Inhibition of Leishmanias But Not Host Macrophages by the Antitubulin Herbicide Trifluralin

MARION MAN-YING CHAN AND DUNNE FONG

The dinitroaniline herbicide trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine), at micromolar concentrations, selectively inhibited both proliferation and differentiation of the parasitic protozoan *Leishmania mexicana amazonensis*. In vitro, radioactive trifluralin showed specific binding to leishmania tubulin but not to mammalian tubulin. Because herbicides such as trifluralin are economical and are considered safe for man and domesticated animals, they may serve as useful sources of potential antiparasitic agents.

LEISHMANIASIS IS ONE OF THE MAJOR parasitic diseases in the developing world. However, the curative and preventive chemotherapy for leishmaniasis is still unsatisfactory (*1–3*). Because tubulin is the most abundant leishmania protein, researchers are actively searching among microtubule inhibitory agents for chemotherapeutic drugs. On the basis of analyses of its DNA sequence, β tubulin of *Leishmania mexicana amazonensis* is more similar to the trypanosomal and plant proteins than animal proteins (*4*); therefore, our focus has been on drugs that disrupt plant microtubules.

Trifluralin (Fig. 1) is normally applied, before emergence, for the control of annual grasses (*5*). Although this compound is toxic to juvenile crawfish and mosquito fish (*6*), the LD_{50} (median lethal dose), when given orally to rats, is 500 mg per kilogram of body weight (*7*). Its inhibitory effect on the cytoskeleton of the coelenterate *Hydra* has been noted during the uptake and transport of the unicellular alga *Chlorella* by *Hydra* in the establishment of symbiosis between the alga and the coelenterate (*8*), in the regeneration of the microtubule-based oral structure of the protozoan *Stentor* (*9*), and in the regeneration of the flagellum of the unicellular alga *Chlamydomonas* (*10*). We studied the effect of trifluralin on the parasitic protozoan *L. m. amazonensis*. The life cycle of this parasite consists of two stages: an extracellular promastigote form with an anterior flagellum, which can be found in the gut of the sand fly, and an intracellular nonmotile amastigote form, which occurs within the phagolysosomes of mammalian macrophages.

Trifluralin inhibited promastigote proliferation by 80 ± 10% at 2.5 μM and by 95 ± 5% at 5 μM (Fig. 2A) (error limits are ±SD throughout the paper). This is comparable to the concentration needed to inhibit the regeneration of the *Chlamydomonas* flagellum (5 μM) (*10*). To measure the drug's effect on intracellular amastigotes, we cultured murine J774 macrophages, approximately 60% of which were infected, with trifluralin and determined the ratio of infected to uninfected cells 3 days later (Fig. 2B). In each case, addition of 5 μM trifluralin reduced the frequency of infection by 50%, as compared to the untreated control (Fig. 2B). The drug probably interfered with amastigote replication, as it had no effect on the viability of purified, nonreplicating amastigotes. In the presence of up to 50 μM trifluralin, these nonreplicating amastigotes were metabolically active (for 1 day at 33°C) as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (*11*).

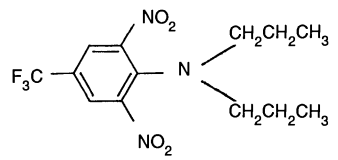

Fig. 1. Molecular structure of trifluralin.

Trifluralin also prevented amastigote-to-promastigote differentiation. Amastigotes, freshly isolated from J774 cells (*12*), were cultured in the presence or absence of trifluralin, and the ratio of promastigotes to amastigotes was determined in each case after 3 days. At 0.5 μM, trifluralin inhibited amastigote-to-promastigote differentiation by 30 to 80% (Fig. 2C). This concentration was lower than that needed to inhibit leishmanial growth.

We also tested the effect of trifluralin on mammalian cells in our in vitro culture system. Murine J774 and P388D1 and human HL-60 lines were cultured in the presence of the herbicide. No significant inhibition of proliferation (90 ± 7%) was observed at 50 μM (Fig. 3), a concentration 20 times the effective dose for the parasites.

To illustrate its mode of action, we demonstrated the specific binding of radioactive trifluralin to leishmania tubulin. Using published methods of tubulin isolation for trypanosomatids and other protozoa (*13, 14*), we partially purified subpellicular tubulin from *L. m. amazonensis* promastigotes, and we isolated mammalian tubulin from rat

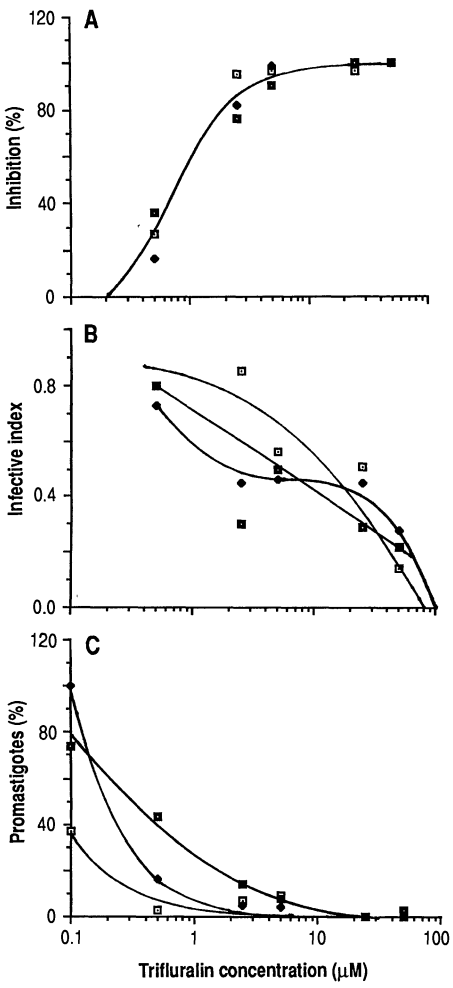

Fig. 2. The effect of trifluralin on *L. m. amazonensis*. (**A**) Promastigotes were cultured at 27°C, in LIT (liver infusion tryptose) medium (*23*), with the indicated concentrations of trifluralin added. After 5 to 6 days, the parasites were counted by hemacytometer, and the inhibition was calculated. Inhibition = 100 − [(number of parasites in experimental)/(number of parasites in untreated)] × 100. (**B**) Trifluralin treatment, at 5 μM, reduced infection by over 50%. Murine J774 macrophages, which had been infected with promastigotes for 3 days, were cultured at 33°C in complete RPMI 1640 plus fetal bovine serum (*12*), with or without trifluralin. After another 3 days, the macrophages were harvested and stained with Giemsa stain, and the number of infected macrophages was determined. Infective index = (percent of infected macrophages in experimental)/(percent of infected macrophages in untreated). The infection in the untreated culture was 60%. (**C**) At 0.5 μM, trifluralin inhibited differentiation of amastigotes to promastigotes. Amastigotes, freshly isolated from infected J774 cells, were cultured in LIT medium with the indicated concentrations of trifluralin. After 3 days at 27°C, the ratio of promastigotes to amastigotes in the cultures was determined. [Each line plotted (each type of symbol) represents a completely independent experiment.]

Bureau of Biological Research and Department of Biological Sciences, Rutgers, The State University of New Jersey, Piscataway, NJ 08855.

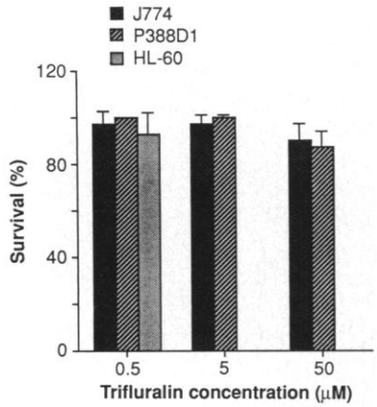

Fig. 3. Trifluralin at 50 μM did not inhibit mammalian cell growth. Murine J774 and P388D1 macrophage lines and human HL-60 cells were cultured at 37°C, in complete RPMI and fetal bovine serum, with the indicated concentrations of trifluralin added. After 5 to 6 days, the number of cells was counted and the percent of survival was calculated. Survival = [(number of cells in experimental)/(number of cells in untreated)] × 100. Standard deviations were calculated for three experiments.

brain (*15, 16*). Leishmania and rat tubulins were quantitated by Coomassie blue G-250 dye binding assay, and the purity was verified by gel electrophoresis (Fig. 4A). Tubulin (30 or 40 μg) was added to 3 μg of ^{14}C-labeled trifluralin (3 mCi/mmol), and the protein was collected by two precipitations with 25% polyethylene glycol–6000 (*17*). The [^{14}C]trifluralin bound to leishmania tubulin quantitatively (258 ± 27 dpm with 30 μg of tubulin and 566 ± 93 dpm with 40 μg) (Fig. 4B). The binding was specific: rat tubulin showed a radioactivity of 71 ± 17 dpm at 30 μg and 51 ± 14 dpm at 40 μg, values similar to those of the control with no protein added (52 ± 19 dpm). This result was comparable to that of [^{14}C]trifluralin binding to the flagellar tubulin of *Chlamydomonas* (942 dpm for 196 μg of protein with 1.03 mCi of [^{14}C]trifluralin per millimole) (*18*). Thus, the selective inhibition of leishmania growth may be due to the specific binding of trifluralin to leishmania tubulin but not to mammalian tubulin.

On the basis of ultrastructural morphology and sequence comparison of small subunit ribosomal RNAs, it has been proposed that trypanosomatids (including *Leishmania* and *Trypanosoma*) and euglenoids (including *Euglena*) are phylogenetically related (*19*). In our earlier study we showed that leishmania tubulin is more similar to trypanosome tubulin than to animal tubulin (*4*). The amino acid sequence of *Leishmania* β tubulin has 80% similarity to that of human tubulin and 93% similarity to that of trypanosomes. *Trypanosoma* β tubulin has 95% similarity to *Euglena* tubulin, which, in turn, is 95% similar to *Chlamydomonas* tubulin and 93% similar to soybean tubulin (*20*). The dinitroaniline herbicides are known to bind to *Chlamydomonas* and rose tubulins (*18, 21*).

Although the exact binding site for trifluralin on leishmanial tubulin is unknown, we have shown that trifluralin selectively binds to leishmania tubulin and interferes with leishmania growth and infection in vitro. Thus, trifluralin may be important for cytoskeleton-targeted chemotherapy against leishmaniasis, and possibly trypanosomiasis. For example, it may be possible to generate a trifluralin-derived drug for topical treatment of cutaneous leishmaniasis. Antimicrotubule drugs, such as thiabendazole, are commercially available as safe, broad spectrum anthelmintics (*22*). There may be other potentially useful antiparasitic agents among the commercially available herbicides.

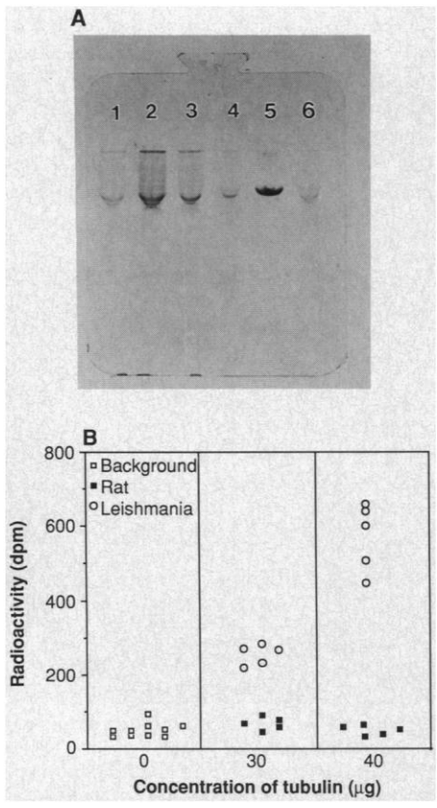

Fig. 4. Isolation of leishmania and rat tubulins and their binding to [^{14}C]trifluralin. (**A**) SDS–polyacrylamide gel electrophoresis of tubulin samples. Lanes 1 and 2, rat tubulin; lanes 3, 4, and 6, leishmania tubulin; lane 5, bovine serum albumin (500 μg/ml). (A 20% gel was run in the PhastSystem of Pharmacia. Different samples were independent preparations.) For leishmania tubulin, phosphate-buffered, saline-washed promastigotes were homogenized in a phosphate-glutamate (PG) buffer containing leupeptin and Triton X-100 (0.5%, Pierce). The detergent-insoluble material was washed in PG buffer, and tubulin was released from the cytoskeleton by $CaCl_2$ (0.5 mM) incubation according to the method of Dolan *et al.* (*14*). For rat tubulin, fresh brains were homogenized in a MES–EGTA–guanosine 5′-triphosphate buffer according to the method of Shelanski *et al.* (*15*), and the microtubules were isolated by the reversible assembly purification method as described by Vallee (*16*). Tubulin from two cycles of microtubule assembly-disassembly was dissolved in PG buffer for drug-binding studies. (**B**) Binding of [^{14}C]trifluralin to leishmania and rat tubulins. At the indicated concentrations, tubulin was added to 0.5 ml of PG buffer containing [^{14}C]trifluralin (3 μg dissolved in 5 μl of dimethyl sulfoxide) and incubated for 30 min at 37°C. We precipitated the protein by adding polyethylene glycol–6000 to a final concentration of 25%. After another 30 min at 4°C, the precipitate was spun in a microfuge at 4°C. The pellet was redissolved in 0.5 ml of PG buffer, transferred to a new microfuge tube, and reprecipitated with polyethylene glycol–6000. After centrifugation, the bottom of the microfuge tube containing the pellet was cut with a scalpel and the pellet dissolved in Beckman Ready Protein Liquid Scintillation Cocktail for the determination of radioactivity.

REFERENCES AND NOTES

1. K.-P. Chang, D. Fong, R. S. Bray, in *Leishmaniasis*, K.-P. Chang and R. S. Bray, Eds. (Elsevier, New York, 1985), pp. 1–30.
2. W. Peters and R. Killick-Kendrick, Eds., *The Leishmaniases in Biology and Medicine* (Academic Press, London, 1987), vols. 1 and 2.
3. D. T. Hart, Ed., *Leishmaniasis* (Plenum, New York, 1989).
4. D. Fong and B. Lee, *Mol. Biochem. Parasitol.* **31**, 97 (1988).
5. J. R. Corbett, K. Wright, A. C. Baillie, *The Biochemical Mode of Action of Pesticides* (Academic Press, London, ed. 2, 1984).
6. S. M. Naqvi and T. S. Leung, *Bull. Environ. Contam. Toxicol.* **31**, 304 (1983).
7. *Merck Index* (Merck, Rahway, NJ, ed. 11, 1989).
8. S. Fracek and L. Margulis, *Cytobios* **25**, 7 (1979).
9. S. Banerjee, J. K. Kelleher, L. Margulis, *ibid.* **12**, 171 (1975).
10. F. D. Hess, *Exp. Cell Res.* **119**, 99 (1979).
11. We found no difference in the metabolism of isolated amastigotes after treatment with trifluralin. Amastigotes (1×10^4) were cultured in RPMI 1640 medium containing 10% fetal calf serum and trifluralin at the indicated concentrations, at 33°C, 5% CO_2, for 48 hours. Viability was determined according to T. Mossman, *J. Immunol. Methods* **65**, 55 (1983). MTT was added 16 hours prior to the measurement of absorbance. In the absence of trifluralin, absorbances in two separate experiments were 0.095 and 0.209. In the presence of 50 μM trifluralin, the absorbances were 0.129 and 0.203, respectively.
12. K.-P. Chang, *Science* **209**, 1240 (1980).
13. L. I. Binder and J. L. Rosenbaum, *J. Cell Biol.* **79**, 500 (1978); G. T. Bramblett, R. Kambadur, M. Flavin, *Cell Motil. Cytoskeleton* **13**, 145 (1989).
14. M. T. Dolan, C. G. Reid, H. P. Voorheis, *J. Cell Sci.* **80**, 123 (1986).
15. M. L. Shelanski, F. Gaskin, C. R. Cantor, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 765 (1973).
16. R. B. Vallee, *Methods Enzymol.* **134**, 89 (1986).
17. B. Konig, P. A. DeNitto, P. M. Blumberg, *J. Cell. Biochem.* **27**, 255 (1985); A. Y. Jeng, N. A. Sharkey, P. M. Blumberg, *Cancer Res.* **46**, 1966 (1986).
18. F. D. Hess and D. E. Bayer, *J. Cell Sci.* **24**, 351 (1977).
19. M. L. Sogin and S. H. Gunderson, *Ann. N.Y. Acad. Sci.* **503**, 125 (1987).
20. M. L. Schantz and R. Schantz, *Nucleic Acids Res.* **17**, 6727 (1989).

21. L. C. Morejohn, T. E. Bureau, J. Mole-Bajer, A. S. Bajer, D. E. Fosket, *Planta* **172**, 252 (1987).
22. E. Lacey, *Int. J. Parasitol.* **18**, 885 (1988).
23. M. Sadigursky and C. I. Brodskyn, *Am. J. Trop. Med. Hyg.* **35**, 942 (1986).
24. We are grateful to R. Herman, A. Y. Jeng, S. M. Peningroth, and J. L. Rosenbaum for advice. We thank M. D. Copeland and K. Polizotto of Eli Lilly for unlabeled and [^{14}C]trifluralin, S. Auerbach for rat brains, E. Cocuzzi for use of the Pharmacia PhastSystem and gel scans, C. McCleery and Y. C. Wu for technical assistance, and T. Miller and L. Weingart for manuscript preparation. Supported in part by grants AI 21364 and CA 49359 from the National Institutes of Health and the Charles and Johanna Busch Memorial Fund.

16 February 1990; accepted 8 June 1990

Cell Alignment Required in Differentiation of *Myxococcus xanthus*

SEUNG K. KIM AND DALE KAISER*

During fruiting body morphogenesis of *Myxococcus xanthus*, cell movement is required for transmission of C-factor, a short range intercellular signaling protein necessary for sporulation and developmental gene expression. Nonmotile cells fail to sporulate and to express C-factor–dependent genes, but both defects were rescued by a simple manipulation of cell position that oriented the cells in aligned, parallel groups. A similar pattern of aligned cells normally results from coordinated recruitment of wild-type cells into multicellular aggregates, which later form mature fruiting bodies. It is proposed that directed cell movement establishes critical contacts between adjacent cells, which are required for efficient intercellular C-factor transmission.

CELL INTERACTIONS ESTABLISH CELL fate during morphogenesis of multicellular organisms (*1–3*). Many cell interactions require correct spatial patterning of cells for proper signaling (*4–6*). A protein called C-factor, which has properties of a morphogenetic paracrine signal, is required for cellular aggregation, spore differentiation, and gene expression induced by starvation of the rod-shaped Gram-negative bacterium, *Myxococcus xanthus* (*7, 8*). C-factor has been purified and identified as the membrane-associated protein product of the *csg*A (ς signal) gene (*7, 8*). Cell motility is required for proper intercellular transmission of C-factor (*9*). Nonmotile cells respond to purified C-factor and produce it at wild-type concentrations, yet they arrest development at a stage similar to *csg*A mutants, which do not produce C-factor. Increased cell density partially restores nonmotile cell sporulation (*10*). These observations suggest that movement might allow cells to establish a spatial pattern that is crucial for subsequent C-factor–dependent sporulation and gene expression. We report here that ordered parallel alignment of nonmotile cells restored both sporulation and developmental gene expression.

On a solid surface, starving *M. xanthus* cells glide to aggregation centers where they build a small, steep-sided mound of about 10^5 cells (*11*). The external simplicity of the mound conceals what scanning electron microscopic studies (*12*) have revealed: cells are patterned inside a mound in organized, coherent arrays. This patterning of cells also can be detected by lower resolution light microscopy (Fig. 1). Coordinated cell movement is apparent early in aggregation, when ridge-like accumulations of gliding cells move with regular periodicity like ripples on a water surface (*13*). Within a circular mound, some cells lyse, while other cells differentiate to ovoid, refractile spores that are resistant to heat and desiccation (*14*). These spores later fill the mature fruiting body. There are similarities in development of myxobacteria and cellular slime molds (*15*).

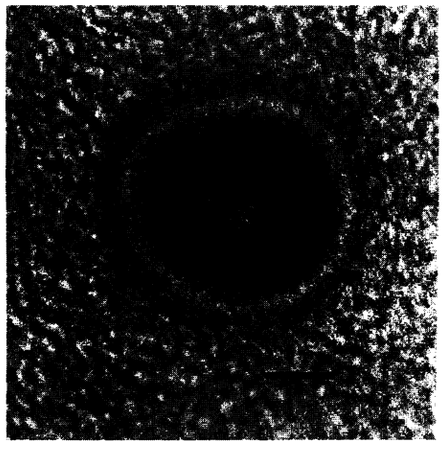

Fig. 1. Internal organization of a nascent *M. xanthus* fruiting body. Wild-type *M. xanthus* strain DK1622 was grown and fruiting body development induced on solid starvation agar (*11, 20*). Aggregated cells were photographed with a Leitz inverted light microscope at 40× magnification. Scale bar, 50 μm.

Control of the development of *M. xanthus* fruiting bodies by cell-cell interactions is implied by four classes of nonautonomous mutants that can sporulate only upon mixture with wild-type cells (*16*). One class of such developmental mutants is the *csg* mutants (*16, 17*), which result from mutation of the *csg*A gene (*18*) and fail to complete aggregation, ripple, lyse, or sporulate (*13*). *csg*A mutants show an altered pattern of developmental gene expression as monitored by transcriptional fusions of *lac*Z to developmentally regulated genes (*19–21*). In *csg*A mutants, expression of *lac*Z fusions is normal for the first 6 hours of development (C-factor–independent expression), but β-galactosidase expression is reduced or abolished after 6 hours (C-factor–dependent) (*21*). All of the developmental defects resulting from mutation of *csg*A are overcome by codevelopment of mutant cells with wild-type cells or by addition of 1 nM purified C-factor (*7, 8*). Biochemical characterization suggests that C-factor is membrane-associated.

Evidence that the transmission of C-factor between cells requires cell movement comes from studies of *M. xanthus* cells that are nonmotile because of mutations in the *mgl*A gene (*22*). Nonmotile cells, like *csg*A mutants, fail to aggregate, ripple, sporulate, or express C-factor–dependent genes (*10*). C-factor (1 nM) purified from wild-type cells restores *mgl*A sporulation and gene expression to wild-type amounts, but admixture of intact wild-type cells does not. Wild-type concentrations of C-factor can be purified from *mgl*A cells, yet intact *mgl*A cells do not rescue *csg*A cells (*9*). These observations support the hypothesis that cells must move for efficient in vivo *csg*A signaling. Proper intercellular C-factor transmission may require a critical spatial orientation achieved only after cells move into the dense aligned cellular organization of a nascent fruiting body.

To test this hypothesis, we simulated the cellular organization found within a fruiting body by artificially aligning nonmotile cells. Nonmotile cells were placed at standard density (5×10^9 per milliliter) on a solid developmental surface that had been scored in one dimension with 5- to 10-μm aluminum oxide abrasive paper to create microscopic grooves. Nomarski optics revealed that cells that settled in these grooves were oriented with their long axes parallel to the

Departments of Biochemistry and Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305.

*To whom correspondence should be addressed.