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Review Article

Cytotoxicity of snake venom enzymatic toxins: phospholipase A_2 and L-amino acid oxidase

Jia Jin Hiu and Michelle Khai Khun Yap

School of Science, Monash University Malaysia, 47500 Bandar Sunway, Malaysia

Correspondence: Yap Michelle Khai Khun (yap.michelle@monash.edu)

The phospholipase A_2 (PLA_2) 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 PLA_2 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 PLA_2 and LAAO contribute to an elevated oxidative stress due to their catalytic actions, for instance, the ability of PLA_2 to produce reactive oxygen species during lipolysis and formation of H_2O_2 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 PLA_2 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 A_2 (PLA_2) and L-amino acid oxidase (LAAO) are ubiquitously found in *Elapidae* and *Viperidae* whereby PLA_2 exists as the most abundant enzymatic toxins, as revealed by venom proteome (Figure 1).

PLA_2 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 PLA_2. Generally, the venom PLA_2 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 Ca^{2+} for their catalytic actions [6]. The venom PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 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, PLA_2 from *Elapidae* venom exists as a monomeric enzyme

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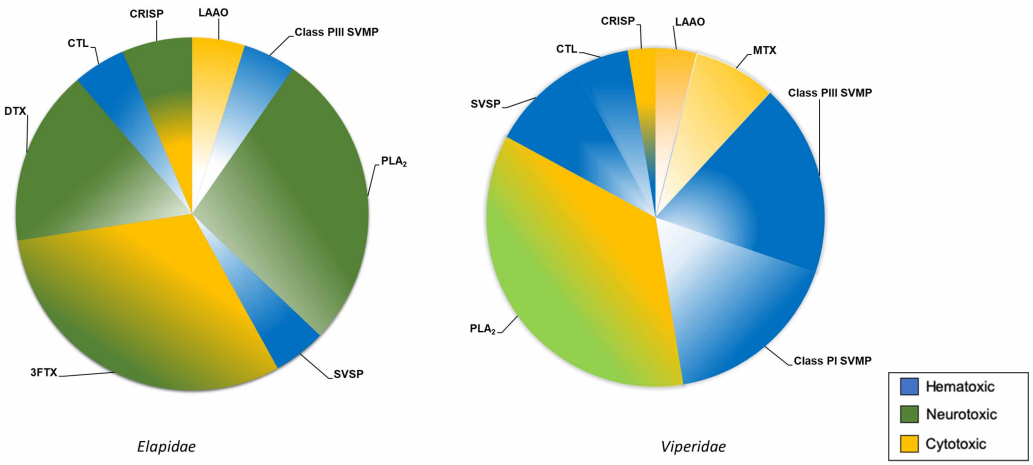

Figure 1. Distribution of different venom toxins from *Elapidae* and *Viperidae*

The venom toxins are coloured according to their respective pharmacological activities, whereby colour intensity indicates the dose-dependent pharmacological actions. On the other hand, different colour distributions within the same toxins correspond to the multiple biological effects exerted by the toxins [90,91]. Of all venom enzymatic toxins, the enzymes LAAO and PLA_2 exhibit cytotoxicity (represented by a yellow colour). Abbreviations: LAAO, L-amino acid oxidase; SVMP, snake venom metalloproteinase; PLA_2, phospholipase A_2; SVSP, snake venoms serine protease; 3FTX, three-finger toxin; DTX, dendrotoxin; CTL, C-type lectin; CRISP, cysteine-rich secretory protein; MTX, myotoxin.

and possesses neurotoxicity while *Viperidae* venom PLA_2 can exist in both monomer and dimer forms. The *Viperidae* monomeric PLA_2 exhibits cytotoxic effects, whereas dimeric PLA_2 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 (H_2O_2). 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 H_2O_2, ammonia, and α-keto acid [24]. The liberated H_2O_2 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 PLA_2 alters plasma membrane integrity in muscle cells to cause myonecrosis [31]. The membrane perturbation by PLA_2 is a secondary process to its catalytic actions on membrane phospholipids [32], indicating that venom PLA_2 exhibits remarkable cytotoxicity. On the other hand, venom LAAO has also been demonstrated to induce cell death due to the generated H_2O_2 [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 phosphatidyl-serine [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 PLA_2 and LAAO. In addition, we also discuss and summarise the cytotoxic effects exerted by PLA_2 and LAAO against different cancer cells with a specific focus on the underlying mechanisms.

Phospholipase A_2

PLA_2 (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

glycerophospholipids 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 PLA_2. The structure of PLA_2 has three major α-helices and two antiparallel β-sheets cross-linked by disulfide bonds [38]. The disulfide-linked α-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 PLA_2 can be classified into two major groups, namely Group I PLA_2 ($GIPLA_2$) and Group II PLA_2 ($GIIPLA_2$) according to the location of disulfide bonds [6,39].

Group I PLA_2 ($GIPLA_2$)

The venom $GIPLA_2$ consists of 115–125 residues with a molecular mass of 13–15 kDa [40]. The $GIPLA_2$ has a single polypeptide chain containing 6–8 disulfide bridges [6]. It contains ∼50% of α-helices and 10% of β-sheets [40]. The venom $GIPLA_2$ has an elapid loop (residues 57–59) that links the α-helices and the β-sheets [41], thus, $GIPLA_2$ is found ubiquitously in elapids venoms. The venom $GIPLA_2$ is different from mammalian pancreatic PLA_2, which the latter enzyme has a pancreatic loop with an additional five amino acid residues at position 62–67 [42]. The $GIPLA_2$ is further divided into Group IA and Group IB for snake venom PLA_2 and mammalian pancreatic PLA_2, respectively. Despite so, Group IB PLA_2 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 α-helix that is identical with mammalian pancreatic PLA_2 [43].

Group II PLA_2s ($GIIPLA_2$)

The venom $GIIPLA_2$ is found exclusively in *Viperidae* venoms. It contains 120–125 amino acid residues and seven disulfide bonds [6]. Unlike $GIPLA_2$, neither the pancreatic nor elapid loops are present in $GIIPLA_2$ enzymes. However, it possesses a C-terminal extension with a different organisation of disulfide bonds, which clearly distinguishes $GIIPLA_2$ from $GIPLA_2$ [44]. In $GIIPLA_2$, the D49 is conserved and contributes to Ca^{2+}-dependent catalytic activity [45]. Thus, $GIIPLA_2$ is also recognised as D49 acidic PLA_2 [46].

Mechanism of cytotoxicity

PLA_2 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 PLA_2 alters the membrane fluidity and causes membrane permeabilisation, which ultimately leads to cell death. The cytotoxic effects of PLA_2 on a different cell are summarised in Table 1.

In general, venom PLA_2 variants can be classified into D49 acidic PLA_2 (Asp-49), K49 basic PLA_2 (presence of Lys-49 instead of Asp-49) and S49 PLA_2 (presence of Ser-49). The basic PLA_2 homologues, K49 and S49 PLA_2s are responsible for many Ca^{2+} independent biological activities and thus they are catalytically inactive [45]. The D49 acidic PLA_2 is less cytotoxic than K49 basic PLA_2, whereby acidic PLA_2 possesses higher IC_{50} than basic PLA_2 (Table 1). On the other hand, S49 PLA_2 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 PLA_2 (IC_{50} = 2.5–12.2 μM). Despite so, S49 PLA_2 demonstrates weaker lipolytic activity compared with K49 PLA_2 [50]. The basic PLA_2 homologues display more pronounced cytotoxic effects in cancer cells.

The C-terminal region of the PLA_2 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 PLA_2 is probably mediated by the interaction between the C-terminal region and the plasma membrane [53–55]. Besides, the PLA_2-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, PLA_2 alters the distribution of different phases in the cell cycle to cause apoptosis [57]. PLA_2 also

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Table 1. The cytotoxicity of different PLA_2 from different snake species on various cell types. The IC_{50} indicates the concentration of venom PLA_2 to kill 50% of the cell populations

Species	Types of PLA_2	Cell types	IC_{50}	References
Bothrops asper	basic PLA_2	Mouse adrenal tumour cells	n.d.	[92]
Bothrops brazili	acidic PLA_2	Jurkat human acute T-cell leukaemia cells	100.0 μg/ml	[53]
Bothrops jararaca	acidic PLA_2	peripheral blood mononuclear cells (PBMC) HL60 human leukaemia cells	n.d. n.d.	[93]
Bothrops jararacussu	Bth TX-1	Jurkat human acute T-cell leukaemia cells Erlich ascitic tumour cells SK-BR-3 human breast cancer cells MCF-7 human breast cancer cells MDAMB231 human breast cancer cells PC-12 rat adrenal medulla pheochromocytoma C2C212 murine muscle cells B16F10 mouse melanoma cells S180 murine sarcoma cells	n.d. n.d. 81.2 μg/ml 104.35 μg/ml >409 μg/ml n.d. n.d. n.d. n.d.	[54,57,94,95]
Bothrops moojeni	acidic PLA_2	Jurkat human acute T-cell leukaemia cells K562-S human immortalised myelogenous leukaemia cells K562-R human immortalised myelogenous leukaemia cells	n.d. 257 μg/ml 191 μg/ml	[96] [55]
Crotalus durissus terrificus	Heterodimeric basic PLA_2	Murine erythroleukemia cells SK-LU-1 human lung cancer cells Hs578T human breast cancer cells KYSE 30 oesophageal cancer cells GAMG human glioblastoma cells HCB151 glioma cells PSN-1 human pancreatic cancer cells PANC-1 pancreatic cancer cells HeLa cervical cancer cells KYSE 270 oesophageal cancer cells U373 glioma cells SiHa cervical cells	3.0–5.0 μg/ml ~4.0 μg/ml ~5.3 μg/ml 1.0 μg/ml <0.5 μg/ml 4.1 μg/ml 0.7 μg/ml <0.5 μg/ml 2.4 μg/ml 8.7 μg/ml 30.2 μg/ml >30.0 μg/ml	[97] [98] [99]
Daboia siamensis	dssPLA_2	SK-MEL-28 human skin melanoma cells	n.d.	[60]
Daboia russelii siamensis	drsPLA_2	SK-MEL-28 human skin melanoma cells	0.90 μg/ml	[62]
Echis carinatus sochureki	Ser49 PLA_2	A549 human adenocarcinoma cells HUVEC human umbilical vein cells	8.5 μM 12.2 μM	[49]
Echis coloratus	Ser49 PLA_2	A549 human adenocarcinoma cells HUVEC human umbilical vein cells	3.5 μM 4.9 μM	
Echis ocellatus	Ser49 PLA_2	A549 human adenocarcinoma cells HUVEC human umbilical vein cells	5.2 μM 5.0 μM	
Echis pyramidum leakeyi	Ser49 PLA_2	A549 human adenocarcinoma cells HUVEC human umbilical vein cells	2.9 μM 2.5 μM	
Micrurus lemniscatus	Myotoxic group I PLA_2 (lemnitoxin)	Rat myocytes	n.d.	[100]
Naja atra	PLA_2	SK-N-SH human neuroblastoma cells	n.d.	[101]
Naja naja	acidic PLA_2	Erlich ascitic tumour cells partially differentiated L6 rat myoblasts platelets from citrated goat blood rat pheochromocytoma PC-12 cells	n.d. n.d. n.d. n.d.	[102] [103]
Naja nigricollis	Nigexine (basic PLA_2)	Epithelial FL cells C-13 T neuroblastoma cells HL60 human leukaemia cells	1.6 mM 2.9 mM 3.1 mM	[104]
Vipera ammodytes ammodytes	neurotoxic secretory PLA_2	Motoneuronal NSC34 cells	n.d.	[7]

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 S180tumour size by 79% in BALB/c mice [54]. In addition, Drs-PLA_2 from *Daboia russelii 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.

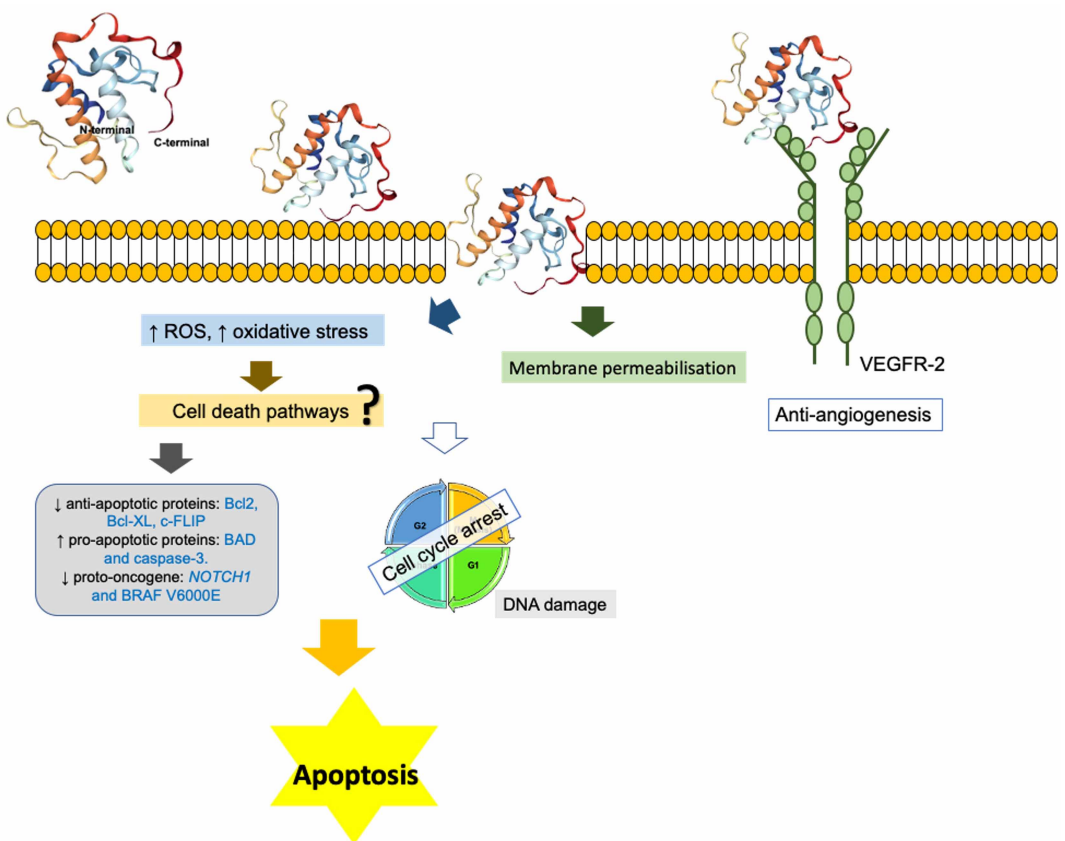

Figure 2. Summary of the cytotoxic effects of venom phospholipase A_2 in cancer cells

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.

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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

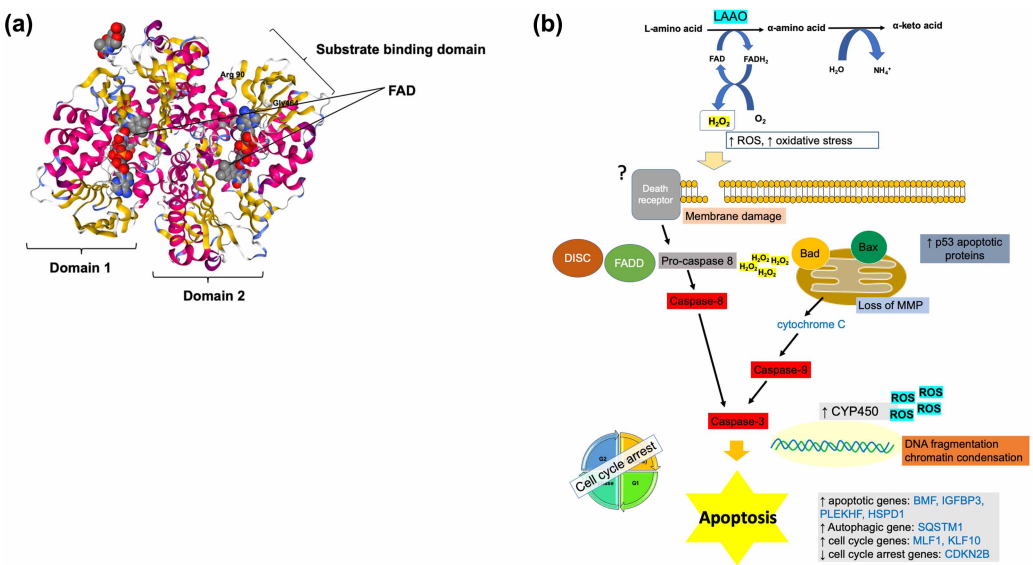

Figure 3. The structural and cytotoxic properties of venom L-amino acid oxidase (LAAO)

A ribbon representation of LAAO (PDD ID: 5Z2G, *Naja atra* venom) is illustrated in (**a**). The LAAO is a homodimeric flavoenzyme containing a substrate-binding domain (yellow colour of mixed β-pleated sheet), a flavin adenine nucleotide (FAD) binding site and a helical domain (red colour). The enzyme catalyses the oxidative deamination of L-amino acid and produces H_2O_2 as the main mediator for its cytotoxicity, as illustrated in (**b**). LAAO exerts apoptosis in cancer cells through extrinsic and intrinsic pathways. It is noteworthy that there is an up-regulation of CYP450 gene families to further enhance the oxidative by producing excessive ROS. On the contrary, the cell cycle arrest gene CDKN2B is down-regulated after exposure to LAAO. The CDKN2B is the main cell cycle regulator that inhibits G1 progression. It explains the role of LAAO in cell cycle arrest at the Go–G1 phase. Abbreviations: DISC-FADD, death-inducing signalling complex and Fas-associated death domain; MMP, mitochondrial membrane potential; BMP, Bcl2 modifying factor; IGFBP3, Insulin-like growth binding protein 3; PLEKHF1, Pleckstrin homology domain containing family F member 1; HSPD1, heat shock 60 kDa protein 1; SQSTM1, Sequestosome 1; MLF1, myeloid leukaemia factor 1; KLF10, Kruppel-like factor 10; CDKN2B, Cyclin-dependent kinase inhibitor 2B.

Table 2. The cytotoxicity of different LAAO from different snake species on various cell types. The IC_{50} indicates the concentration of venomous LAAO to kill 50% of the cell populations

Species	Name of LAAO	Cell type	IC_{50}	References
Agkistrodon acutus	ACTX-6	A549 human lung cancer cells	20 μg/ml	[84]
	ACTX-8	HeLa cervical cancer cells		[75]
Agkistrodon contortrix laticinctus	ACL LAO	HL60 human leukaemia cells	n.d.	[30]
Agkistrodon halys	AhLAAO	L1210 mouse lymphocytic leukaemia	n.d.	[80]
		MOLT-4 human lymphoblastic leukaemia cells		
		HL60 human leukaemia cells		
		RPMI 1788 human peripheral blood		
		A549 human lung cancer cells	n.d.	[105]
Bothrops atrox	BatroxLAAO	HL60 human leukaemia cells	50 μg/ml	[78,83]
		B16F10 mouse skin melanoma	25 μg/ml	
		PC-12 rat adrenal medulla pheochromocytoma		
		Jurkat human acute T-cell leukaemia cells		
		Normal human keratinocytes	5.1 μg/ml	[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 pirajai	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	LmlAAO	AGS gastric adenocarcinoma	22.7 μg/ml	[108]
		MCF-7 human breast cells	1.41 μg/ml	
		VERO normal epithelial monkey kidney	0.83 μg/ml	[35]
		EA. hy926 human umbilical vein		
		HeLa cervical cancer cells		
		MGSO-3 human breast cancer tissue		
		normal human keratinocyte		
Ophiophagus hannah	OH-LAAO	B16F10 murine melanoma	0.17 μg/ml	[109]
		HT-1080 human fibrosarcoma	0.6 μg/ml	
		CHO Chinese hamster ovary cells	0.3 μg/ml	
		murine epithelial cells Balb/3T3	0.45 μg/ml	
		PC3 human prostate cancer cells	0.05 μg/ml	[87]
		MCF-7 human breast cancer cells	0.04 μg/ml	[110]
		A549 human lung cancer cells	0.05 μg/ml	[110]
Trimeresurus flavoviridis	OHAP-1	rat C6 glioma cells RBR 17T	n.d.	[111]
		human glioma U251		
Trimeresurus stejnegeri	TSV-LAO	C8166 human T cell leukaemia	24 nM	[112]
Vipera berus berus	VB-LAAO	HeLa cervical cancer cells	n.d.	[22]
		K562 human leukaemia cells		

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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 isoalloxazine ring. The cofactor $FADH_2$ 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 $FADH_2$ into FAD and at the same time, generating H_2O_2 [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 H_2O_2 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 H_2O_2 activity [34,75,76].

The liberated H_2O_2 accumulates as ROS to cause direct deterioration of the cell membranes. The oxidative stress by H_2O_2 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 requires ligands–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 *Crotalux 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 H_2O_2 levels [81] The ACL-LAAO isolated from *Agkistrodon cntortrix* 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 H_2O_2 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 PLA_2, 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 Go/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 H_2O_2 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, PLA_2 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 PLA_2 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 H_2O_2 through its enzymatic reaction. Therefore, the enzymes are known to cause the accumulation of ROS which eventually leads to cell death. Besides cytotoxicity, PLA_2 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 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 PLA_2 and LAAO are selective to cancer cells only. Nevertheless, before these enzymatic toxins can be developed into chemotherapeutic agent, 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 PLA_2 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 PLA_2 and LAAO interact directly with the surface cell death receptors to induce cytotoxicity.

- Future direction: The potential target actions of PLA_2 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 overexpression 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 PLA_2 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; PLA_2, phospholipase A_2; ROS, reactive oxygen species.

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