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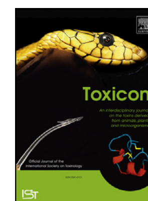

Structural and evolutionary insights into endogenous alpha-phospholipase A_2 inhibitors of Latin American pit vipers

Maria Inácia Estevão-Costa [a, b, 1], Carlos Alexandre H. Fernandes [c, 1], Maurício de Alvarenga Mudadu [a], Glória Regina Franco [b], Marcos Roberto M. Fontes [c, **], Consuelo Latorre Fortes-Dias [a, *]

[a] *Fundação Ezequiel Dias (FUNED), Belo Horizonte, MG, Brazil*
[b] *Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil*
[c] *Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil*

A R T I C L E I N F O

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A B S T R A C T

Phospholipases A_2 are major components of snake venoms ($svPLA_2s$) and are able to induce multiple local and systemic deleterious effects upon envenomation. Several snake species are provided with $svPLA_2$ inhibitors (sbPLIs) in their circulating blood, which confer a natural resistance against the toxic components of homologous and heterologous venoms. The sbPLIs belong to any of three structural classes named α, β and γ. In the present study, we identified, characterized and performed structural and evolutionary analyses of sbαPLIs transcripts and the encoded proteins, in the most common Latin American pit vipers belonging to *Crotalus*, *Bothrops* and *Lachesis* genera. Mutation data indicated that sbαPLIs from Latin American snakes might have evolved in an accelerated manner, similarly to that reported for sbαPLIs from Asian snakes, and possibly co-evoluted with $svPLA_2s$ in response to the diversity of target enzymes. The importance of sbαPLI trimerization for the effective binding and inhibition of acidic $svPLA_2s$ is discussed and conserved cationic residues located at the central pore of the inhibitor trimer are suggested to be a significant part of the binding site of sbαPLIs to acidic $svPLA_2s$. Our data contribute to the current body of knowledge on the structural and evolutionary characteristics of sbPLIs, in general, and may assist in the future development of selective inhibitors for secretory PLA_2 from several sources.

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1. Introduction

Secretory phospholipases A_2 from snake venoms ($svPLA_2s$) are very potent toxins that display a variety of biological activities including neurotoxic, myotoxic, haemolytic, edematogenic, hyperalgesic, pro-inflammatory, hypotensive, platelet-aggregation inhibitory, bactericidal, anticoagulant and cytotoxic actions (reviewed by Gutierrez and Lomonte, 2013). Acquisition of these various effects was achieved by gene duplication and accelerated exon evolution in the protein-coding region, while the mutation rates in cDNAs or genes ensured sufficient molecular plasticity to support the diverse physiological actions of those enzymes (Chuman et al., 2000; Nakashima et al., 1993, 1995; Nobuhisa et al., 1996; Ogawa et al., 1992; Ohno et al., 1998, 2003).

Several snake species possess circulating PLA_2 inhibitors (sbPLIs), which probably co-evolved with venom toxins, aiming at preventing any possible damage from toxins that might have found their way into the blood stream (Kochva et al., 1983). In *Gloydius brevicaudus* (formerly *Agkistrodon b. siniticus*), expression of a particular sbPLI has been shown to be up regulated by venom components (Kinkawa et al., 2010). Other physiological roles have been suggested for sbPLIs in the innate immune system and local regulation of other PLA_2s (Lizano et al., 2003; Ohkura et al., 1999; Okumura et al., 2003). Unique experimental data has been obtained for an sbPLI from *G. brevicaudus* (Shirai et al., 2009, 2010). This specific inhibitor binds to Cyt c and neutralizes the autologous Cyt c released from the dead cells. Hence, the authors suggested an

* Corresponding author.
** Corresponding author.
E-mail addresses: fontes@ibb.unesp.br (M.R.M. Fontes), consuelo.latorre@funed.mg.gov.br (C.L. Fortes-Dias).
[1] Estevão-Costa, M.I. and Fernandes, C.A.H. equally contributed to this study.

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ancestral function for that $svPLA_2$ inhibitor as a Cyt c-binding protein, prior to the $svPLA_2$ inhibition.

Due to their inhibition selectivity against $svPLA_2$s and the sharing of structural similarities with human secretory PLA_2s, the sbPLIs are considered potential molecular models for the development of new inhibitors of the latter. However, to this moment, few studies structural studies were performed with these inhibitors (Fortes-Dias et al., 2014; Okumura et al., 2005; Santos-Filho et al., 2011).

The sbPLIs are grouped into three classes—α, β and γ—based on the presence of characteristic structural domains (Ohkura et al., 1997). Members of different classes can be concomitantly found in a single snake species (Lizano et al., 2000; Nobuhisa et al., 1998; Ohkura et al., 1997; Shirai et al., 2009; So et al., 2011) and even non-venomous snakes may possess this type of molecules (Okumura et al., 1999b, 2002, 2003; Shirai et al., 2009; Thwin et al., 2002). Presence of different sbPLIs in a single specimen appears to ensure effective inhibition of different target $svPLA_2$ whereas, in the non-venomous species, their function is yet to be understood. The typical structural domain of the sbαPLIs is a C-type lectin-like domain (CTLD) (Inoue et al., 1991), although they lack the carbohydrate-binding ability (Ohkura et al., 1993; Okumura et al., 2003, 2005; Santos-Filho et al., 2011). The first and most studied sbαPLIs were purified from the blood plasma of the Asian viperid *Protobothrops* (taxonomically renamed from the former *Trimeresurus*) and *Gloydius* (previously *Agkistrodon*) snakes (Inoue et al., 1991, 1997; Kinkawa et al., 2010; Kogaki et al., 1989; Nishida et al., 2010; Nobuhisa et al., 1997a, b; Ohkura et al., 1993; Okumura et al., 1999a, 2005; So et al., 2011). They are hetero or homotrimers of 20–25 kDa subunits and bind one PLA_2 molecule per trimer (Inoue et al., 1991, 1997; Kogaki et al., 1989; Ohkura et al., 1993, 1997; Okumura et al., 1999b).

Despite purification of a number of sbαPLIs from Latin American viperid snakes—*Bothrops asper* (Lizano et al., 1997), *Cerrophidion godmani* (Lizano et al., 2000) and *Atropoides mexicanus* (Quiros et al., 2007) from Costa Rica; *Bothrops moojeni* (Soares et al., 2003), *Bothrops jararacussu* (Oliveira et al., 2008) and *Bothrops alternatus* (Santos-Filho et al., 2011) from Brazil—few structural and evolutionary studies are currently available. Those inhibitors are generally named MIPs, an acronym for myotoxin inhibitor proteins, because they inhibit Lys^{49} homologues and Asp^{49} myotoxic $svPLA_2$s.

Aiming at a better comprehension of sbαPLs inhibitors and motivated by the rich biodiversity of the herpetological fauna in Latin America, in the present study, we identified sbαPLIs in the most common Brazilian pit vipers belonging to *Bothrops, Crotalus* and *Lachesis* genera. We performed evolutionary and structural analyses of isolated liver transcripts and the corresponding deduced proteins, in order to gain insights into the binding and inhibition of $svPLA_2$s by this class of inhibitors.

2. Materials and methods

2.1. Snake liver collection

Specimens of *Bothrops alternatus, Bothrops erythromelas, Bothrops jararaca, B. jararacussu, B. moojeni, Bothrops neuwiedi, Crotalus durissus terrificus* and *Lachesis muta muta* snakes were obtained alive from the Serpentarium of Fundação Ezequiel Dias in Brazil, with the exception of the latter, which died by natural causes. At the time of sampling, the snakes were euthanized with CO_2 atmosphere, according to the protocol approved by the Committee for Ethics in Animal Use (CEUA FUNED 022/2012). Liver samples were collected in DEPC-treated vials, rapidly frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA and cDNA were prepared as previously described for the sbγPLIs (Estevão-Costa et al., 2008). Briefly, total RNA was isolated from approximately 120 mg of frozen liver tissue with Trizol® (Invitrogen, USA). After integrity checking by electrophoresis on 1% agarose gel, cDNA was synthesized with oligo(dT)$_{12-18}$ primer using the First-Strand Synthesis kit (Invitrogen, USA). The cDNAs encoding for sbαPLIs were obtained by conventional polymerase chain reaction (PCR) in the presence of specific oligonucleotides designed on the basis of published nucleotide sequence encoding for sbαPLIs from Old World snakes—*Protobothrops flavoviridis* (g.i. D87549.1) and *G. brevicaudus* (g.i. AB026666.1) (Nobuhisa et al., 1997a; Okumura et al., 1999b): P1 forward (5′GGAAGGAAAGTACTTTCTCTGGAG3′, in the 5′UTR region), P2 reverse (5′TCATA AAATGAAATAAAACTCACACACGAC3′, in the C-terminal) and P3 forward (5′CATGAGACAGATCCTGACGGA3′), in the N-terminal of the mature protein. Amplification conditions comprised of 5 min at 94 °C; 35 cycles consisting of 3 min at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and an extension period of 7 min at 72 °C, in a Perkin Elmer 2400 Thermo cycler. The pair of primers used was P1/P2 and P1/P3, with expected amplification products of 730 bp and 420 bp, respectively. In the negative controls, DNA and reverse transcriptase were omitted. Aliquots of the amplification reactions were analysed by electrophoresis on 1.0% agarose gel in TBE buffer, in the presence of ethidium bromide.

2.3. DNA cloning and sequencing, primary structure deduction and alignments

DNAs encoding sbαPLIs were processed as previously described (Estevão-Costa et al., 2008). Briefly, fresh PCR products were cloned in a TApCR 2.1 plasmid according to manufacturer's instructions (TA Cloning kit, Invitrogen, USA) and the recombinant plasmids were used to transform *Escherichia coli* strain INVαF'competent cells. DNA from confirmed positive clones was purified using a commercial kit (Wizard Plus Miniprep, Promega, USA) and sequenced by the dideoxy chain termination method (Sanger et al., 1977) on an automated ABI Prism 3110 Genetic Analyser (Perkin Elmer Applied Biosystem, USA), with appropriate oligonucleotides and reagents. Consensus sequences for each clone were obtained from a minimum of four complete reads and all sequences were deposited at the GenBank (www.ncbi.nlm.nih.gov/genbank/).

We used the conventional algorithm of ClustalW for the alignments (Thompson et al., 1994) after similarity search with other sbαPLIs using the Blastn (www.ncbi.nlm.nih.gov/BLAST/). Inhibitors with incomplete primary structures, such as *Ba*MIP (g.i. P81077) from *Bothrops asper* (Lizano et al., 1997), were not included. We performed pairwise alignments of the primary sequences to obtain the percentages of identities and mutations using the BLASTp (NCBI). The sbαPLI from *G. brevicaudus* was used as reference in the sequence alignments. Mutation rates in the nucleotide (nt) sequences were evaluated by the dn/ds ratio, where dn and ds correspond to non-synonymous and synonymous nt mutations, respectively.

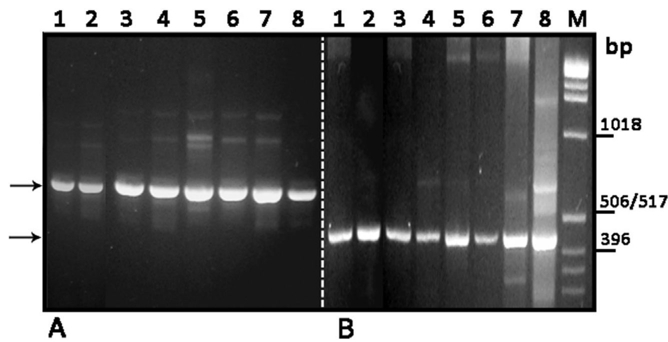

Fig. 1. Electrophoresis on 1.5% agarose gel of the RT-PCR products after amplification of liver tissue from Latin American pit vipers with specific primers for sbαPLIs. The amplification products (730 bp in A and 420 bp in B, depending on the pair of primers used) are indicated by arrows on the right side of the gel. Snake species: 1. *Crotalus durissus terrificus*; 2. *Lachesis muta muta*; 3. *Bothrops alternatus*; 4. *Bothrops erythromelas*; 5. *Bothrops jararaca*; 6. *Bothrops jararacussu*; 7. *Bothrops moojeni*; and 8. *Bothrops neuwiedi*. M. 1 Kb DNA ladder.

2.4. In silico structural analysis

We employed the same methodology as that previously described for the sbγPLIs (Estevão-Costa et al., 2008). In brief, molecular masses and isoelectric points of the deduced primary structures of the sbαPLIs were calculated by the Vector NTI Suite 10.1 software (Invitrogen, EUA). We used the JNET (http://www.compbio.dundee.ac.uk/~www-jpred/jnet/), PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) and SSPRO4 (http://scratch.proteomics.ics.uci.edu/) for secondary structure predictions and the output data was parsed with Perl scripts in order to generate a single consensus result.

The monomer and trimer in silico models of α*Balt*MIP are available at the ModelArchive database (DOI: ma-a2iil and DOI: ma-a4btt, respectively). Accessible solvent area (ASA) was calculated with AREAIMol software (Saff and Kuijlaars, 1997). As previously noted (Santos-Filho et al., 2011), monomer and trimer in silico models of α*Balt*MIP have good overall quality with Z-

Table 1
Average number of nucleotide mutations in sbαPLI transcripts from viperid snakes, with the sbαPLI of *Gloydius brevicaudus* (g.i. AB026666) taken as reference. The transcripts are identified by the species names followed by the clone number. Other published sequences were included, namely *Atropoides mexicanus* (previously *A. nummifer*) (Quiros et al., 2007), *Cerrophidion godmani* (Lizano et al., 2000), *Protobothrops flavoviridis* (Nobuhisa et al., 1997a,b) and *Elaphe quadrivirgata* (Okumura et al., 2003).

Snake species and clone number	Number of nucleotide mutations		
	Non synonymous (dn)	Synonymous (ds)	Total
A. mexicanus	35	8	43
A. mexicanus	34	7	41
C. godmani	37	10	47
B. alternatus_03	30	19	49
B.alternatus_06	30	20	50
B. alternatus_08	30	19	49
B.alternatus_10	30	19	49
B. alternatus_14	30	19	49
B.erythromelas_02	29	20	49
B. erythromelas_04	30	21	51
B.erythromelas_08	29	21	50
B. erythromelas_10	29	21	50
B.erythromelas_12	29	20	49
B. erythromelas_14	29	20	49
B. jararaca_02	29	20	49
B. jararaca_06	29	21	50
B.jararaca_07	29	22	51
B. jararaca_10	30	21	51
B. jararacussu_01	34	25	59
B. jararacussu_05	32	22	54
B. jararacussu_07	34	25	59
B. jararacussu_11	35	24	59
B. moojeni_03	32	22	54
B. moojeni_04	28	18	46
B. moojeni_05	28	20	48
B. moojeni_08	28	21	49
B. moojeni_10	31	22	53
B. moojeni_12	32	22	54
B. neuwiedi_06	35	24	59
B. neuwiedi_08	32	20	52
B. neuwiedi_11	32	20	52
B. neuwiedi_A1	33	25	58
C.d. terrificus_A4	30	16	46
C.d.terrificus_A5	30	16	46
C.d. terrificus_B1	31	16	47
C.d. terrificus_B4	31	16	47
L.m. muta_02	29	11	40
L.m. muta_03	29	11	40
L.m. muta _06	29	11	40
L.m.muta _07	29	11	40
L.m. muta _09	30	11	41
P. flavoviridis	37	18	55
E. quadrivirgata	48	49	97

scores of −4.99 and −4.41, respectively. The percentages of residues distributed in the favoured and allowed regions of Ramachandran plot are 97.9% for the monomer and 94.5% for the trimer.

3. Results and discussion

3.1. SbαPLI transcripts and dn/ds analysis

The RNA integrity of our starting material was confirmed by the unique presence of 18S and 28S bands of ribosomal RNA after electrophoresis on agarose gel (data not shown). Amplification products with the expected sizes—about 420 and 730 bp—after RT-PCR (Fig. 1) confirmed the presence of sbαPLI transcripts in the liver tissue of *Bothrops alternatus, B. erythromelas, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi, C. durissus terrificus* and *L. muta muta* snakes. The transcript sequences were firstly identified by the species name followed by the clone number, before being deposited at the GenBank. A Supplementary Table is included, showing the multiple alignments of the sbαPLI transcripts identified herein—with the clone identification and the corresponding accession number—and the sbαPLIs reported in the literature. The sbαPLI from *G. brevicaudus* (*Gb*αPLI) was taken as reference. The average number of nt mutations per clone was calculated and compared (Table 1). The dn/ds ratios per clone clusterized according to the snake genus or species (Fig. 2).

All the deduced primary structures of the sbαPLIs from the present study contain highly similar signal sequences of 19 amino acids, followed by 147 amino acids in the mature proteins (Table 2), in accordance with similar inhibitors in the literature. The average molecular mass of the mature sbαPLIs described herein is 18,495 ± 20 Da and the isoelectric points vary between 5.26 and 6.65 (data not shown). Important structural segments in the sbαPLIs—signal peptide, CTLD, C-terminal segment, and exon 3—are marked in the aligned primary structures (Table 2). Twenty-six amino acid residues were most frequently mutated in

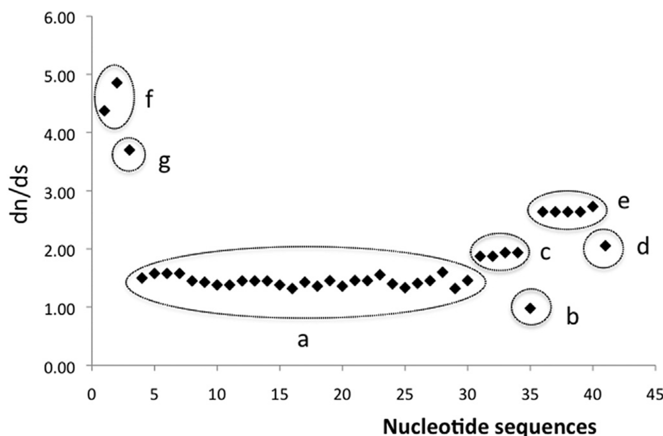

Fig. 2. Nucleotide mutations in sbαPLIs from viperid snakes (present study and literature sources) expressed by the ratio between non-synonymous (dn) and synonymous (ds) mutations. The nucleotide sequences clusterized as indicated: a. *Bothrops genus*; b. *Elaphe quadrivirgata*; c. *Crotalus durissus terrificus*; d. *Trimeresurus flavoviridis*; e. *Lachesis muta muta*; f. *Cerrophidion godmani*; and g. *Atropoides mexicanus*. Each point represents a clone sequence isolated in the present study (a, c, e) or a nucleotide sequence published by other authors (b, d, f, g). The sbαPLI from Asian *Gloydius brevicaudus* was taken as reference.

the deduced amino acid sequences (Table 3), among which nine are concentrated in the exon 3, also called α-helical coiled-coil neck, at the N-terminal of the proteins. It is important to note that the C-terminal segment is highly conserved in the sbαPLIs (Table 2). The predicted secondary structure pattern is greatly conserved as well, with 15% of β-sheets and 33% of α-helices arranged in three well-defined segments (Table 4). The α-helices predicted for the exon 3 are highly conserved, in spite of nine amino acid mutations. The secondary structure predicted is experimentally supported by circular dichroism spectra of the sbαPLIs from *Bothrops jararacussu* (α*Bjussu*MIP) and *Bothrops alternatus* (α*Balt*MIP) (Santos-Filho et al., 2011; Oliveira et al., 2008). SbαPLIs transcripts were identified in the liver of all the species tested herein, hence confirming the wide distribution of this class of PLA_2 inhibitors in Latin American pit vipers. SbαPLIs transcripts were also isolated from the venom glands of these species and exhibited high homology to the liver transcripts (data not shown).

Evolutionary studies based on the dn/ds ratio have been applied to animal toxin cDNAs and/or genes and have led to the proposal of accelerated evolution of $svPLA_2$, based on higher substitution rates in exons compared to introns of the corresponding genes (Ohno et al., 1998). With respect to the sbαPLIs, only the genes encoding for the subunits A and B of the *P. flavoviridis* inhibitor (*Pf*αPLI) have been isolated. Both genes comprised four exons and three introns, with very high identity values, with the exception of the third exon. This exon, which encodes for most of the N-terminal portion, is particularly abundant in non-synonymous mutations with the dn/ds ratio close to four (Nobuhisa et al., 1997a; So et al., 2011). The resulting amino acid substitutions in exon 3 were correlated with the various PLA_2 specificities of sbαPLIs (Nobuhisa et al., 1997a; Okumura et al., 2005). Our cDNA data for Latin American pit vipers are in agreement with the accelerated gene evolution concept, since most of the dn/ds ratios in the deduced primary sequences of the sbαPLIs exceeded 1.0 (Fig. 2). Nobuhisa et al. (1997a) also reported that non-synonymous substitutions were almost four times as frequent as synonymous substitutions in the exon 3 of sbαPLIs from Asian snakes. Hence, the $svPLA_2$ and sbPLI co-evolution proposed for Asian viperid snakes (Nobuhisa et al., 1997a; Shimada et al., 2008; So et al., 2011) may be extended to sbαPLIs (present study) and sbγPLIs (Estevão-Costa et al., 2008) of Latin American pit vipers.

3.2. The role of central pore in the inhibition of acidic PLA_2s

Efforts to elucidate PLA_2-recognition and binding by the sbα PLIs have led to the suggestion of a number of putative candidate regions in the inhibitors. In the first attempt performed with truncated forms of *Pf*αPLI, the CLTD domain, the 37 amino terminal residues and the C-terminal region—particularly the last 12 hydrophobic residues (N^{157} to L^{168})—were found to be critical for the binding of both acidic and basic PLA_2s (Nobuhisa et al., 1998). Examination of the amino acid sequences of the sbαPLIs (Table 2) shows a highly conserved C-terminal portion, suggesting a possible relevance in PLA_2 binding. The importance of the CTLD domain and the C-terminal segment—in particular the hydrophobic core—was subsequently questioned, following the purification of a functionally inactive αPLI-like protein (PLIα-LPs) from non-venomous *Elaphe* snakes (Okumura et al., 2003, 2005; Shirai et al., 2009). PLIα-LPs did not show any inhibition property against several PLA_2s tested, in spite of the conservation of both the hydrophobic core at the C-terminal segment and the CLTD domain. The C-terminal segment of PLIα-LPs has only two mutations—N^{157}D and Y^{164}S—that may be critical for their

Table 2

Deduced primary sequences of sbαPLI transcripts from snake liver, identified by the species name followed by the clone number (present study) or by the accession number in the GenBank. The sbαPLI of *Cerrophidion godmani* was published by Lizano et al. (2000). Structural elements: Signal peptide (shadowed in grey); exon 3 (boxed); conserved motif, according to the MEME software (shadowed in black); C-type lectin-like domain (CTLD) (double line) below the sequences. Most mutated residues are shown in bold font.

```
                                  10        20        30        40        50        60        70        80        90        100
G.brevicaudus|ABO26666      MRLILLSSLLLLGTFLANGHETDPDGHVLNSLMLIVMRLQREFSNLKDGFLTVHKARSFGSGSERLYVTNKEVGNFEGLGEICRQAGGRIPSPQLKNQNKAFA [103]
B.alternatus_03             .......G.....I.......Q....KL....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.alternatus_06             .......G.....I.......Q....KL....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.alternatus_08             .......G.....I.......Q....KL....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.alternatus_10             .......G.....I.......Q....KL....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.alternatus_14             .......G.....I.......Q....KL....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.erythromelas_02           .......G.....I.......Q....K.....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.erythromelas_04           .......G.....I.......Q....K.....IDAL.H....VAK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.erythromelas_08           .......G.....I.......Q....K.....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.erythromelas_10           .......G.....I.......Q....K.....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.erythromelas_12           .......G.....I.......Q....K.....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.erythromelas_14           .......G.....I.......Q....K.....IDAL.H.....AK.RGA....Y......N...........IK...A.RQ..E....H......E....... [103]
B.neuwiedi_A1               .......G.....I.....D.V.RE.K.V...IDAL.H.....AK..GS..I.............M......IK...A.RQ..E..D.H......E....... [103]
B.neuwiedi_06               .......G.....I.......Q....K.....IDAL.D.....AK.RGA....Y......N....M......IK...A.RQ..E..E.H......E....... [103]
B.neuwiedi_08               .......G.....I.......Q....K.....IDAL.D.....AK.RGA....Y......N....M......IK...A.RQ..E..E.H......E....... [103]
B.neuwiedi_11               .......G.....I.....D.V...RKL....IDAL.H.....A...GS..I.............M......IK...A.RQ..E..D.H......E....... [103]
B.jararaca_02               .............I.......Q....K.....IDAL.H.....A..RGA.......................IK...A.RQ..E..E.H......E.H..... [103]
B.jararaca_06               .............I.......Q....K.....IDAL.H.....A..RGA.......................IK...A.RQ..E..E.H......E.H..... [103]
B.jararaca_07               .............I.......Q....K.....IDAL.H.....A..RGA.......................IK...A.RQ..E..E.H......E.H..... [103]
B.jararaca_10               .............I.......Q....K.....IDAL.H.....A..RGA.......................IK...A.RQ..E..E.H......E.H..... [103]
B.jararacussu_01            .......G.....I.....D.V...RKL....IDAL.H.....A...GS..I.............M......IK...A.RQ..E..D.H......E....... [103]
B.jararacussu_05            .......G.....I.......E....K.....IDAL.H.....A...GS..I.............M......IK...A.RD..EK.D.H......E....... [103]
B.jararacussu_07            .......G.....I.....D.V...RKL....IDAL.H.....A...GS..I.............M......IK...A.RQ..E..D.H......E....... [103]
B.jararacussu_11            .......G.....I.....D.V...RKL....IDAL.H.....A...GS..I.............M......IK...A.RQ..EK.D.H......E....... [103]
B.moojeni_03                .............I.......E....K.....IDTL.H..KLYA...YS.....R..........M......IK...A.RQ..E..E.H......E....... [103]
B.moojeni_04                .............I.......E....K.....IDSL.H.....A....A.....R.................IK...A.RQ..E..E.H......E....... [103]
B.moojeni_05                .............I...D.D.V....K.....IYG..H.....A...GA.....R.................IK...A.RQ..E..E.H......E....... [103]
B.moojeni_08                .............I.....D.V....K.....IYG..H.....A...GA.....R.................IK...A.RQ..E..E.H......E....... [103]
B.moojeni_10                .............I.......E....K.....IDTL.H..KLYA...YS.....R.................IK...A.RQ..E..E.H......E....... [103]
B.moojeni_12                .............I.......E....K.....IDTL.H..KLYA...YS.....R..........M......IK...A.RQ..E..E.H......E....... [103]
L.m.muta_02                 .......G............D...E.QM...VIES..I.....A...HA...................S...I.K..A.K...D....H.....FE....... [103]
L.m.muta_03                 .......G............D...E.QM...VIES..I.....A...HA...................S...I.K..A.K...D....H.....FE....... [103]
L.m.muta_06                 .......G............D...E.QM...VIES..I.....A...HA...................S...I.K..A.K...D....H.....FE....... [103]
L.m.muta_07                 .......G............D...E.QM...VIES..I.....A...HA...................S...I.K..A.K...D....H.....FE....... [103]
L.m.muta_09                 .......G............D...E.QM...VIES..I..K..A...HA...................S...I.K..A.K...D....H.....FE....... [103]
C.d.terrificus_A4           .......G........V...DA..E.E....VL.TL.K..K..T...FHA..................S...IK...A.KV..K....Q......E....... [103]
C.d.terrificus_A5           .......G........V...DA..E.E....VL.TL.K..K..T...FHA..................S...IK...A.KV..K....Q......E....... [103]
C.d.terrificus_B1           .......G........V...DA..E.E....VL.TL.K..K..T...FHA..................S...IK...A.KV..K....Q......E....... [103]
C.d.terrificus_B4           .......G........V...DA..E.E....VL.TL.K..K..T...FHA..................S...IK...A.KV..K....Q......E....... [103]
C. godmani                  .......G...........D.K.SEIQL...MIEA..I...D....RYALM...N..........V..S....SK....E...S....H......E....... [103]
A.mexicanus|DQ657241.1      .......G...........D.K.S.VQM...MIEA..I...D.A..RHALM...N.....R............SK....E...S....H......E.....E [103]
A.mexicanus|DQ657242.1      .......G...........D.K.S.VQM...MIEA..I...D.A..RYA.M...N.....G............SK....E...S....H......E.....E [103]
P.flavoviridis|D87548.1     .......G.....I.............Q.MS.MIETL.F..K.YA..RYA.M..NN.............S...IKT..P.K...EE...H......E....... [103]
E.quadrivirgata|ABO30247    .Q............LS.........E.QI....VET.G..EKKIDKVENA.....R................Q....AVRNT..V....H......L.E..... [103]

                                          110       120       130       140       150       160
G.brevicaudus|ABO26666      NVLERHNKEAYLVVGNSANFTNWAEGQPKKADGT--CVKADTHGFWHSTSCDDNLLVVCEFYFIL* [169]
B.alternatus_03             ...............D........A.E.N..A.A--........S....................* [169]
B.alternatus_06             ...............D........A.E.N..A.A--........S....................* [169]
B.alternatus_08             ...............D........A.E.N..A.A--........S....................* [169]
B.alternatus_10             ...............D........A.E.N..A.A--........S....................* [169]
B.alternatus_14             ...............D........A.E.N..A.A--........S....................* [169]
B.erythromelas_02           ..........F....D........A...N..A..--........S...A................* [169]
B.erythromelas_04           ..........F....D........A...N..A..--........S...A................* [169]
B.erythromelas_08           ..........F....D........A...N..A..--........S...A................* [169]
B.erythromelas_10           ..........F....D........A...N..A..--........S...A................* [169]
B.erythromelas_12           ..........F....D........A...N..A..--........S...A................* [169]
B.erythromelas_14           ..........F....D........A...N..A..--........S...A................* [169]
B.neuwiedi_A1               ......G........D........A.E.N..A.A--........S...A................* [169]
B.neuwiedi_06               ......G........D........A.E.N..A.A--........S....................* [169]
B.neuwiedi_08               ......G........D........A.E.N..A.A--........S....................* [169]
B.neuwiedi_11               ......G........D........A.E.N..A.A--........S...A................* [169]
B.jararaca_02               ......G........D........A.E.N..A.A--........S....................* [169]
B.jararaca_06               ......G........D........A.E.N..A.A--........S....................* [169]
B.jararaca_07               ......G........D........A.E.N..A.A--........S....................* [169]
B.jararaca_10               ......G........D........A.E.N..A.A--........S..................Y.* [169]
B.jararacussu_01            ......G........D........A.E.N..A.A--........S...A..............M.* [169]
B.jararacussu_05            ......G........D........A.E.N..A.A--........S...A..............M.* [169]
B.jararacussu_07            ......G........D........A.E.N..A.A--........S...A..............M.* [169]
B.jararacussu_11            ......G........D........A.E.N..A.A--........S...A..............M.* [169]
B.moojeni_03                ......G........D........A.E.N..A..--........S...A................* [169]
B.moojeni_04                ......G........D........A.E.N..A..--........S...A.....R..........* [169]
B.moojeni_05                ......G........D........A.E.N..A..--........S...A................* [169]
B.moojeni_08                ......G........D........A.E.N..A..--........S...A................* [169]
B.moojeni_10                ......G........D........A.E.N..A..--........S...A................* [169]
B.moojeni_12                ......G........D........A.E.N..A..--........S...A................* [169]
L.m.muta_02                 ..............DDP.......A...NE....--........S...A................* [169]
L.m.muta_03                 ..............DDP.......A...NE....--........S...A................* [169]
L.m.muta_06                 ..............DDP.......A...NE....--........S...A................* [169]
L.m.muta_07                 ..............DDP.......A...NE....--........S...A................* [169]
L.m.muta_09                 ..............DDP.......A...NE....--........S...A................* [169]
C.d.terrificus_A4           ..........F....D.G......A...N.....--.....KQ......................* [169]
C.d.terrificus_A5           ..........F....D.G......A...N.....--.....KQ......................* [169]
C.d.terrificus_B1           ..........F....D.G......A...N.....--.....KQ..C...................* [169]
C.d.terrificus_B4           ..........F....D.G......A...N.....--.....KQ.....................*  [169]
C. godmani                  D.......A......D........A.H.NE....--........S...A................* [169]
A.mexicanus|DQ657241.1       D.......A......D........A...NE....--........S...A................* [169]
A.mexicanus|DQ657242.1       D.......A......D........V...NE....--........S...A................* [169]
P.flavoviridis|D87548.1     S.......A......D........A...NE....--........S...A...E............* [169]
E.quadrivirgata|ABO30247    S.......A.....Q...K.....A.E.NN...NKL.....AQ.A...A...ED.......S..*  [169]
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failure to inhibit PLA_2 and could, consequently, play a role in the enzyme binding (Okumura et al., 2003).

A polypeptide fragment close to the N-terminal, which corresponds to the exon 3 in the sbαPLI genes from Asian snakes (Nobuhisa et al., 1997a), was also shown to be critical for both binding and inhibition of acidic PLA_2. It has been later demonstrated that, despite not being a direct binding site for $svPLA_2$, exon 3 is crucial for the formation of the central pore in sbαPLIs trimers. In fact, the central pore constitutes the actual $svPLA_2$binding site (Okumura et al., 2005). Dynamic light scattering experiments and molecular modelling confirmed the central pore as the $svPLA_2$-binding site in α*Balt*MIP (Santos-Filho et al., 2011). Both trimerization and central pore formation depend on the availability of identical helical neck regions in the forming subunits (Nishida et al., 2010). Amino acid mutations in the helical neck, such as those present in the PLIα-LPs from *Elaphe* snakes, cause the formation of atypical pores with impaired functionality (Okumura et al., 2003, 2005; Shimada et al., 2008; Shirai et al., 2009). Based on site-directed mutagenesis and molecular modelling, Okumura et al. (2005) proposed that mutations in the N-terminal portion of exon 3 in the PLIα-LPs—especially N^{45}K, K^{47}E and D^{48}N—could be detrimental to trimerization and could potentially explain their lack of PLA_2 inhibition.

Multiple alignments of deduced amino acid sequences (present study) and deposited sequences from other snake species on the NCBI protein data bank (Table 2) showed that these three residues (N^{45}, K^{47} and D^{48}) are not strictly conserved in the sbα PLIs. Concerning the 47th amino acid position, in particular, it is occupied by basic Lysine or Arginine in almost all sequences of functional sbαPLIs (Table 2). On the other hand, the ^{39}LQ(R/K)EF^{43} peptide located on the alpha-helical neck is conserved in all functional sbαPLIs, with drastic mutations in the PLIα-LPs. Hence, this cluster may be essential in trimer stabilization of the sbαPLIs, whereas N^{45} and D^{48} could be important only for Asian snakes.

Okumura et al. (2005) also proposed that Y^{165}, which is located at the central pore of the *Gb*αPLI trimer, could be essential in PLA_2binding. This amino acid is mutated to S^{165} in the PLIα-LPs. In order to gain further insights into the role of the central pore as PLA_2 binding site, we performed a detailed structural analysis of the in silico trimer model of α*Balt*MIP (Santos-Filho et al., 2011). According to the model, $Y^{165}$is not located at the central pore, but rather above the CTLD domain (Fig. 3). However, an electrostatic surface analysis of the central pore shows highly positive charged residues (Fig. 4A), especially due to presence of basic R^{57}, K^{71}, R^{108} and H^{109} residues, which are conserved in all sbαPLIs (Table 2). Furthermore, these cationic residues display a large accessible area to solvent (ASA), which results in a negatively charged area of ~1600 $Å^2$ exposed to solvent, in the α*Balt*MIP trimer (Table 5).

Electrostatic potential analysis of the crystal structure of BthA-I, an acidic PLA_2 from *Bothrops jararacussu* venom, shows that acidic $svPLA_2$s are likely to possess large negatively charged surfaces (Fig. 4B); hence, the cationic residues found on sbαPLIs are probably involved in the enzyme-inhibitor interaction at the central pore of the trimers. Indeed, the negatively charged surfaces of each BthA-I monomer (Fig. 4B) display a positively charged area of ~1200 $Å^2$. This area is compatible with the negatively charged counterpart found on the α*Balt*MIP trimer.

Interestingly, part of the aforementioned hydrophobic core (L^{158} to V^{161}) is located close to the cationic residues—R^{57}, K^{71}, R^{108} and H^{109}—in the α*Balt*MIP monomer model (Fig. 4C). Site-directed mutagenesis of these hydrophobic residues to alanine affected the binding of acidic PLA_2s (Nobuhisa et al., 1998). These mutations confer flexibility to the hydrophobic core and may lead to structural disturbances in the neighbourhood of the central pore (Fig. 4C). Thus, this hydrophobic region may play a role in ensuring the structural integrity of the negatively charged central pore in the sbαPLIs.

Table 3
Most mutated amino acid residues in the deduced primary structures of mature sbαPLIs. The sbαPLI from *Gloydius brevicaudus* was taken as reference. Mutations that occurred in 100% of the sequences ($n = 43$) are shown in bold font. Only the mutations that occurred in a minimum of eight sequences were considered. The amino acids are numbered according to the sequences presented in Table 2, which include the signal peptides (residues 1 to 19).

Amino acid residue			Clones mutated/total	
Position	Reference	Mutation	No.	%
22	T	K/Q/V/E/A	35/43	81.4
27	**H**	**K/Q/E**	**43/43**	**100.0**
33	**M**	**I/L/V**	**43/43**	**100.0**
34	L	E/D/Y	39/43	90.7
35	**I**	**A/T/S/G**	**43/43**	**100.0**
36	V	L	33/43	76.7
38	R	I/H/D/K/F	42/43	97.7
44	**S**	**A/T/D**	**43/43**	**100.0**
47	K	R/F/E	25/43	58.1
48	D	H/Y/G/N	42/43	97.7
49	**G**	**A/S**	**43/43**	**100.0**
73	V	I	40/43	93.0
74	G	S/K	37/43	86.0
78	G	A/P	41/43	95.3
80	G	E/R/K	42/43	97.7
81	E	Q/D/V/N	35/43	81.4
84	**R**	**S/E/K/V/D**	**43/43**	**100.0**
89	**R**	**H/Q**	**43/43**	**100.0**
96	**K**	**E/L**	**43/43**	**100.0**
119	N	D	42/43	97.7
128	**E**	**A/V**	**43/43**	**100.0**
130	Q	E	24/43	55.8
132	**K**	**N**	**43/43**	**100.0**
135	D	A	29/43	67.4
148	F	S/A	39/43	90.7
152	T	A	28/43	65.1

4. Concluding remarks

One of the main goals of toxinology today concerns the development of therapeutic alternatives for the treatment of snake venom injuries that are not efficiently neutralized by conventional serum therapy. Toxic $svPLA_2$s are among the central proteins responsible for severe consequences of snake accidents. In this context, a full comprehension of their inhibition by sbPLIs may lead to the development of novel molecules to complement the conventional serum therapy. Due to the conserved tertiary structure of secretory PLA_2s, these data could also guide the development of specific inhibitors for human group II PLA_2s involved in inflammatory events (Schaloske and Dennis, 2006). Besides acidic $svPLA_2$, particular sbαPLIs from *Bothrops* snakes have been shown to be able to inhibit several biological activities of basic $svPLA_2$-like proteins, which are highly myotoxic and catalytically inactive (Oliveira et al., 2008; Santos-Filho et al., 2011; Soares et al., 2003). Differently from the acidic $svPLA_2$s, the basic $svPLA_2$-like proteins disturb the membrane by a Ca^{2+}-independent mechanism, which seems to involve an allosteric transition and two different membrane interaction sites at their carboxy-terminal region (Fernandes et al., 2014). The surface of these basic proteins has large positively charged areas (Murakami et al., 2008; Zhou et al., 2008;

Table 4
Consensus of the predicted secondary structures of mature sbαPLIs from Latin American pit vipers, after conducting analyses using three different algorithms. Upper and lower case letters indicate consensus among three and two of the used algorithms, respectively. Legend: C and c-random coil (green); E and e- β-sheet (blue); H and h- α-helix (red); N- no consensus; “-” – gap. Four clones of each species (present study) were randomly selected for the analysis, while accession numbers identify the sequences taken from the GenBank. The exon 3 or helical neck segment is boxed.

Sequence	Positions 1–116 (10 20 30 40 50 60 70 80 90 100 110)	Positions 117–152 (120 130 140 150)
A.mexicanus|DQ657241.1	ccccccNHHHHHHHHHHHHHHHHHHHHHHHHHHHhNhhEcccccNEEEEEccceNcHHHHHHHHHHccceEeNNccHHHHHHHHHHHhhcccEEEEeccccNeeNcccccccccccc	EEEEENcccNNN--ccNccceEEEEEEEENc
A.mexicanus|DQ657242.1	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHhhhhhccccNEEEEEcccNNcHHHHHHHHHHccceENeNccHHHHHHHHHHHHhccceEEEEccccNNNN-cccccccccccc	cEEEEEccccNNc-ccNccccEEEEEEEENc
B.alternatus	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHhhhhHeecccccNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHhccceEEEeccccNeecccccccccccccc	EEEEEccccNNN--ccNccceEEEEEEEENc
B.alternatus	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHhhhhHeecccccNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHhccceEEEeccccNeecccccccccccccc	EEEEEccccNNN--ccNccceEEEEEEEENc
B.alternatus	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHhhhhHeecccccNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHhccceEEEeccccNeecccccccccccccc	EEEEEccccNNN--ccNccceEEEEEEEENc
B.alternatus	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHhhhhHeecccccNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHhccceEEEeccccNeecccccccccccccc	EEEEEccccNNN--ccNccceEEEEEEEENc
B.erythromelas	ccccccNNHHHHHHHHHHHHHHHHHHHHHHHhhhNeeecccccceEEEEEcccNNcHHHHHHHHHHcccceeeeNccHHHHHHHHHHHHcccceEEEEccccceNcccccccccccccc	EEEEEccccNNN--ccNcccceEEEEEEENc
B.erythromelas	ccccccNNhHHHHHHHHHHHHHHHHHHHHHHHhhceEEeccccceEEEEEcccNNchHHHHHHHHHcccceeNeNccHHHHHHHHHHHHccccEEEENccccNNNNcccccccccccc	EEEEEccccNNN--ccNccccEEEEEEEENc
B.erythromelas	ccccccNNHHHHHHHHHHHHHHHHHHHHHHHhhhNeeecccccceEEEEEcccNNcHHHHHHHHHHcccceeeeNccHHHHHHHHHHHHcccceEEEEccccceNcccccccccccccc	EEEEEccccNNN--ccNcccceEEEEEEENc
B.erythromelas	ccccccNNHHHHHHHHHHHHHHHHHHHHHHHhhhNeeecccccceEEEEEcccNNcHHHHHHHHHHcccceeeeNccHHHHHHHHHHHHcccceEEEEccccceNcccccccccccccc	EEEEEccccNNN--ccNcccceEEEEEEENc
B.jararaca	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHhHHhNNNNcNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHHccceEEEEccccNNNNcccccccccccc	EEEEEccccNNN--ccNcccceeEEEEEEENc
B.jararaca	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHhHHhNNNNcNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHHccceEEEEccccNNNNcccccccccccc	EEEEEccccNNN--ccNcccceeEEEEEEENc
B.jararaca	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHhHHhNNNNcNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHHccceEEEEccccNNNNcccccccccccc	EEEEEccccNNN--ccNcccceeEEEEEEENc
B.jararaca	ccccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHHHHhNNNNcNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHHHHccceEEEEccccNNNNcccccccccccc	EEEEEccccNNN--ccNcccceeEEEEEEENc
B.jararacussu	cccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHHHhhhNNccNEEEEEcccNNcHHHHHHHHHHcccNEeNNccHHHHHHHHHHHHHcccEEEEEcccceeNNccccccccccccE	EEEEccccNNcc--ccNcccceeEEEEEEENc
B.jararacussu	ccccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHhhhhhhNccNEEEEEcccNNcHHHHHHHHHHccceEeeNccHHHHHHHHHHHHHccceEEEEccccNNNNcccccccccccc	EEEEEccccNNN--ccNccceEEEEEEEENc
B.jararacussu	cccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHHHhhhNNccNEEEEEcccNNcHHHHHHHHHHcccNEeNNccHHHHHHHHHHHHHcccEEEEEcccceeNNccccccccccccE	EEEEccccNNcc--ccNcccceeEEEEEEENc
B.jararacussu	ccccNNhhHHHHHHHHHHHHHHHHHHHHHHHHHHHhhhNNccNEEEEEcccNNcHHHHHHHHHHccceEeeNccHHHHHHHHHHHHHccceEEEEccccNNNNccccccccccccE	EEEEccccNNNc--ccNcccceeEEEEEEENc
B.moojeni	cccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHHhhhhhNNNNcNEEEEEccceecHHHHHHHHHHcccceeNeNccHHHHHHHHHHHHHccceEEEeccccNNNNccccccccccccE	EEEEccccNNcc--ccNcccceeEEEEEEENc
B.moojeni	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHhHchhcchNNccNEEEEEcccNNcHHHHHHHHHHcccceeNeNccHHHHHHHHHHHHhccceEEEEccccNeeccccccccccccE	EEEEccccNNcc--ccNcccceeEEEEEEENc
B.moojeni	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHHhhHhhhNNccNEEEEEccceNcHHHHHHHHHHcccNENeNccHHHHHHHHHHHHHcccEEEEEccccNeNNccccccccccccE	EEEEccccNNcc--NcNcccceeEEEEEEENc
B.moojeni	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHHhhHhhhNNccNEEEEEccceNcHHHHHHHHHHcccNENeNccHHHHHHHHHHHHHcccEEEEEccccNeNNccccccccccccE	EEEEccccNNcc--NcNcccceeEEEEEEENc
B.neuwiedi	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHHhhhhhNNcccNEEEEEcccNNcHHHHHHHHHHccceENeNccHHHHHHHHHHHHHcccEEEEeccccNNNNccccccccccccE	EEEEccccNNcc--ccNcccceeEEEEEEENc
B.neuwiedi	ccccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHHHhheccccccNEEEEEcccNNcHHHHHHHHHHcccceeeeNccHHHHHHHHHHHHHcccEEEEEccccNNNNcccccccccccc	EEEEEccccNNN--ccNcccceeEEEEEEENc
B.neuwiedi	ccccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHHHhheccccccNEEEEEcccNNcHHHHHHHHHHcccceeeeNccHHHHHHHHHHHHHcccEEEEEccccNNNNcccccccccccc	EEEEEccccNNN--ccNcccceeEEEEEEENc
B.neuwiedi	cccccNhhHHHHHHHHHHHHHHHHHHHHHHHHHHHHhhhhNNccNEEEEEcccNNcHHHHHHHHHHccceEeeNccHHHHHHHHHHHHHccceEEEEccccNNNNccccccccccccE	EEEEccccNNNc--ccNcccceeEEEEEEENc
C.d.terrificus	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHHhchhhhhNNcNEEEEEcccNNcHHHHHHHHHHccceENNNccHHHHHHHHHHHHcccceEEEENcccNNNNccccccccccccE	EEEEccccNNNc--ccNccccceEEEEEEENc
C.d.terrificus	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHHhchhhhhNNcNEEEEEcccNNcHHHHHHHHHHccceENNNccHHHHHHHHHHHHcccceEEEENcccNNNNccccccccccccE	EEEEccccNNNc--ccNccccceEEEEEEENc
C.d.terrificus	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHHhcheeNNNccNEEEEEcccNNcHHHHHHHHHHcccceeNNNccHHHHHHHHHHhcccceEEEEecccccNNccccccccccccE	EEEEccccNNNc--ccNccccceEEEEEEENc
C.d.terrificus	cccccNNhHHHHHHHHHHHHHHHHHHHHHHHHHHhchhhhhNNcNEEEEEcccNNcHHHHHHHHHHccceENNNccHHHHHHHHHHHhcccceEEEENcccNNNNccccccccccccE	EEEEccccNNNc--ccNccccEEEEEEEENc-
L.m.muta	cccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHHhchhhNNNccNEEEEEccceecHHHHHHHHHHccceEeeNccHHHHHHHHHHHHhccceEEEEcccccNeeccccccccccccE	EEEEccccNNNc--NcNccccceEEEEEEENc
L.m muta	cccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHHhchhhNNNccNEEEEEccceecHHHHHHHHHHccceEeeNccHHHHHHHHHHHHhccceEEEEcccccNeeccccccccccccE	EEEEccccNNNc--NcNccccceEEEEEEENc
L.m muta	cccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHHhchhhNNNccNEEEEEccceecHHHHHHHHHHccceEeeNccHHHHHHHHHHHHhccceEEEEcccccNeeccccccccccccE	EEEEccccNNNc--NcNccccceEEEEEEENc
L.m muta	cccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHHhchhhNNNccNEEEEEccceecHHHHHHHHHHccceEeeNccHHHHHHHHHHHHhccceEEEEcccccNeeccccccccccccE	EEEEccccNNNc--NcNccccceEEEEEEENc
G. brevicaudus|AB026666	cccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHhhhhhhhNNcNEEEEEccceecHHHHHHHHHHcccceeNeNccHHHHHHHHHHHHhcccEEEEEccccNeeNccccccccccccE	EEEEccccNNcc--ccNccccEEEEEEEEENc
P.flavoviridis|D87548.1	cccccccHHHHHHHHHHHHHHHHHHHHHHHHHHHhhhhhhNNNccNEEEEEcccNNcHHHHHHHHHHccceENNNccHHHHHHHHHHHhccceEEEeccccNeeNccccccccccccE	EEEEccccNNNc--ccNccccEEEEEEEENc
E.quadrivirgata|AB030247	ccccccNhHHHHHHHHHHHHHHHHHHHHHHHHHHhhhhhhNNNcNEEEEEcccNNcHHHHHHHHHHcccNEeeNccHHHHHHHHHHHHccccEEEEEcccccNeNcccccccccccccc	EEEEEccccNccccccNcccceEEEEEeec-

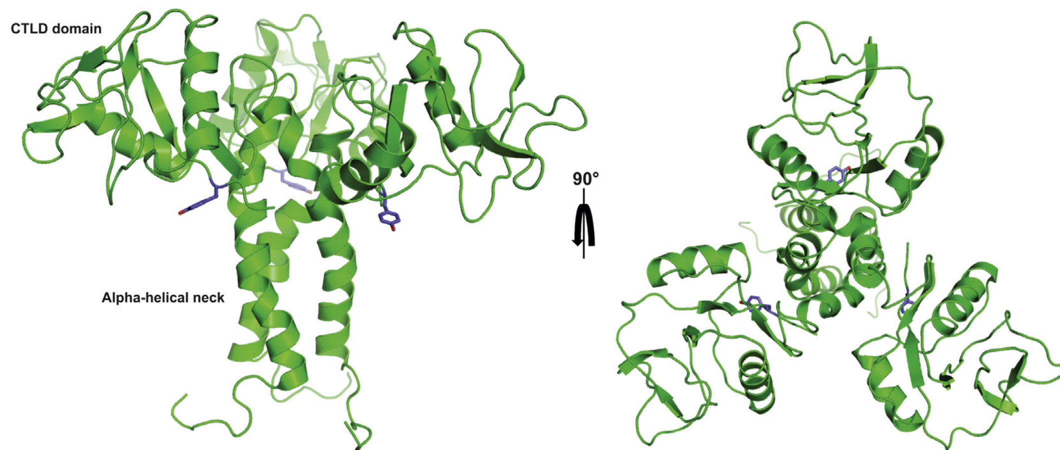

Fig. 3. Location of Y^{165} (purple sticks) on the *in silico* model of α*Balt*MIP trimer, an sbαPLI from *Bothrops alternatus* snakes (available at ModelArchive database under DOI: ma-a4btt). In α*Balt*MIP, Y^{165} is not located at the central pore as previously suggested (Okumura et al., 2005); this residue is located above the CTLD domain instead. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

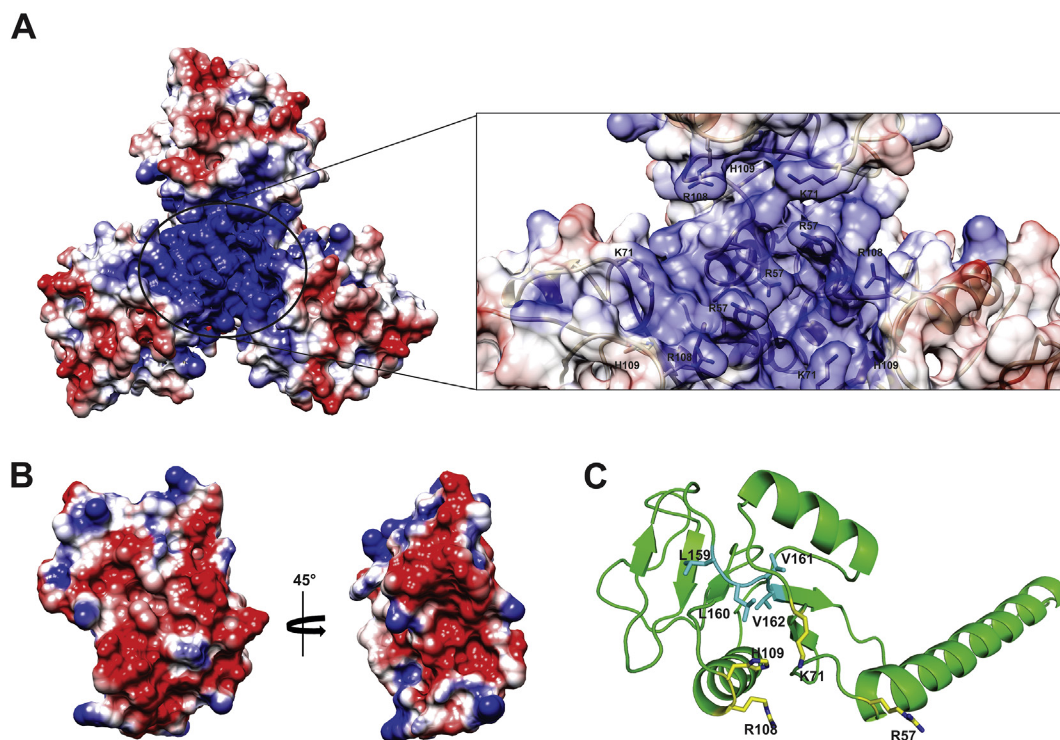

Fig. 4. Electrostatic potential surface analysis of (A) *in silico* model of α*Balt*MIP trimer, a sbαPLI from *Bothrops alternatus* snakes (available at ModelArchive database under DOI: ma-a4btt) and (B) crystallographic structure of BthA-I, an acidic svPLA$_2$ from *Bothrops jararacussu* snake venom (PDB ID 1U73) (Magro et al., 2004). Electrostatic potential surfaces were generated by APBS (Adaptive Poisson-Boltzmann Solver) electrostatic calculations (Baker et al., 2001) which are available in Chimera v.1.9 (Pettersen et al., 2004) after the transformation from a PDB to PQR file using the online server PDB2PQR (Dolinsky et al., 2004), at pH 7.0 and 150 mM NaCl. In the α*Balt*MIP trimer, there is a detailed view of the negatively charged central pore, highlighting (yellow sticks) the four conserved cationic residues R^{57}, K^{71}, R^{108} and H^{109} exposed to the solvent. (C) Location of the hydrophobic core (^{158}L to ^{161}V) on the *in silico* model of α*Balt*MIP monomer (available at ModelArchive database under DOI: ma-a2iil). The residues in the hydrophobic core that may be responsible for the structural integrity of the central pore are highlighted in cyan sticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Dos Santos et al., 2009) that may hamper the interaction at the central pore of sbαPLI trimers. The mechanism of inhibition of basic svPLA$_2$-like proteins by sbαPLIs, however, remains to be better understood.

Table 5
Accessible solvent area (ASA) of cationic residues (Å^2) located on central pore of in silico αBaltMIP trimer model (DOI: ma-a4btt at ModelArchive database) calculated with ArealMol software (Saff and Kuijlaars, 1997).

Residue	Chain			Total
	A	B	C	
Arg57	199.1	129.5	185.8	514.4
Lys71	163	158.5	132.9	454.4
Arg108	159.9	190.5	150.8	501.2
His109	56.6	62.4	41.7	160.7
Total				1630.7

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2016.01.058.

References

Baker, N.A., Sept, D., Joseph, S., Holst, M.J., McCammon, J.A., 2001. Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl. Acad. Sci. U. S. A. 98, 10037–10041.
Chuman, Y., Nobuhisa, I., Ogawa, T., Deshimaru, M., Chijiwa, T., Tan, N.H., Fukumaki, Y., Shimohigashi, Y., Ducancel, F., Boulain, J.C., Menez, A., Ohno, M., 2000. Regional and accelerated molecular evolution in group I snake venom gland phospholipase A_2 isozymes. Toxicon 38, 449–462.
Dolinsky, T.J., Nielsen, J.E., McCammon, J.A., Baker, N.A., 2004. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. Nucleic Acids Res. 32, W665–W667.
Dos Santos, J.I., Soares, A.M., Fontes, M.R., 2009. Comparative structural studies on Lys49-phospholipases A_2 from *Bothrops* genus reveal their myotoxic site. J. Struct. Biol. 167, 106–116.
Estevão-Costa, M.I., Rocha, B.C., de Alvarenga Mudado, M., Redondo, R., Franco, G.R., Fortes-Dias, C.L., 2008. Prospection, structural analysis and phylogenetic relationships of endogenous gamma-phospholipase A_2 inhibitors in Brazilian *Bothrops* snakes (Viperidae, Crotalinae). Toxicon 52, 122–129.
Fernandes, C.A., Borges, R.J., Lomonte, B., Fontes, M.R., 2014. A structure-based proposal for a comprehensive myotoxic mechanism of phospholipase A_2-like proteins from viperid snake venoms. Biochim. Biophys. Acta 1844, 2265–2276.
Fortes-Dias, C.L., Ortolani, P.L., Fernandes, C.A., Lobo, K.R., Amaral de Melo, L., Borges, M.H., Pazin, W.M., Neto Mde, O., Fernandez, R.M., Fontes, M.R., 2014. Insights on the structure of native CNF, an endogenous phospholipase A2 inhibitor from *Crotalus durissus terrificus*, the South American rattlesnake. Biochim. Biophys. Acta 1844, 1569–1579.
Gutierrez, J.M., Lomonte, B., 2013. Phospholipases A_2: unveiling the secrets of a functionally versatile group of snake venom toxins. Toxicon 62, 27–39.
Inoue, S., Kogaki, H., Ikeda, K., Samejima, Y., Omori-Satoh, T., 1991. Amino acid sequences of the two subunits of a phospholipase A_2 inhibitor from the blood plasma of *Trimeresurus flavoviridis*. Sequence homologies with pulmonary surfactant apoprotein and animal lectins. J. Biol. Chem. 266, 1001–1007.
Inoue, S., Shimada, A., Ohkura, N., Ikeda, K., Samejima, Y., Omori-Satoh, T., Hayashi, K., 1997. Specificity of two types of phospholipase A_2 inhibitors from the plasma of venomous snakes. Biochem. Mol. Biol. Int. 41, 529–537.
Kinkawa, K., Shirai, R., Watanabe, S., Toriba, M., Hayashi, K., Ikeda, K., Inoue, S., 2010. Up-regulation of the expressions of phospholipase A_2 inhibitors in the liver of a venomous snake by its own venom phospholipase A_2. Biochem. Biophys. Res. Commun. 395, 377–381.
Kochva, E., Nakar, O., Ovadia, M., 1983. Venom toxins: Plausible evolution from digestive enzymes. Am. Zool. 23, 427–430.
Kogaki, H., Inoue, S., Ikeda, K., Samejima, Y., Omori-Satoh, T., Hamaguchi, K., 1989. Isolation and fundamental properties of a phospholipase A2 inhibitor from the blood plasma of *Trimeresurus flavoviridis*. J. Biochem. 106, 966–971.
Lizano, S., Angulo, Y., Lomonte, B., Fox, J.W., Lambeau, G., Lazdunski, M., Gutierrez, J.M., 2000. Two phospholipase A_2 inhibitors from the plasma of *Cerrophidion (Bothrops) godmani* which selectively inhibit two different group-II phospholipase A_2 myotoxins from its own venom: isolation, molecular cloning and biological properties. Biochem. J. 346, 631–639.
Lizano, S., Domont, G., Perales, J., 2003. Natural phospholipase A_2 myotoxin inhibitor proteins from snakes, mammals and plants. Toxicon 42, 963–977.
Lizano, S., Lomonte, B., Fox, J.W., Gutierrez, J.M., 1997. Biochemical characterization and pharmacological properties of a phospholipase A_2 myotoxin inhibitor from the plasma of the snake *Bothrops asper*. Biochem. J. 326, 853–859.
Murakami, M.T., Kuch, U., Betzel, C., Mebs, D., Arni, R.K., 2008. Crystal structure of a novel myotoxic Arg49 phospholipase A_2 homolog (zhaoermiatoxin) from *Zhaoermia mangshanensis* snake venom: insights into Arg49 coordination and the role of Lys122 in the polarization of the C-terminus. Toxicon 51, 723–735.
Magro, A.J., Murakami, M.T., Marcussi, S., Soares, A.M., Arni, R.K., Fontes, M.R., 2004. Crystal structure of an acidic platelet aggregation inhibitor and hypotensive phospholipase A_2 in the monomeric and dimeric states: insights into its oligomeric state. Biochem. Biophys. Res. Commun. 323, 24–31.
Nakashima, K., Nobuhisa, I., Deshimaru, M., Nakai, M., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, M., Sakaki, Y., Hattori, S., et al., 1995. Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A_2 isozyme genes. Proc. Natl. Acad. Sci. U. S. A. 92, 5605–5609.
Nakashima, K., Ogawa, T., Oda, N., Hattori, M., Sakaki, Y., Kihara, H., Ohno, M., 1993. Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A_2 isozymes. Proc. Natl. Acad. Sci. U. S. A. 90, 5964–5968.
Nishida, M., Okamoto, M., Ohno, A., Okumura, K., Hayashi, K., Ikeda, K., Inoue, S., 2010. Inhibitory activities of the heterotrimers formed from two alpha-type phospholipase A_2 inhibitory proteins with different enzyme affinities and importance of the intersubunit electrostatic interaction in trimer formation. Biochim. Biophys. Acta 1804, 2121–2127.
Nobuhisa, I., Chiwata, T., Fukumaki, Y., Hattori, S., Shimohigashi, Y., Ohno, M., 1998. Structural elements of *Trimeresurus flavoviridis* serum inhibitors for recognition of its venom phospholipase A_2 isozymes. FEBS Lett. 429, 385–389.
Nobuhisa, I., Deshimaru, M., Chijiwa, T., Nakashima, K., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, S., Kihara, H., Ohno, M., 1997a. Structures of genes encoding phospholipase A_2 inhibitors from the serum of *Trimeresurus flavoviridis* snake. Gene 191, 31–37.
Nobuhisa, I., Inamasu, S., Nakai, M., Tatsui, A., Mimori, T., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, S., Kihara, H., Ohno, M., 1997b. Characterization and evolution of a gene encoding a *Trimeresurus flavoviridis* serum protein that inhibits basic phospholipase A_2 isozymes in the snake's venom. Eur. J. Biochem. 249, 838–845.
Nobuhisa, I., Nakashima, K., Deshimaru, M., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Sakaki, Y., Hattori, S., Kihara, H., Ohno, M., 1996. Accelerated evolution of T*rimeresurus okinavensis* venom gland phospholipase A_2 isozyme-encoding genes. Gene 172, 267–272.
Ogawa, T., Oda, N., Nakashima, K., Sasaki, H., Hattori, M., Sakaki, Y., Kihara, H., Ohno, M., 1992. Unusually high conservation of untranslated sequences in cDNAs for *Trimeresurus flavoviridis* phospholipase A_2 isozymes. Proc. Natl. Acad. Sci. U. S. A. 89, 8557–8561.
Ohkura, N., Inoue, S., Ikeda, K., Hayashi, K., 1993. Isolation and amino acid sequence of a phospholipase A_2 inhibitor from the blood plasma of *Agkistrodon blomhoffii siniticus*. J. Biochem. 113, 413–419.
Ohkura, N., Kitahara, Y., Inoue, S., Ikeda, K., Hayashi, K., 1999. Isolation and amino acid sequence of a phospholipase A_2 inhibitor from the blood plasma of the sea krait, *Laticauda semifasciata*. J. Biochem. 125, 375–382.
Ohkura, N., Okuhara, H., Inoue, S., Ikeda, K., Hayashi, K., 1997. Purification and characterization of three distinct types of phospholipase A_2 inhibitors from the blood plasma of the Chinese mamushi, *Agkistrodon blomhoffii siniticus*. Biochem. J. 325, 527–531.
Ohno, M., Chijiwa, T., Oda-Ueda, N., Ogawa, T., Hattori, S., 2003. Molecular evolution of myotoxic phospholipases A_2 from snake venom. Toxicon 42, 841–854.
Ohno, M., Menez, R., Ogawa, T., Danse, J.M., Shimohigashi, Y., Fromen, C., Ducancel, F., Zinn-Justin, S., Le Du, M.H., Boulain, J.C., Tamiya, T., Menez, A., 1998. Molecular evolution of snake toxins: is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? Prog. Nucleic Acid Res. Mol. Biol. 59, 307–364.
Okumura, K., Inoue, S., Ikeda, K., Hayashi, K., 1999a. cDNA cloning and bacterial expression of phospholipase A_2 inhibitor PLI alpha from the serum of the Chinese mamushi, *Agkistrodon blomhoffii siniticus*. Biochim. Biophys. Acta 1441, 51–60.
Okumura, K., Inoue, S., Ikeda, K., Hayashi, K., 2002. Identification of beta-type phospholipase A_2 inhibitor in a nonvenomous snake, *Elaphe quadrivirgata*. Arch. Biochem. Biophys. 408, 124–130.
Okumura, K., Inoue, S., Ikeda, K., Hayashi, K., 2003. Identification and characterization of a serum protein homologous to α-type phospholipase A_2 inhibitor (PLIα) from a nonvenomous snake, *Elaphe quadrivirgata*. IUBMB Life 55, 539–545.
Okumura, K., Masui, K., Inoue, S., Ikeda, K., Hayashi, K., 1999b. Purification, characterization and cDNA cloning of a phospholipase A_2 inhibitor from the serum of the non-venomous snake *Elaphe quadrivirgata*. Biochem. J. 341, 165–171.
Okumura, K., Ohno, A., Nishida, M., Hayashi, K., Ikeda, K., Inoue, S., 2005. Mapping the region of the alpha-type phospholipase A_2 inhibitor responsible for its inhibitory activity. J. Biol. Chem. 280, 37651–37659.
Oliveira, C.Z., Menaldo, D.L., Marcussi, S., Santos-Filho, N.A., Silveira, L.B., Boldrini-Franca, J., Rodrigues, V.M., Soares, A.M., 2008. An alpha-type phospholipase A_2 inhibitor from *Bothrops jararacussu* snake plasma: structural and functional characterization. Biochimie 90, 1506–1514.
Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF Chimera-a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612.

Quiros, S., Alape-Giron, A., Angulo, Y., Lomonte, B., 2007. Isolation, characterization and molecular cloning of AnMIP, a new alpha-type phospholipase A_2 myotoxin inhibitor from the plasma of the snake *Atropoides nummifer* (Viperidae: Crotalinae). Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 146, 60–68.
Saff, E.B., Kuijlaars, A.B.J., 1997. Distributing many points on a sphere. Math. Intell. 19, 5–11.
Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.
Santos-Filho, N.A., Fernandes, C.A., Menaldo, D.L., Magro, A.J., Fortes-Dias, C.L., Estevao-Costa, M.I., Fontes, M.R., Santos, C.R., Murakami, M.T., Soares, A.M., 2011. Molecular cloning and biochemical characterization of a myotoxin inhibitor from *Bothrops alternatus* snake plasma. Biochimie 93, 583–592.
Schaloske, R.H., Dennis, E.A., 2006. The phospholipase A_2 superfamily and its group numbering system. Biochim. Biophys. Acta 1761, 1246–1259.
Shimada, A., Ohkura, N., Hayashi, K., Samejima, Y., Omori-Satoh, T., Inoue, S., Ikeda, K., 2008. Subunit structure and inhibition specificity of alpha-type phospholipase A2 inhibitor from *Protobothrops flavoviridis*. Toxicon 51, 787–796.
Shirai, R., Toriba, M., Hayashi, K., Ikeda, K., Inoue, S., 2009. Identification and characterization of phospholipase A2 inhibitors from the serum of the Japanese rat snake, *Elaphe climacopora*. Toxicon 53, 685–692.
Shirai, R., Gotou, R., Hirano, F., Ikeda, K., Inoue, S., 2010. Autologous extracellular cytochrome c is an endogenous ligand for leucine-rich alpha2-glycoprotein and beta-type phospholipase A2 inhibitor. J. Biol. Chem. 285, 21607–21614.
So, S., Murakami, T., Ikeda, N., Chijiwa, T., Oda-Ueda, N., Kuraishi, T., Hattori, S., Ohno, M., 2011. Identification and evolution of venom phospholipase A_2 inhibitors from *Protobothrops elegans* serum. Biosci. Biotechnol. Biochem. 75, 480–488.
Soares, A.M., Marcussi, S., Stabeli, R.G., Franca, S.C., Giglio, J.R., Ward, R.J., Arantes, E.C., 2003. Structural and functional analysis of BmjMIP, a phospholipase A_2 myotoxin inhibitor protein from *Bothrops moojeni* snake plasma. Biochem. Biophys. Res. Commun. 302, 193–200.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Thwin, M.M., Satish, R.L., Chan, S.T., Gopalakrishnakone, P., 2002. Functional site of endogenous phospholipase A_2 inhibitor from Python serum. Eur. J. Biochem. 269, 719–727.
Zhou, X., Tan, T.C., Valiyaveettil, S., Go, M.L., Kini, R.M., Velazquez-Campoy, A., Sivaraman, J., 2008. Structural characterization of myotoxic ecarpholin S from *Echis carinatus* venom. Biophys. J. 95, 3366–3380.