Chapter II

REVIEW OF LITERATURE

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2.1 A brief review on anticoagulant proteins from cobra venom

Snake venom proteins/toxins exhibit a wide variety of pharmacological effects, including modulation of the hemostatic system. To date, several snake venom proteins affecting hemostasis have been purified and characterized [1-13]. Despite the fact that cobra venoms are primarily neurotoxic, transient coagulopathies leading to increase in whole blood clotting time have been observed in envenomed patients [14,15]. A few anticoagulant proteins have been purified from the venom of different cobras (Table 2.1), which exhibit anticoagulant activity via different modes of action.

Most of the cobra venom anticoagulant toxins belong to enzymatic snake venom protein families of phospholipase A_2 as well as metallo-proteinase. However, to date, only one non-enzymatic anticoagulant three-finger toxin has been purified and characterized from cobra (*Naja nigricollis crawshawii*) venom (Table 2.1). The anticoagulant activity of the cobra venom enzymes may or may not be directly correlated with their enzymatic activity (Table 2.1). Further, some of these anticoagulant proteins have shown significant promise for the development into potent drug prototypes for treatment of thrombosis associated cardiovascular disorders.

2.1.1 Anticoagulant phospholipase A_2 enzymes

Most of the anticoagulant proteins isolated from cobra venom belong to the PLA_2 superfamily of snake venom proteins (Table 2.1). It has been postulated that after binding of snake venom PLA_2s to the target site, they can induce their pharmacological effects through mechanisms that are either dependent on or independent of their enzymatic activity [10,13,16-18]. Similar observations have been reported for anticoagulant PLA_2s where they affect blood clotting either through the enzymatic hydrolysis of pro-coagulant phospholipids of plasma, or by non-enzymatic interaction with plasma phospholipids or blood coagulation factors (Fig 2.1) [10]. Based on their mode of action, the cobra venom PLA_2s are described as follows:

2.1.1.1 Enzymatic mechanism of anticoagulant action of cobra venom PLA_2 enzymes

Plasma phospholipids are an integral part of the 'tenase' complexes formed in the process of activation blood coagulation factors, and therefore, are crucial for a normal coagulation process [19]. Obliteration of these phospholipids surface by snake venom PLA_2s could be the primary mechanism to demonstrate their anticoagulant activity effect [2-4,10-12,20,21]. However, the role of enzymatic activity of venom PLA_2s in their anticoagulant activity is controversial with minimal facts justifying the hypothesis. It is well known that His48 forms the active site in every venom PLA_2 enzymes, and several experiments have been designed and performed to show the association of the catalytic activity of venom PLA_2s with their anticoagulant property [10,21,22]. Alkylation of His48 residue of some venom PLA_2s have led to complete loss of binding to phospholipids as well as enzymatic activity with concomitant loss of their anticoagulant potencies [23]. Further, replacement of hydrolysable phospholipids with non-hydrolysable phospholipids or removal of Ca^{2+} from the reaction site has shown simultaneous forfeiture of enzymatic as well as anticoagulant activities of snake venom PLA_2s [10,21,23]. All these findings suggest that the catalytic activity of the enzyme is obligatory for its anticoagulant property.

The anticoagulant PLA_2s described in Table 2.1 demonstrates that plasma phospholipids hydrolysis is a vital requirement for all cobra venom anticoagulant PLA_2s in order to prolong the clotting time of blood plasma. Furthermore, the penetrability of PLA_2 enzymes into phospholipid monolayers also determines the strength of the anticoagulant effect of PLA_2 enzymes [10,21]. It has been established that strongly anticoagulant PLA_2s demonstrate high penetrating ability, whereas non-anticoagulant PLA_2s show weak penetrability [10,21]. Based on these observations, it was hypothesized that strongly anticoagulant PLA_2 enzymes bind to phospholipid vesicles with enhanced intrinsic fluorescence, whereas poor anticoagulants show little or no effect [10,21].

2.1.1.2 Non-enzymatic mechanism of anticoagulant action of cobra venom PLA_2 enzymes

Contrary to association of enzymatic activity of snake venom PLA_2s with their anticoagulant property, some studies have reported that weak anticoagulant PLA_2s exhibit very strong phospholipid hydrolytic activity [21] or vice-versa [24]. Further, blocking the binding of venom PLA_2s to plasma phospholipids by pre-incubation of the former with specific antibodies (against venom PLA_2s) could reverse the anticoagulant activity of the venom PLA_2, but not its hydrolytic activity, thereby re-instating the dissociation of enzymatic activity from anticoagulant activity [25]. Similar observation are reported where modification of Lys residues cause complete loss of anticoagulant activity but very little loss of enzymatic activity [26]. Otherwise, modification of the carboxylate groups was associated with loss of hydrolytic property but not the anticoagulant effects of venom PLA_2 [27].

The presence of 'pharmacological sites' on PLA_2 enzymes thus have an essential role in targeting themselves to specific proteins of the coagulation cascade in order to disrupt the coagulation process [10]. They also compete with various coagulation factors for binding to plasma phospholipids thus disrupting the tenase complex formation [3,4,10,24]. Based on their specific target of blood coagulation factors, cobra venom PLA_2s can be broadly divided into two sub-groups:

2.1.1.2.1 Cobra venom PLA_2s showing thrombin inhibition

Amongst Elapidae, the first thrombin inhibiting PLA_2 enzyme was purified from the venom of *N. haje* by Osipov and co-workers [24]. Although the PLA_2, TI-Nh, demonstrated weak enzymatic activity, the anticoagulant activity of the enzyme was pronounced by virtue of its non-enzymatic thrombin inhibition property [24]. Thus, TI-Nh provides a crucial example of dissociation of enzymatic activity from anticoagulant activity of venom PLA_2 enzymes.

In another study, Nk-PLA_2β, an acidic anticoagulant PLA_2 was reported from the venom of *N. kaouthia* [4]. Nk-PLA_2β exhibited its anticoagulant property by a combination of its enzymatic and non-enzymatic properties [4]. It demonstrated uncompetitive inhibition of thrombin, its pharmacological target, even in the absence of phospholipids / Ca^{2+} leading to loss of serine protease activity of thrombin [4].

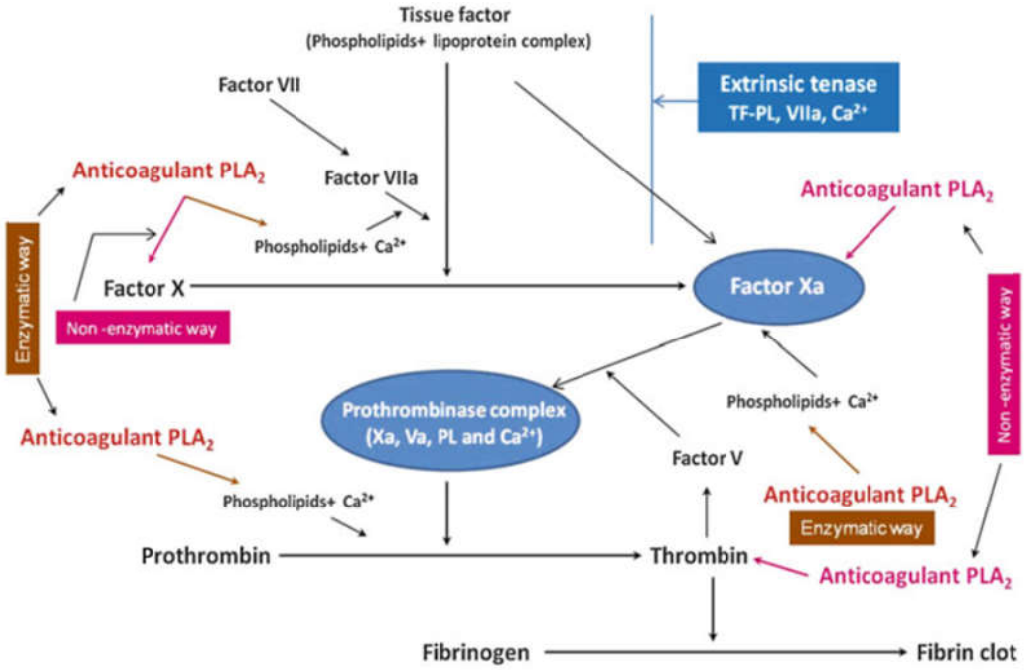

Fig 2.1. Effect of snake venom anticoagulant PLA_2 enzymes in different stages of the extrinsic pathway of blood coagulation [10]. Enzymatic way: Enzymatic way of anticoagulant mechanism of snake venom PLA_2s; Non-enzymatic way: Non-enzymatic way of anticoagulant mechanism of snake venom PLA_2s; Anticoagulant PLA_2: Anticoagulant PLA_2s of snake venom. [Source of image: Saikia and Mukherjee, In: P. Gopalakrishnakone et al. (eds.), Snake Venoms, Toxinology, 2017].

2.1.1.2.2 Cobra venom PLA_2 factor Xa inhibitors

CM-IV, a basic PLA_2 enzyme isolated from *N. nigricollis* demonstrated significant anticoagulant activity by a combination of both enzymatic and non-enzymatic modes of action [28,29]. CM-IV binds to FXa of the prothrombinase complex to disrupt the formation of thrombin from prothrombin [28]. CM-IV competes with factor Va to bind to FXa or replaces bound FVa from FXa-FVa complex thus interfering with the normal extrinsic and common pathways of blood coagulation [28]. CM-IV serves as an essential example of anticoagulant PLA_2 that exerts its anticoagulant effect by protein–protein rather than protein–phospholipid(s) interactions [28].

Mukherjee and co-workers in 2014 [4] isolated another acidic non-toxic PLA_2 isoform named Nk-PLA_2α from the venom of Indian *N. kaouthia*. Like CM-IV, this

PLA_2, Nk-$PLA_2\alpha$, demonstrated anticoagulant activity by a combination of enzymatic activity and non-enzymatic inhibition of FXa [4]. Like Nk-$PLA_2\beta$, Nk-$PLA_2\alpha$ also demonstrated un-competitive mode of inhibition towards its pharmacological target – FXa, without the requirement of phospholipids / Ca^{2+}, thus, impeding the prothrombin activation process of FXa [4].

2.1.2 Anticoagulant metallo-proteinases of cobra venom

Fibrin(ogen)olytic activity is an inherent property of several snake venoms, and is exhibited by the enzymatic actions of serine-proteinase and metallo-proteinsase families of snake venom proteins [1,2,8]. These enzymes degrade either Aα- or Bβ-chains of fibrinogen (and fibrin). Most of the serine-proteinases exhibit either or both fibrinogenolytic and fibrinolytic property, and plasminogen activating property to form plasmin, which in turn degrades fibrin [8]. The metallo-proteinase group of enzymes requires the presence of divalent metal ions to carry out their enzymatic activity [8].

As evident from table 2.1, to date, only three metallo-proteinases have been isolated from cobra venoms. Lahirin, is a very low molecular mass (MW = 6.5 kDa) basic fibrin(ogen)olytic proteinase isolated from the venom of *N. kaouthia* [41]. Reportedly, lahirin is the first low-MW fibrin(ogen)olytic toxin to be characterized from Indian monocled cobra *N. kaouthia* [41]. Incubation of Lahirin with fibrinogen demonstrated preferential digestion of Aα chain, followed by Bβ and γ chains of fibrinogen [41].

In another study by Chanda and co-workers [42], a high molecular mass (66 kDa) basic metallo-proteinase (NKV 66) was characterized from the venom of *N. kaouthia*. The toxin NKV 66 demonstrated α-fibrinogenase activity by degrading the Aα-chain of fibrinogen [42]. Further, NKV 66 demonstrated thrombolytic potency by dissolution of fibrin clots, prolonged re-calcification and thrombin time of blood plasma, and inhibited ADP and collagen induced platelet aggregation in a dose-dependent manner [42]. Interestingly NKV 66 exhibited disintegrin-like activity on A549 cells (adenocarcinomic human alveolar basal epithelial cells) by inhibiting cell adherence to about 40% [42].

Table 2.1 List of anticoagulant proteins isolated from cobra venom.

Name	Source	Charge and Type	Pathophysiology	Mechanism of action	Reference
AC1 AC2	*Naja naja siamensis*	Acidic phospholipase A_2	Prolongs Ca^{2+} clotting and thrombin time of plasma	Enzymatic hydrolysis of plasma phospholipids	[30]
S-VI-3 S-X-3 S-XII-2 S-XIII	*Naja nigricollis crawshawii*	Isoforms of a cardiotoxin	Prolongs re-calcification and prothrombin times of plasma	Unknown	[31,32]
CM-I	*Naja nigricollis*	Basic phospholipase A_2	Weakly anticoagulant; prolongs prothrombin time of plasma	Enzymatic hydrolysis of phospholipids	[29,33]
CM-II	*Naja nigricollis*	Basic phospholipase A_2	Weakly anticoagulant; prolongs prothrombin time of plasma	Enzymatic hydrolysis of phospholipids	[29,33]
CM-IV	*Naja nigricollis*	Basic phospholipase A_2	Strongly anticoagulant; prolongs prothrombin time of plasma; inhibits platelet aggregation	Enzymatic hydrolysis of phospholipids; non-enzymatic and non-competitive inhibition of factor Xa of prothrombinase complex	[28,29,33-35]
Nigexine	*Naja nigricollis*	Basic phospholipase A_2	Strongly anticoagulant; inhibits re-calcification time of platelet suspension	Enzymatic hydrolysis of phospholipids	[36]
Sputatrix PLA_2 AI Sputatrix PLA_2 AII	*Naja naja sputatrix*	Acidic phospholipase A_2	Weakly anticoagulant; prolongs clotting time of blood	Synergism between PLA_2 enzymes and polypeptide anticoagulants	[37]
$sPLA_2$	*Naja naja saggitifera*	Phospholipase A_2, charge not defined	Prolongs clotting time of platelet poor plasma	Undefined	[38]
TI-Nh	*Naja haje*	Phospholipase A_2	Prolongs re-calcification and thrombin time of platelet poor plasma; inhibits thrombin induced platelet aggregation	Enzymatic hydrolysis of phospholipids; and non-enzymatic, mixed mode of thrombin inhibition	[24]

NK-PLA_2-I NK-PLA_2-II	*Naja kaouthia*	Phospholipase A_2	Prolongs re-calcification time of platelet poor plasma; hemolysis	Enzymatic hydrolysis of phospholipids	[20,39]
NK-PLA_2-A	*Naja kaouthia*	Phospholipase A_2	Prolongs re-calcification time of platelet poor plasma	Enzymatic hydrolysis of phospholipids	[40]
NK-PLA_2-B	*Naja kaouthia*	Phospholipase A_2	Prolongs re-calcification time of platelet poor plasma	Enzymatic hydrolysis of phospholipids	[40]
Lahirin	*Naja kaouthia*	Low molecular weight basic metallo-proteinase	Demonstrates fibrinogenolysis	Non-enzymatic degradation of Aα chain of fibrinogen, followed by Bβ- and γ-chains	[41]
Nk-PLA_2α	*Naja kaouthia*	Acidic phospholipase A_2	Prolongs re-calcification, thrombin and prothrombin times of platelet poor plasma	Enzymatic hydrolysis of phospholipids; and non-enzymatic, uncompetitive mode of factor Xa inhibition	[4]
Nk-PLA_2β	*Naja kaouthia*	Acidic phospholipase A_2	Prolongs re-calcification, thrombin and prothrombin times of platelet poor plasma; inhibits thrombin-induced platelet aggregation	Enzymatic hydrolysis of phospholipids; and non-enzymatic, uncompetitive mode of thrombin inhibition	[4]
NKV 66	*Naja kaouthia*	Metallo-proteinase	Demonstrates fibrinogenolysis; thrombolysis of fibrin clots; inhibits ADP and collagen induced platelet aggregation	Enzymatic degradation of α-band of fibrinogen	[42]
NN-PF3	*Naja naja*	Acidic metallo-protease	Demonstrates fibrinogenolysis, and antiplatelet activity	Enzymatic degradation of α-band of fibrinogen and possible cleavage of α2β1 integrin on platelet membrane	[43-45]

NN-PF3 is another single chain high molecular weight (68 kDa) fibrin(ogen)olytic metallo-proteinase isolated from cobra venom, and is also the only metallo-proteinase to be purified and characterized from *N. naja* venom [43-45]. Like NKV 66, NN-PF3 also exhibits α-fibrinogenase activity [43,45] and preferentially degrades α-polymer over Aα-chain of fibrin [45]. Further, NN-PF3 prolonged the clotting time of plasma by virtue of its de-fibrinogenating effect [45]. Unlike other snake venom metallo-proteinases, NN-PF3 did not degrade extracellular matrix proteins [45]; however, it demonstrated considerable inhibition of collagen-induced platelet aggregation by interfering with the binding of collagen (agonist) with the α2β1 integrin (receptor for collagen), possibly by degradation of the latter [44]. Further, it also partially inhibited ADP and epinephrine-induced platelet aggregation by some unknown mechanism [44].

2.1.3 Other anticoagulant and antiplatelet proteins of cobra venom

Apart from the above classes of enzymatic snake venom proteins, there are certain non-enzymatic toxins in cobra venom that exhibit significant anticoagulant and antiplatelet activities. Four isoforms of a cardiotoxin was isolated from *N. nigricollis crawshawii* that demonstrated significant anticoagulant activity by prolongation of the re-calcification and prothrombin time of plasma [32]. Further, a three finger toxin, KT-6.9 (MW = 6.9 kDa) was purified from *N. kaouthia* venom, was responsible for inhibition of ADP, thrombin and arachidonic acid induced platelet aggregation, and its effect was 25 times more pronounced than that of antiplatelet drug clopidogrel [46].

In 1991, Kini and Evans [47] reported the presence of an α-fibrinogenase proteinase F1 from *N. nigricollis* venom. The toxin significantly inhibited platelet aggregation in whole blood by a mechanism which was independent of its action on fibrinogen. Another enzymatic antiplatelet toxin was purified from the venom of *N. naja oxiana*, which was an L-amino-acid oxidase enzyme, and it demonstrated inhibition of ADP and collagen-induced platelet aggregation under controlled conditions [48].

Snake venom PLA_2s are also known to influence platelet aggregation to induce anticoagulant activity. Based on their effects on the platelet function, venom PLA_2s are divided into three major classes [49-51]: group A PLA_2s initiate platelet aggregation,

group B PLA_2s inhibit platelet aggregation, whereas group C PLA_2s exhibit biphasic effects (initiate aggregation at low concentration or with short incubation time but inhibit aggregation at high concentration or with long incubation time). Three isoforms of acidic and cytotoxic PLA_2s purified from *N. n. naja* venom (Table 2.2; described in section 2.3) demonstrated inhibition of epinephrine, collagen, and ADP-induced platelet aggregation by virtue of their enzymatic property, and hence they were classified as group B class of platelet modulating PLA_2s [49].

2.2 A brief appraisal on peptide therapeutics developed from snake venom for treatment of cardiovascular diseases

Undoubtedly, snake venom is a large reservoir of pharmacologically active proteins and peptides. The potency, specificity, and stability of snake venom proteins have made them a valuable source of natural products for drug discovery. It has been estimated that globally, approximately 1 billion people suffer from hypertension, and many of them are being treated with angiotensin converting enzyme (ACE) inhibitors, which has been originally derived from snake venom [52]. The first example of a successful venom-based drug is captopril (Capoten®), which inhibits ACE, an essential enzyme for the production of angiotensin, which is in turn is a vasoconstrictor associated with hypertension [53,54]. It serves as a crucial example to demonstrate how deadly venoms have turned into lifesaving therapeutics. Captopril is an orally available peptidomimetic of 'bradykinin-potentiating peptides' (BPP), first isolated from *Bothrops jararaca* venom as 'bradykinin-potentiating factors' [55-57]. Thereafter, structure –function relationship studies and optimization of the minimal pharmacophore Phe-Ala-Pro led to the development 'Captopril' [53,54,58,59].

Another class of snake venom proteins with anti-hypotensive effects includes natriuretic peptides (NP), which are of four types – atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and urodilatin [60-63]. These NPs are vital regulators of the cardiovascular and renal systems, making them potential therapeutic candidates for treatment of conditions such as hypertension and heart failure. The first snake venom NP was identified from *Dendroaspis angusticeps* and was named the *Dendroaspis* natriuretic peptide (DNP) [64]. Thereafter, presence of NPs has been reported from several other snake venoms [58]. A derivative of DNP

called CD-NP (15 C-terminal residues of DNP were fused with human CNP) [65] showed successful first phase of clinical trials in healthy subjects [66].

Platelet aggregation, an integral part of the hemostatic system, occurs through the interaction of platelet receptor integrin $\alpha_{IIb}\beta_3$ and fibrinogen, which eventually leads to thrombus formation to plug exposed blood vessels. However, thrombus formation in arteries may lead to thrombotic events like myocardial infarction and stroke; thus paving the way for development of antiplatelet agents [67,68]. Disintegrins are a family of cysteine-rich, low molecular weight proteins isolated from viperid snake venoms that contain the integrin-binding tripeptide motif which may be RGD, KGD, MVD, MLD, VGD, ECD, MDG or KTS [58,69,70]. As the RGD and/or KGD tripeptide sequences serve as the principal recognition sites for integrin $\alpha_{IIb}\beta_3$ receptor, therefore this binding thwarts the binding of fibrinogen to the receptor, eventually leading to inhibition of platelet aggregation [71]. Tirofiban (Aggrastat®) and Eptifibatide (Integrillin®) are two commercially successful antagonist drugs designed from snake venom disintegrins. The design for Tirofiban was based on the RGD motif of echistatin, a disintegrin isolated from the venom of *Echis carinatus* [72,73]; while Eptifibatide was designed based on the KGD pharmacophore of barbourin, isolated from *Sistrurus miliarius barbouri*, which is a specific inhibitor of integrin $\alpha_{IIb}\beta_3$ receptor [74,75].

Snake venom thrombin-like enzymes (SVTLEs) are another class of snake venom proteins that find use in the treatment of thrombosis-associated disorders, such as hyperfibrinogenemia [5,53,58,76]. SVTLEs cleaves fibrinogen to release fibrinopeptides A and/or B (like thrombin), however, most SVTLEs cleave only one of the two fibrinogen chains; hence they are classified as classes A (which cleaves Aα), B (which cleaves Bβ) or AB (which cleaves both) [77]. Unlike thrombin, SVTLEs cannot be inactivated by heparin-antithrombin III complex nor can they activate FXIII required for covalent cross-linking of fibrin monomers to form insoluble clots, thereby making them potential defibrinogenating agents [76]. Two such defibrinogenating agents include Ancrod and batroxobin, isolated from the venom of *Agkistrodon rhodostoma* [78] and *Bothrops atrox*, respectively [53,79]. Both of them belong to class A SVTLEs and cleave only fibrinogen Aα, but are unable to activate FXIII or any other coagulation factors. They rapidly catalyze the formation of soluble clot in order to deplete the level

of circulating fibrinogen, thus, preventing formation of insoluble clots in acute thrombosis events [77,80,81]. The procoagulant activity of SVTLEs finds application in sites of injuries or surgeries [58]. For example, Haemocoagulase®, which is a mixture of TLE and thromboplastin-like enzyme from the venom of *B. atrox* [82]. The TLE cleaves fibrinogen into fibrin monomers, while the thromboplastin-like enzyme activates FX, which in turn converts prothrombin into thrombin, leading to coagulation of blood [82].

Alfimeprase, the recombinant form of the metallo-proteinase fibrolase isolated from *Agkistrodon contortrix contortrix* [83] was found to be a clot lysing agent in many animal thrombosis models [84]. However, it failed in phase III clinical trials as it could not meet its primary endpoint and hence, was discontinued [85].

Some other examples of snake venom proteins-based therapeutics currently under development include ProTherapeutics, which is an analgesic peptide derived from a three-finger toxin of *Ophiophagus hannah* [86], and QRx Pharma and Biolink that are prothrombin activators developed from *Pseudonaja textilis* as a procoagulant agent [87]. Only time will tell the fate of these therapeutic, which unfortunately reflects the difficult and time-consuming nature of drug discovery and development [58].

2.3 A brief account on cytotoxic PLA_2s and their complexes in cobra venom

Cobra envenomation is associated with severe local tissue damage causing edema and blistering, ultimately leading to extensive necrosis [14,15,88]. These local symptoms can be attributed to the presence of cytotoxic and myotoxic PLA_2s present in cobra venom (Table 2.2) [89]. As evident from table 2.2, except for the cytotoxic PLA_2s isolated from *N. naja* and *N. kaouthia* venoms, majority of these toxic PLA_2s are basic in nature.

The first toxic PLA_2 from cobra venom was partially purified from the venom of *N. naja*, which was responsible for convulsions when injected in mice [90]. The cytotoxic activity and mechanism of action of this PLA_2 however was not studied. Condrea and co-workers [91] isolated and characterized for the first time a cytotoxic PLA_2 from the venom of *N. nigricollis*, which exhibited direct lysis of mammalian erythrocytes. The effect of this PLA_2 was partially by virtue of its enzymatic hydrolysis

of phospholipids on the membrane of erythrocytes [91,92]. Nigexine is another basic PLA_2 enzyme purified from *N. nigricollis* venom which is responsible for exhibiting differential cytotoxicity towards different mammalian cell lines [93]. The presence of this PLA_2 enzyme not only reduces the cell count, but also inhibits cell proliferation in different cancerous and normal cell lines of mammalian origin [94]. However, the cytolytic property exhibited by nigexine is a combination of its enzymatic hydrolysis of membrane phospholipids as well as a non-enzymatic mechanism of action [94].

The first cytotoxic PLA_2 isolated from the venom of *N. naja* was an acidic PLA_2 (NN-XIa-PLA_2) with a molecular mass of ~15 kDa [95]. The purified PLA_2 exhibited toxicity towards Erlich ascites tumour (EAT) cells with significant release of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) [95]. The PLA_2 was toxic and demonstrated myotoxicity and edema formation when injected into mice [95]. In another report of Rudrammaji and Gowda [96], three acidic PLA_2s were purified and characterized from *N. naja* venom. Similar to NN-XIa-PLA_2, these acidic PLA_2s demonstrated cytotoxicity towards EAT cells, indirect hemolysis towards mammalian erythrocytes, and myotoxicity in the form of edema in mice partly by virtue of its enzymatic activity [96].

In the year 2007, Mukherjee [40] purified two isoforms of *N. kaouthia* acidic PLA_2 – NK-PLA_2-A and NK-PLA_2-B. These two PLA_2s exhibited differential cytotoxicity towards insect and mammalian cell lines, the former being more susceptible than the latter [40]. The differential cytotoxicity exhibited by these PLA_2s can be attributed to the differential hydrolysis of membrane phospholipids of different cells [40]. The cytotoxic effect of these PLA_2s was found to be enhanced in their presence as non-covalent complex with a *N. kaouthia* venom neurotoxin (kaouthiotoxins or KTXs) [97]. The NK-PLA_2:KTX complex reportedly demonstrated significantly higher toxicity towards insect (Sf9 and Tn) and mammalian (VERO and erythrocytes) cell as compared to their individual counterparts [97]. This association of NK-PLA_2-KTX complex serves a fascinating example of protein complementation for augmentation of biological activity by non-covalent interaction of two polypeptides of cobra venom which may play an important role in the pathophysiology of cobra envenomation [97].

Table 2.2 List of cytotoxic and myotoxic PLA_2s reported from cobra venom.

Name of toxin	Source	Charge	Pathophysiology and mechanism	Mechanism	Reference
Cytotoxic PLA_2s					
N. nigricollis phospholipase A_2	*Naja nigricollis*	Basic	Direct hemolysis; LD_{50} dose towards mice is 0.63 mg/kg; showed signs of immobility, cyanosis, lacrimation and exophthalmos, associated with dyspnea and occasional convulsions until death; showed congestion in visceral organs (lung and liver), subserosal petechiae, renal tubular dilatation and albumin casts; multifocal hemorrhages, congestion and alveolar edema in lungs, and mild suppurative chorioditis in brains.	Phospholipid hydrolysis of erythrocyte membrane; toxicity is partly due to enzymatic activity	[91,92,109]
Nigexine	*Naja nigricollis*	Basic	Retards cell proliferation and viability in FL (human), C-13T (murine neuroblastoma), HL 60 (promyelocytic leukemia), MCF-7 (human breast carcinoma), SK-N-SH (human neuroblastoma), X63-Ag8.653 (myeloma of murine B-lymphocyte), BW-5147 (T-lymphocyte myeloma), and CTLL-2 (murine T-lymphocytes) cell lines; hemolyze erythrocytes; lethal to mice with an LD_{50} dose of 30.2 nmol/kg.	Mostly due to phospholipid hydrolysis, but partly by an undefined non-enzymatic mode of action	[93,94]
NN-XIa-PLA_2	*Naja naja naja*	Acidic	Respiratory distress, hind limb paralysis, lacrimation, and myotoxicity in mice; LD_{50} at 8.5 mg/kg body weight of mice; induces mild edema in the foot pads of mice without haemorrhage; cytotoxic to EAT cells with increased levels CK and LDH in serum.	Undefined	[95]
NN-I2c-PLA_2, NN-I2d-PLA_2, NN-I2e-PLA_2	*Naja naja*	Acidic	Cytotoxic to EAT cells; demonstrated indirect hemolysis; induced edema with different potencies in the foot pads of mice without causing haemorrhage.	Partly due to enzymatic activity	[96]
NK-PLA_2-A NK-PLA_2-B	*Naja kaouthia*	Acidic	Highly cytotoxic to insect cell lines like Sf-9 (*Spodoptera frugiperda*) and Tn (*Trichoplusia ni*) cells; mildly cytotoxic to VERO cells (kidney epithelial cells of African green monkey).	Preferential phospholipid hydrolysis of cell membranes	[40]

N. n. atra PLA_2	*Naja naja atra*	Undefined	Cytotoxic to human SK-N-SH neuroblastoma and human histiocytic lymphoma U937 cell line.	Necrosis (for SK-N-SH) and apoptosis (U937) mediated cell death	[98,99]
Myotoxic PLA_2s					
Naja nigricollis III (CMS-5/6)	*Naja nigricollis*	Slightly basic	Highly myotoxic in mice, lethal to mice at LD_{50} of ~1.2 mg/kg).	undefined	[104,110]
Naja nigricollis basic PLA_2 (CMS-9)	*Naja nigricollis*	Basic	Highly myotoxic in mice even at doses <1.0 µg per mouse.	undefined	[104,110]
Naja nivea III-2-3	*Naja nivea*	Basic	Local myonecrosis in mouse skeletal muscle in low doses (less than 2.5 µg per mouse).	undefined	[104]
Naja haje II-2	*Naja haje*	Basic	Myonecrotic lesions observed after 4 h, characterized by various stages of hyaline degeneration of myofibers, ranging from wedge-shaped degenerating areas (delta lesions) to amorphous clumps of fiber mass.	undefined	[104]
NN-XIII-PLA_2	*Naja naja naja*	Basic	Induces myotoxicity, and edema in the foot pads of mice without causing haemorrhage.	undefined	[107]
Naja nigricollis PLA_2 (NG-4)	*Naja nigricollis*	Basic	Rapid and drastic cytotoxicity in cultured muscle cells of mouse origin at very low doses of 1-2 µg/ml; myotoxicity in mice upon injection.	Membrane phospholipid hydrolysis; retardation of transport of amino acid (2-aminoisobutyric acid), and thymidine leading to apparent inhibition of macromolecular syntheses	[105,106]

Another cytotoxic PLA_2 was purified from the venom of *N. n. atra* which demonstrated significant toxicity against SK-N-SH neuroblastoma [98] and human histiocytic lymphoma [99] cell lines. The *N. n. atra* PLA_2 reportedly causes cytotoxicity in mammalian cells by inducing necrosis and apoptosis mediated cell death in SK-N-SH and U937 cells, respectively [98,99]. The cytotoxic property of this enzyme was independent of its enzymatic activity [98,99].

2.4 A brief account on cobra venom myotoxic PLA_2s

Myonecrosis at the site of envenomation is a major concern in cobra envenomed patients [14,100-102]. Extensive studies have been done to identify the myotoxic PLA_2s of snake venoms [103]; however, to date only six myotoxic PLA_2s have been isolated from different cobra venoms (Table 2.2). In 1986, Mebs purified two basic PLA_2s from the venom of *N. nigricollis* which exhibited strong myotoxic property [104]. Severe myonecrotic symptoms were observed in mice injected with *N. nigricollis* III (CMS-5/6) and CM-9 PLA_2s of *N. nigricollis* venom, the latter being more toxic than the former [104]. Apart from myotoxicity, CM-9 exhibited significant lethal potency at a dose < 1 μg/ml [104]. However, the mode of action of these PLA_2 still remains to be unexplored. Another basic PLA_2, NG-4, isolated from the venom of *N. nigricollis* demonstrated very strong *ex vivo* cytotoxicity against cultured mammalian heart and skeletal muscle cells of mouse origin [104]. NG-4 also exhibited myotoxicity in mice injected with the purified PLA_2 [105,106]. Contrary to CM-5/6 or CM-9, NG4 induces myotoxicity by inhibiting protein synthesis in the cells. Along with enzymatic hydrolysis of membrane phospholipids, NG-4 reportedly retarded the amino acid (2-aminoisobutyric acid) and thymidine transport, which in turn inhibited the process of macromolecular synthesis [105].

Another two basic myotoxic PLA_2s were isolated from the venoms of *N. nivea* and *N. n. haje* [104]. Both of them exhibited severe myotoxicity characterized by local necrosis and myonecrotic lesions with hyaline degradation in mouse skeletal muscle cells [104], respectively. The commercial snake antivenoms was reported to neutralize the effect of these myotoxins in *in vitro* conditions [104]; however, the rapid onset of myonecrosis after envenomation limits the efficacy of such antivenoms, especially in snakebite cases with delayed antivenom treatment [104].

A major basic phospholipase A_2 (NN-XIII-PLA_2) was isolated from the venom of southern India *N. n. naja* [107]. It is the only myotoxic PLA_2 isolated from the venom of Indian cobra. Although the mode of action of this PLA_2 enzyme is yet undefined; however, the intramuscular injection of this protein in mice exhibited severe toxicity towards the thigh muscles of mice [107]. Further, edema formation was observed in the foot pads of mice thereby re-instating the role of NN-XIII-PLA_2 in myotoxicity [107].

The cytotoxic and/or myotoxic effects of snake venom PLA_2s are inefficiently neutralized by commercial antivenoms [108]. Although pre-treatment of these toxins could neutralize their pharmacological effects, however, antivenoms are largely ineffective in the neutralization of local toxicity when administered after venom injection and/or envenomation due to the rapid action of venom PLA_2s on muscle cells [108]. Therefore, understanding the pathophysiology of cobra venom PLA_2s exhibiting cytolytic and myotoxic properties can help in the development of alternative strategies of antivenom production in order to combat the characteristic problem of tissue damage and necrosis upon cobra envenomation.

2.5 Understanding the mechanism of snake venom PLA_2-induced myotoxicity

As evident from histological and ultra-structural studies, the effect of venom PLA_2s on skeletal muscle follows a common series of pathological changes [22,89,111-114]:

i. Disruption of plasma membrane;
ii. Formation of 'delta-lesions' (wedge-shaped areas of degeneration) at the periphery of muscle fibers;
iii. Hypercontraction of myofilaments;
iv. Mitochondrial swelling, leading to formation of flocculent densities and rupture of mitochondrial membranes;
v. Disruption of intracellular membrane systems, i.e. sarcoplasmic reticulum and T tubules, and
vi. Pycnosis of nuclei.

Fig 2.2 represents the schematic pathway of membrane disruption followed by myotoxic PLA_2 and the downstream events leading to cell death [89]. Irrespective of the mode of action, i.e. enzymatic or non-enzymatic, the plasma membrane of muscle cells or sarcolemma serves as the primary site of action for myotoxic snake venom PLA_2s [89,113]. Concomitant damage to the plasma membrane is associated with a rapid efflux of membrane cytosolic enzymes which consequently leads to depolarization of muscle cells [89,113]. Further, different cytosolic molecules released by damaged cells triggers danger signals to surrounding tissues, thus contributing extensive tissue damage in the surrounding areas [113,115]. However, there is a dearth of evidence in support of the concept of internalization of cobra venom myotoxic PLA_2 in skeletal muscle cells or if the primary site of action of these enzymes is an intracellular target [89,114].

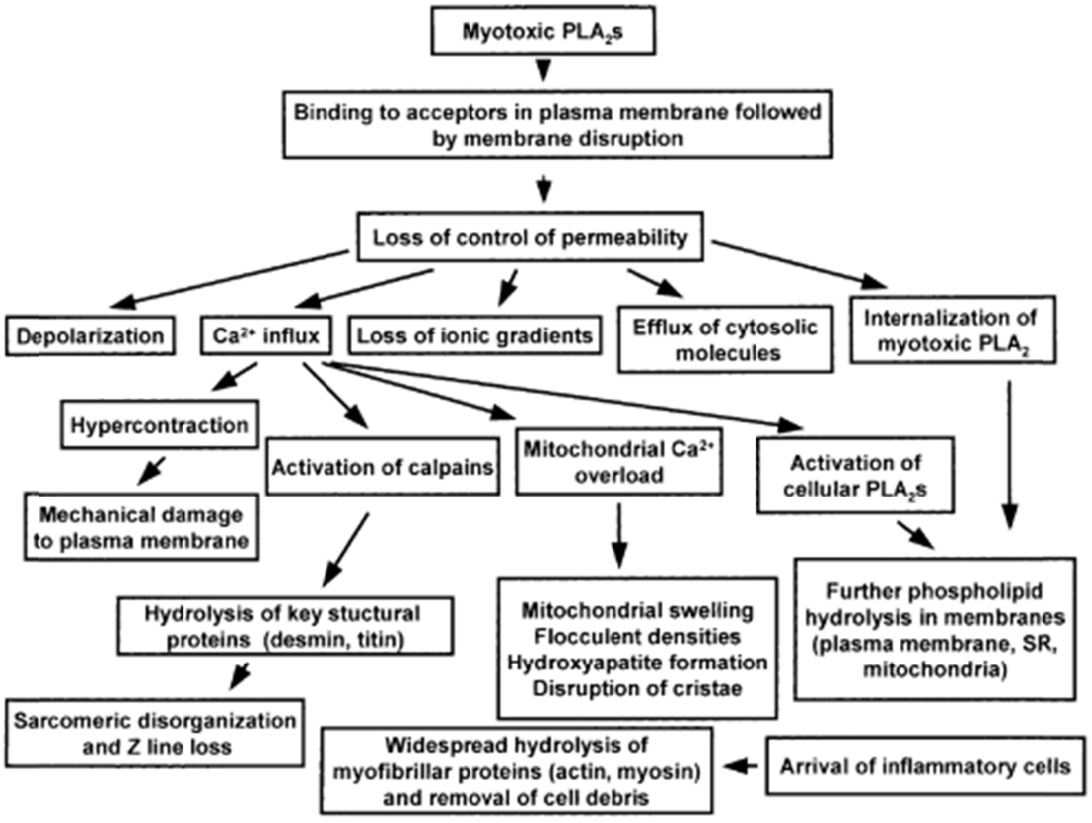

Fig 2.2. Hypothetical sequence of cellular degenerative events in skeletal muscle cells as a consequence of the action of venom myotoxic PLA_2s [89]. After binding to acceptors in the plasma membrane, there is an initial disruption of the integrity of this membrane through catalytically dependent or independent processes. As a consequence, there is loss in the control of permeability to ions and macromolecules. The most critical

event is a prominent Ca^{2+} influx, which is responsible for a large number of degenerative processes which rapidly end in irreversible cell damage. An inflammatory process develops with the release of chemotactic substances and the arrival of inflammatory cells (mainly neutrophils and macrophages) which remove the necrotic debris and set the stage for muscle regeneration. [Source of image: Gutiérrez and Ownby, Toxicon, 2003].

Although the inherent property of phospholipid hydrolysis by myotoxic PLA_2 is a major cause of cytotoxicity in muscle cell wall as other cells [10,40,116]; nevertheless, the existence of specific binding sites on the sarcolemma, such as proteins, may also be responsible for target specific toxicity of PLA_2s towards myogenic cells [16]. It has been previously demonstrated that lipid domains and rafts exist within plasma membranes which suggests that such regions enriched in particular types of glycerophospholipids or glycolipids may function as acceptors of PLA_2s [10,40,89,116]. These negatively charged lipids present on the outer monolayer of muscle plasma membrane probably participate in the anchorage of myotoxic PLA_2s. Once bound to muscle cells, the damage induced by myotoxic PLA_2s to the plasma membrane might be of two main types:

(1) A perturbation in the integrity of the bilayer by a mechanism independent of phospholipid hydrolysis; and
(2) A membrane disruption based on enzymatic phospholipid degradation.

The former type of effect is a characteristic feature of catalytically inactive group II PLA_2 myotoxins isolated from many viperid venoms, such as the Lys49 PLA_2s [117-119]. The cationic residues of these toxins preferentially develop electrostatic interaction with the negatively charged bilayers at temperatures above the phase transition causing membrane destabilization, followed by penetration and disorganization of bilayers, consequently leading to the collapse of their macromolecular organization [10,89,103,113,116,117]. Thus it can be inferred that the enzymatic and toxic activities of snake venom PLA_2s can be dissociated [10,22,113,120].

The second mechanism of action of myotoxic PLA_2s is based on the hydrolysis of plasma membrane phospholipids, which is an inherent characteristic of all enzymatically active, Asp49 group I/II PLA_2s [10,89,103,113]. In yet some other myotoxins, both catalytic and non-catalytic mechanisms may be involved, since inhibition of enzymatic activity reduces, but does not eliminate, myotoxicity [121,122]. Phospholipid hydrolysis is subsequently associated with the generation of free fatty acids and lysophospholipids, which themselves cause further membrane damage through their detergent activity [10,89,103,113,123]. Contrary to the first mechanism, it has been observed in some elapid venom group I myotoxic PLA_2s that abrogation of enzymatic activity completely eliminates myotoxicity, thus implying the positive correlation of myotoxicity with enzymatic activity [111,124].

Disruption of the plasma membrane causes membrane leakiness which is characterized by a rapid efflux of cytosolic molecules (for example, CK, LDH, aspartate aminotransferase, myoglobin and creatine) leading to membrane depolarization [89,103,125]. Notably, heavy influx of Ca^{2+} ions which consequently leads to hypercontraction of myofibrillar apparatus is responsible for the most notorious histological and ultrastructural consequence of myotoxic PLA_2 action in muscle cells [103,111]. The increase in Ca^{2+} ion concentration in the cytoplasm further causes degradation of the muscle proteins desmin and titin, loss of register of sarcomeres, loss of Z-band due to the disaggregation of A- and I-bands, release of loose actin and myosin to the cytosol, and delayed degradation of α-actinin and dystrophin [126-128]. On the other hand, hypercontraction of the muscle cells further promotes damage to plasma membranes in mitochondria, T tubules, sarcoplasmic reticulum, and nuclei [89,129]. Swelling, formation of flocculent densities, vesiculated cristae, dense intracristal spaces, and overt rupture of mitochondrial membranes, hydroxyapatite crystal formation in mitochondria and calcium accumulation are some other important consequential symptoms of Ca^{2+} influx in PLA_2-induced myotoxicity [89,103,130,131].

The characteristic features of plasma membrane rupture, release of cytosolic components, prominent Ca^{2+} influx, mitochondrial Ca^{2+} overload, and pycnotic nuclei, reveals the typical features of necrosis in PLA_2-induced muscle degeneration [111]. However, there are reports of detection of apoptotic nuclei in a number of clinical and

experimental myopathies [132]; nevertheless, no effort has been made to explore the apoptotic role of myotoxic PLA_2s in PLA_2-induced myopathies.

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