Chapter I

INTRODUCTION

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1.1 Introduction

Snakes are one of the most charming and unfathomable animals living on this earth. To date, the sight of a snake is dreadful and traumatic to the common man. In India, from ancient times, snakes are dreaded by human beings on one hand, whereas on the other hand, it is envisaged to be an integral part of their culture. The planet is a home to a more than 3,000 species of snakes distributed across every parts of the globe, except Antarctica [1-4]. This group of elongated, limbless, carnivorous reptile is known to dwell almost under every climatic riff in a diapason of habitats. Based on presence or absence of venom gland this huge group of organism is broadly classified into two groups - venomous and non-venomous. Only about 600 species of snakes are venomous, while most of them are non-venomous in nature [3,5].

Snake venom is a highly effective device used for predation (capturing, immobilization, and digestion of the prey), defense, and competitor deterrence [6,7]. It is a modified form of saliva that consists of a myriad of bio-molecules, primarily enzymatic and non-enzymatic proteins and polypeptides. These proteins / polypeptides are responsible for wide array of pathophysiological symptoms in prey and victims post envenomation [8-11]. Snake venom proteins / polypeptides target a myriad of receptors, ion channels and hemostatic proteins that thus makes them ideal for pharmaceutical research [6,7,12-15]. Furthermore, studies have shown that the composition of snake venom varies from species-to-species (inter-specific) [8] as well as intra-specifically [9,16,17]. Intra-specific venom variation depends on various factors such as age, sex, ontogeny, and locale [9,16,18,19] of the snake. This intra-specific venom difference is one of the major limitations of snakebite treatment with polyvalent antivenoms [17,20,21].

It has been reported that snakes can voluntarily control the amount of venom they inject into their prey [6]. They may either bite aggressively for food or defensively for protection [6,22]. Because only a limited amount of venom is being produced by a snake; therefore, snakes observe economical usage of their venom [23-25] and perhaps

refrain to waste it on non-prey organisms [26]. Notably, about ~50% of bites suffered by humans is defensive in nature and "dry" (without envenomation) [6,26,27].

Naja naja (also known as the Indian Spectacled Cobra) is one of the deadliest snakes distributed widely across the Indian sub-continent. This chapter describes the epidemiology of snake envenomation, venom composition of snakes and its associated clinical manifestations with special reference to *Naja naja*. This chapter emphasizes the pharmacological aspects of snake venom proteins / polypeptides that affect hemostasis and their probable development as drug prototypes for the treatment and / or prevention of cardiovascular diseases. Further, the chapter also describes the emergence of peptides as therapeutics and their advantages over other conventional drugs.

1.2. Taxonomic classification and geographical distribution of snakes

Snakes belong to the sub-order *Serpentes*, order *Squamata*, class *Reptilia* of phylum *Chordata*. Based on their morphological characteristics and mitochondrial DNA sequence similarity, the sub-order Serpentes is classified into two infraorders, viz. Alethinophidia and Scolecophidia [28]. The infra-order Alethinophidia is further subdivided into two groups - Henophidia and Caenophidia, which together comprise of 19 families of snakes [29]. Whereas, the infra-order Scolecophidia comprise of only 3 snake families [29].

The venomous snakes are reported to belong to the infra-order Alethinophidia, group Caenophidia and Colubroidea superfamily [2,30]. The Colubroidea superfamily is subsequently subdivided into four families, viz., Colubridae, Elapidae, Viperidae, and Atractaspididae [28,31]. The largest and multifarious family of snakes, Colubridae, comprises of 8 sub-family of snakes, which together consists of approximately 1,800 species [29]. This family of snake is distributed on all continents throughout the planet, except Antarctica [4].

With about 350 species, the Elapidae family of snakes is another large family of venomous snakes, which is widely distributed across the tropical and sub-tropical regions of America, Africa, the Middle East, Asia, and Australasia [31]. The family is divided into two sub-families – the Elapinae sub-family comprising of coral snakes, cobras, mambas, and kraits, and the Hydrophiinae sub-family comprising of sea snakes.

Elapids are proteroglyphous (front-fanged) and their venom is mostly neurotoxic in nature, thus making them of significant medical importance to humans [2,31]. The Viperidae family of venomous snakes contains around 310 species and is distributed across a range of terrestrial habitats of Americas, Africa, Europe, and Asia (except Siberia, Ireland and Australia) [2,31]. The Viperidae family is further sub-divided into two main sub-families – the Viperinae (pit-less Vipers) and the Crotalinae (pit Vipers) [2,31]. The Viperids are solenoglyphous (hollow-fanged) and their venom is highly hemotoxic in nature and are responsible for extensive human mortality and morbidity across the globe [2,32]. The Atractaspididae family of snakes contains around 30 species which are distributed across Africa and Middle East [31].

1.3. Snakebite: A tropical health hazard

The Earth is a home to 23 families of snakes which comprise of 511 genera and 3378 species [33]. Among them, only about 600 species of snakes are venomous and the remaining are non-venomous [5]. Snake envenomation is an occupational health hazard in tropical and sub-tropical countries like India, where farming is the largest sector of employment. It is estimated that yearly about 1.2 – 5.5 million snakebite cases occur around the world, out of which 421,000 are envenomings, and this figure may go up to as 1,841,000 [3]. Out of these, approximately 20,000 – 94,000 cases results in mortality of the victim while another fraction accounts to severe morbidities [3]. Therefore, snake bite has been re-instated as a "Category A, Neglected Tropical Disease" by World Health Organization (WHO) on June 9, 2017 [34]. However, the number of snakebite cases reported varies from region-to-region across the globe [35-37]. The burden of snakebite lies mostly in South and South-east Asia, sub-Saharan Africa, and Central and South America [3,38] (Fig 1.1). Most of the information on snake envenoming is primarily based on hospital records [39], epidemiological literature [35,36,38], or a combination of epidemiological and WHO mortality data [3,38]. It is sad to note that the agony of snakebite mainly lies with the poor and poverty stricken people of the world [40,41].

1.3.1 Global burden of snakebite

Conservative estimates report that the highest number of envenomings were estimated for South Asia (121,000) followed by Southeast Asia (111,000), and East Sub-Saharan Africa (43,000) [3,37,42]. The lowest numbers were estimated for Central Europe and Central Asia. Kasturiratne and co-workers [3] reports that India had the most envenomings at 81,000 annually, which is followed by Sri Lanka (33,000), Vietnam (30,000), Brazil (30,000), Mexico (28,000), Pakistan (20,000), and Nepal (20,000). Reports suggest that the problem of snakebite is under-appreciated in West Africa [43]. It was estimated that incidences of snakebite envenomation in savannah Nigeria of northwestern Africa is 500 per 100,000 population per year [44]. However, recent estimates suggest that 10,001 to 100,000 snakebite envenomings with a mortality rate of 1,001 to 10,000 deaths per year occurs in the West African sub-region [3]. A more recent study has shown that nearly about 314, 000 bites, 7,300 deaths and 6,000 amputations occur annually in sub-Saharan Africa [45].

On the contrary, incidence of snakebite is a rare event in Europe and North America [35,46]. In North America, approximately 45,000 snakebites occur annually, out of which about 10,000 are inflicted by venomous species, 6,500 require medical intervention, and mortality due to snakebite is approximately 15 per year [47,48]. However, the annual incidence of snakebites in Central and South America is estimated to be at least 300,000; among which 150,000 are envenomations and about 5,000 envenomed individuals die every year [49-51]. Further, incidences of snakebites have reportedly decreased in Australia with a record of 1,000 – 3,000 snakebite cases annually [52,53]. While snakebite numbers are decreasing in temperate Australia, snakebite remains an important cause of morbidity in tropical Australia and of mortality in Papua New Guinea and Irian Jaya [54,55].

1.3.2 Indian scenario of snakebite

India records the highest number of snakebite envenoming cases across the world [3]. The diverse fauna of the country includes more than 250 species and subspecies of snakes, out of which about 50 are highly venomous [56,57]. Majority of the snakebite cases in the country can be accounted to the ‘Big Four’ species of snakes

which includes members of the Elapidae family – Indian Spectacled Cobra (*Naja naja*) and Common Krait (*Bungarus caeruleus*), and Viperidae family – Russell's Viper (*Daboia russelii*) and Saw-scaled Viper (*Echis carinatus*) [58]. Except for the North-east region of India, the Himalayan region and the Andaman and Nicobar Islands, the big four species of snakes are widely distributed throughout the country [58]. However, the above regions of the country are known to have their own distinctive fauna and hence their own species of snakes. Apart from the big four snake species, several other venomous snake species are frequently encountered in India, such as the Central Asian Cobra (*N. oxiana*), Indian Monocellate Cobra (*N kaouthia*), Greater Black Krait (*B. niger*), Banded Krait (*B. jasciatus*), Wall's Krait (*B. walli*), Sind Krait (*B. sindanus*) and Hump-nosed Pit Viper (*Hypnale hypnale*) [57,58]. Some other species of snakes prevalent in India are coral snakes (*Calliophis*, *Sinomicrurus*), sea snakes (*Laticauda*, *Kerilia*, *Lapemis*, *Hydrophis*, *Enhydrina*, *Astrotia*, *Pelamis*), pit Vipers (*Trimeresurus*, *Protobothrops*, *Gloydius*, *Ovophis*) and the King Cobra (*Ophiophagus hannah*) [58].

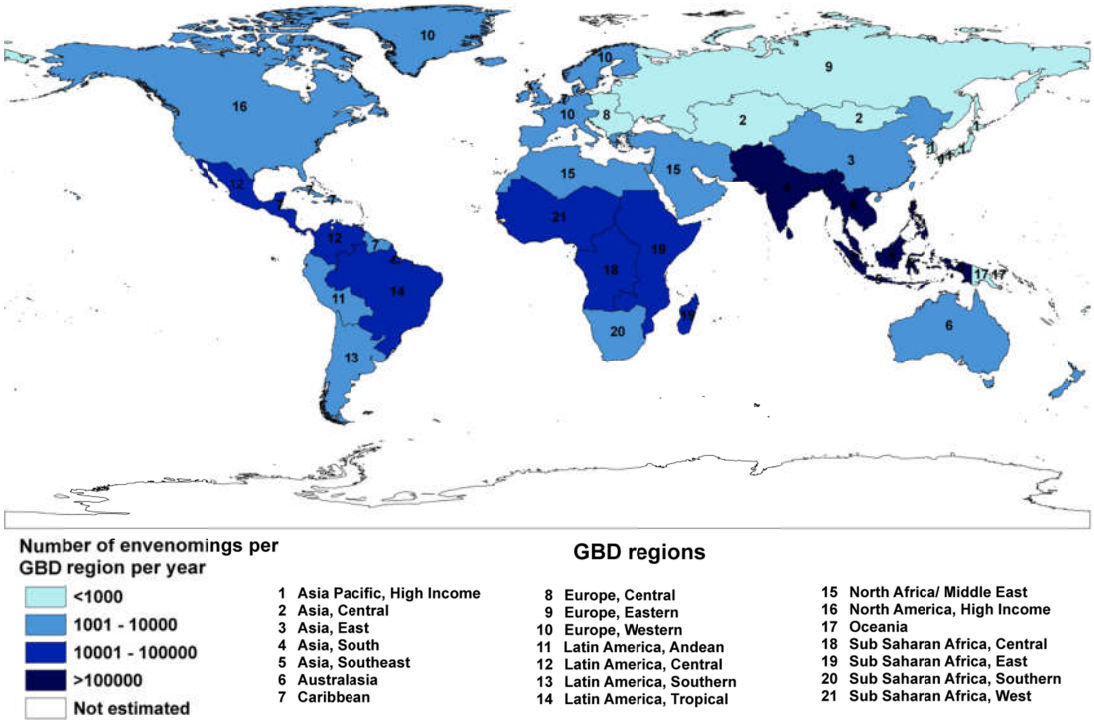

Fig 1.1. Epidemiology of snakebite mortality across the world. Annual estimates of snakebite-induced deaths for 138 countries were obtained from the data published by Kasturiratne et al. [3] and Harrison et al. [40]; the darker a country's colour the greater

the estimated snakebite mortality (Key, left bottom). [Source of figure: Kasturiratne et al., PLoS medicine, 2008].

Approximately 50,000 of the reported snake bite cases per annum results in mortality of victims in India [57,58]. Approximately 97% of snakebite deaths in India are reported in rural areas, wherein the male mortality rate (59%) is higher than that of the females (41%) [57]. Majority of the snake envenomation death occurs during the monsoon months (from June to September) [57]. A study by Mohapatra and co-workers [57] suggested that the annual snakebite deaths is the highest in the states of Uttar Pradesh, Orissa, Andhra Pradesh, and Bihar (4,500) (Fig. 1.2). In yet another report of hospital-based studies, it has been reported that Northern India records a snakebite related mortality rate from 3 – 20% [59]. In Maharashtra, an estimated 10,000 annual venomous snakebites account for 2,000 deaths [60]. Further the states of West Bengal, Tamil Nadu, Chhattisgarh, and Kerala also reports high incidence of snakebite cases [57,59,61] (Fig. 1.2). Despite of being a biodiversity hotspot which harbors a wide variety of venomous snakes, unfortunately, there is no record of snakebite mortality and morbidity cases in the north-eastern states of India.

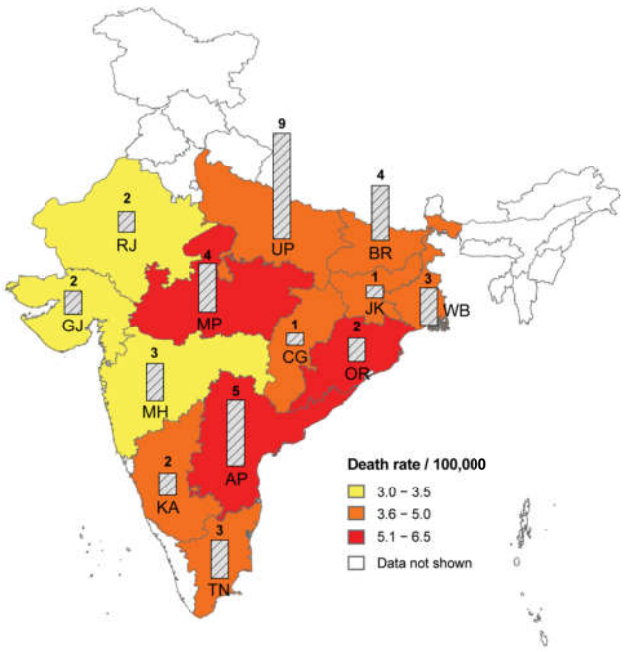

Fig 1.2. Estimated deaths and standardized death rates in states with high prevalence of snakebite deaths, 2005, as published by Mohapatra et al. [57]. The vertical bars represent the state wise estimated deaths (in thousands). Total snakebite

deaths for the 13 states with high-prevalence of snakebite death are 42,800 or 93% of the national total (these states have about 85% of the total estimated population of India). States where the snakebite death rate was below 3/100,000 or where populations are less than 10 million are not shown. The states with high-prevalence of snakebite deaths are: AP-Andhra Pradesh, BR-Bihar, CG-Chhattisgarh, GJ-Gujarat, JH-Jharkhand, KA-Karnataka, MP Madhya Pradesh, MH-Maharashtra, OR-Orissa, RJ-Rajasthan, TN-Tamil Nadu, UP-Uttar Pradesh, WB-West Bengal. [Source of figure: Mohapatra et al., PLoS neglected tropical diseases, 2011].

1.4 The Indian Spectacled Cobra (*Naja naja*)

The Indian Spectacled Cobra (*Naja naja*) was first described by the Swedish physician, zoologist, and botanist Carl Linnaeus in 1758 [62]. The scientific name, *Naja naja* has been derived from the Latinization of the Sanskrit word nāgá (नाग) meaning 'cobra'. The common name (Spectacled Cobra) was given to this snake due to the presence of a spectacled mark on the hood of the snake (Fig 1.3). The snake is also a member of the 'Big Four' snakes of India responsible for a huge number of snake bite mortality and morbidity in this country [56].

Fig 1.3. An Indian Spectacled Cobra (*Naja naja*) with its spreading hood. [Source of figure: http://calphotos.berkeley.edu]

1.4.1 Taxonomic classification

The taxonomic classification of *N. naja* has been summarized in Table 1.1.

Table 1.1. Taxonomic classification of the Indian Spectacled cobra [62].

Kingdom	Animalia
Subkingdom	Bilateria
Infra-kingdom	Deuterostomia
Phylum	Chordata
Sub-phylum	Vertebrata
Infra-phylum	Gnathostomata
Super-class	Tetrapoda
Class	Reptilia
Order	Squamata
Sub-order	Serpentes
Infra-order	Alethinophidia
Family	Elapidae
Genus	*Naja*
Species	*N. naja* (Linnaeus, 1758)

1.4.2 Distinctive features and description

The Indian Spectacled Cobra or *Naja naja* is a heavy bodied species of moderate length, ranging between 1.0 to 1.5 m (3.3 to 4.9 feet) in length [63,64]. The colouration pattern varies widely from region-to-region and hence the snake is identified by its large and impressive hood having a prominent spectacle mark, which it spreads when under threat [63]. This species has a head which is elliptical, depressed, and very slightly distinct from neck. The snout is short and rounded with large nostrils.

1.4.3 Habitat and geographical distribution

The snake is found to be distributed across the Indian sub-continent and found in India, Pakistan, Sri Lanka, Bangladesh, and southern part of Nepal (Fig 1.4) [63]. It inhabits a wide range of habitats throughout its geographical range, and can be found in dense or open forests, plains, agricultural lands, rocky terrain, wetlands, and it can even be found in heavily populated urban areas such as villages and city outskirts, ranging from sea-level to 2,000 meters (6,600 fit) in altitude. This species of snake is absent

from true desert regions. The most preferred habitat of the Indian cobra is often in the vicinity of water, such as holes in embankments, but is also found in tree hollows, termite mounds, rock piles, and small mammal dens [56,63].

Fig 1.4. Geographic range of distribution of the Indian Spectacled Cobra (*Naja naja*). [Source of figure: http://www.toxinology.com/images/snakes/SNML0041.gif]

1.4.4 Venom apparatus of *N. naja*

The snake venom is stored in a specialized parotid gland, known as the venom gland situated behind the maxilla and is connected to elongated and specialized venom delivery structures called fangs. Snake venom delivery apparatus can be broadly divided into two types – front-fanged and rear-fanged [65]. The front-fanged structures are further of two types – proteroglyphous, found in snakes of Elapidae family (Fig 1.5), and solenoglyphous, which is pre-dominant in Viperids and *Atractaspis* genus of Atractaspidids. The rear-fanged or opisthoglyphous snake venom apparatus is found in atractaspidids other than *Atractaspis*, and the venomous group of Colubrids [66,67].

The fangs of Elapid snakes, such as *N. naja*, are shorter in length than that of viperids [68]. They are tubular with a seam connecting the entrance and exit openings for the venom [66]. However, some *Naja* species have a modified exit orifice for the venom which is round, instead of oval, in order to facilitate the defensive behavior of spitting venom at an attacker [69-71].

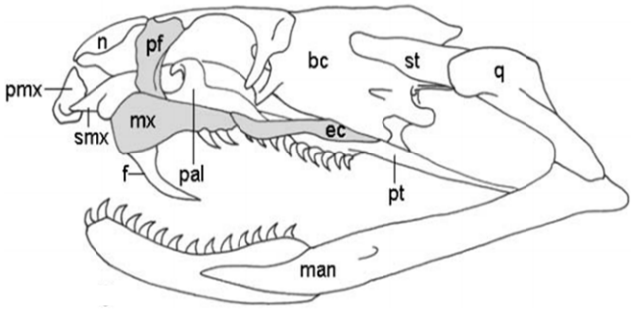

Fig 1.5. Diagrammatic left lateral views of the skull of elapids [66]. The elapids the fangs are frequently followed by several short, solid maxillary teeth. bc, braincase; ec, ectopterygoid; f, fang; man, mandible; mx, maxilla; n, nasal; pal, palatine; pf, prefrontal; pmx, premaxilla; pt, pterygoid; q, quadrate; smx, septomaxilla; st supratemporal. [Source of figure: Deufal and Cundall, Zoologischer Anzeiger, 2006].

1.4.5 Pathophysiology of cobra envenomation

During a bite, the Indian Spectacled Cobra, *N. naja*, injects approximately 170 – 250 mg of venom into its victim, may be determined by the size of the victim [72]. Envenomation by *N. naja* exhibits an array of pathophysiological symptoms in the victims, thereby resulting in permanent morbidities and fatalities [8,73-77]. Owing to the severity of pathophysiological symptoms exhibited upon *N. naja* envenomation, this snake has been classified as a category I medically important snake in India [78,79].

Neurotoxicity, the primary clinical symptom of *N. naja* envenomation is characterized by ptosis, blurring of vision, dysconjugate gaze, and diplopia [73,75]. Symptoms such as drowsiness and irritability are also observed in cobra envenomed patients that may be preceded by corticobulbar tract dysfunction such as dysphonia, dysphagia, absent gag reflex, and respiratory compromise [73,75]. Finally, diaphragmatic breathing followed by ventilatory paralysis may occur. Death may result from respiratory insufficiency causing hypoxia and acidosis [75]. Generalized flaccid paralysis has been noted in patients with respiratory compromise. The mean onset of paralysis, although highly variable, often occurs from 1 to 6 h after being bitten [75].

Besides neurotoxicity, severe local effects such as swelling, edema, and extensive necrosis owing to cytotoxic and / or myotoxic effects are also evident in cobra bite patients [73-75]. Severe local symptoms of envenoming are associated with acute

suffering, prolong hospital stay, permanent scarring, and even death in some patients [73]. Transient coagulopathy characterized by increase in 20 minute whole blood clotting time (20WBCT) test of *N. naja* envenomed patients has also been reported from Sri Lanka and western India [73,74].

Currently, administration of polyvalent antivenom raised against the 'Big Four' snakes of India is the only acceptable therapy to deal with *N. naja* envenomation [73,74,76]. The neurotoxicity exhibited by cobra envenomation may be reversed by immediate administration of antivenom; however, commercial antivenom fails to neutralize the local effects of cobra envenomation such as edema and necrosis [73,74]. This is suggestive of development of alternate strategies for antivenom production to mitigate this long standing problem.

1.5 A brief account on *N. naja* venom

Lyophilized *N. naja* venom is readily soluble in aqueous solvents (buffers such as 20 mM Tris-HCl, pH 7.4 and phosphate buffered saline, pH 7.4). A homogenous solution of *N. naja* venom is clear to slightly yellowish in colour, slightly acidic with a pH range from 6.6–7.0, and relative viscosity is between 1.03 – 1.07 [80]. Like all other snake venoms, the venom of *N. naja* is composed of a 95 – 98% of proteins and peptides that exhibit a variety of pharmacological effects in isolation or combination [81]. Several of such pharmacologically active proteins / polypeptides (toxins) have been isolated, purified, and characterized from the venom of *N. naja* in order to understand their mechanism of action and the pathophysiology exhibited by them upon envenomation [82-97]. Approximately 5% of the venom is composed of non-proteinaceous low molecular mass substances such as amino acids, amines, lipids, nucleosides and nucleotides, carbohydrates and metal ions, most of which are biologically inactive or less active or assist the venom proteins and polypeptides to exert their toxicity [98].

1.5.1 Composition of *N. naja* venom

Like other Elapid venoms, the venom of *N. naja* is neurotoxic in nature [73,76]. On an average, snake venoms are composed of different proteins / polypeptides belonging to approximately 26 different protein families, 12 of which are found in

almost all snake venoms and each exhibiting a distinct functional role [98,99]. The venom proteins can be broadly classified into two classes – the enzymatic proteins and the non-enzymatic proteins / polypeptides [13,15]. The latter class of proteins is predominant in the venom of Indian cobra and is associated with a wide array of pharmacological properties [17,19,21,100].

The predominant non-enzymatic class of *N. naja* venom proteins include the three-finger toxins which constitute approximately 60 – 75% of the total venom proteome [19,21,100,101]. This low molecular mass (6 – 9 kDa) family of snake venom proteins is responsible for a myriad of clinical symptoms such as neurotoxicity, cardiotoxicity, cytotoxicity, and antiplatelet effects [102,103]. The other non-enzymatic proteins of the Indian cobra venom include cystatin, nerve growth factors (NGF), cysteine-rich secretory proteins (CRISP), Kunitz-type serine protease inhibitors (KSPI), cobra venom factors (CVF), natriuretic peptides (NP), ohanin-like proteins (OLP) or vespryns, snaclecs or C-type lectins and vascular endothelial growth factors (VEGF) [19,21,100,101]. CVFs are responsible for complement activation [104], while natriuretic peptides and vespryns are responsible for hypotensive effect [105] and locomotor dysfunction [106], respectively.

The enzymatic proteins of *N. naja* venom consists of phospholipase A_2s (PLA_2), phospholipase Bs (PLB), snake venom metallo- and serine-proteases (SVMP / SVSP), L-amino acid oxidases (LAAO), 5' nucleotidases, phosphodiesterases, aminopeptidases, cholinesterases (including acetylcholinesterases and butyrylcholinesterases) [17,19,21,100,101,107], and hyaluronidases [83]. The neurotoxicity of cobra venom is also exhibited by the cholinesterases and neurotoxic PLA_2s which are post-synaptic neurotoxins and binds reversibly to nerve terminals [76,89,92,108]. PLA_2s, along with metalloproteases and hyaluronidase are responsible for local effects of cobra envenomation such as necrosis and edema [83,87]. The cobra venom metalloproteases and nucleotidases have been reported to affect the hemostatic system [82,95] although the pathophysiology exhibited with this effect is rarely witnessed in *N. naja* envenomation [73,74].

The PLA_2s of cobra venom (other than *N. naja*) are reported to exhibit strong anticoagulant activity [109-112], which suggests the probable role of cobra venom

PLA_2s in transient coagulopathy exhibited post envenomation. In a nutshell, the PLA_2 family of enzymes may be responsible for a considerable amount of toxicity upon *N. naja* envenomation. Hence, this group of proteins has been described in some more detail in section 1.7.

1.5.2 Differential composition of Indian Spectacled Cobra (*Naja naja*) venom: A biochemical approach

In a study by Mukherjee and Maity [16], the differential composition of *N. naja* venom from 3 districts (Burdwan, Purulia and Midnapur) of West Bengal, India and Haffkine Institute, Maharashtra, India were studied by different biochemical techniques. Each of these venoms was decomplexed using size-exclusion chromatography and each fraction obtained along with the whole venoms was subjected to phospholipase, protease, esterase, and nucleotidase enzyme assays. The study reported that *N. naja* venom from Burdwan and Purulia districts of West Bengal exhibited superior phospholipase and protease activities as compared to the other two venoms. Moreover, *in vivo* studies revealed that the venom samples from eastern India (West Bengal) were found to possess higher lethality, edema inducing activity, and hemolysis as compared to the same from western India (Maharashtra), thereby emphasizing on intraspecies differential venom composition owing to geographical difference within the same country. This difference in venom composition renders neutralization of venom components by commercial polyvalent antivenom inefficient for venom of snakes from different locales [16].

In a similar but broader approach, the *N. naja* venom from three different geographical regions [eastern (West Bengal), western (Maharashtra), and southern parts (Tamil Nadu)] of India was studied for their different characteristics and composition [9,20]. Interestingly, the study reported the differential banding patterns of the three venom samples when subjected to sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (SDS-PAGE), wherein it was found that the eastern India *N. naja* venom is mostly predominated with low molecular weight proteins as compared to the other two venom samples [9]. Under identical conditions, eastern India *N. naja* venom showed prominent phospholipase A_2, hyaluronidase, and indirect hemolytic activities as compared to its counterparts from the western and southern regions. Apparently, the

former exhibited higher toxicity as compared to the latter when injected in mice, with a LD_{50} value of 0.7 mg/kg, 1.2 mg/kg, and 2.0 mg/kg for eastern, western and southern *N. naja* venoms, respectively [9,20]. Another interesting finding of this study was that the *N. naja* venom also affected the blood coagulation by affecting the clotting time of blood; both western and southern venoms were found to exhibit anticoagulant activity in contrast to procoagulant activity shown by eastern India *N. naja* venom [9].

The monovalent antivenom raised against eastern India *N. naja* venom failed to neutralize the toxicity induced by *N. naja* venoms from western and southern regions of the country [9,113]. Similar observation was made for polyvalent antivenom from Haffkine Institute which could neutralize the toxic effects of western *N. naja* venom but failed to do the same for eastern and southern venoms. Hence these findings reinstate that differential venom composition due to geographical location causes inefficient neutralization of venom-induced toxicity by polyvalent antivenoms when administered in envenomed patients [113].

1.5.3 Deciphering the composition of Indian Spectacled Cobra (*Naja naja*) venom: A proteomic approach

Proteomics, a technique to identify proteins by mass spectrometry approach, has played a significant role in recent times in deciphering and quantifying complex biological samples and also has contributed for the fast, easy, and efficient identification of snake venom proteome [114-116]. The process involves two different ways to decipher the venom composition, top-down and bottom-up approaches [116,117]. In the top-down approach, the proteins are separated using multidimensional chromatographic techniques and directly subjected to mass analysis [117,118]; whereas, in bottom-up approach or shotgun proteomic analysis, a complex mixture of proteins is subjected to solution proteolysis, followed by chromatographic separation of peptides prior to MS/MS sequencing [117,119]. Snake venom proteins can be decomplexed using multidimensional chromatography (e.g. size-exclusion chromatography, ion-exchange chromatography or reversed-phase high performance liquid chromatography), or electrophoresis (isoelectric focusing and / or one dimensional PAGE based on molecular weight) followed by tandem mass spectrometry analysis [21,120,121]. In 2003, Nawarak et al. [122] performed the first comparative proteomic analysis of 10

different venoms from viperid and elapid snakes by a combination of RP-HPLC, 1D SDS-PAGE, 2D PAGE, and mass spectrometry. Thereafter, studies have been carried out across the globe to study the protein composition of different snake venoms by proteomic analysis.

N. naja venom from different parts of India, Sri Lanka and Pakistan has been studied using shotgun proteomics in order to decipher the venom proteome [19,21,100,123]. Pooled *N. naja* venoms obtained from Pakistan [101,123], Sri Lanka [19], north-western India [19], eastern India (West Bengal) [17,100], western India (Haffkine Institute, Mumbai), [21] and southern India (Irula Snake Catcher's Society, Tamil Nadu) [107] were analyzed for their proteome composition by LC-MS/MS analysis which detected the presence of 28 or 55, 25, 26, 43 or 52, 54, and 81 proteins, respectively, belonging to different families of snake venom proteins. Tandem mass spectrometry analysis followed by quantitative analysis of *N. naja* venom samples from different parts of Indian sub-continent revealed that *N. naja* venom is abundant in three finger toxins [19,21,100,101]. Further, the number of total proteins identified in *N. naja* venom from different locations varied significantly which reinstates the geographical variation in venom composition of this species of snake [21,101]. Further, this difference in venom composition results in differential pathophysiological symptoms exhibited upon *N. naja* envenomation [21,100,101].

1.6 The blood coagulation cascade to understand the anticoagulant mechanism of *N. naja* venom proteins

The blood coagulation cascade is a complex series of events that leads to the formation of stable fibrin clot. Under normal physiological conditions, the process of hemostasis maintains a balance between blood coagulation at sites of vascular injury and keeping blood in a clot-free state for infusion of blood through organs [124,125]. The coagulation system is responsible for the conversion of soluble fibrinogen into fibrin [126]. The fibrin clot reinforces the platelet plug formed during primary hemostasis. The blood coagulation system follows three pathway – the contact factor or intrinsic pathway, the tissue factor or extrinsic pathway, and the common pathway (Fig 1.6) [127].

The intrinsic pathway consists of a cascade of protease reactions initiated by factors that are present within the blood when they come in contact with a negatively charged surface such as glass or the membrane of an activated platelet. A plasma protein called FXII or Hageman factor gets activated to FXIIa in the presence of a high molecular weight kininogen (HMWK). The FXIIa along with HMWK then converts prekallikrein to kallikrein and FXI to FXIa. Thereafter, by a process of positive feedback, kallikrein accelerates the conversion of FXII to FXIIa. The FXIa proteolytically cleaves FIX to FIXa, which is also a protease. FIXa along with FVIIIa (formed by proteolytic cleavage of FVIII by thrombin), Ca^{2+}, and negatively charged phospholipids forming a tenase complex, converts FX to FXa [125].

During a vascular injury, the blood coagulation cascade follows the tissue factor or extrinsic pathway of coagulation [125]. At the very outset, the coagulation factor VII from the plasma comes in contact with tissue factor (TF) on cells which is released outside the vasculature due to a blood vessel injury, and undergoes activation to form factor VIIa [125]. The factor VIIa / TF complex eventually activates the zymogens, factor IX and factor X to their activated forms, factor IXa and factor Xa, respectively [125].

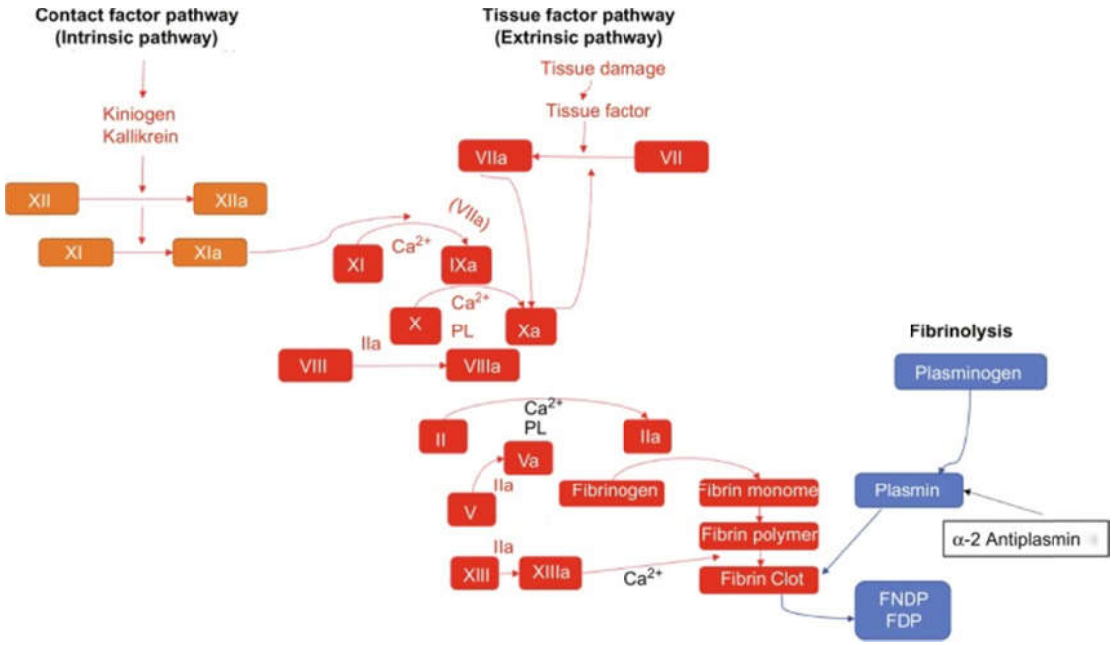

Fig 1.6. A comprehensive representation of the coagulation cascade [127]. [Source of figure: Simmonds et al., In Mechanical Circulatory and Respiratory Support, 2018].

FXa along with factor Va, membrane phospholipids, and Ca^{2+} ions form a prothrombinase complex on the surface of TF-bearing cells which is responsible for the conversion of prothrombin (factor II) to thrombin on the surface of platelets [128,129]. Thrombin, on the other hand, proteolytically cleaves fibrinogen to fibrin which ultimately forms the fibrin clot [125,130]. Besides, thrombin is also responsible for activation of factor V, factor VIII, factor XIII, and platelets to form a platelet plug [131]. Hence, FXa and thrombin are considered as the crucial factors of the coagulation cascade [130,132].

After the blood flow stops, the fibrinolytic system is activated which is a gradual dissolution of the fibrin clot by proteolytic activity of a serine protease, plasmin, which degrades the fibrin network [125,127]. Plasminogen is converted to plasmin by plasminogen activators such as tissue-type plasminogen activator (t-PA) [127]. Haemostatic disorders leading to unwanted thrombus formation in blood vessels may occur due to any minor fault in the fine balance between the coagulation factors, such as thrombin and FXa, and their endogenous inhibitors such as antithrombin III, protein C, and tissue factor pathway inhibitor [125,127].

1.7 Snake venom phospholipase A_2 enzymes

Phospholipase A_2 (PLA_2) enzymes (EC 3.1.1.4) are 119 – 134 amino acid long low molecular weight (13 – 15 kDa) proteins that are ubiquitously present in both intracellular (cytosolic) and extracellular (secretory) forms in almost all animals, including snake venom [133]. PLA_2 specifically hydrolyzes the *sn*-2 ester bond of phospholipids [134] thereby releasing fatty acids and lysophospholipids (Fig. 1.7). Owing to a plethora of pharmacological effects exhibited by snake venom PLA_2 enzymes, they form the most fascinating group of snake venom toxins [133,135-137]. The PLA_2 enzymes initially purified from *N. naja* and *N. tripudians* [138] were called hemolysins due to their ability to rupture erythrocytes. Since then, several snake venom PLA_2 enzymes have been purified and characterized and their primary sequences are reported [133,135,139].

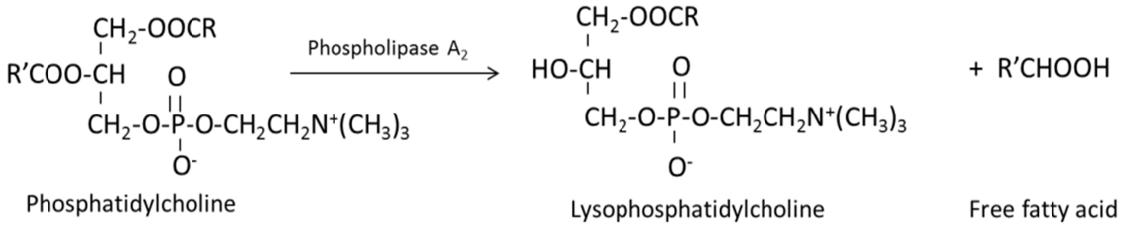

Fig. 1.7. Reaction showing the hydrolysis of phosphatidylcholine by phospholipase A_2.

Although, PLA_2 enzymes exhibit different pharmacological properties, they share 40–99% identity in their amino acid sequences and hence significant similarity in their three dimensional folding (Fig 1.9) [140,141]. Therefore, the functional differences among PLA_2 enzymes cannot be easily correlated to their structural differences [133]. Their structural similarity makes the structure–function relationships in snake venom PLA_2 enzymes elusive, convoluted, and challenging.

1.7.1 Classification of snake venom PLA_2 enzymes

The initial classification PLA_2 enzymes included only 3 groups, viz., secretory PLA_2 ($sPLA_2$), cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), and intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$) [142]. However, another classification of snake venom PLA_2s is based on structural and enzymatic catalysis [133]. There are 15 distinct groups of PLA_2 enzymes present in nature which are broadly clustered under five principal categories – the $sPLA_2$s, the $cPLA_2$s, the $iPLA_2$s, and the PAF acetylhydrolases (PAF-AH), and the lysosomal PLA_2s [143]. The $sPLA_2$s are further divided into 10 groups [143]; however, all snake venom PLA_2s fall only under two groups – Group I and II [143].

1.7.1.1 Group I PLA_2 enzymes

This group of PLA_2 enzyme is predominantly found in mammalian pancreas and venoms of Elapid and Colubrid snakes. They are usually 115 – 120 amino acids long with 7 disulfide bridges. The disulfide bridge between the 11^{th} and 77^{th} cysteine residues is unique to this group. This group of enzyme is further divided into two sub-groups

based on the characteristic surface loop present in snake and mammalian PLA_2 enzymes [139].

Group IA: The characteristic elapid loop in this group of PLA_2 enzymes connect the catalytic α-helix to the β-wing. Most of the elapid venom PLA_2s belong to this group [139].

Group IB: This group of PLA_2 enzymes have an additional 5-amino acid residue (62 – 67) extension, which is called the pancreatic loop, and is found in mammalian pancreas [139]. The groups IB PLA_2s are responsible for digestion of dietery phospholipids [139]. However, this group has also been reported to occur in some snake venoms like *Oxyuranus scutellus* [144], *Pseudonaja textilis* [145], *Notechis scutatus* [146], *Ophiophagus hannah* [147], and *Micrurus frontalis frontalis* [148].

1.7.1.2 Group II PLA_2 enzymes

This sub-group of PLA_2 enzyme is found in the venom of Viperid and Crotalid snakes [139]. Unlike Group I PLA_2s, they do not have a pancreatic or elapid loop, but have an extended C-terminal. The active site in this class of PLA_2 enzymes has an adjacent Cys133 residue which is unique to this group [139].

1.7.2 Structure of PLA_2 enzymes

Snake venom PLA_2s vary in length (119 – 134 amino acids), and are flattened ellipsoids of approximate dimensions 45×30×20 Å [141]. They consist of 14 cysteine residues forming 7 disulfide bonds in order to stabilize the protein structure [149]. The structure of a PLA_2 enzyme comprises of three major α-helices and two antiparallel β-sheets, held by formation of disulfide bridges between them. The N-terminal helix, calcium binding loop, active site, and the β-wing of the PLA_2 structure is conserved in all types of this enzyme (Fig 1.8A-C) [149]. The N-terminal of the enzyme consists of a highly conserved network of hydrogen bonds that stabilize the adjacent β-sheet structure [149]. The strongly hydrophobic catalytic site of the enzyme is characterized by the presence of four key residues: His48, which forms the crucial active site, while it is supported by the hydrogen bonds formed between Asp49, Tyr52, and Asp99 [141]. Two long antiparallel and disulfide linked α-helices form a well-defined, elongated, and

hydrophobic channel around the catalytic site. The calcium binding loop of the PLA_2 enzyme is another highly conserved structure and comprises of residues Tyr28, Gly30, and Gly32 [141].

The His residue hydrogen binds the water molecule used for hydrolysis, whilst Asp49 coordinates and positions the Ca^{2+} ion which binds and polarizes both the phosphate and the *sn*-2 carbonyl groups of the phospholipid molecule during hydrolysis [141]. The calcium-binding loop, comprising of Tyr28, Gly30 and Gly32, in combination with Asp49, binds the Ca^{2+} ion required for catalysis [141]. In addition, there is an interfacial binding surface, which mediates the adsorption of the enzyme onto the lipid-water interface of the phospholipid membrane bilayer, strongly promoted by anionic amphipathic molecules such as fatty acids (FAs) [140]. These features are common to both group I and group II venom PLA_2s. However, they differ only in the position of one of their seven disulfide bonds, by the presence of the pancreatic loop in some of group I enzymes and by an extended C-terminal loop in the group II counterparts [140]. One of the PLA_2 enzyme isoforms (P15445) of *N. naja* venom has been reported to form an oligomeric association with two other molecules of the same enzyme, thereby forming a trimer (Fig 1.8D) [150]. It is noteworthy that the *N. naja* venom PLA_2 monomer (PDB ID: 1A3D) and its corresponding trimer (PDB ID: 1A3F) are the only reported PLA_2 structures reported from the Indian cobra venom (Fig 1.8D) [150].

There is a distinct subgroup of PLA_2 homologue toxins wherein the key Asp49 residue is substituted with a Lys, Arg, Ser, Gln, or Asn residue, among other substitutions, with the consequent loss of Ca^{2+}-binding and enzymatic activity [141]. Most of these PLA_2s are catalytically inert (inactive) and belong to myotoxic Lys49 PLA_2 homologues [151]. The major toxicity determining site in these PLA_2 homologues encompasses residues 115 – 129 in the C-terminal region, which includes a variable combination of positively charged and hydrophobic / aromatic residues [151,152].

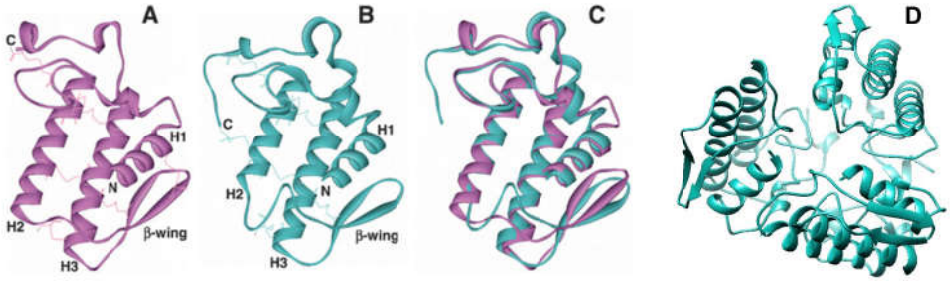

Figure 1.8. Three-dimensional structures of snake venom PLA_2s [141,150]. Ribbon representations of **A.** notexin (1AE7), a group I PLA_2 from *Notechis scutatus scutatus* venom [153] and **B.** D49 basic PLA_2 (1VAP), a group II enzyme from *Agkistrodon piscivorus piscivorus* venom [154]. Disulfide bonds are shown in stick representation. Superposition of both structures **C.** illustrates the conservation in the calcium-binding loop and the three major helices (H1, H2, H3), despite evident deviations in the other interconnecting loops and the β-wing. The C-terminal is extended in the group II enzymes (B) in comparison to group I (A). N- and C-terminals are indicated by N and C, respectively. **D.** Ribbon representation of a trimeric association of group I PLA_2 (accession no. P15445; PDB ID 1A3D) from *Naja naja* venom [150]. [Source of figure: Figures (A-C) have been adapted from Montecucco et al., Cell and Molecular Life Sciences, 2008; figure D has been constructed using PDB structure 1A3F from RCSB database in UCSF Chimera software].

1.7.3 Catalytic property of snake venom PLA_2 enzymes

PLA_2 enzymes catalyze water insoluble phospholipids preferentially at water-phospholipid interfaces, known as 'interfacial catalysis', to liberate free fatty acids (FFAs) and lysophospholipids [155]. The model of interfacial catalysis was proposed by Winget and co-workers [156], according to which the PLA_2 enzyme (E) binds to the substrate (S) at the interface as the E* form resulting in formation of a transient enzyme-substrate (E*-S) interface-bound form. An additional anion activating step occurs at this stage where an anionic phospholipid(s) interacts at the interface of the enzyme, forming the $(E^*\text{-}S)^{\#}$ complex. Once activated, the PLA_2 then catalyze the formation of the enzyme-product (E*-P) complex, and thereafter release the products (lysophospholipids and fatty acids). After release of the product, the E* diffuses in a rushing mode in order to bind with another substrate (S) to initiate the next cycle of catalysis [156].

The active site of the PLA_2 molecule consists of four residues: His48, Asp49, Tyr52, and Asp99 (Fig 1.9) [157]. The side chains of His48 and Asp49 residues are connected to a conserved water molecule through hydrogen bonds, essential for substrate hydrolysis [157]. The mechanism of PLA_2 hydrolysis of phospholipids involves the highly specific interaction between the active site His48, the Ca^{2+} ion as cofactor, conserved water, and the glycerophospholipids substrate [135]. During catalysis, His48, assisted by Asp99, polarize the bound water molecule, which then attacks the *sn*-2 bond of the bound phospholipid to form a tetrahedral oxyanion intermediate [139,140].

Edwards and co-workers have proposed an alternative mechanism of PLA_2 catalysis, where two more water molecules are involved in the formation and breakdown of the tetrahedral intermediate [158]. In both mechanisms, Ca^{2+} ion, coordinated by the oxygen atom of Asp99, serves as an electrophile during catalysis [140,158]. Overall, catalysis by $sPLA_2$ enzymes can be summarized [159] in the following four steps:

1. Binding of Ca^{2+} and substrate;
2. General base-mediated catalysis;
3. Formation and breakdown of tetrahedral intermediate, and
4. Release of the reaction products.

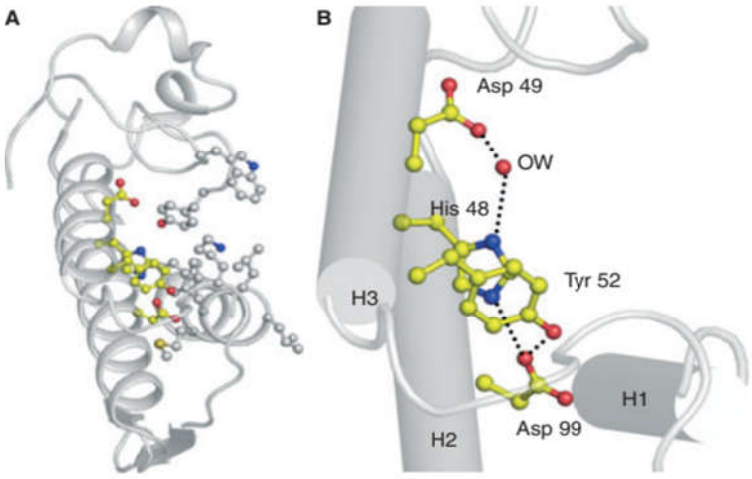

Fig. 1.9. The three-dimensional structure of active site of PLA_2 [157]. A. A view of the PLA_2 structure showing active site residues in yellow. The substrate diffusion channel with hydrophobic residues Leu2, Leu3, Phe5, Ile9, Tyr22, Trp31, and Lys69 is

also seen. **B.** The catalytic network in PLA_2 is shown. OW indicates a water molecule oxygen atom which serves as the nucleophile. The dotted lines indicate hydrogen bonds. [Source of figure: Kang et al., The FEBS Journal, 2011].

1.7.4 Pharmacological effects of snake venom PLA_2 enzymes

Despite sharing 40-99% sequence similarity and having a similar three-dimensional structure, venom PLA_2s exert an amazing variety of toxic and pharmacological effects, which include neurotoxic, myotoxic, hemolytic, edematogenic, hyperalgesic, pro-inflammatory, hypotensive, platelet aggregation inhibitory, anticoagulant, cytotoxic, and bactericidal activities [112,126,133,160-170]. The PLA_2 enzymes post binding to their target(s) can induce several pharmacological effects in victim / prey via different mechanisms that are either dependent or independent of their enzymatic activity [87,89,111,112,133,135,161,165-167,171-175]. By virtue of enzymatic mechanism, PLA_2 enzymes can either hydrolyze intact phospholipids or the released products such as lysophospholipids and fatty acids, in order to cause a pharmacological effect [135,141,176]. The characteristic enzymatic activity can cause phospholipid damage in blood plasma [110,112,162,163,171] or cell membranes [135,160,161,164] and hence, alter the environment of membrane bound proteins, particularly that of the target protein. On the other hand, mechanisms of action that are independent of enzymatic activity, the PLA_2 enzyme can bind to the target protein and exhibit a pharmacological effect by acting as an agonist or an antagonist, or by interfering in the interaction of the target protein with its physiologic ligands [111,112,135,163,171,177]. The myotoxic and anticoagulant pharmacological effects of snake venom PLA_2s are discussed below:

1.7.4.1 Myotoxic effect of PLA_2 enzymes

A number of factors such as membrane surface properties, including membrane phospholipid composition, membrane fluidity, curvature, surface charge, and membrane induced structural changes in the enzyme, determine the strength of interaction, binding to membrane, and the extent of PLA_2 hydrolytic activity on membranes [160,161,164]. The Lys49 PLA_2 myotoxins, devoid of enzymatic activity [140,152,178], comprise of a highly cationic surface [179-181], which preferentially disrupt the integrity of negatively-charged liposomes [181,182] and thus the membrane integrity. There is a

strict connectivity between PLA_2 activity, specificity, and affinity of binding of myotoxic PLA_2s to the 'microdomains' of the sarcolemma, that are particularly sensitive to the presence of lysophospholipids and fatty acids, or where their activity is maximized and causes relevant derangement to the plasma membrane organization [135,141]. Therefore, it may be hypothesized that the negatively-charged microdomains in cell membranes constitute 'acceptor sites' for this group of basic myotoxins [135,141].

When the plasma membrane of muscle fibers is damaged or provoked either by catalytically-dependent or -independent mechanisms of PLA_2s, it triggers a rapid sequence of degenerative events that takes the muscle cells beyond 'the point of no return' within minutes [141]. Such rapid membrane damage with rapid depolarization of muscle fibers [183-186] is accompanied by a prominent influx of Ca^{2+} [187,188], which subsequently promotes a complex series of cellular derangements ultimately leading to necrosis [141]. Cell necrosis and the release of intracellular contents from necrotic cells are accompanied by the onset of a prominent inflammatory reaction, with the synthesis and release of mediators that promote increments in vascular permeability leading to edema and recruitment of neutrophils and macrophages [186]. This opens the possibility that these inflammatory cells contribute to further tissue damage [141].

Apart from phospholipids, studies have also revealed that snake venom PLA_2s bind to certain mysterious PLA_2 receptors or acceptors on the plasma membranes which may or may not be directly involved in the toxic effects [141]. Lambeau and colleagues [173,189,190] demonstrated the high affinity binding of two high molecular weight (180 kDa) monomeric M-type membrane protein (OS1 and OS2) in rabbit myotubes to a PLA_2 from *Oxyuranus scutellatus* venom. These receptors showed similarity to rabbit nerve cell membrane receptors, and their expression is regulated and influenced by factors such as innervation, growth factors, hormones and / or muscle contractile activity [189,190]. Another Lys49 myotoxic PLA_2 from venom of *Agkistrodon piscivorus piscivorus* was shown to bind to a kinase insert domain containing receptor (KDR) [141,191]. However, the exact role of PLA_2-membrane receptors interaction may not always demonstrate a functional relevance and hence their significance in pathophysiology remains unknown till date [192]. In a very recent study by Massimino

and co-workers [193], it was demonstrated that a myotoxic Lys49 PLA_2 (Mt-II) from *Bothrops asper* binds to membrane-bound nucleolin, a protein found in nucleolus, at low temperatures. At physiological temperature, Mt-II bound to nucleolin internalize and co-localize in paranuclear and nuclear areas of myotubes, thereby exhibiting a role of this association in myotoxic property of Mt-II [193].

1.7.4.2 Anticoagulant effect of PLA_2 enzymes

1.7.4.2.1 Types of anticoagulant PLA_2

The anticoagulant activity of snake venom PLA_2 enzyme was first reported by Boffa and Boffa [172]. Based on their anticoagulant potency, PLA_2 enzymes have been classified into strong, weak, and non-anticoagulant enzymes [112,135,171,172,194].

A. Strongly anticoagulant PLA_2 enzymes: These enzymes inhibit blood coagulation at a concentration as low as ~2.0 μg/ml. Such PLA_2 enzymes are reportedly present in the venom of *Naja nigricollis*, *N. m. mossambica*, *N. kaouthia*, *N. haje*, *Vipera berus orientale*, *D. russelii*, *Agkistrodon halys blomhoffi*, and *Crotalus durissus terrificus* enzymes.

B. Weakly anticoagulant PLA_2 enzymes: This group of PLA_2 enzymes shows anticoagulant effects between 3 and 10 μg/ml. Such PLA_2 enzymes have been found to be present in the venom of *N. mossambica*, *N. nigricollis*, *A. h. blomhoffi*, *Enhydrina schistose*, and *Oxyuranus scutellatus*.

C. Non-anticoagulant PLA_2 enzymes: This group of PLA_2 enzymes may have a little effect on the clotting times at concentrations as high as 15 μg/ml. They are reported to be present in the venoms of *N. m. mossambica*, *N. naja*, *N. melanoleuca*, *A. h. blomhoffi*, *Hemachatus haemachatus*, *Bitis gabonica*, *Crotalus admanteus*, *C. durissus terrificus*, *Vipera aspis*, *Notechis scutatus*, and *Bungarus multicinctus*.

1.7.4.2.2 Mechanism of anticoagulant action

The anticoagulant activity of snake venom PLA_2 enzymes is dependent on two mechanisms – (i) the enzymatic mechanism, and (ii) the non-enzymatic mechanism [135,176]. In the former method, the anticoagulant activity is dependent on the plasma

phospholipids hydrolytic activity of the PLA_2 enzyme and this constitutes the primary mechanism of anticoagulant action [135,176]. Plasma phospholipids play an important role in the blood coagulation process as it is an integral part of the tenase complexes (Fig 1.5) [125]. By virtue of its catalytic activity, snake venom PLA_2s hydrolyze the procoagulant plasma phospholipids, thereby obliterating the normal coagulation process [110,162,163,171,176,195,196]. However, the weak anticoagulant enzymes inhibit the extrinsic pathway of blood clotting [109]. The strong anticoagulant PLA_2s are capable of exhibiting a non-enzymatic mechanism of anticoagulation by interacting with blood coagulation factors [109,111,112,163,171]. The strongly anticoagulant PLA_2s bind to the FXa [109,112] or thrombin [111,112,171], thereby hindering the process of normal coagulation. The anticoagulant effect of PLA_2 enzymes can also be correlated with their ability to penetrate phospholipid [194]. Strongly anticoagulant PLA_2 enzymes exhibit high penetrating ability, whereas non-anticoagulant ones show weak penetrating ability [176] and hence cannot hydrolyze the plasma phospholipids.

1.7.5 Pharmacological site and target specificity of snake venom PLA_2

Snake venom PLA_2 enzymes exhibit a diverse set of pharmacological effects [133,135,197]. However, not all PLA_2 enzymes can exhibit different types of pharmacological properties. PLA_2 enzymes bind to target proteins through specific pharmacological sites [133,157,171,177]. Chemical modification studies, polyclonal and monoclonal antibodies, and interaction of inhibitors have supported the presence of pharmacological sites in PLA_2 enzymes [112,135,163,171,198]. The site varies for PLA_2 enzymes exhibiting different pharmacological effects [133].

Thus, the significance of identification of such pharmacological sites of snake venom PLA_2 enzymes can be summarized as – (i) understanding the structure–function relationships of PLA_2 enzymes; (ii) developing strategies to neutralize the toxicity and pharmacological effects by targeting these sites; and (iii) developing prototypes of novel research tools and pharmaceutical drugs [133].

1.7.5.1 Pharmacological site for myotoxic PLA_2

The 'myotoxic site' of snake venom PLA_2s has been predicted to be present at the amino terminal side of helix E of the enzyme with a characteristic cationic charge of

'+00+++00+' [199]. However, Lomonte and co-workers identified the C-terminal cationic / hydrophobic segment (115–129) as the heparin-binding and cytolytic site of K-49 PLA_2 enzymes which is responsible for skeletal muscle necrosis [184,200]. Further Gly30 and Asp49 residues present within or in close proximity to the calcium binding loop of PLA_2 enzymes are also responsible for myotoxic effects, thereby forming the 'myotoxic site' [190].

1.7.5.2 Pharmacological site for anticoagulant PLA_2

A systematic and direct comparison of the amino acid sequences of strong, weak, and non-anticoagulant enzymes demonstrated that the anticoagulant region of PLA_2 enzymes lies within residues 54 and 77 [109,201-203]. Not the overall charge, but the charge of the anticoagulant site determines the anticoagulant potency of a PLA_2 enzyme [109,176]. This anticoagulant site is positively charged in strongly anticoagulant PLA_2 enzymes, and is flanked with a pair of Lys residues on both sides; however, the anticoagulant region is replaced by neutral or negatively charged amino acid residues in weak and non-anticoagulant PLA_2 enzymes [109]. The major replacements of residues in the strong anticoagulant PLA_2 by those in the weak and non-anticoagulant PLA_2 are: (1) negatively charged Glu54 is replaced by neutral residues; (2) positively charged Lys55 is replaced by negatively charged Glu; (3) uncharged Gly57 is replaced by negatively charged Glu; (4) positively charged Lys75 is replaced by Ser or Thr; (5) positively charged Lys77 is replaced by negatively charged Glu or Asp [109,133,176]. The prediction of anticoagulant site of PLA_2 enzymes is strongly supported by chemical modification studies [109], site-directed mutagenesis [204,205], and using synthetic peptides [206].

1.7.6 Evolution of snake venom PLA_2 enzymes

Phylogenetic analysis has shown that snake venom PLA_2 and mammalian pancreatic PLA_2 enzymes share a common origin from a non-toxic ancestor gene [207]. Although, snake venom group I PLA_2 enzymes and pancreatic PLA_2 enzymes (group IB) have evolved through a common series of adaptations [207], however, group II PLA_2 genes evolved separately after species diversification [208,209]. Although both group I and II PLA_2 enzymes demonstrate similar catalytic activity, they differ

structurally in their gene organization [207,210]. Group I PLA_2 genes are approximately 4 kb in size with 4 exons interrupted by introns [207], while group II PLA_2 have 5 exons [211]. The group I and II PLA_2 gene structures show similarity with pancreatic PLA_2 gene and synovial PLA_2 genes, respectively [207,212]. In snake venom group I PLA_2 genes, the exon 3 is smaller as compared to its pancreatic counterpart [213], which is attributed to adaptive evolution of snake venom genes. The exons 3 and 4 of these evolved snake venom PLA_2 genes reportedly accounts for the different pharmacological traits exhibited by snake venom PLA_2 enzymes [210]. The pancreatic loop, encoded by exon 3, is a characteristic feature of mammalian PLA_2 enzymes and some snake venom PLA_2s, while it is absent in majority of snake venom PLA_2 enzymes [213]. The loss of pancreatic loop in snake venom PLA_2 enzymes has followed a Darwinian mode of evolution [139]. The loss of exon 3 in snake venom PLA_2 genes is interpreted to encode for higher adaptability of the gene, resulting in enhanced toxicity and enzymatic activity of snake venom PLA_2 enzymes [210,213].

A snake venom PLA_2 enzyme reportedly consists of several mutational hotspots on the surface which defines the property of the enzyme. As compared to the buried residues, the fully exposed surface residues undergo mutation at a rate 2.6 – 3.5 times higher than the former [133]. In yet another hypothesis, a large set of PLA_2 enzyme gene and protein sequences were compared which confirmed that in group I PLA_2 enzymes gene duplication and diversification occurred after speciation; whereas in group II PLA_2 enzymes, functional diversification occurred before species diversification [214]. Therefore, accelerated evolution of exons, leading to mutations / substitutions in the molecular surface residues contribute directly to formation of different isoforms of the same enzyme with modified molecular surface, and hence differential pharmacological properties [133,212,215].

1.8 Snake venom toxin synergism and PLA_2 complexes

1.8.1 Snake venom toxin synergism

Toxin synergism is an interesting phenomenon observed in snake venom. Lausten has defined toxin synergism as a phenomenon where two or more venom components interact directly or indirectly to potentiate toxicity to levels above the sum

of their individual toxicities. The production of snake venom and its replenishment is an expensive affair for the snake with high metabolic cost [6]. Under such circumstances, toxin synergism directly contributes to enhanced toxicity of the venom [216]. Based on molecular perspective, snake venom toxin synergism is broadly divided into two types – intermolecular synergism and supramolecular synergism [216].

When two or more toxins interact with two or more targets on one or more related biological pathways, causing synergistically increased toxicity, it is referred to as intermolecular synergism (Fig 1.10 A,B,C) [216]. This type of synergism may exist in toxins that may either act on different targets of a physiological process causing a combined synergistic effect (Figure 1.10A) or different physiological processes, which subsequently affect another downstream physiological pathway with enhanced potency (Figure 1.10B). An example of intermolecular synergism is illustrated by the venom of *Dendroaspis polylepis* (black mamba) [217]. The dendrotoxins of *D. polylepsis* venom exhibit an excitatory effect on the neuromuscular system, which is further complemented with a rapid abrogation of neuromuscular function by α-neurotoxins [217]. Another example of toxin synergism is depicted by Asp49 and Lys49 myotoxins of *Bothrops asper* venom, responsible for synergistic myonecrotic effects on myotubes [218]. Such myotoxins reportedly increase Ca^{2+} influx through the plasma membrane thereby causing rapid cell death for myotubes [216,219], which is a scenario depicted in Fig 1.10C.

On the other hand, supramolecular synergism refers to a condition where the toxins form complexes with synergistic effects to create a hyper-potentiated toxin (Fig 1.10D) [216]. Supramolecular synergism is best illustrated by cytotoxins of cobra venom [216]. Cobra cytotoxins are reported to enhance the PLA_2 activity through complex formation and destabilization of cell membranes, causing cellular lysis due to hydrolysis of phospholipids [165,220-224]. Most of the snake venom complexes described in section 1.8.1 represent supramolecular synergism [144,145,216,225-227].

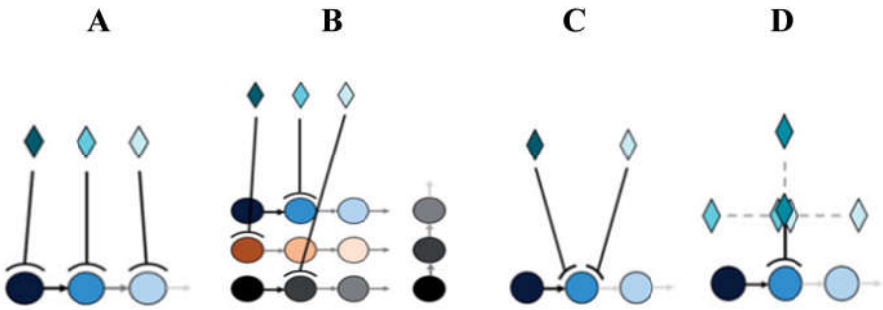

Figure 1.10. Schematic representation of theoretical scenarios in which synergism between toxins occur in snake venom [216]. Models for intermolecular synergism – **A.** when toxins target different targets on the same physiological pathway, **B.** when toxins target different pathways which all regulate or interact with another physiological pathway, **C.** when toxins target the same target in a cooperative manner; and supramolecular synergism – **D.** supramolecular synergism – when toxins combine to form an oligomeric toxin that has a toxicity, which is higher than the combined toxicities of the individual components. [Source of figure: Laustsen, Toxin Reviews, 2016].

1.8.2 Protein complexes of snake venom PLA_2 enzymes

Phospholipase A_2 group of enzymes are one of the most widely studied proteins from snake venom to date [93,112,133,135,139-141,155,160,165,170,228,229]. This class of snake venom proteins occur both in monomeric as well as multimeric forms by forming complexes with other venom proteins, such as same or other PLA_2, serine protease inhibitors (SPI), and three finger toxins (3FTx) [165,220,222,228,230]. Broadly, the snake venom PLA_2 enzymes tend to form two types of protein complexes in nature – the covalent complexes and the non-covalent complexes [228].

1.8.2.1 Covalent complexes of snake venom PLA_2

This class of PLA_2 complexes comprises of two chain – chain A which is a group I PLA_2 enzyme, and chain B which is either a SPI and / or dendrotoxin (Fig 1.11A), that are covalently linked by formation of disulfide bridges between them (Fig 1.11B) [228,231,232]. Isoforms of β-bungarotoxins (Fig 1.11A) isolated from *B. multicinctus* are an example of covalent complex of snake venom PLA_2 isoforms with

SPIs [233]. The β-bungarotoxin binds to the presynaptic site at the neuromuscular junction and gradually disrupts the neurotransmission, finally leading to paralysis [229].

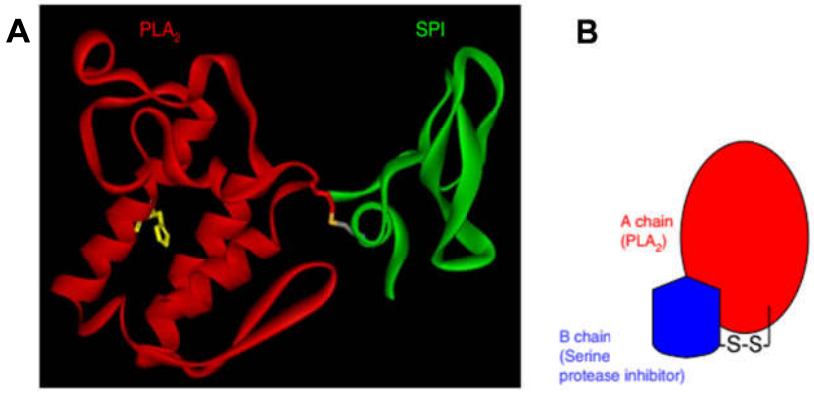

Fig 1.11A. Ribbon model of β-bungarotoxin (β-BuTx) (PDB ID: 1BUN) showing the disulphide linkage between chain A and chain B [228]. B. Schematic representation of β-bungarotoxin, a covalent snake venom PLA_2 complex [228]. [Source of figure: Doley and Kini, Cell and Molecular Life Sciences, 2009]

1.8.2.2 Non-covalent complexes of snake venom PLA_2

Most of the naturally occurring snake venom PLA_2 complexes belong to the non-covalent group of complexes [165,219,221,222,228,234]. In these complexes, the PLA_2 enzymes interact with the other isoform of the same or different PLA_2 and / or other venom toxins via non-covalent interactions [165,228]. The non-covalent PLA_2 complexes may be homodimeric, heterodimeric, heterotrimeric, heteropentameric or oligomeric based on the number of subunits present (Fig 1.12A-D) [228]. Most of these toxins are neurotoxins that exhibit pre- or post-synaptic neurotoxicity in animal models [144,226,228,235-238]. Some examples of such toxins include trimucrotoxin (homodimeric), crotoxin (heterodimeric), taipoxin (heterotrimeric), textilotoxin (heteropentameric) and their isoforms (Fig 1.12A-D) [228]. Nk-PLA_2-I – kaouthiotoxin complex [165] is another example of a non-covalent protein complex that exhibits cell-specific toxicity on different cells [165]. Oligomeric PLA_2 complexes, on the other hand, are non-neurotoxic complexes and mostly act as Ca^{2+} channel blockers, for example taicatoxin (Fig 1.12E) [228].

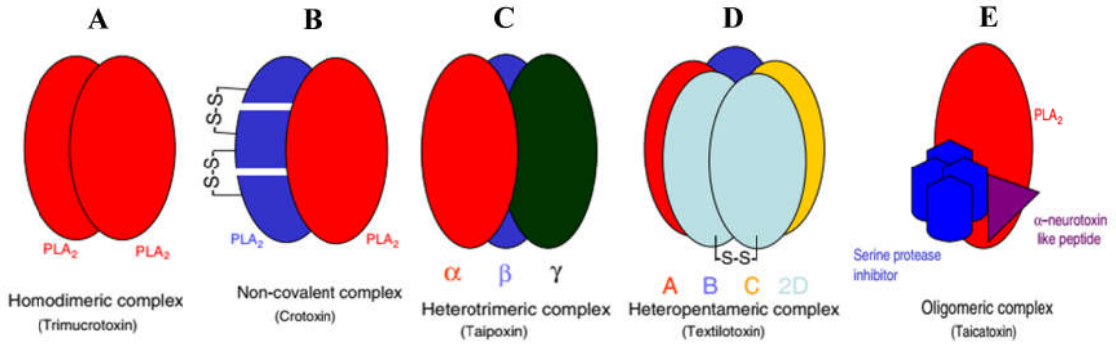

Fig 1.12. Schematic representation of different types of non-covalent snake venom PLA_2 complexes [228]. [Source of figure: Doley and Kini, Cell and Molecular Life Sciences, 2009].

1.9. Thrombosis associated cardiovascular disorders and their treatments

1.9.1 Thrombosis leads to cardiovascular diseases

Thrombosis refers to the medical condition wherein the normal blood flow in the blood vessels is altered due to physiological changes in any two of the three elements of Virchow's triad [239-241]. Rudolf Virchow (1821 – 1902), coined the terms '*thrombosis*' and '*embolism*' and proposed that *de novo* clot formation in veins and arteries is nothing but a myth; instead it is a result of thrombus formation in the peripheral vascular system that leads to clogging of veins and arteries [239]. Henceforth he postulated that thrombi formation is influenced by hypercoagulability (thrombophilia), endothelial injury, and blood stasis [239,240].

The first element, hypercoagulability, occurs when blood starts to clot when flowing through the blood vessels due to certain inheritable conditions such as Factor V Leiden (the most common), prothrombin gene mutation, deficiencies of antithrombin / protein C / S, elevated levels of homocysteine / fibrinogen / factor VIII / IX / XI and abnormal fibrinolytic system, or acquired conditions such as cancer and its medication, surgery / trauma, obesity, pregnancy or use of contraceptive pills, immobility, heparin-induced thrombocytopenia (HIT), HIV infection or AIDS, antiphospholipid syndrome, heart attack and nephritic syndrome [242,243]. The results of these conditions may lead to protein loss, neoplasia, hepatobiliary diseases, hyperadrenocorticism, and systemic inflammation resulting in sepsis or pancreatitis [240,243]. Increased platelet and

leukocyte recruitment in blood vessels, cytokine-mediated tissue factor expression or increased activation / concentration of activated coagulation factors is a result of endothelial injury, which is the second element of the Virchow's triad [244,245]. Alteration in the blood flow stasis, the third element of the triad, results in various physiological conditions such as hypovolemia, vascular anomalies, cardiomyopathy, hyperviscosity disorders (polycythemia, multiple myeloma), and neoplasia [246].

Occurrence of thrombosis in human is associated with extremely high risk of cardiovascular diseases (CVDs) like cerebral and myocardial infarction, ischemic stroke and heart attack [240,247,248] which ultimately may either result in death or paralysis of a part / whole body of the victim. As per the estimates of WHO, approximately 17.9 million people die due to CVDs every year which accounts for 31% of the annual global deaths [249].

1.9.2 Treatment for CVDs and limitations of commercial drugs

Currently available treatment for cardiovascular disease includes administration of antiplatelet, anticoagulant, and / or thrombolytic drugs [245,250]. The antiplatelet drugs target receptors expressed during activation of platelets (Fig 1.13), thereby preventing formation of a stable blood clot [245,251]. On the other hand, the anticoagulant drugs or blood thinners target different factors of the blood coagulation cascade, such as thrombin, factor X / Xa, fibrinogen, factor V / Va and others, thereby inhibiting their activation and delaying the blood coagulation process [245,252,253]. A few anticoagulant drugs and their targets are summarized in a schematic representation in Fig 1.14. The third class of drugs used for treatment of CVDs is thrombolytic or clot-bursting drugs which act by dissolving the fibrin clot formed in the blood vessels [254,255]. They act either directly by breaking the blood clot (for example plasmin) or indirectly by activating plasminogen to plasmin, which in turn causes fibrinolysis (for example, tissue plasminogen activator, urokinase, nattokinase and streptokinase) [254,256].

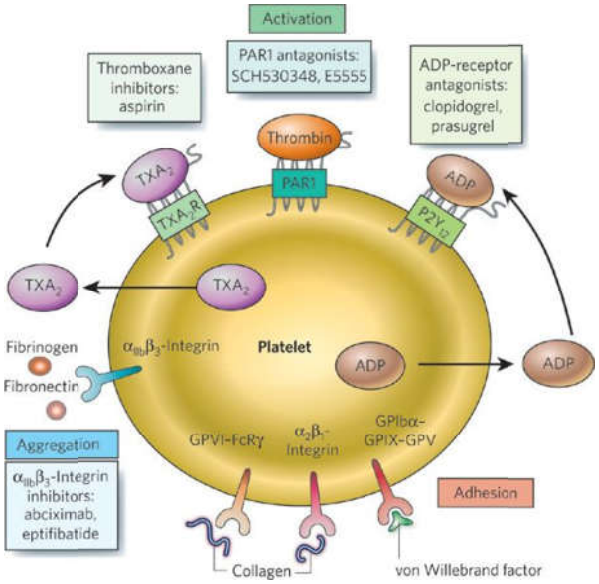

Fig 1.13. A schematic representation of targets of antiplatelet drugs [245]. The ligands for various receptors are shown. Antiplatelet drugs and their targets are also indicated; targets include thromboxane A2 (TXA2), protease-activated receptor 1 (PAR1), the ADP receptor P_2Y_{12} and αIIbβ3-integrin. [Source of figure: Mackman, Nature, 2008].

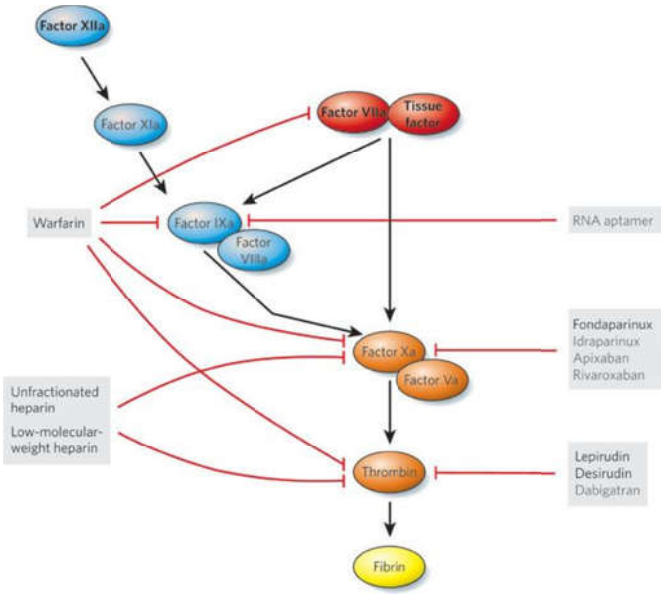

Fig 1.14. A schematic representation of targets of anticoagulant drugs [245]. [Source of figure: Mackman, Nature, 2008].

However, these commercial cardiovascular drugs are often associated with several shortcomings. The antiplatelet and anticoagulant drugs are reported to show

hypersensitivity in administered patients [257]. Antiplatelet drugs such as cyclooxygenase and P_2Y_{12} inhibitors are associated with immediate-type hypersensitivity reactions, for example, gastrointestinal symptoms, headache, drowsiness, dizziness, exacerbation of bronchial asthma, aspirin-exacerbated respiratory disease (AERD), chronic urticarial, anaphylactoid reactions of all degrees of severity like cardiovascular shock, and delayed-type allergic reactions in the form of exanthemas, phototoxic reactions and, rarely, severe bullous reactions [257]. Further, pancytopenia or neutropenia and / or hepatitis, suberythrodermic pustular psoriasis or exanthematous pustulosis have also been reported in patients administered with the antiplatelet drug clopidogrel [257-260]. Further, administration of classical anticoagulants such as heparin and warfarin are associated with several impediments, such as need for intensive coagulation monitoring, wide variation in dose-response relationships, gastrointestinal hemorrhage, long clearance time, multiple drug interactions (in the case of warfarin), and serious immune-mediated thrombocytopenia (in the case of heparin) [261-263].

1.10 Therapeutic application of snake venom anticoagulant proteins

Classical anticoagulants such as heparin and warfarin are associated with several severe complications (section 1.9.2), which therefore leads to a search for alternative anticoagulants from natural resources. Potent anticoagulant proteins and peptides derived from snake venom are known to exhibit thrombin and / or FXa inhibition, such as, PLA_2 [111,112,171,264], C-type lectins [265], KSPIs [234,266], snake venom proteases [267-269], and other low molecular weight polypeptides [270]. Further, there is a constant increase in demand for low / non-toxic anticoagulant alternatives for therapeutic use in the prevention and treatment of thrombosis and associated CVDs.

Snake venom thrombin-like enzymes (SVTLEs) are single chain serine proteases with a catalytic triad of His57-Asp102-Ser195 residues which have found extensive use as ‘defibrinogenating agents’ to achieve ‘therapeutic defibrination’ [267,271]. Most of the characterized SVTLEs cleave the fibrinopeptide A chain from fibrinogen, whereas, there are a few which also cleave the fibrinopeptide B chain like thrombin [271]. Further, SVTLEs are resistant to heparin, due to which they have also found use in detection of fibrinogen dysfunction in normal and heparin-containing blood samples

[272]. The most widely used thrombin-like enzymes - *Bothrops atrox* venom (Batroxobin, Reptilase®) (Pentapharm, Basel, Switzerland) and from *Callosellasma rhodostoma* venom (Ancrod) (Knoll, Ludwigshafen, Germany) are being successfully used to treat ischemic heart diseases, deep vein thrombosis, and peripheral occlusive disorders [271,273].

In addition, fibrinolytic proteases from snake venoms, such as fibrolase have also been explored for their antithrombotic and thrombolytic potential, although its recombinant form failed as a potential drug in the phase II clinical trial [274,275]. The disintegrins, which is a large family of Arg-Gly-Asp (RGD)-containing snake venom proteins are reportedly potential platelet modulators that act by binding to platelet the glycoprotein receptor – GPIIb/IIIa and Ib [271].

A few drugs derived from snake venom for the treatment of thromboembolic disorders are summarized in table 1.2.

Table 1.2. Approved drugs, originating from snake venoms, for treating various thrombo-embolic disorders [276]. Snake venom proteins and their derivatives that are used to treat different haemostatic disorders by targeting coagulation, fibrinolysis or platelet functions.

Protein / Drug name	Structural characteristics	Origin	Mechanism of action	Treatment
Tirofiban / Aggrastat®	Non-peptide synthetic structure mimicking the RGD motif of disintegrin echistatin	*Echis carinatus*	Inhibition of platelet aggregation by blocking $\alpha_{IIb}/\beta_{IIIa}$	Acute coronary syndrome, prevention of thrombotic complications after percutaneous coronary interventions (balloon angioplasty, stenting, etc.)
Eptifibatide / Integrilin®	Cyclic peptide based on KGD structure of	*Sisturus miliarus barbouri*	Inhibition of platelet aggregation by	

Protein / Drug name	Structural characteristics	Origin	Mechanism of action	Treatment
	disintegrin barbourin		blocking $\alpha_{IIb}/\beta_{IIIa}$	
Batroxobin / Defibrase®	SVTLE-A	*Bothrops atrox moojeni*	Conversion of fibrinogen to fibrin – release of fibrinopeptide A	Acute cerebral infarction, unspecific angina pectoris, sudden deafness
Haemocoagulase®	Mixture of two enzymes with thromboplastin-like and thrombin-like activity	*Bothrops atrox*	Clot formation	Prevention and treatment of haemorrhage

1.11 Peptides as drugs: Emergence and advantages

To date, over 7000 naturally occurring peptides having vital physiological roles have been identified, such as hormonal, neurotransmitters, growth factors, ion channel ligands, or anti-infective [277-280]. Peptides are selective and efficient signaling molecules that target various cell surface receptors and ion channels in order to trigger an intracellular signal, and thereby have very few or no off-target side-effects [280,281]. Owing to their attractive pharmacological profile and intrinsic properties, peptides are considered an excellent starting point for the design of novel therapeutics and their specificity has led to translate into excellent safety, tolerability, and efficacy profiles in humans [280,281]. Studies have shown that peptides have gained a wide range of applications in medicine and biotechnology [280-282]. Currently, the peptide research for development of therapeutic agents is experiencing a renaissance for commercialization [280].

With recent advancement in technology, the application of peptides as therapeutics has gained a significant momentum [281]. Peptides as drug are potent, effective, safe, tolerant, highly selective towards their target, and have lower attrition rates [280]. The peptides can be easily synthesized or cloned and expressed in an expression system and can easily be commercialized [280]. In addition to direct application, peptides can be administered in conjugation with other peptides or existing drugs in well-defined formulations [280]. To date, only two antiplatelet drugs have been successfully derived from disintegrins of viperid venom [283] and one bioactive peptide has been derived from *Agkistrodon acutus* venom hydrolysates with potential antithrombotic potency [284]. Although, few synthetic anticoagulant peptides have been derived from hirudin [285]; however, no such attempt has been made to derive such peptides from snake venom anticoagulant proteins.

However, a significant limitation of peptide therapeutics is their short half-lives [281,286]. Peptides generally get cleared from the bloodstream within minutes to hours after administration, due to which it is feared that there can be insufficient exposure in the target tissue to have a significant physiological effect. Short peptide half-lives typically result from fast renal clearance (for peptides <5 kDa) and / or from enzymatic digestion in the blood, kidneys or liver [286]. However, several strategies have been formulated to increase the plasma residence time of peptides such as attachment of inert polymers (e.g. polyethylene glycol or PEG), addition of sugars (trehalose, sucrose, maltose or glucose) or salts (potassium phosphate, sodium citrate or ammonium sulphate), or heparin or chelating agents (EDTA) can enhance the thermal stability of peptides and proteins, cause self-association, modulate solubility, and protect from degradation by proteolytic enzymes [286-288].

Some examples of successful stories of peptide therapeutics include oxytocin (8 aa, labor), calcitonin (32 aa, hypercalcemia, osteoporosis), teriparatide (34 aa, parathyroid hormone analog, osteoporosis), Fuzeon (36 aa, enfuvirtide, antiretroviral), corticotropin releasing hormone / factor (41 aa), and growth hormone releasing hormone / factor (44 aa, lipodystrophy) [281]. Another recent peptide therapeutic to reach the market is Exenatide (Byetta), which is used in the treatment of type 2 diabetes [289].

1.12 Gap in the study

Extensive studies have been performed to identify hemostatically active proteins from snake venom; however, there is a dearth of knowledge on such proteins from the Indian Spectacled Cobra (*N. naja*) venom. As already mentioned, Certain cases of envenomation by *N. naja* are reportedly characterized by transient coagulopathy showing increased 20WBCT. However, minimal effort has been made to identify and characterize the anticoagulant proteins from *N. naja* venom. As a result, there is lack of reports on the neutralization efficiency of the commercially available polyvalent / monovalent antivenoms against the pathophysiological effects of anticoagulant proteins from Indian cobra venom. Such proteins hold good promises to be developed into drug prototypes for the better treatment of cardiovascular diseases. Therefore, there is a need for exploration of the therapeutic potential and biomedical application of *N. naja* venom anticoagulant proteins and peptides derived from them.

1.13 Aims and objectives of the present study

The present study aims to isolate, identify, and characterize a major anticoagulant protein from the venom of *N. naja*. Further, the mechanism of anticoagulant action of the anticoagulant protein would be deciphered which will aid in designing of novel peptides with similar potential. The anticoagulant potential of the protein as well as peptide would be verified by *in vitro*, *ex vivo,* and *in vivo* experiments.

In order to address the issues described in section 1.12, the following four objectives were set for the study:

I. Characterization and elucidation of mechanism of action of a major anticoagulant phospholipase A_2 ($NnPLA_2$-I) purified from Indian cobra *Naja naja* venom.
II. Study on *in vitro* myotoxicity (cytotoxicity towards rat myoblasts) of $NnPLA_2$-I and its acidic cognate complex on rat myoblasts and their neutralization by antivenom.
III. Designing, characterization, and elucidation of mechanism of action of a peptide from the anticoagulant region of $NnPLA_2$-I enzyme.

IV. Assessment of *in vivo* toxicity, anticoagulant, and antithrombotic activity of $NnPLA_2$-I and the anticoagulant peptide in a rodent model.

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