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In Vitro Susceptibilities of *Plasmodium falciparum* to Compounds Which Inhibit Nucleotide Metabolism

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A unique metabolic feature of malaria parasites is their restricted ability to synthesize nucleotides. These parasites are unable to synthesize the purine ring and must therefore obtain preformed purine bases and nucleosides from the host cell, the erythrocyte. On the other hand, pyrimidines must be synthesized de novo because of the inability of the parasites to salvage preformed pyrimidines. Thus, one would anticipate that the blockage of purine salvage or pyrimidine de novo synthesis should adversely affect parasite growth. This premise was tested in vitro with a total of 64 compounds, mostly purine and pyrimidine analogs, known to inhibit one or more steps of nucleotide synthesis. Of the 64 compounds, 22 produced a 50% inhibition of the growth of the human malaria parasite *Plasmodium falciparum* at a concentration of 50 μM or less. Inhibition of the growth of chloroquine-resistant clones of *P. falciparum* did not differ significantly from that of the growth of chloroquine-susceptible clones. Two of the compounds which effectively inhibited parasite growth, 6-mercaptopurine and 6-thioguanine, were found to be potent competitive inhibitors of a key purine-salvaging enzyme (hypoxanthine-guanine-xanthine phosphoribosyltransferase) of the parasite.

The worldwide resurgence of human malaria during the past decade (60) has been due in part to the rapid spread of *Plasmodium falciparum* strains resistant to chloroquine and other commonly used antimalarial drugs (21, 41). In view of this resurgence, there is currently an urgent need to develop and identify additional safe and effective drugs (47).

A rational approach to drug development is to identify drugs which can be directed at unique metabolic targets in the parasite. One such target is the synthesis of purine and pyrimidine nucleotides. Unlike humans, malaria parasites cannot make purines de novo and must therefore obtain purine bases and nucleosides from the erythrocyte host cell and then metabolize these to nucleotides by salvage pathways (54). Another metabolic distinction between these parasites and humans is that *P. falciparum* relies exclusively on pyrimidine synthesis de novo because of its inability to salvage preformed pyrimidines (54). Thus, drugs capable of blocking either purine salvage or pyrimidine de novo synthesis would be expected to show strong antimalarial activity.

In the present study, we measured the in vitro antimalarial activity of 64 compounds known to inhibit one or more aspects of nucleotide or nucleic acid metabolism. A number of these compounds were also tested for their ability to inhibit a key purine-salvaging enzyme (43) of the parasite.

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MATERIALS AND METHODS

Materials. [8-^{3}H]hypoxanthine, [8-^{14}C]guanine, and [8-^{14}C]xanthine were purchased from ICN Biochemicals. The 64 compounds to be tested for antimalarial activity were obtained from the following sources. Thiopurinol, thiopurinol riboside, acyclovir, oxipurinol, and 3′-azido-3′-deoxythymidine were supplied by Burroughs Wellcome Co., Research Triangle Park, N.C. Tiazofurin, toyocamycin, sangivamycin, decoyinine, mycophenolic acid, hadacidin, 8-aminoguanosine, acivicin, 2-fluoroadenine arabinoside, azaserine, 6-diazo-5-oxo-norleucine, *N*-(phosphono-acetyl) aspartic acid, pyrazofurin, dichloroallyl lawsone, L-alanosine, 5-azaorotic acid, ribavirin, suramin, 2′,3′-dideoxycytidine, and 2′,3′-dideoxyadenosine were provided by the National Cancer Institute, Bethesda, Md. J. B. Hynes, University of South Carolina, Charleston, supplied N^{10}-formyl-5,8-dideazafolic acid. M. Loffler, Institut für Physiologische Chemie, Lahnberge, Federal Republic of Germany, provided 5,6-dihydro-5-azaorotic acid. HPA-23 ($NaW_{21}Sb_9O_{86} \cdot 14H_2O$) was supplied by J.-C. Chermann, Institut Pasteur, Paris, France. Warner Lambert Co., Ann Arbor, Mich., was the source of 2′-deoxycoformycin. The remaining test compounds were obtained from commercial sources.

In vitro cultivation of malaria parasites. Two strains of *P. falciparum* were used in these studies. Most of the work was done with clone NC-1 (43) of the FCB strain. FCB is a Colombian strain and is chloroquine resistant and pyrimethamine susceptible (10). Some studies used clone HB-3 of the Honduras I/CDC strain. This strain is chloroquine susceptible (20) and pyrimethamine resistant (37). The Honduras strain was cloned by W. Trager, Rockefeller University, New York, N.Y., and HB-3 was obtained from the Malaria Branch, Centers for Disease Control, Atlanta, Ga., with permission from W. Trager. In our experiments, the 50% inhibitory concentrations (ID_{50}s) of chloroquine and pyrimethamine for the FCB clone were 290 and 18 nM, respectively. The respective ID_{50}s for the Honduras clone were 27 and 8,000 nM. Malaria parasites were grown and maintained in a 2 to 3% suspension of human erythrocytes by the candle jar method (56) as previously described (43). RPMI 1640 growth medium (GIBCO Laboratories, Grand Island, N.Y.) was supplemented with 33 mg of gentamicin per liter, 367 μM hypoxanthine, 2.5 mM L-cysteine, 3 mM L-glutamine, 5% rabbit serum, and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and then buffered to pH 7.4 with 32 mM $NaHCO_3$. Hypoxanthine was

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eliminated from the growth medium 1 week prior to the start of each drug-screening experiment. Parasite cultures were maintained in a partially synchronous state by periodic fractionation with Plasmagel (39).

In vitro drug-screening studies. Parasite growth during drug-screening studies was quantitated by measuring the uptake and incorporation of [^{3}H]hypoxanthine (13). Microtiter plate wells were seeded with 239 μl of complete medium containing a 2% suspension of infected human erythrocytes (0.5% parasitemia) following the addition of a 1-μl sample of a given test compound solution. All compounds were run in triplicate at each concentration. Although no special efforts were made to use synchronous cultures, most experiments were begun with cultures in which about 50% of the erythrocytes were infected with mature trophozoites. After the addition of 0.35 μCi of [^{3}H]hypoxanthine in 10 μl of phosphate-buffered saline to each well, the covered 96-well plates were placed in a modular incubator chamber (Voss), which was then flushed for 1 min with 5% CO_2–95% air. The chamber was placed in a 37°C incubator for 3 days. At the end of the incubation period, the particulate contents of each well were collected on glass fiber filters with a semiautomated cell harvester. The filters were washed thoroughly with distilled water and counted by liquid scintillation. Work by others has shown that the uptake of [^{3}H]hypoxanthine is a reliable measure of parasite growth and development (11, 48).

All drug-screening experiments incorporated three kinds of culture conditions (in triplicate) as controls. These were (i) parasitized erythrocytes not treated with drugs, (ii) parasitized erythrocytes treated with drug solvent (dimethyl sulfoxide) only, and (iii) nonparasitized erythrocytes not treated with drugs. We consistently observed that drug solvent alone did not significantly alter the uptake of [^{3}H]hypoxanthine by parasitized erythrocytes and that nonparasitized erythrocytes took up only minute levels of [^{3}H]hypoxanthine.

The majority of the compounds were dissolved in dimethyl sulfoxide to give a stock solution of 25 mM. Compounds inadequately soluble in dimethyl sulfoxide were dissolved in growth medium without rabbit serum and then sterilized by passage through a 0.2-μm-pore filter. Compounds were initially screened at 1×10^{-4}, 5×10^{-5}, 1×10^{-5}, and 1×10^{-6} M. Those compounds which proved effective on the initial screening (i.e., at least 50% inhibition of growth at 5×10^{-5} M) were retested with an appropriate range of concentrations to determine the ID_{50}s accurately. The ID_{50} was defined as the concentration of drug which reduced [^{3}H]hypoxanthine uptake by 50% as compared with the drug solvent control. This value was obtained graphically from plots of percent uptake versus log of drug concentration. Mean ID_{50}s were derived from at least two experiments.

Synchronization of the Honduras strain. Difficulties were encountered in obtaining a consistent ID_{50} of 5-aminouracil against relatively asynchronous cultures of the Honduras strain. Accordingly, cultures were synchronized by fractionation with Plasmagel (39). This practice allowed two separate kinds of experiments to be set up. In one case, the effect of this drug on parasite development from rings to trophozoites was evaluated with a 28-h study by beginning these experiments with cultures containing about 90% ring forms at 3% parasitemia. In the other case, the effect of this drug on parasite reinvasion of host cells and development to the mature trophozoite stage was evaluated with a 48-h study. In these experiments, about 90% of the parasites (0.8% parasitemia) were initially present at the mature trophozoite stage. In this context, maximal incorporation of [^{3}H]hypoxanthine by malaria parasites occurs as mature trophozoites appear and as schizonts develop, with little if any incorporation by rings (11, 20). Thus, the 48 h-test involved two periods of incorporation as mature trophozoites underwent schizogony, reinvasion, and development to mature trophozoites. On the other hand, the 28-h test provided a single incorporation period associated with the development of rings into mature trophozoites and early schizonts. These experiments differed from the standard 3-day drug screening, in which parasites that were initially rings reinvaded and grew to trophozoites whereas those that were initially trophozoites and schizonts reinvaded twice and grew to rings. Thus, all parasites had a chance to develop from rings to trophozoites and to reinvade in the standard 3-day drug screening.

Enzyme assays and determination of inhibition constants. Experiments were done to examine the ability of those compounds which displayed significant antimalarial activity (ID_{50}, 50 μM or less) to serve as inhibitors of the major purine phosphoribosyltransferase of the parasite. This enzyme (HGX PRTase) is capable of utilizing hypoxanthine, guanine, and xanthine as substrates (43). Enzyme activity was measured with the previously described assay (43, 46), except that the final concentration of [^{3}H]hypoxanthine was reduced to 2 μM. The source of the enzyme in all the above-described experiments was the pooled peak fractions obtained following hydroxylapatite chromatography of extracts of the FCB strain (43). All test compounds were present in the assay mixture at 100 μM. The results were expressed as percent inhibition of enzyme activity.

Apparent inhibition constants were determined for 6-mercaptopurine and 6-thioguanine only. In these experiments, the standard assay was modified by reducing assay times to 10 min and running the reactions at two concentrations of the radiolabeled substrates ([^{3}H]hypoxanthine, 2 and 12 μM; [^{14}C]guanine, 2 and 8 μM; [^{14}C]xanthine, 21 and 62 μM). The concentrations of 6-mercaptopurine and 6-thioguanine ranged from 0.025 to 2 μM. Apparent inhibition constants were obtained from Dixon plots (15) of the kinetic data, with unweighted least-squares analysis.

RESULTS

Of the 64 compounds tested against the FCB strain, 22 showed significant antimalarial activity (ID_{50}, 50 μM or less). These active compounds are listed in Table 1 in decreasing order of potency together with their possible enzymatic sites of action. Although the majority of these sites were identified in mammalian systems, some of the studies referenced in Table 1 were done in parasite systems, mostly protozoan. 5-Fluoroorotic acid and sangivamycin, the most potent of the compounds tested, had ID_{50}s of 42 and 200 nM, respectively. Representative dose-response curves for these two compounds are shown in Fig. 1.

The group of 22 active compounds is composed of about equal numbers of inhibitors of purine metabolism and inhibitors of pyrimidine metabolism (Table 1). Also included in Table 1 are two known antiretroviral agents (HPA-23 and suramin). Tubercidin and cordycepin would be expected to affect both purine and pyrimidine syntheses because of their inhibition of 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase. PRPP participates in both purine salvaging and pyrimidine de novo synthesis. Similarly, 6-diazo-5-oxo-norleucine and azaserine inhibit both purine and pyrimidine syntheses.

TABLE 1. Compounds which effectively inhibited the in vitro growth of *P. falciparum* (FCB strain) and their possible enzymatic sites of action[a]

Compound	ID_{50} (μM)[b]	Site(s) of action	Reference(s)
5-Fluoroorotic acid	0.042 ± 0.015 (0.045)	Orotate PRTase	9, 38, 57
		Thymidylate synthase	7
Sangivamycin	0.20 ± 0.06 (0.058)	Adenosine kinase	33
N^{10}-Formyl-5,8-dideazafolic acid	0.51 ± 0.04	Dihydrofolate reductase	27
		Thymidylate synthase	49
Acivicin	1.1 ± 0.2	Carbamoyl phosphate synthetase II	2, 12
		CTP synthetase	12
Dichloroallyl lawsone	1.4 ± 0.4 (7.4)	Dihydroorotate dehydrogenase	4
6-Diazo-5-oxo-norleucine	3.0 ± 1.1	Carbamoyl phosphate synthetase II	8
		CTP synthetase	36
		GMP synthetase	3
HPA-23	3.6 ± 0.2	Reverse transcriptase	16
Tubercidin	3.7 ± 0.8	Adenosine kinase	31
		PRPP synthetase	26
Mycophenolic acid	4.2 ± 1.3	IMP dehydrogenase	6, 18, 59
5-Fluorouracil	5.2 ± 1.9	Thymidylate synthase	25, 45
		Orotate PRTase	38, 57
6-Mercaptopurine	6.2 ± 0.7 (8.6)	Hypoxanthine-guanine PRTase	30, 58
		Adenylosuccinate synthetase	35
		Adenylosuccinate lyase	35
Cordycepin	6.4 ± 2.4	Adenosine kinase	31
		Adenosine deaminase	1
		PRPP synthetase	26
5-Aminouracil	8.2 ± 1.8 (30)	Orotate PRTase	38
Toyocamycin	9.2 ± 2.4	Adenosine kinase	31, 33
8-Azahypoxanthine	12 ± 1	Hypoxanthine-guanine PRTase	55
6-Thioguanine	18 ± 6 (28)	Hypoxanthine-guanine PRTase	30, 58
Azaserine	20 ± 5	Carbamoyl phosphate synthetase II	8
		CTP synthetase	28
		GMP synthetase	5
Pyrazofurin	24 ± 5 (30)	Orotidylate decarboxylase	14, 51
6-Thioguanosine	24 ± 5	Adenosine deaminase	1
		Purine nucleoside phosphorylase	53
5-Fluorodeoxyuridine	34 ± 5 (48)	Thymidylate synthase	25
Adenine arabinoside	44 ± 1	Adenosine deaminase	1, 8, 50
		Adenosine kinase	42
Suramin	52 ± 17	Reverse transcriptase	34
		Glycerol phosphate oxidase	17

[a] The following compounds were ineffective: acyclovir, L-alanosine, allopurinol, 8-aminoguanosine, 8-azaadenine, 8-azadiaminopurine, 8-azaguanine, 5-azaorotic acid, 6-azauracil, 6-azauridine, 6-azauridine monophosphate, 3'-azido-3'-deoxythymidine, barbituric acid, caffeine, citrazinic acid, cytosine arabinoside, 3-deazauridine, decoyinine, 2'-deoxycoformycin, 2,6-diaminopurine, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine, 5,6-dihydro-5-azaorotic acid, 2-fluoroadenine arabinoside, flucytosine, formycin B, hadacidin, 6-(2-hydroxy-5-nitrobenzyl)-thioguanosine, isoorotic acid, 6-mercaptopurine arabinoside, *N*-(phosphono-acetyl)aspartic acid, 3-oxauracil, oxipurinol, ribavirin, theophylline, thiopurinol, thiopurinol riboside, 2-thiouracil, 6-thioxanthine, thymidine, tiazofurin, and uracil-4-acetic acid.

[b] Mean ± standard error of the mean (ID_{50} against the Honduras strain).

The ineffective compounds (lack of detectable antimalarial activity or ID_{50}s higher than 50 μM) are listed in Table 1, footnote *a*. Although exact metabolic sites of action have not been established for some of these compounds, many are known to inhibit purine or pyrimidine metabolism. In addition, several serve as antiviral agents (acyclovir, 3'-azido-3'-deoxythymidine, ribavirin, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine) and one [6-(2-hydroxy-5-nitrobenzyl)-thioguanosine] is an inhibitor of nucleoside transport in animal cells (40).

A total of 8 of the 22 active compounds were also tested against the Honduras strain, and the observed ID_{50}s are shown in parentheses in Table 1. There was essentially no difference in drug susceptibility between the FCB strain (chloroquine resistant) and the Honduras strain (chloroquine susceptible). The use of relatively synchronous cultures of the Honduras strain showed that 5-aminouracil was much more effective as an antimalarial agent when it was present during reinvasion, i.e., during the development of mature trophozoites to mature trophozoites via a reinvasion step

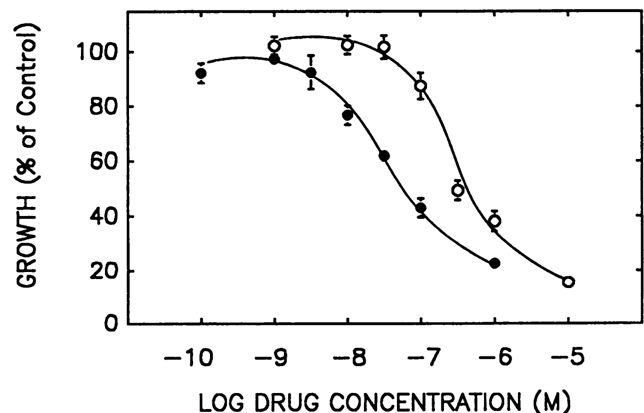

FIG. 1. Inhibition of the in vitro growth of *P. falciparum* (FCB strain) by 5-fluoroorotic acid (●) and sangivamycin (○). Each experimental point is the mean of triplicate determinations, and the error bars represent the standard error of the mean.

(48-h test). The ID_{50} under these conditions was 30 μM (Table 1). On the other hand, during a short test (28 h), the development of rings to mature trophozoites was only minimally affected by this drug, even at a concentration of 10^{-4} M (data not shown). The reason that 5-aminouracil was effective only during the 48-h test is not apparent. One can speculate, however, that 5-aminouracil enters erythrocytes more readily during the reinvasion step. Another possibility is that this pyrimidine exerts its critical effects only during the latter stages of schizogony. Neither reinvasion nor progression to late schizogony would be expected to occur in a 28-h test begun with ring-stage parasites.

The active compounds listed in Table 1 were also tested for their ability to inhibit the major purine phosphoribosyltransferase (HGX PRTase) of the parasite. Our interest in this enzyme stems from our earlier finding (43) that the enzyme may represent a potential target for chemotherapeutic attack because of its unique substrate specificity. At 100 μM, all compounds inhibited the enzyme to some extent, although most served only as weak to moderate inhibitors (data not shown). In contrast, 6-mercaptopurine and 6-thioguanine proved very effective, inhibiting the enzyme by 97 and 96%, respectively.

A more quantitative measure of the inhibitory activity of 6-mercaptopurine and 6-thioguanine was obtained by determining the apparent inhibition constants (K_is). The apparent K_is for 6-mercaptopurine with hypoxanthine, guanine, or xanthine as the purine substrate were 0.47, 0.55, and 0.46 μM, respectively. In view of the close similarity of the apparent K_is for 6-mercaptopurine obtained with the three purine substrates, the apparent K_i for 6-thioguanine was determined only with hypoxanthine. This K_i was 0.42 μM. In all cases, the inhibition was competitive with respect to the purine substrate.

DISCUSSION

Drug-screening studies. As previously noted, malaria parasites have a restricted ability to synthesize nucleotides (54). This is because purine nucleotides can only be synthesized by salvage pathways (absence of de novo synthesis), whereas pyrimidine nucleotides must be synthesized de novo (absence of salvage pathways). With this in mind, it is noteworthy that virtually all of the compounds which effectively inhibited the in vitro growth of *P. falciparum* (Table 1) have been reported to inhibit one or more enzymes of either purine salvage or pyrimidine de novo synthesis. It is evident, therefore, that nucleotide synthesis in malaria parasites represents a prime target for chemotherapeutic attack.

It should be mentioned that several of the target enzymes listed in Table 1 were previously shown by us to be present in *P. falciparum* (46). These enzymes included orotate phosphoribosyltransferase (PRTase), orotidylate decarboxylase, adenosine kinase, hypoxanthine-guanine PRTase, adenosine deaminase, and purine nucleoside phosphorylase. Other studies with *P. falciparum* established the presence of thymidylate synthase (19), dihydroorotate dehydrogenase (24), and carbamoyl phosphate synthetase (22). Indirect evidence for the presence of IMP dehydrogenase, adenylosuccinate synthetase, adenylosuccinate lyase, and GMP synthetase was provided by our finding that exogenous hypoxanthine is metabolized to guanine and adenine nucleotides by a combination of membrane and supernatant fractions of *P. falciparum* (46). On the other hand, PRPP synthetase and CTP synthetase were not detectable in either of these fractions. The apparent absence of these enzymes is puzzling because PRPP is required not only for salvaging purine bases but also for the de novo synthesis of pyrimidines and because the parasite presumably has no other means of synthesizing cytosine nucleotides.

Although Table 1 lists the reported enzymatic sites of drug action, it should be reiterated that these sites were determined for the most part in mammalian systems. Currently there is very little information regarding the actual sites of action in *P. falciparum*. Thus, it is quite possible that some of the compounds have sites of action different from or in addition to those noted in Table 1. This could account for the efficacy of the antiviral agent HPA-23 and of the antiviral and antitrypanosomal agent suramin. On the other hand, the inhibition of orotate PRTase by pyrazofurin has been confirmed in *P. falciparum* (51), as has the inhibition of hypoxanthine PRTase by 6-mercaptopurine and 6-thioguanine (this study, see below).

The compounds which proved to be effective antimalarial agents (Table 1) have not previously been tested in vitro against *P. falciparum*, with the exception of tubercidin, pyrazofurin, and 5-fluoroorotic acid. While the present studies were under way, Scott and co-workers (52) reported ID_{50}s of 0.43 to 0.54 and 6.3 to 10 μM for tubercidin and pyrazofurin, respectively, against two other isolates of the parasite. More recently, Rathod and co-workers (44) found ID_{50}s of 5.8 to 6.0 nM for 5-fluoroorotic acid against Indochina and West Africa clones. These values agree fairly closely with those determined with the FCB and Honduras clones in the present study. 5-Fluoroorotic acid, tubercidin, 6-mercaptopurine, cordycepin, 6-thioguanine, 6-thioguanosine, and adenine arabinoside were reported to have in vitro activity against the simian malaria parasite *P. knowlesi* (32). However, ID_{50}s were not determined.

Enzyme inhibition studies. Because of their inability to synthesize purines de novo, malaria parasites must meet their purine needs by obtaining preformed purines from the erythrocyte host and then metabolizing them to adenine and guanine nucleotides (54). HGX PRTase plays an essential and pivotal role in this process because it is the only parasite enzyme capable of salvaging hypoxanthine, the main purine utilized by malaria parasites (46). In view of its importance to malaria parasites, this enzyme represents a prime target for attack by purine analogs. Two such analogs, 6-mercaptopurine and 6-thioguanine (Table 1), were shown in the present study to serve as potent inhibitors of this enzyme. Interestingly, the K_is that we determined (0.42 to 0.55 μM) are about 1 order of magnitude lower than the K_is found with the corresponding human erythrocyte enzyme (30). This result indicates that HGX PRTase might be preferentially inhibited in vivo at low concentrations of either of these two analogs.

A promising approach for designing more highly selective inhibitors of HGX PRTase was suggested by our finding that this enzyme possesses a unique substrate specificity (43). The enzyme is capable of utilizing hypoxanthine and quanine as well as xanthine, whereas its counterpart in human erythrocytes uses only hypoxanthine and guanine (29). In view of this difference, it seems reasonable to believe that xanthine analogs may preferentially inhibit the malarial enzyme and may therefore represent a new class of highly effective antimalarial agents. Although the few xanthine analogs tested in the present study did not show significant antimalarial activity, it remains important to test a much wider selection of this type of analog.

In conclusion, the present study served to highlight the promise that inhibitors of nucleotide synthesis hold as po-

tentially useful antimalarial agents, especially those agents which adversely affect purine salvage or pyrimidine de novo synthesis. The potential utility of these compounds was further emphasized by the finding that chloroquine-resistant and chloroquine-susceptible clones of *P. falciparum* were equally susceptible to these inhibitors. Clearly, if an agent of this type is to prove clinically useful, it must show a high degree of selectivity for the target enzyme in the parasite. As noted in the present study, such selectivity may be achieved by exploiting differences between the target enzyme and the corresponding host enzyme. For example, it may be possible to exploit differences in tightness of drug binding or, preferably, in substrate specificity. Finally, it may be possible to target the drug into parasite-infected cells. This possibility was raised by the recent finding of Gero et al. (23) that the uptake of nucleosides by *P. falciparum*-infected erythrocytes is much less sensitive to inhibition by nitrobenzylthioinosine than is the uptake by uninfected erythrocytes. It may be feasible, therefore, to deliver cytotoxic nucleosides into parasite-infected cells with some degree of selectivity by coadministration with a nucleoside transport inhibitor such as nitrobenzylthioinosine. The merit of this chemotherapeutic approach was supported by our finding that a similar transport inhibitor [6-(2-hydroxy-5-nitrobenzyl)-thioguanosine] had little if any effect on the in vitro growth of *P. falciparum*.

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