Lead Compounds for Antimalarial Chemotherapy: Purine Base Analogs Discriminate between Human and *P. Falciparum* 6-Oxopurine Phosphoribosyltransferases

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The malarial parasite *Plasmodium falciparum* depends on the purine salvage enzyme hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT) to convert purine bases from the host to nucleotides needed for DNA and RNA synthesis. An approach to developing antimalarial drugs is to use HGXPRT to convert introduced purine base analogs to nucleotides that are toxic to the parasite. This strategy requires that these compounds be good substrates for the parasite enzyme but poor substrates for the human counterpart, HGPRT. Bases with a chlorine atom in the 6-position or a nitrogen in the 8-position exhibited strong discrimination between *P. falciparum* HGXPRT and human HGPRT. The k_{cat}/K_m values for the *Plasmodium* enzyme using 6-chloroguanine and 8-azaguanine as substrates were 50−80-fold and 336-fold higher than for the human enzyme, respectively. These and other bases were effective in inhibiting the growth of the parasite in vitro, giving IC_{50} values as low as 1 μM.

Introduction

Malaria is one of the most widespread infectious diseases in the world today, and the most lethal of the parasites responsible for this disease is *Plasmodium falciparum*.[1] Concerted efforts have been made to curtail the spread of malarial infections but these have only been partly successful. Many strains of *P. falciparum* have developed resistance to the available antiparasitic agents,[2] indicating the need to develop new drugs. Such drugs will be most effective if metabolic differences between the host cell and its parasite invader can be exploited. One potential target is the purine salvage pathway. Humans synthesize their 6-oxopurine nucleoside monophosphates either de novo or by salvage of purine bases. All protozoan parasites studied to date are unable to synthesize the purine ring and possess only the salvage pathway to make purine nucleotides for DNA and RNA synthesis.[3−5]

Hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT[a]) is an essential enzyme in this pathway.[3] HGXPRT synthesizes 6-oxopurine nucleoside monophosphates from the corresponding base and 5-phospho-α-D-ribosyl-1-pyrophosphate (*P*Rib-*PP*). For catalysis, a divalent metal ion must be present, and this is usually Mg^{2+} in vivo (Figure 1). The reaction mechanism for human HGPRT is ordered: *P*Rib-*PP*.Mg^{2+} binds first followed by the purine base.[6] Pyrophosphate then dissociates from the complex and the nucleoside monophosphate is then released, and this is the rate-limiting step. Recently, Sarka et al.[7] have proposed that, for *P. falciparum* HGXPRT, the mechanism is ping-pong and not sequential, with *P*Rib-*PP* being hydrolyzed to ribose-5-phosphate and PP_i with the release of PP_i before the binding of the purine base.

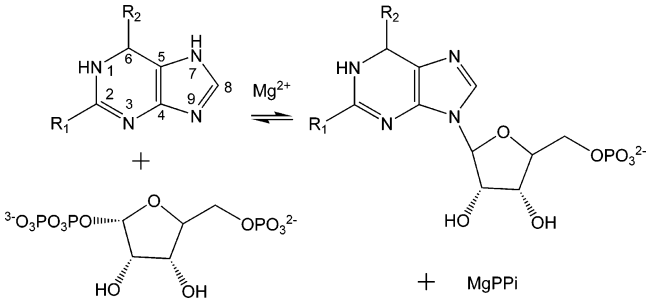

Figure 1. Reaction catalyzed by the 6-oxopurine phosphoribosyltransferases. The naturally occurring purine bases are guanine (R_1 is $-NH_2$), hypoxanthine (R_1 is $-H$), and xanthine (R_1 is $=O$). In all the bases R_2 is $=O$.

One approach to chemotherapy based on the purine salvage pathway is to identify purine base analogs, which are poor or weak substrates for human HGPRT, while being comparatively good substrates for *P. falciparum* HGXPRT. These molecules have the advantage as possible chemotherapeutic agents in that they are able to cross cell membranes.[5,8] Once inside the cell they are then converted by the 6-oxopurine PRTases into toxic nucleotides. The most likely mechanism of action is that these nucleotides are incorporated into DNA and RNA, resulting in the cessation of cell replication. The purine base analog, 6-mercaptopurine, is in use as an anticancer drug, and this is how it exerts its effects.[9] Thus, it can be hypothesized that a base analog that discriminates between the human and the *Plasmodium* 6-oxopurine PRTases would also be effective as an antimalarial agent in the same manner.

Each of the 6-oxopurine PRTases studied to date from mammals, parasites, and bacteria exhibits a different specificity for the naturally occurring purine bases.[10] Previous results at pH 8.5 and in the presence of 110 mM Mg^{2+}, where both the human and *P. falciparum* enzymes are maximally active, showed that human HGPRT utilized both hypoxanthine and guanine efficiently but that xanthine was not a substrate.[11] In contrast,

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[a] Abbreviations: *P*Rib-*PP*, 5-phospho-α-D-ribosyl-1-pyrophosphate; PRTase, phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HGXPRT, hypoxanthine-guanine-xanthine phosphoribosyltransferase; GMP, guanosine 5′-monophosphate; IMP, inosine 5′-monophosphate; HPP, 7-hydroxy[4,3-*d*]pyrazolo pyrimidine.

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P. falciparum HGXPRT utilized guanine, hypoxanthine, and xanthine, though xanthine was a much weaker substrate with k_{cat}/K_m values 112-fold lower for hypoxanthine and 214-fold lower for guanine. The catalytic efficiency (k_{cat}/K_m) of the *Plasmodium* enzyme is lower than for the human HGPRT, with ratios for the two naturally occurring substrates being of the order of 4.[11] This difference is mainly attributable to the fact that, for human HGPRT, the k_{cat} for hypoxanthine is 10-fold higher than for the *Plasmodium* enzyme, while the k_{cat} for guanine was 6-fold higher.[11] Hypoxanthine actually binds better to the *Plasmodium* enzyme (cf. 0.9 μM with 3.1 μM), while there is little difference for the affinity of guanine to the active sites (cf. 1.9 μM with 1.4 μM).[11]

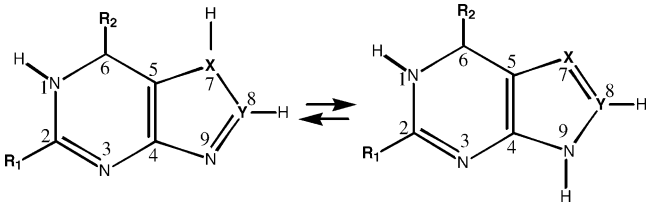
Plasmodium falciparum HGXPRT has been suggested as a potential antimalarial target for several years, but progress in the design of lead therapeutic agents has been slow and limited to bacterial complementation and in vitro susceptibility studies.[12−15] This has been due, in part, to previous difficulties in obtaining sufficient quantities of human and parasite enzymes in pure and stable form. However, the recombinant enzymes are now available, allowing precise measurement of kinetic constants.[7,11,16,17] Such studies provide a framework for the design and synthesis of selective inhibitors or substrates. The crystal structures of both human and *P. falciparum* 6-oxopurine PRTases in complex with immucillinGP or immucillinHP (transition state analogs), pyrophosphate, and Mg^{2+} have been determined.[18,19] Crystal structures of the human enzyme in complex with guanosine 5′-monophosphate (GMP),[20] inosine 5′-monophosphate (IMP),[7] and 7-hydroxy[4,3-d]pyrazolo pyrimidine (HPP)-Mg^{2+}-*P*Rib-*PP*[21] and the structure in the absence of ligands have also been determined.[22] The structures of the human enzyme show the extreme flexibility of the enzyme during the catalytic cycle.[22] The currently available kinetic data suggests that the *P. falciparum* 6-oxopurine PRTase is also very plastic[11,17] and, hence, the difficulty of obtaining structures in the presence of substrates/products or, indeed, in the absence of any ligand. The unliganded enzyme loses activity rapidly unless hypoxanthine and *P*Rib-*PP* are added to the storage buffer.[11,17] The current structures of the two enzymes in the presence of the transition state analogues show little difference in the active site, suggesting that it may not be possible to design discriminatory substrates or inhibitors. However, the similarity in the structures in the presence of the immuncillins is really not all that surprising, as these molecules bind very tightly, with K_i values in the nM range, and perfectly mimic the transition state.

In this report, a number of purine base analogues were examined to test the possibility that differences in the active sites of the human and *Plasmodium* 6-oxopurine PRTases exist and that these could be exploited for development of antimalarial drugs. A number of base analogs were identified that are good substrates for the *Plasmodium* 6-oxopurine PRTase but comparatively weak for the human enzyme. These, and other bases, were then tested for their effect on the growth of the parasite in vitro.

Results and Discussion

Identification of Purine Base Analogs as Substrates for Human and *Plasmodium falciparum* 6-Oxopurine Phosphoribosyltransferases. The possibility that a particular base analog is a substrate for the human or *Plasmodium falciparum* enzyme was initially investigated using reverse-phase HPLC in which the purine base was separated from the nucleoside monophosphate product of the reaction.[23] This technique allowed the rapid screening of base analogs when neither a radiolabeled substrate nor its corresponding product nucleoside monophosphate was available. In all, 11 purine base analogs were tested using this procedure. Eight of these were substrates for *P. falciparum* HGXPRT. Under the same conditions, only three were good substrates for human HGPRT, while two others were very slowly converted to their nucleoside monophosphate (Table 1). 2-Thioxanthine, 8-azaxanthine, and 2-hydroxypurine were not substrates for either enzyme.

Table 1. Structures of the Purine Bases and Purine Base Analogs[a,b]



purine base	R_1	R_2	X	Y	substrate identified by HPLC profile[c] Hu[d]	Pf[e]
Substitutions at the 2-Position						
hypoxanthine	−H	=O	NH	CH	+++	+++
guanine	$-NH_2$	=O	NH	CH	ND[f]	ND[f]
xanthine	=O	=O	NH	CH	ND[f]	ND[f]
2-thioxanthine	−SH	=O	NH	CH	0	0
Substitutions at the 6-Position						
6-mercaptopurine	−H	=S	NH	CH	+++	+++
6-thioguanine	$-NH_2$	=S	NH	CH	+++	+++
6-thioxanthine	=O	=S	NH	CH	0	+++
6-chloroguanine	$-NH_2$	−Cl	NH	CH	0	+++
2-hydroxypurine	=O	−H	NH	CH	0	0
Substitutions at the 8-Position						
8-azahypoxanthine	−H	=O	NH	N	0	+++
8-azaguanine	$-NH_2$	=O	NH	N	+	+++
8-azaxanthine	=O	=O	NH	N	0	0
Substitutions at the 7- and 8-Positions						
allopurinol	−H	=O	CH_2	N	+	+++
Substitutions at the 7-Position						
7-deazaguanine	$-NH_2$	=O	CH_2	CH	0	0

[a] These structures show the predominant tautomeric forms of the purine ring. [b] The substitutions in the ring analyzed for base specificity are shown in bold. [c] These compounds were identified as good (+++), weak (+), or no detectable conversion after incubation for 10 min (0). [d] Human HGPRT (0.02 μM). [e] *Plasmodium falciparum* HGXPRT (0.15 μM). [f] Not determined by HPLC as these are naturally occurring substrates (hypoxanthine was used as a positive control). The enzyme concentrations were adjusted so that the activity toward guanine at pH 8.5 was the same.

Kinetic Analysis and Parasite Susceptibilities of the Purine Base Analogs. The k_{cat} and $K_{m(app)}$ values for purine base analogs identified as substrates by HPLC were measured (Tables 2, 3, and 4) and compared with values for the naturally occurring purine bases. The ability of nonsubstrates to act as inhibitors was tested in spectrophotometric assays using guanine as the substrate. The effects of a number of bases on the uptake of [^{3}H]hypoxanthine into the parasite's nucleic acids and on the growth of parasites in cell culture were then assessed (Figure 2; Table 5). The analogs have been classified into four groups, according to substitutions at the 2-, 6-, 7-, and 8-position in the purine ring (Table 1). The results are discussed below within these groups.

Substitutions at the 2-Position. The structures of the naturally occurring 6-oxopurines hypoxanthine, guanine, and xanthine differ in the substituents at the 2-position of the purine ring (Figure 1). *P. falciparum* HGXPRT can use all three bases as substrates, with k_{cat} values of 0.33 s^{-1} (0.71 s^{-1}) for hypoxanthine, 0.66 s^{-1} (2.1 s^{-1}) for guanine, and 3.3 s^{-1} (3 s^{-1}) for xanthine (pH 7.4, Table 2; values in brackets in the

Table 2. Kinetic Constants for Purine Base Analogs as Substrates for Human and *P. Falciparum* 6-Oxopurine PRTases at pH 7.4[a]

	k_{cat} (s^{-1})		$K_{m(app)}$[b] (μM)	
purine base analog	Hu[c]	Pf[d]	Hu[c]	Pf[d]
hypoxanthine	5.2 ± 0.4	0.33 ± 0.08	3.4 ± 1.0	0.07 ± 0.03[e]
guanine	8.2 ± 0.6	0.66 ± 0.07	1.9 ± 0.4	0.83 ± 0.5
xanthine	NM[f]	3.3 ± 0.2	NM[f]	189 ± 18
6-mercaptopurine	2.4 ± 0.1	0.4 ± 0.02	1.15 ± 0.5	0.02 ± 0.03[e,g]
6-thioguanine	8.7 ± 0.2	0.9 ± 0.02	1.7 ± 0.3	0.75 ± 0.1
6-thioxanthine	NM[f]	0.4 ± 0.1	NM[f]	82 ± 5
6-chloroguanine	NM[f]	1.2 ± 0.06	NM[f]	175.7 ± 2.0
8-azahypoxanthine	NM[f]	9.6 ± 0.3	NM[f]	25.6 ± 2
8-azaguanine	0.4 ± 0.1[h]	4.9 ± 0.08	72 ± 26[h]	12.6 ± 0.23
allopurinol	0.15 ± 0.01	0.6 ± 0.02	47.9 ± 9.2	10.5 ± 0.7

[a] 0.1 M Tris-HCl, 12 mM $MgCl_2$, pH 7.4. [b] $[PRibPP]_0$ = 900−1000 μM. [c] Human HGPRT. [d] *Plasmodium falciparum* HGXPRT. [e] The large errors in these K_m values are due to the limiting sensitivity of the spectrophotometric assay. Accurate values at pH 8.5 are in Table 5. [f] NM = not measurable. Only the k_{cat}/K_m value could be calculated because the maximum $[S]_0$ was [dlt]K_m (see Table 4). See text and Table 4 for estimate of maximum value of k_{cat}/K_m. [g] Lowest concentration of substrate was 3 μM. [h] Maximum concentration of substrate = 71 μM.

Table 3. Comparison of the Catalytic Efficiencies (k_{cat}/K_m) of the Purine Base Analogs for Human and *P. Falciparum* 6-Oxopurine PRTases at pH 7.4[a] and the Specificity Ratio[b]

	k_{cat}/K_m ($M^{-1}s^{-1}$)		k_{cat}/K_m(Pf)/k_{cat}/K_m(Hu)[b]
purine base analog	Hu[c]	Pf[d]	
hypoxanthine	1.5×10^6	4.3×10^6	2.9
guanine	4.3×10^6	8.0×10^5	0.2
xanthine	≤5[e]	1.7×10^4	≥3400
6-mercaptopurine	2.0×10^6	2.0×10^7	10
6-thioguanine	5.1×10^4	1.0×10^6	0.2
6-thioxanthine	≤4[f]	5.0×10^3	≥1250
6-chloroguanine	≤1.3×10^2 [e]	7.0×10^3	≥54
8-azahypoxanthine	1.1×10^3	3.7×10^5	336
8-azaguanine	5.0×10^3	4.0×10^5	80
allopurinol	3.0×10^3	6.0×10^4	20

[a] 0.1 M Tris-HCl, 12 mM $MgCl_2$, pH 7.4. [b] [k_{cat}/K_m (*Plasmodium*)/ k_{cat}/K_m(human)]. [c] Human HGPRT. [d] *P. falciparum* HGXPRT. [e] Numbers indicate maximum possible values for k_{cat}/K_m based on the minimum observable activity.

text correspond to pH 8.5 and 110 mM Mg^{2+}, Table 4). The $K_{m(app)}$ values followed the reverse trend, with hypoxanthine < guanine ≪ xanthine (Tables 2 and 4). Thus, xanthine was a better substrate with respect to k_{cat} but, because of its high K_m value, its catalytic efficiency, as assessed by the k_{cat}/K_m ratio, was lower (Table 4).

The k_{cat} values for guanine and hypoxanthine for human HGPRT at pH 7.4 were 17- and 12-fold higher than for *P. falciparum* HGXPRT (Table 2). The $K_{m(app)}$ values for *P. falciparum* HGXPRT at pH 7.4 could not be measured precisely in the spectrophotometric assay (Table 2) because of the limits of sensitivity of the assay. However, at pH 8.5, these values were higher and could be determined accurately (Table 4). Results confirmed that the human enzyme was a more efficient catalyst than the parasite enzyme with the naturally occurring substrates, hypoxanthine and guanine.

Using xanthine as the substrate, the concentration of the human enzyme in the reaction mixture was increased from 0.03 μM (as used in the normal assay for guanine or hypoxanthine) to 1.3 μM. Even under these conditions, there was no observable change in absorbance at 255 nm, using a concentration of xanthine of 72 μM. Based on an estimate of the minimum observable rate in this assay of 4.7×10^{-10} M s^{-1} (0.004 absorbance units per 30 min), k_{cat}/K_m could be calculated as ≤5 $M^{-1}s^{-1}$. This value can be compared with the measured value of k_{cat}/K_m for the parasite enzyme (1.7×10^4 $M^{-1}s^{-1}$), giving a specificity ratio of ≥3400 in favor of the parasite enzyme (Table 3). The insertion of a 2-oxo group has a very large effect on catalysis by human HGPRT: k_{cat}/K_m at pH 7.4 for hypoxanthine was found to be 1.5×10^6 $M^{-1}s^{-1}$ (Table 3) compared with ≤5 $M^{-1}s^{-1}$ for xanthine, but a much smaller effect on the parasite enzyme (4.3×10^6 $M^{-1}s^{-1}$ for hypoxanthine, 1.7×10^4 $M^{-1}s^{-1}$ for xanthine). As well as not being a substrate for human HGPRT, xanthine was not a competitive inhibitor with respect to guanine. At a xanthine concentration of 60 μM and a guanine concentration of 6 μM, there was no detectable change in the initial velocity of the reaction. Based on the equation for competitive inhibition, the K_i value for xanthine would have to be greater than 650 μM to be consistent with this observation.

In solution, guanine, hypoxanthine, and xanthine exist as mixtures of the N(7)H and N(9)H tautomers in ratios of 50:50 (guanine), 58:42 (hypoxanthine), and 90:10 (xanthine).[24,25] When the purine base is bound in the active site, a hydrogen bond is observed between the N(7)H of the base and the side chain carboxyl of a conserved aspartic acid residue (D137 in human; Figure 3), thereby stabilizing the N(7)H tautomer. The bound base is thus primed for nucleophilic attack by N(9) on the C1 of the ribose ring, regardless of the differences in tautomeric ratio in solution noted above. Hence, it is difficult to see how the tautomeric ratios could explain the observation that xanthine is a substrate for the parasite enzyme but not the human enzyme.

It has been proposed that xanthine is not a substrate for the human enzyme because the 2-oxo exocyclic group would be

Table 4. Kinetic Constants for Purine Base Analogs as Substrates for Human and *P. falciparum* 6-Oxopurine PRTases at pH 8.5[a,b]

	k_{cat} (s^{-1})		$K_{m(app)}$[b] (μM)	
purine base analog	Hu[c]	Pf[d]	Hu[c]	Pf[d]
hypoxanthine	7.4 ± 0.5	0.7 ± 0.3	3.1 ± 0.9	0.9 ± 0.08
guanine	13.4 ± 0.4	2.1 ± 0.2	1.9 ± 0.4	1.4 ± 0.4
xanthine	NM[e]	3.0 ± 0.5	NM[e]	420 ± 0.12
6-mercaptopurine	1.8 ± 0.2	1.4 ± 0.3	3.0 ± 0.6	1.6 ± 0.2
6-thioguanine	7.6 ± 0.4	2.4 ± 0.08	5.9 ± 1.3	1.9 ± 0.5
8-azahypoxanthine	NM[e]	4.5 ± 0.1	NM[e]	145 ± 8[f]
8-azaguanine	NM[e]	2.4 ± 0.06	NM[e]	26.6 ± 4.2[g]
allopurinol	0.08 ± 0.02	1.3 ± 0.4	135 ± 16	11.7 ± 1.3

[a] 0.1 M Tris-HCl, 110 mM $MgCl_2$, pH 8.5. [b] $[PRibPP]_0$ = 900−1000 μM. [c] Human HGPRT. [d] *Plasmodium falciparum* HGXPRT. [e] NM = Not measurable. [f] Maximum concentration of substrate = 234 μM. [g] Maximum concentration of substrate = 71 μM.

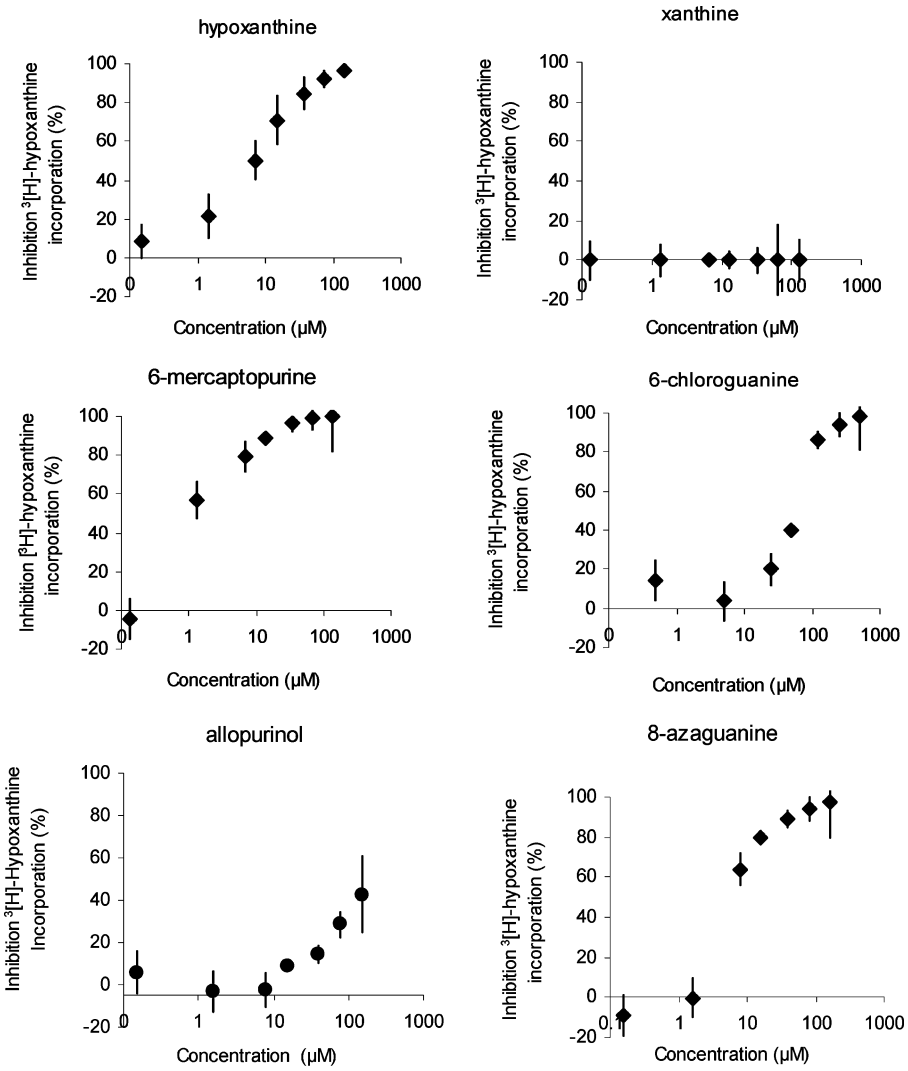


Figure 2. Plots of % inhibition of [^{3}H]hypoxanthine incorporation into *P. falciparum* nucleic acids vs purine base concentration on a logarithmic scale. The purine bases are hypoxanthine, xanthine, 6-mercaptopurine, 6-chloroguanine, allopurinol, and 8-azaguanine.

Table 5. Effect of Purine Base Analogs on the Uptake of [^{3}H]Hypoxanthine into Parasite Nucleic Acids and on the Growth of *P. Falciparum* in Erythrocyte Cell Culture

purine base	IC_{50} (μM) [^{3}H]hypoxanthine[b]	IC_{50} (μM) cell growth[c]	max concn[a] (μM)
hypoxanthine	4.2 ± 0.9	NI[d]	176
guanine	35.3 ± 12	60	185
xanthine	NI[d]	NI[d]	130
2-thioxanthine	NI[d]	NI[d]	125
6-mercaptopurine	1.3 ± 0.3	2	126
6-thioguanine	21.3 ± 6	10	161
6-thioxanthine	NI[d]	NI[d]	120
6-chloroguanine	67 ± 36	10	500
8-azahypoxanthine	90 ± 11	38	136
8-azaguanine	6.6 ± 1.9	18	160
allopurinol	≥150	weak or none	155

[a] The maximum concentration of purine base analog added to the cell culture. [b] IC_{50} value for the inhibition by the base analog of the uptake of [^{3}H]hypoxanthine into parasite cells. These measurements were done in triplicate (see Experimental Section). [c] IC_{50} values for the inhibition of cell growth by the base analog, determined by counting the number of viable parasite cells. [d] No inhibition detected.

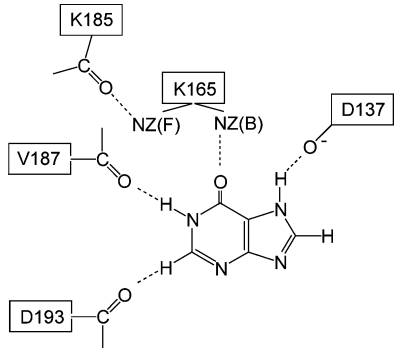


Figure 3. Interactions of the purine base with the amino acid side chains or the backbone carbonyl atoms of human HGPRT. This figure is based on the published crystal structures.[18,20−22] NZ(F) and NZ(B) indicate the different orientations of the NZ atom of the K165 side chain in the absence (F) and presence (B) of the base. On the binding of the base, the side chain of D137 is locked into position, while in the absence of base, it is mobile. These different orientations are not shown in the diagram.

repelled from the active site by electrostatic interactions with the main chain carbonyl oxygen atoms of V187 and D193.[20] Comparison of the structures of *P. falciparum* HGXPRT in complex with immucillinHP.PP_i.Mg^{2+} and human HGPRT in complex with immucillinGP.PP_i.Mg^{2+} shows that the two carbonyl oxygen atoms of V198 and D204 of the parasite enzyme are in the same positions with respect to the purine ring as V187 and D193 in the human enzyme.[17,18] There are no structures of *P. falciparum* HGXPRT with a purine ring containing a 2-exocyclic group. Thus, it is not obvious why

xanthine binds to *P. falciparum* HGXPRT, albeit weakly, but not to human HGPRT. One possible explanation is that *P. falciparum* HGXPRT possesses the ability to flex to accommodate the 2-exocyclic oxygen. Alternatively, xanthine may bind in a slightly different orientation from guanine or hypoxanthine such that catalysis is efficient (increased k_{cat}) but that K_m is greatly increased (Tables 2 and 4). Structures of the two enzymes with *P*Rib-*PP*.Mg^{2+} bound in the active site may provide an explanation for the differences in the enzymes' ability to use xanthine, since it is this complex that binds the purine base.

As expected, both hypoxanthine and guanine inhibited the incorporation of labeled hypoxanthine into the nucleic acids of the parasite (Table 5). This inhibition is due to competition between the unlabeled base and the radiolabeled hypoxanthine (and their subsequent product nucleotides). The IC_{50} values for guanine and hypoxanthine were 35 μM and 4 μM, respectively. The reason for this 10-fold difference is unknown but presumably lies in metabolic differences between hypoxanthine and guanine. The addition of guanine resulted in inhibition of parasite growth (Table 5). The higher concentration of GMP produced by the addition of exogenous guanine may have resulted in the inhibition of another critical enzyme in the pathway such as GMP synthase or adenylosuccinate synthetase and/or may have caused a purine nucleotide imbalance.

Xanthine did not inhibit the uptake of radiolabeled hypoxanthine into the parasite and did not inhibit the growth of the parasite in cell culture. The maximum concentration of xanthine used in the cell culture studies was 100 μM. At this concentration, xanthine would be converted only slowly to XMP (K_m = 189 μM; $k_{cat}/K_m = 1.7 \times 10^4$ $M^{-1}s^{-1}$; Tables 2 and 3). XMP in turn would be converted to GMP by GMP synthase, so that the only likely result of adding exogenous xanthine would be to slightly increase the GMP concentration.

2-Thioxanthine was not a substrate for either the *Plasmodium* enzyme or the human enzyme (Table 2) nor was it a competitive inhibitor. A possible reason for this is that greater repulsion would occur between the exocyclic thiol group and the backbone carbonyl groups than for xanthine, because the sulfur atom has a larger atomic radius than oxygen. As expected, 2-thioxanthine had no effect on the growth of the parasite in cell culture and did not inhibit uptake of [^{3}H]hypoxanthine (Table 5).

In summary, bases with a proton or an amino group in the 2-position of the purine ring were found to be substrates for the human enzyme, whereas bases with an electronegative group (oxygen or sulfur) in this position were not substrates. Bases with an amino group, a proton, or an oxo group, but not a thiol group, were substrates for *P. falciparum* HGXPRT. For these substrates, the K_m increased in the order, $-H < -NH_2 < = O$.

Substitutions at the 6-Position. Adenine, with an amino group at the 6-position of the purine ring, is not a substrate for either the *P. falciparum* or human enzymes.[3] Even at 10 times the concentration of human HGPRT used in the routine assay with guanine as substrate, no change in absorbance was observed at 256 nm, the wavelength used to measure APRT activity.[26] Krenitsky et al.[27] measured a K_i for adenine using human HGPRT of 2.3 mM. In our study, based on a calculation using the concentrations of both substrates in the assay, the K_i had to be $\geq$1 mM, confirming that adenine is an extremely weak competitive inhibitor. The structural (Figure 3) and kinetic data suggest that the exocyclic amino group is sterically blocked from forming a hydrogen bond with the invariant lysine residue (K176 in *P. falciparum* HGXPRT and K165 in human HGPRT). A lysine residue is completely conserved in this position in the amino acid sequence of all 6-oxopurine PRTases studied. The inability to form this bond appears to be sufficient to abolish adenine binding.[20]

The k_{cat} and K_m values for 6-mercaptopurine and 6-thioguanine as substrates for *P. falciparum* HGXPRT and human HGPRT were found to be similar to those for the corresponding 6-oxopurine bases, hypoxanthine and guanine (Tables 2 and 4). Thus, substitution of the exocyclic oxygen by a mercapto group did not have a significant effect. Based on the available structures, the ϵ-amino group of K165 in human HGPRT has sufficient space to form a hydrogen bond with the larger sulfur atom (Figure 3).[21,22]

6-Mercaptopurine and 6-thioguanine were found to be effective antiparasitic agents, with measured IC_{50} values of 1.3 and 21.3 μM, respectively, for uptake of [^{3}H]hypoxanthine, and 2 and 10 μM for inhibition of cell growth (Table 5). These cell growth results are in good agreement with those reported by Queen et al.[12] Schimandle et al.[28] reported that 6-mercaptopurine and 6-thioguanine inhibited the 6-oxopurine phosphoribosyltransferases from *Plasmodium lophurae* and *Plasmodium chabaudi*. Eakin et al.[13] showed that these compounds decreased the growth of *E. coli* SΦ609 cells transformed with the gene coding for *P. falciparum* HGXPRT. They also decreased the growth of the same cells transformed with the human gene.

6-Mercaptopurine and 6-thioguanine are currently in clinical use in maintenance therapy for acute lymphoblastic leukemia.[9,29,30] They are considered to be cytoxic because of their incorporation into DNA.[30] 6-Mercaptopurine has also been used in treatment of Crohn's disease.[31] Adverse side effects occurred in only 10% of the patients and these were reversible. The observation that these 6-thio analogs are good substrates for human HGPRT (Tables 2−4) is consistent with their toxicity to human cells. Because its human toxicity has been extensively studied and therapeutic doses determined, 6-mercaptopurine could possibly be used as an antimalarial drug in life-threatening infections where current treatments are ineffective.

Like xanthine, 6-thioxanthine is a substrate for *P. falciparum* HGXPRT but not for the human enzyme (Table 2). At 120 μM, 6-thioxanthine had no effect on the growth of the parasite (Table 3). This finding is consistent with the relative k_{cat}/K_m values for 6-mercaptopurine (2×10^7 $M^{-1}s^{-1}$), 6-thioguanine (1×10^6 $M^{-1}s^{-1}$), and 6-thioxanthine (5×10^3 $M^{-1}s^{-1}$).

6-Chloroguanine was found to be a substrate for *P. falciparum* HGXPRT (Table 2), with a k_{cat} value similar to guanine. The K_m, however, was 200 times higher than for guanine and 230 times higher than for 6-thioguanine. Thus, the effect of this substitution was to decrease binding affinity. Although the chloro atom is electronegative and would be predicted to form a hydrogen bond to the K176 side chain in the active site, the geometry of the purine ring in 6-chloroguanine is subtly different from that of guanine, perhaps explaining the high K_m. 6-Chloroguanine was an exceedingly poor substrate for human HGPRT. The estimate of k_{cat}/K_m was $\leq 1.3 \times 10^2$ $M^{-1}s^{-1}$ (Table 3), compared with 7×10^3 $M^{-1}s^{-1}$ for the parasite enzyme, giving a specificity ratio of $\geq$54 in favor of the parasite. No inhibition by 6-chloroguanine of human HGPRT acting on guanine could be detected, leading to an estimate of K_i of $\geq$130 μM.

6-Chloroguanine inhibited [^{3}H]hypoxanthine uptake into parasites (IC_{50} = 67 μM) and cell growth (IC_{50} = 10 μM; Figure 2). The difference between these IC_{50} values is not unexpected, because the cell growth assay is affected by the toxicity of the base analog after incorporation into DNA (or RNA), whereas the hypoxanthine incorporation may not be affected to the same extent.

In summary, bases with a chloro atom or a mercapto group in the 6-position were effective in inhibiting parasite growth in vitro. Whereas the 6-mercapto derivatives were substrates for both human and parasite enzymes, 6-chloroguanine was a much better substrate for the parasite enzyme, suggesting that it may be less toxic to human cells than to the parasite.

2-Hydroxypurine and 2-aminopurine have only a proton in the 6-position and are the 6-deoxo analogs of xanthine and guanine, respectively. Consistent with the need for an electronegative substituent at position 6, neither was a substrate (by HPLC; Table 2) nor a competitive inhibitor for either enzyme. Based on the calculation for a competitive inhibitor and using the concentrations of guanine and either of these two analogs in the spectrophotometric assay, the K_i for these bases would have to be greater than 100 μM. As expected, 2-hydroxypurine had no effect on the uptake of labeled hypoxanthine or parasite growth. In contrast to these results, these two compounds were reported to be competitive inhibitors of *Toxoplasma gondii* 6-oxopurine PRTase, with K_i values of 70 and 60 μM, respectively.[32] These findings emphasize the subtle differences between the active sites of the 6-oxopurine PRTases across the different species. For the *Toxoplasma gondii* enzyme, the hydrogen bond between the 6-oxo group and the invariant lysine residue appears not to be essential for binding.

Substitutions at the 8-Position. Both 8-azaguanine and 8-azahypoxanthine were substrates for *P. falciparum* HGXPRT, with much higher k_{cat} values than for the corresponding naturally occurring bases, guanine and hypoxanthine (Table 2). Although the K_m values are higher, these two substrates still bind with reasonable affinity, having K_m values of 12.6 and 25.6 μM, respectively (Table 2). These two analogs were very poor substrates for the human enzyme, however, having k_{cat}/K_m values of 1.1×10^3 $M^{-1}s^{-1}$ (8-azahypoxanthine) and 5×10^3 $M^{-1}s^{-1}$ (8-azaguanine; Table 3). The specificity ratios in favor of *P. falciparum* HGXPRT are 336 and 80 for 8-azahypoxanthine and 8-azaguanine, respectively (Table 3). The published crystal structures of the two enzymes in complex with a transition state analog show that the only amino acid side chain or backbone group in close proximity to, and thus able to interact with, atoms in the purine imidazole ring is the side chain carboxyl of the invariant aspartic acid residue (D137 in human and D148 in *P. falciparum*; Figure 3). Thus, there appears to be no obvious structural reason for the large difference in catalytic efficiency between these enzymes for the 8-aza derivatives. The structural information available also cannot explain the large increases in the K_m values caused by the substitution of a carbon by a nitrogen atom (Tables 2 and 4). It is proposed that the *Plasmodium* enzyme is more flexible such that it can cope better than the human enzyme with variations in the structure of the imidazole component of the purine ring.

8-Azaguanine and 8-azahypoxanthine were more efficient substrates for *P. falciparum* HGXPRT at pH 7.4 than at pH 8.5. The k_{cat} values were higher, and the $K_{m(app)}$ values were lower. Guanine and hypoxanthine also had lower K_m values at pH 7.4, but the k_{cat} values were also lower. 8-Azaxanthine was not a substrate for either enzyme, as assessed by HPLC. It was also not a competitive inhibitor for *P. falciparum* HGXPRT ($K_i \geq 100$ μM). Xanthine is a poor substrate for *P. falciparum* HGXPRT (k_{cat}/K_m value of 1.7×10^4 $M^{-1}cm^{-1}$), and in contrast to guanine and hypoxanthine, the N8 substitution makes 8-azaxanthine a poorer substrate than xanthine.

8-Azaguanine and 8-azahypoxanthine were both effective at inhibiting [^{3}H]hypoxanthine uptake and parasite growth, but 8-azaxanthine had no effect (Table 5). Based on these results, 8-azaguanine in particular has potential as an antimalarial lead compound. It has been reported to be toxic to human cells due to its incorporation into RNA, as opposed to the 6-thio derivatives, which are toxic because of incorporation into DNA.[9]

Allopurinol, which lacks nitrogen in the 7-position but has a nitrogen at the 8-position, was found to be a good substrate for *Plasmodium* HGXPRT, with a similar k_{cat} value to guanine and hypoxanthine though its K_m value was higher (Tables 2 and 4). The k_{cat} value for the human enzyme was 4-fold lower than for the parasite enzyme at pH 7.4 (Table 2) and 16-fold lower at pH 8.5 (Table 4). Allopurinol was a much weaker substrate for the human enzyme than hypoxanthine (k_{cat} was 34-fold lower). The specificity ratio for allopurinol in favor of the parasite enzyme was 20. Like the 8-aza derivatives, allopurinol is a poor substrate for the human enzyme compared to the naturally occurring substrates, hypoxanthine and guanine, but a good substrate for *Plasmodium* HGXPRT. Based on the 8-aza derivatives and allopurinol, changes to the purine imidazole ring have a significant effect on selectivity, the human enzyme being much less tolerant of changes in this region than the *Plasmodium* enzyme.

All of the purine base analogs are planar in the imidazole ring except for allopurinol in which the five-membered ring is not aromatic because of the tetrahedral geometry at C7. The differences in k_{cat} and K_m values between the two enzymes may reflect different binding modes. It is known that the human enzyme undergoes a number of structural changes in this region when the substrates bind, one of which is the movement of the side chain of D137, which allows the formation of a hydrogen bond between OD2 and N(7)H.[22]

Pyrazolopyrimidines such as allopurinol have been used for the treatment of Leishmaniasis (*Leishmania donovani*)[33] and Chagas' disease (*Trypanosoma cruzi*).[34] These parasites are capable of metabolizing allopurinol to pyrazolo[3,4-*d*]pyrimidine nucleotides that are incorporated into RNA and DNA where they exert their toxic effects.[35] Queen et al.[12] observed that allopurinol failed to inhibit the growth of *P. falciparum* in vitro. The data presented here confirm this result (Table 6; Figure 2) although the assay for incorporation of tritiated hypoxanthine showed an IC_{50} value of $\sim$150 μM, indicating a weak effect. This is also consistent with the work of Sarma et al.[36] who showed that the effectiveness of antimalarial treatment was slightly enhanced when allopurinol was co-administered with quinine. One suggested hypothesis to explain this is that allopurinol is an inhibitor of xanthine oxidase, thereby causing an increase in levels of hypoxanthine in red blood cells to counter any negative effects of allopurinol on parasite growth.[37] Another hypothesis is that, as allopurinol is a substrate for other enzymes, in particular purine nucleoside phosphorylase,[38] it can be converted by these alternative pathways into nontoxic compounds. Thus, though allopurinol discriminates between the human and parasite active sites, it is not useful in antimalarial chemotherapy.

Substitutions at the 7-Position. 7-Deazaguanine was not a substrate for either human HGPRT or *P. falciparum* HGXPRT (as assessed by HPLC) nor was it a competitive inhibitor. Therefore, it appears that the N7 atom is a crucial determining factor of the ability of an analog to bind in the active site. However, allopurinol, which also has a carbon in this position, is a substrate for both *P. falciparum* HGXPRT and human HGPRT, as discussed above (Tables 2 and 4). It appears that a nitrogen atom in the 8-position can compensate for the lack of a nitrogen atom in the 7-position.

Structural Considerations of the Purine Base Binding Site. Human HGPRT follows a sequential mechanism with Mg^{2+}. *P*Rib-*PP* binding first and pyrophosphate being the first product

released.[6] The explanation for the different specificities of the two enzymes toward the purine bases may lie in structural or flexibility differences between the enzyme.Mg^{2+}.*P*Rib-*PP* complexes. Such differences would then lead to different effects of structural modification of the purine base on binding (K_m effects) and catalysis (k_{cat} effects). This hypothesis may account for the large differences in both K_m and k_{cat} values between the two enzymes for the naturally occurring substrates, hypoxanthine and guanine. The k_{cat} ratio for hypoxanthine (human/*Plasmodium*) is 16, and for guanine, this ratio is 12. Thus, under physiological conditions, the human enzyme is more efficient in terms of k_{cat}. However, both these substrates bind more tightly to the parasite enzyme with the K_m ratios being 48 (hypoxanthine) and 2.2 (guanine).

The amino acid residues that form bonds with the transition state analog (immucillinHP or GP), Mg^{2+}, and pyrophosphate in the "transition state" complex are completely conserved in human HGPRT and *P. falciparum* HGXPRT.[18,19] The human enzyme undergoes a number of structural changes when the substrates bind and move through the catalytic cycle.[22] Our studies have shown that the *Plasmodium* enzyme can only be stabilized (and reactivated) by the addition of *P*Rib-*PP* and hypoxanthine. NMR (I. Brereton, unpublished) has verified that this stabilization is due only to the presence of these two substrates and that there has been no conversion to the products of the reaction. This is another difference between the two enzymes as, for the human HGPRT, *P*Rib-*PP* can only bind in the presence of divalent metal ions such as Mg^{2+} or Mn^{2+}. However, for catalysis to occur by *Plasmodium* HGXPRT, a divalent metal ion must be present (see NMR above).

Conclusions and Perspectives. The toxicity of a purine base analog to a *Plasmodium* cell is expected to depend upon its uptake by the cell; its conversion by HGXPRT and then by other enzymes to various nucleotides; and to the toxicity of these nucleotides. Incorporation of foreign nucleotides into DNA would result in malfunction and cell death. Purine base analogs are small, uncharged, and relatively nonpolar molecules that may be able to cross cell membranes both spontaneously and/or with the assistance of transporters.[39] Such transporters have been characterized in several protozoan parasites.[39−42]

These studies have found three base analogs, 6-choroguanine, 8-azaguanine, and 8-azahypoxanthine, which are highly selective as well as effective substrates for *P. falciparum* HGXPRT compared with human HGPRT. They also inhibit the growth of *P. falciparum* cells in erythrocytes *in vitro* at micromolar concentrations. Strong discrimination in base specificity between the human HGPRT and *P. falciparum* HGXPRT is achieved by: (i) the presence of an exocylic oxo group at the 2-position; (ii) the presence of a chloro atom in the 6-position; and (iii) replacement of the carbon atom with a nitrogen in the 8-position.

Experimental Section

Kinetic Studies. Recombinant mutant human HGPRT and *P. falciparum* HGXPRT were expressed and purified to homogeneity as previously described.[11] These studies used the triple mutant C22A-C105A-C205A form of human HGPRT, which is highly resistant to oxidation but has kinetic and physical properties identical with those of the wild-type recombinant or erythrocyte HGPRT.[11] Human HGPRT is stored in 0.05 M Tris-HCl, 0.01 M $MgCl_2$, 1 mM DTT, pH 7.4, −70 °C. *P. falciparum* HGXPRT rapidly loses activity except in the presence of 0.01 M phosphate, pH 6.8, 60 μM hypoxanthine, 700 μM *P*Rib-*PP* and 1 mM DTT.[11] The purified and totally activated enzyme can be stored at −70 °C with the addition of 10% glycerol for at least 6 months without loss of activity. For kinetic studies, small aliquots are removed and kept at 5 °C. The activity toward guanine is measured at the commencement of each series of experiments to ensure that the enzyme retains full activity.

Table 6. Molar Absorption Coefficients ($\Delta\epsilon$) for the Conversion of Purine Base Analogs to Their Corresponding Nucleoside Monophosphates

base	λ (nm)	$\Delta\epsilon$ at pH 7.4 ($M^{-1}cm^{-1}$)	$\Delta\epsilon$ at pH 8.5 ($M^{-1}cm^{-1}$)
guanine	257.5	5816 (5817)[a]	5814[a]
6-mercaptopurine[b]	300; 243	3284; 1516	3758; 1270
6-chloroguanine	254	3172	ND[c]
6-thioguanine	355	4310	2162
6-thioxanthine[b]	355; 266	3749; 2399	ND[c]
8-azahypoxanthine	281	2666	6000
8-azaguanine	258	6434	3330
allopurinol	273	1036	3041

[a] Determined by spectral analysis of guanine and GMP. [b] The $\Delta\epsilon$ values for these two bases have been determined at two wavelengths. [c] ND = not determined.

The base analogs (substrates) were separated from their respective nucleoside monophosphates (products) by reverse-phase HPLC.[23] Because human HGPRT and *P. falciparum* HGXPRT exhibit different k_{cat} values for hypoxanthine, the protein concentrations in the assay were adjusted to give the same activity toward hypoxanthine. Kinetic constants, k_{cat} and $K_{m(app)}$ were measured in a continuous spectrophotometric assay at pH 7.4 (0.1 M Tris-HCl, 12 mM $MgCl_2$) to reflect the in vivo conditions or pH 8.5 (0.1 M Tris-HCl, 110 mM $MgCl_2$), where the enzymes are maximally active.[11] The reaction for each base was followed at the wavelength given in Table 6. The $K_{m(app)}$ values for these bases were measured under saturating conditions of *P*Rib-*PP* (0.9−1 mM).[11] The maximum catalytic efficiencies (k_{cat}/K_m) for purine bases were calculated when no change in the absorbance could be detected over 30 min (0−0.05 absorbance range). It was estimated that the limit of the sensitivity of the assay was such that an absorbance change of 0.004 absorbance units over 30 min would be observable, giving a minimum observable value for the initial velocity (v). The equation, $k_{cat}/K_m = v/[S]_0[E]_0$ (assuming K_m [dmt][S]$_0$), then gives the maximum possible value. The $\Delta\epsilon$ values (Table 6) for the conversion of purine base to the reaction product, the corresponding nucleoside monophosphate, were determined spectrophotometrically by following the enzymatic reaction to completion in the assay buffers at pH 7.4 and 8.5. For guanine, the $\Delta\epsilon$ obtained in this way was in excellent agreement with that obtained by running spectra of guanine and GMP (Table 6). The $\Delta\epsilon$ values for the conversion of hypoxanthine to IMP and xanthine to XMP were determined by comparison of the spectra of the base and the nucleotide product of the reaction.[11] These values were 2439 $M^{-1}cm^{-1}$ (pH 7.4 and pH 8.5) for hypoxanthine and, for xanthine, 4685 (pH 7.4) and 3794 $M^{-1}cm^{-1}$ (pH 8.5).

In Vitro Inhibition of *P. falciparum* Growth by Purine Base Analogs. Laboratory-adapted *Plasmodium falciparum* isolate 3D7 was maintained in erythrocyte culture according to the method described by Hamzah et al.[43] The in vitro efficacy of each purine base analog against 3D7 was determined by assessing [^{3}H]-hypoxanthine incorporation into parasites over a 48 h period.[44] Triplicate cultures were exposed to a range of purine base concentrations and 0.5 μCi/well [^{3}H]hypoxanthine. These cultures were then incubated for 48 h, and [^{3}H]hypoxanthine incorporation into the nucleic acids was measured. Parallel experiments without [^{3}H]hypoxanthine were performed to determine the effect of each purine base on parasitemia. These cultures were also incubated for 48 h. Blood smears were then prepared from each culture and stained with Giemsa.[45] Smears were examined under light microscopy, and the level of parasitemia was determined.

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