Inhibition of *Toxoplasma gondii* Replication by Dinitroaniline Herbicides

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*Department of Pathology, Dartmouth Medical School, Hanover, New Hampshire, 03756, U.S.A.; and †Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.

STOKKERMANS, T. J. W., SCHWARTZMAN, J. D., KEENAN, K., MORRISSETTE, N. S., TILNEY, L. G., AND ROOS, D. S. 1996. Inhibition of *Toxoplasma gondii* replication by dinitroaniline herbicides. *Experimental Parasitology* 84, 355–370. Submicromolar concentrations of several dinitroaniline herbicides have been found to specifically inhibit intracellular replication of the protozoan parasite *Toxoplasma gondii*. IC₅₀ concentrations for *T. gondii* survival were ∼100 nM for ethalfluralin and oryzalin and ∼300 nM for trifluralin. Primary human fibroblasts employed as host cells for parasite culture were unaffected at >100-fold higher concentrations. Extracellular parasites were unaffected by these drugs, but within 8 hr after treatment of infected cell cultures, intracellular tachyzoites formed large amorphous bodies containing distorted nuclei. Parasite cytokinesis was completely blocked by drug treatment; nucleic acid synthesis, however, continued at near-normal levels for several days in the continuous presence of drug. All dinitroanilines appear to block nuclear division by inhibition of intranuclear spindle formation, but other cytoskeletal components were differentially affected by the various drugs tested. Subpellicular microtubules were absent in oryzalin-treated parasites, and large fragments of the inner membrane complex were observed throughout the parasite cytoplasm. In contrast, subpellicular microtubules and the inner membrane complex remained intact in ethalfluralin-treated parasites, but the endoplasmic reticulum and nuclear envelope were highly distented. Cytoskeletal elements associated with the conoid were not affected by any of the dinitroanilines tested, and treatment with the Ca²⁺ ionophore A23187 failed to trigger release of drug-treated parasites from infected cells. Mutant parasites resistant to oryzalin, ethalfluralin, or trifluralin were selected by chemical mutagenesis and examined for cross-resistance. An ethalfluralin-resistant mutant displayed cross-resistance to both oryzalin and trifluralin, while a trifluralin-resistant mutant was sensitive to oryzalin and only partially resistant ethalfluralin; an oryzalin-resistant mutant exhibited higher resistance to ethalfluralin and trifluralin than to oryzalin itself. Similarities between Apicomplexan and plant tubulin are discussed. © 1996 Academic Press, Inc.

INDEX DESCRIPTORS: apicomplexa; coccidia; cytoskeleton; microtubule; cytokinesis; tubulin evolution; drug development.

**INTRODUCTION**

The protozoan parasite *Toxoplasma gondii* is a ubiquitous organism capable of infecting a wide range of vertebrate hosts, including man. Approximately one-third of the U.S. population is estimated to be infected, and infection rates range from 10 to 90% elsewhere in the world. Although most infected individuals show no sign of clinical disease, primary infection during pregnancy can lead to serious congenital birth defects (McAuley *et al.* 1994; Daffos *et al.* 1994). Recently, toxoplasmosis has also emerged as a major opportunistic pathogen associated with acquired immunodeficiency syndrome (Luft and Remington 1992; Mariuz *et al.* 1994). Clinical toxoplasmosis has traditionally been treated by blocking folate metabolism with a combination of antifolates (such as pyrimethamine) and sulfonamides (Brooks *et al.* 1987). While this strategy is highly effective in otherwise healthy individuals, the persistence of latent cysts requires that immunocompromised patients be maintained on chemotherapy for life. Unfortunately, the frequent development of allergic reactions to sulfonamides often precludes chronic administration of these drugs, and pyrimethamine alone is generally insufficient to prevent relapse (Leport *et al.* 1988; Tenant-Flowers *et al.* 1991). This situation
lends renewed impetus to the development of novel strategies for dealing with toxoplasmosis (Laughon et al. 1991).

The search for drugs to inhibit T. gondii may reasonably include agents that affect the parasite’s complex cytoskeleton (Nichols and Chiappino 1987). This obligate intracellular parasite is highly motile and actively invades host cells (Sibley 1994). Replication occurs by endodyogeny, a process requiring the assembly of daughter parasites within a cytoskeletal framework inside the mother (Chobotar and Scholtsysek 1982). Although the precise mechanisms underlying these parasite functions are not well understood, the cytoskeleton is suspected to play an important role in motility, invasion, and endodyogeny (Morrissette et al. 1994; Morisaki et al. 1995), and disruption of any of these essential functions might be expected to kill the parasite.

Dinitroanilines have long been known as potent and specific inhibitors of plant microtubules (Hess and Bayer 1977; Ashton and Crafts 1981; Bajer and Molé-Bajer 1986; Morejohn et al. 1987), and their activity has recently been demonstrated against certain parasitic and non-parasitic protozoa (James and Lefebvre 1989; Chan and Fong 1990; Chan et al. 1993a; Stargell et al. 1992; Kaidoh et al. 1995). We have now found that this toxicity extends to the clinically important Apicomplexan parasite T. gondii. Parasites remain metabolically active for a considerable time after herbicide addition, but are unable to divide or produce infectious progeny, probably due to the disruption of spindle-pole body formation during merogony. Other classes of parasite microtubules are differentially affected by the various dinitroanilines tested. The comparatively recent divergence of animals and plants (Baldauf and Palmer 1993; Wainright et al. 1994) relative to the broad spectrum of eukaryotic diversity represented by the protozoa (Sogin et al. 1989; Cavalier-Smith 1993) suggests that herbicides may be a productive source of candidate parasiticidal agents.

**MATERIALS AND METHODS**

**Parasites and host cells.** T. gondii strain RH parasites were grown as tachyzoites in monolayer cultures of human foreskin fibroblasts, in either Eagle’s minimal essential medium or Dulbecco’s modified Eagle’s medium supplemented with 1–3% fetal bovine serum, and were grown at 37°C in a humidified atmosphere containing 5% CO2. Plaque assays were carried out in 25 cm2 tissue culture flasks, and uracil incorporation assays in 24-well tissue culture plates. For details of parasite culture and assays see Roos et al. (1994). Host cell proliferation was assayed by exposing cultures to various drug concentrations for 48 hr, followed by trypsination and counting in a Neubauer hemacytometer.

**Drugs.** The following dinitroanilines were obtained from Drs. John Keeton and Dennis Lade of Lilly Research Laboratories (Indianapolis, IN): oryzalin, 3,5-dinitro-N4,N4-dipropylsulfanilamide; ethalfluralin, N-ethyl-N-(2-methyl-2-propenyl)-2,6-dinitro-4-trifluoromethylbenzenamine; trifluralin, N,N-dipropyl-2,6-dinitro-4-trifluoromethylbenzenamine (see Fig. 1 for structures). Additional samples

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**Fig. 1.** Structure of dinitroaniline herbicides. All three of these compounds inhibit intracellular replication of T. gondii tachyzoites at sub-micromolar concentrations.
were provided by Dr. John Benbow (Lehigh University). All three compounds were stored as 1 mM stocks in DMSO. Dinitroaniline purity was confirmed by HPLC: drugs were diluted to 5 μg/liter in 5% isopropanol (in water), injected into a Waters Nova-Pak C18, 60 Å (4 μm), HPLC column (3.9 mm i.d., 150 mm) using a Waters 510 HPLC pump, eluted using a 1:3: H2O:acetonitrile gradient, and absorbance was measured at 200–245 nm using a Waters 486 tunable absorbance detector (Cabras et al. 1991; D’Amato et al. 1993). Data were analyzed using Millipore Millenium 2010 Chromatography Manager software. Calcium ionophore A23187 was obtained from Sigma (Kansas City, MO) and stored as a 1 mM stock in DMSO.

Mutagenesis. Parasites were chemically mutagenized according to a modification of previously published protocols (Peifferkorn et al. 1992; Roos et al. 1994). Tachyzoites, 2 × 10⁷ (freshly emerged from their host cells) were incubated at 37°C for 2 hr in serum-deficient minimal essential medium containing 90 μg/ml ethylnitrosourea (NIH Human Carcinogen Repository; diluted from stock solution in DMSO). Parasites were washed three times by centrifugation and plated on fresh fibroblast monolayers. Within individual parasitophorous vacuoles, parasites replicate synchronously to produce 2, 4, 8, 16, . . . , tachyzoites (Chobotar and Scholtyseck 1982; Fichera et al. 1995). After intracellular replication to the eight-cell stage (three parasite doublings), tachyzoites were forcibly released from the host cells by passage through a 27-gauge needle, inoculated into fresh cell monolayers in medium containing 1 μM dinitroaniline (ethalfluralin, oryzalin, or trifluralin), and incubated until viable extracellular tachyzoites were evident. Surviving parasites were passaged once more under continued dinitroaniline treatment and cloned by limiting dilution.

Microscopy. Light microscopy was carried out using parasites fixed in methanol and stained with 0.02% Giemsa, or using living cultures observed with phase-contrast optics. For electron microscopy, infected cultures in 35-mm petri dishes were fixed in situ in 0.05 M phosphate buffer (pH 6.3) containing 1% glutaraldehyde and 1% OsO4 (added at room temperature and then placed on ice for 45 min). Samples were rinsed, stained overnight in 0.5% uranyl acetate, dehydrated, and embedded in Epon. Cured plastic blocks were cut, reoriented, and trimmed as required, sectioned with a diamond knife, and examined using a Phillips 200 electron microscope.

Phylogenetic analysis. β-Tubulin sequences for various animal, plant, fungal, and protist species were downloaded from the PIR, GenBank, and EMBL databases using the NCBI RETRIEVE e-mail-server (retrieve@ncbi.nlm.nih.gov) and Network Entrez Web-site (http://www3.ncbi.nlm.nih.gov/entrez/). Predicted protein coding sequence for the entire protein excepting the highly divergent extreme C-terminus (e.g., the last 24 amino acids of the T. gondii sequence [Nagel and Boothroyd 1988]) were aligned using MACAW (Schuler et al. 1991) and ClustalW (Thompson et al. 1994) software, with manual adjustment. Phylogenetic analysis was performed by maximum parsimony (using PAUP 3.0s software [Swofford 1991]) and distance-based methods (using PHYLIP 3.56c software [Felsenstein 1993]). Bootstrap analyses (Hillis and Bull 1993) were based on 100 replicates.

RESULTS

Dinitroaniline herbicides inhibit proliferation of T. gondii. Interest in the complex cytoskeletal apparatus of apicomplexan parasites (Nichols and Chiappino 1992; Morrissette et al. 1994) led us to investigate the effects of dinitroaniline herbicides—which have been reported to inhibit kinetoplastid parasites (Chan et al. 1993a)—against T. gondii. Because previous reports suggested that the effects of these drugs may arise from minor contaminants (Francis et al. 1991; Chan et al. 1993b; Berman 1994), the purity of oryzalin, ethalfluralin, and trifluralin (Fig. 1) was examined by HPLC as described under Materials and Methods. Each drug showed a single major peak containing >99% of the material (not shown). Elution times indicated that oryzalin is the least hydrophobic of these three compounds, trifluralin the most hydrophobic, and ethalfluralin intermediate.

As shown in Fig. 2, all three dinitroanilines inhibited plaque formation by T. gondii parasites in vitro, with IC₅₀ concentrations in the 100–300 nM range. Similar results have also been obtained using microtiter assays, although such experiments are complicated by the dramatic vapor effect of some dinitroaniline compounds. (Experiments must be conducted in sealed chambers, especially when micromolar or higher drug concentrations are employed.) Observation of uninfected host cell fibroblasts by phase-contrast microscopy showed normal mitotic figures, and confluent monolayers exhibit normal morphology. Host cell replication was resistant to ≥50 μg drug (open symbols, broken lines), with inhibition observed only at concentrations where dinitroaniline crystals precipitate on top of the cell monolayer. Pretreatment of either extracellular parasites or uninfected host cells had no effect on parasite infectivity or plaquing efficiency.

Dinitroaniline-treated parasites invade and grow but are unable to divide. To distinguish
between inhibition of parasite invasion vs intracellular proliferation, tachyzoites were added to cultures containing 0.5–1 μM drug (approx three times the IC₅₀) and incubated for 36 hr. After fixation and staining, the percentage of infected fibroblasts and average number of parasites per infected focus was determined and compared with controls. As shown in Table I, host cell invasion was normal in the presence of drug, but it was impossible to accurately determine the number of parasites within infected cells, because intracellular parasite morphology was strikingly abnormal. Instead of ‘‘rosettes’’ (Fig. 3A), parasitophorous vacuoles contained a single swollen blob (Fig. 3B).

In order to characterize this phenomenon more thoroughly, host cell monolayers were inoculated with parasite tachyzoites and incubated for 8 hr. One micromolar drug was then added (8 hr postinfection), and parasite morphology followed over the next 24 hr. At 12 hr postinfection, the vacuoles in untreated controls contained either 2 or 4 tachyzoites (Fig. 4A). Oryzalin-treated cultures (grown for 8 hr in normal medium followed by 4 hr in 1 μM drug) contained vacuoles with either 2 tachyzoites (the progeny of a single tachyzoite which infected and replicated once prior to treatment) or four amorphous blobs (arrowhead in Fig. 4B). After 32 hr, vacuoles in control cultures contained 8, 16, or 32 parasites (Fig. 4C; also note free extracellular parasites which have lysed out of nearby cells), while drug-treated cultures typically contained clusters of four large blobs (Fig. 4D).

Parasites lose viability at the first mitosis, but remain metabolically active. Drug washout experiments were conducted to examine when during the course of dinitroaniline treatment parasites lose their ability to form plaques. As
FIG. 3. Inhibition of intracellular replication by ethalfuralin. (A) 36 hr after infection, parasitophorous vacuoles in untreated cultures contain rosettes of 16 tachyzoites. (B) Parallel cultures incubated in the continuous presence of 1 μM oryzalin contain parasitophorous vacuoles of comparable size to untreated controls, but these vacuoles enclose a single amorphous blob, within which individual parasites cannot be resolved. Samples were fixed in methanol and stained with Giemsa for microscopy. Scale bar, 30 μm.
shown in Fig. 5, the loss of plaque-forming ability in either oryzalin or ethalfluralin was roughly linear with time; virtually no plaques (<1% of control) were found in samples treated for more than 8–12 hr (approximately one replicative cycle [Fichera et al. 1995]). Comparable results were obtained when intracellular parasites were forcibly released by syringe passage and inoculated into fresh cultures for plaque assay, instead of simply washing the culture with drug-free medium (not shown). Because extracellular tachyzoites are an asynchronous population containing parasites at all stages of the cell cycle, this time course is consistent with the interpretation that parasites die when they first enter mitosis in the presence of drug.

In contrast, pyrimethamine (a classic parasitostatic drug) could be removed after 24 hr treatment with minimal loss of parasite viability (broken line in Fig. 5). Incubation of extracellular tachyzoites for 12 hr in 1 μM oryzalin (○) or ethalfluralin (□) had no effect on viability (compared with parallel controls incubated without drug).

**Fig. 5.** Time course of loss of plaquing ability after exposure to dinitroanilines. Infected cultures were treated with 1 μM oryzalin, ethalfluralin, or pyrimethamine for the indicated times, followed by drug removal. Plaque formation was scored as a percentage of untreated controls (average of two independent experiments). The viability of drug-treated parasites declines nearly to 0 within 8–12 hr of dinitroaniline treatment. Incubation of extracellular tachyzoites for 12 hr in 1 μM oryzalin (○) or ethalfluralin (□) had no effect on viability (compared with parallel controls incubated without drug).
Metabolism was followed by measuring the uptake of \[^3\text{H}\]uracil (Pfefferkorn and Pfefferkorn 1977; Roos et al. 1994) in the presence of various dinitroanilines. In contrast to the antifolate pyrimethamine, which rapidly shuts down parasite metabolism, uracil uptake in the presence of oryzalin or ethalfluralin continued virtually unabated for >24 hr (Fig. 6). These observations suggested that dinitroanilines block the replication of infectious parasites by disrupting the spindle–pole body microtubules necessary for division, without inhibiting parasite metabolism per se.

Ultrastructure of dinitroaniline-treated parasites. The pellicle of *T. gondii* tachyzoites normally consists of three bilayer membranes, including the plasma membrane and two membranes of the "inner membrane complex" (Fig. 7). The inner membrane complex is closely apposed to—and possibly connected with—an internal skeleton of subpellicular microtubules. In mitotic parasites, intranuclear spindle–pole microtubules are also observed at the apical end of the nucleus (Chobotar and Scholtyseck 1982). Oryzalin treatment resulted in the loss of subpellicular microtubules, and large sheets of inner membrane complex were found dispersed throughout the cytoplasm (Fig. 8B). The loss of subpellicular microtubules destroys the parasite’s banana-like shape, but Golgi structure was not perturbed, providing a marker for the apical end of the nucleus. No spindle–pole body microtubules were found in any of the oryzalin-treated parasites. Although internal membranes were not swollen in these parasites, prolonged exposure to high concentrations of drug results...
Fig. 8. EM ultrastructure of oryzalin and ethalfluralin-treated parasites. Infected HFF cells were grown in the presence of 1 \( \mu M \) oryzalin (B) or ethalfluralin (A) for 20 hr and prepared for electron microscopy as described under Materials and Methods. The intranuclear spindle–pole body normally present at the apical end of the nucleus of mitotic parasites was absent in all parasites in these samples. Other subcellular components are differentially affected: in oryzalin, the inner membrane complex dissociates from the plasma membrane and can be seen as whorls of membrane within the cytoplasm. In ethalfluralin the Golgi apparatus disappears and other intracellular membranes swell dramatically. g, Golgi apparatus; c, conoid. Scale bar, 1 \( \mu m \).
in swollen mitochondria (possibly as a result of osmotic effects related to Ca\(^{2+}\) transport, as reported in plant systems [Hertel et al. 1980]).

Surprisingly, subpellicular microtubules remained intact in ethalfluralin-treated parasites (Fig. 8A), but spindle–pole body microtubules were nevertheless completely lacking. Parasites caught during the process of replication when herbicide was administered contain long, flaccid buds of inner membrane complex (the scaffolding of developing daughter parasites), but their nuclei show no hint of bifurcation, apparently having retracted from the developing buds into a more stable spheroid conformation upon destruction of the nuclear spindle (not shown). Ethalfluralin treatment is also associated with loss of the Golgi apparatus and a massive expansion of other internal membrane compartments (Roos and Tilney, in preparation).

Response to Ca\(^{2+}\) ionophore. Calcium ionophores act to trigger the release of intracellular tachyzoites from infected host cells, probably by inducing parasite motility (Endo et al. 1982). In order to investigate the effect of dinitroanilines on parasite escape from infected cells, 1 \(\mu M\) A23187 was applied to cultures similar to those shown in Fig. 3 (untreated cultures had formed rosettes containing 8–16 parasites). Ionophore treatment resulted in the complete release of all control parasites within 2 min, but parasites grown in the presence of 1 \(\mu M\) oryzalin, ethalfluralin, or trifluralin remained intracellular. It was interesting to note, however, that the vacuolar space surrounding dinitroaniline-treated parasites (cf. Fig. 3B) shrank within 2 min of ionophore application. Intracellular tachyzoites also became noticeably more refractile when viewed by phase-contrast microscopy (not shown).

Isolation of dinitroaniline-resistant mutants. To further investigate the mechanism of dinitroaniline action in Toxoplasma, parasites were mutagenized with ethylnitrosourea as described under Materials and Methods, and mutants resistant to 1 \(\mu M\) drug were selected using either oryzalin, ethalfluralin, or trifluralin. For each drug under study, five of the most rapidly growing clones were isolated by limiting dilution, and one clone resistant to each drug was chosen for further characterization (designated Orz\(^{R-1}\), Efl\(^{R-1}\), and Trf\(^{R-1}\)).

All three dinitroaniline-resistant mutants exhibited normal morphology when grown in inhibitory concentrations of drug, and the resistance phenotypes of all three clones were stable after passage for at least 25 generations without drug treatment. As indicated in Table II, clone Efl\(^{R-1}\) was resistant not only to the selecting drug (ethalfluralin), but was also cross-resistant to oryzalin and trifluralin. Mutant Orz\(^{R-1}\) was also resistant to all three dinitroanilines, although, curiously, this mutant was more highly resistant to other drugs other than the original selecting agent. Mutant Trf\(^{R-1}\) exhibited cross-resistance to ethalfluralin, but not oryzalin. These patterns suggest that multiple loci may play a role in governing drug resistance.

**DISCUSSION**

We have examined the effect of three dinitroanilines (oryzalin, ethalfluralin, and trifluralin) on invasion and growth of *T. gondii* in *vitro*. All of these herbicides specifically inhibit cytokinesis of intracellular parasites at submicromolar concentrations, without noticeably affecting either the host cells or parasite invasion. The kinetics of plaque inhibition suggest that parasites die when they attempt mitotic replication in the presence of drug. Continued growth in the absence of division produces the bloated parasites observed by light microscopy (Figs. 3 and 4), a phenotype consistent with the swollen, multilflagellated promastigotes observed in oryzalin-treated cultures of *Leishmania* (Chan et al. 1991).

Dinitroanilines have long been known to in-

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<th>Paracies</th>
<th>Oryzalin</th>
<th>Ethalfluralin</th>
<th>Trifluralin</th>
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<tr>
<td>RH (wild-type)</td>
<td>0.3</td>
<td>0.1</td>
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<tr>
<td>Orz(^{R-1})</td>
<td>3.0</td>
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<td>Efl(^{R-1})</td>
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<td>Trf(^{R-1})</td>
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terfere with tubulin polymerization in plants (Hess and Bayer 1977; Ashton and Crafts 1981; Bajer and Molé-Bajer 1986; Morejohn et al. 1987). Trifluralin also binds to *Leishmania* tubulin and inhibits polymerization of subpellicular microtubules (Chan and Fong 1990). In *Plasmodium*, trifluralin binds to and fragments the subpellicular microtubules of gametocytes (Kaidoh et al. 1995) and also inhibits both erythrocytic stages and exflagellation of gametocytes (Nath et al. 1994). We presume that *T. gondii* microtubules also interact with dinitroanilines, although this hypothesis has not been tested directly.

It is interesting to note the differential stability of various parasite cytoskeletal structures upon dinitroaniline treatment. As the *T. gondii* genome is known to contain but a single α-tubulin and a single β-tubulin gene (Nagel and Boothroyd 1988), this heterogeneity is presumed to result from differences in microtubule-associated proteins. Such heterogeneity may underlie the unusual stability of *Toxoplasma* subpellicular microtubules *in vitro* (Morrisette et al. submitted). In mammalian systems, structural integrity of the Golgi apparatus is sensitive to microtubule-disrupting agents (Ho et al. 1989; Turner and Tartakoff 1989). Selective disruption of the Golgi in ethalfluralin (and concomitant distension of other internal membrane components) may have interesting implications for the secretory pathway in *Toxoplasma* (cf. Novick and Schekman 1980).

In order to explore one possible basis for the selectivity of dinitroaniline herbicides for protistan tubulins, we examined tubulin protein sequences from various taxa. As shown in Fig. 9, analysis of unambiguously aligned β-tubulin sequences by maximum parsimony (Swofford 1991) confirms that Apicomplexans and ciliates are sister taxa (Wolters 1991; Gajadhar et al. 1991; Cavalier-Smith 1993; Gagnon et al. 1993; Escalante and Ayala 1995). (No dinoflagellate β-tubulins are available in the database.) Surprisingly, Apicomplexan/ciliate tubulins are distinctly more similar to plant and algal tubulins than to animal tubulins. Distance matrix analyses yield similar results (not shown). Bootstrap analysis (Hillis and Bull 1993) suggests that *T. gondii/plant* tubulin similarity is a global phenomenon (i.e., not restricted to a single protein domain). When analyses are run on datasets including only animals, plants, apicomplexans, and ciliates (with *Giardia lamblia* as an outgroup), the apicomplexan/ciliate clade groups with plants in 93% of bootstrap replicates (not shown). The evolutionary basis of this similarity—which differs from ribosomal gene phylogenies (Sogin 1991; Gagnon et al. 1993; Escalante and Ayala 1995)—remains unclear, although it may simply be the reciprocal aspect of arguments that animals and fungi are sister taxa (Baldauf and Palmer 1993; Wainright et al. 1994). Analysis of α-tubulin and DHFR-TS genes yield phylogenies consistent with the β-tubulin tree (not shown).

According to this view, herbicides may provide a rich source of potential parasiticidal agents. At the very least, as it is now abundantly clear that protozoa are not the “primitive animals” that their name implies, there is no *a priori* rationale for excluding herbicides as candidate parasiticidal drugs. One distinct advantage of herbicides is the availability of toxicological studies. Dinitroanilines in use as commercial herbicides have been extensively studied, and show low mammalian toxicity (Matthews et al. 1986; Garriott et al. 1991); impurities in some preparations may have been responsible for isolated reports of carcinogenicity in animal models (Francis et al. 1991; Berman 1994).

When delivered by topical formulation, trifluralin is active against cutaneous *Leishmania* lesions *in vivo* (Chan et al. 1993a), but given their low solubility it is unlikely that any of the compounds studied here could be effective as a systemic drug. Structure–function studies of several dinitroaniline compounds have shown that at least one of the nitro-groups is required for anti-trypanosomatid activity (Chan et al. 1993b), and further such studies may permit the identification of more soluble compounds (Palmer et al. 1992) which retain their parasiticidal efficacy. Our results for *T. gondii* are also
in agreement with the structure–function studies of Chan et al. (1993b) in finding that ethalfluralin is the most active of the three compounds tested, perhaps because its NO$_2$ group is the most accessible; ethalfluralin is also less hydrophobic than either oryzalin or trifluralin.

A complementary method for the development of more effective dinitroanilines is to identify the molecular target (and specific interactions) for these drugs by genetic means, such as the mutants evaluated in Table II. In this regard, it is interesting to note that while certain tubulin mutations in the green alga _Chlamydomonas reinhardtii_ are resistant to dinitroanilines (Schnibler and Huang 1991; James et al. 1993), most dinitroaniline-resistance mutations in _C. reinhardtii_ map to loci other than tubulin genes (James et al. 1989; James and Lefebvre 1989, 1992; Lux and Dutcher 1991).

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