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Lilian Hernández Alvarez

In silico structural studies on protein targets for drug discovery against neglected tropical diseases

São José do Rio Preto
2022

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biofísica Molecular, junto ao Programa de Pós-Graduação em Biofísica Molecular, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

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Orientador: Profª. Drª. Fátima Pereira de Souza
Coorientador: Pedro Geraldo Pascutti

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A aquellos que están y a los que ya se fueron, a todos los que hacen el camino más ameno…

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“Every day is a new day. It is better to be lucky. But I would rather be exact. Then when luck comes you are ready.”

Ernest Hemingway (The Old Man and the Sea, 1952, p. 32)

RESUMO

As doenças tropicais negligenciadas (NTDs) são infecções prevalentes em áreas remotas e rurais de países pobres da África Subsaariana, Ásia e América, que têm sido historicamente desatendidas e representam o mercado de menor prioridade das grandes indústrias farmacêuticas. As NTDs causadas por parasitas protozoários apresentam altos índices de morbilidade e mortalidade, e os tratamentos disponíveis são tóxicos e ineficientes. Neste estudo, técnicas experimentais e computacionais foram utilizadas para contribuir ao conhecimento das bases moleculares de diversos alvos proteicos dessas doenças com vistas ao desenvolvimento de futuros fármacos. Estratégias computacionais foram empregadas com o objetivo de identificar novos inibidores competitivos da cruzaína, a maior cisteíno protease do *Trypanosoma cruzi*. A avaliação experimental nos permitiu descobrir um novo inibidor competitivo desta protease com um IC_{50} inferior a 15 μM. Além disso, foi determinada a estruturas de raios-X do complexo cruzaína-gallinamida A e métodos computacionais nos permitiram identificar os determinantes atômico-estruturais subjacentes na inibição seletiva desta série de compostos. Por outro lado, as bases moleculares da modulação alostérica exercida sobre fator de iniciação de tradução eucariótica 4E 1 (LmIF4E-1) em *Leishmania major* pela proteína 4E-IP1 (Lm4E-IP1) foram desvendadas utilizando métodos *in silico*. Nesse sentido, foram determinadas as diferenças nos movimentos internos das formas apo e complexadas com Lm4E-IP1 e com cap da LmIF4E-1. Este estudo permitiu detalhar as perturbações dinâmicas ocorridas no sítio principal de ligação da LmIF4E-1 desencadeadas pela união com Lm4E-IP1. Esta analise contribuiu à compreensão do processo da expressão gênica nos tripanossomatídeos e revelou informações cruciais da regulação alostérica nas eIF4Es. Por fim, foi realizada a modelagem computacional do homodímero da esterol 14alfa-desmetilase (AcCYP51) de *Acanthamoeba castellani* inserido numa bicamada lipídica. Nosso modelo revelou que o dímero permanece estável, interagindo com a bicamada ao longo do tempo de simulação. Esses resultados também nos forneceram informações sobre a potência superior do isavuconazol contra esse parasita comparados com outros do mesmo gênero.

Palavras-chave: *Trypanosoma cruzi*. *Leishmania*. *Acanthamoeba castellanii*. Cruzaína. LmIF4E-1. Dímero AcCYP51. Alosteria. Dinâmica molecular. Triagem virtual. Energia livre de ligação. Ensaios de inibição. Modo de ligação. Cristalografia de raios X

ABSTRACT

Neglected tropical diseases (NTDs) are a group of infections that are prevalent in remote and rural areas of low-income countries in Sub-Saharan Africa, Asia, and the Americas. These diseases do not receive enough attention, representing the lowest priority markets for the big pharmaceutical manufacturers. NTDs caused by protozoan parasites are responsible for significant morbidity and mortality and current drug treatments are toxic and prolonged. Here, experimental and computational approaches were employed to contribute to the knowledge of the molecular bases of several targets and the development of future drugs against these diseases. A computational strategy was employed to predict novel competitive inhibitors against the papain-like cysteine protease cruzain. The experimental evaluation of the predicted compounds allowed us to identify a novel competitive inhibitor of this protease with an IC_{50} under 15 μM. In addition, the X-ray structures of the cruzain complexes with gallinamide A and analogs were also determined, and two series of synthetic gallinamide A analogs (23 compounds) were evaluated against cruzain and intracellular *Trypanosoma cruzi* amastigotes. The employed computational techniques allowed us to identify the hot spots and structural determinates underlying the selective inhibition of this compound series. Moreover, *in silico* strategy was developed to elucidate the molecular bases of the previously-reported allosteric modulation in *Leishmania major* exerted by 4E-IP1 (Lm4E-IP1) on eukaryotic translation initiation factor 4E 1 (LmIF4E-1). Here, the differences in internal motions of LmIF4E-1 apo form, cap-bound, and Lm4E-IP1-bound systems were described. This study thoroughly describes the dynamical perturbations of LmIF4E-1 cap-binding site triggered by Lm4E-IP1, providing an understanding of trypanosomatids' gene expression and gaining insight into the allostery of eIF4Es. Finally, the computational modeling of full-length sterol 14alpha-demethylase (AcCYP51) homodimer of *Acanthamoeba castellani* inserted in a lipid bilayer was performed. Our model revealed that AcCYP51 dimer interacts with the membrane lipids and remains stable within the bilayer along the simulation time. These results also provided us the insights into the superior potency of isavuconazole against this parasite.

Keywords: NTDs. Parasite. Chagas. *Leishmania*. *Acanthamoeba castellanii*. Cruzain. LmIF4E-1. AcCYP51 dimer. Allostery. Molecular dynamics simulation. Virtual screening. Free energy. Inhibition assays. Binding mode. Community Analysis. X-ray crystallography.

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ABBREVIATIONS AND SYMBOLS

3D	Three-dimensional
4E-BPs	4E-binding proteins
5-FU	5-fluorouracil
ABF	Adaptive Biasing Force
AIDS	Acquired Immunodeficiency Syndrome
Amberff	Amber Force-field
CADD	Computer-Aided Drug Design
cap	5′ cap (cap-0), a guanine nucleotide connected to mRNA via 5′ to 5′ triphosphate linkage, found on the 5′ end of an mRNA molecule
cap-4	Native trypanosomatids' cap
CARDS	Correlation of all Rotameric and Dynamical States
CASP	Critical Assessment on protein Structure Prediction
CDC	Center for Disease Prevention and Control
CNA	Community network analysis
CYP	Cytochrome P450
CYP51	Sterol 14α-Demethylase
DBLβ12	Duffy binding-like β12 protein
DMSO	Dimethyl sulfoxyde
DMTP	Dimethyl-triphosphate
E64	Trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane
ED	Essential Dynamics
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic initiation factor E
eIF4F	Eukaryotic initiation factor F complex
eIF4G	Eukaryotic initiation factor G
EM	Energy minimization
ER	Endoplasmatic reticulum
FEP	Free energy perturbation
FF	Force-field
ff14SB	AMBER14SB force field
GAFF2	Generalized Amber Force-field 2
GC	Generalized Correlation coefficient
gC1qR	Receptor for the globular heads of complement component C1q
HAT	Human African trypanosomiasis

hCatB	Human cathepsin B
hCatK	Human cathepsin K
hCatL	Human cathepsin L
hCatS	Human cathepsin S
HPLC	High-performance liquid chromatography
IDH2	Isocitrate dehydrogenase 2
kDNA	Kinetoplastids DNA
Ki	Inhibition constant
LIE	Linear Interaction Energy
LmIF4E-1	Eukaryotic translation initiation factor 4E-1 of *Leishmania major*
m^7G	7-methyl-guanosine
m^7GpppA	P1-7-methylguanosine-P3-adenosine-5',5'-triphosphate
m^7GTP	7-methyl-guanosine 5'-triphosphate
MD	Molecular Dynamics
MM-GBSA	Molecular Mechanics Poisson-Boltzmann Surface Area
MTX	Methotrexate
NDH2	Type II NADH dehydrogenase
NPT	Isothermal-Isobaric Ensemble
NTDs	Neglected Tropical Diseases
NVT	Isothermal-Isochoric Ensemble
PBC	Periodic boundary conditions
PCA	Principal Component Analysis
PDB	Protein Data Bank
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PME	Particle Mesh Ewald
PMF	Potential of Mean Force
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
PTR	Pteridine reductase 1
QM	Quantum mechanics
RC	Reaction Coordinates
REL1	RNA-editing ligase 1
RESP	Restrained Electrostatic Potential
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
RPI	Ribose 5-phosphate isomerase

SAR	Structure-activity relationship
SBVS	Structure-Based Virtual Screening
SDS	Sulfate−polyacrylamide
siRNAs	Small interfering RNA molecules
SMD	Steered molecular dynamics
TcIF4E5	Eukaryotic translation initiation factor 4E-*5 of T. cruzi*
TGF-β	Transforming growth factor beta
TI	Thermodynamic Integration
TMH	Transmembrane helix
TR	Trypanothione Reductase
UFF	Universal Force-field
US	Umbrella Sampling
VS	Virtual Screening
WHO	World Health Organization
WISP	Weighted Implementation of Suboptimal Paths
ΔG	Gibbs Free Energy
$\Delta G°_{bind}$	Absolute Binding Free Energy with correction of the standard state.
ΔG_{bind}	Binding Free Energy calculated through MM-GBSA
ΔG_{eff}	Effective Free Energy
ΔGR	Free Energy of releasing the xy orthogonal restraints in the bound state
ΔS_{conf}	Configurational Entropy
$\Delta\Delta G$	Relative Free Energy

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1 INTRODUCTION

In past decades, the efforts to protect the health of the populations affected by Neglected Tropical Diseases have been insufficient. "*People affected by neglected tropical diseases (NTDs) are frequently the target of social stigmatization*," said Hofstraat and van Brakel in their article published in 2016 in the International Health journal [1]. As the main strategies conducted by public health institutions are dedicated to illness prevention via environmental policies controlling vectors and through people education, there will be a greater urgency for new treatments for people in the acute or chronic phase of these infections.

The application of basic research in the study of NTDs has led to the discovery and thorough description of novel molecular phenomena specific to the causative agents of these infections. But at the same time, the use of modern laboratory techniques to study biomolecules and their interactions is a time-consuming and expensive process that generates low short-term profits, mainly in the industry. So, how could academic science continue to contribute to the study of these diseases? As an answer to that question, I have spent most of my Ph.D. course (2017-present) applying cutting-edge computational techniques to study the molecular bases of protein function and dynamics, protein-ligand interactions and protein allostery with molecular targets of several parasites that cause NTDs. In this section, a compilation of the available information about the NTDs of interest and the biological functions related to the studied protein targets is presented. Here, brief attention to the applications of modern computational techniques in the study of macromolecules and cellular processes of parasitic diseases is also given.

1.1 Parasitic diseases

Human parasitic diseases can be defined as a wide range of infections caused by any species that have formed a symbiotic relationship with human hosts. The effects of parasitic infections on humans are also diverse and can range from temporary conditions, such as severe diarrhea or anemia, to more permanent conditions, such as growth retardation, permanent inflammation of limbs and organs, or blindness. These infections are among the most common in the world, affecting billions of people each year [2]. According to the statistical data, one in six people worldwide is believed to be infected with a parasitic disease [3]. However, the burden of these diseases is disproportionately concentrated in economically and socially disadvantaged countries. This significant health burden is reflected in the World Health Organization (WHO) list of NTDs,

7 CONCLUDING REMARKS

The present document compiles a significant amount of results comprising different protein targets of neglected tropical diseases, which were obtained by using a wide range of computational techniques. Cruzain, a key cysteine protease of *T. cruzi*, was thoroughly studied in order to predict new selective inhibitors targeting its active site. A protocol consisting of SBVS of a large compound database against cruzain's active site, followed by several rescoring steps, including the assessment of potential cross-inhibition of human off-targets (hCatL, hCatB, hCatK and hCatS) to remove non-specific ligands, was devised. This protocol led to the successful identification of a novel inhibitor, termed C2, with an IC_{50} of 13 μM against cruzain and no detectable inhibition of hCatL at a compound concentration up to 100 μM. Moreover, *in silico* analyses were performed to identify the most likely binding mode of C2 within cruzain's active site, which yielded a compound conformation consistent with the known molecular determinants of binding to enzyme and a binding free energy close to the experimental value. The predicted structure of the cruzain-C2 complex will be valuable for the optimization of the C2 scaffold in order to obtain more potent derivatives.

We also determined the co-crystal structures of cruzain with gallinamide A, a potent inhibitor first isolated from a collection of marine cyanobacteria, through X-ray diffraction. Subsequently, a series of gallinamide A derivatives was generated and three compounds showed up to 15-fold increase in cruzain inhibition. By combining crystallographic and SAR data obtained for the compound series and computational analyses, we concluded that the N-terminal end of gallinamide A scaffold is loosely bound and that it does not contribute significantly to the compound binding. At the C-terminus, the intramolecular π-π stacking interactions between the aromatic substituents at P1′ and P1 restrict the bioactive conformation of the inhibitors, thus minimizing the entropic loss associated with target binding. Interestingly, MD simulations showed that, in the absence of an aromatic group at P1, the substituent at P1′ interacts with TRP184 of cruzain. The P1-P1′ interactions had no effect on the enzymatic inhibitory activity, whereas anti-*T. cruzi* potency increased by ~5-fold in some cases, likely due to an increase in solubility/permeability of the analogs. Overall, the previous studies set the bases for the rational design of more potent anti-Chagas drugs.

We thoroughly analyzed *in silico* the interactions of LmIF4E-1 and its ligand m^7GpppA and provided new insights into the inhibition of former transcription factor and the regulatory protein Lm4E-IP1. First, it was shown that the m^7G moiety accommodates

between the indole rings of W37 and W83 of LmIF4E-1 during all the simulation time, whereas adenosine remains largely exposed to the solvent. This underscores the importance of the π-π stacking interactions to stabilize m^7G and their contribution to the ligand binding. Remarkably, the ABF free energy calculations accounted for the decrease in the affinity of LmIF4E-1 for the cap in the presence of the Lm4E-IP1, in agreement with experimental results. Furthermore, the allosteric and structural analyses conducted for these systems provided new insights into the allosteric inhibition of LmIF4E-1 by Lm4E-IP1 and identified key residues involved in signaling paths between the dorsal grove and the cap-binding site of the transcription factor. According to our predictions, the regulatory protein binding promotes a rearrangement of LmIF4E-1 W37 and W83 side-chains, thereby leading to a loss of affinity for the cap. Overall, a deep understanding of LmIF4E-1 recognition of the cap and its regulatory protein emerged from our study, which sets the bases for intervention of such process and the discovery of new drugs inhibiting the protein translation in *Leishmania spp.*

Finally, we built a model of AcCYP51 homodimer embedded in a lipid bilayer starting from a crystal structure of this cytochrome lacking the transmembrane regions. Our results revealed the high stability of the modeled protein-lipid system along the MD simulations. Moreover, key interactions at the protein-lipid interface were characterized and hinge regions of CYP51 that are likely to be important for the dimerization process were proposed.

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