ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1997, p. 1922–1925 Vol. 41, No. 9
0066-4804/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Alterations in Ornithine Decarboxylase Characteristics Account for Tolerance of *Trypanosoma brucei rhodesiense* to D,L-α-Difluoromethylornithine

MONIKA ITEN,[1] HELMUT METT,[2] AMANDA EVANS,[2] JOHN C. K. ENYARU,[3] RETO BRUN,[1] AND RONALD KAMINSKY[1]*

Swiss Tropical Institute[1] and Ciba,[2] Basel, Switzerland, and Livestock Health Research Institute, Tororo, Uganda[3]

Received 14 February 1997/Returned for modification 24 March 1997/Accepted 7 July 1997

Ornithine decarboxylase (ODC), the target enzyme of D,L-α-difluoromethylornithine (DFMO), was investigated in four DFMO-tolerant *Trypanosoma brucei rhodesiense* isolates from East Africa and two DFMO-susceptible *T. b. gambiense* isolates from West Africa. Neither drug uptake nor inhibition of ODC activity by DFMO in cellular extracts differed in the two trypanosome subspecies. However, the specific ODC activity of the cellular extracts was three times as high in *T. b. rhodesiense* isolates as in *T. b. gambiense* isolates. Furthermore, a significant difference in the turnover rate of ODC was observed. The time required to induce a 50% reduction of *T. b. rhodesiense* ODC activity under cycloheximide pressure (tentative half-life) was about 4.3 h, whereas that required for *T. b. gambiense* ODC was longer than 18 h. We concluded that the higher specific ODC activity and faster enzyme turnover contributed to a substantial degree to the DFMO tolerance observed in the East African *T. b. rhodesiense* isolates.

D,L-α-Difluoromethylornithine (DFMO; Ornidyl) is one of only two drugs available for the treatment of late-stage sleeping sickness in Africa. DFMO is used mainly in West Africa to cure relapses after failure of melarsoprol (Arsobal) treatment (25), and until now it has been the only alternative to melarsoprol. Only few clinical data are available concerning its efficacy against East African *Trypanosoma brucei rhodesiense* infections (5), and these indicate the failure of DFMO treatment. Furthermore, only 35% of infections with *T. b. rhodesiense* isolates could be cured by DFMO in a rodent model (3, 4). It was shown that Ugandan *T. b. rhodesiense* isolates and West African *T. b. gambiense* isolates respond very differently to DFMO in vitro (12). While *T. b. gambiense* isolates were highly susceptible to DFMO, 32 Ugandan *T. b. rhodesiense* isolates were tolerant to the drug. Since DFMO has never been used in the area of isolation, the nonresponsiveness of these isolates to DFMO is based not on acquired resistance but on innate tolerance.

DFMO is an enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC) (16), one of the key enzymes in the polyamine biosynthetic pathway. As a derivative of ornithine, it serves as a substrate for ODC. Complete inhibition of ODC results in depletion of the polyamines putrescine and spermidine (1), finally causing cessation of the proliferation of DFMO-exposed cells.

The mechanisms which mediate DFMO tolerance in *T. b. rhodesiense* and therefore cause problems in the DFMO treatment of rhodesiense sleeping sickness patients are not known. In this study, DFMO uptake, putrescine uptake, ODC specific activity, the effect of DFMO on ODC in cellular extracts, and the turnover rate of ODC were investigated to identify the characteristics expressed in the DFMO-tolerant phenotype found in *T. b. rhodesiense* isolates from East Africa.

* Corresponding author. Mailing address: Swiss Tropical Institute, P.O. Box 4002, Basel, Switzerland. Phone: 41 61 2848111. Fax: 41 61 2718654.

† Present address: Institute for Botany, Technical University Munich, 80333 Munich, Germany.

MATERIALS AND METHODS

Trypanosomes. The *T. b. rhodesiense* STIB 859 and STIB 863 stocks studied were isolated from sleeping sickness patients from Uganda and propagated in rodents as described by Iten et al. (12). *T. b. rhodesiense* STIB 878 was isolated from a patient in Uganda, *T. b. rhodesiense* STIB 879 was isolated from a patient in Mozambique, and both were stored as frozen stabilates in the KETRI stabilate bank (Kenya Trypanosomiasis Research Institute, Muguga, Kenya) (3). *T. b. gambiense* stocks (STIB 754B and DAL 1355R) were isolated from patients in the Ivory Coast (15).

Cultivation. Bloodstream form trypanosomes were cultivated in minimum essential medium with Earle's salts (GIBCO/BRL no. 072-01100 P) supplemented with 25 mM HEPES, 1 g of additional glucose per liter, 2.2 g of $NaHCO_3$ per liter, and 10 ml of minimum essential medium nonessential amino acids (100×; GIBCO/BRL no. 11140-035) per liter. The medium was further supplemented as described by Baltz et al. (6), with 0.2 mM 2-mercaptoethanol, 2 mM Na-pyruvate, 0.1 mM hypoxanthine, and 0.016 mM thymidine. Heat-inactivated horse serum (15%) was added to the medium for *T. b. rhodesiense* cultures, and 15% normal human serum and 5% heat-inactivated fetal calf serum were added to the medium for *T. b. gambiense* cultures. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Drug susceptibility assays. Trypanosome stocks were tested for susceptibility to DFMO in a long-term viability assay over a period of 10 days as previously described (13), with minor modifications (12).

The influence of putrescine on DFMO susceptibility was investigated by employing the [^{3}H]hypoxanthine incorporation assay as described by Brun and Kunz (8) over a period of 48 h (40 h preincubation and 8 h of incubation with [^{3}H]hypoxanthine).

DFMO uptake experiments. Uptake of [^{3}H]DFMO was measured by using a modification of the method described by Bacchi et al. (4). Briefly, bloodstream trypanosomes were harvested from cultures and resuspended (10^8/ml) in PSG buffer (PSG at 6:4 for *T. b. rhodesiense* and PSG at 4:6 for *T. b. gambiense* [14]) supplemented with D,L-α-[3,4-^{3}H]DFMO (10 μCi/ml, 22.7 Ci/mmol; New England Nuclear, Dupont, Regensdorf, Switzerland) and nonlabelled DFMO to a final concentration of 70 μM. The cells were incubated for 60 min at 37°C in a humidified atmosphere of 5% CO_2 in air. One-milliliter aliquots were centrifuged through oil (90% dibutylphthalate–10% paraffin [vol/vol]) at 14,000 × *g* for 15 s. Tubes were transferred to liquid nitrogen, and the tube tips with the pellet were cut off, placed in 5 ml of scintillation liquid (Ecoscint A; National Diagnostics, Atlanta, Ga.), and counted for 1 min each in a liquid scintillation counter (MR 300; Kontron Instruments, Zürich, Switzerland). Uptake was based on the total DFMO accumulated after 1 h (4).

Measurement of [^{3}H]putrescine uptake was performed with trypanosomes preincubated with DFMO (12.7 μM) for 16 h and with untreated trypanosomes. The cells were prepared as described above. [2,3-^{3}H(*N*)]putrescine at 10 μCi/ml (35 Ci/mmol; New England Nuclear, Dupont) was added to the buffer. The experiments were completed analogously to the DFMO uptake experiments.

Enzyme activities. Trypanosomes were harvested either from cultures by centrifugation, for determination of the ODC turnover rate, or from mouse blood,

TABLE 1. Biochemical characteristics of DFMO-tolerant and -susceptible trypanosomes[a]

Species and stock	DFMO MIC (μM)[b]	Mean uptake (pmol/10^8 cells/h) ± SD[c]		Mean ODC sp act (nmol of CO_2/mg of protein/h) ± SD	Mean IC_{50} of DFMO for ODC (μM) ± SD	Tentative ODC half-life (h)
		DFMO	Putrescine			
T. b. rhodesiense						
STIB 859	211	200.4 ± 9.5	0.27 ± 0.03	76 ± 1.1	27 ± 1.6	4.3
STIB 863	211	212.9 ± 65.2	0.39 ± 0.12	65 ± 3.3	29 ± 0.4	4.3
STIB 878	106	190.5 ± 10.5	0.23 ± 0.01	69 ± 0.6	34 ± 1.2	4.2
STIB 879	106	161.7 ± 34.3	0.27 ± 0.08	83 ± 3.5	24 ± 1.1	4.4
T. b. gambiense						
STIB 754B	7	180.5 ± 26.6	0.27 ± 0.06	25 ± 0.1	31 ± 3.1	18
DAL 1355R	20	207.6 ± 14.2	0.35 ± 0.03	21 ± 0.8	35 ± 0.4	19

[a] The values given are means of three experiments, each performed in duplicate.
[b] The DFMO MIC was determined after an incubation period of 10 days.
[c] Uptake experiments were performed with 70 μM DFMO and 290 nM putrescine.

for determination of ODC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity. After propagation of *T. b. rhodesiense* in Swiss ICR mice and of *T. b. gambiense* in *Mastomys natalensis*, parasites were isolated from blood as previously described (14), with the minor modification that DE53 cellulose was used instead of DE52 cellulose. Purified trypanosomes were suspended in lysis buffer (50 mM Na_2HPO_4-NaH_2PO_4, 0.1 mM EDTA, 2 mM dithiothreitol, 1 mg of aprotinin per ml, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) and homogenized by freeze-thawing. After centrifugation at 10,000 × *g* for 30 min at 10°C, the supernatant was stored in aliquots at −80°C. The ODC activity of cell extracts was determined as previously described (19), and GAPDH activity was determined as described by Hamm et al. (11).

[^{3}H]leucine incorporation into proteins. Trypanosomes were cultivated in the medium described above lacking leucine (GIBCO/BRL no. 041-01895). L-[4,5-^{3}H]leucine (120 to 190 Ci/mmol; Amersham, Rahn AG, Zürich, Switzerland) was added to log phase cultures at a final concentration of 0.5 μCi/ml. After 10 min of preincubation at 37°C, 50 μg of cycloheximide per ml was added. At this concentration, cycloheximide did not show toxic effects on trypanosomes over the observation period of 24 h (unpublished observations). At various time points up to 24 h, 6-ml aliquots were filtered through GF/B filters (Whatman, Maidstone, England). Proteins were precipitated on the filter discs with 10% trichloroacetic acid and then washed with ice-cold 5% trichloroacetic acid. After drying, filters were washed with ice-cold 70% ethanol. Dried filter discs were processed as described above for DFMO uptake experiments.

Northern blot analysis. The ODC DNA probe was generated by PCR with primers based on the previously cloned ODC sequence from *T. b. brucei* (20). A sense primer, 5′-CTGCAGCAACACTGAGATACAACGTGTGAGAGGCA-3′ (amino acid positions 111 to 120), and an antisense primer, 5′-CT GCAGCAAGTGTGAAAGCTGAAGCAACGTAGTACC-3′ (amino acid positions 297 to 306), were constructed. Both contained *Pst*I restriction sites inserted at the 5′ end for subcloning. One microgram of *T. b. brucei* STIB 920 genomic DNA was added to a reaction mixture containing 1× PCR buffer (Boehringer Mannheim), 10% (vol/vol) dimethyl sulfoxide, each deoxynucleoside triphosphate at 200 μM, 1 μg of each oligonucleotide primer, and 2.5 U of *Taq* DNA polymerase in a volume of 100 μl. The reaction mixture was incubated in a thermocycler at 95, 42, and 72°C for 1, 2, and 2 min, respectively, for 40 cycles. Both PCR bands were subcloned into the bluescript KS+ (Stratagene) *Pst*I site, and the sequences were confirmed.

Total RNA was isolated by employing the Trizol reagent (GIBCO/BRL no. 15596-026) in accordance with the product descriptions, and 20 μg was electrophoresed on 1.5% formaldehyde gels and transferred onto Hybond N+ membranes. Filters were hybridized with a ^{32}P-labelled ODC probe (random prime kit; Boehringer Mannheim) at 42°C overnight. The blots were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate with increasing temperature to remove the nonspecifically bound probe. All blots were visualized by autoradiography.

RESULTS

Drug susceptibility. The DFMO susceptibilities of four East African *T. b. rhodesiense* isolates and two West African *T. b. gambiense* isolates were determined in the 10-day feeder layer viability assay (Table 1). The MICs for the four *T. b. rhodesiense* stocks varied from 106 to 211 μM, while those for the West African *T. b. gambiense* stocks were 7 and 20 μM.

Uptake experiments. Both subspecies, the susceptible *T. b. gambiense* isolates and the tolerant *T. b. rhodesiense* isolates, showed similar uptakes of radiolabelled DFMO and putrescine (Table 1). The amount of DFMO taken up varied between 161.7 and 212.9 pmol/10^8 cells/h and was similar to the amount taken up at 4°C, which was between 157.2 and 220.8 pmol/10^8 cells/h (data not shown). The polyamine putrescine was taken up to levels of 0.23 to 0.39 pmol/10^8 cells/h in untreated trypanosomes (Table 1) and 0.21 to 0.39 pmol/10^8 cells/h in trypanosomes pretreated for 16 h with 12.7 μM DFMO (data not shown).

Effect of putrescine on DFMO susceptibility. The effect of DFMO on the *T. b. gambiense* STIB 754B stock was abolished by putrescine (Fig. 1). The same effect of putrescine on DFMO-treated *T. b. gambiense* DAL 1355R was observed (data not shown). When up to 0.1 mM putrescine was used, no effect on DFMO susceptibility could be measured, whereas at concentrations higher than 1 mM, the DFMO effect was fully reversed.

Enzyme activities. ODC and GAPDH activities were analyzed in crude extracts of the six isolates. The ODC specific activity for the *T. b. rhodesiense* isolates was in the range of 65 to 83 nmol of CO_2/mg of protein/h (Table 1), about three times the specific activity found in the *T. b. gambiense* isolates, which was in the range of 21 to 25 nmol of CO_2/mg of protein/h. The GAPDH specific activity was in the range of 3.8 to 4.6 U/mg of

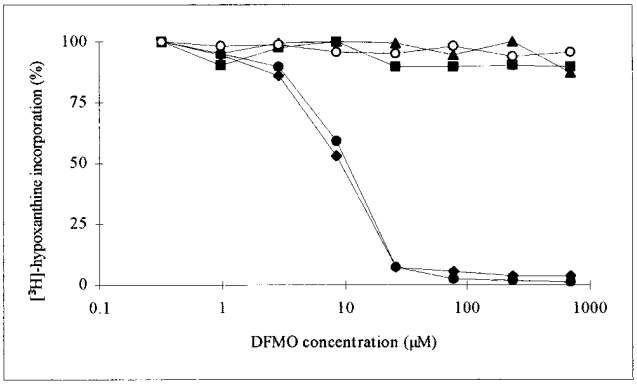

FIG. 1. Effect of putrescine on the DFMO susceptibility of *T. b. gambiense* STIB 754B in vitro determined with the [^{3}H]hypoxanthine incorporation assay (8) over a period of 48 h. Symbols: ●, without putrescine; ◆, in the presence of 0.1 mM putrescine; ▲, 1 mM putrescine; ■, 10 mM putrescine; ○, DFMO-tolerant *T. b. rhodesiense* STIB 863 without putrescine. ^{3}H incorporation is given as a percentage of the ^{3}H incorporation in the control population (without DFMO).

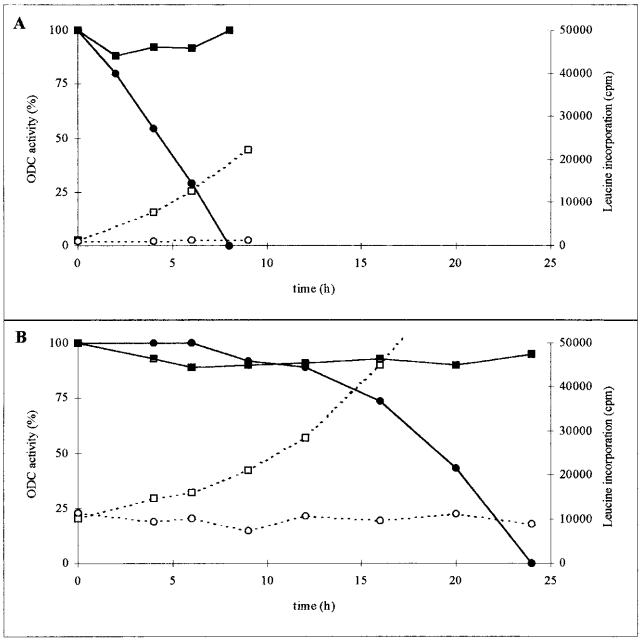

FIG. 2. Tentative half-life of ODC. A, *T. b. rhodesiense* STIB 863; B, *T. b. gambiense* STIB 754B. Symbols: ●, ODC activity in the presence of cycloheximide (50 μg/ml); ■, ODC activity without cycloheximide; ○, [^{3}H]leucine incorporation in the presence of cycloheximide; □, [^{3}H]leucine incorporation without cycloheximide (values for *T. b. gambiense* at 20 and 24 h were 66,000 and 88,000 cpm, respectively). ODC activity is given as a percentage of the activity at time zero. All values are normalized for equal amounts of trypanosomal protein.

protein for both *T. b. rhodesiense* and *T. b. gambiense* (data not shown).

The effect of DFMO on ODC activity was also analyzed in cellular extracts (Table 1). The range of 50% inhibitory concentrations (IC_{50}s) for all isolates was 24 to 35 μM. There was no significant difference between *T. b. rhodesiense* and *T. b. gambiense* regarding inhibition of ODC by DFMO.

Tentative half-life of ODC. The turnover rate of ODC in the different species was determined by measuring the decrease in ODC activity in cells in which protein synthesis had been inhibited by cycloheximide. [^{3}H]leucine incorporation was determined to confirm that protein synthesis had effectively been blocked (Fig. 2). The tentative half-life of ODC was significantly different in *T. b. rhodesiense* and *T. b. gambiense* isolates. The half-life of *T. b. rhodesiense* ODC was 4.2 to 4.4 h, whereas that of *T. b. gambiense* ODC was 18 to 19 h (Table 1 and Fig. 2).

ODC mRNA expression. Northern blot analysis of trypanosomal RNA revealed single transcripts at 2.4 kb for *T. b. rhodesiense* STIB 863 and 2.2 kb for *T. b. gambiense* STIB 754B which hybridized to the ODC probe (Fig. 3).

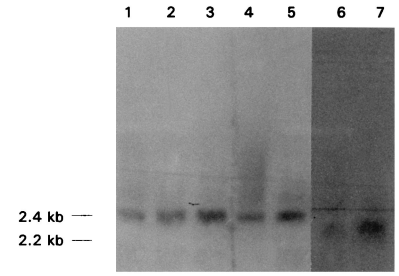

FIG. 3. Northern analysis of ODC mRNA. Lanes 1 to 5 show *T. b. rhodesiense* STIB 863 mRNA after DFMO exposure (12.7 μM) for 0, 8, 16, 24, and 36 h, respectively. Lanes 6 and 7 show *T. b. gambiense* STIB 754B mRNA after drug exposure for 0 and 16 h. Twenty micrograms of total RNA was loaded per lane.

DISCUSSION

The four East African *T. b. rhodesiense* isolates investigated in this study are representative samples of 32 isolates tolerant to DFMO (12). The MICs for the four *T. b. rhodesiense* isolates were >100 μM, which is higher than the DFMO levels in plasma or cerebrospinal fluid reached during treatment (the mean concentration in cerebrospinal fluid was 69 ± 33 μM [17]). The MICs for the West African *T. b. gambiense* isolates were <20 μM, which is below therapeutically achievable levels.

The only known target enzyme for DFMO is ODC (16). Drug uptake and trypanosomal ODC were characterized to analyze the differences in susceptibility between the two *T. b. brucei* subspecies. No differences in DFMO uptake were observed between the tolerant *T. b. rhodesiense* isolates and the susceptible *T. b. gambiense* isolates. In contrast to the observations made on procyclic (tsetse fly midgut-like) forms by Phillips and Wang (21) and Bellofatto et al. (6b), reduced uptake or net accumulation of DFMO, as has been observed in laboratory-induced DFMO-resistant *T. b. brucei* (6b, 21), did not seem to be the reason for drug tolerance in the *T. b. rhodesiense* isolates investigated in this study. Uptake studies performed on ice indicated that DFMO uptake is mediated by diffusion rather than by active transport. These observations confirm earlier findings by Bitonti et al. (7).

The product of the reaction catalyzed by ODC is putrescine. Total inhibition of ODC by DFMO leads to depletion of the product putrescine (1). A possible way to bypass this blockage is the uptake of putrescine. However, in the six strains described here, putrescine uptake did not differ significantly between tolerant *T. b. rhodesiense* and susceptible *T. b. gambiense* (0.23 to 0.39 pmol/10^8 cells/h), which is different from earlier observations by Phillips and Wang (21), who found three- to fourfold increased putrescine uptake in DFMO-resistant procyclic (tsetse fly midgut-like) forms of *T. b. brucei*. The growth-inhibitory effect of DFMO could only be reversed with putrescine concentrations higher than 1 mM. No reversion of DFMO-mediated growth inhibition was detected at a putrescine concentration of 0.1 mM, which is still 500 fold higher than the level achievable in blood (220 nM [9]). Thus, under physiological conditions, blockage of ODC by DFMO is unlikely to be bypassed by increased putrescine uptake.

DFMO inhibited ODC in cellular extracts of all six of the isolates investigated to the same degree with IC_{50}s of 24 to 35 μM, indicating that the affinity of ODC to DFMO did not differ in the two trypanosome subspecies. However, the two subspecies differed with respect to ODC specific activity, which was about three times as high in *T. b. rhodesiense* extracts as in *T. b. gambiense* extracts. The higher specific activity was specific for ODC. As a control, GAPDH did not show higher specific activity in *T. b. rhodesiense*, which was in the range of previously published data (11). The observed differences in ODC specific activity between the two subspecies may be significant regarding DFMO susceptibility of trypanosomes. The intracellular DFMO concentration after 1 h of DFMO exposure can be calculated as 54 to 71 μM, considering an average cell volume of 30 fl and a DFMO uptake of 161.7 to 212.9 pmol/10^8 cells/h (Table 1). Fifty percent inhibition of trypanosomal ODC was achieved with half of the calculated intracellular DFMO concentration (24 to 35 μM; Table 1). The ODC

mRNA transcript of the DFMO-tolerant *T. b. rhodesiense* isolates was the same size as that found in *T. b. brucei* (2.4 kb [20]). However, the ODC mRNA of DFMO-susceptible *T. b. gambiense* was slightly shorter (2.2 kb), which indicates that structural differences may be present at the mRNA level which could have an impact on the regulation of the synthesis and/or degradation of the ODC mRNA.

Human cells have been shown to be little affected by DFMO, because they have a rapid turnover of ODC (half-life, 10 to 20 min [24]) which is efficiently regulated and promptly responds to changing physiological conditions. A reduction of the ODC activity to 50% during cycloheximide treatment was reached within 4.2 to 4.4 h in DFMO-tolerant *T. b. rhodesiense*. This time interval was considerably shorter than that for *T. b. gambiense*, although it was still much longer than the half-life of mammalian ODC. Bacchi et al. (2) determined a 5-h ODC half-life in bloodstream forms of *T. b. rhodesiense* isolates in rodents by using a similar experimental approach. The key role of ODC in the polyamine pathway has been studied extensively in mammalian cells. The very short-lived mammalian ODC (24) is feedback regulated by polyamines. These polyamines, in excessive concentrations, also induce antizyme (18), a noncompetitive inhibitory protein which associates with the ODC, leading to inactivation and degradation of ODC by mammalian 26S proteasome (10). The C-terminal PEST sequence, a proline (P)-, glutamic acid (E)-, serine (S)-, and threonine (T)-rich region (22), is assumed to be involved in the fast degradation, and therefore in the short half-life, of ODC in mammalian cells. In contrast, trypanosomal (*T. b. brucei*) ODC does not contain PEST sequences contributing to a short half-life (7, 20). However, the trypanosomatid *Crithidia fasciculata* has a short half-life of 30 min although it lacks the C-terminal degradation domain (23). The mechanisms mediating the differences in the ODC turnover rates of *T. b. rhodesiense* and *T. b. gambiense* established in this study are not known and remain to be investigated.

The DFMO tolerance of East African *T. b. rhodesiense* isolates cannot be explained by only one mechanism. It appears, rather, that the sum of several factors leads to a DFMO-tolerant phenotype. Two of the factors involved in DFMO tolerance could be identified: (i) higher specific ODC activity and (ii) a shorter tentative half-life of the enzyme. These findings indicate that the failure of DFMO treatment of East African *T. b. rhodesiense* infections is due to an innate lack of susceptibility of the parasite caused by alterations of the target enzyme and/or its regulation.

ACKNOWLEDGMENTS

We are grateful to Cyrus J. Bacchi for the kind gift of the trypanosome stabilates KETRI 2538 and 2708. We thank Terrence O'Reilly for the opportunity to work in his laboratories, Barbara Schacher for technical support, and Jennifer Jenkins for critical reading of the manuscript.

This work was financially supported by the Swiss Development Cooperation (DEH/SDC).

REFERENCES

1. **Bacchi, C. J., J. Garofalo, D. Mockenhaupt, P. P. McCann, K. A. Diekema, A. E. Pegg, H. C. Nathan, E. A. Mullanfy, L. Chunosoff, A. Sjoerdsma, and S. H. Hutner.** 1983. In vivo effects of DL-α-difluoromethylornithine on the metabolism and morphology of *Trypanosoma brucei brucei*. Mol. Biochem. Parasitol. **7:**209–225.
2. **Bacchi, C. J., J. Garofalo, A. Santana, J. C. Hannan, A. J. Bitonti, and P. P. McCann.** 1989. *Trypanosoma brucei brucei*: regulation of ornithine decarboxylase in procyclic forms and trypomastigotes. Exp. Parasitol. **68:**392–402.
3. **Bacchi, C. J., H. C. Nathan, T. Livingston, G. Valladares, M. Saric, P. D. Sayer, A. R. Njogu, and A. B. Clarkson.** 1990. Differential susceptibility to DL-α-difluoromethylornithine in clinical isolates of *Trypanosoma brucei rhodesiense*. Antimicrob. Agents Chemother. **34:**1183–1188.
4. **Bacchi, C. J., J. Garofalo, M. Ciminelli, D. Rattendi, B. Goldberg, P. P. McCann, and N. Yarlett.** 1993. Resistance to DL-α-difluoromethylornithine by clinical isolates of *Trypanosoma brucei rhodesiense*: role of *S*-adenosylmethionine. Biochem. Pharmacol. **46:**471–481.
5. **Bales, J. D., S. M. Harrison, D. L. Mbwabi, and P. J. Schechter.** 1989. Treatment of arsenical refractory Rhodesian sleeping sickness in Kenya. Ann. Trop. Med. Parasitol. **1:**111–114.
6. **Baltz, T., D. Blatz, Ch. Giroud, and J. Crockett.** 1985. Cultivation in a semidefined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. EMBO J. **4:**1273–1277.

6b. **Bellofatto, V., A. A. Fairlamb, G. B. Henderson, and G. A. M. Cross.** 1987. Biochemical changes associated with α-difluoromethylornithine uptake and resistance in *Trypanosoma brucei*. Mol. Biochem. Parasitol. **25:**227–238.

7. **Bitonti, A. J., C. J. Bacchi, P. P. McCann, and A. Sjoedsma.** 1986. Uptake of α-difluoromethylornithine by *Trypanosoma brucei brucei*. Biochem. Pharmacol. **35:**351–354.
8. **Brun, R., and C. Kunz.** 1989. In vitro drug sensitivity test for *Trypanosoma brucei* subgroup bloodstream trypomastigotes. Acta Trop. **46:**361–368.
9. **Cooper, K. D., J. B. Shukla, and O. M. Rennert.** 1978. Polyamine compartimentalization in various human disease states. Clin. Chim. Acta **82:**1–7.
10. **Elias, S., B. Bercovich, C. Kahana, P. Coffino, M. Fischer, W. Hilt, D. H. Wolf, and A. Ciechanover.** 1995. Degradation of ornithine decarboxylase by the mammalian and yeast 26S proteasome complexes requires all the components of the protease. Eur. J. Biochem. **229:**276–283.
11. **Hamm, B., A. Schindler, D. Mecke, and M. Duszenko.** 1990. Differentiation of *Trypanosoma brucei* trypomastigotes from long slender to short stumpy-like forms in axenic culture. Mol. Biochem. Parasitol. **40:**13–22.
12. **Iten, M., E. Matovu, R. Brun, and R. Kaminsky.** 1995. Innate lack of susceptibility of Ugandan *Trypanosoma brucei rhodesiense* to DL-α-difluoromethylornithine (DFMO). Trop. Med. Parasitol. **46:**190–194.
13. **Kaminsky, R., F. Chuma, and E. Zweygarth.** 1989. *Trypanosoma brucei brucei*: expression of drug resistance in vitro. Exp. Parasitol. **69:**281–289.
14. **Lanham, S. M., and D. G. Godfrey.** 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. **28:**521–534.
15. **Mehlitz, D., U. Brinkmann, and L. Haller.** 1981. Epidemiological studies on the animal reservoir of gambiense sleeping sickness. Part I. Review of literature and description of the study areas. Tropenmed. Parasitol. **32:**129–133.
16. **Metcalf, B. W., P. Bey, C. Danzin, M. J. Jung, P. Casara, and J. P. Vevert.** 1978. Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C.4.1.1.17) by substrate and product analogues. J. Am. Chem. Soc. **100:** 2551–2553.
17. **Milord, F., L. Loko, L. Ethier, B. Mpia, and J. Pépin.** 1993. Eflornithine concentrations in serum and cerebrospinal fluid of 63 patients treated for *Trypanosoma brucei gambiense* sleeping sickness. Trans. R. Soc. Trop. Med. Hyg. **87:**473–477.
18. **Murakami, Y., S. Matsufuji, Y. Miyazaki, and S. Hayash.** 1992. Destabilization of ornithine decarboxylase by transfected antizyme gene expression in hepatoma tissue culture cells. J. Biol. Chem. **267:**13138–13141.
19. **Pegg, A. E., and P. P. McCann.** 1982. Polyamine metabolism and function. Am. J. Cell. Physiol. **243:**C212–C221.
20. **Phillips, M. A., P. Coffino, and C. C. Wang.** 1987. Cloning and sequencing of the ornithine decarboxylase gene from *Trypanosoma brucei*. J. Biol. Chem. **262:**8721–8727.
21. **Phillips, M. A., and C. C. Wang.** 1987. A *Trypanosoma brucei* mutant resistant to α-difluoromethylornithine. Mol. Biol. Parasitol. **22:**9–17.
22. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234:** 364–368.
23. **Svensson, F., C. Ceriani, E. L. Wallström, I. Kockum, I. D. Algranati, O. Heby, and L. Persson.** 1997. Cloning of a trypanosomatid gene coding for an ornithine decarboxylase that is metabolically unstable even though it lacks the C-terminal degradation domain. Proc. Natl. Acad. Sci. USA **94:**397–402.
24. **Tabor, C. W., and H. Tabor.** 1984. Polyamines. Annu. Rev. Biochem. **53:** 749–790.
25. **Van Nieuwenhove, S., P. J. Schechter, J. Declercq, G. Bone, J. Burke, and A. Sjoerdsma.** 1985. 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.