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In Vitro Fungicidal Photodynamic Effect of Hypericin on Trichophyton spp

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Abstract Hypericin is a natural photosensitizer used in photodynamic therapy (PDT), which has shown in vitro antifungal effect against *Candida* spp. The aim of this study was to evaluate the in vitro fungicidal effect of hypericin-PDT on dermatophytes. *Trichophyton rubrum* and *Trichophyton mentagrophytes* strains were incubated with different concentrations of hypericin for different times and exposed to light-emitting diode lamp (602 ± 10 nm, 10.3 mW cm^{-2}, and fluence 37 J cm^{-2}). Using the optimal incubation time, 60 min, a 3-log fungicidal effect was achieved with hypericin concentration ranges of 10–20 µM for *T. rubrum* and 20–50 µM for *T. mentagrophytes* ($p = 0.95$). Confocal fluorescence microscopy showed the localization of hypericin inside the dermatophytes diffusely distributed in the cytoplasm of conidia and hyphae and outside the nucleus. In conclusion, hypericin-PDT has a fungicidal effect in vitro on dermatophytes. Hypericin seems to be a promising photosensitizer to treat localized dermatophytic infections such as *tinea pedis* and onychomycosis.

Keywords Photodynamic therapy · Dermatophytes · Hypericin · *Trichophyton rubrum* · *Trichophyton mentagrophytes*

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Introduction

Dermatophytes are a unique group of fungi that infect keratinous tissue and are able to invade the hair, skin, and nails of a living host. Treatment for dermatophytoses involves the use of antifungal agents in either a topical or oral application form or a combination of both. The latter is often applied in case of persistent or extensive mycosis. However, oral antifungal drugs may cause side effects, such as hepatotoxicity, and interaction with other therapies [1].

Regarding the treatment for onychomycosis, there is a 25–30 % relapse rate in the long term for those who have an initial cure [2]. Even this reported success rates appear lower in patients over 65 years of age. Additionally, therapy may be time-consuming and requires considerable patience. Even though new antifungals for onychomycosis are under investigation, such as topical efinaconazole or oral albaconazole, they seem to be very similar to the already available treatments, therefore with high probability to present similar drawbacks in the future.

In the last years, physical therapies have been used with promising results to treat onychomycosis [3]. Antifungal photodynamic therapy (PDT) is an area of increasing interest. Photodynamic inactivation requires microbial exposure to either exogenous or endogenous photosensitizer (PS) molecules, followed by visible light energy, typically wavelengths in the red/near-infrared region. The combination of both with oxygen causes the excitation of the photosensitizers resulting in the production of singlet oxygen and other reactive oxygen species. These compounds react with intracellular components and consequently produce cell inactivation and death [4]. Several clinical reports have treated different infections caused by dermatophytes, most of them using 5-aminolevulinic acid (ALA) or its derivative methyl-aminolevulinate (MAL) as photosensitizers [5].

Hypericin is a naturally occurring polycyclic aromatic naphthodianthrone isolated from certain species of plants from the genus *Hypericum*. Previous reports from our laboratory have revealed a photodynamic fungicidal effect of hypericin against *Candida* spp., especially *Candida albicans* [6]. Therefore, our aim was to study the antifungal effect of hypericin-PDT in vitro against the two species of dermatophytes that produce most of the cases of onychomycosis: *Trichophyton rubrum* and *Trichophyton mentagrophytes*.

Materials and Methods

Chemicals

Hypericin was purchased from HWI Analytik® GMBH (Germany). The Sabouraud medium (SB) was prepared from Sabouraud dextrose agar CM0041 pH 5.6 ± 0.2 (Oxoid®, UK) supplemented with Chloramphenicol at 50 mg L^{-1} (Sigma-Aldrich®, USA). Distilled sterile water was purchased from (Fresenius Kabi®, Spain). Phosphate buffer solution was purchased from (PBS) pH 7.2 ± 0.2 (Bio-Rad®, USA). Dimethyl sulphoxide was purchased from (DMSO) (Panreac®, Spain). CellTracker® Green (CTG) (Ref. C2925) and DAPI® (Ref. D3571) were obtained from Molecular Probes® (Invitrogen™, USA).

Microorganisms and Growing Conditions

Trichophyton rubrum ATCC-28188 and *T. mentagrophytes* ATCC-9533 strains were obtained from the American Type Culture Collection (ATCC, USA). The fungi were grown aerobically for 7–12 days in SB medium at 30 °C. Stock microconidia suspensions were prepared in distilled sterile water and adjusted to 0.5 McFarland turbidimetrically density. Cell viability was estimated counting the number of colony-forming units (CFU) grown on SB after an incubation period of 5 days at 30 °C.

Photosensitizer Solutions

Stock hypericin solution was prepared in DMSO. Work hypericin solutions were diluted with distilled sterile water and stored in the dark at −20 °C before using. Solutions were prepared and handled under light-restricted conditions. The concentrations used ranged from 5 to 200 µM.

Light Sources

Photoinactivation treatment was carried out by using a light-emitting diode (LED) lamp emitting at 602 ± 10 nm with an irradiance of 10.3 mW cm^{-2}. The fluence used was 37 J cm^{-2}.

Photoinactivation Treatment

Starting from 7- to 12-day-old fungi cultures, 0.5 McFarland microconidia suspensions were prepared in distilled sterile water, corresponding to a cell density of >1 × 10^6CFU/mL for *T. rubrum* ATCC-28188 strain and >1 × 10^5 CFU/mL for *T. mentagrophytes* ATCC-9533. These initial suspensions were dropped into microtiter plates where they were then mixed with different concentrations of hypericin in the twofold dilutions ranges 5–20 and 50–200 µM. The

plates were then maintained at 30 °C in the dark for different periods of time. Based on our previous results obtained with *Candida* spp. [6], 0, 15, 30, and 60 min were the preincubation times tested to evaluate the influence of contact time on the outcome of the photodynamic treatments. Afterward, fungi cells were irradiated by means of red LED illumination using 37 J cm^{-2} of fluence. The controls consisted on fungal cultures cultivated under the same conditions with and without hypericin, whether kept in the dark or illuminated. After the treatments, samples and controls were incubated at 30 °C for 5 days, and the antifungal effect was determined by counting the number of CFU/mL [7]. The bactericidal activity criterion, defined as >99.9 % or 3-log_{10} reduction in CFU/mL regarding to the initial inoculum, was used to establish the fungicidal activity. This standard has been previously used by other authors to evaluate the antifungal activity of drugs on *Trichophyton* spp. [8].

All experiments were carried out at least 9 times.

Confocal Fluorescence Microscopy

The 0.5 McFarland standard suspensions were mixed with hypericin obtaining 5 µM samples. This concentration of hypericin was used by our group in recent studies on *Candida* spp. [9]. Samples were incubated for 1 or 30 min. After that, they were centrifuged and washed using PBS at 30 °C three times. The pellets obtained were resuspended in PBS medium at 30 °C, and the fluorescent probe CTG (200 nM) was added. After dark incubation during 40 min at 30 °C, the samples were washed three times as above. The pellets were resuspended in 3 % paraformaldehyde (PFA) solution and further incubated in the dark for 15 min at 30 °C. PFA was removed by washing twice with distilled sterile water. Finally, pellets were resuspended in distilled sterile water and dispensed into a 24-well plate containing a polylysine-treated glass coverslip. The plate was centrifuged in order to stick the cells on the coverslips, which were leaved on glass plates using Mowiol mounting medium containing DAPI at 5 µM [9]. Samples were visualized with an Olympus Fluoview FV10i confocal scanning microscope. Images were acquired using sequential mode with a 60× oil immersion lens and a format of 1,024×1,024 pixels. The confocal pinhole was 1 Airy unit. Images were exported from the FV10i software into Adobe Photoshop CS5.

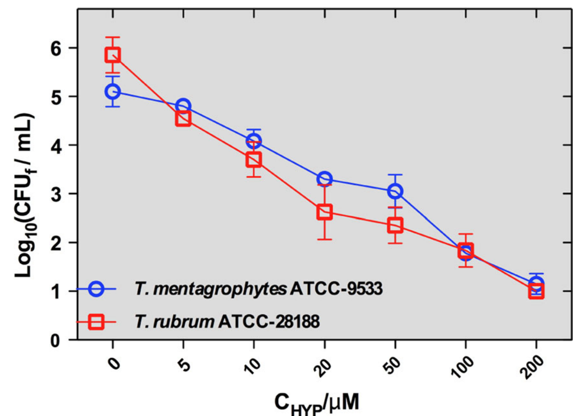

Fig. 1 Hypericin concentration effects on *Trichophyton* spp. photoinactivation starting from McFarland values of 0.5 (initial inocula between 1 × 10^5 and 1 × 10^6 CFU/mL)

Statistics

Hypericin concentration values were expressed by mean ± standard deviation. Differences between two means were analyzed using the Mann–Whitney *U* test. Statistical significance was assumed at a *p* value of <0.05.

Results

In Vitro Photoinactivation

Under the experimental conditions described above, photodynamic treatment with hypericin inhibited the growth of both *Trichophyton* species reaching a fungicidal effect. In the absence of light, hypericin inactivated *Trichophyton* spp. less than 6.5 %. Similarly, the light treatment alone did not exert significant cytotoxicity (around 1.5 % cell viability reduction). The harmless effect of the hypericin solvent (DMSO) on the fungi was also proven.

The effect of preincubation time of *Trichophyton* conidia with the photosensitizer before illumination was studied. 60 min was the optimal time to produce complete photoinactivation in both species using less hypericin concentration than with shorter times. Using this optimal incubation time, a 3-log fungicidal effect was achieved with hypericin concentration ranges of 10–20 µM for *T. rubrum* and 20–50 µM for *T.*

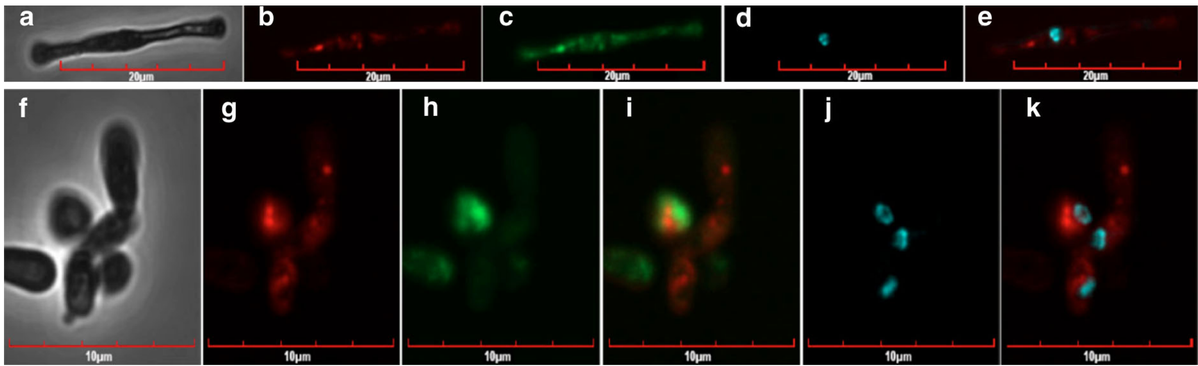

Fig. 2 Localization pattern of hypericin by means of confocal fluorescence microscopy on *T. metagrophytes* ATCC-9533 using a preincubation time with hypericin of 30 min in darkness at 30 °C: **a–e** hypha detail, **f–k** microconidia; **a** and **f** conventional microscopy; **b** and **g** hypericin fluorescence (*red*); **c** and **h** CTG fluorescence (*green*). **i** colocalization between hypericin and CTG; **d** and **j** DAPI® fluorescence (*blue*); **e** and **k** non-colocalization detail between DAPI® and hypericin. (Color figure online)

mentagrophytes, the difference being not statistically significant ($p = 0.95$) (Fig. 1).

Hypericin Localization in Trichophyton spp

Confocal fluorescence microscopy showed colocalization of hypericin with the cellular probe Cell-Tracker® Green (CTG). This colocalization remains at least for the first 30 min of its contact with the dermatophytes. Hypericin distribution was diffuse in the cytoplasm of conidia and hyphae (Fig. 2). No colocalization with the nuclear probe DAPI, either in the conidia or in the hyphae, was found. Control samples without any probe or hypericin showed no fluorescence emission from the own cells.

Discussion

The present study shows, for the first time, that in vitro PDT with hypericin exerts fungicidal effects on *Trichophyton* spp. The ability of in vitro PDT to inhibit dermatophytes microconidia growth was demonstrated by Smijs et al. [10] using porphyrins, such as Sylens B and red light on *T. rubrum*. Other studies were carried out by Amorim et al. [11] and by Rodrigues et al. [12] on *Trichophyton* spp showing the antifungal activity of in vitro PDT with phenothiazines. It is difficult to compare the efficacy of these photosensitizers with hypericin because the experimental conditions were very different.

Regarding the susceptibility of the different *Trichophyton* spp. to hypericin-PDT, even though less concentration of the photosensitizer was needed to photoinactivate *T. rubrum* than *T. mentagrophytes,* the differences were not statistically significant.

Crucial parameters in determining the photocytotoxic activity of any photosensitizer are its cell permeability and subcellular localization. Hypericin seems to be an excellent photosensitizer for antifungal PDT due to its lipophilic characteristics that provide it with better permeabilization in the lipid bilayers of fungi, much better than other photosensitizers with hydrophilic characteristics [4]. We have shown that hypericin is located within the *Trichophyton* spp. microconidia and hyphae due to its colocalization with the cellular probe (CTG). As we demonstrated also in yeasts, hypericin did not localize in the nucleus, which diminishes the possibility to induced mutagenic changes in the fungus [9]. Three important intracellular target sites are prevalent for hypericin, namely the mitochondria, the ER, and to a lesser extent the lysosomes [13]. We did not find any specific location of hypericin inside the dermatophytes.

One of the limitations to extrapolate our results to the clinical setting is that dermatophytic infections are caused by hyphae structures, and our in vitro experiments were made on microconidia. However, dermatophytes are difficult to manage in vitro, and usually, the in vitro studies to test the efficacy of any antifungal treatment are made with microconidia before starting the clinical studies [14]. On the other hand, we have shown that hypericin is equally located within the

Trichophyton spp. microconidia and hyphae, which makes us to presume a similar phototoxic action in both of them. This aspect is very important for a successful PDT in the clinical setting because this means that hypericin-PDT would be effective in destroying either the microconidia formed through germination after spore inoculation and the hyphae, fungal stage which produced the disease.

Our group has showed in previous in vitro studies a selective fungicidal effect of hypericin-PDT on *C. albicans* preserving human skin cells using concentrations of 1.5 μM or less [6]. However, higher concentrations are needed for dermatophytes (10–50 μM) to reach a 3-log reduction. Several studies have used hypericin 0,1 % topically or 40–200 μgr intralesionally to treat different types of skin lesions showing similar phototoxic effects on normal skin to other photosensitizers used in the clinical setting [15, 16].

In conclusion, our study demonstrates the in vitro fungicidal effect of hypericin-PDT on dermatophytes. Hypericin seems to be a promising photosensitizer to use in clinical trials to treat the most common infections caused by dermatophytes such as *tinea pedis* and onychomycosis.

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Conflict of interest None declared.

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