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Discovery of *Plasmodium* (M)TRAP–Aldolase Interaction Stabilizers Interfering with Sporozoite Motility and Invasion

Lauren E. Boucher^{†,‡,¶,¶}, Christine S. Hopp^{‡,§,¶,¶}, Julianne Mendi Muthmja[⊥], Friedrich Frischknecht[⊥], and Jurgen Bosch^{†,‡,¶,¶,*,#}

[†] Department of Biochemistry and Molecular Biology, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, Maryland 21205, United States

[‡] Johns Hopkins Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, Maryland 21205, United States

[§] Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, Maryland 21205, United States

[¶] Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5625 Fishers Lane, Rockville, Maryland 20852, United States

[⊥] Integrative Parasitology, Center for Infectious Diseases, Heidelberg University Medical School, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany

[#] InterRayBio, LLC, 1812 land Avenue, Baltimore, Maryland 21205, United States

Abstract

As obligate, intracellular parasites, *Plasmodium spp.* rely on invasion of host cells in order to replicate and continue their life cycle. The parasite needs to traverse the dermis and endothelium of blood vessels, invade hepatocytes and red blood cells, traverse the mosquito midgut, and enter the salivary glands to continue the cycle of infection and transmission. To traverse and invade

*Corresponding Author: jbosch@case.edu.

¶Author Contributions

L.E.B. and C.S.H. contributed equally to this work. L.E.B. was responsible for preparing the manuscript, enzyme assays, SPR assays, sporozoite trail assays, invasion assays, viability and ImageStream assays, and all primary data analysis. C.S.H. performed the live imaging microscopy, dissected mosquitoes and purified sporozoites, provided protocols and reagents for the sporozoite trail and invasion assays, and edited the manuscript. J.M.M. and F.F. performed replication of the live imaging results and data analysis. J.B. conceived of the idea for this work and analyzed ImageStream data. All authors discussed the results and contributed to the final manuscript.

[¶]J.B.: Pediatric Pulmonology Division, Department of Pediatrics, Case Western Reserve University School of Medicine, 10900 Euclid Ave, Cleveland, OH 44106, USA.

Notes

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsinfecdis.7b00225](https://doi.org/10.1021/acsinfecdis.7b00225). Supplemental Methods: live gliding replicate; Figure S1: MTRAP–aldolase interaction SPR; Figure S2: MMV TRAP–aldolase interaction promoters; Figure S3: MMV MTRAP–aldolase interaction promoters; Figure S4: MMV TRAP–aldolase k_{off} effectors; Figure S5: MMV MTRAP–aldolase k_{off} effectors; Table S1: 24 compounds ordered from Chembridge Hit2Lead; Figure S6: concentration-dependent response for 24 selected compounds TRAP–aldolase; Figure S7: concentration-dependent response for 24 selected compounds MTRAP–aldolase; Figure S8: PfAldolase activity with compounds; Figure S9: trail assay views; Figure S10: flowchart of live imaging analysis; Figure S11: Z-stacks of live imaging movies; Figure S12: replication of live imaging experiments; Figure S13: ImageStream IDEAS gating (PDF)

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cells, the parasite employs an actomyosin motor at the core of a larger invasion machinery complex known as the glideosome. The complex is comprised of multiple protein—protein interactions linking the motor to the internal cytoskeletal network of the parasite and to the extracellular adhesins, which directly contact the host tissue or cell surface. One key interaction is between the cytoplasmic tails of the thrombospondin related anonymous protein (TRAP) and aldolase, a bridging protein to the motor. Here, we present results from screening the Medicines for Malaria Venture (MMV) library of 400 compounds against this key protein—protein interaction. Using a surface plasmon resonance screen, we have identified several compounds that modulate the dynamics of the interaction between TRAP and aldolase. These compounds have been validated *in vitro* by studying their effects on sporozoite gliding motility and hepatocyte invasion. One of the MMV compounds identified reduced invasion levels by 89% at the lowest concentration tested (16 μ M) and severely inhibited gliding at even lower concentrations (5 μ M). By targeting protein—protein interactions, we investigated an under-explored area of parasite biology and general drug development, to identify potential antimalarial lead compounds.

Keywords

malaria; Plasmodium; invasion; drug discovery; protein—protein interaction

According to the World Health Organization (WHO) 2015 Malaria Report,¹ there were 198 million cases of malaria resulting in approximately 584000 deaths in 2014, with most of these deaths occurring in children under the age of 5. Malaria control and elimination programs have managed to lower the number of cases of malaria by 30% and deaths by 47% since 2000.¹ However, a new threat to these programs comes in the form of emerging drug resistance.^{2–5} The first-line treatment for malaria is artemisinin combination therapy (ACT),⁶ and it has been reported over the past decade that there is emerging resistance to artemisinin therapies in Southeast Asia, resulting in delayed parasite clearance.^{5,7,8} As the timeline for new drug development averages 12 years from discovery to US Food and Drug Administration (FDA) approval, new target identification and the development of new antimalarials are priorities.

A Plasmodium parasite must traverse and invade multiple cell types at different stages in its life cycle.⁹ The parasite employs multiple families of extracellular receptors to engage with the host environment and to glide over, invade, traverse, and egress from host cells.^{10,11} Sexual replication in the mosquito requires egress of gametocytes from red blood cells, which is mediated by merozoite-thrombospondin related anonymous protein (MTRAP),^{12,13} and traversal of the midgut wall,¹⁴ which is dependent on CTRP (circumsporozoite-thrombospondin related anonymous protein).^{15,16} Sporozoites employ thrombo-spondin-related protein 1 (TRP1) to exit from oocysts and enter the mosquito hemolymph.¹⁷ Mature

sporozoites rely on TRAP (thrombospondin related anonymous protein) and MAEBL for invasion of the salivary glands.¹⁸ During the mosquito's blood meal, sporozoites are injected into the skin and move through the dermis to invade blood vessels, following which they travel with the blood circulation to the liver, where invasion of hepatocytes initiates the liver stage of infection. Fast gliding of sporozoites is crucial for their migration to the blood vessels and therefore their infectivity.^{9,19} TRAP and TRAP-like protein (TLP)^{20–22} are necessary for parasite motility in the skin, as well as traversal and invasion of hepatocytes, in addition to CSP (circumsporozoite protein) and the apical membrane antigen 1 (AMA1).²³

These extracellular receptors are exported membrane proteins that contain an N-terminal extracellular domain with regions that mediate interactions with ligands on the host cell surface, a transmembrane helix, and a C-terminal cytoplasmic tail. CSP, TRAP, and related proteins contain type-I thrombospondin repeat (TSR) domains, which can bind heparan sulfate proteoglycans (HSPGs) on hepatocytes. TRAPs additionally contain an integrin-like I-domain which can bind to heparin.^{24,25} By their C-terminal cytoplasmic tails, the extracellular receptors connect to an actomyosin motor, which lies beneath the parasite membrane and powers the active process of traversal and invasion.²⁶

The parasite glides and invades cells using power generated by the actomyosin motor which is part of a larger invasion and gliding machinery referred to as glideosome (Figure 1). At the core of the machinery are short filaments of actin, providing the track for myosin A (MyoA) to pull on and move the parasite.²⁷ MyoA is linked to the internal structure of the parasite via the myosin tail interacting protein (MTIP), which binds the C-terminus of MyoA and links the motor to the inner membrane complex (IMC). The IMC is a structure composed of flattened membranous compartments, which contains multiple proteins that interact with the MTIP/MyoA complex on the plasma membrane side of the IMC.²⁷ The structure of the glideosome, including its cytosolic components and extracellular receptors, is detailed in the review by Boucher and Bosch.²⁸

To bridge the cytoplasmic tails of the receptors to the motor, the parasite uses the tetrameric glycolytic enzyme, fructose-1,6- bisphosphate (F1,6P) aldolase, and/or the gliding associated connector (GAC).²⁹ The C-terminal tails of multiple receptors bind in the F1,6P binding pocket of aldolase, while another face of the aldolase tetramer interacts with actin.³⁰ Aldolase has been shown to interact with the C-termini of multiple extracellular receptors, including the TRAP family proteins, and in the related apicomplexan parasite, *Toxoplasma gondii*, the TRAP orthologs MIC2/6/12 (MIC is microneme associate protein) were similarly found to interact with *T. gondii* aldolase.³¹ Additionally, AMA1 and RH1/RH2b/RH4 (RH is reticulocyte homologue protein) have been identified as aldolase interacting partners.^{30–38} Groups have been probing the essentiality of glideosome components and have found that a conditional knockout of aldolase in *T. gondii* did not inhibit invasion when parasites were cultured in a low-glucose medium,³⁹ suggesting that aldolase is dispensable for invasion in apicomplexa. However, a comparable study has not been performed in *P. falciparum* (Pf). Additionally, it had been previously noted that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can also bind adhesin tails and actin³⁷ and could compensate in an aldolase knockout parasite.

Most of the proteins that are part of the glideosome are well conserved across apicomplexa (*Plasmodium spp.*, *T. gondii*, *Eimeria spp.*, *Babesia spp.*, etc.) and represent potential targets for drugs aiming to disrupt this machinery and the parasite. An under-explored area of research in developing novel antiparasitics is to target essential protein–protein interactions. Multiple groups have been working on targeting the MyoA- MTIP protein-protein interaction to disrupt the invasion machinery,^{40,41} and work in our own lab has identified protein–protein interaction stabilizers of the TRAP–aldolase interaction that affect invasion and gliding.⁴² Co-crystallization of the C-terminal TRAP tail with aldolase³³ showed that TRAP binds to the active site of aldolase, sandwiching a conserved tryptophan between two conserved arginines. As invasion is initiated, a tight-junction is formed at the apical end of the parasite. While engaged to the extracellular environment, TRAP is translocated posteriorly, thus moving the sporozoite forward. To allow continuous forward movement, the adhesive attachments to the host cell surface must be released periodically.⁴³ To facilitate this release, the parasite employs a rhomboid protease, which cleaves the canonical rhomboid-cleavage site located in the transmembrane domain of TRAP.⁴³ Mutations to the cleavage site lead to a stop-and-go gliding phenotype *in vitro*⁴³ and a reduction of gliding speed *in vivo*,¹⁹ while showing a dramatic reduction in infectivity,⁴² highlighting the importance of robust gliding for infectivity. We hypothesized that blocking the release of TRAP from aldolase would lead to a similar phenotype.

Since the active site of aldolase is highly conserved across organisms and any compound blocking the TRAP-aldolase interaction would likely also be toxic to human cells, we decided to target the complex interaction rather than block the binding site. In this study, we have screened the Medicines for Malaria Venture's (MMV) Malaria Box⁴⁴ to find stabilizers of the TRAP-aldolase interaction. This library, a subset of the GSK- Novartis-St. Jude's library,⁴⁵ consists of 400 curated compounds that showed activity against blood stage parasites. A previously described surface plasmon resonance (SPR) screen⁴⁶ was employed to investigate the effect of these compounds on the TRAP-aldolase interaction. Compounds promoting the interaction between aldolase and TRAP or MTRAP peptides, as well as those lowering the dissociation rates, were identified. Twenty- four compounds were tested for their activity against sporozoites, studying their effect on gliding motility and invasion phenotypes. These experiments have identified a compound that reduces sporozoite gliding motility and invasion of hepatocytes. As part of the MMV library, which was specifically screened against blood stage parasites, the identified compound has activity at several stages of the parasite life cycle.

RESULTS

Identification of Compounds Targeting the TRAP-Aldolase Interaction.

Recombinant MBP (maltose binding protein) tagged *P. falciparum* TRAP and MTRAP peptides, referred to as MBP-TRAPpep and MBP-MTRAPpep from here on, were produced in *E. coli* and used as analyte in a surface plasmon resonance (SPR) assay as previously described in Boucher and Bosch.⁴⁶ Recombinant aldolase was covalently linked to the chip, and compounds mixed with MBP-(M)- TRAPpep were screened to identify compounds in the MMV library that promoted the MBP-(M)TRAPpep-aldolase interaction. Example SPR

curves for the four expected outcomes, control (dimethyl sulfoxide, DMSO), promoter, inhibitor, or decreased off-rate compound, are shown in Figures 2a-d. The concentration of MBP-(M)TRAPpep used in the screen was selected so that the peptide occupied ~50% of the aldolase binding sites on the chip (Figure 2a). This concentration allowed us to screen for both promoters and inhibitors of the interaction (Figure 2b,c) by identifying compounds that increased or decreased binding of peptides. Kinetic fits were performed to identify compounds that lowered the off rate (k_{off}) of MBP- (M)TRAPpep from aldolase (Figure 2d).

MBP-(M)TRAPpep mixed with DMSO (control) or one of 400 MMV compounds at a final concentration of 10 μM was injected over the aldolase-conjugated chip. Multiple compounds that promoted and inhibited the interaction of MBP-TRAPpep (Figure 2e) or MBP-MTRAPpep (Figure S1) with aldolase were identified. The total response units were normalized to the DMSO control response, and compounds were grouped into promoters (positive response), inhibitors (negative response), and nonbinders (same as baseline). We identified 10 compounds that promoted the MBP-TRAPpep—aldolase interaction (Figure S2) and 10 compounds promoting the MBP-MTRAPpep—aldolase interaction (Figure S3). Compounds that lowered the off rate (k_{off}) of the peptides were also identified (Figures S4 and S5). Twelve MMV compounds identified in the screen were available from the ChemBridge Hit2Lead catalog. We ordered an additional 12 compounds that had similar 2D chemical structures to MMV compounds that were either unavailable from Hit2Lead or shared a common scaffold with the MMV hits, resulting in a new library of 24 compounds for dose-dependent testing *in vitro* (Table S1).

Concentration curves for the 24 compound library were determined via SPR to confirm their ability to promote the MBP- (M)TRAPpep—aldolase interaction in a dose-dependent manner (Figures S6, S7, and 2f). The concentration-dependent effect was assessed for each compound against the MBP-TRAPpep—aldolase interaction (Figure S6) and the MBP-MTRAPpep—aldolase interaction (Figure S7). Of the 24 compounds, 3 displayed concentration-dependent promotion of the MBP- TRAPpep—aldolase interaction. Compounds 3, 4, and 21 promoted the MBP-TRAPpep—aldolase interaction (Figures 2f and S6). The MBP-MTRAPpep—aldolase interaction was promoted in a concentration-dependent manner by 4 compounds. Compounds 1, 3, 4, and 17 promoted the interaction of MBP-MTRAPpep and aldolase (Figure S7). Binding falls off at the highest concentrations of 17. A possible explanation for this behavior is a compound solubility issue or that, at high concentrations, MBP-MTRAPpep displacement occurs. Compound 2 seemed to promote an interaction of MBP-(M)TRAP, however, upon closer analysis of the binding curves, the response was much greater than the expected response, calculated on the basis of chip ligand density. This high level of response indicates that there is either a compound solubility issue or a nonspecific interaction resulting in aberrant binding levels.

Preliminary parasite testing directed our selection of a subset of the 24 compounds for further testing. A sporozoite trail assay (data not shown), performed at a high compound concentration, was used to select 5 of the 24 compounds (**3**, **4**, **17**, **18**, and **23**) for further characterization of their effects on gliding motility and ability to invade cells. The structures and MMV and Chembridge Hit2Lead library IDs for these 5 compounds are found in Table 1, and the concentration-dependent SPR response against the MBP-TRAPpep—aldolase

interaction is shown in Figure 2f. Compounds 18 and 23 were selected for further testing on the basis of a reduction in sporozoite gliding; however, these compounds do not promote the MBP-(M)TRAPpep—aldolase interaction in a concentration-dependent manner (Figure 2f).

We proceeded to test compound **18** because it was the only compound in the top 5 hits not present in the MMV library and was of interest as a potentially new antiparasitic. Compound **17** did not show promotion of the interaction, but we investigated this compound because, at high concentrations, SPR indicated that MBP-(M)TRAPpep was displaced from aldolase. Compound **23** was chosen solely on the basis of initial gliding reduction levels, even though it did not promote the interaction by SPR. Only compounds 3 and 4 displayed concentration-dependent promotion of the MBP-TRAPpep—aldolase interaction via SPR (Figure 2f). These two compounds also displayed a concentration-dependent promotion of the MBP-MTRAP-pep—aldolase interaction (Figure S7). Also, compound 21 had been noted as a MBP-TRAPpep—aldolase interaction promoter in the SPR assay (Figure S6); however, it failed to decrease binding compared to the five selected compounds in the preliminary trail assay (data not shown), so we did not proceed with this compound.

Proceeding with the top 5 compounds from the preliminary trail assay, compounds **3**, **4**, **17**, and **18** were assessed for their ability to inhibit aldolase's enzymatic activity (**23** was not tested due to limited compound availability) in the presence and absence of synthetic *P. berghei* TRAP peptide (PbTRAP6, SEQ:EDNDWN), an ortholog of *P. falciparum* TRAP. TRAP peptides bind in the active site of aldolase, overlapping with the fructose-1,6-bisphosphate (F1,6P) binding site, preventing its catabolism (Figure 3a). Using a coupled enzyme assay system involving triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and nicotinamide adenine dinucleotide (NADH), it was possible to measure aldolase activity in the presence and absence of peptide and/or compound by observing NADH consumption at 340 nm, reflecting F1,6P breakdown (Figure 3b). Aldolase activity levels were normalized to the activity in the presence of PbTRAP6 (set to 100%). Since the PbTRAP6 concentration was chosen to reduce activity by half, removal of PbTRAP6 results in ~200% aldolase activity (Figure 3c).

In the absence of PbTRAP6, no compounds inhibited *P. falciparum* aldolase (Figure S8) or rabbit muscle aldolase (Figure 3d), which shares 98% sequence identity with its human homologue. These results indicate that the compounds alone do not inhibit enzymatic function of human or parasite aldolase and that any observed decrease in PfAldolase function in the presence of compound and PbTRAP6 is due to promotion of PbTRAP6 binding. To test its ability to inhibit in the presence of compound, PbTRAP6 was added to the aldolase reaction at a concentration necessary to obtain a baseline 50% inhibition of the enzyme. Compound was added at 200, 100, and 50 μM to determine its effect in further inhibiting the enzyme. The results showed that compounds 3 and 4 inhibited aldolase activity in a dose-dependent manner in the presence of PbTRAP6, while compound 17 (tested only at 50 μM due to solubility issues) showed no effect (Figure 3c). These results are consistent with those obtained from SPR experiments, indicating that compounds 3 and 4 have a dose-dependent effect on MBP-TRAPpep-aldolase binding, while compound 17 has no effect. Surprisingly, compound 18 increased enzymatic activity in the presence of PbTRAP6 yet did not increase activity of aldolase in the absence of peptide. It is possible

that compound 18 displaces PbTRAP6, while still allowing F16BP binding, leading to higher levels of activity. However, since disruption of MBP-TRAP_{pep} binding to aldolase was not observed in the SPR assay, the compound may be affecting the activity of another enzyme in the coupled assay. Currently, we are unable to fully explain these results and further follow-up studies are needed.

Characterization of Compounds in Sporozoite Trail Assays.

Compounds that were identified via SPR as promoters of the TRAP-aldolase interaction were tested for their effect on sporozoite motility *in vitro*. As sporozoites glide on glass coverslips, they leave behind trails of shed material, which can be visualized by detection of CSP. For this trail assay, *P. berghei* sporozoites were purified from mosquito salivary glands and incubated with the compounds prior to being allowed to glide on glass slides. Following detection of CSP trails and associated sporozoites, parasites were counted and binned into groups of no gliding (0 circles), low gliding (<10 circles), medium gliding (10–20 circles), or high gliding (>20 circles). Results for four different concentrations of the five compounds are shown in Figure 4a, and representative fields of view for sporozoites in the presence of each compound are shown in Figure S9.

In the DMSO-treated control sample, more than 90% of sporozoites deposited trails, with >60% classified as medium or high gliding (>10 trails). All compounds inhibited gliding throughout the concentration range tested (250–31.25 μ M). Compounds 4 and 18 had the greatest effect on gliding motility: at 250 μ M, 4 and 18 reduced total sporozoite gliding levels to below detectable levels, and only at lower concentrations did the sporozoites recover some gliding ability, demonstrating a dose-dependent effect. The most dramatic reduction in gliding was seen with compound 4. When sporozoites were tested at the lowest concentration (31 μ M), less than 60% of the sporozoites deposited trails and, of those, 90% deposited less than 10 circular trails; most sporozoites left 1 incomplete circular trail (Figure S9). For compound 18, less than 25% of sporozoites were able to glide at the lowest compound concentration tested and ~75% of those sporozoites were classified as low gliding, with fewer than 10 trails deposited; however, these low gliding parasites typically deposited more than 1 trail, unlike the low gliding sporozoites in the presence of compound 4, which were unable to glide for more than 1 trail.

These results indicate that the compounds affect sporozoite gliding motility in a concentration-dependent manner with 4 and 18 causing the most significant reduction in gliding. On the basis of these results, compounds 4 and 18 were selected for more in-depth studies of sporozoite gliding motility and hepatocyte invasion assays.

Compounds 4 and 18 Reduce Sporozoite Gliding.

In light of the reduction in gliding motility in the presence of compounds 4 and 18, we decided to perform live imaging studies of sporozoites to further measure the impact of compounds on sporozoite motility. Live imaging allows us to measure additional parameters including gliding velocity and type of gliding motility that could not be assessed using the static sporozoite trail assay. Live imaging of sporozoites gliding on glass slides *in vitro* was performed using *P. berghei* parasites that constitutively express the fluorescent protein

mCherry under control of the *uis4* promoter,¹⁹ and parasites were tracked to determine gliding velocity, radius of circular gliding pattern, and total time gliding (Figure S10). Compounds 4 and 18 were tested at 3.9 and 62.5 μM , respectively. Compound 4 was tested at a lower concentration compared to 18 as it was not possible to obtain a sufficient number of gliding parasites for analysis at higher concentrations. Gliding videos can be found in Movies S1-S9. Live imaging showed that DMSO treated control sporozoites moved at an average velocity of $2.58 \pm 0.05 \mu\text{m s}^{-1}$ (Figure 5c), while compound 18 (62.5 μM) reduced average gliding velocity to $1.88 \pm 0.17 \mu\text{m s}^{-1}$ and compound 4 reduced average gliding velocity to $1.81 \pm 0.09 \mu\text{m s}^{-1}$. These average velocities represent a significant ($p < 0.0001$, unpaired *t* test) reduction in gliding velocity of 27% and 30% for compounds 4 and 18, respectively, compared to the control.

Compounds 4 and 18 Cause Sporozoites To Move in a Stop-and-Go Pattern and Increase Trail Radius.

While performing the live imaging studies, it was observed that parasite motility was more frequently interrupted in compound-treated samples, resulting in a stop-and-go phenotype characterized by pauses between periods of circular gliding. This pattern was reminiscent of the staccato motility described for the TRAP rhomboid cleavage site mutants.^{19,43} To determine if there was a significant increase in stop-and-go movement, videos were analyzed to determine the total amount of time spent gliding for each parasite. The total time spent gliding by a parasite was significantly less for both compounds 4 and 18 ($p < 0.05$ and $p < 0.005$, respectively), at an average of 39 and 25 s of gliding, compared to the control, which averaged 72 s of gliding (Figure 5d). Regarding the fraction of time that sporozoites were paused during gliding, sporozoites treated with either compounds 4 or 18 deviated significantly ($p < 0.0001$ and $p < 0.05$, respectively) from the DMSO control, in which sporozoites paused on average for only 7% of the time during active blocks of gliding (Figure 5e). Compound 4 increased pausing to 32%, while compound 18 increased pauses to 16%.

It was noted while observing both videos and analyzing the trail assay slides that the radius of the circular pattern in the presence of compounds was altered. To determine if there was a significant difference from the control, the radii of the trails were measured in the presence of the five compounds that were tested at 62.5 μM and DMSO in the static trail assay. Higher concentrations of compound could not be used for analysis since there were too few trails for a statistical analysis. The average radius of the circular trail deposited by sporozoites in the trail assay increased in the presence of compounds 4, 17, and 18 ($p < 0.0001$) (Figure 5a). The increase in average trail radius was 3.2 μm (58%), 1.1 μm (20%), and 1.8 μm (33%), for compounds 4, 17, and 18, respectively, compared to the DMSO control. Similarly, in the live imaging experiments, treatment of sporozoites with compounds 4 and 18 resulted in trails with larger radii (Figure 5b); however, the effect change in radius was smaller for compound 4 and less significant ($p < 0.002$). A possible explanation for the discrepancy between trail assay and live gliding is that very slow moving sporozoites are responsible for the large radii observed in the static sporozoite trail assays. The incubation time of 1 h for the trail assays allows for capture and measurement of trails of slow moving parasites, while they are not captured in the 5 min-long live imaging videos.

Sporozoites Treated with Compounds 4 and 18 Exhibit Altered Motility Patterns.

Additional movement patterns have previously been defined⁴⁷ and can be used to classify motile sporozoites into categories such as “circular gliding”, “waving”, and “attached with no movement”. Parasite motility in the live imaging studies was classified manually into one of two categories: “circular gliding” and “waving” (Figure 5f). Waving motility is characterized by parasite attachment at one end and large angular changes in orientation as opposed to a smooth translation along a path. Note that drifting and stationary parasites were not binned in this analysis (Figure S11). Examples of these motility behaviors, as they appear in maximum projections of videos, are shown in Figure 5f. Both compounds 4 and 18 changed the ratio of gliding to waving sporozoites. In the control, nearly 85% of sporozoites are gliding circularly and 15%, waving, whereas 72% and 66% are waving in compound 4 and 18 treated samples, a 6-fold and 4-fold increase, respectively. To ensure our observations were indeed significant, our collaborators in the Frischknecht laboratory repeated a similar live imaging experiment using our compounds with their green fluorescent protein (GFP) expressing *P. berghei* sporozoites and standard assay conditions. They were able to confirm that the ratio of gliding, waving, and attached, but not moving, sporozoites was affected by the compounds (Figure S12).

Characterization of Viability of Compound-Treated Sporozoites.

As we started to think about compounds having an unintended effect on sporozoites, such as cytotoxicity, which may influence the motility changes, we wanted to determine if the compounds were cytotoxic. To assess cytotoxicity, a sporozoite viability assay was performed using propidium iodide (PI), a membrane-permeable DNA-binding molecule in an exclusion assay. Following incubation with DMSO, 85% of control sporozoites were viable, while 250 μ M of compound 4 reduced viability to 79% and compound 18 provided a protective effect, increasing viability to 93% (Figure 6a).

Additionally, a two dye LIVE/DEAD viability assay was used, which allowed for the measurement of both membrane permeability, a marker of death, and enzyme activity, a marker of viability (Figure 6b). While only nonviable cells are permeable to the ethidium homodimer-1, all cells are permeable to the calcein-AM dye; however, this dye is only fluorescent when cleaved by esterases in metabolically active cells. This assay provides a two-color readout of red for cell death and green for live, metabolically active parasites. After incubation with compounds and dyes, 96% of DMSO treated sporozoites were metabolically active. Compounds 4 and 18 at 62.5 μ M reduced sporozoite viability to 82% and 88%, respectively. This decrease in viability in the presence of compound does not account for the large decrease in sporozoite motility.

Finally, imaging flow cytometry with the Amnis ImageStream Mark II was used to quantify sporozoite death using an amine reactive dye that penetrates nonviable parasites, reacts with free amines, and causes nonviable parasites to fluoresce (Figure 6c). The advantage of this protocol was the ability to fix the sporozoites after incubation with the dye and reduce variability due to sample processing delay. Following incubation with DMSO or compounds, it was determined that compounds 4 and 18 did not decrease sporozoite viability compared to the control. This assay was performed using mCherry-expressing sporozoites, and it was

observed that mCherry fluorescence was inversely correlated with sporozoite death, likely due to the sensitivity of mCherry to a possible pH change that may occur upon sporozoite death, suggesting that constitutively expressed mCherry can be used as viability indicator.

While performing viability experiments with the mCherry sporozoites using ImageStream, altered parasite morphology was observed in compound-treated samples. In the control sample, sporozoites typically presented in elongated crescent shapes; however, upon addition of compound, an increased percentage of sporozoites displayed an altered morphology, consisting of an elongated crescent shape with a bulge or membranous vesicle present at one tip (Figure 6e,f). The ImageStream, with its flow assisted cell sorting (FACS) and microscopy ability, allowed us to obtain statistics for this population by gating on the mCherry population (live sporozoites) and performing further gating of sporozoites based on the imaging features such as homogeneity and compactness (Figure S13). We quantified the effects of the compound (Figure 6d) and found that less than 3% of live sporozoites in the control presented with the external membranous vesicles at the tip. While compound **18** had little effect on the morphology of sporozoites at the concentrations tested, compound **4** increased the population of live sporozoites with attached extracellular vesicles. In live gliding assays, at 15.6 μM of compound **4**, 11.5% of live sporozoites displayed external apical vesicles, while at 62.5 μM this population increased to 20% of live sporozoites. The presence of the mCherry signal for these parasites and exclusion of the amine reactive dye indicates that these are not dead sporozoites, though the vesicle does not exclude the dye and this maybe the start of cell death (Figure 6). Alternatively, the composition of the vesicle membrane (if there is one) may be different compared to the sporozoite body and may not be able to exclude the amine reactive dye.

Assessing Reversibility of Compound Treatment Effects.

To determine reversibility of compound effects, the static trail assay was performed with sporozoites that were first treated with compounds for 15 min and then washed and resuspended in fresh media in the absence of compound and subsequently allowed to glide (Figure 4b). The effect of compound **18** was completely reversible at 15.6 μM . After incubation with higher concentrations of compound **18** (31.25 and 62.5 μM), the washed parasites did not recover to control levels. The effects of compound **4** were not reversible at any of the concentrations tested; washed parasites did not recover to glide at the high levels observed in the control. These results indicate that the effect of compound **18** is not reversible at higher concentration, while the effects of compound **4** are not reversible at any concentration.

Interestingly, upon compound **4** washout, the sporozoites that resumed a low level of gliding moved in a weaving gliding pattern (different from the previously mentioned high angular change waving pattern) instead of the typical circular movement (Figure 4c). This weaving pattern of gliding had been previously observed by Kortagere et al.⁴¹ when testing the effects of a pyrazole-urea compound designed to target the MTIP-MyoA interaction, a key protein—protein interaction in the invasion/gliding motility machinery (Figure 1). On the basis of a similar pattern, we anticipate that compound **4** is targeting the invasion machinery, as did the pyrazole-urea compounds, but by promoting the TRAP—aldolase interaction

rather than disrupting the MTIP—MyoA interaction. Both protein interactions are part of the larger glideosome or invasion machinery.

Compounds 4 and 18 Inhibit Sporozoite Invasion of Hepatocytes.

It has been reported that fast gliding of sporozoites is necessary for efficient traversal of the blood vessel and entry into the bloodstream.¹⁹ With the significant reduction in gliding speed of sporozoites in the presence of compounds **4** and **18**, sporozoites may not be able to efficiently invade host cells. To assess whether the observed gliding motility defects would have an impact on hepatocyte invasion, sporozoites were added to a monolayer of hepatocytes (human hepatocyte carcinoma cell line (HepG2)) and allowed to invade in the presence of the compounds. An inside/outside staining protocol⁴⁸ was used to determine the percentage of invaded sporozoites. Two rounds of triplicates were performed with DMSO control and three concentrations each of compounds **4** and **18** (Figure 7a). In the DMSO control sample, 63% of the sporozoites invaded HepG2 cells after 2 h. Both compounds **4** and **18** caused a dose-dependent inhibition of invasion. At the lowest concentration tested (15.6 μ M), compound **4** decreased invasion by 89% compared to the control. At 62.5 μ M, compound **18** decreased invasion by 97%, but at 15.6 μ M, it only decreased invasion by 24%. Viability of HepG2 cells in the presence of compound was assessed using a PI assay (Figure 7b). At the highest compound concentration of 62.5 μ M, compounds **4** and **18** reduced HepG2 viability by 9% and 6%, respectively, which does not account for the large decrease in invasion in the presence of compound.

DISCUSSION

Here, we identified compounds for a nontraditional target, the glideosome, that inhibited parasite motility and invasion. Using SPR, we were able to show that these compounds stabilize the TRAP—aldolase interaction and we further characterized these compounds in *in vitro* parasite motility assays. Of the 24 compounds tested, ~25% displayed a dose-dependent promotion of the (M)TRAPpep and aldolase interaction. We assessed whether compounds could reduce total numbers of sporozoites gliding, levels of gliding, velocity, and time spent gliding. During the analysis phase, unique gliding phenotypes were noted and analyzed including altered movement patterns, ratio of circularly gliding and waving parasites, the radius of sporozoite trails, and unique morphology changes. Viability assays were used to assess possible off-target/cytotoxic effects. Multiple assays confirmed that the compounds were not acting solely through cytotoxic mechanisms. Especially at lower concentrations, the compounds had little to no negative effect on either sporozoite or HepG2 viability but specific effects on sporozoite motility and invasion. Together, these cellular assays and phenotypic changes, along with the SPR interaction data, suggest that compound **18** is affecting the gliding and invasion ability of the parasite, yet may not be doing so through promotion of the TRAP—aldolase interaction as we do not observe dose dependency in SPR or the aldolase activity assay. Compound **18** is not derived from the MMV library and should be further studied for its antiparasitic potential and to determine a mode of action. However, these data suggest that compound **4** is likely targeting the TRAP—aldolase interaction in parasites in a dose-dependent manner.

Compound 4 did not inhibit rabbit aldolase activity and did not inhibit PfAldolase in the absence of TRAP peptide. Static trail assays showed that compound 4 reduced the total number of gliding sporozoites and levels of “high gliding” sporozoites in a dose-dependent manner. In the live imaging studies, 4 reduced the velocity of gliding parasites. More importantly, it shifted the equilibrium of sporozoites circularly gliding to a waving motion, which could be a phenotype of direct invasion machinery effect or a sign of strong local attachment without the ability to release as seen in the TRAP cleavage site mutants. Compound 4 also increased the radius of the sporozoite trails in both the static and live imaging gliding assay, suggesting an effect on parasite cytoskeletal structure and curvature. While compound 4 effects were not fully reversible, we found that, when washed out, sporozoites that resumed gliding did so in a wavy pattern. This pattern is highly similar to a pattern caused by the presence of the MTIP—MyoA inhibiting pyrazole-urea compounds. We hypothesize that removal of the compound from the sporozoite is not completely reversible and that some remains bound to the TRAP—aldolase complex or within the parasite; however, when the free compound is washed away, over time, free aldolase and TRAP that had not already complexed with compound or newly synthesized protein were able to restore gliding.

Another phenotypic abnormality was the stop-and-go movement observed in live imaging studies. A similar effect was recently shown for GFP-tagged LIMP protein.⁴⁹ Compound 4 caused the parasites to begin moving in a stop-and-go manner, increasing the pause between movements. While this pattern could be the result of energy production issues, it could also indicate targeting of the TRAP—aldolase interaction by preventing their dissociation similar to the rhomboid protease resistant TRAP mutant phenotype.⁴³ We speculate that if aldolase is not released from TRAP, it cannot be recycled to bind more receptor tails to continue invasion and gliding. The stop- and-go pattern may be a manifestation of the time that it takes to recruit more aldolase to the machinery. Alternatively, TRAP—aldolase interactions might be needed to produce substrate adhesion, a prerequisite of gliding, and too much adhesion might lead to parasites being stuck on the substrate.^{50–52} Furthermore, as most of TRAP is bound on micronemes, aldolase might bind to TRAP in these vesicles and inhibit their export hence lowering the amount of TRAP available on the surface of the sporozoite.⁵³

The feature of vesicle exocytosis in the presence of compound 4 was unexpected. This feature of attached extracellular vesicles had previously been described by Mota et al.⁵⁴ In that study, apical-regulated exocytosis was observed and sporozoites formed an extracellular vesicle or “cap” at the apical end. This unique morphology could be triggered by addition of ionomycin, a Ca²⁺ ionophore, which raises intracellular calcium levels. Interestingly, the vesicles that were observed stained positive for both EBA-175 and TRAP on the surface. It is possible that the vesicles that we observe are similar. The compound may affect signaling across TRAP (either inside out or outside in),^{52,55} leading to induced exocytosis, or promote exocytosis through another off-target mechanism. Clearly, more work is necessary to understand the action of this small molecule on sporozoites.

SPR and enzyme assays indicated an effective concentration of the compound in the 50–200 μ M range. However, in the sporozoite studies, compounds were effective at much lower

concentrations. Compound 4 showed significant inhibition of invasion at 15 μ M and changes in gliding phenotypes at 4 μ M. It is thus possible that compound 4 has another target in the sporozoite that also has consequences for invasion and gliding. Alternatively, the effective concentration in parasites could be lower than the concentration required in protein assays to see 50% inhibition. It is possible that only a few TRAP–aldolase interactions need to be affected in order for an invasion or gliding motility defect to be observed. Furthermore, a small change in gliding motility and invasion may also be enough to significantly reduce infection.

How these compounds can be applied to stop sporozoites from crossing the skin or invading the liver will require further research. Additionally, while the identified compounds (except 18) have been described to decrease blood stage growth,⁴⁴ future work needs to determine whether the compounds affect merozoite invasion of red blood cells. No targets for compound have been identified according to either PubChem or our knowledge. There are issues to consider when proceeding with this compound for derivatization. Compound 4 is annotated as a tool compound by MMV, indicating that it has properties that exclude it from being “drug-like”. One of these is its dibromoaromatic property, which is not often seen in “drug-like” molecules, due to the fact that bromoaromatics can form reactive metabolites and therefore have safety risks. It may be possible with medicinal chemistry to work on replacing the bromine with a safer chlorine atom. Without a structure of the compound bound to the TRAP–aldolase complex, libraries of compound would likely need to be generated and retested. Due to the rather high concentrations at which the compounds were tested and are apparently effective, it is unclear how much the compound binding can be improved with medicinal chemistry efforts, especially as we envision a multistage invasion inhibitor. However, this work serves as a proof of concept for a compound library screen for promoters of a particular protein–protein interaction, identifying compounds with measurable phenotypic effects.

METHODS

MMV Library Screening via Surface Plasmon Resonance.

PfAldolase was expressed in *E. coli*, purified, and covalently attached to a CM5 sensor chip (GE Healthcare) as previously described.³³ MBP-TRAPpep and MBP-MTRAPpep constructs coding for the 12 and 14 terminal residues of *P. falciparum* TRAP (SEQ: EQFRLPEENEWN) and MTRAP (SEQ: DNKAMDEEEFWALE), respectively, had been previously designed.⁴⁶ The analytes, MBP-TRAPpep and MBP-MTRAPpep, were expressed and purified from *E. coli*, as described previously,⁴⁶ and concentrated to 3 and 1.57 mg/mL, respectively.

The Medicines for Malaria Venture’s (MMV) Malaria Box⁴⁴ compounds were screened against the PfAldolase—MBP-(M)-TRAPpep interactions using a Biacore 3000 (GE Healthcare). The running buffer of 10 mM HEPES, pH 7.5, 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.005% P20, and 1% DMSO was filtered and degassed overnight. MBP-TRAPpep and MBP-MTRAPpep were diluted to 0.03 and 0.018 mg/mL, respectively, in running buffer without DMSO. The compound library stock solutions were diluted to 1 mM in DMSO for a working stock. In a 96-well round-bottom

plate, 1 μL of the compound working stock was added to 99 μL of either MBP- TRAPpep or MBP-MTRAPpep solution. The reaction contained a final concentration of 10 μM of compound and 1% DMSO. For the control reactions, 1 μL of DMSO was added instead of the compound. Additionally, blanks and a DMSO calibration curve were included during the runs. Plates were briefly centrifuged to clear precipitated compound. The screening was performed at room temperature, with a single 50 μL sample injection passed over the CM5- PfAldolase chip at 40 $\mu\text{L}/\text{min}$, with a 70 s contact time and 100 s dissociation time. The aldolase chip was regenerated by injecting 8 μL of 2 M MgCl_2 . All SPR data reduction and analysis was performed using Scrubber 2.0 (BioLogic) using the double referencing method and application of a DMSO calibration curve.

Hit Compound Concentration Dependence via Surface Plasmon Resonance.

Identified compounds were ordered from the ChemBridge Hit2Lead service. Compounds were resuspended in DMSO to a concentration of 50 mM. To test the concentration-dependent effect of the compounds, a 2-fold dilution series of compound was prepared in DMSO and added to reaction buffer containing either MBP-TRAPpep or MBP-MTRAPpep. The final compound concentrations ranged from 200 μM to 195 nM in 11 reactions, with 1% final DMSO concentration, and MBP-TRAPpep and MBP-MTRAPpep final concentrations were 0.06 and 0.17 mg/mL, respectively. Control samples were prepared with DMSO instead of compound, and blanks and DMSO calibration series were included in the run. Triplicate injections of 50 μL were passed across the CM5- PfAldolase chip at 50 $\mu\text{L}/\text{min}$ with a 60 s dissociation time and regeneration with 10 μL of 2 M MgCl_2 between each analyte injection.

Aldolase Coupled Enzyme Assay.

Activity assays were performed using methods previously described.³³ Briefly, assays were performed in 0.2 M glycine buffer titrated with 1 M Tris to pH 7.3. To measure activity, aldolase was added to a reaction mixture containing fructose 1,6 bisphosphate (F1,6P), triose- phosphate isomerase (TIM), glycerophosphate dehydrogenase (GDH), and NADH. Final enzyme concentrations for PfAldolase and rabbit muscle aldolase (Sigma) were 2.5 and 5 nM, respectively. Consumption of NADH, proportional to aldolase activity, was measured at 340 nm using a Spectramax Plus 384 plate reader (Molecular Devices).

For compound testing, PbTRAP peptide (PbTRAP6, SEQ: EDNDWN) was added to the reaction mixture for a final concentration of 250 μM . This concentration provided an approximate 50% reduction in V_{max} of aldolase activity and represented a baseline. Compounds were added to this reaction at final concentrations of 200, 100, and 50 μM to study the dose- dependent effect on TRAP binding. For testing of direct inhibition of PfAldolase and rabbit muscle aldolase by the compounds, reactions were prepared in the absence of PbTRAP. Mean and standard error mean were calculated, and unpaired t test analysis was performed.

Sporozoite Trail Assay.

Sporozoite trail assays were performed as described previously.⁴³ *P. berghei* ANKA GFP sporozoites were dissected from the salivary glands of *Anopheles stephensi* 18–21 days

postblood meal into Dulbecco's Modified Eagle's Medium (DMEM). A 2-fold dilution series of compound or DMSO was prepared in DMEM and added to tubes containing 1.5×10^5 sporozoites each and incubated at 18 °C for 10 min. DMEM containing 2% bovine serum albumin (BSA) and $1 \times$ final compound concentration was added to the reaction resulting in a final concentration of compound ranging from 250 to $31.25 \mu\text{M}$, 0.5% DMSO, and 0.8% BSA (no replicates). The sporozoites were added to glass coverslips coated with mouse anti-CSP mAb 3D11⁵⁶ in a 24-well plate, spun down onto the coverslips, and incubated at 37 °C for 1 h. Sporozoites and CSP trails were subsequently fixed with 4% paraformaldehyde (PFA) and detected with biotinylated mAb 3D11 and streptavidin-fluorescein isothiocyanate (FITC; Invitrogen). Slides were mounted and visualized to count the number of CSP trails. Imaging was performed using a Nikon E600 microscope, and images were acquired with DS-Ri1. Sporozoites were classified into four bins on the basis of the number of trails deposited for 200–250 sporozoites per concentration per compound. The radii of trails were measured for 10–20 fields of view using Fiji,⁵⁷ measuring 130–150 trail radii for parasites in the presence of $62.5 \mu\text{M}$ compound. The mean and standard error mean were calculated, and unpaired *t* test analysis was performed.

Compound washout studies were done at 4 concentrations (2-fold dilutions, 62.5 – $7.8 \mu\text{M}$) in a similar manner. Sporozoites were incubated with compound for 10 min at 18 °C followed by a 10 min incubation at 37 °C (no replicates). Sporozoite samples were split, washed with DMEM, and resuspended in 1% BSA/DMEM with either DMSO for the washout experiment or compound for the nonwashout experiment. Sporozoites were then added to coverslips and allowed to glide for 1 h at 37 °C. Trails for 200–250 sporozoites were detected and counted as previously described. The mean and standard error mean were calculated.

Live Imaging Gliding Motility.

P. berghei ANKA sporozoites expressing mCherry under control of the *uis4* promoter¹⁹ were dissected in DMEM from the salivary glands of *A. stephensi* 18–21 days postblood meal. Sporozoites were preincubated at room temperature with compounds for 10 min and then mixed with DMEM containing 2% BSA and compound ($N = 2$). Five μL of this solution, containing a final concentration of 0.8% BSA, 0.5% DMSO, and 62.5 – $3.9 \mu\text{M}$ of compound, was added to a 14 mm glass bottom dish (MatTek), covered with a round coverslip, and incubated for 5 min at 33 °C to allow sporozoites to settle onto the surface. A Nikon TE200 inverted microscope with a heated stage at 33 °C was used to collect videos, 200 frames (314 s) in length at 1.57 s per frame, for each compound concentration. Sporozoites were tracked using Fiji⁵⁷ and the ToAST module.⁴⁷ Tracking data was analyzed using Excel (Microsoft) and algorithms similar to those in the ToAST module (Figure S10).

Gliding velocity was determined for sporozoites gliding continuously for more than 6 frames (9.4 s). As sporozoites frequently paused and restarted gliding motility, velocity was determined as a weighted average of the average velocity for each block of continuous gliding (>6 frames). A block of active gliding was determined as gliding in one direction with pauses of less than 10 consecutive frames (15.7 s). During a block of active gliding, the amount of time that the sporozoites were paused was measured and used to calculate the

fraction of time that a parasite would spend “stopped”. Gliding radius was determined at each frame by calculating the radius from the positional x , y data from the current frame and previous two frames, using the three points on a circle to calculate the arc radius, and averaged for frames where the parasite was actively gliding (>6 frames). Mean and standard error mean were calculated, and unpaired t test analysis was performed.

To classify sporozoite motility pattern, manual assignment of sporozoite motion was performed by analyzing the maximum projection over time image. Videos were transformed into maximum projection images over time using Fiji⁵⁷ (Figure S11).

HepG2 Invasion Assay.

Invasion assays, fixing, and immunofluorescence detection with sporozoites and HepG2 cells were performed using a previously described method.⁴⁸ Briefly, adherent HepG2 cells were cultured in Complete Medium (DMEM, 2% Pen-Strep, 10% fetal bovine serum (FBS)) at 37 °C with 5% CO₂. Rat tail collagen coated glass coverslips (Neuvitro) were added to 24-well plates, and wells were seeded with 2×10^5 HepG2 cells and incubated for 24 h. The day after seeding, *P. berghei* ANKA sporozoites were dissected from the salivary glands of *A. stephensi* 18–21 days postblood meal into DMEM. 2.5×10^5 sporozoites were added to a tube of Complete Medium containing compound or DMSO, and the mixture was dispensed into wells containing HepG2 cells and incubated for 2 h at 37 °C. The final concentration of DMSO was 0.5%, and compounds were tested at 62.5, 31.25, and 15.6 μ M.

After incubation, the media was gently removed, and 4% PFA/ phosphate buffered saline (PBS) was added and incubated at 37 °C for 20 min. Slides were washed with PBS and blocked with 1% BSA/PBS, and noninvaded sporozoites were detected using anti- CSP mAb 3D11 followed by secondary antibody AlexaFluor 594 conjugated goat antimouse IgG (Invitrogen) in 1% BSA/PBS. After washing with PBS, the coverslips were incubated overnight at –20 °C with ice-cold MeOH for permeabilization. Coverslips were washed with PBS before blocking with 1% BSA/PBS followed by washing with PBS. Both invaded and noninvaded sporozoites were then detected using the 3D11 mAb and secondary AlexaFluor 488 conjugated goat antimouse IgG Ab (Invitrogen) in 1% BSA/PBS. Coverslips were washed with PBS and once with water before mounting using Prolong with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) and cured. The number of invaded sporozoites was determined by quantifying the total number of red and green sporozoites and subtracting of the number of red sporozoites, which were stained prior to HepG2 permeabilization. Mean and standard average mean for replicates (N = 3) were calculated.

Compound Cytotoxicity Assays.

P. berghei ANKA sporozoites were dissected as described previously. 8×10^3 sporozoites were preincubated for 10 min at 18 °C in the presence of 250 μ M of compound or DMSO. Compound or DMSO in DMEM with 2% BSA was added in equal volume to the reaction, resulting in a final concentration of 250 μ M of compound, 1% BSA, and 0.5% DMSO and was incubated at 37 °C for 20 min (no replicates). The sporozoites were then incubated with propidium iodide (PI). Sporozoites were visualized, and the number of sporozoites in phase and red fluorescent were counted to determine viability.

The LIVE/DEAD viability and cytotoxicity testing kit (Life Technologies) was used to determine viability of the *P. berghei* ANKA sporozoites. A working stock solution was prepared in DMEM of 2.5 μ M calcein-AM and 2 μ M ethidium homodimer-1. 5×10^4 sporozoites were incubated in 1% BSA/DMEM with either 62.5 or 31.25 μ M of compound or DMSO, for 15 min at room temperature (no replicates). The sporozoites were centrifuged at 4 °C for 5 min, and the media was removed. The sporozoites were resuspended in calcein/ethidium dye in DMEM and allowed to incubate at room temperature protected from light for 20 min. Another set of reactions were incubated for an additional 15 min at 37 °C and then centrifuged and incubated in dye for 20 min at room temperature. Sporozoites were added to the slides, and the number of red or green fluorescent parasites were counted.

The LIVE/DEAD fixable violet dye kit (Life Technologies) was used to determine the viability of mCherry expressing *P. berghei* sporozoites. A working stock of dye was prepared as 1 μ L in 1 mL of PBS. Sporozoites were incubated in 1% BSA/DMEM with either 62.5 or 15.6 μ M of compound or DMSO for 15 min at room temperature followed by 15 min at 37 °C (no replicates). The compound was then removed, and sporozoites were washed with PBS. Sporozoites were resuspended in dye and incubated for 30 min on ice. After, they were washed in PBS and resuspended in 4% PFA/PBS for 15 min at room temperature. Fixed sporozoites were washed with 100 μ L of 1% BSA/PBS and resuspended in 25 μ L of 1% BSA/PBS. Sporozoite viability was measured using the Amnis MkII ImageStream (EMD Millipore). The violet dye was excited using the 405 nm laser, and mCherry was excited with the 561 nm laser. Signals were collected in the violet and red channels. Data was processed using IDEAS (Amnis).

To assess compound cytotoxicity against HepG2, cells cultured in Complete Medium were used to seed a 6-well plate at 8×10^4 cells per well. Twenty-four hours later, the media was removed and 2 mL of Complete Medium containing compound or DMSO was added; cells were incubated for 2 h at 37 °C (no replicates). After 2 h, the media was removed and cells were washed with DMEM before detachment using 500 μ L of trypsin-EDTA solution for 3 min at 37 °C. One milliliter of DMEM was added to each well, and the cell suspension was transferred to a 1.5 mL tube. Cells were centrifuged for 5 min at 1500g, and the media was removed. Propidium iodide diluted 1:3000 in PBS was used to resuspend the cell pellet. A compensation/negative control was prepared by treating cells with 100% ice-cold MeOH for 90 s. Samples were analyzed using the ImageStream. The laser at 488 nm was used to excite the dye, and fluorescence was detected in the red channel. Data was processed using IDEAS (Amnis).

Graphs, Statistics, and Graphics Generation.

Graphpad (Prism) was used to plot all data and calculate the mean, weighted averages, standard error mean, and *p*-values (unpaired *t* tests) for the various experiments. Fiji (Imagej)⁵⁷ was used to generate Movies S1-S9, the maximum projections over time, and trail assay images. Blender was used to render the glideosome model in Figure 1. PyMOL⁵⁸ was used to render the molecular model in Figure 2. BioChemDraw was used to generate chemical structures. IDEAS was used to analyze ImageStream data and generate FACS images and gating graphs in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACT	artemisinin combination therapy
AMA1	apical membrane antigen 1
BSA	bovine serum albumin
CSP	circum- sporozoite protein
CTRP	circumsporozoite-thrombospondin related anonymous protein; DAPI, 4',6-diamidino-2-phenyl- indole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
F1,6P	fructose-1,6-bisphosphate
FACS	flow assisted cell sorting
FBS	fetal bovine serum
FDA	US Food and Drug Administration
FITC	fluorescein isothiocyanate
GAC	gliding associated connector
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glycerophosphate dehydrogenase
GFP	green fluorescent protein
HepG2	hepatocyte carcinoma cell line
HSPG	heparan sulfate proteoglycan

IMC	inner membrane complex
MBP	maltose binding protein
MIC	microneme associate protein
MMV	Medicines for Malaria Venture
MTIP	myosin tail interacting protein
MTRAP	merozoite-thrombospondin related anonymous protein; MyoA, myosin A
NADH	nicotinamide adenine dinucleotide
Pb	Plasmodium berghei
PBS	phosphate buffered saline
Pf	Plasmodium falciparum
PFA	paraformaldehyde
PI	propidium iodide
RH	reticulocyte homologue protein
SPR	surface plasmon resonance
TIM	triosephosphate isomerase
TLP	TRAP-like protein
TRAP	thrombospondin related anonymous protein
TSR	type-I thrombospondin repeat
WHO	World Health Organization

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Figure 1. Plasmodium invasion machinery complex (glideosome). Structures of proteins from *Plasmodium spp.* and homologous protein structures were combined with the results of biochemical and genetic data to represent a possible glideosome model, depicted here in surface representation. The actin—myosin motor, colored gray/black and magenta/light pink, respectively, is bridged by tetrameric aldolase (multicolor) to the extracellular adhesin TRAP (cyan), which interacts with unknown ligands on the host cell membrane (gray). The motor is anchored to the inner membrane complex (IMC) via its interaction with MTIP

(purple). The IMC contains the glideosome associated proteins including the integral membrane protein GAP40 (orange), the supraalveolar space spanning GAP45 (darkblue), and luminal GAP50 (red). The integral membrane GAPMs (blue) are located on the cytosolic side of the IMC membrane and interact with the alveolins (white/gray), connecting the complex to the parasite's cytoskeletal network.

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Figure 2.

SPR screening of MMV library against the TRAP-aldolase interaction. Example SPR binding and dissociation curves along with experimental schematic for the TRAP-aldolase interaction for the (a) baseline control and compounds (b) promoting binding, (c) inhibiting binding, and (d) stabilizing the off-rate kinetics. Baseline response between TRAP (blue) and aldolase (multicolor) bound to the CM5 chip was set at 25–50% of total possible response. Compound (pink) was passed over chip along with TRAP and could have no effect, block binding, or promote binding. (e) Normalized change in response units (RU) for binding of TRAP to aldolase in the presence of MMV library compound, compared to control in DMSO. (f) Concentration-dependent total binding response in the presence of five compounds selected for further gliding and invasion studies. Mean and standard error mean plotted for each concentration ($N = 3$).

Figure 3.

Aldolase enzyme activity assay. (a) Cartoon and surface representation of aldolase with TRAPpep bound (magenta, sticks) (PDB entry 2pc4³³), overlapping the F1,6P (green, sticks) substrate binding site (PDB entry 4ald)⁵⁹ via PyMOL.⁵⁸ (b) Coupled enzyme assay to measure aldolase activity via NADH consumption as measured at 340 nm. (c) Concentration-dependent effect of compounds ($N=3$) on PfAldolase inhibition in the presence of PbTRAP peptide. Activity is normalized to the PbTRAP control, which reduces V_{max} to 50% of enzyme without PbTRAP. (d) PfAldolase activity in the presence of

compounds to determine direct inhibition of enzyme by the compounds. Activity is normalized to the PfAldolase control measured V_{max} . Mean and standard error mean are plotted for activity assays for each concentration ($N = 3$).

Figure 4.

Quantification of trails deposited by gliding sporozoites in gliding assays and after compound washout. (a) *P. berghei* sporozoites, incubated with either DMSO or the indicated concentrations of compounds, were allowed to glide on mAb 3D11 coated glass slides for 1 h at 37 °C. CSP trails were detected with biotinylated mAb 3D11, and the number of trails were counted for each sporozoite and binned as indicated ($N = 1, 200\text{--}250$ sporozoites). (b) Compound washout experiments were performed by incubating sporozoites with either DMSO or compound followed by a DMEM wash to remove compound. Half of the

compound incubated samples were resuspended with medium containing DMSO, creating the washout population, while the other half were incubated with the original compound. The sporozoites were then allowed to glide on 3D11 coated glass slides for 1 h at 37 °C, and trails were detected, counted, and binned ($N = 1, 200\text{--}250$ sporozites). (c) Representative images from slides of gliding sporozoites after incubation with compound 4 at $7.8\ \mu\text{M}$ followed by compound washout. Parasites display an atypical, wavy gliding phenotype in addition to typical circular gliding patterns, similar to patterns observed for MTIP-MyoA targeting pyrazole-urea compounds studied by Kortagere et al.⁴¹

Figure 5.

Analysis of speed, duration, radius, and phenotype of gliding sporozoites. (a) Radius of sporozoite gliding determined using trail assays by measuring CSP trails deposited by *P. berghei* sporozoites incubated with DMSO or compound at 62.5 aM (concentration N = 1, ~150 sporozoites from 10 to 20 fields of view). (b) The radius of the sporozoite gliding path was measured in live imaging videos of mCherry *P. berghei* sporozoites incubated with DMSO or compound (concentration N = 2). Calculated from the live imaging movies were the (c) gliding velocity, (d) average time spent gliding, and (e) fraction of time that the

parasite was paused during blocks of active gliding, defined as 1–10 frames of cessation of gliding. Mean and standard error mean are plotted, and p-value is calculated using the unpaired t test. (f) Sporozoites could be classified as waving, circularly gliding, attached, or drifting on the basis of maximum projection over time of live imaging frames. The ratio of gliding versus waving sporozoites was determined by observing sporozoites with active movement (not drifting) and manually assigning a waving or gliding phenotype (concentration N =1).

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Figure 6.

Sporozoite viability in the presence of compound. *P. berghei* sporozoite viability was measured in the presence or absence of compound in two unfixed assays and one fixed assay. (a) Measurement of propidium iodide exclusion after incubation with either DMSO or 250 μ M of compound for 15 min at 37 °C. Parasites were counted using a microscope, and viable percentage was determined via the number of nonred parasites compared to total number of parasites counted in phase ($N=1$, ~150 sporozoites). (b) LIVE/DEAD assay using ethidium homodimer as dead indicator and calcein dye as live indicator enables green/

red, live/dead, classification of sporozoites. Parasites were incubated with DMSO or compound for 15 min at RT followed by 15 min at 37 °C before addition of dye. Number of green parasites versus total number of parasites counted on microscope slides indicates viability of sporozoite population ($N = 1$, ~150–200 sporozoites). (c) LIVE/DEAD assay using mCherry expressing parasites and a fixable amine reactive violet dye. Exclusion of violet dye from live cells used to determine viability. Number of violet negative and mCherry positive sporozoites were determined as a percentage of total sporozoite count via the ImageStream ($N = 1$, 200–400 sporozoites). (d) Percentage of live sporozoites from the fixable LIVE/ DEAD violet dye experiment which shows attached extracellular vesicle containing mCherry signal ($N = 1$, 200–400 sporozoites). Representative images showing violet, brightfield, and red channel signal for live sporozoites (e) without and (f) with vesicles present at the apical end.

Figure 7. HepG2 invasion assay and cell viability in the presence and absence of compounds. (a) Percentage of sporozoites that invaded the HepG2 cells. Mean and standard error mean plotted ($N=3$). (b) Viability of HepG2 cells in the presence or absence of compound as determined using a propidium iodide exclusion assay. Mean and standard error mean plotted ($N=1$, ~1000 cells).

Table 1.

Compounds Selected for Parasite Studies

	Hit2Lead MMV ID	Structure
3	5251155/ MMV009108
4	5317392/ MMV007224
17	7078022/ MMV019241
18	7881935
23	67378588/ MMV666069