2. Biochemistry

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2.01 Isolation of bioactive peptides by the action of serine proteases from the venom of *Bothrops jararaca* on endogenous substrates and their actions in cell culture

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Introduction: Venoms are a rich source of proteolytic enzymes. In *Bothrops jararaca* venom, serine proteases and metalloproteases are the main enzymes that act on various tissues and proteins present in the victim. Besides the action on tissues, these proteases could generate some compounds that could have specific actions in cells or display other mechanisms towards the generation of bioactive peptides. Protein precursors of bioactive peptides are generally the target, but nowadays there is new class of proteins that in some conditions may generate bioactive peptides, namely cripteins. **Objectives:** The objectives were to a) identify the bioactive peptides resulting from the action of the serine proteases, trypsin and those from the venom of *Bothrops jararaca* on endogenous substrates, and b) to biochemically isolate and characterize the peptides obtained and determine the possible effects and biological properties of these peptides through several biological tests in vitro and in vivo. **Methods:** A serine protease from *Bothrops jararaca* was separated from the whole venom using an HPLC column for molecular exclusion. The endogenous substrates were also incubated with trypsin and the serine protease from *Bothrops jararaca*. After incubation, proteins and other products were observed by gel electrophoresis with silver nitrate staining and also by means of HPLC profile. The pools of the hydrolysates were tested on cultured fibroblasts and then the purified peaks of these pools. After testing of the peaks on cultured cells, the active peptides were sequenced and synthesized for further testing. **Results and Discussion:** Peaks obtained by HPLC from the hydrolysis of substrates induced cell proliferation in tests with FN-1 cells (fibroblasts) and caused positive membrane lipid peroxidation, and picrosirius red staining showed low stimulation of collagen. These peptides were also found in the proteins that are observed on the cell surface. Therefore, this study suggests that serine proteases are able to generate important biologically active peptides, especially in the interaction of snake venom with human macromolecules.

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2.02 Purification and identification of microRNAs from *Bothrops jararaca* venom glands

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Introduction: The snake venom gland probably evolved from salivary glands of non-venom ancestral species. This epithelium is very specialized in toxin production and is subject to various kinds of regulations, with protein synthesis being initiated by different factors such as reduction of the volume of lumen secretion and other mechanisms dependent on adrenoreceptors. MicroRNAs are small RNAs of 21-23 nt that function as post-transcriptional regulators of gene expression. MicroRNAs have essential roles in development, cell differentiation, cell proliferation, cell death and other functions in plants and animals. **Objectives:** Our aim in this work was to purify and identify microRNAs involved in the regulation of the production and secretion of proteins present in the venom glands of snakes. **Methods:** Our snake model was *Bothrops jararaca*, a Brazilian species belonging to the family Viperidae. In this study, microRNAs were isolated and enriched from total RNA of *B. jararaca* venom glands. After the ligation of suitable clone linkers (mirCAT kit - IDT) the microRNAs, converted into cDNAs were cloned, sequenced, and analyzed by bioinformatics. **Results and Discussion:** The bioinformatics analysis revealed the identity of different small RNAs, with a size range of 13 to 26 nt. Seventeen cloned sequences showed the following sizes: 13 nt (1/17; 6%); 16 nt (4/17; 24%); 18 nt (1/17; 6%); 19 nt (2/17; 12%); 21 nt (1/17; 6%); 22 nt (2/17; 12%); 23 nt (2/17; 12%); 24% (1/17; 6%); 25% (1/17; 6%); 26% (2/17; 12%). The 21-23 nt cloned sequences showed similarity to known microRNAs, such as eca-miR-99a and xtr-miR-99, found in different organisms. We also found significant similarity to piRNAs, a different class of small RNAs, in our 24-26 nt samples. The functions of small RNAs will be analyzed in order to better understand gene regulation in the venom gland. microRNAs represent a new boundary in the knowledge of gene regulation in animals and plants, but the snake microRNA transcriptome set has not yet been studied. We found the first microRNAs and piRNAS in reptiles. Since Brazil is an important repository of this fauna, it is very important to ensure primacy in the study of microRNAs from this taxon.

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2.03 Effects of rLosac on coagulation parameters and comparative studies with RVV-X in the activation of factor X

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Introduction: Losac is the first factor X (FX) activator purified from *Lonomia obliqua* bristles. The mechanism of cleavage is still unknown. On the other hand, the mechanism of RVV-X, a FX activator from *Daboia Russelli,* is well known. RVV-X activates FX through the recognition of the Gla domain, and after this the cleavage of the FX heavy chain occurs through the RVV-X metalloprotease domain. **Objectives:** The aim of the study was to obtain recombinant Losac with enzymatic activity and to compare its activity with RVV-X. **Methods:** The *E. coli* BL21 (DE3) strain was transformed with the recombinant plasmid (pAE-Losac). This system is designed for expression of rLosac with a tail of 6x-His at the N-terminal end. We evaluated the activation of human FX (345 nM) using different concentrations of rLosac and RVV-X in chromogenic assays (0.2 mM S-2765) and also based on the degradation of normal and deglycosylated FX by SDS-PAGE. The specificity of Losac activity for FX was evaluated assaying its activity with several other substrates, including prothrombin (chromogenic assays), fibrinogen and fibrin (SDS-PAGE and fibrin plates, respectively). The capacity of rLosac to activate factor X in plasma was assayed by plasma recalcification time, TTPA and TP. **Results and Discussion:** rLosac and RVV-X activated FX in a dose-dependent manner. rLosac was able to activate deglycosylated FX, although this activity was lower than that with normal FX. Apparently, the protein is specific for FX, since it does not activate prothrombin and does not cleave fibrinogen or fibrin. The normal plasma recalcification time (195.6±37.67s) was shortened after 2 min of incubation with rLosac (70.08 ± 4.80 s) or RVV-X (59.4 ± 3.48 s). Moreover, when we incubated in plasma for 10 min, rLosac, using similar molar concentrations of rLosac or RVV-X, induced a more significant reduction of recalcification time - rLosac:(11.64 ± 4.27.s), RVV-X:(54.06 ± 6.15.s). The TTPA and TP times were reduced when incubated for 2 min with rLosac in comparison with the control - control TTPA:(196.05 ± 2.52), rLosac TTPA:(162.9 ± 5.14), control TP:(16, 6 ± 0.52), rLosac TP:(13.1 ± 0.52). These results demonstrate that the activation of FX by rLosac is different from the one produced by RVV-X. The exact mechanism is not yet known. We are working to understand the mechanism by which rLosac recognizes FX.

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2.04 Characterization of keratinocyte responses to FGF1, FGF2 and FGF7

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Introduction: Keratinocytes, the main epidermal cells, are responsible for the formation of an impermeable barrier that protects the body against injuries and attachment of microorganisms. Therapeutic agents that favor wound healing are of great interest to medicine and have been extensively sought. Studies have suggested the participation of FGF (fibroblast growth factor) family members in wound healing. It is known that these factors promote growth (FGF7 and FGF10, also known as keratinocyte growth factor 1 and 2, respectively) and stimulate the migration of keratinocytes, but their molecular mechanisms and biological role in these cells are not totally established. **Objectives:** The aim of this study was to characterize keratinocyte responses to FGF1, FGF2 and KGF and the expression of endogenous FGFs and FGF receptors (FGFRs). **Methods:** Responses of the HaCaT cell line and keratinocytes immortalized with E6 and E7 (HPV oncogenes) to FGFs were monitored by growth curves, mitotic nuclei counting, MTT assay, migration assay and Western blotting for ERK and Akt phosphorylation. The expression of FGFRs (fibroblast growth factor receptors) and endogenous FGFs were determined by quantitative PCR and PCR, respectively. **Results and Discussion:** All three growth factors, namely, FGF1, 2 and 7, activated the ERK pathway, but only FGF7 promoted keratinocyte proliferation, whereas FGF1 and 2 were more important in triggering migration. Keratinocytes expressed FGFR2, FGFR3 and FGFR4, the last being detected for the first time in this cell type. These cells also expressed endogenous FGF2, which can have a role in paracrine stimulation of dermal fibroblasts and an autocrine role stimulating its own migration. These results suggest that different FGFs trigger different responses in keratinocytes and probably are very important in wound healing, promoting keratinocyte proliferation and migration and, in addition, the stimulation of dermal cells.

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2.05 Antimicrobial activity in whole extracts of two species of Chilopoda

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Introduction: One of the main components of the defense mechanism in vertebrates and invertebrates are the peptides with immune functions. The defensive role of a variety of antibiotic peptides and different types of molecules in multicellular organisms is increasingly recognized. However, the characterization of the molecules with antimicrobial function from chilopods had not begun until recently. For these reasons, it is important to study this subject, not only to understand the success of these factors in the defense mechanisms of these animals, but also to find alternatives to fight the infectious and also parasitic diseases that affect humans. **Objectives:** The aim of this study was the analysis of different molecules in order to identify the antimicrobial factors in the extract of the body of the Brazilian myriapods *Otostigmus* sp. and *Escolopendra viridicornis*. **Methods:** The bodies of the animals were first subjected to maceration and acid extraction and then fractionated in two steps. First, using C18 Sep Pak column cartridge, the hydrophilic and hydrophobic fractions were separated. The hydrophilic fraction was purified in two more steps, first by ion exchange chromatography and then by gel filtration chromatography. The hydrophobic fraction was loaded onto a semi-preparative C18 Jupiter column using a linear gradient of ACN in 0.05% TFA for the second purification step. The column effluent was monitored by absorbance at 225 nm and the antiparasitic and antimicrobial activities were determined by liquid growth inhibition assay. First, characterization was by mass spectrometry (MALDI-TOF). **Results and Discussion:** After HPLC separation of the highly hydrophilic compounds, antimicrobial activity against *Escherichia coli* and *M. luteus* was detected in three fractions (one from *Otostigmus* sp. and two from *E. viridicornis*). These fractions also showed anti-parasite activity against *Leshmania* spp. and *T. brucei*. The analysis with mass spectrometry showed that these fractions have a low molecular weight. In the hydrophobic fraction, seven fractions were detected with antimicrobial activity against *M luteus* (3 fractions) and *E. coli* (4 fractions). Four were from *Otostigmus* sp. and three from *E. viridicornis*. The characterization of these fractions is still in progress.

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2.06 Characterization of major cathepsin L-like proteases from hepatopancreas of the scorpion *Tityus serrulatus*

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Introduction: Cathepsin-L is widely distributed in living organisms and is associated with protein degradation in lysosomes, but some groups of Arthropoda (Hemiptera, Heteroptera, Coleoptera and Acari) show this enzyme related to the digestion of proteins ingested. Our group had found for the first time that the major endopeptidase responsible for protein digestion in the scorpion *Tityus serrulatus* is a cathepsin-L-like cysteine peptidase. **Objectives:** The aim of this study was the characterization of the major cathepsin-L-like component present in the hepatopancreas of the scorpion *Tityus serrulatus*. **Methods:** In order to characterize this enzyme, *Tityus serrulatus* females were fed *Gryllus sp*, dissected and the isolated hepatopancreas was homogenized in a solution of 1 mM MMTS, a reversible inhibitor of cysteine peptidases, dissolved in Milli-Q water. Enzyme source was obtained from crude homogenized samples or partially purified on a HiTrap S column equilibrated in 50 mM citrate-phosphate buffer, pH 5.0, where the elution was obtained with a gradient of 0 – 1 M NaCl in the same buffer. Activity was measured using Z-FR-MCA or Abz-FRQ-EDDnp as substrate in different buffers, all containing 3 mM cysteine and EDTA. **Results and Discussion:** Assays using fluorescent or quenched fluorescent substrates indicated that the enzyme is a cathepsin-L-like hydrolyzing preferentially substrates with a Phe residue at P2. This enzyme showed characteristics similar to another arthropod's digestive cathepsins L, with a pH optimum of 5.5, a molecular weight of 44 kDa (proenzyme) and 26 kDa (active form) determined by Western blotting using an antibody specific for insect digestive cathepsin-L. It was mainly stable in acidic conditions (pH 2.5 to 6.5) at 4°C and 30°C, with thermal stabity showing a half-life of 8 min at 65°C. The isoeletric point calculated for the proenzyme was 5.8 and for the active form 6.5. Also, the K_m values were calculated for two different substrates: Z-FR-MCA (24 μM) and Abz-FRQ-EDDnp (30 nM).

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2.07 Antibiotics from spider eggs

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Introduction: The proliferation of infectious diseases is an important human health problem. Inappropriate use of antibiotics brings about the appearance of bacterial strains resistant to conventional drugs, which means that research is necessary to identify new molecules as alternatives to the currently used drugs. The study of antimicrobial peptides may be advantageous for human use, because its action is faster than conventional antibiotics. Many studies have identified antimicrobial peptides in several species. One example is gomesin, an antimicrobial peptide isolated from the hemocytes of the spider *Acanthoscurria gomesiana,* which possesses a high spectrum of activity against many microorganisms. Lysozymes, 14kDa molecules with antimicrobial activity against Gram-positive bacteria and yeast, have been identified in eggs of some species. Studies on spider silk pointed to the possibility of antimicrobial activity, but no research reported antimicrobial activity from spider eggs. **Objectives:** Our objective was to purify and characterize antimicrobial factors from eggs of the *Phoneutria nigriventer* spider. **Methods:** In order to do so, we used reversed phase high performance liquid chromatography (RP-HPLC) to isolate proteins and peptides, electrophoresis and mass spectrometry techniques to characterize them, and Edman degradation for sequencing **Results and Discussion:** As results, we isolated two antimicrobial peptides from these eggs. The peptides were active against the Gram-positive bacteria *Micrococcus luteus*. Two other fractions, most likely lysozymes, were purified and were active against the yeast *Candida albicans* and the bacterium *M. luteus*. This is the first report of antimicrobial factors from spider eggs.

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2.08 Production of a recombinant platelet aggregation inhibitor from the leech *Haementeria depressa* in *Pichia pastoris*

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Introduction: The saliva of hematophagous animals has substances that maintain blood fluidity during the feeding process, including molecules affecting hemostatic processes. Our group has identified several compounds and determined the profile of transcripts and proteins from the *H. depressa* leech salivary complex through biochemical, transcriptomic and proteomic analysis. In this tissue were detected some clones similar to an inhibitor of platelet aggregation from *H. officinallis* leech named LAPP. LAPP is around 14 kDa and has a pI of 4.0, and it inhibits platelet aggregation by collagen. **Objectives:** The aim of this work was to clone, in an expression vector (pPIC9K), a transcript from the *H.depressa* salivary complex cDNA library which showed identity to LAPP, and to define the best method of expression and purification of this clone for future comparative studies with LAPP. **Methods:** The H06A09_pGEM11Zf was chosen for this study, and after amplification by PCR with specific primers, the product was cloned in pPIC9K vector between *EcoRI* and *NotI* cloning sites. The sequence of the H06A09_pPIC9K clone was confirmed, and it was linearized using *Sac*I digestion. The clone was transformed in *Pichia pastoris* (GS115) and expressed in different conditions. The recombinant protein expressed was submitted to some different purification methods [ultra filtration (Amicon / Millipore 5 and 30kDa); gel filtration (Superdex 75 / GE); anionic exchange (Mono-Q / GE) and reverse phase (C18)]. The different expression and purification steps were analyzed by SDS-PAGE. The platelet-aggregation inhibition assays were performed using collagen as agonist with protein of some purification steps, performed in whole blood. **Results and Discussion:** The H06A09_pPIC9K sequencing showed 93% similarity with LAPP, where all Cys residues were conserved. The best expression method was standardized in BMGY culture medium at 28°C, 260 rpm using 96 h of induction by 0.5% methanol feeding/24 h. The best method for recombinant purification was submitting the culture supernatant to dialysis and concentration by ultra filtration in Amicon 5kDa and then to gel filtration in Superdex 75, followed by reversed phase chromatography on a C18-column in an HPLC system. The recombinant protein was expressed and showed about 20 kDa (SDS-PAGE). Until now, only a few tests of platelet aggregation inhibition with the semi-purified recombinant molecule could be performed, and it showed a low inhibition using collagen as agonist. However, we are purifying a greater amount of protein for the characterization of this inhibitor to be better evaluated.

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2.09 Processing of SVMPs: isolation of the recombinant pro-domain for antibody production

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Introduction: Snake venom metalloproteinases (SVMPs) are abundant enzymes in *Bothrops* venoms responsible for local and systemic symptoms of human poisoning. These enzymes hydrolyze components of the extracellular matrix and modulate the activation of platelets, endothelial cells and the inflammatory system. SVMPs are synthesized as zymogens, and it is believed that enzyme activation is regulated by removing the pro-domain, as occurs in MMPs. However, it is not known exactly how or where the processing of the pro-domain and the activation of SVMPs occur. **Objectives:** In this work we aimed to clone and express the pro-domain of a P-III class SVMP, jararhagin, and produce specific antibodies. These will be used as a tool in immunohistochemistry experiments to identify the exact location where the activation occurs. **Methods:** The sequence of the pro-domain was amplified by PCR from cDNA extracted from venom glands of *Bothrops jararaca.* The gene obtained was cloned into the pAE vector in fusion with 6 histidines. The production of recombinant protein was induced in *Escherichia coli* (BL21 (DE3) star plys S) for 4 h at 37° C by the addition of isopropyl b-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The recombinant pro-domain was purified with immobilized metal affinity chromatography (IMAC), and its size, integrity and purity evaluated by SDS-PAGE. The recombinant protein was injected into mice and rabbits to produce polyclonal antibodies. **Results and Discussion:** Based on the sequences of jararhagin and bothropasin pro-domains, oligonucleotides were synthesized and used to amplify the pro-domain cDNA by PCR. The PCR product was a single band with an estimated size of 500 bp. This band was purified, digested with restriction enzymes and then cloned into the pAE vector. After transfection, colonies were screened by PCR using the vector forward and reverse primers and a positive clone, with a ~500 bp amplification band was found. The insert was sequenced and showed 98% homology with the pro-domain of jararhagin and 97% with the pro-domain of bothropasin. Thus, *E. coli* (BL21 (DE3) star plys S) was transformed with this clone and expression induced by IPTG, resulting in a major protein with a molecular mass of approximately 20 kDa in the soluble extract. The protein was purified by IMAC and injected into rabbits and mice to obtain polyclonal antibodies, which may help to better understand the biosynthesis of venom metalloproteinases and their importance in poisoning.

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2.10 Enzymatic characterization of *Bothriechis schlegelii* snake venom from Colombia and Costa Rica

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Introduction: The *Bothriechis schlegelii* snake (eyelash pit viper, also known as "boracacá") is a relatively small animal (rarely exceeding 75 cm in length) found in the mesic forest in Mexico, Central America and northwest of South America. Patients bitten by *B. schlegelii* show at the bite site pain, edema, ecchymoses and necrosis. Hematuria, gingival bleeding, hipovolemic shock and oliguria are the systemic signs of envenomation. **Objectives:** The aim of this work was to characterize enzymatically *B. schlegelii* snake venom from Colombia (BsCo) and Costa Rica (BsCR). **Methods:** SDS-PAGE (12%) was used to evaluate the protein profile of BsCo and BsCR venoms (5 μg). To analyze the enzymatic activity, zymography was employed using gelatin (2 mg/mL), casein (2 mg/mL), fibrinogen (0.5 mg/mL) and hyaluronic acid (170 μg/mL) as substrate in a polyacrylamide gel (12%). **Results and Discussion:** After SDS-PAGE, under non-reducing conditions, many components with similar molecular masses (150 – 22.5 kDa) were noticed in BsCo and BsCR venoms. However, some bands around 62.5, 33.2, 25.7 and 22.5 kDa were observed exclusively in BsCo venom. No hyaluronidase activity was detected in BsCo and BsCR venoms. Weak fibrinogenolytic activity was observed. BsCo venom showed three bands with MW around 46.8, 42.1 and 30.5 kDa, and BsCR venom displayed components with fibrinogenolytic activity with approximately 43.4 kDa. Different profile for gelatinolytic activity was also observed in BsCR and BsCo venoms. BsCR venom showed bands with intense gelatinolytic activity around 43.1 and 40 kDa. However, BsCo venom showed several components with weak activity around 54, 46.7, 38.1, 31.5 and 25.1 kDa. BsCR and BsCo venoms showed similar profiles for caseinolytic activity located between 50.8 and 23.6 kDa. Both venoms contained an enzyme with higher level of caseinolytic activity around 34.8 kDa. Our results demonstrate that geographic distribution can have an influence on venom composition, which may modify the local and systemic symptoms observed in human envenoming.

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2.11 Anti-mitogenic effects of FGF2 in human cells transformed by the RasV12 oncogene

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Introduction: Our group recently reported that fibroblast growth factor 2 (FGF2) causes cell cycle arrest and induces senescence-associated β-galactosidase in Ras-dependent malignant mouse cells. Aiming to uncover the mechanisms underlying this anti-mitogenic action, we studied the effects FGF2 in human embryonic kidney cells (HEK293 line) stably infected with the construction ER:rasV12, whose product is 4-hydroxy-tamoxifen (4-OHT)-binding domain of the estrogen receptor fused to the oncoprotein H-RasV12. This approach propitiates a molecular switch to turn H-RasV12 on/off, respectively with or without 4-OHT, allowing us to analyze FGF2 effects on HEK293 cell cycle under both normal and malignant phenotypes. **Objectives:** The aim of the study was to determine the effects of FGF2 on HEK293 cells displaying an inducible Ras-dependent malignant phenotype. **Methods:** *Generation of cell sublines.* HEK 293 cells were infected with the pBabe-Neo-ER:rasV12 viral vector and selected for resistance to geneticin (800 μg/ml). Stable poly- and monoclonal sublines were analyzed for integration and inducible expression of H-rasV12 by Western blotting. *Phenotype characterization.* HEK293 sublines were analyzed with respect to growth curves in monolayer cultures (day 0: 3000 cells/cm^2 in 35-mm dishes) and clonogenic assays in suspension cultures of soft-agar (10^3 cells/well in 24-well plates). Cells were grown in 10% FBS-DMEM and treated with 10 ng/ml FGF2 and/or 200 μM 4-OHT. **Results and Discussion:** In 10% FBS-DME, HEK293 sublines displayed a "normal" phenotype growing regularly in monolayer, but not in suspension cultures. FGF2 mitogenically stimulated HEK293 sublines in both monolayer and suspension cultures. Under 4-OHT treatment, HEK293 sublines exhibited morphological transformation in growing monolayers and developed colonies in suspension cultures. These last results have shown that HEK293 cells are not prone to oncogene stress, undergoing malignant-like transformation when submitted to H-RasV12 activation. On the other hand, the proliferation of HEK293 sublines treated with both FGF2 and 4-OHT was drastically inhibited in monolayer and suspension cultures. Thus, like mouse cells, HEK293 sublines could not cope with both FGF2 treatment and H-RasV12 oncogene activation, resulting in severe stress.

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2.12 Effect of FGF2 on human keratinocytes expressing H-RasV12

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Introduction: FGF2 (fibroblast growth factor 2) was initially described as a fibroblast-specific growth factor, but today, it is known for acting in different cell types, triggering signaling pathways involved in proliferation, surveillance, migration and others. Many studies relate FGFs and its receptors to tumorigenesis. Recently, our laboratory reported that FGF2 restores tumor defense mechanisms, inhibiting proliferation and inducing senescence in malignant mouse cells, expressing Ras oncoprotein, but not in immortalized nontumorigenic cell lines. **Objectives:** The aim of this study was to determine if FGF2 also induces antiproliferative effects in human keratinocytes expressing H-RasV12 and if p53 is involved in this phenomenon. **Methods:** HaCaT cells, a human immortalized keratinocyte cell line without functional p53, were infected (retroviral vector) with a construct with the ligand-binding domain of estrogen receptor fused to H-RasV12 (ER:RasV12). This fusion protein allows the activation of H-RasV12 oncoprotein when 4-hydroxy-tamoxifen (4OHT), an estrogen receptor agonist, is added to the culture. **Results and Discussion:** HaCaT sublines responded mitogenically to FGF2; in addition, these sublines displayed traces of malignant phenotype when H-RasV12 was activated with 4OHT. These results imply that HaCaT keratinocytes are not susceptible to oncogene stress under H-RasV12 activation. On the other hand, HaCaT sublines exhibited a severe stress when treated with both FGF2 and 4OHT. Thus, HaCat, like mouse cells, cannot stand FGF2 treatment plus H-RasV12 activation. Furthermore, the stress triggered by FGF2 in Ras-driven human malignant keratinocytes seems to be independent of the tumor suppressor protein p53.

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2.13 Analysis of antithrombin variants from *Bothrops jararaca* snake plasma

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Introduction: Antithrombin, a plasma glycoprotein, is the major inhibitor of the coagulation serine proteinases in mammals and plays a crucial role in the maintenance of normal hemostasis. Fish antithrombins have three polysaccharide chains while mammalian antithrombins show two forms, α-antithrombin, which contains four polysaccharide chains, and β-antithrombin, which lacks one carbohydrate chain situated near the heparin binding site, leading to a higher heparin affinity than the α-form. **Objectives:** The aim of this work was to investigate the presence of glycosylation variants of antithrombin in *Bothrops jararaca* plasma. **Methods:** Antithrombin was isolated from snake plasma with a HiTrap Heparin column using a step-wise gradient with 2 M NaCl. Purified antithrombin was applied to the same column and eluted by a linear gradient from 0.5 to 2 M NaCl. Antithrombin variants would be separated using a HiTrap Heparin column according to their heparin affinity. **Results and Discussion:** Our SDS-PAGE results suggest that glycosylation variants of antithrombin may be present in *Bothrops jararaca* plasma. Further experiments will be carried out to deglycosylate the purified antithrombin from *B. jararaca*. The perspective for this work is to clone the DNA fragment, which encodes for *B. jararaca* antithrombin.

Supported by: FAPESP

2.14 Rondonin: an antifungal peptide isolated from the plasma of the spider *Acanthoscurria rondoniae*

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Introduction: A wide variety of organisms produce antimicrobial peptides as part of their first line of defense. We found an antimicrobial peptide in the plasma of *Acanthoscurria rondoniae* with antifungal activity, and this molecule was characterized by mass spectrometry as a single molecule of 1,236 Da. This peptide has been submitted to "de novo" sequencing, elucidating its primary structure: IIIQYEGHKH, which showed similarity with a hemocyanin fragment and was named rondonin. It has become increasingly clear that, due to the continuous use of antibiotics, the emergence of multi-resistant bacterial strains has occurred all over the world. As expected, antibacterial and antifungal peptides have attracted attention in recent years, in order to find new therapeutic agents. **Objectives:** The objective of this study was to determine the spectrum of activity of rondonin and its analogue: IIIQYEGKH. **Methods:** Synthetic peptide was obtained in an automated bench-top simultaneous multiple solid-phase synthesizer (PSSM 8 system from Shimadzu Co.) using solid phase peptide synthesis by the Fmoc [rocedure. The peptide was purified by reversed-phase chromatography (Shim-pack Prep-ODS, 5μ, 20 mm × 250 mm Shimadzu Co.) semi-preparative HPLC, and the purity and identity of the peptide confirmed by MALDI-TOF mass spectrometry and by analytical HPLC, under the same conditions described above. The range of activity was determined by a liquid growth inhibition assay against three Gram-negative bacteria, three Gram-positive bacteria, two fungi and seven yeasts. The time-kill curve for rondonin was performed using twice the MIC (67 μM) against *Candida albicans* MDM8. The hemolytic activity of these peptides was tested against human red blood cells at an initial concentration of twice the MIC for both peptides. Hemolysis was determined by reading the absorbance at 595 nm of each well in Victor^3 (1420 Multilabel Counter/Victor^3 – Perkin Elmer). **Results and Discussion:** We found that rondonin was active against all yeasts tested and one fungus in a concentration range of from 67 μM to 1.1 μM and its analogue showed antimicrobial activity only against *Pseudomonas aeruginosa* ATCC 27853 with a concentration range from 67 μM to 33.5 μM. The time-kill curve showed us that rondonin is a fungicidal peptide and compared to conventional antifungals, it is more effective. These two peptides have no hemolytic activity at the concentration tested. These results suggest that rondonin could be the first step in the development of new antifungal drugs.

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2.15 Weaving health: the weaving of antimicrobial substances from the ootheca of the spider *Phoneutria nigriventer*

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Introduction: Increasingly, conventional antibiotics are losing their ability to heal, and infectious diseases are responsible for 20 million deaths per year worldwide, where they are among the most common causes of death in the human population. Researchers have demonstrated that bacteria are acquiring resistance to conventional antibiotics rapidly and widely, mainly due to the slow activity of these chemical substances. The conclusion of these facts is that in the future these antibiotics will not be effective and the number of deaths from infectious diseases will just grow. **Objectives:** The objective of this research was to find new antibiotics that drastically reduce the chances of bacteria acquiring resistance. Based on this idea, the hypothesis was proposed that it would be possible to find these antibiotic substances in the *ootheca* (structure made of silk that surrounds the eggs) of the spider *Phoneutria nigriventer*, since it is used to protect the eggs, also from predators, not just physically but also from infectious agents. Besides that, early Brazilian cultures used web silk as a healing substance for deep cuts. **Methods:** With an elaborated hypothesis, and using laboratory methods, we could begin the process of indentifying and characterizing the antimicrobial substances. The samples were purified by high performance liquid chromatography (HPLC) which fractionated the molecules of the ootheca with spectrophotometric monitoring. **Results and Discussion:** The results of the antimicrobial tests showed 8 fractions with antimicrobial and also antifungal activity. Beyond that, two of these molecules were characterized, one with molecular weight of 1338 Da and the other 1194 Da; these two fractions showed antimicrobial activity against *E. coli*. MIC assays based on molar amounts are in process, as well as the sequencing of these molecules.

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2.16 Purification and characterization of a hypothermic component from the venom of *Phoneutria nigriventer*

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Introduction: It is common in *Phoneutria* sp envenomation that there are clinical manifestations with local events. Systemic envenomation is considered severe when causing more severe cardiovascular changes, especially in children. The occurrence of changes in body temperature, mainly hypothermia is related to hemodynamic changes, suggesting that the signs and symptoms occur by the release of norepinephrine at sympathetic nerve terminals. Another possibility is the action on the CNS, due to neurotoxic polypeptides present in the venom. **Objectives:** The aim of the study was the biochemical characterization of fractions obtained from the venom of *Phoneutria nigriventer* and the determination of their biological action on the regulation of body temperature in young rats. **Methods:** The crude venom was purified using gel filtration (Sephadex G-50) and reverse phase HPLC. The body temperature of young rats (140-160 g) was recorded by a subcutaneous probe acquisition system (ML 309 Thermistor Pod - ºC Scale - AdInstruments®) and recorded using the software Powerlab (AdInstruments®) every 0.5 min for 3 h. **Results and Discussion:** Fractionation of the crude venom of *Phoneutria nigriventer* revealed four pools. Pool I caused no changes in body temperature, and the partial results of pools III and IV revealed oscillations, and these pools need to be purified again. Partial results of pool II showed that fraction III causes a very significant fall in body temperature of young rats.The results suggest that the hypothermic effect involves a neurotoxin activity on the thermoregulation of young rats. Purification of the other pools and fractions are underway to determine their activity on the thermoregulation of young rats.

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2.17 Factor X activation mechanism by recombinant Losac (a hemolin from *Lonomia obliqua*): potential inducer of cell proliferation and cell survival

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Introduction: Losac is the first, and so far only, factor X (FX) activator purified from a Lepidoptera secretion. We have shown that Losac is capable of inducing the expression of hemostatic mediators and cell proliferation in HUVECs. However, little is known about the mechanism and structural features implicated in both activities. **Objectives:** The aim of the study was to improve heterologous expression of recombinant Losac (rLosac), to study the FX activation mechanism, and to study the mechanism of cell proliferation and survival. **Methods:** Several *E. coli* strains and methodologies for expression and refolding were applied. Characterization of rLosac: chromogenic assays, SDS-PAGE, fibrin plate and immunoblotting. Cultured cell lines: HUVECs and fibroblast. Cell viability: MTT method. Cell cycle: flow cytometry. Cell signaling pathways: Western blotting. Gene expression: RT-PCR. **Results and Discussion:** rLosac was expressed in *E. coli* BL21(DE3) as inclusion bodies and a molecular mass of 48.6 kDa (containing a His_6-tag N-terminus). rLosac was able to shorten the plasma recalcification time and to activate FX but had no effect on prothrombin, fibrin or fibrinogen, indicating its specificity for FX. rLosac was recognized by antilonomic serum suggesting its role in the envenomation processes. The FX cleavage pattern induced by Losac was similar to that of RVV-X (a well-known factor X activator) and inhibited by PMSF. When a Gla-domainless FX was used the activation was markedly diminished, indicating that the Gla-domain is important for Losac's activity. In an attempt to understand the interaction between Losac and FX, a three-dimensional structure model of Losac was built. Structure comparison analysis identified in Losac's model a probable interaction surface and electrostatic potential quite similar to the Gla-domain FX/RVV-X binding region. Our model of the mechanism of FX predicted that Losac's binding region in domain 2 could be a good candidate for the interaction with the Gla-domain FX followed by the activation through the Losac's catalytic site predicted in domain 1 (Asp^{62}, His^{94} and Ser^{112}). We also determined that rLosac is able to induce proliferation and antiapoptotic effects in HUVECs and fibroblasts submitted to starvation. An increase in the cell cycle (S phase, 48 h) was also observed. Moreover, a significant release of NO and t-PA were modulated by rLosac. Cell signaling studies revealed the activation of survival pathways, and the expression of antiapoptotic genes was confirmed by RT-PCR. Taken together, these results contribute to extending the scope of the biological functions of hemolins.

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2.18 Amblyomin-X induces cell apoptosis and microenvironment alterations by proteasome and NF kappa B modulation

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Introduction: A recombinant protein, with inhibitory effects on factor-X, and characterized as a Kunitz type inhibitor, was obtained from the cDNA library of the salivary gland of the *Amblyomma cajennense* tick. When tested in different cell lines, the protein showed apoptotic activity in tumor cells (Sk Mel-28, Mia Paca-2). Amblyomin-X does not show cytotoxic activity in fibroblasts and melanocytes. **Objectives:** The aim of the study was to evaluate the influence of Amblyomin-X on cell viability and on the microenvironment of cancer cells after treatment. **Methods:** Cell death was evaluated by the MTT assay and flow cytometry. The release of uPA, PAI and tPA/PAI was evaluated by ELISA. The protesomal activity was assessed by fluorimetry, and NF*kappa*B was accessed by Western blotting. **Results and Discussion:** The data obtained showed that Amblyomin-X induces apoptosis in cancer cells but not in normal cells. The cancer cell microenvironment showed changes in uPA and PAI levels. Amblyomin-X also inhibited tryspsin- and chemotrypsin-like activity of proteasomes. Amblyomin-X induced apoptosis in cancer cells but not in normal cells. Furthermore, the treatment with Amblyomin-X decreased the release of uPA and PAI. Our hypothesis is that Amblyomin-X inhibits the activity of the proteasome, thus indirectly inhibiting NF*kappa*B. This disrupts cell cycle control and prevents the transcription of anti-apoptotic proteins, thereby killing cancer cells.

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2.19 Antimicrobial activity of synthetic mygalin and analogues

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Introduction: Mygalin is an acylpolyamine of 417 Da found in blood cells of the mygalomorph spider *Acanthoscurria gomesiana*. Mygalin was identified as bis-acylpolyamine N 1,N 8-bis(2,5-dihydroxybenzoyl)spermidine, in which the primary amino groups of spermidine are acylated with the carboxyl group of 2,5-dihydroxybenzoic acid (gentisic acid). Native mygalin was active against *Escherichia coli* at 85 μM, this activity being completely inhibited by catalase. Therefore, the antibacterial activity of mygalin was attributed to its production of hydrogen peroxide (H_2O_2). **Objectives:** The aim of the study was to synthesize and evaluate the antimicrobial and hemolytic activity of the acylpolyamine mygalin and analogues. **Methods:** Mygalin and its analogues were synthesized using the technique for peptide synthesis. The product of synthesis was analyzed by reversed-phase HPLC on a semi-preparative Jupiter C18 column. The antibacterial activity was determined by a liquid growth inhibition assay against Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Molecular weight and purity of the molecules were analyzed by mass spectrometry (MALDI-TOF). **Results and Discussion:** During the synthesis of mygalin, we obtained three molecules, one of mygalin itself and two analogues. The mygalin showed two gentisic acids on each end of spermidine. The first analogue showed only one gentisic acid and the other analogue had three gentisic acids. Synthetic mygalin was active against *E. coli* at 14 μM, six times more active than the native form. The synthetic molecule and its analogues presented different antimicrobial activities. Synthetic mygalin was active against *E. coli* and *P. aeruginosa* and did not show hemolytic activity. The analogue with one gentisic acid and mass of 281.4 Da, was active against *E. coli* and showed hemolytic activity. The other analogue with three gentisic acids and mass of 553.5 Da, was active against *E. coli* and *Candida albicans*. The determination of the activity spectrum of these three molecules is in progress.

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2.20 Interaction of the antimicrobial peptide longipin with LUV's

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Introduction: Longipin is an antimicrobial peptide (AMP) composed of 18 amino acid residues isolated from the plasma of the harvestman *Acutisoma longipes*. This molecule showed *in vitro* activity against *Micrococus luteus* (Gram-positive bacterium) and *Candida albicans* (yeast). The most common target of AMPs is the lipid membrane. They can form pores or act in a "detergent-like" manner. Otherwise, they can have an internal target. Therefore, in both cases, the antimicrobial peptides must interact with the lipid membrane to exert their actions. **Objectives:** The aim of the study was to investigate the interaction of longipin with large unilamellar vesicles (LUVs) by spectroscopic techniques. **Methods:** The LUVs were extracted through 10 freeze-thaw cycles and were composed of different ratios of palmitoyl-oleoyl phosphatidylglycerol (POPG) and palmitoyl-oleoyl phosphatidylcoline (POPC). LUVs were used in three different assays: (i) binding with different peptide:lipid vesicle ratios (monitoring the fluorescence of tyrosine residues), (ii) dye leakage (using carboxyfluorescein loaded vesicles) to investigate the membrane permeabilization, and (iii) insertion into the bilayer (acrylamide was used as a quencher). The conformational changes of the molecule in the presence of different POPC:POPG ratios in D_2O were analyzed by Fourier transformed infrared spectroscopy. **Results and Discussion:** Longipin showed preferential interaction with POPG-containing vesicles, increasing their permeability. There was a change in the secondary structure of the peptide in contact with POPG:POPC (1:1) when compared to POPC vesicles and the peptide in buffer (10 mM NaCl in D2O). Longipin showed no conformational differences between the POPC vesicles and the buffer systems. Selective activity of antimicrobial peptides can be explained by their preferential binding to the more negatively charged membranes from microorganisms instead of the zwitterionic ones from mammals. Our results elucidate the preferential binding of longipin to negatively charged vesicles (POPG-containing vesicles), increasing their permeability, when compared to zwitterionic vesicles.

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2.21 Purification of Amblyomin-X expressed in *Pichia pastoris*

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Introduction: A cDNA library had been previously constructed with the *Amblyomma cajennense* salivary glands and a clone was chosen to be expressed. The recombinant protein, named Amblyomin-X, inhibits FXa and causes tumor remission in mice implanted with melanoma cells, without, however, affecting the normal cells. The inhibitor was then expressed in the yeast system *Pichia pastoris* using the pPIC9K vector. **Objectives:** The aim of this study was to purify, sequence and obtain parameters of an inhibitor of FXa, obtained from Amblyomin-X. **Methods:** The recombinant protein was separated by distinct processes. Centrifuged materials were first partially purified by ion-exchange chromatography in Source Q (System ÄKTA purifier - GE) and affinity heparin Sepharose. Chromatography (Source Q15 and Heparin Sepharose) used the equilibrium buffer 20 mM Tris- HCl, pH 8.0. Elution buffer was 0 – 500 mM NaCl in 20 mM Tris- HCl, pH 8.0. Flow rate was 10 mL/min. Fractions of 10 mL were collected. Protein profile was monitored at 280 and 214 nm. **Results and Discussion:** Both materials had been partially purified by an ion-exchange chromatography in Source Q (System ÄKTA purifier - GE). The yeast-expressed Amblyomin-X inhibited FXa amidolytic activity using the chromogenic substrate S-2765. Amblyomin-X induced cytotoxicity in both cell lines analyzed, causing morphological changes. The responses were found to be dose- and time- dependent. Therefore, Amblyomin-X is a potential drug for preventing thrombosis and intravascular coagulation in cancer patients.

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2.22 Evaluation of expression in *Pichia pastoris* and purification of Amblyomin-X produced on shaker

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Introduction: A cDNA library had been previously constructed with the *Amblyomma cajennense* salivary glands and a clone was chosen to be expressed. The recombinant protein, named Amblyomin-X, characterized as a Kunitz-type inhibitor, inhibits FXa and causes tumor remission in mice implanted with melanoma cells, but without affecting normal cells. **Objectives:** The aim of this work was to evaluate the expression and purification of Amblyomin-X in *Pichia pastoris*, due to its potential to be of great pharmacological interest. **Methods:** The inhibitor was then expressed in a yeast system *P. pastoris* using the pPIC9K vector. This system offers the advantage of producing an inhibitor to be up-streamed, 8.5 liters of inhibitor had been produced in a shaker. The recombinant protein was separated from the culture medium by centrifugation, filtrations and membrane clarification/concentration (UFP-5-C-4X2MA). The material was partially purified by an ion-exchange chromatography in Source Q (System ÄKTA purifier-GE). The purifications steps were analyzed by 10% SDS-PAGE, Western blotting, and the Schiff method. Also evaluated for specific activities, the cytotoxic activity of FXa against the tumor cell lines Mia-PaCa-2 and Sk-Mel-28. **Results and Discussion:** Amblyomin-X produced in a shaker was about 35 kDa by SDS-PAGE, was identified by anti-Amblyomin-X, inhibited the FXa amidolytic activity using the chromogenic substrate S-2765 (Ki = 0.6 nM), and showed cytotoxic activity in cell lines Mia-PaCa-2 and Sk-Mel-28. Therefore, Amblyomin-X has the potential to prevent thrombosis and intravascular coagulation in cancer patients.

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2.23 FGF2 targets an "Achilles' heel" of Ras-driven mouse malignant cells

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Introduction: Fibroblast growth factor 2 (FGF2) has ordinarily been reported as a mitotic, pro-survivor and sometimes oncogenic signal which stimulates cell growth and proliferation. However, some groups, including ours, have reported that this factor can also selectively trigger unexpected antiproliferative effects in malignant cells. Here, we report that the FGF2/FGFR signaling system targets an "Achilles heel" of a robust K-Ras-dependent malignant cell line. **Objectives:** We aimed to elucidate the cell and molecular mechanisms underlying the antiproliferative stress response triggered by FGF2 in K-Ras-dependent Y1D1 mouse malignant adrenocortical cells. **Methods:** Cell cycle flow cytometry analyses following DNA content and BrdU pulse-labeling, together with a number of other molecular biology techniques, were employed to address the dynamics of FGF2 stress response. **Results and Discussion:** FGF2, in spite of stimulating G0>G1>S transition, selectively caused in malignant cells, a delay in FCS-stimulated DNA synthesis followed by a strong block of the G2/M transition, uncoupling cell growth from cell division. In addition, FGF2 induced specific markers of the DNA damage response in late G1 phase. Furthermore, polyploidization, giant cellsformation and cell death were also late effects of FGF2 treatment. Altogether, these results thus show that FGF2 can uncover a cancer-specific "Achilles heel" in K-Ras-dependent malignant cells, once considered to be highly resistant to cell death.

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