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Comparison of effects of some cyclic chalcone analogues on selected mitochondrial functions

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In earlier studies, cytotoxity of chalcones (**1**) and cyclic chalcone analogues *E*-2-arylidene-tetralones (**2**) and -benzosuberones (**3**) towards various murine and human tumour cells has been tested. Preliminary biochemical investigations showed the compounds to inhibit protein and DNA syntheses. It was also found that the compounds affect the cellular thiol status of the treated cells. In order to gain new insights into the cytotoxic mechanism of the compounds effects of some previously investigated **2** and **3** derivatives on isolated rat liver mitochondria was investigated. It was found that the most cytotoxic compounds **2c** and **3b** significantly decreased the GSH level of the mitochondria. Incubation of the investigated chalcones with reduced GSH under cell-free conditions indicated spontaneous conjugation (non-redox) reaction at pH 7.4 and pH 9.0. Investigation of antioxidant capacity of the compounds by monitoring time course of the Fenton-reaction initiated *in vitro* degradation of 2-deoxyribose showed the compounds to display hydroxyl radical scavenger activity. Investigation of respiratory control ratio of **2c** and **3b** showed the compounds to display an inhibitory effect on respiration, compound **2b**, however, displayed rather an uncoupling effect. The experiments provide further details of cytotoxic effects of the synthetic chalcones displaying dual – cytotoxic and cytoprotective – effects.

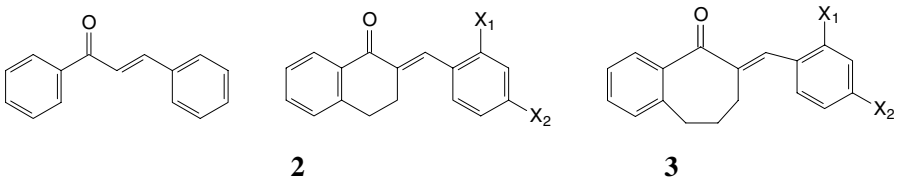
1. Introduction

Chalcones (**1**) are intermediary compounds of the biosynthetic pathway of a very large and widespread group of plant constituents known collectively as flavonoids (Harborne 1986). Among the naturally occurring chalcones (Middleton et al. 2000) and their synthetic analogs (Dimmock et al. 1999a; Go et al. 2005) several compounds displayed cytotoxic (antimitotic, cell growth inhibitor) activity towards cultured tumor cells. Chalcones are also effective *in vivo* as cell proliferating inhibitors, anti-tumor promoting and chemopreventive agents (Middleton et al. 2000; Dimmock et al. 1999a; Go et al. 2005). Chalcones, as α,β-unsaturated carbonyl compounds, have a marked affinity for thiols in contrast to amines (Dimmock et al. 1999a). Since a number of clinically useful anticancer drugs have genotoxic effects due to interaction with the amino groups of nucleic acids, chalcones may be devoid of this important side effect (Dimmock et al. 1999a; Go et al. 2005).

Recently we have investigated *in vitro* antineoplastic activity of several synthetic chalcones and cyclic chalcone analogues. The compounds were evaluated against P388, L1210, Molt 4/C8 and CEM cells as well as against approximately 60 further human tumor cell lines from nine different neoplastic diseases (Dimmock et al. 1999b; 2002; Perjési et al. 2008). Among the compounds investigated *E*-2-(4′-methoxybenzylidene)-1-benzosuberone (**3b**) had the greatest tumor toxicity. Investigation of selectivity of toxicity of *E*-2-(X-benzylidene)-1-benzosuberones to human tumor cell lines compounds **3b** and its methyl substituted analogue **3a** displayed selective toxicity to human breast neoplasms and colon cancers, respectively (Dimmock et al. 1999b).

Structure-activity studies demonstrated that cytotoxicity of the cyclic chalcone analogues were influenced by the shape of the molecules (Dimmock et al. 1999b, 2002; Perjési et al. 2008). Besides the documented tumor cytotoxic effects, some of the above cyclic chalcone analogues were found to display strong CYP1A inhibitor activity (Perjési et al. 2000; Monostory et al. 2003). Accordingly, the compounds represent prototypes of molecules displaying both cytotoxic and cytoprotective (chemopreventive) effects.

Preliminary *in vitro* cytotoxicity studies with Jurkat T cells showed **3a** and **3b** to induce apoptosis and inhibit RNA and protein synthesis (Dimmock et al. 1999b). In a following study cell cycle analysis by flow cytometry of Jurkat T cells exposed to the two compounds were performed. Analysis of the DNA histograms showed that near equitoxic doses of **3a** and **3b** induced apoptosis and aneuploidity. Compound **3b** showed to reduce the total cellular thiol level both under nutrient-free and nutrient-supplemented conditions. Under the latter conditions an increase of the total thiol level of the cells exposed to **3a** could be observed (Rozmer et al. 2006).



1 **2** **3**

a) $X_1 = H,\ X_2 = CH_3$ **b**) $X_1 = H,\ X_2 = OCH_3$ **c**) $X_1 = X_2 = OCH_3$

Cytotoxic effects of α,β-unsaturated carbonyl compounds are frequently associated with their expected reactivity with the essential thiol groups in the living organisms (Dimmock et al. 1999a; Go et al. 2005; Rozmer et al. 2006). Such a reaction can alter intracellular redox status (redox signaling), which can modulate events such as DNA synthesis, enzyme activation, selective gene expression, and regulation of the cell cycle (Powis et al. 1997). An important feature of cells' mitochondrial function is an interaction between three essential cellular processes: oxidative phosphorylation, generation of reactive oxygen species, and initiation of physiological cell death (apoptosis) via mitochondrial permeability transition pores (Wallace 1999). Accordingly, modification of mitochondrial functions by xenobiotics may contribute to their cytotoxic and antineoplastic activities.

As a continuation of our previous studies to investigate cytotoxic mechanisms of the investigated chalcone analoques, in the present work the effect of six tumor cell cytotoxic cyclic chalcone analogs on isolated rat liver mitochondria was investigated. Characterization of mitochondrial respiratory function on enzyme activities as well as redox status of mitochondria administered to compounds of subcytotoxic doses, corresponding to 375 mg/kg/body weight, was conducted. We have investigated effect of the compounds on the reduced glutathione (GSH) content of the exposed mitochondria and characterized *in vitro* reactivity of the compounds with GSH. Since oxidation of hydroxylated metabolites of xenobiotics could generate reactive oxygen species (ROS), the compounds could cause oxidative stress as a result of such a series of reactions (Gregus et al. 2001). To test this possibility we have investigated time dependence of *in vitro* antioxidant (hydroxyl radical scavenger) capacity of the compounds by means of the Fenton-reaction initiated degradation of 2-deoxyribose (Rozmer et al. 2006).

2. Investigations and results

The results of the present study demonstrate that the investigated compounds are able to modify the energy-linked functions of the cells. At the selected concentrations, **2c**, the second most cytotoxic compound tested in this work, displayed an inhibitory effect on ADP-stimulated respiration (state 3) by 27.4%. At the same time, state 4 (controlled respiration) was not affected by the compound (Table 1). On the contrary, compound **3b**, the most cytotoxic compound investigated, had no effect on state 3 respiration, however, increased the rate of oxygen consumption after ADP exhaustion (state 4) by 50.7%. Respiratory control ration (RCR) was significantly decreased by 23.4% and 26.5% with **2c** and **3b**, respectively (Table 1).

In order to clarify whether alteration of oxygen consumption is associated with change in ATP synthesis, activity of the enzyme ATP synthase (ATPase) was investigated. The results of these experiments showed that the dimethoxy substituted derivative **2c** significantly decreased, while **3b** significantly increased ATPase activity. The rest of the compounds practically did not alter activity of the enzyme (Fig.).

Reactivity of the compounds towards cellular thiols was investigated under cell-free conditions. TLC analysis of 1 : 10 molar mixtures of chalcones and GSH incubated at pH 7.4 or pH 9.0 at 50 °C indicated formation of adducts that showed characteristics of both the aromatic and the

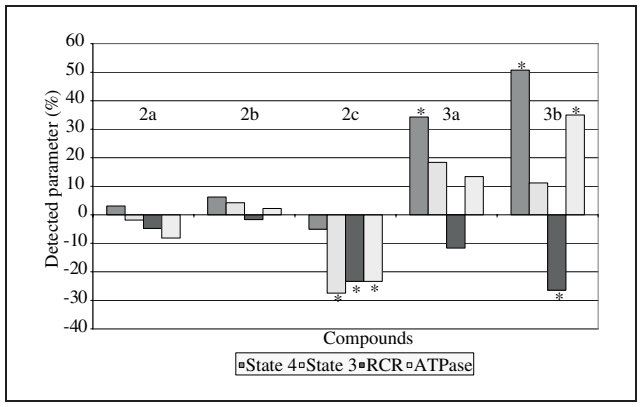


Fig.: Effect of *E*-2-arylidene-1-tetralones (**2a**-**c**) and *E*-2-arylidene-1-benzosuberones (**3a**, **3b**) on mitochondrial functions. (Results are calculated as the relative percentage changes compared to the values of the controls group).
* Mean, significance $p < 0.05$

Table 1: Respiratory functions of mitochondria exposed to *E*-2-arylidene-1-tetralones (2a–c) and *E*-2-arylidene-1-benzosuberones (3a, 3b)

	Toxicity[a] (μM)	O_2 consumption in state 4 (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}{}_p$)	O_2 consumption in state 3 (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}{}_p$)	RCR	ATP-ase (nmol $P_i\ min^{-1} \cdot mg^{-1}{}_p$)
DMSO		11.87 ± 0.83	34.57 ± 0.79	2.91 ± 0.14	0.403 ± 0.017
2a	284.7	12.24 ± 0.32	33.92 ± 1.30	2.77 ± 0.11	0.370 ± 0.014
2b	21.1	12.61 ± 0.69	36.02 ± 0.78	2.86 ± 0.19	0.412 ± 0.015
2c	3.5	11.27 ± 1.04	25.07 ± 1.05*	2.23 ± 0.16*	0.309 ± 0.014*
3a	17.4	15.94 ± 0.66*	40.92 ± 2.78	2.57 ± 0.21	0.457 ± 0.019
3b	0.69	17.89 ± 0.83*	38.44 ± 0.96	2.14 ± 0.10*	0.544 ± 0.021*

[a] Mean of *in vitro* IC_{50} values determined against P388, L1210, Molt 4/C8 and CEM cells (Dimmock et al. 1999b; 2002).
* Mean, significance $p < 0.05$

Table 2: Reduced glutathione (GSH) content of mitochondria and antioxidant capacity data of *E*-2-arylidene-1-tetralones (2a–c) and *E*-2-arylidene-1-benzosuberones (3a, 3b)

	GSH content of mitochondria ($nmol/mg_p$)	Antioxidant capacity (20 min)	Antioxidant capacity (120 min)	Antioxidant capacity (240 minute)
Control	11.69 ± 2.56	0.765 ± 0.021	0.745 ± 0.010	0.759 ± 0.022
2a	26.88 ± 1.62*	0.472 ± 0.039	0.572 ± 0.033	0.468 ± 0.007
2b	11.53 ± 2.88	0.531 ± 0.016	0.435 ± 0.078	0.479 ± 0.063
2c	3.96 ± 2.08*	0.554 ± 0.040	0.523 ± 0.035	0.527 ± 0.043
3a	14.85 ± 2.12	0.641 ± 0.015	0.561 ± 0.007	0.528 ± 0.035
3b	7.41 ± 2.05*	0.591 ± 0.035	0.669 ± 0.006	0.416 ± 0.005
Quercetine		0.291 ± 0.010	0.315 ± 0.08	0.315 ± 0.024

[a] The antioxidant capacity was determined by degradation on deoxyribose with initiation of Fenton reaction. Further experimental details are described in "Materials and methods"
* Mean, significance $p < 0.05$

peptide (GSH) moiety of them even after 1 h incubation. Similar adduct formation could be detected when the incubations were carried out at pH 9.0 at 37 °C. On the contrary, no adduct formation could be detected in incubations carried out at pH 7.4 at 37 °C. Determination of reduced glutathione (GSH) content of incubations free of adduct formation did not indicate redox reaction between the reactants.

Determination of reduced glutathione (GSH) levels in mitochondria exposed to the tested compounds was performed by HPLC. On exposure of mitochondria to **2c** and **3b** significant reduction of GSH level was compared to that of the DMSO-treated control experiments. On the contrary, exposure of mitochondria to **2a** resulted in significantly increased GSH level (Table 2).

Antioxidant capacity and possible pro-oxidant action of the compounds was tested by measurement of time-dependence of Fenton-reaction initiated degradation of 2-deoxyribose (Rozmer et al. 2006). Fenton-reaction initiated degradation of 2-deoxyribose is supposed to be the result of attack of hydroxyl radicals (HO), which results in formation of TBA-reactive substances with absorption maximum at 532 nm. As shown in Table 2, all the tested compounds displayed antioxidant activity ($HO^{\bullet}$ scavenging) both the investigated 20 minute, 120 minute and 240 minute time points.

3. Discussion

We investigated the effects of selected cytotoxic cyclic chalcone analogues on mitochondrial functions. The compounds are of interest since they induce apoptosis of Jurkat cells (Rozmer et al. 2006) and show strong *in vitro* and *in vivo* CYP 1A inhibitory activity (Monostory et al. 2003). Accordingly, the compounds can serve as prototypes for developing antitumor drug with dual – tumor cytotoxic and chemopreventive – activities.

The concentrations of the investigated compounds was set based on their previous *in vitro* cytotoxicity towards various tumor cell lines (Dimmock et al. 1999b, 2002; Rozmer et al. 2006). Decrease of oxygen consumption in the presence of added ADP (state 3) is indicative for inhibition of mitochondrial electron transfer chain of **2c** (Hodnick 1988; Chen 2006). Since the compound does not affect controlled respiration (state 4), the decreased RCR is probably the result of phosphorylation inhibitory effect of the compound or partial uncoupling (Dorta et al. 2005) (Table 1).

Compounds **3a** and **3b** produced a substrate-independent increase in oxygen consumption (respiratory burst) without affecting state 3 activity of the mitochondria (Table 1). Since auto-oxidation of the compounds could not be responsible for the observed oxygen consumption, the consumed oxygen should be the substrate of the mitochondrial electron transfer of the mitochondrial activity (Hodnick et al. 1986; 1988). Such an effect can increase the mitochondrial level of reactive oxygen species (ROS) that can initiate a series of biochemical events, involving cell death (Gregus et al. 2001; Sanz et al. 2006). Both **3a** and **3b** increased ATPase activity but neither compound affected oxygen consumption in the state 3 of mitochondria (Table 1), which is characteristic for an uncoupling effect of the compounds. The best known small molecule uncoupling agents are lipophylic weak acids that dissipate the energized state of mitochondria as heat and no ATP synthesis occur (Dorta et al. 2005, Trumbeckaite et al. 2006). Since neither **3a** nor **3b** is a weak acid *per se*, further work is needed to explore the mechanism of uncoupling action of the compounds.

Several chalcones have been reported to display antioxidant activity (Dimmock et al. 1999a; Go et al. 2005). On the other hand, a variety of chalcones were found to display antibacterial, antifungal, antiviral, and tumor cytotoxic activities (Middleton et al. 2000; Dimmock et al. 1999a; Go et al. 2005). There is also evidence that shows some chalcones to exhibit *in vitro* mutagenicity (Dimmock et al. 1999a). It is presumed that the toxic effects of the compounds might be due to their pro-oxidant activities (Middleton et al. 2000; Dimmock et al. 1999a; Go et al. 2005).

To test this possibility we investigated *in vitro* antioxidant capacity of the compounds by means of the Fenton-reaction initiated degradation of 2-deoxyribose. Fenton-reaction initiated degradation of 2-deoxyribose is supposed to be the result of attack of hydroxyl radicals ($HO^{\bullet}$), which results in formation of TBA-reactive substances with absorption maximum at 532 nm (Rozmer et al. 2006). As shown in Table 2, all the investigated compounds displayed a continuous antioxidant ($HO^{\bullet}$ scavenging) activity at the investigated 20, 120 and 240 minute time points. Although the antioxidant capacity of **3b** showed a minimum at the 120 minute time point, on longer incubations both compounds near equally reduced formation of TBA-reactive deoxyribose degradation products.

Cytotoxic effect of α,β-unsaturated carbonyl compounds are frequently associated with the expected reactivity of the compounds with the essential thiol groups in the living organisms (Dimmock et al. 1999a; Go et al. 2005). Like such compounds, the cyclic chalcone analogs **2** and **3** may be alkylating agents. To study such reactivity of **2** and **3**, the compounds were allowed to react with reduced glutathione (GSH), the main soluble cellular thiol, under cell-free conditions. According to the expectations, **2** and **3** showed intrinsic reactivity towards GSH at higher pH

(pH 9.0) and about physiological pH (pH 7.4) at a higher temperature (50 °C). Analysis of GSH content of the adduct-free incubations did not indicate any measurable redox reactivity of **2** and **3** towards GSH.

In order to investigate whether **2** and **3** can affect the cellular thiol status of the mitochondria, the reduced glutathion (GSH) level was determined. Under cellular conditions the cyclic chalcone analogues **2** and **3** can modify the thiol status by enzyme catalyzed conjugation and/or by oxidation of the reduced thiols to the respective disulfides (Dinkova-Kostova et al. 2001; Galati et al. 2002). On exposure of the mitochondria to the compounds **2c** and **3b** were found to statistically reduce the GSH level compared to the control. This observation suggests greater glutathione-S-transferase-catalyzed reactivity of **2c** and **3b** with GSH (Galati et al. 2002). Under similar conditions **2b** and **3a** did not significantly effect the GSH content of the mitochondria. On the contrary, on exposure of mitochondria to **2a** a statistically significant increase of the GSH level could be observed (Table 2). Earlier elevated cellular thiol level has been reported on exposure of Jurkat T cells to **3a** (Rozmer et al. 2006). Elevated GSH level of the Jurkat T cells exposed to some electrophilic Mannich bases has been supposed to result from de novo synthesis in response to feedback control of GSH (Gul et al. 2002).

In conclusion, it was demonstrated that the investigated compounds effect the cellular thiol status that might be the consequence of their covalent interaction with the main cellular thiol reduced glutathion. They also display pronounced effect on mitochondrial respiration that might contribute to their demonstrated tumor cell cytotoxicity. The experiments provide further details of cytotoxic effects of the synthetic chalcones displaying dual – cytotoxic and cytoprotective – effects (Dimmock et al. 1999b, 2002; Perjési et al. 2008).

4. Experimental

4.1. Materials

The cyclic chalcone analogues **2a–c** and **3a, 3b** were synthesized and purified as described before (Dimmock et al. 1999a, 1999b, 2002). Their structures were characterized by IR and NMR spectroscopy. The purity of the compounds was checked by TLC and GC. Compounds **2** and **3** were dissolved in dimethyl sulfoxide (DMSO) immediately before use.

Male Wistar rats (Velaz, Praha, Czech Republic) weighing 200–250 g were used. Adhering to procedures approved by the University of Košice Animal Care and Use Committee, the animals were sacrificed by cervical displacement and decapitation.

4.2. Methods

Liver mitochondria were isolated from male Wistar rats by the method of Johnson and Lardy (1967). Mitochondrial protein was determined according Lowry after Hartree's modification (Hartree 1972).

Oxygen consumption of isolated mitochondria was measured polarographycally with a Clark electrode (WTW oxi 325 (Germany)) at the of 25 °C. Measurements were carried out in respiratory medium (80 mM KCl, 300 mM KH_2PO_4, 300 mM K_2HPO_4, 15 mM TRIS-HCl, 6 mM $MgCl_2$, 0.78 mM EDTA, pH 7.4) supplemented with 70 nmol of chalcone per mg of mitochondrial protein, 50 mM sodium succinate, 0.5 mM ADP, and 20 mg/ml mitochondrial protein. The test compounds were added in dimethyl sulfoxide (DMSO), a dose that did not affect control rates of respiration. The respiratory rate is expressed as oxygen in nmol of oxygen consumed min^{-1} mg^{-1} of mitochondrial protein (Estabrook 1967). Respiratory control ratio (RCR) was determined as a ratio between oxygen consumption in state 3 (with addition of ADP) and state 4 (after exhaustion of ADP). ATPase activity (EC 3.6.1.3) was determined according Meissner et al. (1974).

Reactivity of chalcones **2** and **3** with reduced glutathione (GSH) was studied by the method described before (Rozmer et al. 2006). The reaction mixtures were kept in a water bath (37 °C and 50 °C) and the progress of the reactions was followed by TLC. on Kieselgel F_{254} plates. The developing solvent used was *n*-butanol : acetic acid : water (40 : 10 : 20, v/v%).

Determination of reduced glutathione in mitochondria (GSH) was performed by the modified method of Abukhalaf et al. (2002). HPLC analyses were performed on a LC-10AD_{VP} liquid chromatograph (Schimadzu, Kyoto, Japan) equipped with an RF-10A_{XL} fluorescence detector (Schimadzu, Kyoto, Japan). The elution gradient profile was as follows: 0 min: 0.5% ACN, 1–5 min: 10% ACN, 6–15 min.: 5% ACN. $R^2 = 0.9906$.

Investigation of antioxidant capacity of chalcones **2** and **3** was performed as described before (Rozmer et al. 2006). Quercetin (200 μM) was used as reference antioxidant. Each value is the average of three independent measurements.

Results are presented as mean ± S.E.M. of at least three independent experiments. Statistical significance was determined by Student's *t*-test with $p < 0.05$ level considered as significant.

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