Biochemical Pharmacology, Vol. 46, No. 3, pp. 471–481, 1993.
Printed in Great Britain.

0006–2952/93 $6.00 + 0.00
Pergamon Press Ltd

RESISTANCE TO DL-α-DIFLUOROMETHYLORNITHINE BY CLINICAL ISOLATES OF *TRYPANOSOMA BRUCEI RHODESIENSE*

ROLE OF *S*-ADENOSYLMETHIONINE

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(*Received* 24 *December* 1992; *accepted* 8 *April* 1993)

Abstract—The ornithine decarboxylase (ODC) inhibitor DL-α-difluoromethylornithine (DFMO) has emerged as a new treatment for West African sleeping sickness but is less effective against East African sleeping sickness. We examined uncloned clinical isolates of *Trypanosoma brucei rhodesiense*, agent of the disease in East Africa, which were refractory to DFMO in laboratory infections, for characteristics that would explain their resistance. None of the isolates were from patients treated with DFMO. Two isolates took up [^{3}H]DFMO at 50–70% lower rates than drug-sensitive strains but ODC activities, K_i values for DFMO, spermidine and spermine uptake rates, polyamine content and inhibition of polymamine metabolism by DFMO were statistically ($P < 0.05$) similar between sensitive and refractory isolates. One cloned strain, continuously passaged *in vivo* under DFMO pressure and included for comparison, had >85% lower ODC activity and up to 14-fold higher putrescine uptake rates than sensitive controls. A statistically important trend was the metabolism of *S*-adenosylmethionine (AdoMet): activities of AdoMet synthetase and AdoMet decarboxylase were 2- to 5-fold and 3- to 40-fold lower in resistant strains, respectively, while intracellular AdoMet pools (AdoMet + decarboxylated AdoMet) that were >60-fold elevated in sensitive strains during DFMO treatment, increased only 9-fold in refractory isolates. The extreme elevation of the AdoMet pool in sensitive isolates from 0.7 to 44 nmol/mg protein and an intracellular pool concentration of ~5 mM may lead to an imbalance in methylation of proteins or other cell constituents as a consequence of DFMO action. These studies indicate that the metabolism of AdoMet is altered significantly in DFMO refractory isolates and suggest that differences in AdoMet metabolism may be responsible for increased tolerance to DFMO.

Treatment of African sleeping sickness in West Africa has improved significantly through the use of the ornithine decarboxylase (ODC§) inhibitor DL-α-difluoromethylornithine (Eflornithine, Ornidyl®, DFMO [1]). This agent, an enzyme-activated inhibitor of the lead enzyme of polyamine biosynthesis, exerts many effects on the causative agents of this disease, *Trypanosoma brucei* ssp. These effects include blockade of polyamine biosynthesis, inhibition of macromolecule synthesis and the elevation of intracellular *S*-adenosylmethionine (AdoMet) and decarboxylated AdoMet [2–6]. Despite significant activity against West African (Gambian) sleeping sickness, DFMO has given sporadic results in the treatment of the East African (Rhodesian) disease. This is of particular concern due to the recent outbreak of arsenical refractory human disease in Uganda [1, 7, 8]. Recently, our laboratory studied fourteen clinical isolates of *Trypanosoma brucei rhodesiense* and found that seven were fully or partially refractory to DFMO in model laboratory infections [9]. These strains had no history of prior exposure to DFMO so the observed resistance did not appear to be due to traditional mechanisms that arise after prolonged exposure of a population to increasing levels of the drug [10, 11]. This study describes biochemical properties of these uncloned clinical isolates and a potential mechanism for naturally occurring drug resistance.

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§ Abbreviations: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine (Ornidyl®); AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; and MDL73811, 5′-{[(*Z*)-4-amino-2-butenyl]} methylamino-5′-deoxyadenosine.

MATERIALS AND METHODS

Trypanosome isolates. The majority of strains described in this study were clinical isolates obtained from the Kenya Institute for Trypanosomiasis Research (KETRI: Muguga, Kenya) through the assistance of its then director, Dr. A. R. Njogu. The origin and drug sensitivities of these strains were described previously [9]. One strain, KETRI 2538, was initially found to be refractory to DFMO in mouse infections [9] but has subsequently become sensitive after mouse passage and serves as one sensitive control strain in this study. Other DFMO refractory isolates (KETRI 243, 269, 1992, 2002, 2285) used in this study were retested in mouse

Table 1. Infectivity of mouse tissues after treatment of *T. b. rhodesiense*-infected mice with DFMO*

Tissue injected	KETRI 243 Number infected/Total	KETRI 269 Number infected/Total
Blood	11/18	15/18
Spleen	5/18	3/18
Liver	2/18	2/18
Brain	1/18	0/18
Heart	0/18	0/18
Lymph nodes (4)	0/18	0/18
Kidney	3/12†	0/18

* Infected mice (groups of three) were treated with 2% DFMO for 3 days. One day after treatment ended, organs from each mouse were excised and macerated in buffer, and 0.1 mL was injected into three clean mice. Data represent two experiments with each strain.

† Kidneys were taken from four of six treated animals.

Table 2. Activity of DFMO *in vitro* against bloodstream forms of *T. b. brucei* and *T. b. rhodesiense* isolates*

Strain	IC_{50} (μM)
T. b. brucei LAB 110 EATRO (S)	10.0 ± 0.7 (2)
KETRI 2538 (S)	12.0 ± 2.0 (2)
KETRI 243 (R)	31.0 ± 7.1 (3)
KETRI 269 (R)	16.5 ± 0.7 (2)
KETRI 2002 (R)	29.5 ± 0.5 (2)

* Trypanosomes were cultured continuously in a monoculture system [17] at 37° in 3% CO_2:97% air in Falcon 24-well plates. One-half the medium was replaced daily, with medium containing appropriate amounts of drug. Hemocytometer counts were made daily and results obtained after 48 hr. Control cells grew to a density of 5.0×10^6 mL^{-1}. Data are expressed as the means ± the range for duplicate determinations, or ±SD where $N > 2$. (S) DFMO sensitive; (R) DFMO refractory. The paired Student's *t*-test indicated that the resistant strains differed ($P < 0.05$) from sensitive strains with respect to IC_{50} values.

model infections and found to be stable in their resistance. For purposes of this study, resistance was taken as the ability of the strain to endure a 3-day oral treatment with 2% DFMO and cause a fatal parasitemia. Two substrains of KETRI 243 (243DFMO, 243As) were also used in some experiments: KETRI 243DFMO was derived by serial passage weekly (9 months) in mice treated with 2% DFMO for 6 days. This strain was cloned by diluting a suspension and infecting mice treated with cytoxan. KETRI 243As is a highly arsenic refractory strain obtained by treating a suspension of KETRI 243 with 100 μM melarsen oxide *in vitro* and infecting mice with the survivors as detailed in Yarlett *et al.* [12]. The resistance level of 243As to DFMO is the same as that of the parent KETRI 243. *Trypanosoma brucei brucei* LAB 110 EATRO was also used as the standard DFMO-sensitive control [9]. Two *T. b. rhodesiense* stabilates, obtained from the American Type Culture Collection (ATCC), were also used as DFMO-sensitive controls: the Wellcome CT strain (ATCC 30027) and the EATRO 105 isolate (ATCC 30119). Mice used in all drug and organ studies were 20 g Swiss–Webster females (Ace Animals, Inc., Boyertown, PA).

DFMO-treated trypanosomes. Large quantities (10^9 cells) of DFMO-treated trypanosomes were obtained by infecting 200 g Wistar rats (Ace Animals) with 2.5×10^5 trypanosomes and allowing a parasitemia of 0.5 to 1.0×10^8 cells mL^{-1} to develop before treating overnight (12–15 hr) with 4% DFMO in the drinking water [2]. Drug consumption was monitored by housing animals separately. Animals so treated consumed 20–40-mL overnight, corresponding to a dose of 4–8 g kg^{-1}. Trypanosomes from DFMO-treated and untreated control animals were harvested by cardiac puncture and were purified by passage of the blood though DEAE columns [13]. Trypanosomes for whole cell studies were washed and suspended in buffer or medium as described below.

Whole cell studies. Bloodstream trypanosomes were isolated as described [2]. For [^{3}H]DFMO uptake studies, purified trypanosomes were suspended (1×10^8 cells mL^{-1}) in uptake medium [RPMI 1640 containing 10% dialyzed fetal bovine serum (FBS), and 50 U of penicillin + 50 μg streptomycin mL^{-1}] to which 5 μCi (50 μM) of [3,4-^{3}H]DFMO (26.6 Ci mmol^{-1}) was added. For polyamine uptake studies, trypanosomes were suspended at 1×10^8 cells mL^{-1}, in PSG–BSA (70 mM phosphate-buffered 43 mM saline, 1% glucose, 1% bovine serum albumin, 50 U mL^{-1} penicillin + 50 μg mL^{-1} streptomycin, pH 7.8) containing 10 μCi of [2,3-^{3}H(N)]putrescine (16.7 Ci mmol^{-1}), 10 μCi [terminal methylenes-^{3}H(N)]spermidine (31 Ci mmol^{-1}), or 1 μCi [tetramethylene-1,4-^{14}C]spermine (77 mCi mmol^{-1}). Cells (3-mL aliquots) were incubated for 60 min in a shaking water bath at 37°. After incubation, 1-mL samples were filtered on Whatman GF/B discs prewashed with uptake medium with either a 1 mM concentration of the appropriate polyamine or 100 μM DFMO added and washed three times with appropriate medium or buffer, dried and counted. Using these conditions, we have found DFMO uptake to be linear for the initial 15 min and then to become hyperbolic for the remaining time. Uptake was based on the total accumulated after 1 hr.

For determination of polyamine biosynthesis, purified and washed bloodstream trypanosomes (2×10^8 mL^{-1}) were suspended in PSG–BSA and incubated for 1 hr at 37° in the presence of 3.3 μCi mL^{-1} of [2,3-^{3}H(N)]ornithine (55 Ci mmol^{-1}). After incubation, cells were washed and extracted, and polyamines were determined by thin-layer chromatography [14].

Cell-free extracts. Purified trypanosomes were suspended at a final concentration of 10^8 mL^{-1} in breakage medium (60 mM sodium phosphate, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 50 μm pyridoxal-5′-phosphate), and homogenates were made by freeze–thawing suspensions twice in a dry ice methanol bath. Homogenates were centrifuged (3900 *g* × 10 min) and stored at −70° [15]. These

Table 3. Summary of ODC activity, uptake of DFMO and its K_i values in DFMO-sensitive (S) and -refractory (R) trypanosome isolates*

Strain	Uptake of [^{3}H]DFMO (pmol mg^{-1} hr^{-1})	ODC activity (nmol mg^{-1} hr^{-1})	K_i DFMO (μM)
LAB 110 EATRO (S)	167.5 ± 34.6 (4)	48.7 ± 24.4 (12)	52.5 ± 26.0 (2)
KETRI 2538 (S)	222.0 ± 34.0 (4)	58.7 ± 40.6 (8)	
Wellcome CT (S)		58.4 ± 18.9 (7)	26.0 ± 1.4 (2)
EATRO 105 (S)		45.9 ± 10.3 (5)	28.3 ± 18.8 (2)
KETRI 243 (R)	150.0 ± 26.4 (3)	57.2 ± 14.5 (7)	22.5 ± 20.5 (2)
KETRI 243DFMO (R)	202.7 ± 20.0 (2)	7.2 ± 5.7 (4)	
KETRI 243As (R)		42.4 ± 19.1 (9)	
KETRI 2002 (R)	72.3 ± 25.4 (3)	35.9 ± 4.4 (3)	
KETRI 2285 (R)	86.5 ± 36.8 (4)	66.0 ± 21.5 (5)	28.0 ± 1.4 (2)
KETRI 269 (R)	120.0 ± 52.0 (3)	10.8 ± 2.7 (6)	
KETRI 1992 (R)	176.5 ± 68.6 (2)	22.8 ± 11.8 (3)	

* Uptake of [^{3}H]DFMO was measured in bloodstream forms harvested from rats and incubated *in vitro* as described in Materials and Methods. ODC was measured as evolution of [^{14}C]O_2 from [1-^{14}C]ornithine in extracts of blood forms [2]. K_i values for DFMO on ODC preparations were measured by time-dependent kinetics [21]. KETRI 243As and KETRI 243DFMO were derived from KETRI 243 as described in Materials and Methods. Data are the means ± the range for duplicate determinations, or ±SD where $N > 2$. Student's t-test ($P < 0.05$ significance) indicated that for [^{3}H]DFMO uptake, only KETRI 2002 and 2285 differed from the sensitive pool. For ODC activity, only KETRI 243DFMO and 269 were reduced significantly. For the K_i for DFMO against ODC none of the values statistically correlated with resistance.

preparations were used for the assay of ODC. Breakage medium for AdoMet decarboxylase preparations was similar except that the final pH was adjusted to 7.0, and aprotinin and soybean trypsin inhibitor were added at 1 mg mL^{-1}. AdoMet synthetase preparations were made from trypanosomes suspended in 10 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol and 1 mg mL^{-1} each of aprotinin and trypsin inhibitor.

Enzyme assays. ODC and AdoMet decarboxylase were assayed by trapping [^{14}C]O_2 released from DL-[1-^{14}C]ornithine (50.3 mCi mmol^{-1}) or *S*-[*carboxy*-^{14}C]AdoMet (47.8 mCi mmol^{-1}), respectively, as described [2]. AdoMet synthetase was assayed according to the method of Kappler *et al.* [16] by measuring production of [*methyl*-^{14}C]AdoMet from L-[*methyl*-^{14}C]methionine (57.4 mCi mmol^{-1}) and ATP. Activities are expressed as nanomoles CO_2 of AdoMet formed per milligram of protein per hour, respectively.

Culture of procyclic (insect midgut) trypanosomes. Cultures of procyclic trypanosomes were obtained by incubating bloodforms of LAB 110 EATRO, KETRI 243 and KETRI 2538 from heavily infected mice at 27° in T2 medium [15]. Transformation took place within 24 hr, and the cultures were passaged at 4- to 6-day intervals thereafter. ODC half-life studies ($T_{1/2}$) were done using procyclic trypanosomes treated with cycloheximide (50 mg mL^{-1}) for 1–6 hr and measuring enzyme activity as described previously [15].

Culture of bloodstream trypomastigotes. Continuous cultures of bloodforms were initiated in a feeder layer-free system by inoculating wells of a 24-well culture dish (Falcon 3047) containing 1 mL of modified Iscove's medium with 10^5 trypanosomes from mouse blood [17]. This medium was identical to that of HMI-18 except that the medium was supplemented with 20% (v/v) horse serum instead of 20% Serum Plus [17]. Plates were incubated in 3% CO_2:97% air at 37°. One-half the volume of medium was replaced daily and trypanosomes achieved peak densities of 5×10^6 mL^{-1} in 4 days. Drug sensitivity tests were done by dissolving the agent in sterile medium and replacing one-half the volume daily with medium containing double strength drug. Cell counts were made daily and IC_{50} values were calculated after 48 hr of exposure.

HPLC procedures. Polyamines, AdoMet and related metabolites were quantitated in whole cell extracts by HPLC procedures using a Perkin Elmer system. Putrescine, spermidine and spermine were analyzed in acid extracts by a reverse phase technique using a C-18 Percosil 10 μm column, and precolumn derivatization with *o*-phthalaldehyde [18]. AdoMet and decarboxylated AdoMet were quantitated from 10% (w/v) perchloric acid extracts using the C-18 column as described [6].

Parasite invasiveness. To determine whether DFMO-resistant parasites were sequestered in tissue spaces inaccessible to drug, the following experiment was done. Mice (3/group) were infected (2.5×10^5 cells) with KETRI 243 or 269 and the infection was allowed to develop for 24 hr. Mice were treated for 3 days with 2% DFMO in drinking water. One day after treatment ceased, tail vein blood films were examined for parasites and those appearing negative (10 fields at 400 magnification) were killed by CO_2 asphyxiation and their tissues tested as sources of trypansosomes by the following procedure. The liver, spleen, heart, brain, kidney and four subcutaneous lymph nodes were excised, rinsed in 50 mM Tris–HCl buffer (pH 7.4) containing 43 mM NaCl, 5.5 mM glucose and 100 U heparin mL^{-1},

with 50 U penicillin and 50 mM streptomycin mL^{-1}, and macerated in 1 mL of the same buffer. Each of the organ macerates along with undiluted blood (cardiac puncture) from each mouse was inoculated into three uninfected mice (0.1 mL per mouse). These mice were observed twice weekly for 60 days for trypanosomes in tail vein blood.

Statistical analysis of data. Results from experiments are expressed as means ± range for duplicate determinations, or ±SD where $N > 2$. The determinations obtained from each strain were subjected to Student's *t*-test to determine 95% confidence limits for each parameter. For each parameter (e.g. ODC activity and DFMO uptake), data for resistant and sensitive isolates were placed into separate pools and Student's *t*-test was used to determine the significance between populations. The paired *t*-test using the sensitive isolates as the normal population ($\bar{x}$) was also applied to each parameter for individual strains within each population (see Tables 2–7). Population parameters that separated with a probability of <0.05 were subsequently compared by use of a linear bar graph with respect to degree of overlap between the ranges of the two populations. This is presented as Fig. 1 in the Discussion.

Protein determinations. Cell extracts and suspensions of lysed (4% sodium lauryl SO_4) trypanosomes were dialyzed for 24 hr versus 1000 vol. of 0.01 M KPO_4 (pH 7.4), and protein content was quantitated by the method of Lowry *et al.* [19]. Cell extracts made in buffer containing proteinase inhibitors were blanked against buffers. Standard curves used bovine serum albumin.

Chemicals. All radiolabeled chemicals were purchased from New England Nuclear (Boston, MA). All other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). DFMO was a gift of the Marion Merrell Dow Research Institute (Cincinnati, OH).

RESULTS

In vivo *and* in vitro *resistance to DFMO.* In most experiments KETRI 243 and 269 were used as DFMO-refractory isolates. These strains killed 100% of mice treated with 2% or 4% DFMO in the drinking water for 3 days, whereas *T. b. brucei* LAB 110 EATRO and *T. b. rhodesiense* strains Wellcome CT, EATRO 105 and KETRI 2538 were 100% cured by 2% DFMO for 3 days. The blood of mice infected with KETRI 243 or 269 and treated with 2% DFMO for 3 days appeared negative or contained $<10^4$ trypanosomes mL^{-1} in thin blood smears for 3–15 days post-treatment. The blood was repopulated with trypanosomes 8–20 days after treatment ceased and the animals died at 12–27 days post-treatment.

To determine whether trypanosomes were sequestered in organs inaccessible to the large dose of DFMO consumed during treatment (5 mL $mouse^{-1}$ day^{-1} for a total dose of 15 g kg^{-1}), organs of mice infected with KETRI 243 and 269 and DFMO treated were excised, washed, macerated and injected into healthy mice. Table 1 indicates that the blood, spleen, liver, and kidney were the primary sources for trypanosomes. Only one of 36 mice infected with brain tissue became parasite positive, and none of the lymph node mice became infected. These studies indicate that sequestering of the parasite in tissue spaces less accessible to drug was not a major factor in reinfection and that a proportion of these trypanosome populations are naturally refractory to DFMO.

Decreased sensitivity of blood forms of these strains was also evident in the growth of strains in the presence of DFMO in the culture system of Hirumi and Hirumi [17]. Table 2 details IC_{50} values obtained with strains testing sensitive or refractory *in vivo*: LAB 110 EATRO and KETRI 2538, both sensitive to 2% DFMO for 3 days, had statistically ($P < 0.05$) lower IC_{50} values than KETRI 243, 269, and 2002.

ODC activity, DFMO uptake and K_i *values.* Common mechanisms of drug resistance in eukaryotic cells include reduced drug uptake, increased activity of the target enzyme or alteration of its inhibition by the drug [20]. DFMO refractory KETRI isolates and cell-free extracts were examined for these properties and compared with drug-sensitive strains (Table 3).

Four of six refractory isolates assimilated DFMO at a rate that was similar to the rates obtained for two sensitive isolates. Two strains (KETRI 2002 and 2285) had reduced DFMO uptake, accumulating [^{3}H]DFMO at 32–59% of the rates obtained with other sensitive and refractory strains. Addition of a 30 μM concentration of the calcium channel antagonists verapamil, diltiazem, nifedipine or desipramine to the incubation medium did not increase the low net uptake rates of these strains (not shown). To determine whether verapamil-independent drug efflux transporters might be operating, blood forms of KETRI 2002 were incubated with [^{3}H]DFMO for 1 hr, washed and suspended in fresh medium with 25–100 μM unlabeled DFMO, and the loss of radiolabel from cells collected on filters was monitored over a 1-hr period. The rates of efflux were compared with those of the DFMO-sensitive strain LAB 110 EATRO, which accumulates DFMO at normal rates, and were found to be essentially the same: LAB 110 EATRO lost 67% of labeled DFMO and KETRI 2285 lost 72% after 1 hr in the presence of 25 μM unlabeled DFMO. Rates of loss were also similar with higher DFMO concentrations.

KETRI 243DFMO is a clone of KETRI 243 derived after continuous passage with DFMO. This strain did not exhibit reduced uptake of DFMO in the *in vitro* uptake studies described above (Table 3).

ODC activity was monitored in eleven isolates and found to be reduced in KETRI 243DFMO and in KETRI 269 (Table 3). ODC activities in cell-free extracts of five isolates were also monitored for sensitivity to DFMO using time-dependent kinetics [21]. The K_i value for the LAB 110 EATRO isolate was 2- to 2.4-fold higher than the other isolates, including DFMO refractory KETRI 243 and 2285. All other K_i values were in the 20–30 μM range.

ODC of mammalian cells has a short half-life, allowing rapid turnover of ODC and decreasing the response to DFMO [22]. The half-life of ODC,

Table 4. Effects of DFMO on polyamine synthesis from [^{3}H]ornithine in trypanosome isolates*

Strain	Treatment	Activity [pmol (mg protein)$^{-1}$ hr^{-1}] Putrescine	Spermidine	Spermine
LAB 110 EATRO (S)	Control	36.03 ± 20.6	3.57 ± 2.1	0.25 ± 0.17
	100 μM DFMO	11.23 ± 7.6	1.62 ± 1.1	0.11 ± 0.09
KETRI 243 (R)	Control	18.1 ± 12.1	3.09 ± 1.5	0.24 ± 0.13
	100 μM DFMO	2.49 + 1.7	1.0 ± 0.5	0.08 ± 0.05
KETRI 2002 (R)	Control	18.6 ± 16.4	1.95 ± 1.7	0.08 ± 0.04
	100 μM DFMO	4.5 ± 3.3	0.7 ± 0.4	0.07 ± 0.02
KETRI 243DFMO (R)	Control	11.11 ± 2.97	4.55 ± 0.42	0.66 ± 0.40
	100 μM DFMO	3.79 ± 1.53	2.89 ± 0.88	0.26 ± 0.08
KETRI 269 (R)	Control	1.64	0.34	0.04
	100 μM DFMO	0.32	0.10	0.02

* Bloodstream trypanosomes were harvested from rats and incubated for 1 hr with 3.3 μCi mL^{-1} of [2,3-^{3}H(N)]ornithine in PSG–BSA as described in Materials and Methods. LAB 110 EATRO and KETRI 243 results are each from three experiments and data are means ± SD. KETRI 2002 and 243DFMO values are from two experiments and are given as means ± range. Analysis of the data using the paired Student's *t*-test ($P < 0.05$ significance) indicated that KETRI 243 and 243DFMO differed from the sensitive pool with respect to lowered putrescine production in both control and DFMO-treated cells. None of the strains was statistically different with respect to per cent inhibition of polyamine production by DFMO.

Table 5. Polyamine content of bloodstream forms of trypanosome isolates*

Strain	Polyamine content [nmol (mg protein)$^{-1}$] Putrescine	Spermidine	Spermine
LAB 110 EATRO (S)	5.1 ± 1.6 (8)	25.3 ± 7.9 (8)	1.5 ± 0.8 (8)
KETRI 2538 (S)	11.7 ± 7.2 (2)	5.9 ± 4.8 (3)	1.2 ± 1.3 (3)
KETRI 2562 (S)	4.1 ± 0.9 (4)	20.6 ± 4.6 (3)	1.3 ± 0.3 (4)
EATRO 105 (S)	4.5 ± 0.4 (6)	14.2 ± 6.7 (6)	1.3 ± 0.2 (7)
Wellcome CT (S)	6.0 ± 1.2 (8)	25.3 ± 11.0 (8)	1.5 ± 0.8 (7)
KETRI 243 (R)	6.1 ± 2.7 (6)	7.6 ± 5.2 (6)	1.6 ± 0.7 (7)
KETRI 2285 (R)	9.6 ± 0.9 (4)	33.5 ± 3.1 (4)	0.6 ± 0.1 (4)
KETRI 2002 (R)	4.7 ± 2.2 (2)	9.9 ± 8.6 (2)	1.0 ± 0.5 (3)
KETRI 1992 (R)	3.5 ± 0.8 (2)	10.8 ± 9.3 (2)	1.4 ± 1.4 (2)
KETRI 269 (R)	3.6 ± 2.0 (5)	5.7 ± 4.9 (5)	1.3 ± 1.0 (5)
KETRI 243DFMO (R)	3.8 ± 1.6 (2)	12.1 ± 4.7 (2)	6.9 ± 1.1 (2)

* Bloodstream trypanosomes were harvested at 72 or 96 hr from infected rats and polyamines were determined using HPLC techniques, as described [18]. Data are means ± range for duplicate determinations, or ±SD where $N > 2$. Analysis of the data using the paired Student's *t*-test ($P < 0.05$ significance) indicated that with respect to putrescine and spermidine content only KETRI 269 was lower than the sensitive strains; with respect to spermidine content, KETRI 2285 was elevated as compared with the sensitive strains.

known to be >4 hr in trypanosomes [15, 23], was also examined in LAB 110 EATRO, KETRI 2538 and KETRI 243 by incubating procyclic (insect midgut) forms in medium plus 50 μg mL^{-1} cycloheximide [15], and monitoring ODC activity over a 6-hr period. The half-life was found to be in excess of 4 hr for each strain (not shown). This study was repeated using blood forms of the same strains harvested from rats and suspended in uptake medium (2.5×10^7 mL^{-1}) containing 50 μg mL^{-1} cycloheximide. Activities of ODC and AdoMet decarboxylase were measured in 10-mL aliquots over 4 hr and found to decline <50% in all cases (not shown).

Inhibition of ODC activity and polyamine synthesis by DFMO. As a further check on the response of sensitive and refractory isolates to DFMO, purified and washed bloodstream trypanosomes were incubated *in vitro* for 1 hr in the presence of 50 μM DFMO (50–300 μM is a physiologically achievable blood dose range [24, 25]), and ODC preparations were made as in Bitonti *et al.* [21]. The inhibition of ODC activity in treated cells was compared and found to be similar in DFMO refractory strains

Table 6. Uptake of exogenous polyamines by bloodstream forms of trypanosomes *in vitro**

Strain	Polyamine uptake [pmol (mg protein)$^{-1}$ hr^{-1}]		
	[^{3}H]Putrescine	[^{3}H[Spermidine	[^{14}C]Spermine
LAB 110 EATRO (S)	1.3 ± 4 (4)	2.2 ± 0.6 (3)	97.2 ± 32.6 (4)
KETRI 2538 (S)	2.9 ± 2.5 (2)	1.4 ± 0.4 (2)	505.0 ± 195 (3)
KETRI 243 (R)	6.1 ± 2.8 (6)	3.3 ± 2.1 (6)	422.0 ± 145 (6)
KETRI 243DFMO (R)	18.9 ± 2.8 (2)	1.0 ± 0.4 (3)	288.4 ± 205.4 (3)
KETRI 2285 (R)	3.7 ± 3.6 (4)	2.0 ± 0.7 (4)	333.7 ± 173.4 (4)
KETRI 1992 (R)	6.6 ± 2.1 (2)	3.7 ± 2.1 (2)	713.4 ± 375.1 (2)
KETRI 2002 (R)	4.6 ± 1.8 (3)	2.7 ± 2.0 (3)	462.0 ± 127 (3)
KETRI 269 (R)	5.1 ± 1.7 (2)	0.7 ± 0 (2)	126.3 ± 11.4 (2)

* Blood form trypanosomes (1×10^8 mL^{-1}) were incubated for 1 hr with labeled polyamine in PSG–BSA as described in Materials and Methods. Data are means ± range for duplicate determinations, or ±SD where $N > 2$. Analysis of the data using the paired Student's *t*-test ($P < 0.05$ significance) indicated that KETRI 243 and 243DFMO differed from the sensitive pool with respect to increased putrescine uptake.

Table 7. Enzymes of AdoMet metabolism in DFMO sensitive and refractory trypanosome isolates*

Strain	AdoMet decarboxylase [nmol (mg protein)$^{-1}$ hr^{-1}]	AdoMet synthetase [nmol (mg protein)$^{-1}$ hr^{-1}]
LAB 110 EATRO (S)	1.27 ± 0.43 (8)	12.60 ± 3.08 (8)
KETRI 2538 (S)	1.32 ± 0.57 (8)	17.34 ± 6.66 (7)
KETRI 243 (R)	0.26 ± 0.18 (7)	3.62 ± 2.44 (8)
KETRI 243DFMO (R)	0.09 ± 0.04 (3)	5.83 ± 3.70 (3)
KETRI 243As (R)	0.67 ± 0.20 (3)	4.60 ± 0.24 (3)
KETRI 269 (R)	0.05 ± 0.01 (4)	4.12 ± 1.66 (3)
KETRI 2002 (R)	0.39 ± 0.23 (3)	6.18 ± 0.98 (2)
KETRI 2285 (R)	0.40 ± 0.24 (3)	

* AdoMet decarboxylase was assayed by trapping $[^{14}C]O_2$ as described in Ref 2. AdoMet synthetase was assayed by trapping the product of [^{14}C]methionine + ATP on filter paper discs as described [16]. All cells were disrupted in the presence of proteinase inhibitors (1 mg mL^{-1} soybean trypsin inhibitor and aprotinin). Data are means ± range for duplicate determinations, or ±SD where $N > 2$. Analysis of the data using the paired Student's *t*-test ($P < 0.05$ significance) indicated that all resistant strains differed from the sensitive pool with respect to both AdoMet decarboxylase and AdoMet synthetase activities.

[KETRI 243 (81%), 2285 (88%)] as well as sensitive isolates [KETRI 2538 (91%), LAB 110 EATRO (90%)] (not shown). Note that KETRI 2285 is a DFMO uptake variant, but still exhibited significant inhibition of ODC activity.

In a similar type of experiment, the effects of DFMO on the ability of strains to synthesize polyamines from [^{3}H]ornithine were also examined (Table 4). In these experiments, washed bloodstream trypanosomes were incubated for 1 hr in PSG–BSA + 3.3 μCi [^{3}H]ornithine in the presence of 100 μM DFMO. After 1 hr the trypanosomes were washed and extracted with dansyl chloride, and the extracts were chromatographed on silica gel plates to allow separation of the polyamines [14]. In a series of experiments comparing LAB 110 EATRO, KETRI 243, KETRI 2002, KETRI 243DFMO, and KETRI 269, putrescine production was reduced 69% in LAB 110 EATRO, 88% in KETRI 243, 77% in KETRI 2002, 66% in KETRI 243DFMO, and 80% in KETRI 269, while spermidine production was decreased 55, 83, 68, 47 and 70%, respectively. These studies indicate that exposure to a clinically achievable drug level inhibits ODC activity and polyamine synthesis in whole cells of DFMO-refractory *T. b. rhodesiense* strains at a rate equal to or greater than that of the sensitive LAB 110 EATRO strain.

KETRI strains 269 and 243 as well as the KETRI 243DFMO clone had lower rates of putrescine synthesis as compared with the LAB 110 EATRO strain, but these were still inhibited to the same extent by DFMO.

Polyamine content and uptake. The polyamine

Table 8. Content of AdoMet and related metabolites in DFMO-treated trypanosomes*

Strain	Treatment	AdoMet	dAdoMet [nmol (mg protein)$^{-1}$]	Total AdoMet
LAB 110 EATRO (S)	Control	0.29	0.81	1.10
	DFMO	21.45 (74)	35.21 (43)	56.66
KETRI 2538 (S)	Control	0.24	0.32	0.56
	DFMO	12.0 (50)	20.30 (63.4)	32.30
KETRI 2538 (S)	Control	0.37	0.11	0.48
	DFMO	15.08 (40.8)	28.12 (255)	43.20
KETRI 243 (R)	Control	0.29	0.71	1.0
	DFMO	2.23 (7.7)	2.85 (4)	5.08
KETRI 243 (R)	Control	1.61	0.27	1.88
	DFMO	4.90 (3.0)	12.10 (44.8)	17.0
KETRI 243 (R)	Control	0.29	0.07	0.36
	DFMO	1.50 (5.2)	5.70 (81)	7.20
KETRI 269 (R)	Control	0.69	0.23	0.92
	DFMO	3.06 (4.4)	4.33 (18.8)	7.39
KETRI 269 (R)	Control	0.92	0.16	1.08
	DFMO	5.88 (6.4)	5.33 (33)	11.21

* Cells were harvested from heavily infected 200 g rats treated for 12–15 hr with 4% DFMO in drinking water. Trypanosomes from three rats were pooled, and cell-free extracts were quantitated for AdoMet and metabolites by HPLC as described [6]. Numbers in parentheses indicate fold-increases over control values.

content of bloodstream trypanosomes harvested from rats was examined using a standard HPLC assay [18]. All the DFMO-sensitive isolates were similar in polyamine content except for KETRI 2538, which had higher putrescine and lower spermidine values (Table 5). Both putrescine and spermidine values appeared to vary in the refractory strains; however, only KETRI 269 was statistically lower in both amines ($P < 0.05$). KETRI 243DFMO apparently had ~4–7 times the spermine levels of any other sensitive or refractory isolate (Table 5); however, this was an average of two values and was not statistically significant.

Uptake of the three major polyamines was examined in bloodforms of DFMO sensitive and refractory isolates. Most strains assimilated low levels of putrescine and spermidine, and all accumulated spermine at 25–200 times the rate of the other polyamines (Table 6). None of the strains that accumulated DFMO at a lower rate (KETRI 269, 2002 and 2285) had statistically ($P < 0.05$) elevated uptake of polyamines. KETRI 243DFMO, however, accumulated putrescine at 2.9 to 13 times the rate of other strains. This response to constant exposure to DFMO has been noted in procyclic forms of *T. b. brucei* continuously cultured in the presence of DFMO [10, 11].

Metabolism of S-*adenosylmethionine.* AdoMet is an important metabolite, serving as the source of aminopropyl groups for spermidine and spermine synthesis and as methyl group donor for most transmethylation reactions [26]. The synthesis and fate of this metabolite have become prominent in the study of DFMO action since it increases dramatically in DFMO-treated trypanosomes [4, 6]. We determined the activities of AdoMet decarboxylase and AdoMet synthetase in DFMO-treated trypanosomes AdoMet synthetase in DFMO sensitive and refractory isolates (Table 7). AdoMet synthetase activity was significantly ($P < 0.05$) higher in sensitive strains ranging from 2-fold higher than KETRI 243As to 29-fold higher than KETRI 269. For each strain, AdoMet decarboxylase activity was far lower than the corresponding AdoMet synthetase activity, ranging from 75-fold lower in KETRI 269 to 10-fold lower for LAB 110 EATRO. DFMO-sensitive strains also had significantly (2- to 4.8-fold) more AdoMet decarboxylase activity than refractory strains. KETRI 269 and KETRI 243DFMO had the lowest activities of all strains tested.

The decreased AdoMet decarboxylase activities in refractory isolates did not appear to be due to destruction or inactivation of the enzyme since, as noted earlier, half-life studies indicated that blood forms of LAB 110 EATRO, KETRI 2538 and KETRI 243 retained >50% of AdoMet decarboxylase activity after a 4-hr incubation in the presence of 50 μg mL^{-1} cycloheximide (not shown).

The differences in activities of enzymes of AdoMet metabolism were also manifested in the intracellular concentrations of AdoMet and decarboxylated AdoMet during DFMO treatment (Table 8). In these experiments, rats infected with DFMO sensitive or refractory strains were treated with DFMO for 12–15 hr, and AdoMet and metabolites were quantitated in trypanosome extracts using HPLC [6]. In each experiment, paired sets of DFMO-treated and control rats were used and trypanosomes from at least three rats were pooled and used for assays.

AdoMet levels increased 40- to 74-fold in 12–15 hr DFMO-treated sensitive isolates. This is a standard response in terms of the magnitude of the increase as well as the final concentration of AdoMet [10–

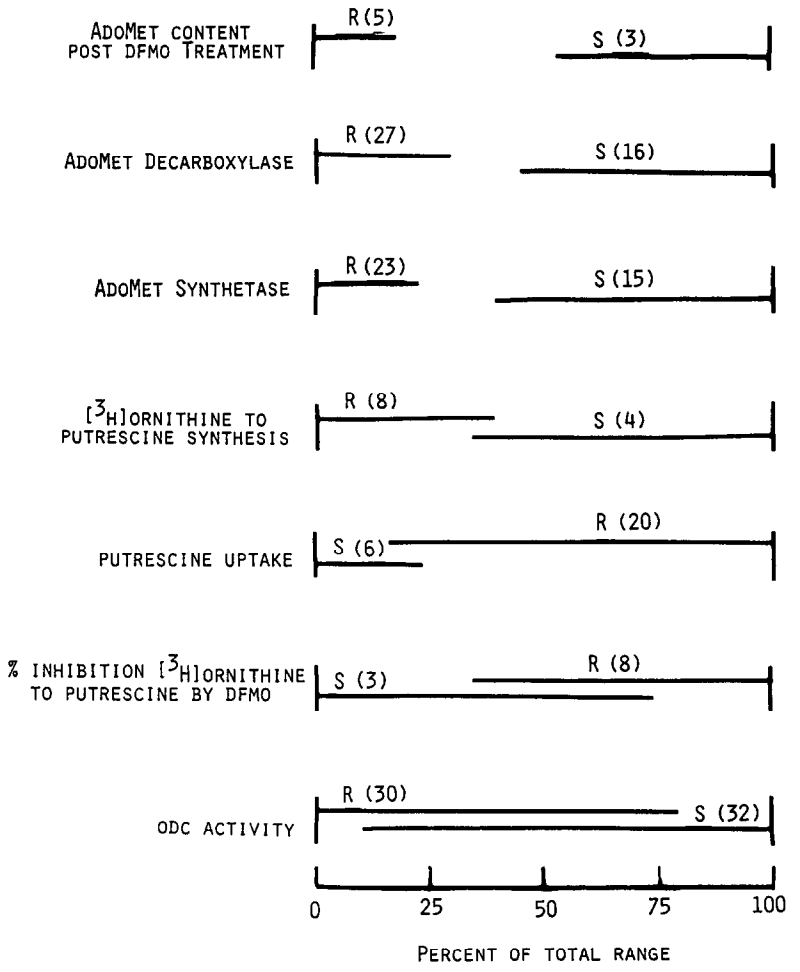

Fig. 1. Analysis of physiological properties of pooled DFMO sensitive and refractory strains showing degree of overlap between ranges of each property. Each pool (S or R) was derived by summing data for each of the strains within the pool. The total range (0–100%) reflects the entire span of values for paired S and R pools. Individual pools were then plotted conforming to the range of values. The degree of overlap in per cent reflects similarity or difference between the pools. The number of values for each range is given in parentheses. Only those properties that differed in a paired Student's *t*-test ($P < 0.05$) are presented. However, ODC activity is included as an example of a property with low separation of means and high overlap of range. Per cent inhibition of [^{3}H]putrescine formation from [^{3}H]ornithine in the presence of 100 μM DFMO illustrates the higher mean and range obtained for the resistant strains and hence the lack of correlation of this property with resistance. The 0–100% ranges for each property were: AdoMet content, 4.89 to 56.1 nmol (mg protein)$^{-1}$ hr^{-1}; AdoMet decarboxylase, 0.053 to 1.790 nmol (mg protein)$^{-1}$ hr^{-1}; AdoMet synthetase, 2.2 to 20.3 nmol (mg protein)$^{-1}$ hr^{-1}; conversion of [^{3}H]ornithine to [^{3}H]putrescine, 3.5 to 57.1 pmol (mg protein)$^{-1}$ hr^{-1}; [^{3}H]putrescine uptake, 0.4 to 12.1 pmol (mg protein)$^{-1}$ hr^{-1}; per cent inhibition of [^{3}H]ornithine to [^{3}H]putrescine conversion by 100 μM DFMO, 59.4 to 84.6%; ODC activity, 21.1 to 79 nmol (mg protein)$^{-1}$ hr^{-1}.

20 nmol (mg protein)$^{-1}$ [6]]. In contrast, increases in AdoMet concentrations in DFMO-treated refractory strains were only 3- to 7.7-fold above controls. Decarboxylated AdoMet also became elevated at 1.6 to 1.9 times that of AdoMet (Table 8). The magnitude of the increase in concentration of these metabolites and final concentrations in DFMO-sensitive strains was not seen in refractory strains. Final concentrations of AdoMet in KETRI 243 and 269 were only 1.5 to ~6 nmol (mg protein)$^{-1}$ after treatment, whereas decarboxylated AdoMet, with the exception of one experiment with KETRI 243, also remained well below 10 nmol (mg protein)$^{-1}$. Total AdoMet pools averaged 44 nmol (mg protein)$^{-1}$ in sensitive strains and 9.6 nmoles (mg protein)$^{-1}$ in resistant strains after DFMO treatment (Table 8), an overall increase of 4.6-fold in sensitive strains. These values were statistically different at a level of $P < 0.05$.

DISCUSSION

The action of DFMO on trypanosomes appears

to be cytostatic in nature, relying on blockade of antigenic variation and a competent immune system for ultimate destruction of the parasite [3, 27, 28]. Maintaining prolonged contact of the parasite with the drug by means of i.v. or multiple oral dosing is therefore a requisite for clinical efficacy [29]. The object of this study was to investigate resistance in clinical isolates. A related goal was to examine the behavior of refractory isolates to determine if invasiveness was related to resistance. In the latter respect, the DFMO-refractory KETRI isolates studied did not appear to establish CNS infections readily in short-term infections. These parasites would have been less accessible to DFMO and required long-term treatment to cure [30]. These data and the *in vitro* data indicate that the refractoriness of the infections to DFMO was due directly to the effects of the drug.

The key metabolic characteristics of DFMO sensitive and refractory isolates which do not show clear correlation with resistance are: ODC activity, the rates of DFMO uptake, K_i values for DFMO, polyamine content, rates of spermidine and spermine uptake, and inhibition of ODC activity and polyamine synthesis during incubation with physiological levels of DFMO. Key aspects of metabolism differing statistically between sensitive and refractory isolates include elevated putrescine uptake, decreased conversion of [^{3}H]ornithine to [^{3}H]putrescine *in vitro*, reduced activity of AdoMet synthetase and AdoMet during exposure to DFMO, all in refractory isolates. The relationships between the pool sizes and degree of overlap for these characteristics are given in Fig. 1.

Figure 1 indicates that for some characteristics, such as enzymes of AdoMet metabolism and total AdoMet pools after DFMO treatment, there was no overlap with the resistant strains consistently present on the lower range of the pool. Hence for these characteristics there was strong correlation with DFMO resistance. With respect to other characteristics, a small (~10%) overlap in the range was present, and there also appeared to be likely characteristics of resistant strains, i.e. elevated putrescine uptake and decreased synthesis of putrescine from ornithine. Other characteristics such as ODC activity and inhibition of putrescine formation from ornithine in the presence of DFMO were shared more completely (40–70%) by both sets of pools and are not likely to influence resistance. Characteristics that completely overlapped (e.g. K_i values for DFMO against ODC and polyamine content) did not influence resistance and are not shown.

These differences, taken together, point to an overall reduction of polyamine synthesis and activity of AdoMet metabolism in refractory strains. In eukaryotic cells, AdoMet links pathways of polyamine production and transmethylation, serving as the aminopropyl group donor for spermidine synthesis and as the methyl group donor for most transmethylation reactions [22, 26, 31]. There is increasing evidence that the metabolism of AdoMet is central to the action of DFMO in trypanosomes: in LAB 110 EATRO, intracellular AdoMet increases >40-fold in cells treated *in vivo* with DFMO [4, 6], while decarboxylated AdoMet increases >1.5-fold above AdoMet levels. These increases serve to raise the combined (AdoMet and decarboxylated AdoMet) intracellular pools from <100 μM in untreated cells to nearly 5 mM in DFMO-treated cells [32]. In mammalian cells treated with DFMO, only minor (under 2-fold) increases in AdoMet are seen as a result of DFMO treatment (33). The increases in the AdoMet pool observed in treated trypanosomes can be attributed to the absence of putrescine, an aminopropyl group acceptor, and to the activity of AdoMet synthetase. In trypanosomes this enzyme is relatively unregulated by its product (K_i 240 μM) as opposed to mammalian cells, in which K_i values of <50 μM are normally found [32]. DFMO refractory isolates had low AdoMet synthetase activity and coincidentally exhibited only marginal increases in AdoMet content during DFMO treatment. This result suggests the presence of differentially controlled AdoMet synthetases in DFMO treatment.

The long-term result of the massive AdoMet buildup in DFMO-sensitive trypanosomes may be hypermethylation of proteins, nucleic acids or lipids, all of which serve as substrates for AdoMet-linked methyltransferases [22, 26]. DFMO-treated LAB 110 EATRO exhibits a 6-fold increase in protein methylase II activity [6, 34]. This enzyme is a carboxymethyltransferase which in mammalian cells is responsible for methylation reactions involving histones and membrane proteins [31, 35]. In recent studies using DFMO-treated blood form trypanosomes *in vitro* [U-^{14}C]methionine was incorporated into protein at approximately ten times the molar rate of [^{35}S]methionine, suggesting a dual role for the universal label in protein synthesis and as a methyl group donor in protein methylation reactions (Bacchi CJ, Garofalo J, Rattendi D, Goldberg B and Yarlett N, unpublished results).

The importance of AdoMet metabolism as a drug target in trypanosomes can be inferred from the activity of MDL 73811, an analog of decarboxylated AdoMet and a potent inhibitor of AdoMet decarboxylase [36, 37]. This agent is curative for the six DFMO-refractory *T. b. rhodesiense* isolates used in the present study [38], and its superior action may be attributed to transport-mediated uptake and the resulting rapid (>20-fold in 1 hr) elevation of AdoMet in trypanosomes treated *in vivo* [4, 39]. In contrast, DFMO elevated AdoMet *in vivo* only after 8–12 hr of treatment ([6]; Yarlett N, Garofalo J, Goldberg B and Bacchi CJ, unpublished results).

Although mechanisms other than aberrant AdoMet synthesis may be contributing to resistance of the *T. b. rhodesiense* isolates, another common mode-of-action is difficult to establish. Although uptake of DFMO was 40–70% lower in two refractory strains (KETRI 2285, 2002) than in other refractory or sensitive isolates, there does not appear to be a correlation between diminished uptake and protection from inhibition of polyamine biosynthesis. Intact cells of KETRI 2002 incubated *in vitro* with [^{3}H]ornithine and DFMO had reduced polyamine synthetic ability comparable with that found in strains with normal uptake (Table 4), and another uptake variant (KETRI 2285) exhibited major

reduction in ODC activity after incubation with DFMO. Moreover, the DFMO levels normally found in blood during treatment (50–300 μM [24, 25, 40]) should ensure that the K_i value is exceeded in the trypanosome and that ODC is inhibited completely even in uptake variants. The uptake of DFMO into *T. b. brucei* was determined to be a non-saturable process, most likely diffusion [41], but the finding of differences in uptake in several strains may indicate that other factors are operating in DFMO entry into some strains.

Because strain KETRI 243DFMO is a clone derived from strain KETRI 243 by drug selection, it would be anticipated that any biochemical changes conferring resistance to the parent strain would be exaggerated in the cloned isolate. Comparison of the results obtained with EATRO 110 (DFMO sensitive), KETRI 243 and KETRI 243DFMO revealed that only ODC activity (8-fold decreased), putrescine uptake (3-fold increased), and AdoMet decarboxylase activity (3-fold decreased) were statistically ($P < 0.001$) different between the KETRI 243DFMO clone and the parent strain. It is interesting to note that only putrescine uptake and AdoMet decarboxylase activity differed between KETRI 243 and the DFMO-sensitive isolate EATRO 110, and further that these two parameters were changed to the same extent (14.5-fold) in KETRI 243DFMO compared with EATRO 110. It is also of interest to note that it is these properties that recur as common differences between all sensitive and resistant isolates, enforcing the importance of these observations.

In one recent study on a procyclic (insect midgut) cell line of *T. b. brucei* made DFMO-refractory *in vitro* by continuous culture with drug, decreased DFMO uptake coupled with increased uptake of putrescine and ornithine were cited as reasons for resistance [11]. In another study using a procyclic cell line derived similarly, decreased DFMO uptake along with elevated ornithine and arginine levels may have formed the basis for resistance [10]. In both studies, the kinetic properties of ODC were not appreciably different from the parent strains. In a third study in which resistance to DFMO was induced by incubating promastigote forms of *Leishmania donovani* with increasing concentrations of drug, enhancement of ODC activity by 15-fold appeared to be the mechanism of resistance [42]. In the present study, ODC activities remained similar in most sensitive and refractory strains, but diminished in KETRI 243DFMO and KETRI 269.

In summary, six DFMO-refractory *T. b. rhodesiense* isolates were studied for mechanism(s) of resistance by examining aspects of polyamine biosynthesis and AdoMet metabolism. Although some strains were uptake deficient for the drug, most had normal uptake and responded to DFMO with significant reductions in ODC activity and polyamine biosynthesis. The AdoMet metabolism of DFMO refractory strains differed from that of sensitive isolates in that synthesis of AdoMet and production of decarboxylated AdoMet was reduced, and refractory strains failed to show appreciable buildup of AdoMet pools during DFMO treatment. This study thus supports other recent work in identifying AdoMet metabolism as an important factor in the mechanism of action of DFMO against African trypanosomes and highlights AdoMet metabolism itself as an important chemotherapeutic target.

Acknowledgements—We thank Karen Alecia, Marcio Costa-Vinhaes, and Laura DelAngel for technical assistance. This work was supported by Grant AI17340 from the National Institutes of Health and by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease (Project 890064).

REFERENCES

1. World Health Organization, *Tenth Programme Report, Tropical Diseases, 1989–1990*. World Health Organization, Geneva, 1991.
2. Bacchi CJ, Garofalo J, Mockenhaupt D, McCann PP, Diekma KA, Pegg AA, Nathan HC, Mullaney EA, Chunosoff LT, Sjoerdsma A and Hutner SH, *In vivo* effects of DL-α-difluoromethylornithine on the metabolism and morphology of *Trypanosoma brucei brucei*. *Mol Biochem Parasitol* **7**: 209–225, 1983.
3. Bitonti AJ, Cross-Doersen DE and McCann PP, Effects of DL-α-difluoromethylornithine on protein synthesis and synthesis of the variant specific glycoprotein (VSG) in *Trypanosoma brucei brucei*. *Biochem J* **250**: 295–298, 1988.
4. Byers TL, Bush TL, McCann PP and Bitonti AJ, Antitrypanosomal effects of polyamine biosynthesis inhibitors correlate with increases in *Trypanosoma brucei brucei S*-adenosyl-L-methionine. *Biochem J* **274**: 527–533, 1991.
5. Fairlamb AH, Henderson GB, Bacchi CJ and Cerami A, *In vivo* effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. *Mol Biochem Parasitol* **24**: 185–191, 1987.
6. Yarlett N and Bacchi CJ, Effect of DL-α-difluoromethylornithine on methionine cycle intermediates in *Trypanosoma brucei brucei*. *Mol Biochem Parasitol* **27**: 1–10, 1988.
7. Mbulamberi DB, Recent epidemic outbreaks of human Trypanosomiasis in Uganda. *Insect Sci Appl* **11**: 289–292, 1990.
8. Bales JD, Harrison SM, Mbwabi DL and Schechter PJ, Treatment of arsenical refractory Rhodesian sleeping sickness in Kenya. *Ann Trop Med Parasitol* **83**: 111–114, 1989.
9. Bacchi CJ, Nathan HC, Livingston T, Valladares G, Saric M, Sayer PD, Njogu AR and Clarkson AB, Jr. Differential susceptibility to DL-α-difluoromethylornithine in clinical isolates of *Trypanosoma brucei rhodesiense*. *Antimicrob Agents Chemother* **34**: 1183–1188, 1990.
10. Bellofatto V, Fairlamb AH, Henderson GB and Cross GAM, Biochemical changes associated with α-difluoromethylornithine uptake and resistance in *Trypanosoma brucei*. *Mol Biochem Parasitol* **25**: 227–238, 1987.
11. Phillips MA and Wang CC, A *Trypansoma brucei* mutant resistant to α-difluoromethylornithine. *Mol Biochem Parasitol* **22**: 9–17, 1987.
12. Yarlett N, Goldberg B, Nathan HC, Garofalo J and Bacchi CJ, Differential sensitivity of *Trypanosoma brucei rhodesiense* isolates to *in vitro* lysis by arsenicals. *Exp Parasitol* **72**: 205–215, 1991.
13. Lanham SM and Godfrey DG, Isolation of salivarian trypanosomes from man and other animals using DEAE cellulose. *Exp Parasitol* **28**: 521–523, 1970.

14. Bacchi CJ, Vergara C, Garofalo J, Lipschik GY and Hutner SH, Synthesis and content of polyamines in bloodstream *Trypanosoma brucei*. *J Protozool* **26**: 484–488, 1979.
15. Bacchi CJ, Garofalo J, Santana A, Hannan JC, Bitonti AJ and McCann PP, *Trypanosoma brucei brucei*: Regulation of ornithine decarboxylase in procyclic forms and trypomstigotes. *Exp Parasitol* **68**: 392–402, 1989.
16. Kappler F, Hai TT and Hampton A, Isozyme-specific enzyme inhibitors. 10. Adenosine 5′-triphosphate derivatives as substrates or inhibitors of methionine adenosyltransferases of rat normal and hepatoma tissues. *J Med Chem* **29**: 318–322, 1986.
17. Hirumi H and Hirumi K, Continuous cultivation of *Trypanosoma brucei* blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers. *J Parasitol* **75**: 985–989, 1989.
18. Yarlett N and Bacchi CJ, Effect of DL-α-difluoromethylornithine on polyamine synthesis and interconversion in *Trichomonas vaginalis* grown in a semi-defined medium. *Mol Biochem Parasitol* **31**: 1–10, 1988.
19. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
20. Hayes JD and Wolf CR, Molecular mechanisms of drug resistance. *Biochem J* **272**: 281–295, 1990.
21. Bitonti AJ, Bacchi CJ, McCann PP and Sjoerdsma A, Catalytic irreversible inhibition of *Trypanosoma brucei brucei* ornithine decarboxylase by substrate and product analogs and their effects on murine trypanosomiasis. *Biochem Pharmacol* **34**: 1773–1777, 1985.
22. Pegg AE, Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res* **48**: 759–774, 1988.
23. Phillips MA, Coffino P and Wang CC, *Trypanosoma brucei* ornithine decarboxylase: Enzyme purification, characterization and expression in *Escherichia coli*. *J Biol Chem* **263**: 17933–17941, 1988.
24. Taelman H, Schechter PJ, Marcelis L, Sonnet J, Kazyumba G, Dasnoy J, Haegele KD, Sjoerdsma A and Wery M, Difluoromethylornithine, an effective new treatment of Gambian trypanosomiasis. *Am J Med* **82**: 607–614, 1987.
25. Van Nieuwenhove S, Schechter PJ, Declerq J, Boné G, Burke J and Sjoerdsma A, Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-α-difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial. *Trans R Soc Trop Med Hyg* **79**: 692–698, 1985.
26. Hoffman RM, Altered methionine metabolism and transmethylation in cancer. *Anticancer Res* **5**: 1–30, 1985.
27. deGee ALW, McCann PP and Mansfield JM, Role of antibody in the elimination of trypanosomes after DL-α-difluoromethylornithine chemotherapy in mice. *J Parasitol* **69**: 818–822, 1983.
28. Bitonti AJ, McCann PP and Sjoerdsma A, Necessity of antibody response in the treatment of African trypanosomiasis with α-difluoromethylornithine. *Biochem Pharmacol* **35**: 331–334, 1986.
29. Schechter PJ and Sjoerdsma A, Therapeutic utility of selected enzyme-activated irreversible inibitors. In: *Enzymes as Targets for Drug Design* (Eds. Palfreyman MG, McCann PP, Temple JG Jr, Sjoerdsma A and Lovenberg W), pp. 201–210. Academic Press, New York, 1989.
30. Bacchi CJ, Nathan HC, Clarkson AB Jr, Bienen EJ, Bitonti AJ, McCann PP and Sjoerdsma A, Effects of the ornithine decarboxylase inhibitors DL-α-difluoromethylornithine and α-monofluoromethyldehydroornithine methylester alone and in combination with suramin against *Trypanosoma brucei brucei* central nervous system models. *Am J Trop Med Hyg* **36**: 46–52, 1987.
31. Van Waarde A, What is the function of protein carboxyl methylation? *Comp Biochem Physiol* **86B**: 423–438, 1987.
32. Yarlett N, Garofalo J, Goldberg B, Ciminelli M, Ruggiero V, Sufrin JR and Bacchi CJ, *S*-Adenosylmethionine synthetase in bloodstream *Trypanosoma brucei*. *Biochim Biophys Acta* **181**: 68–76, 1993.
33. Mamont PS, Danzin C, Wagner J, Siat M, Joder-Ohlenbusch AM and Claverie N, Accumulation of decarboxylated *S*-adenosylmethionine in mammalian cells as a consequence of the inhibition of putrescine biosynthesis. *Eur J Biochem* **123**: 499–504, 1982.
34. Yarlett N, Quamina A and Bacchi CJ, Protein methylases in *Trypanosoma brucei brucei*: Activities and response to DL-α-difluoromethylornithine. *J Gen Microbiol* **137**: 717–724, 1991.
35. Haklai R and Kloog Y, Carboxyl methylation of 21–23 kDa membrane proteins in intact neuroblastoma cells is increased with differentiation. *FEBS Lett* **259**: 233–236, 1990.
36. Casara P, Marchal P, Wagner J and Danzin C, 5′-{[(Z)-4-Amino-2-butenyl]methylamino}-5′-deoxyadenosine: A potent enzyme-activated irreversible inhibitor of *S*-adenosyl-L-methionine decarboxylase from *Escherichia coli*. *J Am Chem Soc* **111**: 9111–9113, 1989.
37. Bitonti AJ, Byers TL, Bush TL, Casara PJ, Bacchi CJ, Clarkson AB Jr, McCann PP and Sjoerdsma A, Cure of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* infections in mice with an irreversible inhibitor of *S*-adenosylmethionine decarboxylase. *Antimicrob Agents Chemother* **34**: 1485–1490, 1990.
38. Bacchi CJ, Nathan HC, Yarlett N, Goldberg B, McCann PP, Bitonti AJ and Sjoerdsma A, Cure of murine *Trypanosoma brucei rhodesiense* infections with an *S*-adenosylmethionine decarboxylase inhibitor. *Antimicrob Agents Chemother* **36**: 2736–2740, 1992.
39. Byers TL, Casara P and Bitonti AJ, Uptake of the antitrypanosoma drug 5′-{[(*Z*)-4-amino-2-butenyl]-methylamino}-5′-deoxyadenosine (MDL73811) by the purine transport system of *Trypanosoma brucei brucei*. *Biochem J* **283**: 755–758, 1992.
40. Abeloff MD, Slavik M, Luk GD, Griffin CA, Hermann J, Blanc O, Sjoerdsma A and Baylin SB, Phase I trial and pharmacokinetic studies of α-difluoromethylornithine—An inhibitor of polyamine biosynthesis. *J Clin Oncol* **2**: 124–130, 1989.
41. Bitonti AJ, Bacchi CJ, McCann PP and Sjoerdsma A, Uptake of α-difluoromethylornithine by *Trypanosoma brucei brucei*. *Biochem Pharmacol* **35**: 351–354, 1986.
42. Coons T, Hanson S, Bitonti AJ, McCann PP and Ullman B, Alpha-difluoromethylornithine resistance in *Leishmania donovani* is associated with increased ornithine decarboxylase activity. *Mol Biochem Parasitol* **39**: 77–90, 1990.