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Abstracts

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Invited Talks

Structural biology in the search for drug candidates against arboviruses circulating in Brazil

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We will describe our structural biology studies of arboviral proteins, including the crystallographic structures of Zika virus NS5 RNA-dependent RNA polymerase and NS3-helicase as well as the structure of yellow fever virus NS3-protease. We will also present the ensuing collaborative efforts towards the identification on inhibitors and development of novel antiviral lead candidates. We will also report our current progresses in the structural and functional studies of flaviviral replication complex and initial structural work with chikungunya virus proteins. These collaborative projects are conducted within the interinstitutional Center for Research and Innovation in Biodiversity and Drug Discovery (CIBFar/CEPID/FAPESP)..

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Sensors and regulators: pliable proteins in bacterial signaling

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Bacteria use different protein machineries as a means to sense environmental and intracellular signals, and respond adaptively. These sensory transduction systems include two-component systems (TCS), one-component systems (OCS), phosphotransferase systems (PTS) and extra-cytoplasmic function (ECF) sigma factors. Our laboratory has been studying TCSs for several ideas, to understand the molecular mechanisms of their information-transmission function.

TCSs are often organized according to a minimal configuration, comprising a sensor histidine-kinase (HK) and a response regulator (RR). The HK has signal-dependent kinase activity, auto-phosphorylating a conserved His at the expense of ATP (Fig. 1). P~HK thereafter transfers the phosphoryl group catalytically, to a conserved Asp residue in the cognate RR.

Based on biochemical and crystallographic evidence obtained from separate HK and RR proteins, as well as from HK:RR complexes, the mechanism of on/off switching has been unveiled (Buschiazzo & Trajtenberg Annu Rev Microbiol 2019). A coiled-coil-driven shifting machine modifies the position of the reactive His and controls the ATP-binding domains' flexibility. We also present results that uncover molecular determinants of directionality in the phosphoryl flow. In prototypical TCSs phosphoryl-transfer generally occurs unidirectionally from the P~His to the RR's Asp. However, different kinds of TCS architectures have emerged during evolution, often resulting in more complex configurations including intermediate components, such as in phosphorelay pathways. In phosphorelays both P~His-to-Asp and P~Asp-to-His phosphoryl-transfer reactions are necessary to walk along the pathway, frequently implying bidirectional flow *in vivo*. The precise configuration of the reaction center in different HK:RR complexes dictates the reversibility/irreversibility of the phosphoryl-transfer, correlated to the distance between the phosphoryl-acceptor and -donor residues (Trajtenberg et al. eLife 2016; and unpublished results). Preliminary simulations with reported constants suggest that directionality is biologically relevant to shape signal outputs.

Our attention has more recently focused also on OCSs that, as TCSs, exhibit allosteric switching mechanisms to control on/off configurations, key for information transmission. Collaborative work focused on a transcription factor from *M. tuberculosis*, revealed the mechanism by which TetR-like OCSs sense effector ligands triggering DNA-dissociation. A flexible to rigid transition is consistent with all the evidence, changing the paradigm of open-closure of HTH DNA-binding domains generally accepted for TetR-like OCSs.

Acknowlegments: ANII (Uruguay) for grant #FCE_1_2017_1_136291 and an MSc scholarship to SL; International Joint Research Unit IMiZA (Inst Pasteur/IPMontevideo); and institutional support from IPMontevideo.

Phospholipase A_2-like toxins: five steps functional mechanism and three different ways for their inhibition - a study based on crystallographic, bioinformatics and biophysics techniques.

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In 2017, the World Health Organization (WHO) added snakebite envenoming to its list of neglected tropical diseases, with a focus on strategies to reduce the ophidian accidents and control the effects of these envenomings. Furthermore, toxin-specific treatments that could be administered at the time of a bite, or where conventional serum therapies are not adequately effective, remain a challenging scientific area of study. Venoms from *Bothrops* snakes are composed of a set of proteins that have different functions. Among venom components, several variants of secreted phospholipases A_2 (PLA_2s) and Phospholipases A_2-like (PLA_2s-like) toxins. PLA_2s-like toxins have been extensively studied by different approaches due to their importance as potential target for drugs against local effects induce by snake envenomations which is not efficiently neutralized by regular antivenom administration. However, despite their conserved tertiary structures compared to PLA_2s, their structural-based biological mechanisms remain incompletely understood. Different oligomeric conformations and "active" sites have been identified or proposed, leading to controversial data in the literature. Based in structural, bioinformatics, biochemical and site-direct mutagenesis studies, we recently proposed a complete functional mechanism composed by five steps, involving an allosteric transition and the participation of two independent interaction sites for docking into and the disruption of the target membrane. More recent, using a combination of structural, affinity, bioinformatics and functional assays, we performed a broad search for natural and synthetic inhibitors of PLA_2-like proteins. These studies revealed diverse neutralization mechanisms that can be classified into three different groups according to the binding region of toxin: (i) Membrane Docking Site (MDoS), (ii) Membrane Disruption Site (MDiS) or (iii) hydrophobic channel. To date, we were able to identify and structurally study at least eight different compounds as potential inhibitors for PLA_2s-like toxins. The results of this long term study further substantiate the current myotoxic mechanism and provide a useful molecular basis for the search of novel neutralizing strategies to improve the treatment of envenomation by viperid snakes.

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Engineering Immunoreactive Proteins for Viral Detection and Neutralization

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Protein engineering is directed towards developing or tuning desired molecular-level properties. Nevertheless, conformational instability of the synthetic protein often leads to loss of native conformation, which leads to degradation and/or aggregation, therefore limiting success rate. To overcome this issue, we have devised a protocol that combines de novo and molecular dynamics techniques to address biomolecular affinity in a predictive manner. The talk will showcase examples in how to design immunoreactive proteins inspired by their specific antigen-antibody interactions. The talk will showcase the *in silico* development and experimental validation of peptidic aptamers capable of binding to a pan-neutralizing epitope of flaviviruses.

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Suggesting Medicines For Chikungunya Fever Through Virtual Screening And Quantum Biochemistry Of The Viral Non-structural Protein nsp2

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The Chikungunya virus (CHIKV) gives rise to the malady whose clinics is associated with fever, headache, joint and muscle pain, joint swelling, and rash. Among the proteins that make up CHIKV, the non-structural protein 2 (nsP2) performs a central proteolytic activity for the maturation of the viral replication complex. The aim of this work was to identify molecules with potential CHIKV inhibitory activity targeting the proteolytic site of nsP2 (pdb: 3TRK). A virtual drug screening of a chemical library comprised of 4999 compounds from the Zinc12 database against the active site of the target protein was performed. For the two main hits identified (ZINC67460397, C18H16N4O2, named LIG1; and ZINC67513969, C14H13N5O4, named LIG2), a more detailed analysis of the interaction with the protein through molecular dynamics was performed. The LIG1-nsP2 and LIG2-nsP2 complexes were further analysed by quantum biochemistry. It was found that, even though they had the same docking score, the compounds presented very different behaviors in the interaction with nsP2. At the simulation time performed, only LIG1 fully stabilizes at the nsP2 binding site. Important conformational adjustments were observed that led to this stabilization. The quantum study of the interactions of this ligand with the protein revealed strong attractive energies with the catalytic site residues (Cys1013 and His1083). The residues that showed the greatest contribution were Cys1013, Tyr1047, Asn1082, Met1238, Asn1011, Ala1046, Met1242, Ala1010, Ser1048, Tyr1079 and His1083. For both ligands, residues with low energy input were also identified, which may guide the rational design of modifications to improve the interaction. The total binding energy calculated up to the 10 Å interaction radius was -81.0 kcal/mol for LIG1 and -60.4 kcal/mol for LIG2. The observations made in this study provide a basis for the development of new drugs against the Chikungunya virus and point to LIG1 and LIG2 as promising candidates. We are now developing efforts for the CHIKV-nsP2 co-crystallization with LIG1 and LIG2 and their structural resolution.

Keywords: chikungunya; nsP2; virtual screening.

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Unravelling the structural basis of a high affinity HIV-1 vaccine antigen: in silico, biophysical and in vitro studies

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The current HIV-1 vaccine development approaches have focused on stimulation of pan-neutralizing antibodies (pnAbs) that recognize functional sites on the HIV-1 Envelope (Env) spike. Although conserved, these epitopes are masked or structurally highly dynamic, thus resulting in weak antibody response in natural infections. The membrane-proximal external region (MPER) of HIV-1 Env is one of those sites and target for the pnAbs 2F5, Z13 and 4E10. We postulate that presentation of a stable conformation of native epitopes to the immune system can stimulate the development and maturation of neutralizing antibodies. To test this hypothesis we have engineered a protein with increased exposure of a stable conformation of the 2F5 epitope. We grafted the 9 residues (ELDKWASLW), corresponding to the core of the 2F5 epitope, into a scaffold protein called Top7, resulting in a chimeric protein called Top7-2F5. Molecular dynamics (MD) simulations were used to engineer the site of the grafting and circular dichroism analysis confirmed the remarkable structural stability of the Top7-2F5. The affinities of the 2F5 pnAb to the isolated 9-mer peptide, 23-mer MPER peptide and Top7-2F5 protein were analyzed by ELISA and confirmed by surface plasmon resonance. According to the results, 2F5 pnAb showed a superior affinity to the Top7-2F5 ($1.4e^{-9}$M) as compared to 9-mer ($7.06e^{-8}$M) and 23-mer MPER ($1.27e^{-8}$M) peptides. MD analysis indicated that the structure of the 9-mer residues grafted into the Top7 is 3-fold more likely to correspond to the native conformation as compared to 23-mer MPER peptide. The reactivity of the Top7-2F5 was also evaluated in 173 HIV-1 positive sera samples by ELISA and 11% of subjects were shown to carry 2F5-specific antibodies. In addition, *in vitro* studies of B-cell activation demonstrated that Top7-2F5 can stimulate patient-derived B cells to proliferate and secrete pnAbs. These results show that MD simulations can help engineer epitope structures with enhanced pnAbs binding affinity and indicate the potential use of structure-guided designed proteins as vaccine antigens to trigger protective and neutralizing responses.

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Prospection, biophysical, and structural characterization of new carbohydrate-active enzymes with biotechnological applications

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Genome analyses and experimental data suggested that thermostable enzymes may afford economic advantages in the production of many biotechnological products based on biomass degradation. The fungus *Thielavia terrestris* plays an important role in the global carbon cycle with enzymes highly capable of hydrolysing all major polysaccharides found in biomass, making it an attractive candidate for industrial applications and bioremediation. From prospection to three-dimensional structures, we foster a deeper understanding of the hydrolysis of vegetal biomass and pollutants, applying modern scientific approaches in macromolecular crystallography associated with biophysical and biochemical studies. In this research, we adopted three different biomasses as a single source of carbon: sugarcane bagasse *in natura* and pre-treated steam explosion, and bleached eucalyptus Kraft pulp. Mass spectrometry analyses of these different biomasses from *T. terrestris* cultivation allowed the identification of 136 carbohydrate-active enzymes (CAZymes), showing highly diversified profile, drawing attention to a distinguished content of oxidative enzymes. We selected all identified CAZymes with possible biotechnological applications for heterologous cloning and subsequent biophysical, biochemical, and structural characterisation. Thus far, an arabinofuranosidase from the GH62 family (*Tt*GH62), a Laccase from the AA1 family (*Tt*Lcc1) and xylanase from the GH10 family (*Tt*GH10) were cloned and are undergoing characterisation. The *Tt*GH62 was characterised in synthetic (pNPAraf) and polymeric substrate (arabinan and arabinoxylan), revealing optimum temperature and pH (for pNPAraf) of 30 °C and 4.5-5.0, respectively. We determined the three-dimensional structure of *Tt*GH62, revealing an exquisite homodimer configuration due to a domain swapping of one beta-propeller blade. The CAZyme *Tt*Lcc1 presented high-temperature stability (Tm of 70 °C) and optimum pH and temperature of 4 and 55 °C, respectively. Characterisation of the biomass-hydrolysing activity of recombinant enzymes suggests that these organisms are highly efficient in biomass decomposition at both moderate and high temperatures. The structural information will form the basis for further studies in site-directed mutagenesis aiming the production of novel enzymes and cocktails with better hydrolytic properties to be used in biotechnological applications.

Keywords: *Thielavia terrestris*, glycoside hydrolase, bioremediation, biomass degradation

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Flavivirus capsid assembly and dynamics: evidence of a structure-driven regulation of protein interaction with intracellular hydrophobic interfaces.

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Dengue (DENV) and Zika (ZIKV) are major arthropod-borne human viral disease, for which no specific treatment is available. They are a worldwide important health concern, which causes neurological disorders and hemorrhagic syndrome. Although the structure of ZIKV and DENV virion has been determined, information on the nucleocapsid is lacking. The most accepted hypothesis is of a disorganized nucleocapsid. Using NMR, we solved the structure and dynamics of full length ZIKV capsid protein (ZIKVC) and the dynamics of DENV capsid protein (DENVC). We showed that the addition of oligonucleotides can form an organized nucleocapsid-like particles (NC-like). The binding to intracellular hydrophobic interfaces, such as endoplasmic reticulum and/or lipid droplets is essential for virus replication. The hydrophobic cleft is the binding site, along with the intrinsically disordered region, and an open-close dynamic of the globular domain that are species-specific. For ZIKVC, helix 1 is smaller and partially occludes protein hydrophobic cleft. Measurements of the dynamics of helix 1, surface exposure and thermal susceptibility of each backbone amide 1H in protein structure revealed the occlusion of the hydrophobic cleft by α1/α1´ and supported a helix 1 position uncertainty. Based on the findings, we propose that the dynamics of flaviviruses structural elements responds for a structure-driven regulation of protein interaction with intracellular hydrophobic interfaces, which would impact in the switches necessary for nucleocapsid assembly. Subtle differences in the sequence of helix 1 impact on its size and orientation and on the degree of exposure of the hydrophobic cleft, suggesting that α-helix 1 is a hotspot for evolutionary adaptation of flaviviruses' capsid proteins.

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Structural analysis of biological macro-molecules by Single-Particle Cryo-EM

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Functional and Structural studies of the Bacteria-Killing Xanthomonadales Type IV Secretion System

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Bacterial secretion systems are large (multi-megaDalton) multiprotein complexes that transport effector proteins through bacterial membranes to the extracellular milieu or directly into the cytoplasm of other cells. One class of these systems, called the Type IV Secretion System (T4SS), mediate the horizontal transfer of genetic material (conjugation) between bacterial cells and the transfer of virulence factors into eukaryotic host cells. Our group has shown that *Xanthomonas citri* and *Stenotrophomonas maltophilia*, both belonging to the Xanthomonadales order of bacteria, carry T4SSs that mediate the transfer of toxic effectors into other Gram-negative bacterial species in a contact-dependent manner. This was the first demonstration of the involvement of a T4SS in bacterial killing and points to this special class of T4SS as an important determinant for bacterial survival during competitive encounters with other bacterial species in the environment. We have since used a variety of approaches, including X-ray crystallography, NMR, cryo-electron microscopy and fluorescence microscopy, to study the structure and function of these systems. These results will be summarized with a special emphasis on the Cryo-EM structure of the 1.3 megaDalton “core complex” of the T4SS from *X. citri*.

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Localizing specific structures, events or molecules in cells by correlative cryo soft X-ray tomography

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Cryo soft X-ray tomography (SXT) of whole cells in the water window energy range can provide relevant structural information of complex cellular phenomena with chemical sensitivity at spatial resolutions of 40 nm [1, 2, 3]. Functional studies can be achieved by correlating this structural information with visible light fluorescence microscopy data on the same cell [2] (see figure below). Cryo-SXT can also be combined with cryo hard X-ray fluorescence microscopy. Kapishnikov and colleagues investigated *Plasmodium falciparum* infected red blood cells to shed light on the heme crystallization process [4] and the mode of action of specific antimalarial drugs [5]. Another recent example deals with the localization and quantification of an Ir organometallic anticancer drug compound in the cellular environment [6]. The lecture aims at presenting the state of the art of cryo-SXT and its capabilities by showing specific research examples.

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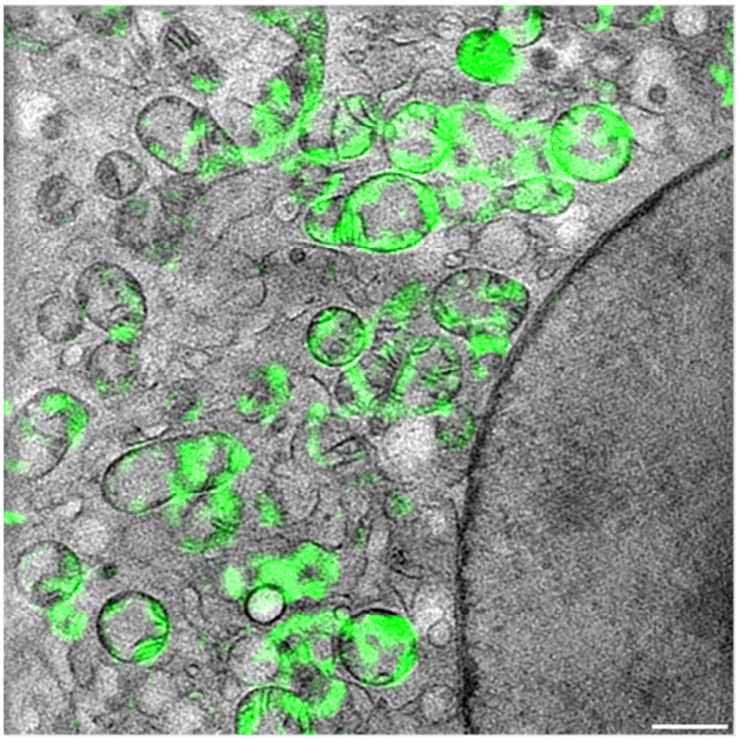

Figure 1: Correlative cryo SIM and cryo SXT on a fibroblast-like cell. Scale bar: 1 µm

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Multimodal Synchrotron Imaging on Cells

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Synchrotron radiation light sources offer a wide spectral "white" source at energies ranging from infrared to hard X-rays. In the last decade, drastic improvements have been achieved in terms of stability, brightness, coherence and low beam divergence. Therefore, numerous synchrotron beamlines are now dedicated to imaging. In the current presentation, imaging techniques using synchrotron radiation will be introduced, illustrating how both scanning imaging and full-field microscopies offer complementary information and allow the mapping of label-free samples at higher spatial resolution. Moreover, at the Synchrotron SOLEIL facility, particular attention is given on the synergy emerging from the coupling of spectroscopies and imaging techniques. Indeed, this approach provides an efficient way to study and characterize complex systems such as found in living cells. Two applications on multimodal synchrotron imaging on cells will be presented:

(1) Influenza A viruses (IAV) represent a major concern for both human and animal health. The diversity of hosts that they can infect and the frequency of inter-species transmission events maintain the threat of the emergence of non-human strain with efficient sustained inter-human transmission that could cause a pandemic. We aim at a better understanding of the emergence of reassortants viruses from the avian reservoir and notably of the implication of the segment 2, encoding PB1, the viral polymerase and the factor of virulence PB1-F2. We have previously demonstrated that the structural behaviour of PB1-F2 i.e. the assembly of amyloid-like fibres in human IAV-infected cells is tightly linked to the pathogenicity of the virus. Thus, we propose to combine different synchrotron techniques (such as IR, UV and soft X-ray) to study the structural behaviour of PB1-F2 expressed by the viruses generated in avian IAV-infected cells.

(2) Recent advances in third-generation synchrotron radiation sources made possible over the last few years to solve the structure of macromolecules from microcrystals of a few microns. This provides new opportunities for innovative crystallogenesis methods such as *in vivo* crystallography, where heterogeneously expressed proteins spontaneously crystallize in living cells. The number of *in vivo* grown protein crystals cases is accumulating in the literature. The expected benefits of this approach make *in vivo* crystallography a promising alternative method for accessing the structure of recalcitrant proteins. In order to understand the poorly investigated *in vivo* crystallization process and turn the cell into a protein crystal factory, we combine multimodal imaging techniques found within synchrotron facilities such as SOLEIL.

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Tracking the fate of *Trypanosoma brucei* cells after knockdown of the RRP44/Dis3 homologue

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Trypanosoma brucei belongs to a group of pathogenic trypanosomatids, which is also known for presenting unique cellular and molecular features. Protein-coding genes are organized into long polycistronic units that are constitutively transcribed. Monocistronic mRNAs are generated by trans-splicing and polyadenylation. Their ribosomes contain trypanosomatid-specific rRNA expansions with implications in the internal structure and possibly also in their activity. In addition, the rRNA equivalent to the 28S rRNA of other eukaryotes is fragmented into 6/7 segments. Our studies on trypanosomatids aim to describe trypanosomatid-specific mechanisms to validate them as targets for new inhibitors. Rrp44 is a conserved modular protein containing endonuclease (PIN) and exoribonuclease (RNB) catalytic domains involved in maturation and quality control of all RNA types. Although Rrp44 functions directly on RNA synthesis and degradation, deficiency in RRP44 causes unexpected phenotypes in different organisms. Yeast mutants of Rrp44 show defective sister chromatid segregation and genome instability. In multicellular model organisms, a conflicting balance between inhibition of development and tumor promotion has been reported for Rrp44 deficiency.

T. brucei RRP44 (TbRRP44) plays an essential function and, differently from the human and yeast counterparts, possesses a second manganese atom bound near the endonuclease catalytic site. Depletion of TbRRP44 causes a series of cellular alterations. We have evaluated these alterations by using DUV microscopy, cryo soft X-ray tomography and fluorescence imaging methods. Cell division is blocked in parallel with increase of cell size. Differences in control and TbRRP44 knockdown cells can be determined by evaluating the autofluorescence of protein fluorophores and the metabolite NAD(P)H. These differences, however, seem not to correlate directly with changes in protein and NAD(P)H content. 3D reconstructions of cryo-SXT images revealed extreme vacuolation of the cytoplasm and a general enlargement of vesicles, including of lipid droplets and calcium-containing vesicles. Nuclei and nucleoli become larger and more diffuse. Mitochondria showed reduced activity and increase of fluorescence in the FAD contribution range. Only approximately 30% of the TbRRP44 knockdown cells are positive for annexin V staining and just a fraction of these cells is positive also for propidium iodide, indicating that only a fraction of the cell population is undergoing canonical programmed cell death. On the other hand, a striking increase of acidic vacuoles is observed in TbRRP44 depletion. Based on the localization of molecular markers, these vacuoles are derived from lysosomes. With depletion time, these vacuoles take up most of the cytoplasm in a process that may be one of the final stages leading to cell death triggered by TbRRP44 depletion.

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Poster Session

New approach to antimicrobial development: structural characterization of vitamin B6 biosynthetic pathway

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Infectious diseases are one of the leading causes of death in the world and the number of antibiotic-resistant infectious is increasing, according to the World Health Organization. For instance, in Brazil and other countries in South America more than 50% of *Staphylococcus aureus* clinical isolates were methicillin-resistant. These data evidence the urgency to identify novel therapeutic targets for antibiotic development. Complete genome sequencing allows the identification of enzymes and pathways that are in many pathogens but not in humans and thus they are promising targets for antimicrobial drug-discovery. An example is the pyridoxal phosphate (vitamin B6) *de novo* synthesis pathway that is carried out by two proteins (Pdx1 and Pdx2) that form an enzyme complex. Pdx2 is a glutaminase which delivers ammonia to Pdx1. This one synthesizes pyridoxal-5-phosphate from ribose 5-phosphate, glyceraldehyde 3- phosphate and ammonia. There are no structural studies of the enzyme complex Pdx1/Pdx2 from *S. aureus*. For this reason, the purpose of this work is to determine the 3D structure of the *S. aureus* enzyme complex. The enzymes SaPdx1 and SaPdx2 were expressed in *E. coli Rosetta* (DE3). Purification was carried out by Ni-affinity chromatography followed by gel filtration. Crystallization was performed with commercial crystal screening kits using the hanging-drop vapor-diffusion. The SaPdx1 crystals was obtained and the diffraction data was collected at the MX2 beamline of the Brazilian Synchrotron (LNLS). The structure of SaPdx1 was solved by molecular replacement. The model building was performed using Phenix AutoBuild and Rosetta. The model was refined up to 2.85 Å using rosetta.refine. The next steps will be site-directed mutagenesis of SaPdx2 and complex crystallographic assays.

Keywords: Staphylococcus aureus, vitamin B6, structural characterization.

Acknowlegments: FAPESP, São Carlos Institute of Physics (IFSC), USP.

Lectin of the seaweed *Codium isthmocladum* (Vickers): Purification and obtaining crystals

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Lectins have a wide range of biotechnological applications. Seaweed lectins, however, still lack in studies of three-dimensional structures. This study aims to purify and obtaining crystals of the lectin of the seaweed *Codium isthmocladum* (CIL).

The specimens were collected at Paracuru beach (Trairi-CE). Protein extractions were performed at a ratio of 1:3 m/v of sliced seaweed and sodium acetate100 mM, 1 M NaCl buffer, pH 5. After 4 h under mechanical stirring, the extract was filtered through nylon tissue and centrifuged at 9000 rpm for 30 min at 4°C. The supernatant was collected and fractionated (F40/100) by ammonium sulfate precipitation. F40/100 was quoted and subjected to ion exchange chromatography (IEC) with DEAE-Sephacel column equilibrated with sodium phosphate 20 mM buffer, pH 7. Different fraction was eluted with successive washes of buffer containing 0.3 and 0.5 M NaCl. The fraction not retained in the column (without NaCl) was concentrated with the use of vivaspin. Hemagglutinating activity (HA) and SDS-PAGE were performed to visualize bands of purified and biologically active CIL. The sparse-matrix method using Crystal Screens I and II supplied by Hampton Research (California, USA) was utilized to perform screening of the crystallization conditions.

F40/100 presented higher AH rabbit blood. IEC presented three peaks (0, 0.3, 0.5 M), F0 presented AH. SDS-PAGE showed a band with 19 kDa. In the absence of β-mercaptoethanol was observated the same band of 19 kDa. Crystals were grown at 295 K by the vapour-diffusion method using 2 ml hanging drops composed of equal volumes of protein solution (12 mg / mL in water) and reservoir buffer (cesium chloride 0.05 M, jeffamine M-600 30%, 0,1 M MES pH 6.5).

A new lectin of green seaweed has been successfully purified and crystallised. It is composed by a monomer of 19 kDa.

Keywords: Lectin, Seaweed, Purification, Cristalization.

Acknowlegments: CNPq; CAPES.

Development of antibody fragments for selectivity assessment of EGF domain proteins

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The factors of the EGF family play important roles in cell proliferation, differentiation, and survival, as well as in carcinogenic and metastatic events. All EGF factors share the conserved EGF structural domain. Considering their structural similarity and low levels in body fluids, highly specific antibodies are needed for their accurate detection and quantification. Hence, the aim of this project is to develop antibody fragments with high selectivity and affinity towards the EGF member, which requires testing selectivity towards all EGF family members. For this purpose, we have established expression systems for the seven EGF factors and a control EGF-domain protein (NRG1α). All factors were produced as Trioredoxin fusion proteins and stably stored at -20ºC. The amino acid sequences of a mouse and a rabbit monoclonal antibodies obtained by mass spectrometry were used to design synthetic genes for single chain fragments of the variable fraction (scFv). The synthetic genes were cloned into the plasmid pET28a. A parallel strategy was based on structural analysis of scFv to select scaffolds suitable for high soluble yield when expressed in *E. coli*. Evaluation of the correlation between structural features and yield was performed with all scFv crystal structures from the PDB describing soluble *E. coli* expression without additional tags. The structures were clustered using structural alignment and analysis of CDRL1 and CDRH3. The computational analysis clustered the scFvs into five groups. Five constructions were designed using scaffolds compatibles for grafting the CDR sequences of the mouse and rabbit monoclonal antibodies sequenced previously by mass spectrometry. Two additional constructions were designed changing the Vernier zone residues to evaluate the antibody affinity. The two original scFv were expressed with and without GFP fusion in *E. coli,* showing low solubility. Only the scFv originating from the rabbit antibody without fusion was expressed in the soluble fraction. On the other hand, three scFvs with scaffolds designed based on structural features, one bearing the mouse and two the rabbit CDRs, showed much higher expression as soluble proteins in *E. coli*. Following purification, binding assays will be performed to evaluate the affinity and selectivity of the different antibody fragments.

Keywords: scFv, EGF domain

Acknowlegments: CAPES, CNPq, FIOCRUZ, UFPR

Mechanism of interaction between eukaryotic initiation factors and poly-A binding proteins in *Trypanosoma cruzi*

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During translation initiation, canonical circularization of a eukaryotic mRNA is mediated by the scaffolding protein eIF4G and the poly-A-binding protein PABP. However, trypanosomatid PABP homologues can interact directly with homologues of the mRNA-cap-binding protein eIF4E. Since a direct eIF4E:PABP interaction has not been described for mammals, this difference between the translation machinery of trypanosomatid and host cells is regarded as a promising target for the development of anti-parasitic drugs. Previous studies described the interaction between the *Leishmania infantum* EIF4E homologue 4 (LiEIF4E4) and LiPABP1. This interaction is mediated by PAM2 motifs found in LiEIF4E4 N-terminal extension and the MLLE domain of LiPABP1. Trypanosomatid EIF4E4 shares the amino-terminal extension containing three PAM2 motifs (“A”, “B” and “C”) with EIF4E3. However, using the two-yeast hybrid assay, we demonstrated the interaction of the two *Trypanosoma cruzi* PABPs (TcPABP1 and TcPABP2) with the eIF4E homologue 3 (TcEIF4E3) but not with TcEIF4E4. Our aim is to validate these interactions in *Trypanosoma cruzi* and further study the mechanism of interaction by reconstituting the protein complexes *in vitro* using recombinant proteins. We are currently working on the immunoprecipitation of the complexes formed *in vivo* by these proteins using polyclonal antibodies in extracts of wild type parasites or anti-GFP antibodies in extracts of GFP-tagged transfectants. In addition, we are mapping the regions mediating the interactions using recombinant truncated constructs. The TcEIF4E3 constructs contain either the entire amino-terminal extension (with the three PAM2 “ABC”) or the “AB” or the “BC” regions in fusion with GFP. The MLLE domain of the two TcPABP homologues are expressed either as thioredoxin or hexa-histidine fusions separated by a TEV protease cleavage site. Although the TcEIF4E3 amino-terminal region is intrinsically disordered, we successfully purified the TcEIF4E3 N-terminal GFP-fusion constructs. The binding affinity between the recombinant proteins will be determined by microscale thermophoresis. This, together with the validation of the interactions in vivo should contribute to the understanding of the trypanosomatid-specific mechanisms of translation, which may represent novel mechanisms of regulation of gene expression.

Keywords: Trypanosoma cruzi, eukaryotic initiation factor, poly-(A) binding protein

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Study of the structure and biochemistry of the *Pseudomonas aeruginosa* PA14_00800 protein

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Pseudomonas aeruginosa is a gammaproteobacterium and an opportunistic pathogen, responsible for a wide range of nosocomial infections. Understanding the bacterium physiology and genes functions are essential to overcome the infections caused by it. Previous studies from our group showed that a mutant in *PA14_00800* is impaired in swarming motility and pyoverdine production. These traits are essential to bacterial infections and denote that PA14_00800 plays a central role in adaptation. To better understand the biological function of PA14_00800, this project aims to solve its 3D structure and to investigate the network of biochemical interactions.

To start the study of PA14_00800 tertiary structure is was necessary to produce and isolate the protein. The recombinant protein was cloned and expressed in *Escherichia coli*, and a combination of gel filtration, ion exchange, and affinity chromatography was used to achieve a purified sample.

The first part of the project was dedicated to finding a good protocol to purify the recombinant protein. That was achieved by successive tests with a combination of different buffers and techniques, which led to a well-optimized protocol, obtaining a cleaner and more stable sample. The NMR spectra recorded during this trial phase indicated that the protein structure varies according to the medium used to produce it. This variability makes this protein a bad candidate for crystallography screenings, as observed in preliminary assays. Furthermore, the NMR assays indicated that the protein structure changes according to the buffer and indicated a possible interaction with metal ions.

PA14_00800 is a previously uncharacterized protein that seems to play a major role in the bacterium physiology, which highlights the importance of uncovering its function. It can assume different forms, depending on the media it is being produced, which indicates a probable sensibility to the environment. Having a good purification protocol in hands and a lot of biochemistry knowledge about the protein, the next step is solving its 3D structure. Joining these results with the phenotype observations done with the mutants will be helpful to uncover this protein's roles.

Keywords: Pseudomonas aeruginosa, NMR.

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Comparative analysis of the interactions between the Importin-α of *Mus musculus* and *Neurospora crassa* with nuclear sequence peptide NIT-2

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Communication between the cell nucleus and the cytoplasm occurs through transport mechanisms that allow molecules to pass through pores present in the nuclear envelope. Among the known transport routes that enable the transport of macromolecules the Classical Nuclear Import Pathway is the best characterized. In this way, the protein Importin-α (Impα) acts in the identification of the proteins to be transported to the nucleus through the recognition of nuclear localization sequences (NLS) present in the cargo proteins. Impα structures have already been elucidated and characterized in some organisms, which it was possible to classify them into three subfamilies. The differences between the Impα proteins of each family show their specificities in the recognition of NLSs, depending on their organism and function. The same NLS peptide may exhibit variations in affinity and binding mode when interacting with Impα from different families. The aim of the study is compare the affinity and binding of the same NLS peptide with Impα proteins from α1 and α2 families. The NLS sequence of the NIT-2 protein, which is a transcription factor of the *Neurospora crassa* fungus was analyzed. Crystallization experiments with the mammalian Impα (MmImpα)/NIT2 NLS complex were performed and the best crystals were submitted to X-ray diffraction, data collection and processing. The structure was elucidated and compared with the previous study with *N. crassa* Impα (NcImpα)/ NIT2 NLS structure. In order to quantify the affinity between these NLS peptides and the MmImpα and NcImpα proteins, Isothermal Titration Calorimetry (ITC) experiments was carried out to provide the dissociation constant, stoichiometry, enthalpy and entropy values. The analysis of NIT-2 binding to minor binding sites of NcImpα and MmImpα receptors reveals interesting differences. The presence of additional interactions of NIT-2-NLS with MmImpα compared to NcImpα may explain the higher affinity of this peptide to MmImpα. Thus, these finds, together with previous results with Impα from other organisms, indicate that the differential affinity of NLSs to minor binding sites may be also responsible for the selectivity of some cargo proteins recognition and transport, particularly for organisms that only have one Impα isoform.

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Crystal structure of the *Trypanosoma cruzi* EIF4E5 translation factor in complex with mRNA cap-4 and molecular specificities of EIF4E homologs in trypanosomatids

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Association of the initiation factor eIF4E with the mRNA cap structure is a key step for translation. Trypanosomatids present six eIF4E homologs, showing a low degree of conservation among themselves and also differing significantly from the IF4Es of multicellular eukaryotes. Trypanosomatids EIF4Es display a distinct set of extensions at N- and C-terminal regions or insertions in specific positions of the eIF4E domain. On the mRNA side, while in most eukaryotes the 5'-end of mature mRNAs contain cap-0 (7-methyl-GTP), trypanosomatids features a cap-4, which is formed by a cap-0, followed by a AACU sequence containing 2′-O-ribose methylations and base methylations on nucleotides 1 and 4. This unique mRNA cap structure strongly anticipate the existence of a trypanosomatid-specific interacting mode for the IF4E-cap-4 complex.

We have described for the first time the crystal structure of a trypanosomatid eIF4E (*T. cruzi* EIF4E5) in complex with cap-4. The TcEIF4E5-cap-4 structure allowed a detailed description of the binding mechanism, revealing the interaction mode for the AACU sequence, with the bases packed in a parallel stacking conformation and involved, together with the methyl groups, in hydrophobic contacts with the protein. This binding mechanism evidences a distinct cap interaction mode in comparison with previously described eIF4E structures and may account for the difference of TcEIF4E5-cap-4 dissociation constant in comparison with other eIF4E homologues.

We have also performed a detailed comparative analysis of kinetoplastid EIF4Es based on the structural comparison of the eIF4E domain and focusing on the insertions which are exclusive of these organisms. The predicted 3D structures of the EIF4E homologs show structural proximity of the S1-S2 and S5-S6 loops to the cap-binding pocket and of the S4-H2 loop to the EIF4G interaction site. The differences in loop length and amino acid composition anticipate different interaction modes for these loops in cap-4 recognition and EIF4G interaction. Quantitative cap affinity assays for *Trypanosoma cruzi* homologs with deleted S4-H2 loop in TcEIF4E1, S1-S2 loop in TcEIF4E2 and N- and/or C-terminal regions indicated molecular features that may account for cap interaction specificities and species-specific functional properties of trypanosomatids EIF4Es.

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Structural and Functional Investigation of Metacaspase-3 in *Trypanosoma cruzi* Apoptosis

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Apoptosis is a highly regulated process of cell death that requires the interaction of several factors. Some therapeutics for parasitic, cardiac and neurological diseases activate apoptosis. Therefore, the study of apoptotic proteins in pathogenic organisms is relevant. However, the molecular mechanism of apoptosis in unicellular organisms remain elusive, despite morphological evidence of its occurrence. In *Trypanosoma cruzi*, the causative agent of Chagas disease, metacaspase-3 (TcMCA3), seems to have a key role in parasite apoptosis. The purpose of this work is to study of structural modifications upon TcMCA3 activation and its action mechanisms in apoptosis pathway. Recombinant TcMCA3 production and purification allowed ligand screening by differential scanning fluorimetry assay and initial crystallization trials. The effects of compound-TCMCA3 on *T. cruzi* viability was evaluated by MTT assay. Flow cytometry with Rhodamin, Annexin and Propidium iodide indicated the occurrence of apoptosis. Recombinant TcMCA3 retains the enzymatic ability of autoprocessing and was able to bind procaspase-activating compound-1(PAC-1). Indeed, PAC-1 reduced *T. cruzi* epimastigote viability with an IC50 of 14.12 μM, induced loss of mitochondrial potential and exposure of phosphatidylserine, features of apoptosis. Chromatographic steps allowed the purification of both the unprocessed and processed states. The structural determination will provide the atomic knowledge of the activation process. Moreover, the crystallographic structure of PAC-1, an apoptosis inductor identified in this work, in complex with TcMCA3 will show the mechanism for apoptosis activation. This work contributes to understand the role of TcMCA3 in *T. cruzi* and to verify the differences in apoptosis between trypanosomatids and metazoans. Above all, it is important to highlight the relevance of this investigation beyond its clinical applications. Identification of a compound that binds to TcMCA3 and promotes apoptosis in *T. cruzi* enables the study of apoptosis in vitro to provide a better understanding of this process.

Keywords: Metacaspase, Trypanosoma cruzi, Apoptosis

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The role of *Trypanosoma brucei* ribonuclease RRP44 in ribosomal RNA processing

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Parasitic trypanosomatids, such as *Trypanosoma brucei*, *T. cruzi* and *Leishmania sp*. present unique features compared with other eukaryotes with regard to RNA processing and maturation. For instance, *T. brucei* ribosomes contain specific rRNA expansions and the 60S subunit is composed of eight rRNAs molecules instead of the three rRNAs found in most eukaryotes. The role of specific endo- and exonucleases in the maturation of the unusual rRNA precursor of trypanosomatids remains largely unknown. One of the nucleases involved in rRNA processing is Rrp44, an exosome associated ribonuclease in yeast, which is not only involved in rRNA processing, but also related to several metabolic RNA pathways. Here we investigate functional and structural aspects of the *T. brucei* ribonuclease RRP44. Recombinant full-length and truncated forms of TbRRP44 were produced for crystallization. *T. brucei* knockdown cells were generated using RNA interference (RNAi) to perform phenotypic characterization. Analysis of pre-ribosomal RNA processing were performed by qRT-PCR and Northern blot. Proliferation curves of knockdown cells confirmed that TbRRP44 is essential for the parasite viability. TbRRP44 depletion causes accumulation of the complete LSU rRNA precursor, in addition to 5.8S maturation impairment. The crystal structure of TbRRP44 endonucleolytic PIN domain was refined at 2.3 Å resolution. Structural comparison with *Saccharomyces cerevisiae* Rrp44 revealed differences which could provide molecular bases for the lack of interaction of RRP44 with the exosome complex in *T. brucei*. A TbRRP44 construction including the exonucleolytic RNB domain was also crystallized. Crystals diffract to 3.1 Å resolution and the structure was determined by molecular replacement. Structural analysis reveals that the exonucleolytic active site is conserved when compared with *S. cerevisiae* Rrp44. In conclusion, we have shown that TbRRP44 is essential for cell viability and correct LSU rRNA maturation. The crystal structure of the TbRRP44 endonucleolytic domain revealed key differences relative to the yeast homologue.

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Comparative study using crystallographic and calorimetric techniques with the importin-alpha and DNA repair nuclear localization sequences

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In the classical nuclear import pathway, proteins containing nuclear localization sequences (NLSs) are imported into the nucleus by the importin-α/β heterodimer. Importin-α (Impα) contains the NLS recognition site, while importin-β mediates transport across the pore of the nuclear membrane. One of the proteins imported by this pathway is MLH1, which acts repairing base mismatch in DNA duplication, being essential for maintaining fidelity in DNA replication. Herein, mutations were performed in different regions of the MLH1 NLS aiming structural and calorimetric studies with the Impα complex and native and mutated MLH1 NLS peptides [(i) ^{466}SSNPRKRHRED^{476}, (ii) ^{466}SSNPRK**K**HRED^{476}, (iii) ^{466}SSNPRKRHR**A**D^{476}, (iv) ^{466}S**A**NPRKRHRED^{476}, (v) ^{466}SSNP**A**KRHRED^{476}] using crystallographic and isothermal titration calorimetry (ITC) techniques. To perform these experiments, protein expression was performed by transformed *E. coli* bacteria containing the vector pET-30a (Novagen) with the *Mus musculus* Impα sequence truncated in its N-terminal domain followed by affinity purification assays. Impα/MLH1 NLS complexes were co-crystallized and X-ray data collection were collect at Brazilian Synchrotron Light Laboratory followed by data processing, structure elucidation, modeling and refinement. Calorimetric experiments were performed using a *MicroCal ITC200* and processed using *Origin* software. The results demonstrated that all NLSs bind to Impα as monopartite NLS. In the case of the native NLS peptide (peptide i), the stoichiometry was two ligands for a protein (1:2), but for the mutated peptide (ii), which an arginine was replaced by lysine, the peptide just bound to one site (stoichiometry 1:1). Regarding to the other mutated peptides (iii-v), which the substitutions were not performed at the fundamental consensus sequences, the stoichiometries were not changed (remain 1:2), but their affinity and enthalpy constants had important changes. Comparing the data obtained from ITC with crystallographic structures, it was possible to better understand the importance of the KR consensus sequence in the P1 'and P2' regions of the peptides in the case of binding at the NLS minor site. It was also possible to get important insights into the understanding the role of each amino acid from the linker region and how the presence of non-polar residues affect the binding NLS peptides and Impα protein.

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Development of activity assays for the ribonuclease Rrp44 from *Trypanosoma brucei*

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Trypanosomatidae comprises a group of protozoan parasites of complex life cycle infecting invertebrates and vertebrates hosts, which includes the species that cause Chagas disease, the various forms of Leishmaniasis and human African trypanosomiasis. Compared to other eukaryotes, trypanosomatids have specificities regarding genomic structure and molecular mechanisms, such as polycistronic transcription, mRNA maturation by trans-splicing and mainly post-transcriptional control of gene expression. Trypanosomatids have also particularities related to their ribosomal RNA (rRNA) structure. The rRNA precursor (pre-rRNA) of most eukaryotes contains the sequences that will be part of mature ribosomes separated by two internal transcribed spacer sequences that are removed during pre-rRNA maturation. However, the pre-rRNA *of T. cruzi* and *T. brucei* contains five, and *Leishmania* spp. six internal transcribed spacers between rRNA segments that will compose mature large subunit. The removal of these additional internal spacers possibly indicates a requirement for additional endoribonuclease activity. *Trypanosoma brucei* has five proteins that possess endoribonuclease domain (PIN) which can act in ribosome maturation. Three of these proteins - TbRrp44, TbUtp24 e TbNob1 - are essential for the parasite viability. Our group has been studying these proteins and has established processes for their purification. This work aims to develop assays to test TbRrp44 activity *in vitro*. The activity assays will be used to map pre-rRNA cleavage sites by testing segments of internal transcribed spacers and verifying if there is an endoribonucleolitic cleavage in each segment. These assays will also be used to test inhibitors for this protein. Besides purifying TbRrp44, the experimental steps include: to define pre-rRNA regions to be cloned, to clone these regions, to generate substrates using *in vitro* transcription or chemical synthesis, to evaluate protein-substrate interactions, and, finally, to evaluate the best conditions to detect endoribonuclease activity for this protein. Currently, several substrates were selected and TbRrp44 was purified, yielding approximately 2,6 mg of protein in one purification cycle. Freezing tests showed that TbRrp44 remains stable after thawing, indicating that this protein can be stored for later use.

Keywords: Ribonuclease. rRNA processing. Trypanosoma brucei.

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Orientation of dimeric coiled-coil human septins probed by solution NMR

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Septins constitute a family of guanine-nucleotide binding protein which is highly conserved in animals and fungi. They are involved in a wide range of cellular processes like cytokinesis, microtubule dynamics and membrane remodeling, mainly because of their ability to self-assemble into linear heterofilaments and high-organized polymers, like bundles and rings. Some septins are related to pathologies in humans, such as cancer, neurodegenerative diseases and male infertility. Human septins are encoded by 13 different genes (*SEPT1* to *SEPT12* and *SEPT14*) and structurally composed by an N-terminal portion, a GTP binding domain and a C-terminal domain often presenting a coiled-coil sequence. The first and only crystallographic structure of a septin heterofilament is a complex of septins SEPT2-6-7 whose the core particle is formed by a hexamer, forming the repeat -(7-6-2-2-6-7)-. Many aspects of these proteins are not yet fully understood, including the role of the C-terminal domain which are absent in this X-ray structure due to poor electron density.

In this study, we used ^{1}H-^{1}H-NOESY NMR spectroscopy to detect the orientation and helix pairing adopted by the homodimeric coiled-coil peptides of SEPT1, SEPT2, SEPT4 and SEPT5 in solution. NOEs analysis, aided by back-calculated spectra using X-ray diffraction models, was able to reveal the relative orientation of each coiled-coil. The only peptide to show an antiparallel structure was SEPT2CC. However, PRE-NMR experiments conducted with SEPT5CC showed the disappearance of specific peaks in the spectrum indicating an antiparallel orientation, contrary to our initial results. A simple evaluation of the coiled-coil heptameric positions, based on the occurrence of each amino acid residue occupying each position, revealed that both orientations are likely to exist despite being far less stable compared to other coiled-coils. Other results from our group also suggest that these peptides could have the ability to form both parallel and antiparallel coiled-coils in certain conditions. We speculate that the coiled-coil antiparallel conformation might be related to cross-linking between filaments, allowing the existence of higher-order filamentous structures.

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STRUCTURAL AND CALORIMETRIC STUDIES WITH THE IMPORTIN-α FROM *Mus musculus* INTERACTING WITH THE NUCLEAR LOCALIZATION SEQUENCE OF THE PROTEIN NEIL3

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DNA damage can occur by endogenous and exogenous genotoxic agents, which may promote instability of the genome and directly lead to diseases. Therefore, these organisms developed a number of DNA repair pathways to maintain the integrity of the genome, and for the proteins involved to perform its function. However, these proteins need to be imported through a nucleus-cytoplasmatic transport. The main importation process is the Classic Nuclear Import Pathway, which is constituted by the heterodimer Importin-α/β. The Importin-β function as the transporter, and the Importin-α operates as an adapter, binding to cargo proteins with activities in the nucleus. These proteins contain the nuclear localization sequences (NLS), which are characterized by one or two clusters of basic amino acids (monopartite and bipartite NLSs, respectively). This study aims to characterize the interaction between the Importin-α and the NLS from the NEIL3 protein using Isothermal Titration Calorimetry (ITC) and crystallographic techniques. The ITC experiments showed that NEIL3 NLS peptide binds with the protein receptor with a stoichiometry of 1:1 (protein:peptide) and dissociation constant compatible with other monopartite structures. A crystal of the Importin-α from *Mus musculus* complexed to the NEIL3 NLS was used to collect X-ray diffraction data using a Synchrotron Radiation Source (LNLS, Campinas, Brazil) which was processed to 2.2 Å resolution. The crystal was isomorphous to the native protein and, then, the Fourier synthesis method was used to get the complex crystal structure. Results from both calorimetric and crystallization experiments show that NEIL3 NLS binds as a monopartite sequence at the MmImpα major binding site. Therefore, it is possible to suggest that the protein NEIL3 is imported by the Classic Nuclear Import Pathway.

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Insights into modes of action of the synthetic peptide *Mo*-CBP3-PEPIII against *Candida spp*. through quantum biochemistry and molecular dynamics simulations

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Candida is the principal group of opportunistic pathogens in humans and is responsible for 8 to 10% of the bloodstream infections at Intensive Treatment Unit. There has been observed a great increase in the microorganism resistance to current strategies, raising a necessity to develop new drugs with a greater spectrum of action. The antimicrobial peptides are indicated as the most promising drug alternative with a better cost-effective, a broad spectrum of action and a low resistance induction. Based in a purified protein from *Moringa oleifera* with high microbial activity, *Mo*-CBP3, our research group designed a synthetic antimicrobial peptide, named *Mo*-CBP3-PEPIII, with antimicrobial activity 130-fold higher compared to *Mo*-CBP3. In this work, we demonstrated the possible mechanism of actions of this synthetic peptide through in silico approaches with keys enzymes: sterol 14-α demethylase, secreted aspartic proteases, β-1,3-glucanase and with realistic membrane of yeast. First, we analyzed the interactions using molecular docking, followed by a simulation of molecular dynamics for 20 ns with the enzymes and 150 ns with the yeast membrane. Finally, we calculated the interaction energies between *Mo*-CBP3-PEPIII and the key enzymes applying quantum biochemistry. Although *Mo*-CBP3-PEPIII presented no interaction with the catalytic site of sterol 1,4-alpha demethylase, it showed interaction with secreted aspartic protease 5 and β-1,3-glucanase in active site, presenting interaction energy of -104.9 kcal/mol and -122.2 kcal/mol, respectively. In the simulation of molecular dynamics was possible to observe the insertion of the *Mo*-CBP3-PEPIII into the yeast membrane. Altogether, the results indicate that the mechanism of action differs from azoles drugs and the interaction with other key enzymes can be an alternative mechanism of action. Thus, it can be inferred that the mechanism of action crucial is the penetration of the *Mo*-CBP3-PEPIII into the membrane and pore formation. *Mo*-CBP3-PEPIII has a great potential to be a new drug with a broad spectrum of action.

Keywords: Mo-CBP3-PEPIII. Mechanisms of action. In silico approaches.

Acknowlegments: Agencies CNPq and CAPES.

Structural characterization of metacaspase TcMCA5 from *Trypanosoma cruzi* and identification of its interaction partners

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Abstract

Cellular death occurs in all organisms, and it means the ending of cell normal metabolism and, consequently, the end of cellular activities. Apoptosis is a well-known process of programmed cellular death (PCD) associated with autophagy that presents peculiar morphological features. There are several treatments inducing PCD, including those for parasitic diseases. This scenario shows that targeting PCD presents potential for drug development. Despite the presence of PCD morphological characteristic, the apoptotic process in unicellular organisms remains incomprehensible. Regarding *Trypanossoma cruzi,* the protozoan that causes Chagas' disease, metacaspases (TcMCA3 and TcMCA5) seem to have a regulatory function on parasitic apoptosis. According to a previous work from our group, TcMCA3 is regulated by interaction with other cell compounds, which induce biochemical events that are characteristics of the apoptotic process. The aims of this work are: (1) the identification of compounds that activate apoptosis in *T. cruzi* through interaction with TcMCA5, (2) the structural characterization of TcMCA5-ligand complexes and (3) the identification of TcMCA5 protein partners. These data are essential to understand the pathway of apoptosis of *T*. *cruzi* and so, if possible, to manipulate the parasites' PCD through the TcMCA5 activation. All these might contribute to the development of new therapeutic approaches and chemotherapy treatments.

Keywords: apoptosis, protozoan, protein-protein interaction.

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Expression of methyltransferases hTrm9L and Trm112 from *Homo sapiens* for future structure solution

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Transporter RNA (tRNA) takes part on the fundamental process of protein synthesis. Its function is to transport aminoacids that will be used during protein synthesis in the ribosome. For this task to be accomplished correctly, many tRNA molecules need chemical modifications, like some nucleoside methylation, to assure their correct folding and its correct decodification[1]. The methylation process is performed by enzymes called methyltransferases (Trm) and uses S-adenosyl-methionine (SAM or AdoMet) as a methyl group donor[2]. The complex Trm9/Trm112 (in which Trm9 is the *de facto* methyltransferase and Trm112 is an auxiliary subunity) is conserved in eukaryotes and Trm9 presents two ortologues in humans, namely, ABH8 e hTrm9L. This work aims at expressing human hTrm9L and Trm112 heterologously for future structure solution.

Escherichia coli BL21(DE3) cells were transformed by electroporation with plasmids pET21b(+) that harbored the coding genes of which subunit. Cells that were effectively transformed were selected by growth in media supplemented with ampicillin. Subsequently, transformed cells were propagated in 5 mL of liquid medium ZYP-5052 (with a composition to specifically express the proteins through self induction)[3] at 37 °C for 22 h. Media were centrifuged and cells were promptly resuspended in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, pH = 7.5); they were sonicated for 5 min. The resulting suspension was centrifuged to separate the cell debris. Samples from total lysis fraction and of the soluble and insoluble fractions were taken for analysis by SDS-PAGE. A Ni^{2+} affinity chromatography of the total lysed fraction was also accomplished. The SDS-PAGE results from expression tests of the Trm112 protein indicated an intense band in the expected position for molecular mass of circa 14 kDa (theoretical mass 14,199 kDa), nevertheless, in the lane that corresponds to the insoluble fraction. Yet, in the SDS-PAGE experiments for subunit hTrm9L, no bands were observed that could be attributed to this enzyme.

We concluded that enzyme hTrm112 is satisfactorily expressed in self induction media and can be purified by means of affinity chromatography. Nevertheless, new tests must be accomplished to stabilize it in solution. Concerning hTrm9L, new cell transformation experiments must be accomplished. Once both proteins are satisfactorily produced, the next step is to solve their structure, either by x-ray crystallography or CryoEM.

Keywords: methyltranferases, transporter RNA, structure determination, protein expression.

Acknowlegments: CAPES.

Structural biology of enzymes involved in the (S)-4-amino-2- hydroxybutyrate (AHBA) biosynthesis from the aminoglycoside butirosin

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Natural products obtained from secondary metabolism of bacteria, plants and fungi represent an important source of antimicrobial agents for the treatment of diverse illness, such as infections. One of these agents is the aminoglycosides, molecules that have precursors from the glycolytic pathway in bacteria, as butirosin, which is produced by *Bacillus circulans*. One of its particularities is the presence of an (S)-4-amino-2- hydroxybutyrate (AHBA) attached to the 2-deoxystreptamine (2-DOS) aglycone ring. The presence of AHBA in butirosin makes this antibiotic less susceptible to bacterial resistance mechanisms through aminoglycosides modifying enzymes. The biosynthesis of the AHBA group in the butirosin pathway is not sufficiently understood and the structures of the six enzymes that participate in its biosynthesis, have not been extensively studied. Herein, we aim to perform a structural analysis of three of these enzymes: BtrJ in charge of the addition of molecules of L-glutamate; BtrK that catalyzes a decarboxylation reaction of glutamate; and BtrH which participates of the final steps transferring the AHBAs group from an acyl carrier protein to a ribostamycin molecule. Genes *btrJ, btrK* e *btrH* were cloned in pET28a plasmid vector. BL21(DE3) or Rosetta competent cells were used for transformation and expression of the protein. Ni^{2+}NTA affinity and size-exclusion chromatography were performed to purify the proteins and subsequent protein crystallography experiments were performed by sitting drop and hanging drop. Crystal X-ray diffraction data collection was performed at Synchrotron Light Source DESy (PETRA III, Germany). Data for BtrK crystals have been obtained at a resolution of about 1,4 Å in the space group P21212. BtrK is a two-domain protein, one of them with a TIM barrel folding which comprises of the PLP active site and possibly, the region that interacts with other substrates (*Figure 1*). BtrK homodimer structure (functional state of the protein) was obtained through symmetry operations in COOT. BtrJ was successfully purified and crystallization assays were developed obtaining crystals with different morphologies; how ever, it was no possible to collect any image after x-ray diffraction. BtrH enzymes experiments are still in progress. Further experiments are proposed to co-crystallize these enzimes with some ligants to see structural changes and elucidate functional features.

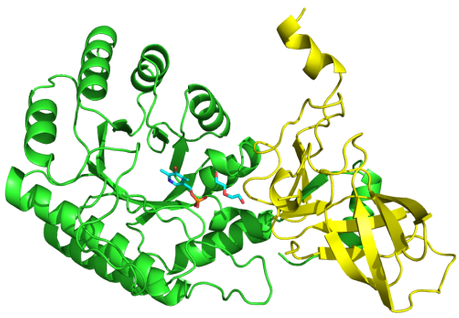

Figure 1. Two domain structure of BtrK protein. TIM barrel domain is identified in green and β-sandwich domain in yellow. PLP active site is represented in stick model.

Keywords: natural products, butirosin, AHBA, decarboxylase, structural biology

Acknowlegments: FAPESP, CAPES, CPNq

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Structural and biochemical studies of cellulosomal thermophilic family 3 β-glucosidase, CtBgl3B from *Clostridium thermocellum*

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The reduction of petroleum reserves, the growing energy demand from emerging countries, indicate the importance to pursuit new sources of renewable energy. Thus, the bioethanol production through hydrolysis of lignocellulosic biomass, has received considerable attention. Due to its potential for development and cost reduction, the enzymatic hydrolysis of cellulose using cellulases can be a key factor for the production of second-generation bioethanol at a competitive cost in the long term. Some thermophilic bacteria, such as *Clostridium thermocellum*, produces an extracellular multi-enzyme protein complex named cellulosome, which has been demonstrating a high ability to efficiently perform cellulosic biomass degradation, especially in the crystalline portion of cellulose. Thereby, cellulase mixtures are commonly used for the saccharification of cellulose in biotechnological applications. The β-glucosidases (BGls, EC 3.2.1.21), which hydrolyzes the β-linkage between two adjacent molecules in dimers and short oligomers of glucose, it has been shown that enhanced levels of BGls in cellulase mixtures may benefit the conversion of cellulose to glucose.

Aiming to characterize cellulosomal BGls from *C. thermocellum*, by structural, biochemical and biophysical techniques herein we describe the CtBgl3B. The cloned CtBgl3B gene had protein expressed in *Escherichia coli* (BL21) and has been successfully purified following affinity and size exclusion chromatography. The purified enzyme has a thermophilic characteristic, showing higher activity at pH 5.5 and 70 °C. Interestingly, it has a melting temperature (Tm) of ≈70 °C, determined by differential scanning fluorimetry (DSF) and circular dichroism (CD).

In addition, kinectic parameters: *Michaelis-Menten* constant Km = 0.45 (mM), catalytic constant k_{cat} = 201 (s^{-1}), and catalytic efficiency k_{cat}/Km = 444 (mM^{-1} s^{-1}). The structure of CtBgl3B was determined by X-ray diffraction at 2.34 Å resolution using molecular replacement, demonstrating a dimeric arrangement in the asymmetric unit, indicators of refinement quality R_{work} and R_{free}, respectively 0.21 and 0.24. The structure has three-domain architecture as observed previously for other glycoside hydrolase family 3 Bgls, and in addition, a fourth domain of unknown function at C-terminal that interact with the partner monomer interfacing near to the catalytic cleft. Multi-angle light scattering (MALS) and small-angle X-ray scattering (SAXS) experiments corroborate for the dimeric arrangement in solution.

Keywords: β-glucosidase (BGls); Clostridium thermocellum; Hydrolysis of ligno-cellulosic biomass; GH3; Cellulosome.

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A Molecular Evolution Strategy for Optimization of Single-Chain Variable Fragments (scFv) Binding to Human Osteopontin

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Osteopontin is a protein mainly produced by osteoclasts, osteoblasts, macrophages and lymphocytes which plays important roles in cell-adhesion, tissue growth and angiogenesis. Osteopontin binds to integrins and CD44 on the cell surface and has been described as a tumor promoting factor in many types of cancers. Blockage of osteopontin binding to integrins by monoclonal antibodies was shown to restrain metastasis and invasion in mice. Therefore, inhibition of osteopontin by antibodies is considered a promising therapy. In this work, we aim to improve the affinity of three scFv antibodies designed to bind to osteopontin and to determine their efficiency to block osteopontin interaction with its receptors. The experimental strategy involves generation of variants of the anti-OPN scFv coding sequences using error-prone PCR followed by cloning into the plasmid pET28a in fusion with SGFP2. This allows for direct selection of positive clones by fluorescence detection when the resulting plasmids are transformed into the *E. coli* BL21-Star (DE3) expression strain plated in medium containing IPTG as inducer. The clones showing higher fluorescence intensity will be selected for determination scFv expression yields and affinity assays. The ability of the scFvs to block osteopontin interaction with either CD44 or integrins will be tested by flow cytometry using Molt-4 and HEK 293 cell lines. Libraries of scFv variants have already been successfully created. Cloning experiments have demonstrated that it is possible to directly transform DNA from ligation reactions into the *E. coli* BL21-Star (DE3) expression strain and to select for positive expression based on GFP fluorescence in medium containing low IPTG concentrations (50 μM). Higher concentrations of IPTG caused inhibition of growth possibly due to toxicity caused by formation of inclusion bodies by the scFv-GFP fusions. Although the expression conditions still need refinement, the colonies displaying fluorescent phenotypes showed a reasonable amount of soluble scFv-SGFP2. Future steps involve selection of a higher number of scFv variants and optimization of the expression conditions to improve recombinant scFv yields. Promising candidates will be purified for analysis of the binding affinity towards osteopontin using microscale thermophoresis. At the end of this project, we expect to obtain scFv variants with a higher affinity to osteopotin and improved solubility.

Keywords: Immunotherapy, Immunology, Cancer.

Acknowlegments: CAPES, CNPq, Fundação Araucária and FIOCRUZ.

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Crystallization and Preliminary X-ray data of a crotoxin B from *Crotalus durissus cumanensis*

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Rattlesnakes are snakes who belongs to the genus *Crotalus* (Viperidae family) and are geographically distributed from Canada to northern Argentina. The most widely distributed species is *Crotalus durissus*, which has 14 described subspecies, such the Colombian rattlesnake *Crotalus durissus cumanensis*, where are distributed in three geographic zones of the country: the Atlantic Coast, the upper valley of Magdalena River and the eastern plains of the Colombian Orinoquía. The venom of the South American rattlesnakes is composed of a complex mixture of peptides, enzymes and toxins. The crotoxin (CTX) is an abundant toxin present in the *Crotalus durissus* genus and the biological effects includes neurotoxicity, systemic myotoxicity, mild edema and acute renal failure. The CTX is a heterodimeric complex formed by a non-enzimatic acidic portion (crotapotin) and the basic PLA_2 (CB), which can make up between 70% and 80% of the toxin contente of the venom. Here, we present the crystallization of a CB single crystal, obtained from *C.d. cumanensis* by a conventional hanging drop vapor-diffusion method. The X-ray diffraction resulted in a set of 720 images. Initial data processing using DIALS v.1.8 software, reveals that the data was diffracted up to 2.75Å resolution and the crystal belongs to centrered monoclinic C2 space group, presenting one molecule in the asymmetric unit. The initial refinement results obtained shows differences in quaternary structure between the CB of this study (monomeric) and the CB from *C.d. terrificus* (tetrameric).

Keywords: *Crotalus durissus cumanensis*, Crotoxin B, Snake venom PLA_2.

Acknowlegments: FAPESP, CNPq and CAPES

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In silico approaches for structural determination of a *Trypanosoma cruzi* Nitroreductase

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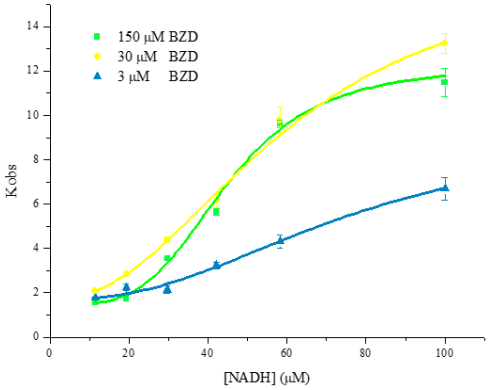
Chagas disease affetcs about 8 million people worldwide, causing death of 7 thousand people/year. Initially, the disease was restricted to low income population, on emergent countries, as in South America. But, due to globalization, the infectant protozoan, *Trypanosoma cruzi*, has spread to developed countries, such USA, where is more than 300 thousand cases estimated. The transmition may be mediated by the blood-sucking bug "kissing bug", or by oral transmition. Benznidazole is capable of treat Chagas disease, but it is very toxic and ineffective. Benznidazole is a prodrug activated by action of a nitroreductase [1], which catalyses the reduction of benznidazole to high nucleophylic metabolites, attacking membranes, proteins and DNA. Despite the drug use since 1970s, its mechanism of action is still unclear. Aiming to develop new drugs nitroredutase targeted, this study is directed to TcNTR (Nitroreductase) structure characterization, and its mechanism of action. This enzyme has an unknown structure, and hasn't been crystallized yet. *In silico* approaches could provide some informations about the ollygomeric state, residues of interaction with cofactor, and also about its catalytic mechanism.

Material and Methods – Kinetic studies were performed with heterologous TcNTR_72 expressed by *E. coli* Rosetta, purified by affinity chromatography, using Ni-NTA resin. Kinetics assays were performed measuring the NADH consumption by fluorescence, in presence of benznidazole. *In silico* studies were required to explain unexpected kinetic profile. Were run the predictors SwissModel, PsiPred, Phobius, TMHMM.

Results – Kinetic assays with benznidazole showed un undescribed sigmoidal character for TcNTR. Since the appearence of the sigmoidal profile, such as autoinhibition of TcNTR on high substrate concentrations, we investigated the possibility of a dimeric organization, not reported. Through the correlation between different predictors and comparission with bacterial nitroreductases, we were able to propose a new model to TcNTR.



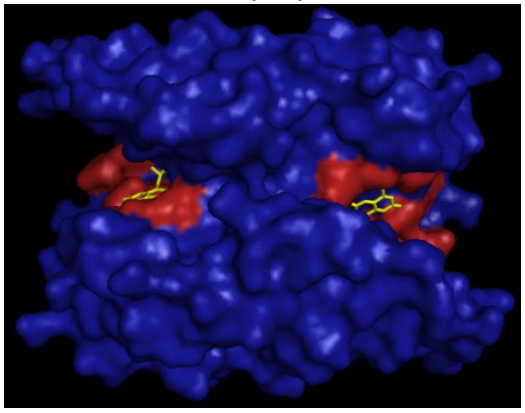


Figure 1a (Left): Enzymatic kinetic assays, of TcNTR_72, showing cooperativity between subunits. BZD at a constant concnetration, varying NADH concentration (μM). Figure 1b (Right): Modelo of TcNTR_72 dimer, based on TtNADH Oxidase (*Thermus thermophilus*), the enzyme with greater structural identity, of 30%.

References: 1 - Hall, B. S., Meredith, et al (2012). Targeting the Substrate Preference of a Type I Nitroreductase To Develop Antitrypanosomal Quinone-Based Prodrugs. *Antimicrobial Agents and Chemotherapy*. **Vol. 56**, 5821-5830.

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Optimization of SmTGR inhibitors using a Fragment-Based Drug Design (FBDD) approach.

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Schistosomiasis is a neglected tropical disease caused by *Schistosoma* spp. Praziquantel is the unique drug used for the treatment of the disease. Although the success of the treatment, the concern about resistance is growing. Thioredoxin glutathione reductase of *Schistosoma mansoni* (SmTGR) is a validated drug target that plays a crucial role in the redox homeostasis of the parasite. An *in silico* fragment derived discovery have been used to identify 32 SmTGR ligands and the search of analogues revealed 196 structurally related compounds was found. In this work an *in silico* fragment-based drug design (FBDD) will be carry out to optimize the compounds with highest inhibitory activity. Linking and growing approach will be use and evaluation of synthetic accessibility and toxicity prediction will be performed.

Keywords: SmTGR, FBDD

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