Discovery of a Small-Molecule Inhibitor of β-1,6-Glucan Synthesis

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It is possible that antifungal drugs with novel modes of action will provide favorable options to treat fungal infections. In the course of our screening for antifungal compounds acting on the cell wall, a pyridobenzimidazole derivative with unique activities, named D75-4590, was discovered. During treatment of Saccharomyces cerevisiae with D75-4590, (i) incorporation of [14C]glucose into the β-1,6-glucan component was selectively reduced, (ii) proteins released from the cell had lost the β-1,6-glucan moiety, and (iii) cells tended to clump, resulting in impaired cell growth. Genetic analysis of a D75-4590-resistant mutant of S. cerevisiae indicated that its primary target was Kre6p, which is considered to be one of the β-1,6-glucan synthases. These results strongly suggest that D75-4590 is a specific inhibitor of β-1,6-glucan synthesis. D75-4590 showed potent activities against various Candida species. It inhibited hyphal elongation of C. albicans as well. Kre6 is conserved in various fungi, but no homologue has been found in mammalian cells. These lines of evidence indicate that D75-4590 is a promising lead compound for novel antifungal drugs. To our knowledge, this is the first report of a β-1,6-glucan inhibitor.

Fungal infections have increased in frequency over the past several decades due to a growing number of immunocompromised patients (8, 9, 35). Present therapeutic options are limited, however, to three classes of compounds: polyenes, azoles, and recently introduced candins (3, 9, 48). The utility of polyenes is limited by their nephrotoxicity (35, 48). Although azoles are safer and most commonly used, the broad usage of these drugs has probably allowed the increase of less-susceptible species of Candida, such as C. glabrata (2, 17). In addition, drug-drug interactions and teratogenicity limit their clinical usage. Candins have solved some of these problems; however, they have not completely satisfied unmet medical needs mainly due to their poor oral absorption and limited spectrum (48). In addition, incidents of resistance to these drugs have also emerged (14, 28). Therefore, development of an orally active antifungal drug with a novel mode of action is desirable.

The fungal cell wall is an attractive target for antifungal agents because it is an essential, fungal-specific organelle that is absent from human cells. The cell wall of Saccharomyces cerevisiae is basically composed of β-1,3-glucan, β-1,6-glucan, chitin, and highly mannosylated glycoproteins, which are interconnected (5, 20). Many fungal-specific enzymes, such as Fks1p, Kre6p, and Chs1p, are involved in the synthesis of β-1,3-glucan, β-1,6-glucan, and chitin, respectively (5). In addition to the synthases of cell wall components, several enzymes have been shown to be involved in the interconnection of these components (5). A number of β-1,3-glucan synthase inhibitors, such as echinocandins, papulacandins, and enfuma-fungin, have already been reported (21, 34, 44). Several chitin synthase inhibitors have been reported as well (13, 42). In contrast, an inhibitor of β-1,6-glucan synthase or the enzymes involved in the interconnection of cell wall components has not been reported.

Genetic analyses of S. cerevisiae and C. albicans have provided us with valuable information regarding β-1,6-glucan synthesis (16, 27, 30, 39). Many proteins encoded by KRE genes, such as KRE6, KRE9, and KRE1, are involved in the biosynthesis in a sequential manner (5). However, no precise functions, either catalytic or regulatory, have been definitively assigned to any KRE gene products. Lack of enzymatic information hampers the discovery of their inhibitors. Structural and biochemical analyses of the yeast cell wall, however, have provided a way to obtain inhibitors. Most cell wall proteins are glycosylphosphatidylinositol (GPI) dependent and are attached to β-1,3-glucan and/or chitin via β-1,6-glucan (19, 22, 23). Recent progress in genetic technology allowed us to attach heterologous protein to the cell wall by constructing a gene of interest fused to a secretion signal and GPI attachment signal (46, 47). Using this technology, we have developed a cell-based assay system for screening various inhibitors of cell wall components, including β-1,6-glucan (A. Kitamura, K. Someya, and R. Nakajima, U.S. patent application 20040091949 [PCT/JP01/03630]). In the course of screening for antifungal compounds using this system, we discovered the compound D75-4590, which has unique activities. To gain a better insight into this compound, we studied the nature of its antifungal activities and its mechanism of action.

MATERIALS AND METHODS

Strains and media. S. cerevisiae YPH850 (MATa ade2 his3 leu2 lys2 trp1 ura3) (40), AY-10 (isogenic strain derived from YPH80; MATa ade2 his3 leu2 trp1 ura3), AY-10k (AY-10 skn1::URA3) [Kitamura et al., U.S. patent application 20040091949 [PCT/JP01/03630]], and the 15 pathogenic fungi listed in Table 1 were used in this study. These pathogenic fungi were purchased from the American Type Culture Collection (Rockville, MD), the Institute for Fermentation Osaka (Osaka, Japan), or the Teikyo Institute of Medical Mycology (Tokyo, Japan).

Sabouraud dextrose agar (SDA; Difco, Detroit, MI), RPMIB (RPMI 1640 supplemented with 1 M sorbitol, 100 mM potassium phosphate buffer [pH 6.5], 2% glucose, 40 μg/ml adenine, 20 μg/ml uracil), YNB (0.67% yeast nitrogen base with amino acids [Difco], 2% glucose), hyphal forming medium 7 (HFM-7; 5 g/liter glucose, 0.26 g/liter Na2HPO4·7H2O, 0.66 g/liter KH2PO4, 0.08 g/liter MgSO4·7H2O, 0.33 g/liter NH4Cl, 16 mg/liter...
The cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer and were incubated with or without drug. After 18 h of incubation with gentle shaking at 30°C, the cell suspensions were examined with a light microscope (Leica model DMLB100; Leica, Solms, Germany). Images were acquired with a digital charge-coupled-device camera (Olympus model DP70).

**Time-kill study.** Exponentially growing cells of *C. glabrata* IPO0622 were suspended in RPMI 1640 with or without drug at a cell density of 1 × 10⁴ cells/ml. Samples were taken after 2, 4, 8, and 24 h of incubation with gentle shaking at 30°C. The samples were diluted appropriately and spread on SDA plates. The number of viable cells in each sample was measured by counting the colonies on each plate after an overnight incubation at 30°C.

**Incorporation studies with growing cells.** The effects of D75-4590 on macromolecular synthesis were evaluated by pulse-labeling the cells with radioactive precursors of specific macromolecules. The assay procedures were conducted based on the methods described by Yamaguchi et al. (49) with some modifications. Exponentially growing cells of *C. glabrata* IPO0622 or *S. cerevisiae* YPH500 were suspended in RPMI 1640 medium to give approximately 0.1 absorbance at 570 nm. Samples were taken after 2, 4, 8, and 24 h of incubation with gentle shaking at 37°C. The samples were diluted appropriately and spread on SDA plates. The number of viable cells in each sample was measured by counting the colonies on each plate after an overnight incubation at 30°C.

**TABLE 1. Antifungal activities of D75-4590 and fluconazole**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC-2 (µg/ml)</th>
<th>MIC-0 (µg/ml)</th>
<th>MFC (µg/ml)</th>
<th>FLC (MIC-2) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> ATCC 24433</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>4</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> YPH500</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>4</td>
</tr>
<tr>
<td><em>C. glabrata</em> IPO0622</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>4</td>
</tr>
</tbody>
</table>

*Note: MIC-0, MIC-2, and MFCs (in micrograms per milliliter) were determined by using the microdilution method described in Materials and Methods. NT, not tested; FLC, fluconazole.*
**RESULTS**

**Discovery of D75-4590.** D75-4590 (Fig. 1) was discovered from our chemical library in a high-throughput manner using our novel cell-based assay system described elsewhere (Kitamura et al., U.S. patent application 20040091949 [PCT/JP01/03630]). Briefly, we constructed two types of yeasts, namely “wall-type arming yeast” and “membrane-type arming yeast,” each having an easily detectable protein (reporter protein) fixed on the cell wall via β-1,6-glucan or on the cell membrane via a GPI anchor by means of genetic engineering (11, 47). Compounds inhibiting the process of protein fixation are expected to accelerate the release of a reporter protein from a cell. Therefore, such compounds can be screened by the detection of a reporter protein in the medium. Although the process of protein fixation to the cell membrane is generally conserved in eukaryotic cells, fixation to the cell wall is considered to be unique to fungi. Therefore, screening for the compounds which accelerate the release of the reporter protein from “wall-type arming yeast” would be an efficient way to find the compounds that inhibit fungus-specific processes (Fig. 2). The results of validation studies of our screening system show that several compounds which act on the cell wall, such as aculeacin A (β-1,3-glucan synthesis inhibitor) and tunicamycin, were considered to be unique to fungi. Therefore, screening for the compounds that inhibit fungus-specific processes (Fig. 3) were counted at intervals using SDA plates. The concentrations of D75-4590 are shown.

**Construction of S. cerevisiae CY-1a and CY-2a.** To construct a plasmid harboring KRE6 of S. cerevisiae CY-10 was disrupted using a method similar to that described by Roemer et al. (37), resulting in S. cerevisiae CY-10c (Kitamura et al., U.S. patent application 20040091949 [PCT/JP01/03630]). S. cerevisiae CY-1a and CY-2a were constructed from S. cerevisiae CY-10c as follows. A HIS3 cassette obtained by digesting pHIS413 (Stratagene) with Spel and PstI was inserted into the HindII and PstI sites of pUC19 to generate pHIS4. Wild-type KRE6 was amplified by PCR (template, S. cerevisiae YPH500 genomic DNA; primers, SCKRE6-Sen3 [5’-CGCGCCCGTAAACAACCAAAACATAGACAAAACCCG-3’] and SCKRE6-An3 [5’-CGAGGGCTTTAGTCTTCTTATGACCGGTTGAA C-3’]) and subcloned into pGEM-T (Promega, Madison, WI) to generate pUAO1. A fragment obtained by digestion of pUAO1 and pRS416-31 with XbaI and NheI was inserted into the SphI site of pUXS-1 to generate pUXS-2. The results of validation studies of our screening system show that several compounds which act on the cell wall, such as aculeacin A (β-1,3-glucan synthesis inhibitor) and tunicamycin, were considered to be unique to fungi. Therefore, screening for the compounds that inhibit fungus-specific processes (Fig. 3) were counted at intervals using SDA plates. The concentrations of D75-4590 are shown.

**Construction of S. cerevisiae CY-1a and CY-2a.** SKN1 (homologue of KRE6) of S. cerevisiae CY-10 was disrupted using a method similar to that described by Roemer et al. (37), resulting in S. cerevisiae CY-10c (Kitamura et al., U.S. patent application 20040091949 [PCT/JP01/03630]). S. cerevisiae CY-1a and CY-2a were constructed from S. cerevisiae CY-10c as follows. A HIS3 cassette obtained by digesting pHIS413 (Stratagene) with Spel and PstI was inserted into the HindII and PstI sites of pUC19 to generate pHIS4. Wild-type KRE6 was amplified by PCR (template, S. cerevisiae YPH500 genomic DNA; primers, SCKRE6-Sen3 [5’-CGCGCCCGTAAACAACCAAAACATAGACAAAACCCG-3’] and SCKRE6-An3 [5’-CGAGGGCTTTAGTCTTCTTATGACCGGTTGAA C-3’]) and subcloned into pGEM-T (Promega, Madison, WI) to generate pUAO1. A fragment obtained by digestion of pUAO1 and pRS416-31 with Spel and NheI (KRE6 without a promoter region) was inserted into the XbaI site (within the region of the KRE6 open reading frame [ORF]) of pUXS-1 to generate pUXS-1a, and pUXS-1b, respectively. pUXS-1a and pUXS-1b were digested with XbaI and were introduced into the chromosomal DNA of S. cerevisiae CY-10c to generate S. cerevisiae CY-1a and CY-2a, respectively. Since the KRE6s of pUXS-1a and pUXS-1b do not have promoter regions, S. cerevisiae CY-1a expresses wild-type KRE6 and CY-2a expresses KRE6 with a resistant mutation.

**Generation and analysis of D75-4590-resistant mutants.** Exponentially growing cells of S. cerevisiae CY-10 were harvest by centrifugation and suspended in fresh RPMB medium to give an absorbance of approximately 0.7 at 595 nm. This cell suspension (150 μl) was mixed with 50 μl of drug solution in 96-well plates and incubated at 30°C for 6 h with occasional shaking. Supernatant from four wells was collected using polyvinylidene difluoride membrane filters (GF/C; Whatman), which were washed with 5% TCA and 95% ethanol and then dried (RNA fraction).

**Glass-fiber filters (GF/C).** Whatman, which were washed with 5% TCA and 95% ethanol and then dried (RNA fraction).

**Acetic acid (precursor of sterol).** [14C]Acetic acid (Daichi Pure Chemicals) was added to the cell suspensions at a concentration of 2.0 μCi/ml, and 5-ml samples were taken at each point. The harvested cells were suspended in 2 ml of a mixture of ethanol and ether (3:1, vol/vol) and incubated at 55°C for 4 h. The supernatant was collected by centrifugation (sterilization fraction).

**Western analysis of proteins released into medium by treatment of drugs.** Exponentially growing cells of S. cerevisiae were harvested by centrifugation and suspended in fresh RPMB medium to give an absorbance of approximately 0.7 at 595 nm. This cell suspension (150 μl) was mixed with 50 μl of drug solution in 96-well plates and incubated at 30°C for 6 h with occasional shaking. Supernatant from four wells was collected using polyvinylidene difluoride membrane filter plates (multiscreen MAHA550; Corning, Corning, NY) and combined. The collected supernatant was concentrated 50 times using ultrafiltration (UFCLTV; Millipore, Tokyo, Japan). Samples were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted to a polyvinylidene difluoride membrane (Millipore). Polyclonal anti-β-1,6-glucan antibodies were generated in rabbits against putalutan (Calbiochem, La Jolla, CA). The immunoglobin G fraction of antiserum was purified via Affi-gel protein A affinity chromatography (Millipore) and was used as the first antibody. Alkaline phosphatase conjugated anti-rabbit immunoglobulin G (goat) antibody was purchased from Sigma and was used as the secondary antibody. Immunodetection of β-1,6-glucan was performed with an alkaline phosphatase detection kit (Bio-Rad, Richmond, CA) according to the manufacturer’s instructions.

**Generation and analysis of D75-4590-resistant mutants.** Exponentially growing cells of S. cerevisiae CY-10 (1 × 10^6 cells) were spread onto RPMB agar containing D75-4590 (64 μg/ml). Plates were exposed to UV irradiation to give approximately 10% survival. After 3 days of incubation at 30°C, 28 colonies had emerged. Genomic DNA was collected from three strains, and their genomic libraries were made using the Xhol, XbaI, HindII, Nhel, Sall, or SphI site of single-copy vector pRS416 (Stratagene, Cedar Creek, TX). They were transformed into S. cerevisiae CY-10, and resistant strains were selected on RPMB agar containing D75-4590 (64 μg/ml). Plasmids were recovered from resistant strains and transformed into E. coli DH5α for amplification. Nucleotide sequencing was done using an Applied Biosystems model 3100 sequencer.

**Production of proteinase K.** Proteinase K was purified from the supernatant of E. coli DH5α, which was used in the experiments.
mannot synthesis inhibitor), significantly accelerate the release of the reporter protein from only “wall-type arming yeast” and that other compounds, including nikkomycin Z, fluconazole, 5-flucytosine, and cycloheximide, slightly accelerated or did not accelerate the release of the reporter protein from both arming yeasts (Kitamura et al., U.S. patent application 20040091949 [PCT/JP01/03630]). It was also found that disruption by KRE6 of “wall-type arming yeast” accelerated the release of the reporter protein (Kitamura et al., U.S. patent application 20040091949 [PCT/JP01/03630]). Therefore, several types of compounds acting on the cell wall should be screened by using our system.

**Antifungal activities of D75-4590.** Table 1 shows the antifungal activities of D75-4590 and fluconazole against various fungi. D75-4590 has activities against a variety of *Candida* species, including fluconazole-resistant strains. Most strains of *C. albicans, C. tropicalis*, and *C. parapsilosis* displayed trailing growth phenomena similar to those observed in the presence of azoles (36). Interestingly, visible morphological changes, which resemble aggregation of *S. cerevisiae*, were observed in these strains at a much lower concentration of D75-4590 than its MIC-2 values (data not shown). No significant effect was found on *Cryptococcus neoformans, Trichosporon asahii*, and *Aspergillus* species even at a concentration of 32 μg/ml. The MICs of D75-4590 against a bacterial strain (*Staphylococcus aureus* 209P and *E. coli* NIHJ) were >32 μg/ml, and its GI50...
C. glabrata IFO0622 treated with D75-4590.

FIG. 6. Effects of D75-4590 and aculeacin A on the incorporation of radioactive precursors into macromolecules in growing cells of S. cerevisiae AY-10 and C. glabrata IFO0622. Growing cells in RPMI medium were treated with or without drug in the presence of radioactive glucose, leucine, uridine, or acetic acid. β-1,3-Glucan, β-1,6-glucan, chitin, mannan, RNA, protein, and sterol fractions were prepared by the methods described in Materials and Methods. The percent changes of the incorporated radioactivities by drug treatment for 1 h (upper panels) or 3 h (lower panels) at each concentration tested are displayed. (a) S. cerevisiae AY-10 treated with aculeacin A. (b) S. cerevisiae AY-10 treated with D75-4590. (c) C. glabrata IFO0622 treated with D75-4590.

To characterize the morphological defects, microscopic analyses of D75-4590-treated cells were performed. First, RPMI medium without serum was used for the culture to determine its effects on yeast-type growing cells. Drug-treated cells of S. cerevisiae, C. glabrata, and C. albicans were clumped; they contained more than one bud and appeared to show incomplete cell separation (Fig. 4). Next, the effect of D75-4590 on hyphal elongation of C. albicans was investigated. As indicated in Fig. 5, static incubation of cells in HFM-7 (18) induces hyphal elongation, and yeast-type cells were rarely seen after 6 h of incubation. The inhibitory effect of D75-4590 on hyphal elongation was observed at a concentration of 1 μg/ml, and yeast-type cells were predominant at a concentration of 16 μg/ml. After 24 h of incubation, cells treated without drugs formed colonies mostly consisting of yeast-type cells with many hyphae on the edges. D75-4590-treated cells also formed colonies, but hyphae on the edges were diminished. Inhibitory effects of D75-4590 on hyphal elongation were also observed when Lee’s medium was used (data not shown).

Biochemical study of the D75-4590 mechanism of action using C. glabrata and S. cerevisiae. Since it was screened by our assay system focusing on the cell wall, it is expected that D75-4590 acts on the cell wall. To obtain information on which components are inhibited, the effects of D75-4590 on the incorporation of radioactive precursors into various macromolecules in growing cells were examined and compared with the effects of aculeacin A (β-1,3-glucan synthesis inhibitor). First, we compared the effects of D75-4590 and aculeacin A on the cell wall components of S. cerevisiae AY-10 (Fig. 6a and b). Aculeacin A strongly reduced the radioactivity in the fraction of β-1,3-glucan and weakly reduced that of β-1,6-glucan. The reduction in the β-1,6-glucan fraction may be a consequence of the inhibition of β-1,3-glucan synthesis, because loss of β-1,3-glucan most likely results in the release of β-1,6-glucan from a cell. In contrast, D75-4590 markedly reduced the radioactivity in the fraction of β-1,6-glucan with only slight or no reduction in the fractions of other cell wall components. A significant reduction was observed at a concentration of 0.078 μg/ml, which is much lower than its MIC-0 value. D75-4590 showed no significant effects on the radioactivity in the fractions of RNA, protein, or sterol at the concentrations tested (Fig. 6b).

A selective reduction in the β-1,6-glucan fraction at a lower concentration of MIC-0 was also observed in the experiment using C. glabrata IFO0622 (Fig. 6c).

Reduction of radioactivity in the fraction of β-1,6-glucan can be explained by either inhibition of β-1,6-glucan synthesis or the release of cell wall protein with β-1,6-glucan from the cells. If D75-4590 inhibits β-1,6-glucan synthesis, the released proteins are expected to have a small or no β-1,6-glucan moiety. To determine the reason for the reduction, the amounts of β-1,6-glucan attached to proteins in the culture medium with or without drug treatment were compared by Western analysis. Anti-β-1,6-glucan antiserum detected β-1,6-glucan attached to proteins in the medium without drug. The reaction was diminished when cells were treated with D75-4590 in a dose-dependent manner (Fig. 7). Since significant reduction was observed at a lower concentration than its MIC-0 value, it is unlikely that
the reduction is caused by a growth defect. These results suggest that D75-4590 most likely inhibits β-1,6-glucan synthesis.

**Genetic study of the D75-4590 mechanism of action using S. cerevisiae.** As many genes are involved in β-1,6-glucan synthesis, we speculated that it could have a mutation in the target enzyme of D75-4590. Genomic libraries of AY-10-R15 were transformed to S. cerevisiae, where we have found a resistant mutation, which indicates that the mutation at position 1654 of KRE6 is solely responsible for the resistance (Table 2). Furthermore, we introduced a resistant mutation of KRE6 into the chromosomal DNA of S. cerevisiae AY-10c (SKN7 null mutant), yielding SKS cerevisiae CY-2a. It was found to be highly resistant to D75-4590, which indicates that the mutation at position 1654 of KRE6 is solely responsible for the resistance (Table 2).

**Homology search of KRE6 among other species.** A homology search in DDBJ, GenBank, and EMBL revealed the existence of KRE6 homologues in many fungal species (C. albicans, C. glabrata, C. tropicalis, Cryptococcus neoformans, Aspergillus fumigatus, Aspergillus terreus, Aspergillus clavatus, and Pneumocystis carinii). We also have cloned some parts of the KRE6 homologues of Candida krusei and C. parapsilosis (data not shown). High homologies are observed among all of these homologues at the C-terminal regions of the transmembrane domains but not at the N-terminal regions. No mammalian gene having significant homology to KRE6 was found. These data confirm that KRE6 is fungus specific and is conserved among various fungi. Interestingly, the amino acid at position 552 in S. cerevisiae, where we have found a resistant mutation, was conserved among Candida species but not among Aspergillus species (Fig. 8).

**Comparison of partial sequences of Kre6p homologues from various fungal species.** The amino acid residues are shown in single-letter amino acid code. The region near the position of the residue (indicated by an arrow) that, when mutated, confers D75-4590 resistance in S. cerevisiae is displayed in the alignment. Abbreviations: SC, S. cerevisiae; CG, C. glabrata; CA, C. albicans; CK, C. krusei; CP, C. parapsilosis; AF, A. fumigatus; AT, A. terreus; AC, A. clavatus.

**TABLE 2. Susceptibilities of S. cerevisiae mutants and the parental strain to various antifungal compounds**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC-0 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D75-4590</td>
</tr>
<tr>
<td>AY-10</td>
<td>2</td>
</tr>
<tr>
<td>AY-10-R4</td>
<td>8</td>
</tr>
<tr>
<td>AY-10-R11</td>
<td>8</td>
</tr>
<tr>
<td>AY-10-R15</td>
<td>&gt;64</td>
</tr>
<tr>
<td>AY-10-R16</td>
<td>4</td>
</tr>
<tr>
<td>CY-1a</td>
<td>8</td>
</tr>
<tr>
<td>CY-2a</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

*Abbreviations: AMB, amphotericin B; SFC, 5-flucytosine; TM, tunicamycin; AC, aculeacin A; CW, calcofluor white; NT, not tested.*

**TABLE 3. Susceptibilities of S. cerevisiae AY-10, transformed with or without plasmid, to various antifungal compounds**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Cloned gene</th>
<th>MIC-0 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D75-4590</td>
<td>AMB</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>pRS416</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>pRS416-13</td>
<td>ADP1</td>
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</tr>
<tr>
<td>pRS416-31</td>
<td>KRE6</td>
<td>32</td>
</tr>
</tbody>
</table>

*Abbreviations: AMB, amphotericin B; SFC, 5-flucytosine; TM, tunicamycin; AC, aculeacin A; CW, calcofluor white.*

FIG. 7. Western analysis of proteins secreted from S. cerevisiae AY-10 treated with D75-4590. Cells were treated with D75-4590 for 6 h, and the extracellular medium was concentrated and used for Western blot analysis with anti-pustulan (β-1,6-glucan) antiserum. Concentrations of D75-4590 added were as follows: 1, 20 µg/ml; 2, 10 µg/ml; 3, 5 µg/ml; 4, 2.5 µg/ml; 5, 1.25 µg/ml; 6, 0.625 µg/ml; 7, 0.313 µg/ml; and 8, 0 µg/ml. M, marker.

FIG. 8. Comparison of partial sequences of Kre6p homologues from various fungal species. The amino acid residues are shown in single-letter amino acid code. The region near the position of the residue (indicated by an arrow) that, when mutated, confers D75-4590 resistance in S. cerevisiae is displayed in the alignment. Abbreviations: SC, S. cerevisiae; CG, C. glabrata; CA, C. albicans; CK, C. krusei; CP, C. parapsilosis; AF, A. fumigatus; AT, A. terreus; AC, A. clavatus.
DISCUSSION

Our findings in biochemical and genetic studies strongly suggest that D75-4590 is a specific inhibitor of β-1,6-glucan synthesis and that its primary target is Kre6p. Many reports have shown that a reduction of β-1,6-glucan leads to partial defects in cell separation, resulting in a multibudding phenotype (24, 25, 29, 38). Lussier et al. reported that homozygous null disruptants of C. albicans KRE9, which are involved in β-1,6-glucan synthesis, failed to form hyphae (24). These phenotypic features were also observed in D75-4590-treated cells, which supports our conclusion that D75-4590 is a β-1,6-glucan inhibitor.

Kre6p is a predicted type II membrane protein localized on the endoplasmic reticulum or Golgi apparatus (30, 38). Kre6p and its functional homologue Skn1p are both present in S. cerevisiae (37). Although neither KRE6 nor SKN1 is essential; disruption of SKN1 has little effect on growth, and disruption of both genes results in extremely slow growth or lethality (37, 38). Since D75-4590 showed a complete growth inhibitory effect against S. cerevisiae, it is likely to inhibit both Kre6p and Skn1p. The essentiality of β-1,6-glucan for normal growth is predicted in Candida species as well (24, 25, 29). Our results showing that a mutation providing resistance against D75-4590 is found in the C terminus of Kre6p indicate that the binding site of D75-4590 is likely to be in its predicted C-terminal luminal domain. Since amino acid sequences in the C-terminal domain are highly conserved, D75-4590 may inhibit the Kre6p of various Candida species. It did actually show activities against various Candida species; however, incomplete inhibition (trailing phenomenon) was observed in some species, including the major pathogen C. albicans. One of the possible explanations is that D75-4590 inhibits only some of the Kre6p homologues in C. albicans. The inhibitory effects of D75-4590 on each Kre6p homologue of C. albicans are under investigation.

It was demonstrated that the cell wall of filamentous fungi, such as Aspergillus species, contains no β-1,6-glucan polymer (10), whereas Kre6p homologues have been found in A. fumigatus, A. terreus, and A. clavatus. The functions of these homologues in Aspergillus species are not well understood, and Henry et al. have suggested that partial silencing of KRE6 expression makes A. fumigatus more susceptible to Congo red (15), which seems to indicate that Kre6p has a role in the cell wall construction in A. fumigatus. To our regret, we did not find any inhibitory or morphological effect of D75-4590 against Aspergillus species, at least under light microscopic observation. One of the possibilities is that amino acid differences contribute to the resistance to D75-4590 in this species. We found a resistant mutation in S. cerevisiae resulting in an amino acid transition from Phe to Ile at position 552. The Kre6p of Aspergillus species has Tyr at a corresponding position, which may be responsible for the resistance (Fig. 8). If D75-4590 or its derivatives inhibit Kre6p of Aspergillus species, analysis of cells treated with the compound may shed light on the role of Kre6p in Aspergillus species. Further work is needed to understand the effect of Kre6p inhibition in Aspergillus species.

Although growth inhibitory effects of D75-4590 are not potent enough in some species to expect in vivo effects, additional effects in vivo can be expected for β-1,6-glucan inhibitors. Cell wall proteins, such as Hwp1p and Als1p, are thought to play important roles in the process of fungal pathogenesis, such as adhesion, hyphal elongation, and biofilm production (1, 12, 33, 41). Most of these proteins are attached to the cell wall via β-1,6-glucan. Although these proteins are not essential for the growth of C. albicans, some of their null mutants have been shown to be avirulent in an animal model. Therefore, a β-1,6-glucan inhibitor is expected to remove these proteins from the cell and reduce the pathogenesis of C. albicans as a result. In fact, there are several reports suggesting that C. albicans mutants with a reduced level of β-1,6-glucan are avirulent (16, 45). Our study clearly shows a potent inhibitory effect of D75-4590 against hyphal elongation as well.

As far as we know, this is the first report of a β-1,6-glucan synthase inhibitor. Since KRE6 is conserved in various fungi, while mammalian cells have neither KRE6 nor β-1,6-glucan polymer, D75-4590 is a promising lead compound for new antifungal agents. However, as its physicochemical profile is not ideal for a drug, the efficacy of D75-4590 in animal models is not promising. Nevertheless, more potent derivatives with ideal physicochemical profiles would be effective. In addition, it is advantageous from the viewpoint of oral administration and chemical modification because it is a small molecule. Derivatization and in vivo studies are now in progress.

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REFERENCES

discovery of \( \beta-1, 6\)-glucan inhibitor


