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Dinitroaniline Activity in *Toxoplasma gondii* Expressing Wild-Type or Mutant α-Tubulin

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The human parasite *Toxoplasma gondii* is sensitive to dinitroaniline compounds which selectively disrupt microtubules in diverse protozoa but which have no detectable effect on vertebrate host cell microtubules or other functions. Replication of wild-type *T. gondii* is inhibited by 0.5 to 2.5 μM oryzalin, but mutant parasites harboring amino acid substitutions in the predicted dinitroaniline binding site confer resistance up to 40 μM oryzalin. However, the precise interaction between dinitroanilines and the binding site in α-tubulin remains unclear. We have investigated the activity of 12 dinitroanilines and the related compound amiprophos methyl on wild-type and dinitroaniline-resistant parasite lines that contain proposed binding site mutations. These data indicate that dinitramine is the most effective dinitroaniline to inhibit *Toxoplasma* growth in wild-type parasites and most resistant lines. Dinitramine has an amino group at the meta position not present in any of the other dinitroanilines tested here that is predicted to form hydrogen bonds with residues Arg2 and Gln133 according to docking data. Remarkably, although the binding site mutation Ile235Val confers increased resistance to most dinitroanilines, it confers increased sensitivity to GB-II-5, a compound optimized for activity against kinetoplastid tubulin. Kinetoplastid parasites have a valine at position 235 of α-tubulin, whereas apicomplexan parasites have an isoleucine at this site. We suggest that this heterogeneity in binding site environment influences relative dinitroaniline sensitivity in distinct protozoan lineages and hypothesize that a mutation that makes the apicomplexan dinitroaniline binding site more like the kinetoplastid site increases sensitivity to a dinitroaniline optimized for activity in the latter parasites.

Although tens of millions of people from the developing world are impacted by diseases caused by parasitic protozoa, the efficacy of available therapeutics is reduced by drug toxicity and the emergence of drug-resistant parasite strains. The demand for new and effective antiparasitic agents is immense. Since parasite microtubules coordinate essential processes such as chromosome segregation during mitosis, compounds that target protozoan but not vertebrate host tubulin would act as parasite-specific antimitotic agents. Dinitroaniline compounds exhibit this selective activity. These small synthetic molecules (oryzalin, ethafluralin, trifluralin, etc.) were discovered in 1960 by researchers looking to inhibit land plant meristematic growth (1). In addition to activity in protozoan parasites also contain plasma membrane-associated microtubules that confer rigidity and a characteristic shape (24, 47). A typical microtubule is a cylindrical polymer formed by 13 longitudinal protofilaments (15, 16, 34). Each protofilament is built by the head-to-tail linkage of α-β-tubulin heterodimers. Microtubules exist in a dynamic equilibrium between tubulin dimer subunits and microtubule polymers that is critical for proper function. Known antimitotic agents bind to specific sites on the tubulin dimer to alter this equilibrium, making tubulin a well-established target for diverse medical conditions ranging from cancer to helminth infections (14, 30, 32, 57).

Both α- and β-tubulins are highly conserved among all eukaryotes (17, 39, 50). However, phylogenetic analysis of the tubulin family has demonstrated that protozoan tubulins (from Alveolates such as Tetrahymena, Plasmodium, and *Toxoplasma* and from Euglenozoa such as Euglena, *Trypanosoma*, and *Leishmania*) form a distinct group from vertebrate and fungal tubulins, and these proteins are most similar to tubulins from land plants (31). The amino acid sequences for α-tubulins from humans, apicomplexans (*Toxoplasma gondii* and *Plasmodium falciparum*) and kinetoplasts (*Trypanosoma cruzi* and *Leishmania major*) share ~83% identity. Conservation within major groups is even higher: kinetoplastid and apicomplexan tubulins are ~86% identical and conservation among apicomplexans or...
among kinetoplastids is ~93% (analysis not shown). Despite a high degree of amino acid sequence conservation, some tubulin-binding compounds are selectively active on phylogenetically restricted tubulin subsets. For example, the benzimidazoles oxfendazole and thiabendazole disrupt nematode and fungal microtubules without affecting human microtubules, making them useful antihelminth and antifungal agents (12, 13, 20, 25, 32, 33, 52, 53). As described above, dinitroaniline compounds selectively disrupt microtubules in diverse protozoa and plants and lack binding to and activity against vertebrate and fungal microtubules (reviewed in references 46, 56, and 57).

Computational data indicate that dinitroanilines bind to a consensus site on protozoan α-tubulin subunits (43, 45). Parallel studies with Bos taurus α-tubulin show that dinitroanilines do not interact with vertebrate tubulin (45). The binding site on protozoan α-tubulins is located under the H1-S2 (N) loop. Since the H1-S2 loop is intimately associated with maintaining protofilament contacts within the microtubule lattice, we hypothesize that dinitroaniline binding interferes with this interaction to disrupt microtubules. Computational studies have established that the dinitroaniline binding site is conserved between apicomplexans (T. gondii and P. falciparum) and kinetoplastids (L. major) and that other dinitroaniline derivatives (GB-II-5 and trifluralin) bind to the same site (43). However, distinct dinitroanilines possess different inhibitory effects. Oryzalin inhibits T. gondii replication at 0.5 μM, but is only effective against Leishmania tubulin at 10 μM (43). Structure-activity relationship (SAR) studies using oryzalin as the lead compound have established that derivatives with altered functional groups attached to the aniline nitrogen or the sulfonylamide nitrogen are more active against kinetoplastid parasites (4, 5, 18, 19, 59, 60). The oryzalin derivative GB-II-5 (N'-phenyl-3,5-dinitro-N,N,N,N-di-n-propylsulfanilamide) has increased potency against kinetoplastids (T. brucei and Leishmania spp.). However, it is much less efficient than oryzalin against the apicomplexans P. falciparum and T. gondii (N. Morrissette and K. Werbovetz, unpublished observations). Protozoan tubulin-dinitroaniline interactions are likely to be influenced by minor species-specific differences in the properties of the binding site that alter interactions with distinct functional groups on different dinitroaniline compounds.

In previous genetic studies of dinitroaniline resistance, we identified and characterized a number of point mutations in Toxoplasma α1-tubulin that confer resistance to oryzalin. A subset of these mutations (Val41Leu, Ser61Le, Phe24His, His28Gln, Leu136Phe, Ile235Lys, Thr239Ile, Arg243Cys, and Arg243Ser) are located in the computationally derived dinitroaniline binding site, and we previously generated Toxoplasma lines harboring allelic replacements of α1-tubulin with genes bearing each of these 11 point mutations (35, 45). Eight of the substitutions (Val41Leu, Ser61Le, Phe24His, His28Gln, Leu136Phe, Thr239Ile, Arg243Cys, and Arg243Ser) are within 4 Å of the bound dinitroaniline, indicating a close association with the compound. Val41Leu, Ser61Le, Phe24His, His28Gln, Leu136Phe, Thr239Ile, Arg243Cys, and Arg243Ser confer higher resistance to oryzalin (7.5 to 40 μM) than the other point mutations identified so far, suggesting that these positions may mediate more important dinitroaniline-ligand interactions. Finally, the 11 mutations are spatially distributed around the dinitroaniline binding site, making them a representative subset with which to study dinitroaniline binding.

Dinitroanilines are important tools to elucidate the properties of selective α-tubulin binding ligands. Optimization of dinitroaniline site ligand activity requires a better understanding of the interaction between tubulin and dinitroanilines. In this study, we investigate the ability of dinitroanilines with different functional groups to inhibit replication in both wild-type parasites (RH strain) and in Toxoplasma lines with predicted binding site mutations. Dinitroaniline resistance is measured as the 50% inhibitory concentration (IC50) for each Toxoplasma line. By assessing the effects of different dinitroanilines on wild-type and binding site mutants, we can identify functional group-binding site interactions that will help us identify improved lead compounds.

MATERIALS AND METHODS

Dinitroaniline compounds. Eleven commercially available dinitroaniline compounds and the related phosphoric amide amiprophos-methyl (APM) were obtained through Sigma-Aldrich: APM [O-methyl O-(2-nitro-p-tolyl)-N-isopropylphosphorothioate] was synthesized by Fluka; butralin (N-sec-butyl-4-tert-butyl-2,6-dinitroaniline), dinitramine (N,N,N,N-diethyl-2,6-dinitro-4-trifluoromethyl-N-phenylenediamine), nitralin (4-methyl-2,6-dinitro-N,N-dipropylaniline), oryzalin (3,5-dinitro-N,N-di-n-propylsulfanilamide), pendimethalin [N-(1-ethylpropyl)-2,6-dinitro-3,4-sydlinidene], and profluralin [N-cyclopropylmethyl-2,6-dinitro-N-propyl-4-trifluoromethylaniline] were synthesized by Riedel-de Haen; benfuralin [N-butylyl-N,N,N,N-tert-butyl-2,6-dinitro-p-toluclidine], ethalfluralin [N-ethyl-N,N,N,N-trifluoro-N-(2-methylallyl)-2,6-dinitro-p-toluclidine], fluchloralin [N,N-di-(2-chloroethyl)-2,6-dinitro-N-propyl-4-(trifluoromethyl)aniline], isopropalin (4-isopropyl-2,6-dinitro-N,N-dipropylaniline), and trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluclidine) were synthesized by Sepulco. GB-II-5 (N'-phenyl-3,5-dinitro-N,N-di-n-propylsulfanilamide) was synthesized as previously described (5).

Culture of Toxoplasma lines. T. gondii tachyzoites (the wild-type RH strain and mutants derived from the RH strain) were grown in human foreskin fibroblast (HFF) cells in DMEM with 10% fetal bovine serum (FBS) as previously described (35, 36, 45). We previously generated T. gondii lines bearing allelic replacements of point mutations Val41Leu, Ser61Le, Phe24His, His28Gln, Leu136Phe, Ile235Lys, Ile235Val, Thr239IIe, Arg243Cys, and Arg243Ser in the α1-tubulin gene that are predicted to fall in the computationally derived dinitroaniline binding site (35, 45). Lines bearing oryzalin resistance mutations were propagated in media containing 0.5 μM oryzalin to prevent the appearance of revertants or suppressor mutations.

Determination of IC50 values. Inhibition of parasite growth in dinitroanilines was measured by a plaque assay modified from an earlier established method (51, 54). Parasites were propagated in HFF cells in DMEM with 10% (FBS). After complete lysis from HFF cells, extracellular parasites were purified from host cell debris by passing lysate through a 3-μm filter (GE Osmonics). Confluent HFF cells in T25 flasks were exposed to culture media that contain a specified concentration of each compound. Equivalent numbers of parasites (~1,000) were inoculated into each flask, and these were left undisturbed for 8 days at 37°C in a humidified 5% CO2 incubator. On the eighth day, each T25 flask was rinsed once with phosphate-buffered saline (PBS), and the remaining host cells were fixed with methanol (5 min at room temperature) and stained with 5× crystal violet solution for 5 min. The crystal violet staining solution consists of 12.5 g crystal violet in 125 ml ethanol mixed with 500 ml 1% ammonium oxalate in H2O. After removal of the crystal violet solution, cells were rinsed with PBS twice and allowed to air dry overnight. Plaques (visualized as irregular clear areas against the violet background produced by the HFF cells) were enumerated for each flask. To generate the kill curve, plaque numbers (represented as a percentage of plaques relative to the no drug control) were plotted versus drug concentration. The IC50 value was extrapolated from the curve as the concentration in which there are 50% of the total plaques compared to the control flask. The results represent the average of three independent IC50 assays ± standard error of the mean.

Immunofluorescence staining. HFF cells on 12-mm circular glass coverslips were inoculated with RH parasites, and these samples were grown overnight in specific dinitroanilines. Intracellular parasites were fixed, permeabilized, and stained as previously described (48). The T. gondii surface was labeled...
with the anti-SAG1 antibody DG52 (7) and detected with an Alexa 594 secondary antibody (Invitrogen), while parasite microtubules were labeled with a Toxoplasma-specific antitubulin antibody (48) detected with an Alexa 488 secondary antibody (Invitrogen). DNA was visualized by DAPI (4',6-diamidino-2-phenylindole) staining (Vector Laboratories). Images were collected on a Zeiss Axioskop using the Axiovision camera and software and exported for manipulation in Photoshop 8.0.

**RESULTS**

Wild-type *Toxoplasma* strains display different sensitivities for distinct dinitroanilines. In previous studies, we used morphological criteria to determine the oryzalin sensitivity of wild-type and dinitroaniline-resistant *Toxoplasma* parasites, including parasites that have amino acid substitutions in the computationally determined binding site. Assays that measure relative growth of *Toxoplasma* in drug by direct proliferation measurements are complicated by the fact that dinitroanilines inhibit replication and invasion by parasite progeny but do not inhibit metabolic activity of parasites in host cells (54). Although plaques in plaque assays become smaller as compound concentrations are increased, we have adapted this standard assay to determine IC_{50} values for 11 structurally distinct dinitroaniline compounds and two related compounds (GB-II-5 and APM) (Fig. 1 and 2). We have also validated that these compounds act on *Toxoplasma* microtubules rather than by some alternative means of toxicity using immunofluorescence microscopy to visualize tubulin in treated cells (Fig. 3). Wild-type *Toxoplasma* tachyzoites treated with dinitramine have disrupted microtubules, inhibited replication, and rounded cell shape, in contrast to elongated and replicating parasites in
matched no-drug controls, consistent with previous observations (48). The IC\textsubscript{50} data indicate that in wild-type parasites dinitramine is the most effective derivative with an IC\textsubscript{50} value of 0.045 \textmu M (Table 1). The next most effective compounds are oryzalin, pendimethalin, and ethalfluralin, which have 5-fold higher IC\textsubscript{50} values of ~0.25 \textmu M. The least effective compound is GB-II-5, which has an IC\textsubscript{50} value of 6.7 \textmu M. Dinitramine is predicted to form an additional hydrogen bond with tubulin.

Dinitramine has significantly higher activity in the IC\textsubscript{50} plaque assay and is distinct from the other dinitroanilines because it has an amine (NH\textsubscript{2}) group at the meta position of the dinitroaniline. In all other compounds save pendimethalin, which has a methyl (CH\textsubscript{3}) group at the meta position, the dinitroanilines are unmodified at this site. In computational studies, dinitramine appears to interact with tubulin, with a similar orientation to the dinitroanilines oryzalin and trifluralin (Fig. 4A). However, docking analysis suggests that the interaction of dinitramine with \textit{Toxoplasma} \alpha-tubulin is strengthened by the addition of two hydrogen bonds which occur between the dinitramine NH\textsubscript{2} and the backbone carbonyl of Arg2 and the side-chain carbonyl of Gln133 (Fig. 4B). This reinforces other hydrogen bonds which are predicted to form between the dinitramine NO\textsubscript{2} and NH\textsubscript{2} groups from Val4 and Arg243, which also exist in other tubulin-dinitroaniline interactions (Fig. 4).

Binding site mutations confer increased resistance to dinitroanilines. We determined IC\textsubscript{50} values for all of the compounds in a set of parasite lines with allelic replacements of each of the 11 \textit{\alpha}1-tubulin point mutations that are located within the computationally determined binding site. Our previous work established that the binding site mutations Leu136Phe, Thr239Ile, and Arg243Ser conferred high levels of oryzalin resistance (~40 \textmu M) relative to other point mutations (35). IC\textsubscript{50} values from the current study confirmed this and established that these mutations conferred high resistance to all compounds tested in the current study, including dinitramine and pendimethalin (Table 1). In addition to these three high resistance mutations, Val4Leu increased IC\textsubscript{50} values (~7 \textmu M) to most compounds tested, with the exception of APM (2.1 \textmu M), dinitramine (3.4 \textmu M), and pendimethalin (3.2 \textmu M). Finally, the Phe24His and His28Gln mutations conferred higher IC\textsubscript{50} values (~10 \textmu M) to a specific subset of compounds. Parasites bearing the Phe24His mutation had IC\textsubscript{50} values of 24.2 \textmu M for butralin, 17.2 \textmu M for GB-II-5, 15.7 \textmu M for benfluralin, and 10.0 \textmu M for APM but the remaining compounds had concentrations under 4.4 \textmu M in this assay. Parasites bearing the His28Gln mutation conferred an IC\textsubscript{50} value of 15.2 \textmu M for benfluralin, 12.4 \textmu M for GB-II-5, and 10.2 \textmu M for butralin but had IC\textsubscript{50} values under 5.4 \textmu M for all remaining compounds.

Dinitroaniline efficacy against binding site mutants. For the 11 binding site mutant lines, dinitramine was the most effective dinitroaniline for 7 mutant lines (Ser6Ile, Phe24His, His28Gln, Ile235Leu, Ile235Thr, Ile235Val, and Arg243Cys) and the second most effective compound for the Val4Leu line (after APM). Pendimethalin ranked first for two binding site mutant lines (Arg243Ser and Thr239Ile) and was the second most

FIG. 2. Determination of IC\textsubscript{50} values for a representative analysis. (A) Representative kill curves for R243S parasites. The individual curves (●, ○, and ▲) represent three independent plaque assays for R243S parasites in profluralin. (B and C) Crystal violet-stained flasks for the control (B) and 20 \textmu M concentration of profluralin (C). As the concentration of dinitroaniline increases, plaques become less frequent and smaller in size.

FIG. 3. Intracellular \textit{Toxoplasma} stained with a \textit{Toxoplasma}-specific antitubulin antibody (green), a surface antigen (SAG-1; red), and DNA labeled with DAPI (blue) indicates that the dinitramine disrupts parasite microtubules. Immunofluorescent images (A and C) and phase-contrast microscopy (B, D) of control parasites (A and B) and parasites treated with 0.25 \textmu M dinitramine (C and D) illustrate that the characteristic crescent shape of tachyzoites is converted into a round and nondividing morphology (arrows) typical of parasites with disrupted microtubules. Scale bar, 10 \mu m.


<table>
<thead>
<tr>
<th>Strain</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td>RH</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>VL</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>PAM</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Butralin</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>Dinitramine</td>
<td>12.0 ± 0.5</td>
</tr>
<tr>
<td>Ethalfluralin</td>
<td>18.0 ± 0.6</td>
</tr>
<tr>
<td>Fluchloralin</td>
<td>24.0 ± 0.7</td>
</tr>
<tr>
<td>GB-II-5</td>
<td>31.0 ± 0.8</td>
</tr>
<tr>
<td>Isopropalin</td>
<td>37.0 ± 0.9</td>
</tr>
<tr>
<td>Nitralin</td>
<td>43.0 ± 1.0</td>
</tr>
<tr>
<td>Oryzalin</td>
<td>49.0 ± 1.1</td>
</tr>
<tr>
<td>Pendimethalin</td>
<td>55.0 ± 1.2</td>
</tr>
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*Results for the most effective compounds are shown in boldface. Results for the second most effective compounds are shown in italics.

TABLE 1. IC50 values for the strains in this study.

**DISCUSSION**

A number of studies have shown that microtubules in protozoan parasites are sensitive to dinitroaniline compounds (2–5, 8–10, 26, 28, 35–38, 40–42, 44–46, 55–59). SAR studies by several groups indicated that modification of functional groups on dinitroanilines can increase antitubulin activity in specific protozoa. Early work modifying functional groups on the triflu-
ralin precursor chloralin established that both NO₂ moieties are critical to inhibit Leishmania promastigotes, as substitutions to groups with less electron-withdrawing activity correspond to loss of antileishmania activity (8). Studies of inhibition of the apicomplexan C. parvum demonstrated that substitution for the aniline propyl chains of oryzalin and trifluralin with two cyclohexanes or one phenyl group retains the antiparasitic activity, but substitutions that increase hydrophilicity of dinitroanilines decrease compound efficacy against parasites (3, 8, 41). More recent work on the relationship between derivatives and kinetoplastid tubulin has optimized activity for the kinetoplastids Leishmania and Trypanosoma (4, 5, 18, 19, 59). Oryzalin is only moderately effective against kinetoplastids, but its derivative GB-II-5 disrupts Leishmania microtubules more efficiently. However, as described above, there is a reciprocal relationship between efficacy of oryzalin and GB-II-5: while GB-II-5 has increased potency over oryzalin in kinetoplastids, it has decreased activity on apicomplexans which are sensitive to oryzalin.

This study characterizes nine compounds with asymmetrical structures (APM, benfluralin, butralin, dinitramine, ethalfluralin, GB-II-5, fluchloralin, pendimethalin, and profluralin) and four symmetrical dinitroanilines (isopropalin, nitralin, oryzalin, and trifluralin). Dinitramine and pendimethalin were the most effective compounds against both wild-type parasites and mutants harboring binding site substitutions. Both compounds feature an additional functional group at the meta position (NH₂ for dinitramine and CH₃ for pendimethalin), suggesting that functionalizing this position augments dinitroaniline binding to /H9251-tubulin. The dinitroaniline prodiamine also has a meta NH₂ (like dinitramine) but like oryzalin possesses two propyl chains at the aniline nitrogen (dinitramine has two ethyl chains). Although we obtained a prodiamine solution to verify that the additional NH₂ group is responsible for the enhanced efficacy of dinitramine as compared to oryzalin, its insolubility in aqueous solution made it impossible to test in a biological setting. Nonetheless, the fact that dinitramine is more potent than all other compounds indicates that the addition of a hydrogen bond donor like NH₂ might strengthen the dinitroaniline-tubulin interaction. Indeed, the dinitramine NH₂ is predicted to provide additional hydrogen bonds by interacting with the backbone carbonyl of Arg2 and the side-chain carbonyl of Gln133. Pendimethalin might also be less effective than dinitramine because it has only one alkyl chain.

FIG. 4. Model of dinitramine docked to Toxoplasma α-tubulin. Docking results for dinitroanilines on Toxoplasma α-tubulin. (A) Common docking orientation of oryzalin (orange), trifluralin (cyan), and dinitramine (yellow) in the binding cleft with amino acids within 3 Å of the docked compounds shown in detail; (B) molecular details for dinitramine showing the two hydrogen-bonding interactions of the NH₂ functional group with the backbone carbonyl of Arg2 and the side-chain carbonyl of Gln133. The Arg243 residue is omitted so not to obscure the hydrogen-bonding interactions of the dinitramine NH₂ with Arg2 and Gln133 (dashed lines).

FIG. 5. (A) IC₅₀ values for oryzalin and GB-II-5 in wild-type parasites and parasites bearing the Ile235Val mutation, which decreases sensitivity to oryzalin but increases sensitivity to GB-II-5. The Ile235Val line is significantly more sensitive to GB-II-5 than the wild type (*, P = 0.02; t test, single tail). (B) Partial alignment of the amino acid sequences for α-tubulin in the apicomplexans Toxoplasma gondii (Tg) and Plasmodium falciparum (Pf) and the kinetoplastids Leishmania major (Lm) and Trypanosoma brucei (Tb). The distinct amino acid at position 235 is highlighted in gray. Toxoplasma carries three distinct α-tubulin genes. The alignment is shown for Toxoplasma α1-tubulin, which appears to be the dominant tubulin; all Toxoplasma dinitroaniline resistance mutations described to date are in the α1-tubulin gene (35, 45, 46). The Val is conserved in α2-tubulin, but α3-tubulin has a Leu at the corresponding position.

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This study characterizes nine compounds with asymmetrical structures (APM, benfluralin, butralin, dinitramine, ethalfluralin, GB-II-5, fluchloralin, pendimethalin, and profluralin) and four symmetrical dinitroanilines (isopropalin, nitralin, oryzalin, and trifluralin). Dinitramine and pendimethalin were the most effective compounds against both wild-type parasites and mutants harboring binding site substitutions. Both compounds feature an additional functional group at the meta position (NH₂ for dinitramine and CH₃ for pendimethalin), suggesting that functionalizing this position augments dinitroaniline binding to α-tubulin. The dinitroaniline prodiamine also has a meta NH₂ (like dinitramine) but like oryzalin possesses two propyl chains at the aniline nitrogen (dinitramine has two ethyl chains). Although we obtained a prodiamine solution to verify that the additional NH₂ group is responsible for the enhanced efficacy of dinitramine as compared to oryzalin, its insolubility in aqueous solution made it impossible to test in a biological setting. Nonetheless, the fact that dinitramine is more potent than all other compounds indicates that the addition of a hydrogen bond donor like NH₂ might strengthen the dinitroaniline-tubulin interaction. Indeed, the dinitramine NH₂ is predicted to provide additional hydrogen bonds by interacting with the backbone carbonyl of Arg2 and the side-chain carbonyl of Gln133. However, addition of a nonpolar group like CH₃ still helps dinitroaniline binding, as evidenced by the higher efficacy of pendimethalin than those of the remaining compounds in this study. Pendimethalin might also be less effective than dinitramine because it has only one alkyl chain.
instead of two at the aniline nitrogen. Butralin also has only one alkyl chain at the same position, and APM does not contain an analogous functional group. Both rank as the most ineffective compounds for wild-type parasites and most binding site mutant lines. Therefore, the presence of two alkyl chains at the aniline nitrogen might stabilize dinitroaniline binding. Overall, the improved efficacy of dinitramine and pendimethalin over other dinitroaniline compounds suggests a novel site for functionalizing dinitroanilines in future SAR studies, as previous research focused only on modifying the nitro groups, the aniline alkyl chains, or the location corresponding to oryzalin sulfonamide (2–5, 8, 18, 19, 41, 59, 60).

The IC_{50} values also suggest the relative contribution of specific binding site residues to the interaction with dinitroanilines. Mutations Leu136Phe, Thr239Ile, Arg243Ser, and Val44Leu are especially important as they confer high resistance relative to other point mutations to most compounds tested, including dinitramine and pendimethalin. This observation suggests the importance of these substitutions for disrupting the dinitroaniline-tubulin interaction in general. Previous studies of the computationally determined binding site show that residues Val4, Leu136, Thr239, and Arg243 are located along the same side of the binding site pocket, facing the α-tubulin core and away from the H1-S2 (N) loop (43). Computational modeling has suggested an identically located binding site for dinitroanilines in kinetoplastid and apicomplexan α-tubulin. However, there are four amino acid differences between the kinetoplastid and apicomplexan binding site residues which might contribute to the differential efficacy of specific dinitroanilines. The residues Val4, Thr41, Leu136, and Ile235 in the apicomplexan site correspond to Ala4, Cys41, Met136, and Val235 in the kinetoplastid site (Clustal alignment not shown) (27). Moreover, distinct residues outside of the binding site could also influence properties of the binding site. GB-II-5 is the least effective compound against wild-type Toxoplasma parasites and binding site mutants. However, the Toxoplasma line harboring the Ile235Val oryzalin resistance mutation is more sensitive to GB-II-5 (IC_{50} value of 4.7 μM) than wild-type parasites (IC_{50} value of 6.7 μM). The mutation Ile235Val converts the apicomplexan residue into a kinetoplastid residue, and this substitution makes GB-II-5 a more effective compound in Toxoplasma. This observation suggests that species-specific differences in tubulin might explain the differential efficacy of dinitroanilines on apicomplexans and kinetoplastids.

Studies of the effect of dinitroaniline derivatives on the related apicomplexan C. parvum are largely consistent with the data presented here. In a short-term in vitro culture assay, fluchloralin, nitratin, oryzalin, pendimethalin, profuralin, and trifluralin had IC_{50} values of 1.81, 4.5, 0.7, 0.19, 1.9, and 1.94 μM (2, 3). Pendimethalin is one of the most effective compounds in both Toxoplasma and Cryptosporidium, while trifluralin and nitratin are much less effective in both organisms. Profuralin is relatively more effective in Cryptosporidium than Toxoplasma, while oryzalin is considerably more effective in Toxoplasma. With the exception of pendimethalin, Toxoplasma is apparently more sensitive to the dinitroaniline derivatives than Cryptosporidium. Another possible explanation for differences in compound activity is that distinct functional groups on different dinitroanilines may influence their relative concentrations inside intracellular Toxoplasma and Cryptosporidium rather than the direct interaction with tubulin. We are currently developing binding assays to test the direct interaction of dinitroanilines with purified tubulin. Such assays will help resolve whether the effects of distinct functional groups are specific to tubulin binding or the nonspecific consequence of differential compound access.

In sum, we have identified dinitramine as the most effective compound in our current study of dinitroaniline derivatives. The additional NH₂ group at the meta position in dinitramine is predicted to provide donors for additional hydrogen bonds, which may explain its increased efficacy over other dinitroanilines. Additionally, the second most effective compound, pendimethalin, also possesses an additional functional group at the same position. Therefore, novel modifications in the meta position of dinitroanilines represent a new strategy to functionalize dinitroanilines and increase dinitroaniline efficacy.

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