

HYPOXANTHINE METABOLISM BY HUMAN MALARIA INFECTED
ERYTHROCYTES : FOCUS FOR THE DESIGN OF
NEW ANTIMALARIAL DRUGS

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OBJECTIVE : To examine the role of hypoxanthine in malaria parasite (*P. falciparum*) purine nucleotide metabolism through the use of specific nucleotide inhibitors.

BACKGROUND : It is the blood stage, or intraerythrocytic (IE) form, of the malaria parasite that produces the major clinical and pathological features of malaria infection. Recently, we began the systematic study of purine metabolism of the IE form of the major human malaria pathogen, *Plasmodium falciparum*, using newly developed techniques for continuous erythrocyte culture (1, 2) and novel chromatographic procedures that permit comprehensive quantitative examination of purine intermediary pathways (3). Purines are essential to the rapidly proliferating malaria parasite for nucleic acid synthesis, energy metabolism and as enzyme cofactors (4).

In this study we report on the metabolism of hypoxanthine by parasitized erythrocytes *in vitro*. Emphasis was placed on identified differences in host and parasite purine pathways (5) and on how through use of specific inhibitors a rational approach to the selective destruction of the IE malaria parasite can be achieved.

METHODS : The FCR-3 strain of *P. falciparum* was maintained in continuous erythrocyte culture (5) (6). Culture flasks containing 5 ml of a 6% erythrocyte suspension in RPMI 1640 media with 10% serum were seeded with parasitized erythrocytes (PRBC) from a maintenance culture to give an initial parasitemia of 0.5% and incubated at 37°C following purging with a gas mixture (CO₂ 5% O₂ 5% N₂ 90%). These cultures produced a 10-fold increase in parasitemia over 48 hours.

Biochemical studies were done as follows. The contents of 48 hour growth cultures were pooled and aliquots transferred to individual flasks to provide a uniform set of test cultures containing 3 ml of parasitized RBC suspension with an adjusted hematocrit of 12%.

Inhibitors (5×10^{-5}) and (³H) hypoxanthine (specific activity 3 Ci/mmol) were added to the test cultures (duplicates) and incubation continued as

described above for 2.5 hours. Inhibitors were added 15 minutes prior to the isotope. Control cultures (triplicates) received isotope only.

Perchloric acid (PCA) extracts were prepared on the cellular and extracellular fractions. Details of these methods have been described elsewhere (5, 6). The clear supernatant containing the acid-soluble purines obtained following PCA precipitation and centrifugation was neutralized and stored frozen (-70°C). The acid-insoluble fraction was solubilized with alkali and an aliquot counted by liquid acintillation spectrometry to measure incorporation of label into nucleic acids (6).

Purine compounds were quantitated by high-performance liquid chromatography (HPLC). These procedures permitted the simultaneous measurement of concentration and radioactivity of purine compounds separated by either reversed-phase (nucleosides and bases) or anion-exchange (nucleotides) gradient HPLC. These PHLC procedures have been described in detail (3).

Statistical comparison between control and inhibitor treated cultures was done using the Student's T-test.

RESULTS : *Incorporation of (^3H) hypoxanthine into purine nucleotides of malaria infected erythrocytes.* Previous studies in our laboratory have demonstrated the pathways for purine salvage and interconversion associated with purine nucleotide synthesis in *P. falciparum* infected erythrocytes (PRBC) (5). The malaria parasite cannot synthesis purines *de novo* (4). Hypoxanthine appears to be the preferred substrate for synthesis of both adensoine and guanosine nucleotides, although adenine and guanine can be used (5). Figure 1 shows a representative nucleotide profile for PCA extracted PRBC following incubation with (^3H) hypoxanthine. Label from hypoxanthine was incorporated first into IMP and then into both adenylates and guanylates. PRBC appear also to synthesize pyridine nucleotide (NAD) from the labelled adenylate pool. Unparasitized (control) BRC can synthesize IMP from hypoxanthine but at a slower rate (34 pmoles/min/ml RBC compared to 196 pmoles/min/ml RBC for PRBC with similar red cell mass (HCT = 12) and a 4.8% parasitemia). Control RBC ~~cannot synthesize~~ adenylates from hypoxanthine and produce only small amounts of guanylates (5, 6).

Effect of specific inhibitors on purine nucleotide synthesis. Table 1 shows the effect of various purine inhibitors on incorporation of (^3H) hypoxanthine into purine nucleotides of PRBC. Control cultures and inhibitor treated cultures were all prepared from a common pooled source of malaria infected BRC and, therefore, have the same initial parasitemia (4.8% PRBC) and red cell mass (HCT = 12).

Hadacidin (N-formyl hydroxy-aminoacetic acid), which is known to inhibit adenylosuccinate synthetase (7), blocked synthesis of adenosine nucleotides from IMP (Table 1). There was a significant decrease (83%, $p < .001$) in newly synthesized ATP and in total adenylates (ΣA). The concentration of ATP - and the level of the adenylate pool - was not decreased. This maintenance of the adenylate pool in the PRBC ~~may~~ reflect a contribution from the red cell via an

alternate source of adenine. There was also a decrease in labelled guanylate in PRBC exposed to hadacidin.

Alanosine [L-2-amino-3-(N-hydroxy, N-nitrosamino) propionic acid], another inhibitor of adenylosuccinate synthetase (7), did not appear to interfere with synthesis of adenosine nucleotides by malaria infected erythrocytes (Table 1). There was, however, an unexpected decrease in labelled GTP and total guanylates (Σ G).

Bredinin (4-carbamoyl-1-B-D-ribofuranyosyl-imidazolium-5-olate), an inhibitor of IMP dehydrogenase (8), interfered with synthesis of guanosine nucleotides from IMP by PRBC (Table 1). There was a decrease (80%, $p < .001$) in newly synthesized GTP and in Σ G. The decrease in GTP concentration was also significant (13%, $p < .005$). There was no apparent effect of bredinin on adenylates of PRBC.

Mycophenolic acid [6-(4-hydroxy-6-, ethoxy-7-methyl-3-oxo-5-pthalanyl)-4-methyl-4-hexenoic acid] has also been shown to inhibit IMP dehydrogenase (9). Mycophenolic acid effectively interfered with the synthesis of guanosine nucleotides from IMP by PRBC (Table 1). The decrease (85%) in labelled GTP and total guanylates was significant ($p < .001$). There was also a moderate ($p < .01$) decrease in the concentration of GTP and in the level of the guanylate pool. Unlike bredinin, mycophenolic acid appeared to produce a decrease in the amount of newly synthesized ATP (21%, $p < .005$).

Effect of inhibitors on nucleic acid synthesis. Table 2 shows the effect of each purine inhibitor on incorporation of (3 H) hypoxanthine into nucleic acids (acid-insoluble fraction) of malaria infected erythrocytes. Two clinically proven antimalarial drugs, chloroquine [7-chloro-4-(4'-diethylamino-methylbutylamino)-quinoline] and mefloquine [α -(2-piperidyl)-2, 8-bis (trifluoromethyl)-4quinolinemethenol hydrochloride], were included for comparison. Hadacidin, bredinin and mycophenolic acid all produced decreases in nucleic acid synthesis by PRBC as measured by incorporation of (3 H) hypoxanthine. Alanosine had no demonstrable effect.

DISCUSSION : These studies confirm the importance of hypoxanthine as a precursor for synthesis of both adenosine and guanosine nucleotides by malaria infected erythrocytes.

Hadacidin, bredinin and mycophenolic acid, each shown to effect specific purine enzymes in other types of cells acted predictably to disrupt purine nucleotide synthesis by PRBC.

The lack of a detectable effect on adenylate synthesis by alanosine may be due to an inability to form the active metabolite, L-alanosyl-AICOR which requires an active *de novo* purine pathway (10).

Each effective purine inhibitor through disruption of nucleotide synthesis interfered with malaria parasite nucleic acid synthesis and thus parasite

growth. We have shown in detailed studies with bredinin that the disruption in nucleic acid synthesis due to a specific block in guanosine nucleotide production is directly correlated with parasite killing (6). Other investigators have also established that radiolabelled nucleic acid precursors are reliable indicators of malaria parasite growth (7, 8).

These studies identify biochemical targets associated with the malaria parasites' metabolism of hypoxanthine which are essential for synthesis of purine nucleotides and nucleic acids (Figure 2). Specific focus on these unique features of parasite purine metabolism and the classes of inhibitors effective against them could lead to the design of new antimalarial drugs.

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Table 1. Effect of inhibitors on incorporation of (³H) hypoxanthine into purine nucleotides of human intraerythrocytic malaria parasites.

Condition	ATP	gtp	IMP	A ^f	G ^g	A/G
Control	nmoles ^a	93	211	1783	109	16
	radioactivity ^b	31 ^d	5	6620	524	13
	specific activity ^c	1562	38170	181		
Hadacidin	nmoles	89	342	1701	106	16
	radioactivity	1493 ^e	42514	1448	166	9
	specific activity	848	124			
Alanosine	nmoles	99	256	1827	114	16
	radioactivity	1606	31460	6282	312	20
	specific activity	4652	123			
Bredinin	nmoles	81	301	1846	93	20
	radioactivity	1603	39772	7062	70	101
	specific activity	5474	132			
Mycophenolic acid	nmoles	82	235	1969	96	21
	radioactivity	1712	38990	6162	80	77
	specific activity	3840	166			

a Nanomoles per ml RBC.

b Integrated radioactive counts (area) per chromatography peak.

c Specific activity = radioactivity per nmole.

d Values are mean ± SEM, n = 3.

e Values are averages of duplicate experiments in which the average deviation of individual measurement from the mean was less than 8 percent.

f ΣA (total adenylates) = AMP + ADP + ATP + NAD.

g ΣG (total guanylates) = GMP + GDP + GTP.

Table 2. Effect of various inhibitors on incorporation of (³H) hypoxanthine into nucleic acids of malaria infected erythrocytes.

Inhibitor ^a	Inhibition (%)
Control ^b	-
Hadacidin	73
Alanosine	0
Bredinin	43
Mycophenolic Acid	67
Chloroquine	79
Mefloquine	76

^a All inhibitors (5 x 10⁻⁵ M)

^b Control (11496 dpm, n = 3)

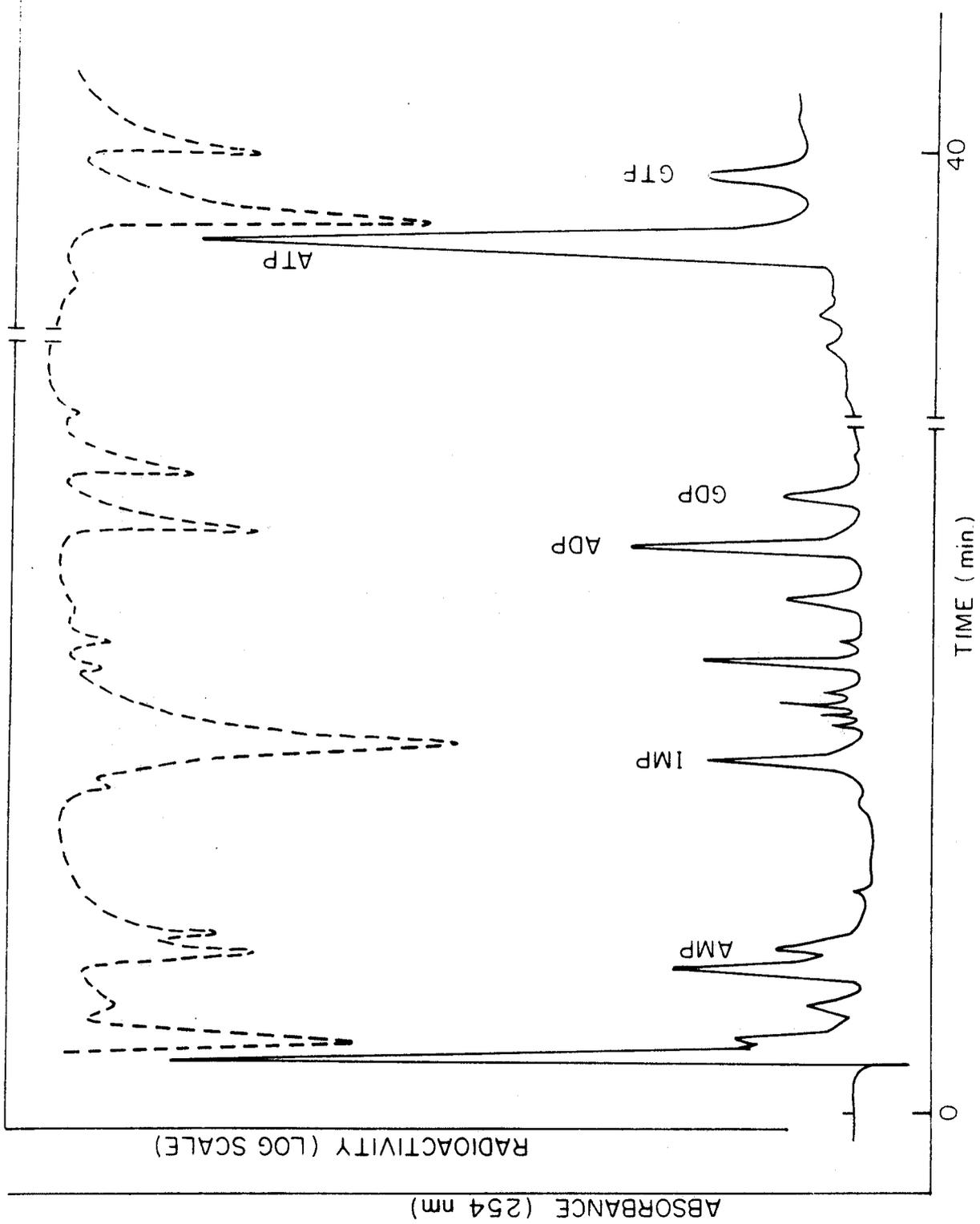


Figure 1.

