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Identification and characterization of thiamine analogs with antiplasmodial activity

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ABSTRACT Thiamine is metabolized into thiamine pyrophosphate (TPP), an essential enzyme cofactor. Previous work has shown that oxythiamine, a thiamine analog, is metabolized by thiamine pyrophosphokinase (TPK) into oxythiamine pyrophosphate within the malaria parasite *Plasmodium falciparum* and then inhibits TPP-dependent enzymes, killing the parasite *in vitro* and *in vivo*. To identify a more potent antiplasmodial thiamine analog, 11 commercially available compounds were tested against *P. falciparum* and *P. knowlesi*. Five active compounds were identified, but only N3-pyridyl thiamine (N3PT), a potent transketolase inhibitor and candidate anticancer lead compound, was found to suppress *P. falciparum* proliferation with an IC_{50} value 10-fold lower than that of oxythiamine. N3PT was active against *P. knowlesi* and was >17 times less toxic to human fibroblasts, as compared to oxythiamine. Increasing the extracellular thiamine concentration reduced the antiplasmodial activity of N3PT, consistent with N3PT competing with thiamine/TPP. A transgenic *P. falciparum* line overexpressing TPK was found to be hypersensitized to N3PT. Docking studies showed an almost identical binding mode in TPK between thiamine and N3PT. Furthermore, we show that [^{3}H]thiamine accumulation, resulting from a combination of transport and metabolism, in isolated parasites is reduced by N3PT. Treatment of *P. berghei*-infected mice with 200 mg/kg/day N3PT reduced their parasitemia, prolonged their time to malaria symptoms, and appeared to be non-toxic to mice. Collectively, our studies are consistent with N3PT competing with thiamine for TPK binding and inhibiting parasite proliferation by reducing TPP production, and/or being converted into a TPP antimetabolite that inhibits TPP-dependent enzymes.

KEYWORDS malaria, *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium berghei*, N3PT, thiamine analogs, antiplasmodial agents, vitamin B_1

Malaria is a significant disease in Africa, several countries in Asia, and in Central and South America. In 2022, malaria infected approximately 249 million people and caused 608,000 deaths (1). The spread of antimalarial drug resistance has been rapid and extensive (2, 3). The limited availability of antimalarials has prompted researchers to develop new treatments (4, 5). During its intraerythrocytic asexual replication, which is exclusively responsible for the clinical symptoms, *Plasmodium* requires various vitamins as cofactors for biochemical processes (6). While nutrients such as amino acids and glucose are obtained from the host plasma or erythrocyte (7), thiamine **1** (vitamin B_1) can either be synthesized by the parasite itself (8) or acquired from the host. Thiamine **1**, being positively charged, is transported into the cytoplasm, where it is converted into the cofactor thiamine pyrophosphate (TPP) **2** by thiamine pyrophosphokinase (TPK; PF3D7_0924300) (Fig. 1). The important role of *Pf*TPK is highlighted by a "likely essential" score in a genome-wide piggyBac transposon mutagenesis screen (9). TPP **2** functions as an essential cofactor for multiple TPP-dependent enzymes, including pyruvate dehydrogenase E1 subunit (EC: 1.2.4.1; PF3D7_1124500; PF3D7_1446400) and

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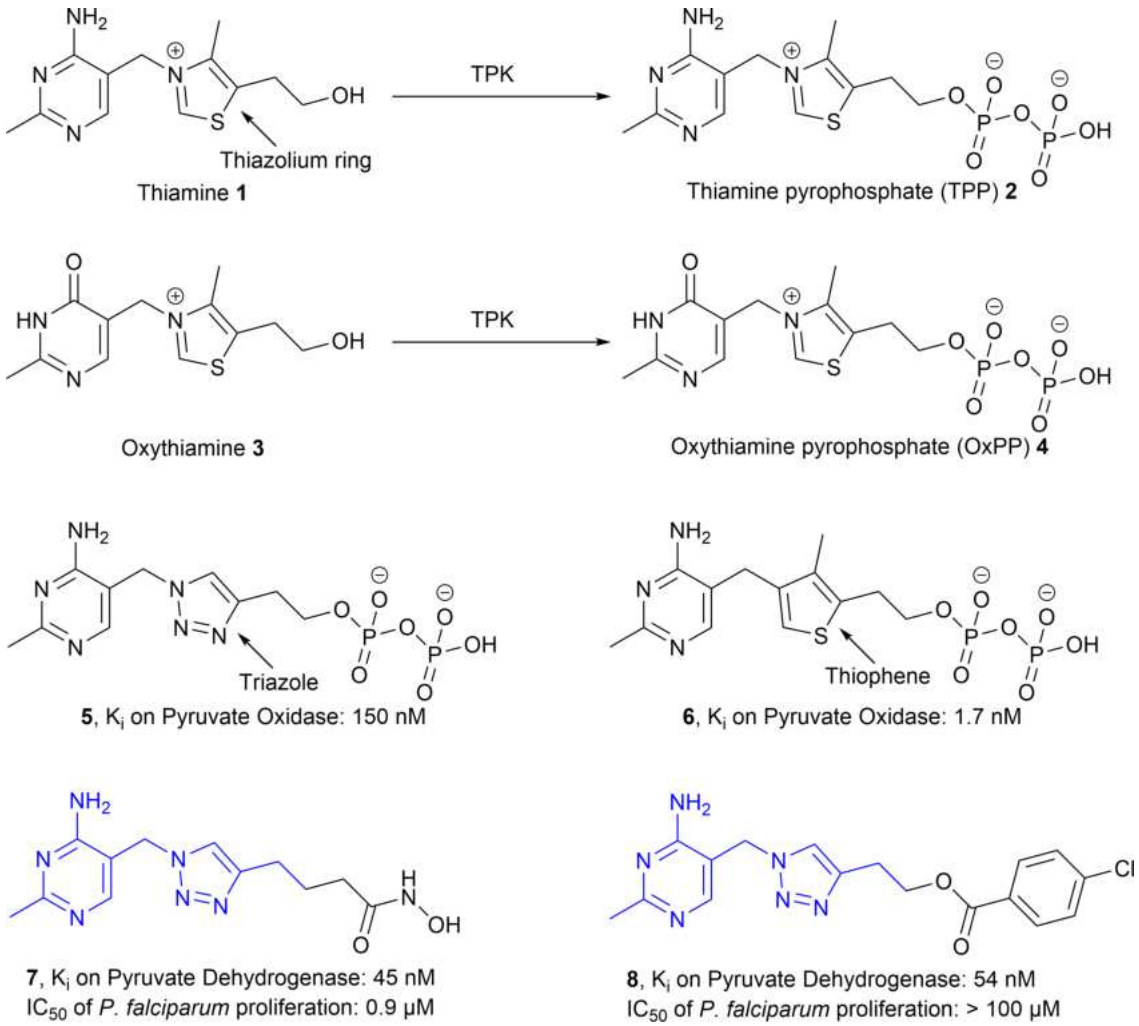


FIG 1 Structures of thiamine, TPP, and their analogs. Key moieties, the thiazolium, triazole, and thiophene rings, are indicated by the arrows. Activity of compounds **5** and **6** against pyruvate oxidase and compounds **7** and **8** against pyruvate dehydrogenase is shown. The reported antiplasmodial activities of compounds **7** and **8** are also included. The common substructure between compounds **7** and **8** is highlighted in blue.

1-deoxy-D-xylulose 5-phosphate synthase (EC: 2.2.1.7; PF3D7_1337200) both located in the apicoplast, oxoglutarate dehydrogenase E1 subunit/alpha-ketoglutarate dehydrogenase (EC: 1.2.4.2; PF3D7_0820700) and branched-chain alpha ketoacid dehydrogenase E1a subunit (EC: 1.2.4.4; PF3D7_1312600) both located in the mitochondrion, and transketolase (EC: 2.2.1.1; PF3D7_0610800) located in the cytoplasm (10–15). Due to the important roles of these enzymes in cellular metabolism, the thiamine/TPP utilization pathway is a promising antimalarial target (16).

Several thiamine analogs with potent antiplasmodial activity have been reported. Oxythiamine **3** is a thiamine analog, where the amino group on the pyrimidine ring is replaced by a hydroxyl group (Fig. 1). Oxythiamine **3** possesses antiplasmodial activity, both *in vitro* and *in vivo*, and has been used to validate the thiamine utilization pathway as an antimalarial drug target (15). As with **1**, **3** is activated by TPK into its pyrophosphate form, oxythiamine pyrophosphate (OxPP; Fig. 1) **4**. Occupying the cofactor pocket (with its PP moiety) but lacking catalytic activity (due to its modified pyrimidine ring), **4** is an inhibitor of TPP-dependent enzymes (17). Previous work has shown that at least two TPP-dependent enzymes may be inhibited by oxythiamine **3** (after conversion to OxPP **4**) as part of its mechanism of action against *P. falciparum* parasites (15).

Replacing the positively charged thiazolium ring of TPP with a neutral ring abolishes the catalytic capability, and we have shown that the resultant TPP analogs are potent inhibitors of TPP-dependent enzymes (18–20). While TPP analogs **5** and **6** are structurally similar (Fig. 1), changing the central ring from triazole to thiophene can lead to >80-fold increase in enzyme inhibition (18). Thiamine analogs **7** and **8,** bearing a common substructure (as highlighted in blue in Fig. 1), are both potent enzyme inhibitors; however, only **7**, featuring a hydroxamate tail group, inhibited *P. falciparum* parasite proliferation (19, 20). Our recent findings collectively suggest that despite being structurally similar, various analogs of **1** and **2** can have disparate biological activities, highlighting the importance of even subtle changes. Therefore, in this study, we investigated the antiplasmodial activity of a range of thiamine analogs in the hope of discovering an on-target thiamine analog with improved properties compared to **3**.

MATERIALS AND METHODS

The following thiamine analogs were purchased from Toronto Research Chemicals Canada or Sigma Aldrich: fursultiamine, cycotiamine, dibenzoyl thiamine, beclotiamine, oxo thiamine, thiotiamine, bisbentiamine. N3-pyridyl thiamine (N3PT) was purchased from MedChemExpress. Allithiamine and acetiamine were kindly provided by the Developmental Therapeutic Programs Cancer of the USA.

Parasite culture

The human malaria parasite *P. falciparum* strain 3D7 (chloroquine sensitive) and the same strain expressing an extra copy of TPK with a GFP-tag (*Pf*TPK-GFP) were maintained in the intraerythrocytic stage essentially as described previously (21). The macaque malaria parasite *P. knowlesi* strain H1 (adapted to human serum) was also maintained in the same way as *P. falciparum*, except that the culture medium was supplemented with 10% heat-inactivated, pooled human serum (22). Fresh human erythrocytes (blood type O^+) were added when parasites were in the trophozoite stage. The parasite cultures were maintained at 37°C inside a shaking incubator and under an atmosphere of 1% oxygen, 3% carbon dioxide, and 96% nitrogen.

In vitro antiplasmodial activity assay

Compounds were tested at different highest final concentrations (between 12.5 and 350 µM) depending on their solubility. Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) or water, followed by dilution in RPMI 1640 medium in the absence of thiamine (formulated in-house from the individual components of RPMI 1640, excluding thiamine) or in the presence of 2.97 µM (the concentration normally present in RPMI 1640) or 297 µM thiamine. The final concentration of DMSO that the parasites were exposed to never exceeded 0.05%, a concentration that has no effect on parasite proliferation (23). Four analogs (fursultiamine, cycotiamine, allithiamine, and ethyl thiamine) in addition to oxythiamine **3** and N3PT **9** were further investigated for their antiplasmodial activity against *P. knowlesi* in the presence of 2.97 µM thiamine. Twofold serial dilutions were performed, with each concentration tested in triplicate. The assay was performed as described, with some modifications (24). Experiments were initiated with parasites in the ring stage with a parasitemia of 0.5% (*P. falciparum*) and mixed stage with a parasitemia level of 1% (*P. knowlesi*) and a hematocrit of 1%. For experiments designed to allow comparison of the activity of the compounds against *P. falciparum* and *P. knowlesi*, asynchronous *P. falciparum* cultures were used to mimic those of *P. knowlesi*. Chloroquine (0.5 µM) was used as the positive control (i.e., complete inhibition of parasite proliferation), and parasites maintained in the absence of any inhibitor represented 100% parasite proliferation. The final volume in each well was 200 µL. Plates were incubated at 37°C under an atmosphere of 96% nitrogen, 3% carbon dioxide, and 1% oxygen.

Parasite proliferation was measured using the SYBR-Safe assay (25), which correlates fluorescence intensity to parasite DNA. Oxythiamine, fursultiamine, and allithiamine appeared to be incompatible with this fluorescence-based assay (there were inconsistent fluorescence intensity readings at some concentrations). For these compounds, the Malstat assay was therefore used instead. The Malstat assay correlates parasite lactate dehydrogenase activity with parasite proliferation during the incubation period (96 h for *P. falciparum* and 54 h for *P. knowlesi*) (26). The concentration at which the compound suppresses parasite proliferation by 50% (IC_{50}) was determined from non-linear regression plots using GraphPad Prism. The data were averaged from three independent experiments.

Generation of parasites expressing *Pf*TPK-GFP

The generation of parasites expressing a copy of *Pf*TPK tagged to GFP, in addition to the endogenous *Pf*TPK, was described previously (20). The transgenic parasites were maintained under WR99210 (10 nM) pressure.

Cytotoxicity evaluation of selected compounds

Cytotoxicity testing of selected compounds was conducted using human foreskin fibroblasts (HFF) as described (27), with some modifications. Dulbecco's Modified Eagle's Medium with 10% heat-inactivated fetal bovine serum was used for the assay. Briefly, the HFF cells were seeded in 96-well plates at a density of about 25×10^4 cells/mL. Cycloheximide (10 µM; a protein synthesis inhibitor) was used as a control to indicate complete inhibition of HFF cell proliferation. Plates were incubated at 37°C in a humidified, 5% carbon dioxide incubator for 96 h. A sample of the supernatant (150 µL) was then carefully aspirated from each well and discarded. The plates were then stored at −80°C. SYBR-Safe assay was used to measure cell proliferation. Briefly, the plates were thawed, and SYBR-Safe lysis solution (150 µL) was added to each well and mixed via pipetting to ensure the HFF cells were detached from the plate and lysed. The plates were then processed as described for the antiplasmodial assay.

Radioactive thiamine uptake assay

[^{3}H]Thiamine accumulation (a combination of thiamine transport and its metabolism into TPP) was measured using a method previously applied for other vitamins (28, 29), with some modifications. *P. falciparum* trophozoites were isolated from erythrocytes using 1% saponin. The isolated parasites were washed twice in 50 mL malaria saline (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 20 mM glucose, 1 mM $MgCl_2$, pH adjusted to 7.1). On average, the number of cells used was 0.8×10^8 cells/mL. [^{3}H]Thiamine, with a final concentration of 0.2 µCi/mL (200 nM), was added to the cell suspension. At predetermined time points (over 30 min), 200 µL of the mixture was added to an oil mix (a 5:4 mix of dibutyl phthalate:dioctyl phthalate). The mixture was centrifuged, washed three times, lysed with 0.1% triton X-100, and precipitated with 5% trichloroacetic acid. A 20 µL aliquot of the supernatant of the first time point was taken to determine the concentration of radioactivity in the extracellular solution. The uptake of [^{3}H]thiamine was expressed as CPM_{in}/CPM_{out}, defining the concentration of [^{3}H]thiamine (and [^{3}H]TPP generated by the parasite) inside the cell relative to that outside of the cell.

Computational docking

The docking of N3PT **9**, oxythiamine **3**, and thiamine **1** to the *Pf*TPK AlphaFold model (AF-Q14RW4-F1) was performed using the CCDC GOLD docking program. To identify the binding pockets and generate a holoenzyme, the model was superimposed onto a holoenzyme of mouse TPK (PDB:2f17), with a metal ion and products of TPK (AMP and pyrithiamine pyrophosphate, an analog of thiamine pyrophosphate that had been used in the crystal structure determination). The thiamine pyrophosphate-binding site was subsequently used to dock **1**, **3**, and **9**, while the AMP-binding site was used to dock

ATP. ATP, **1**, **3**, and **9** were generated using CCDC Mercury. The genetic algorithm runs were conducted with a set of 50 runs. Early termination was not allowed during these runs. To replicate their binding positions, the compounds were subjected to similarity and scaffold constraints based on the original ligands. The docking scoring and rescoring methods employed for analysis were CHEMPLP and GoldScore, respectively (30, 31). For molecular docking using CCDC GOLD, the best-performing ligand from each compound series was chosen as a representative.

In vivo antimalarial activity assay

Female Swiss mice (8 weeks old) were used for this experiment. The study was conducted in strict accordance with the German "Tierschutzgesetz in der Fassung vom 22. Juli 2009" and Directive 2010/63/EU of the European Parliament and Council, which focuses on the protection of animals used for scientific purposes. To assess the effectiveness of N3PT **9** in suppressing parasite proliferation in mice, Peter's 4-day suppression test was used (32). To induce infection, naive mice were injected intravenously with 1×10^4 *P. berghei* parasites, ANKA strain, obtained from an infected donor mouse. The mice were then divided into three groups, each consisting of three individuals. Two hours post-infection, one group received 100 mg/kg and a second group received 200 mg/kg of N3PT **9** dissolved in 100 µL phosphate-buffered saline (PBS) via intraperitoneal injection. The third group of infected mice served as the control and was administered with 100 µL PBS as a vehicle control. Repeat doses were given at intervals of 24, 48, and 72 h after the initial dose. Throughout the duration of the experiment, any signs of toxicity, such as changes in movement and body weight, were monitored and recorded daily. Forty-eight hours after the final drug administration, a small amount of blood was collected from the tail of each mouse to prepare Giemsa-stained slides. The parasitemia of the mice was determined by counting a minimum of 500 erythrocytes. The counting was carried out in a blinded fashion—which parasitemia belonged to which group was only revealed once the counting had been complete.

Statistical analysis

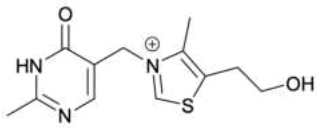
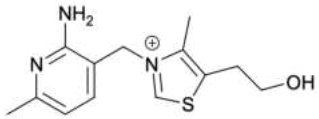
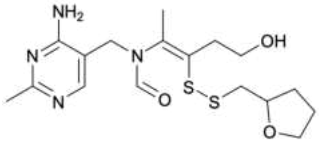
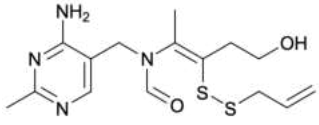
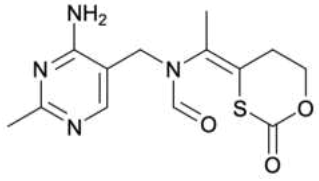
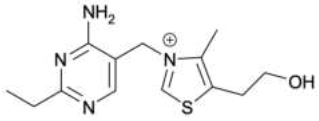
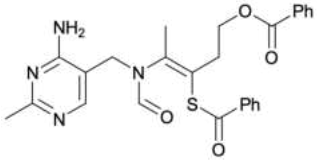
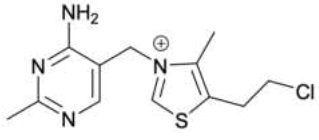
Statistical differences were determined using paired or unpaired, two-tailed Student's *t*-tests, as appropriate. Comparison of mouse weight loss over time between the different groups was carried out using analysis of covariance (ANCOVA).

RESULTS

In vitro antiplasmodial activity and cytotoxicity

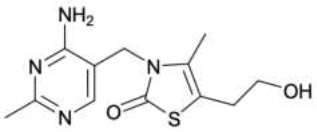
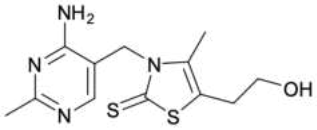
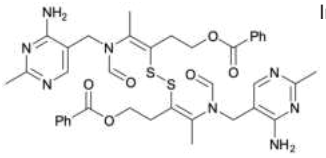
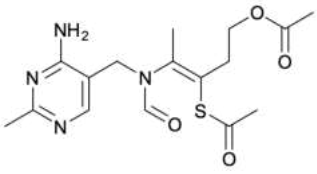
Twelve thiamine analogs, oxythiamine **3** and **9–19,** were tested against the chloroquine-sensitive strain (3D7) of *P. falciparum* in the absence or presence of different extracellular thiamine concentrations (Fig. S1). Alongside oxythiamine **3** [consistent with previous observations (15)], N3PT **9**, fursultiamine **10**, allithiamine **11**, cycotiamine **12**, and ethyl thiamine **13** possessed antiplasmodial activity at the concentrations tested (Table 1). N3PT **9** exhibited significantly more potent inhibition of parasite proliferation compared to oxythiamine **3** at all the extracellular concentrations of **1**: 14-fold lower IC_{50} in thiamine-free medium ($P = 0.04$, paired *t*-test), 62-fold lower in the presence of 2.97 µM thiamine ($P = 0.0006$, paired *t*-test), and 58-fold lower in the presence of 297 µM thiamine ($P = 0.001$, paired *t*-test). Increasing the extracellular thiamine concentration from 0 to 297 µM reduced the antiplasmodial activity of both **3** and **9,** but to different degrees, with oxythiamine **3** showing a 986-fold reduction in activity and N3PT **9** having its activity reduced 220-fold (Table 1; Fig. S2). However, increasing the extracellular thiamine concentration has no effect on the antiplasmodial activity of compounds **10–13**, consistent with them acting via a mechanism unrelated to thiamine utilization. Compounds **9–13** were then tested against *P. knowlesi*, a surrogate of *P. vivax* (33), the second most important *Plasmodium* species in terms of morbidity and mortality (34). All the compounds except for **13** inhibited the proliferation of *P. knowlesi*, albeit with IC_{50}

TABLE 1 Antiplasmodial activity of the thiamine analogs against *P. falciparum* and *P. knowlesi*, and their cytotoxicity against HFF cells[a]

Structure and name	IC_{50} values (μM)				
	P. falciparum			*P. knowlesi*	HFF cells
	Extracellular thiamine concentration (μM)				
	0	2.97	297	2.97	11.6
 Oxythiamine **3**	5.5 ± 0.8	5,425 ± 150	5,559 ± 177	10,800 ± 450	Not tested
 N3PT **9**	0.4 ± 0.1	88 ± 11	96 ± 14	91 ± 10	>200
 Fursultiamine **10**	12.0 ± 0.3	12.0 ± 0.3	12.0 ± 0.6	23 ± 6	38 ± 7
 Allithiamine **11**	75 ± 22	80 ± 26	85 ± 27	84 ± 5	30 ± 15
 Cycotiamine **12**	52 ± 9	53 ± 11	59 ± 16	143 ± 5	>200
 Ethyl thiamine **13**	123 ± 9	127 ± 10	176 ± 22	>1,000	>1,000
 Dibenzoyl thiamine **14**	Inactive at 12.5 μM	Inactive at 12.5 μM	Inactive at 12.5 μM	Not tested	Not tested
 Beclotiamine **15**	Inactive at 325 μM	Inactive at 325 μM	Inactive at 325 μM	Not tested	Not tested

(*Continued on next page*)

TABLE 1 Antiplasmodial activity of the thiamine analogs against *P. falciparum* and *P. knowlesi*, and their cytotoxicity against HFF cells[a] (*Continued*)

Structure and name	IC_{50} values (μM)				
	P. falciparum			*P. knowlesi*	HFF cells
	Extracellular thiamine concentration (μM)				
	0	2.97	297	2.97	11.6
 Oxo thiamine **16**	Inactive at 150 μM	Inactive at 150 μM	Inactive at 150 μM	Not tested	Not tested
 Thiotiamine **17**	Inactive at 200 μM	Inactive at 200 μM	Inactive at 200 μM	Not tested	Not tested
 Bisbentiamine **18**	Inactive at 200 μM	Inactive at 200 μM	Inactive at 200 μM	Not tested	Not tested
 Acetiamine **19**	Inactive at 325 μM	Inactive at 325 μM	Inactive at 325 μM	Not tested	Not tested

[a]Data are averaged from three independent experiments, each carried out in triplicate. Errors represent SEM.

values, in general, being higher than those against *P. falciparum* (Table 1; Fig. S2). They were then also tested against HFF cells to determine if they are cytotoxic. While **10** and **11** showed low micromolar activity against HFF cells, compounds **9**, **12**, and **13** were not cytotoxic (Fig. S3).

Effect of TPK overexpression on parasite sensitivity to thiamine analogs

To determine whether the mode of antiplasmodial action is dependent on TPK, compounds were tested against a *P. falciparum* cell line (termed *Pf*TPK-GFP), which expresses a GFP-tagged copy of TPK, in addition to the endogenous TPK. In both thiamine-free and thiamine-replete conditions, the *Pf*TPK-GFP cell line was hypersensitive to oxythiamine **3** when compared to control parasites transfected with a plasmid expressing only GFP (termed *Pf*3D7-GFP; Fig. 2), consistent with previous observations (15). Having confirmed that the *Pf*TPK-GFP cell line was behaving as expected, a few analogs (**9**, **10** and **12**) were selected, based on their antiplasmodial activity and structural modifications, and tested against these parasites. Fursultiamine **10** and cycotiamine **12** were equally active against the *Pf*TPK-GFP cell line as they were against the control parasites (Fig. S4), consistent with TPK not playing a role in the antiplasmodial activity of these compounds. In contrast, and as observed previously for oxythiamine **3** (15), N3PT **9** was more active against the *Pf*TPK-GFP cell line under thiamine-free conditions (IC_{50} = 0.05 ± 0.02 μM) when compared to *Pf*3D7-GFP parasites (IC_{50} = 0.36 ± 0.03 μM, P = 0.002 , unpaired t-test), although the effect was somewhat less pronounced than that observed with oxythiamine **3** (7-fold vs 13-fold) (Fig. 2). However, in the presence of 297 μM thiamine, the potency of N3PT **9** against both parasite lines

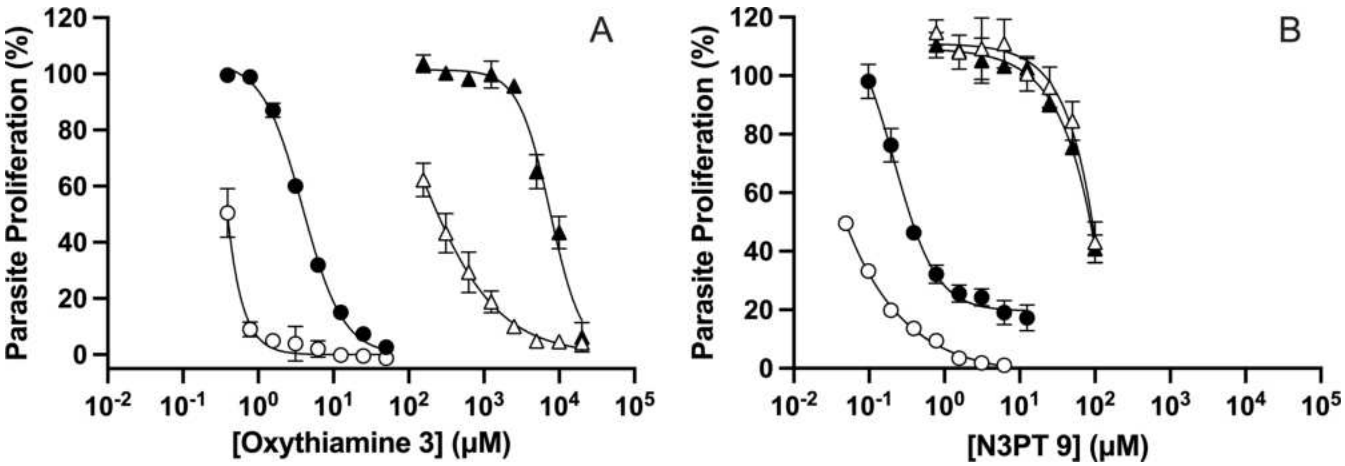


FIG 2 Antiplasmodial activity of oxythiamine **3** (A) and N3PT **9** (B) against *Pf*TPK-GFP (white symbols) and *Pf*3D7-GFP (black symbols). The experiments were carried out in thiamine-free medium (circles) or medium containing 297 µM thiamine (triangles). Data are averaged from three independent experiments, each carried out in triplicate. Error bars represent SEM and, where not visible, are smaller than the symbols.

was comparable (*Pf*TPK-GFP: IC_{50} = 93 ± 4 µM; *Pf*3D7-GFP: IC_{50} = 98 ± 2 µM, P = 0.13 , unpaired *t*-test), unlike what we observed with oxythiamine **3** (*Pf*TPK-GFP: IC_{50} = 275 ± 59 µM; *Pf*3D7-GFP: IC_{50} = 8,220 ± 267 µM, P = 0.0004, unpaired *t*-test) (Fig. 2).

The effect of oxythiamine 3 and N3PT 9 on [^{3}H]thiamine accumulation

A [^{3}H]thiamine accumulation assay was conducted to investigate the effects of compounds **3** and **9** on thiamine uptake into the parasite and its subsequent metabolism into TPP **2** (and any other thiamine-derived metabolites). In the absence of the analogs, isolated parasites accumulated [^{3}H]thiamine and thiamine metabolites at a rate of 0.8 ± 0.3 CPM_{in}/CPM_{out} per minute (measured in the linear part of the time course, 5–30 min). The presence of 100 µM N3PT **9** and a higher concentration of oxythiamine **3** (500 µM; chosen due to its lower antiplasmodial potency when compared to **9**) significantly reduced [^{3}H]thiamine/metabolite accumulation in isolated parasites by more than threefold (0.2 ± 0.04 and 0.21 ± 0.02 CPM_{in}/CPM_{out} per minute, respectively, P ≤ 0.04, paired *t*-test, Fig. 3). These data are consistent with compounds **3** and **9** reducing [^{3}H]thiamine/metabolite accumulation by competing with **1** for thiamine transporters and/or metabolism by TPK.

Docking of oxythiamine 3 and N3PT 9 into the active site of *Pf*TPK

Since N3PT **9** only has a small structural modification in the aminopyrimidine ring when compared to thiamine, computational docking was performed to determine whether it is likely to bind in the active site of *Pf*TPK, supporting the data observed in Fig. 3. With no structure of *Pf*TPK available from either crystallography or cryo-EM, an AlphaFold model (AF-Q14RW4-F1) was used. The model is an apoenzyme consisting of only the tertiary structure of the protein. To identify the binding pockets and generate a holoenzyme with its substrate, the model was superimposed onto a holoenzyme of mouse TPK (PDB:2f17), which has a sequence identity with *Pf*TPK of 33.5%, and has a metal ion, AMP, and pyrithiamine pyrophosphate, an analog of thiamine pyrophosphate, all bound (35) (Fig. 4A). The thiamine pyrophosphate-binding site was subsequently used to dock thiamine **1**, oxythiamine **3**, and N3PT **9**, while AMP was docked into the AMP pocket (Fig. 4B through D). N3PT **9** has been shown to be converted into N3PT pyrophosphate by the human TPK resulting in inhibition of transketolase activity in human colon cancer cells (36). Similarly, oxythiamine **3** is a known substrate of *Pf*TPK (15), and it is oxythiamine pyrophosphate that is likely toxic to parasites. The docking results (Fig. 4) showing that N3PT **9**, thiamine, and oxythiamine **3** have very similar binding poses (Fig. 4B through

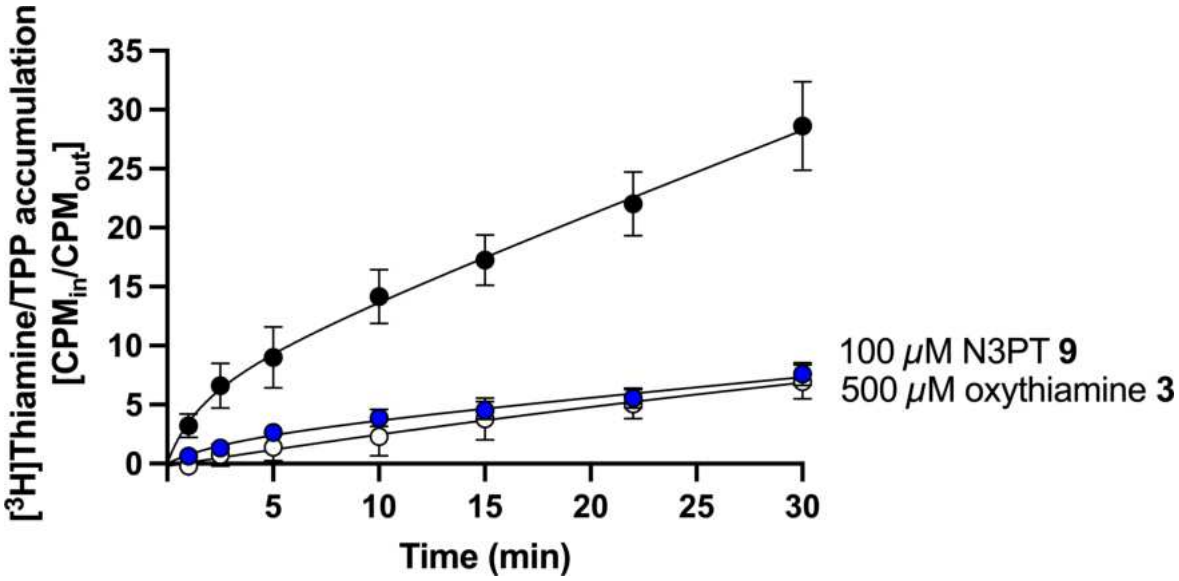


FIG 3 Accumulation of [^{3}H]thiamine and its metabolites in isolated *P. falciparum* trophozoites in the absence (black circles) or presence of 100 µM N3PT **9** (blue circles), or 500 µM oxythiamine **3** (white circles). Data are averaged from three independent experiments, each carried out in duplicate. Error bars represent SEM and, where not visible, are smaller than the symbols.

D; Fig. S5), together with the latter two compounds being known substrates of *Pf*TPK, support the hypothesis that N3PT **9** is also a substrate of *Pf*TPK. The docked model of thiamine has one hydrogen bond from N-1 to the OH of Ser371 and one from the 4-NH_2 group to the main chain C = O of Gln257. However, it seems likely that the side chain of Glu258 would twist round to form a second hydrogen bond to the NH_2 group—this is what happens in PDB:2f17, which has an Asp at the equivalent position. Compared to thiamine's pyrimidine ring, N3PT **9** has a pyridine ring (N-1 replaced by CH), whereas oxythiamine **3** has an oxopyrimidine in place of thiamine's aminopyrimidine (NH_2 replaced by O). These structural differences would mean that N3PT **9** would have one less hydrogen bond interaction with *Pf*TPK when compared to thiamine, and oxythiamine **3** would have one or two less hydrogen bonds. This would mean that **9** and **3** are likely to have lower affinities than thiamine, and **3** may have lower affinity than **9**. Consistent with compound **3** having a lower affinity than compound **9** for *Pf*TPK, a bigger fold-shift in the antiplasmodial IC_{50} value of compound **3** compared to compound **9** was observed in the presence of extracellular thiamine (Table 1). Also consistent with a higher affinity of compound **9** for *Pf*TPK, compared to compound **3**, is the observation that overexpression of *Pf*TPK has no effect on the activity of N3PT **9** in the presence of a high concentration of extracellular thiamine, but the activity of compound **3** is increased under the same conditions (Fig. 2).

In vivo antimalarial activity of N3PT 9

To assess whether N3PT **9** possesses antimalarial activity *in vivo*, we tested it in the murine malaria model, using the *P. berghei* ANKA strain and Peter's Suppressive Test protocol. Two doses of **9** were tested, 100 and 200 mg/kg, administered intraperitoneally. Signs of toxicity, such as weight loss and reduced movement, were monitored from day 0 to day 5 post-infection (Fig. S6). N3PT **9** showed a dose-dependent reduction in the parasitemia of infected mice (P = 0.0015 and P < 0 0.0001 for the 100 and 200 mg/kg doses, respectively; Student's *t*-test) relative to the non-treated group (vehicle only; Fig. 5A). Although the 100 mg/kg dose did not increase the time to symptoms in mice, the 200 mg/kg dose prolonged their time to symptoms by 9 days compared to the untreated mice (Fig. 5B). Treatment with 200 mg/kg N3PT **9** also prevented the mild gradual weight loss associated with progression of *P. berghei* infection (P = 0.0014, ANCOVA difference in

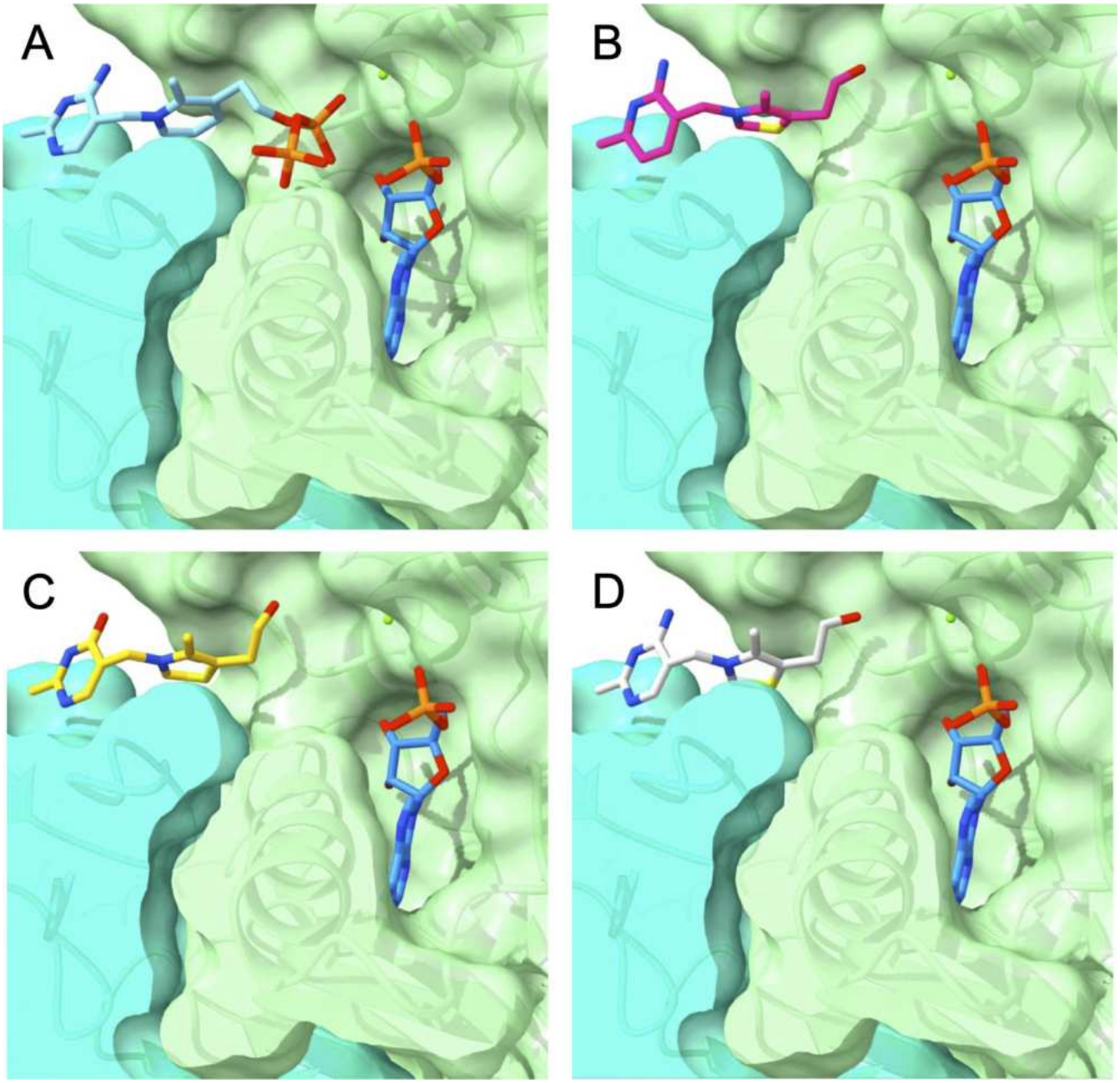


FIG 4 Docking of various compounds into the active site of *Pf*TPK (shown as surface and ribbon) based on an AlphaFold structure (AF-Q14RW4-F1) modeled on the crystal structure of the mouse TPK (PDB:2f17) generated in the presence of AMP. Cyan and green colors represent the two protomers of the putative *Pf*TPK dimer. *Pf*TPK with (A) the ligands in the mouse TPK crystal structure, pyrithiamine pyrophosphate, and AMP, (B) dockings of AMP and N3PT **9** in pink, (C) oxythiamine **3** in yellow, and (D) thiamine **1** in white.

slopes; Fig. S6). This did not happen with 100 mg/kg N3PT **9** ($P = 0.55$, ANCOVA difference in slopes; Fig. S6), consistent with this dose not increasing the time to symptoms.

DISCUSSION

To identify thiamine analogs with better antiplasmodial activity than oxythiamine **3**, 11 compounds **9–19** were evaluated against two *Plasmodium* species alongside oxythiamine **3**. These 12 compounds together sampled a wide chemical space, with 4/12 being positively charged, 3/12 bearing a modified aminopyrimidine ring, 2/12 bearing a neutral

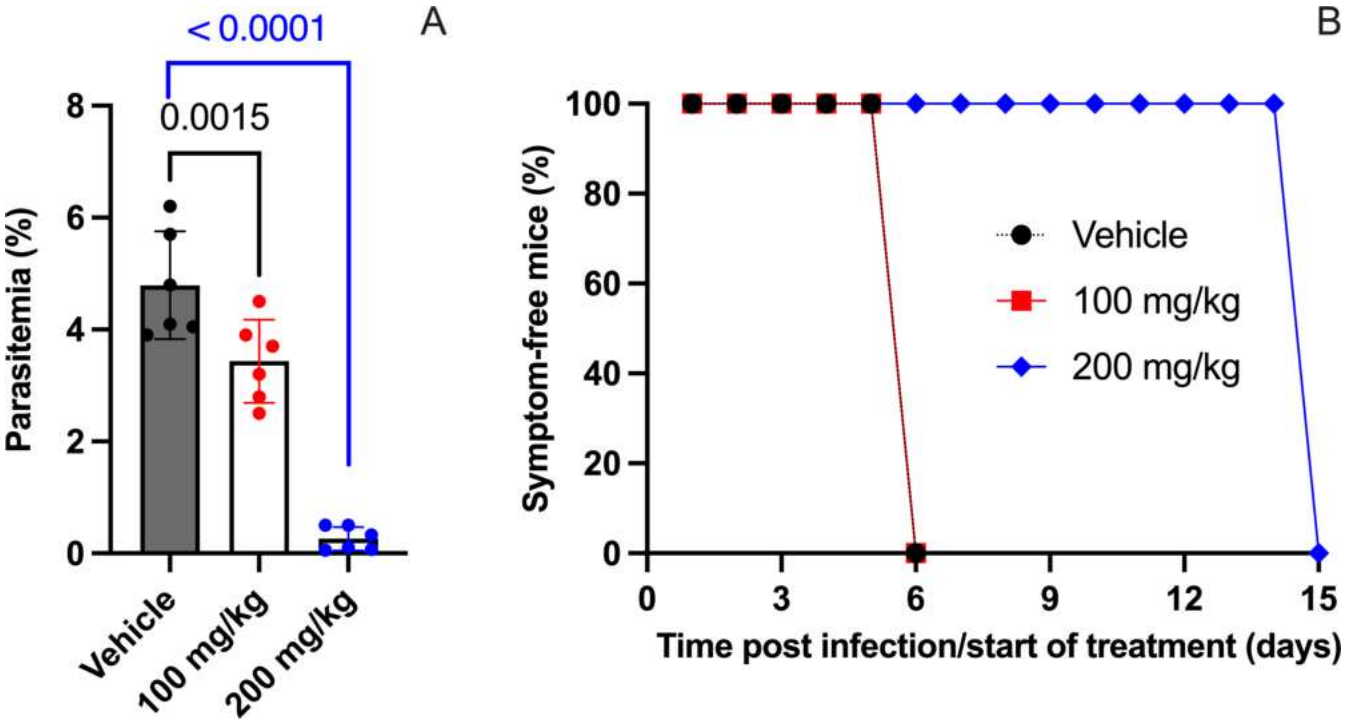


FIG 5 (A) *In vivo* antimalarial activity of 100 and 200 mg/kg N3PT **9**. The data show the average parasitemia at day 5 after infection and daily treatment. Symbols represent data points from individual mice ($n = 6$). Two independent experiments were carried out, each with three mice in each group. Bars represent averaged data ± SD. Student's *t*-test results are indicated above the bars for relevant comparisons. (B) The percentage of symptom-free mice according to treatment: vehicle/PBS (black circles), 100 mg/kg N3PT **9** (red squares), and 200 mg/kg (blue diamonds). $n = 6$, from two biological replicates.

central ring [as for **5–8** that we have previously reported (18–20)], and 6/12 featuring an open scaffold in place of the central ring (Table 1). Compounds **9–13** suppressed *P. falciparum* with IC_{50} values in the micromolar range, while **14–19** were unable to inhibit parasite proliferation by more than 50% at the highest concentration tested (which varied depending on the solubility of the compound). Compounds **13** and **15**, which differ from thiamine's pyrimidine ring and the hydroxyl tail, respectively, may be able to hijack the thiamine transporter for cell entry due to their overall structural similarity. While both compounds may compete with intracellular thiamine for binding to TPK, only ethyl thiamine **13** can be activated into **13**-pyrophosphate, which in turn competes with TPP for the TPP-dependent enzymes' coenzyme pocket. Oxo thiamine **16** and thiotiamine **17** featuring a neutral central ring (as with **7**) failed to inhibit parasite proliferation by more than 30% at the highest concentration tested, probably because they do not have a proper metal-binding group on their tail to enable efficient enzyme binding (18–20). Compounds **10–12**, **14**, **18**, and **19** are all precursors of thiamine and because they are neutral, they may be able to enter cells via simple diffusion. In the cytoplasm, they are chemically transformed into the open-thiol species **20** (Fig. 6), which in turn spontaneously cyclizes into thiamine. They can, therefore, function as thiamine supplements. Because fursultiamine **10** and allithiamine **11** have greater oral bioavailability than the thiamine salt, they have been used to treat beriberi (a disease caused by thiamine deficiency) (37). Upon reduction, fursultiamine **10** and allithiamine **11** produce a corresponding thiol by-product alongside **20**. The observed antiplasmodial effect of these compounds can neither be antagonized by increasing the extracellular thiamine concentration nor can the antiplasmodial activity of fursultiamine **10** be altered by expression of *Pf*TPK-GFP. Accordingly, the inhibitory effect may be due to a non-specific action of their released thiol, which in turn may lead to cell damage, as intracellular thiol oxidation produces hydrogen peroxide (H_2O_2), an oxidizing agent (38). The observed toxic effects in human cells associated with **10** and **11** were a surprising observation,

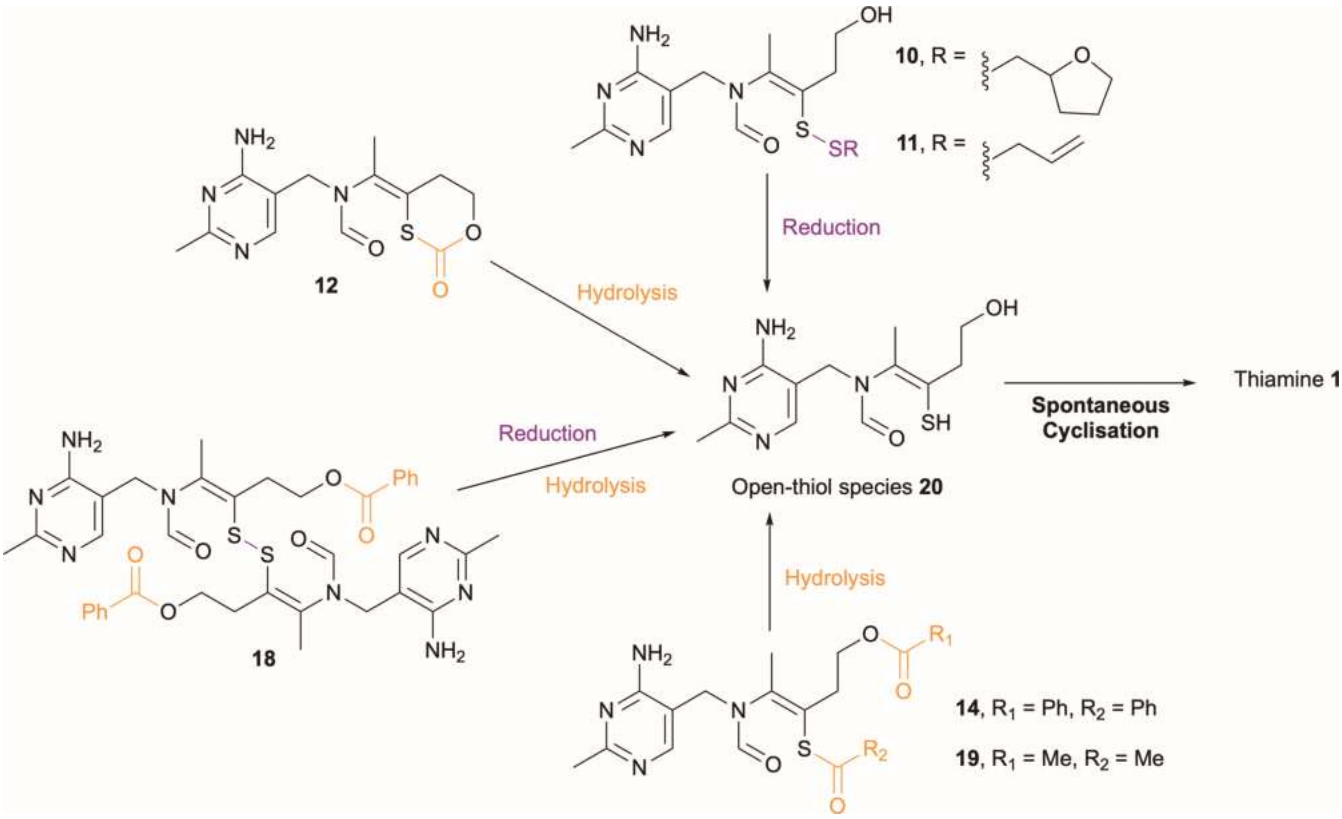


FIG 6 Stepwise transformation of thiamine precursors **10–12**, **14**, **18**, and **19** through an open-thiol species **20** to thiamine **1**. Substructures of the thiamine precursors that will be released in the course of transformation are colored. Reduction and hydrolysis are the two main reaction types, which are highlighted in purple and orange, respectively.

given that they have been used in humans as thiamine supplements and may also be attributed to the released thiol. The transformation of dibenzoyl thiamine **14**, bisbentiamine **18**, and acetiamine **19** into thiamine generates by-products that are likely to be non-toxic to the parasite at the concentrations used (**14** and **18** produce benzoate, and **19** produces acetate). This is consistent with the lack of antiplasmodial activity observed for these compounds. The one exception is cycotiamine **12,** which produces carbonate as a by-product. Carbonate is also expected to be non-toxic to the parasite, so the antiplasmodial action of cycotiamine **12** may be attributed to an off-target mechanism of action by its untransformed form. This notion is corroborated by our findings that its activity cannot be antagonized by increasing the extracellular concentration of thiamine nor is it modified by overexpressing *Pf*TPK.

Upon pyrophosphorylation, N3PT **9** is a known transketolase inhibitor and has shown promising anticancer potential (36, 39). It is the only thiamine analog we tested that showed submicromolar antiplasmodial activity *in vitro* in the absence of extracellular thiamine and was between 10 and 60 times more potent than oxythiamine **3** (depending on the extracellular thiamine concentration). We observed a >200-fold reduction in antiplasmodial activity of N3PT **9** when thiamine was added to thiamine-free medium (Table 1), demonstrating its competitive relationship with thiamine/TPP. The fact that N3PT **9** and ethyl thiamine **13** are both very similar to thiamine **1** but have very different antiplasmodial activity is consistent with the thiamine utilization pathway being sensitive to subtle structural changes in the thiamine analogs. Accordingly, ethyl thiamine **13** was not investigated further. N3PT **9** was also active against *P. knowlesi*, had a high selectivity index (Table 1), and was more active than oxythiamine **3**, at half the dose, when tested *in vivo* (Fig. 5). This is an important stepping stone toward developing an antimalarial compound targeting this pathway. Synthesis of a range of derivatives

of the N3PT **9** lead and systematic *in vitro* testing are warranted to further explore the potential of harnessing the strict dependence of intraerythrocytic *Plasmodium* replication on thiamine.

While the dependence of oxythiamine **3**'s activation by *Pf*TPK has been established here and previously (15), whether N3PT **9** is also metabolized by *Pf*TPK remains to be shown. To address this, we conducted computational modeling to dock oxythiamine **3**, N3PT **9**, and thiamine **1** into the active site of *Pf*TPK. As shown in Fig. 4, the binding mode of N3PT **9** is very similar to that of thiamine **1** and oxythiamine **3**. This is consistent with N3PT **9** being a *Pf*TPK substrate, but the removal of one hydrogen bond from the pyrimidine ring contrasts with the marked higher inhibition of TPK, indicating that for a better understanding of the binding affinity of N3PT **9** to *Pf*TPK, structural data are needed. An interaction between N3PT **9** and *Pf*TPK is further supported by the [^{3}H]thiamine accumulation experiment, in which [^{3}H]thiamine/metabolite (likely to be [^{3}H]TPP) accumulation in isolated parasites was significantly reduced in the presence of 100 µM N3PT **9** (Fig. 3), consistent with N3PT **9** interacting with TPK, either as a substrate or inhibitor. The same effect was observed with oxythiamine **3,** but at a higher concentration, consistent with its lower antiplasmodial activity. In addition, although in the presence of thiamine, the potency of N3PT **9** against parasites expressing *Pf*TPK-GFP is the same as that against control parasites, in thiamine-free medium, *Pf*TPK-GFP-expressing parasites are fourfold more sensitive to N3PT **9** when compared to parasites expressing GFP (Fig. 2), consistent with its antiplasmodial activity, involving, at least in part, *Pf*TPK.

In conclusion, our data identify N3PT **9** as an improved inhibitor over previously reported antiplasmodial thiamine analogs. Further medicinal chemistry efforts to optimize its pharmacological properties may afford a novel antimalarial agent targeting parasite thiamine utilization.

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ETHICS APPROVAL

The protocol received approval from the ethics committee of the Berlin state authority ['Landesamt für Gesundheit und Soziales Berlin', permit number (G029415)].

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (AAC01096-24-s0001.pdf). Figures S1 to S6.

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