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IRREVERSIBLE INHIBITION OF *S*-ADENOSYLMETHIONINE DECARBOXYLASE IN *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES: GROWTH INHIBITION *IN VITRO*

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Abstract—Blocking spermidine and spermine synthesis in *Plasmodium falciparum*-infected erythrocytes with irreversible inhibitors of *S*-adenosylmethionine decarboxylase (AdoMet DC; EC 4.1.1.50), prevented the growth of the parasite *in vitro*. The most potent of these compounds, MDL 73811, inhibited growth of chloroquine-sensitive and -resistant strains of *P. falciparum* equally, with an IC_{50} of 2–3 μM. Other structurally related compounds also inhibited parasite proliferation, but to a lesser degree, determined apparently by their potency for inhibition of AdoMet DC. The growth inhibition by MDL 73811 could be alleviated by incubating infected erythrocytes with spermidine and spermine, but not putrescine. Parasites treated with the drug were arrested at the trophozoite stage of the erythrocytic cycle and had putrescine levels which were elevated by about 3- to 4-fold. Treatment of crude extracts of purified parasites with 1 μM MDL 73811 inhibited AdoMet DC activity by greater than 90%. These biochemical changes in *P. falciparum*-infected cells were consistent with AdoMet DC inhibition being the primary effect of MDL 73811 treatment.

Cell proliferation requires biosynthesis of the polyamines, putrescine, spermidine, and spermine, depletion of which results in cessation of DNA and RNA synthesis [1]. While the absolute role of polyamines in the eucaryotic cell cycle is unclear, they are likely to be involved in stabilizing the structure of newly synthesized chromatin and in the translational efficiency of RNA [2]. One of the most studied polyamine biosynthesis inhibitors is α-difluoromethylornithine (DFMO†), an irreversible inhibitor of ornithine decarboxylase (ODC) [3], which inhibits the growth of certain tumors and parasitic protozoa by depletion of cellular polyamines [4, 5]. ODC activity is regulated by agents which affect proliferation rates, and its content in mammalian cells can be increased or decreased rapidly in response to growth stimuli in part due to the high turnover rate of the protein [1, 5]. This approach has met with some success in the inhibition of the growth of the malarial parasites *Plasmodium falciparum in vitro* and *Plasmodium berghei in vivo* [6, 7] and has led to the development of a novel chemotherapy for *Trypanosoma brucei* infections in mice [8, 9] and *Trypanosoma gambiense* infections in humans [10, 11].

Inhibition of *S*-adenosylmethionine decarboxylase (AdoMet DC; EC 4.1.1.50) also blocks polyamine biosynthesis by preventing the conversion of putrescine to spermidine and spermidine to spermine. The competitive AdoMet DC inhibitor methylglyoxal bis(guanylhydrazone) (MGBG) has been shown to prevent the growth of *P. falciparum in vitro* [12], and therefore it was of interest to test an irreversible inhibitor of AdoMet DC which has been described recently [13], for its effects on *Plasmodia*. This compound, MDL 73811, inhibits the bacterial and trypanosomal enzymes with K_i values of 0.3 and 1.5 μM, respectively [13, 14]. The inhibition is time dependent and irreversible, in that prolonged dialysis of the inactivated enzyme does not restore enzyme activity. Administration of the compound to mice effects cures of *T. brucei* and *T. rhodesiense* infections [14]. Here we show that incubation of chloroquine-sensitive and -resistant *P. falciparum* strains with MDL 73811 and three of its analogs halted the growth of the parasite *in vitro*, arresting the erythrocytic cycle at the trophozoite stage. This inhibition could be prevented by coincubation of the parasites with MDL 73811 and either spermidine or spermine, but not putrescine, consistent with the site of inhibition being AdoMet DC. Additionally, incubation of crude extracts from *P. falciparum*-infected erythrocytes with MDL 73811 irreversibly inhibited AdoMet DC activity.

MATERIALS AND METHODS

Chemicals. [8-^{3}H]Hypoxanthine (10 Ci/mmol), L-[4,5(n)-^{3}H]isoleucine (110.4 Ci/mmol), and *S*-adenosyl-L-[carboxyl-^{14}C]methionine (56.9 mCi/mmol) were from DuPont, NEN Research Products, Boston, MA. Methylbenzethonium hydroxide, putrescine, spermidine, and spermine were from the Sigma Chemical Co., St. Louis, MO. *o*-

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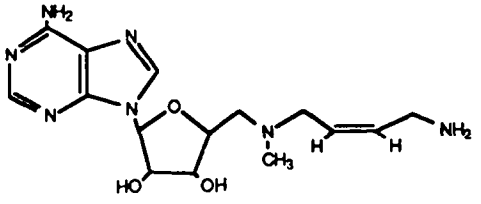
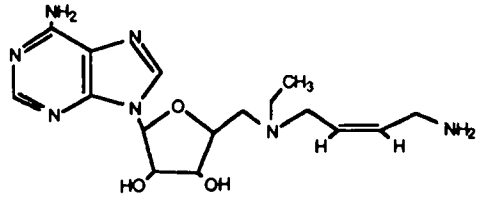
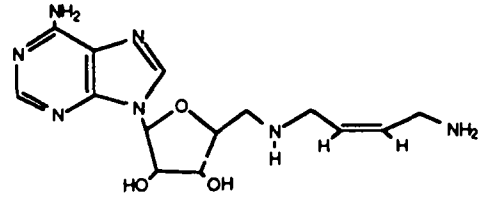
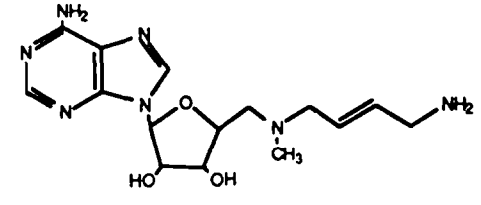
† Abbreviations: AdoMet DC, *S*-adenosylmethionine decarboxylase; DFMO, α-difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone); and ODC, ornithine decarboxylase.

Table 1. Growth inhibition of chloroquine-sensitive and -resistant *P. falciparum* by time-dependent inhibitors of Adomet DC

Inhibitor	Structure	Growth inhibition D6 IC_{50} (μM)	Growth inhibition ITG2 IC_{50} (μM)
MDL 73811		2.3	1.7
MDL 73581		14.5	9.2
MDL 74038		16.2	9.2
MDL 74391		40.0	28.0
Chloroquine		7.3 ng/mL	100.0 ng/mL

The *P. falciparum* strains D6 and ITG2 were incubated with the compounds indicated plus [^{3}H]isoleucine to label newly synthesized protein as described in Materials and Methods. The values are the drug concentrations which inhibited [^{3}H]isoleucine incorporation by 50%.

Phthaldialdehyde was from Calbiochem, San Diego, CA. Octane sulfonate was from the Regis Chemical Corp., Morton Grove, IL. AdoMet DC inhibitors including 5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811, see Table 1) were synthesized at the Marion Merrell Dow Research Institute [13].

P. falciparum *cultures.* *P. falciparum*-infected erythrocytes were maintained in continuous culture [15]. Stock cultures were propagated in human erythrocytes (type O^+) at a 6% hematocrit in RPMI 1640 medium (GIBCO, Grand Island, NY), containing 25 mM HEPES, 50 μg/mL hypoxanthine, 0.2% sodium bicarbonate, and 10% human serum at 37° in a 3% O_2:5% CO_2:92% N_2 atmosphere. The human serum and erythrocytes were from Worldwide Biologicals, Cincinnati, OH. *P. falciparum* strains used included clone D6 (Sierra Leone; [16]), clone W2 (Indochina; [16]), FCR-3 (Gambia, from Dr. J. M. Whuan, Walter Reed Army Institute of Research, Washington, DC), ITG2 (Brazil, from Dr. I. J. Udeinya, Walter Reed Army Institute of Research) and Strain NF54 (obtained from Dr. V. Rosario, Biomedical Research Institute, Rockville, MD).

Macromolecular biosynthesis. Drug effects on *P. falciparum* proliferation were measured as inhibition of [^{3}H]hypoxanthine and [^{3}H]isoleucine incorporation into nucleic acid and protein, respectively, using microtiter cultures [17]. Culture medium lacking hypoxanthine or isoleucine (Selectamine kit, GIBCO) were used in these experiments. Starting parasitemias were 0.5% in a 1% hematocrit for measurement of macromolecule biosynthesis. The cultures were incubated with test compounds for

24 hr; then 1 μCi per well of the appropriate radioisotope was added. Cultures were harvested onto glass fiber filters 18 hr later using an automatic cell harvester, and the radioactivity on each filter was measured by liquid scintillation counting.

Polyamine measurements. *P. falciparum* cultures (1% parasitemia, 2% hematocrit) were set up in 75 cm^2 flasks and then incubated with or without MDL 73811, as indicated, for 48 hr under normal growth conditions. The infected erythrocytes were pelleted by centrifugation and then washed twice with serum-free RPMI 1640 medium. The washed cell pellets were extracted with 5 vol. of 0.4 N perchloric acid. Polyamine levels were determined by high-pressure liquid chromatography [18].

S-Adenosylmethionine decarboxylase assays. Larger scale *P. falciparum* cultures (200–300 mL total volume) were used for preparation of extracts for AdoMet DC assays. Parasites were released from erythrocytes by treating the infected cells with 0.05% saponin solution for 5 min at room temperature [19]. The free parasites were sedimented at 400 *g* and then washed with serum-free RPMI medium. The parasites were lysed in 50 mM sodium phosphate, 0.1 mM EDTA, 2 mM dithiothreitol, 40 μM pyridoxal-5-phosphate (pH 7.4) by three freeze–thaw cycles. The cell lysate was subjected to centrifugation (10,000 *g*, 4°, 30 min) and the supernatant assayed immediately for AdoMet activity. The enzyme activity was measured as described previously [20]. The enzyme reactions included 50 mM $NaPO_4$ (pH 7.4), 2 mM dithiothreitol, 3 mM putrescine, 51 μM [^{14}C]*S*-adenosylmethionine (0.7 μCi) and 150 μg protein in 0.25 mL. The assays were carried out in serum vials at 34° for 30–60 min, after which the reactions were terminated by addition of 0.25 mL of 40% trichloroacetic acid, and incubated for 30 min longer to trap $^{14}CO_2$ on filter papers soaked in methylbenzethonium hydroxide. Radioactivity trapped on the filter papers was measured by scintillation counting in Omnifluor (DuPont, NEN). To test for irreversible inhibition, *P. falciparum* extracts were preincubated with or without MDL 73811 for 5 min, 34°, and then passed through Sephadex G-50 Quick Spin minicolumns (Boehringer-Mannheim, Indianapolis, IN) to separate protein from small molecules. Protein concentrations in the extracts were measured as described previously [21].

RESULTS

The irreversible AdoMet DC inhibitor, MDL 73811 (Table 1), halted growth of several *P. falciparum* strains as determined by incorporation of [^{3}H]hypoxanthine into nucleic acid. The IC_{50} for the drug averaged between 1 and 3 μM for both chloroquine-sensitive (D6, NF54) and chloroquine-resistant (W2, FCR-3, ITG2) organisms. The inhibition was not due to competition for [^{3}H]hypoxanthine uptake by the deoxyadenosine derivative since the same IC_{50} values were obtained when incorporation of [^{3}H]isoleucine into protein was measured (Table 1). Other deoxyadenosine derivatives which inhibit AdoMet DC were somewhat less effective against *P. falciparum* (Table 1), in line with their relative potencies against *T. brucei* AdoMet DC. The K_i and τ_{50} values for these compounds against the trypanosomal AdoMet DC were: MDL 73811, 1.5 μM, 0.3 min [14]; MDL 73581, 3.7 μM, 1.0 min; MDL 74038, 19 μM, 0.8 min; and MDL 74391, 12.5 μM, 2.3 min. The *in vitro* τ_{50} values seemed to approximate the effectiveness of the compounds against *P. falciparum* more closely than did the K_i values. It is interesting to note that the *trans*-butenyl isomer (MDL 74391) was about 15–20 times less effective in inhibiting *P. falciparum* growth than MDL 73811, which is the *cis*-isomer.

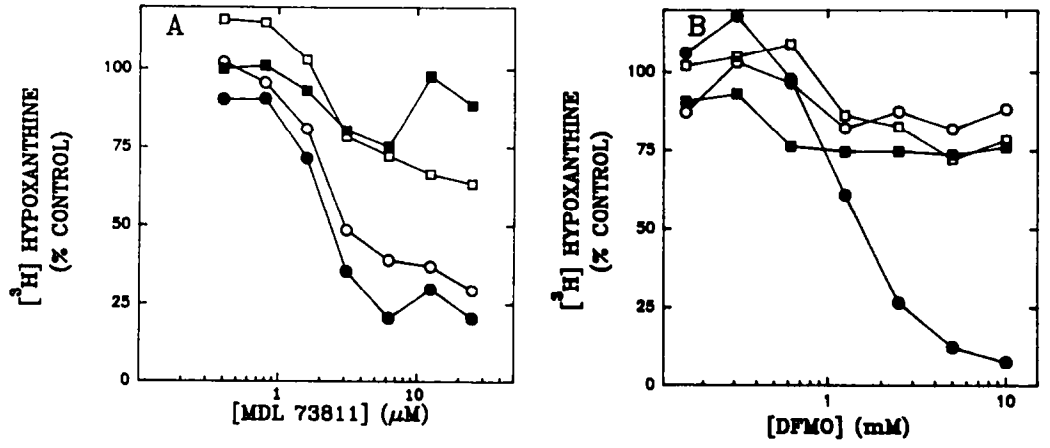


Fig. 1. Alleviation of growth inhibition by MDL 73811 and α-difluoromethylornithine (DFMO) with polyamines. Parasite proliferation was assessed by measuring [^{3}H]hypoxanthine incorporation into nucleic acid as described in Materials and Methods. Cultures treated with serial dilutions of (A) MDL 73811 or (B) DFMO were either not supplemented with polyamines (●) or were supplemented with putrescine (○), spermidine (■), or spermine (□) at 250 μM. Each point is the average from duplicate cultures. Mean control values for [^{3}H]hypoxanthine incorporation for each experiment were 5118 ± 320 cpm (N = 6) and 15,280 ± 2647 cpm (N = 6) for A and B, respectively.

Table 2. Inhibition of progression of *P. falciparum*-infected erythrocytes into schizont stage by MDL 73811

Treatment	% Parasitemia	% Control	Rings/Trophozoites/Schizont*
None	5.0	(100)	0.46/0.38/0.16
	5.9		0.35/0.56/0.09
DFMO	2.7	56	0.10/0.90/0.00
(1.3 mM)	3.4		0.03/0.97/0.00
DFMO	2.5	44	0.13/0.87/0.00
(5.0 mM)	2.2		0.09/0.91/0.00
MDL 73811	3.1	52	0.12/0.88/0.00
(3.0 μM)	2.6		0.26/0.74/0.00
MDL 73811	2.1	41	0.14/0.86/0.00
(10.0 μM)	2.3		0.16/0.84/0.00

P. falciparum-infected erythrocytes (cultures were initiated at 1% hematocrit, 1% parasitemia) were incubated with α-difluoromethylornithine (DFMO) or MDL 73811 at the concentrations indicated for 48 hr. (The concentrations chosen represent the IC_{50} and IC_{90} values for each compound as determined by inhibition of [^{3}H]hypoxanthine into nucleic acid.) Blood smears from these cultures were fixed with methanol and stained with 2% Giemsa. Parasitemias and morphological forms detected in the cultures were scored visually with a 100× oil immersion objective. At least 200 infected erythrocytes were counted for each culture.

* Values represent the fractional portion of each morphological form of parasite in the culture.

Changing the N-methyl group as in MDL 73581 (N-ethyl) or MDL 74038 (N-H) also reduced the effectiveness of the compounds markedly. The metabolite generated during the inactivation of AdoMet DC, 5'-(methylamino-5'-deoxyadenosine [13], had no effect on parasite growth at concentrations up to 80 μM. Supplementation of cultures with up to 100 μM adenosine or adenine did not affect the IC_{50} value for MDL 73811, indicating that the uptake of the compound was not antagonized (data not shown). The growth inhibition by MDL 73811 was alleviated by coincubation of infected erythrocytes with spermidine or spermine, but not putrescine (Fig. 1A). The inability of putrescine to reverse the growth inhibition is consistent with the AdoMet DC-catalyzed reaction being the site of action for the compound. In contrast, all three polyamines effectively reversed the growth inhibition resulting from inhibition of ODC with DFMO (Fig. 1B). DFMO and MDL 73811 did not act synergistically in *P. falciparum* growth inhibition (Cross-Doersen DE and Wright PS, unpublished results).

Effect of MDL 73811 on the erythrocytic cycle and polyamine levels in P. falciparum*-infected erythrocytes.* It has been demonstrated previously that DFMO treatment halts the erythrocytic cycle at the trophozoite stage [6]. Incubation of infected erythrocytes with MDL 73811 had a similar effect (Table 2). No schizont form parasites were visible in Giemsa stained blood smears from cultures incubated for 48 hr with 3 or 10 μM MDL 73811. DFMO treatment of *P. falciparum*-infected erythrocytes leads to a depletion of intracellular polyamines ([7, 22], Table 3). MDL 73811 caused a decrease in spermidine and spermine levels in infected erythrocytes (Table 3), but putrescine levels increased 3- to 4-fold over control values consistent with inhibition of AdoMet DC by MDL 73811. The decrease in spermine was greater in cells treated with MDL 73811 than in those treated with DFMO.

Table 3. Effects of DFMO and MDL 73811 on polyamine levels in *P. falciparum*-infected erythrocytes

Treatment	Polyamines* (pmol/10^9 erythrocytes)		
	Putrescine	Spermidine	Spermine
None	40,50	1100,1280	430,430
DFMO (mM)			
1.3	0,0	70,120	250,250
5.0	0,0	60,60	100,100
MDL 73811 (μM)			
3.0	200,230	250,70	30,30
10.0	130,140	190,190	30,30

The same cultures described for Table 2 were harvested and analyzed for polyamine content as described in Materials and Methods. Each number is a value from the individual cultures.

* Values have polyamine levels measured for uninfected erythrocytes subtracted.

Inhibition of AdoMet DC in P. falciparum *extracts by MCL 73811.* AdoMet DC was inhibited greater than 90% by 5-min incubation of crude cytosolic extracts with 1 μM MDL 73811, and this inhibition was not alleviated by separation of the treated enzyme from small molecules by size exclusion chromatography on Sephadex G-50 spin columns (Table 4). Inhibition of the *T. brucei* AdoMet DC with MGBG, which is a competitive inhibitor of the enzyme, can be reversed by similar size exclusion chromatography [23]. The inability to separate the enzyme from the inhibitory molecules is consistent with MDL 73811 acting on the *P. falciparum* AdoMet DC irreversibility.

Table 4. Inhibition of Adomet DC activity in crude extracts of *P. falciparum* by MDL 73811

[MDL 73811]	Adomet DC activity (pmol/min/mg protein)	% Control
None	6.29 ± 0.37	(100)
1 μM	0.48 ± 0.15	7.6

Crude extracts were prepared from isolated parasites as described in Materials and Methods. The extracts were preincubated at room temperature with or without 1 μM MDL 73811 and then fractionated on G-50 Sephadex into high and low molecular weight fractions. AdoMet DC activity in the high molecular weight fractions was measured. Values are the averages of three determinations ± SD.

DISCUSSION

Inhibition of *P. falciparum* growth by MDL 73811 is apparently through inactivation of the parasite AdoMet DC. MDL 73811 inhibited *P. falciparum* AdoMet DC activity in crude cellular extracts in an apparently irreversible fashion in that separation of protein from small molecules did not alleviate the inhibition. The ability of each deoxyadenosine analog to inhibit nucleic acid or protein synthesis in infected cells correlated more closely with the τ_{50} values than with the K_i values as measured with the *T. brucei* AdoMet DC. (The relatively small amount and instability of the *P. falciparum* enzyme made these experiments prohibitively difficult.) While the *P. falciparum* AdoMet DC may have different kinetic parameters than the *T. brucei* enzyme, we saw nearly complete inhibition of the enzyme activity in 1 μM MDL 73811 (5 min, 20°). Growth inhibition by the drug could be prevented by the addition of the polyamines, spermidine and spermine, which are metabolites produced beyond the affected step. Inhibition of the overall flux through the polyamine synthetic pathway at the AdoMet DC catalyzed step should lead to increased putrescine levels. The cellular levels of polyamines measured followed this prediction as putrescine levels increased 3- to 4-fold while spermidine and spermine levels decreased. The higher concentration of MDL 73811 (10 μM) did not seem to elevate putrescine levels as high as that seen in cultures treated with 3 μM (Table 3). This is likely due to the higher drug concentration killing more of the parasites and resulting in fewer polyamine-containing cells. However, at the higher drug concentration, it appeared that the IC_{90} point was not reached when the parasitemias were determined from stained slides rather than from the incorporation of radiolabeled precursors into the macromolecules of the parasite. This discrepancy in scoring parasites may have arisen because many of the erythrocytes scored visually as infected had either dead or moribund parasites residing within, parasites which could still stain with Giemsa, but not incorporate labeled precursors into protein or nucleic acid.

Sinefungin, an analog of adenosine closely related to *S*-adenosylmethionine, was shown previously to be a potent antimalarial agent (IC_{50} = 0.3 μM) [24]. The primary mode of action for this compound was originally believed to be interference with methylation reactions. Recently, Messika *et al.* [25] have shown that sinefungin is a trophozoite stage specific antimalarial and that depletion of spermidine and spermine was evident in treated cultures. Unlike MDL 73811, sinefungin at levels up to 300 μM did not inhibit AdoMet DC activity in *P. falciparum* extracts. Both MDL 73811 and sinefungin cause depletion of spermidine and spermine levels, albeit via different mechanisms. Consequently, both agents arrest parasites at the trophozoite stage of the erythrocytic cycle, as the polyamines are required for progression into the schizont stage.

Interestingly, the growth of both chloroquine-sensitive and -resistant organisms was inhibited similarly by MDL 73811 and the other deoxyadenosine derivatives described here. It has been demonstrated that *P. falciparum* has multidrug resistance (MDR) genes (designated *pfmdr*1 and *pfmdr*2) which encode P-glycoprotein homologs similar to the proteins which function as broad spectrum transmembrane efflux pumps and are overexpressed in transformed mammalian cells exhibiting the MDR phenotype [26, 27]. Although the absolute role for the MDR genes in chloroquine- (or multidrug-) resistant malarial parasites is not clear [28, 29], our findings nonetheless suggest that the AdoMet DC inhibitors are not substrates for the drug removal systems or putative multidrug-resistant efflux pumps which confer chloroquine resistance in *P. falciparum*-infected erythrocytes [30].

MDL 73811 was about 1000-fold more effective against *P. falciparum*-infected erythrocytes than DFMO. While the inhibitors were effective *in vitro* against *P. falciparum*, administration of MDL 73811 to mice infected with *P. berghei* had no effect on the progress of parasitemia. It is possible that the AdoMet DC of *P. berghei* is not as sensitive to the action of MDL 73811 as is the *P. falciparum* enzyme. Alternatively, the lack of activity against *P. berghei* could be due to the rapid clearance of MDL 73811 in mice; a plasma half-life of about 10 min was found in treated animals (Byers TL and Bitonti AJ, unpublished observations). Thus *P. berghei* is exposed to the drug only transiently, whereas in cultures *P. falciparum* is exposed continuously to the drugs. Efficacy *in vivo* might be enhanced if an MDL 73811 analog could be designed which is concentrated more effectively in the infected erythrocyte. Alternatively, some type of sustained release drug delivery system might be effective, as this mode of delivery enhances the trypanocidal effects of the drug on infections in mice [14].

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