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Antifungal drug response in an *in vitro* model of dermatophyte nail infection

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Despite terbinafine being fungicidal against *Trichophyton rubrum* in standard NCCLS assays and rapidly accumulating in nails *in vivo*, onychomycosis patients require prolonged terbinafine treatment to be cured. To investigate this, we developed a more clinically relevant onychomycosis *in vitro* test model. Human nail powder inoculated with *T. rubrum* and incubated in liquid RPMI 1640 salt medium, which did not support growth alone, developed extensive and invasive mycelial growth. Antifungal drugs were added at different concentrations and cultures incubated for 1 to 4 weeks. Fungal survival was determined by spreading cultures on PDA plates without drug and measuring CFU after 1 to 4 weeks incubation. Drug activity was expressed as the nail minimum fungicidal concentration (Nail-MFC) required for 99.9% elimination of viable fungus. Terbinafine Nail-MFC was 4 μg/ml after 1 week exposure, decreasing to 1 μg/ml after 4 weeks exposure, much higher than MFCs ≤ 0.03 μg/ml determined in standard NCCLS MIC assays. In contrast, other clinically used drugs were unable to kill *T. rubrum* after 4 weeks incubation in this model. Invasive mycelial growth on nail appears to protect *T. rubrum* from the cidal action of systemic drugs, thus providing a rationale for the long treatment periods in onychomycosis.

Keywords antifungals, onychomycosis, *Trichophyton rubrum*

Introduction

Fungal nail infection, onychomycosis, is a common disease. Its prevalence can reach up to 8% in Nordic countries [1,2] and was even higher in a large scale study in North America [3]. Several heterogeneous factors also predispose some population groups to onychomycosis [2,4]. The main causative agents of nail infections are the dermatophytes, of which *Trichophyton rubrum* is the most frequently found [3,5]. These fungi have a characteristic ability to utilise keratin as a nutrient source [6]. Terbinafine is highly effective in treating dermatophyte infections and acts by blocking ergosterol synthesis through the inhibition of squalene epoxidase, resulting in an accumulation of squalene, which is toxic to fungal cells [7]. MIC_{90} values for terbinafine, determined for *T. rubrum* strains according to standard NCCLS procedures, are ≤ 0.06 μg/ml [8–11]. The fungicidal activity of terbinafine against dermatophytes is well established [12–19], with minimum fungicidal concentrations (MFC) of ≤ 0.06 μg/ml against *T. rubrum*, and complete killing requiring not more than a few days [12,15,16,19].

In vivo, terbinafine accumulates rapidly in human nail, where it persists for a long time [20,21]. For example, Faergemann *et al.* [21] reported that a maximum concentration of 0.39 mg/g is reached, and that levels of 0.09 mg/g are still present 55 days after the end of therapy. However, despite its potent cidal activity *in vitro* and its favourable pharmacokinetic properties *in vivo*, standard treatment of toenail infec-

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tions with terbinafine requires 3 months of once-daily oral therapy [22,23]. Other therapies require similar or even longer periods of treatment [24].

The purpose of this study was to develop a nail-based culture model for the testing of antifungal compounds. This model mimics the course of a natural nail fungal infection, with antifungal treatment starting only after mycelial growth is established. In this model, *T. rubrum* is much more resistant to the cidal action of terbinafine than in conventional assays.

Materials and methods

Trichophyton rubrum *strains*

The five *T. rubrum* strains used in this study were clinical isolates from the Novartis Fungal Index (NFI) collection. The strains were from infected nails (NFI 5132 and NFI 5143) or feet (NFI 5139 and NFI 5140), while NFI 5182 corresponds to the ATCC strain 18759, which caused dermatophytosis. To prepare stock inocula, cultures were grown on potato dextrose agar (PDA) (Merck, Whitehouse Station, USA) at 30°C for 1–5 weeks. The conidia and mycelium was then harvested, dispersed in Sabouraud 2% dextrose broth (Merck), and stored at −80°C after addition of 5% (vol/vol) dimethyl sulfoxide (DMSO) as cryoprotectant. Colony forming units (cfu) in these stock inocula was determined, after rapid thawing, by spreading on PDA plates 50 µl from 10-fold serial dilutions in a physiological saline solution, and counting the colonies after 1 week's incubation at 30°C.

Antifungal drugs

Terbinafine, naftifine and itraconazole were synthesized at Novartis, Basel, Switzerland. Fluconazole was extracted and purified from commercial tablets of Diflucan (Pfizer) at Novartis, Vienna, Austria. Amorolfine was from Roche Pharmaceuticals, Basel, Switzerland. Clotrimazole, ciclopiroxolamine and griseofulvin were purchased from Sigma Chemical Co, St Louis, USA. All drugs were dissolved and two-fold serially diluted in dimethyl sulfoxide (DMSO).

Determination of MIC and MFC

MIC were determined in 96-well flat bottom assay plates with a slight modification of the NCCLS microdilution procedure M38-A [25,26]. The final concentration of DMSO was 1% and the size of the inoculum was 2.5×10^4 cfu/ml. Plates were incubated for 4–5 days at 35°C. Growth inhibition was scored visually with the aid of an inverted magnifying mirror from 4 to 0 according to NCCLS M38-A reference method [25]. The MIC corresponded to the lowest concentration giving a score of 1 (equivalent to about 75% growth inhibition) and 100% MIC to the lowest concentration giving a score of 0 (equivalent to 100% growth inhibition). After MIC determination, starting from the last well in which growth was observed up to the highest drug concentration tested, 100 µl was transferred in duplicate onto potato dextrose agar (PDA) medium (Merck) in a 20 ml/9-cm-diameter plate. Plates were incubated for 1 week at 30°C followed by visual inspection of growth. MFC corresponded to the lowest drug concentration (in the assay microtitre plate) at which no more than one colony subsequently grew in the 9-cm plate, corresponding to $\geq 99.9\%$ killing activity.

Preparation and collection of nail powder

Human nail clippings were collected from healthy volunteers within the Novartis Research Institute. Finger and toe nails were pooled and ground to a powder using a stainless steel peppermill (Peugeot, Paris, France) followed by a nail micronizer (Delasco, Council Bluffs, USA). The nail powder was washed with chloroform and then ethanol, prior to being autoclaved at 120°C for 20 min.

Nail model culture system and determination of nail MFC

Autoclaved nail powder was added, at approximately 10 mg per well, to 96-well plates. *T. rubrum*, 5 µl of 1×10^6 cfu/ml, was added directly onto the nail powder. A high cfu/low volume inoculate has been previously reported to give good uniform growth on nail fragments [27]. This was left at 35°C for 1 h, after which 200 µl RPMI select amine medium buffered with 0.165 mol/l MOPS (Invitrogen, San Diego, USA), which is essentially a balanced salts solution, were added (final cfu/ml: 2.5×10^4). The plate was returned to 35°C for 4–5 days, by which time growth was well established. A small volume of drug (10 µl), initially dissolved in DMSO, and then in medium, was added to the desired concentration (final concentration of DMSO 1%) and the culture was returned to 35°C. Drug incubation periods of 1, 2, 3 and 4 weeks were investigated. All experiments were performed in duplicate. After incubation with drug, the content of each well was washed twice with RPMI 1640 (Invitrogen) before being transferred via an inoculating loop to a PDA plate (containing no drug) for nail MFC (Nail-MFC) determination. Plates were incubated at 30°C and were examined and photographed after 1, 2, 3 and 4 weeks. Nail minimum fungicidal concentration was

defined as the lowest drug concentration (in the assay microtitre plate) at which no more than two colonies subsequently grew in the 9-cm plate, corresponding to $\geq 99.9\%$ killing activity.

Results

Nail-MFC assay development with five T. rubrum *strains*

In the presence of nail powder and RPMI select amine medium buffered with 0.165 mol/l MOPS, prominent mycelial growth of *T. rubrum* strains could be seen in each inoculated well after a few days of incubation at 35°C (data not shown). By lifting up the mycelium with forceps, it could be seen that the fungi were firmly attached to the nail particles. In control wells without nail powder the dermatophyte failed to grow (not shown), demonstrating that nail was an essential nutrient source in this assay. Attempts to quantitatively assess mycelium on the nail using fluorescent dyes failed because of the intrinsic, high fluorescence of nail (C. Osborne, unpublished data).

To the mycelium grown on nail for 4–5 days, various concentrations of terbinafine were then added and Nail-MFC determined as described in Materials and methods. Initially, 5 *T. rubrum* strains were tested with a drug incubation time of 1 week and an incubation time on PDA plates without drug of also 1 week. Nail-MFCs were very similar for all five strains tested. However, they were much higher than MFC values obtained by the standard NCCLS MIC assay (Table 1). In cases where fungal outgrowth occurred on the drug-free PDA plates, nascent fungal growth was observed to emanate directly from the nail pieces, suggesting a protective effect of the nail.

Effect of incubation time on terbinafine activity

In view of the similar results obtained with different strains of *T. rubrum*, subsequent experiments were performed with strain NFI 5143. A longer outgrowth incubation time of 4 weeks on drug-free PDA plates after the 1-week's treatment with terbinafine slightly increased the Nail-MFC values from 2 to 4 μg/ml. This more stringent condition (4 weeks incubation on PDA plates) was employed for all subsequent experiments. The influence of incubation time with terbinafine on Nail-MFC was then investigated. The Nail-MFC for terbinafine decreased stepwise with longer exposure to the drug, dropping from 4 μg/ml after a 1-week exposure, to 1 μg/ml after a 4-week exposure (Fig. 1). After 2 and 3 weeks incubation with terbinafine the Nail-MFC was 2 μg/ml.

Table 1 MIC, MFC, and Nail-MFC values of terbinafine against five *Trichophyton rubrum* strains

Strain	MIC[a]	100% MIC	MFC (μg/ml)	Nail-MFC[b]
NFI 5132	0.008	0.016	0.030	2
NFI 5139	0.008	0.016	0.030	2
NFI 5140	0.004	0.008	0.016	2
NFI 5143	0.008	0.016	0.030	2
NFI 5182	0.002	0.004	0.030	1

[a]MIC and 100% MIC correspond to drug concentrations required to inhibit fungal growth by 75% and 100% respectively. [b]1-week incubation with drug followed by 1-week's incubation on PDA plates without drug.

Activity of other antifungals

In contrast to terbinafine, all of the other drugs tested (amorolfine, ciclopiroxolamine, clotrimazole, fluconazole, griseofulvin, itraconazole, and naftifine) were unable to effectively kill *T. rubrum* growing on nail powder. In all cases, prominent fungal growth was observed on PDA plates at the highest concentrations tested (amorolfine 1 μg/ml, ciclopiroxolamine 128 μg/ml, clotrimazole 64 μg/ml, fluconazole 128 μg/ml, griseofulvin 64 μg/ml, itraconazole 4 μg/ml, and naftifine 8 μg/ml) even after a 4-week incubation period with these drugs.

Discussion

The standard NCCLS methodology for antifungal testing makes use of conidial suspensions [25,33], or as used by ourselves, a mixed suspension of mycelial fragments and conidia [26], growing in a rich medium. In contrast, the novel assay described in this report presents the test drug with the challenge of an established dermatophyte mycelium growing on and metabolising its natural substrate, the nail. We postulate that this is much more akin to the clinical situation of onychomycosis in which the drug must overcome an established infection. Terbinafine Nail-MFCs obtained in this model, ≥ 1 μg/ml, were much higher than MFC values obtained after conventional MIC assays, ≤ 0.06 μg/ml [12,15,16,19,26]. The cidal action of terbinafine was also much slower than in conventional assays, requiring 4 weeks incubation to achieve the lowest Nail-MFC value of 1 μg/ml. Nevertheless, terbinafine was the only drug among those tested that was able to eliminate viable *T. rubrum* from nail in this model. Terbinafine was previously reported by Seebacher [34] to have a much weaker killing activity against quiescent conidia-mycelium incubated in a physiological saline, versus growing mycelium incubated in Sabouraud broth. The presence of arthrospores, thought to be

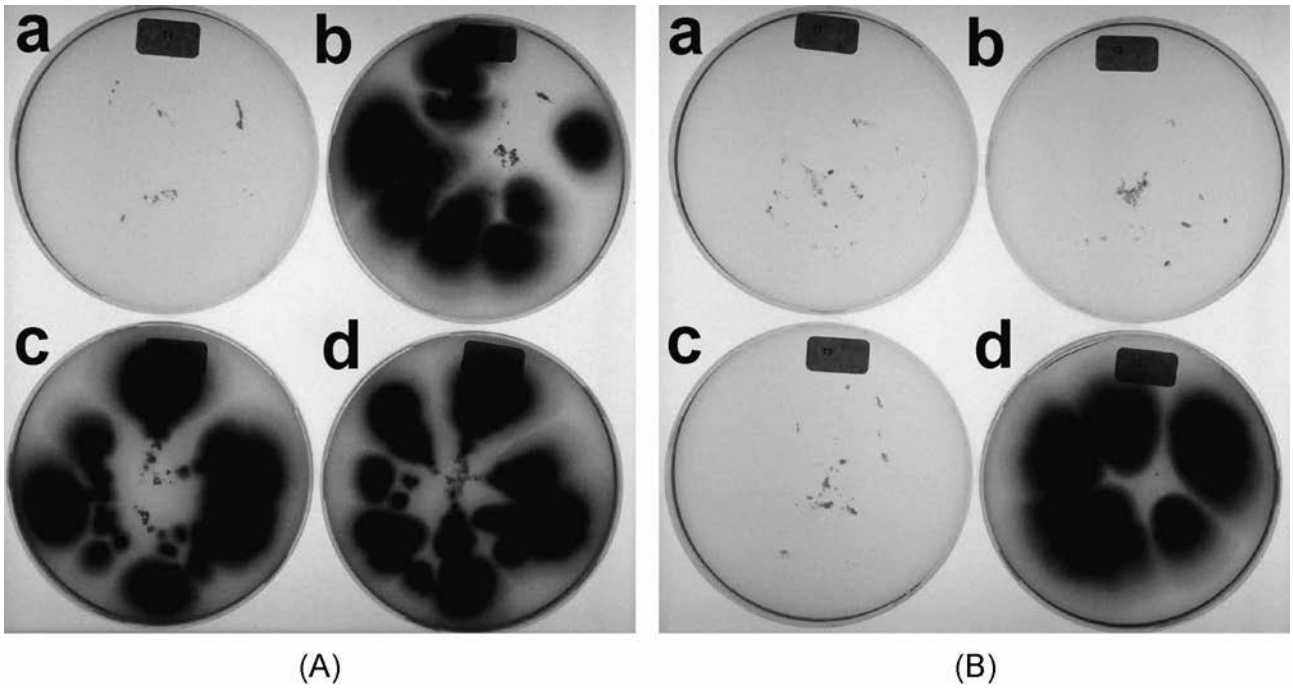

Fig. 1 Influence of incubation time with terbinafine on Nail-MFC. *Trichophyton rubrum* NFI 5143, initially grown on nail without drug for 4 days, was incubated for 1 week (A) or 4 weeks (B) with various concentrations of terbinafine: 4 µg/ml (a), 2 µg/ml (b), 1 µg/ml (c) and 0.5 µg/ml (d), and then transferred to PDA plates without drug and further incubated for 4 weeks.

resistant to drug action [35], was not observed in our growth cultures. The combination of high Nail-MFC values and slow cidal action provides a feasible rationale for the 3-month treatment times in patients [22,23].

The absence of any alternative nutrient supply in the culture medium means that the fungi must utilise the nails as their sole nutrition source by activating the secretion of keratinases [6,27]. Only a few previous studies have used nails as the only source of nutrition [28–30]. Although, these systems have provided useful information, such as fungal morphological changes post drug exposure [28] or the effect of drugs on preventing nail plate invasion [30], none of them has adequately explained the long treatment time required to cure onychomycosis with systemic antifungals. Other models have used keratin particles from human *stratum corneum* as a substrate [31]. However, dermatophytes exhibit differences in their ability to degrade nails versus other types of keratin [32].

In conclusion, the model described here provides an interesting and clinically more relevant tool for evaluating existing or novel antifungals for the treatment of onychomycosis. Future research directions with this model may include the study of additional nail pathogens and rare terbinafine-resistant strains [36], use of drug combinations, and investigation of potential for development of drug resistance during nail infection.

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