An overview of the *Plasmodium falciparum* hexose transporter and its therapeutic interventions

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Abstract
Despite intense elimination efforts, human malaria, caused by the infection of five *Plasmodium* species, remains the deadliest parasitic disease in the world. Even worse, with the emergence and spreading of the first-line drug-resistant *Plasmodium* parasites, therapeutic interventions based on novel plasmodial drug targets are more necessary than ever. Given that the blood-stage parasites primarily rely on glycolysis for their energy supply, blocking glucose uptake, the rate-limiting step of ATP generation, was considered a promising approach to kill these parasites. To achieve this goal, characterization of the plasmodial hexose transporter and development of selective inhibitors have been pursued for decades. Here, we review the identification and characterization of the *Plasmodium falciparum* hexose transporter (PfHT1) and summarize current advances in its inhibitor development.

KEYWORDS
drug design, GLUT, hexose transporter, malaria, PfHT1

1 | INTRODUCTION

Malaria, a life-threatening parasite disease, is caused by the infection of mosquito-borne *Plasmodium* parasites. Five species of *Plasmodium* parasites have been reported as the causal agents of human malaria, including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*.1 Of those parasites, *P. falciparum* represents the deadliest form and accounts for the majority of deaths in African.2 Great efforts have been made to eliminate malaria since the 1990s, which resulted in an approximately 29% reduction of malaria cases over the past two decades.3 Nonetheless, the situation is still alarming as malaria led to an estimated 241 million infections and 627,000 deaths worldwide in 2020.4

Despite intense efforts to generate malarial vaccines, merely one vaccine, the RTS,S, is recommended by the World Health Organization (WHO) for the prevention of *P. falciparum* infection in children,4,5 leaving antimalarial drugs as the current major therapeutic choice.6 To date, more than 20 different antimalarial drugs have been approved for the prophylaxis and treatment of malaria.7 Nonetheless, the rapid emergence and spreading of drug-resistant parasites severely threaten the global malaria control.8–11 Nowadays, all antimalarial drugs have encountered the issue of drug resistance, raising urgent demands for antimalarial agents based on novel therapeutic targets.

The sophisticated life cycle of *Plasmodium* parasites consists of the human liver-stage, human blood-stage, and mosquito-stage. During the blood-stage, *Plasmodium* parasites rapidly replicate asexually inside human erythrocytes, causing the clinical symptoms of malaria.12 To meet the high energy demands for rapid proliferation, blood-stage parasites rely on the fast but less efficient process of glycolysis for generating ATP.13 Consequently, parasitized erythrocytes accelerate their glucose uptake up to 100-fold to support the increased glucose consumption.14–18 Moreover, due to the lack of fructose bisphosphatase, *P. falciparum* cannot synthesize energy stores like glycogen, making continuous glucose uptake an essential step for
parasitic metabolism and survival. Supporting this notion, deprivation of glucose from freed *P. falciparum* led to a reduction in glycolytic flux and a rapid decline in intracellular ATP levels, which severely perturbed its metabolic homeostasis.

Cutting off the energy supply of blood-stage *Plasmodium* parasites through inhibition of their glucose uptake, therefore, represents an enticing strategy in impeding intraerythrocytic parasite proliferation. Accordingly, a major facilitator superfamily (MFS) transporter, PfHT1, was identified from the glucose permeable pathway of blood-stage *P. falciparum*. The PfHT1 is an essential hexose facilitator on parasite plasma membrane (PPM) for the glucose uptake of parasites. Comparative investigation of *P. falciparum* hexokinase (PfHK) and PfHT1 revealed that glucose uptake through PfHT1 is the rate-limiting step of intraerythrocytic parasite glycolysis and inhibiting PfHT1 exhibited parasiticidal effects. Therefore, PfHT1 is considered a novel antimalarial drug target for pharmacological intervention. Herein, we review the identification, characterization and inhibitor development of PfHT1 with emphasis on recent progresses on structural-based rational inhibitor design.

2 | THE DISCOVERY OF PFHT1

Morphological and physical changes of malaria-infected erythrocytes have long been recognized, which result in the perturbation of erythrocyte substrate permeability of sodium, potassium, and amino acids. The concept that glucose permeability of parasitized erythrocytes might also be altered can be dated back to 1966 when Herman and colleagues investigated the $^{14}$C-$\alpha$-glucose utilization of *P. gallinaceum*-infected chicken erythrocytes. They found that the parasitized chicken erythrocytes produced more $^{14}$CO$_2$ derived from $^{14}$C-$\alpha$-glucose and speculated that the uptake of glucose was increased by parasite infection.

The first experimental evidence of accelerated glucose uptake in parasitized erythrocytes was reported in 1974. Through comparing $^{14}$C-labeled $\alpha$-glucose and 3-O-methyl glucose (3-OMG) uptake in *P. lophurae* infected or uninfected duck erythrocytes, Shereman and Tanigoshi proved an increase of glucose uptake in parasitized erythrocytes and reasoned that this phenotype is due to alterations of a simple diffusion component. In the same year, Homewood and Neame published their observations on $\gamma$-glucose uptake by *P. berghei*-infected mouse erythrocytes and concluded that the $\gamma$-glucose only entered the parasitized erythrocytes, but not the uninfected erythrocytes.

Contrary to the diffusion model, Izumo et al. proposed an active transport model in 1989. By comparing $^3$H-labeled 2-deoxy-$\alpha$-glucose (2-DOP) uptake in *P. yoelii*-infected or *P. yoelii*-uninfected mouse erythrocytes, Izumo et al. observed a 8-fold 2-DOP concentration increase inside the parasite compared to the parasitized erythrocyte cytosol. Additionally, the transport of 2-DOP was largely decreased by the supplement of a proton ionophore, carbonylcyanide m-chlorophenylhydrazone (CCCP). They proposed that two components, a simple diffusion system on the erythrocyte plasma membrane (EPM) and a proton-coupled active glucose transporter on the PPM, might be involved in the glucose uptake in parasitized erythrocytes.

Subsequent research from Kirk et al. confirmed the accumulation of $^{14}$C-2-DOP in *P. falciparum*-infected human erythrocytes and discovered that the phosphorylation of 2-DOP, rather than active transport, accounted for the substrate accumulation. Given that the intracellular/extracellular ratios of 3-OMG and $\gamma$-glucose were kept below 1, Kirk et al. concluded that the glucose uptake of parasitized erythrocytes works through an equilibrium manner rather than via active transport. Parallel to this report, Goodyer et al. also raised a similar concern regarding the possibility of 2-DOP phosphorylation in the active transport model. They employed $^3$H-labeled 6-deoxy-$\alpha$-glucose (6-DOG), a non-phosphorylatable glucose analog, for an uptake and efflux assay. Based on kinetic analysis, Goodyer et al. proposed the existence of a saturable glucose transporter on the membrane of parasite compartment. Due to the interference of a possible duct system on parasitophorous vacuole membrane (PVM), the specific location of this glucose transporter could not be distinguished in this research.

To eliminate the interference of the multi-membrane system in malaria-infected erythrocytes, Penny et al. used a Xenopus laevis oocytes heterologous expression system to validate plasmoidal nutrient transporters. By injecting mRNA isolated from asexual stages *P. falciparum* into *X. laevis* oocytes, Penny et al. observed a 7-fold increase in 2-DOP uptake, which therefore confirmed the existence of a *P. falciparum*-encoded glucose transporter. In the following milestone work, Woodrow et al. identified the sequence of *Plasmodium falciparum* hexose transporter, designated PfHT1, by searching the homologous sequence of human glucose transporter 1 (GLUT1). Using the *X. laevis* oocytes expression system, the authors confirmed $\gamma$-glucose and 2-DOP uptake by PfHT1. Additionally, PfHT1 was found to localize on the PPM rather than the EPM by immunofluorescence staining. Henceforth, the approach to cut off glucose uptake for malarial therapies progressed toward the biochemical characterization of PfHT1 and its inhibitor development.

It is noteworthy that the nutrient uptake of intraerythrocytic parasites is much more complicated due to the existence of a PVM and the adaptation changes of parasitic and host membrane systems. For instance, blood-stage parasites are encompassed by PVM, making the passage through PVM as a precondition for glucose entry to PPM. A non-selective channel on PVM is responsible for the permeation of soluble macromolecules up to 1400 Da. Despite no direct evidence for glucose permeation, the PVM channel has been considered a permeable path for glucose uptake given its permeability to a hexose-like polyol glucoronate. Moreover, a duct system on PVM has also been reported to offer direct access to extracellular macromolecules. Additionally, a tubovesicular membrane (TVM) extended from the PVM is relevant to the delivery of nucleosides and amino acids to parasites. Both the duct system and the TVM are undercharacterized and their roles in glucose uptake need further investigations. As for the host erythrocyte membrane, except for GLUT1, a parasite-induced new permeability pathway (NPP) is formed during trophozoite matura-
Nonetheless, glucose penetration of parasitized erythrocytes is dominated by GLUT1-mediated facilitated diffusion.\(^{49}\)

Taken together, the major glucose permeable pathway for intraerythrocytic parasites can be described by a classic three membrane model including EPM, PVM, and PPM (Figure 1). In this model, extracellular glucose is first transported across the EPM by GLUT1 in an equilibrium manner.\(^{50}\) Intracellular glucose then penetrates through PVM via a non-selective channel. Finally, PFHT1 mediates the facilitated diffusion of glucose across the PPM. The subsequent phosphorylation of glucose in glycolysis can maintain the concentration gradient toward parasitic cytosol and drives the glucose influx from extracellular serum.

### 3 | FUNCTIONAL CHARACTERIZATION OF PFHT1

PFHT1 was first identified as a putative transporter gene on \textit{P. falciparum} chromosome 2. As the hybridization of the \textit{P. falciparum} genome with low stringency \textit{PFHT1} sequence cannot yield close sequence, \textit{PFHT1} was defined as a single-copy gene without closed paralogues.\(^{24}\) A 504-amino-acid transmembrane protein, located on the parasite plasma membrane, is encoded by \textit{PFHT1}. \textit{PFHT1} was originally assumed as a glucose transporter for its ability to increase the \(\delta\)-glucose and 2-DOG uptake in \textit{X. laevis} oocytes.\(^{24}\) Further competition binding assays revealed \(\delta\)-fructose as a competitor of \(\delta\)-glucose or 2-DOG uptake, indicating that \(\delta\)-fructose could be a potential substrate for PFHT1. Subsequently, this hypothesis was verified by the PFHT1-mediated \(14\text{C}-\delta\)-fructose uptake in \textit{X. laevis} oocytes.\(^{24,51}\) Therefore, PFHT1 is responsible for both the \(\delta\)-glucose and \(\delta\)-fructose uptake of parasites.

Using the \textit{X. laevis} oocytes heterologous expression system, the transport property and substrate recognition of PFHT1 were analyzed through an in vitro transport and competition binding assay. The glucose and fructose uptake assay revealed PFHT1 as a saturable transporter, whose transport activity is independent of the sodium and proton gradient.\(^{24,52}\) The transport activity of PFHT1 can be inhibited by conventional glucose transporter inhibitors like Phloretin, Phloridizin, and Cytochalasin B (CCB). \(\delta\)-Glucose cannot inhibit the PFHT1-mediated uptake of \(\delta\)-glucose and 2-DOG, classifying PFHT1 as a stereospecific transporter.\(^{53}\) Together with the evidence that glucose uptake of intraerythrocytic parasites is mediated by a saturable transporter in an equilibrium manner, PFHT1 was defined as a stereospecific facilitator to mediate the diffusion of \(\delta\)-glucose and \(\delta\)-fructose across parasite plasma membrane. Kinetic analysis revealed that the affinities (\(K_m\)) of PFHT1 for \(\delta\)-glucose and \(\delta\)-fructose were 0.97 and 11.6 mM, respectively.\(^{52}\) In contrast, its human counterpart GLUT1 facilitates glucose uptake with a \(K_m\) of 3 mM, and another counterpart GLUT5 solely contributes to fructose uptake with a \(K_m\) of 6 mM.\(^{50,53}\) The higher glucose affinity of PFHT1 might help with glucose influx into intraerythrocytic parasites. Notably, despite PFHT1-mediated fructose uptake supporting the parasite survival in vitro, fructose is unlikely the main energy source for blood-stage parasites under physiological conditions, as the physiological fructose concentration is relatively low.\(^{51,54}\)

Stage-dependent expression of \textit{PFHT1} was first characterized by measuring its mRNA levels during the blood-stage.\(^{24}\) \textit{PFHT1} is constitutively expressed throughout the blood-stage with the highest, intermediate, and lowest levels occurring in the early ring stage, trophozoites and meronts, and gametocytes, respectively.\(^{24}\) Consistently, knock-out of \textit{PFHT1} led to a lethal phenotype in intraerythrocytic \textit{P. falciparum}, whereas complementation of episomal \textit{PFHT1} rescued the transgenic parasite.\(^{55}\) These results suggested that PFHT1 is an essential component for the blood-stage \textit{P. falciparum}. To extend the investigation to the whole plasmodial life cycle, \textit{P. berghei}, a rodent malarial species amenable to in vivo life cycle studies, was further characterized to detect the expression of \textit{P. berghei} hexose transporter (PbHT1). By monitoring fluorescence signal from a GFP-tagged PbHT1 or immunofluorescence signal from a hemagglutinin-tagged PbHT1 in transgenic \textit{P. berghei}, PbHT1 was found to be constitutively expressed in both mosquito-stage and liver-stage parasites.\(^{55–57}\) Therefore, \textit{Plasmodium} hexose transporters, like PFHT1 and PbHT1, are not only essential for the blood-stage parasites but may also play important roles in the rest of the plasmodial life cycle.

### 4 | MOLECULAR MECHANISM OF PFHT1 AND ITS SUBSTRATE RECOGNITION

#### 4.1 | Competition analysis and site-directed mutagenesis

Before experimental structures were available, analysis of PFHT1 substrate recognition relied on transport and competition binding assays using an \textit{X. laevis} oocytes heterologous expression system. A variety
of hexose analogs were applied to inhibit PfHT1-mediated D-glucose uptake. The calculated inhibition constant \( K_i \) of the competitors enabled evaluation of the relative binding strengths of different hexose analogs, revealing the structural requirements for PfHT1 substrate recognition.

In aqueous solvents, D-glucose mainly exists in pyranose form as approximately 33% \( \alpha \)-anomer and 66% \( \beta \)-anomer (Figure 2A). To characterize the functionalities of hydroxyl groups on the pyranose form of D-glucose, analogs with hydroxyl group substitutions were first interrogated by competition binding assays. The \( K_i \) of 2-DOG, 5-thio-D-glucose, and 6-DOG are similar to the \( K_m \) of D-glucose. On the other hand, \( K_i \) of 1-DOG and 3-DOG increased substantially, indicating that 1- and 3-hydroxyl groups are involved in stronger interactions with PfHT1 compared to other hydroxyl groups. Interestingly, 3-fluoro-1-deoxy-\( \beta \)-D-glucose (\( \beta \)-anomer) and 1-fluoro-1-deoxy-\( \alpha \)-D-glucose (\( \alpha \)-anomer) restored the inhibition to PfHT1, suggesting that the 3-hydroxyl group of D-glucose might contribute to substrate binding as a hydrogen bond recipient. Consistently, epimer analysis demonstrated that the affinity of C2 epimer (D-mannose) is similar to D-glucose but C3 epimer (D-allose) has a much lower affinity. D-Galactose (C4 epimer) has an intermediate affinity to PfHT1, suggesting that both orientations of 4-hydroxyl group can contribute to the interaction. To access the anomer preference, 1-fluoro-1-deoxy-\( \alpha \)-D-glucose and 1-fluoro-1-deoxy-\( \beta \)-D-glucose were analyzed in the competition binding assay, both of which possess comparable affinities as 1-DOG. Therefore, similar to human glucose transporters, PfHT1 can accommodate both \( \alpha \)- and \( \beta \)-anomer of D-glucose. The two major forms of fructose in water solution include approximately 25% \( \beta \)-fructofuranose and 65% \( \beta \)-fructopyranose (Figure 2B). 2-deoxy-\( \beta \)-fructofuranose, a \( \beta \)-furanose analog, maintained a high affinity to PfHT1, whereas L-sorbofuranose, the \( \beta \)-pyranose analog and C5 epimer of D-fructose, failed to inhibit fructose uptake of PfHT1. These results suggest that PfHT1 transports D-fructose in the \( \beta \)-furanose form.

Site-directed mutagenesis of PfHT1 was employed to analyze Gln169 on the transmembrane segment (TM) 5 and 302SGL triplet motif on TM7; the latter triplet motif was shown to be irrelevant to substrate binding in experimental structures. Q169N variant reserved the ability of D-glucose uptake, but failed to transport D-fructose. Consistently, the \( K_i \) of D-glucose analogs, 1-fluoro-1-deoxy-\( \alpha \)- and 1-fluoro-1-deoxy-\( \beta \)-D-glucose, are comparable between Q169N variant and wild-type PfHT1, whereas the affinity of D-fructose analog, 2-deoxy-\( \beta \)-fructofuranose, to Q169N variant decreased significantly compared to wild-type PfHT1. These results indicated Gln169 is involved in D-fructose binding and transport. Furthermore, a 4-fold decrease in \( K_i \), for 5-thio-D-glucose and 4-fold increase in \( K_i \) for 6-DOG were observed in the Q169N variant, suggesting that Gln169 interacts with 5- and 6-hydroxyl group of D-glucose. To uncover substrate recognition and transport details, a putative three-
dimensional model of PfHT1 (PDB code 1LV1) was further established based on a predicted GLUT1 model (PDB code 1JA5). Limited by the inaccuracy of the initial model and piecemeal strategy for helical assignment and prediction, structural information and mechanistic explanations based on the predicted PfHT1 model are unreliable, and will not be further discussed here.

4.2 Crystal structure of PfHT1 bound with D-glucose

Most recently, we and another group independently reported two crystal structures of D-glucose-bound PfHT1 at 2.6 and 3.65 Å, respectively. The discrepancy of resolution might be due to the different detergents used for protein purification. Nonetheless, PfHT1 molecules in both structures were largely identical with a root-mean-square deviation (RMSD) of 0.444 Å over 364 Cα atoms. Henceforth, I will use our high-resolution structure to elaborate the substrate recognition and transport mechanism of PfHT1.

Similar to other sugar porter (SP) family members like GLUT1 and GLUT3, the overall structure of PfHT1 exhibits a canonical MFS fold with 12 TMs forming two 6-helical bundles. The intervening sequence between the N- and C-domains, together with the C-terminal segment, forms five short helices, which constitute an intracellular helical (ICH) domain (Figure 3A). D-Glucose resides in the central pocket located approximately in the middle of lipid bilayer. Detailed analysis of the central pocket revealed a highly conserved glucose-binding pattern; the glucose-binding residues and their configurations in PfHT1 were almost identical to those of GLUT1 and GLUT3 (Figure 3A). Four residues on the C-domain and one residue on the N-domain form polar contacts with D-glucose. Specifically, the 1-hydroxyl group of D-glucose is stabilized by Gln169 on TM5, Gln305 on TM7a, and Trp412 on TM10. The 2-hydroxyl group interacts with Gln305 on TM7a. The 3- and 4-hydroxyl groups form two hydrogen bonds with...
Asn311 on TM7b. The 6-hydroxy group interacts with Gln169 on TM5 and Asn341 on TM8. An additional hydrogen bond is formed between Gln169 and the hemiacetal oxygen of glucose.

The residues adjacent to the substrate binding site are less conserved between PfHT1 and GLUTs, resulting in a larger central cavity for substrate accommodation in PfHT1. This might be one of the reasons why PfHT1 can transport variety of hexoses. Notably, a series of PfHT1 mutants were generated by the Drew group to rationalize substrate preferences.60 Contrary to the previous report, Q169N variant abolished both the D-glucose and D-fructose transport of PfHT1. The authors identified another two variants, W412A and N435A, that impaired D-fructose transport rather than D-glucose transport. Nonetheless, the molecular mechanism for PfHT1 substrate promiscuity remains unclear.60

Besides the common features like GLUTs, PfHT1 also possesses unique regions. Similar to the GLUT3 structure, the extracellular half of TM1 forms a membrane-parallel helix, designated TM1e in PfHT1; however, the TM1e of PfHT1 is much shorter than that of GLUT3. The intervening sequence between TM1e and TM2 forms a long extracellular loop named L1–2, which tightly coordinates with another long extracellular loop L5–6 through hydrogen bonds between Arg67 on L1–2 and Glu196 and Lys191 on L5–6 (Figure 3B). A disulfide bond between Cys61 and Cys70 connects TM1 and TM2 together, which is important for the D-glucose transport of PfHT1 (Figure 3B). Additionally, TM7b bends to a larger degree toward the substrate-binding site of PfHT1, which seals the central cavity from the extracellular side. Together with the closed intracellular tunnel, the glucose-bound PfHT1 structure presents as an occluded state, which is a missing piece in the alternating access cycle of GLUTs homologue structures (Figure 3C). Combined with other conformational states obtained from GLUTs structures, hexose transport by PfHT1 can be explained by the same alternating access model (Figure 3D).62

5 | INHIBITOR DEVELOPMENT

TARGETING PFHT1

Given its important roles for parasite survival and development in all life cycle stages, pharmacological interventions targeting PfHT1 have long been pursued for novel malarial therapies. Developing selective inhibitors to discriminate PfHT1 and GLUTs is the first step in achieving this goal.

5.1 | Semi-rational basis design

The semi-rational basis approach described the identification of the first synthetic PfHT1 specific inhibitor C3361, whose activity was confirmed through large-scale screening with X. laevis oocytes-based competition binding assay.25,63 This pioneering work was initiated as the substrate requirements of PfHT1 were elucidated. Knowing that O-3 hexose derivatives possess higher potency against PfHT1 than GLUT1, the Krishna group systematically examined O-1 to O-6 glucose derivatives, exemplified by 1-, 2-, 3-, 4-, 5-, and 6-OMG, for their inhibition of PfHT1- or GLUT1-mediated D-glucose uptake.27,51 The 3-OMG exhibited the highest selectivity, while 2- and 4-OMG also presented intermediate selectivity. Longer O-3 substitutions based on 3-OMG were generated for further analysis, which yielded a selective PfHT1 inhibitor 3-O-(undec-10-en)-1-yl-D-glucose (C3361) (Figure 4A). The C3361 selectively inhibited PfHT1 rather than GLUT1 with a selectivity index $S_{GLUT1}/S_{PfHT1}$ of 62. The selectivity between PfHT1 and GLUT5 is higher as inhibition against GLUT5 was not observed.27 Further characterization revealed that C3361 also inhibited hexose transporters from P. vivax (PvHT1), P. knowlesi (PkHT1), and P. yoelii (PyHT1) with comparable potency to PfHT1, suggesting C3361 as a broad-spectrum hexose transporter inhibitor for different Plasmodium spp.27,64

The parasiticidal activity of C3361 was assessed through drug treatments on in vitro cultured blood-stage P. falciparum 3D7 (chloroquine sensitive strain) and K1 (multidrug resistant strain); the growth of both strains was inhibited by C3361.27 A further study revealed that C3361 also inhibited schizont maturation of patient-isolated P. vivax.64 Life-cycle studies using P. berghei demonstrated that C3361 could inhibit both hepatic-stage and vector-parasite-stage parasites, and therefore exhibited transmission blocking activity.54,57,65 Moreover, the in vivo potency of C3361 was evaluated by a P. berghei-infected mouse model where significant suppression of parasitaemia was observed in C3361 treated mouse.27 Therefore, C3361 served as a proof of principle for antimalarial drug development targeting of PfHT1.

Following the establishment of C3361, chemical modifications were employed to explore its structure-affinity relationships. By replacing the aliphatic tail of C3361 with other 3-O-substituents of different length, size and hydrophilicity, the optimum structures that maintained high affinity to PfHT1 were found to be those of 3-O-substituents with C8–C13 lipophilic chains. Meanwhile, none of their derivatives exhibited significant inhibition to GLUT1.66 Subsequently, the optimum position for undec-en-10-yl substituent on D-glucose was investigated. Compared to other O-substitution derivatives, 2-O-(undec-10-en)-yl-D-glucose stood out for its approximately 25-fold higher affinity to PfHT1 than C3361, and negligible inhibition of GLUT1.67

5.2 | Structural-based rational design

Despite the identification and characterization of C3361 shedding light on novel antimalarial drug development, the optimization of further leads has been hampered by insufficient mechanistic insight about its inhibition. A “lollipop” model was proposed by the Krishna group after their discovery of C3361; that is to say, the glucose moiety of C3361 (the ball of the lollipop) occupies the sugar-binding site while the aliphatic chain (the stick) associates with the lipid bilayer.27 This hypothesis was raised based on the following observations: (1) the Q169N mutant of PfHT1 was less susceptible to C3361, indicating that its glucose moiety might interact with amino acids in the
middle of TM5.\(^{27}\) (2) the affinities of C3361 derivatives decreased with the increment of hydrophilicity of their aliphatic tail, which suggested that the aliphatic tail of C3361 is accommodated in a hydrophobic environment.\(^{66}\)

To explore the binding pose of C3361, structural models of PfHT1 were built with homology modeling, followed by molecular docking and dynamic simulations of C3361.\(^{61}\) The best hit model was generated based on homology modeling of the crystal structure of FucP (PDB code 3O7Q). Given that FucP is an Escherichia coli L-fructose/proton symporter, its substrate transport path and central cavity are very different from those of eukaryotic hexose facilitator, PfHT1, which led to inaccurate model building and C3361 docking.\(^{61,68}\) Therefore, the molecular mechanism of C3361 inhibition remained enigmatic until the PfHT1-C3361 structure was determined.

The experimental model of C3361-bound PfHT1 was elucidated by a 3.7 Å resolution crystal structure.\(^{59}\) The overall structure of C3361-bound PfHT1 retains the occluded conformational state that seals the substrate transport tunnel from both extracellular and intracellular sides (Figure 4B). The binding pose of the glucose moiety of C3361 is almost identical to D-glucose in the PfHT1-glucose complex (Figure 4B). Remarkably, conformational changes of PfHT1 were observed in the neighborhood of the C3361 aliphatic chain;
TM1e and TM7b straighten up with C3361 association, while TM2 and TM4 slightly swap away from the center pocket\textsuperscript{59} (Figure 4C). The conformational shifts form a hydrophobic tunnel to accommodate the alkenyl tail of C3361. More importantly, an unprecedented allosteric pocket, encompassed by residues on TM1, 2, 7b, and 11, was induced, encaging the last five carbons of the aliphatic chain with an unoccupied extra space\textsuperscript{59} (Figure 4D). Consistently, the density of the last three carbons on the aliphatic chain cannot be clearly resolved, indicating high mobility at the end of the tail. Notably, no conformational shift was observed between the C3361-bound GLUT3 structure and the D-glucose-bound GLUT3 structure\textsuperscript{69,70} (Figure 4D). Therefore, it is instinctive to pursue a potent and selective PfHT1 inhibitor by simultaneously occupying both the substrate binding pocket and the novel allosteric pocket.

Based on the structural information, dozens of C3361 derivatives were rationally designed with modifications of their sugar moieties, aliphatic linkers and substituents at the end of the aliphatic tail. By introducing aromatic or heteroaromatic groups of various sizes to the end of the aliphatic tail, the quinolin-6-yloxy group was identified as the best substituent to occupy the novel allosteric pocket.\textsuperscript{59} Further structure–activity relationship (SAR) studies of derivatives with different sugar moieties and aliphatic linkers confirmed the following facts: (1) replacement of the D-glucose moiety by D-fructose or L-glucose moiety decreased the potency of the inhibitor;\textsuperscript{59} (2) the optimum length for aliphatic linker is between 8 and 10 carbons;\textsuperscript{59,69} (3) adding a hydrophilic group to the linker negates the inhibition activity of the inhibitor;\textsuperscript{69} (4) O-2 and O-3 sites of the D-glucose moiety are the optimum positions for substitution.\textsuperscript{69} Finally, 3-O-(8-[quinolin-6-yloxy] octyl)-D-glucose, designated HT1-1, was validated as the best hit for its 64-fold higher affinity to PfHT1 compared to C3361\textsuperscript{59} (Figure 4A, E). Proteoliposome-based inhibition assays confirmed that HT1-1 selectively inhibits PfHT1 over GLUT1. Also, HT1-1 exhibits good parasiticidal activity to blood-stage \textit{P. falciparum} 3D7 and Dd2 (multidrug resistant strain) while exhibiting low cytotoxicity in both HEK293T/17 and HepG2 cell lines.\textsuperscript{59} Together, the structural characterization of C3361-bound PHT1 led to the serendipitous discovery of an unexploited allosteric pocket. The following structural-guided rational inhibitor design exploited both the sugar-binding pocket and novel allosteric pocket and succeeded in generating the highly potent and selective inhibitor, HT1-1, paving the way for the development of next-generation orthosteric–allosteric dual inhibitor targeting PfHT1.

### 5.3 HTS for PfHT1 inhibitors

Except for the semi-rational and structural-based rational drug design, high-throughput screens (HTS) provide another powerful path to identifying pre-leads targeting PfHT1. To date, three different systems have been established for HTS, including a PfHT1-complemented \textit{S. cervisiae} system,\textsuperscript{56} a glucose transporter null mutant (\textit{Δlmxgt}) of \textit{Leishmania mexicana} system,\textsuperscript{71} and a fluorescence resonance energy transfer (FRET)-based HTS system.\textsuperscript{72} The PfHT1-complemented yeast system was only validated by its susceptibility to C3361-based growth inhibition, whereas the latter two HTS systems were implemented for PfHT1 inhibitor identification.\textsuperscript{56,72–74}

The \textit{L. mexicana} HTS system was established in a glucose transporter knock out cell line \textit{Δlmxgt}.\textsuperscript{71} Through complementary expression of PfHT1 or GLUT1, the \textit{3H}-D-glucose uptake of \textit{Δlmxgt} was restored, and defined as the complementary null mutant lines, \textit{Δlmxgt[pPfHT1]} and \textit{Δlmxgt[pGLUT1]}. Treatment of C3361 led to specific inhibition of \textit{3H}-D-glucose uptake of \textit{Δlmxgt[pPfHT1]}. To increase throughput, the \textit{3H}-D-glucose was replaced by a live cell permeable dye, alamarBlue, to monitor the viability of \textit{L. Mexicana} in the beginning. Further characterization confirmed that C3361 could specifically inhibit the growth of \textit{Δlmxgt[pPfHT1]} and not \textit{Δlmxgt[pGLUT1]}, which served as a proof of principle for this HTS system.\textsuperscript{71} In practical screening, the indicator was changed to the DNA-binding dye, SYBR green, to monitor cell proliferation.\textsuperscript{73}

Using the \textit{Δlmxgt[pPfHT1/pGLUT1]} \textit{L. mexicana} HTS system, the Landear group screened two “focused” libraries, whose parasitidal effects against \textit{P. falciparum} have been validated in vitro.\textsuperscript{73} The first library, Tres Cantos antimalarial compound set (TCAMS), contained 13 533 compounds. The second, Malaria Box from Medicines for Malaria Venture (MMV), contained 400 compounds. Combined with two rounds of screening of \textit{3H}-D-glucose uptake assays and one round of \textit{3H}-L-proline uptake assays to rule out nonspecific binding, 6 hits from the TCAMS library and 3 hits from Malaria Box were identified.\textsuperscript{73} Further dose–response characterization revealed only two hits, including TCMDC-125163 and MMV009085 (also called GNF-PF-3184), which exhibited high potency to \textit{Δlmxgt[pPfHT1]} and low potency to \textit{Δlmxgt[pGLUT1]} (Figure 5A). The parasitidal effects of TCMDC-125163 and its analogs were confirmed by both \textit{P. falciparum} 3D7 and K1. The cytotoxicities of these compounds were insignificant to human foreskin fibroblasts cells (EC\textsubscript{50} > 25 \textmu M).\textsuperscript{73} However, further optimization of TCMDC-125163 is needed due to its poor pharmacokinetic property.

The FRET-based HTS system was designed to circumvent the weaknesses of the isotope-labeled substrate transport assay and cell death assay, which involve (1) the manipulation of radiolabeled substrate limiting the throughput of the transport assay and (2) both approaches being difficult to distinguish PfHT1 inhibition from the inhibition of other targets.\textsuperscript{72} Therefore, a direct glucose indicator is needed to monitor glucose uptake and its inhibition. FLI\textsuperscript{12}Pglu-700μS6 (FLIP) is a chimeric protein with an architecture of CFP-MglB-YFP.\textsuperscript{75} The intermediate MglB is a glucose-/galactose-binding domain flanked terminally by fluorescence proteins CFP and YFP. Upon glucose binding, conformational change of MglB pulls CFP and YFP together, resulting in a resonance energy transfer from CFP to YFP. By exciting CFP and scanning the emission from YFP, glucose uptake can be directly monitored by the FRET signal of FLIP. HEK293 cells were transfected with plasmids of PfHT1 and FLIP to generate a stable PfHT1-FLIP cell line. Endogenous GLUIT1 expression was further knocked down by shRNA to eliminate background glucose absorption.\textsuperscript{72}
Using the FRET-based PfHT1-FLIP cell HTS system, 400 compounds of Malaria Box from MMV were first screened, yielding 5 primary hits. Combined with the results from $^{14}$C-2-DOG uptake inhibition and the parasite growth inhibition assay, the MMV009085 was identified as a PfHT1 inhibitor.⁷² Inhibition of GLUT1-4-mediated $^3$H-2-DOG uptake demonstrated that MMV009085 has little effect on GLUTs-mediated glucose transport.⁷² Therefore, MMV009085 is a PfHT1 specific inhibitor, consistent with the preliminary finding from the screening using the L. mexicana HTS system.⁷²⁷³ In further research, the Hruz group screened 14,399 compounds from the Maybridge HitFinder collection, yielding 407 hits.⁷⁴ After removing 212 compounds with intrinsic fluorescence, the remaining 195 hits were narrowed down to 6, named WU-1 to WU-6, by their inhibition of a PfHT1-mediated $^3$H-2-DOG uptake assay. Furthermore, the four hits, WU-1, WU-2, WU-3, and WU-5, were characterized for their inhibition of GLUT1-4, revealing WU-1 as a PfHT1 selective inhibitor and WU-2 as a less selective PfHT1 inhibitor. Subsequent parasite growth assays revealed that WU-1 can inhibit P. falciparum 3D7 growth with a lower IC₅₀ than it of WU-2. Given that the IC₅₀ for the glucose uptake inhibition (5.8 ± 0.6 μM) and the parasitic growth inhibition (5.5 ± 0.6 μM) are similar, it was proposed that WU-1 kills P. falciparum by inhibiting PfHT1. Together, WU-1 was validated as a lead that kills P. falciparum by selectively inhibiting PfHT1⁷⁴ (Figure 5A).

5.4 | Other PfHT1 inhibitors

Except for the aforementioned approaches, catechins and lopinavir are another two compound classes that have been identified as PfHT1 inhibitors.
inhibitors through similar routes.\textsuperscript{76} That is to say, preliminary clues from other research revealed antimalarial activity and GLUTs-binding properties for both compounds. It is thus of interest to determine whether catechins and lopinavir can kill the parasites through inhibition of PfHT1.

In the case of catechins, previous research found that catechins could inhibit the asexual blood-stage \textit{P. falciparum} growth.\textsuperscript{77} Also, it was reported that gallated catechins are inhibitors of GLUT1.\textsuperscript{78} To explore their antimalarial mechanism, four catechins extracted from green tea [(\textendash )epicatechin (EC), (\textendash )epicatechin-gallate (EGC), (\textendash )epigallocatechin (EGC) and (\textendash )epigallocatechin-gallate (EGCG)] were assessed with respect to their abilities to inhibit D-glucose uptake via PfHT1 and GLUT1.\textsuperscript{76} The gallated catechins (ECG and EGCG) were identified as PfHT1 and GLUT1 inhibitors with submillimolar \(K_{S}\) (Figure 5B). It was also found that gallated catechins could moderately inhibit GLUT5-mediated D-fructose transport. Further \textit{P. falciparum} 3D7 growth inhibition assays revealed that \(IC_{50}\) of gallated catechins were comparable to their affinities of PfHT1. However, the parasite growth inhibition was not altered by different external D-glucose concentrations. Therefore, while this research identified gallated catechins as nonsel ective inhibitors of PfHT1, GLUT1, and GLUT5, their antimalarial mechanisms remain elusive.\textsuperscript{76}

As for lopinavir, it was originally developed as a human immuno-deficiency virus (HIV) protease inhibitor (PI). Further characterization revealed its antimalarial activity through drug susceptibility assays against \textit{P. falciparum}.\textsuperscript{79} The research of PI-induced insulin resistance revealed that PIs directly interact with GLUT4.\textsuperscript{80} These hints raised the possibility that lopinavir might achieve antimalarial activity through inhibiting PfHT1.\textsuperscript{81} By competing with the\(^{14}\)C-2-DOG uptake of parasites, lopinavir was found to block \textit{P. falciparum} glucose uptake at a submillimolar \(IC_{50}\). Using a PfHT1-overexpression HEK293 cell line, the inhibition of PfHT1-mediated\(^{3}\)H-2-DOG uptake by lopinavir was confirmed. The \(IC_{50}\) of PfHT1 inhibition (\(\sim 14 \mu M\)) is like that of its inhibition of parasite glucose uptake (\(\sim 16 \mu M\)), suggesting that lopinavir suppresses \textit{P. falciparum} growth and glucose uptake by inhibiting PfHT1\textsuperscript{81} (Figure 5B).

\section*{6 | DISCUSSION}

Despite the huge progress that has been made for malaria control, the global malaria burden remains heavy due to the malaria prevalence in Africa and Southeast Asia. New biological threats, exemplified by multidrug-resistant parasites, are compromising the campaign for malaria elimination.\textsuperscript{82} Development of novel antimalarial therapies based on new parasitic targets are urgently needed to conquer drug-resistant variants. Playing an essential role in the hexose uptake of asexual blood-stage \textit{Plasmodium} parasites, PfHT1 represents such a promising and unexploited drug target. Characterization of PfHT1 has uncovered its expression, functionality, enzymatic properties, and structural models, providing opportunities for further antimalarial agent development. Combining the semi-rational design, structural-based rational design, HTS, and other approaches, a series of leads with various chemical scaffolds have been identified or generated.

The first synthetic PfHT1 specific inhibitor, C3361, is the prototype for selective inhibition of PfHT1 compared to GLUTs. Characterization of the in vitro and in vivo parasiticidal activities of C3361 serves as a proof of principle for therapeutic intervention against malaria by inhibiting PfHT1. However, C3361 is not considered a drug-like molecule.\textsuperscript{73,83} Structural characterization of C3361-bound PfHT1 identified unprecedented conformational changes, which lead to the formation of a novel allosteric pocket. By generating C3361 derivatives to occupy the unexploited pocket, an orthosteric–allosteric dual inhibitor, HTI-1, which specifically and potently inhibits PfHT1 with high potency and efficiently suppresses the growth of the asexual blood-stage \textit{P. falciparum}, was rationally designed and generated. Nonetheless, it is unclear whether HTI-1 can kill parasites in vivo. Further optimization is expected to increase the affinity of HTI-1 while retaining its selectivity to PfHT1. Besides, the pharmacokinetics of HTI-1 are not well characterized and require further investigations.

Given the progress from the structural guided rational design of HTI-1, it is clear that structural models are of great importance for the understanding of the inhibition mechanism and optimization of lead compounds. It is therefore intuitive to investigate whether other PfHT1 inhibitors share a similar inhibition mechanism to C3361 and HTI-1. If so, optimization of PfHT1 inhibitors with other chemical scaffolds can be achieved following a similar principle to HTI-1. Otherwise, identifying novel inhibition mechanisms will urgently be needed. Despite the limited insight, hints for the existence of other inhibition mechanisms have been provided via the investigations of lopinavir and WU-1, where both are suggested to bind to an inward-facing state PfHT1.\textsuperscript{74,81} It is therefore worth elucidating the molecular mechanism of PfHT1 binding to new-scaffold inhibitors in future research.

Due to the limitations of current methods, life-cycle studies of PfHT1 rely on the \textit{P. berghei} infected mouse model and proteomic characterization.\textsuperscript{55} The expression and functionality of PfHT1 were mainly characterized in blood-stage parasites. Given that PfHT1 can express in all stages of the plasmodial life cycle, the parasiticidal activities of C3361 against liver-stage and mosquito-stage parasites have been obtained from the \textit{P. berghei} model. Therefore, further characterizations of PfHT1 in the whole life cycle are needed, which could extend the functionality of PfHT1 inhibitors from symptomatic phase treatments to prophylactic and transmission-blocking agents.

In an extended view, blocking the lactate export might be as efficient as inhibiting PfHT1-mediated glucose uptake.\textsuperscript{84,85} Given that glycolysis dominates the ATP generation of asexual blood-stage parasites, the end product, lactate, must be continuously exported through \textit{P. falciparum} lactate transporter (PfFNT) and human monocarboxylate transporter (MCT1) (Figure 1). PfFNT has been intensively interrogated for inhibitor development, which identified another Malarial Box compound, MMV007839, as a PfFNT-specific inhibitor.\textsuperscript{86\textendash 89} MMV007839 and its derivatives can efficiently kill parasites in vitro.\textsuperscript{90} Therefore, therapeutic interventions targeting PfFNT may supplement antimalarial therapies by restricting plasmodial energy supply.
In summary, combined multiple drug design and screening methods, further therapeutic development targeting PfHT1 are promising to generate novel antimalarial agents that may circumvent multidrug-resistant Plasmodium parasites and support the global fight against malaria.

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CONFLICT OF INTEREST
A patent application was filed for HTI-1. The author is one of the inventors for this patent application. Applicant institution: Tsinghua University. Application number: PCT/CN2020/074258. Status of application: not yet published.

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