Dinitroanilines bind α-tubulin to disrupt microtubules

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Protozoan parasites are remarkably sensitive to dinitroanilines such as oryzalin, which disrupt plant but not animal microtubules. To explore the basis of dinitroaniline action, we isolated 49 independent resistant Toxoplasma gondii lines after chemical mutagenesis. All 23 of the lines that we examined harbored single point mutations in α-tubulin. These point mutations were sufficient to confer resistance when transfected into wild-type parasites. Several mutations were in the M or N loops, which coordinate protofilament interactions in the microtubule, but most of the mutations were in the core of α-tubulin. Docking studies predict that oryzalin binds with an average affinity of 23 nM to a site located beneath the N loop of Toxoplasma α-tubulin. This binding site included residues that were mutated in several resistant lines. Moreover, parallel analysis of Bos taurus α-tubulin indicated that oryzalin did not interact with this site and had a significantly decreased, nonspecific affinity for vertebrate α-tubulin. We propose that the dinitroanilines act through a novel mechanism, by disrupting M-N loop contacts. These compounds also represent the first class of drugs that act on α-tubulin function.

INTRODUCTION

Microtubules are polymers constructed from α-β-tubulin heterodimers (Downing and Nogales, 1998a,b). These structures are rapidly assembled and disassembled to create essential components of eukaryotic cells, such as spindles and flagella. The dynamic nature of microtubules makes them susceptible to pharmacological agents. Microtubule-disrupting and microtubule-stabilizing drugs have provided great insight into tubulin and microtubule function; they also have tremendous practical use. Compounds that perturb microtubule dynamics are currently some of the most effective drugs to treat medical conditions, including cancer, gout, and helminth infection (Jordan et al., 1998). Dinitroanilines (oryzalin, ethafluralin, and trifluralin) disrupt the microtubules of plants, ranging from the single-celled alga Chlamydomonas reinhardtii to higher plants such as the monocot Eleusine indica (James et al., 1993; Anthony et al., 1998; Zeng and Baird, 1999). Dinitroanilines also disrupt the microtubules of protozoa, including both free-living species such as Tetrahymena and protozoan parasites such as Trypanosoma spp., Leishmania spp., Entamoeba spp., Plasmodium falciparum, Cryptosporidium parvum, and Toxoplasma gondii (Chan and Fong, 1990; Chan et al., 1991; Gu et al., 1995; Edlind et al., 1996; Stokkermans et al., 1996; Armson et al., 1999; Makioka et al., 2000a,b; Traub-Cseko et al., 2001). Remarkably, the activity of dinitroanilines is restricted to plants and protozoa; these compounds are ineffective against vertebrate or fungal microtubules (Chan and Fong, 1990; Hugdahl and Morejohn, 1993; Murthy et al., 1994; Edlind et al., 1996).

T. gondii is a member of the Apicomplexa, a phylum of parasites that includes several medically and agriculturally significant pathogens (Black and Boothroyd, 2000). Apicomplexans are obligate intracellular parasites; these protozoa only grow and replicate within host cells. Extracellular parasites released by host cell lysis must rapidly invade new host cells to stay alive. Despite these rigorous requirements for parasite survival, apicomplexans are some of the most widespread and damaging pathogens. Human infection by T. gondii can cause life-threatening illness in immunocompromised individuals and birth defects or miscarriage during fetal infection (Hill and Dubey, 2002). Other apicomplexans include Plasmodium and Cryptosporidium, parasites of considerable medical importance, and Theileria and Eimeria, animal pathogens with extensive impact on food production (Levene, 1988).

Parasites of the Apicomplexa are named for their distinctly polarized cell apex that contains a number of unique organelles that coordinate invasion of host cells. Apicomplexan parasites are surrounded by the pellicle, a composite structure formed by association of the plasma membrane with the inner membrane complex, an assemblage of flattened vesicles (Porchet and Torpier, 1977; Dubremetz and Torpier, 1978). There are two populations of microtubules in the invasive stages of apicomplexan parasites: subpellicular microtubules and spindle microtubules (Morrissette and Sibley, 2002a,b). Subpellicular microtubules are nondynamic; they maintain both apical polarity and the characteristic crescent shape of the parasite by interacting with the pellicle (Morrissette et al., 1997). Spindle microtubules form an intranuclear spindle to coordinate chromosome segregation. Both populations are critically important to parasite survival and replication. Although extracellular parasites...
are refractory to the effects of microtubule-disrupting drugs, during intracellular growth parasite microtubules are dynamic and are highly sensitive to disruption (Stokkevarns et al., 1996).

In this work, we demonstrate that Toxoplasma resistance to oryzalin is associated with point mutations to α-tubulin. The point mutations are sufficient for oryzalin resistance when introduced into wild-type (sensitive) parasites. When mapped onto the structure of α-tubulin, most mutations cluster in the core of the protein. Using representative structures of Toxoplasma α-tubulin taken from a molecular dynamics trajectory, we find that oryzalin consistently docks to α-tubulin and binding is altered in several point mutants due to conformational changes. When a similar analysis is carried out on vertebrate α-tubulin, oryzalin has significantly lower affinity and does not bind to the same region.

MATERIALS AND METHODS

Mutagenesis and Selection of Oryzalin-resistant Lines

Toxoplasma tachyzoites (RH strain) were propagated in human foreskin fibroblast (HFF) cells in DMEM with 10% fetal bovine serum. Approximately 10^7 blast (HFF) cells in DMEM with 10% fetal bovine serum. Approximately 10^7

The following primers were used to obtain sequence from

**Sequencing the Drug Assays**

Toxoplasma has two discrete populations of microtubules: spindle microtubules and subpellicular microtubules. The characteristic elongated shape of Toxoplasma is maintained by a 6-μm-long subpellicular microtubules. Shortening these subpellicular microtubules with oryzalin converts parasites to a distinctive round shape. Parasites with shortened microtubules can continue to replicate until they lyse from a host cell. However, round parasites are incapable of invading new host cells and die (Morrisette and Sibley, 2002b). To assess oryzalin resistance in the individual lines, we scored parasite shape and subpellicular microtubule length in increasing concentrations of oryzalin. Parasites were grown overnight in HFF cells on coverslips in increasing concentrations of oryzalin. The coverslips were processed for immunofluorescence staining with a rabbit peptide antibody that specifically recognizes Toxoplasma β-tubulin (Morrisette and Sibley, 2002b). Parasites were scored as having 1) a wild-type or “elongated” phenotype in which the subpellicular microtubules are long and parasites have an elongated shape, or having 2) a “round” phenotype in which the parasites are noninvasive because the subpellicular microtubules are shortened making parasites round (Morrisette and Sibley, 2002b). Resistance to oryzalin was scored as the highest concentration of drug in which intracellular replicating parasites maintained round shape and long subpellicular microtubules. Because the long subpellicular microtubules impart parasites with the capacity to invade host cells, these microtubules are an indicator of the ability to proliferate in oryzalin.

**Amplification of the α-Tubulin Genes from the Oryzalin-resistant Lines**

Genomic DNA was isolated from resistant Toxoplasma lines. The α-tubulin gene was amplified from genomic DNA by using PfU turbo (Strategene, La Jolla, CA) in an MJ research thermocycler with 30 cycles of annealing at 45°C for 30 s followed by 5 min of extension at 68°C. The primers GAGTCTCG-TAGAGAAC AAGC (5’ untranslated region [UTR]) and CGTTTATACCT-GCGTCAAATCTGCAAC; 5’ exon 3: GCGTCAAATCTGCAAC; 5’ exon 3b: GACGACATCAGCAGCGTAC. Sequences were aligned and compared using the Sequencer program (GeneCodes, Ann Arbor, MI).

**Introduction of Novel Restriction Sites**

The QuiChange kit (Strategene) was used to ablate a unique Xhol site in the second intron of α-tubulin and to introduce a novel BamH1 restriction site. The Ser165Pro construct was created with primers Ser165Pro sense GCTGAC-TACCAGAAAGCGGAACGGTACTGGC and Ser165Pro antisense CATAGCTTCTTGCAGAAGACGGTAC. This alteration distinguishes the endogenous tubulin gene from the transgene to discriminate Toxoplasma transfectants with allelic replacement from those with nonhomologous insertions.

**Creation of α-Tubulin Point Mutations**

The mutations Ser165Pro, Ser165Thr, Ser165Ala, ile231Thr, and Thr239Ile were constructed using the QuiChange kit (Strategene) to modify the sequence of wild-type genomic α-tubulin containing the BamH1 restriction site. The Ser165Pro construct was created with primers Ser165Ala sense GGTGACTACCGCAAGAAGG-ACACCAGCCACGCGATCAGGGAGGAGATGACCTG-CAATCAGTC. The Ser165Thr construct was created with primers Ser165Thr sense GGTGACTACCGCAAGAAGG-ACACCAGCCACGCGATCAGGGAGGAGATGACCTG-CAATCAGTC and Ser165Thr antisense CGAACCTTCACTCAGATCTTGCAGAAGACGGTAC.

**Transformation, Selection, and Analysis of Oryzalin-resistant Transfomers**

The linearized transgene constructs with the diagnostic restriction enzyme sites are incapable of invading new host cells and die (Morrissette and Sibley, 1993). Parasites were selected for transformation by amplifying the -tubulin by using electroporation parameters established previously (Soldati and Boothroyd, 1993). Parasites were selected for 0.5 μM oryzalin; resistant tachyzoites were single cell cloned (as described above). Individual clones were assayed for transformation by amplification of the α-tubulin gene by using the primers 5’ coding CAAAATGAGAGAGGTTATCAGC and 3’ coding TTAGTACTCCTGCACCATACGC. Amplified α-tubulin was digested with restriction enzymes or BamH1. Transformed parasites were grown in oryzalin (pig) (gi:15988311) and T. gondii (gi:16937) α-tubulines were modeled based on the analogous bovine -tubulin structure by mutating the completed bovine structure to the -tubulin sequence by using WHAT-IF (Vriend, 1990). The coordinate file for our docking studies, the missing residues (Gln35 to Gln52) were included in the structure of bovine tubulin (1JFF). The α-tubulin sequence was aligned with the α-tubulin sequence in the Swiss-Model Automated Comparative Protein Modeling Server (http://www.expasy.ch/spdbv/).

**Computational Techniques**

To prepare α-tubulin for our docking studies, the missing residues (Gln35 to Lys60) were modeled using Clustal within the Vector NTI suite of programs (Higgins et al., 1996). The amino acid sequences of Toxoplasma α-tubulin (gi:16937) was fit to the structure of bovine tubulin (1JFF) by using the Swiss-Model Automated Comparative Protein Modelling Server (http://www.expasy.ch/swissmod/) in the first approach mode (Guex and Peitsch, 1997). The resulting structure was viewed with SwissPdb Viewer version 3.7 (http://www.expasy.ch/spdbv/).
RESULTS

Oryzalin-resistant Tachyzoites Have Diverse Phenotypes

Although dinitroanilines are potent inhibitors of microtubules in plants and protozoa, their precise mode of action is unknown. To determine the molecular basis of dinitroaniline action, we generated oryzalin-resistant lines by using the chemical mutagen N-nitroso-N-ethylurea (Waldeland et al., 1983; Pfefkerkn, 1984; Dobrowski and Sibley, 1996). Because Toxoplasma is haploid, genetic changes that confer resistance are readily detected, including mutations that are recessive in diploid organisms. We selected for resistance to either 0.5 or 2.5 μM oryzalin. In earlier work, we demonstrated that parasites treated with 0.5 μM oryzalin have shortened, nonfunctional subpellicular microtubules but intact spindle microtubules. In contrast, parasites treated with 2.5 μM oryzalin are missing all microtubules (Morrissette and Sibley, 2002b). The individual oryzalin-resistant Toxoplasma lines displayed distinct drug resistance profiles (Figure 1 and Table 1). Most of the 0.5 μM resistant lines were only moderately resistant to increased oryzalin concentrations. In contrast, the majority of the 2.5 μM resistant lines were resistant to >10-fold increases in oryzalin concentration.

The Oryzalin-resistant Lines Harbor Different Single α-Tubulin Point Mutations

One potential mechanism for resistance to the dinitroanilines would be mutations to tubulin that alter drug binding or microtubule dynamics. The single α-tubulin gene from 23 of the 49 oryzalin-resistant lines was amplified and sequenced to identify base changes conferring amino acid substitutions to tubulin. Each line had a single point mutation to α-tubulin; collectively, there were 21 different amino acid substitutions at 16 positions in α-tubulin (Table 1). Ser165 was a particularly frequent target and was mutated in five of the 23 lines to threonine, proline, or alanine. Either isoleucine or leucine replaced Phe52 and either cysteine or serine replaced Arg243. The Thr239Ile point mutation was also previously identified in E. indica (goosegrass) as a dinitroaniline-resistant α-tubulin mutation (Anthony et al., 1998).

Tubulin Mutations Are Sufficient to Confer Oryzalin Resistance in Toxoplasma

To demonstrate that the α-tubulin mutations were sufficient to confer oryzalin resistance in a wild-type background, they were introduced into the parental Toxoplasma strain. To distinguish wild-type α-tubulin from the α-tubulin transgene, intron 2 was altered to ablate a unique XbaI restriction site and to introduce a unique BamHI restriction site (Figure 2A). The BamHI restriction site is closely linked to the individual point mutations. Five single point mutations (Ser165Pro, Ser165Thr, Ser165Ala, Ile231Thr, and Thr239Ile) were individually introduced into this construct. To test whether the substitutions were additive in combination, the double and triple permutations of these substitutions were also made.

A linear construct of the α-tubulin gene containing the BamHI restriction site and the specific point mutation or mutations was transfected into wild-type (sensitive) Toxoplasma. After 24 h of recovery, the parasites were placed under selection in 0.5 μM oryzalin, the lowest concentration of drug that effectively selects for resistance. Resistant parasites were single cell cloned and expanded. The lines were assayed for allelic integration versus random insertion of the transgene by amplification of the α-tubulin gene from parasite lysate and restriction enzyme analysis of the product (Figure 2B). As a control, the wild-type α-tubulin gene with the restriction site change was transfected into Toxoplasma; resistant lines did not arise after selection in 0.5 μM oryzalin.

Transformation of the tubulin point mutation genes was sufficient to confer resistance to oryzalin in Toxoplasma (Figure 3). In the pseudodiploid (nonhomologous integration) state (Figure 3, black bars), point mutations conferred resistance to lower levels of oryzalin than did allelic replacements of the same constructs (Figure 3, white bars). Allelic replacement of wild-type tubulin with the α-tubulin point mutations conferred resistance to between 0.75 and 5.0 μM oryzalin. The single point mutation Thr239Ile conferred the highest level of resistance. In almost all cases, the double and triple point mutation combinations were less resistant than the single mutations, indicating that these point mutations were not additive in combination (Figure 3, panels 2 and 3). However, the double mutation Ser165Ala/Thr239Ile was highly resistant to oryzalin (>90 μM; Figure 3, panel 4), indicating that these substitutions are synergetic. Oryzalin is...
insoluble in aqueous solutions above ~95 \( \mu \text{M} \), making it impossible to determine the full degree of drug resistance of the Ser165Ala/Thr239Ile double mutation.

In some cases, two of the resistant lines from the mutagenesis screen contained the same \(-\)tubulin point mutation but displayed distinct resistance profiles. The clearest example of this was seen with the Ser165Thr point mutation. Two lines harbored this substitution but showed dramatically different resistance profiles: one line was resistant to 2.0 \( \mu \text{M} \) oryzalin, whereas the other was resistant to >25 \( \mu \text{M} \) oryzalin.

<table>
<thead>
<tr>
<th>Amino acid substitution</th>
<th>Oryzalin selection</th>
<th>Maximum resistance, ( \mu \text{M} )</th>
<th>Codon change</th>
<th>Location in protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>His8Tyr</td>
<td>0.5 ( \mu \text{M} )</td>
<td>10.0</td>
<td>CAC to TAC</td>
<td>( \beta )-sheet 1</td>
</tr>
<tr>
<td>His28Gln</td>
<td>0.5 ( \mu \text{M} )</td>
<td>1.0</td>
<td>CAT to CAA</td>
<td>N loop</td>
</tr>
<tr>
<td>Phe52Ile</td>
<td>0.5 ( \mu \text{M} )</td>
<td>2.5</td>
<td>TTC to ATC</td>
<td>N loop</td>
</tr>
<tr>
<td>Leu136Phe</td>
<td>2.5 ( \mu \text{M} )</td>
<td>&gt;25</td>
<td>TTC to TCT</td>
<td>(( \beta )-sheet 4)</td>
</tr>
<tr>
<td>Asn139Lys</td>
<td>0.5 ( \mu \text{M} )</td>
<td>1.0</td>
<td>AAC to AAA</td>
<td>( \beta )-sheet 4</td>
</tr>
<tr>
<td>Ser165Ala</td>
<td>2.5 ( \mu \text{M} )</td>
<td>5.0</td>
<td>TCG to GCG</td>
<td>( \beta )-sheet 5</td>
</tr>
<tr>
<td>Ser165Pro</td>
<td>0.5 ( \mu \text{M} )</td>
<td>1.0</td>
<td>TCG to GCG</td>
<td>( \beta )-sheet 5</td>
</tr>
<tr>
<td>Ser165Thr</td>
<td>0.5 ( \mu \text{M} )</td>
<td>2.0</td>
<td>TCG to ACG</td>
<td>( \beta )-sheet 5</td>
</tr>
<tr>
<td>Ser165Thr</td>
<td>0.5 ( \mu \text{M} )</td>
<td>&gt;25</td>
<td>TCG to ACG</td>
<td>( \beta )-sheet 5</td>
</tr>
<tr>
<td>Ile231Thr</td>
<td>0.5 ( \mu \text{M} )</td>
<td>1.0</td>
<td>ATC to ACC</td>
<td>( \alpha )-helix 7</td>
</tr>
<tr>
<td>Ile235Val</td>
<td>0.5 ( \mu \text{M} )</td>
<td>1.0</td>
<td>ATC to GTC</td>
<td>( \alpha )-helix 7</td>
</tr>
<tr>
<td>Val252Leu</td>
<td>0.5 ( \mu \text{M} )</td>
<td>&gt;25</td>
<td>GTG to TGT</td>
<td>After ( \alpha )-helix 7</td>
</tr>
<tr>
<td>Ser239Ile</td>
<td>0.5 ( \mu \text{M} )</td>
<td>1.0</td>
<td>GTG to TGT</td>
<td>After ( \alpha )-helix 7</td>
</tr>
<tr>
<td>Tyr24His</td>
<td>C. reinhardtii ( \text{a} )</td>
<td>Near N loop</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr239Ile</td>
<td>E. indica ( \text{b} )</td>
<td>Near M loop</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met268Thr</td>
<td>E. indica ( \text{b} )</td>
<td>Near M loop</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( \text{a} \) From James et al. (1993).

\( \text{b} \) From Yamamoto et al. (1998).

Figure 2. (A) Diagram of the exon-intron structure of \( \alpha \)-tubulin. Endogenous (wild-type) \( \alpha \)-tubulin has a unique XbaI restriction site in intron 2. The transgene was altered to ablate the XbaI site and to introduce a unique BamHI site in intron 2. The point mutations Ser165Pro, Ser165Thr, Ser165Ala, Ile231Thr, and Thr239Ile were introduced in the transgene construct both individually and in double and triple combinations. The \( \alpha \)-tubulin from transformants with oryzalin resistance was amplified with primers (arrows) that are internal to the 5’ and 3’ ends of the transgene and represent the 5’ end of exon 1 and 3’ end of exon 3. (B) Restriction enzyme analysis of the transformed oryzalin-resistant lines gives easily distinguishable patterns after amplification of the \( \alpha \)-tubulin gene. The endogenous (WT) \( \alpha \)-tubulin gene is cut by BamHI and is not cut by XbaI. Conversely, the allelic replacement (AR, homologous integration) is cut by XbaI and is not cut by BamHI. Nonhomologous integration of the transgene creates a pseudodiploid (PD). In this case, both the BamHI and XbaI enzymes cut incompletely.
lin (Table 1). When the Ser165Thr point mutation was introduced as an allelic replacement, the α-tubulin point mutation alone conferred resistance to 2.0 μM oryzalin (Figure 3). Therefore, the line with resistance to >25 μM oryzalin must have an additional mutation(s) superimposed upon the α-tubulin mutation to increase resistance to oryzalin. We hypothesize that lines with high levels of drug resistance (Figure 1) have additional mutations superimposed on the α-tubulin mutation to increase resistance to oryzalin.

Most Tubulin Mutations Localize to the Core of α-Tubulin

The 23 point mutations identified in this study were distributed throughout the linear sequence of Toxoplasma α-tubulin. To investigate how these amino acid mutations might affect tubulin function, we evaluated their conservation in other α-tubulins and their spatial distribution in a structural model of α-tubulin. Figure 4A is a Clustal alignment of S. scrofa α-tubulin (insensitive to the dinitroanilines) with Toxoplasma α-tubulin. Although some point mutations occur in residues that distinguish Toxoplasma (sensitive) from pig (insensitive) α-tubulin, many resistance mutations occur in conserved amino acids. The amino acid sequence of Toxoplasma α-tubulin was fit to the electron diffraction structure of bovine α-tubulin by using the Swiss-Model Automated Comparative Protein Modeling Server Web site in the first approach mode. The mutated amino acids in α-tubulin were identified within the modeled Toxoplasma α-tubulin structure. Two previously identified plant mutations Tyr24His and Met268Thr that confer dinitroaniline resistance were also included in our analysis (James et al., 1993; Yamamoto et al., 1998). The majority of the point mutations clustered within the core of α-tubulin (Figure 4B). Some of the mutated residues were distributed in striking patterns. For example, several of the mutated residues residues (Ile231, Ile235, and Thr239) localized along a single face of helix 7 in α-tubulin (Figure 4C). Arg243 and Leu238 were also in this immediate proximity. In addition, His8, Leu136, and Ser165 occupied essentially linear positions on three strands (β-strands 1, 4, and 5) of a β-sheet (Figure 4C, inset).

Some Mutations Localize to Domains That Coordinate Protofilament-Protofilament Contact

Structural studies have established the critically important role of two tubulin domains (the M loop and the N loop) in microtubule assembly. These loops coordinate lateral interactions between protofilaments to build a microtubule. Studies docking the tubulin dimer structure into high-resolution images of microtubules have established that M loops interact with N loops of laterally adjacent subunits (Li et al., 2002). Moreover, organisms with increased microtubule stability (such as arctic fish) have two amino acid substitutions in the α-tubulin M loop, Ala278Thr and Ser287Thr (Detrich et al., 2000). The oryzalin-resistant point mutation Ile275Thr was an M loop substitution, and three of the point mutations described here (His28Gln, Phe52Ile, and Phe52Leu) were in the N loop (Figure 4). These α-tubulin mutations may compensate for the destabilizing effects of the dinitroanilines by increasing the intrinsic stability of Toxoplasma microtubules.

Identification of the Oryzalin Binding Site

Although our analysis of the resistance mutations implies that oryzalin binds to α-tubulin, the spatial distribution of the mutations and the fact that many mutations were in the
Figure 4. (A) Clustal alignment of oryzalin-sensitive *Toxoplasma* (Tg) and oryzalin-insensitive pig (S. scrofa, Ss) α-tubulins. A yellow background represents amino acid identity, teal denotes residue similarity, and white indicates nonconserved residues. The distribution of α-helices, β-strands, and coils in bovine α-tubulin (black, below Clustal sequence) were adapted from Lowe *et al.* (2001) who solved the structure of bovine tubulin, but mapped it onto the amino acid sequence of pig tubulin. The three red lines (H1, H3, and B5) indicate regions where the predicted secondary structure of *Toxoplasma* α-tubulin deviates from the bovine secondary structure. The boxed residues are mutated to confer oryzalin resistance. The plant mutations at residues 24 and 268 are colored green. The position of the N loop containing the Phe52 mutations is not necessarily accurate because this area was disordered in structural studies and was eliminated from the final model. (B and C) The amino acid sequence of *Toxoplasma* α-tubulin was fit to the structure of bovine α-tubulin by using Swiss-Model automated comparative protein modeling. B shows a ribbon model of *Toxoplasma* α-tubulin (white). The residues that are mutated to confer oryzalin resistance are colored red. The plant mutations at residues 24 and 268 are colored green. The position of the N loop containing the Phe52 mutations is not necessarily accurate because this area was disordered in structural studies and was eliminated from the final model. C shows cut-away views of some of the α-tubulin residues that are mutated to confer oryzalin resistance. Many residues are in striking proximity and show specific orientations. For example, residues Ile231, Ile235, and Thr239 all occur on the same face of α-helix 7. Leu238, Arg243, and Val252 cluster in the same general area. The inset shows a β-sheet formed by β-strands 1, 4, and 5. The mutated residues His8, Leu136, and Ser165 are in a linear pattern across the β-sheet.
The representative structures of locate the binding site (see MATERIALS AND METHODS).

The SO₂ group of oryzalin forms a hydrogen bond with the backbone amide (NH) of Arg64 (red). (B) Two proto (yellow) were mutated in resistant lines; all other binding site residues are colored green. The binding site lies beneath the N loop (translucent Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243, and Phe244. Residues Phe24, His28, Thr239, and Arg243 with equivalent residues in vertebrate trajectories. The results indicated that oryzalin does not interact set of structures obtained from the molecular dynamics trajectory, showed that oryzalin consistently docked to residues Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243, and Phe244 (Figure 5A). The binding affinity at this site was 23 nM. This binding site placed oryzalin beneath the N loop (Figure 5). We also analyzed Toxoplasma α-tubulin containing point mutations that conferred resistance. For the mutants Thr239Ile, Ser165Ala, and Thr239Ile/Ser165Ala, oryzalin binding was eliminated at the site identified in the wild-type Toxoplasma α-tubulin.

In parallel studies, we investigated the interaction of oryzalin with Bos taurus α-tubulin by docking oryzalin to a set of structures obtained from the molecular dynamics trajectories. The results indicated that oryzalin does not interact with equivalent residues in vertebrate α-tubulin. It has a significantly decreased overall affinity (>50-fold) for bovine tubulin and no true consensus binding site. The limited interaction of oryzalin with bovine tubulin does not imply a secondary binding site for oryzalin on bovine α-tubulin but indicates nonspecific, low-affinity interactions in single tubulin snapshots.

**DISCUSSION**

Analysis of oryzalin resistance in T. gondii implicates α-tubulin as the target for dinitroaniline action. We demonstrate here that all Toxoplasma lines with resistance to oryzalin have a point mutation in the single α-tubulin gene. These α-tubulin point mutations are sufficient to confer resistance to oryzalin in Toxoplasma. The preponderance of α-tubulin mutations identified in this study suggests that dinitroanilines bind to and act on α-tubulin. This is unusual, as all characterized compounds that perturb microtubule function bind to and act on β-tubulin (Nogales, 2000). Several of the resistance mutations are located in the M or N loops, regions of tubulin that mediate lateral adhesion of protofilaments (Downing and Nogales, 1998a; Nogales et al., 1998, 1999; Lowe et al., 2001). These mutations are predicted to counteract dinitroaniline action by hyperstabilizing microtubules. However, most mutations were found to be in the core of α-tubulin. We hypothesize that core mutations affect the conformation of the α-tubulin dinitroaniline binding site.

Docking simulations were used to identify the α-tubulin binding site for oryzalin. These simulations were based on a genetic algorithm where the results of individual searches are clustered based on their root mean square deviation. For each α-tubulin conformation, we found the consensus binding site (Figure 5A) was both the largest cluster and contained the binding conformations with the highest affinity. The binding affinity predicted for this site was 23 nM and is in agreement with experimentally determined values of 95–117 nM obtained with purified plant tubulin (Hugdahl and Morejohn, 1993; Murthy et al., 1994). The site identified by our docking simulations is located beneath the N loop and is composed of residues Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243, and Phe244 (Figure 5A). Several resistance mutations (Phe24, His28, Thr239, and Arg243) map to this binding site. We propose that additional mutated residues in the core of α-tubulin also

**Figure 5.** (A) Representative structure of oryzalin bound to α-tubulin as predicted by the docking simulations. The binding site consists of Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243, and Phe244. Residues Phe24, His28, Thr239, and Arg243 (yellow) were mutated in resistant lines; all other binding site residues are colored green. The binding site lies beneath the N loop (translucent red). The SO₂ group of oryzalin forms a hydrogen bond with the backbone amide (NH) of Arg64 (red). (B) Two protofilaments, each consisting of two α-β heterodimers, with bound oryzalin (blue). Oryzalin binds to α-tubulin (silver) beneath the N loop (red); this may interfere with lateral interactions between the N loop and the M loop (yellow) of the adjacent protofilament. The inset shows oryzalin bound beneath the N loop.
Dinitroanilines Bind α-Tubulin

Dinitroanilines are known to bind to microtubules and disrupt their structure, leading to cell death. However, the mechanism of action of these compounds is not fully understood. A recent study investigated the binding of dinitroanilines to α-tubulin, a protein that plays a critical role in microtubule formation.

The study used computational docking to analyze the binding of dinitroanilines to α-tubulin from different species, including Chlamydomonas and tobacco. The results showed that dinitroanilines bind to a specific site on α-tubulin, which is located in the N-loop region. This site is critical for microtubule stability and is also targeted by other microtubule-disrupting drugs such as colchicine.

The study identified multiple points of contact between dinitroanilines and α-tubulin, which explains the high level of resistance observed in some species. The authors propose a binding model for dinitroanilines that involves a specific site on α-tubulin, which could be a target for new antiparasitic drugs.

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