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SUSCEPTIBILITY

# *In Vitro* Resistance and Evolution of Resistance to Tavaborole in *Trichophyton rubrum*

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**ABSTRACT** Tavaborole is currently used in the topical treatment of onychomycosis. In this study, we analyzed the *in vitro* emergence/evolution of resistance against tavaborole in *Trichophyton rubrum*. When *T. rubrum* strains were propagated on media containing the MIC of tavaborole, spontaneous resistant mutants were isolated at a frequency of $10^{-8}$. The frequency was almost 100-fold higher following fungal growth in the presence of a subinhibitory tavaborole concentration (0.5-fold the MIC) for 10 transfers. All collected mutants showed similar 4- to 8-fold increases in the drug MIC. No cross-resistance to other antifungals was evident.

**KEYWORDS** *Trichophyton rubrum*, onychomycosis, tavaborole, resistance, cross-resistance

Onychomycosis is a fungal infection of the nail that is commonly caused by the dermatophyte *Trichophyton rubrum* (1). Several U.S. Food and Drug Administration (FDA)-approved systemic (terbinafine [TRB], itraconazole [ITC]) and topical (amorolfine [AMF], ciclopirox [CPX], efinaconazole [EFI], tavaborole [TVB]) antifungal agents are commonly used for the treatment of onychomycosis (1). The oxaborole drug TVB (AN2690) inhibits the leucyl-tRNA synthetase by trapping the 3′ end of $tRNA^{Leu}$ in the editing site, thus impairing protein synthesis (2–6). TVB displays broad-spectrum activity, being active against dermatophytes, molds, yeasts, and some bacteria, and well penetrates the nail plate for its low molecular weight and hydrophobicity (6–12).

Onychomycosis is often intractable because of the difficulty to reach effective drug levels at the site of infection and the potential acquisition of resistance by fungi, leading to frequent relapses after therapy cessation. In addition, long-term therapy often leads patients to abandon treatments, thus promoting relapses and selection of more-resistant fungal strains (13–15). The proficiency of *T. rubrum* to develop resistance toward several antifungal agents has been documented (16–20).

In this study, we evaluated the natural emergence and evolution of resistance against TVB in *T. rubrum*, which was chosen as a model organism for clinically relevant dermatophytes. We also investigated the potential of TVB to cause cross-resistance toward TRB, ITC, AMF, CPX, and EFI.

Two reference *T. rubrum* strains (ATCC 28188 and ATCC MYA-4438) and two *T. rubrum* clinical isolates (CI-1 and CI-2) were used in this study (17, 20). All of the experiments were repeated three times on separate days, and each strain was tested in duplicate. Conidial suspensions were prepared, and the MICs of TVB, TRB, ITC, AMF, CPX, and EFI were determined by the microtiter broth dilution method as indicated by CLSI standard M38 methodology for susceptibility testing of dermatophytes (21). For all *T. rubrum* strains, the MIC of TVB was 0.63 mg/liter. This value agrees with previously published data (22, 23), but it is lower than the MICs of TVB indicated in other studies for *T. rubrum* (24, 25). As already reported, the MIC value against *T. rubrum* was higher for

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**TABLE 1** Natural TVB-resistant mutants and *in vitro* evolution of resistance in *T. rubrum*

| | Resistance frequency to TVB[a] | | |
|---|---|---|---|
| ***T. rubrum* strain** | **Natural** | **After 5th transfer** | **After 10th transfer** |
| ATCC 28188 | $5.60 \pm 1.37 \times 10^{-8}$ | $1.60 \pm 0.71 \times 10^{-7}$ | $8.10 \pm 0.76 \times 10^{-6}$ |
| ATCC MYA-4438 | $1.81 \pm 0.17 \times 10^{-8}$ | $5.79 \pm 0.98 \times 10^{-7}$ | $1.76 \pm 0.24 \times 10^{-6}$ |
| CI-1 | $2.43 \pm 0.73 \times 10^{-8}$ | $2.45 \pm 0.89 \times 10^{-7}$ | $3.97 \pm 0.91 \times 10^{-6}$ |
| CI-2 | $1.78 \pm 1.03 \times 10^{-8}$ | $4.18 \pm 0.70 \times 10^{-7}$ | $1.87 \pm 0.32 \times 10^{-6}$ |

[a]Data were calculated by dividing the number of CFU grown on the plates containing TVB by the total number of CFU spread on plates. Mean ± SD from three separate experiments.

TVB than for TRB (0.01 mg/liter), ITC (0.08 mg/liter), AMF (0.16 mg/liter), CPX (0.31 mg/liter), and EFI (0.003 mg/liter) (25).

To test the emergence of both natural and induced resistance to TVB in *T. rubrum* isolates, the MIC of the drug was determined by agar dilution assay as previously described (17, 20). For all strains, the MIC values of TVB were 1 mg/liter when $10^8$ CFU/plate were seeded. When $10^5$ CFU/plate were inoculated, the MICs were 0.50 mg/liter for *T. rubrum* ATCC 28188 and 0.25 mg/liter for *T. rubrum* ATCC MYA-4438, CI-1, and CI-2. To isolate naturally occurring spontaneous *T. rubrum* mutants resistant to the drug, aliquots of conidial suspensions ($\sim 10^8$ CFU/plate) were seeded on Sabouraud dextrose agar (SDA) containing 1 mg/liter TVB. To confirm the development of resistance, colonies were transferred to SDA plates containing 2 mg/liter TVB (17, 20). For all strains, the frequency of natural resistance to TVB was $\sim 10^{-8}$ (Table 1). This frequency is comparable to that previously obtained for EFI and lower than that of ITC for the same *T. rubrum* strains but higher than those of TRB, AMF, and CPX (17, 20).

To mimic the conditions of body sites in which low drug levels are reached during therapy, the *T. rubrum* strains were subcultured ($\sim 10^5$ CFU/plate) for 10 sequential transfers on SDA plates containing 0.5-fold the MIC obtained by seeding $10^5$ CFU/plate in the agar dilution assay as previously described (17, 20). For each strain, a confluent growth was obtained at each transfer by using these subinhibitory drug concentrations. At the 5th and 10th transfers, all conidia were collected and seeded on plates containing 2-fold the MIC of TVB. Data analysis was performed by applying one-way analysis of variance followed by Tukey's multiple-comparison test. After the 5th transfer, mutants with increased MIC levels of TVB were isolated from all *T. rubrum* strains at a frequency of $\sim 10^{-7}$ (Table 1). Compared with the frequency of natural resistance and that obtained after the 5th transfer, the frequency observed after the 10th transfer ($\sim 10^{-6}$) was significantly higher for all strains ($P < 0.001$). This resistance frequency was similar to that obtained for TRB in a previous study. In addition, the frequency of resistance to TVB is lower than that of ITC and higher than those of AMF and CPX (17). Although *T. rubrum* isolates collected from patients during a phase 3 clinical trial did not show resistance after repeated exposure to TVB (4, 26), TVB-resistant mutants of *Saccharomyces cerevisiae* and *Escherichia coli* were previously isolated (2, 27, 28). In these organisms, TVB resistance was associated with mutations in the leucyl-tRNA synthetase-encoding gene that alter the editing site or affect the hydrolytic editing activity (2, 27, 28). *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* treated with the TVB derivative AN3365 were also shown to develop resistance to the compound (29–31).

To ensure that resistant phenotypes were genetically stable in the absence of TVB, selected natural and induced mutants were propagated for three transfers on nonselective SDA plates (17, 20). At the end of the transfers, conidia were collected and the MIC of TVB determined by broth microdilution. Natural and induced mutants showed 4- to 8-fold increases in MIC values of TVB versus the parental strains (Table 2). No significant differences were found among the MICs of natural and induced mutants, suggesting that growth in the presence of subinhibitory TVB concentrations does not cause variations in the level of resistance.

The development of resistance to some antifungals can lead to cross-resistance to

**TABLE 2** MIC values of TVB, TRB, ITC, AMF, CPX, and EFI tested toward randomly selected natural and induced *T. rubrum* mutants determined by broth microdilution

| | | MIC (mg/liter)[c] | | | | | |
|---|---|---|---|---|---|---|---|
| Strain[a] | *n*[b] | TVB | TRB | ITC | AMF | CPX | EFI |
| ATCC 28188 | | 0.63 | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| S | 3 | **2.50** | 0.01 | 0.16 | 0.16 | 0.63 | 0.003 |
| I 5th | 4 | **2.50** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| I 10th | 4 | **2.50–5.00** | 0.01 | 0.16 | 0.16 | 0.31 | 0.003 |
| ATCC MYA-4438 | | 0.63 | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| S | 5 | **2.50–5.00** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| I 5th | 4 | **2.50** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| I 10th | 4 | **2.50–5.00** | 0.01 | 0.16 | 0.16 | 0.31 | 0.003 |
| CI-1 | | 0.63 | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| S | 6 | **2.50** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| I 5th | 4 | **2.50** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| I 10th | 4 | **2.50** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| CI-2 | | 0.63 | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| S | 5 | **2.50** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| I 5th | 4 | **2.50** | 0.01 | 0.08 | 0.16 | 0.31 | 0.003 |
| I 10th | 4 | **2.50–5.00** | 0.01 | 0.16 | 0.16 | 0.31 | 0.003 |

[a]S, natural mutants; I 5th, induced mutants after 5th transfer; I 10th, induced mutants after 10th transfer.
[b]Number of tested mutants.
[c]Mode values of three different experiments performed in duplicates. MIC differences of ±1- to 2-fold dilution were not considered significant. Significant differences are in boldface.

other drugs, particularly those sharing a common mechanism of action (16, 17, 20, 32). Therefore, we wondered whether the development of resistance toward TVB could induce cross-resistance to other drugs. The MICs of TRB, ITC, AMF, CPX, and EFI were tested in *T. rubrum* mutants showing increased MIC values to TVB by broth microdilution. No increases in the MICs of these drugs were observed, indicating that the development of lower TVB susceptibility does not induce cross-resistance to other antifungals. This result can be explained by the unique mechanism of action exerted by TVB. In fact, whereas TVB inhibits protein synthesis, TRB, ITC, AMF, and EFI act on the ergosterol biosynthetic pathway, and CPX chelates trivalent cations and impairs fungal cell membrane integrity (1).

In conclusion, natural *T. rubrum* mutants that are resistant to TVB can rarely be isolated. The presence of subinhibitory drug concentrations stimulates the increase of such resistance frequency. However, the good nail penetration of TVB and the absence of cross-resistance in mutants for which TVB MIC levels are elevated suggest that the drug can be successfully used for the treatment of onychomycosis caused by dermatophytes (7, 33–36).

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