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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Effects of a prenyl-baicalein derivative on ER (+) MCF-7 and ER (−) MDA-MB-231 breast tumor cell lines

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Abstract The effect of a prenyl-baicalein derivative in the DNA synthesis of the estrogen-dependent ER (+) MCF-7 and the estrogen-independent ER (−) MDA-MB-231 cell lines was evaluated using both complete and steroid-free medium. The results revealed a biphasic effect in DNA synthesis of ER (+) MCF-7 cells. Moreover, this prenylflavone seemed to reduce the estrogenic effect of 17β-estradiol and to increase the antiestrogenic effect of 4-hydroxytamoxifen and fluvestrant on the ER (+) MCF-7 cells.

Keywords Antiproliferative · Breast adenocarcinoma · Estrogen receptor · Prenylflavones

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Introduction

Estrogens have shown to exert a variety of beneficial effects in men and women providing protection against osteoporosis, menopausal symptoms, cardiovascular diseases, and potentially in some neurodegenerative pathologies. In spite of all these benefits, estrogens are also one of the most important risk factors for breast cancer (Ackerman and Carr, 2002). For this reason, the search for estrogen-like compounds has been an attractive field for the scientific community because of their possible role as health-promoting and disease-preventing agents (Collins-Burow *et al.*, 2000; Milligan *et al.*, 1999; Oh et al., 2006; Osborne et al. 2000; Vaya and Tamir, 2004). Phytoestrogens are non-steroidal plant-derived compounds that functionally mimic mammalian estrogens and therefore are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms, and osteoporosis (Dixon, 2004; Ososki and Kennelly, 2003). This biologic diversity of phytoestrogens is due in part to their ability to act as estrogen agonists and/or antagonists, like selective estrogen receptor modulators (SERM). As estrogen agonists, phytoestrogens mimic endogenous estrogens causing estrogenic effects and as estrogen antagonists, they may block or alter estrogen receptors (ER) preventing estrogenic activity. Consequently, they cause antiestrogenic effects, being not surprising to consider the potential of phytoestrogens as efficient inhibitors of estrogen-mediated growth of human breast cancer (Brzezinski and Debi, 1999; Ososki and Kennelly, 2003; Wang and Kurzer, 1997; Zhao and Mu, 2011).

Flavonoids are an outstanding family of compounds which have displayed a variety of biological activities (Brahmachari and Gorai, 2006; López-Lázaro *et al.*, 2002; Middleton *et al.*, 2000). They represent one of the largest

groups of phytoestrogens with a chemical structure which resembles 17β-estradiol (**1**, Fig. 1) and also the ability to mimic estrogen activity (Oh *et al.*, 2006; Ososki and Kennelly, 2003; Vaya and Tamir, 2004; Wang and Kurzer, 1997). Baicalein (**2**, Fig. 1), a natural occurring flavone, has been reported to have antiestrogenic activity, by inducing MCF-7 cell death making this compound a potential alternative for ER (+) breast cancer prevention and treatment (Po *et al.*, 2002). Among flavonoids, prenylated derivatives have long been recognized for their myriad biological properties, being the antitumor effect one of the most reported in the literature (Botta *et al.*, 2005; Cidade *et al.*, 2009). In fact, it has been demonstrated that isoprenylation of flavonoids significantly improved their growth inhibitory effect on human tumor cell lines (Botta *et al.*, 2005; Cidade *et al.*, 2009; Daskiewicz *et al*. 2005; Neves *et al.*, 2011). Moreover, prenylflavonoids have also been reported as phytoestrogens, especially 8-prenylnaringenin (**3**, Fig. 1) which was found to be more estrogenic than any other known phytoestrogens (Milligan *et al*. 1999; Schaefer *et al.*, 2003). Artelastin (**4**, Fig. 1), isolated from *Artocarpus elasticus* by our research group (Kijjoa *et al.*, 1996), is another example of a prenylflavonoid resembling the effect of phytoestrogens (Pedro *et al.*, 2005; Pedro *et al.*, 2006). Previously, we have reported that compound **4** exhibited a biphasic effect in the DNA synthesis of ER (+) MCF-7 breast tumor cell line which was stimulatory at low concentrations and inhibitory at high concentrations.

During our research work on the search for potential antitumor compounds, several baicalein (**2**) derivatives were synthesized and evaluated for their inhibitory activity against the in vitro growth of several human tumor cell lines (Neves *et al.*, 2011). In particular, a baicalein (**2**) derivative, 5,6-dihydroxy-7-(3-methylbut-2-enyloxy)-2-phenyl-4*H*-chromen-4-one (**5**), revealed to have a potent growth inhibitory effect and to induce cell cycle arrest in G1 phase (Neves *et al*., 2011). Since the antiproliferative effect of flavonoids has already been correlated to ER-dependent mechanisms (Le Bail *et al.*, 1998; So *et al.*, 1997; Davis *et al.*, 2008), in the present study, we investigated the effect of this flavone (**5**) on the proliferation of two breast tumor cell lines (ER (+) MCF-7 and ER (−) MDA-MB-231) in both complete and steroid-free medium (SFM). We further evaluate the effect of this compound in the estrogenic activity of 17β-estradiol (**1**), and in the antiestrogenic activity of 4-hydroxytamoxifen (**6**) and fulvestrant (**7**) in ER (+) MCF-7 cell line, using SFM.

Materials and methods

Chemistry

^{1}H and ^{13}C NMR spectra were taken in $CDCl_3$ at room temperature, on Bruker Avance 300 instruments (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference; HRMS mass spectra were recorded as ESI (electrospray ionization) mode at C. A. C. T. I.-University of Vigo, Spain. Prenyl bromide and baicalein (**2**) were purchased from Sigma Aldrich. Compound **5** was synthesized from baicalein (**2**) according to the previously described procedure (Neves *et al.*, 2011).

*5,6-Dihydroxy-7-(3-methylbut-2-enyloxy)-2-phenyl-4H-chromen-4-one (**5**)*

Yield: 75%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 12.49 (1H, *s*, 5-O*H*), 7.91–7.88 (2H, *m*, H-2′,6′), 7.55–7.51 (3H, *m*, H-3′,4′,5′), 6.69 (1H, *s*, H-3), 6.62 (1H, *s*, H-8), 5.56 (1H, br*t*, $J = 6.8$, H-2″), 5.44 (1H, *s*, 6-O*H*), 4.70 (2H, *d*, $J = 6.8$, H-1″), 1.83 (3H, *s*, H-4″), 1.79 (3H, *s*, H-5″); ^{13}C NMR ($CDCl_3$, 75 MHz): 182.7 (C-4) 164.0 (C-2), 152.1 (C-7), 150.6 (C-8a), 145.6 (C-5), 139.8 (C-3″), 131.5 (C-1′), 131.2 (C-4′), 129.8 (C-6), 129.1 (C-3′, 5′), 126.2 (C-2′, 6′), 118.3 (C-2″), 106.0 (C-4a), 105.4 (C-3), 91.3 (C-8), 66.2 (C-1″), 25.8 (C-4″), 18.3 (C-5″); ESI-HRMS (+) *m/z*: Anal. calc. for $C_{20}H_{19}O_5$ $(M + H)^+$: 339.1227; found: 339.1235.

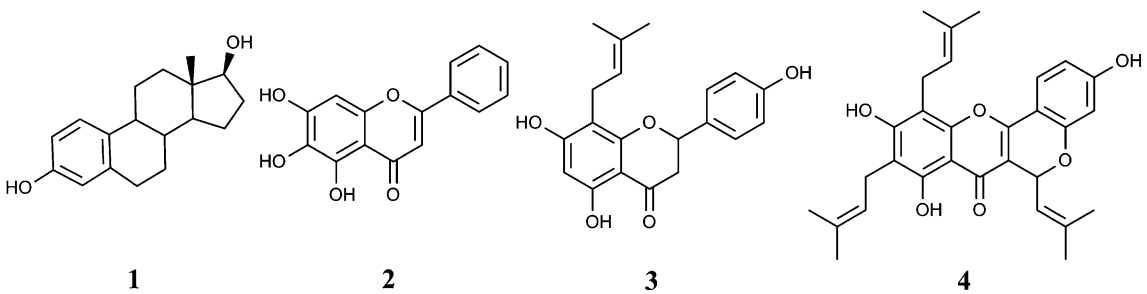


Fig. 1 Structures of 17β-estradiol (**1**) and three natural flavonoids (**2**–**4**) with antiproliferative properties

Biological activity

Reagents and stock solutions of compounds

Fetal bovine serum (FBS), L-glutamine, phosphate buffer saline (PBS), and trypsin were from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 with and without phenol red were from Cambrex (New Jersey, USA). Activated carbon, dextran, dimethylsulfoxide (DMSO), doxorubicin, 17β-estradiol (**1**), ethylenediaminetetracetic acid (EDTA), 4-hydroxytamoxifen (**6**), fulvestrant (**7**), penicillin, streptomycin, sulforhodamine B (SRB), and trypan blue were from SigmaChemical Co. (Saint Louis, MO, USA). Trichloroacetic acid (TCA) and Tris were purchased from Merck (Darmstadt, Germany). [^{3}H] Thymidine was from Amersham (Illinois, USA). The scintillation liquid was from ICN radiochemicals (Irvine, CA, USA). Charcoal-stripped FBS was obtained according to the procedure described elsewhere (Gritzapis *et al.*, 2003). Stock solutions of compounds were prepared in DMSO and stored at −20°C. Appropriate dilutions of the compounds were freshly prepared just prior to the assays.

Cell culture

Two human tumor cell lines were used: MCF-7 (estrogen-dependent breast adenocarcinoma, ECACC, UK) and MDA-MB-231 (estrogen-independent breast adenocarcinoma, ECACC, UK). The human tumor cell lines were grown as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5% (MCF-7) or 10% (MDA-MB-231) heat-inactivated FBS, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For the experiments in SFM, cells were grown as monolayer and routinely maintained in RPMI-1640 medium without phenol red supplemented with 5 or 10% charcoal-stripped fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 (designed thereafter as RPMI-SFM).

Evaluation of the breast tumor cell lines growth inhibition by SRB assay in a complete medium

The effect of compound **5** on the in vitro growth of two human tumor cell lines, ER (+) MCF-7 and ER (−) MDA-MB-231, were evaluated according to the procedure that uses the protein binding dye SRB to assess cell growth (Skehan *et al.*, 1990; Monks *et al.*, 1991). Cells were plated in 96-well plates at appropriate densities to ensure exponential growth throughout the experimental period (1.5×10^5 cells/ml), and then allowed to adhere overnight. Attached cells were exposed for 48 h to five serial dilutions of compound **5** (1.85, 5.56, 16.67, 50.00, and 150.00 μM). Following this incubation period, adherent cells were fixed in situ with TCA, washed and stained with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (Biotek Instruments Inc. PowerWave XS, Winooski, USA). A dose–response curve was obtained for each cell line and the concentration that caused cell growth inhibition of 50% (GI_{50}, corresponding to the concentration of compound that inhibited 50% of the net cell growth), was determined as described elsewhere (Monks *et al.*, 1991). The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in preliminary experiments, by exposing untreated control cells to the maximum concentration of DMSO used in each assay (0.25%). Final concentrations of DMSO did not interfere with the biological activity tested (data not shown). Doxorrubicin was used as positive control and tested in the same manner.

Evaluation of the breast tumor cell lines proliferation in a SFM

The effect of compound **5** on the proliferation of breast ER (+) MCF-7 and ER (−) MDA-MB-231 tumor cells was performed by both SRB and [^{3}H] thymidine incorporation assays. In brief, ER (+) MCF-7 and ER (−) MDA-MB-231 cells were plated in 96-well plates at appropriate densities to ensure exponential growth throughout the experimental period (1.5×10^5 cells/ml), and then allowed to adhere overnight. Attached cells were treated with a range of concentrations of compound **5** (0.10, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, and 100.00 μM) prepared by serial dilution using RPMI-SFM for 72 h. In the SRB assay, adherent cells were fixed in situ with TCA, washed and stained with SRB (Monks *et al.*, 1991). The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (Biotek Instruments Inc. PowerWave XS, Winooski, USA). The percentage of cellular protein content was calculated comparing the absorbance of treated cells with that of untreated control cells. In the [^{3}H] thymidine incorporation assay, 5 μCi/ml of [^{3}H] thymidine was added and cells were incubated for further 4 h at 37°C. Pulsed cells were then harvested on a glass filter 102 × 256 mm (Skatron, Norway) using a semiautomatic cell harvester (Skatron Instruments, Norway) and allowed to dry. Incorporation of radioactive thymidine was determined by liquid scintillation counter LS 6500 (Beckman Instruments, CA, USA) and defined by comparing the arithmetic mean of counts per minute (c.p.m.) of treated cells with that of the untreated control cells. In both methods, 4-hydroxytamoxifen (**6**, 10^{-1} μM in RPMI-SFM) and fulvestrant (**7**, 10^{-1} μM in RPMI-SFM) were used as

positive control for antiestrogenic activity and 17β-estradiol (**1**, 10^{-1} μM in RPMI-SFM) was used as positive control for estrogenic activity. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in preliminary experiments (blank), by exposing untreated control cells to the maximum concentration of DMSO used in each assay (0.25%).

Statistical analysis

All experimental data are presented as means ± SEM from at least three independent experiments (most of them performed in duplicate). Statistical analysis was carried out using a paired Student's *t* test.

Results and discussion

Chemistry

The synthetic approach used to synthesize the prenylated baicalein derivative **5** was by microwave-assisted organic synthesis (MAOS). Therefore, the starting material **2** was submitted to microwave irradiation (200 W, 60°C) for 2 h in presence of prenyl bromide and anhydrous potassium carbonate (Fig. 2) (Neves *et al.*, 2011).

Effect of compound **5** on ER (+) MCF-7 and ER (−) MDA-MB-231 human breast tumor cell lines

The effect of the synthesized prenylated flavone **5** on the in vitro growth of ER (−) MDA-MB-231 (estrogen-independent breast adenocarcinoma cell line) and ER (+) MCF-7 (estrogen-dependent breast adenocarcinoma) was determined according to the procedure that uses the protein binding dye SRB to assess cell growth (Monks *et al.*, 1991; Skehan *et al.*, 1990). Results showed that compound **5** caused a dose-dependent inhibitory effect in both human breast tumor cells (Fig. 3). Noteworthy, compound **5** was significantly ($P < 0.01$) more active in the ER (+) MCF-7 than in ER (−) MDA-MB-231 cells, showing GI_{50} values of 11.2 ± 2.0 μM for ER (+) MCF-7 and of 29.7 ± 2.1 μM for ER (−) MDA-MB-231 cells.

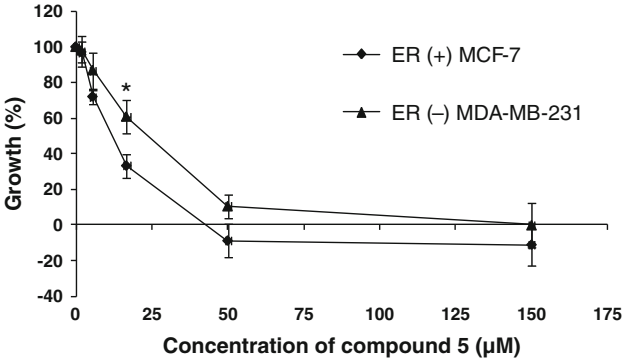
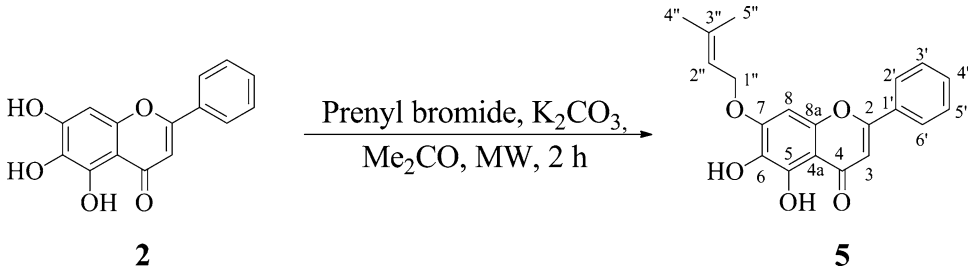


Fig. 3 Effect of compound **5** on the growth of ER (+) MCF-7 and ER (−) MDA-MB-231 cell lines. Cells were treated with several concentrations of compound **5**, with complete medium (blank) or with DMSO control for 48 h. Results represent means ± SEM from at least three independent experiments performed in duplicate. Doxorrubicin was used as positive control (GI_{50} (MCF-7) = 42.8 ± 8.2 nM; GI_{50} (MDA-MB-231) = 10.9 ± 1.3 nM). *$P < 0.01$ (which means that growth inhibitory activity in ER (+) MCF-7 was significantly higher than in ER (−) MCF-7)

Considering that this kind of behavior was already observed for many phytoestrogens (Davis *et al.*, 2008; Le Bail *et al.*, 1998; Pedro *et al.*, 2006), we decided to investigate the possible involvement of the estrogenic receptor (ER) in the observed growth inhibitory effect. First, we have studied the influence of the composition of the culture medium on the growth inhibitory effect of ER (+) MCF-7 and ER (−) MDA-MB-231 cells, since the presence of estrogen-like compounds in RPMI medium has already been described to disclose estrogenic effects of several compounds on breast tumor cell line (Jensen *et al.*, 2003; Journe *et al.*, 2004; Paiva *et al.*, 2011). Therefore, ER (+) MCF-7 and ER (−) MDA-MB-231 cells were cultured in complete medium (RPMI) and also in steroid-free medium (RPMI-SFM), and then treated with a range of the highest concentrations tested (3.13–100.00 μM) of compound **5**. After treatment, cellular growth was assessed using two different methodologies: SRB and [^{3}H] thymidine incorporation assays (data not shown). In the ER (+) MCF-7 cell line, it is possible to observe that compound **5** exhibited a significant more potent effect ($P < 0.05$) in steroid-free conditions (Fig. 4b) than in complete medium (Fig. 4a), suggesting that the growth inhibitory effect of

Fig. 2 Synthesis of compound **5**. *MW* Microwaves



2 **5**

compound **5** could be influenced by the presence of estrogen-like compounds. By contrast, in the ER (−) MDA-MB-231 cells, no significant differences were observed on the cell growth, independent of the medium used (RPMI or RPMI-SFM) (Fig. 4c, d).

In order to investigate if the effects on ER (+) MCF-7 and ER (−) MDA-MB-231 cell lines could be influenced by the concentration of compound **5**, exponentially growing cells were treated with a broad concentration range (0.1–100 μM) of compound **5** for 72 h and DNA synthesis was quantified by thymidine incorporation. To ensure that the observed effects were only due to the tested compound, this assay was carried out using SFM (Fig. 5).

Results presented in Fig. 5 reveal that in the ER (+) MCF-7 cells (Fig. 5a), the prenylated derivative **5** presented a biphasic effect, that is, a growth stimulatory effect at low concentrations (0.10–0.20 μM) and a growth inhibitory effect at high concentrations (3.13–100 μM). In accordance with the results presented with the SRB assay (Fig. 4b), at high concentrations (3.13–100 μM), the tested compound (**5**) exhibited a more potent antiproliferative effect on the ER (+) than on ER (−) cells, as measured by the [^{3}H] thymidine incorporation assay (Fig. 5a, b). These results are in agreement with those reported for phytoestrogens (Pedro *et al.*, 2006), suggesting that the stimulatory/inhibitory effect of compound **5** could be mediated via ER. Compound **5** was further evaluated in the presence of 17β-estradiol (**1**) to determine if flavonoid **5** could inhibit estradiol-induced cell proliferation on MCF-7 cells. ER (+) MCF-7 cells were concomitantly treated with both compounds **1** and **5**, and subsequently evaluated using the [^{3}H] thymidine incorporation assay (Fig. 6). These in vitro experiments were also conducted in SFM to allow for the assessment of estrogenic responses. The results presented in Fig. 6 showed that when ER (+) MCF-7 cells were treated with both 10^{-1} μM of 17β-estradiol (**1**) and compound **5** (0.1–0.78 μM), a significant reduction ($P < 0.05$) of the proliferation effect of 17β-estradiol (**1**) alone or compound **5** alone was observed, suggesting a competition between both compounds for the ER (Fig. 6). This behavior was already described for some flavonoids (Le Bail *et al.*, 1998; So *et al.*, 1997).

In addition, the effect of baicalein derivative **5** in the antiestrogenic activity of the partial antagonist 4-hydroxytamoxifen (**6**) and of the pure antiestrogen fluvestrant (**7**) was investigated using the [^{3}H] thymidine incorporation assay to assess DNA synthesis (Fig. 7). The results showed that when ER (+) MCF-7 cells were treated concomitantly with 4-hydroxytamoxifen (**6**) and compound **5** (3.13 and 6.25 μM), a significant increase ($P < 0.05$) of the

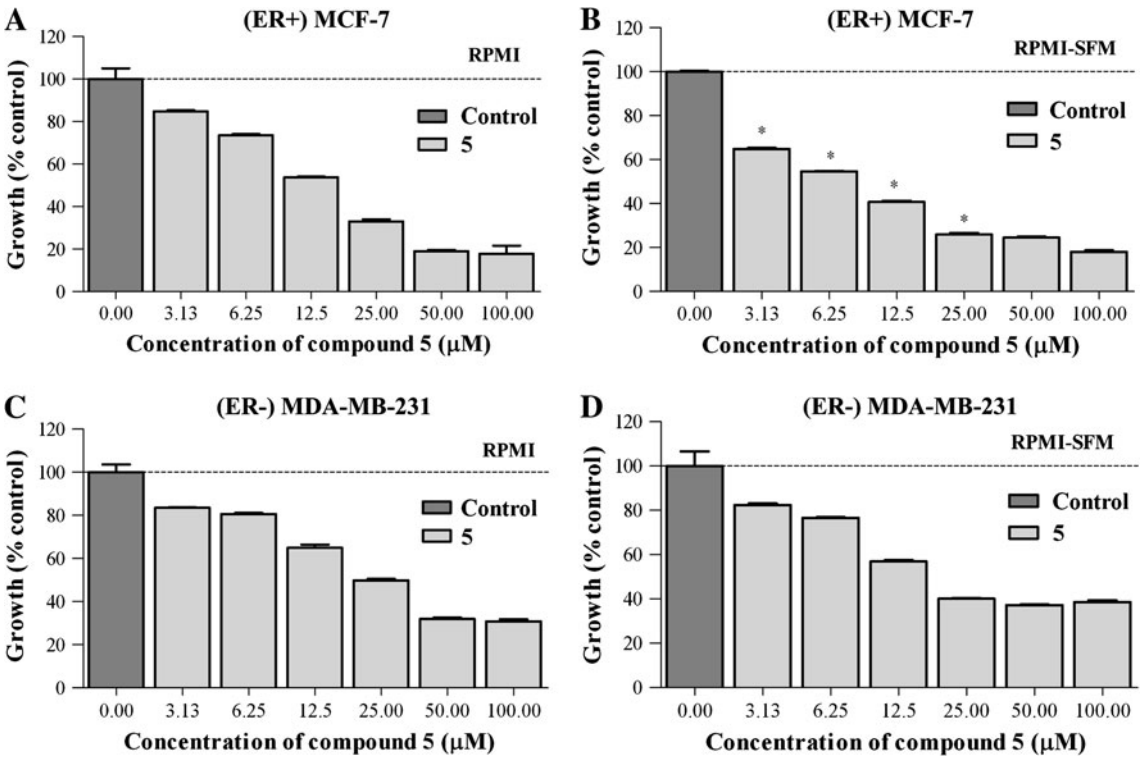


Fig. 4 Effect of compound **5** on the growth of ER (+) MCF-7 (**a**, **b**) and ER (−) MDA-MB-231 (**c**, **d**) cell lines determined by the SRB assay using complete medium (RPMI, **a**, **c**) or steroid-free medium (RPMI-SFM, **b**, **d**). Results are presented as the % relative to untreated control cells and represent means ± SEM from at least three independent experiments performed in duplicate. *$P < 0.05$ (which means that growth inhibitory activity in RPMI-SFM was significantly higher than in RPMI)

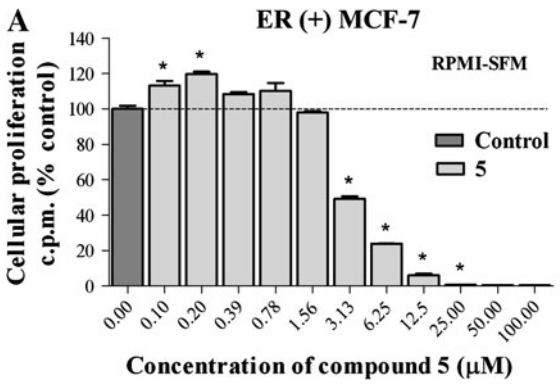


Fig. 5 Effect of compound **5** in the synthesis of DNA of two breast tumor cell lines: ER (+) MCF-7 (**a**) and ER (−) MDA-MB-231 (**b**), determined by the [^{3}H] thymidine incorporation assay using RPMI-SFM. Results are presented as the % relative to untreated control cells and represent means ± SEM from at least three independent experiments performed in duplicate. *$P < 0.05$ (which means that growth stimulatory/inhibitory effects were significantly different in ER (+) MCF-7 and MDA-MD-231 cell lines)

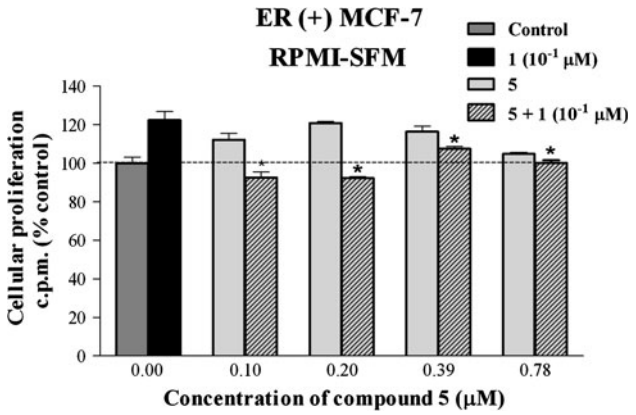


Fig. 6 Effect of compound **5** in the synthesis of DNA of ER (+) MCF-7 cell line in the presence of 17β-estradiol (**1**, 10^{-1} μM) determined by the [^{3}H] thymidine incorporation assay using RPMI-SFM. Results are presented as the % relative to untreated control cells and represent means ± SEM from at least three independent experiments performed in duplicate. *$P < 0.05$ (which means that growth stimulatory effect was significantly lower than with 17β-estradiol (**1**) or compound **5** alone)

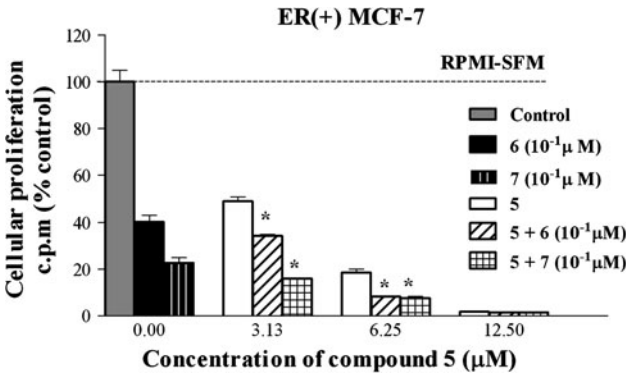


Fig. 7 Effect of compound **5** in the synthesis of DNA of ER (+) MCF-7 cell line in the presence of 4-hydroxytamoxifen (**6**) or fluvestrant (**7**), determined by the [^{3}H] thymidine incorporation assay using RPMI-SFM. Results are presented as the % relative to untreated control cells and represent means ± SEM from at least three independent experiments performed in duplicate. *$P < 0.05$ (which means that the antiproliferative effect was significantly lower than with **6** or **7** alone)

antiestrogenic effect of 4-hydroxytamoxifen (**6**), which was dependent on the concentration of flavone **5**, was observed (Fig. 7). Likewise, a significant increase ($P < 0.05$) of the antiestrogenic effect of fluvestrant (**7**) was observed (Fig. 7). Since the antiestrogenic activity of both ER antagonists (**6** and **7**) on the ER (+) MCF-7 cells was increased in the presence of compound **5**, it is possible to infer that this prenylflavone may act by an additive/synergistic mechanism when in combination with antiestrogen-like compounds.

Conclusions

In conclusion, for the prenylated flavone **5**, the growth inhibitory effect against ER (+) and ER (−) breast tumor cell lines was significantly different, suggesting a possible involvement of the estrogen receptor. This prenylated baicalein derivative **5** revealed to act as an estrogen agonist at low concentrations, whereas it produces estrogen antagonist effects at higher concentrations, being these data reported here for the first time. In addition, compound **5** showed an antiproliferative effect in the presence of both estradiol and estrogen antagonists, 4-hydroxytamoxifen (**6**), and fulvestrant (**7**). Hence, the prenylflavone **5** deserved to be further explored as an antiestrogenic agent given the importance of this effect on the combinatory therapy for ER (+) dependent breast tumors.

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