Efficacy of CS-758, a Novel Triazole, against Experimental Fluconazole-Resistant Oropharyngeal Candidiasis in Mice

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The therapeutic efficacy of CS-758, a novel triazole, was evaluated against experimental murine oropharyngeal candidiasis induced by Candida albicans with various susceptibilities to fluconazole. Against infections induced by strains with various susceptibilities to fluconazole, the efficacy of fluconazole was strongly correlated with the MIC of fluconazole, as measured by the NCCLS method, and agreed with the NCCLS interpretive breakpoints, suggesting that the efficacies of new drugs could be predicted by using this model. The results of the fungal burden study corresponded with the results of the histopathological study. CS-758 exhibited potent in vitro activity (MICs, 0.004 to 0.06 µg/ml) against the strains used in this murine model including fluconazole-resistant strains (fluconazole MICs, 16 to 64 µg/ml). CS-758 exhibited excellent efficacy against the infections induced by all the strains including a fluconazole-resistant strain, and the reductions in viable cell counts were significant at 10 and 50 mg/kg of body weight/dose. Fluconazole was not effective even at 50 mg/kg/dose against infections induced by a fluconazole-resistant strain (fluconazole MIC, 64 µg/ml). These results suggest that CS-758 is a promising compound for the treatment of oropharyngeal candidiasis including fluconazole-refractory infections.

Candida albicans is an opportunistic pathogen that causes both mucosal and hematogenously disseminated infections. Oropharyngeal candidiasis (OPC) is often seen in patients who are immunosuppressed due to AIDS (8, 17, 18) or the use of corticosteroids (19, 20, 21). OPC is the most common fungal infection in patients with AIDS, occurring in as many as 90% of these individuals (8, 13, 17, 18). OPC is usually treated with either topical or systemic antifungal agents (2, 13). In particular, a triazole agent, fluconazole (FLC), is widely used and effective in the treatment of OPC (1, 14). However, recurrent OPC may occur in as many as 50% of OPC patients (13). Moreover, development of FLC resistance, especially in patients with extensive prior FLC use, has increased significantly (9, 11, 15, 16). It is estimated that FLC therapy will eventually fail in approximately 5% of OPC patients with advanced AIDS (16). Thus, it is necessary to develop new antifungal agents that are effective against FLC-resistant strains.

CS-758, formerly R-120758, is a novel triazole, which has potent activity against Candida albicans and also against strains that have low levels of susceptibility to FLC in vitro (5). Previously, we established a new model of experimental murine OPC (6). In this study, we investigated the therapeutic efficacy of CS-758 against experimental murine OPC induced by C. albicans strains that have various susceptibilities to FLC.


MATERIALS AND METHODS

Antifungal agents. CS-758 was synthesized by Sankyo Co., Ltd., for both in vitro and in vivo studies. Itraconazole (ITC) powder was obtained from commercial preparations (Itrizole capsules; Janssen Kyowa Co., Ltd., Tokyo, Japan) by extraction with dichloromethane. The crude extract was purified by silica gel column chromatography (ethyl acetate) and then recrystallized from a mixture of dichloromethane, ethyl acetate, and hexane. The ITC powder (potency, 99.5%) was used for both in vitro and in vivo studies. FLC powder obtained from commercial preparations (Diflucan capsules; Pfizer Pharmaceuticals, Inc., Tokyo, Japan) was extracted with ethanol. After concentration, the crude residue was recrystallized from a mixture of ethanol and ethyl acetate. The FLC powder (potency, 97.8%) was used for the in vitro study, and commercial preparations were used for the in vivo study. All the agents for the in vitro study were dissolved in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For the in vivo study, CS-758 and FLC were suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na; Kanto Chemical Co., Inc., Tokyo, Japan) by extraction with dichloromethane. The crude extract was purified by silica gel column chromatography (ethyl acetate) and then recrystallized from a mixture of dichloromethane, ethyl acetate, and hexane. The ITC powder (potency, 99.5%) was used for both in vitro and in vivo studies. FLC powder obtained from commercial preparations (Diflucan capsules; Pfizer Pharmaceuticals, Inc., Tokyo, Japan) was extracted with ethanol. After concentration, the crude residue was recrystallized from a mixture of ethanol and ethyl acetate. The FLC powder (potency, 97.8%) was used for the in vitro study, and commercial preparations were used for the in vivo study. All the agents for the in vitro study were dissolved in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For the in vivo study, CS-758 and FLC were suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na; Kanto Chemical Co., Inc., Tokyo, Japan). ITC was dissolved in 40% 2-hydroxypropyl β-cyclodextrin (Sigma Chemical Co., St. Louis, Mo.) (3).

Organisms. We used six strains of C. albicans. C. albicans SANK51486 was stored in our laboratory. C. albicans 2010A, 2033A, 2035B, 2054A, and 2085A, which were isolated from the oral cavities of different patients, were provided by Scott G. Fuller of Harbor-University of California, Los Angeles, Research and Education Institute. For the in vitro study, the test organisms were cultured on Sabouraud dextrose agar (SDA; Eiken Chemical Co., Ltd., Tokyo, Japan). For the in vivo study, the test organisms grown on SDA were inoculated into YPG (potency, 97.8%) was used for the in vitro study, and commercial preparations were used for the in vivo study. All the agents for the in vitro study were dissolved in dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For the in vivo study, CS-758 and FLC were suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na; Kanto Chemical Co., Inc., Tokyo, Japan). ITC was dissolved in 40% 2-hydroxypropyl β-cyclodextrin (Sigma Chemical Co., St. Louis, Mo.) (3).

In vitro susceptibility testing. The MICs for the test organisms were determined by the broth microdilution method described in NCCLS document M27-A (10).
TABLE 1. In vitro antifungal activity of CS-758 against C. albicans strains used to induce experimental murine OPC

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>CS-758</td>
</tr>
<tr>
<td>SAMK51486</td>
<td>0.002</td>
</tr>
<tr>
<td>2085A</td>
<td>0.004</td>
</tr>
<tr>
<td>2054A</td>
<td>0.004</td>
</tr>
<tr>
<td>2010A</td>
<td>0.004</td>
</tr>
<tr>
<td>2013A</td>
<td>0.06</td>
</tr>
<tr>
<td>2035B</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Animals. Specific-pathogen-free male ddY mice (age, 4 weeks) were purchased from Japan SLC, Inc., Shizuoka, Japan. The mice were used for the experiments after an acclimation period of 6 days. The mice were immunosuppressed by subcutaneous injection of 4 mg of cortisone acetate (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) per mouse 2 days before, on the day of, and 3 days after inoculation. During the period of immunosuppression the mice were given tetracycline hydrochloride (0.5 mg/ml in drinking water; Achromycin V; Wyeth Laboratories, Inc., Tokyo, Japan) for the prevention of bacterial infection. Mice were given food and water ad libitum throughout the experiments. All animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co., Ltd.

Experimental murine OPC. Experimental murine OPC was induced by a previously reported procedure (6). Test organisms cultured in YPG medium were harvested, washed with sterile physiological saline, and suspended in sterile physiological saline. The suspension was adjusted to 1.0 × 10^7 cells/ml. Before inoculation, the mice were anesthetized by intraperitoneal injection of 27.5 µg of dimorpholamine (Theraptique; Eisai Co., Ltd., Tokyo, Japan), 219 µg of xylazine (Bayer Yukuhin, Ltd., Osaka, Japan), and 1.28 mg of pentobarbital sodium (Nembutal; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) per mouse. Cotton-wool balls (diameter, 3 mm) were saturated with 100 µl of the test organism suspension of 1.0 × 10^7 cells/ml and then placed sublingually in the oral cavity for about 2 h.

Therapeutic efficacy. The test agents were administered orally by gavage once daily for 2 days starting 3 days postinoculation. For the treatment of the infection caused by C. albicans, SAMK51486, CS-758, and FLC were administered at 0.4, 2, and 10 mg/kg of body weight/dose and ITC was administered at 10 mg/kg/dose. For the treatment of the infections caused by the other strains, CS-758 and FLC were administered at 2, 10, and 50 mg/kg/dose and ITC was administered at 50 mg/kg/dose. The control group received 0.2 ml of 0.5% CMC-Na. In our preliminary study, the vehicle for ITC (40% 2-hydroxypropyl β-cyclodextrin) did not influence the viable cell counts compared with those achieved with 0.5% CMC-Na in this model. Each group consisted of six mice. On day 5 postinoculation, the mice were sacrificed and the mandible with the attached tissue was excised. After removal of the bone and teeth, the tissue was homogenized and serial dilutions were cultured on SDA containing 10 µg of chloramphenicol per ml at 35°C for 2 or 3 days. Hereafter, the attached tissue of the mandible without bone or teeth is referred to as “oral tissue.” The viable cell counts in the oral tissue were determined by counting the colonies on the SDA plates.

Histopathological study. For the histopathological study, test agents were administered at 10 mg/kg/dose against experimental murine OPC caused by C. albicans SAMK51486, as described above. On day 5 postinoculation, the mice were sacrificed and the mandibles with the attached tissue were excised. The tissues were fixed in formalin containing 8% sucrose and embedded in paraffin, after which thin sections of the tongues were prepared and stained with periodic acid-Schiff (PAS).

Statistical analysis. The viable cell counts in the oral tissues were compared between the control group and the treated groups by Dunnett’s test. The detection limit was log_{10}5 CFU/g of tissue in this study. When the culture of the oral tissue was sterile, the value of the detection limit was used for the statistical analysis. P values below 0.05 were considered significant. The SAS system for Windows (release 6.12; SAS Institute Inc.) was used for the analysis.

RESULTS

MICs for the test organisms. Table 1 shows the MICs for the test organisms used in this study. According to the guidelines described in NCCLS document M27-A (10), strains SANK51486, 2085A, and 2054A were placed in the FLC-susceptible (FLC-S) category (FLC MICs, ≤8 µg/ml), strains 2010A and 2033A were placed in the FLC-susceptible dose-dependent (FLC-S-DD) category (FLC MICs, 16 to 32 µg/ml), and strain 2035B was placed in the FLC-resistant (FLC-R) category (FLC MICs, ≥64 µg/ml) (10). CS-758 exhibited the most potent activity against these strains, with MICs ranging from 0.004 to 0.06 µg/ml.

Efficacy against experimental murine OPC induced by C. albicans SANK51486. Figure 1 shows the therapeutic efficacies of the test agents against experimental murine OPC induced by strain SANK51486. Treatment with CS-758 and FLC reduced the viable cell counts in the oral tissue in a dose-dependent manner, and the effects were significant at 2 mg/kg/dose (P < 0.001 for CS-758 and P < 0.001 for FLC) and 10 mg/kg/dose (P < 0.001 for CS-758 and P < 0.001 for FLC). At these doses, the reductions were more than 1.5 log_{10} compared with the counts for the control. ITC at 10 mg/kg/dose reduced the viable cell counts in the oral tissue, but the decrease was not significant. Figure 2 shows the PAS-stained specimens of tongues of the control group and the groups treated with CS-758, FLC, and ITC at 10 mg/kg/dose. In the mice treated with CS-758 and FLC, only a few fungal cells were observed. On the other hand, many fungal cells were still observed in the mice treated with ITC, although the area of the infection foci was reduced to some extent compared with that in the control mice.

Efficacy against experimental murine OPC induced by C. albicans strains with various FLC susceptibilities. We next investigated the therapeutic efficacies of the test agents against experimental murine OPC induced by strains 2085A, 2054A, 2010A, 2033A, and 2035B. The FLC MIC for each of these strains was higher than that for strain SANK51486. Therefore, we administered doses that were higher than those used in the experiment with strain SANK51486. Figure 3 shows the therapeutic efficacies of the test agents against experimental murine OPC induced by FLC-S strains 2085A and 2054A. In mice with infections induced by these strains, CS-758 and FLC reduced the viable cell counts in the oral tissue in a dose-dependent manner. In mice with infections induced by strain 2085A, the effects of CS-758 were significant at 2, 10, and 50 mg/kg/
dose ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively), and the effects of FLC were significant at 10 and 50 mg/kg/dose ($P < 0.001$ and $P < 0.001$, respectively). ITC at 50 mg/kg/dose reduced the viable cell counts in the oral tissue significantly ($P < 0.001$). The results for the infection induced by strain 2054A were similar to those for the infection induced by strain 2085A.

Figure 4 shows the therapeutic efficacies of the test agents against experimental murine OPC induced by FLC-S-DD strains 2010A and 2033A and FLC-R strain 2035B. The results against the infection induced by strain 2010A were similar to those against the infection induced by the FLC-S strains. CS-758 and FLC reduced the viable cell counts in the oral tissue significantly: at 2, 10, and 50 mg/kg/dose ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively) for CS-758 and at 10 and 50 mg/kg/dose ($P < 0.05$ and $P < 0.001$, respectively) for FLC. ITC at 50 mg/kg/dose reduced the viable cell counts significantly ($P < 0.001$). In mice with infections induced by strain 2033A, CS-758 reduced the viable cell counts in a dose-dependent manner, and the effects at 10 and 50 mg/kg/dose were significant ($P < 0.001$ and $P < 0.001$, respectively). On the other hand, FLC showed significant effects only at 50 mg/kg/dose ($P < 0.05$). ITC at 50 mg/kg/dose also gave a significant result ($P < 0.01$). In mice with infections induced by strain 2035B, CS-758 reduced the viable cell counts in a dose-dependent manner, and the effects at 10 and 50 mg/kg/dose were significant ($P < 0.01$ and $P < 0.001$, respectively). On the other hand, FLC and ITC were ineffective even at 50 mg/kg/dose.

DISCUSSION

Previously, we reported a new model of OPC in mice (6). That model has been shown to be useful for the evaluation of antifungal agents such as FLC (6) as well as for the investigation of virulence factors (7).

In this study, we first demonstrated that the test agents were effective against experimental murine OPC induced by azole-susceptible strain C. albicans SANK51486 both mycologically and histopathologically. The results of the histopathological analysis from this experiment reflect the results of the fungal burden study, demonstrating that the fungal burden in the oropharynx could be a suitable indicator of therapeutic efficacy.

We next investigated the therapeutic efficacies of triazoles against experimental murine OPC induced by strains with various susceptibilities to FLC. In those experiments in mice, we observed a strong correlation between the MIC and the therapeutic efficacy of FLC. In particular, against the FLC-S-DD strain for which the FLC MIC was 32 μg/ml, FLC reduced the viable cell counts significantly only at 50 mg/kg/dose, and against the FLC-R strain for which the FLC MIC was 64 μg/ml, FLC had no effect even at 50 mg/kg/dose. These results suggest that in vitro resistance to FLC, as measured by the NCCLS method, is significantly correlated with in vivo resistance to FLC in the OPC model.

For ITC, the interpretive breakpoints in NCCLS document M27-A (10) are as follows: susceptible, ≤0.12 μg/ml; suscep-
tible dose dependent; 0.25 to 0.5 μg/ml; and resistant, ≥1 μg/ml. All the strains used in this study were categorized as ITC susceptible or ITC susceptible dose dependent. The therapeutic efficacy of ITC was almost in correlation with its MIC. Although strain 2035B was susceptible to ITC (MIC, 0.12 μg/ml), ITC was not effective in vivo against this strain. The reason for the discrepancy between the MIC and efficacy for ITC remains unknown. Further studies will be needed.

As the efficacy in this model corresponds fairly well to the NCCLS interpretive breakpoints for susceptibility, this model is thought to be useful for prediction of the therapeutic efficacies of new drugs. However, to improve the predictive utility of this model, pharmacokinetic and pharmacodynamic analyses will be needed. Previously, Walsh and colleagues (22) reported on an experimental oropharyngeal and esophageal candidiasis model in rabbits induced by FLC-S and FLC-R C. albicans strains. In their rabbit model, a strong correlation between the in vitro and in vivo activities was described, and it was demonstrated that the FLC concentrations in plasma or esophageal tissue exceeded the MIC for the FLC-S strain at the responsive dose but not that for the FLC-R strain at the unresponsive dose (22). Using the rabbit model, they demonstrated a strong relationship between the fungal burden and the drug concentration in plasma, saliva, and esophageal tissue for V-echinocandin (12). As for our murine OPC model, pharmacokinetic and pharmacodynamic analyses are under way.

In this study, CS-758 was shown to exhibit excellent efficacy against experimental murine OPC including OPC induced by an FLC-R strain. One of the reasons for this excellent efficacy is thought to be the potent in vitro activity of CS-758. In our preliminary study, the plasma drug concentration was sustained above the MICs by at least more than threefold in mice treated with 10 mg of CS-758/kg/dose. In addition, CS-758 was shown to be distributed widely in rat tissue (T. Shibayama, N. Kikuchi, Y. Matsushita, K. Kawai, A. J. John, T. Hirota, and S. Kuwahara, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1090, 2000). The sufficient concentration of CS-758 in plasma and the widespread distribution of the drug in tissue may contribute to the excellent efficacy of CS-758.

Compared with FLC, the large discrepancy was observed in the relationship between the MIC of CS-758 and its efficacy. This discrepancy has been observed in a systemic C. albicans infection model (5). From our preliminary data, the concentration of drug in the plasma of mice administered FLC was approximately 10-fold higher than that in the plasma of mice administered the same dose of CS-758. Additionally, the levels of serum protein binding were 12% for FLC (4) and 99% for CS-758 (unpublished data) in mice. These pharmacokinetic profiles may clarify the discrepancy between in vitro activity and efficacy for CS-758. However, CS-758 has potent in vivo activity, with MICs of 0.25 μg/ml or lower for FLC-R C. albicans strains (FLC MICs, 64 μg/ml) other than the strain used in this study (data not shown). Considering these findings,
CS-758 is a promising compound for the treatment of OPC including FLC-resistant infections.

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REFERENCES


