ORIGINAL ARTICLE

In Vitro Antimicrobial Photodynamic Therapy Against *Trichophyton mentagrophytes* Using New Methylene Blue as the Photosensitizer☆

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Abstract

Introduction and objectives: Antimicrobial photodynamic therapy combines the use of a photosensitizing drug with light and oxygen to eradicate pathogens. *Trichophyton mentagrophytes* is a dermatophytic fungus able to invade the skin and keratinized tissues. We have investigated the use of new methylene blue as the photosensitizing agent for antimicrobial photodynamic therapy to produce the in vitro inactivation of *T mentagrophytes*.

Material and methods: A full factorial design was employed to optimize the parameters for photoinactivation of the dermatophyte. The parameters studied were new methylene blue concentration, contact time between the photosensitizing agent and the fungus prior to light treatment, and the fluence of red light (wavelength, 620–645 nm) applied.

Results: The minimum concentration of new methylene blue necessary to induce the death of all *T. mentagrophytes* cells in the initial suspension (approximate concentration, 10^6 colony forming units per milliliter) was 50 μM for a fluence of 81 J/cm^2 after a contact time of 10 minutes with the photosensitizing-agent. Increasing the concentration to 100 μM allowed the fluence to be decreased to 9 J/cm^2.

Conclusions: Comparison of our data with other published data shows that the susceptibility of *T. mentagrophytes* to antimicrobial photodynamic therapy with new methylene blue is strain-dependent. New methylene blue is a photosensitizing agent that should be considered for the treatment of fungal skin infections caused by this dermatophyte.

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PALABRAS CLAVE
Nuevo azul de metileno;
Terapia fotodinámica antimicrobiana;
Trichophyton mentagrophytes

Terapia fotodinámica antimicrobiana *in vitro* aplicada sobre *Trichophyton mentagrophytes* con nuevo azul de metileno como fotosensibilizador

Resumen

Introducción y objetivos: La terapia fotodinámica antimicrobiana combina el uso de un fármaco fotosensibilizante, la luz y el oxígeno para erradicar microorganismos patógenos. *Trichophyton mentagrophytes* es un hongo dermatofito capaz de invadir la piel y tejidos queratinizados. El objetivo de este trabajo es aplicar la terapia fotodinámica antimicrobiana para la inactivación *in vitro* de *T. mentagrophytes* utilizando el nuevo azul de metileno como agente fotosensibilizador.

Material y métodos: Se aplica un diseño factorial completo para optimizar los parámetros que permiten la fotoinactivación del dermatofito. Se tiene en cuenta la concentración del nuevo azul de metileno, el tiempo de contacto entre el fotosensibilizador y el hongo antes del tratamiento con luz y la fluencia de luz roja aplicada entre 620 y 645 nm.

Resultados: La mínima concentración de nuevo azul de metileno que produce una mortalidad de todas las células de *T. mentagrophytes* de la suspensión inicial (concentración $\sim 10^6$ ufc/ml) es 50 μM para una fluencia de 81 J/cm^{-2} y un tiempo previo de contacto hongo-fotosensibilizador de 10 min. Si se aumenta la concentración a 100 μM la fluencia que se necesita disminuye a 9 J/cm^{-2}.

Conclusiones: La comparación de nuestros datos con otros publicados muestra que la susceptibilidad de *T. mentagrophytes* a la terapia fotodinámica antimicrobiana con nuevo azul de metileno es cepa-dependiente. El nuevo azul de metileno es un fotosensibilizador a tener en cuenta para el tratamiento de las micosis cutáneas causadas por este dermatofito.

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Introduction

Antimicrobial photodynamic therapy (aPDT) eliminates pathogens using visible light in combination with a photoactivatable drug. The interaction of this photosensitizer (PS) with light and oxygen induces the formation of reactive oxygen species (ROS) that kill the infectious agents targeted for eradication. Because no specific cellular target is used, it is very difficult for cells to develop resistance to the PS used.[1] aPDT is especially appealing because of its selectivity, which is a consequence of the use of PSs with a preferential affinity for microbial cells over host tissue and the fact that the photodynamic effect is restricted to the light-treated area.[2]

Trichophyton mentagrophytes is a dermatophytic fungus that invades the stratum corneum of the epidermis as well as keratinized structures such as hair and nails, causing cutaneous mycoses commonly known as tineas.[3,4] Most current treatments are based on the use of antifungal agents, which can cause undesirable side effects, especially when they must be taken orally.[5]

New methylene blue (NMB) is a planar tricyclic phenothiazinium that has phototherapeutic potential due to the fact that its absorption wavelength is in the range of maximum penetration of light in the host tissue (600-850 nm).[6,7] In aqueous buffer solutions, NMB absorbs light at 630 nm and emits fluorescence at 650 nm.

The aim of this study was to optimize the use of aPDT with NMB as the PS for the *in vitro* photoinactivation of *T. mentagrophytes*.

Materials and Methods

Chemical Reagents

NMB and sterile Dulbecco's phosphate-buffered saline (PBS) with a pH of 7.4 were acquired from Sigma-Aldrich, Inc. (St. Louis, MO, United States). Fig. 1 shows the structure of NMB.

The fungal culture medium, Sabouraud agar (SA), was prepared from Sabouraud dextrose agar with a pH of 5.6 ± 0.2 (code CM0041, Oxoid Limited, Basingstoke, United Kingdom).

A stock solution of NMB 1 mM was prepared in PBS (pH 7.4). For the working solutions, the NMB solution was dissolved in PBS in darkness until the desired concentration was reached and stored at 4 °C.
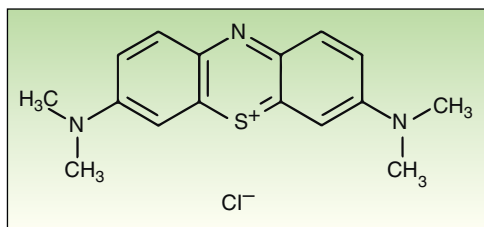
Fungal Strain, Culture Conditions, and Preparation of Cell Suspensions

T. mentagrophytes CECT 2956 were obtained from the Spanish Collection of Type Cultures (Valencia, Spain).

T. mewntagrophytes CETC 2956 cultures were grown on SA and incubated in the dark at 26 °C for 14 days. Once the colonies had grown, they were detached from the surface of the solid culture medium by manual agitation in sterile saline solution with 3 mm glass balls. The cell suspension was filtered through sterile gauze. The working suspensions were adjusted to a concentration level of approximately 10^6 colony-forming units (CFU)/mL in sterile saline solution.

Table 1   Experimental Design for Each Concentration of New Methylene Blue (0, 0.1, 1, 10, 25, 50, 75, 100, and 1000 μM).

	Irradiation Time			
	0 min	10 min	30 min	90 min
Incubation time				
0 min	Experiment 1	Experiment 2	Experiment 3	Experiment 4
10 min	Experiment 5	Experiment 6	Experiment 7	Experiment 8
30 min	Experiment 9	Experiment 10	Experiment 11	Experiment 12
90 min	Experiment 13	Experiment 14	Experiment 15	Experiment 16

Figure 1   Chemical structure of new methylene blue.

Light Source

Cell suspensions of *T. mentagrophytes* were irradiated with a Photocare light-emitting diode (LED) lamp (Sorisa, Sant Quirze del Vallès, Spain). To excite the NMB, the lamp was set to emit red light at between 620 and 645 ± 10 nm.

Experimental Design to Optimize the Photoinactivation Protocol for the *Trichophyton mentagrophytes* Dermatophyte

The protocol for photoinactivation of *T. mentagrophytes* cells by aPDT was optimized using the full experimental design derived from the combination of 3 variables applied at different levels: final NMB concentration (0, 0.1, 1, 10, 25, 50, 75, 100, and 1000 μM), incubation time of the PS in contact with the fungus (0, 10, 30, and 90 min), and irradiation time (0, 10, 30, and 90 min).

Table 1 lists the experiments carried out. In summary, 16 experiments were carried out for each of the 9 NMB concentrations tested, for a total of 144 experiments.

Photoinactivation Experiments With *Trichophyton mentagrophytes*

NMB at one of the concentrations specified above was added to each working suspension (approximate concentration 10^6 CFU/mL) and incubated for 10, 30, or 90 minutes at 30 °C and 120 rpm. After incubation, without previously washing out the excess PS, the samples were irradiated at 620-645 nm using a light source for 10, 30, or 90 minutes, with a fluence of 9, 27, and 81 J/cm^{-2}, respectively, in order to excite the 630 nm band of the NMB.

After being treated with light, 0.1 mL of each suspension was seeded in Petri dishes containing SA and incubated in the dark at 26 °C for 14 days. The colonies formed after the photodynamic treatment were then counted.

For each test, controls for light toxicity (without PS) and for PS toxicity (without light) were carried out under the same experimental conditions.

Results

The *in vitro* optimization of the factors used in aPDT with NMB against *T. mentagrophytes* (PS concentration, contact time, and irradiation time) were assessed according to the decrease in cell viability, expressed as a logarithmic reduction of the number of CFU/mL in relation to the initial cell suspensions of approximately 10^6 CFU/mL. In the absence of PS, exposure of the fungus to light did not inhibit fungal growth at any of the 3 light fluences used (data not shown). The experiment to assess the toxicity of NMB in the dark showed that this PS is only toxic to the dermatophyte when combined with light (red function in Fig. 2).

The results shown in Fig. 2 indicate that concentrations of 0, 0.1, and 1 μM of NMB did not have a fungicidal effect on *T. mentagrophytes*; a confluent culture was obtained after irradiation of the sample for all incubation and irradiation times tested. However, a growth-inhibiting effect was detected with NMB concentrations of 10 and 25 μM. Under some conditions, the reduction in the number CFU/mL was as large as 5 logarithmic units, but complete inhibition of the fungus was not achieved in any case. Eradication of all colonies was achieved using higher PS concentrations (50, 75, and 100 μM) combined with longer incubation and irradiation times. Finally, when samples with a NMB concentration of 1000 μM were irradiated, a complete fungicidal effect was achieved for all experimental combinations.

Therefore, on the basis of the results obtained by applying aPDT to cell suspensions of *T. mentagrophytes* (10^6 CFU/mL), we can deduce that with an agitation and irradiation time of 10 minutes (9 J/cm^{-2}) the lowest concentration of NMB that achieves total cell mortality is 100 μM; however, if the contact time remains the same but the sample is irradiated for 90 minutes (81 J/cm^{-2}), the concentration of NMB can be decreased to 50 μM.

Discussion

Various *in vitro* studies have explored the possibility of PDT as treatment for dermatophytoses, in most cases using 5-aminolevulinic acid or derivatives thereof as
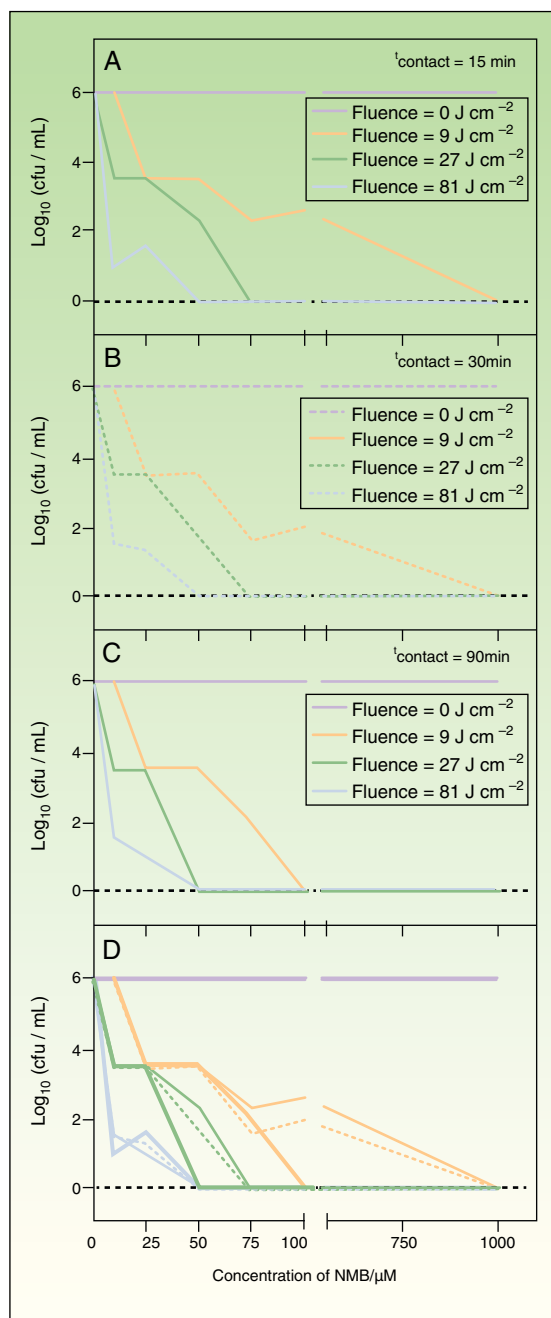
Figure 2 Logarithmic reduction of the number of colony-forming units per mL of *Trichophyton mentagrophytes* after photodynamic therapy with new methylene blue, in which the sample was irradiated at 620-645 nm.
A, contact time 10 minutes. B, contact time 30 minutes. C, contact time 90 minutes. In all panels, the red function indicates the control light; green function, fluence 9 J/cm^{-2}; pink function, fluence 27 J/cm^{-2}; blue function, fluence 81 J/cm^{-2}. D, summary of results for all contact times tested (thin line, 10 minutes; broken line, 30 minutes; thick line, 90 minutes).

the PS.[8] In the case of onychomycosis in particular, *Trichophyton rubrum* has been the most-studied dermatophyte. Various scientific publications have shown that *T. rubrum* can be treated *in vitro* with PDT to

obtain a fungicidal effect with various PSs, including toluidine blue O,[9] 5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride (Sylsens B),[10-14] deuteroporphyrin monomethylester,[10-12] 5-aminolevulinic acid,[14,15] and 1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-opqra]perylene-7,14-dione (hypericin).[16]

However, there have been few studies on the use of aPDT to treat fungal infections caused by *T. mentagrophytes*. In 2010, Jenefar et al.[17] showed that this dermatophyte can be treated with aPDT using acriflavin at a concentration of 0.2 μM, although it should be noted that the authors did not specify the dose of light used. Paz-Cristobal et al.[16] successfully inactivated *T. mentagrophytes* using hypericin at a concentration of 20-50 μM using different doses of light. It is difficult to compare our results with those of these authors because of the very different PSs and experimental conditions used. Dyes from the phenothiazinium family—including NMB—have also been used against *T. mentagrophytes*.[18]

Phenothiazinium dyes have also been found to be useful in PDT against various types of bacteria, including gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*.[19,20] NMB, in particular, has been found to be more effective than methylene blue, toluidine blue O, and dimethylmethylene blue against antibiotic-resistant strains of *Acinetobacter baumannii*.[21] However, the concentration of NMB required to produce a bactericidal effect on *Acinetobacter baumannii* (2 μM with a light dose of 30 J/cm^{-2}) is much lower than that required to inactivate CECT 2956 *T. mentagrophytes*, according to our results in this study (50 vs 100 μM at 81 and 9 J/cm^{-2}, respectively).

Larger cells are known to be less sensitive to aPDT than smaller cells. As a result, eukaryotes are more resistant to photoinactivation than bacteria because they have more targets per cell.[22] Furthermore, in PDT it is generally accepted that the type II mechanism yielding singlet oxygen ($^1O_2$) is the main pathway that causes cell damage. $^1O_2$ is a ROS that does not interconvert with other ROSs, so its main characteristic, in relation to its photodynamic effect, is the length of its lifetime before it returns to its ground state by transferring its energy. The value of this parameter in the cellular environment where $^1O_2$ can react with different biomolecules is unclear. In any case, because of its short useful lifetime, the distance $^1O_2$ can travel from the site of its generation is limited to an estimated 270 nm.[23] This is a very short distance, even at the cellular scale. A typical prokaryote is only a few micrometers long, whereas eukaryotes often reach diameters of 10-30 μm. Specifically, macroconidia of *T. mentagrophytes* that develop in a host can reach a length of 20-50 μm and a width of 6-8 μm. Consequently, the primary reactions of $^1O_2$ in a cell take place within a short distance of where the molecule is generated. Therefore, at the molecular level, the place where $^1O_2$ is generated is very important. The primary reactions of $^1O_2$ with neighboring cells produce secondary ROSs capable of spreading and causing greater oxidative damage in cells.[24]

In the study by Rodrigues et al.,[18] the treatment of *T. mentagrophytes* ATCC 9533 with NMB at a concentration of 10 μM, an incubation time of 30 minutes, and a light dose

of 20 J/cm^{-2} was sufficient to ensure that no surviving dermatophytes developed. In our study, a dose of 10 μM was not sufficient to eradicate the CECT 2956 (ATCC 28443) strain in any case. A comparison of these 2 outcomes suggests that susceptibility to aPDT is strain-dependent because in both cases the incubation time of the suspensions—which underwent similar phototreatment, seeding, and incubation on SA—was 14 days.

Finally, because this study was carried out *in vitro*, its results cannot be directly extrapolated for the treatment of fungal infections in patients. Because of experimental difficulties, we were unable to study the photodynamic effects on the hyphae or macroconidia of *T. mentagrophytes* because laboratory-grown dermatophytes rarely generate these structures. Nevertheless, our results show that NMB is a PS that should be considered in the treatment of cutaneous fungal infections caused by this dermatophyte.

Ethical Disclosures

Protection of persons and animals. The authors declare that no experiments were performed on humans or animals for the purpose of this study.

Data confidentiality. The authors declare that no private patient data appear in this article.

Right to privacy and informed consent. The authors declare that no private patient data appear in this article.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

1. Nonell N, Agut M, Semino C, Mora M, Sagristá ML, Cañete M, et al. Fundamentos físico-químicos de la terapia fotodinámica. In: Gilaberte Y, coordinator. Monografías de dermatología: terapia fotodinámica en dermatología. Madrid: Grupo Aula Médica, S.L.; 2015. pp. 3-10.
2. Rezusta A, López-Chicón P, Paz-Cristobal MP, Alemany-Ribes M, Royo-Díez D, Agut M, et al. *In vitro* fungicidal photodynamic effect of hypericin on *Candida* species. Photochem Photobiol. 2012;88:613–9.
3. Badillet G. Les dermatophytes. Atlas clinique et biologique. Paris: Varia; 1975.
4. Bayó M, Agut M, Calvo MA. Dermatofitosis humanas y caninas: ¿existen dermatofitos zoofílicos y antropofílicos? Rev Reial Academ Med Catalunya. 1994;9:111–8.
5. Pereiro Ferreiros MJJr, Garcia-Martinez FJ, Alonso-Gonzalez J. Update on the treatment of superficial mycoses. Actas Dermatosifilogr. 2012;103:778–83.
6. Wilson BC, Patterson MS. The physics of photodynamic therapy. Phys Med Biol. 1986;31:327–60.
7. Nielsen KP, Juzeniene A, Juzenas P, Stamnes K, Stamnes JJ, Moan J. Choice of optimal wavelength for PDT: The significance of oxygen depletion. Photochem Photobiol. 2005;81:1190–4.
8. Qiao J, Li R, Ding Y, Fang H. Photodynamic therapy in the treatment of superficial mycoses: An evidence-based evaluation. Mycopathologia. 2010;170:339–43.
9. Amorim JCF, Soares BM, Alves OA, Ferreira MVL, Sousa GR, Silveira LB, et al. Phototoxic action of light emitting diode in the in vitro viability of *Trichophyton rubrum*. An Bras Dermatol. 2012;87:250–5.
10. Smijs TG, Bouwstra JA, Schuitmaker HJ, Talebi M, Pavel S. A novel *ex vivo* skin model to study the susceptibility of the dermatophyte *Trichophyton rubrum* to photodynamic treatment in different growth phases. J Antimicrob Chemother. 2007;59:433–40.
11. Smijs TG, Bouwstra JA, Talebi M, Pavel S. Investigation of conditions involved in the susceptibility of the dermatophyte *Trichophyton rubrum* to photodynamic treatment. J Antimicrob Chemother. 2007;60:7550–9.
12. Smijs TG, Schuitmaker HJ. Photodynamic inactivation of the dermatophyte *Trichophyton rubrum*. Photochem Photobiol. 2003;77:556–60.
13. Smijs TG, Mulder AA, Pavel S, Onderwater JJ, Koerten HK, Bouwstra JA. Morphological changes of the dermatophyte *Trichophyton rubrum* after photodynamic treatment: A scanning electron microscopy study. Med Mycol. 2008;46:315–25.
14. Smijs TG, Pavel S. The susceptibility of dermatophytes to photodynamic treatment with special focus on *Trichophyton rubrum*. Photochem Photobiol. 2011;87:2–13.
15. Kamp H, Tietz HJ, Lutz M, Piazena H, Sowyrda P, Lademann J, et al. Antifungal effect of 5-aminolevulinic acid PDT in *Trichophyton rubrum*. Mycoses. 2005;48:101–7.
16. Paz-Cristobal P, Gilaberte Y, Alejandre C, Pardo J, Revillo MJ, Rezusta A. *In vitro* fungicidal photodynamic effect of hypericin on *Trichophyton* spp. Mycopathologia. 2014;178:221–5.
17. Jenefar S, Raynukaa D, Balaji V, Kaviyarasan V, Ebenezer P. Photodynamic control of *Trichophyton mentagrophytes* var. *mentagrophytes* by acriflavin, an acridine dye. J Biosci Res. 2010;1:52–7.
18. Rodrigues GB, Ferreira LKS, Wainwright M, Braga GUL. Susceptibilities of the dermatophytes *Trichophyton mentagrophytes* and *T. rubrum* microconidia to photodynamic antimicrobial chemotherapy with novel phenothiazinium photosensitizers and red light. J Photochem Photobiol B: Biology. 2012;116:89–94.
19. Phoenix DA, Sayed Z, Hussain S, Harris F, Wainwright M. The phototoxicity of phenothiazinium derivatives against *Escherichia coli* and *Staphylococcus aureus*. FEMS Immunol Med Microbiol. 2003;39:17–22.
20. Ragàs X, He X, Agut M, Roxo-Rosa M, Rocha Gonsalves A, Serra AC, et al. Singlet oxygen in antimicrobial photodynamic therapy: Photosensitizer-dependent production and decay in *E. coli*. Molecules. 2013;18:2712–25.
21. Ragàs X, Dai T, Tegos GP, Agut M, Nonell S, Hamblin MR. Photodynamic inactivation of *Acinetobacter baumannii* using

phenothiazinium dyes: *In vitro* and *in vivo* studies. Lasers Surg Med. 2010;42:384–90.

22. Demidova TN, Hamblin MR. Microbial photoinactivation: Effect of cell-photosensitizer binding and cell density. Antimicrob Agents Chemother. 2005;49:2329–35.

23. Moan J. On the diffusion length of singlet oxygen in cell and tissues. J Photochem Photobiol B: Biology. 1990;6:343–7.

24. Davies MJ, FU SL, Dean RT. Protein hydroperoxides can give rise to reactive free radicals. Biochem J. 1995;305:643–9.