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Original Article

Isolation, functional, and partial biochemical characterization of galatrox, an acidic lectin from *Bothrops atrox* snake venom

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Snake venom lectins have been studied in regard to their chemical structure and biological functions. However, little is known about lectins isolated from *Bothrops atrox* snake venom. We report here the isolation and partial functional and biochemical characterization of an acidic glycan-binding protein called galatrox from this venom. This lectin was purified by affinity chromatography using a lactosyl-sepharose column, and its homogeneity and molecular mass were evaluated by high-performance liquid chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. The purified galatrox was homogeneous and characterized as an acidic protein (pI 5.2) with a monomeric and dimeric molecular mass of 16.2 and 32.5 kDa, respectively. Alignment of N-terminal and internal amino acid sequences of galatrox indicated that this protein exhibits high homology to other C-type snake venom lectins. Galatrox showed optimal hemagglutinating activity at a concentration of 100 μg/ml and this effect was drastically inhibited by lactose, ethylenediaminetetraacetic acid, and heating, which confirmed galatrox's lectin activity. While galatrox failed to induce the same level of paw edema or mast cell degranulation as *B. atrox* crude venom, galatrox did alter cellular viability, which suggested that galatrox might contribute to venom toxicity by directly inducing cell death.

Keywords C-type lectin; glycan-binding proteins; snake venom; hemmaglutinating activity

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Introduction

The snake genus *Bothrops*, which belongs to the family Viperidae, is distributed in Central and South America [1]. Envenomation by *Bothrops* spp. can result in severe local tissue damage, including myonecrosis, hemorrhage, and edema [2,3]. Importantly, life-threatening bleeding and coagulopathy can result in renal failure and shock [2,3].

Snake bites arouse a substantial concern for the Brazilian public health. *Bothrops* genus is responsible for high number of envenomation in Brazil. The mortality rate following *Bothrops* species envenomation has been estimated at 2.4%. Importantly, this mortality rate approaches nearly 8% in patients failing to receive medical attention [4]. In particular, the species *Bothrops atrox* can be found in the northern region of Brazil and is responsible for 90% of snakebites in humans in that region [1,5].

Bothrops venom appears to be composed of a variety of biological molecules, including proteins, enzymes, and peptides. Previous studies have suggested that several of these factors may be responsible for the clinical sequela associated with envenomation [3,6,7]. According to Neiva *et al*. [1], the *B. atrox* crude venom from northern Brazil contains several biologically active proteins, such as serine and metalloproteinases, phospholipases A2, L-amino acid oxidases, bradykinin-potentiating peptide, cysteine-rich protein, PL2 inhibitor, and svVEGF.

In addition to enzymes, lipids, and other mediators, lectins, also known as glycan-binding proteins (GBPs), are also present in snake venom. GBPs are proteins that bind

specifically and reversibly to carbohydrates. The sugar-binding activity of these proteins is often located within a distinct polypeptide region designated as the carbohydrate recognition domain (CRD) [8,9]. GBPs are expressed by all animals, plants, fungi, microbes, and most viruses, and participate in diverse biological processes including embryogenesis, development, cell differentiation, migration, activation, apoptosis, regulation of immune response, cancer, immune tolerance, and others [10–12]. Based on the sequence and structural homology of their CRDs, animal lectins are grouped into different families, which include galectins and C-type, I-type, M-type, L-type, P-type, R-type, etc., along with the glycosaminoglycan-binding (GAG-type) proteins [10–13].

Several snake venom lectins have been isolated from different genera, including *Agkistrodon*, *Bitis*, *Bothrops*, *Bugarus*, *Crotalus*, *Dendroaspis*, *Lachesis*, and *Trimeresurus*, and their structures and functions have been characterized [14–29].

In the *Bothrops* genus, different lectins have been purified and characterized. These proteins belong to the C-type (calcium-dependent) lectin family, which have dimeric disulfide-linked subunits with a subunit molecular mass of ~14 kDa and an affinity for β-galactoside residues. The biological activities promoted by these lectins includes hemagglutination, mitogenic activity, formation of paw edema, induction of platelet aggregation, increase in the vascular permeability, renal effects, hypotension, and cytotoxicity toward cell lines [14,15,19,22,29–32].

Gartner *et al*. [14] isolated the first snake venom lectin from *B. atrox* crude venom, called thrombolectin. It was characterized as a disulfide-linked homodimer with an apparent molecular mass of ~28 kDa. The isoelectric point (pI) analysis of the isolated lectin showed a heterogeneous pI of 6.4 and a triplet from 9.5 to 9.7 [14,15]. Thrombolectin promoted hemagglutination in a calcium- and carbohydrate-dependent manner, induced platelet aggregation, and did not have mitogenic activity [15,30,33]. However, the N-terminal amino acid sequence and internal peptide sequence of thrombolectin remained unknown.

In the present study, we aimed to extend the lectin studies on *B. atrox* venom. Therefore, we isolated an acidic galactoside-binding C-type lectin (galatrox) from the venom of *B. atrox*, a species distributed in northern Brazil, and described its biological and biochemical properties, including partial primary structure. This lectin, galatrox, although a potential isoform of thrombolectin, was unique from the previously characterized C-type lectin from *B. atrox*. The results will provide important data for investigating the role of envenomation caused by *B. atrox*, and suggest the potential use of this lectin for general biomedical research.

Materials and Methods

Materials

The crude venom from *B. atrox* was obtained from Serpentarium SANMARU (São Paulo, Brazil) and Institute Butantan (São Paulo, Brazil). The *B. atrox* snake venom used in this study originated from northern Brazil (state of Pará). All chemicals used were of analytical grade.

Animals

Male BALB/c specific pathogen-free mice weighing 18–22 g were provided by the Animal Facilities of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo. Animal care procedures were performed according to COBEA (Brazilian College of Animal Experimentation) guidelines and the experimental protocols approved by the Committee for Ethics on Animal Use (CEUA) from University of São Paulo, USP (Protocol number: 05.1.822.53.8).

Purification of *B. atrox* venom lectin (galatrox)

Crude venom (150 mg) was suspended in phosphate-buffered saline (PBS, pH 7.4) and centrifuged (2500 *g*, 10 min, 25°C) to remove insoluble materials. Then the supernatant was loaded onto a 5-ml bed of lactosyl-sepharose column (Sigma, St. Louis, USA). The column was washed with PBS to elute the unbound material, which we called Lac−. The bound material (Lac+, containing galatrox) was eluted with PBS supplemented with lactose (100 mM). Fractions of 1 ml were collected. A PD-10 column (GE Healthcare, Life Sciences, Little Chalfont, UK) equilibrated in PBS was used to remove lactose from the galatrox preparations. The homogeneity of lactose-free galatrox samples was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and high-performance liquid chromatography (HPLC). This chromatographic procedure was performed using a reverse-phase column ($4.6 \times 150\ mm^2$ ODP 50, Shodex), previously equilibrated with solution A (0.1% trifluoroacetic acid). The sample was eluted with a linear gradient 0–80% of solution B [acetonitrile (ACN) 60%, 0.1% trifluoroacetic acid] at a flow rate of 0.8 ml/min. All chromatographic procedures were monitored by absorbance at 280 nm. The protein concentrations were determined using a microassay based on the Bradford dye-binding procedure [34].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS–PAGE was performed on 12% gels using a Mini V-8.10 Vertical Gel Electrophoresis System (Gibco BRL, Gaithersburg, USA) [35]. Samples were dissolved in

sample buffer containing SDS in the presence or absence of 2-mercaptoethanol (2-ME). After running (80–120 mA, 200 V), the gels were stained with Coomassie brilliant blue G250. Proteins with known molecular mass were used as standards (Amersham Pharmacia Biotech, Little Chalfont, UK).

Isoeletric focusing

The pI of the purified lectin was determined by isoelectric focusing as described by Arantes *et al*. [36]. Briefly, the isoelectric focusing was carried out on sheets of 7% polyacrylamide gel containing BioRad (Hercules, USA) carrier ampholytes (pH 3–14). After prefocusing (30 min, setting: 100 V, 30 mA, 5 W), the samples were applied as drops of liquid on the surface of the gel. The isoelectric focusing was performed at 100–1500 V for ~4 h (setting: 1500 V, 30 mA, 5 W). Focusing was completed when the voltage reached 1500 V and the current was 2 mA or less. As soon as the current was switched off, the pH gradient was determined. Strips of the gel (1 × 2 cm) were cut along the gel sides, immersed individually in 0.5 ml of Milli-Q water for 2 h, and measured for their pH. The remained gel containing the proteins was stained with silver nitrate.

Matrix-assisted laser desorption/ ionization-time-of-flight-mass spectrometry

The molecular mass of galatrox was determined by matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS). MS analysis was performed in MALDI-TOF-MS (Axima Performance; Kratos-Shimadzu, Manchester, UK) linear mode previously calibrated with insulin, cytochrome c, aldolase and bovine serum albumin (BSA). Sample was mixed (1:1) with α-cyano-4-hydroxycinnamic matrix and applied on MALDI target plate by the dried-droplet method. The mass spectrum was collected with an average of 100 scans, 10 laser shots/scan.

In situ gel trypsin digestion and MS analysis

The protein separated by SDS–PAGE was subject to *in situ* gel band, then digested with 0.5 μg of modified trypsin (Promega Co., Madison, USA) as described by Williams and Stone [37]. The tryptic peptides were desalted in a micro tip filled with POROS R2 (Perseptive Biosystems, Foster City, USA) and eluted in 60% methanol in 5% formic acid for MS analysis. The trypsin digestion of protein was dried and re-dissolved in 5 μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid, then 2 μl was applied to the MALDI target using the dried-droplet method, followed by analyzing by MALDI-TOF-MS (MALDI micro MX; Waters, Manchester, UK), which was calibrated with a mixture of angiotensin II, renin, and adrenocorticotropic hormone fragment 17–39 (ACTH 17–39) (mass accuracy <50 ppm). The MS analysis of the tryptic peptides was also carried out in an electrospray triple-quadrupole mass spectrometer Quatro II (Micromass, Manchester, UK) by direct infusion (300 nl/min) under the following conditions: capillary voltage was maintained at 2.8 kV, cone voltage at 40 V, and cone temperature was set to 100°C. The parameters for MS1 precursor ion scanning and production scanning were optimized with synthetic peptide, angiotensin II (Peptide Synthesis Laboratory, UNIFESP, São Paulo, Brazil) for the highest signal-to-noise ratio and MS was calibrated with PEG (50–2000 amu). In the production scanning mode–collision-induced dissociation mass spectrometry (CID-MS/MS), the collision energy was set to 25–35 eV and argon was used as the collision gas with a partial pressure of 3.0×10^{-3} mTorr. The spectrum was collected with an average of 20–50 scans (2–5 s/scan) and processed by the MassLynx software v.3.3 (Micromass). The amino acid sequence of the tryptic peptides was deduced from the series of *b* and *y* ion fragments produced by CID-MS/MS.

N-terminal amino acid sequencing

The elution peak from reverse-phase HPLC containing purified galatrox was lyophilized and submitted to Edman degradation. N-terminal amino acid sequencing of this lectin was performed using a PPSQ-33A automatic sequencer (Shimadzu, Kyoto, Japan). Phenylthiohydantoin derivatives of amino acids were identified by comparing their retention times with the 20 PTH–amino acid standard mixture using an online reverse-phase HPLC.

Amino acid sequence alignment

The amino acid sequences (N-terminal, residues 1–54; tryptic peptide 1, residues 62–73; and tryptic peptide 2, residues 75–84) of the galatrox were aligned with other snake venom C-type lectins using the ClustalX software (version 2.0) [38]. The alignments were based on the similarities between amino acids found in the primary sequence, taking into account conservative exchanges and the position of peptides containing the amino acid sequences of the positions N-terminal to C-terminal to the primary structures aligned.

Hemagglutinating activity assay

To further examine the lectin activity of galatrox, we examined the agglutination potential of this protein. Initially, human blood (ABO-H blood group) was collected with heparin, and the erythrocytes were washed by centrifugation (300 *g*, 10 min) with PBS. The hemagglutination was determined based on the method described by Nowak *et al*. [39]. Assays were performed using microtiter V-well plates with several concentrations of galatrox (0.25–

300 μg/ml). Each well contained 50 μl of 4% suspension of human trypsinized erythrocytes in PBS (pH 7.4), plus 1% (w/v) of BSA [40]. The negative control contained 50 μl of cell suspension and 50 μl of PBS. Following the addition of erythrocytes, the plates were shaken briefly and incubated at room temperature (~25°C) for 2 h. In order to evaluate sugar specificity, galatrox (100 μg/ml) was incubated for 2 h at 25°C with α-lactose, L-(−)-fucose, α-sucrose, D-(+)-mannose, D-(+)-galactose, and D-(+)-rhaminose. All sugars were tested in the same concentration range (1–300 mM). To examine the Ca^{2+} requirements and thermal stability, galatrox (15 μg/ml) was incubated with ethylenediaminetetraacetic acid (EDTA, 5 mM) or heated at 100°C for 15 min, respectively. The formation of an agglutinated erythrocyte mantle was assessed by visual analysis and the results were expressed as hemagglutination arbitrary units (HAUs). One unit was defined as the minimum detectable mantle formation.

Edema-inducing activity assay

To evaluate potential biological properties of galatrox, we next determined whether galatrox could induce paw edema, a previously recognized activity of *B. atrox* venom. The edematogenic effect of galatrox was assessed based on the method described by Levy [41]. Crude venom solution (10 mg/ml), galatrox (0.75 mg/ml), or PBS alone was injected (50 μl) in the subplantar region of three groups of five BALB/c male mice (18–22 g). The contralateral paw received the same volume of PBS. The progression of edema was evaluated with a low-pressure pachymeter (Mitutoyo Co., Tokyo, Japan) at the intervals of 0, 0.5, 1, 2, 4, 12, 24, 48, and 72 h after injection. Edema-inducing activity was expressed as the percentage increase in paw thickness compared with the contralateral paw.

β-Hexosaminidase release assay

Mast cell degranulation was assessed by a β-hexosaminidase release assay using RBL-2H3 cells, a rat mast cell line, as described by Pierini *et al*. [42] and Hoffmann *et al*. [43]. Briefly, RBL-2H3 were maintained as monolayers in microtiter wells containing Dulbecco's modified Eagle's medium, 15% fetal bovine serum, 0.434 mg/ml glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The RBL-2H3 cells were sensitized with IgE anti-DNP (500 ng/ml, Sigma) overnight in a 96-well microplate. After sensitization, the cells were washed twice with PBS–BSA (1 mg/ml, pH 7.4) and then treated with *B. atrox* crude venom or with galatrox at 2.5, 5, and 10 μg/ml for 45 min at 37°C. The positive control was cell stimulated with dinitrophenylated-human serum albumin antigen (50 ng/ml, Sigma-Aldrich). To determine the amount of β-hexosaminidase activity released by the cells, 25 μl of supernatant and 150 μl of 8 mM β-hexosaminidase substrate (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide), in 0.1 M citrate buffer (pH 4.5), were mixed in separate microplates and incubated for 30 min at 37°C. The reaction was quenched by addition of 50 μl of 0.2 M glycine, pH 10. PBS-treated-sensitized RBL-2H3 cells were used to measure spontaneous release of β-hexosaminidase. The total enzyme release was obtained by lysing the cells with 1% Triton-X 100 prior to the removal of the supernatant. The release of β-hexosaminidase was determined by measuring the absorbance of the enzyme reaction product at 405 nm in a microwell plate reader (Molecular Devices, Spectra MAX PLUS) and comparing with the total product associated with cells lysed in 1.0% Triton X-100.

MTT assay

The human cell line HL-60 was obtained from the American Type Culture Collection. Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously [44]. At the end of the 24 h treatment of HL-60 cells with galatrox, MTT (Sigma-Aldrich) solution was added to the culture medium (500 μg/ml final concentration) and the cells were incubated for an additional 4 h. The reaction was stopped by the addition of 100 μl of dimethyl sulfoxide to the cell culture. HL-60 cells were treated with different concentrations of galatrox (100, 125, 150, 175, 200, 225, and 250 μg/ml). Untreated cells were used as a negative control and cyclophosphamide was used as a positive control. MTT reduction was detected by measuring the absorbance at 570 nm. The results were expressed as percentage of MTT reduction activity by cells as an indication of cell viability.

Statistical analysis

Statistical analysis was performed with the Student's *t*-test. $P < 0.05$ was considered statistically significant. Results were expressed as mean ± SEM.

Results

Isolation and purification of galatrox

The purification of galatrox was successfully carried out in one chromatographic step. Galatrox was purified from *B. atrox* crude venom by affinity chromatography on a lactosyl-sepharose column [**Fig. 1(A)**]. The bound material (Lac+), containing galatrox, was submitted to a desalting PD-10 column to remove lactose. The high level of homogeneity of the galatrox preparation was confirmed by HPLC, which showed a single peak [**Fig. 1(B)**]. The SDS–PAGE analysis of the purified galatrox demonstrated a band at ~29 kDa, and when incubated with reducing agents the molecular mass reduced to ~15 kDa, which suggested that this lectin existed as a dimer mediated by

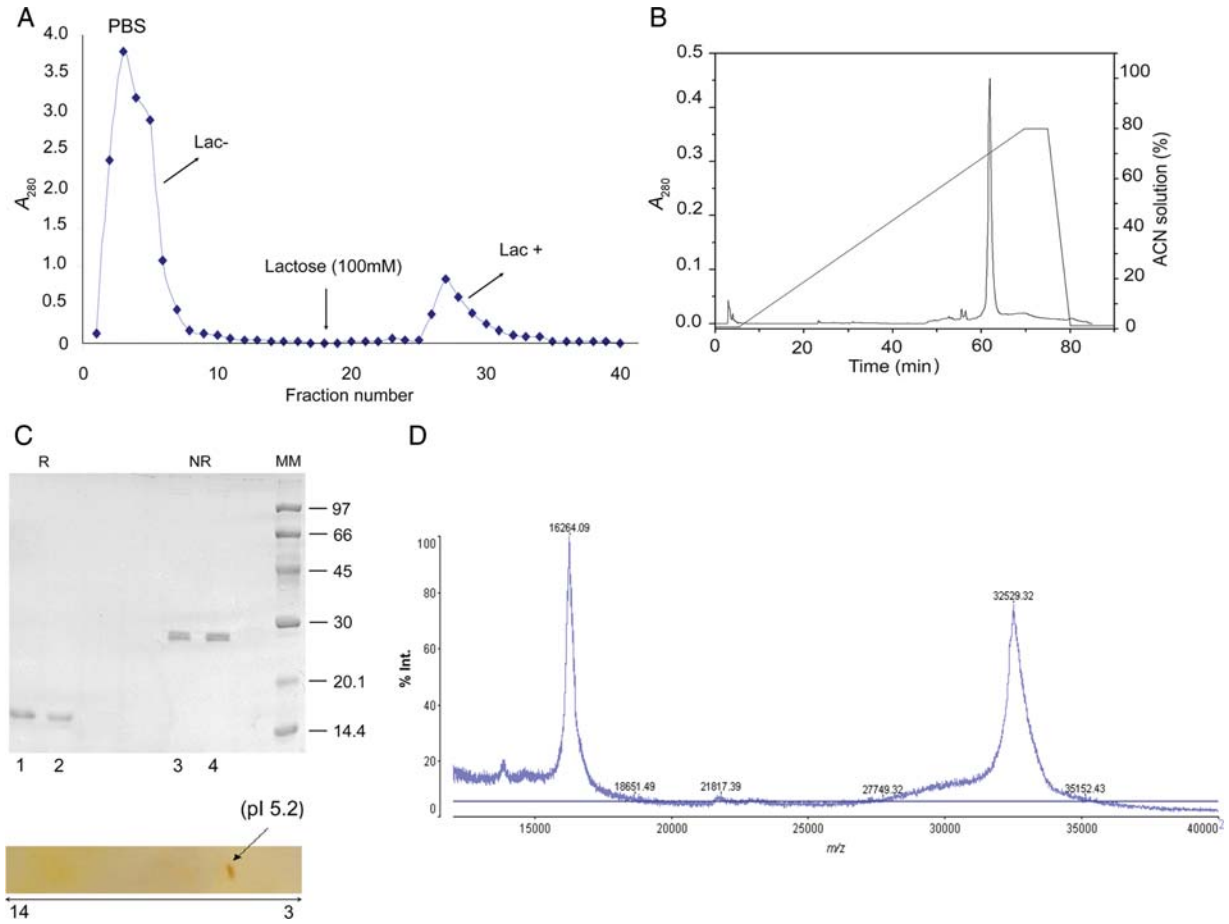

Figure 1 Purification of galatrox from *Bothrops atrox* crude venom (A) Lactosyl-sepharose affinity chromatography column. The unbound material (Lac−) was removed with PBS and the lactose-bound material (Lac+) was eluted with PBS plus lactose (100 mM). The pI of galatrox was determined by isoelectric focusing. (B) Purified galatrox sample was submitted to a HPLC procedure using a reverse-phase column, with an elution linear gradient 0–80% of ACN solution at a flow rate of 0.8 ml/min. All chromatographic procedures were monitored by absorbance at 280 nm. (C) SDS–PAGE analysis of the Lac+ material in reducing (R: lanes 1 and 2) or non-reducing (NR: lanes 3 and 4) conditions. The mass of molecular markers (MM) is indicated in the figure and expressed in kDa. (D) Mass spectrometry analysis of galatrox performed in MALDI-TOF-MS.

disulfide bond formation [**Fig. 1(C)**]. The isoeletric focusing analysis showed that galatrox has a pI value of 5.2 [**Fig. 1(C)**]. The MALDI-TOF mass spectrum analysis of the purified galatrox preparation showed molecular mass monomer of 16264.09 Da and dimer of 32529.32 Da [**Fig. 1(D)**].

Primary structural characterization of galatrox

The N-terminal amino acid sequence of galatrox was obtained by automatic Edman degradation. The N-terminal 54 residues were determined to be NNCPQDWLPMNG LCYKIFDELKAWKDAEMFCRKYKPGCHLASIILYGES PEWAE. In addition, *in situ* trypsin gel digestion of the ~15 kDa band was analyzed by MALDI-TOF-MS and ESI-CID-MS/MS, which resulted in double-protonated ions, *m/z* 1369.710 and *m/z* 1426.875 [**Fig. 2(A)**] whose amino acid sequences were KDFSWEWTDR and GHSEVWLGLWDK, respectively, internal fragment of the primary structure of galatrox [**Fig. 2(B)** and **(C)**]. The two major ions peptide fragments, *m/z* 1369.710 (KDFSWEWTDR) and *m/z* 1241.662 (DFSWEWTDR), detected by mass spectrometry correspond to the same region of galatrox in which one missed trypsin cleavage occurred [**Fig. 2(A)**]. The alignment showed that galatrox N-terminal residues presented high homology to other *Bothrops* spp. lectins (BiL: 95% and BJcuL: 97% identity). Also, in the tryptic peptide 2, the identity was 100% when compared with the C-type lectins from *Bitis*, *Crotalus*, *Bothrops*, and *Trimeresurus*. Particular amino acids were highly conserved (**Fig. 3**), such as cysteine residues involved in disulfide bond formation.

Galatrox-induced agglutination of human erythrocytes

Purified galatrox agglutinated all types of trypsinized human erythrocytes (A^+, B^+, AB^+, and O^+). However, erythrocytes isolated from blood group AB^+ individuals demonstrated the most sensitivity to galatrox-induced agglutination (data not shown). Maximum agglutination occurred at concentrations of nearly 100 μg/ml using AB^+ type group [**Fig. 4(A)**]. To examine the monosaccharide

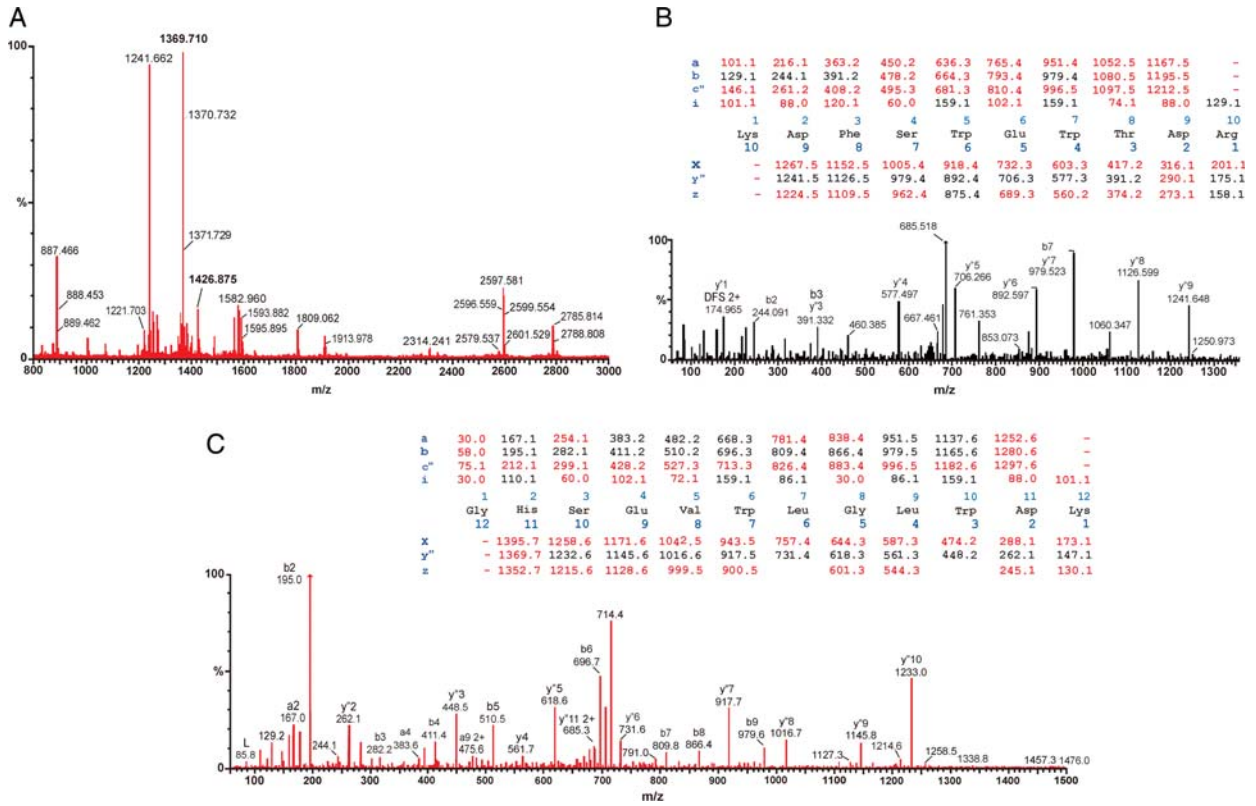

Figure 2 Peptide mass analysis (A) MALDI-TOF-MS peptide mass fingerprint obtained by *in situ* trypsin gel digestion of the ~15 kDa band protein (galatrox). ESI-CID-MS/MS of double protonated ions of (B) *m/z* 685 (1369.710)—KDFSWEWTDR and (C) *m/z* 713 (1426.875)—GHSEVWLGLWDK. The two major ion peptide fragments showed in the figure (1241.662 and 1369.710) represent the same amino acid sequence. The mass difference of 128.048 corresponds to one missed trypsin cleavage (extra lysine residue).

specificity of galatrox, the relative ability of different carbohydrates to inhibit galatrox-induced agglutination was determined. Galatrox exhibited differential sensitivity to inhibition by different carbohydrates with α-lactose displaying the most potent ability to block agglutination [**Fig. 4(B)**]. In addition, the inclusion of EDTA (5 mM) or pre-treatment with heat also prevented galatrox hemagglutination activity [**Fig. 4(C)**]. Importantly, the unbound protein fraction (Lac−) was devoid of hemagglutination activity; however, several concentrations of *B. atrox* crude venom demonstrated this biological property (data not shown).

The lectin from *B. atrox* did not induce paw edema and mast cell degranulation

To accomplish this, galatrox (37.5 μg/mice paw) was injected, following the evaluation of potential paw edema. Although crude *B. atrox* venom induced significant edema, galatrox failed to induce similar changes (**Fig. 5**). Consistent with this, RBL-2H3 mast cells treated with galatrox (2.5–10 μg/ml) showed similar levels of β-hexaminidase release, a surrogate for mast cell activation, compared with negative control (**Fig. 6**). In contrast, RBL-2H3 cells treated with crude venom (2.5–10 μg/ml) released significant levels of β-hexaminidase (**Fig. 6**). Taken together, these results suggest that the ability of crude venom to induce edema likely reflects factors that occur in addition to or independent of galatrox for effective edema formation.

Galatrox was cytotoxic to HL-60 cells

Although galatrox failed to alter paw edema formation or mast cell degranulation, *B. atrox* crude venom also induced severe cell death. We next hypothesized that galatrox might contribute to *B. atrox* crude venom toxicity by inducing cell death. To test this, we examined the potential ability of galatrox to alter the viability of HL-60 cells. Importantly, galatrox induced significant cell death in HL60 cells in a dose-dependent manner, with an IC_{50} close to 250 μg/ml, causing 58% ± 1.4% cell death after 24 h of treatment ($P < 0.05$). However, the maximum cytotoxic effect of galatrox was lower than the positive control (**Fig. 7**). These results demonstrated that galatrox can significantly alter cellular viability and suggest that galatrox may contribute to the *B. atrox* venom-induced pathophysiology by inducing cell death.

Discussion

The evolution of significant number of venomous snakes in the world resulted in a wide variety of venoms, many of

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                -23                    +1                                    37
galatrox          ----------------------NNCPQDWLPMNGLCYKIFDELKAWKDAEMFCRKYKPG
BiL               MGRFIFVSFGLLVVFLSLSGAKGNNCPQDWLPMNGLCYKIFDELKAWKDAEMFCRKYKPG
BJcuL             ----------------------NNCPQDWLPMNGLCYKIFDELKAWKDAEMFCRKYKPG
PAL               ----------------------NNCPPDWLPMNGLCYKIFDELKAWEDAERFCRKYKPG
RSL               ----------------------NNCPLDWLPMNGLCYKIFNQLKTWEDAEMFCRKYKPG
CrL               ----------------------NNCPLDWLPMNGLCYKIFNQLKTWEDAEMFCRKYKPG
BPL               ----------------------NNCPGDWLPMNGLCYKIFNELKAWEDAEMFCRKYKPG
TSL               MGRFIFVSFGLLVVFLSLSGAKGSCCTNDSLPMNGMCYKIFDEPKTWEDAEMFCRKYKPG
                                        . *. * *****:*****:: *:*:*** ********
                                          Peptide-1    Peptide-2
                38                54      62        73 75       84            97
galatrox          CHLASIILYGESPEWAE------ GHSEVWLGLWDK-KDFSWEWTDR------------
BiL               CHLASFHLYGESPEIAEYISDYHKGQSEVWIGLWDKKKDFSWEWTDRSCTDYLSWDKNQP
BJcuL             CHLASIHLYGESPEIAEYISDYHKGQAEVWIGLQDKKKDFSWEWTDRSCTDYLSWDKNQP
PAL               CHLASFHQYGESLEIAEYISDYHKGQAEVWIGLWDKKKDFSWEWTDRSCTDYLTWDKNQP
RSL               CHLASFHRYGESLEIAEYISDYHKGQENVWIGLRDKKKDFSWEWTDRSCTDYLTWDKNQP
CrL               CHLASFHLYGESLEIAEYISDYHKGQDNVWIGLWDKKKDFSWEWTDRSCIDYLNWNKNQP
BPL               CHLASIHIYGESLEIAEYISDYHKGQAEVWIGLWDKKKDFSWEWTDRSCTDYLSWDKNQP
TSL               CHLASFHRLAESLDIAEYISDYHKRQAEVWIGLLDRKKDFSWEWTDRSCTDYLNWDKNQP
                  *****:   .** : **        : :**:** *: **********
                                                                Identity (%)
                98                                      135   N-terminal   Peptide 1   Peptide 2
galatrox          ---------------------------------------
BiL               DHYQNKEFCVELVSDTGYRLWNDQVCESKNAFLCQCKF        95           84          100
BJcuL             DHYQNKEFCVELVSLTGYRLWEDQVCESKNAFLCQCKF        97           67          100
PAL               DHYQNKEFCVELVSLTGYRLWNDQVCGSKNAFLCQCKF        86           75          100
RSL               DHYQNKEFCVELVSLTGYRLWNDQVCESKDAFLCQCKF        82           59          100
CrL               DHYKNKEFCVELVSLSGYRLWNDQVCESKDAFLCQCKF        88           67          100
BPL               DHYENKEFCVELVSLTGYRLWEDQVCESKNAFLCQCKF        84           75          100
TSL               DHYKDKEFCVELVSLTGYHRWNDQVCESKNSFLCQCKF        72           60          100
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Figure 3 Comparison of the amino acid sequence of galatrox with other snake venom galactoside-binding lectins using ClustalX version 2.0 Conserved cysteine residues are denoted in bold '**C**' letters. Gaps are indicated by '−', while '*' indicates the positions which have a single, fully conserved residue, ':' indicates related groups that are strongly conserved, and '.' indicates that related groups with weaker conservation. The N-terminus of the native lectins is located at the position +1 and recombinant lectins at the position −23. The internal tryptic peptides derived from galatrox are indicated in the boxes and positioned based on the amino acid sequence of the following lectins: *B. insularis* lectin (BIL—gi:82126834) [21]; *B. jararacussu* lectin (BJcuL—gi:34922459) [49]; *Bitis arietans* lectin (PAL—gi:34922645) [16]; *Crotalus atrox* lectin (RSL—gi:126130) [70]; *Crotalus ruber* lectin (CRL—gi:118572769) [46]; *Bothrops pirajai* lectin (BPL) [22]; *Trimeresus stejnegeri* lectin (TSL—gi:432509) [71]. The percentage of amino acid residue identity was given by aligning each sequence (N-terminal, Peptide 1 and Peptide 2) individually.

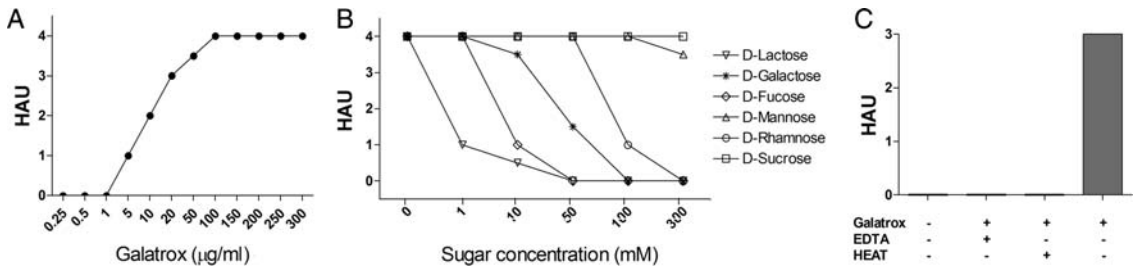

Figure 4 Evaluation of the hemagglutination activity of galatrox (A) Galatrox promoted the hemagglutination of type AB^+ human erythrocytes. (B) Galatrox (100 μg/ml) was tested in the presence or absence of α-lactose, L-(−)-fucose, α-sucrose, D-(+)-mannose, D-(+)-galactose and D-(+)-rhaminose). (C) Galatrox (15 μg/ml) was tested in the presence of EDTA or after heating. PBS was used as negative control (NC). The results were expressed as HAUs (see Materials and Methods section).

which contain highly active toxins composed of a wide range of chemical components, ranging from small molecules to large proteins. These toxins can vary considerably in structure and function among individuals, species, genera, or families [45].

The present work describes the purification and partial biochemical and biological characterization of galatrox, an acidic galactoside-binding C-type lectin from *B. atrox* snake venom. Galatrox was purified by a procedure similar to most galactoside-binding lectins from animal and plant sources, using a single chromatographic affinity step on a lactosyl-sepharose column. The purified galatrox had a high level of homogeneity [**Fig. 1(A)** and **(B)**] [15, 21, 46–49].

The SDS–PAGE analysis indicated that galatrox was a disulfide-linked homodimeric protein with a molecular mass of ~30 kDa under non-reducing conditions, while it showed monomer with an apparent molecular mass of ~15 kDa under reducing conditions [**Fig. 1(C)**]. The selective reaction of 2-ME (reductor agent) to sulfhydryl groups of cysteine residues strongly suggested that monomer formation following reduction likely reflected the cleavage of

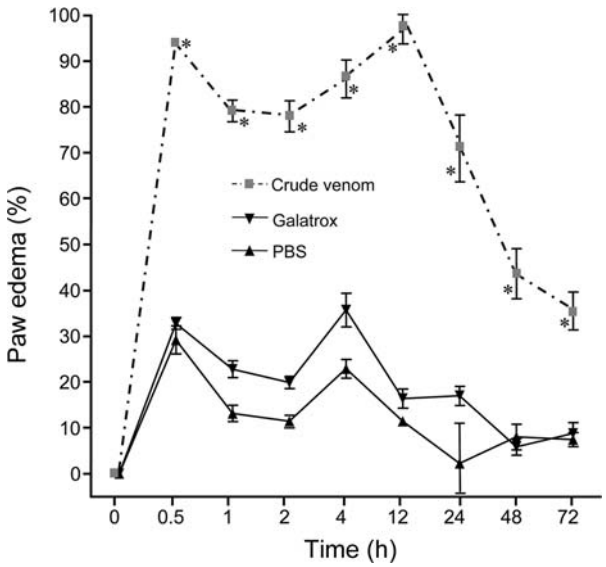

Figure 5 Edematogenic activity in the mice paw Fifty microliters of galatrox (37.5 μg/paw) were injected in the mouse hind paw. PBS was used as negative control and crude venom was used as positive control. The progression of edema was evaluated with a low-pressure pachymeter at 0, 0.5, 1, 2, 4, 12, 24, 48 and 72 h after injection. The results are expressed as the percentage increase in paw thickness compared with the contralateral paw. Values are given as mean ± SEM. $*P < 0.05$ compared with negative control (PBS).

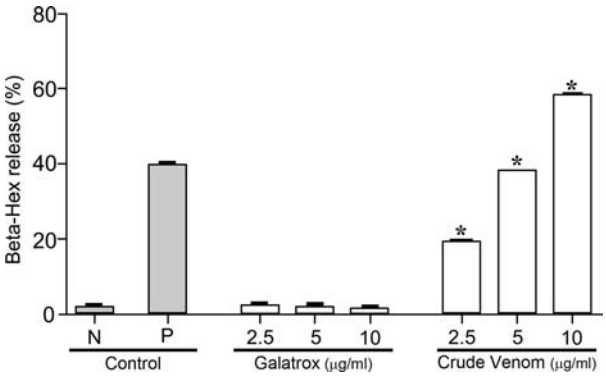

Figure 6 Effects of galatrox treatment on the degranulation of RBL-2H3 cells RBL-2H3 cells sensitized with IgE anti-TNP were stimulated with crude venom or galatrox at the following concentrations (μg/ml): 2.5, 5, and 10. Sensitized RBL-2H3 cells treated with PBS or DNP-HAS antigen were used as negative (N) or positive (P) control, respectively. The β-hexosaminidase activity released was expressed by percentage of total enzyme activity obtained from Triton X-100 cell lysed (see Materials and Methods section). The results are shown as mean ± SEM and are representative of three independent experiments. $*P < 0.0001$ compared with negative control.

disulfide bonds responsible for dimer formation. However, other conformational changes following reduction could account for the prevalence of monomers following reduction.

Previous studies demonstrated that the venom lectins isolated from *B. atrox* (thrombolectin), *Bothrops godmani*, *Bothrops jararacussu*, *Bothrops pirarai*, and *Bothrops*

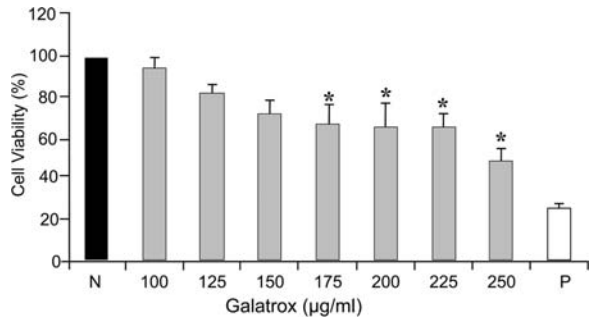

Figure 7 Evaluation of the cytotoxic effect of galatrox The HL-60 cell line was treated with different concentrations of galatrox: 100, 125, 150, 175, 200, 225, 250 (μg/ml) for 24 h. Untreated cells were used as negative (N) control and cyclophosphamide treated cells were used as a positive (P) control. The results were expressed as percentage of cell viability. Values are given as mean ± SEM of the three independent experiments. $*P < 0.05$ compared with negative control.

insularis were partially characterized in regard to their biochemical functions. All venom lectins display a common SDS–PAGE profile with a single band of ~14 and ~28 kDa in reductive and non-reductive conditions, respectively [14,18,19,22,29,32], suggesting lectins are generally conserved among these species. Interestingly, similar to galatrox, the mammalian β-galactoside-binding lectin, galectin-1, is also homodimeric protein with non-covalently linked ~14 kDa monomers [50,51].

The yield of galatrox from crude venom fractionation was ~1.0% of the total protein (data not shown). Generally, snake venom C-type lectins are present in a low amount in crude venoms. The yield of *Lachesis muta* lectin is 0.89% [24], BiL (a lectin from *B. insularis*) 0.5% [21], thrombolectin (*B. atrox*) 1.22% [15], and *B. moojeni* lectin 0.52% [52]. *Bothrops newiedi* lectin presented the highest yield from crude venom (2.48%) [52].

Galatrox presented an acidic pI of 5.2 [**Fig. 1(B)**], which was different from thrombolectin, another lectin isolated from *B. atrox*. The pI of the lectin presented with a single band of 6.4 and a triplet of 9.5–9.7 [15]. This difference may be attributed to the ontogenetic characteristics related to the process of species adaptation. Daltry *et al*. [45] demonstrated that a large intraspecies variation existed in snake venom composition using an isoelectric focusing approach. Interestingly, this variation could be associated with geography and diet.

Amino acid sequencing of galatrox provided a total of 76 residues, 54 from N-terminal determination and 22 from two internal tryptic digestion peptides deduced from their CID-MS/MS fragmentation spectra (**Fig. 2**). Multiple alignments with other galactoside-binding snake venom lectins provided a theoretical positioning of the lectin internal peptides and the composition of its primary sequence (**Fig. 3**). The present data indicated that galatrox shares characteristic amino acid sequences

and a high conservation of cysteine residues with other C-type lectin family members [53].

Núñez *et al*. [54] presented a comparative analysis of the proteomes of the venoms of *B. atrox* specimens from different regions in South America, including Columbia. Among the several proteins studied, a galactose-specific lectin with apparent molecular mass of 14/28 kDa was described. Although no lectin activity or carbohydrate specificity was described for this protein, the N-terminal sequence of 16 amino acids of this protein was identical (100%) to a lactose-binding lectin (BJcuL) isolated from the venom of *B. jararacussu* [49] and galatrox. However, in contrast to our results, Núñez *et al*. [54] did not detect a protein with the same N-terminal sequence in the venom of adult animals of *B. atrox* from Brazil. In order to perform a comparative analysis of crude venom composition by HPLC and SDS–PAGE, we submitted *B. atrox* crude venom derived from northern Brazil (state of Pará), used by our group, to purify galatrox to the same reverse-phase HPLC procedure described by Núñez *et al*. [54] (Supplementary Fig. S1). This approach demonstrated that the galatrox elution time is similar to the galactose-specific lectin described to crude venom of *B. atrox* from Colombia [54], suggesting a similar lectin or isoform of the previously described protein. Interestingly, also in contrast to Núñez *et al.* [54], others authors [1], using a transcriptome analysis, identified the open-reading frame of mRNA of a lactose-specific lectin (C-type lectin) from a cDNA library of the venom gland derived from a sub-adult *B. atrox* specimen from northern of Brazil (Amazonas region). These studies in association with our findings indicated that crude venom of *B. atrox* from northern Brazil could contain a lectin. Based on these considerations, we suggest that differences between our results and previous studies may reflect the age of the animals, gender and geographical variations in the locations from which the snakes were captured [1,54]. Full sequence analyses of the proteins isolated from each group are needed to determine the exact similarities and/or differences between galatrox and other similar previously described proteins.

The β-galactoside-binding lectins have been found in a variety of plants and vertebrate organisms, including snake venom and typically calcium ions are essential to their agglutination activity [8,10,11]. The evaluation of galatrox-induced hemagglutination of type AB^+ human erythrocytes showed that this effect was inhibited preferentially by α-lactose [**Fig. 4(B)**]. Interestingly, L-(−)-fucose displayed more inhibition than D-(+)-galactose, despite the fact that fucose is not a component of lactose (galactose β1–4 glucose). These results coupled with the ability of galatrox to recognize AB^+ erythrotcytes suggested that galatrox can bind cell surface glycoconjugates of human erythrocytes that presents fucosylated glycans with terminal galactose residues, such as blood group antigens. The ability of heat and EDTA to inhibit galatrox-induced agglutination demonstrates a requirement for the native intact protein and a requirement for divalent cations in lectin binding [**Fig. 4(C)**]. In addition, whereas crude venom agglutinated erythrocytes, the Lac fraction did not demonstrate the same activity, indicating that the major component of this venom responsible for this lectin activity was fully adsorbed to the lactosyl-sepharose matrix (data not shown). Therefore, these results suggested that galatrox belongs to the snake venom C-type galactoside-binding lectin group due to its ability to exert carbohydrate recognition activity by binding to galactoside residues in a Ca^{2+}-dependent manner [53].

Galatrox apparently has no significant effect on plasma coagulation *in vitro* nor does it induce platelet aggregation (data not shown). In contrast, lactose-specific lectins from *Lachesis muta*, *Ancistrodon piscivorous leukostoma*, and *Crotalus atrox* stimulated the aggregation of human platelets at 4–76 μg/ml and this effect was inhibited by lactose [33]. However, in the same experiments, thrombolectin occasionally caused aggregation [33]. A C-type lectin purified from the snake venom of *Crotalus ruber* did not induce platelet aggregation even at higher concentrations (73 μg/ml) [33,46]. Thus, the capacity to induce platelet aggregation apparently varies among snake venom lectins due to structural variations [29].

Prominent local edema is a common clinical finding in victims bitten by *Bothrops* sp. This response is mediated by myotoxic phospholipases A2 [55] and metalloproteinases [56]. The contribution of snake venom lectins to this biological event is still unclear [29]. In this study, treatment with 37.5 μg/paw of galatrox did not induce edema compared with PBS and crude venom (**Fig. 5**). Similarly, BTL-2, a small isolectin isolated from *Bryothamnion triquetrum*, did not induce an evident inflammation and/or edematogenic effect after injection into the hind paw of rats [57]. However, BJcuL (a lectin purified from *B. jararacussu*) induced acute phase edema, which was maintained for up to 6 h after injection, and increased vascular permeability in mouse hind paws [29]. In addition, *B. godmani* lectin caused acute edema formation that was inhibited by prior administration of cyproheptadine in mice (a histamine and serotonin receptor antagonist), indicating a possible participation of mast cell degranulation on this event [18]. Although some *Bothrops* venom lectins are able to promote edema formation, these proteins make up only a small percentage of the crude venom, suggesting that these lectins are probably not the major contributor to venom-induced edema [18,29].

Mast cell degranulation can promote protection against snake envenomation by releasing proteases that degrade venom toxins [58,59]. In the literature, it has been described that some animal lectins, such as galectin-3 and

MNCF [60,61], induce mast cell degranulation. Based on these reports, we evaluated the ability of galatrox to induce mast cell degranulation. However, this lectin did not promote the release of β-hexaminidase from RBL-2H3 cells (**Fig. 6**). Also, Aragón-Ortiz *et al*. [27] did not observe a histamine release from mast cells when incubated with *L. muta stenophrys* lectin. Taken together, these results suggested that snake venom lectins are not necessarily important in mast cell envenomation protection.

Several lectins have been found to possess anti-cancer properties. They are used as therapeutic agents, preferentially binding to cancer cell membranes, causing cytotoxicity via inducing apoptosis, autophagy, or necrosis, and inhibiting the tumor growth [62,63]. In this work, we observed that galatrox demonstrates a remarkable cytotoxic activity in HL-60 cells (human promyelocytic leukemia cells), with an IC_{50} around 250 μg/ml (**Fig. 7**). Galectin-1 (300 μg/ml), another animal β-galactoside lectin, binds to HL-60 cells and does not induce apoptosis or inhibition of cell growth after 24–72 h of treatment [64]. In addition, different galectin family members can promote the modulation of cell growth, inhibition of microbial invasion, tumor progression, and metastasis [65–67]. On the other hand, BJcuL, a β-galactoside snake venom lectin, was able to suppress the cell viability of different human tumor cell lines [31]. The effects of lectins on cancer biology and immune response are associated with their specificity in carbohydrate recognition [65–68]. Therefore, further structural and biological studies concerning galatrox involvement in cancer biology are necessary.

Historically, studies examining snake bite envenomation have sought to elucidate the mechanism of venom-induced toxicity as a method of developing potential therapeutics in these patients and possibly identifying a biologically active substance that may be useful in biomedical research [69]. Indeed, toxins isolated from *Bothrops* spp. crude venoms have been used as molecular tools to understand many physiological and pathological events [2]. Therefore, the inhibition of these molecules represents an important strategy in the treatment of snake envenomation [69]. Despite the inability of galatrox to induce edema, mast cell degranulation, or interfere with homeostasis, the ability of galatrox to significantly alter cellular viability suggests that galatrox might contribute to the cell death that can accompany envenomation.

In summary, based on the results obtained from IEF, HPLC, N-terminal amino acid sequence and MALDI-TOF mass spectrometry procedures, we purified a homogenous lectin, galatrox. Our results suggested that galatrox is an acidic protein (pI 5.2) with a monomeric and dimeric molecular mass of 16.2 and 32.5 kDa, respectively. Importantly, this purified lectin from *B. atrox* displayed hemagglutinating activity to human erythrocytes, which was dependent on Ca^{2+} and inhibited by lactose. Biologically, galatrox altered HL-60 cell viability while failing to induce significant mast cell degranulation. Also, this protein is not edematogenic and does not appear to interfere with hemostasis. Based on structural and biological aspects of galatrox, we suggest that galatrox is similar, but not identical to the previously described thrombolectin. Further studies will be necessary to understand the biological role of galatrox during snake envenomation.

Supplementary data

Supplementary data are available at *ABBS* online.

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