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Identification and Characterization of Cruzain Allosteric Inhibitors: A Computer-Aided Approach

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre 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", Campus de São José do Rio Preto.

Orientador: Prof. Dr. Pedro Geraldo Pascutti

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Inscription

A todos aquellos que me inspiran día a día desde
la distancia…

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“My mission in life is not merely to survive, but to thrive; and to do so with some passion, some compassion, some humor, and some style."

— Maya Angelou

RESUMO

Trypanosoma cruzi é o agente causal da doença de Chagas, uma infecção negligenciada que afeta milhões de pessoas nas regiões tropicais. A maioria dos fármacos empregados no tratamento desta doença são altamente tóxicos e geram resistência. Na atualidade, o descobrimento de inibidores alostéricos é um tópico emergente dentro da área de desenho computacional de fármacos, pois promove a acessibilidade a medicamentos mais seletivos e menos tóxicos. Neste trabalho foi desenvolvida uma estratégia para a descoberta computacional de inibidores alostéricos a qual foi aplicada à cruzaína, a principal cisteíno protease do *T. cruzi*. A caracterização molecular da forma livre e ligada da cruzaína foi investigada através do ancoramento molecular, simulações de dinâmica molecular, cálculos de energia livre de ligação e construção de redes de interações entre resíduos. A partir da análise baseada na geometria das estruturas geradas na dinâmica molecular, foram detectados dois potenciais sítios alostéricos na cruzaína. Os resultados sugerem a existência de diferentes mecanismos de regulação exercidos pela ligação de inibidores diferentes no mesmo sítio alostérico. Além disso, foram identificados os resíduos que estabelecem os caminhos de transmissão de informação entre um dos sítios alostéricos identificado e o sítio ativo da enzima. O presente estudo é a primeira aproximação de desenho de inibidores alostéricos da cruzaína e serve para futuras intervenções farmacológicas. Esses resultados constituem uma base para o desenho de inibidores específicos de cisteíno proteases homólogas da papaína.

Palavras-chave: cruzaína, alosteria, triagem virtual, energia livre de ligação, correlação generalizada, redes de proteínas, comunidades, caminho subótimo.

ABSTRACT

Trypanosoma cruzi is the causative agent of Chagas disease, a neglected infection affecting millions of people in tropical regions. There are several chemotherapeutic agents for the treatment of this disease, but most of them are highly toxic and generate resistance. Currently, the development of allosteric inhibitors constitutes a promising research field, since it may improve the accessibility to more selective and less toxic medicines. To date, the allosteric drugs prediction is a state-of-the-art topic in rational structure-based computational design. In this work a simulation strategy was developed for computational discovery of allosteric inhibitors, and it was applied for or cruzain, a promising target and the major cysteine protease of T. cruzi. Molecular dynamics simulations, binding free energy calculations and network-based modelling of residue interactions were combined to characterize and compare molecular distinctive features of the apo form and the cruzain-allosteric inhibitor complexes. By using geometry-based detection on trajectory snapshots we determined the existence of two main allosteric sites suitable for drug targeting. The results suggest dissimilar mechanism exerted by the same allosteric site when binding different potential allosteric inhibitors. Finally, we identified the residues involved in suboptimal paths linking the identified site and the orthosteric site. The present study constitutes the first approximation for designing cruzain allosteric inhibitors and may serve for future pharmacological intervention. These findings are particularly relevant for the design of allosteric modulators of papain-like cysteine proteases.

Keywords: *cruzain, allostery, virtual screening, binding free energy, generalize correlation, protein network, communities, suboptimal pathway.*

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Nomenclature and abbreviations

APBS: Adaptive Poisson-Boltzmann Solver

CC: Cross-correlation

CDC: Centers for Diseases Control

CP: Coordination Propensity

DNA: Deoxyribonucleic Acid

ED: Essential Dynamics

EM: Energy Minimization

FDA: Agency of Food and Drug Administration

FEP: Free Energy Perturbation

GAFF: General AMBER Force Field

GAGs: Glycosaminoglycans

GC: Generalized Correlation

GPCR: G Protein-Coupled Receptors

HAT: Human African Trypanosomiasis

HCatB: Human Cathepsin B

HCatK: Human Cathepsin K

HCatS: Human Cathepsin S

IC50: The half-maximal inhibitory concentration

LIE: Linear Interaction Energy

Lmcps: *Leishmania* cathepsins

MD: Molecular Dynamics

MM-GBSA: Molecular Mechanics/Generalized Born Surface Area

MM-PBSA: Molecular Mechanics/Poisson Boltzmann Surface Area

NF-kB: Nuclear Factor *kappa B*

NMR: Nuclear Magnetic Resonance Spectroscopy

NPT: Constant-pressure ensemble

NTD: Neglected Tropical Diseases

NVT: Constant-volume ensemble

Orthosteric site: Describes the primary site of a receptor (protein)

Orthosteric drug: Drug binding to the orthosteric site

PCA: Principal Component Analysis

PDB: Protein Data Bank

PME: Particle Mesh Ewald method

POVME: POcket Volume MEasurer

RESP: Restricted Electrostatic Potential

RMSD: Root-Mean-Square Deviation

RMSF: Root-Mean-Square Fluctuation

SCA: Statistical Coupling Analysis

TGF-β: Transforming grow factor *beta*

TI: Thermodynamic Integration

VS: Virtual Screening

WHO: World Health Organization

WISP: Weighted Implementation of Suboptimal Paths

ΔG_{bind} :Binding Free energy

ΔG_{eff} : Effective Free Energy

ΔG_{res} :Per-residue Free Energy Contribution

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1. Introduction

Cruzain is the major papain-like cysteine protease of *Trypanosoma cruzi*, the protozoan responsible for Chagas disease. This enzyme is indispensable for the survival and propagation of this parasite and, therefore, is considered as a potential drug target for this disease control (McGrath,Eakin *et al.* 1995;Mark W. Robinson PhD 2011;Sajid,Robertson *et al.* 2011). Toxicity and resistance of the available chemotherapy (Ribeiro,Sevcsik *et al.* 2009;Wilkinson and Kelly 2009;Pena,Pilar Manzano *et al.* 2015), fueled the pursuit of alternative chemotherapeutic agents, leading to the discovery of several cruzain modulators. A tangible evidence of this, is the existence of twenty-five crystal structures of cruzain in complex with several competitive inhibitors on the Protein Data Bank (PDB) (McGrath,Eakin *et al.* 1995;Mott,Ferreira *et al.* 2010;Rogers,Keranen *et al.* 2012;Martinez-Mayorga,Byler *et al.* 2015). On the other hand, several experimental studies and computational predictions have been performed to characterize the cruzain binding site and specificity, facilitating the design of active-site directed drugs (Trossini,Guido *et al.* 2009;Durrant,Keranen *et al.* 2010;Ferreira,Simeonov *et al.* 2010;Rogers,Keranen *et al.* 2012;Martinez-Mayorga,Byler *et al.* 2015). However, the competitive inhibitors of cruzain have demonstrated to be toxic in several clinical trials because the high identity of this enzyme with its host homologues. This fact has encouraged the search for new scaffolds of cruzain competitive inhibitor as well as different strategies for the enzyme inhibition and ultimately novel therapeutic targets.

Allostery phenomenon is an inherent characteristic of most macromolecules (Dokholyan 2016;Nussinov 2016). In recent decades, the design of allosteric drugs has emerged as a promising research field in disease control (Nussinov and Tsai 2013;Lu,Li *et al.* 2014;Liu and Nussinov 2016). Allosteric modulators offer many advantages that make them appropriate as drug candidates. A major benefit of these strategies, compared to those that perturb the active site directly, is that they offer a noninvasive or more specific protein control. Allosteric regulators do not interfere with modulators of active site and do not obstruct its vicinity (Tsai and Nussinov 2014;De Vivo,Masetti *et al.* 2016;Dokholyan 2016).

Several findings pointing to the existence of allosteric regulation in the superfamily of papain-like cysteine proteases (Costa,dos Reis *et al.* 2012;Judice,Manfredi

et al. 2013;Jilkova,Horn *et al.* 2014;Novinec,Lenarcic *et al.* 2014). One example is the work of Novinec *et al.* where several allosteric sites of human cathepsin K (HCatK) and the sectors which mediate allosteric communication in this protein family were identified employing the statistical coupling analysis (SCA) method (Novinec,Korenc *et al.* 2014). Moreover, in that work and in a more recent study, the crystallization of HCatK with allosteric inhibitors, positioned in one of a previously-predicted site, was reported (PDBID: 5J94 and 5JA7) (Novinec,Korenc *et al.* 2014;Novinec,Lenarcic *et al.* 2014;Novinec,Rebernik *et al.* 2016). On the other hand, there are other cases of allosteric modulation in several parasitic cysteine proteases such as Falcipain 2 from *Plasmodium falciparum*, which are allosterically inhibited by heme and suramin compounds (Marques,Esser *et al.* 2013;Marques,Gomes *et al.* 2015).

Interestingly, the role of glycosaminoglycans (GAGs) in allosteric modulation of papain-like cysteine proteases has been widely studied in the past decades. Proteases such as human cathepsins S (HCatS) and B (HCatB), brucipain, cathepsin L of *Leishmania mexicana* and even cruzain present an allosteric modulation by GAGs (Almeida,Nantes *et al.* 1999;Almeida,Nantes *et al.* 2001;Lima,Almeida *et al.* 2002;Li,Yasuda *et al.* 2004;Costa,Batista *et al.* 2010;Costa,dos Reis *et al.* 2012;Judice,Manfredi *et al.* 2013). However, no other biomolecules or chemical compounds have been reported as allosteric modulators of cruzain. Indeed, only one *in silico* characterization of an adjacent subsite to the cruzain orthosteric site was made by Durrant *et al.* (Durrant,Keranen *et al.* 2010).

In recent years, the computer-aided design of allosteric inhibitors has been widely used in the field of rational drug discovery (Dalafave and Dalafave ;Verkhivker,Dixit *et al.* 2009;Novinec,Lenarcic *et al.* 2014;Rastelli,Anighoro *et al.* 2014). The application of techniques such as SCA, molecular docking, molecular dynamics (MD) simulations and allosteric networks has emerged as a valuable complement to experimental methods for the study of allostery (Hertig,Latorraca *et al.* 2016;Nussinov 2016;Papaleo,Saladino *et al.* 2016;Ribeiro and Ortiz 2016;Wagner,Lee *et al.* 2016). These approaches have also allowed the prediction of allosteric sites and the quantification of protein and/or ligand motions in full atomic detail, describing the molecules behavior at high resolution and with the induction of controlled perturbations. (Stanley and De Fabritiis 2015;Zhao and Caflisch 2015;De Vivo,Masetti *et al.* 2016;Hernandez-Rodriguez,Rosales-Hernandez *et al.* 2016).

In this sense, the **main objective** of this work is the following:

To identify allosteric sites and selective allosteric inhibitors of cruzain through *in silico* approaches

In order to accomplish this general objective we will follow the **specific objectives**:

- To select the five crystal structures of cruzain from PDB database.
- To identify putative allosteric sites of this enzyme through structural alignment with HCatK.
- To search for three representative structures of each enzyme through a combination of MD simulations and clustering analysis, in order to perform ensemble virtual screening (VS) experiments with compounds from ZINC database against the putative allosteric sites.
- To re-rank the compounds selected from VS and to refine their binding modes through MD simulations of the enzyme-ligand complexes, and to perform free energy calculations with Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) method based on NPT ensembles extracted from the MD simulations.
- To map the interactions of the selected ligands with the enzyme through per-residue free energy decomposition employing the MM-GBSA method.
- To detect conformational changes in trajectories of apo and holo forms of cruzain evaluating some parameters such as changes in interatomic distances on orthosteric site, coordination propensity and principal component analysis.
- To predict the molecular basis of the cruzain allosteric mechanism by performing cross-correlation analysis of residue-residue distances of apo and holo MD simulations and prediction of the communication pathways between the allosteric and the active sites.

5. Conclusions

Our results constitute a strategy for designing novel allosteric inhibitors of cruzain, the major protease of *T. cruzi*. First, the dynamical survey of previously identified cavities evidenced the presence of one stable pocket with a functional gate (site 3) and one transient pocket (site 1). This characterization of site 1 and site 3 was not be feasible with a simple analysis or superposition of static crystallographic structures of cruzain. Our study on these allosteric sites, which are druggable by small molecules and can modulate the enzymatic activity (Novinec,Lenarcic *et al.* 2014), provides a solid basis for further drug discovery. Secondly, the combination of Autodock Vina score and MM-GBSA free energy calculations allowed to us to propose compounds ZINC00352089 and ZINC83627668 as scaffolds of cruzain allosteric modulators. In addition, we verified that the aliphatic chains of the residues of site 3 mediated the main protein-ligand interactions within the groove. These aspects can be important implications for targeted optimization of lead compounds or for research purposes.

Finally, the allosteric pathways which link site 3 with active site were elucidated by combining MD simulations with correlation of protein motions based in network theory. In this context, no major effects on active site structure were observed due to compound binding (modification of distance and angles between catalytic residues), which indicates that allosteric regulation in cruzain is mediated via alterations of its dynamical properties similarly to HCatK allosteric inhibition (Novinec,Rebernik *et al.* 2016). Mapping communication pathways between site 3 and the catalytic core shows that ZINC83627668 triggers the propagation of dynamics, creating shorter pathways, and stronger correlations. According to our results compound ZINC83627668 disturbs cruzain structure more than ZINC00352089, pointing out that structural dissimilarities between ligands may defined different communication routes, even though they are linked to same allosteric site. In summary, the disruption of this specific network communication could represent a rational approach for designing drugs with improved potency and selectivity against cruzain enzymatic function.

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