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

Osamu KONDOH,*[,a,b] Yukiko INAGAKI,[a,b] Hiroshi FUKUDA,[a,b] Eisaku MIZUGUCHI,[a,b] Yoshikazu OHYA,[c] Mikio ARISAWA,[a,b] Nobuo SHIMMA,[a,b] Yuko AOKI,[a,b] Masahiro SAKAITANI,[a,b] and Takahide WATANABE[b]

[a] *Kamakura Research Laboratories, Chugai Pharmaceutical Co., Ltd. (formerly Nippon Roche Research Center); 200 Kajiwara, Kamakura, Kanagawa 247–8530, Japan:* [b] *Nippon Roche Research Center; 200 Kajiwara, Kamakura, Kanagawa 247–8530, Japan: and* [c] *Department of Integrated Biosciences, Graduate School of Frontier Science, University of Tokyo; Bldg. FSB-101, 5–1–5 Kashiwanoha, Kashiwa, Chiba 277–8562, Japan.*
Received May 5, 2005; accepted August 25, 2005; published online September 7, 2005

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 IC_{50} 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 *Δfks2* 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*,[3—8] and a regulatory subunit encoded by the *RHO1*[9,10] 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:[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*,[6,8] *C. albicans*,[11,12] and *Cryptococcus neoformans*[16]; 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*,[11,12] *Cr. neoformans*,[16] *A. fumigatus*,[17] and *Paracoccidioides brasiliensis*,[18] 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 sphaerosperma*. 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 lipopeptidelactone 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, terpene 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

* To whom correspondence should be addressed. e-mail: kondoosm@chugai-pharm.co.jp

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

Strain	Genotype	Reference/ Source
A451	*MATα ura3 leu2 trp1 can1 aro7*	Refs. 6, 29
Δfks1	*MATα ura3 leu2 trp1 can1 aro7 gsc1::URA3*	Refs. 6, 29
Δfks2	*MATα ura3 leu2 trp1 can1 aro7 gsc2::LEU2*	Refs. 6, 29
YOC1001	*MATa ade2 his3 leu2 lys2 trp1 ura3 fks1::HIS3 fks2::LYS2 ade3::FKS1::TRP1*	Ref. 28
YOC1095	*MATa ade2 his3 leu2 lys2 trp1 ura3 fks1::HIS3 fks2::LYS2 ade3::fks1-28::TRP1*	Y. Ohya
YOC1071	*MATa ade2 his3 leu2 lys2 trp1 ura3 fks1::HIS3 fks2::LYS2 ade3::fks1-1014::TRP1*	Y. Ohya
YOC1077	*MATa ade2 his3 leu2 lys2 trp1 ura3 fks1::HIS3 fks2::LYS2 ade3::fks1-1104::TRP1*	Y. Ohya
YOC1081	*MATa ade2 his3 leu2 lys2 trp1 ura3 fks1::HIS3 fks2::LYS2 ade3::fks1-1125::TRP1*	Y. Ohya
YOC1085	*MATa ade2 his3 leu2 lys2 trp1 ura3 fks1::HIS3 fks2::LYS2 ade3::fks1-1144::TRP1*	Y. Ohya
YOC1089	*MATa ade2 his3 leu2 lys2 trp1 ura3 fks1::HIS3 fks2::LYS2 ade3::fks1-1163::TRP1*	Y. Ohya

YOC1090,[27] respectively. Construction of the temperature-sensitive (ts) strains and a wild-type *FKS1* control YOC1001 in the *Δfks1 Δfks2* background[28] will be described elsewhere (M. Abe, M. Minemura-Asakawa, T. Utsugi, M. Sekiya-Kawasaki, A. Hirata, H. Qadota, K. Morishita, T. Watanabe, and Y. Ohya, unpublished results).

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]

GSI578 [(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 organic phase was dried with magnesium sulfate and concentrated under vacuum. Recrystallization from dichloromethane and hexane gave 2-piperazine-1-yl-benzothiazole. To a solution of 2-piperazine-1-yl-benzothiazole in toluene, 3-bromo-propan-1-ol and diisopropylethylamine were added. The reaction mixture was stirred at 120 °C for 2 h, then added water and extracted with dichloromethane. The organic phase was dried with magnesium sulfate and concentrated under vacuum. The crude material was purified by column chromatography with 2—5% methanol in dichloromethane and recrystallized from dichloromethane and hexane to afford 3-(4-benzo-thiazol-2-yl-piperazine-1-yl)-propan-1-ol. To a solution of 3-(4-benzo-thiazol-2-yl-piperazine-1-yl)-propan-1-ol in tetrahydrofuran, 1,3-difluoro-2-isocyanato-benzene and catalytic amount of pyridine 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.[3] 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-^{3}H]-glucose (222 Bq, Amersham), 75 mM Tris–Cl pH 7.5, 0.75 mM EDTA, 25 mM KF, 20 μM guanosine-5′-(γthio)-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% K_2HPO_4 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 GSI578 [(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 GSI578 against 1,3-β-D-glucan synthase was 0.16 μM, whereas that of aerothricin3 was 0.012 μM.

The inhibitory activity of GSI578 against the growth of various fungal strains was compared to caspofungin, aerothricin3, and amphotericin B (Table 2). GSI578 exhibited antifungal activity against *C. albicans*, *C. glabrata*, and *A. fumigatus* comparative 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

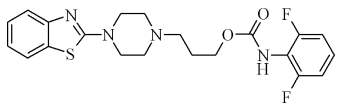

Fig. 1. The Chemical Structure of GSI578

Table 2. Susceptibility of Pathogenic Fungi to GSI578

Strain		MIC [μg/ml]			
		GSI578	Aerothricin3	Caspofungin	AMB
C. albicans	ATCC48130	0.26	0.0078	0.18	0.044
	CY3003	0.83	0.056	0.32	0.089
	CY1123	1.3	0.044	0.36	0.19
	CY1124	0.71	0.0081	0.34	0.088
C. glabrata	ATCC2001	0.17	0.39	0.52	0.35
	IFO0005	0.19	0.70	0.72	0.35
A. fumigatus	CF1083	1.1	0.44	0.35	0.29
	CF1003	0.15	0.19	0.27	0.22
	CF924390	0.47	0.33	0.32	0.28

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

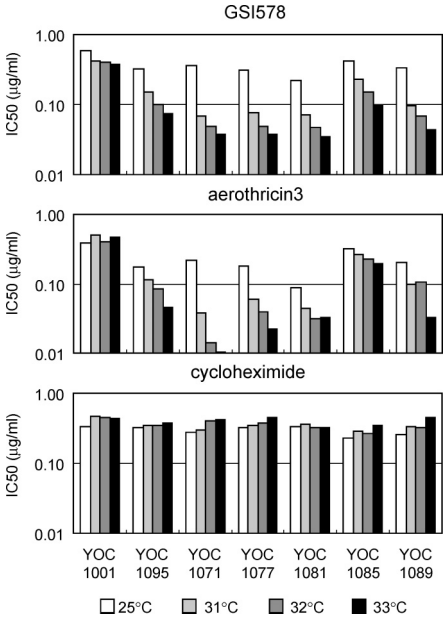

Fig. 2. Susceptibility of *S. cerevisiae* ts Mutants

The yeast growth was measured by absorbance at 600 nm. IC_{50} values at indicated temperatures are the averages of two independent experiments.

mutants exhibited hypersensitive phenotype to GSI578 in a temperature dependent manner, as well as to known 1,3-β-D-glucan synthase inhibitor aerothricin3. 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-D-glucan synthase.

We further explored the effect of the piperazine propanol derivative on 1,3-β-D-glucan synthesis. The *Δfks1* mutant is known to be hypersensitive to aerothricin3 and L-733560, a close analogue of caspofungin, compared to *Δfks2* and their parental strain A451.[8,29] 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-D-glucan synthase inhibition by GSI578 is different from that of cyclic lipopeptide inhibitors such as aerothricin3 and caspofungin.

Table 3. Susceptibility of *S. cerevisiae fks* Null Mutants

Strain	Growth inhibition: IC_{50} [μg/ml]		
	GSI578	Aerothricin3	Cycloheximide
Δfks1	0.50±0.006	0.0033±0.001	0.027±0.001
Δfks2	0.36±0.011	0.16±0.004	0.028±0.002
A451	0.35±0.007	0.12±0.005	0.030±0.001

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

We here identified a novel chemical class of 1,3-β-D-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-D-glucan synthase by an action mode different from known 1,3-β-D-glucan synthase inhibitors. Therefore, the piperazine propanol derivative might achieve 1,3-β-D-glucan synthase inhibition in strains resistant to known 1,3-β-D-glucan synthase inhibitors. Also, complete inhibition of 1,3-β-D-glucan synthase might be possible with our piperazine propanol derivative in combination with known 1,3-β-D-glucan synthase inhibitors.

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

Acknowledgements This work was supported in part by International Research Grant K-1023 from the Japan Health Sciences Foundation. We are grateful to F. Ford for proofreading this manuscript.

REFERENCES

1) Behrens-Baumann W., *Dev. Ophthalmol.*, **32**, 27—51 (1999).
2) Maertens J. A., Boogaerts M. A., *Curr. Pharm. Des.*, **6**, 225—239 (2000).
3) Castro C., Ribas J. C., Valdivieso M. H., Varona R., del Rey F., Duran A., *J. Bacteriol.*, **177**, 5732—5739 (1995).
4) Douglas C. M., Foor F., Marrinan J. A., Morin N., Nielsen J. B., Dahl A. M., Mazur P., Baginsky W., Li W., el-Sherbeini M., Clemas J. A., Mandala S. M., Frommer B. R., Kurtz M. B., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 12907—12911 (1994).
5) Eng W. K., Faucette L., McLaughlin M. M., Cafferkey R., Koltin Y., Morris R. A., Young P. R., Johnson R. K., Livi G. P., *Gene*, **151**, 61—71 (1994).
6) Inoue S. B., Takewaki N., Takasuka T., Mio T., Adachi M., Fujii Y., Miyamoto C., Arisawa M., Furuichi Y., Watanabe T., *Eur. J. Biochem.*, **231**, 845—854 (1995).
7) Ram A. F., Brekelmans S. S., Oehlen L. J., Klis F. M., *FEBS Lett.*, **358**, 165—170 (1995).
8) Mazur P., Morin N., Baginsky W., el-Sherbeini M., Clemas J. A., Nielsen J. B., Foor F., *Mol. Cell. Biol.*, **15**, 5671—5681 (1995).
9) Drgonova J., Drgon T., Tanaka K., Kollar R., Chen G. C., Ford R. A., Chan C. S., Takai Y., Cabib E., *Science*, **272**, 277—279 (1996).
10) Qadota H., Python C. P., Inoue S. B., Arisawa M., Anraku Y., Zheng Y., Watanabe T., Levin D. E., Ohya Y., *Science*, **272**, 279—281 (1996).
11) Mio T., Adachi-Shimizu M., Tachibana Y., Tabuchi H., Inoue S. B.,

Yabe T., Yamada-Okabe T., Arisawa M., Watanabe T., Yamada-Okabe H., *J. Bacteriol.*, **179**, 4096—4105 (1997).
12) Douglas C. M., D'Ippolito J. A., Shei G. J., Meinz M., Onishi J., Marrinan J. A., Li W., Abruzzo G. K., Flattery A., Bartizal K., Mitchell A., Kurtz M. B., *Antimicrob. Agents Chemother.*, **41**, 2471—2479 (1997).
13) Kondoh O., Tachibana Y., Ohya Y., Arisawa M., Watanabe T., *J. Bacteriol.*, **179**, 7734—7741 (1997).
14) Klis F. M., *Yeast*, **10**, 851—869 (1994).
15) Kurtz M. B., *ASM News*, **64**, 31—39 (1998).
16) Thompson J. R., Douglas C. M., Li W., Jue C. K., Pramanik B., Yuan X., Rude T. H., Toffaletti D. L., Perfect J. R., Kurtz M., *J. Bacteriol.*, **181**, 444—453 (1999).
17) Beauvais A., Bruneau J. M., Mol P. C., Buitrago M. J., Legrand R., Latge J. P., *J. Bacteriol.*, **183**, 2273—2279 (2001).
18) Pereira M., Felipe M. S., Brigido M. M., Soares C. M., Azevedo M. O., *Yeast*, **16**, 451—462 (2000).
19) Balkovec J. M., *Opin. Invest. Drugs*, **3**, 65—82 (1994).
20) Kurtz M. B., Douglas C. M., *J. Med. Vet. Mycol.*, **35**, 79—86 (1997).
21) Fujie A., Iwamoto T., Muramatsu H., Okudaira T., Nitta K., Nakanishi T., Sakamoto K., Hori Y., Hino M., Hashimoto S., Okuhara M., *J. Antibiot.*, **53**, 912—919 (2000).
22) Fujie A., Iwamoto T., Muramatsu H., Okudaira T., Sato I., Furuta T., Tsurumi Y., Hori Y., Hino M., Hashimoto S., Okuhara M., *J. Antibiot.*, **53**, 920—927 (2000).
23) Masubuchi K., Okada T., Kohchi M., Sakaitani M., Mizuguchi E., Shirai H., Aoki M., Watanabe T., Kondoh O., Yamazaki T., Satoh Y., Kobayashi K., Inoue T., Horii I., Shimma N., *Bioorg. Med. Chem. Lett.*, **11**, 395—398 (2001).
24) Masubuchi K., Okada T., Kohchi M., Murata T., Tsukazaki M., Kondoh O., Yamazaki T., Satoh Y., Ono Y., Tsukaguchi T., Kobayashi K., Ono N., Inoue T., Horii I., Shimma N., *Med. Chem. Lett.*, **11**, 1273—1276 (2001).
25) Onishi J., Meinz M., Thompson J., Curotto J., Dreikorn S., Rosenbach M., Douglas C., Abruzzo G., Flattery A., Kong L., Cabello A., Vicente F., Pelaez F., Diez M. T., Martin I., Bills G., Giacobbe R., Dombrowski A., Schwartz R., Morris S., Harris G., Tsipouras A., Wilson K., Kurtz M. B., *Antimicrob. Agents Chemother.*, **44**, 368—377 (2000).
26) Pelaez F., Cabello A., Platas G., Diez M. T., Gonzalez del Val A., Basilio A., Martan I., Vicente F., Bills G. E., Giacobbe R. A., Schwartz R. E., Onish J. C., Meinz M. S., Abruzzo G. K., Flattery A. M., Kong L., Kurtz M. B., *Syst. Appl. Microbiol.*, **23**, 333—343 (2000).
27) Dijkgraaf G. J., Abe M., Ohya Y., Bussey H., *Yeast*, **19**, 671—690 (2002).
28) Sekiya-Kawasaki M., Abe M., Saka A., Watanabe D., Kono K., Minemura-Asakawa M., Ishihara S., Watanabe T., Ohya Y., *Genetics*, **162**, 663—676 (2002).
29) Kondoh O., Takasuka T., Arisawa M., Aoki Y., Watanabe T., *J. Biol. Chem.*, **277**, 41744—41749 (2002).