Mutations in α-tubulin confer dinitroaniline resistance at a cost to microtubule function

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Protozoan microtubules are sensitive to disruption by dinitroanilines, compounds that kill intracellular Toxoplasma gondii parasites without affecting microtubules in vertebrate host cells. We previously isolated a number of resistant Toxoplasma lines that harbor mutations to the α1-tubulin gene. Some of the mutations are localized in or near the M and N loops, domains that coordinate lateral interactions between protofilaments. Other resistance mutations map to a computationally identified binding site beneath the N loop. Allelic replacement of wild-type α1-tubulin with the individual mutations is sufficient to confer dinitroaniline resistance. Some mutations seem to increase microtubule length, suggesting that they increase subunit affinity. All mutations are associated with replication defects that decrease parasite viability. When parasites bearing the N loop mutation Phe52Tyr are grown without dinitroaniline selection, they spontaneously acquired secondary mutations in the M loop (Ala273Val) or in an α1-tubulin–specific insert that stabilizes the M loop (Asp367Val). Parasites with the double mutations have both reduced resistance and diminished incidence of replication defects, suggesting that the secondary mutations decrease protofilament affinity to increase parasite fitness.

INTRODUCTION

Microtubules are essential components of diverse structures in eukaryotic cells. These structures include spindles used for chromosome segregation, flagellar/ciliary axonemes used for motility, and cytoplasmic microtubule arrays used as tracks for vesicle transport (Nogales, 2000). Microtubules are built by the polymerization of α/β-tubulin heterodimers, and they typically contain 13 protofilaments, a substructure formed by the longitudinal head-to-tail association of the heterodimers. The lateral association of protofilaments forms a microtubule, which consists of a 24-nm-wide cylinder with a hollow lumen (Downing and Nogales, 1998a,b; Li et al., 2002).

Because α- and β-tubulins are ancestrally related proteins, they contain many domains that have similar structure and function, such as the M and N loops, which coordinate contacts between adjacent protofilaments (Downing, 2000; Lowe et al., 2001; Li et al., 2002). However, α- and β-tubulins also have important differences. For example, although both α- and β-tubulins bind to guanosine triphosphate (GTP), only the β-tubulin GTP is hydrolyzed (Davis et al., 1994; Sage et al., 1995; Anders and Botstein, 2001; Dougherty et al., 2001). Polymerization-dependent β-tubulin GTP hydrolysis is stimulated by a GTPase-activating domain of α-tubulin from the adjacent dimer within the protofilament. When GTP is hydrolyzed, dimers undergo a conformation change that promotes microtubule disassembly (Nicholson et al., 1999; Nogales et al., 2003; Nogales and Wang, 2006a,b).

Appropriate microtubule disassembly is as essential as assembly to the appropriate function of tubulin. Expression of mutant α-tubulin genes that lack a functional GTPase activation domain causes a lethal phenotype in budding yeast associated with extraordinarily long and stable microtubules (Anders and Botstein, 2001). One of the traits that distinguishes α-tubulins from β-tubulins is a conserved eight-amino acid insert (Thr, Val, Val, Pro, Gly, Gly, Asp, Leu) beginning at residue 360 (361 in the Toxoplasma sequence) (Nogales et al., 1998). The structure of stabilized zinc sheets demonstrated that Taxol (paclitaxel) occupies the same region in β-tubulin as the insert does in α-tubulin (Snyder et al., 2001). These results indicate that the α-tubulin M loop has greater protofilament stability than the β-tubulin M loop and that by stabilizing the β-tubulin loop contacts, microtubules are stabilized.

A diverse array of compounds affect microtubule function by shifting the normal equilibrium between tubulin dimers and polymers to destabilize (e.g., colchicines) or stabilize (e.g., paclitaxel) microtubules (Jordan et al., 1998). Because the balanced assembly and disassembly of microtubules is a critically important part of microtubule function, compounds that upset the equilibrium between heterodimer subunits and polymerized microtubules are toxic. Therapeutic strategies have exploited this feature to inhibit the proliferation of fast-growing cancer cells. Other tubulin-binding compounds are used as antiparasitic and antifungal agents and as herbicides. With the exception of the dinitroanilines and peloruiside A, all characterized compounds that alter tubulin equilibrium bind to and act on β-tubulin (Morrisette et al., 2004; Pineda et al., 2004; Jimenez-Barbero et al., 2006; Mitra and Sept, 2006). Because α-tubulin ligands such as the...
Dinitroanilines and peloroside A are only recently identified, they are likely to be important tools for establishing the role of α-tubulin in the tubulin dimer, much as other tubulin binding drugs have helped clarify how the β-tubulin mono-
mer functions.

The activity of dinitroanilines, informally called the “yellow” herbicides, was first reported by scientists from Eli Lilly in 1960 (Alder et al., 1960). Dinitroanilines (oryzalin, trifluralin, and ethafluralin) selectively disrupt plant and protozoan microtubules (Traub-Cseko et al., 2001). These compounds bind to sensitive (plant and protozoan) but not resistant (vertebrate and fungal) tubulins. Protozoan para-
sites, including those broadly grouped as kinetoplastids, apicomplexans and amoeba, are sensitive to dinitroanilines, which inhibit their replication (Chan and Fong, 1990; Kaidoh et al., 1995; Stokkermans et al., 1996; Bogitsh et al., 1999; Werbovetz et al., 1999; Makioka et al., 2000a,b; Traub-Cseko et al., 2001; Bhattacharya et al., 2002, 2004; Morrissette and Sibley, 2002; Werbovetz et al., 2003). Given that the dinitroa-
nilines do not disrupt vertebrate tubulins, dinitroaniline ac-
tivity is of particular interest for the development of new anti-parasitic drugs. We are specifically interested in under-
standing the action of dinitroanilines on apicomplexan para-
sites such as Toxoplasma gondii.

Toxoplasma gondii is a member of the Apicomplexa, a phylum of protozoa that are obligate intracellular parasites (Levene, 1988). Parasites released by host cell lysis must rapidly invade new host cells to stay alive. Despite these stringent growth requirements, apicomplexans are wide-
spread and damaging pathogens with profound direct and indirect impacts on human welfare. Human infection by T. gondii can cause life-threatening illness in immunocompro-
mised individuals and birth defects or miscarriage during fetal infection (Black and Boothroyd, 2000). Other apicom-
exans include Plasmodium (agent of malaria) and Crypto-
sporidium (an opportunistic pathogen), which are parasites of considerable medical importance, and Theileria and Eime-
ria, animal pathogens with extensive impact on food pro-
duction (Levene, 1988).

Apicomplexans are named for their polarized apex that contains several unique organelles that coordinate invasion of host cells. Apicomplexan parasites are surrounded by the pellicle, a composite structure formed by the association of the plasma membrane with the inner membrane complex, an assemblage of flattened vesicles. There are two popula-
tions of microtubules in the invasive stages of apicomplexan para-
sites: subpellicular microtubules and spindle microtu-
bules (Morrissette and Sibley, 2002). Subpellicular microtu-
bules are nondynamic; they maintain both apical polarity and the characteristic crescent shape of the parasite by in-
teracting with the pellicle. Spindle microtubules form an intranuclear spindle to coordinate chromosome segregation.

Both populations are critically important to parasite survival and replication. Wild-type Toxoplasma parasites are resistant to 0.2 μM oryzalin. This value is difficult to measure directly, because parasites in oryzalin continue to undergo protein and DNA synthesis in the absence of microtubule function, making it impossible to measure IC50 values by radiolabel incorporation (Stokkermans et al., 1996). Using drug washout and plaquing values, the approximate IC50 value is 0.1 μM oryzalin, and using morphology (round rather than elongated parasites), parasites begin to round up by ~0.2 μM oryzalin (Stokkermans et al., 1996; Morrissette and Sibley, 2002).

In previous work, we used genetic analysis of oryzalin-
resistant Toxoplasma lines in combination with computa-
tional methods to identify a binding site and mode of action for the dinitroanilines (Morrissette et al., 2004). Additional computational analysis extended these studies to include the interaction of the dinitroanilines oryzalin, trifluralin, and GB-II-5 with Toxoplasma, Leishmania, Plasmodium, and bovine tubulins (Mitra and Sept, 2006). The refined dinitroaniline binding site is located on Toxoplasma, Leishmania, and Plas-
modium α-tubulins beneath the N loop, and it is formed by residues of strand S1 (Arg2, Glu3, Val4, Ile5, Ser6), helix H1 (Cys20, Trp21, Phe24), the N/H1-S2 loop (His28, Met36, Asp39, Lys40, Thr41, Asp47, Pro63, Arg64), strand S2 (Cys65), strand S4 (Leu136), helix H7 (Ile235, Ser236, Thr239, Ala240, Ser241), and the T7 loop (Arg243, Phe244, Asp245). Analysis of vertebrate α-tubulin indicates that dinitroanili-
nines have nonspecific, low-affinity interactions and no consensus binding site, consistent with in vivo and in vitro observations that dinitroanilines do not bind to vertebrate tubulin or disrupt vertebrate microtubules (Morejohn et al., 1987; Hugdahl and Morejohn, 1993; Murthy et al., 1994). When molecular dynamics analysis is used to compare α-tu-
bulin mobility in the presence or absence of bound drug, these simulations suggest that dinitroaniline binding pro-
founds flexibility of the α-tubulin N loop, which is drawn in toward the core of the tubulin dimer (Mitra and Sept, 2006). We hypothesize that when dinitroanilines bind tubulin, the N loop is less able to make contacts with the M loop of the dimer in the adjacent subunit. This prevents tubulin polymerization, destabilizes preexisting microtu-
bules, or both.

In the work presented here, we have generated Toxoplasma parasites that bear homologous integrations of many of the α-tubulin point mutations associated with dinitroaniline re-


dittance. We selected the mutations to test in this study by their predicted mechanism of action: some mutations are expected to stabilize microtubules (a GTPase-activating do-


main mutant and N and M loop mutants), whereas others are anticipated to act by altering the dinitroaniline binding site to lower its affinity for oryzalin. We have characterized the advantageous features of these mutations (dinitroaniline resistance) as well as deleterious aspects (increased replica-
tion defects) to conclude that drug resistance has a fitness cost in T. gondii.

**MATERIALS AND METHODS**

**Culture of Toxoplasma Lines**

Toxoplasma tachyzoites (RH strain) were propagated in human foreskin fibro-


blast (HFF) cells in DMEM with 10% fetal bovine serum as described previ-

ously (Roos et al., 1994). Oryzalin (Riedel-deHaën, Seelze, Germany) stock solutions were made up in dimethyl sulfoxide. Oryzalin-resistant parasite lines were isolated after ethyl-nitrosourea mutagenesis by selection in 0.5 or 2.5 μM oryzalin as described previously (Morrissette et al., 2004).

**Analysis of α-Tubulin Point Mutations**

The α1-tubulin gene was amplified from genomic DNA isolated from individ-


ual oryzalin-resistant parasites by using thermal cycling with primers GAGTCCTCGTAGAAGACACCT (5’ untruncated region [UTR]) and CGTT-


TATACCTTCACCTTTTC (3’ UTR). The amplified 2.3-kb fragment was se-


quenced and analyzed using Sequencher software (Gene Codes, Ann Arbor, MI) to identify point mutations in the coding sequence of the α1-tubulin gene. Primers and methods to sequence the α1-tubulin gene constructs are as described previously (Morrissette et al., 2004). Similar sequencing analysis indicated that none of the resistant lines had mutations in the p1-tubulin gene. We also analyzed the sequences of two recently identified additional Toxo-


plasma α1-tubulin genes (α2-tubulin and α3-tubulin), but we did not identify any mutations associated with these genes in resistant parasites (data not shown).

**Creation of α-Tubulin Point Mutations**

The QuikChange kit (Stratagene, La Jolla, CA) was used to modify a previ-


ously generated construct containing a 2.3-kb insert of the Toxoplasma α1-


tubulin gene where a unique XbaI site in the second intron of α1-tubulin was
Table 1. Primers used to introduce tubulin mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>Val4Leu</td>
<td>Binding site</td>
<td>CGACAAATGAGACCTTGACCATCGACGATCCGAGGCC</td>
</tr>
<tr>
<td>Ser51Leu</td>
<td>Binding site</td>
<td>CGACAAATGAGACCTTGACCATCGACGATCCGAGGCC</td>
</tr>
<tr>
<td>Phe24His</td>
<td>N loop/binding site</td>
<td>GTTTTCCTCCTTTTATTGAGGAGTATGCCGTCGGAGCC</td>
</tr>
<tr>
<td>His28Gln</td>
<td>N loop</td>
<td>GAGAGCAGAGCAGGCAAGGACAGCCAGGAGCC</td>
</tr>
<tr>
<td>Phe49Cys</td>
<td>N loop</td>
<td>GACGACGCTAACAACCTCTTTTGAGCAGAC</td>
</tr>
<tr>
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<tr>
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<td>GACGACGCTAACAACCTCTTTTGAGCAGAC</td>
</tr>
<tr>
<td>Leu136Phe</td>
<td>Binding site</td>
<td>GCTGACAGATCTGACATAGGAGCCAGGTCGAGCC</td>
</tr>
<tr>
<td>Ile235Leu</td>
<td>Binding site</td>
<td>GCTGACAGATCTGACATAGGAGCCAGGTCGAGCC</td>
</tr>
<tr>
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</tr>
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<td>GCTGACAGATCTGACATAGGAGCCAGGTCGAGCC</td>
</tr>
<tr>
<td>Val252Leu</td>
<td>GTP domain</td>
<td>GGTGCGCTCAACGTCGACCTGACTGAGTTCCAGACCACTTTG</td>
</tr>
<tr>
<td>Met268Thr</td>
<td>Near M loop</td>
<td>CTACCCACGCATTCACCTGCTTCTCTCAGTGAGCAGCC</td>
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<tr>
<td>Ala273Val</td>
<td>M loop (secondary)</td>
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</tr>
<tr>
<td>Leu136Phe</td>
<td>Near M loop</td>
<td>GCTGACAGATCTGACATAGGAGCCAGGTCGAGCC</td>
</tr>
<tr>
<td>Met302Leu</td>
<td>Fish, near M loop</td>
<td>CCGCGTCTCTCTGTTTCGATGGTGCGCTCAACGTCGACG</td>
</tr>
<tr>
<td>Met302Phe</td>
<td>Fish, near M loop</td>
<td>CCGCGTCTCTCTGTTTCGATGGTGCGCTCAACGTCGACG</td>
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Dinitroaniline Resistance Mutations

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</tr>
<tr>
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<td>Binding site</td>
<td>GCTGACAGATCTGACATAGGAGCCAGGTCGAGCC</td>
</tr>
<tr>
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<td>Binding site</td>
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replaced with a novel BamHI site. For this study, 21 single point mutations (Val4Leu, Ser51Leu, Phe24His, His28Gln, and Phe49Cys) were introduced into the α1-tubulin gene followed by restriction enzyme analysis of the amplified insert by using XbaI or BamHI enzymes. Parasites harboring an allelic replacement of each point mutation construct were assayed for their drug resistance using increasing concentrations of oryzalin and scoring for changes in morphology as described previously (Morrissette et al., 2004). Individual clones were assayed for homologous integration of the point mutations by amplification of the α1-tubulin gene followed by restriction enzyme analysis of the amplified insert by using XbaI or BamHI enzymes. Parasites harboring an allelic replacement of each point mutation construct were assayed for their drug resistance using increasing concentrations of oryzalin and scoring for changes in morphology as described previously (Morrissette et al., 2004).

Transformation, Selection, and Analysis of Oryzalin-resistant Transformants

Approximately 10° RH strain tachyzoites were transformed with ~8 μg of a linearized construct, selected in 0.5 μM oryzalin, and single cell cloned as described previously (Morrissette et al., 2004). Individual clones were assayed for homologous integration of the point mutations by amplification of the α1-tubulin gene followed by restriction enzyme analysis of the amplified insert by using XbaI or BamHI enzymes. Parasites harboring an allelic replacement of each point mutation construct were assayed for their drug resistance using increasing concentrations of oryzalin and scoring for changes in morphology as described previously (Morrissette et al., 2004).

Quantification of Replication Defects

Toxoplasma were passed into HFF cells without oryzalin and allowed to grow until the host cell monolayer was completely lysed. Extracellular wild-type RH parasites (the parental strain) and parasites bearing homologous integrations of the dinitroaniline resistance mutations were viewed in MatTek dishes with a coverslip inset by using a 63× phase-contrast lens on a Zeiss Axioskop microscope. Images of random fields were captured as tif files and scored by counting. Three independent samples were counted for each cell line. In order not to under-represent the aberrant forms, we counted the number of apical regions to establish total possible parasites lost from the parasite population through replication defects. All aberrant forms were counted together.

RESULTS

We have characterized a diverse group of point mutations in α1-tubulin that are associated with oryzalin resistance in T. gondii (currently 35 different mutations at 25 locations in α1-tubulin). When these mutations are mapped onto a model of Toxoplasma α1-tubulin, many are located in regions that suggest that two mechanisms confer resistance in most cases: mutations that increase dimer–dimer affinity compensate for dinitroaniline disruption of microtubules and mutations that decrease dimer affinity for oryzalin alter the dinitroaniline binding site (Figure 1). We have used an established method to introduce point mutations from these regions into wild-type Toxoplasma by homologous recombination (Figure 2A). These α1-tubulin point mutations are either predicted to increase microtubule stability (mutations located in the N loop, the M loop, and the GTPase-activating domain of α-tubulin) or to affect the dinitroaniline binding site (Figure 1). In most cases, the point mutations confer low levels of oryzalin resistance (0.5–7.5 μM). However, four mutations confer resistance to significantly higher concentra-
trations of oryzalin. Parasites with the Leu136Phe mutation are resistant to 36 μM oryzalin, and those with the Thr239Ile, Val252Leu, or Arg243Ser mutations are resistant to ~40 μM oryzalin (Figure 2B).

In the absence of oryzalin, some of the tubulin mutations result in Toxoplasma that seem to have a thinner and elongated shape relative to the dinitroaniline-sensitive parental strain. This is most obvious in Toxoplasma bearing a homologous integration of the N loop mutation His28Gln (Figure 3). Three-dimensional reconstructions of Toxoplasma subpellicular microtubules by using deconvolution microscopy suggest that microtubule length is increased in parasites with the His28Gln point mutation (Figure 3, C–E). This increased length is likely to result from a shift in the equilibrium between tubulin dimers and microtubule polymers.

To test whether α-tubulin amino acid substitutions that are thought to stabilize microtubules in the Antarctic fish Chiono-dracon rastrosinosus are able to confer resistance to dinitroanilines, we introduced directed changes to Toxoplasma α1-tubulin to make single point mutations at Ser187Ala, Ala273Thr, Met268Val, and Met301Thr. Although some of the Antarctic fish substitutions are very similar to the dinitroaniline resistance mutations (the oryzalin resistance mutation Met301Thr is adjacent to the fish mutations Met302Leu and Met302Phe), we did not recover parasites that had integrated the altered tubulin after selection in oryzalin. Because we only select for α1-tubulin gene integrations with dinitroanilines, we cannot distinguish whether these substitutions do not stabilize Toxoplasma microtubules to confer resistance or whether the resulting microtubules are toxic because they are too stable when expressed in Toxoplasma.

Toxoplasma replicates by endodyogeny, a process of internal budding. We observed an increased proportion of abnormal Toxoplasma “monsters” that are a consequence of aberrant replication (Figure 4). In many cases, these monsters can be attributed to a failure of mutant parasites to synchronize nuclear division (which requires spindle microtubules) and the formation of daughter buds (which requires subpellicular microtubules) during endodyogeny. Toxoplasma actively invades host cells by using actin-based motility in conjunction with a moving junction that forms between the parasite and the host plasma membrane (Soldati and Meissner, 2004). Parasites that lack a rigid elongated crescent shape are incapable of invading host cells (Skermans et al., 1996; Shaw et al., 2000; Morrisette and Sibley, 2002; Morrisette et al., 2004). Because abnormal forms that fail to complete scission (examples 4–11 in Figure 4C) cannot invade new host cells, these are nonviable organisms. There may also be other defective parasites that have lost single chromosomes but seem normal at the level of the light microscope. We attempted to evaluate the frequency of chromosome missegregation using flow cytometry of intracellular (released by syringe passage) parasites stained with SYTOX-green. Toxoplasma has 14 chromosomes; therefore, the loss of a single chromosome would be associated with an ~7% decrease in staining intensity for a small subpopulation of parasites associated with the IN peak. We were not able to resolve any reproducible differences in DNA content.

**Figure 1.** A model of the structure of Toxoplasma α1-tubulin with bound oryzalin (orange) based on previous molecular dynamics and docking results (Morrisette et al., 2004). Binding site residues that occur as point mutants are colored green. The N and M loops are colored mauve and the GTPase-activating domain is highlighted in yellow. (A) The N loop residues His28, Phe49, and Phe52 are mutated in dinitroaniline-resistant Toxoplasma lines. (B) The M loop contains Ala273 and Ile275, which are identified in dinitroaniline resistant α-tubulin genes and other mutations at Met268, Ala273, and Met301 in the N loop. The Ala273 mutation is a second site mutation rather than a resistance mutation (see below). (C) The GTPase activating domain of α-tubulin (yellow) contains a resistance mutation at Val252. Residues Asp251 and Glu254 (asterisks) were shown previously to be essential residues for activating the GTPase of β-tubulin (Anders and Botstein, 2001). (D) The dinitroaniline binding site is defined by several residues, including 11 that are mutated in resistant parasites. These residues are Val4, Ser6, Phe24, His28 (also in the N loop), Leu136, Ile233, Thr239, and Arg243. (E) The entire α-tubulin subunit is displayed with the adjacent α-β heterodimer contact area in the foreground. The β-tubulin subunit of the heterodimer (data not shown) would be behind the α-tubulin structure.
DISCUSSION

Dinitroaniline resistance mutations have been reported in the unicellular green alga _Chlamydomonas reinhardtii_, in higher plants such as _Elesine indica_ (goosefoxtail), and _Setaria viridis_ (green foxtail), and in the protozoans _T. gondii_ and _Chlamydomonas thermophila_ (Schibler and Huang, 1991; James et al., 1993; Gaertig et al., 1994; Anthony et al., 1998; Yamamoto et al., 1998; Delye et al., 2004). In some cases, such as _Thr239Ile_ and _Leu136Phe_, the same α-tubulin mutation has been identified in dinitroaniline-resistant _Toxoplasma, Eleusin-

given the variation associated with this assay and the resolution required for this measurement (data not shown). We demonstrated previously that anucleate _Toxoplasma_ (example 2 in Figure 4C) can invade host cells, but obviously cannot proliferate in the absence of a nuclear genome (Morrisette and Sibley, 2002). Similarly, parasites with inappropriate chromosome content may seem normal and reinvade host cells, but they will be unable to grow and replicate.

When the relative occurrence of grossly abnormal extracellular parasites is quantified for the parental _Toxoplasma_ strain and parasite lines bearing homologous integrations of the α-tubulin point mutations, it is clear that all of the point mutations are associated with some degree of increased rates of replication defects (Figure 5). Extracellular RH (parental strain) _Toxoplasma_ have ~4% of parasites with overt replication defects. Enhanced replication defect rates are particularly obvious in N loop mutants, which have 10–13% of extracellular parasites that are grossly altered and incapable of continued cycles of invasion and replication. The binding site mutation _Arg243Ser_ has high oryzalin resistance (41 μM) tempered by an ~12% rate of replication defects.

Parasites bearing the N loop mutation _Phe52Tyr_ have resistance to 7 μM oryzalin. When this strain was grown in the absence of oryzalin, it spontaneously acquired second site mutations at _Ala273Val_ or _Asp367Val_. Our gene replacement method is limited to introducing α-tubulin transgenes that can be selected for by oryzalin resistance. Despite several attempts to isolate parasite lines bearing _Ala273Val_ and _Asp367Val_ as single mutations to the α-tubulin gene, we were unable to obtain them, suggesting that they do not confer resistance. When combined with _Phe52Tyr_, the _Ala273Val_ and _Asp367Val_ mutations were easily isolated as homologous integrations, and parasite lines with the double mutations (_Phe52Tyr/Ala273Val_ and _Phe52Tyr/Asp367Val_) showed lower resistance than that of parasites with _Phe52Tyr_ mutation alone. We have, therefore, concluded that the _Ala273Val_ and _Asp367Val_ mutations do not confer oryzalin resistance alone, and when combined with the _Phe52Tyr_ mutation, the second site mutations decrease resistance to 2.8 and 3.3 μM oryzalin, respectively (Figure 6A). The degree of replication defects in the single mutation _Phe52Tyr_ (~13%) is higher than that of the double mutations (~6 and 5%), suggesting that in the absence of oryzalin, replication defects are sufficiently deleterious to select for spontaneous secondary mutations that correct this disadvantageous condition (Figure 6B). The location of the second site mutations is consistent with a role in decreased protofilament interactions. The _Ala273Val_ mutation is in the M loop and the _Asp367Val_ mutation is in an α-tubulin insert that stabilizes the α-tubulin M loop. The substitution of Val for Asp at this location disrupts a salt bridge between _Asp367_ and _Arg229_, presumably destabilizing the insert, which in turn destabilizes the M loop protofilament contact (Figure 6C).
of newly invaded parasites stained with a-dimensional projections were created by collecting Z-series images. His28Gln mutant parasites have longer microtubules. These three-dimensional projections were created by collecting Z-series images of wild-type (C) and His28Gln mutant parasites (E) indicate that His28Gln mutant parasites have longer microtubules. These three-dimensional projections were created by collecting Z-series images of newly invaded parasites stained with a Toxoplasma-specific tubulin antibody. The images were processed with Zeiss deconvolution software. An overlay of the wild-type parasite projection (green) on the His28Gln mutant parasite projection (red) illustrates the increased subpellicular microtubule length in the mutant parasites (D).

Figure 3. Phase-contrast microscopy of extracellular wild-type (A) and His28Gln mutant parasites (B) illustrates that Toxoplasma expressing the His28Gln mutation are thinner and longer and that they show an increased frequency of septation defects. (C–E) Projections of wild-type (C) and His28Gln mutant parasites (E) indicate that His28Gln mutant parasites have longer microtubules. These three-dimensional projections were created by collecting Z-series images of newly invaded wild-type parasites stained with a Toxoplasma-specific tubulin antibody. The images were processed with Zeiss deconvolution software. An overlay of the wild-type parasite projection (green) on the His28Gln mutant parasite projection (red) illustrates the increased subpellicular microtubule length in the mutant parasites (D).

In data presented here, we demonstrate that resistance mutations identified in other species function to confer resistance in Toxoplasma. The Met268Thr mutation observed in Eleusine confers dinitroaniline resistance in Toxoplasma as does a Phe24His substitution that is analogous to the Tyr24His mutation in Chlamydomonas (James et al., 1995; Anthony et al., 1998; Yamamoto et al., 1998). However, when we attempted to introduce substitutions that are hypothesized to confer cold stability to the microtubules of Antarctic fish (Detrich et al., 2000), they do not confer oryzalin resistance. Because we select for transformation with dinitroanilines, we are unable to distinguish whether these substitutions do not confer dinitroaniline resistance in Toxoplasma or whether they are lethal when expressed because the microtubules are too stable.

The observations presented in this article 1) that M and N loop mutations have relatively low resistance and the highest incidence of replication defects and 2) that a subset of binding site mutations have extremely high resistance are consistent with our working model that invokes two major mechanisms of dinitroaniline resistance. In this study, we selected a subset of tubulin mutations to analyze from a larger collection of substitutions conferring dinitroaniline resistance based on the location of the altered residues in the structure of α-tubulin. In previous work, we characterized homologous integrations of five resistance mutations that were selected from the larger set of mutations. Three of the previously studied mutations were distinct substitutions (Ala, Pro, Thr) to Ser165: these mutations conferred relatively low dinitroaniline resistance (1–2 μM oryzalin). Other researchers subsequently identified Ser165Pro as an α-tubulin mutation associated with resistance to HTI-286 (a hemiasterlin derivative) in human carcinoma cell lines (Poruchynsky et al., 2004). Because the hemiasterlin binding site has been located in a distinct region of the tubulin dimer at the α-β-subunit interface, predominantly in β-tubulin, we expect that the Ser165Pro mutation is associated with general microtubule stability (Mitra and Sept, 2004; Ravi et al., 2005). The other two previously studied point mutations were Thr239Ile, which had been previously identified in dinitroaniline-resistant goosegrass and Ile231Thr, which is in the same face of α-helix 7 as Thr239 (Morrissette et al., 2004). The original Thr239Ile line did not survive freezing and we recently discovered that in two new and independent integrations, the Thr239Ile confers resistance to 40 μM oryzalin (Figure 2). This suggests that the previous line was an incorrectly genotyped nonhomologous integration. Our corrected observation is consistent with the other data presented here that suggests that mutations to residues in the binding site are associated with high resistance (Figure 2).

Previous researchers have demonstrated the importance of the M and N loop residues in microtubule function and stability by using both natural mutations and directed substitutions to these domains in α-tubulin. Alanine scanning studies in budding yeast have identified α-tubulin N loop mutations that are benomyl resistant, presumably due to increased microtubule stability, or benomyl supersensitive, presumably due to decreased microtubule stability (Richards et al., 2000). Alanine scanning analysis also indicates that M loop substitutions are associated with benomyl supersensitivity, revealing that these microtubules have decreased stability (Richards et al., 2000). The α-tubulin N loop mutation Glu55Lys and the M loop mutation His283Tyr confer cold-mid and vinblastine resistance in human cells (Hari et al., 2003).

Dynamic instability of microtubules depends on the polymerization-stimulated hydrolysis of GTP bound to β-tubulin, and it is necessary for microtubule disassembly (Davis et al., 1994; Sage et al., 1995; Anders and Botstein, 2001; Dougherty et al., 2001). Experiments in budding yeast indicate that two residues (Asp252 and Glu255) in α-tubulin activate the β-tubulin GTPase in the adjacent dimer (Richards et al., 2000; Anders and Botstein, 2001). When these residues are mutated to alanine, the resulting dimers are poisonous and are lethal as a minor component of the total tubulin population (Anders and Botstein, 2001). Budding yeast with induced expression of mutant α-tubulins (Asp252Ala, Glu255Ala, and Asp252Ala/Glu255Ala) have aberrant bundled microtubules. These microtubules are not dynamic, and microtubule-disrupting drugs are incapable of disrupting the microtubules. The dinitroaniline-resistance mutation in Toxoplasma α1-tubulin at Val252Leu is not the equivalent residue to the Asp252 residue in Saccharomyces cerevisiae. The Toxoplasma Val252 residue is equivalent to Leu253 in yeast, and so it is immediately adjacent to the critically important residue (Figure 1C). We hypothesize that the Val252Leu mutation may decrease the activity of the GTPase-activating domain to increase microtubule stability. This mutation is associated with very high dinitroaniline resistance, but it does not seem to cause the parasites to have obviously longer and more stable microtubules.

Our analysis of the effect of tubulin mutations on replication indicates that all of the resistance mutations confer increased rates of defects to negatively influence parasite...
fitness. As shown in Figure 5, substitutions predicted to hyperstabilize microtubules (M and N loops) are generally associated with the highest rates of replication defects. This may be associated with defective chromosome segregation, because previous studies have associated decreased microtubule dynamics with chromosome loss (Beinhauer et al.,...
For example, altering residues of the GTP binding domain of tubulin function, such as tubulin folding or subunit flexibility, perhaps as a consequence of altered tubulin folding (Richards et al., 1997; Asakawa et al., 2000). In contrast, several mutations in yeast α-tubulin have been shown to alter folding or other aspects of subunit function to influence drug resistance or hypersensitivity and microtubule sensitivity to heat or cold. For example, altering residues of the GTP binding domain of α-tubulin (such as the triple mutation Lys97Ala/Glu98Ala/Asp99Ala) confers cold sensitivity and benomyl supersensitivity, perhaps as a consequence of altered tubulin folding (Richards et al., 2000). In contrast, several mutations in yeast β-tubulin (Glu108Ala/Glu111Ala, Glu123Ala, and Arg282Ala/Glu288Ala) are cold sensitive but benomyl resistant (Reijo et al., 1994). Ultimately, the significant issue is whether resistant parasites could be sufficiently fit to be spread throughout natural populations in competition with wild-type parasites in the absence of drug selection. This might be achieved by high resistance due to multiple mutations or through multiple mutations that confer resistance but maintain fitness.

Toxoplasma parasites bearing the N loop mutation Phe52Tyr have resistance to 7 μM oryzalin, but ~13% of the parasite population has overt replication defects, possibly due to microtubules with increased stability. When this parasite strain was grown in the absence of oryzalin, it spontaneously acquired secondary mutations in the M loop (Ala273Val) or in an α-tubulin-specific insert that stabilizes the M loop (Asp367Val). The Asp367Val point mutation eliminates a salt bridge between Asp367 and Arg229. This salt bridge stabilizes the α-tubulin insert, and in turn, the insert stabilizes the α-tubulin M loop (Figure 6C). We expect that elimination of the salt bridge ultimately decreases the strength of the α-tubulin M loop contact within the protofilament, akin to the action of the Ala273Val mutation, which is directly in the M loop. We hypothesize that the secondary mutations increase Toxoplasma fitness by decreasing microtubule stability. Consistent with this hypothesis, parasites with the double mutations have both decreased dinitroaniline resistance and decreased incidence of replication defects. It is likely that the Phe52Tyr strain has a high rate of chromosome segregation defects that provide a strong selective pressure for second site mutations to correct for tubulin function in the absence of dinitroaniline selection. Previous work in the fission yeast Schizosaccharomyces pombe has indicated that tubulin mutations (the α-tubulin mutation Val260lle) or the loss of the EB-1 homologue MAL3 are associated with less dynamic microtubules and increased rates of chromosome loss (Beinhauer et al., 1997; Asakawa et al., 2006).

Collectively, the results presented in this article suggest that mutations to α1-tubulin confer dinitroaniline resistance at a cost to microtubule function and parasite fitness. Since a number of novel mutations confer resistance, it is important to evaluate whether parasites treated with microtubule-disrupting drugs would rapidly acquire resistance mutations if used in a therapeutic setting. Studies that have exploited growth competition assays to assess the fitness of Toxoplasma strains bearing wild-type or pyrimethamine-resistant dihydrofolate reductase genes have concluded that even strains that behave identically in vitro can display growth defects in vivo (Fohl and Roos, 2003). Our dinitroaniline-resistant strains have overt replication defects in vitro, suggesting that they have reduced fitness and would fare poorly in similar competition assays. Therefore, drugs that selectively target parasite microtubules remain a compelling option for the development of new antiparasitic therapies.

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