Chapter VII

CONCLUSIONS AND FUTURE PERSPECTIVES

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7.1 Conclusions and future perspectives

The present study investigates the Biochemical and pharmacological properties of a major acidic phospholipase A_2 enzyme ($NnPLA_2$-I) isolated and purified from the venom of Indian Spectacled Cobra, *Naja naja*. Further, extensive studies have been done to demonstrate the pathophysiological significance of $NnPLA_2$-I and its cognate complex. Moreover, a 7-mer peptide, derived from the pharmacological site of $NnPLA_2$-I was designed, synthesized, and its anticoagulant property was characterized and compared with wild-type of enzyme and commercial anticoagulants. The mechanism of action of this protein/peptide was elucidated in order to explore its possible therapeutic applications as drug prototype.

The first finding of this study is the purification, characterization, and elucidation of anticoagulant mechanism of a 14.2 kDa major acidic PLA_2 enzyme of *N. naja* venom, named $NnPLA_2$-I. $NnPLA_2$-I exhibited *in vitro* and *in vivo* strong anticoagulant property as well as *in vitro* anti-platelet activity. Even at a dose of 4.0 mg/kg, the purified PLA_2 was found to be non-toxic towards Wistar strain albino rats, albeit it prolonged the re-calcification, prothrombin, activated partial thromboplastin, and thrombin time of blood plasma in the treated animals at a dose of 0.4 mg/kg. $NnPLA_2$-I demonstrated its anticoagulant activity by a combination of its enzymatic and non-enzymatic thrombin inhibition properties. To the best of our knowledge, $NnPLA_2$-I is the first thrombin inhibitor PLA_2 to be isolated from the venom of Indian Spectacled Cobra (*Naja naja*). Low molecular weight heparin (LMWH), on the other hand, inhibits the anticoagulant activity of $NnPLA_2$-I, but does not interfere with the antiplatelet activity of this enzyme suggesting the therapeutic application of a low dose of LMWH in treating cobra bite patients.

The second, yet interesting finding of this study is that 'vimentin', an intermediate filament protein of the cytoskeleton, may serve as a cell membrane acceptor for cobra venom PLA_2 and its acidic cognate complex on rat myoblasts. To the best of our knowledge, this study is the first report demonstrating the binding of cobra

venom PLA_2 with membrane-bound vimentin. In nature, $NnPLA_2$-I was also found to exists in the form of a cognate complex with a long chain neurotoxin (LNTx) and a cytotoxin (CTx) in an approximate molar stoichiometric ratio of PLA_2:LNTx:CTx = 1:2:1 and contain a trace quantity of NGF. The binding of $NnPLA_2$-I cognate complex to membrane-bound vimentin along with phospholipid micro-domains of the plasma membranes, may be responsible for exerting cytolytic and/or cytotoxic effects on rat myogenic cells. Nevertheless, the presence of trace quantity of NGF in the cognate complex was associated with augmented toxicity and enhanced binding to vimentin which was demonstrated by cytotoxicity assessment towards myoblasts and *in silico* analysis, respectively.

The binding of $NnPLA_2$-I with rat myoblasts is followed by internalization of the purified PLA_2 into the cells, although the effect of this phenomenon remains to be explored in the future. Further, commercial polyvalent antivenoms failed to neutralize the cytotoxic effect of the cognate complex when incubated with rat myoblasts pre-treated with $NnPLA_2$-I cognate complex; thus suggesting the need for alternative strategies of antivenom production for better management of local effects such as, edema and necrosis, in cobra bite patients.

In silico studies on thrombin-$NnPLA_2$-I interaction, led us to the third major finding of this study. Out of the 12 peptides designed from the thrombin interaction regions/residues of $NnPLA_2$-I, only one peptide (ACR9) demonstrated prominent anticoagulant activity. The 7-mer peptide, ACR9 (775.85 Da), at a dose of 4.0 mg/kg, was devoid of toxicity when administered in Wistar strain rats. However, it showed considerable anticoagulant activity by dual inhibition of thrombin and factor Xa at a 10-fold lower dose of 0.4 mg/kg. Superior inhibition of thrombin by ACR9 compared to argatroban as well as inhibition of FXa is suggestive of its future development as an anticoagulant drug for treatment of cardiovascular diseases by prevention of thrombosis. The INR values obtained for prothrombin and activated partial thromboplastin times by the peptide were within the optimal range, thus dismissing the risks of internal bleeding. Further, the peptide demonstrated antithrombotic property even at a lower dose of 0.2 mg/kg, which was characterized by inhibition of κ-carrageenan induced thrombus formation in the rat tail veins.

This study warrants the development of this peptide prototype into a successful therapeutic for treatment of occlusive thrombosis. The primary future prospect of this study includes a long-term pre-clinical study to determine the enduring effects of ACR9 in animal model, along with determination of pharmacokinetic and pharmacodynamic profile of the peptide. In addition, an analysis on the inhibitory effect of ACR9 on upstream coagulation factors of blood is also necessary. Further, designing alternate delivery mechanisms for administration of ACR9 would be another future goal of this study.