Molecular Basis for Fungal Selectivity of Novel Antimitotic Compounds

Thomas Lila,* Thomas E. Renau,† Lori Wilson, Jay Philips,‡ Georges Natsoulis,§
M. Jamie Cope,|| William J. Watkins, and Jerry Buysse||

Essential Therapeutics, Mountain View, California 94043

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Compounds that selectively disrupt fungal mitosis have proven to be effective in controlling agricultural pests, but no specific mitotic inhibitor is available for the treatment of systemic mycoses in mammalian hosts. In an effort to identify novel mitotic inhibitors, we used a cell-based screening strategy that exploited the hypersensitivity of a yeast α -tubulin mutant strain to growth inhibition by antimitotic agents. The compounds identified inhibited yeast nuclear division and included one structural class of compounds shown to be fungus specific. MC-305,904 and structural analogs inhibited fungal cell mitosis and inhibited the *in vitro* polymerization of fungal tubulin but did not block mammalian cell microtubule function or mammalian tubulin polymerization. Extensive analysis of yeast mutations that specifically alter sensitivity to MC-305,904 structural analogs suggested that compounds in the series bind to a site on fungal β -tubulin near amino acid 198. Features of the proposed binding site explain the observed fungal tubulin specificity of the series and are consistent with structure-activity relationships among a library of related compounds.

Cell-based screening strategies have identified numerous classes of compounds that specifically inhibit mammalian cell mitosis (9, 16, 18). The most commonly identified molecular target of these antimitotic agents is the major structural component of the mitotic spindle, tubulin. Tubulin exists as an obligate α/β heterodimer *in vivo*, and antimitotic compounds are known to interfere with dynamic assembly and disassembly of microtubule structures by a variety of mechanisms. Studies using compound cross-linking, competitive binding, protein crystallography, and analysis of compound effects on tubulin polymerization provide good evidence that at least three classes of compounds have distinct binding sites on mammalian β -tubulins: vinca alkaloids, colchicines, and taxanes (3, 17, 20). In general, however, these compounds are inactive against fungal tubulins (4, 5).

Antimitotic agents in the benzimidazole class, in contrast, may show strong specificity for fungal tubulin or may inhibit both fungal and mammalian tubulin polymerization *in vitro* (e.g., carbendazim or nocodazole, respectively [12]). The differing species specificities of known tubulin-targeting agents suggest that despite the high degree of amino acid sequence homology in this protein family, it may be possible to exploit small differences in tubulin structure between mammalian and fungal groups for therapeutic purposes. The widespread use of benzimidazoles in agricultural and veterinary settings demonstrates the effectiveness of inhibiting tubulin function in the control of fungal and nematode pathogens, but no agent in this

class has been developed for use in treating systemic human fungal infections. Many benzimidazole antimitotics suffer from liabilities such as poor aqueous solubility and non-tubulin-related toxicity; identifying novel compounds that target tubulin might provide solutions to these problems.

The paucity of tubulin in fungal cells relative to their mammalian counterparts makes obtaining sufficient protein to perform direct biochemical screens for fungal tubulin disruptors impractical. However, mutations affecting both α -tubulin and β -tubulin genes that hypersensitize mutant cells to tubulin depolymerizing compounds such as benomyl have been described (22). In work presented here, we have exploited the specific hypersensitivity of a *Saccharomyces cerevisiae* α -tubulin mutant to antimitotic agents as a means of screening a library for novel compounds with related modes of action. Using an integrated genetic, medicinal chemistry, and structural modeling platform, we have characterized the structural basis of fungal selectivity for a novel class of tubulin polymerization inhibitors designated the MC-305,904 series.

MATERIALS AND METHODS

Fungal culture and strain construction. Genotypes of fungal strains used in this study are shown in Table 1. Sensitivity testing was performed in 96-well plates in YPD medium (1% Bacto-yeast extract, 2% Bacto Peptone, 2% dextrose). Fifty microliters of a suspension containing 1×10^4 cells/ml was added to 50- μ l volumes of serially diluted compounds, followed by incubation for 40 h at 30°C. Growth was assessed by measuring the optical density of the culture in each well at 600 nm (OD₆₀₀). Yeast transformation and plasmid selection were performed using standard methods (1).

S. cerevisiae strains SC1712 and SC1463 were constructed by targeted gene replacement of the *TUB1* and *ACT1* loci, respectively. For each mutant strain, two PCR products were first generated. *S. cerevisiae* genomic DNA containing the coding sequence of the gene targeted for replacement plus 200 bp downstream of the stop codon of the gene of interest was amplified under mutagenic conditions to generate the first product, and a second product corresponding to the *Kluveromyces lactis* *URA3* coding sequence plus 200 bp of 3' and 5' sequence was also amplified. PCR primers were designed to result in homology of 20 bp between the 3' end of product 1 and the 5' end of product 2 as well as homology of 45 bp between the 3' end of product 2 and the sequence immediately 3' of the

* Corresponding author. Present address: Cytokinetics Inc., 280 E. Grand Ave., South San Francisco, CA 94080. Phone: (650) 624-3270. Fax: (650) 624-3010. E-mail: tlila@cytokinetics.com.

† Present address: Roche Bioscience Neurobiology Unit, Palo Alto, CA 94304.

‡ Present address: Epigenomics, Inc., Seattle, WA 98101.

§ Present address: Iconix Pharmaceuticals, Mountain View, CA 94043.

|| Present address: Symyx Therapeutics, Santa Clara, CA 95051.

TABLE 1. Strains used in this study

Strain ^a	Genotype	Origin (reference)
AF46645	Wild type	46645, American Type Culture Collection
SC5314	Wild type	SC5314 (7)
SC760	<i>MATa ura3-52 lys2 trp1Δ1</i>	F760, Gerald Fink, Whitehead Institute
SC1210	<i>MATα ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1</i>	YP102, Phil Hieter, Univ. British Columbia
SC1463	<i>MATa ura3-52 lys2 trp1Δ1 act1-105::K. lactis URA3</i>	Directed mutagenesis
SC1712	<i>MATa ura3-52 lys2 trp1Δ1 tub1-1712::K. lactis URA3</i>	Directed mutagenesis
SC2164	<i>MATα ura3-52 his4-619 tub2-150 (T238A mutation)</i>	DDY292 (15)
SC2233	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS316-TUB2]</i>	This study
SC2236	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-H6Y]</i>	MC-305,904 selection
SC2237	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-H6Y, G82E]</i>	MC-305,904 selection
SC2238	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-G17S]</i>	MC-305,904 selection
SC2239	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-A19K]</i>	Directed mutagenesis
SC2240	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-G26D]</i>	Directed mutagenesis
SC2241	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-E27D]</i>	MC-305,904 selection
SC2243	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-G132D]</i>	MC-305,904 selection
SC2245	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-F200Y, D161Y]</i>	MC-305,904 selection
SC2247	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-M163I]</i>	Directed mutagenesis
SC2248	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-A165D]</i>	MC-305,904 selection
SC2249	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-A165N]</i>	Directed mutagenesis
SC2250	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-A165N,F200Y]</i>	Directed mutagenesis
SC2251	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-F167Y]</i>	MC-305,904 selection
SC2252	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-D197A]</i>	Directed mutagenesis
SC2253	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-F200Y]</i>	Directed mutagenesis
SC2255	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-L257M]</i>	Directed mutagenesis
SC2256	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-TUB2]</i>	This study
SC2257	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-CaTUB2]</i>	This study
SC2258	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-E198A]</i>	CUY729 (21)
SC2260	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-A165V]</i>	Directed mutagenesis
SC2261	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-E198Q]</i>	Directed mutagenesis
SC2262	<i>MATα ura3 his3Δ1 leu2 lys2 Δtub2::kanR [pRS313-tub2-L253V]</i>	DDY008 (<i>tub2-201</i>), David Drubin, University of California—Berkeley

^a Strain AF46645 is *A. fumigatus*, SC5314 is *C. albicans*, all others are *S. cerevisiae*.

stop codon of the gene targeted for replacement. Wild-type strain SC760 was cotransformed with both PCR products, and *URA*⁺ transformants were selected. These were expected to result from replacement of all or part of the chromosomal coding sequence for the targeted gene along with integration of the *K. lactis URA3* gene at a site 200 bp downstream. Primary isolates were counter-screened for phenotypes of interest (e.g., cold-sensitive [11°C] growth and nuclear division arrest phenotypes in the case of SC1712), and genetic linkage of the phenotypes and the *K. lactis URA3* gene was confirmed by crossing and segregation analysis. SC1712 was selected for compound library screening based on results of sensitivity testing against a panel of antifungal compounds.

A set of isogenic *S. cerevisiae* strains containing mutant *tub2* alleles were constructed by a plasmid shuffle strategy using strain SC2233. This strain carries a chromosomal *tub2* null allele and is viable due to a *URA3*⁺-marked *TUB2*⁺ plasmid (pRS316-*TUB2*). After transformation with pRS313-based (26) *HIS3*⁺-marked plasmids carrying the mutant *tub2* alleles, pRS316-*TUB2*⁺ was counter-selected by replica plating *HIS*⁺ colonies onto agar medium containing 1 mg of 5-fluoroorotic acid (Sigma)/ml, yielding strains in which the mutant *tub2* genes encode the only source of β-tubulin. Molecular cloning was performed using standard methods (24). The *TUB2* gene was amplified from strain SC760 genomic DNA using forward primer T2F (GAC TCA CTA TAG GGC GAA TTG GAG CTC CTC AAA ACT GGT GCA CTT) and reverse primer T2R (GCT TGA TAT CGA ATT CCT GCA GCC CGG GAA TTA CAT ACT TCA GTA GGG AAT). The resultant product was digested with restriction endonucleases *SacI* and *SmaI* and cloned into pRS316 to yield plasmid pRS316-*TUB2*. The *tub2-L253V*, *tub2-T238A*, and *tub2-E198A* mutant alleles were amplified from genomic DNA of the preexisting strains indicated in Table 1 and cloned into pRS313 as above. Site-directed *tub2* mutants were constructed via a two-step PCR process. Divergent overlapping primers encoding the mutant lesion were used in combination with either T2F or T2R to separately amplify 5' and 3' segments of the mutant gene. The two products were then annealed, and the full-length mutant genes were amplified with primers T2F and T2R. Products were cloned as *SacI/SmaI* fragments into pRS313 (*HIS3*⁺), and DNA sequence was verified by the chain termination method (25). Plasmid pRS313-*CaTUB2* was constructed in a similar fashion, except that three overlapping PCR products

were generated: one product consisted of the *S. cerevisiae TUB2* 5' region through the codon for amino acid 30, a second product encoded *Candida albicans TUB2* amino acids 30 to the stop codon, and the third product corresponded to the *S. cerevisiae TUB2* 3' noncoding sequences. The three products were annealed and amplified with T2F and T2R to yield the chimeric *TUB2* gene.

Nuclear division analysis. Cell division cycle arrest in *S. cerevisiae* was evaluated by inoculating 96-well plates containing compound dilutions in YPD medium with a log phase culture strain SC760 to 1×10^5 cells/ml. After 5 h of exposure to compounds, an aliquot was fixed with 3.7% formaldehyde. The remaining culture was grown for 24 h and used to assess the minimum growth inhibitory concentration (MIC) for each compound, defined as 80% reduction in OD₆₀₀ compared to untreated control wells. Fixed samples corresponding to the MIC for each compound were mounted in glycerol containing 1 μg of 4'-6-diamidino-2-phenylindole (DAPI)/ml to stain nuclear DNA. Cell cycle arrest was assessed by scoring the number of large-budded cells (those in which daughter cell diameter is >1/2 of the diameter of the mother cell) in a 100- to 200-cell sample viewed by phase-contrast microscopy. The presence of a single nuclear body in cells of morphologically arrested populations (defined as a population consisting of >2/3 large-budded cells) was confirmed by fluorescence microscopic examination of DAPI-stained DNA. For *C. albicans* strain SC5314, external morphology alone was not an accurate indicator of nuclear division state, so nuclear staining in the large-budded cells was analyzed directly. Samples were prepared as above, except that cells were exposed to compound for 3 to 4 h before fixation. Fifty to 100 large-budded cells (defined as cells in which the daughter cell's longest dimension is >2/3 of the longest dimension of the mother cell) were identified by phase-contrast microscopy, and the number of DAPI-stained nuclear bodies was then scored. Arrested populations were defined as populations having >50% large-budded cells with 1 nucleus, compared with an average of 8% for untreated populations (standard deviation, 4.6% in three trials).

Flow cytometry. For yeast flow cytometry, a log-phase SC760 culture was diluted to an OD₆₀₀ of 0.05 in YPD containing final concentrations of 1 mg of hydroxyurea/ml or 50 μg of nocodazole or MC-05,991/ml. Two-milliliter samples were incubated for 5 h at 26°C and fixed overnight at 4°C in 70% ethanol. More

than 75% of cells in the compound-treated samples had large buds, indicative of nuclear division cycle arrest. Cells were washed in 0.2 M Tris buffer (pH 7.5) and incubated for 1 h in the presence of 1 μ g of RNase A/ml at 37°C. Samples were resuspended in 1 ml of phosphate-buffered saline (PBS) containing 1 μ g of propidium iodide/ml, and nuclear DNA fluorescence was quantitated with a BD Biosciences FACS Vantage SE flow cytometer.

Human cell flow cytometry was performed on ECR293 kidney epithelial cells grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 50 μ g each of penicillin/ml and streptomycin/ml. Semiconfluent plates were exposed to 0.25% trypsin in PBS, and cells were harvested, replated at a 1:8 dilution in fresh medium, and incubated for 16 h at 37°C. Medium was then aspirated and replaced with fresh medium containing 2 μ g of nocodazole/ml or 64 μ g of MC-305,904 analogs/ml. After the times indicated in the legend to Fig. 1, cells were fixed in ice cold 70% ethanol for 1 h, washed in PBS, and resuspended in PBS plus 5 μ g of propidium iodide/ml and 100 μ g of RNase A/ml. Samples were incubated for 1 h at ambient temperature and filtered through a 100- μ m nylon mesh to remove aggregates, and nuclear DNA fluorescence was quantitated with a BD Biosciences FACS Vantage SE flow cytometer.

Isolation of MC-305,904-resistant strains. Strain SC1210 was grown to saturation in YPD medium and diluted to 1×10^7 cells/ml in medium containing 64 μ g of MC-305,904/ml. Separate cultures containing 1×10^8 total cells were incubated for 24 h at 30°C and then diluted 10-fold in YPD medium containing 64 μ g of MC-305,904/ml. After an additional 72 h of incubation, cells were diluted to 1×10^4 cells/ml and sensitivity to MC-305,904 was determined by broth microdilution as above. One-half of the cultures gave rise to isolates capable of growth in 64- μ g/ml MC-305,904. Isolates from different initial cultures were streaked to single colonies and mated to SC1463, and the resultant diploid was sporulated. Segregation (2:2) of the marked *ACT1* gene and the MC-305,904 resistance phenotype among progeny from this cross established close linkage to the *TUB2* locus (1.4 kb separates the *K. lactis URA3* marker from the 5' end of the *TUB2* coding sequence in SC1463). The presence of *tub2* mutations in original MC-305,904-resistant isolates was confirmed by amplifying *TUB2* genes from genomic DNA, cloning into plasmid pRS313, and sequencing as described above. In order to eliminate possible effects of genetic modifiers that could complicate further analysis and comparison (Tables 5 and 6), isogenic *tub2* mutant strains were constructed by introducing plasmid-borne *tub2* alleles into strain SC2233 via a plasmid shuffle strategy as described above.

Tubulin polymerization assays. Bovine brain tubulin (Cytoskeleton, Inc., Denver, Colo.) polymerization assays were performed in 80- μ l volumes in 96-well microtiter plate format. Two milligrams of tubulin/ml was incubated in PEM buffer (100 mM PIPES [pH 6.9], 2 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 10% glycerol) at 35°C. OD₃₅₀ was measured every 30 s for 45 min. Compounds were initially assayed at three concentrations—6 to 12 μ M, 30 to 60 μ M, and 120 to 250 μ M—depending on the molecular weight of the compound. Assays in which compound solubility limitations caused baseline optical density increases of >50% of the polymerization-induced optical density increase of control assays were disregarded. Additional measurements were performed on compounds of particular interest, such as those in Table 4.

S. cerevisiae tubulin was prepared by the method of Davis et al. (6), and polymerization assays were performed at Cytoskeleton Inc. Briefly, 3.5- μ l samples containing approximately 12.5 μ M tubulin in PEM buffer were incubated for 30 min at 30°C, fixed with 1% glutaraldehyde, mounted on Formvar-coated grids (Pella Inc.), and processed for uranyl acetate negative staining. Two fields were photographed for each sample.

Cytotoxicity assays. K562 human chronic myelogenous leukemia cells (American Type Culture Collection no. 243-CCL) were adjusted to 1×10^5 cells/ml in macrophage serum-free medium (Gibco BRL). Ninety-six-well plates containing 200- μ l cell culture plus compound dilutions were incubated for 72 h at 37°C. Cells were then harvested by centrifugation, and 100 μ l of a 1:1 mixture of culture medium and 4 mg of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)/ml in PBS was added to each well. After 4 h at 37°C, 200 μ l of dimethyl sulfoxide was added to each well, and absorbance at 540 nm was measured.

Tubulin structure modeling. As an aid in rationalizing the effects of β -tubulin mutations on MC-305,904 series sensitivity, homology models of the *S. cerevisiae* and *C. albicans* tubulin dimers were built using the structure of bovine brain tubulin as a template. Particular attention was given to the region surrounding amino acid E198. MODELLER (23) was used to build a preliminary model, and further refinement was performed using CHARMm (Accelrys Inc.). The WHATCHECK suite of software (<http://www.sander.embl-heidelberg.de/whatcheck>) was used to check stereochemistry, and models were superposed and compared using tools available in QUANTA (Accelrys Inc.).

Tubulin sequence analysis. Evaluation of β -tubulin amino acid conservation was based on CLUSTALW alignment of sequences from *S. cerevisiae* (GenBank NP_011149), *C. albicans* (translation of Stanford *Candida* genome project contig 6), *Aspergillus fumigatus* (AAL01593), *Aspergillus flavus* (A43794), *Aspergillus nidulans* (1312295B), *Neurospora crassa* (PO5220), *Schizosaccharomyces pombe* (PO5219), *Pneumocystis carinii* (P24637), pig brain (PO2554), human brain (AAC28654), human prostate (Q13509), mouse bone marrow (A25437), mouse brain (D25437), and a ubiquitously expressed mouse isotype (NP_035785).

RESULTS

Identification of compounds having fungal antimitotic activity. A library of 4,464 compounds that inhibit the growth of at least one of four fungal species (*S. cerevisiae*, *C. albicans*, *A. fumigatus*, or *Cryptococcus neoformans*) was screened for fungal antimitotic activity. An *S. cerevisiae* strain that is selectively hypersensitive to the benzimidazole class of tubulin-depolymerizing compounds was used to perform the antimitotic screen. SC1712, which carries a cold-sensitive α -tubulin mutation (*tub1-1712*), was found to be 8-fold and 16-fold supersensitive to benomyl and nocodazole, respectively, but was not hypersensitive to a variety of other antifungal agents.

The SC1712 and the wild-type parent were exposed in parallel to 4, 8, 16, and 32 μ g of each compound screened/ml. Seven compounds (0.16% of the library) inhibited growth of the mutant, but not an isogenic wild-type strain, at two or more of the concentrations tested and were selected for further analysis. One of these seven compounds, MC-291,734, is a benzimidazole carbamate with a structure similar to the known antifungal agent carbendazim (Fig. 1). The other six compounds were not structurally related to known tubulin-binding compounds and thus were considered potentially novel antimitotic agents.

In order to further evaluate the compounds for putative antimitotic activity, their ability to arrest the tubulin-dependent process of nuclear division was examined. Table 2 shows the percentages of cells treated with growth-arresting concentrations of the compounds that accumulate with a single nucleus and large-budded morphology indicative of nuclear division arrest. All seven compounds identified by *tub1-1712* hypersensitivity screening were capable of effectively arresting the nuclear division cycle of the *tub1-1712* strain, while in contrast, 80 control compounds with no known effect on tubulin did not arrest nuclear division. Only one of the compounds identified in the screen, MC-305,904, was able to arrest the cell cycle of the wild-type parent strain. The lack of wild-type cell cycle arrest for the other compounds was attributed to non-tubulin-related modes of action that predominate at the high compound concentrations required to inhibit wild-type growth; poor solubility of the compounds in aqueous media may underlie this phenomenon. Alternatively, some compounds may interact more effectively with the mutant tubulin than with the normal protein.

Several observations served to focus attention on MC-305,904. First, it is a relatively potent inhibitor of yeast cell growth. Second, it can arrest nuclear division of the wild-type strain. Third, the collection of screening hits contains a close structural analog, MC-253,166. Both MC-305,904 and MC-253,166 were poorly soluble in aqueous solution. A number of close structural analogs were synthesized that overcame this liability (e.g., solubility of MC-305,904 was 5 μ g/ml in 50 mM

<i>tub1-1712</i> hypersensitivity screen		Additional MC-305904 analogs	
MC-226,728	MC-253,637	MC-05,905	MC-06,011
MC-239,300	MC-291,734	MC-05,991	MC-06,285
MC-247,136	MC-305,904	MC-06,307	MC-06,341
MC-253,166		MC-06,380	
benzimidazoles			
thiabendazole	benomyl	nocodazole	

FIG. 1. Compounds used in this study.

phosphate buffer at pH 7 compared with 262 $\mu\text{g/ml}$ for MC-06,341) and allowed more accurate measurements in further studies described below.

MC-305,904 series mode of action and fungal tubulin specificity. Compounds that disrupt mammalian tubulin function are typically highly cytotoxic, particularly against sensitive cell lines such as the human lymphoma line K562 (18). Evaluating the fungal specificity of compounds in this study was begun by comparing the concentrations of each compound resulting in fungal growth inhibition, K562 cytotoxicity, and inhibition of bovine brain tubulin polymerization in vitro (Table 3). Two compounds with relatively poor antifungal activity, MC-226,728 and MC-291,734, are highly toxic to the human

cell line ($<0.2 \mu\text{M}$) and are potent inhibitors of mammalian tubulin polymerization ($<10 \mu\text{M}$). In contrast, two members of the MC-305,904 series (MC-305,904 and MC-06,341) were relatively potent in inhibiting fungal growth, were relatively non-cytotoxic toward cultured human cells, and lacked measurable activity against mammalian tubulin in vitro. These compounds were selected for further evaluation. Other compounds, such as MC-239,300, MC-247,136, or MC-253,637, were not studied further due to relatively poor antifungal activity.

A set of experiments further demonstrated the fungal specificity of compounds in the MC-305,904 series and showed that the compounds act by disrupting the polymerization of fungal tubulin. Flow cytometric results displayed in Fig. 2A to D

TABLE 2. *tub1-1712* mutant hypersensitivity and cell cycle arrest of compound-treated *S. cerevisiae*

Compound	MIC ($\mu\text{g/ml}$) against:		Large-budded arrest (%)	
	SC760 (<i>TUB1</i> ⁺)	SC1712 (<i>tub1-1712</i>)	SC760 (<i>TUB1</i> ⁺)	SC1712 (<i>tub1-1712</i>)
No compound			29 (7.5) ^a	33 (7.6) ^a
Cerulenin	1.5	3	24	24
Cycloheximide	0.2	0.2	10	38
Fluconazole	16	16	30	17
Nocodazole	8	0.5	89	97
MC-226,728	256	8	37	90
MC-239,300	256	64	37	97
MC-247,136	64	16	42	91
MC-253,166	256	16	34	94
MC-253,637	256	64	43	96
MC-291,734	>256	16	46	95
MC-305,904	8	0.25	84	92

^a Values in parentheses are standard deviations from three trials.

showed that yeast cells exposed to a soluble MC-305,904 analog were arrested with an increased nuclear DNA content identical to that caused by the benzimidazole class tubulin depolymerizer nocodazole, establishing that the nuclear division arrest occurred during the mitotic phase. Flow cytometric analysis of human cells treated with cytotoxic concentrations of MC-305,904 analogs, including MC-06,341, did not cause increase in nuclear DNA content (Fig. 2E to G), confirming the lack of mammalian tubulin-related activity seen in vitro. Cultured cell toxicity among MC-305,904 analogs varies but not in proportion to antifungal potency.

Tubulin polymerization assays using purified *S. cerevisiae* as well as mammalian tubulin further characterized the fungal specificity of these compounds and established that fungal tubulin is the molecular target for the series. None of the six MC-305,904 analogs tested were effective inhibitors of bovine brain tubulin assembly in vitro, as judged by light-scattering measurements under polymerization-inducing conditions (Table 4). In contrast, the compounds with more potent antifungal activity, namely, MC-06,341 and MC-06,307, completely prevented the in vitro polymerization of purified *S. cerevisiae* tu-

bulin at 50 μM compound concentrations. Overall, the ability of compounds tested to inhibit yeast tubulin polymerization (Table 4) paralleled the compounds' yeast growth-inhibitory potency. Representative results of the electron microscopic assay used are shown in Fig. 3. The lower limit of the MC-06,341 concentration required to inhibit yeast tubulin polymerization in vitro has not been determined, but the data in Table 4 demonstrate greater-than-20-fold-higher selectivity of the compound for yeast tubulin over that for mammalian tubulin.

MC-305,904-resistant mutants. In order to understand the interaction of MC-305,904 series compounds with tubulin, we generated mutant *S. cerevisiae* isolates capable of growth in medium containing 64 μg of MC-305,904/ml. Genetic crosses showed that compound resistance in eight independent isolates was recessive and attributable to a single locus closely linked to the *TUB2* gene. *TUB2* encodes the sole β -tubulin gene in *S. cerevisiae*, and mutations in β -tubulin genes have been previously associated with resistance to benzimidazole class tubulin depolymerizing agents in both fungal and nematode species (see Discussion). We sequenced the *TUB2* genes from each of the resistant isolates and found that each carried one or more distinct point mutations. Each of the sequenced *tub2* mutant alleles was transferred into a naïve genetic background (see Materials and Methods) and was shown to confer MC-305,904 resistance on the resultant strain, confirming the role of the characterized mutations in the compound resistance phenomenon (Tables 1 and 5, MC-305,904 selection). None of the mutant *tub2* genes alters susceptibility to compounds, such as fluconazole, that act by mechanisms unrelated to tubulin. Since the solubility limit of MC-305,904 is near the concentration required to inhibit wild-type cell growth, a concern was that limitations on the maximum achievable compound concentration could limit the ability to distinguish various degrees of compound resistance. Therefore, resistance to soluble analogs, including MC-06,341, was also examined, and differing degrees of resistance for different mutant strains were apparent.

In examining the locations of the affected amino acids with respect to the available crystal structure of bovine brain tubulin (14), one region of the protein in particular prompted further investigation (Fig. 4). The most robust resistance to MC-06,341 was conferred by two mutations affecting closely localized amino acids, A165 and F167. A third MC-305,904 resistance mutation affected both D161 and F200, also in close three-dimensional proximity to A165 and F167. Amino acids at all of these positions are conserved among fungal species, but the counterparts of A165 and F200 are conserved as N and Y, respectively, in known mammalian β -tubulins. The effects of changing these two residues to their mammalian tubulin counterparts on resistance to MC-305,904 analogs were tested. The A165N mutant alone or in combination with F200Y conferred >16-fold-greater resistance to MC-06,341, while an F200Y mutation caused 4-fold-greater resistance (Table 5). An A165V mutation resulted in MC-06,341 resistance nearly equal to that of less conservative perturbations. Thus, primary amino acid sequence differences between fungal and mammalian β -tubulins in the vicinity of A165 are sufficient to explain the fungal specificity of the MC-305,904 series. The specific yet dramatic effects of conservative β -tubulin mutations on MC-305,904 series sensitivity suggest that these mutations may alter a binding site for the compounds. This possibility was further

TABLE 3. Comparative antifungal, cytotoxic, and mammalian tubulin polymerization inhibitory concentrations for *tub1-1712* mutant-hypersensitive compounds

Compound	MIC (μM) against wild- type yeast ^a	Cytotoxicity, as TC ₅₀ (μM) against human K562 cells ^b	IC ₅₀ (μM) against in vitro BBT ^c
MC-226,728	>752	0.13	<7
MC-291,734	>1,000	0.18	<10
MC-305,904	29	67	>100
MC-06,341	39	340	>1,000
MC-239,300	1,312	>34	>50
MC-247,136	355	412	>200
MC-253,637	1,137	>30	>56
Nocodazole	27	≤ 0.3	<2
Colchicine	641	0.01	<5

^a *S. cerevisiae* strain SC760.

^b Concentration resulting in 50% reduction in MTT conversion (see Materials and Methods).

^c Concentration resulting in 50% reduction in bovine brain tubulin polymerization.

FIG. 2. Representative results of flow cytometric analysis of cell ploidy upon exposure to soluble MC-305,904 analogs. (A to D) Propidium iodide staining fluorescence intensity from log-phase *S. cerevisiae* strain F760 in the absence of compound (A) or after 6 h of exposure to growth-inhibitory concentrations of the DNA replication inhibitor hydroxyurea (B), the microtubule polymerization inhibitor nocodazole (C), or the MC-305,904 analog MC-05,991 (D). (E to G) Propidium iodide staining fluorescence intensity from growing populations of ECR293 human kidney epithelial cells in the absence of compound (E) or after 24 h of exposure to cytotoxic concentrations of MC-06,341 (F) or nocodazole (G).

investigated by expanding the analysis of mutations that affect amino acids near A165 as well as mutations that affect other parts of the β -tubulin protein.

A prominent feature of the spatial region surrounding β -tubulin residue A165 is a clustered group of acidic amino acids, including D197 and E198 (Fig. 4). The possibility that D197 and/or E198 plays a role in determining MC-305,904 sensitivity was probed by constructing mutations that affect the two res-

idues. A D197A mutation had little effect on sensitivity to MC-305,904 or MC-06,341, while an E198A mutation caused compound resistance. The importance of the acidic side chain of E198 is further illustrated by the robust MC-305,904 resistance of an E198Q mutant. The acidic side chain of E198 may participate in high energy binding interactions with MC-305,904 and perhaps with other classes of compounds as well (see Discussion). Two additional mutations affecting residues within 8 Å of the E198 carboxyl carbon, namely, M163I and L253V, rendered mutant strains specifically supersensitive to MC-305,904 series compounds. The effects of other β -tubulin mutations are shown in Table 5 and Fig. 4 and are discussed below.

Antifungal spectrum of MC-305,904 analogs. While MC-305,904 lacks whole cell activity against the major fungal pathogens *C. albicans* and *Aspergillus fumigatus*, a subset of (more soluble) analogs bearing a methyl substituent in place of the MC-305,904 phenyl ring trifluoromethyl group (Table 6, inset) does inhibit the growth of one or both pathogens (a detailed account of observed structure-activity relationships in a library of 650 analogs will be the focus of a subsequent report). Table 6 illustrates that among these compounds, substitutions on the C₄ position of the thiazoline ring strongly influence the antifungal species spectrum. A set of MC-305,904 analogs having increasingly large, hydrophobic C₄ substituents trends toward diminishing activity against the yeast pathogen *C. albicans*, while the same compounds trend toward increasing

TABLE 4. MC-305,904 analog inhibition of yeast and mammalian tubulin polymerization

Compound	MIC (μ g/ml) ^a	In vitro tubulin polymerization	
		Mammalian (IC ₅₀ [μ M]) ^b	Yeast ^c
Nocodazole	8	<2	—
MC-06,341	8	>1,000	—
MC-06,307	32	>500	—
MC-05,905	64	>1,000	+/-
MC-06,380	128	>1,000	+
MC-06,285	>256	>1,000	+
MC-06,011	>256	>1,000	+
Colchicine	256	<5	+

^a *S. cerevisiae* strain SC760.

^b Concentration resulting in 50% reduction in bovine brain tubulin polymerization.

^c Electron microscopic tubulin assembly assay in the presence of 50 μ M compound; —, no polymer formation; +, polymer abundance similar to that of the wild type; +/-, intermediate results. See Fig. 3 for representative fields.

FIG. 3. MC-305,904 series inhibition of *S. cerevisiae* tubulin polymerization in vitro. Electron micrographs of 12.5 μ M tubulin assembled in the absence of compound (A) or in the presence of 50 μ M nocodazole (B), MC-06,341 (C), or MC-05,905 (D). Magnification, $\times 11,500$.

activity against the mold pathogen *A. fumigatus*. Differential growth sensitivity assays were used to evaluate whether these opposing trends are due to differences in the tubulin proteins or, alternatively, due to differences in compound access or efflux.

Two *S. cerevisiae* strains were generated—one in which the *TUB2* gene encodes a protein essentially identical to *C. albicans* *TUB2* (SC2257) and one in which the *TUB2* gene was modified to reflect a conserved amino acid sequence difference between yeast and mold species (SC2255). Alignment of predicted β -tubulin protein sequences from the yeasts *S. cerevisiae* and *C. albicans* and the mold species *A. fumigatus*, *A. nidulans*, *A. flavus*, and *N. crassa* was performed, and the positions of amino acid sequence differences between the species were analyzed with respect to the location of the presumed MC-305,904 series binding site near *S. cerevisiae* residue 198. A conserved difference between yeast and mold β -tubulins affects the helix 8 region of the tubulin structure proposed to form one face of the MC-305,904-binding site; leucine 257 of yeast species is replaced by methionine in mold tubulins. No amino acid sequence differences between *S. cerevisiae* and *C. albicans* β -tubulins affect residues in the E198 region. Based on these observations, a *tub2-L257M* mutant strain of *S. cerevisiae* (SC2255) was constructed.

Table 6 compares growth inhibition sensitivities of the *S. cerevisiae* strains bearing the endogenous, *Candida*-like, and *Aspergillus*-like *TUB2* genes when exposed to compounds with

TABLE 5. Relative sensitivities of *tub2* mutant strains to growth inhibition by MC-305,904 analogs

Strain	Mutation(s)	Fold resistance to ^a :		
		MC-305,904	MC-06,341	Fluconazole
SC2236	H6Y	>8	4	1
SC2237	H6Y,G82E	>8	4	1
SC2238	G17S	>8	2	1
SC2239	A19K	1	1	1
SC2240	G26D	1	1	1
SC2241	E27D	>4	2	1
SC2243	G132D	>8	1/2	1
SC2247	M163I	1/8	1/16	1
SC2248	A165D	>8	>16	1
SC2249	A165N	>8	>16	1
SC2250	A165N, F200Y	>8	>16	1
SC2260	A165V	>8	16	1
SC2251	F167Y	>4	16	1
SC2252	D197A	1/2	1	1
SC2258	E198A	>8	>16	1
SC2261	E198Q	>8	>16	1
SC2253	F200Y	>4	4	1
SC2245	F200Y, D161Y	>4	2	1
SC2164	T238A	>8	4	1
SC2262	L253V	1/32	1/32	1
SC2255	L257M	1/2	1/2	1

^a Growth inhibitory concentrations for MC-305,904, MC-06,341, and fluconazole against an isogenic wild-type strain (SC2256) were 32, 16, and 32 μ g/ml, respectively.

various activities toward the wild-type organisms. In all cases, the sensitivities of the wild-type strain (SC2256) and the strain bearing the *Candida*-like *TUB2* gene (SC2257) were identical, as might be predicted based on the identity of amino acids in the MC-305,904-binding pocket. The *Aspergillus*-like *tub2-L257M* mutant strain (SC2255), in contrast, was selectively hypersensitive to compounds that had growth-inhibitory activity against wild-type *A. fumigatus*. The magnitude of *tub2-L257M* hypersensitivity varied independently of wild-type *S. cerevisiae* activity but in approximate proportion to wild-type *A. fumigatus* activity. Hypersensitivity of the *tub2-L257M* mutant to the soluble benzimidazole thiabendazole was also observed. Overall, these results suggest that the potency of analogs toward *C. albicans* is strongly influenced by compound access or efflux issues, while differences in *Aspergillus* potencies more closely reflect differential interactions with an MC-305,904-binding site on the β -tubulin protein.

DISCUSSION

A mutant strain of yeast was used in this study to identify novel inhibitors of fungal mitosis. Important factors in the success of the screening paradigm were the specificity of both the initial screening (*tub1-1712* mutant hypersensitivity) and secondary validation (cell cycle arrest) assays. In a convenient differential growth inhibition screen, the mutant was used to preselect compounds for more cumbersome cell biological validation procedures. In addition, the sensitivity of the *tub1-1712* strain to antimitotic compounds allowed detection of cryptic antimitotic activity that may be obscured by unrelated or non-specific modes of action. This is underscored by data in Table 2 which suggest that six of seven compounds could not have

FIG. 4. Proposed binding site of MC-305,904 series compounds on tubulin based on analysis of compound sensitivity-altering mutations. (A) Homology model of yeast tubulin E198 region. The solvent-exposed side chain of E198 is shown at the center of a hydrophobic pocket in the β -tubulin protein near the α/β interface. Amino acids lining the pocket include residues affected by point mutations conferring resistance to MC-305,904 class compounds (green). Other residues in the region are altered in MC-305,904-supersensitive mutant strains (yellow) or in mutant strains that are differentially responsive to structurally distinct subsets of MC-305,904 analogs (blue). (B) Analogous region of bovine brain tubulin based on the crystal structure of Lowe et al. (14). The hydrophobic pocket present in the yeast tubulin homology model is partly filled by larger, polar side chains of N165 and Y200. Residue numbering reflects β -tubulin primary sequence and differs from coordinates used by Lowe et al. in some instances. See the text and Table 5 for further details.

been identified even if the entire library were assessed for wild-type strain cell cycle arrest.

While the definition of a validated lead compound used here (*tub1-1712* hypersensitivity plus nuclear division arrest) does not necessarily describe only compounds acting directly on the tubulin protein, at least four of the seven compounds identified in the initial screen did target tubulin. Evidence presented shows that two compounds are potent inhibitors of mammalian tubulin polymerization; one of these is of the benzimidazole class. Other compounds include MC-305,904 and MC-253,166,

which are close structural analogs of compounds shown here to inhibit fungal tubulin polymerization. Other compounds identified in the mutant hypersensitivity screen may be weak inhibitors of fungal and mammalian tubulin polymerization, may be fungus-specific inhibitors that do not effectively enter the cell, may act upon tubulin via a mechanism other than inhibition of polymerization, or may affect the function of non-tubulin targets with important roles in the nuclear division cycle.

Evidence suggesting the site of MC-305,904 binding to tubulin. The well-characterized mammalian tubulin-binding

TABLE 6. MC-305,904 analog thiazoline ring substituent effects on activity toward pathogenic fungal species

Cc1ccc(NC2=CNCCS2)cc1 < C4

Compound	C ₄ substituent ^a	MIC (μg/ml) against:			Fold hypersensitivity of mutant:	
		<i>C. albicans</i> SC5314	<i>A. fumigatus</i> AF46645	<i>S. cerevisiae</i> TUB2 ⁺ SC2256	<i>S. cerevisiae</i> CaTUB2 SC2257	<i>S. cerevisiae</i> tub2-L257M SC2255
1	(CH ₃) ₂	64	>128	128	1	1
2	CH ₃	32	>128	8	1	2
3	H	128	128	128	1	4
4	CH ₂ CH ₃	128	32	16	1	8
5	CH ₂ (CH ₃) ₂	>128	8	16	1	32
Thiabendazole	NA ^b	>128	4	128	ND ^c	256

^a Compounds 4 and 5 are racemic mixtures with respect to the C₄ chiral carbon substituent, while compound 2 (MC-06,341) is the R isomer only.
^b Not applicable (benzimidazole).
^c Not determined.

compounds colchicine, taxol, and vinblastine are all thought to interact primarily with sites on the β -tubulin subunit. Yeast strains selected for MC-305,904 resistance all contained mutations in the *TUB2* gene, suggesting that the β -tubulin protein may be the primary molecular target of this compound series as well. Three general aspects of the compound sensitivity studies summarized in Table 5 combine to provide strong evidence that mutations in the region immediately surrounding A165/E198 affect a binding site for MC-305,904 series compounds.

First, some of the most highly compound-resistant strains are associated with point mutations in the E198 region that are likely to have minimal distal effects on tubulin structure. These include mutations affecting E198 itself (E198Q) or nearby amino acids (A165V, F167Y). Second, conserved differences between fungal and mammalian β -tubulins in the A165/E198 region suggest a structural basis for the fungal tubulin selectivity of the MC-305,904 series; compound resistance resulting from A165N and F200Y mutations validates this hypothesis. Comparison of the bovine brain tubulin crystal structure with a model of the A165/E198 region of *S. cerevisiae* β -tubulin reveals that the majority of the mutations affecting MC-305,904 sensitivity lie in or close to a pocket facing the exterior of the microtubule and near the α/β interface. This pocket is roughly the correct size to accommodate a ligand such as MC-305,904 and lies between two subdomains of β -tubulin that have been proposed to undergo a conformational shift upon polymerization (2). In light of this, it seems plausible that a compound binding to this site could interfere with the conformational shift and prevent tubulin from polymerizing. Finally, mutations affecting certain amino acids in the region had differing effects on sensitivity to structurally distinct tubulin depolymerizing agents. These effects are most simply explained as resulting from local perturbation of a compound-binding site, as opposed to general effects on tubulin stability. Mutations of interest include L253V, which confers resistance to benzimidazole antimetabolites (22; data not shown) but up to 32-fold hypersensitivity to MC-305,904 analogs (Table 5), and the A165V mutant, which confers resistance to MC-305,904 series compounds but 8-fold hypersensitivity to benomyl (data not shown). Others have noted similarly opposite sensitivity of an *A. nidulans* strain bearing an A165V mutation toward different structural groups within the benzimidazole class (10). Other mutations discriminated among different structural subsets of the MC-305,904 series. For example, the G132D mutant was mildly supersensitive to analogs having a methyl substituent at the R₂ position of the phenyl ring (e.g., MC-06,341 [Table 5]) but was resistant to compounds having certain other substituents at the same position (e.g., MC-305,904, CF₃ group). Another strain carrying the *tub2*-L257M mutation was constructed based on simple, rational comparison of the proposed yeast and *Aspergillus* binding site amino acid composition and was differentially hypersensitive to compounds in proportion to their *Aspergillus* whole-cell activity. Since the G132D and L257M mutations affect amino acids that lie on opposite sides of the proposed binding pocket and are differentially responsive to structural modifications affecting opposite ends of the MC-305,904 core structure, the behavior of these mutants suggests an orientation of the compounds within the binding site. The

proposed MC-305,904-binding site on β -tubulin is illustrated in Fig. 4.

Other *tub2* mutations examined—H6Y, G17S, E27D, and T238A—affect amino acids located relatively far (>10 Å) from the E198 side chain and thus have effects not easily explained by direct alteration of the proposed binding site. All four mutations conferred relatively mild (two- to fourfold) resistance to soluble MC-305,904 analogs. The amino acids affected by these mutations map to two regions of the bovine brain tubulin crystal structure. Side chains of residues corresponding to E27 and T238 hydrogen bond near the α/β contact region, and H6 and G17 are within van der Waals distance from each other in the interior of the protein. The effects of two additional mutations representing conserved differences between mammalian and fungal tubulins nearest to each of the above mutant pairs were also examined. Unlike analogous mutations in the E198 region, A19K and G26D mutations had no noticeable effect on sensitivity to MC-305,904 analogs (Table 5). Overall, the four mutations affecting amino acids distal to the E198 region exhibited none of the behaviors of mutations near the binding site proposed in Fig. 4. These mutations may exert their relatively mild effects indirectly, perhaps by stabilizing tubulin polymers or altering the MC-305,904-binding site at a distance.

Evidence for similarity in MC-305,904 and benzimidazole-binding sites. In a number of fungal species, the majority of reported benzimidazole-resistant mutants also affect β -tubulins and include mutations similar to those shown here to alter sensitivity to MC-305,904. Previously described mutations affect amino acid residues including H6, A165, F167, E198, and F200 (8, 10, 11, 13). In *S. cerevisiae*, systematic benomyl sensitivity studies implicate a region of the dimer near the α/β interface (8, 13, 21, 22), with the highest levels of resistance resulting from mutations affecting amino acids near β -tubulin E198. The similarities in the set of mutations known to affect sensitivity toward benzimidazoles and MC-305,904 analogs imply that both classes of compounds may interact with tubulin at a similar or overlapping binding site, and indeed, several groups have suggested that benzimidazoles bind to β -tubulin in the vicinity of E198 (10, 13, 19). Both classes of compounds have basic nitrogen-containing regions that could participate in compound binding via a high-energy interaction with acidic residues such as E198. Perhaps more intriguing than the similarity in the MC-305,904 and benzimidazole-binding sites suggested by these studies, however, are the differences implied by some of the mutations that cause opposite effects on sensitivity. A deeper understanding of the structural basis underlying the interactions of compounds with this region of tubulin may aid in the development of improved tubulin polymerization inhibitors capable of exploiting more of the available binding interaction sites.

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