A phospholipase A₂ inhibitor has been purified from the serum of *Notechis ater* using DEAE-Sephaloc chromatography. The inhibitor was found to be composed of two protein subunits (α and β) that form the intact complex of approximately 110 kDa. The α-chain is a 30-kDa glycoprotein and the β-chain a nonglycosylated, 25-kDa protein. N-terminal sequence analysis reveals a high level of homology to other snake phospholipase A₂ inhibitors. The inhibitor was shown to be extremely pH and temperature stable. The inhibitor was tested against a wide variety of phospholipase A₂ enzymes and inhibited the enzymatic activity of all phospholipase A₂ enzymes tested, binding with micromole to nanomole affinity. Furthermore, the inhibitor was compared with the Eli-Lilly compound LY311727 and found to have a higher affinity for human secretory nonpancreatic phospholipase A₂ than this chemical inhibitor. The role of the carbohydrate moiety was investigated and found not to affect the *in vitro* function of the inhibitor.

For many years research on PLₐ₂ enzymes has centred on snake venom PLₐ₂s, largely because of the fact that PLₐ₂ enzymes are extremely abundant in snake venoms and are easily purified. PLₐ₂ enzymes have also been identified and purified from bovine, porcine, and human pancreas (1–3) and in snake venom PLA₂s, largely because of the fact that PLA₂ enzymes have centred on structural and sequence characteristics. Pancreatic PLₐ₂ enzymes belong to group I, whereas the human secretory nonpancreatic PLₐ₂ is a group II enzyme. PLₐ₂ enzymes from snake venom belong to group I or II (6–8).

Recently PLₐ₂ enzymes have been implicated as playing a role in a range of diseases including rheumatoid and osteoarthritis, asthma, acute pancreatitis, psoriasis, multiple organ failure, septic shock, and adult respiratory distress syndrome (9–11). Several review articles (9, 10, 12–14) have examined the physiological and potential disease role of hsPLₐ₂-II. Of major importance are the reaction by-products of PLₐ₂ activity, a free fatty acid and a lysophospholipid. The fatty acid is often arachidonic acid, one of the main constituents of the cell membrane in several tissues (15–17). Arachidonic acid is the precursor for inflammatory mediators such as thromboxanes, leukotrienes, and prostaglandins (18,19). Lysophospholipids are the precursor of platelet activating factor (20).

The suggestion that PLₐ₂ enzymes play a role in diseases process has intensified research on PLₐ₂ inhibitors. Many investigators have purified and characterized PLIs from numerous sources including plant, fungi, and bacteria (21–28). A large research effort has gone into the design of synthetic compounds, such as the Eli-Lilly compound LY311727, which specifically inhibits hsPLₐ₂-II (29). Additionally, a number of PLIs have been purified from the serum of elapid and crotalid snakes. These PLIs have all been shown to be homologous or heterologous complexes that interact with PLₐ₂ enzymes to inhibit enzymatic activity (30–43).

We now report the purification and functional characterization of a PLI from *Notechis ater* (Australian tiger snake) serum. The inhibitor termed, *N. ater* inhibitor (NAI) has been shown to inhibit the enzymatic activity of all PLₐ₂ enzymes that it was tested against. Additionally, the affinity of the interaction was in the nanomole to micromole range dependent on the PLₐ₂ examined.

**EXPERIMENTAL PROCEDURES**

**Materials**—Notexin from *Notechis scutatus* (Common tiger snake) and taipoxin from *Oxyuranus scutellatus* (Australian taipan) were purchased from Venom Supplies (Tanunda, South Australia). Bee venom PLA₂ (*Apis mellifera*), bovine pancreatic PLA₂ (*Bos taurus*), and *Crotalus atrox* PLₐ₂ (Western diamondback rattlesnake) were purchased from Sigma. Recombinant human secretory human type II PLₐ₂ was generously donated by the Garvan Institute (Darlinghurst, New South Wales, Australia). The PLₐ₂ inhibitor LY311727 was generously donated by Eli-Lilly and Co. (Indianapolis, Indiana). Pooled *N. ater* serum was obtained from World Tiger Snake Farm (Launceston, Tasmania). It should be noted that the serum was pooled from two very similar subspecies of *N. ater*, namely *N. ater seroventyi* (Chappell Island tiger snake) and *N. ater humphreysi* (Tasmanian tiger snake).

**Purification and N-terminal Sequencing**—NAI (which elutes in the 0.5 M peak) was purified from whole *N. ater* serum using a DEAE-Sephacel (Amersham Pharmacia Biotech) column (1.5 × 15 cm). Serum (10 mg of total protein) was loaded onto the column that had been equilibrated in 0.1 M ammonium acetate, pH 7.0, at a flow rate of 0.5 ml/min. Proteins were eluted with an increasing concentration of 0.1, 0.25, 0.5, and 1.0 M ammonium acetate, pH 7.0, at a flow rate of 0.5 ml/min. The eluant was monitored at 280 nm (ISCO, UA-6 UV-visible Detector), and fractions were collected (ISCO, Retriever II). The 0.5 M peak (referred to as the semi-purified preparation or SPP) was used for functional characterization of NAI. The α- and β-chains of NAI were purified on a Amersham Pharmacia Biotech smart system fitted with a Amersham Pharmacia Biotech Sephasil C8 5 μm (2.1 × 100 mm) column. The column was equilibrated in 0.1% (v/v) trifluoroacetic acid in water (buffer B) at a flow rate of 100 μl/min. The SPP collected form the DEAE-Sephacel column was loaded onto the C8 column and eluted with a gradient of 0.08% (v/v) trifluoroacetic acid, 80% (v/v) acetonitrile in water (buffer B). The percentage of buffer B was increased as follows: 0–2 min 0%, 2–10 min 0–55%, 10–50 min 55–70%, and 50–60 min 70–100%. The eluant was monitored at 214 and 280 nm. Software controlled peak fractionation was used to collect peaks with the integrated fraction collector. Following reduction and alkylation the pro-
Functional Characteristics of NAI

Proteins were N-terminally sequenced with a Perkin-Elmer Applied Biosystems Procise 494 protein sequencer.

Size Exclusion Chromatography—The molecular mass of NAI was assessed by size exclusion chromatography according to the method of Andrews (44). A Superose 12 HR 10/30 (Amersham Pharmacia Biotech) column was calibrated with; aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and lysozyme (14.4 kDa) at a flow rate of 0.5 ml/min in 0.1 M ammonium acetate, pH 7.0. The SPP was chromatographed under identical conditions, and the native mass of NAI was estimated using the elution

![Fig. 1. DEAE-Sephacel purification of NAI](image)

Whole *N. ater* serum (10 mg of total protein) was loaded onto a DEAE-Sephacel column (1.5 × 15 cm) that had been equilibrated in 0.01 M ammonium acetate, pH 7.0, at a flow rate of 0.5 ml/min. Protein was eluted with increasing salt concentrations as indicated on the figure. NAI elutes in the 0.5 M peak (P4).

![Fig. 2. SDS-polyacrylamide gel electrophoresis analysis of DEAE-Sephacel separated *N. ater* serum](image)

Peaks were collected (as shown in Fig. 1) and analyzed by SDS-polyacrylamide gel electrophoresis, and the gel was silver-stained. Lanes 1 and 8, molecular mass markers (sizes as indicated, kDa); lane 2, whole serum; lane 3, peak 1; lane 4, peak 2; lane 5, peak 3; lane 6, peak 4; lane 7, peak 5 from Fig. 1.
volume of the peak containing NAI.

Phospholipase A₂ Assay—Two PLA₂ assays were utilized, either the spectrofluorometric assay of Radvanyi et al. (45) or a mixed micelle assay based on the method of Seilhamer et al. (46). Assays were carried out as described except that enzymes were dissolved in saline/0.1% (w/v) bovine serum albumin. For the spectrofluorometric assay substrates utilized were either 1-hexadecanoyl-2-(1-pentadecanoyl)-sn-glycero-3-phosphocholine (10pPC, Molecular Probes, Inc.) or 1-hexadecanoyl-2-(1-pentadecanoyl)-sn-glycero-3-phosphoglycerol, ammonium salt (10pPG, Molecular Probes, Inc.). Phosphatidyl ethanolamine, L-α-1-palmitoyl-2-arachidonoyl, [arachidonoyl-L-¹⁴C] (NEN Life Science Products) (specific activity, 2.035 Gbq/nmol) was used as substrate in the mixed micelle assay.

Temperature and pH Studies—The pH stability was investigated by altering the pH of the solution in which the SPP (10 μg/assay) was dissolved and testing this in the fluorometric PLA₂ assay. The assay was performed using N. scutatus venom as the PLA₂ source (50 ng/assay) at room temperature. The pH values tested were 2, 4, 6, 7, 8, 9, 10, and 12. The SPP was made up to the appropriate pH and incubated overnight at 4 °C prior to testing. Temperature stability was assessed in the same manner as the pH stability. Samples were heated or cooled at the appropriate temperature for 30 min and immediately tested in the fluorometric PLA₂ assay at room temperature. The temperatures examined were 4, 25, 37, 50, 60, 70, 80, 90, and 100 °C. For both experiments the assay was performed at room temperature. Also, samples were not preincubated with venom because the stability of the PLA₂ under varying pH and temperature values could not be assured. All samples were performed in triplicate with appropriate positive and negative controls. Results are expressed as the percentage of inhibition relative to control values. The α- and β-chains were purified by RP-HPLC (see above) after deglycosylation and analyzed by mass spectrometry using a Micromass Platform II single quadrupole instrument. The mass of the reverse phase purified proteins were also measured before deglycosylation.

IC₅₀ Determination—All enzymes except hsPLA₂-II and bovine pancreatic PLA₂ were tested in the fluorometric assay using 10pPC as substrate. Bovine pancreatic PLA₂ and hsPLA₂-II were tested in the fluorometric assay using 10pPG as substrate and in the mixed micelle assay. The amount of enzyme added for the fluorometric assay was as follows; notexin, 16.7 ng; taipoxin, 800 ng; C. atrox PLA₂, 40 ng; bee venom PLA₂, 10 ng; bovine pancreatic PLA₂, 6250 ng. For the mixed micelle assay 100 ng of bovine pancreatic PLA₂ or 5 ng of hsPLA₂-II were used. All enzymes were dissolved in saline/0.1% (w/v) bovine serum albumin, except bovine pancreatic PLA₂ and hsPLA₂-II, which were dissolved in 20 mM Tris, pH 8.0, and 1 mM NaCl, 10 mM Tris, pH 7.5,

Figure 3. RP-HPLC purification of NAIα and NAIβ. The SPP (∼80 μg) was purified with an Amersham Pharmacia Biotech Sephasil C8 (2.1 × 100 mm) column at a flow rate of 100 μl/min. The column was equilibrated in 0.1% (v/v) trifluoroacetic acid, water, and the proteins were eluted with an increasing concentration of buffer B (0.08% (v/v) trifluoroacetic acid, 80% acetonitrile, water) as indicated on the graph.

| Chain       | N-terminal sequence                           |
|-------------|-----------------------------------------------|
| α-Chain     | HSCEICHNFRGDCQSDDEEECASPDQCG                 |
| β-Chain     | LECEICIGLGECAHNTKTCDAQQTCV                   |

Table I: N-terminal sequence of NAIα and NAIβ

Following RP-HPLC separation and reduction and alklylation, both chains were N-terminally sequenced.
respectively. The IC\textsubscript{50} was determined by preincubating varying concentrations of NAI (10.5 mg/mL) in a constant volume, against a constant amount of enzyme (see above and Refs. 49–51). The concentration of NAI was varied between 30 \( \mu \)M and 70 pM (PLA\textsubscript{2}- and assay-dependent) until a sigmoidal dose response curve was generated allowing calculation of the IC\textsubscript{50} value. For comparison, the IC\textsubscript{50} for the Lilly compound LY311727 was determined using the mixed micelle assay with hsPLA\textsubscript{2}-II. All samples were performed in triplicate. Results were analyzed by nonlinear regression with GraphPad Prism (version 2.01) and expressed as the percentage of inhibition relative to control values.

**RESULTS**

**Purification and N-terminal Sequencing**—NAI was purified from whole \textit{N. ater} serum utilizing a DEAE-Sephacel column (Fig. 1). All peaks were collected, concentrated, and tested for inhibitory activity against \textit{N. scutatus} venom in the fluorometric assay. Peaks 3 and 4 inhibited; however, when samples were diluted to a equal protein concentration, inhibition of PLA\textsubscript{2} enzymatic activity was only detected in peak 4. The purity of NAI is approximately 90\% as judged by SDS-polyacrylamide gel electrophoresis analysis (Fig. 2). NAI is composed of two proteins, an \( \alpha \)-chain (\( \sim 30 \) kDa) and a \( \beta \)-chain (\( \sim 25 \) kDa). These proteins are noncovalently associated because a similar separation pattern is observed when the gel is run under nonreducing conditions (not shown). The native mass of NAI was estimated at 110 kDa by size exclusion chromatography (not shown). The \( \alpha \)– and \( \beta \)-chains can be separated by RP-HPLC (Fig. 3), and it is apparent that isoforms of the \( \alpha \)-chain are present. By measuring the area under the curve for the \( \alpha \)– and \( \beta \)-chains at 280 nm and correcting for their relative absorbance using extinction co-efficients calculated from the amino acid sequence, the NAI complex is suggested to be composed of two \( \alpha \)-chains and one \( \beta \)-chain. The N terminus of the purified chains were sequenced (Table I). Two N termini were present in the peak containing NAI\( \alpha \), demonstrating that isoforms were partially separated under the RP-HPLC conditions utilized (Fig. 3).

**Deglycosylated NAI**—The glycosylation status of NAI\( \alpha \) and NAI\( \beta \) was investigated with the digoxigenin glycan detection system. After determining that only the \( \alpha \)-chain was glycosylated, the type of linkage was analyzed, using enzymes specific for N-linked or O-linked sugars (52, 53). All carbohydrate moieties were removed by N-glycosidase F but were not affected by O-glycosidase (not shown). Mass spectrophotometric analysis of the native and deglycosylated \( \alpha \)-chain (not shown) indicated mass differences of 2207 and 2864 Da, which are consistent with carbohydrate masses of biantennary sugars (54). After deglycosylation, NAI (in the form of the SPP) was tested for inhibitory activity against whole \textit{N. scutatus} venom, \textit{A. bilineatus} venom, and bee venom PLA\textsubscript{2} using the fluorometric assay (Fig. 6). It is apparent that deglycosylated NAI is as active on

![Fig. 4. Temperature stability of NAI. NAI was incubated at varying temperatures for a period of 30 min and tested in the fluorometric assay for inhibitory activity against \textit{N. scutatus} venom. Results are represented as the averages \pm S.E.]
Functional Characteristics of NAI

**FIG. 5.** pH stability of NAI. NAI was incubated in solutions of varying pH overnight and tested in the fluorometric assay for inhibitory activity against *N. scutatus* venom. Results are represented as the averages ± S.E.

**FIG. 6.** Activity of deglycosylated NAI. SPP (containing NAI) was deglycosylated with N-glycosidase and tested for inhibitory activity against *N. scutatus* venom, *A. bilineatus* venom, and bee venom PLA₂ using the fluorometric assay. DG, deglycosylated NAI; Native, native SPP (control).
**IC50 Determination**—The IC50 values determined (with 95% confidence interval) using the fluorometric assay (Fig. 7) with 10pPC as substrate for notexin, taipoxin, *C. atrox* PLA2, and bee venom PLA2 enzymes are as follows: 0.13 nM (0.05–0.28 nM), 0.89 nM (0.53–1.5 nM), 2.4 nM (1.4–3.9 nM), and 0.16 nM (0.085–0.31 nM), respectively. The IC50 for bovine pancreatic PLA2 (Fig. 8) calculated using the fluorometric assay with 10pPG as substrate (the enzyme is not active on 10pPC) was 32.3 nM (21.8–47.7 nM). Using the mixed micelle assay the IC50 values for bovine pancreatic PLA2 and hsPLA2-II (Fig. 9) were 12,050 nM (2,030–71,490 nM) and 287.3 nM (146.4–563.9 nM), respectively. The IC50 using LY311727 (Fig. 10) on hsPLA2-II in the mixed micelle assay was calculated at 808 nM (551.4–1,184 nM).

**DISCUSSION**

**Characteristics of NAI**—N-terminal sequencing revealed two isoforms of NAIα that could be partially separated under the conditions utilized. These isoforms have high homology to other PLIs, with 67–70% homology to the 30-kDa chain of the PLI from *Naja naja kaouthia* (Thailand cobra), depending on which sequence was used for comparison. Only one sequence was present for NAIβ, which also shows high homology (84%) to the 25-kDa chain of the PLI purified from *N. naja kaouthia* (31). A number of PLIs have been isolated from either elapid or crocidaid serum. Many of these PLIs have been sequenced and show homology to two protein families. We propose that PLIs with homology to urokinase plasminogen activator receptor, Ly-6, and CD59 will be classified as Class A PLIs. Those with homology to the carbohydrate recognition domain of C-type lectins will be classified as Class B PLIs, whereas other PLIs will be classified as Class C PLIs.

Experiments were designed to investigate the pH stability and thermostability of NAI (Figs. 4 and 5). NAI was found to be extremely pH- and temperature-resistant with greater than 89% inhibition recorded for temperatures between 4 and 90 °C and greater than 94% inhibitory activity retained between pH 4 and 12. These experiments also serve to demonstrate that NAI is an effective inhibitor whether it is added before the PLA2 (IC50 studies) or added after the PLA2 (pH or temperature studies). When NAI was added after the PLA2 inhibition was immediate and as effective as when NAI was preincubated with the PLA2 enzyme. Preincubation was used for the IC50 studies because the assay was empirically determined to be more robust when performed in this fashion.

Carbohydrate moieties are common on snake PLIs and have been detected or presumptive evidence for their existence has been found on at least one chain for all snake PLIs characterized (30, 33, 34, 36–43). The common occurrence of carbohydrate moieties raises the question as to its role in vivo or in vitro. Because NAI was found to cross-react with a range of PLA2 enzymes, deglycosylated NAI was tested for inhibitory activity against group I, II, and III venom PLA2 enzymes (Fig. 6). The results demonstrated that the inhibitory activity of NAI is not affected in vitro by the glycosylation status of the α-chain and that deglycosylated NAI is still as active as native NAI on all PLA2 enzymes tested. A similar finding was reported for the PLI isolated from *Laticauda semifasciata* serum (42). From this we can postulate that carbohydrate moieties present on the α-chain play little or no role in the in vitro association of NAI with the inhibited PLA2 enzyme.

**IC50 Determination**—To gain quantitative data on the affinity of NAI for PLA2 enzymes, IC50 values were determined for notexin, taipoxin, *C. atrox* PLA2, bee venom PLA2, bovine pancreatic PLA2, and hsPLA2-II (Figs. 7–10). It must be considered that some experiments were performed under varying assay conditions.
conditions. The calculated IC$_{50}$ values for bovine pancreatic 
PLA$_2$ were vastly disparate depending on the assay utilized 
(32.3 nm fluorometric assay and 12,050 nm mixed micelle). This 
in itself reveals something about the interaction between NAI 
and the inhibited PLA$_2$. The difference in the results cannot be 
explained because of the enzyme concentration used in the 
assay, because more enzyme was used in the fluorometric assay 
(6,250 ng/assay) than the mixed micelle assay (100 ng/assay). 
If enzyme concentration was the cause, one would expect the 
opposite finding with the IC$_{50}$ values.

However, it is clear that NAI is able to inhibit all PLA$_2$ 
enzymes against which it was tested, using a variety of sub-
strates and assay conditions. Direct comparisons can be made 
for the IC$_{50}$ calculated for hsPLA$_2$-II and bovine pancreatic 
PLA$_2$ (Fig. 9) using the mixed micelle assay, with NAI having a 
much higher affinity for hsPLA$_2$-II. Clearly the only variable 
is the enzyme itself with hsPLA$_2$-II, a basic protein, belonging 
to group II (55), whereas bovine pancreatic PLA$_2$ is a group I 
neutral PLA$_2$ enzyme (56). As such, it is possible that a charge 
interaction between NAI and the PLA$_2$ is important for binding 
or that the C-terminal extension present in group II PLA$_2$ 
enzymes may play a role in increasing the affinity of the 
interaction.

Examination of IC$_{50}$ data obtained with the fluorometric 
assay reveals a similar pattern. In order of increasing IC$_{50}$ 
values results were: notexin (basic, monomer, group I), bee 
venom PLA$_2$ (basic, monomer, group III), taipoxin (heteroti-
mer, group I), and $C$. atrox PLA$_2$ (acidic, homodimer, group II; 
Refs. 57–63). With the exclusion of taipoxin, these results 
appear to confirm the observation made for hsPLA$_2$-II and 
bovine pancreatic PLA$_2$. That is, basic PLA$_2$ enzymes are 
bound with higher affinity, whereas neutral or acidic PLA$_{28}$ are 
bound with lower affinity. However, the interaction is not that 
simple because the three-dimensional structure of bee venom 
PLA$_2$ is markedly different from that of notexin or hsPLA$_2$-II 
(64–66). Additionally, $C$. atrox PLA$_2$ is an acidic homodimer, 
and the affinity of NAI for this enzyme is still quite high. The 
decrease in affinity from notexin to $C$. atrox PLA$_2$ is certainly 
not comparable with that seen for hsPLA$_2$-II (basic) versus 
bovine pancreatic PLA$_2$ (neutral). It must be remembered the 
location of the charges and three-dimensional structure of the 
inhibited PLA$_2$ enzyme may be important for the affinity of 
NAI. Finally, taipoxin, a heterotrimer approximately 46 kDa is 
size, is strongly bound by NAI, and is composed of basic, acidic, 
and a neutrally charged chains (59–61). Clearly, NAI is work-
ning via a mechanism that all PLA$_2$ enzymes have in common.

For comparison with a chemical inhibitor, the IC$_{50}$ values for 
hsPLA$_2$-II using NAI or LY311727, were determined (Fig. 10). 
LY311727 was designed using a structure based approach by 

Fig. 8. Inhibition curve for NAI versus bovine pancreatic PLA$_2$ enzyme. For experimental details see "Experimental Procedures." The 
IC$_{50}$ value was determined for bovine pancreatic PLA$_2$ using the fluorometric assay with 10pPG as substrate. Results are represented as the 
averages ± S.E.

For comparison with a chemical inhibitor, the IC$_{50}$ values for 
hsPLA$_2$-II using NAI or LY311727, were determined (Fig. 10). 
LY311727 was designed using a structure based approach by 
crystallizing the inhibitor with hsPLA$_2$-II and then examining 
the structure and identifying interactions between the inhibi-
tor and PLA$_2$. After examination, the inhibitor was redesigned 
to maximize interactions and hence increase the efficacy of 
binding (29). The IC$_{50}$ values were 808 and 287.3 nm for 
LY311727 and NAI, respectively. As such, NAI represents a 
potent natural inhibitor of hsPLA$_2$-II, exceeding the affinity of 
LY311727, despite the fact that LY311727 was specifically 
developed to inhibit hsPLA$_2$-II. Additionally, NAI is cross-re-
active against all PLA$_2$ enzymes it has been tested against, 
whereas LY311727 is specific for hsPLA$_2$-II (29).

Generally, Class A and C PLIs tend to be broadly inhibitory, 
whereas Class B PLIs display a limited inhibition range. Naja 
PLI (Class A) purified from $N$. naja kaouthia (Thailand cobra) 
serum (30) is similarly constructed to NAI (Class A) but lacks
Fig. 9. inhibition curves for NAI versus group I PLA_2 enzymes. For experimental details see “Experimental Procedures.” IC_{50} values were determined for hsPLA_2-II and bovine pancreatic PLA_2 using the mixed micelle assay with phosphatidyl ethanolamine as substrate. Results are represented as the averages ± S.D.

Fig. 10. inhibition curves for NAI versus Lilly LY311727 for hsPLA_2-II. For experimental details see “Experimental Procedures.” IC_{50} values were determined for rhPLA_2 using the mixed micelle assay with phosphatidyl ethanolamine as substrate. NAI and Lilly LY311727 were used as inhibitors. Results are represented as the averages ± S.D.
the broad inhibitory repertoire, exhibiting poor inhibition against crotalid PLA₂ enzymes (67). NAI is the only PLI to inhibit the enzymatic activity of the hsPLA₂-II enzyme. Crota-
lus neutralizing factor, purified from Crotalus durissus terrifi-
cus (South American rattlesnake) serum (32–34), was tested to
neutralize the enzymatic activity of the hsPLA₂ enzyme.
PLI, purified from Flavomitra flavoviridis (11). Michaels, R. M., Chang, Z. L., and Beezhold, D. H. (1994) Biochim. Biophys. Acta 1247, 93–100.

The mode of action of NAI is as yet unknown, apart from the
suggestion that basic charges present on the enzyme and the
three-dimensional structure of the inhibited PLA₂ play a role in
the interaction with NAI. Nor is it clear why NAI displays such
a diverse inhibitory profile while other PLIs tend to be limited in
the range of PLA₂ enzymes they inhibit. It is certainly
interesting to speculate that NAI or a derivative thereof may
prove useful in the treatment of snake bite victims or more
importantly in the treatment of the many human diseases in
which PLA₂ enzymes have been implicated.

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