



Insights into the local pathogenesis induced by fish toxins: Role of natterins and nattectin in the disruption of cell–cell and cell–extracellular matrix interactions and modulation of cell migration

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ABSTRACT

Combined proteomic and transcriptomic approaches to study the composition of the venom of *Thalassophryne nattereri* venomous fish revealed the primary structures of the major toxins as a family of proteases natterins, never described on venoms and a C-type lectin nattectin. To gain new insights into the mechanisms of venom pathogenesis and to further elucidate the role of its major toxins, the natterins and nattectin, we undertook *in vitro* investigations using these isolated toxins. Here we demonstrated the specific ability of the nattectin to bind types I and V collagen and natterins to bind and cleave type I collagen as well as type IV collagen, disrupting cell attachment and HeLa cells survival. Natterins have cytotoxic effect on both adherent cells or at in suspension, showing direct induction of necrosis that is followed by cell detachment. Nattectin improves integrin-mediated HeLa cell adhesion and resistance to apoptosis by its binding to RGD-dependent integrins, especially the β1 subunit. Based on our studies we now report that extracellular matrix (ECM) components as well as the integrin β1 subunit are targets for the natterins and nattectin. The ECM degradation or remodeling activities exerted by these toxins affect cell–cell and cell–ECM adhesion and survival and impair inflammatory cell migration into inflamed tissues.

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1. Introduction

Thalassophryne nattereri (niquim) is a venomous fish of the Batrachoididae family and, in Brazil, it is known by the severity of the accidents provoked in fishermen and bathers (Fonseca and Lopes-Ferreira, 2000; Faco et al., 2003). Its venomous apparatus is composed of two dorsal and two lateral canaliculated spines covered by a membrane connected to venomous glands at the base of the fins. The venom displays proteolytic and myotoxic activities, but it is devoid of phospholipase A₂ activity (Lopes-Ferreira et al., 1998).

The local pathogenesis induced by the venom includes edema, erythema and severe pain followed by intense necrosis and a markedly poor healing response (Lopes-Ferreira et al., 2001). Using a mouse model, Lopes-Ferreira et al. (2002) demonstrated that the venom action on the endothelium contributes to blood stasis and to the formation of platelet and fibrin thrombi, with consequent ischemia. Corroborating the findings, recent studies from our laboratory demonstrated increased levels of TNF-α, IL-1β and IL-6 in footpad homogenates from venom injected-mice associated with a very low inflammatory cellular influx into local lesions (Lima et al., 2003), the latter being likely the consequence of an impaired blood flow in venules at injured tissue and the cytotoxic effect of the venom components upon inflammatory cells. Moreover,

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Pareja-Santos et al. (2009) showed that *T. nattereri* venom alters the extracellular matrix structure of mouse footpad tissue by the activation of matrix metalloproteinases (MMP-2 and MMP-9), in addition to decreasing collagen fiber content during the healing phase. It was also shown that the venom affects the cytoskeleton organization and pseudopodia formation of epithelial cells.

This scenario indicates an ambiguous role of the venom in the inflammatory process. On the one hand it displays a potent pro-inflammatory activity illustrated by the detected chemoattractants upregulation, and on the other hand, it affects the ability of tissue healing due to the extracellular matrix disorganization caused by MMP up regulated activity, which impairs the infiltration of inflammatory cells.

Combined proteomic and transcriptomic approaches applied to analyze *T. nattereri* venom complexity revealed the identity of the major toxins as a family of new proteins displaying kininogenase activity, the natterins. The transcriptomic analysis of this protein family showed five related sequences, named natterin 1–4 and P, which did not show any significant similarity to tissue kallikreins or any other proteinase. Besides releasing kallidin from low molecular weight kininogen and cleaving kininogen derived synthetic peptides, the natterins show nociceptive and edema-inducing effects similar to that presented by the whole venom (Lopes-Ferreira et al., 2004; Magalhães et al., 2005). The venom also contains a galactose-specific lectin belonging to the family of C-type lectins named natterectin, which showed a Ca^{2+} -independent hemagglutinating activity and induced persistent neutrophil mobilization in mice, indicating that marine organisms are source of immunomodulator agents (Lopes-Ferreira et al., 2011).

To gain new insights into the mechanisms of venom pathogenesis and to further elucidate the role of its major toxins, the natterins and natterectin, we undertook *in vitro* and *in vivo* investigations using these isolated toxins. Based on our studies we now report that extracellular matrix components as well as the integrin $\beta 1$ subunit are targets for the natterins and natterectin. The extracellular matrix degradation or remodeling activities exerted by these toxins affect cell–cell and cell–extracellular matrix adhesion and survival and impair inflammatory cell migration into inflamed tissues.

2. Experimental procedures

2.1. Venom and toxins

T. nattereri fish venom was obtained from fresh captured specimens at the Northeastern coast of Brazil (IBAMA 16221-1); natterins and natterectin were isolated as described before (Lopes-Ferreira et al., 2004). Endotoxin Detoxi-Gel TM (Pierce, Perbio, Northumberland, UK) was used according to manufacturer's instructions to remove the contaminating LPS in venom or toxin solutions. LPS level was detected using Limulus Amoebocyte Lysate (BioWhittaker Inc., Walkersville, MD) as <0.8 pg/ml.

2.2. Reagents, chemicals, and antibodies

Eagle medium and newborn calf serum were obtained from Invitrogen (Merelbeke, Belgium), laminin (354232,

B&D), type I (234149) and type IV (234154) collagens (Calbiochem, La Jolla, California). Mouse anti-venom of *T. nattereri*, anti-natterins and anti-natterectin were produced by immunization of mouse with 10 μg of venom or toxins according to Piran-Soares et al. (2007). Anti-type I collagen (PA1-27396), anti-type IV collagen (SC9302), and anti-laminin (PA116730) antibodies were from Santa Cruz Biotechnology, USA. PE-armenian hamster IgG anti-mouse CD29 (integrin $\beta 1$, 120291), purified rat IgG2ak anti-mouse CD49e (integrin $\alpha 5$, 553318), and FITC mouse anti-rat IgG (H+L, 114811) were purchased from eBioscience (San Diego, CA).

2.3. Solid-phase binding assays

Assays with one ligand adsorbed to plastic microtitre wells were carried out according to Buzza et al. (2005) using established ELISA-type protocols. Microtitre plates (96-well; Costar, Cambridge, MA, USA) were coated with type I collagen (3 $\mu\text{g}/\text{mL}$), type IV collagen (3 $\mu\text{g}/\text{mL}$), laminin (2 $\mu\text{g}/\text{mL}$) or BSA (1 $\mu\text{g}/\text{mL}$) (negative control) in PBS for 18 h at 4 °C. For blocking, the wells were washed with PBS, and incubated with 200 μL of 10% BSA in PBS for 3 h at 37 °C. After washing, *T. nattereri* venom (1 $\mu\text{g}/\text{mL}$) was added to each well for 3 h at 37 °C. Primary antibodies (mouse anti-*T. nattereri* venom, anti-natterins, anti-natterectin, at 1 $\mu\text{g}/\text{mL}$) or goat anti-type I collagen, anti-type IV collagen, and anti-laminin (all at 1 $\mu\text{g}/\text{mL}$) were added to the plates and incubated for 1 h at room temperature. After washing and incubation for 1 h at room temperature with second antibodies (1/2000) anti-mouse or anti-goat IgG conjugated to horseradish peroxidase HRP (Amersham Biosciences) the absorbance of the specific binding was analyzed by spectrometry at 492 nm.

2.4. Culture of HeLa cells

The human adenocarcinoma cell line HeLa (CCL-2) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 in Eagle medium supplemented with 10% fetal calf serum according to Kalantarov and Acevedo (1998). For experiments, cells were used at 60–80% confluence in medium without supplements. Cells were detached with 0.05% trypsin/0.5 mM EDTA in PBS.

2.5. Effect of venom or toxins on cell adhesion to ECM components

Microtitre plates (96-well; Costar, Cambridge, MA, USA) were coated overnight at 4 °C with 100 μL of PBS containing purified type I collagen (3 $\mu\text{g}/\text{mL}$), type IV collagen (3 $\mu\text{g}/\text{mL}$), or laminin (2 $\mu\text{g}/\text{mL}$) according to Buzza et al. (2005). After washing, *T. nattereri* venom (1 $\mu\text{g}/\text{mL}$), natterins (1 $\mu\text{g}/\text{mL}$) or natterectin (1 $\mu\text{g}/\text{mL}$) were added to each well and further incubated for 3 h at 37 °C. HeLa cells (5×10^5 cells/mL) were added to the plate and allowed to adhere and reach confluence (1–2 days). The wells were washed five times with PBS, and the adherent cells were fixed with 10% (w/v) formaldehyde in PBS. The bound cells were stained

with 0.05% (w/v) crystal violet for 30 min. The unbound dye was washed from the plates, and the stained cells were lysed with 1% (w/v) SDS for 60 min, and the absorbance of the wells was measured using a microtitre plate reader at 540 nm.

2.6. Effect of venom or toxins on cell adhesion

According to Rigot et al. (1998), HeLa cell suspensions (1×10^6 cells/mL) were allowed to adhere and reach confluence at 37 °C, 5% CO₂. Non-adherent cells were removed by washing twice with PBS and further treated for 24 h at 37 °C with 1 µg/mL of *T. nattereri* venom, natterins or natterectin. Non-adherent cells were removed by washing with PBS and the number of adherent cells was assessed as described above.

2.7. Detection of apoptosis or necrosis

HeLa cell suspensions (1×10^6 cells/mL) were incubated with 1 µg/mL of *T. nattereri* venom, natterins or natterectin for 24 h at 37 °C, 5% CO₂. After washing and centrifugation, the culture supernatant was discarded and the cell pellets were resuspended and viability was analyzed using a propidium iodide and FITC-annexin V binding assay (BD Biosciences, Mississauga, ON, Canada) according to Michie et al. (2003). The intensity of fluorescence of stained cells was acquired using a BD FACSCalibur flow cytometer and data were analyzed with CellQuest software (BD Biosciences, Mississauga, ON, Canada).

2.8. Cleavage of ECM proteins by natterins

According to Bonnefoy and Legrand (2000), natterins at 1 µg were incubated with 3 µg of type I collagen, or 2 µg of laminin, or 3 µg of type IV collagen in 20 µL of PBS for 24 h at 37 °C as were controls of matrix proteins and natterins. Reactions were stopped by addition of Laemmli sample buffer and submitted to electrophoresis on 8% or 4–20% SDS-polyacrylamide gels at 20 mA for 2 h. Gels were silver stained.

2.9. Effect of natterectin upon integrin-mediated functions in tumor cells

HeLa cells (1×10^6 cells/mL) were pre-incubated with a mixture of 10 µg/mL anti- α_5 and anti- β_1 mAbs for 30 min in ice. Cells were then added to 96-well microtitre plates coated with 1 µg/mL natterectin and allowed to adhere for 24 h at 37 °C, 5% CO₂. Adherent cells were stained with crystal violet, as described above, and viability was evaluated by the MTT assay (Mosmann, 1983). Data were expressed as a percentage of adhesion in the absence of toxins.

2.10. Detection of the interaction of natterectin to HeLa cell integrins

HeLa cells (1×10^6 cells/mL) incubated in the presence of 1 µg/mL natterectin at 4 °C for 4 h were stained with antibodies anti- β_1 (Purified armenian hamster IgG2* γ 1

anti-mouse CD29) or anti- α_5 mAb (Purified rat IgG2ak anti-mouse CD49e) for 30 min on ice. PE anti-armenian hamster IgG and FITC mouse anti-rat IgG were used as second antibodies. The cell surface expression of integrin subunits was determined by flow cytometry analysis according to Nobumoto et al. (2008). The mean of fluorescence intensity of stained cells was acquired using a BD FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada) and data analyzed with CellQuest software (BD Biosciences, Mississauga, ON, Canada).

2.11. Statistical analysis

All values were expressed as mean $\pm$ SEM. Parametric data were evaluated using analysis of variance, followed by the Tukey test for multiple comparisons. 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. Natterins and natterectin alter the HeLa adhesion to immobilized ECM components

First we identified the ability of natterins and natterectin to bind extracellular matrix proteins (laminin, types I and IV collagen). In Fig. 1A–C, we only observed high recognition of untreated ECM components by antibodies direct against type I collagen, laminin or type IV collagen; and an insignificant binding was reached by anti-venom, anti-natterins or anti-natterectin antibodies. After treatment of ECM components with *T. nattereri* venom (3 h, 37 °C), high levels of binding of natterins and natterectin were demonstrated to type I collagen (Fig. 1D), and of natterectin to type IV collagen (Fig. 1F). Natterins or natterectin showed weak binding affinity to laminin (Fig. 1E).

To determine whether binding of toxins to ECM components altered the adherent properties, HeLa cells were incubated in dishes coated with types I and IV collagen and laminin, all previously treated with venom or toxins. HeLa cells that exhibit anchorage-independent cell growth (Aplin et al., 1998) showed similar adhesion levels to types I or IV collagen and laminin-coated dishes, which did not differ to adhesion levels of HeLa cells to plastic (the first two columns on the left in Fig. 2A–C). As shown in the last column of Fig. 2A and C, adhesion of HeLa cells was not hampered by binding of natterectin to types I or IV collagen, while venom and mainly natterins treatments inhibited the adhesion of cells on dishes coated with types I and IV collagen (third and fourth columns in the Fig. 2A and C). In Fig. 2B, adhesion levels of HeLa cells to laminin were similar after venom or toxins treatments.

3.2. Proteolytic and cytotoxic effects of natterins

Based on the results that show natterins possess protease activity (Lopes-Ferreira et al., 2004) we investigate whether treatment of natterins directly degrade ECM components. For this, SDS-polyacrylamide gel

electrophoresis after 24 h at 37 °C of incubation with natterins was carried out. Under reducing conditions, soluble type IV collagen appears in two forms, full-length (>250 kDa) and a 120 kDa form, which was degraded by natterins (Fig. 3, lanes 7–8). The high molecular forms of

type I collagen above 250 kDa were also cleavage by natterins (Fig. 3, lanes 3–4). No proteolytic activity of natterins was observed to laminin (Fig. 3, lane 5–6).

The knowledge that natterins act degrading ECM components which is associated to detachment-induced

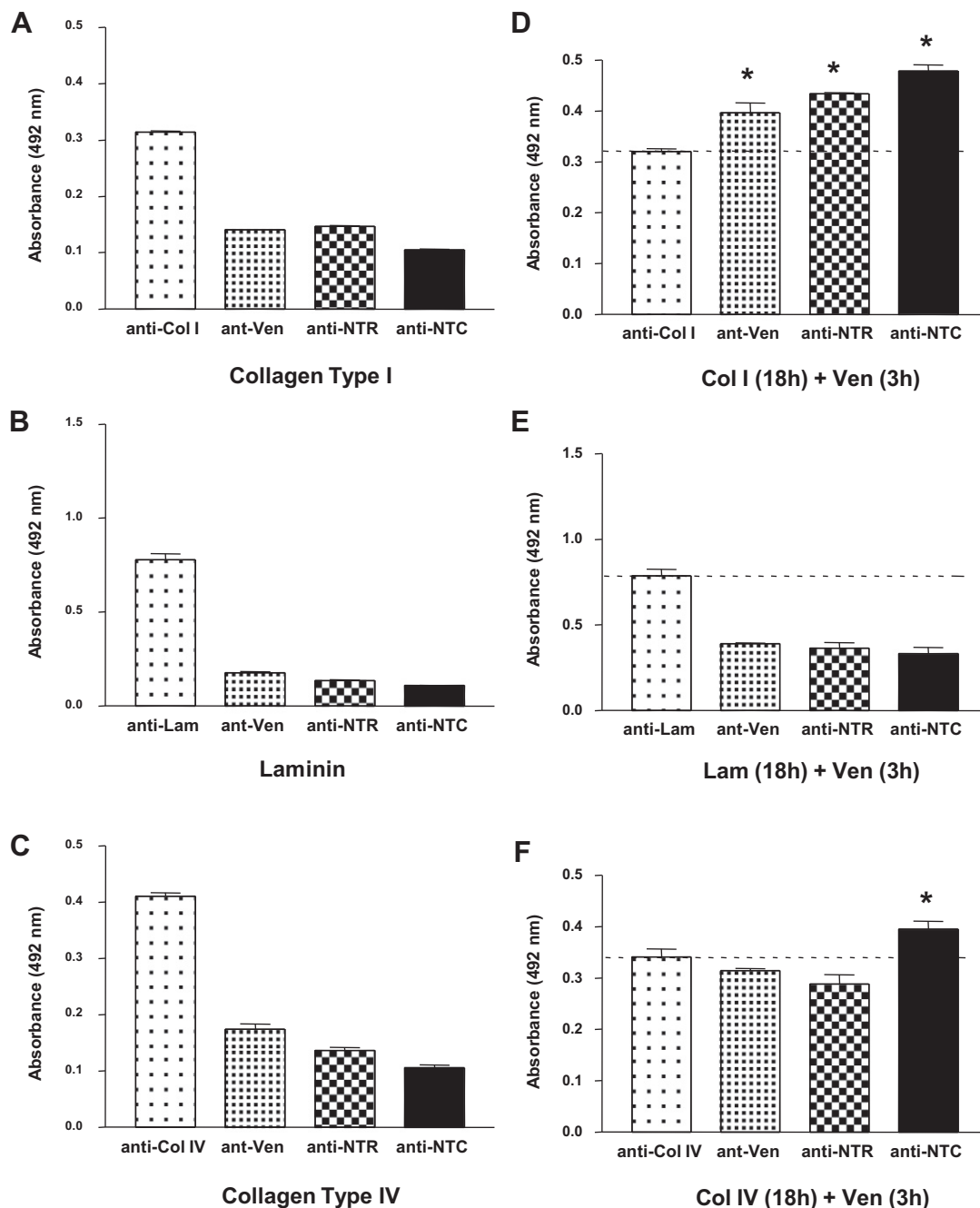


Fig. 1. Interaction of natterins and natterectin with ECM components. Wells were coated with types I and IV collagen (3 µg/mL), and 2 µg/mL laminin in PBS for 18 h at 4 °C. After blocking with BSA and washing, *T. nattereri* venom at 1 µg/mL was added for 3 h, at 37 °C. Bound toxins were quantified by ELISA using mouse anti-venom of *T. nattereri*, anti-natterins, anti-natterectin (all at 1 µg/mL, D–F) and ECM components were detected using specific goat anti-type I collagen, anti-type IV collagen, and anti-laminin (all at 1 µg/mL) as primary and an anti-goat IgG conjugated to horseradish peroxidase (A–C). The absorbance of the specific binding in triplicate wells was analyzed by spectrometry at 492 nm. **p* < 0.05 compared to specific binding.

cell death (anoikis) (Meredith et al., 1993) the next step was to investigate whether toxins induce death following cell detachment. As shown in Fig. 4A, treatment of adherent HeLa cells with venom or natterins, and not nattertin, resulted in a dramatic loss of adherent cells, which is consistent with the increased LDH leakage observed (data not shown). In addition, venom or natterins greatly affected total viability of cells at suspension, inducing cell death; and nattertin rescues cells from death by apoptosis (Fig. 4B). These results demonstrate that natterins act on the matrix to induce cell detachment and on cells to induce death; and nattertin is an important factor for survival of cells.

3.3. Nattertin inhibits integrin-mediated functions in tumor cells

The integrin $\alpha 5 \beta 1$ is the major integrin expressed by HeLa cells (at 2700–3200 receptors/cell), which bind to the 70 kDa amino terminal region of fibronectin, which contains the RGD sequence (Pankov and Yamada, 2002). Because cell surface levels of $\beta 1$ integrins are linked to the ability of HeLa cells to adhere to the ECM, we determined the effects of nattertin on cell adhesion using neutralizing antibodies to subunits $\beta 1$ and also to $\alpha 5$. In Fig. 5A, we observed a slight increase (13%) in adherent cells to dishes coated with nattertin (column 2) compared to plastic (column 1). Then, the blocking of $\alpha 5 / \beta 1$ subunits results in the 39% of inhibition of the cell adhesion to nattertin-adsorbed dishes (column 4). In addition, HeLa cells loss the viability after blocking of $\alpha 5 / \beta 1$ subunits when plated on nattertin-coated dishes (Fig. 5B).

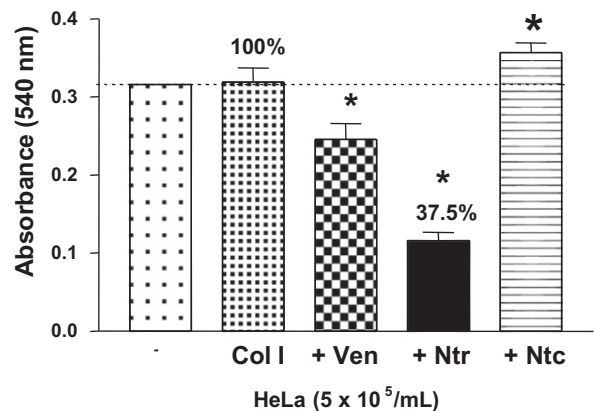
3.4. HeLa cell integrin is binding target for nattertin

Adhesion molecules play a pivotal role in cell adhesion and resistance to death. In order to clarify whether nattertin binds to $\alpha 5$ or $\beta 1$ subunits on cell surface, HeLa cells were exposed to nattertin for 4 h at 4 °C and then stained with antibodies to integrin subunits. We found a significant decrease of CD29 expression in HeLa cells treated with nattertin, showing that this lectin significantly binds the $\beta 1$ integrin subunit, and no binding was observed to $\alpha 5$ subunit (CD49e, Fig. 5C). These results mean that the adherence and viability of HeLa cells to nattertin are mostly because of the binding the $\beta 1$ integrin subunit.

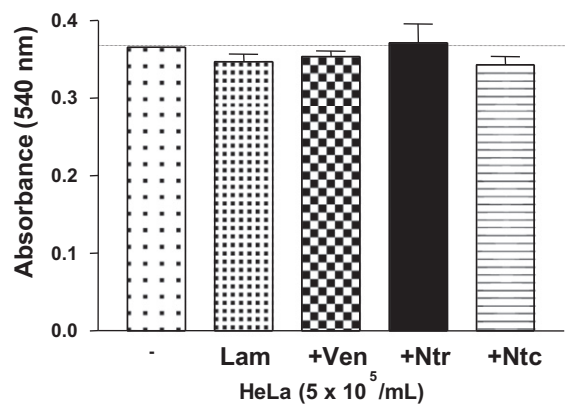
4. Discussion

Combined proteomic and transcriptomic approaches to study the composition of the venom of *T. nattereri* venomous fish (Magalhães et al., 2006) revealed the primary structures of the major toxins as a family of proteases natterins, never described in venoms and a C-type lectin nattertin. Natterins presents nociceptive, edema-inducing and kininogenase activity similar to that presented by the whole venom (Lopes-Ferreira et al., 2004; Magalhães et al., 2005) and nattertin, which contains the QPD (Gln-Pro-Asp) sequence in the carbohydrate recognition domain recognizes Gal- β (1–3)-N-acetylgalactosamine. Here we now report that extracellular matrix components

A Type I Collagen



B Laminin



C Type IV Collagen

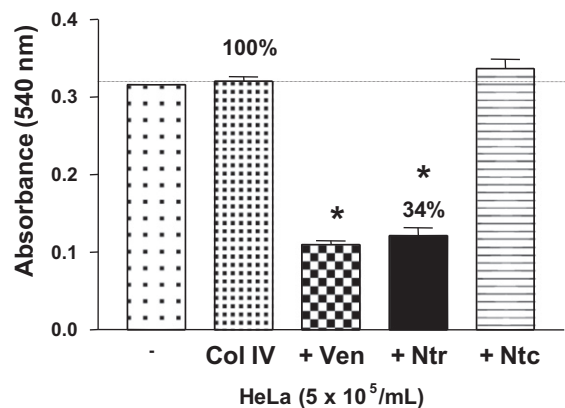


Fig. 2. Natterins detach cells from type I and IV collagens and nattertin induces cell adhesion. Coated wells with types I (A) and IV collagen (C) (3 $\mu\text{g/mL}$), and 2 $\mu\text{g/mL}$ laminin (B) were blocked with BSA and treated for 3 h at 37 °C with venom or toxin solutions at 1 $\mu\text{g/mL}$. HeLa cells were plated onto treated matrices in supplemented Eagle medium and allowed to adhere and reach confluence. For quantitation of remaining adherent cells, cells were fixed with formaldehyde and incubated with 0.05% crystal violet for 30 min under constant agitation. Deposited crystals were solubilized by 1% SDS and detected at 540 nm. * $p < 0.05$ compared to adhered HeLa cells to ECM components.

as well as the integrin $\beta 1$ subunit are targets for the natterins and nattectin. The extracellular matrix degradation or remodeling activities exerted by these toxins affect cell–cell and cell–extracellular matrix adhesion and survival and impair inflammatory cell migration into inflamed tissues.

The importance of extracellular matrix, including fibronectin, collagen, and laminin, to cellular growth and differentiation of normal and malignant cells has been known for many decades. Here we demonstrated the specific ability of the nattectin to bind type I collagen, basic constituent of the extracellular matrix and type V collagen, the integral structural component of venular basement membrane. In addition, natterins only bind the type I collagen. Previous reports have shown binding of snake venom metalloproteinases (SVMP) to collagen fibers, as occurs with crovidisin (Liu and Huang, 1997), catrocollastatin (Zhou et al., 1995), and jararhagin (Moura-da-Silva et al., 2008). After binding to collagen, the proteolytic activity of these SMVP persists and cleaves extracellular matrix components, resulting in disruption of capillary vessels and strong local hemorrhage.

Based on the previous results that show natterins have protease activity (Lopes-Ferreira et al., 2004) we provide evidence that the binding of natterins to type I collagen results in its proteolytic degradation. Our findings show that natterins can degrade *in vitro* type I collagen as well as type IV collagen, suggesting that these matrix components are more susceptible to natterins attack and can expose available sites for recognition and cleavage. This activity was also demonstrated by other enzymes such as kallikrein and plasmin, human serine proteases (Ledesma et al., 2000; Yousef and Diamandis, 2002), which present extensive cleavage activity that in turn release bioactive peptides and elicit various biological responses. Furthermore, the ability of natterins to cleave ECM proteins and also to inhibit the cell–ECM adhesion excludes the possibility of generation of pro-adhesive peptides by natterins.

Although the natterins cleavage sites in collagens are yet to be determined, given its ability to efficiently disrupt integrin-mediated HeLa adhesion to these matrices, natterins probably cleaves these proteins at the integrin–interaction site. Recently Buzza et al. (2005) demonstrate that human granzyme B (GrB) cleaves vitronectin and fibronectin in the RGD integrin-binding motif, explaining its ability to detach primary and transformed human cell lines.

Also, natterins have potential cytotoxic effect on adherent cells or cells in suspension, showing direct induction of cell death that is followed by cell detachment. Thus, the cooperation between degradation of ECM components and induction of cell death helps to explain the intense necrosis and a markedly inefficient healing response seen in *T. nattereri* victims (Lopes-Ferreira et al., 2001) and the very low inflammatory cellular influx into footpad lesions of mice (Lima et al., 2003).

Cell–ECM interactions are mediated by numerous adhesion receptors, of which integrins are the most prominent (Hynes, 1999). $\beta 1$ integrin can pair with the $\alpha 3$, $\alpha 6$ or $\alpha 7$ integrin subunit to form heterodimeric laminin receptors, with $\alpha 5$, αV or $\alpha 8$ to form fibronectin receptors, or with $\alpha 1$, $\alpha 2$, $\alpha 10$ or $\alpha 11$ to form collagen receptors

(Hynes, 2002). Naturally occurring integrin inhibitors have been found in venoms of snakes, leeches and insects. Most of the known integrin inhibitors are disintegrins (White et al., 2001), which bear a functional Arg–Gly–Asp (RGD) sequence in a flexible loop; and C-type lectin proteins (CLPs) (Eble and Tuckwell, 2003; Pilonet et al., 2007; Saray et al., 2007).

Consistent with the idea that C-type lectins can bind integrins and inhibit its binding to ECM components we further confirm the role of nattectin in cell attachment and viability. We found that nattectin in contrast, improves integrin-mediated cell adhesion presumably binding to glycoproteins at the cell surface via its lectin-domain. As previously reported, galectins as Gal-8 acts via interaction with integrins on adherence and spread of several types of normal and transformed cells (Liu and Rabinovich, 2005). To determine whether nattectin binds to $\alpha 5$ or $\beta 1$ integrin subunits on cell surface we searched for binding by means of flow cytometry, using incubation of HeLa cells with nattectin for 4 h at 4 °C and then stained with antibodies to integrin subunits.

Ours results show that the improvement of the adherence of HeLa cells mediated by nattectin can be attributed to its binding to RGD-dependent integrins, especially the $\beta 1$ subunit. It is not possible speculate that nattectin contain an RGD integrin-binding motif (Arg–Gly–Asp) since the peptide sequence of nattectin is now entirely known and does not contain any RGD site (Lopes-Ferreira et al., 2011). Interestingly, the finding that the anti- $\alpha 5$ /anti- $\beta 1$

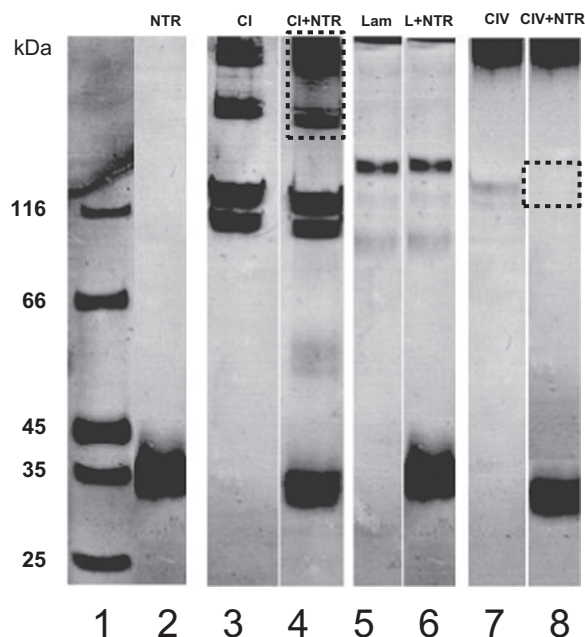


Fig. 3. Natterins cleave the major ECM components. Natterins at 1 μ g were incubated with 3 μ g of type I collagen, 2 μ g of laminin, or 3 μ g type IV collagen in PBS as were control matrix proteins and natterins. Reactions were stopped by addition of Laemmli sample buffer and submitted to electrophoresis on 8% or 4–20% SDS-polyacrylamide at 20 mA for 2 h. Gels were silver stained.

antibodies did not eliminate all the binding suggests that other adhesive proteins on the HeLa cells may be important to cell adhesion of these cells.

Altogether, our data that show the binding of nattertin in a carbohydrate-dependent manner with glycoprotein structures on the cell surface (as $\beta 1$ integrin subunit) and with glycosylated components of the ECM lead us to hypothesize the requirement of two CRD's or dimerization of nattertin. Almost all galectins either contain CRD's or dimers, and bivalence is a prerequisite for Gal-3 (Ahmad et al., 2004) and Gal-9 activities by their interactions and formation of lattices (Matsushita et al., 2000).

Furthermore, the increased survival of HeLa cells induced by nattertin can be correlated to the possible binding of nattertin to $\beta 1$ integrin. This interaction could

result in the generation of intracellular signaling (Mitra and Schlaepfer, 2006), in the modulation of the affinity or avidity of the $\beta 1$ integrins for their ligands; and finally with the generation of counter-balance proteins with anti-(Bcl-2, Mcl-1, Bcl-X_L) or pro-apoptotic (Bad) activities.

In conclusion, we have defined the point of divergence between natterins and nattertin and shown that natterins act as protease degrading extracellular matrix components and promoting necrosis and cell detachment; and that nattertin improves both integrin-mediated cell adhesion and resistance to apoptosis, via its lectin function. The extracellular matrix degradation or remodeling activities exerted by these toxins affect cell–cell and cell–extracellular matrix adhesion and survival and impair inflammatory cell migration into inflamed tissues.

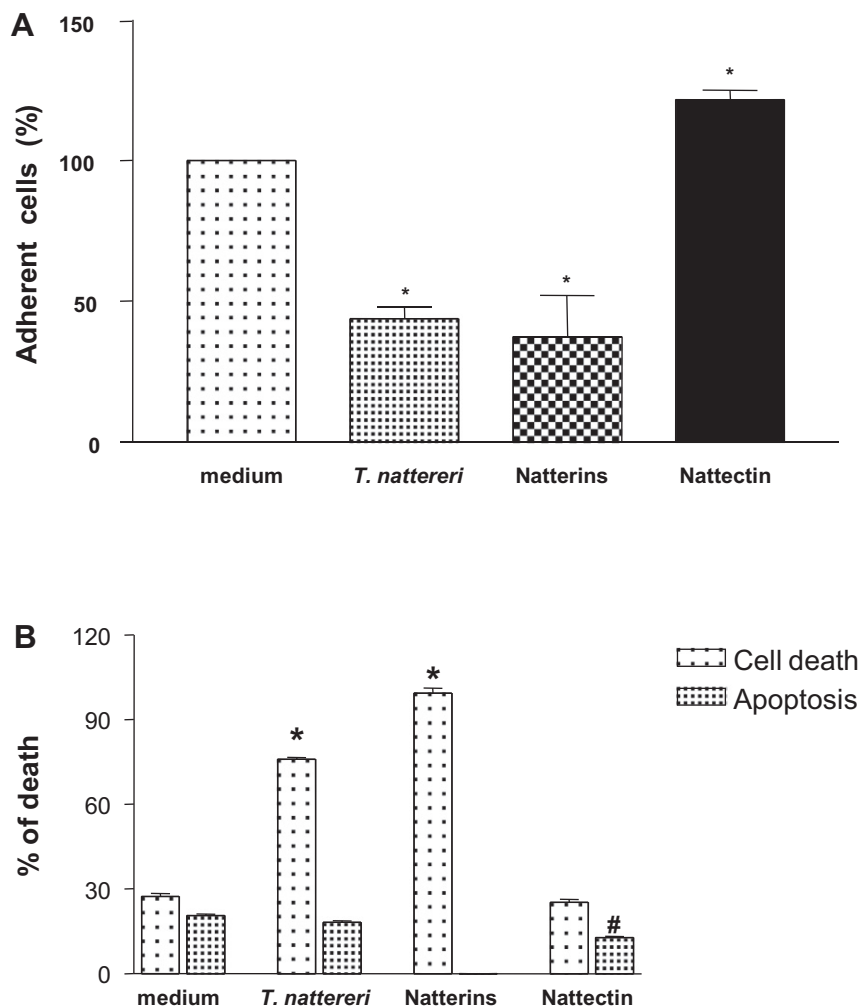


Fig. 4. Detachment and death of adherent cells exposed to Natterins. Confluence HeLa cells were treated with 1 $\mu\text{g/mL}$ of venom or toxins in supplemented Eagle's medium for 24 h (A). Non-adherent cells were removed to assess the number of adherent cells as described in Fig. 2. HeLa cell suspensions (1×10^6 cells/mL) were incubated with 1 $\mu\text{g/mL}$ of venom, natterins and nattertin for 24 h at 37 °C, 5% CO₂. After washing and centrifugation, the culture supernatant was discarded and the cell pellet was resuspended and viability was analyzed using a propidium iodide and FITC-annexin V binding assay (B). The intensity of fluorescence of stained cells was acquired using a BD FACSCalibur flow cytometer and data analyzed with CellQuest software. FITC-annexin V and PI negative show viable cells, or no measurable apoptosis; FITC-annexin V positive and PI negative show early apoptosis, membrane integrity is present; and finally FITC-annexin V and PI positive show end stage apoptosis and cell death. * $p < 0.05$ compared to un-stimulated cells and # $p < 0.05$ compared to control apoptosis.

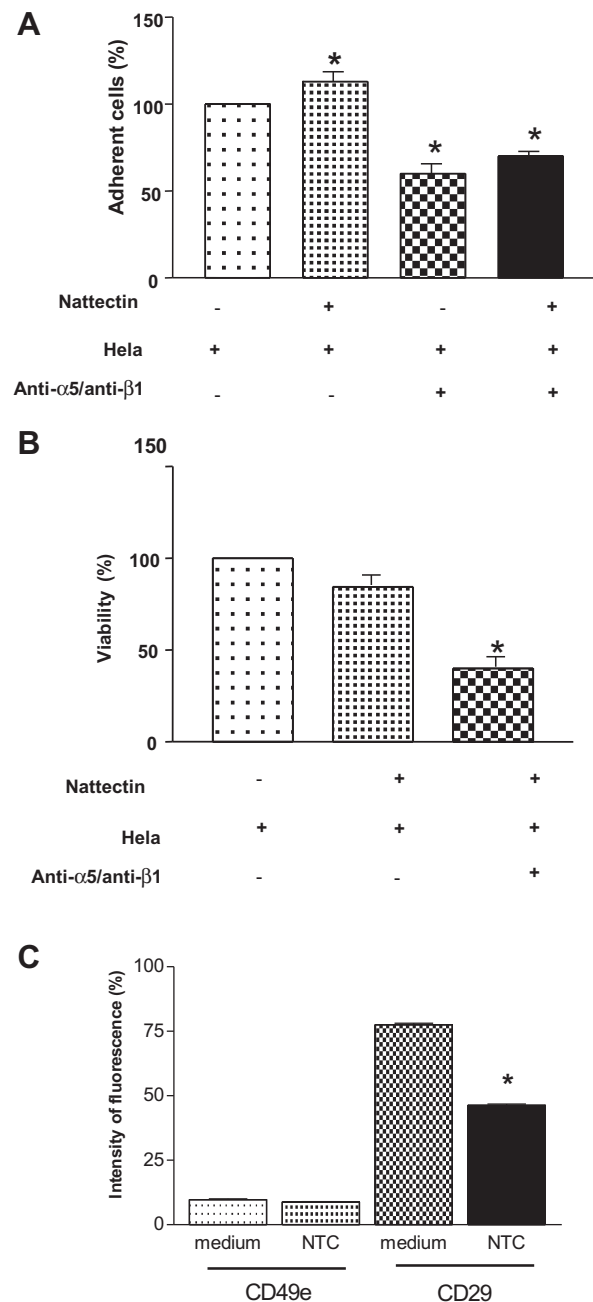


Fig. 5. Nattectin inhibits integrin-mediated functions in tumor cells. HeLa cells (1×10^6 cells/mL) were pre-incubated with a mixture of 10 μ g/mL anti- $\alpha 5$ mAb and anti- $\beta 1$ for 30 min in ice. Cells were then added to 96-well microtitre plates coated with 1 μ g/mL nattectin (NTC) and allowed to adhere for 24 h at 37 °C, 5% CO₂. Adherent cells were stained with crystal violet (A) and, viability was determined by MTT assay (B). The binding of nattectin on HeLa (C) was determined by flow cytometric analyses after stain of cells with antibodies anti- $\beta 1$ (CD29) and anti- $\alpha 5$ mAb (CD49e) for 30 min. PE anti-american hamster IgG and FITC mouse anti-rat IgG were used as second antibodies. The intensity of fluorescence of stained cells was acquired using a BD FACSCalibur flow cytometer and data analyzed with CellQuest software. * $p < 0.05$ compared to untreated cells.

Conflict of interest statement

None of the authors has any potential financial conflict of interest related to this manuscript.

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References

- Ahmad, N., Gabius, H.J., Andre, S., Kaltner, H., Sabesan, S., Roy, R., Liu, B., Macaluso, F., Brewer, C.F., 2004. Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *J. Biol. Chem.* 279, 10841–10847.
- Aplin, A., Howe, A., Alahari, S., Juliano, R., 1998. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol. Rev.* 50, 197–263.
- Bonnefoy, A., Legrand, C., 2000. Proteolysis of subendothelial adhesive glycoproteins (fibronectin, thrombospondin, and von Willebrand factor) by plasmin, leukocyte cathepsin G, and elastase. *Thromb. Res.* 98, 323–332.
- Buzza, M.S., Zamurs, L., Sun, J., Bird, C.H., Smith, A.I., Trapani, J.A., Froelich, C.J., Nice, E.C., Bird, P.I., 2005. Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. *J. Biol. Chem.* 280, 23549–23558.
- Eble, J.A., Tuckwell, D.S., 2003. The $\alpha 2\beta 1$ integrin inhibitor rhodocetin binds to the A domain of the integrin $\alpha 2$ subunit proximal to the collagen-binding site. *Biochem. J.* 376, 77–85.
- Faco, P.E., Havt, A., Barbosa, P.S., Nobre, A.C., Bezerra, G.P., Menezes, D.B., Fonteles, M.C., Lopes-Ferreira, M., Monteiro, H.S., 2003. Effects of *Thalassophryne nattereri* fish venom in isolated perfused rat kidney. *Toxicon* 42, 509–514.
- Fonseca, L.A., Lopes-Ferreira, M., 2000. Clinical and experimental studies regarding poisoning caused by a fish *Thalassophryne nattereri* (niquim). *An. Bras. Dermatol.* 75, 435–443.
- Hynes, R.O., 1999. Cell adhesion: old and new questions. *Trends Cell Biol.* 9, 33–37.
- Hynes, R.O., 2002. Integrins: bidirectional, allosteric signaling machines. *Cell* 110, 673–687.
- Kalantarov, G., Acevedo, H.F., 1998. Demonstration of dose dependent cytotoxic activity in cancer cells by specific human chorionic gonadotropin monoclonal antibodies. *Cancer* 83, 783–787.
- Ledesma, M.D., Da Silva, J.S., Crassaerts, K., Delacourte, A., De Strooper, B., Dotti, C.G., 2000. Brain plasmin enhances APP alpha-cleavage and Abeta degradation and is reduced in Alzheimer's disease brains. *EMBO Rep.* 1, 530–535.
- Lima, C., Clissa, P.B., Piran-Soares, A.A., Tanjoni, I., Moura-da-Silva, A.M., Lopes-Ferreira, M., 2003. Characterisation of local inflammatory response induced by *Thalassophryne nattereri* fish venom in a mouse model of tissue injury. *Toxicon* 42, 499–507.
- Liu, C.Z., Huang, T.F., 1997. Crovidisin, a collagen-binding protein isolated from snake venom of *Crotalus viridis*, prevents platelet-collagen interaction. *Arch. Biochem. Biophys.* 337, 291–299.
- Liu, F.T., Rabinovich, G.A., 2005. Galectins as modulators of tumour progression. *Nat. Rev. Cancer* 5, 29–41.
- Lopes-Ferreira, M., Bárbaro, K.C., Cardoso, D.F., Moura-da-Silva, A.M., Mota, I., 1998. *Thalassophryne nattereri* fish venom: biological and biochemical characterization and serum neutralization of its toxic activities. *Toxicon* 36, 405–410.
- Lopes-Ferreira, M., Emim, J.A., Oliveira, V., Puzer, L., Cezari, M.H., Araújo, M.S., Juliano, L., Lapa, A.J., Souccar, C., Moura-da-Silva, A.M., 2004. Kininogenase activity of *Thalassophryne nattereri* fish venom. *Biochem. Pharmacol.* 68, 2151–2157.
- Lopes-Ferreira, M., Magalhães, G.S., Fernandez, J.H., Junqueira-de-Azevedo, I.L.M., Ho, L.P., Lima, C., Valente, R.H., Moura-da-Silva, A.M., 2011. Structural and biological characterization of Nattectin, a new C-type lectin from the venomous fish *Thalassophryne nattereri*. *Biochimie* 93, 971–980.
- Lopes-Ferreira, M., Moura-da-Silva, A.M., Piran-Soares, A.A., Ângulo, Y., Lomonte, B., Gutiérrez, J.M., Farsky, S.H., 2002. Hemostatic effects induced by *Thalassophryne nattereri* fish venom: a model of endothelium-mediated blood flow impairment. *Toxicon* 40, 1141–1147.

- Lopes-Ferreira, M., Núñez, J., Rucavado, A., Farsky, S.H.P., Lomonte, B., Ângulo, Y., Moura-da-Silva, A.M., Gutierrez, J.M., 2001. Skeletal muscle necrosis and regeneration after injection of *Thalassophryne nattereri* (niquim) fish venom in mice. *Int. J. Exp. Pathol.* 82, 55–64.
- Magalhães, G.S., Junqueira-de-Azevedo, I.L., Lopes-Ferreira, M., Lorenzini, D.M., Ho, P.L., Moura-da-Silva, A.M., 2006. Transcriptome analysis of expressed sequence tags from the venom glands of the fish *Thalassophryne nattereri*. *Biochimie* 88, 693–699.
- Magalhães, G.S., Lopes-Ferreira, M., Junqueira-de-Azevedo, I.L.M., Spencer, P.J., Araújo, M.S., Portaro, F.C.V., Ma, L., Valente, R.H., Juliano, L., Fox, J.W., Ho, P.L., Moura-da-Silva, A.M., 2005. Natterins, a new class of proteins with kininogenase activity characterized from *Thalassophryne nattereri* fish venom. *Biochimie* 87, 687–699.
- Matsushita, N., Nishi, N., Seki, M., Matsumoto, R., Kuwabara, I., Liu, F.T., Hata, Y., Nakamura, T., Hirashima, M., 2000. Requirement of divalent galactoside-binding activity of ecalectin/galectin-9 for eosinophil chemoattraction. *J. Biol. Chem.* 275, 8355–8360.
- Meredith, J.E., Fazeli Jr., B., Schwartz, M.A., 1993. The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* 4, 953–961.
- Michie, J., Akudugu, J., Binder, A., Van Rensburg, C.E., Böhm, L., 2003. Flow cytometric evaluation of apoptosis and cell viability as a criterion of anti-tumour drug toxicity. *Anticancer Res.* 23, 2675–2679.
- Mitra, S.K., Schlaepfer, D.D., 2006. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr. Opin. Cell Biol.* 18, 516–523.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63.
- Moura-da-Silva, A.M., Ramos, O.H., Baldo, C., Niland, S., Hansen, U., Ventura, J.S., Furlan, S., Butera, D., Della-Casa, M.S., Tanjoni, I., Clissa, P. B., Fernandes, I., Chudzinski-Tavassi, A.M., Eble, J.A., 2008. Collagen binding is a key factor for the hemorrhagic activity of snake venom metalloproteinases. *Biochimie* 90, 484–492.
- Nobumoto, A., Nagahara, K., Oomizu, S., Katoh, S., Nishi, N., Takeshita, K., Niki, T., Tominaga, A., Yamauchi, A., Hirashima, M., 2008. Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices. *Glycobiology* 18, 735–744.
- Pankov, R., Yamada, K.M., 2002. Fibronectin at a glance. *J. Cell Sci.* 115, 3861–3863.
- Pareja-Santos, A., Saraiva, T.C., Costa, E.P., Santos, M.F., Zorn, T.T., Souza, V. M., Lopes-Ferreira, M., Lima, C., 2009. Delayed local inflammatory response induced by *Thalassophryne nattereri* venom is related to extracellular matrix degradation. *Int. J. Exp. Pathol.* 90, 34–43.
- Pilorget, A., Conesa, M., Sarraz, S., Michaud-Levesque, J., Daoud, S., Kim, K. S., Demeule, M., Marvaldi, J., El Ayeb, M., Marrakchi, N., Beliveau, R., Luis, J., 2007. Lebectin, a *Macrovipera lebetina* venom-derived C-type lectin, inhibits angiogenesis both in vitro and in vivo. *J. Cell. Physiol.* 211, 307–315.
- Piran-Soares, A.A., Komegae, E.N., Souza, V.M.O., Fonseca, L.A., Lima, C., Lopes-Ferreira, M., 2007. Neutralizing antibodies obtained in a persistent immune response are effective against deleterious effects induced by the *Thalassophryne nattereri* fish venom. *Toxicon* 49, 920–930.
- Rigot, V., Lehmann, M., Andre, F., Daemi, N., Marvaldi, J., Luis, J., 1998. Integrin ligation and PKC activation are required for migration of colon carcinoma cells. *J. Cell Sci.* 111, 3119–3127.
- Sarraz, S., Delamarre, E., Marvaldi, J., El Ayeb, M., Marrakchi, N., Luis, J., 2007. Lebectin and lebecetin, two C-type lectins from snake venom, inhibit $\alpha 5\beta 1$ and αv -containing integrins. *Matrix Biol.* 26, 306–313.
- White, J.M., Bigler, D., Chem, M., Takahashi, Y., Wolfsberg, T.G., 2001. In: Beckerle, M.C. (Ed.), *Cell Adhesion*, vol. 39. Oxford University Press, Oxford, pp. 189–216 (Chapter 6).
- Yousef, G.M., Diamandis, E.P., 2002. Human tissue kallikreins: a new enzymatic cascade pathway? *Biol. Chem.* 383, 1045–1057.
- Zhou, Q., Smith, J.B., Grossman, M.H., 1995. Molecular cloning and expression of catrocollastatin, a snake-venom protein from *Crotalus atrox* (western diamondback rattlesnake), which inhibits platelet adhesion to collagen. *Biochem. J.* 307, 411–417.