Antiproliferative activity and p53 upregulation effects of chalcones on human breast cancer cells

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ABSTRACT
Chalcones are valuable structures for drug discovery due to their broad bioactivity spectrum. In this study, we evaluated 20 synthetic chalcones against estrogen-receptor-positive breast cancer cells (MCF-7 line) and triple-negative breast cancer (TNBC) cells (MDA-MB-231 line). Antiproliferative screening by MTT assay resulted in two most active compounds: 2-fluoro-4'-aminochalcone (11) and 3-pyridyl-4'-aminochalcone (17). Their IC50 values ranged from 13.2 to 34.7 μM against both cell lines. Selected chalcones are weak basic compounds and maintained their antiproliferative activity under acidosis conditions (pH 6.7), indicating their resistance to ion-trapping effect. The mode of breast cancer cells death was investigated and chalcones 11 and 17 were able to induce apoptosis rather than necrosis in both lines. Antiproliferative target investigations with MCF-7 cells suggested 11 and 17 upregulated p53 protein expression and did not affect Sp1 protein expression. Future studies on chalcones 11 and 17 can define their in vivo therapeutic potential.

Introduction
Breast cancer is the most common type of cancer that affects women around the world, corresponding to 25% of cases. It is also the main cause of cancer death among women1–3. Its classification is based on the presence of cellular receptors: (i) Hormone-Receptor (HR), with Estrogen (ER) and/or Progesterone-Receptors (PR); (ii) Human Epidermal Growth Factor-2 receptor (HER2); and (iii) Triple-Negative Breast Cancer (TNBC), which does not express ER, PR, or HER2 receptors4–6. Chemotherapy choices for breast cancer are made according to its classification aiming to reach specific targets. ER-positive cancer treatments include ER modulators, such as tamoxifen or aromatase inhibitors. HER2-positive cancer is treated with monoclonal antibodies, such as trastuzumab, which is administered alongside tyrosine-kinase inhibitors7–9. TNBC cancer treatment is focused on cytotoxic agents, such as taxanes or doxorubicin. This cancer has poor response to chemotherapy, particularly at metastatic sites, with survival rates below 2 years. Altogether, TNBC cancer has been considered the most severe and difficult to treat due to a lack of targeted therapy10–12.

Chalcones are valuable structures for drug discovery due to their broad bioactivity spectrum, as well as their versatile and simple synthesis. Their structures, bearing two benzene rings (A and B), are linked by an enone bridge. They have demonstrated antiproliferative activity against cancer cells, including breast cancer13–15. Mai et al. described chalcone was effective against ER-positive breast cancer cells (MCF-7 line) targeting 20 apoptotic markers16. Iftikhar et al. reported that chalcone bearing chlorine at position 2 was effective against breast cancer cells (CAL-51 line) and induced accumulation of p53 protein17. Silva and coauthors described unsubstituted chalcone increased p53 protein activity in osteosarcoma cells (U2OS line) through the induction of heat shock protein DNAJB118.

In our ongoing search for anticancer compounds with structures based on chalcone framework, we evaluated the antiproliferative activity of 20 chalcones against ER-positive cells (MCF-7 line) and TNBC (MDA-MB-231 line). The two most active chalcones (11 and 17) were selected to antiproliferative evaluation under acidosis conditions (pH 6.7) and pro-apoptotic activity in MCF-7 and MDA-MB-231 lines. In addition, we investigated tumour molecular targets of 11 and 17 in MCF-7 line, which were able to upregulate p53 protein expression.

Materials and methods
Chemical procedure for synthesis of chalcones 1–20
Reagents and solvents were purchased from Merck® (Kenilworth, NJ). Series of 20 chalcones was synthesised by Claisen–Schmidt aldol condensation reaction, according to protocol described by...
Santos and coauthors, with minor modifications\textsuperscript{19,20}. Reactions were carried out at room temperature using 3.0 mmol of 4\textsuperscript{-}aminoaceto-phenone and 3.0 mmol of aldehydes, which were dissolved in ethanol (30 mL). Sodium hydroxide in ethanol (1.0 mol/L) was added as catalyst solution. Reagents conversion was monitored using thin layer chromatography. Crude product was poured onto ice (from distilled and deionised water) and filtered. All compounds were purified over silica gel chromatography column eluted with mixture of hexane and ethyl acetate (3:2). Melting points were determined in TecнопонПФМ-II\textsuperscript{TM} apparatus (М5 Технопон Инструментация, Piracica, Brazil) and were uncorrected. Structure of compounds was confirmed by \textsuperscript{1}H and \textsuperscript{13}C nuclear magnetic resonance (NMR) spectra analyses. Spectral data were obtained in Bruker Avance III\textsuperscript{TM} (14 Tesla, 600 MHz) equipment (Bruker Corporation, Billerica, MA) using deuterated dimethyl sulfoxide (DMSO-\textit{d}_6) as solvent. Chalcones had their UV–vis spectra and chromatograms obtained in High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) Agilent Technologies\textsuperscript{TM} 1220 Infinity equipment (Agilent Technologies, Palo Alto, CA) coupled with a photo- diode array system (1260-Infinity\textsuperscript{TM}) and Agilent Zorbax Eclipse Plus C\textsubscript{18}\textsuperscript{TM} column (250 mm × 4.6 mm, 5 \textmu m), using methanol:water (3:1) as mobile phase (1.0 mL/min).

**Antiproliferative activity of chalcones 1–20**

Human breast cancer cell lines MCF-7 (HTB-22) and MDA-MB-231 (HTB-26) were purchased from American Type Culture Collection (ATCC). Both cell lines were cultured in DMEM (Gibco\textsuperscript{TM}, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS-LGC\textsuperscript{TM}), peni- cillin-streptomycin (100 \textmu g/mL, Merck\textsuperscript{TM}, Kenilworth, NJ). Both cell lines were incubated at 37°C under humidified atmosphere with 5% CO\textsubscript{2} (Thermo Fischer Scientific\textsuperscript{TM} Incubator, Waltham, MA).

Antiproliferative activity of chalcones 1–20 was evaluated by MTT assay\textsuperscript{21–23}. Cells were seeded in 96-well plate, with an initial cell density of 1 × 10\textsuperscript{4} and 2.5 × 10\textsuperscript{4} cells/well of MCF-7 and MDA-MB-23, respectively. Cells were cultured with DMEM supplemented with 10% FBS and compounds at 20 \textmu M for 48 h at 37°C under 5% CO\textsubscript{2} humidified atmosphere. Cells were incubated with MTT (Sigma Aldrich\textsuperscript{TM}, St. Louis, MO) solution (1 mg/mL) for 40 min. Formazan crystals were solubilised in dimethylsulfoxide (DMSO, Sigma Aldrich\textsuperscript{TM}, St. Louis, MO), and absorbance rates were measured at 562 nm in ThermoPlate\textsuperscript{TM} TP Reader. Chalcones 1, 6, 8, 9, 11, 17, 19, and 20 had the highest antiproliferative activity and were assayed against both cell lines at seven concentrations, ranging from 1.25 to 80 \textmu M for IC\textsubscript{50} values determination.

**Antiproliferative activity of selected chalcones 11 and 17 under acidosis**

Cell viability was evaluated under acidosis conditions by MTT assay, using 4-morpholine-ethanesulfonic acid to produce a pH value of 6.7\textsuperscript{24,25}. Cells were treated with selected chalcones 11 and 17 at their respective IC\textsubscript{50} values. All procedures were performed in triplicate and three independent experiments.

**Pro-apoptotic activity of selected chalcones 11 and 17**

Pro-apoptotic activity of selected chalcones 11 and 17 was evaluated using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen\textsuperscript{TM}, BD Biosciences, Franklin Lakes, NJ) by flow cytometry\textsuperscript{26}. MCF-7 and MDA-MB-231 cells were seeded and cultivated in 6-well plates (6.5 × 10\textsuperscript{5} cells/well), for 24 h, and treated with 40 \textmu M selected chalcones in DMEM containing 10% FBS for 48 h. As negative control 0.1% DMSO was used. Adherent and floating cells were collected, submitted to centrifugation, and washed with PBS (Sigma Aldrich\textsuperscript{TM}, St. Louis, MO). Apoptotic cells were determined by flow cytometry in BD FACScalibur\textsuperscript{TM} Flow Cytometer (BD Biosciences\textsuperscript{TM} Franklin Lakes, NJ), after double staining (Annexin V/propidium iodide) using FITC Annexin V Apoptosis Detection kit I (BD Pharmingen\textsuperscript{TM}, BD Biosciences, Franklin Lakes, NJ) according to instructions of the manufacturer. All procedures were performed in triplicate and in three independent experiments.

**Effect of selected chalcones 11 and 17 on Sp1 and p53 proteins expressions**

Protein expression was assessed using western blot assay. Breast cancer cells (MCF-7) were seeded in 6-well plates at 7.5 × 10\textsuperscript{5} cells/well, treated with selected chalcones 11 and 17 at 10 and 20 \textmu M or with negative control (0.1% DMSO) for 24 h\textsuperscript{26}. Cells were suspended in PBS solution, collected in Radioimmunoprecipitation Assay buffer – (RIPA buffer at 4°C), supplemented with proteinase inhibitors, sonicated for 20 min, and washed with PBS, and were centrifuged (14,000 rpm, 4°C, 20 min, Eppendorf\textsuperscript{TM}, Hamburg, Germany). Protein total concentration was obtained using Pierce\textsuperscript{TM} BCA Protein Assay reagent (Thermo Scientific\textsuperscript{TM}, Waltham, MA). Total proteins (30 \mu g) in sodium dodecyl sulphate (SDS) buffer were warmed at 95°C, cooled at 4°C and submitted to sodium dodecyl sulphate–polyacrylamide gel electrophoresis SDS-PAGE (12%, 100 V, 360 mA) for 90 min at room temperature, and were transferred to nitrocellulose membrane (PALL Corporation\textsuperscript{TM}, Port Washington, NY). Membranes were blocked using TBST buffer (25 mM Tris, 3 mM KCl, 0.14 M NaCl, 0.05% Tween 20) containing 5% nonfat milk at room temperature for 1 h, followed by overnight incubation at 4°C, with primary antibodies to Sp1 (MilliPore\textsuperscript{TM}, Burlington, MA), p53, and β-actin (Santa Cruz Biotechnology\textsuperscript{TM}, Dallas, TX). Subsequently, membranes were washed with TBST (3×) and incubated with peroxidase-conjugated secondary antibodies diluted in TBST buffer 5% nonfat milk (1:5000) at room temperature and washed several times with TBST buffer. Proteins were detected through chemiluminescence using Enhanced Chemiluminesence (ECL\textsuperscript{TM}) western blotting detection reagent (Amersham Biosciences\textsuperscript{TM}, Little Chalfont, UK) in LAS 500 luminescence analyser (GE Healthcare\textsuperscript{TM}, Chicago, IL)\textsuperscript{28}. Bands intensity in image was quantified using ImageJ\textsuperscript{TM} software (NIH Image J system, Bethesda, MD).

**Statistical analysis**

Statistical analyses were carried out using one-way ANOVA multiple comparison tests followed by Tukey’s HSD test\textsuperscript{19,27}. Statistical significance difference was considered if \textit{p} < 0.05.

**Results and discussion**

**Chemistry**

Chalcones 1–20 were synthesised by Claisen–Schmidt reaction with yields of 30–93% after chromatography purification. Series of compounds has chalcones with amino at position 4’ (ring A) and benzene ring B substituted by electron-withdrawing (EWG) and electron-donating groups (EDG). Also, aryl analogues with ben- zene ring B replaced for furan, thiophene, pyridine, and naphthyl rings were synthesised (Scheme 1).
All NMR parameters, including hydrogen and carbon chemical shifts (δH and δC in ppm), integrations, multiplicities, and coupling constants (J in Hz), corresponded to proposed structures of chalcones 1–20. Two main signals in 1H NMR spectra were diagnosed: (i) a pair of doublets with J ranging from 15 to 16 Hz (7.43–8.10 ppm), attributed to methyne hydrogens of trans carbon–carbon double bonds and (ii) broad singlets between 6.18 and 6.27 ppm related to geminal hydrogens of amino group. In 13C NMR spectra, signals in 185.9–186.1 ppm were related to a pivotal chromophore of chalcone framework (conjugation between ring B and enone bridge). HPLC-DAD analyses of peak areas integrations indicated chalcones purity of 94.0–99.9%. All chromatograms and spectra are presented in supplemental data (Figures S1–S62).

Antiproliferative activity of chalcones 1–20

Preliminary antiproliferative activity of chalcones 1–20 was evaluated by MTT assay for 48 h against MCF-7 (ER) and MDA-MB-231 (TNBC) lines. Percentages of metabolically viable cells (%MVC) are summarised in Table 1. Series of compounds demonstrated active (e.g. 20) and inactive (e.g. 3) chalcones, suggesting molecular variations were relevant for antiproliferative screening against both cell lines. Experiments at 20 μM allowed us to derive preliminary structural features related to antiproliferative activity.

![Scheme 1. Claisen-Schmidt reaction for synthesis of chalcones 1–20.](image)

### Table 1. Percentage of metabolically viable cells (%MVC) treated with chalcones 1–20 (at 20 μM) and IC50 values (in μM) of selected chalcones.

| Cpd. | Ar             | %MVC MCF-7 (ER) | IC50 MCF-7 (ER) | %MVC MDA-MB-231 (TNBC) | IC50 MDA-MB-231 (TNBC) |
|------|----------------|-----------------|-----------------|------------------------|------------------------|
| 1    | Phenyl         | 97.3 ± 6.0      | >100            | 82.6 ± 4.0             | 60.3 ± 2.9            |
| 2    | 4-Nitrophenyl  | 97.3 ± 4.9      | nd              | 104.1 ± 3.8            | nd                     |
| 3    | 4-Trifluoromethylphenyl | 98.3 ± 6.6 | nd              | 100.7 ± 6.7            | nd                     |
| 4    | 4-Cyanophenyl  | 98.2 ± 2.1      | nd              | 99.7 ± 6.3             | nd                     |
| 5    | 4-Fluorophenyl | 93.3 ± 5.7      | nd              | 92.8 ± 4.6             | nd                     |
| 6    | 4-Chlorophenyl | 74.6 ± 5.2      | >100            | 81.4 ± 7.3             | 69.0 ± 8.5            |
| 7    | 4-Bromophenyl  | 70.1 ± 1.5      | nd              | 97.1 ± 5.5             | nd                     |
| 8    | 3-Fluorophenyl | 72.8 ± 5.1      | >100            | 73.3 ± 5.4             | 74.1 ± 1.2            |
| 9    | 3-Chlorophenyl | 52.5 ± 6.7      | 34.2 ± 6.4      | 97.7 ± 4.8             | >100                   |
| 10   | 3-Bromophenyl  | 68.3 ± 4.8      | nd              | 100.1 ± 4.4            | nd                     |
| 11   | 2-Fluorophenyl | 50.3 ± 7.8      | 13.2 ± 3.5      | 84.6 ± 3.2             | 34.7 ± 5.2            |
| 12   | 2-Chlorophenyl | 69.0 ± 6.4      | nd              | 88.7 ± 3.6             | nd                     |
| 13   | 4-Methylphenyl | 88.7 ± 4.2      | nd              | 100.2 ± 5.3            | nd                     |
| 14   | 4-Methoxyphenyl| 87.9 ± 7.5      | nd              | 95.9 ± 6.4             | nd                     |
| 15   | 2-Furyl        | 104.5 ± 3.6     | nd              | 100.1 ± 7.1            | nd                     |
| 16   | 2-Thiophenyl   | 99.4 ± 5.7      | nd              | 95.6 ± 5.3             | nd                     |
| 17   | 3-Pyridyl      | 51.8 ± 2.5      | 15.7 ± 5.9      | 75.7 ± 6.3             | 33.9 ± 7.1            |
| 18   | 4-Pyridyl      | 61.3 ± 3.4      | nd              | 108.7 ± 2.4            | nd                     |
| 19   | 1-Naphthyl     | 53.4 ± 4.3      | 143.7 ± 2.9     | 88.9 ± 1.7             | >100                   |
| 20   | 1,4-Biphenyl   | 44.2 ± 3.3      | 22.7 ± 6.0      | 95.9 ± 11.2            | >100                   |

**Dox**

- nd: not determined

**Notes:**
- Different letters indicate different values with statistical significance p < 0.05 in Tukey’s multiple comparisons test; dox: doxorubicin (reference antineoplastic drug).
- ±: standard deviation.
- Ar: aromatic ring.
53.4 ± 4.3 and 44.2 ± 3.3, respectively. Despite effect against both lines, MCF-7 line (ER) was more sensitive to chalcones than MDA-MB-231 line (TNBC) (Table 1).

The five most antiproliferative compounds against MCF-7 were 9, 11, 17, 19, and 20 (%MVC values lower than 60). Against MDA-MB-231, the five most potent ones were 1, 6, 8, 11, and 17 (%MVC values lower than 85). These seven compounds were selected to determine IC50 values for both cell lines (Table 1). Chalcones 1, 6, and 8 were inactive against MCF-7 (IC50 > 100 μM) and active against MDA-MB-231, with IC50 values of 60.3, 69.0, and 74.1 μM, respectively. Chalcones 19 and 20 were active against MCF-7 with IC50 values of 14.3 and 22.7 μM, respectively, and inactive against MDA-MB-231 (IC50 > 100 μM). Chalcones 11 and 17 were active against both cell lines, displaying IC50 values of 13.2–34.2 μM and were selected for additional bioassays.

**Antiproliferative activity of selected chalcones 11 and 17 under acidosis**

In normal cells, intracellular and extracellular pH values are 7.2 and 8.4, respectively. On the other hand, in breast tumour cells, intracellular and extracellular pH values are 7.1–7.4 and 6.5–7.1, respectively. This extracellular acidosis has been related to Warburg effect, in which tumour cells are in intense anaerobic metabolism, producing and exporting acid compounds through transporters. The biological central interest in acidosis is due to metabolism, producing and exporting acid compounds through transporters. The biological central interest in acidosis is due to metabolically active cancer cells. The Cationic form exhibits reduced passive permeation through membrane phospholipids when compared to neutral form. Doxorubicin is antineoplastic and weak basic drug and has demonstrated low efficacy in acidosis microenvironment due to ion-trapping effect. This phenomenon is caused by protonation of basic functionalities, converting neutral into cationic compounds.

Chalcones are known to induce apoptosis in several cancer cells, being able to up regulate more than 15 pro-apoptotic markers expression, such as Bad, Bax, Bid, Bim, CD40, Fas, IGFBP-5, IGFBP-6, p21, and sTNF-R116. Hsu and Bortolotto and their respective collaborators have demonstrated pro-apoptotic activity of unsubstituted chalcone against MCF-7 breast cancer cells38,39. These authors have described intrinsic apoptotic pathway induced by unsubstituted chalcone, with inhibition of Bcl-2 and induction of ataxia telangiectasia mutated (ATM) mammals.

**Effect of selected chalcones 11 and 17 on Sp1 and p53 proteins expression**

MCF-7 and MDA-MB-231 lines have demonstrated wild and mutant p53 protein, respectively40. Thus, we selected MCF-7 to conduct molecular target experiments. Western blot assay was performed to evaluate effect of chalcones 11 and 17 on p53 and Sp1 proteins expression in MCF-7 line for 24 h at 10 and 20 μM (Figure 3).

Sp1 protein is transcription factor involved in cell proliferation and differentiation. In breast cancers, it acts on invasion and metastasis processes41,42, and has been classified as marker for...
poor prognosis. Chalcones 11 and 17 were not able to modulate Sp1 protein expression, at either concentration, presenting similar effect to DMSO 0.1% (negative control).

p53 protein is tumour suppressor and its mutated status has been related to several types of cancers. Expression of p53 in breast cancer cells varies according to its classification. ER-positive and ER-negative cancer types have wild-type p53 and mutated protein forms, respectively. Activation and stabilisation of wild-type p53 induce cell-cycle arrest and cell death through apoptosis, reducing cancer progression. This cell pathway has been recognised as attractive target to simple and low-weight-molecular compounds with promising antineoplastic potential. Chalcones 11 and 17 induced 5-fold upregulation of p53 expression in MCF-7 cells (Figure 3), indicating these compounds are able to activate and stabilise p53 protein expression. This result is the first evaluation of low-molecular-weight compounds against breast cancer cells. In this context, methoxychalcones and naphthylchalcones have been described as agents of p53 activation and stabilisation in prostate cancer and osteosarcoma cell lines, respectively.

Conclusions
In summary, we reported activity of series of 20 chalcones against two types of breast cancer cells, ER-positive (MCF-7 line) and TNBC (MDA-MB-231 line). Preliminary investigations suggested halogens on ring B and additional benzene rings play central role in antiproliferative activity. Basic chalcones 11 and 17 were antiproliferative agents under acidosis (at pH 6.7), displaying resistance to iontrapping effect. These compounds induced apoptosis rather than necrosis in both cells, upregulating p53 expression in ER-positive cells (MCF-7 line).

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Disclosure statement

No potential conflict of interest was reported by authors.

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