Cytotoxicity of Natural Hydroxyanthraquinones: Role of Oxidative Stress

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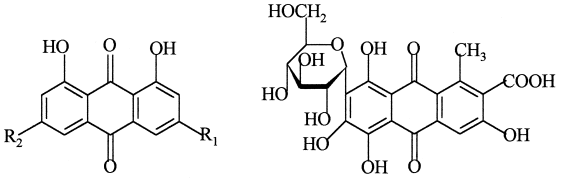
In order to assess the role of oxidative stress in the cytotoxicity of natural hydroxyanthraquinones, we compared rhein, emodin, danthron, chrysophanol, and carminic acid, and a series of model quinones with available values of single-electron reduction midpoint potential at pH 7.0 (E^1_7), with respect to their reactivity in the single-electron enzymatic reduction, and their mammalian cell toxicity. The toxicity of model quinones to the bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK), and HL-60, a human promyelocytic leukemia cell line, increased with an increase in their E^1_7. A close parallelism was found between the reactivity of hydroxyanthraquinones and model quinones with single-electron transferring flavoenzymes ferredoxin: $NADP^+$ reductase and NADPH: cytochrome P-450 reductase, and their cytotoxicity. This points to the importance of oxidative stress in the toxicity of hydroxyanthraquinones in these cell lines, which was further evidenced by the protective effects of desferrioxamine and the antioxidant *N*,*N*′-diphenyl-*p*-phenylene diamine, by the potentiating effects of 1,3-bis-(2-chloroethyl)-1-nitrosourea, and an increase in lipid peroxidation.

Introduction

Natural 1,8-dihydroxyanthraquinones rhein, danthron, emodin (Fig. 1) are used in pharmacy as laxatives, other hydroxylated anthraquinones are used in industry as textile dyes or food colourants, e. g. carminic acid (Fig. 1). Although hydroxylated anthraquinones were considered as potential antitumour agents (Koyama *et al.*, 2001), their intake may be associated with increased colon cancer and adenoma risk (Schörkhuber *et al.*, 1998). These compounds also possess immunosuppressive (Huang *et al.*, 1992), photosensitizing (Rahimipour *et al.*, 2001), mutagenic and cytotoxic properties (Mian *et al.*, 1991; Müller *et al.*, 1996). The enzymatic redox cycling and subsequent oxidative stress (Mian *et al.*, 1991; Bironaitė and Öllinger 1997, Kågedal *et al.*, 1999), the inhibition of topoisomerase II (Müller *et al.*, 1996) and protein kinase C (Chan *et al.*, 1993), and the inhibition of glycolysis and mitochondrial functions (Floridi *et al.*, 1989, 1990) are considered as potential mechanisms of the cytotoxicity of hydroxyanthraquinones. The relative importance of these mechanisms is insufficently understood so far, although it may be important in view of the diverse fields of hydroxy-anthraquinone use, as well as of some similarity of their structure to other important quinones, anthracycline antibiotics adriamycin and



Danthron: R_1= -H, R_2 = -H
Rhein: R_1= -COOH, R_2 = -H
Chrysophanol: R_1 = -CH_3, R_2 = -H
Emodin: R_1= -CH_3, R_2 = -OH

Carminic acid

Fig. 1. The structures of hydroxyanthraquinones used in this study.

Abbreviations: E^1_7, single-electron reduction midpoint potential at pH 7.0; cL_{50}, the concentration of compound for 50% cell survival; FNR, ferredoxin:$NADP^+$ reductase; P-450R, NADPH: cytochrome P-450 reductase; Q, quinone; k_{cat}, catalytic constant; $k k_{cat}/K_m$, bimolecular rate constant; DPPD, *N*,*N*′-diphenyl-*p*-phenylene diamine; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea.

daunorubicin, whose mechanisms of cytotoxicity remain the subject of considerable controversy (Gewirtz, 1999).

Frequently, the aerobic cytoxicity of quinones or nitroaromatic compounds, the other important group of prooxidants, increases with an increase in their single-electron reduction midpoint potential at pH 7.0 (E^1_7) with a relationship $\Delta\log cL_{50}/\Delta E^1_7 \sim -10\ V^{-1}$, where cL_{50} is the concentration of compound for 50% cell survival (Guissany *et al.*, 1990; O'Brien, 1991). This points to the oxidative stress as to the main factor of cytotoxicity, since, as a rule, the rates of single-electron reduction of quinones or nitroaromatics by flavoenzymes initiating redox cycling increase with E^1_7 of oxidants (Butler and Hoey, 1993; Anusevičius *et al.*, 1997). However, the studies of natural hydroxyanthraquinones in this direction are hampered by the absence of E^1_7 values for a number of important compounds (Wardman, 1989; Rath *et al.*, 1996).

In this work, we have compared the cytotoxicity of several natural hydroxyanthraquinones (Fig. 1) with their reactivity towards single-electron transferring flavoenzymes NADPH:cytochrome P-450 reductase (EC 1.6.2.4) and ferredoxin: $NADP^+$ reductase (EC 1.18.1.2). Taken together with the analogous data of the model quinone compounds with available E^1_7 values, our results demonstrate that the oxidative stress may be the main factor of the cytotoxicity of hydroxyanthraquinones.

Materials and Methods

Hydroxyanthraquinones (Fig. 1) and other reagents were obtained from Sigma or Aldrich, and used as received. The kinetic measurements were carried out spectrophotometrically using a Hitachi-557 spectrophotometer in 0.1 M K-phosphate buffer (pH 7.0) containing 1 mM EDTA at 25 °C. NADPH: cytochrome P-450 reductase (P-450R) from pig liver was prepared as described previously (Yasukochi and Masters, 1976), the enzyme concentration was determined using $\varepsilon_{460} = 22\ mM^{-1}cm^{-1}$. The activity of P-450R using 50 μM cytochrome *c* as an electron acceptor (NADPH concentration, 100 μM) was 77 μmol mg^{-1} min^{-1}. The reduction of cytochrome *c* was monitored using $\Delta\varepsilon_{550} = 20\ mM^{-1}cm^{-1}$. Ferredoxin: $NADP^+$ reductase (FNR) from *Anabaena* was prepared as described previously (Pueyo and Gomez-Moreno, 1991) and was a generous gift of Dr. M. Martinez-Julvez and Professor C. Gomez-Moreno (Zaragoza University, Spain). The enzyme concentration was determined using $\varepsilon_{459} = 9.4\ mM^{-1}cm^{-1}$. The activity of FNR using 1 mM ferricyanide as electron acceptor (concentration of NADPH, 200 μM) was equal to 330 μmol mg^{-1} min^{-1}. The catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of quinone reduction correspond to the reciprocal intercepts and slopes of plots [E]/v *vs.* 1/[Q], where [E] is the enzyme concentration, and [Q] is the concentration of quinone. k_{cat} is the number of NADPH molecules oxidized by the single active center of an enzyme per second. The rate of oxygen consumption during enzymatic reactions was monitored using a Clark electrode.

The culture of bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) was grown and maintained in Eagle's medium supplemented with 10% fetal bovine serum at 37 °C as described previously (Nemeikaitė and Čėnas, 1993). HL-60, a human promyelocytic leukemia cell line, was cultured in RPMI-1640 medium with 10% fetal serum (Dičkancaitė *et al.*, 1997). In the cytotoxicity experiments, cells (3.0×10^4/ml, FLK, or 3.0×10^5/ml, HL-60) were grown in the presence of various amounts of quinones for 24 h, and counted using a hematocytometer with viability determined by exclusion of Trypan blue. Before the count, FLK cells were trypsinized. After the 24 h incubation of the cells with quinones, lipid peroxidation was monitored according to the formation of malondialdehyde, using the thiobarbituric acid test (Ramanathan *et al.*, 1994).

Results and Discussion

The single-electron reduction of quinones by flavoenzymes ferredoxin: $NADP^+$ reductase and NADPH:cytochrome P-450 reductase is extensively documented (Butler and Hoey, 1993; Anusevičius *et al.*, 1997;). Therefore, we used FNR and P-450R as model systems for the evaluation of the redox cycling activity of natural hydroxyanthraquinones. Their reduction by FNR and P-450R was analogous to the single-electron reactions of other low-potential quinones under aerobic conditions, i.e., quinones oxidized excess NADPH, with the

consumption of a stoichiometric amount of O_2 per mole of NADPH. For example, in the presence of 150 μM NADPH and 20 nM P-450R, 10 μM 5-hydroxy-, 5,8-dihydroxy-, or 2-methyl-1,4-naphthoquinone, or 9,10-phenanthrene quinone oxidized 50–70 μM NADPH in 1.5 min, whereas 10 μM danthron, rhein, emodin, chrysophanol, or carminic acid oxidized 30–60 μM NADPH in 50 min. FNR catalyzed the reduction of added cytochrome *c* (50 μM) by hydroxyanthraquinones, at the rate of 180–190% NADPH oxidation rate. The reduction of cytochrome *c* was inhibited by 80–90% by 30 μg/ml superoxide dismutase. Taken together, our data show that natural hydroxyanthraquinones undergo P-450R- and FNR-catalyzed redox cycling with the formation of superoxide (reactions (1,2), where Q is quinone, and $Q^{\cdot-}$ is semiquinone):

$$2Q + NADPH \xrightarrow{\text{P-450R, FNR}} 2Q^{\cdot-} + NADP^+ + H^+ \quad (1)$$

$$Q^{\cdot-} + O_2 \longrightarrow Q + O_2^{\cdot-} \quad (2)$$

We were unable to determine the k_{cat} values of the reaction, since at their concentrations above 30 μM, danthron, chrysophanol and carminic acid inhibited P-450R, and emodin, chrysophanol and danthron inhibited FNR. In the other cases, the reaction rates followed the linear dependence on the hydroxyanthraquinone concentrations up to 150–200 μM. The molecular reduction rate constants (k_{cat}/K_m) of hydroxyanthraquinones by FNR and P-450R are given in Table I, together with k_{cat}/K_m of model quinones (partly determined in the present study, and partly taken from our previous work (Anusevičius *et al.*, 1997)). Among hydroxyanthraquinones used in this study, only the E^1_7 value of danthron has been available so far (Table I). It is evident that in the reactions with P-450R and FNR, danthron follows the same parabolic log k_{cat}/K_m dependence on E^1_7 as other quinones (Fig. 2). The k_{cat}/K_m of rhein, carminic acid, chrysophanol, and emodin are close to or below the k_{cat}/K_m of danthron and above the k_{cat}/K_m of 2-hydroxy-1,4-naphthoquinone with E^1_7 = −0.41 V (Table I).

For the cytotoxicity experiments, we used the bovine leukemia virus-transformed lamb kidney fibroblast line FLK used in our previous studies (Nemeikaitė and Čėnas, 1993; Čėnas *et al.*, 2001), and HL-60, a human promyelocytic leukemia cell line. Table I shows the concentrations of hydroxyanthraquinones for 50% cell survival (cL_{50}), and the cL_{50} values for model compounds (partly determined in the present study, and partly taken from our previous works (Nemeikaitė and Čėnas, 1993; Dičkancaitė *et al.*, 1997)). It is evident, that the cytotoxicity of model quinone compounds and danthron towards both cell lines increases with an increase in their E^1_7 (Fig. 3A). The linear dependences of log cL_{50} vs. E^1_7 are characterized by r^2 = 0.9592 (FLK), and r^2 = 0.9040 (HL-60). For the quantitative analysis of the cytotoxicity of hydroxyanthraquinones with unavailable values of E^1_7, we applied the approach used in our previous work for the description of the proxidant cytotoxicity of nitroaromatic explosives with unavailable E^1_7 (Čėnas *et al.*, 2001), namely the geometrical mean of the reactivity of hydroxyanthraquinones and model quinone compounds in FNR- and P-450R-catalyzed reactions (log k_{cat}/K_m (FNR) + log k_{cat}/K_m (P-450R)/2 (Table I) as a correlation parameter. It is evident, that the low cytotoxicity of hydroxyanthraquinones is consistent with their low redox cycling ability (Fig. 3B). For all the examined compounds, the linear dependences of log cL_{50} vs. (log k_{cat}/K_m (FNR) + log k_{cat}/K_m (P-450R)/2 are characterized by r^2 = 0.9492 (FLK), and r^2 = 0.8838 (HL-60). The antioxidant N,N′-diphenyl-*p*-phenylene diamine (DPPD) (Miccadei

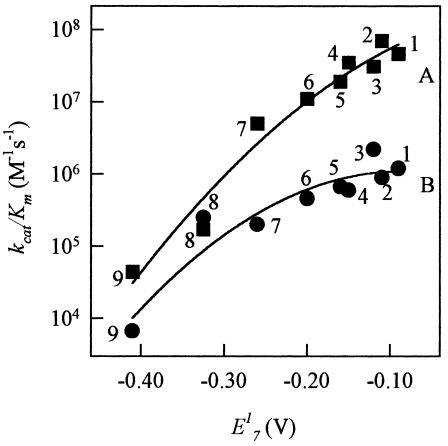


Fig. 2. The dependence of bimolecular rate constants of reduction (k_{cat}/K_m) of model quinones and danthron by NADPH:cytochrome P-450 reductase (A) and ferredoxin:$NADP^+$ reductase (B) on their single-electron reduction midpoint potentials (E^1_7). The numbers of compounds are taken from Table I.

Table I. Midpoint potentials of single-electron reduction of quinones (E^1_7), their bimolecular rate constants of reduction (k_{cat}/K_m) by ferredoxin:NADP$^+$ reductase (FNR) and NADPH:cytochrome P-450 reductase (P-450R), and quinone concentrations for 50% survival of FLK and HL-60 cells during a 24-h incubation (cL_{50}).

No. Compound	E^1_7 [V][a]	k_{cat}/K_m [$M^{-1}s^{-1}$] a) FNR[b]	b) P-450R	cL_{50} [μM] a) FLK	b) HL-60
1. 5-Hydroxy-1,4-naphthoquinone	−0.09	$1.2 \pm 0.1 \times 10^6$	$4.6 \pm 0.3 \times 10^7$	0.50 ± 0.10	1.70 ± 0.20[c]
2. 5,8-Dihydroxy-1,4-naphthoquinone	−0.11	$9.0 \pm 1.0 \times 10^5$	$7.0 \pm 0.6 \times 10^7$	0.11 ± 0.02[d]	1.05 ± 0.20[c]
3. 9,10-Phenanthrene quinone	−0.12	$2.2 \pm 0.1 \times 10^6$	$3.1 \pm 0.4 \times 10^7$	0.70 ± 0.08	3.00 ± 0.27
4. 1,4-Naphthoquinone	−0.15	$6.0 \pm 0.7 \times 10^5$	$3.5 \pm 0.2 \times 10^7$	1.60 ± 0.10	1.13 ± 0.15
5. 2-Methyl-5-hydroxy-1,4-naphthoquinone	−0.16	$6.7 \pm 0.7 \times 10^5$	$1.9 \pm 0.1 \times 10^7$	1.50 ± 0.10	3.60 ± 0.30[c]
6. 2-Methyl-1,4-naphthoquinone	−0.20	$4.6 \pm 0.3 \times 10^5$	$1.1 \pm 0.1 \times 10^7$	3.50 ± 0.30	55.0 ± 12.0
7. Tetramethyl-1,4-benzoquinone	−0.26	$2.0 \pm 0.2 \times 10^5$	$5.0 \pm 0.2 \times 10^6$	16.0 ± 3.0[d]	61.0 ± 11.0
8. Danthron	−0.325[e]	$2.5 \pm 0.2 \times 10^5$	$1.7 \pm 0.1 \times 10^5$	120 ± 15.0	150 ± 12.0
9. 2-Hydroxy-1,4-naphthoquinone	−0.41	$6.7 \pm 0.4 \times 10^3$	$4.4 \pm 0.3 \times 10^4$	700 ± 100	1000 ± 120[c]
10. Rhein	–	$3.3 \pm 0.2 \times 10^5$	$6.7 \pm 0.4 \times 10^4$	150 ± 20.0	100 ± 10.0
11. Emodin	–	$1.3 \pm 0.1 \times 10^4$	$1.3 \pm 0.1 \times 10^5$	155 ± 15.0	120 ± 11.0
12. Chrysophanol	–	$1.5 \pm 0.1 \times 10^4$	$2.5 \pm 0.3 \times 10^5$	150 ± 15.0	178 ± 19.0
13. Carminic acid	–	$2.0 \pm 0.2 \times 10^4$	$4.8 \pm 0.3 \times 10^4$	350 ± 52.0	720 ± 81.0

[a] From Wardman (1989).
[b] Bimolecular rate constants of reduction of compounds 1–7,9 taken from Anusevičius *et al.* (1997).
[c] From Dičkancaitė *et al.* (1997).
[d] From Nemeikaitė and Čėnas (1993).
[e] From Rath *et al.* (1996).

et al., 1988) and the iron-chelating agent desferrioxamine, the latter preventing the Fenton reaction, gave significant protection from the toxicity of danthron to FLK cells (Fig. 4A), and rhein and emodin to HL-60 cells (data not shown). This is analogous to the protective effects of DPPD and desferrioxamine against the cytotoxicity of model quinones and rhein to rat hepatocytes (Öllinger and Brunmark, 1991; Bironaitė and Öllinger, 1997), and model quinones to HL-60 cells (Dičkancaitė *et al.*, 1997). 1,3-Bis-(2-chloroethyl)-1-nitrosourea (BCNU), which inactivates the antioxidant flavoenzyme glutathione reductase (EC 1.6.4.2) and depletes intracellular reduced glutathione (Öllinger and Brunmark, 1991), potentiated the toxicity of danthron (Fig. 4B). The other evidence of oxidative stress was an increase in the intracellular content of the lipid peroxidation product malondialdehyde. After 24 h incubation with 150 μM danthron or 500 μM carminic acid that resulted in the death of 80–85% FLK cells, the content of malondialdehyde was equal to 2.1 ± 0.4 nmol/10^6 cells, whereas in the untreated cells it was equal to 0.5 ± 0.1 nmol/10^6 cells.

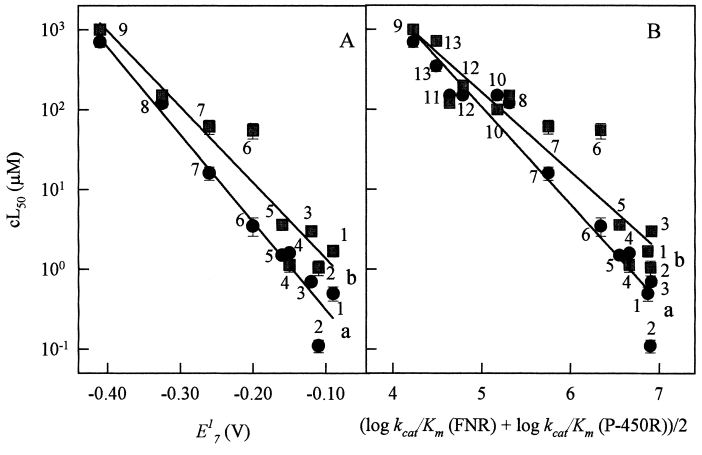


Fig. 3. (A) The dependence of cytotoxicity of model quinones and danthron to FLK (a) and HL-60 cells (b) on the single-electron reduction midpoint potential of quinones (E^1_7). (B) The dependence of cytotoxicity of model quinones and natural hydroxyanthraquinones to FLK (a) and HL-60 cells (b) on their reactivitity towards ferredoxin:NADP$^+$ reductase and NADPH: cytochrome P-450 reductase ((log k_{cat}/K_m (FNR) + log k_{cat}/K_m (P-450R)/2). The numbers of compounds are taken from Table I.

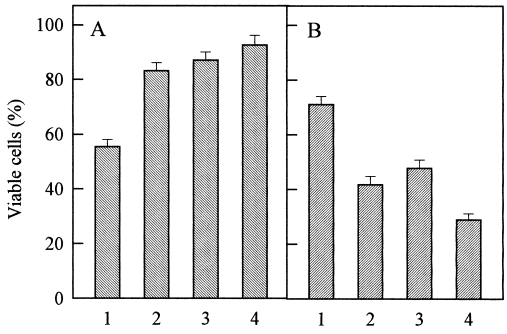


Fig. 4. (A) The protecting effects of DPPD (2 μM) and desferrioxamine (300 μM) in the cytotoxicity of 100 μM danthron to FLK cells. Additions: danthron (1), danthron + DPPD (2), danthron + desferrioxamine (3), danthron + DPPD + desferrioxamine. (B) The potentiating effects of 25 μM BCNU in the cytoxicity of danthron to FLK cells. Additions: 50 μM danthron (1), 50 μM danthron + BCNU (2), 100 μM danthron (3), 100 μM danthron + BCNU (4). Cell viability in control experiments, 96 ± 3%; DPPD, desferrioxamine and BCNU decreased cell viability by 1–3%, $n = 3–4$.

Although the involvement of oxidative stress in the cytotoxicity of rhein and other dihydroxyanthraquinones has been demonstrated in previous studies (Mian *et al.* 1991; Bironaitė and Öllinger 1997, Kågedal *et al.*, 1999), their redox cycling properties and cytotoxicity were not compared to other quinones with available E^1_7 values. This leads to the uncertainty of the relative importance of oxidative stress and other potential cytotoxicity mechanisms. For example, although daunorubicin-induced apoptosis and necrosis in HL-60 cells are partly prevented by DPPD and desferrioxamine, the comparison with model quinones shows that daunorubicin is much more toxic than one may expect from its low reduction potential, −0.34 V (Dičkancaitė *et al.*, 1997). It indicates that in addition to the oxidative stress (Powis, 1989), the other mechanisms of daunorubicin cytotoxicity, e.g. topoisomerase inhibition (Gewirtz, 1999), may be equally or even more important. In the present study, the correlations between the cytotoxicity and the redox cycling ability of natural hydroxyanthraquinones and model quinones with a wide range of E^1_7 values (Fig. 3A,B), supported by the effects of the antioxidants and BCNU (Fig. 4A,B), show that the toxicity of hydroxyanthraquinones to FLK and HL-60 cells is mainly determined by their redox cycling reactions with formation of superoxide (reactions (1,2), and, subsequently, other reactive oxygen species like H_2O_2 and hydroxyl radical, $OH^{\cdot}$ (Powis, 1989; Hippeli and Elstner, 1997). It appears that the rate constants of the single-electron enzymatic reduction of natural hydroxyanthraquinones as well as of other groups of prooxidant compounds with unavailable E^1_7 values may serve as a useful tool for the quantitative description of their cytotoxity with the involvement of oxidative stress.

Acknowledgements

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