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Anticoagulant and cytotoxic assessment of L-amino acid oxidase from Indian cobra (*Naja naja*) venom

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ABSTRACT

Introduction: Snake venom L-amino acid oxidase (SV-LAAO) has promising therapeutic prospects because of its effects on various biological functions. Purified Naja naja venom enzyme L-amino acid oxidase (NnLAAO) was studied to examine its anticoagulant and cytotoxic properties.

Methods: Activated partial prothrombin time (APPT) assay and prothrombin time (PT) both of these assays are used to access the anti-coagulant property of NnLAAO on human blood sample. Sulforhodamine B (SRB) protocol is performed on MDA MB-231and HCT 116 cell, which are treated with different doses (0.4µg-100µg) of NnLAAO for 48h incubation time, respectively, and the percentage of inhibition was compared with oxaloplatin as positive control.

Results: NnLAAO significantly prolonged the APPT and showed a negligible effect on the PT. NnLAAO inhibited cytotoxicity in a dose-dependent manner in both breast cancer MDA MB-231 and colon adenocarcinoma (HCT-116) cell lines with 60% and 78% of inhibition respectively.

Conclusion: The NnLAAO enzyme of Indian cobra venom is a promising anticoagulant as well an anticancer agent for various cell lines because of its efficiency and specificity, as it will offer better treatment and response with fewer side effects.

Introduction

Indian Cobra (*Naja naja*), belongs to Elapidae family. *Naja naja* was first described by Carl Linnaeus. It is also known as the spectacled Cobra or Asian Cobra. Indian cobra is a medium sized snake with smooth, shiny scales, wide neck and head, with a distinctive hood mark on top of the head. It is mostly found in thick forests and cultivated farms, in search of prey. *Naja naja* venom contains several enzymatic and non-enzymatic components including neurotoxins and cardiotoxins. Elapidae venoms are thus neurotoxic, with known protease activity, but also have hemostatic variations (1). Snake bite is considered as an important medical emergency due to its rapidity in causing local and systemic damage to the victim. Venomous snakes possess a complex blend of biological toxins in their venom, which causes death worldwide. Snake venom contains a mixture of proteins, peptides, nucleotides, enzymes and other pharmacological bioactive molecules. The chemical composition of snake venoms are enormously different in snake species due to geographical variation (2). Snake venom forms the richest source of pharmacological bioactive molecules and hence, exhibits a wide range of biochemical, physiological and toxicological properties; thereby assessment of the potency of snake venom has become an interesting topic for scientists and researchers all over the world. The snake venom L-amino acid oxidase (SV-LAAO) has evident therapeutic properties due to its effects on various biological functions including platelet aggregation, cell apoptosis and cytotoxicity (3). It is also believed to have anti-coagulant, anti-microbial, anti-leishmaniasis, anti-tumor and anti-HIV activities. SV-LAAO is also being explored for its potential in cancer therapy. L-amino acid oxide, which is a homodimeric flavoprotein contains non-covalently bound FAD as a cofactor. LAAOs are multifunctional enzymes, which exhibit anti-coagu-

lant and anti-bacterial properties, platelet aggregation, apoptosis and edema. These effects are attributed to the release of high amounts of H2O2, a known reactive oxygen species (ROS), during the reaction. According to Ande et al. (4), ROS is formed extracellularly and acts directly by altering cell membrane permeability, and is also involved in cell apoptosis. Anticoagulant proteins have a major contribution in the mechanism of blood coagulation. Thrombosis and hemostasis are the major targets of snake venom proteins and can provide potential for designing and developing new drugs to prevent/treat blood clotting disorder. Few in vitro studies have reported the possible involvement of snake venom proteins and their components, in inhibiting blood clot formation (5). Sakurai et al. reported that purified LAAO from *A. h. blomhoffii* venom possesses anticoagulant activity (6). Several venom proteins including PLA2, LAAO and proteases exhibit their enzymatic anticoagulant properties though different mechanisms. Snake venom proteins with molecular masses between 6 kDa and 35 kDa are said to prolong coagulation and inhibit blood coagulation (7). Cancer is one of the leading causes of death worldwide, and hence there is an urgent need for a better treatment for cancers. The search to cure cancer using natural resources has been in practice for long time as surgery, radiotherapy and chemotherapy do not provide adequate protection against cancer cells. Current cancer treatment methods simultaneously affect normal cells along with cancerous cells, causing more serious side effects. Calmette in 1993 first reported with the use of snake venom as a treatment for cancer in laboratory animals (8). Venoms from *elapidae*, *viperidae* and *crotalide* were found to have cytotoxic properties against B16F10 melanoma cell lines. The cytotoxicity was stronger in *elapidae* venom compared to *viperidae* and *crotalide* in causing cell aggregations (9). But this anticancer agent involves various side effects, that are toxic to normal cells and thus decreases its therapeutic indexes (10). This finding led the research for identifying other agents for cancer treatment from naturally available products. The aim of the present study was to further investigate the anticoagulant properties and the mechanism of cytotoxicity of the purified enzyme – L-amino acid oxidase (*Nn*-LAAO) from the Indian cobra (*Naja naja*) venom, belonging to the western part of the Indian subcontinent, against two human cancer cell lines, including Human colorectal carcinoma (HCT 116) and breast cancer cell lines (MDA MB-231).

Methods

Purified *Naja naja* venom enzyme namely *Nn*LAAO was obtained based on the report by Neema et al. (11). The HCT 116 and MDA MB 231 cell lines were preserved and studied at the Department of Biochemistry, JSS University Mysore. Fresh human blood samples were collected from healthy volunteers. All chemicals were of analytical grade.

Re-calcification time

Re-calcification time (RT) was determined according to the procedure described by Condrea et al., (12). RT was performed by mixing fresh human blood sample with 0.11M tri-sodium citrate at the ratio of 9:1. The mixture was centrifuged for 15 min at 3000 rpm. Clotting was initiated by adding 30µl of CaCl2 (10mM final concentration) into a pre-warmed mixture of 100 µl of platelet poor plasma (PPP) and 100µl of 50mM Tris-HCl, 0.1M NaCl, pH 7.4 (with different concentrations of *Nn*LAAO). The time between appearance of visible clot from the addition of CaCl2 was recorded in sec. For control experiments Tris-HCl buffer alone was added instead of *Nn*LAAO.

Anticoagulant property

Activated partial prothrombin time (APPT)

APPT was determined using APPT reagent (AGAPEE kit, India) according to manufacturer's protocol. Blood samples were collected from 10 healthy individuals in tubes containing 3.2% tri-sodium citrate (9:1 v/v). Plasma was extracted by immediate centrifugation of blood samples at 2000 rpm for 15 min at 4oC. Normal citrated human plasma (100µl) was pre-incubated with *Nn*LAAO in a dose-dependent manner (2-10µg/ml) for 5 min at 37oC in 10µl of 10mM Tris-HCl buffer, at the pH of 7.2. A portion of obtained blood samples was also immediately centrifuged at 2000 rpm, for 15 min at 4oC to obtain plasma. The reaction mixture was activated by adding 0.2ml of cephaloplastin. Plasma clotting was initiated by adding 100µl of 0.02 M CaCl2, and the clotting time was recorded. Relative clotting factor (RCF) with respect to standard Heparin was calculated based on the following formula;

Prothrombin time (PT)

Fresh citrated human plasma (100µl) was collected similar to the above-mentioned procedure. Samples were incubated with various concentrations of *Nn-LAAO (2-10*µg) and crude *Naja naja venom (2-10*µg) for 10 min at 37oC in 10µl of 10mM Tris-HCl buffer, at the PH of 7.2. After adding an optimal amount (200µl) of PT reagent-brain thromboplastin to the reaction mixture, the plasma clotting time was recorded in seconds. One unit of anticoagulant activity corresponds to an increase of 20 sec in normal plasma coagulation called the International Normalized Ratio (INR). The International Sensitivity Index was 1 for the kit used in the present study. PT value for each concentration was calculated and compared to the value of the control.

Experiments were repeated once more and the mean of two measurements were recorded.

Cytotoxic property

Cytotoxicity was assessed using one or more human cell lines, which were in active growing condition and undergoing mitotic cell division. The reason behind this activity is the production of H2O2 through the enzymatic reaction (13). To analyze the cytotoxic effect of *Nn*LAAO on human colorectal carcinoma (HCT 116) and breast cancer cell lines (MDA MB-231), these cell lines were incubated for 48h in a dose dependent manner following Sulforhodamine B (SRB) protocol.

Measurement of cell viability using SRB assay

Sulforhodamine B (SRB) assay was performed according to the method described by Skehan et al., (14). Experimentally, cells were fixed in 1/4th volume of cold 50% (w/v) TCA at 4oC. After 1h the media was removed and the wells were washed with water (200µl X 4 times) to wash out the TCA and serum proteins. The plates were dried, incubated with 100µL 0.4% SRB for 30 minutes to stain the cellular proteins and were washed quickly with 1% acetic acid (200µl X 4 times) to remove unbound SRB. The bound SRB was solubilized in 10 mM Tris base solution (100µl/well) and the absorbance was measured at 490 nm in a Bio-Rad plate reader. The percentage of cell growth inhibition was calculated by comparing the OD values with control DMSO vehicle treated cells.

Results

The direct anti-coagulant effects of *Nn*LAAO on the blood samples was analyzed using the following assays – recalcification time, APPT and PT. The present study showed that, purified protease *Nn*LAAO from the venom of *N. naja,* significantly delayed the onset and progress of blood coagulation, prolonged the APPT at concentrations between 2 and 10µg, but had negligible effect on the PT (table 1). APPT was prolonged when compared to the control and the crude *N. naja* venom. Similarly, PT assay showed negligible results. PT did not show further activity even when the enzyme concentration was increased. The negative results of prothrombin activation assay were expected as *Nn*LAAO was found to be an anticoagulant protein rather than a procoagulant, as all prothrombin activators were isolated, thus far were exhibiting procoagulant activity. With the establishment of *Nn*LAAO as an anticoagulant protein, we further constructed a concentration-dependent response for recalcification time to determine its effective clinical range. Figure 1 shows recalcification time in seconds for both purified *Nn*LAAO and crude *N. naja* venom concentrations (0.25-2.5µg) for a prolonged time. Cytotoxicity assay relies on the ability of the SRB to bind cellular protein components and measure the total biomass. The result of SRB assay determined that *Nn*LAAO killed the HCT 116 and MDA MB-231 cell lines in a dose dependent manner. Figures 2 and 3 show the response of MDA MB-231and HCT 116 cell treated with different doses (0.39µg-100µg) of *Nn*LAAO for 48h incubation time, respectively, and the percentage of inhibition was calculated in comparison to oxaloplatin as positive control. *Nn*LAAO (100µg) inhibited only 60% of breast cancer (MDA MB-231) cell line and 78% inhibition was observed in human colon adenocarcinoma (HCT-116) cell line when compared to 49.7% inhibition using crude *Naja naja* venom and 80% inhibition with oxalopolantin as positive control.

Discussion

LAAO is one of the hemorrhagic toxins that are present in snake venoms. The present study demonstrates, that *Nn*LAAO purified from the *Naja naja* (Indian

Table 1. Prothrombin time assay for NnLAAO and crude N. naja venom samples

Concentration of NnLAAO (µg)	Control*	NnLAAO*	crude N. naja*
0	1.00±0.1	1.00±0.3	1.00±0.4
1	1.06±0.8	1.12± 0.4	1.29±0.3
2	1.32±0.2	1.54±0.3	1.65±0.2
3	1.89±0.5	2.01±0.8	2.21±0.8
4	2.09± 0.4	2.23±0.2	2.35±0.1
5	2.19±0.2	2.26±0.1	2.38±0.5

* International normalized ratio (INR) for each value was calculated comparing to the value of the control. Presented values were the mean for two independent experiments.

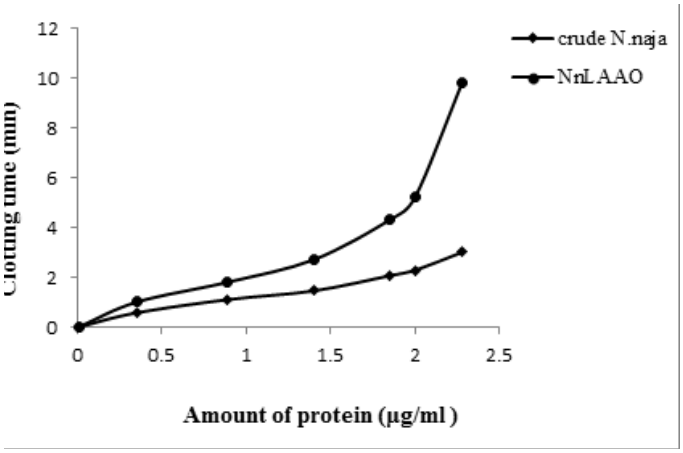

Figure 1. Recalcification times of NnLAAO and crude N. naja venom

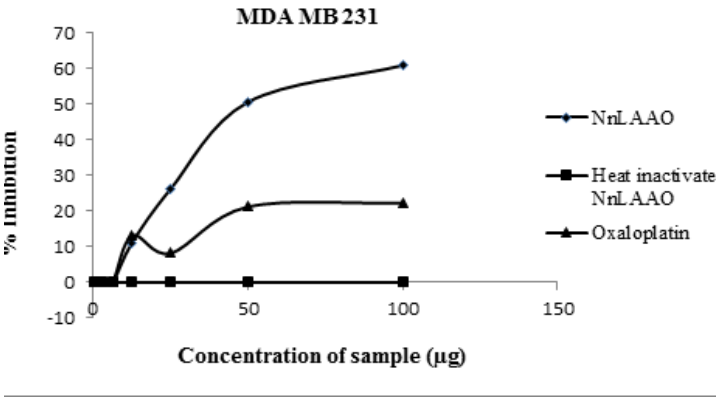

Figure 2. Cytotoxic activity of NnLAAO on MDA MB-231

cobra) venom, found in the Indian subcontinent, possesses a strong anticoagulant and cytotoxic activities. Both crude *Naja naja* venom and *Nn*LAAO showed strong anticoagulant activity. In addition, *Nn*LAAO enzyme dose-dependently prolonged the activated partial thromboplastin time (aPTT), but had little effect on the prothrombin time (PT). According to APTT assay, in the presence of calcium ions, cephaloplastin activates coagulation factors of intrinsic pathway in plasma, leading to clot formation. Clotting time is proportional to the concentration of factors VIII, IX, XI and XII as well as common pathway factors II, V and X. Whereas PT measures the time taken by the citrated plasma to clot, in the presence of tissue thromboplastin and Ca++ which activates extrinsic pathway of human blood coagulation cascade. This fact assists in identifying the cause and extent of a hemorrhagic disorder. When thromboplastin reagent is added to citrated plasma, clotting cascade is initiated, forming gel clot. The time required for clot formation would be prolonged if there is a reduced factor(s) activity in the extrinsic pathway of coagulation cascade. The use of chemotherapeutic and surgery in cancer therapy remains the predominant option for clinical control. These therapies provide inadequate effect in addition to their adverse effects on the normal cells as well as on cancer cells. Furthermore, one of the major problems in chemotherapy is the resistance developed after initial treatment. These drawbacks have led to the increased demand for using anticancer drugs developed from natural resources. The SRB cell cytotoxicity assay is said to be one of the most widely used methods applied to detect cell viability, and can induce cytotoxicity in various cancer cell lines in a dose-dependent manner. The SRB cell proliferation assay indicates that, cell proliferation is inhibited by treatment of different cancer cell lines with various concentrations of snake venom protease (SVP). toxins isolated from various sources implement their effect through physiological alterations including modulation of the activity of the membrane enzymes, inhibition of the platelet aggregation, hemolytic activity, cardiac arrest, cytotoxicity, etc. The apoptotic effect of LAAO was also reported in human prostate adenocarcinoma (PC-3) model through an increase in caspase-3/7 cleavage (16). On the other hand, various other snake species (*N. Haje ,N. Oxiana, and N. Kaouthia*) have shown cytotoxic properties. These venoms can readily penetrate into living cancer cells as human lung adenocarcinoma A549 and promyelocytic leukemia HL60 and thereby cause lysosomal leakage and plasma membrane injury (17).

Conclusion

Pharmacological characteristics of Indian cobra venom indicate that, *Nn*LAAO has an anticoagulant activity, suggesting its principal activity was mediated via the intrinsic coagulation pathway. The SV-LAAO is the most effective and safe compound (than its crude venom) to be used as antitumor agent or in combination with chemotherapeutic drugs. Purified *Nn*LAAO showed comparatively suitable inhibition against human colorectal carcinoma (HCT 116) cell line compared to crude *Naja naja venom,* but had a considerably less effect on breast cancer cell lines *(MDA MB-231). More in vitro* studies are required to identify safe and suitable therapeutic dose of these venomous compounds or their combinations as well as their possible side effects on normal cells. Furthermore, trials are recommended to deliver these compounds in a suitable pharmaceutical form (nanoparticle) that could minimize their toxicity, target the tumor cells and facilitate their distinct effect individually or in combination with other chemotherapeutic agents. The observed efficacy and specificity make *Nn*LAAO a good candidate to be used as anticoagulant and anti-thrombotic agent as well

as anti-tumor, due to the lower cost, better response and fewer side effects.

Ethical disclosure

Informed consent was collected from the subjects. No additional tests were done by blood samples.

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Author contributions

All authors contributed equally in performing this project.

Conflict of interest

There is no conflict of interest.

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