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Preliminary Structural Studies of the Kinetoplastida Target Enzyme and Foundation for Subsequent Studies

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Abstract: Kinetoplastida, a class of flagellated protists, includes several pathogenic genera responsible for neglected tropical diseases. These parasites rely on unique biochemical pathways, such as those involved in glycolysis, which are absent in their human hosts, making them attractive targets for selective drug development. This study presents a preliminary structural and functional analysis of a key target enzyme from Kinetoplastida, laying the foundation for further mechanistic studies and drug screening. Using a combination of homology modeling, molecular docking, and sequence alignment, we identify conserved active site residues and potential ligand binding regions. The results provide valuable insights for future structure-based drug design efforts aimed at treating Trypanosoma and Leishmania infections.

Keywords: Kinetoplastida; enzyme structure; homology modeling; drug discovery; neglected tropical diseases; molecular docking; Trypanosoma; Leishmania

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1. Introduction

Pathogens, a diverse group of biological agents, are responsible for diseases in hosts, spanning across various classifications like viruses, bacteria, and both single and multicelled eukaryotic organisms [1]. They target a wide array of hosts, each exhibiting unique interactions due to different dependencies and pathogenic mechanisms [2]. They have a high capacity for transmission and a rapid rate of replication, and their metabolic processes are flexible and variable [3]. Pathogens can cause a wide range of conditions, from minor health problems to serious illness and even death. They have a significant socioeconomic and healthcare impact, particularly in a globalized world where their spread is accelerated [4]. The rising challenge of drug resistance complicates the control of once manageable diseases [5]. Historically, pathogens have been linked to major epidemics, such as the Black Death and the more recent COVID-19, altering the course of human history, civilizations, migrations, warfare, and social constructs. Their effects extend to both aquatic and terrestrial ecosystems [6].

Specifically, when focusing on Trypanosomes, a class of flagellate protozoan parasites, they are known to cause a variety of diseases. A specific example, Trypanosoma brucei, a unicellular flagellate parasite with an organism that uniquely encloses glycolysis in an organelle called a glycosome, is predominantly found in sub-Saharan Africa [7-10]. Its distribution is governed by the presence of the tsetse fly (Glossina spp.). Responsible for the majority of sleeping sickness cases, Trypanosoma brucei's impact is twofold, with early symptoms like fever and joint pain, and later advancing to severe neurological issues following the parasite's penetration of the blood-brain barrier, ultimately leading to death,

which can be very damaging to humans [11,12]. Therefore, the study of pathogens, their surveillance, and the development of preventive and therapeutic strategies are important topics in medicine and public health today.

In this study, with the help of databases and PyMOL, the structures of ligands and binding sites were compared between mammalian and parasitic enzymes, so as to provide a basis for the screening of suitable drug targets in parasitic glycolytic enzymes and to achieve the goal of blocking glycolysis in parasites without affecting human enzymes [13].

2. Special Features of Trypansomid Glycolysis in Bloodstream

Glycolysis plays an essential role in Trypanosomes, being a primary energy source within these parasites. As the parasite's bloodstream-form relies on glycolysis as its sole source of ATP production, trypanosoma-specific glycolytic enzyme inhibitors are targeted as an effective treatment strategy [14-16]. This process is facilitated by enzymes located in specialized organelles called glycosomes [17]. The adaptability of Trypanosomes, necessary for survival in varied environmental conditions, is largely attributed to glycolysis, allowing survival in both oxygen-rich and deficient conditions. The structural integrity of the glycosomes, and the role of glycosomal enzymes are critical for the parasite's growth, survival, and pathogenicity, making them potential targets for therapeutic intervention [18].

This study investigates the first seven enzymes of the glycolytic pathway in Trypanosomes, examining differences in enzyme localization, structural homology, regulatory mechanisms, and kinetics compared to mammalian systems. These distinctions are explored for their potential in developing targeted therapeutics against parasites, minimizing the impact on the host organism.

3. Selection Criteria of Target Enzyme

In molecular-targeted drug discovery, the emphasis is placed on the uniqueness and structural variations of enzymes between pathogens, such as Trypanosoma species, and their human hosts [19]. This aspect serves as a critical criterion for determining an enzyme's potential as a therapeutic target. Enzymes that display substantial structural variances between the parasite and humans are deemed more promising for drug development. This is because these structural variations can be utilized to devise drugs that specifically target the parasite's enzymes while minimally affecting analogous enzymes in humans, thereby reducing the likelihood of adverse effects [20]. When selecting the most promising rational drug design from a set of proteins, in addition to considering the underlying biological importance and disease relevance, based on the types of drug discovery in molecular modeling discussed in this course, the following steps can be used to screen the target proteins:

First, structural and functional characterization should be based on the three-dimensional structural information of the protein, especially the detailed structure of the active site or other potential drug-binding regions, to determine whether there are known drug-binding sites or potential drug-intervention sites. This information can help ensure the success of subsequent binding studies. Second, homology and selectivity should be assessed. If the protein shows a high degree of similarity to proteins in humans and other species, it will aid in evaluating the potential drug selectivity and side effects; available data on drugs and compounds, if the compound or drug interaction data with the protein, including the effectiveness of the inhibitor or agonist is known, i.e., there is a larger data base for the protein, either from the internet or database, which will help in the side-by-side comparisons of the subsequent studies [21].

In the context of the seven key enzymes in the glycolytic pathway of Trypanosoma, including Hexokinase, Glucose Phosphate Isomerase, Phosphofructokinase, Fructose Diphosphate Aldolase, Triose Phosphate Isomerase, Glyceraldehyde Phosphate Dehydrogenase, and Phosphoglycerate Kinase, a structural comparison between Trypanosoma

and human counterparts is crucial. The foremost screening indicator is the structural identity and similarity between these enzymes from the parasite and humans. To achieve targeted inhibition of the parasite's enzymes with minimal impact on the host's enzymes, lower values of identity and similarity are preferred. Initial screening suggests Hexokinase and Phosphofructokinase as prospective targets due to their relatively lower identity and similarity percentages, which stand at approximately 8.7%/13.7% and 12.9%/21.3% (Table 1) respectively (sequences identity and similarity can be calculated with the help of ebi.ac.uk).

Table 1. Summary Information Gained from Online Data Bases Supporting the Selection of Targeted Enzyme.

	Enzyme from (PDB)	Mammal	Enzyme from Pathogen (PDB)
Hexokinase	1DGK		5BRF
Quality	Satisfying		Moderate
Identity and similarity	8.7%/13.7%		
Phosphofructokinase	4U1R		6QU3
Quality	Moderate		Moderate
Identity and similarity	12.9%/21.3%		

This contrasts with the 40%-50% range observed in the remaining five enzymes. Then, follow the general steps given in the former graph, comparing the available data on drugs and compounds, it is suggested that there are more data supporting studies of Hexokinase as a drug target than Phosphofructokinase in the last five years; homology and selectivity, a preliminary comparison of parasite and mammal enzymes in PYMOL show that Hexokinase has a relatively large RMSD (5.527690) when using the cealign command (Figure 1.), indicating that the enzyme structure of the parasite and mammal is likely to be quite different; moreover, Hexokinase functions in the early stages of the glycolytic pathway, and inhibition of Hexokinase may have a more direct effect on the parasite's energy metabolism; so Hexokinase was selected rather than Phosphofructokinase.

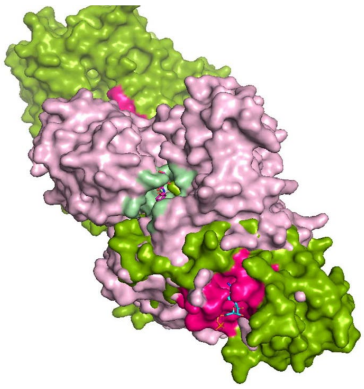

Figure 1. General Presentation of Enzymes from Two Sources and Active Site Location. (1DGK is colored in green with palegreen colored active sites; 5BRFis colored in pink with hotpink colored active sites.).

4. Modelling Comparison of Mammal & Parasite Enzyme

A general comparison of the similarity of from two different sources is presented in Figure 1, where it is observed that the overlap between the two is not very good and there are no compatible parts, proving that they are poorly similar and can be used for subsequent studies of targeted parasite drugs.

Comparing the ligand binding sites, the following similarities are noted: hydrogen bonding, hydrophobic region interaction, and charge complementarity are common in the

ligand-protein binding of 1DGK and 5BRF, which are selected from mammals and parasites, especially hydrogen bonding. After observing the structure and binding of the three ligands, it is revealed that the GLC and PO4 binding sites are less exposed, making it difficult to advance the study of the active site. Therefore, the main focus here is on discussing and comparing the ADP ligand in enzymes of mammalian origin and the 4V5 ligand binding site in enzymes of pathogen origin (Figure 2, Figure 3).

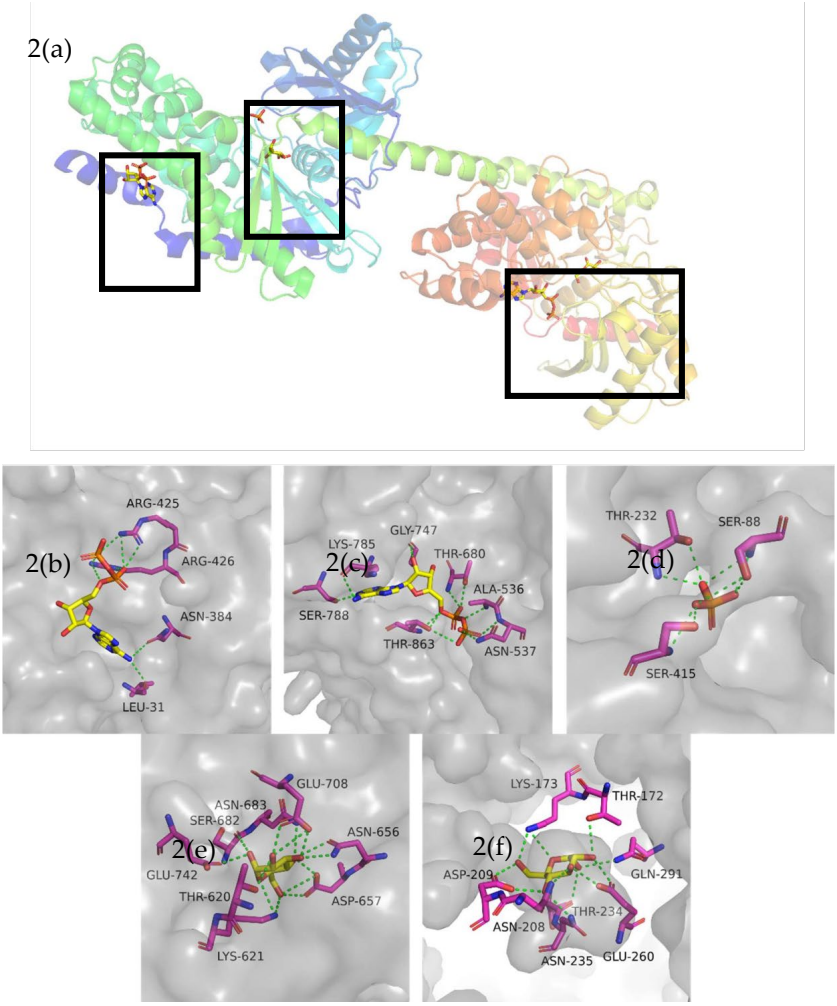

Figure 2. Architecture of the 1DGK Target and Structure of the Complex with Adp, Glc and PO4 Ligands.

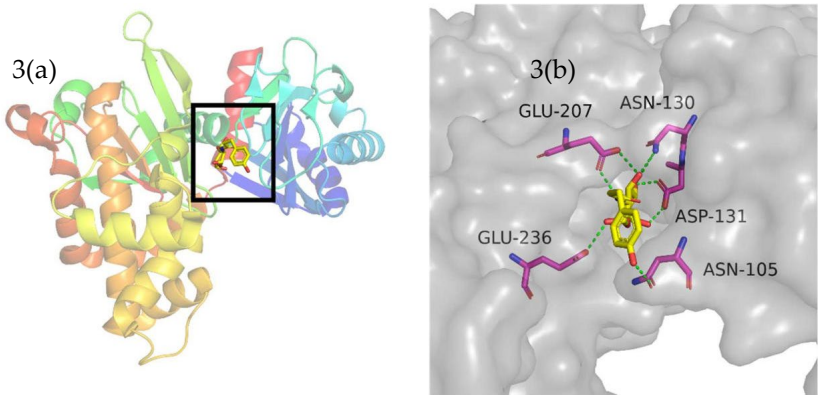

Figure 3. Architecture of the 5BRF Target and Structure of the Complex with 4v5 Ligand.

Figure 2 is the architecture of the 1DGK target and structure of the complex with ADP, GLC and PO4 ligands for mammal.

a) General structure of 1DGK. The chains making the structure are colored respectively; the active site with ligands (ADP, GLC and PO4 ligands, colored by elements) are boxed.
b) The boxed region is blown up to show the detailed information of binding pocket complexed with ADP. Amino acids that interact to form hydrogen bonds are labelled.
c) Similar with b, the boxed region shows the detailed information of binding pocket complexed with ADP in the backward sequence. Amino acids that interact to form hydrogen bonds are labelled.
d) The boxed region shows binding pocket complexed with PO4.
e) The boxed region shows binding pocket complexed with GLC. Amino acids that interact to form hydrogen bonds are labelled.
f) Similar with e, the boxed region shows binding pocket complexed with GLC in the forward sequence, amino acids that interact to form hydrogen bonds are labelled.

Figure 3 is the architecture of the 5BRF target and structure of the complex with 4V5 ligand for pathogen.

a) General structure of 5BRF. The chains making the structure are colored respectively; the active site with 4V5 ligands is boxed.
b) The boxed region is blown up to show the detailed information of binding pocket complexed with 4v5. Amino acids that interact to form hydrogen bonds are labelled.

Regarding the binding sites and with the help of PyMOL, it can be observed that compared to 1DGK, the entrance to the binding site in 5BRF is relatively narrower and the ligand is more tightly covered (it is considered that the ligand binding may be responsible for the enzyme conformational change) [22]. Moreover, it can be observed that the hydrogen bond in 1DGK, where the ligand binds to the active site, is shorter than that in 5BRF, which is important for the strength and stability of intermolecular interactions. In general, the shorter the hydrogen bond, the stronger it is, and shorter hydrogen bonds imply denser and stronger electrostatic interactions between hydrogen bond donors and acceptors; moreover, in biomolecules, shorter and stronger hydrogen bonds enhance intermolecular interactions, which in turn affects the molecule's structural stability and biological function; Shorter hydrogen bonds are often considered better drug-target interactions in drug design because they provide stronger binding and higher selectivity.

5. Details of Known Ligands & Interactions

The active sites and bound ligands of the two are then compared separately. In 1DGK, there are three specific ligands of interest: ADP, GLC, PO4.

For ADP, it appears to be a nucleotide or its analogue, which has multiple features that can interact with protein binding sites. As a ligand, its potential protein binding sites need to have the following properties: hydrogen bond interaction sites, where the amino group of adenine and other nitrogen atoms on the ring can serve as hydrogen bond donors or acceptors to form hydrogen bonds with amino acid residues in the protein, such as arginine (Arg) and glutamic acid (Glu); Charge interaction sites, where the phosphate group of a nucleotide is negatively charged and needs to interact with a positively charged amino acid residue, as lysine (Lys) or arginine (Arg) at the protein binding site; hydrophobic interaction region, the hydrophobic part of adenine group may form hydrophobic interaction with hydrophobic amino acid residues, as phenylalanine (Phe), leucine (Leu) in the protein binding site (Figure 2b, Figure 2c). For GLC, a sugar molecule containing multiple hydroxyl groups, its binding site may need to have the following characteristics: the ability to form hydrogen bonds, as the multiple hydroxyl groups on the ligand can act

as hydrogen bond donors or acceptors. Therefore, binding sites may need to contain amino acid residues that can participate in hydrogen bond formation, as aspartate (Asp), glutamate (Glu), serine (Ser), threonine (Thr); Complementarity of size and shape; Charge complementarity; The binding site needs to be specific enough to recognize the stereochemistry of the sugar (Figure 2e, Figure 2f).

For PO4, it is a phosphate ($PO4^{2-}$) ion with two negative charges. If the phosphate group is used as a ligand, its possible protein binding sites need to have the following properties: positively charged regions, requiring positively charged amino acid residues to promote charge interactions, therefore, it include lysine (Lys), arginine (Arg) under this condition; metal ion coordination sites; phosphate ions often form coordination bonds with metal ions; polar environment; phosphate ions are highly hydrophilic, so the binding site should have a polar environment that can form hydrogen bonds with multiple oxygen atom; Size and shape complementarity, the size and shape of the binding site should be complementary to the shape of the phosphate ion to ensure tight binding, etc (Figure 2d).

Among the ligands of 5BRF, 4V5 is worth noticing, as it could be useful in drug design and development (Figure 3). According to the provided image of the molecular structure, this ligand contains multiple functional groups, and based on the characteristics of this ligand, its binding site may need to have the following properties: hydrogen bond donor and acceptor, there are multiple hydroxyl (-OH) and carbonyl (=O) groups on the ligand that may form hydrogen bonds, so the binding site may need to have amino acid residues that are able to form hydrogen bonds with these functional groups, as shown in the figure, aspartic acid (Asp), Glutamic acid (Glu), Asparagine (Asn) presence contributes to hydrogen bond formation; hydrophobic region, the benzene ring on the ligand suggests that the binding site may require a hydrophobic region to facilitate hydrophobic interactions, which may involve hydrophobic amino acid residues; charge interactions; size and shape complementarity, the geometry of the binding site will need to be complementary to the ligand's size and shape to ensure high affinity binding; polar and non-polar regions, the binding site may contain polar and non-polar regions to match the distribution of polar and non-polar functional groups on the ligand; dynamic flexibility, the binding site may require a certain degree of flexibility to accommodate the binding of the ligand and maximize the contact area.

Therefore, hydrogen bonding, hydrophobic interactions, and charge complementarity are observed in both 1DGK and 5BRF, selected from mammals and parasites in ligand-protein binding, with a particular emphasis on hydrogen bonding.

It's worth mentioning that 4V5 is found uniquely in 5BRF, and based on that active site, s series of ligands are designed. According to D'Antonio and his team, the idea and method of how to design a ligand is summarized: in order to bind efficiently to the targeted enzyme [22]. A phenol group is added to the tail of HPOP-GlcN (Hydroxyphenyloxopropyl glucosamine), which allows for van der Waals interactions between the phenol moiety and the side chains of the non-polar amino acid residues of the protein; the phenol group aromatic ring in the tail allows π-stacking interactions with the side chains of aromatic amino acid residues in the active site of TcGlcK; hydrophobic interactions, etc.

HPOP-GlcN was designed with the dynamics and plasticity of the enzyme binding site in mind. The following aspects are mainly mentioned in the paper: induced fitting: when HPOP-GlcN is close to the active site of targeted enzyme, the structure of the enzyme may undergo minor changes to better fit the ligand; adaptation of the binding pocket; and exploitation of the dynamics. Therefore, dynamic simulations, predicting and analyzing the dynamic changes in enzyme-ligand binding are important in the drug design stage.

These feasible approaches give insight to the design of ligands targeted 5BRF, which will be further illustrated in ligand design strategies.

6. Ligand Design Strategies

As mentioned above, the active site of our targeted enzyme is surrounded by many polar amino acids (the addition of -OH, -NS, -S groups might help a ligand bind more tightly to this enzyme); hydrophobic groups can also be added for possible hydrophobic region interaction. In addition to this, when protein structures are known and potential drug molecules are searched for, structure optimization can be carried out on molecules known to have desirable pharmacological activity and good characterization. Subsequently, guided by structure-activity relationship studies, the activity was optimized by structural modifications; Anderson and his team optimize the crystallographic methods, which can significantly improve sequence accuracy and identify functionally important residues through crystallographic refinement [23,24].

Apart from that, with a known three-dimensional structure of protein, SBDD can be used during this procedure. Structure-Based Drug Design (SBDD) is an approach to drug design based on the three-dimensional structure of a target protein [25,26]. It relies on understanding the protein structure and using this information to discover or optimize molecules that interact with it.

The structure of an effective ligand is known, on the basis of which it is possible to use the Ligand-Based Drug Design [27,28]. This approach is particularly suitable for those cases where the 3D structure of the protein is unknown or difficult to obtain, where the active site properties of the protein are deduced from the known ligand information, and where new compounds are designed using drug analogue databases and drug design software [29-32] (e.g. ChemDraw or MOE).

7. Conclusion

The field of computer-aided drug design (CADD) is advancing and can be combined with artificial intelligence (AI) and machine learning (ML) technologies. This combination enables CADD to process and analyze large amounts of biomedical data more efficiently, thereby improving the efficiency of discovery and optimization of new drug candidates.

Specifically, when performing drug screening and optimization, ML algorithms can be used for virtual screening to quickly identify potentially bioactive compounds, thereby accelerating drug candidate discovery, and AI can assist in the optimization of drug molecules by predicting the bioactivity and pharmacokinetic properties of compounds to improve their performance, greatly reducing errors caused by manual manipulation; pharmacophore and QSAR modelling, ML technology plays an important role in the creation and optimization of pharmacophore models and QSAR (quantitative structure-activity relationship) models. These models can be used to predict the activity and properties of new compounds, providing valuable guidance for drug design; in addition, not only experimental and prospective coverage in vitro, but also prediction of ADMET properties in vivo; biomarker discovery and research on disease mechanisms, AI technology can analyze complex biomarker data, helping to identify key factors related to disease and providing clues for drug target discovery; personalized medicine and precision therapy, AI and ML technologies show great potential in personalized medicine and precision therapy, being able to design customized treatment plans based on patients' genetic and biomarker information.

In conclusion, the timeliness of drugs for specific diseases, such as epidemics, is crucial. The combination of novel CADD technology with AI and ML helps identify targets more efficiently and accurately, leading to the design of corresponding drugs. This significantly shortens the time required for a drug to reach the market, ultimately benefiting humanity.

In this research, a comparative analysis of the structural attributes of various enzymes, including aspects like enzyme quality and supporting data, is undertaken. This led to the selection of one enzyme out of seven for in-depth study. Furthermore, for finding and ranking of targets in drug development against Trypanosoma and other parasites,

methodologies involving Metabolic Control Analysis (MCA) and metabolic pathway modeling can be employed. MCA and kinetic modeling help measure how much each enzyme controls the flow (flux control coefficients) and the amounts of substances in the process (concentration control coefficients). Saavedra and his team find that usually two or three enzymes mainly control the flow in these processes. Stopping these main enzymes affects the process more than stopping the ones controlling the flow the most.

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