ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1993, p. 1909–1913
0066-4804/93/091909-05$02.00/0

Vol. 37, No. 9

Structure-Function Analysis of Antimicrotubule Dinitroanilines against Promastigotes of the Parasitic Protozoan *Leishmania mexicana*

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Received 14 January 1993/Returned for modification 1 March 1993/Accepted 30 June 1993

Although leishmaniasis is a major tropical disease, the currently available drugs are toxic and inadequate. We show that the antimicrotubule herbicide trifluralin has antileishmania activity. The present study aimed at deducing the relationship between the structure of the molecule and its antiprotozoan activity. Nine dinitroanilines, all of which were analogs of trifluralin, were compared. We found that pendimethalin was 2.5-fold more potent than trifluralin, and the higher efficacy may be correlated with molecular structural features that increase the accessibility to one nitro group. This association was further supported by molecular modeling. Moreover, trifluralin samples from two sources differed in their activities by more than threefold, and gas column chromatography showed that impurities were present in the more potent sample.

Leishmaniasis is a major tropical disease affecting 12 million people (33). Pentavalent antimonial agents and pentamidine have been the recommended treatment for this disease since the second world war (8). However, they cause severe adverse side effects and failure of treatment is not uncommon; therefore, better, less toxic therapeutic drugs are urgently needed. We discovered that trifluralin and oryzalin, antimicrotubule dinitroaniline herbicides, inhibit leishmania proliferation and infectivity (4–7). Moreover, in addition to being effective against *Leishmania* spp., trifluralin is effective against the in vitro proliferation of *Trypanosoma brucei* trypomastigotes (6, 14) and the intraerythrocytic forms of the malaria parasite *Plasmodium falciparum* (22). Thus, trifluralin is effective against several species of protozoan parasites.

Trifluralin and oryzalin belong to a class of compounds known as the dinitroanilines (Fig. 1 and Table 1). As herbicides, the dinitroanilines cause multinucleation, accumulation of cells at the metaphase, and the loss of microtubules from root cells. They bind to plant but not animal tubulins. Trifluralin and many of its analogs have been commercially available since the 1960s and have been widely used for weed control on lands with fruit trees and vegetable crops. Trifluralin and related compounds are inexpensive to manufacture, and their traits are well characterized, including their toxicities and shelf-lives. In its purified form, trifluralin is generally shown to be noncarcinogenic, nonteratogenic, and nonmutagenic, as shown by various standard in vitro mutagenic studies and in vivo genotoxicity assays with bacteria, yeasts, mammalian cells, and animals (1, 9, 11, 12, 18, 24, 34). Thus, this group of herbicides includes promising lead compounds for antiparasitic agents. Moreover, they may be economical for the treatment of the domestic animal reservoirs that play a major role in the epidemiology of parasitic diseases (such as dogs for leishmaniasis in some countries [see references 15 and 25]).

To pursue the goal of developing dinitroanilines for leishmaniasis therapy, we studied the analogs of trifluralin in an attempt to deduce the crucial chemical structures needed for activity. We found that pendimethalin is a more potent compound than trifluralin, and we detected an impurity in trifluralin that could lead to a novel and highly potent antileishmania agent.

MATERIALS AND METHODS

Dinitroanilines. Trifluralin and benfluralin were obtained from two sources: Dow Elanco and Riedel-de Haen. Oryzalin and ethalfluralin were provided by Dow Elanco, and dinitramine and prodiamine were provided by Sandoz. Fluchloralin, nitralin, pendimethalin, and profluralin were purchased from Riedel-de Haen through the U.S. distributor Crescent Chemicals (Hauppauge, N.Y.).

Promastigote assay. Dinitroanilines were dissolved according to the following protocol. The compounds were first dissolved in acetone as a 100 mM solution. Then they were diluted in 0.1% acetone-liver infusion tryptose (27) medium that had been prewarmed to 65°C. Solutions of 1 mM (1/100 dilution) and 0.5 mM (1/2 dilution) were made from the acetone stocks and then were made to the final concentrations by using 10-fold serial dilutions. The compounds were added at the indicated concentrations to equal volumes of *Leishmania mexicana amazonensis* LV 78 promastigotes (10^5/ml). The cultures, 2 ml per well in 24-well tissue culture plates, were incubated at 27°C. After 5 to 6 days, the parasites were counted with a hemacytometer, and the inhibition was calculated. Inhibition (in percent) = 100 − [(number of parasites remaining after treatment)/(number of parasites in control)] × 100] (5). For each experiment, the data were plotted and then the effective doses in which there was 50% inhibition of promastigote proliferation (ED_{50}s) were determined. Three replicates were performed for each analog, and then the ED_{50}s were averaged and standard deviations were calculated.

Statistical analysis. The program MyStat (Macintosh) was used for performing the Student *t* test comparisons.

Gas chromatography analysis. The dinitroaniline was in-

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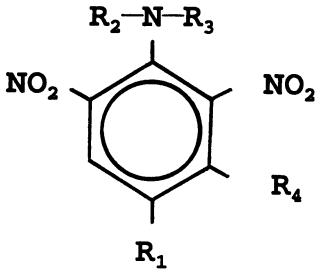

FIG. 1. General structural formula of dinitroaniline. The substituents R1, R2, R3, and R4 are given in Table 1.

jected directly into a gas chromatograph, at 100 mM in acetone, in a volume of 1 μl, for purity analysis. A Varian 3400 gas chromatograph equipped with a fused silica capillary column (60 m by 0.25 mm [inner diameter]; thickness, 0.32 μm; DB-1; J&W Inc.) and a flame ionization detector was used. The operation conditions were as follows: injector temperature, 270°C; detector temperature, 300°C; helium carrier flow rate, 1 ml/min; and temperature program, 40 to 260°C at 4°C/min. A split ratio of 50:1 was used.

Molecular modeling. The molecular modeling programs Insight (version 2.0) and Discover (version 2.7) (Biosym Technologies, Inc.) were used to build and minimize the energies of the dinitroaniline molecules.

RESULTS

Differences between trifluralin preparations from Dow Elanco and Riedel-de Haen. Trifluralin from two different sources varied in their ED_{50}s against *L. mexicana*. Trifluralin from Dow Elanco (ED_{50}, 4.6 μM) was more potent than that from Riedel-de Haen (ED_{50}, 14.2 μM), a threefold difference (Table 2). Consistent results were obtained with three batches of trifluralin from each company (Dow Elanco lots 573AP8, 518AP9, and 990AP0; Riedel-de Haen lots 02320, 10870, and 91650). Thus, we suspected the presence of impurities in the herbicide samples.

TABLE 1. Chemical structures of dinitroanilines

Dinitroaniline	Constituent at: R1	R2	R3	R4
Trifluralin	$-CF_3$	$-C_3H_7$	$-C_3H_7$	$-H$
Nitralin	$-S(=O)_2-CH_3$	$-C_3H_7$	$-C_3H_7$	$-H$
Oryzalin	$-S(=O)_2-NH_2$	$-C_3H_7$	$-C_3H_7$	$-H$
Pendimethalin	$-CH_3$	$-CH(C_2H_5)_2$	$-H$	$-CH_3$
Profluralin	$-CF_3$	$-CH_2-\triangleleft$	$-C_3H_7$	$-H$
Benfluralin	$-CF_3$	$-C_2H_5$	$-C_4H_9$	$-H$
Ethalfluralin	$-CF_3$	$-C_2H_5$	$-CH_2-C(CH_3)=CH_2$	$-H$
Fluchloralin	$-CF_3$	$-C_3H_7$	$-CH_2CH_2Cl$	$-H$
Dinitramine	$-CF_3$	$-C_2H_5$	$-C_2H_5$	$-NH_2$
Prodiamine	$-CF_3$	$-C_3H_7$	$-C_3H_7$	$-NH_2$

TABLE 2. Efficacies of trifluralin and related analogs

Compound and comparison group	Source[a]	ED_{50} (μM)[b]	SD[b]
Trifluralin	R	14.2	1.9
Trifluralin	D	4.6[c]	1.5
Comparisons of analogs with trifluralin from Riedel-de Haen			
Fluchloralin	R	4.0[c]	0.0
Pendimethalin	R	5.6[c]	2.2
Profluralin	R	11.2	3.3
Dinitramine	S	24.3	4.0
Nitralin	R	23.4[c]	4.0
Prodiamine	S	>50	
Benfluralin	R	>50	
Comparisons of analogs with trifluralin from Dow Elanco			
Benfluralin	D	6.6	2.9
Ethalfluralin	D	7.6	1.9
Oryzalin	D	17.3[d]	6.4

[a] Sources: R, Riedel-de Haen; S, Sandoz; D, Dow Elanco.
[b] The ED_{50}s and standard deviations (SDs) were averaged and determined from three independent experiments.
[c] Indicates significant difference from trifluralin (Riedel-de Haen) ($P < 0.05$) by the Student t test.
[d] Indicates significant difference from trifluralin (Dow Elanco) ($P < 0.05$) by the Student t test.

Impurities in the dinitroaniline compounds. Gas chromatography analysis showed that the more potent trifluralin samples contained impurities (Fig. 2A and B). By quantitative analysis, the largest impurity peak constituted approximately 0.6% of the trifluralin peak. In addition, two other Dow Elanco compounds, benfluralin and ethalfluralin, also had the same minor impurity peaks (data not shown) and their ED_{50}s, 6.6 and 7.6 μM, respectively, were similar to that of trifluralin with the contaminant. Fluchloralin from Riedel-de Haen, the ED_{50} of which was comparable to those of the compounds from Dow Elanco (4 μM), also had similar impurity peaks (Table 2 and Fig. 2C). Thus, the importance of these impurities was emphasized.

Active structures. We attempted to deduce the structure-function relationship of analogs against *L. mexicana*. Comparisons were made among the analogs which did not contain the impurities. The results indicated that higher efficacy was associated with an increase in the level of accessibility to the nitro group; access to either one of the two nitro groups was sufficient.

Pendimethalin (ED_{50}, 5.6 μM), with a hydrogen instead of a propyl group (at the R3 position; see Table 1), was more effective than trifluralin from Riedel-de Haen (ED_{50}, 14.2 μM) and had an efficacy comparable to that of the compound from Dow Elanco (ED_{50}, 4.6 μM). On the other hand, benfluralin, with an extra carbon at the R3 position, was ineffective (ED_{50}, >50 μM). Correspondingly, profluralin, with a cyclopropylmethyl group that is similar in size to the propyl group of trifluralin, was similar in efficacy (ED_{50}, 11.2 μM) to trifluralin. Apparently, a hydrophobic region of maximum volume corresponding to a propyl group at R2 or R3 is required. This interpretation was further supported by the findings with prodiamine and dinitramine. With prodiamine, an amino group at R4 decreased the hydrophobicity

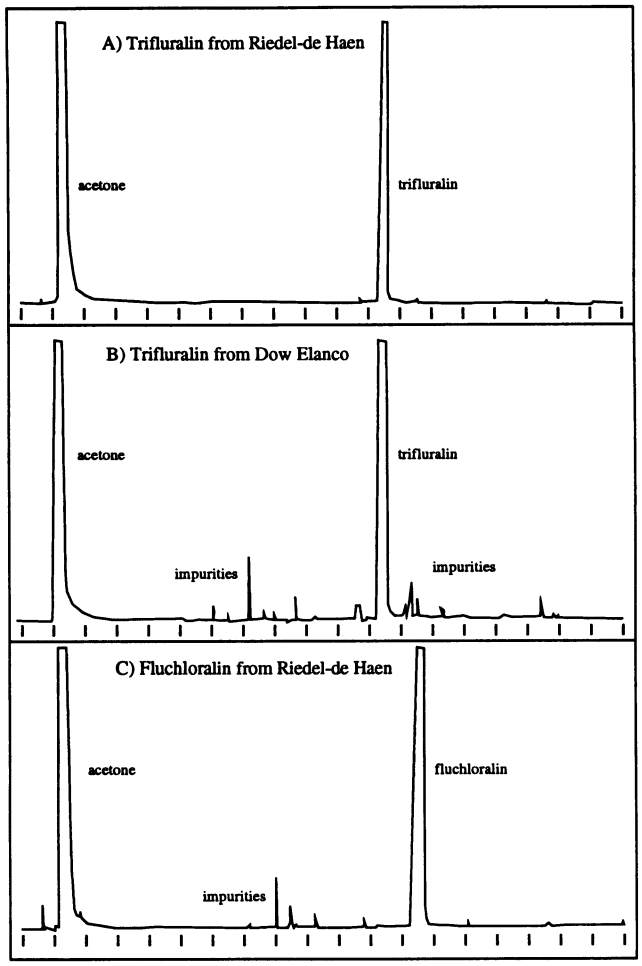

FIG. 2. Gas chromatograms of trifluralins and fluchloralin.

on one side of the molecule and aborted its efficacy (ED_{50}, >50 μM); however, this effect may be compensated for by reducing the bulkiness at R3 by one carbon, as seen in dinitramine (ED_{50}, 24.3 μM).

The structures of the trifluralin analogs were approximated by molecular mechanics, using the Insight II molecular modeling package. The models show that the dialkyl-substituted amino and, possibly, the *o*-nitro groups must rotate out of the plane of the benzene ring. Even though the exact positions await crystallographic analysis, the molecular structures of these compounds in the immediate vicinity of the central benzene ring are so compact and constrained sterically that only minor variations from predicted molecular structures are likely. From the models presented in Fig. 3, the accessibility of the nitro groups can be estimated. In comparison with trifluralin, one of the nitro groups of pendimethalin is more accessible, whereas the hydrophobic region of prodiamine is reduced by the presence of an amino group at R4. Comparing prodiamine and dinitramine, the ethyl groups in dinitramine are replaced by the propyl groups in prodiamine, which appears to be a positive effect, compensating for the negative effects of the amino group at the R4 position. Comparing trifluralin and benfluralin, at the R3 position, the propyl group of trifluralin yields an effective compound, whereas the bulkier butyl group of benfluralin does not.

In addition, we found that substitution of a sulfonyl group for a trifluoromethyl group at R1, an alternate method of decreasing the hydrophobicity of the molecule, caused the compounds to be less effective. Nitralin (ED_{50}, 23.4 μM) was 1.6-fold less effective than trifluralin (ED_{50}, 14.2 μM) (Table 2).

DISCUSSION

In our attempt to determine the crucial chemical structures needed for leishmania inhibition, we compared the efficacies of different trifluralin analogs and found that trifluralin from different sources varied in ED_{50}. Gas chromatography analysis showed that the more potent preparations contained impurities. Since trifluralin is a photolabile compound, the impurities may be photodegraded products or they may be synthesis by-products (18, 32). We are in the process of isolating the impurities from trifluralin so that the antileishmania activity of the impurity may be assessed directly.

Comparing the efficacies of different trifluralin analogs, we also found that the increase in efficacy correlated with the accessibility of one of the nitro groups and to the presence of a hydrophobic region of specific volume. Molecular modeling of the analogs supported this conclusion drawn from the experimental results. Pendimethalin was more effective than trifluralin against *L. mexicana* and was not inhibitory to human monocytes. Moreover, this increase in efficacy was not associated with an increase in toxicity for mammalian cells. As with trifluralin (5), no significant inhibition of proliferation was observed at a concentration that was 12.5-fold greater than the effective dose for the parasites (data not shown).

The action of dinitroanilines on plants and protozoans may be due to similarities among their tubulin proteins (10, 30). Silflow (29) has discussed the fact that the ciliate and flagellate protozoans seem to lack diversity in their tubulins and suggested that the reason may be the stringent and constrained requirements for the axoneme structure of these unicellular organisms. This is an interesting point for contemplation. However, many multicellular organisms also have cells with cilia and flagella.

Experiments performed by us and others have indicated that dinitroanilines are active against several groups of protozoans, namely, the flagellates, the ciliates, and the apicomplexans. For the flagellates, oryzalin inhibits leishmania differentiation and in vitro microtubule polymerization of *Leishmania* tubulin (7). Trifluralin binds *Leishmania* tubulin and inhibits *Leishmania* differentiation; it also binds *Chlamydomonas* flagellar tubulin and inhibits flagellar regeneration of *C. eugametos* (5, 16, 17). Trifluralin even modulates the chemosensory response of the dinoflagellate *Crypthecondinium cohnii* (20). For the ciliates, trifluralin inhibits microtubule-based oral structure regeneration of *Stentor coeruleus*; it inhibits cell division of *Paramecium tetraurelia* (3, 23). Oryzalin even increases α-tubulin mRNA transcription of *Tetrahymena thermophila* (31). For the apicomplexans, trifluralin inhibits both the growth and the differentiation of *P. falciparum*, especially at the exflagellation stage (22). Previously, Bajer and Mole-Bajer (2) have designated oryzalin, together with another herbicide, amiprophos-methyl (APM), as "'the colchicines' of the plant kingdom." This designation can now be extended to include the protozoans.

Referring to trifluralin, Morejohn and Fosket (21) have stated: "Since protoctists (protozoans) generally are resis-

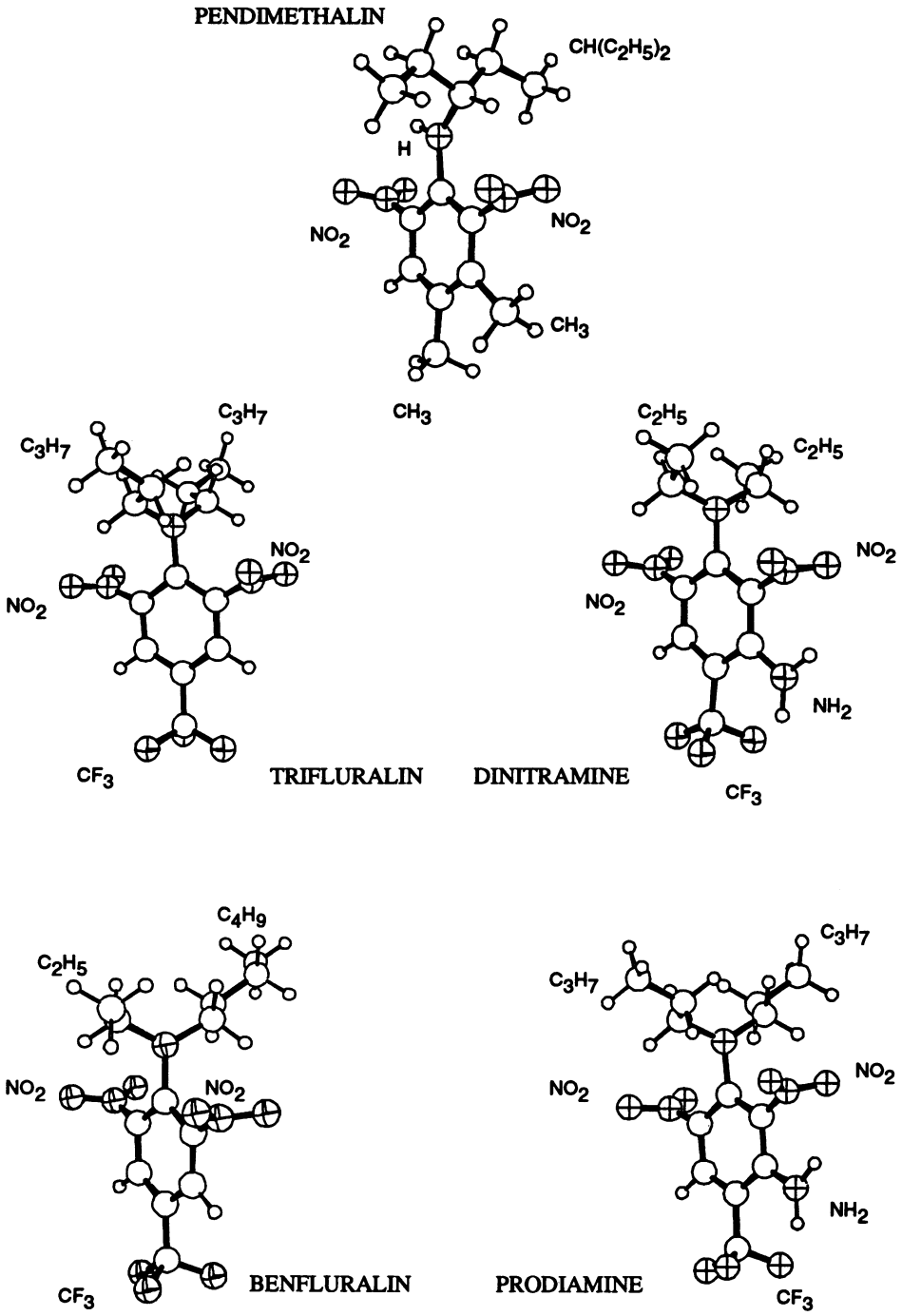

FIG. 3. Molecular modeling results for five dinitroanilines.

tant to colchicine treatment, the anti-microtubule herbicide may become valuable probes for the analysis of microtubule function in these organisms." Most dinitroaniline compounds are commercially available; this is an advantage over APM, which is not commercially available. They are useful compounds for studying microtubule functions in both free-living and parasitic protozoans. Oryzalin has been chosen because of its solubility. However, if the protozoans can tolerate a low dose of organic solvent (1% acetone, in our case, for *L. mexicana*, or solvents such as dimethyl sulfoxide), other, possibly more potent dinitroanilines (such as pendimethalin for *L. mexicana*) can be used for such studies.

For the parasitic protozoan *L. mexicana*, targeting of drugs at unique organelles such as the kinetoplast and glycosomes has been suggested (13). Our data, as presented here, have demonstrated that microtubules are also promising targets. Drugs with microtubules as their targets have previously been used successfully, notably, the antihelminthic benzimidazoles and the anticancer compounds such as vinblastine, vincristine, and taxol (19, 26, 28). Likewise,

drugs based on dinitroanilines and related compounds definitely have potential to be economic and effective antileishmania chemotherapeutic agents.

ACKNOWLEDGMENTS

We thank Robert Herman for reading the manuscript, Chiann-Chyi Chen for excellent technical assistance, and Dow Elanco and Sandoz for herbicide samples.

This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (grants 910024 and 920134). The work was also supported in part by grant R01 CA49359 from the National Institutes of Health and by the Charles and Johanna Busch Memorial Fund.

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