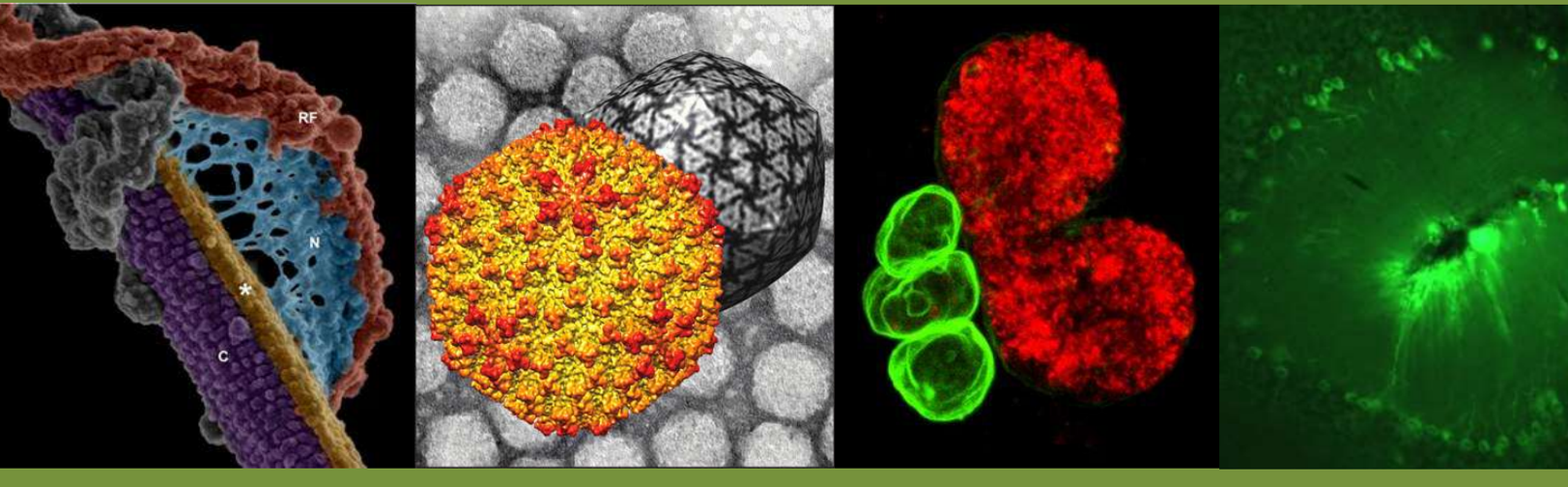
BIENNIAL REPORT
2011 – 2012

INBEB

Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem



inct institutos nacionais de ciência e tecnologia

CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico

FAPERJ Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro

DECIT Departamento de Ciência e Tecnologia

CAPES

Headquarters:

UFRJ - Universidade Federal do Rio de Janeiro

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Instituto de Bioquímica Médica, UFRJ

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Instituto de Biofísica Carlos Chagas Filho, UFRJ

SUMMARY

Introduction

PRESENTATION

The National Institute of Science and Technology for Structural Biology and Bioimaging is a pioneering initiative with a mission to create and consolidate a scientific-technical infrastructure that allows the study of structures of biological systems, from the macromolecular level to the whole organism, making use of the most advanced analytical techniques and images of the highest possible resolution.

Thus, the Institute promotes inter- and multidisciplinary activities, integrating conventional areas such as biophysics, parasitology, microbiology, immunology, biochemistry, pharmacology, chemistry and computational biology as well as extending their boundaries. This allows for greater interaction between different groups to solve biological problems. We have become increasingly aware of the need to integrate studies on the structure of macromolecules and how they combine to form biological units, which in turn are organized into different cell types, constituting the different tissues and organs that make up a living being.

Understanding biological structures at different levels, from the macromolecular to the whole-organism level, is the central goal that has led us to assemble a nucleus of research groups with proven leadership in biomedical and biotechnological research in Brazil. In addition, our mission is to create conditions in which this infrastructure can be integrated into similar but less complex initiatives in different regions of the country, through the involvement of a large number of smaller institutions.

We have also extended our interaction with the private sector through a partnership with the Instituto D'OR (IDOR) in order to reinforce and expand our ability to do translational research. Through this partnership, IDOR researchers have access to the small-animal bioimaging infrastructure at the INBEB and the AL researchers have access, when needed, to an array of imaging equipment for human subjects in the Rede D'OR.

Another key objective of the Institute is to contribute to the training of researchers in Structural Biology and Bioimaging at various levels (from undergraduate to post-doctoral).

Facilities

The headquarters of the INBEB are located on the main campus of the Universidade Federal do Rio de Janeiro (UFRJ). The equipment is available not only to the groups that belong to the INBEB, but also to the general scientific community both within Brazil and abroad. The instruments are frequently utilized by our Mercosur colleagues, who are developing projects that take advantage of our state-of-the-art infrastructure and central location.

The INBEB facilities (equipment, animal-care facilities and research core labs) are housed in three units, each one with its own headquarters building, which constitute the National Bioimage Centers (CENABIOs):

1) CENABIO I, or Centro Nacional de Ressonância Magnética Nuclear Jiri Jonas (Jiri Jonas National Center for Nuclear Magnetic Resonance);

2) CENABIO II, which houses the equipment for small-animal bioimaging;

3) CENABIO III, which is in the last phase of construction with funds provided by Pro-INFRA at UFRJ (FINEP/MCT). This unit will house the equipment for electronic, confocal, multiphoton, and atomic force microscopy.

- Cenabio I

This unit, which focuses on the elucidation of macromolecular structure, combines the early NMR equipment (originally part of the CNRMNwith a Bruker DRX 600 MHz spectrometer, upgraded to the digital system AVANCE, both acquired with funds awarded for INBEB equipment. This combination now allows for full use of four channels, inverse triple resonance probes, and the inverse triple resonance cryoprobe. With this upgrade, the NMR equipment is now state-of-the-art and its sensitivity and resolution are equivalent to that of a new spectrometer.

The Bruker Avance III 800 MHz spectrometer, which has four channels and an inverse triple resonance probe, has also been amplified and diversified. Resources from the INBEB have allowed for maintenance of the spectrometers and and acquisition of supplementary equipment (e.g. backup power supplies, unit upgrades, probes, high-pressure NMR equipment, air

conditioners, etc.). These additions were crucial to our ability to keepthe NMR Center open 24 hours per day for use by Associate Laboratory members and by a large number of researchers, who are not affiliated with the INBEB. The Bruker DRX 400-MHz wide-bore instrument, which is equipped with three channels, inverse triple resonance probes, a broadband inverse probe, and magic angle spinning (MAS) for investigation of solid samples, has also been widely used. Two new spectrometers just purchased (700 and 500 MHz) will extend the range of applications to solid-state NMR.

Figure 1: NMR SPECTROMETERS ROOM (CENABIO I). PROFESSORS IN CHARGE: FÁBIO ALMEIDA AND ANA PAULA VALENTE.

- CENABIO II

The division for small-animal imaging is the one that has received the greatest investment during the consolidation phase. The construction of CENABIO II was completed in May, 2010, and INBEB resources were used to install the electrical wiring and to purchase equipment required to support the imaging instruments. The CENABIO II building (Figure 2) brings together a broad range of bioimaging instruments for small animals.

FIGURE 2: CENABIO II BUILDING.

The 7-Tesla magnet used for magnetic resonance imaging (MRI) of small animals (Figure 3) has been available for use since May, 2010. This equipment allows for morphological and functional analyses of organs and systems in live animals (especially mice and rats, the experimental animals most often used in biomedical research). Nuclear magnetic resonance imaging is a non-invasive, non-destructive technique that allows investigators to monitor the morphology and in some cases the organ function of animals over time without sacrificing the experimental animal.

FIGURE 3: 7 TESLA, 210 BORE ACTIVELY SCREENED REFRIGERATED MAGNET SYSTEM. VARIAN, INC. NMR SYSTEMS. PROFESSOR IN CHARGE: FERNANDA TOVAR MOLL.

Other bioimaging equipment previously acquired using other funds, has now been moved to the new CENABIO II building, which contains the appropriate supporting infrastructure for efficient use by INBEB researchers and external users. A 110-KVA generator and backup power supplies protect the equipment and ensure that there are no interruptions in the operations.

The following equipment is completely installed:

FIGURE 4: EQUIPMENT I: VEVO 770-120. VISUAL SONICS, TORONTO, CANADÁ / EQUIPMENT II: MYLAB® 30 CV. BIOSOUND ESAOTE, INC. USA / EQUIPMENT III: CARIS PLUS. BIOSOUND ESAOTE, INC. USA. PROFESSOR IN CHARGE: EMILIANO MEDEI.

High-resolution ultrasound equipment (Figure 4), designed to acquire high-resolution images from small animals, enabling for example the

visualization of embryonic development in mice by monitoring organs such as the heart, liver, and kidneys;

A bioluminescence and fluorescence detection system for use in live animals that allows us to visualize cells labeled with enzymes (such as luciferase) that activate luminescent molecules, such as luciferin, or fluorescent labels. Use of labeled cells or pathogens makes it possible to track their dissemination when injected into live animals.

FIGURE 5: IVIS LUMINA SYSTEM. XENOGEN CORP, CA, EUA. PROFESSOR IN CHARGE: EMILIANO MEDEI.

Scintigraphy equipment for small animals (PET/SPECT/CT) was recently acquired with federal and state funds (MS-Decit/FAPERJ).. This equipment allows us to detect radiolabeled molecules and cells at a resolution of 2 millimeters. It is coupled to a computerized tomography scanner, which will allow us to overlay 3D SPECT images (single photon emission computerized tomography) with the computerized tomography images in real time. This equipment is particularly useful in studying biodistribution of labeled molecules, a technique that is especially important in evaluating new drugs.

FIGURE 6: MODEL: TRIUMPH® II – PET/SPECT/CT SYSTEM, GE.
PROFESSOR IN CHARGE: ALYSSON RONCALLY CARVALHO.

Other instruments available in Cenabio II include a FACSAria flow cytometer (Figure 7), which is used for cell sorting in awide range of applications. Cenabio II also houses about 1300 animals in 387 microisolator cages for mice, 60 microisolator cages for transgenic mice and 72 cages for rats.

FIGURE 7: BD FACSARIA™.
PROFESSOR IN CHARGE: EMILIANO MEDEI.

- Cenabio III

The microscopy division will collect under one roof a variety of equipment that is currently spread out among several UFRJ laboratories, including:

A conventional scanning electron microscope (Figure 8);

FIGURE 8: SCANNING ELECTRON MICROSCOPE JEOL JSM 5310, WITH MAXIMUM VOLTAGE 25 KV, MAGNIFICATION RANGE FROM 35 TO 100,000 X.
PROFESSOR IN CHARGE: KILDARE MIRANDA. TECHNICIANS: NOÊMIA RODRIGUES AND THIAGO LUIZ DE BARROS MOREIRA.

A high-resolution scanning electron microscope with a cryo-stage (Figure 9);

FIGURE 9: SCANNING ELECTRON MICROSCOPE JEOL JSM 6340, HIGH RESOLUTION WITH FIELD EMISSION GUN. EQUIPPED WITH CRYO-STAGE, DETECTOR OF BACKSCATTERED ELECTRONS AND SECONDARY ELECTRONS.
PROFESSOR IN CHARGE: KILDARE MIRANDA. TECHNICIANS: DANIEL IUCIF AND THIAGO LUIZ DE BARROS MOREIRA.

Two conventional transmission electron microscopes (Figures 10 and 11);

FIGURE 11:TRANSMISSION ELECTRON MICROSCOPE ZEISS 902 60-80 KV WITH MAGNIFICATION UP TO 250,000 X. EQUIPPED WITH POWER FILTER FOR PERFORMING ENERGY LOSS SPECTROSCOPY AND OBTAINING ELECTRON SPECTROSCOPIC IMAGING. IT HAS A MEGAVIEW G2 CAMERA (14-BIT).
PROFESSOR IN CHARGE: KILDARE MIRANDA. TECHNICIAN: THIAGO LUIZ DE BARROS MOREIRA.

FIGURE 10: TRANSMISSION ELECTRON MICROSCOPE ZEISS 900, 50-80 KV WITH MAGNIFICATION UP TO 280,000 X. IT HAS A MEGAVIEW III CAMERA (12-BIT).
PROFESSOR IN CHARGE: KILDARE MIRANDA. TECHNICIANS: NOÊMIA RODRIGUES AND THIAGO LUIZ DE BARROS MOREIRA.

Two analytical transmission electron microscopes, which provide for collecting an X-ray emission spectrum for x-ray microanalysis (Figure 12);

FIGURE 12: TRANSMISSION ELECTRON MICROSCOPE JEOL 1200 EX WITH ENERGY-DISPERSIVE X-RAY MICROANALYSIS, 60 TO 120 KV (80KV PREFERABLY) WITH MAGNIFICATION RANGE FROM 600 TO 500,000 X. EQUIPPED WITH SECONDARY ELECTRON DETECTOR, TRANSMITTED ELECTRON DETECTOR AND X-RAY DETECTOR FOR ELECTRON MICROANALYSIS AND MAPPING. IT HAS A CAMERA MEGAVIEW III (12 BIT). PROFESSOR IN CHARGE: KILDARE MIRANDA. TECHNICIAN: THIAGO LUIZ DE BARROS MOREIRA.

A transmission electron microscope capable of operating at 200 KV (Figure 13);

FIGURE 13: TRANSMISSION ELECTRON MICROSCOPE TECNAI G20, D2114, FEI COMPANY, OPERATING AT 200 KV. EQUIPPED WITH TOMOGRAPHY HOLDER, ABLE TO OBTAIN IMAGES AROUND ITS Y AXIS, AT THE ANGULAR RANGE OF -70º TO +70º. CONFIGURED FOR ELECTRON TOMOGRAPHY OF BIOLOGICAL SPECIMENS 200-300 NM THICK AND ALLOWS THE ACQUISITION OF TOMOGRAPHIC SERIES FOR ALIGNMENT AND 3D RECONSTRUCTION IN SPECIFIC SOFTWARES.
PROFESSOR IN CHARGE: KILDARE MIRANDA. TECHNICIAN: THIAGO LUIZ DE BARROS MOREIRA.

An environmental scanning electron microscope, acquired with INBEB resources (Figure 14).

FIGURE 14: SCANNING ELECTRON MICROSCOPE QUANTA 250, FEI COMPANY BRAND. TENSION BETWEEN 1-30 KV, EQUIPPED WITH A PELTIER SAMPLE COOLER (ENVIRONMENTAL MODE) AND BEAM DECELERATOR. ABLE TO WORK IN HIGH (10-2 A 10-4 PA), LOW VACUUM (10 A 130 PA) AND ENVIRONMENTAL MODE(10, 400 PA), ALLOWING THE OBSERVATION OF NON-CONDUCTIVE AND / OR HYDRATED MATERIALS. HAS AN X-RAY DETECTOR FOR ELECTRON MICROANALYSIS AND MAPPING, AND A DETECTOR OF BACKSCATTERED ELECTRONS AND SECONDARY ELECTRONS.
PROFESSOR IN CHARGE: KILDARE MIRANDA. TECHNICIANS: RACHEL RACHID AND THIAGO LUIZ DE BARROS MOREIRA.

In addition, there are several smaller, special-purpose microscopes:

- Two confocal microscopes;
- A multiphoton microscope;
- A multiphoton microscope with a fluorescence correlation spectroscopy system (FCS);
- A total internal reflection fluorescence (TIRF) microscope;
- Three atomic-force microscopes, including two new state-of-the-art instruments recently acquired with INBEB funds.

With this vast array of equipments, the CENABIO III is the most complete microscopy facility in Latin America, and allows for visualizing

proteomic structures such as amyloid fibers, viral particles, bacteria, and protozoans. It also makes it possible to track a single viral particle within a living cell, enabling us to determine the route it takes during the infectious process.

Members

The INBEB consists of 20 associate laboratories (ALs) at 20 institutions in seven Brazilian states.

- Brazilian Institutions

1. Pará

Universidade Federal do Pará (UFPA).

2. Pernambuco

Universidade Federal de Pernambuco (UFPE, PE);
Centro de Pesquisas Aggeu Magalhães (CPQAG - FIOCRUZ);
Centro de Tecnologias Estratégicas do Nordeste (CETENE).

3. Bahia

Universidade Federal da Bahia (UFBA, BA).

4. Minas Gerais

Universidade Federal do Triangulo Mineiro (UFTM).

5. São Paulo

Universidade Estadual de Campinas (Unicamp, SP).

6. Rio de Janeiro

Universidade Federal do Rio de Janeiro (UFRJ);
Universidade Federal Fluminense (UFF);
Universidade Estadual do Rio de Janeiro (UERJ);
Universidade Estadual do Norte Fluminense (UENF);
Universidade Santa Úrsula (USU);
Centro Universitário Estadual da Zona Oeste (UEZO);
Bio-Manguinhos (FIOCRUZ);
Instituto de Pesquisa Clínica Evandro Chagas (IPEC, FIOCRUZ);
Instituto Nacional de Metrologia (INMETRO);
Instituto Militar de Engenharia (IME);
Instituto Nacional de Cardiologia (INC);
Instituto D'Or de Ensino e Pesquisa (IDOR)

7. Santa Catarina

Universidade Federal de Santa Catarina (UFSC, SC).

- Associate Laboratories

The ALs are directed by leading researchers in many different fields:

AL1. Associate Laboratory of Virus and Cancer Structural Biology
Coordinator: Jerson Lima Silva, Instituto de Bioquímica Médica/UFRJ.

AL2. Associate Laboratory of Structural Biology of Cardiac and Amyloidogenic Proteins
Coordinator: Débora Foguel, Instituto de Bioquímica Médica/UFRJ.

AL3. Associate Laboratory of Protein Structure Determination by NMR
Coordinator: Fábio Almeida, Instituto de Bioquímica Médica, UFRJ.

AL4. Associate Laboratory of Pharmacologic Proteomics
Coordinator: Russolina Zingali, Instituto de Bioquímica Médica, UFRJ.

AL5. Associate Laboratory of Nuclear Magnetic Resonance, Organic Synthesis and Molecular Modeling
Coordinator: José Daniel Figueroa Villar, Instituto Militar de Engenharia (IME)

AL6. Associate Laboratory of Proteins and Proteomic Heterologous Expression
Coordinator: Hernán Terenzi, Universidade Federal de Sta Catarina (UFSC)

AL7. Associate Laboratory of Protein Biochemistry
Coordinator: Carlos H. Inácio Ramos, Universidade Estadual de Campinas (UNICAMP)

AL8. Associate Laboratory of Macromolecules Crystallization
Coordinator: Marcelo Santos Castilho, Universidade Federal de Bahia (UFBA)

AL9. Associate Laboratory of Cellular Ultrastructure Hertha Meyer
Coordinator: Wanderley de Sousa, Instituto de Biofísica Carlos Chagas Filho (UFRJ)

AL10. Associate Laboratory of Genomics, Proteomics, Modeling and Nanoscopy of Biological Systems
Coordinator: Paulo Mascarello Bisch, Instituto de Biofísica Carlos Chagas Filho (UFRJ)

AL11. Associate Laboratory of Microscopy
Coordinator: Thaís Cristina Souto Padrón, Instituto de Microbiologia Prof Paulo de Goes (UFRJ)

AL12. Associate Laboratory of Cellular Ultrastructure
Coordinator: Marlene Benchimol, Universidade Santa Ursula (USU)

AL13. Associate Laboratory of Structural Biotechnology
Coordinator: Celso B. Sant'Anna Filho, Instituto Nacional de Metrologia (INMETRO)

AL14. Associate Laboratory of Structural Biology

Coordinator: Edilene Oliveira da Silva, Universidade Federal do Pará (UFPA)

AL15. Associate Laboratory of Microscopy CETENE
Coordinator: Christina Alves Peixoto, Fundação Oswaldo Cruz and Centro de Tecnologias Estratégicas do Nordeste (FIOCRUZ, CETENE - Pernambuco)

AL16. Associate Laboratory of Molecular and Cellular Cardiology
Coordinator: Antonio Campos de Carvalho, Instituto de Biofísica Carlos Chagas Filho (UFRJ)

AL17. Associate Laboratory of Ion Transport Physiology in Health and Disease
Coordinator: Adalberto Vieyra, Instituto de Biofísica Carlos Chagas Filho (UFRJ)

AL18. Associate Laboratory of Immunology
Coordinator: Júlio Scharfstein, Instituto de Biofísica Carlos Chagas Filho (UFRJ)

AL19. Associate Laboratory of Cellular and Molecular Neurology
Coordinator: Rosalia Mendez Otero, Instituto de Biofísica Carlos Chagas Filho (UFRJ)

AL20. Associate Laboratory of Inflammation and Metabolism
Coordinator: Fernando Augusto Bozza, Instituto de Pesquisa Clínica Evandro Chagas (IPEC-FOC)

- Steering committee:

The steering committee is composed of coordinators from seven Associate Laboratories:

- Jerson Lima da Silva- AL1 (IBqM, UFRJ);
- Wanderley de Souza- AL 09 and 13 (IBCC/UFRJ and INMETRO);
- Antonio Carlos Campos de Carvalho- AL 16 (Instituto Nacional de Cardiologia and IBCCF/UFRJ);
- Carlos Ramos- AL 7 (UNICAMP);
- Hernán Terenzi- AL 6 (UFSC);
- Edilene Oliveira da Silva- AL 14 (UFPA)
- Adalberto Ramon Vieyra- AL 17 (IBCCF/UFRJ).

- Promoting interactions:

The aim of the Institute is to promote productive interactions among its ALs and with external collaborators, by creating the infrastructure needed to integrate studies in different areas. Material support for a multidisciplinary approach enables interactions that are both more efficient and more creative in solving biological problems.

We have become increasingly aware of the need to integrate studies about the structure of macromolecules, how they combine to form multifaceted biological structures and macromolecular complexes; as well as how those are organized into different cell types constituting the different tissues and organs that make up a living being. Understanding the formation of biological structures at their different levels, from the macromolecular to the whole organism level, is the central goal that has led us to assemble a significant number of research groups with proven leadership in biomedical research in Brazil.

INBEB also have an important interaction with the business sector through a partnership with the D'Or Institute for Research and Teaching (IDOR) and the Rede Labs-D'Or, in order to expand our ability to do translational research. This has been crucial in initiating research activities related to small animal magnetic resonance imaging at the INBEB.

This partnership was established in June 2009, with Dr. Fernanda Tovar Moll, the IDOR's research director and a member of AL1. Dr. Moll coordinates the activities and use of the 7-Tesla magnet for MRI studies and is responsible for implementing IDOR-INBEB collaborative projects, especially those that involve MRI *studies in human beings (located at IDOR) and small animals (located at the INBEB). Through this partnership, IDOR researchers have access to the small animal bioimaging infrastructure at the INBEB and the AL researchers have access, when necessary, to an array of human imaging equipment in the Rede D'OR.*

We also maintain a regular lecture program with participation of members from different ALs, and we promote one-day or half- meetings and roundtables on special topics. Moreover, the Annual Meetings organized by the Institute allow our Associate Laboratories to show their work to other members of the INBEB and to external researchers. These activities tend to promote new and enjoyable opportunities to interact, improve, and establish new partnerships. In the following chapters, we will show these results in detail.

Another important tool to promote the integration among INBEB members is the institute's website: www.inbeb.org.br. This site provides information about how to access the INBEB facilities, courses, lectures, news from members and INBEB achievements. In later chapters we present more details about the meetings, lectures and outreach strategies.

Finally, since its inception, the Institute has created a network of collaboration among the different Associate Laboratories and the Multiuser Facilities. The interactions have increased mainly as a function of the exchange between students and researchers, which can be appreciated in the list of publications in the following chapter. There, it is possible to observe the co-authoring of articles by junior and senior INBEB members from different groups.

- Cooperation with a Nobel chemistry laureate

The latest addition to the INBEB community is Dr Kurt Wüthrich, a Swiss chemist/biophysicist and Nobel Chemistry laureate from 2002. Dr Wüthrich develops Nuclear Magnetic Resonance (NMR) methods for studying biological macromolecules and will serve for the next three years as a visiting researcher at the INBEB and the Federal University of Rio de Janeiro (UFRJ) – with support from the Brazilian federal exchange program"Science without borders".

KURT WÜTHRICH AT THE INBEB LABORATORY OF NUCLEAR MAGNETIC RESONANCE. ***CREDIT: CECILIA ACIOLI / "O GLOBO" NEWSPAPER.***

According to Wüthrich, the collaboration is only possible due to the infrastructure offered by INBEB. Our facilities allow him to work with the same equipment (unique in Latin America) that he uses in his laboratories at Scripps Research Institute, USA, and the Federal Institute of Technology from Zurich. “It would make no sense to come to Brazil if there were no laboratories and equipments here”, he told the Brazilian press. “Historically, Brazil, and other Latin American countries, faced ups and downs in science. Now, however, the country is in a time of strong growth in investments in science, which makes it the right time to combine big spending with a step change in scientific production. We must be realistic: Without this basic investment, nothing would happen.”

Two Brazilians - a PhD student and a postdoctoral fellow - will have their research work supervised directly by the Nobel winner. The doctoral thesis, developed by Leonardo Vasquez, a student at UFRJ, is about the synthesis of proteins within the ribosome. The postdoctoral fellow, Luana Heimfarth, from the Federal University of Rio Grande do Sul (UFRGS), studies the application of nuclear magnetic resonance techniques in the field of neurobiology. “I hope my coming will bring a new way of thinking in science education and infrastructure in Brazil, besides expanding the options for the next generation of Brazilian scientists”, says Wüthrich. “Again, if I did not think that Brazil was on the right track, I would not be here”.

Science highlights

OVERVIEW

In the biennial of 2011-2012, the members of the INBEB ALs have published several papers, and some of these resulted from extensive collaborations between researchers within a single AL or collaboration between different ALs.

The following is a brief summary of the results from each Associated Laboratory.

AL 1

ASSOCIATE LABORATORY OF STRUCTURAL BIOLOGY OF VIRUSES, PRION AND CANCER

Coordinator: Jerson Lima Silva - IBqM/UFRJ.

Members:

Andréa Cheble de Oliveira – IBqM/UFRJ
Andre Marco de Oliveira Gomes – IBqM/UFRJ
Monica Santos Freitas - IBqM/UFRJ
Patrícia Souza dos Santos - IBqM/UFR J
Davis Fernandes Ferreira – IMPPG/UFRJ
Yraima Moura Lopes Cordeiro – FF/UFRJ
Theo Luiz Ferraz de Souza - FF/UFRJ
Rafael Braga Gonçalves - UNIRIO
Claudia Vitoria M. Gallo - UERJ
Luciane Pinto Gaspar - Bio-Manguinhos (FIOCRUZ)
Fernanda Tovar Moll - ICB/UFRJ e I'Dor

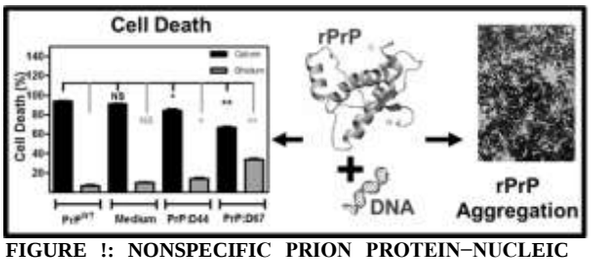


FIGURE !: NONSPECIFIC PRION PROTEIN–NUCLEIC ACID INTERACTIONS LEAD TO DIFFERENT AGGREGATES AND CYTOTOXIC SPECIES. LEFT: CYTOTOXICITY ASSAY. RIGHT: TEM SHOWING DNA-INDUCED PRP AGGREGATION.

The central subject of our research is to understand the mechanisms of protein folding, protein misfolding, protein-protein interactions and supramolecular assembly. Our aim is to decode how these processes are related to the normal physiological function of the proteins and to the development of diseases, such as virus infections, prion and other neurodegenerative diseases and cancer. Exploiting spectroscopic tools such as fluorescence and NMR, our work with high pressure in biochemistry and structural biology has yielded a wealth of new data and testable models concerning new concepts for the folding and association of proteins, virus assembly, protein misfolding and aggregation. We have demonstrated that the entropic nature of protein interactions and the changes in hydration are crucial in the assembly of virus particles and amyloid aggregates. The studies of the stability of virus particles using high pressure have resulted in a new method for obtaining antiviral vaccines and other applications. Combining biophysical (high pressure) and structural biology tools, we have found that changes in hydration and cavity distribution in the interior of the proteins play a key role in different biological processes such as viral membrane fusion, prion conversion and loss of function of the tumor suppressor protein. In the case of prion protein, involved in transmissible spongiform encephalopathies, the prion protein becomes less hydrated when converts to the aggregated, scrapie-like isoform and we have demonstrated that this process can be catalyzed by nucleic acids. Among our most important findings is the description that prions have other accomplices, such as nucleic acids and glycosaminoglycans, which chaperone their activity in converting the PrP^C into the disease-causing isoform.

We highlight below the most important findings of our Associated Laboratory:

1. Prion Diseases. Transmissible Spongiform Encephalopathies (TSE) embody a group of neurodegenerative diseases that affect humans and other mammals. They occur when the native prion protein (PrP^{C}), an alpha-helical rich protein, is converted into an infectious misfolded isoform. This isoform, the scrapie PrP (PrP^{Sc}), forms aggregates, leading to neurodegeneration. It has been proposed that the spontaneous conversion from PrP^{C} to PrP^{Sc} is prevented by a high energetic barrier and changes in the activation energy, like the presence of a catalyst, would lead to prion conversion. Among the proposed catalysts, our group has characterized nucleic acid molecules as effective inducers of such process.

FIGURE 2!: SCHEMATIC MODEL OF PROTEIN-LOW MOLECULAR WEIGHT HEPARIN INTERACTION. FROM JACS 2011.

More recently, we characterized the interaction of the prion protein with distinct nucleic acid sequences by biophysical techniques and investigated the toxicity of such complexes using cellular biology approaches. We found that, although prion protein:DNA interactions seem to be unspecific, they lead into different aggregated species that may be toxic to cells in culture, depending on the DNA sequence. In addition, Glycosaminoglycans, usually, heparan sulfate has been suggested to be the PrP^{Sc} cellular recptor and its interaction to murine rPrP 23-231 was followed by local conformational changes triggering protein aggregation. The PrP-Heparin interaction seems to be pH dependent and it is related to histidine patterns of protonation at the octapeptide and C-terminal region. Those informations were published in 2011 at the Journal American Chemistry Society.

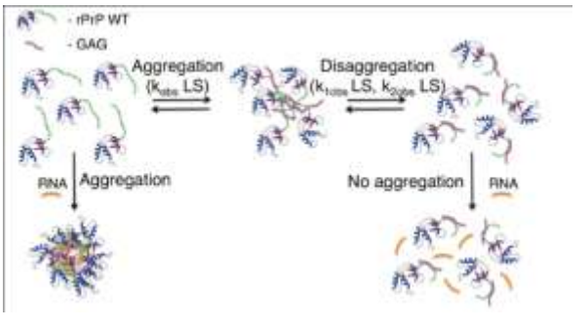


FIGURE 2: A TP53 MUTATION ELECTROPHEROGRAM FROM A BREAST MALIGNANT TUMOR. THE ARROW INDICATES THE BASE CHANGE OF THE MISSENSE MUTATION P.V173M.

2. Mutational analysis of the tumor suppressor gene TP53 in breast cancer and the association of the mutant protein with pathological characteristics and therapy. p53 is a tumor suppressor protein implicated in fundamental cellular processes of maintaining genomic integrity. tp53 mutations have been considered one of the most frequent mutational events in cancer (douglas hanahan and robert a. weinberg, 2011) and they are frequent in breast cancer which is one of the most important malignant diseases, responsible for the most part of women death by cancer in brazil. Our work aims to analyze the different tp53 mutations in breast cancer cases in association with the structural aspects of the mutant p53 and pathophisiological features of tumors and breast cell lines. we present illustrative obtained results.

3. Prions And Cancer: The Prionoid Behavior of p53

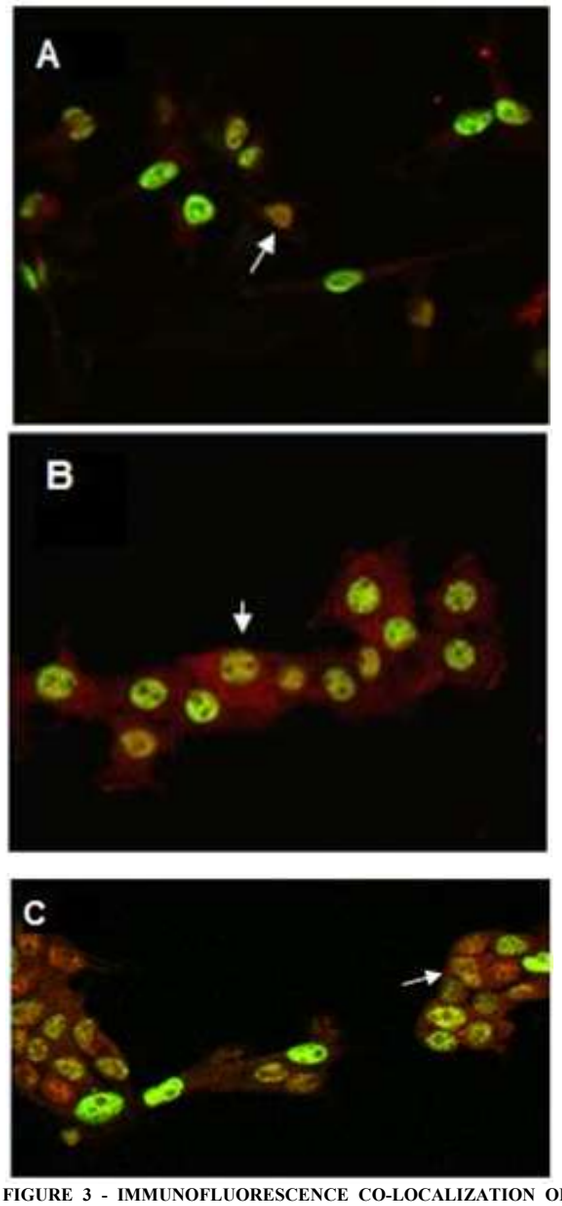


FIGURE 3 - IMMUNOFLUORESCENCE CO-LOCALIZATION OF P53 (GREEN) AND PROTEIN AGGREGATES (RED) IN BREAST CANCER CELL LINES (40X): (A) MDA-MB-231 (MUTANT P.R280K); (B) T-47D (MUTANT P.L194F); (C) MCF-7 (WILD P53).THE ARROW INDICATES DE CO-LOCALIZATION OF P53 AND PROTEIN AGGREGATES (YELLOW).

Over 50% of all human cancers lose p53 function. To evaluate the role of aggregation in cancer, we asked whether wild-type (wt) p53 and the hot-spot mutant R248Q could aggregate as amyloids under physiological conditions and whether the mutant could seed aggregation of the wild-type form. The central domains (p53c) of both constructs aggregated into a mixture of oligomers and fibrils. R248q had a greater tendency to aggregate than wt p53. Full-length p53 aggregated into amyloid-like species that bound thioflavin T. The amyloid nature of the aggregates was demonstrated using x-ray diffraction, electron microscopy, FRIR, dynamic light scattering, cell viabilility assay, and anti-amyloid immunoassay. The x-ray diffraction pattern of the fibrillar aggregates was consistent with the typical conformation of cross β-sheet amyloid fibers with reflexions of 4.7 å and 10 å. A seed of R248Q p53c amyloid oligomers and fibrils accelerated the aggregation of wt p53c, a behavior typical of a prion. The R248Q mutant co-localized with amyloid-like species in a breast cancer sample, which further supported its prion-like effect. A tumor cell line containing mutant p53 also revealed massive aggregation of p53 in the nucleus. We conclude that aggregation of p53 into a mixture of oligomers and fibrils sequestrates the native protein into an inactive conformation that is typical of a prionoid. this prion-like behavior of oncogenic p53 mutants provides an explanation for the negative dominance effect and may serve as a potential target for cancer therapy.

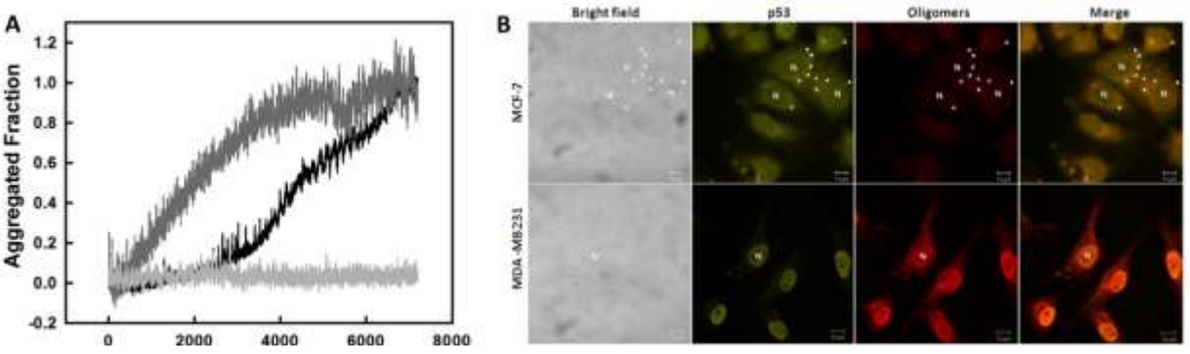


Figure 4 shows the seeding of wild-type p53 aggregation by aggregated R248Q and the presence of native and aggregated p53 in breast cancer cell lines. (A) Aggregation was monitored by thioflavin T fluorescence emission over time at 37 °C. Wild-type p53 at 10 µM (black line) or R248Q at 20 µM was incubated at 37 °C for 30 min, and after 10-fold dilution, the protein was added to 10 µM wild-type p53 (dark gray line). Also, R248Q was seeded alone at 2 µM as a control (gray line). There was a clear suppression of the lag phase of wild-type p53 aggregation (dark gray line), demonstrating the significant seeding potential of the aggregated mutant protein. (B) MCF-7 (wild-type p53) and MDA-MB 231 (mutated p53) cells were labeled with anti-p53 and anti-oligomer primary antibodies. The first column shows the bright field images, the second column shows p53-labeling, the third column shows the labeling of aggregates, and the last column shows the merged images of p53 labeling and aggregate labeling. We demonstrated that tumor cells containing mutant p53 revealed massive aggregation of p53 in the nucleus.

4. Resveratrol Anti-Tumor Properties Are Rescued Through P53 Transfection in p53 Defective Cancer Cell Lines

Resveratrol is a naturally occurring dietary compound found in grapes, berries, and peanuts. The compound was discovered in red wine in 1939 but by large did not attract the attention of the scientific community. More recently, pre-clinical studies have revealed the many beneficial properties of resveratrol. These include antidiabetic, cardioprotective, and chemopreventive effects. The latter has been associated to resveratrol antioxidant and anti-inflammatory proprieties. A number of studies show that resveratrol induces cell death in different types of cancers, both in humans and animal models. p53 has been suggested to play a role in the anticancer properties of this compound. We investigated resveratrol-induced cytotoxicity in H1299 cells, which are non-small lung cancer cells that have a partial deletion of the gene that encodes the p53 protein. The results for H1299 cells were compared with those for three cell lines that constitutively express wild-type p53: breast cancer MCF-7, adenocarcinomic alveolar basal epithelia A549 and non-small lung cancer H460. Cell viability assays revealed that resveratrol reduced the viability of all four of these cell lines in a dose- and time-dependent manner. MCF-7, A549 and H460 cells were more sensitive to resveratrol than were H1299 cells when exposed to the drug for 24 h at concentrations above 100 µM. Resveratrol also increased the p53 protein levels in MCF-7 cells without altering the p53 mRNA levels, suggesting a post-translational modulation of the protein. The resveratrol-induced cytotoxicity in these cells was partially mediated by p53 and involved the activation of caspases 9 and 7 and the cleavage of PARP. In H1299 cells, resveratrol-induced cytotoxicity was less pronounced and (in contrast to MCF-7 cells) cell death was not accompanied by caspase activation. These findings are consistent with the observation that MCF-7 cells were positively labeled by TUNEL following exposure to 100 µM resveratrol whereas H1299 cells under similar conditions were not labeled by TUNEL. The transient transfection of a wild-type p53-GFP gene caused H1299 cells to become more responsive to the pro-apoptotic properties of resveratrol, similarly to findings in the p53-

positive MCF-7 cells. Our results suggest a possible therapeutic strategy based on the use of resveratrol for the treatment of tumors that are typically unresponsive to conventional therapies because of the loss of normal p53 function.

5. Virus Structure, Assembly and Cellular Interatcions. Arboviruses (arhtropod-borne viruses) include the alphaviruses, flaviviruses and (-) strand RNA bunyaviruses, among them there are over 700 viruses currently known and considered a major problem of animal and human health worldwide.

The group of Davis Ferreira, in collaboration with Dr. Dennis Brown from North Carolina State University was attempting to obtain a more virulent strain of Dengue virus for the studies of virus penetration and infection. While improving purification methods for Dengue we have observed by electron microscopy a previously unidentified virus. This virus was different from all known flaviviruses and could not be detected by any common procedure. A sequence of the genome was produced by *de novo* assembly and was not found to match to any known viral sequence. The composition and three dimensional structure of this virus, named Espeirito Santo virus (ESV) were presented and its sequence compared to other members of the Birnavirus family.

This new virus was classified as a new Entomobirnavirus which infect insect cells. Interestingly, ESV was found to grow better upon co-infection with a virulent strain of Dengue-2 and to replicate in c6/36 insect cells but not in mammalian Vero cells. Interestingly, during this co-infection, Dengue viral proteins can be detected, but no viral particles are assembled. The uniqueness of this relationship remains to be elucidated. This paper was published in the journal of virology (Vancini et. al, 2012 below) and the work was selected to be the cover of the issue.

Yellow fever is another mosquito-borne disease, caused by a member of the *Flavivirus* genus. The disease is greatly neglected, despite its high mortality rate and the fact that approximately 900 million people live in high-risk areas around the world. Different flaviviruses induce cell death by apoptosis during their replication, but little is known about the mechanisms underlying the cell death induced by yellow fever virus (YFV). Our group evaluated the role of the mitochondrial death pathway in YFV-induced apoptosis. We observed that cell death induced by YFV correlates with loss of mitochondrial membrane potential, a marker of activation of the mitochondrial death pathway. Blockage of this route avoided cell death, indicating a central role of mitochondria in YFV-induced apoptosis. Our results also suggest that this mechanism is caspase-dependent. This work intends to improve the current understanding of the process underlying the cell death observed in YFV and other flaviviruses infection and may help in developing an effective strategy for therapeutic intervention in yellow fever and other flavivirus-induced diseases.

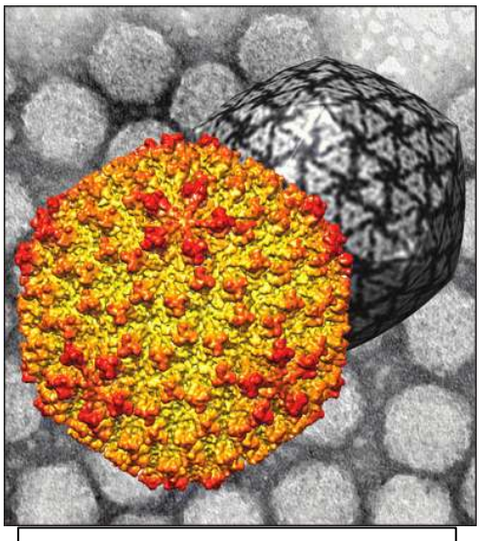
Membrane fusion is a crucial step in flavivirus infections and a potential target for antiviral strategies. Lipids and proteins play cooperative roles in the fusion process, which is triggered by the acidic pH inside the endosome. This acidic environment induces many changes in glycoprotein conformation and allows the action of a highly conserved hydrophobic

sequence, the fusion peptide (FP). Despite the large volume of information available on the virus-triggered fusion process, little is known regarding the mechanisms behind flavivirus–cell membrane fusion. Our group evaluated the contribution of a natural single amino acid difference on two flavivirus FPs, FLAG and FLAH, and investigated the role of the charge of the target membrane on the fusion process. We used an *in silico* approach to simulate the interaction of the FPs with a lipid bilayer in a complementary way and used spectroscopic approaches to collect conformation information. We found that both peptides interact with neutral and anionic micelles, and molecular dynamics (MD) simulations showed the interaction of the FPs with the lipid bilayer. Mild differences between FLAG and FLAH were observed according to the pH and the charge of the target membrane model. The MD simulations of the membrane showed that both peptides adopted a bend structure, and an interaction between the aromatic residues was strongly suggested, which was also observed by circular dichroism in the presence of micelles. As the FPs of viral fusion proteins play a key role in the mechanism of viral fusion, understanding the interactions between peptides and membranes is crucial for medical science and biology and may contribute to the design of new antiviral drugs. Those data were published in 2012 at PLoS One.

Alphaviruses are enveloped arboviruses. The viral envelope is derived from the host cell and because this envelope contains glycoproteins involved in cell recognition and entry, its integrity is critical for the success of the early events of infection. Differing levels of cholesterol in different hosts leads to the production of alphaviruses with distinct levels of this sterol loaded in the envelope. Using Mayaro virus, a New World alphavirus, we have investigated the role of cholesterol on the envelope of alphavirus particles assembled in either mammalian or mosquito cells. Our results show that although quite different in their cholesterol content, Mayaro virus particles obtained from both cells share a similar high level of lateral organization in their envelopes. This organization, as well as viral stability and infectivity, is severely compromised when cholesterol is depleted from the envelope of virus particles isolated from mammalian cells, but virus particles isolated from mosquito cells are relatively unaffected by cholesterol depletion. We suggest that it is not cholesterol itself, but rather the organization of the viral envelope, that is critical for the biological activity of alphaviruses. These data were published in 2011 at JBC.

We have also studied an avian Influenza virus and its inactivation induced by hydrostatic pressure. High pressure is very effective in dissociating large assemblies such as microtubules, amyloid aggregates, amorphous aggregates and inclusion bodies. Because viral structure is highly dependent on protein-protein interactions, hydrostatic pressure has been a valuable tool for assessing viral structure-function relationships. Also, pressure has been suggested as an approach for viral inactivation and vaccine development. Because high pressure does not introduce exogenous substances into a vaccine and is often selective in its action on macromolecular structures, its use usually results in highly immunogenic preparations. Influenza virus poses a serious global health threat,

particularly in light of newly emerging strains such as avian virus H5N1. Recently, we used H3N8 avian influenza virus that had been inactivated by hydrostatic pressure as a vaccine. Our goal was to assess the immunogenic and protective capacity of the pressurized virus in a Balb/c mouse model. After vaccination, the mice were challenged and monitored for virus-specific antibodies, clinical symptoms and death. Following immunization, there was an increase in IgG1 and IgG2a levels in the serum of the mice and in IgA levels in nasal lavage specimens. A viral neutralization assay showed that neutralizing antibodies were produced. After challenge, the control group, which was immunized with saline, showed all of the examined clinical signs of disease, whereas animals that were vaccinated did not develop any clinical signs. The results indicated that the animals presented a satisfactory humoral response after vaccination and were protected against viral challenge. Those results were published in 2012 at Procedia in Vaccinology.



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AL 2

ASSOCIATE LABORATORY OF STRUCTURAL BIOLOGY OF CARDIAC AND AMYLOIDOGENIC PROTEINS

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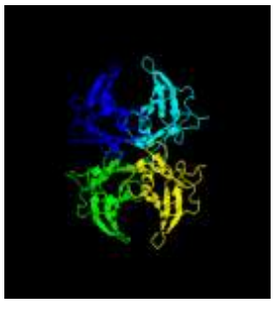


FIG 1: TTR QUATERNARY STRUCTURE. EACH MONOMER IS SHOWN IN A DIFFERENT COLOR.

Protein Misfolding Diseases caused by Transthyretin (TTR):

Protein misfolding diseases include a broad range of pathologies in which proteins fail to fold properly or to remain in their folded state. Many protein misfolding diseases, generically termed amyloidoses, are characterized clinically by the presence of proteinaceous insoluble amyloid material, the amyloid fibril. Amyloid fibrils share a common conformation, rich in cross-β structure formed by intertwined layers of β-sheets extending parallel to the fibril axis. Our group has been studying transthyretin (TTR), a 55-kDa homotetrameric protein composed of identical 127-residue subunits with a predominantly β-sheet structure and it is found in human plasma and cerebrospinal fluid (CSF) (Fig.1). Wild-type TTR (wt-TTR) is responsible for senile systemic amyloidosis, a disease that affects 10% of people over 80-years old and is characterized by heavy amyloid deposits in the heart. More than 80 point mutations of TTR are involved in familial amyloidotic polyneuropathy (FAP), familial amyloidotic cardiomiopathy (FAC) and central nervous system amyloidosis (CNSA). Among the variants of TTR, V30M and L55P are the most important because of their high frequency of occurrence and the aggressiveness of the symptoms they evoke. A25T, on the other hand, is one of the few TTR mutations associated with a rare type of amyloidosis that is restricted to the CNS and is characterized by amyloid fibril deposition in leptomeningeal and subarachnoid vessels. T119M is a non amyloidogenic variant alleviating the symptoms of the V30M mutation.

Several hypotheses have been proposed to explain the amyloidogenic properties of TTR. The most accepted one presupposes the dissociation of the tetramers into a monomeric, partially folded state, which is aggregation

prone. Our group has characterized an altered tetramer of TTR (T4*, purple circles) which is aggregation prone.

Below are summarized the main findings of our group regarding TTR and other amyloid proteins and other amyloid proteins.

Leptomeningeal Amyloidosis: identifying protein partners that co-aggregate during A25T-TTR fibrillogenesis in cerebrospinal fluid:

Deposition of amorphous aggregates and fibrils of TTR in leptomeninges and subarachnoid vessels is a characteristic of leptomeningeal amyloidosis (LA), a currently untreatable cerebral angiopathy. We reported the X-ray structure of the A25T homotetramer of TTR, a natural mutant described in a patient with LA. The structure of A25T-TTR is indistinguishable from that of wild-type TTR (wt-TTR), indicating that the difference in amyloidogenicity between A25T-TTR and wt-TTR cannot be ascribed to gross structural differences. Using pressure-induced dissociation of the tetramer, we show that A25T-TTR is 3 kcal/mol less stable than L55P-TTR, the most aggressive mutant of TTR described to date. After incubation for 15 days at 37 °C (pH 7.3), A25T-TTR forms mature amyloid fibrils. To mimic the environment in which TTR aggregates, we investigated aggregation in cerebrospinal fluid (CSF). Unlike L55P-TTR, A25T-TTR rapidly forms amyloid aggregates in CSF that incorporated several protein partners. Utilizing a proteomics methodology, we identified 19 proteins that copurified with A25T-TTR amyloid fibrils. We confirmed the presence of proteins previously identified to be associated with TTR aggregates in biopsies of TTR amyloidosis patients, such as clusterin, apolipoprotein E, and complement proteins. Moreover, we identified novel proteins, such as blood coagulation proteins. Overall, our results revealed the in vitro characterization of TTR aggregation in a biologically relevant environment, opening new avenues of investigation into the molecular mechanisms of LA.

Characterizing the interaction between TTR fibrils and inflammatory cells:

Since 1973, some groups have described the presence of inflammatory markers in association with amyloid fibrils from many diverse amyloidoses, including the TTR-related amyloidoses. Some of these markers originate from neutrophilic granules, such as elastase. Recently a novel death mechanism has been described in neutrophils in which chromosomal DNA decorated with elastase is extruded from the cells and serves as traps for microorganism. Notably, we showed that amyloid fibrils are able to induce this mechanism through PI3K-Akt-mTOR signaling pathway. In addition to neutrophils, A25T fibrils are able to activate microglia, the brain resident macrophage, inducing neurodegeneration in an animal model of TTR-related amyloidosis. We also are studying the role of inflammatory cells and its mediators in patients diagnosed with FAP. These patients possess higher levels of proinflammatory cytokines which correlates with disease progression. In conclusion, our data shows that inflammation plays an important role in TTR-related amyloidoses.

Characterizing amyloid-aggregation inhibitors:

Until now, TTR-related diseases have been untreatable, although a new drug called Tafamidis has been approved only in Europe to specifically treat V30M patients. Thus, new strategies are still necessary to treat FAP caused by other variants of TTR. TTR has two channels in the dimer interface that bind to the hormone thyroxin and that have been used to accommodate anti-amyloidogenic compounds. These compounds stabilize the tetramers, rendering TTR less amyloidogenic. We showed that three non-steroidal anti-inflammatory compounds-sulindac (SUL), indomethacin (IND) and lumiracoxib (LUM)-as tetramer stabilizers and aggregation inhibitors. WT-TTR and the very aggressive TTR variant L55P were used as models. These compounds were able to stabilize TTR against high hydrostatic pressure (HHP), increasing the ΔGf by several kcal. They were also effective in inhibiting WT-TTR and L55P acid- or HHP-induced aggregation; in particular, LUM and IND were very effective, inhibiting almost 100% of the aggregation of both proteins under certain conditions. The species formed when aggregation was performed in the presence of these compounds were much less toxic to cells in culture. The crystal structures of WT-TTR bound to the three compounds were solved at high resolution, allowing the identification of the relevant protein:drug interactions. We characterized the ligand-binding features of LUM, IND and SUL to TTR, emphasizing the critical interactions that render the protein more stable and less amyloidogenic.

Parkinson's disease (PD) is a movement disorder characterized by the loss of dopaminergic neurons in the substantia nigra and the formation of intraneuronal inclusions called Lewy bodies, which are composed mainly of α-synuclein (α-syn). Selegiline (Sel) is a noncompetitive monoamino oxidase B inhibitor that has neuroprotective effects and has been administered to PD patients as monotherapy or in combination with l-dopa. Besides its known effect of increasing the level of dopamine (DA) by monoamino oxidase B inhibition, Sel induces other effects that contribute to its action against PD. We evaluated the effects of Sel on the in vitro aggregation of A30P and wild-type α-syn. We showed that Sel delays fibril formation by extending the lag phase of aggregation. In the presence of Sel, electron microscopy reveals amorphous heterogeneous aggregates, including large annular species, which are innocuous to a primary culture enriched in dopaminergic neurons, while their age-matched counterparts are toxic. The inhibitory effect displayed by Sel is abolished when seeds (small fibril pieces) are added to the aggregation reaction, reinforcing the hypothesis that Sel interferes with early nuclei formation and, to a lesser extent, with fibril elongation. NMR experiments indicate that Sel does not interact with monomeric α-syn. Interestingly, when added in combination with DA (which favors the formation of toxic protofibrils), Sel overrides the inhibitory effect of DA and favors fibrillation. Additionally, Sel

blocks the formation of smaller toxic aggregates by perturbing DA-dependent fibril disaggregation. These effects might be beneficial for PD patients, since the sequestration of protofibrils into fibrils or the inhibition of fibril dissociation could alleviate the toxic effects of protofibrils on dopaminergic neurons. In nondopaminergic neurons, Sel might slow the fibrillation, giving rise to the formation of large nontoxic aggregates.

Neurotrophic Factors:

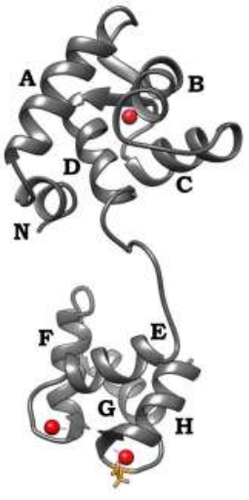
Parkinson's disease (PD) is a neurodegenerative disorder that is caused by the death of midbrain dopaminergic neurons. Current therapies for PD do not halt the neurodegeneration nor repair the affected neurons. Therefore, search for novel neurotrophic factors (NTF) for midbrain dopaminergic neurons, which could be used in novel therapeutic approaches, is highly wanted. In 2007, a potent NTF for dopaminergic neurons was described as the conserved dopamine neurotrophic factor (CDNF). Single doses of this protein protect and restore dopaminergic neurons in experimental models of PD. CDNF has two domains; an N-terminal saposin-like domain, which may bind to membranes; and a presumably intrinsically unstructured C-terminal which contains an internal cysteine bridge in a CXXC motif similar to that of thiol/disulphide oxidoreductases and isomerases, and may thus reduce the endoplasmic reticulum stress caused by incorrectly folded proteins. We show for the first time the nuclear magnetic resonance assignment of N-terminal domain of recombinant CDNF (residues 1–105) by solution 2D and 3D NMR spectroscopy. We were able to obtain a nearly complete resonance assignment, which is the first step toward the solution structure determination of this neurotrophic factor. More recently, the solved the structure of full-length CDNF and showed that this NTF protects domapinergic neurons in culture against toxic oligomers of a-synuclein.

Cardiomyopathies Caused by Defective Muscle Proteins (Sorenson laboratory):

The main focus of the muscle research group of AL2 (Sorenson laboratory) is on defective proteins that lead to cardiomyopathies, as well as associated mechanisms of action of normal contractile and regulatory proteins.

An extensive study of the cardiomyopathy-associated TnC mutant D145E carried out under standard laboratory conditions (20^{o}C) (publications submitted at the time of a previous report for AL 2 and now published, see list below) led to the conclusion that this mutant causes defects in cardiac thin-filament regulation that are quite small, even with all the proteins of the regulatory system present. PhD student T. Veltri has now shown that folding of the mutant protein is remarkably unstable in the physiological temperature range (30-45^{o}C), and that the instability is correlated with a substantial increase in sensitivity to Ca^{2+}, an important hallmark of the hypertrophic cardiomyopathies. These findings mean that future studies of TnC mutants will likely need

to include experiments at or near physiological temperatures, where the full range of their functional defects can be explored. A new set of experiments is underway to explore other structural characteristics of D145E and related cardiomyopathy mutants, in collaboration with G de Oliveira in Rio and Dr JR Pinto at Florida State University.



CARDIAC TNC WITH D145E INSERTED IN C-DOMAIN (YELLOW).

In another project related to regulation of contraction in cardiac fibers, doctoral student C. Figueiredo-Freitas has described a profile of negative effects of nitrosative stress on the physiology of cardiac myofilaments, associated with S-nitrosylation of reactive thiols to form R-SNO in key sarcomeric proteins. Specific sites in myosin, actin, troponin I and C, myosin-binding protein C and titin were identified by mass spectrometry (LC/MS-MS). Our experiments with different concentrations of CysNO, an NO donor, in living myocytes indicate that some –SNO sites, reactive to low CysNO concentrations, may be regulated by S-nitrosylation, while others react only to much higher concentrations. We will now attempt to use Cys-less mutants of some of these proteins to pinpoint the sites responsible for the observed inhibitory effects on maximum force, Ca^{2+} sensitivity, myofibrillar ATPase and rate of relaxation.

An interesting offshoot of the cardiac S-nitrosylation experiments arose during an INBEB seminar in which Dr A Vieyra (AL 17) described protection against ischemia-reperfusion injury of the kidney by prior injection of stem cells into the cortex. C. F.-Freitas´s suggestion that S-nitrosylation of key proteins might be the mechanism of protection, as it is in the heart, led to new experiments that demonstrated a large increase in mitochondrial protein R-SNO following the injection (Beiral et al., 2013, online). Further details in the AL 17 report.

A third project from the Sorenson laboratory is related to cytoplasmic motors, specifically myosin V. Using confocal microscopy, PhD student LT Oliveira has demonstrated that very low levels of the $A\beta_{42}$ peptíde, one of the villains in Alzheimer´s disease, interfere with intracellular transport in neurons (Oliveira et al, 2012). The nature of the organelles being transported is under investigation.

Biological and Medicinal Chemistry of Degenerative Diseases (Luis Mauricio's Laboratory, for Pharmaceutical Biotechnology at the School of Pharmacy at UFRJ):

***Diabetes* and amyloidosis** – Amylin is a peptide secreted by islet beta cells along with insulin, and it is involved in the regulation of several key metabolic functions. We have shown that s.c. amylin can regulate the glycemia in both normal and T1DM mice, in a very narrow concentration range,

independently from insulin. Reposition of amylin in diabetic individuals has been hampered by the limited solubility and highly amyloid character of human amylin. We have designed and produced a confined systems comprising polymeric nanoparticle dopped with amylin, which allows the sustained release of bioactive amylin *in vivo*. These data suggest that modulation of amylin stability by strategies such as molecular confinement and bioconjugation may serve as strategies for the production of stable amylin pharmaceutical formulation for therapeutic use.

Coagulopathies – Blood hemostasis control affects several diseases. On of the most important enzymes in the blood cascade is thrombin. One of our projects focuses in the characterization of thrombin ligands as lead compounds in the treatment of coagulopathies. Thrombin is a large protein and its structure has not been solved in solution yet. Despite the hundreds of crystallographic structures, no effective ligand has been designed up to the present for clinical therapeutic use. We have recently characterized the dynamics of thrombin in solution by the use of structural biology approaches such as SAXS and molecular dynamic simulation. Through these approaches we observed large conformational deviations from the crystal structure, which may account for distinct behaviors in solution compared to solid phase. Further characterization is being conducted in order to understand the structural basis for ligand interaction in solution. Consequences are evident, such as allowing the design of lead compounds targeting thrombin.

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AL 3

ASSOCIATE LABORATORY OF PROTEINS STRUCTURE DETERMINATION BY NMR – CNRMN

Jiri Jonas

Coordinator: Fabio Almeida – IBqM/CNRMNJJ/UFRJ.

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Ana Paula Valente – IBqM/UFRJ
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Ronaldo Mohana Borges – IBCCF/UFRJ
Marcius S. Almeida – IBqM/UFRJ

X **LA3 is directly related to CNRMN and the use of nuclear magnetic resonance as a tool to solve structure and dynamics of biomolecules, probe interactions and in the development of biologically active compound.**

During this period two major developments are in course. The methodology for structure determination of excited states, through the collaboration with the University of Connectiut Health Center with Prof. Dmitry Korzhnev and the implementation of fast-acquisition methods that will enable the automation of the resonance assignments and structural determination. This is being achieved by the collaboration with Scripps Research Institute with Prof. Kurt Wuthrich, Nobel Laureate in chemistry in 2002 (implementation of APSY) and through the collaboration with Prof. Martin Billeter from University of Gothenburg (implementation of PRODECOMP). All these new methods will enable the research groups that are part of LA3 to improve the efficiency of solving biologically relevant problems.

As a group, from January, 2011 to the Apr, 2013, we have published 19 papers and one patent deposit.

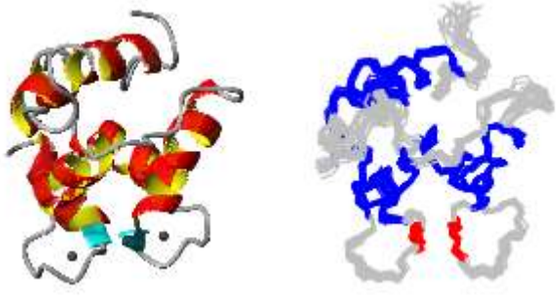
Laboratory for Nuclear Magnetic Resonance of Biomolecules – BioNMR, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro (Ana Paula Valente and Fábio C. L. Almeida)

^{1}H, ^{13}C and ^{15}N resonance assignments and second structure information of Gad m 1: a β-parvalbumin allergen from Atlantic cod (Gadus morhua).

Gad m 1 is the major allergen from Atlantic cod. It belongs to β-parvalbumin protein family and is characterized by the presence of two calcium-binding sites so called EF-hand motifs. β-Parvalbumins such as Gad m 1 are the most important fish allergens and their high cross-reactivity is the cause of the observed polysensitization to various fish species in allergic patients. Despite extensive efforts, the complete elucidation of β-parvalbumin-IgE complexes has not been

achieved yet. Allergen structural studies are essential for the development of novel immunotherapy strategies, including vaccination with hypoallergenic derivatives and chimeric molecules. Here, we report for the first time the NMR study of a β-parvalbumin: Gad m 1. This report includes: (1)H, (13)C and (15)N resonance assignments of Gad m 1 as well as the second structure information based on the (13)C chemical shifts.

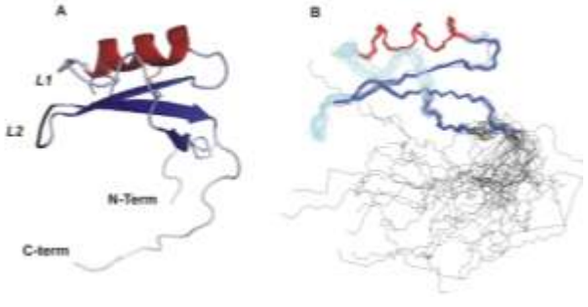
The structure of Gad m 1 is now solved. We are now mapping the interaction with IgE to better understand the allergy pathogenesis.



STRUCTURE OF GAD M 1, THE THE MOST IMPORTANT FISH ALLERGEN.

Portrayal of complex dynamic properties of sugarcane defensin 5 by NMR: multiple motions associated with membrane interaction.

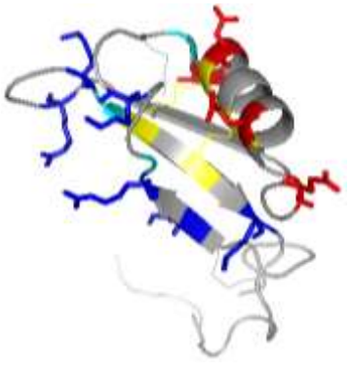
Defensins are essentially ancient natural antibiotics with potent activity extending from lower organisms to humans. Sd5 is a recently described antifungal defensin that appears to be the result of a recent gain of function. We reported here the solution NMR structure of Sd5 and characterized the backbone dynamics in the free state and in the presence of membrane models. (15)N relaxation dispersion measurements indicate intrinsic conformational exchange processes, showing two clear distinct k(ex), 490 and 1800 s(-1). These multiple motions may be related to transient twisting or breathing of the α helix and β sheet. The stages of membrane recognition and disruption by Sd5 over a large timescale range were mapped and demonstrated that Sd5 in solution sampled an ensemble of different conformations, of which a subset is selected upon membrane binding. Defensins share similar structures, but we demonstrated here that their dynamics can be extremely diverse.



STRUCTURE OF SD5 – PLANT DEFENSIN DISPLAYING COMPLEX DYNAMIC PROPERTY

Characterization of the Excited State of Sd5 by relaxation dispersion data.

Our group is now studying the excited state of SD5 using relaxation dispersion. The low populated excited state B is more compact than the highly populated low energy state (A). The entalpic interactions are the most important forces that mediate the A/B transition. Mostly polar residues stabilizes the protein. Excited state involves reorientation of the α-helix relative to the β-sheet. Possibly, there is a partial unfolding of the helix at the excited state.

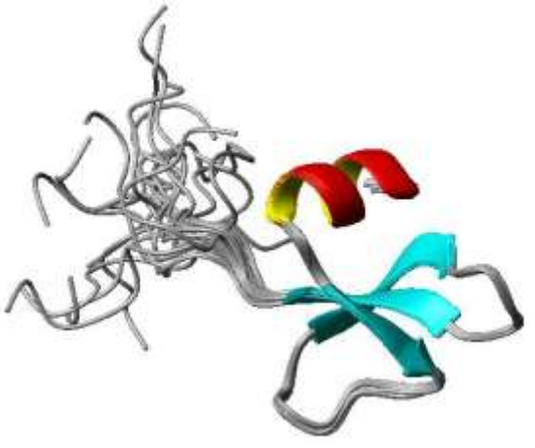


IN COULOR ARE THE RESIDUES PARTICIPATING IN THE TRASITION BETWEEN THE GROUND (A) AND EXCIETED STATE (B) OF SD5. POSITIVE RESIDUES ARE IN BLUE, NEGATIVE IN RED AND IN YELLOW ARE THE TWO DISULFIDE BONDS INVOLVED. MOST OF THE RESIDUES ARE POLAR.

Structure of Human β-Defensins – HBD6

Human β-defensins (HbDs) are small cationic antimicrobial proteins produced by epithelial cells. They are a component of the innate immune system and communicate with the adaptive immune system by interacting with specific chemokine and Toll-like receptors, resulting in the modulation of immune-competent cell responses in the host. HbDs can selectively chemoattract immature dendritic cells and memory T cells through two G-protein-coupled receptors, CCR2 and CCR6. HbDs are involved in several cellular functions, such as regulating epithelial cell proliferation, enhancing wound healing, stimulating chemokine production, inhibiting angiogenesis, promoting chemotaxis, and modulating host cell gene expression.

HbDs are believed to function as alarm molecules that stimulate the adaptive immune system when a threat is present. When HbDs are unable to recognize the threat, the delay in activating the adaptive response would provide the invader with a significant advantage. We utilized NMR for the structure determination of HbD6 and studied its interaction with tumor cell microvesicles (MVs). HbD6 showed a stronger interaction with MCF-7 MVs then with MDA-MB-231, suggesting an inverse correlation with the aggressiveness potential of the cell. The structure is shown bellow.



STRUCTURE OF HUMAN □-DEFENSINS 6.

The disordered N-terminal region of dengue virus capsid protein contains a lipid-droplet-binding motif.

Dengue is the major arthropod-borne human viral disease, for which no vaccine or specific treatment is available. We used NMR, zeta potential measurements and atomic force microscopy to study the structural features of the interaction between dengue virus C (capsid) protein and LDs (lipid droplets), organelles crucial for infectious particle formation. C protein-binding sites to LD were mapped, revealing a new function for a conserved segment in the N-terminal disordered region and indicating that conformational selection is involved in recognition.

The results suggest that the positively charged N-terminal region of C protein prompts the interaction with negatively charged LDs, after which a conformational

rearrangement enables the access of the central hydrophobic patch to the LD surface. Taken together, the results allowed the design of a peptide with inhibitory activity of C protein-LD binding, paving the way for new drug development approaches against dengue.

This work resulted in a patent deposition as following: **DENV - Derived peptides for the inhibition of the flavivirus replication, 2012.** ALMEIDA, F. C. L., Dapoian, A.T., Santos, N.C., Martins, I.C. Country: Brasil. Deposit Number: 020120047785., 28/05/2012.

Heat stability of Proteobacterial PII protein facilitates purification using a single chromatography step.

The P(II) proteins comprise a family of widely distributed signal transduction proteins that integrate the signals of cellular nitrogen, carbon and energy status, and then regulate, by protein-protein interaction, the activity of a variety of target proteins including enzymes, transcriptional regulators and membrane transporters.

We have previously shown that the P(II) proteins from Azospirillum brasilense, GlnB and GlnZ, do not alter their migration behavior under native gel electrophoresis following incubated for a few minutes at 95°C. This data suggested that P(II) proteins were either resistant to high temperatures and/or that they could return to their native state after having been unfolded by heat.

Here we used (1)H NMR to show that the A. brasilense GlnB is stable up to 70°C. The melting temperature (Tm) of GlnB was determined to be 84°C using the fluorescent dye Sypro-Orange. P(II) proteins from other Proteobacteria also showed a high Tm. We exploited the thermo stability of P(II) by introducing a thermal treatment step in the P(II) purification protocol, this step significantly improved the homogeneity of A. brasilense GlnB and GlnZ, Herbaspirillum seropedicae GlnB and GlnK, and of Escherichia coli GlnK.

Only a single chromatography step was necessary to obtain homogeneities higher than 95%. NMR(1) and in vitro uridylylation analysis showed that A. brasilense GlnB purified using the thermal treatment maintained its folding and activity. The purification protocol described here can facilitate the study of P(II) protein family members.

Identification of regions involved in substrate binding and dimer stabilization within the central domains of yeast Hsp40 Sis1.

Protein folding, refolding and degradation are essential for cellular life and are regulated by protein homeostatic processes such those that involve the molecular chaperone DnaK/Hsp70 and its co-chaperone DnaJ. Hsp70 action is initiated when proteins from the DnaJ family bind an unfolded protein for delivery purposes. In eukaryotes, the DnaJ family can be divided into two main groups, Type I and Type II, represented by yeast cytosolic Ydj1 and Sis1, respectively.

Although sharing some unique features both members of the DnaJ family, Ydj1 and Sis1 are structurally and functionally distinct as deemed by previous studies, including the observation that their central

domains carry the structural and functional information even in switched chimeras.

In this study, we combined several biophysical tools for evaluating the stability of Sis1 and mutants that had the central domains (named Gly/Met rich domain and C-terminal Domain I) deleted or switched to those of Ydj1 to gain insight into the role of these regions in the structure and function of Sis1. The mutants retained some functions similar to full length wild-type Sis1, however they were defective in others.

We found that: 1) Sis1 unfolds in at least two steps as follows: folded dimer to partially folded monomer and then to an unfolded monomer. 2) The Gly/Met rich domain had intrinsically disordered characteristics and its deletion had no effect on the conformational stability of the protein. 3) The deletion of the C-terminal Domain I perturbed the stability of the dimer. 4) Exchanging the central domains perturbed the conformational stability of the protein. Altogether, our results suggest the existence of two similar subdomains in the C-terminal domain of DnaJ that could be important for stabilizing each other in order to maintain a folded substrate-binding site as well as the dimeric state of the protein.

Moniliophthora perniciosa necrosis- and ethylene-inducing protein 2 (MpNep2) as a metastable dimer in solution: structural and functional implications.

Understanding how Nep-like proteins (NLPs) behave during the cell cycle and disease progression of plant pathogenic oomycetes, fungi and bacteria is crucial in light of compelling evidence that these proteins play a role in Witches` Broom Disease (WBD) of Theobroma cacao, one of the most important phytopathological problems to afflict the Southern Hemisphere.

The crystal structure of MpNep2, a member of the NLP family and the causal agent of WBD, revealed the key elements for its activity. This protein has the ability to refold after heating and was believed to act as a monomer in solution, in contrast to the related homologs MpNep1 and NPP from the oomyceteous fungus Phytophthora parasitica.

Here, we identify and characterize a metastable MpNep2 dimer upon over-expression in Escherichia coli using different biochemical and structural approaches. We found using ultra-fast liquid chromatography that the MpNep2 dimer can be dissociated by heating but not by dilution, oxidation or high ionic strength. Small-angle X-ray scattering revealed a possible tail-to-tail interaction between monomers, and nuclear magnetic resonance measurements identified perturbed residues involved in the putative interface of interaction. We also explored the ability of the MpNep2 monomer to refold after heating or chemical denaturation. We observed that MpNep2 has a low stability and cooperative fold that could be an explanation for its structure and activity recovery after stress. These results can provide new insights into the mechanism for MpNep2's action in dicot plants during the progression of WBD and may open new avenues for the involvement of NLP-oligomeric species in phytopathological disorders.

Thermodynamic and structural characterization of zwitterionic micelles of the membrane protein solubilizing amidosulfobetaine surfactants ASB-14 and ASB-16.

Surface tension and isothermal titration calorimetry (ITC) were used to determine the critical micelle concentration (cmc) of the zwitterionic amidosulfobetaine surfactants ASB-14 and ASB-16 (linear-alkylamidopropyldimethylammoniopropanosulfonates) at 25 °C. The cmc and the heat of micellization were determined from 15 to 75 °C by ITC for both surfactants.

The increase in temperature caused significant changes in the enthalpy and in the entropy of micellization, with small changes in the standard Gibbs energy (ΔG(mic)), which is consistent to an enthalpy−entropy compensation with a compensatory temperature of 311 K (ASB-14) and 314 K (ASB-16). In the studied temperature range, the heat capacity of micellization (ΔC(p)(mic)) was essentially constant. The experimental ΔC(p)(mic) was lower than that expected if only hydrophobic interactions were considered, suggesting that polar interactions at the head groups are of significant importance in the thermodynamics of micelle formation by these surfactants. Indeed, a NMR NOESY spectrum showed NOEs that are improbable to occur within the same monomer, resulting from interactions at the polar head groups involving more than one monomer.

The ITC and NMR results indicate a tilt in the polar headgroup favoring the polar interactions. We have also observed COSY correlations typical of dipolar interactions that could be recovered with the partial alignment of the molecule in solution, which results in an anisotropic tumbling. The anisotropy suggested an ellipsoidal shape of the micelles, which results in a positive magnetic susceptibility, and ultimately in orientation induced by the magnetic field.

Such an ellipsoidal shape was confirmed from results obtained by SAXS experiments that revealed aggregation numbers of 108 and 168 for ASB-14 and ASB-16 micelles, respectively. This study characterizes an interesting micelle system that can be used in the study of membrane proteins by solution NMR spectroscopy.

Effort on metabolomics and metabonomics by NMR

Nuclear magnetic resonance is a powerful technique to the global understanding of cell metabolism and pathogenesis. The BioNMR laboratory is using NMR to understand the metabolism of saliva and its correlation with diverse pathogenesis, including the formation of caries. We also did NMR studies lung cancer cells.

Salivary metabolite signatures of children with and without dental caries lesions

A metabolomic approach was used to analyze endogenous metabolites and to correlate with a specific biological state. The analysis of salivary metabolites is a growing area of investigation with potential for basic and clinical applications.

Analyses of children's saliva in different dentitions and with or without caries could poten- tially reveal a specific profile

related to oral disease risk. Nuclear Magnetic Resonance (NMR) is well suited for mixture analysis followed by Principal Component Anal- ysis combined with Linear Regression (PCA-LR) statistics and was used to identify differences in the salivary metabolites. The classificatory analysis was performedusing PCA-LR based on 1,000 cross-validation bootstrap runs from both classifiers in order to increase the data information from a small sample size. The PCA-LR presented a statistically good classificatory performance for children with and without caries with an accuracy of 90.11 % (P\0.001), 89.61 %sensitivity (P\0.001), and 90.82 % specificity (P\0.001). Children with caries lesions presented higher levels of several metabolites, including lactate, fatty acid, acetate and n-butyrate.

Saliva from subjects with different dentition stages was also analyzed. Although the salivary samples were poorly classified, permanent dentition presented increased levels of acetate, saccharides and propionate. The NMR data and PCA-LR were able to classify saliva from children with or without caries, with performance indexes comparable to the partial least-squares regression discriminant analysis (PLS-DA) results also performed. Our data also showed similar salivary metabolite profiles for healthy subjects despite the differences in their oral hygiene habits, socio-economic status and food intake.

This work is collaboration with Dental School of the Federal University of Rio de Janeiro.

Energy metabolism in H460 lung cancer cells: effects of histone deacetylase inhibitors.

Tumor cells are characterized by accelerated growth usually accompanied by up-regulated pathways that ultimately increase the rate of ATP production. These cells can suffer metabolic reprogramming, resulting in distinct bioenergetic phenotypes, generally enhancing glycolysis channeled to lactate production. In the present work we showed metabolic reprogramming by means of inhibitors of histone deacetylase (HDACis), sodium butyrate and trichostatin. This treatment was able to shift energy metabolism by activating mitochondrial systems such as the respiratory chain and oxidative phosphorylation that were largely repressed in the untreated controls.

Various cellular and biochemical parameters were evaluated in lung cancer H460 cells treated with the histone deacetylase inhibitors (HDACis), sodium butyrate (NaB) and trichostatin A (TSA). NaB and TSA reduced glycolytic flux, assayed by lactate release by H460 cells in a concentration dependent manner. NaB inhibited the expression of glucose transporter type 1 (GLUT 1), but substantially increased mitochondria bound hexokinase (HK) activity. NaB induced increase in HK activity was associated to isoform HK I and was accompanied by 1.5 fold increase in HK I mRNA expression and cognate protein biosynthesis. Lactate dehydrogenase (LDH) and pyruvate kinase (PYK) activities were unchanged by HDACis suggesting that the increase in the HK activity was not coupled to

glycolytic flux. High resolution respirometry of H460 cells revealed NaB-dependent increased rates of oxygen consumption coupled to ATP synthesis.

Metabolomic analysis showed that NaB altered the glycolytic metabolite profile of intact H460 cells. Concomitantly we detected an activation of the pentose phosphate pathway (PPP). The high O(2) consumption in NaB-treated cells was shown to be unrelated to mitochondrial biogenesis since citrate synthase (CS) activity and the amount of mitochondrial DNA remained unchanged.

NaB and TSA induced an increase in mitochondrial function and oxidative metabolism in H460 lung tumor cells concomitant with a less proliferative cellular phenotype

Laboratory for Structural Genomics of Kinetoplastids, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro (Prof. José Ricardo M. Pires)

(1)H, (15)N and (13)C assignments of a putative peptidyl prolyl cis-trans isomerase FKBP12 from Trypanosoma brucei.

TbFKBP12 is a putative peptidyl prolyl cis-trans isomerase from Trypanosoma brucei, causative agent of the African trypanosomiasis or sleeping sickness. It interacts with the immunosuppressive drug rapamycin inhibiting the formation of TORC2 complex leading to parasite death by inhibiting cell proliferation through cytokinesis blockade. Moreover, RNAi silencing of TbFKBP12 revealed essential function in both procyclic and bloodstream forms. Both facts make TbFKBP12 an attractive target for ligand development and thus structural data is desirable. In this work we report the NMR resonance assignments for (1)H, (15)N and (13)C nuclei in the backbone and side chains of the TbFKBP12 as basis for further studies of structure, backbone dynamics, interaction mapping and drug screening.

Purification and characterization of Hb 98-114: a novel hemoglobin-derived antimicrobial peptide from the midgut of Rhipicephalus (Boophilus) microplus.

The antimicrobial activity of hemoglobin fragments (hemocidins) has been reported in a variety of models. The cattle tick Rhipicephalus (Boophilus) microplus is a blood sucking arthropod from where the first in vivo-generated hemocidin was characterized (Hb 33-61).

In the present work we identified a novel antimicrobial peptide from the midgut of fully engorged R. (B.) microplus females, which comprises the amino acids 98-114 of the alpha subunit of bovine hemoglobin, and was designated Hb 98-114. This peptide was active against several yeast and filamentous fungi, although no activity was detected against bacteria up to 50μM of the synthetic peptide. Hb 98-114 was capable of permeabilizing Candida albicans cell membrane and had a fungicidal effect against this yeast. Circular dichroism (CD) and nuclear magnetic resonance (NMR) experiments showed that Hb 98-114 has a random conformation in aqueous solution but switches to an alpha-helical conformation in the presence of sodium dodecyl sulfate (SDS).

This alpha helix adopts an amphipathic structure which may be the mechanism of cell membrane permeabilization. Importantly, Hb 98-114 may play an important role in defending the tick midgut against fungal pathogens and is the first hemocidin with specific antifungal activity to be characterized.

Spectroscopic characterization of oligoaniline microspheres obtained by an aniline-persulfate approach.

This paper investigates the structure of the products obtained from the polymerization of aniline with ammonium persulfate in a citrate/phosphate buffer solution at pH 3 by resonance Raman, NMR, FTIR, and UV-vis-NIR spectroscopies. All the spectroscopic data showed that the major product presented segments that were formed by a 1,4-Michael reaction between aniline and p-benzoquinone monoimine, ruling out the formation of polyazane structure that has been recently proposed.

The characterization of samples obtained at different stages of the reaction indicated that, as the reaction progressed, phenazine units were formed and 1,4-Michael-type adducts were hydrolyzed/oxidized to yield benzoquinone. Raman mapping data suggested that phenazine-like segments could be related to the formation of the microspheres morphology

Laboratory for Structural Genomics of Cancer, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro (Prof. Marcius Silva Almeida).

Parkinson's disease (PD) is a neurodegenerative disorder that is caused by the death of midbrain dopaminergic neurons. Current therapies for PD do not halt the neurodegeneration nor repair the affected neurons. Therefore, search for novel neurotrophic factors (NTF) for midbrain dopaminergic neurons, which could be used in novel therapeutic approaches, is highly wanted. In 2007, a potent NTF for dopaminergic neurons was described as the conserved dopamine neurotrophic factor (CDNF).

Single doses of this protein protect and restore dopaminergic neurons in experimental models of PD. CDNF has two domains; an N-terminal saposin-like domain, which may bind to membranes; and a presumably intrinsically unstructured C-terminal which contains an internal cysteine bridge in a CXXC motif similar to that of thiol/disulphide oxidoreductases and isomerases, and may thus reduce the endoplasmic reticulum stress caused by incorrectly folded proteins.

We show for the first time the nuclear magnetic resonance assignment of N-terminal domain of recombinant CDNF (residues 1-105) by solution 2D and 3D NMR spectroscopy. We were able to obtain a nearly complete resonance assignment, which is the first step toward the solution structure determination of this neurotrophic factor.

The structure of CDNF is now solved. The Figure is shown bellow.

Structure of CDNF

Heparin binding by murine recombinant prion protein leads to transient aggregation and formation of RNA-resistant species.

The conversion of cellular prion protein (PrP(C)) into the pathological conformer PrP(Sc) requires contact between both isoforms and probably also requires a cellular factor, such as a nucleic acid or a glycosaminoglycan (GAG).

Little is known about the structural features implicit in the GAG-PrP interaction. In the present work, light scattering, fluorescence, circular dichroism, and nuclear magnetic resonance (NMR) spectroscopy were used to describe the chemical and physical properties of the murine recombinant PrP 23-231 interaction with low molecular weight heparin (LMWHep) at pH 7.4 and 5.5. LMWHep interacts with rPrP 23-231, thereby inducing transient aggregation. The interaction between murine rPrP and heparin at pH 5.5 had a stoichiometry of 2:1 (LMWHep:rPrP 23-231), in contrast to a 1:1 binding ratio at pH 7.4. At binding equilibrium, NMR spectra showed that rPrP complexed with LMWHep had the same general fold as that of the free protein, even though the binding can be indicated by significant changes in few residues of the C-terminal domain, especially at pH 5.5.

Notably, the soluble LMWHep:rPrP complex prevented RNA-induced aggregation. We also investigated the interaction between LMWHep and the deletion mutants rPrP Δ51-90 and Δ32-121. Heparin did not bind these constructs at pH 7.4 but was able to interact at pH 5.5, indicating that this glycosaminoglycan binds the octapeptide repeat region at pH 7.4 but can also bind other regions of the protein at pH 5.5. The interaction at pH 5.5 was dependent on histidine residues of the murine rPrP 23-231. Depending on the cellular milieu, the PrP may expose different regions that can bind GAG. These results shed light on the role of GAGs in PrP conversion. The transient aggregation of PrP may explain why some GAGs have been reported to induce the conversion into the misfolded, scrapie conformation, whereas others are thought to protect against conversion. The acquired resistance of the complex against RNA-induced aggregation explains some of the unique properties of the PrP interaction with GAGs.

Laboratório de Genômica Estrutural, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (Ronaldo Mohana-Borges)

Mapping the Interactions of Dengue Virus NS1 Protein with Human Liver Proteins Using a Yeast Two-Hybrid System: Identification of C1q as an Interacting Partner.

Dengue constitutes a global health concern. The clinical manifestation of this disease varies from mild febrile illness to

severe hemorrhage and/or fatal hypovolemic shock. Flavivirus nonstructural protein 1 (NS1) is a secreted glycoprotein that is displayed on the surface of infected cells but is absent in viral particles. NS1 accumulates at high levels in the plasma of dengue virus (DENV)-infected patients, and previous reports highlight its involvement in immune evasion, dengue severity, liver dysfunction and pathogenesis.

In the present study, we performed a yeast two-hybrid screen to search for DENV2 NS1-interacting partners using a human liver cDNA library. We identified fifty genes, including human complement component 1 (C1q), which was confirmed by coimmunoprecipitation, ELISA and immunofluorescence assays, revealing for the first time the direct binding of this protein to NS1.

Furthermore, the majority of the identified genes encode proteins that are secreted into the plasma of patients, and most of these proteins are classified as acute-phase proteins (APPs), such as plasminogen, haptoglobin, hemopexin, α-2-HS-glycoprotein, retinol binding protein 4, transferrin, and C4.

The results presented here confirm the direct interaction of DENV NS1 with a key protein of the complement system and suggest a role for this complement protein in the pathogenesis of DENV infection.

High mobility group box 1 protein as an auxiliary biomarker for dengue diagnosis.

Despite the availability of many methods for rapid and early diagnosis of dengue, there is still a need to develop new approaches that not only combine low cost, specificity, and sensitivity, but also are capable of accurately detecting secondary infection in the early stages of the disease. We report the potential of the high mobility group box 1 protein as an auxiliary biomarker for early dengue diagnosis. We tested a 205-sample serum panel that included negative and positive samples from primary and secondary dengue cases, as well as samples from patients with dengue-like symptoms. We observed that high mobility group box 1 protein was generally detected only in dengue-positive samples for persons with primary and secondary infections. These results highlight the possibility of using this endogenous molecule as an auxiliary biomarker to aid in dengue detection and improve current methods for early diagnosis of dengue.

Elevated serum levels of high mobility group box 1 (HMGB1) protein in dengue-infected patients are associated with disease symptoms and secondary infection.

The aim of this study was to evaluate whether HMGB1 levels were altered in DENV-infected patients' sera and whether this augment correlated with disease pathogenesis. Samples from DENV-infected patients were collected from different days after the onset of symptoms and from patients experiencing primary or secondary infection. The circulating HMGB1 concentration was measured in healthy blood donors as well as in donors with primary and secondary cases of DENV infection by a quantitative capture ELISA assay.

We observed that the HMGB1 concentration in DENV-infected patients was significantly higher than in healthy patients. HMGB1 levels reached the highest concentration in the first day after the onset of symptoms and decreased throughout the course of the infection. Moreover, we observed that the HMGB1 concentration was augmented during secondary infection as well. We hypothesize that HMGB1 levels correlate with disease pathogenesis, specifically with the clinical symptoms and secondary infection, implicating a pro-inflammatory cytokine role for HMGB1 in DENV infection. This is the first report assessing the circulating levels of HMGB1 during DENV infection.

The dengue vector Aedes aegypti contains a functional high mobility group box 1 (HMGB1) protein with a unique regulatory C-terminus.

The mosquito Aedes aegypti can spread the dengue, chikungunya and yellow fever viruses. Thus, the search for key molecules involved in the mosquito survival represents today a promising vector control strategy. High Mobility Group Box (HMGB) proteins are essential nuclear factors that maintain the high-order structure of chromatin, keeping eukaryotic cells viable. Outside the nucleus, secreted HMGB proteins could alert the innate immune system to foreign antigens and trigger the initiation of host defenses.

In this work, we cloned and functionally characterized the HMGB1 protein from Aedes aegypti (AaHMGB1). The AaHMGB1 protein typically consists of two HMG-box DNA binding domains and an acidic C-terminus. Interestingly, AaHMGB1 contains a unique alanine/glutamine-rich (AQ-rich) C-terminal region that seems to be exclusive of dipteran HMGB proteins. AaHMGB1 is localized to the cell nucleus, mainly associated with heterochromatin. Circular dichroism analyses of AaHMGB1 or the C-terminal truncated proteins revealed α-helical structures.

We showed that AaHMGB1 can effectively bind and change the topology of DNA, and that the AQ-rich and the C-terminal acidic regions can modulate its ability to promote DNA supercoiling, as well as its preference to bind supercoiled DNA. AaHMGB1 is phosphorylated by PKA and PKC, but not by CK2. Importantly, phosphorylation of AaHMGB1 by PKA or PKC completely abolishes its DNA bending activity. Thus, our study shows that a functional HMGB1 protein occurs in Aedes aegypt and we provide the first description of a HMGB1 protein containing an AQ-rich regulatory C-terminus.

Dengue virus capsid protein binding to hepatic lipid droplets (LD) is potassium ion dependent and is mediated by LD surface proteins.

Dengue virus (DENV) affects millions of people, causing more than 20,000 deaths annually. No effective treatment for the disease caused by DENV infection is currently available, partially due to the lack of knowledge on the basic aspects of the viral life cycle, including the molecular basis of the interaction between viral components and cellular compartments.

Here, we characterized the properties of the interaction between the DENV capsid (C) protein and hepatic lipid droplets (LDs), which was recently shown to be essential for the virus replication cycle. Zeta potential analysis revealed a negative surface charge of LDs, with an average surface charge of -19 mV. The titration of LDs with C protein led to an increase of the surface charge, which reached a plateau at +13.7 mV, suggesting that the viral protein-LD interaction exposes the protein cationic surface to the aqueous environment. Atomic force microscopy (AFM)-based force spectroscopy measurements were performed by using C protein-functionalized AFM tips.

The C protein-LD interaction was found to be strong, with a single (un)binding force of 33.6 pN. This binding was dependent on high intracellular concentrations of potassium ions but not sodium. The inhibition of Na(+)/K(+)-ATPase in DENV-infected cells resulted in the dissociation of C protein from LDs and a 50-fold inhibition of infectious virus production but not of RNA replication, indicating a biological relevance for the potassium-dependent interaction. Limited proteolysis of the LD surface impaired the C protein-LD interaction, and force measurements in the presence of specific antibodies indicated that perilipin 3 (TIP47) is the major DENV C protein ligand on the surface of LDs.

Induction of a protective response in mice by the dengue virus NS3 protein using DNA vaccines.

The dengue non-structural 3 (NS3) is a multifunctional protein, containing a serino-protease domain, located at the N-terminal portion, and helicase, NTPase and RTPase domains present in the C-terminal region. This protein is considered the main target for CD4+ and CD8+ T cell responses during dengue infection, which may be involved in protection. However, few studies have been undertaken evaluating the use of this protein as a protective antigen against dengue, as well as other flavivirus.

In the present work, we investigate the protective efficacy of DNA vaccines based on the NS3 protein from DENV2. Different recombinant plasmids were constructed, encoding either the full-length NS3 protein or only its functional domains (protease and helicase), fused or not to a signal peptide (t-PA). The recombinant proteins were successfully expressed in transfected BHK-21 cells, and only plasmids encoding the t-PA signal sequence mediated protein secretion. Balb/c mice were immunized with the different DNA vaccines and challenged with a lethal dose of DENV2. Most animals immunized with plasmids encoding the full-length NS3 or the helicase domain survived challenge, regardless of the presence of the t-PA.

However, some mice presented clinical signs of infection with high morbidity (hind leg paralysis and hunched posture), mainly in animal groups immunized with the DNA vaccines based on the helicase domain. On the other hand, inoculation with plasmids encoding the protease domain did not induce any protection, since mortality and morbidity rates in these mouse groups were similar to

those detected in the control animals. The cellular immune response was analyzed by ELISPOT with a specific-CD8+ T cell NS3 peptide. Results revealed that the DNA vaccines based on the full-length protein induced the production of INF-γ, thus suggesting the involvement of this branch of the immune system in the protection.

Characterization of a porin channel in the endosymbiont of the trypanosomatid protozoan Crithidia deanei.

Crithidia deanei is a trypanosomatid protozoan that harbours a symbiotic bacterium. The partners maintain a mutualistic relationship, thus constituting an excellent model for studying metabolic exchanges between the host and the symbiont, the origin of organelles and cellular evolution.

According to molecular analysis, symbionts of different trypanosomatid species share high identity and descend from a common ancestor, a β-proteobacterium of the genus Bordetella. The endosymbiont is surrounded by two membranes, like Gram-negative bacteria, but its envelope presents special features, since phosphatidylcholine is a major membrane component and the peptidoglycan layer is highly reduced, as described in other obligate intracellular bacteria. Like the process that generated mitochondria and plastids, the endosymbiosis in trypanosomatids depends on pathways that facilitate the intensive metabolic exchanges between the bacterium and the host protozoan.

A search of the annotated symbiont genome database identified one sequence with identity to porin-encoding genes of the genus Bordetella. Considering that the symbiont outer membrane has a great accessibility to cytoplasm host factors, it was important to characterize this single porin-like protein using biochemical, molecular, computational and ultrastructural approaches. Antiserum against the recombinant porin-like molecule revealed that it is mainly located in the symbiont envelope. Secondary structure analysis and comparative modelling predicted the protein 3D structure as an 18-domain β-barrel, which is consistent with porin channels.

Electrophysiological measurements showed that the porin displays a slight preference for cations over anions. Taken together, the data presented herein suggest that the C. deanei endosymbiont porin is phylogenetically and structurally similar to those described in Gram-negative bacteria, representing a diffusion channel that might contribute to the exchange of nutrients and metabolic precursors between the symbiont and its host cell.

Polyclonal antibodies against properly folded Dengue virus NS1 protein expressed in E. coli enable sensitive and early dengue diagnosis.

The non-structural 1 (NS1) protein plays an important role in dengue diagnosis because it has been detected as a soluble serum antigen in both primary and secondary infections. The NS1 protein was expressed in Escherichia coli cells, and the efficiency of four different refolding protocols was tested. All of the protocols generated dimeric NS1 in a conformation similar to that of the protein expressed by eukaryotic cells.

A polyclonal antibody produced from the properly folded E. coli recombinant NS1 (rNS1) protein proved to be a useful tool for the diagnosis of Dengue virus because it detected 100% of the Dengue virus 2 (DENV2) in infected patients' sera and 60% of the DENV IgM-positive sera not detected by commercial NS1-based diagnostic kits. These data suggest a high-efficiency method for correctly folding rNS1 that maintains its structural and immunogenic properties.

In addition, a detection method using the polyclonal antibody against correctly folded rNS1 seemed to be more sensitive and efficient for NS1 detection in serum, highlighting its usefulness for developing a high-sensitivity diagnostic kit (Alonso et al., 2011).

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Marcius Silva Almeida

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AL 4

ASSOCIATE LABORATORY OF PHARMACOLOGIC PROTEOMIC

Coordinator: Russolina Zingali - IBqM/UFRJ.

Members:

Robson Queiroz Monteiro – IBqM/UFRJ
Márcia Regina Soares da Silva – IQ/UFRJ
Bianca Cruz Neves – IQ/UFRJ

The Proteomic Unity has been serving many laboratories from INBEB for the determination of mass from recombinant proteins to peptides in order to confirm correct expression and/or synthesis (Laboratories from Dr Foguel, Dr Almeida, Dr da Silva, Dr Bisch, etc). Furthermore, the unit continued to collaborated in projects that envisage the proteomic characterization of biological processes such as, the characterization of the ventrar disc of the Giardia lamblia; the interaction of the Dengue virus with hepatic cells; the characterization of the golgi apparatus of Thrichomonas faetus etc... (Dr. Souza, Dr. Benchimol, Dr. Bisch, Dr. Soares, Dr Souto-Padron).

Main research projects where the group participates:

1. Proteomics, Genomics and Bioinformatics in the study of the interaction between pathogenic and non-pathogenic microorganisms with their hosts.
2. Prospective proteomics.

Results obtained for these projects:

Study of Gluconacetobacter diazotrophicus interaction with plant

In the previous report we have shown some results on the interacion of *Gluconacetobacter diazotrophicus* and the plant *Arabidopis thaliana*.

Arabidopsis thaliana was chosen as study model, because its *genome* was fully sequenced. *Arabidopsis* plants were inoculated with *Gluconacetobacter* in hydroponic medium. After 3,7 and 10 days of inoculation the growth of colonies in plate was tested and confirmed the presence of the bacterium within the plant tissues that showed an increased colonization of bacteria on leaves compared with roots. Also some morphological changes were measured, an increase in the development of lateral roots and root hairs of plants in the presence of the bacteria were observed. The extracts of both segments were submitted to 1D SDS-PAGE. The extracts of inoculated leaves showed to be enhanced by three major proteins bands between 50–20 KDa. The trypsinized proteins bands have been analyzed using proteomics technologies. and revealed a total of 283 proteins identified in leaves and 81 in roots in the absence of the bacteria, 262 in leaves and 101 in roots in its presence. Identified proteins, in leaves with bacteria, such as, ribulose-1,5-biphosphate

carboxylase may improve plant growth through increased photosynthetic rates. The results suggest that the *G. diazotrophicus* is able colonizing *A. thaliana* plants tissues, contributing to the development of the plant, through the formation of lateral roots and root hairs. Changes the pattern of protein expression in both tissues suggest that this interaction may be beneficial to plant.

Dengue virus infection

In the previous report we show our first results on the analysis of the secreted proteins of HepG2 cells infected or not with the Dengue virus (Higa *et al*., 2008). We now continued the analyses on the peptidomic (<10kDa)., A different group of strategies with more sensitive equipments permited the identification of more then 50 peptides. Most of than were the result of higher molecular weight proteins.

Snake venom proteomics

The antiserum therapy is the most effective treatment available to save envenomations by venomous animals. The productions of this immunobiologicals have been done for the same principle from their discovery, immunizing animals with whole venom. Our group has studied the immunochemistry of snake venoms and antivenoms to identify molecules that escape of antibodies recognition and neutralization. Researche in this field can bring improvements and effectiveness in these products and treatment of snakebite. In this line, to gain clues for outlining an alternative antiserum generation strategy based on immunization we characterized the proteome and the venom gland transcriptome of *coral snakes, Micrurus altirostris and M. corallinus*. The transcripts codifying for 3FTx represent 80% of gland product in *Micrurus altirostris* followed by *Micrurus corallinus* counting 54% of venom transcrips. All 3FTX share a common structure of three loops organized by anti-parallel β-sheets. A common feature of these toxins is their loops cross-linked by four conserved disulfide bridges. *Micrurus corallinus'* 3FTXs sequences were organized in tree subtypes groups comprehending α-neurotoxins, non-conventional 3FTx and cardiotoxins (Fig. 1). The eppitope mapping of a α-neurotoxin from *Naja nigricollis* highlight the second loop as most important region to eppitope content (Tenette-Souaille and Smith, 1998). Based in this report we analyzed the immunogenicity of peptides stretches covering from the second cysteine of first loop until the second cysteine of second loop (Fig 1). The peptides selected representing α-neurotoxins (Fig. 1A) cardiotoxins (Fig. 1B) and non conventional 3FTx of *M. corallinus* venom were synthesized for further utilization as antigens. For *Micrurus altirostris* three peptides were selected to cover the 3FTx subtypes of its venom. The α-neurotoxins were organized in two groups by homology. We called altirostrotoxin-I (Fig. 2A) the sequences identified on proteoma in the first fraction of reverse phase separated chromatography in *M. altirostris* showing homology of 97% with D1 of *M. pyrrhocryptus* and MS-1 of *M. surinamensis*, both first peaks in reverse phase chromatography separation of these venoms. This conserved toxin is followed by altirostrotoxins II (Fig. 2B), the α-neurotoxin showing 85% of homology with frontotoxin II of *M. frontalis*. Its important

to mention that the homology inner this two types of α-neurotoxin is less than 28%. The third group of 3FTXs used to peptide stretch design identified on proteome and transcriptoma surveys was that of week neurotoxins (Fig. 2C). We produced three polyclonal antibodies (serum A, B, C), serum A and B by encapsulating synthetic peptides in liposome vesicles, with and without addiction of lipopolissacaride (LPS) in formulation. The serum C was produced linking the peptides with a carrier protein KLH. Each serum was evaluated by ELISA and the efficacy to recognize "native" antigens of *M. altirostris* venom was measured (Fig.: 3). The commercial antiserum against coral snake produced by Butantan Institute was analyzed as control and data comparison. The serum B showed highest immune-reactivity with native proteins when compared with serum A and C (Fig. 3A). In this line the serum B reach a half of recognition (DO ~ 0,5) of commercial anti-coral (DO ~1). These results represent the first step to design an antivenom based on structural similarities of major toxic molecules found on *Micrurus* venom. The capability of the antiserum to neutralize the toxic activities of *M. altirostris* venom is being investigated in our laboratories.

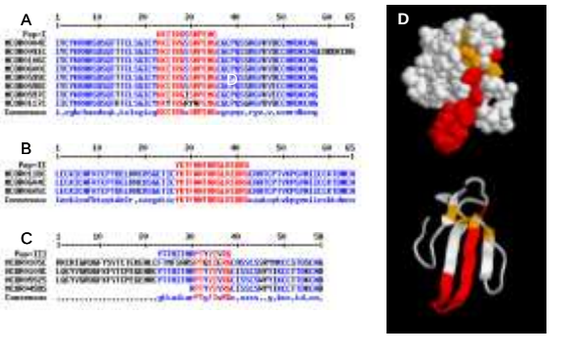

FIGURE 1: SEQUENCE ALIGNMENT OF THREE MAJOR EXPRESSED 3FTX OF *M. CORALLINUS* VENOM. THE SEQUENCES WERE FROM TRANSCRIPTOME STUDIES OF *M. CORALLINUS* (LEÃO ET AL.,) SHOWING HOMOLOGY WITH A-NEUROTOXINS (A), NONCONVENTIONAL 3FTX (B) AND CARDIOTOXINS (C). THE MOLECULAR MODEL (D) IS HIGHLIGHT THE SURFACE SELECTED (IN RED) TO ORGANIC SYNTHESIS. THE MODELS WERE GENERATED USING CRYSTALLOGRAPHIC DATA FROM ERABUTOXIN PDB NUMBER XX.

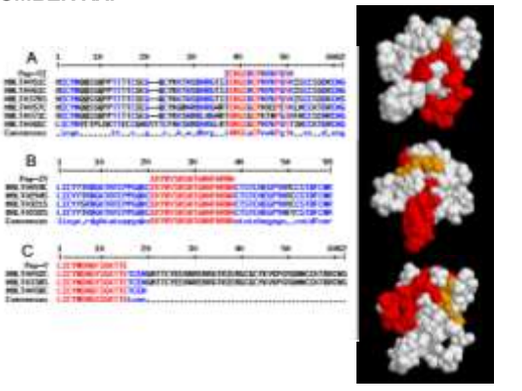

FIGURE 2: SEQUENCE ALIGNMENT OF THREE MAJOR EXPRESSED 3FTX OF *M. CORALLINUS*. THE SEQUENCES WERE FROM TRANSCRIPTOME ANALYSIS OF *M. ALTIROSTRIS* (CORREA NETTO ET. AL., 2011) SHOWING HOMOLOGY WITH A-NEUROTOXINS CALLED HEREIN ALTIROSTROTOXINAS-I (A), ALTIROSTROTOXINAS-II (B) AND WEEK NEUROTOXINS CALLED ALTIROSTROTOXINAS-III (C). THE MOLECULAR MODEL (1D) ARE HIGHLIGHT THE SURFACE SELECTED (IN RED) TO ORGANIC SYNTHESIS BY F-MOC . THE MODELS WERE GENERATED USING CRYSTALLOGRAPHIC DATA FROM ERABUTOXIN PDB NUMBER XX.

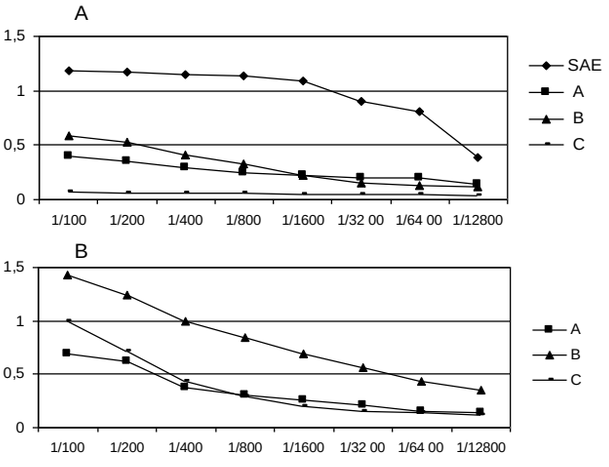

FIGURE 3: DIRECT-BIDING ELISA EVALUATING THE AFFINITY OF ANTIBODIES TO WHOLE VENOM (PANEL A) AND THREE-FINGER-TOXINS (PANEL B). THE SERA WERE RAISED IN RABBIT WITH 3FTX-PEPTIDES AS ANTIGEN ENCAPSULATED IN LIPOSOME WITH (A) AN WITHOUT (B) LPS. (C) THE IMMUNE-REACTIVITY OF ANTI-SERA PRODUCED BY 3FTX-PEPTIDES CONJUGATED WITH KLH.

Proteomic Profile of membrane Proteins from HCC-1954 Breast Cancer Cell line

Breast cancer can disseminate to regional lymph nodes and establish distant metastases, preferentially in bone, lung, and liver tissues, resulting in poor outcome and high

mortality. The molecules closely associated with these processes are predominantly present at the cell surface and in the extracellular space, establishing the first contacts with the target tissue. In our laboratory we have started an analyses of a breast cancer cell line HCC1954.

The HCC1954 is a hormone receptor negative, ERBB2 positive, poorly differentiated breast cancer cell line derived from a primary stage IIA, grade 3, invasive ductal carcinoma, which has been the subject of several large-scale genome and transcriptome analysis. In order to identify the cell surface proteins present in HCC1954 cell line that can be involved in the development of invasive ability and metastases, the cell membrane proteins were biotinylated and total protein extract were fractionated using the cell surface protein isolation kit (Pierce®). The proteins were identified via 1-D gel electrophoresis (Figure 4) followed by a 1-D reversed-phase chromatography coupled to an ESI-Q-TOF or a 2-D SCX/RP chromatography coupled to a Synapt HDMS mass spectrometer (Waters, Farmington, MI, USA). More than 1500 proteins of the HCC-1954 breast cancer cell line were identified (Figure 5). The enriched membrane fraction (eluted fraction) enabled the identification of 3 times more plasma membrane, receptor activity and cell adhesion proteins by both proteomic identification methodologies (Figure 6). Among them, molecules that plays important roles in cell migration and angiogenesis such as integrins α-2, α-3, α-v, molecules involved in cell adhesion, cell junction and cell-cell interactions as catenin α-1/cadherin-1, CD44, CD166, CEAM5, were identified.

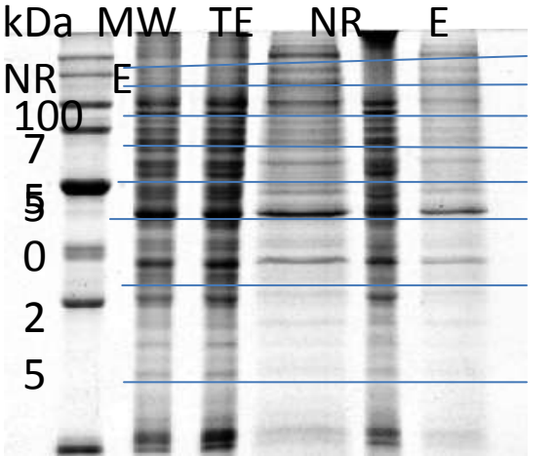

FIGURE 4 - 1-D GEL ELECTROPHORESIS FROM HCC-1954 CELL LINEAGE. PROTEINS WERE FRACTIONATED USING THE CELL SURFACE PROTEIN ISOLATION KIT (PIERCE®). 30 µG OF TOTAL PROTEINS FROM EACH FRACTION (TE – TOTAL EXTRACT; NR - NOT RETAINED; E – ELUTED) WERE APPLIED IN AN 12% SDS-PAGE GEL. THE GEL SECTIONS WERE SUBMMITED TO MASS SPECTROMETRY. NR AND E FRACTIONS WERE DONE IN TWO BIOLOGICAL REPLICATES.

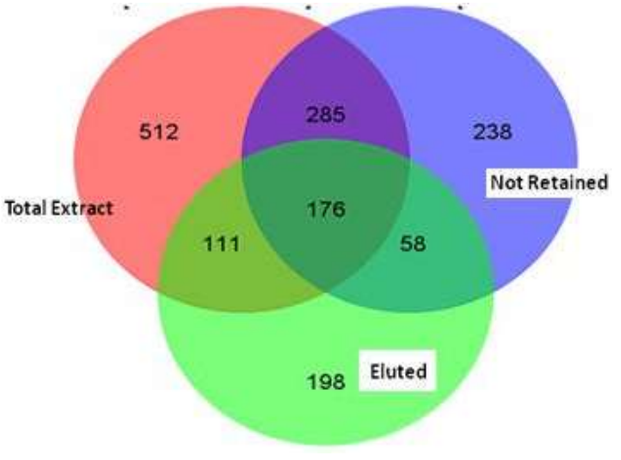

FIGURE 5 – VENN DIAGRAM GENERATED BY PEAKS PROGRAM VERSION 6.0. COMPARISON OF TOTAL PROTEIN IDENTIFIED IN EACH FRACTION TOTAL EXTRACT, NOT RETAINED (NON BIOTINYLATED) AND ELUTED (BIOTINYLATED) PROTEINS OF HCC-1954 CELL LINE BY A 1-D REVERSED-PHASE CHROMATOGRAPHY COUPLED TO AN ESI-Q-TOF MASS SPECTROMETER .

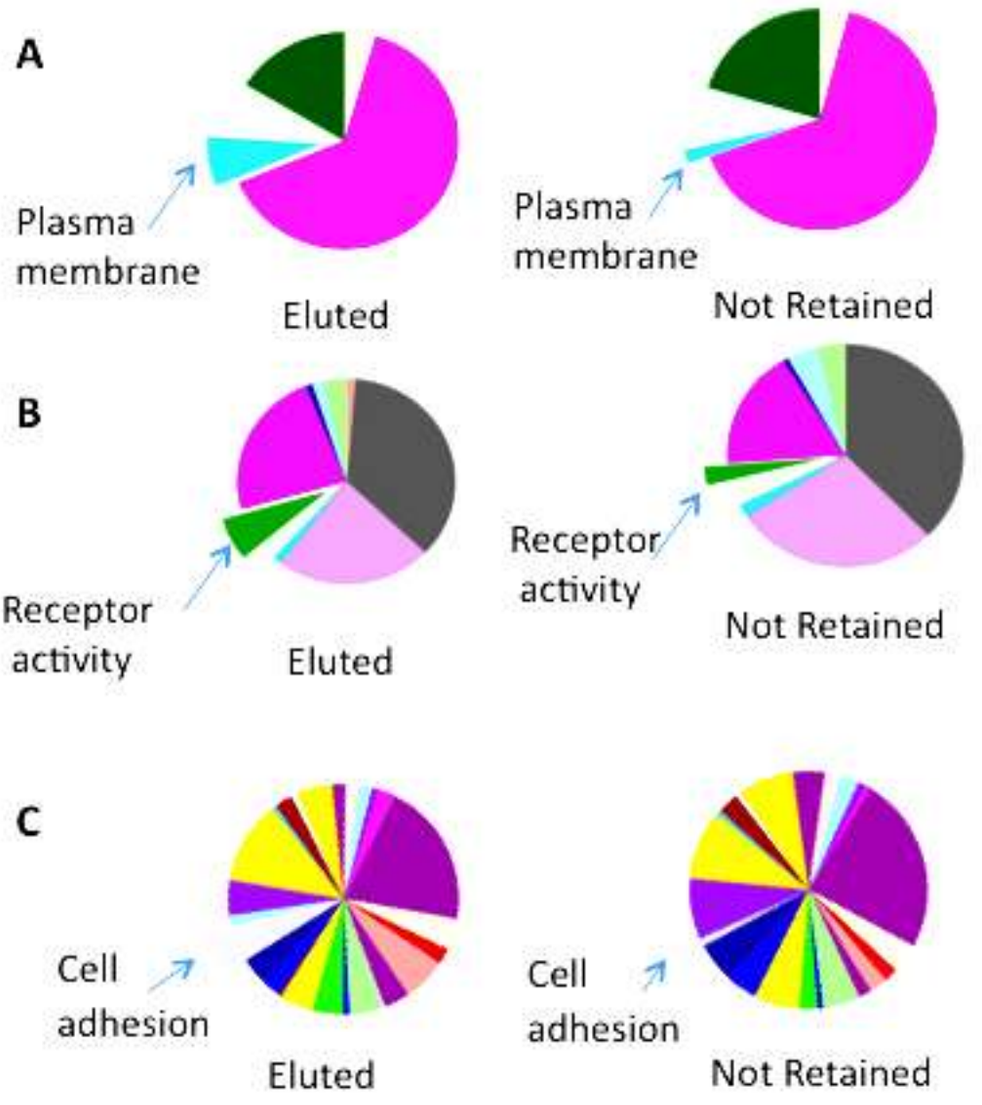

FIGURE 3 - GENE ONTHOLOGY MAPPING PERFORMED USING PANTHER TOOLS. CELLULAR COMPONENT (A), MOLECULAR FUNCTION (B) AND PROTEIN CLASS (C) OF THE PROTEINS IDENTIFIED IN THE ELUTED (BIOTINYLATED) AND NOT RETAINED FRACTIONS (NON BIOTINYLATED) FROM THE HCC-1954 HUMAN BREAST CANCER CELL LINE.

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AL 5

ASSOCIATE LABORATORY OF NUCLEAR MAGNETIC RESONANCE, ORGANIC SYNTHESIS AND MOLECULAR MODELING

COORDINATOR: JOSÉ DANIEL FIGUEROA VILLAR - IME.

MEMBERS:

LUZINEIDE W. TINOCO – NPPN/UFRJ
PEDRO GERALDO PASCUTTI – IBCCF/UFRJ

Development of Acetylcholinesterase Inhibitors and Reactivators for Alzheimer Disease and Defense Against Chemical Weapons.

The enzyme acetylcholinesterase is fundamental for diverse toxicology and health problems, with emphasis on Alzheimer's disease (AD). This enzyme is also the main target for neurotoxic organophosphorus compounds used as chemical weapons or pesticides, which cause more than a million intoxications every year world wide.

Inhibitors and reactivators of this enzyme are designed by molecular modeling (quantum mechanics, docking and dynamics), synthesized and tested using the Ellman test and NMR. The developed NMR method for testing these compounds is the best procedure and can be applied with any type of compounds (Figure 2A).

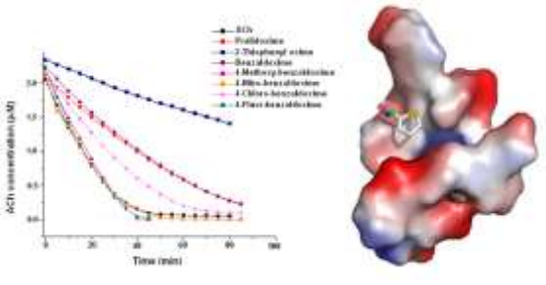

FIGURE 2A: NMR TEST INHIBITION OF ACHE BY NEUTRAL OXIMES IN COMPARISON WITH PRALIDOXIME.

The neutral inhibitors and reactivators are more important because have better conditions for blood brain barrier (BBB) penetration. We also found that some neutral oximes are appropriate phosphylated AChE reactivators, as shown in Table 1.

Comp	% AChE React.	pKa	ΔE (au)
2-PAM	83	7.68	-0.406
24	93	10.63	-0.560
17	62	11.39	-0.537
18	58	11.39	-0.535

TABLE 1: OXIMES REACTIVATION CAPACITY OF ACHE INHIBITED WITH ETHYL PARAOXON AND COMPARISON WITH THE PKA VALUES AND THE CALCULATED ENERGY DIFFERENCE (ΔE) BETWEEN THE NEUTRAL AND THE DEPROTONATED FORM OF EACH OXIME.

Development of New Bioactive Compounds from Benzylidene Barbiturates and Analogues.

This project is focused on design, synthesis and evaluation of new compounds for different diseases. One example is tranquilizers without sleeping effects. These compounds, hich are novel structures are planned as GABA receptors antagonists. The results with rotarod are shown in Figure 2B, which indicate that the tested compounds 3E

and 3F are effective tranquilizers in comparison with diazepam.

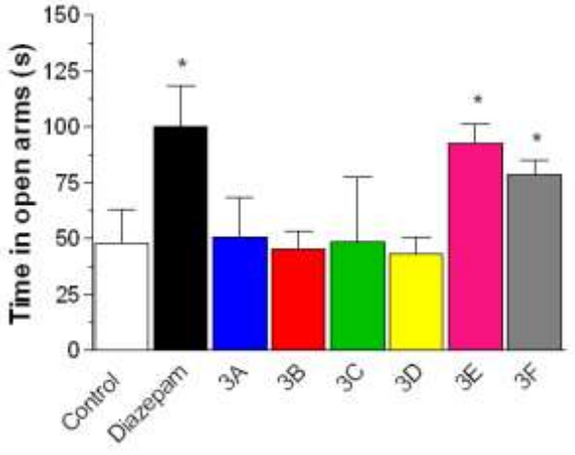

FIGURE 2B: ROTAROD TEST OF DESIGNED, SYNTHESIZED AND TESTED NEW COMPOUNDS.

The conformational and structural details of these molecules were calculated with quantum mechanics, indicating that the *ortho*-substituted compounds are the most effective ones. This information lead to design, synthesis and evaluation of new compounds with better water solubility and more effective CNS effects (Figure 2C).

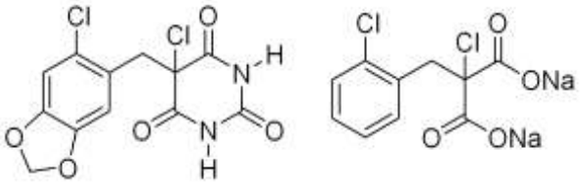

FIGURE 2C: EXAMPLE OF NEW CNS DEPRESSANTS.

Benzylidene barbiturates have also been used to demonstrate that NMR chemical shifts, atomic charges and Hammett equation can be used to predict their polarization, reactivity and mechanisms of reactions, as shown in Figure 2D.

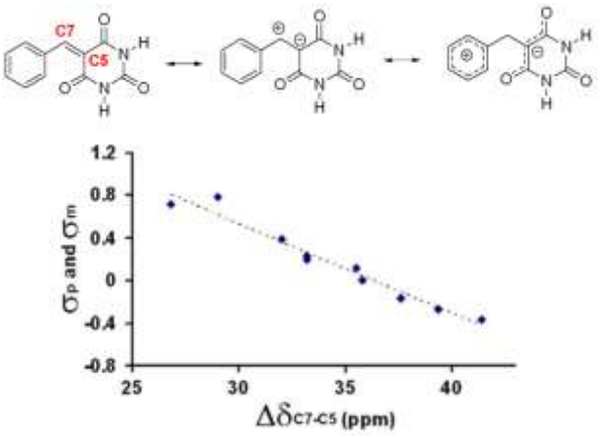

FIGURE 2D: CORRELATION OF BENZYLIDENE BARBITURATES POLARIZATION WITH HAMMETT σ AND δ_{C7-C5}.

These intermediates are being used to prepare new potential drugs for the treatment o malaria, Chagas disease and leishmaniose, as well as new antibiotics and antiviral compounds.

Analysis and Environmental Control of Poly-aromatic Compounds.

Metrological methods are being prepared and applied for the analysis and control of toxic poly-aromatic compounds, which are produced by fuel consumption and diverse burning processes, and their presence in the atmosphere leads to several problems of cancer, pulmonary intoxication and death. The analysis method by chromatography and by NMR leads to confirmation of the method quality, as shown in Figure 3E.

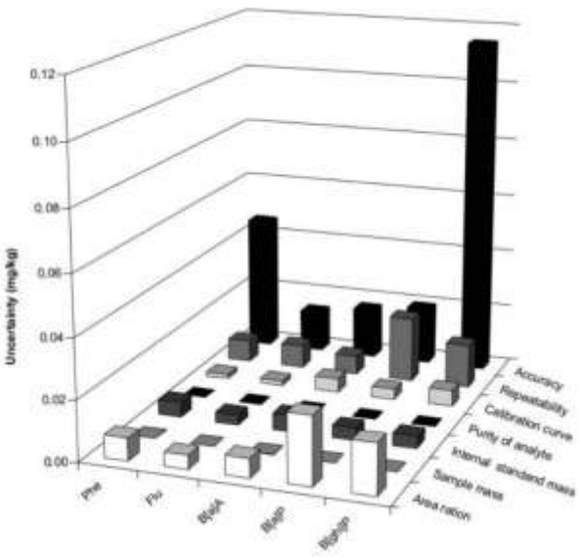

FIGURE 3E: CONFIRMATION OF CONTRIBUTION OF THE UNCERTAINTY SOURCES USED IN THE EXPERIMENTS.

Now is being developed methodologies to eliminate these compounds from the atmosphere.

Design of New Inhibitors of Nucleoside Hydrolase (NH) of *Plasmodium chagasi*.

In previous works there was discovered quinolone derivatives as good inhibitors of *L. donovani* NH. The molecular modeling and dynamics studies of these compounds lead to the design and synthesis of new potential drugs for treatment of visceral leishmaniasis. The prepared compounds are more stable than the previous quinolones, being resistant to hydrolysis by NH and other hydrolytic enzymes. L. chagasi is important because is the responsible protozoa for visceral leishmaniasis in Brazil. The preparation of the new compounds is shown in Figure 3F.

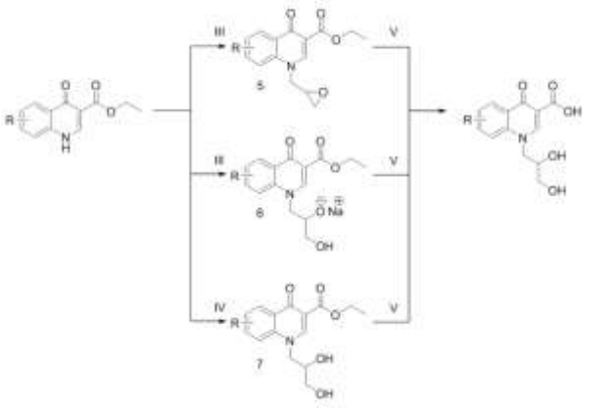

FIGURE 3F: BASIC PROCEDURES FOR CONVERSION OF QUINOLONE TO NEW INHIBITORS OF NH.

These compounds are now being tested as inhibitors of NH by the NMR method.

Design of New Photosensitive DNA Intercalates to Decrease the Irradiation Dose for Cancer Treatment. DNA intercalates, when posses photo sensitivity, were shown to be effective to decrease 50% of the irradiation dose for cancer in order to decrease damages caused by higher irradiation doses. These compounds were designed by molecular modeling and studied by docking. Some examples of the new products are shown in Figure 2G.

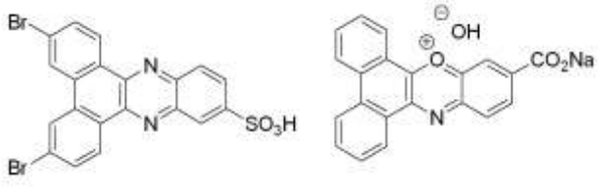

FIGURE 2G: NEW PHOTOSENSITIVE DNA INTERCALATES FOR CANCER TREATMENT.

These compounds are being tested as DNA intercalates by NMR. The structure of DNA fragments are determined by NMR, as well as all the ^{1}H and ^{13}C chemical shifts. The interaction studies are executed using relaxation times (T_1 and T_2), diffusion

coefficients (D), chemical shifts (δ) and saturated transfer difference (STD). The type of interaction is shown in Figure 2H.

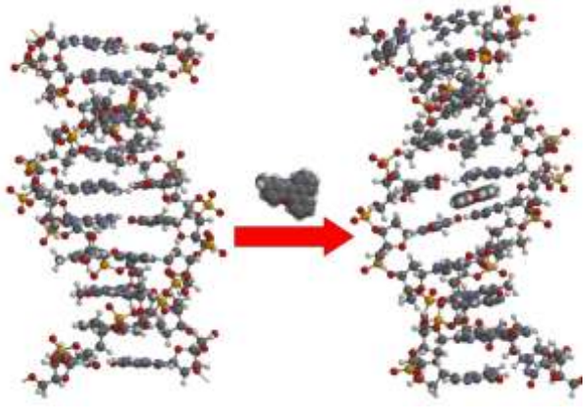

FIGURE 2H: INTERCALATION OF LIGAND GK OF THE FRAGMENT OF B TYPE DNA FRAGMENT CGAATTAACG.

The structure of GK, as well as all the synthesized and tested compounds was determined by NMR with complete and definitive ^{1}H and ^{13}C assignment, as shown in Figure 2I.

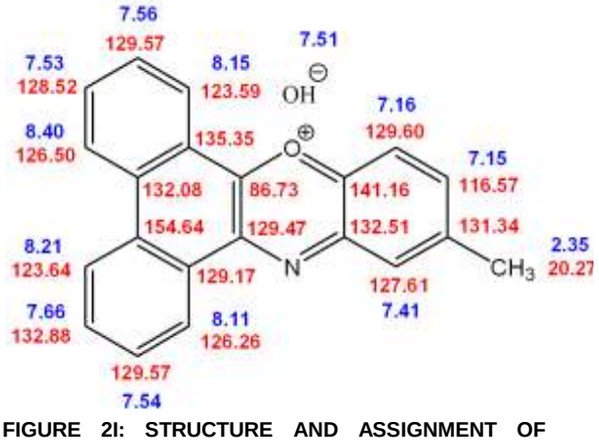

FIGURE 2I: STRUCTURE AND ASSIGNMENT OF COMPOUNDS GK.

Identification of novel target enzyme inhibitors for the treatment of diseases neglected.

The enzyme nucleoside hydrolase from Leishmania donovani (NHLd), expressed in E. coli and purified by affinity, was used as target for the treatment of visceral Leishmaniasis. Nucleoside analogs, pterocarpanoquinonas and flavonoid quercetrin were tested as possible NHLd inhibitors. For these tests we used a methodology developed in the group using NMR analysis (Renno, 2012). The use of NMR in these tests had the advantage of clear differentiation between substrate and product, which in the spectrophotometric method was only possible in enzymatic assays using the enzyme hypoxanthine oxidase. Also, we used the STD (Saturation Transfer Difference) technique to do a rapid identification of ligands for this enzyme for the development of high-affinity inhibitors. More than 70 fragments were analyzed, with identification of three molecular fragments that bind to this enzyme. The STD and DOSY techniques have also been used successfully in our group and enabled the identification and mapping of the topology of ligands binding to enzymes cruzipain and triosephosphate isomerase from T. cruzi (Guzman, 2012 and Merlino, 2012)

Development and evaluation of Protective Agents against Yersinia pestis (Bubonic Plague).

Our goal was characterize Yersinia pestis Plasmin protein (Pla) and their interaction with Human Plasminogen peptide (PK2). Yersinia pestis is classified in category A according with CDC (Center for Disease Control and Prevention, USA), because of it capacity of contamination and mortality.

The structural characterization of PK2 is important to plan mimetic peptides with potential to inhibit the interaction Pla-Plg, and abort the process of invading Y. pestis in

mammalian cells. CD studies showed structural modifications by Pla in the presence of PK2, indicating Pla-PK2 interaction (Figure 1A).

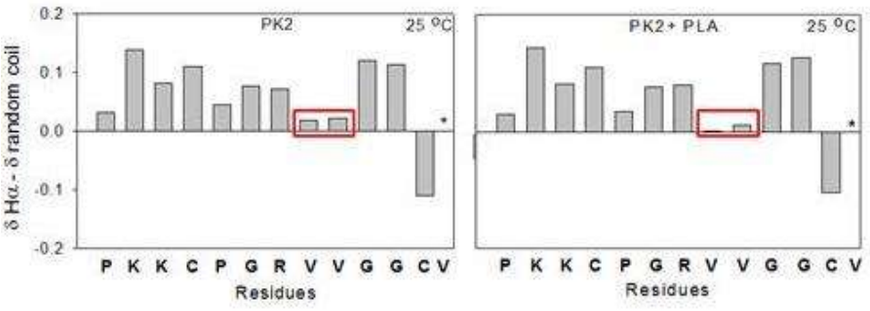

Figure 1C: Chemical shift difference between PK2 and PK2-Pla. The positive bars indicate that PK2 is in β-conformation and the Pla promotes a slight modification in VV region (red rectangle).

This observation it was corroborated by NMR by the increase of the intensity of all the peaks, suggesting lower flexibility of Pla in the presence of PK2 (Figure 1B). Pla cleaves the R561-V562 bond in the sequence PGRVVGG for plasminogen activation. However, only a slight chemical shift perturbation was observed for H□ of VV residues of PK2 in the presence of Pla (Figure 1C), suggesting that Pla-PK2 interaction is affecting mainly the RVV region. Gel filtration chromatography show that Pla is dimeric at pH 6.5 and monomeric at pH 8.0 (Figure 1D).

So, at the pH used for NMR analyses (pH 5.0) Pla was probably in the dimeric form, explaining the absence of large modification on the NMR spectra of PK2-Pla. We are performing CD and NMR analyses of PK2-Pla interaction at pH 6.5 and pH 8.0 to evaluate the effect of pH in the Pla structure and PK2-Pla interaction.

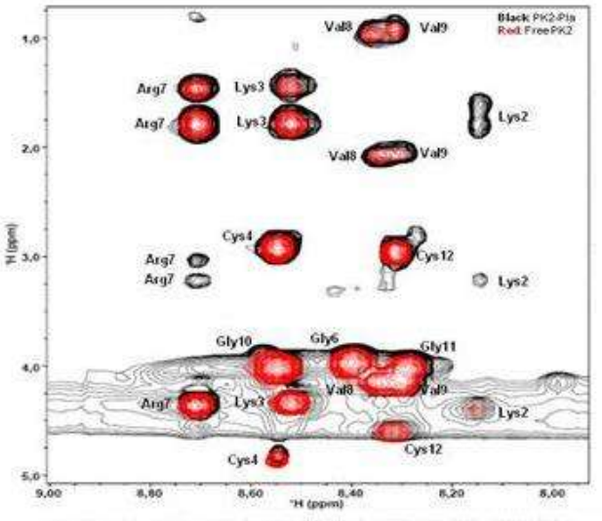

Figure 1B: TOCSY spectra of free PK2 (red) and PK2-Pla (black). In the presence of Pla were observed stronger cross peaks and some cross peaks that were absent in the PK2 free spectra Suggesting that PK2 is less flexible due the interaction with Pla.

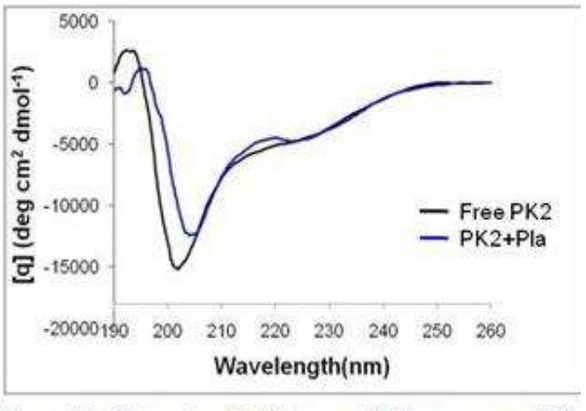

Figure 1A: CD spectra of PK2 free and in the presence of Pla

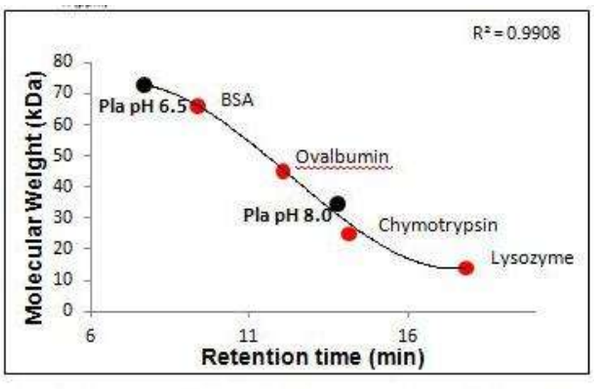

Figure 1D: Gel filtration chromatography of Pla at pH 6.5 and pH 8.0 (black). Proteins used for calibration (red): BSA (66 kDa), Egg albumin (45 kDa), Chymotrypsin (25 kDa) and Lysozyme (14 kDa).

NMR analysis of a neuroblast specific site of MARCKS and their interaction with the antibody mAb 3C3.

Here we addressed the structural characterization of a peptide containing S25, recognized by the monoclonal antibody 3C3 (mAb 3C3). Phosphorylated (S25p) and unphosphorylated (S25up) peptides, comprising this serine, were analyzed by Surface Plasmon Resonance (SPR), Circular Dichroism (CD) and Nuclear Magnetic Resonance (NMR) spectroscopy.

SPR showed that mAb 3C3 bound to the S25p peptide and the whole protein with a similar affinity, albeit different kinetics. NMR and CD spectroscopy data indicated that S25 phosphorylation and antibody interaction do not cause extensive modifications on the peptide structure.

NMR study reveals that both peptides are able to self-aggregate. Sharper lines, absence of multiple spin systems, and higher conformational stability under dilution and temperature increase observed for the phosphorylated peptide suggested a more ordered structure. (manuscript in preparation).

It was described that MARCKS is transported by sphingosine-1-phosphate to the cell membrane. This process is related to the protection of the endothelial cell barrier. Dysfunction on endothelial cell barrier results in the increased vascular permeability observed in inflammation, tumor metastasis, angiogenesis, and atherosclerosis. Therefore, we are currently studying by CD and NMR the interaction of sphingolipids with MARCKS.

New project.

More recently, the group has introduced a new line of research focused on the analysis of complex mixtures by NMR and HPLC-NMR. Analyzes were made of samples of green tea (Camellia sinensis) from various sources in South America. 12 components were identified in the samples of tea, whose presence and content vary with respect to their country of origin. For example, myo-inositol was only observed in the sample of Peru. In the analyzes by NMR, ranging from green tea of concentration in aqueous solution of 1-100 mg / ml, it was observed that increasing the concentration of the tea favors the formation of molecular aggregates, as already observed for pure catechins. In the analyzes by HPLC-NMR in addition to the characteristic components of green tea that had been identified, signs of other substances that co-elute with the chromatographic peaks, but have never been described (manuscript in preparation).

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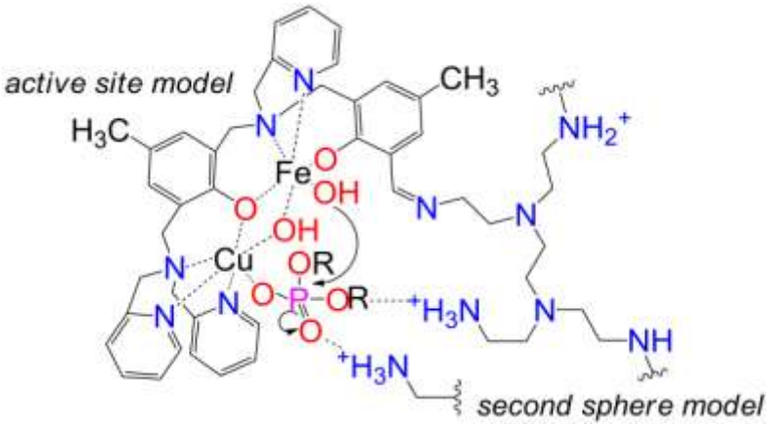
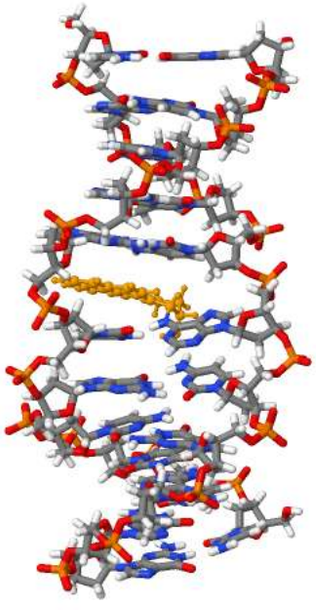
AL 6

ASSOCIATE LABORATORY OF PROTEINS AND PROTEOMIC HETEROLOGOUS EXPRESSION

COORDINATOR: HERNÁN TERENZI – UFSC.

Polyethyleneamine (PEI) strategy
(electrostatic interactions)





Pyrene strategy
(intercalation)

Chemical Hydrolases

In the last decade our studies proved that small metal compounds mimicking the active site of phosphohydrolases or oxidases could act as an artificial nuclease, i.e. able to hydrolyze the phosphodiester bond of nucleic acids. This activity, however, was kinetically far lower than that observed in natural nucleases (enzymes). Many features of the polypeptide chain contribute to enhance the hydrolysis efficiency of these enzymes including: the correct positioning of nucleophile to P-O bond, the stabilization of transition state and, obviously, the tight binding of the protein to the nucleic acid structure.

Most complexes studied so far lack ancillary DNA-binding motifs that do not participate in the strand scission mechanism directly. For example, the best small metal complex studied is Cu(phen)2 (phen= 1,10-phenanthroline) reported by Sigman and co-workers (1979). The phen ligand both act as DNA-binding motif, an as a chemical core of the copper(I) center. In other words, the ligand phen was not intended to exclusively acts as a binding motif.

On the other hand, Dervan and colleagues extensively used polyamine derivatives, such netropsin and distamycin that bind to the minor groove of AT-rich DNA strands, as intended DNA binding motifs. The

attachment of this motif as ancillary ligand to a chelated metal center (like EDTA, for example) was very reasonable to promote specific (esite-directd) cleavage of diffusive reactive oxygen species induced by redox active metals (specially, Iron). Although effective, this strategy was employed on non-hydrolytic artificial nucleases and the proposal was not to enhance the catalytic efficiency but to induce site-directed nucleic acid cleavage.

Our recent work highlights two new strategies to enhance the DNA cleavage ability of two heterobinuclear metal compounds focusing the improvement of the DNA-complex interaction without changes in the catalytic mechanism of strand breakage. The first one is the covalent attachment of a FeIIICuII metal complex (containing the unsymmetrical ligand H2L =

2-bis[{(2-pyridylmethyl)aminomethyl}-6-{(2-hydroxybenzyl)

(2-pyridylmethyl)}aminomethyl]-4-methylphenol) into the dendrimeric-like polymer polyethyleneimine (PEI). PEI is a high molecular weight polymer (~28 kDa) with several amine motifs behaving as a polycationic polymer in solution, which strongly interact with the anionic portion of the nucleic acid structure (i.e the phosphodiester bonds) by electrostatic interactions. For this reason, PEI is extendedly used as a transfection agent in gene delivery. In addition, the amine motifs serve as bonding group between PEI and an aldehyde present in the complex structure to form a stable imine bond (Schiff base). Our recent work highlighted that PEI-FeIIICuII system has the same catalytic mechanism and turnover of the parent complex whithout PEI when assayed with an artificial substrate (BDNPP, bis-dinitrophenyl phosphate), but with the nucleic acid substrate (plasmid DNA) an association four-fold higher. These results indicate that the catalytic efficiency of the complex FeIIICuII was successfully enhanced by a factor of 4-fold maintaining its particular cleavage mechanism.

The second strategy is attaching the well-known intercalative DNA-binding motif pyrene in the complex ligand. We choose a previously established model of hydrolytic metal compound FeIIIZnII (containing the same unsymmetrical ligand H2L as FeIIICuII) to prove our strategy. The FeIIIZnII complex, although presenting a hydrolytic mechanism of DNA cleavage, presents a low reaction rate. The addition of one pyrene motif enhanced the cleavage rate around 140-fold when compared to the same complex without pyrene. In addition, as we have seen with PEI, the addition of this type of DNA-binding motif does not alter the mechanism of DNA hydrolysis. These two examples are new evidences to support our new strategy to improve the catalytic properties of metal complexes in terms of DNA cleavage.

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AL 7

ASSOCIATE LABORATORY OF PROTEINS BIOCHEMISTRY

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STRUCTURAL INSIGHTS ON TWO HYPOTHETICAL SECRETION CHAPERONES FROM *Xanthomonas axonopodis* pv. *citri*

Several Gram-negative bacterial pathogens have developed type III secretion systems (T3SSs) to deliver virulence proteins directly into eukaryotic cells in a process essential for many diseases. The type III secretion processes require customized chaperones with high specificity for binding partners, thus providing the secretion to occur. Due to the very low sequence similarities among secretion chaperones, annotation and discrimination of a great majority of them is extremely difficult and a task with low scores even if genes are encountered that codify for small (20 kDa) proteins with low pI and a tendency to dimerise. Concerning about this, herein, we present structural features on two hypothetical T3SSs chaperones belonging to plant pathogen Xanthomonas axonopodis pv. citri and suggest how low resolution models based on Small Angle X-ray Scattering patterns can provide new structural insights that could be very helpful in their analysis and posterior classification.

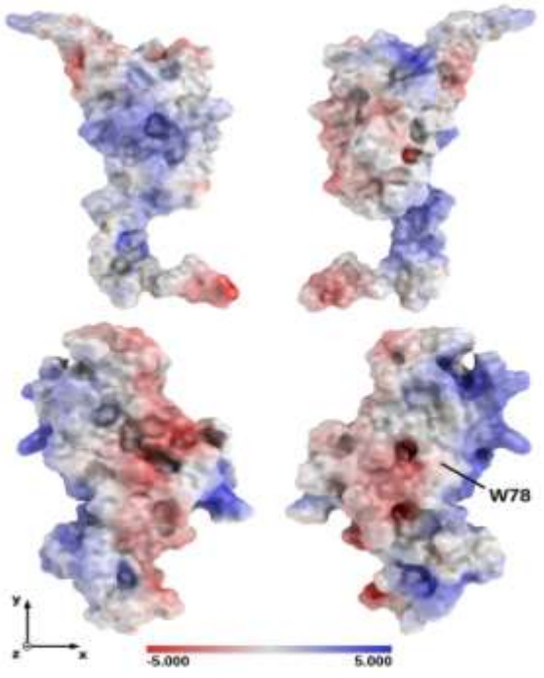

FIGURE 1. **THE ELECTROSTATIC SURFACE POTENTIALS (310 K) FOR THE PREDICTED MODELS OF XAC0419 (TOP ROW) AND FLGN (BOTTOM ROW). THE RIGHT COLUMN IS ROTATED CLOCKWISE BY 180^{O} AROUND THE Y-AXE. ELECTROSTATIC POTENTIALS ARE SHOWN AS MULTIPLES OF KT/E, WHERE K IS THE BOLTZMANN'S CONSTANT, T IS THE TEMPERATURE AND E IS THE ELECTRON CHARGE. THE SURFACE REGION OCCUPIED BY THE SINGLE TRYPTOPHAN RESIDUE (W78) IN FLGN IS MARKED IN THE FIGURE (LOWER RIGHT).**

Due to the intrinsic low resolution of the SAXS technique, atomic coordinate models are normally used to interpret the resulting envelopes, whether derived by high-resolution experimental techniques or predicted by computational methods. In the present case, experimental high-resolution models were not available, for either of the proteins under study or for homologues.

Although efforts were made, attempts to obtain samples at higher concentrations were unsuccessful, which limited the use of high-resolution techniques. For these reasons, the protein structure prediction was employed in order to obtain coordinate models through the I-TASSER server.

It is very interesting that the best fitting to the experimental envelopes were not obtained for the I-TASSER models with the highest scores, but with the second and third best models for XAC0419 and FlgN, respectively. Remarkably, except for probable mobile or intrinsically unordered regions in the proteins, an impressive similarity between the envelopes and the predicted models was observed in both cases. An excellent agreement is observed for the core region comprising the □-helices and □-sheets predicted for XAC0419, although loop regions may assume a more globular, compact shape in solution than what was anticipated by the predicted atomic model. In the case of FlgN, the compact domain composed of □-helices superposes very well onto a core portion of the experimental envelope and, at the same time, an unordered, although globular region seems to be present, based on the predicted atomic model. It is important to mention that the experimental low-resolution envelopes and the coordinate models were obtained by completely independent approaches. Furthermore, as in the present case, there are various examples were SAXS data have been used as a valuable tool to compare and select compatible models of proteins.

Although there are some structural data on secretion chaperones, almost all of them present class I T3SS chaperones as dimers. Surprisingly, our results indicate that XAC0419 and FlgN are monomeric in solution, which is an opening for further structural investigations on this kind of proteins. Our results indicate that FlgN is a helical protein, whose three-dimensional folding assumes a prolate shape in agreement to the data expected for the most flagellar chaperones, with crystallographic data on FliS from *Aquifex aeolicus*, and with size-exclusion chroma- tography data on FliT from *Salmonella*. On the other hand, the hypothetic XAC0419 protein also elongated in shape and with 2α-3β fold (α–ββ–α), looks very similar in structure to known class I T3SS chaperones.

STRUCTURE-ACTIVITY RELATIONSHIP OF SOME 4-ANILINOQUINAZOLINES AS POTENTIAL INHIBITORS OF ADENOSINE KINASE

The Adenosine Kinase (ADK) is an important enzyme that might be related to several diseases, as stroke, epilepsy and infarct. For these reasons, ADK inhibition is an important target in these maladies treatment. In order to inhibit ADK functioning, a serie of 8-methoxy-4-anilinequinazolines was synthesized, with different substituents in the positions 3’ and 4’ of the aniline ring. Nevertheless, only the three best results are shown. The synthesized compounds were characterized, and their interactions with ADK were studied by spectroscopic techniques and bioinformatics assays. Fluorescence emission experiments were executed (tryptophan

emission), and the obtained data revealed that these compounds interact with ADK binding sites, allowing the calculation of dissociation constant (K_d). In order to better understand the binding mechanism, *in silico* assays were performed, especially docking experiments. All these results indicate that the new compounds may be very promising in ADKs inhibition.

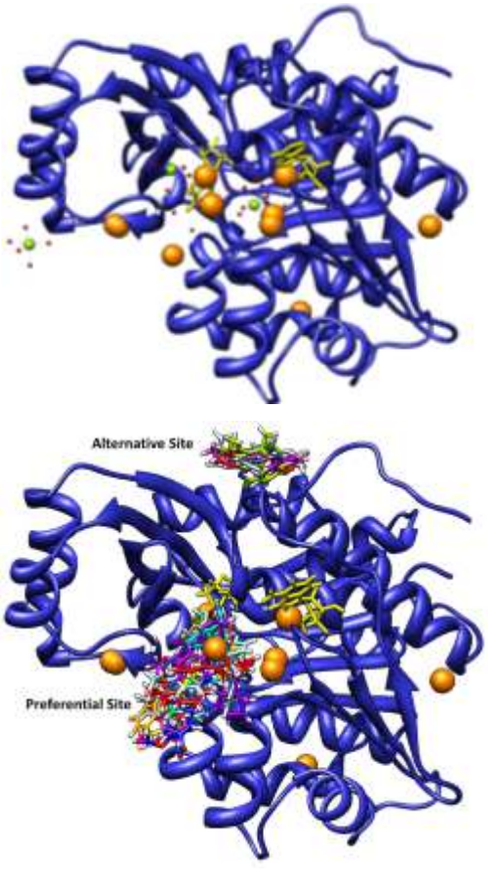

FIGURE 2. **POTENTIAL ADK'S BINDING SITES (ORANGE SPHERES) AND NATURAL SUBSTRATES ADO (YELLOW). MAGNESIUM ATOMS ARE SHOWN IN GREEN, WATER MOLECULES DEPICTED AS RED SPHERES. DOCKING RESULTS FOR THE SYNTHESIZED COMPOUNDS (COLORED STRUCTURES) ON THEIRS PREFERENTIAL AND ALTERNATIVE BINDING SITES .**

A closer investigation of the docking assays shows that the compounds have a tendency to bind at the entrance of ATP enzyme's pocket, thus preventing substrate binding to the enzyme, and so, inhibiting the ADK function. These results for the three best systems are illustrated in Fig.2.

A widely explored technique in the investigation of interactions between a protein and ligands is the emission fluorescence spectroscopy. This technique, when applied to protein/substrate systems, is based on proteins intrinsic fluorescence, or even better, on proteins tryptophan (Trp) intrinsic fluorescence. In order to study and evaluate how ADK behave upon compounds binding, fluorescence assays were conducted. The quenching assays are based on the decrease of proteins fluorescence when inhibitor is added. So, by titrating the enzyme with the compounds, it was possible to observe if fluorescence quenching has occurred, its dimension, and also if there were changes in Trp conformation. The obtained fluorescence emission spectra showed a pronounced fluorescence quenching. For 8MEO-4I, 8MEO-3BR and 8MEO-4ACET 99% of quenching was achieved. The red-shifts in fluorescence maxima, and the $\square\square_{max}$ for 8MEO-4I, 8MEO-3BR and 8MEO-4ACET were 46 nm, 25 nm and 4 nm respectively, what indicate that the enzyme's tryptophans, after inhibitors binding, are more exposed to solvent than before compounds addition. The calculation of K_d was performed through a Non Linear Least Squares Fit (NLLS), considering the observed fluorescence quenching and the correspondent ligand concentration. The fluorescence emission data allowed the calculation of dissociation constants values, besides conformational changes upon binding. The literature states a value of 38.0 $\mu mol.L^{-1}$ for ATP and since the synthesized compounds bind on ADK's ATP binding site entrance, ATP's K_d value is a

good reference value. It was determined that the three compounds, 8MEO-4I, 8MEO-3BR and 8MEO-4ACET show K_d values much lower than ATP's K_d value of 38.0 µmol L^{-1}. This indicates that these compounds have a greater affinity for ADK than ATP, and therefore, binds to ADK more strongly; characteristic that put them in a condition of good candidates for adenosine kinase inhibitors. Another important factor related to fluorescence quenching assays is the occurance of RET. In order to RET happens, it is necessary to have overlap of the spectra of energy donor specie (in ADK's case, tryptophan) and the spectra of an energy acceptor, such as the quinazoline derivatives. This spectral overlap is result of a static quenching mechanism, in which there is the formation of a specific binding non-fluorescent complex between ADK and the compounds. RET occurrence is another valuable indication that a specific binding has occurred.

STRUCTURE AND FUNCTION OF MOLECULAR CHAPERONES

Cells require a protein quality control (PQC) system to obtain a correct balance between folding and the degradation of incorrectly folded or misfolded proteins. This system maintains protein homeostasis and is essential for life. Key components of the pqc are molecular chaperones, which compose a ubiquitous class of proteins that mediate protein quality control by aiding in both the correct folding of proteins and the elimination of proteins that are misfolded due to cellular stress or mutation. The majority of the available experimental data regarding molecular chaperones are from prokaryotes. Any therapeutic strategy for human conformational diseases or cancers that involve chaperones will require knowledge about the conformation of these proteins and how they interact with each other. Therefore, a better understanding of the mechanism of action of eukaryotic molecular chaperones will truly provide insight into cellular function under normal and stressed conditions. That is our goal and we are involved in studying the relationship between structure and function of chaperones from human, plants and protozoa.

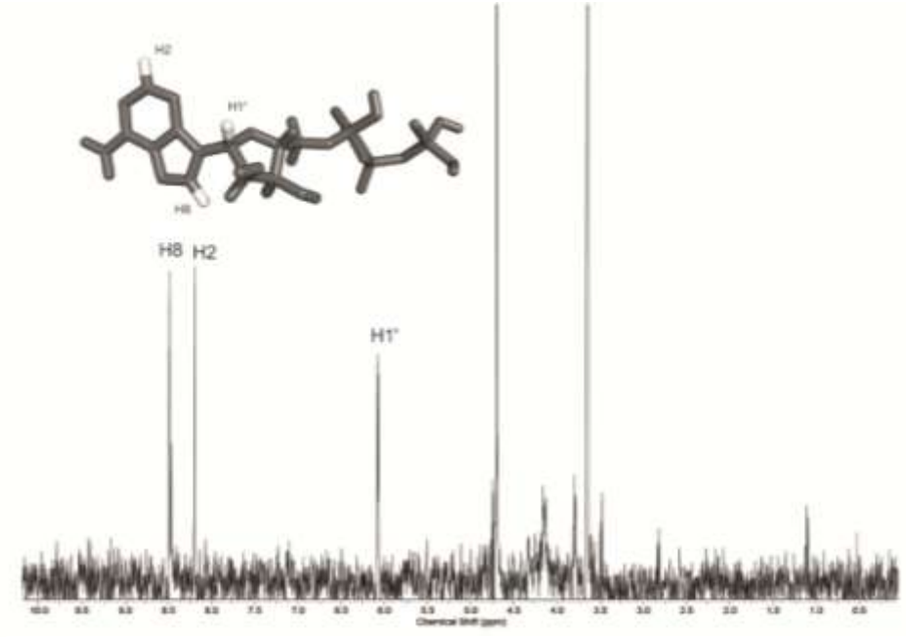

FIG. 3. **PLANT HSP101 IS A GOOD MODEL FOR UNDERSTANDING THE RELATIONSHIP BETWEEN THE STRUCTURE AND FUNCTION OF HSP100 PROTEINS AND TO INVESTIGATE THE ROLE OF THESE CHAPERONES IN DISAGGREGATION PROCESSES. HERE, WE PRESENT THE CLONING AND PURIFICATION OF A SUGARCANE ORTHOLOG, SHSP101, WHICH IS EXPRESSED IN SUGARCANE CELLS AND IS A FOLDED HEXAMER THAT IS CAPABLE OF BINDING NUCLEOTIDES AS SHOWED BY STD EXPERIMENTS. SEE: CAGLIARI ET AL, 2011.**

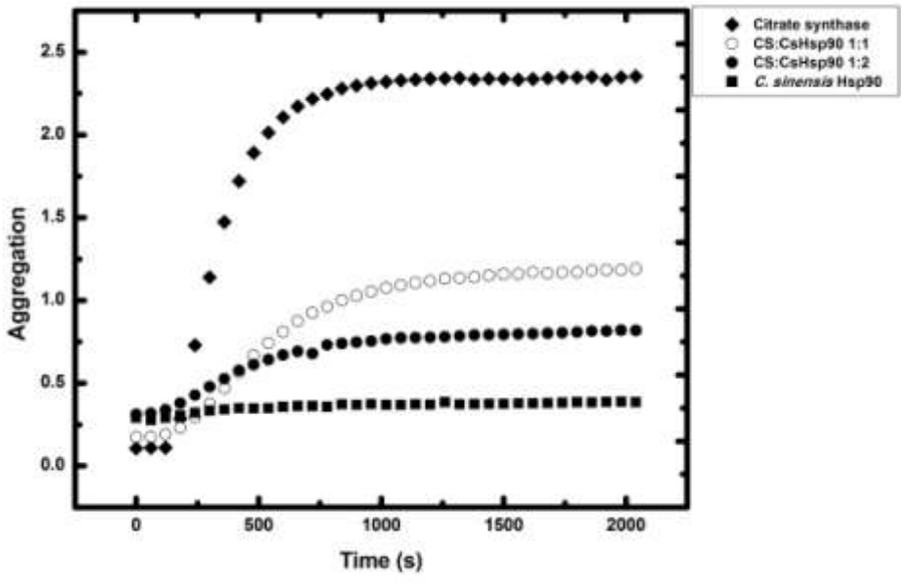

FIG. 4. **HSP90 IS A PRIMARY FACTOR IN THE MAINTENANCE OF HOMEOSTASIS IN PLANTS. HSP90 FROM *CITRUS SINENSIS* (SWEET ORANGE) WAS PRODUCED AND MEASURED BY CIRCULAR DICHROISM, INTRINSIC FLUORESCENCE SPECTROSCOPY AND DYNAMIC LIGHT SCATTERING. THE CHAPERONE FORMED A DIMER IN SOLUTION WITH A STOKES**
RADIUS OF APPROXIMATELY 62 Å. IN ADDITION, IT WAS RESISTANT TO THERMAL UNFOLDING, WAS ABLE TO PROTECT CITRATE SYNTHASE FROM AGGREGATION (AS SHOWED IN THE FIGURE), AND WESTERN BLOT ANALYSIS DEMONSTRATED THAT CSHSP90 WAS CONSTITUTIVELY EXPRESSED IN *CITRUS SINENSIS* CELLS. SEE MENDONÇA ET AL, 2012.

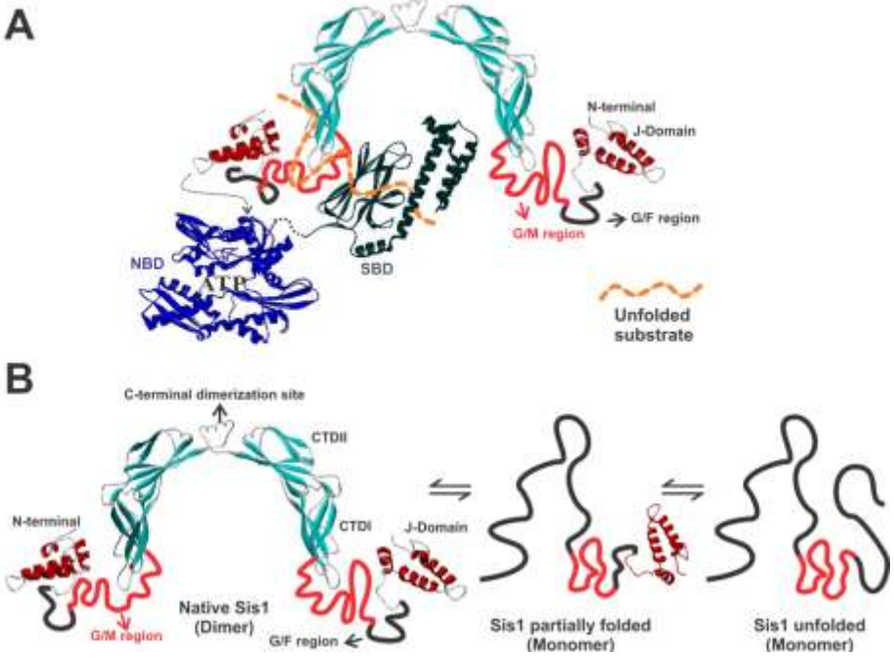

FIG. 5. **PROTEINS FROM THE DNAJ CHAPERONE FAMILY BIND AND PROTECT AN UNFOLDED PROTEIN FOR DELIVERY PURPOSES. WE EVALUATE THE STABILITY OF SIS1 AND MUTANTS THAT HAD THE CENTRAL DOMAINS DELETED OR SWITCHED TO GAIN INSIGHT INTO THE ROLE OF THESE REGIONS IN THE STRUCTURE AND FUNCTION. WE FOUND THAT: 1) SIS1 UNFOLDS IN AT LEAST TWO STEPS AS FOLLOWS: FOLDED DIMER TO PARTIALLY FOLDED MONOMER AND THEN TO AN UNFOLDED MONOMER. 2) THE GLY/MET RICH DOMAIN HAD INTRINSICALLY DISORDERED CHARACTERISTICS AND ITS DELETION HAD NO EFFECT ON THE CONFORMATIONAL STABILITY OF THE PROTEIN. 3) THE DELETION OF THE C-TERMINAL DOMAIN I PERTURBED THE STABILITY OF THE DIMER. ALTOGETHER, OUR RESULTS SUGGEST THE EXISTENCE OF TWO SIMILAR SUBDOMAINS IN THE C-TERMINAL DOMAIN OF DNAJ THAT COULD BE IMPORTANT FOR STABILIZING EACH OTHER IN ORDER TO MAINTAIN A FOLDED SUBSTRATE-BINDING SITE AS WELL AS THE DIMERIC STATE OF THE PROTEIN AS SHOWED IN THE FIGURE. SEE BORGES ET AL., 2012.**

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6.Borges, J.C., Ramos, C.H.I. (2011). Analysis of molecular targets of *mycobacterium tuberculosis* by analytical ultracentrifugation. *Current medicinal chemistry*. 18, 9, 1276-1285

7.Tiroli-Cepeda, A., Ramos, C.H.I. (2011). An overview of the role of molecular chaperones in protein homeostasis. *Protein pept. Lett.* 18, 101-109.

8.Olivier Julien, Pascal Mercier, Claire Allen, Olivier Fisette, Carlos H. I. Ramos, Patrick Lagüe, Tharin m. A. Blumenschein, and Brian d. Sykes (2011). Is there nascent structure in the intrinsically disordered region of troponin i? *Proteins: Struct. Funct. Bioinfo.* 79, 1240-1250.

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10.Gabriela v Meirelles, Julio Silva, Yuri Mendonca, Carlos H. I. Ramos, Iris Torriani and Jörg Kobarg (2011). Human nek6 is a monomeric mostly globular kinase with an unfolded short n-terminal domain. *Bmc Struct. Biol.* 11, 12.

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12.GAVA, L.M., Gonçalves, d.c., Borges, j.c., Ramos, C.H.I. (2011). STOICHIOMETRY AND THERMODYNAMICS OF THE INTERACTION BETWEEN THE C-TERMINUS OF HUMAN 90 KDA HEAT SHOCK PROTEIN HSP90 AND THE MITOCHONDRIAL TRANSLOCASE OF OUTER MEMBRANE TOM70. *Arch. Biochem. Biophys.* 513, 119-125.

13.Silva, j.c., Borges, j.c., Cyr, D.M., Ramos, C.H.I., Torriani, I (2011). Central domain deletions affect the saxs solution structure and function of yeast hsp40 proteins sis1 and ydj1. *Bmc Struct. Biol.* 11, 40.

14.Rodrigues, A.G.; Ping, L.Y.; Marcato, P.D.; Alves, O.L.; Silva, M.C.P.; Ruiz, R.C.; Melo, I.S.; Tasic, L.; de Souza, A.O. Biogenic antimicrobial silver nanoparticles produced by fungi. *Applied Microbiology and Biotechnology* 2012, *95*, 1-8.

15.Mendonça, Y.A., Ramos, C.H.I. (2012). CLONING, PURIFICATION AND CHARACTERIZATION OF A 90 KDA HEAT SHOCK PROTEIN FROM CITRUS SINENSIS (SWEET ORANGE). *Plant physiol. Biochem.* 50, 87-94.

16.da Silva, v.c., Ramos, C.H.I. (2012). THE NETWORK INTERACTION OF HUMAN 90 KDA HEAT SHOCK PROTEIN HSP90: A TARGET FOR CANCER THERAPEUTICS. *J. Proteomics.* 75, 2790-2802

17.Júlio c. Borges, Thiago v. Seraphim, David z. Mokry, Fabio c. L. Almeida, Douglas m. Cyr, Carlos H.I. Ramos. (2012). Identification of regions involved in substrate binding and dimer stabilization within the central domains of yeast hsp40 Sis1. PLOS One, v.7 n.12 e50927

AL 8

ASSOCIATE LABORATORY OF MACROMOLECULES CRYSTALLIZATION

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Associate laboratory number 8, from Universidade Federal da Bahia, has shifted its research interests towards enzymes that are considered as good targets for either antifugal therapy or neglected disease drug development. In the last two years, several efforts have been made to clone and express lanosterol 14-alfa demethylase from fluconazole resistant *Cryptococcus. neoformans* strains, *Schistosoma mansoni* dihydrofolate reductase; *Leishmania donovani* Pteridine reductase and superoxide dismutase from *Trypanosoma brucei*. Some work has also been carried out with *S. mansoni* purine nucleoside phosphorylase in collaboration with Dr. Adriano D. Andricopulo (INBEQMeDI) and with farnesyltransferase from *Plasmodium falciparum*. As these two projects underscore the importance of integrated analysis of structural data along with chemometric approaches to uncover the structure-activity relashionship of lead compounds towards their macromolecular targets, special attention will be paid to them in this report. A detailed discussion of the results summarized below can be found in Postigo et al 2011 and Freitas et al 2012.

Schistosomiasis, caused by *S. mansoni*, is a parasitic infection that affects roughly 200 million people worldwide. Currently, praziquantel and oxaminiquine are the only drugs available to treat shistosomiasis, however none of them is capable to prevent re-infection and recent results indicated that significant resistance to the drug may be present in different geographic locations. It is well known that *S. mansoni* lacks the de novo pathway for purine biosynthesis, thus depending entirely on the salvage pathway for its purine requirements. Taking into account that purine nucleoside phosphorylase plays an important role in the purine salvage pathway, it has been identified as an attractive target for schistosomiasis chemotherapy. Moreover, the difference in K_M values for inosine (human PNP= 41 μM Vs *Sm*PNP=6,4 μM) suggest that inhibition requirements for the parasite enzyme are rather dissimilar from those for the human enzyme. Kinetic studies with 9-deaza analogs show that apparent value of K_M changes with increasing inhibitor concentration (Figure 1A), thus supporting a competitive mechanism of inhibition. X-ray studies confirm this information for BCX34, one of the most potent ground state inhibitors identified thus far (Figure 1B).

Further analysis of crystallographic structure indicates that potency is related to 9-

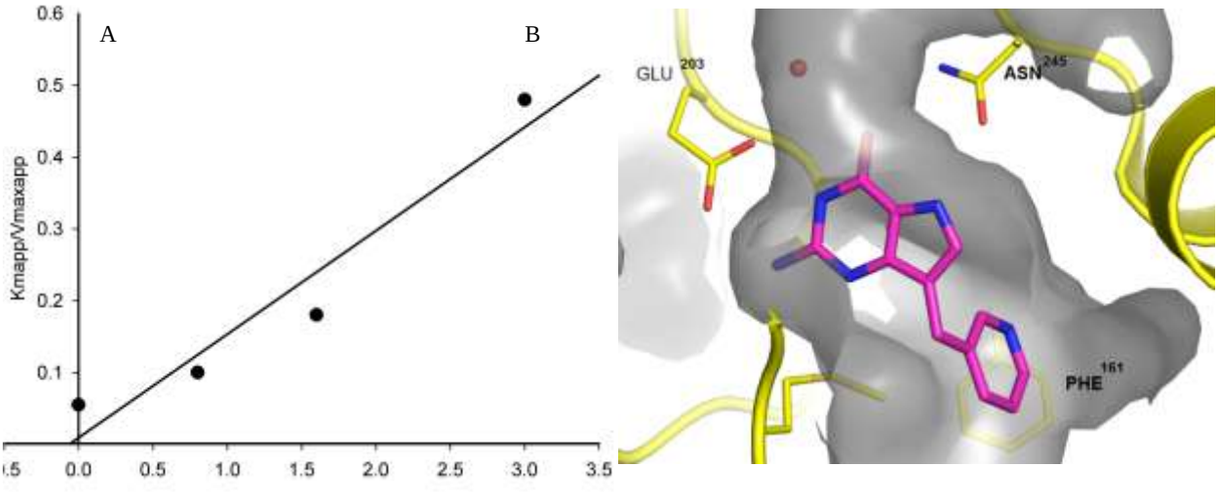

FIGURE 1 – KINECTIC AND STRUCTURAL CHARACTERIZATION OF BCX-34 BINDING PROFILE TOWARDS *S. MANSONI* PNP. A) INCREASING VALUES OF AFFINITY FOR INOSINE (KM APP) NOT ONLY PROVE THE COMPETITIVE MECHANISM OF INHITION FOR BCX-34 , BUT ALSO ALLOWS ITS KI VALUE TO BE DETERMINED (0.45□M). B) THE CRYSTALLOGRAPHIC STRUCTURE OS S. MANSONI PNP IN COMPLEX WITH BCX-34 (PDB CODE: 3DJF)REVEAL THE BINDING PROFILE IN THE ACTIVE SITE AND HIGHLIGHTS THAT HYDROPHOBIC INTERACTIOS WITH PHE[161] ARE CRUCIAL TO INHIBITORY POTENCY

position substituent features, such as hydrophobic interactions with Phe[161].In order to investigate this matter, hologram QSAR models were developed for a series of 25 ground-state *Sm*PNP inhibitors. The most predictive HQSAR model (r^2_{pred} = 0.80) reveal that the purine ring might have opposing effects toward potency (Figure 2), depending on the features of 9-position moiety. Compounds possessing aryl groups in the 9 position of the purine ring are correctly placed to bind the hydrophobic pocket nearby Phe[161] and thus have green colored purine rings, whereas compounds with shorter and non-planar chains bind loosely to this pocket, As a consequence, thse molecules do not tightly H-bond to Asn[245] and Glu[203] (reddish colored purine ring)

Malaria is responsible for the death of over 1 million people every year and the prognostic is getting even worse as *P. falciparum* develops resistance to existing drugs (chloroquine, mefloquine, sulfadoxime/pyrimethamine). Aiming at circumvent such problems novel macromolecular targets have been explored, such as the protein farnesyl transferase (PFT). This enzyme prenylates other proteins targeting them to the membrane. In the human host, protein prenylation is mediated by three different enzymes: PFT, protein geranylgeranyl transferase type I (PGGT-I) and protein geranylgeranyl transferase type II (PGGT-II), whereas the parasite lacks PGGT-I. This fact prompted many reaseach groups to develop PFT inhibitors. For instance, tetrahydroquinoline and ethylenediamine derivatives have proved effective alternatives against the parasite *in vitro*. Aiming at further understand the structural and physico-chemical properties that govern structure-activity relationships for 192 inhibitors, different chemomectric approaches were undertaken. Principal component analysis show that the

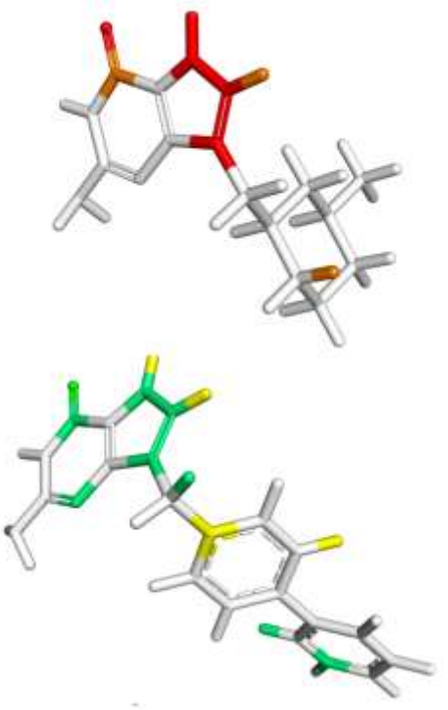

FIGURE 2 – HOLOGRAM QSAR MODEL SHOWS THAT PURINE RING CAN HAVE EITHER POSITIVE (GREEN/YELLOW) OU DETRIMENTAL EFFECTS TOWARDS POTENCY (RED/ORANGE) DEPENDENDING ON WICH MOIETY IS FOUND IN POSITION 9

molecules have completely different chemical properties, hinting that their structure-activity relationships might be dissimilar. As expected, no 2D QSAR could be built for the whole dataset. Instead, descriptor-based and fragment-based 2D-QSAR models were built for subgroups of the complete dataset. The analysis of regression vector for the best descriptor-based QSAR model (r^2= 0.78, q^2= 0.75, 2 PCs, r^2_{pred}= 0.77) of group A molecules (118) hints that steric features have a negative effect over the biological activity. More interesting though it the information provided by the best fragment-based QSAR model (A/B/Ch r^2= 0.82, q^2= 0.72, 3 PCs, r^2_{pred}= 0.79): the steric ballast can be found even in the most potent compounds of group A PFT inhibitors (Figure 3). On the other hand, descriptor-based QSAR model (r^2= 0.79, q^2= 0.74, 2 PCs, r^2_{pred}= 0.57) for group B molecules (74) suggests that electronic features play an essential role towards potency. Moreover, fragment-based QSAR models (A/B/C/Ch r^2= 0.86, q^2= 0.74, 4 PCs, r^2_{pred}= 0.75) highlight that this property has already been optimized for all compounds within this group (Figure 3). Taken together, the data collected from the chemometric studies points out that different properties should be modulated for each series of inhibitors.

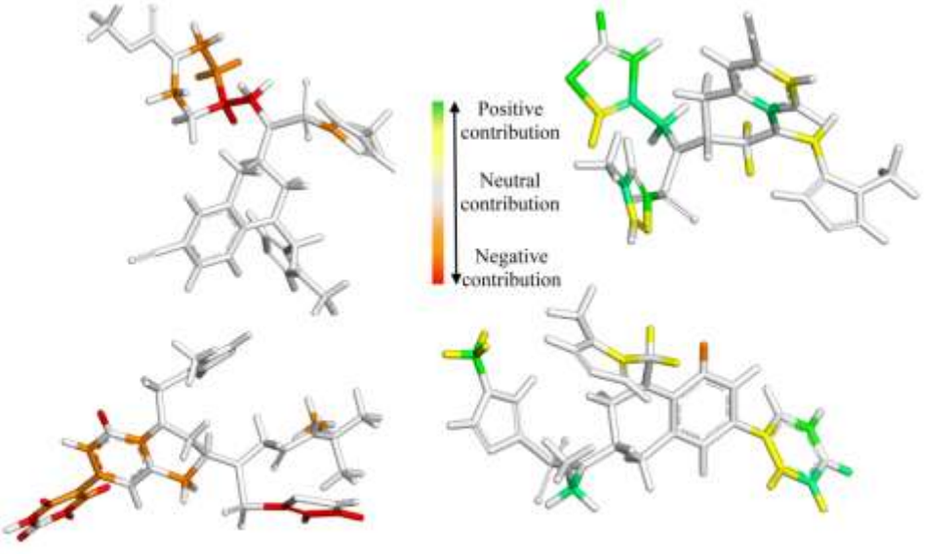

FIGURE 3 – 2D DESCRIPTOR-BASED (LEFT PANEL) AND FRAGMENT-BASED (RIGHT PANEL) QSAR MODELS FOR HYDROXY-ETHYLAMINE (HEA) DERIVATIVES THAT INHIBIT BACE-1. BOTH MODELS SHOW GOOD STATISTICAL PARAMETERS AND PREDICTIVE POWER

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AL 9

ASSOCIATE LABORATORY OF CELLULAR ULTRASTRUCTURE HERTHA MEYER

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I. Introduction

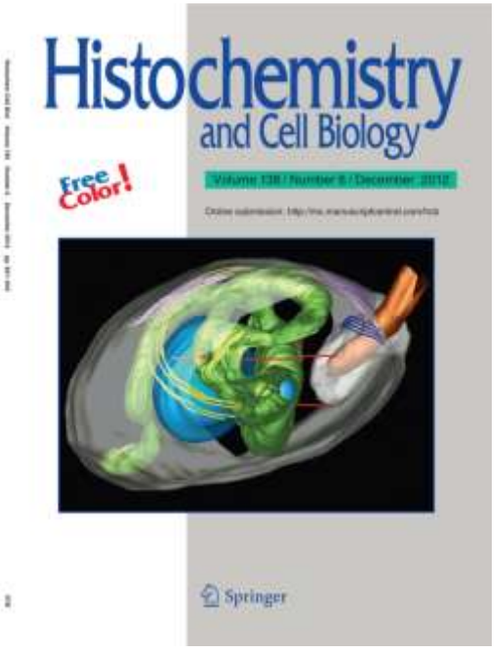
There are three main areas of research in the Laboratory: Structural Organization of Parasitic Microorganisms (Protozoa and Fungi) and Helminthes; Interaction of parasites with host cells, and Experimental Chemotherapy against Parasites. These areas will be briefly covered below

II. Structural Organization of Parasitic Microorganisms, and Fungi

II.1. Acidocalcisomes and Contractile vacuole

Understanding the mechanisms involved in ion homeostasis and osmoregulation control in protozoan parasites has been challenge for many research groups. Over the past few years, a number of key players in osmoregulatory signaling in protozoans, including trypanosomatids (Leishmania and Trypanosoma genus) and malaria parasites have been identified. Among these, cyclic AMP (cAMP) has been shown to play a key role in osmoregulation in Trypanosoma cruzi, where the regulatory volume decrease occurs through a mechanism that involves a cAMP-dependent pathway that leads to the efflux of osmolytes across the parasite surface, and water elimination through a contractile vacuole complex (CVC). In T. cruzi, the contractile vacuole system is formed by a central vacuole (bladder) surrounded by a collection of interconnected vesicles and tubules that undergo dynamic changes upon osmotic stress.

The functionality of the CVC has for long been demonstrated but only recently part of the signaling process responsible for the



THREE-DIMENSIONAL MODEL OF NA AMASTIGOTE FORM OF *TRYPANOSOMA CRUZI*. *AUTHORS: WENDELL GIRARD-DIAS AND KILDARE MIRANDA*

osmoregulatory response, a mechanism that involves participation of other organelles, has been described. In this regard, a unique characteristic of this system is the presence of acidic calcium-rich organelles named acidocalcisomes, whose structural organization, chemical properties and physiological activity may vary upon events of osmotic stress. Biochemical and molecular data have shown that the sequence of events that take place in cells submitted to hyposmotic stress leads to an increase in cAMP levels, stimulating the traffic of an aquaporin from acidocalcisomes to the CVC through a fusion mechanism. This has been revealed by electron tomography of cryofixed cells exposed to hyposmotic treatments. Acidocalcisomes contain basic amino acids and high levels of cations and polyphosphate, a content that once released within the contractile vacuole, leads to an increase in the osmotic pressure towards the lumen of the

organelle and stimulates water transport across the CVC membrane. Functional analysis of mutant parasites that overexpress enzymes involved in the control of cAMP levels, such as the T. cruzi phosphodiesterase TcPDE C2, showed alterations in the regulatory volume decrease (RVD), when compared to wild type cells. In addition, mutants that overexpress a Class III PI3 kinase showed a large and functional CVC and were more efficient in volume recovery when submitted to severe hyposmotic stress. Taken together, our data show dynamic changes in the osmoregulatory system of T. cruzi, governed by signaling events that involve a unique mechanism of interaction of the CVC with acidocalcisomal components. Whether or not this mechanism can be extended to other cell models is currently under investigation in our laboratory.

II.2. Endosymbiosis in Trypanosomatids

The characterization of a porin-like channel in the endosymbiont of Crithidia deanei considering ultrastructural, biochemical and molecular aspects

Some trypanosomatids harbor a symbiotic bacterium, which maintains an obligatory association with the host, thus constituting an excellent model to study organelle origin and cellular evolution. The presence of porins, which are typical outer membrane proteins, in the endosymbiont envelope would confirm its Gram-negative ancestral, since previous molecular analysis classified this bacterium in subdivision of Proteobacteria. In this study, we used biochemical and molecular methods to identify porins in the C. deanei endosymbiont envelope. Search on the endosymbiont genome annotation database identified a sequence that shares homology with porin genes of Gram-negative bacteria, especially with those from Bordetella genus. Subsequently, primers were designed and a PCR amplification produced a 1,2 Kb DNA fragment that was cloned in the pET21dHis-Tev vector to overexpress the recombinant protein. We purified the recombinant porin using Ni2+ column and ASB-14 was used for the protein solubilization and antiserum production. Immunolocalization by fluorescence microscopy and transmission electron microscopy techniques, using affinity purified antibodies against the recombinant protein, showed labeling in the symbiont, especially in the bacterium envelope. Taking together, data indicate that the outer membrane of the C. deanei endosymbiont presents prokaryotic origin.

II.3. Coordinated division between the symbiotic bacterium and the host cell structures as seen by 3D reconstruction

In this study, we used light, electron microscopy and tri-dimensional reconstruction approaches to observe the endosymbiont shape and division during Angomonas deanei and Strigomonas culicis cell cycle. We found that the bacterium divides independently of the appearance of the new flagellum, but always before the kinetoplast and the nucleus segregation. In addition, the endosymbiont is usually found close to the host cell nucleus, presenting different shapes during the protozoan cell cycle. Considering that the endosymbiosis in trypanosomatids is a mutualistic relationship, which resembles

organelle acquisition during evolution these results can contribute to the understanding of the mechanisms for establishment of organelles in eukaryotic cells.

In the case of S. culicis the symbiotic bacterium presents different formats and different positions in relation to other cell structures. Thus, at the beginning of the protozoan cell cycle, the endosymbiont presents a constricted form that becomes more elongated until the bacterium division that occurs before the kinetoplast and nucleus segregation. During cytokinesis, symbionts are positioned close to nuclei to ensure that each daughter cell will inherit a single copy bacterium.

II.4. Identification of gene sequences which encode proteins involved in phosphatidylcholine biosynthesis in A. deanei and its symbiont

In fact we performed the complete analysis of the predicted protein of two symbiont bearing trypanosomatids and their respective symbiotic bacteria.These results were obtained after sequencing and analysis of protozoa genomes. n an effort to better understand the symbiosis in trypanosomatid protozoa, we used DNA pyrosequencing and a reference-guided assembly to generate reads that predicted 16,968 and 12,170 open reading frames (ORFs) in two symbiont-bearing trypanosomatids, Angomonas deanei (previously named as Crithidia deanei) and Strigomonas culicis (first known as Blastocrithidia culicis), respectively. Identification of each ORF was based primarily on TriTrypDB using tblastn, and each ORF was confirmed by employing getorf from EMBOSS and Newbler 2.6 when necessary. The monoxenic organisms revealed conserved housekeeping functions when compared to other trypanosomatids, especially compared with Leishmania major. However, major differences were found in ORFs corresponding to the cytoskeleton, the kinetoplast, and the paraflagellar structure. The monoxenic organisms also contain a large number of genes for cytosolic calpain-like and surface gp63 metalloproteases and a reduced number of compartmentalized cysteine proteases in comparison to other TriTryp organisms, reflecting adaptations to the presence of the symbiont. The assembled bacterial endosymbiont sequences exhibit a high A+T content with a total of 787 and 769 ORFs for the Angomonas deanei and Strigomonas culicis endosymbionts, respectively, and indicate that these organisms hold a common ancestor related to the Alcaligenaceae family. Importantly, both symbionts contain enzymes that complement essential host cell biosynthetic pathways, such as those for amino acid, lipid and purine/pyrimidine metabolism. These findings increase our understanding of the intricate symbiotic relationship between the bacterium and the trypanosomatid host and provide clues to better understand eukaryotic cell evolution.

II.5. Endocytosis in Trypanosoma cruzi

In 2011-2012 we have published the isolation and characterization of the lipid inclusions of Trypanosoma cruzi reservosomes (Pereira MG et al, PLoS One 6:e22359, 2011), demonstrating that the inclusions are formed

by the accumulation of cholesterol, acquired from human LDL added to culture medium, in an acidic environment, forming crystal like inclusions. Unlike mammalian cells, T. cruzi epimastigotes are able to mobilize and distribute crystalized cholesterol. The cholesterol distribution route and kinetics from reservosomes to other organelles and plasma membrane were also investigated, the manuscript is in preparation. We have also demonstrated that Leishmania amazonensis promastigotes take up and metabolize human LDL particles in both a time and dose-dependent manner (De Cicco NN et al., Exp. Parasitol. 130:330, 2012). This mechanism implies the presence of a true LDL receptor because the uptake is blocked by both low temperature and by the excess of non-labelled LDL. This receptor is probably associated with specific microdomains in the membrane of the parasite, such as rafts, because this process is blocked by methyl-b-cyclodextrin. Cholesteryl ester fluorescently-labeled LDL (BODIPY-cholesteryl-LDL) was used to follow the intracellular distribution of this lipid. After uptake it was localized in large compartments along the parasite body. The accumulation of LDL was analyzed by flow cytometry using FITC-labeled LDL particles. Together these data show for the first time that L. amazonensis is able to compensate for its lack of lipid synthesis through the use of a lipid importing machinery largely based on the uptake of LDL particles from the host.

The use of electron tomography has revealed some new aspects of T. cruzi epimastigotes endocytic pathway, as the proximity of ribosomes and reservosomes (Girard-Dias et al., Histochem Cell Biol. 138:821. 2012) and unknown cytoplasmic microtubules, among other interesting cell features described in other sections of this report. The cytostome and the cytopharinx in its entire extension were also reconstructed using serial electron tomography, emphasizing the origin and disposition of the microtubules that go along the cytopharinx, as well as the close apposition of cargo containing vesicles (manuscript in preparation).

We have demonstrated that part of the fluorescent transferrin that T. cruzi epimastigotes uptake from medium returns to cell culture supernatant after reaching reservosomes. Transferrin can be collected from supernatant both intact and partially degraded. If transferrin is coupled to colloidal gold particles, however, we cannot find gold in the culture supernatant. Electron microscopy images of these epimastigotes show gold containing vesicles laterally fusing with the cytopharinx (manuscript in preparation).

II.6. Secretory Organelles of Toxoplasma gondii

Secretory organelles of Toxoplasma gondii (micronemes, rhoptries and dense granules) are crucial for host cell invasion and formation of the parasitophorous vacuole (PV). We examined whether their relative volumes change during the intracellular cycle. Stereological analysis of random ultrathin sections taken at 5 min of interaction, 7 and 24 h post-infection demonstrated that the relative volume of each type of organelle decreases just after the respective peak of secretion. Micronemes are radially arranged below the

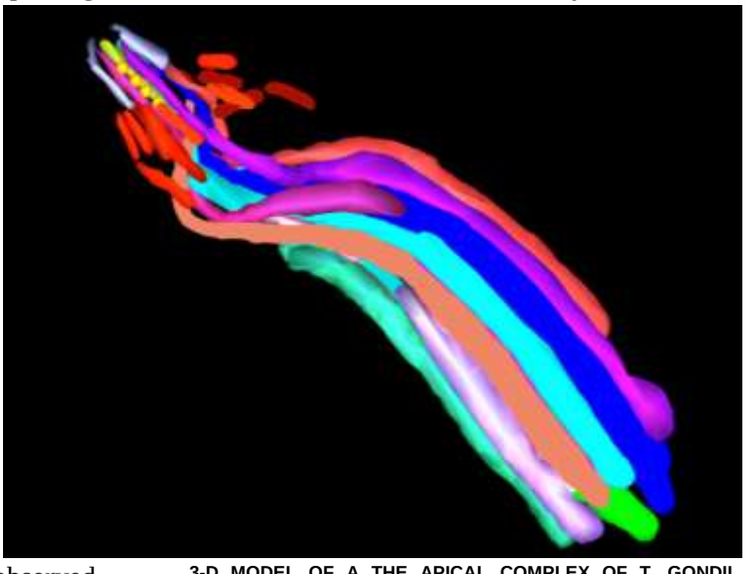
polar ring, while rhoptries converge to but only a few reach the inside of the conoid. In contrast to the apical and polarized organelles, dense granules were found scattered throughout the cytoplasm, with no preferential location in the parasite cell body. Extensive observation of random sections indicated that each organelle probably secretes in a different region. Micronemes secrete just below the posterior ring and probably require that the conoid is extruded. The rhoptries passing through the conoid secrete at a porosome-like point at the most apical region. Dense granules secrete laterally, probably at fenestrations in the inner membrane complex. Immunocytochemistry showed that there are no subpopulations of rhoptries or dense granules, as a single organelle can contain more than one kind of its specific proteins.

The vacuolar-like profiles observed at the apical portion of parasites just after invasion were confirmed to be empty rhoptries, as they were positively labeled for rhoptry proteins. These findings contribute for a better understanding of the essential behavior of secretory organelles.

II.7. The Cytoskeleton of Toxoplasma gondii

Speculations on the motility of the conoid of Toxoplasma gondii based on ultrastructural data

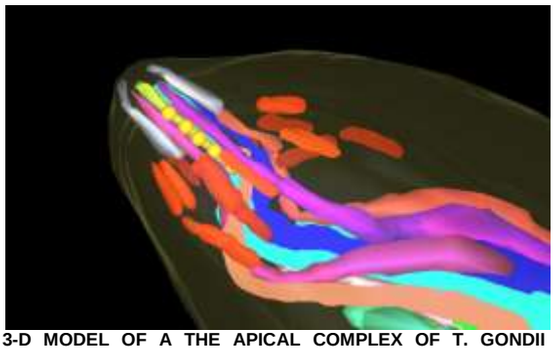
The conoid is a structure of the cytoskeleton of several Apicomplexa, including Toxoplasma gondii. It is located at the apical portion of the cell body and is an elaborated structure formed by extremely stable microtubules in a non canonic assembly of 9 comma shaped protofilaments. The conoid crowns the microtubular cytoskeleton, composed by 22 subpellicular microtubules regularly disposed around a polar ring. As the parasite glides along a substrate, adheres and finally invades a host cell, the conoid is seen quickly moving up and down above the polar ring, although little is known about the mechanics involved in its motility.



3-D MODEL OF A THE APICAL COMPLEX OF T. GONDII TACHYZOITE RECONSTRUCTED FROM SLICE AND VIEW SECTIONS OBTAINED IN THE HELIOS DUAL BEAM FIB SCANNING MICROSCOPE (FEI CO.). THE CONOID IS IN GRAY, AROUND IT MICRONEMES (RED) AND PASSING THROUGH THE CONOIDAL CHANNEL THREE RHOPTRIES (VARIOUS COLORS) AND LIGNED WITH THE INNER MICROTUBULES (GREEN) 5 VESICLES (YELLOW) ARE OBSERVED. THE PLASMA MEMBRANE IS TRANSPARENT AND COVERS THE WHOLE ASSEMBY. (MARCIA ATTIAS/TATIANA C. PAREDES-SANTOS)

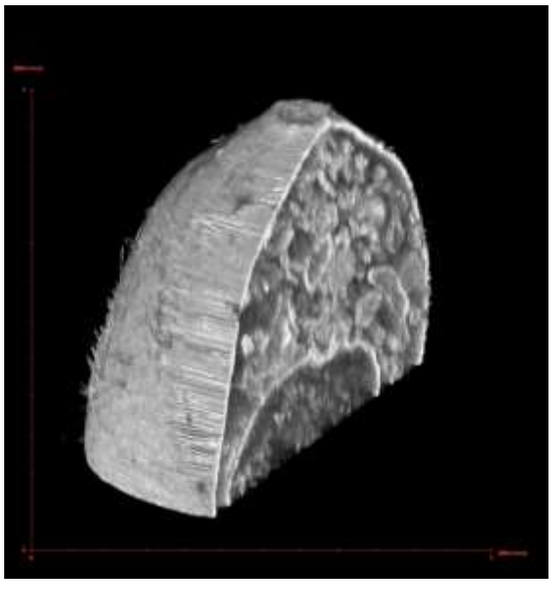
New information on the ultrastructure of the conoid and related structures were obtained using Field Emission Scanning Electron Microscopy to observe tachyzoites submitted to membrane extraction protocols with detergents. The posterior and apical polar rings were seen connected to the conoid through tiny bridges. Electron-dense material was observed over the microtubules of the conoid fibers. These structures can be correlated to proteins described in the literature

such as motor proteins that could take part in its up and down motility. As it moves, the shape of the conoid does not change. However, the apical portion of the parasite appears thinner and stretched during its extrusion.



3-D MODEL OF A THE APICAL COMPLEX OF T. GONDII TACHYZOITE RECONSTRUCTED FROM SLICE AND VIEW SECTIONS OBTAINED IN THE HELIOS DUAL BEAM FIB SCANNING MICROSCOPE (FEI CO.). THE CONOID IS IN GRAY, AROUND IT MICRONEMES (RED) AND PASSING THROUGH THE CONOIDAL CHANNEL THREE RHOPTRIES (VARIOUS COLORS) AND LIGNED WITH THE INNER MICROTUBULES (GREEN) 5 VESICLES (YELLOW) ARE OBSERVED. THE PLASMA MEMBRANE IS TRANSPARENT AND COVERS THE WHOLE ASSEMBY. (MARCIA ATTIAS/TATIANA C. PAREDES-SANTOS)

These observations indicate that the conoid moves up and down associated to motor proteins and not due to stretching of its spiraled fibers, as previously believed. In another approach, electron tomography of 200nm thick sections of tachyzoites fixed by high pressure freezing followed by freeze substitution confirmed the results obtained with FE-SEM and also shed more light on the association of rhoptries and micronemes with the conoid. Although it is common knowledge that microneme secretion precedes rhoptry secretion, both are believed to liberate its contents though the conoidal channel. With tomography and 3-D reconstruction of the tomograms, it is clear that only a single rhoptry docs at the tip of the conoidal channel and secrete, although many other rhoptries can be counted, confirming the morphometric analisys and random sections observations (see precedent section). Another detail is that rhoptries are not aligned with the main axis of the parasite: they always bend to one side. With respect to the micronemes, they are shorter than the rhoptries, but they are more rhomboid and a set of micronemes crowns around the polar ring, while other sets are more randomly distributed in the cytosol. Taken together, these informations and the previous data of the literature and of our group, led us to propose a model for the secretion for these organelles. Is short: micronemes, as well as conoid motion is calcium dependent while rhoptry secretion is not.



3-D MODEL OF A T. GONDII TACHYZOITE RECONSTRUCTED IN THE SOFTWARE AMIRA FROM SLICE AND VIEW SECTIONS OBTAINED IN THE HELIOS DUAL BEAM FIB SCANNING MICROSCOPE (FEI CO.). THE APICAL PORTION, INNER ORGANELLES AND THE NUCLEUS ARE SEEN. (MARCIA ATTIAS/ DAVID WALL, ACKNOWLEDGEMENT TO FEI COMPANY).

So, micronemes set around the polar ring would fuse with the plasma membrane upon the elevation of the conoid and release its secretion. Rhoptries, on the other side, would secrete its contents passing through the conoidal channel, releasing its contents directly into the nascent parasitophorous vacuole.

II.8. The anterior flagella of Giardia lamblia

Giardia duodenalis, a protozoan parasite that causes intestinal disorders known as giardiasis, presents a life cycle involving two developmental stages: cysts and trophozoites. The trophozoite contains four pairs of flagella emerging from the cell in strategic points so that the protozoan exhibits a peculiar motility. In addition to motility, the protozoan uses the flagella to complete the intricate cell division at the same time that it is able to attach to the surface of intestinal epithelial cells. In addition to the flagella, the protozoan also presents other microtubule-containing structures such as the adhesive disk, the median body and the funis. Flagella-associated structures have also been identified. Here we further analyze the structural organization of the anterior flagella of G. duodenalis. High resolution scanning electron microscopy of detergent-extracted trophozoites revealed novel aspects of the interaction of the axonemes of the anterior flagella with the marginal plates. Images of the marginal plates, known to be part of the ventrolateral flange, showed that it had a "boomerang-like" shape and was located in the anterior region of the parasite, above the crossing point of the axonemes of the anterior flagella towards the periphery of the cell. Two well distinguished structures were seen associated with the anterior flagella. The first one correspond to the 'dense rods', located just below the axoneme. The second one is a system of filaments located in the upper portion of the flagellum, facing the marginal plates and connecting these two structures. The thickness of the filaments is around 18nm and they are spaced at intervals of 4-32nm (average 18nm). The length of the filaments may vary from 33 to 240 nm. We used an indirect approach, involving the use of some antibodies and immunofluorescence microscopy, to get some information on the structures found. For this, two antibodies were used: the first one recognizes some proteins found in the paraflagellar rod (PFR) of trypanosomatids, which is a structure that also presents filamentous bridges connecting cytoskeletal structures; and a second one, primarily developed to identify the tight junction protein claudin-1 in epithelial cells. Both antibodies predominantly labeled the portion of the anterior flagella where the filamentous bridges are located. Electron microscopy immunocytochemistry of negatively stained cells also confirmed labeling at this region. Western blots analyses showed that all the antibodies used recognize some protozoan proteins.

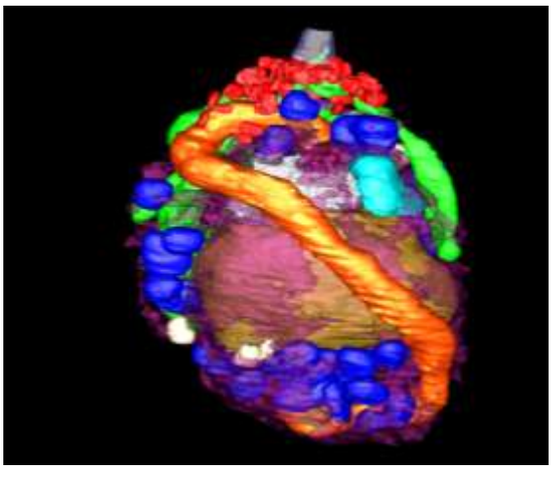
II.9. The Paraflagellar Rod (PFR) Structure of Trypanosomatids

The study of the paraflagellar rod was continued in order to obtain more information regarding its structure and its participation in the flagellar beating. At present we are isolating the flagellum and the PFR to obtain proteomic information and the identification of new proteins involved in the structure of the PFR.

II.10. The cystogenesis in Toxoplasma gondii

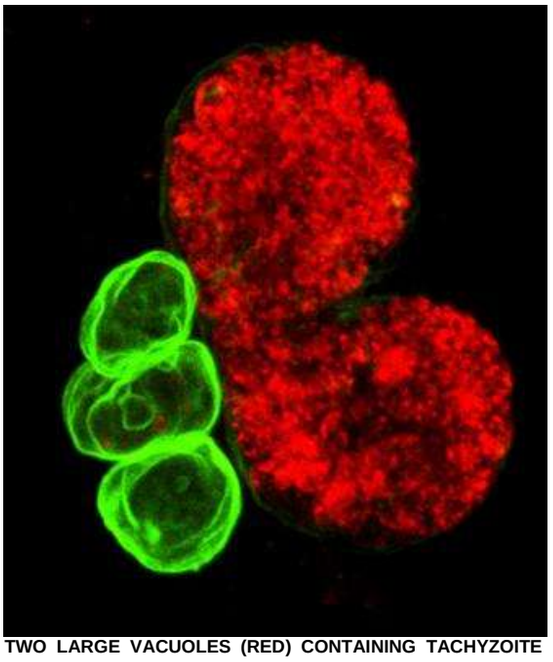
The encystation process is a key step in Toxoplasma gondii life cycle, allowing the parasite to escape from the host immune

system and the transmission among the hosts. A detailed characterization of the formation and structure of the cyst stage is essential for a better knowledge of toxoplasmosis. Here we isolated cysts from mice brains and analysed the cyst wall structure and cyst matrix organization using different electron microscopy techniques. Images obtained showed that the cyst wall presented a filamentous aspect, with circular openings on its surface.



3-D MODEL OF THE INNER ORGANELLES OF A COMPLETE T. GONDII TACHYZOITE RECONSTRUCTED FROM SLICE AND VIEW SECTIONS OBTAINED IN THE HELIOS DUAL BEAM FIB SCANNING MICROSCOPE (FEI CO.). THE CONOID IN THE UPPER PORTION IS SURROUNDED BY MICRONEMES (RED), RHOPTRIES APPEAR IN GREEN AND THE MITOCHONDRION IN ORANGE. DENSE GRANULES ARES NAVY BLUE AND THE GOLGI COOMPLEX (WHITE) ABOVE THE NUCLEUS (PURPLE) AND THE APICOPLAST (LIGHT BLUE) ARE SEEN..(MARCIA ATTIAS/ TATIANA C. PAREDES-SANTOS).

The filaments were organized in two layers: a compact one, facing the exterior of the whole cyst and a more loosen one, facing the matrix. Within the cyst wall, we observed tubules and a large number of vesicles. The cyst matrix presented vesicles of different sizes and tubules, which were organized in a network connecting the bradyzoites to each other and to the cyst wall. Large vesicles, with a granular material in their lumen of glycidic nature were observed. Similar vesicles were also found associated with the posterior pole of the bradyzoites and in proximity to the cyst wall.



TWO LARGE VACUOLES (RED) CONTAINING TACHYZOITE FORMS OF *TOXOPLASMA GONDII* OCCUR SIMULTANEOUSLY WITH BRADYZOITES CONTAINING VACUOLES (GREEN) IN LLC-MK2 CELLS INFECTED WITH THE EGS STRAIN.GREEN- DOLICHUS BIFLORUS LECTIN, THAT LABELS THE CYST WALL. RED- SAG1 ANTIBODY. CONFOCAL MICROSCOPY. (TATIANA SANTOS/ ROSSIANE VOMMARO)

We also investigated the in vitro cystogenesis and behavior of the EGS strain, isolated from human amniotic fluid of a patient with toxoplasmosis and corresponds to a recombinant I/III strain. We observed that tachyzoites of the EGS strain converted to intracellular cysts spontaneously in LLC-MK2 epithelial cells, HSFS fibroblasts and C6 glial cell lineage. The peak of conversion occurred in the LLC-MK2 cells after 4 days of infection. Using specific markers against bradyzoite, tachyzoite and cyst wall components, we confirmed stage conversion and distinguished immature from mature cysts. It was also observed that the deposition of cyst wall components occurred before the total conversion of parasites. Transmission electron

microscopy confirmed the fully conversion of parasites presenting the typical characteristics of bradyzoites as the posterior position of the nucleus and the presence of amylopectin granules. A thick cyst wall was also detected. Besides, the scanning microscopy revealed that the intracyst matrix tubules were shorter than those from the parasitophorous vacuole intravacuolar network and were immersed in a granular electron dense material. The EGS strain spontaneously forms high burden of cysts in cell culture without artificial stress conditions, and constitutes a useful tool to study this stage of the T. gondii life cycle.

II.11. Physical and structural studies of Cryptococcus neoformans polysaccharide capsule

Capsule production is common among bacterial species, but relatively rare in eukaryotic microorganisms. Members of the fungal Cryptococcus genus are known to produce capsules, which are major determinants of virulence in the highly pathogenic species Cryptococcus neoformans and Cryptococcus gattii. Although the lack of virulence of many species of the cryptococcus genus can be explained solely by the lack of mammalian thermotolerance, it is uncertain whether the capsules from these organisms are comparable to those of the pathogenic cryptococci. The polysaccharide (PS) capsule of C. neoformansis the hallmark of this global fungal pathogen. It contributes to infection by protecting the cell against a variety of host immune defenses, interfering with phagocytosis and suppressing both cellular and humoral immunity. Because of this, the capsule is considered a main virulence determinant and remains a major target for the development of therapeutic strategies against cryptococcal disease. Although ps capsule critical role in pathogenesis is well understood, many fundamental aspects about its structure, dynamics, regulation, and mechanical properties are poorly understood, given its complex composition and conformation. By taking a physicochemical approach and combining multiple techniques, we determined that cryptococcal capsular ps exhibits polymer solution characteristics consistent with branching. This structural property appears to be an important determinant of capsular ps biological activity, and presents implications for capsule synthesis, assembly, and capsular-based vaccine development.

We compared the characteristic of the capsule from the non-pathogenic environmental yeast Cryptococcus liquefaciens with that of C. neoformans. Microscopic observations revealed that C. liquefaciens has a capsule visible in india ink preparations that was also efficiently labeled by three antibodies generated to specific C. neoformans capsular antigens. Capsular polysaccharides of C. liquefaciens were incorporated onto the cell surface of acapsular C. neoformans mutant cells. Polysaccharide composition determinations in combination with confocal microscopy revealed that C. liquefaciens capsule consisted of mannose, xylose, glucose, glucuronic acid, galactose and n-acetylglucosamine. Physical chemical analysis of the C. liquefaciens polysaccharides in comparison with C. neoformans samples revealed significant differences in viscosity,

elastic properties and macromolecular structure parameters of polysaccharide solutions such as rigidity, effective diameter, zeta potential and molecular mass, which nevertheless appeared to be characteristics of linear polysaccharides that also comprise capsular polysaccharide of C. neoformans. The environmental yeast, however, showed enhanced susceptibility to the antimicrobial activity of the environmental phagocytes, suggesting that the C. liquefaciens capsular components are insufficient in protecting yeast cells against killing by amoeba. These results suggest that capsular structures in pathogenic Cryptococcus species and environmental species share similar features, but also manifest significant difference that could influence their potential to virulence.

Several monoclonal antibodies (mabs) generated against the capsule have shown to be protective against in vivo models of cryptococcal disease. We determined that protective but, not non-protective, mabs were able to affect budding of encapsulated yeasts by trapping daughter cells inside the mother's capsule. This effect is determined by the ability of the mab to increase the elastic properties of the capsule via cross-linking of ps molecules. Results obtained from these studies are important for understanding the direct microbial-effects of capsular mabs and the mechanism of mab-mediated protection. In general, the structural and physical properties of the ps capsule described here authenticate its complex design and virulent nature. Our findings provide insights about fundamental capsular features important for better comprehension its biology and function. More importantly, we provide basic information that might be crucial for the design and development of therapeutic strategies against C. neoformans and for the study of other encapsulated pathogens.

III. Interaction of Parasites with Host Cells

III.1. Toxoplasma gondii egress: On the trail of calcium

Following invasion, Toxoplasma gondii replicate inside a non-fusogenic parasitophorous vacuole (PV) and, after multiple cycles of division by endodiogeny, leave the cell, and invade new ones and, by doing so, spread the infection. Infection by this protozoan seems to requires the participation of dynamin, crucial for the pinching off of vesicles from plasma membrane. During invasion we could observe this GTPase in the locus between the parasitophorous vacuole and host cell plasma membrane. The mechanisms involved are not entirely elucidated, and may rely on the cooperation with actin filaments. The involvement of dynamin in the parasite intracellular development and egress and is being further investigated. As previous studies shown [Caldas et al. 2007], the premature release of parasites from host cell results in unsuccessful infections in permissible host cells. A kinetic study was performed in order to determine the average time range necessary for T. gondii intracellular maturation, which enables the infection. For reinfection assay, parasite egress from LLC-MK2 cells was induced at 2, 6, 8 and 24 hours post-infection (hpi). After harvesting, they were submitted to interaction with permissible cells of the same

lineage. The assay indicates that the minimum average time for parasite maturation is between 6 and 8hpi.

Natural egress, as well as endodiogeny, was also observed by a kinetic videomicroscopy, integrating the ongoing studies of natural egress.

III.2. Trypanosoma cruzi host cell interaction:

Trypanosoma cruzi is an intracellular parasite that, like some other intracellular pathogens, targets specific proteins of the host cell vesicular transport machinery, leading to a modulation of host cell processes that results in the generation of unique phagosomes. In mammalian cells, several molecules have been identified that selectively regulate the formation of endocytic transport vesicles and the fusion of such vesicles with appropriate acceptor membranes. Among these, the GTPase dynamin plays an important role in clathrin-mediated endocytosis, and it was recently found that dynamin can participate in a phagocytic process. We used a compound called dynasore that has the ability to block the GTPase activity of dynamin. Dynasore acts as a potent inhibitor of endocytic pathways by blocking coated vesicle formation within seconds of its addition. Here, we investigated whether dynamin is involved in the entry process of T. cruzi in phagocytic and non-phagocytic cells by using dynasore. With this aim, peritoneal macrophages and LLC-MK2 cells were treated with increasing concentrations of dynasore before interaction with trypomastigotes, amastigotes or epimastigotes. We observed that, in both cell lines, the parasite internalization was drastically diminished (by greater than 90% in LLC-MK2 cells and 70% in peritoneal macrophages) when we used 100 µM dynasore. The T. cruzi adhesion index, however, was unaffected in either cell line. Analyzing these interactions by scanning electron microscopy and comparing peritoneal macrophages to LLC-MK2 cells revealed differences in the stage at which cell entry was blocked. In LLC-MK2 cells, this blockade is observed earlier than it is in peritoneal macrophages. In LLC-MK2 cells, the parasites were only associated with cellular microvilli, whereas in peritoneal macrophages, trypomastigotes were not completely engulfed by a host cell plasma membrane. Taken together our results demonstrate that dynamin is an essential molecule necessary for cell invasion and specifically parasitophorous vacuole formation by host cells during interaction with Trypanosoma cruzi .

We also analysed one of the potential evasion mechanism that would enable Trypanosoma cruzi to establish infection. It has been shown that virus and parasitic protozoa, including Leishmania amazonensis and Toxoplasma gondii, are capable of mimicking mammalian apoptotic cell death by the exposure of phosphatidylserine (PS). This process, called “apoptotic mimicry”, justify the occurrence of apoptotic features in a unicellular pathogen. DaMatta et al (2007) demonstrated that the exposure of PS by a population of Trypanosoma cruzi trypomastigotes, but not epimastigotes or intracellular amastigotes, leads to the deactivation of macrophages through a TGF-b

signaling pathway. We investigated if the presence of PS modulates trypomastigotes' entry process into macrophages. In order to quantify the percentage of PS+ trypomastigotes, we used flow cytometry and a low percentage was found. Then, we separated PS- and PS+ trypomastigotes, using an Annexin V microbead kit, which allowed us to interact separately these subpopulations with macrophages for 1, 24 and 48 hours, always comparing with total population (PS- and PS+). The subpopulations' separation through this Kit showed a consonance with the percentage of PS+ trypomastigotes found through flow cytometry, validating this method. We could observe that the PS+ parasite's internalization was drastic diminished (higher than 90%) within 24 and 48 hours of interaction. The T. cruzi's adhesion index was not altered. Besides that, PS- parasites showed an infection's progression of 60% higher when compared with total population within 48 hours. By field emission scanning electron microscopy, we observed that PS- parasites enter in macrophages preferentially by anterior region, but further quantifications will be done to confirm this data. Together, our findings suggest the participation of PS in trypomastigotes' entry process into peritoneal macrophages, indicating cooperation between PS- and PS+ parasites in the establishment of the infection.

III.3. Macropinocytosis as an additional mechanism of invasion of cell by Toxoplasma gondii.

Toxoplasma gondii entry in host cells is described as an active process, necessary for the successful formation of parasitophorous vacuole. On the other hand, when phagocytosed by the host cell, the parasite is destroyed by the phagolysosomal pathway. Lately, a third form of invasion in host cells has been described for other protozoan parasites, e.g., T. cruzi. That is macropinocytosis, an endocytic process where cells internalize large amounts of solutes. To investigate whether Toxoplasma gondii entry into peritoneal macrophages and LLC-MK2 epithelial cells can be also mediated through a macropinocytosis-like process, we used several different inhibitors of macropinocytosis presently available and confirmed that these inhibitors partially blocked internalization of Toxoplasma gondii by host cells. This hypothesis is supported by the numbers obtained in the internalization index assay and by observations with immunofluorescence microscopy and scanning electron microscopy. After treatment with the inhibitors: Amiloride and IPA-3, there was a significant decrease in the number of parasites internalized. Proteins such as Pak1, which participate in macropinosome formation, were localized in Toxoplasma gondii parasitophorous vacuole. We also observed co-localization between the parasite and an endocytic fluid phase marker. All together, these results indicate that Toxoplasma gondii is able to use one more mechanism of penetration into host cell, and that is macropinocytosis.

III.4. Interaction of Plasmodium chabaudi with erythrocytes

Plasmodium, the causal agent of malaria, interacts with the erythrocyte

modifying its cytoplasm and surface. These modifications have been characterized mainly through microscopy techniques, where electron microscopy has played a key role. At the ultrastructural level, the three-dimensional architecture of Plasmodium has been characterized by different reconstruction methods that comprise mainly the 3D reconstruction from serial sections and electron tomography. Different methods for three-dimensional visualization of biological structures at the ultrastructural level have been developed and extensively applied by different research groups. In the field of electron microscopy, a new technique that has emerged is the use of a focused ion beam and scanning electron microscopy for 3D reconstruction at nanoscale resolution. The resolution achieved so far with this method is getting close to that achieved with electron tomography. However, the higher extent of volume that can be reconstructed with this instrument represent one of the main benefits of this technique, which can provide statistically relevant 3D morphometrical data. As the life cycle of Plasmodium species is a process that involves several structurally complex developmental stages that are responsible for a series of modifications in the erythrocyte surface and cytoplasm, a high number of features within the parasites and the host cells has to be sampled for the correct interpretation of their 3D organization. In the current project, we used FIB-SEM to visualize the 3D architecture of multiple erythrocytes infected with Plasmodium chabaudi and analyzed their morphometrical parameters in a 3D space. Plasmodium parasites divide within a parasitophorous vacuole and interact with their host cells through secretion and internalization processes that induce modifications on the erythrocyte cytoplasm and surface. The development of P. falciparum inside the red blood cell is generally accompanied by morphological alterations on the surface of the infected erythrocyte, including surface knobs, structures that may be involved in cytoadherence and cerebral malaria. Despite the importance of such alterations in human malaria models, a detailed description of the structural changes on the surface of RBCs infected with other Plasmodium species had not yet been reported. We analyzed and quantified alterations on the host cells, such as the variety of shapes and sizes of their membrane clefts and parasite internal structures. 3D modeling showed the organization of membrane clefts, a tubovesicular network (TVN) originating from the parasitophorous vacuole and a polymorphic organization of hemoglobin-filled tubules within the parasite. We also characterized the surface of erythrocytes infected with Plasmodium chabaudi, a rodent malaria parasite considered a model for cytoadherence studies in the absence of knobs. Analyses of infected RBCs (iRBC) by different microscopy techniques revealed structural changes on host cell surfaces, including large deformations due to the presence of the parasite, surface invaginations that generate endocytic vesicles within the host cell cytoplasm, and formation of knob-like structures, previously described only in other malaria models. Alterations of the intracellular organization, such as budding of micro and small vesicles subsequently

spreading throughout the cytoplasm, were also observed. Altogether, the results suggest that a broad spectrum of modified structures are involved in the interaction of Plasmodium chabaudi with the host cell and provide evidence for the presence of knob-like structures on the surface of P. chabaudi in RBC, a characteristic that may be relevant for the study of the cytoadherence mechanisms.

IV. Experimental Chemotherapy against Parasites.

IV.1. Chagas disease chemotherapy

Chagas disease, caused by the protozoan Trypanosoma cruzi, is an endemic illness in Latin America. Efforts have been made by several groups to develop new effective and safe anti-T. cruzi drugs. We observed that thiazolidine LPSF SF29 inhibited growth of the epimastigote and amastigote forms and caused lysis in the trypomastigote form of T. cruzi, leading to death of the protozoan. Mitochondrial dysfunction was also observed. The thiazolidine induced ultrastructural alterations such as detachment of the flagellar membrane, intense mitochondrial swelling, formation of myelin-like figures and the appearance of autophagosomes. Taken together, these results suggest that this new thiazolidine is active against T. cruzi and constitutes a promising drug for the therapy of Chagas disease .

The antifungal posaconazole (PCZ) is the most advanced candidate for the treatment of Chagas disease, having potent anti-Trypanosoma cruzi activity in vitro and in animal models of the disease as well as an excellent safety profile in humans. Amiodarone (AMD) is the antiarrhythmic drug most frequently used for the symptomatic treatment of chronic Chagas disease patients, but it also has specific anti-T. cruzi activity. When used in combination, these drugs exhibit potent synergistic activity against the parasite. Electron microscopy was used to analyse the effects of both compounds, acting individually or in combination, against T. cruzi. The 50% inhibitory concentration (IC50) against epimastigote and amastigote forms was 25 nM and 1.0 nM for PCZ and 8 □M and 5.6 □M for AMD, respectively. The antiproliferative synergism of the drugs (fractional inhibitory concentration < 0.5) was confirmed and the ultrastructural alterations in the parasite induced by them, leading to cell death, were characterized using electron microscopy. These alterations include intense wrinkling of the protozoan surface, swelling of the mitochondrion, shedding of plasma membrane vesicles, the appearance of vesicles in the flagellar pocket, alterations in the kinetoplast, disorganization of the Golgi complex, accumulation of lipid inclusions in the cytoplasm, and the formation of autophagic vacuoles, the latter confirmed by immunofluorescence microscopy. These findings indicate that the association of PCZ and AMD may constitute an effective anti-T. cruzi therapy with low side effects.

We also analysed the effect to sirtuins inhibitors, especially 3-arilideneindoin-2-onas (TFMDI).The sirtuins are histone deacetylases enzymes present in prokaryote and eukaryote cells, where are associated with stress resistance, longevity,

genomic stability and energy metabolism. In this study we evaluated the effect of 3-arilideneindolin-2-onas (TFMDI), a sirtuins inhibitor, against T. cruzi. This compound inhibited epimastigote and amastigote forms proliferation, showed IC50 of 7 µm and 1.1 µm, respectively. Against trypomastigote form, the compound exhibited a LD50 of 1.1 µm. tfmdi also demonstrated low potential toxicity to peritoneal macrophages, with cc50 of 90 µm, being more selective for amastigote (around 81 times) than macrophages. K-DNA disorganization was observed in epimastigote treated with tfmdi by giemsa staining using optical microscopy. Moreover, the compound inhibited parasite cell division, especially during cytokinesis. These alterations also were confirmed by scanning electron microscopy. In addition, the transmission electron microscopy (TEM) showed epimastigote with loss of chromatin condensation, presence of several electron-lucent vacuoles and autophagic vacuoles, Golgi apparatus disorganization, and K-DNA disorganization. The same kdna alterations were observed in treated trypomastigote, when analyzed by met. Furthermore, loss of cytoplasm organelles, presence of swelling mitochondrial and myelin-figures also were visualized. Taken together, our work showed that sirtuins inhibitors such as TFMDI had antiproliferative effect against epimastigote and amastigote, and lytic activity against trypomastigote forms of T. cruzi, interfering mainly on cellular cycle and in mechanism of cell death, being a promising drug for treatment of chagas disease.

IV.2. Chemotherapy for the treatment of leishmaniasis

Our group is involved in study new compounds with potential activity against Leishmania sp, focus at this moment mainly in the effects of different inhibitors in Leishmania amazonensis, an important specie for the epidemiology of leishmaniasis in Brazil. Thus, three different classes of compounds have been studied in the last two years: 1) Ergosterol Biosynthesis Inhibitors (EBIs) alone or in combination; 2) Phospholipid analogues; 3) Histone Deacetylases Inhibitors.

Results obtained with Ergosterol Biosynthesis Inhibitors (EBIs):

We have studied four EBIs: amiodarone, posaconazole, itraconazole and E5700. Amiodarone (AMIO) is an antiarrhythmic drug used to treat chronic Chagas disease that inhibits the squalene epoxidase. Posaconazole (POSA) and itraconazole (ITZ) are known azoles that inhibit the C14α-demetilase; and E5700 is a squalene synthase inhibitors. We have investigated the effect of these compounds on the proliferation, ultrastructure and mitochondrial physiology. AMIO was also tested in murine model of cutaneous leishmaniasis by infection of Balb/C mice with Leishmania amazonensis. For AMIO, The IC50 values were 4.21 and 0.46 µM against promastigotes and intracellular amastigotes, respectively, indicating high selectivity for the clinically relevant stage. E5700, ITZ and POSA alone produced a marked reduction in the viability of L. amazonensis promastigotes, with MIC (minimum inhibitory concentration) values of 30 nM, 1 µM, and 1 µM,

respectively. Several combinations were tested and the most efficient was the combination of 1.25 nM E5700 with 40 nM ITZ or 0.625 nM E5700 with 5 nM POSA, which resulted in FIC (fractional inhibitory concentration) values of 0.082 for the combination of E5700 with ITZ and 0.026 for E5700 with POSA, indicating a very potent synergistic effects. Against intracellular amastigotes, the MICs for E5700, ITZ and POSA alone were 30 nM, 1 µM, and 1 µM, respectively. The results indicated strong synergism, with MICs of 2.5 nM E5700 plus 20 nM ITZ (FIC=0,103) and 2.5 nM E5700 plus 2.5 nM POSA (FIC=0,085). We also found that treatment with AMIO leads to a collapse of the mitochondrial membrane potential ($\Delta\Psi$) and to an increase in the production of reactive oxygen species, in a dose-dependent manner. Fluorescence microscopy of cells labeled with JC-1, a marker for mitochondrial energization, and transmission electron microscopy confirmed severe alterations of the mitochondrion, including intense swelling and modification of its membranes. These alterations were also observed for the treatments with POSA and ITZ. Differential interference contrast microcopy (DIC) revealed a significant alteration on the shape of promastigotes after treatment with E5700 in combination with POSA, more than that observed with ITZ. Transmission electron microscopy of treated-parasites showed several alterations such as: 1) Presence of lipid bodies; 2) Intense mitochondrial swelling followed by the loss of matrix content; and 3) Presence of autophagosome-like structures. In summary, our results indicate that combinations of EB inhibitors acting at different steps of the pathway have synergistic activity against L. amazonensis and open up the possibility of a novel combination therapies for the treatment of leishmaniasis.

Results obtained with phospholipid analogues:

We evaluated the effect of a novel alkyl phosphocholine-dinitroaniline hybrid molecule, TC95, against Leishmania amazonensis promastigotes and intracellular amastigotes. Antiproliferative assays indicated that TC95 is a potent inhibitor of promastigotes and intracellular amastigotes with IC50 values of 2.6 µM and 1.2 µM, respectively. Fluorescence microscopy with anti-α-tubulin antibody revealed changes in the cytoskeleton, whilst scanning electron microscopy showed alterations in the shape, plasma membrane, length of the flagellum, and cell cycle. Flow cytometry confirmed the cell cycle arrest mainly in G1 phase, however a significant population appeared in sub G0/G1 and super-G2. The alterations in the plasma membrane integrity were confirmed by fluorometric analysis using Sytox Blue. Transmission electron microscopy also revealed an accumulation of lipid bodies, confirmed by fluorescence microscopy and fluorometric analysis using Nile Red. Important lesions were also observed in organelles such as mitochondrion, endoplasmic reticulum and Golgi complex. In summary, our study suggests that TC95, an alkyl phosphocholine-trifluralin hybrid molecule, is a promising novel compound against Leishmania amazonensis.

Results obtained with histone deacetylases inhibitors:

Histone desacetilases inhibitors are new compounds studied against gastric tumors, inducing alterations on the gene expression that promoting apoptosis in the treated-cells. Thus, the aim of this work was study the effects of a novel histone desacetilases inhibitors, against Leishmania amazonensis promastigotes and intracellular amastigotes. The effects induced by TFMDI were evaluated using different techniques such as: growth curve, immunofluorescence microscopy, scanning and transmission electron microscopy, western blotting, and fluorimetry. For promastigotes, the IC50 value was 2 μM. Against intracellular amastigotes, the effect was more pronounced after 48h of treatment and the IC50 value was around 3 μM. Immunofluorescence, DIC and scanning electron microscopy revealed an alteration on the promastigote' shape, that presented elongated and thinner, and an increase in the expression of acetylated tubulin. Western blotting using an anti-tubulin acetylated antibody confirmed this increase. Furthermore, transmission electron microscopy revealed several ultrastuctural alterations, such as: 1) Mitochondrial swelling followed by the formation of many vesicles inside the matrix; 2) Presence of many lipid bodies randomly distributed through the cytoplasm; 3) Abnormal chromation condensation; and, 3) Formation of blebs on the plasma membrane. Fluorimetric analysis with Nile Red confirmed the increase in the number of lipid bodies. New TFMDI analogues have been tested with activity similar against promastigotes and intracellular amastigotes. All these results together indicated that this compound is a promising molecule against leishmaniasis, however new studies are necessary to understand better this mechanism of action.

IV.3. Experimental Chemotherapy in Toxoplasmosis

Previous studies from our group have demonstrated the high susceptibility of Toxoplasma gondii tachyzoites to the sterol analogues 22,26-azasterol and 24,25-(R,S)-epiminolanosterol. In this work we present data on testing in vitro three novel azasterols as potential agents for the treatment of toxoplasmosis. The three compounds inhibited parasite growth at micromolar concentrations, in a dose-dependent manner. Electron microscopy analysis of intracellular tachyzoites after treatment with the most effective compound showed drastic mitochondrion swelling associated with the appearance of an electron-lucent matrix and disrupted cristae. Parasite lysis also took place. The appearance of electron dense cytoplasmic structures similar to amylopectin granules distributed throughout the parasite suggests that azasterols might be inducing differentiation of those tachyzoites which were not lysed to the bradyzoite stage.

We also tested novel ester prodrugs of ciprofloxacin, which were synthesized and tested for their antitoxoplasma activity. These new compounds proved to be extremely efficient against the tachyzoite form of these parasites, inhibiting parasite growth with IC50 values at nanomolar range. Kinetic studies

revealed that the novel fluoroquinolones blocked tachyzoites proliferation within 24 h of exposure. After 48 h of exposure the IC50 were found to be lower from those observed at 24 h, suggesting that novel fluoroquinolones exert also a slight “delayed death ” effect in T. gondii parasites.

We also verified an efficient palladium-catalyzed Suzuki–Miyaura cross-coupling protocol effected in a mixture of DME/water (2:1) that enables the reaction of sterically hindered and electron rich 6-chloro or 6-bromo-1,4-benzoxazines(ones) with a variety of aryl, vinyl or alkylboronic acids. Coupling is effected with catalyst loading of 5 mol % using sealed-vessel microwave processing. The resulting compounds exhibit potent activity against Toxoplasma gondii tachyzoite proliferation. Derivatives of benzoxazines inhibited T. gondii tachyzoite proliferation showing IC50 values in the low micromolar range (0.86 and 4.5 mM). The most active compounds were 6-(3-methoxyphenyl)-5,7,8-trimethyl-2-phenyl-3,4-dihydro-2H-1,4-benzoxazine and 6-(4-butylphenyl)-5,7,8-trimethyl-2-phenyl-3,4-dihydro-2H-1,4-benzoxazine with IC50 of 1.21±0.04 mM and 0.86±0.4 mM, respectively, after 72 h.

Nanoencapsulation

An innovative product based on the nanoencapsulation of pyrimethamine (PYR), currently used in the toxoplasmosis chemotherapy, aiming an improvement of drug efficacy was proposed. The in vitro cytotoxicity effect of encapsulated pyrimethamine (PYR) and pyrimethamine - colloidal suspension was concomitantly evaluated against LLC-MK2 lineage and mouse peritoneal macrophage showing that the cells had similar tolerance for both PYR encapsulated or in the aqueous suspension. CF1 mice acutely infected with tachyzoites of Toxoplasma gondii RH strain treated with different doses (5.0-10 mg/kg/day) of PYR-nanocapsules had survival rate higher than the animals treated with the same doses of non-encapsulted PYR. Thus encapsulation of PYR improved the efficacy of this drug against an acute model of toxoplasmosis in mice and can be considered an alternative for reducing the dose of PYR, which, in turn, would also reduce the side effects associated to the treatment.

IV.4. Effects miltefosine on the proliferation, ultrastructure and phospholipid composition of Angomonas deanei (previously named as Crithidia deanei) and its symbiotic bacterium

Miltefosine promoted a low effect on cellular proliferation, when compared to other drugs that also disturb Phosphatidylcholine (PC) biosynthesis pathways. However, miltefosine treated protozoa presented intense ultrastructural alterations as plasma membrane shedding and blebbing, mitochondrial swelling and convolutions of the endosymbiont envelope. The use of 32Pi as tracer revealed that the protozoan phospholipid synthesis was affected by miltefosine since decreased amounts of PC, cardiolipin (CL) and phosphatidylethanolamine (PE) were observed, while phosphatidylinositol (PI) production was unaltered. PI, which participates in signaling mechanisms, may somehow contribute to the

maintenance of satisfactory levels of phospholipids during the miltefosine treatment. Mitochondrion fractions obtained from protozoa treated with miltefosine presented a decrease in PC, PI and CL production, while isolated symbionts showed a diminished synthesis of PC, PE and PI, reinforcing the idea that an intensive metabolic exchange occurs between the host trypanosomatid and structures of symbiotic origin.

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AL 10

ASSOCIATE LABORATORY OF GENOMIC, PROTEOMIC, MODELING AND NANOSCOPY OF BIOLOGICAL SYSTEMS

Coordinator: Paulo Mascarello Bisch – IBCCF/UFRJ.

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Lílian Ayres Sá
Camila Bayer

Collaborators:
Nice Americano
Francisco Lopes

Introduction

The laboratory is devoted to the development of new techniques on large scale and high-trough-put "omics" biological sciences. Covering also molecular modeling and nano-scale biophysics. Most of the effort is focused on the investigation of the control mechanisms of gene expression in bacteria as model organisms. Studding specific target for drug design for infectious and cancer diseases we follow many biomedical applications. Plant–bacteria association and abiotic plant stress are also investigates at our laboratory. In the context of the CENABIO (INBEB) Institute we are mostly responsible for the AFM facility and the relation of this technique with the more general aspects of the structural biology.

CENABIO has recently acquired two atomic force microscopes, one directly bought from Bruker Nanno Inc., an Arizona Corporation and the other by a Loan Agreement dated as of march 2012, also with Bruker Nanno Inc. Both equipments are under the responsibility of AL10.

A) Dimension FastScan Scanning Probe Microscope

Work 10s of times faster with fast scanning rates in air or fluid, automated laser and detector alignment, comprehensive work flow and smart engaging. Built-in measurement automation software ScanAsyst™ provide an easy approach for beginners. TappingMode™ images can be achieved at 20Hz and ScanAsyst images at 6Hz

B) BioScope Catalyst

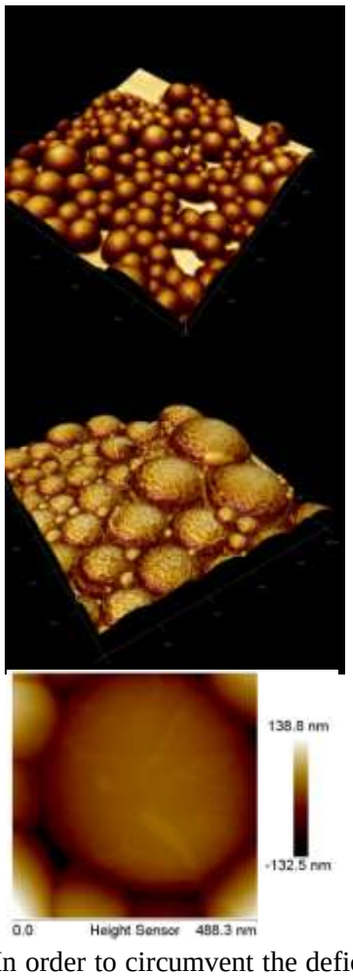
Catalyst system is mounted on a Zeiss Axiovert 200 inverted light microscopy to optimize the measurement of biological samples. Both systems share the same control software and basic capabilities and imaging modes.

Polymeric Nanocapsules Characterization

Nanocapsules are a promising tool with remarkable behavior as they can be pre-programmed to have specific functions according to some special characteristics as small size (from a few to some thousandth of nanometers) and special surface chemistry. Because of those characteristics it is possible to obtain special effects like controlled distribution, specific transportation and controlled activity. This conjunct of characteristics will have a huge impact in all fields of research: basic research, health, industry process and environment.

The nanopharmacology is a growing branch promising properties such as controlled, prolonged and sustained release of the active drug, reduction of the required dose for therapeutic effect and toxic effects and also biotargeting. This type of technology is being highly study for the treatment of cancer, a worldwide concern.

The two types of highest incidence, breast and prostate cancer, lead with high frequency to bone metastasis. Despite having no cure, palliative treatments improve the quality of life and increase survival time. Among them treatment with radiopharmaceuticals stands out. Radiopharmaceuticals consist of a biological vector linked to a radionuclide emitter of beta particles (for treatment) and/or gamma (for diagnosis). The [bis(phosphonomethyl)amino]methylphosphonic acid attached to Samarium-153 (^{153}Sm-EDTMP) is known to have the most clinical benefits, and around 70-80% of patients show a clear improvement. The major disadvantage of this radiopharmaceutical is its superficial acting, which requires, in almost all cases, multiple doses.



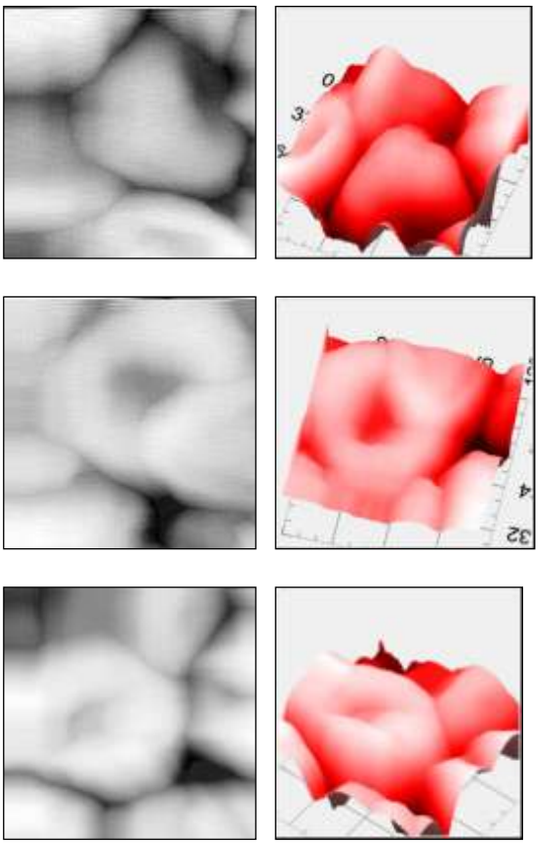
In order to circumvent the deficiencies of the radiopharmaceutical ^{153}Sm-EDTMP and obtain a controlled and sustain release, in this work, polymeric nanocapsules were linked with EDTMP by the double emulsification method. The produced nanoparticles of PLA/PVA/EDTMP were characterized with Atomic Force Microscopy (AFM), which allows the observation of no only the medium size of the nanoparticles (230nm), but also the size dispersion of them (range 100-500nm). Also it was possible to characterize some physical-chemicals properties of the materials that compose this nanoparticles.

Preliminary biodistribution assays were performed with Wistar rats. PLA/PVA/EDTMP nanocapsules were compared with PLA/PVA ones and with an EDTMP solution. All samples were labeled with ^{99m}Tc and images were acquired on a gamma camera. The amount of nanocapsules with EDTMP in bones is the same as unencapsulated EDTMP. However, they remain in larger quantities for a period of 2h30min, suggesting a more prolonged action compared to unencapsulated EDTMP. Nanocapsules of PLA/PVA are not found in the bones, as expected. The results suggest potential benefits for a formulation with nanocapsules.

Fast Scanning AFM – a new perspective for biological imaging

A fast-scanning AFM (FSAFM) prototype was developed, and this new conception allows the acquisition of tridimensional images at higher speeds when compared to any conventional equipment, where now time is the 4th dimension. The FSAFM pototype can produce images up to 256 x 256 pixels, one per second. At this rate, images of *in vitro* biological cell surfaces can be visualized in terms of their dynamic behavior, opening new perspectives for the understanding and characterization of complex mechanisms from the visualization and measurement points of view in micro and nanoscales.

The grayscale images below (with their tridimensional representations beneath) show the sequence of (5 x 5) μm^2 (128 x 128 pixels) images obtained from a healthy red blood cell sample at 1 image/sec, in contact mode.



AFM image restoration using the Tikhonov regularization method implemented in GPU

The Atomic Force Microscope (AFM) is a technology that allows imaging at the nanoscale of almost any surface that includes non-conductive materials such as polymers, ceramics and biological samples. However, the images obtained can exhibit poor signal-to-noise ratios, caused either by the blurring effects of the probe sensor and the mechanical and/or electromagnetic external interferences. In order to treat those images or, at least, minimize the effects of the degradation, it is necessary to use restoration techniques such as mathematical morphology, Fast Fourier Transform (FFT) or deconvolution, This work proposes the use of the Tikhonov regularization method implemented in a Graphic Processing Unit (GPU) platform. A drastic image restoration time was obtained, opening a new perspective for the treatment of images at acquisition time, specially in Fast Scanning AFM experiments.

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AL 11

ASSOCIATE LABORATORY OF MICROSCOPY

Coordinator: Thaïs Cristina B. S. Souto Padrón - IMPPG/UFRJ.

Members:

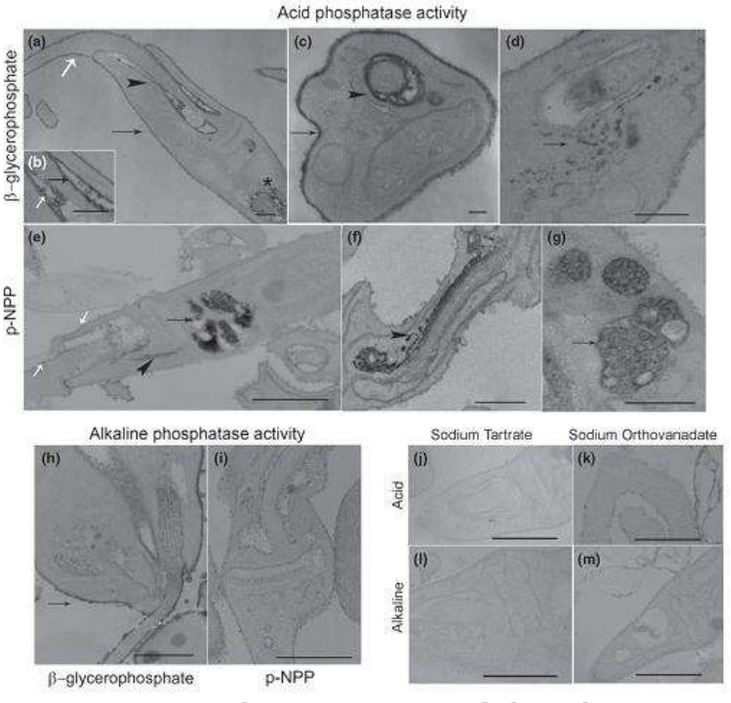
Ulisses Lins - IMPPG/UFRJ.

Our group consists of 2 laboratories involved in structural and cellular biology:

1 - Laboratório de Biologia Celular e Ultraestrutura - coordinated by Dr. Thaïs Souto-Padrón focus its attention to the study of the structural organization of parasitic protozoa such as Trypanosoma cruzi and Leishmania and their interaction with host-cells.

The main subjects of our interest are:

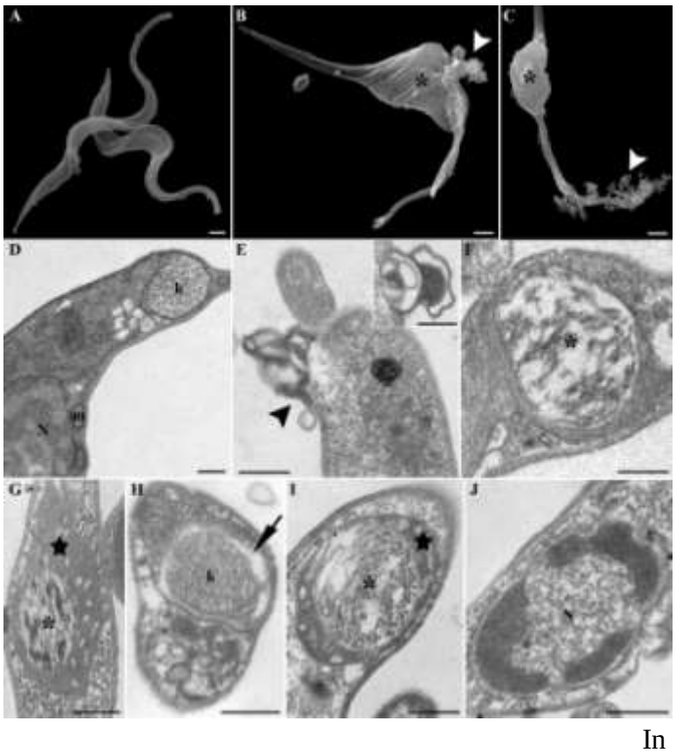
a) Effect of drugs that interfere in the endocytic/exocytic pathways in T. cruzi and Leishmania and their potential effects in the modulation of cell surface molecules. This subject is complemented by the studies of the effect of surface enzymes in parasite-host cell interaction. During the period of the present report we characterize the presence of different secreted phosphatase activities in Leishmania amazonensis, the influence of the substrate in cytochemical labeling and the potential involvement of secreted phosphatase activity in both PV maturation and amastigote survival. This study was accepted for publication in FEMS Micobiology Letters. (Figure 1)



b) Experimental chemotherapy in trypanosomatids. We analyze the effect of natural compounds such as snake and bee venons in the proliferation and ultrastructure in search to define kind of cell death process involved in parasite death. This topic main developed by Camila Marques Adade that presented her PhD theses in July 2010 and continues in the laboratory as a Pos-Doc student. Camila and some graduated students were involved in the study of Appis melifera venom and derived compounds such as melittin. Recently we had a paper accepted for publication in Toxicon (Ref. No.: TOXCON-D-12-00468R1) entitled Melittin peptide kills

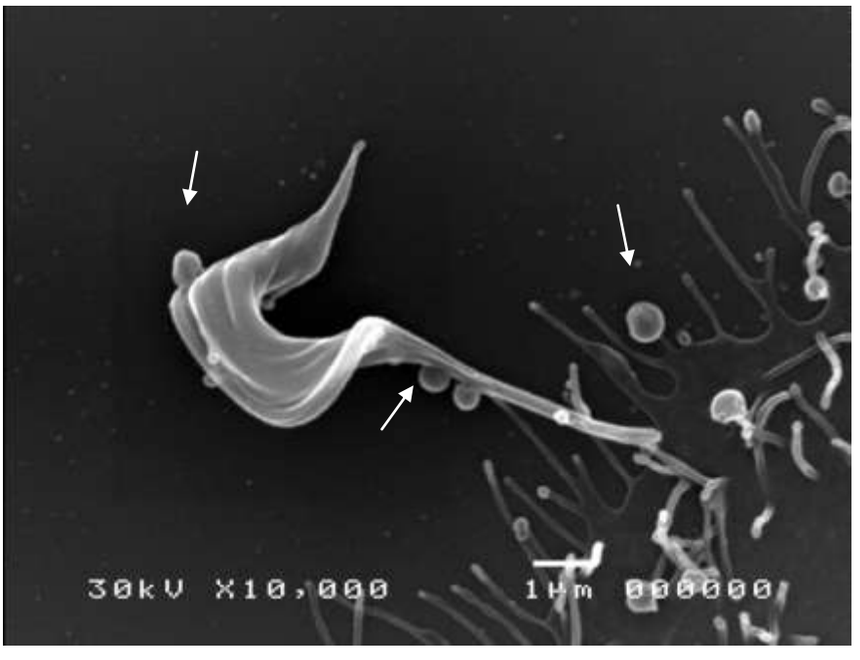
Trypanosoma cruzi parasites by inducing different cell death pathways.

Authors: Camila M Adade, Ph.D.; Isabelle R Oliveira, graduate student; Joana A Pais, Graduate student; Thaïs Cristina Baeta Soares Souto-Padrón (Figure 2).

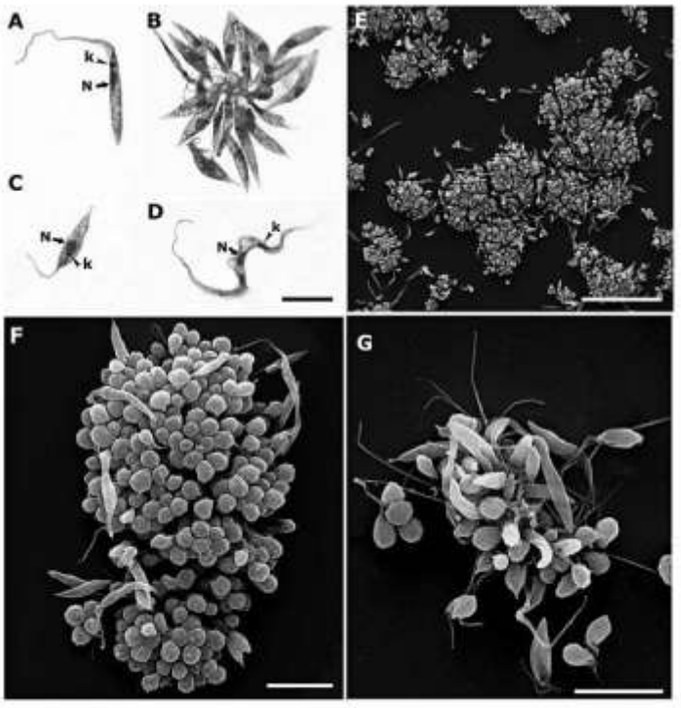


c) The analysis of shedding process in T. cruzi. The ultrastructure and immunocytochemical detection of the components of the shedding vesicles from different strains of the parasite. In this topic we analyze the ultrastructure, composition (presence of proteases and antigens), and signaling pathways involved in the process of shedding of different vesicles by trypomastigote and amastigote forms of T. cruzi. This topic is developed by Roberta Ferreira Cura das Neves, a PhD student. During the period of this report our laboratory and together with Dr. Wanderley de Souza's laboratory entered into a partnership with the Institut Pasteur in Montevideo for the structural and molecular biology analysis of shedding vesicles released by T. cruzi. We had a visit from a PhD student from Urugay, Rosa Maria Gracia-Silva, that worked with Roberta gaving rise to a study that was submitted for publication (Figure 3).

d) Ultrastructural analysis of trypanosomatids isolated from fishes and toads. The student Moara Lemos, presented her PhD thesis in February 2012 and had submitted two studies for publication: the first, titled "Isolation and In Vitro Culture of Trypanosomes From

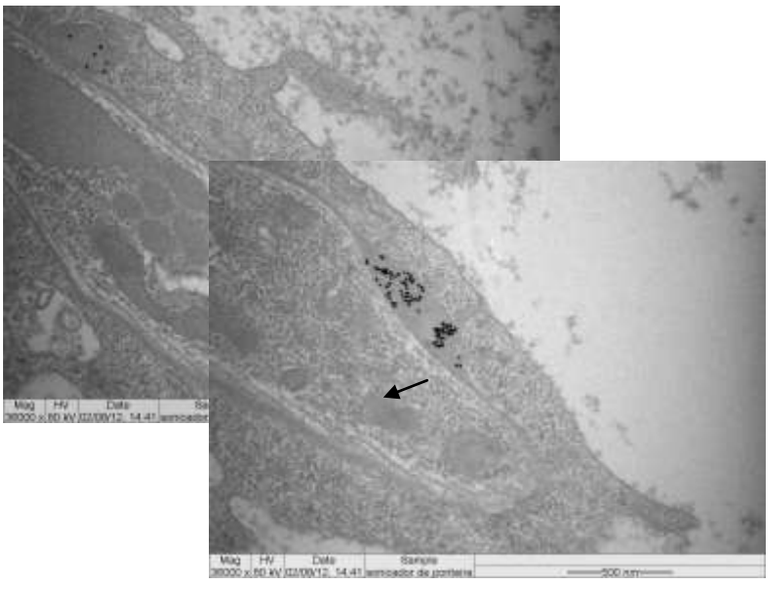


THE FIGURE SHOWS SHEDDING VESICLES BEING RELEASED FROM THE SURFACE OF TRYPOMASTIGOTES OF CLONE CL BRENER. THE SAME VESICLES CAN BE OBSERVED ADHERED TO THE SURFACE OF LLC-MK2 CELLS (WHITE ARROWS).



Leptodactylus ocellatus From the Atlantic Forest in a New Experimental Culture Medium" has been accepted for publication in the Journal of Parasitology. The second study was about the Isolation, ultrastructural characterization and phylogenetic analysis of a new fish trypanosome in Brazilian armored catfishes Hypostomus affines and Hyposotmus luetkeni and are in the process of answering to referees. This is the first study in Brazil to address the morphology, in vitro culture and taxonomy of a fish trypanosome. Moara continues in the laboratory as a Pos-Doc student in the same line of investigation. (Figure 4).

e) Parasite-vector interaction. It was developed in collaboration with Dra. Angela Lopes. Our student Thiago Luis Alves e Silva presented his PhD thesis in August 2012 and recently we submitted a study for publication entitled "The function of phytophagous insect Oncopeltus fasciatus hemocytes in the immune response towards the tomato parasite Phytomonas serpens". In this report, we found that during the infection of O. fasciatus with P.serpens, these parasites multiply in the hemolymph and modify their morphology originating "gigantic" parasites, a common morphotype observed during the life cycle of Phytomonas sp. Additionally, we observed that the infection induces the activation of cellular responses. These responses included an increase in the number of circulating hemocytes (hemocytes present in the hemolymph); the formation of nodules, which trapthe parasites and adhere to insect organs removing parasites from the circulation and phagocytosis (Figure 5).



INSECTS WERE ANESTHETIZED ON ICE AND GOLD-LABELED BSA PARTICLES SOLUTION (10 NM DIAMETER) DILUTED 1:5 IN PBS WAS INJECTED. GOLD-LABELED BSA WAS USED AS A MARKER FOR LYSOSOMES. AFTER 30 MIN, THE INSECTS WERE CHALLENGED WITH *P. SERPENS*. AFTER 2, 6 AND 72 H POST INFECTION THE HEMOLYMPH WAS EXTRACTED AND THE HEMOCYTES WERE THEN COLLECTED VIA CENTRIFUGATION AT 1,500 X *G* AND WASHED TWICE IN PBS. THEN, THE CELLS WERE PROCESSED FOR TRANSMISSION ELECTRON MICROSCOPY. IN SOME SECTIONS, IT WAS POSSIBLE TO OBSERVE GOLD PARTICLES CLOSE TO THE PARASITE INDICATING THAT PRE-LABELED LYSOSOMES FUSED WITH TO THE VACUOLE WHERE PARASITES WERE (BLACK ARROW).

2 - Laboratório de Ultraestrutura e Biologia Celular de Procariotos - coordinated by Dr. Ulysses Lins focus its attention to the study of the biology, diversity and biomineralization in magnetotactic bacteria.

The main subjects of our interest are:

1) Biology and diversity of magnetotactic bacteria.

During the period, we advanced in the description of magnetotactic bacteria in extreme environments. To achieve that goal we analyzed samples collected from sediments with extreme conditions: high temperature, low temperature, high salinity and high sulfur content. At least four new types of magnetotactic bacteria were discovered and their morphological (ultrastructure, mineral analysis of the magnetosomes) and phylogenetic (16S rDNA sequencing) characteristics were described.

2) Cultivation of magnetotactic bacteria.

Magnetotactic bacteria are fastidious microorganisms. But, to advance in the understanding of the cell biology it is mandatory to grow these cells in pure cultures. For that we established a collaboration effort with Professor Dennis Bazylinski from University of Nevada, LV, EUA. Dr. Bazylinski is one of the world leading experts in magnetotactic microorganisms. So far, we have been able to cultivate at least three strains of marine and freshwater magnetotactic bacteria. We isolated the first magnetotactic bacterium capable of biomineralization of greigite (Fe_3S_4) magnetosomes (see Figure below). Also, we are now trying the isolate and grow one strain from sediments collected in Brazil.

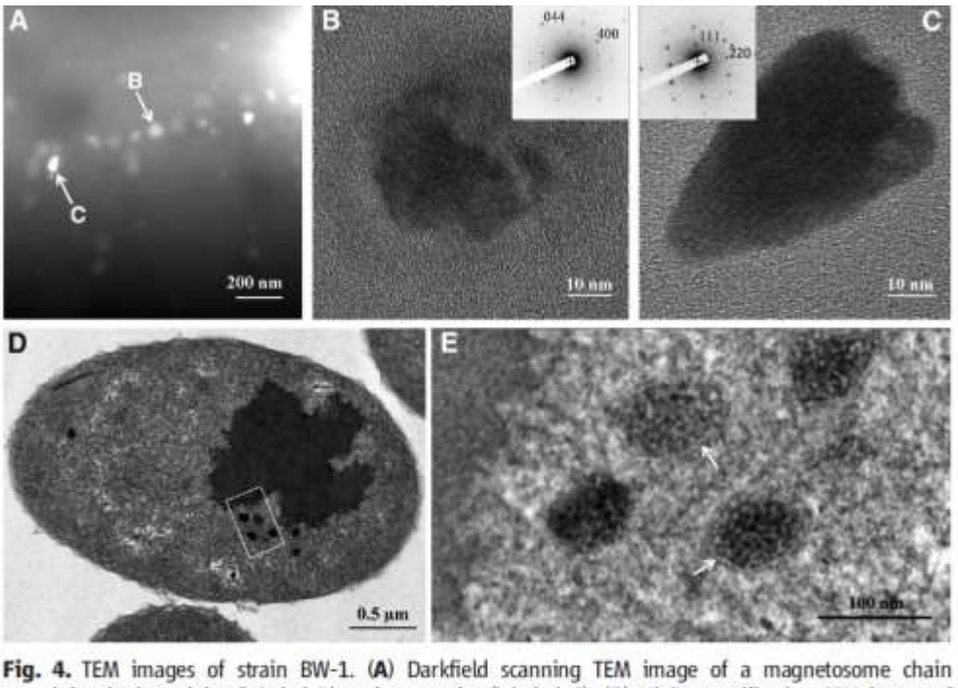


Fig. 4. TEM images of strain BW-1. (**A**) Darkfield scanning TEM image of a magnetosome chain containing both greigite (labeled B) and magnetite (labeled C). (**B**) High-magnification TEM image of greigite crystal labeled in (A). (Inset) SAED of crystal viewed along the [0 −1 1] zone axis. Pattern is consistent with greigite. (**C**) High-magnification TEM image of magnetite crystal labeled in (A). (Inset) SAED pattern of crystal viewed along the [−1 −1 2] zone axis. (**D**) TEM image of a stained thin-section of a cell of BW-1 showing several magnetosomes aligned in the cell. Dark, intracellular, electron-dense mass represents large cell inclusion that is also visible by use of light microscopy. (**E**) High-magnification TEM image of magnetosomes in (D) showing that an electron-dense layer surrounds the greigite crystals, suggesting the presence of a magnetosome membrane.

AL11 publications (2011-2012):

1. Abreu, Fernanda ; Cantão, Mauricio E ; Nicolás, Marisa F ; Barcellos, Fernando G ; Morillo, Viviana ; Almeida, Luiz GP ; do Nascimento, Fabrícia F ; Lefèvre, Christopher T ; Bazylinski, Dennis A ; R de Vasconcelos, Ana Tereza ; Lins, Ulysses . Common ancestry of iron oxide- and iron-sulfide-based biomineralization in magnetotactic bacteria. The ISME Journal (Print), p. 1-7, 2011.

2. Adade CM, Chagas GSF, Souto-Padrón T. Apis mellifera venom induces different cell death pathways in Trypanosoma cruzi. 2012 Parasitology 139: 1444-1461, 2012.

3. Adade CM, Cons BL, Melo PA, Souto-Padrón T. Effect of Crotalus viridis viridis snake venom on the ultrastructure and intracellular survival of Trypanosoma cruzi. Parasitology. 138: 46-58, 2011.

4. Andrade, Leonardo R. ; Lins, Ulysses ; Farina, Marcos ; Kachar, Bechara ; Thalmann, Ruediger . Immunogold TEM of otoconin 90 and otolin relevance to mineralization of otoconia, and pathogenesis of benign positional vertigo. Hearing Research, v. 292, p. 14-25, 2012.

5. De Araujo, Lívia Vieira ; Abreu, Fernanda ; Lins, Ulysses ; Anna, Lídia Maria de Melo Santa ; Nitschke, Márcia ; Freire, Denise Maria Guimarães . Rhamnolipid and surfactin inhibit Listeria monocytogenes adhesion. Food Research International, v. 44, p. 481-488, 2011.

6. Dias FA, Santos ALS, Lery LMS, Oliveira MM, Bisch PM, Saraiva EM, Souto-Padrón T, Lopes AH. Evidence that a laminin-like insect protein mediates early events in the interaction of a phytoparasite with its vector's salivary gland. PLoS One. 7(10):e48170, 2012.

7. Fortes GB, Alves L, Oliveira R, Dutra FF, Rodrigues D, Fernandez PL, Souto-Padrón T, Kelliher M, Chan FKM, Golenbock D, Bozza MT. Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. Blood 119 (10): 2368-2375, 2012.

8. Jovane, L. ; Florindo, F.; Bazylinski, Dennis A ; Lins, U. Prismatic magnetite magnetosomes from cultivated Magnetovibrio blakemorei strain MV-1: a magnetic fingerprint in marine sediments?. Environmental Microbiology Reports, v. 4, 2012.

9. Kalirai, Samanbir S. ; Lam, Karen P. ; Bazylinski, Dennis A. ; Lins, Ulysses ; Hitchcock, Adam P. . Examining the chemistry and magnetism of magnetotactic bacterium Candidatus Magnetovibrio blakemorei strain MV-1 using scanning transmission X-ray microscopy. Chemical Geology, v. 300-301, p. 14-23, 2012.

10. Lefevre, C. T. ; Frankel, R. P. ; Abreu, F. ; Lins, Ulysses ; Bazylinski, D. Culture-independent characterization of a novel, uncultivated magnetotactic member of the Nitrospirae phylum. Environmental Microbiology (Print), v. 13, p. 538-549, 2011.

11. Lefevre, C. T. ; Menguy, N. ; Abreu, F. ; Lins, U. ; Posfai, M. ; Prozorov, T. ; Pignol, D. ; Frankel, R. B. ; Bazylinski, D. A. A Cultured Greigite-Producing Magnetotactic Bacterium in a Novel Group of Sulfate-Reducing Bacteria. Science (New York, N.Y.), v. 334, p. 1720-1723, 2011.

12. Lefevre, C. T. ; Posfai, M. ; Abreu, F. ; Lins, Ulysses ; Frankel, R. P. ; Bazylinski, D. . Morphological features of elongated-anisotropic magnetosome crystals in magnetotactic bacteria of the Nitrospirae phylum and the Deltaproteobacteria class. Earth and Planetary Science Letters, v. 312, p. 194-200, 2011.

13. Marques, Joana Montezano ; Almeida, Fernando Pereira ; Lins, Ulysses ; Seldin, Lucy ; Korenblum, Elisa . Nitrate treatment effects on bacterial community biofilm formed on carbon steel in produced water stirred tank bioreactor. World Journal of Microbiology & Biotechnology, v. 28, p. 2355-2363, 2012.

14. Martins, J. L. ; Silveira, T.S. ; Abreu, Fernanda; Almeida, Fernando Pereira ; Rosado, Alexandre Soares ; Lins, U. . Spatiotemporal distribution of the magnetotactic multicellular prokaryote Candidatus Magnetoglobus multicellularis in a Brazilian hypersaline lagoon and in microcosms. International Microbiology, v. 15, p. 141-149, 2012.

15. Pereira ACTC, Leite FGG, Brasil BSAF, Andrade LG, Pimenta PFP, Souto-Padrón T, Ferreira PCP, Traktman P, Kroon EG, Bonjardim CA. A Vacccinia virus-driven interplay between the MKK4/7-JNK1/2 pathway and cytoskeleton reorganization. The Journal of Virology 86(1):172-184, 2012.

16. Pereira ACTC, Soares-Martins JAP, Leite FGG, Da Cruz AFP, Torres AA, Souto-Padrón T, Kroon EG, Ferreira PCP, Bonjardim CA.SP600125 Inhibits orthopoxviruses replication in a jnk1/2 - independent maner: implication as a potential antipoxviral. Antiviral Research 93(1): 69-77, 2012.

17. Portela M, Das Chagas MS, Cerqueira DF, De Souza IP, Souto-Padrón T, De Araújo Soares R, De Araújo Castro GF. Differential collagenolytic activity of Candida albicans isolated from oral mucosa and dentinal carious lesions of HIV-infected children. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology 113(3):378-383, 2012.

18. Silva ALN, Adade CM, Shoyama FM, Neto CPS, Souto-Padrón T, Almeida MV, Rezende CAM, Silva CV, Souza MA.In vitro leishmanicidal activity of N-dodecyl-1,2-ethanediamine. Biomedicine & Pharmacotherapy, 66(3):180-186, 2012.

19. Sobrinho, Rodrigo Lima ; Lins, Ulysses ; Bernardes, Marcelo Corrêa . Geochemical Characteristics Related to the Gregite-Producing Multicellular Magnetotactic Prokaryote Candidatus Magnetoglobus multicellularis in a Hypersaline Lagoon . Geomicrobiology Journal, v. 28, p. 705-713, 2011.

AL 12

ASSOCIATE LABORATORY OF CELLULAR ULTRASTRUCTURE

COORDINATOR: MARLENE BENCHIMOL – USU.

The main purposes of the project were followed, as shown below:

1) Important new drugs, such as metronidazol (the drug of choice for trichomoniasis), BPQ-OH and mitelfosina were used to compare the behavior of the T. vaginalis under drug treatment and after the interaction with host-cells. In addition, human cells in cultures were also used to see the drugs effects on human cells. HeLa cells and Caco cells were used for comparison. The most significant alterations were (1) membrane blebbing and disruption, (2) cell wrinkling and (3) the formation of cell clusters. In addition, autophagic vacuoles, and smaller hydrogenosomes were also observed. Nonspecific cytotoxicity assays using the cultured mammalian cell lines using the new drugs, but with metronidazole the effects were severe. We have two articles published under submission.

2) We have followed the behavior of pseudocysts, a form of trichomonas, which internalize the flagella under stress conditions. For this, we have obtained several T. foetus strains and tested the cytotoxicity when under interaction with different cells lineages. The results were published in one article where we have shown the high infectivity of the pseudocyst form, which added important new date to literature.

3) We have obtained fresh isolates of T. foetus, from Argentina cattle. We observed that pseudocysts are present in vivo and are very infective. We have published one articles about this e other findings in this matter.

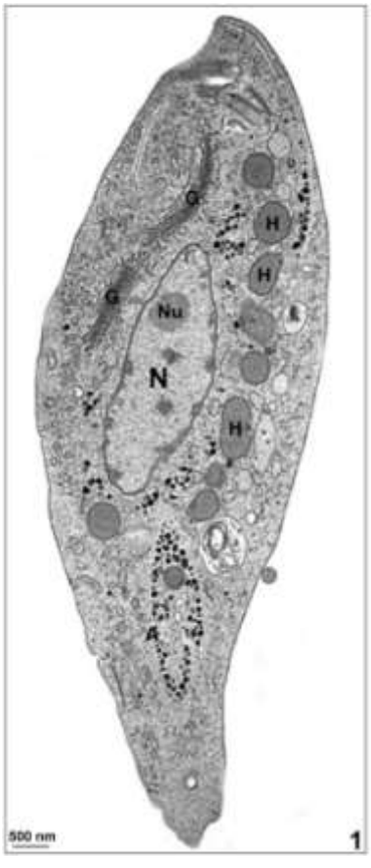


FIGURE 1. A GENERAL VIEW OF *T. FOETUS* IN A ROUTINE LONGITUDINAL THIN SECTION. NOTE THE WELL-DEVELOPED GOLGI (G) WITH SEVERAL BUDDING VESICLES, THE HYDROGENOSOMES (H) AND THE NUCLEUS (N) WITH ITS NUCLEOLUS (NU). BAR, 500 NM.

4) We have removed the plasma membrane of T. foetus and analyzed using new electron microscopy, such as the Magellan and

Quanta. We have discovered a new accessory filament that follows the costa, a periodic structre of this parasite, and we were able for see, the ultrastructural aspects of the bands that form the costa, and their measurements. An article is under submission.

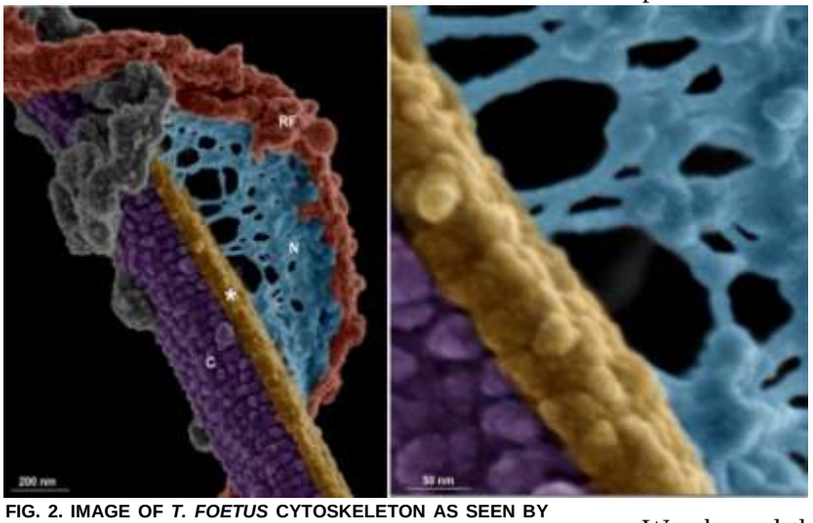


FIG. 2. IMAGE OF *T. FOETUS* CYTOSKELETON AS SEEN BY SCANNING ELECTRON MICROSCOPY OF EXTREME HIGH RESOLUTION (XHR-SEM). A FILAMENTOUS NETWORK IS SEEN LOCATED UNDER THE UNDULATING MEMBRANE, CONNECTING A NEW ACCESSORY FILAMENT OF THE COSTA TO THE RECURRENT FLAGELLUM (A). AT HIGHER MAGNIFICATION IS POSSIBLE TO OBSERVE FILAMENTOUS AND GLOBULAR STRUCTURES FORMING THIS NETWORK (B).

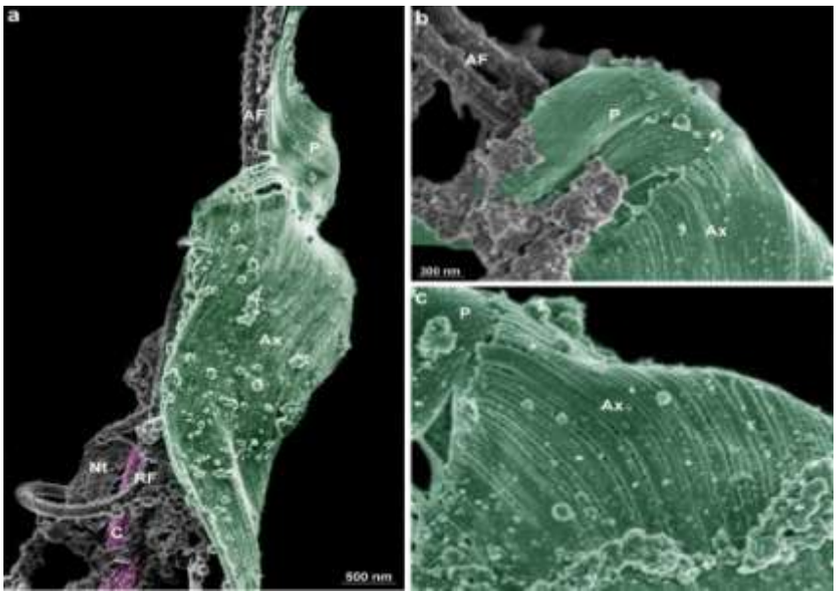


FIG. 3. SCANNING ELECTRON MICROSCOPY OF EXTREME HIGH RESOLUTION (XHR-SEM) OF THE PELTA-AXOSTYLAR SYSTEM OF THE PARASITE *T. FOETUS*. (A) AN OVERVIEW OF THE PELTA (P) THAT IS LOCATED ON ANTERIOR REGION, SUPPORTING THE FLAGELLAR CANAL FOR THE ANTERIOR FLAGELLA (AF) EXIT AND THE AXOSTYLE (AX). A FILAMENTOUS NETWORK (NT) IS SEEN CONNECTING THE COSTA (C) TO THE RECURRENT FLAGELLUM (RF)(B-C) THE PELTA-AXOSTYLAR JUNCTION. NOTE THAT EACH MICROTUBULE IS CLEARLY VISUALIZED AND THEY ARE ORGANIZED IN TWO DISTINCT GROUPS.

5) We have performed cell fractionation to obtain isolated organelles from T. foetus. The aim was to obtain a pure fraction of the costa of trichomonas, and visualize by electron microscopy and perform proteomics. One article is in preparation, showing the specific proteins found in the costa of trichomonas.

6)We have tested if trichomonads are species-specific, because the literature believed that they are. Thus, we incubated T. vaginalis with cells of different animals and T. foetus with human cells. We showed that the parasites are not species-specific, contrary what was previously published in the literature. We have published um paper about this.

7)Concerning Giardia lamblia we have obtained a major advance on the process of encystation in vitro. We have used immunocytochemistry to compare the behavior of the encystation specific vesicles (ESV) and we were able to find the granules responsible for the carbohydrates portion of the cyst wall. Using immunofluorescence we detected two granules populations, one of them, which is a new organelle. We have published one paper that received good compliments from the referees.

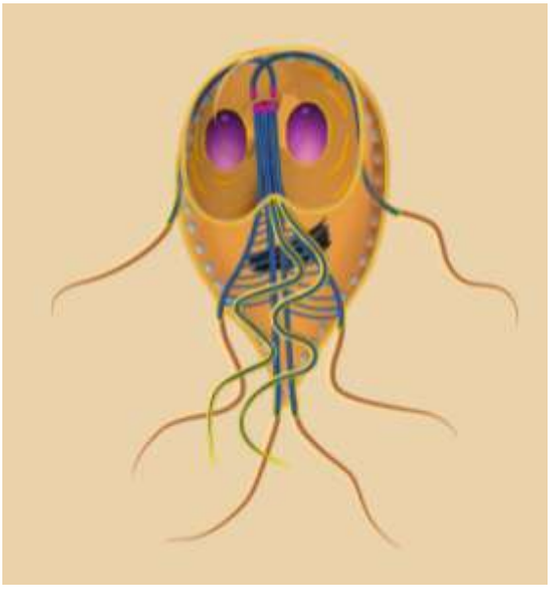


FIGURE 4. SCHEME OF *GIARDIA LAMBLIA* SHOWING THE EIGHT FLAGELLA, TWO NUCLEI, THE VENTRAL DISC AND MEDIAN BODIES.

8) In a report with an international collaboration we have used specific antibodies to follow the behavior of legumain in T. vaginalis, using recombinant molecules. We have published a paper about these results.

9) In a report with an international collaboration we have used tracers to detect new proteins in the cytoskeleton of T. vaginalis. An article has been published about this result.

10) We also demonstrated that IL-10 releases by bovine epithelial cells when cultured with Trichomonas vaginalis and Tritrichomonas foetus and an article has been published.

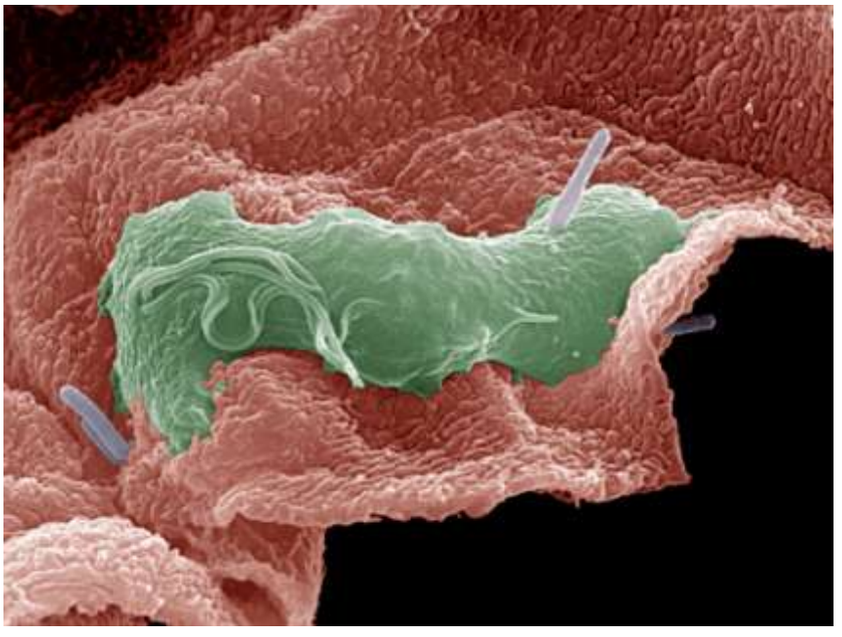


FIGURE 6. OVERVIEW OF ONE *T. VAGINALIS* (GREEN) INFECTING ONE VAGINAL EPITHELIAL CELL (RED). NOTE THAT THE PARASITE INSERT CELL EXTENSIONS IN THE CELL BODY OF THE HOST. BACTERIA ARE SEEN IN BLUE.

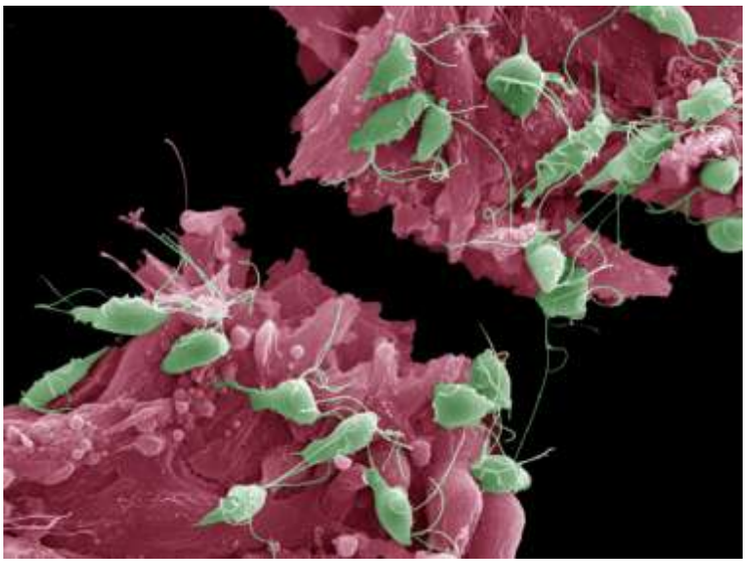


FIGURE 7. INTERACTION OF SEVERAL CATTLE PARASITES (*TRITRICHOMONAS FOETUS* IN GREEN COLOR) DURING INTERACTION WITH VAGINAL EPITHELIAL CELLS (RED).

AL12 publications (2011-2012):

1) NEVES NETO, A. P., BENCHIMOL, M. Cytotoxicity by *Tritrichomonas foetus* pseudocysts. Protist (Jena. Print). , v.10.101, p.1 - 10, 2011.

2) PEREIRA NETO, A., CAMPERO, C., Alfredo Martínez, BENCHIMOL, M. Identification of *Tritrichomonas foetus* pseudocysts in fresh preputial secretion samples from bulls. Veterinary Parasitology (Print). , v.175, p.1 - 8, 2011.

3) VILELA, R., BENCHIMOL, M.

Trichomonas vaginalis and *Tritrichomonas foetus* with keratin: an important relationship. Memórias do Instituto Oswaldo Cruz (Impresso). , v.106, p.701 - 704, 2011.

4) ROSA, I., Rocha D., DESOUZA, W., Júlio Urbina, BENCHIMOL, M. Ultrastructural alterations induced by Sterol methyltransferase inhibitors on *Trichomonas vaginalis*. FEMS Microbiology Letters. , v.315, p.72 - 78, 2011.

5) Midlej V, PEREIRA NETO, A., BENCHIMOL, M. Ultrastructural features of *Tritrichomonas mobilensis* and comparison with *Tritrichomonas foetus*. Veterinary Parasitology (Print). , v.182, p.171 - 180, 2011.

6)Neves Neto, AP, Nascimento LF (IC) BENCHIMOL, M. Cytotoxic effects exerted by *Tritrichomonas foetus* pseudocysts. Protist (Jena. Print).  , v.163, p.529 - 543, 2012.

7)Vilela, R. BENCHIMOL, M. IL-10 release by bovine epithelial cells cultured with *Trichomonas vaginalis* and *Tritrichomonas foetus*. Memórias do Instituto Oswaldo Cruz (Impresso), 2012.

8) Jorge González, Christian Muñoz, Mauricio Pérez, Patricio R. Orrego, Luis Osorio, Bessy Gutiérrez, Hernán Sagua, Juan L. Castillo, Jose Martínez-Oyanedel, ARROYO, R., Jose Franco da Silveira, Midlej V, BENCHIMOL, M., Esteban Cordero, Patricio Morales. A protein phosphatase 1 gamma(PP1γ) of the human protozoan parasite *Trichomonas vaginalis* is involved in proliferation and cell attachment to the host cell.. International Journal for Parasitology, v.42, p.715 - 727, 2012.

9) D. Esdras Teixeira (PG), Crepaldi P. H., BENCHIMOL, M., DESOUZA, W. Interactive multimedia to teach the life cycle of *T. cruzi*, the causative agent of Chagas disease. Plos Neglected Tropical Diseases. , v.12, p.DOI: 10.1371/j - , 2012.

10) RENDON-GANDARILLA, F. J., RAMON-LUING, L. L. A., ORTEGA-LOPEZ, J., ARROYO, R., ROSA, I. (PG), BENCHIMOL, M. The TvLEGU-1, a Legumain-Like Cysteine Proteinase, Plays a Key Role in *Trichomonas vaginalis* Cytoadherence. Journal of Biomedicine and Biotechnology (Online). , v.2012, p.ID 561979 - , 2012.

11)VILELA, R. (PG), BENCHIMOL, M. *Trichomonas vaginalis* and *Tritrichomonas foetus*: interaction with fibroblasts and muscle cells - new insights in host cell parasite cytotoxicity. Memórias do Instituto Oswaldo Cruz (Impresso).  , v.107, p.1 - 8, 2012.

12) Midlej, V. Isadora Peixoto Meinig (IC), DESOUZA, W., BENCHIMOL, M.A new set of carbohydrate-positive vesicles in encysting Giardia lamblia. Protist (Jena. Print), 2013 Protist. 2012 Jul;163(4):529-43. doi: 10.1016/j.protis.2011.11.005. Epub 2012 Feb 5.

Books and Book Chapters:

1) Book Chapter entitled: *Trichomonas vaginalis* – in the book entitled Protozoologia Médica- *Wanderley de Souza (editor)- Editora Rubio- chapter 19. Rio de Janeiro-RJ (in press).*

2) *A*tlas on the Structural Organization of Human Pathogenic Protozoa (E-Book)- Wanderley de Souza (editor).

3) Book Chapter entitled: *Trichomonas vaginalis* - Bentham Books – Paquistão (in Press)

4) Book Chapter entitled: “Hydrogenosome” in the book entitled 'Advances in Photosynthesis and Respiration' book series. Organized by Martin F. Hohmann-Marriott. Publisher: Springer, Dordrecht, The Netherlands

5) Book: Atlas entitled The Life cycle of *Trypanosoma cruzi*. Rio de Janeiro : F. Cecierj, 2011, v.1. p.50. D. Esdras Teixeira, BENCHIMOL, M., Crepaldi P. H., DESOUZA, W.

AL 13

ASSOCIATE LABORATORY OF STRUCTURAL BIOTHECNOLOGY

Coordinator: Celso B. Sant'Anna Filho – INMETRO.

Members:

Ana Paula Gadelha – INMETRO.
Danielle Pereira Cavalcanti – INMETRO.
Emile Santos Barrias – INMETRO.
Rafael de Cássio Bernardi – INMETRO.
Roberta Fernandes Pinto – INMETRO.

The Structural Biology Laboratory, at National Institute of Metrology, Quality and Technology– INMETRO, has a group composed by 8 researchers, 8 technicians, 2 graduate students and 10 undergraduate students, which include biologists and physicists. The focus of our group is mainly based on bionanometrological and biofuels studies, as described below.

Renewable energy sources are developed worldwide, owing to high oil prices and to limit greenhouse gas emissions. Currently, there are many international efforts aimed to finding renewable, sustainable, and environment friendly energy sources to overcome these problems. Biofuels (bioethanol and biodiesel) have attracted considerable attention during the past decade as renewable source fuel with environmental benefits. However, concerns exist about the source of feedstocks, including the impact it may have on biodiversity and land use and competition with food crops. As bioethanol is considered a viable energy source for the future, it is expected to form a sustainable basis, meet socio-economic concerns, providing greater security for energy supply and reduce the environmental impacts associated with fossil fuels.

Plant cell wall (PCW) is a high complex structure mainly composed of polysaccharides (cellulose and hemicelluloses) and lignin. Lignocellulosic biomass, including sugarcane, has been considered as potential source to second generation biofuel production. The technology used to conversion of fermentable sugar in bioethanol involves pretreatment, which aim to improve digestibility of biomass, such as acidic and thermal degradation. The PCW molecular architecture remains unclear and it has been related to recalcitrance of biomass to deconstruction. We have applied high resolution microscopy methodologies to have a detailed analysis of sugarcane cell wall architecture, as well as to analyze its deconstruction after pretreatments, focusing on the effect in lignin (Figure 1). Also, using 1D and 2D-PAGE and mass spectrometry, we have also analyzed the protein of PCW sugarcane, to get some information for improving the process of ethanol saccharification (Figure 1D).

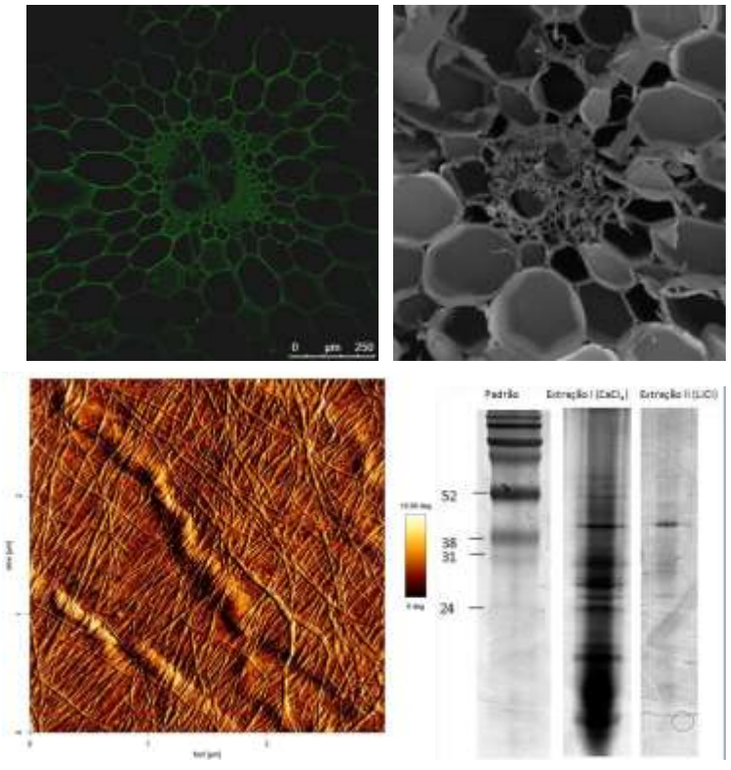


FIGURE 1. SUGARCANE CELL WALL OBSERVED BY DIFFERENT TECHNIQUES. (A) CONFOCAL LASER SCANNING MICROGRAPH; (B) SCANNING ELECTRON MICROGRAPH; (C) ERROR SIGNAL OF ATOMIC FORCE MICROSCOPY. (D) 1D-PAGE OF PROTEINS OF SUGARCANE CELL WALL.

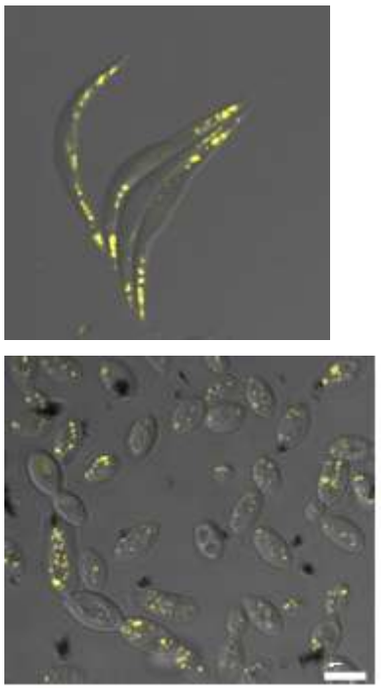


FIGURE 2. CONFOCAL LASER SCANNING MICROGRAPHS OF MICROALGAE *ANKISTRODESMUS* (A) AND YEAST (B). NILE RED STAINING SHOWS THE LIPID BODIES (YELLOW) INSIDE THESE MICRORGANISMS.

The feedstocks usually adopted for biodiesel production are vegetable oils. Recently, much attention has been paid to the exploration of microbial oils. The oil productivity of microorganisms greatly exceeds the vegetable productivity and non-arable land is used for production. We isolated some oleaginous yeasts and microalgaes and analyze the oil content using Nile Red staining and Confocal Laser Scanning Microscopy (Figure 2).

Other focus of our work is the study of the protozoa members of the Trypanosomatidae family, which are characterized by the presence of specific and unique structures that are involved in different cell activities. The mitochondrial or kinetoplast DNA (kDNA), the flagellum and the Paraflagellar Rod (PFR), a complex array of filaments connected to the flagellar axoneme, are examples of these structures.

To understand the detailed structural organization of the kinetoplast DNA, we developed a procedure to analyze intact isolated kDNA networks of trypanosomatids using Atomic Force Microscopy (AFM). This analysis allows the examination of kDNA at high resolution, enabling us to identify regions of overlapping kDNA molecules and sites where several molecules cross forming rosettes at the kDNA periphery (Figure 3). Currently, our group has used the AFM to study the action of DNA intercalating drugs on the kDNA structure.

The role performed by the PFR is not well established. In order to obtain detailed

information about the PFR structure during flagellar beating, we used atomic force microscopy (Figure 4a-b) and transmission electron microscopy analysis of replicas obtained by quick-freezing, freeze-fracture and deep-etching technique (Figure 4c). The images obtained show that the PFR of Trypanosoma cruzi is not a fixed and static structure. Measurements of the distances between the PFR filaments and the filaments that connect the PFR to the axoneme, as well the angles between the intercrossed filaments supporting this idea. Based on the information obtained and using graphic computation, we proposed an animated model for the PFR structure during flagellar beating, providing a new way to observe the PFR filaments during this process. A stationary frame of the flagellum in straight state can observed in Figure 4d.

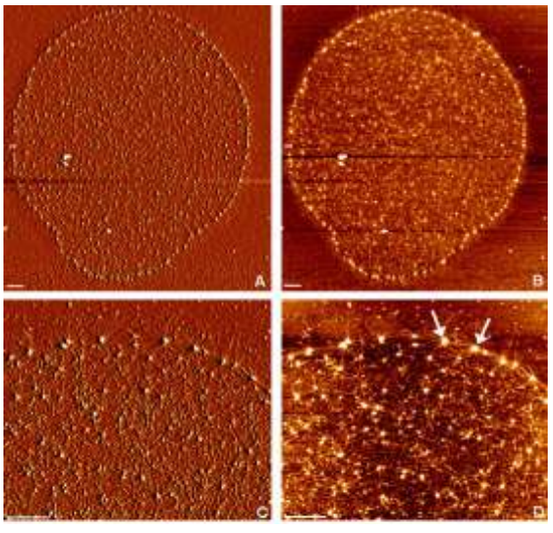


FIGURE 3: AFM ANALYSIS OF ISOLATED KDNA NETWORK OF TRYPANOSOMATID *CRITHIDIA FASCICULATA*. THE ENTIRE NETWORK IS SHOWN IN (A) AND (B) AND DETAILS OF THIS STRUCTURE IS OBSERVED IN (C) AND (D). CLUSTERING OF DNA MOLECULES FORM ROSETTES AROUND ALL NETWORK (ARROWS). (A) AND (C) AMPLITUDE SIGNAL AND (B) AND (D) TOPOGRAPHY SIGNAL IMAGES. BARS = 0.5 µM (A, B) AND 0.25 µM (C–D).

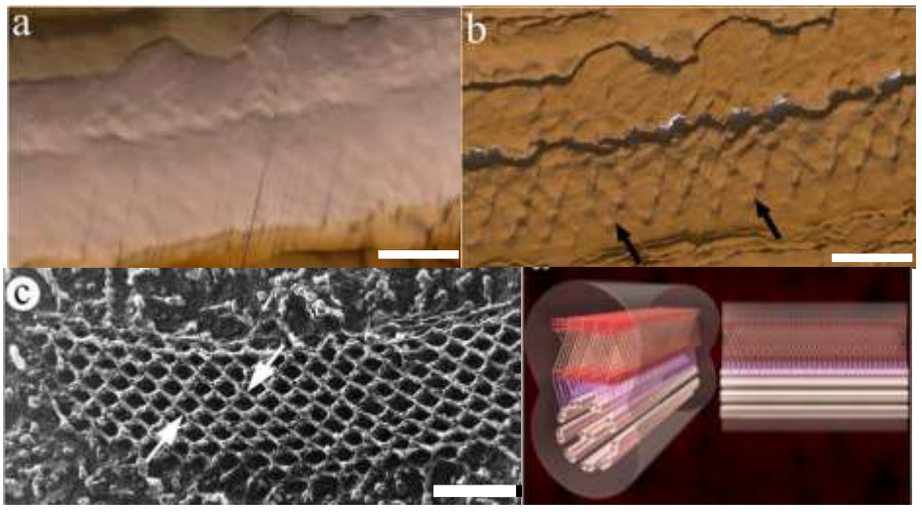


FIGURE 4. (A-B) AFM INTERMITTENT CONTACT MODE IMAGE OF A STRAIGHT STATE FLAGELLUM OF *TRYPANOSOMA CRUZI*. (A) TOPOGRAPHIC 3D VIEW OF PART OF THE FLAGELLUM. (B) PHASE IMAGE OF THE FLAGELLUM SHOWING THE LATTICE ORGANIZATION OF THE FILAMENTS OF THE PFR (ARROWS). (C) DEEP-ETCHING REPLICA IMAGE OF PFR FILAMENTS (ARROWS) SHOWING A LONGITUDINAL FRACTURE OF THE INTERMEDIATE DOMAIN. (D) FRAME VIEW OF PFR ANIMATION DURING FLAGELLAR BEATING. IN THIS STRAIGHT STATE, THE INTERCROSSED FILAMENTS REVEAL A REGULAR DIAMOND STRUCTURE. AXONEME – LIGHT PINK; FILAMENTS THAT LINK THE PFR TO THE AXONEME – PURPLE; PROXIMAL AND DISTAL DOMAINS OF THE PFR – RED; AND, THE INTERMEDIATE DOMAIN – SALMON. BARS – A-B – 200 NM; C – 250 NM.

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4. Freitas-Junior, Paulo Roberto Gonçalves, Catta-Preta, Carolina Moura Costa, Andrade, Iamara da Silva, Cavalcanti, Danielle Pereira, Souza, Wanderley, Einicker-Lamas, Marcelo, Motta, Maria Cristina M. Effects of miltefosine on the proliferation, ultrastructure and phospholipid composition of Angomonas deanei, a trypanosomatid protozoan that harbors a symbiotic bacterium. FEMS Microbiology Letters. , v.333, p.129 - 137, 2012.

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6- Veiga-Santos, P.; Barrias, E.S.; Santos, J.F.C.; De Barros Moreira, T.L.; Carvalho, T.M.U. Urbina, J. A.; De Souza, W. Effects of amiodarone and posaconazole on the growth and ultrastructure of Trypanosoma cruzi. International Journal of Antimicrobial Agents (Print) , v. 39, 2012.

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12. Zuma, Aline Araujo, Cavalcanti, Danielle Pereira, Maia, Marina C.P., de Souza, Wanderley, Motta, Maria Cristina M. Effect of topoisomerase inhibitors and DNA-binding drugs on the cell proliferation and ultrastructure of Trypanosoma cruzi. International Journal of Antimicrobial Agents (Print). , v.37, p.449 - 456, 2011.

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AL 14

ASSOCIATE LABORATORY OF STRUCTURAL BIOLOGY

COORDINATOR: EDILENE OLIVEIRA DA SILVA – UFPA.

Immunomodulatory activity induced by Bioproducts in vitro

One of the main research projects is focuses on the immunomodulatory activity in vitro based on bioproducts research from Amazon´s biodiversity. The monocytes are mononuclear phagocytes originate from bone marrow, presents in the peripheral blood and are able to differentiate into macrophages and dendritic cells. The differentiation process started when monocytes migrates from blood circulation to surrounding tissues. During the migration process, occurs a release of many citokines, as M-CSF and IL-6 that help in the differentiation process. In vitro, this process happens after five days in culture environment, even without any stimulating factors.

Macrophages play specific role during inflammatory processes, which is essential for the innate response to the clearance of the microorganisms. During the process of differentiation the monocytes suffered several morphological changes. Blood monocytes have lower number of philopodium, small cytoplasm area, less Golgi complex and endoplasmatic reticulum.

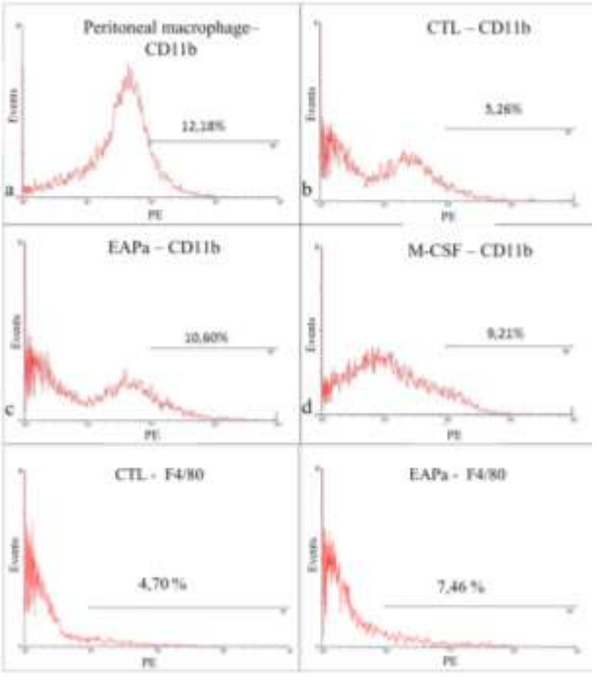

FIGURE 1: DETECTION OF THE SURFACE MARKERS CD11B AND F4/80 BY FLOW CYTOMETRY IN BONE MARROW CELLS MAINTAINED IN CULTURE FOR 96 HOURS. (A) PERITONEAL MACROPHAGES LABELED WITH CD11B (MACROPHAGES), POSITIVE CONTROL. B) CONTROL NON TREATED. C) BONE MARROW CELLS TREATED WITH 100 MG/ML OF EAPA. (D) BONE MARROW CELLS TREATED WITH M-CSF. (E) CONTROL NON-TREATED LABELED WITH F4/80. (F) CELLS TREATED WITH EAPA AND MARKED WITH F4/80. WE OBSERVED A SIGNIFICANT INCREASE IN POSITIVE MARKING FOR F4/80 AND CD11B IN CELLS TREATED WITH EAPA COMPARED TO CONTROL NON-TREATED. ANOVA WAS USED, STUDENT T. P <0.05.

However, cells in the process of differentiation or differentiated cells (macrophages) presented many cytoplasm projections that increased the area, with higher spreading ability and a lot of Golgi complex and endoplasmatic reticulum characteristic of increasing in the protein synthesis. Furthermore, macrophages derived of monocytes express some surface molecules that characterize macrophage differentiation,

as EMR1-F4/80, CD11b and CD80. Natural products from plants and microorganisms represent an important alternative source of new immunomodulators agents. Physalis angulata is an annual herb distributed in tropical and subtropical regions of the world.

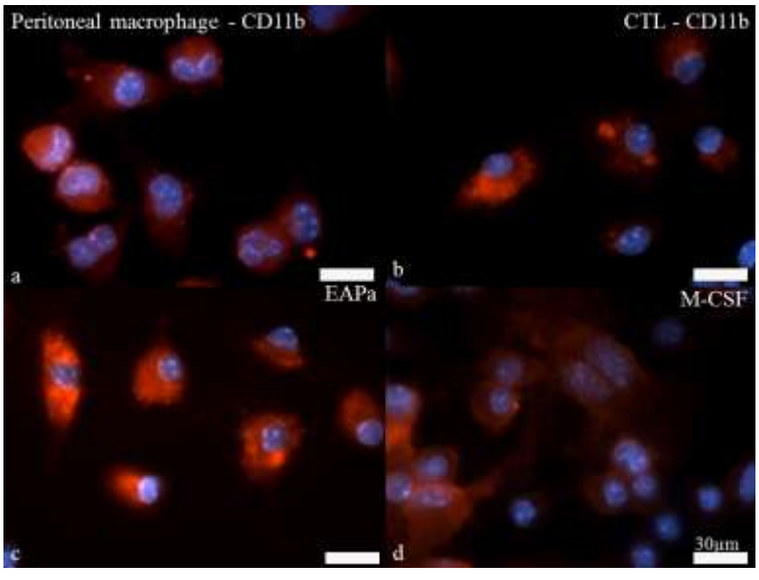

FIGURE 2: DETECTION OF THE SURFACE MARKER CD11B IN BONE MARROW CELLS MAINTAINED IN CULTURE AND TREATED WITH 100 MG/ML OF EAPA FOR 96 HOURS. (A) PERITONEAL MACROPHAGES, AS POSITIVE CONTROL (B) CONTROL NON-TREATED (C) BONE MARROW CELLS TREATED WITH 100 MG/ML OF EAPA (D) BONE MARROW CELLS TREATED WITH M-CSF. NOTE THAT EAPA TREATED CELLS MARKING POSITIVE WITH CD11B PRESENT.

In Amazonia, is popularly known as "camapu". Extracts from this plant is widely used in popular medicine as analgesic, antirheumatic, antinociceptive and anti-inflammatory. In addition, P. angulata has compounds called physalins, which showed antileishmanial activity in vitro and in vivo for cutaneous leishmaniasis.

Thus, we consider interesting to analyze the effects of the aqueous extract obtained from roots of P. angulata on host cell. For this we used monocytes obtained from murine bone marrow. We have recently demonstrated that aqueous extract obtained from P. angulata (EAPa) effectively stimulates the process of cell adhesion, also were observed on treated cell as the increase of cytoplasm and spreading ability, cytoskeleton alterations and high number of cytoplasmatic projections.

Furthermore, EAPa it not promotes the proliferation of lymphocytes and polymorphonuclear leukocytes, nor the increased number of activated macrophages and resident along the culture time. In immunophenotyping performed by immunofluorescence and flow cytometry, labeling CD11b and F4/80, a marker specific for mononuclear phagocytes revealed that EAPa seems to stimulate the differentiation of bone marrow cells in macrophages (Figure 1 - 2).

In immunophenotyping using the CD11c marker, specific of dendritic cells, showed that EAPa did not stimulate differentiation of the treated cells into dendritic cells. No cytotoxic effect was observed in cells treated with EAPa when compared to the untreated control. Thereby, these results demonstrate that EAPa can promote the differentiation of bone marrow cells into macrophages with activation in just 96 hours of culture and can be used as an immunomodulator agent.

Other bioproduct analyzed is the 5-hydroxy-2-hydroxymethyl-γ-pyrone (HMP), which is produced by some species of Aspergillus fungi, has bacteriostatic activity and inhibition effect of the tyrosinase enzyme in the process of melanin biosynthesis. However, relatively little is known about their role on immune cells.

Thus, the aim of this study was to evaluate the effects of HMP on the cell viability and differentiation of human blood monocytes in vitro. Human peripheral leucocytes were obtained from blood bag donated from Fundation Hemocenter of Para State. Cell isolation was performed using HISTOPAQUE® 1077-density-gradient. Monocytes were treated for 24, 48 and 72 hours with 50 and 100 μg/mL of HMP. The ultrastructural analysis of treated monocytes showed spreading ability, high number of cytoplasmatic projections and vacuoles, features that are often observed in activating cells (Figure 3).

Immunofluorescence analysis of the expression of surface protein specific for the macrophage (F4/80), demonstrated that human monocytes treated with 50 and 100 μg/mL for 48 and 72 h showed the similar pattern of expression of proteins to that of human monocytes differentiated by macrophage colony-stimulating factor (M-CFS). The viability test used showed that HMP has no citotoxicity effect on human monocytes when treated with 50 and 100 μg/mL of HMP.

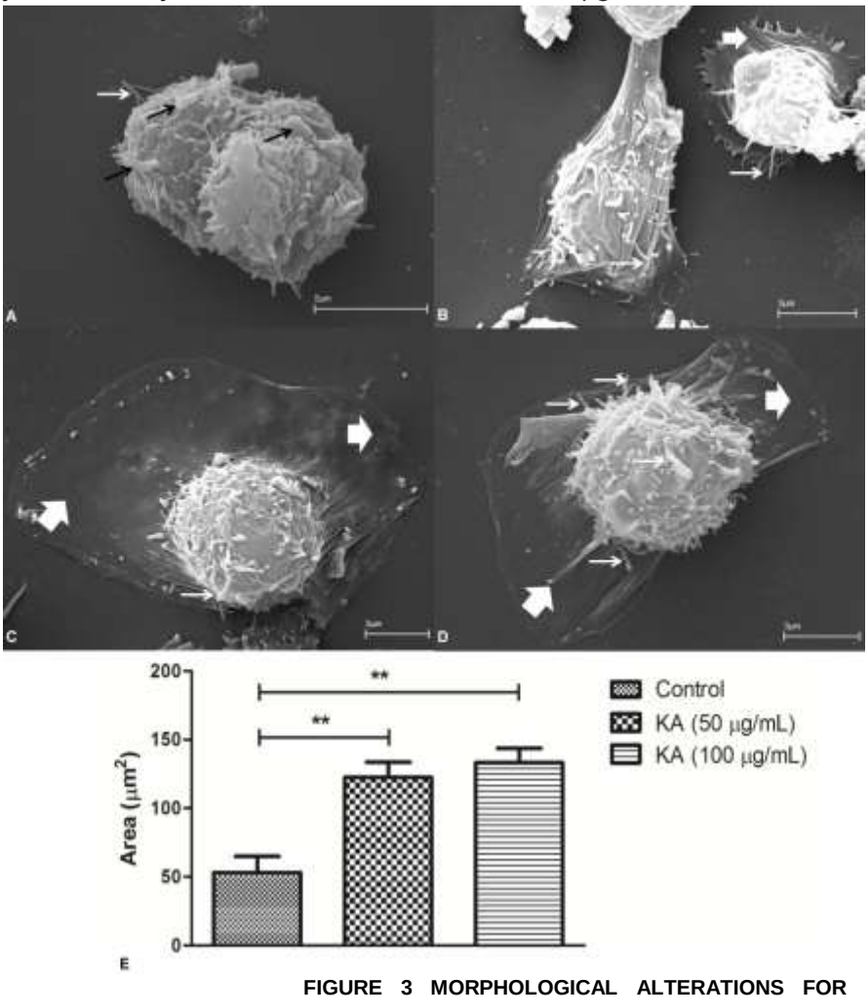

FIGURE 3 MORPHOLOGICAL ALTERATIONS FOR SEM IN HUMAN BLOOD MONOCYTES EXPOSED TO 50 AND 100 μG/ML HMP FOR 48 H. (A) CONTROL MONOCYTE CULTURED FOR 48 H WITHOUT ANY CYTOKINES. SOME TYPICAL MONOCYTE FEATURES CAN BE SEEN, NAMELY: A FEW MICROVILLOUS PROJECTIONS (WHITE ARROWS) WITH RUFFLES AND BLEBS OF THE CELL MEMBRANE CLOSE TO THE CELL BODY (BLACK ARROWS). IN GENERAL, THE CELL MAINTAINED THE SHAPE AND CHARACTERISTICS FOUND IN MONOCYTES. (B) MACROPHAGES TREATED WITH M-CSF (50 NG/ML) TO INDUCE DIFFERENTIATION. (C, D) TREATED CELLS WITH CYTOPLASMIC PROJECTIONS (WHITE ARROWS THIN) AND INCREASED CYTOPLASM AND SPREADING ABILITY IN COMPARISON WITH UNTREATED CELLS (WHITE ARROWS WIDE). (E) INCREASE IN MONOCYTE CTOPLASM AREA TREATED USING MORPHOMETRY. ANOVA WAS USED, STUDENT T. P <0.05.

These results demonstrate a new role for HMP as an immunomodulator agent, inducing the differentiation of monocytes into

macrophages. In addition, we analyzed the effects of HMP on the cell viability and activation of human blood neutrophils in vitro. Neutrophils are phagocytic cells from the innate immune system with developed mechanisms for intracellular digestion of phatogens, immune complexes and cell debris.

The search for drugs at low doses, with low cytotoxicity, which stimulate microbicidal response of phagocytic cells and that, is still capable of destroying an intracellular microorganism is constant. Thus, neutrophils were treated for 1 hour with 50 and 100 µg/mL of HMP. Cytometric analysis was executed for measurement of viability of neutrophils by JC-1 and IP.

In addition, the viability test using colorimetric method and neutral red showed that HMP has no citotoxicity effect on human neutrophils when treated with 50 and 100 µg/mL of HMP. The morphological analysis by optical microscopy and scanning electron microscopy of treated neutrophils showed extensive lamellipodia formation, pseudopodia extention, high spreading ability and increase of cell volume in comparison with untreated cells (Figure 4).

These results demonstrate that HMP promoted several morphological changes that suggest cell activation without cell injury. Thus, both HMP and the extract from Physalis angulata root could be useful as alternative agents to induce monocytes differentiation and neutrophils activation, indicating a possible potential role modulating innate immunity and to be effective during infections with pathogens.

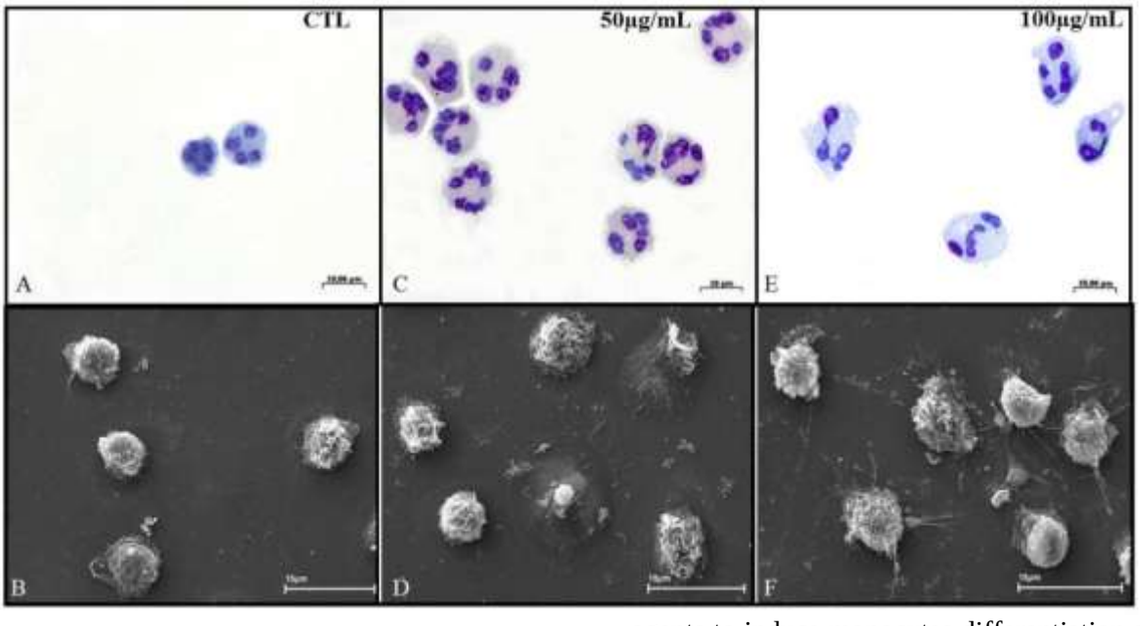

FIGURE 4: THE MORPHOLOGICAL ANALYSIS BY OPTICAL MICROSCOPY AND SCANNING ELECTRON MICROSCOPY. (A-B) CONTROL CELLS WITH TYPICAL MORPHOLOGY (C-D) NEUTROPHILS TREATED WITH 50 µG/ML OF HMP. (E-F) NEUTROPHILS TREATED WITH 100 µG/ML OF HMP. NOTE TREATED CELLS WITH EXTENSIVE LAMELLIPODIA FORMATION, PSEUDOPODIA EXTENTION AND INCREASE OF CELL VOLUME.

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2. Oliveira, DMS; Saraiva, EM; Ishikawa, EAY; Sousa, AAA; **Silva, EO**; Da Silva, I Distribution of phlebotomine fauna (Diptera:Psychodidae) across an urban-rural gradient in an area of endemic visceral leishmaniasis in northern Brazil. Memórias do Instituto Oswaldo Cruz 106:1039-1044 (2011)

Accepted Manuscript:

3. Farias, LHS; Rodrigues, APD; Saraiva, E; Seabra; S, Silveira, FT; DaMatta, RA; Silva, EO**.**Phosphatidylserine exposure and surface sugars in two *Leishmania (Viannia) braziliensis* strains involved in cutaneous and mucocutaneous leishmaniasis.In press The Journal Infectious Disease

AL 15

ASSOCIATE LABORATORY OF MICROSCOPY CETENE

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Ana Karolina de Santana Nunes – CETENE.
Bruna Santos da Silva – UFPE.
Catarina Rapôso Dias Carneiro – UFPE.
Dilênia de Oliveira Cipriano Torres – UFPE.
Edlene Lima Ribeiro – UFPE.
Fabiana Oliveira dos Santos Gomes – UFPE.
Gabriel Barros Rodrigues – UFPE.
Ingrid Tavares Fragoso – UFPE.
Karla Patricia de Sousa Barbosa – UFPE.
Laise Aline Martins dos Santos – UFPE.
Maria Gabriela Duque Spínola – UFPE.
Maria Eduarda Rocha de França – UFPE.
Mariana Aragão Matos Donato – UFPE.
Rayana Leal de Almeida Luna – UFPE.
Shyrlene Meiry da Rocha Araújo – UFPE.
Sura Wanessa Santos Rocha – UFPE.

Pharmacological studies have demonstrated that Diethylcarbamazine (DEC) affects the metabolism of arachidonic acid, blocking a number of steps in both the cyclooxygenase (COX) and lipoxygenase pathway, thereby acting as an anti-inflammatory drug [1].

Studies developed in our lab demonstrated that DEC can be a potential drug for the treatment of chronic inflammation induced by chronic alcoholism and a hepatoprotective drug in reducing lesions in mice malnourished [2,3].

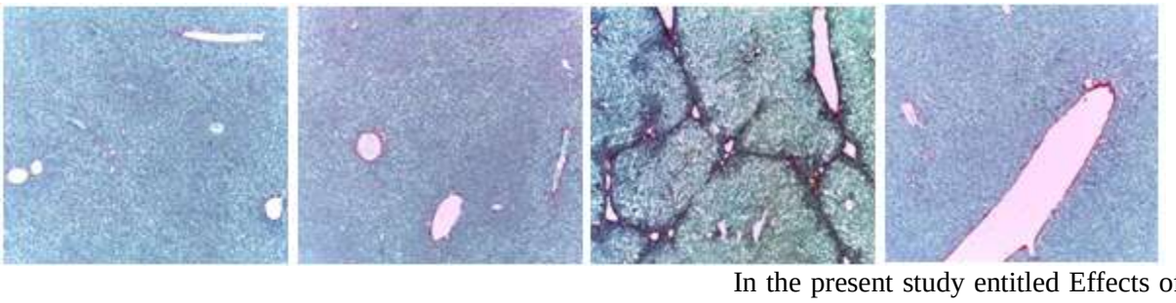


FIGURE 1 - MICROGRAPH OF HEPATOCYTES. A- CONTROL GROUP, B- DEC50 GROUP, C- CCL4 GROUP, D- CCL4+DEC GROUP. BLOOD VESSEL (V), FIBROSIS (ASTERISK), COLLAGEN (ARROWHEAD), SIRIUS RED STAINING, BAR = 100MM.

In the present study entitled Effects of Diethylcarbamazine on the inflammatory process in chronic liver C57Bl/6J mice wild type we investigated the effect of DEC on

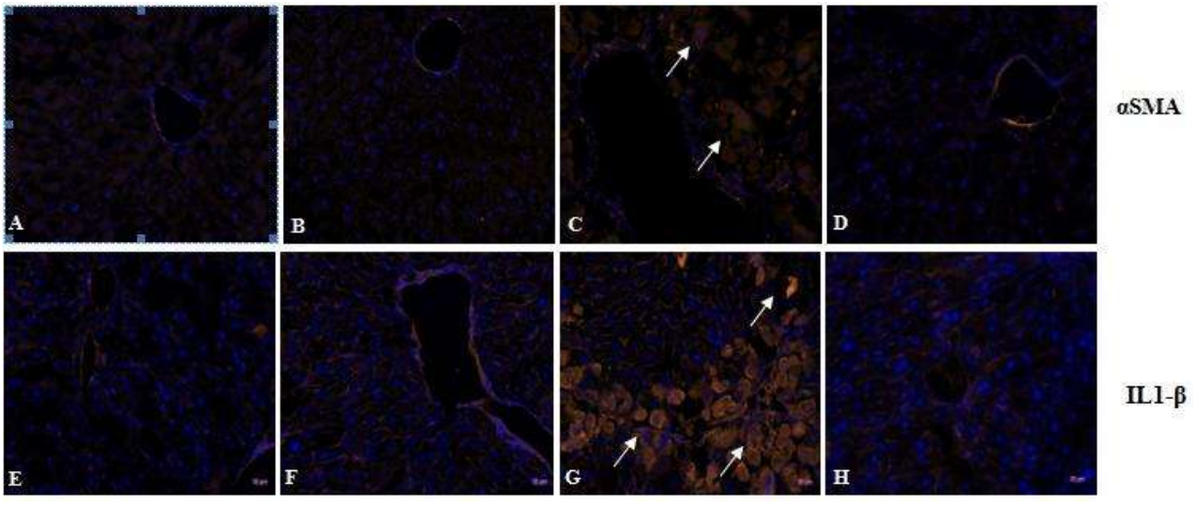
chronic liver inflammation induced by carbon tetrachloride (CCl4). Forty C57BL/6J male mice were separated in groups (n=10): control group, DEC 50mg kg group, CCl4 group and CCl4 + DEC group. DEC (50mg/kg) was administered in bottle for 12 days. CCl4 (0.5 ml/g) was administered for 6 weeks (2 injections per week). Liver fragments were processed for histological (HE and Sirius red), immunofluorescence and molecular analysis.

In the group of animals exposed to CCl4 was observed striking cytoplasmic and nuclear degeneration, with the presence of fibrosis, inflammatory infiltrates and hemorrhagic foci. The animals treated with CCl4 + DEC showed a decrease of all lesions observed in CCl4 group. The CCl4 showed intense staining specific for collagen in fibrotic areas. However, CCl4 + DEC group showed reduced collagen labeling.

Results of immunofluorescence revealed strong increase of inflammatory markers such as IL-1β and α-SMA in fibrotic areas, and a reduction of immunoreactivity of these markers after treatment with DEC. Western blot analyzes showed increased expression of COX-2, IL-1β and NF-kB in the group subjected to chronic liver injury and also a significant decrease after treatment with DEC. The present results, confirm that DEC is a possible alternative treatment for chronic liver inflammation.

[1] Maizels RM & Denham 1992.
[2] S.W.S. Rocha, et al. 2012..
[3] S.W.S Rocha, et al. Journal, 2012.

FIGURE 2 – IMMUNOFLUORESCENCE ANALYSES FOR ASMA AND . A,E- CONTROL GROUP; B,F- DEC50 GROUP; C,G- CCL4 GROUP; D,H- CCL4+DEC GROUP. MACROPHAGES (ARROW), REDUCED STAINING AFTER 50MG/KG DEC-TREATED GROUP. BAR = 20MM.



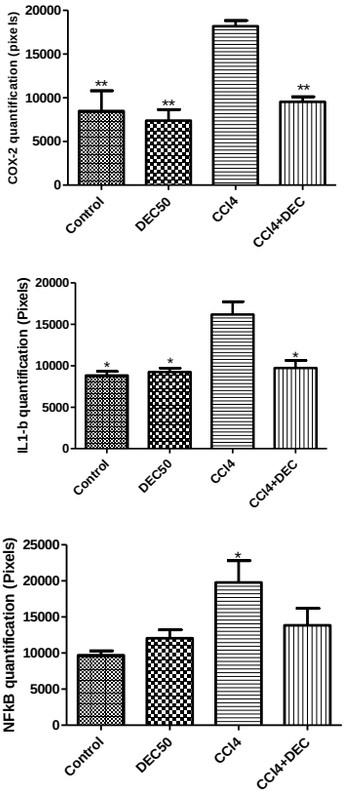
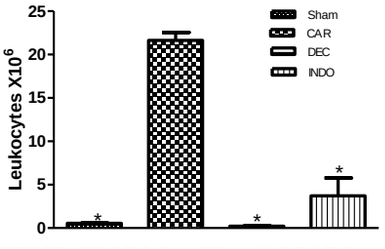


Figure 3 **– WESTERN BLOT ANALYSES OF LIVER EXPRESSION. A- COX-2 QUANTIFICATION, B- IL1-B QUANTIFICATION, C- NFKB QUANTIFICATION.**

***P< 0,05, **P< 0,02. THE COLUMNS REPRESENT THE MEAN ± S.D. OF THE PROTEIN BLOT INVESTIGATED (N= 4 ANIMALS).**

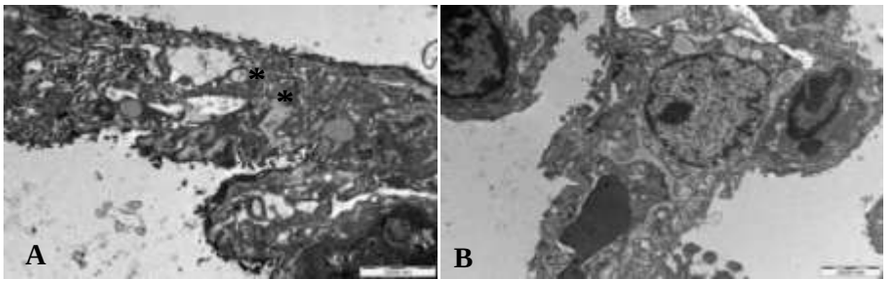
In another study entitled Effects of Diethylcarbamazine on the process of acute lung inflammation in mice we aimed to increase knowledge of the mechanisms of anti-inflammatory DEC using the model of carrageenan-induced pleurisy. Carrageenan-induced inflammation is a model of local acute inflammation commonly used to evaluate the activity of anti-inflammatory drugs [1] and assess the contribution of cells and mediators to the inflammatory process [2].

Forty swiss Webster male mice, 30-day-old and weighting 15-20g, were used in all experiments. Four groups were studied: group Sham: Saline + Saline (control); group CAR: Saline + carrageenan (1% in saline); group DEC: Carrageenan + DEC 50 mg/Kg and group INDO: Carrageenan + indometacin 5mg/Kg (gold standard) were treated orally with indomethacin and DEC 3 days before induction of pleurisy with carrageenan.



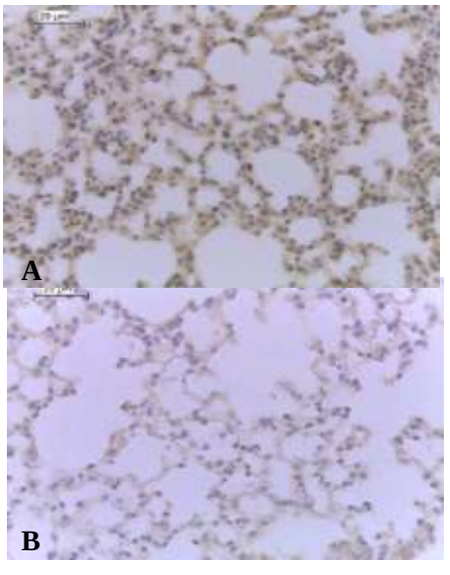
EFFECT OF DEC ON CELL MIGRATION IN THE INITIAL PHASE (4 H) OF THE INFLAMMATORY REACTION INDUCED BY CARRAGEENAN IN MICE; DATA EXPRESSED AS MEAN ± SEM OF 10 MICE FOR EACH GROUP; *P < 0.05 VS. CARRAGEENAN.

The control group received only water during the experimental period. The injection of carrageenan into the pleural cavity induced the accumulation of fluid containing a large number of polymorphonuclear cells (PMNs) as well as infiltration of PMNs in lung tissues and



(A) LUNG SECTIONS FROM MICE WITH CARRAGEENAN-INDUCED PLEURISY SHOWING ENHANCED THICKNESS OF THE INTERSTITIAL SPACE FILLED WITH COLLAGEN FIBERS, MYELIN BODIES, VACUOLES AND LAMELLAR BODIES CONTAINING ELECTRODENSE GRANULES. (B) LUNG TREATED WITH DEC PRESENTING PRESERVED PNEUMOCYTES.

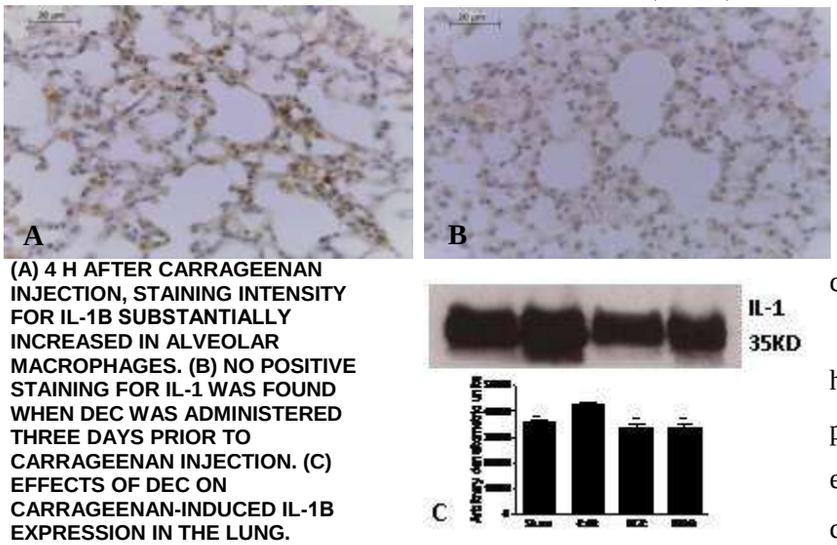
increased production of nitrite, increased expression of interleukin-1β, cyclooxygenase (COX-2). In conclusion the present study demonstrate that the administration of DEC in a model of acute inflammation induced by carrageenan led to reduction in lung injury,



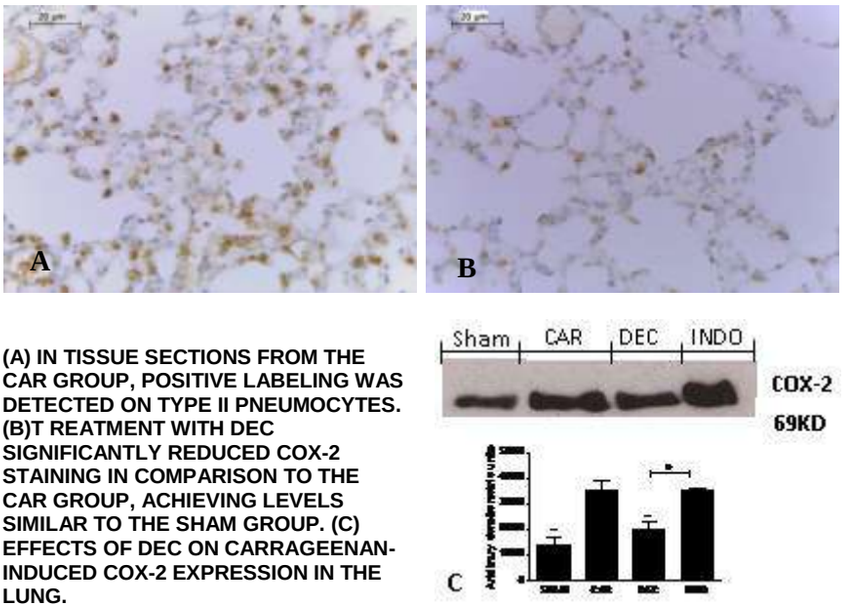
(A) IN TISSUE SECTIONS OBTAINED FROM THE CAR GROUP, POSITIVE STAINING FOR TNF-A WAS MAINLY LOCATED IN INFLAMMATORY CELLS. (B) AFTER TREATMENT WITH DEC, THE DEGREE OF POSITIVE STAINING FOR TNF-A WAS REDUCED IN THE LUNG TISSUE

PMNs migration, the release of pro-inflammatory cytokines and COX-2, thereby confirming previous observations that DEC effectively acts through the NOS/COX mechanism.

[1] Impellizzeri D, et. al. 2011.
[2] Tomlinson A, et. al. 1994.



(A) 4 H AFTER CARRAGEENAN INJECTION, STAINING INTENSITY FOR IL-1B SUBSTANTIALLY INCREASED IN ALVEOLAR MACROPHAGES. (B) NO POSITIVE STAINING FOR IL-1 WAS FOUND WHEN DEC WAS ADMINISTERED THREE DAYS PRIOR TO CARRAGEENAN INJECTION. (C) EFFECTS OF DEC ON CARRAGEENAN-INDUCED IL-1B EXPRESSION IN THE LUNG.



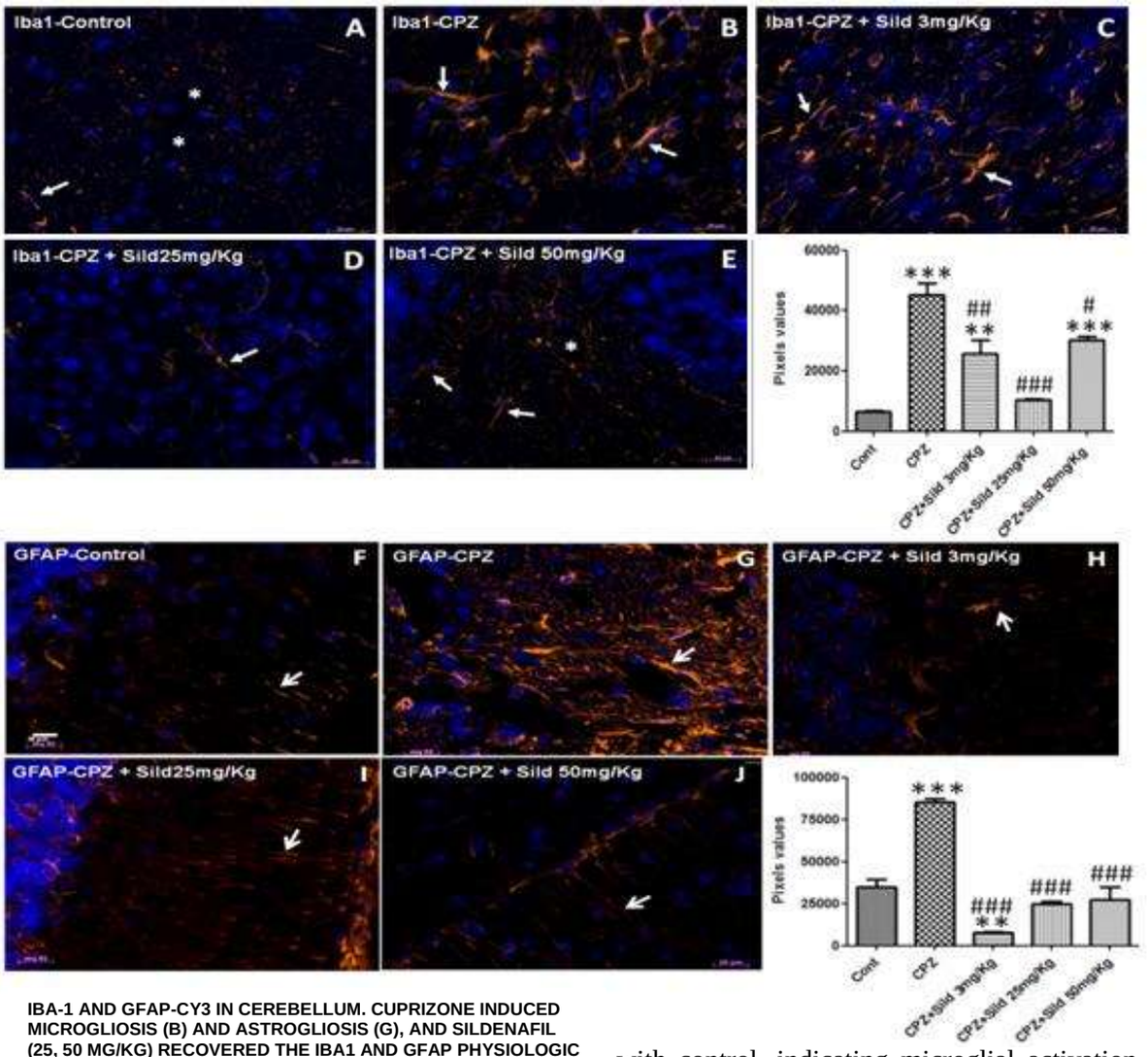
(A) IN TISSUE SECTIONS FROM THE CAR GROUP, POSITIVE LABELING WAS DETECTED ON TYPE II PNEUMOCYTES. (B)T REATMENT WITH DEC SIGNIFICANTLY REDUCED COX-2 STAINING IN COMPARISON TO THE CAR GROUP, ACHIEVING LEVELS SIMILAR TO THE SHAM GROUP. (C) EFFECTS OF DEC ON CARRAGEENAN-INDUCED COX-2 EXPRESSION IN THE LUNG.

Another drug studied by our laboratory is Sildenafil that is an important inhibitor of of cGMP-specific phosphodiesterase type 5 (PDE5) enzyme. Neuroinflammation plays a crucial role in the multiple sclerosis (MS) pathogenesis, an autoimmune disorder characterized by demyelination and progressive psychomotor impairment [1].

The current MS-treatment options are partially effective and need a parenteral route of administration. Sildenafil (Viagra®) induces cyclic 3'5'guanosine monophosphate (cGMP) accumulation through phosphodiesterase-5 (PDE5) inhibition. Cerebellum presents constitutively PDE5, and it has been shown that cGMP-pathways modulates micro- and astroglial (neuroinflammatory resident cells) reaction [2].

Sildenafil despite having an excellent tolerability profile, it has used only for erectile dysfunction and currently for pulmonary hypertension treatment. Here, in the study entitled Sildenafil (Viagra®) Prevents Demyelination and Neuroinflammation in a Multiple Sclerosis Model, the effects of sildenafil in the neuroinflammation were investigated in a MS animal model. Five C57BL/6 mice, 7-weeks-old, were used/group.

The groups received: 1) Cuprizone (0.2%) mixed into a chow/4 weeks, 2) Cuprizone into a chow while sildenafil (Viagra®) 3, 25 or 50 mg/kg in the drinking water, or 3) Controls received pure chow/water. After perfusion, the cerebella were processed for western blotting, immunohistochemistry/immunofluorescence and luxol fast blue staining. Cuprizone significantly increased the GFAP levels and decreased myelination staining intensity. Sildenafil-25/50 mg/Kg groups showed GFAP-



IBA-1 AND GFAP-CY3 IN CEREBELLUM. CUPRIZONE INDUCED MICROGLIOSIS (B) AND ASTROGLIOSIS (G), AND SILDENAFIL (25, 50 MG/KG) RECOVERED THE IBA1 AND GFAP PHYSIOLOGIC EXPRESSION (D, I, J). ARROWS SHOW IBA1 AND GFAP POSITIVE PROCESSES (ORANGE), THICKER AND IN GREATER NUMBER AFTER CUPRIZONE, BUT SIMILAR TO CONTROL AFTER SILDENAFIL. NUCLEI ARE COUNTERSTAINED WITH DAPI (BLUE). THE QUANTIFICATION (FIVE ARBITRARILY SELECTED AREAS) WAS DONE USING GIMP2 SOFTWARE. **P<0.01, ***P<0.001 COMPARING WITH CONTROL; #P<0.05, ##P<0.01, AND ###P<0.001 COMPARING WITH CUPRIZONE. ONE-WAY ANOVA/TUKEY; MEAN±S.E.M.. BARS = 20 µM.

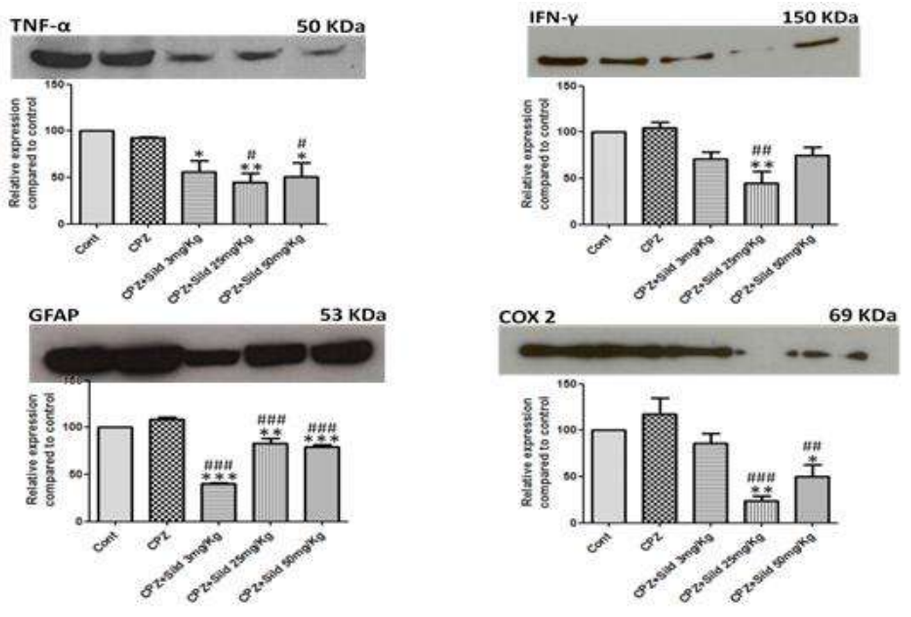
astrocytic expression close to baseline; there was no demyelination.

There was a significant COX-2, IL-2 and IL-1β increase after cuprizone treatment, compared to control group. Cuprizone did not induce changes in IFN-γ and TNF-α expression. After sildenafil administration, all cytokines evaluated and COX-2 expression significantly decreased, compared with control/cuprizone groups. Iba1 levels increased in the cuprizone-treated animals compared with control, indicating microglial activation. Animals treated with sildenafil-25 mg/Kg showed decreased Iba1 expression, compared to cuprizone group.

The increased levels of cGMP, by PDE5 inhibition, probably act as a neuroinflammation modulator, regulating cytokine levels and protecting myelin, astrocytes and microglia. Therefore, after well-designed clinical trials, Sildenafil may be a future drug compatible with daily oral administration for people with MS and other neuroinflammatory / neurodegenerative diseases, providing additional benefits to current treatments.

[1] Block ML, Zecca L, Hong JS. 2007.
[2] Frank-Cannon TC et al.. 2009.

Our laboratory also evaluate new thiazolidine derivatives synthetized by the Department of Antibiotics of the Federal University of Pernambuco.



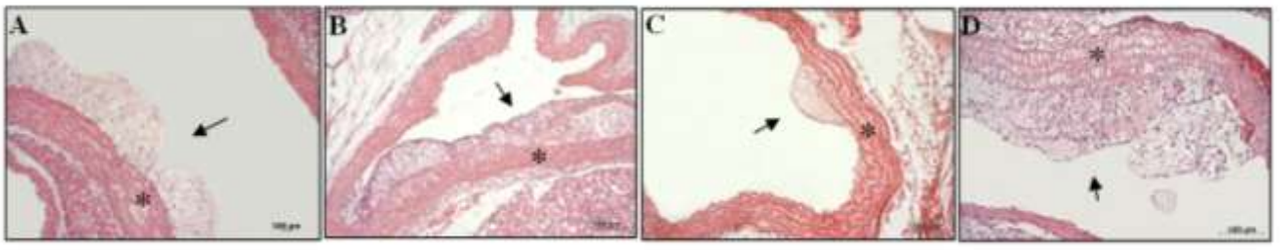
IMMUNOBLOTS. PICTURES SHOW BANDS IN CONTROL AND TREATED GROUPS; PIXELS VALUES WERE QUANTIFIED USING IMAGE J SOFTWARE (DEMONSTRATED IN GRAPHS). ALL BLOTS WERE NORMALIZED WITH B-ACTIN .*P<0.05, **P<0.01 AND ***P<0.001 COMPARING WITH CONTROL; #P<0.05, ##P<0.01, AND ###P<0.001 COMPARING WITH CUPRIZONE. ONE-WAY ANOVA/TUKEY; MEAN±S.E.M

Thiazolidinediones (TZDs) are used to enhance sensitivity to insulin and have demonstrated a protective effect over a variety of cardiovascular markers and risk factors. Controversially, the TZDs are associated with the development of heart failure. Thus, lines of research have invested in the search for new molecules in order to obtain more selective and less harmful treatment alternatives for the pathogenesis of atherosclerosis and its risk factors.

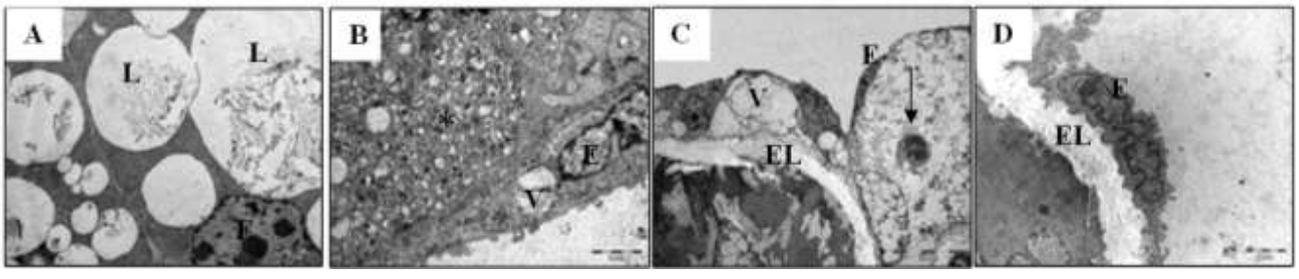
The present study was designed for Evaluation of thiazolidine derivatives LPSF/GQ-02 and LPSF/GQ-16 on atherosclerotic lesions in LDL receptor-deficient mice (LDLR-/-). Animals were fed a diet rich in fat for 10 weeks. In the last two weeks, animals received either Pioglitazone, LPSF/GQ-02 or LPSF/GQ-16 daily through gavage. At the end of the treatment, aortas were dissected for subsequent analyses.

Morphometric analyses revealed that neither pioglitazone or LPSF/GQ16 led to satisfactory effects over atherosclerosis. However, LPSF/GQ-02 led to a reduction in area of the atherosclerotic lesions (figure 1).

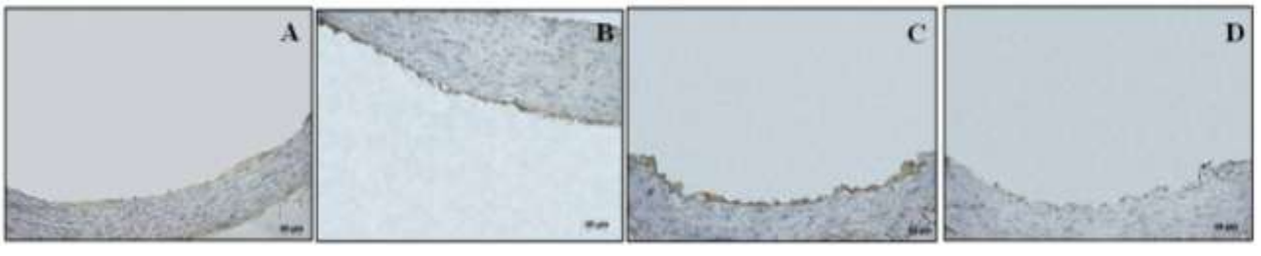
Ultrastructural analyses revealed extensive degeneration of the endothelium and an increase in apoptotic cells in the subendothelial space following the use of pioglitazone and LPSF/GQ-16. However, LPSF/GQ-02 caused minimal cell alterations in the aortic endothelium (figure 2). Regarding markers endothelial nitric oxide synthase (eNOS) and Matrix Metalloproteinase 9 (MMP-9), LPSF/GQ-16 and pioglitazone exerted similar effects, increasing the expression of MMP-9 and has no effect on the expression of eNOS compared with the control group. On the other hand, LPSF/GQ-02 was effective in reducing the expression of MMP-9 and increase eNOS significantly (figure 3 and 4). The results suggest that the new thiazolidine derivative LPSF/GQ-02 is a promising candidate for the treatment of atherosclerosis.



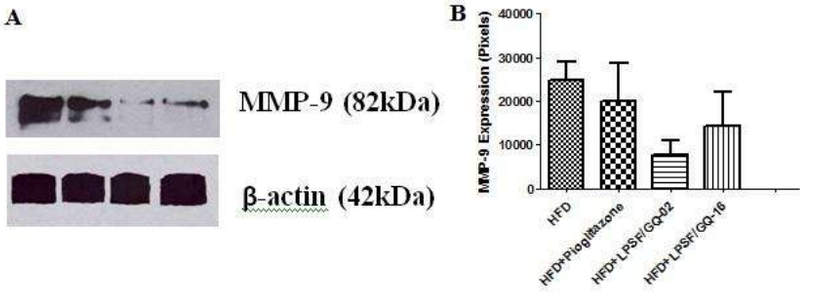
HISTOLOGICAL CUTS OF AORTA WITH ATHEROSCLEROTIC LESIONS (A, B, C, D); FIG. 1 A – AORTA OF CONTROL GROUP SHOWING ARTERIAL WALL THICKNESS, WITH DISORGANIZATION OF SMOOTH MUSCLE CELLS (ASTERISK) AND ATHEROSCLEROTIC LESIONS (ARROW); TREATMENT WITH PIOGLITAZONE (FIG. 1 B) AND LPSF/GQ-16 (FIG. 1 D) DID NOT REVERT CONDITIONS CAUSED BY FAT-RICH DIET WITH REGARD TO ARTERIAL WALL THICKNESS (ASTERISK) AND DISORGANIZATION OF SMOOTH MUSCLE CELLS (ARROW); LPSF/GQ-02 REVERTED CONDITIONS CAUSED BY FAT-RICH DIET, PRESERVING ARTERIAL WALL (ASTERISK) AND REDUCING ATHEROSCLEROTIC LESIONS (ARROW) (FIG. 1 C); MAGNIFICATION 20X.



ULTRASTRUCTURE OF ASCENDING AORTA OF LDLR$^{-/-}$ MICE WITH ATHEROSCLEROTIC LESIONS (A, B, C, D); FIG. 2 A – FOAMY CELL FROM CONTROL GROUP REPLETE WITH LIPIDS; FIG. 2 B - GROUP TREATED WITH PIOGLITAZONE SHOWING VACUOLIZED ENDOTHELIAL CELLS, SUBENDOTHELIAL SPACE WITH APOPTOTIC CELLS (ASTERISK) FIG. 2 C – GROUP TREATED WITH LPSF/GQ-16 SHOWING DESTRUCTION OF SUBENDOTHELIAL SPACE WITH INFLAMMATORY CELLS (ARROW); FIG. 2 D – GROUP TREATED WITH LPSF/GQ-02 SHOWING CONTINUOUS ELASTIC LAMINA WITH WELL-PRESERVED ENDOTHELIAL CELLS; E – ENDOTHELIAL CELL; F – FOAMY CELL; V – VACUOLES; EL – ELASTIC LAMINA; L – LIPIDS



IMMUNOHISTOCHEMICAL ANALYSES FOR ENOS IN LDLR$^{-/-}$ MICE (A,B,C,D,E). FIG. 3A – CONTROL GROUP, SHOWED LOW MARKUP FOR ENOS IN ENDOTHELIAL CELLS. FIG. 3B –TREATMENT WITH PIOGLITAZONE DID NOT INCREASED EXPRESSION OF ENOS SIGNIFICANTLY. FIG. 3C - SIMILARLY, THE GROUP TREATED WITH LPSF/GQ-16 DID NOT SHOW CHANGES IN THE EXPRESSION OF ENOS. FIG. 3D – ON THE OTHER HAND, ANIMALS TREATED WITH LPSF/GQ-02 HAD A SIGNIFICANT INCREASE IN THE EXPRESSION OF ENOS



WESTERN BLOT ANALYSIS OF AORTA EXPRESSION. (A) WESTERN BLOT SHOWING THE EFFECT OF PIOGLITAZONE, LPSF/GQ-02 AND LPSF/GQ-16 ON MMP-9 LEVELS IN ATHEROSCLEROSIS. (B) MMP-9 CONTENT MEASURED BY PIXELS QUANTIFICATION OF WESTERN BLOT BANDS SHOWED SIGNIFICANT DIFFERENCE BETWEEN LPSF/GQ-02 AND CONTROL GROUPS. *P<0.05. DATA WERE ANALYZED USING STUDENT'S T-TEST. THE COLUMNS REPRESENT THE MEAN ± S.D. OF THE PROTEIN INVESTIGATED; THE RESULTS WERE CONFIRMED IN THREE SETS OF EXPERIMENTS (N = 5 ANIMALS).

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AL 16

ASSOCIATE LABORATORY OF MOLECULAR AND CELLULAR CARDIOLOGY

Coordinator: Antonio Campos de Carvalho – IBCCF/UFRJ.

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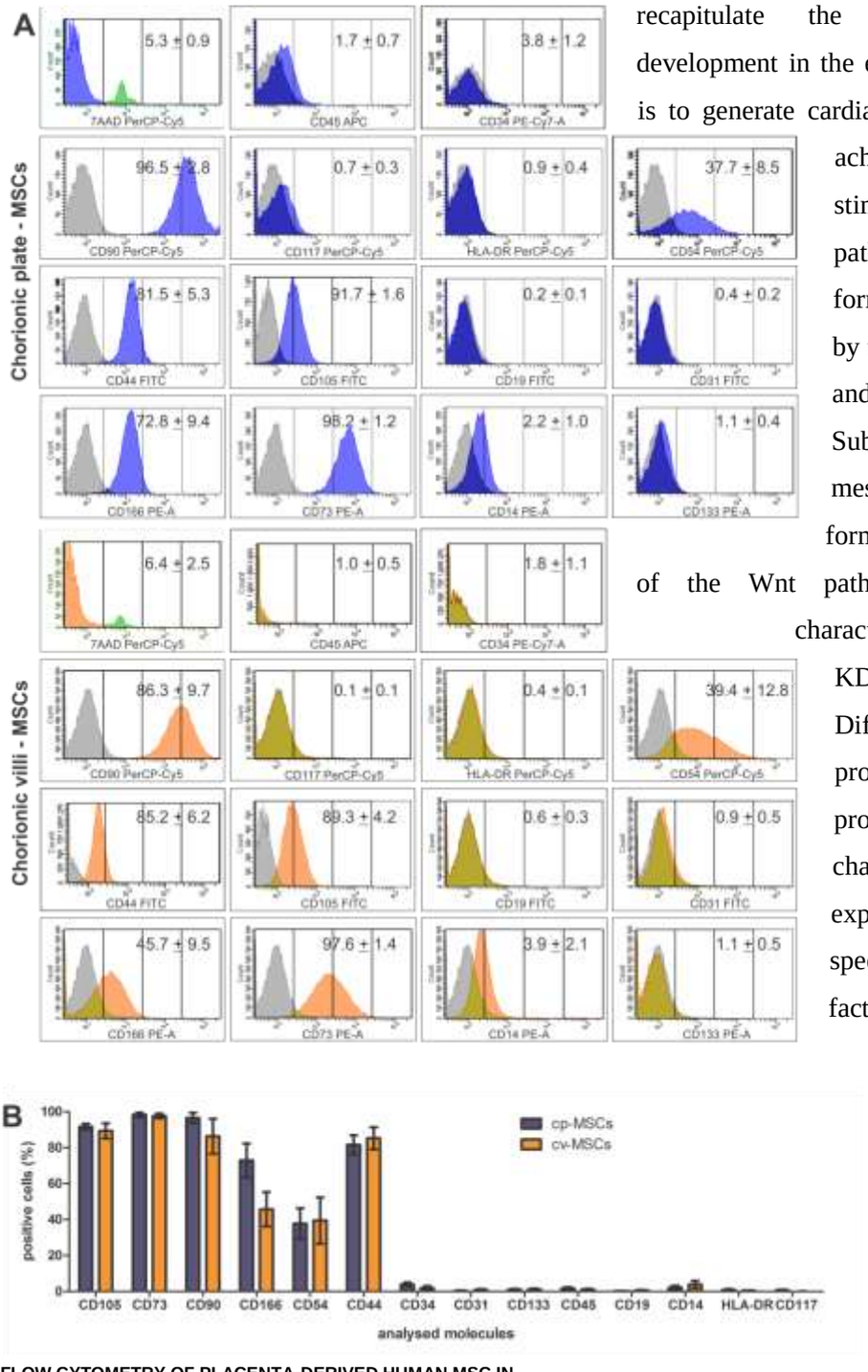
Aldo Rogélis Aquiles Rodrigues - UFTM

Our group has been working on the isolation and characterization of pluripotent and multipotent human and murine stem cells. We have developed methods for cultivating human mesenchymal stem cells (MSC) from neonatal tissues (amniotic fluid, placenta, Wharton's jelly and the walls from umbilical cord artery and vein). These MSC have been isolated, characterized immunophenotypically and differentiated into osteoblasts, chondrocytes and adipocytes. Attempts to differentiate these cells into cardiomyocytes have failed. We have also isolated Cardiosphere Derived Cells (CDC), a heterogeneous cell population, which is supposed to contain cardiac stem cells. CDC have been isolated, expanded in cultured and characterized by flow citometry. Although morphologically similar to MSC from adult and neonatal tissues, these cells display a distinct phenotype. Attempts to differentiate CDC into cardiomyocytes, endothelial and smooth muscle cells have also failed.

Regarding the pluripotent cells, we have produced induced pluripotent stem cells (iPSC) from MSC derived from menstrual blood. The menstrual blood MSC have yielded iPSC-like cells after transduction with lentiviral vectors containing the Yamanaka factors. In fact we have been able to produce iPSC-like cells using only three factors (Klf4, Sox2, Oct3/4), without the need for c-Myc, with great efficiency and in shorter times (first colonies detected at 4 days after transduction). We attribute this improvement to the endogenous expression of some of the pluripotency factors in MSC from menstrual blood. Differentiation of the iPSC-like cells into cell types of ecto, meso and endodermal origin has been shown.

We are also working on direct differentiation of human and mouse fibrobasts into cardiomyocytes. This project is still at a preliminary stage. We have succeded in expressing cardiac transcription factors using Srivastava's vectors in mouse embryonic fibrobasts and are now trying to increase

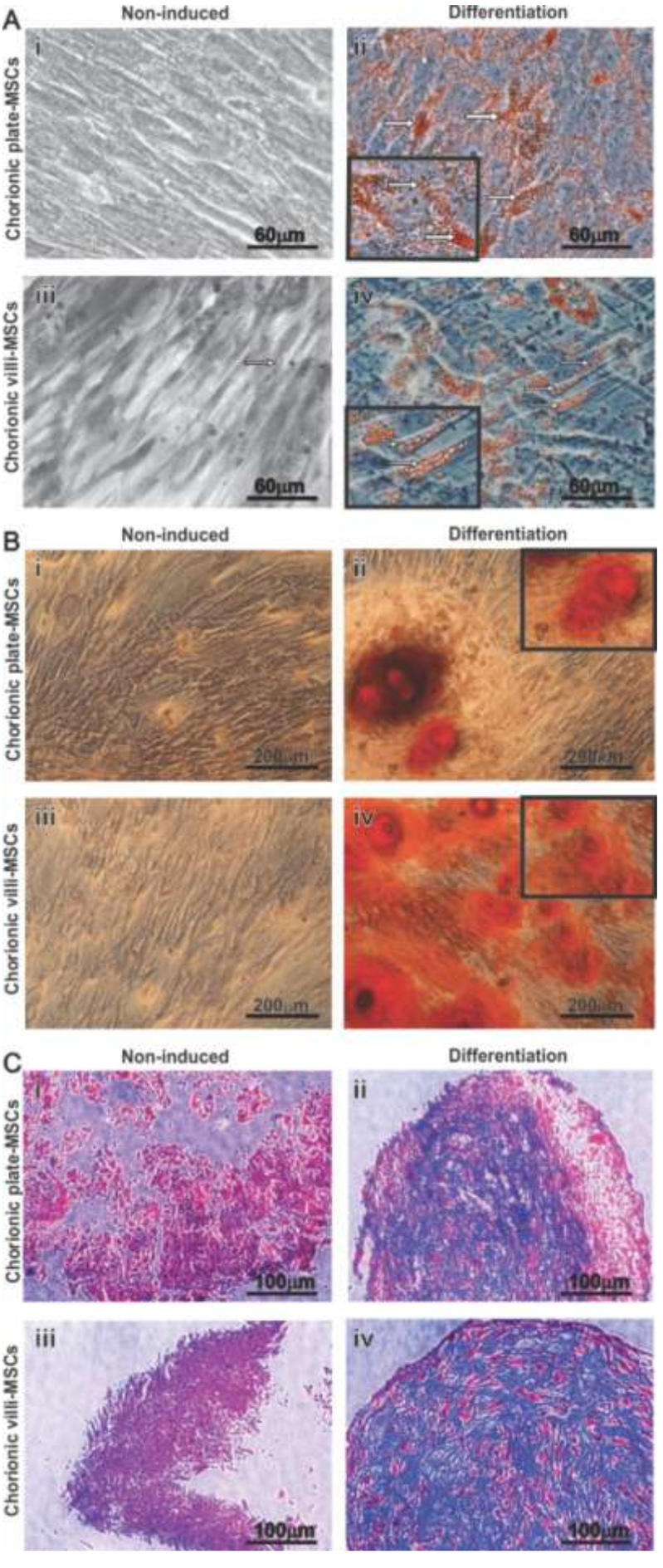
efficiency using additional transcription factors that play prominent roles during cardiogenesis.



FLOW CYTOMETRY OF PLACENTA-DERIVED HUMAN MSC IN THIRD PASSAGE. (A) IN BLUE, THE EXPRESSION OF MESENCHYMAL, HEMATOPOIETIC AND ENDOTHELIAL CELL MARKERS ARE SHOWN FOR CHORIONIC PLATE-DERIVED CELLS. IN YELLOW, THE SAME MARKERS ARE SHOWN FOR CHORIONIC VILLI-DERIVED CELLS. ISOTYPE CONTROLS ARE SHOWN IN GREY. (B) QUANTIFICATION OF FLOW CYTOMETRY DATA SHOWING THAT MSC ARE POSITIVE FOR CD105, CD73, CD90, CD166, CD54 AND CD44, AND NEGATIVE FOR HEMATOPOIETIC AND ENDOTHELIAL CELL MARKERS.

Importantly, we are also working in differentiation of pluripotent stem cells into cardiomyocytes. This complex process must recapitulate the steps of cardiac development in the embryo. The first stage is to generate cardiac mesoderm, which is achieved through the stimulation of Wnt pathway. Mesoderm formation can be detected by the expression of CD56 and PDGFRα. Subsequenty, the cardiac mesoderm needs to be formed through inhibition of the Wnt pathway. This stage is characterized by expression of KDR and PDGFRα. Differentiation then progresses to the cardiac progenitor stage, characterized by the expression of the cardiac specific transcription factor Nkx2.5, and, finally, to the formation of beating cardiomyocytes.

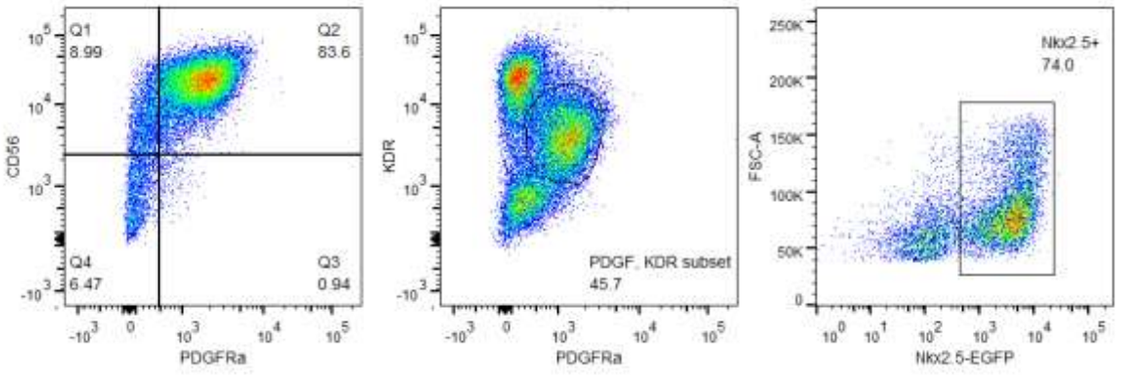
All our in vitro experiments are geared towards generating cells that can be used for cardiac repair. In our in vivo experiments we are using four animal disease models of cardiovascular alterations: a myocardial infarction model, a chronic chagasic cardiomyopathy model, a pulmonary hypertension model, and an induced diabetes



MESODERMAL DIFFERENTIATION OF PLACENTA-DERIVED MSCS. (A) OIL RED O STAINING AFTER ADIPOGENIC DIFFERENTIATION. CYTOPLASMIC LIPID DROPLETS WERE OBSERVED IN THE INDUCED (II) CP-MSC AND (IV) CV-MSCS, BUT WERE NOT PRESENT IN THE NON-INDUCED (I) CP-MSCS AND (III) CV-MSCS CULTURES. WHITE ARROWS INDICATE LIPID DROPLETS IN THE CYTOPLASM. (B) ALIZARIN RED STAINING AFTER OSTEOGENIC DIFFERENTIATION. CALCIUM DEPOSITS IN THE EXTRACELLULAR MATRIX WERE OBSERVED IN THE INDUCED (II) CP-MSC AND (IV) CV-MSCS, BUT WERE NOT PRESENT IN THE NON-INDUCED (I) CP-MSCS AND (III) CV-MSCS CULTURES. (C) HISTOLOGICAL ANALYSIS OF 3-WEEK CULTURED PELLETS. THE MICROMASS, FORMED AFTER CHONDROGENIC DIFFERENTIATION, WAS EMBEDDED IN PARAFFIN, SECTIONED AND STAINED WITH ALCIAN BLUE. NUCLEI WERE COUNTERSTAINED WITH NUCLEAR FAST RED. PROTEOGLYCANS, STAINED IN BLUE, WERE OBSERVED IN THE INDUCED (II) CP-MSC AND (IV) CV-MSCS. NON-INDUCED (I) CP-MSCS AND (III) CV-MSCS DID NOT PRESENT PROTEOGLYCAN STAINING.

model. These models are well characterized in our lab and validated by electro and echocardiography. In testing the validity of cell therapies in each of these models we are using different cell types (MSC, CDC, ESC) that are injected intravenously or in some instances by echo-guided intramyocardial injection. The injected cells are transduced with luciferase and serially tracked using a bioluminescence apparatus. Cardiac function is serially evaluated by ECG and echocardiography and, after animal sacrifice, histopathology is performed. In the diabetes model we also measure vascular reactivity in isolated aorta. We have been

able to record MRI images of the hearts of these animals, using the multi-imaging facility that was created by our Institute.



Cardiac differentiation of pluripotent stem cells. The first dot blot shows differentiation into mesoderm in the population that is double-positive for CD56 and PDGFRα. The second dot blot shows the presence of cardiac mesoderm, which is KDR low and PDGFRα positive. The KDR high and PDGFRα negative population are blood progenitors which are also formed from the mesoderm. The third dot blot shows the presence of cardiac progenitors, which are positive for Nkx2.5.

A new project was added to our Associated Laboratory by the incorporation of Prof. Adriana B Carvalho to the group. We are currently studying the expression of microRNAs (mIRs) in a rat model of myocardial infarction and in patients with ischemic cardiomyopathy. miR-208a is a MyomiR expressed specifically in the heart as it is located in one of the introns of the α-myosin heavy chain gene. This miR is one of the regulators of the expression of GATA4, a major transcription factor in the maintenance of cardiac homeostase. The role of miR-208a as a therapeutic target for the treatment of heart failure is being currently investigated in our laboratory. Additionally, mIRs are being screened for their capacity to induce or improve efficiency in the generation of iPSC or of induced cardiomyocytes (iCM).

We have also finished clinical trials performed in patients with Chagasic and dilated cardiopathies and in stroke patients, using bone marrow derived mononuclear cells.

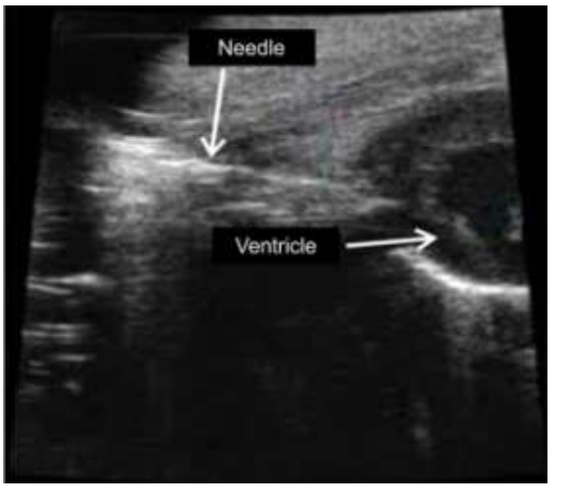


IMAGE SHOWS THE NEEDLE USED TO INJECT CELLS DIRECTLY INTO THE MYOCARDIUM OF AN INFARCTED MOUSE HEART, USING THE HIGH RESOLUTION ECHOCARDIOGRAM.

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1. Oliveira, Patricia Fidelis ; Werneck-de-Castro, Joao P. S. ; Pinho-Ribeiro, Vanessa ; Shalom BCM ; Nascimento, J. S. ; Costa-e-Sousa, Ricardo H. ; da Cruz, I. C. ; Rangel R R;Goldenberg, Regina C. dos Santos ; CAMPOS DE CARVALHO A. C. . Soluble factors from multipotent mesenchymal stromal cells have antinecrotic effect on cardiomyocytes in vitro and improve cardiac function in infarcted rat hearts. Cell Transplantation, v. 21, p. 1011-1021, 2012.

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4. Dias da Silva, V. J. ; Gnecchi Ruscone, T. ; Bellina, V. ; Oliveira, M. ; Maciel, L. ; CAMPOS DE CARVALHO A. C. ; Salgado, H. C. ; Bergamaschi, C. M. ; Tobaldini, E. ; Porta, A. ; Montano, N. . Acute adenosine increases cardiac vagal and reduces sympathetic efferent nerve activities in rats. Experimental Physiology (Print), p. online, 2012.

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AL 17

ASSOCIATE LABORATORY OF ION TRANSPORT PHYSIOLOGY IN HEALTH AND DISEASE

Coordinator: Adalberto Vieyra – IBCCF/UFRJ.

Members:

Celso Caruso Neves – IBCCF/UFRJ
José Roberto Meyer-Fernandes – IBqM/UFRJ
Marcelo Einicker Lamas – IBCCF/UFRJ
Jennifer Lowe – IBCCF/UFRJ
Lucienne S. Lara – ICB/UFRJ
Elaine Gomes Quintana – IBCCF/UFRJ
Rafael H. F. Valverde – IBCCF/UFRJ
Luiz Roberto Leão Ferreira – IB/UFF
Aloa Machado de Souza - IFRJ

Laboratory 17 of the National Institute of Science and Technology for Structural Biology and Bioimaging (INBEB) comprises different research groups focused on the studies of: (i) transport processes and their regulation in health and disease, and (ii) the biochemistry of parasites (including transport processes).

The Laboratory has an associated group at the Federal University of Pernambuco (UFPE). During the last 2 years, the Laboratory has broadened its internal collaborations and interactions with researchers from Laboratories 2 and 16 of the INBEB. The internal collaborations have contributed to describing – for the first time – the mechanisms of acquisition of phosphate, a key element in energy metabolism, by *Trypanosoma cruzi* and *Trypanosoma rangeli*.

Collaboration with Laboratory 2 has

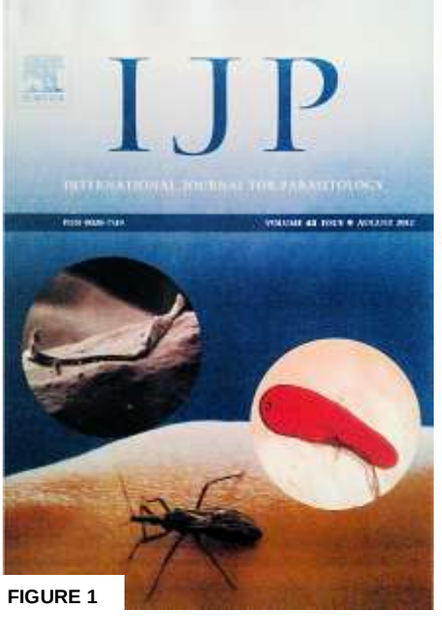

FIGURE 1

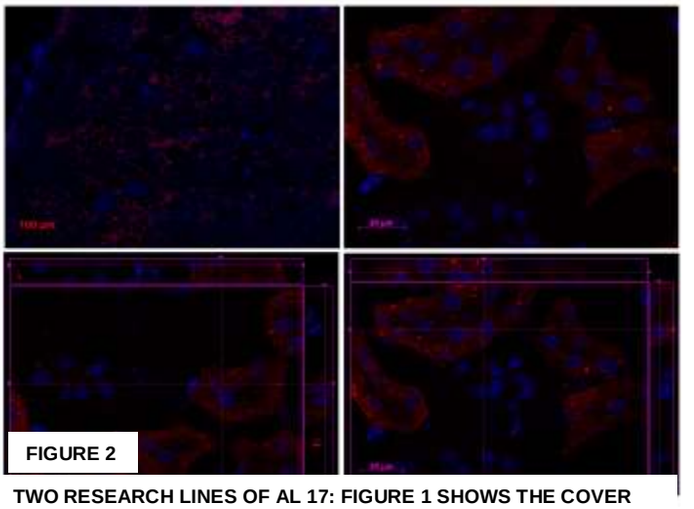

FIGURE 2

TWO RESEARCH LINES OF AL 17: FIGURE 1 SHOWS THE COVER ARTICLE IN THE INTERNATIONAL JOURNAL FOR PARASITOLOGY, ABOUT THE INTERACTION OF *TRYPANOSOMA RANGELI* WITH THE SALIVARY GLAND OF THE CHAGAS DISEASE'S INSECT VECTOR. FIGURE 2 SHOWS HOW STEM CELLS ESTABLISH THEMSELVES IN AN AREA DAMAGED BY ISCHEMIA

enabled us to demonstrate the key role of *S*-nitrosylation of proteins in the repair mechanisms elicited by stem cells in ion-transporting processes in episodes of ischemia/reperfusion (I/R), which are very common in acute renal and cardiac diseases.

Partnership with the National Cancer Institute (CEMO Laboratory) was essential for our proteomics studies, which are unraveling the key signaling pathways affected by stem cells in I/R.

Thanks to a more recent association established at the Institute of Biomedical Sciences, University of Sao Paulo (supported by the Ministry of Health and the Brazilian National Research Council), Laboratory 17 began to implement a national network for the study of applications of stem cells in kidney diseases.

Our partnership with Laboratory 16 and the associated group at the UFPE has enabled us to elucidate the common mechanisms underpinning the severe changes in renal and cardiac transporters induced by chronic malnutrition, including sudden death.

The maintenance of international associations with the Tulane University School of Medicine, the Hypertension and Renal Center of Excellence at Tulane University, the Division of Nephrology (Medicine) and the Johns Hopkins University School of Medicine (all in the USA) has continued to be fruitful for studies regarding renal ion transport processes in health and disease.

Finally, the continuation of years of association with the Laboratoire de Chimie et Biologie des Métaux at the Commissariat à l'Energie Atomique (France) opened new directions for elucidating the regulatory mechanisms of copper ATPases in the "neglected" metabolic disorders of Wilson and Menkes.

All these contributions involve several graduate and undergraduate students, as well as post-doctoral trainees.

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AL 18

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Traditionally referred as the contact/intrinsic pathway of coagulation, the Kallikrein-Kinin-System (KKS) was for many years regarded as a proteolytic mechanism that is irrelevant for hemostasis, since the clotting time is not markedly delayed in individuals with genetic deficiencies of FXII, FXI and High Molecular Weight Kininogen. More recently, awareness that transgenic mice deficient of FXII or high molecular weight kininogen are relatively resistant to thrombosis has boosted interest in this research field. Another important development was the characterization of polyphosphates (released by activated platelets or mast cells), heparin (released from mast cell granules) and DNA (contained in extracellular traps of neutrophils) as the endogenous activators of the KKS/contact system in vivo. Whether implicated in fibrin-driven stabilization of microthrombi and/or in the propagation of inflammation through the proteolytic generation of bradykinin, the KKS is now regarded as a hub-like proteolytic network that reciprocally couples innate immunity to pro-thrombogenic and inflammatory pathways.

In the past decade, our group demonstrated that TLR2-driven innate immunity and inflammatory edema propagated by the KKS operate in intertwined manner in three different infection models: Chagas disease, Visceral Leishmaniasis (VL) and Periodontitis. We chose these infectious diseases as models because their etiologic agents, respectively Trypanosoma cruzi, Leishmania donovani and L. chagasi, and the gram-negative periodontal bacterium Porphyromonas gingivalis, express kinin-

releasing proteases. Focusing on experimental models of Chagas disease, our group was the first to link the TH1-directing function of IL-12-producing dendritic cells (DCs) to the activation of G-protein coupled bradykinin B2 receptors (BK2R).

Subsequent studies in experimental models of VL and Periodontitis reinforced the view that pathogen-induced generation of bradykinin in peripheral and/or intralymphoid tissues provides a bridge between inflammation and immunity In this progress report, we will list our publications (full articles, reviews, book chapters and short communication) in the last period of this grant (2011-2012). In other to facilitate the critique of external reviewers, the published information was grouped according to each specific research theme. When pertinent, the citations of published work will be followed by brief comments on the relevance of these discoveries, and how they have influenced current research activities. In the last section of this report, we will select a few images from published work (schemes), along with the corresponding legends.

KKS in experimental Chagas disease.

Trypanosoma cruzi invades host cells through the activation of endothelin and bradykinin receptors: a converging pathway leading to chagasic vasculopathy.

In previous studies, we showed evidence that T. cruzi induces a neutrophil-dependent edema via a trans-cellular "cross-talk" involving TLR2/CXCR2 (inflammation initiated by innate sensing of pathogens) and BK2Rs/ETRs (amplification, at expense of proteolytic activation of the KKS). In this article we showed evidences that vasoactive kinins and endothelins, acting cooperatively through the signaling of their cognate GPCRs, may influence host/parasite balance and pathogenic outcome in experimental Chagas disease. In the first part of this study, we analyzed the roles of endothelin receptors (ETaR/ETbR) and bradykinin B2 receptors (BK2R) in infection-associated microvasculopathy.

Using intravital microscopy (Hamster cheek pouch-HCP), we found that specific antagonists of ETaR, ETbR and BK2R were able to nullify plasma leakage responses and cancel neutrophil accumulation in HCP microvascular beds induced by the topically applied trypomastigotes. Another example of BKRs/ETRs cooperation emerged from studies of host cell-parasite interaction in culture systems. Whether using GPCR antagonists or RNA silencing, these experiments showed that trypomastigotes invade more efficiently their target host cells (neonatal murine cardiomyocytes, human (primary) endothelial cells or human smooth muscle cells) through the interdependent signaling of BK2R/ETaR/ETbR.

Moreover, we showed that parasite uptake via the ETR/BKR pathway was abolished by cholesterol-depleting drugs, thus suggesting that BK2R and ETRs may physically associate in lipid rafts/caveolae or assembly in other cholesterol-rich microdomains of plasma membrane. Collectively, these studies support the concept that T. cruzi might take advantage of the

transient formation of an intracardiac edema to proteolytically generate infection-promoting peptides, such as bradykinin- in the inflamed myocardium. In a more recent review, we have further refined this hypothesis, in light of recent evidence that BKRs, ETRs, most likely associated to other GPCR partners, may act as "gateways" for invasion of cardiovascular cells (Scharfstein et al., Frontiers in Immunology, 2013; Andrade et al., abstract, SBPZ, 2012).

Observation: the original article by Andrade et al., 2012 was object of special commentary by the editors of BJP (D 'Orléans-Juste P, Bkaily G, Rae GA. Endothelin and bradykinin: 'brothers-in-arms' in Chagas vasculopathies? Br J Pharmacol. 165:1330-32, 2012.

Intracardiac injection of Dm28c Trypanosoma cruzi provides a model of infection-associated myocarditis and heart fibrosis that depends critically on cooperative activation of the kallikrein/kinin system and the endothelin pathway.

This innovative model of intracardiac infection allowed us to test the hypothesis that T. cruzi trypomastigotes takes advantage of an intracardiac edema to invade heart cells via activation of BKRs/ETRs. Briefly, we injected T. cruzi trypomastigotes in the left ventricle of naïve mice using high resolution echocardiography [#]. Shortly before infection, the mice were injected (i.v.) with dextran-TRICT, a macromolecular tracer of intracardiac edema (measured 2 h p.i.). After detecting formation of interstitial edema in the heart tissues of wt mice, we showed that microvascular leakage was (i) prevented in wt mice pretreated with a single dose of specific antagonists of ETR or BK2R. or (ii) inconspicuous in the heart of BK2R-deficient mice.

Notably, the reduction of edema formation sought by ETR or BK2R antagonists lead to marked decreased in heart parasitism (measured by qPCR, 3 days after parasite injection). Strikingly, the early treatment with a single dose of BK2R or ETR antagonist had long term impact in the progression of myocardiopathy, because these mice were protected from myocarditis and heart fibrosis, assessed 30 d after parasite injection. As a follow up of these studies, we are now studying the functional interplay between cardiac mast cells and the KKS in chronic chagasic pathology.

[#] available in CENABIO- these experiments were made in collaboration with members of the team headed by Dr. Antonio Campos de Carvalho, also linked to INBEB (AL 16).

Mast cells propagate inflammation in peripheral sites of Trypanosoma cruzi infection through the activation of the kallikrein-kinin cascade.

Using intravital microcopy in the HCP model, Dr. Clarissa Nascimento, Dr. Erik Svensjo and colleagues showed evidence that the degranulation of perivascular mast cells is an essential step in the progression of the KKS-driven inflammatory edema elicited by T. cruzi trypomastigotes. Motivated by these findings, we then verified whether these premises hold true in the intracardiac model of

chagasic infection. Indeed, we found that that mast cell stabilizers such as cromolyn inhibited KKS-driven intracardiac edema and the ensuing heart parasitism. Ongoing studies may clarify whether the intracardiac activation of the KKS/contact coagulation depends on mast cell secretion of (i) polyphosphates (ii) heparin or (iii) DNA contained in extracellular traps.

Exploring the inflammatory role of the mast cell/kallikrein-kinin cell pathway in the modulation of T cell responses against Trypanosoma cruzi.

As mentioned above, our group have previously demonstrated that Dm28c trypomastigotes activate the kinin system through activation pathways involving trans-cellular cross-talk between TLR2 and BK2R and that this signaling contributes to host immune resistance, since that BK2R-/- mice succumb to acute systemic infection with T. cruzi (Monteiro et al., Journal of Immunology, 2006; Monteiro et al., PLoS Pathogens, 2007).

Here we studied the role of BK1R in chronic chagasic infection. Histopathological studies demonstrate that transgenic mice deficient in BK1R are less vulnerable to myocarditis and cardiac remodelling. In other words, BK2R (constitutively expressed) and BK1R (induced in inflamed tissues) seem to play opposite roles if the pathogenesis of Chagas disease. Preliminar studies suggest that KKS activation in the parasitized heart is critically dependent on mast cell degranulation by endothelins.

KKS/Visceral Leishmaniasis

In an earlier study performed with insect-stage promastigotes of the L. donovani complex (Svensjo et al, Microbes and Infection, 2006; reviewed by Scharfstein and Svensjo, see reference for our chapter in the book “Kinins” edited by M. Bader, 2012), we reported that these parasites (etiologic agents of Visceral Leishmaniasis) induce inflammatory edema and infect macrophages via signaling of bradykinin B2 receptors (BK2R). These studies showed that kinins (released via parasite cysteine proteases) have dichotomous role in the host/parasite relationship: in the interaction with resident macrophages, activation of BK2R stimulates promastigotes uptake and fuel amastigote outgrowth.

However, infection assays performed with inflammatory macrophages revealed an opposite phenotype, i.e., activation of the kinin/BK2R pathway upregulates microbicidal responses, constraining intracellular outgrowth of Leishmania. Given the precedent that BK2R-/- mice succumbed to acute systemic challenge with T. cruzi (Monteiro et al, PLoS Pathogens, 1997). In this key paper, we linked the susceptibility of BK2R-/- to impaired capacity to generate protective effector T cells (type 1) as the infection progressed. Furthermore, the primary immunological dysfunction was ascribed to deficient activation of BK2R-/- DCs in the splenic compartment.

In Nico et al. (2102) [#], we subjected WT and BK2R-/- mice to an intravenous challenge with L. chagasi amastigotes. Our results showed that BK2R-/- mice displayed increased liver parasitism and

worsened hepatosplenic pathology. Partial analysis of the immune responses of L. chagasi –infected WT and BK2R-/- mice revealed that Th1 responses were impaired in the transgenic line. As yet, it is not known whether kinins, presumably released in the liver/spleen of VL mice, stimulate protective Th1-responses through the triggering of BK2R+/+ DCs, as shown in T. cruzi infection, and/or whether they act as innate stimuli, enhancing the microbicidal power of activated WT (BK2R+/+) macrophages. Irrespective of the mechanisms underlying the susceptible phenotype of L. chagasi-infected BK2R-/- mice, this study sets another precedent that activation of the kinin/BK2R pathway promotes host resistance against infection by intracellular parasites.

#: the mouse model of LV used in this work was already established in the laboratory of Dr. C. Palatnik (Institute of Microbiology). Immunological studies were performed under the supervision of Prof. Alexandre Morrot, of the same institute.

Leishmaniasis: Effect of sandfly saliva components on the host microcirculation.

It has been reported that Lutzomyia longipalpis, the sand fly vector of Leishmania chagasi promastigotes, affects the pathogenic outcome of Leishmania infection through the activity of multiple vasoactive and immunomodulatory substances. For example, maxadilan, a potent vasodilator component of salivary gland homogenates [SGH), has been shown to enhance infectivity of several Leishmania species via mechanisms that that are not sufficiently well understood. In a previous study, we have shown that SGH and maxadilan are equally potent in increasing plasma leakage (Svensjö et al. 2009). As an extension of these studies, here we investigated whether maxadilan and pituitary adenylate cyclase-activating peptide (PACAP-38) may cause plasma leakage through the activation of leukocytes via triggering of PAC1 and CXCR1/2 receptors. Our intravital microscopy studies in the HCP suggest that both maxadilan and PACAP-38 increased plasma leakage via stimulation of PAC1-receptors. Assays with specific GPCR blockers revealed a positive correlation between plasma leakage and activation/accumulation of leukocytes in microvascular beds. In vitro migration of neutrophils was also stimulated to the same degree by maxadilan and IL-8. In order to determine whether the maxadilan effect was due to a release or induced synthesis of IL-8, we asked whether these effects were blocked by reparixin (selective inhibitor of CXCR1/2-receptor). Indeed, reparixin blocked maxadilan and IL-8 induced neutrophil migration in vitro and reduced plasma leakage and leukocyte accumulation in vivo. Our findings suggested that maxadilan may affect host/parasite balance in the sites of Leishmania infection through the induction of neutrophil-driven edema via the PAC1R/CXCR1/2 pathway.

KKS in experimental infection with P.gingivalis.

In a previous study, we reported that bradykinin release in BALB/c subgingival tissues exposed to the periodontal bacterium P.gingivalis induces IL17-producing and INF-γ producing T cells in gingipain-dependent

manner (Monteiro et al., J.Immunol. , 2009). Here, we showed evidence that bone marrow derived DCs exposed to P.gingivalis (W83) versus gingipain-deficient mutants (KRAB) differentially produce IL-23, i.e., a key Th17-polaring cytokines. Strikingly, we found that DC production of IL-23 is upregulated by mouse gingival fibroblasts (FB), in gingipain-dependent manner.

Interestingly, the recognition of the bacterium by gingival fibroblasts did not affect at all CCR7 expression by DCs. Our results suggest that gingival fibroblasts guide the functional responses of immature DCs exposed to P.gingivalis, shifting the cytokine production to the IL-23/IL-17 axis. Ongoing studies should determine whether the KKS has a critical role in this process, as previously proposed, on the basis of findings obtained in the settings of in vivo infection.

KKS and Arbovirus infection of endothelial cells (Subsidiary Project)

Infection-associated vasculopathies caused by arbovirus, such as Dengue, can be life-threatening. Considering that Sindbis virus induces infection-associated inflammatory and vasoactive responses in humans, causing rash and arthritis, here we examined the role of KKS in the outcome of endothelial infection in vitro. This issue was addressed by incubating Sindbis-infected Human endothelial cells (HBMECs) with low concentrations of bradykinin (BK), a potent inducer of inflammatory edema in a broad range of infectious diseases. First, we found that Sindbis upregulated the expression bradykinin B2 receptors (BK2R) expression in infected HBMECs. Second, we showed that BK reduced SINV-induced apoptosis and enhanced virus replication in HBMECs via the BK2R/PI3 kinase/ERK signaling pathway. Strikingly, intracerebral infection of mice in the presence of a BK2R antagonist reduced the viral load in the CNS.

Our data suggest that Sindbis infection renders human endothelial cells hypersensitive to BK, which then increases host cell survival and viral replication through the activation of BK2R. Ongoing studies should clarify if the deregulation of the kinin pathway may increase endothelium damage in Sindbi infection - as well as in other life-threatening arbovirus infections, such as Dengue.

KKS and vaccination: novel strategies for developing adjuvants.

In 2003, we have submitted a request for patent registration to INPI (2003, final decision still pending) based on the idea that the endogenous release of bradykinin in intralymphoid tissues might potentiate the efficacy of vaccine adjuvants. The groundwork leading to this proposition was the discovery that the incorporation of synthetic bradykinin combined to antigens in alum-based emulsions may stimulate Th1 responses via the BK2R/IL-12 pathway through the activation of immature DCs (Aliberti et al., J.Immunol, 2003; Monteiro et al., 2006; Monteiro et al., PLoS Pathogens, 2007. In a key finding, we found that Th1 induction in mice immunized with [alum/BK/ovalbumin] emulsion was potentiated by a single-dose treatment with captopril, the anti-hypertensive drug (generic)

that prevents degradation of bradykinin by angiotensin-converting enzyme (ACE).

The idea to combine the use of exogenous BK/alum to Captopril treatment proved successful when we performed vaccination experiments in mice immunized with soluble T. cruzi extract (unpublished data). In the present work, we explored the possibility that exogenous activators of the KKS, such as dextran sulfate, might serve as vaccine adjuvants. Before testing the immunostimulatory effects of dextran sulfate (DXS), Dr. Clarissa Nascimento and Dr. Erik Svensjo used intravital microscopy to investigate the dynamics of KKS activation in peripheral tissues. Using hamster cheek pouch topically exposed to DXS, they noted that DXS-induced microvascular leakage progresses as a bi-phasic process. After a prolonged "lag" period of ~30 min characterized by absence of microvascular leakage, DXS induces an potent edema reaction that is initially confined to a few post-capillary venules in the HCP microcirculation. Within minutes, there is a transition from minute extravasation- involving a few post capillary venules- to the explosive phase of inflammation. Notably, the expansion of the inflammatory wave is critically dependent on activation of the mast cell/KKS axis. Based on these findings, we are currently re-designing strategies to deliver DXS and vaccine antigens altogether into the draining lymph nodes, in ways that prevent premature release of nociceptive BK in peripheral tissues. In contrast, by targeting DXS to the intralymphoid compartment, we might be able to activate DCs via BK2R, ultimately harnessing development of Th1 effector T cells.

Role of MyD88 in modulation of adaptive immunity during Chagas Disease (Subsidiary Projects headed by Prof. AC. Oliveira).

In the last decade, several studies have shown that MyD88-deficient mice are highly susceptible to infection with different intracellular parasites, including Trypanosoma cruzi. Recently, the lab of Maria Bellio observed that, despite the greater susceptibility, IFN-gamma production and cytotoxicity mediated by CD8 T cells are intact in MyD88-deficient mice infected with this parasite (Oliveira et al., PLoS Pathogens, 2010). Since MyD88 is an essential adaptor molecule for signaling through TLR, IL-1R and IL-18R, this group aimed to investigate the intrinsic role of MyD88 expression in CD4 and CD8 T cells during infection with this parasite. In order to answer this question, the production of IFN-gamma and cytotoxic mediators by CD4 and CD8 T cells, as well as T cell migration to heart, in mixed (WT + MyD88KO→WT) bone marrow chimeric mice infected with T. cruzi were analyzed by intracellular staining and flow cytometry (FACS).

AL18 publications (2011-2012):

1. *Andrade D, Serra R, Svensjö E, Lima AP, Ramos ES Jr, Fortes FS, Morandini AC, Morandi V, Soeiro M de N, Tanowitz HB, Scharfstein J.* Trypanosoma cruzi invades host cells through the activation of endothelin and bradykinin receptors: a converging pathway leading to chagasic vasculopathy. Br J Pharmacol. 165:1333-47, 2012.

2. *Nico D, Feijó DF, Maran N, Morrot A, Scharfstein J, Palatnik M, Palatnik-de-Sousa CB.* Resistance to visceral leishmaniasis is severely compromised in mice deficient of bradykinin B2-receptors. Parasit Vectors. 14;5:26-32, 2012.

3. *SVENSJÖ E, SARAIVA EM, AMENDOLA RS, BARJA-FIDALGO C, BOZZA MT, LERNER ET, TEIXEIRA MM AND SCHARFSTEIN J*. MAXADILAN, THE *LUTZOMYIA LONGIPALPIS* VASODILATOR, DRIVES PLASMA LEAKAGE VIA PAC1-CXCR1/2-PATHWAY. *MICROVASCULAR RESEARCH 83:185-193, 2012*.

4. *Rust NM, Papa MP, Scovino AM, da Silva MM, Calzavara-Silva CE, Marques ET Jr, Peçanha LM, Scharfstein J, Arruda LB*. Bradykinin enhances Sindbis virus infection in human brain microvascular endothelial cells. Virology. 422: 81-91, 2012

Invited Reviews:

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7. *RODRIGUES, M. M.; OLIVEIRA, A.C. AND BELLIO, M.* THE IMMUNE RESPONSE TO *TRYPANOSOMA CRUZI*: ROLE OF TOLL-LIKE RECEPTORS AND PERSPECTIVES FOR VACCINE DEVELOPMENT. JOURNAL OF PARASITOLOGY RESEARCH, V. 2012, P. 1-12, 2012.

Book Chapters:

8. *Scharfstein, J and Svensjo E*. Chapter 20. The Kallikrein-Kinin System in Parasitic Infections. In: "Kinins". Edited by M. Bader. De Gruyter GmbH7 Co. KG, Berlin/Boston, 2012.

AL 19

ASSOCIATE LABORATORY OF CELLULAR AND MOLECULAR NEUROBIOLOGY

Coordinator: Rosália Mendez Otero – IBCCF/UFRJ.

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Marcelo Felippe Santiago – IBCCF/UFRJ

Main Research Lines and Objectives:

The main lines of research in our group aim to establish animal models of neurological diseases which will allow us to test the safety and efficacy of therapy with stem cells, steps necessary for clinical studies with stem cells in neurological patient. The isolation and characterization of the stem cells to be used in the therapies is also an important component of our research. It is also important to be able to label the cells in order to investigate the migration and homing of these cells after transplantation into the animal models and patients. In this respect, we have investigated labeling techniques which could be used both in pre-clinical and clinical studies. During the period covered by this report (January 2011- Dez 2012) we were able to conclude some of the goals of our proposal and the main results of each specific objective will be summarized below:

Specific Objectives/Goals: progress reached in this period

Evaluate the effectiveness of stem cell therapy with multipotent (mesenchymal cells, endothelial progenitors and neural stem cells) and pluripotent (embryonic and inducible) in animal models of neurological diseases:

We have investigated the functional benefit of cell therapy with multipotent stem cells in several models of neurological disorders. In some of the models we showed that that cell therapy with the mononuclear fraction or with mesenchymal stem cells reduces the functional deficits generated by the lesion to the nervous system. In addition, we have investigated the cellular and molecular mechanisms involved in this improvement and demonstrated that multipotent stem cells released factors that resulted in neuroprotection and also reduced the response of the reactive microglia. For example, in the animal model of optic nerve lesion (a model of lesion to the central nervous system) we compared the effect of mononuclear cells and

mesenchymal cells obtained from the bone marrow and we were able to show an beneficial effect on ganglion cell survival and axonal regeneration. We have also concluded the study in a model of Huntington disease and the results revealed that there is a decrease in neuronal death in the animals treated with mesenchymal stem cells. In this model we were able to show that the injected cells can be traced in vivo for several weeks using RMI. We have concluded during this period several other projects and the results have been published (Jasmin et al., 2012a, b; Mendez-Otero and Campos de Carvalho, 2012; Miguel et al., 2012; Ribeiro-Resende et al., 2012; Moraes et al., 2012; Mesentier-Louro et al., 2012; Friedrich et al., 2012; Giraldi-Guimaraes et al., 2012; Vasconcelos-dos-Santos et al., 2012; Pimentel-Coelho et al., 2012; Miyakoshi et al., 2012; Jasmin et al., 2011; de Azevedo-Pereira et al., 2011; Battistella et al., 2011; Zaverucha-do-Valle et al., 2011).

Other studies are still in progress. It is important to mention that the results from the pre-clinical studies allowed us to propose a Phase I clinical study to evaluate the safety of cell therapies with multipotent stem cells from the bone marrow in patients with ischemic stroke. The phase I study was concluded and published and the phase II/III study to evaluate efficacy was approved and will start to recruit patients in 2013.

Test the labeling of different types of stem and progenitor cells with superparamagnetic iron oxide nanoparticles (SPION) – in vitro and invivo;

Establish protocols for incorporation of nanoparticles by different types of stem cells through reaction for detection of SPIO;

Investigate the effects of incorporated SPIONs on the proliferation, differentiation and cell death in vitro and in vivo;

Develop new coatings to increase the capacity of incorporation of nanoparticles by cells and/or by specific sub-population:

In the clinical studies, we have used stem cells labeled with ^{99m}Technetium in order to analyze the migration and homing of the transplanted cells to the lesioned region in the patients. However, the half-life of this radioactive compound is of approximately 6 hs which gives us only a maximum of 24 hs to visualize migration and homing of the injected cells. To solve this problem we have investigated the possibility of labeling different stem cells (pluri and multipotent) with commercial available SPIONs and also with SPIONs specially generated by our group. We were able to establish protocols for each cell type and for the different SPIONs. Using these protocols we have also tested the proliferation, viability and differentiation capacity of the labeled cells and concluded that the incorporation of SPIONs does not affect any of these cellular functions. We have now used these protocols to labeled different types of cells with SPIONs and we were able to follow the homing of these cells in vivo using RMI in the animal models of neurological diseases.

Evaluate the safety and effectiveness of different types of labeled cells in different animal models of disease (nervous system,

heart and kidney) with respect to toxicity and limit of detection;

Verify whether the transplanted cells migrate to the lesion sites through the same reactions as well as monitor their destination by MRI at different times after the transplant;

Evaluate the possible role of labeled cells in animal models of cell therapy:

These are ongoing projects and we are still in the process of performing the experiments and analyzing the results. We have however preliminary results with respect to toxicity and limit of detection. We have found that we can detect 100.000 mesenchymal stem cells labeled with SPIONs using a 7 T RMI. The cells were injected into the spinal cord of an adult mouse and the signal was still present 77 days after the injection (Fig 1).

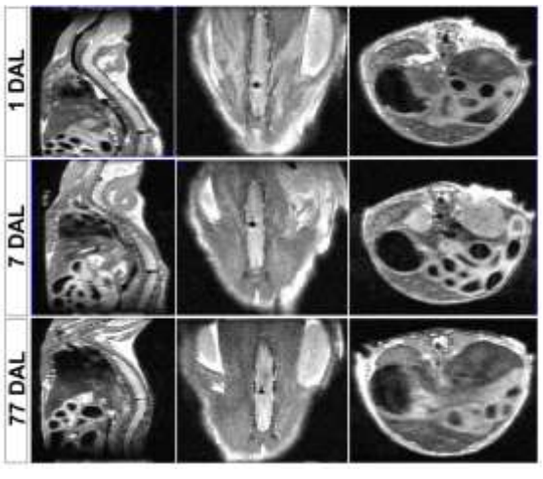

Fig 1. MSC labeled with SPIONs were injected into the spinal cord of an adult mouse. We can see the hipointense signal 1, 7 and even 77 days after the injection.

New strategies to recover cognition in aging and neuropsychiatric disorders:

Cognition involves a group of mental process by which we process information and apply knowledge, to define preferences and how we see the world. Cognitive impairments develop with aging and are present in most of the neuropsychiatric disorders, including Alzheimer disorder, depression and schizophrenia. Cognitive impairments are not well treated by current therapies and are major contributors to disability. The group of Prof. Rogerio Panizzutti study new strategies to recover cognition in aging and in neuropsychiatric disorders. Since the interaction between genes and environment are in the origin of most of these conditions the group has been study the effect of environmental stressors on mice from different genetic background. In the last year the group described the effect of acute stress on different cognitive tests and observed that acute stress induces deficits in sensorimotor gating and memory in mice. Decreased levels of the neuromodulator D-serine in the cerebral cortex and hippocampus accompanied the cognitive impairment. Interestingly, the acute administration of D-serine to the stressed mice reverted the impairment almost completely. Now the group is studying the effect of stress on the cognition of mutant mice that are deficient in D-serine, and in rats subjected to maternal experimental infection, an environmental factor associated to neuropsychiatric disorders.

In the CENABIO the group successfully installed and operated the first equipment in Rio de Janeiro to study the pre-pulse inhibition of the startle response (PPI) in rodents. PPI is a neural response that measure sensorimotor gating and is affected in neuropsychiatric disorders. The equipment was installed in the CENABIO and has been used

by different research groups from the Medical Biochemistry Institute and Biomedical Sciences Institute.

Using the PPI the group studied the neural effect of the application of pulsed electromagnetic fields (PEMF). PEMF has been used for the treatment of depression and the group asked whether PEMF would be useful for the treatment of cognitive deficits present in schizophrenia. Remarkably, PEMF application completely reverted the deficit in PPI present in an animal model of cognitive impairment in schizophrenia. Now the group is studying the effect of PEMF on other cognitive tests and the brain regions affected by PPI and PEMF by means of immunohistochemistry of the immediate early gene c-fos (Figure 1). The next step will be to translate these pre-clinical studies to patients in other to establish new PEMF applications.

NEURONAL ACTIVATION IN THE CEREBELLUM AFTER PPI RESPONSE. IMMUNOHISTOCHEMISTRY FOR C-FOS REVEALS NEURONS WITH THE TYPICAL SHAPE OF PURKINJE CELLS THAT WERE ACTIVATED FOLLOWING THE PPI PROTOCOL.

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2. Global update: Brazil. Mendez-Otero R, Campos de Carvalho AC. Regen Med. 2012 Nov;7(6 Suppl):144-7.

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9. Bone marrow mononuclear cells and mannose receptor expression in focal cortical ischemia. Giraldi-Guimarães A, de Freitas HT, Coelho Bde P, Macedo-Ramos H, Mendez-Otero R, Cavalcante LA, Baetas-da-Cruz W. Brain Res. 2012 May 3;1452:173-84. doi: 10.1016/j.brainres.2012.03.002. Epub 2012.

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16. Bone marrow mononuclear cells increase retinal ganglion cell survival and axon regeneration in the adult rat. Zaverucha-do-Valle C, Gubert F, Bargas-Rega M, Coronel JL, Mesentier-Louro LA, Mencalha A, Abdelhay E, Santiago MF, Mendez-Otero R. Cell Transplant. 2011;20(3):391-406. doi: 10.3727/096368910X524764.

AL 20

ASSOCIATE LABORATORY OF INFLAMMATION AND METABOLISM

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Members:

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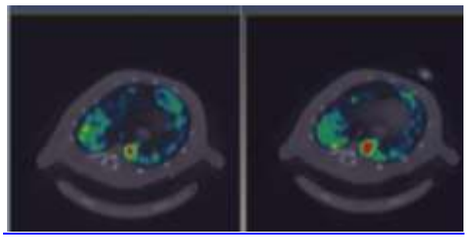
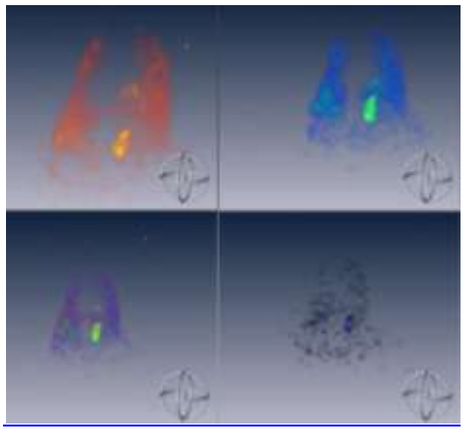
Antonio Giannella Neto – PEB/UFRJ
Hugo C. Castro Faria Neto - Fiocruz/RJ
Marcus F. Oliveira – IBqM/UFRJ
Rosana Souza Rodrigues – ID'OR/HUCFF-UFRJ
Walter Araújo Zin - IBCCF/UFRJ

In this brief report, we will address the main research areas of the Laboratory of Inflammation and Metabolism from the INCT of Structural Biology and Bioimaging, Lab 20. In summary, two are the main research areas in our group. Despite both of them deal with applications of biomedical imaging in different problems and fields of research, the main focus is on the metabolism repercussions of a given inflammatory process. In the first, the uptake pattern of 18-fluorodeoxyglucose (18FDG) in the lungs at the very early stage of acute lung injury is the main field of interest. In the second one, the application of biomedical imaging in the field o neuroinflammation and aging is the main topic. Each area will be briefly described as follows.

18-FDG uptake in early acute lung injury

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), are syndromes of acute respiratory failure that result in acute pulmonary edema and inflammation. ALI and ARDS are a major problem in critically ill patients because their high incidence and, despite advances in supportive therapy, their mortality remains unacceptably elevated. The diagnosis of ALI and ARDS is based on clinical, radiological and gas exchange parameters, but those are late events occurring after molecular signaling and fluid accumulation in the lung. Traditional methods of imaging (chest x-rays and computed tomography) have small sensitivity and specificity in the early diagnosis. Positron emission tomography (PET) with 18-FDG has been considered a noninvasive and highly sensitive imaging technique that can be used to quantify pulmonary inflammation.





In the Laboratory of Inflammation and Metabolism we are interested in describing the pattern of 18F-FDG uptake in the lung parenchyma of experimental models of ALI in rats and mice (Figure 1). Additionally, we are interested in the early uptake pattern and in the kinetics of glucose incorporation by the lung parenchyma of rodents with ALI, as well as in the molecular mechanisms responsible for such event.

Imaging and Neuroinflammation

Another important research field of the Laboratory of Inflammation and Metabolism is the application on biomedical images in the study of neuroinflammation, aging and dementia. For decades, magnetic resonance imaging (MRI) has provided non-invasive assessment of many neurological disorders, as well as acute neuroinflammatory diseases. MRI allows macrostructural, cellular and metabolic measurements. T1-weighted gradient echo images show gray and white matter abnormalities. T2-weighted spin echo images highlight fluid from neuroinflammation. Advanced diffusion-weighted imaging (DWI) shows demyelination, axonal damage and remyelination. Phosphorous MR spectroscopy (31P-MRS) provides simultaneous in vivo bioenergetic assessments, such as ATP, PCr (phosphocreatine) and Pi (inorganic phosphate). Intracellular pH can be assessed by the chemical shift of Pi relative to PCr. Longitudinal assessments may reflect changes in energy metabolism and mitochondrial function due to pathological processes.

More sophisticated MRI techniques may have applicability to severe neuroinflammatory conditions, such as sepsis, and measurements of reactive oxygen species (ROS). Perivascular edema, and spectroscopic abnormalities can be demonstrated by MRI and 1H-MRS in a mouse sepsis model (Bozza et al., JCBFM 2010). The detection of oxidative damage and reactive oxygen species (ROS) by MR techniques is now possible through the use of Gd-based spin trapping contrast agents that track the formation of protein radicals. It is unknow whether characteristic MR abnormalities define subjects with age-associated chronic low-level neuroinflammation, or whether disease activity may be assessed by either traditional or novel MR techniques.

Positron emission tomography (PET) is used for evaluation of dementia, seizures, and for assessment of recurrent tumor versus radiation necrosis, and may be applicable to neuroinflammatory disorders. Activated microglia, monocytes and macrophages show an increase in expression of peripheral benzodiazepine receptors (PBR). PBR binding ligands, such as [(11)C]PK11195 are currently under development and investigation and may play a future role in assessing neurodegenerative where inflammation plays a role. 18F fluorodeoxyglucose (FDG) PET shows increased uptake in acute cerebral inflammation, and decreased uptake in the late stages of the diseases. Increased uptake of glucose analogs is a very early event (< 6h) in experimental sepsis, possibly from excitotoxity or activation of microglial orastrocytes (Figure 2). Decreased FDG uptake in neocortical regions of the brain 24h after endotoxin, and could be due to neuronal injury or dysfunction. FDG PET may

provide assessment of both the acute and chronic phases of neuroinflammation.

In AD, decreased uptake of FDG occurs in the parieto-temporal, cingulate, and medial temporal cortices. MR findings of AD show early hippocampal and medial temporal volume loss. The discrepancy between FDG PET and MRI findings may be due to technical limitations of PET in measuring small structures. Many patients with mild cognitive impairment (MCI) show similar, but less-severe, regional hypometabolism. This might lead to the speculation that pre-clinical AD can be diagnosed by FDG PET, but this has not been supported by longitudinal studies. MRI and FDG PET may provide useful indicators that MCI has progressed into frank AD, particularly when longitudinal studies can be performed. However, the utility of MRI or FDG PET for characterizing MCI and chronic neuroinflammation has not been studied.

The brains of AD patients reveal beta-amyloid plaques and neurofibrillary tangles, which contain beta-amyloid peptides (Abeta) and highly phosphorylated tau proteins. Abeta deposition leads to an increase in beta-amyloid plaques, the initial neuropathological change in AD. Radiotracers for in vivo imaging beta-amyloid in brain is an important focus of research development. The most widely used and studied of these agents is N-methyl-[(11)C]2-(4'-methylaminophenyl)-6-hydroxybenzothiazole ([(11)C]PIB). (11C)PIB positron emission tomography (PET) has been validated as showing increased binding in subjects with AD, compared to normals. (11)C]PIB binding also occur in subjects with mild cognitive impairment (MCI) and also some elderly normal patients without neurocognitive effects. Abeta deposition, the primary pathological feature of AD, has also been shown occur in response to lipopolysaccharide-induced neuroinflammation in animal models. (11C)PIB uptake may, in some cases, indicate a population of individuals with ongoing neuroinflammation due to chronic or recurrent low level systemic inflammation. However, this has not been studied.

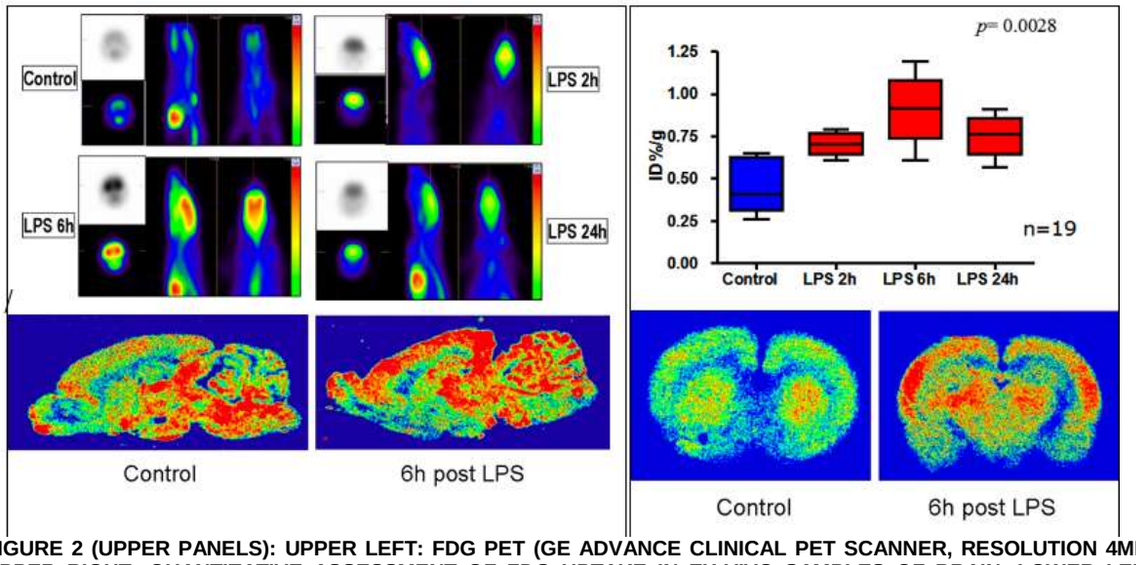


FIGURE 2 (UPPER PANELS): UPPER LEFT: FDG PET (GE ADVANCE CLINICAL PET SCANNER, RESOLUTION 4MM). UPPER RIGHT: QUANTITATIVE ASSESSMENT OF FDG UPTAKE IN EX-VIVO SAMPLES OF BRAIN. LOWER LEFT: DIGITAL FLUORESCENCE AUTORADIOLOGRAPHY (BAS-3000, RESOLUTION 40 UM) OF NBDG. LOWER RIGHT: PHOSPHOR IMAGER (BAS-5000) DIGITAL AUTORADIOGRAPHY (RESOLUTION 25 UM) OF 14C-2DG. IMAGES SHOW A SIMILAR PATTERN, WITH INCREASED CORTICAL UPTAKE OF GLUCOSE ANALOGS AT EARLY TIME POINTS POST LPS, WHICH DECREASES BY 24H.

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9. Antonaglia, V.; Ferluga, M.; Capitanio, G.; Lucangelo, U.; Piller, F.; Roman-Pognuz, E.; Biancardi, B.; Caggegi, G.D.; Zin, W. A. Respiratory mechanics in COPD patients who failed non-invasive ventilation: role of intrinsic PEEP. Respiratory Physiology & Neurobiology, v. 184, p. 35-40, 2012.

10. Araujo, C. V. ; Freitas, V. E. ; Tibirica, E. V. ; Bozza, P. T. ; Castro-Faria-Neto, H. C. ; Silva, A. R. . Ppar Gamma Activation Protects The Brain Against Microvascular Dysfunction In Sepsis.. Microvascular Research (Print), V. 84, P. 2, 2012.

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Scientific meetings and training of human resources

INBEB Annual Meetings

The Annual Meetings organized by the Institute also allows our AssociateLaboratories to show their work to other members and external researchers. Thereby, INBEB groups can enjoy an opportunity to interact, improve their work and establish new partnerships.

- 3rd Annual Meeting

The **Third** Annual Meeting of INBEB occurred on 28 to 30 November, 2011, in the Auditorium Rodolpho Paul Rocco, at the Federal University of Rio de Janeiro. The event presented the scientific results of the 20 Associate Laboratories from INBEB, including various lectures and about 200 posters.

This meeting was also the occasion for inauguration of new equipment, the PET / SEPCT / Micro-CT for small animals. This tool, derived from applications in nuclear medicine, is capable of generating three-dimensional functional images of organs, systems or whole animal body. The inauguration was preceded by a roundtable on bioimaging applications.

JERSON SILVA, THE GE REPRESENTATIVE, AND PROFESSOR ALYSSON (of AL20) EXPLAINING THE EQUIPMENT FEATURES.

STUDENTS PRESENTING THEIR WORK. *CREDIT: MAURICIO CASTRO.*

- 4TH ANNUAL MEETING

The Fourth Annual Meeting of INBEB occurred on 8, 9 and 10 of May, 2013, in the Professor Hélio Fraga Auditorium, at the Federal University of Rio de Janeiro. The event also presented the scientific results of the 20 Associate Laboratories from INBEB, including various lectures and about 200 posters.

This meeting was This meeting was attended by the Swiss researcher Kurt Wüthrich, Nobel Prize winner for chemistry in 2002. During the event, it was inaugurated a room-office for Wüthrich at the INBEB headquarters. The Nobel Prize winner also presented the main conference: "Historical Development and Current Trends of NMR in Structural Biology and Biotechnology".

Wüthrich maintains a partnership with the Institute through the federal program Science Without Borders, and has served as a visiting professor at INBEB / UFRJ since July 2012, which guides the work of doctoral and post-doctoral.

PROFESSOR WÜTHRICH AT THE CONFERENCE AND AT HIS OFFICE.

FAPERJ SCIENCE FAIR

The INBEB attended the science fair organized by the Rio de Janeiro's Foundation for Research Support (FAPERJ). It took place on 29 and 30 June 2011, at the Cultural Center for Citizen Action, at the city´s newly remodeled Seaport Zone.

INBEB STAND AT THE 2011 FAPERJ SCIENCE FAIR.

SCIENCE COURSES AND MEETINGS

In the last two years, the Institute took part in or promoted various events and courses. Some of them are listed below.

- WORKSHOP ON MICROSCOPY

Advanced Techniques in Correlation Spectroscopy and Fluorescence Microscopy – Spectroscopy through the microscope.

Speakers: Michelle Digman and Enrico Gratton (Laboratory for Fluorescence Dynamics - University of California at Irvine). Coordinator: Professor Andre Gomes.

1st day 14/03 - Introduction to Fluorescence Correlation Spectroscopy (FCS).

2nd day 15/03 - Advanced Techniques in Raster Imaging Correlation Spectroscopy / RICS e Number and Brightness / N & B).

3rd day 16/03 - Images of Fluorescence Lifetime and its analysis through "Phasors".

The lectures were given from 9:00 am to 12:30 pm at the Auditorium Marcos Moraes, UFRJ.

- Hands-on workshop on fast protein NMR

Organizers: Fabio C. L. Almeida - Federal University of Rio de Janeiro, Jonas Fredriksson - Federal University of Rio de Janeiro, Martin Billeter - Chemistry & Molecular Biology, University of Gothenburg, Sweden. Date: March 21-23, 2012

The course provided an introduction to fast NMR techniques and their application to protein studies. Lectures were complemented by exercises, instrument demonstrations, and a hands-on computer lab.

Topics included relations between experiment time, spectral resolution, number of dimensions and sensitivity. An overview described non-uniform sampling schedules as well as experimental and processing aspects, together with illustrative examples. Concepts presented included pulse sequences, random sampling, projections, decompositions, and optimized sampling. Various algorithms were discussed (e.g. GFT, PR, APSY, MDD, TAD...). The Projection-Decomposition approach (PRODECOMP) was explained with regard to assignment and 3D structure, and further demonstrated in a computer lab.

The course was held at the Federal University of Rio de Janeiro.

- Site training on high pressure NMR

On May 28, 29 and 30, 2012, the INBEB held a training course in the use of high pressure coupled to nuclear magnetic resonance, which featured two lectures given by Professor Professor Joshua Wand/Benjamin Rush Professor of Biochemistry & Biophysics/Department of Biochemistry & Biophysics/University of Pennsylvania Perelman /School of Medicine and the Chief Executive of Daedalus Innovations LLC, Ronald W. Peterson..

5/28/12, 4 p.m.: "Recent Advances in NMR Spectroscopy of Encapsulated Proteins in Low Viscosity Fluids".

5/29/12,4 p.m.: "The dark energy of proteins comes to light: Protein motion, conformational entropy & protein function“.

- Workshop: small animal imaging and translational imaging

On September 27/28, 2012 this Workshop was offered by the INBEB and the Institute D'Or. The event featured a cycle of lectures and hands-on equipment training for bioimaging in small animals: USG, Bioluminescence, MRI and PET-CT. The talks took place in the Auditorium Hélio Fraga at UFRJ.

- Mini Workshop on prions

On October 23, 2012, the INBEB organized a Mini Workshop on "Prions and Protein Misfolding Diseases in Amyloids." The event featured the following conferences:

- Dr. Claudio Soto, Visiting Professor / Department of Neurology / University of Texas Health Science Center at Houston: "Prions, transmissible neurodegenerative diseases and proteins."

- Dr. Rafael Giraldo, Professor / Centro Biológico de Investigaciones / Department of Cell and Molecular Biology / CSIC / Madrid / Spain: "Mad bacteria: untangling amyloid proteinopathies through a minimal synthetic prionoid."

- Science without Borders in Structure Determination of Excited States

On November 22/23, 2012, the Nuclear Magnetic Resonance Laboratory of Biomolecules (BioNMR) organized this event

to celebrate the arrival, as Special Visiting Professor at UFRJ for the next three years, of the researcher Dmitry Korzhnev, who pioneered the development of technology for determination of high-resolution structures of proteins in excited states.

- II Brazilian meeting on research integrity, science and publication ethics (II BRISPE)

Coordinated by AL 2 members, with IBqM,COPPE/UFRJ; Fiocruz, USP, PUC/RS. May 28 – June 1, 2012.

Research integrity and responsible conduct of research (RI/RCR) have been high on the policy agenda of the countries responsible for most activities in science, technology and innovation. Latin America, including Brazil, has had little visibility in this international debate, as the approach to RI/RCR in the region is at its early stages.

The 2nd Brazilian Meeting on Research Integrity, Science and Publication Ethics (II BRISPE) was organized to broaden the participation of the Brazilian academic community in these international conversations and to stimulate the interest of researchers, educators and policy makers in dialogues that include science & society, accountability in science, public trust in science and research excellence. Meetings and conferences were held in Rio, São Paulo and Porto Alegre. Site: http://www.iibrispe.coppe.ufrj.br/. This meeting resulted in an invitation to submit a proposal for Brazil to host the 4th World Conference on Research Integrity, in 2015 or 2016.

Related publication: Vasconcelos SM, Steneck NH, Anderson M, Masuda H, Palacios M, Pinto JC, **Sorenson MM.** (2012) The new geography of scientific collaborations. Changing patterns in the geography of science pose ethical challenges for collaborations between established and emerging scientific powers. EMBO Rep. 13:404-407

- Other symposia, courses and congresses organized

By AL 9 members:

Scientific Meetings (Organizing Committee):

- Simpósio Sul Brasileiro de Microscopia e Microanálise, Maringá, PR. 2012 (Marcia Attias, Wanderley de Souza, Kildare Rocha de Miranda).

- IX Congresso Interamericano de Microscopia- CIASEM 2011 . 25-29 setembro, 2011 Advisory Committee (Marcia Attias, Wanderley de Souza).

- XXIII Congresso da Sociedade Brasileira de Microscopia e Microanálise. Armação dos Búzios. 15 a 18 de outubro de 2011. Organizing

Committee. (Wanderley de Souza, Marcia Attias, Kildare Rocha de Miranda).

Courses offered:

- 1º Curso Teórico-Prático de Microscopia Eletrônica da UFES-Departamento de Morfologia e Pós-Graduação em Biotecnologia do Centro de Ciências da Saúde. Universidade Federal do Espírito Santo 04 a 15 de julho de 2011 (Marcia Attias e Kildare Miranda).

- Three dimensional reconstruction of biological samples - Márcia Attias, IX Congresso Interamericano de Microscopia, CIASEM 2011

- Microanalysis applied to biomedical sciences- Kildare Miranda, IX Congresso Interamericano de Microscopia - CIASEM 2011.

- Cryotechinques applied to Biomedical Sciences Marlene Benchimol. IX Congresso Interamericano de Microscopia- CIASEM 2011

- Advances in SEM and its applications in Biomedical Sciences Wanderley de Souza. IX Congresso Interamericano de Microscopia-CIASEM 2011.

By AL 12 members:

Marlene Benchimol- Conferences At The XXXI International Congress Of Protistology / XXVI Annual Meeting of The Brazilian Society Of Protozoology / XXXVII Annual Meeting On Basic Research In Chagas Disease, The Round Table Discussion On Cell Therapy In The Treatment Of Chagas Disease.

Annotation of Centrioles. Imagens on line. , 2011. PEREIRA, A. N., BENCHIMOL, M.

Museum Exposition at Fiocruz about reproduction –participation

BENCHIMOL, M., ANDREATA, R., E F Albuquerque VIII Congresso do Progrma Institucional de Bolsas de Iniciação Científica-PIBIC, 2012. (Congresso, Organização de evento)

BENCHIMOL, M. 10th International Congress on Cell Biology, 2012. (Congresso, Organização de evento)

D. Esdras Teixeira, Crepaldi P. H., BENCHIMOL, M. Nascer um fenômeno comum a todos, 2011. (Exposição, Organização de evento)

By AL 18 members:

XIV. Open Teaching Courses (vacation Activities) for High-Students:

Theme: Immunological and microbiological aspects of oral health

Tema: “Parâmetros imunológicos e microbiológicos na saúde oral”

July 2011; Location: CCS- Bloco D.

Comment: Funded by FAPERJ. Students (n=25) were introduced to basic concepts in oral microbiology, immunology/inflammation for one week. The teaching activities were coupled to free-dental care services and personal advises on oral care at the Faculty of Odontology.

Organizer: Prof. J. Scharfstein, Prof. E. Feres-Filho (Faculty of Odontology, HU-UFRJ) and Prof. R. Hirata (UERJ). Collaborator: Dr. E. Svensjo.

XVI. Honor Lecture: Prof. Julio Scharfstein. Closing Conference at the XXVIII Annual Congress of the Brazilian Society of Protozoology, Caxambu, October 2012.

Closing Lecture: "Roles of the Kallikrein-Kinin System in the Pathogenesis of Chagas(Heart) Disease: New Lessons from Experimental Models" ,

XXVIII Annual Meeting of the Brazilian Society of Protozoology/XXXIX Annual Meeting on Basic Research in Chagas' Disease, Caxambu, October 2012.

REGULAR SEMINARS

The Institute promotes regular seminars at the Federal University of Rio de Janeiro. In the biennium 2011 and 2012, 14 lectures were organized, as listed below:

23/10/2012:

- Dr. Claudio Soto, Visiting Professor/Department of Neurolgy/University of Texas Health Science Center at Houston: "Prions, neurodegenerative diseases and transmissible proteins”.

- Dr. Rafael Giraldo, Professor from Centro de Investigaciones Biológicas/Departamento de Biologia Celular e Molecular/CSIC/Madrid/Espanha: “Mad bacteria: Untangling amyloid proteinopathies through a minimal synthetic prionoid”.

17/09/2012:

- Dr. Deborah Schechtman, Professor from Departamento de Bioquímica/IQ/USP: "Proteina quinase C e seus substratos em células tronco indiferenciadas".

22/08/2012:

- Dr. David Veesler, Research Associate/The structural Virology lab - Johnson's lab/Department of Molecular Biology/The Scripps Research Institute: "Life in the extremes: atomic structure of Sulfolobus Turreted Icosahedral Virus".

20/08/12:

- Dr. Ovidiu Radulescu/DIMNP (Dynamique des Interactions Membranaires Normales et Pathologiques/Université de Montpellier/FR: "Stochastic fluctuations of gene expression: making sense out of noise".

18/05/2012:

- Professor Sir Alan Fersht, Emeritus Herchel Smith Professor of Organic Chemistry,Department of Chemistry, MRC Laboratory of Molecular Biology, Cambrigde University:"The tumour suppressor p53: from structural biology to drug discovery"

- Dr. Christopher Jaroniec, Associate Professor, Department of Chemistry, The Ohio State University: "Atomic-resolution studies of protein structure and dynamics by magic-angle spinning solid-state NMR spectroscopy".

12/03/2012:

- Prof. Neil Cashman, Univ. British Columbia - Scientific Director, PrioNet/Canada: "Protein Misfolding Diseases".

05/03/2012:

- Dr. Marco A.M. Prado, professor from Department of Physiology & Pharmacology and Department of Anatomy & Cell Biology at University of Western Ontario, Canadá: "Dissecting neuronal and astrocyte communication using genetically-modified mice: implications for neurodegenerative diseases".

13/09/2011:

- Dr. Fernando Palhano, professor from Instituto de Bioquímica Médica da UFRJ: "Patologia e funcionalidade das fibras amilóides".

30/08/2011:

- Dr. Göran Hedenstierna, professor from Uppsala University-Stockholm, in Sweden: "Airway closure and atelectasis during anesthesia: causes of hypoxemia".

14/06/2011:

- Dr. Sotiris Missailidis, professor from Open University in England: "Design and use of aptamers as novel inhibitors, therapeutic, imaging and diagnostic agents in cancer and inflammatory disease".

29/04/2011:

- Dr. Dimitry Korzhnev, Microbial and Structural Biology (MMSB) at the University of Connecticut Health Center. "NMR studies of transiently populated protein folding intermediates: from detection to high-resolution structure".

29/03/2011:

- Professor Rogério Panizzutti, from Instituto de Ciências Biomédicas da Universidade Federal do Rio de Janeiro (UFRJ): "Cognição, plasticidade e treinamento cerebral na esquizofrenia: do modelo animal ao paciente (e vice-versa)".

21/02/2011:

- Dr. Pierre Hainaut, form International Agency for Research on Cancer, Lyon, France: "Lung cancer: new insights into mechanisms through genomics".

11/01/2011:

- Dr. Fabiana Munhoz, post-doctoral at the Fresnel Institute, in Marseille, France: “Microscopia CARS e aplicações”.

Training and teaching human resources

The INBEB members are integrated into graduate programs, several of which were given a grade of 6 or 7 by the CAPES evaluation. To date, a total of 80 master's dissertations and 60 doctoral theses have been completed by INBEB members, as shown below:

- Doctoral theses completed:

AL 1

Danielly Cristiny Ferraz da Costa. Aspectos Celulares e Estruturais da Modulação da Proteína Supressora de Tumor p53 por Resveratrol. 2011. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Advisor: Jerson Lima da Silva.

Elington Lannes Simões. Neuroplasticidade em Amputados: Um Estudo de Neuroimagem por Ressonância Magnética. 2012. Tese (Doutorado em Ciências Morfológicas) - Universidade Federal do Rio de Janeiro, . Advisor: Fernanda Freire Tovar Moll.

Luiza da Cunha Stankevicins. microRNAs in breast cancer progression and DNA damage response. 2012. Tese (Doutorado em Fisiopatologia Clínica e Experimental) - Universidade do Estado do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Advisor: Cláudia Vitória de Moura Gallo.

Mariana Pierre de Barros Gomes. Interação PrP-RNA: aspectosestruturais e estudos de toxicidade. 2011. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Fundação Carlos Chagas Filho de Amparo à Pesq. do Estado do Rio de Janeiro. Advisor Jerson Lima da Silva.

Marcelo Barbosa de Accioly Mattos. Estudo da prevalência de anticorpos contra Aa e bacteriofagos em pacientes com Periodontite. 2012. Tese (Doutorado em Odontologia) - Universidade do Estado do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Advisor: Davis Fernandes Ferreira.

Shana Priscila Coutinho Barroso. Avaliação da capacidade imunogênica do vírus da Influenza aviária H3N8 inativado por pressão hidrostática. 2008-2012. 2012. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Advisor Andrea Cheble de Oliveira.

AL 2

Adriana Fonseca Marques. Regulação Alostérica de Cisteíno-Protease de Plasmodium: potencial uso terapêutico. 2011. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Orientador: Luis Mauricio Trambaioli da Rocha e Lima.

Priscila dos Santos Ferreira da Silva. Estudos Estruturais e Celulares com a Proteína Amiloidogênica Transtirretina: Da Proteína à Doença. 2013. Tese (Doutorado em Quimica Biológica) - Instituto de Bioquímica Médica, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Orientador: Debora Foguel.

Vivian de Almeida Silva. Bases estruturais e termodinâmicas do enovelamento proteico e reconhecimento molecular: modelos transtirretina e trombina. 2011. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, . Orientador: Luis Mauricio Trambaioli da Rocha e Lima.

AL 3

Carolina Cruzeiro da Silva. Determinação da estrutura e dinâmica de tioredoxinas. 2011. 0 f. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Co-Orientador: Ana Paula Canedo Valente. Orientador: Fabio Ceneviva Lacerda Almeida.

Carolina Galvão Sarzedas. Determinação da estrutura ligada a membrana do peptídeo de fusão do virus VSV. 2011. 0 f. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Co-Orientador: Ana Paula Canedo Valente. Orientador: Fabio Ceneviva Lacerda Almeida.

Rodolpho do Aido Machado. Estudos estruturaisl da proteína FKBP12 de Trypanosoma brucei. 2012. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Orientador: Jose Ricardo Murari Pires.

AL 4

Ana Cristina Ferraz Nogueira. Identificação E Caracterização Do Sítio De Interação E Das Mudanças Conformacionais Da Trombina Induzidas Pela Botrojaracina. 2012. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Orientador: Russolina Benedeta Zingali.

Carlos Correa Netto. Venômica e antivenômica; uma relação intrínseca no desenho e controle de qualidade de soros antiofídicos.. 2011. Tese (Doutorado em Quimica Biologica) - Instituto de Bioquímica Médica /CCS / UFRJ, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Orientador: Russolina Benedeta Zingali.

Daniella de Moraes Mizurini. Estudo da atividade anitrombótica da Nitroforina 2 e Aegyptina: inibidores exógenos da via intrínseca/via de contato da coagulação sanguínea. 2011. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Orientador: Robson de Queiroz Monteiro.

Luize Gonçalves Lima. Envolvimento de microvesículas contendo fator tecidual em diferentes aspectos da biologia tumoral. 2012. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Orientador: Robson de Queiroz Monteiro.

Tatiana Corrêa Carneiro Lobo. Ixolaris: um inibidor exógeno do Complexo Fator VIIa/Fator Tecidual bloqueia a sinalização celular mediada pelo receptor ativado por protease PAR-2 humano. 2011. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Orientador: Robson de Queiroz Monteiro.

Vivian de Almeida Silva. Bases estruturais e termodinâmicas do enovelamento proteico e reconhecimento molecular: modelos transtirretina e trombina. 2011. Tese (Doutorado em Química Biológica) - Universidade Federal do Rio de Janeiro, . Co-Orientador: Robson de Queiroz Monteiro.

AL 5

Samuel Silva da Rocha Pita. Estudo de Complexos da Tripanotiona Redutase de Trypanosoma cruzi com Inibidores Peptídeomiméticos. 2011. Tese (Ciências Biológicas (Biofísica)) - Universidade Federal do Rio de Janeiro, bolsa CNPq.

AL 6

MANUEL SEBASTIAN REBOLLO COUTO. Secretoma de micoplasmas. 2012. Tese (Doutorado em Pós Graduação em Bioquímica) - Universidade Federal de Santa Catarina, . Orientador: Hernan Francisco Terenzi.

AL 7

Alessandra Prando, Estudos biofísicos de chaperonas de secreção e de interações proteína-ligante. 2012. Tese (Doutorado em Química) - Universidade Estadual de Campinas. Advisor: Ljubica Tasic.

Juliana Fattori, Resolução estrutural de proteínas hipotéticas, chaperonas de secreção, da bactéria *Xanthomonas axonopodis* pv. *citri*. 2011. Tese (Doutorado em Química) -

Universidade Estadual de Campinas. Advisor: Ljubica Tasic.

Lisandra Marques Gava. Caracterização e interação do comínio c-terminal da chaperona hsp90 humana e das co-chaperonas tom70 e hop. 2011. Tese (doutorado em biologia funcional e molecular) - Universidade Estadual de Campinas. Advisor: Carlos Henrique Inacio Ramos.

Yuri de Abreu Mendonça. Clonagem e caracterização de uma hsp90 de citrus sinensis potencialmente envolvidos processo infectivo do fitopatógeno xanthomonas citri.. 2011. Tese (doutorado em biologia funcional e molecular) - Universidade Estadual de Campinas. Advisor: Carlos Henrique Inacio Ramos.

AL 9

Aline Cristina de Abreu Moreira de Souza. O Envolvimento dos Receptores P2Y na Reativação de *Toxoplasma gondii*. Início: 2012. Tese (Doutorado em Ciências Biológicas (Biofísica)) - Universidade Federal do Rio de Janeiro.

Eduardo José Lopes Torres. ASPECTOS MORFOLOGICOS E ULTRAESTRUTURAIS DA RELAÇÃO *Trichuris sp*. E *Trichuris muris* COM SEU HOSPEDEIRO VERTEBRADO. 2011. Tese (Ciências Biológicas (Biofísica)) - Universidade Federal do Rio de Janeiro

Erica dos Santos Martins Duarte. Estudo de Novos Compostos com Atividade anti-*Toxoplasma gondii* Utilizando modelos in vitro e in vivo. 2011. Tese (Doutorado em Ciências Biológicas (Biofísica)) - Universidade Federal do Rio de Janeiro.

FABIO MENDONÇA GOMES. Ultraestrutura, Expressão Gênica e Mobilização de Polifosfato em Lagartas Anticarsia gemmatalis. 2012. Tese (Doutorado em Ciência Biológicas (Biofísica)) - Universidade Federal do Rio de Janeiro

Gladys Corrêa da Silva. O papel do receptor P2X7 durante a infecção por *Toxoplasma gondii*. 2011. Tese (Doutorado em Ciências Biológicas (Biofísica)) - Universidade Federal do Rio de Janeiro.

Lia Carolina Almeida Soares de Medeiros. Organização estrutural e reconstrução tri-dimensional de protozoários do filo Apicomplexa (*Eimeria spp* e *Plasmodium chabaudi*). 2011. Tese (Doutorado em Ciência Biológicas (Biofísica)) - Universidade Federal do Rio de Janeiro

Miria Gomes Pereira. Reservossomos de *Trypanosoma cruzi* estocam e mobilizam colesterol. 2011. Tese de Doutorado em Ciências Biológicas (Biofísica) - Universidade Federal do Rio de Janeiro.

AL 10

Augusto Garcia Almeida. Restauração de Imagens de Microscopia de Força Atômica com o Uso da Regularização de Tikhonov via Processamento em GPU. 2013. Tese (Doutorado em Modelagem Computacional) - Universidade do Estado do Rio de Janeiro, . Co-Orientador: Geraldo Antônio Guerrera Cidade.

AL 11

Karen Tavares Silva. Otimização da produção de magnetossomos pelo vibrio magnetotáctico Candidatus Magnetovibrio blakemorei . 2012. Universidade Federal do Rio de Janeiro.

Moara Lemos. "Tripanossomas de peixes brasileiros: Isolamento, taxonomia, caracterização ultra-estrutural e interação parasito-hospedeiro" - Instituto de Microbiologia Paulo de Góes – UFRJ – Fevereiro 2012.

Thiago Luiz Alves e Silva. "Aspectos morfológicos, fisiológicos e genéticos da interação de tripanossomatídeos heteroxênicos com seus hospedeiros invertebrados e implicações no processo de transmissão" - Instituto de Microbiologia Paulo de Góes – UFRJ 31 de Agosto 2012.

AL 15

Karla Patrícia de Sousa Barbosa. Avaliação da ação anti-inflamatória de novos derivados tiazolidínicos em modelo de pleurisia induzida por carragenina em camundongos. 2012. Tese (Doutorado em Ciências Biológicas) - Universidade Federal de Pernambuco, Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco. Co-Orientador: Christina Alves Peixoto.

Mariana Aragão Matos Donato. ESTUDO SOBRE O MECANISMO DE AÇÃO DO CITRATO DE SILDENAFIL NO DESENVOLVIMENTO FOLICULAR DE CAMUNDONGOS C57BL/6. 2013. Tese (Doutorado em Ciências Biológicas) -

Universidade Federal de Pernambuco, Fundação de Amparao a Pesquisa do Estado de Pernambuco. Orientador: Christina Alves Peixoto.

AL 16

Jasmin. Caracterização, Biodistribuição e Efeito das Células Mesequimais de Medula Óssea Maracadas com Nanopartículas Injetas INtravenosamente em um Modelo Murino de Doença de Chagas. 2012. Tese (Doutorado em Biofisica) - Instituto de Biofisica Carlos Chagas Filho, Conselho Nacional de Desenvolvimento Científico e Tecnológico. Orientador: Antonio Carlos Campos de Carvalho.

Juliana Amaral Passipieri. Caracterização e Potencial Terapêutico de Células Mesenquimais da Placenta Humana em Modelo Murino de Insuficiência Cardíaca. 2012. Tese (Doutorado em Ciencias Biologicas - Fisiologia) - Universidade Federal do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Orientador: Antonio Carlos Campos de Carvalho.

Luiz Fernando Quintanilha de Mesquita. Comparação Entre Modelos Experimentais de Lesão Hepática Induzidos por Radiação Ionizante, Ressecção Tecidual e Intoxicação por Tetracloreto De Carbono. 2011. Tese (Doutorado Em Medicina (Radiologia) - Universidade Federal Do Rio De Janeiro, Coordenação De Aperfeiçoamento de Pessoal De Nível Superior. Orientador: Regina Coeli Dos Santos Goldenberg.

AL 17

André Luiz Araújo dos Santos. Ecto-Fosfatases em Trypanosoma rangeli e a sua participação na proliferação e adesão às glândulas salivares de Rhodnius prolixus. 2012. Química Biológica Course – Universidade Federal do Rio de Janeiro. Supervisor: José Roberto Meyer Fernandes.

André Luiz Fonseca de Souza. Ecto-enzimas de Trypanosoma rangeli envolvidas com a disponibilização / aquisição de fosfato inorgânico. 2011. Química Biológica Course – Universidade. Supervisor: José Roberto Meyer Fernandes.

André Meyer Alves de Lima. Dança e ciência: estudo acerca de processos de roteirização e montagem coreográfica baseados em formas e padrões de organização biológicos a partir dos fundamentos da dança de Helenita Sá Earp. 2012. Química Biológica Course – Universidade Federal do Rio de Janeiro. Supervisor: Adalberto Ramon Vieyra.

Daniela Cosentino Gomes. Caracterização de uma proteína desacopladora em Trypanosoma rangeli e a participação deste parasito na modulação das defesas antioxidantes de Rhodnius prolixus. 2012. Química Biológica Course – Universidade Federal do Rio de Janeiro. Supervisor: José Roberto Meyer Fernandes.

Diogo Vives da Costa. Mecanismos renais envolvidos na gênese da hipertensão em ratas na posmenopausa. 2011. Ciências Biológicas/Fisiologia Course – Universidade Federal do Rio de Janeiro. Supervisor: Celso Caruso Neves.

Flavia Axelband. Interações regulatórias entre angiotensina II, angiotensina-(3-4) e bradicinina na Ca2+-ATPase de túbulos proximais renais. 2012. Ciências Biológicas/ Fisiologia Course – Universidade Federal do Rio de Janeiro. Supervisor: Adalberto Ramon Vieyra.

Hellen Jannisy Vieira Beiral. Descobrindo as ações de células-tronco no nível de fluxo de elétrons e translocação de prótons em mitocôndrias renais. 2011. Ciencias Biológicas/Fisiologia Course – Universidade Federal do Rio de Janeiro. Supervisor: Adalberto Ramon Vieyra.

Humberto Muzi Filho. Efeito da desnutrição multifatorial no controle da homeostasia intracelular do íon Ca2+ no ducto deferente de rato. 2011. Farmacologia e Química Medicinal Course – Universidade Federal do Rio de Janeiro. Supervisor: Valéria do Monti Nascimento Cunha. Co-Supervisor: Lucienne da Silva Lara.

Leucio Duarte Vieira Filho. Transportadores de sódio e mecanismos de sinalização no rim de ratos submetidos à desnutrição intrauterina e tratamento com alfa-tocoferol. 2011. Bioquímica e Fisiologia Course – Universidade Federal de Pernambuco. Supervisor: Ana Durce de Oliveira Paixão. Co-Supervisor: Adalberto Ramon Vieyra.

Naira Ligia Lima Giarola. Resistência a drogas em Leishmania amazonensis e choque térmico em Trypanosoma cruzi: duas condições de estresse modulando as atividades ecto-ATPásicas desses protozoários. 2012. Química Biológica Course – Universidade Federal do Rio de Janeiro. Supervisor: José Roberto Meyer Fernandes.

Ricardo Luiz Luzardo Filho. A desnutrição protéica durante a lactação programa alterações morfofuncionais e no transporte renal de Na com consequente estabelecimento de hipertensão arterial sistêmica na vida adulta. 2011. Ciencias Biológicas/Fisiologia Course – Universidade Federal do Rio de Janeiro. Supervisor: Adalberto Ramon Vieyra.

Sharon Landgraf Schlup. Doença renal na hipertensão: causa ou conseqüência. 2011. Ciências Biológicas/Fisiologia Course – Universidade Federal do Rio de Janeiro. Supervisor: Celso Caruso Neves.

AL 18

Erivan Schneider Ramos Jr. Papel do sistema calicreína-cinina (scc) na ativação de células sentinelas do sistema imune inato infectadas com porphyromonas gingivalis. Início 2011. Tese (ciências biológicas, biofísica) - universidade federal do rio de janeiro.

Juliana Pandini Castelpoggi. Cooperação entre receptores da imunidade inata: decifrando as vias inflamatórias envolvidas no desenvolvimento de abscessos induzidos por bacteroides fragilis. Inicio 2012. Tese (ciências biológicas, biofísica) - universidade federal do rio Comments: these findings integrate the phd thesis work of juliana p. Castelpoggi. The main objective of this work is to elucidate the proinflammatory pathways (with special emphasis on the role of inflammassome) in host resistance to peritonitis in mice challenged with the commensal bacteria bacteroides fragili. De janeiro.

Larissa Nogueira De Almeida. O sistema calicreína-cinina (scc) na infecção chagásica experimental: impacto da ativação de receptores de cininas sobre o perfil funcional de células dendríticas, macrófagos e neutrófilos. Início 2011. Tese (ciências biológicas, biofísica) - universidade federal do rio de janeiro. Erivan Schneider Ramos Jr. Papel do sistema calicreína-cinina (scc) na ativação de células sentinelas do sistema imune inato infectadas com porphyromonas gingivalis. Início 2011. Tese (ciências biológicas, biofísica) - universidade federal do rio de janeiro.

AL 19

Andreia de Vasconcelos dos Santos. Terapia com células de medula óssea em modelo de acidente vascular encefálico isquêmico. 2011. Tese (Doutorado em Biofisica) - Universidade Federal do Rio de Janeiro, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior. Orientador: Rosalia Mendez-Otero.

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Science education and outreach activities

Nucleus for Science Education and Outreach

Apart from being a reference center for biomedical and biotechnological research in Latin America, the National Institute for Structural Biology and Bioimage (INBEB) also plays a role in education and popularization of science to the public.

To increase INBEB´s interaction with society, we established in 2012 the Center for Education and Science Communication (NEDiCi, in the Portuguese acronym). Its main goals are to disseminate the scientific results from our groups, as well as to promote activities (courses, lectures, workshops, tours, videos and others) designed for teachers and students from primary and secondary schools.

The NEDiCi is currently coordinated by researcher Patricia S. dos Santos, who has extensive experience in organizing courses for grade school students, and teachers, and for undergraduates. Since 1996, Dr Santos participates in teaching summer courses offered by the Institute of Medical Biochemistry at UFRJ and is currently developing a project to assess the creativity and learning of students participating in activities organized by NEDiCi. This project includes the participation of the UFRJ undergraduate students Andressa Bezerra de Mello, Natalia Sant'Anna da Silva and Raquel Cotrim.

The Center also enjoys the collaboration of other INBEB researchers, including Professor Emiliano Medei (from AL 16, and IBCCF/UFRJ), who had two projects in science education and outreach approved by FAPERJ that are developed in partnership with INBEB.

Also, the journalist Marina Verjovsky collaborates in maintaining the website and dealing with public relations.

the following offer more details of some of the activities developed by the Center:

- Vacation (winter and summer) courses

The researcher Patrícia Souza dos Santos coordinates the INBEB vacation courses for students and teachers in public schools.

In the last biennium, 11 courses were conducted, reaching a total of 203 school students and 56 teachers. They were:

January 2011 and July 2011

1st and 2nd. "From grape to wine" - The 1st edition had 19 school students and 5 teachers, the 2nd had 20 school students and 5 teachers.

January 2012

3rd. "Pepper, parsley and garlic: Spices for life? To frighten vampires? What can they do to me? "- Taught at UFRJ for 18 school students and 3 teachers.
4th. "Inside the food" – Course offered to 18 school students in São Pedro da Serra / Nova Friburgo, coordinated by researcher Emiliano Medei (IBCCF / UFRJ).

July 2012

5th. "Inside the food" – Offered to 15 school students in São Pedro da Serra / Nova Friburgo (by professor Emiliano Medei, IBCCF / UFRJ).
6th. "What the market "Ver-o-peso" has to do with health?" – Course offered to 43 high school students from State College Manoel Antonio de Castro and 5 teachers of basic education from Igarapé Miri / PA.
7th. "Food: in sickness and in health" – Taught at UFRJ for 27 school students and 12 teachers.

December 2012:

8th. "Inside the food" - Offered to 15 school students from São Pedro da Serra / Nova Friburgo (by professor Emiliano Medei, IBCCF / UFRJ).

January 2013:

9th. "Food: in sickness and in health" Taught at UFRJ for 14 school students and 10 teachers.
10th. "Mosquitoes: Science and Myth" - Taught at UFRJ for 15 school students and 14 teachers.
11th. "Science and gastronomy: are birds of a feather (and flock together)?" - Special Course for eight high school students who have already done some vacation course before (Collaboration NEDiCi / INBEB with the Institute of Nutrition Josué de Castro, UFRJ).

SCHOOL TEACHERS PARTICIPATING IN A COURSE.

SCHOOL STUDENTS PARTICIPATING FROM A COURSE.

- Financial support from FAPERJ

These courses count on the support of FAPERJ, for the following projects:

- **"O pão nosso de cada dia: um encontro da Biologia, da Física, da Matemática e da Química com a Nutrição"** (Coordinated by professor Eliane Fialho de Oliveira);

- **"*Little bite of science:* o que os mosquitos e os alimentos podem nos ensinar sobre Ciência?"** (Coordinated by professor Mário Alberto Cardoso da Silva Neto);

- **“Interação Universidade-Escola-Governo: contribuindo para a formação de professores através de uma proposta de curso de férias”** (Coordinated by professor Andrea Thompson da Poian);

- **“Da sala de aula à bancada: aprendendo ciência com as mãos”** (Coordinated by professor Emiliano Medei).

- Vacation courses as a research subject

In addition to vacation courses, Dr Patricia S. Santos coordinates a research that aims to assess the creativity and learning of students participating in activities organized by NEDiCi. This project includes the participation of the UFRJ undergraduate students Andressa Bezerra de Mello, Natalia Sant'Anna da Silva and Raquel Cotrim.

The main questions of this research are: Do the vacation courses promote an environment that fosters creativity? What do the school students and teachers who participate think about it? These participants will be asked through semi-structured interviews and thereplies will be evaluated through the analysis of the Collective Subject Discourse (Lefevre, Lefevre and Teixeira 2000). This methodology is a qualitative way to represent the thought of a collectivity, adding in a speech-synthesis the discursive content of similar meaning emitted by different people.

- INBEB’s partner school wins scientific award

Students from State School Manoel Antônio de Castro (EEMAC), located in the municipality of Igarapé-Miri in Pará, won the 1st place prize in the Health Sciences category at the event MILSET National (Movement for International Scientific and Technical Recreation), held in Fortaleza, Ceará. Among the projects with the highest overall score of the meeting, the students gained credentials to participate in the international version of the event, that will happen in September 2013 in Abu Dhabi, United Arab Emirates.

The paper "Natural cream for hand hygiene: using biodiversity of the Amazon to take care of our health" is developed by the students Mayra Carolina Oliveira and Janaina Santos, both of the 3rd year of high school, and guided by teachers Josineide Pantoja and Hélio Júnior. The project investigated the microbicidal action of 13 Amazonian plants, in order to

obtain a natural cream for sanitizing hands, with an effect similar to alcohol. The cream is already being tested in vitro and will be tested in vivo with mice, in a partnership with the Federal University of Pará (UFPA).

This is one of severel achievements reached by those students, as a result of the teachers efforts, especially by Josineide Pantoja, who is also a PhD student at the Structural Biology and Parasitology Program at UFPA. Thus, the school established a partnership with the Associate Laboratory 14 of INBEB, coordinated by professor Edilene Oliveira da Silva, an researcher at UFPA and headleader of the Structural Biology and Parasitology Laboratory.

In July 2012, the EEMAC participated (43 students and 7 teachers) of the INBEB vacation course "What the market *Ver-o-Peso* has to do with health?", Which included the presence of monitors and researchers from the Federal University of Rio de Janeiro at the school. In addition, four students from the school were invited to participate in the IV INBEB Annual Meeting, held in April 2013, where they presented their scientific work, along with undergraduates, masters and doctorate from various Brazilian states. As a result, INBEB awarded 2 airline tickets for students to participate in national conferences.

In return, Pantoja publicly thanked at her page on facebook: “I thank the whole INBEB team in the person of Dr. Jerson Lima Silva for all your commitment deposited in Basic Education students, not only from Pará, but throughout Brazil. I reiterate that every encouragement INBEB gave to our students has been of fundamental importance for every achievement that we are having in Scientific Initiation”.

- Adoption of a school

The professor Emiliano Medei (IBCCF/UFRJ) had two grants from FAPERJ, for Dissemination and Popularization of Science and Support for Public Schools of Rio de Janeiro. In these projects, the multidisciplinary team promotes interactions among scientists and high school students from public schools located about 180km from the Federal University of Rio de Janeiro, in São Pedro da Serra, Municipio de Friburgo – Rio de Janeiro. The projects bring these students to visit research laboratories at the Federal University of Rio de Janeiro, where they can observe how some equipment and techniques are used to answer scientific questions. The project also leads scientists to the school in Friburgo, where they contributed to the construction of a teaching laboratory.

STUDENTS VISITING THE INBEB FACILITIES, AT UFRJ .

The science teaching laboratory was inaugurated in the State School José Martins da Costa on November 19, 2011. Unique in the region, it has several new pieces of equipment that provide students with the opportunity to learn science by doing various kinds of experiments, and analyzing water and food. The facility also already receives the INBEB vacation courses.

So far, around 60 students had participated in the activities and the results can already be noted. The school achieved excellent placement in the Index of Basic Education Development (IDEB) 2011, reaching the highest average among all state schools in the region. The school director, Gleici Heringer, cited the role of INBEB / UFRJ / Faperj projects for this result (read the news report, in Portuguese, at: www.avozdaserra.com.br/ noticia/20694/colegio-estadual-jose-martins-da-costa-em-sao-pedro-da-serra-tambem-se-destaca-no-ideb).

STUDENTS EXPERIMENTING AT THE LABORATORY IN JOSE MARTINS DA COSTA STATE SCHOOL, SAN PEDRO DA SERRA, RJ.

At the end of the activities, the students answered a questionnaire about their impressions about science and scientists. All of the students positively evaluated the activities, which they considered the topics discussed to be important and interesting. 54% of students rated the activity as very interesting, 45% as interesting and only 1% felt it was unattractive. Most of them had never personally known a scientist before (76%), and had

never visited a university before (64%). In addition, when asked if the activities had changed their feelings for science, the vast majority (75%) said yes, for the better.

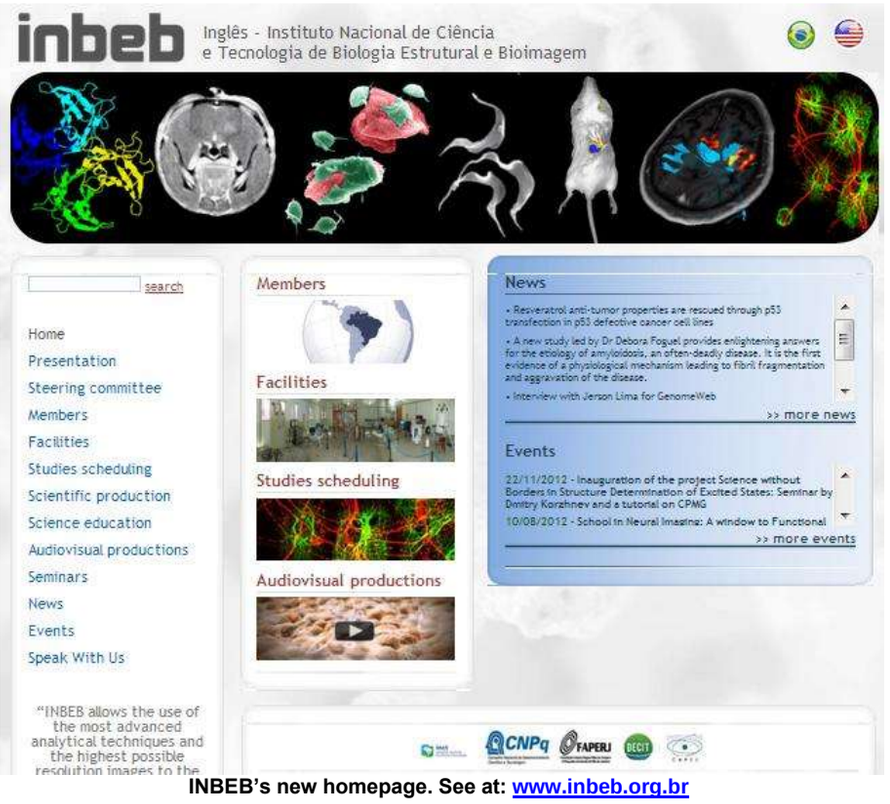
STUDENTS, TEACHERS AND COORDINATORS TOGETHER AT THE JOSE MARTINS DA COSTA STATE SCHOOL, SAN PEDRO DA SERRA, RJ.

These initiatives aim to refresh and stimulate the critical thinking skills of our youth, bring them closer to the university and to stimulate their interest in scientific knowledge. The goal is to show them that biomedical sciences go far beyond names and technical terms found in textbooks.

- INBEB WEBSITE AND SOCIAL MEDIA

The journalist Marina Verjovsky works in collaboration with NEDiCi on education projects and establishing a bridge between researchers and society through dissemination of INBEB activities on the website and in the press.

In early 2012, the INBEB redesigned its website: www.inbeb.org.br. It became more attractive and dynamic, allowing the public easy access to INBEB information and reports on its infrastructure. Furthermore, the researchers and collaborators are now able to register their projects and schedule studies in Cenabio units through the website.



INBEB's new homepage. See at: www.inbeb.org.br

Another novelty is the INBEB page on facebook, that is one more step toward improving information sharing and strengthening of ties with the general public and the academic community.

INBEB'S NEW FACEBOOK PAGE. SEE AT:
WWW.FACEBOOK.COM/PAGES/INBEB/128028600698542

Other initiatives from INBEB members

- Audiovisual productions

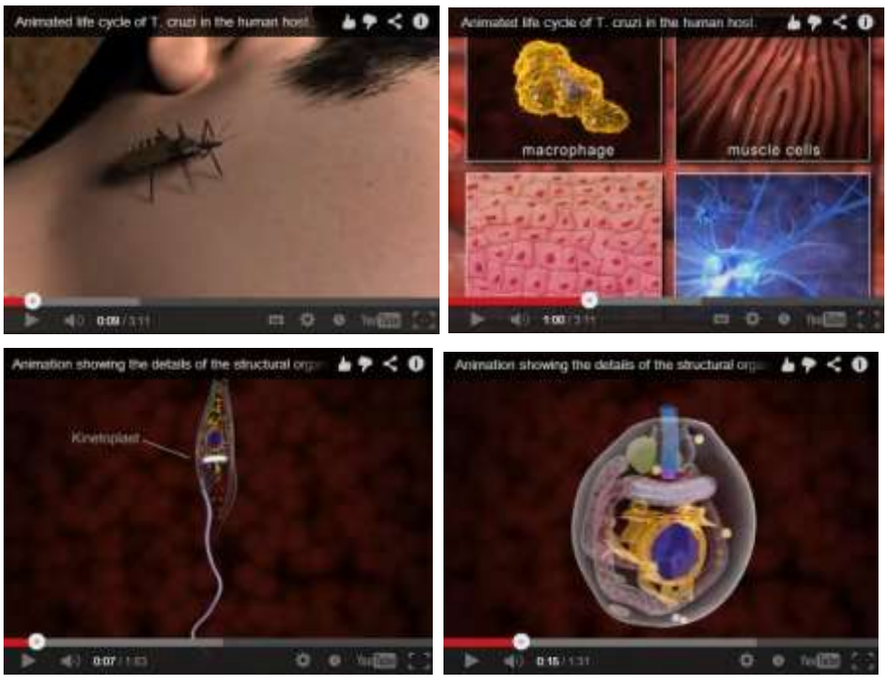
Groups AL9 and AL 12 are involved in several activities related with scientific dissemination and continuing education for teachers of the basic education. Interactive media to teach the life cycle of Trypanosoma cruzi and Leishmania have been produced. Besides printed material containing text and updated color schemes, animated models and 3D animations about the life cycle, developmental stages in the different hosts, and the organelles and structures of these protozoan parasites were also created. This is a powerful tool to contribute to the better understanding of students, teachers and also researchers, and any member of the general public about dynamic processes in parasitology and also in cell Biology. Two books in Portuguese were published, one in 2012 on the T. cruzi life cycle, and the other, on Leishmania, is in press and will be released in March, 2013. These books are being distributed free of charge, mainly in public grade schools and universities. Two other books are in preparation: one about the life cycle of Toxoplasma gondii and the other on Plasmodium.

Another activity on this topic is a new graduate course for Biology teachers: the Professional Masters in Science for Biology Teachers from the Federal University of Rio de Janeiro (UFRJ). This graduate course was authorized by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) in 2011 and currently has 75 students, most of them teachers in Brazilian public schools. This is an initiative organized by several faculty professors from Biophysics, Biochemistry and Biology Institutes, who teach at the Xerém campus of UFRJ, and Inmetro, in the interior of the state of Rio de Janeiro. The main goals of this program are:

1) Scientific formation, focusing on the expansion of the scientific method experience of the students integrating labs from the UFRJ and Inmetro, in order to modify their global vision toward biological themes, facilitating the use of scientific method to teach Biology and Sciences in grade schools and high schools;

2) Theoretical and practical update, since the students have access to new concepts through classes with experts on several areas of Biology;

3) Development and evaluation of methods, materials, and practical experiments for teaching Biology at the grade school and high school levels.



SOME SNAPSHOTS FROM THE VIDEOS

Those videos have extraordinary visual and didactic quality and are also available at the INBEB Youtube channel (www.youtube.com/videosinbeb) and at the INBEB website (www.inbeb.org.br, in the main menu "Audiovisual productions").

Perspectives and future developments

Our National Center for Structural Biology and Bioimaging – INBEB/CENABIO/UFRJ has its embryo in the Jiri Jonas National Center for Nuclear Magnetic Resonance in 1996. Among different supports, the participation in the Millenium Institute Program as a Millennium Institute for Structural Biology, Biotechnology and Biomedicine was crucial to consolidate a national facility in NMR, with three state-of-the-art NMR instruments (400, 600 and 800 MHz) and two new instruments for solid-state NMR (500 and 700 MHz – installation in September 2013). In the last 4 years, with the support of the National Institute Program, the INCT for Structural Biology and Bioimaging has been consolidated by creating two more units, one dedicated to biomaging of small animals (INBEB/CENABIO/UFRJ-2), inaugurated in 2010, and the other in Microscopy. The bioimaging facility has instruments that are at the forefront of knowledge in this field, some of them the only ones in Latin America, such as 7 Tesla MRI, besides PET/CT/ SPECT, ultrasound and *in-vivo* high resolution luminescence/fluorescence animal unit. The third unit, which will come online in August of this year, is in the final stages of construction, and will house a vast park of microscopy equipment. Thus, the three Units of CENABIO / UFRJ are accommodated in a total area of 2,200 m^2. It is noteworthy that the INBEB/CENABIO was recently recognized as a new supplementary organ of UFRJ in February 28, 2013.

The creation of all this infrastructure has increased interaction both among INBEB researchers and among researchers from different Brazilian and international institutes, including the INCTs. Organization of the individual research groups into a National Institute for Science and Technology has made it possible to create synergistic and increasingly productive interactions. As can be seen in this report, during these years we have constructed a multidisciplinary program to approach a great number of relevant scientific questions.

Gradually, we have strengthened our ties with IDOR (Instituto D´Or for Research and Teaching), thereby closing a

gap between basic and clinical research (translational research). The construction of CENABIO III, a new building to house the microscopy equipment, will be finished in the next months. With this, we will be creating the largest and most advanced collection of equipment in Latin America for NMR of macromolecules, imaging of small animals and microscopy.

The catalytic effect of having formal collaborations among the different Associate Laboratories has been mutually beneficial, leading to synergistic actions that combine structural, dynamics, molecular biology, and micro- and macro-imaging techniques. Especially gratifying is to see that the younger members of the groups (graduate students and post-docs) demonstrate enormous enthusiasm and creativity, which holds great promise for a new generation of imaginative leaders in these areas. One of the main goals of INBEB is to support these young professors as they build up their own research groups.

Several publications and thesis dissertations have incorporated a great number of approaches in the frontier technologies of structural biology, cell biology and bioimaging, as well in some cases translational medicine. The training of undergraduate and graduate students as well as post-docs is crucial if we want to produce high-quality science with publications in high-impact journals. It is important to emphasize that the participation of our Institute in the "Science without Borders" Program is also contributing to improving the quality of our research. Researchers in INBEB have a strong association with researchers and international institutions. We maintained a high degree of interaction with almost all of the foreign researchers initially listed in the project when it was submitted. It is worth noting that we have incorporated Professor Kurt Wüthrich, Nobel Prize in Chemistry, as a Special Visiting Researcher (CsF) of the INBEB. He has an office and lab facilities where he is the formal advisor of a graduate student and of a post-doc. They will also visit Professor Wüthrich at the Scripps Research Institute in the

next 12 months. We also host, as a CSsF Special Visiting Researcher, Prof. Dmitry Korzhnev, (University of Connecticut Health Center), a great expert on the studies of excited states of proteins by NMR. In addition, we have several undergraduate, graduate and post-doc students visiting international institutions for periods from 6 to 12 months.

We believe that we have been able to consolidate a highly multidisciplinary program in the biomedical and biotechnological area. More important than the more than 900 articles published in peer-reviewed journals over the last four years is that they reflect a highly collaborative research effort. Not counted in this publication are the many studies conducted by researchers not belonging the INCT network, but who use the facilities of INBEB. Not less important is the fact that the research conducted in INBEB resulted in 174 new Masters and 115 new PhDs. We forecast that the upcoming years will be very productive and display the advantages of a new way to do research in the environment of INBEB/CENABIO.