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Improving treatment of chromoblastomycosis: the potential of COP1T-HA and antimicrobial photodynamic therapy against *Fonsecaea monophora in vitro*

Xinyao Liu[a,b*], Zhi Zhang[a,c,d*], Jitong Sun[a,c,d], Renjie Fang[e], Xin Ran[a,c,d], Yuzhou Liu[e,f] and Yuping Ran[a,c,d]

[a]Department of Dermatovenereology, West China Hospital, Sichuan University, Chengdu, China; [b]Center for Pathogen Research, West China Hospital, Sichuan University, Chengdu, China; [c]Laboratory of Dermatology, Clinical Institute of Inflammation and Immunology (CIII), Frontiers Science Center for Disease-related Molecular Network, West China Hospital, Sichuan University, Chengdu, China; [d]Academician Workstation of Wanqing Liao, West China Hospital, Sichuan University, Chengdu, China; [e]ShenYunZhiHe Company, Qidian BLDG, Beijing, China; [f]School of Chemistry, Beihang University, Beijing, China

ABSTRACT

Fonsecaea monophora is one of the common pathogenic species of Chromoblastomycosis (CBM). Antimicrobial photodynamic therapy (aPDT) shows promise as a new treatment for CBM. In this study, we evaluated the effectiveness of COP1T-HA which is a porous organic cage and aPDT against *Fonsecaea* spp. *in vitro*.

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1. Introduction

Chromoblastomycosis (CBM) is a chronic granulomatous skin disease caused by various dematiaceous fungi invading the skin and subcutaneous tissues. Diagnosis primarily depends on identifying the presence of brownish-black, thick-walled spores known as sclerotic bodies within the infected tissue (Queiróz-Telles et al. 2009). The genus *Fonsecaea*, including *Fonsecaea pedrosoi*, *F. monophora*, *F. nubica*, and *F. pugnacius*, are the most common pathogenic fungi causing CBM (Zhong et al. 2024). CBM primarily occurs in tropical and subtropical regions, with southern China also being an endemic area for this disease. *Fonsecaea monophora* is the main pathogenic fungus causing chromoblastomycosis in southern China. It was first identified as a new species in 2004, differentiated from *F. pedrosoi*. Studies have shown that melanin is an important virulence factor of *F. monophora*, which helps the fungus evade the oxidative response of phagocytic cells, thereby facilitating persistent infection (Li et al. 2022). Current treatment options include systemic antifungal drugs, localised cryotherapy, heating therapy, surgical excision, CO_2 laser therapy, photodynamic therapy, immunotherapy, etc. However, it is still challenging to achieve a complete cure, and highly prone to recurrence. For instance, triazole antifungals, commonly used for fungal infections, require long-term administration that may lead to drug resistance (Deng et al. 2015) and adverse reactions such as potential hepatotoxicity. In addition, surgical excision may not be viable for extensive or localised lesions, and immunotherapy remains experimental and costly. Therefore, CBM remains a significant challenge in clinical practice.

Antimicrobial photodynamic therapy (aPDT) utilises non-toxic photosensitisers (PS) and an appropriate light source to induce the generation of reactive oxygen species (ROS), effectively eliminating microbial pathogens (Cieplik et al. 2018). It has been widely used to treat superficial and subcutaneous mycosis (Wu et al. 2022). Indeed, PDT can be considered as a compelling alternative treatment for CBM. However, challenges related to water solubility and aggregation pose significant obstacles in the application of photosensitisers (PSs)-based aPDT. Recently, we have successfully developed a porous organic cage photosensitiser – COP1T-HA (Liu et al. 2022a). This

CONTACT Yuping Ran ranyuping@vip.sina.com Department of Dermatovenereology, West China Hospital, Sichuan University, Chengdu, China; Yuzhou Liu liuyuzhou@deepchem.cn School of Chemistry, Beihang University, Beijing, China

*Xinyao Liu and Zhi Zhang make the equal contribution to this work.

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remarkable PS not only addresses the above challenges but also significantly enhances the fungicidal efficiency of conventional photosensitisers against multidrug-resistant *Candida* spp., while broadening its absorption spectrum.

In this study, we identified a clinical isolate as *F. monophora* (HXFM001), using ITS sequencing and phylogenetic analysis (Figure S1, Table S1, S2), then evaluated the *in vitro* efficacy of COP1T-HA against HXFM001 through the agar plate dilution method (also including two additional clinical strains, one is *F. monophora* and the other is *F. nubica*), colony growth and slide culture observation.

2. Case study

2.1. Strains and cultures

The strains used in this study were isolated from the skin lesion exudates of three clinical CBM patients. Sabouraud dextrose agar (Difco, Detroit, USA) was used in this study to culture these strains at 30 °C for 14 d. After being identified and cultured, the strains were stored at −80 °C in our lab.

2.2. Morphological observation

After culturing the skin lesion specimen on SDA plate at 30 °C for 14 d, the colony exhibited a dark brown to black colour and velvety appearance, with noticeably slow growth (Figure 1(a,b)). Under magnification with a dermoscope, the colony morphology became more clearly visible (Figure 1(e)). Several round, thick-walled, brownish, septate muriform cells were observed in PAS (Figure 1(c)) and GMS (Figure 1(d)) staining in the biopsy tissue from a female patient. From SEM observation, the strain has the characteristic beaked-brachysporium type conidiophore, with oval-shaped conidia that are arranged in chains of 2 to 3 at the tip of the conidiophore (Figure 1(f)). Under high magnification, conidia can be observed with loose branching patterns and prominent tooth-like projections (Figure 1(g,h)). During its natural growth cycle, we observed the conidiophore approaching an upright position, with the apex gradually developing dense branching (Figure S2).

2.3. Preparation of COP1T-HA aPDT

For the PDT, spores were harvested and suspended in phosphate buffer solution (PBS). The suspension was sequentially filtered using 4 layers of sterile gauze to eliminate the mycelium. The spores were diluted to 1×10^6 CFU/mL in PBS and then mixed with COP1T-HA [It was prepared as previously reported (Liu et al. 2022a)].

2.4. The time-dependent antifungal activity of COP1T-HA aPDT

To determine the antifungal activity of COP1T-HA aPDT, the agar plate dilution method was employed for the strain HXFM001. Specifically, each solution

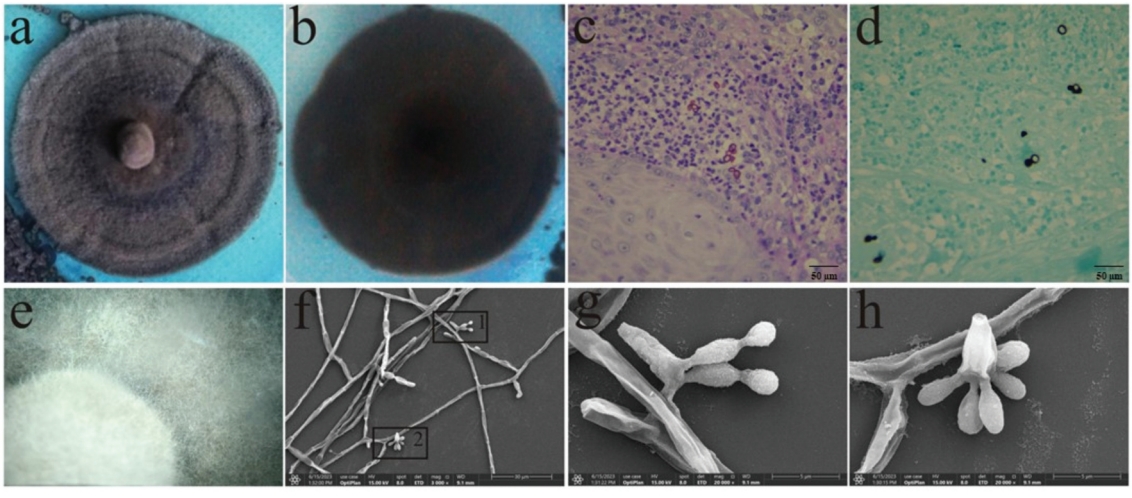

Figure 1. Morphological observation. (a) The front side of colony. (b) The reverse side of colony. (c) PAS staining. (d) GMS staining of patient's tissue. (e) Dermoscopic observation. (f) SEM observation of the conidiophore under low magnification. (g, h) SEM observation of the conidia under high magnification.

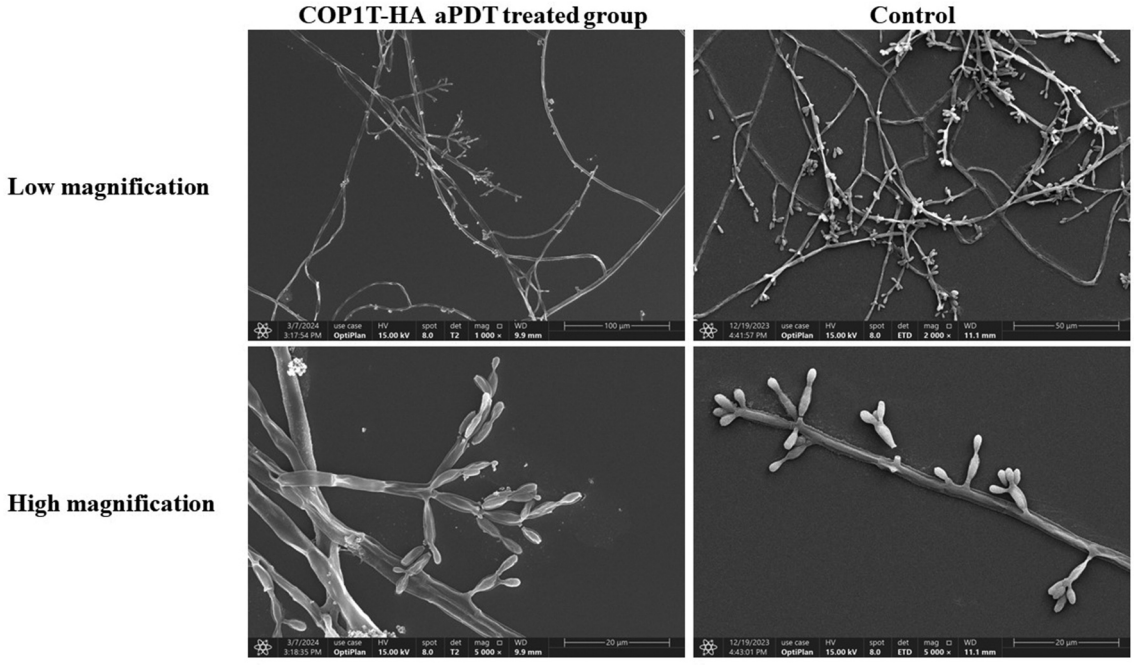

Figure 2. *In vitro* antifungal activities of COP1T-HA towards the slide culture of *Fonsecaea monophora* observed by SEM. COP1T-HA aPDT causes mycelial depression and shrinkage, as well as shedding of conidia of *F. monophora*. The SEM observation of *F. monophora* growth with (left side) or without (right side) COP1T-HA aPDT treatment. The concentration of COP1T-HA is 100 μg/mL.

(200 μL) of the fungal diluent that contained COP1T-HA (100 μg/mL) was added to a 96-well plate ($n = 3$). Next, the plate was incubated at 30 °C under a 470 nm laser (MDL-III-470 nm, 100 mW/cm^2) for different time (0 min, 5 min, 10 min, 15 min, 20 min). Afterwards, the cultures were kept in the dark and incubated at 30 °C for 3 h. Subsequently, approximately 10^4 CFU/mL fungal suspension was spread onto YPD agar plate after aPDT treatment. The agar plates were cultured at 30 °C in the dark after spreading. All experiments were independently repeated in triplicate. Our previous study has already proven that the COP1T lacks antimicrobial activity. Therefore, we did not repeat it here.

The results in Figure S3 showed the effect of different treatment durations of COP1T-HA (100 μg/mL) on the growth of *F. monophora*, indicating that fungal cells were inhibited in a time-dependent manner in response to COP1T-HA under 470 nm laser exposure (100 mW/cm^2). It can significantly inhibit fungal growth in only 5 min of COP1T-HA aPDT treatment. On the contrary, the fungal viability with or without COP1T-HA treatment remained similar in the dark, suggesting the nontoxicity of COP1T-HA under dark conditions.

2.5. The concentration-dependent antifungal activity of COP1T-HA aPDT

We further utilised the comparable method to explore the correlation between the antifungal activity of COP1T-HA and its concentration. Each solution (200 μL) comprised the fungal diluent (100 μL) and COP1T-HA (100 μL), with the final concentrations adjusted sequentially to 400 μg/mL, 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL, and 12.5 μg/mL. Both components were added to a 96-well plate. Then the plate was incubated at 30 °C under the 470 nm laser (MDL-III-470 nm, 100 mW/cm^2) for 5 min. The subsequent steps were identical to the previous ones. Three distinct strains of *Fonsecaea* spp. were tested in this experiment. We found that at the concentration of 100 μg/mL, with an exposure time of 5 min, COP1T-HA could effectively inhibit the growth of nearly all three *Fonsecaea* spp. (Figure S4).

2.6. Morphological changes of F. monophora before and after COP1T-HA aPDT

Taking the strain of *F. monophora* (HXFM001) as an example, we observed the ability of COP1T-HA aPDT on colony growth of *F. monophora*. Initially, fresh

mycelia of the *F. monophora* were cultured on SDA plates at 30 °C for 7 d. A plug (5 mm diam.) consisting of the actively growing culture and adhering mycelia were then immersed in a PBS dilution containing COP1T-HA (100 μg/mL). Next, the plate was incubated at 30 °C under a 470 nm laser (MDL-III -470 nm, 100 mW/cm^2, 20 min) and kept in the dark for 3 h. Finally, the plug was transferred to fresh SDA medium and incubated at 30 °C for another 7 d for observation and the diameter of the colony was measured. All experiments were independently repeated in triplicate. The fungal colonies HXFM001 in COP1T-HA aPDT treatment group displayed a markedly reduced growth rate compared to the untreated group, with average diameters of 1.5 mm and 0.8 mm (SDA, 30 °C, 7 d) respectively (Figure S5).

The ability of COP1T-HA aPDT on slide culture of *F. monophora* (HXFM001) was observed by microscopy (200 ×). Firstly, the slide culture of *F. monophora* was conducted on potato dextrose agar (Difco, Detroit, USA) at 30 °C for 7 d and observed by microscopy. Then, COP1T-HA (100 μg/mL) was added dropwise onto the slide. Subsequently, the slide was illuminated at 30 °C under a 470 nm laser (MDL-III-470 nm, 100 mW/cm^2, 20 min). After COP1T-HA aPDT treatment, the slide was washed twice with PBS and observed by microscopy again. We observed a thinning of fungal hyphae and blurring of septa of *F. monophora* in COP1T-HA aPDT treated group, suggesting that COP1T-HA aPDT disrupted the morphology and structure of fungal hyphae (Figure 2 & S6).

3. Discussion

PDT has shown potential in eradicating various fungi, such as *Candida* species (Liu et al. 2022b), Dermatophytes (Kamp et al. 2005), *Aspergillus* (Friedberg 2001), also including *Fonsecaea* and *Cladophialophora* species. For example, Hu et al. (2019) showed that a combination of PDT and antifungal drugs enhanced the antifungal activity against *Fonsecaea* species, including *F. monophora* and *F. pedrosoi*. Similar to our results, these results highlight the potential of PDT as a therapeutic approach against *Fonsecaea* infections. However, traditional photosensitisers are hindered by problems such as poor water solubility, limited active ingredients, and high cost, which have restricted their clinical applications.

To overcome the deficiency of traditional photosensitisers, Iyer et al. (2021) reported a novel nanoplatform to treat *Cryptococcus neoformans* infections. The nanoplatform allowed targeted delivery of the drugs to the fungal cells, which showed improved fungicidal activity against *C. neoformans in vitro* and in an animal model of cryptococcal meningitis, which is more susceptible to infection in immunocompromised individuals (Chen et al. 2016). In our previous work (Liu et al. 2022a), we developed a nanoplatform COP1T-HA (a new strategy to prepare powerful PSs for efficient aPDT by introducing porous cage compound COP1T, which could facilitate the transportation of O_2 and ROS species; the hydroxyl groups of HA are attached to the carboxylic acid groups of COP1T by esterification; hydrophilic group polyethylene glycol 2,000 groups were introduced for improving water solubility of HA), which demonstrated excellent antifungal effects against drug-resistant *Candida* species, improving the aggregation and poor water solubility issues commonly observed in photosensitisers. Additionally, our unpublished data suggests that COP1T-HA exhibited promising results in treating dermatophyte infections, including *Trichophyton rubrum*, *Microsporum canis*, and *Trichophyton mentagrophytes*.

In conclusion, we identified a clinical isolate as *F. monophora* using morphological observation, ITS sequencing and phylogenetic analysis. We have demonstrated that COP1T-HA exhibited significant fungicidal effects against *Fonsecaea* spp. *in vitro* through the agar plate dilution method, colony growth and slide culture observation. Interestingly, the antifungal ability of COP1T-HA shows a concentration-dependent relationship on *Fonsecaea* spp., and it can significantly inhibit fungal growth in only 100 μg/mL. Additionally, we find that aPDT has a certain synergistic effect with traditional antifungal treatment.

While aPDT holds promise as a potential therapeutic approach for managing CBM, further research is crucial, especially in exploring nanoplatform-based photosensitisers for improved efficacy against *Fonsecaea* species both *in vitro* and *in vivo*. Moreover, the complexity of fungal infections extends beyond CBM, particularly in immunocompromised individuals, including those suffering from haematological malignancies (Arastehfar et al. 2019). This complexity emphasises the need for versatile and robust treatments like aPDT, capable of targeting a broad spectrum of fungal pathogens. The development of such advanced therapies, effective

against a wide range of fungal species, is not just a medical necessity but also a step towards revolutionising the CBM treatment landscape. By expanding the therapeutic arsenal with such targeted and effective antifungal options, we open new avenues for combating these challenging infections.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Yuzhou Liu http://orcid.org/0000-0002-1109-7704
Yuping Ran http://orcid.org/0000-0002-9382-0571

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