Underlying mechanisms of the effect of minocycline against *Candida albicans* biofilms

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Abstract. Minocycline (MH) is a broad-spectrum antimicrobial agent and semisynthetic tetracycline derivative, which has been widely used in the clinic due to its efficacy. Having the strongest anti-microbial effect, MH exceeded the traditional scope of antibiotics and its previously unknown antifungal activity is also gradually being discovered. To preliminarily investigate the inhibitory effect of MH on *Candida albicans* (*C. albicans*), changes of cell growth, hyphal formation and transition, biofilm production and signaling pathway gene expression of *C. albicans* in the presence of MH were assessed in the present study. An XTT reduction assay was performed to quantitatively detect the metabolic activity of biofilms and evaluate the inhibition of MH on this. The results suggested that biofilm formation was clearly inhibited by 67% (P<0.0001) in the presence of 250 μg/ml MH, while mature biofilms were not significantly affected. In addition, MH inhibited the transition from yeast to hypha in a dose-dependent manner. Furthermore, reverse transcription-quantitative PCR revealed that several hyphae- and adhesion-specific genes associated with the Ras/cyclic (c)AMP/protein kinase A (PKA) pathway were differentially expressed following MH treatment, including downregulation of ras family GTPase (*RAS1*), adenylyl cyclase-associated protein 1 (*CAP1*), thiamin pyrophosphokinase 1 (*TPK1*), adenylate cyclase (*CDC35*), transcription factor (TEC1), agglutinin-like protein 3 (*ALS3*) and hyphal wall protein 1 (*HWP1*) and upregulation of *EFG1* (enhanced filamentous growth protein 1 gene) and *PDE2*

(high-affinity phosphodiesterase gene). The most obviously changed genes were *TPK1*, *HWP1* and *RAS1*, downregulated by 0.33-, 0.48- and 0.55-fold, respectively. It was suggested that MH is associated with alterations in the morphology of *C. albicans*, such as the repression of hypha and biofilm formation of cells, and MH affected the Ras/cAMP pathway to regulate the expression of cAMP-associated genes.

Introduction

Candida albicans (C. albicans) is considered the most common opportunistic fungal pathogen in humans due to its widespread distribution in the environment. It has a high propensity to cause invasive infections on mucosal surfaces and is particularly life-threatening in individuals with immunodeficiency and dysbacteriosis, as systemic candidiasis has a high mortality rate in such patients (1,2). Fungi continue to vary and mutate in response to environmental and body stimuli and the antifungal drugs available are simply insufficient for the clinical/pharmacological treatment of patients. In addition, the incidence of *C. albicans* resistance increased rapidly due to the widespread and long-term usage of limited antifungal drugs, causing additional difficulty in treatment and ultimately leading to refractory fungal infections (3-5).

One of the morphological characteristics that distinguish *C. albicans* from other organisms is polymorphism and pleomorphism. *C. albicans* may have three distinct phenotypic characteristics: A yeast-like form at 25˚C, and additional pseudohyphae and hyphae forms at 37˚C, which are commonly referred to as filamentous morphologies (6,7). All three forms may coexist and transform with the change of environmental stimuli. *C. albicans* biofilms, as an important barrier, are considered significant for pathogenicity and virulence, as well as adhesion and infectivity, which are contributing factors in the process of infecting the host and are able to withstand the stimulation of the external environment and drugs. Therefore, numerous clinical antifungal drugs are mainly resistant to biofilms. The major conventional antifungal drugs currently in clinical use are fluconazole and amphotericin B. Studies have demonstrated that the frequent and excessive use of antifungal drugs led to the notorious resistance of *C. albicans* to a wide variety of clinical antifungal drugs, including the aforementioned two (8,9).

The contradiction between effective antifungal therapy and finite available clinical antifungal drugs is increasingly

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prominent. Therefore, novel antifungal agents are urgently required in order overcome the resistance of *C. albicans* biofilms. Recent scientific studies have re-evaluated tetracycline (TE) derivatives, such as minocycline (MH), TE, doxycycline (DO), tigecycline (TGC), oxytetracycline and demethylchlortetracycline, for their potential antifungal activity as well as old traditional antibiotics combined with antifungal drugs to achieve an outstanding effect against fungal infections (10-13).

MH, a TE derivative, is a traditional antimicrobial agent that inhibits bacterial protein synthesis, which is extensively used in the clinic due to its large spectrum of antibiotic activity. MH has a strong effect on gram-positive bacteria, including *Staphylococcus aureus*, with the effect on gram-negative bacilli being slightly weaker. When MH is combined with other antifungal agents, such as fluconazole, the antifungal effect of combination treatment is significantly stronger than that of MH used alone (10,11,14,15). Previous studies have reported that MH is able to mediate several biological phenomena and is associated with the capacity of morphological transformation from yeast to hypha in *C. albicans* (14).

The RAS/cyclic (c)AMP/protein kinase A (PKA) signaling pathway has an impact on cell growth, differentiation, protein secretion and transport. Hogan and Sundstrom (16) and Phillips and Crowe (17) determined separately that in *C. albicans*, the Ras signaling pathway mediates the morphological transition and biofilm formation, and contains certain vital hypha-specific genes, including ras family GTPase (*RAS1*), thiamin pyrophosphokinase 1 (TPK1), enhanced filamentous growth protein 1 (*EFG1*), *TEC1*, *CAP1* (adenylyl cyclase-associated protein 1), phosphodiesterase 2 (*PDE2*) and adenylate cyclase (*CDC35*), as well as certain adhesion-specific genes, including agglutinin-like protein 3 (*ALS3*) and hyphal wall protein 1 (*HWP1*). Based on the results of these studies, MH may be a promising and effective antifungal agent. It is able to alleviate the urgent problem of drug resistance and short supply of antifungal drugs, which may provide a major breakthrough in the clinical treatment of fungal infections. However, in order to understand the antifungal activity of MH thorough and systematic evaluation is required and the effect of MH on *C. albicans* biofilms and the mechanisms underlying this require further investigation. In the present study, it was hypothesized that the antifungal effect of MH on *C. albicans* is mainly associated with biofilm formation and its regulation is closely associated with the RAS/cAMP/PKA pathway. In the present study, the *in vitro* antifungal activity of the widely used antimicrobial MH in *C. albicans* was assessed, and whether its potential antifungal mechanism is associated with the Ras/cAMP signaling pathway was systematically explored to verify the validity of this hypothesis.

Materials and methods

Strains, antibacterial agents and growth conditions. A total of four TE derivatives were examined in the present study: MH, TE, DO and TGC. The TH drug susceptibility test (TE, 30 μg; DO, 30 μg; TGC, 15 μg) was obtained from BioMérieux and MH drug papers (30 μg) from Merck KGaA. MH was dissolved in DMSO. A high concentration of the original solution was prepared, stored at -20˚C away from light and then diluted to the desired final concentration prior to use. The final content of DMSO was <2% and had no effect on cells in the present study.

The reference strain *C. albicans* ATCC 90028 was a gift obtained from Renmin Hospital of Wuhan University. All clinically isolated yeast strains used in the present study were isolated at the People's Hospital of China Three Gorges University (Yichang, China) and stored in the central laboratory. All strains used in the present study were identified according to the standard morphological criteria (18). Strains were routinely cultured on Sabouraud dextrose agar (SDA) plates at 28˚C for 24-48 h, then streaked out onto Yeast Extract-Peptone-Dextrose (YPD) liquid medium and grown at 30˚C in a shaking incubator for 24-48 h until they reached the logarithmic growth phase and the absorbance was measured at 600 nm. Hyphal inductions were performed at 37˚C in liquid medium, and Spider and RPMI-1640 medium were prepared as previously described (19).

Effect of TE derivatives on the growth of yeast strains (Kirby-Bauer disc diffusion method). All strains were adjusted to 0.5 McFarland bacterial suspensions and then evenly spread to a Mueller-Hinton agar plate (Thermo Fisher Scientific, Inc.). Once the surface of the plate was dry, sensitivity test papers containing one of the 4 TE antibiotics were applied. All plates were incubated for 24 h at 28˚C and the diameter of the inhibition ring was measured.

Effect of MH on planktonic cells in C. albicans. Antifungal susceptibility testing was performed in 96-well tissue culture plates (Corning, Inc.) according to the protocol of the Clinical and Laboratory Standards Institute (20). In brief, *C. albicans* ATCC 90028 was prepared with initial cells at 1x10^5 CFU/ml and cultured in YPD liquid medium for 48 h at 37˚C. To evaluate the effect of MH on the cell growth in *C. albicans* ATCC 90028, an inoculum concentration of yeast cells at ~1x10^6 cells/ml was added into liquid RPMI 1640 medium containing MH at concentrations of 0, 125, 250, 500 and 1,000 μg/ml, incubated in 96-well tissue culture plates at 37˚C and shaken at 200 rpm.(2.2 x g). The cells were monitored every 4 h and counted at the predetermined time-points (0, 4, 8, 12, 16, 20, 24, 28 and 32 h) using a Multiskan GO Microplate reader (Thermo Fisher Scientific, Inc.) to measure the absorption/optical density (OD) at 600 nm, and the background optical densities were already subtracted. A total of three independent experiments were performed (21).

Hyphal formation assessment. To further estimate whether the MH affects bud-to-hypha transition, a fixed amount of *C. albicans* cells (1x10^6 cells/ml) was cultured in several nutritious hypha transition media. The spider and RPMI 1640 media (Sangon Biotech Co., Ltd.) were supplemented with 20% fetal bovine serum (FBS; Cytiva) in 24-well tissue culture plates containing various concentrations of MH and incubated at 37˚C for 3 h, and hyphal formation was evaluated microscopically to count 200 cells and calculate the percentage of hyphal formation, as previously described (14).

Antifungal activity against mature biofilms. The *C. albicans* cells in the logarithmic growth phase were collected and

centrifuged, RPMI 1640 medium was added to adjust the concentration to $1x10^6$ CFU/ml and 100 μl bacterial liquid was added to the 96-well culture plate for 90 min of adhesion at 37˚C. The medium was gently sucked off with a sample gun, non-adherent planktonic cells were removed, 100 μl fresh RPMI 1640 medium was added and the plate was incubated at 37˚C for 24 h until the mature biofilm was formed [20]. The biofilm supernatant was then aspirated, RPMI 1640 with MH was added to for a 24-h incubation, the growth of the biofilm was observed under a microscope and an XTT kit (Sangon Biotech Co., Ltd.) was used to evaluate the formed mature biofilm. Similarly, in order to detect the effect of MH on the formation of biofilms, MH was added to fresh RPMI 1640 after 90 min of adhesion; subsequent methods were described above until biofilm formation to observe the anti-biofilm effect of MH [20,22].

Gene expression analysis of C. albicans-specific genes. Following MH treatment overnight, *C. albicans* cells were washed and collected, and a Fungal RNA out kit (Sangon Biotech Co, Ltd.) was used to extract and isolate the total RNA of *C. albicans* according to the manufacturer's protocol and the standard method. The OD was measured at 260-280 nm using a Nanodrop 2000 Ultraviolet spectrophotometer (Thermo Fisher Scientific, Inc.) to test the nucleic acid concentration and purity of the RNA. The integrity of the RNA was assessed using denatured electrophoresis through a 1% agarose-3-(N-morpholino) propanesulfonic acid gel (Sangon Biotech Co., Ltd.). A reverse transcription kit (Takara Bio, Inc.) was used to reverse-transcribe single-stranded RNA (1 μg) into cDNA. Next, reverse transcription-quantitative PCR (RT-qPCR) was performed using a 7500 real-time PCR system (Thermo Fisher Scientific, Inc.); hyphal-specific and biofilm-associated genes of *C. albicans*, as well as their transcriptional regulators, were analyzed in the present study, and the primers are listed in Table I. The associated genes were as follows: RAS1, a major transcription factor involved in the MAPK pathway; EFG1, transcription factor in the cAMP/PKA pathway; GAP1, which encodes a general amino acid permease; ALS3 and HWP1, which encode agglutinin-like protein 3 and hyphal wall protein 1, respectively, and are activated by EFG1; CDC35 (coding for adenylyl cyclase); TPK1 (PKA catalytic subunits); PDE2 (high-affinity phosphodiesterase gene); TEC1, a transcription factor; ACT1, a housekeeping gene that has the ability to express stably and consistently in organisms, and was chosen as an endogenous control gene for correcting the loading inaccuracies [23]. The final values were calculated by the $2^{-\Delta\Delta Cq}$ method [24].

The PCR conditions were programmed and divided into three steps according to the experimental conditions and purposes. The first step was initial denaturation at 95˚C for 5 min, where double-stranded DNA templates break hydrogen bonds under thermal action to form single-stranded DNA. The second step was annealing at 55-65˚C, with the continuous gradient temperature set at a heating rate of 0.1˚C/sec to match the appropriate suitable solution curve temperature, the system temperature was lowered and the primer bound to the DNA template to form a local double strand. This step eventually went through 40 cycles of amplification and quantification at 95˚C for 10 sec, 55˚C for 30 sec and 72˚C for 15 sec with a

Table I. PCR primers used for detection of gene expression in *Candida albicans*.

Primer	Sequence (5'-3')
CAP1-F	GAACCACCATCAACATCA
CAP1-R	CAACCCATCAATATCAAGT
TPK1-F	AGAAGTTCAAGATGTGACTTAT
TPK1-R	CATCATCAGAACCACCTTGT
CDC35-F	TCCATGTCAAATATGCCAACG
CDC35-R	CTTCACATCCCAACTTTCAGG
EFG1-F	TCCATGTCAAATATGCCAACG
EFG1-R	TGGATTCATACCGTATTGGTCATTA
TEC1-F	TGGATTCATACCGTATTGGTCATTA
TECI-R	TCGGGCAATCCTTTGAATAAA
RAS1-F	GTGGTGGTGTTGGTAAATC
RAS1-R	TTCTTGTCCAGCAGTATCT
PDE2-F	TGCTGTGGGACATTGGAG
PDE2-R	GGCGGAAATTATGGAACG
ALS3-F	GTGATGCTGGATCTAACGGTATTG
ALS3-R	GTCTTAGTTTTGTCGCGGTTAGG
HWP1-F	CGGAATCTAGTGCTGTCGTCTCT
HWP1-R	CGACACTTGAGTAATTGGCAGATG
ACT 1-F	TTGATTTGGCTGGTAGAG
ACT 1-R	ATGGCAGAAGATTGAGA

F, forward primer; R, reverse primer; CAP1, adenylate cyclase-associated protein 1; TPK1, thiamin pyrophosphokinase 1; CDC35, adenylate cyclase; EFG1, enhanced filamentous growth protein 1; Tec1, transcription factor; RAS1, ras family GTPase; PDE, 2,3',5'-cyclic-nucleotide phosphodiesterase; ALS3, agglutinin-like protein 3; HWP1, hyphal wall protein 1; ACT 1, actin related gene 1.

continuous single fluorescence measurement and end. The last step was a final extension maintained at 72˚C for 5-10 min. dNTP was used as raw material and primers started from the 3'end, extending from the 5' to 3' end, to synthesize DNA strands complementary to the template, followed by a cooling step at 42˚C.

Statistical analysis. Values are expressed as the mean fold change ± standard deviation of three independent experiments. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). One-way analysis of variance with Tukey's post hoc test was used to evaluate the statistical significance of differences among the treatment groups. $P<0.05$ were considered to indicate a statistically significant difference.

Results

Inhibitory effects of TE derivatives on yeasts. The results of the drug sensitivity test are presented in Fig. 1. MH had the most obvious inhibition circle among the four TE derivatives (ME, TE, DO and TGC). By measurement and quantification, the ratio of the inhibition zone diameter ≥14 mm for MH on *C. albicans* was determined to be 80%, that on *C. tropicalis* was 96% and that on *C. Portuguese* was 87%. TE, DO and
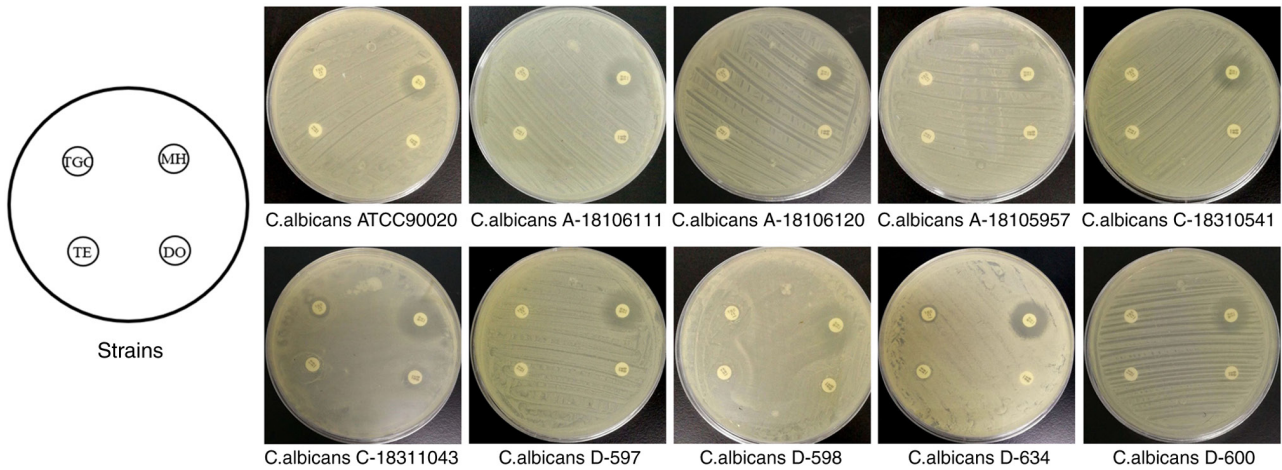
Figure 1. Antifungal effects of 4 TE derivatives. MH had a marked antifungal activity against *C. albicans* and DO had a slight antifungal activity against *C. albicans*, while TE and TGC had no antifungal activity. TE, tetracycline; MH, minocycline; DO, doxycycline; *C. albicans*, *Candida albicans*; TGC, tigecycline.

TGC exhibited no sufficient antifungal activity against *C. albicans* with the inhibition zone diameter being 6-10 mm, <14 mm in all strains.

Effects of MH on C. albicans growth. In the presence of MH at the concentrations of 125, 250, 500 and 1,000 μg/ml, a significant growth inhibition of *C. albicans* was observed in the treatment group as compared to the control group without MH treatment (Fig. 2). At 250 μg/ml, MH exhibited the most potent inhibitory activity on *C. albicans* growth among all concentrations used. After 8 h of incubation in liquid culture, the growth curves of *C. albicans* began to diverge and exhibit visible differences. After 32 h, the growth was significantly restricted in RPMI-1640 medium with MH at 125 μg/ml, leading to a 59.9% reduction (P<0.01), and in the presence 250 μg/ml MH, the maximum inhibition was 68.0% (P<0.01) as compared to the control group. However, with further increases in the concentration of MH, the growth inhibition of planktonic *C. albicans* decreased.

MH regulates the hyphal formation process of C. albicans. Morphological changes were observed under light microscopy following incubation for 4 h and the quantitative results suggested that MH inhibited hyphal growth induction at all tested concentrations. More specifically, the maximum hypha formation inhibition occurred at the lowest concentration of MH (125 μg/ml) in both RPMI-1640 and Spider media and the degree of hypha inhibition slightly decreased with the increase of the MH concentration (Fig. 3A). The hypha rate of ~200 cells for each sample was determined using light microscopy (Fig. 3B). In drug-free medium, >70% of *C. albicans* cells converted to the hyphal morphology after 4 h of incubation, with only a small percentage retaining their yeast form. However, following incubation with 125 and 250 μg/ml MH, the hypha formation rate was only 11.75±2.86% with a significant inhibition of hyphae in all media (P<0.001). Following incubation with 500 μg/ml MH, the hypha rates were 46.00±5.29% in RPMI-1640 medium and 23.00±2.64% in Spider medium, both were significantly different from the control group (P<0.001). Following incubation with 1,000 μg/ml MH, the
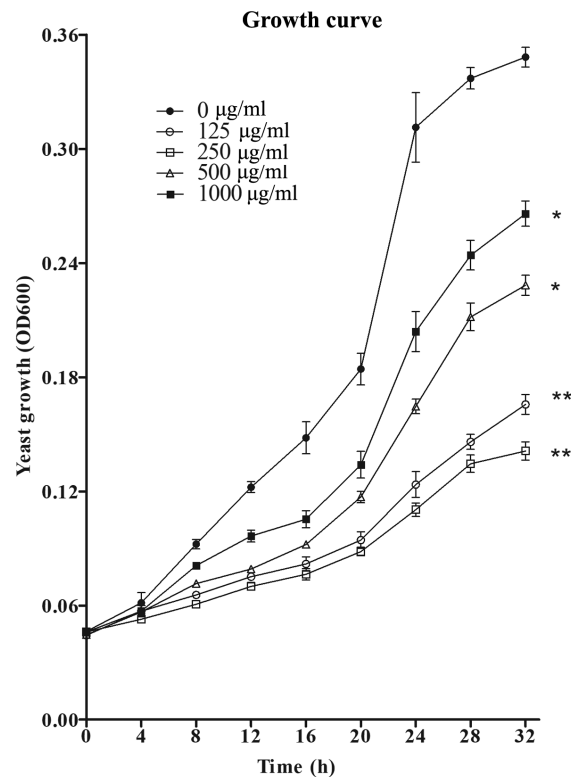

Figure 2. Effects of different concentrations of MH on fungal cell growth of the *Candida albicans* strain ATCC 90028. *P<0.05; **P<0.01. OD, optical density; MH, minocycline.

hypha rates were 54.33±4.04% in RPMI-1640 medium and 35.33±2.51% in Spider medium, both were significantly different from the control group (P<0.01).

MH inhibits C. albicans biofilms. An XTT reduction assay was performed to examine the potential of MH to inhibit biofilm formation *in vitro*. As presented in Fig. 4, the biofilms formed by *C. albicans* decreased in an MH dose-dependent manner in terms of their density and thickness. More specifically, MH inhibited biofilm formation by 52% at 125 μg/ml (P<0.001
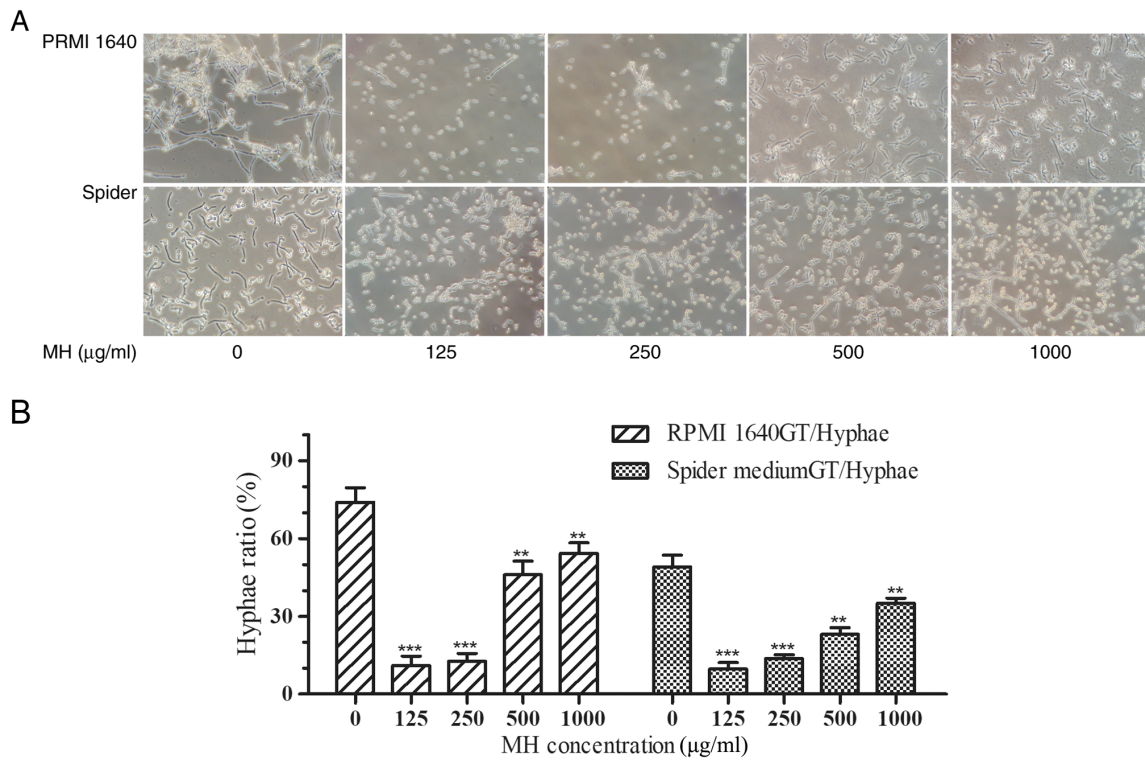
Figure 3. (A) Effects of MH on hyphal formation in *C. albicans*. Cells (1x10⁶ cells/ml) were grown in Spider or RPMI 1640 media supplemented with 20% FBS. Photomicrographs of *C. albicans* ATCC 90028 grown at 37˚C for 4 h. MH had an obvious trend to inhibit filamentous growth as compared with cells incubated in drug-free medium (magnification, x400). (B) The hyphae rate was counted in a minimum of 200 cells from each sample following 4 h of cultivation. The results indicated that MH was able to inhibit hyphae formation at all concentrations, with the effects of 125 and 250 µg/ml MH being most significant. **P<0.01; ***P<0.001 vs. control. MH, minocycline; *C. albicans*, *Candida albicans*; FBS, fetal bovine serum.
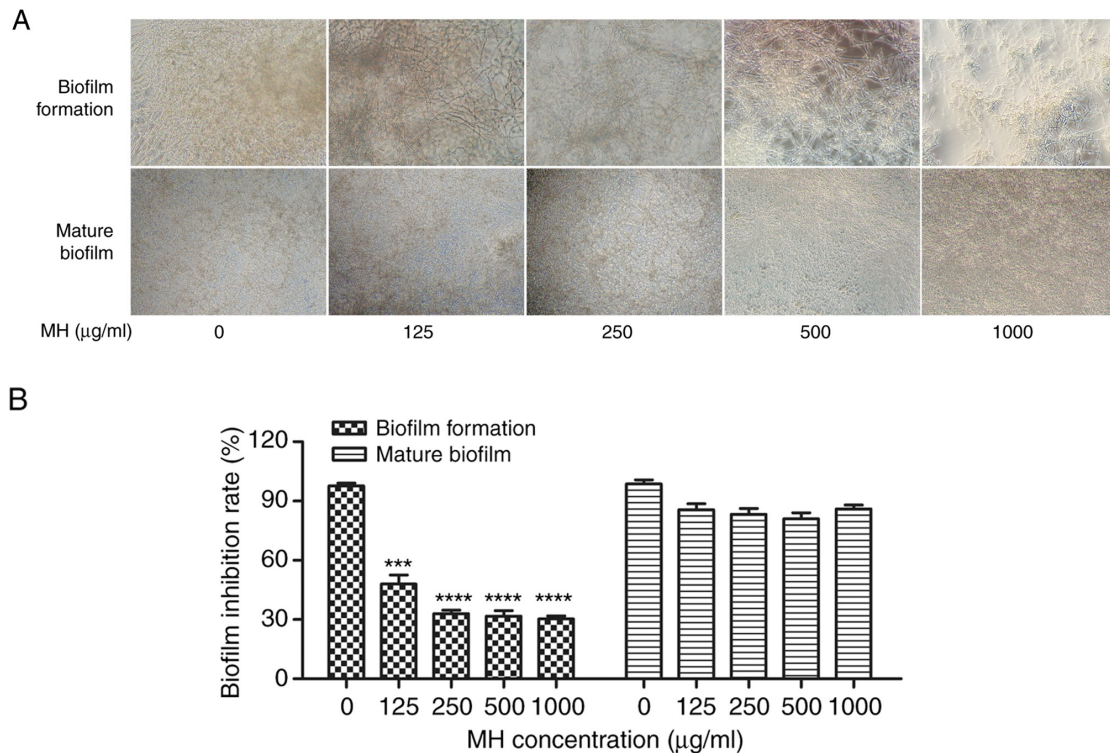

Figure 4. (A) Light microscopy images of biofilm formation and mature biofilm. With the increase in the MH concentration, the inhibition of biofilm formation was more obvious. At 250 µg/ml MH, there was a marked decrease in the density of biofilms. By contrast, in the presence of MH, mature biofilms exhibited no changes obvious changes observed by standard light microscopy (magnification, x1,000) (B) Effects of MH on biofilm formation and mature biofilms. MH inhibited *C. albicans* biofilm formation *in vitro*. Biofilm inhibition was evaluated using an XTT reduction assay and the results are presented as the percentage of MH-treated biofilms relative to control biofilms that were formed without drug treatment. ****P<0.0001 and ***P<0.001 vs. control. MH, minocycline; *C. albicans*, *Candida albicans*.
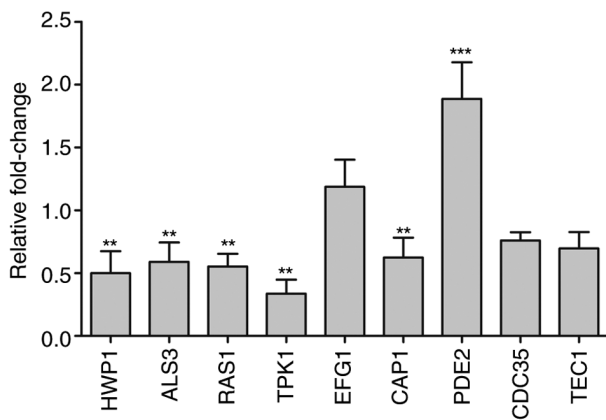
Figure 5. Gene expression levels in the *C. albicans* strain ATCC 90028. Fold change values of the expression of each target gene are shown relative to the values of the control group containing untreated *C. albicans*. The housekeeping gene ACT1 was used as an internal reference gene. The expression level of each target gene in the MH-free sample was set as 1. [**]P<0.01; [***]P<0.001. *C. albicans*, *Candida albicans*; MH, minocycline.

vs. control), and with the increase of the MH concentration, the inhibitory effect became more pronounced. MH inhibited biofilm formation by 67% at 250 µg/ml (P<0.001 vs. control). However, MH had no marked activity against the mature biofilms, which exhibited no obvious/significant changes following treatment with any of the MH concentrations. In conclusion, the tendency of MH to inhibit biofilm formation was confirmed.

MH regulates the expression of cAMP pathway genes. The expression levels of 9 genes were monitored by RT-qPCR. Hypha-specific genes from the adhesion process, *HWP1* and *ALS3*, which are downstream components of the cAMP/PKA pathway, are positively regulated by EFG1 (25), and they were downregulated by 0.48- and 0.59-fold, respectively, following 250 µg/ml MH treatment. Certain hyphal transcriptional regulation genes from the cAMP/PKA pathway, including *RAS1, TPK1, EFG1, CAP1, PDE2* and *CDC35,* were also examined (Fig. 5). The upregulated genes included *EFG1* and *PDE2*. The expression levels of EFG1 were slightly, though not significantly upregulated by MH treatment in the present study, while the upstream components *RAS1, TPK1* and *CAP1* were downregulated by 0.55-, 0.33- and 0.62-fold, respectively. *CDC35* and *TEC1* were downregulated by 0.76- and 0.69-fold, respectively. Taken together, the results suggested that genes downregulated following MH treatment, including *HWP1, ALS3, RAS1* and *TPK1*, were all associated with the AMP pathway and regulated by the Ras/cAMP pathway (16).

Discussion

The results of the present study demonstrated that the antifungal activity of MH was mediated by regulatory factors in the Ras/cAMP pathway, which was conducive to the reduction of biofilm formation and limiting of hyphae formation and long-term maintenance of *C. albicans*.

It was indicated that MH had different inhibitory effects on different strains of fungus. It had a strong antifungal activity

on yeasts such as *C. albicans*, *C. tropicalis* and *C. lusitaniae*, with the inhibition zones being >14 mm and demonstrating a significant activity against *C. albicans*. MH had no inhibitory activity against *C. pseudosmooth*, *C. glabrata* and *C. parapsilosis*, while TE, DO and TGC had no significant antifungal properties. The antifungal effect of MH was marked as compared to other TE derivative agents. This may be linked to the morphological transformation and virulence in fungi (26); further research is required in the future to confirm this.

In addition, several studies have verified that the length and thickness during the growth of filamentous fungi directly affects the pathogenicity of hyphae, with the two being positively correlated (27,28). The yeast form of *C. albicans* is less pathogenic and has a better adhesion capacity than the hyphae form due to its small and single form, at least in part because the yeast form is more easily eliminated by the defense system of the host than the hyphae form (29). In Spider and RPMI-1640 media, nutrient-rich conditions were added with 20% FBS in order for *C. albicans* to switch to the hyphae form more rapidly. However, in the present study, MH was able to maintain the yeast form for longer and inhibited the formation of hyphae, while MH clearly affected the hypha growth of *C. albicans* at the concentrations of 125 and 250 µg/ml. In RPMI-1640 medium and Spider medium, the hypha rate (%) of 125 MH treatment was the most significant, 11.00±3.61 and 9.67±2.51, respectively. These results are consistent with those by Shi *et al* (10), who reported that when MH was combined with fluconazole, the minimum inhibitory concentration on *C. albicans* was decreased as compared with that of fluconazole used alone. Although there have been numerous reports on the phenomenon of antifungal agents used alone or combined with antimicrobial agents through *C. albicans* biofilms (13-15), few have investigated the particular antifungal characteristics of antimicrobial agents in *C. albicans*. To explore the potential mechanisms, the interference ability of MH to penetrate biofilms was first assessed.

C. albicans biofilms have a complex and intricate three-dimensional network architecture, with the barrier characteristics of *C. albicans* biofilms mainly reflected in an increased tolerance to traditional antifungal therapy. An XTT reduction assay was performed to demonstrate the suppressive function of MH on biofilms, and it was indicated that MH had a distinct inhibitory effect against *C. albicans* biofilms. The results suggested that 250 µg/ml MH significantly depressed biofilm formation by 67% (P<0.001). With the increase in concentration, the inhibitory activity of MH tended to be stable and changes showed no significant difference, indicating that the optimal inhibitory effect had been achieved or approached at 250 µg/ml. Of note, it exhibited no activity to impair the maintenance of mature biofilms. In conclusion, MH had a strong antibiofilm effect against *C. albicans in vitro*, which appears to be attributable to its anti-morphological transition activities.

According to the inhibition of *C. albicans* filamentous growth and biofilm formation, the transcription levels of genes associated with mycelium growth, adhesion and biofilm formation in *C. albicans* were determined to further clarify the molecular mechanism of the anti-biofilm effects of MH. The analysis revealed that several important genes were differentially expressed following MH treatment. *CAP1,*

TPK1, CDC35, TEC1, RAS1, ALS3 and *HWP1* were down-regulated, and *EFG1* and *PDE2* were upregulated following treatment with 250 μg/ml MH. In this assay, MH was applied at 250 μg/ml, since at this concentration had a significant anti-biofilm activity. It should be noted that these downregulated genes are all important components of the RAS1/cAMP/PKA signaling pathway; the present hypothesis that a direct interaction between the pathways occurs at the transcriptional level was confirmed. Previous studies have reported that several signaling pathways are linked to morphogenesis and biofilms in *C. albicans*, with the MAPK and cAMP/PKA signaling pathways being the most prominent (16,30,31). The cAMP/PKA pathway has attracted extensive attention in fungal pathogens such as *C. albicans*, *Trichophyton*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (32,33), due to its vital function in the specific morphology development of fungal biofilms. The Ras/AMP/PKA pathway is involved in the regulation of various traits of *C. albicans* and responds to specific environmental stimuli and cell subtype combinations (30).

In the present study, the most significantly downregulated genes were RAS1 and TPK1 (0.55- and 0.33-fold, respectively). RAS1 has a vital role in the morphological switch and biofilm formation of yeast and hypha (34,35). It is a soluble small GTPase that transmits signals to stimulate filamentous growth in the Ras1/cAMP/PKA signaling pathway as a transcription factor (36-38). Despite RAS1 gene mutation and deletion of alleles, which resulted in serious hyphal growth defects, such as deformation and irregularity in response to serum, and inhibition of pseudohyphal development (34), studies have indicated that the addition of sufficient exogenous cAMP or overexpressed components of MAP kinase cascade in the growth media is able to correct the above morphological defects and make mycelium return to normal (35,39). Evidence suggested that the RAS1 gene is an intermediate factor in the anti-fungal regulation of MH, which is involved in signaling pathway regulation through the *RAS1* gene to interfere with biofilms (14).

Recent research has demonstrated that the deletion of TPK1 results in hyphal formation defects on solid media in *C. tropicalis* (40). In GlcNAc-inducing liquid medium, *TPK1* was associated with the appearance of germ-tube, with the mRNA levels of *TPK1* increasing gradually with cell growth and peaking at the onset of germ-tube emergence (41). Lin and Chen (40) reported that PDE2 tightly regulated the intracellular cAMP level. Deficiency of PDE2 led to the activation of cAMP, which decreased the thickness of the cell wall and cell membrane of the fungus, therefore reducing the virulence of the fungus and making cells more sensitive to antifungal agents. High expression of PDE2 inhibited cAMP synthesis and limited hypha production (42), while all the defects observed in hyphal morphogenesis *in vitro* were reversible and rectifiable. The present results suggested that the increase in PDE2 mRNA following MH intervention was most significant, which was consistent with the results of previous study (43), which further explained the inhibition of PDE2 on hypha and biofilm growth in *C. albicans*.

EFG1 is a well-characterized transcription factor and important component of the Ras/cAMP pathway, which may both bidirectionally regulate genes that participate in the essential processes and stages of cellular growth, such as white-opaque transition in morphogenesis, and glycolysis and respiration in cell metabolism (44,45). Furthermore, EFG1 acts upstream of TEC1 and is located in the middle stream of the RAS1/cAMP-PKA signaling pathway, which regulates and controls the upstream and downstream components, including HWP1, TCE1 and ALS3 (42). Mutational analysis suggested that deficiency of the EFG1 gene disrupted normal morphological transformation, impeded the growth of mycelium, induced hyphae in a pseudorevertant strain and was dependent on the EFG1 transcription factor that has a regulatory role in the downstream of protein kinase A (46). TEC1, located downstream of EFG1, also regulates the growth of hyphae-specific genes, with the EFG1/efg1 mutants resulting in the overexpression of TEC1 (47). Deletion of TEC1 resulted in thinner biofilms and the observed disordered growth of short spores on their surface. In the present study, a slight elevation of EFG1 was observed and the modulating effect of MH on EFG1 was not significant. In the present results, most aberrant genes were downregulated genes, which were all important components of the RAS1/cAMP/PKA signaling pathway, while fewer genes were upregulated. These results suggested that the signaling pathways of MH may be dominated by negative regulation and that the RAS1/cAMP/PKA signaling pathway may have a crucial role in the regulation of hypha and biofilm by MH.

However, the present study had certain limitations. For instance, due to the limited resources and funding available, no resistant strain analysis was performed and the effects of MH on drug-resistant strains of *C. albicans* were not analyzed. In addition, animal studies are required to verify the drug toxicity and feasible doses of MH. Further experiments, such as western blot analysis to detect changes in the protein levels, may be performed, or changes in electrolytes in the biofilm, such as ionic calcium, may be assessed in order to investigate the mechanism of action of MH. Validation using clinical samples is also required. However, despite these limitations, the results of the present study may provide novel insight that may lead to the development of clinical medication and treatment.

In conclusion, the present study demonstrated that MH exhibits a strong anti-biofilm effect against *C. albicans* selectively, and the predominant mechanisms are associated with the Ras/cAMP pathway. Therefore, the hypothesis of the present study, that the antifungal effect of MH on *C. albicans* is mainly associated with biofilm formation and its regulation is closely associated with the RAS/cAMP/PKA pathway was validated. These results indicated that MH may be an effective antifungal agent for *C. albicans* infections. However, clinical application is not yet possible, as more prospective studies using strains and animal models are required, and treatment strategies for drug-resistant *C. albicans* infection require to be developed to further verify the clinical applicability of MH. This is the direction of our future research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ and QD designed the experiments and drafted the manuscript. ZM performed the experiments and analyzed the data. TG and BZ performed the RT-qPCR experiments. QD reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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