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UNIVERSITY OF CALIFORNIA SAN DIEGO

Target Identification and Validation of the Imidazolopiperazine Class of Antimalarials

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Krypton Izon Carolino

Committee in charge:

Professor Elizabeth Winzeler, Chair
Professor Ethan Bier
Professor Victor Nizet
Professor Suresh Subramani

2024

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The dissertation of Krypton Izon Carolino is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

DEDICATION

This dissertation is dedicated with my utmost profound gratitude to Gil and Yolanda Carolino, Karizma and Krislyn Carolino, Renee Corpus, Casper, Maxx, my friends and mentors.

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LIST OF SUPPLEMENTAL DATASETS

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carolino_genome_analysis

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Chapter 2, in part, is currently being prepared for submission for publication of the material. Carolino, Krypton; Blauwkamp, James; Ghidelli-Disse, Sonja; Laureano De Souza, Mariana; Morano, Alexander; Chen, Daisy; Farre, Jean-Claude; Jose Lafuente-Monasterio, Maria; Absalon, Sabrina; Gamo, Francisco-Javier; Dvorin, Jeffrey; Winzeler, Elizabeth. The dissertation author is the primary researcher and author of this material.

Chapter 3, in part, contains material that may be prepared for publication at a future time with Dr. Elizabeth Winzeler. The dissertation author is the primary investigator and author of this material.

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Chemical Genetics
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Biochemistry
Drug Discovery

Research supervised by Professor Elizabeth Winzeler

ABSTRACT OF THE DISSERTATION

Target Identification and Validation of the Imidazolopiperazine Class of Antimalarials

by

Krypton Izon Carolino

Doctor of Philosophy in Biology

University of California San Diego, 2024

Professor Elizabeth Winzeler, Chair

The antimalarial resistance arms race is alive and well. In 2022, the *Plasmodium* genus of parasites were responsible for 249 million cases of malaria, 608,000 of which were fatal (WHO, 2023). This increase in cases is attributed to lack of an effective vaccine and restricted access to preventative and treatment measures coming after the COVID-19 pandemic, but especially to the ongoing development of antimalarial resistant parasites. Thus, there is a dire need to create medicines that possess novel modes of action. And if malaria were ever to be eradicated, we need those that inhibit parasite growth at every stage of their life cycle. So, how do we know which compounds exhibit antimalarial activity in a novel way?

This dissertation highlights and applies strategies of antimalarial target identification. Chapter 1 describes omics approaches: *in-vitro* evolution and whole genome analysis, proteomic affinity chromatography, cellular thermal shift assay, metabolomic profiling. Chapter 2 uses these methods on imidazolopiperazines, a new class of antimalarials that possess a novel mode of action and are active against multiple stages of the parasite life cycle. Previous work evolving parasites against the GNF179 analog only presented multidrug resistance mechanisms; metabolomic profiling with the KAF156 analog did not present clear perturbations. Using proteomic affinity chromatography, thermal shift assay, and surface plasmon resonance, we identify GNF179 interaction with a putative dynamin-like GTPase. Having only been predicted to be essential in parasites, we also confirm that it is an essential *P. falciparum* gene via conditional knockdown. Molecular docking and GTPase activity assay suggest imidazolopiperazine binding to the N-terminal GTPase domain, yet a nonsense mutation that removes residues from the C-terminal end confers resistance. In Chapter 3, we investigate the significance of the transmembrane domains and C-terminal tail of this GTPase protein to imidazolopiperazine interaction. Through this research, we can better guide imidazolopiperazine development and anticipate resistant alleles.

Chapter 1 | The antimalarial resistome – finding new drug targets and their modes of action

Abstract

To this day, malaria remains a global burden, affecting millions of people, especially those in sub-Saharan Africa and Asia. The rise of drug resistance to current antimalarial treatments, including artemisinin-based combination therapies, has made discovering new small molecule compounds with novel modes of action an urgent matter. The concerted effort to construct enormous compound libraries and develop high-throughput phenotypic screening assays to find compounds effective at specifically clearing malaria-causing *Plasmodium* parasites at any stage of the life cycle has provided many antimalarial prospects, but does not indicate their target or mode of action. Here, we review recent advances in antimalarial drug discovery efforts, focusing on the following ‘omics’ approaches in mode of action studies: IVIEWGA, CETSA, metabolomic profiling.

Main Text

Introduction

Tropical diseases remain a prevalent source of morbidity and mortality globally. Malaria, arguably the most widespread parasitic infection in humans, is mainly caused by protozoan parasite species *Plasmodium falciparum* and *P. vivax*. Humans contract malaria through the bite of an infected female mosquito, releasing *Plasmodium* sporozoites that first travel to the host liver, where they mature into schizonts. Schizonts rupture and release merozoites back into the bloodstream, where they invade red blood cells. Merozoites mature into schizonts that in turn generate more merozoites that will invade other red blood cells when they burst out of their current cells. Aside from the asexual blood stage, there is the sexual blood stage in which merozoites mature into gametocytes that are taken up by mosquitoes taking a blood meal, where they develop into sporozoites (Cowman et al., 2016). These sporozoites are then transmitted to the next human by the next mosquito blood feed, thus continuing the life cycle.

In 2018, there were an estimated 228 million cases of malaria worldwide (an increase of nine million cases over 2017), 405,000 of which resulted in death (WHO, 2018). Malaria remains a significant burden despite preventative measures (currently chemoprevention, insecticides, and insecticide-treated bed nets). Parasite resistance to current antimalarial drugs (including first-line artemisinin-based combination therapies, ACTs), as well as mosquito resistance to insecticides (whether behavioral, physiological, or biochemical (Ndiath, 2019)), has threatened malaria eradication efforts. Developing innovative vector control measures like CRISPR-based gene drives (Kyrou et al., 2018), as well as novel treatment/prevention strategies such as monoclonal antibodies (Kisalu et al., 2018), vaccines (Draper et al., 2018), or antimalarial drugs with novel modes of action are needed more than ever.

One approach to developing new drugs involves identifying small molecules with antiparasitic activity using phenotypic screening – testing compound directly against living parasites. The concerted efforts by many labs to design high-throughput phenotypic screening methods for the entire *Plasmodium* life cycle and assemble chemical libraries have led to the discovery of many compound candidates effective in parasite clearance (Yahiya et al., 2019). This approach has led to new clinical candidates such as KAE609 (cipargamin) and KAF156 (ganaplacide), which are currently in phase IIb clinical trials. A recent screen of over 500,000 compounds yielded 681 candidates with submicromolar efficacy at the liver stage (Antonova-Koch et al., 2018). Another screen of almost 70,000 compounds yielded 17 candidates with transmission blocking activity (Delves et al., 2018). A limitation to these screens is that they do not specify targets. Thus, many candidate hits are being re-screened in specific assays to determine putative mechanism of action. The Tres Cantos Antimalarial Compound Set was re-screened in assays specific for transmission blocking: TCMDC-135461 and TCMDC-137173 target cytochrome bc1; TCMDC-134114 targets dihydrofolate reductase, and TCMDC-125849 targets *Pfs25* (Delves et al., 2019). It was also re-screened in assays specific for respiratory inhibition (Gomez-Lorenzo et al., 2018) and proteasome inhibition (Mata-Cantero et al., 2019).

Additionally, as the rate of discovery of new antimalarial chemotypes decreases, many are reconsidering target-specific screens, like a recent biochemical screen designed to identify inhibitors of lysyl-tRNA synthetase (Baragana et al., 2019). Such screens can be less expensive to run, do not require specialized access to parasites, and could theoretically give leads with improved potency and selectivity, especially when combined with knowledge of the target's structure. Two promising compounds in clinical trials, DSM265 and P218, were found to target DHODH and DHFR, respectively, using this method. The current bottleneck now lies in identifying high quality

targets, such as lysyl-tRNA synthetase. A bioinformatics approach can be taken to predict alternative binding sites in *Plasmodium* aminoacyl tRNA synthetases that have low homology to those in humans, thus making them potential druggable targets (Nyamai and Tasthan, 2019). Here, the computational approach is expanded as we discuss current methods for discovering antimalarial drug targets with novel modes of action through ‘omics’ approaches – genomics, proteomics, metabolomics.

In-vitro evolution and whole genome analysis unveils novel target genes

A promising method for discovering new targets involves *in-vitro* evolution and whole genome analysis (IVIEWGA). This method comprises exposing *P. falciparum* parasites to sublethal doses of a compound until an upward shift in IC₅₀ is observed in the culture, indicative of resistant parasites. Sequenced genomes of the resistant parasites are compared to those of the drug-naïve parent to reveal genetic changes such as single nucleotide polymorphisms (SNPs) and copy number variants (CNVs). These genomic changes can point to genes encoding drug targets, but can also point to genes encoding multidrug resistance, such as the *P. falciparum* multidrug resistance 2, *PfMDR2* (Morita et al., 2019), or improving the fitness of a drug-selected line. They may even be just random background mutations. Distinguishing whether a gene encodes a target or a resistance mechanism can be challenging, especially if the mutation is in a currently uncharacterized genes, as was the case with the *P. falciparum* Niemann-Pick Type C1-Related protein, *PfNCR1*, now deemed important for parasite plasma membrane composition and digestive vacuole biogenesis (Istvan et al., 2019). Recently, this method has uncovered or reconfirmed additional new antimalarial drug targets, notably: the proteasome (Xie et al., 2018), thymidylate synthase, farnesyltransferase (*PfFTB*), dipeptidyl aminopeptidase 1 (*PfDPAP1*),

aminophospholipid-transporting P-type ATPase (*PfATPase2*) (Cowell et al., 2018), bifunctional farnesyl/geranylgeranyl diphosphate synthase (*PfFPPS/PfGGPPS*) (Gisselberg et al., 2019), cGMP-dependent protein kinase (*PfPKG*) (Baker et al., 2017), and cyclin-dependent-like kinase 3 (Alam et al., 2019) (Figure 1.1a). Other drug targets discovered through IVIEWGA are summarized in this review (Luth et al., 2018). In a few cases, resistant lines have been created, but it is unclear which mutation gives resistance, potentially because resistance is conferred by a mutation in an AT-rich intergenic region. Whole genome sequencing is advancing to allow deeper coverage and longer reads (that can help repetitive intergenic tracts) and bioinformatics pipelines are improving to better detect SNPs and CNVs. Machine-learning may be used to predict which of these are likely to confer resistance without laborious manual confirmation via CRISPR-Cas9 genome editing or inducible TetR-apptamer systems (Goldfless et al., 2014).

‘Irresistible’ compounds are those that have failed to generate resistant *P. falciparum* lines. They are interesting because if developed into drugs, they may be less likely to fail early from acquired drug resistance. For these compounds, IVIEWGA could be attempted in closely related organisms, like *Toxoplasma gondii* (Rosenberg, 2019). Other methods are required to study these irresistibles, such as proteomics and metabolomics discussed below. Furthermore, as molecular biology techniques advance, generation of *Plasmodium* overexpression libraries will be feasible, allowing for more efficient target identification via IVIEWGA.

Affinity-based and protein-stability assays can directly identify and validate target genes

Proteomics has been used to study *Plasmodium* protein expression and post-translational modifications, integral in finding regulatory mechanisms that can be exploited for antimalarial drug discovery (Swearingen and Lindner, 2018). It can also be utilized to remedy limitations of

IVIEWGA through approaches like affinity-based or protein-stability assays (Drewes and Knapp, 2018). In the popular pull-down method, a tag bound covalently to a compound of interest attaches to interacting protein targets, which can then be isolated from other proteins via centrifugation, magnetic separation, or click chemistry, depending on the tag used. Interacting proteins, now enriched, are prepared for liquid chromatography followed by mass spectrometry (LC/MS–MS) so they can be identified. Recently, an alkyne tag on a photoactivatable group-diaminoquinazoline compound probe was used to bind its molecular target via UV-activated click chemistry; LC/MS–MS identified 104 candidate target genes (Lubin et al., 2018). Because pull-downs can yield false positives through non-specific binding, they should also be used in competition assays. *P. falciparum* cell extracts are incubated with heavy Sepharose beads having an analog compound, like MMV666845, or adenosine triphosphate (ATP)-competitive kinase inhibitors (Kinobeads) attached; as the compound of interest, like MMV390048, increases, the target protein should bind less to the beads (Figure 1.2). And by cross-referencing pull-down data with that from IVIEWGA, candidates can be narrowed down to one target, as seen with the MMV390048-phosphatidylinositol 4-kinase (*PfPI4K*) interaction (Paquet et al., 2017). Moreover, approaches like protein-protein immunoprecipitation studies can be used in elucidating mechanisms of action, as seen with *P. falciparum* histone deacetylase 1, *PfHDAC1*, interacting with 26 complex proteins (Engel et al., 2019). Though, a limitation to pull-downs is synthesis of these tag-bound small molecules and subsequent testing for efficacy, as the tag may neutralize affinity or decrease potency, requiring structure-activity relationship (SAR) optimization.

To circumvent compound alteration, one can utilize the emerging label-free cellular thermal shift assay (CETSA), or thermal proteome profiling (TPP), another method that does not require previous knowledge on compound mode of action. This experiment relies on biophysical

properties of proteins, while in their native environments even, to unravel interactions with small molecules. When cells are treated with a compound of interest, the compound binds to its protein target. When challenged with heat, unbound proteins denature and precipitate out of solution, whereas compound-bound proteins are stabilized and thus require higher temperature to precipitate, relative to the vehicle control. A protein, identified via LC/MS-MS, with an increased temperature shift suggests that it is binding to the compound and possibly the direct target (Reckzeh et al., 2019) (Figure 1.3). This method has recently been developed for parasites, like *Leishmania donovani* (Corpas-Lopez et al., 2019) and *P. falciparum*. Quinine and mefloquine were found to bind to purine nucleoside phosphorylase, *PfPNP* (Dziekan et al., 2019) (Figure 1.1b). The drawback of this assay is that it has currently been developed to identify soluble proteins only, and detection is not always possible when endogenous protein levels are low (Dziekan et al., 2020). If compounds have an alternative mechanism of action responsible for their activity, such as binding hemoglobin, RNA, or lipids, protein-binding events may be real but obscure the true mechanism of action. Again, specificity of drug target interactions, along with downstream stability fluctuations, can yield false positives. Thus, this method still requires additional validation. There is promise for high-throughput CETSA that utilizes NanoLuciferase reporter and AlphaLISA assay to identify compounds directly targeting a specific protein (Henderson et al., 2020). Also, there are other stability-based proteomics techniques waiting to be harnessed in malaria research (Nguyen et al., 2017).

Metabolomic profiling and transcriptomics narrows down the mode of action

Metabolomic profiling comprises treating *P. falciparum* parasites with a compound of interest, extracting and analyzing the metabolites via LC–MS, and comparing the metabolomics

fingerprint, or metaprint, to that of untreated parasites (Allman et al., 2016). This method may not provide the exact target, but it narrows down the mode of action, valuable for target validation or characterization. One caveat is that metabolite changes could be attributed to non-specific or non-viable phenotypes, but this can be overcome with testing at various concentrations or over a period of time (Allman et al., 2016). Recently, MMV000787, MMV021735, and MMV030666 showed a profile akin to DSM265 and atovaquone, which disrupt the mitochondrial electron transport chain via inhibition of dihydroorotate dehydrogenase and cytochrome bc1, respectively, observed through an increase in pyrimidine precursors dihydroorotate and *N*-carbamoyl-L-aspartate. Additionally, KAE609 and MMV019017 showed a profile of decreasing nucleoside diphosphate and triphosphate levels, a signature of inhibiting Na⁺-dependent/H⁺-dependent ATPase *PfATP4* (Murithi et al., 2019). Another study found that pantothenamides, like MMV689258, sequesters coenzyme A to block acetyl-CoA anabolism, observed through CoA analog buildup (Schalkwijk et al., 2019) (Figure 1.1c). A limitation is the possibility that compounds do not act through metabolic pathway disruption. And as with other big data sets, metabolomics studies are susceptible to confounding variables, such as host contamination and the normalization approach, which need to be addressed (Carey et al., 2018).

The pre-erythrocytic stage remains of particular interest for antimalarial drug discovery because, with fewer parasites, resistance is less likely to emerge at this stage. Recently, genome-wide RNA-seq performed for *Plasmodium berghei* exoerythrocytic stages provided insight into the metabolic networks of these parasites during development in the liver (Caldelari et al., 2019). When combining these findings with those from a whole life cycle phenotypic screen of barcoded knockout mutant *P. berghei* parasites, a liver-stage metabolic model was generated, which indicated that type II fatty acid synthesis and elongation, tricarboxylic acid, amino sugar, heme,

lipoate, and shikimate metabolism to be essential for parasite proliferation and thus potential drug targets in the liver stage (Stanway et al., 2019). Moreover, transmission-blocking agents are also of interest to prevent new malaria cases. Combining RNA-seq and proteomics in characterizing oocyst and salivary gland sporozoites of *Plasmodium yoelii* and *P. falciparum* provides insight in the mechanism of parasite transmission, which can be targeted for novel action drug discovery (Lindner et al., 2019).

Conclusion

Taking an omics approach is the future of novel antimalarial drug discovery (Cowell and Winzeler, 2019). Millions of compounds have been screened for antimalarial activity. The bottleneck to drug discovery occurs in drug target identification and mode of action. The most used, genomic method of IVIEWGA generates lists of target genes that are muddled by background noise. Therefore, complementary assays are required, those investigating the *Plasmodium* transcriptome, proteome, and metabolome (Sexton et al., 2019). Involving CETSA in target validation, the potential to develop drugs with novel mechanisms within the near future is more feasible. Metabolomic profiling further helps prioritize compounds for lead optimization. Compounds targeting the food vacuole, metabolic processes, mitochondria, and transport pathways have been discovered (Kumar et al., 2018). Since not every compound can go to clinical trials, mechanistic and pharmacokinetic studies can help identify and prioritize compounds (Chaparro et al., 2018).

Though the *P. falciparum* genome has been fully sequenced, many genes still lack functional predictions, in part because of the dearth of malaria researchers. With the release of Malaria Cell Atlas (Howick et al., 2019), there is now an initial and growing source for gene

function and mechanism information for the parasite across its complex life cycle, rather than just focusing on the asexual blood stage. This atlas can serve as a reference for understanding functions of genes targeted by various compounds throughout the parasite life cycle. These omics approaches should be further developed for *P. vivax*, the other species responsible for a majority of malaria cases worldwide, and one with a unique hypnozoite stage of development (Roth et al., 2018). Additionally, with many natural products shown to have antimalarial activity (Tajuddeen and Van Heerden, 2019), it would be beneficial to identify their target and mechanism of action through these omics approaches. Lastly, not only are antimalarial drugs with novel modes of action needed but also vaccines, or novel vector control methods to reach malaria eradication (Laurens, 2018).

Figures

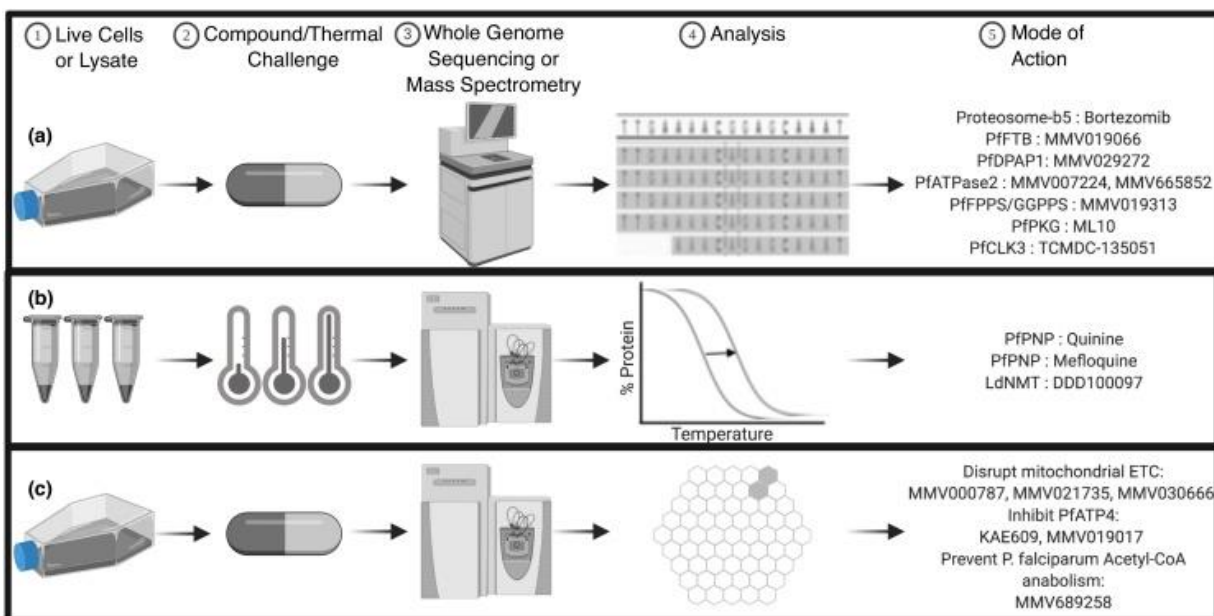


Figure 1.1 | Overview of IVIEWGA, CETSA, and metabolomic profiling processes

a. IVIEWGA comprises exposing drug-naïve *P. falciparum* parasites to sublethal doses of a compound until resistance is observed via an upwards shift in IC_{50} concentration. Parasites are prepared for whole genome sequencing, followed by call variant comparisons to the parental strain. **b.** CETSA consists of mixing compound, or vehicle, with *P. falciparum* parasite lysates and exposing mixtures to a range of temperatures, from 37C to 73C. Samples are prepared for LC/MS–MS, yielding melting curves for each protein. **c.** In metabolomic profiling, *P. falciparum* parasites are treated with compound, or vehicle, followed by metabolite extraction. Samples are prepared for LC/MS–MS, generating a metabolic fingerprint (metaprint) for each compound. Each individual hexagon represents a metabolic pathway; the shading determines fold change.

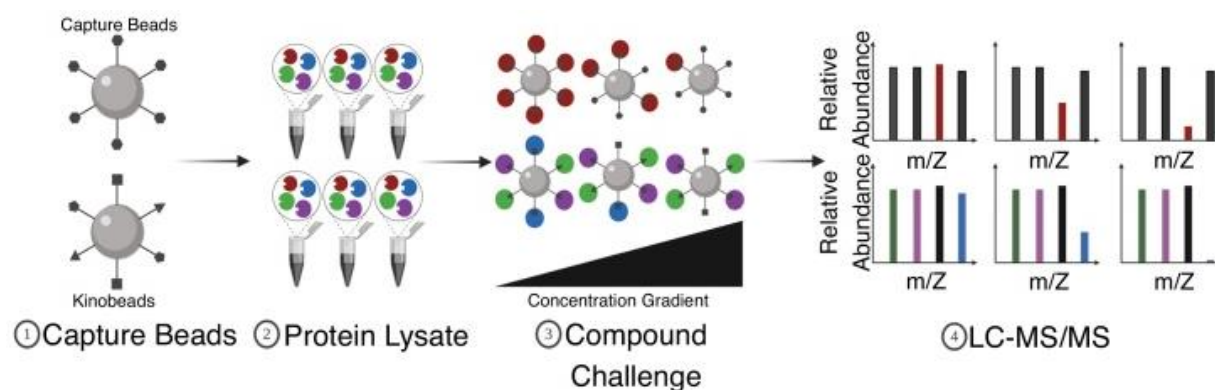


Figure 1.2 | Overview of chemoproteomics pull-down competition approach

Capture beads are prepared by covalently immobilizing an analog compound (top) to Sepharose beads; should ATP-competitive kinase inhibitors be bound, one would generate Kinobeads (bottom). These beads are added to *P. falciparum* lysates, with increasing amounts of compound of interest. As compound of interest increases, it will compete with ligands attached to the beads for the target protein. Upon analysis of proteins bound to the beads via LC/MS–MS, there should be a decrease in the abundance of the target protein, as seen between MMV66685 and PfPI4K when MMV390048 is added (Paquet et al., 2017).

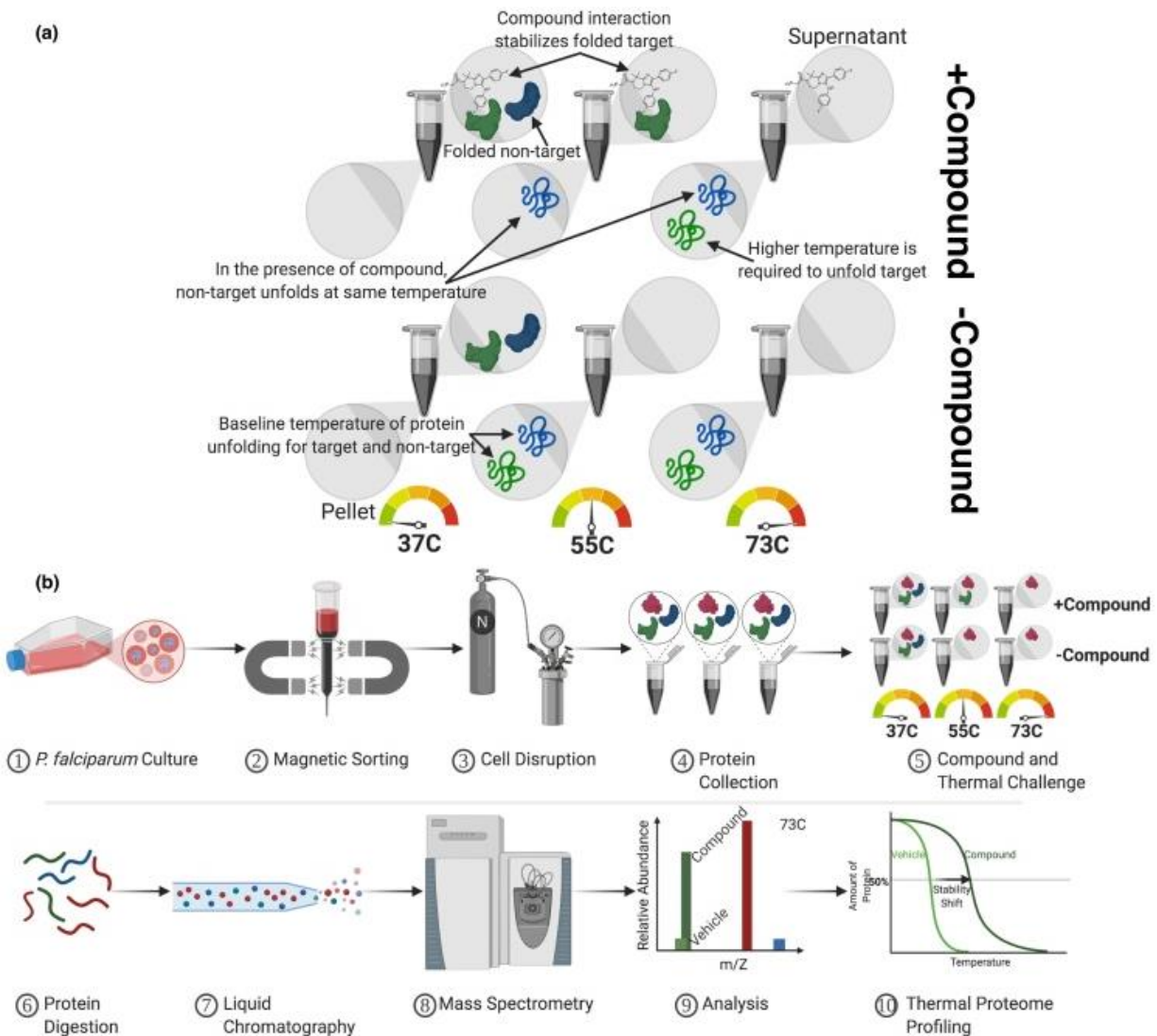


Figure 1.3 | Schematic of the cellular thermal shift assay

a. Late-stage *P. falciparum* parasites are concentrated via magnetic isolation to minimize protein masking by hemoglobin. Protein lysates generated after nitrogen cavitation and cell disruption are subject to treatment with compound, or control, followed by exposure to a range of temperatures to determine protein stabilities. Sample supernatants are digested and TMT-labeled for LC/MS–MS processing. Thermal proteome profiling comprises generating stability shift curves for each protein detected, looking for increased temperature to denature 50% of a protein. **b.** Presence of the compound during compound and thermal challenge stabilizes the target protein, requiring a higher temperature to denature 50% of that protein. Stabilization keeps more target protein in the supernatant relative to the pellet.

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Chapter 2 | *Plasmodium SEY1* is a novel druggable target that contributes to the imidazolopiperazine mechanism of action

Abstract

The precise mode of action of ganaplacide (KAF156), a phase III antimalarial candidate, remains elusive. Here we employ omics-based methods with the closely related chemical analog, GNF179, to search for potential *Plasmodium* targets. Ranking potential targets derived from chemical genetics and proteomic affinity chromatography methodologies identifies *SEY1*, or Synthetic Enhancement of YOP1, which is predicted to encode an essential dynamin-like GTPase implicated in homotypic fusion of endoplasmic reticulum (ER) membranes. We demonstrate that GNF179 decreases *Plasmodium* SEY1 melting temperature. We further show that GNF179 binds to recombinant *Plasmodium* SEY1 and subsequently inhibits its GTPase activity, which is required for maintaining ER architecture. Using ultrastructure expansion microscopy, we find GNF179 treatment changes parasite ER and Golgi morphology. We also confirm that *SEY1* is an essential gene in *P. falciparum*. These data suggest that *SEY1* may contribute to the mechanism of action of imidazolopiperazines and is a new and attractive druggable target.

Main Text

Introduction

Malaria, caused by the *Plasmodium* genus of organisms, remains the most common infectious parasitic disease worldwide. In 2022, there were 249 million cases, an annual increase of two million; of these cases, 608,000 were fatal (WHO, 2023). Despite public health efforts to monitor malaria transmission (Pattanshetty et al., 2024) and increase access to prevention and treatment (Mao et al., 2023), the disease remains a significant burden due to ineffective vaccines (Duffy, 2022) and the ever-increasing insecticide and antimalarial resistance, including the gold standard artemisinin-based combination therapies (Siqueira-Neto et al., 2023). Resistance has been reported against nearly all antimalarial classes – endoperoxides (Dondorp et al., 2009), quinolines (Sidhu et al., 2002), antifolates, naphthoquinones (Antony & Parija, 2016). Thus, there is a dire need for medicines possessing novel modes of action (MOA) to combat the antimalarial resistance arms race. If malaria were ever to be eradicated, antimalarials that inhibit growth at all stages of the complex parasite life cycle is ideal (Luo et al., 2023).

Imidazolopiperazines (IZPs), discovered through target-agnostic phenotypic screens conducted by the Genomics Institute of the Novartis Research Foundation in 2007 (Meister et al., 2011), are a new generation of antimalarial compounds that possess a novel mode of action. The potency against *Plasmodium* and the physiochemical and pharmacokinetic properties of the initial screening hit was improved through medicinal chemistry efforts, leading to the development of ganaplacide, or KAF156 (Wu et al., 2011). This class is the first next-generation compound series to be highly active against multiple stages of the parasite life cycle – asexual blood stage, liver stage (Meister et al., 2011), sexual blood stage, and parasite transmission (Kuhlen et al., 2014; Ouologuem et al., 2022) – while maintaining low toxicity against human host cells. The series is

even active against artemisinin-resistant parasites (Dembale et al., 2018; Yipsirimetee et al., 2022). Human clinical trials support its properties of quick absorption, long half-life, and minimal adverse effects (Leong et al., 2014), and its ability to clear acute *P. falciparum* and *P. vivax* malaria infections (White et al., 2016). IZPs also display prophylactic activity in controlled human malaria infection studies (Kublin et al., 2021). KAF156 has been shown to work effectively in combination with piperazine (Leong et al., 2018) and lumefantrine (Ogutu et al., 2023), and it is currently in the phase III patient exploration stage alongside the latter (Siqueira-Neto et al., 2023).

Despite the detailed clinical studies, IZP mode of action remains poorly characterized. Profiling of 113 metabolites in *P. falciparum* treated with KAF156 did not present clear metabolomic perturbations (Allman et al., 2016). *In-vitro* evolution and whole genome analysis (IVIEWGA) of individual *P. falciparum* clones evolved to confer resistance to closely related chemical analog GNF179 has consistently yielded single nucleotide variants (SNVs) in three genes: *PfCARL* (PF3D7_0321900), cyclic amine resistance transporter (LaMonte et al., 2016); *PfUGT* (PF3D7_1113300), UDP-galactose transporter; *PfACT* (PF3D7_1036800), acetyl-CoA transporter (Lim et al., 2016). *PfCARL* and *PfUGT* are predicted to be essential in *Plasmodium* through a lack of gene disruptions by *piggyBac* transposon insertions (Zhang et al., 2018). Although mutations in *PfUGT* are rare, 13 unique mutations in *PfCARL* have been identified, many of which were further validated via CRISPR/Cas9 genetic engineering (LaMonte et al., 2016). *PfCARL* yeast ortholog, *Saccharomyces cerevisiae* EMP65, protects folding polypeptides from promiscuous degradation within the endoplasmic reticulum (ER) lumen (Zhang et al., 2017), while human ortholog *HsTAPT* is implicated in Golgi morphology and trafficking (Symoens et al., 2015). *PfUGT* encodes a solute carrier 35 (SLC35) member that assists in sugar import to the ER (Ishida & Kawakita, 2004). *PfACT* imports acetyl-CoA to the ER for use in lysine acetylation of

some newly synthesized proteins (Nyonda et al., 2022). Unfortunately, mutations in these genes also confer resistance to unrelated compound classes, suggesting that they encode multidrug resistance transporters rather than IZP targets (Imlay et al., 2023; Lim et al., 2016; Magistrado et al., 2016). These results predict IZPs to inhibit protein trafficking, protein transport, and establishment of new permeation pathways (LaMonte et al., 2020).

Further IVIEWGA experimentation with GNF179 on the *S. cerevisiae* model organism yielded adaptive mutations in genes also functioning within the ER, specifically implicated in ER-based lipid homeostasis and autophagy: *ScELO2* contributes to sphingolipid biosynthesis; *ScSUR2* and *ScLCB4* are involved in sphingolipid metabolism; *ScATG15* is induced in proteasome-independent ER expansion caused by misfolded protein aggregation; *ScSEC66* is part of the *ScSEC63* complex involved in protein targeting and import into the ER. However, *ScSUR2* and *ScELO2* also appear to be multidrug resistance genes in yeast, and some of these gene orthologs are nonessential in *Plasmodium* (LaMonte et al., 2020). Thus, the search continues.

Here we identify and validate *SEY1* as a possible biological target of IZPs. *SEY1*, or Synthetic Enhancer of YOP1, is predicted to be an essential dynamin-like GTPase implicated in homotypic fusion of ER membranes. It is structurally conserved, including within the druggable GTPase domain. We detect elevated levels of *PfSEY1* in pulldown experiments with GNF179 and elevated levels of GNF179 on *PvSEY1*-coated sensor chips in surface plasmon resonance experiments. GNF179 also reduces *PvSEY1* melting temperature, adding further evidence of interaction. GNF179 inhibits *PvSEY1* GTPase activity, supporting molecular docking predictions of binding to conserved GTPase domain motifs. These results may explain our observed parasite ER morphology changes upon GNF179 treatment. We also demonstrate that *Plasmodium SEY1*

overexpression confers resistance to GNF179, while *Plasmodium SEY1* knockdown confers sensitivity to the compound. Finally, we confirm that *PfSEY1* is an essential parasite gene.

Results

PfSEY1 is pulled down abundantly by GNF179-linked beads

To further investigate the MOA of IZPs and potentially identify a target, we first utilized proteomic affinity chromatography derived from the Cellzome platform (Bantscheff et al., 2007). Beads were linked to the amino group in GNF179, and to the 5-position of benzodiazol in MMV007564, a negative control compound that confers resistance to *PfCARL* mutations (Magistrado et al., 2016), generating Compound X. Both GNF179 and Compound X maintained their inhibitory activity with IC₅₀ measures of 0.006μM and 3.42μM, respectively (Figure 2.1a). These compounds were tested in a series of 12 experiments, comprising biological duplicates of six different conditions. Low, membrane-preserving 0.02% NP40 protein extractions were mixed with 1mM GNF179 or Compound X, while standard 0.8% NP40 protein extractions were mixed with three different on-bead concentrations of GNF179 (1mM, 0.2mM and 0.1mM); mixed human cell (HEK293/K-562/Placenta/HepG2) extractions were also tested against 1mM GNF179 to rule out potential off-targets. For each experiment, tandem mass spectrometry (MS/MS) data were collected for 10 sample conditions (Table 2.1).

Amongst the proteins detected (carolino_affinity_chromatography), *HsFLOT1* and *HsFLOT2*, ubiquitous human structural proteins involved in lipid raft and vesicle formation (Babuke et al., 2009), were most affected by GNF179. As a representative example, 0.2mM GNF179 on-bead conditions displayed IC₅₀ averages of 0.56μM and 0.62μM for *HsFLOT1* and *HsFLOT2*, respectively (Figure 2.1b). We also measured apparent dissociation constants (K_{dapp})

by considering protein depletion by the beads. Depletion factors are a function of the affinity of inhibitors to captured proteins and the concentration of tagged ligands. *HsFLOT1* and *HsFLOT2* were able to compete GNF179 off the beads, displaying K_{dapp} averages of 0.34 μ M and 0.39 μ M for *HsFLOT1* and *HsFLOT2*, respectively (Figure 2.1b). *HsFLOT1* and *HsFLOT2* *Plasmodium* orthologs, *PfRH2a* and *PfSTOML*, respectively, were also identified, with the latter having generated IC_{50} and K_{dapp} averages of 4.35 μ M and 3.17 μ M, respectively (Figure 2.1b). Both proteins lack clear small molecule binding pockets and are predicted to be nonessential in *P. falciparum* (Zhang et al., 2018). Although *HsFLOT1* and *HsFLOT2* cannot be ruled out, they are unlikely candidates for an inhibitor with high parasite cell selectivity and low human cell toxicity.

Given that no compelling candidates were identified (*i.e.*, essential parasite proteins with low K_{dapp}), we reanalyzed this data alongside those derived from bioinformatic analyses of genes mutated during *in-vitro* evolution of *S. cerevisiae* and *P. falciparum*, ranking targets based on evidence of interaction with known determinants of resistance, druggability, predictability of essentiality in *P. falciparum* (Table 2.2), and localization. Many targets have evidence of ATP- and GTP-binding domains. Regarding the major *P. falciparum* resistance mechanisms of KAF156 and GNF179, *PfCARL* was not identified in the proteomic pulldowns, but spectra for *PfACT* and *PfUGT* were detected (carolino_affinity_chromatography).

PfSEY1 (PF3D7_1416100) was one of the highest ranked proteins, being druggable, pulled down abundantly, and parasite-specific; it is also predicted to be essential (Zhang et al., 2018). SEY1, or Synthetic Enhancer of YOP1, has been identified in *S. cerevisiae* as a dynamin-like GTPase, playing a role in homotypic ER membrane fusion (Figure 2.2a): two ScSEY1 proteins in different ER membranes bind GTP, initiating dimerization and subsequent membrane tethering upon GTP hydrolysis; GDP then releases, the proteins dissociate, and the process repeats (Yan et

al., 2015). In *Fusarium graminearum* and *Candida albicans*, *SEY1* deletion reduces fungal virulence (Chong et al., 2020; Yan et al., 2015). Importantly, *SEY1* is structurally conserved (Figure 2.2b), with the high-quality crystal structure (minus transmembrane domains) of *C. albicans* *SEY1* (Yan et al., 2015) displaying mirror symmetry with the *PfSEY1* model (Figure 2.2c). There is also a druggable catalytic region, the GTPase domain, which comprises conserved motifs – the P-loop, Walker A, Walker B, and guanosine-binding sites (Hu et al., 2009).

In contrast to other *P. falciparum* proteins that appeared in almost all conditions (e.g., HSP90, eIF4A, elongation factor 1-alpha), *PfSEY1* was specific to GNF179-linked beads, as more of this protein was pulled down by the GNF179-linked beads (Figure 2.1c) in the low detergent parasite extraction relative to the Compound X-linked beads (Figure 2.1d). This pulldown of *PfSEY1* by GNF179 is further corroborated with tests on the standard detergent parasite extraction (Figure 2.1e). *PfSEY1* also displayed a dose dependency with GNF179, as less protein was bound at lower on-bead concentrations; this contrasts with proteins that were uniformly detected in all GNF179 on-bead concentrations, such as *PfHSP90* (Figure 2.1f). Moreover, *SEY1* human orthologs within the atlastin family ranked lower (carolino_affinity_chromatography), suggesting parasite specificity. Unfortunately, *PfSEY1* showed little evidence of being competed off GNF179-linked beads, with its relative abundance remaining consistent across all sample conditions (carolino_affinity_chromatography).

ScSEY1 mutations confer resistance to GNF179 in yeast

SEY1 attracted our attention because previous *S. cerevisiae* selections with GNF179 identified a *ScSEY1*(S437*) nonsense mutation (Figure 2.2d), albeit the mutation was found amongst additional mutations (LaMonte et al., 2020). To confirm the impact of this SNV, we

utilized homologous recombination to replace native *ScSEY1* with myc-tagged full-length or truncated *ScSEY1* in the haploid Green Monster, or GM, that was used in the original selections (Figure 2.3a). This attenuated strain has its 16 ATP-binding cassette (ABC) multidrug transporter genes replaced with modified *Aequorea victoria GFP* (Suzuki et al., 2011), thus requiring lower dosage for growth inhibition. Successful integration was confirmed by Western blot, displaying the expected 92kDa and 52kDa signals for GM+*ScSEY1-myc* and GM+*ScSEY1(S437*)-myc*, respectively (Figure 2.3b). Dose-response testing in which wild-type GM and the GM+*ScSEY1-myc* mutant cells were grown in YPD media with increasing GNF179 concentrations revealed similar IC₅₀ measures of 32.8µM +/- 3.0µM and 34.1µM +/- 3.3µM, respectively, indicating that the fusion tag and selection marker had no confounding effects. Contrarily, for the GM+*ScSEY1(S437*)-myc* mutant, we observed a 1.4-fold increase in IC₅₀ to 47.8µM +/- 2.6µM (Figure 2.3c). As a control, all strains were treated with fluconazole and miconazole, antifungals not expected to involve *SEY1*, and we observed similar IC₅₀ measures between the strains (Figure 2.3d).

Additional SEY1 copies also confer resistance to GNF179

Copy number variations (CNVs) have also been associated with adaptation and drug resistance in parasites (Qidwai, 2020). Thus, we tested whether additional copies of *SEY1* affects GNF179 IC₅₀ using the yeast model. To do so, we engineered BY4741, the parental strain of GM, that was already expressing the *ScSEY1-TAP* construct to also express an additional *ScSEY1-myc* construct (Figure 2.3a); this mutant was subsequently confirmed via Western blot, displaying the expected 110kDa and 92kDa signals for TAP- and myc-tagged *ScSEY1*, respectively (Figure 2.3b). The mutant with two copies of the *ScSEY1* gene displayed a 1.5-fold increase in GNF179

IC₅₀ to 240µM +/- 10µM, indicating a resistance phenotype (Figure 2.3e). In contrast, wild-type BY4741 and the BY4741+*ScSEY1-TAP* mutant treated with GNF179 displayed similar IC₅₀ measurements of 163µM +/- 8µM and 164µM +/- 6µM, respectively, again indicating the absence of any fusion tag cassette effects. The IC₅₀ measures are elevated here as the strains are expressing all ABC transporters. As expected, no strains showed increased resistance to the fluconazole and miconazole controls (Figure 2.3f).

We also determined if the *Plasmodium SEY1* ortholog were sufficient to confer resistance against IZPs. Myc-tagged *P. vivax SEY1* under the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter was transformed into wild-type *Komagataella phaffii* GS115, a yeast strain frequently used for recombinant protein expression (Figure 2.3a); *PvSEY1* was used as it contains fewer low-complexity regions relative to *P. falciparum*. Integration was subsequently confirmed via Western blot, showing the expected 105kDa signal (Figure 2.3b). Additionally, whole genome sequencing of these two lines confirmed the integration and showed no additional genomic changes outside of the added *PvSEY1-myc* construct (carolino_genome_analysis). Compared to the parent, addition of *PvSEY1* yielded a 3.0-fold increase in GNF179 IC₅₀ to 119µM +/- 8µM, from 38.9µM +/- 2.3µM (Figure 2.3g). No differences in IC₅₀ measures were observed between the strains when treating with fluconazole or miconazole (Figure 2.3h). As a control, we also transformed myc-tagged *S. cerevisiae SEY1* under the *GAPDH* promoter into the GS115 strain. Of the eight mutant colonies tested via Western blot probing with anti-myc antibody, two of them displayed the expected 93kDa signal (Figure 2.4a). Conducting the same ten-point dose response testing with GNF179 on the verified mutants generated an average IC₅₀ measure of 115.7µM, a measure more resembling that of the GS115+ *PvSEY1-myc* mutant than the parent (Figure 2.4b).

These results from two species of yeast and two orthologs of *SEY1* confirm that SEY1 mutations confer resistance to IZPs, but not to antifungals.

GNF179 destabilizes SEY1 in protein lysates

Though targets of antimalarials are expected to be essential, the presence of a nonsense mutant, as opposed to a missense one, remained perplexing. We thus sought to further assess the GNF179-SEY1 interaction through cellular thermal shift assay (CETSA). This approach can be used against a specific candidate for target confirmation, relying on the principle that proteins bound to a compound may be protected from denaturation after temperature challenge (Dziekan et al., 2019). We first utilized this method to determine if ScSEY1 interacts with GNF179. Since ScSEY1 is a membrane protein, it needs to first be solubilized. By adding 1% digitonin detergent to the lysis step of protein extraction, the protein was detected in the supernatant rather than the pellet (Figure 2.4c). To gauge the protein stability of ScSEY1, aliquots of the protein lysate were treated with DMSO and exposed to a range of temperatures, from 30°C to 60°C. The subsequent samples were cleared of protein aggregates caused by thermal challenge and analyzed via Western blot, probing with anti-myc antibody. The experiment indicated protein denaturation occurring at 60°C (Figure 2.4d). For the thermal shift assay, the protein was challenged to a range of temperatures in the presence of GNF179 or tavaborole antifungal at a 100:1 compound-to-protein ratio. ScSEY1-myc denaturation profiles for DMSO and tavaborole were similar after normalization against ScATP2, a common loading control protein in yeast (Snyder et al., 1999), and one not expected to interact with GNF179. Unexpectedly, for GNF179, a statistically significant denaturing protein profile was observed: at 55°C, 40.0% and 37.5% of the protein remained in solution for DMSO and tavaborole, respectively, but only 15.5% of the protein for

GNF179. This significant decline was also seen at 60°C, in which 18.4%, 17.9%, and 0.5% of the protein remained in solution for DMSO, tavaborole, and GNF179, respectively (Figure 2.4e).

Protein lysate containing *PvSEY1-myc* was also used in thermal shift assay. To first solubilize the membrane protein, protein lysates were treated with digitonin or Triton X-100 detergents, at 1% or 1.5% concentrations. The addition of either detergent at concentrations greater than 1% resulted in solubilization of the protein into the supernatant (Figure 2.5). Aliquots of the protein lysate were then challenged to a range of temperatures in the presence of GNF179 or artemisinin control at a 100:1 compound-to-protein ratio, cleared of protein aggregates, and analyzed via Western blot, probing with anti-myc antibody. *PvSEY1-myc* denaturation profiles for DMSO and artemisinin were again similar after normalization against *KpATP2*. But, for GNF179, an even more severely denatured protein profile was observed: at 45°C, 89.2% and 90.4% of the protein remained in solution for DMSO and artemisinin, respectively, but only 50.7% of the protein for GNF179. Similar decline was also seen at 50°C (80.2%, 80.6%, and 40.6% for DMSO, artemisinin, and GNF179, respectively), 55°C (41.6%, 40.5%, and 11.8%), and 60°C (23.7%, 23.8%, and 2.0%) challenges (Figure 2.6a). Interestingly, the denaturation profile for *PvSEY1-myc* against GNF179 was more drastic relative to that for *ScSEY1-myc* against GNF179 (Figure 2.4e). Perhaps there is a species difference in which GNF179 more affects the *Plasmodium* ortholog. However, no difference in GNF179 IC₅₀ was observed between the GS115+*PvSEY1-myc* and GS115+*ScSEY1-myc* mutants (Figure 2.4b).

Although the premise of CETSA is that proteins are stabilized by compound binding, there are exceptions. A previous study showed that human multidrug-resistance transporter 1 (*HsMDR1*) was destabilized by elacridar by blocking ATP binding that normally stabilizes the transporter. Thus, in some situations, destabilization may be viewed as evidence of drug-target interaction

(Reinhard et al., 2015). It is possible that GNF179 prevents SEY1 homodimerization, resulting in a lower melting temperature.

KAF156 is predicted to dock onto conserved *PvSEY1* GTPase motifs

Since GNF179 destabilization of *PvSEY1* was observed, we hypothesize that GNF179 may block GTP binding that stabilizes *PvSEY1*, akin to elacridar blocking of ATP binding that stabilizes *HsMDR1* (Reinhard et al., 2015). We sought to address this by identifying potential binding sites through molecular docking, a technique that predicts binding between ligands and receptor residues. Without published crystal structures for *PvSEY1*, we utilized ColabFold (Mirdita et al., 2022) to predict its structure, using truncated *CaSEY1* (RCSB: 5CA9) as a template. Using MGLTools (Eberhardt et al., 2021; Trott & Olson, 2010), KAF156 and GTP were fit into the GTPase domain of the monomeric *PvSEY1* homology model. Hydrogen bond interactions were predicted between GTP and *PvSEY1* Ser48, Thr72, Arg184, and Asp185 residues, conserved motifs of the GTPase domain (Yan et al., 2015). Hydrogen bond interactions were also predicted between KAF156 and the *PvSEY1* Thr72 residue that was observed with GTP. These results suggest that KAF156 docks onto the Walker A site of the GTPase domain. Additionally, the binding energies for GTP and KAF156 onto *PvSEY1* are -8.09 and -7.10 kcal/mol, respectively, further supporting the interaction (Figure 2.6b).

We also constructed a theoretical protein structure for *ScSEY1*, again using truncated *CaSEY1* (RCSB: 5CA9) as a template. KAF156 and GTP were then fit into the GTPase domain of the monomeric *ScSEY1* homology model. Hydrogen bond interactions were predicted between GTP and *ScSEY1* Lys50 and Asp181 residues, conserved motifs of the GTPase domain (Yan et al., 2015). Hydrogen bond interactions were also predicted between KAF156 and the *ScSEY1*

Asp181 residue that was observed with GTP. These results suggest that KAF156 docks onto the GTP-binding site of the GTPase domain. Furthermore, the binding energies for GTP and KAF156 of -7.45 and -7.68 kcal/mol, respectively, supports the interaction (Figure 2.4f). To fully determine if PvSEY1 homodimerization is affected, analytical ultracentrifugation, which sediments proteins based on size, should be conducted (Edwards et al., 2020; Schuck et al., 2023; Yan et al., 2015).

Recombinant *Pk*SEY1 interacts weakly with GNF179

We further explore *Plasmodium* SEY1 interaction with IZPs through direct binding affinity experiments. Surface plasmon resonance (SPR) is a tool that elucidates physical protein-ligand interactions, with the benefit of measuring an equilibrium dissociation constant, K_D , as observed for the inhibition of *Pf*HSP70 and *Pf*HOP by 2-phenylthynesulfonamide (Muthelo et al., 2022). Of note, we used recombinant *Plasmodium knowlesi* SEY1, as ganaplacide was observed to be even more potent against this species (van Schalkwyk et al., 2021). His-tagged *Pk*SEY1 was immobilized onto an NTA-coated sensor chip, and then GNF179 was flowed over the sensor chip at varying concentrations; plane-polarized light was then directed towards the metal surface of the chips and the resonant angle measured, as refractive indexes change with molecular interactions (Figure 2.7). As GNF179 concentration increased, there was a greater spike in response differences. A K_D of 144 μ M was measured for *Pk*SEY1-His (Figure 2.6c), suggestive of weak binding affinity.

GNF179 inhibits PvSEY1 GTPase activity in protein lysates

If IZPs were to bind to the GTPase domain, then there should be a decrease in GTPase activity. To test this, we developed a GTPase activity assay for *Plasmodium* SEY1 based on an

existing assay measuring free phosphate production by *ScSEY1* (Anwar et al., 2012). Protein lysate containing *PvSEY1*-myc-His was extracted from *Escherichia coli* and passed through a Ni-NTA column; the protein of interest is only detected by anti-myc antibody upon IPTG induction (Figure 2.8a). Each reaction comprising 1 μ M GTPase-containing eluate, 125 μ M artemisinin or GNF179, and 0 μ M to 250 μ M GTP was incubated for 30 minutes before measuring absorbance at 360nm wavelength. In the absence of GTP, no free phosphates were produced, as expected. Also, free phosphate levels between the DMSO and artemisinin conditions remained similar at all concentrations of GTP: 15.2 μ M $\pm$ 3.19 μ M and 15.6 μ M $\pm$ 4.1 μ M, respectively, at 125 μ M GTP, and 29.8 μ M $\pm$ 3.5 μ M and 29.9 μ M $\pm$ 2.7 μ M, respectively, at 250 μ M GTP. However, for the GNF179 condition, free production was hindered: 6.02 μ M $\pm$ 1.49 μ M at 125 μ M GTP and 12.2 μ M $\pm$ 3.5 μ M at 250 μ M GTP (Figure 2.6d). As a control, *PvSEY1* with a Walker A (switch1) mutation that inhibits GTPase activity (Yan et al., 2015) was expressed and tested (Figure 2.8a). Mutated *PvSEY1*(T7A) hydrolyzed significantly less GTP relative to *PvSEY1* – 3.79 μ M $\pm$ 3.11 μ M vs. 14.8 μ M $\pm$ 4.3 μ M with 250 μ M GTP and 5.63 μ M $\pm$ 2.18 μ M vs. 27.1 μ M $\pm$ 1.4 μ M with 500 μ M GTP – indicating enzymatically active *PvSEY1* in lysate (Figure 2.8b). This also supports possible effects by the predicted IZP docking onto this residue (Figure 2.6b). *E. coli* biotin ligase, which was the initial gene in the vector, was also tested for GTPase activity. Minimal background signal was observed through the 500 μ M GTP condition (Figure 2.8c). These results indicate a reduction of *PvSEY1* GTPase activity by GNF179.

GNF179 induces *P. falciparum* ER and Golgi morphology defects

Previous studies have shown that mutations in *ScSEY1* cause defects in ER formation and, to an extent, Golgi morphology (Okamoto et al., 2012). If GNF179 were to affect SEY1, then there

could be a resultant disturbance to ER morphology, and possibly to the associating Golgi. We examined the phenotype of GNF179-treated *P. falciparum* using ultrastructural expansion microscopy, a method established for isotopically increasing the size of cell structures while maintaining the integrity of the proteome (Gambarotto et al., 2019; Liffner et al., 2023). Asexual blood stage parasites were synchronized and treated two to four hours post-invasion with 25nM GNF179 for 16 hours, and then subsequently analyzed using this high-resolution method with antibodies to both the ER (*PfBIP*) and the Golgi (*PfERD2*). Several clear phenotypic changes were evident. In DMSO, the ER expanded to encompass the nucleus, whereas in GNF179, the ER signal around the nucleus was reduced: the mean ER area for DMSO and GNF179 were $33.4\mu\text{m}^2 \pm 10.9\mu\text{m}^2$ and $22.0\mu\text{m}^2 \pm 6.9\mu\text{m}^2$, respectively (Figure 2.9a). This contrasts the ER expansion observed in previous GNF179-treated *P. falciparum* cells (LaMonte et al., 2020). Perhaps nucleus area also diminished upon GNF179 treatment, but this was not the case (Figure 2.9a). Upon GNF179 treatment, the Golgi was also affected, appearing detached from the nucleus: the mean Golgi distance from the nucleus for DMSO and GNF179 were $0.466\mu\text{m} \pm 0.591\mu\text{m}$ and $1.46\mu\text{m} \pm 0.96\mu\text{m}$, respectively (Figure 2.9b). These observations were statistically significant, confirming a morphology defect in the parasite ER and Golgi in response to GNF179 treatment.

Conditional knockdown of *PfSEY1* confirms that it is essential

Drug targets need to be essential, and *PfSEY1* has only been predicted to be an essential gene (Zhang et al., 2018). We sought to confirm its essentiality by performing conditional knockdown experiments with the tetracycline repressor protein-development of zygote inhibited-aptamer system (TetR-DOZI aptamers) to test parasite viability. In the absence of anhydrotetracycline (aTc), the TetR-DOZI chimeric protein binds to the ten aptamers on the

transcripts, resulting in their conversion to inactive messenger ribonucleoprotein particles (mRNPs); in the presence of aTc, the chimeric protein preferentially binds to this molecule, thus allowing for translation to proceed (Rajaram et al., 2020). The TetR-DOZI aptamers tag was transfected into the 3' UTR of *PfSEY1* using CRISPR/Cas9 (Figure 2.9c). Western blot probing with V5 antibody for the included smV5 tag (Viswanathan et al., 2015) confirmed the 155kDa signal when aTc is present (Figure 2.9d). Parasites were grown with (+aTc) and without (-aTc) aTc, and their parasitemia was measured daily. After 24 hours, parasite growth was already diminished in the -aTc condition; in contrast, in the +aTc condition, in which *PfSEY1* is still being translated, growth appeared normal (Figure 2.9e). This observation of decreasing viability is associated with diminishing *PvSEY1* levels (Figure 2.9d). Thus, *PfSEY1* is an essential gene.

As a final step, we determined the IC₅₀ against GNF179 for this *PfSEY1-smV5^{Tet}* knockdown mutant over a range of aTc concentrations. It was not possible to generate a reproducible dose response curve at low concentrations of aTc (e.g., 5nM aTc), as growth was already substantially suppressed prior to the added suppression observed over a range GNF179 concentrations relative to the vehicle control; using 25nM or 50nM aTc mitigated this growth attenuation (Figure 2.10), while still reducing *PfSEY1* levels (Figure 2.9d). Dose response experiments at 25nM and 50nM aTc generated an IC₅₀ measure of 1.96nM +/- 0.45nM and 1.66nM +/- 0.28nM, respectively; compared to the 500nM condition (2.72nM +/- 0.55nM), this indicates a small but reproducible shift increase in sensitivity to GNF179 (Figure 2.9f), consistent with SEY1 playing a role in the mechanism of action of GNF179.

Discussion

Here we present evidence that *Plasmodium SEY1* is a critical antimalarial drug target and may play a role in the mechanism of action of IZPs.

Although data from *S. cerevisiae* guided our analysis, GNF179 is over 1000-fold less potent against this species, relative to *P. falciparum* (LaMonte et al., 2020). In yeast, *ScSEY1* is a nonessential gene (Hu et al., 2009), as mutants devoid of *ScSEY1* do not show a growth defect nor defects in ER morphology; only when additional genes – *ScYOP1*, *ScRTN1* – are missing will aberrant ER morphology occur (Anwar et al., 2012). Furthermore, there are redundant pathways for ER membrane fusion: retrograde SNAREs can fuse ER membranes in the absence of *ScSEY1* (Rogers et al., 2013), or possibly when *ScSEY1* is sequestered by IZPs. Treatment of the GS115 strain of yeast with 400µM GNF179 overnight did not affect its ER morphology, as observed with the *KpSEC61-mCherry* ER marker (Figure 2.11). This could explain the higher GNF179 IC₅₀ measures with yeast (Figure 2.3c-h). The fact that a nonsense mutation that removes *ScSEY1* transmembrane domains confers resistance to IZPs suggests that presence of a GNF179/*ScSEY1* complex is more toxic than having no *ScSEY1* at all.

We had previously shown that parasites are more sensitive to GNF179 when protein export is altered. Additionally, GNF179 misfolding of proteins in the ER could trigger the ERAD(II) pathway of degradation, which depends on autophagy and lysosomal trafficking (LaMonte et al., 2020). In *Dictyostelium discoideum*, amoeba mutants devoid of *SEY1* demonstrate a constitutive activation of the unfolded protein response pathway (Husler et al., 2021). Furthermore, mutating *SEY1 Arabidopsis thaliana* ortholog Root Hair Defective 3 (*AtRHD3*) changes Golgi distribution and motility within the plant (Chen et al., 2011). *PfSEY1* has many characteristics of a predicted target of IZPs, and we provide additional data implicating protein trafficking in IZP function.

Since GNF179 resistance was observed with the *ScSEY1*(S437*) nonsense mutant, we cannot ignore possible direct binding sites of IZPs to the C-terminal tail of the protein. However, docking KAF156 onto the C-terminal region of *PvSEY1* did not yield any interactions (Figure 2.6b). The C-terminal tail may still be important to IZP binding. Mutating the phosphorylated residues within the C-terminal tail of *AtRHD3* aberrates tubule formation (Ueda et al., 2016). *SEY1* human ortholog atlastin-1 (*HsATL-1*) possesses an amphipathic helical domain at the C-terminus that assists in facilitating membrane fusion (Faust et al., 2015). IZPs could also misfold *SEY1* in a manner preventing its C-terminal TM domains from embedding into specific lipid membranes, which is required in *ScSEY1*-dependent ER membrane fusion (Sugiura & Mima, 2016).

Although our data indicate a genetic interaction between *SEY1* and GNF179, as well as protein-ligand binding, the measured K_D (Figure 2.6c) may not be sufficient to account for the low nanomolar inhibitory activity of IZPs, unless there is potentiation of activity in the cellular environment that cannot be reproduced *in-vitro*. For example, the ER membrane or specific lipids could be required to facilitate binding (Sugiura & Mima, 2016; Wang & Rapoport, 2019). There could also be additional regulation, such as C-terminal tail phosphorylation, that is needed to fully capture binding. To further elucidate this, experiments involving *Plasmodium* *SEY1*-GNF179 protein crystallography and *in-vivo* tracking must be conducted.

Like other drug targets, *PfSEY1* is predicted to be an essential gene in *P. falciparum* (Zhang et al., 2018). This was confirmed with our *PfSEY1-smV5^{Tet}* knockdown experiments, in which cell growth ceased within 24 hours of aTc-dependent translation inhibition (Figure 2.9e). Like other drug targets, it contains a known druggable pocket, the nucleoside triphosphate hydrolase domain. Previous studies have shown kinases and tRNA synthetases possessing this domain to be inhibited

by antimalarials (Arendse et al., 2021; Xie et al., 2023). IZPs could still target a yet to be discovered GTPase involved in protein trafficking. Even if *PfSEY1* were not the target of IZPs, it is still a compelling target for future drug development.

IZPs remain the leading antimalarial candidate, being farthest along the drug development pipeline with a novel mode of action. Currently, no SNPs conferring IZP resistance have been found in African samples (Foguim et al., 2019). Moreover, previous studies have shown that they inhibit many species of *Plasmodium* (Aniweh et al., 2023; Dembele et al., 2021; van Schalkwyk et al., 2021). Ultimately, investigating IZP effects on different SEY1 orthologs strengthens this new compound-protein interaction. Through target identification, resistance to IZPs can be better anticipated, toxicity can be further remedied, and synergistic drug research can be more guided.

Methods

Proteomic affinity chromatography

Setup is summarized in Table 2.1. GNF179- and Compound X-coated beads were mixed with *P. falciparum* protein lysates (extracted using low 0.02% or standard 0.8% detergent) and subsequently competed with free GNF179; mixed human cell extracts were also tested. Resulting ten samples were analyzed in parallel (TMT 10-plex) to generate values for the affinity of the beads to the bound proteins (“depletion” values, four samples) and to generate IC₅₀ values (six samples) in a single experiment. Sample 1 and 2 represent the vehicle control; sample 3 and 4 were done in the same way, but while the beads were discarded after the first incubation step the extract was incubated with fresh beads to measure how much protein could rebind to the fresh beads (was depleted from the extract by first bead-binding). Apparent dissociation constants were determined by considering the protein depletion by the beads. Sample 5-10 were used to generate IC₅₀ values

by adding GNF179 over a range of concentrations (maximum of 20 μ M, 1:3 dilutions). MS/MS was utilized for quantification. MS1 score analysis was done using Prism 10.

Strain construction

For *S. cerevisiae* transformation, 8 OD₆₀₀ early-log phase cells (0.8 OD₆₀₀ mL⁻¹) were washed twice with cell-grade water and then twice with lithium acetate buffer (100mM lithium acetate, 10mM Tris-HCl pH 7.5, 1mM EDTA). Approximately 100 μ g boiled salmon sperm DNA and 500ng PCR-generated linear DNA were added, followed by six-fold volume of 40% v/v polyethylene glycol in lithium acetate buffer. The sample was incubated at 30°C for 30 minutes, treated with 10% v/v DMSO, and subsequently heat-shocked at 42°C for 15 minutes. Cells were sedimented and resuspended in 1mL YPD (1% yeast extract, 2% peptone, 2% dextrose), allowing overnight recovery at 30°C before plating on YPD+Zeocin (50 μ g mL⁻¹) agar plates. SDS-PAGE and subsequent Western blot with mouse anti-myc (Invitrogen, R950-25, 1:1000) or rabbit anti-TAP (Invitrogen, CAB1001, 1:1000) antibodies detected the proteins of interest; rabbit anti-ScATP2 (gifted by the Subramani Lab at UCSD, 1:2000) was included as a loading control. HRP-linked goat anti-mouse (Bio-Rad, 1706516, 1:5000) and goat anti-rabbit (Bio-Rad, 1706515, 1:5000) antibodies were the secondary antibodies used for chemiluminescence. The mutant strains used in this study can be found in Table 2.3.

The three linear DNA constructs transformed into *S. cerevisiae* were created using oligos summarized in carolino_DNA_sequences. Myc-tagged full-length ScSEY1 was generated using oligos oKC1 and oKC2 on pPICZ-C plasmid (gifted by the Subramani Lab). Myc-tagged truncated ScSEY1(S437*) was generated using oligos oKC2 and oKC3 on the same plasmid. Oligos oKC4 and oKC5 were used on genomic DNA (gDNA) from the GM+ScSEY1-myc mutant to obtain the

additional *ScSEY1* gene construct that was transformed into BY4741+*ScSEY1-TAP*, which is from the Yeast-TAP Tagged ORF Library (Ghaemmaghami et al., 2003).

For *K. phaffii* (gifted by the Subramani Lab) transformation to express *P. vivax* or *S. cerevisiae SEY1*, 50 OD₆₀₀ early-log phase cells were resuspended in 5mL YPD containing 20mM HEPES pH 8.0 and 25mM 1,4-Dithiothreitol and then incubated at 30°C for 15 minutes (with orbital rotation). After, cells were washed three times with cold sterile, and then with cold 1M sorbitol; the cells were incubated in 500µL cold 1M sorbitol for one hour. Exactly 100µL of cells were mixed with 500ng linearized plasmid DNA. After 10 minutes on ice, the sample was placed in a 2mm cuvette and electroporated (50µF, 200Ω, 7500kV cm⁻¹). Cells were plated on SD+CSM-His (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% dextrose, 0.077% CSM-His) agar plates. Strains underwent quality assurance using SDS-PAGE and subsequent Western blot with mouse anti-myc and HRP-linked goat anti-mouse antibodies. For controls, rabbit anti-*ScATP2* and mouse anti-GAPDH (Invitrogen, 1:1000, MA5-15738) were used. The mutant strains used in this study can be found in Table 2.3.

The DNA plasmid containing *PvSEY1* transformed into *K. phaffii* was generated by amplifying myc-tagged codon-optimized *PvSEY1*, which was ordered from IDT, with oligos oKC6 and oKC7. The pIB2 plasmid (gifted by the Subramani Lab) was digested with *EcoRI* and *KpnI* restriction enzymes. Gibson assembly (NEB, E2611) was used to combine these two gene fragments. The complete plasmid was linearized with *StuI* restriction enzyme.

The DNA plasmid containing *ScSEY1-myc* used in *K. phaffii* transformation was generated by amplifying *ScSEY1* (from the BY4741 genome) with oligo oKC8 and oKC9 and vector (from pIB2+*PvSEY1-myc*) with oligo oKC10 and oKC11. The two gene fragments were combined via

Gibson assembly. The complete plasmid was linearized with *Stu*I. The oligos are summarized in *carolino_DNA_sequences*.

For transformation of Rosetta 2 DE3 (Novagen, 71400) and DH5-alpha strains of *E. coli* (NEB, C2987), 50µL of chemically competent cells were mixed with 10ng circular plasmid or 10% of a Gibson reaction. Cells were incubated on ice for 30 minutes before heat-shocking at 42°C for 30 seconds. After incubation on ice for five minutes, cells were added to 950µL SOC and incubated at 37°C for an hour (with shaking at 225rpm). Cells were plated on LB+Ampicillin (0.5% yeast extract, 1% tryptone, 1% NaCl, 100µg mL⁻¹ ampicillin) plates. Plasmids were extracted using miniprep kits (Promega, A1460) and sequenced by Plasmidsaurus for accuracy.

For construction of the *PfSEY1-smV5^{Tet}* line, 25µg homology-directed repair (HDR) plasmid was linearized by digestion, purified, and then co-transfected with 20µg guide RNA (gRNA) plasmid (containing *Streptococcus pyogenes Cas9*) into *P. falciparum* 3D7 parasites (synchronized as schizonts) using the Amaxa 4D system. Parasites were cultured with 500nM anhydrotetracycline (aTc) from the onset of transfection. One day post transfection, 2.5nM WR99210 (Jacobus Pharmaceuticals) drug pressure was applied.

HDR (pAM115) and gRNA plasmids (pAM116, pAM117, pAM118): 3'- and 5'-homology regions (HR) of *PfSEY1* were amplified from *P. falciparum* 3D7 gDNA using oligos oJDD6152/6153 and oJDD6146/6147, respectively. A codon-altered region for the last 88 amino acids was generated by IDT and amplified using oligos oJDD6148/6149. The smV5^{Tet}-Dozi-DHFR drug cassette region and pGEM backbone were amplified from pPG03 (Gurung et al., 2024) using oligos oJDD6150/6151 and oJDD6154/6155, respectively. Fragments were joined using the NEBridge Golden Gate method (with NEB *Bsa*I-HF v2) by New England Biolabs. To generate the three *PfSEY1* gRNA plasmids, oligos corresponding to each guide were annealed and ligated

into BpiI-digested pRR216 (Rudlaff et al., 2019), which encodes *SpCas9* and a U6 guide cassette. For pAM116, oligos oJDD6156/6157 were used. For pAM117, oligos oJDD6158/6159 were used. For pAM118, oligos oJDD6160/6161 were used (carolino_DNA_sequences).

Protein extraction

Protein lysate containing functional ScSEY1-myc was extracted from *S. cerevisiae* or *K. phaffii*, while protein lysate containing functional PvSEY1-myc was extracted from *K. phaffii*. Approximately 400 OD₆₀₀ early-log phase cells were washed twice with sterile water and incubated in 8mL zymolyase buffer (500mM KCl, 5mM MOPS-KOH pH 7.2, 10mM Na₂SO₃, 5000U Zymolyase-100T) for 30 minutes at 30°C (orbital rotation). Cells were then pelleted and resuspended in 4mL homogenization buffer (5mM MES, 1M sorbitol, 5mM NaF, 20mM EDTA, 1mM PMSF, 1x protease inhibitor cocktail) and lysed using a Dounce homogenizer. Cell debris was removed by centrifuging at 1,000g for 10 minutes. ScSEY1 was solubilized in 1% digitonin, while PvSEY1 protein was solubilized in 1% Triton X-100, for two hours at 4°C (with orbital rotation). Solubilized proteins were clarified by centrifugation at 100,000g for 20 minutes. Proteins were detected via Western blot using mouse anti-myc, rabbit anti-TAP, mouse anti-GAPDH, and rabbit anti-ScATP2 antibodies, along with the corresponding HRP-linked secondary antibodies.

Full-length functional PvSEY1-myc-His protein was extracted from *E. coli*. Approximately 1L of early-log phase cells were harvested after induction with 1mM IPTG at 16°C for 16 hours (with shaking at 160rpm). Cells were lysed in 20mL lysis buffer (1% lysozyme, 1% protease inhibitor cocktail, 1% Triton X-100, 1mM PMSF, 20mM imidazole, 2500U endonuclease in B-PER reagent) at 4°C for two hours (with orbital rotation). The cell lysate, cleared by chilled centrifugation at 20,000g for 20 minutes, was incubated with Ni-NTA beads at 4°C for 16 hours

(with orbital rotation) before passing them through a column. The beads were washed three times with wash buffer (500mM NaCl, 20mM Tris-HCl pH 7.9, 20mM imidazole). Then, proteins were eluted off the beads using two column volumes of elution buffer (500mM NaCl, 20mM Tris-HCl pH 7.9, 300mM imidazole). Protein samples were concentrated using Pierce protein concentrators (Thermo Scientific, 88502) and quantified via Qubit (Invitrogen, Q33211). Proteins were detected via Western blot, using mouse anti-myc and HRP-linked goat anti-mouse antibodies.

For quantifying *Pf*SEY1-smV5 abundance in knockdown experiments, whole cell lysates were extracted from *P. falciparum* cells that were harvested from red blood cells via saponin lysis. The parasites were boiled in Laemmli buffer containing beta-mercaptoethanol for five minutes; cell debris was removed via centrifugation at max speed for five minutes. Protein concentration was measured using Qubit before SDS-PAGE and Western blot with mouse anti-V5 (Invitrogen, R960, 1:1000), mouse anti-*Pf*HSP70 (Genscript, SC1320A 1:1000), and goat anti-mouse antibodies.

IC₅₀ determination

For proteomic affinity chromatography experiments, inhibition of intraerythrocytic *P. falciparum* growth was determined by a modified *in-vitro* [3H]-hypoxanthine incorporation method (Desjardins et al., 1979). Briefly, a culture of parasitized red blood cells (0.5% parasitemia with over 70% of total parasites in ring stage, 2.0% hematocrit) in RPMI-1640, 5% AlbuMAX, and 5μM hypoxanthine was exposed to drug serial dilutions. 96-well plates (Costar, #3894) were incubated for 24 hours at 37°C, 5% CO₂, 5% O₂, and 95% N₂. After, [3H]-hypoxanthine was added, and plates were incubated for an additional 24 hours. Then, plates were harvested on glass fiber filters (Wallac, #1450-421) using a cell harvester (Tomtec 96, PerkinElmer). Filters were

dried, and MeltiLex A melt-on scintillator sheets (PerkinElmer #1450-441) were used to determine [3H]-hypoxanthine incorporation. Radioactivity was measured using a MicroBeta counter (PerkinElmer). Data were normalized using the incorporation by the positive control (infected red blood cells without drug). IC₅₀ values were determined using Excel and Grafit 5. Human biological samples (placenta in pull-downs, red blood cells in IC₅₀ determination) were sourced ethically, and their use in the research was in accordance with terms from an IRB/REC-approved protocol.

For yeast experiments, a single colony was grown in YPD overnight at 30°C to early-log phase. Exactly 0.0005 OD₆₀₀ of cells were grown in 100µL YPD and exposed to a ten-point dose response of GNF179 or antifungals. After incubation at 30°C for 16 hours (with shaking at 500rpm), OD₆₀₀ was measured on a plate reader (BioTek Synergy). Percent growth was normalized against the vehicle-only condition (1% DMSO) and plotted against log₁₀ compound concentration using Prism 10, generating curves and IC₅₀ measures. Experiments were conducted in 96-well format, with at least two biological and technical replicates. Background strains tested include GM, a mutant *S. cerevisiae* strain devoid of 16 ABC multidrug transporter (Suzuki et al., 2011), *S. cerevisiae* BY4741, and *K. phaffii* GS115 (Table 2.3). GNF179, miconazole, and fluconazole were tested.

For experiments with *P. falciparum* *PfSEY1-smV5^{Tet}*, parasites were cultured in human O⁺ red blood cells at 2% hematocrit (BioIVT, Hicksville, NY) in prewarmed parasite growth media (RPMI-1640 with L-glutamine and 25mM HEPES, supplemented with 2g L⁻¹ NaHCO₃, 2.6g L⁻¹ AlbuMAX II, 13.6mg L⁻¹ hypoxanthine, and 50mg L⁻¹ gentamicin). Selection of the mutant was maintained with 2.5nM WR99210, and normal wild-type levels of *PfSEY1* were maintained with 500nM aTc. Parasitemia was assessed by examining thin blood smears fixed with methanol and subsequently stained with Giemsa under an upright microscope (Olympus CX33). Asynchronous

parasites in the presence of aTc and WR99210 were treated with a 12-point dose response of GNF179 (0.003-500nM) in 384-well V-bottom plate format. After 72 hours, growth media was removed and 2 μ M Syto-61 (Invitrogen, S11343) was added. Plates were read using a flow cytometer (BD FACSCanto) 20 minutes later. Data was visualized using FlowJo (BD Biosciences) and analyzed with Prism 10, generating curves and IC₅₀ values. Experiments were conducted in biological and technical replicates and normalized against the DMSO-only condition.

Whole genome sequencing

Yeast cells were grown overnight to early-log phase and gDNA was extracted using the DNeasy Blood and Tissue kit, following manufacturer's protocol (Qiagen, 69504). DNA was diluted to 300ng mL⁻¹ for DNA library preparation with Nextera XT (Illumina, FC-131-1024), barcoding with DNA/RNA UD Indexes Set A (Illumina, 20027213), and purification with AMPure XP beads (Beckman, A63880). Resulting purified tagmented DNA was pooled and submitted to the IGM Genomics Center at UCSD for Illumina MiSeq sequencing. Paired-end raw sequence reads were aligned to *K. phaffi* GCF_000027005.1 reference genome using BWA-MEM (Li & Durbin, 2009) and preprocessed following standard GATK version 3.5 protocols (McKenna et al., 2010; Van der Auwera et al., 2013). SNVs and indels were called with GATK HaplotypeCaller and filtered based on the following exclusion criteria: quality score (QUAL) <500, filtered depth (DP) <7.

Thermal shift assay

Solubilized protein lysate from 400 OD₆₀₀ early-log phase yeast cells was divided into three microcentrifuge tubes – one containing 20mM GNF179, another containing 20mM artemisinin or

tavaborole, and a third containing DMSO. Each tube was then further divided into six PCR tubes, one for each temperature condition (40°C-65°C). Each tube was heated for two minutes, and then chilled for two minutes. Centrifugation at 100,000g for 20 minutes in 4°C removed denatured protein aggregates. Analysis of the supernatants was done by SDS-PAGE and subsequent Western blot with mouse anti-myc and rabbit anti-ScATP2 antibodies. Intensities were quantified using ImageJ and analysis was done using Prism 10.

Molecular docking

To generate the theoretical *PvSEY1* or *ScSEY1* structures, protein sequences were processed in ColabFold v1.5.1 (Mirdita et al., 2022), using *CaSEY1* (RCSB: 5CA9) as a template. The highest scoring model was then analyzed using MGLTools (Eberhardt et al., 2021; Trott & Olson, 2010). The protein structure was stripped of water molecules, then given Kollman charges and polar hydrogen atoms. Molecular structure for GTP or KAF156 was then uploaded. Autogrid 4 was executed around the GTPase domain of both proteins, as well as the C-terminal tail of the predicted *PvSEY1* structure. Autodock 4 was then used to fit the ligand into these regions, generating a score for each position. PyMol was used to create protein models, noting hydrogen bonds between ligand and amino acid residues within five angstroms.

Surface plasmon resonance

Full-length *P. knowlesi* SEY1 (His-tagged) purchased from MyBioSource was processed on a Biacore 3000 by Profacgen. *PkSEY1*-His ligand was immobilized on NTA sensor chips (washed with 350mM EDTA and 500mM NiSO₄ prior) and washed with 0.05% surfactant P20 in 1x PBS to reach 1300 resonance units (RU). Various concentrations of the GNF179 analyte were

then injected using the following parameters: 120 second contact time, 0.5 second dissociation contact time, 30 $\mu\text{L min}^{-1}$ flow rate. To obtain the K_D , data were analyzed using BIAevaluation software.

GTPase activity assay

GTPase activity was measured by quantifying free phosphate production using the EnzChek Phosphate assay kit (Invitrogen, E6646). The 100 μL reactions comprise 50 μL reaction buffer (20% glycerol, 2mM EDTA, 4mM β -mercaptoethanol, 5mM MgCl_2 , 50mM HEPES, 200mM KCl), 20 μL MESG, and 1 μL PNP; the remaining 29 μL is composed of the following: GTP, compound or vehicle, 1 μM GTPase. A_{360} measurements were taken after 30 minutes on a plate reader (BioTek Synergy), kept at 37°C. Free phosphate generated was plotted against increasing GTP or compound concentration. Assays were done in 96-well format, with biological and technical replicates. Analysis was done using Prism 10.

Ultrastructural expansion microscopy

P. falciparum 3D7 parasites expressing Cas9 were tightly synchronized using a combination of density separation by 60% Percoll gradient and subsequent selective osmotic lysis after two hours of incubation to select for newly invaded parasites. Cultures were then treated with 25nM DMSO or GNF179 for 16 hours before samples at approximately 1% hematocrit were settled on poly-d-lysine-coated 12mm round coverslips for 30 minutes at 37°C, and then fixed by 4% PFA in PBS for 20 minutes at 37°C. The coverslips were washed three times in PBS before treating with 500 μL of 1.4% v/v formaldehyde and 2% v/v acrylamide in PBS. Samples were incubated overnight at 37°C. Gelation, denaturation, staining, and expansion of the gels were

performed as previously described (Liffner & Absalon, 2021). Gels were stained with rabbit anti-*Pf*BIP (Dvorin Lab at Harvard Medical School, 1:2000) for ER or rabbit anti-*Pf*ERD2 (Muralidharan Lab at the University of Georgia, 1:2000) for Golgi. Secondary antibody staining with goat anti-rabbit Alexa Fluor 488 (Thermo Scientific, A-11034, 8µg mL⁻¹), NHS Ester Alexa Fluor 405 (Thermo Scientific, A30000, 8 µg mL⁻¹), and SYTOX Deep Red (Thermo Scientific, S11380, 1µM) were used to stain proteins and DNA, respectively. Stained gels were imaged using a Zeiss LSM900 AxioObserver with an Airyscan 2 detector and a 63x Plan-Apochromat objective lens with numerical aperture of 1.4. Images were analyzed using Zen Blue 3.5 software (Zeiss, Oberkochen, Germany) using 3D Airyscan processing at moderate filter strength. Images shown are maximum intensity projections of between 10 and 30 z-slices of the entire image. Nucleolus and ER area was calculated by taking the area of the projection that is showing the largest surface area of the respective signal. Distance of Golgi to nucleus was calculated by measuring the 3D distance of the closest point of the Golgi signal to that of the nuclear signal. Analysis was done on Prism 10.

Parasite growth assay

A culture of *P. falciparum PfSEY1-smV5^{Tet}* grown to approximately 10% parasitemia, under 2.5nM WR99210 selection pressure and 500nM anhydrotetracycline (aTc) induction, was washed with prewarmed parasite growth media three times to remove aTc. Quantitative growth assays were performed in 96-well V-bottom plates using asynchronous parasites set up in triplicate and cultured with 5, 25, 50 or 500nM aTc. All cultures, which started at 1% parasitemia, were checked daily by blood smears.

Fluorescence microscopy

The GS115+*KpSEC61-mCherry* mutant of yeast is a gift from the Subramani Lab. A single colony is grown in YPD overnight at 30°C to early log phase concentrations. The culture is diluted with fresh YPD and then split into two conditions: DMSO (at 1%) and GNF179 (at 10x IC₅₀). The cultures were placed back into shakers for overnight treatment. The cells were placed on a microscope slide and checked for fluorescence of tagged proteins.

Figures and Tables

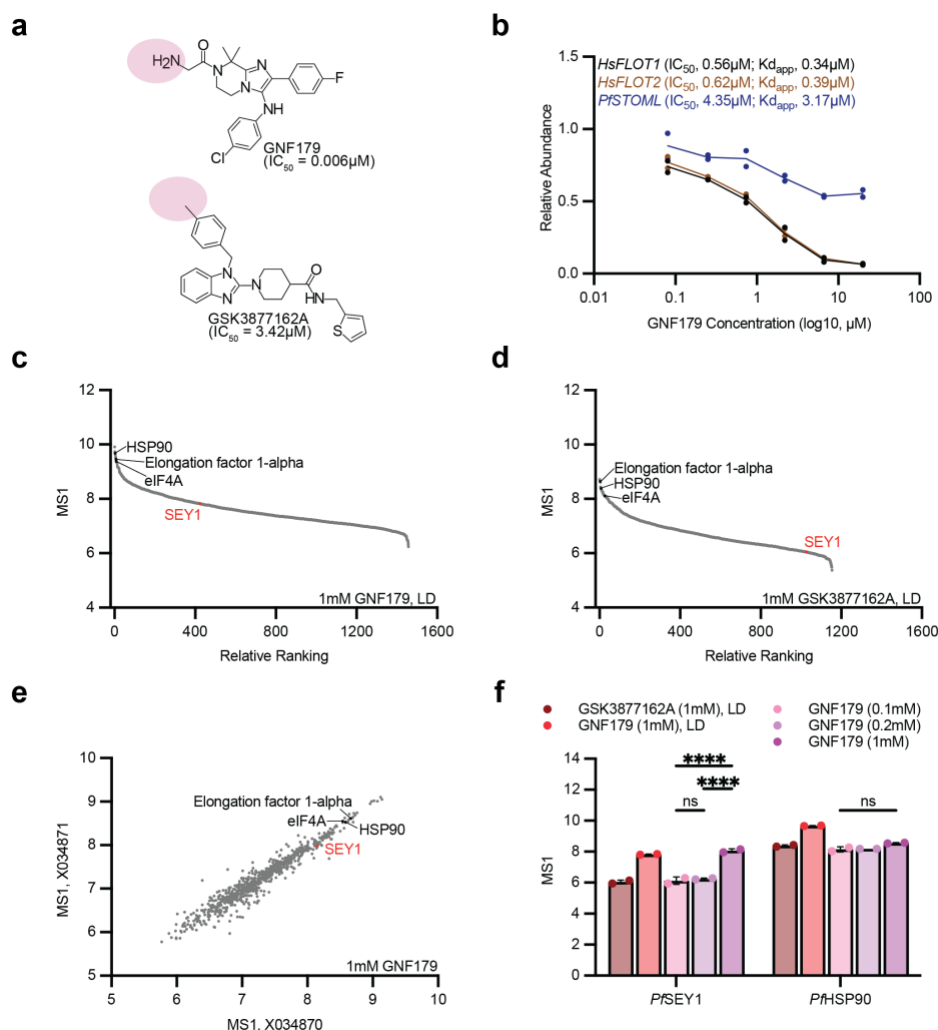


Figure 2.1 | *PfSEY1* is identified in proteomic affinity chromatography with GNF179

a. Compound structures of GNF179 and Compound X that were linked to beads for proteomic affinity chromatography; location of the linker is highlighted in pink. **b.** Six-point dose response (with GNF179) to quantify GNF179 IC_{50} for *HsFLOT1*, *HsFLOT2*, and *PfSTOML* in experiments using beads coated with 0.2mM GNF179 (X035929 and X035930). **c.** Relative ranking of proteins by decreasing average MS1 scores for beads coated with 1mM GNF179 mixed with low detergent (LD) parasite protein extract (X035927, X035928); only the 1458 proteins detected in both experimental replicates are shown. **d.** Relative ranking of proteins by decreasing average MS1 scores for beads coated with 1mM Compound X mixed with low detergent (LD) parasite protein extract (X035202, X035203); only the 1154 proteins detected in both experimental replicates are shown. **e.** Distribution of MS1 scores for proteins detected with beads coated with 1mM GNF179 mixed with standard detergent protein extract (X034870, X034871); only the 774 proteins detected in both experimental replicates are shown. **f.** Distribution of MS1 scores for *PfSEY1* and *PfHSP90* across different bead conditions as described in Supplemental Table 2.

Table 2.1 | Setup of Proteomic Affinity Chromatography

Each of the 12 experiments comprised 10 treatments: vehicle control (n=1), 0 μ M; vehicle control (n=2), 0 μ M; re-binding of non-bound fraction (NBF) to fresh beads (n=1), 0 μ M; rebinding of NBF to fresh beads (n=2), 0 μ M; dose response competition over six concentrations – 20.0 μ M, 6.67 μ M, 2.22 μ M, 0.74 μ M, 0.25 μ M, and 0.08 μ M.

<u>Experiment number</u>	<u>Compound immobilized on beads (on-bead concentration)</u>	<u>"Free" compound added to extract for competition</u>	<u>Material for protein extract preparation</u>
<u>X034870</u>	<u>GNF179 (1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (standard 0.8% NP40)</u>
<u>X034871</u>	<u>GNF179 (1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (standard 0.8% NP40)</u>
<u>X035202</u>	<u>Compound X (1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (low detergent 0.02% NP40)</u>
<u>X035203</u>	<u>Compound X (1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (low detergent 0.02% NP40)</u>
<u>X035857</u>	<u>GNF179 (1mM)</u>	<u>GNF179</u>	<u><i>Human: HEK293/K-562/Placenta/HepG2</i></u>
<u>X035858</u>	<u>GNF179 (1mM)</u>	<u>GNF179</u>	<u><i>Human: HEK293/K-562/Placenta/HepG2</i></u>
<u>X035927</u>	<u>GNF179 (1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (low detergent 0.02% NP40)</u>
<u>X035928</u>	<u>GNF179 (1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (low detergent 0.02% NP40)</u>
<u>X035929</u>	<u>GNF179 (0.2mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (standard 0.8% NP40)</u>
<u>X035930</u>	<u>GNF179 (0.2mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (standard 0.8% NP40)</u>
<u>X035931</u>	<u>GNF179 (0.1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (standard 0.8% NP40)</u>
<u>X035932</u>	<u>GNF179 (0.1mM)</u>	<u>GNF179</u>	<u><i>Plasmodium falciparum</i> (standard 0.8% NP40)</u>

Table 2.2 | Genes identified through genetic and proteomic experiments

Allelic changes occur in the species (*S. cerevisiae* or *P. falciparum*) used in the genetic screen; asterisks refer to nonsense mutations, and “fs” notations refer to frame shifts (insertion or deletion events). Target likelihood is based on predicted essentiality in *P. falciparum*, as well as presence of known ligand-binding domains.

Protein description	Genetic screen species	Gene name in <i>P. falciparum</i>	Allelic change	Potential as target
Heat shock protein 90	<i>S. cer.</i>	PF3D7_0708400	S17I	Likely
Karyopherin beta	<i>S. cer.</i>	PF3D7_0524000	G427R	No
Protein SEY1 homolog	<i>S. cer.</i>	PF3D7_1416100	S437*	Likely
Calcium-dependent protein kinase 1	<i>S. cer.</i>	PF3D7_0217500	K365T	Likely
Ras-related protein Rab-11A	<i>P. fal.</i>	PF3D7_1320600	D139Y	Likely
Uncharacterized protein	<i>P. fal.</i>	PF3D7_1366300	Y3425fs G3416fs	No
Uncharacterized protein	<i>P. fal.</i>	PF3D7_0410800	M360I	No
Bifunctional dihydrofolate reductase-thymidylate synthase	<i>S. cer.</i>	PF3D7_0417200	I279S	Likely
Gas41 homologue, putative	<i>P. fal.</i>	PF3D7_0807000	S24Y	Likely
Acetyl-CoA transporter, putative	<i>P. fal.</i>	PF3D7_1036800	E272fs M487fs S242* A94T R108K S110R L5fs I411N L253* C193* D165N G559R*	No
Uncharacterized protein	<i>P. fal.</i>	PF3D7_0727900	R789I	No
Uncharacterized protein	<i>P. fal.</i>	PF3D7_1136600	S1088N	No
UDP-galactose transporter, putative	<i>P. fal.</i>	PF3D7_1113300	F37V	No
Pfmc-2TM Maurer's cleft two transmembrane protein	<i>P. fal.</i>	PF3D7_0222100	M44I	No
Nucleolar GTP-binding protein 2	<i>S. cer.</i>	PF3D7_1423000	R468R	Likely

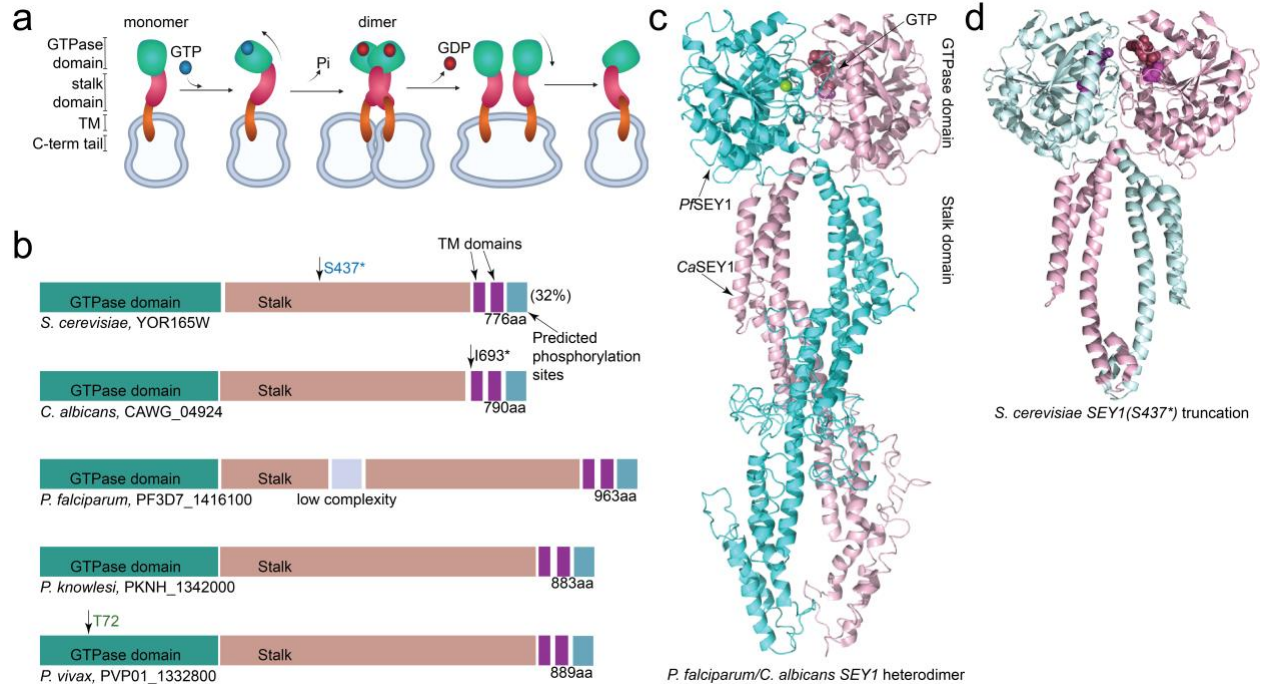


Figure 2.2 | SEY1 expresses a structurally conserved GTPase protein

a. Model recapitulating *S. cerevisiae* SEY1-mediated ER membrane fusion (Yan et al., 2015). The N-terminal GTPase domain is followed by the stalk domain, two transmembrane (TM) domains anchoring into ER membranes, and then the C-terminal tail. When guanosine triphosphate (GTP) is hydrolyzed, phosphate (Pi) is released, resulting in guanosine diphosphate (GDP). **b.** Domain structures for different SEY1 orthologs discussed in this paper. Arrows point to transmembrane (TM) domains, predicted phosphorylation sites within the C-terminal tail, truncation sites associated with GNF179 resistance (blue text), and MGLTools-predicted KAF156-interacting sites (green text); I693* refers to the nonsense mutation displayed in the *C. albicans* SEY1 crystal structure (PDB: 5CA9). **c.** Dimer structure between aforementioned truncated *C. albicans* SEY1 (CaSEY1) and *P. falciparum* SEY1 (PfSEY1) SWISS-derived homology model displays mirror symmetry. **d.** Predicted dimer structure of *S. cerevisiae* SEY1 that displays the S437* nonsense mutation conferring GNF179 resistance (LaMonte et al., 2020).

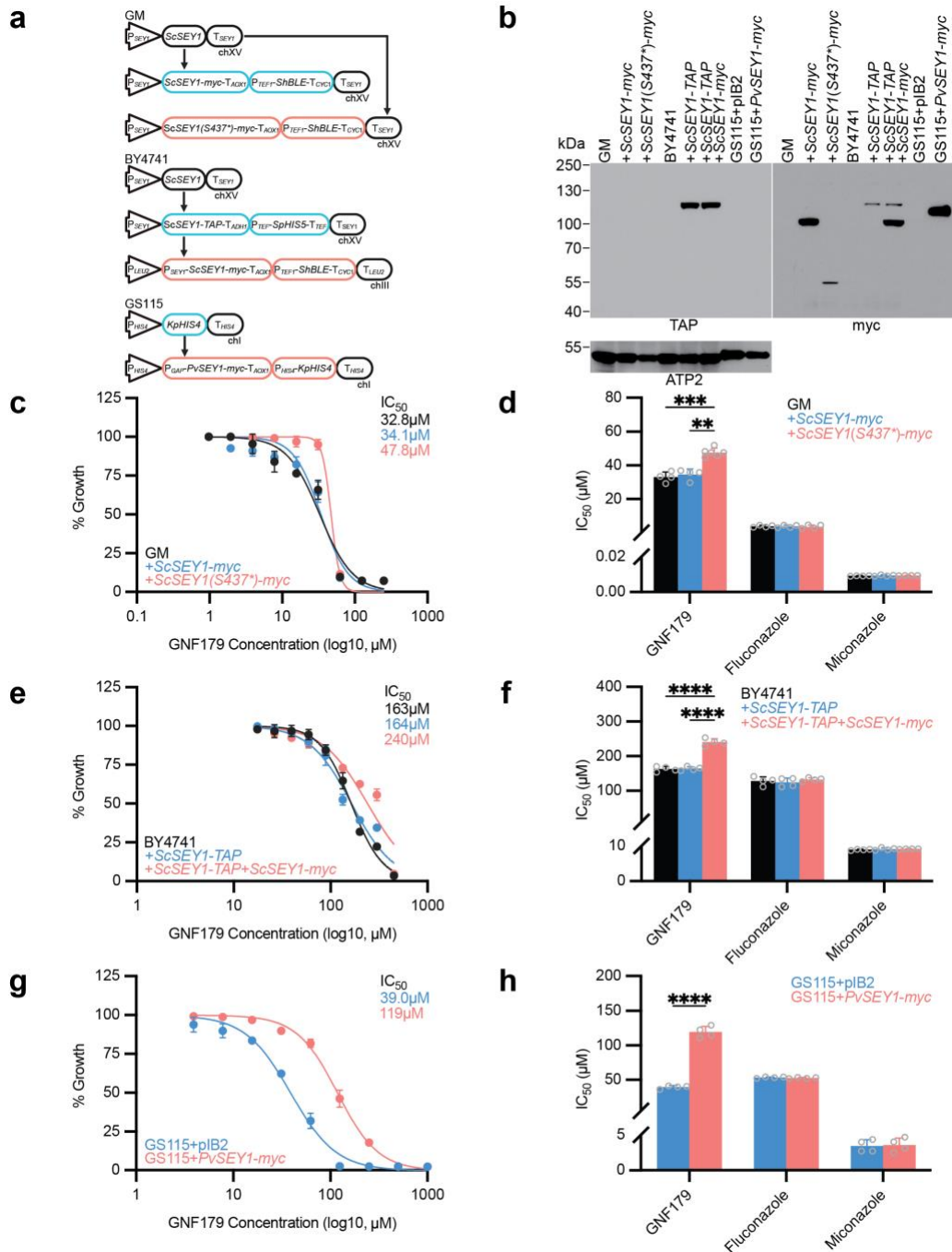


Figure 2.3 | SEY1 mutations confer resistance to GNF179

a. Diagram of yeast mutants used in this study. **b.** Western blot of protein lysates from the yeast mutants used in this study. **c, d.** Dose response curve for GM+ScSEY1(S437*)-myc mutant treated with GNF179; average IC₅₀ is presented. Bar graph summarizing IC₅₀ averages for this mutant treated with GNF179 or antifungals. **e, f.** Dose response curve for BY4741 mutant expressing two copies of ScSEY1 treated with GNF179; average IC₅₀ is presented. Bar graph summarizing IC₅₀ averages for this mutant treated with GNF179 or antifungals. **g, h.** Dose response curve for GS115+PvSEY1-myc mutant treated with GNF179; average IC₅₀ is presented. Bar graph summarizing IC₅₀ averages for this mutant treated with GNF179 or antifungals.

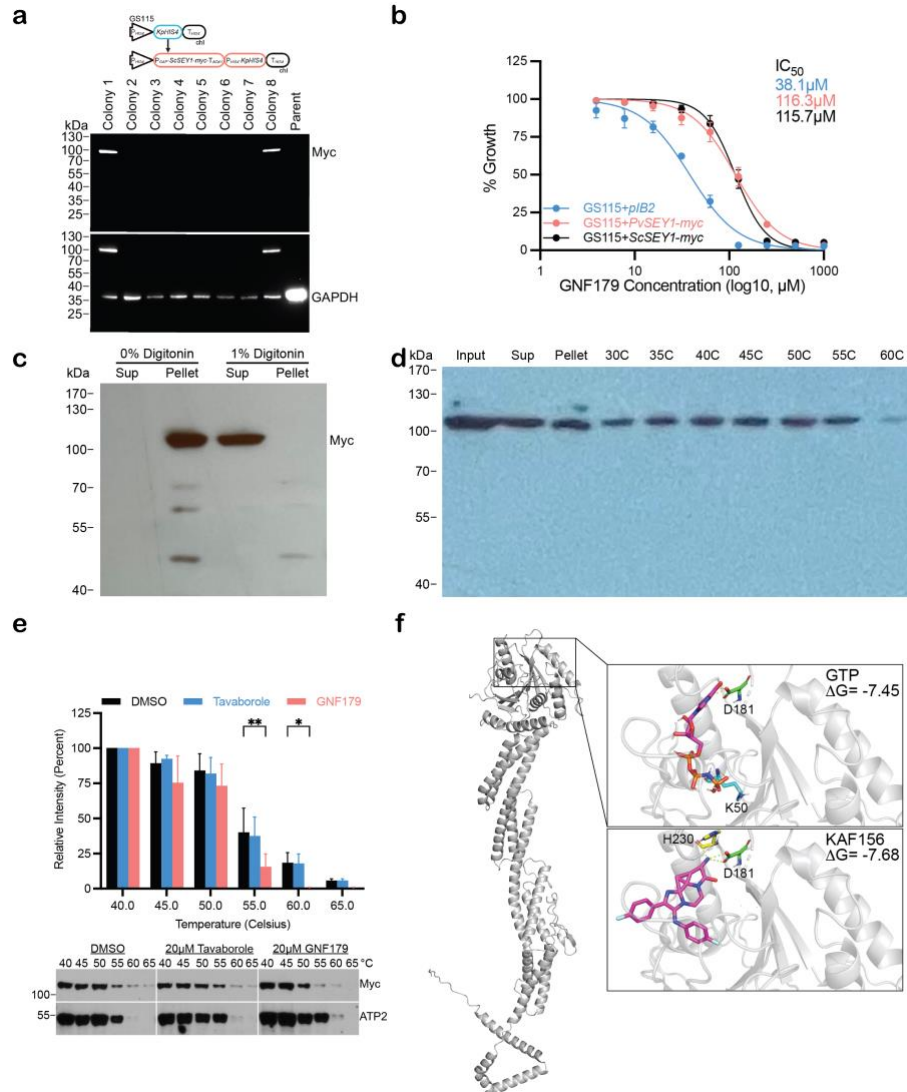


Figure 2.4 | GNF179 destabilization of ScSEY1 could be explained by the predicted binding to its GTPase domain

a. The *ScSEY1-myc* construct was integrated into the *his4* locus (in chromosome I) of the GS115 strain of *K. phaffii*. Mutant colonies were confirmed to express the fusion protein via Western blot, by probing for a 93kDa signal. *KpGAPDH*, a 36kDa protein, was also probed as a loading control.

b. Dose response curve for GS115+*ScSEY1-myc* mutant treated with GNF179; average IC₅₀ is presented.

c. Western blot probing with anti-myc antibody confirms solubilization of *ScSEY1-myc* protein when it is detected in supernatant (Sup) rather than pellet.

d. Digitonin-treated protein lysate containing *ScSEY1-myc* challenged to a range of temperatures, from 30°C to 60°C. Baseline *ScSEY1-myc* stability was established for each temperature by Western blot. Input refers to whole cell lysate prior to detergent treatment. Sup and Pellet refer to fractions obtained after detergent treatment.

e. Representative Western blot of the abundance of *ScSEY1-myc* remaining in protein lysates from GM+*ScSEY1-myc* after treatment with 20 μ M tavorole or GNF179, and subsequent thermal challenge. Data from biological duplicates are normalized against *ScATP2* loading control protein.

f. Docking analysis of GTP and KAF156 onto the GTPase domain of the *ScSEY1* homology model derived from cytosolic *CaSEY1* (PDB: 5CA9). GTP interacts with residues in cyan, whereas KAF156 interacts with residues in yellow; green residues interact with both ligands.

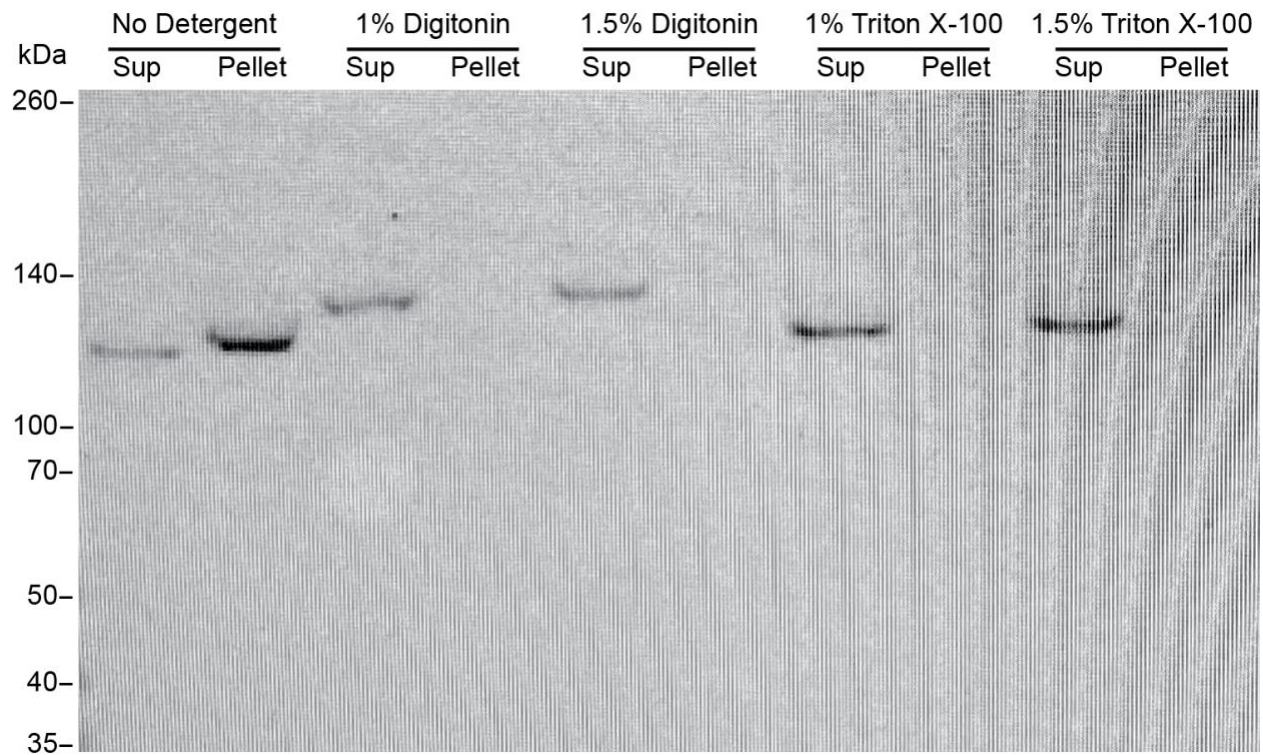


Figure 2.5 | Western blot of *PvSEY1* solubilization by digitonin or Triton X-100

Recombinant *PvSEY1-myc* is expressed in the GS115 strain of *K. phaffii*, and subsequently lysed by dounce homogenization. Resulting protein lysate is treated with detergent at two concentrations and centrifuged to obtain supernatant (Sup) and pellet fractions. Protein was detected in each fraction by probing with anti-myc antibody.

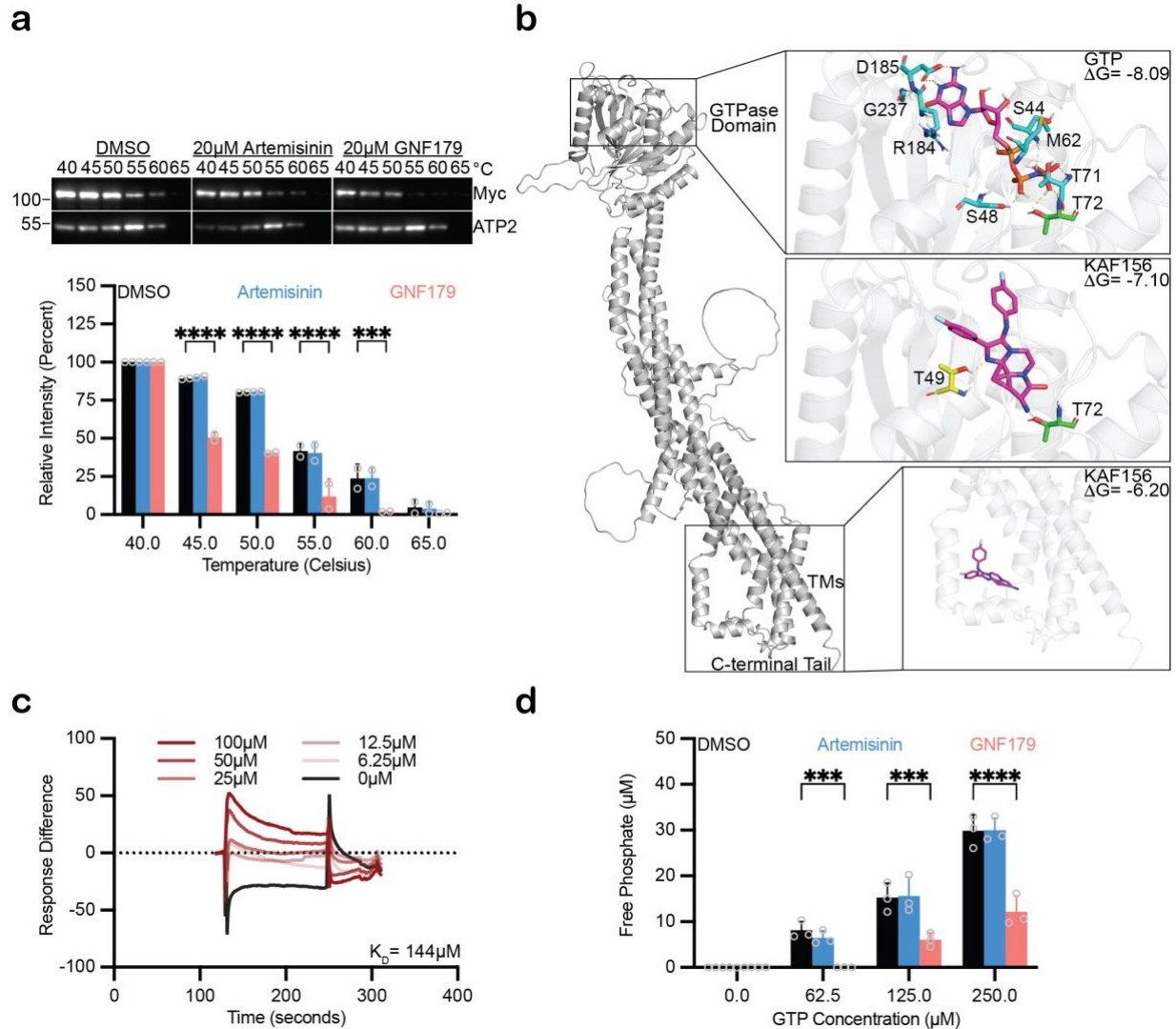


Figure 2.6 | GNF179 interacts with *Plasmodium* SEY1, destabilizing the protein and blocking its GTPase activity

a. Representative Western blot of the abundance of *Pv*SEY1-myc remaining in whole cell lysates from GS115+*Pv*SEY1-myc after treatment with 20μM artemisinin or GNF179, and subsequent thermal challenge from 40°C to 65°C. Data from biological duplicates are normalized against *Kp*ATP2 loading control protein and analyzed using Dunnett test, noting comparisons with p-value<0.05. **b.** Docking analysis of KAF156 and GTP onto the GTPase domain of the *Pv*SEY1 homology model derived from *Ca*SEY1 (PDB: 5CA9). GTP interacts with residues in cyan, whereas KAF156 interacts with residues in yellow; green residues interact with both ligands. Yellow dashes show interacting atoms, all within five angstroms. KAF156 was also docked on the C-terminal tail of the *Pv*SEY1 homology model. **c.** Surface plasmon resonance analysis for the interaction between recombinant His-tagged *Pk*SEY1 and increasing GNF179 concentration; K_D is presented. **d.** GTPase activity of *Pv*SEY1-myc in protein lysates from *E. coli* is measured by quantifying free phosphate production in the presence of 125μM GNF179 or artemisinin. Data represent biological triplicates analyzed using Tukey test, noting comparisons with p-value<0.05.

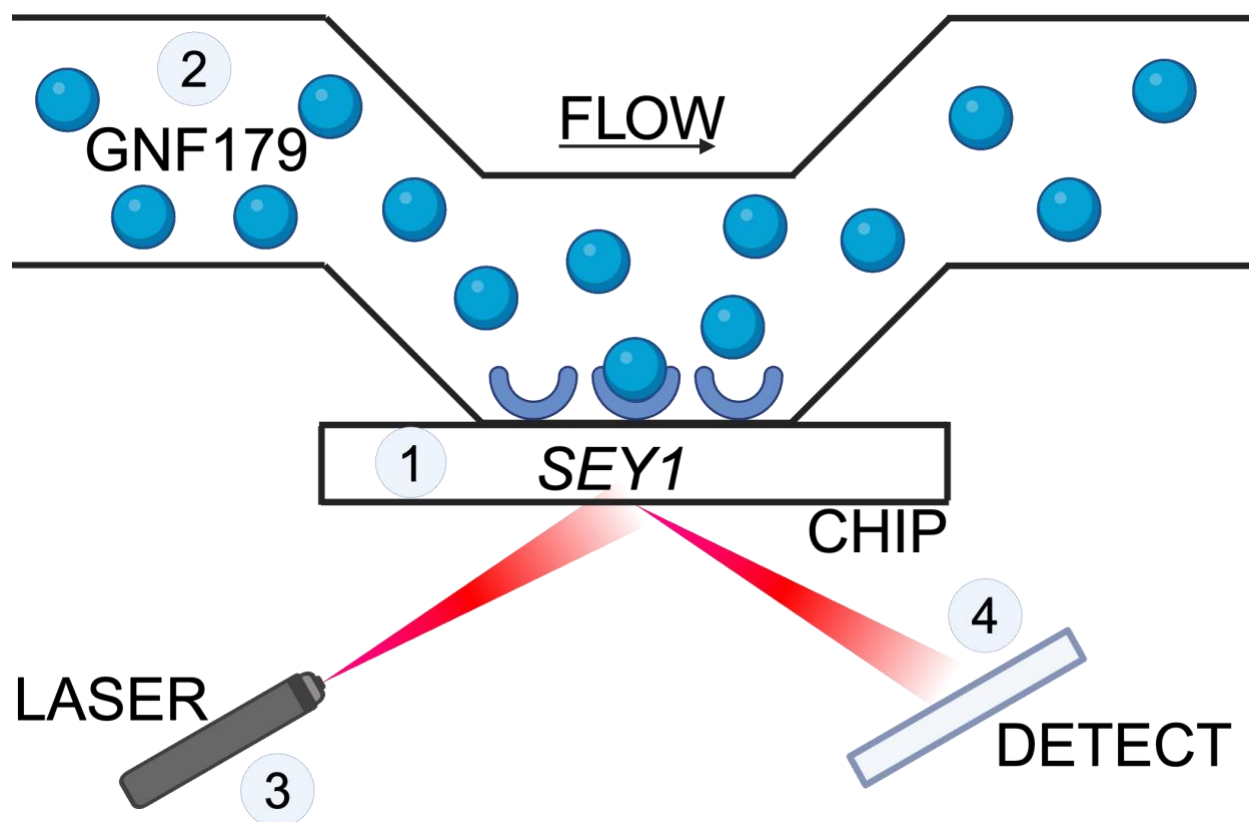


Figure 2.7 | Schematic for surface plasmon resonance

In the first step, the ligand or protein of interest, such as SEY1, is bound to the sensor chip. Second, the analyte or compound of interest, such as GNF179, is flowed over the surface of the sensor chip. Third, a laser is shined through a prism beneath the sensor chip, generating a total-internal-reflection condition. In the fourth step, the angles of reflected light are detected. The shift in reflective angle is dependent on the amount of mass bound on the surface of the sensor chip (i.e., a shift occurs when ligand-analyte interaction is present).

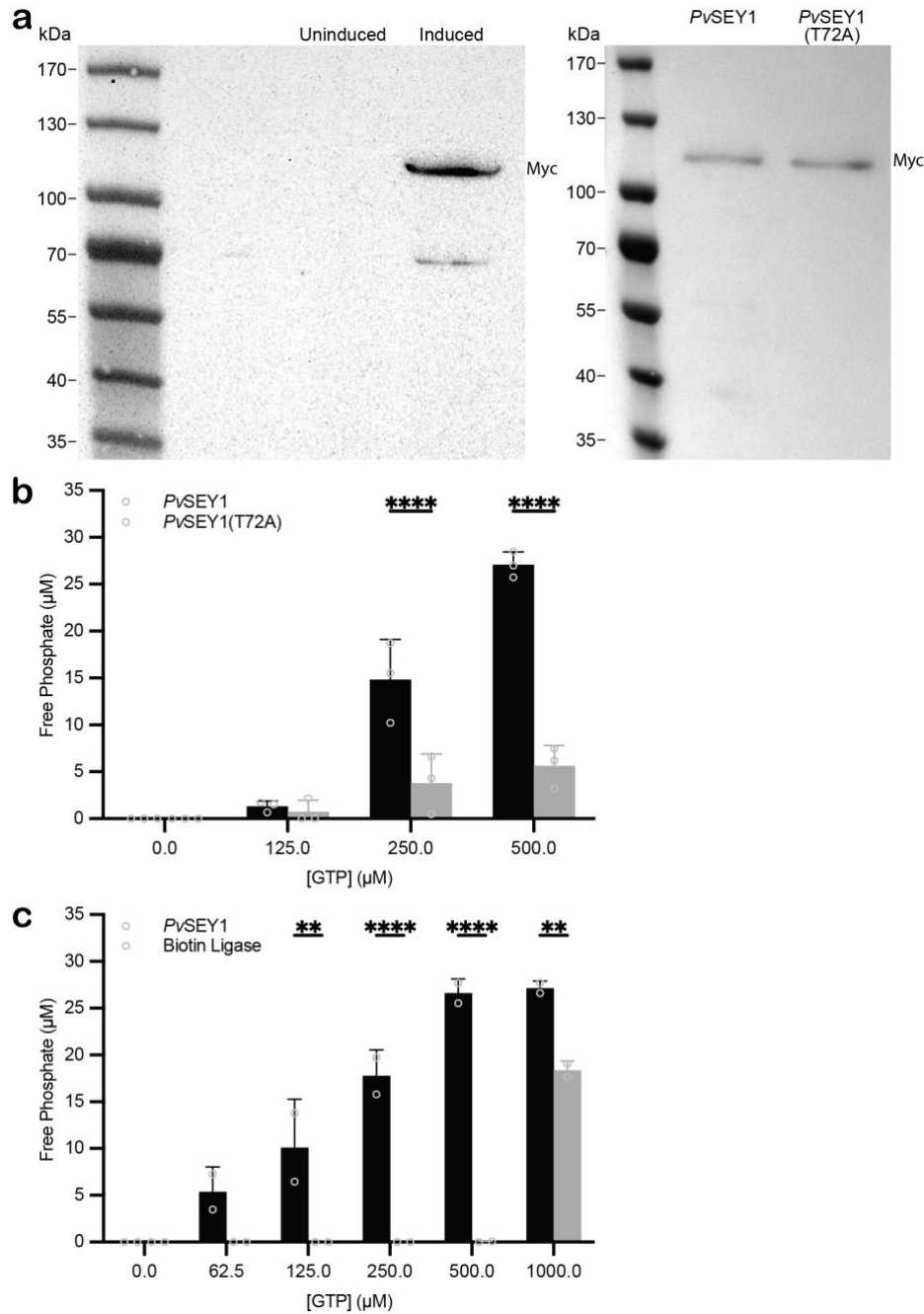


Figure 2.8 | Wild-type *PvSEY1*, but not *PvSEY1(T72A)*, possess GTPase activity

a. Western blot of the 106kDa *PvSEY1*-myc protein upon IPTG induction (left); *PvSEY1*-myc-His and *PvSEY1(T72A)*-myc-His are detected in protein eluates off the Ni-NTA column (right).

b. Protein lysates containing *PvSEY1*-myc-His or *PvSEY1(T72A)*-myc-His are tested for GTPase activity by measuring free phosphate production with increasing GTP concentrations; data represents biological duplicates analyzed using Sidak test, noting comparisons with $p\text{-value} < 0.05$.

b. Protein lysates containing His-tagged *PvSEY1*-myc or Biotin ligase are tested for GTPase activity; data represents biological duplicates analyzed using Sidak test, noting comparisons with $p\text{-value} < 0.05$.

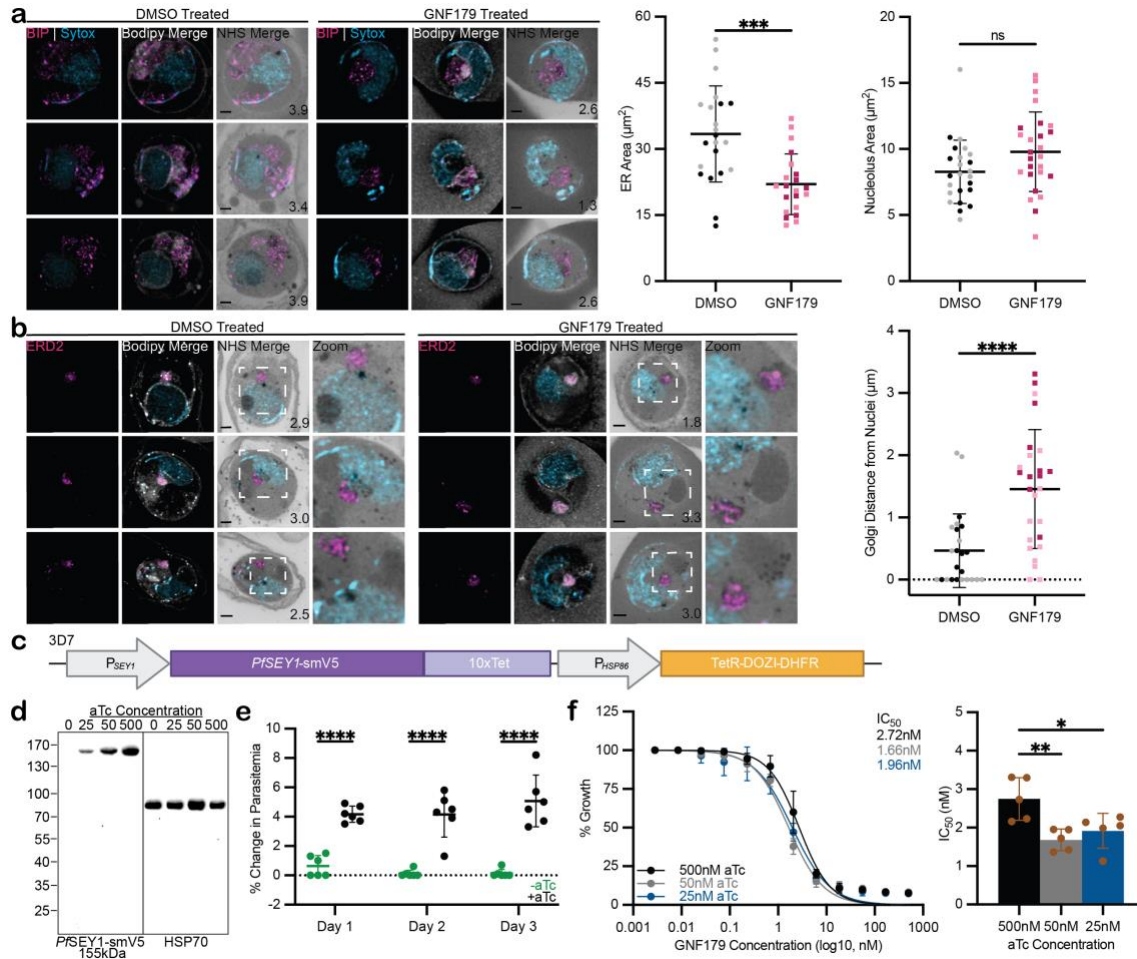


Figure 2.9 | GNF179 affects *Plasmodium* ER and Golgi morphology

a, b. Ultrastructural expansion microscopy experiments tracking ER (*Pf*BIP), Golgi (*Pf*ERD2), and nucleus (Sytox) in the presence or absence of GNF179. ER area and nucleolus area (**a**) and Golgi distance from the nucleus (**b**) are measured for at least 20 cells from biological duplicates (represented by two-tone colors) and analyzed using unpaired t-test. Scale bar is 2 μm ; image depth is in microns indicated by values in the bottom right corner of NHS-merged images. **c.** TetR-DOZI aptamers construct is integrated into the *PfSEY1* locus for knockdown studies. *PfSEY1* is fused with the smV5-tag, followed by the 10xTet domain. **d.** Western blot of *PfSEY1*-smV5 abundance in whole cell lysates of *P. falciparum* after treatment with increasing aTc concentrations. Right panel depicts the 155kDa constructed protein detected with anti-smV5 antibody; on the left panel, anti-*Pf*HSP70 antibody is used to detect the 70kDa loading control protein. **e.** Growth curve of the *PfSEY1* knockdown mutant. Cultures containing no aTc (-aTc) or 500nM aTc (+aTc) started at 1% parasitemia on Day 0 and change in their parasitemia were measured by subtracting previous-day parasitemia from current-day parasitemia. Data represent six biological replicates analyzed using Sidak test, noting comparisons with $p\text{-value} < 0.05$. **f.** GNF179 IC₅₀ measures for the *PfSEY1*-smV5 mutant treated with 25nM, 50nM, or 500nM aTc. Dose response data (left) are summarized in the bar graph (right); five biological replicates were analyzed using Dunnet test, noting comparisons with $p\text{-value} < 0.05$.

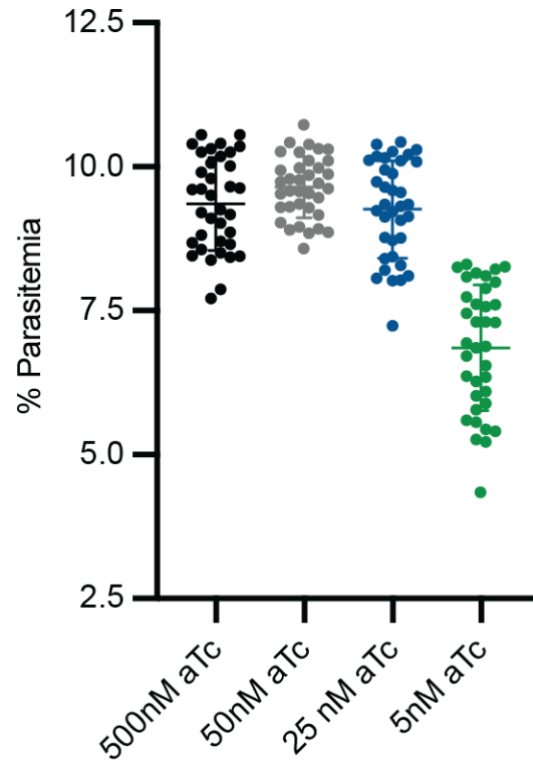


Figure 2.10 | Low concentration of aTc reduces viability of the TetR-DOZI-based *PfSEY1* knockdown mutant

The *PfSEY1* knockdown mutant was grown under normal translation conditions (500nM aTc), washed, split to 1% parasitemia, and then grown with various aTc concentrations, from 5nM to 500nM aTc. Each biological duplicate was tested in technical triplicates; each triplicate was divided into twelve wells. Parasitemia was measured after 48 hours for each of the 36 samples within each aTc condition.

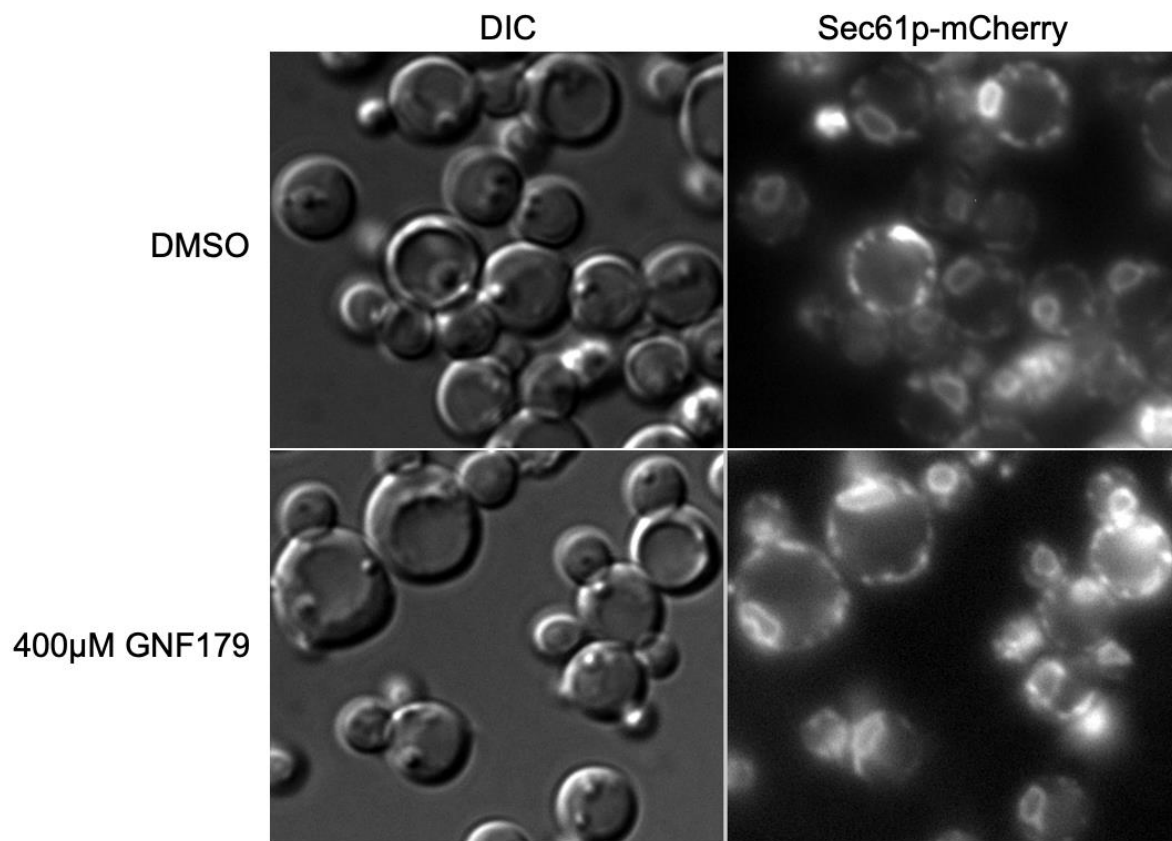


Figure 2.11 | Yeast ER morphology remains unaffected upon GNF179 treatment

GS115 strain of yeast expressing *KpSEC61*-mCherry is treated with DMSO or GNF179 at 10x IC_{50} concentration. Even after overnight incubation, there is no difference in the morphology of the ER, as observed with the *KpSEC61*-mCherry ER marker.

Table 2.3 | Yeast strains used in this study

For species, *Sc* and *Kp* refer to *S. cerevisiae* and *K. phaffii*, respectively.

Strain	Species	Genotype
GM	<i>Sc</i>	MATa <i>adp1Δ snq2Δ ycf1Δ pdr15Δ yor1Δ vmr1Δ pdr11Δ nft1Δ bpt1Δ ybt1Δ ynr070wΔ yol075cΔ aus1Δ pdr5Δ pdr10Δ pdr12can1Δ::GMToolkit-a lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i> (deletions for the ABC transporter genes are marked with [tetO2pr-GFP, URA3]).
GM+ <i>ScSEY1-myc</i>	<i>Sc</i>	MATa <i>adp1Δ snq2Δ ycf1Δ pdr15Δ yor1Δ vmr1Δ pdr11Δ nft1Δ bpt1Δ ybt1Δ ynr070wΔ yol075cΔ aus1Δ pdr5Δ pdr10Δ pdr12can1Δ::GMToolkit-a lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i> (deletions for the ABC transporter genes are marked with [tetO2pr-GFP, URA3]) <i>SEY1::ScSEY1-myc, BleoR</i> .
GM+ <i>ScSEY1(S437*)-myc</i>	<i>Sc</i>	MATa <i>adp1Δ snq2Δ ycf1Δ pdr15Δ yor1Δ vmr1Δ pdr11Δ nft1Δ bpt1Δ ybt1Δ ynr070wΔ yol075cΔ aus1Δ pdr5Δ pdr10Δ pdr12can1Δ::GMToolkit-a lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0</i> (deletions for the ABC transporter genes are marked with [tetO2pr-GFP, URA3]) <i>SEY1::ScSEY1(S437*)-myc, BleoR</i> .
BY4741	<i>Sc</i>	MATa <i>his3Δ1 met15Δ0 ura3Δ0 leu2Δ0</i> .
BY4741+ <i>ScSEY1-TAP</i>	<i>Sc</i>	MATa <i>his3Δ1 met15Δ0 ura3Δ0 leu2Δ0 SEY1::ScSEY1-TAP, HIS3</i> .
BY4741+ <i>ScSEY1-TAP</i> + <i>P_{SEY1}-ScSEY1-myc</i>	<i>Sc</i>	MATa <i>his3Δ1 met15Δ0 ura3Δ0 leu2Δ0::P_{SEY1}-ScSEY1-myc, BleoR SEY1::ScSEY1-TAP, HIS3</i> .
GS115+pIB2	<i>Kp</i>	<i>his4::HIS4</i> .
GS115+ <i>P_{GAPDH}-PvSEY1-myc</i>	<i>Kp</i>	<i>his4::P_{GAPDH}-PvSEY1-myc, HIS4</i> .
GS115+ <i>P_{GAPDH}-ScSEY1-myc</i>	<i>Kp</i>	<i>his4::P_{GAPDH}-ScSEY1-myc, HIS4</i> .

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Chapter 3 | Implications of the *Plasmodium* SEY1 C-terminus on imidazolopiperazine binding

Abstract

The imidazolopiperazine class of compounds, which includes the KAF156 analog that is currently in FDA trials, exhibits antimalarial activity by targeting the GTPase domain of *Plasmodium* SEY1, which is an essential dynamin-like protein implicated in homotypic fusion of endoplasmic reticulum membranes. In anticipation of resistance alleles, we investigate protein mutations that may prevent imidazolopiperazine binding. KAF156 is not predicted to bind to the GTPase domain if *P. vivax* SEY1 is devoid of its transmembrane domains and C-terminal tail. Surface plasmon resonance experiments do not indicate an interaction between recombinant cytosolic PvSEY1(1-2403) and the GNF179 analog. The GTPase activity of this truncated protein is unaffected by GNF179. Additionally, analytical ultracentrifugation experiments show that homodimerization of PvSEY1(1-2403) is unaffected by GNF179. Overall, these data suggest that the C-terminus of PvSEY1 may be integral to imidazolopiperazines resistance.

Main Text

Introduction

Malaria, caused by the *Plasmodium* parasite, continues to be a global issue. In 2022, rates of malaria cases and deaths were at 58.4 per 1000 at-risk people and 14.3 per 100,000 at-risk people, respectively, which are far behind the milestones instilled by the Global Technical Strategy for Malaria (WHO, 2023). Attempts to remedy this include development of vaccines (e.g., monoclonal antibodies), vector control strategies against insecticide-resistant mosquitoes, diagnostic kits to detect elusive *pfhrp2* and *pfhrp3* parasites, and antimalarials with novel mode of action (MOA). The imidazolopiperazine (IZP) class of antimalarial compounds are of interest due to its highly selective activity towards parasites, regardless of their life cycle stage (Kuhlen et al., 2014; Meister et al., 2011; Ouologuem et al., 2022). The KAF156 analog is currently in the phase III patient exploration stage of FDA trials alongside lumefantrine (Siqueira-Neto et al., 2023).

Despite detailed clinical studies demonstrating IZP efficacy (Kublin et al., 2021; Leong et al., 2014), their MOA remains poorly characterized. Metabolomic profiling of *P. falciparum* treated with KAF156 did not present clear gene expression changes (Allman et al., 2016). *In-vitro* evolution and whole genome analysis (IVIEWGA) of *P. falciparum* that are resistant to the GNF179 analog yielded single nucleotide variants (SNVs) in genes localizing in the endoplasmic reticulum (ER): *PfCARL*, PF3D7_0321900; *PfUGT*, PF3D7_1113300; *PfACT*, PF3D7_1036800 (LaMonte et al., 2016; Lim et al., 2016). *PfCARL* and *PfUGT* are predicted to be essential in *Plasmodium* (Zhang et al., 2018), but mutations in these genes confer resistance to unrelated compound classes, suggestive of encoding multidrug resistance transporters (Imlay et al., 2023; Lim et al., 2016; Magistrado et al., 2016). Additional IVIEWGA testing with the *S. cerevisiae* yeast model organism yielded more adaptive mutations in genes that function within the ER;

however, genes such as *ScSUR2* and *ScELO2* also appear to be multidrug resistance genes (LaMonte et al., 2020).

Through proteomic affinity chromatography, *P. falciparum* SEY1, PF3D7_1416100, was identified as a potential IZP target (Figure 2.1). Studies on *S. cerevisiae* Synthetic Enhancer of YOP1, or *ScSEY1*, suggest it encodes an ER-localized dynamin-like GTPase implicated in homotypic fusion of ER membranes (Yan et al., 2015). *Plasmodium* SEY1 is structurally conserved, comprising a druggable GTPase domain, stalk region, two transmembrane domains, and a C-terminal tail (Figure 2.2c), and it is an essential protein (Figure 2.9e). The interaction between IZPs and *Plasmodium* SEY1 is further validated through thermal shift assay (Figure 2.6a) and surface plasmon resonance (Figure 2.6c).

Here, in anticipation of *Plasmodium* SEY1 mutations conferring resistance to imidazolopiperazines, we investigate mutations within the protein that can disrupt IZP binding. Mutations to the GTPase domain are omitted as that will also inhibit SEY1 activity. Although a nonsense mutation at the S437 residue of *ScSEY1* can confer resistance to GNF179 (Figure 2.3c), mutations to the stalk region are omitted as that will inhibit SEY1 fusion activity (Yan et al., 2015). However, removal of the *ScSEY1* transmembrane domains and C-terminal tail do not affect its homodimerization (Anwar et al., 2012), thus GTP binding and fusion activity are unaffected. KAF156 is not predicted to bind to the C-terminal end of *PvSEY1* (Figure 2.6c), but the C-terminal end may still be integral. In *Arabidopsis thaliana*, mutations to the phosphorylated residues on the C-terminal tail of the Root Hair Defective 3 (*AtRHD3*) ortholog aberrates tubule formation (Ueda et al., 2016). SEY1 human ortholog atlastin-1 (*HsATL-1*) possesses an amphipathic helical domain at the C-terminus that assists in facilitating membrane fusion (Faust et al., 2015). We show evidence of the *PvSEY1* C-terminal end being integral to IZP binding. KAF156 is not predicted

to bind to the GTPase domain of *PvSEY1* that is devoid of its transmembrane domains and C-terminal tail. This observation is supported by surface plasmon resonance studies between this truncated protein and GNF179. Furthermore, its GTPase activity and homodimerization are not affected. Overall, these data provide insight into possible mutations that confer resistance to IZPs.

Results

GNF179 is not predicted to bind to the GTPase domain of *PvSEY1*(1-2403)

To first explore potential implications of the C-terminus of *Plasmodium SEY1* on imidazolopiperazine binding, we utilized molecular docking to predict changes to GTPase domain binding upon removal of the C-terminus. *PvSEY1*, PVP01_1332800, is a 2,667 base pair gene that expresses a protein comprising 889 amino acids (Figure 2.2). The last 88 amino acids of *PvSEY1*, which encompasses its two transmembrane domains and C-terminal, were removed, generating a cytosolic protein. Using Colabfold (Mirdita et al., 2022), we simulated the protein structure of *PvSEY1*(1-2403), still using *CaSEY1* (RCSB: 5CA9) as a template. Through MGLTools (Eberhardt et al., 2021; Trott & Olson, 2010), we then attempted to fit GTP and KAF156 into the GTPase domain of the monomeric model. Hydrogen bond interactions were predicted between GTP and cytosolic *PvSEY1* Gly41, Gly46, Arg184, and Asp185 residues (Figure 3.1); Gly41 and Gly46 are conserved motifs in the P-loop site of the GTPase domain, whereas Arg184 and Asp185 are conserved motifs in the guanosine-binding site of the GTPase domain (Hu et al., 2009; Yan et al., 2015). GTP interactions with the guanosine-binding site was also observed with the full-length *PvSEY1* model (Figure 2.6b). Clearly, GTP is still interacting with the GTPase domain of cytosolic *PvSEY1*. For KAF156, no hydrogen bond interactions were predicted between cytosolic *PvSEY1* and the compound (Figure 3.1); recall, KAF156 was predicted to bind to the Walker A site of full-

length *PvSEY1* (Figure 2.6b). Additionally, the highest rated positioning does not place KAF156 in the same vicinity as GTP (Figure 3.1). Perhaps removal of the transmembrane domains and/or the C-terminal tail changes folding of the protein.

PvSEY1(1-2403) does not interact with GNF179

We then confirm the predicted loss of binding between imidazolopiperazines and *PvSEY1* with surface plasmon resonance. To obtain recombinant *PvSEY1*(1-2403), the 264 base pairs corresponding to the transmembrane domains and C-terminal tail were removed from the pET21+*PvSEY1* plasmid that was used in GTPase assays (Figure 2.8a), generating pET21+*PvSEY1*(1-2403)-6xHis (Figure 3.2a). The new plasmid was transformed in BL21 DE3 bacteria, a commonly used strain for protein expression, and allowed to express the protein upon addition of IPTG to remove repression by the lac operon. Cells were lysed, and the protein lysate was purified through a Ni-NTA column, washing away other proteins and subsequently eluting the recombinant protein with imidazole. Recombinant *PvSEY1*(1-2403) was immobilized onto a CM5 sensor chip, and then GNF179 was flowed over the sensor chip at varying concentrations. Analysis of the response differences from refractive angle measures indicates that GNF179 did not bind to the protein that was immobilized on the chip (Figure 3.2b). It is possible that positively charged molecules were interacting non-specifically to the carboxyl groups on the sensor surface. Despite counteracting this by doubling PBS concentration to increase ionic strength, no physical binding was observed between GNF179 and cytosolic *PvSEY1* (Figure 3.2c).

GNF179 does not affect the GTPase activity of *PvSEY1*(1-2403)

If IZPs do not bind to *PvSEY1*(1-2403), then GTPase activity should remain unaffected. To test this, we conducted GTPase activity assays. Purified *PvSEY1*(1-2403), at 1 μ M, was added to reactions containing 125 μ M GNF179 or DMSO and incubated for 30 minutes with increasing concentrations GTP before measuring absorbance at 360nm wavelength. In the absence of GTP, no free phosphates were produced, as expected. As the GTP concentration increased, so did the free phosphate concentration (Figure 3.2d). Regarding the DMSO condition, the truncated protein remains active and able to hydrolyze GTP. The difference in free phosphate production between the two conditions were not statistically significant: as an example, 18.1 μ M and 18.2 μ M of free phosphates were measured for the DMSO and GNF179 treatments, respectively. When full-length *PvSEY1* was tested, GNF179 inhibition of GTPase activity was observed (Figure 2.6d).

It is possible that GNF179 that was used in the GTPase assay lost its potency. To test this, GM+*ScSEY1*(*S437**)-*myc* mutant, a BY47471 strain of *S. cerevisiae* devoid of 16 ABC transporters (Suzuki et al., 2011) but expressing a truncated *ScSEY1* gene, was cultured in YPD overnight at 30°C. After growth to early-log phase, aliquots of the culture were treated to a ten-point dose response curve with the same GNF179 sample that was prepared for the GTPase activity assay (Figure 3.2d). After overnight growth at 30°C, OD₆₀₀ was measured for each treatment sample. Analysis of the curve generated an IC₅₀ measure of 45.7 μ M (Figure 3.3), in line with the IC₅₀ measure of 47.8 μ M that was observed in confirming GNF179 resistance by yeast expressing the *ScSEY1*(*S437**) nonsense mutation (Figure 2.3c). Thus, these results indicate a lack of cytosolic *PvSEY1* GTPase activity inhibition by GNF179.

GNF179 does not affect the homodimerization of *PvSEY1*(1-2403)

If GTP, but not imidazolopiperazines, can bind to *PvSEY1*(1-2403), then the protein should still undergo homodimerization. To test this, we conducted analytical ultracentrifugation (AUC), a method of identifying interacting biomolecules in as close to a native state as possible by separating them by molecular mass, size, or shape (Edwards et al., 2020). Previously, AUC had been applied to demonstrate the GTP-dependent homodimerization of cytosolic *ScSEY1*: the yeast ortholog forms dimers in the presence of nonhydrolyzable GTP, but not in the presence of GDP (Yan et al., 2015). Four reactions were set up: 200 μ M *PvSEY1*(1-2403) alone, with 200 μ M nonhydrolyzable GTP, with 200 μ M GNF179, or with both molecules at 200 μ M. Each sample was centrifuged at 40,000rpm for 24 hours and analyzed using SEDFIT software (Schuck et al., 2023). Firstly, as expected for cytosolic *PvSEY1* alone, the protein remained as monomer: Peak 3, which corresponds to a protein with an apparent molecular weight of 95 kDa (size of *PvSEY1*(1-2403), encompassed 52.8% of the protein detected in the sample (Figure 3.4a, Table 3.1). Other peaks were observed, and they may correspond to other forms of cytosolic *PvSEY1*, complexes formed with cytosolic *PvSEY1*, or just other proteins that were purified alongside cytosolic *PvSEY1*.

The AUC profile for the GTP condition depicts proteins of larger molecular weight (Figure 3.4b). Peak 4, which contains 31.4% of the protein detected, possessed an apparent molecular weight of 221kDa (Table 3.2). With the expected weight of cytosolic *PvSEY1* to be 190kDa, most likely this peak refers to dimeric *PvSEY1*. Peak 4 in the *PvSEY1*(1-2403)-alone condition is only 4.5% of the detected proteins (Table 3.1). Additionally, there is a decrease in monomeric *PvSEY1*: Peak 2 (71kDa) and Peak 3 (121kDa) now encompassed 22.0% and 11.6% of the detected protein, respectively (Table 3.2). It is possible that these peaks refer to monomeric *PvSEY1* that is bound to GTP and monomeric *PvSEY1* that is not bound to GTP. Furthermore, there is a shift in

dimerization K_d from 104μM (Figure 3.4a) to 3.6μM (Figure 3.4b). Thus, this data suggest that PvSEY1(1-2403) still exhibits GTP-induced homodimerization.

The AUC profile for the GNF179 condition depicts proteins of lower molecular weight (Figure 3.4c). With 57.3% of the detected protein in Peak 3, which corresponds to a protein of 94kDa, most cytosolic PvSEY1 remained a monomeric (Table 3.3). Peak 4, proteins of 182 kDa, only covered 7.9% of the detected proteins (Table 3.3). Also, the dimerization K_d weakened to 38μM (Figure 3.4c). Thus, GNF179 does not induce homodimerization of cytosolic PvSEY1.

As for the AUC profile derived from treating PvSEY1(1-2403) with both GTP and GNF179, it more resembled that of the GTP condition rather than that of the protein-alone condition. The homodimerization K_d improved to 4.6μM (Figure 3.4d). Most protein detected (36.4%) are at the 206kDa size (Peak 4), closer size of cytosolic PvSEY1 in a dimer state (Table 3.4). And, as observed with the GTP condition, Peak 2 (69kDa) and Peak 3 (116kDa) cover 23.1% and 10.0% of the detected proteins, respectively (Table 3.4). Thus, this data suggests that GNF179 is not inhibiting GTP-induced homodimerization of cytosolic PvSEY1.

Discussion

Here we present evidence of the C-terminus of *Plasmodium* SEY1 being required for the binding of imidazolopiperazines.

Our analysis of molecular docking studies on PvSEY1 that is missing transmembrane domains and C-terminal tail led us to believe a protein folding change may disrupt IZP binding (Figure 3.1). However, the protein structure is simulated, albeit based on the crystal structure of the cytosolic *Candida albicans* ortholog (Yan et al., 2015). To fully elucidate the implications of the PvSEY1 C-terminus to IZP binding, the protein, in both full-length and truncated forms, must

be crystallized and analyzed for binding. Subsequently, the two protein forms can be overlapped for identification of any protein folding changes.

We had previously shown GNF179 effects on full-length *Plasmodium* SEY1. GNF179 was observed to bind to full-length *Plasmodium knowlesi* SEY1 (Figure 2.6c), but not to truncated PvSEY1 (Figure 3.2c). To best compare these surface plasmon resonance experiments, the SEY1 proteins should come from the same *Plasmodium* species, especially with IZPs being more potent against *P. knowlesi* (van Schalkwyk et al., 2021). GNF179 was observed to inhibit the GTPase activity of full-length PvSEY1 (Figure 2.6d), but not that of the cytosolic form (Figure 3.2d). It is possible that cytosolic PvSEY1 is not fully functioning if it were not tethered to the ER membrane (Sugiura & Mima, 2016), or if it did not have the effects brought by the C-terminal tail (Ueda et al., 2016). Furthermore, we are unsure if cytosolic SEY1 affects ER morphology. In *S. cerevisiae*, absence of the ortholog has minimal adverse effects to ER morphology, as it is a nonessential gene (Anwar et al., 2012; Rogers et al., 2013). *Plasmodium falciparum* SEY1 is essential (Figure 2.9e), so *in-vivo* tracking and organelle observation studies of the parasite should be conducted.

The analytical ultracentrifugation experiments further demonstrate the lack of interaction between cytosolic PvSEY1 and GNF179. The GTPase domain remained able to bind GTP, and the stalk region remained able to dimerize (Figure 3.4). Firstly, the full-length PvSEY1 protein must also undergo AUC experiments to be fully confident that homodimerization is unaffected when the transmembrane domains and/or the C-terminal tail is absent. The experiment was also set up for competition between non-hydrolyzable GTP and GNF179 for the GTPase domain. Mixing both molecules together indicates that PvSEY1 has a greater affinity for GTP. The next step here is to treat cytosolic PvSEY1 with GNF179 prior to the addition of GTP. This could create steric hindrance around the GTPase domain or denature the protein altogether.

Imidazolopiperazines, specifically KAF156, remain the leading antimalarial candidate, being farthest along the drug development pipeline and possessing antimalarial activity at multiple stages of the *Plasmodium* life cycle. Currently, no SNPs conferring IZP resistance have been found in African samples (Foguim et al., 2019). Ultimately, investigating IZP effects on different parts of *PvSEY1* can help identify resistance alleles of this new generation class of antimalarials.

Methods

Molecular Docking

To generate the predicted protein structure of cytosolic *PvSEY1*, 88 amino acids were removed from the *PvSEY1* protein sequence (PVP01_1332800) and uploaded onto ColabFold v1.5.1 (Mirdita et al., 2022), using *CaSEY1* (RCSB: 5CA9) as a template. The highest scoring model was then processed using MGLTools (Eberhardt et al., 2021; Trott & Olson, 2010). The protein structure was stripped of water molecules and given Kollman charges and polar hydrogen atoms. Autogrid 4 was executed to place GTP and KAF156 in the GTPase domain of the theoretical cytosolic *PvSEY1* structure. Autodock 4 was then used to identify interactions between compound and amino acids. PyMol was used to visualize the interactions, noting hydrogen bonds between ligand and amino acid residues within five angstroms.

Protein expression and purification

The pET21+*PvSEY1*(1-2403)-6xHis plasmid, derived by removing 264 base pairs off the C-terminal end of the *PvSEY1* gene in the pET21+*PvSEY1*-6xHis plasmid, was transformed into Rosetta 2 DE3 (Novagen, 71400), following manufacturer protocol. Cells were plated on LB+Ampicillin (0.5% yeast extract, 1% tryptone, 1% NaCl, 100µg mL⁻¹ ampicillin) plates. A

single colony of the mutant bacteria was placed in 1L LB+Ampicillin media and grown to early-log phase. IPTG at 1mM was then added, and protein was expressed for 16 hours at 16°C (with shaking at 160rpm). Cells were harvested and lysed in 20mL lysis buffer (1% lysozyme, 1% protease inhibitor cocktail, 1% Triton X-100, 1mM PMSF, 20mM imidazole, 2500U endonuclease in B-PER reagent) at 4°C for two hours (with orbital rotation). The cell lysate, cleared by chilled centrifugation at 20,000g for 20 minutes, was incubated with Ni-NTA beads at 4°C for 16 hours (with orbital rotation) before passing them through a column. The beads were washed three times with wash buffer (500mM NaCl, 20mM Tris-HCl pH 7.9, 20mM imidazole). Then, proteins were eluted off the beads using two column volumes of elution buffer (500mM NaCl, 20mM Tris-HCl pH 7.9, 300mM imidazole). Protein samples were concentrated using Pierce protein concentrators (Thermo Scientific, 88502) and quantified via Qubit (Invitrogen, Q33211).

Surface plasmon resonance

Recombinant cytosolic *P. vivax* SEY1 was processed on a Biacore 3000 by Profacgen. PvSEY1-6xHis ligand was immobilized on a CM5 sensor chip (washed with 350mM EDTA and 500mM NiSO₄ prior) and washed with 0.05% surfactant P20 in 1x PBS or 2x PBS to reach 3036 resonance units (RU). Various concentrations of the GNF179 analyte were then injected using the following parameters: 120 seconds of contact time, 30 seconds of dissociation contact time, 30μL min⁻¹ flow rate. Data were analyzed using BIAevaluation software.

GTPase activity assay

GTPase activity was measured indirectly by quantifying free phosphate production using the EnzChek Phosphate assay kit (Invitrogen, E6646). The 100μL reactions comprise reaction

buffer (20% glycerol, 2mM EDTA, 4mM β -mercaptoethanol, 5mM MgCl_2 , 50mM HEPES, 200mM KCl), MESG, PNP, 1 μ M GTPase, 125 μ M GNF179 (or 1% DMSO), and increasing concentrations of GTP. Absorbance (A_{360}) measurements were taken using a plate reader (BioTek Synergy) after 30 minutes of incubation at 37°C., and subsequently converted to free phosphate concentrations using a standard curve. Data, representative of biological duplicates, were visualized and analyzed using Prism 10 software.

IC₅₀ determination

A single colony of the GM+*PvSEY1(S437*)-myc* mutant was grown in YPD overnight at 30°C to early-log phase; GM, or Green Monster, refers to the BY4741 strain of *S. cerevisiae* that has 16 ABC transporters replaced with GFP (Suzuki et al., 2011). Exactly 0.0005 OD₆₀₀ of cells were grown in 100 μ L YPD and exposed to a ten-point dose response of GNF179 (maximal 250 μ M, 1:2 serial dilution). After incubation at 30°C for 16 hours (with shaking at 500rpm), OD₆₀₀ was measured on a plate reader (BioTek Synergy). Percent growth was normalized against the vehicle-only condition (1% DMSO) and plotted against log₁₀ compound concentration and analyzed using Prism 10 software. Technical duplicates were conducted in 96-well plate format.

Analytical ultracentrifugation

Purified cytosolic *PvSEY1* (200 μ M GTPase in 25mM HEPES pH 7.0, 500mM NaCl, 5% glycerol, 2mM DTT) was mixed in a 500 μ L reaction with 50mM Tris pH 8.0, 300mM NaCl, and 5mM MgCl_2 , and 5% DMSO. For the GTP condition, 200 μ M nonhydrolyzable GTP was added to the reaction. For the GNF179 condition, 200 μ M GNF179 in 5% DMSO was added in place of just DMSO. For the condition containing both GTP and GNF179, 200 μ M nonhydrolyzable GTP and

200 μ M GNF179 in 5% DMSO were used in the reaction. The samples were added to the Proteome Lab XL-1 machine (Beckman), using interference for detection. Loading was through 2-channel cells with the sapphire window. Sedimentation was conducted using an An-50 Ti 8-place rotor (Beckman) that was spun at 40,000rpm for 24 hours, at 30°C. Data were analyzed using the SEDFIT software.

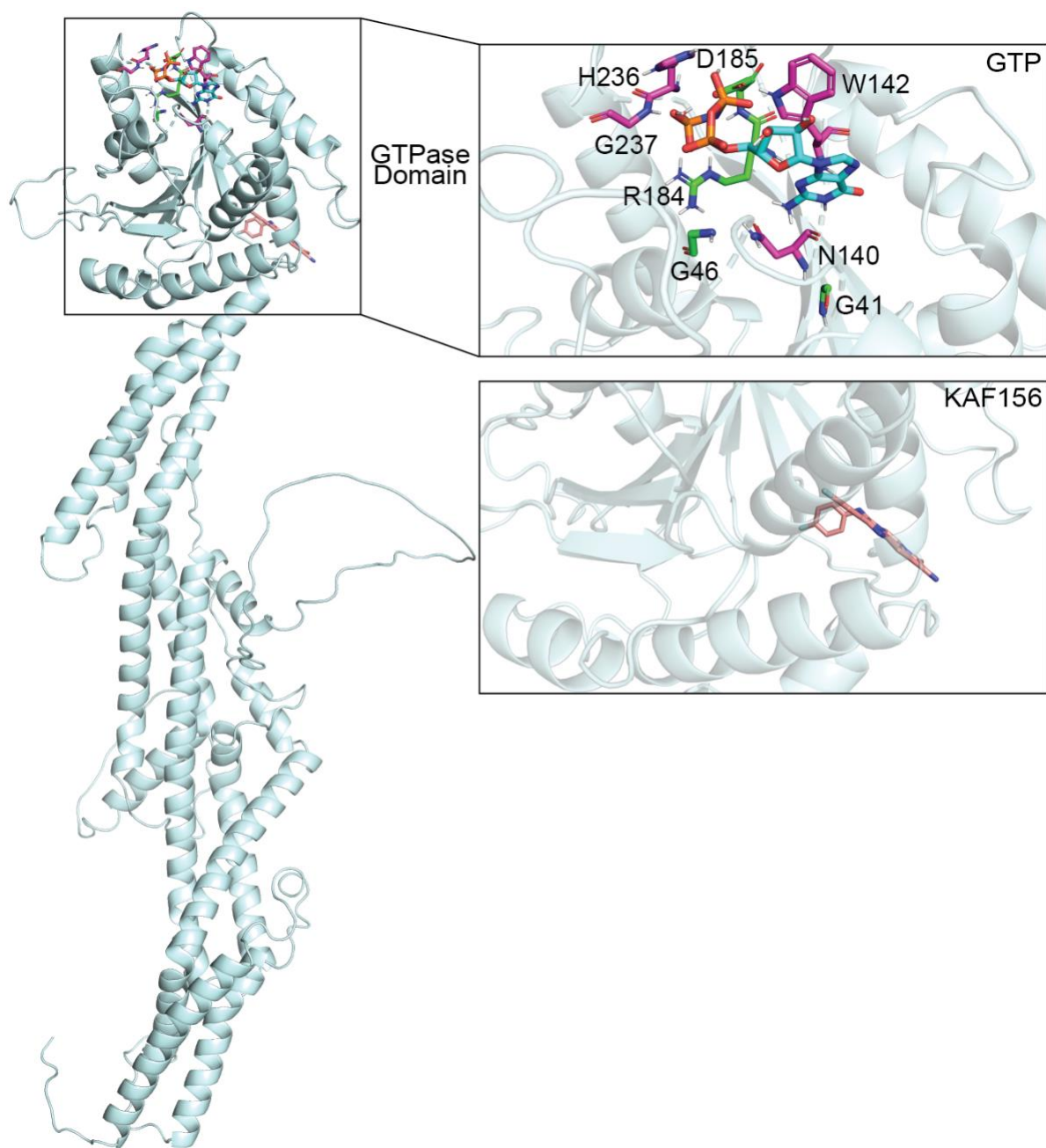


Figure 3.1 | GNF179 is not predicted to bind to the GTPase domain of cytosolic *PvSEY1*

Docking analysis of GTP (cyan molecule) and KAF156 (orange molecule) onto the GTPase domain of cytosolic *PvSEY1* (light cyan structure), which was derived from crystallized *CaSEY1* (PDB: 5CA9). Residues in magenta are interacting non-conserved GTPase motifs, whereas residues in green are conserved GTPase motifs; all interacting residues are within five angstroms.

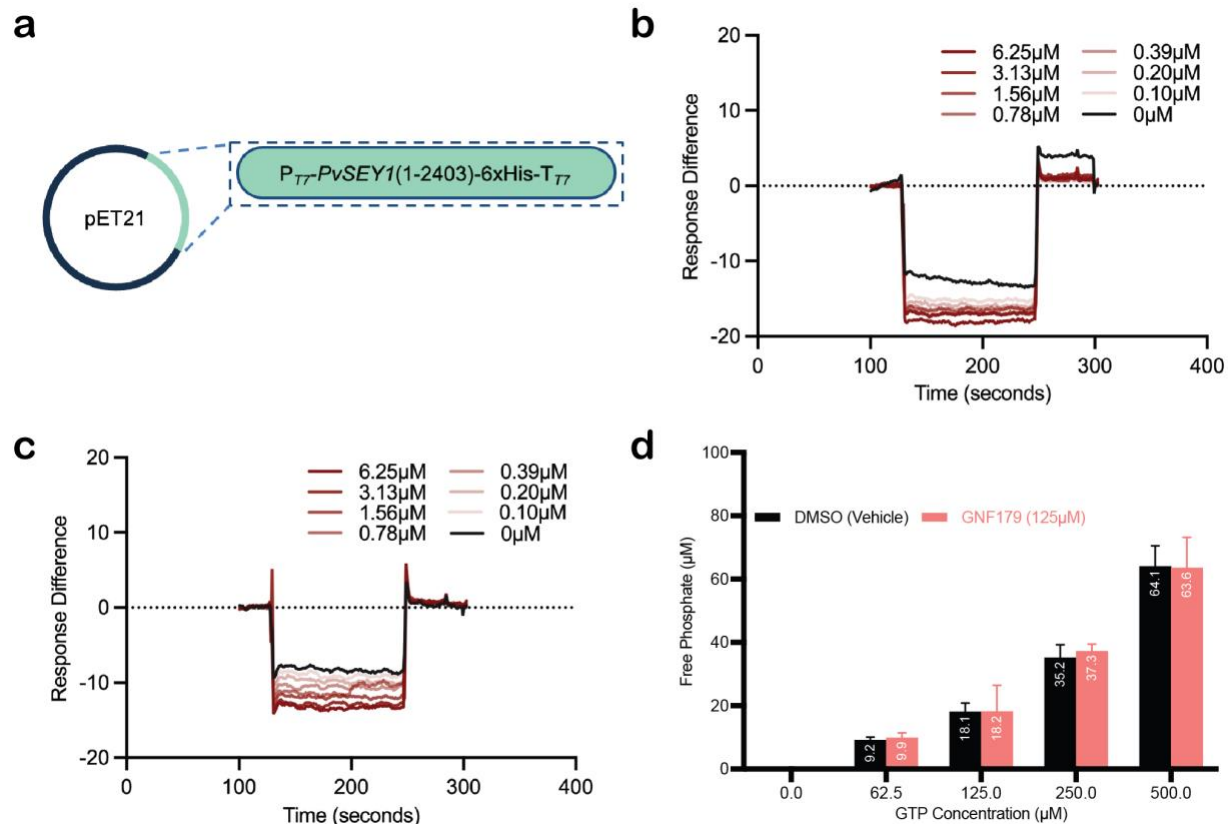


Figure 3.2 | GNF179 does not affect GTPase activity of cytosolic *PvSEY1*

(a) Design of the pET21 plasmid possessing the cytosolic *PvSEY1* gene, which is tagged with 6xHis and under the T7 promoter. Gene expression is induced by IPTG as it removes inhibition by the lac operon. **(b)** SPR analysis for the interaction between recombinant cytosolic *PvSEY1* and increasing GNF179 concentration, using standard 1x PBS. **(c)** SPR analysis for the interaction between recombinant cytosolic *PvSEY1* and increasing GNF179 concentration, using increased ionic strength 2x PBS. **(d)** GTPase activity of cytosolic *PvSEY1* is measured by quantifying free phosphate production in the presence of 125μM GNF179, with increasing GTP concentrations. Data represent biological duplicates analyzed using Tukey test, noting comparisons with p-value<0.05.

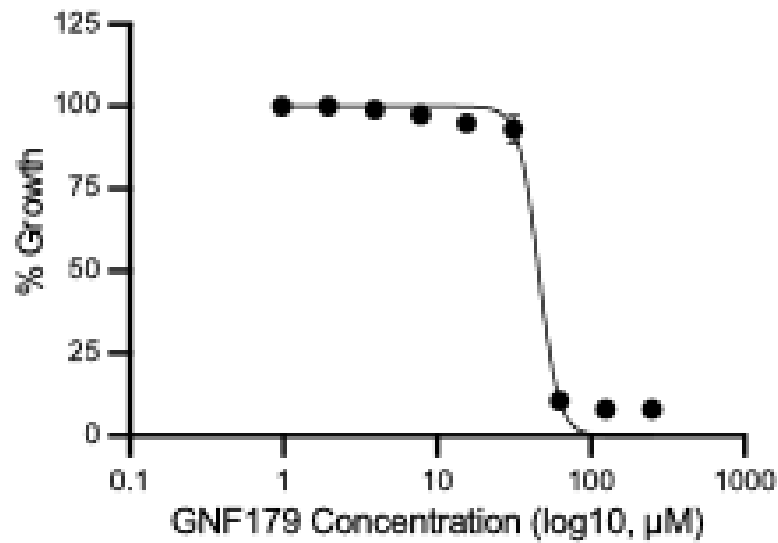


Figure 3.3 | GNF179 dose-response curve for GM+ScSEY1(S437*)-myc

The GM+ScSEY1(S437*)-myc mutant of *S. cerevisiae* yeast was treated with GNF179, from 0 μM to 250 μM , to test the potency of the compound. The IC_{50} measured was 45.7 μM . Technical duplicates are shown here.

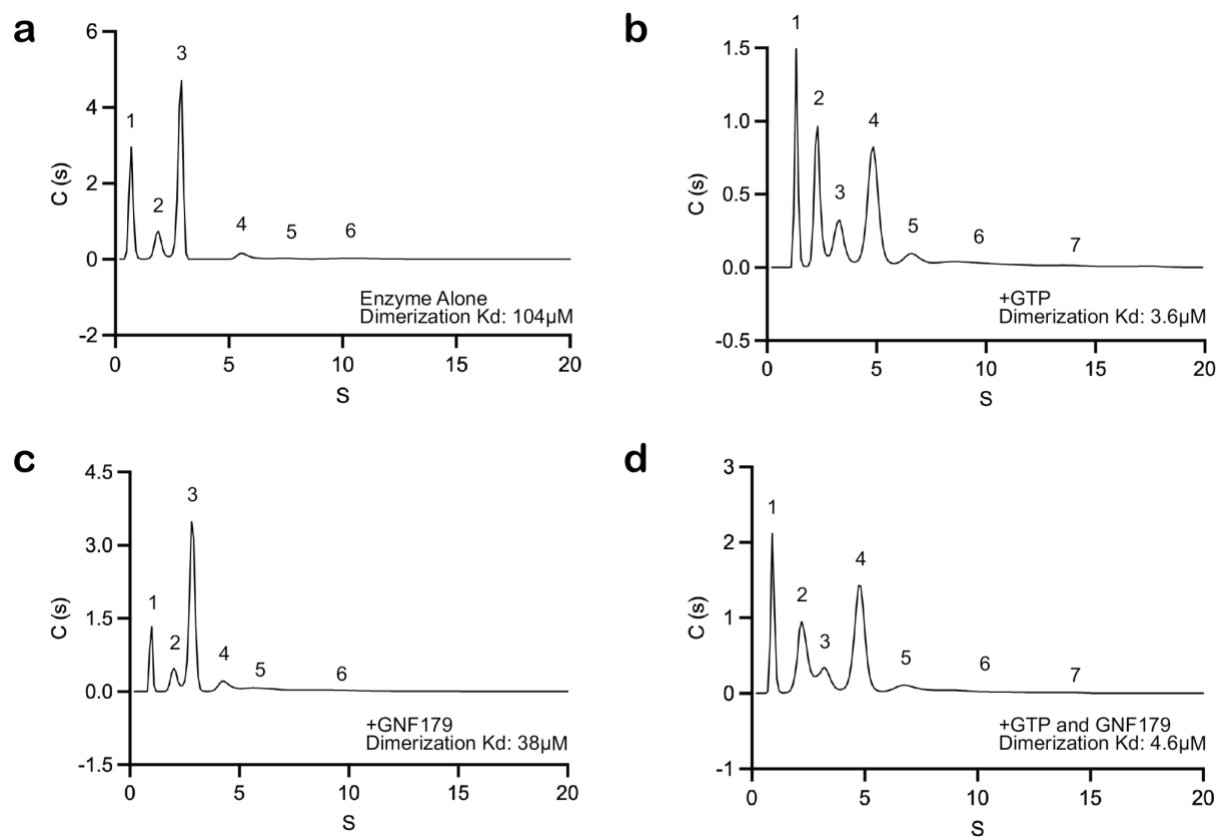


Figure 3.4 | GNF179 does not affect GTP-induced homodimerization of cytosolic *PvSEY1*

AUC results for cytosolic *PvSEY1* alone **(a)**, or treated with GTP **(b)**, GNF179 **(c)**, or both GTP and GNF179 **(d)**. Each numbered peak is reflective of a protein of increasing size. Dimerization Kd measures are displayed.

Table 3.1 | Summary of AUC results for cytosolic PvSEY1 alone

Parameters (experimental S, relative amount of protein (%), and apparent molecular weight) are calculated for each peak in the C(s) distribution.

Peak	S	Relative Amount (%)	Apparent MW (kDa)
1	0.698	26.7	11
2	1.906	11.9	52
3	2.856	52.8	95
4	5.735	4.5	270
5	7.557	1.4	409
6	10.732	2.7	692

Table 3.2 | Summary of AUC results for cytosolic PvSEY1 treated with GTP

Parameters (experimental S, relative amount of protein (%), and apparent molecular weight) are calculated for each peak in the C(s) distribution.

Peak	S	Relative Amount (%)	Apparent MW (kDa)
1	1.319	16.8	30
2	2.354	22.0	71
3	3.349	11.6	121
4	4.858	31.4	211
5	6.777	6.9	347
6	9.845	8.8	608
7	14.346	2.5	1,070

Table 3.3 | Summary of AUC results for cytosolic PvSEY1 treated with GNF179

Parameters (experimental S, relative amount of protein (%), and apparent molecular weight) are calculated for each peak in the C(s) distribution.

Peak	S	Relative Amount (%)	Apparent MW (kDa)
1	0.974	12.1	19
2	2.042	9.3	57
3	2.835	57.3	94
4	4.402	7.9	182
5	6.095	7.1	296
6	9.684	6.3	593

Table 3.4 | Summary of AUC results for cytosolic PvSEY1 treated with GTP and GNF179

Parameters (experimental S, relative amount of protein (%), and apparent molecular weight) are calculated for each peak in the C(s) distribution.

Peak	S	Relative Amount (%)	Apparent MW (kDa)
1	0.928	17.7	18
2	2.297	23.1	69
3	3.261	10.0	116
4	4.784	36.4	206
5	7.096	7.3	372
6	10.169	4.6	639
7	13.940	0.9	1,025

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Chapter 3, in part, contains material that may be prepared for publication at a future time with Dr. Elizabeth Winzeler. The dissertation author is the primary investigator and author of this material.

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