Mechanism of Action of Efinaconazole, a Novel Triazole Antifungal Agent

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The mechanism of action of efinaconazole, a new triazole antifungal, was investigated with Trichophyton mentagrophytes and Candida albicans. Efinaconazole dose-dependently decreased ergosterol production and accumulated 4,4-dimethylsterols and 4α-demethylsterols at concentrations below its MICs. Efinaconazole induced morphological and ultrastructural changes in T. mentagrophytes hyphae that became more prominent with increasing drug concentrations. In conclusion, the primary mechanism of action of efinaconazole is blockage of ergosterol biosynthesis, presumably through sterol 14α-demethylase inhibition, leading to secondary degenerative changes.

Ergosterol is an important structural component of fungal cell membranes, maintaining membrane fluidity and a permeability barrier, and is essential for fungal cell viability (1–3). Several classes of antifungal drugs target ergosterol biosynthesis. Among these, triazole antifungals (e.g., itraconazole) and imidazole antifungals (e.g., clotrimazole and miconazole) inhibit sterol 14α-demethylase (14-DM) in the ergosterol biosynthesis pathway (4). The consequent ergosterol depletion affects cell membrane integrity and function and is believed to inhibit fungal cell growth and affect morphology.

Efinaconazole, a novel triazole antifungal drug currently under development as a topical treatment for onychomycosis, has demonstrated efficacy in patients with toenail onychomycosis in two phase 3 clinical trials (5). Onychomycosis and other superficial mycoses are caused mainly by dermatophytes (e.g., Trichophyton rubrum and Trichophyton mentagrophytes) and yeast (e.g., Candida albicans). Efinaconazole possesses similar or higher antifungal activity against T. rubrum and T. mentagrophytes (MIC range, 0.00098 to 0.031 μg/ml) and a broader spectrum of activity than those of currently marketed antifungals used in onychomycosis (6). We investigated the effects of efinaconazole on fungal ergosterol biosynthesis and dermamphyte hyphal morphology.

In the present study, T. mentagrophytes strain SM-110 and C. albicans strain ATCC 10231 were used. The MICs of efinaconazole (Kaken Pharmaceutical), itraconazole, and clotrimazole (Sigma-Aldrich) were determined by the broth microdilution method using morpholinoalanilsonic acid (MOPS)-buffered RPMI 1640, as described in CLSI documents M38-A2 (7) and M27-A3 (8), using visual endpoint readings of 80% growth inhibition at 4 days and 50% growth inhibition at 48 h, respectively. Efinaconazole was 4-fold more active than itraconazole against T. mentagrophytes SM-110 (MICs of 0.0039 and 0.016 μg/ml, respectively). Similarly, efinaconazole was 8-fold more active than clotrimazole against C. albicans ATCC 10231 (MICs of 0.00098 and 0.0078 μg/ml, respectively). The two strains showed typical susceptibilities to the antifungals tested, consistent with previous findings for these species (6, 9).

Ergosterol biosynthesis assays were conducted by modifying the methods of Vanden Bossche et al. (10) and Ryder et al. (11). T. mentagrophytes (2 × 10⁸ microconidia/ml) and C. albicans (1 × 10⁵ cells/ml) were exposed to drugs in MOPS-buffered RPMI 1640 medium containing [1,2-14C]sodium acetate at 0.4 μCi/ml and cultured at 35°C for 24 h with shaking. After culture, cells were subjected to a saponification treatment with a 50% ethanol solution containing 15% potassium hydroxide and 0.1% pyrogallol at 80°C for 2 h. Nonsaponified lipids were then extracted and separated by thin-layer chromatography using heptane-diisopropyl-ether-acetic acid (60:40:4 [vol/vol/vol]) as a developing solvent. Incorporation of radioactivity into cell membrane lipids (i.e., 4-desmethylsterols [ergosterol], 4,4-dimethylsterols [lanosterol], 4α-methylsterols, and squelane) was measured using a liquid scintillation counter. In both fungal strains, efinaconazole decreased the radioactivity of the 4-demethylsterol fraction and increased the radioactivity of the 4,4-dimethylsterol fraction in a dose-dependent manner at concentrations below its MICs (Fig. 1A). The increase in radioactivity of the 4α-methylsterol fraction was minimal, while there was essentially no change in squelane fraction radioactivity. The increase in 14-DM substrates (4,4-dimethylsterols and 4α-methylsterols) and decrease in 4-desmethylsterols suggest that efinaconazole, like other azoles, inhibits 14α-demethylase (4). Efinaconazole was 4.9 times more active than itraconazole in inhibiting ergosterol biosynthesis in T. mentagrophytes (Fig. 1B); the 50% inhibitory concentrations (IC₅₀) were 0.0070 (1.8× MIC) and 0.0338 (2.2× MIC) μg/ml, respectively. Similarly, efinaconazole was 7.3 times more active than clotrimazole in C. albicans (Fig. 1B); the IC₅₀ of 0.0040 (0.41× MIC) and 0.0029 (0.37× MIC) μg/ml, respectively. Efinaconazole IC₅₀ in the two fungal species were comparable to their corresponding MICs. Therefore, the potent in vitro antifungal activity of efinaconazole makes it a promising new drug for the treatment of onychomycosis and other superficial mycoses.
Efinaconazole can be explained by its strong inhibition of ergosterol biosynthesis. Ergosterol depletion and 14α-methylsterol accumulation resulting from 14-DM inhibition interfere with the "bulk" functions of ergosterol in fungal cell membranes (12). This leads to alterations in membrane fluidity and activities of several membrane-bound enzymes (e.g., chitin synthase) (12), which may induce cell membrane damage and morphological changes. The effect of efinaconazole on T. mentagrophytes hyphal morphology was therefore investigated. T. mentagrophytes (2 × 10⁶ microconidia/ml) was cultured in Sabouraud dextrose broth at 30°C for 24 h with shaking. After conidia germinated, efinaconazole was added to the medium, and cultures were incubated for an additional 24 h. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis of the hyphae was performed as described by Nishiyama et al. (13), with minor modifications, using 2.5% glutaraldehyde for sample fixation and osmium tetroxide for SEM sample coating. Efinaconazole induced notable morphological and ultrastructural changes in hyphae (Fig. 2A and B). At concentrations ranging from 0.001 to 0.01 μg/ml, shortening of interseptal distance, globular swelling, and thickening of the cell wall were observed. Additionally, at concentrations ranging from 0.1 to 10 μg/ml, nonuniform widths and flattening of hyphae, separation of plasma membrane from the cell wall, accumulation of electron-dense granules in the space between the cell wall and the plasma membrane, discontinuity of the plasma membrane, and degeneration of organelles were observed. These changes became more prominent with increasing drug concentrations. Except for globular swelling, these changes have been previously found in Trichophyton species treated with other azoles, including bifonazole (14), itraconazole (15), and lanoconazole (16). Globular swelling was unique to efinaconazole treatment; however, its biological significance is unknown.

Morphological and ultrastructural changes were observed at much lower efinaconazole concentrations than the previously reported MIC (0.2 μg/ml) for this test strain in Sabouraud dextrose broth (17). The highly electron-dense granules observed in the space between the cell wall and plasma membrane were considered to be lipid materials accumulated as a result of 14-DM inhibition, as suggested with itraconazole (15), lanoconazole (16), and neticonazole (18). These observations may be related to ergosterol biosynthesis inhibition.

Further, hyphal flattening, plasma membrane discontinuity, and degeneration of intracytoplasmic organelles produced at efinaconazole concentrations of 0.1 μg/ml or higher are likely associated with loss of viability and cell death. Indeed, we have previ-
ously reported that efinaconazole was fungicidal against 28 *T. mentagrophytes* strains in Sabouraud dextrose broth, with an 80% minimum fungicidal concentration (MFC80) of 0.25 \(\mu\)g/ml (19).

Antifungals such as miconazole (20, 21), clotrimazole (21), sertaconazole (22), and butenafine (23) have been reported to quickly damage fungal cell membranes through direct physico-chemical interactions (within 1 h of drug treatment). However, because these effects were generally observed at concentrations several thousand times higher than the drugs’ MICs, they are believed to be unrelated to the primary mechanism of action. To examine if efinaconazole could directly damage fungal cell membranes via the same mechanism, intracellular phosphate (Pi) leakage was measured using the method previously described by Iwatai et al. (23), with minor modifications. The efinaconazole concentrations for this assay were set at 0.1 to 100 \(\mu\)g/ml to evaluate the contribution of direct-membrane damage to the *T. men-
Efinaconazole hyphal changes observed at 0.1 to 10 μg/ml. *T. mentagrophytes* (1 × 10⁷ hyphae/ml) was exposed to efinaconazole or miconazole (LKT Laboratories) in physiological saline at 30°C for 2 h with shaking. *C. albicans* (1 × 10⁶ cells/ml) was exposed to efinaconazole or butenafine (Kaken Pharmaceutical) in distilled water at 35°C for 1 h with shaking. The positive controls, miconazole and butenafine, caused significant leakage of intracellular Pi at 40 and 100 μg/ml, respectively (Fig. 3), consistent with previous results (21, 23). In contrast, efinaconazole produced minimal Pi leakage at concentrations as high as 100 μg/ml (Fig. 3), suggesting that it does not have a direct fungal cell membrane-damaging effect. The short incubation time point (2 h) was needed to determine the existence of a direct, acute damage to the cell membrane. The electron microscopy observations after treatment with efinaconazole (≤10 μg/ml) for 24 h suggest an effect on cell membrane integrity, and thus, Pi leakage cannot be excluded at later time points as an indirect effect of ergosterol biosynthesis inhibition.

In conclusion, the mechanism of antifungal action of efinaconazole is blockage of ergosterol biosynthesis by inhibiting 14-DM. The resulting ergosterol depletion and accumulation of precursor sterols may secondarily affect cell membrane integrity and function, leading to degenerative morphological changes.

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REFERENCES


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**FIG 3** Effect of efinaconazole and positive-control drugs on Pi leakage from *T. mentagrophytes* and *C. albicans* cells. Each bar represents the mean ± SEM for triplicate cultures of *T. mentagrophytes* or data for a single culture of *C. albicans*.