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In Vitro Antimalarial Activity of Nucleic Acid Precursor Analogues in the Simian Malaria *Plasmodium knowlesi*[1]

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Analogues of nucleic acid precursors were screened for antimalarial activity in *Plasmodium knowlesi* by using an in vitro culture system. Activity was assessed by the degree of inhibition of incorporation of L-[*methyl*-^{14}C]methionine into protein and of [8-^{14}C]adenosine and [6-^{14}C]orotic acid into ribonucleic acid and deoxyribonucleic acid. The incorporation of adenosine or orotic acid was effectively inhibited by many of the compounds, including 3′ analogues of purine nucleosides, many of the 6-position analogues of purine bases and nucleosides, and 5-position analogues of orotic acid. Only a few compounds inhibited methionine incorporation into protein, and in each instance adenosine or orotic acid incorporation also was inhibited. Some compounds inhibited adenosine or orotic acid incorporation into both ribonucleic acid and deoxyribonucleic, whereas other analogues inhibited incorporation into one nucleic acid only. The qualitative and quantitative differences suggest that this experimental system may be appropriate for investigation of metabolic pathways of the malaria parasite, as well as for demonstration of antimalarial activity of candidate antimalaria drugs.

The emergence of drug-resistant strains of *Plasmodium falciparum* malaria has increased the need for chemicals with antimalarial activity (11). New compounds are synthesized by workers following leads obtained primarily from in vivo studies in which host factors such as absorption, metabolic alteration, and toxicity may obscure the relationship between the parasite and the chemical under investigation. Studies with free parasites have not been feasible because efforts to prepare suitable isolates generally have been unsuccessful (3), although the investigations of Trager et al. (15) and the recently described techniques of Prior and Kreier (10) may resolve this problem. An intermediate approach, in which parasites remain within red blood cells but are removed from the host and studies with in vitro culture techniques, has been useful in studies of activities of several classes of drugs (2, 6). In the present study this system employing the simian malaria *Plasmodium knowlesi* has been applied to the measurement of antimalarial activity of potential nucleic acid precursor antimetabolite analogues (orotic acid, purine bases, and purine nucleosides). The parameters measured were the incorporations of L-[*methyl*-^{14}C]methionine into protein and [8-^{14}C]adenosine and [6-^{14}C]-orotic acid into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

MATERIALS AND METHODS

The technique has been described in detail previously (2, 6) but is described generally here.

Incubation procedure: P. knowlesi. Parasitized blood was obtained from infected rhesus monkeys (*Macaca mulata*). Incubation was done in minimal essential medium (12.6 ml per culture tube) containing no folic acid and with added fetal bovine serum. Compounds being tested for antimalarial activity were added in 1 ml of aqueous solution to give a final concentration of 10 mg/liter. Each sample tube contained either 0.65 μCi of L-[*methyl*-^{14}C]methionine (10.5 mCi/mmol), 0.25 μCi of [8-^{14}C]adenosine (45.5 mCi/mmol), or [6-^{14}C]orotic acid (56.6 mCi/mmol) in one of two preparations: 5 μCi plus 1 mg of unlabeled orotic acid (Sigma Chemical Co.) or, when analogues of orotic acid were studied, 2 μCi with no added unlabeled orotic acid. All labeled compounds were obtained from New England Nuclear. Triplicate samples were prepared for each combination. Blood cells (approximately 10% parasitized) were added as 0.2 ml of a 15% suspension of packed cells in the medium. Samples with uninfected blood were prepared similarly. During 20 h of incubation, the parasites matured from early trophozoite to late schizont stages. Growth was stopped by centrifugation, decanting of

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the supernatant solutions, and freezing of the remaining cell buttons.

Analytical procedure. The cells were thawed and treated with 10% trichloroacetic acid, and the resulting precipitates were separated into fractions representing RNA, DNA,, and protein by alkaline and acidic digestions. Radioactivity was measured by scintillation counting technique. Observed counts per minute were averaged for triplicate samples, and values for uninfected blood samples were subtracted. Radioactivity of samples containing test compounds was compared to that of samples containing no drug and the comparison was expressed as percentage; a value of 100% would indicate no inhibitory activity, whereas 0% would result from complete inhibition.

Materials. Compounds which were tested for antimalarial activity were received from commercial, individual, and institutional sources as indicated in Tables 1 to 3.

RESULTS

The radioactivity incorporated by the parasites in samples without any drug usually resulted in measured activities of approximately 2,500 counts per min for L-[*methyl*-^{14}C]methionine in protein. For [8-^{14}C]adenosine, the usual activities were approximately 12,000 and 4,000 counts per min in RNA and DNA, respectively. For [6-^{14}C]orotic acid, the values were 600 and 400 counts per min, and in orotic acid analogue studies they were 1,000 and 800 counts per min in RNA and DNA, respectively. Compounds were arbitrarily separated into groups based on relative effectiveness. Those compounds that allowed less than 20% of the control incorporation of any of the precursors into protein, DNA, or RNA were considered to be effective as antimalarial drugs in this system and are presented in Table 1. A second group of less effective compounds with values of 20 to 50% of control incorporation is presented in Table 2. Compounds which had values above 50% of control were considered to be ineffective and are listed in Table 3 for purposes of comparison.

The results allow grouping of some compounds on the basis of structure and effectiveness. Analogues of adenosine with modification involving the 3′ position were very effective. Cordycepin (3′-deoxyadenosine) inhibited precursor incorporation into both DNA and RNA, confirming the report of Trigg et al. (16). Xylosyladenine (the 3′ structural analogue) and 3′-amino-3′-deoxyadenosine were perhaps even more effective than cordycepin as reflected by the decreased methionine incorporation into protein. Arabinosyladenine, the 2′ structural analogue, did not have appreciable activity in this system (Table 2), although activity against the rodent malaria *P. berghei* has been reported (5). 5′-Deoxyadenosine was effective in respect to adenosine incorporation, but other 5′ analogues exhibited only moderate inhibitory activity. These included the stereoisomer (lyxosyladenine), the 5′-carboxylate (adenineribofuranosyluronic acid), and the 5′-chloro and 5′-tosyl analogues.

Analogues of purine bases and ribosides with a sulfhydryl group at the 6 position also constituted a group of effective compounds. The several compounds with a substituted mercapto group at the 6 position were less effective.

Most of the compounds tested which had other constituents at the 6 position were effective against at least one parameter. Compounds of this type which were not effective at the 20% incorporation level included 6-chloroguanosine and all of the 6-dimethylamino analogues except puromycin. The effectiveness of puromycin in this system, thus, would seem not to depend on the substitutent at its 6 position, but rather to derive from the modification of the 3′ position.

Addition of substituents at the 8 position of adenosine and guanosine did not result in inhibition. The three 1,3-dimethyl-2-oxy-6-thio-8-mercaptopurine analogues were effective, but this may be attributable to the 6-thio rather than the different 8-mercapto substituents. Alterations within the base ring structure gave inconclusive results: 8-azaguanine was effective but 8-azaadenine and allopurinol were not.

The orotic acid analogues tested were almost exclusively those with alterations at the 5 position. 5-Fluoroorotic acid has been reported to be an effective antimalarial drug in respect to orotic acid incorporation into DNA in vitro (6) and in this study was effective for both RNA and DNA with adenosine and orotic acid. Dihydro-5-fluoroorotic acid and 5-nitroorotic acid were effective in respect to DNA but not RNA for both adenosine and orotic acid. 5-Bromoorotic acid was effective only for orotic acid incorporation into DNA.

DISCUSSION

The purposes of present study were to test the applicability of the in vitro test system for the study of antimetabolites in the nucleic acid metabolism of malaria parasites and to screen compounds of this type for antimalarial activity. Compounds with a variety of structural features were employed, and the results indicate that the experimental system was sensitive to activities of a number of nucleic acid precursor analogues. It was beyond the scope of the

study to establish definite sites of activity, but there was encouraging evidence that further study to that end may be accomplished. The results demonstrated that more than one mode of action was accessible to drugs that were effective against the malaria parasite. Compounds exhibiting inhibition in both RNA and DNA cannot have acted in the same fashion as did compounds exhibiting inhibition in DNA but not RNA. Some compounds showed effec-

TABLE 1. *Compounds effective against P. knowlesi malaria in vitro*[a]

Analogue	Source[b]	Incorporation (%)				
		[^{14}C]methionine	[^{14}C]adenosine		[^{14}C]orotic acid	
			RNA	DNA	RNA	DNA
Purine nucleoside						
Purine riboside	17592	3	0	0	10	11
3′-Deoxyadenosine	[c]	80	2	0	0	0
3′-Amino-3′-deoxyadenosine	174270	20	0	0	1	4
Adenine xyloside	180984[d]	6	2	0	0	0
5′-Deoxyadenosine	[d]	110	17	20	60	35
1-Methyladenosine	[c]	70	25	10	70	35
N′-oxoadenosine	10369[d]	7	5	0	13	0
N^2-dimethylguanosine	[c]	80	110	18	70	20
2-Chloroadenosine	[c]	60	40	8	60	15
6-Chloropurine riboside	24883[d]	12	1	0	15	3
6-Mercaptopurine riboside	10240[d]	15	20	0	18	0
6-Mercaptopurine-2′-deoxyriboside	[c]	20	3	0	35	18
6-Mercaptoguanosine	18143[c]	50	30	15	60	4
6-Methoxypurine riboside	[c]	80	18	16	100	60
6-Methoxyguanosine	58213	100	19	10	120	35
6-Methylaminopurine riboside	[c, d]	90	25	18	80	40
Puromycin	3717	0	0	0	8	0
Formycin	[e]	25	0	0	3	4
Kinetin	[c]	60	40	10	45	8
Tubercidin	139008	1	0	0	1	0
Purine base						
6-Mercaptopurine	2785	30	5	0	6	1
6-Thioguanine	1141	20	25	3	90	6
8-Azaguanine	18676	60	80	5	110	7
Imuran	153994	18	12	1	20	2
1,3-Dimethyl-2-oxy-6-thiopurine derivatives:						
-8-(3-Hydroxy-*n*-propyl)	28344	4	0	0	1	0
-8-(2-Aminoethyl)	28342	25	18	2	9	3
-8-Acetomethyl	28365	130	0	0	17	1
Orotic acid						
5-Fluoroorotic acid	152520	25	13	0	8	1
5,6-Dihydro-5-fluoroorotic acid	[f]	35	9	0	35	0
5-Azaorotic acid	178337	70	100	12	50	5
5-Nitroorotic acid	35877	60	120	1	140	1
5-Bromoorotic acid	46579	110	140	140	110	18
4-Thioorotic acid	178939	60	60	35	39	9

[a] The compounds allowed incorporation of less than 20% of the amount of incorporation in the absence of any drug, for at least one parameter. The compounds were present at 10 mg per liter during the incubations with *P. knowlesi*-infected red blood cells. The values are averages from triplicate analyses.

[b] Numbers are Walter Reed numbers assigned to compounds obtained through U.S. Army Antimalarial Drug Program, Division of Medicinal Chemistry, Walter Reed Army Institute of Research (WRAIR).

[c] Purchased from Sigma Chemical Co., St. Louis, Mo.

[d] Obtained from R. A. Olsson, Division of Medicine, WRAIR.

[e] Generously supplied by M. Earl Balis, Sloan-Kettering Institute for Cancer Research.

[f] Prepared by B. J. Boone, Division of Biochemistry, WRAIR.

tiveness with adenosine and not with orotic acid, with the reverse seen also.

The results with methionine indicated that the success of nucleic acid precursor analogues generally was in nucleic acid metabolism and not in that of protein. Only nine of the compounds inhibited methionine incorporation into protein, and in no case was this inhibition seen without concomitant effect against nucleic acid metabolism.

Although, for purposes of comparison, the compounds were classified by activity on an arbitrary basis, it is recognized that the single concentration by weight (10 mg/liter), which is employed when drugs are screened, does not allow overall comparison of activities in a strict sense. It is appreciated that compounds with smaller molecular weights had higher molar concentrations. The orotic acid analogues had a molar concentration range of 4.3×10^{-5} to 6.4×10^{-5} M, whereas the purine bases and nucleosides had ranges of 3.4×10^{-5} to 7.4×10^{-5} M and 1.8×10^{-5} to 4.0×10^{-5} M, respectively. The ranges within the groups are reasonably narrow, and thus the arbitrary classification is probably a useful approximation of relative activity.

Modes of action of some of the compounds have been investigated in considerable detail in other systems. However, the metabolic pathways of malaria parasites have not been characterized sufficiently as to allow knowledge gained from study of antimetabolites in other organisms to be applied with confidence. The malaria parasite probably does not accomplish all of the metabolic sequences found to be sites of activity of drugs in other systems.

As a particular example, the de novo synthesis of purines has been found not to occur in rodent *P. berghei* (18) and avian *P. lophurae* malarias (19). Thus, in the malaria parasite, pseudofeedback inhibition of the purine biosynthetic pathway at the point of phosphoribosylamine production cannot be considered to be the mode of action of 6-mercaptopurine and other compounds which do have this action (as their metabolite nucleotides [14]) in other systems (13).

Malaria parasites do possess an active pyrimidine biosynthetic pathway. Exogenous orotic

TABLE 2. *Compounds less effective against P. knowlesi malaria in vitro*[a]

Analogue	Source[b]	Incorporation (%)				
		[^{14}C]methionine	[^{14}C]adenosine		[^{14}C]orotic acid	
			RNA	DNA	RNA	DNA
Purine nucleoside						
Formycin B	180149	90	80	30	130	30
Adenine lyxoside	[c]	70	20	25	50	25
6-(2-Pyridyl)-methylmercaptopurine-arabinoside	73512[d]	70	60	20	90	40
Adenine ribofuranosyl uronic acid	[c]	90	70	60	100	35
5'-Tosyladenosine	[d]	60	90	60	70	45
Adenine arabinoside	135078[c]	80	110	40	70	50
5'-Chloro-5'-deoxyadenosine	[c]	120	45	25	100	60
8-Mercaptoguanosine	[d]	80	90	40	90	35
8-Bromoadenosine	[d]	90	70	60	80	30
6-Dimethylaminopurine riboside	46876[c]	110	80	30	80	30
6-(*p*-Nitrobenzyl)-thioguanosine	[c]	110	110	100	45	50
6-Chloroguanosine	[c]	80	25	35	140	60
Orotic acid						
5-Methylorotic acid	25852	130	140	140	150	35
5-Chloroorotic acid	114872	120	120	110	45	50
5,6-Dihydroorotic acid	[e]	70	110	90	120	30

[a] Conditions were described in Table 1. These compounds allowed incorporation of 20 to 50% incorporation in the absence of any drug, for at least one parameter.

[b] Numbers are Walter Reed numbers assigned to compounds obtained through U.S. Army Antimalarial Drug Program, Division of Medicinal Chemistry, Walter Reed Army Institute of Research (WRAIR).

[c] Obtained from R. A. Olsson, Division of Medicine, WRAIR.

[d] Purchased from Sigma Chemical Co., St. Louis, Mo.

[e] Purchased from Calbiochem, La Jolla, Calif.

TABLE 3. *Compounds ineffective against P. knowlesi malaria in vitro*[a]

Analogue	Source[b]
Purine nucleoside	
2',3'-Isopropylidene adenosine	15419[c]
Psicofuranine	[c]
3'-Deoxy-3'-acetylamino-6-dimethylaminopurine riboside	75919
Puromycin aminonucleoside	[d]
1-Methylguanosine	[d]
6-Methylmercaptopurine riboside	3408[b]
7-Methylguanosine	[d]
8-(2-Carboxyethylmercapto)-adenosine	[c]
8-Dimethylaminoadenosine	180145
Purine base	
8-Azaadenine	15189[c]
9-(5-Chloro-*n*-pentyl)-adenine	123192
Allopurinol	4251
Orotic acid	
2-Thioorotic acid	440
5-Aminoorotic acid	[e]
5-Methoxyorotic acid	135808

[a] Conditions were described in Table 1. These compounds allowed more than 50% incorporation in the absence of any drug.

[b] Numbers are Walter Reed numbers assigned to compounds obtained through U.S. Army Antimalarial Drug Program, Division of Medicinal Chemistry, Walter Reed Army Institute of Research (WRAIR).

[c] Obtained from R. A. Olsson, Division of Medicine, WRAIR.

[d] Purchased from Sigma Chemical Co., St. Louis, Mo.

[e] Purchased from K & K Laboratories, Plainview, N.Y.

acid is utilized by the parasite for nucleic acid biosynthesis (9) and has been used as an experimental entry point into parasite metabolism for study of drug effects (6, 7). The conversion of orotic acid to orotidylic acid involves 5-phosphoribosyl pyrophosphate. A drug which blocked production of 5-phosphoribosyl pyrophosphate thus would have an effect against the formation of orotidylic acid, and the subsequent production of RNA and DNA would be inhibited because the parasite does not utilize exogenous pyrimidine sources other than orotic acid in this experimental system. This inhibition should be reflected by reduced incorporation of both adenosine and orotic acid into RNA and DNA, and this was observed with compounds which have been reported to inhibit 5-phosphoribosyl pyrophosphate synthesis: 6-mercaptopurine and its riboside (13), xylosyladenine (4), and 3-deoxyadenosine (cordycepin) (8, 17).

Reduced incorporation of both orotic acid and adenosine into RNA could also result from inhibition of RNA polymerase, an activity also reported for cordycepin.

Analogues of orotic acid may be metabolized to the corresponding analogues of normal nucleotides and incorporated into nucleic acids; the resulting "fraudulent" RNA or DNA may cause derangement in subsequent metabolism. This has been observed with 5-fluoroorotic acid and, in addition, its metabolite 5-fluoro-2'-deoxyuridylate has a specific site of action, inhibiting the enzyme thymidylate synthetase and, thereby, subsequent DNA production (1).

In investigations utilizing intact cell systems, permeability must be considered. The results in this study may reflect alterations in permeability brought about by some or all of the compounds; elucidation of this will require specific studies. Permeability does complicate investigation of antimetabolic activity but is itself an important area for study in the malaria parasite with respect to development of effective antimalarial drugs. The experimental system employed in this study should be appropriate for further study in this area.

Although studies in vitro do not yield information concerning such important factors in drug activity in vivo as possible limited absorption, metabolic alterations of the compound before arrival at the site of action, or toxicity to the host, these factors themselves complicate investigations of new drugs in vivo. A structural alteration might be an important innovation for establishment of antimalarial activity, but this could be masked by toxicity and be unnoticed. In this respect, study in vitro allows a clearer view of the direct effect of a compound upon the parasite.

The results obtained with these analogues of nucleic acid precursors indicate that this procedure employing incubation in vitro is applicable to study of compounds of this type for antimalarial activity and should be useful in identification of metabolic pathways utilized by the parasite, in determination of structure-activity relationships, and in prediction of structural configuration for effective new antimalarial drugs.

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