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Redox properties of chrysanthemic acid esters of hydroxynaphthoquinones: implications for their toxicity in mammalian cells

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Because the chrysanthemic acid derivatives comprise a class of natural insecticides, the synthesis of new compounds of this group and the studies of their action in various cell types deserve certain interest. We have found that the reactivity of juglonyl chrysanthemate and isonaphthazarinyl chrysanthemate towards the model single-electron transferring flavoenzyme ferredoxin : $NADP^+$ reductase and two-electron-transferring DT-diaphorase correlated with the electron-accepting and sterical properties of analogous naphthoquinones. Their toxicity in primary mice splenocytes and bovine leukemia virus-transformed lamb kidney fibroblasts (line FLK) also correlated with the single-electron-transfer properties of model quinones which possess no chrysanthemate substituent. The cytotoxicity was partly protected by antioxidants and potentiated by the prooxidant 1,3-bis-(2-chloroethyl)-1-nitrosourea and by the inhibitor of DT-diaphorase, dicumarol. This implies their 'oxidative stress'-type cytotoxicity. Taken together, these data indicate that the chrysanthemate group may not introduce any additional factor(s) of quinone cytotoxicity in mammalian cells.

Key words: chrysanthemic acid, quinone, mammalian cell cytotoxicity, ferredoxin : $NADP^+$ reductase, DT-diaphorase

Abbreviations: Q, quinone; $Q^{\cdot-}$, semiquinone; FNR, ferredoxin : $NADP^+$ reductase; NQO1, DT-diaphorase; k_{cat}/K_m, bimolecular rate constant in enzymatic steady-state reactions; E_7^1, single-electron reduction potential at pH 7.0; $O_2^{\cdot-}$, superoxide; cL_{50}, compound concentration for 50% cell death.

INTRODUCTION

The esters of 3-(1-isobutenyl)-2,2-dimethylcyclopropanecarboxylic acid (chrysanthemic acid, Fig. 1) are essential building blocks of pyrethroids, a class of natural insecticides [1]. Allethrin (2-methyl-4-oxo-3-(2-propanyl)-2-cyclopenten-1-yl-2-2-dimethyl-3-(2-methyl-1-pro-penylcyclopropane)-carboxylate) is used worldwide against a wide variety of insects involved in vector-borne diseases. Its insecticidal activity is based on the inhibition of Na^+ channels of insects [2]. Several hundred tonnes per year of allethrin and its analogues are manufactured and used throughout the world [3]. In this aspect, it is important to obtain new derivatives of chrysanthemic acid, as well as to study their action mechanisms. Among other approaches, it is interesting to examine the properties of chrysanthemic acid-substituted quinones which may possess both quinone- and chrysanthemate-dependent action modes. Typically, the cytotoxicity of quinones (Q) is caused by their single-electron reduction by flavoenzymes dehydrogenases-electrontransferases, e. g., mammalian NADPH : cytochrome P-450 reductase (P-450R, EC 1.6.2.4) or plant / algal ferredoxin : $NADP^+$ reductase (FNR, EC 1.18.1.2)

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which initiate the redox cycling of their anion-radicals ($Q^{\cdot-}$) with the subsequent formation of superoxide ($O_2^{\cdot-}$) and other activated oxygen species [4, 5]. The resulting 'oxidative stress-type' cytotoxicity increases upon an increase in the single-electron reduction potential of quinones, E^1_7, and should be insignificantly influenced by their particular structure [6]. The toxicity of quinones in mammalian cells may be decreased by the action of flavoenzyme NAD(P)H : quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2), which performs their two-electron reduction to relatively stable hydroquinones conjugated by glucuronic acid or sulphate and are more readily excreted from the cell [4].

In this paper, we analyse the flavoenzyme-catalyzed single- and two-electron reduction of two chrysanthemic acid-substituted naphthoquinones and several their analogues (Fig. 1) as well as the impact of these reactions on their cytotoxicity towards two mammalian cell lines. It has been found that mammalian cell cytotoxicity of chrysanthemate-substituted quinones in general is the same as that of their model compounds which possess no chrysanthemate group, thus making them suitable candidates for further insecticidal studies.

EXPERIMENTAL

Chrysanthemic acid (Fig. 1) and its ethyl ester (the ratio of cis- and trans-isomers, 1 : 1.7), isonaphthazarin (2,3-dihydroxy-1,4-naphthoquinone), juglonyl acetate, juglonyl chrysanthemate (Fig. 1, the ratio of cis- and trans-isomers, 1 : 2), and isonaphthazarinyl chrysanthemate (Fig. 1, trans-isomer) were obtained by to the conventional methods [7, 8]. The structure of the compounds was identified by means of elemental analysis, NMR (Bruker AMX-400), IR (UR-20), and m/s (Finnigan CQ). All other compounds were obtained from Sigma, unless specified otherwise.

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. Ferredoxin : $NADP^+$ reductase (FNR, EC 1.18.1.2) from *Anabaena* was prepared as described [9] and was a generous gift of Dr. Marta Martines-Julvez and Professor Carlos Gomez-Moreno (Zaragoza University, Spain). Recombinant rat NAD(P)H : quinone oxidoreductase (DT-diaphorase, NQO1, EC 1.6.99.2) was obtained from Sigma. The enzyme concentrations were determined spectrophotometrically using ε_{459} = 9.4 mM^{-1} cm^{-1} (FNR), and ε_{460} = 11 mM^{-1} cm^{-1} (NQ01). The rates of FNR- or NQO1-catalyzed oxidation of 100 μM NADPH by quinones corrected for the intrinsic NADPH-oxidase activity of enzymes were determined according to $\Delta\varepsilon_{340}$ = 6.2 mM^{-1} cm^{-1}. The catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of the reduction of compounds were calculated from the Lineweaver–Burk plots. k_{cat} is the number of NADPH molecules oxidized by a single active center of FNR or NQO1 per second.

The primary mice splenocytes were obtained from BALB / c mice as described previously [10]. These experiments were approved by the Lithuanian Veterinary and Food Service (License No. 0171, 2007). The splenocytes (10^6/ml) were suspended in RPMI 1640 medium with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml); their viability was determined after 24 h incubation of the splenocytes with the examined compounds according to the Trypan blue exclusion test. The compounds were dissolved in DMSO whose final concentration in the medium, 0.6%, did not affect the splenocyte viability. 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 and antibiotics as described previously [11]. In the cytotoxicity experiments, cells (2.5×10^4/ml) were seeded on glass slides in 5 ml flasks in the presence or in

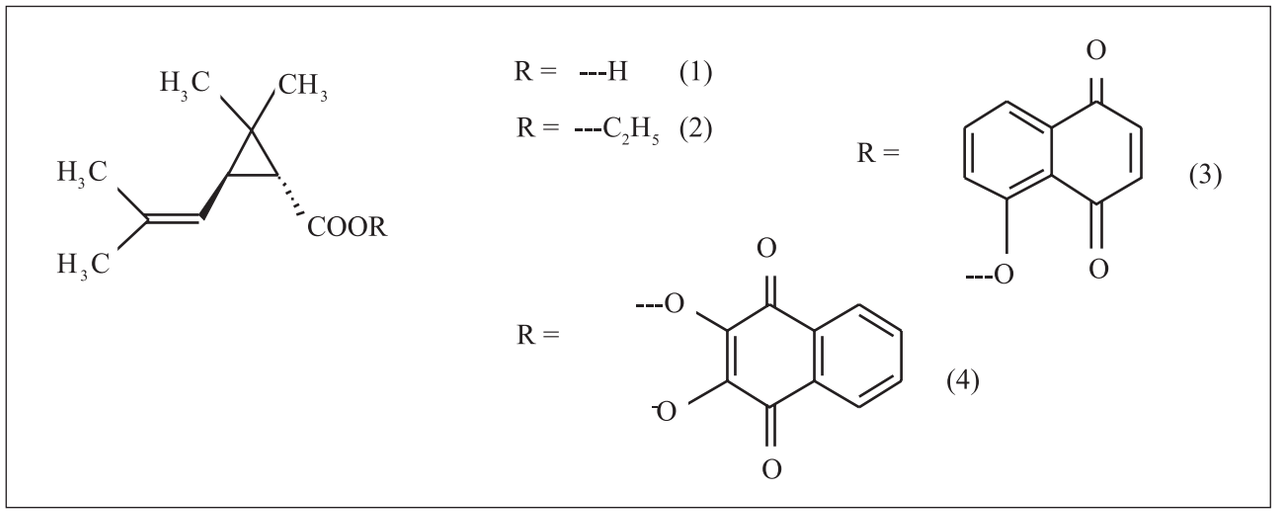

Fig. 1. Structural formulae of derivatives of chrysanthemic acid (trans-isomer): chrysanthemic acid (1), ethyl chrysanthemate (2), juglonyl chrysanthemate (3), and isonaphthazirinyl chrysanthemate (4)

the absence of compounds, and were grown for 24 h. Further, the slides were rinsed 3–4 times by phosphate buffer saline and stained with Trypan blue. The cells on the slides were counted under a light microscope. The statistical analysis was performed using Statistica (version 4.3, StatSoft, 1993).

RESULTS AND DISCUSSION

Because the cytotoxicity of chrysanthemic acid-substituted quinones may be related to their flavoenzyme-catalyzed single- and two-electron reduction, we have examined their coresponding enzymatic reactions. Typically, the reactivity of quinones towards the single-electron-transferring ferredoxin : $NADP^+$ reductase (FNR) increases with an increase in their single-electron reduction potential (E^1_7) with the coefficient $\Delta\log (k_{cat}/K_m) / \Delta E^1_7 \sim 8.0\ V^{-1}$, where k_{cat}/K_m is the bimolecular reaction rate constant not too much specific towards their particular structure [5]. This is in accordance with an 'outer-sphere' mechanism of the single-electron transfer [12]. Thus, FNR may serve as a model system to predict the 'oxidative stress'-type cytotoxicity of quinones in mammalian cells. One may suggest that the E^1_7 values for isonaphthazarine and isonaphthazarinyl chrysanthemate may be close to that of 2-hydroxy-1,4-naphthoquinone, –0.41 V because the presence of the 2(3)-hydroxy- or 2(3)-acetoxy-group in the quinone ring may not too significantly influence the electron-donating properties of the anionic 2(3)-hydroxy group which is the main determinant of the low E^1_7 value for 2-hydroxy-1,4-naphthoquinone (Fig. 1, Table). Besides, the E^1_7 values for juglonyl chysanthemate and juglonyl acetate may be lower than the E^1_7 value for juglone (–0.09 V) and may be similar to that of menadione (2-methyl-1,4-naphthoquinone, E^1_7 = –0.20 V) because the esterification of 5-hydroxy- group disrupts its H-bond with the carbonyl moiety of quinone. This interaction increases the redox potential of quinone / anionic semiquinone couple of corresponding 5(8)-hydroxy-1,4-naphthoquinones, e. g., juglone or naphthazarine, despite the electron-donating properties of the 5(8)-hydroxy- group [13]. In general, the k_{cat}/K_m values of FNR-catalyzed reduction of the investigated compounds (Table) agree with our assumptions. On the other hand, NAD(P)H : quinone oxidoreductase (NQO1) may decrease the toxicity of quinones because it catalyzes their two-electron reduction into relatively stable hydroquinones which are efficiently excreted from the cells by their conjugates [4]. The reactivity of NQO1 also increases with an increase in E^1_7 of quinones; however, it also decreases with an increase in their van der Waals volume [5]. Again, the k_{cat}/K_m values for investigated quinones agree with our suggestions for NQO1-catalyzed reactions [5] because the reactivity of juglone derivatives is higher than those of isonaphthazarine derivatives (Table). However, the reactivity of juglonyl chrysanthemate seems to be slightly higher than expected (Table) because chrysanthemate possesses a larger van der Waals volume than does the ethyl group.

Next, we have studied the cytotoxicity of compounds in primary mice splenocytes and bovine leukemia virus-transformed lamb embryo kidney fibroblasts (line FLK), the latter comprising a reference base for our mammalian cell cytotoxicity studies [6]. We have found that chrysanthemic acid and its ethyl ester are moderately cytotoxic in splenocytes and in FLK cells (Table). The cL_{50} values for juglonyl chrysanthemate and juglonyl acetate in both cell lines are similar to those of juglone or menadione (Table). The cytotoxicity of isonaphthazirinyl chrysanthemate and isonaphthazarine is higher than that of 2-hydroxy-1,4-naphthoquinone, but lower than those of juglone derivatives (Table). This is more or less consistent with their assumed electron-accepting properties and their reactivities towards the model enzyme, FNR [5]. We have also found that juglonyl chrysanthemate exhibited an oxidative stress-type cytotoxicity in FLK cells: its action has been partly prevented by an antioxidant *N, N*-diphenyl-*p*-phenylene diamine and the Fe-ion chelator desferrioxamine, the latter preventing the Fenton reaction. Cytotoxicity has also been potentiated by 1,3-bis-(2-chloroethyl)-1-nitrosourea (Fig. 2) which inactivates the flavoenzyme glutathione reductase (EC 1.6.4.2) [14]. Glutathione reductase serves as a source of an important intracellular antioxidant, reduced glutathione.

Table. **Bimolecular reduction rate constants (k_{cat}/K_m) of the examined quinones by ferredoxin : $NADP^+$ reductase (FNR) and DT-diaphorase (NQO1), their single-electron reduction potentials determined by pulse-radiolysis (E^1_7), and their concentrations for 50% mammalian cell death (cL_{50})**

No.	Compound	k_{cat}/K_m ($M^{-1}s^{-1}$)		E^1_7 (V, vs. NHE)[a]	cL_{50} (μM)	
		a) FNR	b) NQO1		a) Splenocytes	b) FLK
1.	Juglonyl chrysanthemate	$2.5 \pm 0.3 \times 10^5$	$1.0 \pm 0.1 \times 10^7$	–	50 ± 6.5	1.9 ± 0.1
2.	Isonaphthazirinyl chrysanthemate	$5.7 \pm 0.4 \times 10^3$	$5.2 \pm 0.3 \times 10^5$	–	280 ± 40	100 ± 14
3.	Juglonyl acetate	$7.6 \pm 0.6 \times 10^5$	$1.1 \pm 0.1 \times 10^7$	–	75 ± 6.0	1.1 ± 0.1
4.	Isonaphthazarine	$8.3 \pm 1.0 \times 10^4$	$3.7 \pm 0.3 \times 10^6$	–	350 ± 45	63 ± 6.0
5.	Juglone (5-hydroxy-1,4-naphthoquinone)	$1.2 \pm 0.1 \times 10^{6a}$	$2.4 \pm 0.3 \times 10^{8a}$	–0.09	4.0 ± 07	0.50 ± 0.1[b]
6.	2-Methyl-1,4-naphthoquinone	$4.6 \pm 0.5 \times 10^{5a}$	$8.4 \pm 0.7 \times 10^{7a}$	–0.20	10 ± 1.4	3.5 ± 0.3[b]
7.	2-Hydroxy-1,4-naphthoquinone	$6.7 \pm 0.5 \times 10^{3a}$	$5.9 \pm 0.5 \times 10^{6a}$	–0.41	1000 ± 130	700 ± 100[b]
8.	Chrysanthemic acid	–	–	–	1500 ± 100	850 ± 120
9.	Ethyl chrysanthemate	–	–	–	1650 ± 120	720 ± 90

[a] From Ref. [5]. [b] From Ref. [6].

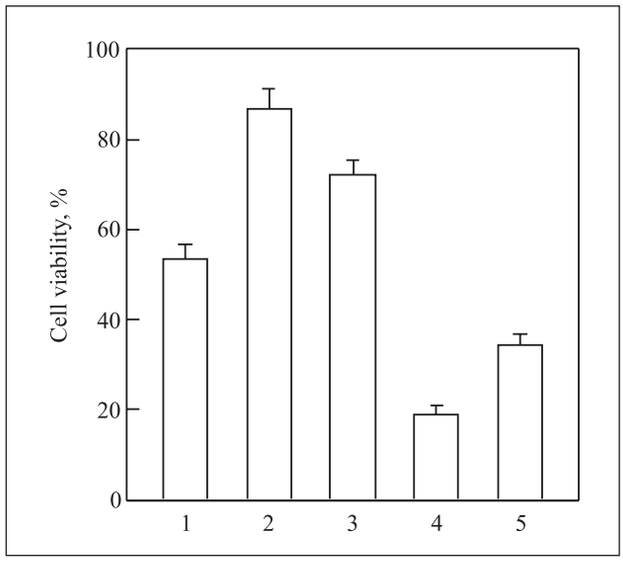

Fig. 2. Viability of FLK cells in the presence of 2.0 μM juglonyl chrysanthemate. Additions: none (1), 3.0 μM *N, N*-diphenyl-*p*-phenylene diamine (2), 300 μM desferrioxamine (3), 20 μM dicumarol (4), or 20 μM 1,3-bis-(2-chloroethyl)-1-nitrosourea (5), $n = 3$, $p < 0.02$ for (1) vs. (2–5)

Besides, cytotoxicity was enhanced by an inhibitor of NQO1, dicumarol (Fig. 2), showing that the two-electron reduction of juglonyl chrysanthemate by NQO1 may be responsible for its detoxification. Analogous effects have been also observed in the action of juglonyl chrysanthemate in splenocytes (data not shown).

CONCLUSIONS

The data of our work show that chrysanthemic acid and its ethyl ester are modestly toxic in two mammalian cell lines. The introduction of a chrysanthemate group into naphthoquinone moiety does not specifically increase their cytotoxicity, which is similar to that of model quinones lacking a chrysanthemate group and possesing similar electron-accepting properties. Thus, chrysanthemic acid esters of hydroxynaphthoquinones deserve further studies as potential insecticidal agents, taking into account their predictable cytotoxicity in mammalian cells.

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CHRIZANTEMŲ RŪGŠTIES HIDROKSINAFTOCHINONŲ ESTERIŲ REDOKS SAVYBĖS: JŲ RYŠYS SU JUNGINIŲ TOKSIŠKUMU ŽINDUOLIŲ LĄSTELĖSE

S a n t r a u k a

Kadangi chrizantemų rūgšties dariniai yra gamtiniai insekticidai, yra svarbu sukurti naujus šios klasės junginius ir ištirti jų poveikį įvairių tipų ląstelėse. Nustatėme, kad juglonil- chrizantemato ir izonaftazarinilchrizantemato reaktingumas flavininio fermento ferredoksin : $NADP^+$ reduktazės katalizuojamoje vienelektroninėje redukcijoje ir DT-diaforazės katalizuojamoje dvielektroninėje redukcijoje koreliavo su kitų naftochinonų, turinčių panašias elektronoakceptorines ir sterines savybes, reaktingumu. Jų toksiškumas pirminiuose pelių splenocituose ir galvijų leukemijos virusu transformuotuose ėriuko inkstų fibroblastuose (linija FLK) taip pat koreliavo su modelinių chinonų, neturinčių chrizantemato grupių, bet pasižyminčių panašiomis elektronoakceptorinėmis savybėmis, toksiškumu. Citotoksiškumas buvo nulemtas oksidacinio streso, kadangi jį slopino antioksidantai, o jį skatino prooksidantas 1,3-bis-(2-chloretil)-1-nitrozokarbamidas ir DT-diaforazės inhibitorius dikumarolas. Šie duomenys rodo, kad chrizantemato grupė chinonų struktūroje nedidina chinonų citotoksiškumo žinduolių ląstelėse.