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 $\alpha 1$ -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 $\alpha 1$ -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 α -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 peloruside 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

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dinitroanilines and peloruside 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 monomer 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 parasites, 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; Morrisette and Sibley, 2002; Werbovetz *et al.*, 2003). Given that the dinitroanilines do not disrupt vertebrate tubulins, dinitroaniline activity is of particular interest for the development of new antiparasitic drugs. We are specifically interested in understanding the action of dinitroanilines on apicomplexan parasites 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 widespread and damaging pathogens with profound direct and indirect impacts on human welfare. Human infection by *T. gondii* can cause life-threatening illness in immunocompromised individuals and birth defects or miscarriage during fetal infection (Black and Boothroyd, 2000). Other apicomplexans include *Plasmodium* (agent of malaria) and *Cryptosporidium* (an opportunistic pathogen), which are parasites of considerable medical importance, and *Theileria* and *Eimeria*, animal pathogens with extensive impact on food production (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 populations of microtubules in the invasive stages of apicomplexan parasites: subpellicular microtubules and spindle microtubules (Morrisette and Sibley, 2002). Subpellicular microtubules are nondynamic; they maintain both apical polarity and the characteristic crescent shape of the parasite by interacting 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 $<0.2 \mu\text{M}$ 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 IC_{50} values by radiolabel incorporation (Stokkermans *et al.*, 1996). Using drug washout and plaquing values, the approximate IC_{50} value is $0.1 \mu\text{M}$ oryzalin, and using morphology (round rather than elongated parasites), parasites begin to round up by $\sim 0.2 \mu\text{M}$ oryzalin (Stokkermans *et al.*, 1996; Morrisette and Sibley, 2002).

In previous work, we used genetic analysis of oryzalin-resistant *Toxoplasma* lines in combination with computational methods to identify a binding site and mode of action

for the dinitroanilines (Morrisette *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 (Mittra and Sept, 2006). The refined dinitroaniline binding site is located on *Toxoplasma*, *Leishmania*, and *Plasmodium* 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, Ile42, Asp47, Pro63, Arg64), strand S2 (Cys65), strand S4 (Leu136), helix H7 (Ile235, Ser236, Thr239, Ala240, Ser 241), and the T7 loop (Arg243, Phe244, Asp245). Analysis of vertebrate α -tubulin indicates that dinitroanilines 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 -tubulin mobility in the presence or absence of bound drug, these simulations suggest that dinitroaniline binding profoundly limits flexibility of the α -tubulin N loop, which is drawn in toward the core of the tubulin dimer (Mittra 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 microtubules, 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 resistance. 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 domain 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 replication 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 fibroblast (HFF) cells in DMEM with 10% fetal bovine serum as described previously (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 $2.5 \mu\text{M}$ oryzalin as described previously (Morrisette *et al.*, 2004).

Analysis of $\alpha 1$ -Tubulin Point Mutations

The $\alpha 1$ -tubulin gene was amplified from genomic DNA isolated from individual oryzalin-resistant parasites by using thermal cycling with primers GAGTCTCGTAGAGAACAAAGC (5' untranslated region [UTR]) and CGTTTATACCTTCACCTTTTC (3' UTR). The amplified 2.3-kb fragment was sequenced and analyzed using Sequencher software (Gene Codes, Ann Arbor, MI) to identify point mutations in the coding sequence of the $\alpha 1$ -tubulin gene. Primers and methods to sequence the $\alpha 1$ -tubulin gene constructs are as described previously (Morrisette *et al.*, 2004). Similar sequencing analysis indicated that none of the resistant lines had mutations in the $\beta 1$ -tubulin gene. We also analyzed the sequences of two recently identified additional *Toxoplasma* α -tubulin genes ($\alpha 2$ -tubulin and $\alpha 3$ -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 previously generated construct containing a 2.3-kb insert of the *Toxoplasma* $\alpha 1$ -tubulin gene where a unique XbaI site in the second intron of $\alpha 1$ -tubulin was

Table 1. Primers used to introduce tubulin mutations

Mutation	Location	Primer
Val4Leu	Binding site	CGACAAAATGAGAGAGCTTATCAGCATCCACGTCGGCCAGGCC
Ser6Ile	Binding site	CGACAAAATGAGAGAGGTTATCATCATCCACGTCGGCCAGGCCGG
Phe24His	Binding site	GTTTTCTCTCTTCTTTTATAGGAGCTCCACTGCCTGGAACATGG
His28Gln	N loop/binding site	GGAGCTCTTCTGCCTGGAACAAGGTATCCAGCCG
Phe49Cys	N loop	GGTGGTGACGACGCCTGCAACACCTTCTTTTCCGAGACAGGC
Phe52Ile	N loop	GACGACGCCTTCAACACCCTCTTTTCCGAGACAG
Phe52Leu	N loop	GACGACGCCTTCAACACCCTCTTTTCCGAGACAG
Phe52Tyr	N loop	CGACGCCTTCAACACCCTACTTTTCCGAGACAGG
Leu136Phe	Binding site	GCACTGGTCTCCAGGGTTTCTTCATGTTCAACGCCGTCGGC
Ile235Leu	Binding site	CCTGAACAGACTGATTGCCAGGTCACCTCCTCCCTGACCGCG
Ile235Thr	Binding site	CCTGAACAGACTGATTGCCAGGTCACCTCCTCCCTGACCGCG
Ile235Val	Binding site	CCTGAACAGACTGATTGCCAGGTCGTCTCCTCCCTGACCGCG
Thr239Ile	Binding site	GCCAGGTCATCTCTCCTGATCGCGTCTCTCCG
Arg243Cys	Binding site	CGCGTCTCTCTGTTTCGATGGTGCGCTCAACGTCGACG
Arg243Ser	Binding site	CGCGTCTCTCAGTTTCGATGGTGCGCTCAACGTCGACG
Val252Leu	GAP domain	GGTGCGCTCAACGTCGACCTGACTGAGTTCAGACCAACTGG
Met268Thr	Near M loop	CTACCCACGCATTCACTTCACGCTCTCATCGTATGCG
Ala273Val	M loop (secondary)	CTCTCATCGTATGCGCCATCATCAGCGCAGAGAAGG
Ile275Thr	M loop	CTCATCGTATGCGCCACCATCAGCGCAGAGAAG
Ala295Val	Near M loop	CTGAGATCACCAACTCGGTTTTTCGAGCCCGC
Met301Thr	Near M loop	CGAGCCCGCGAGCAGATGGCCAAAGTGTGATCC
Asp367Val	α -Insert (secondary)	CCCCTGTGGTTCCTGGTGGTGTCTTGGCCAAAGTTCATGCGCGCC
Ser187Ala	Fish	GCAGTTGTGGAACCGTACAACGCCGTCCTTTCCACTCACTCTCTGTTGG
Ala278Thr	Fish, M loop	CGTATGCGCCCATCATCAGCACAGAGAAGGCGTACCACGAGCAG
Ser287Thr	Fish, M loop	GGCGTACCACGAGCAGTTGACTGTGCGTGAGATCACC
Met302Leu	Fish, near M loop	CGAGCCCGCGAGCATGTTGGCCAAAGTGTGATCTCCG
Met302Phe	Fish, near M loop	CGAGCCCGCGAGCATGTTTCGCCAAAGTGTGATCTCCG

replaced with a novel BamHI site. For this study, 21 single point mutations (Val4Leu, Ser6Ile, Phe24His, His28Gln, Phe49Cys, Phe52Ile, Phe52Leu, Phe52Tyr, Leu136Phe, Ile235Leu, Ile235Thr, Ile235Val, Arg243Cys, Arg243Ser, Val252Leu, Met268Thr, Ala273Val, Ile275Thr, Ala295Val, Met301Thr, and Asp367Val) and two double mutations (Phe52Tyr/Ala273Val and Phe52Tyr/Asp367Val) were introduced into the *Toxoplasma* α 1-tubulin gene. We also introduced five point mutations that are observed as differences in the α -tubulin genes of Antarctic fish (Ser187Ala, Ala278Thr, Ser287Thr, Met302Leu, and Met302Phe) into the *Toxoplasma* α 1-tubulin gene (Detrich *et al.*, 2000). The mutagenesis primers used to generate these mutant tubulins are listed in Table 1. All α -tubulin constructs were verified by sequencing as described previously (Morrisette *et al.*, 2004).

Transformation, Selection, and Analysis of Oryzalin-resistant Transformants

Approximately 10^7 RH strain tachyzoites were transformed with $\sim 8 \mu\text{g}$ of a linearized construct, selected in $0.5 \mu\text{M}$ oryzalin, and single cell clones as described previously (Morrisette *et al.*, 2004). Individual clones were assayed for homologous integration of the point mutations by amplification of the α 1-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 (Morrisette *et al.*, 2004). In our previous study, we isolated and characterized individual clones from a single transfection and selection of 10^7 parasites. Because it is possible that these clones are siblings rather than independent lines, we have altered our approach during this study to isolate allelic replacements from parasites derived from more than one independent transfection and selection. Parasites bearing allelic replacements of the mutations His28Gln, Thr239Ile, Arg243Ser, and Arg243Cys obtained from two independent transfections and selections yielded consistent results in this study.

Immunofluorescence of Parasites

Intracellular parasites on 12-mm circular glass coverslips were fixed, permeabilized, and stained as described previously (Morrisette and Sibley, 2002). They were mounted in VECTASHIELD mounting media with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images of newly invaded parasites stained with a *Toxoplasma* specific anti- β -tubulin antibody (Morrisette and Sibley, 2002) and an Alexa 488 secondary antibody (Invitrogen, Carlsbad, CA) were collected on a Zeiss Axioskop using the AxioVision camera and software (Carl Zeiss, Thornwood, NY). Z-series images were

processed using Zeiss deconvolution software and images were exported and manipulated in Photoshop 8.0 (Adobe Systems, Mountain View, CA).

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 suspension in MatTek dishes with a coverslip inset by using a $63\times$ 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 α 1-tubulin that are associated with oryzalin resistance in *T. gondii* (currently 35 different mutations at 25 locations in α 1-tubulin). When these mutations are mapped onto a model of *Toxoplasma* α 1-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 α 1-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 – $7.5 \mu\text{M}$). However, four mutations confer resistance to significantly higher concen-

Figure 1. A model of the structure of *Toxoplasma* α 1-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, Ala295, and Met301 are nearby the M 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, Ile235, 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.

trations of oryzalin. Parasites with the Leu136Phe mutation are resistant to 36 μ M oryzalin, and those with the Thr239Ile, Val252Leu, or Arg243Ser mutations are resistant to $\sim$ 40 μ M 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 *Chionodraco rastrospinosus* are able to confer resistance to dinitroanilines, we introduced directed changes to *Toxoplasma* α 1-tubulin to make single point mutations at Ser187Ala, Ala278Thr, Ser287Thr, Met302Leu, and Met302Phe. 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 α 1-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 (Stokkermans *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 $\sim$ 7% decrease in staining intensity for a small subpopulation of parasites associated with the 1N peak. We were not able to resolve any reproducible differences in DNA content

Figure 2. (A) Diagram of the exon-intron structure of $\alpha 1$ -tubulin used for the allelic replacement strategy. Endogenous (wild-type) $\alpha 1$ -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 individual point mutations were introduced in the transgene construct and electroporated into *Toxoplasma*. Parasites with a homologous integration of the transgene are identified by conversion of the XbaI restriction site to a BamHI site. (B) Oryzalin resistance of *Toxoplasma* with allelic integration of the $\alpha 1$ -tubulin gene containing the individual point mutations in the binding site (BS), N loop (NL), M loop (ML), and GTPase-activating domain (GD). Resistance conferred by the predicted binding site mutation Thr239Ile was previously reported to be 5 μ M, which is in error. We have generated two new independent integrations of the Thr239Ile construct that have resistance to 41 μ M oryzalin, as illustrated here.

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 $\alpha 1$ -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 μ M) tempered by an ~12% rate of replication defects.

Parasites bearing the N loop mutation Phe52Tyr have resistance to 7 μ M 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 $\alpha 1$ -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 $\alpha 1$ -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 μ M 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).

DISCUSSION

Dinitroaniline resistance mutations have been reported in the unicellular green alga *Chlamydomonas reinhardtii*, in higher plants such as *Eleusine indica* (goosegrass) and *Setaria viridis* (green foxtail), and in the protozoans *T. gondii* and *Tetrahymena 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*, *Eleus-*

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 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 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).

ine, and *Setaria*. 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.*, 1993; 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 μ M oryzalin). Other researchers subsequently identified Ser165Pro as an α -tubulin mutation associated with resistance to HTI-286 (a hemiassterlin derivative) in human carcinoma cell lines (Poruchynsky *et al.*, 2004). Because the hemiassterlin 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 (Mittra 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 (Morrisette *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 μ M 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 colcemid 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* α 1-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

Figure 4. Hyperstabilized microtubules affect the fidelity of parasite replication (A) *Toxoplasma* parasites replicate by endodyogeny, a process of internal budding that creates two internal daughter parasites within a mother parasite. This process permits an apically polarized “mother” parasite to undergo mitosis while building two polarized daughter buds, each consisting of inner membrane complex, subpellicular microtubules, and apical organelles around the daughter nuclei. When the daughter parasites are mature, they emerge from the mother, adopting her plasma membrane. (B) Illustration of the aberrant forms of extracellular parasites: (i) anucleate and (ii) diploid parasites result from completion of cytokinesis in the absence of nuclear division; (iii) parasites that complete cytokinesis in the absence of nuclear division can exclude nuclear material; two-headed (iv) or multiple headed (v) parasites result from arrest during cytokinesis in the absence of nuclear division; (vi) and parasites with late completion of nuclear division can fail to complete septation (cytokinesis). (C) Phase-contrast images of malformed extracellular parasites. The large panel shows an apparently wild-type parasite (1) next to an anucleate parasite (2) and a grossly enlarged parasite (3). The anucleate and enlarged parasites (2 and 3) likely result from cytokinesis without nuclear division as illustrated in B iv, which shows similar parasites joined by incomplete cytokinesis. Incomplete septation of one (5), two (6), three (7), or four (8 and 9) daughter buds from a residual body containing some or all nuclear material. Parasites in 10 and 11 have completed nuclear division and daughter cell formation, but they have not completed septation.

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.*,

Figure 5. Quantification of gross replication defects in the individual α -tubulin lines. Relative proportion of replication defects in wild-type parasites and the individual homologous integrations. The parental RH strain has ~4% aberrant extracellular parasites. The binding site (BS), N loop (NL), M loop (ML) and GTPase-activating domain (GD) mutants all have some degree of increased replication defects.

Figure 6. Dinitroaniline resistance is sufficiently deleterious that in the absence of oryzalin selection, *Toxoplasma* parasites harboring the Phe52Tyr mutation acquire compensatory second site mutations at either Ala273Val (the M loop) or Asp367Val. (A) Oryzalin resistance of the single mutant Phe52Tyr (7 μ M) is greater than that of the double mutants Phe52Tyr/Ala273Val (2.8 μ M) and Phe52Tyr/Asp367Val (3.3 μ M). The single substitutions Ala273Val or Asp367Val do not confer any oryzalin resistance. (B) Replication defects associated with the single mutant Phe52Tyr are greater (~13%) than those in the double mutants Phe52Tyr/Ala273Val (~6%) and Phe52Tyr/Asp367Val (~5%). (C) Substitution of Val for Asp at residue 367 disrupts a salt bridge that links Arg229 (blue) to Asp367 (red) within the α -tubulin-specific insert (green). The eight amino acids of the insert stabilize the M loop (purple) of α -tubulin.

1997; Asakawa *et al.*, 2006). Because we do not think that the putative binding site mutations influence microtubule stability, we hypothesize that they alter some other aspect of tubulin function, such as tubulin folding or subunit flexibility, in *Toxoplasma*. In budding yeast, alanine scanning mutations in 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 μ M 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 micro-

tubule 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 Val260Ile) 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 α 1-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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REFERENCES

- Alder, E. F., Wright, W. L., and Soper, G. F. (1960). Control of seedling grasses in turf with diphenylacetone nitrile and a substituted dinitroaniline. *Proc. North Centr. Weed Control Conf.* 17, 23–24.
- Anders, K. R., and Botstein, D. (2001). Dominant-lethal α -tubulin mutants defective in microtubule depolymerization in yeast. *Mol. Biol. Cell* 12, 3973–3986.
- Anthony, R. G., Waldin, T. R., Ray, J. A., Bright, S. W., and Hussey, P. J. (1998). Herbicide resistance caused by spontaneous mutation of the cytoskeletal protein tubulin. *Nature* 393, 260–263.
- Asakawa, K., Kume, K., Kanai, M., Goshima, T., Miyahara, K., Dhut, S., Tee, W. W., Hirata, D., and Toda, T. (2006). The V260I mutation in fission yeast α -tubulin Atb2 affects microtubule dynamics and EB1-Mal3 localization and activates the Bub1 branch of the spindle checkpoint. *Mol. Biol. Cell* 17, 1421–1435.
- Beinhauer, J. D., Hagan, I. M., Hegemann, J. H., and Fleig, U. (1997). Mal3, the fission yeast homologue of the human APC-interacting protein EB-1 is required for microtubule integrity and the maintenance of cell form. *J. Cell Biol.* 139, 717–728.
- Bhattacharya, G., Herman, J., Delfin, D., Salem, M. M., Barszcz, T., Mollet, M., Riccio, G., Brun, R., and Werbovetz, K. A. (2004). Synthesis and antitubulin activity of N1- and N4-substituted 3,5-dinitro sulfanilamides against African trypanosomes and leishmania. *J. Med. Chem.* 47, 1823–1832.
- Bhattacharya, G., Salem, M. M., and Werbovetz, K. A. (2002). Antileishmanial dinitroaniline sulfonamides with activity against parasite tubulin. *Bioorg. Med. Chem. Lett.* 12, 2395–2398.
- Black, M. W., and Boothroyd, J. C. (2000). Lytic cycle of *Toxoplasma gondii*. *Microbiol. Mol. Biol. Rev.* 64, 607–623.
- Bogitsh, B. J., Middleton, O. L., and Ribeiro-Rodrigues, R. (1999). Effects of the antitubulin drug trifluralin on the proliferation and metacyclogenesis of *Trypanosoma cruzi* epimastigotes. *Parasitol. Res.* 85, 475–480.
- Chan, M. M., and Fong, D. (1990). Inhibition of leishmanias but not host macrophages by the antitubulin herbicide trifluralin. *Science* 249, 924–926.
- Davis, A., Sage, C. R., Dougherty, C. A., and Farrell, K. W. (1994). Microtubule dynamics modulated by guanosine triphosphate hydrolysis activity of beta-tubulin. *Science* 264, 839–842.
- Delye, C., Menchari, Y., Michel, S., and Darmency, H. (2004). Molecular bases for sensitivity to tubulin-binding herbicides in green foxtail. *Plant Physiol.* 136, 3920–3932.
- Detrich, H. W., 3rd, Parker, S. K., Williams, R. C., Jr., Nogales, E., and Downing, K. H. (2000). Cold adaptation of microtubule assembly and dynamics. Structural interpretation of primary sequence changes present in the α - and β -tubulins of Antarctic fishes. *J. Biol. Chem.* 275, 37038–37047.
- Dougherty, C. A., Sage, C. R., Davis, A., and Farrell, K. W. (2001). Mutation in the beta-tubulin signature motif suppresses microtubule GTPase activity and dynamics, and slows mitosis. *Biochemistry* 40, 15725–15732.
- Downing, K. H. (2000). Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics. *Annu. Rev. Cell Dev. Biol.* 16, 89–111.
- Downing, K. H., and Nogales, E. (1998a). New insights into microtubule structure and function from the atomic model of tubulin. *Eur. Biophys. J.* 27, 431–436.
- Downing, K. H., and Nogales, E. (1998b). Tubulin structure: insights into microtubule properties and functions. *Curr. Opin. Struct. Biol.* 8, 785–791.
- Fohl, L. M., and Roos, D. S. (2003). Fitness effects of DHFR-TS mutations associated with pyrimethamine resistance in apicomplexan parasites. *Mol. Microbiol.* 50, 1319–1327.
- Gaertig, J., Thatcher, T. H., Gu, L., and Gorovsky, M. A. (1994). Electroporation-mediated replacement of a positively and negatively selectable beta-tubulin gene in *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. USA* 91, 4549–4553.
- Hari, M., Wang, Y., Veeraraghavan, S., and Cabral, F. (2003). Mutations in alpha- and beta-tubulin that stabilize microtubules and confer resistance to colcemid and vinblastine. *Mol. Cancer Ther.* 2, 597–605.
- Hugdahl, J. D., and Morejohn, L. C. (1993). Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. *Plant Physiol.* 102, 725–740.
- James, S. W., Silflow, C. D., Stroom, P., and Lefebvre, P. A. (1993). A mutation in the alpha 1-tubulin gene of *Chlamydomonas reinhardtii* confers resistance to anti-microtubule herbicides. *J. Cell Sci.* 106, 209–218.
- Jimenez-Barbero, J., Canales, A., Northcote, P. T., Buey, R. M., Andreu, J. M., and Diaz, J. F. (2006). NMR determination of the bioactive conformation of peloruside A bound to microtubules. *J. Am. Chem. Soc.* 128, 8757–8765.
- Jordan, A., Hadfield, J. A., Lawrence, N. J., and McGown, A. T. (1998). Tubulin as a target for anticancer drugs: agents which interact with the mitotic spindle. *Med. Res. Rev.* 18, 259–296.
- Kaidoh, T., Nath, J., Fujioka, H., Okoye, V., and Aikawa, M. (1995). Effect and localization of trifluralin in *Plasmodium falciparum* gametocytes: an electron microscopic study. *J. Eukaryot. Microbiol.* 42, 61–64.
- Levene, N. D. (1988). The protozoan phylum Apicomplexa, Boca Raton, FL: CRC Press.
- Li, H., DeRosier, D., Nicholson, W., Nogales, E., and Downing, K. (2002). Microtubule structure at 8 Å resolution. *Structure* 10, 1317.
- Lowe, J., Li, H., Downing, K. H., and Nogales, E. (2001). Refined structure of alpha beta-tubulin at 3.5 Å resolution. *J. Mol. Biol.* 313, 1045–1057.
- Makioka, A., Kumagai, M., Ohtomo, H., Kobayashi, S., and Takeuchi, T. (2000a). Effect of dinitroaniline herbicides on the growth of *Entamoeba histolytica*. *J. Parasitol.* 86, 607–610.
- Makioka, A., Kumagai, M., Ohtomo, H., Kobayashi, S., and Takeuchi, T. (2000b). Effect of the antitubulin drug oryzalin on the encystation of *Entamoeba invadens*. *Parasitol. Res.* 86, 625–629.
- Mitra, A., and Sept, D. (2004). Localization of the antimitotic peptide and decapeptide binding site on beta-tubulin. *Biochemistry* 43, 13955–13962.
- Mitra, A., and Sept, D. (2006). Binding and interaction of dinitroanilines with apicomplexan and kinetoplastid alpha-tubulin. *J. Med. Chem.* 49, 5226–5231.
- Morejohn, L. C., Bureau, T. E., Mole-Bajer, J., Bajer, A. S., and Fosket, D. E. (1987). Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. *Planta* 172, 252–264.
- Morrisette, N. S., Mitra, A., Sept, D., and Sibley, L. D. (2004). Dinitroanilines bind α -tubulin to disrupt microtubules. *Mol. Biol. Cell* 15, 1960–1968.
- Morrisette, N. S., and Sibley, L. D. (2002). Disruption of microtubules uncouples budding and nuclear division in *Toxoplasma gondii*. *J. Cell Sci.* 115, 1017–1025.
- Murthy, J. V., Kim, H. H., Hanesworth, V. R., Hugdahl, J. D., and Morejohn, L. C. (1994). Competitive inhibition of high-affinity oryzalin binding to plant tubulin by the phosphoric amide herbicide amiprofos-methyl. *Plant Physiol.* 105, 309–320.
- Nicholson, W. V., Lee, M., Downing, K. H., and Nogales, E. (1999). Cryo-electron microscopy of GDP-tubulin rings. *Cell Biochem. Biophys.* 31, 175–183.
- Nogales, E. (2000). Structural insights into microtubule function. *Annu. Rev. Biochem.* 69, 277–302.
- Nogales, E., and Wang, H. W. (2006a). Structural intermediates in microtubule assembly and disassembly: how and why? *Curr. Opin. Cell Biol.* 18, 179–184.
- Nogales, E., and Wang, H. W. (2006b). Structural mechanisms underlying nucleotide-dependent self-assembly of tubulin and its relatives. *Curr. Opin. Struct. Biol.* 16, 221–229.
- Nogales, E., Wang, H. W., and Niederstrasser, H. (2003). Tubulin rings: which way do they curve? *Curr. Opin. Struct. Biol.* 13, 256–261.
- Nogales, E., Wolf, S. G., and Downing, K. H. (1998). Structure of the alpha beta tubulin dimer by electron crystallography. *Nature* 391, 199–203.
- Pineda, O., Farras, J., Maccari, L., Manetti, F., Botta, M., and Villarrasa, J. (2004). Computational comparison of microtubule-stabilising agents laulimalide and peloruside with Taxol and colchicine. *Bioorg. Med. Chem. Lett.* 14, 4825–4829.
- Poruchynsky, M. S., Kim, J. H., Nogales, E., Annable, T., Loganzo, F., Greenberger, L. M., Sackett, D. L., and Fojo, T. (2004). Tumor cells resistant to a microtubule-depolymerizing hemiasterlin analogue, HTI-286, have mutations in alpha- or beta-tubulin and increased microtubule stability. *Biochemistry* 43, 13944–13954.
- Ravi, M., Zask, A., and Rush, T. S., 3rd. (2005). Structure-based identification of the binding site for the hemiasterlin analogue HTI-286 on tubulin. *Biochemistry* 44, 15871–15879.
- Reijo, R. A., Cooper, E. M., Beagle, G. J., and Huffaker, T. C. (1994). Systematic mutational analysis of the yeast beta-tubulin gene. *Mol. Biol. Cell* 5, 29–43.
- Richards, K. L., Anders, K. R., Nogales, E., Schwartz, K., Downing, K. H., and Botstein, D. (2000). Structure-function relationships in yeast tubulins. *Mol. Biol. Cell* 11, 1887–1903.
- Roos, D. S., Donald, R. G., Morrisette, N. S., and Moulton, A. L. (1994). Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods Cell Biol.* 45, 27–63.

- Sage, C. R., Dougherty, C. A., Davis, A. S., Burns, R. G., Wilson, L., and Farrell, K. W. (1995). Site-directed mutagenesis of putative GTP-binding sites of yeast beta-tubulin: evidence that alpha-, beta-, and gamma-tubulins are atypical GTPases. *Biochemistry* 34, 7409–7419.
- Schibler, M. J., and Huang, B. (1991). The colR4 and colR15 beta-tubulin mutations in *Chlamydomonas reinhardtii* confer altered sensitivities to microtubule inhibitors and herbicides by enhancing microtubule stability. *J. Cell Biol.* 113, 605–614.
- Shaw, M. K., Compton, H. L., Roos, D. S., and Tilney, L. G. (2000). Microtubules, but not actin filaments, drive daughter cell budding and cell division in *Toxoplasma gondii*. *J. Cell Sci.* 113, 1241–1254.
- Snyder, J. P., Nettles, J. H., Cornett, B., Downing, K. H., and Nogales, E. (2001). The binding conformation of Taxol in beta-tubulin: a model based on electron crystallographic density. *Proc. Natl. Acad. Sci. USA* 98, 5312–5316.
- Soldati, D., and Meissner, M. (2004). Toxoplasma as a novel system for motility. *Curr. Opin. Cell Biol.* 16, 32–40.
- Stokkermans, T. J., Schwartzman, J. D., Keenan, K., Morrisette, N. S., Tilney, L. G., and Roos, D. S. (1996). Inhibition of *Toxoplasma gondii* replication by dinitroaniline herbicides. *Exp. Parasitol.* 84, 355–370.
- Traub-Cseko, Y. M., Ramalho-Ortigao, J. M., Dantas, A. P., de Castro, S. L., Barbosa, H. S., and Downing, K. H. (2001). Dinitroaniline herbicides against protozoan parasites: the case of *Trypanosoma cruzi*. *Trends Parasitol.* 17, 136–141.
- Werbovetz, K. A., Brendle, J. J., and Sackett, D. L. (1999). Purification, characterization, and drug susceptibility of tubulin from leishmania. *Mol. Biochem. Parasitol.* 98, 53–65.
- Werbovetz, K. A., Sackett, D. L., Delfin, D., Bhattacharya, G., Salem, M., Obrzut, T., Rattendi, D., and Bacchi, C. (2003). Selective antimicrotubule activity of N1-phenyl-3,5-dinitro-N4,N4-di-n-propylsulfanilamide (GB-II-5) against kinetoplastid parasites. *Mol. Pharmacol.* 64, 1325–1333.
- Yamamoto, E., Zeng, L., and Baird, W. V. (1998). Alpha-tubulin missense mutations correlate with antimicrotubule drug resistance in *Eleusine indica*. *Plant Cell* 10, 297–308.