**In Vitro** Antifungal Activity of ME1111, a New Topical Agent for Onychomycosis, against Clinical Isolates of Dermatophytes

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The treatment of onychomycosis has improved considerably over the past several decades following the introduction of the oral antifungals terbinafine and itraconazole. However, these oral agents suffer from certain disadvantages, including drug interactions and potential liver toxicity. Thus, there is a need for new topical agents that are effective against onychomycosis. ME1111 is a novel selective inhibitor of succinate dehydrogenase (complex II) of dermatophyte species, whose small molecular weight enhances its ability to penetrate the nail plate. In this study, we determined the antifungal activity of ME1111 against dermatophyte strains, most of which are known to cause nail infections, as measured by the MIC (n = 400) and the minimum fungicidal concentration (MFC) (n = 300). Additionally, we examined the potential for resistance development in dermatophytes (n = 4) following repeated exposure to ME1111. Our data show that the MIC₉₀ of ME1111 against dermatophyte strains was 0.25 μg/ml, which was equivalent to that of the comparators amorolfine and ciclopirox (0.25 and 0.5 μg/ml, respectively). ME1111 was fungicidal at clinically achievable concentrations against dermatophytes, and its MFC₉₀s against Trichophyton rubrum and Trichophyton mentagrophytes were 8 μg/ml, comparable to those of ciclopirox. Furthermore, ME1111, as well as ciclopirox, did not induce resistance in 4 dermatophytes tested. Our studies show that ME1111 possesses potent antifungal activity and suggest that it has low potential for the development of resistance in dermatophytes.

Among superficial fungal infections, by far the most difficult to cure is toenail onychomycosis, which is responsible for 50% of all nail disease (1). Onychomycosis, a fungal nail infection affecting up to 13% of the general population (2–8) and 25% of the geriatric and diabetic population (7, 9, 10), is more than just a cosmetic problem. More importantly, onychomycosis has been reported to cause chronic pain associated with prolonged standing or walking and acute pain from footwear and cutting of the nails (11–13).

The greatest predisposing risk factor for developing onychomycosis is advanced age, as the risk is reported to be 18.2% in patients 60 to 79 years of age compared to 0.7% in patients younger than 19 years of age. Further, men are up to 3 times more likely to have onychomycosis than women, though the reasons for this gender difference are not clear (5, 14). Other risk factors include diabetes and conditions contributing to poor peripheral circulation (15). In fact, onychomycosis may represent an important predictor for the development of diabetic foot syndrome and foot ulcers (16). Patients who are immunosuppressed, as those with HIV infection and those undergoing cancer therapy, are also predisposed to fungal nail infection (17).

Several nonclinical risk factors also affect a person’s chance of developing fungal nail infections. For example, toenail onychomycosis is not prevalent in tropical climates, presumably because people in those areas are not in the habit of wearing occlusive footwear that creates a warm, moist environment for the proliferation of fungi. Further, the spread of foot infections, including tinea pedis (athlete’s foot), may occur in places such as shower stalls, bathrooms, or locker rooms, where floor surfaces often are wet and people are barefoot (18). Nail trauma will also increase the risk of fungal infection of the affected nail, especially in the geriatric population (5, 17).

The treatment of onychomycosis has improved considerably over the past several decades following the introduction of the oral antifungals terbinafine and itraconazole. However, these drugs may have side effects such as liver damage or drug interactions, which are particularly relevant in the elderly population (19). Unfortunately, currently available topical agents such as ciclopirox 8% and amorolfine 5% have low efficacy (approximately 5 to 12%) (20, 21). This low efficacy can be attributed mainly to the inability of the drug to penetrate through the nail plate to the nail bed, where the infection resides (22). Additionally, thickened nails, extensive involvement of the entire nail, lateral disease, and yellow spikes (hyperkeratotic bands extending to the nail matrix) contribute to a poor response to topical treatment (17). Thus, there remains a need for new topical agents that are effective against onychomycosis. ME1111 is a novel antifungal discovered by Meiji Seika Pharma Co., Ltd. (Tokyo, Japan), and its small molecular mass (202.25 g/mol) enhances its ability to penetrate the nail plate (Fig. 1). The investigational drug is currently undergoing clinical evaluation. In this study, we determined the antifungal activity of ME1111 against dermatophyte species known to cause onychomycosis, as measured by MIC and minimum fungicidal concentration (MFC). Additionally, we examined the potential for resistance development in dermatophytes following repeated exposure to ME1111.
MATERIALS AND METHODS

Test strains. MICs were determined against 100 recent clinical strains each of Trichophyton rubrum, T. mentagrophytes, T. tonsurans, and Epidermophyton floccosum, mostly isolated in the United States (Table 1). The test panel included seven T. rubrum strains with elevated terbinafine MICs (≥8 μg/ml) and nine T. mentagrophytes strains with elevated itraconazole MICs (≥1 μg/ml). MFCs were determined against all strains with the exception of T. tonsurans. Two reference strains, T. rubrum ATCC MYA-4438 and T. mentagrophytes ATCC MYA-4439, were included as quality control strains in MIC tests. Two clinical isolates each of T. rubrum (23490 and 23576) and T. mentagrophytes (19826 and 19893), from the culture collection at the Center for Medical Mycology, were used in the resistance development studies.

Test articles. ME1111 was supplied by Meiji Seika Pharma Co., Ltd. The comparators used were amorolfine hydrochloride (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and ciclopirox olamine (Sigma-Aldrich Co., St. Louis, MO). All test articles were supplied in powder form and dissolved in dimethyl sulfoxide. Serial dilutions were prepared in RPMI 1640 buffered with MOPS [3-(N-morpholino)propanesulfonic acid] (Hardy Diagnostics, Santa Maria, CA) in a range of 0.06 to 32 μg/ml for ME1111, 0.001 to 0.5 μg/ml for amorolfine, and 0.008 to 4 μg/ml for ciclopirox in the MIC testing.

MIC of ME1111 and comparators. The MICs of ME1111, amorolfine, and ciclopirox against each isolate were determined according to the Clinical and Laboratory Standards Institute (CLSI) document M38-A2 methodology (23). Isolates were subcultured onto potato dextrose agar (PDA; Fisher Scientific, Hampton, NH) or cerea agar (for T. rubrum strains) and incubated at 30°C until good conidiation was achieved, usually within 7 days. Conidia were harvested to sterile saline by swabbing the colony surface with a sterile swab and were allowed to settle for 10 to 15 min. Conidium counts were then standardized using a hemacytometer, and the suspension was adjusted to 1 × 10^4 to 3 × 10^6 CFU/ml in RPMI 1640.

Serial dilutions of drug and inoculum were combined in 96-well round-bottom microtiter plates and incubated at 35°C for 4 days. The MIC endpoint was defined as the lowest concentration that inhibited 80% of fungal growth compared to the growth control. MIC values within 2 dilutions were considered equivalent.

MFCs of ME1111 and comparators. Because there is no standardized methodology for determining MFCs of antifungals, we conducted preliminary experiments and optimized the testing method using several different parameters, including inoculum size and growth medium. The following testing conditions produced reproducible MFC values: MIC microdilution assays against all strains were performed in Sabouraud dextrose broth (SDB) (Fisher Scientific) using an inoculum of 10^6 CFU/ml. Microdilution trays were incubated at 35°C without shaking. After 7 days of incubation, the entire contents of each clear well were subcultured onto a PDA plate. To avoid antifungal carryover, the aliquots were allowed to soak into the agar and were streaked for isolation once dry, thus removing the cells from the residual drug. The MFC was defined as the lowest concentration that resulted in complete inhibition of growth upon subculture (>99.9%).

Development of resistance assay. Two strains each of T. rubrum (23490 and 23576) and T. mentagrophytes (19826 and 19893), tested in the MIC experiment above, were chosen for this study because of their low ME1111 MIC values, making it easier to detect any rise in MIC following development.

TABLE 1 MIC data for ME1111 and comparators against dermatophyte strains, performed in RPMI 1640 broth (CLSI M38-A2)

<table>
<thead>
<tr>
<th>Species (n) and MIC measured</th>
<th>Yr isolated</th>
<th>Country (no. of isolates)</th>
<th>MIC (μg/ml) result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ME1111</td>
</tr>
<tr>
<td>T. rubrum (n = 100)</td>
<td>2007–2011</td>
<td>USA (98), Iceland (2)</td>
<td>&lt;0.06–0.5</td>
</tr>
<tr>
<td>MIC range</td>
<td></td>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>MIC_50</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>T. mentagrophytes (n = 100)</td>
<td>2006–2011</td>
<td>USA (88), Japan (12)</td>
<td>0.06–0.5</td>
</tr>
<tr>
<td>MIC range</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>MIC_50</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>T. tonsurans (n = 100)</td>
<td>2009–2011</td>
<td>USA (100)</td>
<td>&lt;0.06–0.5</td>
</tr>
<tr>
<td>MIC range</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>MIC_50</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>E. floccosum (n = 100)</td>
<td>1998–2011</td>
<td>USA (69), Australia (13), Japan (7), Mexico (5), Iceland (4), Canada (2)</td>
<td>0.06–1</td>
</tr>
<tr>
<td>MIC range</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>MIC_50</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>All species (n = 400)</td>
<td></td>
<td></td>
<td>&lt;0.06–1</td>
</tr>
<tr>
<td>MIC range</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>MIC_50</td>
<td></td>
<td></td>
<td>0.25</td>
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</tbody>
</table>
TABLE 2 MIC data for ME1111 and comparators against dermatophyte strains with elevated terbinafine or itraconazole MICs in RPMI 1640 broth

<table>
<thead>
<tr>
<th>Species</th>
<th>ME1111</th>
<th>AMF</th>
<th>CIC</th>
<th>TRB</th>
<th>ITR</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. rubrum</td>
<td>0.06–0.25</td>
<td>0.002–0.25</td>
<td>0.125–0.5</td>
<td>≥8</td>
<td>ND</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. mentagrophytes</td>
<td>0.125–0.25</td>
<td>0.001–0.004</td>
<td>0.06–0.125</td>
<td>ND</td>
<td>≥1</td>
</tr>
<tr>
<td>(n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Performed according to the methodology detailed in CLSI document M38-A2. Abbreviations: AMF, amorolfine; CIC, ciclopirox; TRB, terbinafine; ITR, itraconazole; ND, not determined.

Repeated exposure to ME1111. MIC microtiter plates for ME1111, amorolfine, and ciclopirox against each isolate were set up according to the CLSI M38-A2 methodology. To determine the inoculum to be used for this assay, the contents of the microdilution wells at 0.5 × MIC, 1 × MIC, and 2 × MIC were transferred to a PDA plate and streaked for isolation. Growth subcultured from the highest concentration (0.5 × MIC, 1 × MIC, or 2 × MIC) was used in subsequent passages as described below.

For serial passages, conidia were harvested and an inoculum was prepared to a concentration of 10^7 CFU/ml. Four concentrations of each antifungal (0.5 × MIC, 1 × MIC, 2 × MIC, and 4 × MIC) were prepared in 10 ml of RPMI 1640 using sterile 15 ml centrifuge tubes. Each tube was inoculated with 0.5 ml of inoculum and incubated for 4 days at 35°C. Tubes were subsequently centrifuged for 10 min at 1,338 × g, and the supernatant was decanted. Sterile saline (0.6 ml) was then added, and the tubes were vortexed. For the second passage, another set of tubes was prepared (0.5 × MIC, 1 × MIC, 2 × MIC, and 4 × MIC), to which 100 μl of cell sediment was added. The passaged cells were incubated for 4 days at 35°C. An additional 100 μl of cell sediment was plated onto the surface of a PDA or cereal agar plate and spread with a loop to make a “lawn.” The subcultured plates were incubated for 4 days at 35°C, and conidia were harvested for MIC testing as described above. The entire passage procedure was repeated for a total of 15 passages, with MIC testing performed after each passage.

RESULTS

Table 1 shows the data for ME1111 and comparators against the dermatophyte strains tested, while Table 2 shows the data against the seven T. rubrum strains with elevated terbinafine MICs and nine T. mentagrophytes strains with elevated itraconazole MICs. The ME1111 MIC (defined as the lowest concentration to inhibit 50% of the strains tested) and MIC90 (defined as the lowest concentration to inhibit 90% of the strains tested) against T. rubrum strains were 0.125 and 0.25 μg/ml, respectively. The MIC50 and MIC90 values of ME1111 against the remaining isolates were all 0.25 μg/ml. The cumulative MIC50 and MIC90 values of ME1111 were 0.25 and 0.25 μg/ml, respectively, against all strains. This was equivalent to the results for amorolfine (0.125 and 0.25 μg/ml, respectively) and ciclopirox (0.25 and 0.5 μg/ml, respectively). More importantly, no isolates with ME1111 MICs greater than 8 μg/ml were observed in the dermatophytes tested (n = 400).

Table 3 shows the MFC values for strains subcultured from MIC assays conducted in SDB. ME1111 demonstrated an MFC50 (defined as the lowest concentration to demonstrate fungicidal activity against 50% of the strains tested) and an MFC90 (defined as the lowest concentration to demonstrate fungicidal activity against 90% of the strains tested) of 4 and 8 μg/ml, respectively, against the T. rubrum strains tested. These MFC50 and MFC90 values were similar to those of ciclopirox (8 and 8 μg/ml, respectively) but higher than those of amorolfine (0.25 and 0.5 μg/ml, respectively).

ME1111 demonstrated MFC50 and MFC90 of 2 and 8 μg/ml, respectively, against the T. mentagrophytes strains tested. These MFC50 and MFC90 values were similar to those of ciclopirox (16 and 16 μg/ml, respectively) but higher than those of amorolfine (0.125 and 0.25 μg/ml, respectively). ME1111 demonstrated MFC50 and MFC90 of 4 and 32 μg/ml, respectively, against the E. floccosum strains tested, which again were similar to those of ciclopirox (8 and 16 μg/ml, respectively) but higher than those of amorolfine (0.125 and 0.25 μg/ml, respectively).

As can be seen in Table 4, there was no increase in MIC greater than 3 dilutions following 15 passages with ME1111 or comparators against any of the 4 strains tested.

Exposure of T. rubrum 23576 to ME1111 at 2 × MIC and 4 × MIC resulted in no growth after passage 12, while exposure of T. rubrum 23576 to ME1111 at 0.5 × MIC and 1 × MIC resulted in no growth after passage 15. However, exposure of T. rubrum 23576 to amorolfine at 4 × MIC resulted in a 3-dilution increase in MIC values after the 15th passage, suggesting development of resistance.

DISCUSSION

ME1111 is a novel selective inhibitor of succinate dehydrogenase (complex II) of dermatophyte species, with a small molecular mass that enhances its ability to penetrate the nail plate (24). In fact, in vitro penetration studies conducted in Franz cell assays using human nail as well as a guinea pig model demonstrated that ME1111 concentrations in the nail following single and/or multiple applications were several orders of magnitude greater than the MFC50 and MFC90 determined in this study for three major dermatophytes species causing onychomycosis (25, 26).

Our in vitro data show that ME1111 demonstrated potent antifungal activity against all of the dermatophyte strains tested. Importantly, ME1111 had potent activity against the 16 dermatophyte strains tested with elevated terbinafine or itraconazole MICs. Further, the MICs of ME1111 were equivalent to that of...
amorolfine and ciclopirox, with MICs of values of all three antifungals in the range of 0.125 to 0.5 μg/ml. Additionally, our data show that ME1111 has fungicidal activity against T. rubrum and T. mentagrophytes (MFC90 values of 8 μg/ml) that is comparable to that of ciclopirox.

Regarding the potential of dermatophytes to develop resistance to ME1111, our findings show that there was no increase in MICs greater than 3 dilutions after repeated exposure to ME1111 or comparators in the Trichophyton strains tested. T. rubrum 23576, when exposed to ME1111 at ×2 MIC and ×4 MIC, showed no growth after passage 12, while repeated exposure of the same strain to ME1111 at 0.5× MIC and 1× MIC resulted in no growth after passage 15. This failure to grow from subcultures may be an indication of extensive morphological or structural damage to the hyphal cells by repeated exposure to ME1111, owing to its fungicidal effect. The accepted definition of resistance development is 15, which indicates that this antifungal, unlike ME1111, has the potential to induce resistance. From this study, it is suggested that, similar to ciclopirox, ME1111 has low potential for inducing drug resistance in T. rubrum and T. mentagrophytes, the organisms that account for >98% of nail infections.

In conclusion, ME1111 shows promise of high efficacy as a topical treatment for onychomycosis.

ACKNOWLEDGMENT

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REFERENCES


