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A novel *in vitro* potency assay of antisera against Thai *Naja kaouthia* based on nicotinic acetylcholine receptor binding

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Snake envenomation is an important medical problem. One of the hurdles in antivenom development is the *in vivo* assay of antivenom potency which is expensive, gives variable results and kills many animals. We report a novel *in vitro* assay involving the specific binding of the postsynaptic neurotoxins (PSNTs) of elapid snakes with purified *Torpedo californica* nicotinic acetylcholine receptor (nAChR). The potency of an antivenom is determined by its antibody ability to bind and neutralize the PSNT, thus preventing it from binding to nAChR. The PSNT of *Naja kaouthia* (NK3) was immobilized on microtiter wells and nAChR was added to bind with it. The *in vitro* IC_{50} of *N. kaouthia* venom that inhibited 50% of nAChR binding to the immobilized NK3 was determined. Varying concentrations of antisera against *N. kaouthia* were separately pre-incubated with 5xIC_{50} of *N. kaouthia* venom. The remaining free NK3 were incubated with nAChR before adding to the NK3 coated plates. The *in vitro* and *in vivo* median effective ratio, ER_{50}s of 12 batches of antisera showed correlation (R^2) of 0.9809 ($p < 0.0001$). This *in vitro* assay should be applicable to antisera against other elapid venoms and should reduce the use of live animals and accelerate development of life-saving antivenoms.

Snake envenomation is an important medical problem especially in the developing world. It has been estimated that around 421,000–2.5 million people are envenomed annually with about 20,000–94,000 fatalities[1]. Antivenoms (AVs) are the rationale and the most effective therapy of snake envenomation. However, this serious public health problem has so far been neglected and effective, affordable antivenoms remain unavailable in many parts of the developing world. Recently, efforts from a number of research institutions are underway to solve this problem[2].

In the development and production of an AV, at least two major steps are involved: an effective immunization program and the pre-clinical testing to assess the neutralizing potential of the AV against the lethal effects of homologous and heterologous venoms. The accepted AV potency assay is the standard murine lethality assay to determine the median lethal dose (LD_{50}) that estimates the lethality of the venom and the median effective dose (ED_{50}) of the AV[3,4]. For this *in vivo* assay, three to five mice per venom dose are used and the total of about 6 different doses are tested. Thus, about 30 mice are needed for the determination of the LD_{50} of a venom. Similarly, about 30 mice are needed for the ED_{50} determination of an AV against a venom. Therefore, a large number of mice will be used for the *in vivo* neutralization assays. For example, the number of mice required by European Pharmacopoeia using this method to test the activity of one European viper venom antiserum (LD_{50} and ED_{50} tests combined) against five venoms, is 374 mice per batch of antivenom[5]. Using this figure, testing a pan-specific AV against 27 different venoms[6] would require about 2,020 mice. The assay is very costly, laborious and can give

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highly variable results. Moreover, some lethality studies have been shown to be inconsistent, suggesting that rodent death may not measure relevant efficacy outcomes in humans[7]. Lastly, witnessing the suffering and death of a large number of animals is the most difficult part of the experiment for many. In Buddhist countries like Thailand, most, if not all, laboratory personnel and graduate students refuse to do such experiments. Thus, it is becoming increasingly difficult to perform the *in vivo* assay for ethical and religious, as well as regulatory reasons.

For the above reasons, various types of *in vitro* neutralization assays have been developed to be used in place of, or to reduce the number of the *in vivo* assay. It has been reported that venom toxicity and effectiveness of AV can be studied using the chick biventer cervicis preparation[8–10]. This assay was used for screening AVs against the neurotoxic effects of venoms[11]. However, this *in vitro* assay requires the preparation of chick biventer cervicis muscles and the assay is laborious and time-consuming. *In vitro* neutralization of some venom enzymatic activities have been studied for use in AV potency assay[12]. It was shown that the neutralization of phospholipase A_2 activities by antivenom against *Micrurus nigrocinctus* highly correlated with the *in vivo* neutralization activity. Also, the inhibition of indirect hemolytic activity induced by phospholipase A_2 was also shown to correlate well with the *in vivo* potency of a polyspecific antivenom[13]. However, these assays are applicable only to antivenoms directed against venoms with enzymatic activities that parallel the lethality of the venoms.

Numerous investigators have studied and reported the use of ELISA for AV potency determinations together with the correlation between the results of the *in vitro* and *in vivo* neutralization assays[14–20]. However, the "neutralizing potency" described as "*in vitro* ELISA titer" occasionally did not correlate well with the *in vivo* neutralizing activity. For example, Ibrahim and Farid[21] studied the lethality-neutralizing potency, ELISA antibody level and the avidity indexes of a polyvalent antivenom against seven snake venoms. They showed poor correlation between the *in vivo* and *in vitro* assays with the *in vitro* assays always giving high values. ELISA has at times been criticized on the grounds that the antigen-antibody *'binding'* reaction measured cannot be assumed to be the same as the *'neutralization'* reaction of the antigen.

Elapid snakes (cobras, kraits and mambas) produce the lethal postsynaptic neurotoxins (PSNTs) which bind specifically and quasi-irreversibly with nicotinic acetylcholine receptor (nAChR) at the muscle end-plate[22,23]. This binding results in the inhibition of neuromuscular transmission which can lead to respiratory arrest and death[23]. Thus, it should be possible to develop an *in vitro* functional assay to test antivenom against elapid venoms based on the ability of the antivenom antibodies to inhibit the binding of PSNTs to nAChR. Such an *in vitro* assay would closely mimic the lethality reactions of PSNTs of the elapids *in vivo*, in particular cobras (*Naja* sp.). Cobras are in general listed as Category 1 of medically important snakes throughout most parts of Asia and Africa[4,24].

An *in vitro* potency assay based on PSNT binding to nAChR was previously studied for the venom of coral snake *Micrurus nigrocinctus*[12]. It was found that, the ED_{50} of the horse antisera against *M. nigrocinctus* in neutralizing the lethal effect of the venom did not correlate with the antivenom ability to inhibit the nAChR-binding activity but correlated well with the inhibition of the venom phospholipase A_2 activity.

By using a different reaction scheme and conditions from that studied above[12], we report here the development of a novel *in vitro* potency assay of monospecific antisera against the venom of the Thai monocled cobra *Naja kaouthia* based on nAChR binding. The assays gave excellent correlation ($R^2 = 0.9809$; $p < 0.0001$) with the corresponding *in vivo* assay using mice. This *in vitro* assay should be useful in reducing or partially replacing the *in vivo* assays used to test antivenoms against *N. kaouthia* and other elapid venoms.

Results

Development of the *in vitro* neutralization assay using nAChR-PSNT binding. *The optimal concentrations of NK3, nAChR, rat anti-nAChR antibody and goat-anti-rat HRP conjugate in the in vitro potency assay.* The optimal concentrations of NK3, nAChR, rat anti-nAChR antibody and goat-anti-rat HRP conjugate used in the *in vitro* potency assay were studied as described in Materials and Methods (step: pre-incubation 2). The results are shown in Fig. 1. As the concentration of NK3 used to coat the microtiter plate increased, the signal as measured by the OD_{450nm} increased. This was also observed when the concentration of nAchR used to bind the immobilized NK3 was increased. To economize on the nAChR available while obtaining reasonably high OD_{450nm} signal, it was decided to use 15 µg/ml of NK3 for coating the plates and 0.707 µg/ml of nAChR for binding to the NK3 coated plate. A 1:1600 dilution of rat anti-nAchR serum and a 1:4500 dilution of goat anti-rat-IgG conjugated HRP were used.

Inhibition of nAChR binding to NK3-coated plate by N. kaouthia venom or N. kaouthia cytotoxin. Crude *N. kaouthia* venom was used to determine the 50% inhibition of nAChR binding (*in vitro* IC_{50}). In the first step, crude *N. kaouthia* venom at various concentrations was incubated with the purified nAChR (0.707 µg/ml) for 1 hr at 25 °C. The solution was then transferred to NK3 coated plates. The concentration of the crude venom that reduced nAchR binding by 50% was defined as the IC_{50}. The results (Fig. 2) showed that the IC_{50} of *N. kaouthia* was 0.0281 µg/ml.

To study the specificity of the nAChR binding, *N. kaouthia* cytotoxin I at various concentrations (0.4875 to 0.0152 µg/ml) was tested as described above. It was shown (Fig. 2) that the cytotoxin had no effect on the nAChR binding. Thus, the inhibition reaction was specific to the postsynaptic neurotoxins.

Neutralization of N. kaouthia venom by horse monospecific antisera as determined by nAChR binding to the NK3-coated plate. Twelve horse monovalent anti-*N. kaouthia* sera were serially diluted 2-fold from 1:500 to 1:512,000 and the dilutions were separately incubated with $5xIC_{50}$ of *N kaouthia* venom (1.4029 µg/ml) in the 'Pre-incubation 1' experiment. After ultrafiltration and 'Pre-incubation 2', the reaction mixtures were added to the NK3-coated plates. The binding of the free nAChR to the plate was measured at OD_{450nm} and the results are shown in Fig. 3.

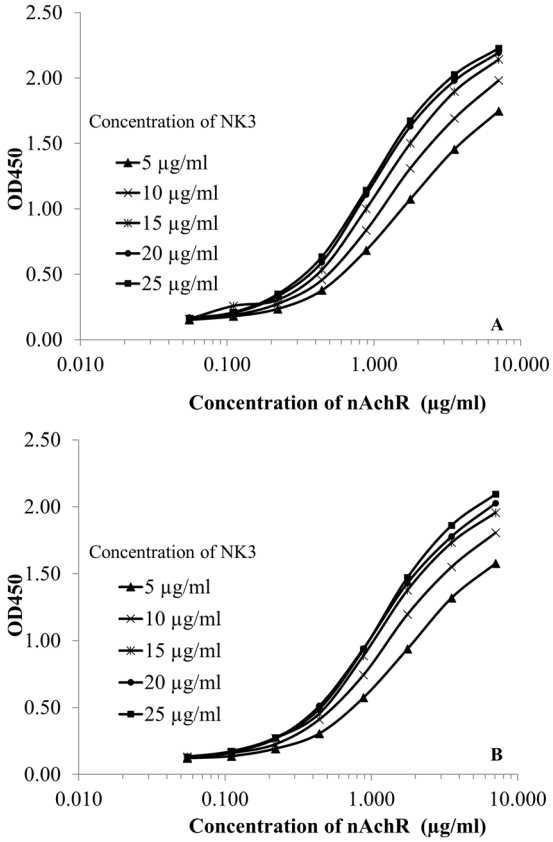

Figure 1. Determinations of optimal concentrations of NK3, purified nAChR, rat anti-nAChR serum and goat anti-rat IgG conjugated HRP. A: goat anti-rat IgG-HRP conjugate at 1:4500, B: goat anti-rat IgG-HRP conjugate at 1:6000.

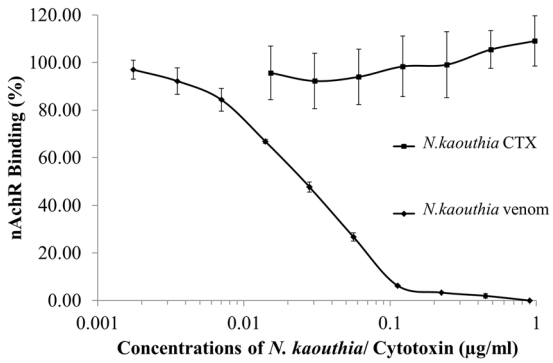

Figure 2. The inhibition of nAChR binding to the NK3 coated-plate by *N. kaouthia* venom and by *N. kaouthia* cytotoxin I. nAChR binding were expressed as mean ± S.D. of 4 determinations.

The *in vitro* median effective ratio (ER_{50}s), expressed as µg of venom neutralized per µl of antiserum, and the *in vivo* ER_{50}s (mg of venom neutralized per ml of antiserum) of the 12 horse antisera are shown in Table 1. The correlation coefficient, *R*, between the *in vitro* ER_{50}s and the *in vivo* ER_{50}s was 0.9904, and the coefficient of determination for the regression model was $R^2 = 0.9809$ ($p < 0.0001$), as shown in Fig. 4.

Discussion

It is reported here the first successful development of an *in vitro* potency assay for antiserum against an elapid snake based on nicotinic acetylcholine receptor binding. The reactions employed in the *in vitro* assay closely mimicked those of the *in vivo* toxicological reactions of the elapid postsynaptic neurotoxins. Unlike the ELISA which at times gave poor correlation with *in vivo* assay and has often been criticized in that the antibody binding did not necessarily result in toxin neutralization, the present *in vitro* assay involved the binding and neutralization by the antisera antibodies of the lethal snake toxins thus preventing them from binding to nAchR. The correlation

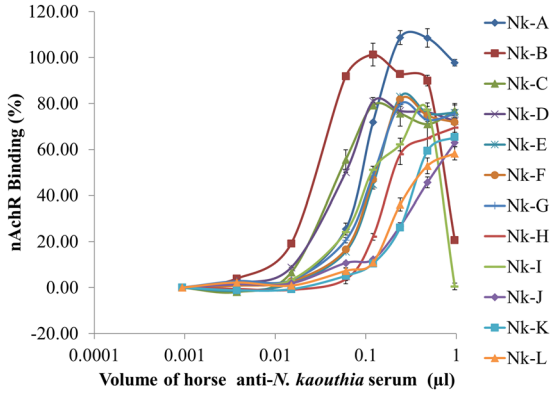

Figure 3. Effects of horse anti-*N. kaouthia* sera in neutralizing *N. kaouthia* venom as determined by nAChR binding to NK3-coated plate. nAChR binding were expressed as mean ± S.D. of 4 determinations.

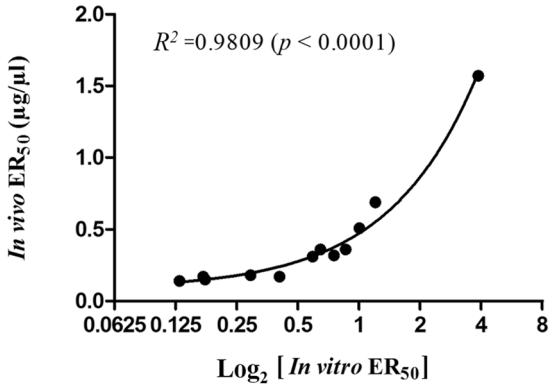

Figure 4. Regression between the nicotinic binding efficacy (Log_2 [*in vitro* ER_{50}]) and the lethality neutralization efficacy (*in vivo* ER_{50}). R^2: Coefficient of determination. *In vitro* ER_{50} values were expressed as mean ± S.D. (µg venom/µl antiserum) of 4 determinations. *In vivo* ER_{50} values were expressed as median dose ± 95% C.I. from serial dose-response study in mice (n = 4–5 mice per dose). Footnote: For each batch of antiserum, the *in vitro* ER_{50} was mean ± S.D. from 4 determinations while the *in vivo* ER_{50} was median ± 95% C.I. (C.I. = confidence level).

Horse	*In vitro* ER_{50} ± S.D. (µg venom/µl antiserum)	*In vivo* ER_{50} (mg venom/ml antiserum)
Nk-A	0.8598 ± 0.1089	0.36 (0.24–0.54)
Nk-B	3.8693 ± 0.2887	1.57 (1.05–2.36)
Nk-C	1.2039 ± 0.2074	0.69 (0.46–1.03)
Nk-D	1.0055 ± 0.1368	0.51 (0.34–0.76)
Nk-E	0.7526 ± 0.2438	0.32 (0.21–0.48)
Nk-F	0.5911 ± 0.0433	0.31 (0.21–0.47)
Nk-G	0.6456 ± 0.0727	0.36 (0.24–0.53)
Nk-H	0.2921 ± 0.0564	0.18 (0.12–0.27)
Nk-I	0.4060 ± 0.1322	0.17 (0.11–0.26)
Nk-J	0.1307 ± 0.0467	0.14 (0.09–0.20)
Nk-K	0.1748 ± 0.0031	0.15 (0.10–0.23)
Nk-L	0.1712 ± 0.0530	0.17 (0.12–0.26)

Table 1. *In vitro* and *in vivo* ER_{50}s of horse anti-*N. kaouthia* sera in neutralizing *N. kaouthia* venom. For each batch of antiserum, the *in vitro* ER_{50} was mean ± S.D. from 4 determinations while the *in vivo* ER_{50} was median ± 95% C.I. (C.I. = confidence level).

between the *in vivo* assay using mice and the developed *in vitro* assay was very high as supported by the correlation coefficient of $R = 0.9904$.

An *in vitro* assay based on PSNT binding to nAchR was studied by Stiles[25,26] and Alape-Giron *et al.*[12,27]. Using an ELISA format, the bindings of purified nAchR from *T. californica* to the immobilized long and short PSNTs were shown to be specific. Furthermore, these researchers showed that horse antivenom against *M. nigrocinctus nigrocinctus* venom contained antibodies that inhibited the binding of the venom α-neurotoxins to purified nAchR, and reversed the binding of the toxins already complexed with the receptor[12]. The antivenom ED_{50} in neutralizing the lethal effect of the venom was shown not to correlate with the antivenom's ability to inhibit the nAchR-binding activity ($r = 0.34$; $p > 0.05$) but correlated well with the inhibition of phospholipase A_2 activity. From these results, they concluded that the lethality of the venom was the result of the combined actions of various toxins[12] and recent proteomics results have shown that the short-chain α-neurotoxins are likely to play a leading role in the lethality induced by this venom[28].

The *in vitro* assay reported here involved reaction schemes that were different from those reported by Alape-Giron *et al.*[12]. Two salient features of the present assay protocol were as follows.

First, the two crucial reactions i.e., the antibody-venom toxins reaction, and the toxin-nAChR reaction, were carried out in solution rather than on solid surface. This was to allow for total exposure of the reactants' surface residues resulting in more complete binding with their counterparts, and also to avoid any possible steric interactions between the two high molecular weight reactants (IgG and nAchR) which are likely to be more pronounced on solid surface.

Second, after the venom-antibody reaction in 'Pre-incubation 1', the antibodies, free or toxin bound, were removed from the reaction mixture by ultrafiltration. This was important in that if these antibodies were not removed, any free excess antibody remaining in the 'Pre-incubation 1' reaction could react with the immobilized toxins in the final reaction resulting in reduced binding of nAChR (added in the later step) to the immobilized toxins. Furthermore, since the dissociation constant of toxin-antibody complexes are usually in the micromolar range while the toxin-nAchR dissociation constant is closer to nanomolar range[29], it is conceivable that, without the ultrafiltration to remove the antibodies, the antibody-bound toxin might dissociate and form tighter complex with nAChR. These reactions would shift the equilibrium of the toxin-antibody reaction, and the measured amount of nAChR bound to the immobilized toxin would also be reduced.

The described assay procedure was thought to improve the reactions involved and to eliminate or minimize any inaccuracy of reactant concentrations measured; leading to highly correlated *in vitro* and *in vivo* results.

It should be noted in Fig. 3 that at higher concentrations of the antisera Nk-A Nk-B and Nk-I, the nAChR bindings were decreased. This phenomenon, often observed in immunoassays, is known as the 'prozone phenomenon' or 'hook effect' where, at excess concentration of antibody, immunochemical reactions e.g., hemagglutination, were inhibited or become less pronounce[30,31].

The *in vitro* potency assay described here should be applicable to antivenoms against most, if not all, elapids whose venoms contain mainly or exclusively postsynaptic neurotoxins as major lethal components. However, the usefulness of the assay for some elapids producing other lethal toxins e.g., some *Bungarus* venoms may contain, in addition to PSNTs, presynaptic neurotoxins (β-neurotoxins) which are highly lethal[32,33]. Thus, this *in vitro* nAchR binding assay which worked well with antisera against the *Naja* venoms might not work as well with some of the *Bungarus* venoms, depending on the abundance of the β-neurotoxins present in the particular venom and their role to the overall neurotoxicity.

Since effective, affordable antivenoms against snake venoms remain unavailable in many parts of the world[2], studies were being made to produce pan-specific antivenoms that cover multiple snake venoms from wide geographical areas[6]. Such a pan-specific antivenom could be produced in large volume and, due to the economy of scale, could be produced at low cost. However, in the development of such pan-specific antivenoms, a large number of mice would be needed to assay its efficacy against many homologous and heterologous venoms, and over the years, the cumulative number of mice used will be even more perturbing considering the need to repeat the assay from batch to batch of antivenom. With the developed *in vitro* assay described here, the development and production of poly-specific or pan-specific AVs should become easier and simpler. This should eventually result in saving the lives of mice and the victims of snake envenomation.

In conclusion, the assay should reduce the use of mice for potency assays for example, during the immunization program and/or fractionation process of antivenom production. In some cases, it may even replace the *in vivo* assay. The *in vitro* assay is less expensive, less biologically variable and could avoid the ethical and religious issues involved. The *in vitro* assay could facilitate the development and production of new and effective antivenoms, especially the pan-specific antivenoms which usually employ a large number of mice. The availability of new antivenoms combined with the reduction in production cost could, in turn, save the lives of more snakebite victims, which are mostly from the poorer regions of the world[34].

Materials and Methods

Materials. Electroplaque tissue from *Torpedo californica* (Pacific electric ray) was obtained from Dr. Charles Winkler, Aquatic Research Consultants (San Pedro, CA, USA). *Naja kaouthia* (NK, formally known as *Naja naja siamensis*) venom from pool of several adult snakes of Thai origin and horse monovalent antisera against *N. kaouthia* was purchased from Queen Soavabha Memorial Institute (QSMI). Benzoquinonium dibromide was purchased from Santa Cruz (Dallas, TX, USA). Goat against rat IgG conjugated with horse radish peroxidase (HRP) was purchased from Abcam (SF, USA). *N. kaouthia* postsynaptic toxin 3 (NK3, formally known as *N. n. siamensis* toxin 3) was purified as described by Karlsson *et al.*[35]. *N. kaouthia* cytotoxin (CTX-I) was purified as described by Tan *et al.*[36]. N-hydroxysuccinamide-Sepharose (NHS-Sepharose) was from GE Health Care. All other reagents were from Sigma Chemical, St Louis. Missouri, unless stated otherwise.

Methods. *Purification of nAChR and production of anti-nAChR antibody in rats.* Purification of nAChR from *T. californica* electroplaque was carried out as described by Lindstorm *et al.*[37]. The purified receptor (10 µg) in 0.1 ml phosphate buffer saline (PBS) pH 7.4 was emulsified with Complete Freund adjuvant and injected subcutaneously into each of the eleven Wistar rats. The second and third immunizations were carried out using the receptor emulsified in Incomplete Freund adjuvant and alum as the adjuvant, respectively. Blood of each rat was collected from the heart at the end of the experiment.

In vivo neutralizing activity of horse monospecific antisera against N. kaouthia venom. The intravenous median lethal dose, LD_{50}, of *N. kaouthia* venom, 0.18 (0.12–0.27) µg/g, was adapted from Tan *et al.*[38] of the same laboratory using the same batch of venom as with the current work. Neutralization of lethality was conducted as described by Ramos-Cerrillo *et al.*[39]. Briefly, a challenge dose of the venom constituting 5 LD_{50} in 50 µl saline was pre-incubated at 37 °C for 30 min with varying dilutions of the pooled horse sera in normal saline, to give a total volume of 250 µl. The venom-antiserum mixture was subsequently injected into the caudal vein of the mice. The mice were allowed free access to food and water *ad libitum* and the number of survival after 48 h was recorded. The effective dose-50 (ED_{50}) was determined as the volume of antiserum that protected 50% of the challenged mice from death using probit analysis. The neutralizing efficacy of the antiserum was also expressed as median effective ratio ($ER_{50} \pm 95\%$ C.I. where C.I. is confidence interval) in mg venom/ml antiserum that gave 50% survival of the mice tested.

Development of the *in vitro* neutralization assay using nAChR-PSNT binding. *Optimal conditions of nAChR, rat anti-nAChR antibody and goat-anti-rat HRP conjugate binding to NK3 coated microtiter plate.* This assay was the basic assay format for *in vitro* binding of solubilized, purified nAChR to the elapid PSNTs immobilized on the microtiter plate. Briefly, purified NK3 at various concentrations were coated to the microtiter wells (Polystylene High Binding 3590, Costar). After washing with 0.05% TWEEN 20 in phosphate buffered saline (PBST), the plate was blocked with 200 µl/well of PBST and 1% BSA for 2 hr. The purified nAChR (in PBS containing 0.05% Tween 20 and 0.15 BSA) at various concentrations were added to bind the immobilized NK3 by incubation at 25 °C for 1 hour. After 3 time washings to remove the unbound nAChR, rat anti-nAChR serum at various concentrations was added and incubated at 25 °C for 1 hr; this was followed by addition of goat-anti-rat-HRP conjugate (ab7097, Abcam) and incubated for 1 hr at room temperature. After 4 washes with PBST, 100 µl/well of freshly prepared substrate solution (0.01% w/w 3,3′,5,5′-tetramethyl benzidine and 0.003% hydrogen peroxide in 0.075 M citrate buffer, pH 5.0) was added. The plate was allowed to stand in the dark for 30 min at 25 °C and the reaction was stopped by adding 25 µl of 4 N sulfuric acid. The absorbance of 450 nm was read against blank using an ELISA reader (Multiskan Go, Thermo Scientific). Optimal concentrations of NK3 (used for coating the plate), nAChR, rat anti-nAChR antibody and goat-anti-rat-HRP conjugate were estimated and used in the experiments that followed.

Inhibition of nAChR binding to the NK3 coated plate by N. kaouthia venom. The ability of an elapid venom (*N. kaouthia)* which contains PSNTs to inhibit the binding of nAChR to NK3 immobilized plate was studied and was expressed as IC_{50} (venom concentration inhibiting 50% of the nAChR binding). In this assay, *N. kaouthia* crude venom at various concentrations was pre-incubated (25°C for 1 hr) with a fixed and optimal concentration of nAChR before the mixture was added to the NK3-coated plate and incubated at 25°C for 1 hour. This was followed by additions of rat anti-nAChR serum at 1:1600 dilution and incubated at 25°C for 1 hr, followed by 1:4500 diluted goat-anti-rat-HRP conjugate (Abcam) and incubated for 60 min at 25 °C. A parallel experiment using purified NK3 as the reference standard in place of the venom was also carried out. The concentration of the tested venom used in the pre-incubation step that inhibited 50% of the nAChR binding to the immobilized NK3 was the median inhibitory concentration (IC_{50}) of that venom.

Inhibition of the N. kaouthia venom PSNTs from binding to nAChR by horse antisera. Using a format similar to that described above, an *in vitro* assay of horse antiserum potency (*in vitro* ED_{50}) was carried out. Horse sera at various amounts (0.94 nl–0.96 µl) were pre-incubated at 37 °C for 1.5 hr with a fixed amount ($5 \times IC_{50}$) of *N. kaouthia* venom in 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.05% TWEEN20, 0.1% w/v BSA in a total volume of 480 µl. This was referred to as 'Pre-incubation 1'. The mixture was then filtered through a 100 kDa MWCO ultrafiltration membrane (Amicon®) to remove antibody-toxin complexes, free antibodies and some other high molecular weight horse serum proteins. The filtrates (126 µl) containing the remaining free venom PSNTs were then incubated with an optimal amount of nAChR (14 µl in the same buffer) at 25° C for 1 hr as described above and this was referred to as 'Pre-incubation 2'. The mixtures containing any remaining free nAchR were then added to the microtiter wells immobilized with NK3, followed by the rat anti-nAChR antibody, goat-anti-rat HRP conjugate, etc. The reaction products were then processed as described above. Wells incubated with a non-immune horse serum in place of antisera were included as background control.

The percentage of nAChR binding was then determined using the following formula:

$$\%\text{nAChR binding} = \frac{(\text{OD sample} - \text{OD Ag control}) \times 100}{(\text{OD max} - \text{OD Ag control})}$$

'OD max' represented the binding of nAChR (optimal amount) which was not pre-incubated with the venom or antiserum.

'OD Ag control' represented the binding of nAChR after being pre-incubated with 5 folds of IC_{50} of *N kaouthia* venom (and without antiserum in 'Pre-incubation 1').

'OD sample' represented the binding of nAChR after nAChR (optimal amount) was pre-incubated with filtrate from 'Pre-incubation 1' (where 5 folds of IC_{50} of *N. kaouthia* venom was pre-incubated with various amount of antiserum).

From the results, dose–response curves of horse sera volumes vs percents of nAChR binding were constructed. The *in vitro* neutralizing activities (ED_{50}s) represented the horse antiserum volumes at which the nAChR binding was inhibited by 50 percent compared to wells incubated with buffer in place of antisera. The *in vitro* median effective ratio, ER_{50}, represented μg venom/μl antiserum that the nAChR binding was inhibited by 50% was calculated. The results of the *in vitro* study on nAChR binding for every batch of the horse antisera (Nk-A to Nk-L) were presented as means ± S.D. of 4 determinations.

Ethics approval. Experiment involving rats was reviewed and approved by the Animal Care and Use Committee of the Faculty of Veterinary Science, Mahidol University, Protocol no. MUVS-2014–29 in accordance with the Guidelines of the National Research Council of Thailand. The protocol of animal study on mice was based on the guidelines given by the Council for International Organizations of Medical Sciences (CIOMS) and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Malaya (Ethical clearance No. 2014-09-11/PHAR/R/TCH).

Miscellaneous procedures. Protein concentration was determined by the procedure described by Lowry *et al.*[40] and by BCA Protein assay Kit (Pierce™) using bovine serum albumin as the standard. IC_{50} and ED_{50} values were determined using GraphPad Prism 5.0 program and BioStat 2009 version 5.8.3.0, respectively. The correlation analysis was evaluated by linear regression using GraphPad Prism 5.0 software. In brief, the correlation coefficient R was determined from the linear regression model, and R^2 (coefficient of determination) is the square of the correlation coefficient. An R^2 of 0.8–1.0 indicates that the regression line well fits the data in correlation. The statistical significance of the correlation test was set at $p < 0.05$.

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Author Contributions

K.R. and C.H.T. designed the study; P.S., K.P., K.Y.T., S.E., B.C. and W.W. undertook the laboratory studies; K.Y.T., C.H.T., K.R., P.S. and S.Y. undertook the analysis; K.R., P.S., C.H.T. and N.H.T. drafted the manuscript and all authors contributed to the final version; K.R. and C.H.T. are responsible for the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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