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ANTIMALARIAL ACTIVITY OF A 4′,5′-UNSATURATED 5′-FLUOROADENOSINE MECHANISM-BASED INHIBITOR OF *S*-ADENOSYL-L-HOMOCYSTEINE HYDROLASE

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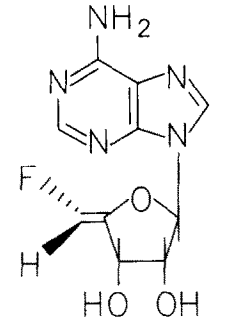
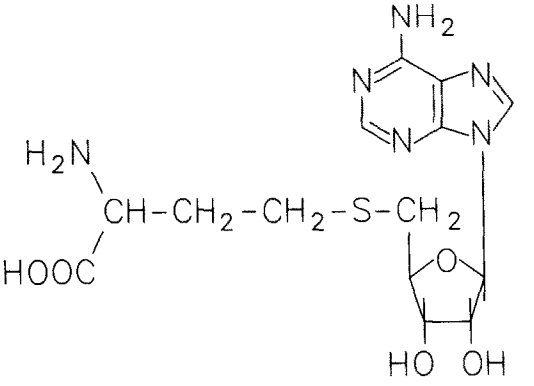
Abstract—A 4′,5′-unsaturated 5′-fluoroadenosine inhibitor of *S*-adenosyl-L-homocysteine hydrolase (SAH hydrolase; EC 3.3.1.1), MDL 28842, was found to inhibit markedly the growth of *Plasmodium falciparum in vitro* and *Plasmodium berghei* in mice. Inhibition of *P. berghei* growth was associated with a large increase in the concentration of *S*-adenosyl-L-homocysteine (SAH) in the erythrocytes of the mice treated with MDL 28842. This increase in SAH was due apparently to inhibition of the mouse erythrocyte SAH hydrolase activity, because SAH hydrolase activity was undetectable in either *P. berghei* or *P. falciparum* isolated from infected erythrocytes, although enzyme activity was readily detected in mouse erythrocyte extracts. Therefore, MDL 28842 probably inhibits plasmodial growth indirectly by adversely changing the milieu of the host erythrocyte. SAH hydrolase represents a worthwhile target for the future development of potent inhibitors for the chemotherapy of malaria.

New agents for the chemotherapy of malaria, which remains a leading cause of death worldwide, are urgently needed because of the spreading resistance of *Plasmodium falciparum* to the currently available drugs such as chloroquine [1]. One recent approach to the development of new antimalarial drugs involves the inhibition of transmethylation reactions in the malaria parasite with various adenosine analogs [2, 3]. Many of these compounds are thought to exert their inhibition of transmethylation indirectly by way of inhibition of *S*-adenosyl-L-homocysteine hydrolase (SAH hydrolase; EC 3.3.1.1). Inhibition of this enzyme leads to a marked increase in the intracellular levels of *S*-adenosyl-L-homocysteine (SAH), which in turn is a potent inhibitor of transmethylation [4]. In fact, a recent study showed that the antimalarial activity of neplanocin A, a potent inactivator of SAH hydrolase, is correlated with perturbations in the intracellular levels of SAH [5].

In the present study, we have examined the effects of a novel 4′,5′-unsaturated 5′-fluoroadenosine mechanism-based inhibitor of SAH hydrolase (MDL 28842; Fig. 1) [6] on the growth of malaria parasites *in vitro* and *in vivo*, the changes in SAH levels in infected and uninfected erythrocytes, and the kinetics of inhibition of SAH hydrolase by the inhibitor.



MDL 28,842



S-ADENOSYL-L-HOMOCYSTEINE

Fig. 1. Chemical structures of MDL 28842 and *S*-adenosyl-L-homocysteine.

METHODS

Culture of P. falciparum *and* in vitro *drug testing.* *P. falciparum* (clone D-6 of the Sierra Leone strain [7]) was maintained in continuous culture in human erythrocytes as described [8] using the methods of Trager and Jensen [9]. Drug testing *in vitro* was

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carried out exactly as described previously [8] using standard procedures [10]. In 96-well microtiter cultures, *P. falciparum* was at a starting parasitemia of approximately 0.5% in a 1% suspension of erythrocytes in a total volume of 0.2 mL. The SAH hydrolase inhibitor was dissolved in culture medium and then was filter-sterilized. [^{3}H]Hypoxanthine (1 μCi per well) was added 24 hr after initiation of cultures, and the incorporation of [^{3}H]hypoxanthine into RNA and DNA was measured by harvesting the organisms onto glass fiber filters 18 hr later using an automatic cell harvester. The radioactivity on the filters was measured subsequently by liquid scintillation counting.

Plasmodium berghei *infections*. A *P. berghei* model infection [11] was used to study the blood schizonticidal activity of the SAH hydrolase inhibitor. Outbred CD-1 mice (Charles River Breeding Laboratories) weighing 20–22 g at the time of infection were infected by intravenous injection of 10^7 parasitized erythrocytes from a mouse with a rising parasitemia. The NK65 strain of *P. berghei* was used for these studies.

The SAH hydrolase inhibitor, MDL 28842, was administered by intraperitoneal injection in 0.2 mL of 50% dimethyl sulfoxide three times per day (8:30 a.m., 12:30 p.m. and 4:30 p.m.) for 3 days. The drug was given in divided doses because no pharmacokinetic data were available to suggest that a single daily administration would ensure that the drug was present in plasma long enough at sufficient concentrations to inhibit *P. berghei* growth. Additionally, preliminary experiments showed that the divided doses were tolerated better than single high doses. Approximately 18 hr after the last dose of MDL 28842, degrees of parasitemia were determined using thin smears of venous blood stained with Giemsa. Results are expressed as the percentage of erythrocytes infected with at least one parasite.

Measurement of intracellular SAH concentrations. Measurement of SAH in erythrocytes entailed first removing leukocytes from the whole blood [12]. Blood (approximately 1 mL/mouse) was taken by cardiac puncture, and 0.05 mL of a solution of 0.1 M EDTA (pH 7.5) was added as an anticoagulant. The blood was centrifuged for 10 min at 1500 *g*, and the plasma and buffy coat were discarded. To the erythrocytes was added 0.5 mL of a solution of 100 mM potassium phosphate (pH 7.5) containing 5 mM $MgCl_2$, 138 mM NaCl and 0.4 mM EDTA. The suspended erythrocytes were then applied to a 5-mL column of Servacel (sulphoethyl cellulose; Serva) that had been equilibrated in the suspending buffer. Erythrocytes were then eluted from the column with 4 mL of the same buffer and collected in a tube containing an additional 0.05 mL of 0.1 M EDTA to prevent coagulation. This procedure generally removed 99% of leukocytes and greater than 90% of platelets as determined by automated blood counting. Erythrocytes were finally sedimented at 1500 *g* for 10 min and after removal of the supernatant fraction, the pellet (packed cell volume of 0.4 mL) was extracted with 1 mL of 0.4 M perchloric acid. The protein precipitate was removed by centrifugation at 15,000 *g*, and the supernatant fraction was filtered through a 0.45 μm filter in preparation for analysis by HPLC. Intracellular SAH concentrations were determined by HPLC analysis of the perchloric acid extracts following a published procedure [13].

Assay of erythrocyte and plasmodial SAH hydrolase activity and determination of inhibition kinetics of MDL 28842. Erythrocytes were purified from whole mouse or human blood on sulfoethyl cellulose columns as described above. The erythrocytes were then lysed in 1 mM potassium phosphate (pH 7.6) containing 2 mM dithiothreitol and 1 mM EDTA and centrifuged at 17,000 *g* for 20 min. SAH hydrolase activity was precipitated by the addition of ammonium sulfate to 35% saturation. The precipitate was dissolved in a small amount of the above buffer and dialyzed overnight. These enzyme preparations contained 20 mg protein/mL from human and mouse erthrocytes and were stored frozen at −20° for 4 weeks without loss of activity.

For determination of time-dependent inactivation kinetics for MDL 28842 against SAH hydrolase, 1 mg of mouse erythrocyte protein or 2 mg of human erythrocyte protein was incubated at 37° or 30°, respectively, in a solution of 50 mM potassium phosphate (pH 7.6) containing 1 mM EDTA, 2 mM dithiothreitol, 10 mM D,L-homocysteine and 10% glycerol in a final volume of 0.5 mL. At the times indicated in the figures, 0.05-mL samples were taken from the enzyme/inhibitor incubation and diluted 10-fold into the same ice-cold buffer and kept on ice until all samples had been collected, and then SAH hydrolase activity was assayed.

SAH hydrolase activity was initiated by the addition of 10 μM [^{3}H]adenosine (0.1 μCi) to the enzyme samples on ice. After 10-min incubations at either 37° for the mouse enzyme or at 30° for the human enzyme, the reaction was terminated by adding 1 mL of 50 mM HCl, and the [^{3}H]SAH which was formed was separated from the substrate by chromatography on SP-Sephadex G-25 columns (Pharmacia) as described [14]. Analysis of the inhibition kinetics was carried out as described by Kitz and Wilson [15].

P. falciparum and *P. berghei* were separated from infected erythrocytes by a saponin lysis technique [16]. The parasites were lysed by three cycles of freezing and thawing, and supernatant fractions were collected by centrifugation at 15,000 *g* for 10 min. SAH hydrolase activity in supernatant fractions was assayed for 10 min as described above using 7 μg protein from *P. falciparum* and 165 μg protein from *P. berghei*.

Chemicals. [2,8-^{3}H]Adenosine (30 Ci/mmol) and [8-^{3}H]hypoxanthine (10 Ci/mmol) were purchased from ICN and New England Nuclear, respectively. MDL 28842 was synthesized at the Merrell Dow Research Institute [6].

RESULTS

Inhibition of P. falciparum *and* P. berghei *growth*. MDL 28842 was found to be an inhibitor of *P. falciparum* growth *in vitro*, with an IC_{50} (concentration of drug which inhibited the incorporation of [^{3}H]hypoxanthine by 50%) of 4.7 μM (Fig. 2). A similar IC_{50} value was obtained when parasite counts

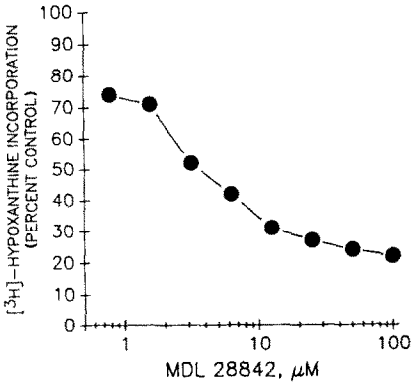

Fig. 2. Inhibition of *P. falciparum* growth by MDL 28842. MDL 28842 was added to *P. falciparum* cultures, and parasite growth was assessed by measuring the incorporation of [^{3}H]hypoxanthine as described in Methods. Each point is the mean incorporation of [^{3}H]hypoxanthine in duplicate wells. The experiment was repeated twice with similar results. Control value for [^{3}H]hypoxanthine incorporation: 2688 cpm.

Table 1. Suppression of *P. berghei* infections in mice during administration of MDL 28842

Treatment	Dose (mg/kg)	% Parasitemia	N
Vehicle		26.0 ± 6.8	5
MDL 28842	1	13.0 ± 4.5	5
MDL 28842	3	8.6 ± 2.7	5
MDL 28842	10	6.2 ± 2.4	5

Mice were infected with *P. berghei*, and were treated with MDL 28842 three times per day for a 3-day period. MDL 28842 was dissolved in 50% dimethyl sulfoxide. Percent parasitemia was determined as described in Methods. Values are means ± SD.

were made on Giemsa-stained blood smears from parallel cultures treated with MDL 28842 but not exposed to [^{3}H]hypoxanthine. Inhibition of growth did not appear to be stage-specific because the surviving parasites observed in these blood smears were found not to be of one predominant stage. Because of the marked inhibition of *P. falciparum* growth *in vitro*, the drug was administered to mice that were infected with a lethal strain of *P. berghei* to examine the effects of the compound *in vivo*. It was found that there was a 50% suppression of parasitemia in mice when they were administered 1 mg MDL 28842/kg three times per day for a 3-day period (Table 1). At the highest dose of MDL 28842 which was well-tolerated by the mice, 10 mg/kg, parasitemia was suppressed 77%. Complete clearance of parasitemia was not seen in any of the mice, and treated mice did not survive any longer than mice treated with the vehicle control.

SAH levels in mouse erythrocytes. Levels of SAH in erythrocytes were measured in both uninfected mice and mice infected with *P. berghei*. During treatment with 10 mg MDL 28842/kg for 3 days, the intraerythrocytic levels of SAH were found to increase dramatically (Table 2): an apparent 26-fold increase in the erythrocytes from *P. berghei*-infected mice and an apparent 70-fold increase in the uninfected mice. The difference in the increases in infected and uninfected mice is probably not significant since SAH was below the limits of detection in a number of the untreated mice in both experiments shown in Table 2, making interpretation of the control levels difficult.

Inhibition of SAH hydrolase. Because MDL 28842 was effective at inhibiting the growth of *P. falciparum in vitro* and *P. berghei in vivo* and increased the levels of SAH in erythrocytes of mice treated with MDL 28842, it was of interest to determine the inhibition kinetics of the inhibitor against the SAH hydrolase of the *Plasmodia* and against the same enzyme from erythrocytes.

SAH hydrolase activity could not be detected in either *P. falciparum* or in *P. berghei* which had been separated from infected erythrocytes. This finding suggested that the target for MDL 28842 must be the SAH hydrolase of the host erythrocyte and that any effects of the compound on the parasite must be secondary to this inhibition.

SAH hydrolase from both human and mouse erythrocytes proved to be exquisitely sensitive to inhibition by MDL 28842. The data in Fig. 3 show that the inactivation of mouse erythrocyte SAH hydrolase by MDL 28842 was time dependent. A $K_I = 0.18\,\mu M$ and $T_{1/2}$ at saturating inhibitor concentration of 0.6 min were obtained (Fig. 4). An identical K_I was obtained for the human erythrocyte SAH hydrolase (not shown). The K_I values for inhibition of the SAH hydrolase from both types of erythrocytes were similar to the K_I reported previously for the inhibition of rat liver SAH hydrolase [6].

DISCUSSION

A 4′,5′-unsaturated 5′-fluoroadenosine mechanism-based inhibitor of SAH hydrolase, MDL 28842, was found to inhibit markedly the growth of both *P. falciparum in vitro* and *P. berghei in vivo*. In each instance, concentration– or dose–response curves were relatively flat. Suppression of *P. falciparum* growth *in vitro* appeared to level off at about 80%, a value which is comparable to the 77% inhibition of *P. berghei in vivo* with the maximum tolerated dose of MDL 73811. The growth inhibition in mice was associated with a large increase in the intraerythrocytic concentration of SAH. This increase in SAH was apparently due solely to inhibition of erythrocyte SAH hydrolase by MDL 28842 because SAH hydrolase activity was not detected in *P. berghei* separated from its erythrocyte host. SAH hydrolase activity was undetectable in *P. falciparum*, as well, although the enzyme activity was detected readily in human erythrocyte extracts. Previously it was reported that treatment of human patients with either 2′-deoxyadenosine [17] or adenine arabinoside [18, 19] also resulted in inhibition of erythrocytic SAH hydrolase activity and increased concentrations of SAH. MDL 28842 had apparent K_I values against mouse and human erythrocyte SAH hydrolase of

Table 2. Increased *S*-adenosylhomocysteine in mouse erythrocytes during treatment with MDL 28842

Treatment	*S*-Adenosylhomocysteine (nmol/10^9 erythrocytes)	*S*-Adenosylmethionine	N
Experiment 1			
None	0.02 ± 0.02	1.73 ± 0.21	6
MDL 28842	0.52 ± 0.13	1.76 ± 0.15	6
Experiment 2			
None	0.01 ± 0.01	ND*	6
MDL 28842	0.71 ± 0.18	ND	5

Mice, either uninfected (Experiment 2) or infected with *P. berghei* (Experiment 1) as described in Methods, were treated with 10 mg MDL 28842/kg three times per day for 2 days and twice on day 3. One hour after the last treatment, erythrocytes were purified from whole blood, and SAH and *S*-adenosylmethionine were determined by HPLC analysis as described in Methods. Values are means ± SD.

* ND = not determined.

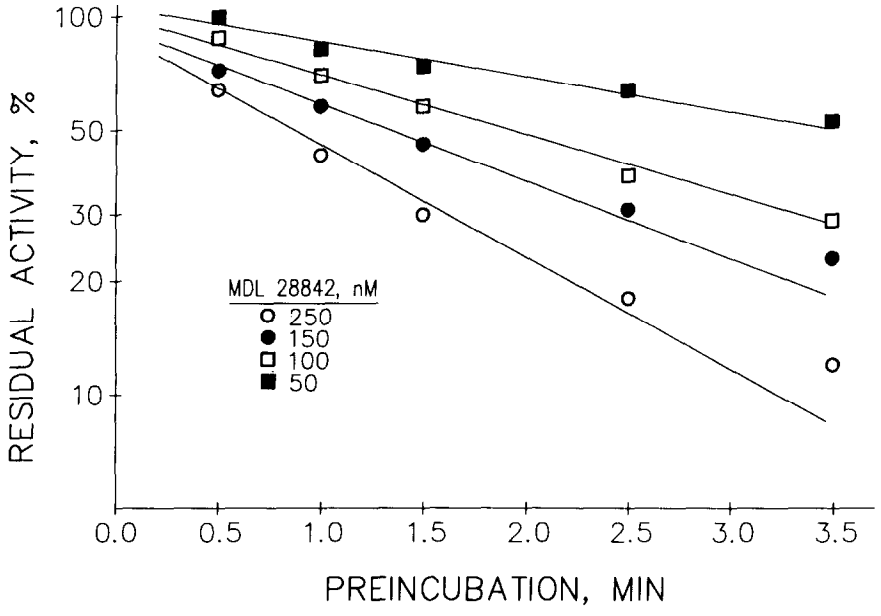

Fig. 3. Time-dependent inactivation of mouse erythrocyte SAH hydrolase by MDL 28842. Mouse erythrocyte SAH hydrolase (1 mg protein) was incubated at 37° with the indicated concentrations of MDL 28842. At the indicated times, samples were removed and diluted 10-fold in ice-cold buffer and stored on ice until residual SAH hydrolase activity was determined as described in Methods.

0.18 and 0.19 μM, respectively, and inhibited the enzyme from both types of cells in a time-dependent and irreversible manner (not shown). These facts taken together suggest that the effects of MDL 28842 against malaria parasites are largely, if not completely, indirect. Disturbing the intraerythrocytic milieu with a drug-induced increase in SAH results in slower growth of the parasites. SAH is known to inhibit transmethylation reactions in other cells and tissues [4]. The high concentrations of SAH induced by treatment with MDL 28842 may inhibit transmethylation reactions in the *Plasmodia*. Alternatively, plasmodial growth may be dependent on the salvage of the adenosine formed as a product of SAH hydrolase activity in the host erythrocyte.

The indirect nature of the antimalarial effects of MDL 28842 may limit the usefulness of the drug as a chemotherapeutic agent. Complete eradication of the intraerythrocytic parasite may be impossible without lowering SAH hydrolase activity to a level that is incompatible with the well-being of the animal host. In fact, the observed toxicity of MDL 28842 to the *P. berghei*-infected mice may be related to inhibition of host SAH hydrolase activity. However, it is also possible that the toxicity of MDL 28842 to the host is not related solely to its inhibition of SAH hydrolase activity and that any nonspecific component of toxicity could be avoided through the design of a more specific inhibitory agent.

The potency of MDL 28842 for inhibition of *P.*

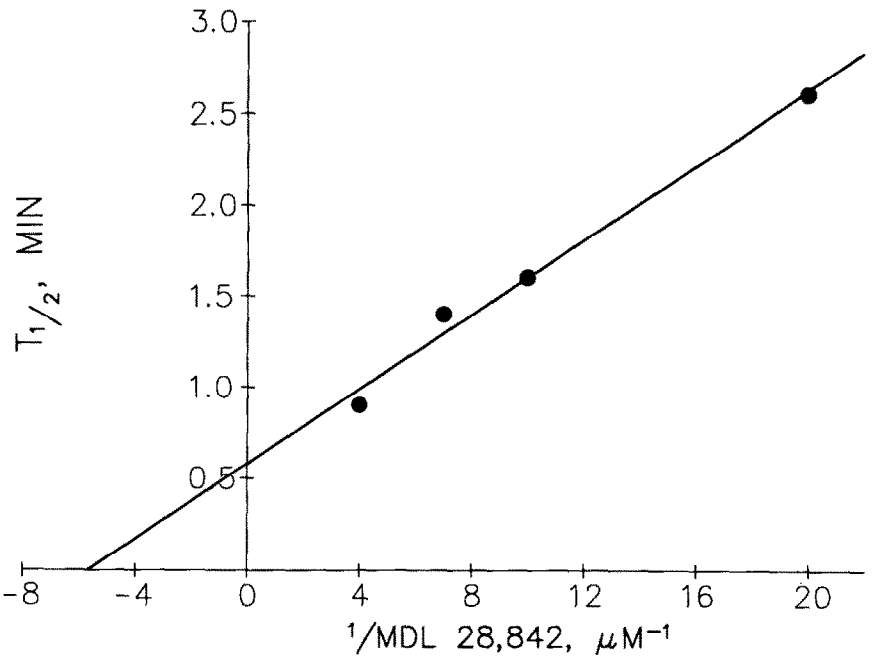

Fig. 4. Inhibition kinetics for MDL 28842 against mouse erythrocyte SAH hydrolase. The half-life of SAH hydrolase activity was determined in the absence and presence of various concentrations of MDL 28842, as shown in Fig. 3. From the plot of half-life versus 1/[MDL 28842], an apparent K_I and half-life at infinite inhibitor concentration were determined.

falciparum growth *in vitro*, $IC_{50} = 4.7\ \mu M$, is approximately the same as the potency of neplanocin A reported previously [5]. In that study, neplanocin A caused a rise in both SAH and *S*-adenosylmethionine in infected human erythrocytes treated with the drug *in vitro*. The rise in SAH in that study was only about 2-fold. Our results differ in two respects from the earlier work: (1) erythrocyte *S*-adenosylmethionine levels were unchanged by treatment of mice with MDL 28842, and (2) SAH levels in mouse erythrocytes increased to a much greater extent. This may be because of a higher specificity of MDL 28842 for inhibition of SAH hydrolase as compared to neplanocin A, which may have other effects in the treated cell. Neplanocin A was found to be converted to *S*-neplanocinylmethionine and also to *S*-neplanocinyl nucleotides. These metabolites may interfere with other cell functions and thus result in less specific cytotoxicity. In contrast, MDL 28842, because of its substituents at the 5′-position, cannot form the corresponding methionine-containing nucleoside and cannot be phosphorylated to the corresponding nucleotides. Therefore, a more specific site of action of MDL 28842 as compared to neplanocin A can be deduced from the present study.

It is also of interest that *P. falciparum* and *P. berghei* apparently lack SAH hydrolase activity because this enzyme activity was readily detected by us in another protozoan parasite, *Leishmania mexicana mexicana* (R. J. Baumann, unpublished results). This enzyme activity in *Leishmania* promastigote cell extracts was potently inhibited by MDL 28842, and 0.1 mM MDL 28842 caused 50% inhibition of the growth of the promastigote form of this parasite in axenic cultures.

SAH hydrolase would appear to be a potential target for chemotherapy of malaria parasites residing in erythrocytes, especially with a compound such as MDL 28842 which is an irreversible inhibitor of the enzyme. Reactivation of the enzyme after inhibition with MDL 28842 is dependent upon resynthesis of protein, and since erythrocytes cannot synthesize proteins, the enzyme would remain inactivated indefinitely. A more pronounced effect on *P. berghei* in mice possibly could have been obtained with higher doses of MDL 28842, but doses above 10 mg/kg three times per day were increasingly toxic to the mice. Although MDL 28842 was not curative for *P. berghei* infections, inhibitors of SAH hydrolase less toxic to the mammalian host may yet prove to be useful chemotherapeutic agents.

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