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Preliminary communication

Synthesis and antioxidant potential of novel synthetic benzophenone analogues

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ABSTRACT

Considering that oxidative stress is strongly implicated in the toxicity of chemotherapy, much effort is focused on the research of diverse antioxidants as protective agents. An efficient synthesis of three novel benzophenones containing 1,3-thiazol moiety (**6a**–**c**) is described. Their antioxidant power was evaluated in vitro and in three cell lines (the cancerous MCF7 and the non-cancerous hTERT-HME1 mammary cells, and the H9c2 cardiomyoblastic cells). One analogue 5-(2,5-dihydroxybenzoyl)-2(3*H*)-benzothiazolone (**6c**), displayed an important antioxidant activity, a low cytotoxicity, and could decrease reactive oxygen species production generated by *tert*-butyl hydroperoxide (*t*BHP) in all three cell lines. Interestingly, **6c** was able to protect the non-cancerous cells against *t*BHP-induced death. Further studies are underway to determine its relevance as an adjuvant in oxidative stress inducing chemotherapy.

1. Introduction

Toxicity, as well as the development of resistance, is a major obstacle for successful chemotherapy and radiotherapy. Multiple organ systems can be affected, with both acute and chronic side effects. Cytoprotection, or protection of normal cells, is a strategy now being investigated in preclinical and clinical models [1]. The ideal protective agent would possess selectivity (only healthy tissue protection), broad spectrum activity (protection of numerous tissues against a wide variety of cytotoxic agents), and a favourable side effect profile (well tolerated) [2]. It would prevent all toxic side effects, from non-life threatening (alopecia) to potentially fatal (severe cardiomyopathy, severe thrombocytopenia), without diminishing the antitumor efficacy of the cancer therapy [3]. Considering the fact that oxidative stress is strongly implicated in the toxicity of chemotherapy, much effort has been focused on the research of diverse antioxidants as potential chemoprotective agents [4,5]. Thus, the phenolic antioxidants which are effective agents against oxidative stress-mediated disorders (cardiovascular dysfunctions, inflammatory diseases, neurodegenerative diseases, diabetes…) [6–8] are also protectors in cancer therapy [9,10]. Their pharmacological action is mostly ascribed to free radical scavenging, chelation of redox active metal ions, modulation of gene expression and interaction with cell signalling pathways [11]. Among phenolics, natural hydroxybenzophenones are biologically active metabolites present in plants and especially in Guttiferae family [12,13]. Radical scavenging activity represents one of their most relevant functions. For example, garcinol, a polyisoprenylated benzophenone isolated from *Garcinia indica* suppressed hydroxyl radical more strongly than DL-α-tocopherol in the Fenton reaction system [14]. Some of the benzophenone glycosides isolated from *Hypericum annulatum* showed a free radical scavenging activity and exerted substantial protection of bone-marrow cells against anticlonogenic effects induced by epirubicine [15]. Based upon these natural models, different benzophenone derivatives were synthesized. For example, the antioxidant properties of synthetic benzophenone and its trihydroxy-derivative were reported to have similar characteristics to those of phenol and resorcinol [16]. Polyhydroxybenzophenones displayed significant scavenging effects on the superoxide anion and prevented cell death in menadione-treated myoblasts [17].

These interesting biological effects led us to design and synthesize novel benzophenones containing a benzothiazolone moiety which could adjoin important pharmacological activities. Indeed, 2(3*H*)-benzothiazolone derivatives are pharmacophores largely used in medicinal chemistry [18]. In particular, they display anti-inflammatory properties [19–21] and can inhibit Cox pathway [22]. These two tightly linked properties are strongly related to their antioxidant activity.

In order to develop antioxidant adjuvants for chemotherapy, this preliminary study is devoted to the design and synthesis of three novel benzophenone derivatives, i.e. 5-(hydroxybenzoyl)-2(3*H*)-benzothiazolones. Their antioxidant activity was assessed in

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vitro, and their toxicity was verified in cell cultures. These two analyses allowed the choice of the most effective of these analogues, named **6c**, whose effect on cell redox status and efficacy against oxidative stress induced by *tert*-butyl hydroperoxide (*t*BHP) was explored in two human mammary cell lines (a cancerous one, MCF7, and a non-cancerous one, hTERT-HME1) and in a cardiomyoblast cell line, H9c2. We suggest that this compound could be a potential candidate as a chemoprotective agent.

2. Results and discussion

2.1. Chemistry

The general synthetic method shown in Scheme 1 was employed for the preparation of the new benzophenones containing 1,3-thiazol moiety. The preparation involved a straightforward reaction sequence: (i) Friedel–Crafts acylation of methoxybenzenes **1a**–**c**, (ii) reduction of the nitro group, (iii) formation of thiazol ring, (iv) replacement of the thione group by a carbonyl group and (v) demethylation of aryl methyl ethers to afford the desired hydroxyl substituted benzophenones **6a**–**c**.

As the Friedel–Crafts acylation of 2(3*H*)-benzothiazolone was previously studied and was found to proceed with high regioselectivity in position 6 [23–25], the access to the 5-benzoyl derivatives of 2(3*H*)-benzothiazolone was realized by us using an alternative route (Scheme 1).

In the first step the Friedel–Crafts acylation of activated benzenes such as anisole (**1a**), veratrole (**1b**), and 1,4-dimethoxybenzene (**1c**) was realized. Acylation was carried out with freshly prepared 4-chloro-3-nitrobenzoyl chloride in dichloromethane in the presence of $AlCl_3$ as a catalyst [26]. Selective reduction of the nitro group in compounds **2a**–**c** was accomplished with tin(II) chloride according to a published procedure [27] to obtain the *o*-chloroanilines **3a**–**c** in high yields. These compounds underwent smooth cyclization with ethyl potassium xanthate in dimethylformamide (DMF) as solvent to afford 5-aroyl-2(3*H*)-benzothiazolethiones (**4a**–**c**). Thiones **4a**–**c** were oxidized with $KMnO_4$ under basic conditions to the corresponding benzothiazole-2-sulfonic acids, which were used in the next stage without isolation. After acidic hydrolysis 5-aroyl-2(3*H*)-benzothiazolones (**5a**–**c**) were obtained. The preparation of target compounds **6a**–**c** was achieved by demethylation of the methoxy groups on compounds **5a**–**c** by treatment in refluxing 48% aqueous HBr.

2.2. Pharmacology

2.2.1. In vitro antioxidant capacity

The ferric reducing antioxidant power (FRAP) method [28] was used for assessing the antioxidant potential of **6a**–**c** derivatives.

The highest antioxidant activity was observed for **6b** (6.1 nmol Fe^{2+}/nmol compound), while **6a** lacked any ability to reduce Fe^{3+} (Fig. 1). The antioxidant capacity of **6b** and **6c** was, respectively, 2 and 1.5 times higher than that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) a water soluble derivative of vitamin E, used as a reference antioxidant.

2.2.2. Cytotoxicity

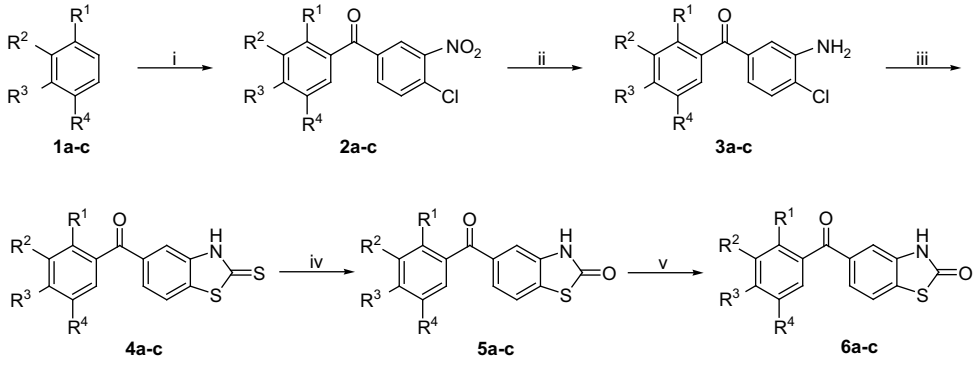
The cytotoxicity of benzophenone derivatives was tested on the cancerous MCF7 and the non-cancerous hTERT-HME1 mammary cell lines, following 48 or 72 h exposure, using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay [29]. MTT was used here as a first intention screening method in order to eliminate compounds that could present any cell toxicity.

As evident from IC_{50} values summarized in Table 1, the most cytotoxic compound was **6b**, while **6c** did not show noteworthy cytotoxic effects below 100 μM on both cell lines. Thus, **6c** which displayed a high in vitro antioxidant potential, without significant toxicity in a non-cancerous cell line was selected for further studies. The concentration of 100 μM was chosen for further experiments according to IC_{20} values.

2.2.3. Effect on cellular redox status

In order to verify whether **6c** could play a functional role in biological systems, the levels of intracellular reduced glutathione (GSH) were quantified by high-performance liquid chromatography (HPLC) using a post-column reaction with *ortho*-phthaldehyde [30].

In both cell lines, a significant increase of GSH levels was observed after a 48 h-incubation (88.9 ± 7.2 vs 71.3 ± 2.5 for MCF7, $p < 0.05$ vs control and 59.4 ± 1.9 vs 43.3 ± 1.1 for hTERT-HME1, $p < 0.001$ vs control) (Fig. 2). In non-cancerous cells, a significant increase was also detected after 72 h of incubation.



Scheme 1. Schematic representation of the synthesis and chemical structures of the tested 5-(hydroxybenzoyl)-2(3*H*)-benzothiazolones (**6a**–**c**). Reagents and conditions: (i) 4-chloro-3-nitrobenzoyl chloride, $AlCl_3$, CH_2Cl_2, 45 °C; (ii) $SnCl_2 \cdot 2H_2O$, 37% HCl, C_2H_5OH; (iii) $C_2H_5OC(S)SK$, DMF, reflux; (iv) $KMnO_4$, KOH, 60–70 °C; (v) 48% HBr, CH_3COOH, *n*-$Bu_4N^+Br^-$, reflux.

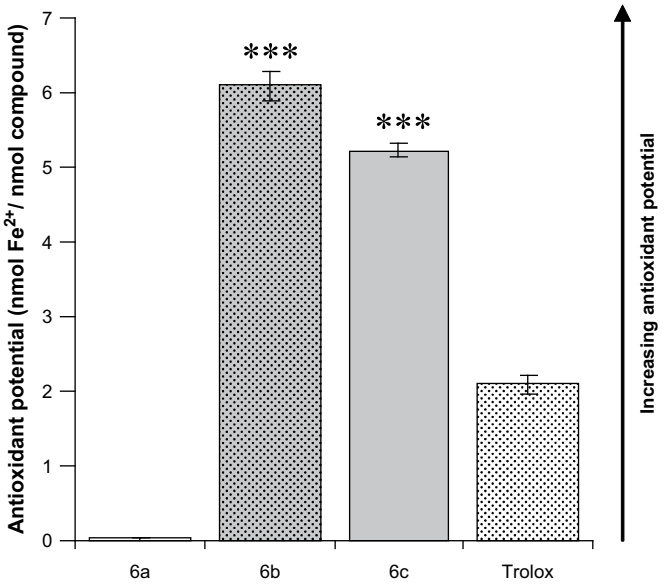


Fig. 1. Antioxidant capacity of benzophenone derivatives (**6a**–**c**) (FRAP method). Values are expressed as antioxidant ratio (nmol Fe^{2+}/nmol compound). Data are means ± SD ($n = 3$), $^{***}p < 0.001$ with respect to Trolox.

Moreover, ROS levels were detected by flow cytometric analysis using H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) as described previously [31]. H_2DCFDA is capable of crossing the plasma membrane to enter a cell interior, where esterases hydrolyze its acetyl moiety to produce 2′,7′-dichlorodihydrofluorescein (H_2DCF). The de-acetylated form of the probe is then susceptible to oxidation, generating a fluorescent product, 2′,7′-dichlorofluorescein (DCF).

In MCF7 cells, during the first 24 h of incubation, **6c** was a more powerful reducer of H_2O_2 production than Trolox (Fig. 3). After 24 h, **6c** did not act as a radical scavenger, while Trolox continuously inhibited ROS production by 20–30% in both cell lines.

2.2.4. Cytoprotective action

Many biological functions of polyphenols have been attributed to their free radical scavenging and antioxidant properties [6]. The new benzophenone **6c** could be particularly efficacious due to its high antioxidant in vitro efficacy (FRAP assay). Thus, it was interesting to test the protective potential of this compound against oxidative-induced stress in cells. For that, *tert*-butyl hydroperoxide (*t*BHP) was used as a membrane-permeant pro-oxidant agent [32]. To evaluate protection against oxidative stress, MCF7 and hTERT-HME1 cells were pretreated with 100 μM **6c** for 4 h (a time treatment compatible to an antioxidant activity as shown by Fig. 3), and thereafter exposed to 0.5 mM *t*BHP.

The most significant production of reactive oxygen species (ROS) in cells was measured after 0.5 and 1.5 h of *t*BHP treatment. During this length of time, a pre-treatment with **6c**, at the concentration of 100 μM, significantly lowered the ROS levels down to levels for controls not exposed to *t*BHP in both cell lines (Fig. 4A).

In parallel, cell survival was evaluated using a flow cytometric analysis with H_2DCFDA and PI (propidium iodide). H_2DCFDA enters viable cells freely where it is converted to the green fluorescent dichlorofluoresceine, whereas the red fluorophore PI only enters altered membrane-cells. Therefore, viable cells are identified with a high green and a low red fluorescence [33]. *t*BHP dramatically induced cell death in hTERT-HME1, while this effect was discrete in MCF7. Compound **6c** efficiently protected the non-cancerous cell line against cell death provoked by *t*BHP, as shown by the increase in cell survival (43–59%) observed in the co-treated (**6c** + *t*BHP) cells (Fig. 4B).

Since the sensitivity of cardiac cells to oxidative stress limits anthracyclin-based chemotherapy, we evaluated whether **6c** could also protect the cardiomyoblastic cells, H9c2 exposed to *t*BHP.

Fig. 5A shows that H9c2 cells are extremely sensitive to *t*BHP. Indeed, a 0.5 h treatment by *t*BHP 0.5 mM induced cell mortality of 89% vs 6% in the control group (DMSO). To avoid such an overwhelming mortality, and allow realistic evaluation of **6c** to protect from oxidative stress, we decreased the dose of *t*BHP. H9c2 cells were treated by very low doses of *t*BHP (0.05 mM). Fig. 5B and C shows that **6c** induced an important decrease of ROS levels (up to 7-fold) produced by *t*BHP and saved cell viability, indicating that the benzophenone was able to efficiently protect these cells against *t*BHP-induced toxicity.

Table 1
Cytotoxic activity of benzophenone analogues (**6a**–**c**) on MCF7 and hTERT-HME1 breast cell lines after 48 and 72 h of incubation (MTT assay)

Compound	Breast cancer cell line, MCF7		Non-cancer breast cell line, hTERT-HME1	
	IC_{20} (μM)	IC_{50} (μM)	IC_{20} (μM)	IC_{50} (μM)
48 h				
6a	44.5 ± 4.7	120.9 ± 2.1	73.1 ± 3.7	109.2 ± 3.4
6b	12.1 ± 3.9	43.6 ± 4.7	43.5 ± 4.4	155.3 ± 4.9
6c	93.5 ± 6.8	167.6 ± 2.4	149.9 ± 4.3	182.2 ± 6.2
72 h				
6a	37.8 ± 4.5	95,9 ± 5,1	78.4 ± 2.1	110.6 ± 4.8
6b	8.3 ± 3.2	28.1 ± 2.7	33.8 ± 3.6	116.3 ± 4.7
6c	126.0 ± 2.3	173.2 ± 4.1	144.6 ± 4.7	167.4 ± 3.8

IC values are the mean of 8 assays ± SD.

3. Conclusion

Many adverse effects of chemotherapy agents are related to the rise of oxidative stress levels in multiple organ systems. In order to develop novel antioxidant molecules as chemoprotectants for non-cancerous cells, we designed new molecules associating two chemical structures that could be efficient against oxidative stress (a benzophenone and a benzothiazolone moiety). This paper describes the successful synthesis of three derivatives, 5-(hydroxybenzoyl)-2(3*H*)-benzothiazolones (**6a**–**c**), and the evaluation of the pharmacological properties of the lead candidate, i.e. (**6c**) [5-(2,5-dihydroxybenzoyl)-2(3*H*)-benzothiazolone]. Compound **6c** exhibited evident antioxidant properties in vitro. A screening on human mammary cell lines – the cancerous MCF7 and the non-cancerous hTERT-HME1 showed that **6c** did not exert any cytotoxic effect. Moreover, this benzophenone derivative displayed a potent free radical scavenging activity and was able to efficiently protect cells against oxidative stress provoked by *tert*-butylhydroperoxide. These properties could be due to the significant increase of GSH level observed in **6c** treatments. This study must now be extended by comparing the antioxidant properties of the two moieties of the molecule with those of the bifunctional resulting compound.

Moreover, the protective action of **6c** was also evident in H9c2, a cardiomyoblast cell line. These results are of particular relevance considering that the cardiotoxicity of doxorubicin, a drug often used in breast cancer treatment, is due to oxidative stress generation. Thus, the protective effect of **6c** against doxorubicin toxicity has now to be tested in cancer cells and in cardiac cells in order to appreciate its eventual relevance in therapeutics.

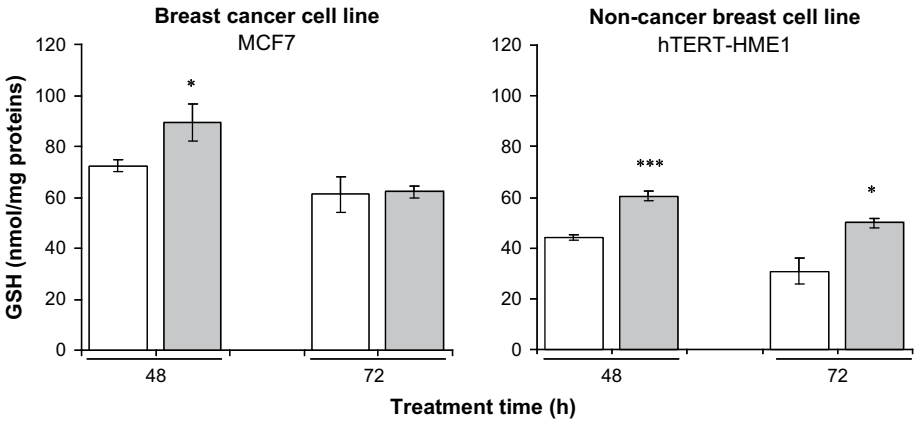


Fig. 2. Intracellular GSH levels in MCF7 and hTERT-HME1 breast cells after 48 and 72 h of incubation with 100 μM **6c** (▩). Data are means ± SD ($n = 3$), $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to respective control cells (□), which were performed with DMSO.

4. Experimental protocol

4.1. Chemistry

Melting points were determined on a Boetius hot-stage microscope and are uncorrected. IR spectra were recorded in nujol on a Specord 71 spectrometer. ^{1}H NMR spectra were obtained with a Bruker DRX 300 spectrometer operating at 300 MHz in DMSO-d_6. Chemical shifts were recorded in parts per million (ppm, δ) downfield from TMS as an internal standard. Elemental analyses were performed on a Vario EL III microanalyzer (Elementar Analysensysteme GmbH, Germany). Obtained results were within 0.4% of theoretical values. Thin layer chromatography (TLC) was carried out on silica gel plates (Merck 60 F_{254}) using toluene–chloroform–ethylacetate (3:1:1) as eluent; zones were detected visually under ultraviolet irradiation (254 nm).

4.1.1. 4-Chloro-4′-methoxy-3-nitrobenzophenone (**2a**)

Anisol (2.7 ml, 25 mmol) was added to a solution of freshly prepared 4-chloro-3-nitrobenzoyl chloride (6.05 g, 27.5 mmol) in dichloromethane (25 ml). The resulting solution was cooled and then aluminum chloride (3.67 g, 27.5 mmol) was slowly added at 0–5 °C. After complete addition, the mixture was allowed to stir at room temperature for 1 h, and then was heated up to 45 °C for 4 h. The reaction mixture was poured onto ice (250 g) and conc. HCl (15 ml). The crude product was isolated by extraction with dichloromethane. The combined organic layers were washed with 10% aqueous $NaHCO_3$, water, dried over Na_2SO_4 and evaporated in vacuum. The residue was purified by recrystallization from diisopropyl ether. Yield 4.88 g (67%). M.p. 103 °C. IR (nujol, cm^{-1}): 1640, 1540, 1340, 1270. Anal. Calcd. for $C_{14}H_{10}ClNO_4$: C 57.65; H 3.46; N 4.80. Found: C 57.84; H 3.49; N 4.82.

4.1.2. 4-Chloro-3′,4′-dimethoxy-3-nitrobenzophenone (**2b**)

Compound **2b** was synthesized as for **2a** starting from veratrole. Recrystallization from ethanol gave 2.70 g (34%). M.p. 157–158 °C. IR (nujol, cm^{-1}): 1650, 1510, 1370, 1270. Anal. Calcd. for $C_{15}H_{12}ClNO_5$: C 56.00; H 3.76; N 4.35. Found: C 56.35; H 3.78; N 4.35.

4.1.3. 4-Chloro-2′,5′-dimethoxy-3-nitrobenzophenone (**2c**)

Compound **2c** was synthesized analogously as for **2a** starting from 1,4-dimethoxybenzene. Recrystallization from ethanol gave 4.80 g (60%). M.p. 112–113 °C. IR (nujol, cm^{-1}): 1670, 1540, 1350, 1270. Anal. Calcd. for $C_{15}H_{12}ClNO_5$: C 56.00; H 3.76; N 4.35. Found: C 56.37; H 3.79; N 4.31.

4.1.4. 5-(4-Methoxybenzoyl)-2(3H)-benzothiazolethione (**4a**)

To a stirred suspension of **2a** (2.33 g, 8 mmol) in ethanol (20 ml), a hot solution of $SnCl_2 \cdot 2H_2O$ (7.24 g, 32 mmol) in conc. HCl (16 ml) was slowly added. The stirring was continued for 30 min at room temperature. The obtained clear solution was treated with 30% aqueous NaOH until pH was strongly basic and the amino derivative **3a** was isolated in 85% yield. Then, a mixture of **3a** (2.61 g, 10 mmol) and ethyl potassium xanthate (3.59 g, 22 mmol) in DMF (20 ml) was refluxed for 3 h. After heating, the reaction mixture was poured onto water (50 ml) and then was acidified with 10% HCl. The obtained precipitate was filtered, washed with water and

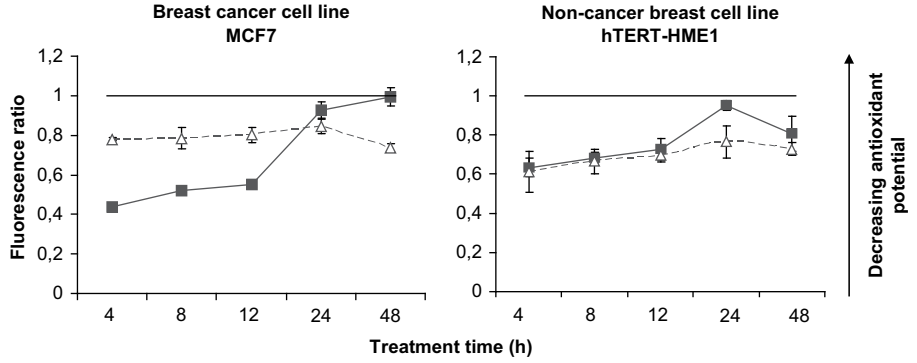


Fig. 3. Effect of **6c** on redox status of MCF7 and hTERT-HME1 cells in culture. Cells were treated for 4, 8, 12, 24 and 48 h with **6c** (—■—) and Trolox (– –△– –) (100 μM). ROS levels were detected by flow cytometry as dichlorofluoresceine (DCF) fluorescence ratio between treatment and DMSO control (—). Data are means ± SD ($n = 3$).

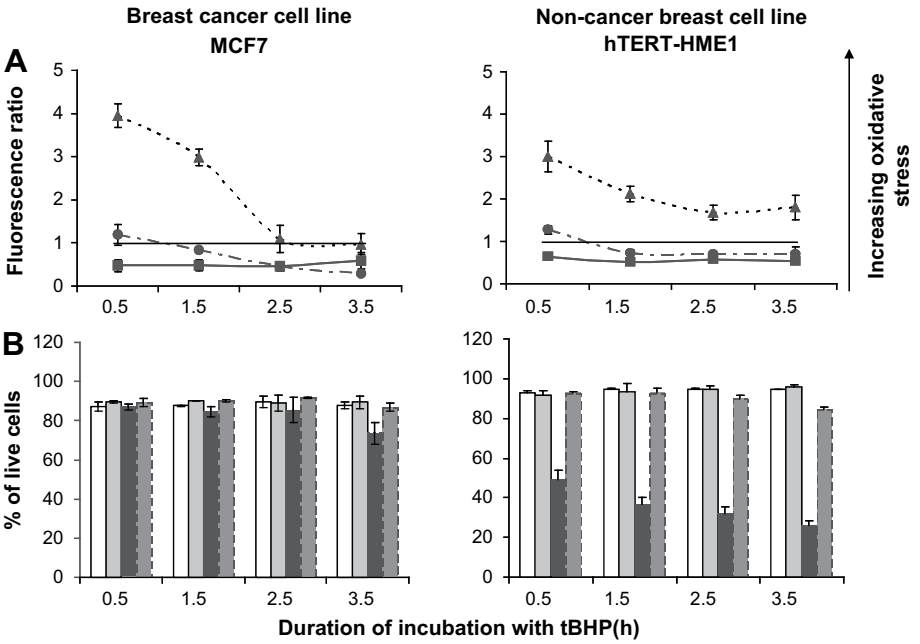


Fig. 4. Evaluation of the protective potential of **6c** against oxidative stress induced by *tert*-butyl hydroperoxide (*t*BHP) in MCF7 and hTERT-HME1 cells. (A) ROS production was assessed by flow cytometric analysis as DCF fluorescence ratio between treatment and control. Co-treated cells (—●- -) were pre-incubated with **6c** for 4 h, followed by a subsequent incubation (0.5–3.5 h) with *t*BHP (0.5 mM). Three controls were performed for testing the effects of a 4 h pre-incubation with DMSO (—) or **6c** (—■—), and for testing the effects of a 0.5–3.5 h incubation with *t*BHP alone (- -▲- -). (B) Cytotoxicity was assessed by flow cytometric analysis with PI and H_2DCFDA. Co-treated cells (▩) were pre-incubated with **6c** for 4 h, followed by a subsequent incubation (0.5–3.5 h) with *t*BHP (0.5 mM). Three controls were performed for testing the effects of a 4 h pre-incubation with DMSO (□) or **6c** (▩), and for testing the effects of a 0.5–3.5 h incubation with *t*BHP alone (■). Results are the mean of triplicate assays ± SD.

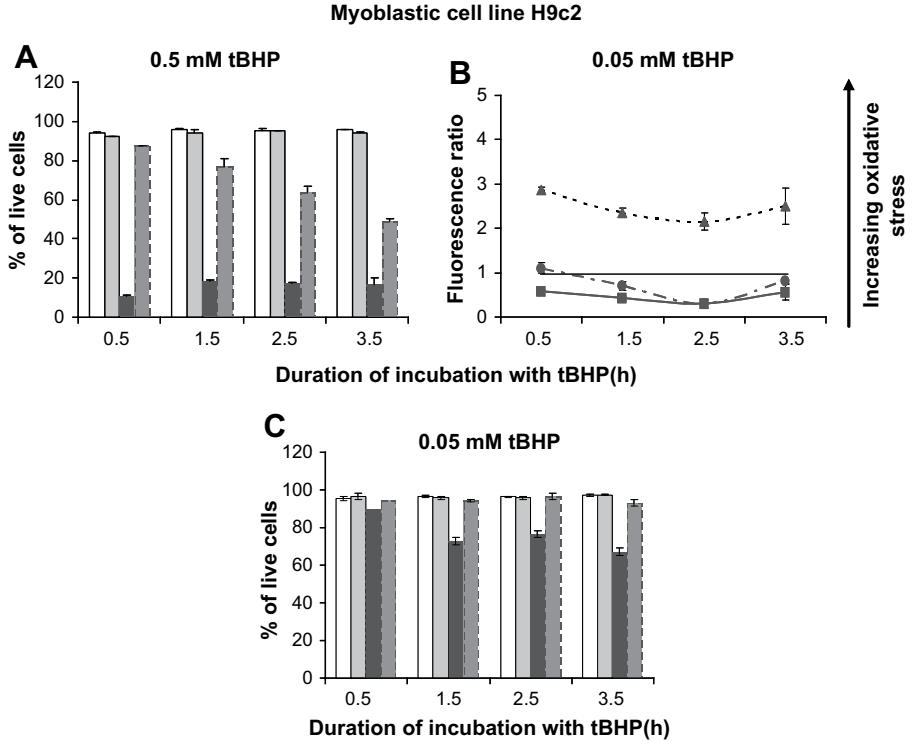


Fig. 5. Evaluation of the protective potential of **6c** against oxidative stress induced by *tert*-butyl hydroperoxide (*t*BHP) in cardiomyoblastic cells H9c2. (A,C) cytotoxicity was assessed by flow cytometric analysis with PI and H_2DCFDA. Co-treated cells (▩) were pre-incubated with **6c** for 4 h, followed by a subsequent incubation (0.5–3.5 h) with *t*BHP (0.5 mM (A) or 0,05 mM (C)). Three controls were performed for testing the effects of a 4 h pre-incubation with DMSO (□) or **6c** (▩), and for testing the effects of a 0.5–3.5 h incubation with *t*BHP alone (■). (B) ROS production was assessed by flow cytometric analysis as DCF fluorescence ratio between treatment and control. Co-treated cells (—●- -) were pre-incubated with **6c** for 4 h, followed by a subsequent incubation (0.5–3.5 h) with *t*BHP (0.05 mM). Three controls were performed for testing the effects of a 4 h pre-incubation with DMSO (—) or **6c** (—■—), and for testing the effects of a 0.5–3.5 h incubation with *t*BHP alone (- -▲- -). Results are the mean of triplicate assays ± SD.

dried to yield 2.85 g (95%) of **4a**. Recrystallization from 2-methoxyethanol gave 2.38 g (79%). M.p. 225–228 °C. IR (nujol, cm^{-1}): 3000–3100, 1640, 1250. Anal. Calcd. for $C_{15}H_{11}NO_2S_2$: C 59.78; H 3.68; N 4.63; S 21.28. Found: C 60.12; H 3.58; N 4.33; S 21.43.

*4.1.5. 5-(3,4-Dimethoxybenzoyl)-2(3H)-benzothiazolethione (**4b**)*

Compound **4b** was synthesized as for **4a** starting from **2b**. Recrystallization from 2-methoxyethanol gave 1.06 g (32%). M.p. 243–244 °C. IR (nujol, cm^{-1}): 3200, 1640, 1250. Anal. Calcd. for $C_{16}H_{13}NO_3S_2$: C 57.99; H 3.95; N 4.23; S 19.35. Found: C 58.15; H 3.91; N 4.21; S 19.65.

*4.1.6. 5-(2,5-Dimethoxybenzoyl)-2(3H)-benzothiazolethione (**4c**)*

Compound **4c** was synthesized as for **4a** starting from **2c**. Recrystallization from ethanol gave 1.35 g (41%). M.p. 176–179 °C. IR (nujol, cm^{-1}): 3000–3100, 1640, 1280. Anal. Calcd. for $C_{16}H_{13}NO_3S_2$: C 57.99; H 3.95; N 4.23; S 19.35. Found: C 58.13; H 4.09; N 4.35; S 19.60.

*4.1.7. 5-(4-Methoxybenzoyl)-2(3H)-benzothiazolone (**5a**)*

To a stirred solution of **4a** (1.50 g, 5 mmol) in 10% aqueous KOH, a hot solution of 10% $KMnO_4$ was slowly added for 20–30 min. The end of the oxidation reaction was determined by the excess of $KMnO_4$ in reaction mixture. The precipitate of MnO_2 was filtered and washed with hot water. The filtrate was acidified to pH = 2 with conc. HCl and refluxed until the evolving of SO_2 finished. The obtained precipitate of **5a** was collected by filtration, washed with water and dried. Recrystallization from dioxane gave 0.93 g (65%). M.p. 214–215 °C. IR (nujol, cm^{-1}): 3000–3100, 1680, 1640, 1270. ^{1}H NMR (300 MHz, DMSO-d_6): 3.86 (s, 3H, OCH_3), 7.09 (d, 2H, ArH, J = 8.8 Hz), 7.39 (d, 1H, ArH, J = 1.5 Hz), 7.44 (dd, 1H, ArH, J = 1.5 Hz, J = 8 Hz), 7.74–7.78 (m, 3H, ArH), 12.10 (br s, 1H, NH). Anal. Calcd. for $C_{15}H_{11}NO_3S$: C 63.14; H 3.89; N 4.91; S 11.24. Found: C 63.47; H 3.68; N 4.66; S 11.55.

*4.1.8. 5-(3,4-Dimethoxybenzoyl)-2(3H)-benzothiazolone (**5b**)*

Compound **5b** was synthesized as for **5a** starting from **4b**. Recrystallization from ethanol gave 0.50 g (32%). M.p. 232–235 °C. IR (nujol, cm^{-1}): 3000–3100, 1680, 1640, 1250. ^{1}H NMR (300 MHz, DMSO-d_6): 3.83 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 7.11 (d, 1H, ArH, J = 8.4 Hz), 7.35 (dd, 1H, ArH, J = 1.8 Hz, J = 8.3 Hz), 7.38 (d, 1H, ArH, J = 1.8 Hz), 7.54 (d, 1H, ArH, J = 1.3 Hz), 7.60 (dd, 1H, ArH, J = 1.3 Hz, J = 8.2 Hz), 7.83 (d, 1H, ArH, J = 8.4 Hz), 12.25 (br s, 1H, NH). Anal. Calcd. for $C_{16}H_{13}NO_4S$: C 60.94; H 4.16; N 4.44; S 10.17. Found: C 60.68; H 4.30; N 4.33; S 9.87.

*4.1.9. 5-(2,5-Dimethoxybenzoyl)-2(3H)-benzothiazolone (**5c**)*

Compound **5c** was synthesized as for **5a** starting from **4c**. Recrystallization from ethanol gave 1.06 g (67%). M.p. 210–214 °C. IR (nujol, cm^{-1}): 3000–3100, 1680, 1640, 1270. ^{1}H NMR (300 MHz, DMSO-d_6): 3.61 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 6.89 (d, 1H, ArH, J = 2.8 Hz), 7.09 (dd, 1H, ArH, J = 2.8 Hz, J = 8.8 Hz), 7.13 (d, 1H, ArH, J = 8.8 Hz), 7.40 (d, 1H, ArH, J = 1.5 Hz), 7.45 (dd, 1H, ArH, J = 1.5 Hz, J = 8.2 Hz), 7.71 (d, 1H, ArH, J = 8.2 Hz). Anal. Calcd. for $C_{16}H_{13}NO_4S$: C 60.94; H 4.16; N 4.44; S 10.17. Found: C 60.59; H 4.12; N 4.48; S 10.05.

*4.1.10. 5-(4-Hydroxybenzoyl)-2(3H)-benzothiazolone (**6a**)*

To a suspension of **5a** (0.29 g, 1 mmol) in acetic acid (2 ml), an aqueous solution of 48% HBr (7 ml) and tetrabutylammonium bromide (5 mg) was added. The mixture was refluxed for 4 h and after cooling was poured onto water (50 ml). The precipitate was collected, washed with water and dried. Recrystallization from ethanol gave 0.14 g (52%). M.p. 297–299 °C. IR (nujol, cm^{-1}): 3000–3250, 1680, 1640. ^{1}H NMR (300 MHz, DMSO-d_6): 6.89 (d, 2H, ArH, J = 8.7 Hz), 7.36 (d, 1H, ArH, J = 1.5 Hz), 7.42 (dd, 1H, ArH, J = 1.5 Hz, J = 8.1 Hz), 7.66 (d, 2H, ArH, J = 8.7 Hz), 7.73 (d, 1H, ArH, J = 8.1 Hz), 10.42 (br s, 1H, OH), 12. 04 (br s, 1H, NH). Anal. Calcd. for $C_{14}H_9NO_3S$: C 61.98; H 3.34; N 5.16; S 11. 82. Found: C 61.78; H 3.20; N 5.01; S 11.47.

*4.1.11. 5-(3,4-Dihydroxybenzoyl)-2(3H)-benzothiazolone (**6b**)*

Compound **6b** was synthesized as for **6a** starting from **5b**. Recrystallization from acetone gave 0.12 g (43%). M.p. 309–310 °C. IR (nujol, cm^{-1}): 3050–3350, 1690, 1640. ^{1}H NMR (300 MHz, DMSO-d_6): 6.84 (d, 1H, ArH, J = 8.2 Hz), 7.10 (dd, 1H, ArH, J = 2.1 Hz, J = 8.2 Hz), 7.22 (d, 1H, ArH, J = 2.1 Hz), 7.35 (d, 1H, ArH, J = 1.3 Hz), 7.41 (dd, 1H, ArH, J = 1.4 Hz, J = 8.1 Hz), 7.72 (d, 1H, ArH, J = 8.1 Hz), 9.48 (br s, 1H, OH), 9.83 (br s, 1H, OH), 12.05 (br s, 1H, NH). Anal. Calcd. for $C_{14}H_9NO_4S$: C 58.53; H 3.16; N 4.88; S 11.16. Found: C 58.18; H 3.46; N 4.85; S 10.81.

*4.1.12. 5-(2,5-Dihydroxybenzoyl)-2(3H)-benzothiazolone (**6c**)*

Compound **6c** was synthesized as for **6a** starting from **5c**. Recrystallization from ethanol gave 0.12 g (42%). M.p. 240–242 °C. IR (nujol, cm^{-1}): 3400, 3000–3150, 1710, 1660. ^{1}H NMR (300 MHz, DMSO-d_6): 6.71 (d, 1H, ArH, J = 2.9 Hz), 6.80 (d, 1H, ArH, J = 8.8 Hz), 6.86 (dd, 1H, ArH, J = 2.9 Hz, J = 8.8 Hz), 7.40 (d, 1H, ArH, J = 1.1 Hz), 7.46 (dd, 1H, ArH, J = 1.1 Hz, J = 8.1 Hz), 7.72 (d, 1H, ArH, J = 8.2 Hz), 9.08 (s, 1H, OH), 9.64 (s, 1H, OH), 12.03 (br s, 1H, NH). Anal. Calcd. for $C_{14}H_9NO_4S$: C 58.53; H 3.16; N 4.88; S 11.16. Found: C 58.88; H 3.23; N 4.84; S 10.79.

4.2. Pharmacology

4.2.1. Ferric reducing antioxidant power (FRAP)

The FRAP assay [28] depends upon the reduction of a ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex to the ferrous tripyridyltriazine (Fe^{2+}–TPTZ) by a reductant at low pH. Fe^{2+}–TPTZ has an intensive blue color and was monitored at 593 nm. Trolox was used as standard antioxidant. Working FRAP reagent was prepared as required by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3 \cdot 6H_2O$ solution. Freshly prepared FRAP reagent was warmed at 37 °C for 30 min and added to the samples every 30 s. Absorbance readings were taken after 30 min every 30 s.

4.2.2. Cell lines and cell culture

Human breast adenocarcinoma cell line MCF7 (ATCC) was grown in phenol red-free RPMI-1640 medium, supplemented with 10% heat inactivated fetal bovine serum, 10,000 U/ml penicillin and 10,000 μg/ml streptomycin, 2 mM L-glutamine, 1.25 mM sodium pyruvate, amino acids and vitamins (Eurobio, France). Human breast epithelial cell line hTERT-HME1 (ATCC) was grown in a mixture of RPMI-1640 (90%) and Mammary Epithelial Growth Medium (10%) (Cambrex, USA). The cell lines were cultivated at 37 °C in a humidified atmosphere containing 5% CO_2. Rat myoblast embryonic cell line H9c2 (ATCC) was cultured in standard DMEM medium containing 3.7 g/L sodium bicarbonate, 4.5 g/L glucose supplemented with 10% fetal bovine serum and 4 mM L-Glutamine at 37 °C in a humidified atmosphere of 10% CO_2.

4.2.3. Cell viability determination (MTT assay)

Cell survival was detected by MTT reduction as described by Mosmann [29]. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and were incubated 24 h. After 48 or 72 h exposure to benzophenones, the medium with tested compounds was removed and fresh media with MTT (final concentration of 0.5 mg/ml) was added. The incubation continued for 3 h at 37 °C. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the spectrophotometric determinations were done at 550 nm. Determination of inhibitory concentrations was performed using generalized linear models as described previously [34].

4.2.4. Determination of intracellular levels of GSH

Intracellular GSH levels were determined by a modified HPLC method based on Lenton et al. [30] using *ortho*-phtalaldehyde. The cells were seeded at a density of 0.75×10^6/Petri dish, incubated 24 h and exposed to the benzophenones for 48 or 72 h. The cells were collected in 10% perchloric acid and ethylenediaminetetraacetic acid (EDTA) on ice and centrifuged at 12,000 × g for 5 min. The supernatant was stored at −80 ° for HPLC analysis and the pellet was used for protein determination. The retention time for GSH was about 3 min.

4.2.5. Flow cytometry analysis

ROS generation was measured as described previously [31], with H_2DCFDA which is trapped within cells and becomes fluorescent when oxidized. Mammary cell lines were plated at density of 0.75×10^6 and cardiomyoblasts at 0.5×10^6 in 100-mm dishes. After 24 h incubation and exposure to different treatments, the cells were incubated with 50 μM H_2DCFDA at 37 °C for 15 min. Both detached and adherent cells were resuspended in PBS and detected in FL1 (green) on a FACScan cytofluorometer (Beckton–Dickinson).

Estimation of cell viability by flow cytometry was assessed by dual color fluorescence analysis, using both H_2DCFDA and PI (propidium iodide) according to Ross [33]. Viable cells are identified with a high green and a low red fluorescence.

4.2.6. Statistics

All experiments were repeated at least three times. Results were presented as mean ± SD and analyzed using the Student's *t*-test to assess the statistical significance. Treatment efficacy was considered as statistically significant for $p < 0.05$.

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