Discovery of Potent Inhibitors of *Plasmodium* PK5

by

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Steven W. Baldwin

Thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in the Department
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ABSTRACT

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Abstract

Malaria is one of the oldest and deadliest diseases in the world, affecting approximately 200 million people annually. The role of protein phosphorylation in the complex life cycle of the malaria parasite, *Plasmodium*, as well as the promising therapeutic values of protein kinase inhibitors have sparked increasing interest in understanding the *Plasmodium* kinome. Although many protein kinases have been shown to be essential for *Plasmodium* survival, their functions remain unknown. Protein kinase 5 (PK5) is a putative cyclin dependent kinase (CDK)-like protein in the malaria parasite, and it is thought to be essential for blood stage proliferation in *P. falciparum*. In the present study, biochemical binding assays were used to identify potent and selective inhibitors of *Pf*PK5. Two compounds were found to selectively bind to *Pf*PK5 over the human analog, *Homo sapiens* CDK2. In addition, a known CDK inhibitor was used in the development of a chemical probe to identify potential macromolecules essential to parasite survival. Here, we report important structural moieties potentially involved in *Pf*PK5 binding. Elucidation of the biological targets through the use of our chemical probe may aid in further understanding of *Plasmodium* biology.

Dedication

To Kevré Hendricks and my parents, Ruth and Mario Perkins for their unwavering support on this journey.

To Dr. Emily Derbyshire and the entire Derbyshire lab for continuously believing in me and being an excellent example of the new generation of scientists.

To my brother and soldier Jose Rosario.

Finally, to every child who has ever been told that they wouldn't make it.

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List of Abbreviations

ACT – Artemisinin combination therapies

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

CDK – Cyclin dependent kinase

DMEM – Dulbecco's Modified Eagle Medium

DMF – Dimethylformamide

DMSO - Dimethylsulfoxide

DNA – Deoxyribonucleic acid

GST – Glutathione S-transferase

HPLC – High performance liquid chromatography

Hs – Homo sapiens

IC_{50} – Half maximal inhibitory concentration

IPTG – Isopropyl β-D-1-thiogalactopyranoside

K_d – Dissociation constant

PCR – Polymerase chain reaction

PDB – Protein data bank

Pf – Plasmodium falciparum

PK5 – Protein kinase 5

WHO – World Health Organization

Acknowledgements

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I am also grateful to my collaborators Dr. Nobutaka Kato (Broad Institute) and Dr. Paul Sanschagrin (Harvard Medical School) for whom this work would not be possible. In addition, I would like to thank Dr. David Gooden (Duke) who graciously assisted with many aspects of synthesizing compounds and Dr. George Dubay who lent his expertise in spectroscopy.

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Chapter 1

Introduction

Malaria is an infectious disease that plagues approximately 100 countries worldwide, affecting predominantly young children and pregnant women (1). The causal parasitic agent of malaria comes from the genus *Plasmodium.* Although there have been significant advances in malaria research in the last several decades, there are still many aspects of P*lasmodium* biology that remain unknown.

Protein kinases are emerging drug targets in many diseases, most notably cancer, and have gained attention in developing malaria therapeutics. Protein kinases in other systems are known to be essential for the regulation of most cellular pathways, including proliferation and differentiation in eukaryotic cells. It is likely that *Plasmodium* protein kinases have similar important functions. As the malarial kinome continues to be characterized, the importance of these protein kinases in parasitic survival and development becomes more evident.

1.1 Malaria: A global health problem

Malaria has remained a devastating disease, affecting approximately 200 million people annually in poor tropical and subtropical areas. Despite successful eradication in

more developed countries, it continues to severely impact developing countries in Asia, South America, and Africa (**Figure 1**). Sub-Saharan Africans suffer the most from malaria infection, and it is reported that 90% of cases are diagnosed in Africa, mainly among children under the age of five and pregnant women (1). As reported by the World Health Organization (WHO), malaria is responsible for one of five deaths among African children under the age of five. According to the 2014 World Malaria Report, 3.3 billion people, which amounts to half the world's population, live in areas at risk of malaria transmission (1). Confounding the problem of disease control, the parasites rapidly develop resistance when new antimalarials, such as artemisinin, are introduced. This necessitates continual development of new drugs, but there are only a few compounds in the pipeline currently, making it increasingly more difficult to treat the disease particularly in these underdeveloped regions (1).

In addition to obvious health consequences, malaria has substantially impacted the economy of areas where the disease is severely implicated. This disease has inflicted considerable costs to individuals and governments alike. According to WHO, the estimated direct and indirect annual cost of malaria in Africa is equivalent to more than 2.5 billion US dollars, while the Center for Disease Control estimates the direct cost worldwide to exceed 12 billion US dollars annually.

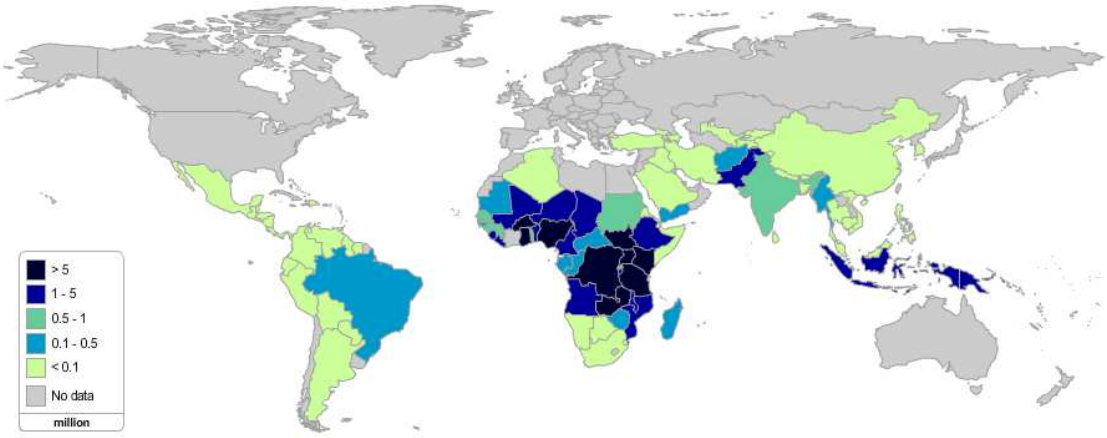


Figure 1: Map of reported malaria cases by country reported by the World Health Organization in 2014.

1.2 Plasmodium life cycle

Malaria is caused by a protozoan parasite of the *Plasmodium* genus. There are currently five species of *Plasmodium* that can infect human hosts, *P. falciparum*, *P. vivax*, *P. knowlesi, P. ovale*, and *P. malariae*. Both the severity and length of disease symptoms can vary greatly depending on which strain has infected its human host.

The parasite has a complex life cycle that begins with a one-time asymptomatic liver stage followed by a cyclic symptomatic blood stage (**Figure 2**). During the blood meal of an infected female *Anopheles* mosquito, *Plasmodium* sporozoites from the mosquito salivary gland enter the human's blood stream and travel into the liver, infecting hepatocytes (2, 3). After entering the liver cells, these sporozoites rapidly

divide asexually and mature into schizonts. These schizonts rupture the cells and release merozoites, which enter the blood stream and invade red blood cells. Once these merozoites establish erythrocytic infection, they rapidly replicate over several days, also leading to the bursting of these cells. This rupture of red blood cells releases new merozoites into the blood stream which can infect new red blood cells, starting the cyclical blood stage. This continuous bursting of red blood cells leads to malaria's characteristic symptoms, including fevers, anemia and fatigue.

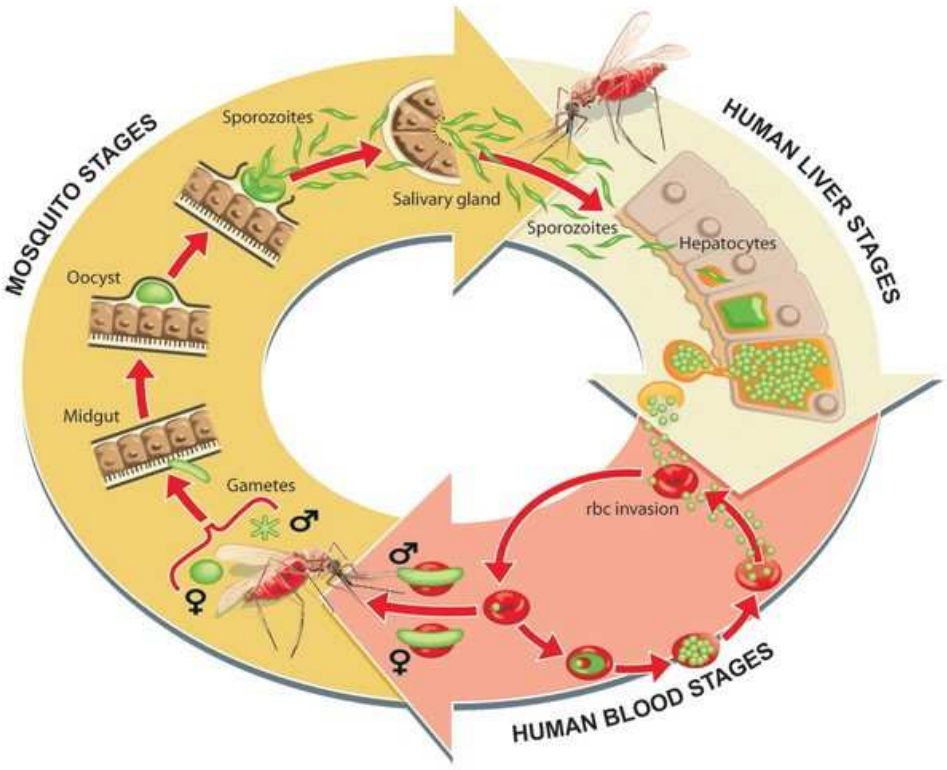


Figure 2: *Plasmodium* life cycle (image by Moore's graphics)

During the blood stage, a small fraction of the merozoites develop into gametocytes which are unable to reproduce in their human hosts. These gametocytes reproduce once they are extracted by another female *Anopheles* mosquito during a blood meal and they differentiate into sporozoites within the mosquito's gut. The resulting sporozoites then travel to the salivary glands, continuing the life cycle of the parasite.

1.3 Plasmodium kinome

The mechanisms by which malaria hijacks its host's resources for survival remain unknown. As previously discussed, these parasites have a complex life cycle that involves multiple hosts and distinct cells within these hosts (3-5). Throughout this complex life cycle, protein kinases (phosphorylating enzymes) are known to be essential for growth and development and likely regulate complex biological processes. The importance of protein phosphorylation for the progression of the *Plasmodium* life cycle has established the importance of protein kinases as druggable target (5-8).

Plasmodium kinome contains 65 predicted protein kinases which accounts for approximately 1.2% of the parasitic genome, for which kinase functions are predominantly unknown (4-6). Bioinformatic analyses of the parasite kinome was completed to produce phylogenetic trees of protein kinases from *P. berghei* (**Figure 3**) and *P. falciparum,* where only four features were found to distinguish the kinomes of

both species (9). This homology is indicative of how highly conserved this class of proteins is, making elucidating their individual functions particularly difficult.

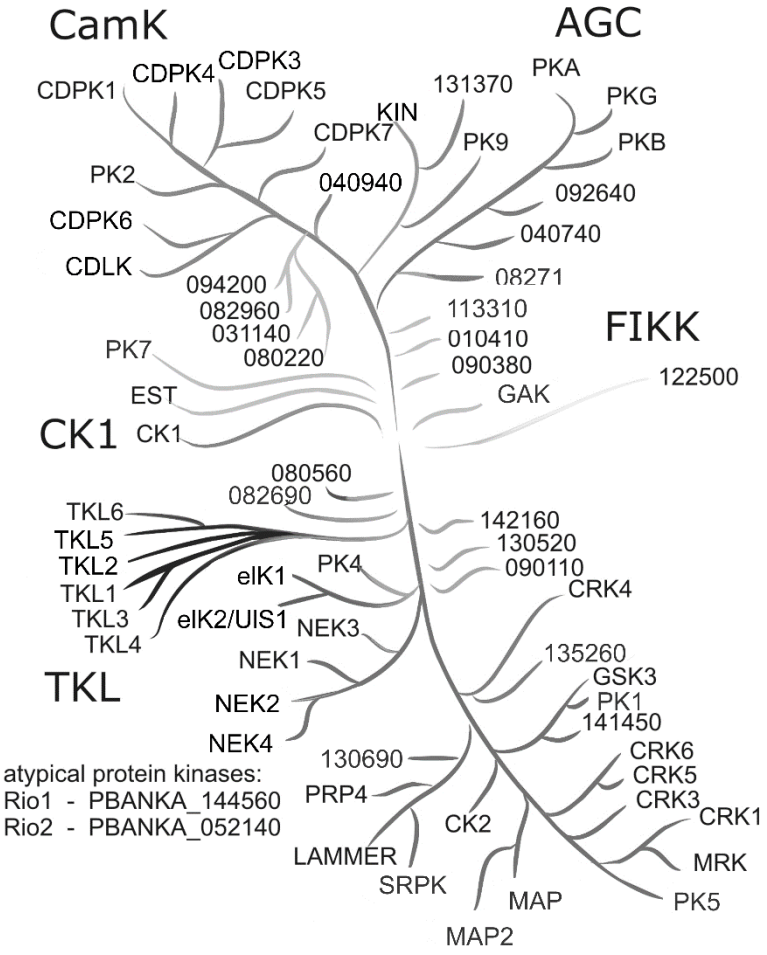


Figure 3: Phylogenetic tree of *P. berghei* kinases (9)

Previous genetic studies have established several protein kinases that are essential for the survival of the *Plasmodium* parasite (5, 6, 9, 10). One genetic study involved double crossover homologous recombination in order to employ a reverse

genetic strategy to knock out *P. berghei* (rodent malaria) protein kinases and identify them as essential proteins. For example, CRK1-, a cyclin-dependent like kinase and CDPK1, a calcium-dependent protein kinase have both been reported essential for asexual growth and thus parasite survival (9). However, since protein kinases are notorious for functional redundancies, it is often not feasible to ascertain function from a single gene knock out. Elucidating the role of a protein family using small-molecule probes offers many advantages as an alternative to traditional genetic studies such as genetic knock outs (4). These molecules often exhibit promiscuity, meaning they target many proteins with similar function and sometimes they target an entire protein family.

1.4 Current antimalarial therapies

Drugs that are currently on the market as potent antimalarials are facing the emergence of drug resistance (11). In fact, the World Health Organization (WHO) launched an emergency response against the increase in anti-malarial drug resistance in 2011. Resistance to chloroquine, a potent antimalarial active against the blood stage of *P. vivax, P. malariae* and *P. falciparum*, first emerged in the 1950s (1). Chloroquine binds to heme and disrupts the membrane function of the parasite (12). Also widely prescribed, artemisinin and its derivatives are used as prodrugs of the biologically active metabolite dihydroartemisinin against blood stage malaria (13). Artemisinin combination therapies

(ACTs) are currently the first line of defense worldwide due to their rapid action against *P. falciparum* (1). However, in 2008, clinical evidence of resistance to ACTs was reported in Southeast Asia (14), and the resistance is continuing to spread across more malaria infected regions. As parasites continues to evolve to evade drug therapies, there is an urgent need to identify a variety of essential molecular targets and develop more effective antimalarials. The vast majority of past therapeutic development, however, has largely focused on the cyclic blood stage of malaria.

A strategy for combating drug resistance is the identification of dual stage inhibitors. By targeting the asymptomatic liver stage in combination with the frequently targeted blood stage, more effective pharmaceuticals can be developed (2, 3, 15). An example of this is the commercially available drug Malarone which is a combination of atovaquone and proguanil. Due to the dual-stage activity against both blood and liver stage parasites, Malarone is used as a prophylactic treatment against malaria. Atovaquone was previously shown to target the cytochrome bc_1 complex of the mitochondrial respiratory chain in *Plasmodium* (16), while proguanil is a known antifolate that targets dihydrofolate reductase after conversion to its active metabolite, cycloguanil (17). Although resistance to Malarone was reported in 2003 (18), this class of dual-stage drugs has great potential as antimalarial agents, as targeting both stages reduces the likelihood of resistance in the parasites.

1.5 Polypharmacology

With the increasing resistance to common drugs, there is an urgent need to discover new antimalarial targets. However, our limited knowledge of *Plasmodium* biology makes these targets difficult to ascertain. One way of alleviating this issue is by targeting the parasite kinome through polypharmacology, a concept that focuses on drugs that target multiple macro molecules. Kinase inhibitors work well for this approach because they are able to target multiple ATP-binding proteins due to structural similarity in the ATP binding pocket required for activity (4, 5, 7, 8). Using polypharmacology allows for inhibition of multiple targets. In the case with malaria polypharmacology often generates dual inhibitors that target both the liver stage and blood stage of the parasite, potentially delaying drug resistance.

The development of new drugs to combat malaria has also been costly (1). This is particularly challenging with disease of the poor such as malaria. By developing treatments that inhibit multiple targets and potentially delaying drug resistance the financial burden can be lowered in these endemic areas.

1.6 Cyclin dependent kinases and Plasmodium

A recent screen that targeted kinases with small molecules found this class of molecules to be potent inhibitors of malaria parasites. In particular, the screen revealed

that inhibitors reported to bind to the cyclin dependent kinase (CDK) were the most common hits (4). CDKs are a family of relatively small multifunctional enzymes that function by binding to regulatory subunits known as cyclins. CDKs play a vital role in the cell cycle regulation of eukaryotes and phosphorylate their protein substrates by transferring phosphate groups from ATP to serine and threonine amino acids residues (19). Much of what is currently known about CDK activation is based on the CDK2/cyclin A complex. In the malaria parasite, *Plasmodium falciparum* protein kinase 5 (*Pf*PK5) has the highest sequence identity to *Hs*CDK1 and *Hs*CDK2, providing a starting point for understanding parasite protein kinases (**Figure 4a)** (20, 21). The genetic knock out of PK5 in the erythrocytic stages of *P. berghei* (9) did not produce any obvious phenotypes, however the protein is likely to be essential for the erythrocyte asexual cycle in *P. falciparum* (22).

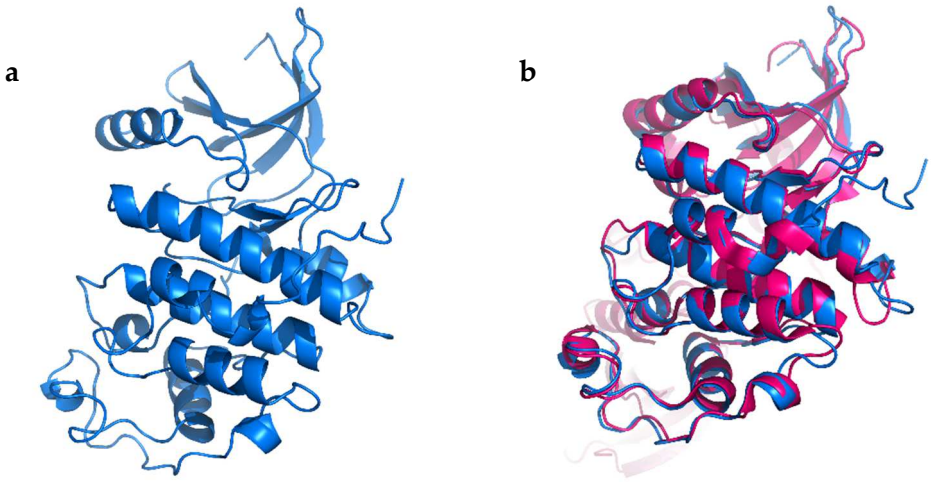


Figure 4: (a) Crystal structure of *Hs*CDK2 (PDB ID: 1AQ1) (b) Crystal structure of *Pf*PK5 (PDB ID: 1OB3) superimposed on *Hs*CDK2.

*Pf*PK5 has been previously cloned from *Plasmodium falciparum* and has 60% sequence identity to human CDK1 (6, 21). However, the structure of *Pf*PK5 activated by a cyclin subunit, in complex with a known inhibitor, resembles the activated *Hs*CDK2. *Pf*PK5 has previously been co-crystallized with three inhibitors exposing sites where sequence identity between the protein and the human analogs differs significantly (20) (**Figure 4b**). The difference in sequence identity between *Pf*PK5 and *Hs*CDK2 near and within the active site, may be exploited to identify and develop selective inhibitors of parasite proteins over their human host analogs.

1.7 Focus of this research

Understanding the role of protein kinases in *Plasmodium* biology is essential to learning more about parasite biology and identifying new druggable targets in the fight against malaria. Thousands of known human kinase inhibitors were previously screened in both the liver and blood stages of malaria to discover parasite inhibitors. Several molecules were identified as antimalarials able to inhibit parasite load in both stages. Further, using biochemical assays, many of these compounds were found to bind to *Pf*PK5 directly in the μM range, but all also bound the human CDK2 in pM to nM ranges. We aimed to engineer compounds that inhibit *Pf*PK5 but not *Hs*CDK2. This work is presented in Chapter 2. Chemical informatics of these known inhibitors predicted that they bind protein kinases, but possible off-targets are unknown (4). To address this issue, chemical probes are needed to enable target discovery and verification. Elucidation of the biological target(s) through the use of these probes will aid in understanding *Plasmodium* biology. The development of this chemical probe is presented in Chapter 3.

Chapter 2[1]

In silico screen to discover inhibitors of *Pf*PK5

There are many unique characteristics of the *Plasmodium* cell cycle. However, it is expected that cyclin dependent kinases (CDKs) will still be crucial to cell cycle progression in the parasite as seen in other eukaryotes. Similar to other CDK-like proteins, *Pf*PK5 is essential to the cell cycle regulation and consequently, the survival of the *Plasmodium* parasite. This enzyme is known to be activated during nuclear division in the cell cycle of the malaria parasite, making it a potential target for malaria treatment (6, 21).

Small molecule inhibitors of human kinases were recently profiled to evaluate the malaria kinome during the parasite's liver stage (23). In this study, inhibitors of the CDK family most frequently generated activity against *P. berghei* infection of liver cells by reducing parasite load in hepatocytes. These compounds were subsequently tested for binding to *Pf*PK5 and *Hs*CDK2 due to their relatively high structural homology. Many of these compounds bind to *Pf*PK5 in the μM range, but also bind to the human

[1] The work described in this chapter was done in collaboration with Amber Harold in Prof. Emily Derbyshire's lab (Duke University), Dr. Nobutaka Kato (Broad Institute) and Dr. Paul Sanschagrin in Prof. Piotr Sliz's lab (Harvard Medical School).

CDK2 in pM to nM ranges, indicating that they are selective for the *Hs*CDK2. As such, these compounds cannot be used as malaria therapeutics because the human host targets would be inhibited with higher potency than parasite targets. Thus, we began a search to engineer compounds that inhibit *Pf*PK5 but not *Hs*CDK2 to provide the desired selectivity.

For the following experiments, blood stage secondary assays were conducted in collaboration with Dr. Nobutaka Kato at the Broad Institute and Dr. Derbyshire and Amber Harold assisted with liver stage malaria assays. *In silico* screening and molecular docking studies were conducted by Dr. Paul Sanschagrin in collaboration with Dr. Piotr Sliz at Harvard Medical School.

2.1 Experimental

Our strategy to discover selective inhibitors, shown in **Figure 5,** began with an *in silico* screen to predict inhibitors of *Pf*PK5 and was completed by Dr. Paul Sanschagrin in Dr. Piotr Sliz's lab from Harvard Medical School. Based on these computational studies, the top 184 virtual hits were cherry picked (ICCB-Longwood Screening Facility, Harvard Medical School) and subsequently tested in a target-based primary screen that measured binding to *Pf*PK5 and *Hs*CDK2. From this primary screen, three compounds were identified as actives based on selective binding for *Pf*PK5 over *Hs*CDK2. These actives were purchased from ChemBridge Corp and used in a secondary assay to determine

their respective dissociation constants (K_d). To improve affinity and selectivity, we then completed a structure-activity relationship analysis on commercially available structural analogs of the most selective compound.

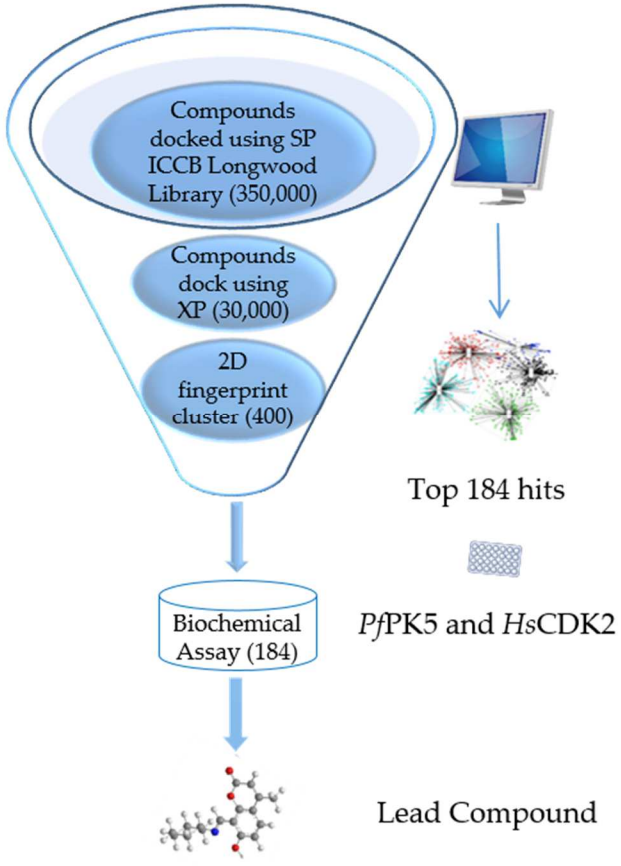


Figure 5: Scheme of strategy to discover selective inhibitors of *Pf*PK5

2.1.2 *In silico* studies

Molecular docking studies were conducted to predict potential inhibitors of *Pf*PK5. Three hundred and fifty thousand readily accessible compounds were docked

onto *Pf*PK5 (PDB ID: 1V0O) from a virtual library using Glide, a high throughput virtual screening program. Compounds were first docked using standard precision (SP) followed by extra precision (XP) mode to eliminate false positives. Finally, 2D fingerprint clustering was used to further eliminate false positives by estimating differences in sub-structural fragments in each compound. The top 184 predicted binders were selected based on docking scores and structural diversity. Screening compounds were then cherry picked from diverse libraries and obtained through the ICCB-Longwood Screening Facility (Harvard Medical School) for further assessment.

2.1.3 Overexpression of *Pf*PK5

A construct of *Pf*Pk5 with a terminal GST affinity tag was generously provided by Dr. Jane Endicott from the Northern Institute for Cancer Research. The construct was transformed into *E. coli* BL21-CodonPlus cells by Dr. Derbyshire. Isolated DNA was verified by sequence analysis (Genewiz). For expression, transformed *E. coli* BL21-CodonPlus cells were plated on Luria Broth (LB) Agar supplemented with 200 μg/mL ampicillin and 25 μg/mL chloramphenicol to select for cells containing the plasmid of interest and grown overnight at 37 °C. A single transformant (colony) was then grown overnight in 5 mL of LB supplemented with 200 μg/mL ampicillin and 25 μg/mL chloramphenicol. This culture was used to inoculate a 1 L culture of LB with 200 μg/mL ampicillin and 25 μg/mL chloramphenicol and then grown at 37 °C shaking at 250 rpm until the OD_{600} reached 0.4-0.5. Expression was then induced with isopropyl-β-D-1-

thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and the culture was grown overnight at 20 °C. The culture was harvested by centrifugation and the cell pellet was stored at -80 °C until purification. *Pf*PK5 was purified by glutathione affinity and gel filtration chromatography by Dr. Derbyshire and purity was assessed to be >95% by SDS-PAGE analysis. This protein was used to develop an ATP-dependent assay to measure compound affinity that is not discussed in this work.

2.1.4 *P. falciparum* PK5 and *H. sapiens* CDK2 assays

A target-based assay was used to verify binding of the 184 compounds from the *in silico* screen. These compounds were tested for binding to *Pf*PK5 and *Hs*CDK2 at 12 μM employing an active site-directed competition binding assay by DiscoveRx using KINOMEscan™ Technology. As shown in **Figure 6**, the assay utilized a DNA tagged kinase, and an immobilized ligand in order to determine the amount of kinase captured on a solid support using ultra-sensitive quantitative real-time PCR (qPCR). Additionally, interactions between inhibitors and enzymes are quantified as a function of inhibitor concentration without the use of ATP. The absence of ATP allows for the determination of true thermodynamic interactions permitting the measurement of dissociation constants (K_d). First tests were done with compounds from the ICCB-Longwood Screening Facility (Harvard Medical School) and then compounds that showed binding at 12 μM were selected and purchased from ChemBridge Corporation for K_d

determination (DiscoveRx). Results were plotted using Excel for the primary screen and GraphPad Prism for K_d determination in the secondary screen.

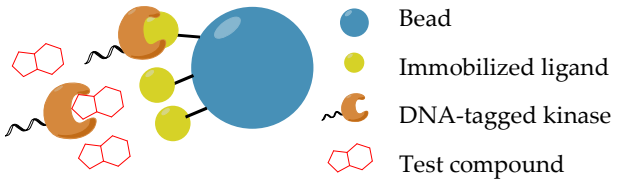


Figure 6: Active-site directed competition binding assay used to identify inhibitors of *Pf*PK5 and *Hs*CDK2.

2.1.5 Cell-based studies

Liver stage malaria assay. HepG2 (human liver) cells (ATCC) were maintained in DMEM (Invitrogen), 10% (vol/vol) FBS (Sigma), and 1% (vol/vol) antibiotic–antimycotic (Invitrogen) in a standard tissue culture incubator (37 °C, 5% CO_2). *Plasmodium*-infected *Anopheles stephensi* mosquitoes were obtained from the New York University Langone Medical Center Insectary.

P. berghei ANKA liver stage parasites expressing a luciferase reporter (24) were used to infect HepG2 cells similar to a previously published report (25). In the initial screen HepG2 cells were seeded in a 384-well microplate at 15,000 cells/well using a multichannel pipette (Rainin). After 18–24 hr at 37 °C the media was exchanged and then 184 compounds (600 nL) were added to the plate in duplicate using a D300 liquid dispenser (Hewlett Packard). The final concentration of DMSO was 2.0% (vol/vol) and

compounds were at 8 μM. DMSO (2% vol/vol) was used as a negative control and halofuginone (1 μM) was used as a positive control for parasite inhibition. After 1 hr, *P. berghei* ANKA parasites obtained from freshly dissected *A. stephensi* mosquitoes were added to the plates at 4,000 parasites/well, the plates were spun for 10 min at 800 rpm, and then incubated at 37 °C. The final assay volume was 30 μL. After a 43-hr incubation at 37 °C, CellTiter-Fluor (Promega) was added to the plate, incubated for 30 min and fluorescence was measured to assess relative HepG2 viability. After the fluorescence measurement Bright-Glo (Promega) was added to the plate and luminescence was measured to assess relative parasite load in the HepG2 cells. The relative fluorescence and luminescence signal intensities of the plate were evaluated with an EnVision (PerkinElmer) system. Results were plotted using Microsoft Excel. These experiments were done with Dr. Derbyshire and Amber Harold.

Blood stage malaria assays. Compounds that were identified as actives and selective for *Pf*PK5 over the human analog were subsequently tested for activity against blood stage malaria parasites. Red blood cells were cultured and maintained in a standard tissue culture incubator at 37 °C in the presence of 5% CO_2 and 4% O_2. Blood stage screens were completed using *P. falciparum* 3D7 parasites in the red blood cells following a previously reported protocol (26). These assays were completed by Dr. Nobutaka Kato at the Broad Institute. Results were plotted using GraphPad Prism.

2.1.6 Structure-activity relationship analyses

Once a lead compound was identified using biochemical and *in vitro* assays, a structural homology search was conducted to identify commercially available analogs of the identified lead compound. The lead compound was also docked to the crystal structure of *Pf*PK5 by Dr. Paul Sanschagrin. Using both the interactions predicted from this docking and the structural similarity search, we identified and purchased structural analogs from the ChemBridge Corporation. These new compounds were subsequently assessed for binding in the *Pf*PK5 and *Hs*CDK2 competition binding assays as well as malaria inhibition using *in vitro* assays previously described. All results were plotted using GraphPad Prism.

2.2 Results and discussion

Initial *in silico* docking studies identified 400 structurally diverse potential inhibitors of *Pf*PK5. The top 184 compounds were chosen for further analysis and cherry picked from diverse libraries. Hits from the *in silico* screen were obtained through the ICCB-Longwood Screening Facility (Harvard Medical School) and tested, as described in the sections below.

2.2.1 Primary biochemical assays

Predicted inhibitors were screened for binding to *Pf*PK5 and *Hs*CDK2 in an active-site-directed competition binding assay. This primary biochemical screen

included 184 compounds identified from the *in silico* screen. The percent bound to *Hs*CDK2 versus *Pf*PK5 was plotted in **Figure 7a**. This primary screen revealed three compounds that preferentially bind *Pf*PK5 over *Hs*CDK2. Structures for these primary screening actives **3268K07**, **821F18** and **738O07** are shown in **Figure 7b**.

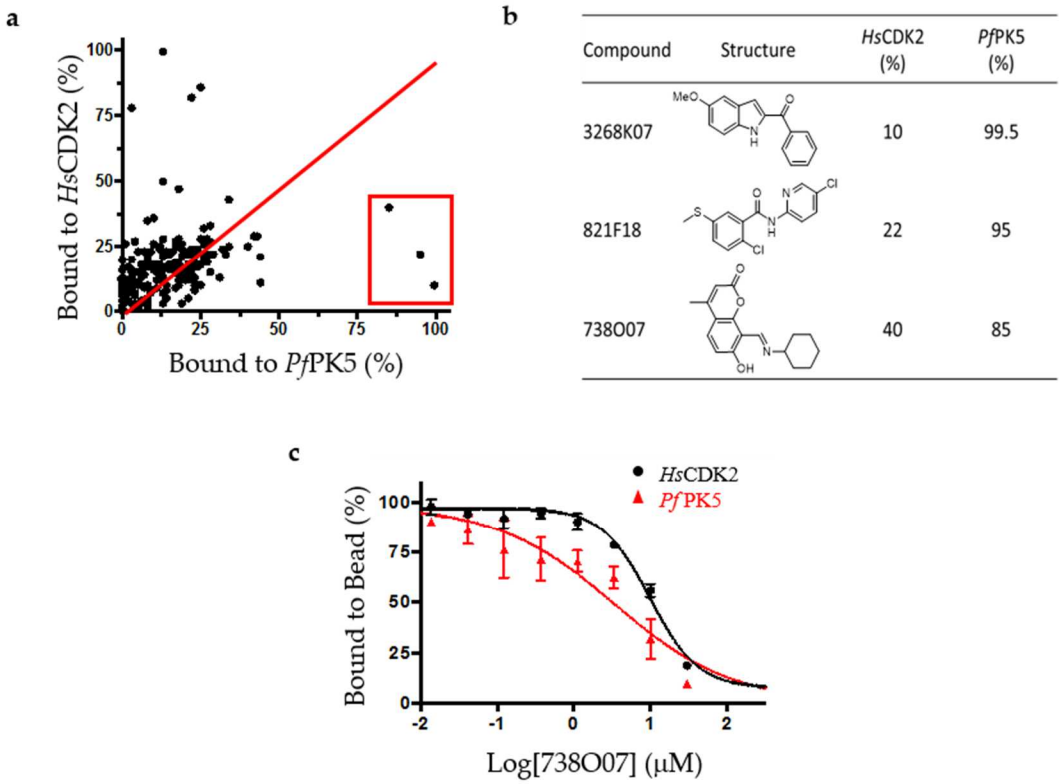


Figure 7: (a) Initial 12 μM screening of 184 compounds showing percent bound to *Hs*CDK2 versus percent bound to *Pf*PK5. (b) Table displaying structures and initial screening results of three compounds that were selective for *Pf*PK5 over *Hs*CDK2 (c) Competitive binding plots of 738O07 to *Pf*PK5 (red curve) and *Hs*CDK2 (black curve).

Screening actives were purchased and tested at additional concentrations (0 to 30 μM) to generate binding curves and estimate K_d values. Analysis of the results show that purchased **3268K07** and **821F18** were inactive against both *Pf*PK5 and *Hs*CDK2. This

suggested that the compounds originally obtained from the screening facility were different from those obtained from ChemBridge. To test this possibility all samples were analyzed by LC-MS but no significant differences were observed between compounds from ICCB verses ChemBridge. Studies are currently underway to further analyze these compounds and to determine if they were false positives. However, **738O07** showed greater than 2-fold selective binding to *Pf*PK5 (K_d of 5 μM) over *Hs*CDK2 (K_d of 11 μM), as seen in the competitive binding plot in **Figure 7c**.

2.2.2 Primary phenotypic screen

The inhibitory activity of cherry picked compounds against the *Plasmodium* infection of liver cells using the high-throughput liver stage malaria screen was completed as previously described (section 2.1.3). Compounds were also tested for liver cell toxicity using a fluorescence reporter assay kit, Cell-Titer Fluor (Promega). Active compounds in this phenotypic assay were identified as compounds that decreased parasite load in liver cells by ≥ 90% compared to the negative control, DMSO, but did not inhibit liver cell viability. Actives are shown in blue in **Figure 8a**. Many compounds inhibit liver cells and thus malaria parasites, but only a small subset are selective for parasites.

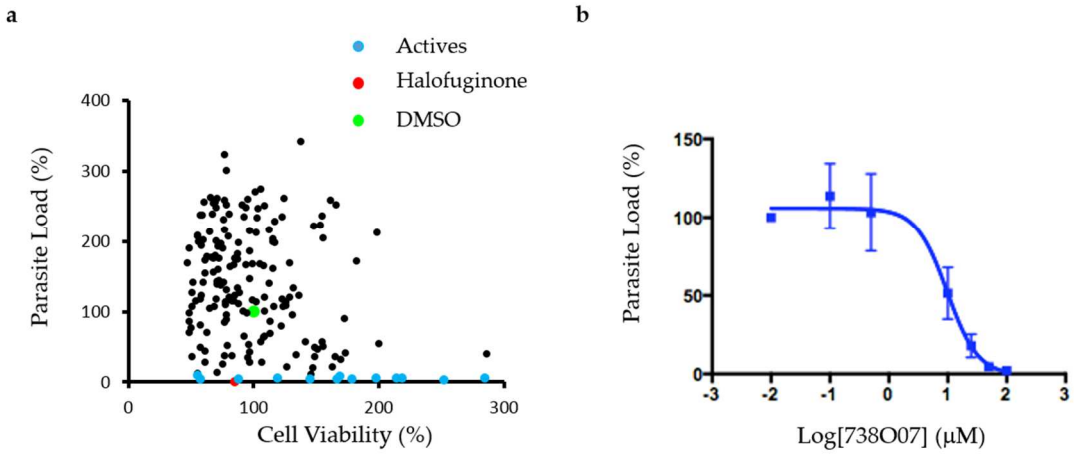


Figure 8: (a) Primary phenotypic screen shown as relative parasite load versus HepG2 cell viability relative to negative control (DMSO). (b) Representative dose-response curve for *P. berghei* ANKA liver stage for 738O07.

2.2.3 Secondary phenotypic screen

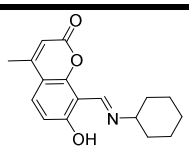
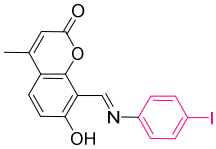
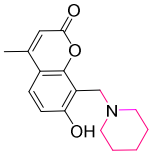
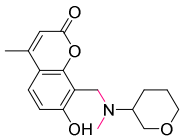
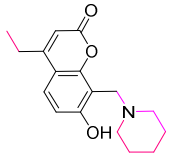
Although **738O07** was not identified as a screening active in the primary phenotypic screen, its selectivity for *Pf*PK5 in the competition binding assay motivated further analysis. Therefore, we used this compound to measure dose-dependent activity against liver-stage *P. berghei.* This dose response curve was generated for parasite inhibition in HepG2 (IC_{50} of 9.5 μM) and plotted in **Figure 8b. 738O07** may inhibit liver stage parasites by binding to *Pf*PK5 and *Hs*CDK2 or by inhibition of another target.

2.2.4 Structure activity relationship analyses

To further assess **738O07**, four structural analogs were chosen for biochemical testing. These four were chosen based on 1) predicted interactions from the molecular docking of **738O07** in to the *Pf*PK5 ATP binding site, 2) a compound structural similarity

search of 80% or greater and 3) commercial availability to facilitate fast results. Analogs were purchased to identify important structural moieties for protein binding. Structural analogs were profiled in the *Pf*PK5 and *Hs*CDK2 active-site directed competition binding assays. These commercially available compounds were also assessed in liver and blood stage cell-based malaria assays as previously described.

Table 1: Structure-activity relationship analysis of 738O07

Compound	Structure	*Hs*CDK2 K_d(μM)	*Pf*PK5 K_d(μM)	Liver Stage Malaria[a] IC_{50}(μM)	Blood Stage Malaria[b] IC_{50}(μM)
738O07		11	5	17	>20
5311879		28	14	19	>20
5257904		>30	>30	>10	>20
6315619		>30	>30	>10	~20
9203871		>30	>30	>10	~20

[a] IC_{50} determined for *P. berghei* ANKA (liver stage) and [b]*P. falciparum* 3D7 (blood stage).

Compound structures and their respective activities are reported in **Table 1.** The compound **5311879** is the only structural analog that binds to either the human or *P. falciparum* kinase. Importantly, this compound exhibited selectivity for *Pf*PK5 (K_d of 14 μM) over *Hs*CDK2 (K_d of 28 μM) similar to the parent compound, **738O07.** However, this binding is two-fold less potent than the parent compound. **5311879** and **738O07** showed similar activity against liver-stage parasites with an IC_{50} ~18 μM. The ability of **5311879** to bind to *Pf*PK5 suggests that the imine moiety is important with respect to *Pf*PK5 activity. This may be partially due to the orientation of the sp^2 hybridized nitrogen with respect to the *Pf*PK5.

Here we report the first successful example of an *in silico* model used to identify malaria inhibitors. **738O07** and **5311879** are also the first inhibitors to date that have selectivity for *Pf*PK5 over *Hs*CDK2. More work around this scaffold may improve this selectivity to yield more potent *Pf*PK5 binders with minimum affinity for *Hs*CDK2. This selectivity will be important for the generation of chemical probes to elucidate the function of cyclin dependent kinases in *Plasmodium* parasites.

Chapter 3[1]

Development of a potent *Plasmodium* chemical probe

Throughout the complex life cycle of *Plasmodium*, protein kinases are known to be essential for growth and development and likely regulate complex biological processes; however, the role of many protein kinases in regulating these biological processes remain to be determined. Identifying small molecule inhibitors that will elucidate the roles of essential kinases involved in *Plasmodium* infection is important for understanding the biology of this complex organism.

In 2013, over 1300 small molecule kinase inhibitors were examined to evaluate the role of the malaria kinome during *P. berghei* infection of liver cells, using a whole cell-phenotypic screen (4). Those with nanomolar efficacies were then tested for activity against blood stage malaria parasites, in order to identify any dual stage inhibitors.

Dual stage inhibition by these small molecules indicates their targets are essential to multiple parasites life stages. These efforts identified the small molecule named CDK 1/2 inhibitor III as a potent inhibitor of multiple parasite stages. Chemical informatics

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[1] The work described in this chapter was done in collaboration with Prof. Jiyong Hong (Duke University) for synthetic probe design and Dr. Nobutaka Kato (Broad Institute) for blood stage assays.

predicts that this inhibitor binds to cyclin dependent protein kinases 1 and 2, but the precise target in *Plasmodium* is unknown. Identification of the biological target will aid in understanding *Plasmodium* biology which may eventually lead to the development of new therapeutics (7, 8, 25, 27, 28).

3.1 Experimental

The development of chemical probes is necessary to enable target discovery of leads generated from high-throughput screening efforts. The strategy to develop a chemical probe for malaria proteins first began with the identification of a potent antimalarial, CDK 1/2 inhibitor III. Since then, the parent compound and structural analogs were synthesized following a previous protocol (29).

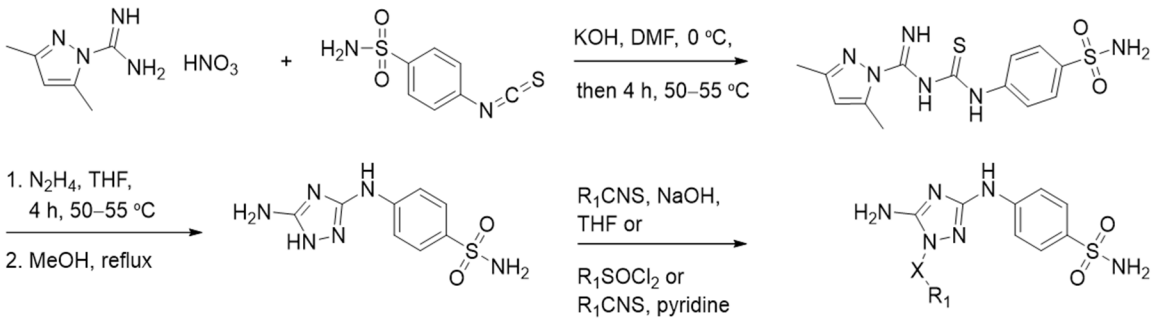
The synthesized CDK 1/2 inhibitor III (**MMP03**) and related structural analogs were tested for binding to *Pf*PK5 and *Hs*CDK2 using Kinome Scan technologies. In addition, compounds were tested for inhibition of blood and liver stage parasites.

3.1.1 Synthesis of structural analogs

CDK 1/2 inhibitor III and related structural analogs (**Table 2**) were synthesized using previously published protocols outlined in **Scheme 1**. Compound purification was modified for each of the final compounds tested in biochemical and cell-based assays. **MMP03**, **MMP06** and **MMP07** were purified using a Teledyne-ISCO Combiflash® RF unit, while **MMP04** and **MMP005** were purified using flash column

chromatography methods. All compound purifications were completed using a 0-10% (v/v) gradient of methanol in dichloromethane (DCM) to achieve >95 % purity.

Scheme 1: Synthesis of CDK 1/2 inhibitor III and related structural analogs[a]



[a](a) KOH in DMF, 50–55 °C , 5 h (b) Hydrazine in THF 4 h then reflux in MeOH (c) for MMP03 and MMP07 R_1CNS, 1 M NaOH, THF, room temperature; for MMP04–05, R_1COCl, anhydrous pyridine, room temperature, overnight; for MMP06 R_1SO_2Cl, anhydrous pyridine, room temperature, overnight (29).

Table 2: Modified structural moieties of CDK 1/2 inhibitor III and related structural analogs

	X	R_1
MMP03	CS	$NH(2,6\text{-}F_2)C_6H_3$
MMP04	CO	C_6H_5
MMP05	CO	$(2,6\text{-}F_2\text{-}3\text{-}Me)C_6H_2$
MMP06	SO_2	$(2,6\text{-}F_2)C_6H_3$
MMP07	CO	$NH(2,6\text{-}F_2)C_6H_3$

3.1.2 Biochemical assay

Synthesized compounds were tested for binding to *Pf*PK5 and *Hs*CDK2 using the active site-directed competition binding assay by DiscoveRx using KINOMEscan™

Technology previously described. Binding curves were generated to determine dissociation constants (K_d). Results were plotted using GraphPad Prism.

3.1.3 Cell-based studies

Liver stage malaria assay. Huh7 cells (Duke Cell Culture Facility) were maintained in DMEM (Invitrogen) containing 10% (v/v) FBS (Sigma), and 1% (vol/vol) antibiotic–antimycotic (Invitrogen) in a standard tissue culture incubator (37 °C, 5% CO_2). *Plasmodium*-infected *A. stephensi* mosquitoes were obtained from the New York University Langone Medical Center Insectary.

P. berghei ANKA liver stage parasites expressing a luciferase reporter (24) were used to infect Huh7 cells similar to a previously published report and the final assay volume was 30 µL. Cells (7,000 cell/well) were infected with parasites (4,000 parasites/well), and after a 42-48-hr incubation CellTiter-Fluor (Promega) was added to the plate and fluorescence was measured to assess relative Huh7 viability. After the fluorescence measurement Bright-Glo (Promega) was added to the plate and luminescence was measured to assess relative parasite load. The relative signal intensity of the plate was evaluated with an EnVision (PerkinElmer) system. This work was done in collaboration with Dr. Derbyshire and Amber Harold. Results were plotted using GraphPad Prism.

Blood stage malaria assays. Compounds were tested for activity against blood stage parasites. Blood stage screens were completed using *P. falciparum* 3D7 parasites in

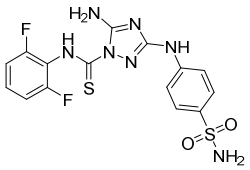
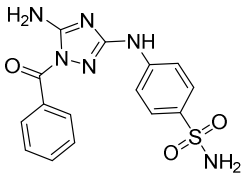
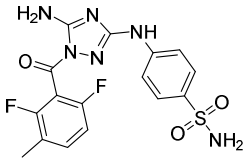
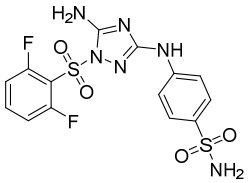
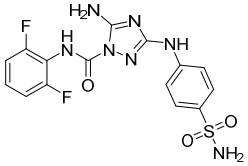
red blood cells following a previously reported protocol (26). These assays were completed by Dr. Nobutaka Kato at the Broad Institute. Results were plotted using GraphPad Prism.

3.2 Results and discussion

Here we report the analysis of a potent inhibitor of *Hs*CDK2 that was recently discovered to bind to the essential *Plasmodium* protein kinase, *Pf*PK5. Although there has been significant advances in malaria research over the past few decades, the role of protein kinases in regulating important *Plasmodium* biological processes remains an active area of research. The research reported in this chapter was completed to aid in the development of a chemical probe that can be used for target identification.

We first began with the synthesis of CDK1/2 inhibitor III, referred to here as **MMP03**, and its related structural analogs that were previously assessed for activity against various human kinases (29). Due to the structural similarity between *Pf*PK5 and *Hs*CDK2, we first profiled these compounds for the ability to bind to both of these proteins. We subsequently assessed whether these compounds were inhibitors of liver stage and blood stage parasites using cell-based assays to assess their ability to reduce parasite load. **Table 2** summarizes the *Pf*PK5, *Hs*CDK2, and cell-based activity data for CDK 1/2 inhibitor III (**MMP03**) and related analogs.

Table 3: Structure-activity relationship analysis of CDK 1/2 inhibitor III (MMP03)

Compound	Structure	*Hs*CDK2 K_d(μM)	*Pf*PK5 K_d(μM)	Liver Stage malaria[a] IC_{50}(μM)	Blood Stage malaria[b] IC_{50}(μM)
MMP03		0.00039	0.37	0.43	0.14
MMP04		0.013	23	35	22.7
MMP05		0.013	2.4	0.79	0.73
MMP06		2.1	>30	51	20.4
MMP07		0.13	>30	38	1.7

[a] IC_{50} determined for *P. berghei* ANKA (liver stage) and [b]*P. falciparum* 3D7 (blood stage)

Based on this structure activity relationship analysis we were able to identify some important structural moieties for binding to *Pf*PK5 and activity against blood stage and liver stage parasites for **MMP03**. The sulfanomide moiety on the benzoyl group is

important for *Pf*PK5 binding as it increases the *Pf*PK5 affinity by greater than 100-fold when compared to the carbonamide moiety containing compound, **MMP07 (**K_d of >30 μM**)**. The 3-position on the benzoyl group can tolerate the addition of a methyl substituent as **MMP005** is equipotent with respect to activity against blood stage (IC_{50} of 0.73 μM) and liver stage parasites (IC_{50} of 0.79 μM). However, **MMP05** binding to *Pf*PK5 (K_d of 2.5 μM) was reduced 18-fold when compared to **MMP03** (K_d of 0.14 μM). This reduction in binding to *Pf*PK5 but not *in vitro* activity suggests that **MMP05** may be targeting other important biological molecules that are likely essential for *Plasmodium* survival.

In summary, we have identified **MMP05**, a structural analog of CDK 1/2 inhibitor III as a dual stage inhibitor of *Plasmodium* that binds to *Pf*PK5. Although this compound is less potent than CDK 1/2 inhibitor III (**MMP03**), we believe that it may target other essential *Plasmodium* proteins. Developing chemical probes from both **MMP03** and **MMP05** can therefore be beneficial for the elucidation of protein kinase functions that are essential to the survival of the parasites.

Appendix A

Experimental Details of Compounds Reported

General. ^{1}H spectra were obtained at 400 MHz on a Varian INOVA multinuclear NMR spectrometer. Flash column chromatography was done using SiliaFlash® silica gel F60 (230-400 mesh). Standard solvents from Sigma Aldrich were used as received without any further purification. Thin-layer chromatography was performed on pre-coated glass plates with silica gel 60 F_{254} from the EMD Millipore Corporation. Yields were not optimized. **MMP03**, **MMP06** and **MMP07** were purified using a Teledyne-ISCO Combiflash® RF unit with a 0-10% (v/v) gradient of methanol in dichloromethane.

Mass electrospray positive spectra (MS) and HPLC quantitative purity analysis were carried out by Duke Mass Spectrometry Facility on an Agilent LC/MS equipment with a Zorbax 50x2mm C8 column or an Ace SuperC18 2x50mm column. The method was ran using 100%/0.2% (v/v) H2O/formic acid as mobile phase A and 98%/2%/0.3% (v/v/v) CAN/H2O/formic acid as mobile phase B and based on the absorption at 254 nm.

Synthesis of analogues. Synthesized using previously published protocols (29).

3,5-dimethyl-*N*-((4-sulfamoylphenyl)carbamothioyl)-1*H*-pyrazole-1-carboximidamide (compound **MMP01):**

Suspension A: 4 mL of DMF was added to a flask containing potassium hydroxide (3.734 mmol) and 1-Amadino-3,5-dimethylpyrazole nitrate (3.734 mmol) to create a suspension at 0℃. Solution B: 2 mL of DMF was added to isothiocyanate (1.867

mmol) (Oakwood Chemical Products) to create a solution. Solution B was then added to Suspension A at 0℃. After 30 minutes the reaction mixture was brought up to room temperature then stirred at 50-55℃ for 6 hours. 150 mL of icy water was then poured into reaction mixture. Resultant solid was filtered using vacuum filtration, rinsed with water and dried *in vacuo* to give intermediate as a yellow powder. ^{1}H NMR (400 MHz, acetone-d_6) δ 10.81 (s, 1H), 9.92 (s, 1H), 8.43 (s, 1H), 7.98-7.71 (m, 4H), 6.56 (s, 2H), 6.10 (s, 1H), 2.21 (s, 3H), 2.20 (s, 3H); MS (ESI) m/z: M+H$^+$: 255

4-((5-amino-1*H*-1,2,4-triazol-3-yl)amino)benzenesulfonamide (compound **MMP02):**

Hydrazine (5.67 mmol) was added to solution containing **MMP01** (0.567 mmol) intermediate in THF (6.5 mL). Reaction was stirred vigorously for 4-6 hours at 50-55℃. Reaction was evaporated *in vacuo* and the residue was then refluxed in methanol (6 mL) over night and cooled down to room temperature. The resultant solid was triturated with methanol to produce intermediate **MMP02** as a gray solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.3 (s, 1H), 9.2 (s, 1H), 7.79-7.42 (m, 4H), 7.04 (s, 2H), 5.96 (s, 2H); MS (ESI) m/z: 353.1 (M+H$^+$)

5-amino-*N*-(2,6-difluorophenyl)-3-((4-sulfamoylphenyl)amino)-1*H*-1,2,3-triazole-1-carbothioamide (compound **MMP03)**

2,6 difluorophenyl isothiocyanate (0.4 mmol) in THF (0.4 mL) was added to **MMP02** in 1 M NaOH (0.8 mL) at 0℃. Reaction was stirred for 30 minutes at 0℃ then brought up to room temperature and stirred for 5-7 hours. Reaction was neutralized

with 1 M HCl at 0℃. Neutralized material was washed in a 125 mL separatory funnel with 30mL of ethyl acetate and subsequently washed with 50 mL of brine solution three times. The organic layer was collected, dried over sodium sulfate and dried *in vacuo.* Product was purified using Teledyne-ISCO Combiflash Rf system with 0-10% MeOH in DCM to give final product as a white powder. ^{1}H NMR (400 MHz, acetone-d_6) δ 10.42 (s, 1H), 8.99 (s, 1H), 8.27 (s, 2H) 7.9-7.86 (d, 2H), 7.77-7.72 (d, 2H), 7.53 (m, 1H) 7.22-7.14 (m, 2H) ,6.42 (s, 2H); MS (ESI) m/z: 426.1 ($M+H^+$); HRMS: calc'd 426.0613 ,found 426.0612

4-((5-amino-1-benzoyl-1*H*-1,2,4-triazol-3-yl)amino)benzenesulfonamide (compound **MMP04)**

Benzoyl chloride (0.472 mmol) was added to **MMP02** (0.395 mmol) in anhydrous pyridine (3.16 mL) at 0℃ and stirred for 30 minutes. Reaction was brought up to room temperature and stirred overnight. Pyridine was dried over nitrogen gas and product was then dried *in vacuo*. Chromatography purification of the crude product on silica gel with 2-10% methanol/dichloromethane gave **MMP04** as a white powder. ^{1}H NMR (400 MHz, acetone-d_6) δ 8.83 (s, 1H), 8.3 (d, 2H), 7.75 (m, 7H), 7.51 (s, 2H), 6.39 (s, 2H); MS (ESI) m/z: 359 ($M+H^+$); HRMS: calc'd 359.0921; found 359.0922

4-((5-amino-1-(2,6-difluoro-3-methylbenoyl)-1*H*-1,2,4-riazol-3-yl)aminobenzene sulfonamide (compound **MMP05**)

2,6-difluoro-3-methylbenzoyl chloride (0.395 mmol) was added to **MMP02** (0.395 mmol) in anhydrous pyridine (3.16 mL) at 0℃ and stirred for 30 minutes. Reaction was

brought up to room temperature and stirred overnight. Crude material was washed in a 125 mL separatory funnel with 30 mL of ethyl acetate and subsequently washed with 50 mL of brine solution three times. The organic layer was collected, dried over sodium sulfate and dried *in vacuo.* Chromatography purification of the crude product on silica gel with 2-10% methanol/dichloromethane in a gradient gave product **MMP05**. ^{1}H NMR (400 MHz, acetone-d_6) δ 8.82 (s, 1H), 7.67-7.59 (m, 5H), 7.51 (s, 2H), 7.14 (td, J = 8.8, 1.4 Hz, 2H), 6.36 (s, 2H), 2.33 (s, 3H); MS (ESI) m/z: 409 ($M+H^+$); HRMS: calc'd 409.0889; found 409.0888

4-((5-amino-1-2((2,6-difluorophenyl)sulfonyl)-1*H*-1,2,4-triazol-3-yl)amino)benzene sulfonamide (compound **MMP06)**

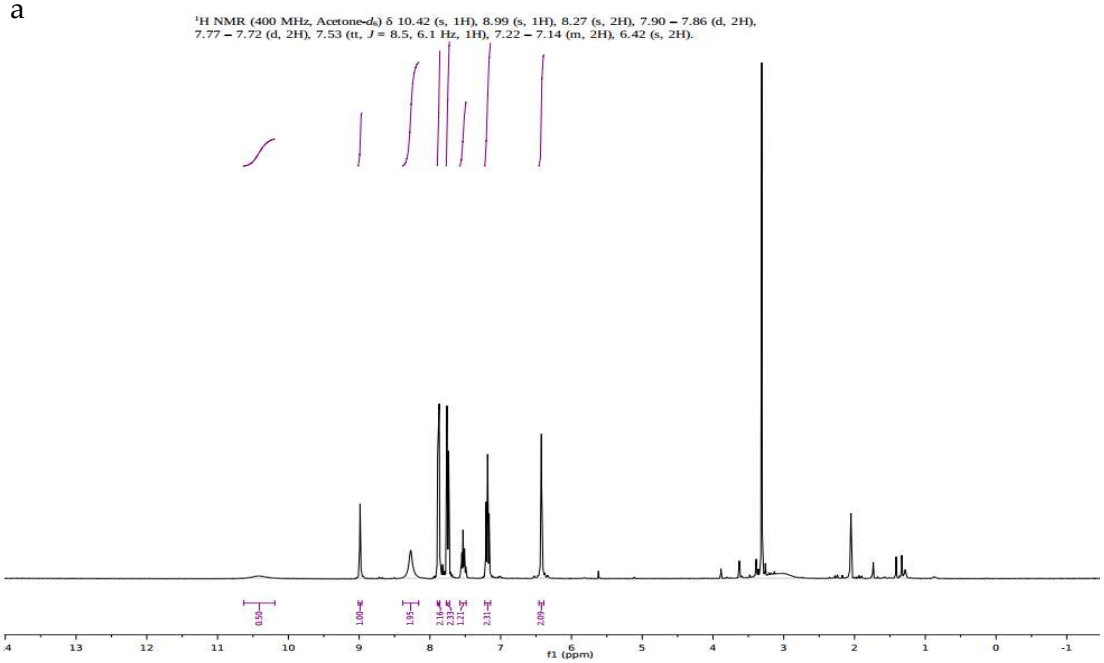
2,6-difluorobenzenesulfonyl chloride (0.470 mmol) was added to **MMP02** (0.395 mmol) in anhydrous pyridine (3.16 mL) at 0℃ and stirred for 30 minutes. The reaction was brought up to room temperature and stirred overnight. Crude material was washed in a 125 mL separatory funnel with 30 mL of ethyl acetate and subsequently washed with 50 mL of brine solution three times. The organic layer was collected, dried over sodium sulfate and dried *in vacuo.* Product was purified using Teledyne-ISCO Combiflash Rf system with 0-10% MeOH in DCM to give the final product as a white powder. ^{1}H NMR (400 MHz, acetone-d_6) δ 8.80 (s, 1H), 7.87 (tt, J = 8.5, 6.0 Hz, 2H), 7.79-7.70 (m, 2H), 7.70 – 7.59 (m, 2H), 7.02 (s, 2H), 7.40- 7.23 (m, 2H), 7.02 (s, 2H), 6.40 (s, 2H); MS (ESI) m/z: 431 ($M+H^+$); HRMS: calc'd 431.0402; found 431.0404

5-amino-*N*-(2,3-difluorophenyl)-3-((4-sulfamoylphenyl)amino-1*H*-1,2,4-triazole-1-carboxamide (compound **MMP07)**

2,6 difluorophenyl isocyanate (1 mmol) in THF (1 mL) was added to **MMP02** in 1 M NaOH (2 mL) at 0℃. Reaction was stirred for 30 minutes at 0℃ then brought up to room temperature and stirred for 5-7 hours. Reaction was neutralized with 1 M HCl at 0℃. Neutralized material was washed in a 125 mL separatory funnel with 30 mL of ethyl acetate and subsequently washed with 50 mL of brine solution three times. The organic layer was collected, dried over sodium sulfate and dried *in vacuo.* Product was purified using Teledyne-ISCO Combiflash Rf system with 0-10% MeOH in DCM to give the final product as a yellow solid. ^{1}H NMR (400 MHz, acetone-d_6) δ 9.16 (s, 1H), 8.82 (s, 1H), 7.87-7.84 (m, 2H), 7.75 (dt, J = 6.7, 2.3 Hz, 2H), 7.48-7.44 (m, 1H), 7.17 (dd, J = 8.2, 2.4 Hz, 2H), 7.05 (s, 2H), 6.39 (s, 1H); MS (ESI) m/z: 410 ($M+H^+$); HRMS: calc'd 410.0841; found 410.0838

Appendix B

a



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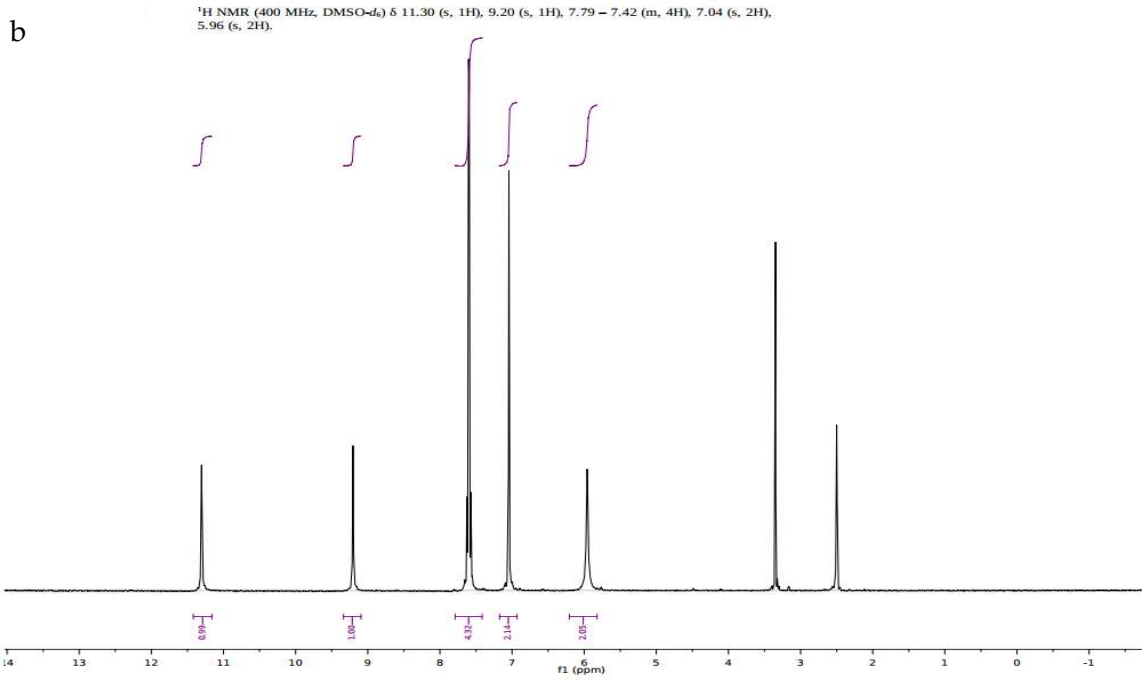


Figure S1: (a) ^{1}H NMR MMP01 and (b) ^{2}H NMR MMP02

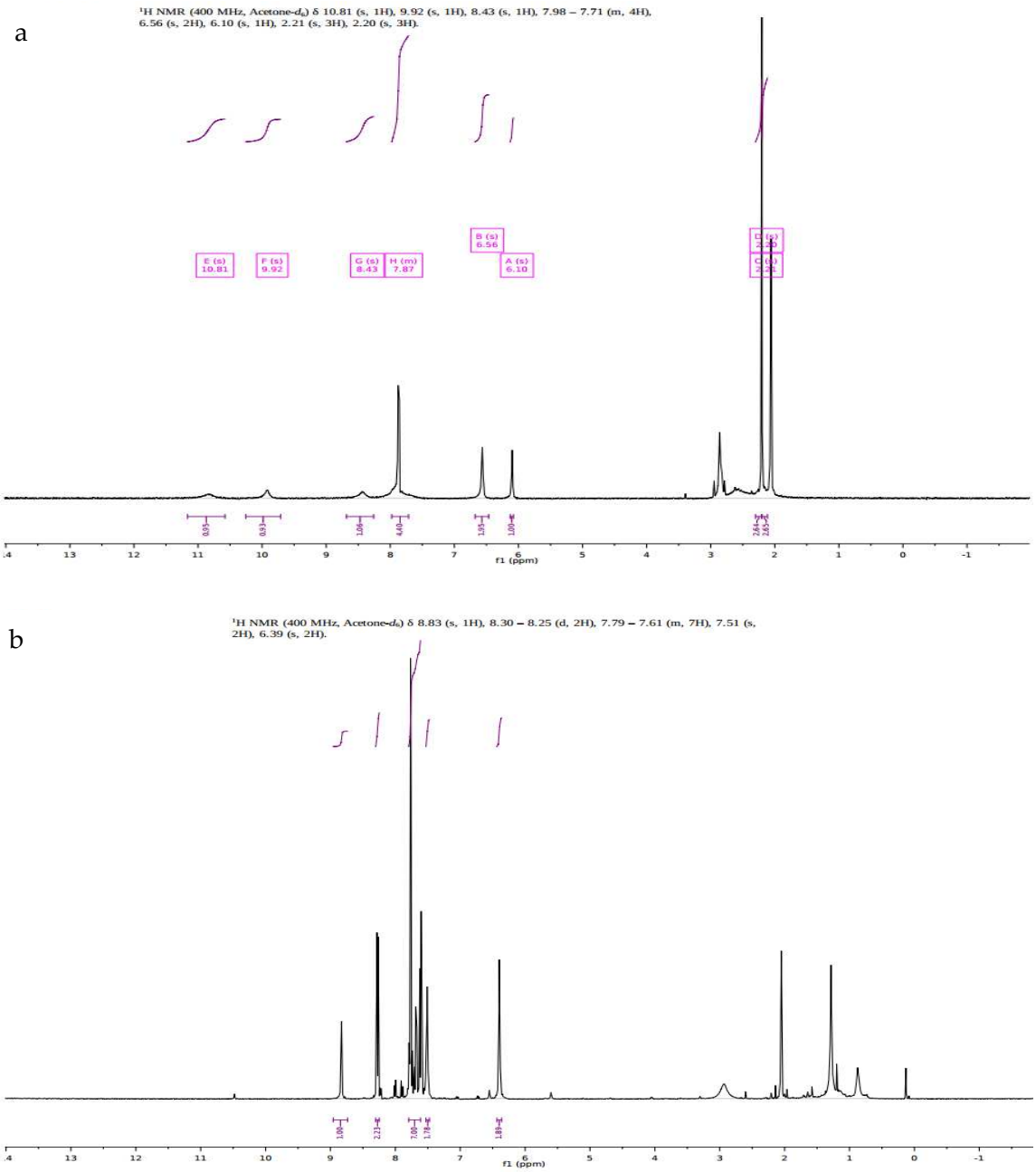
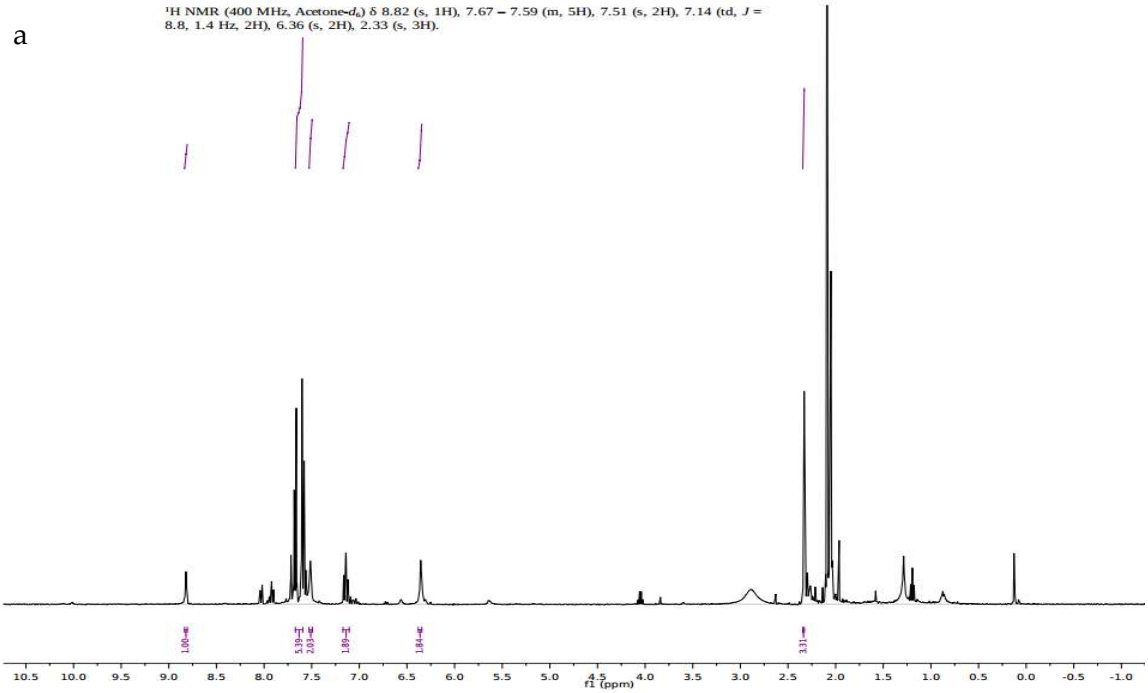


Figure S3: (a) ¹H NMR MMP03 (b) ¹H NMR MMP04

a



b

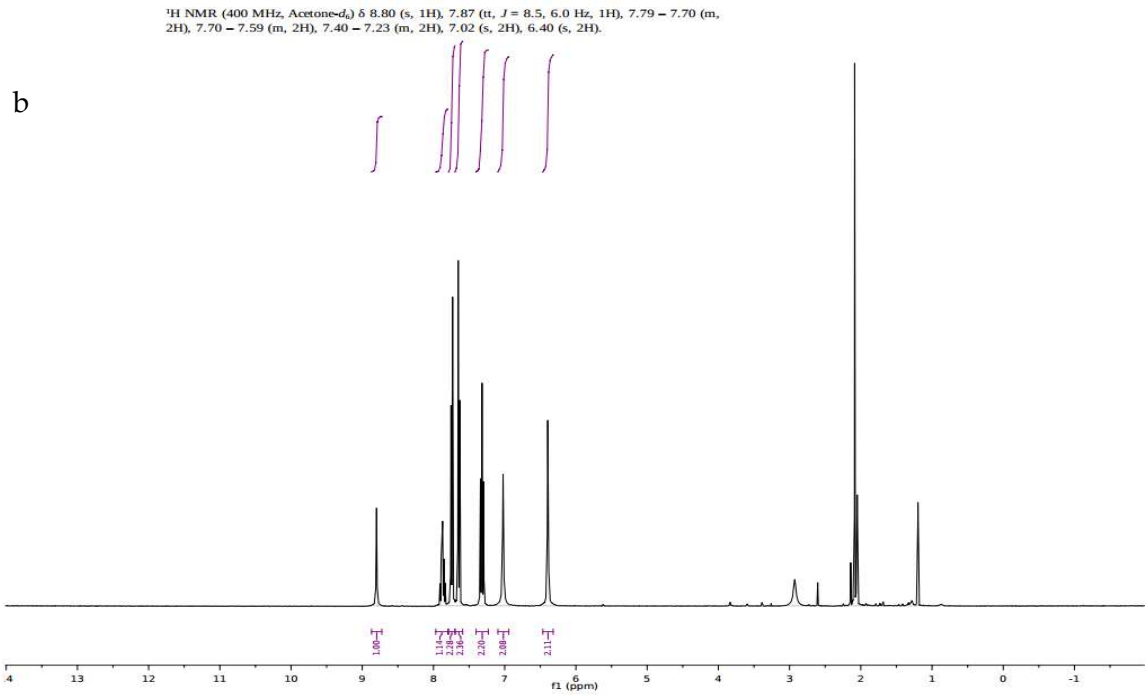


Figure S4: (a) ¹H NMR MMP05 (b) ¹H NMR MMP06

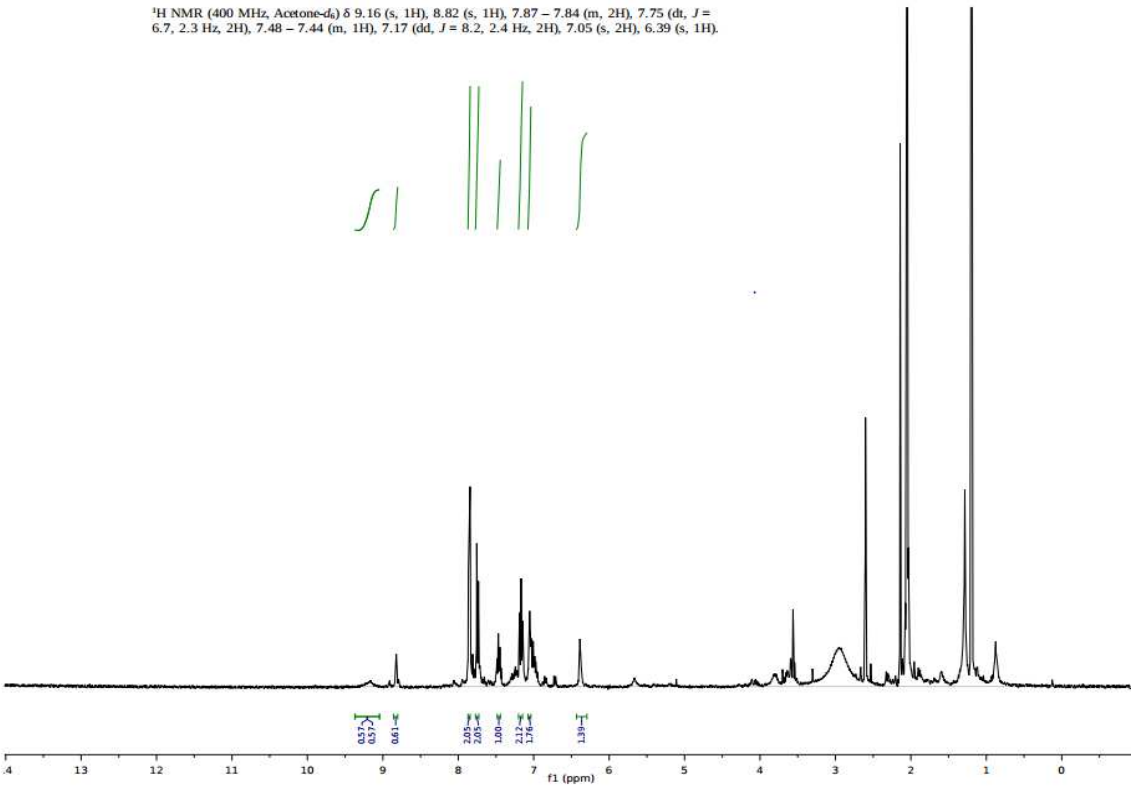


Figure S4: ^{1}H NMR MMP07

Table S1: HRMS and HPLC analyses of compounds

Compound	Formula	HRMS (MH^+)	HPLC
MMP03	$C_{15}H_{13}F_2N_7O_2S_2$	Calc'd 426.0613 Found 426.0612	98.1%
MMP04	$C_{15}H_{14}N_6O_3S$	Calc'd 359.0921 Found 359.0922	>99%
MMP05	$C_{16}H_{14}F_2N_6O_3S$	Calc'd 409.0889 Found 409.0888	98.9%
MMP06	$C_{14}H_{12}F_2N_6O_4S_2$	Calc'd 431.0402 Found 431.0404	>99%
MMP07	$C_{15}H_{13}F_2N_7O_3S$	Calc'd 410.0841 Found 410.0838	97.3%

References

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