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Effect of diterpenoid kaurenoic acid on genotoxicity and cell cycle progression in gastric cancer cell lines

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ABSTRACT

The goal of our study was to evaluate the effect of kaurenoic acid, obtained from copaiba oil resin, in gastric cancer (GC) and a normal mucosa of stomach (MNP01) cell lines. The compound was tested at concentrations of 2.5, 5, 10, 30 and 60 μg/mL. Comet and micronucleus assays were used to access its potential genotoxicity *in vitro*. Moreover, we evaluated the effect of kaurenoic acid in cell cycle progression and in the transcription of genes involved in the control of the cell cycle: *MYC*, *CCND1*, *BCL2*, *CASP3*, *ATM*, *CHK2* and *TP53*. Kaurenoic acid induced an increase on cell DNA damage or micronucleus frequencies on GC cell lines in a dose-dependent manner. The GC and MNP01 cell lines entering DNA synthesis and mitosis decreased significantly with kaurenoic acid treatment, and had an increased growth phase compared with non-treated cells. The treatment induced apoptosis (or necrosis) even at a concentration of 2.5 μg/mL in relation to non-treated cells. GC cell lines presented reduced *MYC*, *CCND1*, *BCL2* and *CASP3* transcription while *ATM*, *CHK2* and *TP53* increased in transcription in relation to non-treated cells, especially at a concentration above 10 μg/mL. The gene transcription in the MNP01 (non-treated non-cancer cell line) was designated as a calibrator for all the GC cell lines. In conclusion, our results showed that kaurenoic acid obtained from *Copaifera* induces DNA damage and increases the micronuclei frequency in a dose-dependent manner in GC cells, with a significant genotoxicity observed above the concentration of 5 μg/mL. Moreover, this compound seems to be able to induce cell cycle arrest and apoptosis in GC cells.

1. Introduction

Gastric cancer (GC) remains the third leading cause of cancer-related mortality worldwide, and invasion and metastasis of gastric cancer represents the major reason for its poor prognosis [1]. Multimodal therapies have proven to benefit patients with cancer. However, without curative surgery, variations and combinations of chemotherapy and/or radiation cannot bring clinically meaningful success nowadays [2].

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One of the main problems in cancer treatment is gradual resistance of cancer cells against treatment [3]. Therefore, the aim of immunopharmacological studies has been to improve cancer treatment results [4]. The alternate solution for the harmful effects of synthetic agents is the use of phytotherapy [5]. Bioactive compounds from plants have a lot of biological activities and may present great potential in the development of new drugs for the treatment of human diseases [6].

Reports about medicinal plants in the Brazilian Amazonian region have been documented in the literature, especially concerning the oil resin extracted from the trunk of *Copaifera langsdorffii* Desfon (Leguminosae) [7,8]. This oil resin is a reputed folk remedy used by the natives of Brazil for the treatment of several diseases such as sore throat, urinary and pulmonary infections, and to hasten ulcer and wound healing [9]. Usually, this oil resin is administered orally *in natura*, or applied in ointment form [10–12]. This substance has great social and economic representation in the Amazon region, since it represents about 95% of the entire oil resin production countrywide [13].

Several studies have demonstrated that the *Copaifera* oil resin has manifold therapeutic properties, including anti-inflammatory, antitumoral, antimelanoma, antiulcerogenic, antilipoperoxidation and antioxidant [10,12,14–16]. Furthermore, the diterpenes, kaurenoic and polyaltic acids present in the pure oil seem to present healing and anti-inflammatory properties [7,8].

Kaurenoic acid (*ent*-kaur-16-en-19-oic acid) (Fig. 1) is classified as a diterpene and considered an intermediate of the gibberellin plant growth hormone biogenesis [17]. Some studies showed that kaurenoic acid presents in vitro anti-parasitic and anti-microbial activities [18–23]. Moreover, this diterpene has anti-proliferative action in CEM leukemic cells, MCF-7 breast and HCT-8 colon cancer cells [24]. Additionally, kaurenoic acid is also able to reduce human sperm motility when directly tested in sperm samples, but was only weakly spermicidal [25].

While *Copaifera* oil resin has its herbal properties widely reported, there are few studies in the scientific literature that evaluated the effects of this oil resin components, especially in GC cell lines. Thus, the present study aims to assess the genotoxicity of kaurenoic acid and the effect of this compound in the cell cycle and in the transcription of genes involved, among other functions, in GC cell lines.

2. Materials and methods

2.1. General experimental procedures

The ACP02, ACP03 and AGP01 cell lines, previously established and characterized by our research group, were used in the present study [26,27]. The ACP02 cell line was established from a diffuse-type GC, and ACP03 and AGP01 were from an intestinal-type GC. ACP03 is able to start a tumorigenesis process in *Cebus paella* [28]. We also used the GC cell line PG100 obtained from Rio de Janeiro Cell Bank, Brazil, that was previously cytogeneticly characterized by our group [29].

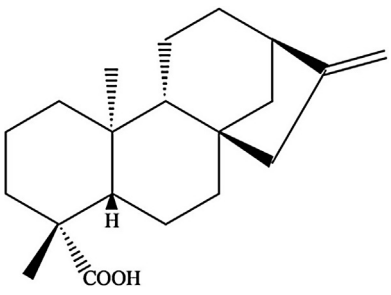

Fig. 1. Molecular structure of kaurenoic acid (ent-kaur-16-en-19-oic acid).

Cells lines were cultivated under standard conditions in Modified Eagle's medium salts, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin [30]). Cells were maintained in 25 cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland) at 37 °C in a humidified atmosphere containing 5% CO_2 and were harvested by treatment with 0.15% trypsin and 0.08% EDTA in phosphate-buffered saline (PBS). Cells (3×10^5) were seeded in 5 mL of complete medium and grown for 2 days prior to treatment with the test substance.

Cells were treated with different concentrations of kaurenoic acid (2.5, 5, 10, 30 and 60 μg/mL) for 3 h. After this, the cells were washed with ice-cold PBS and trypsinized with 100 μL trypsin (0.15%) and were resuspended in complete medium. The final concentration of DMSO in the culture medium was kept constant, below 0.1% (v/v). The negative control cell cultures were not exposed to kaurenoic acid, while the methyl methanesulfonate (Sigma Aldrich Co, St. Louis, MO, USA) was used as a positive control (4×10^{-5} M). All cell treatments were carried out with three replicates.

2.2. Chemical isolation

In this study, kaurenoic acid (CAS 6730-83-2) (Sigma Chemical Co., St. Louis, MO, USA) was diluted under sterile conditions in dimethyl sulfoxide (DMSO) and the concentrations used in this study were based on preliminary cytotoxicity assays performed by our research group (unpublished data). Kaurenoic acid was obtained from the oil resin of *C. langsdorffii* that grows abundantly in the Amazon region of Northern Brazil [31].

2.3. Genotoxicity assay

The Comet assay (alkaline version) was performed as described by Singh et al. [32] with some modifications [33,34]. After cell treatment, the cells were washed with ice-cold PBS and trypsinized with 100 μL trypsin (0.15%) and were resuspended in complete medium. An aliquot (450 μL) of the cell suspension from each experimental group was taken and centrifuged at 1.000 rpm for 5 min in a microcentrifuge (Eppendorf). The resulting pellet was homogenized with 300 μL of a low melting point agarose (0.8%), spread onto microscope slides pre-coated with a normal melting point agarose (1.5%) and covered with a coverslip. After 5 min at 4 °C, the cover slip was removed and the slides were immersed in cold lysis solution (2.5 M NaCl; 100 mM EDTA; 10 mM Tris, 10% DMSO and 1% Triton-X, pH: 10) for one week. After lysis, the slides were placed in an electrophoresis chamber and covered with freshly made electrophoresis buffer (300 mM NaOH; 1 mM EDTA, pH > 13).

The electrophoresis was run for 25 min (34 V and 300 mA). Afterward, the slides were neutralized by submersion in distilled water (4 °C) for 5 min and fixed in 100% ethanol for 3 min. Staining of the slides was performed immediately before the analyses using ethidium bromide dye (20 μg/mL). Slides were prepared in duplicate, and 100 cells were analyzed per sample (50 cells from each slide) using a fluorescent microscope (Olympus BX41) at 40× magnification.

The damage index (DI) is based on the length of migration and the amount of DNA in the tail. It is considered a sensitive measure of DNA. Five categories (0–4) were used here: class 0 (no damage), class 1 (slight damage with a tail length shorter than the diameter of the nucleus), class 2 (medium damage with a tail length one or two times the diameter of the nucleus), class 3 (significant damage with a tail length greater than two times the diameter of the