Cytotoxicity of snake venom enzymatic toxins: phospholipase A2 and L-amino acid oxidase

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The phospholipase A2 (PLA2) and L-amino acid oxidase (LAAO) are two major enzymes found in the venoms from most snake species. These enzymes have been structurally and functionally characterised for their pharmacological activities. Both PLA2 and LAAO from different venoms demonstrate considerable cytotoxic effects on cancer cells via induction of apoptosis, cell cycle arrest and suppression of proliferation. These enzymes produce more pronounced cytotoxic effects in cancer cells than normal cells, thus they can be potential sources as chemotherapeutic agents. It is proposed that PLA2 and LAAO contribute to an elevated oxidative stress due to their catalytic actions, for instance, the ability of PLA2 to produce reactive oxygen species during lipolysis and formation of H2O2 from LAAO catalytic activity which consequently lead to cell death. Nonetheless, the cell-death signalling pathways associated with exposure to these enzymatic toxins are not fully elucidated yet. Here in this review, we will discuss the cytotoxic effects of PLA2 and LAAO in relationship to their catalytic mechanisms and the underlying mechanisms of cytotoxic actions.

Introduction
Snake venom is a complex mixture of proteins and polypeptides with a diverse array of pharmacological activities. The proteins and polypeptides constitute ~95% of the dry weight of the venom [1]. Significant differences in venom composition have been reported between closely related species or even between the same species from different geographical origins [2,3]. Among all the venom toxins, the enzymatic toxins phospholipase A2 (PLA2) and L-amino acid oxidase (LAAO) are ubiquitously found in Elapidae and Viperidae whereby PLA2 exists as the most abundant enzymatic toxins, as revealed by venom proteome (Figure 1).

PLA2 is one of the most extensively studied enzymatic toxins in snake venoms [4]. Snake venoms are the major source of Group 1 and Group II secretory PLA2. Generally, the venom PLA2 is a small protein with the molecular mass of ~13–15 kDa. The enzyme catalyses the hydrolysis of phospholipids at sn-2 positions to produce lysophospholipids and free fatty acids [5]. It requires Ca2+ for their catalytic actions [6]. The venom PLA2 possesses presynaptic or postsynaptic neurotoxicity [7,8], systemic or local myotoxicity [9,10], cardiotoxicity [11], platelet aggregation inhibition [12], anticoagulant [13] and oedema inducing activities [14]. The venom-induced neurotoxicity has been suggested to be attributed to the β-neurotoxin, a PLA2 enzyme in nature that inhibits pre-synaptic neuromuscular transmission [15]. Although the molecular mechanism is not well characterised, studies have shown that the neurotoxic effects exerted by venom PLA2 are presumably due to the influx of cytosolic calcium ions when binding to the voltage-gated ion channels on the neuronal membrane [16,17]. Besides, the PLA2 can cause mitochondrial membrane disruption in the respiratory muscle as a result of phospholipid hydrolysis [18,19]. These events further lead to acute neuromuscular weakness, followed by flaccid paralysis [20]. In general, PLA2 from Elapidae venom exists as a monomeric enzyme.
and possesses neurotoxicity while Viperidae venom PLA2 can exist in both monomer and dimer forms. The Viperidae monomeric PLA2 exhibits cytotoxic effects, whereas dimeric PLA2 possesses cytotoxic effects at a lower dose and neurotoxicity at a higher dose ([21], Figure 1).

LAAO is a flavoenzyme that catalyses the oxidative deamination of L-amino acid to α-keto acid and produces hydrogen peroxide (H2O2). Snake venom LAAOs display various pharmacological activities. Some enzyme LAAOs exhibit potent platelet inhibitory actions [22] while other LAAO isoforms induce platelet aggregation [23]. The antiplatelet mechanism of LAAO is attributed to the elevated production of H2O2, ammonia, and α-keto acid [24]. The liberated H2O2 affects ADP-induced platelet formation and distorts the interactions between blood coagulation factors [25,26]. In addition, LAAO also possesses antimicrobial actions [27], oedema [28], haemolysis [29] and haemorrhage [30].

Although both enzymatic toxins demonstrate various pharmacological effects, they share a similar feature whereby the products from their catalytic actions pose potent cytotoxic agents. For example, venom PLA2 alters plasma membrane integrity in muscle cells to cause myonecrosis [31]. The membrane perturbation by PLA2 is a secondary process to its catalytic actions on membrane phospholipids [32], indicating that venom PLA2 exhibits remarkable cytotoxicity. On the other hand, venom LAAO has also been demonstrated to induce cell death due to the generated H2O2 [33–35]. Cancer is characterised by an uncontrolled cells proliferation, the ability to escape apoptosis and evading growth suppressors with active metastasis. Cancer cells differ from normal cells not only in the cellular metabolism but the lipid compositions on plasma membranes. Cancer cells have asymmetry in their membrane lipid compositions such as extracellular accumulation of phosphatidylserine [36] and higher lipid concentrations than normal cells [37]. Both enzymatic toxins exert their effects on the plasma membrane, it is thus suggested that cancer cells are more susceptible to toxins’ actions.

In this review, we outline our current understanding of the structural properties and catalytic actions of both PLA2 and LAAO. In addition, we also discuss and summarise the cytotoxic effects exerted by PLA2 and LAAO against different cancer cells with a specific focus on the underlying mechanisms.

Phospholipase A2
PLA2 (EC 3.1.1.4) is an enzyme belongs to a family of lipolytic enzyme esterase which specifically catalyses the hydrolysis of the ester linkages in glycerophospholipids at the sn-2 position. The hydrolysis of
glycero-phospholipids liberates free fatty acid, such as arachidonate and the release of lysophosphatidic which are the mediators in various biological processes.

The Ca^{2+} is a crucial cofactor for catalysis, thus the Ca^{2+} binding loop structure is highly conserved in most of the venom PLA2. The structure of PLA2 has three major \( \alpha \)-helices and two antiparallel \( \beta \)-sheets cross-linked by disulfide bonds [38]. The disulfide-linked \( \alpha \)-helices (residues 37–54 and residues 90–109) form a hydrophobic channel catalytic site which facilitates the binding of phospholipid substrates [31]. The four key residues in the active site involves in the coordination of the Ca^{2+}, are His48, Asp49, Tyr52 and Asp99 via hydrogen bond formation and coupling interaction [6]. The venom PLA2 can be classified into two major groups, namely Group I PLA2 (GIPLA2) and Group II PLA2 (GIIPLA2) according to the location of disulfide bonds [6,39].

**Group I PLA2 (GIPLA2)**
The venom GIPLA2 consists of 115–125 residues with a molecular mass of 13–15 kDa [40]. The GIPLA2 has a single polypeptide chain containing 6–8 disulfide bridges [6]. It contains ~50% of \( \alpha \)-helices and 10% of \( \beta \)-sheets [40]. The venom GIPLA2 has an elapid loop (residues 57–59) that links the \( \alpha \)-helices and the \( \beta \)-sheets [41], thus, GIPLA2 is found ubiquitously in elapid venoms. The venom GIPLA2 is different from mammalian pancreatic PLA2, which the latter enzyme has a pancreatic loop with an additional five amino acid residues at position 62–67 [42]. The GIIPLA2 is further divided into Group IA and Group IB for snake venom PLA2 and mammalian pancreatic PLA2, respectively. Despite so, Group IB PLA2 enzymes have also been identified in the venoms from Oxyuranus scutellatus, Micrurus frontalis frontalis, Notechis scutatus and Ophiophagus hannah due to the presence of the \( \alpha \)-helix that is identical with mammalian pancreatic PLA2 [43].

**Group II PLA2s (GIIPLA2)**
The venom GIIPLA2 is found exclusively in Viperidae venoms. It contains 120–125 amino acid residues and seven disulfide bonds [6]. Unlike GIPLA2, neither the pancreatic nor elapid loops are present in GIIPLA2 enzymes. However, it possesses a C-terminal extension with a different organisation of disulfide bonds, which clearly distinguishes GIIPLA2 from GIPLA2 [44]. In GIIPLA2, the D49 is conserved and contributes to Ca^{2+}- dependent catalytic activity [45]. Thus, GIIPLA2 is also recognised as D49 acidic PLA2 [46].

**Mechanism of cytotoxicity**
PLA2 catalyses the cleavage of the ester bond of phospholipids at the sn-2 site by nucleophilic attack [47]. Calcium ion, on the other hand, stabilises the negatively charged transition state by coordinating the phosphate oxygen and a carbonyl group during the catalysis [48]. Most of the biological membranes are composed of phospholipids, it is believed that PLA2 alters the membrane fluidity and causes membrane permeabilisation, which ultimately leads to cell death. The cytotoxic effects of PLA2 on a different cell are summarised in Table 1.

In general, venom PLA2 variants can be classified into D49 acidic PLA2 (Asp-49), K49 basic PLA2 (presence of Lys-49 instead of Asp-49) and S49 PLA2 (presence of Ser-49). The basic PLA2 homologues, K49 and S49 PLA2s are responsible for many Ca^{2+} independent biological activities and thus they are catalytically inactive [45]. The D49 acidic PLA2 is less cytotoxic than K49 basic PLA2, whereby acidic PLA2 possesses higher IC_{50} than basic PLA2 (Table 1). On the other hand, S49 PLA2 variants have been isolated from the venoms of saw-scaled vipers *Echis* sp. [49] which also exhibit Ca^{2+} independent biological activities with potent cytotoxic effects than K49 PLA2 (IC_{50} = 2.5–12.2 \( \mu \)M). Despite so, S49 PLA2 demonstrates weaker lipolytic activity compared with K49 PLA2 [50]. The basic PLA2 homologues display more pronounced cytotoxic effects in cancer cells.

The C-terminal region of the PLA2 is believed to be responsible for compromised membrane integrity and interacts with vascular endothelial growth factor receptor-2 (VEGFR-2) [51,52]. The C-terminal region of the enzyme could also bind to VEGFR-2 to inhibit angiogenesis, an essential process in cancer metastasis. Therefore, the cytotoxicity of PLA2 is probably mediated by the interaction between the C-terminal region and the plasma membrane [53–55]. Besides, the PLA2-induced cytotoxicity might involve the liberated reactive oxygen species (ROS) during its phospholipid metabolism, further increases intracellular oxidative stress. Elevated oxidative stress leads to the activation of cell death pathways. Although there is no establishment of the exact pathways, it might involve the down-regulation of anti-apoptotic proteins such as Bcl2, Bcl-XL and c-FLIP [56]. There is also an increase in pro-apoptotic BAD expression and the activation of caspase 3 [56].

Moreover, PLA2 alters the distribution of different phases in the cell cycle to cause apoptosis [57]. PLA2 also

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Table 1. The cytotoxicity of different PLA₂ from different snake species on various cell types. The IC₅₀ indicates the concentration of venom PLA₂ to kill 50% of the cell populations.

| Species           | Types of PLA₂ | Cell types                                      | IC₅₀   | References     |
|-------------------|---------------|-------------------------------------------------|--------|----------------|
| Bothrops asper    | basic PLA₂    | Mouse adrenal tumour cells                      | n.d.   | [92]           |
| Bothrops brazили | acidic PLA₂   | Jurkat human acute T-cell leukaemia cells       | 100.0 μg/ml   | [53]         |
| Bothrops jararaca | acidic PLA₂   | peripheral blood mononuclear cells (PBMC)       | n.d.   | [93]           |
|                  |               | HL60 human leukaemia cells                      | n.d.   |                |
| Bothrops jaracussu| Bth TX-1      | Jurkat human acute T-cell leukaemia cells       | n.d.   | [54,57,94,95]  |
|                  |               | Erlich ascitic tumour cells                     | 104.35 μg/ml  |               |
|                  |               | SK-BR-3 human breast cancer cells               | 81.2 μg/ml  |               |
|                  |               | MCF-7 human breast cancer cells                 | 104.35 μg/ml  |               |
|                  |               | MDAMB231 human breast cancer cells              | >409 μg/ml  |               |
|                  |               | PC-12 rat adrenal medulla pheochromocytoma      | n.d.   |                |
|                  |               | C2C212 murine muscle cells                      | n.d.   |                |
|                  |               | B16F10 mouse melanoma cells                     | n.d.   |                |
|                  |               | S180 murine sarcoma cells                       | n.d.   |                |
| Bothrops moojeni  | acidic PLA₂   | Jurkat human acute T-cell leukaemia cells       | n.d.   | [96]           |
|                  |               | K562-S human immortalised myelogenous leukaemia cells | 257 μg/ml | [55]       |
|                  |               | K562-R human immortalised myelogenous leukaemia cells | 191 μg/ml |               |
| Crotalus durissus| Heterodimeric | Murine erythroleukaemia cells                   | 3.0–5.0 μg/ml  | [97]       |
|                  | basic PLA₂    | SK-LU-1 human lung cancer cells                 | n.d.   |                |
|                  |               | Hs578T human breast cancer cells                | n.d.   |                |
|                  |               | KYS30 human lung cancer cells                   | 1.0 μg/ml  |               |
|                  |               | GAMG human glioblastoma cells                   | <0.5 μg/ml  |               |
|                  |               | HCB151 glioma cells                             | 4.1 μg/ml  |               |
|                  |               | PSN-1 human pancreatic cancer cells             | 0.7 μg/ml  |               |
|                  |               | PANC-1 pancreatic cancer cells                  | <0.5 μg/ml  |               |
|                  |               | HeLa cervical cancer cells                      | 2.4 μg/ml  |               |
|                  |               | KYSE 270 oesophageal cancer cells               | 8.7 μg/ml  |               |
|                  |               | U373 glioma cells                               | 30.2 μg/ml |               |
|                  |               | SiHa cervical cells                             | >30.0 μg/ml |               |
| Daboia siamensis | dssPLA₂       | SK-MEL-28 human skin melanoma cells             | n.d.   | [60]           |
| Daboia russelli | dmsPLA₂       | SK-MEL-28 human skin melanoma cells             | 0.90 μg/ml | [62]        |
| Echis carinatus | Ser49 PLA₂    | A549 human adenocarcinoma cells                 | 8.5 μM   | [49]          |
|                  |               | HUVEC human umbilical vein cells                | 12.2 μM  |               |
| Echis coloratus  | Ser49 PLA₂    | A549 human adenocarcinoma cells                 | 3.5 μM   |               |
|                  |               | HUVEC human umbilical vein cells                | 4.9 μM   |               |
| Echis ocellatus  | Ser49 PLA₂    | A549 human adenocarcinoma cells                 | 5.2 μM   |               |
|                  |               | HUVEC human umbilical vein cells                | 5.0 μM   |               |
| Echis pyramidum | Ser49 PLA₂    | A549 human adenocarcinoma cells                 | 2.9 μM   |               |
|                  |               | HUVEC human umbilical vein cells                | 2.5 μM   |               |
| Micrurus lemniscatus | PLA₂     | Rat myocytes                                    | n.d.   | [100]        |
|                  | (lemnitoxin)  |                                              |        |                |
| Naja atra        | PLA₂         | SK-N-SH human neuroblastoma cells               | n.d.   | [101]         |
| Naja naja        | acidic PLA₂  | Erlich ascitic tumour cells                     | n.d.   | [102]         |
|                  |               | partially differentiated L6 rat myoblasts       | n.d.   | [103]         |
|                  |               | platelets from citrated goat blood              | n.d.   |                |
|                  |               | rat pheochromocytoma PC-12 cells                | n.d.   |                |
| Naja nigricollis | Nigexine (basic PLA₂) | Epithelial FL cells | 1.6 mM | [104]         |
|                  |               | C-13 T neuroblastoma cells                      | 2.9 mM   |               |
|                  |               | HL60 human leukaemia cells                      | 3.1 mM   |               |
| Vipera ammodytes | neurotoxic  | Motoneuronal NSC34 cells                       | n.d.   | [7]           |
|                  | secretory PLA₂|                                              |        |                |
exerts genotoxic effects to induce cytotoxicity in human lymphocytes [58]. In addition, PLA$_2$ induces cytotoxicity through DNA damage and the formation of micronuclei [58]. The PLA$_2$ also significantly ameliorates the expression of proto-oncogene NOTCH1 and BRAF V600E genes in SK-MEL-28 cells [59]. As revealed by Annexin V-Propidium iodide double-staining flow cytometry, apoptosis remains as the predominant cell death mechanism in PLA$_2$-associated cytotoxicity [60]. It is noteworthy that, the venom PLA$_2$ exhibits time-dependent and dose-dependent cytotoxicity in cancer cells without any effects on normal cells [61]. Besides, the venom PLA$_2$ has been reported for its in vivo antitumour properties. The PLA$_2$ from Bothrops jararacussu, BthTX-1 could reduce the S180 tumour size by 79% in BALB/c mice [54]. In addition, Drs-PLA$_2$ from Daboia russellii siamensis has also been found to reduce tumour nodules by 65% in BALB/c mice [62]. So far, only crotoxin, a PLA$_2$ from Crotalus durissus terrificus venom undergoes phase I clinical trials which shows the objective partial response in cancer patients [63]. The cytotoxicity of PLA$_2$ is described in a schematic diagram (Figure 2).

**L-amino acid oxidase**

LAAO (EC. 1.4.3.2) is a homodimeric flavoenzyme with covalently linked-flavin adenine dinucleotides (FADs) contributes to a yellow appearance in snake venom. Each subunit in LAAO possesses a molecular mass of 50–70 kDa.

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**Figure 2. Summary of the cytotoxic effects of venom phospholipase A$_2$ in cancer cells**

An example of the three-dimensional structure of a K49 basic PLA$_2$ from Bothrops flavoviridis venom is shown [Protein Data Bank accession (PDB) ID: 6AL3]. The C-terminal of PLA$_2$ interacts directly with the cell membrane to produce membrane perturbating effects. Accumulation of reactive oxygen species (ROS) occurs due to catalytic actions of PLA$_2$ on membrane phospholipids which causes cell death. The venom PLA$_2$ reduce the expression of anti-apoptotic proteins, for example, Bcl2, Bcl-XP, c-FLIP and proto-oncogene such as NOTCH1 and BRAF V600E. On the contrary, venom PLA$_2$ increases the expression of pro-apoptotic proteins BAD and caspase-3. At the same time, venom PLA$_2$ triggers cell cycle arrest in cancer cells. Altogether, the findings imply that apoptosis is the predominant cell death mode in PLA$_2$-induced cytotoxicity.
The enzyme has a molecular mass of 110–159 kDa under a native state [26,64]. The LAAO consists of three major domains, which are a substrate-binding domain, a FAD-binding domain and a helical domain ([65], Figure 3a). The substrate-binding domain is characterised by seven strands of mixed β-pleated sheet forming a pocket for substrate binding.

The FAD-binding domain is composed of two conserved motifs, including the FAD-binding motif and the GG motif, with a consensus sequence of three glycine residues (Gly) residues [66]. The first Gly is highly conserved and contributes to the positioning of the second Gly. The second Gly allows a proximity of the main chain to the negatively charged pyrophosphate of the FAD. The second Gly residue of the GG motif plays an important role in interacting with the ribose of the FAD molecule Whereas, the third Gly promotes the close packing of α-helix and β-sheets of the motifs [67]. In brief, these interactions stabilise the tight binding of the FAD cofactor to the LAAO [68].

The helical domain forms a funnel-shaped entrance protruding into the protein core near the flavin cofactor, where the active site is located. This funnel-shaped helical domain facilitates the entry orientation of amino acid substrates through electrostatic interaction with the carboxylic groups (–COOH) of the substrates [65]. It appears that the key residues involved in the interaction with substrates are Arg90 and Gly 464 [65,69]. Besides, there are also two residues, His223 and Arg 322 which present at the active site to involve in the catalytic mechanisms of LAAO [69]. The LAAO exhibits high stereospecificity and enantioselectivity towards the oxidative deamination of L-amino acids due to the presence of a helical domain specifically in LAAO [70].

A catalytic reaction of LAAO comprises a reductive half reaction and the oxidation half reaction (Figure 3b). During the first half of the reduction reaction, FAD plays an important role as a cofactor. The reductive half reaction involves the abstraction of a proton from the amino group of the L-amino acid substrate by a basic His223.
Table 2. The cytotoxicity of different LAAO from different snake species on various cell types. The IC₅₀ indicates the concentration of venomous LAAO to kill 50% of the cell populations.

| Species Name       | LAAO Type  | Cell Type                                      | IC₅₀ (μg/ml) | References |
|--------------------|------------|-----------------------------------------------|--------------|------------|
| *Agkistrodon acutus* | ACTX-6     | A549 human lung cancer cells                  | 20           | [84]       |
|                    | ACTX-8     | HeLa cervical cancer cells                    |              | [75]       |
| *Agkistrodon contortrix laticinctus* | ACL LAO | HL60 human leukaemia cells                    | n.d.         | [36]       |
| *Agkistrodon halyx* | AhLAAO     | L1210 mouse lymphocytic leukaemia MOLT-4 human lymphoblastic leukaemia cells HL60 human leukaemia cells RPMI 1788 human peripheral blood A549 human lung cancer cells | n.d.         | [80]       |
| *Bothrops atrox*   | BatroxLAAO | HL60 human leukaemia cells                    | 50           | [78,83]    |
|                    |            | B16F10 mouse skin melanoma                    | 25           |            |
|                    |            | PC-12 rat adrenal medulla pheochromocytoma    |              |            |
|                    |            | Jurkat human acute T-cell leukaemia cells     |              |            |
|                    |            | Normal human keratinocytes                    | 5.1          | [33]       |
| *Bothrops insularis* | BILAO | Tubular                                        |              | [106]      |
| *Bothrops jararaca* | BjarLAAO-I | Ehrlich ascites tumour cells                  | n.d.         | [107]      |
| *Bothrops leucurus* | BI-LAAO    | MKN-45 gastric cancer cells                   | n.d.         | [34]       |
|                    |            | HuTu human duodenocarcinoma                   |              |            |
|                    |            | RKO human colorectal cells                    |              |            |
|                    |            | LL-24 human fibroblast cells                  |              |            |
| *Bothrops moojeni* | BmooLAAO-I | EAT cells                                     |              | [23]       |
|                    |            | HL60 human leukaemia cells                    |              |            |
| *Bothrops piraia*  | BpirLAAO-I | HL60 human leukaemia cells                    | n.d.         | [79]       |
|                    |            | BCR-ABL human leukaemia cells                 |              |            |
|                    |            | HL60 human leukaemia cells                    |              |            |
|                    |            | Jurkat human acute T-cell leukaemia cells     | n.d.         | [76]       |
|                    |            | SKBR-3 human breast cancer cells              |              |            |
|                    |            | S180 murine sarcoma                           |              |            |
|                    |            | Ehrlich ascites tumour cell                   |              |            |
| *Bungarus fasciatus* | BF-LAAO | A549 human lung cancer cells                  | n.d.         | [28]       |
| *Calloselasma rhadostoma* | CR-LAAO | Jurkat human acute T-cell leukaemia cells     | n.d.         | [85]       |
| *Crotalus atrox*   | Apoxin I   | HL60 human leukaemia cells                    | n.d.         | [81]       |
|                    |            | A2780 human ovarian cancer cells              |              |            |
|                    |            | 293T human embryonic kidney cells             |              |            |
|                    |            | KN-3 odontoblast cells                        |              |            |
| *Eristocophis macmahoni* | LNV-LAO | MM6 human monocytic cells                    |              | [64]       |
| *Lachesis muta*    | LmiLAAO    | AGS gastric adenocarcinoma                     | 22.7         | [108]      |
|                    |            | MCF-7 human breast cells                      | 1.41         |            |
|                    |            | VERO normal epithelial monkey kidney          | 0.83         | [35]       |
|                    |            | EA. hy926 human umbilical vein                |              |            |
|                    |            | HeLa cervical cancer cells                    |              |            |
|                    |            | MGSO-3 human breast cancer tissue             |              |            |
|                    |            | normal human keratinocytes                    |              |            |
| *Ophiophagus hannah* | OiLAAO | B16F10 murine melanoma                        | 0.17         | [109]      |
|                    |            | HT-1080 human fibrosarcoma                    | 0.6          |            |
|                    |            | CHO Chinese hamster ovary cells               | 0.3          |            |
|                    |            | murine epithelial cells Balb/3T3              | 0.45         |            |
|                    |            | PC3 human prostate cancer cells               | 0.05         | [87]       |
|                    |            | MCF-7 human breast cancer cells               | 0.04         | [110]      |
|                    |            | A549 human lung cancer cells                  | 0.05         | [110]      |
| *Trimeresurus flavoviridis* | OHAP-1 | rat C6 glioma cells RBR 17T human glioma U251 | n.d.         | [111]      |
| *Trimeresurus stejnegeri* | TSV-LAO | C8166 human T cell leukaemia                  | 24 nM        | [112]      |
| *Vipera berus berus* | VB-LAAO | HeLa cervical cancer cells                    | n.d.         | [22]       |
residue [65]. Concomitantly, an imino intermediate is formed when a hydride is transferred from α carbon of the substrate to the N5 of the FAD isalloxazine ring. The cofactor FADH2 is produced in this reaction. The imino acid is further hydrolysed non-enzymatically into α-keto acid and ammonia [71]. The second oxidative half reaction involves the oxidation of the FADH2 into FAD and at the same time, generating H2O2 [72]. This reaction completes the LAAO catalytic cycle as the FAD cofactor is regenerated for subsequent cycles [73].

**Mechanism of cytotoxicity**

Extensive studies have demonstrated that snake venom LAAOs induce cytotoxic effects, particularly on cancer cell lines (Table 2). However, the actual cytotoxic mechanism is poorly understood. Most of the hypotheses are based on the accumulated H2O2, generated during the LAAO catalytic activity, which leads to oxidative stress [22,74,75]. This theory is further supported by a few studies which have demonstrated a reduction in the cytotoxic effect of LAAO upon exposure to glutathione (GSH) or catalase, which inhibit the H2O2 activity [34,75,76].

The liberated H2O2 accumulates as ROS to cause direct deterioration of the cell membranes. The oxidative stress by H2O2 could also lead to the dissipation of MMP to induce translocation of cytochrome c to cytosol [77]. Cytochrome c then activates caspase-9, an initiator caspase presence in the intrinsic mitochondrial-mediated apoptosis. The p53 apoptotic proteins are found to be substantially expressed in the presence of LAAO, followed by translocation of the cytoplasmic Bax protein to mitochondria to activate the downstream apoptotic pathways [75]. Furthermore, LAAO has been reported to activate another initiator caspase-8 in the extrinsic death-receptor apoptosis before downstream activation of caspase-3 (the executioner phase of apoptosis) [78,79]. Extrinsic apoptosis involves ligand–death receptor interactions to form DISC–FADD, followed by cleavage of pro-caspase 8 to active caspase-8. However, it is uncertain if LAAO interacts with the death receptors for the occurrence of the extrinsic pathway. On the other hand, caspase-3 is responsible for the end-point apoptotic features such as chromatin condensation (karyorrhexis) and DNA fragmentation. The findings thus conclude that LAAO exerts apoptosis through extrinsic and intrinsic pathways.

Besides, LAAO from *Agkistrodon halys* venom displays cytotoxicity on murine lymphoblastic leukaemia cells (L1210) with prominent apoptotic features such as DNA fragmentation [80]. Similarly, apoxin 1, a type of LAAO from *Crotalus atrox* venom also induces DNA fragmentation in human umbilical endothelial cells, HL-60 (human leukaemia) A2780 (human ovarian carcinoma) and NK-3 (rat endothelial cells) due to elevated H2O2 levels [81]. The ACL-LAAO isolated from *Agkistrodon contortrix* venom has also been demonstrated to cause DNA fragmentation in HL60 cells [30]. On the other hand, LAAO from *Ophiophagus hannah* venom was found to alter several apoptotic, autophagic and cell cycle-related genes, as a result of accumulated H2O2 released from the enzyme action [82]. Furthermore, the LAAO also significantly up-regulates cytochrome P450 genes to further increase intracellular ROS levels [82]. Similar to PLA2, venom LAAO also induces cell cycle arrest in cancer cell lines. In a study on *Bothrops atrox* snake venom LAAO treated HL-60 cells, the BatroxLAAO exerts an arrest in the G0/G1 phase with a decrease in S and G2/M phases [83]. Another LAAO from *Agkistrodon acutus* venom, namely ACTX-6 also elicits cell cycle arrest in A549 cells [84]. Collectively, these findings suggest that venom LAAO activates both intrinsic and extrinsic apoptotic pathways (Figure 3).

In addition to apoptosis, the venom LAAO exhibits a dose-dependent transition of apoptosis to necrosis when its concentration increases [22,33,80,83]. This is presumably related to the levels of H2O2 produced by the enzyme, as the treatment with catalase significantly reduced the number of necrotic cells [85].

Although apoptosis remains as the predominant cell death mode in LAAO-induced cytotoxicity in cancer cells, the venom LAAO is able to cause autophagy in normal human keratinocyte [33]. Autophagy refers to a self-degenerative cell death process in which cellular components are degraded in autophagic vacuoles of dying cells [86]. The LAAO-induced cytotoxic effects are dose dependent and follow a sequential manner of cells undergoing autophagy, apoptosis to necrosis within 24 h [33,35]. On the other hand, preclinical trials of LAAO from *Ophiophagus hannah* revealed that LAAO suppresses PC-3 Solid Tumour Growth in a tumour xenograft mouse model [87]. The venom LAAO exhibits selectivity towards cancer cells and relatively non-toxic to normal cells [79,87–89].

**Conclusion**

The enzymatic toxins, PLA2 and LAAO from snake venoms, exhibit pronounced cytotoxic effects mainly on cancer cells. They suppress cancer cells proliferation, induce apoptosis and cell cycle arrest, although necrosis and autophagy cell death are also observed. The C-terminal region of PLA2 is suggested to contribute to its
cytotoxicity upon interaction with the cell membranes. On the other hand, LAAO is known to produce notable levels of H₂O₂ through its enzymatic reaction. Therefore, the enzymes are known to cause the accumulation of ROS which eventually leads to cell death. Besides cytotoxicity, PLA₂ and LAAO also possess anticoagulant activity which could be promising candidates in cancer research as venous thromboembolism is often observed in cancer. The exact modes of cell death elicited by the enzymes, especially the potential agonistic actions on the death receptors, are not well established. Therefore, elucidation of the possible enzymes–receptors interactions is required in future studies. While considering the potential anticancer effects of both enzymes, we must not forget to ascertain the selectivity of the enzymes towards cancer cells only. Since non-cancer cells are less susceptible to both enzymes, it is most likely that the cytotoxic actions of PLA₂ and LAAO are selective to cancer cells only. Nevertheless, before these enzymatic toxins can be developed into chemotherapeutic agents, their efficacy, potency, and safety need to be established while considering new approaches for targeted delivery, these include formulation into nanoparticles or conjugation with ligands or monoclonal antibodies which recognises targeted cancer cells.

**Perspectives**

- **Importance of the field:** Although both enzymatic toxins exhibit various pharmacological actions, we should not neglect their cytotoxic properties on cancer cells. Both PLA₂ and LAAO produce oxidative stress and trigger cell cycle arrest and apoptosis in cancer cells, thereby suggesting their potential applications as anticancer lead molecules.

- **Current status:** Despite well documented structural and catalytic properties of both enzymes, their cytotoxic actions remain superficial without in-depth analysis on the specific cell–death signalling pathways. It remains ambiguous if both PLA₂ and LAAO interact directly with the surface cell death receptors to induce cytotoxicity.

- **Future direction:** The potential target actions of PLA₂ and LAAO on cell surface death receptors remain poorly understood. Cancer cells possess abnormalities in cell surface death receptors, for instance, down-regulation of TRAIL receptor DR4, mutated DR5 as well as over-expression of TRAIL decoy and Fas decoy. Thus, investigation of enzymes–death receptor interaction will distinguish the selectivity of the enzymes targeting cancer cells. This is attainable via *in-silico* docking analysis and chemical cross-link mass spectrometry to detect enzyme–receptor interactomes which enables the annotation of signalling pathways targeted by enzymes PLA₂ and LAAO during cytotoxicity.

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**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.

**Author Contribution**

J.J.H. and M.K.K.Y. wrote the manuscript draft, M.K.K.Y. edited the manuscript. All authors approved the final article.

**Abbreviations**

FADs, flavin adenine dinucleotides; LAAO, L-amino acid oxidase; PLA₂, phospholipase A₂; ROS, reactive oxygen species.

**References**

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