Biochemical Pharmacology, Vol. 34, No. 10, pp. 1773–1777, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

CATALYTIC IRREVERSIBLE INHIBITION OF *TRYPANOSOMA BRUCEI BRUCEI* ORNITHINE DECARBOXYLASE BY SUBSTRATE AND PRODUCT ANALOGS AND THEIR EFFECTS ON MURINE TRYPANOSOMIASIS*

ALAN J. BITONTI,† CYRUS J. BACCHI,‡ PETER P. MCCANN and ALBERT SJOERDSMA

Merrell Dow Research Institute, Cincinnati, OH 45215; and ‡Haskins Laboratories and Biology Department of Pace University, New York, NY 10038, U.S.A.

(*Received* 31 *July* 1984; *accepted* 17 *September* 1984)

Abstract—Ornithine decarboxylase from *Trypanosoma brucei brucei* was inhibited by several substrate (ornithine) and product (putrescine) analogs both *in vitro* and *in vivo*. Since α-difluoromethylornithine is effective for the treatment of experimental and clinical African trypanosomiasis, it was possible that the more potent ornithine and putrescine analogs might be more active in treating the disease. However, only α-monofluoromethyldehydroornithine methyl ester was more potent than α-difluromethylornithine against mouse trypanosomiasis and warrants further study in model infections.

Ornithine decarboxylase (ODC: L-ornithine carboxylyase, EC 4.1.1.17) catalyzes the conversion of ornithine to putrescine [1]. Putrescine was well as the polyamines spermidine and spermine are important regulators of cell growth, proliferation and differentiation [2]. Inhibition of ODC activity and consequent reduction in polyamine synthesis leads to a reduction in cell proliferation in a wide variety of cell types [3–6]. Inhibition of ODC in *Trypanosoma brucei brucei*, a parasite of livestock in Africa, has especially pronounced effects on cell proliferation [7–10]. Murine laboratory infections of *T. b. brucei* can be cured by the administration of α-difluoromethyl-ornithine (DFMO), an enzyme-activated, irreversible inhibitor of ornithine decarboxylase [7, 10]. DFMO has also been used successfully in the treatment of human trypanosomiasis [11, 12], although large doses of the drug are required. Consequently, it seemed desirable to find an agent which was as effective as DFMO at a lower dose.

Recently, a number of other analogs of ornithine and putrescine have been synthesized [13–17] in an attempt to discover more potent irreversible inhibitors of ODC. We detail here the characteristics of these new compounds on *T. b. brucei* ODC activity *in vitro* and in whole cells and the effects of the drugs on a model *T. b. brucei* infection in mice.

MATERIALS AND METHODS

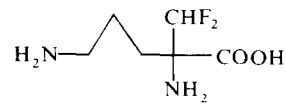
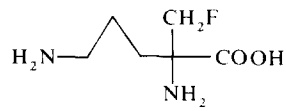
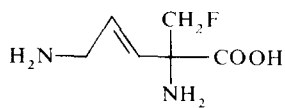
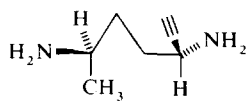
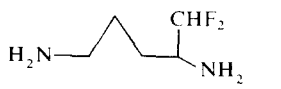
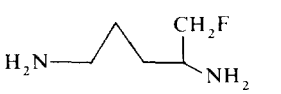
Preparation and assay of trypanosomal ornithine decarboxylase. Trypanosoma brucei brucei (EATRO 110) was maintained by passage through 250–300 g male Wistar rats. Infected rat blood was collected in EDTA by cardiac puncture from CO_2/ether-anesthetized rats, and trypanosomes were separated from blood elements on columns of DEAE-cellulose [18] using a buffer containing 90 mM Tris (pH 7.8), 2% glucose and 50 mM NaCl for equilibration and elution of the columns. Purified trypanosomes were washed twice with the elution buffer and then sonicated in a solution containing 50 mM Tris (pH 7.0), 2 mM dithiothreitol, 1 mM EDTA and 50 μM pyridoxal phosphate. Broken cells were centrifuged for 60 min at 100,000 *g* and the supernatant fraction (6–10 mg protein/ml), used for the determination of ornithine decarboxylase activity, was stored frozen at −20° without measurable loss of activity for up to 2 months. ODC activity was determined by measuring $^{14}CO_2$ release at 37° over a 60-min period as described previously [3]. The standard assay mixture contained 80 mM Tris (pH 7.0), 1 mM ornithine, 2 mM dithiothreitol, 50 μM pyridoxal phosphate, 2.5 μCi [1-^{14}C] ornithine, and 100–150 μg protein in a final volume of 1 ml.

Inhibition kinetics for ornithine decarboxylase. Time-dependent, irreversible inhibition of ornithine decarboxylase was determined as described [3]. Trypanosomal ornithine decarboxylase was incubated at 20° or 37° in 100 mM Tris (pH 7.0), 1 mM dithiothreitol, 50 μM pyridoxal phosphate with various concentrations of inhibitor. At selected times, 67 μl aliquots of the enzyme incubation were removed and transferred to reaction vessels in which ornithine decarboxylase activity was to be determined. These flasks contained all the components of the ornithine

* This research was supported in part by Grant AI 17,340 from the National Institute of Allergy and Infectious Diseases and by the U.N.D.P./World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases (C.J.B).

† Correspondence should be directed to: Alan J. Bitonti, Ph.D., Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, OH 45215.

Table 1. Activity of ornithine and putrescine analogs against *T. b. brucei* ornithine decarboxylase*

		K_i (μM)	$T_{\frac{1}{2}}$ (min)	$T_{\frac{1}{2}}$ at 10 μM (min)	Ref.†
Ornithine analogs					
α-Difluoromethylornithine		130	1.0	12	[10]
α-Monofluoromethylornithine		104	1.1	12	[15]
α-Monofluoromethyldehydroornithine		14	1.1	2.6	[13]
Putrescine analogs					
(R,R)-δ-Methyl-α-acetylenicputrescine		5	1.0	1.6	[17]
α-Difluoromethylputrescine		140	2.4	35	[14]
α-Monofluoromethylputrescine		25	2.8	10	[14, 16]
α-Monofluoromethyldehydroputrescine		11	1.5	3	[13]

* *T. b. brucei* ornithine decarboxylase was prepared and assayed as described in Materials and Methods.

† Reference numbers refer to original references which detail the synthesis of the inhibitors and determination of inhibitory activity using rat liver ornithine decarboxylase.

decarboxylase reaction (including unlabeled ornithine) except [1-^{14}C]ornithine. The reaction vessels were held at 0° until all time points were taken and then the ornithine decarboxylase assay was initiated by the addition of [1-^{14}C]ornithine. Dilution of the enzyme incubation into the reaction vessels at 0° was assumed to stop the inhibition reactions.

In vitro *polyamine synthesis*. Polyamine synthesis in blood stream trypomastigotes was determined as described [9]. Trypanosomes, separated as above, were washed twice in PSG (70 mM phosphate-buffered 43 mM saline + 1% glucose, pH 8.0 [18]); and resuspended to 2×10^8 cells/ml in PSG + 1% bovine serum albumin + 50 μmoles penicillin + 50 μg/ml streptomycin sulfate (PSG-BSA). The formation of putrescine and spermidine was measured at 37° in PSG-BSA by incubating 2×10^8 trypanosomes/ml in the presence of 3.3 μCi/ml [^{3}H]ornithine. After 1 hr of incubation the cells were collected by centrifugation, washed and extracted with 0.4 N perchloric acid, and polyamines were determined as described [19].

Protein concentrations in all experiments were determined by the method of Lowry *et al.* [20].

Chemicals. The ODC inhibitors were synthesized at the Merrell Dow Research Institute, Strasbourg, France. Their inhibitory properties towards rat liver ODC have been described [13–17]. D,L-[1-^{14}C]ornithine (56 mCi/mmole) was purchased from Amersham (Arlington Heights, IL) and L-[2,3-^{3}H(N)]ornithine from New England Nuclear Boston, MA).

RESULTS

Kinetics of ornithine decarboxylase inhibition. Kinetic analysis of ODC inhibition by the ornithine and putrescine analogs studied was done as described [21, 22]. From plots of half-life of enzyme activity versus the inverse of concentration of an inhibitor it was possible to determine an apparent dissociation constant (K_i) for each inhibitor and half-life ($T_{\frac{1}{2}}$) of the enzymes activity at an infinite concentration of inhibitor (Table 1). Inhibition of enzyme activity by each analog was time dependent and irreversible.

Table 2. Inhibition of putrescine and spermidine synthesis in intact trypanosomes by inhibitors of ornithine decarboxylase*

Inhibitor	Concn (μM)	% Inhibition	
		Putrescine	Spermidine
α-Difluoromethylornithine	50	61	42
	100	73	54
α-Monofluoromethyldehydroornithine	50	52	19
	100	70	47
α-Monofluoromethyldehydroornithine, methyl ester	50	56	34
	100	63	39
α-Monofluoromethyldehydroornithine, ethyl ester	50	65	50
	100	75	58
(R,R)-δ-Methyl-α-acetylenicputrescine	0.5	40	40
	1.0	60	53
	2.5	77	75
	5.0	85	83
	10.0	91	92

* Trypanosomes (2×10^8/ml) were incubated for 1 hr at 37° in the presence of [^{3}H]ornithine, and the formation of [^{3}H]putrescine and [^{3}H]spermidine was determined as detailed in Materials and Methods. Putrescine formation in cells not exposed to inhibitors was 4×10^5 cpm/hr/mg trypanosomal protein. Spermidine formation in control cells was 1×10^5 cpm/hr/mg trypanosomal protein.

The most potent compound was (R,R)-δ-methyl-α-acetylenicputrescine with a $K_i = 5$ μM. α-Difluoromethylornithine was found to be a less potent inhibitor of trypanosomal ODC ($K_i = 130$ μM).

Inhibition of ornithine decarboxylase in intact cells. The effects of DFMO, (R,R)-δ-methyl-α-acetylenicputrescine, α-monofluoromethyldehydroornithine, α-monofluoromethyldehydroornithine methyl ester and α-monofluoromethyldehydroornithine ethyl ester on the formation of putrescine and spermidine were determined in suspensions of intact trypanosomes (Table 2). Marked inhibition of putrescine and spermidine synthesis was obtained with all the compounds tested, but again the most potent was (R,R)-δ-methyl-α-acetylenicputrescine which was 40% inhibitory at 0.5 μM.

Effects of inhibitors in murine trypanosomiasis. α-Difluoromethylornithine at 2% in drinking water protected 100% of mice with the acute laboratory strain of trypanosomiasis (Table 3). DFMO was also effective at 0.75% in drinking water, curing four of five animals. The only other compound giving a significant percentage of cures was α-monofluoromethyldehydroornithine methyl ester. The latter compound at 0.5% in drinking water cured fourteen of seventeen animals and five of five animals when given at 0.75% in drinking water. Administration of (R,R)-δ-methyl-α-acetylenicputrescine by intubation resulted in a prolongation of life but did not effect cures. Doses of (R,R)-δ-methyl-α-acetylenicputrescine higher than 25 mg/kg proved to be too toxic for evaulation of antitrypanosomal effects. Large doses of any of the compounds administered by intraperitoneal injection four times a day were uniformly ineffective.

DISCUSSION

Trypanosoma brucei brucei is exquisitely sensitive to DFMO [7] and represents a major novel lead to chemotherapy of African trypanosomiases based on its lack of significant toxicity even when administered in large doses (up to 30 g/day) to comatose sleeping sickness patients [11, 12]. The analogs of DFMO described in this paper are potent inhibitors of mammalian ornithine decarboxylase activity [13–17] and were studied to determine their potential to inhibit trypanosomal ornithine decarboxylase and to assess their efficacy against a model trypanosome infection.

The kinetics of inhibition showed that the trypanosomal ODC is not drastically different from mammalian ODC. The most potent of the compounds tested, (R,R)-δ-methyl-α-acetylenicputrescine, had an apparent dissociation constant (K_i) of 5 μM which is quite similar to that obtained with rat liver ODC [17]. The apparent K_i obtained for DFMO was 130 μM, significantly higher than previously reported [23] and somewhat higher than the K_i of 39 μM reported for rat liver ODC [24].

It appears, then, that the apparent activity of DFMO in growth inhibition of trypanosomes is not related to a uniquely sensitive enzyme in the parasite. Rather, the inhibitory effects of DFMO appear to center on the likelihood of mediated drug uptake by trypanosomes [9] with a resultant rapid intracellular concentration of drug (up to 2.5 mM; not shown). Mammalian cells take up DFMO by passive diffusion only and attain millimolar intracellular concentrations of drug only after prolonged incubation with high extracellular concentrations of DFMO [25]. Trypanosomes may also be more dependent on polyamines than most mammalian cells because of their extremely rapid doubling time in the blood stream (6–8 hr).

Kinetic differences among the ODC inhibitors were not necessarily reflected in the potency of the compounds when polyamine synthesis was studied *in vitro* using intact trypanosomes. DFMO was as potent as α-monofluoromethyldehydroornithine in inhibiting putrescine and spermidine formation in

Table 3. Effects of ornithine decarboxylase inhibitors on *T. b. brucei* infections in mice*

Inhibitor	Dose	Average survival beyond control (days)	No. cured/No. treated
α-Difluoromethylornithine	0.2%	0	0/5
	0.5%	13.2	2/20
	0.75%	27	4/5
	2.0%	>30	24/24
	375 mg/kg, i.p.†	7.3	0/20
α-Monofluoromethylornithine	0.1%	<1	0/6
	0.5%	2	0/6
	1.0%	4	0/6
	2.0%	13	1/5
α-Monofluoromethyldehydroornithine	0.1%	5.5	0/5
	0.2%	7.0	0/5
	0.5%	7.5	0/20
	0.75%	3.4	0/5
	375 mg/kg, i.p.†	2.7	0/15
α-Monofluoromethyldehydroornithine, methyl ester	0.1%	5	0/5
	0.2%	13.8	1/5
	0.5%	26.6	14/17
	0.75%	>30	5/5
	375 mg/kg, i.p.†	11.6	1/5
α-Monofluoromethyldehydroornithine, ethyl ester	0.1%	0	0/5
	0.2%	8	0/5
	0.5%	6.8	0/10
	0.75%	11.8	1/6
	375 mg/kg, i.p.†	6.2	0/14
(R,R)-δ-Methyl-α-acetylenicputrescine	0.1%	0	0/5
	0.25%	1	0/10
	10 mg/kg, i.p.	1.2	0/30
	25 mg/kg, i.p.	0	0/20
	25 mg/kg, i.p.†	0	0/5
	10 mg/kg, p.o.	11.3	0/10
	25 mg/kg, p.o.	0	0/5
	10 mg/kg, p.o.†	15	0/5
	10 mg/kg, i.v.	1.3	0/10
	25 mg/kg, i.v.	2.5	0/5

* Mice were injected intraperitoneally with 2–5 × 10^5 trypanosomes and the infection was allowed to develop for 24 hr. Animals were then divided into groups and the administration of drugs was begun and continued for 72 hr. Animals surviving longer than 30 days were confirmed to have no parasitemia and were considered cured. The average survival time beyond controls was calculated using only those mice that died before 30 days. Doses listed as percentages represent the percent (w/v) of drug dissolved in the drinking water. Each mouse drank approximately 5 ml of water/day so total dosages of each drug can be calculated. Drug doses administered intraperitoneally (i.p.), intravenously (i.v.) or *per os* (p.o.) were given once a day for 3 consecutive days except those doses designated with a dagger (†) which were given every 6 hr for 3 consecutive days.

whole cells even though the apparent K_i for DFMO was 9-fold greater than the apparent K_i for α-monofluoremethyldehydroornithine. (R,R)-α-Methyl-α-acetylenic putrescine, on the other hand, had both a lower K_i than DFMO (5 μM vs 130 μM) and was distinctly more potent against polyamine biosynthesis in the whole cell.

Two other compounds, α-monofluoromethyldehydroornithine methyl ester and α-monofluoromethyldehydroornithine ethyl ester [13], appeared to be equal in potency to DFMO in the intact parasite. No kinetic data are presented in Table 1 for the two esters of α-monofluoromethyldehydroornithine because these compounds do not inhibit ODC activity in a time-dependent manner *in vitro*, but probably are prodrugs which are dependent on hydrolysis to the active acid.

When the inhibitors were tested for their effects on the progression of trypanosome infections in mice, a marked disparity between *in vitro* data and *in vivo* potency was evident. Only one compound other than DFMO was consistently curative. α-Monofluoromethyldehydroornithine methyl ester appeared to be more effective than DFMO in the model infection, with a cure rate of 82% at 0.5% in drinking water, a dose at which DFMO was only 10% curative. α-Monofluoromethylornithine and (R,R)-δ-methyl-α-acetylenicputrescine which were approximately equal to DFMO or much more potent than DFMO, respectively, against ODC *in vitro* were not effective

against trypansosome *in vivo*, even when various doses were administered by several routes.

Several specific comments can be made concerning the activities of DFMO and its analogs: (1) *T. b. brucei* ODC is kinetically similar to mammalian ODC with respect to the inhibitors tested, (2) the apparent K_i of an inhibitor does not necessarily indicate its potency with respect to inhibition of polyamine synthesis in whole cells or in curing trypanosome infection in mice, and (3) α-monofluoromethyldehydroornithine methyl ester is apparently more active than DFMO against *T. b. brucei in vivo* and further investigations into its usefulness as an alternate antiparasitic drug are warranted. A final general point is that the *in vivo* bioavailability of a particular compound is probably of crucial importance. Thus, for example, the ethyl and methyl esters of α-monofluoromethyldehydroornithine was well as the potent irreversible inhibitors α-monofluoromethyldehydroornithine and δ-methyl-α-acetylenicputrescine all effectively inhibit putrescine biosynthesis in the *T. b. brucei* cell, but only the methyl ester of α-monofluoromethyldehydroornithine was curative *in vivo*.

Acknowledgements—The authors thank Susan E. Kelly, Joanne Garofalo and Diane Mockenhaupt for technical assistance.

REFERENCES

1. A. E. Pegg and H. G. Williams-Ashman, in *Polyamines in Biology and Medicine*, (Eds. D. R. Morris and L. J. Marton), pp. 3–42. Marcel Dekker, New York (1981).
2. A. E. Pegg and P. P. McCann, *Am. J. Physiol.* **243**, C212 (1982).
3. A. J. Bitonti, P. P. McCann and A. Sjoerdsma, *Biochem. J.* **208**, 435 (1982).
4. P. S. Mamont, P. Bohlen, P. P. McCann, P. Bey, F. Schuber and C. Tardif, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1626 (1976).
5. A. Sjoerdsma, *Clin. Pharmac. Ther.* **30**, 3 (1981).
6. G. D. Luk, G. Goodwin, L. J. Marton and S. B. Baylin, *Proc. natn. Acad. Sci. U.S.A.* **78**, 2355 (1981).
7. C. J. Bacchi, H. C. Nathan, S. H. Hutner, P. P. McCann and A. Sjoerdsma, *Science* **210**, 332 (1980).
8. P. P. McCann, C. J. Bacchi, W. L. Hanson, G. D. Cain, H. C. Nathan, S. H. Hutner and A. Sjoerdsma, *Advances in Polyamine Research* (Eds. C. M. Caldarera, V. Zappia and U. Bachrach), Vol. 3, pp. 97–110. Raven Press, New York (1981).
9. C. J. Bacchi, J. Garofalo, D. Mockenhaput, P. P. McCann, K. A. Diekema, A. E. Pegg, H. C. Nathan, E. A. Mullaney, L. Chunosoff, A. Sjoerdsma and S. H. Hutner, *Molec. biochem. Parasit.* **7**, 209 (1983).
10. B. W. Metcalf, P. Bey, C. Danzin, M. J. Jung, P. Casara and J. P. Vevert, *J. Am. chem. Soc.* **100**, 2551 (1978).
11. A. Sjoerdsma and P. J. Schechter, *Clin. Pharmac. Ther.* **35**, 287 (1984).
12. A. Sjoerdsma, J. A. Golden, P. J. Schechter, J. L. R. Barlow and D. V. Santi, *Trans. Ass. Am. Phycns.* **XCVII**, 70 (1984).
13. P. Bey, F. Gerhart, V. VanDorsselaer and C. Danzin, *J. med. Chem.* **26**, 1551 (1983).
14. C. Danzin, P. Bey, D. Schirlin and N. Claverie, *Biochem. Pharmac.* **31**, 3871 (1982).
15. J. Kollonitsch, A. A. Patchett, S. Marburg, A. L. Maycock, L. M. Perkins, G. A. Golduras, D. E. Duggan and S. D. Aster, *Nature, Lond.* **274**, 906 (1978).
16. A. Kallio, P. P. McCann and P. Bey, *Biochem. J.* **204**, 771 (1982).
17. C. Danzin, P. Casara, N. Claverie, B. W. Metcalf and M. J. Jung, *Biochem. biophys. Res. Commun.* **116**, 237 (1983).
18. S. M. Lanham and D. G. Godfrey, *Expl. Parasit.* **28**, 521 (1970).
19. C. J. Bacchi, C. Vergara, J. Garofalo, G. Y. Lipschik and S. H. Hutner, *J. Protozool.* **26**, 484 (1979).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. R. Kitz and I. B. Wilson *J. biol. Chem.* **237**, 3245 (1962).
22. M. J. Jung and B. W. Metcalf, *Biochem. biophys. Res. Commun.* **67**, 301 (1975).
23. J. Garofalo, C. J. Bacchi, S. D. McLaughlin, D. Mockenhaupt, G. Trueba and S. H. Hutner, *J. Protozool.* **29**, 389 (1982).
24. P. Bey in *Enzyme-Activated Irreversible Inhibitors* (Eds. N. Seiler, M. J. Jung and J. Koch-Weser), pp. 27–41. Elsevier/North Holland Biomedical Press, Amsterdam, The Netherlands (1978).
25. B. G. Erwin and A. E. Pegg, *Biochem. Pharmac.* **31**, 2820 (1982).