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In vitro cytotoxicity of L-amino acid oxidase from the venom of *Crotalus mitchellii pyrrhus*

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ABSTRACT

L-amino acid oxidase isolated from *Crotalus mitchellii pyrrhus* (Cmp-LAAO) exhibited cytotoxicity against LNCaP prostate adenocarcinoma cells. The viability of LNCaP cells decreased in a concentration- and time-dependent manner upon administration of Cmp-LAAO. Cmp-LAAO induced apoptosis as evidenced by AnnexinV/PI staining using flow cytometry. An increase in caspase-9 and caspase-3 activity were also observed. The damaging effect of LAAO appears to be due to its enzymatic activity, that produces hydrogen peroxide which can then induce oxidative stress within the cells. As expected, the level of oxidative stress in LNCaP cells increased with Cmp-LAAO treatment as confirmed by 2′, 7′-dichloro-fluorescin diacetate (DCFDA) fluorescence assay. Co-treatment with catalase significantly reduced the cytotoxic effect of Cmp-LAAO, thereby affirming that hydrogen peroxide is probably the main mediator of Cmp-LAAO cytotoxicity. Hence, Cmp-LAAO may be a potential cancer therapeutic for prostate cancer.
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1. Introduction

Among cancer deaths in men, prostate cancer is the second leading cause in the United States. According to the American Cancer Society, the United States is estimated to have 161,360 new cases of prostate cancer cases in 2017 (American Cancer Society, 2017). Older men are more likely to contract prostate cancer when compared to younger men, where the chance of contracting prostate cancer increases rapidly beyond the age of 50, although incidence to mortality ratio is high for prostate cancer. Radiation therapy and radical prostatectomy have been shown to be the main stay of treatment for prostate cancer patients (Silberstein et al., 2017). In recent years, new treatment options such as cryotherapy and high-intensity focused ultrasound have also been introduced (Ouzzane et al., 2016). Chemotherapy using docetaxel is usually reserved for metastatic castrate-resistant prostate cancer (Fizazi et al., 2015). Other FDA-approved drugs for prostate cancer treatment include bicalutamide (which is used in combination with luteinizing hormone releasing hormone agonist) (Patel et al., 2016) and abiraterone acetate (Rathkopf et al., 2017).

Normal organ homeostasis is maintained by a balance between cell proliferation and programmed cell death. Inhibition of apoptosis has been implicated in the development of tumour as well as chemoresistance. Promoting apoptosis in cancer cells may lead to the regression of cancer cells and is one of the most direct way of treating cancer (Bruckheimer and Kyprianou, 2000). Thus, any agent that induce apoptosis may be useful for chemotherapy against cancer (Le Goff et al., 1992).

L-amino acid oxidase (LAAO) is one of the toxins found in snake venom. The proportion of LAAO in snake venom can be as high as 25% (More et al., 2010). Of late, there has been an increasing interest in its anticancer potential. (Burin et al., 2016; Guo et al., 2015; Teixeira et al., 2016; Zhang and Wei, 2007). AkbuLAAO from *Agkistrodon blomhoffii ussurensis* venom has been shown to possess anti-tumour effect by downregulating TGFβ signalling pathway in HepG2 cells (Guo et al., 2015). CR-LAAO isolated from *Calloselasma rhodostoma* venom was proposed to be an anticancer agent for its acute inflammation-inducing effect *in vivo* (Costa et al., 2017). LAAO is known to catalyze the oxidative deamination of L-amino acid, resulting in the production of hydrogen peroxide (H_2O_2), which is believed to be the cytotoxic agent that contributes to its potential

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antitumor property (Suhr and Kim, 1996), although some authors have also suggested the involvement of other mechanisms. For instance, rusvinoxidase (an LAAO) inactivated by two freeze-thaw cycles remained cytotoxic towards MCF7 breast cancer cells (Mukherjee et al., 2015) Murakawa et al. (2001) found that apoptosis-inducing protein isolated from parasite-infected fish (which was identified to be LAAO) induced apoptosis using two different mechanisms: a fast, H_2O_2-dependent mechanism and a slow mechanism mediated through L-amino acid depletion.

In the present study, we evaluated the anti-cancer potential of LAAO isolated from a Southwestern speckled rattlesnake, *Crotalus mitchellii Pyrrhus* (Cmp-LAAO), against prostate cancer by using the LNCaP prostate adenocarcinoma cell line as the *in vitro* model. The cytotoxic effect of Cmp-LAAO was evaluated in both LNCaP and RWPE-1 (normal prostate epithelial cells) cell lines. Annexin V (AV)/propidium iodide (PI) staining followed by analysis with flow cytometry. Caspase activity were checked to verify the mechanism of cell death. DCFDA staining was used to assess the level of intracellular oxidative stress upon treatment with Cmp-LAAO. Catalase co-treatment was done to demonstrate hydrogen peroxide as the agent from Cmp-LAAO activity that induced cell death.

2. Materials and methods

2.1. Materials

Hyclone™ RPMI 1640 media (SH30255.01) and Hyclone™ fetal bovine serum (FBS) (SH30088.02) were from GE Healthcare (Chicago, IL, USA). Annexin V-Alexa Fluor 647 (A23204), MitoProbe™ $DiIC_1(5)$ assay kit (M34151) and keratinocyte-SFM medium (17005042) were from ThermoFisher (Waltham, MA, USA). Cell Proliferation Assay MTS (G3580) was Promega (USA). Caspase-3 (L00289) and Caspase-9 (L00304) colorimetric assay were from GenScript (Piscataway, NJ, USA). 2′, 7′-dichlorofluorescin diacetate (D6883) was from Sigma-Aldrich (USA).

2.2. Methods

2.2.1. Bioassay-guided isolation of Cmp-LAAO

Crude CMP venom powder (purchased from NNTRC, US) was dissolved in 50 mM ammonium bicarbonate, pH 8.0 (ABC). The venom solution was passed through a gel filtration chromatography column (Superdex G-75) equilibrated with ABC. The fractions collected were pooled according to the peaks and tested for their cytotoxic effects on LNCaP cells. The active fraction was buffer-exchanged with 50 mM sodium acetate (pH 5.0) using an AMICON filter and subjected to a cation exchange column equilibrated with 50 mM sodium acetate (pH 5.0). The column was then eluted with an increasing volume of elution buffer (1.0 M sodium chloride in 50 mM sodium acetate, pH 5.0). The active fraction was buffer-exchanged with ultrapure water and subjected to a reverse phase column in 0.1% trifluoroacetic acid (TFA). The proteins were subsequently eluted with increasing concentration of elution buffer (0.1% TFA in 80% acetonitrile).

2.2.2. Mass spectrometry identification

The fraction obtained from reverse phase chromatography was separated by 4–20% BioRad Precast Gel, stained with GelCode blue stain and digested with trypsin (Shevchenko et al., 2006). Digested peptides were loaded into the column (Acclaim PepMap™ RSLC, C18, 100 Å, 2 μm, 75 mm × 15 cm) equilibrated with buffer A (0.1% formic acid, 2% acetonitrile) which was then separated using Easy-nLC nanoflow high-performance liquid chromatography system. The peptides were eluted with a 120-min gradient at a flow rate of 250 nL/min from 8 to 40% buffer B (0.1% formic acid, 80% acetonitrile). In positive ion mode, survey full scan MS spectra (m/z 310–1400) were acquired with a resolution of $r = 60{,}000$, an AGC target of 1e6, and a maximum injection time of 700 ms. In each survey scan, 10 most intense peptide ions with ion intensity >2000 counts and a charge state ≥2 were isolated sequentially to a target value of 1e4 and fragmented in the linear ion trap by CID using 35% normalized collision energy. A maximum exclusion list of 500 was applied, with one repeat count and exclusion duration of 30 s. MaxQuant version 1.5.0.30 was used in database searches of the MS data with tryptic specificity (maximum two missed cleavages) and initial mass tolerance: 6 ppm for precursor ions and 0.5 Da for fragment ions. N-acetylation and oxidized methionine were searched as variable modifications while cysteine carbamidomethylation was searched as a fixed modification, and. Maximum false discovery rate was fixed to 0.01 for both protein and peptide. A protein is accepted as positively identified if it has more than 10 unique peptides with a minimum length of 7 amino acids and % sequence coverage >20%.

2.2.3. Cell culture

LNCaP (human prostate carcinoma) cells were cultured in RPMI 1640 culture medium (supplemented with 10% FBS), while normal RWPE-1 prostate epithelial cells were grown in keratinocyte-SFM medium (supplemented with EGF 1-53 and BPE) in T-75 flask at 37 °C, 5% CO_2 and humidified air. Cells were detached with 0.25% (w/v) trypsin-0.53 mM EDTA when they reached 80% confluency. Both cell lines were sub-cultured at a sub-cultivation ratio of 1:5. The seeding density for LNCaP cells in 6-well and 96-well plates were 450,000 cells/well and 7000 cells/well, respectively. While that for RWPE-1 cells in 96-well plate was 15,000 cells/well. After seeding, cells were allowed to grow for 36 h before treatment.

2.2.4. Cell viability assay

MTS assay was used to assess cell viability according to manufacturer's instruction. LAAO was freshly prepared in complete RPMI and complete keratinocyte-SFM medium at various concentrations (0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg/mL). Cells were treated with 100 μL of LAAO at various concentrations and for different time points (3, 6, 12, 24 and 48 h). At the end of each time point, 20 μL of MTS reagent was added into each well before re-incubation at 37 °C, 5% CO_2 and humidified air in the dark for 2 h.

The absorbance readings were taken at 490 nm with a spectrophotometer (Tecan Infinite F200 PRO). The % cell viability was calculated as follow:

$$\%\ cell\ viability = \frac{Treatment\ group - blank\ reading}{Control\ group - blank\ reading} \times 100\%$$

2.2.5. Flow cytometry detection of apoptosis

Briefly, cells were pelleted and re-suspended in 1X binding buffer. Then, 3.0 μL of AV-FITC and PI were added and the mixture was incubated at room temperature for 15 min before flow cytometry analysis.

2.2.6. Caspase activity assay

Caspase activity was assessed using the colorimetric assay kit from GenScript. Briefly, 50 μL of cold Lysis Buffer was added to the cells and kept on ice for 30 min, followed by centrifugation at 16,000 × g for one minute at 4 °C. Protein quantification (Bradford assay) was done on the supernatant. The same protein sample was used to assay for both caspase-3 and caspase-9 activities. About 200 μg of protein was taken from the supernatant and topped up to

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