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In Silico, In Vitro and In Vivo Toxicological Assessment of BPP-BrachyNH$_2$, A Vasoactive Proline-Rich Oligopeptide from *Brachycephalus ephippium*

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Abstract BPP-BrachyNH$_2$ is a proline-rich oligopeptide (PRO) firstly identified in skin secretion of the frog *Brachycephalus ephippium*, which possess in vitro inhibitory activity of angiotensin-I converting enzyme (ACE) and endothelium-dependent vasorelaxant activity. Considering its potential application in the treatment of cardiovascular diseases, the present work assessed the toxicological profile of the BPP-BrachyNH$_2$. The in silico toxicity prediction was performed from the best model obtained through the optimization of the FASTA query peptide. This prediction study revealed that BPP-BrachyNH$_2$ induced high predicted LD$_{50}$ values for both humans and rats, and then is well-tolerated in the recommended range. The MTT assay was applied for the in vitro cytotoxic evaluation in murine macrophages. In this assay, a decrease of cell viability was not observed. The in vivo acute toxicological study was performed after the intraperitoneal administration of BPP-BrachyNH$_2$ at doses of 5 and 50 mg/kg. After intraperitoneal administration, no death, alterations in behavioral parameters or weight gain curve was observed, as well as none in the serum biochemical parameters, and gross pathological and histopathological analyses. These observations demonstrates an acceptable safety profile for BPP-BrachyNH$_2$, leading towards further studies focused on investigation of pharmacological and therapeutical applications for this peptide.

Keywords Cytotoxicity · MTT · In silico · pkCSM · Proline-rich oligopeptide · Toxicological

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Introduction

Proline-rich oligopeptides (PROs) represent a class of peptides firstly isolated from the *Bothrops jararaca* venom, which present proline residues at their C-terminal portions and inhibitory activity of the angiotensin-I converting enzyme (ACE) as well as the potentiation of bradykinin effects (Camargo et al. 2012; Morais et al. 2013). Furthermore, secretions and venoms from other animals, such as amphibians and scorpions have also been described as a potential source of PROs with potential applications (Conceição et al. 2007; Verano-Braga et al. 2008).

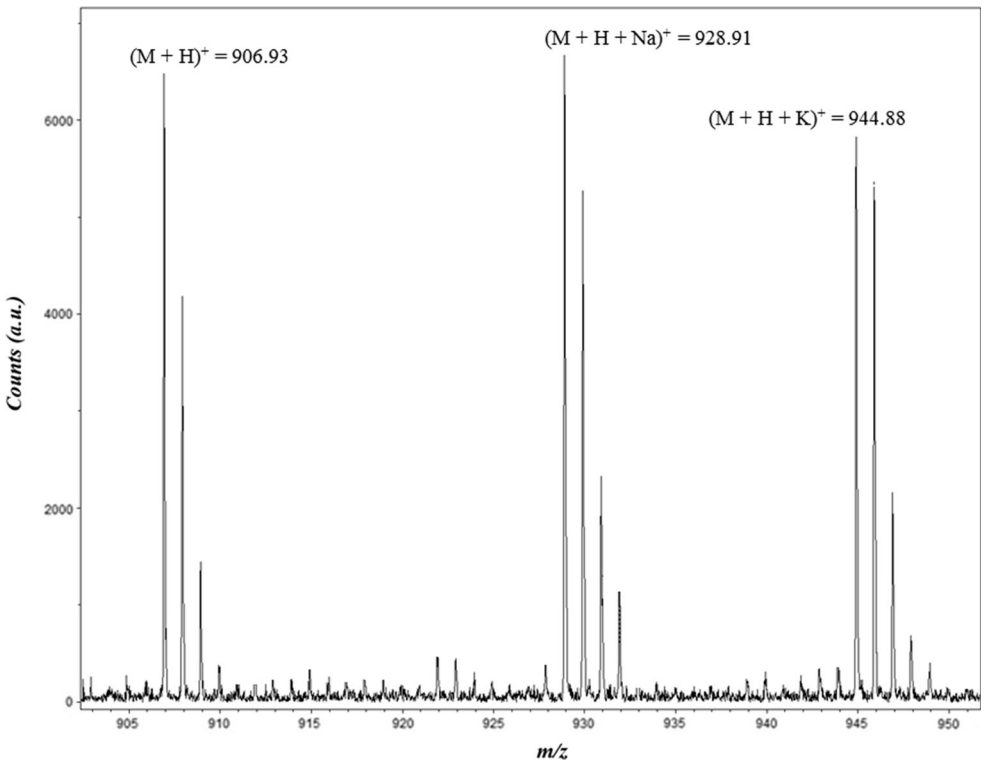

Fig. 1 MS spectra of BPP-BrachyNH$_2$, $[M+H]^+ = 906.93$

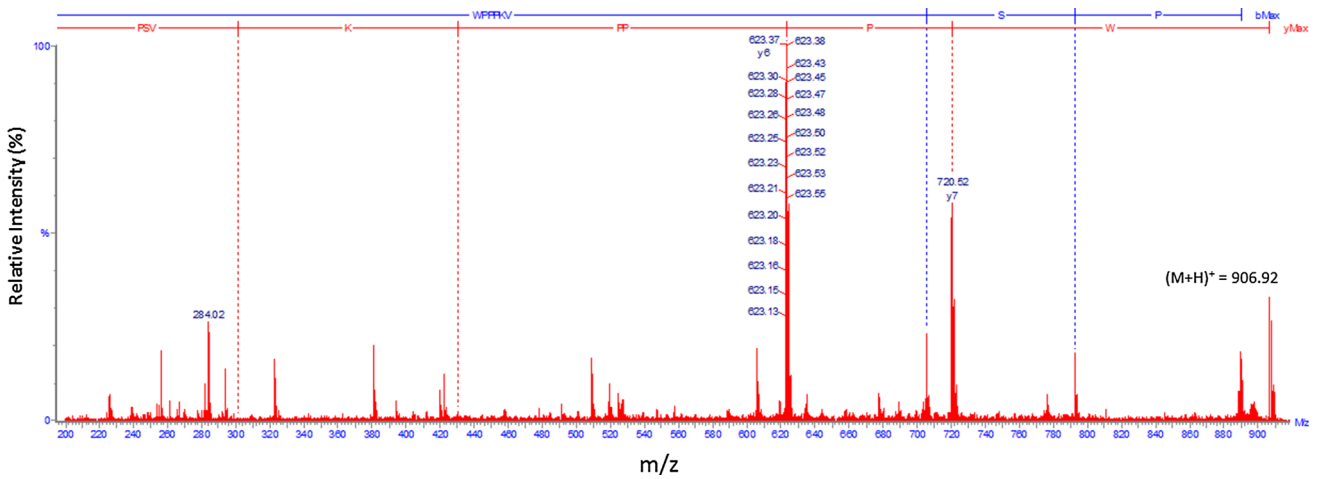

Fig. 2 MS/MS spectra of BPP-BrachyNH$_2$, $[M+H]^+ = 906.92$, acquired in an UltrafleXtreme MALDI-TOF/TOF

Interestingly, the peptide BPP-BrachyNH$_2$ (Figs. 1, 2) obtained from the skin secretion of the frog *Brachycephalus ephippium* induces endothelium-dependent vasorelaxant effect mediated by nitric oxide and in vitro inhibitory activity of ACE (Arcanjo et al. 2015).

The application of in silico (computer-based) modeling in the search for lead compounds is a promising endeavor in drug discovery, since it often accelerates the process and cuts down costs (DiMasi et al. 2003). Virtual screening methods are useful because, in principle, they narrow down the number of compounds to be actually tested in biological assays. This is practicable when the in silico scoring methods are sufficiently able to discriminate between active and inactive ones (Kubinyi 1998; Klebe 2006).

Acute toxicity studies in animals are commonly applied in order to attend a sort of requirements related to controlled risks for human health and the environment. An estimated lethal dose in 50% of animals in an experiment (LD_{50}) usually accomplishes the classification requirements for all regulatory authorities in chemicals, pharmaceuticals, consumer goods in general and pesticides. The

information obtained in these studies are useful to set doses in repeat-dose studies, and preliminarily identify some possible target organ of toxicological effects, as well as to provide relevant information for selection of doses in Phase 1 studies or overdose in humans (CDER 1996; OECD 2002a).

Considering the potential therapeutic applications of BPP-BrachyNH$_2$, its toxicological profile was assessed by in silico, in vitro and in vivo approaches, and reported in this study.

Experimental

Synthesis, Purification and Characterization of BBP-Brachy-NH$_2$

The synthesis of the octapeptide BPP-BrachyNH$_2$ (WPPPKVSP) was carried out manually, with a standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry (Fields and Noble 1990) starting from a Rink-amide-MBHA resin (0.59 mmol/g, Peptides International, Louisville, KY, USA). F-moc-protected amino acids (Peptides International, Louisville, KY, USA) were used in four-fold molar excess relative to the nominal scale of synthesis (1.2 mmol). Couplings were performed with 1,3-diisopropylcarbodiimide/ethyl 2-cyano-2-(hydroxyimino) acetate (DIC/Oxyma) in *N*,*N*-dimethylformamide (DMF) for 2–3 h. Side chain protected groups were *tert*-butyl for Ser, and Boc for Lys and Trp. Amino groups deprotections were conducted by 4-methylpiperidine/DMF (1:4, v:v) for 20–30 min. Removal of side chain protection and cleavage of the peptide from the resin were performed by the use of 10.0 mL TFA:water:tioanisol:ethanodithiol:triisopropylsilane (86:5.0:5.0:2.5:1.0, v:v:v:v:v) with addition of 1 g phenol for 90 min at room temperature under shaking. After solvent evaporation under nitrogen, the peptide was precipitated by addition of cold diisopropyl ether, collected by filtration and washed four times with cold diisopropyl ether. Extraction was performed with 200 mL H_2O:ACN (1:1, v:v) and crude peptide was lyophilized. Purification was performed using a preparative HPLC system (LaPrep Sigma), with LP1100 Quaternary LPG pump injection with fractionation valve. The elution conditions consisted of a linear gradient from 10 to 30% of acetonitrile in water. The eluent was monitored at the absorbance of 220 nm, and products corresponding to absorbing peaks were collected. Purity and molecular mass determination of the synthetic peptide (BPP) were performed using a MALDI-TOF/TOF mass spectrometer (UltrafleXtreme, Bruker Daltonics) operating under LIFT™ mode for MS/MS experiments (Figs. 1, 2). Purified peptide was obtained with 96% based on starting resin. Stock peptide solutions were prepared in water and their concentrations were determined according to tryptophan molar absorptivity (5550 M^{-1}/cm) at 280 nm (Arcanjo et al. 2015).

In Silico Toxicity Prediction

The toxicity analysis were performed by using pkCSM (Pires et al. 2015a), an in silico tool web server maintained by VLS3D (Cambridge University). The entry was the best model obtained through the optimization of the FASTA query peptide by AMMP (Fig. 3), a full-featured molecular dynamics program to manipulate both small molecules and macromolecules including proteins, nucleic acids and other polymers (Pedretti et al. 2004). The structure received the Gasteiger charges and AMBER field force and submitted to minimization using a genetic algorithm, through 3000 steps. The conformational search was realized by using the Boltzmann jump method, with flexible and psi torsions, at 300 K and RMSD equal to 60.00. The Gibbs free energy to the best conformer generated was calculated through Swiss-PDBViewer (Kaplan and Littlejohn 2001). Other physicochemical properties were also calculated, such as molecular weights (MW), lipophilicity (logP), rotatable bonds (NRB), polar surface area (PSA), number of hydrogen bond acceptors (HBA) and number of hydrogen bond donors (HBD).

Animals

Swiss and BALB/c female mice (25–30 g, 2 months) were used. The animals were maintained throughout the study period at 12 h light/dark cycle and temperature of 23 ± 2 °C), with free access to water and food (Purina-

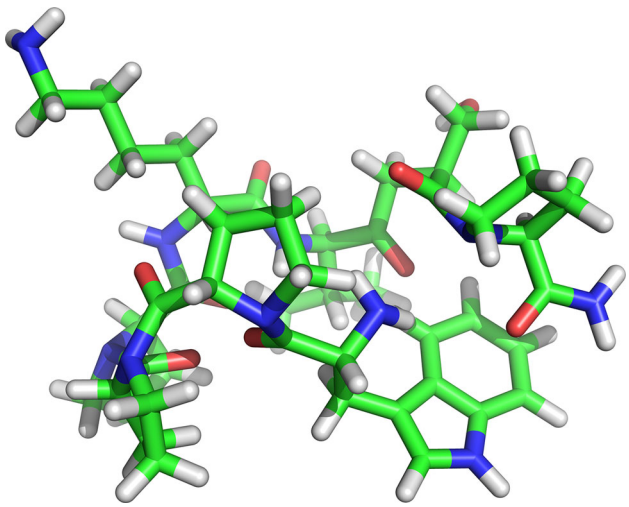

Fig. 3 Best optimized conformation of the peptide BPP-BrachyNH$_2$ in solution. The molecular physicochemical properties are described as follows: MW (g/mol) = 906.119; LogP = −2.206; NRB = 26; HBA = 11; HBD = 7; PSA = 380.105; and Gibbs free energy = 414.412 kJ/mol

Nestlé, São Paulo, SP, Brazil). The experimental procedures were performed according to recommendations of the Organization for Economic Co-operation and Development (OECD), and performed with approval by the Ethics Committee for Animal Experimentation from the Federal University of Piauí, Brazil (permission no. 008/2012).

Obtention and Cultive of Murine Peritoneal Macrophages

Peritoneal macrophages were obtained from male BALB/c mice. Three days after intraperitoneal administration of 1.5 mL of 3.0% thioglycollate, animals were euthanized, and immersed in 70% alcohol for 1 min. Then, 8 mL of sterile PSS was added in the peritoneal cavity, and the solution containing peritoneal macrophages was aspirated and transferred to a sterile tube, and then submitted to two centrifugations at 1500 rpm and 4 °C during 10 min, with repeated washings with sterile saline (0.9% NaCl). Afterwards, the supernatant was discarded and the cells re-suspended in 2 mL of RPMI 1640 medium supplemented with fetal bovine serum (10% FBS), penicillin (10,000 IU/mL), and streptomycin (10 mg/mL). The counting of viable macrophages was carried out in a Neubauer chamber and Blue Trypan staining, for analysis of cell viability (Rodrigues et al. 2015).

In Vitro Cell Viability of Murine Macrophages by MTT Assay

The evaluation of the cytotoxic activity of BPP-BrachyNH$_2$ in BALB/c murine macrophages was performed by MTT assay. Briefly, macrophages (1×10^6/well) were incubated in 96-well plates with 100 µL of RPMI 1640 medium (Sigma, St. Louis, USA) at 37 °C and 5% CO_2 during 4 h. The BPP-BrachyNH$_2$ was incubated at different concentrations (10^{-12}–10^{-5} M) during 48 h, and 10 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added in each well from a stock solution at 5 mg/mL in PBS, and was incubated during 4 h. Rightly after, the supernatant was discarded and 100 µL of DMSO was added to each well. The plate was stirred during 30 min in order to completely dissolve the formazan salt. The absorbances were read at 550 nm (absorbance microplate reader ELx800™, BioTek® Instruments, USA). The results were expressed as CC_{50} (mean cytotoxic concentration for 50% of the cells) with the confidence intervals calculated by non-linear regression (Rodrigues et al. 2013, 2015).

In Vivo Assessment of Acute Toxicity in Mice

The toxicological evaluation of BPP-BrachyNH$_2$ was performed based on the Acute Toxic Class Method (OECD no. 423), with modifications (OECD 2002b). Female Swiss mice were divided in three groups with three animals each, one control group intraperitoneally treated with saline (0.1 mL/10 g body weight), and two other groups intraperitoneally treated with BPP-BrachyNH$_2$ at doses of 5.0 and 50 mg/kg (5.5 and 55 µmol/kg), respectively. The doses were chosen based on previous studies with other PROs, where the highest active doses up to 0.75 µmol/kg (ca. 73-fold lower than the chosen doses) were reported (Camargo et al. 2012; Morais et al. 2013). After the first week of observations, the treatments were repeated on other three groups of three animals each according to the presence or absence of death, totaling six animals per treatment.

Evaluation of Clinical and Behavioral Parameters

After administration of BPP-BrachyNH$_2$, the animals were monitored during the first 8 h in order to observe the occurrence of death. After 24 h, the observation time for each group was around 30 min to 1 h. The animals were weighed daily, as well as clinical and behavioral parameters were evaluated according to the Guidance Document on the Recognition, Assessment and Use of Clinical Signs from OECD (OECD 2002c). The main evaluated endpoints are listed in Table 1.

Evaluation of Serum Biochemical Parameters

After 14 days of observation, animals were anesthetized with sodium thiopental (45 mg/kg), and then blood samples were collected from orbital plexus by a glass capillary tube and collecting tubes with coagulation activator and separator gel (Vacuette, Greiner Bio-One, Germany). The material was centrifuged at 3500 rpm during 10 min, and then the following biochemical parameters were evaluated: glucose, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), total cholesterol, and triglycerides. The tests were performed using commercial kits following the manufacturer's instructions (LABTEST® Pleno; Labtest, Lagoa Santa, MG, Brazil).

Evaluation of Body and Relative Organ Weights

The animals were daily weighed in order to obtain the dose–effect curve. After 14 days, the animals were anesthetized and then euthanized with a lethal dose of sodium thiopental (100 mg/kg, i.p.). Right after, the organs (heart, lung, stomach, spleen, liver and kidneys) were removed for gross pathology analysis (texture, consistency and color). Then, the organs were weighed in order to calculate the relative weight, as follows:

Table 1 Computer-aided toxicity parameters and recommended ranges to oral active compounds

Model name	Unit	Preferred	Predicted value
Hepatotoxicity	Categorical	No	Yes
Skin sensitisation	Categorical	No	No
AMES toxicity	Categorical	No	No
Max. tolerated dose (human)	log mg/kg day	Up to 0.477	0.255
hERG I inhibitor	Categorical	To be interpreted relative to the bioactive concentration	No
hERG II inhibitor	Categorical	To be interpreted relative to the bioactive concentration	Yes
Oral rat acute toxicity (LD_{50})	mol/kg	To be interpreted relative to the bioactive concentration	2.694
Oral rat chronic toxicity (LOAEL)	log mg/kg day	To be interpreted relative to the bioactive concentration	3.547

Table 2 Clinical and behavioral signs assessed after acute administration of BPP-BrachyNH_2 at doses of 5.0 and 50 mg/kg i.p.

Evaluated signs	Time (min)				
	30	60	120	180	240
Stimulant					
Hyperactivity	0	0	0	0	0
Aggressiveness	0	0	0	0	0
Tremors	0	0	0	0	0
Convulsion	0	0	0	0	0
Piloerection	0	0	0	0	0
Depressive					
Ptosis	0	0	0	0	0
Sedation	0	0	0	0	0
Anesthesia	0	0	0	0	0
Ataxia	0	0	0	0	0
Righting reflex	0	0	0	0	0
Catatonia	0	0	0	0	0
Analgesia	0	0	0	0	0
Loss of corneal reflex	0	0	0	0	0
Loss of auricular reflex	0	0	0	0	0
Autonomic nervous system-related					
Diarrhea	0	0	0	0	0
Constipation	0	0	0	0	0
Lacrimation	0	0	0	0	0
Salivation	0	0	0	0	0
Cyanosis	0	0	0	0	0
Others					
Ambulation	0	0	0	0	0
Self-cleaning	0	0	0	0	0
Climb	0	0	0	0	0
Vocalization	0	0	0	0	0
Abdominal writhes	0	0	0	0	0
Deaths	0	0	0	0	0

(0) Without effect

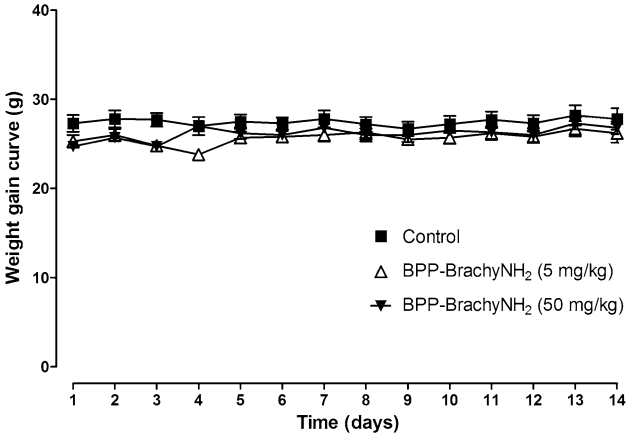

Fig. 4 Evolution of the weight gain of the animals from control group (*filled square*) or intraperitoneally treated with BPP-BrachyNH_2 at doses of 5.0 (*triangle*) and 50 (*filled inverted triangle*) mg/kg. Data represented as mean ± SEM, n = 6 animals per group

$$[\text{body weight (g)}/\text{animal weight on the day of necropsy (g)}] \times 100.$$

Histopathological Analyses

After euthanasia, the organs (liver, spleen, kidney and lung) were carefully removed and fixed in 10% formaldehyde solution during 72 h. For the histopathological analysis, the fixed organs were dehydrated in solutions with increasing concentrations of ethanol (70, 80, 90 or 99.9%), diaphanized in xylene (3×), impregnated and embedded in warm paraffin at 60 °C. Then, the histological sections were obtained by a microtome (5 μm thick). Then, histological sections were submitted to the hematoxylin and eosin staining method. Histopathological analyzes were performed by a single observer and the photographic record made in digital camera coupled to an optical microscope.

Table 3 Serum biochemical parameters of female Swiss mice 14 days after the treatment with single intraperitoneal dose of BPP-BrachyNH$_2$ (5 and 50 mg/kg, i.p.)

Parameters	Control	BPP-BrachyNH$_2$ (i.p.)	
		5 mg/kg	50 mg/kg
Glucose (mg/dL)	186.0 ± 18.7	132.8 ± 9.0*	136.5 ± 6.9*
Urea (mg/dL)	63.5 ± 8.5	64.6 ± 3.4	50.7 ± 3.1
Creatinine (mg/dL)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
AST (U/mL)	96.2 ± 11.0	120.0 ± 26.0	85.5 ± 16.6
ALT (U/mL)	58.0 ± 8.2	76.6 ± 6.3	48.7 ± 6.0
ALP (U/mL)	88.0 ± 13.6	87.4 ± 4.4	114.8 ± 9.5
GGT (U/mL)	2.3 ± 1.4	1.1 ± 0.5	1.6 ± 0.7
LDH (U/mL)	648.7 ± 70.9	899.2 ± 130.3	599.0 ± 40.1
Total cholesterol (mg/dL)	82.3 ± 6.4	78.8 ± 6.5	77.3 ± 3.0
Triglycerides (mg/dL)	83.3 ± 9.7	77.4 ± 11.3	72.7 ± 1.3

The values are expressed as mean ± SEM (n = 6/group)

* $p < 0.05$ when compared with control; Student t test for non-paired samples

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *GGT* gamma-glutamyl transferase, *LDH* lactate dehydrogenase

Table 4 Relative organ weights of female Swiss mice 14 days after the treatment with single intraperitoneal dose of BPP-BrachyNH$_2$ (5 and 50 mg/kg, i.p.)

Organs (g)	Control	BPP-BrachyNH$_2$ (i.p.)	
		5 mg/kg	50 mg/kg
Lungs	0.656 ± 0.042	0.674 ± 0.042	0.610 ± 0.037
Heart	0.478 ± 0.026	0.555 ± 0.030	0.463 ± 0.020
Liver	4.909 ± 0.084	5.036 ± 0.429	4.824 ± 0.293
Spleen	0.395 ± 0.037	0.362 ± 0.034	0.348 ± 0.048
Kidneys	1.227 ± 0.052	1.282 ± 0.075	1.185 ± 0.049

The values are expressed as mean ± SEM (n = 6/group)

Statistical Analyses

Values are expressed as mean ± standard error of the mean (SEM). Differences between groups were determined by Student's *t* test analysis for unpaired samples. The results were considered statistically significant when $p < 0.05$.

Results and Discussion

The distributions of the peptide MW, log*P*, number of HBA and number of HBD were evaluated for the peptide and represented in Fig. 3. In addition, the data of NRB and PSA, which have been used as an additional criterion to test for favorable metabolism and pharmacokinetics outcomes, were determined (Ntie-Kang 2013). The Gibbs free energy calculated for BBP-BrachyNH$_2$ was 414.412 kJ/mol.

In the study of the prediction of peptide toxicity profile, several predictors including human maximum tolerated dose, oral rat acute toxicity (LD_{50}), oral rat chronic toxicity, human ether-à-go-go related gene (hERG) inhibitors, hepatotoxicity, skin sensitization and mutagenicity were used. These parameters and descriptors are presented in Table 1. Together, the toxicity profile of the peptide revealed that BPP-BrachyNH$_2$ is well-tolerated by humans and rats, with the values in the recommended range. Likewise, the AMES mutagenicity prediction revealed negative for the peptide, indicating absence of BPP-BrachyNH$_2$-induced mutagenic activity, as well as absence of skin sensitization.

The pkCSM hepatotoxicity prediction is based on the human liver-related side effects of 531 different compounds. If the compound is related with at least one liver pathological or physiological event, the prediction returns positive result (Fourches et al. 2010; Pires et al. 2015b). Although the in silico study predicted hepatotoxicity for the BPP-BrachyNH$_2$, no alteration was observed in the in vivo serum biochemical parameters, such as AST, ALT, ALP, and GGT, as well as in the liver histopathological analysis (described below).

The blockage of the voltage-dependent ion channel encoded by hERG may affect the cardiac repolarization, leading to drug-induced QT interval prolongation, which is a critical side-effect of non-cardiovascular therapeutic agents (Vandenberg et al. 2012; Wang et al. 2013). The pkCSM prediction of hERG inhibition is based on two models. The hERG I model is based on the IC_{50} values of 368 compounds, and classify the compounds as strong inhibitors ($IC_{50} < 1$ - μM), and non-blockers exhibiting moderate (1–10 μM) or weak ($IC_{50} \geq 10$ μM) inhibition (Marchese Robinson et al. 2011). The hERG II model is based on the preliminary data of 806 compounds, also considering molecular parameters,

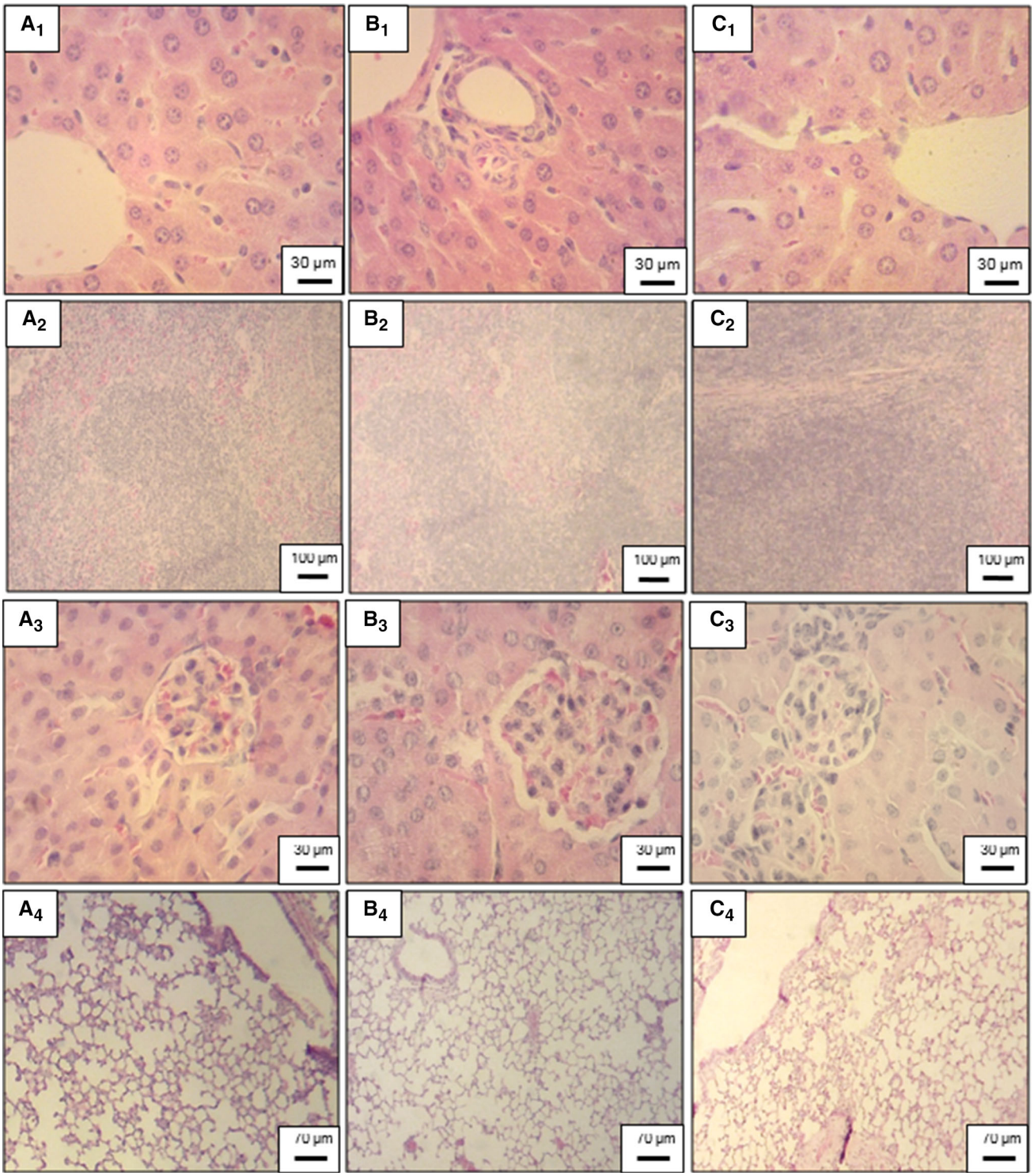

Fig. 5 Photomicrographies of histological sections obtained from the liver ($\mathbf{a}_1$, $\mathbf{b}_1$, $\mathbf{c}_1$), spleen ($\mathbf{a}_2$, $\mathbf{b}_2$, $\mathbf{c}_2$), kidney ($\mathbf{a}_3$, $\mathbf{b}_3$, $\mathbf{c}_3$) and lung ($\mathbf{a}_4$, $\mathbf{b}_4$, $\mathbf{c}_4$) of animals (n = 3) intraperitoneally treated with vehicle (**a**) or BPP-BrachyNH_2 at doses of 5.0 (**b**) and 50 (**c**) mg/kg. After 14 days, the animals were euthanized, and the organs were removed and processed to histopathological analysis. The results showed that the treatment with BPP-BrachyNH_2 did not show any changes compared with vehicle. Hematoxylin and eosin staining

such as solubility and MW (Wang et al. 2012). Our in silico results revealed a positive result for hERG II but not for the hERG I. Although these results suggest a possible BPP-BrachyNH_2-induced cardiotoxic effect, the differences between the applied models should be considered, as well as the different applied datasets in the same model could show

variability in the final results (Marchese Robinson et al. 2011).

In vitro cytotoxicity assays are essential in the early stages of the development of new drugs, because they are able to estimate the starting dose and the range of concentrations to be used in the in vivo non-clinical stages (OECD 2010). In this study, a decrease of cell viability induced by BPP-BrachyNH$_2$ in murine macrophages was not observed in MTT assay, suggesting the absence of cytotoxicity for this peptide.

Regarding the in vivo toxicological study, the BPP-BrachyNH$_2$ (5 and 50 mg/kg) did not cause any death after intraperitoneal administration in Swiss female mice, indicating absence of toxicity. Interestingly, in a previous study, captopril was not able to induce death up to the concentration of 2000 mg/kg, with LD_{50} of 3255 mg/kg in female Swiss mice (Imai et al. 1981). Therefore, captopril is also considered as a safe drug. Besides, during the first 24 h after treatment and throughout 14 days, no clinical or behavioral sign of toxicity was observed (Table 2). The general activity of mice was not altered during the period of study, demonstrating a good health condition.

The evaluation of the body weight curve of the animals is an important indicator for the toxicological assessment of a substance, generally related to alterations in the consumption of water and food (OECD 2002c). The acute administration of BPP-BrachyNH$_2$ did not promote alterations in the body weight curve of mice throughout the treatment period (Fig. 4). Besides, a previous study report that captopril up to 4000 mg/kg is not able to alters the body weight of female Swiss mice (Imai et al. 1981).

Furthermore, no marked alterations were observed in the serum biochemical parameters when compared with the reference ranges observed for animals from different standardized animal houses (Santos et al. 2010). However, a mild alteration of blood glucose levels was observed when compared with vehicle (Table 3), suggesting further studies for a possible BPP-BrachyNH$_2$-induced hypoglycemic effect in experimental models of diabetes, thereby enhancing the potential application of this peptide in the treatment of cardiovascular diseases (Arcanjo et al. 2015).

The evaluation of the organ weight is a useful screening tool in the characterization of effects related to general toxicity studies (Michael et al. 2007). After treatment with BPP-BrachyNH$_2$, no changes were also found in organ weights (Table 4). Besides, no significant change was observed in the gross pathological examination of the organs (lungs, heart, liver, spleen and kidneys), as well as morphological changes were not observed in the histological sections (Fig. 5).

Conclusions

The in silico toxicity prediction indicated a well-tolerated toxicological profile for the BPP-BrachyNH$_2$. Besides, this peptide did not presented any signal of in vivo acute toxicity after single dose intraperitoneal administration. This study indicates an acceptable safety profile for studies in animal experimental models focused on the investigation of the potential pharmacological, biotechnological and therapeutic applications of this peptide. In this sense, these findings reinforce further studies regarding the toxicological effects of in vivo chronical exposure to BPP-BrachyNH$_2$.

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Compliance with Ethical Standards

Conflict of interest JRSAL on behalf of all authors declare that this article content has no conflicts of interest.

Ethical Approval The experimental procedures were performed with approval by the Ethics Committee for Animal Experimentation from the Federal University of Piauí, Brazil (Permission No. 008/2012). The animals were handled and euthanized in accordance with Resolution No. 1000 (2012) of the Brazilian Federal Council of Veterinary Medicine, in order to minimize suffering.

Research Involving Human Participants This article does not contain any studies with human participants.

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