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CR-LAAO antileukemic effect against Bcr-Abl$^+$ cells is mediated by apoptosis and hydrogen peroxide

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ABSTRACT

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by the presence of the Bcr-Abl tyrosine kinase protein, which confers resistance to apoptosis in leukemic cells. Tyrosine kinase inhibitors (TKIs) are effectively used to treat CML; however, CML patients in the advanced (CML-AP) and chronic (CML-CP) phases of the disease are usually resistant to TKI therapy. Thus, it is necessary to seek for novel agents to treat CML, such as the enzyme L-amino acid oxidase from *Calloselasma rhodostoma* (CR-LAAO) snake venom. We examined the antitumor effect of CR-LAAO in Bcr-Abl$^+$ cell lines and peripheral blood mononuclear cells (PBMC) from healthy subjects and CML patients. CR-LAAO was more cytotoxic towards Bcr-Abl$^+$ cell lines than towards healthy subjects' PBMC. The H_2O_2 produced during the enzymatic action of CR-LAAO mediated its cytotoxic effect. The CR-LAAO induced apoptosis in Bcr-Abl$^+$ cells, as detected by caspases 3, 8, and 9 activation, loss of mitochondrial membrane potential, and DNA damage. CR-LAAO elicited apoptosis in PBMC from CML-CP patients without TKI treatment more strongly than in PBMC from healthy subjects and TKI-treated CML-CP and CML-AP patients. The antitumor effect of CR-LAAO against Bcr-Abl$^+$ cells makes this toxin a promising candidate to CML therapy.

Abbreviations: AC, accelerated phase; ANOVA, analysis of variance; AP, advanced phase; Bcr-Abl$^+$, cell lines positive for the *BCR-ABL*1 oncogene; CML, chronic myeloid leukemia; CML-AP, chronic myeloid leukemia patients in the advanced phase of the disease; CML-CP/T, chronic myeloid leukemia patients in the chronic phase of the disease treated with imatinib mesylate; CML-CP/WT, chronic myeloid leukemia patients in the chronic phase of the disease without drug treatment; CML-CP, chronic myeloid leukemia patients in the chronic phase of the disease; CP, chronic phase; CR-LAAO, L-amino acid oxidase from *Calloselasma rhodostoma* venom; CT, control; DAS, dasatinib; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; HFS, hypotonic fluorescent solution; IM, imatinib mesylate; LAAO, L-amino acid oxidase; NIL, nilotinib; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PI, propidium iodide; SV-LAAO, L-amino acid oxidase from snake venom; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor; TMRE, tetramethylrhodamine ethyl ester perchlorate; VP-16, etoposide; $\Delta\psi m$, mitochondrial membrane potential.

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1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cells characterized by the presence of a reciprocal translocation between chromosomes 9 and 22 *t*(9;22) [1], which results in the *BCR-ABL1* oncogene that encodes a Bcr-Abl fusion oncoprotein [2]. This abnormal oncoprotein exhibits constitutive tyrosine kinase (TK) activity, which upregulates cell growth and induces apoptosis resistance in CML cells [3,4].

Apoptosis, a programmed cell death mechanism important to maintain tissue homeostasis, can be triggered via two main pathways: the extrinsic pathway, which is mediated by death receptors interaction with their respective ligands followed by caspase 8 activation; and the intrinsic pathway, whose activation depends on the release of mitochondrial factors to the cytosol and subsequent caspase 9 activation [5,6]. Both pathways promote the enzymatic activation of caspase 3 and PARP cleavage and result in the characteristic features of apoptosis [7,8].

Some molecular therapies for CML target the Bcr-Abl fusion oncoprotein, including the selective TK inhibitor (TKI) imatinib mesylate (IM) [9,10]. Despite the effectiveness of IM to treat CML, many CML patients in the advanced (AP) or chronic (CP) phase of the disease (CML-AP and CML-CP, respectively) are resistant to its therapeutic effects, especially those bearing the T315I mutation [11]. The majority of the IM-resistant CML patients have benefited from treatment with the second-generation TKIs, such as dasatinib (DAS) and nilotinib (NIL) [12]. However, DAS and NIL are not effective in CML patients who present the T315I Bcr-Abl-specific mutation [13,14]. To overcome the aforementioned limitations, scientists have investigated the antitumor effect of new compounds that can help to develop effective drugs to treat CML patients who do not respond to the existing drugs. In this sense, the snake venom toxins have emerged as promising sources of molecules for anticancer drug development [15]. Most snake venoms are composed of toxic molecules, such as the L-amino acid oxidases (LAAOs) [16]. LAAOs are flavoenzymes that catalyze the oxidative deamination of L-amino acids to α-keto acids and produce ammonia and H_2O_2 as by-products. These enzymes usually are FAD-binding homodimeric glycoproteins of 110–150 kDa [17].

LAAOs from snake venom (SV-LAAOs) have attracted great scientific interest over the last decades due to their pharmacological potential [18,19]. In addition to the antibacterial and antiparasitic properties, these molecules display cytotoxic and apoptosis-inducing effect in MCF7 (breast tumor), A549 (lung adenocarcinoma), and MKN 45 (stomach tumor) tumor cell lines [20–23]. Our research group has recently reported that a LAAO from *Bothrops pirajai* snake venom induces apoptosis in HL-60 (human promyelocytic leukemia) and HL-60.Bcr-Abl (chronic myeloid leukemia) cells [24]. The mechanisms by which SV-LAAOs act have not been clearly elucidated yet, but there are evidences that the H_2O_2 produced during the enzymatic reaction mediates their antitumor effect [25,26].

LAAO is the major component of *Calloselasma rhodostoma* venom. *C. rodhostoma* is a terrestrial snake found in Asia, also known as Malayan pit viper or *Agkistrodon rhodostoma*. The structural properties of the LAAO isolated from *C. rhodostoma* (CR-LAAO) snake venom have been extensively studied [27,28], but there are few reports on its pharmacological and biological actions. This toxin was cytotoxic against yeast, as well as against the Jurkat (acute T cell leukemia) and HepG2 (hepatocarcinoma) tumor cell lines [29–31].

In the present study, we examined whether CR-LAAO exerts cytotoxic effect towards one Bcr-Abl$^-$ tumor cell line (HL-60), three Bcr-Abl$^+$ tumor cell lines – HL-60.Bcr-Abl, K562, and KCL22 –, as well as towards healthy subjects' and CML patients' peripheral blood mononuclear cells (PBMC). We also assessed the antitumor effectiveness of CR-LAAOs in combination with IM, DAS, and NIL. CR-LAAO exerted apoptosis-inducing and genotoxic effects on CML cells, and potentiated the effect of TKIs on Bcr-Abl$^+$ cells.

2. Material and methods

2.1. Cell lines

The following tumor cell lines were used in this study: HL-60 (human promyelocytic leukemia cells); HL-60.Bcr-Abl, obtained by infection of HL-60 cells with a retrovirus carrying the *BCR-ABL1* gene; K562 (ATCC n. CCL 243) and KCL22, obtained from CML patients in blast crisis phase bearing the b3a2 and b2a2 translocations, respectively. The HL-60, HL-60.Bcr-Abl, and KCL22 cell lines were kindly provided by Dr. João P. G. Amarante-Mendes, from The Biomedical Sciences Institute of University of São Paulo, Brazil.

All cell lines were cultured in complete RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin, under an atmosphere of 5% CO_2 and 95% air, at 37 °C.

2.2. Peripheral blood mononuclear cells (PBMC) isolation

Peripheral blood was collected into vacuum tubes containing anticoagulant and the PBMC were isolated by the Ficoll-Hypaque density gradient centrifugation method, using Histopaque-1077 (Sigma Diagnostics, Inc., MO, USA). This study enrolled ten healthy subjects (control group) of both sexes, aged between 29 and 65 years, and ten CML patients followed at Ribeirão Preto Medical School Hospital of the University of São Paulo (HCFMRP-USP), Brazil. Patients were divided into two groups, according to the CML disease phase: chronic phase (CP; $n = 5$) and advanced phase (AP; $n = 5$, including patients in the accelerated phase (AC) and blast crisis). The patients' features are presented in Table S1. The Human Research Ethics Committee of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil, approved the study protocol, which was registered under the code CEP 243/2012.

2.3. CR-LAAO toxin

2.3.1. Determination of purity

CR-LAAO was isolated from *C. rhodostoma* snake venom and purified according to the method described by Macheroux et al. [32]. The purity of the sample was analyzed by reversed-phase high-performance liquid chromatography (Shimadzu®) using a C18 column (4.6 mm *i.d.* x 25 cm, Shim-pack CLC-ODS, Shimadzu®) and 12% SDS-PAGE, as described by Laemmli [33], but using non-reducing conditions. The molecular weight standards used were: Unstained Protein Molecular Weight Marker (14.4–116 kDa) (Thermo Fischer Scientific, Waltham, MA, USA).

2.3.2. Enzymatic activity

The purified enzyme was lyophilized and stored at 4 °C. Prior to use, CR-LAAO was solubilized in PBS (phosphate buffered saline), and the total protein concentration in the sample was determined by the microbiuret method [34] To ascertain whether the protein was active – which is a requisite for the biological assays – the enzymatic activity of CR-LAAO was determined in 0.1 M Tris–HCl buffer pH 7.2, at 25 °C, using the conjugated enzyme assay reported by Pessatti et al. [35].

2.4. Cytotoxicity evaluation

The cell lines and healthy subjects' PBMC were cultured in 180 μL of RPMI 1640 complete medium in 96-well plates (2×10^4 cells per well), in the presence of CR-LAAO (0.05–2.50 μg/mL) for 24 h, at 37 °C, and under an atmosphere of 5% CO_2. To assess