Characterization of Protein Drug Targets and Application of Protein Therapeutics in Parasitic Protozoa

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Abstract

This proposal examines several novel routes of protein drug design, their therapeutic applications and potential drug targets in several parasitic protozoa. By comparing similar known proteins and assessing potential associations based on previous research, this paper attempts to discern the function of *Plasmodium falciparum* serine-repeat antigen 5 and metacaspases in *Trypanosoma brucei* and *Leishmania major*. Additionally, compound 9, an improved version of inhibitor GSK3494245/ DDD01305143/compound 8 of 20S protease in *L. major*, is proposed; by suggesting the development of a more selective inhibitor, increased affinity to specific subunits of 20S protease will improve its potential as a therapeutic agent. Lastly, a novel "conoid cap" therapy is outlined—a protein complex that inhibits mechanical organelles used in penetration of host cells—by assessing the chemical properties such as its resistance to secretory enzymes and identifying/supporting potential binding partners for the cap. Although many of these parasites are neglected by research in the United States, they are still relevant because of their world-wide prevalence and severe effects. This proposal provides a novel perspective on protozoan therapies by investigating modern strategies—such as the use of noncanonical amino acids and BLASTp data—in immunology and new technology in protein characterization.

1 Introduction

With technology regarding protein characterization and analysis evolving, this review aims to propose leading research in discerning drug targets and designing drug candidates in protozoan parasites. Protozoan parasites are an overlooked subject in research in the United States, even though they are estimated to affect over one third of the world's population. [AKG+19, SNDM+18] Inspecting essential proteins in protozoa through various scientific assays and tests can

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provide insight into their chemical characteristics. Examining the structure and composition of these essential proteins can help to compare their similarities with known proteins, in order to consider their possible functions. In combination with understanding the fundamental functions and processes of these proteins, their homologies can pave the way toward developing novel therapies. By using knowledge of known proteins that are chemically similar to these essential proteins in protozoa, we can suggest potential drug targets, hypothesize the structure of therapeutics, and have direction in designing said proteins. The contents of this proposal include potential targets to protein quality control proteases in protozoa and the development of theoretical "cap" to the apical end of apicomplexan parasites.

Apicomplexans, a phylum of unicellular protozoan parasites that use an apical complex structure to penetrate a host cell, are the primary group of organisms that are discussed in this proposal. Firstly, *Toxoplasma gondii*, an obligate intracellular apicomplexan. *T. gondii* affects over 40 million people in the United States alone. Additionally, it is believed that over 1 billion people throughout the world are infected, too. [AKG+19] *T. gondii* can cause severe infection in immunocompromised individuals, but primarily resides latently in hosts, unknowingly. Typically, *T. gondii* is spread through ingestion of cyst in contaminated red meats, however it can be congenitally contracted and cause lethal birth defects. [LBCV+21] Like other apicomplexans, *T. gondii* invades a host cell through mechanical and secretory organelles, replicates within a parasitophorous vacuole, and exits the host cell through a cell lysing egress. [SNDM+18, MMS+18] Its vacuolous anatomy renders it extremely resistant to immune response. *T. gondii* is especially common in developing nations and is infrequently studied in the United States. However, *T. gondii* should be more thoroughly studied because of its adverse affects on immunocompromised and pregnant patients. Also, nearly 1.1 million U.S pregnancies per year [JPF14] and approximately 60 million individuals in the United States have some form of toxoplasmosis. [ZSG+20]

Next, *Plasmodium* is a genus of apicomplexans that is an agent of malaria. The main vector and transmission of the parasite is through the bite of a female *Anopheles* mosquito. *Plasmodium falciparum*, the deadliest species of *Plasmodium*, typically penetrate erythrocytes and follow a similar lytic cycle to *T. gondii* with invasion, growth, replication and egress. [HBS18] Dissimilar to its relative, *Plasmodium* causes lethal infection in many hosts. While malarial vaccines are a common subject of investigation, decades of research and a variety of diverse, novel candidates have undergone clinical assessment. Yet, malaria is still an extremely prevalent issue in developing nations. [DSK+18]

African Trypanosomiasis, commonly known as sleeping sickness, is caused by the infection of an intracellular flagellate protozoan parasite that is contracted through the bite of a tsetse fly. [MHC+20] It affects the central nervous system causing discomforting symptoms that are sometimes lethal. They occupy the blood stream of warm-blooded animals. [MHC+20] *Leishmania major* is another neglected tropical parasite that is transmitted through the bite of sand flies that inject promastigotes into humans. Visceral leishmaniasis affects the internal

organs and, in severe cases, can be fatal. It is also intracellular and causes black fever. [WBT+19]

The kinds of proteins that will be discussed throughout this proposal will all be linked to the protein quality control system: a variety of proteases, zymogens and caspases. They are all types of proteases—enzymes that typically break down proteins and peptides—but zymogens are inactive enzymes that require activation by another enzyme. [AKG+19]

2 PfSERA5 Pseudo-Zymogen

P. falciparum serine-repeat antigen 5 contains a papain-like triad active site much like other cysteine proteases. [VDP16] It is similarly folded like cathepsin K and L and Caricain. [SCL+20] Cathepsin L has a major role in antigen processing and turnover of intracellular and secreted proteins in growth regulation and bone resorption. Cathepsin K has a main function to mediate bone resorption. [DWC+20] Caricain is a proteolytic enzyme that is involved with hydrolysis of a broad spectrum of peptide bonds, similar to those of papain. Papain helps break proteins down into smaller protein fragments of peptides and amino acids. [VDP16] It is believed that the product of PfSERA5 could induce antibodies that either protected against blood-stage infection in vivo or interfered with egress or invasion in vitro [HLN+13] which could make PfSERA5 a potential vaccine candidate against *P. falciparum*.

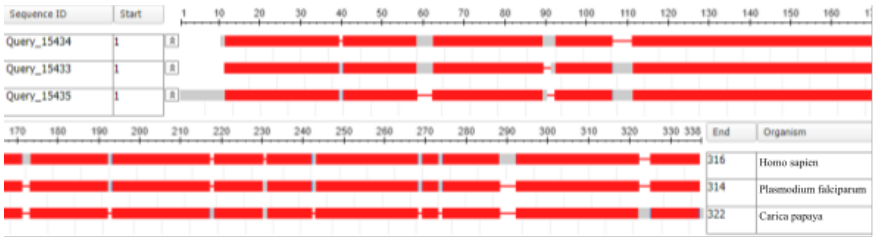

Figure 1: A BLASTp alignment on NCBI of Cathepsin K (Query_15434), PfSERA5 (Query_15433), and Caricain (Query_15435). [AGM+90, GS93] The two cathepsins display extremely similar homologous sequences. In particular, between residues 111 and 171, they show nearly identical sequences with PfSERA5

It is known that PfSERA5 plays an essential role in egress, but its activation and regulation is poorly understood. Because it has structural similarities to Papain and Caricain, which are involved in proteolysis [SCL+20], I propose that it is involved in hydrolysis of membrane proteins in erythrocytes upon invasion and/or egress. Additionally, it could play a role in dismantling other parts of the cellular membrane, similar to osteoclasts [DWC+20] which use cathepsin K and L to degrade collagen and other matrix proteins during bone resorption. In order to test the efficacy of PfSERA5's role in collagen proteolysis, merozoite

cells and collagen will be labeled with fluorophores to reveal structural changes during cell egress. Observing the concentration of PfSERA5 could potentially indicate its relation to egress.

Firstly, PfSERA5 willbe labelled with noncanonical amino acids—which will be described more in the FRET assay. Then, surface proteins will be tagged with fluorescein isothiocyanate antibodies (FITC) [SCKBK17] and collagen will be probed with green fluorescent protein (GFP) by genetic insertion following the promoter gene of membrane collagen, facilitated by CRISPR/CAS9. [KQL$^+$17] It is expected that PfSERA5 will play a prevalent role in proteolysis in membrane proteins during egress or invasion—either as a proteolysis catalyst or binding partner to other proteins. If PfSERA5 is involved, as it associates with collagen or surface protein there would be a change in the wavelength of the fluorophores.

Alternatively, a knockout study could provide insight into PfSERA5's potential association with collagen and surface protein degradation by evaluating the difference between their hydrolysis in control vs. knockout groups. If PfSERA5 is associated with collagen and surface protein degradation, then the hydrolysis in control groups would be significantly greater than in knockout groups.

PfSERA5 and PfSERA6 could be binding partners, too. This is because, normally precursory cleavage of the prodomain occurs in zymogens when a ligand binds to their active site, activating the catalytic properties of the enzyme. However, PfSERA5 does not cleave its prodomain from its central domain. [SCL$^+$20] All serine-repeating antigens in *P. falciparum* have known ligands which cleave their prodomains, besides PfSERA5 and 6. Also, PfSERA5's catalytic triad near the surface (Ser596, His762, and Asn787) is homologous to PfSERA6's (Cys596, His762, and Asn787). [SCL$^+$20] This does not indicate that PfSERA5 and 6 could be binding partners, however, their similarity indicates that they could have similar binding partners. PfSERA5's structure could still be related to proteolysis while acting as or being regulated by similar or the same compounds as PfSERA6.

In order to test if PfSERA5 and 6 have an affinity to one another, a FRET assay of concentrations of each protein could be used to indicate if they form a dimer. In FRET, the fluorescence wavelength and intensity of a fluorescent amino acid changes when it gets close to another fluorescent amino acid. [AMLS$^+$20] I suggest placing distinct fluorophores, such as a genetically inserted or crosslinked noncanonical amino acid [NHSF$^+$20], oriented away from the active site. The fluorescence of the fluorophore on PfSERA5 will change if bound to PfSERA6, indicating if they form a dimer. If they do form a dimer, it is likely that their functions are not only interchangeable, but they are catalytically inactive in small concentrations because of decreased binding and, therefore, less prodomain cleavage. Then a knockout study of PfSERA5 or 6 would indicate their regulatory properties on one another and their effects on egress/invasion.

If PfSERA5 and PfSERA6 are in fact binding partners, creating an inhibitor using to the catalytic triad Cys/Ser596, His762, and Asn787 would be an effective route in drug development.

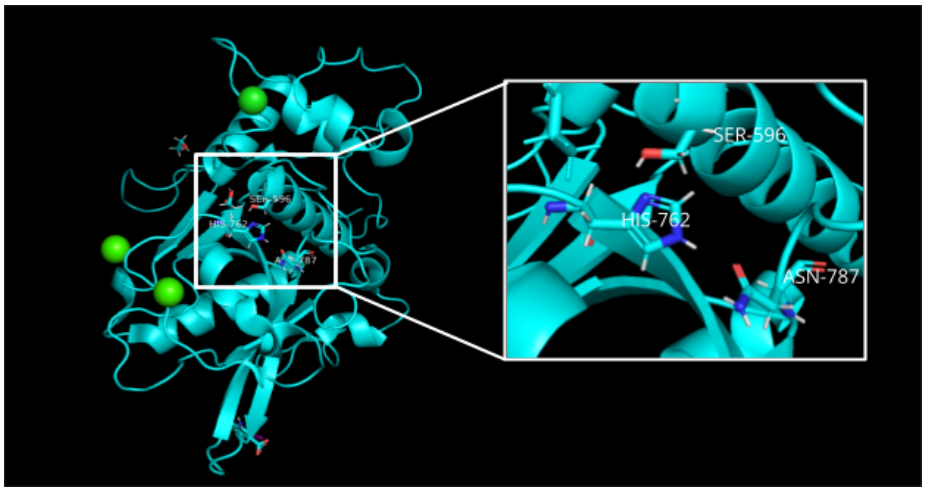

Figure 2: PfSERA5's catalytic triad near the surface (Ser596, His762, and Asn787).

3 Compound 9 an Inhibitor for 20S Protease

GSK3494245/DDD01305143/compound 8 is an inhibitor for 20S protease that targets between the $\beta 4$ and $\beta 5$ subunits. It was bioengineered through mutagenesis and modification of potential ligands for 20S protease during observation of various assays. [DWC$^+$20] Compound 8 is currently in preclinical testing as a treatment for visceral leishmaniasis and has been shown to inhibit chymotrypsin-like activity associated with the proteasome. 20S protease is prevented from repairing proteins and this induces an accumulation of ubiquitylated proteins in *Leishmania*. [WBT$^+$19,TBDR$^+$21] Compound 8 has also shown to be a relevant compound in other related parasites.

However, it has been briefly mentioned compound 8 can bind to the wrong subunits of the protease. In such circumstances, for example in $\beta 2$ [WBT$^+$19, TBDR$^+$21], it has no effect on the function of the protease. I propose screening a variety of compounds similar to compound 8 as a method to produce compound 9, an improved compound that both has the inhibitory capabilities of compound 8 and prevents binding to $\beta 2$ subunit. Various compounds that have a decreased affinity for $\beta 2$ can then be tested for $\beta 4$ and $\beta 5$ subunit binding sites. In order to facilitate this screening, concentrations of $\beta 2$ subunit will be separated from 20S protease, then the compounds will be separately tested on $\beta 4$ and $\beta 5$ subunits. Additionally, there are a few mutant strains of visceral leishmaniasis (T30A, G197S, G197C) that are potentially immune to the effects of compound 8. The production of compound 9 with less hydrophilic residues near the alpha carbon of G197 by substituting hydrophilic residues for valine, leucine or alanine could combat G197S and C mutations. Changing glutamine to asparagine or threonine at Q222 would attempt to combat T30A mutation, and prevent the formation

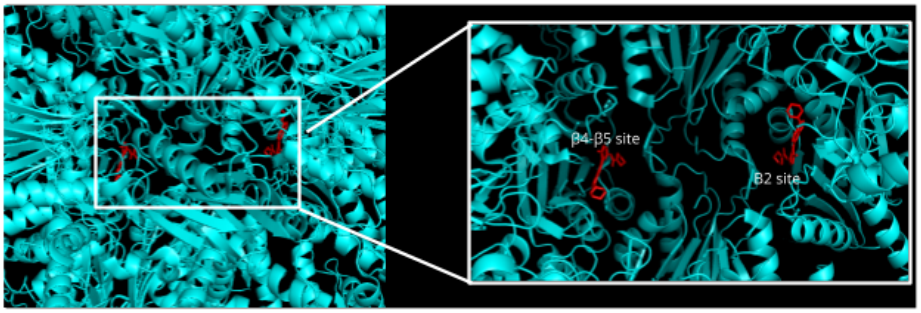

Figure 3: The active binding of compound 8 between $\beta 4 - \beta 5$ subunit sites and the $\beta 2$ subunit site.

of a pyrrolidine pocket. The administration of such modification to the genome of *Leishmania* could be through CRISPR/CAS9 or mRNA in an attempt to target the population by treating mutated strains.

4 Metacaspases

Metacaspases (MCAs) show clear correlation between cell growth and proliferation along with apoptosis, meaning they are likely regulators of the cell cycle as a whole. [VDT$^+$19] For instance, in *Trypanosoma brucei* *Tb*MCA3-5 are involved in cytokinesis, cell proliferation and apoptosis at the same time. Much evidence suggests that MCAs have positive feedback systems like other cell cycle regulators like p53 [VDT$^+$19], however, it is possible that *Tb*MCA3-5 causes apoptosis to occur due to mitotic catastrophe. More specifically, they could be related to mitotic catastrophe in the mitochondrion. This is evidenced by the fact that the orthologue *Lmj*MCA in *Leishmania major* is located in the mitochondrion and associated with the mitotic spindles. Additionally, the mitochondria in MCA-induced apoptosis are often amorphous or degenerate. [CMBCM12]

In order to utilize MCAs as a potential therapeutic target, development of an inhibitor would be a possible route of therapy. Most MCAs are involved in cell cycle, so inhibition of the expression of MCAs as a whole could prevent the maturation of protozoans with MCAs. On the other hand, upregulation of MCA production is another route of therapy that will be pursued. This would need to be organism specific by using a protein specific to the target protozoa and either administers a concentration of an MCA or induces the overproduction of an MCA. The reason the overproduction of an MCA is a potential therapy worth investigation is because it is found the MCA-induced programmed cell death is significantly less likely to recover during anastasis. [VDT$^+$19] A knockout study of *Tb*MCAs and *Lmj*MCAs would indicate which are worth investigating for the previously mentioned therapies. If all of the proposed functions of *Tb*MCAs and *Lmj*MCAs are unaffected by a knockout study, then the efficacy of the proposed functions is unlikely.

5 Conoid "Cap"

The conoid is found at the apical end of apicomplexan parasites. It is an organelle that is crucial to host cell invasion and its cone shape aids in penetration of the cellular membrane. It is formed by an extensive network of microtubules that connects the apical end of the parasite to secretory organelles such as the rhoptries. The conoid secretes digestive and proteolytic enzymes which facilitate degradation of the host cellular membrane. [LNH$^+$20] I propose the design of a protein complex (conoid cap) with complementary side chains to a protein located at the conoid of *T. gondii* in an attempt to inhibit its secretory function and penetration of host cells. [LADS17] Because the structure of the conoid has similar orthologues among apicomplexans such as the *Plasmodium* [LNH$^+$20], the development of a conoid cap could benefit research therapies for other parasites, too.

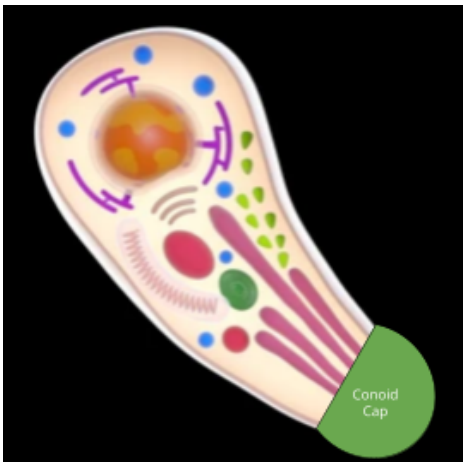

Figure 4: The general structure and shape of the conoid cap protein scaffold.

The conoid in all apicomplexans contain similar microtubules and surface proteins. [LNH$^+$20] Therefore, the conoid cap of *T. gondii* can be a baseline scaffold for many other apicomplexans. The general concept behind the conoid cap would be to create a cone-shaped protein scaffold nearly 1 micrometer in diameter given the conoid dimensions are roughly 1 micrometer in width. [PTK$^+$20] The protein scaffold would have a complementary pocket or antigen which binds to a targeted protein and either permanently binds or inhibits the function of the selected protein target.

In the discussion of developing such a conoid cap, there are a variety of surface proteins that can be targeted. Along with a network of microtubules, composed of filaments and fibers, there is a diverse body of unique proteins that will be mentioned below that can facilitate a protein-protein interaction at various points on the conoid cap. The following proteins and protein complexes are located at the conoid and have been discovered to play crucial roles in

motility, penetration and stability in cell invasion of host cells for *T. gondii*; all of these proteins could be potential candidates for a target of the conoid cap.

5.1 Conoid Protein Hub 1

Firstly, the conoid protein hub 1 (CPH1) is a recently discovered protein that has been localized at the base of the conoid. While the function of the protein is yet to be determined, it has been observed to be related to maintenance of the surface proteins at the conoid of *T. gondii*. [LADS17] When depleted, it causes the conoid to shorten and collapse. [PTK$^+$20] Its structure contains ankyrin repeats, a commonly found structure in eukaryotic organisms. Ankyrin repeats have a rigid helix-turn-helix-turn three-dimensional structure that creates a spiraling hemispherical shape. [LADS17] They exist exclusively to promote specific folding in proteins and mediate protein-protein interaction. [LMT06]

Although many ankyrin repeat antibodies and binding proteins exist, such as DARPins [SDB20], the structure of a hypothetical conoid would have to include side chains and general secondary/tertiary structure which make it specific to CPH1 and extremely selective. DARPins, which do exhibit great target specificity, are based on naturally occurring ankyrin-repeat domains. The rigid scaffold of the ankyrin repeat domain fold achieves a high affinity and specificity in DARPin antibody production, making the scaffold a great starting point for developing a conoid cap. [SDB20] These side chains of the complementary domain would need to prevent interaction between other ankyrin proteins—that would normally adhere to themselves—found elsewhere in the biological systems of host cells.

5.2 Doublecortin-Domain Protein

Next, doublecortin-domain protein (TgDCX) [LNH$^+$20] is also a newly characterized protein complex that is found along the microtubules of the surface of the conoid complex. I plan to utilize TgDCX as a specific binding candidate for conoid caps and antibody targets. TgDCX generates and stabilizes the microfilaments along the subpellicular level, but it also affects microtubules at the head of the conoid which open while protruded. Because of its essential role in repair and development of microtubules which facilitate penetration, not only could TgDCX be a target for the conoid cap but is also a good target for a permanently bound antibody which prevents TgDCX from developing the conoid. Additionally, because DCX is a protein expressed in the genomes of all apicomplexans and many other protozoans, creating a therapeutic that uses doublecortin as a target will aid in the development of therapies for many protozoans.

In humans, the DCX gene expresses the production of doublecortin, a neuronal migration protein. Doublecortin has two DCX domains, whereas in *T. gondii*, TgDCX has one DCX domain and one P25α domain. DCX has significant homologies to P25α (30% similarity and 22% identity) [LNH$^+$20] which not only indicates that the P25α has a similar role in TgDCX as DCX has to

doublecortin in humans, but also, it alludes to the efficacy of using TgDCX as a drug target. Because the P25α domain in TgDCX is unique to *T. gondii*, cross reactivity is less of a concern in targeting TgDCX. [LNH$^+$20] Covalently bound antibodies to TgDCX will be developed via proximity cross-linking noncanonical amino acid incorporation and modification of anti-doublecortin antibodies in humans.

5.3 Apical Cap-Binding Proteins (TLAP3 and TrxL1)

Thioredoxin-like-associated protein 3 (TLAP3) is a subpellicular microtubule binding-protein (SPMT) that coats the apical cap region and intraconoidal microtubules of all stages of *T. gondii*. While knockouts for TLAP proteins did not result in defects in SPMT generation [PTK$^+$20], TLAP3's active site/antigens will be examined for inspiration for the design of the conoid cap. Because TLAP3 coats all of the apical cap proteins (ACs) not only could the said TLAP3-based conoid cap potentially inhibit the penetration mechanism of the conoid, but it could alternatively inhibit the functions of the AC proteins which are all related to motility mechanisms, egress and penetration. TrxL1 also has the ability to bind to apical cap proteins, however, its structure and sequence are less certain. [LNH$^+$20]

At the point of the conoid there are a pair of intraconoidal microtubules which seemingly assist in the discharge of secretory enzymes. However, the function of this section of the conoid is relatively unknown. The structure of protein has antigens located at the direct point of the protein.

5.4 Conoid Cap Administration and Development

In development of the conoid cap, any of the listed above proteins that have knowledge of structures could be used in development of the binding site of the cap. Antibodies for the listed proteins are also another basis for the binding site of the cap. For proteins that are less understood, a protein scaffold that has a general shape similar to the conoid cap could be developed through a series of directed evolution. For instance, this alternative method can be potential achieved by using the DARPins protein scaffold for CPH1 (PDB). [PTK$^+$20, SDB20] The design of the cap could either be a complement to the sequence of residues that are facing the extracellular matrix, or could fit into the active site or antigens of the proteins.

To increase the affinity of mutated protein scaffolds, spectroscopy; X-ray crystallography; MD simulations and protein predicting software may be useful to increase the understanding of basic sequence to dynamic structure of the scaffold. A consensus sequence can be determined from either NMR or X-Ray crystallography to be submitted to RoseTTaFold to get a general prediction of the protein. From there, MD simulations between the scaffold and the conoid cap will increase the understanding of cross-linking interactions and binding affinity.

Potential routes of therapy administration will be introducing mRNA of designed caps to parasites. The parasite would produce the cap at the conoid. Alternatively, mRNA could be introduced to host cells to produce caps intracellularly; during cell invasion, the invasive parasite would not be able to penetrate another cell after infecting a cell that applies a cap to the conoid. This kind of mRNA could be taken supplementally or as a vaccine that aims to prevent *T. gondii* infection for small windows of time. Another potential route of therapy could deliver the caps would be delivering conoid via injection into the bloodstream. This could be particularly effective in infection of erythrocytes which could be potentially applicable to *Plasmodium*.

Some potential difficulties with the approach of using conoid caps would be creating caps that are resilient to secretory enzymes that are secreted at the conoid. These enzymes are typically used to denature/degrade the cellular membrane of host cells, so these enzymes create nonoptimal environments for phospholipids and membrane proteins. [LBCV$^+$21] However, DARPins protein scaffolds have extremely stable structures, more resilient than proteins naturally found in membranes. [SDB20] This suggests that targeting the CPH1 will be a contentious candidate for investigation and inclusion in some form as a constituent in a conoid cap scaffold.

6 Conclusion

With all of the options presented above, factors such as accessibility to equipment or, even, cross reactivity among potentially developed therapeutics may prevent the proposed research. Overall, this proposal was meant to shed light on an extremely prevalent issue in developing nations that is often overlooked in the research community of the United States.

As modern methodology advances, it is important to investigate novel strategies in therapeutic and drug design. Although scientists' understanding of many of these new remedies is vague and limited, it is all worthwhile to explore new paths. Proteins are increasingly more important to a variety of fields, immunology included, because more research is constantly shedding new light on these essential macromolecules. Additionally, information provided in this proposal is culminated from leading research and can provide a unique perspective on creating therapeutics for protozoans and apicomplexans.

7 Materials and Methods

7.1 Sequence Alignment and Molecular Modeling

Sequences for all SERA genes were obtained from Protein Data Bank and PlasmoDB. Sequence alignments were performed using the BLASTp Database of NCBI. [AGM$^+$90, GS93] PfSERA5PE (PDB: 6X44), PfSERA6PE (PlasmoDB gene ID: PF3D7_0207500), ProCathepsin L (PDB: 1CS8), ProCathepsin K (PDB: 1BY8), ProCaricain (PDB: 1PCI).

7.2 Molecular Modeling

Different molecular models of PfSERA5PE (PDB: 6X44), were generated using the PyMol Molecular Graphics System, Version 2.0.

8 Acknowledgments

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