CHAPTER III

METHODOLOGY

1. Research design : The study will be divided into two parts, analytical design (prospective study) and experimental design in animal models.

2. Design overview : The design of study is important because it governs how data are to be analyzed (Rothman and Greenland, 1998; Weisberg, Krosnick and Bowen, 1996). The types of design include characteristics;

   2.1. *Cross-sectional.* A random sample of individuals from a population is taken at one point in time. Individual people included in the sample are examined for the presence of disease and their status with regard to other risk factor type of study is useful for describing the situation at the time of data collection and it allows determining prevalence. Cross-sectional design is easy to accomplish, but do not establish the temporal sequence of events needed for drawing causal inferences.

   2.2. *Retrospective (case- control).* This type of design can be used effectively for the study of low incidence diseases as well as of conditions developing over a long time, looked back to determine past exposure and no incidence or prevalence. It allows an investigation of preliminary causal hypotheses. It is quick and has a relatively low cost.

2.3. *Prospective (cohort).* Disease-free persons are classified on exposure at beginning of follow-up period, then followed for some period of time to ascertain the occurrence of disease in each group. Prospective (Cohort) are yield for incidence rates and estimates of risk (better evidence of temporal sequence).

2.4. *Experiment.* An intervention study is research design in which the investigator manipulates a factor and measures the subsequent outcome. It gives strongest evidence for etiology.

3. **Consideration of choice in study design.**

3.1. Stage of hypothesis development

- Generation : case series, ecologic, cross-sectional

- Hypothesis testing and estimation of effect : case-control, cohort, experimental studies.

3.2. Nature of disease

If incidence is

- High (common disease) : cohort or cross – sectional

- Low (rare disease) : case – control

3.3. Nature of exposure

If occurrence is

- Rare : cohort

- Common : case – control or cross – sectional

If it requires measurement : cohort

3.4. Nature of study population

- High attrition rate (e.g., transient population , mobile population) : case – control or cross – sectional studies.

3.5. Context of research

(i) If financial resources are

- High : cohort

- Low : case – control or cross – sectional

(ii) Ethical constraints : case – control or cross – sectional studies

(iii) Time allowable

- Short : cross – sectional or case – control studies

- Long : cohort

In this study, we will look for the factors causing tissue necrosis after *N. kaouthia and C. rhodostoma* bites. It is a hypothesis generation since a systematic study to generate a hypothesis about the predictors of tissue necrosis has not been documented. Therefore, a prospective study of all cases admitted to hospitals will be carried out. There are cases without necrosis at recruitment.

4. Prospective study .

The design is based on selecting two groups of non-diseased people, one exposed to a factor postulated to cause a disease and another on unexposed to the factor. They are followed over time and their change in disease status is recorded during the study period. The prospective study is the most effective observational study for investigation of causal hypotheses with respect to disease occurrence. It provides

disease incidence estimates (i.e., tissue necrosis incidence) which are more meaningful than prevalence data for establishing cause-effect relationships. Advantages and disadvantages of prospective are more summarized below (Bowing, 1998).

Advantages	Disadvantages
• Determination causal direction	• Take too long and /or expensive if outcome is rare or has a long lag time between exposure and outcome
• Less subject of recall and other bias	• Hard to do if non-exposure is rare or if most people have the exposure
• Easier to measure and control for potential confounders	• Not as definite as interventional study, especially randomized control trial
• Measurement of true prevalence and incidence of exposure and diseases or other outcome	• Some interventions are too expensive , too hard or just unethical to do

Because tissue necrosis will occur within a few days after snakebites, the disadvantages of the prospective study summarized above do not apply here. In this study, all patients bitten by *N. kaouthia* or *C. rhodostoma* snakes will be followed until his(her) discharge from the hospitals. All signs and symptoms of victims will be observed at least three times; the first day of admission, the second or the day of worst clinical conditions and finally on the day of discharge from hospital. While staying in the hospital, patients will be frequently observed so that the day of worst clinical

conditions is determined. Two investigators will document of the manifestations of patients independently from standardized instrument and each assessment data as documented will be evaluated independently.

5. Retrospective study

In retrospective studies, also known as case-control studies, the investigator selects cases with a specific disease, and appropriate controls without the disease, and obtains data regarding past exposure to possible etiologic factors in both groups. The rates of exposure of the two groups are then compared. A case-control approach is preferred when studying rare diseases, such as most cancers, because a very large number of individuals would be needed in order to draw conclusions in a prospective study. Although it is possible to detect the association of multiple exposures or factors with a particular disease, retrospective studies are generally used to study diseases that have some unique and specific cause, such as infectious agents, in order to avoid the problem of confounding etiologic factors Advantages and disadvantages of retrospective study are shown in table below (Bowing, 1998).

Advantages	Disadvantages
• Less expensive than cohort because of decreased number of subjects required	• Records for identifying past exposure may be missing or incomplete
• Quicker results	• Bias can be introduced by an inappropriate control group
• Best for rare diseases in the general population	• Responders may inaccurately recall events in the past
• Possible to obtain more detailed information on exposure and confounding factors	• Recall Bias: Cases may recall events better than controls because of vested interest
	• Not effective for rare exposures that account for only a small proportion of cases
	• Can not calculate relative risk (use odds ratio)

6. Experimental study

The other design of study is experimenal. An intervention and the basic principles are that it involves deliberately changing population parameters and assessing the effect. The experiment study has the following advantages and disadvantages (Bowing, 1998).

Advantages	Disadvantages
• Prospective direction	• Contrived situation
• Ability to randomized subjects	• Impossible to control animal behavior
• Appropriate temporal sequence of cause and effect	• Ethical constraints
• Ability to control extraneous variables	• External validity still uncertain
• Best evidence of causality	• Expensive in terms of time, personnel, facilities and cost

The protocol will be worked in animal models for preliminary study of searching drugs or reagents to protect and to limit tissue necrosis occurrence from *N. kaouthia* or *C. rhodostoma* bites. Mice will be supplied from National Laboratory Animal Center, Mahidol University where controlled ethical of experimental methods in animal models is controlled.

7. Population

Target population is the entire group a researcher is interested in; the group about which the researcher wishes to draw conclusions (Toma, 1999). The target population of this study is snakebite victims from *N. kaouthia and C. rhodostoma* bitten in Thailand of any age, gender , race and status of patients.

The accuracy records to determine the exact epidemiology or even the number of snake bite cases are also generally unavailable because the individuals bitten by snakes firstly consult traditional practitioners before visiting a medical center. However,

the incidence of herbal traditional treatments after snakebite in patients has declined resulting from urbanization in Thailand. Most of snakebite patients have been in hospitals and health care centers around Thailand (Ponchanugool, Limthongkul and Wilde, 1997).

8. Sample

The *sample population* consists of snakebite patients bitten by cuprit snakes, and admitted to all hospitals and health care centers in Thailand. *A sample* which is a subset of population selected for the purpose of studying certain characteristics of an entire population of interest therefore must represents the population. The sample will be selected from ten provincial hospitals which had a high prevalence of *N. kaouthia* or *C. rhodostoma* bites, such as Nakornsawan, Prachaubkirikhan, Surathani, Nakornsrithammarat, Songkra, Trang, Ratchaburi, Lopburi, Nakornratchasrima and Lumpang provinces. The number of snakebite victims of each provincial hospital from top ten areas are approximately 5-10 % of total snakebites patients reported to Division Epidemilogy of Ministry of Public Health (Division of Epidemiology, 1999).

A decision on the required sample size has to be based on different calculations depending on whether estimates for a categorical/ or a continuous variable are to be calculated. The factors to take into consideration include the accuracy required, the sampling method to be used, the size of the smallest subgroup and the actual variability of the variable of interest in the population.

8.1. Sample size for prevalence

In this study , sample size will be calculated using prevalence formula $(Z^2 pq / L^2)$, because the objective is to estimate the incidence of tissue necrosis in victims bitten by *N. kaouthia* or *C. rhodostoma..* The formula is related to the following variables.

Z = value of confidencial level. The z values to be used in the formula shown are 1.65 , 1.96 and 2.58 for 90 %, 95 % and 99% confidence levels respectively. Confidence intervals indicate the precision of evidence; where confidence intervals are wide, they indicate less precise estimates of effect. The larger the trial's sample size, the larger the number of outcome events and the greater becomes the confidence that the true incidence is close to the value stated. Thus the confidence intervals narrow and " precision" is increased. In a "positive finding" study the lower boundary of the confidence interval, or lower confidence limit, should still remain important or clinical significant if the results are to be accepted. In a "negative finding" study, the upper boundary of the confidence interval should not be clinically significant if you are to confidently accept this result.

p = expected prevalence. The value will be estimated from the incidence of severity of tissue necrosis in patients after culprit snakebites.

q = 1 – p

L = precision level. The level of precision *L* has to be decided. This parameter is the distance of the sample estimate in either direction from the true population proportion considered acceptable by the investigator. It can be expressed either as a

number of percentage points (absolute precision) or a percentage of the expected value (relative precision).

The sample size of patients bitten by *N. kaouthia* is estimated to be 80 cases as shown below by using the variations to be :

$Z = 1.96$ for a 95 % confidence.

$p = 30\% = 0.3$. The prevalence of tissue necrosis from *N. kaouthia* bite were approximately 30 %.

$q = 1 - p = 1 - 0.3 = 0.7$

$L = 10 \% = 0.1$

Sample size = $\{ (1.96)^2 \times 0.3 \times 0.7 \} / (0.1)^2$

  = 81 cases

The sample size of victims of *C. rhodostoma* bites is calculated to be 81 cases as shown below by letting.

$Z = 1.96$ for a 95 % confidence

$p = 70 \% = 0.7$.   Tissue necrosis are occurred 70 % of total patients in *C. rhodostoma* bites.

$q = 1 - p = 1 - 0.7 = 0.3$

$L = 10 \% = 0.1$

Sample size = $\{(1.96)^2 \times 0.7 \times 0.3 \} / (0.1)^2$

  = 81 cases

8.2 Sample size for Relative Risk (RR)

Another hypothesis will be looked for is the factors affecting on tissue necrosis (or the prediction) after *N. kaouthia* or *C. rhodostoma* injection. It is usually expressed as the Relative Risk (RR) which is the ratio of the probability of developing, in a specified period of time, an outcome among those receiving the treatment of interest or exposed to a risk factor or predictor, compared with the probability of developing the outcome if the risk factor or predictor or intervention is not present. Relative Risk is determined by first establishing a baseline, an accounting of how common a disease (or condition) is in the general population. If RR yields a ratio of 1.0, there is no increased risk at all. An increase in risk would result in a number larger than 1.0. A decrease in risk would result in a lower RR (i.e., less than 1), and indicates a protective effect. An Relative Risk of at least 2.0 is necessary to indicate a cause and effect relationship, and an Relative Risk of 3.0 is preferred. The magnitude of effect is usually expressed as one of the following.

Experimental event rate (EER), The percentage of intervention/exposed group who experienced outcome in question.

Control event rate (CER), the percentage of the control/nonexposed group who experienced outcome in question.

Relative Risk = (EER) / (CER)

The distance between fang marks (possibly indicating the size of the snake), the factors association with tissue necrosis of *N. kaouthia* bites will be used to calculate Relative Risk. The average distance between fang marks was 1.33 cm will be used as a cut of point.

In a previous study, the distance between fang marks more than 1.3 cm was 23 cases in total 68 cases, was 10 cases less than 1.3 cm (Pochanugool, Limthongkul and Wilde, 1997).

The distance between fang marks	Tissue necrosis	No tissue necrosis	Total
Distance between fang marks less than 1.3 cm	13	22	35
Distance between fang marks more than 1.3 cm	8	2	10
Distance between fang mark 1.3 cm (cut of point)			23
Total			68

Relative Risk = $\{(8/10) / (13/35)\}$ = 2.16

Sample size will be calculated in the next step. The statistical of comparison two independent groups consists of the following,

- State the null hypotheses and alternative (H_0 vs H_A).

- Identify the test statistic, the value with the choice of accepting H_A.

- Specify the decision-making rule. The null hypothesis is considered to be true if the calculated probability (based on the test statistic) is greater than desired level of significance (α), and false if the calculated probability is less than or equal to the significance level. It is commonly accepted that a result is considered to be significant if the calculated probability is less than 0.05.

Decision	True situation	
	H₀ is true	*H₀ is false*
H₀ is accepted	Correct decision	Type II error (β)
Hₐ is rejected	Type I error (α)	Correct decision

- Probability of type I error equals to the level of significance (α).

- The power of testing a statistical hypothesis is the probability when H_0 is rejected when is one minus the probability of the Type II error (1-β)

- Probability of type II error (β) is the probability of not reject the null hypothesis when it is false.

However, the relationship between Type I error and Type II error is such that decreasing the probability of Type I error increases the probability of Type II error, and decreasing the probability of Type II error increases the probability of Type I error.

The sample size , N, is calculated as follows.

$$N = \frac{[Z_{\alpha/2}\sqrt{2PQ} + Z_\beta \sqrt{P_1(1+R-P_1(1+R^2))}]^2}{(P_1(1-R))^2}$$

$Z_{\alpha/2}$ = 1.96 for a two-tailed test with 95% significance level,

Z_β = 1.28 with the power of testing is chosen to be 1-β = 0.8

P_1 : The proportion of tissue necrosis in control , if there is an indication of distance between fang marks less than 1.3 cm : 13/35 = 0.371

R = Relative Risk = 2.16

$$P = (P_1(1+R)) / 2 = (0.191\ (1+2.16)) / 2 = 0.30$$

$$Q = 1-P = 1-0.30 = 0.7$$

$$\text{Sample size} = \frac{[(1.96 \times \sqrt{2 \times 0.30 \times 0.70}) + (1.28\ \sqrt{[0.371 \times (1+2.16-0.371(1+2.16)]})]^2}{(0.371 \times (1-2.16))^2}$$

$$= 33 \text{ cases}$$

Thus, a sample size of 33 snakebite victims from *N. kaouthia* is required. For *C. rhodostoma* bites, no report about distance between fang marks on tissue necrosis are aviable. Another factor, tourniquets application, is selected for sample calculation. After application of the tourniquet average 1.6 hours (cut of point), mean venom levels were different before and after release in 2/6 cases (less tan 1.6 hours) (Ho et al., 1986).

Tourniquet application	Number of differences of venom level before and after release tourniquet	No differences of venom level before and after release tourniquet	Total
Tourniquet application less than 1.6 hours	2	4	6
Tourniquet application more than 1.6 hours	4	2	6
Tourniquet application 1.6 hours (cut of point)			1
Total			13

Relative Risk = (4/6) / (2/6) = 2

P_1 : The incidence of differences of venom antigen level before and after release in control , application tourniquet less than 1.6 : 2/6 = 0.333

R = Relative Risk = 2

P = (P1(1+R)) /2 = (0.153 (1+2)) / 2 = 0.229

Q = 1-P = 1-0.229 = 0.771

$$\text{Sample size} = \frac{[(1.96 \text{ X } \sqrt{2 \text{X} 0.229 \text{X } 0.771} + (1.28 \sqrt{(0.333(1+2-0.333(1+2)^2}]^2}{(0.333 \text{ X } (1-2))^2}$$

= 35 cases

The number of victims of *C. rhodostoma* injection is requried to be 35 cases .

In summary , 81 patients will be collected for *C. rhodostoma*, 81 for *N. kaouthia* bites respectively. All patients will be selected from 10 provincial hospitals mentioned. The data will included any age, gender, education and fulfill the eligibility criteria of the study.

9. Eligible Criteria

The snakebite victims from *N. kaouthia* or *C. rhodostoma* bites will be selected if they fulfil the inclusion criteria. Poisonous snakes including neurotoxic or hematoxic effects have many types of each genus, therefore, doctors hardly diagnoses the type of cuprit snake if the criteria are not obviously shown.

The inculsion criteria are:

i)   The patients who are admitted to hospitals.

ii)   All victims who report *N. kaouthia* or *C. rhodostoma* bites to doctors at hospitals.

iii)   Victims who bring *N. kaouthia* or *C. rhodostoma* snakes to hospitals or primary health care centers because the snake is the supportable evidence to present the snake type before doctor diagnosis.

iv)   Victims with signs and symptoms are clearly shown to be *N. kaouthia* or *C. rhodostoma.* In Thailand, many types of snakes are neurotoxic, however the severity of signs/symptoms of *N. kaouthia* is less neurotoxic than king cobra and *Bungarus* genus , and hematotoxicity effects in *C. rhodostoma* are stronger than other snakes in the same genus.

v)   Patients who agree to participate by signing informed consent.

Exclusion criteria. The conditions which preclude entrance of candidates into an investigation even if they meet the inclusion criteria. if snakebite patients die after the first day of admission in hospitals or health care centers. They will be excluded because the investigator cannot classify tissue necrosis and no progress of treatment.

10. Preparation for data collection

All snakebite victims will be admitted to Medical wards in hospitals or health care centers. Doctors and nurses will explain the study protocol to the participants and secure some understanding before starting the project. In each hospital, only one

hospital staff will be the direct contact person with the principle investigator. This contact person will work with a team to ensure adherence to protocol, collecting specimen for quality control of biochemical and hematological parameter and complete of data. Patients will be recorded for clinical manifestations at least three times, the first day of admission, the day when clinical conditions get worse and the last day of discharge from hospitals or health care centers.

The progress of the signs and symptoms will be assessed on a periodic basis including progression beyond the site of bite and systemic manifestations.

For *C. rhodostoma* bite, the following signs should be documented;

- Pain, swelling , or echymosis involving more than 7.5 cm. from the bite site.

- Coagulation parameter, e.g., abnormal ; Prothrobin Time (PT) < 20 s to 55 seconds , Partial Throbin Tim (PTT) < 50 to 75 seconds ; platelets , 50,000 – 100, 000/ml ; Venous Clotting Time (VCT) > 30 min.

N. kaouthia bite should be assessed for;

- Pain, swelling , or echymosis involving more than 7.5 cm. from the bite site.

- Tachycardia 26 to 40 breaths/minute ; accessory muscle use , poor vision or ptosis, dysphagia.

11. Data collection overview

Questionnaires are the mainstay for epidemiologists though the other methods can be just as useful and each has its strength and limitations. They will be used for finding some confounding factors which predict the severity of tissue necrosis after *N. kaouthia* and *C. rhodostoma* bites. Questionnaires will be completed by nurses or doctors who are the staffs of the project. They are composed of two parts: general data and clinical information. The clinical syndrome are divided into 6 categories in 4 scores. Data included vital signs (Temperature, Pulse, Respiratory Rate) and blood chemistries (Prothrombin Time, Partial Throbin Time, Fibrinogen, Platelet).

In retrospective study, all records from existing database of clinical manifestations 6 areas will be reviewed by nurse (contacting person) in provincial hospitals. The all of categories will be used to abstract data about tissue necrosis, clinical syndrome and associated factors from the hospital record.

12. Baseline data on possible confounder.

Confounding variable or Confounder is a variable that can modify the outcome of interest, is not an intermediate variable, and is associated with the factor under investigation. A confounding variable may be due chance or bias. Unless it is possible to adjust for confounding variables, their effects cannot be distinguished from those of factor(s) being studied. *Confounder in this study include demographic data, geographic data , factor related to snakebites and factors related to treatment. There are factors effecting on severity of tissue necrosis after N. kaouthia or C. rhodostoma bites within the framework of the current research proposal.*

12.1 Baseline data

12.1.1 Demorgraphic data include gender, age, education salary and occupation.

12.1.2. *Geographic data* covers province , urban, rural area and place of bite (indoor, outdoor).

12.1.3. *Factors related to snakebites* are divided into

- ❑ Season , site of bite and time of bite

- ❑ Predisposing factors including barefoot or darkness

- ❑ Distance between fang marks

12.1.4. Factors related to treatment of snakebite.

- ❑ Duration between bite and seeking medical device

- ❑ Hospitalization

- ❑ Treatment with antivenoms(the number of vials of antivenoms)

- ❑ Prognosis (dressing, debridgement , amputation)

- ❑ First aid treatment (tourniquet using)

13. Bias

Bias is the systemic error, deviation of results or inferences from the truth , or processes leading to such deviation. In this experiment, *bias will be minimized resulting;*

- • Clear inclusion criteria to screen snakebite victims.

- • Independent comparision of clinical data recorded by two investigators.

- Double check of laboratory (blood chemistry) results, by performing some duplicated blood sample at a reference laboratory at Chulalongkorn Hospital, Faculty of Medicine. If there is a significant discrepancy between local and reference value, the issue will be discussed with people involved to solve the discrepancies.

14. Feasibility

Feasibility is capable of being done or practicability. The snakebite severity score (SSS) could be done in victims either *C. rhodostom* or *N. kaouthia*, because it had been set up by the physician expert of snakebite and tested in many snake types. The SSS covers most human systems and the clinical manifestations of each system are clearly to determine. Therefore, the SSS evaluation is a good questionnaire for evaluation of the severity of tissue necrosis and progression of signs/symptoms of victims.

1. Chemicals and biochemicals

Chemicals. Chemicals were of reagent grade and were purchased from Sigma Chemical Company., St. Louis, Missouri, U.S.A. excepted as indicated.

Venoms. Crude venoms of *Naja kaouthia* (NK) and *Calloselasma rhodostoma* (CR) in fresh form were purchased from Queen Saovabha Memorial Institute, Bangkok. The venoms were kept at -20°C until used.

2. Assay of phospholipase A_2 and metalloproteinase activities

2.1. Phospholipase A_2 activity was determined by a method described by Gul, Khara and Smith, 1974. Crude venom (13 μg/ml of NK venom or 100 μg/ml of CR venom) was added to a mixture of 0.2 ml 5 % packed sheep red cells and 1 ml of egg yolk solution (dilution 1:60) into a glass tubes. The reaction was allowed to proceed for 15 min at 37 C, at the end of which time 5 ml of ice cold 0.9% NaCl containing 2 mM EDTA was added to stop the reaction and ghost red blood cells were removed by centrifugation at 5000 xg for 5 min. The release of hemoglobin was measured at 540 nm and expressed as percent of total hemoglobin contained in 0.2 ml of red cells when totally hemolysed with water.

2.2. Protease activity was determined by a modified method of Kunitz (1946) using casein as the substrate. The reaction mixture contained 0.1 ml of 0.1% casein solution in 0.15 M. PBS buffer, 8 mM $CaCl_2$, pH 7.3. into which snake venom (5 mg/ml of NK or 50 μg/ml of CR) was added in 0.2 ml of 0.15 M PBS. After 30 min incubation at 37°C , 300 μl of 5 % ice-cold trichloroacetic acid was added to the

mixture and the precipitate was removed by centrifugation at 10,000 xg for 5 min. The amount of acid soluble peptide was measured in 1 ml of the supernatant by the method of Lowry et al (1951).

2.3. Metalloproteinase activity was determined as the EDTA-inhibitable protease activity of the venom. In preliminary experiments, it was found that inhibition of protease activity of NK venom reached plateau at 2 mM EDTA while for CR venom, 5 mM EDTA was needed. Thus, the 100 % MP activities in NK and CR venoms were the protease activity inhibited by 2 mM and 5 mM EDTA respectively. The inhibition by each MP inhibitor was compared with that by EDTA.

3. Inhibition of venom phospholipase A_2 and metalloproteinase by various enzyme inhibitors

3.1. Enzyme inhibitors were divided into 2 groups : water soluble and water-insoluble inhibitors. The water-soluble inhibitors (EDTA, TEPA, DFO and Deferiprone) were prepared in 0.15 M. PBS, pH 7.3. The water insoluble inhibitors (quinine, mefloquine hydrochloride, para-bromophenacyl bromide and N-phenylglycine) were dissolved in dimethylsulfoxide (DMSO) before adding to the enzyme reaction mixture in which the final concentration of DMSO was 10% or less.

- Phospholipase A_2 inhibitors studied were : EDTA (0.1 – 5 mM), quinine (1mM – 10mM), mefloquine (0.125 mM– 0.50 mM) and para-bromophenacyl bromide (0.125 mM – 0.50 mM)

- Metalloproteinase inhibitors studied were: Desferrioxamine (1mM–20 mM), Deferiprone (1 mM – 10 mM), Tetraethylenepentamine (1 mM – 20 mM) and N-phenylglycine (1 mM – 20 mM).

- Hyaluronidase inhibitor studied was 10 mM Sodium aurothiomalate.

3.1.1. Assay of phospholipase A_2 inhibitors. The inhibitions of phospholipase A_2 of NK or CR venom by various inhibitors were studied by preincubation of each enzyme inhibitor with the crude venom for 0, 5, 10 min at room temperature prior to assay of the phopsholipase A_2 activity.

3.1.2. Assay of metalloproteinase inhibitors. The inhibitions of metalloproteinase of NK or CR venom by various inhibitors were studied by preincubation of each enzyme inhibitor with the crude venom for 0 , 5, 10 min at room temperature prior to assay of the metalloproteinase activity.

4. Determination of survival time of mice injected with NK or CR venom in the presence and absence of various enzyme inhibitors

Swiss albino mice weighing 20±2 gm supplied by the National Laboratory Animal Center, Mahidol University, were used.

The effect of PLA_2 or metalloproteinases inhibitors on the survival time of mice receiving snake venom was determined according to the modified method of Kuppusamy and Das (1991). The venom was prepared in sterile normal saline solution (NSS) at doses ranging from 0.3 – 0.7 µg/gm mouse for NK venom or 40-80 µg/gm mouse for CR venom. The venom solution (50 µl) was mixed with the inhibitor solution

(50 μl) at room temperature for 5 min. For water insoluble inhibitors, the final concentration of DMSO in the mixture was 2.5% (v/v). The mixture was injected subcutaneously (s.c.) into groups of 5 mice. The volume of injection was kept constant at 100 μl/ 20 gm mouse and all injections were done using a 250 μl Hamilton syringe. Control mice were injected with venom in NSS and 2.5%(v/v) DMSO. The survival time (time between injection and death) was then monitored. Results are expressed as mean survival time ± standard error (S.E).

5. Determination of local tissue damage induced by snake venoms

5.1. Determination of edema

Edema induced by NK or CR venom injection was determined according to the method of Chaves et al (1995). The venom was prepared in sterile NSS at a final concentration of 2.5 μg/20 gm mouse for NK venom and 2 μg/20 gm mouse for CR venom. Two types of experiments were carried out.

(A) Pre-incubation experiments. The venom solution was preincubated with various concentrations of enzyme inhibitor(s) for 5 min at room temperature. Then, 50 μl of each mixture was injected subcutaneously into the right footpads of groups of four mice. The left footpads were injected with 50 μl of NSS. Control mice were injected with venom alone in the right footpads. After 1 hr, the mice were killed by ether inhalation. Edema-forming activity was estimated according to Yamakawa et al (1976). Edema was expressed as the increment in weight of the right footpad comparing with the left footpad.

(B) <u>Independent inoculation experiments</u>. Groups of four mice were injected subcutaneously in the right footpads with 25 μl of venom solution. Then, enzyme inhibitor(s) in 25 μl was injected at the same site after 1, 3 or 10 min. The left footpad was injected with 50 μl of NSS. After 1 hr, the mice were killed by ether inhalation. Edema-forming activity was estimated as described above.

5.2. Determination of hemorrhage

Hemorrhage induced by NK or CR venom was determined according to the method of Gutierrez et al (1985). Two types of experiments were carried out .

(A) <u>Pre-incubation experiments</u>. A constant amount of CR venom (10 μg/mouse) was prepared (50 μl) in NSS and preincubated with 50 μl of enzyme inhibitor(s) or for 5 min at room temperature. Then, 100 μl of each mixture was injected intradermally (i.d.) in the abdominal region of groups of four mice. Control mice were injected with venom alone or NSS alone. Mice were sacrificed by ether inhalation 2 hr after injection. The skin was removed and the diameters of hemorrhagic spots were measured and compared to those of control mice. Result was expressed as mean hemorrhagic diameter (mm) $\pm$ standard error (S.E).

(B) <u>Independent inoculation experiments</u>. The venom solution (50 μl) was injected intradermally (i.d.) in the abdominal region of groups of four mice. After venom injection, a 50 μl solution of the enzyme inhibitor(s) was injected at the same site at different time intervals (1, 3, or 10 min). The total volume of injection was kept constant at 100 μl. The mice were killed by ether inhalation after 2 hr. The skin was

removed and the diameter of hemorrhagic spot was measured and compared to those of control mice. Result was expressed as mean hemorrhagic diameter (mm) $\pm$ standard error (S.E.).

5.3. Determination of myonecrosis

Myonecrosis induced by NK or CR venom was determined according to the method described by Leon et al., (2000) with some modifications.

(A) <u>Pre-incubation experiments</u>. The venom was prepared in sterile normal saline solution at a final concentration of 5 µg/mouse for NK venom and 25 µg /mouse for CR venom. The venom solution (25 µl) was preincubated with 25 µl of enzyme inhibitor(s) for 5 min at room temperature. Then, 50 µl of each mixture was injected intramuscularly in the right gastrocnemius of groups of four mice. Control mice were injected with either venom alone or NSS alone. Mice were sacrificed by ether inhalation 3 hr after injection. A blood sample was collected from the heart and centrifuged at 8,000 xg for 2 min using Backman Microfuge B, USA. Plasma was collected and assayed for creatine phosphokinase (CPK) activity using the Sigma CPK kit no.520.

(B) <u>Independent inoculation experiments</u>. Groups of four mice were injected intramuscularly in the right gastrocnemius with venom (5 µg of NK or 25 µg of CR), dissolved in 50 µl NSS. The enzyme inhibitor(s) was injected at the same site after 1, 3 or 10 min. In the case of inhibitor mixture, equal volume of 5 mM EDTA , 5 mM N-phenylglycine and 10 mM were mixed before injection into mice. The total volume of injection was kept constant at 50 µl. After 3 hr, the mice were sacrificed by ether

inhalation. A blood sample was collected from the heart and then centrifuged at 10,000 xg for 2 min using Backman Microfuge B, USA. Plasma was collected and assayed for creatine phosphokinase (CPK) activity using the Sigma kit no.520.

5.4. Determination of creatine phosphokinase activity

The creatine phosphokinase activity was determined according to the procedure described in Sigma Diagnostics Kit Manual (1999) using creatine standard (no.520-60) as reference. The reaction was carried out in polyvinyl microtiter plate (Polyvinyl ELISA plate, Costar, Cambridge). An aliquot of 2 μl plasma (from #5.3) was added to 10 μl of phosphocreatine solution used as substrate. The reaction was started by adding 4 μl of ADP-glutathione solution and the mixture was incubated at 37°C for 30 min. Following incubation, 4 μl of p-hydroxymercuribenzoate solution was added to stop the reaction. Then to each well was added 20 μl of α-napthol solution, 20 μl of 0.05% diacetyl solution and 140 μl of distilled water. The contents of the tubes were mixed immediately after each reagent addition. The pink color developed after 15 min at 37°C. The optical density was read against appropriate blanks at 540 nm using Multiskan MCC/340 MK II (Flow Laboratories, Irvine, Ayshire, Scotland). All assays were performed in triplicates. The creatine phosphokinase activity was calculated and expressed in term of Sigma units/ml.

One Sigma unit of creatine phosphokinase activity was defined as enzyme that can transfer 1 nmol of phosphate from phosphocreatine to ADP per min at 25°C.

5.5. Histological analysis

Calloselasma   rhodostoma venom (25 µg)   was preincubated with a mixture of various inhibitors (EDTA 5 mM , TEPA 5 mM , N-phenylglycine 5 mM) for 5 min before injection into the thigh muscle of a mouse. After 3 hr the mouse was killed by ether inhalation , and thigh muscle was dissected, fixed in 10 % formalin and embedded in paraffin. Sections were stained with hematoxylin/eosin stain. Myotoxic activity were studied under light microscope (Mebs, 1986) .