ABSTRACT

ABSTRACT

Snake envenomation is a major occupational health hazard in tropical and sub-tropical countries of South and South-east Asia, sub-Saharan Africa, Central and South America, and Oceania. Conservative estimates have reported that yearly about 1.2 – 5.5 million snakebite cases occur around the world, out of which 421,000 are envenoming cases and approximately 20,000 – 94,000 cases results in mortality of the victims. Therefore, on June, 2017, the World Health Organization (WHO) has reinstated that snake bite is a "Category A, Neglected Tropical Disease". The snakebite scenario in India is more pathetic with approximately 49,500 deaths reported due to snake envenoming every year.

The Indian cobra or Spectacled Cobra (*Naja naja*) is a member of the 'Big Four' venomous snakes of India and a category I medically important snake of the world. This is one of the most dreaded species of snakes to inhabit the Indian sub-continent that is responsible for a large number of snakebite fatalities and morbidity. The *N. naja* venom is a complex mixture of various enzymatic and non-enzymatic toxins, predominated by non-enzymatic low molecular weight three finger toxins. The primary pathophysiology associated with envenomation by *N. naja* is neurotoxicity; however, local symptoms such as inflammation, blistering, and edema leading to tissue necrosis at the site of bite, or a part / entire limb are also characteristic clinical features of cobra envenomation. Nevertheless, some cobra envenomed patients of western India have also demonstrated transient coagulopathy by increasing the blood clotting time, which suggests in *in vitro* anticoagulant activity of *N. naja* venom. Except one metallo-proteinase, other anticoagulant proteins of *N. naja* venom have not been characterized to date, albeit they may hold potential for biomedical application as a drug prototype in the treatment of cardiovascular diseases such as unwanted thrombosis.

Snake venom phospholipase A_2s (E.C. 3.1.1.4) is one of the major components of snake venom including cobra venom and are known to exhibit a plethora of pharmacological effects such as neurotoxicity, myotoxicity, hemolysis, edema, hyperalgesia, inflammation, hypotension, inhibition of platelet aggregation, anticoagulation, cytotoxicity, and bactericidal activity. This class of snake venom toxins causes enzymatic hydrolysis of phospholipids at their target site to aid the

pathophysiology of cobra envenomation. For anticoagulant and cytotoxic / myotoxic activities of venom PLA_2s, binding to and hydrolysis of plasma phospholipids and cell membrane phospholipids, respectively, are the primary mode of action. However, not all of the clinical symptoms associated with snake venom PLA_2s are dependent on their enzymatic activity, as some also demonstrate non-enzymatic mode of action to elicit a pharmacological symptom. In nature, these snake venom PLA_2s either occur in isolation or in a complex with other venom toxins, which consequently leads to synergistic potentiation of their biological activities.

The process of hemostasis maintains a fine balance between 'blood coagulation' at sites of vascular injury and 'fibrinolysis' to keep blood in a clot-free state for infusion through organs. Alterations in blood coagulability, endothelial injury and / or blood stasis gives rise to a medical condition called 'thrombosis'. Formation of thrombus in the arteries supplying blood to vital organs, such as the heart or brain, can cause myocardial infarction or stroke, respectively. Although several commercial anticoagulants (warfarin, heparin, argatroban) are available for treatment of thrombosis and associated cardiovascular diseases; however, these drugs have been found to be associated with various adverse effects. Apart from eliciting higher immune response, the classical anticoagulants are associated with higher risk of bleeding, thrombocytopenia, residual effect interaction with other drugs, and narrow therapeutic index. Therefore, a search for alternative anticoagulants to be developed from natural resources, such as snake venom proteins / peptides or their derivatives, has gained a momentum in the recent years.

Peptide-based therapy has been a major break-through in the field of biomedical research. Low molecular mass peptides have certain potential advantages over chemical molecules as anticoagulant drugs. Notably, low molecular mass peptides as compared to low molecular weight synthetic drugs possess higher affinity towards their target, have lower toxicity, and have high tissue penetration ability, albeit some complications are also associated. However, several strategies have been developed in recent years to overcome such limitations.

For an easy understanding, this thesis is divided into following seven chapters and salient features of each chapter is described below.

Chapter I. The first chapter of the thesis provides an introduction to the global burden of snakebite including the Indian scenario. The chapter also provides a brief appraisal on Indian cobra *N. naja*, its venom composition, and pathophysiology of envenomation. The chapter also briefly describes the structural and functional aspects of snake venom phospholipase A_2 with special reference to anticoagulant and cytotoxic / myotoxic PLA_2s. Further, this chapter provides a brief account on the importance and applications of peptide-based therapeutics derived from natural sources, including snake venom. The aims and objectives of the study are described in this chapter.

Chapter II. This chapter reviews the published literature on anticoagulant, cytotoxic and myotoxic PLA_2s from cobra venom. This chapter also reviews the different therapeutics and drug prototypes developed from snake venom for treatment of cardiovascular diseases.

Chapter III. This chapter describes the various methods employed for purification of a major anticoagulant PLA_2 and its myotoxic complex from *N. naja* venom. The chapter also provides a detailed methodology employed to perform different biochemical and pharmacological assays for characterization and elucidation of mechanism of actions of the purified anticoagulant PLA_2, its cytotoxic cognate complex and an anticoagulant 7-mer peptide developed from the anticoagulant region of this enzyme. Further, the chapter describes in detail the procedures of LC-MS/MS and MALDI-ToF analyses for identification of different venom proteins in isolation as well in protein complexes. This chapter also provides an account of the methodology implemented for performing different protein-protein interaction studies such as fluorescence spectrofluorometry, surface plasmon resonance, immunological studies (such as ELISA and western blotting), affinity chromatography, and *in silico* docking analyses. Further, the chapter also describes the *in vivo* experiments for toxicity assessment and validation of therapeutic potential of the anticoagulant PLA_2 and the anticoagulant 7-mer peptide.

Chapter IV. This chapter includes the results and discussion on purification of an acidic anticoagulant PLA_2 enzyme ($NnPLA_2$-I) of *N. naja* venom, its biophysical and biochemical characterization, *in silico* analysis, and *in vitro* and *in vivo* pharmacological characterization in order to elucidate the mechanism of action of $NnPLA_2$-I. In this

chapter the future application of this PLA_2 for development of drug prototypes for treatment of thrombosis-associated diseases has also been suggested.

Chapter V. This chapter describes the results and discussion on cognate complex formation of $NnPLA_2$-I with cobra venom three finger toxins, along with toxicity assessment of the complex on L6 rat myoblasts.

Chapter VI. This chapter describes the designing, synthesis and characterization of a 7-mer peptide designed from the non-catalytic anticoagulant region of $NnPLA_2$-I, and also manifests the future application of this peptide as an antithrombotic agent.

Chapter VII. This chapter includes the major findings of this work and envisions the future scopes of the findings of this study.

The present study describes the purification and characterization of an acidic phospholipase A_2 ($NnPLA_2$-I), with a molecular weight of 14.2 kDa, from Indian Spectacled Cobra (*N. naja*) venom. LC-MS/MS analysis of the purified PLA_2 demonstrated the presence of conserved putative domains of phospholipase A_2 superfamily of snake venom toxins and showed 100% sequence similarity with a previously uncharacterized PLA_2 of *N. naja* venom (accession no. P15445). $NnPLA_2$-I preferentially hydrolyzed phosphatidylcholine (PC), over phosphatidylserine (PS) or phosphatidylethanolamine (PE) with *Km* and *Vmax* values of 0.72 mM and 29.3 µmol min^{-1} mg^{-1}, respectively. The anticoagulant activity of $NnPLA_2$-I was found to be higher than that of commercial anticoagulants heparin / AT-III or warfarin in both *in vitro* and *ex vivo* conditions. Alkylation of the His47 residue at the active site of $NnPLA_2$-I or pre-incubation of the PLA_2 with commercial anti-snake venoms differentially inhibited the catalytic, anticoagulant, and platelet deaggregation activities of $NnPLA_2$-I. Low molecular weight heparin (LMWH) was found to inhibit the anticoagulant activity of $NnPLA_2$-I, albeit it did not inhibit the catalytic and platelet deaggregation activity of the enzyme. $NnPLA_2$-I demonstrated a non-enzymatic, mixed inhibition of thrombin with a *Ki* value of 9.3 nM. To the best of our knowledge, $NnPLA_2$-I is the first thrombin inhibitor PLA_2 from *N. naja* venom. *In silico* analysis showed binding of $NnPLA_2$-I to thrombin with an atomic contact energy of -99.66, and identified the residues of $NnPLA_2$-I responsible for binding to thrombin. It was found that 11 of the 19 identified

residues were present in the anticoagulant pharmacological site of $NnPLA_2$-I. In addition, $NnPLA_2$-I showed antiplatelet activity by inhibiting the aggregation of platelets in platelet rich plasma (PRP), and also inhibited the collagen and thrombin-induced aggregation of PRP. However, $NnPLA_2$-I failed to demonstrate antiplatelet activity in washed platelet suspension, although supplementing the reaction mixture with platelet poor plasma or other phospholipid (PC or PS) demonstrated deaggregation of washed platelets, suggesting that the antiplatelet activity of $NnPLA_2$-I is dependent on the enzymatic property of the enzyme. Alkylation of histidine residue of $NnPLA_2$-I resulted in 95% reduction of its platelet deaggregation property, but heparin could not inhibit the antiplatelet activity of $NnPLA_2$-I. Except for marginal toxicity towards mammalian platelets, the purified PLA_2 was non-toxic to selected mammalian normal and cancerous cell lines. Further, $NnPLA_2$-I, at a dose of 4.0 mg/kg, was also devoid of toxicity when tested in Wistar strain rat model.

In the venom of *N. naja*, $NnPLA_2$-I interacts with three finger toxins (cytotoxin and neurotoxin) present in the venom to form cognate complexes that greatly potentiate its cytotoxicity towards L6 rat myoblasts. $NnPLA_2$-I forms an acidic and a basic complex in *N. naja* venom, which was identified by two dimensional electrophoresis followed by tandem mass spectrometry analysis of 2D spots. $NnPLA_2$-I forms an acidic non-covalent complex with a long chain neurotoxin (accession no. P25671) and a cytotoxin (accession no. P86538) in a molar stoichiometric ratio of 1:2:1 (PLA_2: LNTx:CTx). The mass of the complex corresponds to ~36 kDa as determined by one-dimensional electrophoresis and size exclusion chromatography. The LC-MS/MS analysis further confirmed that $NnPLA_2$-I as well as its acidic cognate complex binds to vimentin, an intermediate filament protein of the cytoskeleton, expressed on the surface of rat myoblasts. This study reports for the first time the involvement of membrane-bound vimentin as an acceptor for cobra venom PLA_2 and its complex. ELISA, immunoblots and spectrofluorometric analyses showed greater binding of $NnPLA_2$-I cognate complex to vimentin as compared to the binding of $NnPLA_2$-I. The immunofluorescence study evidenced the internalization of $NnPLA_2$-I to L6 myoblasts post binding with vimentin in a time-dependent manner. Pre-incubation of polyvalent antivenom with $NnPLA_2$-I cognate complex demonstrated better neutralization of myotoxicity as compared to exogenous addition of polyvalent antivenom 60 – 240 min

post treatment of myotubes with cognate complex suggesting clinical advantage of early antivenom treatment to prevent cobra venom-induced myonecrosis. Docking analysis showed that 19 – 22 residues, of $NnPLA_2$-I preferentially binds with the rod domain (99 – 189 and 261 – 335 regions) of vimentin. However, $NnPLA_2$-I cognate complex showed greater binding with the same regions of vimentin which corroborates well with wet lab experiment. This result indicates the pathophysiological significance of cognate complex in cobra venom-induced myonecrosis.

A synthetic 7-mer peptide with the sequence E^1-K^2-I^3-S^4-G^5-G^6-W^7 (MW = 775.85 Da) was designed from the residues of the thrombin-binding region of $NnPLA_2$-I. The peptide was devoid of any enzymatic property but demonstrated strong anticoagulant activity by mixed and uncompetitive inhibition of thrombin and factor Xa, respectively. Although $NnPLA_2$-I was only a thrombin inhibitor, however, the 7-mer peptide was a dual inhibitor of thrombin and FXa, two vital factors of the blood coagulation cascade. *In silico* analysis showed binding of this 7-mer peptide to thrombin and FXa with atomic contact energies of -224.28 and -39.86, respectively. The docking experiments were validated by *in vitro* analysis where the peptide exhibited higher thrombin inhibition with IC_{50} and *Ki* values of 2.0 μM and 0.39 μM, respectively, as compared to IC_{50} and *Ki* values of 19.6 μM and 1.0 μM, respectively, against FXa. The thrombin inhibition property of the 7-mer peptide surpasses than that of commercial drug argatroban in both *in vitro* and *ex vivo* conditions. Docking studies demonstrated binding of the 7 residues of the 7-mer peptide to 19 residues of thrombin, including His57 and Ser195, the active site residues of thrombin, which ultimately leads to inhibition of the catalytic activity of thrombin. Further, 6 residues of the peptide interacts with 10 residues of the heavy (D) chain of FXa; however, none of these residues belong to the active site of FXa, which corroborates with the non-competitive inhibition of FXa exhibited by the peptide. Docking results were also validated by spectrofluorometric analysis. Surface plasmon resonance (SPR) analysis demonstrated that the K_D value of interaction between the 7-mer peptide and thrombin was 3.8 μM. This 7-mer peptide did not show hemolytic activity, cytotoxicity against mammalian cells, and did not arrest the cell cycle of treated MCF-7 cells. In *in vivo* and *ex vivo* conditions, the 7-mer peptide exhibited superior anticoagulant ($p<0.05$) activity as compared to $NnPLA_2$-I, argatroban, and heparin when administered in Wistar strain

albino rats. Further, the optimum INR (international normalized ratio) value of PT and APTT suggested that this 7-mer peptide may not produce the risk of internal bleeding disorder. Moreover, at a dose of 0.2 mg/kg the antithrombotic potency of the 7-mer peptide was found to be superior as compared to argatroban which is highly encouraging for therapeutic application of this 7-mer peptide in the prevention of thrombosis. Administration (i.v.) of the 7-mer peptide at a dose of 4.0 mg/kg did not show adverse pharmacological effects and toxicity in treated rats thus suggesting its pre-clinical safety and high therapeutic index.