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In vitro antitumor effect of a lignan isolated from *Combretum fruticosum*, trachelogenin, in HCT-116 human colon cancer cells

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ABSTRACT

The use of natural products in therapeutics has been growing over the years. Lignans are compounds with large pharmaceutical use, which has aroused interest in the search for new drugs to treat diseases. The present study evaluated the cytotoxicity of (−)-trachelogenin, a dibenzylbutyrolactone type lignan isolated from *Combretum fruticosum*, against several tumor and non-tumor cell lines using the MTT assay and its possible mechanism of action. (−)-Trachelogenin showed IC_{50} values ranging of 0.8–32.4 μM in SF-295 and HL-60 cell lines, respectively and IC_{50} values > 64 μM in non-tumor cell lines. (−)-trachelogenin persistently induced autophagic cell death, with cytoplasmic vacuolization and formation of autophagosomes mediated by increasing LC3 activation and altering the expression levels of Beclin-1.

1. Introduction

The use of plant bioactive compounds with a potential role in the prevention or treatment of diseases has increased over the years (Isoda et al., 2014; Melo et al., 2007). The Combretaceae family, consisting of grasses, shrubs and trees, comprises about 20 genera and 600 species widely distributed in the tropical and subtropical regions of Africa and Brazil. The largest genera belonging to this family are *Combretum* and *Terminalia*, with about 370 and 200 species, respectively (Lima et al., 2012). The plants belonging to these genera are used in popular medicine to treat a variety of maladies including stomach diseases, bacterial infections, cancer, dysmenorrhea, fever, gastric ulcers, heart disease, pneumonia, and skin diseases (Eloff et al., 2008).

Combretum fruticosum is a creeper plant found in tropical and subtropical forests from Mexico to Argentina (Braga et al., 2007). This species, still poorly studied, has biological activities including inhibition of the angiotensin converting enzyme (Braga et al., 2007; Serra et al., 2005) and antitumor effect (Nascimento et al., 1985). A phytochemical study of the genus *Combretum* demonstrated many classes of components including triterpenes, flavonoids, nonprotein amino acids and lignans (Lima et al., 2012).

Lignans are a well-known class of compounds with wide pharmaceutical use due to their diverse biological activities, including antitumor, antiviral, hepatoprotective, platelet activating factor antagonist, and other properties. Despite the wide spectrum of biological activities related to lignans, the literature emphasizes the antioxidant properties and the role of these metabolites in cancer treatment and prevention. Research in this area has revealed several mechanisms of action related to the control of cell growth. (Lee and Xiao, 2003; Macrae and Towers, 1984).

Trachelogenin, a dibenzylbutyrolactone-type lignan, has been shown to be a Ca^{2+} antagonist with potent antihypertensive activity (Ichikawa et al., 1986), antagonist of platelet activating factor (PAF) (Iwakami et al., 1992) and to possess antileukemic and anti-HIV activity (Páska et al., 1996; Schroder et al., 1990; Trumm and Eich, 1989). More recently it showed effect against hepatitis C virus (Qian et al., 2016), active component on barrier function in intestinal epithelial cells (Shin et al., 2015), cytotoxicity, antiproliferative and chemopreventive activities (Mervai et al., 2015; Sólyomváry et al., 2015; Sólyomváry et al., 2014; Kang et al., 2007; Zhu et al., 2013). However, there are few

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studies related to the mechanism of action of the antitumor activity from this molecule.

According to the American Cancer Society (2016), colorectal cancer (CRC) is the third most frequent cancer, excluding skin cancers, in both men and women in the United States, with an estimated 65,270 new cases of colon cancer and 39,220 new cases of rectal cancer in 2016, with an estimated mortality rate of 49,190 for the same year. In Brazil, the incidence of new colorectal cancer cases is on average 17,000 per 100,000 people in both men and women (Instituto Nacional do Câncer (INCA), 2016). CRC is an uncontrollable disease that develops slowly, starting with polyps on the epithelial lining of the colon or rectum. It is an aggressive tumor which can lead to death (Bardhan and Liu, 2013).

Thus, the aim of this study was to evaluate the cytotoxicity of (−)-trachelogenin isolated from the stalks of *Combretum fruticosum* against several tumor and non-tumor cell lines and to determine the possible mechanism of action in HCT-116 colon carcinoma human cells.

2. Material and methods

2.1. Plant material

The *Combretum fruticosum* stalks were collected in July 2008 in the County of Caucaia, Ceará, Brazil. The plant material was identified by Dra. Maria Iracema Bezerra Loiola, botanist from the Departamento de Biologia of the Universidade Federal do Ceará (UFC). A sample specimen (registration number: 43.054) has been deposited at the Herbário Prisco Bezerra-UFC.

2.2. Extraction, fractionation, isolation and identification

The *C. fruticosum* stalks (3.1 kg) were air-dried, powdered and then extracted with EtOH (3 × 10 L) at room temperature for 24 h. The resulting solution was evaporated under reduced pressure to yield 64.7 g of crude extract, which was solubilized in a mixture of MeOH-H_2O (7:3), and partitioned with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH, to yield the correspondent fractions CFEH (5.2 g), CFED (10.2 g), CFEA (21.5 g) and CFEB (20.6 g), respectively. The CH_2Cl_2 fraction (10.2 g) was subjected to a silica gel column chromatography using CH_2Cl_2, CH_2Cl_2-EtOAc (7:3, 4:6 v/v), EtOAc, EtOAc-MeOH (7:3, 4:6) and MeOH as eluents, providing 7 fractions (T1-T7), after TLC analysis. The fraction T3 (1.1 g) was chromatographed on a silica gel column eluted with CH_2Cl_2, CH_2Cl_2-MeOH (99:1, 97:3, 95:5, 1:1) to furnish 47 subfractions of 8 mL each. The subfractions F8–15 (98.4 mg) and F28–40 (256.9 mg) were analyzed by HPLC using a solvent system of H_2O (0.1% TFA)-MeOH- (6:4), with a 3.60 mL/min flow rate, resulting in the isolation of the compound identified as (−)-trachelogenin (16.5 mg, RT = 8.6 min).

The (−)-trachelogenin was isolated as a white amorphous solid (m.p. 200–203 °C, $[\alpha]_D^{20}$ 46.4) (*c* 0.25, MeOH). Its ^{1}H NMR spectrum (500 MHz, Py-d_5) displayed signals corresponding to two AMX systems, one at δ_H 6.85 (d, J = 8.5 Hz, H-5), 6.71 (d, J = 2.0 Hz, H-2) and 6.57 (d, J = 7.9 Hz, H-6) and other at δ_H 6.83 (d, J = 8.5 Hz, H-5′), 6.69 (br s, H-2′) and 6.70 (d, J = 2.0 Hz, H-6′). In addition, three singlets at δ_H 3.77 (s, OCH_3–3), 3.77 (s, OCH_3–3′) and 3.78 (s, OCH_3–4′), were observed, corresponding to three methoxy groups. The ^{13}C NMR-CPD spectrum (125 MHz, Py-d_5) displayed signals for 20 carbon atoms, including one signal at δ_C 180.7 (C-9), corresponding to carboxyl lactone, in addition to 12 signals sp^2 carbons for two benzene rings. The signals at δ_C 146.8 (C-3), 150.6 (C-4), 149.5 (C-3′) and 150.6 (C-4′) were indicative of carbon bearing oxygen atoms. The analysis of all spectroscopic data allowed the identification of (−)-trachelogenin (Fig. 1A), a lignan previously isolated from *Glycydendron amazonicum* (Euphorbiaceae) (John and Tinto, 1992), but first reported in the genus *Combretum*.

2.3. Cell cultures

The human tumor cell lines used in this work were HL-60 (promyelocytic leukemia), OVCAR-8 (ovarian), HCT-116, HCT-8 (colon), PC-3 (prostate) and SF-295 (brain), which were kindly provided by the National Cancer Institute (Bethesda, MD, USA). The murine non-tumor cell lines 3T3-L1, L929 (murine fibroblast) and human non-tumor cell line HEK293 (embryonic kidney) was obtained from ATCC and deposited in the Rio de Janeiro cell bank. The cells were maintained in appropriate medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO_2. Peripheral blood mononuclear cells (PBMC) were also tested.

Heparinized blood from healthy, non-smoking donors who had not taken any drugs for at least 15 days prior to sampling was collected. The PBMCs were isolated via a standard method of density-gradient centrifugation over Ficoll–Hypaque. PBMCs were washed and re-suspended at a concentration of 1 × 10^6 cells/mL in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO_2. Phytohemagglutinin (4%) was added at the beginning of the experiment. After 24 h of culture, the cells were treated with either test compound dissolved in dimethyl sulfoxide (DMSO) or DMSO alone and cells were analyzed at the specified time points.

2.4. MTT assay - inhibition of tumor cell proliferation

The cytotoxicity of (−)-trachelogenin was tested against above mentioned cell lines using the MTT assay. Cells were plated in 96-well plates and treated with increasing concentrations of (−)-trachelogenin, or with DMSO alone as negative control. Doxorubicin was used as a positive control. HCT-116 cells were incubated with (−)-trachelogenin for 24, 48 and 72 h to verify time-dependent cytotoxicity. At the end of incubation, the plates were centrifuged and the supernatant was removed and replaced with fresh medium (150 µL) containing MTT (0.5 mg/mL). Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product by metabolically active cells (Mosmann, 1983). Three hours later, the plates were centrifuged, the MTT formazan product was dissolved in 150 µL DMSO, and the absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada) at 595 nm. Growth inhibition was estimated as the reduction in values as compared to a DMSO alone control.

2.5. Analysis of the mechanisms involved in the cytotoxic activity

The following experiments were performed to elucidate the mechanism involved in the cytotoxic action of (−)-trachelogenin (2.5, 5 and 10 µM) in HCT-116 cells. These concentrations were chosen based on dose-response curves obtained by MTT assay after 48 h of treatment. Doxorubicin (0.4 µM) was used as a positive control.

2.5.1. Cell viability and cell morphological changes

Cell viability was examined in triplicate using the trypan blue dye exclusion test. Cells were plated in 24-well plates and treated with (−)-trachelogenin for 24 and 48 h. Aliquots were removed from cultures, and the cells were stained with trypan blue and viable and non-viable cells were counted in a Neubauer chamber. To evaluate cell morphology, cells were harvested, transferred to cytospin slides, fixed with methanol and stained with quick panoptic kit (Laborclin, Brazil). Evaluation of cell morphological changes was made by light microscopy (Olympus, Tokyo, Japan).

2.5.2. Morphological analysis using confocal microscope

HCT-116 cells were treated with (−)-trachelogenin for 48 h.