α-Tubulin Mutations Alter Oryzalin Affinity and Microtubule Assembly Properties To Confer Dinitroaniline Resistance\textsuperscript{\textdagger}†

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An extraordinary number of small molecules target the eukaryotic α-β tubulin dimer. Compounds that shift the normal equilibrium between free dimers and polymers to destabilize or stabilize microtubules are exploited for diverse applications, ranging from cancer chemotherapy to treatment of helminth infections (31, 32). Although many compounds interact with almost all tubulin isotypes, some small molecules are selectively active against phylogenetically restricted subsets of tubulins. For example, helminth and fungal tubulins are selectively sensitive to several benzimidazoles (benomyl, albendazole, and mebendazole) that require the presence of “susceptible” amino acids (E198 and F200) in β-tubulin (33, 36, 37). Dinitroanilines represent another group of selective small molecules. They are synthetic compounds that inhibit microtubules in plants and protozoa but are inactive against the microtubules of vertebrates and fungi (reviewed in references 50, 53, and 70). These molecules (e.g., oryzalin and trifluralin) have been used in commercial herbicide formulations for over 40 years (53). Dinitroaniline binding studies using plant, protozoan, and vertebrate tubulins established that only sensitive tubulins bind dinitroanilines (9, 28, 49, 72). The ability of dinitroanilines to selectively disrupt the microtubules of protozoan parasites without affecting vertebrate microtubules suggests the exciting possibility that we may be able to develop novel antiparasitic agents by understanding the mechanism of action of these compounds on sensitive tubulins.

Resistance to microtubule-disrupting or -stabilizing drugs is often associated with point mutations to α- or β-tubulin that alter polymerization or binding site properties of tubulin heterodimers. Genetic studies of a wide variety of dinitroaniline-sensitive organisms have identified mutations to α-tubulin associated with development of resistance. Studies using the unicellular green alga Chlamydomonas reinhardtii identified the Y24H mutation, and work with the higher land plants Eleusine indica (goosegrass) and Setaria viridis (green foxtail) identified the mutations T239I and M268T (goosegrass) and L136F and T239I (foxtail) (6, 11, 30, 79). Research from our group using the apicomplexan parasite Toxoplasma gondii identified 35 unique α-tubulin point mutations that confer oryzalin resistance (45, 52). The T. gondii mutations include the substitutions L136F and T239I, akin to the plant mutations. We were able to convert sensitive parasites into resistant lines by homologous integration of α-tubulin transgenes bearing individual mutations identified in our screen. Moreover, we were able to confer resistance with α-tubulin transgenes bearing the M268T or F24H (equivalent to Y24H) mutations, which we did not identify in our resistance screen but were associated with dinitroaniline resistance in other organisms (45). This suggests that resistance mechanisms are conserved in plants and protozoa and that there is most likely a common dinitroaniline binding site and mechanism of action.

Computational studies support a model in which the dini-
troaniline binding site is located in the α-tubulin subunit beneath the H1-S2 loop, and compound binding disrupts prototifilament contacts in the microtubule lattice (48, 52). Specifically, studies using flexible dinitroaniline docking to Toxoplasma, Leishmania, Plasmodium, and bovine tubulin structures have identified a consistent binding site on protozoan but not vertebrate α-tubulin. Molecular dynamics simulations suggest that dinitroaniline binding profoundly limits flexibility of the α-tubulin H1-S2 loop, which is drawn toward the core of the protozoan tubulin dimer (48). Based on docking studies and the location of the mutations on the α-tubulin subunit, we developed a model for how these mutations confer dinitroaniline resistance. This model predicts that (i) mutations within or near the binding site reduce tubulin dimer-dinitroaniline ligand affinity and (ii) mutations to the M or H1-S2 loops or to the GTPase-activating domain increase prototifilament affinity within the microtubule lattice to compensate for the destabilizing effect of dinitroanilines (53).

In order to test our model of dinitroaniline resistance mechanisms, we required purified tubulin so that we could measure assembly properties and the dinitroaniline avidities of dimers bearing resistance mutations. Purification of biochemical quantities of tubulin from T. gondii is constrained by the fact that it is an obligate intracellular parasite with a minimal (but critically important) microtubule cytoskeleton. Traditionally, researchers have exploited Escherichia coli expression to produce proteins for biochemical and structural analysis. Tubulin folding requires the TCP-1 chaperones, which are specific to eukaryotes, and to date no one has expressed polymerization-competent α-β dimers in bacteria (67, 77). Although some researchers have used bacterially expressed tubulin monomers for drug binding studies, we strongly believe that such studies must be carried out on polymerization-competent α-β dimers. Simple eukaryotic organisms, such as Saccharomyces cerevisiae and Tetrahymena thermophila, have reduced-copy-number, genetically tractable tubulin genes and provide the opportunity to assess the effects of amino acid changes in the context of genetically homogeneous tubulin (8, 17, 23, 24, 76).

*T. thermophila* is a free-living ciliate that is classified as an alveolate, along with the apicomplexans and dinoflagellates (13, 38). It has a single α-tubulin gene and two identical β-tubulin genes (19, 47) that encode tubulin, which is incorporated into diverse microtubule populations. The primary source of heterogeneity in these ciliates arises from posttranslational modifications (PTMs) to α- and β-tubulins (16, 68). Since *T. thermophila* is amenable to bulk culture, it is genetically tractable, and is related to *T. gondii*, we chose to exploit it to study the effects of dinitroaniline resistance mutations on the properties of protozoan tubulin.

In this study we expressed α-tubulins bearing the L136F or I252L mutation in *T. thermophila* as the sole source of α-tubulin. These mutations correspond to the L136F and V252L mutations in *T. gondii*. The L136F mutation is located in the computationally determined binding site for dinitroanilines, and we predicted that it would reduce affinity for oryzalin. The V252L mutation is located in the GTPase activating domain. It should be noted that the lethal mutation D252A in budding yeast does not correspond to this position, since*S. cerevisiae* has an earlier single amino acid insertion; I/V252 corresponds to yeast tubulin L253, which is located between the essential GAP residues D252 and E255 (2, 60). We hypothesized that the V252L mutation decreases activation of the β-tubulin GTPase, leading to slowed hydrolysis of GTP and consequently a larger GTP cap. However, the data presented here indicate that both the L136F and I252L point mutations alter the properties of tubulin in unanticipated ways to modify dinitroaniline affinity and its critical concentration (*C*ₜₜ).
buffer (PME [0.1 M PIPES (pH 6.9), 1 mM MgCl₂, 1 mM EGTA] plus protease inhibitors [1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 25 μg/ml leupeptin]) on ice and sonicated (Bio-Rad) with 10 30-s bursts at 25 W with a 2-min cooling interval between each burst (61). The sonicated suspensions were cooled on ice for 30 min and then centrifuged at 17,000 × g for 40 min at 4°C. The resulting supernatant was filtered through glass wool, and a peristaltic pump was used to load a 10-ml column of a previously equilibrated mixture (two volumes of PME + P buffer and DEAE-Sepharose Fast Flow matrix [GE Healthcare]) at a rate of 2.5 ml/minute. The column was washed with two column volumes of PME + P buffer followed by four column volumes of PME + P containing 0.1 M KCl and 0.25 M glutamate (pH 6.9). Tubulin was eluted with two column volumes of PME + P buffer containing 0.3 M KCl and 0.75 M glutamate (pH 6.9). Fractions (2.5 ml) were collected after the start of the 0.3 M KCl–0.75 M glutamate elution. Fractions containing tubulins were identified by SDS-PAGE and pooled, and GTP, dimethyl sulfoxide (DMSO), and additional MgCl₂ were added to final concentrations of 10 mM MgCl₂, 8% (vol/vol) DMSO, and 2 mM GTP. The tubulin solution was then incubated at 37°C for 30 min for assembly and then spun at 17,000 × g for 40 min at 4°C. The supernatant containing heterodimeric tubulin was stored at −80°C in 100-μl aliquots. We assessed the quality of tubulin from each preparation by SDS-PAGE. As assessed by Coomassie blue staining, our preparations were consistently free of detectable levels of contaminating proteins.

**Tryptophan-quenching dinitroaniline binding assay and calculation of KＤ values.** Quenching of intrinsic tubulin fluorescence by oryzalin was based on an established method (71, 72). Tubulin samples (0.5 or 1 μM) were mixed with various concentrations of oryzalin as indicated in the figures (0 to 80 μM). Tubulin tryptophan fluorescence was excited at 290 nm, and emission was recorded every 5 nm from 310 to 340 nm. Intensity at 325 nm was taken for calculation of quenching, following correction for a (minor) inner filter effect based on a control experiment with the same drug additions to a solution of 10 μM N-acetyl-tryptophanamide. Fractional quenching of fluorescence was fitted to a single-site binding model using the Prism software from GraphPad.

**Critical concentration assays.** To determine the C₅₀ values for the different tubulins, we carried out assembly assays for each tubulin sample. Tubulin was assembled for 30 min under the assembly conditions described above, in a 100-μl reaction volume. Assembled tubulin was pelleted for 20 min in an airfuge (Beckman) at maximal speed. A 30-μl aliquot from the middle of the tube was removed to measure the free tubulin concentration by bicinchoninic acid (BCA) assay. After the remaining supernatant was removed, the pellet was suspended in 100 μl ice-cold 1× PME and quantified by BCA assay. Polymerized tubulin in the pellet was plotted as a function of the total tubulin in the reaction mixture. Extrapolation of this plot to zero polymer gave the maximum tubulin concentration in the absence of polymer (i.e., the C₅₀).

**RESULTS**

*T. thermophila* has abundant, dinitroaniline-sensitive tubulin. The free-living ciliate *T. thermophila* is an excellent source of abundant dinitroaniline-sensitive tubulin, and we investigated it as a system for expressing functional mutant tubulins for biochemical characterization. Previous work from our group had defined a large number of point mutations in α-tubulin that confer dinitroaniline resistance in *T. gondii*. Since *T. gondii* is an obligate intracellular parasite, it is not a feasible source of material for biochemical studies. However, *T. thermophila* α- and γ-tubulins share 95% identity, which corresponds to 22 amino acid differences between *T. thermophila* Atu1p and *T. gondii* α1-tubulin, 12 of which are conservative substitutions (see Fig. S1 in the supplemental material). Wild-type *T. thermophila*, including the wild-type strain for these studies, is sensitive to dinitroanilines such as oryzalin (18). The wild-type (control) strain used here exclusively expresses a wild-type *T. thermophila* α-tubulin sequence with a carboxy-terminal His6 tag, and growth of this strain is inhibited by increasing concentrations of oryzalin (Fig. 1A). When *T.
from cultures grown in the presence of oryzalin were labeled with an antitubulin antibody, the abundant axonemes that typically cover the cell surface (Fig. 1B) were lost (Fig. 1C). Interestingly, some other highly stable tubulin structures, such as the cortical microtubules, were largely retained. Oryzalin-treated *T. thermophila* cells also appear smaller, perhaps as a function of reduced nutrient uptake due to a reduction or disruption of oral cilia.

**Mutant *T. thermophila α-tubulin genes complement the ATU1 deletion.** The heterokaryon strains AAKO2.7 and AAKO5.5 each contain a deletion of the single α-tubulin gene ATU1 in the “germ line” micronucleus (25, 26). Essential expression of Atu1p α-tubulin is provided by an intact copy of ATU1 in the “somatic” macronucleus. Mating the AAKO2.7 and AAKO5.5 strains triggers destruction of their macronuclei, and reconstituted macronuclei (derived from zygotic micronuclei) lack the ATU1 gene. In the absence of a complementing gene supplied in trans, loss of α-tubulin leads to cell death. After mating the ATU1 knockout heterokaryons, we attempted to rescue lethality by using *T. thermophila* Atu1p α-tubulin transgenes containing H28Q, L136F, R243S, T239I, or I252L mutations. These point mutations correspond to substitutions that confer dinitroaniline resistance in *T. gondii* and exhibit aberrant morphology (Fig. 1). We recovered viable strains expressing the L136F or I252L mutations as the sole source of α-tubulin in *T. thermophila*. Curiously, these residues are located within 5 Å of each other within the tubulin dimer, although only L136 is a proposed binding site residue. The mutant strains showed comparable growth rates to the wild-type strain in the absence of oryzalin (Fig. 2A) and expressed nearly identical levels of tubulin per cell (50.2 ± 1.0 pg of protein; mean ± standard deviation [SD]) (Fig. 2B). Although we did not recover strains with the H28Q, T239I, or R243S mutations, the complementation experiments were only performed once. We do not have any evidence to suggest that tubulin bearing any of these mutations would not be functional in *Toxoplasma*. In fact, since the mutations were originally identified in the sole copy of an essential α-tubulin gene in *Toxoplasma*, they most likely represent viable substitutions in tubulin.

**Mutant α-tubulins alter *T. thermophila growth in oryzalin.** *T. thermophila* strains that express wild-type α-tubulin exhibited growth inhibition in 7.5 to 15 μM oryzalin (Fig. 1A). The L136F and I252L strains were less sensitive to oryzalin, as illustrated by their continued growth in increasing concentrations of this compound. The L136F strain was essentially insensitive to 15 μM oryzalin (Fig. 3A, top). Growth of the I252L strain was somewhat attenuated in 15 μM oryzalin, as the slope of log-phase growth was reduced and the cells appeared to reach stationary phase at a lower cell density (Fig. 3A, bottom). Unfortunately, due to the hydrophobicity of oryzalin and other dinitroanilines, we could not reliably assay the growth of these *T. thermophila* strains with higher compound concentrations, as it was apparent that the dinitroanilines precipitate out of SPP growth medium when present above ~20 μM. When *T. thermophila* grown in 7.5 μM oryzalin was examined by immunofluorescence, cells expressing wild-type tubulin lost most cilia and exhibited aberrant morphology (Fig. 3B). These defects included increased numbers of cells with two micronuclei, shrunken macronuclei, a rounded cell shape, a smaller size, or loss of cilia from the somatic cortex and oral cavity. When *T. thermophila* cells expressing L136F and I252L mutations in α-tubulin were stained after growth in 7.5 μM oryzalin, the cell population was composed of healthy-looking, growing, and ciliated cells (Fig. 3D and E).

**Purification of wild-type and mutant tubulins.** *Tetrahymena* strains expressing wild-type, L136F, and I252L tubulins were grown in bulk culture (~6 liters) for purification of tubulin. After optimizing protocols which exploit DEAE chromatography and cycles of induced polymerization and depolymerization to isolate functional tubulin dimers (61), we obtained highly purified samples that were free of any discernible high-molecular-weight microtubule-associated proteins (MAPs) or other contaminating proteins (Fig. 4A). These samples exhibited GTP-dependent assembly, which could be observed as increased light scattering (increased optical density over time at 350 nm). In order to rule out denatured protein aggregation as a source of increased light scattering, we verified that substitution of GDP for GTP prevented a change in optical den-

![FIG. 2. Complemented *T. thermophila* strains show normal growth and express similar levels of tubulin. (A) *T. thermophila* wild-type (WT) and mutant strains grow with similar kinetics at 30°C in the absence of oryzalin. (B) Immunoblot analysis of cell lysates equivalent to ~9,750 cells per well indicated that the three *T. thermophila* strains (the wild-type complemented strain and strains expressing tubulin with the L136F or V252L point mutation) express essentially equivalent concentrations of tubulin per cell (30 pg). This blot was probed with the anti-α-tubulin monoclonal antibody 1-5-2.](image)
Electron microscopy of negatively stained samples showed that the tubulins form microtubules and a few other polymeric forms in the presence of GTP (Fig. 4C). These samples were used to investigate differences between wild-type tubulin and L136F or I252L mutant tubulins. 

*Thermophila* α-tubulin mutants have reduced oryzalin affinity. The *Tetrahymena* tubulin dimer has three tryptophans within the α-subunit polypeptide and four tryptophans within the β-subunit polypeptide. These confer tubulin in solution with inherent fluorescence emission at 325 nm. In cases where small-molecule ligands act as fluorescence resonance acceptors, tryptophan quenching at 325 nm is a straightforward method to measure compound binding to a protein (12, 71, 72). Oryzalin binding brings this small-molecule ligand into sufficiently close proximity to resonance quench tryptophan emission, and it therefore reduces the absolute fluorescence from the sample in proportion to oryzalin binding. While a vertebrate tubulin control exhibited minimal quenching at high concentrations of oryzalin, wild-type *Thermophila* tubulin showed substantial tryptophan quenching at 1 to 2 μM oryzalin, indicating avid binding. The L136F and I252L *Thermophila* tubulins have dramatically reduced quenching in oryzalin. Tryptophan quenching data can be used to calculate $K_d$ values for the interaction of tubulin with oryzalin (Fig. 5 and Table 1). It should be noted that it is possible to measure quenching at 80 μM oryzalin because of the solubility of the PME buffer used for this assay than in *Tetrahymena* SPP growth medium. The maximal quenching for all *Thermophila* tubulin samples was remarkably similar, indicating that oryzalin bound in the same location to these three samples. Vertebrate tubulin has a much lower affinity for oryzalin, as reflected in a much higher $K_d$, and it also has a different maximal fluorescence quenching from what was observed with *Thermophila* tubulin. These data indicate that wild-type *Thermophila* tubulin has a $K_d$ of 0.44 μM for oryzalin, in contrast to vertebrate tubulin, which has a $K_d$ of 77 μM for oryzalin. Both L136F and I252L mutant tubulins have reduced oryzalin affinities: L136F tubulin has a $K_d$ of 11 μM and I252L tubulin has a $K_d$ of 6.7 μM. 

**L136F tubulin has a decreased critical concentration.** As tubulin heterodimers in solution assemble into microtubules the turbidity increases, which can be observed as a change in the optical density at 351 nm. Samples at a concentration of 7 μM tubulin were assayed for assembly in the presence or absence of 5 μM oryzalin. In this assay, microtubule assembly is defined as an increase in turbidity above 0.02 absorbance units. All three tubulin samples (wild-type, L136F, and I252L) assembled in the absence of oryzalin (Fig. 6A, black traces). However, in 5 μM oryzalin, wild-type *Thermophila* tubulin did not polymerize, although both L136F and I252L mutant tubulins assembled (Fig. 6A, gray traces). Since L136F tubulin appears to avidly assemble over a range of concentrations (~8 to 17.5 μM) (data not shown), we determined the $C_c$ values for...
I252L tubulin. In contrast, L136F tubulin has a decreased 0.09 mg/ml for wild-type tubulin and 0.56/H11006 tubule lattice, permitting assembly at lower tubulin concentration.

tubulins have essentially identical Cc wild-type and mutant T. thermophila tubulins (Fig. 6B and Table 1). The tubulin Cc defines the concentration of tubulin required for equilibrium between microtubule assembly and disassembly. Above the Cc, tubulin will polymerize until the tubulin pool reaches the Cc, and below the Cc, microtubules will depolymerize into free tubulin subunits. A lower Cc value indicates that subunits have greater affinity within the microtubule lattice, permitting assembly at lower tubulin concentrations. Our assays indicated that wild-type and I252L T. thermophila tubulins have essentially identical Cc values: 0.55 ± 0.09 mg/ml for wild-type tubulin and 0.56 ± 0.06 mg/ml for I252L tubulin. In contrast, L136F tubulin has a decreased Cc of 0.25 ± 0.07 mg/ml.

**DISCUSSION**

Although the structure of the tubulin dimer within the microtubule lattice has been determined (20, 21, 42, 44, 54, 55, 57), precise biochemical roles for many amino acids in both α- and β-tubulins remain ill-defined. The situation is complicated by natural sequence variations: there are species-specific differences in tubulins and multiple isotypes of both α- and β-tubulins in multicellular organisms (22, 39–41, 65, 69, 75). Adding to this complexity, directed or selected mutations to associated proteins rather than by directly influencing tubulin biochemistry, it is unlikely that they have an overt effect on the assembly or drug binding properties of purified tubulin.

Although it is impossible to control the tubulin PTMs, it is possible to reduce complexity in tubulin samples by exploiting

![FIG. 4. Purified tubulin is free of discernible MAPs and shows GTP-dependent assembly. (A) An overloaded Coomassie blue-stained SDS-PAGE gel illustrates the purity of DEAE-purified, cycled L136F tubulin used for biochemistry experiments; the absence of contaminating proteins (particularly any high-molecular-weight microtubule-associated proteins) is typical of all our protein purifications. (B) Assembly of I252L tubulin (as well as the wild-type and L136F samples [data not shown]) is dependent upon the presence of GTP (circles). There was no change in optical density when the assembly reaction mixture contained GDP (squares), indicating that the light scattering reflects assembly rather than aggregation of denatured protein. (C) Electron microscopy of negatively stained samples demonstrated that tubulins polymerize to microtubules and other polymeric forms in the presence of GTP. The wild-type tubulin sample is shown, but all tubulins assembled into similar polymers in this assay. Bar, 200 μm.](image)

![FIG. 5. Binding curves for association of tubulin with oryzalin. Oryzalin binding was modeled using a one-site binding, nonlinear fit. Wild-type T. thermophila tubulin, L136F T. thermophila tubulin, and I252L T. thermophila tubulin all have similar maximal quench values, indicating that oryzalin interacts in the same fashion with each of the three tubulins. The binding curve and maximal quenching are different for vertebrate tubulin. These data indicate that the Kd values for oryzalin are 77 μM for vertebrate tubulin and 0.44 μM for wild-type T. thermophila tubulin, which is reduced to 11 μM for L136F T. thermophila tubulin and 6.7 μM for I252L T. thermophila tubulin.](image)

**TABLE 1. Biochemical properties of T. thermophila tubulins**

| α-Tubulin type | Assembly in oryzalin | Oryzalin Kd<sup>a</sup> (μM) | Critical concen<sup>b</sup> (μM) |
|----------------|----------------------|-----------------------------|-------------------------------|
| Wild type      | Sensitive            | 0.44 ± 0.1                 | 5.5 ± 0.9                     |
| L136F          | Resistant            | 11.0 ± 2                   | 2.5 ± 0.7                     |
| I252L          | Resistant            | 6.7 ± 1                    | 5.6 ± 0.6                     |

<sup>a</sup> The standard error associated with a specific Kd value was generated within the analysis program and reflects an estimate of the fit for the data set (each of which was generated from three different samples).

<sup>b</sup> Variations reported for the critical concentrations reflect the standard errors of the means from three experiments.
genetically tractable simple organisms with single genes for α- and/or β-tubulin. These organisms provide the opportunity to prepare monospecific samples to compare the activities of distinct amino acid sequences in tubulin. For example, *S. cerevisiae*, which has two α-tubulin genes, TUB1 and TUB3, and a single β-tubulin gene, TUB2 (63, 64, 66), has been exploited to dissect the roles of isotype differences and binding site residues. Under wild-type transcriptional control, the TUB1 α-tubulin gene, but not the TUB3 α-tubulin gene, is sufficient for survival, although tub3 null yeast cells have increased benomyl sensitivity (63, 64). If TUB3 expression levels are increased, this α-tubulin is sufficient to support growth of a tub1 null (8). When TUB1p and TUB3p dimers are individually isolated from tub3 and tub1 null strains, it is clear that the 39-amino-acid differences make TUB3p-containing microtubules less dynamic than TUB1p-containing microtubules (8). This is consistent with the increased sensitivity of tub3 null strains to benomyl. Researchers have also introduced directed mutations into the sole β-tubulin gene (TUB2) to analyze the requirements for Taxol sensitivity. *S. cerevisiae* is innately insensitive to the microtubule-stabilizing drug Taxol, due both to drug efflux (plasma membrane transporters confer multidrug resistance) and to small differences in β-tubulin residues required for Taxol binding. Introduction of five substitutions to β-tubulin is sufficient to confer Taxol sensitivity (24). When this mutant β-tubulin gene is introduced into a transporter knockout strain, Taxol exposure causes cells to develop long microtubules and arrest during cell division, similar to the effects of Taxol on vertebrate cells (14).

We are interested in understanding the basis of the selective action of dinitroanilines on plant and protozoan tubulin subsets and the influence of resistance mutations on the properties of tubulin. In both plants and protozoa, dinitroaniline resistance is associated with amino acid substitutions to the α-tubulin subunit of the tubulin dimer (3–6, 11, 30, 45, 52, 79). We identified 35 different single point mutations to the α1-tubulin gene that confer dinitroaniline resistance in *T. gondii* (45, 46). In some, but not all, cases the substitution makes the tubulin more like insensitive vertebrate tubulin. For example, although the V252L mutation in *Toxoplasma* converts valine to leucine, the residue typically seen in vertebrate tubulins, the L136F mutation converts a residue that is conserved between *Toxo- plasma* and vertebrate tubulins into an atypical residue at this position. The resistance mutations cluster into distinct domains, which led us to propose that they act by distinct mechanisms: (i) to increase microtubule stability or (ii) to decrease tubulin affinity for dinitroanilines (53). Although in some cases we could observe overt phenotypes associated with the expression of a mutant tubulin in *T. gondii*, such as lengthened, hyperstabilized microtubules associated with the H28Q mutation, in most cases the only detectable phenotypes associated with mutant tubulins in parasites were dinitroaniline resistance
and reduced fitness in the absence of drug selection (45, 46). In order to characterize the biochemical properties of tubulin heterodimers bearing these amino acid substitutions, we needed to exploit a genetically tractable, dinitroaniline-sensitive organism for expression of altered polymerization-competent tubulin.

We previously demonstrated that α-tubulin transgenes bearing M268T or F24H (equivalent to Y24H) mutations, which are associated with dinitroaniline resistance in other organisms, also confer resistance in T. gondii (45). This suggested that substitutions to T. thermophila α-tubulin residues would confer resistance, even in cases where the mutated residue was different between wild-type T. gondii and wild-type T. thermophila tubulin. We engineered an established T. thermophila α-tubulin gene construct to individually contain the mutations H28Q, L136F, R243S, T239I, and I252L. With the exception of residue 252, all sites of mutation occurred in amino acid residues that are conserved between T. thermophila and T. gondii (see Fig. S1 in the supplemental material). However, the residue at position 252 is a valine in T. gondii and an isoleucine in T. thermophila. Although substitution of a leucine for an isoleucine in T. thermophila is a subtler alteration than substitution of a leucine for a valine, the I252L mutation conferred dinitroaniline resistance in T. thermophila.

We determined that the $C_d$ value for wild-type T. thermophila tubulin is 0.56 mg/ml. This is within the range of values that have been observed for tubulins isolated from other eukaryotes. Although tubulins isolated from sea urchin (Strongylocentrotus purpuratus), budding yeast, and the protozoan parasite Leishmania amazonensis have $C_d$ values in the range of 0.1 to 0.2 mg/ml, tubulin from bovine brain has a $C_d$ of 0.56 mg/ml and tubulin from corn (Zea mays) has a $C_d$ of 0.83 mg/ml (8, 10, 27, 34, 35, 71). It is important to note that buffer ionic strength and additives such as Taxol or DMSO influence relative $C_d$ values, so that a close comparison of values from different studies is not informative. However, in this research, we used identical tubulin assembly conditions to measure $C_d$ values for T. thermophila tubulin with L136F or I252L substitutions. These results can be directly compared and indicate that I252L tubulin assembly is essentially identical to that of wild-type T. thermophila tubulin, while L136F tubulin has a dramatically reduced $C_d$, indicating that it assembles with greater avidity.

Previous researchers have used several different techniques to measure the affinity of plant or protozoan tubulin dimers for the dinitroaniline oryzalin. Tubulins isolated from higher land plants have $K_d$ values of 0.095 μM (Zea mays) and 2.59 μM (suspension cultures of rose tissue) (28, 49). Tubulin isolated from L. amazonensis has a $K_d$ of 19 μM (71). However, Leishmania and other kinetoplastid parasites are relatively insensitive to oryzalin. In comparison, they have a $K_d$ value of 1.7 μM for the dinitroaniline analog GB-II-5, which is a much more effective inhibitor of kinetoplastid tubulin. Our measurements indicated that wild-type T. thermophila tubulin has an oryzalin $K_d$ of 0.44 μM. Oryzalin affinity is reduced in the L136F and I252L mutant T. thermophila tubulins, which have $K_d$ values of 11 and 6.7 μM, respectively. These data indicate that single mutations to α-tubulin can dramatically change oryzalin sensitivity, although resistant T. thermophila tubulins are still more sensitive to oryzalin than bovine brain tubulin, which has a $K_d$ of 77 ± 25 μM for oryzalin.

The biochemical properties of wild-type tubulin and tubulins bearing the L136F and I252L substitutions indicate that we cannot precisely anticipate the mechanism of resistance in mutant tubulins by location of point mutations within the structure of the tubulin dimer. We hypothesized that I252L tubulin would produce hyperstabilized microtubules due to reduced activity of the GTPase activating domain. Our experiments indicate that it has a $C_d$ value similar to wild-type tubulin and most likely works by allosterically altering the drug binding site conformation to reduce affinity for dinitroanilines. This mechanism is much more consistent with its ability to confer high levels of oryzalin resistance (~40 μM) in T. gondii (45). We predicted that the L136F mutation would decrease the affinity of tubulin for dinitroanilines. Our binding data indicate that this is the case, but we also discovered an unanticipated reduction in the $C_d$ associated with this substitution, most likely an indication that L136F tubulin also has increased subunit affinity within the microtubule lattice. Previous observations of the H28Q mutation, which is located both in the H1-S2 loop and in the proposed dinitroaniline binding site, had suggested that it is not always possible to assign a single mechanism (dinitroaniline or dimer-dimer affinity) for resistance mutations (45). The observations presented here corroborate this conclusion. In this report we demonstrate that mutations that confer dinitroaniline resistance in T. gondii (L136F and I252L) and green foxtail (L136F) confer resistance in T. thermophila. These data suggest that the drug binding site and mechanism of disruption are conserved in diverse, dinitroaniline-sensitive organisms. These studies are also proof of concept that expression of altered tubulin in T. thermophila is an excellent method for dissecting the precise role of single amino acid changes in tubulin function.

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