Evaluation of the Ability of a Novel Miconazole Formulation to Penetrate Nail Using Three Nail *In Vitro* Models

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ABSTRACT

Objective: The aim of this study was to evaluate the ability of topical miconazole to penetrate the nail. **Methods**: Bovine hoof, a healthy human nail TurChub® zone of inhibition model, and an infected nail model were used in this study. In the hoof model, miconazole was applied to hoof slices subsequently placed on a *Trichophyton mentagrophytes* seeded agar plate. In the TurChub® zone of inhibition assay, topical miconazole (10%) was applied to full thickness human nails, using *T. rubrum* as the test organism. The infected nail model, using ChubTur® test systems, was performed using full thickness human nails infected with *T. rubrum* prior to the application of miconazole (10%). Drug penetration was assessed by zones of inhibition (ZOI) and ATP recovery, respectively. **Results**: In the hoof model, miconazole showed significantly larger ZOI after 60 minutes of penetration compared to 8% ciclopirox nail lacquer ($P < 0.05$). Similar results were achieved with the TurChub® assay comparing topical miconazole to 8% ciclopirox ($P \leq 0.05$). The observed ZOI for 10% efinaconazole and the base formulations containing either 10% miconazole or 10% fluconazole were equivalent to total kill (ZOI of 3 - 4 cm) of *T. rubrum* in the TurChub® cells, indicating high/therapeutic levels of drug permeation. Base formulations with miconazole, fluconazole, and efinaconazole were all statistically ($P < 0.05$) superior to 8% ciclopirox. In the infected nail model, infected nails treated with topical miconazole demonstrated a significantly ($P \leq 0.05$) lower percentage ATP recovery (i.e. less viable organism) when compared to 8% ciclopirox, indicating greater antifungal efficacy. **Conclusion**:

Miconazole is capable of nail penetration when applied topically as either a previously marketed formulation or in the penetration enhancing formula.

INTRODUCTION

Onychomycosis, a fungal infection of the nail most commonly caused by the dermatophytes *Trichophyton rubrum*, *T. mentagrophytes*, and *Epidermophyton floccosum* [1-3], is a condition that can cause discomfort and lead to permanent disfigurement. [4] In turn, it can lead to diminished self-esteem and negative effects on a patient's quality of life. [5] Onychomycosis is estimated to affect 2% to 13% of the general population. [6-10] Its high incidence and prevalence makes it an important public health problem. [11]

Onychomycosis leads to nail hyperkeratosis and onycholysis of the nail bed [12], factors that make drug treatment difficult. [13] Nail thickening makes drug penetration particularly difficult and lifting of the nail plate from the nail bed creates an air space in which the fungus can proliferate and in which the drug cannot permeate. [14-15]

Treatment of onychomycosis is challenging and cure rates are low [16-18], mostly secondary to poor patient compliance from prolonged treatment regimens, poor results, and high re-infection rates. [1, 13-14, 19] Oral agents can result in significant side effects and potential drug interactions [20-21], particularly in patients with diabetes, the immunocompromised, and the elderly. [20, 22-26] To avoid such issues,

topical medications were developed; however many of these agents have lacked efficacy. [1, 21] It is thought that the poor therapeutic results of such agents are a consequence of formulation development based on skin drug penetration [27], not taking into account the physical and chemical differences between nail and skin [1, 15, 27-30], and the nail changes secondary to infection. [14, 31] The need to improve nail drug delivery has led to the discovery of penetration enhancers that could aid in drug delivery through nail [32-34], thereby improving outcomes in the treatment of onychomycosis.

In this study, we demonstrate the ability of miconazole nitrate to penetrate the nail employing *in vitro* nail penetration assays. Initially, we employed the bovine hoof model, which uses bovine hooves to simulate the human nail. The hoof model was chosen for initial compound screening for several reasons: 1) human nails have a limited availability, 2) human nails have large variability in thickness, and 3) the bovine hoof has been shown to be similar in structure and permeability to human nail. [35] Once efficacy in this hoof model was established, the TurChub® zone of inhibition assay and infected nail models were employed using healthy and infected human nails, respectively. In the bovine hoof model, the ability of miconazole nitrate to penetrate the nail was compared to 8% ciclopirox nail lacquer. In the TurChub® zone of inhibition and infected nail models, the ability of 10% miconazole nitrate combined with a penetration enhancer formulation, was compared to 10% fluconazole, 8% ciclopirox, and 10% efinaconazole.

MATERIALS AND **METHOD**

Test organisms

In the bovine hoof model, *T. mentagrophytes* ATCC 24953, taken from the culture collection at the Center for Medical Mycology, Cleveland, OH was used. *T. mentagrophytes* was selected as it is one of the major causes of onychomycosis. Furthermore, *T. mentagrophytes* produces conidia consistently and reproducibly. To obtain conidia for use in the penetration assay, subcultures were inoculated onto potato dextrose agar plates (PDA) and incubated for 5-7 days at 30 °C. Next, 5 mL of normal sterile saline solution (NS, 0.85% sodium chloride) was added to each plate and the conidia were harvested, washed three times with sterile saline, and then resuspended in 5 mL of saline. A hemacytometer was used to determine the cell concentration in diluted suspension. The working suspension of *T. mentagrophytes* conidia was standardized to a final concentration of 2-5 x 10^5 conidia/mL.

In the TurChub® zone of inhibition and infected nail models, a *T. rubrum* strain isolated from a patient suffering from onychomycosis was used to prepare a conidial suspension. The test isolate was sub-cultured onto Sabouraud dextrose agar (SDA), and PDA and incubated at 20-25 °C for 7days. The fungal colonies were then covered with 5 mL of Ringer's solution and suspensions of conidial and hyphal fragments were made. The suspension was then filtered through sterile gauze to remove

mycelium. The density of the suspension was assessed spectrophotometrically at 600 nm, and the spore suspension adjusted to approximately 1 x 10^7 spores/mL by diluting with Ringer's solution.

Test formulations

Three penetration enhancing formulations were supplied by Humco Pharmaceuticals (Austin, TX, USA) containing a novel base formulation comprised of: acetylcysteine, alcohol, camphor, EDTA, eucalyptus oil, hydroxypropylcellulose, hydroxypropyl starch phosphate, magnesium aluminum silicate, menthol, propylene carbonate, propylene glycol, purified water, sodium hydroxide, sodium thioglycolate, rtrontium chloride, tea tree oil, thymol, and urea. One of the formulations was a placebo comprised of the base formulation only, while the other two formulations contained either fluconazole at 10% or miconazole at 10%. Miconazole nitrate 2% was also tested in the bovine hoof model, whereas 8% ciclopirox topical solution and efinaconazole at 10% were investigated in the TurChub® zone of inhibition and infected nail models.

Treatment groups

In the bovine hoof model, treatment groups included miconazole nitrate 2% (applied for 30 minutes), miconazole nitrate 2% (applied for 60 minutes), 8% ciclopirox nail lacquer, and untreated control.

In the TurChub® zone of inhibition and infected nail models, treatment groups included infected control (agar inoculated with organism but not dosed with test

sample), non-infected control (control not inoculated with test organism or dosed with test sample), base formulation with no drug, base formulation with 10% miconazole, and base formulation with 10% fluconazole, 10% efinaconazole, or 8% ciclopirox solution.

Bovine hoof model

Due to the limited availability of human nails and the large variability in their thickness, a nail penetration model using a bovine hoof was developed as bovine hooves are comparable in structure and permeability to human nail. Hooves were obtained from Mahan Packing Co. (Bristolville, OH). After the flesh was removed, the hooves were cleaned with antibacterial soap and washed with water to remove the soap. Cleaned hooves were subsequently sliced using a band saw. The thickness of each slice was measured with an electronic digital caliper and slices 0.5-1.0 mm thick (mimicking human nails thickness) were autoclaved. Prior to the application of test formulation, autoclaved slices were soaked in sterile water for 2 hours to prevent cracking when discs were cut. Three hundred microliters of the test formulation was applied to the top of the hoof slice and allowed to penetrate for 30 or 60 minutes. Subsequently, excess formulation was removed and 3 discs cut using an 8 mm biopsy punch. The discs were then placed, surface up, on a 4 mm thick PDA plate seeded with *T. mentagrophytes* ATCC 24953 (2-5 x 10^5 conidia/mL). The plates were incubated at 35 $^{\circ}$C for 4 days at which time the diameter of the zone of inhibition (ZOI) was measured and the effective zone calculated. The effective ZOI is defined as the zone diameter minus the diameter of the disk.

TurChub® zone of inhibition

The TurChub® ZOI assay uses a modified Franz cell, in which sections of human nail serve as the barrier through which the drug initially penetrates prior to reaching an agar-filled receptor chamber where the dermatophytes grow.

Distal nail clippings were obtained from volunteers' toenails which had been grown to a minimum length of 3 mm. Prior to acceptance of the nails, all nail donors were required to have not used nail varnish or polish on their toenails within 6 months and to have no visible signs of damage or disease to their nails. Using scissors, nail clippings were cut into pieces, which were a minimum of 3 mm x 3 mm. The nail clippings were initially placed in water and heated to 60 °C for 15 min followed by immersing in a 70% ethanol in water solution and vortex mixed for one minute at ambient room temperature to disinfect the nails. This process of washing and mixing by vortex was repeated once. The ethanol solution was then decanted and replaced with sterile Ringer's solution, vortex mixed for 1 min, and decanted. This process of washing with Ringer's solution was carried out a total of three times. Once the washing process was complete, the nail clippings were placed into a sterile Petri dish without a lid and air dried for 30 min at room temperature in a laminar flow cabinet. The thickness of all the nail sections was measured using a caliper.

Preparation of the TurChub® cells was performed as described in Traynor *et al.* [32] Briefly, the receiver compartment of each TurChub® was filled with agar (PDA) ensuring complete contact with the agar in the receiver compartment and the

underside of the nail. The *T. rubrum* organism suspension was pipetted onto the agar surface within individual TurChub® cells and then left to dry. The surface of the nail mounted in the gasket section of a TurChub® cell was dosed with 100 µL of test sample and the TurChub® cell was occluded and incubated at 20-25 °C for a total of 14 days. Infected and non-infected controls were also included.

Infected nail model

The onychomycosis nail model uses infected human nail mounted in the gasket section of a modified Franz cell (ChubTur® cells). In the onychomycosis model, distal nail clippings infected with *T. rubrum* were mounted into the validated ChubTur® gasket system. The receiver compartment of each ChubTur® was then partially filled with an inert sterile humidity control medium (Ringer's solution). The cells were then incubated at 20-25 °C for 14 days to allow full growth of the organism on the nail. After establishing the growth of the organism on the nails, the nails were dosed daily for 7 days with 2 µL of the test formulations. Additional samples were also set up as an infected control to monitor the growth of *T. rubrum* on the nail samples over the incubation period, and control nails without any infection were also set up and incubated to ensure the absence of contamination. The effectiveness of each formulation was determined after removing the nail sample from the cell (24 h after the final dose). The presence of viable microorganisms was measured by a validated bioluminescence ATP method, in which the amount of luminescence measured from the infected nails is directly proportional to the amount of ATP concentration. In turn, the level of ATP detected is an indication of the

viability of *T. rubrum* in the onychomycosis nail.

To ensure the test formulations were compatible with the ATP assay; the direct effect of the formulations on the ATP assay itself was investigated independently of the nails and organisms. There was no substantial interference with any of the formulations with the ATP assay, in which the percentage recovery was within ± 10% of the ATP standard. Therefore, the ATP assay was found to be 'fit for purpose' for the quantification of samples in the infected nail investigation.

Statistical Analysis

For analyses, the mean effective ZOI ± the standard deviation was calculated from the obtained data and compared between different treatment groups. The one-way ANOVA with a Bonferroni post-hoc test was employed in determining significance. All statistical analyses were performed using Statistical Package for Social Science (SPSS) for Windows, version 16.0 (Chicago, IL). A *P*-value of ≤ 0.05 was considered statistically significant.

The statistical analysis of the infected nail investigation was performed for all test formulations as a complete population using SPSS for Windows, version 19.0 (Chicago, IL). Statistical comparison was performed using a one-way ANOVA with a Tukey's post-hoc test, where a *P*-value of ≤ 0.05 was considered statistically significant (95% confidence level).

RESULTS

Table 1 and Figure 1 show the average effective ZOI for each treatment group in the bovine hoof model. As expected, the untreated controls showed no ZOI (average zone size of 0.0 mm). Miconazole nitrate 2%, 30 and 60 minute exposure, demonstrated significant activity when compared to the untreated control ($P < 0.05$) indicating that miconazole nitrate was able to penetrate the hoof material and cause inhibition of *T. mentagrophytes.* Importantly, miconazole nitrate 2%, following 60 minutes exposure, showed significantly larger ZOI when compared to 8% ciclopirox nail lacquer ($P < 0.05$). Miconazole nitrate 2%, following 30 minutes exposure, showed a trend towards increased ZOI, as compared to 8% ciclopirox nail lacquer ($P = 0.07$) albeit not statistically significant. Miconazole nitrate showed significantly larger zone sizes when left for 60 minutes, as compared to 30 minutes ($P < 0.05$).

In the TurChub® ZOI model, following application of a single dose (100 µL) of the base penetration enhancer formulations containing 10% miconazole and 10% fluconazole for 14 days, the mean ZOI of *T. rubrum* observed were 3.57 ± 0.30 and 3.48 ± 0.15 cm, respectively (Figure 2). The mean ZOI for the cells dosed with 100 µL of 10% efinaconazole was 3.58 ± 0.20. There were no ZOI present after dosing of the nails with the 100 µL of the placebo base penetration enhancer formulation or 8% ciclopirox. The observed ZOI for the 10% efinaconazole and the base penetration enhancer formulations containing either 10% miconazole or 10% fluconazole were equivalent to total kill (ZOI of 3 - 4 cm) of *T. rubrum* in the TurChub® cells, which is indicative of potency and high/therapeutic levels of drug permeating through the nail. The base formulations with 10% miconazole and 10% fluconazole, and the marketed

product with 10% efinaconazole, were all statistically ($P < 0.05$) superior to 8% ciclopirox.

Following the infected nail investigation whereby the nails were treated daily for 7 days with 2 µL of the test formulations (Figure 3), the greatest decrease in percentage ATP recovery compared to the infected control was observed for the 10% efinaconazole solution (3.33%) and the base penetration enhancing formulations with 10% w/w miconazole (4.75%) and 10% w/w fluconazole (6.57%). The aforementioned ATP levels, which were all statistically similar ($P > 0.05$), could be considered baseline, and therefore equivalent to total kill of the organism. The ATP following treatment with 8% ciclopirox was 20.02% compared to the infected control, indicating this test formulation was significantly less efficacious than the base penetration enhancing formulation with 10% w/w miconazole and the 10% efinaconazole solution ($P \leq 0.05$). Moderate anti-fungal efficacy was observed from the placebo base penetration enhancing formulation (79.96% ATP recovery compared to the infected control); however, this was significantly less than all of the active test formulations ($P \leq 0.05$).

Discussion

The data showed that miconazole with and without penetration enhancer can penetrate the nail as demonstrated using several nail models. In the infected nail model, the data showed the greatest reduction in ATP recovery (indicating potent antifungal activity) compared to the infected control, following treatment with the

efinaconazole solution and miconazole or fluconazole in the penetration enhancing formulation (3.33%, 4.75% and 6.57% ATP recovery, respectively). There was no statistical difference between the percentage ATP recovery for the three aforementioned formulations ($P > 0.05$), and the results reflect those observed in the TurChub® investigation where there was complete kill of *T. rubrum* in the test systems following treatment with the same three formulations. The highest percentage ATP recovery (indicating least antifungal activity) compared to the infected control was observed following treatment with 8% ciclopirox (20.02% ATP recovery). A significantly ($P \leq 0.05$) higher percentage ATP recovery was observed after the application of 8% ciclopirox compared to the base formulation with miconazole and the base formulation with efinaconazole; however, there was no statistical difference between the 8% ciclopirox and the base formulation with fluconazole ($P > 0.05$). These results indicate that miconazole and efinaconazole were statistically superior than 8% ciclopirox in the treatment of *T. rubrum* infected full thickness nails, whereas no statistical differentiation was seen between fluconazole and 8% ciclopirox. Moderate antifungal efficacy was observed from the vehicle base formulation (79.96% ATP recovery), which was significantly less than that of each of the active formulations [$P \leq 0.05$].

Previously published data supports our findings. In a Franz cell diffusion ZOI assay which was similar to our TurChub® zone of inhibition assay, 5% efinaconazole demonstrated average zone sizes ± SD of 2.52 ± 0.42. [36], while ciclopirox demonstrated no zones of inhibition. These findings support our data in which 10%

efinaconazole showed mean zone sizes ± SD of 10% was 3.58 ± 0.20, and ciclopirox 8% showed no zones of inhibition.

CONCLUSION

In the hoof model assay, it was shown that miconazole can penetrate hoof material and exhibit antifungal activity. The findings of the hoof model were then further explored using human nail models, which are more clinically relevant. Data from the TurChub® ZOI and infected nail models showed the ability of miconazole nitrate to penetrate human nail and inhibit fungal growth as measured by ZOI and ATP recovery, respectively. The data indicates that 10% miconazole nitrate in the penetration enhancing formulation is equivalent to 10% efinaconazole and superior to 8% ciclopirox, suggesting that miconazole nitrate may be effective in the topical treatment of onychomycosis.

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Table 1. Bovine hoof model effective zones of inhibition

	Average Effective Zone of Inhibition ± SD (mm)	*P*-values against Untreated Control
Miconazole Nitrate 2% (60 minutes)	26.5 ± 9.7	< 0.0001
Miconazole Nitrate 2% (30 minutes)	12.7 ± 8.1	0.0010
8% Ciclopirox Nail Lacquer	2.8 ± 3.4	1.0000
Untreated Control	0.0 ± 0.0	-----

Figure 1. The effective zone in the bovine hoof model was assessed by subtracting the 8 mm diameter of the bovine hoof disc from the zone of inhibition diameter (arrow).

Figure 2. Mean ZOI of *T. rubrum* on PDA in TurChub® cells that were mounted with distal nail clippings and treated with a single 100 µL dose of test formulation. The cells were incubated at 20-25 °C for 7 days after inoculation with *T. rubrum* (mean ± SD, n=6

active, n=3 placebo).

Figure 3. The amount of ATP recovered from the nail 24 h after the final treatment, presented as a percentage of the *T. rubrum* infected control, following daily treatment (2 μL) for 7 days using the base penetration enhancing formulations (with 10% w/w miconazole, 10% fluconazole and the placebo), the 10% efinaconazole solution and the 8% ciclopirox topical nail lacquer (mean ± SD, n=6 active, n=3 placebo/control).