Plasmodium falciparum Purine Nucleoside Phosphorylase Is Critical for Viability of Malaria Parasites*§

Received for publication, September 17, 2008, and in revised form, October 22, 2008. Published, JBC Papers in Press, October 28, 2008, DOI 10.1074/jbc.M807218200

Dennis C. Madrid†1, Li-Min Ting‡, Karen L. Waller§2, Vern L. Schramm§, and Kami Kim†§3

From the †Department of Microbiology and Immunology, the §Division of Infectious Diseases, Department of Medicine, the ‡Division of Cardiology, Department of Medicine, and the †Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

Human malaria infections resulting from Plasmodium falciparum have become increasingly difficult to treat due to the emergence of drug-resistant parasites. The P. falciparum purine salvage enzyme purine nucleoside phosphorylase (PfPNP) is a potential drug target. Previous studies, in which PfPNP was targeted by transition state analogue inhibitors, found that those inhibiting human PNP and PfPNPs killed P. falciparum in vitro. However, many drugs have off-target interactions, and genetic evidence is required to demonstrate single target action for this class of potential drugs. We used targeted gene disruption in P. falciparum strain 3D7 to ablate PNP expression, yielding transgenic 3D7 parasites (Δpfpnp). Lysates of the Δpfpnp parasites showed no PNP activity, but activity of another purine salvage enzyme, adenosine deaminase (PfADA), was normal. When compared with wild-type 3D7, the Δpfpnp parasites showed a greater requirement for exogenous purines and a severe growth defect at physiological concentrations of hypoxanthine. Drug assays using immucillins, specific transition state inhibitors of PNP, were performed on wild-type and Δpfpnp parasites. The Δpfpnp parasites were more sensitive to PNP inhibitors that bound hPNP tighter and less sensitive to MT-ImmH, an inhibitor with 100-fold preference for PfPNP over hPNP. The results demonstrate the importance of purine salvage in P. falciparum and validate PfPNP as the target of immucillins.

Each year, Plasmodium species infect 300 to 500 million people and cause nearly two million deaths, mostly in children under the age of five in sub-Saharan Africa (1). Most deaths are due to infection with Plasmodium falciparum. Only AIDS and tuberculosis are more lethal human infectious diseases. No vaccine is available, despite intensive international efforts. At present, control of malaria is dependent on prevention with bed nets, insecticides or chemoprophylaxis, and chemotherapeutic treatment of clinical cases. However, chemotherapy for malaria has been complicated by recent increases in mortality and morbidity due to the emergence of drug-resistant strains (2).

Because parasitic protozoa are unable to synthesize purines de novo, purine salvage has been proposed as a potential target for chemotherapy of protozoan parasite infections, including those caused by Plasmodium spp. Malaria parasites are obligate intracellular parasites that carry out their asexual cycle in erythrocytes. Unlike most mammalian cells, erythrocytes have no biochemical machinery for de novo purine synthesis, but act as a rich source of purine salvage enzymes, particularly purine nucleoside phosphorylase (PNP) and adenosine deaminase (ADA). The purine salvage pathway of Plasmodium begins with the deamination of adenosine to inosine by ADA, followed by conversion of inosine to hypoxanthine by PNP. The final enzyme in the pathway is hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT). Hypoxanthine is a precursor for all purines and is a central metabolite for nucleic acid synthesis in P. falciparum. Interestingly, P. falciparum is able to survive in both PNP- and ADA-deficient erythrocytes, suggesting that Plasmodium enzymes, PfADA and PfPNP, are sufficient for survival of parasites within the erythrocyte (3, 4).

Prior studies have shown that PfPNP and PfADA have an additional specificity for 5′-methylthiopurines, and that salvage of 5′-methylthioadenosine (MTA), a dead-end molecule of polypurine synthesis, is through the malarial purine salvage enzymes (5). Although humans and other Apicomplexa, such as Toxoplasma and Eimeria, have a redundant pathway for purine salvage via adenosine kinase (6), PNP, PfPNP, and PfHGXPRT appear to be the only pathway by which purines are salvaged in Plasmodium. Because P. falciparum expresses fewer enzymes in the pathways for purine nucleoside and MTA salvage than its host, these pathways are attractive targets for antibiotic design. Disruption of PfPNP expression or activity may not only affect purine salvage, but could potentially perturb homeostasis of the polypurine pathway.

Immucillins are powerful transition state inhibitors of PNP that kill P. falciparum in vitro by inducing purine-less death (7, 8). Most immucillins tested in malaria cultures, such as ImmH,

* This work was supported, in whole or in part, by National Institutes of Health Grants NSRA 5 F31 AI056665-4 (to D. C. M.), T32-CM007288 (AECOM Medical Science Training Program), R01 AI049512 (to V. L. S.), and R21-AI052469 (to K. K.). This work was also supported by United States Army Research Grant W81XWH-05-2-0025 (to K. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Table S1.

1 Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.

2 Present address: Dept. of Microbiology, Monash University, Victoria 3800 Australia.

3 To whom correspondence should be addressed: 1300 Morris Park Ave., Ullmann 1225, Bronx, NY 10461. Tel.: 718-430-2611; Fax: 718-430-8968; E-mail: kkim@aecom.yu.edu.

4 The abbreviations used are: PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase; HGXPRT, hypoxanthine-guanine phosphoribosyl transferase; MTA, methylthioadenosine; WT, wild type.
bind with a higher specificity to human PNP than PfPNP, and it could not be determined whether inhibition of PfPNP alone was capable of causing parasite starvation in the parasite (7, 8). The discovery of a novel purine-recycling pathway in malaria, with additional specificity for 5′-methylthiopurines, led to the development of a second generation of immucillins with 5′-methylthio modifications. MT-ImmH shows a 100-fold preference for PfPNP and kills *P. falciparum in vitro* with a similar IC₅₀ to ImmH (5). The additional specificity of PfPNP for 5′-methylthiopurines permits specific targeting of the malarial purine salvage pathway and perhaps the polyamine pathway by inhibiting a single enzyme.

Many drugs have off-target interactions, and genetic evidence is required to demonstrate the importance of the proposed target. To further explore the importance of PfPNP we used single crossover homologous recombination (9) to genetically disrupt *pfpnp* in *P. falciparum*. Immucillin drug sensitivity profiles of both wild-type (WT) parasites and transgenic parasites lacking PfPNP (∆pfpnp) were altered, confirming the importance of inhibition of PfPNP for immucillin efficacy. The ∆pfpnp lines have a greater requirement for exogenous purines and are unable to thrive at physiological concentrations of hypoxanthine. These results illustrate the importance of purine salvage enzymes for *P. falciparum* viability.

**EXPERIMENTAL PROCEDURES**

**P. falciparum Culture**—Human erythrocytes were collected from local volunteers under protocol CCI 99–240 of the Albert Einstein College of Medicine. *P. falciparum*, strain 3D7, was grown in RPMI supplemented with 0.5% (w/v) Albumax II (Invitrogen Corporation) and 50 mg/liter of hypoxanthine (∼370 μM) at 4% hematocrit (10).

**Vector Construction and Parasite Transfection**—The *pfpnp* allelic exchange fragment was PCR-amplified from 3D7 genomic DNA, using the primer combination p10/p11 (see supplementary materials). This yielded a 0.5-kb fragment that was cloned into the plasmid, pBSDmini, which contains the *bsd* gene that encodes resistance to blasticidin, flanked by promoter and terminator elements from *cam* (calmodulin) and *hrpII* (histidine-rich protein II), respectively (11, 12). The resulting 5.1-kb transfection plasmid, pBSDmini/pfpnp, was electroporated into the 3D7 *P. falciparum* ring stage parasites according to established protocols (9, 13, 14). In short, plasmid-transfected parasites were selected by the addition of 2.5 nM blastidicidin (InvivoGen, San Diego, CA) to the culture medium, starting 48 h post-transfection. Parasite clones were obtained by two rounds of limiting dilution using 500 μM hypoxanthine in the culture media, and identified using the MALSTAR assay specific for *P. falciparum* lactate dehydrogenase (15, 16).

**Nucleic Acid Analysis**—Genomic DNA of *P. falciparum* was purified as previously described (17). Plasmid integration into the *pnp* locus was detected by PCR, using the primer combinations p1/p4 and p3/p6 (see supplemental materials and Fig. 1, A and B). Integration into the *cam* and *hrpII* loci was assessed using primer combinations p7/p4 and p8/p9, respectively (see supplemental materials and data not shown). Amplification of non-recombinant, endogenous *pfpnp* sequence was assayed using primers p1/p2 (see supplemental material and Fig. 1, A and B). PCR conditions included a primer extension temperature of 62°C to account for the high A-T content in *P. falciparum* genomic DNA (18).

For Southern blot analysis, genomic DNA was digested with EcoRI or HindIII. It was electrophoresed overnight at 2.5 V/cm in 0.8% (w/v) agarose gels (1.5 mg of DNA/lane), alongside the digoxigenin-labeled DNA molecular mass ladder (Roche Applied Science) and 2 μg of transfection plasmid digested with the appropriate enzyme. The DNA was then transferred to Nylon membranes, and cross-linked by UV irradiation. An internal 0.5-kb region of *pfpnp* and a 0.3-kb region of *bsd* were PCR-amplified using primers p12/p13 and p9/p10, respectively (see supplementary materials), and labeled with digoxigenin (Roche Applied Science). Hybridizations were performed at 60°C, and membranes were washed at a maximum stringency of 0.3% SSC, 0.1% SDS (w/v) at 60°C, prior to CDP-Star detection, and autoradiography.

For stage-specific reverse transcriptase-PCR assays, cultures were doubly synchronized by sorbitol treatment. Total RNA was prepared using TRIzol® (Invitrogen). Cultures with >95% trophozoites were utilized for RNA extraction. First strand cDNA was synthesized from 5 μg of total RNA, using the power shot pre-amplification system (BD Biosciences) with random hexamer priming.

**Western Blots and PfPNP and PfADA Antibody Production**—The manufacturer’s protocols were followed to raise mouse polyclonal antibodies, using the adjuvant TiterMax® Gold (TiterMax, Norcross, Georgia). Briefly, BALB/c mice were injected with 100 μg of purified recombinant His₆-tagged PfPNP or PfADA emulsified in adjuvant. Mouse serum was collected 4, 6, and 8 weeks post-immunization. Western blots were performed on 0.1% (w/v) saponin-lysed parasite pellets that were washed with phosphate-buffered saline and resuspended in SDS-PAGE loading buffer. Blots were probed with 1:1000 dilutions of each primary antibody and detected using Renaissance Western blot Chemiluminescence Reagents (PerkinElmer Life Science).

**Drug Assays**—*In vitro* drug responses were calculated using 72-h [³H]ethanolamine incorporation assays (19, 20). Levels of incorporation were measured using a 1450 Microbeta Liquid Scintillation and Luminescence Counter (PerkinElmer Life Science). MT-ImmH, ImmH, 2′-deoxy ImmH, and 2′-deoxy ImmG were synthesized as previously described (7, 21, 22). Culture media for studies with various immucillins contained no hypoxanthine supplement. Immucillins were dissolved in water to make stock solutions, and diluted with media prior to addition to cultures. Following incubation with inhibitor for 18 h, 200-μl cultures in 96-well plates were supplemented with 1 μCi of [³H]ethanolamine (Amersham Biosciences; 25 Ci/mmol). After 54 h, cultures were frozen overnight and thawed to lyse the cells. The lysates were harvested on glass-fiber filters and washed with 1.2 ml of H₂O. Filters were dried and counted in a Winspectral 1414 Scintillation Counter. Experiments were carried out multiple times with six replicate wells for each drug concentration. Drug inhibition concentrations were calculated by regression analysis of dose-response curves. S.D. ± mean was calculated for each IC₅₀ value using KaleidaGraph version 4.03 (Synergy Software, Reading, PA). Individual data points
more than two mean ± S.D. were discarded. The IC_{50} values ±
the mean ± S.E. were calculated from the IC_{50} values from 3 to
12 independent experiments, and for each drug tested a one-way
analysis of variance analysis in combination with a Bonferroni
post hoc test was performed to determine the p values of the
various group pairs using KaleidaGraph version 4.03 (Synergy
Software, Reading, PA). Drug dilution series using the
immucillins were carried out to expand the data set between
concentrations of 1 × 10^{-8} and 1 × 10^{-6} M. These data are
included in the determination of the IC_{50} values. For some
experiments, parasitemias were counted on Giemsa-stained
smears of cultures treated in parallel.

Enzymatic Assays—Parasite lysates were obtained from para-
sites harvested from 60 ml of culture. The cell pellet was lysed
using 0.1% (w/v) saponin and washed twice with STE, followed by
two washes with 50 mM potassium phosphate pH 7.4. The pellets
were resuspended in 300 μl of 50 mM potassium phosphate pH 7.4,
then frozen and thawed five times. Insoluble material was removed
by centrifugation and the protein concentration of cell lysates
determined using the Bradford method. 50 μg of protein (30–50
μl lysate) was used to assay for enzyme activity. Assays were
performed in triplicate. Hypoxanthine produced by PfPNP was
assayed by coupling to the xanthine oxidase reaction and meas-
uring uric acid formation at 293 nm over time (7). PfADA action
was assayed by coupling to the xanthine oxidase reaction and meas-
uring lactate dehydrogenase assay, as described in Ref. 23.

Purine Requirements—Sorbitol-synchronized parasites were
used, and each experiment was seeded with 1% parasitemia,
with >95% rings and at 1% hemocrit. Assays were carried out in
200-μl volumes in 96-well plates. Stock solutions of 2 mM
purines (hypoxanthine, adenosine, and inosine) were used for
subsequent serial dilutions. Before beginning the experiment,
the parasites were washed three times in purine-free culture
media, resuspended in purine-free media, and added to the
plates, which already contained a 2× concentration of each
purine in an equal volume of media (i.e. 1× final concentra-
tion). Parasites were allowed to grow for 48 h with the corre-
sponding purine at each concentration. After 48 h, 100 μl of
media was removed from each well, and 100 μl of media with
the appropriate purine concentration and 1 μCi of [3H]ethanol-
amine (GE Healthcare) was added. After 24 h of incubation
with the radiolabel, cell cultures were frozen overnight and
thawed to disrupt cells. The mixtures were then harvested on
glass-fiber filters and washed with 1.2 ml of H2O. Filters were
thawed to disrupt cells. The mixtures were then harvested on
glass-fiber filters and washed with 1.2 ml of H2O. Filters were
dried and counted in a Winspectral 1414 Scintillation Counter.
The experiment was performed with six replicate wells for each
purine concentration. Mean ± S.D. was determined, and two-
tailed unpaired t tests were carried out to determine the statis-
tical significance for each disrupted line as compared with the
wild-type control line using KaleidaGraph version 4.03 (Synergy
Software). These results were also confirmed using the
lactate dehydrogenase assay, as described in Ref. 23.

Doubling Time—Highly synchronized parasites were used,
and each experiment was seeded with 1% parasitemia, with
>95% rings and at 4% hemocrit. All parasites were washed
three times in purine-free medium and resuspended in 5, 10, or
100 μM hypoxanthine. Giemsa-stained thin smears were taken
every 24 h. The medium was changed every 24 h while main-
taining the appropriate hypoxanthine concentration. Cultures
were cut back to 1% when they reached 3–6% parasitemia (usu-
ally every 48 h). Smear identities were blinded and parasitemias
counted in triplicate. Parasite -fold increases were calculated by
correcting the parasitemias for the -fold dilution. Data were
plotted using KaleidaGraph version 4.03, and curve fit analysis
was carried out using the equation y = N_0 e^{kx} (where N_0 =
initial population, and k = growth constant). Growth constants
(k) were used to determine doubling times.

RESULTS

Production of PNP "Knock-out" Clones—A transfection plas-
mid, pBSDmini/pfpnp, was designed to disrupt full-length
pfpnp in the chromosomal locus (9, 11). A truncated portion of
the pnp open reading frame was cloned into pBSDmini (12).
Integration of pBSDmini/pfpnp into the parasite genome via
homologous recombination and single site crossover was pre-
dicted to generate a pnp locus that was disrupted by the inser-
tion of the plasmid, producing two non-functional fragments
of pnp (Fig. 1A). The pBSDmini/pfpnp plasmid lacked the
sequences for 10 amino acids at the N terminus and 53 amino
acids at the C terminus. The 5’-non-functional fragment would
lack Asp^{206}, and the 3’-non-functional fragment would lack
His^{7}, both of which are critical for catalytic activity of PPNP (21).

Electroporation of pBSDmini/pfpnp into 3D7 parasites gen-
erated episomally transformed lines that were maintained by
selection with blastidicin (InvivoGen). Passaged lines were
screened by PCR for homologous recombination and plasmid
integration into the pnp, hrpII, or cam loci (data not shown).
Bulk cultures from two independent transfection events tested
positive for integration into the pfpnp locus at day 20 post-
electroporation, and clones were immediately generated from
this line by two rounds of limiting dilution (16). Initially, peri-
odic cycling on and off blastidicin selection was performed
before cloning; however, pnp disruptants were lost from cul-
tures, suggestive of a growth disadvantage for pnp disruptants.
Immediate cloning and maintenance of the parasites selected
with blastidicin at high concentrations of hypoxanthine
allowed us to successfully identify three pnp integrants from
two independent transfections, as well as several hrpII integ-
ratRs. The hrpII integrants were used as wild-type controls,
because they had integrated the transfection plasmid but did not
have a defect in PPNP and could be maintained under
similar blastidicin drug selection as the Δpfpnp clones. Prior
studies have shown that integration into the hrpII locus is not
deleterious (15, 16).

The Δpfpnp clones were confirmed by PCR analysis to be
recombinant solely for pnp integration, and were PCR-negative
for wild-type pnp (Fig. 1B). The Δpfpnp clones were PCR posi-
tive for intact plasmid, which would be expected if multiple
copies of the plasmid were integrated into the locus. It is not
uncommon for plasmids to form concateners before inte-
grating into wild-type loci (24). DNA isolated from the
cloned parasite lines was transformed into Escherichia coli,
and no colonies were observed, consistent with only integrated copies of the transfection plasmid present in the clones (data not shown).

Southern blot hybridization confirmed that the endogenous pnp locus had been disrupted (Fig. 1C). Using a probe for pnp, hybridizing bands of ~26, 5, and 7 kb, respectively, were detected upon EcoRI and HindIII digestion of genomic DNA from the Δpfpnp clones. These bands contrasted with the 2.3- and 8.5-kb bands generated by EcoRI and HindIII digests of untransfected 3D7 genomic DNA. The EcoRI restriction pattern was consistent with integration of multiple plasmids into the pnp locus. Integration of a single copy of the plasmid would generate a 7.4 kb band upon EcoRI digestion; however, a band at ~26 kb is indicative of at least four concatameric copies of the plasmid. HindIII cuts the plasmid once, linearizing it. The 7-kb band in the HindIII digests is in accordance with the expected plasmid fragments connected to the 5'- and 3'-truncated gene and promoter regions in the locus. Integration was also con-
PfPNP Is Critical for Viability of Malaria Parasites

Expression Levels in Knock-out Mutants—To verify disruption of the pfpnp locus abrogated pfpnp expression, we performed reverse transcriptase-PCR analyses on synchronized trophozoite-stage RNA preparations from the Δpfpnp clones and the 3D7 wild-type parental line. All Δpfpnp clones lacked pnp transcripts, but expressed normal levels of ada transcripts (Fig. 2A). To compare PfPNP protein expression levels between parental and recombinant lines, Western blots were performed on lysates of synchronized parasite cultures, using anti-PfPNP and anti-PfADA antibodies (Fig. 2B). PfPNP protein was absent in the Δpfpnp clones, whereas a band of ~26 kDa was observed in the WT line. With longer exposures a cross-reacting band of ~30 kDa was visible in all lines (as seen in Δpfpnp 1 in Fig. 2B). All lines possessed equivalent PfADA protein levels indicated by the band migrating at ~42 kDa. To confirm the absence of enzymatically active PfPNP, protein lysates of wild-type (3D7 and hrpII integrants) and Δpfpnp derived from saponin-lysed infected erythrocytes were tested for PNP and ADA activity. All three Δpfpnp lines had ADA activity, but only the wild-type and hrpII integrant lines had detectable PNP activity (Table 1).

Purine Requirements of the Δpfpnp Lines—The growth requirement of the Δpfpnp lines for exogenous purines was analyzed by measuring [3H]ethanolamine incorporation over time (19). Ethanolamine incorporation has been shown to be proportional to parasite number, and its incorporation should not be affected by perturbation of parasite metabolism. Hypoxanthine is the major purine in human serum with concentrations reported in the low micromolar range (0.4–6 μM) (25, 26). The Δpfpnp lines exhibited a statistically significant reduction in incorporation of [3H]ethanolamine compared with the WT control at physiological concentrations of purine (0.4–6 μM) (Fig. 3, A–C). The requirement for higher levels of exogenous purines in the Δpfpnp lines was also confirmed by using the lactate dehydrogenase assay (Fig. 3D), in which parasite lactate dehydrogenase activity is used as a surrogate of growth. Therefore, the Δpfpnp lines have a greater need for exogenous purines than the WT control. There was a slight preference for hypoxanthine over inosine, and for inosine over adenosine as a source of exogenous purine; however, the difference was minimal (supplemental Fig. S1). Under these conditions erythrocyte metabolism can convert adenosine and inosine to hypoxanthine.

Growth and Doubling Times of Δpfpnp Lines in Various Concentrations of Hypoxanthine—At physiological concentrations of hypoxanthine (5–10 μM), the Δpfpnp lines displayed a growth defect relative to the hrpII integrant WT control as monitored by counting parasitemias of infected erythrocytes (supplemental Fig. S2). There was no significant difference in the -fold increase of the WT control and the Δpfpnp lines at supra-physiological concentrations of hypoxanthine. Doubling times were derived from the plots of parasite growth and are represented in Table 2. The WT control exhibited a small difference in doubling times at 10 and 100 μM hypoxanthine: 9:1 and 7:0 h, respectively. In contrast, doubling times of the Δpfpnp lines at the same concentrations were ~24 and 7:5 h (Table 2). Thus, the WT parasites showed little impairment of growth at 10 μM purine, whereas the growth of Δpfpnp lines was significantly compromised.

Response of Δpfpnp Lines to Inhibitors of PNP, Immucillins—ImmH is a powerful inhibitor of host and parasite PNP and MT-ImmH is more specific for PpPNP than human PNP (5). Both immucillins kill P. falciparum in vitro and have IC50 values determined to be in the 50 nM range (5). Unlike host PNP, PpPNP has a preference for ribonucleosides over deoxyribonucleosides. Thus, 2′-deoxy ImmH and 2′-deoxy ImmG are potent inhibitors of hPNP, but not PpPNP with IC50 values in the 500 nM range (7). Prior studies also suggested that inhibition of PpPNP was essential for anti-parasitic efficacy of immucillins (5, 7).

The Δpfpnp lines are more sensitive to ImmH, a more potent inhibitor of hPNP than PpPNP (Fig. 4A), and less sensitive to MT-ImmH (Fig. 4B), a PNP inhibitor with ~100-fold tighter binding to PpPNP (5). Drug assays and IC50 determinations using these immucillins showed that the Δpfpnp lines were ~11 times less sensitive to MT-ImmH than wild-type 3D7 and the hrpII integrant control, ~6 times more sensitive to ImmH, ~42 times more sensitive to 2′-deoxy ImmH, and ~88 times more sensitive to 2′-deoxy ImmG (Table 3). Growth curves are shown relative to total ethanolamine incorporation of

| Parasite line | PNP | ADA |
|---------------|-----|-----|
| 3D7           | 23.0 ± 5.1 | 16.1 ± 3.8 |
| hrpII integrant | 21.8 ± 4.5 | 14.8 ± 3.2 |
| Δpfpnp 1      | ND  | ND  |
| Δpfpnp 2      | 82.5 ± 6.0 | 42.3 ± 9.0 |
| Δpfpnp 3      | 112 ± 15   | 155 ± 13    |

* p = 0.23.
* ND, not detectable.
PfPNP Is Critical for Viability of Malaria Parasites

ununtreated controls. As illustrated in Fig. 4C (and Fig. 3), ∆pfpnp lines grow poorly at low concentrations of hypoxanthine with less overall ethanolamine incorporation than wild-type parasites. Drug assays were performed without hypoxanthine supplementation. The ∆pfpnp lines showed a hierarchy similar to the disassociation constant (Fig. 4D) of each inhibitor for each disrupted line as compared with the wild-type control line (Δpfpnp lines showed a greater need for exogenous hypoxanthine (∗), inosine (B), and adenosine (C). Error bars represent the mean ± S.D., and two-tailed unpaired t tests were carried out to determine the statistical significance for each disrupted line as compared with the wild-type control line (hrpl1 integrant). *p < 0.0001; **, p < 0.001. The need for exogenous purines is confirmed using the lactate dehydrogenase assay (D), where the intensity of blue color reflects parasite lactate dehydrogenase activity, which is an indication of parasite growth.

TABLE 2

| Purine | Normal | WT | Ino | Ado |
|--------|--------|----|-----|-----|
| Hx     | 25     | 12.5 | 6  | 3   |
| Ino    | 25     | 12.5 | 6  | 3   |
| Ado    | 25     | 12.5 | 6  | 3   |
| Hx     | 25     | 12.5 | 6  | 3   |
| Ino    | 25     | 12.5 | 6  | 3   |
| Ado    | 25     | 12.5 | 6  | 3   |
| Hx     | 25     | 12.5 | 6  | 3   |
| Ino    | 25     | 12.5 | 6  | 3   |
| Ado    | 25     | 12.5 | 6  | 3   |

FIGURE 3. Purine requirements for ∆pfpnp lines. Using [3H]ethanolamine as an indicator of growth, the ∆pfpnp lines show a greater need for exogenous hypoxanthine (A), inosine (B), and adenosine (C). Error bars represent the mean ± S.D., and two-tailed unpaired t tests were carried out to determine the statistical significance for each disrupted line as compared with the wild-type control line (hrpl1 integrant). *p < 0.0001; **, p < 0.001. The need for exogenous purines is confirmed using the lactate dehydrogenase assay (D), where the intensity of blue color reflects parasite lactate dehydrogenase activity, which is an indication of parasite growth.

DISCUSSION

The research presented herein indicates that blocking the production of hypoxanthine, via targeted genetic disruption or biochemical block at PfPNP, causes a growth defect in P. falciparum. This is likely to occur before schizogony, as expression data indicate that transcripts of purine salvage enzymes are increased during the trophozoite stage, as the parasite prepares to replicate its genome and produce merozoites (27–29). Examination of blood smears of immucillin-treated parasites shows a prolongation of the replication cycle as evidenced by accumulation of trophozoite forms and delayed appearance of rings. Thus, absence of DNA precursors prolongs the cell cycle and eventually results in purine-less death of the parasite. These data collectively and definitely demonstrate the importance of PfPNP to P. falcipa- runum viability. This growth defect may also be affected by a perturbation in the levels of polyamines (5), however, this mechanism has yet to be established.

Disruption of essential genes in P. falciparum is not possible, due to the haploid chromosome state of the blood-stage parasite. However, we were able to achieve a conditional disruption of PfPNP by hypoxanthine supplementation of media for our ∆pfpnp clones. In vitro P. falciparum culture media usually contains 50 mg/liter of hypoxanthine (~370 μM), and maintenance of the ∆pfpnp clones at such high, non-physiological levels restored normal growth rates. However, hypoxanthine is reported to be present in human serum at much lower concentrations (ranging from 0.4 to 6 μM) (25, 26). Culture of ∆pfpnp parasites at these physiological concentrations resulted in severe growth retardation and eventual death of the ∆pfpnp parasites.

The ∆pfpnp lines required higher levels of exogenous purines, although no specific purine (i.e. hypoxanthine, inosine, or adenosine) appears to be of particular importance. Adenosine and inosine can be converted into hypoxanthine by ADA and PNP in the human erythrocyte cytosol and then used by the parasite to circumvent the block at PNP. In addition, ATP is present at millimolar concentrations in the erythrocyte cytosol and may be a purine source for parasites. Depending upon the concentrations of ribosyl phosphates within the erythrocyte cytosol, hypoxanthine may be converted to inosine by PNP. At
PfPNP is Critical for Viability of Malaria Parasites

Lower concentrations of adenosine, erythrocyte adenosine kinase activity will predominate to generate AMP (K_m 50 nM versus 70–90 μM for hADA (30, 31)).

A parasite line with a knock-out in the nucleoside transporter, PfNT1, has been reported and has a similar impairment of growth at physiological purine levels as Δpfpnp (23). For both Δpfpnp and Δpfnt1 parasite lines, growth is rescued by high concentrations of purines. The exact attributes of the PfNT1 transporter have been somewhat controversial (32–36), but it appears that PfNT1 (also known as PfENT1) is the major purine transporter of the parasite plasma membrane and is capable of transport of hypoxanthine, adenosine, inosine as well as other purines and pyrimidines (37).

El Bissati et al. (23, 37) have postulated that hypoxanthine is the major purine transported by PfNT1 and that PfPNP is unlikely to be important for parasite viability. Barrington et al. (23) have illustrated that PfPNP is primarily active in the erythrocyte cytosol (see Table 1 where PfPNP was measured on saponin-lysed-infected erythrocytes depleted of host cytosol), it seems likely that other purines are taken up by PfNT1 and that the activity of PfPNP is of similar importance to PfNT1.

Thus it is probable that parasite uptake of both purine nucleosides and nucleobases is important for optimal viability of Plasmodium. Although hypoxanthine is the most abundant purine in serum, the low K_m of PfHGXPRT (<1.0 μM) (38) and the lower K_m of PfPNP versus hPNP (5 versus 40 μM (7)) enables the parasite to metabolically trap any available purines, including adenosine, inosine, or hypoxanthine (37). As erythrocyte and Plasmodium purine transporters are equilibrative, steady state concentrations of purines will be equivalent in serum, the erythrocyte cytosol, and parasite cytosol. Any available purines will be rapidly utilized by the parasite.

The activity of PfPNP on 5′-methylthioinosine may also be critical when purine levels are low. This activity allows the parasite to recycle purines from MTA derived from polyamine synthesis (5).

A unified model that integrates the phenotype of Δpfpnp and Δpfnt1 is presented in Fig. 5. In this model, the low affinity purine transporter PfNT1 is responsible for equilibrative transport of adenosine, inosine, and hypoxanthine (as well as guanine, guanosine, and xanthine). These purines are incorporated into parasite nucleotide pools via the action of PfADA, PfPNP, and PfHGXPRT. If PfNT1 is not functional, purines can be taken up by alternative transport pathways. At low purine concentrations, in Δpfnt1, purines are poorly transported, whereas

TABLE 3
IC₅₀ values of wild type versus Δpfpnp using various immucillins

Parasite lines were incubated with drug in media without hypoxanthine for 48 h and then further incubated with radiolabel for 24 h. For each line, 3–12 independent experiments were carried out. The error is reported as mean ± S.E. To determine the statistical significance between the IC₅₀ values of the wild type and disrupted lines for a particular inhibitor the Bonferroni test was used to calculate the p value for every combination of group pairs.

| Parasite line | 3D7 | hpil/Δpfpnp 1 | Δpfpnp 2 |
|---------------|-----|---------------|----------|
| IC₅₀ (nM)     |     |               |          |
| ImmH          | 48 ± 5 | 840 ± 62      | 900 ± 90 |
| MT-ImmH       | 48 ± 8 | 62 ± 10       | 6.0 ± 2.8 |
| 2′-deoxy-ImmH | 8.5 ± 3.5 | 16 ± 7       | 6.4 ± 0.8 |
| 2′-deoxy-ImmG | 9.0 ± 1.6 | 5.9 ± 0.6      |          |

*P < 0.0001.

*P < 0.001.
in \( \Delta pfpnp \), purines are transported but must be converted to hypoxanthine in the erythrocyte cytosol to be utilized by the parasite. Low levels of hypoxanthine are available from the erythrocyte cytosol purine pools so \( \Delta pfpnp \) are impaired but viable.

The dramatic increase in doubling time and severe retardation of development observed in the \( \Delta pfpnp \) lines at physiological concentrations of hypoxanthine provide evidence of the vital role that purine salvage plays in the parasite life cycle. The difference in drug response of the \( \Delta pfpnp \) lines compared with wild type is also noteworthy. Uncoupling the host and parasite pathways caused a decrease in sensitivity to the parasite-specific PNP inhibitor (MT-ImmH), and an increase in sensitivity to the PNP inhibitors that target both host and parasite PNP (ImmH, 2'-deoxy ImmH, and 2'-deoxy ImmG). Together, these results demonstrate the importance of PfPNP, even when host PNP is abundant, and thus validate PNP as the specific target of the immucillins.

The in vitro culture system does not completely mimic conditions in an infected human, but these studies indicate PfPNP is likely to play a critical role in vivo in malaria-infected human hosts. In parallel studies, we have demonstrated that \( P. yoelii \) parasites lacking PyPNP are attenuated in mice (39), providing further genetic evidence for the importance of \( P. yoelii \) PNP in vivo.

The attenuated phenotype of the \( \Delta pfpnp \) parasites validates further studies to pharmacologically target purine pathways for malaria treatment. Immucillins have been extensively investigated in primate and human models; there is a wealth of information about their oral availability, toxicity, and efficacy as a treatment for certain types of cancer (40, 41). Further studies in the human host or primate models are required to determine whether immucillins kill the parasite or attenuate the parasite sufficiently to allow the human host to successfully clear the infection. Thus our study supports further investigation of immucillins, or related transition-state inhibitors of the purine salvage pathway, as novel chemotherapeutic agents for the fight against malaria.

Acknowledgments—We gratefully acknowledge Peter C. Tyler and Gary B. Evans of Industrial Research Limited, New Zealand, for immucillin synthesis and many stimulating discussions during the course of this work.

REFERENCES
1. Greenwood, B., and Mutabingwa, T. (2002) Nature 415, 670–672
2. Trape, J. F., Pison, G., Spiegel, A., Enel, C., and Rogier, C. (2002) Trends Parasitol. 18, 224–230
3. Daddona, P. E., Wiesmann, W. P., Lambros, C., Kelley, W. N., and Webster, H. K. (1984) J. Biol. Chem. 259, 1472–1475
4. Daddona, P. E., Wiesmann, W. P., Milhouse, W., Chern, J. W., Townsend, L. B., Hershfield, M. S., and Webster, H. K. (1986) J. Biol. Chem. 261, 11667–11673
5. Ting, L. M., Shi, W., Lewandowicz, A., Singh, V., Mwakingwe, A., Birck, M. R., Ringia, E. A., Bench, G., Madrid, D. C., Tyler, P. C., Evans, G. B., Furneaux, R. H., Schramm, V. L., and Kim, K. (2005) J. Biol. Chem. 280, 9547–9554
