Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 205-210

INHIBITION OF GROWTH OF MYCOPLASMA DISPAR BY DL-α-DIFLUOROMETHYLLYSINE, A SELECTIVE IRREVERSIBLE INHIBITOR OF LYSINE DECARBOXYLASE, AND REVERSAL BY CADAVERINE (1,5-DIAMINOPENTANE)

Hannu Pösö,[1] Peter P. McCann*,[3]
Raili Tanskanen,[2] Philippe Bey[3] and Albert Sjoerdsma[3]

[1]Department of Pharmacology and Toxicology
[2]Department of Microbiology and Epizootology
College of Veterinary Medicine
SF-00550 Helsinki 55, Finland

[3]Merrell Dow Research Institute
2110 E. Galbraith Road
Cincinnati, Ohio 45215

Received October 19, 1984

DL-α-Difluoromethyllysine was shown to be a potent inhibitor of lysine decarboxylase from Mycoplasma dispar. The inhibition appeared to be specific since neither difluoromethylornithine nor difluoromethylarginine, known to inhibit other decarboxylases, inhibited the reaction catalyzed by lysine decarboxylase in extracts of M. dispar. Inhibition was irreversible since extensive dialysis could not overcome the inhibitory effect exerted by difluoromethyllysine. Difluoromethyllysine (1 mM) also totally blocked the growth of M. dispar when added at the beginning of the growth period, while 1 mM cadaverine, the product of the reaction, reversed this inhibitory effect when added to the culture medium. When difluoromethyllysine was added during the logarithmic growth phase of Mycoplasma, it inhibited the increase of the growth; 1 mM cadaverine again partially reversed this inhibitory action.

Difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC; EC 4.1.1.17; 1), depresses the synthesis of the physiologically occurring polyamines, i.e. putrescine (1,4-diaminobutane), spermidine, and spermine (2) which are necessary for normal and malignant growth (3,4) in various growth conditions (3-8). As such DFMO potentially may have value in the treatment of cancer (9,10) and already has been shown to have clinical efficacy for some protozoan infections (10-12).

*To whom reprint requests should be addressed.

0006-291X/84 $1.50

Difluoromethylarginine (DFMA) is an irreversible inhibitor of bacterial arginine decarboxylases (13,14). Used in conjunction with other inhibitors of bacterial polyamine biosynthetic enzymes, i.e. monofluoromethylornithine, an irreversible inhibitor of bacterial ODC, (15), and dicyclohexylamine, an inhibitor of bacterial spermidine synthase, (16,17), DFMA slows the growth of bacteria such as E. coli and Pseudomonas aeruginosa, by lowering their polyamine levels (15,18).

We now report studies with difluoromethyllysine (DFML) which demonstrate that it is a novel, specific, irreversible inhibitor of lysine decarboxylase (LDC; EC 4.1.1.18) from M. dispar (19), with consequent blockade of the biosynthesis of cadaverine (1,5-diaminopentane) and concomitant retardation of the growth of this organism.

MATERIALS AND METHODS

Chemicals. L-(1-^{14}C)Ornithine (57 mCi/mmol) was purchased from New England Nuclear (Boston., MA., U.S.A.) and L-(U-^{14}C)lysine (324.7 mCi/mmol) from Amersham (Bucks., U.K.). DL-α-difluoromethylornithine (DFMO; MDL 71,782), DL-α-difluoromethylarginine (DFMA; MDL 71,897) and DL-α-difluoromethyllysine (DFML; MDL 71,837) were synthesized at Merrell Dow Research Institute (Cincinnati, Ohio., U.S.A. and Strasbourg, France) as previously described (20). All other chemicals were obtained from Sigma Chemical Co., (St. Louis, MO., U.S.A.).

Cultivation of M. dispar. M. dispar (strain 462/2) was obtained from FAO/WHO Collaborative Centre for Animal Mycoplasmas (Aarhus, Denmark). The organism was cultivated in glucose/calf serum (GS) broth as described by Gourlay and Leach (19) with the following modifications: Hartley's digest broth and fetal calf serum in the Gourlay and Leach medium were replaced by Bacto PPLO Broth w/v CU (Difco, Detroit, MI., U.S.A.), and newborn calf serum (Gibco). In addition, the medium was supplemented by fresh yeast extracts (8%) prepared according to the method of Hers and Masurel (21). Penicillin was replaced by ampicillin (0.5 mg/ml). For enzymatic studies Mycoplasmas were grown in 2 liters of the GS broth and the cells were harvested during the logarithmic growth phase (at day 3) by centrifugation (10 min at 8000 g) and washed once with 25 mM Tris-HCl (pH 7.5) containing 5 mM dithiothreitol and 0.1 mM EDTA (standard buffer). After centrifugation the pellet was suspended in 1 ml of standard buffer and cells were disrupted by sonication with a MSE PG-742 cell disruptor. After centrifugation at 42000 g for 45 min, the supernatant was used as the source of LDC. For determination of the growth of M. dispar, triplicate serial tenfold dilutions were prepared in broth media (1.8 ml) and incubated for 10 days at 37°C. The growth was determined by using a color changing unit (CCU) as the measure of the growth (22).

Analytical methods. The activity of LDC was measured by using the same conditions as those for ODC (23) but replacing unlabeled and labeled ornithine with the corresponding lysines. Protein was measured by the method of Bradford (24).

RESULTS AND DISCUSSION

We started these experiments by analyzing two strains of Mycoplasma, M. bovirhinis and M. dispar, with respect to the decarboxylation of ornithine and lysine. While M. bovirhinis demonstrated both ODC and LDC activity, M. dispar had only LDC activity (not shown) and consequently this organism was selected for further studies with DFML.

Table 1 shows the effect of different concentrations of DFML on LDC activity from M. dispar. When extracts were preincubated for 30 min with DFML, the activity of LDC was irreversibly lost as judged by the lack of recovery of enzyme activity after exhaustive dialysis of the samples. DFML 10 μM caused 50% inhibition of the activity of LDC, and 1 mM DFML totally inactivated the enzyme. As seen in Table 1, partial protection against the inhibition of LDC by 100 μM DFML was provided by 1 mM lysine, similar to the protective ability of ornithine (1) and arginine (13) in the case of DFMO and DFMA, respectively. Table 1 also shows that neither DFMO (1 mM) nor DFMA (1 mM) had any effect on LDC activity. This suggests that LDC is highly specific in utilizing lysine as a substrate. This is in contrast to mammalian ODC which may use a substrate, either ornithine (yielding putrescine) or, under certain circumstances, lysine (yielding cadaverine) (25,26).

TABLE 1 **Effect of DFML, DFMO and DFMA on the activity of Lysine Decarboxylase from M. dispar**

Treatment of the Enzyme	LDC Activity (pmoles/mg of protein/60 min)	Remaining Activity (% Control)
Control	186	100
+ 10 μM DFML	92	50
+ 100 μM DFML	8	4
+ 1 mM DFML	4	2
+ 100 μM DFML + 1 mM Lysine	124	67
+ 1 mM DFMO	189	102
+ 1 mM DFMA	200	107

LDC was obtained from 250 mg of M. dispar grown as described in "Materials and Methods". LDC samples were incubated with various compounds at concentrations indicated in the table in the presence of 40 μM pyridoxal phosphate for 30 min at 37°C and then dialyzed 24 h against 1000 vol of standard buffer to remove any unbound difluoro derivatives of amino acids or to control the effect of the exhaustive dialysis on the activity of the enzyme. The activity of the untreated control before dialysis was 205 pmoles of ^{14}C formed during one hour incubation. The results are the average of three experiments.

TABLE 2 Effect of 1 mM Difluoromethyllysine on the growth of M. dispar

Treatment	Final Titer of the Growth of M. dispar (CCU/0.2 ml)
GS-broth	$1 \quad X_a 10^8$
GS-broth + 1 mM DFML	----
GS-broth + 1 mM DFML + 1 mM Cadaverine	5.5×10^8
GS-broth + 1 mM Cadaverine	5.1×10^8

DFML was added at the beginning of the growth period. Duplicate serial tenfold dilutions of refrigerated and thawed stock cultures of M. dispar (10^8 CCU/0.2 ml) were prepared in GS-broth, GS-broth with 1 mM DFML, GS-broth with 1 mM DFML + 1 mM cadaverine and GS-broth with 1 mM cadaverine and incubated for 10 days.
[a] Below the sensitivity of the method used (22).

We then studied the effect of DFML on the growth of M. dispar. Table 2 shows that 1 mM DFML had a dramatic effect on the growth of M. dispar when added at the time of the inoculation. No growth of the organism was observed when the series of dilutions of the stock culture of M. dispar (10^8 CCU/0.2 ml) was prepared in GS-broth with 1 mM DFML and incubated for 10 days. Cadaverine (1 mM) completely reversed the effect of DFML on Mycoplasma (Table 2). When cadaverine (1 mM) was added alone it did not stimulate the growth of Mycoplasma any more than DFML and cadaverine together (Table 2).

Since the growth of M. dispar was totally inhibited when DFML was added at the beginning of the culture period (Table 2), another experiment was done wherein M. dispar was allowed to reach logarithmic growth phase and after two days 1 mM DFML, alone and in combination with 1 mM cadaverine, was added to the cultures. As shown in Fig. 1, DFML prevented any further increase in growth but did not seem to have a cytotoxic effect on the Mycoplasma. Again 1 mM cadaverine partially reversed the DFML-caused inhibition of growth and apparently prolonged the growth period of M. dispar under our experimental conditions.

Our results therefore suggest that DFML alone could be a useful drug in some Mycoplasma infections even though polyamine metabolism in a number of other Mycoplasmas is apparently quite complex (27) and would seemingly require simultaneous inhibition of more than one decarboxylase. Thus, M. dispar could

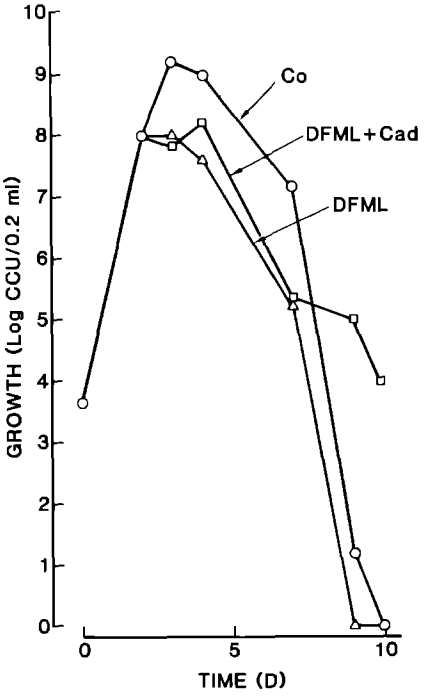

Fig. 1. Effect of DFML on the growth of M. dispar at the end of logarithmic growth phase.
30 ml of GS-broth was inoculated with 1.05 X 10^7 CCU of M. dispar (day 0), divided 2 days later into three equal parts and 1.0 mM DFML or 1.0 mM DFML + 1.0 mM cadaverine (Cad) was then added to the cultures (day 2). (O; Co): Control culture, (Δ; DFML): 1.0 mM DFML; (□; DFML + Cad): 1.0 mM DFML + 1.0 mM cadaverine. (Note: The rate of growth is shown in a logarithmic scale.)

be an example of a Mycoplasma, which synthesizes cadaverine rather than putrescine as no ODC activity was found. This would also explain the potent effect of DFML, since the apparent inhibition of cadaverine biosynthesis prevents growth (Table 2). Direct measurement of cadaverine and other cellular polyamines are needed before the exact mechanism of the effect of DFML on the growth of M. dispar can be determined. Further studies to be done would be also to see if DFML has some effect in vivo on the colonization of the respiratory tract (28,29) or on pneumonia of calves caused by M. dispar infection (30).

ACKNOWLEDGEMENTS

This study received financial support from the Wellcome Foundation and from the Research Council for Medical Sciences of the Academy of Finland.

REFERENCES

1. Metcalf, B.W., Bey, P., Danzin, C., Jung, M.J., Casara, P. Vevert, J.P. (1978) J. Amer. Chem. Soc. 100, 2551-2553.
2. Tabor, C.W. and Tabor, H. (1976) Annu. Rev. Biochem. 45, 285-306.
3. Jänne, J., Pösö, H. and Raina, A. (1978) Biochim. Biophys. Acta 473, 241-306.
4. Pegg, A.E. and McCann, P.P. (1982) Am. J. Physiol. 243, C212-C221.
5. Mamont, P.S., Duchesne, M.C., Grove, J. and Bey, P. (1978) Biochem. Biophys. Res. Commun. 81, 58-66.
6. Höltta, E., Jänne, J. and Hovi, T. (1979) Biochem. J. 178, 109-117.
7. Fozard, J.R., Part, M.-L., Prakash, N.J., Grove, J., Sjoerdsma, A. and Koch-Weser, J. (1980) Science 208, 505-508.
8. Pösö, H. and Pegg, A.E. (1983) Biochim. Biophys. Acta 747, 209-214.
9. Jänne, J., Alhonen-Hongisto, L, Seppanen, P. and Siimes, M. (1981) Med. Biol. 59, 448-457.
10. Sjoerdsma, A. and Schechter, P.J. (1984) Clin. Pharmacol. Ther. 35, 287-300.
11. McCann, P.P., Bacchi, C.J., Clarkson, A.B., Sedd, J.R., Nathan, H.B., Amole, B.O., Hutner, S.H. and Sjoerdsma, A. (1981) Med. Biol. 59, 434-440.
12. McCann, P.P., Bacchi, C.J., Hanson, W.L., Cain, C.D., Nathan, H.C., Hutner, S.H. and Sjoerdsma, A.H. (1981) Adv. Polyamine Res. 3, 97-110.
13. Kallio, A., McCann, P.P. and Bey, P. (1981) Biochemistry 20, 3163-3166.
14. Paulin, L. and Pösö, H. (1983) Biochim. Biophys. Acta 742, 197-205.
15. Bitonti, A.J., McCann, P.P. and Sjoerdsma, A. (1982) Biochem. J. 208, 435-441.
16. Paulin, L., Vehmaanperä, J., Nykanen, I. Pösö, H. (1983) Biochem. Biophys. Res. Commun. 114, 779-784.
17. Pegg, A.E., Bitonti, A.J., McCann, P.P. and Coward, J.K. (1983) FEBS Letters, 155, 192-196.
18. Bitonti, A.J., Kelly, S.E. and McCann, P.P. (1984) Life Sci. 34, 1513-1520.
19. Gourlay, R.N., Leach, R.H. (1970) J. Med. Microbiol. 3, 111-123.
20. Bey, P., Vevert, J.P., VanDorsellaer, V. and Kolb, M. (1979) J. Org. Chem. 44, 2732-2742.
21. Hers, J.F. and Masurel, N. (1967) Ann. NY Acad. Sci. 143, 447-460.
22. Rodwell, A.W. and Whitcomb, R.F. (1983) The Work Methods in Mycoplasmalogy, vol. 1, (Razin, S. and Tully, J.G., eds.) pp. 188-190, Acad. Press, New York.
23. Pegg, A.E., Lockwood, D.H. and Williams-Ashman, H.G. (1970) Biochem. J. 117, 17-31.
24. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
25. Henningson, S., Persson, L. and Rosengren, E. (1976) Acta Physiol. 98, 445-449.
26. Pegg, A.E. and McGill, S. (1979) Biochim. Biophys. Acta 568, 416-427.
27. Alhonen-Hongisto, L., Veijalainen, P., Ek-Kommonen, C. and Jänne, J. (1982) Biochem. J. 202, 267-270.
28. Tanskanen, R. (1983) Acta Vet. Scand. 24, 334-336.
29. Tanskanen, R. (1984) Acta Vet. Scand., in press.
30. Gourlay, R.N., Howard, C.J., Thomas, L.H. and Wyld, S.G. (1979) Res. Vet. Sci. 27, 233-237.