Chemico-Biological Interactions 222 (2014) 112–125

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Morphological and biochemical alterations activated by antitumor clerodane diterpenes

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ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form 8 October 2014
Accepted 15 October 2014
Available online 27 October 2014

Keywords:
Casearia sylvestris
Cytotoxicity
Antiproliferative action
Apoptosis
Human cells
Murine cells

ABSTRACT

Casearia sylvestris Swartz (Salicaceae) is a plant commonly widespread in the Americas. It has oxygenated tricyclic bioactive clerodane diterpenes with antimicrobial, antiulcer, larvicidal, chemopreventive, anti-inflammatory, antioxidant and antiproliferative properties. Due to this requirement for the developing of new anticancer drugs, it was initially evaluated the cytotoxic activity of a fraction with Casearins (FC) and its clerodane diterpenes Casearin B (Cas B), D (Cas D), X (Cas X) and Caseargrewiin F (Cas F) isolated from *C. sylvestris* leaves against 7 tumor cell lines, Sarcoma 180 cells (S180) and on normal peripheral blood mononuclear cells (PBMC). All substances tested showed cytotoxic potential. Cas F and X were the most active compounds. Cell death analyzes with Cas F (0.5 and 1 μM) and Cas X (0.7 and 1.5 μM) using the HL-60 leukemia line as experimental model showed DNA synthesis and membrane integrity reduction, DNA fragmentation and mitochondrial depolarization, specially after 24 h exposure, cell cycle arrest in G_0/G_1 phase caused by Cas X, activation of the initiator -8/-9 and effector -3/-7 caspases and phosphatidylserine externalization, all biochemical features of apoptosis corroborated by chromatinic condensation, karyorrhexis, cytoplasmic vacuolation and rarefaction and cellular shrinkage, morphological findings specially observed after 12 and 24 h of incubation. Therefore, Cas X and F were the most functional molecules with more pronounced lethal and discriminating effects on tumor cells and antiproliferative action predominantly mediated by apoptosis, highlighting clerodane dipertenes as promising lead antineoplastic compounds.

1. Introduction

Between 1981 and 2010, out of 1073 new chemical entities (NCE) approved as novel medicines by the Food and Drug Administration (FDA), only 36% can be classified as truly synthetic; 64% are unmodified NCEs, derived or synthetic molecules that mimic or are based on natural compounds. Then, instead of the interest in molecular modeling, combinatorial chemistry and other chemical synthesis techniques, natural products, and particularly, medicinal plants, remain an important source of new therapeutic agents against infectious diseases (bacteria or fungi), insects, cancer, dyslipidemia and immunomodulation [1–6].

Casearia sylvestris Swartz (Salicaceae), popularly known as "guaçatonga", "café silvestre", erva-de-lagarto", "língua-de-tiú", "cafezinho-do-mato" and "corta-lengua", is a plant distributed in tropical and temperate regions around the world and commonly widespread in the Americas. In Brazil, it is present from Amazonas (Tapajós river region) to Rio Grande do Sul states [7,8].

Different parts of *C. sylvestris* have shown antimicrobial [9–11], antiulcer [12–14], larvicidal [15], chemopreventive [16], anti-inflammatory and antioxidant properties [14,17,18]. Most of these properties are attributed to the different secondary metabolites

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http://dx.doi.org/10.1016/j.cbi.2014.10.015
0009-2797/

isolated from *C. sylvestris* belonging to the casearin, caseargrewiin and casearvestrin classes, oxygenated tricyclic bioactive clerodane diterpenes which have presented also excellent cytotoxic potential [4,10,19,20–23]. Thus, we firstly analyzed the *in vitro* antiproliferative action of a fraction with casearins (FC) and its isolated compounds Casearin B (Cas B), Casearin D (Cas D), Casearin X (Cas X) and Caseargrewiin F (Cas F). Secondly, it was studied the mechanism involved in the antiproliferative activity using HL-60 leukemia line as experimental model.

2. Methods

2.1. Chemicals, isolation of the compounds and structure identification

Leaves of *C. sylvestris* were collected at the Parque Estadual Carlos Botelho (São Miguel Arcanjo, São Paulo, Brazil). The plant was identified by Dr. Ines Cordeiro (Instituto Botânico do Estado de São Paulo, São Paulo, Brazil). Voucher specimens (numbers AGS04, AGS05, AGS06, AGS13 and AGS19) were deposited at the Herbarium Maria Eneida P. Kaufmann (Instituto Botânico do Estado de São Paulo, São Paulo, Brazil). Dried and powdered leaves of *C. sylvestris* were extracted with ethanol in a stainless steel extractor with solvent reflux for ca. 24 h at 40 °C. The crude extract was concentrated under reduced pressure (rotary evaporator) and dried in desiccators over silica gel under reduced pressure to yield a dry residue. The structures of Cas B, D, X and F were determined by spectrometric data (nuclear magnetic resonance, ultraviolet, infrared and mass spectrometry) and compared to the spectral report available in the literature [23,24] (Fig. 1).

Fetal calf serum was purchased from Cultilab (Campinas, SP), RPMI 1640 medium, trypsin–EDTA, penicillin and streptomycin were purchased from GIBCO® (Invitrogen, Carlsbad, CA, USA). Propidium iodide (PI), acridine orange (AO), ethidium bromide (EB) and Rhodamine 123 (Rho-123) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Doxorubicin (Doxolem®) was purchased from Zodiac Produtos Farmacêuticos S/A, Brazil.

2.2. Animals

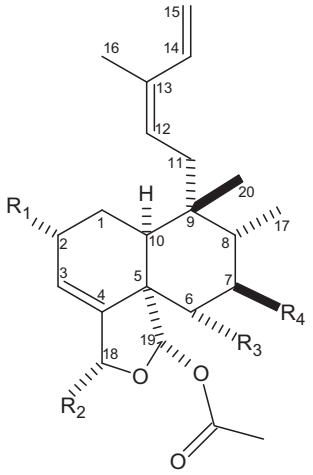
Adult female Swiss mice (*Mus musculus* Linnaeus, 1758) were obtained from the animal facilities of the Universidade Federal do Piauí (UFPI), Teresina, Brazil. They were kept in well-ventilated cages under standard conditions of light (12 h with alternate day and night cycles) and temperature (27 ± 2 °C) and were housed with free access to commercial rodent stock diet (Nutrilabor, Campinas, Brazil). All procedures were approved by the Committee on Animal Research at the UFPI (Process nº 102/2011) and followed the Brazilian (*Colégio Brasileiro de Experimentação Animal* – COBEA) and International Standards on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council).

2.3. In vitro antiproliferative assays

The cytotoxic potential of the FC, Cas B, D, X and F was assessed after 72 h exposure using leukemia (HL-60), breast (MDA-MB/231, Hs578-T, MX-1), prostate (PC-3, DU-145) and skin (B16/F-10) tumor lines, Sarcoma 180 cells (S180) and normal peripheral blood mononuclear cells (PBMC). Cell culture was performed 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. Quantification of cell proliferation was spectrophotometrically determined using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter). Control groups (negative and positive) received the same amount of DMSO (0.1%). Doxorubicin (Dox, 0.01–8.6 μM) was used as positive control.

2.3.1. Antiproliferative study on tumor cells evaluated by MTT assay

The cytotoxicity against HL-60, MDA-MB/231, Hs578-T, MX-1, PC-3, DU-145, and B16/F-10 cancer cells was determined by MTT assay [25], which analyzes 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. Briefly, cells



	R_1	R_2	R_3	R_4
Casearin B	CH_3O	CH_3CO_2	CH_3CO_2	n-$C_3H_7CO_2$
Casearin D	OH	n-$C_3H_7CO_2$	OH	n-$C_3H_7CO_2$
Casearin X	n-$C_3H_7CO_2$	n-$C_3H_7CO_2$	OH	H
Caseargrewiin F	n-$C_3H_7CO_2$	CH_3CO_2	OH	H

Fig. 1. Chemical structures of the molecules isolated from *Casearia sylvestris* leaves.