Effect of β-1,6-Glucan Inhibitors on the Invasion Process of Candida albicans: Potential Mechanism of Their In Vivo Efficacy

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Received 31 March 2009/Returned for modification 29 May 2009/Accepted 5 July 2009

β-1,6-Glucan is a fungus-specific cell wall component that is essential for the retention of many cell wall proteins. We recently reported the discovery of a small molecule inhibitor of β-1,6-glucan biosynthesis in yeasts. In the course of our study of its derivatives, we found a unique feature in their antifungal profile. D21-6076, one of these compounds, exhibited potent in vitro and in vivo antifungal activities against Candida glabrata. Interestingly, although it only weakly reduced the growth of Candida albicans in conventional media, it significantly prolonged the survival of mice infected by the pathogen. Biochemical evaluation of D21-6076 indicated that it inhibited β-1,6-glucan synthesis of C. albicans, leading the cell wall proteins, which play a critical role in its virulence, to be released from the cell. Correspondingly, adhesion of C. albicans cells to mammalian cells and their hyphal elongation were strongly reduced by the drug treatment. The results of the experiment using an in vitro model of vaginal candidiasis showed that D21-6076 strongly inhibited the invasion process of C. albicans without a significant reduction in its growth in the medium. These evidences suggested that D21-6076 probably exhibited in vivo efficacy against C. albicans by inhibiting its invasion process.

Modern advances in treatment, especially for patients with immune deficiencies, have led to a larger population of those being susceptible to opportunistic pathogens, thereby increasing the importance of Candida species as pathogens (16, 39). In spite of the recent progress of antifungal drugs, the mortality rate for systemic candidiasis remains significantly high. Moreover, the management of candidiasis is complicated by the limited treatment options, resulting in the emergence of various problems in medical care, such as recurrence and biofilm (5, 24, 30). Drugs with a new mode of action could offer more-preferable options. In recent years, a great deal of effort has been made to identify essential and fungus-specific targets. In addition, the invasion process of candidiasis has become the focus as a potential target of novel antifungal drugs (8, 14, 17, 35).

We recently discovered a specific inhibitor of β-1,6-glucan synthesis named D75-4590 (11) (A. Kitamura, K. Someya, and R. Nakajima, U.S. patent application 20040091949; international patent application PCT/JP1/03630 [2003]) (Fig. 1). Genetic studies suggested that its primary target is Kre6p, which is conserved in various fungi (Kitamura et al., U.S. patent application 20040091949; international patent application PCT/JP1/03630 [2003]). D75-4590 shows activity against most Candida species but not against Cryptococcus neoformans or Aspergillus species in a conventional in vitro antifungal test (11). Since β-1,6-glucan is thought to be an essential component for yeast and neither Kre6p nor β-1,6-glucan exists in mammalian cells, D75-4590 is expected to be a promising lead for antifungal drugs (15, 19, 25). From a different point of view, since it is the first inhibitor of β-1,6-glucan synthesis, it would be a variable tool to investigate the role of β-1,6-glucan in various fungi for their growth as well as their pathogenesis, which is the main focus of this study. One of our interests lies in our hypothesis that the β-1,6-glucan inhibitors could show in vivo efficacy not only by inhibiting the growth of fungi but also by attenuating their pathogenesis (11).

Although D75-4590 does not have potent activity and good physicochemical properties to show significant efficacy in animal models, the chemical modifications of D75-4590 have enabled us to obtain compounds with more-preferable profiles and to investigate the various effects of β-1,6-glucan inhibitors in vitro as well as in vivo. We have started this study with two of these compounds, D11-2040 and D21-6076.

MATERIALS AND METHODS

Strain and media. The Saccharomyces cerevisiae strains used in this study are listed in Table 1. Four pathogenic fungal strains, Candida albicans ATCC 24433, C. albicans ATCC 90028, Candida glabrata ATCC 48435, and Candida krusei ATCC 44507, were purchased from the American Type Culture Collection; the Institute for Fermentation, Osaka; or Teikyo Institute of Medical Mycology. All the strains were stored at −80°C and were cultured in YNB (0.67% yeast nitrogen base with amino acid, 2% glucose) plus requirements and 2% agar or Sabouraud dextrose agar (SDA; Difco, Detroit, MI) prior to use. All the strains were grown at 30°C, unless otherwise specified. The mediums used were MOPS (morpholinepropanesulfonic acid)-buffered RPMI 1640 (21), YNB, minimum essential medium (MEM) (0.01 g/liter biotin [pH 7.0]; Sigma, St. Louis, MO), Lee’s medium (13), Spider medium (3), and RP medium (RPMI 1640 [Sigma], 2.5% fetal calf serum, 20 mM HEPES, 16 mM sodium hydrogen carbonate [pH 7.0]) (38). Requirements were added when necessary. Escherichia coli DH5α was used for the propagation of plasmids and was grown in Luria broth or agar (Difco) with 100 µg/ml ampicillin (Sigma) when appropriate.

[Received 31 March 2009/Returned for modification 29 May 2009/Accepted 5 July 2009]
Chemicals. D75-4590 (2-ethyl-2′, N′-DEAE)-aminoo-3-methylpyridyl [1,2-α-benzimidazole-4-carbonitrile], D11-2040 (1-353-3′, N′-dimethylaminopyrrolidin-1-yl)-3-methyl-2-phenylpyridinyl[1,2-α-benzimidazole-4-carbonitrile], D21-6076 (1-{353-3′, N′-dimethylaminopyrrolidin-1-yl}-8-methyl-7-phenylpyridinyl[1,2-α; 2′, 3′-d]-imidazole-9-carbonitrile), (their structures are shown in Fig. 1), and nine other derivatives were synthesized in our laboratories. The drugs were dissolved in dimethyl sulfoxide and were used for the biological test with the final concentration of dimethyl sulfoxide at less than 1%.

**Construction of CY-3a, CY-4a, and CY-5a.** SKN1 (a homologue of SKN6) of **C. albicans** and SKN1, was obtained from our KRE6 pUXS4. ScHIS3 cassette obtained by digesting pRS403 (Stratagene, Cedar Creek, TX) so that they were translated into serine in parts of ScKRE6, which code for the N-terminal region of ScCASKN1-MP2 for pUAD9, yielding pUAD12 and pUAD11, respectively. The **Fig. 2**). First, we constructed a YIp-type plasmid containing a pUAS7 to generate pUAE3, pUAE4, and pUAE5, respectively. A fragment of pUAD7 to generate pUAE3, pUAE4, and pUAE5 did not have a promoter region. **S. cerevisiae** CY-1a expresses only wild-type ScKRE6, and CY-3a, CY-4a, and CY-5a express only chimeric KRE6.

**MIC determination.** The MIC for the yeast strain was measured by the microdilution method, reported by the National Committee for Clinical Laboratory Standards (NCCLS), except that the incubation temperature was 30°C (21). The initial cell densities were from 1 × 10^3 to 3 × 10^5 cells/ml in all tests. The lowest MIC producing an optically clear well (MIC-0) was used as an end point for the experiments with **S. cerevisiae**. The lowest MIC producing a prominent reductivity in turbidity (MIC-2) was used for the experiments with **Candida** species. To gain reproducible and precise MIC-2 values, an oxidation-reduction indicator, Alamar Blue (Biosource, Camarillo, CA), was added to MOPS-buffered RPMI medium (33). MIC-2 is defined as a 50% reduction compared with that of a drug-free control, with absorbance at 570 nm. In the test using MDM, SDB, YNB, or Spider or Lee’s medium, Alamar Blue was not used, and 50% reduction was measured spectrometrically (optical density at 600 nm [OD600]). OD600 ODwater was measured with a Wallac 1420 ARVOx (Wallac, Tokyo, Japan) multilabel counter. All experiments were performed in duplicate. When the results were not consistent, another experiment was conducted on a different day to determine the result.

**In vivo study.** Five-week-old Slc:ddY female mice (Japan SLC, Inc., Shizuoka, Japan) were used. All the experiments with animals were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Daiichi Pharmaceutical Co., Ltd. Systemic infections with **C. albicans** ATCC 90028 and **C. glabrata** ATCC 48435 were induced in neutropenic mice. Transient immunosuppression was induced by intraperitoneal treatment with 200 mg/kg of body weight of cyclophosphamide 4 days before and 1 day after the infection. Fungal cells grown overnight in SDA were collected, and suspensions were prepared with 0.1% (vol/vol) Tween 80 (Wako) in saline. Infections were induced by the injection of **C. albicans** (2.6 × 10^6 cells) or **C. glabrata** (2.4 × 10^6 cells) via the tail vein. The drugs were administered orally three times daily for 1 day, starting 1 hour after inoculation at a dose of 3.3 or 10 mg/kg of body weight for D21-6076. In all the experiments, each group contained 10 mice, and the control group received 0.2 ml of 5% glucose solution with 1% (vol/vol) lactic acid. The mortality of the mice was recorded for 14 or 30 days after infection.

**Inhibition of β-1,6-glucan synthesis in whole cells.** The effects of D21-6076 on β-1,6-glucan synthesis were evaluated by the method described previously (11). Simply, exponentially growing cells of **C. albicans** ATCC 90028, **C. glabrata** ATCC 48435, or **C. krusei** ATCC 44507 were suspended in RPMI 1640 medium to give approximately 0.6 absorbance at 595 nm. After drug solution and [14C]glucose were added, the reaction tubes were incubated at 30°C with occasional shaking. After 3 h of incubation, samples were taken, and crude fractions of (1,3)-β-glucan, chitin, mannan, and (1,6)-β-glucan were prepared as follows. The harvested cells were extracted with 3% NaOH at 80°C for 1 h. Mannan fractions were prepared from the supernatant using Fehling’s reaction. Insoluble materials were washed and digested with Zymolyase 100T (Seikagaku Kougyou) overnight. After digestion, insoluble material was harvested as a chitin fraction. The supernatants were taken as glucan fractions [(1,3)-β-glucan fraction plus (1,6)-β-glucan fraction] and were dialyzed overnight. After dialysis, samples were taken as (1,6)-β-glucan fractions. The radioactivity of each fraction was counted.

**TABLE 1. S. cerevisiae strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPH500</td>
<td>mata ade2 his3 leu2 lys2 trp1 ura3</td>
<td>28; Kitamura et al., U.S. patent application 20040091949; international patent application PCT/JP01/03630 (2003)</td>
</tr>
<tr>
<td>AY-10c</td>
<td>∆skn1::URA3 ade2 his3 leu2 in YPH500 background</td>
<td></td>
</tr>
<tr>
<td>CY-1a*</td>
<td>∆kre6::HIS3 pUAE1 (ScKRE6) in AY-10c</td>
<td>This study</td>
</tr>
<tr>
<td>CY-3a*</td>
<td>∆kre6::HIS3 pUAE3 (ScKRE6-CaSKN2) in AY-10c</td>
<td>This study</td>
</tr>
<tr>
<td>CY-4a*</td>
<td>∆kre6::HIS3 pUAE4 (ScKRE6-CaKRE6) in AY-10c</td>
<td>This study</td>
</tr>
<tr>
<td>CY-5a*</td>
<td>∆kre6::HIS3 pUAE5 (ScKRE6-CaKRE6) in AY-10c</td>
<td>This study</td>
</tr>
</tbody>
</table>

*The strains were constructed by the methods illustrated in Fig. 2. CY-1a, CY-3a, CY-4a, and CY-5a are designed to express ScKre6p or the fusion protein of ScKre6p and CaSKN1.
TABLE 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Purpose</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCKRE6-Sen3</td>
<td>SckRE6</td>
<td>PCR</td>
<td>Forward</td>
<td>5'-CGGGCGCGTAAACAAACAGCAGACAAAAACCG-3'</td>
</tr>
<tr>
<td>SCKRE6-Anti3</td>
<td>SckRE6</td>
<td>PCR</td>
<td>Reverse</td>
<td>5'-CGAGGGCTTTGTTCCATAGGCCCATTTGGA-3'</td>
</tr>
<tr>
<td>CASKN1-Sen2</td>
<td>CaSKN1</td>
<td>PCR</td>
<td>Forward</td>
<td>5'-GCAGGATCTGATCTAGGAGATG-3'</td>
</tr>
<tr>
<td>CASKN1-Ant2</td>
<td>CaSKN1</td>
<td>PCR</td>
<td>Reverse</td>
<td>5'-GCCCGCGCTAATATAGGGGGTTTGTTT-3'</td>
</tr>
<tr>
<td>CAKRE6-Sen4</td>
<td>CaKRE6</td>
<td>PCR</td>
<td>Forward</td>
<td>5'-CCTTCAAAATTACATCAGC-3'</td>
</tr>
<tr>
<td>CAKRE6-Anti4</td>
<td>CaKRE6</td>
<td>PCR</td>
<td>Reverse</td>
<td>5'-CGGGATCCCGGACTGCCAGGAGC-3'</td>
</tr>
<tr>
<td>CASKN1-mp1</td>
<td>CaKRE6</td>
<td>Mutagenesis</td>
<td></td>
<td>5'-GGTACATTAGAATATGCGCTTTTACAAATATTG-3'</td>
</tr>
<tr>
<td>CASKN1-MP2</td>
<td>CaKRE6</td>
<td>Mutagenesis</td>
<td></td>
<td>5'-GGGGAAATTTGCGAAATTACCCCGG-3'</td>
</tr>
<tr>
<td>CASKN1-MP1</td>
<td>CaSKN1</td>
<td>Mutagenesis</td>
<td></td>
<td>5'-CCAAGGGTATCCTGCAATGAGTTTGGC-3'</td>
</tr>
</tbody>
</table>

with a toluene scintillator. The radioactivity of each β,1-3-glucan fraction was calculated by subtracting the radioactivity of the β,1,6-glucan fraction from that of the glucan fraction.

Fluorescent microscopy and TEM. C. albicans ATCC 90028 (1 × 10⁶ cells/ml) was treated with or without 1 µg/ml D11-2040 in MOPS-buffered RPMI for 6 h with shaking. The cells were harvested and were chemically fixed in 3% glutaraldehyde (EM Science, Tokyo, Japan)-0.1 M phosphate buffer (Kanto Chemical) for 2 h and then washed three times with 0.1 M phosphate buffer. To observe its effects on the mannan layer, parts of the cells were harvested and suspended in phosphate buffer containing concanavalin A-fluorescein conjugate (Molecular Probes, Eugene, OR) at the concentration of 10 µg/ml (34). After being stained for 30 min, the cells were washed with phosphate buffer and examined using a fluorescent microscope (Leica model DMLB100; Solms, Germany). Images were acquired using a digital charge-coupled-device camera (Olympus model DP70; Tokyo, Japan). The rest of the cells were further prepared for transmission electron microscopy (TEM). The samples were postfixed in 2% osmium tetroxide prepared in the same buffer for 2 h. This was followed by several washes and dehydration, and finally, the cells were embedded in Spurr's low-viscosity resin (28). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a TEM (Hitachi H-500; Tokyo, Japan).

Adherence assay. The adherence assay was performed fundamentally as described by Fratti et al. (7) using A549 human lung cancer cells. The human cells were grown to confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C (5% CO₂) in a 6-well culture plate. C. albicans cells were treated with D21-6076 in MOPS-buffered RPMI at 30°C overnight without shaking. The established monolayers of A549 cells were sequentially washed twice with 2 ml of Dulbecco’s phosphate-buffered saline (DPBS), overlaid with either 100 or 200 drug-treated C. albicans cells in 1 ml of DPBS, and incubated at 37°C for 45 min in an atmosphere of air containing 5% CO₂. Following incubation, monolayers of cells were washed twice with 2 ml of warm DPBS to remove nonadhering cells, and they were then covered with 2 ml of warm SDA. Yeast colonies appearing after 48 h of growth at 30°C were counted. The experiments were conducted in triplicate.

Effect on hyphal growth. Exponentially growing cells of C. albicans ATCC 90028 were suspended in HFM-7. Cell suspensions with or without drugs were cultured on type I collagen-coated 24-well plates (Iwaki, Tokyo, Japan) to let the cells tightly adhere to the bottom of the wells. After 6 or 18 h of incubation without shaking at 37°C, cells were examined using a light microscope (Olympus model LE7). Images were acquired using a digital charge-coupled-device camera. In order to quantitate the extent of hyphal growth, a crystal violet staining assay was carried out using the methods reported by Wakabayashi et al. (38), with slight modifications. Yeast-form cells of C. albicans at 1 × 10⁶ cells/ml were cultured in RPM medium with or without drugs using a 96-well flat-bottom microplate. After static incubation at 37°C in a 5% CO₂ atmosphere for 24 h, the medium in the wells was gleyly discarded, and the adherent Candida mycelia were sterilized by treatment with 70% ethanol, followed by washing with 0.25% sodium dodecyl sulfate (SDS) and water. The remaining mycelia were stained with 0.02% crystal violet and washed with 0.25% SDS and water. After the microplates were dried, 150 µl of isopropanol containing 0.04 N HCl and 50 µl of 0.25% SDS were added to the wells and mixed. The OD₄₉₀ was measured with a Wallac 1420 ARVOxx multilabel counter. The IC₅₀ was defined as the lowest drug concentration that results in a 50% decrease in absorbance compared with that of the drug-free control.
RHVE and model of vaginal candidiasis. The human epithelium used for the in vitro model of vaginal candidiasis was supplied by SkinEthic Laboratories (Nice, France). It was obtained by culturing transformed human keratinocytes of cell line A431 derived from a valval epidermoid carcinoma (26). Infection experiments were performed by the procedure described by Schaller et al. (27). Reconstituted human vaginal epithelium (RHVE) was infected with 2 experiments were performed by the procedure described by Schaller et al. (27). Reconstituted human vaginal epithelium (RHVE) was infected with 2 × 10⁶ cells of C. albicans ATCC 90028, and various concentrations of D21-6076 were added to the maintenance medium of the epithelial culture. After 24 h of incubation at 37°C in a 5% CO₂ atmosphere, a part of each specimen was fixed with formaldehyde. Semithin sections were studied with a light microscope equipped with a digital camera after being stained with p-aminosalicylic acid and methylene blue.

RESULTS

In vitro and in vivo activities of D21-6076. The antifungal activities of D11-2040 and D21-6076 against Candida strains were measured by the conventional NCCLS method. The results are summarized in Table 3. D11-2040 and D21-6076 showed potent activities against C. glabrata and C. krusei, which are 8 to 64 times stronger than D75-4590. The MICs of both compounds for C. glabrata are lower than those for C. krusei. Although slight growth reductions with significant morphological changes were visible at a wide range of drug concentrations, the MICs of both compounds obtained for C. albicans strains were >32 μg/ml. To comprehend the effects of the growth medium on their antifungal activity, their MICs for all the species tested in the following increasing order of activity, as well (data not shown). Next, the cell wall defects caused by the inhibition of β-1,6-glucan synthesis due to drug treatment were microscopically examined. C. albicans cells growing in a budding form were treated with D11-2040 for a longer amount of time (6 h) to clearly observe the cell wall defect and were evaluated by TEM. As expected, the cell walls of the drug-treated cells lack the darkly stained outer layer which is thought to be primarily composed of mannoproteins (Fig. 5A and B). A similar phenotype was observed in the KRE6 null mutant of S. cerevisiae as well (25). To confirm the degradation of the mannan layer, the drug-treated cells were stained with fluorescein-conjugated concanavalin A (34) and observed by fluorescent microscopy. A significant decrease in the fluorescence level was seen by drug treatment at concentrations of 0.25 μg/ml or more (Fig. 5C).

The effects of D21-6076 on the invasion process of the Candida cell. We next investigated the effects of D21-6076 on each process of the invasion. First, its inhibitory effects on yeast cells adherent to the monolayer of mammalian cells (A549) were

### Table 3. MICs of the compounds in NCCLS methods

<table>
<thead>
<tr>
<th>Strain</th>
<th>D75-4590</th>
<th>D11-2040</th>
<th>D21-6076</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans ATCC 24433</td>
<td>8</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>C. albicans ATCC 90028</td>
<td>16</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>C. glabrata ATCC 48435</td>
<td>1</td>
<td>0.016</td>
<td>0.125</td>
</tr>
<tr>
<td>C. krusei ATCC 44507</td>
<td>16</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* MICs were determined using MOPS-buffered RPMI as the medium, and MIC-2 was used as the end point.

### Table 4. MICs of the compounds against C. albicans ATCC 24433 in various media

<table>
<thead>
<tr>
<th>Medium</th>
<th>D11-2040</th>
<th>D21-6076</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>MEM</td>
<td>&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>SDB</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>YNB</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Lee’s</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>Spider</td>
<td>0.063</td>
<td>0.125</td>
</tr>
</tbody>
</table>

* MIC-2 was used as the end point. Abbreviations: RPMI, MOPS-buffered RPMI; YNB, yeast nitrogen base.
measured. As shown in Fig. 6, D21-6076 strongly inhibited the adherence of all tested fungal cells to mammalian cells in the following increasing order of activity: *C. glabrata*, *C. albicans*, and *C. krusei*. Second, the effect of D21-6076 on the hyphal elongation of *C. albicans* was microscopically observed using Lee’s medium (13). As shown in Fig. 7, D21-6076 clearly suppressed hyphal elongation at a concentration of 0.25 μg/ml. Similar inhibitory effects were observed when serum-containing medium (HFM-7) or Spider medium was used to induce hyphal elongation. Finally, to confirm its effect on the invasion process comprehensively, we assessed its efficacy in a vaginal candidiasis model based on RHVE. In a no-drug control well, *C. albicans* cells had attached to the epithelial cells and invaded into the RHVE, with hyphal formation within the first few hours after the infection. Extensive penetration of *Candida* cells was observed after 1 day of infection, along with vegetative growth in the medium above the RHVE. *Candida* cells were also detected in the medium below the RHVE as well. In contrast, in the medium with D21-6076, few *Candida* cells were attached to the mammalian cells, and no invasion was observed (Fig. 8). Although the fungal cells grew well in the medium above the RHVE, no cells were detected in the medium below the RHVE. These results suggested that D21-6076 had the potential to inhibit the invasion process of *C. albicans* cells into mammalian tissue. Similar results were observed in another experiment using reconstituted tissue consisting of KMST-6 and Caco-2 cells (data not shown).

The relationship between inhibition of β-1,6-glucan synthesis, adherence, and hyphal elongation. In theory, β-1,6-glucan inhibitors promote the release of the cell wall proteins, leading to a defect of *C. albicans* cells in adhesion and hyphal formation. If this is the case, positive correlations should be observed among the activities of inhibitors against these events. To confirm this, the activities of eight derivatives of D21-6076 on each event were compared. The inhibitory activities on hyphal elongation were quantified by a crystal violet staining assay (38). As shown in Fig. 9, good correlations were observed between the inhibitory effects on the β-1,3-glucan synthesis and adherence as well as that on the β-1,6-glucan synthesis and hyphal elongation. In addition, the strength order of the activity of D21-6076 against *C. glabrata*, *C. albicans*, and *C. krusei* is consistent in β-1,6-glucan inhibition and adherent tests, which also supports the contention that the inhibition of β-1,6-glucan contributes to the loss of the adherent nature of fungal cells.

Inhibitory effects of D21-6076 on each KRE6 homologue of *C. albicans*. One of the questions that remains unsolved is why...
D21-6076 does not inhibit the budding growth of *C. albicans* in spite of its potent inhibitory activities on β-1,6-glucan synthesis. Mio et al. isolated two homologues of *ScKRE6* in *C. albicans*, namely, *CaKRE6* and *CaSKN1*, and their involvement in β-1,6-glucan synthesis (18). We have also isolated two homologues of *ScKRE6* in *C. albicans*, one of which is *CaKRE6*, but the other was found to be different from *CaSKN1*. Since Northern blotting revealed that the mRNA of the novel *KRE6* homologue was detectable but was much lower than that of *CaKRE6* (data not shown), we tentatively named it *CaSKN2* (the nucleotide sequence is available in NCBI under accession number XM_714950). The existence of three homologues in the genome of five strains of *C. albicans* was confirmed by PCR amplification using a specific primer for each homologue. Although Mio et al. expected that *CaKRE6* would be an essential gene because they never achieved a homozygous *CaKRE6* null
Correlation coefficient, \( R^2 \), does not inhibit all of the Kre6p homologues of C. albicans (the construction is shown in Fig. 2) and compared its susceptibility against that of the \( \beta-1,6 \)-glucan inhibitors. Although these transformants grow significantly slower than the parent strain, the inhibitory effects of D21-6076 can easily be measured. As shown in Table 5, the MICs of D21-6076 against these mutants (CY-3a, CY-4a, CY-5a) were similar to that against the parent strain, suggesting that D21-6076 most likely inhibits all of the Kre6p homologues of C. albicans.

**DISCUSSION**

*C. albicans* is a polymorphic fungus capable of converting its cell shape from a budding yeast into a filamentous form. The pathogenicity of *C. albicans* has been attributed to several factors that enable the pathogen to damage and penetrate tissues, to escape host immune systems, and to establish systemic infections (12, 17). Three important factors for pathogenicity are adherence to mammalian cells, hyphal elongation, and protease secretion. That is, yeast cells adhere to mammalian cells and then invade tissue by switching their growth from a unicellular yeast form into pseudohyphae or hyphae, with proteases attacking the host cell membranes throughout the process (17). Genetic analyses have provided a great deal of information about the genes involved. Some of the proteins encoded by *ALS* and the *CRH* family are essential for adhesion (2, 23). Hwp1p is a hypha-specific protein which is a substrate for mammalian transglutaminases and mediates covalent attachment (22, 31). Secretory aspartic protease (SAP) and the phospholipase (PLD) family are involved in the invasion into tissue (1, 32). Most of the proteins which play an important role in this process are not essential for budding growth, and null mutants lacking such important proteins for the invasive process have been shown to be much less virulent in an animal model. The important fact for our study is that most of these proteins have the typical features of glycosylphosphatidylinositol–anchored proteins, with a signal peptide, a serine- and threonine-rich region, and a potential COOH-terminal domain for glycosylphosphatidylinositol anchor attachment, and are most likely to covalently link to the \( \beta-1,3 \)-glucan–chitin network via \( \beta-1,6 \)-glucan (6). Hence, the lack of \( \beta-1,6 \)-glucan

### Table 5. MICs of the compounds against *S. cerevisiae* expressing various KRE6 homologues

<table>
<thead>
<tr>
<th>Strain</th>
<th>KRE6 homologue expressed</th>
<th>MIC (( \mu )g/ml) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D75-4590</td>
</tr>
<tr>
<td>AY-10c</td>
<td>ScKRE6</td>
<td>8</td>
</tr>
<tr>
<td>CY-1a</td>
<td>ScKRE6</td>
<td>8</td>
</tr>
<tr>
<td>CY-3a</td>
<td>ScKRE6-CaSKN2</td>
<td>8</td>
</tr>
<tr>
<td>CY-4a</td>
<td>ScKRE6-CaKRE6</td>
<td>16</td>
</tr>
<tr>
<td>CY-5a</td>
<td>ScKRE6-CaSKN1</td>
<td>16</td>
</tr>
</tbody>
</table>

* a MICs were determined using MOPS-buffered RPMI as the medium. MIC-0 was used as the end point.
would result in the release of these proteins from fungal cells, making them avirulent. Indeed, it is reported that the genes involved in β-1,6-glucan synthesis, such as BIG1 and KRE5, are not essential for budding growth but are essential for the full virulence in C. albicans (9, 36).

Considering the fact given above, it is reasonable that D21-6076, which potently inhibited β-1,6-glucan synthesis, also inhibited C. albicans to adhere to the mammalian cell and to allow hyphal elongation to occur. D21-6076 prolonged the survival of mice infected by C. albicans with serum concentrations less than 1 μg/ml. The inhibitory effects of D21-6076 on its invasion processes were observed at a concentration of 1 μg/ml or less, while it only slightly affected the budding growth of C. albicans, even at a concentration of 32 μg/ml. Although there remain many issues to be addressed, it seems reasonable to think that D21-6076 showed efficacy against C. albicans in an animal model, due mainly to its inhibition of the invasion process.

Although much attention has been focused on the invasion process as a target for new antifungal agents (1, 4, 23, 31, 32), almost nothing is known about the actual potency of such drugs in vivo due to the lack of an actual drug. From this point of view, D21-6076 and its derivatives could be valuable tools. One of the concerns is that a drug without activities against budding growth may only temporally suppress the progress of infection, and removal of the drug may result in treatment failure soon afterward. It is true that treatment with D21-6076 showed 100% survival of mice infected with C. glabrata, while it gave only a partial response in those infected with C. albicans. However, this difference is reasonable considering that D21-6076 showed stronger activity against C. glabrata than C. albicans in all the in vitro evaluations, including an adherence test. The fact that 1-day treatment of D21-6076 gave 100% survival of mice infected with C. albicans even on day 8 indicated that it may act in more ways than just by inhibiting the progress of infection. Moreover, a 5-day treatment with another derivative, which has a similar in vitro antifungal profile, leads to 100% survival, even at 30 days after infection (data not shown). These data suggested that a β-1,6-glucan inhibitor by itself could treat systemic candidiasis. Still, more-comprehensive and detailed studies are needed to further understand the effect of such a compound in vivo.

Although indirectly, our study suggested that D21-6076 inhibited all Kre6p homologues of C. albicans. Therefore, the most simple and reasonable explanation for the poor activity of D21-6076 against yeast-type C. albicans cells is that KRE6 genes are not essential for its budding growth. Several other explanations, however, are possible. One possibility is that D21-6076 inhibits the interaction of Kre6p and other proteins or indirectly inhibits Kre6p. As we previously reported, our assumption that the primary target of these derivatives is Kre6p is based on the fact that a mutation in KRE6 confers resistance to the drugs with S. cerevisiae (11). Meanwhile, Kre6ps are suggested to be phosphorylated proteins and to have interaction with other proteins, including Keg1p (20). Therefore, it is possible that D21-6076 inhibits the proteins interacting with Kre6p and that a mutation in KRE6 confers resistance because it affects the conformation of the binding site of D21-6076. Studies are under way to demonstrate the direct interaction between these compounds and Kre6p.

REFERENCES


