Targeting a Novel *Plasmodium falciparum* Purine Recycling Pathway with Specific Immucillins*

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*Plasmodium falciparum* is unable to synthesize purine bases and relies upon purine salvage and purine recycling to meet its purine needs. We report that purines formed as products of polyamine synthesis are recycled in a novel pathway in which 5'-methylthioinosine is generated by adenosine deaminase. The action of *P. falciparum* purine nucleoside phosphorylase is a convergent step of purine salvage, converting both 5'-methylthioinosine and inosine to hypoxanthine. We used accelerator mass spectrometry to verify that 5'-methylthioinosine is an active nucleic acid precursor in *P. falciparum*. Prior studies have shown that inhibitors of purine salvage enzymes kill malaria, but potent malaria-specific inhibitors of these enzymes have not been described previously. 5'-Methylthio-immucillin-H, a transition state analogue inhibitor that is selective for malarial relative to human purine nucleoside phosphorylase, kills *P. falciparum* in culture. Immucillins are currently in clinical trials for other indications and may also have application as anti-malarials.

Malaria continues to be a major cause of morbidity and mortality throughout the world with more than 1 million deaths per year primarily in children in sub-Saharan Africa (1). In addition, travelers and soldiers are at significant risk for exposure to malaria. Drug resistance is increasing even to newer anti-malarials such as mefloquine. This has led to an urgent need for new anti-malarials both for chemotherapy and prophylaxis.

*Plasmodium falciparum* lacks de novo purine synthesis, but its human hosts can synthesize purines by de novo pathways. Thus purine salvage pathways have been proposed as malaria-specific targets. Efforts to understand purine metabolism have been complicated by the presence of purine salvage enzymes in both the host and the parasite and limited understanding of purine salvage enzymes in the parasite. Early studies indicated that hypoxanthine was the major purine precursor in malaria, but more recently it has been assumed that *Plasmodium* species, like *Toxoplasma gondii*, another pathogenic apicomplexan, are able to salvage purines via redundant purine salvage pathways (2, 3).

Polyamine biosynthesis pathways have also been explored as targets for treatment of parasitic infections including malaria (4). The polyamine pathway is the target for difluoromethylornithine, a mechanism-based inhibitor of ornithine decarboxylase, in use for treatment of sleeping sickness caused by *Trypanosoma* (4). The pathway forms two molecules of methylthioadenosine (MTA) for the synthesis of each spermine molecule. In most organisms, MTA is recycled into adenine and methionine pools. Because erythrocytes do not synthesize polyamines, synthesis of polyamines is likely essential for malaria parasites, and enzymes of the polyamine pathway are being investigated as anti-malarial targets (4). Most surprisingly, genes encoding the enzymes normally associated with purine salvage and recycling of MTA are not present in the recently completed genome sequences of *P. falciparum* and *Plasmodium yoelii* (5, 6) (www.plasmodb.org).

To understand more fully the purine pathways of malaria, we have characterized the activities of adenosine deaminase (PfADA) and purine nucleoside phosphorylase (PfPNP) from *P. falciparum*. PfADA and PfPNP have catalytic specificities that allow them to use methylthiopurines and therefore to function in both purine salvage and methylthiopurine recycling. By using accelerator mass spectrometry (AMS), we show this pathway is active in *P. falciparum* cultured in human erythrocytes. MT-immucillinH (MT-1mmH), a specific inhibitor of PfPNP (7), kills malaria in culture. Therapeutic agents to target the methylthio specificity of PfADA or PfPNP have potential for rapid development and application to malaria.

MATERIALS AND METHODS

Reagents—Calf spleen ADA, xanthine oxidase, adenosine, MTA, inosine, and S-adenosylhomocysteine were purchased from Sigma. Coformycin was obtained from Calbiochem. Deoxycoformycin was a gift of Dr. David C. Baker, Department of Chemistry, the University of Tennessee. Erythrose-9-12-hydroxy-3-onyladenine hydrochloride was purchased from Sigma. Human PNP and PfPNP (chromosome 5 of *P. falciparum*)

1 The abbreviations used are: MTA, 5'-methylthioadenosine; PNP, purine nucleoside phosphorylase; PfPNP, *P. falciparum* PNP; MTI, 5'-methylthioinosine; ADA, adenosine deaminase; PfADA, *P. falciparum* ADA; 1mmH, immucillin-H; MT-1mmH, 5'-methylthio-immucillin-H; MTP, methylthioadenosine phosphorylase; APRT, adenosine phosphoribosyltransferase; AK, adenosine kinase; MTRA, methylthioadenosine nucleosidase; MTRK, methylthioborobase kinase; AMS, accelerator mass spectrometry; RP-HPLC, reverse phase-high performance liquid chromatography; MTR-P, 5-methylthioribose-1-PO₄.
were overexpressed in *Escherichia coli* using the methods described (7–9). ImmH and MT-ImmH were synthesized as described (7, 10, 11). [8-14C]Uric acid was purchased from Moravek Biochemicals (Brea, CA). [8-14C]ATP was purchased from Amersham Biosciences. 1mM thione was synthesized from [8-14C]ATP (Amersham Biosciences) and methionine (Sigma), using S-adenosylmethionine synthetase as described previously (12). S-Adenosylmethionine was converted to MTA by incubating S-adenosylmethionine at pH 3–5 and at 70 °C for 3.5 h. All labeled compounds were purified by reverse phase-HPLC (RP-HPLC) with a 15-μm, 7.8 × 300-mm C18 Delta pak column (Waters Associates), eluting isocratically with 50 mM ammonium acetate, pH 5.0, and 25% methanol at 1 ml/min. Product elution was monitored at 260 nm.

**Cloning and Expression of *P. falciparum* ADA—**PIADA was identified on chromosome 10 from the Malarial Parasite Genome Project (5) as a protein of 367 amino acids and 42.5 kDa. PIADA was amplified from strain 3D7 genomic DNA using the PCR primers 5’-AATTGTAA-GAATATGATGACTATGAGA-3’ (sense) and 5’-AAATATTT-TATCTATTATTTTATCTTG-3’ (antisense). The coding region was placed in the pTrcHis2-TOPO vector (Invitrogen) and expressed in *E. coli* strain TOP 10. Recombinant PIADA was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 10 h and purified by Ni2+ chromatography. The expressed PIADA was >95% homogeneous by denaturing gel electrophoresis.

**Cloning and Expression of *E. coli* PNP—**The coding sequence of *E. coli* PNP (gene) was amplified by PCR from *E. coli* (strain TOP 10) genomic DNA with the primers 5’-ATGGCTACCTCAGGACA-TATTAGGCTTCCAGGCTTCTCAGGC-3’ (sense) and 5’-CTAGTATGTAGTATGTAGGCCTTCT-TATGCCGCAACAGAA-3’ (antisense), which introduced a His6 tag (underlined) before the stop codon. The PCR product was cloned into the pTrcHis2-TOPO vector (Invitrogen) and transformed into *E. coli* strain TOP 10. The recombinant PNP with a C-terminal His6 tag was expressed by induction of the bacterial culture with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 10 h and purified using nickel affinity chromatography.

**Enzymatic Assays and Inhibition Studies—**Hypoxanthine produced by PNP was assayed by coupling to the xanthine oxidase reaction and measuring uric acid at 265 nm. Coupled PNP assays contained 106 pmoles MTA, 50 mM NaPO4, pH 7.5, 61 millinits of ADA, 2 millinits of PNP, and 60 millinits of xanthine oxidase. Reactions were initiated by the addition of MTA. Inhibition studies measured both initial and slow onset rates to establish both the initial dissociation constant (Kᵢ) and the steady-state dissociation constant (Kₛ) as described previously (9). Kₛ is the lower of these values.

**Product Identification—**NMR and IR spectra were used to identify the MTA isolated in this way. Fourier transform infrared spectroscopy was carried out on the protein using a Fourier transform infrared spectrometer (ThermoNicolet, Madison, WI) using an MCT detector. IR revealed the C=O stretch (1672 cm⁻¹) of the product to be identical to that of free bases. NMR experiments in D₂O were performed at 25 °C on a Bruker DRX 300 MHz spectrometer. NMR was used to identify the 5′-methylthio group.

**Gene Search Strategy—**Human and *E. coli* HGPRT, PNP, ADA, AK, human and yeast MTAP, Klebsiella MTRK, and *E. coli* MTAN were used as search queries to identify potential orthologues in malaria species using the tblastn function (using default settings) at the NCBI eukaryotic genomes data bases (searching against all Apicomplexa and kinetoplastids; www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?data base= 18045&organism = eu&blastprogram = blastn) at PlasmoDb (www.plasmodb.org) websites. Leishmania APRTase and AK and *T. gondii* AK were also used as search queries. *P. falciparum* hypoxanthine-guanine phosphoribosyltransferase, PNP, and ADA were readily identified, but no potential homologues for AK, APRT, MTAP, MTAN, or MTRK were identified in any malaria species. Alternative purine salvage enzymes, such as inosine-guanoine kinase (using bacterial enzymes as queries), were also not identified. It should be noted that PIADA was recently proven to be functional (13). Earlier biochemical reports of AK and APRTase activity in extracts of *Plasmodium* isolated from human erythrocytes gave activities not significantly greater than in the erythrocytes (14).

PlasmoDB annotations were searched with text queries of the blast output and queries using EC numbers to identify AK, APRTase, MTAP, MTAN, or MTRK homologues. Gene ontology biological function, gene ontology biological process, and gene ontology cellular component assignments, and metabolic pathway annotations were also reviewed. No CCI 99-240 or the Albert Einstein College of Medicine. Sorbitol-synchronized cultures of *P. falciparum* strain 3D7 were grown in RPMI supplemented with 0.5% Albumax II (Invitrogen). Culture media for studies with ImmH and MT-ImmH contained no hypoxanthine supplement. Drugs were dissolved in water and diluted with water or media prior to addition to cultures. Following incubation with ImmH or MT-ImmH for 18 h, the 200-μl cultures in 96-well plates were supplemented with 1 μCi of [3H]ethanolamine (Amersham Biosciences, 25 Ci/mmol). After 48 h, cell cultures were frozen and thawed to disrupt cells, and the mixtures were harvested on glass fiber filters and washed with 1.2 ml of H₂O. Filters were dried and counted in a Windspectral 1414 scintillation counter. Experiments were done twice with six replicate wells for each experiment. Individual data points may not exceed two standard deviations from the mean were discarded. For some experiments, parasite uptake of 3H was counted on Giemsa-stained smears of cultures treated in parallel.

**Purine Metabolism in *P. falciparum* by Accelerator Mass Spectrometry (AMS)—***P. falciparum* was cultured in human erythrocytes (1% hematocrit) to 1% parasitemia as indicated above. Cultures were synchronized at the ring stage by sorbitol lysis and allowed to recover for 48 h prior to use in experiments. After washing three times in hypoxanthine-free medium, cultures were incubated for 24 h in hypoxanthine-free media following the addition of 1 nM carrier-free [8-14C]hypoxanthine, [8-14C]inosine, [8-14C]uracil, [8-14C]MTA, or [8-14C]MTI to 200-μl cultures (~20 rpm/culture or 200 fmol). [8-14C]MTI was produced from [8-14C]MTA by using PIADA (50 μM Tris-HCl, pH 7.4, 37 °C) and purified by RP-HPLC (peak was identified by retention time). In experiments with ImmH and MT-ImmH contained no hypoxanthine and were synchronized at the ring stage by sorbitol lysis and allowed to recover for 48 h prior to use in experiments. After washing three times in hypoxanthine-free medium, cultures 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 done twice with six replicate wells for each experiment. Individual data points may not exceed two standard deviations from the mean were discarded. For some experiments, parasite uptake of 3H was counted on Giemsa-stained smears of cultures treated in parallel.

**RESULTS**

**The Plasmodium Genome Lacks Genes for Adenine Salvage and Recycling—**Genome sequence data for *P. falciparum*, *P. yoelii*, and partial genome sequences of other Plasmodium species were searched for orthologues of purine salvage enzymes present in mammals and *E. coli*. The closest protozoan orthologues (usually from *T. gondii*, a bacterial orthologue from *Saccharomyces cerevisiae* orthologue, and a mammalian orthologue were used as search queries (see “Materials and Methods”). *P. falciparum* and all other malaria genomes lack APRT, AK, MTAN, MTRK, and MTAP. We were also unable to orthologues of other potential purine salvage enzymes that have been reported in other organisms but not in the *Plasmodium* species. Other groups have independently come to similar conclusions (13, 15).

**PADA Converts MTA to MTI—**An unusual feature of *P. falciparum* is the expression of PIADA, PIAPA, and hypoxanthine-guanine-xanthine phosphoribosyltransferase at 4–700 times the specific activity found in human erythrocytes, far exceeding the rate of purine salvage in this purine auxotroph (14). *P. falciparum* grows in ADA- or PNP-deficient erythrocytes.
cytes, establishing the competence of parasite-expressed enzymes for purine salvage (18, 19). We hypothesized that \( P. falciparum \) evolved dual substrate specificities in both PfPNP and PfADA to permit the conversion of MTA to hypoxanthine and \( P. falciparum \) evolved dual substrate specificities in both PfPNP and PfADA to permit the conversion of MTA to hypoxanthine and \( P. falciparum \) evolved dual substrate specificities in both PfPNP and PfADA to permit the conversion of MTA to hypoxanthine and 8-aminopurine rings in its substrates, nucleosides with adenine rings (including MTA) being neither substrates nor inhibitors for the enzyme. The action of PfPNP on a 5’-methylthio-containing nucleoside would therefore require recognition of MTI. Formation of this substrate could hypothetically be accomplished by deamination of MTA to MTI, but a MTA deaminase activity has not been reported previously.

PfADA was expressed in \( E. coli \) to determine its specificity and to define its role in purine cycling. Human, \( E. coli \), and PfADAs share only 12.8% combined identity in the amino acid sequence (47% of 367 amino acids), but every amino acid interacting with the catalytic site \( \text{Zn}^{2+} \) ion and with transition state analogues in the crystal structure of mouse ADA (22) is conserved in \( E. coli \) and \( P. falciparum \) ADAs (Fig. 1). PfADA was able to catalyze the deamination of both adenosine and MTA (Table I). MTA and adenosine were equivalent substrates (\( k_{cat}/K_m \)) for purified recombinant PfADA enzymatic activity with MTA, PfADA had no activity with MTA. PfADA has an N-terminal extension not present in human ADA, but deletion of the first 27 amino acids of PfADA did not affect its ability to recognize MTA (data not shown). The 5’-hydroxyl group of adenosine analogues is in contact with His-17 and Asp-19 of mouse ADA, residues that are conserved in PfADA (22) (Fig. 1).

Because MTI had not been described previously as a metabolic intermediate, we confirmed the chemical identity of the product of the PfADA reaction on MTA. HPLC confirmed a single UV-active species with a different retention time from MTA (Fig. 2A). The product was confirmed as MTI using NMR (not shown) and infrared spectrometry following RP-HPLC purification (Fig. 2B).

**PfADA Is Inhibited by Inhibitors of ADA—Coformycin, deoxycoformycin, and the 1-ribosyl analogues of the coformycins are tight-binding inhibitors of both mammalian and \( P. falciparum \) ADAs (18, 22). Coformycin and deoxycoformycin have comparable activity against bovine ADA and PfADA (Table I). Experimentally equivalent \( K_d \) values were obtained for coformycin and deoxycoformycin inhibition using adenosine or MTA substrates, supporting a single catalytic site for both deaminations. Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride is a potent inhibitor of mammalian ADA (\( K_d \) of 50 nM) but a weak inhibitor of PfADA (\( K_d \) of 180 \( \mu \)M), establishing significant catalytic site differences in these enzymes (Table I). ADA activity obtained from cell lysates of \( P. falciparum \)-infected ADA-deficient human erythrocytes has been reported to have properties similar to our cloned PfADA (18). Details of the binding site that accommodates both MTA and adenosine in PfADA await structural analysis.

**MTI Is Actively Metabolized in \( P. falciparum \)—**We used AMS to measure incorporation of trace amounts of 8-\( ^{14} \)C-labeled purine precursors in \( P. falciparum \) cultured in human erythrocytes. AMS sensitivity is \( -1 \) amol of 8-\( ^{14} \)C/mg of carbon (with 1 dpm equivalent to 10,000 amol of 8-\( ^{14} \)C (23)). ATP, the primary purine source in erythrocytes, is 20,000 nM in malaria cultures maintained at 1% hematocrit, and the amounts of tracer added (\( \leq \)1 nM) would not be expected to perturb the physiological balance of purines (ATP ↔ ADP ↔ AMP ↔ adenosine ↔ inosine ↔ hypoxanthine; Fig. 3). \( P. falciparum \) was cultured in erythrocytes for 24 h without hypoxanthine and then incubated for another 48 h with 1 nM of 8-\( ^{14} \)CMTI (200 fmol; Table II and Fig. 3). Control cultures contained 1 nM [8-\( ^{14} \)C]hypoxanthine, 8-\( ^{14} \)Cinosine, 8-\( ^{14} \)Curic acid, 8-\( ^{14} \)Cadenine, or 8-\( ^{14} \)CMTA. After 48 h, 8-\( ^{14} \)C incorporation into acid-insoluble nucleic acids was 16.7, 9.1, and 10.8 fmol of 8-\( ^{14} \)C from hypoxanthine, inosine, and MTI, respectively. Labeled MTA and adenine were incorporated less efficiently, reflecting dilution into the erythrocyte purine pools and lack of a malarial APRT (Fig. 3). The control

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**Fig. 1. Adenosine deaminase of \( P. falciparum \).** Alignment of \( P. falciparum \), \( E. coli \), and human ADA protein sequences. Amino acids common to all three are shown in **green** and those common to any two are shown in **yellow**. Ligands to the catalytic site \( \text{Zn}^{2+} \) of the mammalian ADA are marked with an **asterisk**, and those in contact with a transition state analogue in crystals of mouse ADA (22) are marked with a #. Note that catalytic site residues are completely conserved in the three species, suggesting all are \( \text{Zn}^{2+} \)-containing deaminases. The N-terminal region of mammalian ADA contacts the 5’-hydroxyl group of adenosine analogues.
of MTI and MTA structures).

Similar with inosine (2'-hydrochloride. has only 2.5% of the catalytic activity (kcat/Km) values were obtained using MTA as the substrate for PfADA (data not shown). EHNA is erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride.

The sample of MTI was isolated by RP-HPLC following hydrolysis of MTA with PfADA. The peak at 1672 cm\(^{-1}\) (arrow), due to the C=O vibration in the purine ring of both inosine and MTI, is absent in the spectrum of MTA (see Fig. 4).

**TABLE I**

| Substrate or inhibitor | Calf spleen ADA | P. falciparum ADA |
|------------------------|----------------|------------------|
|                        | K_m  | k_cat  | K_d  | K_m  | k_cat  | K_d  |
| Adenosine              | 56 \pm 7 | 65 \pm 3 | <0.02 | 29 \pm 3 | 1.8 \pm 0.1 | 16 \pm 3 \times 10^{-10} |
| Methylthioadenosine    | 7 \pm 4 \times 10^{-11} | 29 \pm 4 \times 10^{-10} | 49 \pm 5 \times 10^{-9} |
| S-Adenosylhomocysteine | 4 \times 10^{-11} | 1.8 \pm 0.1 | 16 \pm 3 \times 10^{-10} |
| Coformycin             | 4 \times 10^{-11} | 15 \pm 0.9 | 18 \pm 6 \times 10^{-5} |
| EHNA                   | 4 \times 10^{-11} | 15 \pm 0.9 | 18 \pm 6 \times 10^{-5} |

**Fig. 2.** PfADA converts MTA to MTI. a, HPLC showing the conversion of MTA to MTI, 10 min after addition of PfPNP. Duplicate reactions were separated on a reverse phase C18 Deltapak column before and 10 min after addition of PfADA to MTA. b, infrared absorption spectrum of MTA (green), MTI (red), and inosine (blue). The sample of MTI was isolated by RP-HPLC following hydrolysis of MTA with PfADA. The peak at 1672 cm\(^{-1}\) (arrow), due to the C=O vibration in the purine ring of both inosine and MTI, is absent in the spectrum of MTA (see a for comparison of MTI and MTA structures).

The action of PfPNP on MTI is not a general property of hexameric PNPs. We overexpressed and tested *E. coli* PNP, which has been extensively characterized structurally and enzymatically (20, 21). *E. coli* PNP has activity against inosine and adenosine but has no detectable activity toward MTA or MTI.

**MT-ImmH, a Selective Inhibitor of PfPNP, Kills Malaria in Culture—MT-ImmH, a methylthio derivative of ImmH, exhibits increased specificity for PfPNP by mimicking a transition state specific to the parasite enzyme (Fig. 5A) (7). MT-ImmH binds better to PfPNP (K_d 2.7 \times 10^{-11} M) than to human PNP (K_d 303 nM) by a factor of 112. In contrast, the discrimination factor for ImmH (K_d human PNP/K_d PfPNP) is 0.065. The methylthio group of MT-ImmH provides specificity for inhibition of PfPNP, although MT-ImmH binds 3-fold less well to PfPNP than ImmH (K_d \(2.7 \text{ and } 0.9 \text{ nM, respectively}\)).

MT-ImmH was compared with ImmH for the ability to kill cultured parasites (Fig. 5B). *P. falciparum* strain 3D7 cultures were treated with variable amounts of ImmH or MT-ImmH followed by an assay for viability based on incorporation of \(^{3}H\)ethanolamine (24). The IC_{50} values for ImmH and MT-ImmH were 63 and 50 nM, respectively.

The *P. falciparum* survival curves yield a sharp dose response with ImmH and a shallow response to MT-ImmH. MT-ImmH binds to PfPNP better than to host PNP, and initial inhibition of parasite growth is seen at lower concentrations of
drug. In contrast, ImmH binds best to host PNP, and inhibition of parasite growth is not seen until host PNP is fully inhibited. Human PNP concentration is substantial (\( \approx 2000 \text{ nM} \)) in erythrocytes, and at \( 1\% \text{ hemocrit} \approx 20 \text{ nM} \) ImmH is required for inhibition of the host enzyme in experimental cultures (25). Inhibition of host PNP by ImmH is near-stoichiometric because of the 56 pM \( K_d \) values (8). Inhibition of \( P. falciparum \) growth occurs at drug concentrations where both erythrocyte and Pf-PNP are expected to be inhibited by ImmH (\( K_d \) values of 56 and 860 pM respectively). With MT-ImmH, inhibition of parasite growth occurs at concentrations where PfPNP but not host PNP is strongly inhibited.

Differences in transport of ImmH and MT-ImmH could also contribute to differences in dose response. Initial characterization of \( P. falciparum \) purine transporters has been reported, but malaria parasite transport is not yet fully characterized (26–28). Inhibition of the polyamine pathway through product (MTA) inhibition is unlikely to be the cause of parasite death, because MTA is converted to MTI by PfADA.

Microscopic examination of treated cell cultures revealed that parasite numbers decreased as \( [\text{3H}] \) ethanolamine incorporation decreased, and no viable parasites were detected in cultures with full inhibition of \( [\text{3H}] \)ethanolamine incorporation (Fig. 5B). Thus, both ImmH and MT-ImmH kill parasites rather than inducing stasis. \( P. falciparum \) cultures are rescued from the toxic effects of both Imm-H and MT-ImmH by hypoxanthine (Ref. 25 and data not shown). The 5'-methylthio group of MT-ImmH precludes 5'-phosphorylation or incorporation into nucleic acids, further establishing that the metabolic block of immucillins is at hypoxanthine formation (25).

**TABLE II**

Detection of \( ^{14}\text{C} \)-labeled nucleic acids by AMS

\( P. falciparum \) cells were cultured in 96-well plates as discussed under “Materials and Methods.” 48 hours after sorbitol synchronization, cultures were equilibrated in hypoxanthine-free media (24 h for experiment 1 and 12 h for experiment 2). Labeling was initiated by addition of 200 fmol (experiment 1) or 100 fmol (experiment 2) of \( ^{8-14}\text{C} \)-carrier-free purine to each culture. \( ^{14}\text{C} \)-Labeled nucleic acids were quantitated by AMS in quadruplicate (experiment 1) or triplicate (experiment 2) after the indicated labeling period. S.D. for each group is given. ND means not done.

| 8-\( ^{14}\text{C} \)-Labeled precursor | Exp. 1 (48-h labeling), 200 fmol | Exp. 2 (12-h labeling), 100 fmol |
|---------------------------------|---------------------------------|---------------------------------|
| Hypoxanthine                    | 16.7 ± 3.1                      | 4.7 ± 2.2                       |
| Methylthioinosine               | 10.8 ± 1.2                      | 2.8 ± 0.2                       |
| Inosine                         | 9.1 ± 1.5                       | 3.0 ± 0.7                       |
| Adenine                         | 1.3 ± 0.1                       | 0.2 ± 0.1                       |
| Methylthioadenosine             | 1.5 ± 0.2                       | 0.6 ± 0.2                       |
| Uric acid                       | 0.06 ± 0.01                     | ND                              |

**FIG. 3.** Purine pathways of malaria-infected erythrocytes. MTI is incorporated into nucleic acids of \( P. falciparum \) as detected by AMS (Table II). The purine salvage and purine recycling pathways in human erythrocytes infected with \( P. falciparum \) are shown. Human erythrocytes have AK, APRT, and MTAP, enzymes not identified in \( P. falciparum \). Both species have ADA, PNP, and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity. MTI, MTA, inosine, adenine, adenine, xanthine, and hypoxanthine metabolism are depicted with MTI metabolism shown in red. MTA can either enter erythrocyte purine pools or enter the parasite to be hydrolyzed by PfADA to form MTI (not shown).

**FIG. 4.** PfADA and PfPNP act in concert to convert MTA to hypoxanthine. The coupled reactions MTA \( \rightarrow \) MTI \( \rightarrow \) hypoxanthine \( \rightarrow \) uric acid are catalyzed by PfADA + PfPNP (xanthine oxidase) but not when either enzyme is substituted with the same quantity of its mammalian counterpart. cADA and hPNP are calf ADA and human PNP, respectively. Hypoxanthine formed by the sequential action of PfADA and PfPNP is converted to uric acid by xanthine oxidase. The formation of uric acid was detected by measuring absorbance at 293 nm.
**DISCUSSION**

*P. falciparum* is remarkable because of its small number of purine salvage enzymes despite the complete reliance on this pathway (Fig. 6). The purine salvage enzymes PfADA and PfPNP each have two roles in the parasite and replace the functions of PNP, ADA, MTAP, APRT, and AK in mammals. The action of these two enzymes permits the parasite to form hypoxanthine from erythrocyte purine pools and to recycle hypoxanthine from polyamine synthesis within the parasite. Hypoxanthine is a precursor for all purines and is a central metabolite for nucleic acid synthesis in *P. falciparum*.

AK, APRT, MTAP, MTAN, and MTRK cannot be identified *in silico* by homology searches in any *Plasmodium* genome. Although some of these activities have been reported as being present in lysates from *P. falciparum*-infected erythrocytes, the activities were low and may be due to small amounts of host enzymes associated with isolated parasites (14). The expression pattern suggests that PfADA, PfPNP, and hypoxanthine-guanine-xanthine phosphoribosyltransferase form the major path for purine salvage in *P. falciparum*. Other groups have independently drawn similar conclusions (13, 15). Purine salvage in malaria is unlike that in most other protozoa, including other Apicomplexa such as *T. gondii* (2, 3) and *C. parvum* (13) that are rich in AK and rely on adenosine salvage to AMP for a major purine source. As expected, ImmH is not effective against *T. gondii* at doses up to 10 μM, but it is effective against *T. gondii* with disruption of AK.

Erythrocytes do not synthesize polyamines, so an intact polyamine pathway is important for viability of malaria parasites.

Enzymes of this pathway have been identified and characterized as potential targets for anti-malarials (4, 29–31). MTA is a dead-end molecule, and its recycling is essential for purine and methionine conservation (32). In mammals, adenine and MTR-1-P are recycled to ATP and methionine to regenerate S-adenosylmethionine (32, 33). In malaria, MTA is converted to MTI, a metabolite that is unknown in other organisms but is readily converted to hypoxanthine and incorporated into nucleic acids in the parasite.

The only reaction to form adenine in humans is the conversion of MTA to adenine and MTR-1-P. Lacking both MTAP and APRT, *P. falciparum* can neither produce nor salvage adenine. APRT is found in most organisms, reflecting the need for recycling MTA formed in polyamine synthesis. *T. gondii*, like malaria, does not appear to have an APRT or MTAP and has a PNP whose sequence is similar to that of PfPNP (15). *T. gondii* does not have an obvious ADA, but ADA are less conserved than other purine salvage enzymes (15). Thus it remains to be determined whether *T. gondii* PNP also utilizes MTI or has a purine recycling pathway similar to *P. falciparum*.

A pathway for conversion of MTR-1-P to methionine is expressed in bacteria and mammals, although the pathway is not fully characterized in any organism (16, 34) (shown as a dashed arrow in Fig. 6). The genes for recycling the methylthio group of MTA to methionine have been proposed for *B. subtilis*, but the pathway is not apparent in the *P. falciparum* genome (16). An exception is α-ketomethylthiobutyrate transaminase, located on chromosome 2 of the *P. falciparum* genome (35), but this enzyme can operate independently in transamination reactions and need not be linked to methylthio group salvage. Recycling of MTA to IMP spares *P. falciparum* from higher rates of purine salvage, but the constant supply of methionine...
in human blood may be sufficient to meet the requirement for this essential amino acid. The pathway of methylthioribose salvage by *P. falciparum* remains to be established.

Evolution of PIPNP and PfADA to serve as dual specificity enzymes in *P. falciparum* has streamlined the metabolic pathways to take advantage of the nutrient-rich environment of human blood. *P. falciparum* expresses fewer enzymes in the essential pathway for purine nucleoside and MTA salvage than human blood.

ImmH and other PNP inhibitors previously tested in malaria cultures all bind tighter to human PNP than PIPNP, and previously we could not establish if inhibition of PIPNP alone was capable of causing purine starvation in the parasite (25). Our current studies indicate that inhibition of PIPNP is critical. Preclinical trials with ImmH in mammals indicate oral availability and low toxicity, useful features for anti-malarial trials (36). Although ImmH is not toxic to erythrocytes, in theory, a malaria-specific immucillin would be preferable to avoid potential toxicity to host cells. MT-ImmH binds less tightly to PIPNP than ImmH (7). Refinements in inhibitor design based upon analysis of the transition state and crystal structures of PIPNP (7, 8) may permit improved PIPNP specificity and potency.

A similar strategy can be explored for PfADA. A single dose of deoxycoformycin dramatically reduced parasitemia in primates with *Plasmodium knowlesi* (37), but deoxycoformycin is highly toxic in mammals and not specific for malarial ADA. Nonetheless, the comparison of the inhibition profiles of PfADA and mammalian ADA (Table I) suggests that there are differences in catalytic features that can be exploited for the synthesis of malaria-specific ADA inhibitors. PIPNP-specific immucillins with PfADA-specific inhibitors may create combinations that are more potent and less toxic than single drug therapy. The evolution of catalysts with substrate specificity for two pathways is a unique method of providing genomic economy for *Plasmodia* but also introduces an Achilles heel into parasite metabolism.

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Targeting a Novel Plasmodium falciparum Purine Recycling Pathway with Specific Immucillins

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