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Research Article

TONS504-Photodynamic Therapy Against *Trichophyton mentagrophytes* Infection: *In vitro* culture and *In vivo* Animal Model Study

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Abstract

Despite the presence of various topical and systemic antifungal agents, few reports of photodynamic therapy (PDT) are available. In order to determine the antifungal effect of PDT, *In vitro* and *In vivo* analyses against *Trichophyton mentagrophytes* (*T. mentagrophytes*) were performed. *T. mentagrophytes* were cultured *In vitro* and also inoculated on the plantar skin of guinea pigs. They were irradiated with a diode laser of 670 nm in the presence of a novel cationic photosensitizer, TONS504. TONS504-PDT showed antifungal effect against *T. mentagrophytes* both *In vitro* and *In vivo*. The effect was irradiation dose- and TONS504 concentration-dependent and the maximal effect was observed at 100 J/cm^2. TONS504-PDT showed a significant antifungal effect and might be applicable against *T. mentagrophytes*-related skin diseases.

Keywords: Anti-Fungal Effect; PDT; *Trichophyton mentagrophytes* ; TONS504; Therapy

Abbreviations:

PDT	: photodynamic therapy;
T. mentagrophytes	: *Trichophyton mentagrophytes*

Introduction

Skin fungal infection is prevalent especially in the elderly people [1]. However, currently available topical or systemic treatments either alone or in combination are occasionally not successful. Furthermore, systemic treatments may not be applicable for patients having various commodities or due to adverse effects, such as liver and kidney dysfunction and due to drug interactions.

Photodynamic therapy (PDT) is a new therapeutic modality for skin tumors including actinic keratosis and Bowen disease, and 5-aminolevulinic acid (ALA)-PDT has been approved for the treatment of actinic keratosis in the US [1].

TONS504,13,17-bis(1-carboxyethyl)carbamoyl(3-methylpyridine)-3-(1,3-dioxane-2-yl)methylidene-8- ethenyl-2-hydroxy-2, 7, 12, 18-tetramethyl chlorine, diN-methy iodide (MW:1370) is a novel cationic chemical,. Our recent

study disclosed anti-fungal effect of TONS504-PDT for *Malassezia furfur In vitro* [2, 3]. In the present study, we investigated the antifungal effect of TONS504-PDT on *Trichophyton mentagrophytes* using both *In vitro* culture and *In vivo* animal model.

Materials and Methods

Chemicals

TONS504,13,17-bis(1-carboxyethyl)carbamoyl(3-methylpyridine)-3-(1,3-dioxane-2-yl)methylidene-8- ethenyl-2-hydroxy-2, 7, 12, 18-tetramethyl chlorine, diN-methy iodide s(MW:1370) was synthesized by Photochemical Co. Ltd, Okayama, Japan. TONS504 was dissolved in 1-menthol solution (lotion) at a concentration of 1mg/ml.

Laser units

A diode laser (LD670-05, Hamamatsu Photonics K.K., Hamamatsu, Japan) was used as a light source for TONS504-PDT. The diode laser is a continuous-wave laser operating at wavelength 660 nm with 6 mm spot size.

Culture of *Trichophyton mentagrophytes* and antifungal effect of PDT

Trichophyton mentagrophytes (*T. mentagrophytes*) isolated NTM-105 strain was kindly provided by Dr. A. Matsuda (Public Health Research Institute of Kobe City, Kobe, Japan). The first culture of *T. mentagrophytes* was carried out on Kimming-Plates (Merk, Darmstadt, Germany) for 3-4 weeks at room temperature. Then liquid cultures of *T. mentagrophytes* were prepared by inoculation of Sabouraud glucose (2%) broth (Heipha Diagnostika, Heidelberg, Germany). Liquid culture was continuously shaken at 50 rpm on a shaker Promax 2020 (Heidolph, Schwabach, Germany). Then *T. mentagrophytes* was cultured in Sabouraud dextrose broth with 0.001% olive oil containing various doses (0-1 μg/ml) of TONS504 for 4 hours under shielded condition, and was inoculated in a 2 cm-sized culture dish. Then the culture dish was irradiated with LD670-05 diode laser at 0-100 J/cm^2, single pulse, 6 mm spot size, and 1mm overlap between the spots. Viability area of the fungal growth was traced with clear films and was measured.

Animals and *In vivo* inoculation procedure

Young adult male Hartley strain guinea pigs weighing 350-500 g were used in *In vivo* experiments. The inoculation was performed on the plantar part of the hind feet according to the method by Fujita et al [4].

Examination of infection processes

Seven days after the inoculation, the 5 x 5 x 1 mm inoculation paper disc was removed and the animal feet were free from wrapping tapes. The feet were visually inspected for clinical findings for 7 days. Microscopic examination of scales was performed following 15% potassium hydroxide treatment on the 7 day after the removal of disc. Percent of positive culture is 64/70 guinea pigs.

Photodynamic therapy (PDT)

At 14 days after removal of the disc, guinea pigs were anesthetized with sodium pentobarbital (350 mg/kg), and TONS504 ointment was applied onto the skin for 3 hours. Sixty guinea pig plantar skin was irradiated with LD670-05 diode laser (150mW/cm^2, 670 nm) at 0-100 J/cm^2, one to pulse, 6 mm spot size, and 1mm overlap between the spots. The PDT was performed at one to four times every 7 days and, the treated feet were visually examined for clinical findings. Microscopic examination of scales was performed using 15% potassium hydroxide solution. Furthermore, scales from the plantar part were cultured on Sabouraud glucose (2%) broth plate and was examined for the growth of *T. mentagrophytes*.

Results and Discussion

Results

TONS504-PDT suppresses *T. mentagrophytes* growth *In vitro*

Diode laser irradiation was performed on *T. mentagrophytes* after the incubation of 1 μg/ml TONS504. The antifungal effect was irradiation dose-dependent and the maximal effect was observed at 100 J/cm^2 (Figure 1A). Irradiation alone did not affect the viability of *T. mentagrophytes*. In order to determine the optimal concentration of TONS504, PDT with various concentrations of TONS504 was performed. The antifungal effect was observed in a concentration-dependent manner and the maximal effect was detected at 1 μg/ml (Figure 1B).

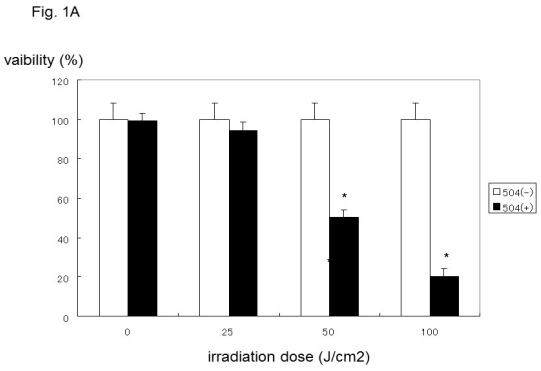

Figure 1. TONS504-PDT suppresses viability of *T. mentagrophytes*

in an irradiation dose-dependent manner

T. mentagrophytes was incubated on culture plates in the presence or absence of 1mg/ml TONS504 for 4 hours. Following the treatment, the plates were irradiated with various doses of LD670-5 diode laser. Note that *T. mentagrophytes* is mostly viable by 100 J/cm^2 and 50 J/cm^2 irradiation withit TONS504. (B) Viability of *T. mentagrophytes* at the indicated concentration dose. □:PDT in the absence of TONS504, ■:PDT in the presence of 1mg/ml TONS 504 (504(+)). *P<0.01 compared with that in the absence of TONS504(504(-)).

TONS504-PDT suppresses *T. mentagrophytes* growth *In vivo*

After the application of 0.1% (weight/weight) TONS504 ointment for 4 hours in occlusion dressing, 100 J/cm^2 irradiation was performed once a week for up to four weeks. The clinical examination showed remarkable improvement of tinea pedis of guinea pig skin (Figure 2A). In order to determine the effect of antifungal effect of PDT, fungal culture of guinea pig scales was performed. The antifungal effect depended on the irradiation number of times and culture-positive feet were mostly undetectable following 4 times of PDT treatment at 100 J/cm^2 (Figure 2B). Irradiation alone did not affect the viability. The antifungal effect was irradiation dose-dependent and the maximal effect was observed at 100 J/cm^2 (Figure 2C). No adverse effect such as burn or scar formation was detected.

Fig. 2A

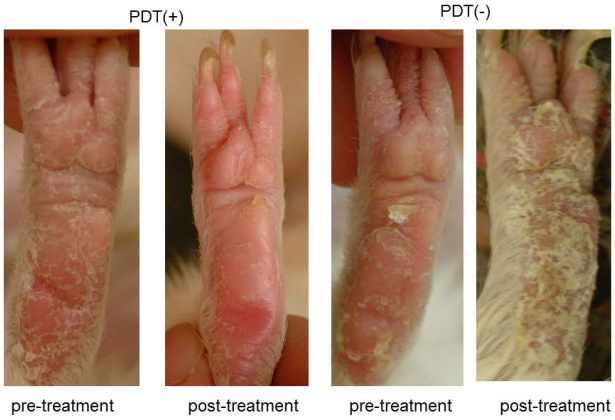

Fig. 2B

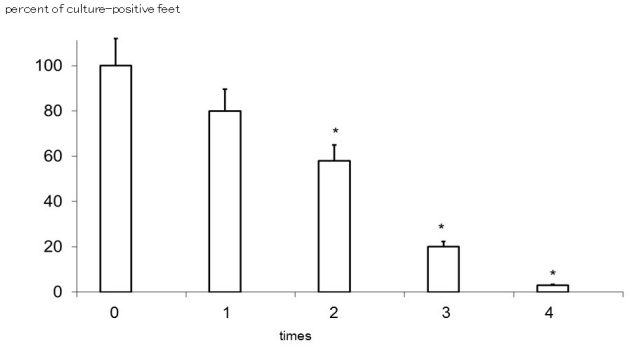

Fig. 2C

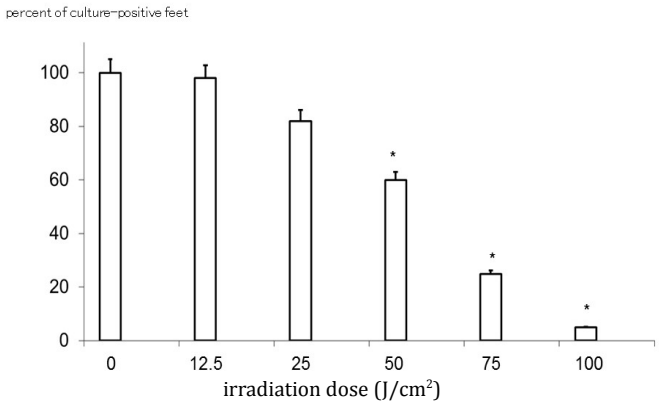

Figure 2. TONS504-PDT suppresses growth of *T. mentagrophytes In vivo*.

(A) *T. mentagrophytes* was incubated on the guinea pig skin and TONS504-PDT was performed.

(B). After the application of TONS504 ointment, the guinea pig skins were irradiated with various number of LD670-5 diode laser (100 J/cm^2) treatment times once a week for up to 4 times. Growth of *T. mentagrophytes* at the indicated number of treatment times of PDT was measured.*P<0.01 compared without PDT.

(C). After the application of TONS504 ointment, the guinea pig skins were irradiated with various doses with LD670-5 diode laser once a week for 4 times. Growth of *T. mentagrophytes* at various doses of PDT was measured.*P<0.01 compared without PDT.

Discussion

Topical PDT has proved to be useful for various skin tumors [5]. Moreover, PDT for infective viral, bacterial, and fungal skin diseases are described [6-8]. Kamp et al demonstrated 5-aminolevulinic acid (ALA)-PDT suppresses the growth of *T. rubrum In vitro* [7]. Because of its anionic nature, cell and bacterial membranes show high affinity to cationic substances. The cationic photosensitizer, TONS504, was much more effective than anionic ALA, and TONS504-PDT potently suppressed *Malassezia furfur* growth [2], suggesting that electric polarity is more critical for the attachment of photosensitizer to fungi. Our study demonstrated that TONS504-PDT also decreased *T. mentagrophytes* viability *In vitro*. However, the antifungal effect of PDT against *T. mentagrophytes* was slightly less than that against *M. furfur.* This might be due to less efficient TONS504 penetration into the fungal cell wall and membranes (data not shown).

Experimental dermatophytosis model inoculating *T. mentagrophytes* on the back of guinea pigs has been established by Bloch [9]. However, the model is not equivalent to human tinea pedis lesion. The experimental guinea pig model results in spontaneous resolution within 4 weeks. In contrast, infecting fungi were constantly observed in the stratum corneum of all inoculated feet more than 6 months in our experimental guinea pig model [4]. In the present study, TONS504-PDT effectively improved the tinea pedis of guinea pig pad

demonstrating the antifugal efficacy against tinea pedis.

It has been suggested that one out of 5-6 Japanese population are suffering from tinea pedis. Some patients do not respond to topical agents, and systemic treatments may not be applicable because of adverse effects or the patients' comorbidities. The PDT might be useful for these cases. Furthermore, Watanabe et al [8] showed successful ALA-PDT treatment against toe nail onychomycosis suggesting TONS504-PDT might be also effective against onycomycosis. The limitation of the present study is that it is essentially the analysis of guinea pig model. If a patient does not respond to topical antifungal agents, the PDT response might be different from a naïve guinea pig, because of various factors such as thick stratum corneum of human sole, low adherence to TONS504, infected environment that causes reinfection, etc. Thus it is necessary to perform the TONS504 PDT analysis in human model *In vivo*.

Conclusion

Our results indicate that TONS504-PDT could be a novel therapeutic modality for the treatment of *T. mentagrophytes*-related diseases. Further study would be required to establish the clinical efficacy of PDT against various fungal diseases.

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