ARTICLE

The human muscarinic acetylcholine receptor antagonist, Dicyclomine targets signal transduction genes and inhibits the virulence factors in the human pathogen, Candida albicans

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Abstract

Dicyclomine is a human muscarinic acetylcholine receptor antagonist used for the treatment of abdominal cramps. We are reporting here that dicyclomine can inhibit the in vitro growth and virulence factors of the human pathogen *Candida albicans* very effectively. Dicyclomine inhibited adhesion, early biofilm, mature biofilm, and planktonic growth. Yeast to hyphal form transition of *C. albicans* in various inducer media such as serum, proline, glucose, and *N*-acetylglucosamine was inhibited. Dicyclomine also could kill *C. albicans* cells within 15 min of exposure. Dicyclomine appears to inhibit the yeast to hyphal conversion by affecting signal transduction pathway. The expression of selected genes associated with yeast to hyphal form transition in serum in presence of dicyclomine was studied using real-time polymerase chain reaction (RtPCR). The RtPCR analysis showed that dicyclomine targets both cAMP pathway as well as MAPK cascade. Eight genes were upregulated. Out of these, three major upregulated genes were *Bcy1*, *Tup1*, and *Mig1*. Dicyclomine downregulated *Ume6*, *Ece1*, and *Pde2* genes which are involved in cAMP signaling pathway and also downregulated the DNA binding protein gene, *Rfg1*. Dicyclomine significantly upregulated the master negative regulator of hyphal formation, *Tup1*. Based on this study we suggest that the muscarinic acetylcholine receptor antagonist, dicyclomine could be repositioned as a potential anti-*Candida albicans* as well as anti-virulence agent.

Introduction

Candida albicans is a part of the normal microbiome of healthy humans. Under immunocompromised conditions, it can become pathogenic [1]. *C. albicans* can form biofilms on biotic as well as abiotic surfaces [2]. Abiotic surfaces include implanted devices in the body like urinary catheters, venous catheters, voice prostheses, dentures etc [2–4]. *Candida* biofilms are highly resistant to most of the currently used drugs [5]. Seventy percentages of microbial infections in humans involve biofilm formation [3, 6, 7]. As such there is a necessity for developing novel drugs which can target biofilms. One of the strategies for anti-*Candida* drug development is the inhibition of

virulence factors and repositioning of drugs. Repositioning studies have shown promise in the recent years [8, 9]. In this study we are reporting the repositioning of a drug, called dicyclomine. Dicyclomine is an antispasmodic agent [10–12]. It is an inexpensive medicine and is commonly used for intestinal hypermotility treatment. Also, it can reduce irritable bowel syndrome. Dicyclomine relieves muscle spasms and cramps in the gastrointestinal tract, which emerges during infections by blocking the activity of acetylcholine on muscarinic receptors located on the surface of smooth muscle cells. Muscarinic acetylcholine receptors are known to play many fundamental roles in human physiology. They are typical G-protein-coupled receptors (GPCRs) which can facilitate signal transduction via heterometric G-protein to downstream second messengers. A number of drugs target muscarinic acetylcholine receptors. Dicyclomine is reported to target human muscarinic acetylcholine M1 receptor as well as M3 receptor [13–15]. Many hyphal-specific genes mediate yeast to hyphal transformation through two pathways, Ras1-cAMP-PKA pathway and Ras1-MAPK cascade [16]. In Ras1-cAMP-PKA pathway, when Ras1 is activated, it can

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Table 1 The MIC of pure and commercial dicyclomine against virulence factors of *Candida albicans* (ATCC 90028 and GMC-16)

Sr.no	Molecule name	MIC mg/ml				Planktonic assay	Adhesion	developing biofilm	Mature biofilm	MFC
		Morphogenesis induced by								
		Serum	Pro	Glc	GlcNAc					
2	Dicyclomine (commercial)	0.062	0.062	0.125	0.25	0.125	0.031	0.062	0.25	0.25
3	Dicyclomine (pure)	0.062	0.062	0.125	0.25	0.5	0.25	0.062	0.25	1

stimulate adenylyle cyclase (*Cyr1*) that leads to increase in cAMP production. This results in the binding of cAMP to *Bcy1*. *Bcy1* is the subunit regulator of protein kinase A (PKA) [17]. *Bcy1* may activate either *Tpk1* or *Tpk2*. *Tpk1* and *Tpk2* are PKA catalytic subunits which can activate the transcription factor *Efg1* [18, 19]. The activated *Efg1* is required for virulence which can cause systemic candidiasis in mouse model [20]. *Ece1* and *Ume6* are downstream genes of the activated *Efg1* [21, 22]. To know the mechanism through which dicyclomine can inhibit *C. albicans* morphogenesis, we studied the expression of selected hyphal genes that are involved in Ras1-cAMP-PKA and MAPK signal pathways. Our real time PCR study has identified potential targets of dicyclomine in *C. albicans*. In this manuscript, we are reporting that dicyclomine can inhibit virulence factors of the human pathogen, *C. albicans*, raising the possibility of repositioning dicyclomine as an antifungal agent.

Results

Inhibition of yeast to hypha in C. albicans by dicyclomine

C. albicans is affected by many environmental cues which are contributed in yeast to hyphal switching. Here, we tested four factors that can induce yeast to hyphal form transition. Dicyclomine inhibited this transition in two strains of *C. albicans* (ATCC90028 and GMC-16) induced by 5% serum, 1% proline, 1% glucose, and 1% *N*-acetlglucosamine at 0.062, 0.062, 0.125, 0.25 mg/ml respectively (Table 1; Figs. 1a, 2a–d).

Dicyclomine inhibits Candida albicans growth

Dicyclomine at concentration of 0.5 mg/ml reduced *Candida* growth by 50% (Table 1 and Fig. 1b (A)). The minimum fungicidal concentration (MFC) was observed at 1 mg/ml (Table 1 and Fig. 3a). Commercial dicyclomine also reduced *Candida albicans* growth by 50% [minimun inhibitory concentration (MIC)] at 0.125 mg/ml (Table 1 and Fig. 1b (A)) and MFC was at 0.25 mg/ml (Table 1 and Fig. 3b).

Dicyclomine inhibits adhesion of Candida albicans to polystyrene

The effect of pure dicyclomine on adhesion cells was observed at 0.25 mg/ml where 50% of adhesion was inhibited. This concentration was considered as the minimum inhibitory concentration (MIC). MIC of the commercial dicyclomine on adhesion cells was 0.031 mg/ml (Table 1 and Fig. 1c).

Dicyclomine inhibits biofilm formation of Candida albicans on polystyrene surface

Both pure and commercial dicyclomine inhibited developing biofilm at 0.062 mg/ml after 48 h of incubation at 37 °C (Table 1; Figs. 1d (A), 4a, b). Mature biofilm was inhibited at 0.25 mg/ml and above this concentration after 48 h of incubation at 37 °C (Table 1 and Fig. 1d (B)).

Dicyclomine kills C. albicans cells

Both pure and commercial dicyclomine were highly toxic to *Candida albicans* (ATCC 90028) cells. Pure dicyclomine killed 100% of inoculum within 15 min of exposure at MFC 1 mg/ml, whereas commercial dicyclomine killed 100% of cells within 15 min of exposure at MFC of 0.25 mg/ml. But in case of *C. albicans* (GMC-16), both the pure and commercial dicyclomine killed 100% of cells within 4 h of exposure (Fig. 1b (B)).

Dicyclomine inhibits biofilm growth of C. albicans on silicon discs

Candida albicans formed biofilm on silicon rubber air way discs characterized by a thick network of hyphal and yeast cells. Pure and commercial dicyclomine caused considerable inhibition of biofilm (Fig. 4c).

Dicyclomine regulates hyphal genes in yeast to hyphal form conversion in C. albicans

To discover the basic mechanism by which the muscarinic receptor antagonist, dicyclomine inhibited yeast to hyphal form transformation induced by serum, we studied the

Fig. 1 **a** Effect of dicyclomine on *Candida albicans* (ATCC 90028 and GMC-16) morphogenesis induced by various media; 5% Serum, 1% v/v Proline, 1% v/v *N*-acetylglucosamine and 1% v/v glucose. **b** Effect of dicyclomine on; **A** *Candida albicans* (ATCC 90028 and GMC-16) planktonic growth; **B** *Candida albicans* (ATCC 90028 and GMC-16) growth in time-dependent kill curve assay. **c** Effect of dicyclomine on adherence of *Candida albicans* on polystyrene surface. **d** Effect of dicyclomine on *Candida albicans* (ATCC 90028 and GMC-16) biofilms; **A** Developing biofilm; **B** Mature biofilm (Color figure online)
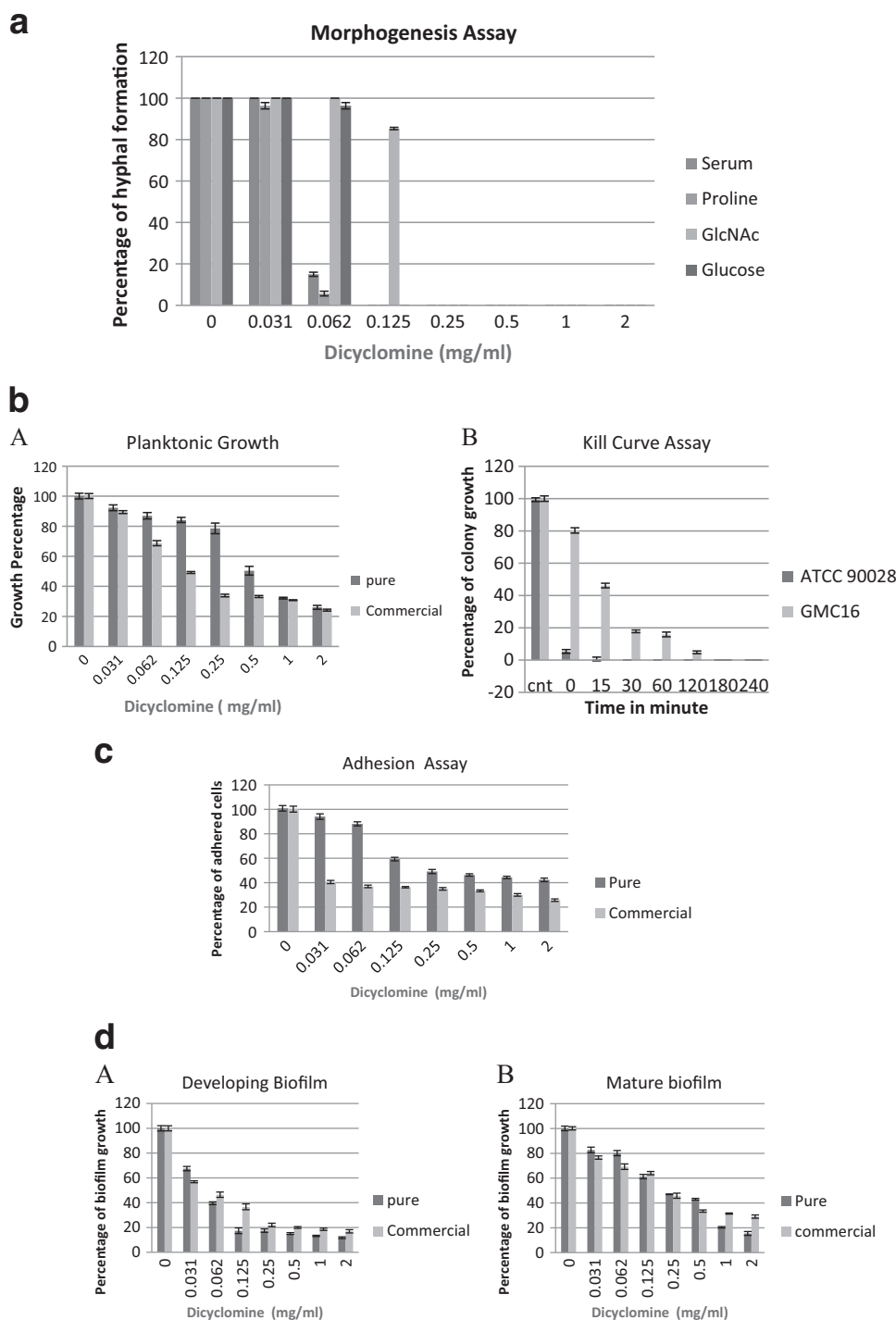
expression of some genes involved in cAMP-PKA and MAPK pathways using real time-PCR analysis. Dicyclomine affected both these pathways. It altered the expression of genes involved in cAMP-PKA pathway where the sub-unit regulator of cAMP-dependent PKA, *Bcy1* and the transcription factor, *Efg1* were upregulated by 42 and 15 folds, respectively. The expression of downstream hyphal genes of cAMP-Efg1 pathway, *Ece1* and *Ume6* were downregulated by 4.7 and 4.9 folds, respectively. The cAMP regulator, *Pde2* also downregulated up to 1.3 fold with dicyclomine treatment. The transcriptional suppressor factor, *Tup1* was significantly overexpressed by 38 folds and Tup1-dependent transcriptional repressor, *Mig1* was also upregulated by 37 folds. The Tup1-dependent transcriptional repressor, *Rfg1* was downregulated up to 3.2 folds. Dicyclomine also could affect the expression of genes

Fig. 2 a Effect of dicyclomine on 5% serum induced yeast to hyphal form transition in *Candida albicans* (ATTC 90028); **A** Control; **B** 0.031 mg/ml; **C** 0.062 mg/ml; **D** 0.125 mg/ml; **E** 0.25 mg/ml; **F** 0.5 mg/ml; **G** 1 mg/ml; **H** 2 mg/ml. **b** Effect of dicyclomine on 1% proline induced yeast to hyphal form transition in *Candida albicans* (ATTC 90028); **A** Control; **B** 0.031 mg/ml; **C** 0.062 mg/ml; **D** 0.125 mg/ml; **E** 0.25 mg/ml; **F** 0.5 mg/ml; **G** 1 mg/ml; **H** 2 mg/ml. **c** Effect of dicyclomine on 1% *N*-acetylglucosamine induced yeast to hyphal form transition in *Candida albicans* (ATTC 90028); **A** Control; **B** 0.031 mg/ml; **C** 0.062 mg/ml; **D** 0.125 mg/ml; **E** 0.25 mg/ml; **F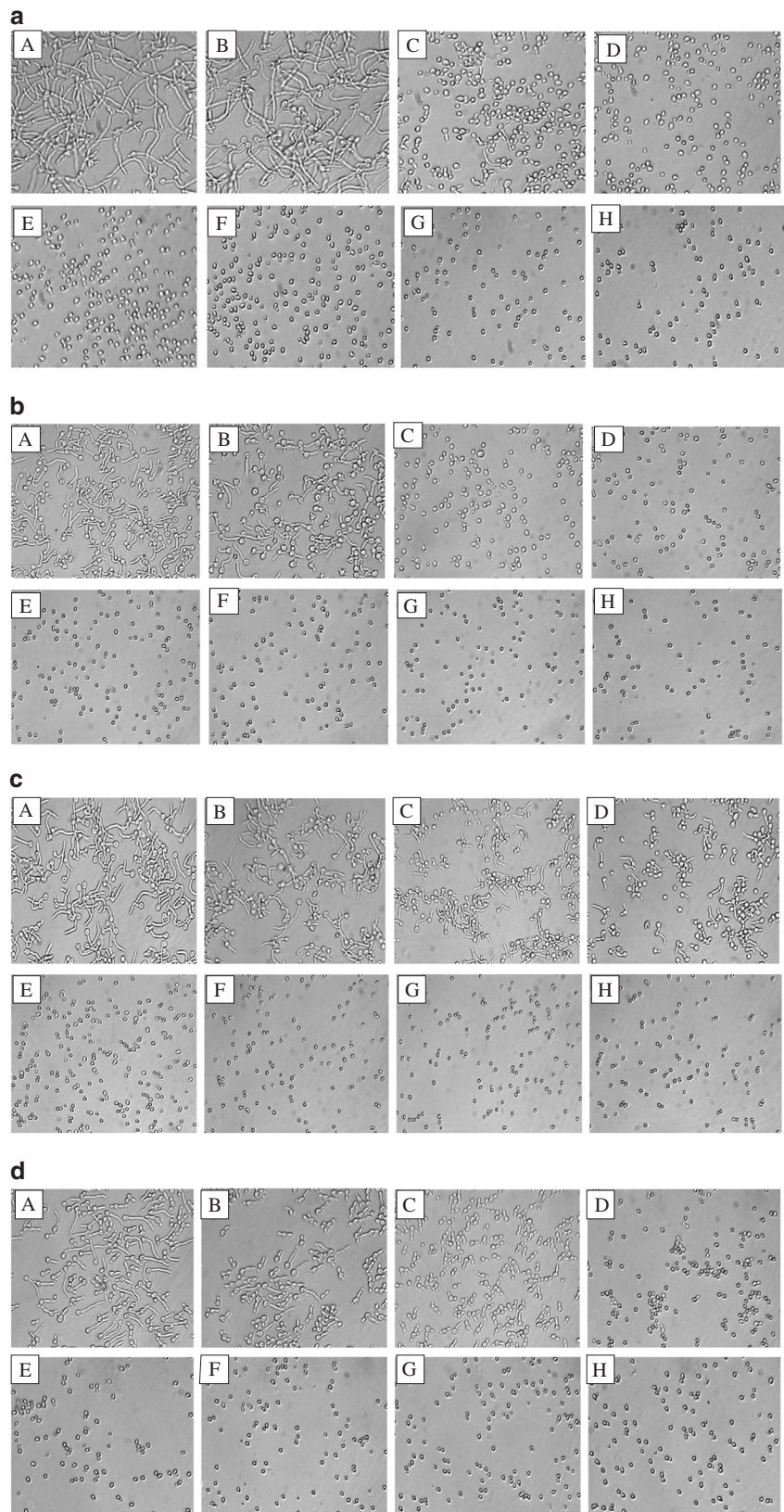** 0.5 mg/ml; **G** 1 mg/ml; **H** 2 mg/ml. **d** Effect of dicyclomine on 1% glucose induced yeast to hyphal form transition in *Candida albicans* (ATTC 90028); **A** Control; **B** 0.031 mg/ml; **C** 0.062 mg/ml; **D** 0.125 mg/ml; **E** 0.25 mg/ml; **F** 0.5 mg/ml; **G** 1 mg/ml; **H** 2 mg/ml
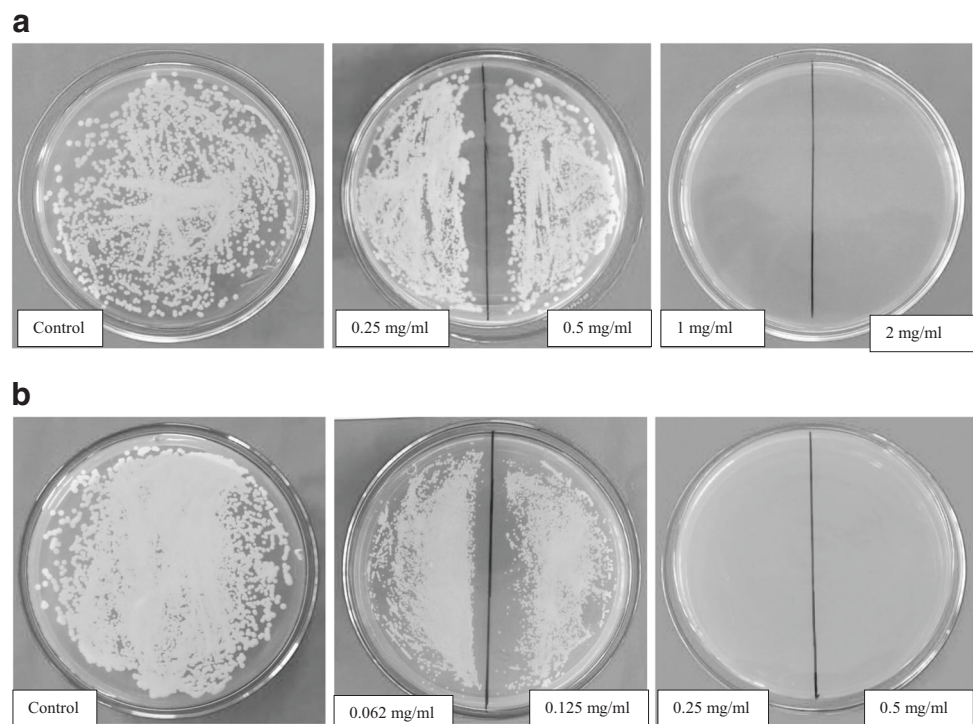
involved in MAPK pathway. The expression of *Hst7*, *Cek1*, *Tec1*, and *Cst20* was upregulated by 12, 2.8, 2.5, and 1.4 folds, respectively (Figs. 5 and 6).

Discussion

Various drugs are repositioned as anti-*Candida albicans* agents [9, 23, 24]. These studies suggest that *Candida albicans* may share a number of targets with humans, since both humans and *Candida* share a common eukaryotic heritage. The human muscarinic receptors (M1, M2, M3, M4, and M5) are typical GPCRs and play significant roles in signal transduction. In this manuscript, for the first time we are proposing repositioning dicyclomine as an anti-*Candida albicans* agent. Dicyclomine is an anticholinergic and antispasmodic agent, widely used as a drug for stomach cramps. This drug effectively inhibited the growth and virulence factors of *Candida albicans* (Table 1; Figs. 1, 2a–d, 3a, b, 4a–c). It also caused considerable inhibition of drug-resistant biofilm of *C. albicans* (Table 1; Figs. 1d (A, B), 4a–c). Dicyclomine killed *Candida* cells within 15 min of exposure (Fig. 1b (B)). These results suggest that dicyclomine could be used as a potential anti-*Candida* agent. Hyphal formation of *C. albicans* in serum is through the Ras1 signaling pathway [16]. The expression of selected genes involved in Ras1-cAMP-PKA and MAPK signal pathways was studied in presence of serum and dicyclomine. We found that the transcriptional suppressor of

hyphal genes, *Tup1* was upregulated (Figs. 5 and 6) that can block filamentous growth and repress biofilm formation in *C. albicans* [25–27]. Deletion of *Tup1* is reported to cause constitutive hyphal formation [25]. The overexpression of *Tup1* leads to the downregulation of hyphal genes, *Ece1 Ume6*, and the cAMP regulator, *Pde* [26]. Dicyclomine upregulated *Tup1* which led to the downregulation of *Ece1*, *Ume6*, and *Pde2* expression (Figs. 5 and 6). *Ece1* and *Ume6* are involved in the extension and growth of hypha, adherence, and biofilm formation [28–32]. The overexpression of *Ece1* may be involved mainly in adhesion or biogenesis and may have contributed in hyphal and biofilm formation [33]. In *C. albicans*, *Ume6* encodes a key filament-specific transcriptional regulator of hyphal development and virulence [30]. *Ume6* is a downstream target of multi-signaling pathways involved in hyphal growth [22]. Mutation of *Ume6* causes defects in hyphal extension and biofilm formation [30]. *Pde2* encodes a high affinity−phosphodiesterase regulating cAMP level and is required for hyphal growth, virulence and induction of biofilm formation on rat catheter [34–36]. Deletion of *Pde2* leads to increase cAMP level which causes constitutive activation of cAMP signaling pathway. This results in hyperfilamentation, but without enhancing virulence [35, 37]. Dicyclomine downregulated *Pde2* which may cause overexpression of the subunit regulator of cAMP-dependent-PKA, *Bcy1* (Figs. 5 and 6) [17]. *BCY1* has a crucial role in the regulation of cell differentiation and death. It has an essential role in cell viability in *C. albicans* [17].

Fig. 4 a Effect of pure dicyclomine on developing biofilm of *Candida albicans* (ATTC 90028); **A** Control; **B** 0.031 mg/ml; **C** 0.062 mg/ml; **D** 0.125 mg/ml; **E** 0.25 mg/ml; **F** 0.5 mg/ml; **G** 1 mg/ml; **H** 2 mg/ml. **b** Effect of commercial dicyclomine on developing biofilm of *Candida albicans* (ATTC 90028); **A** Control; **B** 0.031 mg/ml; **C** 0.062 mg/ml; **D** 0.125 mg/ml; **E** 0.25 mg/ml; **F** 0.5 mg/ml; **G** 1 mg/ml; **H** 2 mg/ml. **c** Scanning electron micrograph (SEM) of *Candida albicans* biofilm formation on silicon discs. **A** Control; **B** Biofilm treated with 1 mg/ml of pure dicyclomine; **C** Biofilm treated with 1 mg/ml of commercial dicyclomine
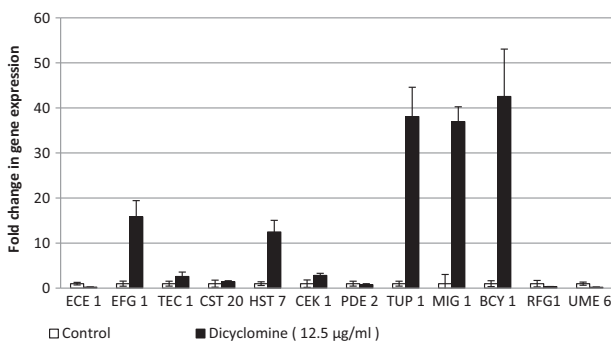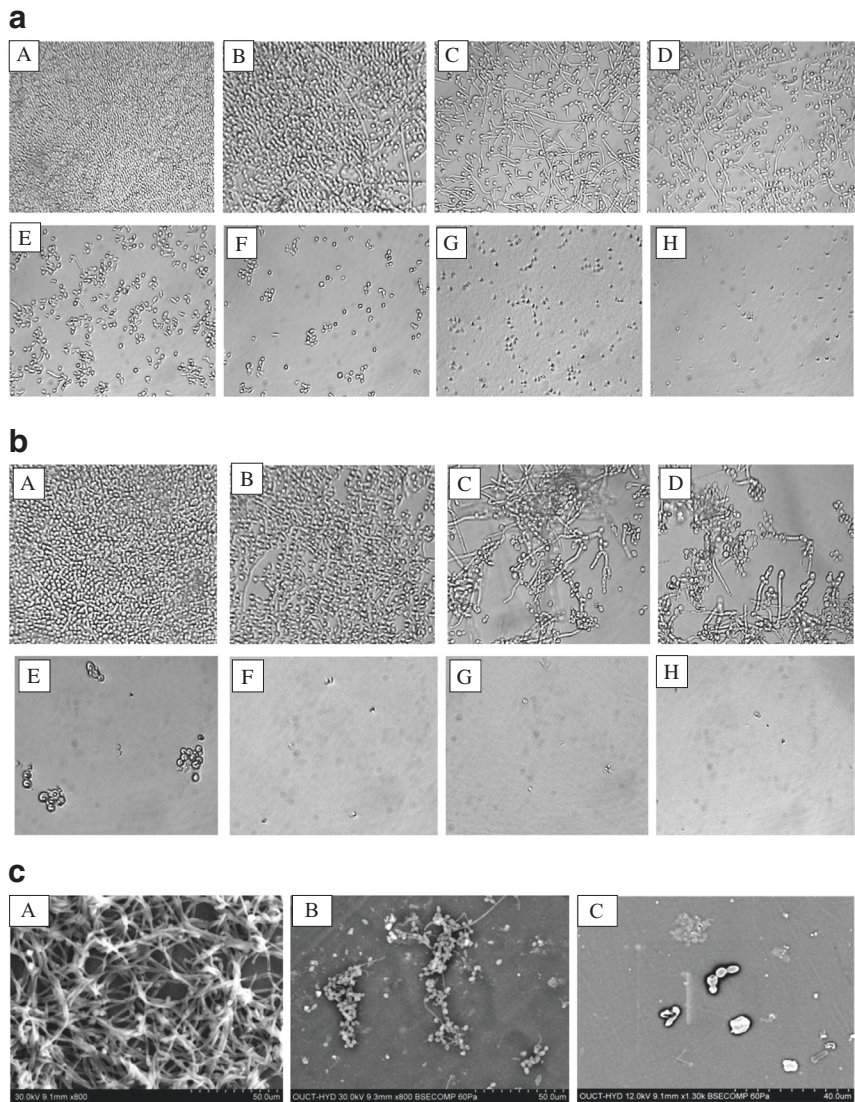

Fig. 5 Modulation of gene expression in *Candida albicans* by dicyclomine during yeast to hyphal form conversion induced by serum

Deletion of *Bcy1* leads to multiple cellular morphologies and enhances the development of filaments [17]. *Rfg1*, a Tup1-dependent suppressor [38] was downregulated by treatment with dicyclomine. This may be due to *Mig1*

upregulation (Figs. 5 and 6). The key regulator transcription factor *Efg1* [18, 19] may be upregulated as a result of *Bcy1* overexpression. Dicyclomine altered the expression of *Cst20*, *Hst7*, *Cek1*, and *Tec1* that are involved in MAPK pathway (Figs. 5 and 6). In *C. albicans*, a MAP kinase pathway is involved in controlling of hyphal development [37] and mating processes. The deletion of the genes, *Cph1*, *Cst20*, *Hst7*, and *Cek1* cannot block filament growth induced by serum and does not dramatically affect the transcription profile of the yeast to hyphal form transition. Deletion of *CPH1* causes delay in hyphal formation [39]. The upregulation of *Tec1* may be due to the *Efg1* upregulation with dicyclomine treatment (Figs. 5 and 6). The mutation of *tec1* exhibits suppressed filament formation in liquid serum-containing media [16]. This study indicates that a protein-like muscarinic receptor may have an important function in the yeast to hyphae formation in *C. albicans* and it may influence both cAMP and MAPK
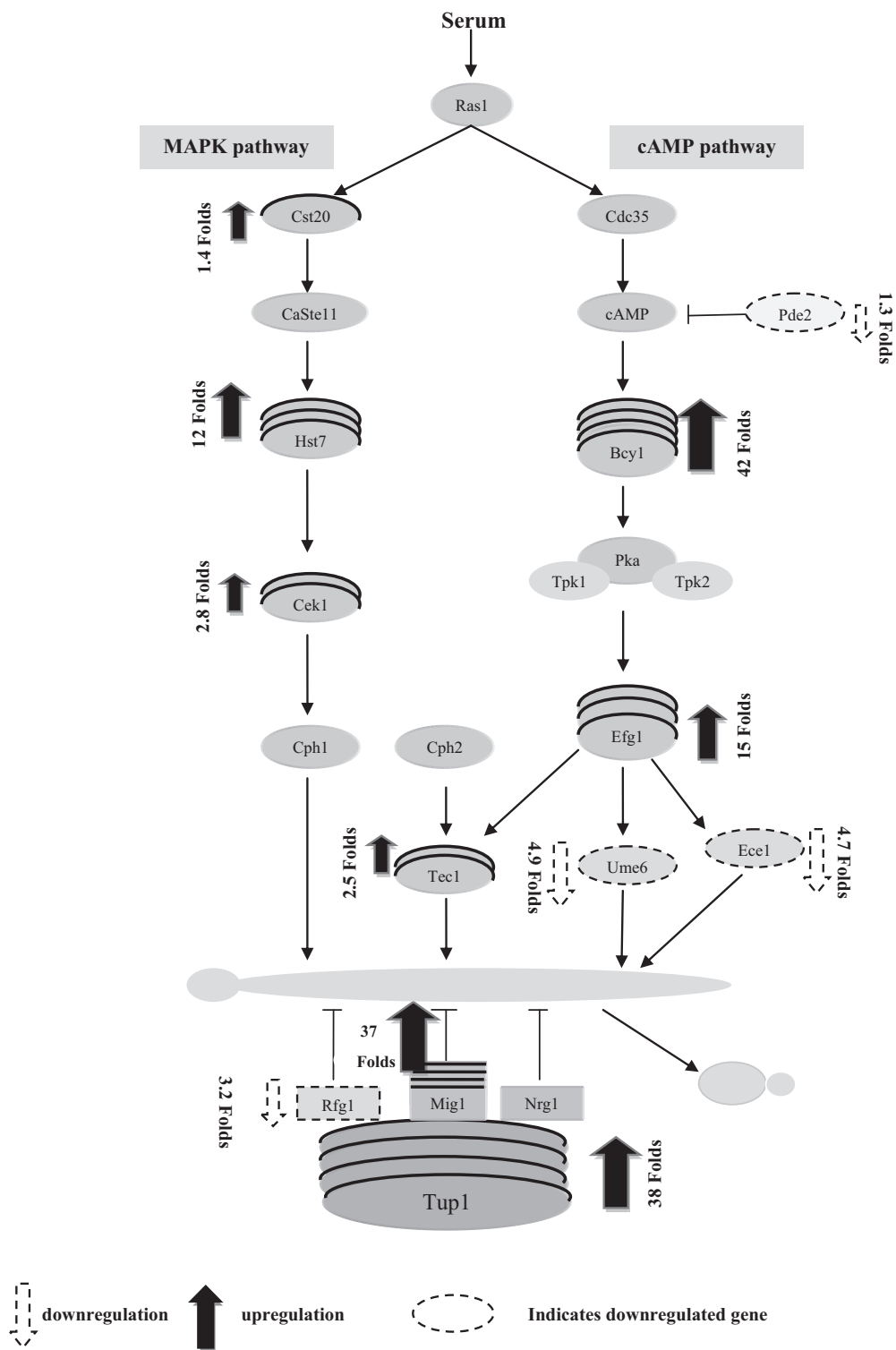
Fig. 6 Dicyclomine targets signal transduction pathways in *C. albicans*
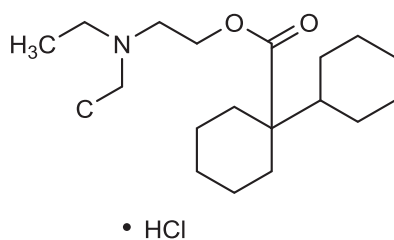
Fig. 7 Structure of Dicyclomine hydrochloride

signaling pathways. Dicyclomine may inhibit yeast to hyphal form conversion by targeting multiple genes.

Methods

Media and chemicals

Dicyclomine hydrochloride (Fig. 7) was purchased from Sigma-Aldrich Chemicals Ltd, Mumbai, India. Dicyclomine hydrochloride injection form was purchased from a local medical store. Menadione and 2, 3-bis (2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were purchased from Sigma-Aldrich Chemicals Ltd. All other media components and plates were purchased from HiMEDIA Chemicals Ltd, Mumbai, India.

Culture of Candida albicans

A standard strain of *Candida albicans* ATCC 90028 was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India. GMC-16 was obtained from Government Medical College, Nanded, MH, India. The culture was maintained on yeast peptone dextrose (YPD) agar slant at 4 °C and propagated by inoculating a single colony from the YPD agar plates into 50 ml YPD broth in 250-ml conical flask. Flasks were incubated overnight at 30 °C at 100 rpm on an orbital shaking incubator. The cells were harvested by centrifugation at 2000 rpm and washed thrice with sterile 0.1 M phosphate-buffered saline, pH 7.4 and the cell density was determined using a haemocytometer count.

Hyphal formation assay

Yeast to hyphal form transition assay was carried out in 96-well microtitre plates [40]. *Candida albicans* cells stock was diluted to 1×10^6 cells/ml in several inducer media including 5% serum, 1% v/v *N*-acetylglucosamine, 1% v/v proline, and 1% v/v glucose. Various concentrations of dicyclomine hydrochloride were prepared and added in each well. Wells without drugs were kept as a control. The final volume was kept at 200 µl in each well. The microtitre plates were incubated at 37 °C at 120 rpm on an orbital shaker incubator for 2 h. After incubation period, cells were

observed microscopically by using an inverted light microscope (Metzer, India). Hundred cells were counted and numbers of yeast and hyphal forms were noted. All the experiments were done in triplicates.

Antifungal activity

Minimum inhibitory concentration (MIC)

MIC was determined by using the standard methodology M27 A2 as per CLSI guidelines [23]. Various concentrations of dicyclomine were prepared in RPMI-1640 medium by double dilution manner in the 96-well microtitre plates. 1×10^3 cells/ml from *Candida albicans* cells stock was prepared. The final volume of RPMI-1640 medium kept in each well was 200 µl. The wells without addition of dicyclomine served as control. The microtitre plates were incubated at 35 °C for 48 h, and the absorbance was recorded spectrophotometrically at 620 nm using a microplate reader (Multiscan Ex, Thermo Electronic Corp, USA). The concentration of dicyclomine which caused a 50% reduction in the absorbance compared to the control was considered as the MIC. All the experiments were done in triplicates.

Minimum fungicidal concentration (MFC)

The wells of MIC of dicyclomine and above MIC against *Candida albicans* growth were selected for determination of MFC. Ten microliters of cell suspension was taken from the wells and spread on YPD agar plates. The plates were incubated at 30 °C for 24 h and observed for the presence of colonies. The absence of colonies growth was considered as the fungicidal effect. The lowest concentration of dicylomine that showed no growth was considered as the MFC. All the experiments were done in triplicates.

Adhesion assay

The effect of dicyclomine on adhesion of cells on the polystyrene surface was studied using 96-well plates. Double dilutions of various concentrations of dicyclomine were prepared in sterile phosphate-buffered saline (PBS) and put in each well [41]. Fifty microliters of prepared cells was put in each well to obtain 1×10^7 cells/ml and control kept without dicyclomine. The plates were incubated at 37 °C for 90 min at 100 rpm in an orbital shaker incubator. After incubation, the wells were washed by sterile PBS to remove non adhered cells. Density of the adhered cells was analyzed by using XTT metabolic assay. The color formation was measured by using microtitre plate reader at 450 nm. More than 50% reduction compared to the control was considered as MIC. All the experiments were done in triplicates.

Biofilm formation

Biofilm formation of *Candida albicans* was developed in an in vitro on the surface of 96-well polystyrene plates as per standard method [41]. 1×10^7 cells/ml were prepared and 100 µl of cell suspension was added in each well. The microtitre plates were incubated at 37 °C for 90 min at 100 rpm. Non adhered cells were aspirated and removed using sterile PBS gently to avoid biofilm disruption. For early biofilm formation, serial concentrations of dicyclomine were prepared by double dilution in RPMI-1640 and 200 µl was added in each well. Then, the plates were incubated at 37 °C for 48 h to undergo biofilm formation. After incubation period, the plates were washed thrice with sterile PBS and observed under an inverted light microscope (Metzer, India) for the presence or absence of biofilm. Confirmation and quantitation of biofilm formation was studied by the XTT metabolic assay. For mature biofilm formation, 1×10^7 cells/l were prepared and 100 µl of cell suspension was added in each well. The microtitre plate was incubated at 37 °C for 90 min at 100 rpm. Non adhered cells were removed by washing with sterile PBS. Then 200 µl of RPMI-1640 was added in each well and microtitre plates incubated at 37 °C for 24 h. After incubation period, the biofilms were washed with sterile PBS. Various concentrations of dicyclomine in RPMI-1640 were prepared by double dilution and 200 µl was added in each well. The plates were incubated at 37 °C for 48 h. The wells were again washed by sterile PBS after incubation period. The biofilm formation was quantitated by the XTT metabolic assay. All the experiments were done in triplicates.

XTT assay for biofilm quantitation

The early and mature biofilm formation was quantitated by using a XTT metabolic assay. One milligram of XTT powder was dissolved in 1 ml distilled water and sterilized by using a bacteriological filter. Menadione was prepared in acetone, then 10 µl of menadione was added to the XTT to the final concentration of 4 µM. The wells were washed with sterile PBS. Eighty microliters of PBS was added in each well followed by adding 20 µl of XTT-menadione reagent. The plates were incubated in dark condition at 37 °C for 3 h to develop color formation. The color formation was measured at 450 nm using a microplate reader (Multiskan EX, Thermo Electron Corp. USA). Wells without test drugs were used as controls. Those without biofilm were kept as blanks. The concentration of dicyclomine which caused ≥50% lowering of growth was considered as the MIC for biofilm formation. All the experiments were done in triplicates.

Kill curve assay

Activity of dicyclomine against the growth of *Candida albicans* (ATCC 90028 and GMC-16) was studied by time-dependent kill curve assay. 2.5×10^3 cells/ml were prepared in 10 ml sterile RPMI-1640 along with MFC of dicyclomine and were incubated at 30 °C at 100 rpm. From this mixture, 0.5 ml was taken at different time intervals (0, 15, 30 min, 1, 2, 4, 5, 6, 7 and 8 h), and was washed twice with sterile PBS to remove the drug carryover effect. The pellet was re-suspended in 50 µl sterile PBS and spread on YPD agar plates. The plates were incubated at 30 °C for 48 h. The formed colonies on the plates were counted and compared with control plate lacking dicyclomine. Plates were kept in triplicates.

Scanning electronic microscopy of biofilm

Candida albicans biofilm was developed on sterile oropharyngeal silicon rubber airway discs seeding in 12 microtitre plates [40]. A 1 mg/ml concentration of dicyclomine was prepared in RPMI-1640. RPMI-1640 without dicyclomine was kept for control. Two mililiters of 1×10^7 cells/ml was inoculated in each well containing sterile oropharyngeal silicon rubber airway disc and was incubated at 37 °C for 90 min at 100 rpm to allow adhesion of cells on discs. After incubation the discs were washed thrice using sterile PBS. The discs were transferred to sterile wells and dicyclomine −RPMI-1640 solution was added in each well. The discs without dicyclomine were considered as control. The plates were incubated at 37 °C for 48 h at 60 rpm. Then, the discs were washed again using sterile PBS and were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline, pH 7.4 for 24 h at 4 °C. After 24 h, the discs were post-fixed in 2% aqueous solution of osmium tetra-oxide for 4 h, then dehydrated in a series of graded alcohols and were finally dried to a critical drying point with a critical point dryer unit. The discs were held over stubs and gold coating was performed using an automated gold coater (Model: JOEL JFC-1600, JOEL Limited, Akishima, Tokyo, Japan) for 5 min. Photographs were taken using a scanning electron microscope (Model: JOEL-JSM 5600, JOEL Limited, Akishima, Tokyo, Japan). All the experiments were done in triplicates. Representative pictures are presented.

Real-time PCR Analysis

Hyphal genes expression of yeast to hyphal form transition in serum induction was studied using Real Time PCR. *C. albicans* yeast cells (1×10^6 cells/ml) were inoculated in 20% serum containing 12.5 µg/ml of dicyclomine and incubated for 90 min. The total RNA was isolated using RNeasy® Mini Kit (QIAGEN). Then, cDNA was built by

Table 2 Gene-specific primers used in qRT-PCR

Primers	Sequence (5′→3′)
ACTIN-F	5′ ATGGACGGTGAAGAAGTTGC 3′
ACTIN-R	5′ ACCTCTTTTGGATTGGGCTTCA 3′
ECE1F	5′ CCCTCAACTTGCTCCTTCACC 3′
ECE1R	5′ GATCACTTGTGGGATGTTGGTAA 3′
EFG1F	5′ TATGCCCCAGCAAACAACTG 3′
EFG1R	5′ TTGTTGTCCTGCTGTCTGTC 3′
TEC1F	5′ AGGTTCCCTGGTTTAAGTG 3′
TEC1R	5′ ACTGGTATGTGTGGGTGAT 3′
CST20F	5′ TTCTGACTTCAAAGACATCAT 3′
CST20R	5′ AATGTCTATTTCTGGTGGTG 3′
HST7F	5′ ACTCCAACATCCAATATAACA 3′
HST7R	5′ TTGATTGACGTTCAATGAAGA 3′
CEK1F	5′ AGCTATACAACGACCAATTAA 3′
CEK1R	5′ CATTAGCTGA ATGCATAGCT 3′
PDE2F	5′ ACCACCACCACTACTACTAC 3′
PDE2R	5′ AAAATGAGTTGTTCCTGTCC 3′
TUP1F	5′ GAGGATCCCATGTATCCCCAACGCACCCAG 3′
TUP1R	5′ GGCGACGCGTCGTTTTTTGGTCCATTTCCAAATTCTG 3′
MIG1F	5′CTTCAACTAGCCTATATTCCGATGG 3′
MIG1R	5′-CTTTCTGTAGGTACCAACAACTAC 3′
BCY1F	5′ CCCAAGCTTATGTCTAATCCTCAACAGCA 3′
BCY1R	5′ GGGCTGCAGTTAATGACCAGCAGTTGGGT 3′
RFG1F	5′-CACACATAGGTACCCCAATACAC-3′
RFG1R	5′CACTTTAAACAGATAAACTCGAGGATATG-3′
UME6F	5′ TCTACTTCTAATCCAATGGTG 3′
UME6R	5′ TATCATTACTTGATTTTTTCCGAG 3′

using SuperScript® III for first strand synthesis (Invitrogen, Life Technologies, USA). PCR reactions were carried out using KAPA SYBR® Fast qPCR Kit Master Mix (2×) (BIOSYSTEMS, South Africa) in 96-well PCR plates with preliminary denaturation for 3 min at 95 °C and were followed by 32 amplifications cycles of denaturation at 95 °C for 30 s. The annealing step was done at 60 °C for 20 s and primer extension at 72 °C for 30 s (CFX 96 Real time System, Bio-Rad, USA). Primers were purchased from Eurofins Genomics India Pvt. Ltd. They are tabulated in Table 2. Actin gene was used as an internal control and the transcript levels of these genes were calculated using the formula $2^{-\Delta\Delta CT}$ [42]. All the experiments were performed in triplicates.

Statistical analysis

The mean with standard deviations of values were obtained from three different observations. Values in the control and treatment groups were compared using Student's t test. A value of $P < 0.05$ was considered statistically significant.

Compliance with ethical standards

References

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