Redox Properties of Novel Antioxidant 5,8-Dihydroxycoumarin: Implications for its Prooxidant Cytotoxicity

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The aim of this work was to characterize the redox properties of the new antioxidant 5,8-dihydroxycoumarin (5,8-DHC), isolated from sweet grass (*Hierochloë odorata* L.), and to determine its impact on its cytotoxic action. Reversible electrochemical oxidation of 5,8-DHC at pH 7.0 was characterized by the midpoint potential ($E_{p/2}$) of 0.23 V vs. the normal hydrogen electrode. 5,8-DHC was slowly autoxidized at pH 7.0, and it was active as a substrate for peroxidase (POD, EC 1.11.1.7) and tyrosinase (TYR, EC 1.14.18.1). Oxidation of 5,8-DHC by POD/H_2O_2 yielded the product(s) which reacted with reduced glutathione and supported the oxidation of NADPH by ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2) and NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2). The concentration of 5,8-DHC for 50% survival of bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) during a 24-h incubation was (60 ± 5.5) μM. Cytotoxicity of 5,8-DHC was decreased by desferrioxamine, catalase, the antioxidant *N*,*N*'-diphenyl-*p*-phenylene diamine, and potentiated by 1,3-bis-(2-chloroethyl)-1-nitrosourea and dicumarol, an inhibitor of NQO1. This shows that 5,8-DHC possesses the oxidative stress-type cytotoxicity, evidently due to the action of quinodal oxidation product(s). The protective effect of isoniazide, an inhibitor of cytochrome P-450 2E1, points to hydroxylation of 5,8-DHC as additional toxification route, whereas the potentiating effect of 3,5-dinitrocatechol, an inhibitor of catechol-*o*-methyltransferase (COMT, EC 2.1.1.6), points to the *o*-methylation of hydroxylation products as the detoxification route.

Key words: Hydroxycoumarins, Antioxidants, *Hierochloë odorata* L.

Introduction

Natural polyphenolic compounds are considered to be dietary antioxidants. This property is derived from their free radical and activated oxygen scavenging ability (Rice-Evans *et al.*, 1996; Bors and Michel, 2002). However, apart from their beneficial effects, these compounds show a wide and contradictory spectrum of action, involving antitumor, antiviral, antibacterial, cardioprotective, pro- and antimutagenic activity (Williams *et al.*, 2004, and references therein). Interestingly, polyphenols possess the oxidative stress-type cytotoxicity in mammalian cells (Dičkancaitė *et al.*, 1998; Sergedienė *et al.*, 1999; Awad *et al.*, 2002; Moridani *et al.*, 2003). This is attributed to the polyphenol autoxidation in cell growth media with the production of extracellular H_2O_2 (Nakagawa *et al.*, 2004, and references therein), intracellular generation of reactive oxygen species (Galati and O'Brien, 2004, and references therein), and depletion of intracellular reduced glutathione (GSH) or other –SH compounds by their quinone/quinomethide-type oxidation products (Metodiewa *et al.*, 1999; Boersma *et al.*, 2000).

Recently, natural and synthetic hydroxycoumarins emerged as a promising group of antioxidant compounds (Bailly *et al.*, 2004). These compounds are also considered as potential anticancer agents

Abbreviations: 5,8-DHC, 5,8-dihydroxycoumarin; POD, peroxidase; TYR, tyrosinase; FNR, ferredoxin:$NADP^+$ reductase; NQO1, DT-diaphorase; COMT, catechol-*o*-methyltransferase; $E_{p/2}$, midpoint potential; NHE, normal hydrogen electrode; SCE, saturated calomel electrode; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant; GSH, reduced glutathione; cL_{50}, compound concentration for 50% cell survival; DPPD, *N*,*N*'-diphenyl-*p*-phenylene diamine; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea.

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(Kawaii *et al.*, 2001; Chu *et al.*, 2001; Finn *et al.*, 2002; Kim *et* al., 2005; Yim *et al.*, 2005). The mechanisms of mammalian cell cytotoxicity and/or apoptosis induction by hydroxycoumarins involve the inhibition of kinases, activation of caspases, and mobilization of cytochrome *c* from mitochondria (Chu *et al.*, 2001; Kim *et al.*, 2005; Yim *et al.*, 2005). However, the possibility of the prooxidant events in the cytotoxicity of hydroxycoumarins has not been addressed.

Recently, a new representative of hydroxycoumarins, 5,8-dihydroxycoumarin (5,8-DHC), has been isolated from sweet grass (*Hierochloë odorata* L.), and has been shown to be the main antioxidant substance of this plant (Bandonienė *et al.*, 2000; Pukalskas *et al.*, 2002). The redox properties, enzymatic reactions, and cytotoxicity of this compound has not been studied so far. The aim of this work was to characterize the redox properties and mammalian cell cytotoxicity of 5,8-DHC with emphasis on the possibility of the oxidative stress-type cytotoxicity.

Materials and Methods

5,8-DHC was purified from *Hierochloë odorata* as described (Pukalskas *et al.*, 2002) and a yellow crystalline material with m.p. of 216 °C has been obtained. Ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2) from *Anabaena* was prepared as described by Pueyo and Gomez-Moreno (1991); it was a generous gift of Professor C. Gomez-Moreno (Zaragoza University, Spain). Rat liver NAD(P)H:quinone reductase (DT-diaphorase, NQO1, EC 1.6.99.2) was prepared as described by Prochaska (1988). Mushroom tyrosinase (TYR, EC 1.14.18.1), horseradish peroxidase (POD, EC 1.11.1.7), NADPH and other compounds were obtained from Sigma and used as received. The enzyme concentrations were determined spectrophotometrically using ε_{459} = 9.4 mM^{-1} cm^{-1} (FNR), ε_{460} = 11 mM^{-1} cm^{-1} (NQO1), ε_{402} = 102 mM^{-1} cm^{-1} (POD), and ε_{345} = 8.5 mM^{-1} cm^{-1} (TYR).

Enzymatic reactions were studied spectrophotometrically by the use of a Hitachi-557 UV-VIS spectrophotometer in 0.1 M K-phosphate (pH 7.0) with 1 mM EDTA at 25 °C. The activity of FNR using 1 mM ferricyanide as electron acceptor (the concentration of NADPH, 200 mM) was determined using $\Delta\varepsilon_{420}$ = 1.0 mM^{-1} cm^{-1}, and it was equal to 330 μmol mg^{-1} min^{-1}. The activity of NQO1 determined according to the rate of menadione-mediated reduction of 50 μM cytochrome *c* (concentration of NADPH, 100 μM; concentration of menadione, 10 μM) was equal to 3300 mmol mg^{-1} min^{-1}. To characterize the spectral changes during enzymatic oxidation, the spectra of 50–200 μM 5,8-DHC were recorded at 250–600 nm before and after the addition of 0.1 μM POD and 500 μM H_2O_2 (incubation time, 5 min). The most pronounced absorbance decrease was observed at 300 nm, giving $\Delta\varepsilon_{300}$ = 5.05 mM^{-1} cm^{-1}. Catalytic constants (k_{cat}) and bimolecular rate constants of oxidation of 5,8-DHC (k_{cat}/K_m) were determined from the plots [E]/*v vs.* 1/[5,8-DHC], where [E] stands for the enzyme concentration, and *v* stands for the reaction rate. k_{cat} is the number of molecules of 5,8-DHC oxidized by a single active center of enzyme per second. In POD-catalyzed reactions, 200 μM H_2O_2 was used as oxidant. The oxidation of catechol (0.1–1.0 mM) by tyrosinase was monitored according to the oxygen consumption rate, using a Clark electrode.The activity of the oxidation product(s) of 5,8-DHC in supporting FNR-or NQO1-catalyzed NADPH oxidation was studied as follows: 5,8-DHC (10–250 μM) was oxidized by 0.1 μM POD and 600 μM H_2O_2. After the reaction, excess H_2O_2 was decomposed by 50 U/ml catalase. Subsequently, 100 μM NADPH and 40 nM FNR or NQO1 were introduced into the reaction mixture, and the NADPH oxidation rate was determined using $\Delta\varepsilon_{340}$ = 6.2 mM^{-1} cm^{-1}.

Cyclic voltammograms were recorded using a PA2 polarograph and a 4103-XY recorder (Laboratorni Pristroje, Czech Republic) under anaerobic conditions in 0.1 M K-phosphate (pH 7.0) with 1 mM EDTA at 25 °C (Sergedienė *et al.*, 1999). A glassy carbon SU-1500 electrode served as a working electrode, a saturated calomel electrode [SCE, 0.244 V *vs.* normal hydrogen electrode (NHE)] was used as the reference, while a Pt electrode (56 mm^2) was used as an auxiliary electrode.

Bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) were grown and maintained as described (Dičkancaitė *et al.*, 1998). Cells (2.5×10^4/ml) were grown in 5-ml glass flasks in Eagle's medium with 10% fetal bovine serum and antibiotics at 37 °C for 24 h in the absence of the compounds examined and in their presence. The amount of viable (adherent) cells was calculated under a light microscope. The adherent cells were found by 98–99% viable using the Trypan blue

exclusion test. In contrast, the detached cells were found by 98–99% nonviable.

Statistical analysis was performed using Statistica (version 4.3) software (StatSoft Inc., 1993). The octanol/water partition coefficients (log *P*) were calculated using an ACD logP (version 1), a generous gift of Advanced Chemistry Development Inc. (Toronto, Ont., Canada).

Results and Discussion

In this work we examined the following redox properties of 5,8-DHC which may be relevant to its antioxidant activity and/or the oxidative stress-type cytotoxicity: i) the assessment of midpoint potential; ii) the enzymatic oxidation by peroxidase (POD) and tyrosinase (TYR) [these enzymes are used as model systems to generate the quinone/quinometide products of oxidation of polyphenols (Metodiewa *et al.*, 1999; Boersma *et al.*, 2000; Moridani *et al.*, 2001)]; and iii) the ability of 5,8-DHC oxidation product(s) to support the enzymatic oxidation of NADPH, catalyzed by ferredoxin:NADP$^+$ reductase (FNR) and NAD(P)H:quinone reductase (NQO1). FNR is used as a model enzyme to study the single-electron reduction of quinones to their anion-radicals and their redox cycling (Anusevičius *et al.*, 1997; Čėnas *et al.*, 2004). NQO1 performs two-electron reduction of quinones to hydroquinones in mammalian cells, and may protect from the cytotoxicity of quinone-type products of polyphenol oxidation (Moridani *et al.*, 2001, 2003).

Cyclic voltammograms of 5,8-DHC show the reversible oxidation and subsequent re-reduction peaks at 0.010 V and – 0.030 V *vs.* SCE, respectively (potential scan rate, 50 mV/s, data not shown). Because both oxidation and reduction peak currents show the linear dependence on the square root of potential scan rate, the electrochemical reactions are controlled by the diffusion. We failed to observe other oxidation or reduction peaks between 0.8 to – 0.5 V *vs.* SCE. Thus, 5,8-DHC is characterized by $E_{p/2}$ = – 0.01 V *vs.* SCE at pH 7.0, which corresponds to $E_{p/2}$ = 0.23 V *vs.* NHE. The latter value is more positive than $E_{p/2}$ = 0.04 V of the structural analogue of 5,8-DHC, 1,4-naphthohydroquinone (Čėnas *et al.*, 2004), possibly due to the electron-accepting properties of the pyrone ring.

Peroxidase oxidizes 5,8-DHC with $k_{cat} \geq 3000\ s^{-1}$, and $k_{cat}/K_m = (3.5 \pm 0.4) \times 10^7\ м^{-1}\ s^{-1}$. Thus 5,8-DHC may be qualified as a very efficient substrate for POD, most probably due to its low oxidation potential (Folkes and Candeias, 1997, and references therein). In contrast, TYR oxidizes 5,8-DHC with $k_{cat} \geq 1\ s^{-1}$, and $k_{cat}/K_m = (2.0 \pm 0.2) \times 10^3\ м^{-1}\ s^{-1}$. These rate constants are much lower than those of the specific substrate for tyrosinase, catechol, $k_{cat} = (222 \pm 15)\ s^{-1}$, and $k_{cat}/K_m = (4.4 \pm 0.2) \times 10^5\ м^{-1}\ s^{-1}$. Low activity of 5,8-DHC in TYR-catalyzed reaction may be explained by the enzyme preference for the *o*-hydroquinone structure (Jolley *et al.*, 1974). Although the identification of the product(s) of 5,8-DHC enzymatic oxidation was beyond the scope of this work, there exists some evidence that quinone-type product(s) may be formed: a) the addition of reduced glutathione (GSH) causes the spectral changes of 5,8-DHC oxidation product(s) (Fig. 1A), showing that quinone-SG adducts may be formed; b) the oxi-

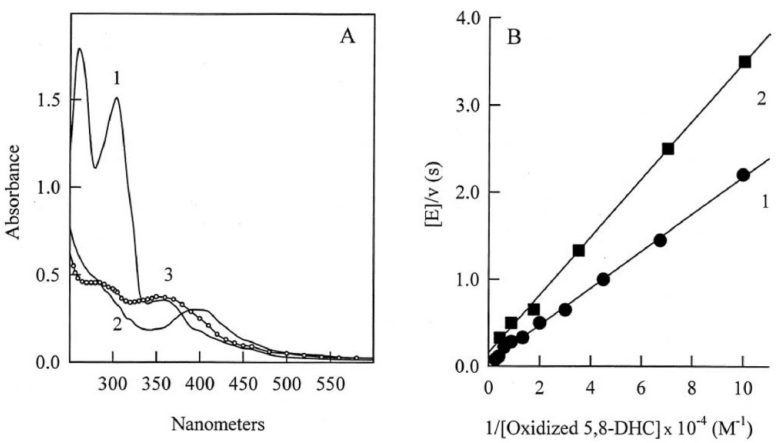

Fig. 1. (A) Spectra of 220 мм 5,8-DHC before oxidation (1), after oxidation by 0.1 μм peroxidase and 600 μм H_2O_2 and addition of catalase (2), and after subsequent addition of 10 мм reduced glutathione (3). (B) Rate of NADPH oxidation in the presence of oxidized 5,8-DHC and ferredoxin:NADP$^+$ reductase (1) or DT-diaphorase (2), pH 7.0, 25 °C.

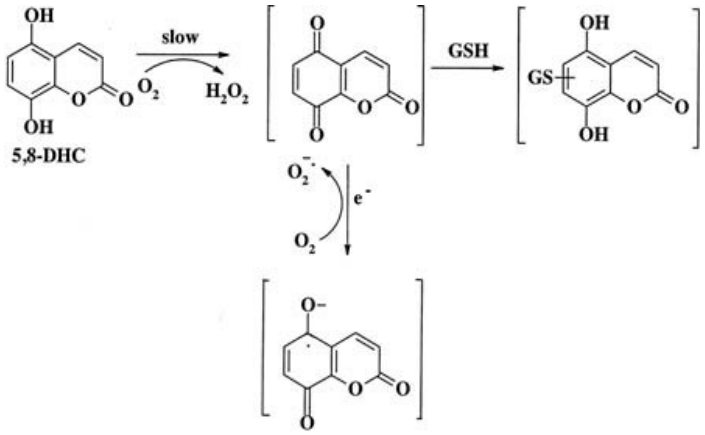

Fig. 2. Proposed scheme of oxidative transformation of 5,8-DHC relevant to its prooxidant cytotoxicity.

dation product(s) are active as substrates for ferredoxin:$NADP^+$ reductase and NQO1 (Fig. 1B), which requires the presence of the quinone structure (Anusevičius *et al.*, 1997; Čėnas *et al.*, 2004). In FNR/NADPH-catalyzed reduction of 5,8-DHC oxidation product(s), more than 3 mol NADPH were oxidized per mol 5,8-DHC. The reaction was accompanied by O_2 consumption. It shows that the single-electron reduction of oxidized 5,8-DHC by FNR leads to its redox cycling, *i.e.*, the reoxidation of free radical formed by oxygen with the formation of superoxide and parent quinone (Ollinger and Brunmark, 1991; Anusevičius *et al.*, 1997, and references therein). It is important to note, that 5,8-DHC slowly autoxidizes in aqueous media, with $t_{1/2}$ ~ 3 h at pH 7.0 and 25 °C, and $t_{1/2}$ ~ 45 min at pH 7.4 and 37 °C. The use of a Clark electrode monitoring the autoxidation of 5,8-DHC showed that the process is accompanied by the formation of H_2O_2, because the addition of 100 U/ml catalase caused the partial return of oxygen. The UV-VIS spectra of the autoxidation product(s) closely resembled those given in Fig. 1A, with similar absorbance changes occurring after the addition of GSH (data not shown). In addition, the product(s) of 5,8-DHC autoxidation supported the FNR- and NQO1-catalyzed NADPH oxidation, although at 3–4 times slower rates than given in Fig. 1B (data not shown). Our findings summarized in Fig. 2, imply that 5,8-DHC may undergo the following reactions relevant to the oxidative stress-type cytotoxicity: a) oxidation of 5,8-DHC with the formation of H_2O_2 and quinone product(s); b) reactions of quinone product(s) with GSH; and c) redox cycling of quinone product(s).

Previously we have shown that flavonoids exhibit the oxidative stress-type cytotoxicity in FLK cells (Dičkancaitė *et al.*, 1998). We found that the concentration of 5,8-DHC for 50% survival of FLK cells (cL_{50}) is equal to (60 ± 5.5) μM (24 h incubation). Antioxidant *N,N'*-diphenyl-*p*-phenylene diamine (DPPD) (Ollinger and Brunmark, 1991) and Fe ion chelator desferrioxamine partly protected against the cytotoxicity of 5,8-DHC (Fig. 3A). Importantly, the cytotoxicity was also decreased by exogenous catalase (Fig. 3A). This shows that the formation of extracellular H_2O_2, *i.e.* the autoxidation of 5,8-DHC in cell culture media, contributes to its cytotoxicity. In contrast, the prooxidant 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) which inactivates the antioxidant flavoenzyme glutathione reductase (EC 1.6.4.3) (Ollinger and Brunmark, 1991), potentiated the cytotoxicity of 5,8-DHC (Fig. 3A). Taken together, these data indicate that 5,8-DHC possesses the oxidative stress-type cytotoxicity in FLK cells. It has been suggested that NOQ1 may protect against their oxidative stress-type cytotoxicity, because the NQO1-catalyzed reduction of their quinone-type oxidation products may divert them from the reactions with GSH and/or redox cycling (Fig. 2) (Moridani *et al.*, 2001, 2003), although this concept has been challenged in a recent study (Boots *et al.*, 2005). We found that the inhibitor of NQO1, dicumarol, potentiated the cytotoxicity of 5,8-DHC (Fig. 3B), thus showing that NQO1 may protect from its cytotoxicity. In control experiments, dicumarol did not affect the cytotoxicity of H_2O_2 [cL_{50} = (50 ± 6.0) μM]. Among the other pathways of polyphenol metabolism, one may mention their cytochrome P-450-catalyzed hydrox-

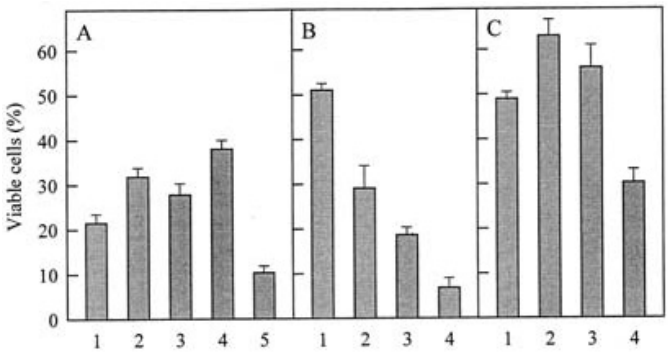

Fig. 3. Effects of antioxidants and prooxidants (A), the inhibitor of DT-diaphorase, dicumarol (B), and the inhibitors of cytochromes P-450 and catechol-*o*-methyltransferase (C) on the cytotoxicity of 5,8-DHC in FLK cells. (A) Additions: 120 μM 5,8-DHC (1), 120 μM 5,8-DHC + 2.5 μM DPPD (2), 120 μM 5,8-DHC + 300 μM desferrioxamine (3), 120 μM 5,8-DHC + 100 U/ml catalase (4), 120 μM 5,8-DHC + 20 μM BCNU (5), $n = 3-4$, $p < 0.05$ for 2, 3 against 1, and $p < 0.01$ for 4, 5 against 1. (B) Additions: 60 μM 5,8-DHC (1), 60 μM 5,8-DHC + 20 μM dicumarol (2), 120 μM 5,8-DHC (3), 120 μM 5,8-DHC + 20 μM dicumarol (4), $n = 3-4$, $p < 0.02$ for 2 against 1, $p < 0.05$ for 4 against 3. (C) Additions: 60 μM 5,8-DHC (1), 60 μM 5,8-DHC + 1 mM isoniazide (2), 60 μM 5,8-DHC + 5 μM α-naphthoflavone (3), 60 μM 5,8-DHC + 5 μM 3,5-dinitrocatechol, $n = 3-4$, $p < 0.05$ for 2 against 1, and $p < 0.005$ for 4 against 1.

ylation (Moridani *et al.*, 2001, 2003). These reactions may contribute to their cytotoxicity, because the hydroxylation of hydroxybenzenes increases their autoxidation rate. In contrast, the catechol-*o*-methyltransferase (COMT, EC 2.1.1.6)-catalyzed methylation of catechol-type hydroxylation products may decrease their cytotoxicity (Moridani *et al.*, 2002). In accordance with this, we found that isoniazide, an inhibitor of cytochrome P-450 2E1, partly protected against the cytotoxicity of 5,8-DHC, whereas the efferct of α-naphthoflavone, an inhibitor of cytochromes P-450 1A1/A2, was less expressed (Fig. 3C). In contrast, a COMT inhibitor, 3,5-dinitrocatechol, potentiated the cytotoxicity (Fig. 3C). It shows that cytochrome P-450-catalyzed hydroxylation of 5,8-DHC is its additional toxification route, whereas the *o*-methylation of its hydroxylation products may be their detoxification route.

In conclusion, the observed oxidative stress-type cytotoxicity of 5,8-DHC in FLK cells (Figs. 1, 3) is in line with the data on the analogous cytotoxicity mechanism of other groups of polyphenolic antioxidants, flavonoids and hydroxybenzenes, in several mammalian cell lines (Dičkancaitė *et al.*, 1998; Sergedienė *et al.*, 1999; Moridani *et al.*, 2001, 2002, 2003; Nakagawa *et al.*, 2004). In FLK cells, the cytotoxicity of flavonoids increased with a decrease in their $E_{p/2}$ and an increase in their lipophilicity, log *P* (Dičkancaitė *et al.*, 1998). The FLK cell cytotoxicity of 5,8-DHC correlated with the cytotoxicity of flavonoids, taking into account the $E_{p/2}$ value for 5,8-DHC, 0.23 V, and its log *P*, 2.85. Our findings also argue for the possible importance of the oxidative stress in the cytotoxicity of other hydroxycoumarins, because 6,7- or 7,8-dihydroxycoumarins, possessing easily an oxidizable catechol structure, displayed an enhanced cytotoxicity in several cell lines as compared to their 7-hydroxy- or 6,7-dimethoxy-analogues (Kawaii *et al.*, 2001; Finn *et al.*, 2002).

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