Alterations in Ornithine Decarboxylase Characteristics Account for Tolerance of Trypanosoma brucei rhodesiense to D,L-\(\text{-}\alpha\)-Difluoromethylornithine

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Ornithine decarboxylase (ODC), the target enzyme of D,L-\(\alpha\)-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-\(\alpha\)-Difluoromethylornithine (DFMO; Ornidy) 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.

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 stablites in the KETRI stablitate bank (Kenya Trypanosomiasis Research Institute, Muguga, Kenya) (3). T. b. gambiense stocks (STIB 754B and DAL 1359R) 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 NaHCO3 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% CO2 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 [3H]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 [3H]hypoxanthine).

DFMO uptake experiments. Uptake of [3H]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 (107/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-\(\alpha\)-[3,4-3H]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% CO2 in air. One-milliliter aliquots were centrifuged through oil (90% dibutylphthalate–10% paraffin [vol/vol]) at 14,000 × g for 15 s. The tubes were transferred to liquid nitrogen, and the tube tips with the pellet were cut off, placed in 5 ml of scintillation liquid (Ecosint 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 [3H]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. [2,3-3H]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,

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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 NaH2PO4-Na2HPO4, 0.1 mM EDTA, 2 mM dithiothreitol, 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).

**TABLE 1. Biochemical characteristics of DFMO-tolerant and -susceptible trypanosomes**

<table>
<thead>
<tr>
<th>Species and stock</th>
<th>DFMO MIC (µM)</th>
<th>Mean uptake (pmol/10^8 cells/h) ± SD</th>
<th>Mean ODC sp act (nmol of CO2/mg of protein/h) ± SD</th>
<th>Mean IC50 of DFMO for ODC (µM) ± SD</th>
<th>Tentative ODC half-life (h)</th>
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</thead>
<tbody>
<tr>
<td>T. b. rhodesiense</td>
<td></td>
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<tr>
<td>STIB 859</td>
<td>211</td>
<td>200.4 ± 9.5</td>
<td>0.27 ± 0.03</td>
<td>76 ± 1.1</td>
<td>27 ± 1.6</td>
</tr>
<tr>
<td>STIB 863</td>
<td>211</td>
<td>212.9 ± 65.2</td>
<td>0.39 ± 0.12</td>
<td>65 ± 3.3</td>
<td>29 ± 0.4</td>
</tr>
<tr>
<td>STIB 878</td>
<td>106</td>
<td>190.5 ± 10.5</td>
<td>0.23 ± 0.01</td>
<td>69 ± 0.6</td>
<td>34 ± 1.2</td>
</tr>
<tr>
<td>STIB 879</td>
<td>106</td>
<td>161.7 ± 34.3</td>
<td>0.27 ± 0.08</td>
<td>83 ± 3.5</td>
<td>24 ± 1.1</td>
</tr>
<tr>
<td>T. b. gambiense</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>STIB 754B</td>
<td>7</td>
<td>180.5 ± 26.6</td>
<td>0.27 ± 0.06</td>
<td>25 ± 0.1</td>
<td>31 ± 3.1</td>
</tr>
<tr>
<td>DAL 1355R</td>
<td>20</td>
<td>207.6 ± 14.2</td>
<td>0.35 ± 0.03</td>
<td>21 ± 0.8</td>
<td>35 ± 0.4</td>
</tr>
</tbody>
</table>

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

**FIG. 1.** Effect of putrescine on the DFMO susceptibility of 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 CO2/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 CO2/mg of protein/h. The GAPDH specific activity was in the range of 3.8 to 4.6 U/mg of...
inhibited by cycloheximide. [3H]leucine incorporation was de-

determined to confirm that protein synthesis had effectively been

an effect of DFMO on ODC activity was also analyzed in

cellular extracts (Table 1). The range of 50% inhibitory con-

centrations (IC50s) for all isolates was 24 to 35

cell volume of 30 fl and a DFMO uptake of 161.7 to 212.9

cpm, respectively). ODC activity is given as a percentage of the activity at time

The only known target enzyme for DFMO is ODC (16).

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 (IC50s) 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. [3H]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).

**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.

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/10⁸ 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 IC50s 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/10⁸ 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 isolate 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.

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