Studies on glutathione conjugation reactions of some cyclic chalcone analogs to seek reactivity-biological activity relationships

Doctoral (Ph.D.) Thesis

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1. Introduction

1.1. Chalcone

Chalcones (1,3-diphenyl-2-propen-1-ones) are intermediary and precursor compounds found in flavonoid biosynthesis. Chalcones, among others, have been proven to show cytotoxic, anti-inflammatory, and anthelmintic effects. Some chalcones have been approved for clinical use. The ease of structural modification and the wide variety of biological and pharmacological activities have led to high diversity in chalcone derivatives and, in turn to popularity in drug research and development. Several biological effects of chalcones and their synthetic analogs are associated with their spontaneous and enzyme-catalyzed reactivities. Different mechanisms of action were suggested for these activities, but the exact molecular target is not known in many cases.

Chalcones can interact with cellular macromolecules through non-covalent interactions associated with lower toxicity and genotoxicity. Previously it was demonstrated that chalcones and their analogs show high intrinsic reactivity toward thiol groups. Therefore, studying the interaction of chalcones and sulfur-containing structures may be the key to unraveling the mechanism of action of such compounds. GSH protects the reductive intracellular state by reducing reactive species, usually with the help of the glutathione peroxidase (GPx) enzymes. Moreover, it participates in glutathione S-transferase enzyme (GST)-catalyzed reactions in detoxifying xenobiotic metabolism and oxidative stress-related reactive products. GSH also plays a vital role in keeping essential antioxidants such as vitamin C and vitamin E in their reduced form resulting in maintaining redox balance. Finally, GSH can reversibly bind to protein thiol residues (glutathionylation) to prevent their oxidative damage. Non-enzymatic biotransformation is a significant component of the biotransformation of xenobiotics as the enzymatic ones due to spontaneous reactions of electrophilic species. Highly reactive electrophiles such as the endogenous 4-hydroxy-2-noneal or the exogenous NAPQI, the well-known metabolite of paracetamol, can react with GSH before their possible reaction with important cellular components such as DNA, RNA, and protein. These reactive electrophilic compounds are metabolized before exerting toxic effects in the cells, and less than expected attention has been given to non-enzymatic biotransformation.

1.2. Thiols

1.2.1. Glutathione

L-gamma-glutamyl-L-cysteinyl-glycine (glutathione, GSH) is a major tripeptide found and synthesized in significant (1-10 mM) concentrations in most mammalian cells. Among the

essential cellular functions of glutathione are (a) antioxidant activity, (b) metabolism and elimination of xenobiotics, (c) maintaining the cellular redox potential, and playing an important role in (d) cell proliferation, (e) fatty acid biosynthesis and (f) signal transduction of gene expressions should be mentioned.

1.2.2. N-acetylcysteine

N-acetylcysteine (NAC) is a non-toxic compound and precursor of glutathione biosynthesis. Glutathione does not have good oral absorption, and to increase its concentration thus, NAC supplementation is advised. The most important use of NAC in medical practice is against acetaminophen poisoning. It also involves a sulfhydryl functional group which can closely mimic the mechanism of glutathione in reaction with chalcone analogs.

1.2.3. Glutathione S-Transferases

Glutathione transferases possess three main functions in living organisms: (a) catalytic activities, (b) binding of non-substrate ligands, and (c) involvement in protein-protein interactions. The substrates of the GST-catalyzed reactions have three common properties: a) they are hydrophobic, b) they have an electrophilic (electron-deficient) atom (center), and c) they have spontaneous reactivity towards GSH.

1.2.4. Glutathione in cancer

Reactive oxygen species (ROS) play an important role in tumor cell signaling, tumorigenesis, and cancer progression. Its production is considerably higher in cancerous cells due to higher growth and proliferation rate. Therefore, such cells commonly show higher expression of antioxidant systems such as Nrf2 and other enzymes related to GSH synthesis and utilization. Moreover, some tumors promote upregulation of GSH synthesis and turnover, such as ovarian, breast, and lung cancer. Given the vital role of GSH in the survival and drug resistance of cancerous cells provides an exciting perspective on cancer treatment. While the elevation of GSH and GST was considered a sign of a bad prognosis until recently, exploring the dual nature of GSH reactivity is interesting.

2. Aims

The general aim of this Ph.D. work was to investigate the thiol (reduced glutathione (GSH) and N-acetylcysteine (NAC)) reactivity of 4/4'-substituted chalcones and seven-membered cyclic chalcone analogs *in vitro*. The specific aims are as follows

- Investigation of thiol-reactivity of chalcones (**1**) and seven-membered chalcone analogs (**4**) to study how
  (a) the 4(4')-aromatic substitution, and
  (b) incorporation of a seven-membered aliphatic ring into the chalcone moiety
  can affect the initial thiol reactivity of the derivatives.
- To study how the pH of the incubation medium affects the initial thiol-reactivity of the 4(4')-CH_3- and the 4(4')-OCH_3-substituted **1** and **4** derivatives,
- To study the stereochemical outcome of the thiol addition reactions onto the 4(4')-CH_3- and the 4(4')-OCH_3-substituted **1** and **4** derivatives at different pH,
- To study the metabolic stability of the **4c** derivative using rat liver microsomes, and
- To seek correlations between the observed thiol-reactivity and previously published cancer cell cytotoxic effects of some **1** and **4** derivatives.

3. Material and Methods

3.1. In vitro chalcone-GSH incubations

The starting (*E*) isomers of the chalcones were synthesized using a previously described method. The purity was tested using TLC and HPLC-UV. The structure of both the (*E*) and (Z) isomers and those of the formed conjugates were verified using LC-MS measurements. Reduced glutathione and *N*-acetylcysteine were obtained from Sigma-Aldrich (Budapest, Hungary). The methanol CHROMASOLV gradient for HPLC was obtained from Honeywell (Hungary). Trifluoroacetic acid HiperSolve CHROMANORM was obtained from VWR (Budapest, Hungary) and formic acid from Fischer Chemicals. Deionized water was purified at the Institute of Pharmaceutical Chemistry (University of Pécs) for use in HPLC and HPLC-MS measurements by Millipore Direct-Q^{TM}. Mobile phases used for HPLC measurements were degassed by an ultrasonic water bath for 5 minutes before use. Other chemicals used were of the analytical grade available.

To evaluate the reactivity of the investigated chalcones and their analogs with thiols, two solutions containing reduced glutathione (GSH) and *N*-acetylcysteine (NAC) were prepared as

follows: Each solution was prepared at three different pH values (3.2, 6.3, and 8.0). The pH was set using freshly prepared 1M NaOH solution.

Solution (a) Solutions of GSH and NAC were prepared to a final volume of 1.5 cm^3 in deionized water with a concentration of 2.0×10^{-1} $mol \cdot L^{-1}$ (0.3 mmol thiol).

Solution (b) Chalcone solutions were freshly prepared before incubation to a 4.6 cm^3 volume of HPLC-grade methanol with a concentration of 6.5×10^{-3} $mol \cdot L^{-1}$ (0.03 mmol chalcone).

Solution (c) The GSH/NAC and chalcone solutions were pre-incubated in a 37 °C water bath for 15 minutes in the dark. Then, solutions (a) and (b) were mixed, resulting in a mixture of the thiol and the chalcone in a molar ratio of 10:1.

Solution (c) was kept in the dark in a temperature-controlled (37 °C) water bath for 315 min. To monitor the reaction by RP-HPLC and to qualitatively characterize the progress of the addition processes, the composition of the incubation mixtures was analyzed at the 15, 45, 75, 105, 135, 165, 195, 225, 255, 285, and 315 min time points by HPLC-UV.

To evaluate the initial (0 min) peak area of chalcones (**1a, 1b)** and the cyclic analogs (**4a, 4b**), 4.6 cm^3 methanolic solution of each was prepared in the same way as above (Solution (b)), and the solutions were diluted with 1.5 mL of aqueous solution with the respective pH before analysis. Before mixing, the solutions were pre-incubated at 37 °C for 30 min.

To compare the products of the previously proven light-initiated (*E*)/(*Z*) isomerization of the parent compounds [64] with those of the non-light (retro-Michael reaction)-initiated isomerization, solutions of chalcone solutions (**1a-4b**) were prepared by method (b), and the solutions were subjected to scattered laboratory light for one week. The solutions were analyzed by HPLC-UV and HPLC-MS.

3.2.Microsomal measurements

The starting (*E*) isomers of the chalcones were synthesized using a previously described method. The purity was tested using TLC and HPLC-UV. The structure of both the (*E*) and (Z) isomers and those of the formed conjugates were verified using LC-MS measurements. Male rat pooled liver microsome 10 mg (M9066), magnesium chloride ($MgCl_2$), 25mm syringe filters nylon membrane, pore size 0.45µm, and ß-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (ß-NADPH) were purchased from Sigma Aldrich (Budapest, Hungary). Alamethicin, and reduced L-glutathione BioChemica were obtained from Cayman Chemicals and ITWreagents. Methanol CHROMASOLV HPLC grade was obtained from Honeywell (Hungary). Millipore Direct-Q™ at the Institute of Pharmaceutical Chemistry (University of Pécs) was used to provide deionized water for the measurements. Phosphate

buffered Saline (PBS) pH 7.4 was prepared freshly on the day of incubation based on the 2014 Cold Spring Harbor Laboratory press method.

490µL PBS was mixed with 50µL of rat liver microsome (20 mg/mL protein) and 10 µL of alamethicin in methanol (50 µg/mg protein) and was left on ice for 15 minutes for microsomal activation. Then 100µL of NADPH solution (2.0 µmol/mL final concentration) was added, and the solution was vortexed to mix well. 25µL of a freshly prepared acetonitrile solution of **4b** (final concentration 0.25 µmol/mL) and 100µL of $MgCl_2$ solution (final concentration of 5.0 µmol/mL) were added. After each addition step, the solution was vortexed. The incubation volume was set to a total of 980 µL by the addition of 205 µL of PBS and left in a 37°C water bath for 3 minutes. Then 20 µL of GSH solution in PBS (final concentration of 5.0 µmol/mL) was added to bring the final volume to 1.0 mL. The mixture was vortex mixed for 10 seconds, taken to a shaking water bath, and kept in it for the incubation period (120 minutes). 150 µL samples were taken at 0, 30, 60, and 90-minute time points. (The 0-minute time points were considered the time of placement of the incubates into the water bath.) 150 µL ice-cold methanol was used to stop the reaction at each time point. The 300 µL sample was centrifuged at 6000 rpm for 5 min; the supernatant was collected using a syringe and passed through a 0.45µm nylon membrane syringe filter. Control incubation containing all the constituents except the liver microsome was analyzed at 0, 30, 60, and 90-minute time points.

3.3.HPLC-UV measurements

The measurements were performed on an Agilent 1100 HPLC system coupled with a UV–VIS detector. The wavelength was set at 260 nm. The separation of the components was carried out in a reversed-phase chromatographic system. A Zorbax Eclipse XBD-C8 column (150 mm × 4.6 mm, particle size 5 µm; Agilent Technologies, Waldbronn, Germany) was used to separate the components. The injection volume was 10 µL. The column oven was set at room temperature (25 °C) during the measurement. Data were recorded and evaluated using Agilent ChemStation (B.03.01). Gradient elution was performed at a flow rate of 1.2 mL/min; the mobile phase consisted of (A) water and 0.1% trifluoroacetic acid and (B) methanol and 0.1% trifluoroacetic acid. The gradient profile was as follows: an isocratic period of 8 min of 40% mobile phase B, followed by a linear increase to 60% in 4 min, a second linear gradient to 90% in 3 min, and a 5 min isocratic period of 90%. The column was then equilibrated to the initial conditions with a 2 min linear gradient to 40%, followed by 3 min of the isocratic period.

3.4.HPLC-MS Measurements

HPLC HESI-MS analyses were performed on an Ultimate 3000 liquid chromatograph (Dionex, Sunnyvale, CA, USA) coupled with a Thermo Q Exactive Focus quadrupole-Orbitrap hybrid

mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The scan monitored *m/z* values ranging from 100 to 1000 Da. Data was acquired using Q Exactive Focus 2.1 and Xcalibur 4.2 software (Thermo Fisher Scientific). Analysis of compounds and adducts was performed in HESI positive and negative ionization modes with the following parameters: spray voltage, 3500 V; vaporizer temperature, 300 °C; capillary temperature, 350 °C; spray and auxiliary gas flows, 30 and 10 arbitrary units, respectively; resolution, 70,000 at 200 *m/z*; and fragmentation, 20 eV.

HPLC separation was performed on an Accucore C18 column (150 mm × 2.1 mm, particle size 2.6 µm), and an Accucore C18 guard column (5 mm × 2.1 mm, particle size 2.6 µm) was also used. The injection volume was 5 µL; the flow rate was 0.4 mL/min. Data analysis and evaluations were performed using Xcalibur 4.2 and FreeStyle 1.7 software. A binary gradient of eluents was used, consisting of mobile phases A and B.

The gradient parameters in chalcones were (A) water and 0.1% formic acid and (B) methanol and 0.1% formic acid. The gradient elution was as follows: isocratic elution for 1 min to 20% eluent B, continued by a linear gradient to 100% in 9 min, followed by an isocratic plateau for 2 min. Then, the column was equilibrated back to 20% in 0.5 min and continued isocratically for 2.5 min. The sampler was at room temperature, and the column oven was at 40 °C.

The parameters of the gradient in the case of adducts were (A) water and 0.1% formic acid and (B) methanol and 0.1% formic acid. The gradient elution was as follows: isocratic elution for 1 min to 10% eluent B, continued by a linear gradient to 95% in 13 min, followed by an isocratic plateau for 3 min. Finally, the column was equilibrated to 10% in 0.1 min and continued isocratically for 2.9 min. The sampler was at room temperature, and the column oven was at 40 °C. The diode array detector was also set at 260 nm wavelength alongside MS analysis.

3.5. Molecular modeling

The structures **1c, 4c, CH_3SH,** and **CH_3S^-** were constructed using the Gaussview 6.0 software. Theoretical calculations were performed by DFT, implemented in the G16 software package. The molecules were optimized using the hybrid exchange and correlation functional with long-range correction, M06-2X, combined with the basis set 6-311++G(d,p) in the gas phase. Frontier molecular orbitals (FMO) were obtained. Molecular electrostatic potential maps contributed to the global electrophilicity analysis through their electronic isodensity surfaces. MEP maps provide a visual representation of the electrostatic potential on the surface of a molecule, which can reveal regions of high and low electron density. The electrostatic potential V(**r**) at point **r** is defined as.

$$V(\mathbf{r}) = \sum_{\alpha} \frac{Z_A}{|\mathbf{r}_\alpha - r_A|} - \int \frac{\rho(\mathbf{r})}{|\mathbf{r}_\alpha - \mathbf{r}|} d\mathbf{r} \quad (1)$$

where Z_A is the charge of nuclei α at point $\boldsymbol{r}_\alpha$ and $\rho(\boldsymbol{r})$ is the charge density at point $\mathbf{r}$.

The local electrophilicity of the molecules was determined by the Fukui function, and then it was possible to predict the molecular site selectivity.

$$f(\mathbf{r}) = \left[\frac{\partial \rho(\mathbf{r})}{\partial N}\right]_v, \quad (2)$$

where N is the number of electrons in the system, and the constant term v in the partial derivative is external potential.

Multiwfn 3.6 program was used to calculate the Fukui. In addition, the *py*SiRC – a machine-learning computational platform- was used to simulate oxidation reactions facilitated by free-radical compounds. To imitate the oxidation impact induced by a radical attack, the hydroxyl radical (˙OH) was chosen as the archetype system of degradation reactions. The reaction rate constant of the oxidative attack caused by the hydroxyl radical on chalcones compounds was predicted using the XGBoost ML algorithm, and the MACCS fingerprint was employed as a structural descriptor.

4. Results

4.1.Open chain Chalcones

4.1.1. Reactions performed under pH 8.0/7.4 conditions

This pH was chosen to mimic the GST-catalysed reaction considering the pK_a values of reduced GSH and NAC, 8.83 and 9.52, respectively. In this pH, 3.6% of molecules in the case of GSH and 0.75% of NAC molecules exist in an ionized form which is the more reactive form of the thiols. Plotting the change of chromatographic peak areas of the parent compound **1a** and **1b** as a function of incubation time is indicating the composition of an equilibrium. By the end of the incubation period (315 min) with GSH, the initial area of the HPLC peak corresponding to the parent compounds **4a** and **4b** was reduced to 3.7% and 5.2%, respectively. In the case of the incubations with NAC, the initial areas of **1a** and **1b** was decreased to 5.2% and 9.8%, respectively.

Based on the previously explained mechanism of the Michael addition reaction, a new chiral center is formed in the case of the open-chain chalcone analogs; in the case of the cyclic analogs, two new chiral centers will arise. Furthermore, in the case of the open chain analogs and considering the inherent chirality of the two thiols, the formation of two diastereomeric adducts

was expected. However, using our HPLC conditions, the **GSH-1** and **GSH-2** conjugates were not separated (Table 1).

Table 1. Retention times (t_R)[1] and integrated peak areas (A) of the investigated chalcones (**1a** and **1b**) and their GSH adducts (**GSH-1** and **GSH-2**)[2].

pH [3]	*Compound*	t_R *(E)-Chalcone*	*Area Ratio* [4] A_{315}/A_0	t_R *(Z)-Chalcone*	*Area (Z)-Chalcone*	t_R *GSH–1*	*Area GSH–1*	t_R *GSH–2*	*Area GSH–2*
3.2	**1a**	16.4	0.81	16.2	<100	13.8	4245	N/D [5]	-
3.2	**1b**	15.9	0.96	15.7	<100	11.9	3352	N/D [5]	-
6.3	**1a**	16.3	0.09	16.0	<100	13.2	16,571	N/D [5]	-
6.3	**1b**	15.8	0.21	15.5	<100	11.3	17,160	N/D [5]	-
8	**1a**	16.3	0.04	16.1 [6]	<100	13.3	17,419	N/D [5]	-
8	**1b**	15.7	0.08	15.5	<100	11.0	20,387	N/D [5]	-

[1]Retention times in minutes; [2]data refer to the average of two independent measurements at the 315 min time point; [3]pH value of the aqueous thiol solution; [4]ratios of peak areas measured at 0 and 315 min; [5]not detectable.

In the case of NAC incubations, the formed **NAC-1** and **NAC-2** adducts were present; however only partially separated. Based on the integration of the two overlapping peaks, the ratio of the two diastereomeric adducts was unequal and showed a (1.7–1.2 times) excess of the less polar diastereomers (Table 2).

Table 2. Retention times (t_R)[1] and integrated peak areas (A) of the investigated chalcones (**1a** and **1b**) and their NAC adducts (**NAC-1** and **NAC-2**)[2].

pH [3]	*Compound*	t_R *(E)-Chalcone*	*Area Ratio* [4] A_{315}/A_0	t_R *(Z)-Chalcone*	*Area (Z)-Chalcone*	t_R *NAC–1*	*Area NAC–1*	t_R *NAC–2*	*Area NAC–2*
3.2	**1a**	16.3	0.89	16.1	<100	15.2	1260	15.3	2173
3.2	**1b**	15.8	0.98	15.5	<100	14.1	1156	14.2	1507
6.3	**1a**	16.3	0.24	16.0	<100	15.1	4906	15.2	6457
6.3	**1b**	15.8	0.47	15.5	<100	14.1	4712	14.2	5422
8	**1a**	16.2	0.05	16.0	<100	15.1	6167	15.2	8875
8	**1b**	15.7	0.10	15.5	<100	14.1	7167	14.2	8975

[1]Retention times in minutes; [2] data refer to the average of two independent measurements at the 315 min time point; [3]pH value of the aqueous thiol solution; [4]ratios of peak areas measured at 0 and 315 min; [5]not detectable.

The structure of the parent chalcones (**1a** and **1b**), as well as their GSH and NAC conjugates, were verified by HPLC-MS.

The AUC of GSH-conjugate (**GSH-1**) of compounds **1a** and **1b** plotted as a function of time shows a mild but constant increase, while in the case of NAC adducts (**NAC-1** and **NAC-2**)

function of the integrated HPLC peak areas (AUCs) over time shows that the formation of the NAC-conjugates of **1a** increased in the first 45 min and remained the same over incubation. In the case of the NAC-conjugates of **1b**, the kinetic product (**NAC-1**) was rapidly formed in the first 15 min. After that, however, the **NAC-1** isomer of **1b** was trans-isomerized to the thermodynamic product (**NAC-2**), reaching the equilibrium composition by the 105 min time point.

In addition to the data above, it is worth mentioning that during incubations with both NAC and GSH, some minor new peaks with a slightly lower retention time than the **1a** and **1b** peaks (those of the unreacted chalcones) were formed. Our previous results suggest that these peaks are the (*Z*) diastereomers of the parent (*E*)-chalcones. Since these peaks do not arise in incubations performed without thiols, the formation of such peaks can be considered as a result of a retro-Michael addition reaction, a non-light-initiated isomerization of chalcones and their analogs. To identify the structure of the expected (*Z*) diastereomers, light-initiated isomerization of **1a** and **1b** was performed. The results were compared with those of the non-light-initiated isomerization experiments. The formed compounds were identified as the respective (*Z*) isomers.

4.1.2. Reactions performed under pH 6.3/6.8 condition

Reactions under slightly acidic conditions mimic the cellular milieu of cancer cells since their metabolism shifts to support their very costly rapid proliferation, migration, and survival. Most of the glucose is converted into pyruvate and, in turn, lactic acid, even in the presence of oxygen, in contrast to the normal aerobic glucose metabolism, where pyruvate is further oxidized to carbon dioxide and water. Accumulating lactic acid causes the pH of cancer cells to be slightly more acidic than normal cells. Under these experimental conditions (pH 6.3/6.8), only about 0.9% of GSH and 0.2% of NAC molecules exist in the more reactive thiolate form. The progress of the reactions under such conditions was more restricted than that observed at pH 8.0/7.4. In the GSH incubations, the initial area of the parent compounds **1a** and **1b** was reduced to 9.4% and 21.4% by the end of the investigated period (Table 1). The respective figures for the NAC incubations were 24.4% and 46.8% (Table 2).

Progress curves of the reactions indicated that the percentage figures represent compositions close to equilibrium. Similar to the results obtained under pH 8.0/7.4 conditions, the formation of a small amount of (*Z*) isomers was detected in the incubation mixtures.

Progress curves of the formation of the chalcone-GSH and chalcone-NAC adducts showed two parallel concave curves with finite limits.

The progress curve of the diastereomeric peak-1 of compounds **1a** and **1b** in reaction with GSH shows a constant increase in the AUC. However, the AUC of the final time point is smaller in the case of this pH in comparison to pH 8.0/7.4 by 4.9% and 15.8% for compounds **1a** and **1b,** respectively.

The **NAC-1** and **NAC-2** show similar behavior to the **GSH-1** peak, where the AUC increases to the last time point, indicating that the equilibrium state has not been reached. The AUC of peaks-1 and 2 has been reduced by 20.4% and 27.2% in the case of **1a** and 34.2% and 39.6% in the case of **1b**.

4.1.3. Reactions performed under pH 3.2/3.7 condition

Reactions under stronger acidic conditions proceeded to a much lower extent than those under the above two conditions. It could be explained based on the stronger acidic conditions; the thiol function of both GSH and NAC exists exclusively in protonated (neutral) form. Although protonated thiols can act as nucleophilic reagents, their reactivity is much lower than their deprotonated (negatively charged) counterparts.

Only a small amount of adducts were detected in each chalcone–GSH/NAC incubates case. The chromatographic peak area values of the (Z) isomers were similar to those in the respective incubates at pH 8.0/7.4 and 6.3/6.8 (Tables 1 and 2).

Progress curves of the reaction of chalcones with GSH showed a linear downhill shape. A similar linear reduction in the chromatographic peak areas was also observed in the NAC incubations.

Over the incubation period, the chromatographic peak areas of the chalcone–GSH and chalcone-NAC diastereomers continuously increased.

4.2. (*E*)-2-benzylidinebenzosuberones

4.2.1. Reactions under Slightly Basic (pH 8.0/7.4) Conditions

Initially, we investigated the reactions of **4a** and **4b** under basic conditions. Considering the p*K*a values of GSH (8.83) and NAC (9.52), about 3.6% of the GSH and 0.75% of the NAC molecules are under pH 7.4 conditions. The reaction with GSH and NAC showed that both cyclic chalcones have intrinsic reactivity with the investigated thiols. By the end of the incubation period (315 min) with GSH, the initial area of the HPLC peak corresponding to the parent compounds **4a** and **4b** was reduced by 43.5% and 26.3 %, respectively (Table 3). While the compounds were incubated with NAC, the respective figures were 7.9% and 7.6% (Table 4). Changes in the chromatographic peak areas of the starting chalcones as a function of the

incubation time indicated that the compositions reflect the equilibrium only in the case of the NAC incubation.

Table 3. Retention times (t_R)[1] and integrated peak areas (A) of the investigated cyclic chalcone analogs (**4a** and **4b**) and their GSH adducts[2].

pH [3]	*Compound*	*t_R (E)-Chalcone*	*Area Ratio* [4] *A_{315}/A_0*	*t_R (Z)-Chalcone*	*Area (Z)-Chalcone*	*t_R GSH–1*	*Area GSH–1*	*t_R GSH–2*	*Area GSH–2*
3.2	**4a**	17.1	0.89	16.8	55.1	14.8[5]	74.9	15.2[5]	111.5
3.2	**4b**	16.6	0.95	16.3	136.1	N/D[5]	-	N/D[5]	-
6.3	**4a**	17.0	0.84	16.7	446.6	14.6	297.2	15.1	331.8
6.3	**4b**	16.9	0.91	16.7	513.4	14.2	233.6	14.8	256.4
8.0	**4a**	17.4	0.57	17.1	302.8	15.0	2847.0	15.4	3216.3
8.0	**4b**	16.8	0.74	16.5	412.0	13.9	2584.9	14.6	2785.0

[1]Retention times in minutes; [2] data refer to the average of two independent measurements at the 315 min time point; [3] pH value of the aqueous thiol solution; [4] ratios of peak areas measured at 0 and 315 min; [5] not detectable.

Table 4. Retention times (t_R)[1] and integrated peak areas (A) of the investigated cyclic chalcone analogs (**4a** and **4b**) and their NAC adducts[2].

pH [3]	*Compound*	*t_R (E)-Chalcone*	*Area Ratio* [4] *A_{315}/A_0*	*t_R (Z)-Chalcone*	*Area (Z)-Chalcone*	*t_R NAC–1*	*Area NAC–1*	*t_R NAC–2*	*Area NAC–2*
3.2	**4a**	17.1	0.76	16.8	124.1	N/D[5]	-	N/D[5]	-
3.2	**4b**	16.6	0.88	16.3	126.9	N/D[5]	-	N/D[5]	-
6.3	**4a**	17.5	0.93	17.2	118.9	16.3	60.0	16.5	513.9
6.3	**4b**	16.7	0.91	16.4	184.5	15.3	61.8	15.6	392.1
8.0	**4a**	17.5	0.92	17.2	467.5	16.3	477.7	16.5	913.4
8.0	**4b**	17.0	0.92	16.8	541.9	15.7	347.5	15.9	624.2

[1]Retention times in minutes; [2] data refer to the average of two independent measurements at the 315 min time point; [3] pH value of the aqueous thiol solution; [4] ratios of peak areas measured at 0 and 315 min; [5] not detectable.

As a result of the addition reactions, two new chiral centers are formed. Considering the inherent chirality of the two thiols, the formation of four diastereomeric adducts was expected. However, only two separate peaks could be detected under the present chromatographic conditions. The analysis showed a slight excess of the less polar diastereomers in both cases. The structure of the parent chalcones and their GSH and NAC conjugates were verified by HPLC-MS.

The time course of increase of the two separated peaks, **GSH-1** and **GSH-2**, and **NAC-1** and **NAC-2** showed some characteristic differences. In the case of the GSH adducts of **4a** and **4b**, the peak areas almost linearly increased over time. The progress curves of formation of the

adducts, however, showed somewhat different slopes, especially from the 105-minute time point. In the case of the NAC-adducts, the progress curves deviate from linearity. The curvatures of the concave curves differ from the 75-minute time point. By the end of the incubation period (315 min), the ratio of the two peaks of the GSH incubations remained close to unity (1.13 and 1.08 for **4a** and **4b**, respectively). In the case of the NAC incubations, the respective ratios were 1.91 and 1.80 (Tables 3 and 4). The formation of (*Z*)-chalcones could be detected in all four incubations. In the case of the NAC incubations, the area of the (*Z*)-peaks is comparable with those of the chalcone-NAC adducts (Tables 3 and 4).

4.2.2. Reaction under Slightly Acidic (pH 6.3/6.8) Conditions

As mentioned previously, the reaction of the cyclic chalcones with the two thiols under slightly acidic conditions (virtual pH 6.8) mimics the cellular milieu of the cancer cell. Under such conditions, about 0.9% of the GSH molecules and 0.2% of the NAC molecules exist in the more reactive thiolate form. The change in the concentration (chromatographic peak areas) of the starting chalcones **4a** and **4b** show parallelism in both reactions. By the end of the incubation period (315 min) with GSH, the initial area of the HPLC peak of **4a** and **4b** was reduced by 16.1% and 9.1%, respectively. While the compounds were incubated with NAC, the respective figures were 8.9% and 7.1%. These latter figures are very close to those obtained under slightly basic conditions (Tables 3 and 4).

In the GSH incubations, the separated HPLC peak areas of the **4a-GSH** and **4b-GSH** diastereomers increased closely parallel over time.

At the end of the incubation period, the ratio of the area of two separated peaks of the chalcone-GSH adducts is close to unity (1.12 and 1.10 for **4a** and **4b**, respectively) (Table 3).

A similar tendency could be observed for the **NAC-2** peak (peak with the higher retention time) area of **4a** and **4b**. At the same time, the chromatographic peak area of the **NAC-1** peak of **4b** remained practically unchanged, but that of **4a** slightly increased.

As a result, the ratio of the **NAC-2**/**NAC-1** areas at the 315 min time point was 8.57 and 6.34 for **4a** and **4b**, respectively. In all four incubations, the peak areas of the (*Z*)-chalcones are comparable to those of the formed adducts. Since the only source of the (*Z*) isomers under the experimental conditions is the retro-Michael reactions, it is reasonable to presume that the observed diastereomeric distributions do not reflect the results of the kinetics-controlled reactions.

4.2.3. Reaction under Acid (pH 3.2/3.8) Conditions

Under stronger acidic conditions, the thiol function of both GSH and NAC exists exclusively in protonated (neutral) form. Although the protonated thiols can act as nucleophilic reagents, their reactivity is much lower than their deprotonated (negatively charged) counterparts.

In the chalcone-GSH incubations, progress curves of the reactions (reduction of the initial area of the chalcones) showed a very slight downhill linear shape. At the end of the incubations, the initial values of the peak areas of **4a** and **4b** were reduced by 10.6% and 5.3%, respectively. At the same time, a linear increase in the peak area of the **4a-GSH** adducts (peaks 1 and 2) could be observed in parallel. The peaks corresponding to the respective **4b-GSH** adducts could not be detected. The ratio of the **4a-GSH** isomeric peaks (315 min time point) was 1.48. The areas of the respective (*Z*) isomers were much lower than in the pH 8.0 and pH 6.3 incubations (Table 3).

In the chalcone-NAC incubations, the reduction of the initial area of the chalcones showed a very slight downhill linear shape with somewhat different slopes. The initial peak area of **4a** and **4b** was reduced by 23.7% and 12.1% by the 315 min time point (Table 4). However, no **4-NAC** peaks could be identified. HPLV-UV analysis of the incubates showed the formation of several small peaks, more polar than the parent **4a** and **4b**. HPLC-MS investigations indicated the expected adduct formation, but it was impossible to identify them in the HPLC-UV chromatograms. In both incubations, the formation of the (*Z*) isomers could be seen (Table 4).

4.2.4. Molecular modeling analysis

The values for molecular properties of **4a**, **4b,** methanethiol (**CH_3SH**), and deprotonated methanethiol (**CH_3S^-**) were calculated. The highest occupied molecular orbital energy (E_{HOMO}) reflects the ability of a molecule to donate electrons, the lowest unoccupied molecular orbital energy (E_{LUMO}) demonstrates the ability to accept electrons, and the gap energies ($\Delta E_{LUMO-HOMO}$) are related to the chemical stability of molecules.

The chemical potential, chemical hardness, and electrophilicity are defined as ($\boldsymbol{\mu} = \left(\frac{\partial E}{\partial N}\right)_v$), ($\boldsymbol{\eta} = \frac{1}{2}\left(\frac{\partial^2 E}{\partial N^2}\right)_v$), and ($\boldsymbol{\omega} = \frac{\mu^2}{2\eta}$), respectively. $\boldsymbol{\eta}$ indicates the resistance of the molecule to alter its electronic density distribution and is higher for **4c**. On the other hand, $\boldsymbol{\mu}$ indicates the change in free energy when electrons are added or removed from the molecule. At the same time, $\boldsymbol{\omega}$ is a measure of a molecule's tendency to act as an electrophile. The value of $\boldsymbol{\omega}$ increased for **1c,** and $\boldsymbol{\mu}$ decreased when compared to **4c**.

In order to depict the distribution of electric charge on the molecular surface, a molecular electrostatic potential (MEP) map was generated. The red spots on the MEP surface represent the electron-rich sites and are susceptible to electrophilic attack. In contrast, the blue spots represent the electron-depleted regions and are sites susceptible to nucleophilic attack. For **CH_3S^-**, the MEP is reddish due to the unit negative charge resulting from deprotonation.

The k_{OH} rate constants were calculated for chalcone compounds, and the results indicate that the compounds with the highest k_{OH} values point to greater reactivity. The order of reactivity potential was observed as **CH_3SH** (9.15×10^9 M^{-1} s^{-1}) **1c** (9.01×10^9 M^{-1} s^{-1}) > **4c** (7.85×10^9 M^{-1} s^{-1}) > **CH_3S^-** (5.48×10^9 M^{-1} s^{-1}).

4.2.5. Microsomal incubation

Microsomal incubation of **4b** was performed using alamethicin-activated rat liver microsomes. Alamethicin is a peptide antibiotic that forms voltage-dependent channels in the lipid bilayers by oligomerizing various alamethicin molecules. In the first three minutes of the experiments, **4b** were incubated with rat liver microsomes in the presence of alamethicin. Since the parent compound has no hydroxyl substituent, only the oxidation and reduction reactions were expected over this period. Then, GSH was added to the mixtures. From then on, a reaction with GSH of the parent compound and its possible oxidative metabolites could also occur. These latter derivatives could be formed in spontaneous or GST-catalyzed reactions.

HPLC-MS investigation of the control incubates (without adding microsomes) indicated the formation of the expected **4b-GSH** adducts (Table 5). Under the present chromatographic conditions, similar to the previous results, two separated chalcone-GSH peeks appeared in the chromatograms. The structure of the **4b-GSH**-adducts (**4b-GSH-1** and **4b-GSH-2**) was verified by positive mode HR-MS

Besides the GSH conjugates, formation of the respected (*Z*)-isomer of the parent **(*E*)-4b** was observed. Since the reaction mixtures were kept in the dark, the formation of the **(*Z*)-4b** can only be explained due to the retro-Michael reaction of the formed adducts. The structure of the **(*E*)-4b** and **(*Z*)-4b** was verified based on the positive mode HR-MS of the isomeric mixture obtained by light isomerization of the pure **(*E*)-4b**.

The sum of the AUC of the two diastereomeric **4b-GSH** peaks was higher at each time point than in the respective control incubate. Although the ionization response factors of the four diastereomers are not known, the significant differences between the AUCs measured in the control and the microsomal incubates strongly support that the alamethicin-activated microsomes accelerate the chalcone-GSH conjugation reaction (Table 5).

Table 5. HPLC-MS peak areas (AUCs) of the **4b**, the **4b-GSH** isomeric peaks, and that of the oxidative metabolites of **4b** as a function of incubation time.

Compound	t_R *(min)*	*Control 0 min*	*Control 90 min*	*Microsome 0 min*	*Microsome 90 min*
(E)-4b	13.8	4,093,677,464	2,215,039,743	8,297,984,438	3,815,982,269
(Z)-4b	13.32	495,826,994	299,494,818	856,426,548	550,348,953
4b-GSH-1	10.47	1,466,946	18,724,489	2,210,842	50,714,479
4b-GSH-2	10.86	2,493,046	12,017,553	4,293,434	61,264,226
Nor-4b	12.25	N/D	N/D	7,206,428	81,441,916
4b+O	11.93	N/D	N/D	11,740,789	44,057,081

In analyzing the total ion MS spectrum of microsomal incubates, two products were found whose formation can be explained by CYP-catalyzed reactions: an oxygenated **4b (4b+O)** and the demethylated derivative (**nor-4b**). The AUC of both peaks increased over the incubation time (Table 5). The exact structure of the formed oxygenated metabolite needs further investigation. Lack of formation of a GSH-adduct of the possible epoxide metabolite; however, it is reasonable to suppose that the formed oxygenated metabolite is a hydroxyl derivative of **4b**. The demethylation reaction of **4b** resulted in the respective 4'-OH derivative. HPLC-MS analysis did not indicate the presence of a signal of the corresponding alcohol.

5. Discussion

Michael-type thiol reactivity of chalcones and related compounds is frequently associated with biological activities. In contrast, several examples demonstrate that non-covalent interactions of chalcones with cellular macromolecules can play an important role in the biological effects of the compounds. In a QSAR study, Katsori et al. found the clog*P* parameter to play an important part in the QSAR relationships. The authors found that the electronic effects are comparatively unimportant in the anticancer effect of the investigated chalcones. However, the open-chain **1a** and **1b** possess weaker cytotoxicity than the respective **4a** and **4b**. Furthermore, cytotoxicity and cell cycle modulating effects of **4a** and **4b** showed characteristic differences.

Investigation of the spontaneous reactivity of chalcones **1a**, **1b** and their seven-membered cyclic analog **4a**, **4b** demonstrated that both GSH and NAC react with the investigated chalcones under acidic (pH 3.2/3.7, pH 6.3/6.8) and basic (pH 8.0/7.4) conditions. However, the rate of the initial reactions and the composition of the equilibriums was affected by the nature of the reactants and the pH of the incubation mixtures.

Analysis of the effect of the 4-substituents under basic (pH 8.0/7.4) or slightly acidic (pH 6.3/6.8) conditions showed the 4-methyl substituted **1a** and **4a** to display the higher initial reactivity. ^{13}C NMR shifts – indicating the electron density around the particular nuclei – of the C10 atom of **1a** (144.9 ppm), **1b** (144.6 ppm), **4a** (138.0 ppm), and **4b** (137.7 ppm) were reported to be very similar. The observed difference in the reactivity of the chalcones with different substituents can be explained by the stability of the thiol-adducts. An early work of Humphlett et al. demonstrated that the activity of the α-hydrogen atom of the chalcone adduct, the resonance stabilization of the enone formed by cleavage, and the anionic stability of the thiolate ion are the determining factors of the reverse process. The authors found the α-keto and the ß-phenyl substitutions as determining factors in the effective reverse reactions. Since the 4-methoxy substitution can more effectively increase the electron density on the carbon-carbon double bond, and the formed chalcone is resonance-stabilized, the elimination process is more effective in the case of **1b (4b)** than **1a (4a)**. The observation further strengthens the previously suggested view that the different reactivities can be (at least partly) the result of the different stability of the thiol adducts. Similar conclusions were withdrawn by d'Oliveira et al. while investigating a few chalcones and their rigid quinolone analogs.

The results obtained in the pH 6.3/6.7 incubations are similar to those of the pH 8.0/7.4 (Tables 3 and 4). Under such conditions, the composition of both incubations represents equilibrium mixtures. Under both conditions, the conversion of **4a** is somewhat higher in the case of both thiols.

The ratio of the area of the two separated peaks in the GSH incubates (315 min time point) was close to the unity for **4a** and **4b** under both pH (pH 8.0/7.4 and 6.3/6.8) conditions (Table 3). On the contrary, HPLC analysis of the reactions of **1a** and **1b** with NAC showed different (1.8-8.57 times) excess of the least polar diastereomer (Table 4). The observed diastereoselectivity was affected by the nature of the 4-substituent and the pH. Thus, the methyl–substituted **4a** showed higher diastereoselectivity at both pH values. Diastereoselectivity was increased as the pH was reduced (Table 4). It is worth mentioning, however, that the observed diastereoselectivities do not reflect the diastereoselectivity of the addition reactions (retro-Michael reactions).

Under the acid conditions (pH 3.2/3.8), the formation of the respective conjugates is exclusively due to the nucleophilic addition of the protonated thiol forms onto the polarized carbon-carbon double bounds. A comparison of the respective compositions of the GSH incubates showed that the derivatives with the same substituent possess similar GSH reactivities (Table 3). However,

different results were obtained in the case of the reactions with NAC. The 315-minute percent conversion was found to be higher for **4a** (23.7%) and **4b** (12.1%) than those of the corresponding open-chain chalcones **1a** and **1b** (10.9% and 1.5%, respectively) (Table 6). However, no **4-NAC** adducts could be identified in the HPLC-UV chromatograms. Instead, several small, unidentified peaks appeared. HPLC-MS analysis could identify the expected conjugates.

Table 6. Percent reduction of initial chalcone HPLC-UV peaks in the 315-minute GSH and NAC incubation mixtures of the series **1** and **4**. *Calculated based on data published in [77].

Compound	*pH*	*Reagent thiol*	*Reduction of initial peak area (t=315 min) (%)*	*Reagent thiol*	*Reduction of initial peak area (t=315 min) (%)*
1a	8.0/7.4	GSH	96.3*	NAC	94.8*
4a	8.0/7.4	GSH	43.5	NAC	7.6
1b	8.0/7.4	GSH	92.1*	NAC	90.2*
4b	8.0/7.4	GSH	26.3	NAC	7.9
1a	6.3/6.7	GSH	90.6*	NAC	75.6*
4a	6.3/6.7	GSH	16.1	NAC	7.1
1b	6.3/6.7	GSH	78.3*	NAC	53.3*
4b	6.3/6.7	GSH	9.1	NAC	9.0
1a	3.2/3.7	GSH	19.3*	NAC	10.9*
4a	3.2/3.7	GSH	10.6	NAC	23.7
1b	3.2/3.7	GSH	4.2*	NAC	1.5*
4b	3.2/3.7	GSH	5.3	NAC	12.1

Concerning the effect of the cyclic structure, incorporation of the seven-membered ring into the chalcone moiety reduced the spontaneous thiol-reactivity (Table 6). Since the nature of thiols and the aromatic substituents are the same, the ring structure can explain the observed differences in reactivities of the two series. Amslinger et al. investigated the thiol reactivity of chalcones with various substituents in their α-position. The kinetics of thiol reactivities of the derivatives were correlated with some of their biological effects directly connected to their Michael acceptor ability. For example, α-methyl substitution of 2',3,4,4'-tetramethoxychalcone (TMC) decreased, and α-cyano substitution substantially increased the thiol reactivity of the nonsubstituted TMC. Based on these earlier observations, it is reasonable to suppose that the reduced reactivity of the benzosuberone derivatives **4a** and **4b** is the consequence of added effects of the α-alkyl substitution and the conformational strain caused by the cyclic structure of the starting enone and the reaction intermediate. Further research is needed to numerically characterize the electronic and stereochemical effects of the ring.

As a result of the GSH-addition reaction in the case of the open-chain compound **1a** and **1b**, formation of two diastereomeric adducts are possible. While the chromatographic conditions could not separate the GSH adducts, both diastereomers were detected during the NAC reaction. In these reactions, diastereoselectivity was observed; the less polar diastereomer was 1.7-1.2 times more favored than the other adduct (Table 2). The selectivity was inversely proportional to the pH and varied with the substituents.

The above findings provide further evidence to support the formation of a six-membered cyclic intermediate stabilized by hydrogen bonds as an intermediate compound during the reaction of chalcones with protonated thiols. This notion was previously proposed in the reaction of GSH with 4'-hydroxychalcone bis-Mannich derivatives. As per our earlier explanation, the *Re*-side of the planar enone group is attacked by the protonated thiol, forming a six-membered intermediate where the bulky aryl ring occupies a pseudoequatorial position.

As a result of the addition reactions to the cyclic chalcones **4a** and **4b**, formation of four diastereomeric adducts is possible. Because of the inherent chirality of GSH and NAC, two *cis* adducts and two *trans* adducts are expected to be formed

Earlier, Armstrong et al. reported on the stereochemistry of the GSTM 4-4-catalyzed reaction of GSH and the open-chain chalcone analogs (*E*)-(4'-X-phenyl)-3-butene-2-ones (PBO). In the reactions, a higher amount of the more polar GSH-adducts were formed. Based on the results of HPLC separation of the diastereomeric pairs of the PBO-GSH and the **4-GSH** adducts, we can presume that the two separated peaks formed in the present reactions correspond to the diastereomeric *cis* and *trans* adducts.

The retro-thia-Michael reactions can result in the formation of the respective (*Z*)-isomers as well. Therefore, to obtain authentic reference (*Z*) isomers, the stereochemically homogeneous (*E*) isomers of **1** and **4** were submitted to light-initiated isomerization, as published before. As a result, HPLC-MS data agreed with the respective (*Z*) isomers. Since all experiments were performed in the dark, the retro thia-Michael reaction could be the only source of the (*E*)/(*Z*) isomerization.

In the case of **4a** and **4b** reactions with both thiols relatively high amount of (*Z*)-isomer was detected, and the disappearance of starting compounds was witnessed in the progression curve of the incubations due to the net change of reversible reaction under both slightly basic and slightly acidic conditions. However, the detected HPLC peak areas of the (*Z*)-isomer in the case of **1a** and **1b** were significantly small (<100mAU at 315 min).

To obtain physicochemical properties insights into different reactivities of chalcones (**1**) and their seven-membered cyclic analogs (**4**), HOMO and LUMO molecular orbital energy and some electrophilic reactivity parameters of **1c**, **4c**, - and as model thiols –**CH_3SH** and **CH_3S^-** were calculated (Table 6). According to the Hard and Soft, Acids and Bases (HSAB) theory, nucleophilic-electrophilic reactions occur preferably between electrophiles and nucleophiles of similar hardness or softness. In the case of the α,ß-unsaturated ketones, the carbonyl oxygen atom withdraws electrons from the C_2=C_{10} bond – generating an electron deficiency at C_{10} – the most likely site to receive nucleophilic attacks. In methanethiol, nucleophilic attacks can occur at the sulfur atom. In compounds **1c** and **4c**, the carbonyl O has a high negative charge density, indicating its Lewis base behavior. On the other hand, regions of lower charge density, which appear in blue, indicate the Lewis acid behavior of the molecules.

A comparison of the LUMO energies showed that **1c** (-35.98 kcal/mol) is more acidic than **4c** (-28.44 kcal/mol. The LUMO energy of **CH_3SH** is (-2.979 kcal/mol), which increases to 77.99 kcal/mol in its deprotonated form (**CH_3S^-**). These characters are also reflected by all the other determined parameters (Table 6). Therefore, molecular orbital calculations provided data to support the experimental findings. The equilibrium (close-to-equilibrium) compositions of **1a** and **1b** show a higher product ratio than the cyclic chalcone analog **4a** and **4b**.

During the microsomal incubations, the **4b-GSH** proceeded to form in confirmation of the results of the *in vitro* incubations. The sum of the conjugates at each sampling time point was significantly larger than those of the control incubate; one may conclude that the alamethicin-activated microsome accelerated the chalcone-GSH conjugation. Since compound **4b** is a relatively lipophilic chalcone analog, it could be the substrate of both the microsomal glutathione transferases (MGSTs) and the so-called microsome-associated GST enzymes. These latter GST forms are associated with the outer microsomal membranes, and their characteristics resemble those of the cytosolic GSTs.

In analyzing the total ion MS spectrum of microsomal incubates, two products were found whose formation can be explained by CYP-catalyzed reactions: an oxidized **4b** (**4b+O**) and the demethylated derivative (**nor-4b**). The AUC of both peaks increased over the incubation time. The exact structure of the formed oxidized metabolite needs further investigation. Lack of formation of a GSH-adduct of the possible epoxide metabolite; however, it is reasonable to suppose that the formed oxidized metabolite is a hydroxyl derivative of **4b**. The demethylation reaction of **4b** resulted in the respective 4'-OH derivative. Earlier studies on the 4'-OH-metabolite (**nor-4b**) showed the compound to have a pronounced effect on the

modulation of mitochondrial respiratory functions. Phosphorylation inhibitory and/or partial uncoupling effect of the compound stimulated mitochondrial activity and increased the formation of ROS. Formation of a high amount of (*Z*)-isomer in the microsomal incubation might be a contributing molecular event in the observed biological effects of the compound. Furthermore, the GSH-reactivity of **4b** can also take part in the previously reported apoptotic effect of the compound.

6. Conclusion

Both chalcones **1a**, **1b** and their seven-membered cyclic analogs (**4a**, **4b**) showed intrinsic reactivity towards GSH and NAC under physiological (pH 7.4) conditions. The reactivity of the open-chain chalcones was higher than that of the cyclic ones. Furthermore, the reactivity of the compounds is also affected by the aromatic substituent found in position-4 of the benzylidene moiety. These observations serve as a basis for planning and synthesizing other chalcone/cyclic chalcone derivatives with optimized thiol-reactivity.

Microsomal biotransformation of **4b** showed the compound to be metabolized by the CYP and the GST enzymes. As a result of CYP-catalyzed transformations, one monooxygenated (**4b+O**) and the demethylated (**nor-4b**) metabolites were identified. In addition, a noticeable amount of **(*Z*)-4b** was also identified in incubates. Since the (*Z*)-isomer of the parent compound has a different three-dimensional structure, degree of conjugation, and lipophilicity, the formation of this isomer might be a molecular event that plays a role in the observed biological effects.

Furthermore, the GSH-reactivity of **4b** can affect the compound's bioavailability. The reactions that change the cellular GSH-status also take part in the previously reported apoptotic effect of this class of compounds. It should be mentioned, however, that while the thiol reactivity of **4a** and **4b** is comparable, there is a two-order of magnitude difference in the cytotoxicity against most of the investigated cancer cell lines.

The anticancer potential of chalcones is correlated with their ability to act on various molecular targets such as ABCG2, tubulin, activated nuclear B cell growth (NF-κB), vascular endothelial growth factor (VEGF), tyrosine kinase receptor (EGFR), mesenchymal-epithelial transition factor (MET), 5-α reductase, ACP-reductase, histone deacetylase, p53, CDC25B (protein tyrosine phosphatase), retinoic acid receptors, estrogenic topoisomerase receptors, and MDM2. Considering the present and our previous results, it is reasonable to suppose that the

molecular basis of the different biological effects of **4a** and **4b** is related to the non-covalent interactions of the compound.

7. Publications and Presentations

Number of publications related to the subject of the thesis: 4

Number of publications not related to the subject of the thesis: 2

Number of oral and poster presentations related to the subject: 6

The sum of impact factors from publications related to the topic of Ph.D. thesis: 11.135

Publications related to the topic of the Ph.D. thesis:

1. Perjési, P.; Caridad, N.P.; Aline, B.; Giulio, D. d'Oliveira; Kenari, F. The Chemistry of GST-Catalyzed Reactions. In *Glutathione: Biosynthesis, Functions and Biological Implications*; Perjési, P., Ed.; Nova Science Publishers: Hauppauge, New York, **2019**; pp. 373–403. ISBN 978-1-5361-4740-7.

2. Kenari, F.; Molnár, S.; Perjési, P. Reaction of Chalcones with Cellular Thiols. The Effect of the 4-Substitution of Chalcones and Protonation State of the Thiols on the Addition Process. Diastereoselective Thiol Addition. *Molecules* **2021**, 26, 4332. doi:10.3390/molecules26144332.

3. Kenari, F.; Molnár, S.; Pintér, Z.; Bitaraf, S.; Perjési, P. (*E*)-2-Benzylidenecyclanones: Part XVII. An LC-MS Study of Microsomal Transformation Reactions of (*E*)-2-[(4'-Methoxyphenyl) Methylene]-Benzosuberon-1-One: A Cyclic Chalcone Analog. *J. Pharm. Biopharm. Res*. **2023**, 4, 326–339, doi:10.25082/JPBR.2022.02.004.

4. Kenari, F.; Molnár, S.; Borges, I.D.; Napolitano, H.B.; Perjési, P. (*E*)-2-Benzylidenecyclanones: Part XVIII Study the Possible Link between Glutathione Reactivity and Cancer Cell Cytotoxic Effects of Some Cyclic Chalcone Analogs A Comparison of the Reactivity of the Open-Chain and the Seven-Membered Homologs. *Int. J. Mol. Sci.* **2023**, *24*, 8557. https://doi.org/10.3390/ijms24108557.

Other publications not related to the topic of the Ph.D. thesis:

1. Sadighpour, T.; Mubarak, M.; Sabaeifard, P.; Saeifar, S.; Kenari, F. COVID-19 and Renal Involvement; Evolving Role of Thromboinflammation, Vascular and Glomerular Disease in the Pathogenesis. *J. Nephropathol*. **2021**, 10. doi:10.34172/jnp.2021.23.

2. Rozmer, Z.; Kenari, F.; Tyukodi, L.; Kulcsár, G.; Huber, I.; Perjési, P. A PTE GYTK Gyógyszerészi Kémiai Intézetben folyó kutatásokról I. Szerkezet-reaktivitás és szerkezet-hatás vizsgálatok. *Magy. Kém. Foly. Kém. Közl*. **2022**, 128, 53–59. doi:10.24100/MKF.2022.02.53.

Conference oral and poster presentations related to the Ph.D. thesis:

1. Kenari, F.; Perjési, P. Study on Interaction of Reduced Glutathione (GSH) with Cyclic Chalcone Analogs.; [s.n.], Ed.; Pécsi Tudományegyetem Általános Orvostudományi Kar: Pécs, 2018; p. [36]-[36].

2. Kenari, F.; Perjési, P. Study of Interaction of Reduced Glutathione (GSH) with Chalcone and Some Cyclic Chalcone Analogues. *Acta Pharm. Hung*. **2017**, 87, 156–157.

3. Perjési, P.; Almási, A.; Kenari, F.; Kuzma, M.; Fliszár-Nyúl, E. Non-Enzyme Catalyzed Metabolic Transformations of Xenobiotics. *Acta Pharm. Hung*. **2017**, 87, 113.

4. Kenari, F.; Molnár, S.; Kulcsár, G.; Perjési, P. Study of Interaction of Reduced Glutathione (GSH) with Some Chalcone Analogues. In VIII. Interdiszciplináris Doktorandusz Konferencia 2019: Absztrakt kötet. 8th Interdisciplinary Doctoral Conference 2019: Book of Abstracts; Bódog, F., Csiszár, B., Eds.; Pécsi Tudományegyetem Doktorandusz Önkormányzat, Pécs, 2019; pp. 122–122. ISBN 978-963-429-374-3.

5. Kenari, F.; Bernardes, A.; Noda, PC.; Molnár, S.; Kulcsár, G.; Perjési, P. Study of Interaction of Reduced Glutathione (GSH) with Some Chalcone Analogs in Vitro and in Vivo. *Acta Pharm. Hung*. **2020**, 90, 123–124.

6. Kenari, F.; Perjési, P. Study on Interaction of Reduced Glutathione (GSH) with Cyclic Chalcone Anaglogues.; Magyar Gyógyszerésztudományi Társaság (MGYT): Budapest, 2018; p. abstract.

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