine splittings were 0.643 ± 0.014; 1.196 ± 0.062 and 0.905 ± 0.010 G for NPPN-3223, NPPN-3222 and NPPN-3226 seminaphthofuranquinones, respectively. Panel B shows the remnant radical 10 min after the beginning of the reaction, indicating that the three seminaphthofuranquinones were

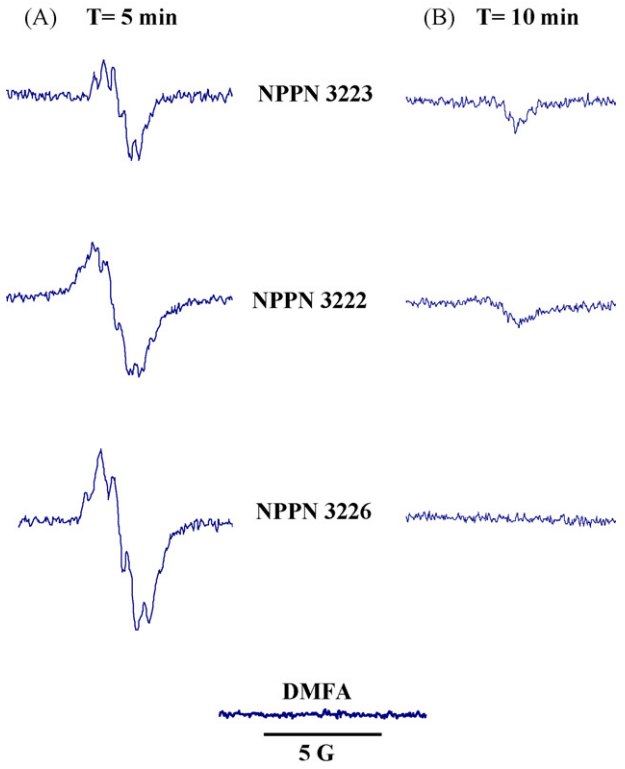

Fig. 5. ESR spectrum of NPPN seminaphthofuranquinones reduction by the liver microsomal NADPH–P450 reductase system. Signals were obtained 5 min (A) and 10 min (B) after initiated the reaction. The reaction mixture contained 5 mM NPPN, 10 mM NADPH, NADPH-generating system, liver microsomes (6.0 mg/ml protein), 50 mM Tris–HCl, pH 7.4, and 10% (v/v) DMFA, under nitrogen. DMFA is a control in the same conditions that A, except for the NPPN. Other experimental conditions were as described under Section 2.

unstable, being the NPPN-3226 semiquinone the shortest lived product. The values obtained after 5 min of initiated the reaction were 1.2, 5.2 and 4.6 nM for NPPN-3223, 3222 and 3226, respectively.

4. Discussion

Quinone inhibition of lipid peroxidation is a well-known phenomenon, although its mechanism has not been properly determined. The results presented in this paper provide information on the mechanism of the inhibition of rat liver microsomal lipid peroxidation by the NFQs. We demonstrated that these compounds: (a) inhibited NADPH-dependent, but not hydroperoxide-dependent microsomal lipid peroxidation; (b) stimulated NADPH oxidation and microsomal oxygen uptake, increased superoxide anion formation and generated seminapthofuranquinone radical. On the other hand, NPPN-3226 inhibited ascorbate-dependent lipid peroxidation. Our results indicate that the quinone redox-cycling, catalyzed by the microsomal NADPH–cytochrome P450 reductase, contributes to the NFQs inhibitory effects. Comparing the three compounds, NPPN-3226 was more effective on lipid peroxidation inhibition (Table 1). Reducing equivalents are consumed by quinone reduction and by microsomal lipid peroxidation. In close agreement with Wills [38], Dubin et al. [24] and Fernández Villamil et al. [39] quinones reduction by NADPH and semiquinone oxidation by dioxygen divert electrons that would otherwise catalyse lipid peroxidation, thus preventing it, in accordance with reactions (1)–(3), where Q, QH• and QH_2 are the quinone, semiquinone and hydroquinone, respectively.

$$NADPH + H^+ + Q \rightarrow NADP^+ + QH_2 \quad (1)$$

$$QH_2 + Q \rightleftarrows 2\,QH^{\bullet} \quad (2)$$

$$QH^{\bullet} + O_2 \rightleftarrows Q + {}^{\bullet}O_2{}^- + H^+ \quad (3)$$

It was also of interest to assess lipid peroxidation in a system where NADPH was omitted. For this experiment, *t*-BuOOH was chosen as the trigger of lipid peroxidation. The results in Table 2 demonstrate no inhibition of propagation by the NFQs unless NADPH was added to the incubation mixture, suggesting inhibition of lipid peroxidation by these compounds in its reduced form.

In addition, our results have shown that NPPN-3226 inhibited ascorbate–lipid peroxidation in a system where microsomal enzymes were inactivated (Table 2). Furthermore, ascorbate ($AscH^-$) reduces quinone to semiquinone followed by the oxidation of semiquinone by molecular oxygen generating superoxide anion (reactions (4) and (5)) [40] and, according to the ascorbate–iron system, NPPN-3226 reduction products inhibit lipid peroxidation (Table 2), as demonstrated by Talcott et al. [41] for menadione, Kostyuk and Lunets [42] for *o*-benzoquinone derivatives, and Dubin et al. [24] for *o*-naphthoquinones and related compounds. In close agreement with Talcott et al. [41] we propose that an antioxidant effect independent of electron diversion may contribute to the inhibitory effect of the NFQs. The most important inhibition of microsomal lipid peroxidation, observed in the presence of NPPN-3226 (Table 1), could be partially ascribed to the antioxidant effect demonstrated in ascorbate–lipid peroxidation (Table 2). Antioxidant effects for the NFQs *per se* were discarded since no effects were observed when these compounds were assayed with the stable free radical DPPH (data not shown) and by *t*-BuOOH–lipoperoxidation when NADPH was not included in the reaction mixture (Table 2).

$$AscH^- + Q \rightarrow {}^{\bullet}Q^- + {}^{\bullet}Asc^- + H^+ \quad (4)$$

$${}^{\bullet}Q^- + O_2 \rightleftarrows Q + {}^{\bullet}O_2^- \quad (5)$$

Hydroquinones are naturally occurring chain-breaking antioxidants, whose reactions with peroxyl radicals yield semiquinone radicals [43]; in fact, reduced NFQ would inhibit free radical reactions, being oxidized to the original NFQ, which is incorporated into a new cycle of redox conversion. We suggest that the hydroquinones and/or semiquinones compounds formed by NFQs reduction would be capable of lipid peroxidation inhibition, interacting with the lipid peroxide radicals, leading to an antioxidant effect (reactions (6) and (7)). Cadenas [44] previously classified hydroquinones in: (I) redox-stable, (II) redox-labile that subsequently autoxidize with the formation of reactive oxygen species, and (III) those that readily rearrange to potent electrophiles undergoing biological alkylating reactions. Under our experimental conditions, we hypothesize the formation of a redox-stable hydroquinone for NPPN-3226. The disappearance of the semiquinone signal after incubation for 10 min (Fig. 5) suggests, in the presence of NPPN-3226, an equilibrium displacement of reaction (2) towards the hydroquinone formation; therefore, leaving less semiquinone available. The latter result is associated to a minor ascorbate oxidation in the presence of NPPN-3226 (Fig. 2). In addition, the semiquinone signals of NPPN-3222 and NPPN-3223 for 10 min (Fig. 5) would be attributed to remnant semiquinone and/or the result of labile hydroquinone formation with subsequent semiquinone production (reaction (2)); all these results, in aerobic conditions, would lead to increase the superoxide anion formation as demonstrated for both NPPN-3222 and NPPN-3223 as compared to NPPN-3226 (reaction (3) and Fig. 3). On the other hand, the rates of autoxidation of the various hydroquinones were strongly dependent upon structure, as proposed by Munday [45]. According to this author, methyl naphthohydroquinones were slowly oxidized at pH 7.4 as compared to halogenated naphthohydroquinones. The results here presented propose the importance of a methyl group on NPPN-3226 structure, suggesting that the inductive properties of this substituent may serve to stabilize forms that are important for antioxidant activity, as described by Talcott et al. [41] for menadione. According to da Silva et al. [22], NPPN-3222 and NPPN-3223 showed the highest activity on bloodstream trypomastigotes of *T. cruzi*, among the assayed NFQs, revealing that the presence of an iodine atom led to an increase in their activity. The latter results are in agreement with the observed increase in superoxide formation in the presence of the iodinated compounds (Fig. 3). According to Cadenas [44], the inherent chemical reactivity of the formed hydroquinone is independent from the type of catalysis and is strictly an expression of its functional chemistry groups. Our results would agree with a possible antioxidant activity for NPPN-3226; however, we cannot exclude antioxidant activities for both NPPN-3222 and NPPN-3223 at higher drug concentrations.

$$QH_2 + LOO^{\bullet} \rightarrow QH^{\bullet} + LOOH \quad (6)$$

$$QH^{\bullet} + LOO^{\bullet} \rightarrow Q + LOOH \quad (7)$$

The data here presented enhance previous observations with *o*-naphthoquinones, such as β-lapachone and several related compounds [24,39]. These results support the view that the NFQs reduction by NADPH–cytochrome P450 reductase and semiquinone oxidation by molecular oxygen divert electrons, preventing microsomal lipid peroxidation. In addition, we propose that reduced forms of NPPN-3226 would exert antioxidant activity. Further research will be necessary to elucidate the influence of the structure of the studied NFQs on lipid peroxidation and other possible mechanisms involved in that process. These studies would contribute to the development of new drugs against Chagas disease for which, up to now, an efficient and safe chemotherapy is not available.

4.1. Conclusions

Our results support the view that the naphthofuranquinones reduction by NADPH–cytochrome P450 reductase and semiquinone oxidation by molecular oxygen divert electrons, preventing microsomal lipid peroxidation. On the other hand, we propose that reduced forms of β-methyl naphthofuranquinone would exert antioxidant activity.

Conflicts of interest statement

There are no conflicts of interest to be disclosed by any of the authors on this manuscript.

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