Identification of a Novel Inhibitor Specific to the Fungal Chitin Synthase

INHIBITION OF CHITIN SYNTHASE 1 ARRESTS THE CELL GROWTH, BUT INHIBITION OF CHITIN SYNTHASE 1 AND 2 IS LETHAL IN THE PATHOGENIC FUNGUS CANDIDA ALBICANS

Masayuki Sudo†, Toshikazu Yamazaki†, Kazunao Masubuchi§, Mikio Taniguchi§, Nobuo Shimma§, Mikio Arisawa§, and Hisafumi Yamada-Oka††

From the †Department of Mycology and Oncology and the §Department of Chemistry, Nippon Roche Research Center, Kanagawa 247-8530, Japan

As in Saccharomyces cerevisiae, the pathogenic fungus Candida albicans harbors three chitin synthases called CaChs1p, CaChs2p, and CaChs3p, which are structurally and functionally analogous to the S. cerevisiae ScChs2p, ScChs1p, and ScChs3p, respectively. In S. cerevisiae, ScCHS1, ScCHS2, and ScCHS3 are all non-essential genes; only the simultaneous disruption of ScCHS2 and ScCHS3 is lethal. The fact that a null mutation of the CaCHS1 is impossible, however, implies that CaCHS1 is required for the viability of C. albicans. To gain more insight into the physiological importance of CaCHS1, we identified and characterized a novel inhibitor that was highly specific to CaChs1p. RO-09-3143 inhibited CaChs1p with a Ki value of 0.55 nM in a manner that was non-competitive to the substrate UDP-N-acetylglucosamine. RO-09-3143 also hampered the growth of the C. albicans cells with an MIC50 value of 0.27 µM. In the presence of RO-09-3143, the C. albicans cells failed to form septa and displayed an aberrant morphology, confirming the involvement of the C. albicans Chs1p in septum formation. Although the effect of RO-09-3143 on the wild-type C. albicans was fungistatic, it caused cell death in the cachs2Δ cachs3Δ null mutants but not in the cachs3Δ null mutants. Thus, it appears that in C. albicans, inhibition of CaChs1p causes cell growth arrest, but simultaneous inhibition of CaChs1p and CaChs2p is lethal.

Candida albicans is an opportunistic pathogen and is one of the most common pathogens in humans. In healthy individuals, it remains in the oral cavity, gastrointestinal tract, and genitai; however, it colonizes and invades various tissues and organs and causes systemic fungal infections in neutropenic individuals, such as AIDS patients and those undergoing cancer chemotherapy or immunomodulation therapy for organ transplantation. Polymers and azoles are used to treat systemic Candida infections, but the adverse effects of polyenes and the expression of CaCHS1 remains low in both the yeast and hyphal forms (3). Although the cachs3Δ null mutants retained the ability to develop hyphae in vitro (11), a null mutation of CaCHS3 significantly attenuated the virulence (11), suggesting that the cell wall chitin plays important roles in pathogenicity. Whereas neither CaCHS2 nor CaCHS3 is essential for viability, the fact that a null mutation of CaCHS1 is impossible implies that CaCHS1 is essential for the growth of C. albicans (14).

To understand more about the physiological importance of C. albicans chitin synthase 1 and to also develop a valuable antifungal drug, we identified a novel chitin synthase inhibitor that is highly specific to CaChs1p. The inhibitor designated RO-09-3143 inhibited the septum formation and the growth of C. albicans cells. Further characterization of the compound, using the null mutant strains of the chitin synthase genes, showed that the inhibition of CaChs1p only arrested the cell growth, but the inhibition of CaChs1p and Chs2p was lethal in C. albicans.

MATERIALS AND METHODS

Synthesis of RO-09-3143 Inhibitor—RO-09-3143 was synthesized from 8-amino-4H-benz[1,4]oxazin-3-one by successive reductive N-alkylations with 6,6-dimethylhepta-2,4-diynal and then with formalin. One gram of 8-amino-4H-benz[1,4]oxazin-3-one (6.1 mmol) and 2.9 g of 6,6-dimethylhepta-2,4-diynal (21.64 mmol) were stirred in 100 ml of methanol/acetic acid (98% MeOH, 2% AcOH) containing 960 mg of NaBH4CN (15.28 mmol) at room temperature for 15 h. Thereafter, 1.73 ml of 37% formalin (21.34 mmol) and 960 mg of NaBH4CN (15.28 mmol) were added to the reaction mixture, and the reaction was further stirred at room temperature for 3 h. The reaction mixture was evaporated under reduced pressure, and the materials were partitioned between CH2Cl2 and water. The organic layer was washed with saturated NaCl solution dried over anhydrous sodium sulfate, filtered through a filter paper, and concentrated under reduced pressure. Purification of RO-09-3143 was carried out by silica gel column chromatography. After the crude residue was applied onto a silica column (150 g of SiO2, 4 × 24 cm),...
RO-09-3143 was eluted with 700 ml of CH$_2$Cl$_2$/acetone (20:1), which yielded 1.25 g of RO-09-3143 (with an overall yield of 69%). 8-Amino-4H-benz[1,4]oxazin-3-one was synthesized according to the method of Newbery and Phillips (15).

Cloning and Expression of CaCHS1, CaCHS2 and ScCHS2—The chitin synthase activities of CaChs1p, CaChs2p, and ScChs2p were determined by expressing them under the control of the GAL1 promoter in S. cerevisiae. The entire open reading frames of CaCHS1 and CaCHS2 were amplified by polymerase chain reaction with genomic DNA isolated from the wild-type CAI4. The resulting DNA fragments were cloned at the Xhol site located downstream of the GAL1 promoter of YPL plasmid (16). YpL-CaCHS1, YpL-CaCHS2, and YpL-ScCHS2 were cloned at the NcoI site located downstream of the chromosomal ScCHS1 and the construction of YpL-ScCHS2 are already described in a previous paper (16). YpL-CaCHS1, YpL-CaCHS2, and YpL-ScCHS2 were then transformed into the haploid S. cerevisiae strain, RRA400-1U (MATa his3 leu2 trp1 ura3 chs1::HIS3), in which a 2.1-kilobase NcoI-Ncol region of the chromosomal ScCHS1 and a 1.1-kilobase BglII-BglII region of the chromosomal ScCHS2 were replaced by URA3 and HIS3, respectively (16). The Leu$^+$ transformants were isolated and cultured at 30 °C in yeast nitrogen base (Difco) supplemented with glucose and the necessary amino acids. CaChs1p, CaChs2p, and ScChs2p were expressed by culturing the S. cerevisiae cells bearing YpL-CaCHS1, YpL-CaCHS2, or YpL-ScCHS2 in medium containing galactose at 30 °C for 12 h. DNA sequences of the cloned CaCHS1 and CaCHS2 were confirmed as described elsewhere (17). Primers used to amplify CaCHS1 were 5'-GCCGGCGTCTAGAATGCAACACATTAAACATGT-3' and 5'-GGGGCGGCTCTAGAATTATTTGTAGT-3', and the primers used to amplify CaCHS2 were 5'-GGGCTAGAATGTTAACAATCCC-3' and 5'-CTCTAGATTATCTGCGATGTTCATTGC-3'.

Determination of Susceptibilities of C. albicans and S. cerevisiae Cells to Compounds—Susceptibilities of C. albicans cells to RO-09-3143 and other compounds were determined as MIC$_{50}$ values, which were calculated from the $A_{600}$ obtained after culturing 10$^6$ cells of the wild-type strain CAI4 (18) in 0.1 ml of YPD medium at 30 °C for 24 h in the presence or absence of various concentrations of the indicated compounds. The fungicidal effects of RO-09-3143 on C. albicans and S. cerevisiae cells were also examined by counting the number of colonies derived from the viable cells. Approximately 10$^8$ C. albicans cells from the wild-type strain CAI4 (18), the cach2Δ null mutant (14), and the cach3Δ null mutant (14) and the same number of S. cerevisiae cells from ECY36-3D (MATa leu2 trp1 ura3 cach2Δ cach3Δ) (9, 10) and ECY36-3C (MATa leu2 trp1 ura3 cach1Δ cach2Δ cach3Δ::LEU2) (9, 10) were cultured in 0.1 ml of YPD medium in the presence or absence of the indicated concentrations of RO-09-3143 and nikkomycin Z. At the indicated time points, the cultures were spread onto YPD medium agar plates. After incubation at 30 °C for 2 days, the number of colonies appearing on the plates, which represented the number of viable cells in the cultures, was counted.

Assays of Chitin Synthases—Total membranes were prepared from S. cerevisiae RRA400-1U cells carrying YpL-CaCHS1, YpL-CaCHS2, or YpL-ScCHS2 as described previously (16). To determine the activities of each compound to inhibit CaChs1p activity, the cultures were harvested, washed with water, treated with 100 µg/ml Calcofluor White (Sigma), and examined by fluorescent microscopy.

RESULTS

Inhibition of CaChs1p Activity and C. albicans Growth by RO-09-3143—Because a null mutation of CaCHS1 is impossible (14), CaCHS1 may be required for the growth of the C. albicans cells. As a result, a specific inhibitor for CaChs1p can be developed as a novel antifungal drug. To identify such inhibitors, we expressed CaChs1p in S. cerevisiae RRA400-1U cells, which harbors neither ScCHS1 nor ScCHS2, under the control of the S. cerevisiae GAL1 promoter (16). As mentioned above, the endogenous ScChs2p activities of the total membranes of RRA400-1U cells were negligible (16). By screening the chemical libraries with the total membranes of S. cerevisiae cells overexpressing CaChs1p, we identified RO-41-0986 as a novel chitin synthase inhibitor (Fig. 1A). RO-41-0986 inhibited CaChs1p more strongly than did nikkomycin Z; the $K_i$ values of RO-41-0986 and nikkomycin Z to CaChs1p were 0.63 µM and 0.27 µM, respectively. Although RO-41-0986 structurally resembles terbinafine, which is a specific inhibitor of fungal squalene epoxidase, RO-41-0986 did not inhibit C. albicans squaene epoxidase even at the concentration of 100 µM.

The abbreviation used is: MIC$_{50}$, minimum inhibitory concentration.
Next RO-41-0986 was chemically modified to improve its potency as a CaChs1p inhibitor. Among several hundred derivatives, RO-09-3143 was found to be one of the strongest inhibitors of CaChs1p (Fig. 1A) (21). Interestingly, RO-09-3143 was rather specific for CaChs1p; it inhibited CaChs1p with a $K_i$ value of 0.55 nm. ScChs2p was much less susceptible to RO-09-3143; the $K_i$ value of ScChs2p to RO-09-3143 was 1 $\mu$M, which is about 2000 times greater than that for CaChs1p (Fig. 2). Neither CaChs2p nor ScChs1p activity was affected by the compound at the concentration of 100 $\mu$M. Inhibition kinetics demonstrated that RO-09-3143 inhibited CaChs1p and ScChs2p in a manner that was non-competitive to the substrate, UDP-GlcNAc, whereas nikkomycin Z displayed its feature as a competitive inhibitor to the substrate (Fig. 2). Although an enzyme assay for CaChs3p has not yet been established, RO-09-3143 did not significantly inhibit ScChs3p, which is functionally analogous to CaChs3p even at the concentration of 100 $\mu$M.

During the course of chemical modification of RO-41-0986 and identification of RO-09-3143, we also clarified the structure-activity relationship of the compound with respect to its inhibition of CaChs1p. The structure-activity relationship of RO-09-3143 was described as follows: 1) the $t$-butylacetylene moiety of the side chain was essential, 2) the replacement of the N-unsaturated amide moiety of the quinolone ring was required to keep the potency, and 3) the introduction of an oxygen or sulfur atom into the lactam ring at the 4-position sustained the potency (Fig. 1B). Additional results of the structure-activity relationship have been published separately (21).

Inhibition of C. albicans Growth by RO-09-3143—If CaCHS1 is an essential gene, RO-09-3143 should also abrogate the growth of the C. albicans cells. As expected, RO-09-3143 inhibited the growth of C. albicans cells with the MIC$_{50}$ value of 0.27 $\mu$M. Because CaCHS1 has been shown to be involved in septum formation (14), we also asked whether RO-09-3143 affects the septum formation. The wild-type CAI4 cells were treated with a non-lethal dose of RO-09-3143 and then stained with Calcofluor White. In the presence of 10 $\mu$M RO-09-3143, the cells still somehow continued to divide and increase in size but failed to form septa. Mother and daughter cells did not separate, and there was no or only faint fluorescence at the boundaries of these cells (Fig. 3, B and C); however, cell separation occurred normally in the absence of RO-09-3143, and strong fluorescence was detected at every septum of the dividing cells (Fig. 3A).

Essentially the same effects by RO-09-3143 were observed with the cachs2Delta/cachs3Delta double null mutant. Untreated, these cells clearly sustained the ability to create septa as judged by the fluorescence from Calcofluor White, but RO-09-3143 abolished the fluorescence at their septa and caused a failure of completion of cell separation (Fig. 3, D–F). All of these results demonstrate that RO-09-3143 inhibited the CaChs1p activity even at a cellular level and arrested the cell growth through a blockage of the CaChs1p function, that is septum formation. The fact that it was impossible to create the C. albicans mutant lacking CaCHS1 and that ScChs2p, although less susceptible than CaChs1p, was also affected by RO-09-3143 prompted us to further explore the mode of action of RO-09-3143 with the S. cerevisiae mutants deficient in the chitin synthase genes. In the cells of ECY36-3C that are defective in both ScCHS1 and ScCHS2, the strong fluorescence of Calcofluor White was detected at bud neck but not at septa (Fig. 4D). On the contrary, in the cells of ECY36-3D lacking ScCHS1 and ScCHS3 there was strong fluorescence at septa but not at bud neck (Fig. 4A), confirming that ScChs3p was responsible for chitin synthesis at bud neck, whereas ScChs2p was required for septum formation (10). The fluorescence at bud neck in the ECY36-3C cells remained even in the presence of a non-lethal dose (200 $\mu$M) of RO-09-3143 (Fig. 4, E and F), but the fluorescence from the septa of ECY36-3D cells almost completely disappeared after the addition of 200 $\mu$M RO-09-3143 (Fig. 4, B and C). Thus, it appears that in C. albicans and S. cerevisiae, RO-09-3143 specifically interferes with the chitin synthesis that is dependent on the activities of CaChs1p and ScChs2p.

Although the above results indicate that CaCHS1 is essential for the growth of C. albicans cells, it still remains unclear as to whether inhibition of CaChs1p is lethal or not. Because the complete shut-off of the CaCHS1 expression has not yet been established, we addressed this possibility by using RO-09-3143. As shown in Fig. 5, RO-09-3143 only arrested the growth of the wild-type CAI4 cells. The number of viable cells did not change until 20 h in the presence of RO-09-3143; the cell number gradually increased and reached a plateau by 80 h demonstrating that the blockage of the CaChs1p function resulted in cell growth arrest in C. albicans. Interestingly, however, RO-09-3143 caused the death of the C. albicans cells in...
FIG. 3. Effects of RO-09-3143 on the septum formation in C. albicans. C. albicans cells of the wild-type CAI4 (A–C) and those of the cachs2Δ/cachs3Δ double null mutant (D–F) were cultured for 24 h in the presence (B, C, E, and F) or absence (A and D) of 10 μM of RO-09-3143. Thereafter, the cells were stained with Calcofluor White. Septa and bud necks, where chitin was accumulated heavily thereby producing strong fluorescence, were detected under a fluorescent microscope.

The presence of nikkomycin Z, a substrate analog of chitin synthase (22, 23). In S. cerevisiae, nikkomycin Z preferably inhibits ScChs1p and ScChs3p (24); the defects in both ScCHS2 and ScCHS3 are lethal in S. cerevisiae (10). Therefore, one explanation for the above result may be that the dual inhibition of CaChs1p and CaChs3p is lethal in C. albicans. We validated this possibility by examining the effects of RO-09-3143 on the cachs2Δ and cachs3Δ null mutants. Although there was a transient decrease in the number of viable cells, RO-09-3143 did not kill the cachs3Δ null mutant. The transient decrease in the number of the viable cachs3Δ null mutant cells after the addition of RO-09-3143, however, would be an artifact of the colony assay because the cachs3Δ null mutant cells became highly aggregated and sometimes tended to form hyphae, especially in the presence of RO-09-3143 (data not shown). On the contrary, RO-09-3143 caused cell death in the cachs2Δ null mutant; more than 99.9% of the cachs2Δ null mutant cells were thought to be dead 6 h after addition of RO-09-3143 (Fig. 5). Thus, it appears that inhibition of CaChs1p can only arrest cell growth, but simultaneous inhibition of CaChs1p and CaChs2p is lethal in C. albicans.

As RO-09-3143 inhibited the ScChs2p activity and septum formation in ECY36-3D (scchs1Δ/scchs3Δ mutant) cells, we also examined the effects of the compounds on the viability of these cells. The number of the viable ECY36-3D cells but not ECY36-3C (scchs1Δ/scchs2Δ mutant) cells decreased by about 90% at 24 h after the addition of 300 μM RO-09-3143. As in the C. albicans cachs3Δ null mutant cells, the decrease in the number of viable ECY36-3D cells was transient; at 72 h there was no significant difference in the number of the viable cells between ECY36-3D and ECY36-3C cells (data not shown). Further increase in the concentration of RO-09-3143, however, resulted in precipitation of the compound and did not cause cell death of the ECY36-3D cells.

DISCUSSION

In this study, we identified RO-09-3143 as a highly potent and specific inhibitor of C. albicans Chs1p. Although its effect on ScChs2p was remarkably weaker than its effect on CaChs1p, RO-09-3143 also inhibited the activity of S. cerevisiae Chs2p (a functional homolog of CaChs1p) but not the activity of ScChs1p that is a homolog of CaChs2p. This suggests that RO-09-3143 is interactive only with chitin synthases that belong to the family of S. cerevisiae Chs2p. In vivo, RO-09-3143 abrogated septum formation and arrested the growth of C. albicans cells. This result of RO-09-3143 suggests that septum formation is an essential process of cell division in C. albicans. In fact, RO-09-3143 reduced the fungal burden in kidneys and prolonged the survival of mice that had been systemically infected with a lethal dose of C. albicans. As RO-09-3143 inhibited the ScChs2p activity and septum formation in ECY36-3D (scchs1Δ/scchs3Δ mutant) cells, it also delays the cell growth and causes aberrant morphology in S. cerevisiae (8, 10).

On the other hand, RO-09-3143 caused cell death in the cachs2Δ null mutant but not in the cachs3Δ null mutant in C. albicans. This was a rather unexpected result because in S. cerevisiae a double disruption of ScCHS1 and ScCHS2 is still viable, and only the simultaneous inactivation of ScCHS2 and ScCHS1 is still lethal (25).

FIG. 4. Effects of RO-09-3143 on the septum formation in S. cerevisiae. S. cerevisiae cells of ECY36-3D (scchs1Δ/scchs3Δ mutant) (A–C) and those of ECY36-3C (scchs1Δ/scchs2Δ mutant) (D–F) were cultured for 24 h in the presence (B, C, E, and F) or absence (A and D) of 20 μM RO-09-3143. Thereafter, the cells were stained with Calcofluor White. Septa and bud necks, where chitin was accumulated heavily thereby producing strong fluorescence, were detected under a fluorescent microscope.

FIG. 5. Effects of nikkomycin Z and RO-09-3143 on the viability of C. albicans. C. albicans cells of the wild-type CAI4, the cachs2Δ null mutant (chas2Δ/−/−), and the cachs3Δ null mutant (chas3Δ/−/−) were cultured in the presence or absence of 10 μM nikkomycin Z and 20 μM RO-09-3143. At the indicated times, samples of the cultures were diluted and spread onto agar plates. The number of viable cells in the culture was determined by counting the number of colonies appearing on the agar plates. □, none; ○, nikkomycin Z; ×, RO-09-3143; Δ, nikkomycin Z and RO-09-3143.

* M. Sudoh, T. Yamazaki, M. Arisawa, and H. Yamada-Okabe, manuscript in preparation.
ScCHS3 is lethal (10). Furthermore, the addition of RO-09-3143 and nikkomycin Z to the wild-type C. albicans cells also led to cell death, although neither compound was a fungicidal agent for C. albicans when used alone. By using the S. cerevisiae chitin synthases, nikkomycin Z has been shown to be rather selective to ScChs1p and ScChs3p. The $K_{i}$ value of nikkomycin Z to ScShe2p is 4000 times greater than the $K_{i}$ value of nikkomycin Z to ScChs1p and 700 times greater than the $K_{i}$ value of nikkotoxin Z to ScChs3p (24). Also in C. albicans, we observed that CaChs2p was highly susceptible to nikkomycin Z. Although the enzyme assay for CaChs3p and the susceptibilities of CaChs3p to nikkomycin Z remain to be established, the above results strongly suggest that the lethality caused by the addition of RO-09-3143 and nikkomycin Z is also a result of the dual inhibition of CaChs1p and CaChs2p. In S. cerevisiae, RO-09-3143 also transiently reduced the number of viable cells of EY36-3D (scchs1Δ/scchs3Δ mutant), but it failed to kill these cells. The failure of RO-09-3143 to cause cell death of EY36-3D presumably may be because of the lower susceptibility of ScChs2p to RO-09-3143 and also to a limited water solubility of the compound.

Although RO-09-3143 transiently reduced the number of viable cells of the C. albicans cachs3Δ null mutant, we considered that this would be the consequence of the highly clumpy feature of the cachs3Δ null mutant cells, and thereby, an artifact of the colony assay. Another possibility may be that the stability of RO-09-3143 influenced the killing kinetics of C. albicans cachs2Δ and cachs3Δ null mutants differently. In fact, in an animal model the initial stage of the major RO-09-3143 metabolism was N-demethylation, which drastically reduced the potency as a CaChs1p inhibitor; the MIC50 value of the demethylated form of RO-09-3143 was about 1000 times greater than that of the intact RO-09-3143. However, this possibility is unlikely because doubling times in the YPD medium of the cachs2Δ and cachs3Δ null mutants were similar, and the half-life of RO-09-3143 in the C. albicans conditioned medium was longer than 5 h, which is sufficient to cover several cell cycles of these mutant cells. In fact, further addition of RO-09-3143 at 12 and 24 h after the first addition of RO-09-3143 did not cause cell death, and it only delayed recovery of the growth of the cachs3Δ null mutant cells.

Whereas the dual inhibition of CaChs1p and CaChs2p appears to be lethal in C. albicans, the function of CaChs2p is still obscure despite the fact that its protein level is supposed to be much higher than that of the other two chitin synthases (13). In S. cerevisiae, ScChs1p, the functional homolog of CaChs2p, is believed to repair the cell wall at the bud scar during and/or after bud separation (7). We and others (12–14) also disrupted CaCHS2 in C. albicans and found that the disruption of CaCHS2 slowed down the germ tube formation but did not affect the morphology, cell growth in vitro, septum formation, or virulence. Moreover, a decrease of the chitin contents in the hyphae of the cachs2Δ null mutants depended on the methods used to extract chitin from the cells (13). Nevertheless, the results of this study imply that CaChs2p has some unknown function that may in part be overcome by CaChs1p.

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