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

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Abbreviations: PNP, purine nucleoside phosphorylase; PfPNP *P. falciparum*PNP; MTA, 5' -methylthioadenosine; MTI, 5'-methylthioinosine; ADA, adenosine deaminase; ImmH, Immucillin-H; MT ImmH, 5'-methylthio-Immucillin-H; MTAP, methylthioadenosine phosphorylase; APRT, adenine phosphoribosyltransferase; AK, adenosine kinase; MTAN, methylthioadenosine nucleosidase; MTRK, methylthioribose kinase; AMS, accelerator mass spectrometry

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Abstract:

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 the polyamine pathway 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 previously been described. 5'-methylthio-Immucillin-H, a transition state analogue inhibitor that is selective for malarial over human purine nucleoside phosphorylase, kills *P. falciparum* in culture. Immucillins are currently in clinical trials for other indications and may have application as antimalarials.

Malaria continues to be a major cause of morbidity and mortality throughout the world with more than one million deaths per year primarily in children in sub Saharan Africa. In addition, travellers and soldiers are at significant risk for exposure to malaria. Drug resistance is increasing even to newer antimalarials such as mefloquine. This has led to an urgent need for new antimalarials 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 ^{1,2}.

Polyamine biosynthesis pathways have also been explored as targets for treatment of parasitic infections including malaria ³. The polyamine pathway is the target for difluoromethylornithine (DFMO), a mechanism-based inhibitor of ornithine decarboxylase, in use for treatment of sleeping sickness caused by *Trypanosoma* ³. The pathway forms two molecules of methylthioadenosine (MTA) for the synthesis of each spermine molecule. In most organisms, MTA is recycled into purine and methionine pools. 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 *P. yoelii* ^{4,5} (<http://www.plasmodb.org>).

We show that *P. falciparum* has evolved purine salvage enzymes that also function in recycling of MTA. Adenosine deaminase (PfADA) and purine nucleoside phosphorylase (PfPNP) from *P. falciparum* have catalytic specificities that allow them to use methylthiopurines

and therefore to function in both purine salvage and methylthiopurine recycling. Using accelerator mass spectrometry (AMS) we show that 5'-methylthioinosine (MTI) formed from MTA is an active precursor for nucleic acid synthesis in *P. falciparum* cultured in human erythrocytes.

The unique methylthiopurine specificity of PfPNP was explored for novel inhibitor design. 5'-methylthio-Immucillin-H (MT-ImmH) is a transition state analogue inhibitor of PfPNP that mimics MTI and was designed to be specific for PfPNP⁶. We compare MT-ImmH to Immucillin-H (ImmH), a potent inhibitor of both human PNP and PfPNP, in cultured *P. falciparum*^{7,8}. MT ImmH and ImmH are both effective in killing *P. falciparum* cultured in human erythrocytes by preventing formation of hypoxanthine from either inosine or MTI. This approach suggests novel agents based upon the unique specificity of PfADA and PfPNP for methylthiopurines have potential as novel antimalarial chemotherapy. One transition state analogue inhibitor of PNPs, Imm H, is currently in clinical trials for other indications⁹. Therapeutic agents to target the PfPNP step therefore have potential for rapid development and application to malaria.

RESULTS

The *Plasmodium* genome lacks genes for purine salvage and purine 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, the *Saccharomyces cerevesiae* orthologue and a mammalian orthologue were used as search queries (see methods). *P. falciparum* and all other malaria genomes lack adenine phosphoribosyltransferase (APRT), adenosine kinase (AK), methylthioadenosine nucleosidase (MTAN), methylthioribose kinase (MTRK) and methylthioadenosine phosphorylase (MTAP) (Fig. 1a).

An unusual feature of *P. falciparum* is the expression of PfADA, PfpNP and hypoxanthine-guanine-xanthine phosphoribosyl-transferase (HGXPRT) at 4 to 700 times the specific activity found in human erythrocytes, far exceeding the rate of purine salvage in this purine auxotroph¹⁰. *P. falciparum* grows in ADA or PNP-deficient erythrocytes, establishing the competence of parasite-expressed enzymes for purine salvage^{11,12}. We hypothesized that *P. falciparum* evolved dual substrate specificities in both PfpNP and PfADA to permit the conversion of MTA to hypoxanthine and MTR-1-P and thereby eliminate the need for expression of MTAP, MTAN, MTRK, AK or APRT (Fig. 1a).

The PfpNP sequence has greater similarity to *E. coli* than to human PNP⁷, and its structure is hexameric as is the *E. coli* enzyme^{6,13,14}. PfpNP however, has unique substrate specificity when compared to either *E. coli* or mammalian PNPs^{7,14}. PfpNP is specific for 6-oxopurine 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 5'-methylthioinosine (MTI). Formation of this substrate could hypothetically be accomplished by deamination of MTA to MTI, but a MTA deaminase activity had not been reported previously (Fig. 1a).

PfADA and PfpNP are active with methylthiopurines

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 amino acid sequence (47 of 367 amino acids), but every amino acid interacting with the catalytic site Zn²⁺ ion and with transition state analogues in the crystal structure of mouse ADA¹⁵ is conserved in *E. coli* and *P. falciparum* ADAs (Fig. 1b). PfADA was able to catalyze the deamination of both adenosine and MTA (Table 1). MTA and adenosine were equivalent substrates (k_{cat}/K_m) for purified recombinant PfADA enzyme. Calf spleen ADA has no activity with MTA. PfADA has an N-terminus extension not present in human ADA, but deletion of the first 27 amino acids of PfADA does not affect its ability to recognize MTA (data not shown). The 5'-hydroxyl group of

ADA inhibitors is in contact with His17 and Asp19 of mouse ADA, residues that are conserved in PfADA¹⁵ (Fig. 1*b*).

Because MTI has not previously been described 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. 1*c*). The product was confirmed as MTI using NMR (not shown) and infrared spectrometry following RP-HPLC purification (Fig. 1*d*).

Characterization of PfADA inhibition profile.

Coformycin, deoxycoformycin and the L-ribosyl analogues of the coformycins are tight-binding inhibitors of both mammalian and *P. falciparum* ADAs^{11,15}. Coformycin and deoxycoformycin have comparable activity against bovine ADA and PfADA (Table 1). 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. EHNA is a potent inhibitor of mammalian ADA (K_d of 50 nM) but a weak inhibitor of PfADA (K_d of 180 μ M), establishing significant catalytic site differences in these enzymes (Table 1). ADA activity obtained from cell lysates of *P. falciparum* infected ADA-deficient human erythrocytes has been reported to have similar properties to our cloned PfADA¹¹. 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 accelerator mass spectrometry (AMS) to measure incorporation of trace amounts of [8-¹⁴C] labelled purine precursors in *P. falciparum* cultured in human erythrocytes.

AMS sensitivity is approximately 1 amol/mg carbon (with 1 dpm equivalent to 10,000 amol ^{14}C ; ^{16,17}). ATP, the primary purine source in erythrocytes, is 20,000 nM in malaria cultures maintained at 1% hematocrit, and the amounts of tracer added (<1nM) would not be expected to perturb the physiological balance of purines (ATP ↔ ADP ↔ AMP ↔ adenosine ↔ inosine ↔ hypoxanthine; Fig. 2a).

P. falciparum was cultured in erythrocytes for 24 hours and then incubated for another 48 hours with 1 nM of [8- ^{14}C] MTI (200 fmol; Table 2 and Fig. 2a). Control cultures contained 1 nM [8- ^{14}C]-hypoxanthine, [8- ^{14}C]-inosine, [8- ^{14}C]-uric acid, or [8- ^{14}C] MTA. After 48 hr, [^{14}C]-incorporation into acid-insoluble nucleic acids was 16.7, 9.1 and 10.8 fmol of ^{14}C from hypoxanthine, inosine and MTI, respectively. Labelled MTA was incorporated less efficiently, reflecting dilution into the erythrocyte purine pools (Fig. 2a). The control of uric acid showed <0.06 fmol incorporation. A second labelling experiment in which malaria cultures were labelled for 12 hours gave similar results (Table 2). MTI is equivalent to inosine as a nucleic acid precursor suggesting that incorporation is via a similar pathway.

PfPNP activity is high in infected erythrocytes (5 $\mu\text{mol/hr/mg}$ protein; ¹⁰). Both MTI and inosine are good substrates for PfPNP with K_m values of 4.7 and 16 μM and k_{cat} values of 1.7 and 1.5 sec^{-1} for inosine and MTI, respectively ⁶. Human PNP has only 2.5% of the catalytic activity (k_{cat}/K_m) with MTI as with inosine (2×10^4 vs $8 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$), but this activity is unlikely to be of physiological significance since MTI is not formed in mammals (Fig. 2a).

A reaction mixture containing purified PfADA and PfPNP converted MTA efficiently to hypoxanthine (MTA → MTI → hypoxanthine; Fig. 2b). Replacement of either PfADA or PfPNP with equivalent units of its mammalian counterpart, based on the activity with adenosine or inosine as substrates, was not efficient at this conversion. Adding larger amounts of mammalian PNP resulted in hypoxanthine formation as predicted by the kinetic constants (data

not shown). Therefore, both PfADA and PfPNP exhibit catalytic competence for methylthio substrates, an activity diminished in human PNP and absent from calf ADA.

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^{13,14}. *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 enzyme⁶. MT ImmH binds better to PfPNP (K_d 2.7 nM) 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 the K_d of MT-ImmH is 2.7 nM making MT-ImmH a less potent inhibitor of PfPNP than ImmH (K_d 860 pM;⁶).

MT-ImmH was compared to ImmH for the ability to kill cultured parasites (Fig. 2c). *P. falciparum* strain 3D7 cultures were treated with variable amounts of ImmH or MTI-ImmH followed by an assay for viability based on incorporation of [³H]-ethanolamine¹⁸. The IC₅₀ values for ImmH and MT-ImmH were 63 nM 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 PfPNP better than 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 (~ 2000 nM) in erythrocytes and at 1% hemocrit ~20 nM ImmH is

required for inhibition of the host enzyme in experimental cultures⁸. Inhibition of host PNP by ImmH is near-stoichiometric because of the 56 pM K_d ¹⁹. Inhibition of malaria cultures occurs at drug concentrations where both erythrocyte and PfPNP are expected to be inhibited by ImmH (K_d values of 56 pM and 860 pM respectively). With MT-ImmH, inhibition of PfPNP alone reduces parasite growth, and the combination of host and cellular PNP inhibition is lethal.

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 purine transport is not yet fully characterized²⁰⁻²². Inhibition of the polyamine pathway through product (MTA) inhibition would not be the cause of parasit death, because MTA is converted to MTI by PfADA.

Microscopic examination of treated cell cultures revealed that parasite numbers decreased as [³H]-ethanolamine incorporation decreased, and no viable parasites were detected in cultures with full inhibition (Fig. 2c). 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 hypoxanthine⁸ (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 formation⁸.

DISCUSSION

P. falciparum is remarkable because of its small number of purine salvage enzymes despite the complete reliance on this pathway. 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 by homology searches in any malaria 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 parasites¹⁰. The expression pattern suggests that PfADA, PfPNP and HGXPRT form the major path for purine salvage in *P. falciparum*. Purine salvage in malaria is unlike that in most other protozoa including other Apicomplexa such as *T. gondii*^{1,2} and *Cryptosporidium parvum*²³ that are rich in AK and rely on adenosine salvage to AMP for a major purine source.

MTA is a dead-end molecule, and its recycling is essential for purine and methionine conservation²⁴. Adenine and 5-methylthioribose-1-PO₄ (MTR-1-P) are recycled to ATP and methionine to regenerate S-adenosylmethionine^{24,25}. In malaria, MTA is converted to MTI, a metabolite that is unknown in other organisms.

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 (data not shown). This suggests that *T. gondii* may also generate and use MTI in a 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^{26,27} (shown as a dashed arrows in Fig. 1a). The genes for recycling the methylthio group of MTA to methionine have been proposed for *Bacillus subtilis* but the pathway is not apparent in the *P. falciparum* genome²⁷. An exception is α -ketomethylthiobutyrate transaminase, located on chromosome 2 of the *P. falciparum* genome²⁸, 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 PfPNP 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. *Plasmodium falciparum* expresses fewer enzymes in the essential pathway for purine nucleoside and MTA salvage than its host, making these pathways attractive targets. Inhibition of the dual-specificity PfPNP permits metabolic disruption of two pathways by targeting a single enzyme.

Imm-H and the analogues previously tested in malaria cultures all bind tighter to human PNP than PfPNP, and we could not establish if inhibition of PfPNP alone was capable of causing purine starvation in the parasite⁸. Our current studies indicate that inhibition of PfPNP is critical. Preclinical trials with Immucillin H in mammals indicate oral availability and low toxicity, useful features for antimalarial trials⁹. MT ImmH is the first Immucillin analogue to show high specificity for malarial PNP. While more specific for malarial PNP, MT-Imm-H binds less tightly to PfPNP than Imm-H⁶. Refinements in inhibitor design based upon analysis of the crystal structure of PfPNP⁶ may assist in inhibitor design for improved PfPNP specificity and potency.

A similar strategy can be explored for PfADA. A single dose of deoxycoformycin cured primates with *P. knowlesi*²⁹, but deoxycoformycin is highly toxic in mammals and not specific for malaria ADA. Nonetheless, the comparison of the inhibition profiles of PfADA and mammalian ADA (Table 1) suggests that there are differences in catalytic features that can be exploited for the synthesis of malaria-specific ADA inhibitors. PfPNP-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, but also introduces an Achilles heel into parasite metabolism.

MATERIAL AND METHODS

Enzymes and inhibitors.

Calf spleen ADA, xanthine oxidase, nucleoside substrates, coformycin, buffers and chemicals were purchased as the highest purity available. Deoxycoformycin was a gift of David C. Backer, Department of Chemistry, the University of Alabama. Human PNP and PfPNP (chromosome 5 of *P. falciparum*) were overexpressed in *E. coli* using the methods described described^{6,7,19}.

PfADA was identified on chromosome 10 from the Malarial Parasite Genome Project⁴ as a protein of 367 amino acids and 42.5 kDa. PfADA was amplified from strain 3D7 DNA using the PCR primers 5'-AATTGTAAGAATATGGATACTTCATATGAGA-3' (sense) and 5'-AAAATATTTACTTATAATTTTATTTTTTATATCTGG-3' (antisense). The coding region was placed in a pTrcHis2-TOPO vector (Invitrogen) and expressed in *E. coli* strain TOP 10. Recombinant PfADA was induced with 1 mM IPTG at 37 °C for 10 hr and purified by Ni²⁺ chromatography. The expressed PfADA was > 95% homogeneous by denaturing gel electrophoresis.

The coding sequence of *E. coli* PNP (PNPI; *deoD* gene) was amplified by PCR from *E. coli* (strain TOP 10) genomic DNA with the primers 5'-ATGGCTACCCCACACATTA-3'(sense) and 5'-TCATGATGATGATGATGATGCTCTTTATCGCCAGCAGAA-3'(antisense) which introduced a 6xHis tag (underline) 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 6xHis tag was expressed by induction of the bacterial culture with 1 mM IPTG at 37°C for 10 hours and purified using nickel affinity chromatography.

ImmH and MT-ImmH were synthesized as described^{30,31}.

Assays, inhibition and product identification.

Hypoxanthine produced by PNP was assayed by coupling to the xanthine oxidase reaction and measuring uric acid formation at 293 nm⁷. ADA action on adenosine or MTA was measured by the absorbance change at 265 nm. Coupled ADA/PNP assays contained 106 μM MTA, 50 mM NaPO₄ pH 7.5, 61 mU ADA, 2 mU PNP and 60 mU 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 (K_i) and the steady-state dissociation constant (K_i^*) as previously described⁷. K_d is the lower of these values.

Adenosine, MTA, inosine, MTI and hypoxanthine were purified by reverse phase HPLC (RP-HPLC) with a Water's Deltapak C₁₈ 15 μm, 7.8 mm × 300 mm column. Purines were eluted with 100 mM ammonium acetate and 50% methanol. The reaction was monitored at 260 nm.

NMR and IR spectra were used to identify the MTI isolated in this way. FTIR (Fourier Transform Infrared) spectroscopy was performed on a Nicolet Magna-IR 760 Fourier transform spectrometer (ThermoNicolet, Madison, WI) using a MCT detector. IR revealed the C=O stretch (1672 cm⁻¹) of the product to be identical to that of inosine. 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 and PlasmoDb websites. *Leishmania* APRTase and AK and *T. gondii* AK were also used as search queries. *Plasmodium falciparum* HGXPRTase, PNP, and ADA were readily identified, but no potential homologues for AK, APRT, MTAP, MTAN or MTRK were identified in any malaria species (Plasmodb 4.0). It should be noted that PfPNP was originally annotated as uridine phosphorylase

in Plasmodb (<http://www.plasmodb.org>) but its function and activity have been confirmed ⁷. *Toxoplasma gondii* AK ², as well as a potential *C. parvum* AK were identified using these search strategies. The *C. parvum* AK was recently proven to be functional ²³. 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 ¹⁰.

PlasmoDb annotations were searched with text queries of the blastx output and queries using E.C. numbers to identify AK, APRTase, MTAP, MTAN or MTRK homologues. Gene ontology (GO) biological function, GO biological process, GO cellular component assignments, and metabolic pathway annotations were also reviewed. No additional candidate orthologues were found. In addition, no potential homologues were found for the *B. subtilis* proteins expressed by the *mtnK*, *mtnY*, *mtnW*, *mtnX*, *mtnZ* and *mtnV* genes that are proposed to constitute the methionine salvage pathway ²⁷.

***P. falciparum* culture and immucillin kill curves.**

Human erythrocytes were collected from local volunteers under protocol CCI 00-31 or CCI 99-240 of the Albert Einstein College of Medicine. *P. falciparum* strain 3D7 was grown in RPMI supplemented with 10% human AB serum (Gemini Bioproducts) or 0.5% Albumax II (Invitrogen). Culture media for studies with ImmH and MT-ImmH contained no hypoxanthine supplement. Following incubation with ImmH or MT-ImmH for 48 hr, the 200 μ L cultures in 96-well plates were supplemented with 1 μ Ci [³H]-ethanolamine (Amersham, 25 Ci/mmol). After 18 hr, cell cultures were frozen and thawed to disrupt cells, and the mixtures harvested on glass-fiber filters and washed with 1.2 ml 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 more than two standard deviations from the mean were discarded. For some experiments, parasitemias were counted on Giemsa-stained smears of cultures treated in parallel.

Purine metabolism in *P. falciparum* by accelerator mass spectrometry (AMS).

Plasmodium falciparum were cultured in human erythrocytes (1% hematocrit) to 1% parasitemia as indicated above. After washing 3x in hypoxanthine-free medium, cultures were incubated 24 hr in hypoxanthine-free media followed by the addition of 1 nM carrier-free [8-¹⁴C]-hypoxanthine, [8-¹⁴C]-inosine, [8-¹⁴C]-uric acid, [8-¹⁴C]-MTA or [8-¹⁴C]-MTI to 200 µL cultures (approximately 20 dpm/culture or 200 fmoles). [8-¹⁴C]-MTI was produced from [8-¹⁴C]-MTA by hydrolysis with PfADA and purified by RP-HPLC (peak was identified by retention time). After labelling 48 hr, cells were washed 3x with unlabelled fresh medium, precipitated and pellets rinsed 3x with 6% trichloroacetic acid, extracted with acetone and dried. Three mg sucrose was added as carbon carrier, samples converted to graphite and analyzed for ¹²C/¹⁴C ratio by AMS at the Livermore National Laboratory AMS facility³².

In a second AMS experiment, *P. falciparum* cultures were incubated 12 hr in hypoxanthine-free media and labelled with 100 fmol [8-¹⁴C]-purine for 12 hours. Labelled cells were washed 3x with cold PBS and nucleic acids precipitated and washed with 3% cold perchloric acid.

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References:

1. Donald, R.G., Carter, D., Ullman, B. & Roos, D.S. Insertional tagging, cloning, and expression of the *Toxoplasma gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. Use as a selectable marker for stable transformation. *J Biol Chem* 271, 14010-9. (1996).
2. Sullivan, W.J., Jr. et al. Insertional tagging of at least two loci associated with resistance to adenine arabinoside in *Toxoplasma gondii*, and cloning of the adenosine kinase locus. *Mol Biochem Parasitol* 103, 1-14. (1999).
3. Muller, S., Coombs, G.H. & Walter, R.D. Targeting polyamines of parasitic protozoa in chemotherapy. *Trends Parasitol* 17, 242-9 (2001).
4. Gardner, M.J. et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419, 498-511. (2002).
5. Carlton, J.M. et al. Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 419, 512-9. (2002).
6. Shi, W. et al. *Plasmodium falciparum* purine nucleoside phosphorylase: Crystal structures, immucillin inhibitors and dual catalytic function. *J Biol Chem* (2004).
7. Kicska, G.A. et al. Transition state analogue inhibitors of purine nucleoside phosphorylase from *Plasmodium falciparum*. *J Biol Chem* 277, 3219-25. (2002).
8. Kicska, G.A. et al. Purine-less death in *Plasmodium falciparum* induced by immucillin-H, a transition state analogue of purine nucleoside phosphorylase. *J Biol Chem* 277, 3226-31. (2002).
9. Kilpatrick, J.M. et al. Intravenous and oral pharmacokinetic study of BCX-1777, a novel purine nucleoside phosphorylase transition-state inhibitor. In vivo effects on blood 2'-deoxyguanosine in primates. *Int Immunopharmacol* 3, 541-8 (2003).
10. Reyes, P. et al. Enzymes of purine and pyrimidine metabolism from the human malaria parasite, *Plasmodium falciparum*. *Mol Biochem Parasitol* 5, 275-90. (1982).
11. Daddona, P.E., Wiesmann, W.P., Lambros, C., Kelley, W.N. & Webster, H.K. Human malaria parasite adenosine deaminase. Characterization in host enzyme-deficient erythrocyte culture. *J Biol Chem* 259, 1472-5 (1984).
12. Daddona, P.E. et al. Expression of human malaria parasite purine nucleoside phosphorylase in host enzyme-deficient erythrocyte culture. Enzyme characterization and identification of novel inhibitors. *Journal of Biological Chemistry* 261, 11667-73 (1986).
13. Mao, C. et al. The crystal structure of *Escherichia coli* purine nucleoside phosphorylase: a comparison with the human enzyme reveals a conserved topology. *Structure* 5, 1373-83 (1997).
14. Bennett, E.M., Li, C., Allan, P.W., Parker, W.B. & Ealick, S.E. Structural basis for substrate specificity of *Escherichia coli* purine nucleoside phosphorylase. *J Biol Chem* 278, 47110-8 (2003).
15. Wilson, D.K., Rudolph, F.B. & Quioco, F.A. Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. *Science* 252, 1278-84 (1991).
16. Lappin, G. & Garner, R.C. Current perspectives of ¹⁴C-isotope measurement in biomedical accelerator mass spectrometry. *Anal Bioanal Chem* 378, 356-64 (2004).
17. Lappin, G. & Garner, R.C. Big physics, small doses: the use of AMS and PET in human microdosing of development drugs. *Nat Rev Drug Discov* 2, 233-40 (2003).

18. Elabbadi, N., Ancelin, M.L. & Vial, H.J. Use of radioactive ethanolamine incorporation into phospholipids to assess in vitro antimalarial activity by the semiautomated microdilution technique. *Antimicrob Agents Chemother* 36, 50-5. (1992).
19. Lewandowicz, A. & Schramm, V.L. Transition state analysis for human and *Plasmodium falciparum* purine nucleoside phosphorylases. *Biochemistry* 43, 1458-1468 (2004).
20. Carter, N.S., Landfear, S.M. & Ullman, B. Nucleoside transporters of parasitic protozoa. *Trends Parasitol* 17, 142-5. (2001).
21. Carter, N.S. et al. Isolation and functional characterization of the PfNT1 nucleoside transporter gene from *Plasmodium falciparum*. *J Biol Chem* 275, 10683-91. (2000).
22. Parker, M.D. et al. Identification of a nucleoside/nucleobase transporter from *Plasmodium falciparum*, a novel target for anti-malarial chemotherapy. *Biochem J* 349, 67-75 (2000).
23. Striepen, B. et al. Gene transfer in the evolution of parasite nucleotide biosynthesis. *Proc Natl Acad Sci U S A* In press(2004).
24. Trackman, P.C. & Abeles, R.H. Methionine synthesis from 5'-S-Methylthioadenosine. Resolution of enzyme activities and identification of 1-phospho-5-S methylthioribulose. *J Biol Chem* 258, 6717-20 (1983).
25. Kelley, W.N., Levy, R.I., Rosenbloom, F.M., Henderson, J.F. & Seegmiller, J.E. Adenine phosphoribosyltransferase deficiency: a previously undescribed genetic defect in man. *J Clin Invest* 47, 2281-9 (1968).
26. Dai, Y., Pochapsky, T.C. & Abeles, R.H. Mechanistic studies of two dioxygenases in the methionine salvage pathway of *Klebsiella pneumoniae*. *Biochemistry* 40, 6379-87 (2001).
27. Sekowska, A. & Danchin, A. The methionine salvage pathway in *Bacillus subtilis*. *BMC Microbiol* 2, 8 (2002).
28. Berger, L.C., Wilson, J., Wood, P. & Berger, B.J. Methionine regeneration and aspartate aminotransferase in parasitic protozoa. *J Bacteriol* 183, 4421-34 (2001).
29. Webster, H.K., Wiesmann, W.P. & Pavia, C.S. Adenosine deaminase in malaria infection: effect of 2'-deoxycoformycin in vivo. *Adv Exp Med Biol* 165 Pt A, 225-9 (1984).
30. Evans, G.B. et al. Addition of lithiated 9-deazapurine derivatives to a carbohydrate cyclic imine: convergent synthesis of the aza-C-nucleoside immucillins. *J Org Chem* 66, 5723-30 (2001).
31. Evans, G.B., Furneaux, R.H., Schramm, V.L., Singh, V. & Tyler, P.C. Targeting the Polyamine Pathway with Transition-State Analogue Inhibitors of 5'-Methyladenosine Phosphorylase. *J. Med. Chem.* In press(2004).
32. Ognibene, T.J., Bench, G., Vogel, J.S., Peaslee, G.F. & Murov, S. A high-throughput method for the conversion of CO₂ obtained from biochemical samples to graphite in septa-sealed vials for quantification of ¹⁴C via accelerator mass spectrometry. *Anal Chem* 75, 2192-6 (2003).

Figure legends

Figure 1

a. Pathways for recycling MTA and adenosine in the polyamine and methylthio transfer pathways in humans, *E. coli* and *P. falciparum*. Abbreviations are: PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase; MTAP, methylthioadenosine phosphorylase; APRT, adenine phosphoribosyltransferase; AK, adenosine kinase; MTAN, methylthioadenosine nucleosidase; MTRK, methylthioribose kinase. PfADA catalyzes deamination of both adenosine and MTA and PfPNP catalyzes phosphorolysis of both inosine and MTI.

b. Alignment of *P. falciparum*, *E. coli* and human ADA protein sequences. Amino acids common to all three are marked in green and those common to any two are marked in yellow. Ligands to the catalytic site Zn^{2+} of the mammalian ADA are marked with an asterisk (*) and those in contact with a transition state analogue in crystals of mouse ADA¹⁵ are marked with a hash (#). Note that catalytic site residues are completely conserved in the three species, suggesting all are Zn^{2+} -containing deaminases. The N-terminal region of mammalian ADA contacts the 5'-hydroxyl group of adenosine analogues.

c. HPLC chromatogram showing the conversion of MTA to MTI, 10 minutes after addition of PfPNP. Duplicate reactions were separated on a reverse phase C18 μ Deltapak column before and 10 minutes after addition of PfADA to MTA.

d. 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 panel c for comparison of MTI and MTA structures).

Figure 2

a. MTI is incorporated into nucleic acids of *P. falciparum* as detected by AMS. 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 present in *P. falciparum*. Both species have ADA, PNP and HGPRT activity. MTI, MTA, inosine, adenosine, 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). Results of two separate labelling experiments are shown in Table 2.

b. PfADA and PfPNP convert MTA to hypoxanthine. The coupled reactions $\text{MTA} \rightarrow \text{MTI} \rightarrow \text{hypoxanthine} \rightarrow \text{uric acid}$ are catalyzed by PfADA + PfPNP 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 is converted to uric acid by xanthine oxidase.

c. Action of ImmH and MT ImmH on the growth and survival of *P. falciparum* cultured in human erythrocytes. Cultures with 1% parasitemia were incubated 48 hr in the presence of ImmH or MT-ImmH followed by an additional 18 hr in the presence of 1 μCi [^3H]-ethanolamine¹⁸. IC_{50} for ImmH was 63 nM. IC_{50} for MT-ImmH was 50 nM. Parasitemias of cultures harvested and counted in parallel are as indicated next to each datapoint. Background incorporation for erythrocytes was subtracted from all values.

Table 1. Kinetic properties of mammalian and *P. falciparum* ADA

Substrate or inhibitor	<u>Calf spleen ADA</u>			<u><i>P. falciparum</i> ADA</u>		
	K_m (μM)	k_{cat} (s^{-1})	K_d (M)	K_m (μM)	k_{cat} (s^{-1})	K_d (M)
adenosine	56 ± 7	65 ± 3		29 ± 3	1.8 ± 0.1	
methylthioadenosine		<0.02		170 ± 16	15 ± 0.9	
S-adenosylhomocysteine		<0.02		>1000	<0.04	
coformycin			$7 \pm 4 \times 10^{-11}$			$16 \pm 3 \times 10^{-10}$
deoxycoformycin			$29 \pm 4 \times 10^{-10}$			$12 \pm 4 \times 10^{-10}$
EHNA			$49 \pm 5 \times 10^{-9}$			$18 \pm 6 \times 10^{-5}$

Reactions were performed as discussed in the materials and methods. K_d values were obtained using adenosine as the substrate in the reaction. Similar K_d values were obtained using MTA as the substrate for PfADA (data not shown).

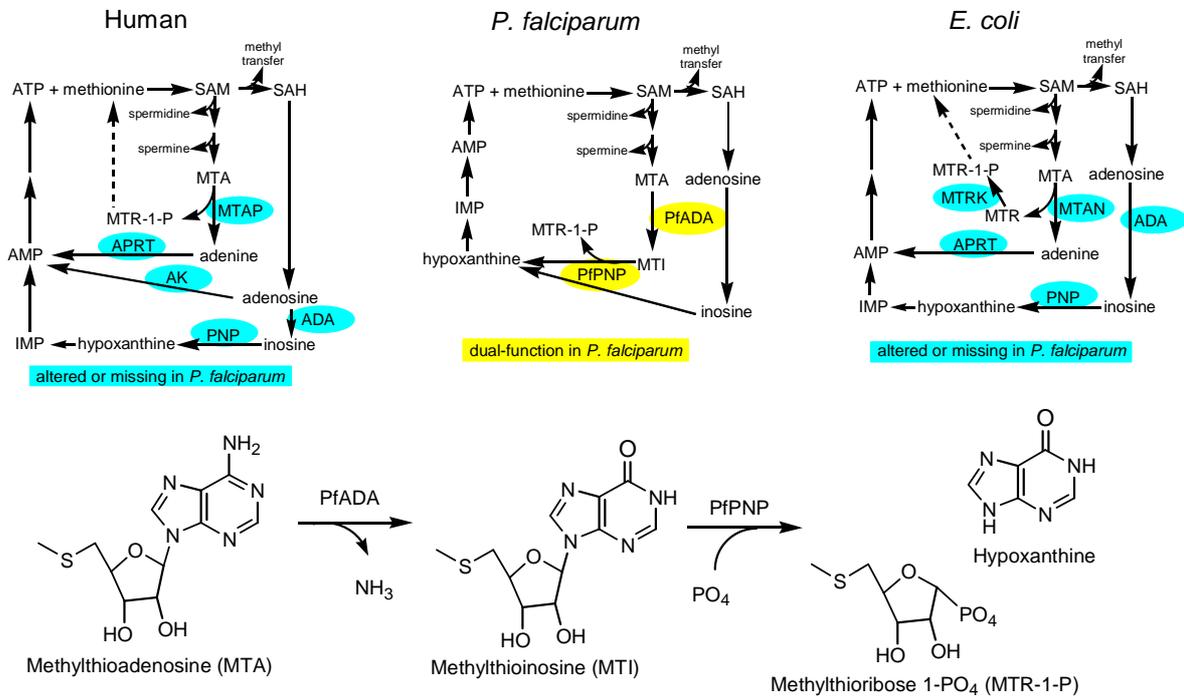
Table 2. Detection of [¹⁴C]-labelled Nucleic Acids by AMS

	<u>Expt 1 (48 h labelling)</u>	<u>Expt 2 (12 h labelling)</u>
[8- ¹⁴ C]-labelled Precursor	fmol ¹⁴ C	fmol ¹⁴ C
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
methylthioadenosine	1.5 ± 0.2	0.6 ± 0.2
uric acid	.06 ± .01	ND

P. falciparum were cultured in 96 well plates as discussed in the materials and methods. 200 fmol (Expt 1) or 100 fmol (Expt 2) of [8-¹⁴C] carrier-free purine was added to each culture. [¹⁴C]-labelled nucleic acids were quantitated by AMS in quadruplicate (Expt 1) or triplicate (Expt 2). Standard deviation for each group is given. ND means not done.

Figure 1

a.



b.

Adenosine deaminase

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* * #
Pf MNCKNMDTSYEIINYLTKDELIDLSCMDKKERYK IWKRLPKCELHCHLDVCFVSDFFL NVIRK YNI 67
Hu -----MAQTPAFDCKPKVELHVHLDGSIK PETILYGR RRG I 36
Ec -----MIDTTLPLTDIHRHLDGNIRPQTI LELGRQ YNI 33

Pf QPNMSDEE-IIDYYLFSKPGKSLDEFVEKALRLTDIYIDYTVVVDLAKHAVFNKYKEGVVLM EFRY 132
Hu ALPANTA ELLN-VIGMDKPLTLPDFLAKFDYYMPAIAAGCREAIKRIAYEFVEMKAKEGVVYVEVRY 102
Ec SLPAQSL ETLIPHVQVIANEPDLVSFLTKLDWGVKVLASLDACRRVAFENIEDAARHGLHYVELRF 99

Pf SPSFMSFKHNLDKDL-----IHEAIVKGLNEAVALLEYKIQVGLLCTGDGGLSHERMKEAA 188
Hu SPHELLANSKVEPIPWNAEGDLTPDEVVALVGGQLQEGER--DFGVKARSILCCMRHQPNWSPKVVVE 167
Ec SPGYMAMAHLPVAG-----VVEAVIDGVR EGR--TFGVQAKLIGIMSR TFGAEACQQL 153

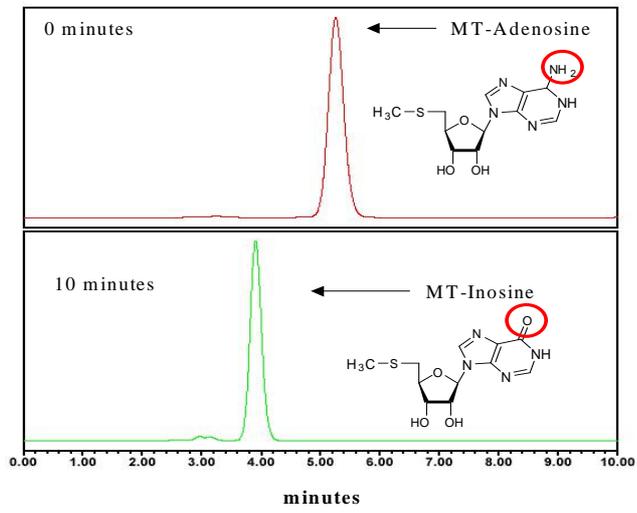
Pf EFCIKHKKDFVGYDHAGHEVDLDPF-----KDI FDNIREEGISLSVHAGEDVSIPLNLSLYTAINL 249
Hu LCKNYQQQTVVAIDLAGEDET-IPGSLLPGHVQAYQEA VSKGIHRTVHAGEVGS A---EUVKEAVDI 230
Ec EAFLAHRDQITALDLAGDELGFPGSLFLS-----HFNRARDA GWHITVHAGEAAGP---ESIWQAIRE 213

Pf LHVKRIGHGIRVSESQELIDLVKEKDI LLEVCPI SNVLLNNVKSMDTHPIRMLYDAGVKVSVNSDDP 316
Hu LKTERLGHGYHTLEDQALYNRLRQENMHFEICPWSSYLTGAWKPDTEHAVIRLKNQDQANYSLNTDDP 297
Ec LGAERIGHGVKAI EDRALMDFLAEQQIGIESCLTNSNIQTSTVAELAAHPLKTFLEHGIRASINTDDP 280

Pf GMFLTNIITDNYEELYTHLNF T LADFMKMN LWAVQKSFVDPDIK NKIISKYF----- 367
Hu LIKSTLDTDYQMTKRDMGFT EEFKRLNINA AKSSFLPQDEKRELLDLLYKAYGMPPSASAGQL 363
Ec GVQGVDI IHEYTVAAAPAGLSREQIRQAQINGLEMAFLSAEEKRALREKVAAK----- 333

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c.



d.

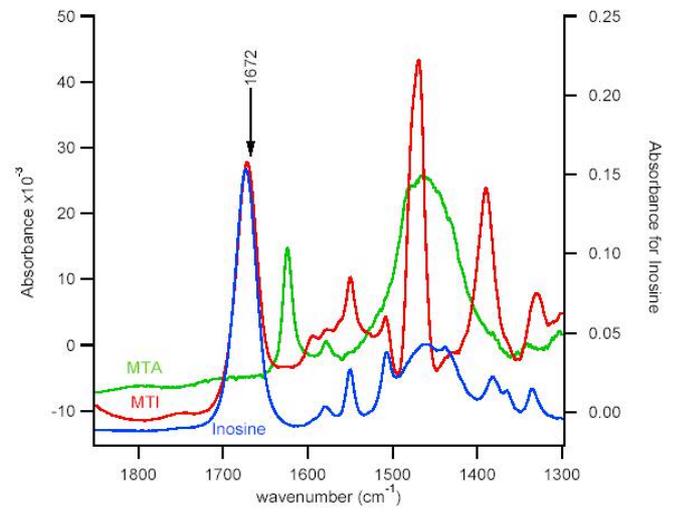
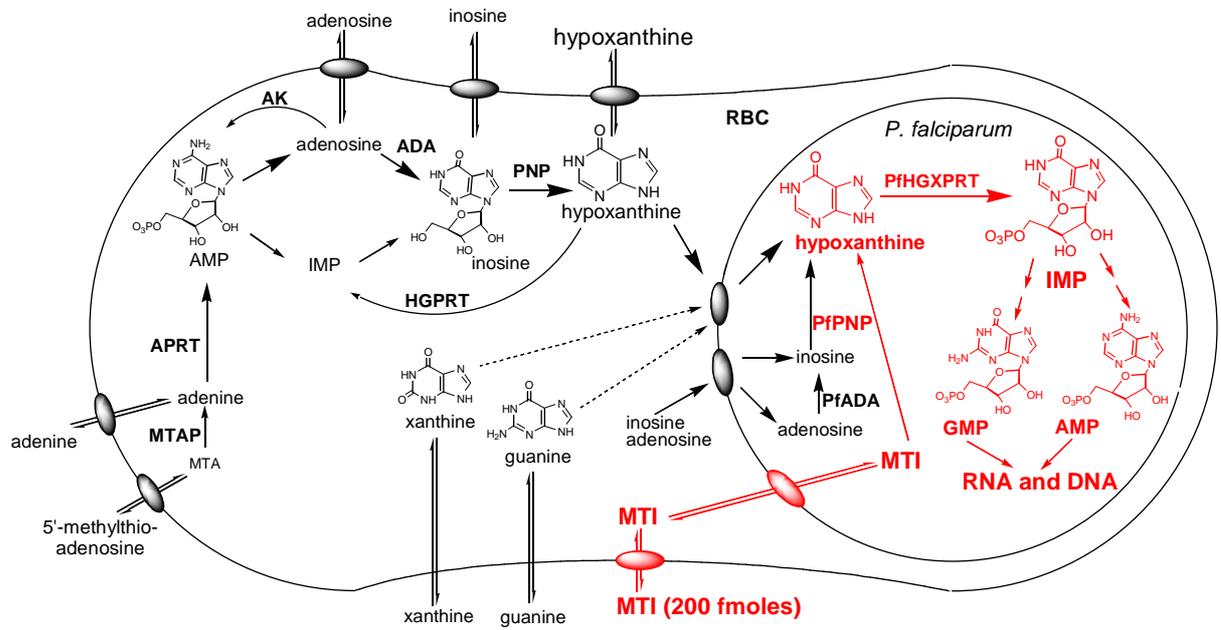
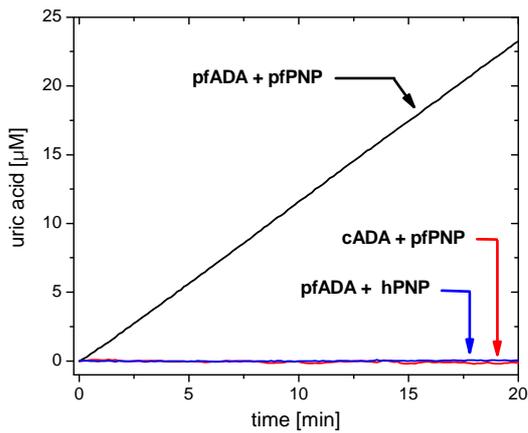


Figure 2

a.



b.



c.

