

## An examination of quinone toxicity using the yeast *Saccharomyces cerevisiae* model system

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### Abstract

The toxicity of quinones is generally thought to occur by two mechanisms: the formation of covalent bonds with biological molecules by Michael addition chemistry and the catalytic reduction of oxygen to superoxide and other reactive oxygen species (ROS) (redox cycling). In an effort to distinguish between these general mechanisms of toxicity, we have examined the toxicity of five quinones to yeast cells as measured by their ability to reduce growth rate. Yeast cells can grow in the presence and absence of oxygen and this feature was used to evaluate the role of redox cycling in the toxicity of each quinone. Furthermore, yeast mutants deficient in superoxide dismutase (SOD) activity were used to assess the role of this antioxidant enzyme in protecting cells against quinone-induced reactive oxygen toxicity. The effects of different quinones under different conditions of exposure were compared using IC<sub>50</sub> values (the concentration of quinone required to inhibit growth rate by 50%). For the most part, the results are consistent with the chemical properties of each quinone with the exception of 9,10-phenanthrenequinone (9,10-PQ). This quinone, which is not an electrophile, exhibited an unexpected toxicity under anaerobic conditions. Further examination revealed a potent induction of cell viability loss which poorly correlated with decreases in the GSH/2GSSG ratio but highly correlated ( $r^2 > 0.7$ ) with inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), suggesting disruption of glycolysis by this quinone. Together, these observations suggest an unexpected oxygen-independent mechanism in the toxicity of 9,10-phenanthrenequinone. © 2004 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** Yeast; Quinone toxicity; Redox cycling; Covalent bond; 9,10-Phenanthrenequinone

### 1. Introduction

Quinones are a class of compounds of substantial toxicological and pharmacological interest. For exam-

ple, benzene-derived quinones have been implicated in bone marrow toxicity, quinones generated from the oxidation of polycyclic aromatic hydrocarbons can form adducts with DNA and RNA, and a variety of quinone compounds have been developed as anti-cancer drugs (Brunmark and Cadenas, 1989; O'Brien, 1991; Monks and Lau, 1992; Bolton et al., 2000). Quinones may also contribute to environmental toxicity because of their

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presence in air pollution (Schuetzle et al., 1981; Allen et al., 1997). Numerous studies have shown that diesel exhaust particles (DEPs), which represent a significant contributor to urban airborne particulate matter, exhibit toxicity consistent with quinone-based chemistry (Sagai et al., 1993; Bai et al., 2001).

The chemical basis for the toxicity and pharmacology of quinones has been examined extensively and two primary mechanisms have been proposed to account for their actions. Quinones can act as electrophiles via 1,4-addition chemistry leading to covalent modification of biological molecules at their nucleophilic sites. Alternatively, quinones can “redox” cycle in the presence of a biological reductant and dioxygen ( $O_2$ ) to generate reactive oxygen species (ROS) such as superoxide ( $O_2^-$ ) or hydrogen peroxide ( $H_2O_2$ ) which can be deleterious (O’Brien, 1991; Monks et al., 1992; Kumagai et al., 2002). Although the ability of quinones to act as either electrophiles or redox cycling agents is well established, the degree to which these properties contribute to their overall toxicity is unclear and will be highly dependent on their chemical properties and the conditions of cellular exposure. Determination of the contribution of these two mechanisms to the overall toxicity of many quinones is difficult since under most experimental conditions both mechanisms can operate. Moreover, alternate mechanisms of quinone toxicity may be masked by the assumption that all quinone-mediated cell toxicity is due to these two mechanisms. For example, if a quinone is found to generate reactive oxygen species through redox cycling, it is often assumed that this is responsible for all of its toxicity while other as yet uncharacterized mechanisms may contribute as well.

In an attempt to validate established mechanisms of quinone toxicity in a whole cell in vivo system, to determine the contribution of each mechanism on the overall toxicity, and to assess participation of new and/or alternative mechanisms of toxicity, we have begun to examine the effects of quinone exposure on the viability of the yeast *Saccharomyces cerevisiae*. The yeast model system is ideal for these studies since (1) yeast are a simple eukaryotic organism with significant homology to mammalian systems (vida infra); (2) the yeast genome is fully sequenced allowing facile manipulation of their genetics to predictably control their susceptibility/resistance to

quinone toxicity; (3) yeast can survive under a variety of conditions including anaerobic conditions and varying pH, which allows an evaluation of growth conditions/cell environment on toxicity; and (4) yeast manipulation is simple and inexpensive. Most important to studies of redox active toxins, however, is the ability of yeast to grow anaerobically. Herein, we have used this feature of yeast in studies of quinone toxicity, and found an oxygen-independent pathway of 9,10-phenanthrenequinone (9,10-PQ) toxicity.

## 2. Materials and methods

### 2.1. Materials

1,4-Benzoquinone (1,4-BQ), 1,2-naphthoquinone (1,2-NQ), 1,4-naphthoquinone (1,4-NQ), 9,10-phenanthrenequinone, and 9,10-anthraquinone (9,10-AQ) were purchased from Aldrich (Milwaukee, WI). The quinones were dissolved in dimethylsulfoxide (DMSO) to give a 50 mM stock solution. Serial dilutions were then performed to achieve the desired final concentration in a 10 ml suspension of yeast cells. The final concentration of DMSO was 0.2%. All other chemicals and solutions were purchased from commercial suppliers and were of the highest purity available.

### 2.2. Yeast strains and growth condition

Yeast *S. cerevisiae* strain EG103 (MAT $\alpha$  leu2-3, 112 his $\Delta$ 1 trp1-289 ura3-52 GAL $^+$ ), EG118 (EG103 with *sod1* $\Delta$ A::URA3) (Longo et al., 1996), and EG133 (EG103 with *sod1* $\Delta$ A::URA3 *sod2* $\Delta$ ::TRP1) were gifts from Dr. Edith Gralla (Department of Chemistry and Biochemistry, UCLA). EG103, EG118, and EG133 strains were grown on YPDG (1% yeast extract/2% peptone/0.1% glucose/3% glycerol) plates to avoid selecting respiration deficient mutants (Sherman, 1991). Single colonies of EG103, EG118, and EG133 were grown overnight at 30 °C and 275 rpm in complete synthetic dextrose (SDC) medium, SD-uracil, and SD-tryptophan, respectively (Longo et al., 1996). Aliquots of the resulting cultures were then diluted with the same medium to a final optical density at 600 nm (OD<sub>600</sub>) of 0.25 in a 10 ml volume. After addition of the test compound,

cultures were grown at 30 °C for 6–8 h and growth was monitored hourly by OD<sub>600</sub> measurements.

### 2.3. Anaerobic experiments

The procedure for performing anaerobic experiments has been described previously (Shinyashiki et al., 2000). Briefly, 10 ml of diluted cultures were transferred into 25 ml Erlenmeyer flasks equipped with gas tight rubber septa. Deoxygenation was performed by purging the cultures with nitrogen gas, using a syringe needle through the septa, for 45 min at 30 °C with shaking prior to addition of test compound. Oxygen levels as low as 0.9% of the air saturated value were achieved, as measured with a YSI biological oxygen monitor (Yellow Springs, OH). Aerobic experiments were performed in parallel, with the exception that the flasks were not swept with nitrogen and the septa were opened frequently to introduce air into the headspace.

### 2.4. Data analysis

Growth, monitored as an increase in optical density with time, can be fitted to an exponential function as shown in Eq. (1):

$$OD = OD_0 \exp(kt). \quad (1)$$

The effect of a quinone on the growth rate constant,  $k$ , is shown in Eq. (2):

$$k = k_0 + \beta[Q] \quad (2)$$

where  $[Q]$  is the quinone concentration and  $\beta$  is a value that reflects the potency of the quinone in inhibiting growth.

Conversion of (1) to natural logarithms and substitution of (2) for  $k$  yields

$$\ln(OD) = \ln(OD_0) + k_0 t + \beta[Q]t. \quad (3)$$

The parameters  $k_0$  and  $\beta$  can be estimated from a linear regression analysis of the optical density measurements at different times for quinone-free and quinone at different concentrations. The IC<sub>50</sub> value is estimated from the expression:

$$IC_{50} = \frac{-k_0}{2\beta}. \quad (4)$$

All of the data for a specific quinone under a given condition, e.g., anaerobic growth, were pooled and fitted by linear regression to Eq. (3) in SAS Proc REG (SAS Institute). The resulting estimates and variance/co-variance information for  $\beta$  and  $k_0$  were used to estimate IC<sub>50</sub> and their 95% confidence limits according to procedures previously described (Fieller, 1954; Laska et al., 1997; Heitjan, 2000).

### 2.5. Cell viability

A single colony of strain EG103 was grown overnight in SDC medium. An aliquot of this culture was then used to inoculate about 100.0 ml of fresh SDC medium. The resulting diluted culture was then grown for several hours to a final OD<sub>600</sub> of 1.0. After centrifugation (850 ×  $g$ , 4 °C, 5 min), the resulting cell pellet was re-suspended in 100.0 ml of sterilized phosphate buffer (0.1 M, pH 6.20) and 8.0 ml aliquots were exposed to different concentrations of 9,10-PQ for 1 h at 30 °C. Samples were then centrifuged (850 ×  $g$ , 4 °C, 5 min) and the cell pellet re-suspended in 1.0 ml of sterilized water. Following counting using a hemocytometer, cells were diluted and approximately 150–200 cells were plated on rich YPD medium. Colonies were grown for 2–3 days in an incubator at 30 °C and the percentage of survivors determined.

### 2.6. GAPDH activity

Washed cell pellets were re-suspended in 350  $\mu$ l of phosphate buffer (0.1 M, pH 7.4) and 100  $\mu$ l of glass beads (0.5 mm). Cells were then lysed by vortexing vigorously six times for 30 s at 1 min intervals. Samples were then centrifuged (15,000 ×  $g$ , 4 °C, 5 min) and the resulting supernatant was assayed for GAPDH activity according to the spectrophotometric method described by Dagher and Deal (1982), based on the reduction of the cofactor NAD<sup>+</sup>. In brief, in 1.0 ml volumes containing 66.5 mM phosphate buffer (pH 7.4), 1.0 mM NAD<sup>+</sup>, 3.0 mM arsenate, and 0.5 mM glyceraldehyde-3-phosphate (G-3-P), NADH formation was monitored at 340 nm every 12 s for 1 min using a double beam Uvikon spectrophotometer. The blank cuvette contained all of the components, except for G-3-P, which was used to start the reaction. GAPDH activity was normalized to protein levels, as determined by the Bradford method (Bradford, 1976),

and expressed as nanomoles of NADH formed per second per milligram of protein utilizing an extinction coefficient of  $6290 \text{ M}^{-1} \text{ cm}^{-1}$ .

### 2.7. Glutathione analysis

Washed cell pellets were vigorously vortexed for 3 min in a mixture containing 4.8% perchloric acid,  $5.0 \mu\text{M}$  EDTA, and  $200 \mu\text{l}$  of glass beads ( $0.5 \text{ mm}$ ). Following the addition of a previously determined volume of KOH ( $7.0 \mu\text{l}$ ,  $30 \text{ M}$ ) to bring the pH to about 2, samples were vortexed for an additional minute. After centrifugation ( $20,000 \times g$ ,  $4^\circ\text{C}$ , 10 min), the resulting supernatant of each sample was collected and analyzed for glutathione.

Glutathione analysis was performed using a HP-1090 liquid chromatograph coupled with an electrochemical LC-44 amperometric detector (Bioanalytical Systems Inc.). The simultaneous electrochemical detection of reduced (GSH) and oxidized (GSSG) glutathione was achieved at an oxidation potential of  $+1.350 \text{ V}$ . Samples were chromatographed using a  $5 \mu\text{m}$ ,  $0.4 \text{ cm} \times 20 \text{ cm}$  ODS2 column (Column Engineering Inc.) and a mobile phase consisting of  $50 \text{ mM}$   $\text{NaH}_2\text{PO}_4$ ,  $10 \mu\text{M}$  octane sulfonic acid, and 0.6% acetonitrile, as described by Lakritz and Buckpitt (1997). The retention times for GSH and GSSG were approximately 4.5 and 8.4 min, respec-

tively. Calibration curves were constructed from known amounts of authentic GSH (0, 39.1, 78.1, 156.2, 312.5, and  $625.0 \text{ pmol}$ ) and GSSG (0, 39.1, 78.1, and  $156.2 \text{ pmol}$ ) standards. The limit of detection for GSH and GSSG was estimated at  $32.9 \text{ pmol}/10 \mu\text{l}$  and  $39.1 \text{ pmol}/10 \mu\text{l}$ , respectively. Intracellular concentrations of GSH and GSSG were estimated using a volume of  $70.0 \mu\text{m}^3$  per yeast cell (BIO101, 1991).

### 3. Results

The quinones examined in this study are shown in Fig. 1. Menadione is a toxic quinone whose actions have been primarily attributed to its redox activity (Jamieson, 1992; Flattery-O'Brien et al., 1993; Flowers-Geary et al., 1993). 1,4-Benzoquinone was chosen as an agent whose toxicity derives primarily from its actions as an electrophile. These compounds were used for comparison with the other quinones which were selected due to their presence in air pollution particles (Cho et al., 2004). Yeast cells were exposed to the quinones under both aerobic and anaerobic conditions and the effects of exposure on growth were determined by optical density determinations at hourly intervals during the first 6–8 h of growth. The growth rate of yeast during this time interval is exponential in nature and can be analyzed mathe-

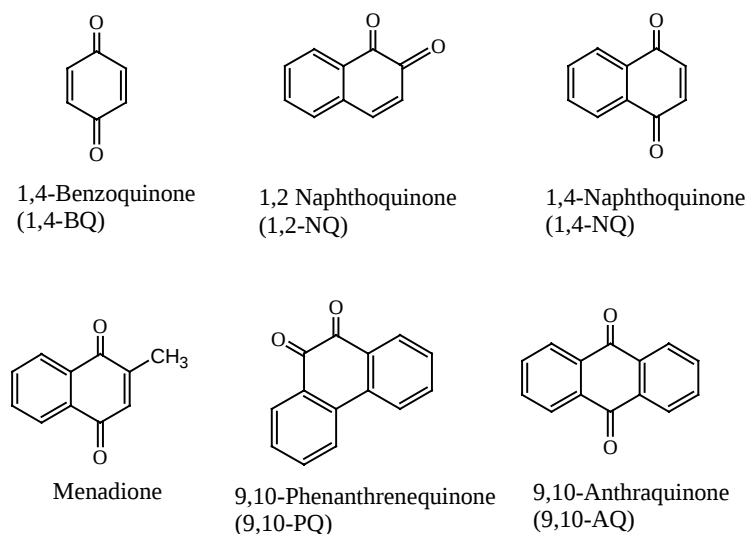


Fig. 1. Quinones used in this study.

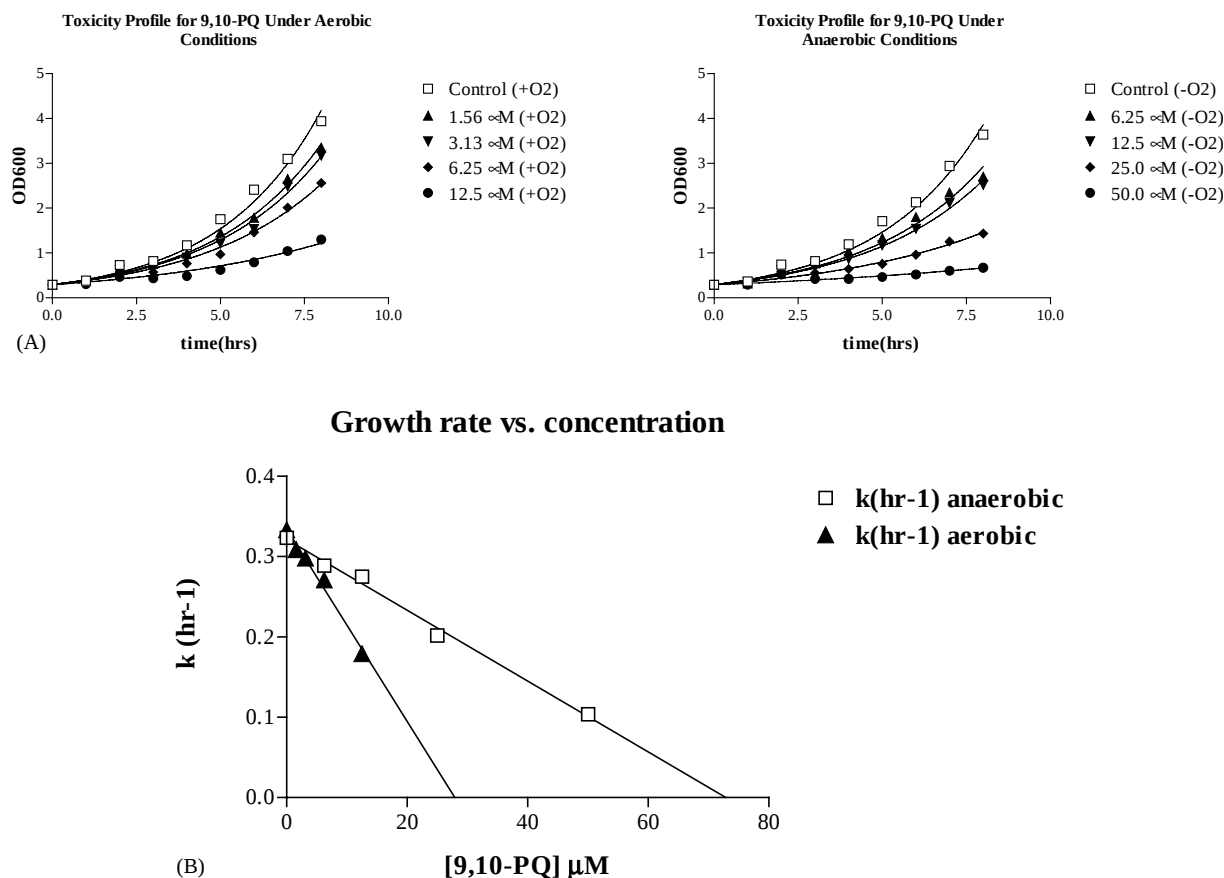


Fig. 2. (A) Aerobic and (B) anaerobic growth curve in the presence of 9,10-phenanthrenequinone. The growth rate was monitored by hourly optical density measurements at 600 nm. The results for each concentration were analyzed for their fit to an exponential growth expression by nonlinear regression analysis (GraphPad, San Diego, CA). The value of  $k$ , the rate constant for exponential growth, is plotted against quinone concentration and analyzed by linear regression. The line represents the best fit straight line for aerobic and anaerobic growth.

matically using an exponential model. Representative aerobic and anaerobic growth curves for 9,10-PQ are shown in Fig. 2. IC<sub>50</sub> values (Table 1) were estimated by linear regression analysis of the growth rate con-

stants over the concentrations used, as described in Section 2 (see Fig. 2). As shown in Table 1, the toxicity of some quinones (1,2-NQ, 1,4-NQ, and 9,10-PQ) was significantly greater in the presence of O<sub>2</sub>. The

Table 1  
Inhibition of growth by different quinones under different conditions

| Quinone   | Aerobic IC <sub>50</sub> (μM) | Anaerobic IC <sub>50</sub> (μM) | SOD1 <sup>-</sup> IC <sub>50</sub> (μM) | SOD1 <sup>-</sup> ,2 <sup>-</sup> IC <sub>50</sub> (μM) |
|-----------|-------------------------------|---------------------------------|-----------------------------------------|---------------------------------------------------------|
| 1,2-NQ    | 61.85 (58.30–65.81)           | 92.69 (81.76–107.07)            | 21.18 (19.32–23.36)                     | 12.64 (10.73–15.14)                                     |
| 1,4-NQ    | 17.16 (16.26–18.15)           | 29.12 (27.29–31.19)             | 12.34 (11.34–13.54)                     | 6.39 (5.98–6.87)                                        |
| 1,4-BQ    | 48.75 (43.22–56.01)           | 29.50 (26.18–33.88)             | 22.89 (20.26–26.08)                     | 33.10 (29.44–37.72)                                     |
| 9,10-PQ   | 13.81 (13.35–14.29)           | 36.00 (33.64–38.70)             | 3.26 (3.01–3.55)                        | 2.79 (2.33–3.52)                                        |
| Menadione | 34.36 (32.01–37.05)           | 143.56 (125.77–167.77)          |                                         |                                                         |

The IC<sub>50</sub> values estimated by linear regression analysis of optical density vs. time data according to the procedure described in Section 2.4. SOD1<sup>-</sup> and SOD1<sup>-</sup>,2<sup>-</sup> refer to yeast mutants deficient in one or both superoxide dismutases, copper–zinc–SOD (CuZnSOD) and manganese–SOD (Mn–SOD), respectively (Longo et al., 1996).

increase in toxicity in the presence of  $O_2$  presumably reflects their ability to act as catalysts for the generation of ROS, such as superoxide ( $O_2^-$ ). Of particular note is the lack of toxicity exhibited by 9,10-AQ. This quinone was found to be relatively non-toxic in our initial studies (no effect on growth at 100  $\mu M$ ) under either aerobic or anaerobic conditions, and therefore, was not tested further.

The generation of ROS is a key feature of quinone toxicity, and therefore, it may be expected that yeast lacking superoxide dismutase (SOD), the enzyme primarily responsible for degrading  $O_2^-$ , would be more susceptible to the toxicity of these quinones. To test this notion, yeast mutants lacking the major SOD enzyme, namely, copper zinc superoxide dismutase (CuZnSOD), were exposed to quinones under aerobic conditions and the  $IC_{50}$  values determined. Deletion of CuZnSOD increased the toxicity of all quinones tested (Table 1) with the greatest proportional increase exhibited by 9,10-PQ. Interestingly, a less dramatic increase in sensitivity was observed in yeast lacking both CuZnSOD and MnSOD, consistent with CuZnSOD being the predominant  $O_2^-$  degrading enzyme (Sturtz et al., 2001).

The observed toxicity of 9,10-PQ in this yeast model system is somewhat enigmatic. Lacking an electrophilic site, 9,10-PQ was expected to be strictly a redox cycling agent and exhibit only oxygen-dependent toxicity. However, 9,10-PQ was also potent in inhibiting cell growth anaerobically with an  $IC_{50}$  value of 36.0  $\mu M$  (Table 1). In contrast, the toxicity of 1,4-BQ, as a strictly electrophilic quinone exhibited little dependence on oxygen. In fact, this quinone was more toxic under anaerobic conditions. Although the latter effect would suggest oxygen involvement in aerobic toxicity, it is likely to be indirect (see Section 4).

In an attempt to gain further insight into the anaerobic toxicity of 9,10-PQ, different growth phases of yeast were examined for their sensitivity to 9,10-PQ toxicity. Several metabolically distinct phases of growth are observed when yeast are grown in glucose-based medium. These include early-, mid-, late-exponential, and post-diauxic (Werner-Washburne et al., 1993). During the exponential phase, yeast cells grow primarily by glycolysis. As glucose becomes exhausted, cells undergo the diauxic shift and adapt to respiratory metabolism in the presence of molecular oxygen. Therefore, during the

post-diauxic phase of growth, cells are predominantly dependent on energy exclusively provided by respiration (Werner-Washburne et al., 1993). The effect of 9,10-PQ on essential metabolic processes such as glycolysis and respiration can be differentially examined in yeast by simply evaluating toxicity at different growth phases. Thus, yeast cells grown and harvested at early-, mid-exponential, and post-diauxic phases of growth were examined for their cell viability loss following 1 h exposure to 9,10-PQ. As shown in Fig. 3A, the growth phases were differentially susceptible to 9,10-PQ, with the early exponential phase exhibiting the greatest sensitivity, followed by late exponential, and post-diauxic phases. Further examination of this differential toxicity revealed a strong correlation between cell viability loss and inhibition of the important glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 3B). In particular, the greater sensitivity to 9,10-PQ exhibited by the early exponential growth phase was highly correlative ( $r^2 > 0.85$ ) with GAPDH inhibition by this quinone (Fig. 3C). On these basis, this growth phase was selected for examining the role of oxygen in the toxicity of 9,10-PQ. As shown in Fig. 4A and 4B, comparable correlation ( $r^2 > 0.72$ ) between cell viability loss and GAPDH inhibition was also observed under anaerobic conditions. Furthermore, the  $EC_{50}$  value for GAPDH activity loss approximates that for cell viability loss under anaerobic conditions (Table 2).

Since redox cycling is considered the primary mechanism of toxicity of 9,10-PQ, intracellular levels of reduced and oxidized glutathione were measured in early exponential cells and correlated with cell viability following exposure to 9,10-PQ. Reduced glutathione

Table 2

Effective concentrations ( $EC_{50}$ ) of 9,10-PQ for cell viability loss and GAPDH inhibition of early exponential yeast under aerobic and anaerobic conditions

|                     | Aerobic $EC_{50}$ ( $\mu M$ ) | Anaerobic $EC_{50}$ ( $\mu M$ ) |
|---------------------|-------------------------------|---------------------------------|
| Cell viability loss | 1.60 $\pm$ 0.14               | 11.43 $\pm$ 0.56                |
| GAPDH inhibition    | 4.89 $\pm$ 2.02               | 14.91 $\pm$ 3.61                |

Effective concentrations for 50% cell viability loss and GAPDH inhibition ( $EC_{50}$ ) were estimated by plotting percent of control values vs. concentration and analyzed by non-linear regression for fit to the equation  $y = B_{max}[9,10-PQ]/EC_{50} + [9,10-PQ]$  (Graphpad, San Diego, CA). Values represent the average of at least triplicate determinations  $\pm$  S.E.

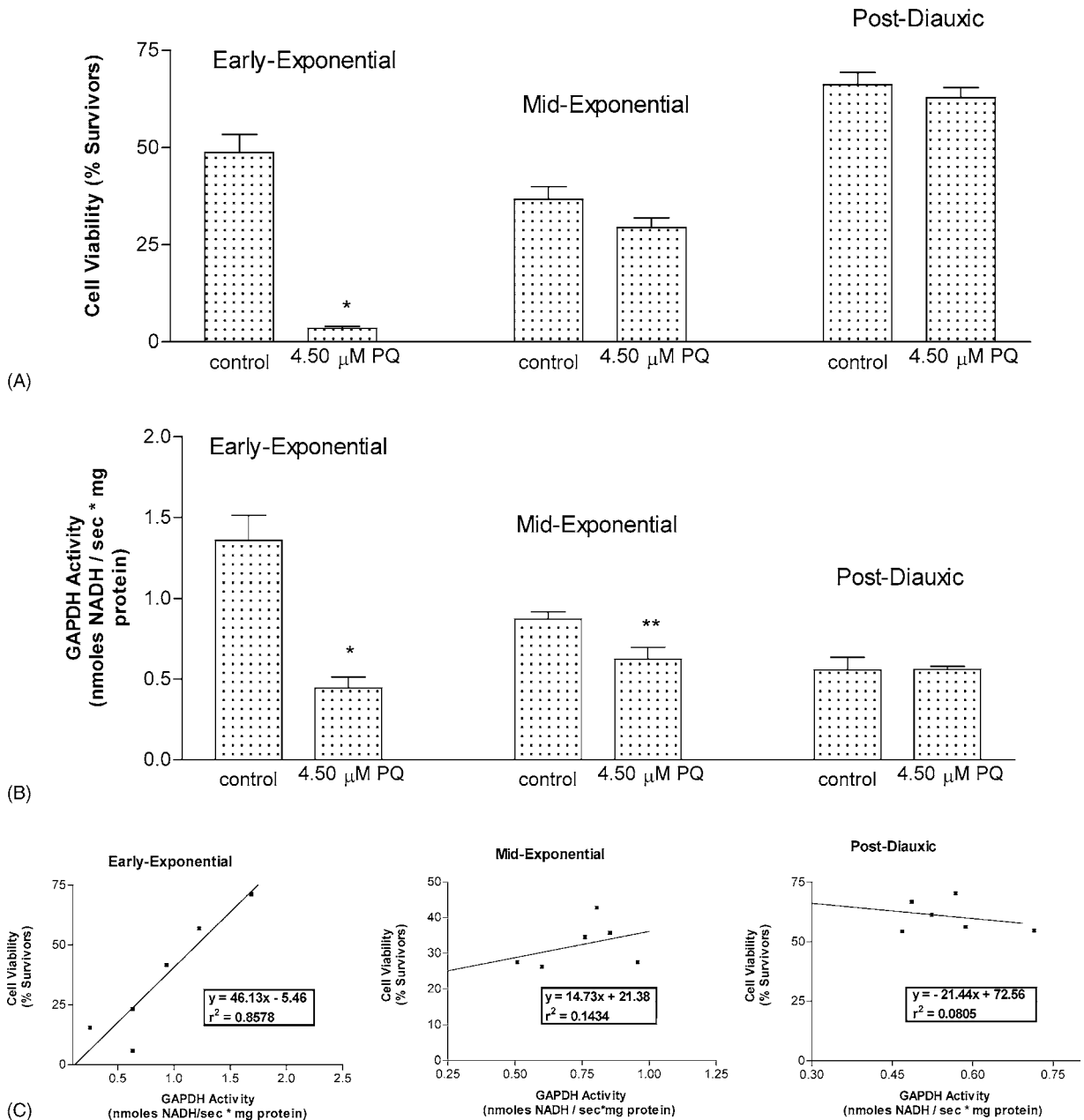


Fig. 3. Cell viability (A) and GAPDH activity (B) of yeast at different growth phases following exposure to 9,10-PQ under aerobic conditions. (A) Yeast cells (EG103) were grown in SDC medium and harvested at early-, mid-exponential, and post-diauxic phase. Following 1 h exposure to 9,10-PQ at 30 °C, cells were plated on rich YPD medium and the percent survivors determined after 2–3 days. Values shown represent the average of at least triplicate determinations  $\pm$  S.E. (\*)  $P < 0.0001$  vs. control. Other growth phases were not significantly different. (B) GAPDH activity was determined by lysing cell pellets in phosphate buffer (0.1 M, pH 7.4) and measuring NADH formation spectrophotometrically in a 1.0 ml volume. Values shown represent the average of at least triplicate determinations  $\pm$  S.E. (\*)  $P < 0.0005$  vs. control. (\*\*)  $P < 0.05$  vs. control. (C) Linear regression analysis of cell viability and GAPDH activity.

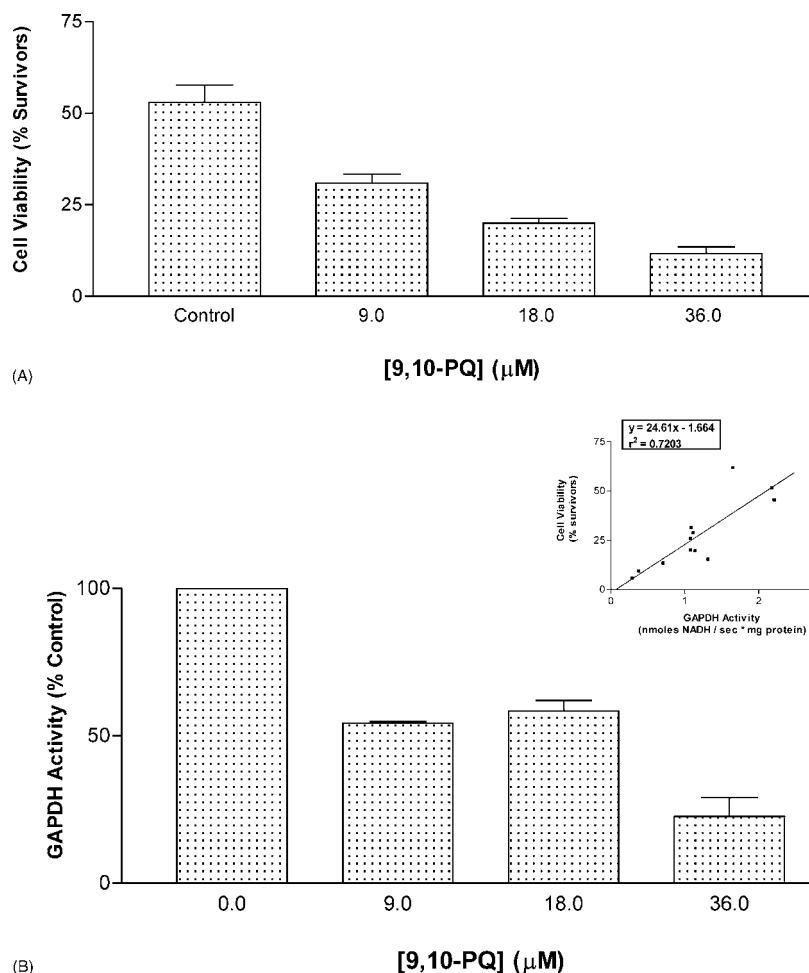


Fig. 4. Cell viability (A) and GAPDH activity (B) of early exponential yeast following exposure to 9,10-PQ under anaerobic conditions. Early exponential yeast cells were deoxygenated for 2 h and exposed to 9,10-PQ. Cell viability and GAPDH activity were determined as previously described. Values shown represent the average of at least triplicate determinations  $\pm$  S.E.

plays a vital role as the major antioxidant molecule in the cell in buffering the cell against oxidative stress, at reported intracellular concentrations ranging from 1 to 10 mM (Schafer and Buettner, 2001; Dickinson and Forman, 2002; Filomeni et al., 2002). Thus, the ratio between reduced glutathione, GSH, and its oxidized form, GSSG, is considered a good reflection of the redox environment of the cell. As a strictly redox cycling agent, 9,10-PQ would be expected to induce toxicity by disrupting the natural redox environment of the cell through the catalytic generation of GSSG at the expense of GSH. As shown in Table 3, 9,10-PQ, at a concentration of 4.50  $\mu$ M induced significant GSH

depletion and an associated generation of GSSG. However, redox cycling does not appear to be the primary mechanism of cytotoxicity of 9,10-PQ, since diamide induced a greater decrease in the GSH/GSSG ratio, but no loss in cell viability. Diamide directly oxidizes GSH to GSSG and represents a good model compound to study the effects of oxidative stress. On the other hand, the effects of 1,4-BQ on GSH levels are attributed primarily to its electrophilic properties, i.e., direct Michael addition chemistry. Indeed, 1,4-BQ at a concentration of 30.0  $\mu$ M induced the expected significant GSH depletion with no concomitant generation of GSSG so that the net effect on the GSH/GSSG

Table 3

Cell viability and intracellular glutathione levels of early exponential yeast following 1 h exposure to 9,10-PQ, 1,4-BQ, and diamide

| Treatment       | Cell viability (% survivors) | Intracellular GSH (mM) | Intracellular GSSG (mM) | GSH/2GSSG |
|-----------------|------------------------------|------------------------|-------------------------|-----------|
| Control         | 50.17 ± 2.57                 | 2.31 ± 0.14            | 0.017 ± 0.004           | 67.94     |
| 4.50 $\mu$ M PQ | 3.47 ± 0.57                  | 0.42 ± 0.11            | 0.433 ( <i>n</i> = 2)   | 0.48      |
| 30.0 $\mu$ M BQ | 37.23 ± 2.00                 | 0.33 ± 0.07            | 0.015 ± 0.001           | 11.00     |
| 1.0 mM diamide  | 49.71 ± 5.06                 | 0.20 ( <i>n</i> = 2)   | 0.478 ( <i>n</i> = 2)   | 0.21      |

Early exponential yeast cells exposed to 9,10-PQ for 1 h at 30 °C were lysed and analyzed for their GSH and GSSG content as described in Section 2. Intracellular concentrations were determined using a yeast cell volume of 70.0  $\mu$ m<sup>3</sup>. Cell viability studies were performed as previously described. Values represent at least triplicate determinations ± S.E., unless otherwise noted.

ratio was less than that for diamide. Together, these data suggest that disruption of the cell's redox environment through decreases in the GSH/GSSG ratio is not the primary mechanism of toxicity of 9,10-PQ.

#### 4. Discussion

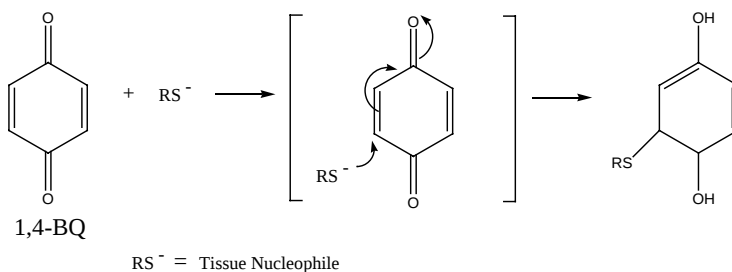
Quinones are thought to express their toxicity by one or both of two chemical processes, a stoichiometric process involving 1,4-addition reactions with nucleophilic functions such as thiols on proteins (Fig. 5A) or through the catalytic reduction of oxygen with electrons which would have otherwise been used for other purposes (electron transport in mitochondria, metabolism, etc.) (Fig. 5B). The first reaction, the Michael addition reaction, reflects the reactivity of the quinone as an electrophile in which nucleophilic functional groups on proteins and other macromolecules are alkylated. The modified protein or macromolecule would be expected to lose its normal function and initiate changes in the cell reflecting that loss. The redox reaction (Fig. 5B) generates reactive oxygen species through the reduction of oxygen and these ROS can induce oxidative damage to the cell. Toxicity due to this redox process differs from covalent bond-based toxicity because of the catalytic nature of the quinones. One equivalent of quinone can generate multiple equivalents of superoxide and in this way overwhelm the protective, antioxidant systems in the cell. It should also be noted that a quinone covalently linked to a macromolecule (Fig. 5A) may be capable of redox cycling if it has access to reducing electrons. In this way, the redox cycling quinone catalyst is "fixed" to a protein and may be longer-lived or may exhibit toxicity local to that protein (Monks and Lau, 1992).

As a probe for assessing each process, yeast have a particular advantage because of their ability to grow under aerobic and anaerobic conditions. Inhibition of growth is a convenient test for toxicity and the IC<sub>50</sub> values obtained here provide a quantitative assessment of the toxicity of each compound. As a first approximation in the analysis of toxicity, the IC<sub>50</sub> value obtained in the absence of oxygen would be expected to reflect covalent-based toxicity and that obtained for aerobic growth conditions would be a sum of both covalent and redox-based toxicity (Table 1). The contributions of each reaction to the overall toxicity of a given quinone will depend on its reactivity, i.e., the electrophilicity of the quinone function on the one hand, and its redox potential on the other.

A quinone that is capable of redox cycling in the cell must have the ability to accept an electron from a biological reducing agent and then transfer that electron to O<sub>2</sub>. The reduction potential of a quinone will, therefore, be a critical factor in its ability to perform this chemistry. The likely one-electron reductants for quinones in biological systems are flavoproteins. Since reduction potentials of reduced flavins are in the range of −0.270 to −0.320 V (versus normal hydrogen electrode, NHE) (O'Brien, 1991; Buettner, 1993), a favorable electron transfer from a reduced flavin such as that in cytochrome P450 reductase requires a quinone reduction potential greater than −0.270 V (for example, see O'Brien, 1991). Moreover, for a thermodynamically favorable reduction of O<sub>2</sub> by a reduced quinone species to occur, the quinone reduction potential should be around −0.16 V or less. Therefore, the optimal reduction potential range for redox cycling would be expected to be between −0.27 and −0.16 V.

1,4-BQ has a reduction potential that far exceeds the range for significant redox cycling (0.1 V versus NHE) so its toxicity should not be redox-based but

### A. Electrophilic 1,4-addition Reaction



### B. Generation of reactive oxygen species (redox cycling)

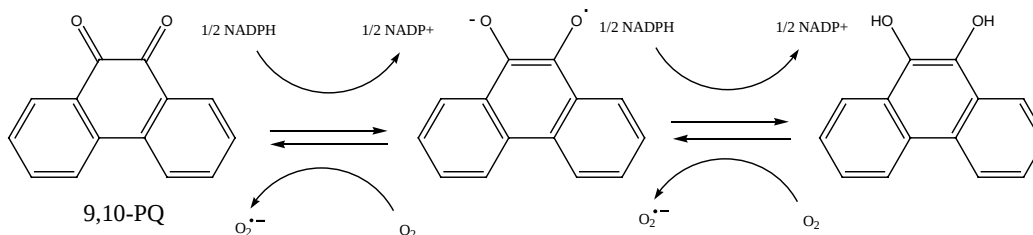


Fig. 5. Toxic reaction pathways of quinones. (A) 1,4-Benzoquinone (1,4-BQ) and (B) 9,10-phenanthrenequinone (9,10-PQ) are used as examples for electrophilic and redox active quinones, respectively.

due to its electrophilic properties. Thus, the toxicity of this quinone should be  $O_2$ -independent and indeed, 1,4-BQ toxicity is largely independent of  $O_2$  (Table 1). The redox potential of 9,10-AQ is too low for electron transfer ( $E^0 = -0.390$  V), making its reduction by biological reductants apparently difficult, and as its double bonds are aromatic, it is a very poor Michael acceptor. Based on these arguments, 9,10-AQ should not exhibit toxicity associated with either the redox cycling or electrophilic mechanisms and we have found this to be the case. This compound was not toxic at concentrations as high as  $100 \mu\text{M}$  and could not be examined further because of solubility problems.

Oxygen-dependent toxicity, through the generation of  $O_2^-$ , should be much greater when the antioxidant enzyme superoxide dismutase is deleted. The quinones that show evidence of redox cycling-mediated toxicity, 1,2-NQ, 1,4-NQ, and 9,10-PQ, are significantly more toxic in the SOD-deleted yeast mutant, as indicated by decreases in the  $IC_{50}$  values (Table 1). In contrast,

1,4-BQ toxicity is independent of oxygen; in fact, it is less toxic aerobically. We have no explanation for this observation at this time.

Based on its reduction potential, 9,10-PQ ( $E^0 = -0.12$  V) will likely be a redox cycling quinone and should have a much lower  $IC_{50}$  under aerobic conditions. Moreover, the ratio of  $O_2$ -dependent versus  $O_2$ -independent toxicity should be greatest for this compound since it is a poor electrophile. In contrast, the naphthoquinones, 1,2-NQ and 1,4-NQ, should exhibit toxicity under aerobic and anaerobic conditions, with greater toxicity exhibited under aerobic conditions when ROS generation and electrophilicity can operate. Indeed, the resulting growth inhibition profiles are in good agreement with this mechanistic understanding. The  $IC_{50}$  values for the naphthoquinones increased under anaerobic conditions by factors of 1.5 and 1.7 for 1,2-NQ and 1,4-NQ, respectively. Interestingly, 9,10-PQ, whose low electrophilicity would suggest minimal anaerobic toxicity, exhibited an  $IC_{50}$  of  $36.0 \mu\text{M}$  under

such conditions, an increase of only a factor of 2.6 (Table 1). The significant toxicity of 9,10-PQ by an oxygen-independent mechanism is noteworthy because it may represent the ability of this quinone to inhibit GAPDH.

The observed inhibition of GAPDH by 9,10-PQ, to our knowledge, is a new finding that may explain the unexpected anaerobic toxicity of this particular quinone. Although 9,10-PQ undergoes significant redox cycling, as reflected by GSSG generation, the resulting change in the cell's redox environment is not sufficient to induce the cell viability loss when compared to the actions of diamide and 1,4-BQ (Table 3). The actions of the latter compounds suggest that viability loss, measured under these conditions, is not related to GSH depletion. The observed loss in cell viability may in fact reflect the ability of this quinone to inhibit glycolysis, an oxygen-independent metabolic process. Indeed, GAPDH has been proposed to share some of the control over glycolysis with the major regulators, phosphofructokinase and hexokinase, since its inhibition results in inactivation of glycolysis and subsequent cell viability loss (Hyslop et al., 1988; Choi et al., 1995). The greater loss in cell viability under aerobic conditions may reflect the ability of 9,10-PQ to induce oxidative modification of glycolytic proteins, in particular GAPDH, through redox cycling. In support of this, GAPDH inactivation in response to elevated levels of hydrogen peroxide has been extensively documented (Thomas et al., 1995; Grant et al., 1999; Klatt and Lamas, 2000). The chemical mechanism by which 9,10-PQ inhibits GAPDH under anaerobic conditions is currently unknown, but it may be speculated that PQ competes for the NAD<sup>+</sup> binding site of GAPDH in a similar manner as it competes for the NADP<sup>+</sup> binding site of neuronal nitric oxide synthase (nNOS) and glutathione reductase (Bironaite et al., 1989; Kumagai et al., 1998). Studies to address such questions are currently underway in our laboratory.

In summary, these studies demonstrate the utility of yeast as a probe to assess redox and electrophile-based toxicities. More importantly, the yeast model system serves as a tool for the discovery of other, as yet unestablished, mechanisms of quinone-based toxicity. Our results demonstrate that other pathways, independent of oxygen and electrophilic chemistry, may operate for 9,10-PQ.

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