Mechanism of Action of the Benzimidazole Fungicide on *Fusarium graminearum*: Interfering with Polymerization of Monomeric Tubulin But Not Polymerized Microtubule

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ABSTRACT

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Tubulins are the proposed target of clinically relevant anticancer drugs, anthelmintic, and fungicide. β_2 -tubulin of the plant pathogen *Fusarium graminearum* was considered as the target of benzimidazole compounds by homology modeling in our previous work. In this study, α_1 -, α_2 -, and β_2 -tubulin of *F. graminearum* were produced in *Escherichia coli*. Three benzimidazole compounds (carbendazim, benomyl, and thiabendazole) interacted with the recombinant β_2 -tubulin and reduced the maximum fluorescence intensity of 2 μ M β_2 -tubulin 47, 50, and 25%, respectively, at saturation of compound-tubulin complexes. Furthermore, carbendazim

significantly inhibited the polymerization of α_1 -/ β_2 -tubulins and α_2 -/ β_2 -tubulins 90.9 ± 0.4 and $93.5 \pm 0.05\%$, respectively, in vitro. A similar result appeared with benomyl on the polymerization of α_1 -/ β_2 -tubulins and α_2 -/ β_2 -tubulins at $89.9 \pm 0.1\%$ and $92.6 \pm 1.2\%$ inhibition ratios, respectively. In addition, thiabendazole inhibited $81.6 \pm 1\%$ polymerization of α_1 -/ β_2 -tubulins, whereas it had less effect on α_2 -/ β_2 -tubulin polymerization, with $20.1 \pm 1.9\%$ inhibition ratio. However, the three compounds cannot destabilize the polymerized microtubule. To illuminate the issue, mapping the carbendazim binding sites and β/α subunit interface on β/α -tubulin complexes by homology modeling showed that the two domains were closed to each other. Understanding the nature of the interaction between benzimidazole compounds and *F. graminearum* tubulin is fundamental for the development of tubulin-specific anti-*F. graminearum* compounds.

Benzimidazole derivatives are of wide interest because of their diverse biological activity and applications; they are remarkably effective compounds with respect to both their inhibitory activity and their favorable selectivity ratio (Ansari and Lal 2009; Hosamani and Shingalapuri 2011; Navarrete-Vázquez et al. 2006). The benzimidazole ring shared by benzimidazole compounds displays an important heterocyclic pharmacophore in drug discovery. These compounds, carrying different substituents in the benzimidazole structure, are associated with a wide range of biological activities, including anticancer, antiviral, antibacterial, antifungal, antihelminthic, anti-inflammatory, antihistaminic, proton pump inhibitor, antioxidant, antihypertensive, and anticoagulant properties (Tunçbilek et al. 2009). Initial studies on the mechanism of action of the benzimidazoles focused on DNA and RNA synthesis. It soon became clear that the observed inhibition of DNA synthesis was a secondary effect, and that blockage of nuclear division was primarily responsible for this effect (Clemons and Sisler 1971; Davidse 1973; Hammerschlag and Sisler 1973). In arresting nuclear division of fungi, the benzimidazoles show a striking resemblance to the secondary plant metabolite colchicine, which disrupts mitosis and meiosis in animal and plant cells by inactivating the spindle. Biochemical studies on the mechanism of action of benzimidazole compounds have shown that the antimetabolic action of MBC in fungi is probably mediated via binding to fungal tubulin (Davidse 1975). In addition, more evidence of

the mechanism of action of the benzimidazoles on the fungi was derived from latter biochemical studies (Davidse 1987).

Benzimidazole derivatives play an important role in controlling various fungal pathogens. The most popular fungicide is carbendazim (Fig. 1). The fungicide is used to control plant diseases in cereal and fruit. Binding experiments using [14 C] carbendazim and crude mycelial extract of three strains of *Aspergillus nidulans* indicated that binding to a cellular protein, with characteristics typical for tubulin, was involved in the mechanism of action of benzimidazole fungicides (Davidse and Flach 1977). Competitive inhibition of [14 C] carbendazim binding by colchicine and nocodazole supported the idea that the carbendazim binding protein was fungal tubulin. Colchicine and nocodazole effectively inhibit microtubule assembly of tubulin from mammalian sources, whereas carbendazim only slightly affects this process (Friedman and Platzer 1978; Hoebeke et al. 1976). Assembly of yeast tubulin, however, is sensitive to both nocodazole and carbendazim. These in vitro studies strongly support the idea that the cytological effects of benzimidazoles in fungi are caused by an interference with the normal functioning of microtubules because benzimidazoles bind to tubulin. Interference with microtubule assembly in vivo has been convincingly demonstrated by Howard and Aist (1977, 1980), who studied the various effects of carbendazim on hyphal tip cells of *Fusarium acuminatum* with light and electron microscopy.

F. graminearum is a filamentous fungus that causes the serious infectious disease Fusarium head blight (FHB) on wheat, maize, and barley, and can cause severe yield and economic losses in these crops worldwide (Goswami and Kistler 2004). Benzimidazoles, including carbendazim, are a group of broad-spectrum, systemic fungicides which have been used to control FHB by interacting with β -tubulin and stopping hyphal growth (Hollomon et al. 1998).

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In recent studies from the authors' laboratory (Chen et al. 2008, 2009), point mutations in the β_2 -tubulin gene at residues 167 (Phe to Tyr), 198 (Glu to Lys), and 200 (Phe to Tyr) were detected in benzimidazole-resistant field strains of *F. graminearum*. In addition, these sites constituted a hydrophobic pocket in β_2 -tubulin by homology modeling, which identified the possible binding domain for benzimidazoles (Qiu et al. 2011). In addition, benomyl (Delp and Klopping 1968) (conversion products carbendazim and butyl iso-cyanate) and thiabendazole (Staron et al. 1964) were introduced as fungicides. Thiabendazole was also applied as anthelmintic (Brown et al. 1961). Both benomyl and thiabendazole belong to benzimidazole compounds.

Although the mechanisms of action of benzimidazoles on the fungi, parasites, and mammal cells have been extensively studied, most of the research was derived at the biochemical level but not the molecular level. The potency with which presumed antimicrotubule drugs inhibit microtubule assembly can be determined by in vitro assays using partially purified tubulin. In initial studies, only mammalian brain cells could be used as the tubulin source because of the high concentration of tubulin needed. The tubulin content was as high as 30% in porcine brain cells (Castoldi and Popov 2003). In contrast, the content of tubulin in fungal cells was below 1%; a yield of tubulin at 0.5 mg/liter was purified by overexpression of tubulin in *A. nidulans*, which was the first report of the purification of assembly-competent tubulin from a filamentous fungus (Yoon and Oakley 1995). Recently, one-step purification of assembly-competent tubulin from diverse eukaryotic sources was developed but purified tubulin was 0.1% of the total protein in yeast extract (Widlund et al. 2012). Furthermore, expression and purification of tubulin fused with maltose-binding protein in *Escherichia coli* have been reported but the recombinant tubulin cannot be polymerized in vitro (Hollomon et al. 1998). The purpose of this study was to overcome the difficulties in preparing sufficient quantities of fungal tubulin by purification of recombinant α - and β_2 -tubulins of *F. graminearum*. Furthermore, effects of carbendazim, benomyl, and thiabendazole on the polymerization of *F. graminearum* tubulins were investigated using turbidity and homology modeling, providing more information of the mechanism of action of benzimidazoles. This work will promote structural and functional research into fungal tubulin. In future, the *F. graminearum* tubulins will be used as a tool in screening of antimicrobial compounds.

MATERIALS AND METHODS

Strains and plasmids. *F. graminearum* strain 2021 (Chen et al. 2007), which was isolated from wheat in Zhejiang Province and sensitive to carbendazim, reserved in our lab, was used as the original strain to amplify the α -tubulin genes. Plasmid *pET30a*⁺ was purchased from Novagen (Merck). *E. coli* strain DH5a was used as the recipient for subcloning, and strain BL21 (DE3) pLysS was used for protein expression.

Fig. 1. Structures of benzimidazole compounds of carbendazim, benomyl, and thiabendazole.

Construction of expression plasmids. For preparation of RNA from vegetative hyphae, each strain was grown in liquid potato sucrose (Dingguo) for 36 h. Total RNA was extracted using Trizol REAGENT (Invitrogen) following the manufacturer's protocol and was quantified by spectrophotometry at 260/280 nm. The RNA was then used as the template for reverse-transcription polymerase chain reaction (RT-PCR). The α_1 -tubulin (FGSG_00639) and α_2 -tubulin (FGSG_00397) genes were amplified by PCR using the cDNA of *F. graminearum*. The primers (Table 1) were α_1 -30F: 5'-GCACATATGCGTGAGGTCATTAG-3' (the recognition site of *NdeI* is underlined); α_1 -30R: 5'-GTAGAATTCCCGTACTCAGCCTCCAA-3' (the recognition site of *EcoRI* is underlined); α_2 -30F: 5'-CGCGGATCCATGAAGGGCGAGATTC-3' (the recognition site of *BamHI* is underlined); and α_2 -30R: 5'-CATGCGGCCCGCC TAGTACTCGAGTTCTTCT-3' (the recognition site of *NoI* is underlined). The primers used to amplify the β_2 -tubulin gene (FGSG_06611.3) were β_2 -30F: 5'-CGTCATATGCGTGAGATTG-3' (the recognition site of *NdeI* is underlined) and β_2 -30R: 5'-TACCATAAGCTTTCCACCC-3' (the recognition site of *HindIII* is underlined).

The PCR products were subcloned into the expression vector *pET30a*⁺ to obtain expression constructs *pET30a*- α_1 tubulin, *pET30a*- α_2 tubulin, and *pET30a*- β_2 tubulin. The recombinant expression plasmids were confirmed by restriction enzyme analysis and sequence.

Expression, refolding, and purification of recombinant *F. graminearum* α_1 -, α_2 -, and β_2 -tubulins. *E. coli* strain BL21 (DE3) pLysS was used as a host for expression of the target genes. The transformation of *pET30a*- α_1 -, α_2 -, and β_2 -tubulin into *E. coli* was performed according to the manufacturer's instructions (Merck). The induction procedure for gene expression was as follows: 2 ml of Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of H₂O) containing kanamycin at 50 μ g ml⁻¹ and chloramphenicol at 34 μ g ml⁻¹ was inoculated with a bacterial colony (BL21 [DE3] pLysS strain transformed with expression constructs) and incubated for 14 h at 225 rpm and 37°C. A 1-ml aliquot of the overnight culture was transferred into a flask containing 50 ml of LB medium with the same antibiotic, and the flask was agitated at 225 rpm at 37°C until the culture reached an optical density at 600 nm (OD₆₀₀) = 0.4 to 0.6. Isopropyl β -D-thiogalactoside was added to a final concentration of 1 mmol liter⁻¹, and the culture was incubated at 225 rpm and 37°C for 6 h. After incubation, the bacterial cells were harvested by centrifugation at 6,000 rpm for 10 min at 4°C and frozen at -80°C. A noninduced culture was used as a negative control.

E. coli cells overexpressing recombinant *F. graminearum* tubulins were resuspended in lysing buffer composed of 20 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl, and 1% (vol/vol) Triton X-100 and disrupted by ultrasonication. Cell-free extract was centrifuged at 12,000 $\times$ g for 20 min at 4°C and the pellets (inclusion bodies) were refolded, as previously reported (Jang et al. 2008). The pellets obtained after sonication was dissolved in binding buffer (6 M guanidine hydrochloride, 20 mM Tris, 300 mM NaCl, 20 mM imidazole, and 1 mM β -mercaptoethanol, pH 8.0) and the supernatant was collected by centrifugation at 12,000 $\times$ g for 10 min at 4°C. Supernatant containing tubulin was loaded onto a Ni-charged sepharose column preequilibrated with the binding buffer. The column was washed with binding buffer to base line, then with the

TABLE 1. Polymerase chain reaction primers used in this study^a

Primer	Sequence (5' to 3')	Recognition site
α_1 -30F	GCACATATGCGTGAGGTCATTAG	<i>NdeI</i>
α_1 -30R	GTAGAATTCCCGTACTCAGCCTCCAA	<i>EcoRI</i>
α_2 -30F	CGCGGATCCATGAAGGGCGAGATTC	<i>BamHI</i>
α_2 -30R	CATGCGGCCCGCTAGTACTCGAGTTCTCT	<i>NoI</i>
β_2 -30F	CGTCATATGCGTGAGATTG	<i>NdeI</i>
β_2 -30R	TACCATAAGCTTTCCACCC	<i>HindIII</i>

^a Recognition sites are underlined.

washing buffer (6 M urea, 20 mM Tris, 300 mM NaCl, 40 mM imidazole, and 1 mM β -mercaptoethanol, pH 8.0). The bound protein was refolded on-column using a decreasing gradient of urea (6 to 0 M), starting with the washing buffer and finishing with the refolding buffer (20 mM Tris, 300 mM NaCl, 20 mM imidazole, and 1 mM β -mercaptoethanol, pH 8.0). The refolded protein was eluted using elution buffer (20 mM Tris, 300 mM NaCl, 0.5 M imidazole, and 1 mM β -mercaptoethanol, pH 8.0). Fractions containing the target protein were dialyzed against polymerization buffer (0.1 mM Pipes [pH 6.8], 0.1 mM NaOH, 1 mM EGTA, 1 mM MgSO_4 , and 5% glycerol), concentrated, quantified using the Bradford method (Bradford 1976), and stored at -80°C . The purity of tubulin was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and the integrity of tubulin was confirmed by Western blot using an anti-His antibody (Zen BioScience), as previously reported (Jang et al. 2008).

Drug-tubulin binding assay. The interaction of each test compound (carbendazim, benomyl, and thiabendazole) with recombinant *F. graminearum* β_2 -tubulin were measured as the amount of reduction in the intrinsic fluorescence of tryptophan residues following incubation with each compound (Eftink and Ghiron 1981). Equilibrium binding was determined by measuring the reduction in fluorescence intensity of recombinant *F. graminearum* β_2 -tubulin following incubation with a range of concentrations of each compound. Each concentration of compound (2, 8, 20, and 40 μM) was incubated with 2 μM tubulin for 30 min at 37°C and fluorescence was immediately measured with excitation of 280 nm and emission of 340 nm (Giles et al. 2009; MacDonald et al. 2004). Emission spectra were corrected for inner filter effects due to drug addition according to previously published equation (Mertens and Kägi 1979) $F(\text{corr}) = F(\text{obs}) \times \text{antilog}[(A_x + A_m)/2]$, where $F(\text{corr})$ is the corrected fluorescence intensity, $F(\text{obs})$ is the observed intensity, and A_x and A_m are the measured absorbance at the wavelengths of excitation and emission, respectively. The corrected fluorescence

intensity values from three independent tests were then used to determine the percent reduction of fluorescence intensity.

Tubulin polymerization in vitro. Tubulin polymerizations were assayed at A_{350} using a Molecular Devices VersaMax microplate reader equipped with temperature controllers. The tubulin monomers were polymerized using a standard polymerization assay, as previously reported (Dong et al. 2004; Jang et al. 2008; Koo et al. 2009b).

The reaction mixture included 4 μM β_2 -tubulin plus 4 μM α_1 - or α_2 -tubulin isotypes mixed with Pipes buffer to give a final concentration of 2 μM for each tubulin isotype. Then, guanosine-5'-triphosphate, disodium salt (GTP) (Dingguo) was added to a final concentration 2 mM in the total volume (Oxberry et al. 2001). OD value was measured at 350 nm at 37°C , every 5 min for 1 h (Koo et al. 2009a, 2010). At this wavelength (350 nm), scattering of light produced during the polymerization process causes an increase in absorbance proportional to the formation of tubulin aggregates.

Using this polymerization procedure, the effects of carbendazim, benomyl, and thiabendazole at a final concentration of 20 μM (which had been dissolved in dimethyl sulfoxide) on α - and β_2 -tubulin polymerization were assessed. Compounds were added to the polymerization mixture before and after incubation at 37°C for 1 h to investigate the effect of compounds on monomeric and polymerized tubulins, respectively. The degree of polymerization was assessed by subtracting the initial absorbance reading from the final reading obtained after 1 h. The inhibition ratios of benzimidazole compounds on polymerization were calculated using the formula inhibition ratio = $(Pa - Pb/Pa) \times 100$, where Pa was the polymerization degree of α - plus β_2 -tubulins without adding benzimidazole compounds and Pb was the polymerization degree after adding the compounds. All tests were repeated three times. The average inhibition ratios of benzimidazole compounds on polymerization are the means of the three experiments, and significant differences among the experiments were calculated using Fisher's least significant difference test.

Fig. 2. Production of recombinant α_1 -, α_2 -, and β_2 -tubulins in *Escherichia coli*. **A**, Lane 1: total protein of BL21 (DE3) pLysS transformed with *pET30a+*. Lanes 2 to 5: samples of α_1 -tubulin; lanes 2 and 3: total protein without and with isopropyl β -D-thiogalactoside (IPTG) induction, respectively; lanes 4 and 5: supernatant and precipitation fraction after induction, respectively. Lanes 6 to 9: samples of α_2 -tubulin; lanes 6 and 7: total protein without and with IPTG induction, respectively; lanes 8 and 9: supernatant and precipitation fraction after induction, respectively. **B**, Lane 1: total protein of BL21 (DE3) pLysS transformed with *pET30a+*. Lanes 2 to 5: samples of β_2 -tubulin; lanes 2 and 3: total protein without and with IPTG induction, respectively; lanes 4 and 5: supernatant and precipitation fraction after induction, respectively. **C**, Lanes 1 to 3: purified α_1 -, α_2 -, and β_2 -tubulins, respectively. **D**, Lanes 1 to 3: Western blot analysis of α_1 -, α_2 -, and β_2 -tubulins, respectively, using an anti-His antibody; lane M: protein marker. Arrowheads indicate expected or observed positions of respective protein bands.

Homology modeling of *F. graminearum* α - and β -tubulins. The homology model of *F. graminearum* α - and β -tubulins was constructed using the online server at I-TASSER (Roy et al. 2010; Zhang 2008). Visualization and display of the amino acid residues on the homology structure were carried out using the PyMOL Molecular Graphics System (version 1.5.0.4; Schrödinger, LLC).

RESULTS

Expression, refolding, and purification of recombinant *F. graminearum* tubulins. The α_1 -, α_2 -, and β_2 -tubulins were inserted into expression vector *pET30a+* and confirmed by double digestion reaction and sequencing (data not shown). All the constructs were transformed into *E. coli* strain BL21 (DE3) pLysS for protein expression. All the recombinant tubulins (α_1 -, α_2 -, and β_2 -tubulins) were expressed as inclusion bodies (Fig. 2A and B) and refolded on the columns (see Materials and Methods). The yields of recombinant α_1 -, α_2 -, and β_2 -tubulins were approximately 6, 5, and 3 mg/liter, respectively. In addition, the purified recombinant α_1 -, α_2 -, and β_2 -tubulins were above 90% pure (Fig. 2C). Western blot was performed on purified fusion-protein detected bands of the expected sizes for each recombinant protein, and indicated that the structural integrity of the tubulin fusion proteins was preserved throughout the expression and purification processes (Fig. 2D).

Fig. 3. Equilibrium binding of test compounds with recombinant *Fusarium graminearum* β_2 -tubulin; the graph illustrates the proportion of tubulin fluorescence that was quenched following the addition of increasing concentrations of carbendazim, benomyl, and thiabendazole. Line bars in each point denote standard errors of three experiments.

Drug-tubulin binding assay. All three of the benzimidazole compounds interacted with recombinant *F. graminearum* β_2 -tubulin and quenched fluorescence in a concentration-dependent manner (Fig. 3). Saturation of compound-tubulin complexes was achieved at concentrations of approximately 20 μ M, as demonstrated by the plateau in the curves. A return in fluorescence occurred following saturation point due to the high concentrations of compounds exhibiting strong intrinsic fluorescence or conformational changes occurring in β_2 -tubulin, causing dissociation of compounds. Carbendazim and benomyl at 20 μ M induced high reductions in fluorescence intensity of β_2 -tubulin (47 and 50%, respectively). In contrast, thiabendazole induced lower reductions in fluorescence intensity of β_2 -tubulin (25%).

Effects of carbendazim on *F. graminearum* tubulin polymerization in vitro. *F. graminearum* β_2 -tubulin was proposed as the target for benzimidazole fungicides but how the drug interferes with tubulin polymerization was not clear. Each tubulin subunit (2 μ M α_1 / β_2 -tubulin and 2 μ M α_2 / β_2 -tubulin) was polymerized in the presence of 2 mM GTP for 1 h at 37°C (Fig. 4). In addition, increasing A_{350} of α_1 / β_2 -tubulin was 0.14 while that of α_2 / β_2 -tubulin was 0.26, which indicated that β_2 -tubulin preferred binding to α_2 -tubulin rather than α_1 -tubulin.

To investigate the effects of carbendazim on tubulin polymerization in vitro, 20 μ M carbendazim was added to the polymerization mixture containing α - plus β_2 -tubulins, and the A_{350} was measured after adding 2 mM GTP to initiate the polymerization. The polymerizations for α_1 / β_2 -tubulins and α_2 / β_2 -tubulins both were significantly suppressed by the carbendazim at 91 and 93%, respectively (Fig. 4A and B). However, almost no depolymerization was observed when α - plus β_2 -tubulins (α_1 / β_2 -tubulins and α_2 / β_2 -tubulins) were polymerized for 1 h at 37°C followed by adding 20 μ M carbendazim (2 and 5% depolymerization ratios of α_1 / β_2 -tubulins and α_2 / β_2 -tubulins, respectively), which indicated that the polymerized tubulins were not affected by the carbendazim (Fig. 4A and B). In addition, in the absence of carbendazim, increasing A_{350} of α_1 / β_2 -tubulin was 0.14 while that of α_2 / β_2 -tubulin was 0.26, which indicated that β_2 -tubulin preferred binding to α_2 -tubulin rather than α_1 -tubulin.

Inhibitions of benzimidazole compounds on *F. graminearum* tubulin polymerization in vitro. To study whether some other benzimidazole compounds (benomyl and thiabendazole) had a similar mechanism of action on the *F. graminearum* tubulin polymerization, benomyl and thiabendazole were tested. Similarly to carbendazim, benomyl and thiabendazole did not have the ability to depolymerize polymerized tubulins (α_1 / β_2 -tubulins and α_2 / β_2 -tubulins) in vitro (data not shown). Furthermore, 20 μ M carbendazim could cause a significant decrease in the amount of α_1 / β_2 -tubulin and α_2 / β_2 -tubulin polymerization mixtures (90.9 ± 0.4 and $93.5 \pm 0.05\%$ inhibition ratios, respectively) (Table 2). The similar result also appeared with

Fig. 4. Effects of carbendazim on polymerization of A, α_1 / β_2 -tubulin and B, α_2 / β_2 -tubulin. Symbols: ♦ = no carbendazim in polymerization buffer, ▲ = adding 20 μ M carbendazim after polymerization at 37°C for 60 min, and ■ = adding 20 μ M carbendazim before polymerization at 37°C. Absorbance at 350 nm was determined for 60 min. Points = means of triplicate measurements at each time point.

the adding of 20 μM benomyl in the α_1/β_2 -tubulin and α_2/β_2 -tubulin polymerization mixtures (89.9 ± 0.1 and $92.6 \pm 1.2\%$ inhibition ratios, respectively) (Table 2). In addition, thiabendazole inhibited the polymerization of α_1/β_2 -tubulin polymerization greatly ($81.6 \pm 1\%$ inhibition ratio), whereas it had less effect on α_1/β_2 -tubulin polymerization ($20.1 \pm 1.9\%$ inhibition ratio) (Table 2). Thiabendazole had lower inhibition ratios of α_2/β_2 -tubulin polymerization than carbendazim and benomyl. In addition, the concentration resulting in 50% inhibition of mycelial growth (EC_{50}) of the three compounds for *F. graminearum* were 2.46, 2.10, and 5.61 μM , respectively, which were converted from published data of EC_{50} ($\mu\text{g ml}^{-1}$) dividing by each molecular weight. The inhibition ratio and EC_{50} (μM) of the three compounds ranked carbendazim $\approx$ benomyl < thiabendazole. This result indicated that EC_{50} ($\mu\text{g ml}^{-1}$) of the three compounds was negatively correlated with the inhibition ratios of *F. graminearum* α - plus β_2 -tubulin polymerizations. Furthermore, these results suggest that the interference of the three compounds with tubulin polymerization was the main mechanism of action.

Mapping the carbendazim binding sites and β_2/α sub-unit interface on β_2/α -tubulin complexes. To illuminate why the benzimidazole compounds inhibit polymerization of the monomeric tubulins but not the polymerized microtubule, we

built two three-dimensional homology models of *F. graminearum* α_1 -/ β_2 -tubulin and α_2 -/ β_2 -tubulin protein, respectively, using an online platform for protein structure and function predictions, I-TASSER, based on multiple threading alignments. Superposition of homology structures of *F. graminearum* α_1 -/ β_2 -tubulin (yellow/red) dimer and α_2 -/ β_2 -tubulin (yellow/red) dimer on the crystal structure of *Saccharomyces cerevisiae* α -1-/ β -tubulin (blue/green) dimer (PDB accession 4FFB), respectively, showed a remarkable similarity of overall structure-folding between the two structures (Fig. 5A and B). Carbendazim binding pocket (cyan), consisting of amino acid positions 165, 167, 198, and 200, and the β/α -tubulin interface (blue), consisting of amino acid positions 2, 131, 245 to 247, 251, 256, 258 to 260, 322 to 324, 327, and 347 to 351, were shown on *F. graminearum* β_2 -tubulin (Fig. 5C and D).

DISCUSSION

Microtubules are built from $\alpha\beta$ -tubulin heterodimers, which are targets of many antitumor drugs, anthelmintics, and fungicides. Attempts to understand the molecular basis of mechanism of benzimidazole reagents, related to β_2 -tubulin in *F. graminearum*, have been hampered by the inability to purify tubulin from filamentous fungi on a large scale. In other ways, the resulting preparations (tubulins) from natural tissues were contaminated by microtubule-associated proteins and microtubule motor proteins; in addition, it was difficult to separate different tubulin subtypes due to their similar physico-chemical properties. In the present study, by using the *E. coli* expression system, we were able to produce enough *F. graminearum* α_1 -, α_2 -, and β_2 -tubulins (recombinant tubulins at 6, 5, and 3 mg/liter, respectively) with high purity (>90%) and free of microtubule-associated proteins. In contrast, the best yield of tubulin purified from fungi in the literature was <1.0 mg/liter (Yoon and Oakley 1995). In our study, the recombinant tubulins mainly formed inclusion bodies which were consistent with previous reports of tubulins from other species expressed in *E. coli* (Blackhall et al. 2006; Koo et al. 2009a; Lubega et al. 1993). The recombinant tubulins of *F. graminearum* were refolded in column and demonstrated to have the biological function of polymerizing into a

TABLE 2. Effect of benzimidazole compounds on the *Fusarium graminearum* and tubulin polymerization in vitro^a

Benzimidazole compounds	Inhibition ratio of α_1 - and β_2 -tubulin polymerization (%)	Inhibition ratio of α_2 - and β_2 -tubulin polymerization (%)	EC_{50} (μM)
Carbendazim	90.9 ± 0.4	93.5 ± 0.05	2.46
Benomyl	89.9 ± 0.1	92.6 ± 1.2	2.10
Thiabendazole	81.6 ± 1.0	20.1 ± 1.9	5.61

^a Inhibition ratios were measured by adding 20 μM compounds followed by polymerizing at 37°C. Values are means of three experiments and differences among the experiments were not significant (i.e., $P > 0.05$, Fisher's least significant difference test). EC_{50} indicates the concentration of compound resulting in 50% inhibition of mycelial growth. EC_{50} (μM) were converted from data of EC_{50} ($\mu\text{g ml}^{-1}$) published in a previous report (Qiu et al. 2011).

Fig. 5. Homology models of α - and β_2 -tubulins of *Fusarium graminearum* complexes. **A** and **B**, superposition of homology structures of *F. graminearum* α_1 -/ β_2 -tubulins (yellow and red, respectively) dimer and α_2 -/ β_2 -tubulins (yellow/red) dimer on the crystal structure of *Saccharomyces cerevisiae* α -1-tubulin/ β -tubulin (blue/green) dimer, respectively. The homology model of α - plus β_2 -tubulin complexes of *F. graminearum* were constructed by I-TASSER. **C**, Carbendazim binding pocket (cyan) and β_2/α_1 -tubulin interface (blue) were shown on *F. graminearum* β_2 -tubulins. **D**, Carbendazim binding pocket (cyan) and β_2/α_2 -tubulin interface (blue) were shown on *F. graminearum* β_2 -tubulins (red).

microtubule. Although it is desirable to obtain pure native tubulin protein for characterization studies, refolded tubulins have been applied to polymerization (Koo et al. 2009a) and drug–tubulin interaction (Lubega et al. 1993) in early research.

Carbendazim has been proposed to bind to β_2 -tubulin of *F. graminearum* using homology modeling (Qiu et al. 2011), while no evidence of the carbendazim-tubulin physical interaction was raised. In this study, interaction between three benzimidazole compounds with β_2 -tubulin of *F. graminearum* was demonstrated by fluorescence quenching (Fig. 3). This result indicated that the β -tubulin was the binding site for benzimidazole, which has been reported in fungal and invertebrate hosts. Furthermore, the fluorescence quenching ratios of the three benzimidazole compounds (carbendazim, benomyl, and thiabendazole) to β_2 -tubulin was negatively correlated with the EC_{50} values (2.46, 2.1, and 5.61 μ M, respectively), indicating that the binding affinity between the drug and β_2 -tubulin may be the main mechanism of the action of benzimidazole compounds. In addition, this result was consistent with previous reports (Davidse 1986; Davidse and Flach 1977; Hollomon et al. 1998). Point mutation in β -tubulin can dramatically reduce carbendazim sensitivity in *F. graminearum*, which also supports the conclusion above (Qiu et al. 2011).

Studies with the classical antimitotic agent colchicine have shown that this compound binds to tubulin prior to its polymerization (Sackett and Varma 1993; Uppuluri et al. 1993). Inhibition of microtubule assembly appears to be achieved through addition of colchicine-bearing tubulin to the end of a growing microtubule and loss of the ability of these subunits to accept other tubulin molecules for further microtubule growth. Data obtained from fluorescence spectroscopy and labeling experiments have indicated that the colchicine binding site is on the β -tubulin monomer, obviously close to the α/β subunit interface (Uppuluri et al. 1993). In this study, the mechanism underlying the action of the benzimidazole compounds against *F. graminearum* was investigated using turbidimetric methods, as previously reports (Fourest-Lieuvain 2006; Minoura et al. 2013). In the absence of carbendazim, the A_{350} of polymerization mixtures increased from 0.25 to 0.4 and 0.34 to 0.6, indicating the polymerization of α_1 -/ β_2 -tubulin and α_2 -/ β_2 -tubulin, respectively (Fig. 4). This result indicated that β_2 -tubulin preferred binding to α_2 -tubulin rather than α_1 -tubulin. By contrast, tiny changes of A_{350} of polymerization mixtures of α_1 -/ β_2 -tubulin and α_2 -/ β_2 -tubulin were observed if carbendazim was added into the mixtures before the polymerization; this result indicated that the carbendazim significantly inhibited the polymerization of tubulins. This result was consistent with the mechanism of the action of colchicine. Furthermore, in order to investigate whether carbendazim interfered with microtubule dynamics by directly destabilizing the microtubule, polymerized tubulins were treated with carbendazim and no changes of A_{350} of polymerization mixtures of α_1 -/ β_2 -tubulin and α_2 -/ β_2 -tubulin were observed. This result indicated that carbendazim cannot affect the assembled microtubule. Why would the benzimidazole compounds inhibit polymerization of the monomeric tubulins but not the polymerized microtubule? The carbendazim binding pocket in β_2 -tubulin of *F. graminearum*, consisting of amino acids at positions 165, 167, 198, and 200, was close to the α_1/β_2 - and α_2/β_2 -tubulin subunit interfaces by homology modeling (Fig. 5). In addition, the carbendazim binding pocket was located inside the β_2 -tubulin structure. Based on these results, we can assume that formation of the drug- β tubulin complex resulted in a conformation change in the α/β_2 subunit interface followed by loss of the ability of these subunits to accept other tubulin molecules for further microtubule growth. In contrast, polymerized microtubule may have a more compact structure which destroyed the accessibility of the drug to tubulins.

The mechanism underlying the activity of the benzimidazole compounds against *F. graminearum* was investigated in this study. The inhibition ratios of three benzimidazole compounds (carbendazim, benomyl, and thiabendazole) on the polymerization of α - plus

β_2 -tubulins were determined and the correlation of inhibition ratios between EC_{50} of compounds for *F. graminearum* were analyzed (Table 2). Inhibition ratio of 20 μ M carbendazim on α_1 - and β_2 -tubulin polymerization and α_2 - and β_2 -tubulin polymerization were 90.9 ± 0.4 and $93.5 \pm 0.05\%$, respectively. Similarly, the inhibition ratios of 20 μ M benomyl on α_1 -/ β_2 -tubulin polymerization and α_2 -/ β_2 -tubulin polymerization were 89.9 ± 0.1 and $92.6 \pm 1.2\%$, respectively. Therefore, carbendazim has almost the same inhibition ratio as benomyl on tubulin polymerizations. This result can be explained because it is well established that, in either aqueous solutions or soil, benomyl (one molecular) rapidly degrades to its breakdown product carbendazim (one molecular) (Chiba and Cherniak 1978; Tang et al. 1992). In addition, this speculation was confirmed by the similar EC_{50} of carbendazim and benomyl on *F. graminearum* (2.46 and 2.10 μ M, respectively). Butyl iso-cyanate (the other breakdown product of benomyl) also contributed to the drug function in vivo (Tsu and Wold 1973). However, inhibition ratios of 20 μ M thiabendazole on α_1 -/ β_2 -tubulin polymerization and α_2 -/ β_2 -tubulin polymerization were 81.6 ± 1 and $20.1 \pm 1.9\%$, respectively, which were remarkably lower than that of carbendazim and benomyl. This corresponds to the larger EC_{50} of 5.61 μ M thiabendazole on *F. graminearum*. We concluded that the higher the inhibition ratios of the three benzimidazole compounds on *F. graminearum* tubulin polymerization, the lower the EC_{50} (μ M). Combined with the results of fluorescence quenching ratios of the three benzimidazole compounds (carbendazim, benomyl, and thiabendazole) to β_2 -tubulin, the main mechanism of action (although they have others) of the three benzimidazole compounds consisted of benzimidazole compounds primarily binding to the β -tubulin, then disturbing the microtubule dynamic (Davidse 1986; Jamieson et al. 2011; Thelwingani et al. 2009).

Understanding the nature of the interaction between benzimidazole compounds and *F. graminearum* tubulin is fundamental for the development of tubulin-specific anti-*F. graminearum* compounds with selective activity, appropriate pharmacokinetic properties, and low human toxicity. Most notably, the results of this study provide better understanding of the mechanism of action of benzimidazole compounds.

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LITERATURE CITED

- Ansari, K. F., and Lal, C. 2009. Synthesis, physicochemical properties and antimicrobial activity of some new benzimidazole derivatives. *Eur. J. Med. Chem.* 44:4028-4033.
- Blackhall, W. J., Drogemuller, M., Schnieder, T., and von Samson-Himmelstjerna, G. 2006. Expression of recombinant β -tubulin alleles from *Cylocyclus nasutus* (Cyathostominae). *Parasitol. Res.* 99:687-693.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Brown, H. D., Matzuk, A. R., Ilves, I. R., Peterson, L. H., Harris, S. A., Sarett, L. H., Egerton, J. R., Yakstis, J. J., Campbell, W. C., and Cuckler, A. C. 1961. Antiparasitic drugs. IV. 2-(4'-thiazolyl)-benzimidazole, a new anthelmintic. *J. Am. Chem. Soc.* 83:1764-1765.
- Castoldi, M., and Popov, A. V. 2003. Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. *Protein Expr. Purif.* 32:83-88.
- Chen, C., Bi, C., Yu, J., Wang, J., Li, H., Luo, Q., and Zhou, M. 2008. Carbendazim-resistance and its molecular mechanism in *Gibberella zeae*. *J. Plant Pathol.* 90:S2:17-77.
- Chen, C.-J., Yu, J.-J., Bi, C.-W., Zhang, Y.-N., Xu, J.-Q., Wang, J.-X., and Zhou, M.-G. 2009. Mutations in a β -tubulin confer resistance of *Gibberella zeae* to benzimidazole fungicides. *Phytopathology* 99:1403-1411.
- Chen, Y., Wang, J., Zhou, M., Chen, C., and Yuan, S. 2007. Vegetative compatibility of *Fusarium graminearum* isolates and genetic study on their carbendazim-resistance recombination in China. *Phytopathology* 97:1584-1589.

- Chiba, M., and Cherniak, E. A. 1978. Kinetic study of reversible conversion of methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate (benomyl) to methyl 2-benzimidazole carbamate (MBC) and butyl isocyanate (BIC) in organic solvents. *J. Agric. Food Chem.* 26:573-576.
- Clemons, G. P., and Sisler, H. D. 1971. Localization of the site of action of a fungitoxic benomyl derivative. *Pestic. Biochem. Physiol.* 1:32-43.
- Davidse, L. C. 1973. Antimitotic activity of methyl benzimidazol-2-yl carbamate (MBC) in *Aspergillus nidulans*. *Pestic. Biochem. Physiol.* 3:317-325.
- Davidse, L. C. 1975. Mode of action of methyl benzimidazol-2-yl-carbamate (MBC) and some biochemical aspects of acquired resistance against this fungicide in *Aspergillus nidulans*. In *System Fungicide*. Akademie Verlag, Berlin.
- Davidse, L. C., and Flach, W. 1977. Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. *J. Cell Biol.* 72:174-193.
- Davidse, L. C. 1986. Benzimidazole fungicides: Mechanism of action and biological impact. *Annu. Rev. Phytopathol.* 24:43-65.
- Davidse, L. C. 1987. Biochemical aspects of benzimidazole fungicides-action and resistance. Pages 245-257 in: *Modern Selective Fungicides—Properties, Applications, Mechanisms of Action*. H. Lyr, ed. Longman, London.
- Delp, C., and Klopping, H. L. 1968. Performance attributes of a new fungicide and mite ovicide candidate. *Plant Dis. Rep.* 52:95-99.
- Dong, H., Li, Y.-Z., and Hu, W. 2004. Analysis of purified tubulin in high concentration of glutamate for application in high throughput screening for microtubule-stabilizing agents. *Assay Drug Dev. Technol.* 2:621-628.
- Eftink, M. R., and Ghiron, C. A. 1981. Fluorescence quenching studies with proteins. *Anal. Biochem.* 114:199-227.
- Fourest-Lieuvin, A. 2006. Purification of tubulin from limited volumes of cultured cells. *Protein Expr. Purif.* 45:183-190.
- Friedman, P. A., and Platzer, E. G. 1978. Interaction of anthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. *BBA-Gen. Subjects.* 544:605-614.
- Giles, N. L., Armson, A., and Reid, S. A. 2009. Characterization of trifluralin binding with recombinant tubulin from *Trypanosoma brucei*. *Parasitol. Res.* 104:893-903.
- Goswami, R. S., and Kistler, H. C. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* 5:515-525.
- Hammerschlag, R. S., and Sisler, H. D. 1973. Benomyl and methyl-2-benzimidazole carbamate (MBC): Biochemical, cytological and chemical aspects of toxicity to *Ustilago maydis* and *Saccharomyces cerevisiae*. *Pestic. Biochem. Physiol.* 3:42-54.
- Hoebcke, J., Van Nijen, G., and De Brabander, M. 1976. Interaction of oncodazole (R17934), a new anti-tumoral drug, with rat brain tubulin. *Biochem. Biophys. Res. Commun.* 69:319-324.
- Hollomon, D. W., Butters, J. A., Barker, H., and Hall, L. 1998. Fungal beta-tubulin, expressed as a fusion protein, binds benzimidazole and phenyl-carbamate fungicides. *Antimicrob. Agents Ch.* 42:2171-2173.
- Hosamani, K. M., and Shingalapuri, R. V. 2011. Synthesis of 2-mercapto-benzimidazole derivatives as potential anti-microbial and cytotoxic agents. *Arch. Pharm. (Weinheim)* 344:311-319.
- Howard, R. J., and Aist, J. R. 1977. Effects of MBC on hyphal tip organization, growth, and mitosis of *Fusarium acuminatum*, and their antagonism by D₂O. *Protoplasma* 92:195-210.
- Howard, R. J., and Aist, J. R. 1980. Cytoplasmic microtubules and fungal morphogenesis: Ultrastructural effects of methyl benzimidazole-2-ylcarbamate determined by freeze-substitution of hyphal tip cells. *J. Cell Biol.* 87:55-64.
- Jamieson, J. D., Smith, E. B., Dalvie, D. K., Stevens, G. J., and Yanochko, G. M. 2011. Myeloperoxidase-mediated bioactivation of 5-hydroxythiabendazole: A possible mechanism of thiabendazole toxicity. *Toxicol. In Vitro* 25:1061-1066.
- Jang, M.-H., Kim, J., Kalme, S., Han, J.-W., Yoo, H.-S., Kim, J., Koo, B.-S., Kim, S.-K., and Yoon, M.-Y. 2008. Cloning, purification, and polymerization of *Capsicum annuum* recombinant α and β tubulin. *Biosci. Biotechnol. Biochem.* 72:1048-1055.
- Koo, B.-S., Jang, M.-H., Park, H., Kalme, S., Park, H.-Y., Han, J.-W., Yeo, Y.-S., Yoon, S.-H., Kim, S.-J., Lee, C.-M., and Yoon, M.-Y. 2010. Characterization of *Capsicum annuum* recombinant α - and β -tubulin. *Appl. Biochem. Biotechnol.* 160:122-128.
- Koo, B., Kalme, S., Yeo, S., Lee, S., and Yoon, M. 2009a. Molecular cloning and biochemical characterization of α - and β -tubulin from potato plants (*Solanum tuberosum* L.). *Plant Physiol. Biochem.* 47:761-768.
- Koo, B.-S., Park, H., Kalme, S., Park, H.-Y., Han, J.-W., Yeo, Y.-S., Yoon, S.-H., Kim, S.-J., Lee, C.-M., and Yoon, M.-Y. 2009b. α - and β -tubulin from *Phytophthora capsici* KACC 40483: Molecular cloning, biochemical characterization, and antimicrotubule screening. *Appl. Microbiol. Biotechnol.* 82:513-524.
- Lubega, G. W., Geary, T. G., Klein, R. D., and Prichard, R. K. 1993. Expression of cloned β -tubulin genes of *Haemonchus contortus* in *Escherichia coli*: Interaction of recombinant β -tubulin with native tubulin and mebendazole. *Mol. Biochem. Parasitol.* 62:281-292.
- MacDonald, L. M., Armson, A., Thompson, R. A., and Reynoldson, J. A. 2004. Characterisation of benzimidazole binding with recombinant tubulin from *Giardia duodenalis*, *Encephalitozoon intestinalis*, and *Cryptosporidium parvum*. *Mol. Biochem. Parasitol.* 138:89-96.
- Mertens, M. L., and Kägi, J. H. 1979. A graphical correction procedure for inner filter effect in fluorescence quenching titrations. *Anal. Biochem.* 96:448-455.
- Minoura, I., Hachikubo, Y., Yamakita, Y., Takazaki, H., Ayukawa, R., Uchimura, S., and Muto, E. 2013. Overexpression, purification, and functional analysis of recombinant human tubulin dimer. *FEBS Lett.* 587:3450-3455.
- Navarrete-Vázquez, G., Rojano-Vilchis, M. de M., Yépez-Mulia, L., Meléndez, V., Gerena, L., Hernández-Campos, A., Castillo, R., and Hernández-Luis, F. 2006. Synthesis and antiprotozoal activity of some 2-(trifluoromethyl)-1H-benzimidazole bioisosteres. *Eur. J. Med. Chem.* 41:135-141.
- Oxberry, M. E., Geary, T. G., Winterrowd, C. A., and Prichard, R. K. 2001. Individual expression of recombinant α - and β -tubulin from *Haemonchus contortus*: Polymerization and drug effects. *Protein Expr. Purif.* 21:30-39.
- Qiu, J., Xu, J., Yu, J., Bi, C., Chen, C., and Zhou, M. 2011. Localisation of the benzimidazole fungicide binding site of *Gibberella zeae* β 2-tubulin studied by site-directed mutagenesis. *Pest Manage. Sci.* 67:191-198.
- Roy, A., Kucukural, A., and Zhang, Y. 2010. I-TASSER: A unified platform for automated protein structure and function prediction. *Nat. Protoc.* 5:725-738.
- Sackett, D. L., and Varma, J. K. 1993. Molecular mechanism of colchicine action: Induced local unfolding of β -tubulin. *Biochemistry* 32:13560-13565.
- Staron, T., Allard, X., and Gug, M. 1964. Propriétés antifongiques des 2-(4'-thiazolyle) benzimidazole ou thiabendazoles. *Phytopathol. 13*:163-168.
- Tang, C., Yanagihara, K., and Zhang, Y. 1992. 1-butyl isocyanate from aqueous Benlate® formulations. *Arch. Environ. Contam. Toxicol.* 23:270-272.
- Thelingwani, R. S., Zvada, S. P., Dolgos, H., Ungell, A. B., and Masimirembwa, C. M. 2009. In vitro and in silico identification and characterization of thiabendazole as a mechanism-based inhibitor of CYP1A2 and simulation of possible pharmacokinetic drug-drug interactions. *Drug Metab. Dispos.* 37:1286-1294.
- Tunçbilek, M., Kiper, T., and Altanlar, N. 2009. Synthesis and in vitro antimicrobial activity of some novel substituted benzimidazole derivatives having potent activity against MRSA. *Eur. J. Med. Chem.* 44:1024-1033.
- Twu, J. S., and Wold, F. 1973. Butyl isocyanate, an active-site-specific reagent for yeast alcohol dehydrogenase. *Biochemistry* 12:381-386.
- Uppuluri, S., Knipling, L., Sackett, D. L., and Wolff, J. 1993. Localization of the colchicine-binding site of tubulin. *Proc. Nat. Acad. Sci. USA* 90:11598-11602.
- Widlund, P. O., Podolski, M., Reber, S., Alper, J., Storch, M., Hyman, A. A., and Drechsel, D. N. 2012. One-step purification of assembly-competent tubulin from diverse eukaryotic sources. *Mol. Biol. Cell* 23:4393-4401.
- Yoon, B., and Oakley, B. R. 1995. Purification and characterization of assembly-competent tubulin from *Aspergillus nidulans*. *Biochemistry* 34:6373-6381.
- Zhang, Y. 2008. I-TASSER server for protein 3D structure prediction. *BMC Bioinf.* 9:40.