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Original article

Cytotoxicity of *trans*-chalcone and licochalcone A against breast cancer cells is due to apoptosis induction and cell cycle arrest

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ABSTRACT

Chalcones are precursors of flavonoids that exhibit structural heterogeneity and potential antitumor activity. The objective of this study was to characterize the cytotoxicity of *trans*-chalcone and licochalcone A (LicoA[1]) against a breast cancer cell line (MCF-7) and normal murine fibroblasts (3T3). Also the mechanisms of the anti-cancer activity of these two compounds were studied. The alkaline comet assay revealed dose-dependent genotoxicity, which was more responsive against the tumor cell line, compared to the 3T3 mouse fibroblast cell line. Flow cytometry showed that the two chalcones caused the cell cycle arrest in the G1 phase and induced apoptosis in MCF-7 cells. Using PCR Array, we found that *trans*-chalcone and LicoA trigger apoptosis mediated by the intrinsic pathway as demonstrated by the inhibition of Bcl-2 and induction of Bax. In western blot assay, the two chalcones reduced the expression of cell death-related proteins such as Bcl-2 and cyclin D1 and promoted the cleavage of PARP. However, only *trans*-chalcone induced the expression of the CIDEA gene and protein in these two experiments. Furthermore, transient transfections of MCF-7 using a construction of a promoter-luciferase vector showed that *trans*-chalcone induced the expression of the CIDEA promoter activity in 24 and 48 h. In conclusion, the results showed that *trans*-chalcone promoted high induction of the CIDEA promoter gene and protein, which is related to DNA fragmentation during apoptosis.

1. Introduction

Despite advances in cancer research, breast cancer is a leading cause of death among women. Surgery, radiotherapy and chemotherapy administered separately or in combination are the most common treatments for breast cancer, but are not always effective and may cause undesired side effects [1]. Doxorubicin is a chemotherapeutic agent commonly used for the treatment of breast cancer, but has limitations because of its cardiotoxicity [2] and selection of resistant tumors. Resistance to cytotoxic drugs can be caused by the reduced expression of tumor suppressor genes and by the overexpression of proto-oncogenes [3,4]. The resistance in tumor cells may prevent apoptosis by altering the modulation of essential genes involved in programmed cell death pathways, such as those of the Bcl-2 family [5]. In this regard, there is a continuous search for alternatives of cytotoxic compounds that exhibit a better therapeutic response than the classical chemotherapeutic agents.

Some examples of alternative molecules under research are the chalcones, which are open-chain polyphenols that consist of two aromatic rings joined by one α, β-unsaturated propenone. These compounds occur naturally as precursors of flavonoids and possess anti-microbial, anti-oxidant and anti-inflammatory properties, among others [6]. There are numerous reports of natural and synthetic chalcones with potent anti-tumor activity against different cell lines [7], resulting from the activation of apoptotic pathways and anti-proliferative action [6–8,12,14–16].

In the present study, *trans*-chalcone and licochalcone A (LicoA) were found to be cytotoxic and considerably genotoxic, caused cell cycle arrest, induced apoptosis, and modulated apoptotic genes through the inhibition of Bcl-2 and induction of Bax in the breast cancer cell line MCF-7. Western blot assay confirmed the two chalcones reduced the expression of cell death-related proteins such as Bcl-2 and cyclin D1 and promoted the cleavage of PARP at

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[1] LicoA = licochalcone A.

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48 h. However, only *trans*-chalcone induced the expression of the CIDEA gene and protein, which is involved in the apoptotic mechanism in the MCF-7 cell.

2. Materials and methods

2.1. Materials

The two tested compounds, *trans*-chalcone and LicoA, and some of the main reagents including MTT (4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), trypsin, doxorubicin hydrochloride, dimethyl sulfoxide (DMSO), DMEM, penicillin, kanamycin, streptomycin and LDH assay TOX7 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was obtained from Cultilab (Campinas, SP, Brazil), and Triton® X-100 was purchased from USB (Cleveland, OH, USA). The flow cytometry kits Cycletest Plus DNA Reagent and FITC Annexin V Apoptosis Detection I were purchased from BD Biosciences (San Jose, CA, USA). The materials used for gene expression were RNeasy® Mini Kit, RNase-free DNase Set, RT^2 SYBR® Green ROX™ qPCR Mastermix, RT^2 First Strand Kit, and RT^2 Profiler™ PCR Array PAHS-012Z (Qiagen, Germany). The BCA protein assay was purchased from Thermo Scientific (Rockford, IL, USA), jetPRIME from Polyplus-transfection (New York, NY, USA), and the 1X passive lysis buffer and DualGlo Luciferase Assay Kit from Promega (Madison, WI, USA).

2.2. Cell culture and assay conditions

The cell lines 3T3 (mouse fibroblasts) and MCF-7 [human breast cancer (estrogen receptor (ER)-positive)] were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL kanamycin and 100 μg/mL streptomycin and maintained in a humidified 5% CO_2 atmosphere at 37 °C. Doxorubicin was used at 2.5 μg/mL (4.31 μM) as the positive control for all the tests, except for the comet assay in which a concentration of doxorubicin of 0.8 μg/mL (1.38 μM) was used. Culture medium containing 0.25% DMSO was used as the solvent control.

2.3. Growth inhibition

The inhibition of cell viability by the two chalcones was evaluated by the MTT assay. Each cell line (MCF-7 and 3T3) was plated at a density of 3×10^4 cells/well in 96-well plates and incubated for 24 h. The cells were then treated with 5 different concentrations of the chalcones (25, 20, 15, 10, and 5 μg/mL) for 24 and 48 h. Next, 20 μL MTT solution (5 mg/mL) was added per well, and the plates were incubated for 4 h. The plates were centrifuged at $1800 \times g$ for 5 min, the culture medium was removed, and formazan dye was solubilized in 200 μL DMSO. Absorbance was read in a Thermoplate TP-Reader at 550 nm. The experiment was repeated at least three times in triplicate. The percent inhibition of cell viability was determined by the cell death formula of Szliszka et al. [8]. The estimated averages were used to calculate the IC_{20}, IC_{50} and IC_{80} values by nonlinear regression.

2.4. Lactate dehydrogenase assay

For the determination of lactate dehydrogenase (LDH) leakage, 3×10^4 MCF-7 cells/well were incubated in 96-well plates for 24 h and then treated with IC_{50} and IC_{80} (48-h MTT) of the chalcones for 24 and 48 h. Triton X-100 (1%, USB) was used as control. Next, 50 μL/well of the cell-free supernatant was collected and mixed with 100 μL/well reagent mixture (TOX7 Kit from Sigma-Aldrich) according to manufacturer instructions. The plates were covered with opaque material and incubated at room temperature for 30 min. Absorbance was read in a microplate reader (ELx 800 UV Bio-Tek Instruments, Inc.) at 490 nm. LDH leakage was calculated according to Grosse et al. [9]. The experiments were performed three times in triplicate.

2.5. Comet assay

The alkaline comet assay was used to determine the genotoxic potential of the chalcones against MCF-7 and 3T3 cells. Briefly, 3×10^5 cells/well were seeded in 6-well plates and incubated for 24 h. The cells were then treated with the lowest concentrations of the chalcones used in the MTT assay (15, 10, and 5 μg/mL) for 6 h. The treated cells were fixed on slides and submitted to lysis and electrophoresis, followed by neutralization, according to Liao et al. [10]. The slides were stained with 20 μg/mL ethidium bromide per slide. On fluorescence microscopy, 100 cells/slide were randomly selected and scored visually into five classes of DNA damage according to Cavalcanti et al. [11]. The DNA damage index (DDI) was calculated using the following formula: $DDI = (0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)$. The experiments were performed three times in duplicate.

2.6. Flow cytometry

Cell cycle and apoptosis were analyzed in a FACSCanto II flow cytometer (BD Biosciences). Twenty-four well plates with 1×10^5 cells/well were used in both experiments. The tests were done using the IC_{50} of the chalcones determined by the 24-h MTT test in the MCF-7 cell line.

2.6.1. Apoptosis analysis using annexin V-FITC/PI

Apoptosis was evaluated using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to manufacturer recommendations, with slight modifications. After incubation for 24 h, cells were treated with the inhibitory concentrations (IC_{50} and IC_{20}) of the chalcones for 24 h. After this period, the cells were trypsinized, centrifuged ($201 \times g$ for 5 min at 8 °C), washed twice with cold 1X PBS, and resuspended in 500 μL of 1X binding buffer. Next, 5 μL PI and 5 μL annexin V-FITC were added and the tubes were incubated at room temperature in the dark for 15 min before analysis in the flow cytometer. The experiments were performed three times in duplicate treatments.

2.6.2. Cell cycle analysis

After incubation for 24 h, the treated cells with IC_{50} and IC_{20} (24-h MTT) were collected, centrifuged, and washed as described above for apoptosis analysis. The supernatant was decanted and the cells were stained with the Cycletest™ DNA Reagent Kit (BD Biosciences) according to the protocol of the manufacturer. The test was performed once in triplicate and the results were analyzed using the ModFit LT program (BD Biosciences).

2.7. RNA extraction, cDNA conversion and RT^2 Profiler PCR Array

The real-time PCR Array was used to screen for apoptotic genes related to chalcone-induced cell death. This quantitative real-time PCR Array permits to analyze the expression of 84 apoptosis-related genes. First, MCF-7 cells were seeded at a density of 2.5×10^6 in 25-cm^2 flasks and incubated for 24 h. Next, the cells were treated with the IC_{50} (24-h MTT) of the chalcones for 6 and 24 h. Total RNA was extracted using the RNeasy® Mini Kit and RNase-free DNase Set (Qiagen). The integrity of the samples was assessed in a 2100 Bioanalyzer (Agilent) (RIN ≥ 8). Next, 500 ng of each RNA sample were converted into cDNA using the RT^2 First Strand Kit (Qiagen). Each cDNA sample was mixed with RT^2 SYBR® Green ROX™ qPCR Mastermix (Qiagen) and a 25-μL aliquot of the mixture (cDNA and Mastermix) was added per well of the RT^2