

rBaltMIP, a recombinant alpha-type myotoxin inhibitor from *Bothrops alternatus* (*Rhinocerophis alternatus*) snake, as a potential candidate to complement the antivenom therapy

Norival A. Santos-Filho ^{a, d, 1}, Tiago S. Sousa ^{a, 1}, Johara Boldrini-França ^a,
 Ludier K. Santos-Silva ^b, Danilo L. Menaldo ^a, Flávio Henrique-Silva ^b, Adélia C.O. Cintra ^a,
 Helen J. Laure ^e, Carla C.N. Mamede ^c, Fábio Oliveira ^c, Thalita B. Riul ^a,
 Marcelo Dias-Baruffi ^a, José C. Rosa ^e, Suely V. Sampaio ^{a, *}

^a Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, FCFRP-USP, Ribeirão Preto, SP, Brazil

^b Departamento de Genética e Evolução, Universidade Federal de São Carlos, UFSCar, São Carlos, SP, Brazil

^c Departamento de Ciências Fisiológicas, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, MG, Brazil

^d Departamento de Bioquímica e Tecnologia Química, Instituto de Química, Univ. Estadual Paulista, Araraquara, SP, Brazil

^e Departamento de Biologia Celular e Molecular e Bioagentes Patogénicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, FMRP-USP, Ribeirão Preto, SP, Brazil

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ABSTRACT

Phospholipase A₂ inhibitors (PLIs) are important targets in the search and development of new drugs. This study aimed at evaluating the potential of an alpha-type phospholipase A₂ inhibitor from *Bothrops alternatus* (*Rhinocerophis alternatus*) snake in its recombinant form (rBaltMIP) to complement the conventional antivenom therapy. Biochemical experiments showed that rBaltMIP presented pI 5.8 and molecular masses of ~21 kDa by SDS-PAGE and 19.57 kDa by MALDI/TOF MS. After tryptic peptides sequencing, the results were compared with other PLIs available in databases, showing 100% identity between rBaltMIP and its native inhibitor BaltMIP and from 92% to 96% identity with other inhibitors. Myotoxic activities of BthTX-I and BthTX-II toxins were measured via plasma CK levels, showing myotoxic effective concentrations (EC50) of 0.1256 µg/µL and 0.6183 µg/µL, respectively. rBaltMIP neutralized the myotoxicity caused by these two toxins up to 65%, without promoting primary antibody response against itself. Nevertheless, this recombinant PLI was immunogenic when standard immunization protocol with Freud's adjuvant was used. In paw edema assays, EC50 of 0.02581 µg/µL and 0.02810 µg/µL, respectively, were observed with edema reductions of up to 40% by rBaltMIP, suggesting its use as an additional antivenom. In addition, myotoxicity neutralization experiments with the myotoxin BthTX-I showed that rBaltMIP was more effective in inhibiting muscle damage than the conventional antivenom. Thus, considering the severity of envenomations due to *Bothrops alternatus* (*Rhinocerophis alternatus*) and the low neutralization of their local effects (such as myotoxicity) by the current antivenoms, rBaltMIP is a promising molecule for the development of novel therapeutic strategies for clinical applications.

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1. Introduction

Snakebites represent a social, economic and public health

problem, causing serious debilitating consequences to the victims (Habib, 2013; Hifumi et al., 2015). Although the lethality induced by snake envenomations is low, the frequency of sequelae related to

* Corresponding author. Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café, s/n, Monte Alegre, CEP 14040-903 Ribeirão Preto, São Paulo, Brazil.

E-mail address: suvilela@usp.br (S.V. Sampaio).

¹ These authors contributed equally in this article.

local complication is higher. In 2009, the World Health Organization included snake envenomations in the list of neglected tropical diseases (Cruz et al., 2009; Harrison et al., 2009; Scheske et al., 2015; WHO, 2012). In Latin America (Gutiérrez, 2014) and in Brazil (Chippaux, 2015) envenomations still be a costly public health problem.

Nowadays, serum therapy using antisera composed of specific immunoglobulins is the only treatment for snake envenomations, but there are ongoing issues with availability, effectiveness and dosing (Scheske et al., 2015). These antivenoms neutralize the toxicity and lethality of specific venoms, but their administration is often associated with significant clinical side effects, such as serum sickness and renal failure (Morais and Massaldi, 2009; Zolfagharian and Dounighi, 2015). Additionally, the production of antivenoms is associated with high costs related to animal maintenance and also comes across animal welfare concerns, which instigates the search for innovative products for snakebite therapy (Kriki et al., 1999; Prado et al., 2016).

The search for natural inhibitors that neutralize snake venom toxins is of extreme importance for the production of more efficient antivenoms, especially considering that several toxins induce weak immunogenic responses, making traditional serum therapy unable to inhibit local effects of envenomations, such as the myotoxicity induced by phospholipases A₂ (PLA₂s) and PLA₂-like enzymes (Kulkeaw et al., 2007; Santos-Filho et al., 2015a).

Venomous and non-venomous snakes present proteins in their blood, called phospholipase A₂ inhibitors (PLIs) (Marcussi et al., 2007; Oliveira et al., 2008; Santos-Filho et al., 2015a). These PLA₂ inhibitors are classified into three types: α , β and γ , according to their structural aspects, and they can neutralize several effects of snake venoms, including phospholipase, myotoxic, neurotoxic, inflammatory and edema-inducing activities (Lizano et al., 2003; Marcussi et al., 2007; Santos-Filho et al., 2015a).

To date, several α PLIs were purified from the blood of different snake species (Faure, 2000; Fortes-Dias, 2002; Lizano et al., 2003; Marcussi et al., 2007). One of these inhibitors is α BaltMIP, which was isolated from *Bothrops alternatus* (*Rhinocerophis alternatus*) snake plasma by Santos-Filho et al. (Santos-Filho et al., 2011). This inhibitor was characterized as a glycosylated oligomeric protein with subunits of Mr around 24,000, and had its N-terminal sequence determined. α BaltMIP inhibitory activities were also assayed in relation to the toxic action of different PLA₂s, such as bothropstoxins I and II (BthTX-I and -II) from *Bothrops jararacussu* venom, piratoxins I and III (PrTX-I and -III) from *B. pirajai* venom, and crototoxin B (CB) from *Crotalus durissus terrificus* venom, showing potential to inhibit the anticoagulant, hemolytic, cytotoxic and myotoxic activities of the tested toxins.

Subsequently, Santos-Filho et al. (Santos-Filho et al., 2014) expressed a recombinant α -type inhibitor from *B. alternatus* (*Rhinocerophis alternatus*) snake, called rBaltMIP, using a heterologous system in *Pichia pastoris*, also conducting structural, biochemical and functional characterization experiments. The expressed protein was tested regarding its ability to inhibit the phospholipase activity of different PLA₂s and was also effective in decreasing the myotoxic activity of different Asp49 (BthTX-II and PrTX-III) and Lys49 (BthTX-I and PrTX-I) enzymes. The inhibition of the myotoxic activity was also performed without prior incubation of myotoxins/inhibitor. Furthermore, the cytotoxic activity of Asp49 PLA₂s and Lys49 PLA₂-like enzymes was decreased after incubation with rBaltMIP.

Considering that conventional antivenoms are usually inefficient against the local effects induced by myotoxins after envenomations, the present study aims at investigating the possible use of rBaltMIP as a complement for the traditional serum therapy. Furthermore, in order to supplement the previous findings on this

inhibitor, we also described further biochemical and functional characteristics and evaluated the immunogenic potential of rBaltMIP.

2. Materials and methods

2.1. Animals

Male Swiss (18–22 g) and BALB/c (18–22 g) mice were provided by the Animal Facilities of University of São Paulo, Campus Ribeirão Preto-SP. All procedures involving animals were approved by the Ethics Committee on Animal Use of University of São Paulo - Campus Ribeirão Preto (CEUA no 13.1.276.53.1).

2.2. Venom, antivenom and toxins

Crude venom was acquired from the Center for Extraction of Animal Toxins (CETA) (Morungaba – SP, Brazil). Commercial polyvalent veterinary antivenom (AV) was purchased (Rasg & Apik; lema bioLOGIC do Brasil Ltda) and used in a molar ratio of 1:1 (rBaltMIP:BthTX-I). BthTX-I and II were isolated from *B. jararacussu* venom using Sephadex G-75 and CM-Sepharose columns as previously described (Cintra et al., 1993; Homsi-Brandeburgo et al., 1988), with slight modifications. The homogeneity of all isolated proteins was confirmed by SDS-PAGE and RP-HPLC.

2.3. Expression and purification of rBaltMIP

The induction of expression and purification of the recombinant inhibitor was accomplished as previously described by Santos-Filho et al. (Santos-Filho et al., 2014).

2.4. Reverse phase chromatography

In order to analyze the homogeneity of the toxins and the recombinant inhibitor, a reverse phase high performance liquid chromatography (RP-HPLC) was performed using a C18 column (250 × 4.6 mm, CLC-ODS, Shimadzu, Japan) previously equilibrated with solvent A [0.1% trifluoroacetic acid (TFA)] and eluted with a concentration gradient of solvent B [70% acetonitrile (ACN) and 0.1% TFA] at a flow rate of 1 mL/min. Chromatography was performed on a HPLC Shimadzu system and fractions were monitored at 280 nm.

2.5. Biochemical characterization

Chromatography fractions, isolated toxins and the recombinant inhibitor were monitored by SDS-PAGE (Laemmli et al., 1970). Molecular masses were estimated by the interpolation of a linear logarithmic curve of relative molecular mass of standard proteins (14.4–116 kDa, unstained low range standard, Thermo Scientific) versus the distance of migration of the analyzed proteins in the gel. Isoelectric focusing of rBaltMIP was carried out according to Vesterberg (Vesterberg, 1972), with minor modifications. For protein quantification, sample solutions were analyzed using the micro-biuret method (Itzhaki and Gill, 1964) or the method described by Scopes (Scopes, 1974).

2.6. Molecular mass determination by mass spectrometry

The molecular mass of rBaltMIP was determined in a MALDI-TOF/TOF mass spectrometer (AXIMA PERFORMANCE, Shimadzu Biotech – Launchpad 2.8.3 software; Kratos-Shimadzu, Manchester, UK), using sinapinic acid (10 mg/mL in 50% acetonitrile/0.1% TFA) as matrix at 1:1 ratio (sample:matrix). MS spectra were obtained in linear positive mode and the detected ions had their

masses determined in relation to a calibration standard (Protein MALDI-MS Calibration Kit; Sigma, MSCAL-1-1 KT, 057k6815) containing the following proteins: insulin (bovine), cytochrome C (horse), aldolase (rabbit) and BSA (bovine serum albumin). The molecular mass range evaluated was of 5000–100,000 m/z.

2.7. Amino acid sequencing of rBaltMIP tryptic peptides by tandem mass spectrometry

2.7.1. In situ tryptic hydrolysis

rBaltMIP sample was diluted in 0.1 M ammonium bicarbonate, reduced with 45 mM DTT and alkylated with 100 mM iodoacetamide (Sigma-Aldrich) prior to the addition of a trypsin solution (0.5 µg in 20 µL of 0.1 M ammonium bicarbonate) and incubation at 37 °C for 24 h. The reaction was stopped by adding 5 µL of formic acid. Thereafter, the tryptic peptides were desalted in a micro reversed phase column (POROS R2, Perceptive Biosystems, USA) previously activated with methanol and equilibrated with 0.2% formic acid. Samples were loaded onto the column and purified from salts and other hydrophilic components by 3 washes with 100 µL of 0.2% formic acid, followed by elution of peptides with 30 µL of a solution containing 60% methanol and 5% formic acid. Then, samples were dried in a vacuum centrifuge to be ready for mass spectrometry analysis.

2.7.2. ESI Q-TOF

The peptide samples were diluted with 20 µL of a solution of 0.1% formic acid and 5% acetonitrile (ACN), being subjected to separation by UPLC-NanoAcquity (Waters, Beverly, MA) using a C18 reverse phase capillary column, directly coupled to the ionization source. The solutions used were 5% ACN in 0.1% formic acid (A) and 95% ACN in 0.1% formic acid (B) at a flow rate of 300 nL/min with an increasing linear gradient of solution B from 5% to 80% in 55 min. Peptides were analyzed in an electrospray source coupled to a Q-TOF spectrometer (ESI-Q-TOF-MS, Ultima, Waters), using a voltage of 3.5 kV for the ionization source, a cone voltage of 40 V and a source temperature of 100 °C. The spectra were collected in data-dependent-acquisition mode, and processed by the MassLynx 4.1 software. Collision-induced dissociation (CID-MS/MS) was performed automatically, selecting the most intense top 3 ions for a given time of chromatography. The peak list was generated by MassLynx software and submitted to Mascot server 2.4.1.

2.7.3. Identification of peptides

ESI Q-TOF spectra were processed and submitted to a database for identification of proteins using the Mascot software (version 2.4.1) against the NCBI database (NCBInr_20140323. fasta) following the parameters: no taxonomy, trypsin enzyme with loss of 1 cleavage (missed cleavage) and variable modifications to methionine oxidation and carbamidomethylation of Cys. Precursor ion tolerance was set to 1.2 Da and MS/MS fragments to 0.8 Da. False-positive identifications (FDR) was set to 1%, the mass spectra were submitted to the database in reverse mode, with a level of statistical significance of p < 0.05 corresponding to a permissible error of 5%.

2.8. Myotoxic activity

Myotoxic activity was performed by measuring the levels of creatine kinase (CK) in the plasma of male BALB/c mice (18–22 g). Animals (groups n = 3) were injected into the gastrocnemius muscle with 50 µL of phosphate-buffered saline (PBS) alone (negative control) or containing different concentrations of Lys49 and Asp49 myotoxins (BthTX-I and II from *B. jararacussu*). After 3 h of injection, mice tail tips were cut and the blood was collected on

heparinized capillary, which were immediately centrifuged at 3000×g for 10 min. The activity of the enzyme creatine kinase was determined using 4 µL of plasma and 1 mL of the reagent from a CK-UV K010 kinetic kit (Bioclin), determining the absorbance at 340 nm after 3 min of reaction at 37 °C. The CK activity was expressed in units/L, with one unit representing the phosphorylation of one nmol of creatine per minute.

2.8.1. Determination of BthTX-I and II's EC50 for myotoxicity

The half maximal effective concentrations (EC50) of the toxins BthTX-I and II were calculated by a dose versus response curve. Animal groups (n = 3) received samples containing different concentrations of the myotoxins (0.05; 0.1; 0.2; 0.4; 0.8 and 1.6 µg/µL) in 50 µL of PBS (the higher concentration was used only for BthTX-II). The control group was injected with 50 µL of PBS. After 3 h, blood samples were collected and the plasma CK activity was determined as described in section 2.8. Three independent experiments were performed using triplicates for each toxin concentration. Graphs were plotted using the GraphPad Prism 5 software.

2.9. Edema-inducing activity

Male Swiss mice (18–22 g, n = 3) had their right paws measured with a low pressure caliper (Mitutoyo) and then received subplantar injection of 50 µL of PBS (control group) or different concentrations of toxins (BthTX-I and II) solubilized in 50 µL of PBS. After 30 min, the paw size was measured again and the percentage of edema was calculated by the increased paw size compared to the initial measurements (before injection). Three independent experiments were carried out in triplicate for each concentration of both toxins.

2.9.1. Determination of BthTX-I and II's EC50 for edema

EC50 for the edema-inducing activity of BthTX-I and II were calculated by a dose versus response curve. For that, animals were subdivided in groups (n = 3), with the control receiving only PBS and the others receiving different concentrations of the toxins (0.0078; 0.0156; 0.0312; 0.0625; 0.125; 0.25; 0.5; 1.0 and 2.0 µg/µL).

2.10. Neutralization of myotoxicity and edema by rBaltMIP

These experiments were carried out as described in sections 2.8 and 2.9. The molar ratios of toxin/inhibitor were calculated using the EC50 values obtained for each toxin (2xEc50). The molar ratios used were 1:1 and 1:2 (toxin/inhibitor), with preincubation for 30 min at 37 °C prior to administration in animals. In addition, we evaluated the neutralization of the myotoxic activity induced by *B. jararacussu* crude venom and BthTX-I using a commercial veterinarian snake antivenom (AV) (Rasg & Aplik), alone or supplemented with rBaltMIP. Three independent experiments were performed in triplicate for each concentration of toxin (BthTX-I and II) and each assay.

2.11. Histopathology of muscle fibers

After collecting blood for the assessment of plasma CK levels, mice were euthanized by carbon dioxide inhalation followed by cervical dislocation. A section of the central region of their gastrocnemius muscle was then removed and soaked in fixation solution (10% formaldehyde in PBS, v/v). The material was dehydrated using increasing concentrations of ethanol and subsequently embedded in paraffin. The resulting blocks were cut into thick sections of 2.5 µm, stained with hematoxylin-eosin (0.25% w/v) and examined under an optical microscope (Santos-Filho et al., 2008, 2014).

2.12. Characterization of the immunogenic potential of rBaltMIP

2.12.1. Immunization of animals

Initially, three female BALB/c mice (18–22 g) were anesthetized and blood samples (200 µL) were collected by puncture of the orbital plexus to obtain pre-immune serum (negative control). Subsequently, mice were immunized via the intraperitoneal route with 10 µg of antigen (rBaltMIP) diluted in 150 µL of sterile PBS and emulsified with 150 µL of complete Freund's adjuvant (CFA, Difco Laboratories Inc., Detroit, MI, USA). Fifteen days later, a second immunization was performed using incomplete Freund's adjuvant (IFA) instead of CFA. After another 15 days, a blood sample (200 µL) was collected by puncture of the orbital plexus. After additional 15 days, mice were anesthetized for complete blood collection by cardiac puncture. Serum was separated from clots by centrifugation at 1500×g for 10 min, aliquoted and stored at –20 °C. Afterwards, all sera samples were diluted (1:1000 to 1:8000 in PBS) and analyzed by ELISA. This immunization procedure was developed based on the protocols described by Harlow and Lane (Harlow and Lane, 1988).

Elicitation of anti-rBaltMIP antibodies was also evaluated in the experiments of neutralization of myotoxicity (section 2.10), using the inhibitor rBaltMIP (50 µg) in the presence or absence of BthTX-I (50 µg) and without CFA or IFA. Four groups of BALB/c mice ($n = 4$) received intramuscular injections (50 µL) of PBS (vehicle control), BthTX-I, rBaltMIP or BthTX-I plus rBaltMIP (previously incubated for 30 min at 37 °C). After 15 days, mice were anesthetized for complete blood collection by cardiac puncture, obtaining serum as mentioned above. Sera samples from each experimental group were analyzed by ELISA in the dilution of 1:4000.

2.12.2. Detection of anti-rBaltMIP antibodies by ELISA

The efficiency of animal immunization with rBaltMIP was monitored by ELISA. Initially, rBaltMIP was dissolved in PBS (200 µg/mL) and 50 µL of this protein solution was loaded into the wells (1 µg per well) of a 96 wells polystyrene microtiter plates, followed by overnight incubation at 4 °C (antigen immobilization). Sequentially, each well was loaded with blocking buffer (3% gelatin, 0.5% Tween 20 in PBS, 200 µL per well) and incubated for 2 h at 37 °C (blocking step). After this period, samples of preimmune- or immune-sera (50 µL/well), diluted 1:1000, 1:2000, 1:4000 and 1:8000 in PBS, were loaded to the plate and incubated for 1 h at 37 °C (primary antibody incubation). Then, wells were loaded with 50 µL of horseradish peroxidase conjugated anti-mouse IgG rabbit antibody (Jackson ImmunoResearch, USA) diluted 1:5000 in PBS followed by incubation for 1 h at 37 °C (secondary antibody incubation). All of the above steps were followed by washing with PBS containing 0.1% Tween (200 µL per well). Sequentially, TMB solution (tetramethylbenzidine, Organon Teknica, Boxtel, NL) in the presence of hydrogen peroxide was loaded into each well. Finally, after 10 min, this reaction was stopped by adding 50 µL of 2 N sulfuric acid solution per well, followed by reading the absorbance at 450 nm in a spectrophotometer (Spectramax Plus – Molecular Devices).

2.13. Statistical analyses

The results were expressed as means ± standard deviation (SD). The significance of differences was determined by One-way ANOVA followed by Tukey post-test, with $p < 0.05$ considered significant. All the analyses were performed using the GraphPad Prism 5 program.

3. Results and discussion

Adequate treatments for snakebite envenomations are critically dependent on the ability of antivenoms to reverse venom-induced coagulopathy, myotoxicity, hypotensive shock and other effects (Fusco et al., 2015). It is known that, although antivenom therapy is effective against systemic toxic effects of envenomations, the conventional antivenoms present several limitations, such as the low efficacy against local effects induced by PLA₂ and PLA₂-like myotoxins, poorly neutralizing these toxins mainly due to the discrepancies between the pharmacokinetic profiles of low molecular mass toxins and antivenoms (Gutiérrez et al., 2003; Prado et al., 2016). Thus, improvements in the conventional serum therapy could be made with molecules such as PLIs that would be able to neutralize the local damages induced by snake toxins, without inducing toxic or immunogenic effects when administered in snakebite victims.

Although many studies have been investing in the characterization of natural inhibitors of snake toxins, such researches have to face challenges such as the necessity of finding new inhibitors (from plants, mammals, snakes, etc), the low availability and the difficulties in obtaining such inhibitors in sufficient quantities to allow functional, structural and biochemical studies. In the case of snakes, for example, obtaining PLIs from their blood come across ethical and environmental issues, since the animals need to be sacrificed in order to get these materials, and yet the amount of PLIs obtained is often insufficient for their full characterization. Thus, the heterologous expression of PLIs by molecular biology techniques greatly contributes to their studies, allowing their obtainment in larger quantities independently of snake serum.

In our previous studies, the heterologous expression of rBaltMIP was successfully achieved, making possible to obtain the purified recombinant inhibitor in larger quantities when compared to the native protein (α BaltMIP) and in its active form, as shown by its capacity to partially reduce the PLA₂ and cytotoxic activities of both Asp49 and Lys49 enzymes and their *in vivo* myotoxicity (Santos-Filho et al., 2014). Nevertheless, some important biological and structural questions remained unanswered, such as: Would rBaltMIP be efficient in complementing conventional antivenoms? Would it diminish the myotoxic effects of snake venoms? Would it cause antibody responses by itself?

Considering this, the present study aimed to further characterize rBaltMIP. Using the same protocol described in our previous work (Santos-Filho et al., 2014), we obtained the recombinant inhibitor and described some new biochemical and biological features of this molecule. rBaltMIP presented molecular mass of ~21 kDa by SDS-PAGE and 19.5 kDa by MALDI/TOF MS and pI of 5.8 (data not shown), demonstrating an acidic character like other α PLIs described in the literature, such as BaMIP from *B. asper* and α BjussuMIP from *B. jararacussu* snake (Lizano et al., 1997; Oliveira et al., 2008). Additionally, tryptic digestion of rBaltMIP resulted in 7 peptides (Table 1) that showed 100% sequence identity to the native inhibitor α BaltMIP previously described by Santos-Filho et al. (Santos-Filho et al., 2011), confirming the efficiency of the heterologous expression described by Santos-Filho et al (Santos-Filho et al., 2014), and used in the present study to obtain the recombinant inhibitor.

It has been reported that α PLIs inhibit a variety of PLA₂ activities, such as enzymatic, myotoxic, cytotoxic, edema-inducing, among others (Lizano et al., 2003; Marcussi et al., 2007). Prior to the inhibition tests involving rBaltMIP and *B. jararacussu* myotoxins, we determined the effective concentrations (EC50) for the induction of myotoxicity and edema by BthTX-I (0.1256 µg/µL and 0.02581 µg/µL, respectively) and BthTX-II (0.6183 µg/µL and 0.02810 µg/µL, respectively) (data not shown). These values were used to evaluate

Table 1

rBaltMIP tryptic peptides determined by LC-ESI-Q-TOF mass spectrometry.

Peptide	m/z	Mr exp	Charge	Mr calc	Pep_delta	Pep_score	Pep_seq
1	449,75	897,48	2	897,50	-0,0168	31,65	GAFLTVYK
2	563,33	1124,64	2	1124,63	0,0012	33,85	GAFLTVYKAR
3	460,25	918,48	2	918,49	-0,0144	43,34	AFANVLER
4	560,31	1118,61	2	1118,61	0,0059	37,43	EIKNFEALR
5	818,96	1635,91	2	1635,91	0,0008	80,53	LLNSLIDALMHILQR
6	750,03	2247,06	3	2247,09	0,0326	42,58	QICEKADGHIPSPQLENQNPK
7	1177,08	2352,15	2	2352,10	0,0434	99,13	EAYLVVGDSANFTNWAAGEPNK

*Data obtained using Mascot program v.2.4.1 and NCBI nr databank.

the inhibitory potential of rBaltMIP, using two toxin:inhibitor molar ratios (1:1 and 1:2 for myotoxicity, and 1:2 and 1:7 for edema) and considering the total activity of each myotoxin alone as 100%.

Regarding the myotoxicity experiments, control groups that received only PBS showed basal CK levels (around 6%) (Fig. 1A and B). BthTX-I in the presence of rBaltMIP at 1:1 and 1:2 M ratios showed significantly lower myotoxic activities, with inhibitions around 53 and 62%, respectively (Fig. 1A), while for BthTX-II, the inhibition levels were 55 and 57%, respectively (Fig. 1B). The inhibitor by itself did not induce myotoxicity in comparison to the control group (Fig. 1A and B).

As discussed in our previous work (Santos-Filho et al., 2011), some authors pointed the C-terminal region as the main responsible for the toxic effects of Lys49 PLA₂s (Lomonte et al., 2003a, 2003b; Núñez et al., 2001; Santos-Filho et al., 2015b). However, the existence of other motifs that can participate or complement the action of the toxic effector site should not be discarded (Lomonte et al., 2003b). However, C-terminal peptides of Asp49 PLA₂s present no direct membrane damage activity, suggesting that the toxic mechanisms exerted by these proteins involves different pathways from that used by myotoxic Lys49 PLA₂s, probably inducing muscle cell damage by affecting the integrity of plasma membranes, thereby leading to hyper contraction and other intracellular effects (Fuly et al., 2003; Núñez et al., 2001; Santos-Filho et al., 2008).

In the edema-inducing experiments, PBS control groups showed

an increase in paw thickness around 30% in comparison to the initial measurements (Fig. 1C and D). When BthTX-I was injected along rBaltMIP at 1:2 and 1:7 M ratios, inhibitions of 17 and 20%, respectively, were observed (Fig. 1C), while the inhibitions for BthTX-II were of 20 and 40%, respectively (Fig. 1D). The inhibitor by itself did not induce edema in comparison to the control group (Fig. 1C and D).

Edema formation is the result of a synergism between various inflammatory mediators that increase the vascular permeability or the blood flow (Landucci et al., 2012). Other studies have also shown the ability of α PLIs to inhibit edema and myotoxic activities induced by different PLA₂s (Asp49 PLA₂s) and PLA₂-like enzymes (Lys49 PLA₂s) (Lizano et al., 2000, 2003; Thwin et al., 2000). Our results showed that rBaltMIP had similar inhibitory effects on the myotoxicity induced by both BthTX-I and II, but was more efficient in inhibiting the edema induced by BthTX-II, an Asp49 PLA₂, than that induced by BthTX-I, a Lys49 PLA₂. This is somehow different from what was described for other α PLIs, such as α BjussuMIP from *B. jararacussu* snake plasma, which showed higher inhibitory effects on the edema induced by Lys49 molecules in comparison to the Asp49 ones (Oliveira et al., 2008). BmjMIP, an α PLI from *B. moojeni*, also presented higher inhibitory potential on Lys49 PLA₂s (BthTX-I, PrTX-I, MjTX-I and II) in comparison to Asp49 PLA₂s (PrTX-III and BthTX-II) (Soares et al., 2003). BaMIP from *B. asper* also inhibited the edema-inducing activity of both Asp49 and Lys49 PLA₂s, with higher inhibition of the latter (Lizano et al., 1997).

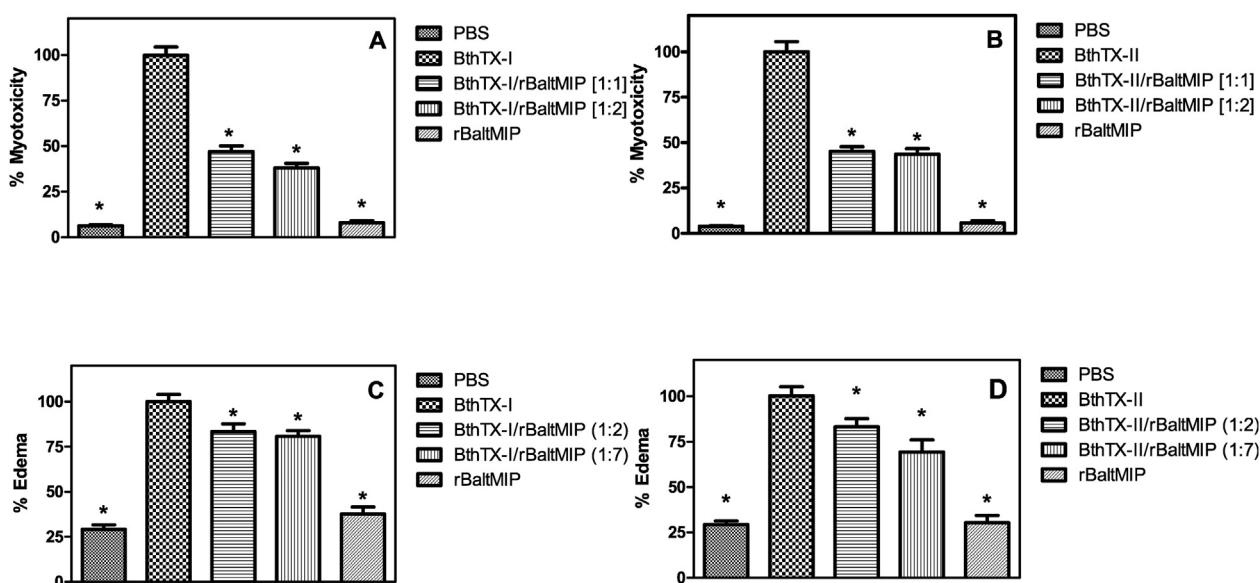


Fig. 1. Inhibition of the myotoxic and edema-inducing activities of BthTX-I and II by rBaltMIP. Myotoxins were preincubated with the inhibitor in different molar ratios at 37 °C for 30 min before administration in the animals. The activity of each toxin alone was considered as 100%. Inhibition of the myotoxic activity of BthTX-I (A) and BthTX-II (B) by rBaltMIP. Inhibition of the edema-inducing activity of BthTX-I (C) and BthTX-II (D) by rBaltMIP. The results of three individual experiments for each toxin were expressed as means \pm S.D. ($n = 3$) and statistically significant differences ($p < 0.05$) were marked with (*).

According to several works, the main structural domain responsible for the toxic effects of Lys49 PLA₂-like enzymes is the C-terminal region between the amino acid residues 115–129 (Lomonte et al., 2010). However, the mechanism of toxicity exerted by Asp49 myotoxins probably involves damage by affecting the integrity of the plasma membranes (Fuly et al., 2003; Santos-Filho et al., 2008). On the other hand, the probable mechanisms for edema induction by catalytically active Asp49 PLA₂s are related to the inflammatory reaction that takes place due to the hydrolysis of membrane phospholipids through the release of arachidonic acid or through activation of intracellular processes in target cells (Zuliani et al., 2005). Corroborating this hypothesis, studies have shown that chemical modification of His48 residue in the catalytic site by p-bromophenacyl bromide (BPB) induces loss of enzymatic activity and toxicity of snake venom PLA₂s (Santos-Filho et al., 2008; Soares and Giglio, 2003; Soares et al., 2004). However, the association between PLA₂ enzymatic activity and edema formation is complex, once that enzymatically inactive Lys49 PLA₂-like also elicits edema-forming activity, indicating that PLA₂ could have pharmacological domain independent of the catalytic site (Bonfim et al., 2009; Kini and Evans, 1989; Nunes et al., 2011), which can interact with cellular membranes to induce cellular activation leading to a cytokine-dependent inflammatory reaction (Zuliani et al., 2005). As could be observed, the mechanisms by which PLA₂s induce paw edema are not clear and further studies are needed to investigate it (Bonfim et al., 2009).

Another interesting result is that rBaltMIP was more able to inhibit the myotoxic than edema-inducing activity. These results endorse the hypothesis that PLA₂s could induce edema and myotoxicity by different mechanisms. Probably, rBaltMIP binds to

myotoxic domains of myotoxins, avoiding toxicity; however, following this same hypothesis, rBaltMIP is not able to efficiently prevent edema because it does not bind to possible edema pharmacological sites.

It is important to cite that, the mechanism of inhibition of α PLIs is not totally clear. Several research groups hypothesized that the interaction between the inhibitor and proteins seems to occur in different ways (Estevão-Costa et al., 2016; Nishida et al., 2010; Okumura et al., 2005; Quirós et al., 2007; Santos-Filho et al., 2011, 2014). In a recent study, Estevão-Costa and co-workers (Estevão-Costa et al., 2016) suggested that the central pore, which is composed by positive charged residues, especially R57, K71, R108 and H109, could be a significant part of the binding site of α PLIs to acidic PLA₂s. However, the positive surface of the basic PLA₂s could prevent the PLA₂/PLI interaction at the central pore, and according to these authors, the mechanism of inhibition of basic PLA₂s by α PLIs remains to be understood.

The main focus of our study was to evaluate rBaltMIP as a potential complement of the conventional serum therapy usually applied to treat snake envenomations. For that purpose, a polyvalent veterinary antivenom was purchased to be evaluated in addition to the recombinant inhibitor against the toxic effects of BthTX-I and *B. jararacussu* venom. The commercial antivenom was able to neutralize around 30% of the myotoxic activity of *B. jararacussu* venom (Fig. 2A) and BthTX-I (Fig. 2B), whereas rBaltMIP inhibited approximately 50% of the myotoxicity of the venom (Fig. 2A) and 60% of that induced by BthTX-I (Fig. 2B). When the antivenom was complemented with rBaltMIP, an inhibition of around 60% was observed for both samples tested. The veterinary antivenom and rBaltMIP were also evaluated alone and did not

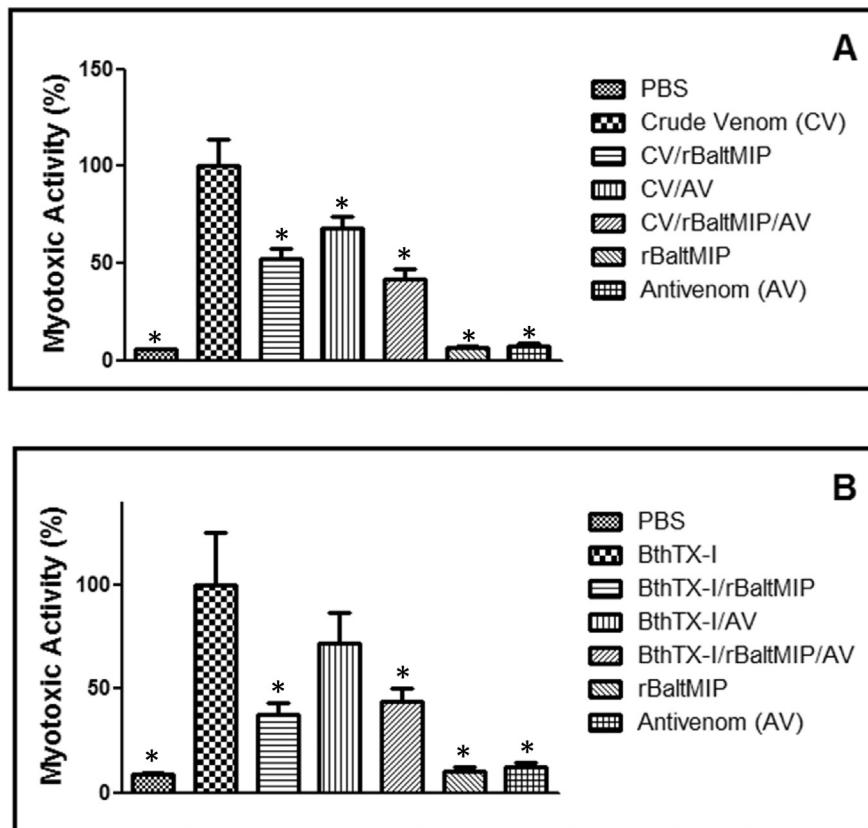


Fig. 2. Inhibition of the myotoxic activity of *B. jararacussu* venom (A) and BthTX-I (B) by rBaltMIP, commercial antivenom or the combination of both. Samples were preincubated with inhibitor, antivenom or the combination of both for 30 min at 37 °C before injection at 1:1 M ratio. The activity of each sample alone was considered as 100%. Results were expressed as means ± S.D. ($n = 3$) and statistically significant differences ($p < 0.05$) were marked with (*).

induce myotoxicity on animals (Fig. 2).

Histopathological analysis showed that BthTX-I and *B. jararacussu* crude venom are capable of inducing damage to muscle fibers in animals, recruiting leukocyte infiltrates and forming an intense accumulation of intercellular fluid (Fig. 3A and B). In case of the crude venom, an intense hemorrhage was also induced, as illustrated by the presence of large amounts of red blood cells in the muscle (Fig. 3B).

When BthTX-I was preincubated with rBaltMIP, a decrease in its local damage capacity could be noticed by the integrity of muscle fibers and the decreased number of leukocyte infiltrates (Fig. 3C).

Also, rBaltMIP greatly inhibited the myotoxic activity of BthTX-I when compared to the antivenom (Fig. 3E). When *B. jararacussu* crude venom was previously incubated with rBaltMIP, a higher preservation of muscle fibers and decreased leukocyte infiltrates could also be observed, but the red blood cells were still present (Fig. 3D). This hemorrhage was only inhibited by preincubation of crude venom with the commercial antivenom (Fig. 3F), while the preservation of muscle fibers was more evident after preincubation with rBaltMIP.

Trying to validate the hypothesis that rBaltMIP can complement the conventional antivenom, the inhibitor was added to the

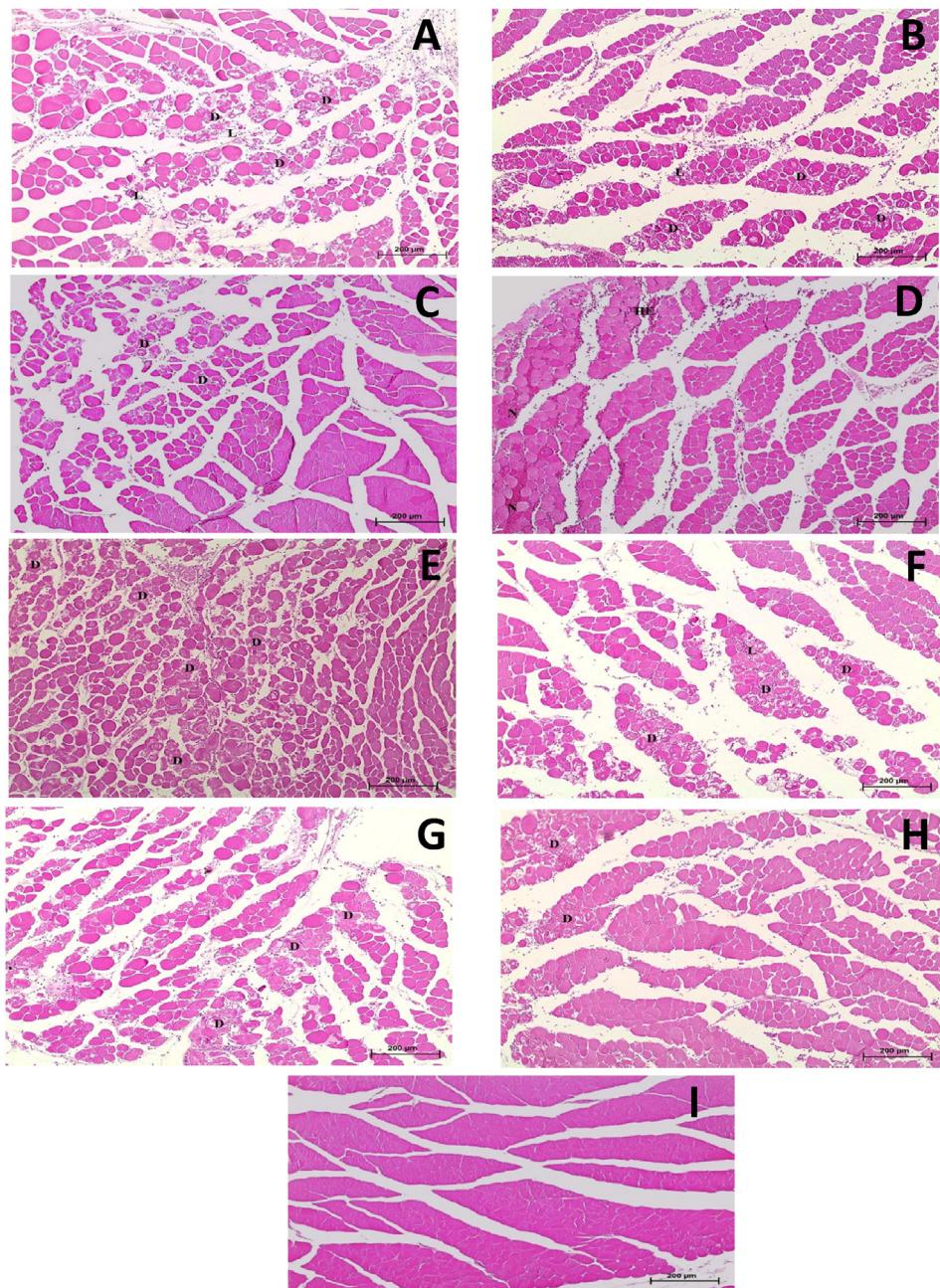


Fig. 3. Histopathological analyses to evaluate the potential of rBaltMIP as a complement of traditional serum therapy. Light microscopy of gastrocnemius muscles of mice that were injected with: (A) BthTX-I (50 µg), (B) *Bothrops jararacussu* crude venom (50 µg), (C) BthTX-I (50 µg) preincubated for 30 min at 37 °C with rBaltMIP at 1:1 M ratio, (D) crude venom preincubated with rBaltMIP, (E) BthTX-I (50 µg) preincubated with polyvalent antivenom, (F) crude venom (50 µg) preincubated with polyvalent antivenom in the ratio recommended by the manufacturer, (G) BthTX-I (50 µg) preincubated with antivenom solution supplemented with rBaltMIP, (H) crude venom (50 µg) preincubated with antivenom supplemented with rBaltMIP, (I) PBS (negative control). Letters indicate the presence of necrosis (N), fiber degradation (D), red blood cells (HE) and infiltrated leukocytes (L). Bar = 200 µM.

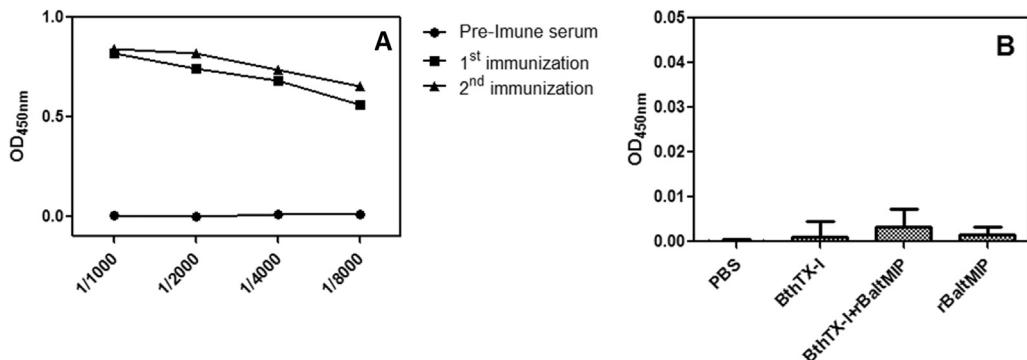


Fig. 4. Immunogenic potential of rBaltMIP. (A) Detection of antibodies against the antigen rBaltMIP by ELISA, showing the mean optical density obtained for sera from three animals at dilutions of 1:1000; 1:2000; 1:4000 and 1:8000. The high reactivity observed indicates an immunogenic potential of rBaltMIP to produce anti-rBaltMIP polyclonal antibodies when administered intraperitoneally in repeated doses at intervals of 15 and 30 days and when emulsified with Freund's Complete and Incomplete Adjuvant, respectively. (B) Production of anti-rBaltMIP antibodies in sera of mice treated intramuscularly with rBaltMIP. Using the myotoxicity assays and injecting the gastrocnemius muscle of mice with BthTX-I and rBaltMIP solution (1:1, molar ratio), no significant production of anti-rBaltMIP antibodies in the serum of animals could be observed after 15 days by ELISA.

commercial antivenom and incubated with BthTX-I or *B. jararacussu* crude venom for 30 min prior to injection. Histopathology of muscle fibers that were inoculated with BthTX-I preincubated with the antivenom supplemented with rBaltMIP (Fig. 3G) showed that complemented antivenom solution was able to reduce muscle damage compared to BthTX-I, and especially to BthTX-I previously incubated only with antivenom. In addition, the crude venom preincubated with the antivenom supplemented with rBaltMIP (Fig. 3H) induced less muscle damage in comparison to the crude venom alone or previously incubated only with the antivenom, and also induced less hemorrhage, suggesting an apparent synergistic effect.

The synergic effect of rBaltMIP with commercial antivenom was more evident when *B. jararacussu* crude venom was used. When the crude venom was injected in mice muscle, muscle fiber damage and intense hemorrhage could be observed (Fig. 3B), evidencing the local effects induced by myotoxins (among them Lys49 PLA₂-like) and also hemorrhage caused by other proteins (such as metalloproteases). This hemorrhage was inhibited after preincubation of crude venom with the commercial antivenom, although little preservation of the muscle fibers was observed (Fig. 3F). Other interesting observation could be made when rBaltMIP was preincubated with crude venom, since muscle fibers were preserved, but hemorrhagic activity was still present. However, when the crude venom was preincubated with the antivenom supplemented with rBaltMIP, an apparent synergic effect could be noted, since muscle damage (caused by myotoxins, which was specifically inhibited by rBaltMIP) and hemorrhage (inhibited by antivenom) were both decreased (Fig. 3H).

Several authors have suggested supplementation of antiphidian serum with natural inhibitors (Lizano et al., 2003; Oliveira et al., 2011; Santos-Filho et al., 2015a, 2014, 2011). Lizano et al. (Lizano et al., 2003) evaluated and confirmed the hypothesis that natural α PLIs could complement the conventional serum therapy. However, some questions were raised by these authors. The first one was about the limitation related to the availability of inhibitors, since they are isolated from snake blood, which are insufficient and ecologically unacceptable sources of inhibitors for commercial purposes. As mentioned earlier, the key to this problem would be the heterologous expression of proteins by molecular biology techniques, thus being a resource used to obtain greater quantities of active biomolecules. The second important question raised by Lizano et al. (Lizano et al., 2003) was about the toxicity and the immunogenic potential of α PLIs. According to them, after PLIs administration, an immune response would initiate, which could

lead to anaphylactic reactions and inhibition of its therapeutic effects.

Considering this and aiming at the possible commercial use of plasma inhibitors in complementing conventional snake antivenoms, the immunogenic potential of rBaltMIP was evaluated in BALB/c mice using a conventional intraperitoneal immunization protocol in the presence of Freud's adjuvant. Polyclonal antibodies against rBaltMIP were detected at all tested serum dilutions (1:1000, 1:2000, 1:4000 and 1:8000) by ELISA, and, as expected, pre-immune sera did not present anti-rBaltMIP antibodies (Fig. 4A). These results suggest that rBaltMIP is immunogenic when administered intraperitoneally in repeated doses (0, 15 and 30 days) in the presence of adjuvants. In the literature, there has been reported that a robust primary antibody response against proteins might occur even through a single injection without adjuvants (Gore et al., 2004). Interestingly, alarmins released by necrotic tissue, such as high-mobility group box 1 (HMGB1), are potent endogenous adjuvants that could enhance the primary antibody response to poorly immunogenic antigens (Rovere-Querini et al., 2004). Based on this information, we decided to check whether the immunogenic property of rBaltMIP could also be detected in the sample sera of mice that were used in the test of myotoxicity neutralization. For this, a myotoxicity inhibition test was conducted by injecting rBaltMIP with BthTX-I at 1:1 M ratio, and collecting and separating the animal's blood after 15 days, evaluating sera by ELISA for the production or not of anti-rBaltMIP antibodies. Interestingly, we did not detect the production of anti-rBaltMIP antibodies when the inhibitor was used as a treatment against the myotoxicity induced by BthTX-I (Fig. 4B). This result could be partially explained by experimental differences between those approaches: i) use of Freud's adjuvant or not; ii) distinct antigen doses; iii) number of antigens injections; iv) immunization route; v) tissue microenvironment. Nonetheless, further studies are needed to better understand the cellular and molecular mechanisms associated with the immunogenicity of this recombinant alpha-type myotoxin inhibitor, including the mapping of its functional site, in order to find peptides from this inhibitor with the capacity of neutralizing BthTX-I and -II with low immunogenicity, as described for another PLI (Thwin et al., 2002).

4. Conclusion

In conclusion, our studies showed important functional features for rBaltMIP, suggesting possible applications that could be useful not only for therapeutic purposes, but also in biotechnological

industries studying the mechanisms of snake toxins, in the design of synthetic inhibitors and in the comprehension of other functional and structural characteristics of myotoxins. Moreover, considering that the anti-myotoxic effect of rBaltMIP is not associated with a primary antibody response against itself, it is possible to suggest its viable utilization as an important recombinant molecule to design additional antivenom therapy against *Bothrops alternatus* (*Rhinocerophis alternatus*) envenomation.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical statement

The authors Norival A. Santos-Filho, Tiago S. de Sousa, Johara Boldrini-França, Ludier K. Santos-Silva, Danilo L. Menaldo, Flávio Henrique-Silva, Adélia C.O. Cintra, Helen J. Laure, Carla C. N. Mamede, Fábio de Oliveira, Thalita B. Riul, Marcelo Dias-Baruffi, José C. Rosa and Suely V. Sampaio declare that manuscript named "rBaltMIP, a recombinant alpha-type myotoxin inhibitor from *Bothrops alternatus* snake, as a potential complement of antivenom therapy" complies with the Elsevier Ethical Guidelines for Journal Publication.

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Transparency document

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