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The metabolism of 9-chloro-β-lapachone and its effects in isolated hepatocytes. The involvement of NAD(P)H:quinone oxidoreductase 1 (NQO1)

Silvia H. Fernandez Villamil [b], Patricia H. Carrizo [a], Maria E. Di Rosso [a], Maria P. Molina Portela [a], Marta Dubin [a,*]

[a] Centro de Estudios Farmacológicos y Botánicos(CEFYBO), Facultad de Medicina, Universidad de Buenos Aires-CONICET, Paraguay 2155, piso 16, C1121ABG Buenos Aires, Argentina
[b] Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina

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ABSTRACT

A β-lapachone analogue (3,4-dihydro-2,2-dimethyl-9-chloro-2H-naphtho[1,2*b*]pyran-5,6-dione) (9-chloro β-lapachone), named CGQ, with antitumoral, antiviral and antitrypanocidal activities was assayed for cytotoxic effects on isolated rat hepatocytes. The incubation of hepatocytes with this *o*-naphthoquinone showed (a) decreased adenylate energy charge, as a result of a decrease in ATP, and an increase in AMP levels; (b) increased $NADP^+$ content, with a concomitant decrease of NADPH, NADH and NAD^+ content; (c) decreased GSH content, accompanied by an increase in GSSG formation; (d) stimulated oxygen uptake as well as increased superoxide anion production and hydrogen peroxide formation; (e) inhibited lipid peroxidation; (f) hepatocyte viability was not reduced unless the NQO1 inhibitor dicoumarol was present. We hypothesize that the cytotoxicity of CGQ in dicoumarol-treated hepatocytes was the result of inhibition of the NQO1 detoxification pathway, thus allowing more quinone to be metabolized towards the one-electron pathway to form reactive semiquinones and/or reactive oxygen species. The results obtained indicate a protective role of NQO1 in preventing CGQ cytotoxicity in isolated rat hepatocytes.

1. Introduction

Quinones are widely distributed in nature and many clinically important drugs contain the quinone nucleus [1,2]. In cells, naphthoquinones can undergo metabolism either via one-electron reduction to form the semiquinone [3,4], a reaction catalysed by NADPH–cytochrome P-450 reductase, NADH–cytochrome b_5 reductase or NADH–ubiquinone reductase, or via a two-electron reduction to form the hydroquinone [5], a reaction catalysed by the cytosolic flavoprotein DT-diaphorase (NAD(P)H (quinone acceptor) oxido-reductase), NQO1. The consequences of these reductions are physiologically important since the semiquinone redox-cycle generates reactive oxygen species (ROS) [1,6]. In addition to these reactions, some naphthoquinones arylate intracellular thiols or other nucleophils, including thiol proteins, an effect that contributes to their cytotoxicity [7,8].

Although the metabolism of several *p*-naphthoquinones has been extensively studied in isolated cell systems and in sub-cellular fractions [9,10] little is known about the biological fate of *o*-naphthoquinones. These compounds are interesting because of their electronic structure, which makes them more reactive than *p*-naphthoquinones in biological systems [11,12]. Among *o*-naphthoquinones stand β-lapachone and analogous compounds with modifications at position 2 of the pyran ring or at position 8 of the benzene ring (the CGQ quinones) [13,14]. These quinones induce the semiquinone, superoxide anion and hydrogen peroxide production by redox-cycle in the presence of adequate electron donors [13,15,16]. Studies on β-lapachone in cancer chemotherapy revealed topoisomerases I and II in apoptosis as its biochemical targets [17–19] and NQO1 overexpression in pancreatic cancer as an active mechanism for ROS production in the presence of this quinone [20]. In addition, β-lapachone inhibited RNA-dependent DNA polymerase and replication of Rauscher leukemia virus [14] and myeloblastosis virus as well as the expression of HIV gene long repeat [21] and the growth of trypanosomatids, including *Trypanosoma cruzi*, the agent of Chagas' disease [1,22,23].

It was reported that *o*-naphthoquinonoid compounds represent a group of substances with great potential for screening against cancer cell lines and some infectious diseases like trypanosomiasis.

Abbreviations: CGQ, 3,4-dihydro-2,2-dimethyl-9-chloro-2H-naphtho[1,2*b*]-pyran-5,6-dione; SOD, superoxide dismutase; ROS, reactive oxygen species; NQO1, NAD(P)H quinone oxidoreductase 1; DMFA, *N*,*N*-dimethylformamide; TBA, thiobarbituric acid; MDA, malondialdehyde; dicoumarol, 3,3′-methylenebis[4-hydroxycoumarin].

* Corresponding author. Tel.: +54 114508 3680x107; fax: +54 114508 3680.
E-mail address: dubinmarta@yahoo.com.ar (M. Dubin).

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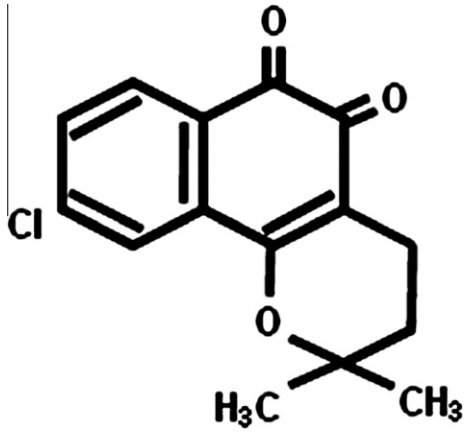

Fig. 1. Chemical structure of CGQ: 3,4-dihydro-2,2-dimethyl-9-chloro-2H-naphtho[1,2-*b*]pyran-5,6-dione.

On these grounds, β-lapachone has been proposed for clinical use in cancer therapy [18]. However, *o*-naphthoquinones cytotoxicity on tumor cells might be associated with adverse effects for the human host. Since chemotherapeutic activity of lipophilic *o*-naphthoquinones would depend on the quinone toxicity, it seemed of interest to investigate the effect of a single lipophilic *o*-naphthoquinone on a suitable biological target such as rat hepatocytes. For this reason, we investigated the action of a β-lapachone analogue (Fig. 1) (3,4-dihydro-2,2-dimethyl-9-chloro-2H-naphtho[1,2*b*]pyran-5,6-dione) (9-chloro β-lapachone) CGQ, which proved in our laboratory to be very active in inducing oxidative damage in trypanosomatids [13,24] and in inhibiting the growth rate of *T. cruzi in vitro* (unpublished results). Accordingly, in the present study we have investigated the action of CGQ on several metabolic parameters in isolated rat hepatocytes: (a) the cellular content of adenine nucleotides and pyridine nucleotides, (b) the cellular content of thiol compounds, especially GSH, (c) lipid peroxidation and (d) cell viability. In this context we analyzed the role of the enzyme NQO1 in normal hepatocytes and the competition between the activating and detoxifying pathways in the presence of the quinone CGQ.

2. Materials and methods

2.1. Animals

Male Wistar rats (220–250 g) were used in the experiments. Animals were fed a Purina-like rat chow. The protein content of the diet was 23% and included all the essential aminoacids.

2.2. Chemicals

CGQ was obtained from Novartis, Basle, Switzerland. Collagenase A from *Clostridium hystolyticum* was purchased from Boehringer Mannheim GmbH, Germany. D-glucose, sucrose, NAD^+, NADH, $NADP^+$, NADPH, ATP, ADP, AMP, potassium phosphoenolpyruvate, pyruvate kinase, adenylate kinase, luciferase–luciferin, DMFA, HEPES, bovine serum albumin (A-4503), 3-aminobenzamide, TCA, EDTA, EGTA, menadione, glucose oxidase, glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, dicoumarol, GSH, *o*-phthaldialdehyde, trizma base, acetylated cytochrome *c* and Trypan blue were purchased from the Sigma Chemical Co., St. Louis MO, USA. Other reagents were obtained from the suppliers indicated and were of the highest purity available.

2.3. Hepatocytes isolation and incubation

Hepatocytes were isolated from the liver of fed rats, which were anesthetized i.p. with 20 μmol sodium pentobarbital (5 mg/100 g body weight). The liver cells were isolated by the method of Seglen [25]. Briefly, after isolation and filtration on Nytrel TI 500 (Ets Desjobert, Paris, France), the suspension obtained from one liver was incubated for 4 min at 37 °C and filtered on nylon (150 μm, Filtration Armentières, Paris, France). The suspension was diluted in medium C containing (mM): 130 NaCl; 5.2 KCl; 0.9 $MgSO_4$; 0.12 $CaCl_2.2H_2O$; 20 Tris–HCl, and 3.0 Na_2HPO_4–KH_2PO_4; pH 7.4, saturated with O_2 and centrifuged at 50 g for 90 s. The cells were resuspended in 50 ml of medium C, allowed to settle for 10 min at 0 °C, and resuspended in the same medium to make a suspension of approximately 5×10^6 hepatocytes/ml. The subsequent steps of the isolation–purification procedure were performed as described before [26]. Cells were counted under the microscope with a haemocytometer. Hepatocyte viability was estimated by the Trypan blue exclusion method. Hepatocytes showing an initial viability greater than 95% were used for the experiments for no more than 4 h after isolation and were kept under 95% O_2 and 5% CO_2. The Trypan blue exclusion assay was used also for determining the effects of CGQ and dicoumarol on hepatocyte viability ($3x10^6$ cells/ml).

Hepatocyte suspensions were diluted with Krebs–Henseleit buffer-5.0 mM glucose solution (pH 7.4) to a concentration of about 3×10^6 cells/ml. Duplicate samples were incubated in Erlenmeyer flasks, with additions as indicated under results. Incubations were performed in a New Brunswick Gyrotory Water Bath Shaker, model G76, at 37 °C, at a rate of 90 strokes/min, under carbogen atmosphere. CGQ, dissolved in DMFA, was added at zero-time. Controls received the same volume of solvent (DMFA), the concentration of which did not exceeded 1% (v/v). For measuring biochemical parameters, aliquots of incubation mixtures were taken as indicated in results.

2.4. Analytical methods

2.4.1. Adenine nucleotide assay

Adenine nucleotide levels in hepatocytes were measured by the Luciferase–Luciferin method [27], using a Packard Pico-Lite Luminometer. Samples (0.2 ml) of the incubation mixture (6×10^5 - hepatocytes) were used in these experiments.

2.4.2. Pyridine nucleotide assay

Pyridine nucleotides levels in hepatocytes were assayed essentially as described by Bernofsky and Swan [28]. Pure nucleotides were added to control samples in order to establish that recovery was greater than 95%.

2.4.3. GSH and GSSG assay

Aliquots of the cell suspension (2×10^6 cells/ml) were removed and diluted 1:2 in 1 M TCA/10 mM EDTA. Proteins were removed by centrifugation and the supernatant fraction diluted 1:100 was assayed for GSH and GSSG by the fluorometric *o*-phthaldialdehyde method of Hissin and Hilf [29]. Results are expressed as GSH equivalents.

HPLC analysis of adduct production from CGQ and GSH was performed using a Spherorb C_{18} 250 × 4 mm reverse phase C_{18} column (Pharmacia LKB, Piscataway, NJ, USA) attached to a Pharmacia LKB system consisting of UV detector VWM 214, HPLC pump 2248 and LKB 2221 Integrator. The mobile phase was 10 mM, KH_2PO_4–K_2HPO_4, pH 6.0, 60% (v/v) methanol degassed with He, and the flow rate was 0.5 ml/min, at 2000–2300 psi. Absorbance was measured at 258 nm. The analyzed reaction mixture consisted of CGQ and GSH (2:1) which was examined during a 1 h period, at 10 min intervals, either under air or under helium.

2.4.4. Superoxide anion assay and oxygen uptake

Superoxide anion formation in hepatocytes was measured employing acetylated cytochrome *c*. Hepatocytes were suspended in Hanks'-Hepes buffer (10^6 cells/ml) containing acetylated cytochrome *c* (3–4 mg/ml). The reaction was started by CGQ addition and the change in absorbance values/min was measured. The rate of cytochrome *c* reduction sensitive to addition of 100 U/ml SOD was considered rate of $O_2^{\cdot-}$ production [13]. 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 hepatocytes (2×10^6 cells/ml) in Krebs–Henseleit buffer mixture supplemented with 12.5 mM Hepes pH 7.4, was measured in a 1.8 ml closed and magnetically stirred glass chamber in the presence or in the absence of 25 μM antimycin.

2.4.5. Catalase and glutathione peroxidase assay

Catalase and glutathione peroxidase were measured spectrophotometrically at 240 nm and 340 nm, respectively, as described in the corresponding references [30,31].

2.4.6. Lipid peroxidation assay

NADPH-dependent lipid peroxidation in hepatocytes (3×10^6 cells/ml) was determined by measuring the *in vitro* formation of TBA-reactants, essentially as described by Dubin et al. [15].

2.4.7. Hydrogen peroxide assay

Hydrogen peroxide production was measured by *p*-hydroxylphenyl acetate peroxidation in the presence of horseradish peroxidase (HRP) [32]. The reaction mixture contained 3×10^6 hepatocytes/ml in Hanks'-HEPES medium, pH 7.4, 100 μM CGQ, 12 U/ml HRP and 2.5 mM *p*-hydroxyphenyl acetic acid. Fluorescence was measured at 317 nm and 414 nm (excitation and emission, respectively), using an SRSLM Aminco spectrofluorometer.

2.5. Statistical analysis

Unless stated otherwise, CGQ effect was calculated by taking as control value that of the DMFA-containing sample, at the same incubation time. GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA) was used to calculate standard error of independent experiments involving duplicate analyses for each sample condition. Statistical analysis was performed using two-way analysis of variance (ANOVA) test and Bonferroni post-tests. Significance was accepted at $p < 0.05$.

3. Results

3.1. Effect of CGQ on hepatocyte adenylate charge

Hepatocytes contain adenine nucleotide pools characterized by (a) a relatively high level of ATP, as compared with ADP and AMP, and (b) relatively constant nucleotide levels in samples incubated for no longer than 1 h in the absence of oxidizable substrates. Table 1 shows the time dependence of the effects of CGQ on ATP, ADP and AMP levels in hepatocytes. Under the given experimental conditions, it can be seen that (a) in control hepatocytes, nucleotide levels did not significantly change with time; (b) in the presence of 100 μM CGQ, ATP level decreased progressively over the time, reaching a reduction by 39% at 60 min of incubation. Similarly, the well-known ROS generating system glucose-glucose oxidase decreased ATP by 14.4%, 20.0%, 27.6% and 44.3%, after 5, 15, 30 and 60 min of incubation, respectively. In contrast to ATP, CGQ did not change ADP level, whereas the AMP level also increased in a time-dependent manner up to twofold of the initial value after 60 min of incubation. Accordingly, the adenylate energy charge (AC, the ratio [ATP + 0.5ADP]/[ATP + ADP + AMP]) of control hepatocytes was not modified during the incubation period, whereas in CGQ-treated cells, this parameter significantly decreased after 30 and 60 min of incubation. Similar results were observed with the glucose–glucose oxidase system.

3.2. Effect of CGQ on pyridine nucleotide levels

Table 2 illustrates the effect on NAD(P)(H) levels in rat hepatocytes after 15 min exposure to 100 μM CGQ. The $NADP^+$ level increased about fourfold and significantly exceeded NADPH depletion (6.15 vs. 2.70 nmol/10^6 cells, respectively). NAD^+ and NADH levels decreased by about 34.5% and 54.5%, respectively. NAD^+ depletion was not prevented by 3-aminobenzamide, a known PARP inhibitor (data not shown). The net increase in $NADP^+$ was very close to the sum of decreases in NADPH, NAD^+ and NADH. In this way, there was little or no change in the total content of pyridine nucleotides.

3.3. Effect of CGQ on hepatocyte thiols

Exposure of hepatocytes to CGQ caused a significant decrease of the GSH level (Fig. 2). It can be seen that after 5-min incubation with the quinone, GSH fell by about 75% as compared to its initial level, and the depletion was followed by a slow recovery to reach a

Table 1
Effect of CGQ and glucose oxidase on the levels of ATP, ADP and AMP in hepatocytes.

Sample		Incubation time (min)			
		5	15	30	60
Control	ATP	16.90 ± 0.76	16.75 ± 0.67	16.90 ± 0.38	16.98 ± 0.28
	ADP	3.60 ± 0.20	3.58 ± 0.21	3.60 ± 0.12	3.57 ± 0.14
	AMP	0.71 ± 0.05	0.83 ± 0.04	0.92 ± 0.05	0.91 ± 0.05
	AC	0.88 ± 0.03	0.86 ± 0.02	0.87 ± 0.03	0.87 ± 0.02
CGQ	ATP	15.88 ± 0.24*	13.91 ± 0.51***	13.32 ± 0.28***	10.47 ± 0.36***
	ADP	3.62 ± 0.12	3.61 ± 0.17	3.59 ± 0.22	3.71 ± 0.16
	AMP	0.75 ± 0.04	1.10 ± 0.12**	1.56 ± 0.08***	2.05 ± 0.11***
	AC	0.87 ± 0.02	0.84 ± 0.02	0.82 ± 0.02*	0.76 ± 0.02***
Glu + GOx	ATP	14.46 ± 0.37**	13.39 ± 0.32***	12.23 ± 0.14***	9.45 ± 0.13***
	ADP	3.46 ± 0.13	3.71 ± 0.10	3.69 ± 0.12	3.77 ± 0.15
	AMP	0.77 ± 0.04	1.25 ± 0.05***	1.80 ± 0.31***	2.22 ± 0.11***
	AC	0.87 ± 0.04	0.83 ± 0.02	0.79 ± 0.03***	0.73 ± 0.03***

Experimental conditions are described in Section 2. Values are expressed in nmol nucleotide/10^6 cells and are the mean ± SEM of 4 independent determinations, each in duplicate. Nucleotide levels at time 0 min: ATP: 17.19 ± 0.98, ADP: 3.55 ± 0.14, AMP: 0.74 ± 0.04. Control: sample containing DMFA, CGQ: 100 μM; Glu + GOx: glucose 5 mM + glucose oxidase 5 U/ml. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as compared to the control sample at the same time. AC: adenylate charge [(ATP) + 0.5(ADP)]/[(ATP) + (ADP) + (AMP)]. AC at time 0 min: 0.88 ± 0.03.

Table 2
Effect of CGQ (100 μM) on pyridine nucleotides balance in rat hepatocytes.

Incubation time (min)	Pyridine nucleotides (nmol/10^6cells)			
	$NADP^+$	NADPH	NAD^+	NADH
0	1.49 ± 0.17	3.85 ± 0.60	5.74 ± 0.45	1.12 ± 0.15
15	7.64 ± 1.44***	1.15 ± 0.15**	3.76 ± 0.38**	0.51 ± 0.06*
Net change	+6.15	−2.70	−1.98	−0.61
Initial total pyridine nucleotides			12.20 nmol/10^6 cells	
Total after 15 min			13.06 nmol/10^6 cells	
Increase in $NADP^+$			6.15 nmol/10^6 cells	
Loss of NADPH + NADH + NAD^+			5.29 nmol/10^6 cells	

Experimental conditions are described in Sections 2 and 3. Values are the mean ± SEM of 4 independent determinations, each in duplicate.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as compared to respective sample at 0 min.

final level of about 41% of the initial one after 60 min incubation. In control hepatocytes the GSH level changed to a lesser extent, the maximal depletion observed being 20% of the initial value. On the other hand, in CGQ treated hepatocytes, intracellular GSSG content showed an increase in a time-dependent manner. HPLC analysis of mixtures of CGQ with GSH (100 μM, 20 mM, respectively) for the presence of GS-quinone adducts yielded negative results as expected from this quinone structure. Absorption spectrum of the solution containing GSH and CGQ (both at 2 mM) in the 400–500 nm range did not change during a 60 min incubation.

3.4. Effect of CGQ on oxygen uptake in hepatocytes

Fig. 3 shows the concentration-dependent effect of CGQ on antimycin-sensitive and insensitive hepatocyte respiration. Antimycin A is an inhibitor of complex III in the mitochondrial electron transport chain. Both activities were increased by CGQ, but the effect was greater in antimycin A-inhibited hepatocytes as expressed by the ratio of control and quinone-treated hepatocytes. These results suggest a CGQ redox-cycle operating in this system. Moreover, the addition of 25 μM dicoumarol, a well-known inhibitor of NQO1[33], significantly stimulated antimycin A-independent oxygen consumption by 100 μM CGQ-treated hepatocytes (69.2 ± 4.5 to 99.1 ± 3.2 nanoatoms O/min/10^6 cells).

We also studied $O_2^{\cdot-}$ production in cells incubated with 100 μM CGQ, and observed an increase in superoxide anion generation (0.17 ± 0.05 to 2.69 ± 0.60 nmoles $O_2^{\cdot-}$/min/10^6 cells), measured as acetylated cytochrome *c* reduction.

3.5. Hydrogen peroxide production as affected by CGQ in isolated hepatocytes

Since spontaneous dismutation of $O_2^{\cdot-}$ yields small amounts of hydrogen peroxide, we studied H_2O_2 production in hepatocytes incubated with CGQ. Fig. 4 shows that hepatocytes incubation with 100 μM CGQ caused a time-dependent increase in H_2O_2 production. Hydrogen peroxide production was confirmed by adding sodium azide, an inhibitor of catalase, to the hepatocyte suspension. It should be noted that azide is also a known inhibitor of cytochrome c oxidase [34], thus H_2O_2 produced when azide was added to the reaction mixture could not only be due to catalase inhibition but also due to inhibition of mitochondrial respiration, or both. Results obtained for DMFA control were subtracted from the respective sample. In close agreement with its function, azide increased hydrogen peroxide production in hepatocytes, reaching after

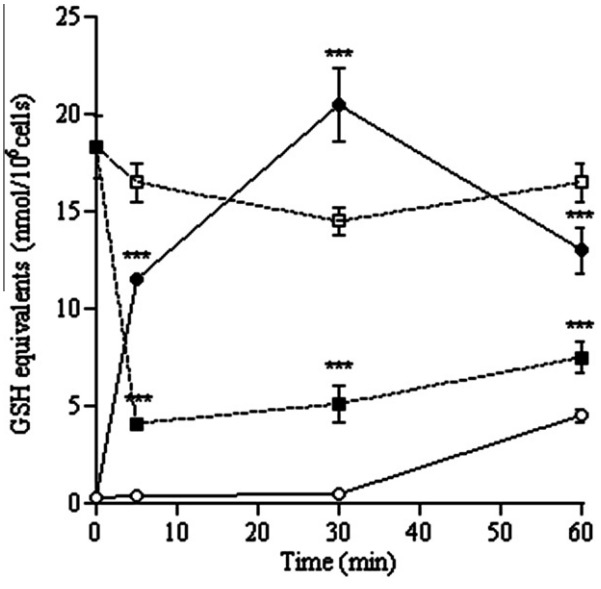

Fig. 2. Time course of GSH and GSSG levels (GSH equivalents) in isolated hepatocytes exposed to CGQ. Hepatocytes (2x10^6 cells/ml) were incubated in the absence (GSH: –□–; GSSG: —○—) or in the presence (GSH:–■–; GSSG: —●—) of CGQ 100 μM. Aliquots were taken at the indicated times. GSH and GSSG were determined as described in Section 2. Results are the means ± SEM of 5 independent experiments. Significantly different from respective control; ***$p < 0.001$.

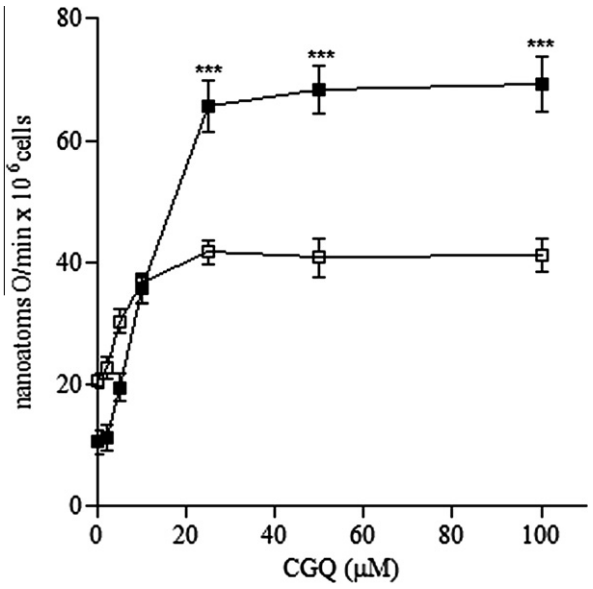

Fig. 3. Effect of CGQ on the respiration of hepatocytes. Hepatocyte respiration was measured polarographically as described in Section 2 either in the presence (—■—) or in the absence (—□—) of antimycin 25 μM. Values are the mean ± SEM of 4 independent experiments. Significantly different from control; ***$p < 0.001$.

5 min of incubation a highly significant level, compared to the respective sample incubated in the absence of sodium azide.

3.6. Lipid peroxidation

Lipid peroxidation processes play an important role in liver damage produced by many toxic agents. Accordingly, the effect of CGQ on MDA production by hepatocytes was investigated. The results presented in Fig. 5 indicate that the NADPH-initiated lipid peroxidation was significantly inhibited in a CGQ concentration-dependent manner (25 to 100 μM).

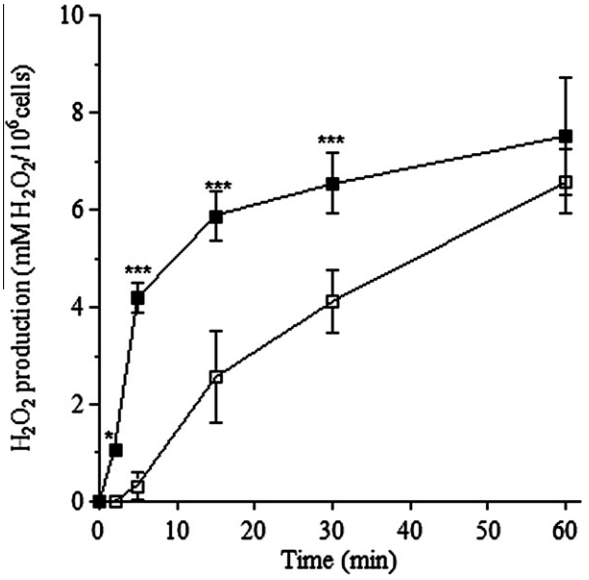

Fig. 4. Time course of H_2O_2 level in isolated hepatocytes suspension exposed to CGQ. Hepatocytes ($3x10^6$ cells/ml) were incubated with 100 μM CGQ in the absence (—□—) or in the presence (—■—) of 1 mM sodium azide. Aliquots were taken at the indicated times. Extracellular H_2O_2 was measured as described in Section 2. Sample values, from which the DMFA control values have been subtracted, are given as the mean ± SEM of 4 independent experiments. Significantly different from each sample incubated in the absence of sodium azide; $^{*}p < 0.05$ and $^{***}p < 0.001$.

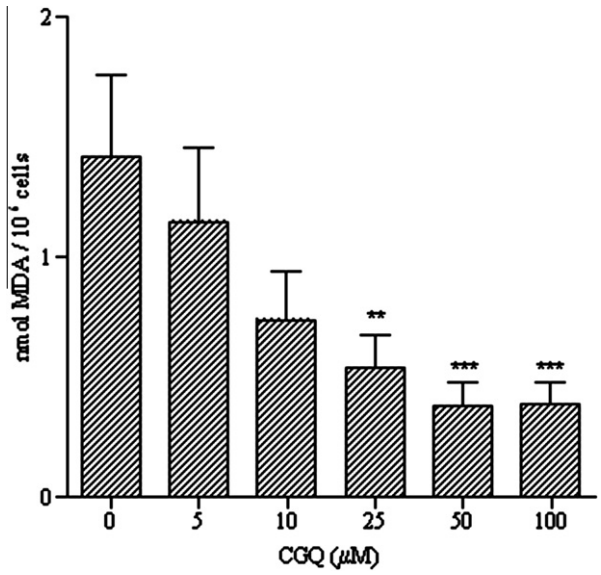

Fig. 5. Effect of CGQ on MDA production in hepatocytes. Experimental conditions were as indicated under Section 2.Values are the means ± SEM of 5 independent experiments. Significantly different from control; $^{**}p < 0.01$ and $^{***}p < 0.001$.

3.7. Viability

The effect of CGQ on cell viability is shown in Table 3. Under our experimental conditions neither control conditions nor dicoumarol addition, induced significant changes in cell viability, as determined by the Trypan blue exclusion assay. CGQ addition induced no significant cell death up to 4 h. However, addition of dicoumarol (20 μM) to CGQ-treated hepatocytes caused a marked decrease of cell viability in a concentration and time-dependent manner, with 60% and 64% loss of viability, compared to control, incubated for 4 h with 50 and 100 μM CGQ, respectively.

4. Discussion

It is well-known that *o*-naphthoquinones produce oxidative stress [6,13,17,22,35–38] and that there are activating and detoxifying pathways regulating the effects caused by quinonoid drugs. However, the metabolic pathway of the *o*-naphthoquinone CGQ in hepatocytes remains unclear. Changes in the levels of pyridine nucleotides, ATP, GSH and viability are closely related phenomena in hepatocytes [39]. One of the most important parameters in the intracellular homeostasis is the adenylate energy charge, as calculated by the Atkinson relationship [40]. The observed changes in ATP and AMP levels over 1 h in the presence of CGQ 100 μM (Table 1) were not sufficient to cause cell death. A reduction of the energy charge to a value below approximately 0.25 was reported to be inevitably followed by the induction of cell death [39].

The CGQ-induced changes in the levels of NADPH, $NADP^+$, NADH and NAD^+ did not lead to cell death up to four hours. Similar results were observed by other authors [41] in cultured rat hepatocytes treated with tert-butyl hydroperoxide or menadione [42]. The increase in $NADP^+$ exceeded the loss of NADPH and equaled the sum of the losses of other pyridine nucleotides namely NADPH, NADH and NAD^+. The conversion of NADH to NAD^+ and then NAD^+ to $NADP^+$ probably accounts for the increase in $NADP^+$ over the one resulting from the oxidation of NADPH. It was reported that NAD^+ kinase is inhibited by NADPH [42]. In this way, as under our experimental conditions NADPH was depleted, activation of NAD^+ kinase remains as a good possibility. The reduction of $NADP^+$ to NADPH was not detected since NADPH might have been used up by other NADPH-dependent flavoenzymes in an important attempt to counteract the effects of quinone-induced oxidative stress [43]. The depletion of NAD^+ was not prevented by 3-aminobenzamide, suggesting that the NAD^+ hydrolysis was not mediated by PARP activation [44] and supporting the postulated pyridine nucleotide interconvertion.

Naphthoquinone-dependent depletion of hepatocellular glutathione may be due to several mechanisms including (a) direct conjugation [45], or (b) redox cycle-generated ROS such as superoxide anion and hydrogen peroxide, which can be detoxified by glutathione peroxidase, with concomitant formation of oxidized glutathione. Our investigation of GS-quinone adducts by HPLC analysis and absorption spectrum of CGQ–GSH mixtures in the 400–500 nm range yielded negative results (data not shown), as expected from this quinone structure, in which the two electrophilic positions on the quinonoid nucleus are blocked by C=O and pyran [46]. Similar results were found by other authors [45] using 2,3-dimethyl-1,4-naphthoquinone. Under our experimental conditions, the lost GSH was almost completely recovered as GSSG, suggesting that almost all the depleted glutathione is oxidized and not arylated or conjugated [47].

Table 3
Effect of dicoumarol on cell viability of hepatocytes exposed to CGQ.

Sample	Incubation time (h)			
	1	2	3	4
Control	87.74 ± 2.26	87.67 ± 1.45	87.41 ± 2.97	86.45 ± 4.06
Control + Dic	85.87 ± 2.07	84.47 ± 1.44	87.00 ± 2.00	87.67 ± 2.33
CGQ 5 μM	87.98 ± 1.97	85.67 ± 2.33	86.56 ± 2.65	86.68 ± 4.09
CGQ 5 μM + Dic	84.00 ± 0.58	81.00 ± 0.58*(4)	78.00 ± 0.58***(10)	66.33 ± 1.20***(24)
CGQ 10 μM	88.26 ± 1.28	87.33 ± 1.45	88.65 ± 0.97	84.37 ± 0.95
CGQ 10 μM + Dic	81.00 ± 0.58*(6)	77.00 ± 0.58***(9)	66.33 ± 1.20***(24)	61.00 ± 0.58***(30)
CGQ 25 μM	89.72 ± 3.45	88.33 ± 3.28	87.19 ± 2.91	86.38 ± 1.87
CGQ 25 μM + Dic	76.73 ± 0.89***(11)	67.63 ± 0.34***(20)	54.36 ± 1.21***(37)	43.97 ± 0.99***(50)
CGQ 50 μM	89.26 ± 1.41	87.33 ± 1.45	86.43 ± 1.46	82.29 ± 3.24
CGQ 50 μM + Dic	72.40 ± 0.55***(16)	54.43 ± 1.64***(36)	47.80 ± 1.25***(45)	35.13 ± 1.05***(60)
CGQ 100 μM	89.83 ± 1.74	81.43 ± 1.79	81.33 ± 0.88	80.23 ± 0.54
CGQ 100 μM + Dic	71.03 ± 0.64***(17)	49.43 ± 0.43***(41)	39.47 ± 0.32***(55)	31.90 ± 1.35***(64)

Experimental conditions are described in Sections 2 and 3. Dic: dicoumarol (20 μM). Values are expressed as percentage of viable cells and represent the mean ± SEM of 5 independent experiments, each in duplicate. Control viability at time 0 min: 93.98 ± 0.95. Significantly different from the respective control sample; $^{*}p < 0.05$ and $^{***}p < 0.001$. Percent inhibition of viability is given in parentheses.

Although some quinones can be reduced directly by intracellular reductants, such as ascorbate and GSH, quinones can undergo either (a) enzymatic one-electron reduction catalyzed by microsomal NADPH–cytochrome P-450 reductase to the semiquinone radical which, in the presence of molecular oxygen, can transfer an electron and form the superoxide anion radical $O_2^{\cdot-}$ [5,9,13,24,48–51]; or (b) two-electron reduction to the hydroquinone, catalyzed by cytosolic NQO1, (Fig. 6) [5,7]. Unlike most other cellular reductases, NQO1 reduces quinones bypassing the unstable and highly reactive semiquinone intermediate. In the present study, we have shown that CGQ increased extracellular oxygen uptake, superoxide anion generation and hydrogen peroxide formation in isolated rat hepatocytes, confirming the participation of a redox-cycle in this *o*-naphthoquinone metabolism. The mechanism involving GSH, one-electron reduction of the *o*-naphthoquinone or the semiquinone, could not be dismissed. One-electron reduction of CGQ yields the semiquinone that may react with GSH, or with other thiols, yielding the corresponding thiyl radicals. The radical character is transferred according to reactions 1–4 and, finally, via disproportionation to a non radical product [7].

$$Q^{\cdot-} + GS^{-}(H^{+}) \rightarrow QH_2 + GS^{\cdot} \quad (1)$$

$$GS^{\cdot} + GS^{-} \rightarrow GSSG^{\cdot-} \quad (2)$$

$$GSSG^{\cdot-} + O_2 \rightarrow GSSG + O_2^{\cdot-} \quad (3)$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^{+} \xrightarrow{SOD} H_2O_2 \quad (4)$$

These reactions fit in well with GSH depletion and GSSG production observed after incubation of hepatocytes with CGQ. Moreover, NADPH depletion and $NADP^{+}$ production under the same experimental conditions are easily understood considering the pyridine nucleotide specificity of both NQO1 and NADPH–cytochrome P-450 reductase. As result of the different reactions previously described for CGQ in hepatocytes, $O_2^{\cdot-}$ and H_2O_2 are produced and, therefore, cell damage by oxidative stress could be possible. Nevertheless, hepatocytes contain powerful antioxidant systems such as the glutathione peroxidase system and catalase, which remained unaffected under our experimental conditions (data not shown), and thus able to limit the quinone-generated reactive species so they do not reach the level to induce cell death. It is worth mentioning that after a 4 h-incubation with CGQ no significant decrease of cell viability was observed, which, however, does not exclude that cell death by apoptosis could be starting [52]. Since CGQ inhibited lipid peroxidation, no significant role could be attributed to lipid peroxidation products as intermediates of CGQ action. Similar results were reported in rat liver microsomes incubated with CGQ [15].

The obligatory two-electron reducing cytosolic NQO1 can reduce a wide spectrum $O_2^{\cdot-}$ quinones and thereby compete with the one-electron reductases for the quinone substrate. However, this will depend upon the relative affinity of quinones for the different flavoenzymes. Evidence for this phenomenon has been well demonstrated in menadione-treated hepatocytes [51]. On the other hand, NQO1 may either prevent or promote the redox cycling of quinones [7]. Which process is applied will depend upon the ratio between the level of NQO1and the concentration of quinone within the tissue, upon the rate of reduction of the particular quinone by the enzyme, and upon the stability of the hydroquinone that is produced. If the hydroquinone is stable, it will remain available for conjugation. However, if it is easily autoxidized, the efficiency of conjugation will be compromised, and quinone reduction may constitute an activation process rather than detoxification with formation of semiquinone and ROS [53]. It was established that NQO1 activity is an important determinant of β-lapachone cytotoxicity in breast cancer cells, NQO1-overexpressing cells, suggesting that the hydroquinone form of β-lapachone is unstable and rapidly undergoes autoxidation to the parent quinone, which can again serve as substrate for reduction by NQO1 [49]. Furthermore, previous observations with β-lapachone and related *o*-naphthoquinones in cytosol preparations indicate that the corresponding hydroquinones may act as redox-labile [13]. Under our experimental conditions, in spite of the fact that hydroquinones can be unstable substances, a redox-stable CGQ-hydroquinone could be formed and detoxification could be promoted by factors that might stabilize the naphthohydroquinone, thus preventing its autoxidation [7,54,55].

We observed that oxygen consumption and cell death significantly increased in the combined presence of CGQ and dicoumarol. In this context, we conclude that the cytotoxicity by CGQ, in dicoumarol-treated hepatocytes, is the result of the inhibition of the NQO1 detoxification pathway, thus allowing more quinone to be metabolized via the one-electron toxic pathway to form reactive semiquinones and/or reactive oxygen species (Fig. 6). The results here obtained show a protective role of NQO1 in preventing CGQ cytotoxicity in isolated hepatocytes and demonstrate the importance of competition between activating and detoxifying pathways to regulate the oxidative stress caused by this *o*-naphthoquinone.

It is possible that *in vitro* experiments on NQO1-induced redox cycling could provide a useful screen for identification of potential anti-tumor quinones [56,57]. It is interesting to note that promo-

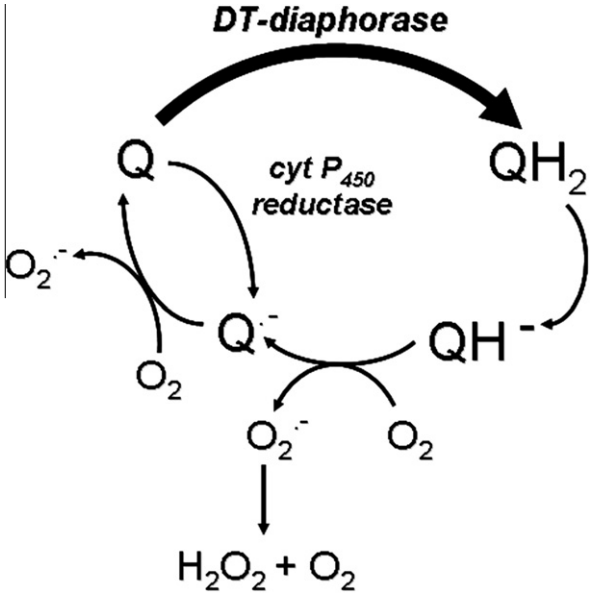

Fig. 6. A schematic representation of CGQ metabolism in isolated hepatocytes. Q: CGQ; $Q^{\cdot-}$: semiquinone; QH_2: hydroquinone; QH^-: ionized hydroquinone.

tion of redox cycling by NQO1 is a common feature of certain anticancer drugs such as β-lapachone and streptonigrin [35]. On the basis of structural similarities, this behavior is also to be expected from CGQ.

Conflicts of interest statement

None declared.

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References

[1] S.L. de Castro, D.G. Batista, M.M. Batista, W. Batista, A. Daliry, E.M. de Souza, R.F. Menna-Barreto, G.M. Oliveira, K. Salomao, C.F. Silva, P.B. Silva, N. Soeiro, M. de, Experimental chemotherapy for chagas disease: a morphological, biochemical, and proteomic overview of potential *Trypanosoma cruzi* targets of amidines derivatives and naphthoquinones, Mol. Biol. Int. 2011 (2011) 306928.
[2] M. Dubin, S.H. Fernandez Villamil, A.O. Stoppani, Cytotoxicity of beta-lapachone, an naphthoquinone with possible therapeutic use, Medicina (Buenos Aires) 61 (3) (2001) 343–350.
[3] T. Iyanagi, I. Yamazaki, One-electron-transfer reactions in biochemical systems V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase), Biochim. Biophys. Acta 216 (2) (1970) 282–294.
[4] G. Powis, P.L. Appel, Relationship of the single-electron reduction potential of quinones to their reduction by flavoproteins, Biochem. Pharmacol. 29 (19) (1980) 2567–2572.
[5] C. Lind, P. Hochstein, L. Ernster, DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation, Arch. Biochem. Biophys. 216 (1) (1982) 178–185.
[6] Y. Kumagai, Y. Shinkai, T. Miura, A.K. Cho, The chemical biology of naphthoquinones and its environmental implications, Annu. Rev. Pharmacol. Toxicol. 52 (2012) 221–247.
[7] E. Cadenas, Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism, Biochem. Pharmacol. 49 (2) (1995) 127–140.
[8] L. Rossi, G.A. Moore, S. Orrenius, P.J. O'Brien, Quinone toxicity in hepatocytes without oxidative stress, Arch. Biochem. Biophys. 251 (1) (1986) 25–35.
[9] H. Kappus, H. Sies, Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation, Experientia 37 (12) (1981) 1233–1241.
[10] P.J. O'Brien, Molecular mechanisms of quinone cytotoxicity, Chem. Biol. Interact. 80 (1) (1991) 1–41.
[11] A. Boveris, R. Docampo, J.F. Turrens, A.O. Stoppani, Effect of beta-lapachone on superoxide anion and hydrogen peroxide production in *Trypanosoma cruzi*, Biochem. J. 175 (2) (1978) 431–439.
[12] A. Boveris, A.O. Stoppani, R. Docampo, F.S. Cruz, Superoxide anion production and trypanocidal action of naphthoquinones on *Trypanosoma cruzi*, Comp. Biochem. Physiol. C 61 (C2) (1978) 327–329.
[13] S. Fernandez Villamil, A.O. Stoppani, M. Dubin, Redox cycling of beta-lapachone and structural analogues in microsomal and cytosol liver preparations, Methods Enzymol. 378 (2004) 67–87.
[14] K. Schaffner-Sabba, K.H. Schmidt-Ruppin, W. Wehrli, A.R. Schuerch, J.W. Wasley, Beta-Lapachone: synthesis of derivatives and activities in tumor models, J. Med. Chem. 27 (8) (1984) 990–994.
[15] M. Dubin, S.H. Fernandez Villamil, A.O. Stoppani, Inhibition of microsomal lipid peroxidation and cytochrome P-450-catalyzed reactions by beta-lapachone and related naphthoquinones, Biochem. Pharmacol. 39 (7) (1990) 1151–1160.
[16] N.V. de Witte, A.O. Stoppani, M. Dubin, 2-Phenyl-beta-lapachone can affect mitochondrial function by redox cycling mediated oxidation, Arch. Biochem. Biophys. 432 (2) (2004) 129–135.
[17] A.G. Ravelo, A. Estevez-Braun, H. Chavez-Orellana, E. Perez-Sacau, D. Mesa-Siverio, Recent studies on natural products as anticancer agents, Curr. Top. Med. Chem. 4 (2) (2004) 241–265.
[18] A.B. Pardee, Y.Z. Li, C.J. Li, Cancer therapy with beta-lapachone, Curr. Cancer Drug Targets 2 (3) (2002) 227–242.
[19] B. Frydman, L.J. Marton, J.S. Sun, K. Neder, D.T. Witiak, A.A. Liu, H.M. Wang, Y. Mao, H.Y. Wu, M.M. Sanders, L.F. Liu, Induction of DNA topoisomerase II-mediated DNA cleavage by beta-lapachone and related naphthoquinones, Cancer Res. 57 (4) (1997) 620–627.
[20] L.S. Li, E.A. Bey, Y. Dong, J. Meng, B. Patra, J. Yan, X.J. Xie, R.A. Brekken, C.C. Barnett, W.G. Bornmann, J. Gao, D.A. Boothman, Modulating endogenous NQO1 levels identifies key regulatory mechanisms of action of beta-lapachone for pancreatic cancer therapy, Clin. Cancer Res. 17 (2) (2011) 275–285.
[21] C.J. Li, L.J. Zhang, B.J. Dezube, C.S. Crumpacker, A.B. Pardee, Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication, Proc. Natl. Acad. Sci. USA 90 (5) (1993) 1839–1842.
[22] A.V. Pinto, S.L. de Castro, The trypanocidal activity of naphthoquinones: a review, Molecules 14 (11) (2009) 4570–4590.
[23] S.C. Bourguignon, D.F. Cavalcanti, A.M. de Souza, H.C. Castro, C.R. Rodrigues, M.G. Albuquerque, D.O. Santos, G.G. da Silva, F.C. da Silva, V.F. Ferreira, R.T. de Pinho, C.R. Alves, Trypanosoma cruzi: insights into naphthoquinone effects on growth and proteinase activity, Exp. Parasitol 127 (1) (2011) 160–166.
[24] M.P. Molina Portela, S.H. Fernandez Villamil, L.J. Perissinotti, A.O. Stoppani, Redox cycling of o-naphthoquinones in trypanosomatids.Superoxide and hydrogen peroxide production, Biochem. Pharmacol. 52 (12) (1996) 1875–1882.
[25] P.O. Seglen, Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion, Exp. Cell Res. 82 (2) (1973) 391–398.
[26] M. Dubin, P.H. Carrizo, A.M. Biscardi, S.H. Fernandez Villamil, A.O. Stoppani, Effect of 5-nitroindole on adenylate energy charge, oxidative phosphorylation, and lipid peroxidation in rat hepatocytes, Biochem. Pharmacol. 48 (7) (1994) 1483–1492.
[27] A. Lundin, M. Hasenson, J. Persson, A. Pousette, Estimation of biomass in growing cell lines by adenosine triphosphate assay, Methods Enzymol. 133 (1986) 27–42.
[28] C. Bernofsky, M. Swan, An improved cycling assay for nicotinamide adenine dinucleotide, Anal. Biochem. 53 (2) (1973) 452–458.
[29] P.J. Hissin, R. Hilf, A fluorometric method for determination of oxidized and reduced glutathione in tissues, Anal. Biochem. 74 (1) (1976) 214–226.
[30] D.E. Paglia, W.N. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J. Lab. Clin. Med. 70 (1) (1967) 158–169.
[31] A. Claiborne, Catalase activity, in: R.A. Greenwald (Ed.), Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, Florida, 1985, pp. 283–284.
[32] P.A. Hyslop, L.A. Sklar, A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes, Anal. Biochem. 141 (1) (1984) 280–286.
[33] R.M. Phillips, Inhibition of DT-diaphorase (NAD(P)H:quinone oxidoreductase, EC 1.6.99.2) by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and flavone-8-acetic acid (FAA): implications for bioreductive drug development, Biochem. Pharmacol. 58 (2) (1999) 303–310.
[34] D.F. Wilson, B. Chance, Reversal of azide inhibition by uncouplers, Biochem. Biophys. Res. Commun. 23 (5) (1966) 751–756.
[35] R. Munday, Activation and detoxification of naphthoquinones by NAD(P)H:quinone oxidoreductase, Methods Enzymol. 382 (2004) 364–380.
[36] M. Ough, A. Lewis, E.A. Bey, J. Gao, J.M. Ritchie, W. Bornmann, D.A. Boothman, L.W. Oberley, J.J. Cullen, Efficacy of beta-lapachone in pancreatic cancer treatment: exploiting the novel, therapeutic target NQO1, Cancer Biol. Ther. 4 (1) (2005) 95–102.

[37] E.A. Bey, M.S. Bentle, K.E. Reinicke, Y. Dong, C.R. Yang, L. Girard, J.D. Minna, W.G. Bornmann, J. Gao, D.A. Boothman, An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by beta-lapachone, Proc. Natl. Acad. Sci. USA 104 (28) (2007) 11832–11837.
[38] E.A. Hillard, F.C. de Abreu, D.C. Ferreira, G. Jaouen, M.O. Goulart, C. Amatore, Electrochemical parameters and techniques in drug development with an emphasis on quinones and related compounds, Chem. Commun. (Camb.) (23) (2008) 2612–2628.
[39] F.A. Redegeld, R.M. Moison, A.S. Koster, J. Noordhoek, Depletion of ATP but not of GSH affects viability of rat hepatocytes, Eur. J. Pharmacol. 228 (4) (1992) 229–236.
[40] D.E. Atkinson, The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers, Biochemistry 7 (11) (1968) 4030–4034.
[41] K. Yamamoto, J.L. Farber, Metabolism of pyridine nucleotides in cultured rat hepatocytes intoxicated with tert-butyl hydroperoxide, Biochem. Pharmacol. 43 (5) (1992) 1119–1126.
[42] C.R. Stubberfield, G.M. Cohen, Interconversion of NAD(H) to NADP(H). A cellular response to quinone-induced oxidative stress in isolated hepatocytes, Biochem. Pharmacol. 38 (16) (1989) 2631–2637.
[43] W.A. Morgan, Pyridine nucleotide hydrolysis and interconversion in rat hepatocytes during oxidative stress, Biochem. Pharmacol. 49 (9) (1995) 1179–1184.
[44] C.R. Stubberfield, G.M. Cohen, NAD+ depletion and cytotoxicity in isolated hepatocytes, Biochem. Pharmacol. 37 (20) (1988) 3967–3974.
[45] M.G. Miller, A. Rodgers, G.M. Cohen, Mechanisms of toxicity of naphthoquinones to isolated hepatocytes, Biochem. Pharmacol. 35 (7) (1986) 1177–1184.
[46] T.W. Gant, D.N. Rao, R.P. Mason, G.M. Cohen, Redox cycling and sulphydryl arylation; their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes, Chem. Biol. Interact. 65 (2) (1988) 157–173.
[47] C. Toxopeus, I. van Holsteijn, J.W. Thuring, B.J. Blaauboer, J. Noordhoek, Cytotoxicity of menadione and related quinones in freshly isolated rat hepatocytes: effects on thiol homeostasis and energy charge, Arch. Toxicol. 67 (10) (1993) 674–679.
[48] M. Nakamura, T. Hayashi, One- and two-electron reduction of quinones by rat liver subcellular fractions, J. Biochem. 115 (6) (1994) 1141–1147.
[49] J.J. Pink, S.M. Planchon, C. Tagliarino, M.E. Varnes, D. Siegel, D.A. Boothman, NAD(P)H:quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity, J. Biol. Chem. 275 (8) (2000) 5416–5424.
[50] G. Powis, B.A. Svingen, P. Appel, Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and other cells, Mol. Pharmacol. 20 (2) (1981) 387–394.
[51] H. Thor, M.T. Smith, P. Hartzell, G. Bellomo, S.A. Jewell, S. Orrenius, The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes.A study of the implications of oxidative stress in intact cells, J. Biol. Chem. 257 (20) (1982) 12419–12425.
[52] L.M. Lopez, M. Dubin, P.H. Carrizo, M.H. Burgos, A. Pellegrino de Iraldi, A.O. Stoppani, Apoptogenic effect of the lipophilic o-naphthoquinone CG 10–248 on rat hepatocytes: light and electron microscopy studies, Biocell 27 (2) (2003) 213–224.
[53] R. Munday, Inhibition of naphthohydroquinone autoxidation by DT-diaphorase (NAD(P)H-[quinone acceptor] oxidoreductase), Redox Report 3 (3) (1997) 189–196.
[54] R. Munday, Autoxidation of naphthohydroquinones: effects of metals, chelating agents, and superoxide dismutase, Free Radic. Biol. Med. 22 (4) (1997) 689–695.
[55] A. Brunmark, E. Cadenas, Redox and addition chemistry of quinoid compounds and its biological implications, Free Radic. Biol. Med. 7 (4) (1989) 435–477.
[56] D. Ross, J.K. Kepa, S.L. Winski, H.D. Beall, A. Anwar, D. Siegel, NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms, Chem. Biol. Interact. 129 (1–2) (2000) 77–97.
[57] R.J. Riley, P. Workman, DT-diaphorase and cancer chemotherapy, Biochem. Pharmacol. 43 (8) (1992) 1657–1669.