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Cytotoxic properties of iron–hydroxynaphthoquinone complexes in rat hepatocytes

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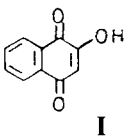
The mechanisms of toxicity to isolated rat hepatocytes of Fe(II) and Fe(III) complexes of two structurally related naphthoquinones have been studied. All complexes were found to show a dose-dependent toxicity which precedes cell death. Within the naphthoquinone series the order of toxicity is Fe(II) > parent naphthoquinone > Fe(III). The iron complexes of 5-OH-1,4 naphthoquinone (5-OH-1,4 NQ; Juglone) are more toxic than the iron complexes of 2-OH-1,4 naphthoquinone (2-OH-1,4 NQ; Lawsone) indicating that the mechanisms of toxicity are different. Electrochemical studies on these complexes shows that 5-OH-1,4 NQ facilitates formation of stable semiquinone species while 2-OH-1,4 NQ does not. The low redox potential of 2-OH-1,4 NQ makes it a poor substrate for metabolism by reductases.

Keywords: iron–naphthoquinone complexes, cytotoxicity, hepatocytes, redox cycling, glutathione, cyclic voltammetry

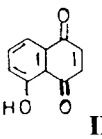
Introduction

Naturally occurring quinones such as mitomycins, aziridinylquinones, mitoxanthrones and doxorubicinones have been investigated in detail because of their cytotoxic or cytostatic properties as anticancer compounds (Carter 1975, Carter & Crooke 1979, Arcamone 1982, Young *et al.* 1981). Acute toxicities of these quinonoidal compounds appear to be related to their interactions with reduced glutathione and the subsequent disturbance of cellular energy homoeostasis and calcium homoeostasis (Di Monte *et al.* 1984a,b). Although many of these quinonoidal anticancer compounds have a cofactor requirement of some specific metal ion for biological activity, their metal complexes are not evaluated for their ability in altering the glutathione levels in the cell. It has recently been shown that alterations in the GSH levels correlate with the production of oxygen radicals by these compounds (Ernster 1984) and the induction of AP-1-mediated GST Ya gene expression (Pinkus *et al.* 1995).

Lawsone (**I**) and Juglone (**II**) represent a pair of naturally occurring isomeric naphthoquinones which are the active principles of two plant families, i.e. *Lawsonia alba* (Lal & Dutt 1933) and *Juglans regia* (Morton 1965). The former



I



II

has been used as a dye to color hair, nails and biological tissues since ancient times (Thompson 1971), while the latter is known for its allelopathic properties (Soderquist 1973). Inspite of their isomeric nature the compounds exhibit striking differences in their metal complexation properties as well as biological activities (Joshi 1975). Doherty *et al.* (1987) have shown that **II** is more toxic than **I** to rat hepatocytes by an order of magnitude which has been attributed to the potential of these compounds to induce conditions of oxidative stress and depletion of reduced GSH. Since both compounds are capable of forming metal chelates, especially with iron which has been implicated in the oxidative stress (Halliwell & Gutteridge 1984), it motivated us to undertake a study of the interaction of the iron complexes of **I** and **II** with intracellular GSH in rat hepatocytes, and to examine its correlation with the redox cycling capabilities of these compounds determined by cyclic voltammetry. Our present work reveals that the cytotoxicities of the parent quinones are enhanced upon metal complexation, especially with ferrous ions, and that reversible redox cycling observed for **II** seems to facilitate the oxidation of GSH.

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Materials and methods

The ligands Lawsone **I** (2-OH-1,4 naphthoquinone) and Juglone **II** (5-OH-1,4 naphthoquinone) were synthesized according to literature methods (Jasiatis & Krantz 1972). Tetraethyl ammonium perchlorate (TEAP) and dimethylsulfoxide (DMSO) solvent were commercial products of the highest available grade of purity. Iron complexes (both ferrous and ferric) of the two ligands were prepared by the interaction of aqueous solutions of corresponding metal salts (sulfate in the case of ferrous compounds and chloride in the case of ferric compounds) with methanolic solutions of the ligands in the molar ratio of 1:2 (for the ferrous compound) and 1:3 (for the ferric compound) at 40°C for 4 h under a Schlenk assembly maintained under nitrogen atmosphere. The pH of the reaction mixture was maintained between 5 and 6 with a few drops of an aqueous 10% sodium acetate solution. All the solutions were de-gassed with N_2 for 10 min prior to their use. The reaction mixture was stored in a refrigerator overnight, after which the solvent was stripped off on a rotavapor and the separated microcrystalline product was filtered. All of the complexes were washed with water and cold methanol, and then dried under vacuum at room temperature. The elemental analysis and relevant structural parameters are included in Table 1.

Cyclic voltammetric (CV) profiles of the synthesized complexes were obtained on a BAS CV-27 electrochemical system as reported previously (Kumbhar *et al.* 1991).

Isolation and treatment of hepatocytes

Male Sprague-Dawley rats, treated with phenobarbital (1 mg/ml in drinking water for 5 days before use), which were allowed food and water *ad libitum*, were used. Hepatocytes were isolated by collagenase perfusion of the liver as described (Moldeus *et al.* 1978). Incubations were performed at 37°C using 10^6 cells/ml in Krebs–Henseleit buffer, supplemented with 12.6 mM HEPES (pH 7.4). The reactions were terminated by the addition of 70% perchloric acid (50 ml) at various times or by the separation of pellet and supernatant by centrifugation with subsequent acid addition. The viability of the cells was determined by Trypan blue exclusion during the course of the experiment. The effects of pre-treatment of the ligands and their metal complexes was followed by resuspension of the cells in fresh Krebs–Henseleit medium. All compounds were dissolved in DMSO and the final concentration of this solvent in the incubation medium was 0.7 mM. Samples were analyzed for GSH and GSSG contents by the HPLC method described earlier (Reed *et al.* 1980).

Results and discussion

Structural characterization of the synthesized iron complexes has shown that the overall geometry of these compounds is octahedral with the general formula as $[Fe^{2+}(L)_2(H_2O)_2]$ or $[Fe^{3+}(L)_3]$ where L is the anion of 2- or 5-hydroxynaphthoquinone (Table 1).

The toxicity of parent isomeric naphthoquinone ligands, i.e. **I** and **II**, to freshly isolated hepatocytes was assessed at 37°C for various time intervals (Figure 1). It was observed

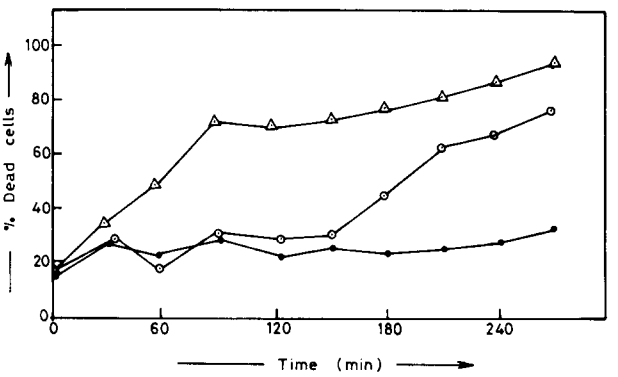

Figure 1. Time course of toxicity to isolated rat hepatocytes. Control (●) or presence of 25 μM Lawsone (⊙) and 25 μM Juglone (△). Values are representative of results obtained from three separate cell preparations.

Table 1. Analytical and structural data for iron complexes of Lawsone and Juglone

Compound	Elemental analyses [found (calc.) (%)]			BM[a]		g[b]	$E_{1/2}$[c] (V)
	C	H	Fe	300 K	5 K		Fe^{2+}/Fe^{3+}
$[Fe^{2+}(Lawsone)_2(H_2O)_2]$	54.74 (54.82)	3.11 (3.22)	12.65 (12.74)	5.2	4.8	—	+0.35
$[Fe^{3+}(Lawsone)_3]$	62.50 (62.63)	2.57 (2.63)	9.58 (9.71)	4.3	2.8	2.8	—
$[Fe^{2+}(Juglone)_2(H_2O)_2]$	54.73 (54.82)	2.96 (3.22)	12.53 (12.74)	3.8	2.2	—	+0.41
$[Fe^{3+}(Juglone)_3]$	62.61 (62.63)	2.51 (2.63)	9.63 (9.71)	4.3	2.5	2.0	—

[a] Units of Bohr Magneton.
[b] Lande splitting factor from ESR spectra.
[c] Versus Ag/AgCl.

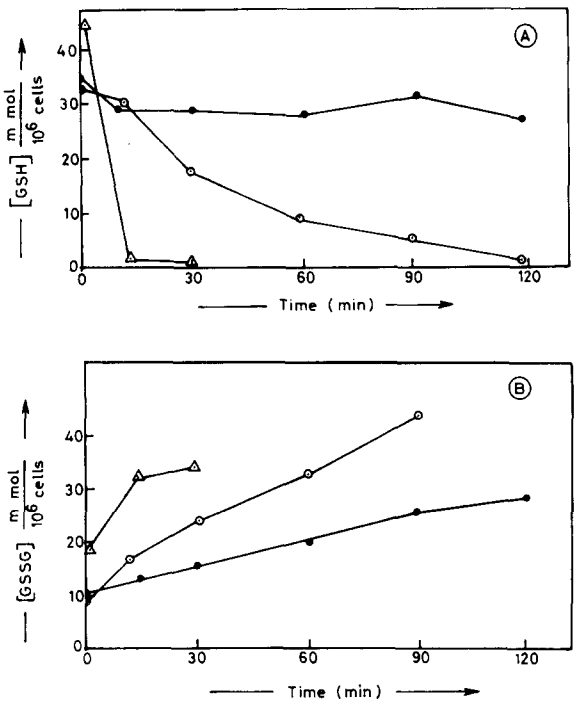

Figure 2. (**A**) Intracellular GSH depletion induced by Lawsone and Juglone in isolated rat hepatocytes. Control (●), 25 μM Lawsone (⊙) and 25 μM Juglone (△). (**B**) Generation of oxidized glutathione in isolated rat hepatocytes after incubation. Control (●), 25 μM Lawsone (⊙) and 25 μM Juglone (△). Values are representative of results obtained from three separate cell preparations.

that the latter was significantly more toxic than **I** at this temperature where cell death occurred in almost all cells after about 4 h when exposed to 25 μM concentration. Much higher concentrations (350 μM) were required to achieve the same results for **I**, which indicates that **II** is approximately an order of magnitude more toxic to isolated hepatocytes than **I**.

In further experiments it was observed that the addition of either of the ligands to hepatocytes caused a concentration-dependent depletion of intracellular levels of GSH (Figure 2) which precedes cell death. The rapid loss of GSH in the presence of Juglone but not Lawsone was attributed to the direct chemical reaction of this naphthoquinone with GSH (Doherty *et al.* 1987), although no specific reason for this behavior was provided. It had earlier been established in our laboratory that differences in the reactivity patterns of these two isomeric naphthoquinones originate from the nature of hydrogen bonding interactions present in them. For example, it was shown that the presence of strong intramolecular hydrogen bonding interactions present in **II** results in its higher ionization constant, lower melting point, lower dipole moment (Padhye & Kulkarni 1975a), faster chromatographic elution (Padhye & Kulkarni 1975b) and stronger chelation with biologically relevant metal ions (Bottei & McEachern 1970). On the other hand, the presence of intermolecular hydrogen bonding in **I** promoted through its conjugate base form results in its lower ionization constants (resembling those of carboxylic acids), much weaker interactions with biometals and very mild antimicrobial activities.

The observed toxicity of **II** resembled that exhibited by other naphthoquinones such as 2,3-dimethyl-1,4-naphthoquinone, which has been shown to be capable of redox cycling and generating the corresponding naphthosemiquinone moiety. Recently, the reaction products between menadione (2-methyl-1,4-naphthoquinone) and GSH have been shown to involve the generation of H_2O_2, oxidized glutathione (GSSG), menadione–GSH conjugate, and a number of menadione-thiol and oxygen-derived radicals (Ross *et al.* 1985). It has been proposed that the direct chemical interaction between menadione and GSH leading to the generation of reactive oxygen species plays a crucial role in the menadione-induced biochemical changes in the cellular systems. Although it is difficult to distinguish the contribution of the direct chemical reaction of **II** with GSH or protein thiols from that of redox cycling, the cyclic voltammetry studies carried out on the two naphthoquinones

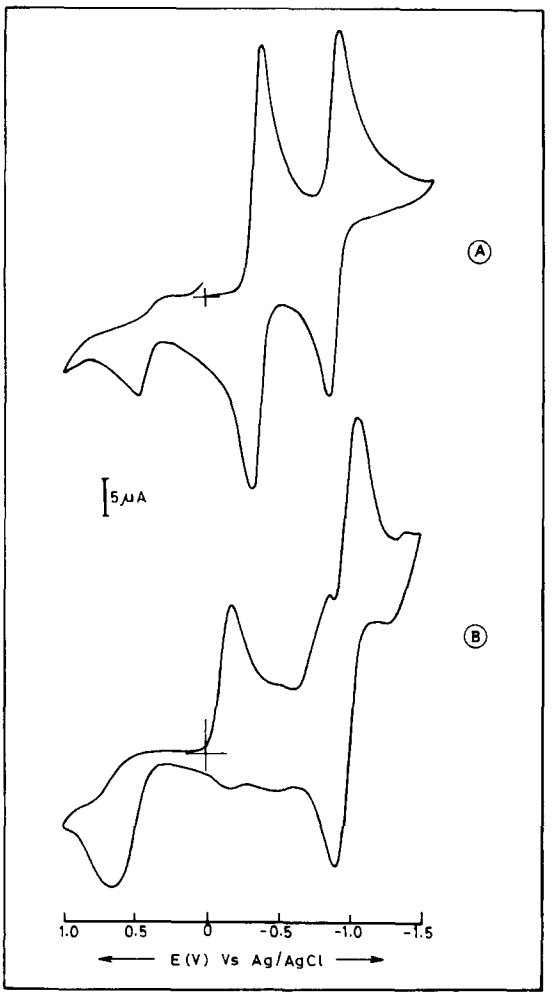

Figure 3. Cyclic voltammogram of (**a**) Juglone and (**b**) Lawsone in DMSO with TEAP as supporting electrolyte.

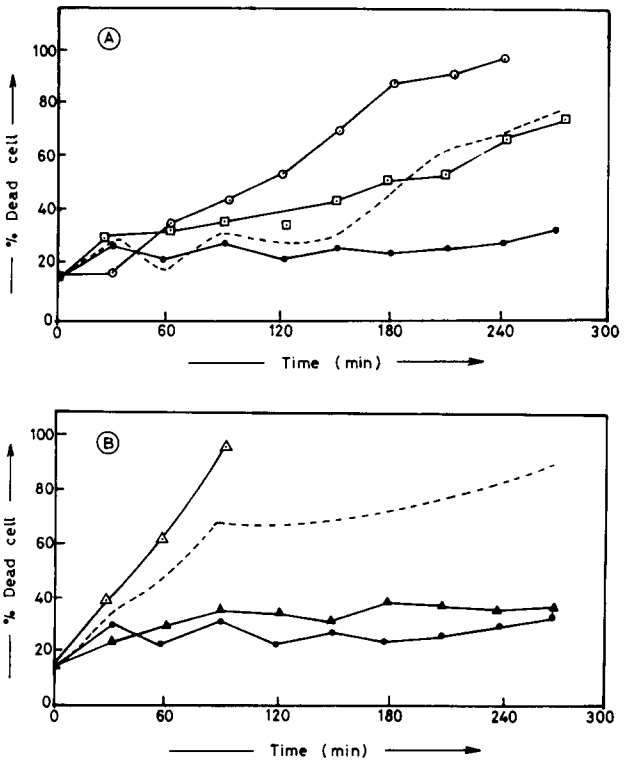

Figure 4. Time course of toxicity to isolated rat hepatocytes by Lawsone and Juglone. Control (●). **(A)** In the presence of 350 μM $[Fe^{2+}(Lawsone)_2(H_2O)_2]$ (⊙), 350 μM $[Fe^{3+}(Lawsone)_3]$ (□) and 25 μM Lawsone (– –). **(B)** In the presence of 25 μM $[Fe^{2+}(Juglone)_2(H_2O)_2]$ (△), 25 μM $[Fe^{3+}(Juglone)_3]$ (▲) and 25 μM Juglone (– –). Values are representative of results obtained from three separate cell preparations.

clearly reveal the differences in redox cycling behavior of the two isomers. For example, cyclic voltammogram of **II** reveals two completely reversible one-electron reduction waves (Figure 3a) corresponding to the semiquinone and semiquinone anion formation, respectively. On the other hand, **I** exhibits an irreversible reduction peak at −0.50 V and a quasi-reversible peak centered at −1.26 V, respectively (Figure 3b). The appearance of a single irreversible cathodic peak for **I** may be attributed to the two tautomeric forms for it that undergo chemical reactions on deprotonation which are not reversible.

When isolated hepatocytes were treated with different concentrations of ferrous and ferric complexes of **I** and **II**, respectively, it was seen (Figure 4) that the ferrous compounds are the most lethal species, due perhaps to their ability to undergo Fenton-type reactions leading to active oxygen species. The ferrous–juglonate complex is especially remarkable as it brings about nearly 100% cell death in about 80 min at a substantially lower (25 μM) concentration. The ferric complexes, on the other hand, exhibit no enhancement in their activities. It has been shown that reduction of the ferric species to the ferrous species is the pre-requisite for their possible implications in the cyto-toxicities (Aust & Miller 1990). The electrochemical profile (Figure 5) of the $Fe(Juglone)_2(H_2O)$ shows two reversible reductions centered at −0.32 and −1.05 V, which are similar to those observed for the ligand, albeit the second peak is shifted by 0.15 V to the negative side. A reversible oxidation peak centered at +0.41 V which corresponds to the Fe^{2+}/Fe^{3+} couple is also observed. It is known that

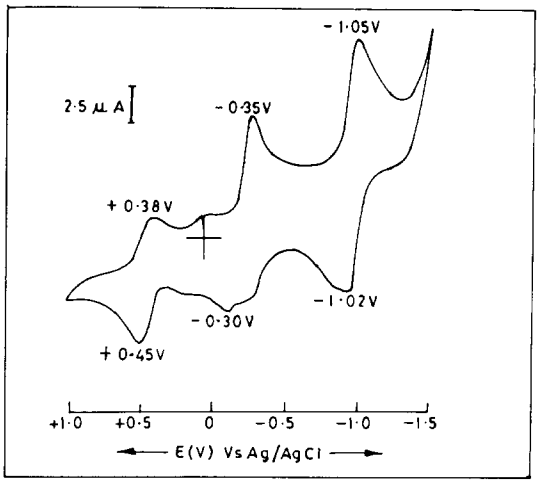

Figure 5. Cyclic voltammogram of 10 mM $[Fe^{2+}(Juglone)_2(H_2O)_2]$ in DMSO with TEAP as supporting electrolyte.

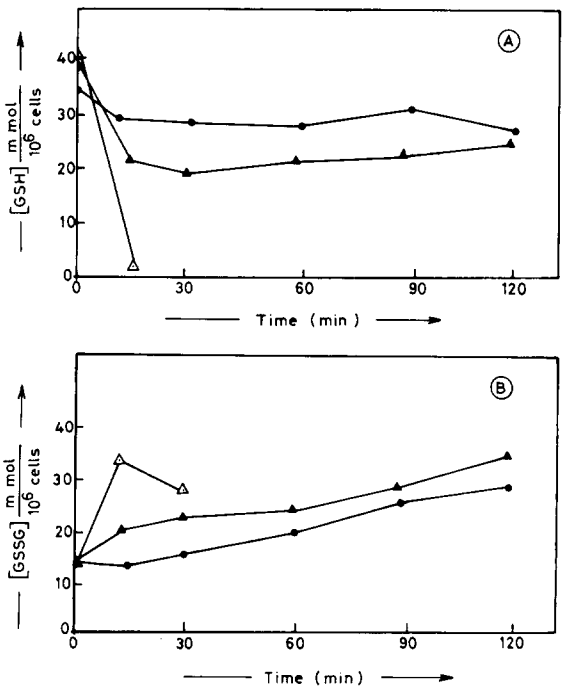

Figure 6. **(A)** Intracellular GSH depletion induced by iron complexes in isolated rat hepatocytes. Control (●) and in the presence of 350 μM $[Fe^{2+}(Juglone)_2(H_2O)_2]$ (△), 350 μM $[Fe^{3+}(Juglone)_3]$ (▲) and 25 μM Juglone (– –). **(B)** Generation of oxidized glutathione in rat hepatocytes in the presence of 25 μM $[Fe^{2+}(Juglone)_2(H_2O)_2]$ (△), 25 μM $Fe^{3+}(Juglone)_3]$ (▲) and 25 μM Juglone (– –). Values are representative of results obtained from three separate cell preparations.

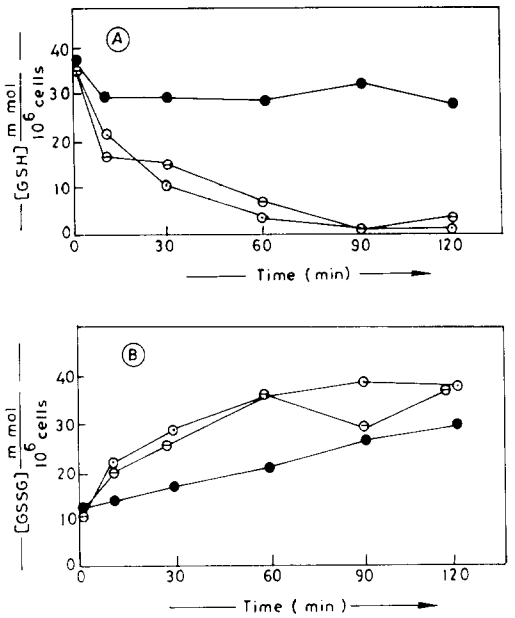

Figure 7. (**A**) Intracellular GSH depletion induced by iron complexes in isolated rat hepatocytes. Control (●) and in the presence of 350 μM $[Fe^{2+}(Lawsone)_2(H_2O)_2]$ (⊙) and 350 μM $[Fe^{3+}(Lawsone)_3]$ (○). (**B**) Generation of oxidized glutathione in rat hepatocytes in the presence of 25 μM $[Fe^{2+}(Lawsone)_2(H_2O)_2]$ (⊙) and 25 μM $[Fe^{3+}(Lawsone)_3]$ (○). Values are representative of results obtained from three separate cell preparations.

chelating agents greatly influence the extent of Fenton-type reactions. For example, chelation with EDTA shifts the Fe^{2+}/Fe^{3+} couple from -0.77 to -0.12 V and citrate shifts it to -0.33 V (Aust & Miller 1990). This indicates that the Fe(II) to Fe(III) conversion is very facile in $Fe(Juglone)_2(H_2O)_2$ which leads to its enhanced toxocity. This condition obviously becomes difficult to comply with upon metal complexation as no reversible Fe^{2+}/Fe^{3+} redox couple can be observed in the respective ferric compounds.

Since the quinone-based redox couple may be inter-related to the oxidation of intracellular thiols like GSH, the intracellular levels of GSH were measured (Figures 6 and 7). It was observed that the depletion of GSH preceeded cell death. The subsequent generation of the oxidized glutathione (GSSG) was also measured. Of particular interest was the rapid loss of GSH which occurred when hepatocytes were treated with ferrous–juglonate complex. The initial rapid depletion of hepatocellular GSH levels may indeed be due to direct interaction of the quinone with GSH (Ross *et al.* 1985). Again depletion was found to be maximum in the case of the ferrous complexes for both series. Substantial generation of GSSG was observed in the case of the Fe(II) complexes as compared with the Fe(III) complexes and parent ligands, indicating that one-electron-reduced quinone species generated by corresponding metal ions may play a crucial role in the generation of active oxygen species, which in turn may be responsible for the production of the GSSG moieties.

The present work has thus shown that ferrous complexes of peri-hydroxynaphthoquinones are highly cytotoxic to rat hepatocytes, where toxicity is promoted by their facile interaction with intracellular components like GSH, resulting in the conditions of oxidative stress. It is likely that complexation with metal ions like ferrous species results in the stabilization of the intermediate semiquinone species, which promotes redox cycling with glutathione. A similar observation had recently been made in the case of ferrous–bleomycin compounds (Guajardo & Mascharak 1995).

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