Ph.D. thesis

Investigation of molecular mechanism of cytotoxic and chemoprotective effects of cyclic chalcone analogues

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I. Introduction

The chemistry and biological activities of chalcones (1,3-diphenyl-2-propenones) (**1**) have been of interest for a long time. Chalcones are intermediary compounds of the biosynthetic pathway of the naturally flavonoids. The wide range of biological activities of both naturally occuring and synthetic analogues, among others cytotoxic, antitumor, antiinflammatory and chemopreventive properties are well documented in the literature.

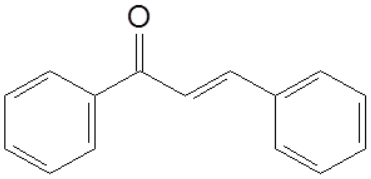
The benefit of synthesis and comparative investigation of biological activity of synthetic chalcone analogues with similar structure is the possibility to study the structure-activity relationship. The main objective of investigations of synthetic chalcone derivatives is to develop more effective analogues applicable mainly in tumor therapy.

Chalcones are α,β-unsaturated ketones and representatives of this class of compounds have demonstrated a preferential reactivity towards thiols in contrast to amino and hydroxyl groups. Thus interactions with nucleic acids may be absent which could eliminate the important genotoxic side effect which have been associated with certain anticancer drugs.

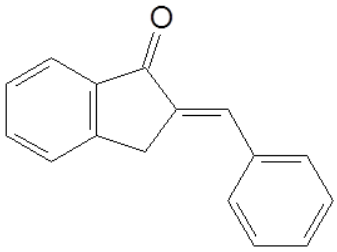
Biologic effects of α,β-unsaturated carbonyl compounds, like chalcones, are frequently associated with the nucleophilic addition of cellular thiol groups onto the polarized carbon-carbon double bound (Michael-reaction). Such a reaction can alter intracellular redox status, which can modulate events such as DNA synthesis, enzyme activation, selective gene expression and regulation of cell cycle. Several biological effects of chalcones, for example NQO1 (NADPH:quinone reductase) inducer effect, anti-inflammatory effect or GST (glutathione S-transferase) P1-1 inhibitory effect is associated with their Michael-type reactivity towards reduced glutathione (GSH).

Previous structure-activity studies demonstrated that cytotoxicity of the synthetic chalcone analogues is influenced by the shape of the molecules. Although chalcones are conjugated molecules they are flexible enough to exist in several conformations in solutions, therefore investigation of open chain chalcones can not provide the analysis of relationship between activity and the optimal shape of the molecule. To study the mentioned relationship cyclic chalcone analogues (**2**-**4**) were synthetized, and their chemical reactivity, molecular structure and biological activity were investigated.

Several cyclic chalcone analogues with various subtituents at different positions have been synthetized and their in vitro antineoplastic activity (IC_{80} values) has been investigated against murine and human cancer cell lines. According to the results the biological activity is influnced mainly by the ring size, the bensosuberone derivatives - containing a seven membered ring - were the most effective compounds. It was also found that cytotoxicity of the individual compounds greatly varied as a function of nature and position of substituents in the particular derivatives. Among the compounds investigated the bensosuberone analogues bearing a methoxy or dimethylamino substitutent in *para*-position showed the greatest tumor toxicity. Besides the documented tumor cytotoxic effects, some cyclic chalcone analogues were found to display emergent CYP1A inhibitor activity. Accordingly, the compounds represent prototypes of molecules displaying both cytotoxic and chemoprevetive effects.



chalcone (1)



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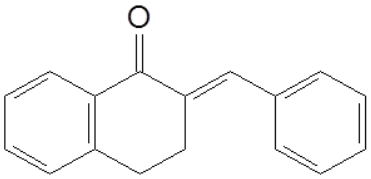

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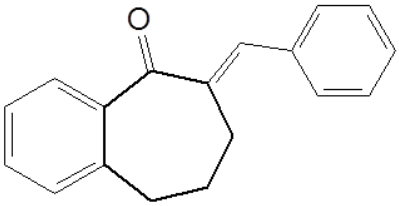

(*E*)-2-benzylidene-1-benzosuberone (4)

II. Aims

Our main goals were to investigate the physico-chemical properties of cyclic chalcone derivatives and a better understanding of molecular mechanism of action resulting the cytotoxic/cytoprotectiv activity.

- The objective was to extend a reversed-phase thin layer chromatographic method to determinate the lipophilicity of biologically active chalcones and cyclic analogues, to complete a data base for future QSAR investigations.

To expand our knowledge on the mechanism of the observed cytotoxic effect we selected two analogues for the measurments: (*E*)-2-(4'-methoxybenzylidene)-1-benzosuberone (**4a**), which showed the greatest tumor toxicity in previous *in vitro* tests, and its less active 4'-methyl substituted analogue (**4b**). For this purpose our main aims were the following:

- In order to detect the changes in the proliferation of untreated and treated Jurkat T cell samples determination of cell viability and total cell number using the Trypan Blue dye exlusion test, and determination of the ratio of living, early apoptotic and late apoptotic or necrotic cells analysed by flow cytometry was planed.
- Flow cytometric analysis of the cell-cycle progression of Jurkat T lymphocytes after exposure to equitoxic doses of the compounds for 8, 24 and 48 hours, corresponding to 1/3-, 1- and 2-times the cell-doubling time of the cells.

- Investigation of time dependence of *in vitro* antioxidant (hydroxyl radical scavanger) capacity of the analogues by means of the Fenton-reaction initiated degradation of 2-deoxyribose.
- Study of the antioxidant/prooxidant nature of the compounds and the effect on redox status of Jurkat T cells, investigating their effect on ROS production using the dichlorofluorescein test.
- Investigation of the effect of the chalcone derivatives on isolated rat liver mitochondria, characterization of mitochondrial respiratory function and activity of the enzyme ATP synthase.
- Investigation of possible interaction of the analogues with cellular thiols and glutathione. Determination of the ratio and amount of reduced and oxidized glutathione in Jurkat T cells after exposure with the compounds.
- Investigation of the reactivity of the compounds with reduced glutathione under cell free conditions. Application of thin-layer chromatography to indicate the formation of the chalcone-glutathione adducts. Application of RP-HPLC method coupled with mass spectrometry to investigate the chalcone-glutathione reaction under cellular conditions.

III. Methods and results

III.1. Determination of log*P* of cyclic chalcone analogues

Log*P* values of 29 biologically active chalcone and cyclic chalcone analogues have been determined by an optimized and validated reversed-phase thin layer chromatographic method. RP-TLC was performed on silanized silica gel $60F_{254}$ as stationary phase with methanol-water, 60 + 40 (*v*/*v*) as mobile phase. The samples, compounds of the calibration and validation set were dissolved in 1:1 methanol-chloroform (2 mg/ml), and these solutions were spotted on the plate (2 µl). After development the plates were dried and the chromatograms were assessed visually under UV illumination (λ = 254 nm) and after spraying with iodine.

For RP-TLC determination of log*P* a calibration plot ($\log P = aR_M + b$) should be constructed by use of a properly selected set of compounds with known $\log P_{\text{shake flask}}$ values. This equation serves for calculation of the $\log P_{TLC}$ values of the highly lipophilic compounds investigated, on the basis of their chromatographic retention ($R_M = \log(1/R_f - 1)$ values.

The compounds investigated are highly lipophilic, but the range of the log*P* values spans almost three orders of magnitude (log*P* = 2.53 – 5.35), which might have an important effect on their biological activity.

Increasing the ring size of respective cyclic derivatives was accompanied by the expected higher lipophilicity. Increasing the ring size from five (**2**) to six-membered (**3**) was accompanied by somewhat greater $\log P_{TLC}$ enhancement than that observed on increasing the

ring size from zero (open chain) (**1**) to five (**2**), and from six (**3**) to seven (**4**). The average log*P* increment of a methylene group was found to be 0.4 log units. These experimental observations reflect the importance of intra- and intermolecular interactions affecting lipophilicity.

The order of lipophilicity observed for compounds with *para* substituents was:

$$OH < CN < NO_2 < H < F < OCH_3 < CH_3 < Cl < N(CH_3)_2 \approx Br$$

The effects of the position (*ortho*, *meta* or *para*) of the aromatic substituents followed the order $\log P_{TLC}(orto) \leq \log P_{TLC}(para) \leq \log P_{TLC}(meta)$ among the benzosuberone derivatives (**4**) investigated. In contrast to the data obtained for the compounds in series **4**, for series **2** and **3** the lipophilicity of derivatives with the same substituents in the *ortho*, *meta* and *para* positions does not follow the regularity given above. This observation also indicates the complex effect of steric and electronic factors on the lipophilicity of the compounds.

III.2. Investigation of mechanism of action of cyclic chalcone derivatives

The cyclic chalcone analogues (**4a** and **4b**) were synthesized and purified as described before. All the chemicals used were of the analytical grade available. Jurkat human lymphocyte leukemia cell line (Clone E6-1, ATCC TIB-152) was obtained from LGC Standards. Jurkat T cells were cultured under conventional conditions (37°C humidified atmosphere of 95% air and 5% CO_2) in RPMI 1640 medium, pH 7.4 supplemented with 10% heat-inactivated foetal calf serum (FCS) with addition of penicillin (100 U/ml) and streptomycin (100 μg/ml).

Exponentially growing Jurkat T cells (5 x 10^5 cells in 1.0 ml RPMI 1640 cell culture medium) were exposed to the previously determined IC_{80} concentrations of **4a** (5,6 μM) and **4b** (23,3 μM) for 1, 4, 8, 24 or 48 hours, respectively. The compounds were added in 10 μl DMSO solution to the cells suspended in 1.0 ml medium (1%). Control A cells were untreated; only vehicle, 10 μl DMSO (1 *v/v*%), was added to the controll B cells.

III.2.1. Investigation of the effect of two cyclic chalcone analogues on cell proliferation and cell cycle of Jurkat T cells

Cell number and cell viability was determined by exclusion of Trypan Blue dye and a haemocytometer after cells were exposed to **4a** or **4b** for 8, 24 and 48 hours. Cell number in case of untreated control A cells and control B cells (1% DMSO) doubled within 24 hours, wich corresponds to the doubling time of Jurkat T cells. This result suggests that DMSO used as solvent for the chalcones does not influence the cell number in the applied concentration. The respective incubations exposed to **4a** and **4b** contain similar total number of cells at all the three time points. The total number of viable cells at the 8 hour time point is close to that of in the control incubations. Thus, the 8 hour treatment could be interpreted as specifically

related to proliferation. Inhibitory effect of the compounds on cell proliferation is remarkable after 24 and 48 hours.

To test cytotoxicity of **4a** and **4b** in the Jurkat T cell line a flow cytometric method was performed by double staining with fluorescein isothiocyanate (FITC)-labeled Annexin V (AVF) and propidium iodide (PI), to distinguish non-apoptotic live cells ($AVF^{negative}$-$PI^{negative}$), early apoptotic cells ($AVF^{positive}$-$PI^{negative}$) and late apoptotic or necrotic cells ($AVF^{positive}$-$PI^{positive}$). Jurkat T cells were exposed to **4a** and **4b** for 8, 24 and 48 hours.

At the 8 h time point neither **4a** nor **4b** exhibited toxic effect. At the 24 h time point the percentage of living cells was statistically lower (57.7%) after exposure to **4a** than after exposure to **4b** (72.7%). The rest of cells could be detected in the early apoptotic (**4a**: 22.8%; **4b**: 7.8%) or the late apoptotic/necrotic (**4a**: 16.8%; **4b**: 17.1%) state. At the 48 h time point only PI staining was applied to determinate the percentage of the living ($PI^{negative}$) and the late apoptotic/necrotic ($PI^{positive)}$ cells. At this time point the total amount of live cells decreased to 42.0% and 48.5% after exposure to **4a** and **4b**, respectively. Consequently, the compounds investigated inhibit proliferation and cause apoptosis in the applied concentration.

The effects of **4a** and **4b** on the cell-cycle distribution of Jurkat T cells at the 8, 24 and 48 h time points were studied by DNA content analysis by flow cytometry. After exposure cells were stained with propidium iodide (PI). PI intercalates into double-stranded nucleic acids and can be excited by 488 nm laser light, therefore the intensity of the fluorescent signal is proportional to the DNA amount in the cells. The data were obtained by separation of cell-cycle phases, compared with the phases of the cell-cycle distribution of untreated cells.

Early (8 h) effect of **4a** appeared primarly as an increase in the proportion of cells in the G_2/M phase (36.9%) accompanied by a decrease in the G_0/G_1 phase cells. Characteristic cell cycle disturbance of cells exposed to **4b** could not be observed at this time point. Longer (24 h) exposure to **4a** lead to further decrease of proportion of G_0/G_1 cells, while the percentage of cells in the G_2/M phase (44.8%) and the area of the Sub-G_0 (apoptotic and necrotic cells) peak further increased. At this time interval histogram of cells exposed to **4b** showed a moderate decrease in proportion of the G_0/G_1 phase (31.7%) cells accompanied by a slight increase in the G_2/M phase cells and of the Sub-G_0 peak. The different cell cycle effect of **4a** and **4b** at this time point can also be characterized by the $(G_2/M)/(G_0/G_1)$ ratios, which are 0.47, 6.1 and 0.85 for the control, the **4a**-treated, and the **4b**-treated incubations, respectively. In the case of prolonged (48 h) exposure further increase of the area of the Sub-G_0 peak and formation hyperdiploid cells could be observed in the case of both treatments.

Since the 8 h time period is only about 1/3 of the cell-doubling time, the results indicate that **4a** lifts the G_1 block that largely prevents duplication of genetic errors, and induces G_2/M arrest, which is a well documented pathway of cell cycle disruption caused by flavonoids and chalcones. The observations suggest that **4a** could cause a shortly developed DNA damage, which is frequently linked to G_2/M arrest. Such a remarkable attenuation of the G_1 checkpoint and induction of G_2/M arrest could not be observed in cells exposed to equitoxic dose of **4b**.

G_1 arrested cells can undergo cell death by apoptosis or necrosis, which is an important feature of ensuring genomic integrity. On longer (48 h) exposure to **4a** the percentage of cells

in the G_0/G_1, S and G_2/M phases drastically dropped. Concurrent increase of Sub-G_0 and hyperdiploid cells indicates loss of G_2 checkpoint stringency. Such a delayed cell death, also known as „mitotic death", is characteristic of many p53 mutated tumors.

III.2.2. Investigation of *in vitro* antioxidant effect of two cyclic chalcone derivatives

Antioxidant capacity and possible pro-oxidant action of the compounds was tested by measurment of time-dependence of Fenton-reaction initiated degradation of 2-deoxyribose. Degradation of 2-deoxyribose is supposed to be the result of attack of the Fenton-reaction generated hydroxyl radicals ($HO^{\cdot}$), which results in formation of carbonyl fragments that – on heating with TBA at low pH – generate a pink chromogen with absorption maximum at 532 nm. Testing of antioxidant effect (hydroxyl radical scavenging potential) is based on the competitive reaction of dexyribose and the tested antioxidant with the reactive hydroxyl radicals. For comparison, we have performed the experiments in the presence of and without addition of ethylendiaminetetraacetic acid (EDTA) to the incubation mixture. Addition of EDTA results in formation of iron(II)-EDTA complex, whose Fenton-activity could result in degradation of EDTA. In the absence of EDTA, a portion of the iron ions is complexed by deoxyribose. In these so called „site-specific" Fenton reactions hydroxyl radicals react immediately at the place where there are formed. Accordingly, compounds with ligand properties compete for the iron ions with the deoxyribose molecules and thus decrease degradation caused by the iron-catalyzed hydroxyl radical attack.

The level of the TBA-reactive substances in control incubations was found to be almost constant over 240 minute period. In experiments performed withouth addition of EDTA both compounds displayed a continuous antioxidant activity during the investigated 240 minute time period. Although the antioxidant capacity of **4a** showed a minimum about the 120 minute time point, on longer incubations both compounds reduced formation of TBA-reactive deoxyribose degradation products near equally.

Degradation of deoxyribose and therefore the amount of TBA-reactive compounds (the measured absorbance) is significantly lower in incubations performed in the presence of EDTA than that of formed withouth addition of EDTA. Level of the TBA-reactive substances of the incubations of **4a** showed an increase over the 60-120 minute period followed by significant decrease over the rest of the incubation time. The methyl derivative (**4b**) also showed a pronounced antioxidant effect under this conditions during the investigated time period.

The results demonstrated that the investigated compounds showed antioxidant effect in short term experiments, but the antioxidant capacity is significantly influenced by the experimental conditions in the long term experiments. Chemical transformation of the tested substances can result formation of derivatives that can initiate further redox activities under the experimental conditions.

III.2.3. Investigation of the effect of chalcone analogues on cellular redox status

In order to investigate the effect of the chalcone analogues on intracellular ROS production in Jurkat T cells, oxidation-sensitive 2',7'-dichlorofluorescin diacetate (DCFH-DA) was used. This compound is a nonfluorescent, cell-diffusible dye. Intracellular esterases cleave the acetyl groups from the molecule to produce nonfluorescent 2',7'-dichlorofluorescin (DCFH). This is trapped inside the cell and int he presence of ROS, DCFH is subsequently modified to the fluorescent DCF, which can be detected by flow-cytometry.

Jurkat T cells were incubated with **4a** and **4b** for 1 and 4 h, respectively. ROS production level was evaluated using FACS analysis. ROS induction was markedly decreased in cells treated with **4b** both after 1 and 4 h exposure (80.20 ± 10.35% és 71.35 ± 16.51% of control, respectively); however, compound **4a** did not inluence the peroxide level compared to the untreated control cells at any of the two timepoints.

Generation of ROS was evaluated to determine whether oxidative stress was involved in chalcone induced cell death. The findings indicate that the compounds tested did not increase the intracellular ROS production under the experimental conditions; moreover compound **4b** decreased the peroxide level showing antioxidant activity. This latter result is in accordance with those of investigation of antioxidant capacity of the compounds by monitoring time course of the Fenton reaction initiated *in vitro* degradation of 2-deoxyribose.

Effects of the cyclic chalcone derivatives on isolated rat liver mitochondria, including mitochondrial respiration and ATPase activity, was investigated. Oxygen consumption of isolated mitochondria was measured polarographycally with Clark electrode at 25 °C. Measurments were carried out in respiratory medium supplemented with chalcone, sodium succinate, ADP and mitochondrial protein. The respiratory rate was expressed as oxygen in nmol x min^{-1} x protein mg^{-1}. Respiratory control ratio (RCR) was determined as a ratio between oxygen consumption in state 3 (with addition of ADP) and state 4 (before addition of ADP). The compounds investigated had no effect on state 3 respiration, however increased the rate of oxygen consumption in state 4 by 50.7%. RCR was significantly decreased by **4a**. 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. Such an effect may alter the redox state of mitochondria.

In order to clarify whether alteration of oxygen consumption is associated with change in ATP synthesis, activity of the enzyme ATP synthase was investigated according to Meissner. The results showed that compound **4a** significantly increased ATPase activity without affecting oxygen consumption in state 3. The detected uncoupling effect is characteristic for small, lipophylic weak acids, when oxygen consumption increases even though no ATP synthesis occurs. Since neither **4a** nor **4b** is a weak acid *per se*, further work is needed to explore the mechanism of uncoupling action of the compounds.

To investigate possible interaction of **4a** and **4b** with cellular thiols, effect of the compounds on the total thiol content of the exposed Jurkat T cells was investigated under nutrient-free and nutrient-supplemented mediums. After 1 h (in PBS), 4 h and 8 h (RPMI with 10% FCS) exposure, the free cellular thiol content of the celles was determined using the Ellmann reagent. The results were normalized to protein concentration. On exposure of cells to the compounds for 1 h in nutrient-free PBS **4a** statistically reduced the cellular SH-level compared that of the DMSO-treated control cells. Under similar conditions **4b** did not affect the free thiol content. In a further set of experiments Jurkat cells were exposed to **4a** and **4b** in cell culture conditions with 10% FCS, where nutrients were available and the exposure periods (4 h and 8 h) were long enough for the cells to react to the possible change in the GSH levels. Under such conditions compound **4a** reduced the total cellular thiol level of the cells at both time points. On the contrary, on exposure of cells to **4b** a slight increase of the SH-level at the 4 h but not at the 8 h time point could be observed.

Under cellular conditions the cyclic chalcone analogues **4a** and **4b** can modify the thiol status by enzyme catalyzed conjugation and/or by oxidation of the reduced thiols to respective disulphides. The observations suggest greater glutathione-S-transferase-catalyzed reactivity of **4a** and glutathione.

As a continuation of our work towards a better understanding of the SH-reactivity of the chalcone analogues, effects of compounds **4a** and **4b** on reduced (GSH) and oxidized (GSSG) glutathione levels of Jurkat T cells exposed to **4a** and **4b** for 4 h was investigated using a reversed phase HPLC method after derivatization with *ortho* phthalic aldehyde (OPA). Reduced glutathione reacts with OPA to form a stable, highly fluorescent tricyclic derivate at pH 8, while GSSG reacts with OPA only at pH 12. While measurment of GSSG, the thiol function of GSH was chemically modified by *N*-ethylmaleimide (NEM) to non-OPA-reactive derivative.

Concentration of GSH and GSSG in the samples was determined by means of standard curves. The results were normalized to protein concentration. Effect of the investigated compounds were different under the applied conditions. Compound **4a** was found not to influence the GSH level, but significantly increased the GSSG level in the cells (from 0.67 ± 0.26 nmol/mg protein to 1.15 ± 0.33 nmol/mg protein). Chalcone **4b** enhanced the GSH level (from 35.3 ± 6.6 nmol/mg protein to 42.4 ± 7.2 nmol/mg protein), whereas did not markedly change the GSSG level, compared to untreated and DMSO-treated cells.

The GSH system is one, but probably the most important cellular defence mechanism that exists in the cell. Glutathione does not only act as an ROS scavanger but also functions in regulation of intracellular redox state. The ability of cells to generate GSH (either by reduction of GSSG or *de novo* synthesis of GSH) is an important factor in efficiency of managing oxidative stress. The methyl analogue (**4b**) increased the reduced glutathione level of the cells. Earlier, elevated cellular thiol level has been reported on exposure of Jurkat and HeLa cell lines to some electrophilic Mannich bases. Elevated GSH level of Jurkat T cells might have resulted from *de novo* synthesis in response to feedback control of GSH.

In contrast, chalcone **4a** induced an increase in GSSG content, which can be evidence that this compound induces oxidative stress. GSSG is a physiological indicator of intracellular defence system activity against ROS, and it can be used to monitor oxidative stress *in vivo*. This observation is in accordance with the previous results showing compound **4a** to increase oxygen consumption without affecting state 3 activity of rat liver mitochondria.

III.2.4. Investigation of the conjugation reaction of cyclic chalcone analogues with reduced glutathione

Alteration of cellular glutathione status can also been associated with the Michael-type reactivity of α,β-unstaurated carbonyl compounds towards reduced glutathione and other essential cellular thiols in addition to the redox reaction.

Firstly, spontaneous reactivity of cyclic chalcone derivatives towards reduced glutathione, the main soluble cellular thiol, was investigated under cell-free conditions. Thin layer chromatographic analysis of equimolar mixtures of chalcones (**4a** and **4b**) 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 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. TLC was carried out on Kieselgel F_{254} plates, the developing solvent used was *n*-butanol : acetic acid : water (40:10:20 *v/v*%). For visualization, the chromatograms were illuminated by 254 nm UV light and subsequently subjected to ninhydrin solution.

Under slightly acidic conditions – characteristic for tumor cells – compounds **4a** and **4b** did not show intrinsic activity with GSH at 37 °C.

The non-enzyme catalyzed reaction of GSH and the chalcone analogues was also investigated by reversed-phase HPLC analysis. In the incubation mixture the concentration of GSH was 5×10^{-2} M and the concentration of the chalcone was 5×10^{-3} M, respectively. The pH of the reaction mixture was set to pH 8.0. After incubation (150 and 330 min) the components were analysed by HPLC method using UV detection. As a result, in addition to the peak of the chalcone, two new chromatographic peaks appear, indicating the formation of two diastereomeric GSH adducts of compounds **4a** and **4b**. The structural assignments were supported by MALDI-TOF-MS and HPLC-MS with electrospray ionization measurments. The chromatograms gave evidence of the formation of two diastereomeric GSH adducts of **4a** and **4b** with the protonated molecular masses of 586.18 and 570.23, respectively.

To investigate whether chalcone-GSH conjugation reaction undergoes under cellular conditions as well, analysis of cell supernatants and sediments after treatment (4 h) of Jurkat T cells with compounds **4a** and **4b**, the developed RP-HPLC method coupled with ESI-MS was applied. The selected ion chromatograms and the ESI mass spectra of the analysed cell preparations give evidence of presence of the protonated molecule of **4a** (m/z 279.2) both in cell supernatant and cell sediment. Presence of diastereomeric GSH adducts of **4a** – formed in *in vitro* incubations of **4a** and GSH – could not be observed. The results of similar

investigations of compound **4b** were the same as those obtained with chalcone **4a**. The observations indicate that chalcones penetrate the cell membrane and affect intracellular biochemical events. However, chalcone-GSH adducts could not be observed either in the cell supernatant or the cell sediment. This observation might be the consequence of reversible addition of GSH onto the chalcones' polar carbon-carbon double bond, which can result in a retro-Michael-type decomposition of the adducts when the excess of GSH is reduced.

IV. Conclusions, novel findings

- LogP values of a series of biologically active chalcone and cyclic chalcone analogues variously substituted have been determined by an optimized and validated RP-TLC method.

- The *in vitro* cytotoxic and antiproliferative effect of the chalcone analogues against Jurkat T cells were invastigated using flow cytometric methods. It was demonstrated, that equitoxic doses of both compounds induced apoptosis. The more effective compound **4a** caused an immediate derivative G_1 lift and G_2/M arrest, which was followed by cell death accompanied by formation of hyperdiploid cells.
- The *in vitro* antioxidant effect of the compounds was demonstrated by the ability to inhibit the toxicity of hydroxyl radicals. The possible pro-oxidant effect was investigated by monitoring time course of Fenton-reaction initiated degradation of 2-dezoxyribose. Compound **4b** showed antioxidant activity under the applied conditions, the methoxy analogue (**4a**) showed antioxidant activity in short term analysis, but temporary pro-oxidant effect in the long term analysis, particularly in the presence of EDTA.
- The effect of the chalcone derivatives on the redox status of Jurkat T cells was investigated, and it was demonstrated that the compounds do not increase the intracellular ROS activity, the methylanalogue (**4b**) significantly decreases the ROS-level and showes antioxidant activity.
- The effect of the analogues on isolated rat liver mitochondrial functions was investigated. It was demonstrated that compound **4a** increased the mitochondrial oxygen consumption and enhanced the activity of ATPase enzyme. Alteration of mitochondrial functions may play a role in the cytotoxic effect of the molecules investigated.
- The reactivity of cyclic chalcone analogues with cellular thiols, including reduced glutathione was examined. While compound **4a** increased the oxidated glutathione level and did not influence the reduced glutathione content, compound **4b** enhanced the reduced glutathione (and the total cellular thiol) level. The latter result may explain the previously experienced antioxidant activity (decrease of ROS production) of **4b**. The GSSG increasing effect of **4a** may take part in the induction of apoptosis.

- Incubation of **4a** and **4b** with reduced glutathione under cell free conditions - in neutral or slighly alkaline medium, at higher temperature - indicated spontaneous (non-enzyme catalyzed) conjugation (non-redox) reaction. Formation of chalcone-GSH adducts were proved by thin-layer chromatographic and RP-HPLC methods, the structural assignments were supported by HPLC-MS with electrospray ionization measurments.
- The developed HPLC-MS method provided the appropriately selective and sensitive tool to investigate the chalcone-GSH reactions under cellular conditions as well. After treatment of Jurkat T cells only the investigated compounds could be detected from the samples, but the presence of the adducts could not be proved. This observation might be the consequence of reversible addition of GSH onto the chalcones' polar carbon-carbon double bond, which can result in retro-Michael-type decomposition of the adducts when the excess of GSH is reduced.

In conlclusion, it was demonstrated that the less active 4-methylanalogue (**4b**) induces apoptosis in Jurkat T cells, but the cell-cycle influencing effect is not pronounced compared to that of compound **4a**. The methyl analogue displays antioxidant activity both in the applied *in vitro* test and under cellular conditions, which can be associated with the ability to increase the reduced glutathione level in the cells.

The cytotoxic compound **4a** showed to alter the cell-cycle mechanism drastically, induces apoptosis and the formation of hyperdiploid cells. The compound influences mitochondrial functions, cellular thiol-level, increases the oxidated glutathione content of the cells. The interference with the cellular antioxidant defence system might contribute to its demonstrated tumor cell toxicity.

Alteration of the cell-cycle mechanism, the G_2/M phase block might be a result of the effect of the chalcones on the polimerization-depolimerization process of tubulin. The cytotoxic effect might be a consequence of the non-covalent interaction between the cyclic chalcone derivatives and cellular macromolecules (tubulin, DNA).

The results of our research provide new insights into the dual – cytotoxic and chemopreventive – effects of the cyclic chalcone analogues.

V. List of publications

Publications related to the present PhD thesis

1. **Zs. Rozmer**, T. Berki, G. Maász, P. Perjési: Different effects of two cyclic chalcone analogues on redox status of Jurkat cells. *Toxicology in Vitro*. (2014) 28, 1359-1365. IF: 3.207
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