CHAPTER 8

CONCLUSION

8.1 The toxinology and proteome of of *N. sumatrana* venom

Naja sumatrana venom exhibited the common enzymatic activities of Asiatic cobra venoms: low protease, phosphodiesterase, alkaline phosphomonoesterase and L-amino acid oxidase activities, moderately high acetylcholinesterase and hyaluronidase activities and significantly high activity of phospholipase A_2.

The venom composition of the three Southeast Asian spitting cobras (*N. sumatrana, N. siamensis* and *N. sputatrix*) were compared using Resource® S ion exchange chromatographic fractionation, as these three species of spitting cobras were formerly considered as one species, *i.e. N. sputatrix*, the Malayan cobra. The results showed that venoms of these three cobras were indeed distinctly different, though all three venoms contain pharmacologically active basic phospholipase A_2 and large amount of cardiotoxins. This is in contrast to the non-spitting *N. kaouthia* venom, which only has acidic phospholipase A_2 and much smaller amount of cardiotoxins.

The proteome of *N. sumatrana* venom was further investigated using two approaches: Shotgun LC-MS/MS and multi-dimensional chromatography-2DE approaches. Shotgun analysis revealed the presence of 50 individual proteins in the venom, with three finger toxins (both neurotoxins and cardiotoxins) and phospholipases A_2 constituted about 38% and 36%, respectively of the types of proteins identified. Other proteins identified included thaicobrin, aminopeptidase, Cysteine rich secreting protein (CRISP), cobra venom factor, complement depleting factors, zinc metalloproteinase-disintegrin, nerve growth factor, NADH dehydrogenase, cobra serum albumin and the natriuretic peptide.

The multi-dimensional chromatography-2DE approach involved Resource® S ion exchange chromatography, reverse phase HPLC, Sephadex® G-50 gel filtration chromatography and 2-dimensional electrophoresis. The ion exchange and reverse phase HPLC revealed the presence of basic venom proteins including 17 identified protein toxins (8 phospholipases A_2, 4 neurotoxins and 5 cardiotoxins) as well as 20 very minor protein fractions that could not be identified. Seven of the identified toxins (a long neurotoxin, a short neurotoxin, two phospholipases A_2 and 3 cardiotoxins) together account for 87% of total venom protein and they are primarily responsible for the toxic actions of the *N. sumatrana* venom. The two neurotoxins together constituted 12.2% of total venom protein, and are the dominant toxins of the venom because of their very high lethality.

The ion exchange/reverse phase chromatographic separations failed to identify the high molecular weight proteins of the venom. A Sephadex® G-50-2DE approach was used to investigate these high molecular weight venom proteins. The 2DE of a high molecular weight venom protein fraction isolated by Sepahdex® G-50 chromatography of *N. sumatrana* venom yielded 31 protein spots. The proteins identified included cobra venom factor, complement depleting factor, serum albumin precursor, phosphodiesterase, 5'-nucleotidase, hemorrhagic metalloproteinase, serine proteinase, natrin and venom nerve growth factor. These proteins and also those proteins identified by shotgun analysis mentioned above have also been identified in the proteome of a few other Asiatic cobra venoms, suggesting that these might be common constituents of Asiatic *Naja* venoms.

8.2 The pharmacokinetics of cobra venoms and venom toxins

A comparative study on the pharmacokinetics of *N. sumatrana* and *N. sputatrix* venom in rabbits showed that the two venoms exhibited similar pharmacokinetics characteristics: 1) intravenous serum venom concentration-time profile of both venoms displayed a biexponential pattern that was best fitted into two compartment model of pharmacokinetics; 2) both venoms possessed similar initial distribution half-life ($T_{1/2\alpha}$), terminal elimination half-life ($T_{1/2\beta}$), and volume of distribution by area ($V_{d,area}$); 3) values of the ratio of inter-compartmental transfer rate constants (k_{12} and k_{21}) of both venoms suggested that at equilibrium, amount of venom antigens in central compartment were similar to that of peripheral compartment; 4) the intramuscular bioavailability ($F_{i.m.}$) of *N. sumatrana* and *N. sputatrix* venoms were comparable, both at about 40%. These features were generally similar to data obtained for African cobra venoms and it is concluded that this may be the general pattern of pharmacokinetics for most *Naja* venoms.

To further understand the pharmacokinetics of venom toxin components, specific ELISAs for the three major venom constituents of *N. sumatrana* venom (phospholipase A_2, neurotoxin and cardiotoxin) were developed to study the pharmacokinetics of the three toxins in rabbits injected with the toxins.

The serum venom concentration-time profiles of all three toxins injected intravenously were best fitted to an open two-compartment pharmacokinetic model with a faster distribution half-life during initial phase compared to when whole venom was injected intravenously, reflecting a rapid distribution of the toxins upon entering into systemic circulation. The phospholipase A_2 has a higher k_{12}/ k_{21} ratio compared to neurotoxin and cardiotoxin, suggests that at

equilibrium, the amount of phospholipase A_2 antigens in peripheral compartment is larger than in the central compartment, which indicates phospholipase A_2 exhibited a stronger affinity for target tissues to exert pharmacology activities. When toxins were injected intramuscularly, neurotoxin and cardiotoxin antigens reached the maximal toxins antigens concentration within 30 min, which was much faster than phospholipase A_2 and the whole venom, reflecting a very rapid absorption of neurotoxin and cardiotoxin from the site of injection to systemic circulation. The rapid absorption of neurotoxin and cardiotoxin suggests rapid onset of systemic symptoms upon cobra bites. The systemic clearance of neurotoxin and cardiotoxin are both significantly higher than phospholipase A_2 and whole venom, indicating that neurotoxin and cardiotoxin were eliminated from systemic circulation more quickly than phospholipase A_2 and whole venom. The faster clearance of neurotoxin and cardiotoxin is presumably related to their smaller size and implies more rapid clinical recovery from these systemic effects. It is also interesting to note that neurotoxin had an intramuscular bioavailability ($F_{i.m.} = 81.5\%$) higher than phospholipase A_2 and cardiotoxin, indicating a nearly complete absorption of the neurotoxin from site of injection. The incomplete absorption of phospholipase A_2 and cardiotoxin may account for the local symptoms including necrosis, local inflammation and pain upon cobra envenomation, which may involve synergistic interactions of cardiotoxin and phospholipase A_2. The high bioavailability and short T_{max} of neurotoxin when injected intramuscularly explained why neurotoxic effect is the dominant symptom in most cobra bites.

The effect of NPAV on the pharmacokinetics of *N. sputatrix* venom in rabbits was also examined and it was found that a multiple dosing regimen '4 + 2 ml' of NPAV was more effective in reducing the serum venom antigen concentrations

and preventing the transient resurgence of the serum venom antigen levels, which occurred subsequent to the initial dose of NPAV. The results also showed that it is necessary to administer larger amount of antivenom than that is indicated by the *in vitro* and *in vivo* neutralization potentials in rodent model, in order to eliminate the injected venom antigens effectively.

8.3 Future works on the toxinology and proteome of *Naja* venoms

The present works showed that full knowledge (including quantitative information) of venom protein composition can be achieved by combination of various chromatographic methods and mass spectrometry, and the information obtained could contribute to deeper understanding of the pathophysiological actions of cobra venom. At the moment, only the proteomes of venoms from a few Asiatic *Naja* have been elucidated but mostly without quantitative data. The proteomes of venoms from more Asiatic *Naja* should be investigated (with quantitative studies) to enable a comprehensive understanding of the spectrum of pathophysiological actions of Asiatic *Naja* venoms. This knowledge could give insight to the design of a pan-specific antivenom against Asiatic *Naja* venoms.

8.4 Future works on pharmacokinetics of *Naja* venoms and venom toxins

The present works on pharmacokinetics of *N. sputatrix* and *N. sumatrana* venoms showed that their pharmacokinetics characteristics were similar, with intramuscular bioavailability of approximately 40% and elimination half-lives ($T_{1/2\beta}$) of between 12-15 h. Presumably, the pharmacokinetics of other *Naja* venoms are similar to those described here. The present works on pharmacokinetics of the three major toxins from *N. sumatrana* venom represent the only study on pharmacokinetics of all the major venom components. The study yielded several very interesting information, including the high intramuscular bioavailability of neurotoxin compared to cardiotoxin or phospholipase A_2, the rapid absorption of the neurotoxin and cardiotoxin when the toxins were injected intramuscularly. Similar studies on the pharmacokinetics of the major lethal toxins of other *Naja* venoms should be carried out to establish if these interesting pharmacokinetic characteristics are common features of cobra venom toxins.