Piperazine Propanol Derivative as a Novel Antifungal Targeting 1,3-β-D-Glucan Synthase

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1,3-β-D-Glucan synthase, which synthesizes a main component of fungal cell wall, is one of the promising targets for antifungal agents. In order to identify novel chemical classes of 1,3-β-D-glucan synthase inhibitors, we screened a chemical library monitoring inhibition of the Candida albicans 1,3-β-D-glucan synthase activity. The piperazine propanol derivative GSI578 [(2,6-difluoro-phenyl)-carbamic acid 3-(4-benzothiazol-2-yl-piperazine-1-yl)-propyl ester] was identified as a potent inhibitor against 1,3-β-D-glucan synthase with an IC₅₀ value of 0.16 μM. GSI578 exhibited in vitro antifungal activity against pathogenic fungi including C. albicans and Aspergillus fumigatus. Temperature-sensitive mutations of the FKS1 gene in the BBY2 background of Saccharomyces cerevisiae, where FKS1 and FKS2 encode putative catalytic subunits of 1,3-β-D-glucan synthase, altered sensitivity to GSI578. This suggests that the antifungal activity of the piperazine propanol derivative has an effect on 1,3-β-D-glucan synthase inhibition. Results of our initial evaluation suggest that the piperazine propanol derivative is a novel chemical structure of the class of antifungals which inhibit fungal cell growth by inhibiting fungal 1,3-β-D-glucan synthase.

Key words cell wall; piperazine propanol; 1,3-β-D-glucan synthase; antifungal

Antifungal agents, polyenes, and azoles are available for the treatment of serious and life-threatening fungal infection, mainly caused by Candida albicans and Aspergillus fumigatus. However, their clinical uses are restricted due to toxicity (polyenes), fungistatic activity (azoles), and the emergence of resistant isolates (azoles).1,2 To overcome these problems, novel antifungal agents with a different mode of action are in demand.

1,3-β-D-Glucan synthase (UDP-glucose: 1,3-β-D-glucan 3-β-D-glucosyltransferase; EC 2.4.1.34), catalyses the synthesis of 1,3-β-D-glucan, the most abundant component of the fungal cell wall. 1,3-β-D-Glucan synthase is composed of a putative catalytic subunit with sixteen putative transmembrane domains encoded by a pair of closely related genes, FKS1/GSC1/CWH53/ETG1/CND1/PBR1/YLR342W and FKS2/GSC2/G4074/YGRO32W, and a regulatory subunit encoded by the RHO1/RHO10 in Saccharomyces cerevisiae. In C. albicans, the catalytic and regulatory subunits are encoded by CaFKS1/GSC1,11,12 and CaRHO1,13 respectively. 1,3-β-D-Glucan synthase has three features of a promising target for an antifungal agent:1,2,14,15 1) its function is essential for growth, proven by the fact that the disruption of the genes for the catalytic subunit of 1,3-β-D-glucan synthase is a lethal event in S. cerevisiae, C. albicans, and Cryptococcus neoformans; 2) mammalian cells have no comparable cell wall, indicating that a 1,3-β-D-glucan synthase inhibitor would be highly selective to fungal cells; and 3) genes for catalytic subunits have been identified from several pathogenic fungi, such as C. albicans, Cr. neoformans, A. fumigatus, and Paracoccidioides brasiliensis, and are highly homologous to each other. It is likely that 1,3-β-D-glucan synthase inhibitors might possess a broad spectrum of antifungal activity.

Several 1,3-β-D-glucan synthase inhibitors have been identified, such as echinocandins and papulacandins.19,20 Papulacandins are liposaccharide inhibitors isolated from Papularia sphaeroasperma. The echinocandins, including cilofungin, aculeacins, and pneumocandins, are cyclic hexapeptides with a lipophilic side chain such as a linoleoyl or myristoyl moiety. From this chemical class, caspofungin and micafungin have been launched recently, and anidulafungin is being developed. Aerothricin3/FR901469, a cyclic lipopeptidolactone composed of twelve amino acids and a 3’-hydroxypalmitoyl moiety, is another class of lipopeptide inhibitors recently isolated.21-24 More recently, another class of inhibitors, terpenic glycosides, have been identified from natural sources.25,26 We describe here a novel piperazine propanol derivative of 1,3-β-D-glucan synthase inhibitor identified from a chemical library. In vitro antifungal activity of this inhibitor was assessed. Susceptibility analysis of S. cerevisiae fks temperature-sensitive mutants supports the conclusion that the piperazine propanol derivative inhibits fungal cell growth by inhibiting fungal 1,3-β-D-glucan synthase.

MATERIALS AND METHODS

Fungal Strains As reference strains, ATCC strains from the American Type Culture Collection (Rockville, MD, U.S.A.) and IFO strains from the Institute for Fermentation Osaka (Osaka, Japan) were purchased. Other clinical isolates were obtained from hospitals in Japan. The S. cerevisiae strains used in this study are listed in Table 1 and were cultivated in YPD medium (2% Bacto peptone, 1% Bacto yeast extract, and 2% glucose). The YOC1071, YOC1077, YOC1081, YOC1085, and YOC1089 have the same FKS1 alleles as YOC1072, YOC1078, YOC1082, YOC1086, and...
were added. The reaction mixture was stirred at 70°C for 1 h then concentrated under vacuum. The crude material was purified by column chromatography (2% methanol in dichloromethane) to afford (2,6-difluoro-phenyl)-carbamic acid 3-(4-benzothiazol-2-yl-piperazine-1-yl)-propyl ester.

**Purification of 1,3-β-D-Glucan Synthase and Measurement of Its Inhibition** Membrane preparation and partial purification of 1,3-β-D-glucan synthase were described previously.11,13) 1,3-β-D-Glucan synthase activity measurement was performed as previously described.31 Briefly, the membrane fraction was prepared from late-log phase cells and then the enzyme was partially purified by product entrapment. The partially purified enzyme was mixed with 0.1 mM UDP-[6-3H]-glucose (222 Bq, Amersham), 75 mM Tris–Cl pH 7.5, 0.75 mM EDTA, 25 mM KF, 20 μM guanosine-5’-(ythio)triphosphate, 0.1% BSA, and 7.8% Glycerol. Test compounds were serially diluted and added. The reaction was carried out in 100 μl at 25 °C for 30 min. After filtration and twice washing with 70% ethanol, the radiolabeled glucose incorporated into the polymerized glucan on the filter was quantified by measuring the radioactivity.

**In Vitro Antifungal Activity** Antifungal susceptibility assay was performed by the NCCLS M27-A2 microdilution method using modified media: Yeast Nitrogen Base (YNB) supplemented with 1% glucose and 0.25% K2HPO4, was used for the Candida spp. and, when solidified with 0.2% low-melting temperature agarose, used for *A. fumigatus*. The inoculum size for all strains was 1 to 3×10^4 conidia/ml in final concentration. 96-well plates were incubated at 35 °C for 1 d for *C. albicans* and *A. fumigatus*, 2 d for *C. glabrata* ATCC2001, and 3 d for IFO0005. MICs were determined by the concentration of drug that produced an 80% reduction of turbidity compared to that of the drug free control measured by spectrophotometer at an optical density of 600 nm. The susceptibility of *S. cerevisiae* strains was measured the same, except for temperatures of 25 °C for null mutants and 25 °C, 31 °C, 32 °C, and 33 °C for ts mutants. IC_{50} values refer to the compound concentrations that gave 50% inhibition of cell growth compared with the control.

**RESULTS AND DISCUSSION**

In order to identify novel chemical classes of antifungals, screening of a chemical library was conducted by monitoring the inhibition of 1,3-β-D-glucan synthase activity. 1,3-β-D-Glucan synthase was partially purified by product entrapment from the pathogenic fungus *C. albicans* IFO1060.11,13) We identified GS1578 [(2,6-Difluoro-phenyl)-carbamic acid 3-(4-benzothiazol-2-yl-piperazine-1-yl)-propyl ester] as a potent 1,3-β-D-glucan synthase inhibitor (Fig. 1). The IC_{50} value of GS1578 against 1,3-β-D-glucan synthase was 0.16 μM, whereas that of aerothricin3 was 0.012 μM.

The inhibitory activity of GS1578 against the growth of various fungal strains was compared to caspofungin, aerothricin3, and amphotericin B (Table 2). GS1578 exhibited antifungal activity against *C. albicans*, *C. glabrata*, and *A. fumigatus* comparable to the reference antifungals.

To explore the inhibition of 1,3-β-D-glucan synthesis by the piperazine propanol derivative, we measured the susceptibility of fks1 ts mutants in the Δfks2 background at semipermissive and permissive temperatures. Figure 2 shows that ts

### Table 1. *S. cerevisiae* Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/ Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A451</td>
<td>MATα ura3 leu2 trp1 can1</td>
<td>Refs. 6, 29</td>
</tr>
<tr>
<td>Δfks1</td>
<td>MATα ura3 leu2 trp1 can1</td>
<td>Refs. 6, 29</td>
</tr>
<tr>
<td>Δfks2</td>
<td>MATα ura3 leu2 trp1 can1</td>
<td>Refs. 6, 29</td>
</tr>
<tr>
<td>YOC1001</td>
<td>MATα ade2 his3 leu2 lys2 trp1 ara3 fuse::URA3 ade3::FKS1::TRP1</td>
<td>Ref. 28</td>
</tr>
<tr>
<td>YOC1095</td>
<td>MATα ade2 his3 leu2 lys2 trp1 ara3 fuse::URA3 lacZ::TRP1 ade3::fxl-1014::TRP1</td>
<td>Y. Ohya</td>
</tr>
<tr>
<td>YOC1071</td>
<td>MATα ade2 his3 leu2 lys2 trp1 ara3 fuse::URA3 lacZ::TRP1 ade3::fxl-1125::TRP1</td>
<td>Y. Ohya</td>
</tr>
<tr>
<td>YOC1077</td>
<td>MATα ade2 his3 leu2 lys2 trp1 ara3 fuse::URA3 lacZ::TRP1 ade3::fxl-1144::TRP1</td>
<td>Y. Ohya</td>
</tr>
<tr>
<td>YOC1081</td>
<td>MATα ade2 his3 leu2 lys2 trp1 ara3 fuse::URA3 lacZ::TRP1 ade3::fxl-1163::TRP1</td>
<td>Y. Ohya</td>
</tr>
<tr>
<td>YOC1085</td>
<td>MATα ade2 his3 leu2 lys2 trp1 ara3 fuse::URA3 lacZ::TRP1 ade3::fxl-1182::TRP1</td>
<td>Y. Ohya</td>
</tr>
<tr>
<td>YOC1089</td>
<td>MATα ade2 his3 leu2 lys2 trp1 ara3 fuse::URA3 lacZ::TRP1 ade3::fxl-1163::TRP1</td>
<td>Y. Ohya</td>
</tr>
</tbody>
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**Chemicals** The chemical library of compounds was obtained from Alanex Corp. (CA, U.S.A.). Amphotericin B (AMB) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aerothricin3 was prepared as previously described.29)

GS1578 [(2,6-Difluoro-phenyl)-carbamic acid 3-(4-benzothiazol-2-yl-piperazine-1-yl)-propyl ester] was synthesized as follows. To a solution of 2-chloro-benzothiazole in toluene, piperazine and diisopropylethylamine were added. The reaction mixture was stirred at 120 °C for 18 h, then added water and extracted with dichloromethane. The partially purified enzyme was mixed with 0.1 mM UDP-[6-3H]-glucose (222 Bq, Amersham), 75 mM Tris–Cl pH 7.5, 0.75 mM EDTA, 25 mM KF, 20 μM guanosine-5’-(ythio)triphosphate, 0.1% BSA, and 7.8% Glycerol. Test compounds were serially diluted and added. The reaction was carried out in 100 μl at 25 °C for 30 min. After filtration and twice washing with 70% ethanol, the radiolabeled glucose incorporated into the polymerized glucan on the filter was quantified by measuring the radioactivity.

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deletion mutants of growth by inhibiting fungal 1,3-
dependently of the temperature. These results indicated that
cloheximide, which targets protein synthesis, inhibits inde-
glucan synthase inhibitor aerothricin3. The control drug cy-
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b temperature dependent manner, as well as to known 1,3-
mutants exhibited hypersensitive phenotype to GSI578 in a
temperature derivative on 1,3-
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Table 3. Susceptibility of S. cerevisiae fks Null Mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth inhibition: IC50 [μg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSI578</td>
</tr>
<tr>
<td>Δfks1</td>
<td>0.50±0.006</td>
</tr>
<tr>
<td>Δfks2</td>
<td>0.36±0.011</td>
</tr>
<tr>
<td>A451</td>
<td>0.35±0.007</td>
</tr>
</tbody>
</table>

The yeast growth was measured by absorbance at 600 nm. Values are the averages of three independent experiments and standard deviations.

The growth of fungi was measured by absorbance at 600 nm.

mutants exhibited hypersensitive phenotype to GSI578 in a temperature dependent manner, as well as to known 1,3-β-glucan synthase inhibitor aerthricin3. The control drug cycloheximide, which targets protein synthesis, inhibits independently of the temperature. These results indicated that piperazine propanol derivative GSI578 inhibits fungal cell growth by inhibiting fungal 1,3-β-glucan synthase.

We further explored the effect of the piperazine propanol derivative on 1,3-β-glucan synthesis. The Δfks1 mutant is known to be hypersensitive to aerthricin3 and L-733560, a close analogue of caspofungin, compared to Δfks2 and their parental strain A451.358 Therefore, the susceptibility of FKS deletion mutants of S. cerevisiae to piperazine propanol GSI578 was measured. Interestingly, GSI578 exhibited similar inhibitory activity to Δfks1, Δfks2, and their parental strain (Table 3) as well as the control drug cycloheximide. These results considered with the abovementioned results on the ts mutants, suggest that the mechanism of 1,3-β-glucan synthase inhibition by GSI578 is different from that of cyclic lipopeptide inhibitors such as aerthricin3 and caspofungin.

We here identified a novel chemical class of 1,3-β-glucan synthase inhibitors which exhibits potent in vitro antifungal activity. Susceptibility analysis of the fks mutant strains suggested that the piperazine propanol derivative inhibits 1,3-β-glucan synthase by an action mode different from known 1,3-β-glucan synthase inhibitors. Therefore, the piperazine propanol derivative might achieve 1,3-β-glucan synthase inhibition in strains resistant to known 1,3-β-glucan synthase inhibitors. Also, complete inhibition of 1,3-β-glucan synthase might be possible with our piperazine propanol derivative in combination with known 1,3-β-glucan synthase inhibitors.

The piperazine propanol GSI578 represents the first non-natural product inhibitor of 1,3-β-glucan synthase. With pharmacological assessment and chemical modification of the piperazine propanol derivative, therapies for fungal infections may possibly be expanded.

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REFERENCES