In vitro susceptibility testing of amorolfine in pathogenic fungi isolated from dermatomycosis patients in China

In vitro Empfindlichkeitsprüfung mit Amorolfin an pathogenen Pilzen von Patienten mit Dermatomykosen in China

R.-Y. Li,1 Z. Wan,1 A.-P. Wang,1 Y.-N. Shen,2 C.-M. Lu,3 M. Li,2 L.-Y. Xi,3 W.-D. Liu 2 and F.-Q. Zeng3

1Department of Dermatology, Peking University First Hospital, Research Center for Medical Mycology, Peking University, Beijing, 2Institute of Dermatology, Chinese Academy of Medicine, Nanjing and 3Department of Dermatology, Second Hospital, Zhongshan University, Guangzhou, China

Summary

The antifungal susceptibility of isolates from Chinese dermatomycosis patients to amorolfine was investigated following National Committee for Clinical Laboratory Standards (NCCLS) protocols. In total, 383 isolates were tested, including 132 strains from tinea pedis, 148 strains from tinea corporis/cruris, and 103 strains from onychomycosis. The minimum inhibitory concentration (MIC) of amorolfine against dermatophytes ranged from 0.01 to 0.08 µg ml⁻¹. The MIC₅₀ and MIC₉₀ of amorolfine for Trichophyton rubrum were both equal to 0.04 µg ml⁻¹; for T. mentagrophytes these MICs were 0.04 µg ml⁻¹ and 0.08 µg ml⁻¹ respectively; and for Epidermophyton floccosum they were 0.02 µg ml⁻¹ and 0.04 µg ml⁻¹ respectively. The MIC range of amorolfine against Candida parapsilosis was 0.5–16 µg ml⁻¹. MIC₅₀ and MIC₉₀ for C. parapsilosis were 0.5 and 2 µg ml⁻¹. MIC ranges of amorolfine against Scopulariopsis spp. and Acremonium spp. were 0.5–4 and 2–8 µg ml⁻¹, respectively. Candida albicans, Fusarium solani and Aspergillus flavus required relatively higher concentrations of amorolfine to inhibit their growth (MIC 0.125–64 µg ml⁻¹, MIC₅₀ and MIC₉₀ were 4 and 64 µg ml⁻¹). The results demonstrated that amorolfine is the only topical agent that has such a potent antifungal activity and a broad spectrum against a wide range of pathogenic fungi.

Zusammenfassung

Die antimykotische Empfindlichkeit von Isolaten chinesischer Patienten mit Dermatomykose auf Amorollin wurde mittels NCCLS Protokollen untersucht. Im ganzen wurden 383 Isolate getestet, bestehend aus 132 Stämmen von Tinea pedis, 148 Stämmm von Tinea corporis/cruris und 103 Stämmm von Onychomykosen. Die MHK von Amorollin gegen Dermatophyten reichte von 0,01 µg ml⁻¹ bis 0,08 µg ml⁻¹. Die MHK₅₀ und MHK₉₀ von Amorollin gegen T. rubrum waren beide identisch mit 0,04 µg ml⁻¹; für T. mentagrophytes lagen beide MHKs bei entsprechend 0,04 µg ml⁻¹ und 0,08 µg ml⁻¹; und für E. floccosum betrugen sie 0,02 µg ml⁻¹ and 0,04 µg ml⁻¹. Das MHK-Intervall von Amorollin für C. parapsilosis reichte von 0,5 bis 16 µg ml⁻¹. MHK₅₀ und MHK₉₀ für C. parapsilosis lagen jeweils bei 0,5 µl ml⁻¹ und 2 µg ml⁻¹. MHK-Intervalle von Amorollin für Scopulariopsis spp. und Acremonium spp. reichten jeweils von 0,5 µg ml⁻¹ bis 4 µg ml⁻¹ und 2 µg ml⁻¹ bis 8 µg ml⁻¹.

Correspondence: Dr Ruoyu Li, Department of Dermatology, Peking University First Hospital, Research Center for Medical Mycology, Peking University, No. 8 Xishiku St, West District, Beijing 100034, China, Tel: 86-10-66551122-3056. Fax: 86-10-66551216. E-mail: lrywdh@public.bta.net.cn

Accepted for publication 7 May 2003
C. albicans, Fusarium solani und Aspergillus flavus verlangten relativ höhere Konzentrationen von Amorolfin, um in ihrem Wachstum gehindert zu werden (MHK zwischen $0.125 \mu g \cdot ml^{-1}$ und $64 \mu g \cdot ml^{-1}$, MHK$_{50}$ und MHK$_{90}$ betrugen $6 \mu g \cdot ml^{-1}$ und $64 \mu g \cdot ml^{-1}$). Die Ergebnisse zeigten, dass Amorolfin das einzige topische Mittel ist, dass eine solch starke antimykotische Aktivität und ein breites Spektrum gegen pathogene Pilze aufweist.

**Key words:** dermatophytes, yeasts, molds, antifungal susceptibility, amorolfine.

**Schlüsselwörter:** Dermatophyten, Hefen, Schimmelpilze, Empfindlichkeitsprüfung, Amorolfin.

**Introduction**

Amorolfine is a member of morpholine antymycotic agents that in a previous *in vitro* study demonstrated an activity against a wide range of pathogenic fungal strains. The nail lacquer formulation of amorolfine, a topical preparation (brand name: Loceryl nail lacquer), is currently used for the treatment of onychomycosis and was recently launched in China. A cream formulation is also available for the treatment of different kinds of dermatomycoses. However, up to today no clinical data of the antifungal activity of amorolfine in Chinese clinical isolates exist.

Hence, the purpose of the present study was to investigate the antifungal activity of amorolfine against common clinical isolates from Chinese dermatomycosis patients by respecting the National Committee for Clinical Laboratory Standards (NCCLS) guidelines with its accepted modification for dermatophytes, yeasts and molds. These guidelines have recently been developed with accepted modification by the US NCCLS, and are today internationally accepted as being the ‘gold standard’, for the testing of dermatophytes, yeasts and molds.

**Materials and methods**

**Isolates**

All strains were isolates from clinical specimens obtained by clinicians of the Peking University Research Center for Medical Mycology, the Institute of Dermatology and the Zhongshan University Secondary Hospital.

In total, 383 isolates were tested, including 132 strains from tinea pedis, 148 strains from tinea corporis/cruris, and 103 strains from onychomycosis. Among these, 334 strains of dermatophytes were tested (252 strains of *Trichophyton rubrum*, 49 strains of *T. mentagrophytes* and 18 strains of *Epidermophyton floccosum*, five strains of *T. tonsurans*, *T. violaceum* and *Microsporum canis*), 30 strains of yeasts (15 strains of *Candida albicans*, 10 strains of *C. parapsilosis*, three strains of *C. guilliermondii* and one strain of *C. tropicalis* and *C. glabrata*) and 19 strains of molds (six strains of *F. solani*, five strains of *Aspergillus flavus* and *Acremonium* spp., three strains of *Scopulariopsis* spp.). Isolates were identified in the species level by conventional methods and were routinely maintained on Sabouraud glucose agar (SDA) slants. Yeasts were incubated at 35 °C, dermatophytes and other molds were incubated at 27 °C. American Type Culture Collection (ATCC) quality control organisms (*C. parapsilosis* ATCC 22019) were used.

**Preparation of amorolfine solution**

Amorolfine powder was supplied by Galderma Laboratories (Fontenay-sous-bois, France; permitted code: 010063-1, concentration: 100.9%). To prepare the stock solution (1600 $\mu g \cdot ml^{-1}$), 6.4 mg amorolfine powder was dissolved in 4 ml 100% dimethyl sulfoxide (DMSO), 0.5 ml of this solution was dispensed into each tube, and stored at $+20$ °C.

**Susceptibility testing**

The minimum inhibitory concentrations (MICs) of amorolfine were determined separately according to the protocols of NCCLS M-38P (modified for dermatophytes) and M-27A assay.

**Medium**

The RPMI 1640 containing L-glutamine but no sodium bicarbonate and buffered at pH 7.0 with 3-(N-morpholino) propanesulfonic acid, monosodium salt (MOPS), was used as medium for the broth microdilution susceptibility testing.
**Broth microdilution testing**

In accordance with the NCCLS reference method microdilution plates were used. According to the NCCLS proposed standard serial twofold dilutions were prepared.

A volume of 0.2 ml stock solution of amorolfine (1600 μg ml⁻¹) was added into the first tube and 0.2 ml of RPMI 1640 was dispensed to each tube from the second to the 10th. 0.2 ml amorolfine stock solution was added to the second tube and twofold diluted in turn and 9.8 ml RPMI 1640 medium was dispensed into each tube.

Aliquots of 100 μl each of the antifungal agent medium mixtures were dispensed to each well of the 96 flat-bottomed microplate. The first 10-wells were considered as drug concentration gradients with a MIC range for amorolfine of 0.03–16 mg l⁻¹, the 11th was the growth control, and the 12th tube was the vacuity control with a solvent control performed at the same time.

**Inoculum preparation**

The tested dermatophytes and molds were cultured at 27 °C on potato dextrose agar (PDA) at an interval of 7–10 days. Yeasts were cultured on SDA 48 h at 35 °C. A standardized inoculum was prepared by counting microscopically the microconidia. The culture was gently swabbed with a cotton tip applicator to dislodge the conidia from the hyphal mat.

The suspension was transferred to a sterile centrifuge tube, and with sterile normal saline the volume was adjusted to 5 ml. The resulting suspension was counted on a hemocytometer and was diluted in RPMI 1640 to the desired concentration.

The inocula were prepared according to the final concentration of dermatophytes and other molds: 0.5–2.5 × 10⁵ cfu ml⁻¹; yeasts: 0.5 × 10²–2.5 × 10³ cfu ml⁻¹. Column 1 was filled with 200 μl of medium as a sterility control. Columns 2 through 11 were filled with 100 μl of the inoculum and with 100 μl of the serially diluted antifungal agent. Column 12 was filled with 200 μl of the inoculum and used as a growth control.

**Incubation time and temperature**

The microdilution plates were incubated at 35 °C for yeasts and 27 °C for dermatophytes and other molds and were visually read after 1–7 days of incubation.

**Reading of the microplates**

Dermatophytes could be read after 5–7 days of incubation. Other non-dermatophyte molds could be read after 2–3 days.

Yeasts could be read after 24–48 h of incubation. For yeasts, the MIC was defined as the moment, at which, compared with the growth rate in the control well 80% of the organism were inhibited. For molds, the MIC was defined as the time-point at which 50% of the organisms were inhibited compared with that of the growth in the control well.

All isolates were run in twice and the results were read visually. Additionally, MICs at which 50 and 90% inhibitions were used.

### Table 1

**Minimum inhibitory concentration (MIC) distribution of amorolfine against clinically common isolates (μg ml⁻¹).**

<table>
<thead>
<tr>
<th>Dermatophytes</th>
<th>&gt;0.08</th>
<th>0.08</th>
<th>0.04</th>
<th>0.02</th>
<th>0.01</th>
<th>0.005</th>
<th>0.0025</th>
<th>0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichophyton rubrum (252)</td>
<td></td>
<td>1</td>
<td>145</td>
<td>105</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. mentagrophytes (49)</td>
<td>1</td>
<td>2</td>
<td>40</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. tonsurans (5)</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. violaceum (5)</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermophyton floccosum (18)</td>
<td>3</td>
<td>14</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsporum canis (5)</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeasts and molds</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Candida albicans (15)</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis (10)</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. guilliermondii (3)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. tropicalis (1)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. glabrata (1)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium solani (6)</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus flavus (5)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scopulariopsis spp. (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acremonium spp. (5)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
of tested strains were inhibited were calculated, referred hereafter as MIC_{50} and MIC_{90}.

**Results**

Table 1 shows the distribution of MICs for amorolfine against 383 strains of common clinical isolated dermatophytes, yeasts and molds. Table 2 shows the range of MICs, MIC_{50} and MIC_{90} of amorolfine against these isolates.

The MIC of amorolfine against *T. rubrum*, *T. mentagrophytes*, *E. floccosum* and other tested dermatophytes ranged from 0.01 to 0.08 μg ml^{-1}. The MIC_{50} and MIC_{90} of amorolfine for the *T. rubrum* were both equal with 0.04 μg ml^{-1}, for *T. mentagrophytes* MIC_{50} and MIC_{90} were 0.04 μg ml^{-1} and 0.08 μg ml^{-1} respectively, for *E. floccosum* they were 0.02 μg ml^{-1} and 0.04 μg ml^{-1} respectively.

The MIC of amorolfine against *C. parapsilosis* ranged from 0.5 to 16 μg ml^{-1}. MIC_{50} and MIC_{90} for *C. parapsilosis* were 0.5 and 2 μg ml^{-1} respectively. MIC ranges of amorolfine against three strains of *Scopulariopsis* spp. and five strains of *Acremonium* spp. were 0.5–4 μg ml^{-1} and 2–8 μg ml^{-1} respectively.

The MIC range of amorolfine against *C. albicans* was 0.125–64 μg ml^{-1}; MIC_{50} and MIC_{90} were 4 and 64 μg ml^{-1}. The MIC range of amorolfine against other rarely isolated molds from the nail such as *F. solani* was 4–64 μg ml^{-1} and for *A. flavus* >64 μg ml^{-1}.

**Discussion**

Tinea pedis, tinea corporis/cruris as well as onychomycosis are very common fungal infections in the Chinese population. An epidemiological survey showed that dermatophytes are the dominant pathogens, accounting for 70–80% of the causative agents. Most commonly described are dermatophyte species, notably *T. rubrum*, *T. mentagrophytes* as well as *E. floccosum*. Yeasts and other molds could be isolated from the infected area especially from nails and mixed infections caused by the above pathogens could also be noticed.

Because of inadequately effective agents the treatment of onychomycosis was a challenge for a long time for clinicians in China. Today various oral antifungal treatments have become available in China. However, a significant proportion of treatment failures and/or relapses after treatment in patients were expected. Moreover, some patients are reluctant to use oral antifungal therapy due to the limitation of their general health condition, hence new treatment modalities and drugs are of need.

Amorolfine is a morpholine antimycotic agent, active against a wide range of pathogenic fungi. Uchida et al. reported the average MIC values of amorolfine against clinical isolates of *T. mentagrophytes*, *M. canis*, and *E. floccosum* were 0.0267, 0.0079, and 0.0018 μg ml^{-1}, respectively. MIC range of amorolfine against isolates of *C. albicans* was 0.01–10 μg ml^{-1}.

The reason of amorolfine having such a strong activity against these above-mentioned strains compared with all other available agents lies in its specific action mode of inhibiting two enzymes (614-reductase and 87, 88-isomerase) of the ergosterol biosynthetic pathway at distinct and separate points.

International standard methods of antifungal susceptibility testing were not available for a long time. Today the NCCLS has developed internationally accepted methods. But only one previous *in vitro* study used
NCCLS recommendations to assess MICs of amorolfine. In the present study, NCCLS methods with accepted modification were used to test in vitro the susceptibility to amorolfine for dermatophytes, yeasts and molds. The results of this study showed that most of the pathogenic fungi, especially dermatophytes, were very susceptible to amorolfine. In addition, the study showed that amorolfine is also very effective against the most common isolated molds from nails. However, several strains of C. albicans, F. solani (MIC 4–64 μg ml⁻¹) and A. flavus and other rarely isolated molds from the nail required relatively higher concentrations of amorolfine to inhibit their growth.

The present study demonstrated that results of the MICs for amorolfine against dermatophytes, yeasts and molds were similar to those shown in a clinical study described by Evans, concluding that amorolfine is a topical agent having a potent antifungal activity and a broad spectrum against a wide range of fungi isolated from Chinese dermatomycosis patients.

Amorolfine’s antifungal effect has not been reduced and no resistance strains have been found during the past 10 years. Although the result is valuable, we still need to do further investigations in vitro and in vivo to evaluate the antifungal activity of amorolfine in Chinese patients.

Acknowledgments
Authors address their sincere thanks to Drs Zhao Zuotao and Huang Xiaojie for their technical assistance and the Galderma Laboratories for their support.

References