



Research paper

Structural and biological characterization of Nattectin, a new C-type lectin from the venomous fish *Thalassophryne nattereri*

Mônica Lopes-Ferreira^{a,*}, Geraldo Santana Magalhães^b, Jorge Hernandez Fernandez^c,
Inácio de Loiola M. Junqueira-de-Azevedo^d, Paulo Le Ho^d, Carla Lima^a,
Richard H. Valente^e, Ana Maria Moura-da-Silva^b

^a Laboratório Especial de Toxinologia Aplicada (CEPID/FAPESP), Instituto Butantan, Avenida Vital Brazil, 1500. Butantan. São Paulo, Brazil

^b Laboratório de Imunopatologia, Instituto Butantan, São Paulo, Brazil

^c Laboratório Nacional de Computação Científica, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Brazil

^d Centro de Biotecnologia, Instituto Butantan, São Paulo, Brazil

^e Laboratório de Toxinologia, Instituto Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil

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ABSTRACT

Lectins are glycan-binding receptors that recognize glycan epitopes on foreign pathogens and in the host systems. They can be involved in functions that include innate immunity, development, immune regulation and homeostasis. Several lectins have been purified and characterized from fish species. In this work, using cation-exchange chromatography, a galactose-specific lectin belonging to the family of C-type lectins was isolated from the venom of the Brazilian venomous fish *Thalassophryne nattereri*. Nattectin is a basic, non-glycosylated, 15 kDa monomeric protein. It exhibits hemagglutination activity that is independent of Ca^{2+} . We also demonstrated a lectin activity for Nattectin in the innate immune system, especially in neutrophil mobilization in mice, indicating that marine organisms are source of immunomodulator agents.

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1. Introduction

Lectins are widely distributed from *Caenorhabditis elegans* to *Homo sapiens*, that bind specifically and reversibly to carbohydrate structures, particularly the sugar moiety of glycoconjugates and glycoproteins. C-type lectins are proteins that contain a carbohydrate recognition domain (CRDs) and bind carbohydrate structures in a Ca^{2+} -dependent manner. Calcium ions are directly involved in ligand binding, as well as in maintaining the structural integrity of the CRD that is necessary for the lectin activity, but many C-type lectins are Ca^{2+} -independent and may not necessarily bind to sugar ligands [1].

The specificity of C-type lectins can be generally divided into two categories: a) the mannose (Man) specific C-type lectins, such as DC-SIGN, that possesses specificity for Man and/or fucose (fuc) terminated glycans, b) and galactose- (Gal) specific C-type lectins, such as macrophage galactose lectin, that recognize Gal- and N-acetylgalactosamine (GalNAc) terminated glycans [2].

Since the identification of Electrolectin, the first galectin-specific lectin isolated from the electric organs of the eel *Electrophorus electricus* [3], a substantial number of lectins have been identified in other fish [4]. These lectins are distributed in the epidermis and also in the epithelia of the upper digestive tract and gills, fluids and cells where they exhibit agglutinating or opsonizing activity against the fish pathogen *Vibrio anguillarum* [5,6]. The Indian catfish (*Clarias batrachus*) expresses a Ca^{2+} -dependent galactose-specific serum lectin that significantly alters the viability and pathogenicity of various strains of the bacteria *Aeromonas* and produces a dose-dependent increase in bactericidal activity of fish macrophages [7].

Fish lectins have been extensively described within tissues and cells associated with host defensive functions (branchial epithelium, renal interstitium, hepatic sinusoidal, intestinal sub-mucosal granular layer, skin and particularly within circulating granulocytes), however only recently, a tetrameric lectin belonging to the family of mannose-binding B-type was isolated from the mucus and sting of *Scorpaena plumieri* venomous fish [8].

Thalassophryne nattereri venomous fish is a member of the Batrachoididae family and, in Brazil, it is known by the severity of the accidents provoked in fishermen and bathers [9,10]. Its venomous

* Corresponding author. Fax: +55 11 3726 1024.

E-mail address: lopesferreira@butantan.gov.br (M. Lopes-Ferreira).

apparatus presents two dorsal and two lateral canaliculated spines covered by a membrane, all connected to venomous glands at the base. Envenoming symptoms are readily evident, including local edema, erythema and severe pain followed by intense necrosis and a markedly inefficient healing response [11]. *T. nattereri* venom is composed of proteins endowed with proteolytic and myotoxic properties, but devoid of phospholipase A₂ activity [12]. Recently, we demonstrated that the venom has a family of toxins, named natterins with kininogenase activity, which contributes to the pain and edema induced by the venom, since these effects were diminished with kininogenase-specific antagonists [13].

In this work, we report that a galactose-specific lectin belonging to the family of C-type lectins was isolated from the venom of the Brazilian fish *T. nattereri* in a single step using a cation-exchange chromatography. Nattectin is a monomeric non-glycosylated protein with an experimentally determined molecular mass of 15,087 Da and a calculated basic isoelectric point of 9.73. Its carbohydrate specificity corresponds to core and terminal galactose: Gal-β(1–3)-N-acetylgalactosamine and Nattectin exhibits hemagglutination activity, which was Ca²⁺ independent. We also demonstrated a lectin activity for Nattectin in the innate immune system, especially in neutrophil mobilization in mice, indicating that marine organisms are source of immunomodulator agents.

2. Material and methods

2.1. Animals

Male 8- to 10-week-old Swiss mice were purchased from the Butantan Institute (São Paulo, Brazil). Animals were housed in a laminar flow holding unit (Gelman Sciences, Sydney, Australia) in autoclaved cages on autoclaved bedding, in an air-conditioned room on a 12-hour light/dark cycle. Irradiated food and acidified water were provided *ad libitum*. All the procedures involving animals were in accordance to the guidelines provided by the Brazilian Government's ethical and animal experiment regulations.

2.2. Cloning and sequencing of Nattectin cDNA

A cDNA library was constructed from mRNA prepared from 9 glands extracted from 3 specimens of the fish *T. nattereri* collected at Mundaú lagoon, State of Alagoas, Brazil, and expressed sequence tags – ESTs were generated and sequenced from this cDNA library as described [14]. The generated ESTs were compared with GenBank nucleotide and protein databases using Blastn and Blastx programs at NCBI, allowing the identification of clones encoding a lectin-like protein. The complete cDNA sequence of Nattectin was obtained by selecting the clone with the largest cDNA insert from the contig. The complete cDNA sequence was achieved by primer walking and deposited in GenBank under accession number AAU11827. Related C-type lectin sequences were cited from the GeneBank database. A multiple sequence alignment was made with CLUSTAL W (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). For better alignment, all fish C-type lectins had its signal peptide predicted by SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) and removed afterwards.

2.3. Isolation and biochemical characterization of Nattectin

T. nattereri venom was collected from 40 specimens collected at Mundaú lagoon, State of Alagoas, Brazil (IBAMA, 14693-1). Venom was extracted from the openings at the tip of the spines by applying pressure at their bases. After centrifugation, venom was pooled and stored at –80 °C before use. For toxin purification, 3 mg venom was diluted in 500 µL of 50 mM Tris/HCl pH 8.0 and subjected to

fractionation in a Mono-S FPLC column (Amersham Biosciences, Uppsala, Sweden) equilibrated with the same buffer, and eluted with a linear gradient from 0 to 2 M NaCl at a flow rate of 1 mL min^{–1}, monitored at 280 nm. Protein concentration was measured following the method of Bradford using BSA as the standard. Fractions were screened for hemagglutinating activity of human erythrocytes and purity level of the isolated lectin was accessed by sodium-polyacrylamide gel electrophoresis at 12% (SDS-PAGE), according to the method of [15], under reducing and non-reducing conditions.

For N-terminal sequencing, Nattectin was adsorbed on PVDF in a ProSorb cartridge (Applied Biosystems, USA) and submitted to Edman sequencing on a 494 Procise protein sequencer (PerkinElmer, USA) according to the manufacturer's instructions for PVDF samples. Molecular mass analyses of the fractions and purified peptides were performed on a Q-TOF Ultima API (Micromass), under positive ionization mode and/or by matrix assisted laser desorption ionization-time of flight – MALDI-TOF mass spectrometry on an Ettan MALDI-TOF/Pro system (Amersham Biosciences), using -CHCA or sinapinic acid as matrices. Alternatively, for internal peptide sequencing, 2D gel spots containing Nattectin were submitted to reduction, alkylation and trypsinization followed by nanoLC-nanoESI ION TRAP mass spectrometry.

2.4. Production of antiserum against *T. nattereri* venom and purified Nattectin

Mouse antisera raised against *T. nattereri* venom and Nattectin were produced by immunization of mouse with 10 µg of such proteins according to Piran-Soares et al. [16]. Mice ($n = 5/\text{group}$) were injected with 10 µg of protein venom or Nattectin at day 0. Forty-two days after the first injection mice were boosted with the same dose of venom or lectin. Blood samples from the mice were obtained at days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, and 77 after immunization by retroorbital bleeding, and individual mouse sera were tested for antibody response by enzyme-linked immunosorbent assay (ELISA).

2.5. Homology modeling of Nattectin structure

The structures of galactose-specific lectin from *Crotalus atrox* venom (1ZJN) and type II antifreeze protein from *Crotalus arengus* (2PY2) were used to model the structure of Nattectin (GenBank: AAU11827). The atomic coordinates of the 2PY2 and 1ZJN, solved at 1.7 and 2.2 Å resolution respectively, were used as a template in comparative modeling by satisfaction of spatial restraint [17], implemented in the program Modeller 9v7 [18]. Galactose coordinates were imported from 1ZJN. Twenty-five different models were generated in this experiment. The quality of each predicted fold was evaluated using the Discrete Optimized Protein Energy function (DOPE) of the variable target function [19]. The stereochemical quality of the 3 best scoring models was assessed by Procheck program [20] at 2.0 Å resolution. Final model was selected based on the overall stereochemical quality for further energy minimization, relaxation and molecular simulation experiments using Gromacs 3.3.1 [21].

2.6. Carbohydrate-binding specificity

For identification of Nattectin carbohydrate specificity, a competition assay was carried out using the DIG glycan differentiation kit (Roche Diagnostics, Mannheim, Germany) according to de Santana Evangelista et al. [8]. The control glycoproteins carboxypeptidase Y, transferrin, fetuin and asialofetuin were run on 12% SDS-PAGE, and proteins electroblotted onto a nitrocellulose membrane. Membranes were then incubated with Nattectin (1 µg/mL) before adding the

digoxigenin-labeled GNA, SNA, PNA, DSA, and MAA lectins. The membrane was then washed and incubated with anti-DIG antibodies conjugated with alkaline phosphatase, and 5-bromo-4-chloro-3-indolylphosphate was used as alkaline phosphatase substrate, with enzyme catalysis resulting in a dark color. The specificities of labeled lectins are: GNA: recognizes terminal mannose, (1–3), (1–6) or (1–2) linked to mannose; thus it is suitable for identifying “high mannose” N-glycan chains or O-glycosidically linked mannoses in yeast glycoproteins. SNA: recognizes sialic acid linked (2–6) to galactose; thus it is suitable for identifying complex, sialylated N-glycan chains in combination with the lectin MAA; correspondingly linked sialic acids in O-glycan structures. MAA: recognizes sialic acid linked (2–3) to galactose; in combination with SNA (see above) it is suitable for identifying a) complex, sialylated carbohydrate chains and b) type of sialic acid linkage. Lectin MAA identifies (2–3)-linked sialic acids in O-glycans too. PNA: recognizes the core disaccharide galactose (1–3) N-acetylgalactosamine and is thus suitable for identifying O-glycosidically linked carbohydrate chains (with exception of yeast glycoproteins). DSA: recognizes Gal-(1–4) GlcNAc in complex and hybrid N-glycans, in O-glycans and GlcNAc in O-glycans.

2.7. Hemagglutination and inhibition assay

The hemagglutinating activity of Nattectin was assayed using human A type erythrocytes. Nattectin (2.5–10 µg) was subjected to serial two-fold dilutions in 50 µL phosphate-buffered saline (PBS, pH 7.4) in microtiter U-well plates. Next, 50 µL of a 2% suspension of human erythrocytes were added and the plates were shaken briefly and incubated at 37 °C for 3 h. The hemagglutination inhibition assay was performed by incubating Nattectin and EDTA, D-galactose or D-mannose (at 10 and 30 mM) with the same volume of suspension of erythrocytes in the same buffer solution.

2.8. Induction of local inflammatory reaction by Nattectin

Leukocyte migration was assessed in the footpad of mice according to Lima et al. [22]. Animals were injected in the intraplantar region of the right hind footpad with Nattectin (10 µg protein

in 30 µL sterile saline) or with 30 µL sterile saline alone. After periods of 2 h, 6 h, 24 h, 48 h, and 7, 14 or 21 days animals were killed and the right paws were amputated; the tissue was dismembered with scissors and homogenized with a glass piston in PBS containing 10 mM EDTA. Samples were immediately centrifuged at 3000 rpm, 4 °C, for 20 min. The supernatants were stored at –20 °C for cytokine, chemokine, lipidic mediators, and matrix metalloproteinases (MMPs) determination. Cell pellets were resuspended in PBS containing 0.1% newborn calf serum (Sigma) for cell counting using a hemocytometer and cytocentrifuge slides were prepared, air dried, fixed in methanol, and stained (Wright-Giemsa, Scientific Products, Chicago, IL). For differential cell counts, 300 leukocytes were enumerated and identified as mononuclear cells and neutrophils on the basis of staining and morphologic characteristics.

2.9. Quantification of cytokines, chemokines and eicosanoid

Cytokines and chemokines were measured in footpad tissue homogenates by a specific two-site sandwich ELISA, using the OptEIA Kit (BD-Pharmingen, San Diego, CA, USA). Detection limits were 7.8 pg/mL for IL-1β, IL-6, TNF-α, and MCP-1 or 15.62 pg/mL for KC. Concentrations of LTB4 and PGE2 were measured in the footpad homogenates collected 6 h after Nattectin or saline injection, by a specific enzymatic immunoassay, using a commercial kit (Cayman Chemicals, MI, USA). After addition of the substrate, the absorbance was recorded at 412 nm and concentration of the eicosanoid was estimated from a standard curve.

2.10. Determination of matrix metalloproteinases activity by gelatin zymography

Gelatinase activity in footpad homogenates of mice was analyzed on 12% SDS-polyacrylamide gels containing 1 mg/mL gelatin under non-reducing conditions. After electrophoresis, the gel was incubated for 30 min at room temperature on a rotary shaker in 0.05 M Tris-HCl, pH 7.4, containing 2.5% Triton X-100 to remove traces of SDS. The gel was washed with deionized water to remove excess of Triton X-100 and then incubated in zymography incubation buffer

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-30  tccagagcttctcatcgaaccatcacaagatgcatcagttccacatttactgtgttc
      M A S V P H F T V F
30   ctctttctggcctgtgcttttagggattggagcaaatgtgacgcgtagagcaaccagttcc
    L F L A C A L G I G A N V T R R A T S S
90   tgtccgaaagggttgactcaccatggctctcgtgtttcacgttccatcgcggtagtagt
    C P K G W T H H G S R C F T F H R G S M
150  gactggcgagtgagaggtgcttgcattcgtaaagggtgggaacctggcctccattcac
    D W A S A E A A C I R K G G N L A S I H
210  aaccgacgtgagcaaaacttcatcacacactgattcacaactgagcgggtgaaacaga
    N R R E Q N F I T H L I H K L S G E N R
270  agaacctggattggaggcaacgacgcagtcaggaggatgtggtttgtctgatggg
    R T W I G G N D A V K E G M W F W S D G
330  tccaagttaactacaaagggtgaaaaaggccagcctgacaacatgtcccagctgaa
    S K F N Y K G W K K G Q P D K H V P A E
390  cactgagcagagaccaacttcaaaggagccttctggaataatgccctctgtaaaagtgaag
    H C A E T N F K G A F W N N A L C K V K
450  aggtctttcttctggtgcaaaaatctgtgattctgctcaggatgtgttgatgatgtcagg
    R S F L C A K N L ***
510  cctcgatgacatcagcagcagcttgactgagagttaaataccactttctgttcagccaat

570  aagctcttacatgcataatgcttgtgaattctgaataaaagtaccaaagtatgtgacat

630  ttgggaaacaataaaaaaaaaaaaaaaaaaaaaa
  
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Fig. 1. Complete nucleotide and deduced amino acid sequence of Nattectin. The predicted signal peptide is indicated in bold type and the N-terminal sequence of the mature protein determined by Edman degradation is highlighted in dark gray. The internal peptides determined by MS/MS spectrometry sequencing are highlighted in light gray. The stop codon is indicated by asterisks and the polyadenylation signal is underlined.

(0.05 M Tris–HCl, pH 8.0, 0.15 M NaCl, 0.01 M CaCl_2 , 0.02% CHAPS) at 37 °C for 12 h. The gel was stained with Coomassie Blue and destained. Gelatin digestion was identified as clear zones of lysis against a blue background. The molecular mass (kDa) of the gelatinases was estimated against markers of known recombinant proteins MMPs (R&D Systems, Minneapolis, MN, USA).

2.11. Statistical analysis

Non-parametric data were assessed using the Mann–Whitney test. Differences were considered statistically significant at $p < 0.05$.

The SPSS statistical package (Release 8.0, Standard Version, 1997) was employed.

3. Results

3.1. Identification of Nattectin by cDNA cloning

A cDNA library was constructed and the ESTs obtained from 5' ends were clustered using highly stringent parameters (>95% identity). Each cluster was then searched with Blastn and Blastx programs for similar sequences in DNA and protein databases. One

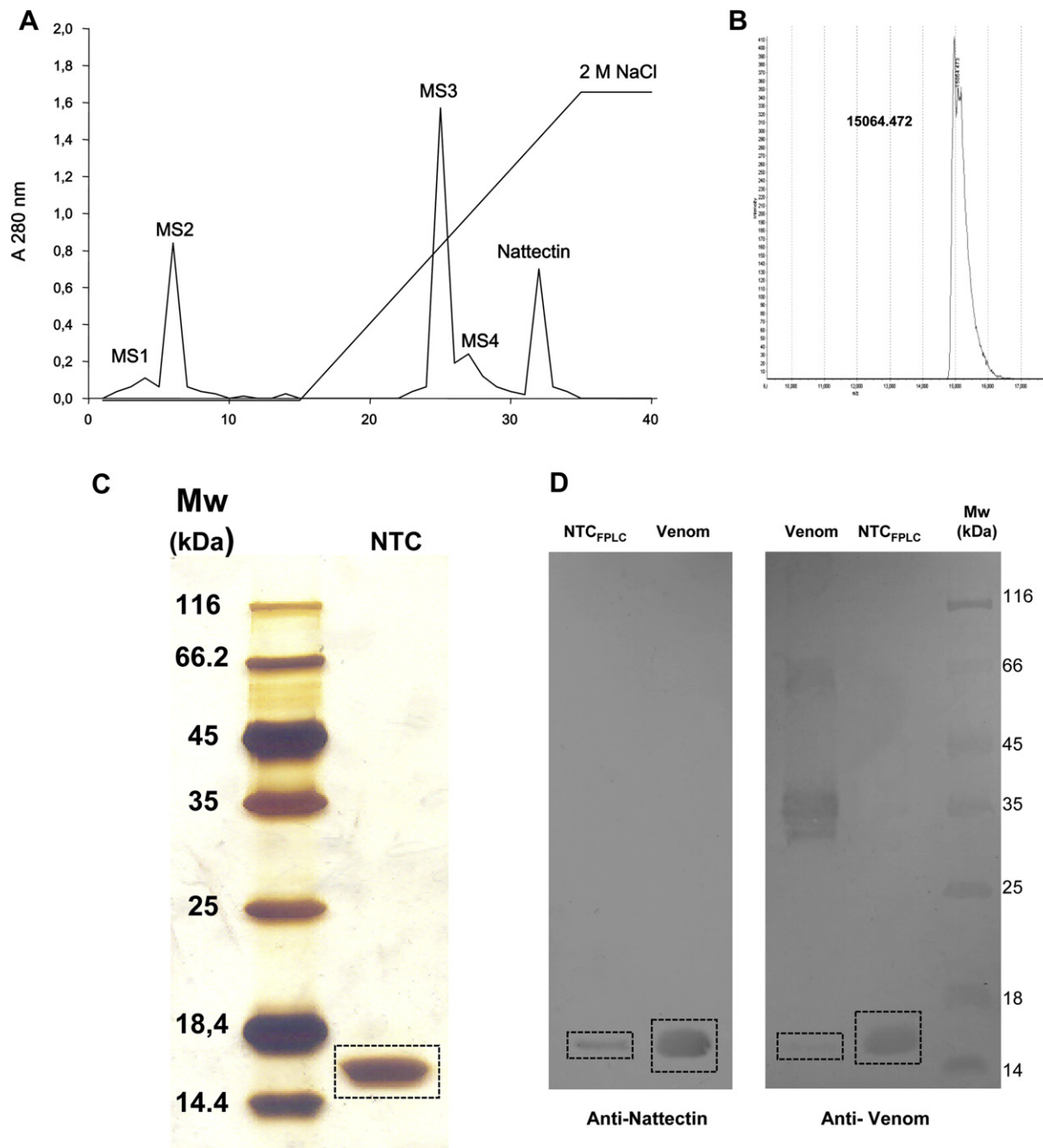


Fig. 2. Purification of Nattectin from *T. nattereri* crude venom. (A) Cation-exchange chromatography (Mono S, FPLC-column, pH 8.0) of crude *T. nattereri* venom monitored at 280 nm. The column was pre-equilibrated with 50 mM Tris–HCl buffer, pH 8.0 and the proteins eluted with a linear gradient from 0 to 2 M NaCl. The analysis of Nattectin by MALD-TOF revealed a mass of 15,064 Da (B). (C) SDS-PAGE of Nattectin eluted from cation-exchange chromatography under non-reducing conditions. (D) Western immunoblot analysis using mouse polyclonal antisera against venom of *T. nattereri* or Nattectin.

cluster, containing 6 ESTs showed significant similarity with C-type lectins from other sources, particularly with C-type lectin from non venomous fish such as *Spirinchus lanceolatus* and *Salmo salar* with identities around 34%, and with C-type lectins found in snake venoms such as *Trimeresurus stejnegeri* and *Lachesis muta* with identities around 30%. Since the 6 ESTs showed to be identical in their region of alignment, the longest EST from this cluster was then subjected to a complete sequencing and the resulting product was named Nattectin (GenBank accession: AAU11827).

The complete nucleotide sequence (Fig. 1) was 641 bp in length (excluding the poly (A) tail) and contained a 474 bp open reading frame (ORF). The 5' untranslated region (5'UTR) is 30 bp long while the 3'UTR is 162 bp long. The polyadenylation signal was detected 92 bp downstream the stop codon. The open reading frame predicted a precursor protein with 159 amino acid residues with a signal of 21 residues (Fig. 1, bold) followed by a pro-peptide of 5 residues NVTRR. The N-terminal sequence determined by Edman sequencing is shown in dark gray in Fig. 1. The secreted mature protein would then consist of 133 residues, with a calculated molecular mass of 15,094 Da and an isoelectric point of 9.73.

3.2. Isolation of Nattectin from the venom of *T. nattereri*

Methods for purifying lectins differ from one to another according to the source of the lectins, but classical protein

purification methods, such as salting out and different chromatographic methods are still the basic methods used to purify lectins. Venom of *T. nattereri* was fractionated using cation-exchange chromatography (FPLC, Mono-S column, pH 8.0) and fractions screened for hemagglutinating activity of human erythrocytes. Five fractions were isolated: two acidic/neutral fractions (MS1, MS2) collected before the initiation of the 0–2 M NaCl gradient and three basic (MS3, MS4 and MS5) fractions collected with NaCl concentrations of approximately 1 M, 1.15 M and 1.67 M, respectively (Fig. 2A). Hemagglutinating activity was observed only in fraction MS5 (data not shown), which was characterized by SDS-PAGE with apparent molecular mass of 15 kDa as a single band, under reducing (data not shown) and non-reducing conditions (Fig. 2C – lane 2).

The purified lectin was subjected to N-terminal amino acid sequencing by Edman degradation, which yielded the sequence ATSSCPKGWTHHGSR, identical to the peptide predicted by the cDNA sequence of Nattectin (Fig. 1- highlighted in dark gray). We undertook the analysis of internal peptides by MS/MS spectrometry, and the readable sequences obtained represented 5 peptide sequences which are highlighted in light gray on Fig. 1. Taken together, N-terminal and internal peptides consist of 51 residues (38.3%) identical to the ones predicted by the cDNA sequence, thus confirming that this protein is coded by Nattectin cDNA and that Nattectin is present in the venom secretion.

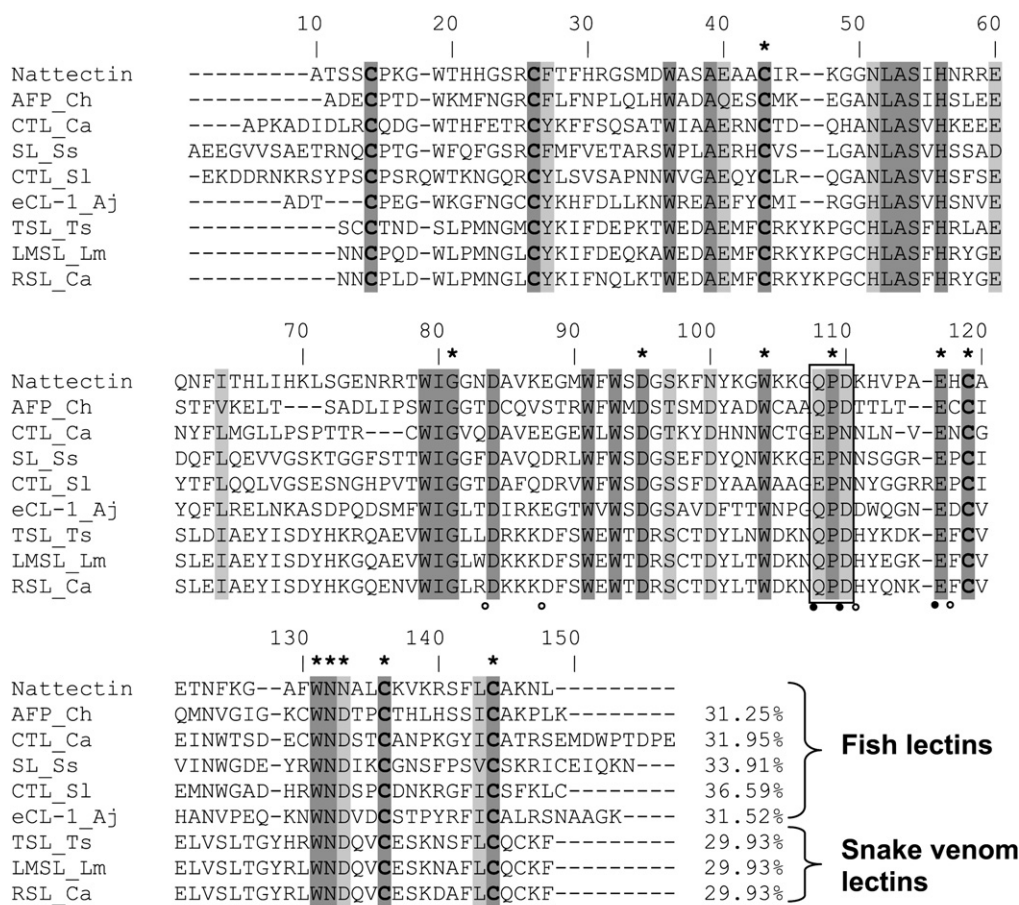


Fig. 3. Amino acid sequence alignment of nattectin and other fish and snake venom C-type lectins. Abbreviations and accession numbers are as follows: AFP_Ch, antifreeze protein from *Clupea harengus* (AAA49200); AFP_Ch, C-type lectin from *Carassius auratus* (AAN75192); SL_Ss, serum lectin from *Salmo salar* (AAO43605); CTL_Sl, C-type lectin from *Spirinchus lanceolatus* (BAE45333); eCL-1_Aj, C-type lectin 1 from *Anguilla japonica* (BAC54022); TSL_Ts, Galactose-binding lectin from *Trimeresurus stejnegeri* (Q9YGP1); LMSL_Lm, lectin-like protein from *Lachesis muta* (AAB49518); RSL_Ca, Galactose-specific lectin from *Crotalus atrox* (P21963). Identical and conserved amino acids are denoted in dark gray and light gray, respectively. Conserved cysteines residues are in boldface and stars (*) indicate the amino acids common with the invariant residues of CRDs. Residues forming the first and second Ca^{2+} binding site are indicated with white and black circles, respectively. Gaps (-) have been introduced to optimize identity. The percentage identity between Nattectin and each protein is shown on the right.

The analysis of Nattectin by MALD-TOF revealed a mass of 15,064 Da (Fig. 2B), which is in agreement with the theoretical mass (15,094) for the mature protein deduced from the cDNA sequence. Considering the molecular mass determined by MALDI-TOF and those observed by SDS-PAGE, under reducing and non-reducing conditions, we suggest that Nattectin is a monomeric molecule that is not subjected to post-translational modifications during its biosynthesis. However, the formation of oligomers via noncovalent binding cannot be excluded.

The mouse polyclonal antiserum raised against Nattectin detected the 15 kDa band in Western blots of lectin separated by FPLC chromatography and in the venom of *T. nattereri* (Fig. 2D, lanes 1 and 2, respectively). A western blot analysis of the HPLC purified protein probed with the antiserum raised against *T. nattereri* venom showed one predominant band (lane 4 – Fig. 2D), similar of the 15 kDa band present in the venom (lane 3 – Fig. 2D).

3.3. Structural relationships among Nattectin and other animal lectins

The first approach to understand the structure/function relationships was to align Nattectin sequence with a series of known C-type lectin sequences present both in fish and snake venoms (Fig. 3). The overall identity with fish lectins was around 34%, while with snake lectins was about 30%, but all the six cysteine residues of mature Nattectin were conserved in all aligned sequences (boldface). The stars show twelve of eighteen invariant residues of carbohydrate-recognition domains (CRDs) found in the C-type lectins.

An important feature observed in these alignments was a substitution on the Nattectin carbohydrate-binding motif. Apart from lectins from *Carassius auratus*, *S. salar* and *S. lanceolatus*, all the other, including Nattectin, contained the QPD (Gln-Pro-Asp) motif (boxed) that is considered to confer galactose-ligand specificity; while all others C-type lectins contain the EPN motif (Glu-Pro-Asn) which is known to bind mannose. It is also recognized that a Trp

(W) residue next to the QPD motif is important for high-affinity galactose binding with pronounced selectivity, and this was also present in Nattectin sequence, suggesting that the fish venom lectin is a galactose-specific lectin.

In order to determine amino acid residues involved in Ca^{2+} binding for Nattectin, we compared its sequence with a characterized rat mannose-binding protein (MBP). MBP-A CRD's is known to binds to two Ca^{2+} atoms, one at residues D¹⁶¹, E¹⁶⁵, D¹⁸⁸ and D¹⁹⁴, and the other at residues E¹⁸⁵, N¹⁸⁷, E¹³⁴, N¹⁹³, and D²⁰⁵. In Fig. 3, the position for the first and second Ca^{2+} binding site for all sequences when compared to MBP are indicated by a white and black circles, respectively. In contrast to rat MBP-A, Nattectin lacked two of the corresponding residues at the first Ca^{2+} binding site and 3 residues at the second. The absence of those residues may suggest the independence for Ca^{2+} binding in the lectin activity of Nattectin.

3.4. Structural model for galactose recognition by Nattectin

To obtain a detailed model for Nattectin and to study the molecular basis of galactose recognition we performed homology-modeling procedures using Modeller9v7 having the structures of C-type lectins from snake venom (1JZN), mouse scavenger receptor (2OX9), and type II antifreeze protein from *C. arengus* (2PY2) for templates (Fig. 4A). Although the sequence identity of these proteins was low (32–37%), the obtained structural superposition for these structures shows a conserved structure with r.m.s.d. varying from 1.3 to 3.2 Å. It is important to remark that loops surrounding the second Ca^{2+} binding site (Fig. 3) and related to galactose recognition showed important differences in structural packing and length. For modeling of Nattectin- Ca^{2+} -galactose interaction, coordinates of the Ca^{2+} ion trapped on the second site, and Gal residue from the 1JZN were imported to our model. In the solvated and relaxed model (500 ps of simulations), the analysis of Ca^{2+} -coordination sphere, forming the second Ca^{2+} binding site (motif Q-X-D-(X)₄-E-(X)₁₃N-D) shows an Asn₁₆₁ residue replacing the last Asp of the motif (Fig. 4B).

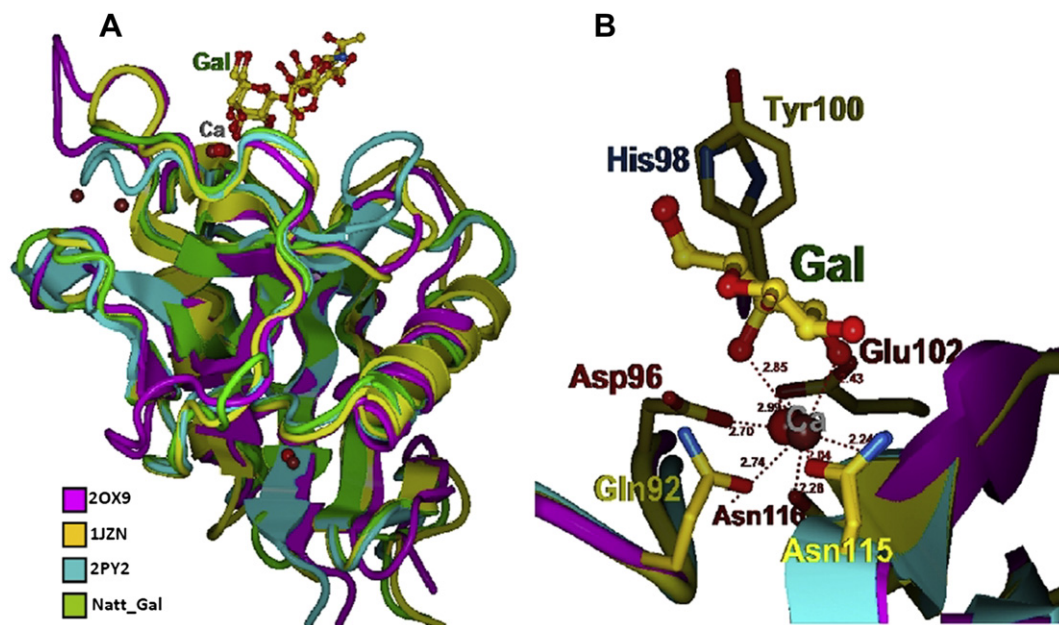


Fig. 4. Molecular model for galactose recognition site in Nattectin structure. (A): Structural superposition of mouse C-type lectin (2OX9), Galactose-specific lectin from *C. atrox* venom (1JZN) and Nattectin (Natt_Gal) and antifreeze protein type II form *C. arengus* (2PY2). All the structures are very similar, differing only in both loops surrounding the Ca^{2+} recognition site. (B): Structural alignment of residues coordinating Ca^{2+} ion in our initial model. The only remarkable substitution in the Ca^{2+} binding site is the Asp to Asn116 (134 position in the Fig. 3 alignment).

3.5. Functional activities of Nattectin

The functional activity of Nattectin was first accessed by its ability to agglutinate human red blood cells. Agglutination of human erythrocytes was provoked by Nattectin isolated by cation-exchange chromatography with the minimal agglutinating concentration of 2.5 µg/well. Nattectin-induced agglutination was not suppressed by increasing concentrations of mannose or the chelating agent EDTA even at 30 mM, but completed inhibited by galactose (Table 1). These results suggest that the Ca^{2+} -activities of Nattectin are not essential for this lectin to induce hemagglutination and corroborate the modeling indication that Nattectin–galactose interaction is not mediated by this divalent cation.

The carbohydrate-binding specificity was then evaluated by a competition assay with standard lectin/glycoprotein pairs. Nattectin strongly inhibited the binding of PNA to asialofetuin (Fig. 5, lane 4), indicating that its carbohydrate specificity corresponds to core and terminal galactose: Gal-β(1–3)-N-acetylgalactosamine.

Inhibitions of binding were not observed when Nattectin competed with the following pairs: GNA/carboxypeptidase Y; SNA/transferrin; MAA/fetuin; DSA/asialofetuin, ruling out the binding of Nattectin to mannose (1–2), (1–3), or (1–6) linked to mannose; sialic acid linked (2–6) to galactose; sialic acid linked (2–3) to galactose; or Gal-(1–4)-N-acetylglucosamine in complex and hybrid N-glycans, in O-glycans and GlcNAc in O-glycans (lanes 1, 2, 3, and 5).

3.6. Pattern of leukocyte recruitment and inflammatory mediators production induced by Nattectin

Here we investigate the effects of Nattectin on cellular recruitment in a murine model of inflammation. In Fig. 6A, the injection of Nattectin in footpad of mice provoked a persistent recruitment of leukocytes in all time-point analyzed, characterized by an elevated number of neutrophils until 21 days after injection (Fig. 6B). Macrophages were also recruited into footpad of mice at 24 h after injection of Nattectin (Fig. 6C).

Critical mediators of monocyte and neutrophil trafficking were analyzed in footpad of mice injected with Nattectin. In Fig. 6D–F we observed that Nattectin induced the production of the lipid mediator LTB4 at 6 h pos injection, the pro-inflammatory cytokine IL-1β at 24 h, and the chemokine KC was seen at 7 d after injection. Nattectin did not induce detectable amounts of IL-6, TNF-α or MCP-1 in footpad of mice (data not shown).

It is known that pro-inflammatory cytokines as TNF-α, IL-8 (or KC) and IL-1β are capable of inducing MMP secretion. MMP-2 with proteolytic activity was induced after Nattectin injection at 6 h, 7, 14 and 21 days (Fig. 7, lanes 5–8). Comparisons with rMMP-9 standard (lane 2) indicated that high MMP-9 activity was detected in footpad supernatant of mice injected with Nattectin at 2 h (Fig. 7, lane 4).

Table 1
Inhibition of hemagglutination activity of Nattectin.

	Minimum concentration for inhibition of hemagglutination
Saccharides	
D-Galactose	0.78 mM
D-Mannose	NI _a NI _b
Chelating agent	
EDTA	NI _a NI _b

NI_a: No inhibition at 10 mM.

NI_b: No inhibition at 30 mM.

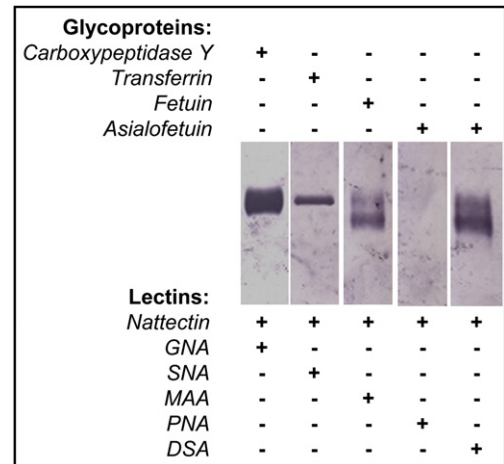


Fig. 5. Carbohydrate specificity of Nattectin. The control glycoproteins carboxypeptidase Y, transferrin, fetuin and asialofetuin were ran on 12% SDS-PAGE, and proteins electroblotted onto a nitrocellulose membrane. Membranes were then incubated with Nattectin (1 µg/mL) before adding the digoxigenin-labeled GNA, SNA, PNA, DSA, and MAA lectins. The membrane was then incubated with anti-DIG antibodies conjugated to alkaline phosphatase and revealed with 5-bromo-4-chloro-3-indolylphosphate.

These results suggest that Nattectin acts a pro-inflammatory agent, inducing sustained leukocyte mobilization and chemoattractant mediators into the site of injection.

4. Discussion

During the course of molecular evolution regarding venomous animals' toxins, a wide variety of sequence motifs have been generated. These motifs are essential for targeting important physiological components with such a high affinity that suffices to kill or immobilize preys and predators. Amongst venomous animals, several species of fish can produce severe human envenoming and a few toxins have already been isolated from their venoms. Despite the reduced number of reports, interesting fish toxins have been described [23–26], usually adding previously unknown primary structures to the literature [27,28].

These assumptions are also adequate to *T. nattereri* venom. Combined proteomic and transcriptomic approaches to study the venom composition revealed polypeptides never described in venoms or unique unknown primary structures like the major toxin, natterins, which present nociceptive, edema-inducing and kininogenase activity similar to that presented by the whole venom [29]. Besides these unique proteins, we detected another cluster encoding a putative lectin protein in *T. nattereri* venom gland transcriptome [14].

In the present report we show the complete characterization of this cDNA sequence and the biochemical and functional properties of the corresponding venom-secreted protein. Nattectin is a basic monomeric protein, non-glycosylated, galactose-specific lectin belonging to the family of C-type lectins with 15 kDa. It exhibits hemagglutination activity that is independent of Ca^{2+} with remarkable pro-inflammatory activity, inducing neutrophil mobilization in mice.

Analysis of Nattectin precursor sequence shows that twelve of the eighteen invariant residues of carbohydrate-recognition domains that participate in the interaction with the galactose-ligand in most C-type lectins are conserved, retaining the galactose-binding activity of Nattectin. Moreover, Nattectin displays jellyroll topology typical of legume lectins, as evident from homology-modeling using prototypic C-type lectins (snake venom – 1JZN, mouse scavenger

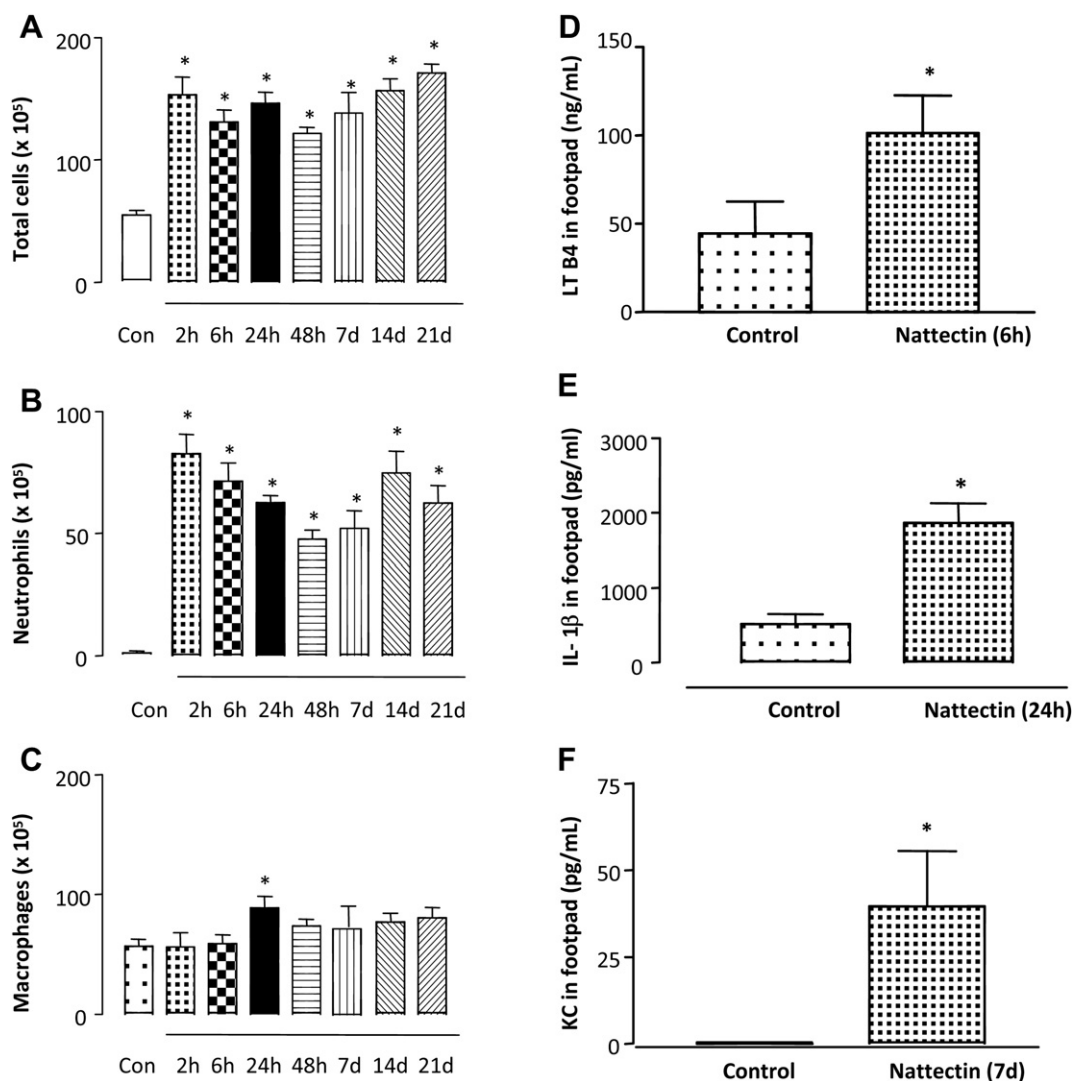


Fig. 6. Induction of footpad inflammation by Nattectin. Swiss mice were injected with Nattectin (10 μ g of protein in 30 μ L of sterile saline) in the intraplantar region of the right hind footpad. Animals injected with 30 μ L sterile saline were considered as control. Two, 6, 24, and 48 h or 7, 14, and 21 days after injection animals were killed and the right paws were amputated; the tissue was dismembered with scissors and homogenized with a glass piston in PBS + 10 mM EDTA. After centrifugation, the cell pellets were resuspended for total and differential cell counts (A–C). Chemoattractant mediators (D–F) were measured in footpad tissue homogenates of mice injected with Nattectin by a specific two-site sandwich ELISA. * $p < 0.05$ compared to control group.

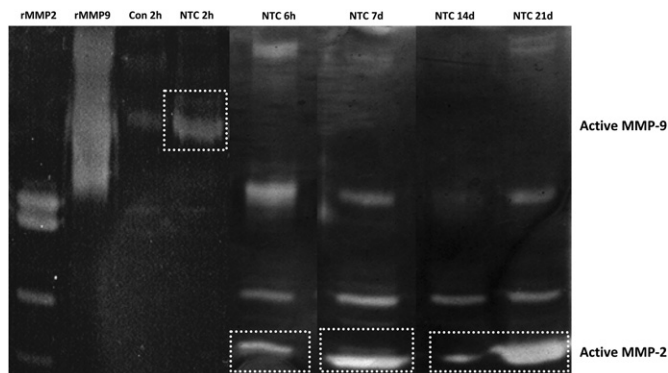


Fig. 7. Nattectin induces active MMPs in inflamed tissue. Enzymatic activity in footpad homogenates of mice injected with Nattectin was analyzed by zymography gel containing 1 mg/mL gelatin. After electrophoresis, the gels were stained with Coomassie Brilliant blue and destained bands were visualized. The molecular mass (kDa) of the gelatinases was estimated against markers of MMP-2 and MMP-9 recombinant proteins (lanes 1 and 2).

receptor – 2OX9, and type II antifreeze protein from *C. arengus* – 2PY2). Despite the binding site of the Nattectin being structurally similar to the aforementioned lectins, the differences in important amino acid residues imply that Nattectin may interact with galactose residues, with consequent specific downstream effects. In addition, the absence of one canonical Asn-X-Thr tripeptide acceptor sequence for N-glycosylation suggests that Nattectin is not glycosylated.

The eCL-1 sequence shows the same carbohydrate-binding motif, QPD, observed in Nattectin, while AJL-2 shows the EPN motif as many others fish C-type lectins. The sequence similarity was also observed between Nattectin and type II anti-freeze proteins (AFP) from several fish species. In these sequences, the presence of the QPD motif is common and it was firstly suggested that the ice-binding site of the type II AFP corresponded to this carbohydrate-binding site of the lectins [30]. However, type II AFPs present several substitution at these sequence positions and recent data indicate that the ice-binding site of AFPs consists of Asp⁹⁴, Thr⁹⁶, Thr⁹⁸, and Glu⁹⁹ forming a relatively flat surface to interact with ice, which evolved from the sugar-binding mechanism of lectins,

developing a potentially larger flat ice-binding surface similar to that of other types of AFPs, that ensures strong interactions with the ice lattice even in the absence of the Ca^{2+} ion [31].

Half of amphioxus C-type lectins consist solely of a CRD domain. In a recent study, Yu and co-workers [32] characterized three C-type lectins with a single CRD domain from the Chinese amphioxus, one of which possesses direct microbial killing activity by binding their microbial targets via interaction with peptidoglycan (PGN) and glucan carbohydrates. However, C-type lectins are expressed as dimers or even in more complex assemblies. One important structural feature observed for Nattectin is that it is present in venom secretion as a 15 kDa monomeric form. Conceptually, lectin–ligand interactions are relatively weak compared with other immune recognition molecules, but high avidity for the target is achieved by the association of peptide subunits into oligomeric structures in which multiple CRDs simultaneously interact with the ligand [33]. The monomeric nature of Nattectin showed by SDS gel electrophoresis and the capacity to agglutinate erythrocytes prompts us to suggest that multivalence via noncovalent association can also be achieved, as occurs with galectin-3 [34].

Nattectin isolated from the venom presented highly conserved residues of carbohydrate-recognition domains found in the C-type lectins, but lacked important residues stabilizing Nattectin–galactose to bind Ca^{2+} . Substitution of D-N₁₆₁ may implicate in important electrostatic properties with subsequent loss of the Ca^{2+} pentagonal-bipyramidal coordination, which may explain its independency of this ion in the hemagglutination activity. While most of the C-type lectin molecules bind with sugars through CRD domains in a Ca^{2+} -dependent fashion, some of the newly identified C-type lectin molecules bearing similar CRD domains do not demonstrate this ability due to a lack of the conserved residues required for Ca^{2+} -coordination [35]. For example, mouse dectin 1 expressed on DC and macrophage and the insect imulectin-2 did not require calcium for its binding activity [32,36].

Despite the importance of conserved Ca^{2+} binding site in the stabilization of the carbohydrate-lectin binding, additional contacts with the surface of the protein, direct hydrogen bonds with side chains of the protein, as well as water-mediated hydrogen bonds and long-range electrostatic interactions can together account for the increase in the selectivity of the binding site.

C-type lectins, as pattern recognition receptor, play an important role in innate immunity, recognizing exogenous ligands, such as glycans on the surface of virus, bacteria, fungi, and protozoa, thereby acting as nonself recognition molecules and engaging effector functions such as immobilization, opsonization, and phagocytosis of the potential pathogens. Considering the large number of bacteria in the aquatic environment and the severe diseases in fish, the presence of soluble Nattectin within the venom gland of the *T. nattereri* can be primarily involved in fish immunity against various pathogens [37]. In another way, the capacity of Nattectin binds sugar moiety of glycoconjugates and glycoproteins expressed in cell surfaces and tissues licensing Nattectin to share with natterins, the responsibility for the induction of severe local symptoms induced by the venom of *T. nattereri*.

In this regard, the presence of lectin proteins in venoms of different species may indicate that the same ancestor protective mechanism has been selected to evolve into different models of self-defense, generating anti-microbial and cytotoxic agents, anti-freeze proteins and molecules affecting homeostatic mechanisms of vertebrates.

Attention is given to lectins regarding their application as tools in biotechnology or in immunotherapies [38]. Here, we show that Nattectin possesses the ability to bind to glycan epitopes and induce inflammatory reaction in mice. Nattectin has potent pro-inflammatory properties, promoting sustained leukocyte

recruitment into footpad of mice, mainly neutrophilic inflammation. Neutrophils play a central role in innate immunity to bacterial infection. Recruitment of circulating neutrophils to an affected site proceeds through defined steps: (i) circulating cells roll on the stimulated endothelium; (ii) surface-bound activating signal (chemokine) cause activation of neutrophil $\beta 2$ integrins, which immobilize the cells on the endothelium; and (iii) cells migrate through the endothelial cell layer and interstitial space by degrading the extracellular matrix.

Our results confirm that Nattectin acts on endothelial cell membranes inducing activation and subsequent release of chemotactic factors as IL-1 β , LTB₄, KC, MMP-9, MMP-2 able to recruit neutrophils, consistent with lectin functions, or directly promotes the adhesion of neutrophils by activation of integrin αM , as suggested by Nishi and co-workers [39]. These authors demonstrated that the lectin galectin-8 modulates neutrophil function via interaction with integrin αM ($\alpha M\beta 2/\text{Mac}-1$), and Gal-3 can function as a chemokine, inducing migration of human monocytes and macrophages [40].

The major chemoattractant for neutrophil during inflammation is CXC chemokine IL-8 (CXCL8) in humans and KC (CXCL1) in mice. We found here that the sustained neutrophil recruitment to the inflamed tissue induced by Nattectin does not only occur through the neutrophil CXCL1 signaling, once the production of this chemokine by Nattectin is restricted at 7 d of pos-injection. Thus, our data suggest that Nattectin can be active in acute inflammation and in chronic situations, where MMPs are up regulated and cells continue to infiltrate despite a lack of sustained chemokine signal.

In summary, marine organisms as venomous fish are source of bioactive components with immunomodulator properties and their identification are of great importance for the elucidation of envenomation induced in human victims and consequently the identification the better pharmacological treatment. Further, in this work, we revealed the crucial role of Nattectin in the innate immune system and chronic manifestations, especially in neutrophil mobilization. This newly recognized activity of Nattectin open up the possibility of their use as adjuvant, inducing chemoattractants secretion in the vaccination site that in turn leads to a potent recruitment of additional immune cells to amplify the immune response to the vaccine antigens.

Acknowledgments

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