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A Novel Suicide Inhibitor Strategy for Antiparasitic Drug Development

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Abstract DL-α-Difluoromethylornithine (DFMO), a suicide inhibitor of eukaryotic ornithine decarboxylase (ODC), has therapeutic activities against African trypanosomiasis. The K_i value of DFMO for ODC of *Trypanosoma brucei* is somewhat higher than that for mouse ODC. The therapeutic efficacy of DFMO cannot therefore be attributed to a preferential inhibition of the parasite enzyme. The *T. brucei* gene encoding ODC was cloned and sequenced, and the derived amino acid sequence has 61.5% homology with that of mouse ODC, except that the C-terminal 36 amino acids of the mouse enzyme are missing from the parasite enzyme. The cloned *T. brucei* and mouse ODC genes were expressed in ODC-deficient Chinese hamster ovary cells (CHO) where the *T. brucei* enzyme was stable, but mouse ODC was unstable. Thus, the observed difference in intracellular stability is a property of the ODC protein itself, rather than of the cellular environment in which it is expressed. A chimeric ODC composed of the amino terminus of trypanosome ODC and the C-terminus of mouse ODC also was rapidly degraded in CHO cells, suggesting that peptide sequences in the mouse ODC carboxy-terminus determine its stability.

The relatively slow turnover of the parasite enzyme constitutes the basis of selective antitrypanosomal action of DFMO. By this same token, many other proteins known to perform crucial functions in bacteria, fungi, protozoa, helminths, etc., also may have shorter half-lives in the mammalian hosts than in parasites. Suicide inhibitors of these proteins may have desirable characteristics as good chemotherapeutic agents. This new approach could provide an additional strategy for controlling infectious diseases.

Key words: *Trypanosoma brucei*, ornithine decarboxylase, in vivo half-life, PEST hypothesis, DL-α-Difluoromethylornithine

The formation of polyamines required for cellular proliferation is controlled by ornithine decarboxylase (ODC) in eukaryotes [1]. In the actively dividing long-slender bloodstream trypomastigotes of African trypanosomes, putrescine and spermidine constitute the main pool of polyamines [2]. They are mainly synthesized from ornithine in *Trypanosoma brucei* and are taken up only very slowly and rather sparingly from extracellular sources [3]. DL-α-Difluoromethylornithine (DFMO), a catalytic irreversible inhibitor of mammalian ODC, has recently demonstrated good therapeutic activities against African trypanosomiasis in animal models as well as in humans [4]. It acts on *T. brucei* by inhibiting the ODC of the parasite. The physiological consequence of such enzyme inhibition turns out to be quite interesting. The actively dividing long-slender form of *T. brucei* is transformed to the non-dividing short-stumpy form by DFMO treatment both in vivo [5] and in vitro [6]. The non-dividing form is apparently incapable of changing its variant surface glycoprotein coat and is eventually caught up by the host immune response [7]. This drug-induced transformation bears a close resemblance to the natural transformation of *T. brucei* from long-slender to short-stumpy forms, during which the mitochondrial genes encoding cytochrome b, the cytochrome oxidase subunits, and NADH dehydrogenase are transcribed at high levels [8]. However, during the natural transformation of a pleomorphic *T. brucei* strain in infected mice, there was no sign of decrease of ODC activity in the parasite. Instead, there was a gradual increase of the specific enzyme activity from 16 to 35 nmoles CO_2/h/mg protein (unpublished observation). This discrepancy suggests that the drug-induced transformation is not the natural process leading to the short-stumpy forms of *T. brucei*. In fact, during the entire cycle of development of *T. brucei*, there appears to be always a

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constant level of ODC mRNA and a fluctuating but substantial level of ODC activity in the parasite (unpublished observation). The ODC function thus must be essential for the continuous development of *T. brucei*. This may explain why DFMO has anti-trypanosomal activity.

Our interest in DFMO is not to understand its detailed mechanism of inhibition of *T. brucei* ODC, but to find out why it is so toxic to the parasite but so harmless to the mammalian host [9]. By conventional wisdom, DFMO could be a potent inhibitor of *T. brucei* ODC but a much weaker inhibitor of mammalian ODC. In order to test this possibility, we purified the ODC from the bloodstream monomorphic *T. brucei* EATRO 110 to homogeneity via ammonium sulfate fractionation, pyridoxamine affinity column chromatography, and MonoQ FPLC [10]. It has a smaller subunit molecular weight of 45 KD when compared with the 53 KD subunit of mouse ODC [11]. When the K_i values of DFMO were estimated against the ODCs of *T. brucei* and mouse, they turned out to be 220 μM and 39 μM, respectively [10]. The parasite enzyme is thus less susceptable to DFMO than to mouse ODC. There must be another difference(s) between these two enzymes constituting the basis of preferential antitrypanosomal action of DFMO.

THE PRIMARY STRUCTURE OF *T. BRUCEI* ORNITHINE DECARBOXYLASE

A cloned mouse lymphoma S49 cDNA of ODC was successfully employed as a heterologous probe to identify and clone the *T. brucei* gene encoding ODC [11]. It is present in the parasite in a single copy. The sequence of this gene bears two major discrepancies from that of the Balb/c mouse ODC when translated into open reading frames (Fig. 1). The parasite enzyme appears to have either an extra 20 amino acid peptide or a shortage of two amino acids at the N-terminus depending on which one of the two initiating codons is used for translation. It lacks the 36 amino acid peptide at the C-terminus of the Balb/c mouse enzyme. There is 61.5% homology between the residues #21–#445 of *T. brucei* ODC and the residues #1–#425 of Balb/c mouse ODC. When the homologous amino acid substitutions are discounted, the percentage of homology reaches 90%. Clearly, these two enzymes are very similar to each other, which agrees with our previous observations [11].

The deletion of mouse C-terminal 36 amino acid peptide in the *T. brucei* enzyme is of considerable interest because MaCrea and Coffino [12] were able to make C-terminal deletions of the mouse ODC by cDNA excision, cloning, and expression in an *Escherichia coli* ODC deletion mutant HT289. They found that the mouse enzyme C-terminus could be deleted up to 38 amino acid residues without any appreciable decrease in enzymatic activity. Thus, the *T. brucei* enzyme with only 36 amino acid deletion from the C-terminus of the mouse enzyme must still be a functional enzyme. This has been subsequently verified by our successful expression of the *T. brucei* ODC gene in *E. coli* [10]. The recombinant protein thus generated was purified to homogeneity and found to possess the same specific ODC activity as does the authentic *T. brucei* enzyme.

THE IN VIVO HALF-LIFE OF *T. BRUCEI* ORNITHINE DECARBOXYLASE

The mouse enzyme C-terminal #423–#449 peptide is also classified as a PEST region of the protein with a PEST score of 5.2 [13]. The definition of a PEST region is a peptide very rich in proline, glutamic acid, serine, threonine, and aspartic acid and is flanked by basic amino acids. These sequences are commonly found in many eukaryotic proteins with in vivo half-lives shorter than 2 h but not in those with longer half-lives [13]. According to the PEST hypothesis, the PEST sequences may represent substrates for caesin kinase II, which would phosphorylate the serine residues in the PEST region. The phosphorylated peptides, representing potential Ca^{++} binding sites, may elicit intracellular protein degradation by Ca^{++} -activated cytoplasmic proteases such as calpain. Mammalian ODC is one of the most rapidly turning-over eukaryotic proteins with an estimated in vivo half-life of 20 min [14]. It was assigned to two PEST regions at the C-terminal #423–#449 and the middle #295–#307 of mouse ODC by Rogers et al. [13]. *T. brucei* ODC does not have the mouse enzyme C-terminus #426–#461, and its sequence corresponding to the mouse ODC sequence #295–#307 does not have the appearance of a PEST region (see Fig. 1). Thus, by the PEST theory, *T. brucei* ODC should have a relatively long half-life in vivo. This elucidation was later confirmed

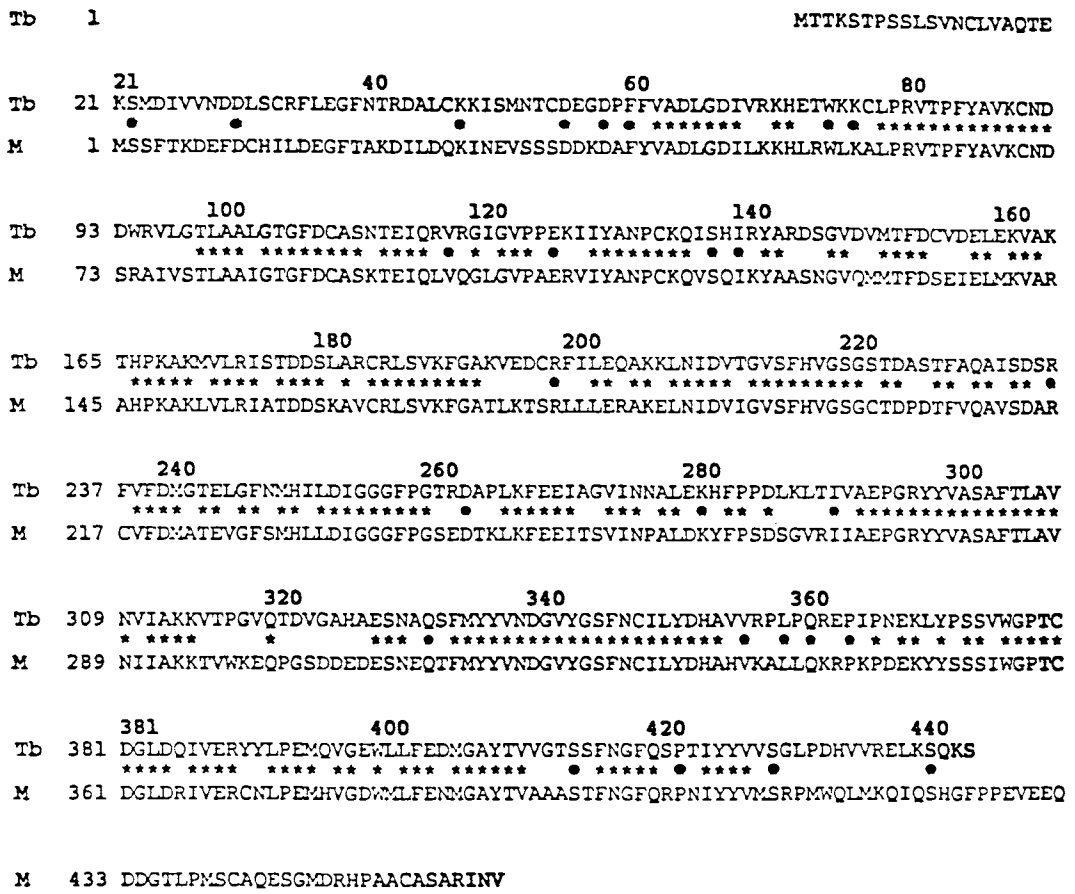

Fig. 1. Comparison of the amino acid sequences between *T. brucei* ODC and Balb/c mouse lymphoma S49 ODC. The identical segments between the *T. brucei* sequence (Tb) and the mouse sequence (M) are marked by stars (★).

by our observations that *T. brucei* EATRO 110 procylic forms, kept under an inhibited state of protein synthesis by cycloheximide (50 μg/ml), maintained a constant level of ODC activity for at least 6 h [10].

THE INTRACELLULAR STABILITY OF *T. BRUCEI* ORNITHINE DECARBOXYLASE IS A PROPERTY OF THE PROTEIN ITSELF

The possibility that the intracellular stability of *T. brucei* ODC could be attributed to the absence of the C-terminal 36 amino acid peptide found in mouse ODC has prompted Ghoda et al. [15] to examine the stability of a mutant mouse ODC with 37 amino acids deleted from the carboxy-terminus. A mammalian expression vector consisting of cDNA encoding the truncated mouse ODC, flanked by the SV40 early promoter and the hepatitis B virus polyadenylation signal sequence, was constructed and employed to transfect the ODC-deficient C55.7 Chinese hamster ovary (CHO) cells [16]. Cells expressing the wild-type mouse ODC lost the activity with a half-life of approximately 1 h. Cells expressing the truncated protein, however, retained full activity for at least 4 h under 100 μg/ml cycloheximide [15]. Thus, the carboxy-terminal domain is indeed responsible for the rapid intracellular degradation of mouse ODC.

In order to verify whether the stability of *T. brucei* ODC is an inherent property of the enzyme protein itself or due to a special intracellular environment provided by the parasite, we have cloned the *T. brucei* ODC gene into the same mammalian expression vector and expressed it in the CHO mutant cells [17]. The experimental results indicated that the *T. brucei* ODC activity remained constant in the CHO cells for at least 4 h under 100 μg/ml cycloheximide [17]. Thus, the parasite enzyme has re-

mained stable within the intracellular environments of both *T. brucei* trypomastigotes and CHO cells. It is most likely that the stability is attributable to the property of *T. brucei* ODC itself.

An additional experiment was performed, in which a *T. brucei* ODC gene fragment encoding amino acid residues #1–#384 and a mouse ODC cDNA fragment encoding amino acids #375–#461 were combined via a complementary adapter. The adapter, encoding the last base of codon 383 through the second base of codon 394 of trypanosome ODC followed by the third base of codon 374 through codon 375 of mouse ODC, joined the two ODC gene fragments without adding or deleting any amino acid residues between the two ODC sequences. The resulting chimeric ODC gene, coding for amino acids 1–395 of trypanosome ODC and 376–461 of mouse ODC, represents an exact match, because the numberings of *T. brucei* ODC are 20 ahead of those of mouse ODC (see Fig. 2). This chimeric gene, flanked by the SV40 early promoter and the HBV polyadenylation signal in the mammalian expression vector, was expressed in the CHO mutant cells.

The half-life of the chimeric ODC in transfected CHO cells was estimated to be shorter than 1 h [17]. Thus, the added C-terminus from mouse ODC must be responsible for this rapid turnover of *T. brucei* ODC. The mechanism of this rapid turnover must be one of targeting rather than of disruption of a protein structure. However, since much of the rest of the peptide sequences are homologous between the ODCs from *T. brucei* and mice, it is still possible that the C-terminus alone may not suffice, but that there are common sequences in conjunction with the C-terminus necessary for rapid protein degradation. It is not clear what role, if any, the other PEST sequence in mouse ODC (amino acids #293–#333) may play. Apparently, its presence in the truncated mouse protein does not bring about rapid degradation of the latter, nor does its absence from the chimeric protein prevent rapid degradation. It may not, therefore, play a significant role in ODC turnover at all.

THE BASIS OF THERAPEUTIC ACTIVITY OF DFMO AGAINST AFRICAN TRYPANOSOMIASIS

The apparent intracellular stability of *T. brucei* ODC has provided a major distinction between the ODC of the parasite and that of the mammalian host. This distinction may very well be the basis of therapeutic utility of DFMO as an antitrypanosomal agent. While DFMO is a potent, irreversible inhibitor of mammalian ODC, the fast rate of in vivo drug metabolism and excretion as evidenced by the need of frequent dosings [18], coupled with continuous rapid synthesis of new ODC enzyme molecules in the mammalian host, may quickly nullify the drug inhibitory effect. But the same enzyme activity in *T. brucei* will remain inhibited even after the removal of DFMO from the mammalian host because of the slower turnover of parasite ODC. A state of polyamine deficiency is achieved in DFMO-treated *T. brucei* both in vitro and in vivo [6]. The polyamine level in mammalian blood is very low due to the presence of high activity of polyamine oxidase in the plasma [19]. Thus, the state of polyamine deficiency is caused not only by the effective inhibition of parasite ODC, but also by the very low polyamine content in the plasma of the infected host where the parasites reside. Thus, no adequate polyamine uptake can be depended on to circumvent the consequence of ODC inhibition. This analysis may help to explain why, among the many species of parasitic protozoa, the African trypanosomes are the only family of protozoa susceptible to DFMO treatment. They are the only parasites staying in the bloodstream of mammalian hosts where the polyamine levels are low. The others are in the tissues or inside rapidly proliferating cells where the polyamine contents

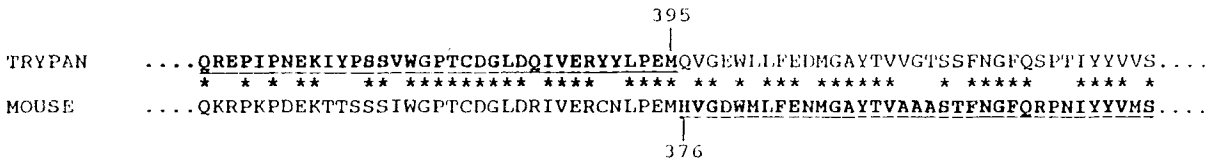

Fig. 2. Amino acid sequences of trypanosome, mouse, and trypanosome-mouse chimeric protein. The **top line,** TRYPAN, represents the sequences flanking amino acid 395 of *T. brucei* ODC. The **bottom line,** MOUSE, represents the sequences flanking amino acid 376 of mouse ODC.

are relatively high. ODC is thus no longer the only provider of polyamines. It can be bypassed.

PROSPECTS

By a fortuitous combination of relatively slow ODC turnover in African trypanosomes vs. the mammalian host, and low polyamine levels in mammalian blood, a modest, nondiscriminating, irreversible inhibitor of ODC, DFMO, becomes a selective antitrypanosomal agent. By the same token, other rapidly turning-over proteins in mammals may reveal worthwhile intervention targets in parasites for this same therapeutic strategy. If the enzymes turn out to have substantially longer half-lives in parasite over host and perform indispensable functions for survival of the parasites, they also may be qualified as potential targets for antiparasitic chemotherapy through design of irreversible inhibitors.

HMG-CoA reductase is one example of a prime candidate. It has a half-life estimated at 1.5 to 3 h in the cytoplasm of mammalian cells [20]. This enzyme apparently performs an important function in the human trematode parasite *Schistosoma mansoni* by supplying isoprenoid compounds necessary for schistosome egg formation [21]. When mice infected with *S. mansoni* were treated with a highly selective and potent inhibitor of HGM-CoA reductase, mevinolin, egg production in adult worms was decreased [21]. The cDNA encoding *S. mansoni* HMG-CoA reductase has been cloned and sequenced recently [22]. The deduced peptide sequence suggests a hydrophilic carboxyl terminus sharing 48–52% sequence identity with the carboxyl termini of other HMG-CoA reductases in a region that contains the catalytic domain [22], but the hydrophobic amino terminus bears little resemblance to that of human HMG-CoA reductase that consists of three designated PEST sequences [13]. None of the three PEST sequences can be identified in *S. mansoni* HMG-CoA reductase, suggesting a substantially longer in vivo half-life for the latter. Although the PEST hypothesis needs to be validated in every individual case, it should be relatively straightforward to examine the in vivo stability of *S. mansoni* HMG-CoA reductase to test the theory and possibly provide another target for a suicide inhibitor approach to antiparasitic chemotherapy.

It is thus probably reasonable to suggest at this point that computer-assisted searches for PEST sequences in databases for mammalian enzymes be carried out in order to facilitate identification of new potential target enzymes. This process could be accelerated substantially through the work ongoing in the international human genome program, which may serve to illustrate one set of potentially valuable results that may be gleaned from this program.

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