In vitro Evaluation of Photodynamic Effects Against Biofilms of Dermatophytes Involved in Onychomycosis

Borui Chen1,2, Yi Sun3, Jinyan Zhang2, Ruijun Chen2, Xiurong Zhong4, Xiaomo Wu2, Libao Zheng2* and Jingjun Zhao1*

1 Department of Dermatology, Tongji Hospital, Tongji University School of Medicine, Shanghai, China, 2 Dermatology Hospital of Fuzhou, Fuzhou, China, 3 Department of Dermatology, Jingzhou Central Hospital, The Second Clinical Medical College, Yangtze University, Jingzhou, China, 4 Electron Microscopy Laboratory, Fujian Medical University, Fuzhou, China

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Dermatophytes are the most common cause of onychomycosis, counting for 90% fungal nail infection. Although dermatophyte pathogens are normally susceptible to antifungal agents, onychomycosis often results in refractory chronic disease, and the formation of biofilms frequently underlines the inadequate responses and resistance to standard antifungal treatment. Numerous in vitro and in vivo antimicrobial photodynamic therapy (aPDT) studies have shown biofilm eradication or substantial reduction, however, such investigation has not yet been expanded to the biofilms of dermatophytes involved in onychomycosis. To shed a light on the potential application of aPDT in the clinic management of onychomycosis, in particular with the manifestation of dermatophytoma, we investigated photodynamic effects on the viabilities and the drug susceptibilities of the biofilm of dermatophytes in vitro. Here, methylene blue at the concentration of 8, 16, and 32 µg/ml applied as photosensitizing agent and LED (635 ± 10 nm, 60 J/cm2) as light source were employed against six strains of Trichophyton rubrum, ten strains of Trichophyton mentagrophytes and three strains of Microsporum gypseum isolated from clinical specimens. Our results indicated highly efficient photodynamic inhibition, exhibiting CFU (colony forming unit) reduction up to 4.6 log10, 4.3 log10, and 4.7 log10 against the biofilms formed by T. rubrum, T. mentagrophytes, and M. gypseum, respectively. Subjected biofilms displayed considerable decreases in SMICs (sessile minimum inhibitory concentrations) to multiple antifungal agents when compared with untreated groups, indicating the biofilms of dermatophytes became more susceptible to conventional antifungal drugs after aPDT. Additionally, the obliteration of biofilm after aPDT could be observed as shattered and ruptured structures being evident in SEM (Scanning Electron Microscopy) images. These findings suggest that aPDT is an attractive alternative treatment holding great promise for combating recalcitrant onychomycosis associated with the biofilm formation.

Keywords: dermatophytes, biofilm, onychomycosis, aPDT, dermatophytoma
INTRODUCTION

Onychomycosis is the most prevalent onychopathy that comprises 50% of nail disorders worldwide (Gupta et al., 2017). Dermatophytes are the predominant pathogens, followed by non-dermatophyte molds and yeasts responsible for approximately 10% of onychomycosis. Although dermatophytic pathogens are normally susceptible to antifungal agents, it is estimated that only 25–50% of patients with onychomycosis are cured after the standard treatment (Evans and Sigurgeirsson, 1999; Sigurgeirsson et al., 2002; Gupta et al., 2004; Baran et al., 2007). The presence of biofilm is considered to be a major contributing factor to the recalcitrance of chronic dermatophytic infection refractory to conventional antifungal regimes (Burkhart et al., 2002; Warshaw et al., 2005; Nusbaum et al., 2012; Costa-Orlandi et al., 2014).

Biofilm is a sessile microbial community in which microbes are embedded in highly compacted self-produced matrix of extracellular polymeric substances (EPS), composed of polysaccharides, proteins, extracellular DNA, membrane vesicles, etc (Al-Fattani and Douglas, 2006; Martins et al., 2010; Rajendran et al., 2013). The formation of biofilm is crucial for the microbial survival, sheltering microbes from a variety of environmental assaults, such as desiccation, UV-irradiation, antibiotics, and host immune system (Ceri et al., 2001; Ramage et al., 2012; Costa-Orlandi et al., 2014). Comparing to free-floating planktonic cells of the same species, biofilm pathogens can tolerate as much as 1000-fold higher levels of antimicrobial agents (Hawser and Douglas, 1995; Donlan and Costerton, 2002; Marsh, 2004) and microbial biofilms thereby account for more than 60% of all fungal and bacterial infections in humans (Cieplik et al., 2018).

Antimicrobial photodynamic therapy (aPDT) had been recently proposed to combat biofilms clinically (Lyon et al., 2011; Cieplik et al., 2014; Baltazar et al., 2015). As a non-antibiotic approach, aPDT employs non-toxic photosensitizers (PSs) and visible light at specific wavelength to generate reactive species of oxygen (ROS) and nitrogen (RNS), which are capable of killing microbes (Hamblin and Hasan, 2004; Alves et al., 2014; Taraszkiewicz et al., 2015). Notably, cytotoxic radicals produced by aPDT have extremely short half-lives and react only in their sites of formation, which reduces the cytotoxicity to adjacent normal tissues (Dai et al., 2009; Baltazar et al., 2015). Numerous in vitro as well as some in vivo aPDT studies have demonstrated aPDT has a broad-spectrum of activity against the biofilms and susceptible fungal pathogens and bacterial species include yeast (Candida spp.), non-dermatophyte molds ( Fusarium spp., Exophiala spp.), G+ bacteria (S. aureus, E. faecalis, and Streptococcus spp.) and G- bacteria (P. aeruginosa and Aggregatibacter. actinomycetemcomitans) (Gilaberte et al., 2011; Junqueira et al., 2012; Seth et al., 2013; Beirao et al., 2014; Mannucci et al., 2014; Orlandi et al., 2014; Al-Ahmad et al., 2016; Gao et al., 2016; Carvalho et al., 2018).

However, the application of aPDT to biofilms formed by dermatophytes is less studied (Ali et al., 2016; Toukabi et al., 2018) and treatment with high efficacy remains challenging in clinic (Burkhart et al., 2002; Arrese and Pierard, 2003; Sigurgeirsson, 2010). Moreover, there are increasing rates of antimicrobial resistance among dermatophytes, especially for Trichophyton rubrum, the most frequent etiologic agent for onychomycosis (Baltazar et al., 2015). In an attempt to gain insight into the potential clinical implementation of aPDT tackling the dermatophytic biofilms implicated in onychomycosis, we investigated photodynamic effects on the viabilities, and the drug susceptibilities of the biofilm of dermatophytes, ranging from T. rubrum, T. mentagrophytes to M. gypseum.

MATERIALS AND METHODS

Fungal Strains

Six strains of T. rubrum (Nos. 16463, 16355, 41452, 41467, 16618, and 41453), ten strains of T. mentagrophytes (Nos. 7240, 5614, 16446, 16339, 16494, 16077, MYA-4439, 8395, 8396, and 8397), and three strains of M. gypseum (Nos. 13789, 8305, and 8397), and three strains of M. gypseum (Nos. 13789, 8305, and 8825) were supplied by the research center of medical mycology of Peking University (RCCMPU). All analyzed clinical isolates of dermatophytes, ranging from T. rubrum, T. mentagrophytes to M. gypseum, were included as control strains.

FIGURE 1 | SEM images of the biofilms of T. mentagrophytes. SEM provided three-dimensional images for biofilm structural assessment, with low magnification of x100 displayed in (A) and high magnifications of x500 and x5000 displayed in (B) and (C), respectively. Two types of peculiar ECM architecture can be observed: (I) an extremely thin “blanket-like” layer covering the areas between hyphae (B); (II) very fine “mesh-like” layer wrapping the filaments of hyphae (C).
Antifungal Agents
All antifungal drugs including terbinafine (TRB; purity $\geq 98\%$, SIGMA), itraconazole (ITC; purity $\geq 99\%$, SIGMA), cyclopirox (CLO; purity $\geq 99\%$, European Pharmacopoeia Reference Standard), and fluconazole (FLU; purity $\geq 98\%$, SIGMA) were purchased in powder form from Sigma Chemical Co., St. Louis, MO and prepared as outlined in the clinical and laboratory standards institute (CLSI) broth microdilution method M38-A2. The working concentration ranges of drugs were $0.0009785 \sim 0.5 \mu g/ml$ for TRB, $0.03125 \sim 16 \mu g/ml$ for ITC and CLO and $0.125 \sim 64 \mu g/ml$ for FLU.

Biofilm Preparation in 96-Well Microtiter Plates
Biofilm formation assay was performed in 96-well microtiter plates based on the method described by Costa-Orlandi et al. (2014) and further verified by Brilhante and Toukabri (Brilhante et al., 2017; Toukabri et al., 2018). The strains were grown on oatmeal agar (BD company) and incubated at $28^\circ C$ for 14 days until sporulation. The inoculum was prepared by covering the cultures with $0.01M$ PBS (PH 7.2) adjusting to a final concentration of $1 \times 10^6$ CFU/ml. Then, 100 µl of inoculum were added to 96-well plates (Corning 3599). The plates were incubated without agitation at $37^\circ C$ for 3 h for biofilm pre-adhesion. Then, the supernatant was gently removed from the wells and the cells were washed three times with $0.01M$ PBS (PH 7.2) for removing non-adherent cells. Following that, 100 µl of RPMI 1640 medium were added and the plates were incubated at $37^\circ C$ for 72 h. The media were then carefully extracted without disturbing the biofilm. The 96-well plate was washed with sterile PBS for three times to remove detached spores.

Scanning Electron Microscopy
For SEM analysis, the preparation of biofilms was conducted on Thermanox coverslips (Thermo Fisher Scientific) instead of microtiter plates (Costa-Orlandi et al., 2014; de Aguiar Cordeiro et al., 2015). After 72 h incubation, the biofilms were fixed with 500 µl of 2.5% glutaraldehyde at $4^\circ C$ overnight. Then biofilms were washed with cacodylate buffer twice, followed by 10 min dehydration with ethanol at each ascending concentrations (50, 70, 80, 95, and 100% ethanol) and drying for 30 min at $28^\circ C$. After drying, samples were dried in CO$_2$, coated with gold and observed in a FEI Quanta 250 scanning electron microscope (FEI, Netherlands).

Colony Forming Unit Counting
Colony forming unit is a cellular viability metric, measured by raw counts of clones growing in a standard sized Petri dish. Briefly, 200 µl of sterile water was added into each well of 96-well plate after the biofilm formation, followed by vigorous washing to thoroughly suspend the biofilm cells. The suspensions were then diluted 100 times by taking 2 µl of suspension diluted into 198 µl sterile water, after which half of the diluted suspension was used for inoculating a SDA plate for colony counting.

XTT Reduction Colorimetric Assay
A semiquantitative measure of biofilm formation was calculated by using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay, adapted from previous reports. XTT was prepared in a saturated solution at
TABLE 1 | Effects of photodynamic inhibition on the biofilms of dermatophytes.

| Strain   | Mock (CFU/ml) | 16 μg/ml M (CFU/ml) | Light (CFU/ml) | Light (CFU/ml) | 32 μg/ml MB with light (CFU/ml) | 32 μg/ml MB with light (CFU/ml) | 16 μg/ml MB with light (CFU/ml) | 16 μg/ml MB with light (CFU/ml) | 8 μg/ml MB with light (CFU/ml) |
|----------|---------------|---------------------|----------------|----------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------|
| T. rubrum | RCMMPU-16483  | 1.11 × 10^6         | 7.67 × 10^5    | 8.42 × 10^5    | 5 × 10^3                     | 5 × 10^3                     | 2.02 × 10^5                   |                               |
|          | RCMMPU-16355  | 9.75 × 10^5         | 5.82 × 10^5    | 7.7 × 10^5     | 6.67 × 10^4                  | 5.5 × 10^4                  | 2.32 × 10^5                   |                               |
|          | RCMMPU-41452  | 1.24 × 10^6         | 1.64 × 10^6    | 9.92 × 10^5    | 0                            | 1.67 × 10^3                 | 3.42 × 10^5                   |                               |
|          | RCMMPU-41467  | 1.3 × 10^6          | 1.33 × 10^6    | 1.18 × 10^6    | 2.83 × 10^5                  | 5.88 × 10^5                 | 8 × 10^5                      |                               |
|          | RCMMPU-16618  | 4.5 × 10^4          | 2.33 × 10^4    | 2.45 × 10^5    | 0                            | 0                           | 1.38 × 10^5                   |                               |
|          | RCMMPU-41453  | 9.35 × 10^5         | 3.25 × 10^5    | 4.33 × 10^5    | 0                            | 0                           | 6.67 × 10^3                   |                               |
| T. mentagrophytes | RCMMPU-7240 | 1.47 × 10^6         | 1.87 × 10^6    | 1.32 × 10^6    | 1.43 × 10^5                  | 2.92 × 10^5                  | 4.43 × 10^5                   |                               |
|          | RCMMPU-5614   | 1.96 × 10^6         | 1.81 × 10^6    | 1.48 × 10^6    | 8.17 × 10^4                  | 1.15 × 10^5                  | 3.1 × 10^5                    |                               |
|          | RCMMPU-16446  | 1.21 × 10^6         | 1.13 × 10^6    | 8.52 × 10^5    | 4.17 × 10^4                  | 3.93 × 10^5                  | 5.53 × 10^5                   |                               |
|          | RCMMPU-16339  | 1.1 × 10^6          | 9.42 × 10^5    | 7.78 × 10^5    | 4 × 10^4                     | 1.5 × 10^5                   | 1.55 × 10^5                   |                               |
|          | RCMMPU-16494  | 3.1 × 10^5          | 6.07 × 10^5    | 3.77 × 10^5    | 0                           | 0                           | 0                            |                               |
|          | RCMMPU-16077  | 7.1 × 10^5          | 5.53 × 10^5    | 7.85 × 10^5    | 0                           | 0                           | 0                            |                               |
| M. gypseum | MYA-4439     | 1.6 × 10^6          | 1.64 × 10^6    | 1.09 × 10^6    | 3.83 × 10^4                  | 3.87 × 10^5                  | 4.35 × 10^5                   |                               |
|          | RCMMPU-8395   | 6.95 × 10^5         | 6.33 × 10^5    | 8.52 × 10^5    | 0                           | 0                           | 1 × 10^5                     |                               |
|          | RCMMPU-8396   | 3.08 × 10^5         | 2.15 × 10^5    | 6.07 × 10^5    | 0                           | 0                           | 0                            |                               |
|          | RCMMPU-8397   | 3.62 × 10^5         | 2.12 × 10^5    | 3.3 × 10^5     | 0                           | 0                           | 2.87 × 10^5                   |                               |
|          | RCMMPU-13789  | 4.67 × 10^4         | 1.67 × 10^3    | 5.5 × 10^4     | 0                           | 0                           | 0                            |                               |
|          | RCMMPU-8305   | 2 × 10^4            | 7.67 × 10^4    | 3.5 × 10^4     | 6.67 × 10^3                  | 8.33 × 10^3                 | 0                            |                               |
|          | RCMMPU-8825   | 2.15 × 10^5         | 1.88 × 10^5    | 1.2 × 10^5     | 0                           | 0                           | 0                            |                               |

Data are mean values from three replicate experiments.

0.5 g/liter in Ringer’s lactate. The solution was filter sterilized through a 0.22-µm-pore-size filter, aliquoted, and stored at −70°C. Prior to each assay, an aliquot of stock XTT was thawed, and menadione (Sigma; 10 mM prepared in acetone) was added to a final concentration of 1 µM. A 100 µl aliquot of the XTT-menadione solution was then added to each prewashed biofilm and to control wells (for the measurement of background XTT-reduction levels). The plates were then incubated in the dark for up to 2 h at 37°C. The activity of the fungal mitochondrial dehydrogenase reduces the tetrazolium salt XTT to formazan salts, resulting in a colorimetric change that correlates with cell viability. The colorimetric change was measured using an ELISA reader (Microplate Reader iMarkTM; BIO-RAD) at 490 nm. In all experiments, RPMI 1640 medium free of biofilm formation was included as a negative control (Mowat et al., 2007; Pitangui et al., 2012).

Photodynamic Treatment
The PDI technique with modifications in the volume used, the incubation time, and the concentrations of methylene blue was described by Lyon et al. (2013). The methylene blue was tested at concentrations of 32 µg/ml (T1), 16 µg/ml (T2) and 8 µg/ml (T3), with 100 µl of each concentration added into 96-well plates containing biofilms. After incubation in dark for 3 h at 37°C, the biofilms were irradiated using a LED with an irradiance of 100 mW/cm² at a wavelength of 635 ± 10 nm and a distance of 1 cm for 600 s (60 J/cm²). Control conditions were conducted as biofilms in PBS without irradiation (C1), biofilms with methylene blue (16 µg/ml) and without irradiation (C2), biofilms in PBS, and irradiated (C3).

SMICs Determination
The values of SMICs were experimentally determined in this study using XTT-reduction colorimetric assay. The working concentrations of TRB, ITC, CLO, and FLU were prepared by a series of twofold dilutions (dilution range, 0.5–0.0009785 µg/ml for TRB; 16 to 0.03125 µg/ml for ITC and CLO; 64 to 0.125 µg/ml for FLU). The SMIC80s of TRB, ITC, CLO, and FLU were defined as the concentration at which 80% decrease in optical density would be detected in comparison to the mock controls in the absence of antimicrobial agents (Pierce et al., 2008). All tests were performed in triplicate.

Statistical Analysis
Data were presented as the mean ± SD and analyzed with PRISM software package version 7.0 (XSTAT Addinsoft, Paris, France). Three independent experiments were performed for all measurements. The differences between two groups were analyzed with Student's t-test. Two-way ANOVA analysis was used to determine statistical differences among multiple groups. p < 0.05 was considered as statistical significance.

RESULTS
Biofilm Morphology
Biofilm formation using the strain of T. mentagrophytes 7240 isolated from clinical specimen was prepared on coverslip according to the method described by Costa-Orlandi et al. (2014). SEM providing three-dimensional images for in-depth structural assessment revealed that T. mentagrophytes 7240...
produced noticeably robust biofilms with branched hyphae forming a mycelial network (Figure 1A), firmly attached to the coverslips. In particular, two types of peculiar extracellular matrix (ECM) architecture could be observed: (i) an extremely thin “blanket-like” layer covering the areas between hyphae (Figure 1B); (ii) very fine “mesh-like” layer wrapping the filaments of hyphae (Figure 1C). The biofilm morphology of high resolution and magnification was investigated and imaged by SEM technique to confirm the biofilm-formation in this study and the SEM images obtained are similar to those reported previously (Brilhante et al., 2017; Vila et al., 2017; Guzel Tunccan et al., 2018).

aPDT Reducing the Viability of the Biofilms of Dermatophytes

The aPDT with LED (InGaAlP, 100 mW/cm²) exhibited CFU reduction by 2.0 log₁₀, 4.3 log₁₀, and 4.6 log₁₀ against the biofilms formed by *T. rubrum* at the concentrations of MB 8, 16, and 32 µg/ml, respectively (Figure 2 and Table 1), demonstrating photodynamic inactivation in MB concentration dependent manner. The biofilms of *T. mentagrophytes* displayed the same pattern as to that of *T. rubrum*, with the CFU reductions at 3.3 log₁₀, 4.0 log₁₀, and 4.3 log₁₀, accordingly. Interestingly, in contrast to *T. rubrum* and *T. mentagrophytes*, the CFU reduction of *M. gypseum* was more efficient at the concentration of MB 8 µg/ml (4.7 log₁₀) than that of 32 µg/ml or 16 µg/ml (4.26 log₁₀ and 4.25 log₁₀, respectively). Such observation was probably due to generally high susceptibility of *M. gypseum* to aPDT and fewer strains have been tested in this study. Subsequently, no significant differences in CFU reductions were observed at the chosen concentrations of MB for *M. gypseum*. However, the efficiencies of aPDT against the biofilms of dermatophytes were lower than that of the biofilms formed by *Fusarium* spp., previously reported as 5.6 log₁₀ in reduction with the same aPDT regimen (Gao et al., 2016), suggesting the biofilms of dermatophytes may be relatively more resistant to aPDT than other fungal pathogens associated biofilms. Nevertheless, with the CFU reductions ranging from 2 log₁₀ to 4 log₁₀, aPDT proved to be a highly effective approach against the biofilms of dermatophytes in *vitro*.

aPDT Increasing the Susceptibilities of Biofilms to Conventional Antimicrobial Agents

SMIC ranges of terbinafine (TRB), itraconazole (ITC), cyclopixiro (CLO), and fluconazole (FLU) against biofilms with or without aPDT (T1 regimen) were summarized in Figure 3 and Table 2. The susceptibilities of *T. rubrum*, *T. mentagrophytes*, and *M. gypseum* biofilms to these antifungal agents were variable, but TRB was consistently more efficient against fungal growth in comparison to ITC, CLO, and FLU in all three species tested. The biofilms that were subjected to aPDT exhibited significant reductions in SMIC80 when compared with aPDT untreated groups, indicating that the treatment of aPDT effectively increased the susceptibilities of *T. rubrum*, *T. mentagrophytes* and *M. gypseum* to these conventional antimicrobial drugs. Furthermore, aPDT exerted comparable effects on increasing the susceptibility of *T. rubrum*, *T. mentagrophytes* to TRB, ITC, CLO, and FLU as shown in the Figure 3D. In contrast, the susceptibility of *M. gypseum* to TRB after aPDT was less affected, where the SMIC80 of TRB without aPDT was just four times higher than that with aPDT, much lower than 64-fold increase observed with ITC and FLU.

Obliteration of Biofilm Resulted From Photodynamic Therapy

To investigate the morphological alteration of dermatophyte biofilm after aPDT (T1 regimen), SEM images of *M. gypseum* biofilm were obtained following the photodynamic treatment. As shown in the Figure 4, comparing to aPDT untreated specimen (Figure 4A) in which dense entangled hyphae exhibited regular morphology with uniform diameter and smooth surfaces as well as the characteristic “blanket-like” membrane and fine “mesh-like” wrapping layer were present, the mycelia of *M. gypseum* biofilm after aPDT treatment were fractured and shattered with raptured hyphae and fragmented “blanket-like” membranes (Figures 4B–D).

DISCUSSION

Onychomycosis is the most common nail infective disorder (Piraccini and Alessandri, 2015) and is caused primarily by anthropophilic dermatophytes, in particular by *Trichophyton rubrum*, followed by *Trichophyton mentagrophytes* var.
The methylene blue was tested at concentrations of 32 µg/ml as the regimen of T1. The biofilms were irradiated using a LED with an irradiance of 100 mW/cm² at a wavelength of 635 ± 10 nm and a distance of 1 cm for 600 s (60 J/cm²). Data are mean values from three replicate experiments.

### TABLE 2 | Photodynamic effects on the SMIC80 of dermatophytic biofilms.

| Strain            | SMIC80 (µg/ml) | TRB | ITC | CLO | FLU |
|-------------------|----------------|-----|-----|-----|-----|
|                   | −PDT           | +PDT| −PDT| +PDT| −PDT| +PDT|
| T. rubrum         | ROMMPU-16463   | 0.146| 0.022| 3.000| 0.042| 16.000| 6.667| 64.000| 0.708 |
|                   | ROMMPU-16355   | 0.500| 0.011| 6.333| 0.729| 1.000| 0.031| 1.417 | 0.125 |
|                   | ROMMPU-41452   | 0.012| 0.006| 1.833| 4.000| 13.333| 1.333| 48.000| 3.333 |
|                   | ROMMPU-41467   | 0.500| 0.336| 13.333| 0.042| 1.333| 0.031| 53.333| 0.125 |
|                   | ROMMPU-16618   | 0.500| 0.001| 9.333| 2.010| 12.000| 1.677| 22.750| 0.250 |
|                   | ROMMPU-41453   | 0.029| 0.001| 5.510| 5.427| 16.000| 2.000| 42.833| 0.375 |
| T. mentagrophytes | ROMMPU-7240    | 0.375| 0.001| 16.000| 16.000| 1.000| 0.031| 64.000| 13.500 |
|                   | ROMMPU-5614    | 0.500| 0.001| 16.000| 5.750| 1.000| 0.031| 25.333| 0.125 |
|                   | ROMMPU-16446   | 0.417| 0.001| 6.167| 0.031| 0.667| 0.031| 10.833| 0.125 |
|                   | ROMMPU-16339   | 0.500| 0.002| 6.833| 0.385| 16.000| 0.031| 3.667 | 0.125 |
|                   | ROMMPU-16494   | 0.500| 0.500| 1.396| 5.354| 1.042| 0.031| 32.000| 21.833 |
|                   | ROMMPU-16077   | 0.500| 0.500| 6.167| 5.354| 8.667| 0.031| 48.000| 42.708 |
|                   | MYA-4439       | 0.500| 0.011| 11.000| 0.031| 1.750| 0.031| 43.333| 0.458 |
|                   | ROMMPU-8395    | 0.073| 0.001| 16.000| 0.031| 5.333| 2.667| 16.000| 0.125 |
|                   | ROMMPU-8396    | 0.027| 0.001| 0.229| 0.031| 2.167| 0.042| 64.000| 0.125 |
|                   | ROMMPU-8397    | 0.014| 0.001| 0.333| 0.031| 1.667| 1.344| 32.000| 24.333 |
| M. gypseum        | ROMMPU-13789   | 0.172| 0.084| 16.000| 0.698| 6.708| 0.031| 42.667| 24.333 |
|                   | ROMMPU-8305    | 0.013| 0.001| 10.833| 0.031| 0.875| 0.062| 32.000| 0.167 |
|                   | ROMMPU-8825    | 0.005| 0.001| 5.542| 0.063| 8.167| 1.021| 64.000| 0.125 |

The methylene blue was tested at concentrations of 32 µg/ml as the regimen of T1. The biofilms were irradiated using a LED with an irradiance of 100 mW/cm² at a wavelength of 635 ± 10 nm and a distance of 1 cm for 600 s (60 J/cm²). Data are mean values from three replicate experiments.

**FIGURE 4** | SEM images of the biofilms of M. gypseum after photodynamic treatment. (A) M. gypseum not subjected to photodynamic treatment. (B–D) M. gypseum subjected to photodynamic treatment. (I) Formation of a relatively complete membrane-like structure. (II) Biofilms appeared to have a "hole" in their surface, with a tearing appearance. (III) Perforated ECM surrounded the macroconidia. (IV) Mycelia were fractured, sections of hyphae were broken.
interdigitale (Faergemann and Baran, 2003). The non-dermatophyte molds, such as Fusarium spp., Acremonium spp., Alternaria spp., Scopulariopsis brevicaulis, Aspergillus spp., can also be involved in the pathogenesis with estimated 10% prevalence worldwide (Gupta and Nakrieo, 2014; Gasser et al., 2016; Gupta et al., 2016; Motamedi et al., 2016). Yeasts, like Candida albicans and Candida parapsilosis, represent the third cause of nail fungal infection, but only occurring when predisposing factors are present, mainly immunosuppression, and diabetes (Arrua et al., 2015; Gasser et al., 2016).

Developing novel therapeutic approach against the biofilms of dermatophytes implicated in recalcitrant onychomycosis presents a pressing need in the clinical management, especially the onychopathic condition with dermatophytoma (Sigurgeirsson, 2010). Initially established as a successful modality for malignancies and age-related macular degeneration (Dougherty et al., 1978; Orenstein et al., 1996), photodynamic inactivation has been shown as an effective alternative strategy for combating biofilms. The antimicrobial effects of aPDT have been observed on bacterial, non-dermatophytic, and yeast biofilms in vitro as well as in vivo using various animal models (Friedberg et al., 2001; Giroldo et al., 2009; Lyon et al., 2011; Soares et al., 2011; Takahashi et al., 2014; da Silva et al., 2018). However, the aPDT effect on dermatophytic biofilms has been less investigated due to the lack of reliable models. Fortunately, Costa-Orlandi et al. have recently successfully established the procedure to in vitro form the biofilms of dermatophytes using the stains of T. rubrum ATCC 28189 and T. mentagrophytes ATCC 11481 within 72 h, providing a valuable in vitro model to facilitate the investigation of photodynamic effect on dermatophytic biofilms (Costa-Orlandi et al., 2014). In addition to ATCC strains, the dermatophytic isolates obtained from clinical onychomycosis specimens ranging from T. rubrum, T. mentagrophytes to M. gypseum were examined and selected for their capability of biofilm-forming in vitro in our study. Due to the generally low sporulation of T. rubrum, only 6 out of 70 initial clinical isolates were identified capable of forming biofilm after 72 h of cultivation. Ultimately, six strains of T. rubrum, ten strains of T. mentagrophytes and three strains of M. gypseum capable of biofilm formation were subsequently subjected to aPDT based assays, enabling us to gain an in-depth insight into the application of aPDT against dermatophytic biofilms implicated in clinical onychopathic infections.

Photodynamic inactivation of aPDT requires the application of photosensitizer (PS) and subsequent irradiation with visible light corresponding to the specific absorption wavelength of photosensitizer (Castano et al., 2005; Plaetzer et al., 2009). A variety of PSs have been previously used in antifungal photodynamic inactivation, including MB, toluidine blue, 5-aminolevulinic acid, and so on (Cormick et al., 2009; Calzavara-Pinton et al., 2012; Dai et al., 2012). The photosensitizer MB used in our investigation has an absorption wavelength over 600 nm, which has been shown capable of exerting substantial reduction on the biofilms of dermatophytes tested. However, T. rubrum appeared more resistant than T. mentagrophytes and M. gypseum and the MB concentration dependence was more evident in T. rubrum than that of T. mentagrophytes and M. gypseum, presumably due to T. rubrum being equipped with abundant red pigments that may interfere the absorption of MB's chromophore.

The effect of aPDT on the susceptibility of dermatophytic biofilms to clinically applied antifungal agents was examined and the biofilms subjected to aPDT exhibited significant reductions in SMIC80, meaning aPDT effectively increased the susceptibilities of T. rubrum, T. mentagrophytes and M. gypseum to these conventional antimicrobial drugs, including terbinafine, itraconazole, cyclopinox, and fluconazole. The mechanism underlying aPDT-induced disruption rendering biofilms more susceptible could be multi-factorial, involving photodynamic action targeting multiple cellular components, such as fractionating plasma membrane, triggering ion imbalance leading to intolerable changes in osmotic pressure and pH, and DNA breaking. The observed sensitization of dermatophytes to the antifungal agents resulted from the decreased biofilm viability in this study, however, in the future, more investigation with sub-lethal dose of aPDT could be conducted to have a better understanding of how different levels of oxidative and nitrosative stresses affecting the susceptibility of dermatophytic biofilms to antimicrobials and to facilitate the optimization of combination therapies.

In conclusion, our results suggest that photodynamic approaches hold great promise for combating the biofilm of dermatophytes involved in onychomycosis. In vitro photodynamic treatment with methylene blue and LED was found to be highly efficient in inactivating dermatophytic biofilms of T. rubrum, T. mentagrophytes, and M. gypseum. When aPDT applied alongside antifungal agents, it has the potential to reduce drug dosages, drug toxicity, and treatment times. Further investigation is needed to address if such efficacy could be ultimately obtained in vivo and it is important to optimize treatment protocols to cope with constrained drug permeation and light attenuation through nail plates in human studies.

**AUTHOR CONTRIBUTIONS**

BC, YS, and JGZ conceived and designed the study. BC, JYZ, RC, and XZ performed all the experiments. BC and XW analyzed the data and wrote the manuscript. JGZ, LZ, and XW provided the general guidance and revised the manuscript.

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