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Evaluation of the Morphological Effects of TDT 067 (Terbinafine in Transfersome®) and Conventional Terbinafine on Dermatophyte Hyphae *in vitro* and *in vivo*

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Running Title: Morphological Effects of TDT 067

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ABSTRACT

TDT 067 is a novel, carrier-based dosage form of terbinafine in Transfersome® (1.5%) formulated for topical delivery of terbinafine to the nail, nail bed, and surrounding tissue. We examined the effects of TDT 067 and conventional terbinafine on the morphology of dermatophytes. *Trichophyton rubrum* hyphae were exposed to TDT 067 or terbinafine (15 mg/ml) and examined under white light, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Subungual debris from patients treated with TDT 067 in a clinical trial was also examined. Exposure of *T. rubrum* hyphae to TDT 067 led to rapid and extensive ultrastructural changes. Hyphal distortion was evident as early as 4 hours after exposure to TDT 067. After 24 hours, there was complete disruption of hyphal structure with few intact hyphae remaining. Exposure to terbinafine resulted in similar morphological alterations as those seen with TDT 067; however, the effects of TDT 067 were more extensive, whereas a portion of hyphae remained intact after 24 hours' exposure to terbinafine. Lipid droplets were observed under TEM following 30 minutes' exposure to TDT 067, which after 24 hours had filled the intracellular space. These effects were confirmed *in vivo* in subungual debris from patients with onychomycosis who received topical treatment with TDT 067. The Transfersome® in TDT 067 may potentiate the action of terbinafine by delivering terbinafine more effectively to its site of action inside the fungus. Our *in vivo* data confirm that TDT 067 can enter fungus in the nail bed of patients with onychomycosis and exert its antifungal effects.

INTRODUCTION

Onychomycosis is a common fungal disease of the nail, with an overall estimated prevalence of 10-20% (13). Predisposing factors include increasing age, immunosuppression, poor peripheral circulation, diabetes mellitus, nail trauma, tinea manuum, and tinea pedis (11, 24, 27, 29). Dermatophytes are the major cause of nail infection, with the most frequently detected dermatophyte species being *Trichophyton rubrum* (91%) and *T. mentagrophytes* (7.7%) (7, 9, 28). The treatment of onychomycosis has improved considerably following the introduction of the oral antifungals terbinafine and itraconazole (21). However, drug−drug interactions and hepatotoxicity have been associated with these oral antifungals (1, 15, 20), and there are still a proportion of patients who do not achieve efficacy (25). Elderly patients are at increased risk of onychomycosis (27). As the elderly are often receiving multiple medications, topical treatment, which avoids the potential for drug interactions and systemic effects, may be preferred by physicians and patients. Topical agents approved for the treatment of mild to moderate fungal nail infection without matrix involvement include formulations based on ciclopirox or amorolfine. However, the rates of efficacy reported for these formulations are generally low (5), and this is considered to be due to poor penetration of the antifungals through the nail (18). Consequently, there is still a need for new topical treatments which can penetrate the nail sufficiently for antifungal activity at the site of infection in the nail bed.

TDT 067 (terbinafine 15 mg/ml in Transfersome®) is a carrier-based dosage form of terbinafine that has been formulated for topical delivery of terbinafine to the nail plate, nail bed, and surrounding tissue to treat onychomycosis. The Transfersome® is an ultra-deformable lipid vesicle consisting of a phospholipid bilayer. The inclusion of the membrane-softening agent TWEEN-80 facilitates the deformability of the Transfersome®, allowing it to pass intact through the intercellular spaces in the skin. The vesicles have high surface hydrophilicity and their movement across the skin is driven by the

transcutaneous water gradient, resulting in delivery of high levels of drug to subdermal tissue. (3).

In vitro experiments have demonstrated that TDT 067 has potent inhibitory and cidal activity against dermatophytes, and has enhanced antifungal activity compared with conventional terbinafine preparations (10). The excipients of TDT 067 are widely used in pharmaceutical and cosmetic products for topical application, and clinical studies involving more than two thousand patients with different diseases have shown that Transfersome® preparations are well tolerated. (2, 16, 22). Furthermore, in two phase II clinical studies of TDT 067 in patients with onychomycosis, treatment was well tolerated and most patients did not experience application-site reactions (6, 26). Systemic exposure to TDT 067 was negligible in both studies and there were no clinically relevant hepatic laboratory abnormalities reported (6, 26).

In this study, we investigated the mechanisms underlying the *in vitro* activity of TDT 067 by comparing the effects of TDT 067 and conventional terbinafine on the morphology of *T. rubrum* (the predominant cause of onychomycosis) *in vitro* using different microscopic tools, including white light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Additionally, the *in vivo* effects of TDT 067 on the morphology of fungi present in subungual debris collected from subjects with onychomycosis enrolled in a Phase II clinical trial and treated with TDT 067 were examined by white light microscopy and TEM and compared with fungi present in nail samples from untreated patients.

MATERIALS AND METHODS

Fungal isolates

T. rubrum MRL 10754, a clinical strain isolated from a patient with onychomycosis that has been shown to be susceptible to terbinafine (minimum inhibitory concentration [MIC] = 0.016 µg/ml), was

taken from the culture collection at the Center for Medical Mycology, Cleveland OH, and was used for the *in vitro* analyses.

Subungual nail samples

Subungual nail samples were collected from subjects with bilateral onychomycosis of the toenail who were enrolled in an open-label Phase II maximal dose study of TDT 067 (study CL-067-II-02). Patients received treatment with TDT 067 twice daily, applied to the infected toenail(s) and surrounding skin as a spray (total daily dose of terbinafine 21 mg), for 28 days. Nail samples were collected at screening, on Days 1, 7, 14, and 28 during the treatment period, and on Day 35 following a 7-day washout period. Subungual debris was also collected from an untreated patient with onychomycosis for use as a control.

Antifungals

TDT 067 liquid formulation (concentration of terbinafine 15 mg/ml) was provided by Celtic Pharma Development Services Bermuda Ltd. and terbinafine powder (conventional terbinafine i.e. not in Transfersome®; Sigma-Aldrich, St. Louis, MO) was provided by the Center for Medical Mycology, Cleveland, OH. Terbinafine powder was dissolved in dimethyl sulfoxide.

White light microscopy

To determine the effects of TDT 067 on the hyphal morphology of *T. rubrum,* an inoculum containing 3 x 10^3 conidia/ml was prepared in RPMI-1640 buffered with MOPS (Hardy Diagnostics, Santa Maria, CA), added to the wells of the microtiter plates (100 µl aliquots) and incubated at 35ºC for 2−3 days until good hyphal growth was achieved. TDT 067 or conventional terbinafine test solutions (15 mg/ml) were prepared in RPMI-1640, added to the wells of microtiter plates (100 µl aliquots) and re-incubated

at the same temperature. At pre-determined time intervals (5, 10, 15, and 30 minutes, 1, 4, 8, 12, 24, 48, 72, and 96 hours and once weekly thereafter), a loopful of hyphal growth from the bottom of each well was transferred to a glass microscope slide containing a drop of potassium hydroxide (KOH). Subungual debris from target nail samples and the untreated control was analyzed on a glass microscope slide containing a drop of KOH (14). Photomicrographs from a representative field visualized under white light microscopy were recorded using an AxioCam MRN camera.

Scanning electron microscopy

To examine the effect of the TDT 067 and conventional terbinafine on the surface topography of *T. rubrum* hyphae using SEM, *T. rubrum* conidia were allowed to form hyphae as described above. TDT 067 and conventional terbinafine (at a concentration of 15 mg/ml) were then added to the wells of the microtiter plates (100 µl aliquots) and re-incubated. After 2, 4, 8, and 24 hours, aliquots were removed from the microtiter plates and processed for SEM as described by Chandra et al. (4). Briefly, organisms were fixed with 2% glutaraldehyde for 2 hours and washed with sodium cacodylate buffer. Washed organisms were then treated with 1% osmium tetraoxide followed by treatment with 1% tannic acid and 1% uranyl acetate. Samples were washed with distilled water or sodium cacodylate buffer between treatments. Treated samples were then dehydrated through an ethanol series. The untreated control hyphal elements were processed in parallel for SEM analyses. Prepared samples were sputter coated with Au/Pd (60/40) and viewed under a scanning electron microscope (model XL3C ESEM Philips microscope).

Transmission electron microscopy

The temporal effects of TDT 067, compared with conventional terbinafine and untreated controls, on the ultrastructure of *T. rubrum* hyphae were evaluated using TEM. Briefly, fully formed hyphae were exposed to TDT 067, conventional terbinafine (both at 15 mg/ml), or left untreated, and samples were collected at different time points (5, 30 minutes, 2, 4, and 24 hours). Following treatment, hyphal

elements were removed and processed for TEM as described by Hanaichi et al. (12). The hyphae were fixed in 2.5% glutaraldehyde and 2% potassium permanganate and washed with distilled water. Hyphae were subsequently exposed to 1% potassium dichromate and 1% uranyl acetate. The fungal elements were washed with distilled water, embedded in agar, and cut into small cubes (0.5−1 mm^3) which were dehydrated through an ethanol series. The 100% ethanol was replaced with propylene oxide and the samples were embedded in Epon. Samples of subungual debris from patients were embedded into an agar pellet, fixed, and processed as previously described (12). Sections were obtained using an ultramicrotome, counterstained with lead citrate, and observed under a transmission electron microscope (model 1200EX, JEOL, Japan).

RESULTS

White light microscopy analysis of in vitro *effects*

Examination of untreated control *T. rubrum* hyphae under light microscopy showed healthy septate hyphae (Figure 1-A). In contrast, exposure of hyphae to TDT 067 resulted in morphological changes in which the filaments appeared stunted and contained discrete vacuole-like bodies within them. These changes were evident as early as 30 minutes following exposure to TDT 067 (Figure 1-B), and were observed consistently at every time point from 8 hours through the duration of the study in hyphae exposed to TDT 067. Exposure of *T. rubrum* hyphae to conventional terbinafine (15 mg/ml) treatment did not result in the formation of the vacuole-like structures seen with TDT 067-treated cells. Conventional terbinafine also affected the hyphal morphology, with filaments showing evidence of thinning and breakage. However, over the course of the study, TDT 067 treatment resulted in fewer hyphal elements compared with those treated with conventional terbinafine. By Week 7, no hyphae were observed in samples exposed to either of these drugs.

Scanning electron microscopy analysis of in vitro *effects*

SEM analysis was used to investigate the effect of TDT 067 and conventional terbinafine (15 mg/ml) on *T. rubrum* topography. Untreated control *T. rubrum* hyphae exhibited typical structures of healthy hyphal elements, with filaments of uniform width, cylindrical shape and a smooth outer appearance (Figure-2A and 2D). In contrast, morphological changes, including twisting and bulging of hyphae, were evident in *T. rubrum* hyphae exposed to TDT 067 for as little as 2 hours. After 4 hours of exposure to TDT 067, severe shriveling and distortion of the hyphal elements was observed (Figure-2B). In addition, breakage and collapsing of the hyphae became visible. At this time point, hyphal/cytoplasmic debris could be seen dispersed on the surface of the hyphae. Exposure of *T. rubrum* hyphae to terbinafine for the same period (4 hours) also showed significant cell collapse and twisting of hyphal elements. Additionally, a minimal amount of cytoplasmic debris, relative to TDT 067-treated hyphae, was noted with cells exposed to terbinafine (Figure-2C).

Exposure of *T. rubrum* hyphae to TDT 067 for a longer period of time (24 hours) resulted in complete disruption of hyphal structures, with hyphae almost fully collapsed, twisted, and shriveled, with evidence of breakage (Figure-2E). No intracellular materials were evident and only the cell wall remained. Hyphae exposed to conventional terbinafine for the same time period showed some bulging, breaking, and twisting; however there also appeared to be some healthy hyphal elements (Figure 2F).

Transmission electron microscopy analysis of* in vitro *effects

TEM analysis showed that untreated control *T. rubrum* hyphae exhibited the expected cellular structures, including nuclei, mitochondria, vacuoles, and intact cell membranes and cell walls (Figure-3A). In contrast, after as little as 5 minutes of exposure to TDT 067, disruption of intracellular material was evident and there was a decreased presence of mitochondria (Figure-3B). Lipid droplets were visible within the hyphae at 4 hours (Figure-3C). After 24 hours of exposure to TDT 067 (Figure-3D), the cell membrane had deteriorated and the entire intracellular space was filled with lipid droplets. Similar disruption of the intracellular matrix was observed following exposure to

conventional terbinafine after 24 hours (Figure-3E). There was evidence of separation of the cytoplasm from the outer cell envelope, though the cell wall appeared to be intact. Dark electron-dense areas were visible at 24 hours in cells treated with conventional terbinafine (Figure-3E); however, no lipid droplets were observed in hyphae exposed to conventional terbinafine.

In vivo *effects of TDT 067 on fungal morphology in clinical samples*

Nail samples from seven patients who were treated with TDT 067 were analyzed under white light microscopy. Lipid droplets were seen within the hyphae in subungual debris from all seven patients with onychomycosis after only 7 days of treatment with TDT 067 (Figure-4A), and were evident after one day of treatment (i.e., two applications of TDT 067) in samples from one patient. Gross morphological changes, such as hyphal swelling and distortion were evident in samples from four patients after 14 days' treatment (Figure-4B). Samples obtained from an untreated control patient showed the expected hyphal morphology and intracellular structures in the TEM analysis (Figure-5A). Samples from five patients demonstrating the most hyphae were analyzed by TEM. The hyphae were misshapen and the intracellular structures of the hyphae had been replaced by lipid droplets by Day 7 (Figure-5B).

DISCUSSION

In this study, we showed that exposure of *T. rubrum* hyphae to TDT 067 led to rapid and extensive ultrastructural changes to these filaments, including hyphal distortion and destruction of intracellular material. Distinct differences were evident in the rapidity and nature of the effects of TDT 067, compared with conventional terbinafine.

The effects of TDT 067 and conventional terbinafine were studied comprehensively using three different microscopic techniques (white light microscopy, SEM and TEM). White light microscopy and SEM studies showed that hyphae exposed to both TDT 067 and conventional terbinafine *in vitro*

demonstrated several morphological changes, which were more extensive following TDT 067 exposure. These morphological changes were evident as early as 4 hours after exposure to TDT 067. Hyphae exposed to TDT 067 also showed the presence of lipid droplets which appeared to disrupt the intracellular matrix and, by 24 hours, had filled the entire intracellular space, threatening the integrity of the hyphae. These lipid droplets were observed *in vitro* under white light microscopy and TEM studies, and their presence was also confirmed *in vivo* in subungual debris from patients with toenail onychomycosis who received topical TDT 067 treatment in a Phase II clinical study.

These data indicate that TDT 067 is more effective than conventional terbinafine in damaging the fungal cellular morphology. Our data also suggest that the Transfersome® in TDT 067 potentiates the action of terbinafine, with enhanced fungicidal activity compared with conventional terbinafine. The onset of action is also enhanced, as the Transfersome® delivers terbinafine more effectively to its site of action inside the fungus where it disrupts the intracellular matrix. The uptake mechanism of Transfersome® into fungus is not fully understood, but may be an active transport process promoted by the natural affinity of the phospholipids of the Transfersome® and the lipids of the fungal cell. Further investigations on this are ongoing. Although the lipid droplets have not been thoroughly characterized, one possibility is that they are lipid inclusions that accumulate following the penetration of the Transfersomes® through the hyphal cell wall, and may, therefore, be of Transfersome® origin. This conclusion is supported by our observations in other studies in which similar vacuoles were seen in *T. rubrum* hyphae exposed to the Transfersome® vehicle (i.e., Transfersome® without terbinafine; data not shown). An alternative hypothesis is that the lipid droplets are derived from organelles in which the membranes have been disrupted by the antifungal and surfactant effects of TDT 067 within the fungal cell. It is known that terbinafine acts at least in part through ergosterol inhibition (8, 23), and ultrastructural studies have reported the presence of large numbers of lipid droplets in the cytoplasm and adjacent to the cell membrane and cell wall in hyphae exposed to the precursor of terbinafine,

naftifine (17). Further studies are ongoing to investigate the origin of the lipid droplets observed following TDT 067 treatment.

The morphological changes seen in hyphae in subungual debris obtained from subjects enrolled in a Phase II study who were treated with TDT 067 demonstrate that, following topical application of TDT 067, terbinafine is able to reach the infecting organisms residing in the subungual debris where it exerts its antifungal effects. Furthermore, these effects were evident only 7 days after the start of treatment. In addition, the observation of concentric lamellar lipid structures within hyphae and in the adjacent areas of the fungal cells seen in the subungual debris provides direct evidence that TDT 067 reached the fungi. This is, to our knowledge, the first study to confirm the *in vitro* morphological effects of antifungals in clinical samples. Our findings are exciting since previous studies have shown that conventional terbinafine tends to be bound to nail material thereby failing to penetrate the nail plate (19) and exert its antifungal potential. Pharmacokinetic analysis from this TDT 067 clinical study demonstrated that systemic exposure to terbinafine was negligible following 28 days' treatment with TDT 067, and consistent with this no treatment-related systemic adverse events or hepatic laboratory abnormalities were reported (26). The ability of TDT 067 to deliver terbinafine to the site of infection with minimal systemic exposure increases its potential utility as a topical antifungal.

In summary, our data show that TDT 067 has potent antifungal activity that is manifested by drastic morphological effects on fungal cells that are distinct from those seen with conventional terbinafine. Additionally, we have confirmed that TDT 067 is able to reach the fungi in the nail bed in patients with nail infections and exert its antifungal effects. These data suggest that TDT 067 may have clinical utility for the treatment of onychomycosis in patients without nail matrix involvement. An ongoing Phase III study of 48 weeks of treatment with TDT 067 will establish the efficacy and safety in patients with onychomycosis.

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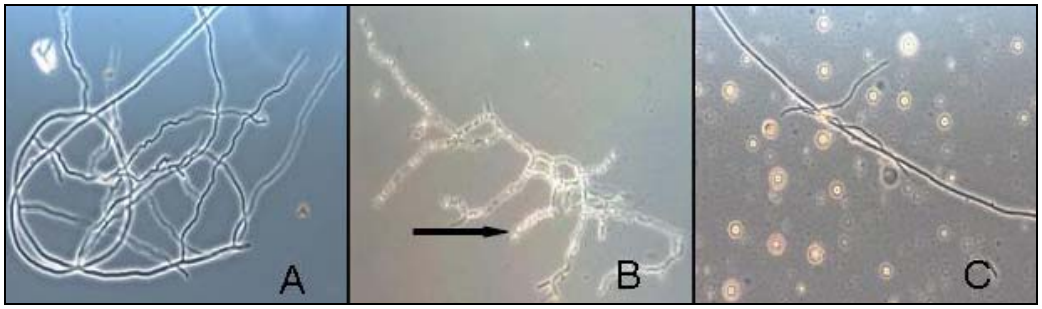
Figure 1. White light microscopy analysis of dermatophyte hyphae following 8 hours' exposure to TDT 067 or conventional terbinafine (15 mg/ml).



Growth control (A); TDT 067 at 8 hrs (B); conventional terbinafine (15 mg/ml) at 8 hours (C).

The arrow in (B) indicates lipid droplets.

Magnification 40X.

374 **Figure 2. Scanning electron microscopy analysis of dermatophyte hyphae following 4 and 24**

375 **hours' exposure to TDT 067 or conventional terbinafine (15 mg/ml)**

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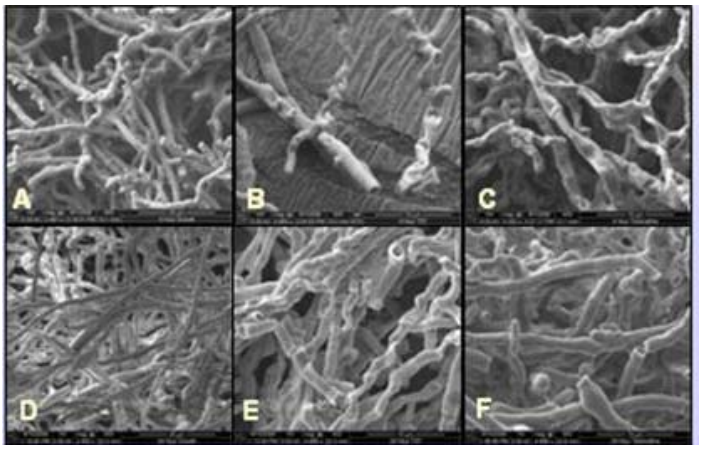

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379 Growth control at 4 hours (A) and 24 hours (D). TDT 067 at 4 hours (B) and 24 hours (E).

380 Conventional terbinafine at 4 hours (C) and 24 hours (F).

381 Magnification 4999X.

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384 **Figure 3. Transmission electron microscopy analysis of dermatophyte hyphae following 4 and 24**
385 **hours' exposure to TDT 067 or conventional terbinafine (15 mg/ml)**
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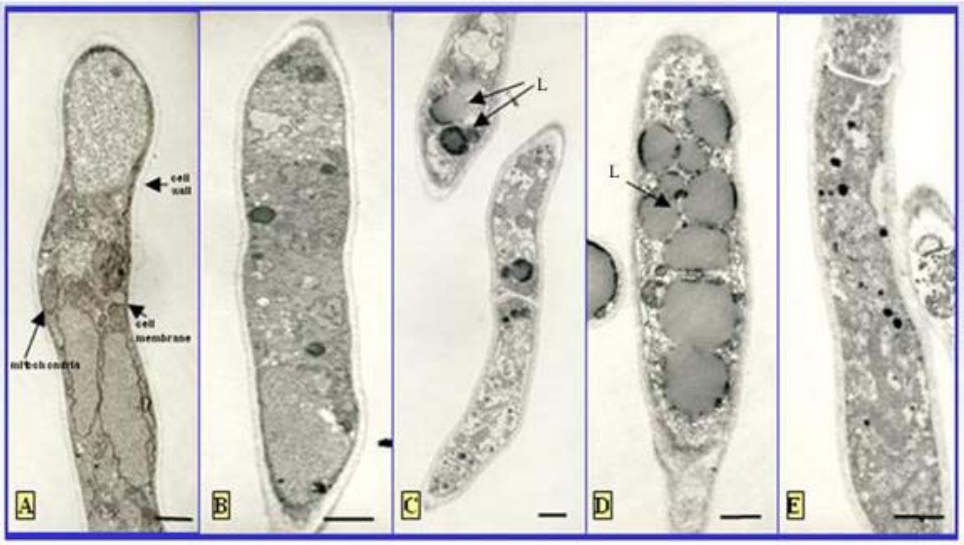

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389 A) Growth control. B) Cross section of hyphae exposed to TDT-067 for 5 minutes, C) 4 hours, and D)

390 24 hours. E) Cross-section of hyphae exposed to conventional terbinafine for 24 hours.

391 Bar = 1μm; L = lipid droplets.

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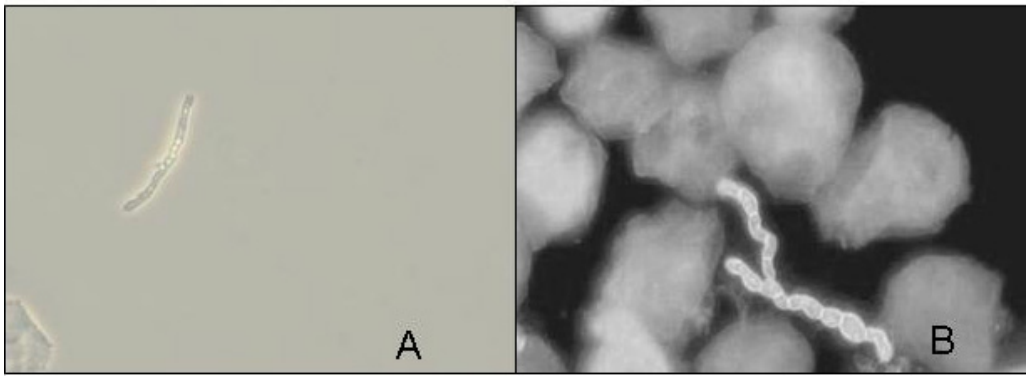
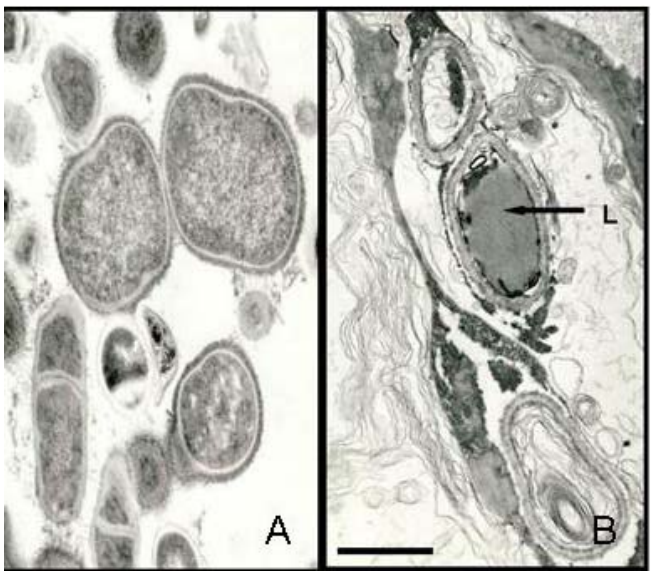
Figure 4. White light microscopy analysis of dermatophyte hyphae within subungual debris from patients with toenail onychomycosis who received topical treatment with TDT 067 (terbinafine in Transfersome®) twice daily for 28 days.



Hyphae with lipid globules after 7 days' treatment (A). Magnification 20X. Swollen hyphae evidenced after 7 days' treatment (B). Magnification 40X.

Figure 5. Transmission electron microscopy analysis of dermatophyte hyphae within subungual debris from patients with toenail onychomycosis who received topical treatment with TDT 067 (terbinafine in Transfersome®) twice daily for 7 days.



A) Untreated nail; B) nail treated for 7 days with TDT 067.

Bar = 1µm; L = lipid droplets.