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

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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 Epidermphyton 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). 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 antymykotische Empfindlichkeit von Isolaten chinesischer Patienten mit Dermatomeskose auf Amorollin wurde mittels NCCLS Protokollen untersucht. Im ganzen wurden 383 Isolate getestet, bestehend aus 132 Stämmen von Tinea pedis, 148 Stämmen von Tinea corporis/cruris und 103 Stämmen von Onychomycosen. 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 und 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 µl 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. 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.

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C. albicans, Fusarium solani und Aspergillus flavus verlangten relativ höhere Konzentrationen von Amorolfin, um in ihrem Wachstum gehindert zu werden (MHK zwischen 0.125 μg ml⁻¹ und 64 μg ml⁻¹, MHK₅₀ und MHK₉₀ betrugen 6 μg ml⁻¹ und 64 μg 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, amorolfin.

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

**Introduction**

Amorolfin 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.¹ The nail lacquer formulation of amorolfin, 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 amorolfin in Chinese clinical isolates exist.

Hence, the purpose of the present study was to investigate the antifungal activity of amorolfin 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.²

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 amorolfin solution**

Amorolfin powder was supplied by Galderma Laboratories (Fontenay-sous-bois, France; permitted code: 010063-1, concentration: 100.9%). To prepare the stock solution (1600 μg ml⁻¹), 6.4 mg amorolfin 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 amorolfin were determined separately according to the protocols of NCCLS M-38P (modified for dermatophytes) and M-27A assay.²⁻⁴

**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 × 10\(^5\) cfu ml\(^{-1}\); yeasts: 0.5 × 10\(^2\)–2.5 × 10\(^3\) 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%
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. flocosum 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. flocosum 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 onychomycoses 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. flocosum. 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. flocosum 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 (Δ14-reductase and Δ7, Δ8-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 amorol- 
fine. In the present study, NCCLS methods with 
accepted modification were used to test in vitro the 
susceptibility to amorolfine for dermatophytes, yeasts 
and molds.2

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,10 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.

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