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A Synthetic Chalcone as a Potent Inducer of Glutathione Biosynthesis

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Abstract

Chalcones continue to attract considerable interest due to their anti-inflammatory and antiangiogenic properties. We recently reported the ability of 2',5'-dihydroxychalcone (2',5'-DHC) to induce both breast cancer resistance protein-mediated export of glutathione (GSH) and c-Jun N-terminal kinase-mediated increased intracellular GSH levels. Herein, we report a structure–activity relationship study of a series of 30 synthetic chalcone derivatives with hydroxyl, methoxyl, and halogen (F and Cl) substituents and their ability to increase intracellular GSH levels. This effect was drastically improved with one or two electrowithdrawing groups on phenyl ring B and up to three methoxyl and/or hydroxyl groups on phenyl ring A. The optimal structure, 2-chloro-4',6'-dimethoxy-2'-hydroxychalcone, induced both a potent NF-E2-related factor 2-mediated transcriptional response and an increased formation of glutamate cysteine ligase holoenzyme, as shown using a human breast cancer cell line stably expressing a luciferase reporter gene driven by antioxidant response elements.

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Supporting Information

Characterization of chemical compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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INTRODUCTION

Glutathione (GSH) is a tripeptide present at high concentrations in the cell (5–10 mM) with diverse functions including modulation of cell proliferation, antioxidant defense, and detoxification of xenobiotics.^{1,2} The rate-limiting enzyme for GSH synthesis is glutamate cysteine ligase (GCL), which is composed of a catalytic subunit (glutamate cysteine ligase catalytic subunit, GCLC) and a regulatory subunit (glutamate cysteine ligase regulatory subunit, GCLM).³ The second step of GSH synthesis is catalyzed by glutathione synthase (GS). Low levels of GSH have been associated with a variety of diseases including diabetes, pulmonary fibrosis, and epilepsy.^{2,4–6} Along with a number of phase II antioxidant enzymes such as heme oxygenase-1 (HO-1), the enzymes involved in GSH synthesis are tightly regulated by several transcription factors including NF-E2-related factor 2 (Nrf2), activator protein-1 (AP-1), and nuclear factor κ B (NF- κ B).^{2,3} In the case of GCL, post-transcriptional factors have also been reported.^{3,7}

Chalcones are both biosynthetic precursors of flavonoids and end products associated with a variety of biological activities.^{8,9} Anti-inflammatory and antiangiogenic properties of naturally occurring and synthetic chalcones continue to attract considerable interest.^{10–13} While their toxicity has been associated with the production of reactive oxygen species (ROS) and mitochondrial dysfunction,^{14,15} nontoxic concentrations have been reported to inhibit the synthesis of the inducible nitric oxide synthase, induce the expression of HO-1, and trigger GSH synthesis and export.^{14,16–20} We recently reported the ability of the naturally occurring chalcone 2',5'-dihydroxychalcone (2',5'-DHC) to induce breast cancer resistance protein (BCRP)-mediated export of GSH²¹ and c-Jun *N*-terminal kinase (JNK)-mediated increased levels of cellular GSH,¹⁴ suggesting that chalcones could be used to modulate cellular levels of GSH.

Herein, we report a structure–activity relationship study of a series of 30 chalcone derivatives with hydroxyl, methoxyl, and halogen (chlorine and fluorine) substituents (Figure 1) in their ability to increase intracellular levels of GSH (iGSH). This study was carried out using a human breast cancer cell line stably expressing a luciferase reporter gene driven by antioxidant response elements [MCF-7/antioxidant response element (AREc32), referred as AREc32 cells in this study], which also allowed us to examine the chalcone-induced Nrf2 transcriptional activity.²²

Chemistry

The chalcones assessed in this study were synthesized following the classical method shown in Scheme 1. An acetophenone derivative was condensed with 1 equiv of a benzaldehyde derivative in the presence of a solution of KOH in a mixture H₂O:MeOH.

RESULTS

Chalcone-Induced Increased Levels of iGSH

A series of chalcone analogues with hydroxyl, methoxyl, and halogen groups in both phenyl rings A and B were tested at 10 μ M in their ability to elevate iGSH levels in AREc32 cells

(6 and 24 h treatments) (Figure 1 and Table 1). The most active compound was chalcone **23** with a 3-fold increase in iGSH levels as compared to control (24 h treatment) followed by chalcones **28**, **24**, and **21**. The positive role of the chlorine on ring B can be clearly seen by comparing chalcones **21**, **23**, **24**, and **28** to their unsubstituted analogues (**14**, **22**, and **26**). The higher activity of chalcone **23** vs **15** and **16** shows the importance of three methoxyl and/or hydroxyl groups on ring A. Comparison with **24** shows that one chlorine on ring B has a higher effect than two chlorines. However, this is not a general rule since **21** was more active than **16**, showing that the optimal number of electrowithdrawing groups on ring B depends on the number of substituents on ring A. Adding a third chlorine (**29** vs **28**) appeared to decrease the effect. The higher activity of **16** (one Cl in position 2) as compared to **19** (one Cl in position 3) reveals the importance of the position of the chlorine for optimal activity. To determine whether the electrowithdrawing effect of the chlorine was responsible for the higher activity, we synthesized and evaluated chalcones substituted with fluorines and a nitro group. As shown in Table 1, the introduction of fluorines (**15** vs **16**) and a nitro group (**27** vs **28**) did not enhance the activity, indicating that the electrowithdrawing effect is not the only factor mediating the higher activity of the chlorine analogues. Chlorines on ring A (**30**) had no increased effect. It is also worth noting that the chalcone with no halogens that had the optimal effect was chalcone **7**, with two methoxyl groups in positions 2 and 6 on ring B, and that adding another methoxyl group in position 4 (**8**) abolished the effect.

Chalcone **23** Induces GSH Biosynthesis

To determine whether the **23**-induced increase in iGSH was due to increased GSH synthesis, AREc32 cells were cotreated with **23** (10 μ M) and buthionine sulfoximine (BSO, 20 μ M), an inhibitor of GCL.²³ As shown in Figure 2A, **23**-induced increased GSH levels were blocked by BSO. Comparison of the effects of **23** to those of the known inducers of GSH biosynthesis, sulforaphane (SFN, mixture of *D* and *L* stereoisomers) and *tert*-butylhydroquinone (tBHQ), indicates that **23** is significantly more potent (Figure 2A).^{24,25} Moreover, both **3** (2',5'-DHC) and **23** (20 μ M, 6 h of treatment) induced an increase in GCL holoenzyme formation (Figure 3, bottom panel). Interestingly, this was not associated with an increase in either GCLC or GCLM protein levels (Figure 3, top panels). The formation of GCL holoenzyme using **3** was harder to detect with a longer treatment time (24 h).¹⁴ To examine the signal transduction pathways mediating **23**-induced GSH synthesis, pharmacological inhibitors of the JNK and p38 mitogen-activated protein (MAP) kinase pathways were employed, namely, anthrapyrazolone (SP600125, 20 μ M) and 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1*H*-imidazol-5-yl]pyridine (SB203580, 20 μ M), respectively.^{14,26–28} Both compounds showed only a weak inhibitory effect on **23**-induced increased iGSH levels (Figure 2B). Because ROS have also been associated with increased GSH synthesis, the catalytic antioxidant manganese-(III) *meso*-tetrakis(*N,N'*-diethylimidazolium-2-yl)porphyrin (MnTDE-1,3-IP⁵⁺, 20 μ M) was tested as well, showing a similar weak effect (Figure 2B).^{14,29}

Chalcone **23** Induces Nrf2 Transcriptional Activity

Constitutive and inducible GSH biosynthesis has been associated with Nrf2 transcriptional activity, and recent studies demonstrate that chalcones with electrowithdrawing groups on

phenyl ring B induce Nrf2 activity.^{2,10} Selected compounds of our series, that is, chalcone **23**, **28** and **3**, were examined at 5 μ M for their ability to induce Nrf2-dependent ARE-luciferase activity in AREc32 cells (18 h of treatment) and compared to the activities of the known Nrf2 activators SFN and tBHQ (5 μ M). The **23**-induced luciferase activity was ~4–5-fold higher than **3** and SFN and ~10-fold higher than tBHQ (Figure 4A). In an attempt to determine the pathways involved in mediating this Nrf2 transcriptional response, AREc32 cells were preincubated with 20 μ M SP600125 (JNK inhibitor), SB203580 (p38MAPK inhibitor), or the catalytic antioxidant MnTDE-1,3-IP⁵⁺ before treatment with **23**. While SP600125 and MnTDE-1,3-IP⁵⁺ showed a slight inhibitory effect (yet not statistically significant), SB203580 had no effect on **23**-induced ARE-luciferase activity (Figure 4B).

Chalcone **23**-Induced Cytotoxicity

Because **23** has a markedly higher ability to induce GSH synthesis than **3**, we compared their relative toxicity in AREc32 cells (0–60 μ M, 48 h of treatment). As shown in Figure 5, **23** had a slight higher toxicity than **3** (IC₅₀ around 25 and 33 μ M, respectively), suggesting a correlation between toxicity at high concentrations and the ability to induce both the Nrf2 transcriptional activity and the GSH synthesis at nontoxic concentrations.

DISCUSSION

Among the chalcones tested, chalcone **23** showed the highest activity in inducing GSH biosynthesis. It also showed a strong ability to trigger the Nrf2 transcriptional response. These effects were significantly higher than those of sulforaphane, a well-known inducer of Nrf2-transcriptional activity and GSH biosynthesis.^{24,30} While the JNK pathway seemed to play a significant role in mediating 2',5'-DHC-induced Nrf2 activation and GSH synthesis,¹⁴ this role appeared to be rather weak in the case of **23**. Also, results from studies employing **23** in combination with the catalytic antioxidant MnTDE-1,3-IP⁵⁺ suggest an insignificant role for ROS in triggering this Nrf2 response. Similar to **3**, **23** enhanced GCL holoenzyme formation. Intriguingly, this was not the result of increased GCL subunit expression since **23** did not increase either GCLC or GCLM protein expression. This and previous results suggest a post-transcriptional and post-translational effect of **23** and **3** on GCL holoenzyme formation.¹⁴ Taken together, our results show that chalcone-induced GSH synthesis does not rely exclusively on Nrf2 activation, despite an apparent cause–effect relationship. Further studies are needed to determine the precise mechanism by which **23** promotes increased GCL holoenzyme formation.

We have previously reported that chalcones induce a bimodal response in iGSH levels overtime.¹⁴ The initial drop in iGSH levels at 6 h of treatment is due to chalcone-induced efflux of GSH, and we recently demonstrated that BCRP mediates this phenomenon.²¹ The most active compounds in inducing GSH biosynthesis showed no decrease in iGSH levels at 6 h of treatment, suggesting that the synthesis of GSH compensates for the loss of GSH early in treatment. On the other hand, some chalcones have been found to inhibit BCRP-mediated drug efflux.^{31–33} The fact that the combination of **23** and BSO resulted in lower iGSH levels than BSO alone (Figure 2A) suggests that **23** is able to induce GSH efflux as well. It is worth noting that BCRP expression has been associated with Nrf2 activation.³⁴ It

remains to be determined whether there is a correlation between the abilities of chalcones to inhibit BCRP and to induce GSH transport.

New compounds that are able to induce GSH biosynthesis might have beneficial effects in a number of diseases that have been associated with low levels of GSH.^{4–6} The chemoprotective effects of sulforaphane have been studied in a number of models, including cancer and neuroprotection.^{24,30,35} Some triterpenoids have also raised interest as potent Nrf2 activators with antioxidant and anti-inflammatory activities.³⁶ Mounting data support that, rather than being “antioxidants” per se, flavonoids and other polyphenols induce an antioxidant adaptive response, most likely by triggering cell-signaling pathways.^{26,37} In this regard, chalcones in particular appear to be very interesting.¹⁴ Another salient aspect of this study is our finding that chalcones post-translationally enhance GCL holoenzyme formation. Although **23** was slightly more toxic than **3**, **23** was markedly more efficient in inducing both the Nrf2 transcriptional response and the GSH synthesis at nontoxic concentrations. To conclude, **23** is a potent new activator of Nrf2 transcriptional activity that has the ability to modulate intracellular levels of GSH.

EXPERIMENTAL SECTION

Chemicals and Reagents

Chemicals and reagents for chalcone synthesis were obtained from either Sigma-Aldrich (St. Louis, MO) or Acros (Geel, Belgium). Chalcone (**1**), 2',5'-DHC (**3**), and 2',4',6'-trimethoxychalcone (**26**) were purchased from Indofine Chemicals Company, Inc. (Hillsborough, NJ). BSO, D,L-sulforaphane (SFN), *tert*-butylhydroquinone (tBHQ), L-GSH, pyruvate, NADH, and *meta*-phosphoric acid were purchased from Sigma-Aldrich. SB203580 was purchased from Biomol (Plymouth Meeting, PA) and SP600125 from Calbiochem (San Diego, CA). MnTDE-1,3-IP⁵⁺, prepared as previously described in U.S. patent #6,544,975B1, was a kind gift from Aeolus Pharmaceuticals (Mission Viejo, CA).

Synthesis of Chalcones: Typical Procedure

To a stirred solution of acetophenone derivative (1 mmol) and a benzaldehyde derivative (1 mmol) in MeOH (10 mL) was added KOH (50% aqueous solution, 1 mL). The solution was heated at 70 °C for 3–5 h, MeOH was evaporated, and the residue was dissolved in CH₂Cl₂/H₂O (50 mL, 4:1). The organic layer was washed with brine and evaporated, and then, column chromatography was carried out over silica gel (AcOEt/hexane 1:2). In the case of fluorinated chalcones, KOH (25%) was used. The structural analysis and characterization are provided in the Supplementary Information.

Analytical Methods

¹H NMR spectra were recorded on a Bruker AC-400 instrument (400 MHz). Chemical shifts (δ) are reported in ppm relative to Me₄Si (internal standard). Electrospray ionization ESI mass spectra were acquired by the Analytical Department of Grenoble University on an Esquire 300 Plus Bruker Daltonis instrument with a nanospray inlet. Combustion analyses were performed at the Analytical Department of Grenoble University, and all tested compounds had a purity of at least 95%. Thin-layer chromatography (TLC) used Merck

silica gel F-254 plates (thickness 0.25 mm) and flash chromatography used Merck silica gel 60, 200–400 mesh.

Cell Line and Culture Conditions

Transformed human breast cancer cells stably expressing a luciferase reporter gene driven by AREs (MCF-7/AREc32) were obtained from Dr. Joe M. McCord (University of Colorado, Aurora, CO) and were grown in DMEM (low glucose) supplemented with 10% fetal bovine serum (FBS), 1% pen/strep (10000 unit, Cellgro), and Geneticin (400 mg/500 mL) at 37 °C and 5% CO₂-supplemented air atmosphere.²²

Intracellular Levels of GSH

Intracellular GSH levels were determined by HPLC with electrochemical detection (HPLC-EC).³⁸ Cultured AREc32 cells from 24-well plates were washed once with 1 mL of PBS, resuspended in 0.5 mL of PBS, and sonicated. Ten percent *meta*-phosphoric acid (25 μ L) was then added to the samples (1% v/v final concentration), the samples were centrifuged at 20000g for 10 min, and the supernatants were used for HPLC analysis. The HPLC column used was a Synergi 4u Hydro-RP 80A (150 mm $\times$ 4.6 mm) from Phenomenex (Torrance, CA), and the mobile phase was sodium phosphate buffer (125 mM sodium phosphate monobasic, pH adjusted to 3 with phosphoric acid) and 0.9% methanol. The flow rate was 0.5 mL/min. The retention time for GSH under these conditions was 7.0 min. The HPLC instrument was from ESA, Inc. (Chelmsford, MA) and was equipped with an autosampler (model 540) and a Coul array detector (model 5600A). The potential applied was +0.75 V vs H/Pd electrode, and the injection volume was 5 μ L. The remaining 0.1 mL sample was used to measure protein content using Coomassie plus protein assay reagent (Thermoscientific, Rockford, IL).

Immunoblotting of GCLC, GCLM, and GCL Holoenzyme

AREc32 cells were grown in six-well plates treated and washed with PBS. Cells were then sonicated in 200 μ L of PBS. Cell debris was spun down, and the supernatant volume was reduced to approximately 30 μ L by evaporation using a speed vacuum system. GCLC, GCLM, and GCL holoenzyme levels were detected as described previously.⁷ α -Glyceraldehyde-3-phosphate dehydrogenase (α -GAPDH) was used as an internal standard.

Luciferase Activity

AREc32 cells were cultured in 12-well plates to ~60% confluence and incubated in the presence of 5–10 μ M compound for 18 h. In some experiments, the cells were preincubated with the catalytic antioxidant MnTDE-1,3-IP⁵⁺, the SP600125 JNK inhibitor, or the SB203580 p38MAPK inhibitor for 30 min before exposure to **23**. The treated cells were washed with PBS and lysed with cell lysis buffer (BD Biosciences, San Jose, CA). The cell lysate was centrifuged at 20000g for 15 min, and the supernatant was used for the firefly luciferase assay. Luciferase assays were carried out using enhanced luciferase assay kit (BD Biosciences) on a Moonlight 2010 luminometer.³⁹

Assessment of Cytotoxicity

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay is commonly used to measure cancer cell survival; yet, it has revealed artifacts when measuring the cytotoxicity of pro-oxidant agents.⁴⁰ Another simple method to evaluate drug-induced cytotoxicity is using membrane integrity as an index, which can be assessed by monitoring the release of cytosolic lactate dehydrogenase (LDH). AREc32 cells were grown in 24-well plates, and LDH activity was measured after 48 h of treatment in both culture medium and cell lysates (50 mM HEPES, 0.5% Triton X-100, pH 7) using a plate reader format as previously described.⁴¹ Briefly, 5 μ L of either cell culture supernatant or lysates were incubated with 0.24 mM NADH in a Tris/NaCl, pH 7.2, buffer in 96-well plates for 5 min at 25 °C. The reaction was started by the addition of 9.8 mM pyruvate, and the consumption of NADH was followed at 340 nm for 5 min at 30 °C. The percent LDH release was calculated by the following equation: $\text{supernatant LDH}/(\text{supernatant LDH} + \text{lysate LDH}) \times 100$.

Statistical Analysis

Data are presented as means $\pm$ standard errors. Each experimental group consisted of 3–4 wells, and the results were repeated at least once. Data were subsequently analyzed for significant differences using ANOVA analysis coupled with a Tukey's range test where significance was preset at $P < 0.05$ (Prizm v.4, GraphPad, San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

AP-1	activator protein-1
ARE	antioxidant response element
BCRP	breast cancer resistance protein
BSO	L-buthionine sulfoximine
2',5'-DHC	2',5'-dihydroxychalcone
α-GAPDH	α -glyceraldehyde-3-phosphate dehydrogenase
GCLC	glutamate cysteine ligase catalytic subunit
GCLM	glutamate cysteine ligase regulatory subunit

GSH	reduced glutathione
HO-1	heme oxygenase-1
HPLC-EC	HPLC with electrochemical detection
JNK	c-Jun <i>N</i> -terminal kinase
LDH	lactate dehydrogenase
MAP	mitogen-activated protein
MnTDE-1,3-IP⁵⁺	manganese(III) <i>meso</i> -tetrakis(<i>N,N'</i> -diethylimidazolium-2-yl)porphyrin
NF-κB	nuclear factor κ B
Nrf2	NF-E2-related factor 2
ROS	reactive oxygen species

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Figure 1.
General structure of the chalcones tested.

Scheme 1.
Synthetic Procedure of Chalcones

Figure 2.

Chalcone-induced increases in iGSH levels in AREc32 cells. (A) Chalcone **23**-induced increase in iGSH (10 μ M) was higher when compared to **3** (10 μ M), **28** (10 μ M), sulforaphane (SFN, 10 μ M), and *tert*-butylhydroquinone (BHQ, 10 μ M) (24 h treatment) and was inhibited by BSO (20 μ M), an inhibitor of GSH synthesis. (B) The SP600125 and SB203580 inhibitors of JNK and p38MAPK, respectively, and the catalytic antioxidant MnTDE-1,3-IP⁵⁺ (SP, SB, and MnP, 20 μ M, respectively) (24 h of treatment) had a weak inhibitory effect on **23**-induced increase in iGSH. Bars with different letters were statistically different from one another ($n = 3$, $P < 0.05$).

Figure 3.

Chalcone **23**-induced formation of GCL holoenzyme in AREc32 cells. As shown by immunoblotting protein extracts of treated AREc32 cells (6 h treatment), both **3** and **23** (20 μ M, respectively) increased the formation of GCL holoenzyme as compared to control (native PAGE), whereas no increase in GCLC nor GCLM was detected (SDS PAGE). α -GAPDH was used as an internal standard. The experiment was repeated once.

Figure 4.

Chalcone **23**-induced Nrf2 transcriptional response in AREc32 cells. (A) Using 5 μ M concentrations, **23**-induced luciferase activity was ~4–5-fold higher than 3 and SFN (18 h treatment). (B) A 10 μ M concentration of **23**-induced luciferase activity was slightly decreased (statistically insignificantly) by the SP600125 inhibitor of JNK (SP, 20 μ M) and by the catalytic antioxidant MnTDE-1,3-IP⁵⁺ (MnP, 20 μ M). The SB203580 inhibitor of p38MAPK (SB, 20 μ M) had no inhibitory effect. Bars with different letters were statistically different from one another ($n = 4$, $P < 0.05$).

Figure 5.

Cytotoxicity of chalcones **23** and **3** in AREc32 cells. Chalcone **23** was more toxic than **3** (IC_{50} of 25 and 33 μM , respectively) using the percentage of LDH release as an index of cytotoxicity (48 h of treatment, $n = 3$).

Table 1

Induction of iGSH Increased Levels in AREc32 Cells^a

chalcone	substituents on phenyl A						substituents on phenyl B						iGSH (%)	
	2'	3'	4'	5'	6'		2	3	4	5	6		6 h	24 h
1													103	115
2							OCH ₃				OCH ₃		111	189
3	OH			OH									82	177
4	OH		OCH ₃				OCH ₃		OCH ₃				100	174
5	OH		OCH ₃				OCH ₃		OCH ₃				114	160
6	OH		OCH ₃				OCH ₃			OCH ₃			100	153
7	OH		OCH ₃				OCH ₃						90	231
8	OH		OCH ₃				OCH ₃		OCH ₃				91	104
9	OH						Cl				Cl		108	180
10	OH		OCH ₃				Cl				Cl		107	193
11	OH					OCH ₃	Cl				Cl		88	183
12	OCH ₃		OCH ₃				Cl						109	232
13	OCH ₃		OCH ₃					F					78	173
14	OCH ₃					OCH ₃							87	158
15	OCH ₃					OCH ₃	F						103	195
16	OCH ₃					OCH ₃	Cl						105	211
17	OCH ₃					OCH ₃							106	204
18	OCH ₃					OCH ₃		F					82	171
19	OCH ₃					OCH ₃		Cl					88	165
20	OCH ₃					OCH ₃	F		F				104	182
21	OCH ₃					OCH ₃	Cl				Cl		122	256
22	OH		OCH ₃			OCH ₃							101	191
23	OH		OCH ₃			OCH ₃	Cl						107	299
24	OH		OCH ₃			OCH ₃	Cl				Cl		119	250
25	OH		OCH ₃			OCH ₃	F				F		135	226

chalcone	substituents on phenyl A						substituents on phenyl B					iGSH (%)	
	2'	3'	4'	5'	6'		2	3	4	5	6	6 h	24 h
26	OCH ₃		OCH ₃	OCH ₃	OCH ₃							91	209
27	OCH ₃		OCH ₃	OCH ₃	OCH ₃	F	F				F	118	223
28	OCH ₃		OCH ₃	OCH ₃	OCH ₃	Cl	Cl				Cl	122	261
29	OCH ₃		OCH ₃	OCH ₃	OCH ₃	Cl	Cl		Cl		Cl	119	246
30	Cl				Cl	OCH ₃	OCH ₃				OCH ₃	93	120
31		Cl	Cl			OCH ₃	OCH ₃		OCH ₃		OCH ₃	98	88

^a Cells were exposed to chalcones (10 μ M) tested in their ability to increase cellular levels of GSH (iGSH as a percentage as compared to control, 6 and 24 h of treatment, margin error $\pm 10\%$, $n = 3$).