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Short Communication

Aripiprazole repurposed as an inhibitor of biofilm formation and sterol biosynthesis in multidrug-resistant *Candida albicans*

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ABSTRACT

Drug repurposing is an anticipative chemotherapeutic strategy that accentuates the inadequacy of antifungal drugs. The study identifies an antipsychotic drug, aripiprazole, as a biofilm and hyphal inhibitor of *Candida albicans*. Microtitre plate biofilm inhibition, metabolic activity and hyphal inhibitory assays were used to assess the potency of aripiprazole; and filipin staining, reactive oxygen species staining, cAMP rescue, propidium iodide staining, computational studies and qRT-PCR assays were used to elucidate its mode of action. The study revealed aripiprazole functioned in a manner similar to standard azoles, particularly the imidazole, ketoconazole, by inhibiting pseudohyphal formation during the early stages of hyphal development. The action of aripiprazole on *C. albicans* was dose-dependent and it exhibited varied mechanisms of action at low and high dosages. At low dosage, aripiprazole outperformed ketoconazole in terms of inhibiting biofilm formation, hyphal filamentations, and yeast flocculation, whereas at higher dosage it mimicked ketoconazole. This study illustrates the anti-candidal potential and mechanistic activities of aripiprazole, and indicates the future use of this drug as an anti-biofilm agent.

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1. Introduction

The armamentarium of drugs available for treating fungal infections is woefully inadequate, and three-quarters of antifungal drugs are azoles [1]. Topical and systemic antifungal imidazoles and triazoles are frontline defences against invasive fungal infections. Almost all azoles function by inactivating lanosterol 14-alpha-demethylase (CYP51) of *C. albicans*, which plays a prominent role in sterol biosynthesis by converting lanosterol to ergosterol [2]. Reduced production of ergosterol impedes cell wall synthesis, and renders fungal cell membranes porous, which leads to leakage of intracellular contents, an inability to reproduce, and death.

Resistance to azoles has been reported for several clinical strains of pathogenic yeasts and has been attributed predominately to mutations in the sterol biosynthesis pathway and/or to upregulation of drug efflux pumps [3,4]. *Candida albicans* is the most common pathogenic yeast that readily grows as a biofilm on implanted medical devices and human body parts [5]. Preventing biofilm growth is a challenge [1,6]. Most azoles exhibit fungicidal effects but these drugs should also eradicate biofilm growth (an intrinsic trait of these pathogens) to prevent reappearance of drug-resistant persister cells that cause superinfections with cataclysmic consequences.

Drugs that annihilate *C. albicans* biofilms have recently been screened, synthesised and researched [5]. Much effort has been expended on determining the antibacterial potential of drugs from pharmacologically distinct families [5,7]. Several antipsychotic drugs have been studied with a view to repurposing them for antimicrobial chemotherapies. The third-generation atypical antipsychotic, aripiprazole is one of the more recent additions to this repurposing list. Aripiprazole is clinically approved by the European Medicines Agency and the Food and Drug Administration for the treatment of acute mania and schizophrenia, depression, and bipolar disorder, and is regarded as a catalyst to encourage further research. Aripiprazole was recently reported to be ineffective against bacterial pathogens [7]; however, we found it highly effective against *C. albicans* biofilms. The present study was conducted to compare the efficacies of aripiprazole and antifungal azoles and determine the mode of action of aripiprazole against *C. albicans*.

2. Materials and methods

2.1. Yeast strains and culture conditions

Yeast strains of *C. albicans* (DAY 185, ATCC 10231, ATCC 24433, and ATCC 18804) were procured from the Korean Culture Centre

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of Microorganisms or from the American Type Culture Collection. Yeasts were maintained in potato dextrose agar (PDA) or potato dextrose broth (PDB) and/or Roswell Park Memorial Institute medium-1640 (RPMI). Aripiprazole, itraconazole, ketoconazole, and amphotericin B were purchased from Sigma Aldrich (USA) and dissolved in dimethyl sulphoxide (DMSO).

2.2. Polystyrene plate biofilm inhibition assay

Biofilm assays were performed in 96-well microtitre plates using the crystal violet staining method as previously described [8]. Briefly, *C. albicans* were inoculated into PDB and incubated overnight at 37 °C with shaking. Cultures were re-inoculated into fresh PDB and treated with aripiprazole, itraconazole, or ketoconazole (0-50 μg/mL). Microtitre plates were incubated at 37 °C without shaking for 24 h, and biofilms that adhered to plate bottoms were stained with 0.1% crystal violet for 20 min, washed repeatedly with sterile distilled water, and resuspended in 95% ethanol. Plates were read at 570 nm. For assessing cell viability, XTT assay was conducted as previously described [9] (detailed procedure and other assays of yeast-hyphae-transition, colony morphology, and yeast flocculation are provided in the Supplementary materials).

2.3. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) of aripiprazole and ketoconazole were estimated using the microdilution method using 14 mL polystyrene tubes (SPL Life Sciences, Korea) [10]. Briefly, overnight cultures of *C. albicans* (DAY 185, ATCC 10231, ATCC 24433, or ATCC 18804) were treated with various concentrations (0-1000 μg/mL) of aripiprazole or ketoconazole and incubated at 37 °C for 24 h with constant shaking at 250 rpm. Broth turbidity was then measured at 620 nm. Results were recorded as MIC_{90} (concentration that killed 90% of yeast cells [11]).

2.4. Sterol staining, cAMP rescue, ROS and gene expression assays

Filipin is a chemical that stains sterols and is frequently used to study sterol distribution in *C. albicans*. Briefly, overnight cultures of *C. albicans* ATCC 10231 were inoculated into fresh PDB (in 14 mL polypropylene tubes) supplemented with aripiprazole (50 μg/mL) or ketoconazole (50 μg/mL). Tubes were then incubated for 90 min at 37°C with constant shaking (250 rpm) when pseudohyphal elements were stained with filipin (20 μg/mL) (Sigma-Aldrich, USA) for 30 min. Imaging was performed using a DAPi filter in an iRiS™ Digital Cell Imaging System (Logos Bio Systems, Korea). Other mechanism-based assays (qRT PCR assays with the gene specific primers [Table S4], cAMP rescue and reactive oxygen species [ROS]) are provided in the Supplementary materials.

2.5. Propidium iodide (PI) staining

Propidium iodide (PI) is a universal marker for studying loss of membrane integrity [12]. *C. albicans* ATCC 10231 grown in PDB with or without aripiprazole (250 μg/mL) or ketoconazole (250 μg/mL) was pelleted, and stained with PI (final concentration 30 μM) for 1 h. Cells were then de-stained in distilled water and visualized under a fluorescent microscope (iRiS™ Digital Cell Imaging System) under a green ultraviolet LED light.

2.6. Molecular docking assay

Computational studies were conducted to elucidate the binding modes of ligands (aripiprazole derivatives or standard antifungal azole) with a standard azole target as previously described [9]. Interactions between ligands and lanosterol 14-alpha-demethylase (CYP51) of *C. albicans* retrieved form Protein Data Bank (PDB: 5V5Z, DOI: 10.2210/pdb5V5Z/pdb) were studied using Schrödinger Maestro 11.4 (Schrödinger Software Solutions, USA). The detailed lists of ligands and docking protocols are provided in the Supplementary materials.

2.7. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test using SPSS version 23 (SPSS Inc., Chicago, IL, USA). All experiments were conducted in triplicate, and results are expressed as means ± standard deviations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results and discussion

3.1. Aripiprazole restrained C. albicans biofilm formation

Aripiprazole prevented biofilm formation by *C. albicans* on the polystyrene surface (Fig. 1a). A significant reduction (~60%) in biofilm formation was noted for aripiprazole at 10 μg/mL, and 80% and 90% reductions were noted at 25 and 50 μg/mL, respectively (Fig. 1a). However, aripiprazole had no candicidal effect at 10-50 μg/mL under static conditions (Figure S1). In contrast, ketoconazole did not noticeably reduce biofilm formation at these concentrations (Fig. 1b), although it had a marginal effect at 50 μg/mL (Fig. 1b). Ketoconazole concentration-dependently affected the growth profile of ATCC 10231 strain, which indicates a candicidal effect (Figure S1). However, the strain was wholly resistant to itraconazole, which showed no effect on yeast growth or biofilm formation (Fig. S1). Aripiprazole also exhibited anti-biofilm activity against another drug-resistant *C. albicans* strain, DAY 185, at concentrations as low as 5 μg/mL without compromising the growth profile (Fig. S2). The results were further confirmed by XTT assay (Figs. S1 and S2).

3.2. Aripiprazole and ketoconazole had similar effects on the growth profiles of C. albicans

MICs of aripiprazole and ketoconazole against *C. albicans* strains were determined. Both agents exhibited equivalent anti-candidal activities at higher concentrations, and the MIC_{90} of both was >500 μg/mL (Figs. 1c and 1d). Both drugs were highly effective against other *C. albicans* strains (ATCC 24403, and ATCC 18804) and showed similar MIC readings (≤100 μg/mL) (Table S1). These results indicate aripiprazole might adopt the mechanism of action of ketoconazole at higher dosages. In addition, we tested the efficacy of antifungal amphotericin B against *C. albicans* ATCC 10231 biofilms and growth. The drug was effective against *C. albicans* biofilms because of its fungicidal activity, with an MIC of 0.5 μg/mL (Fig. S3).

3.3. Aripiprazole and ketoconazole restrained hyphal protrusions in solid and liquid media

The hyphal form of *C. albicans* is considered a prerequisite of pathogenicity in hosts. Aripiprazole-treated colonies displayed smooth edges and no hyphal protrusions, whereas 25 μg/mL ketoconazole-treated colonies were smaller and had shrunken edges (Fig. 1e). Top views of control yeast colonies showed inconsistent wrinkle patterns, which were not observed when aripiprazole or ketoconazole were used (Fig. 1f). Wrinkled colonies are known to be frequently associated with biofilm formation in

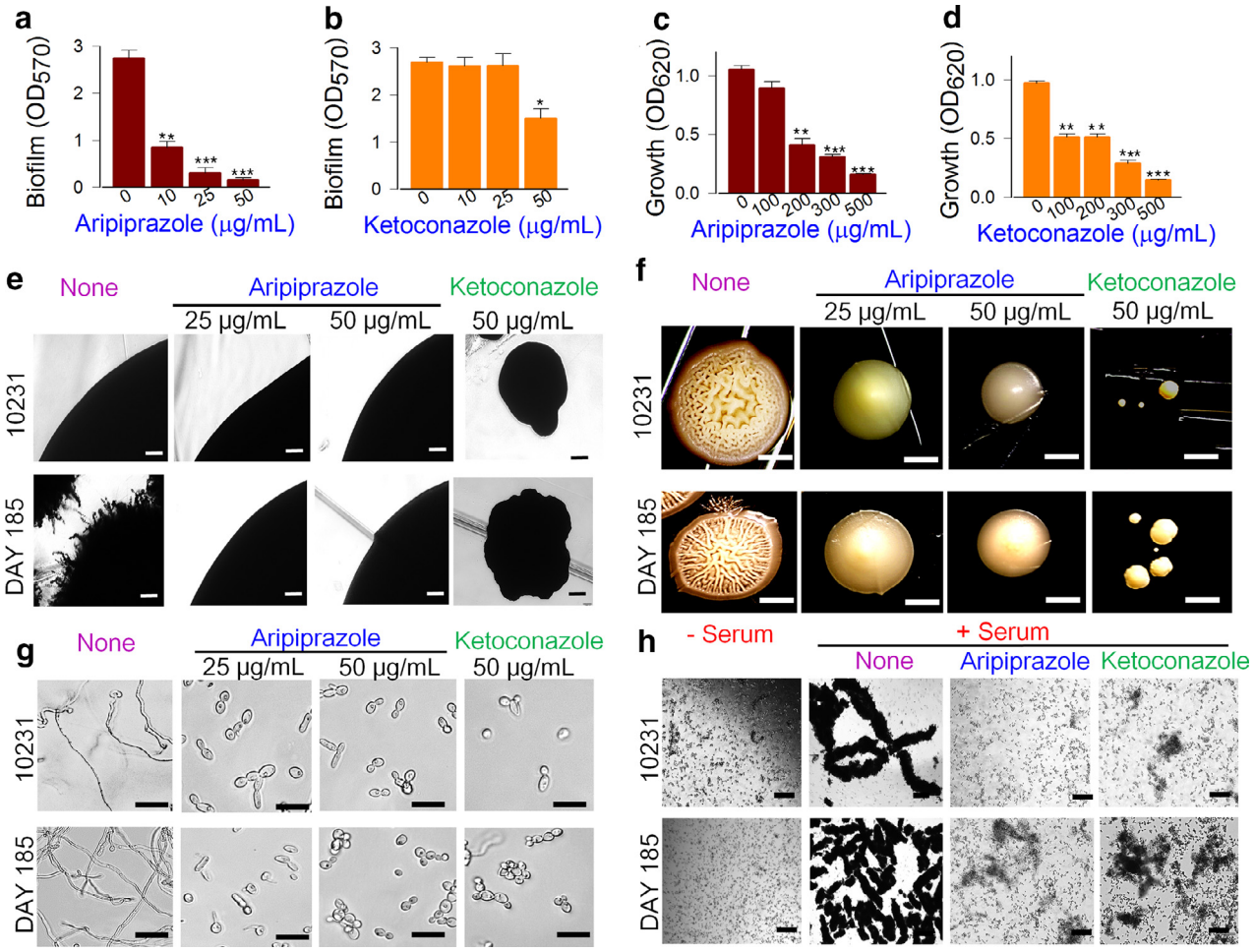

Fig. 1. Effects of aripiprazole and ketoconazole on *C. albicans* ATCC 10231. Crystal violet quantification of *C. albicans* biofilms treated with (a) aripiprazole, and (b) ketoconazole, growth inhibitory profiles of *C. albicans* treated with (c) aripiprazole, and (d) ketoconazole, (e) inhibition of hyphal protrusions from embedded colonies by aripiprazole and by ketoconazole following 7 d of incubation at 37 °C, scale bar=100 μm, (f) disruption of colony wrinkles by aripiprazole and by ketoconazole following 7 d of incubation at 37 °C, (g) inhibition of yeast-to-hyphal transitions under anaerobic conditions by aripiprazole or ketoconazole, scale bar=20 μm, and (h) inhibition of yeast flocculation by aripiprazole or ketoconazole under serum-induced conditions (3% human serum), scale bar=100 μm. Graphs are plotted as means ± SEMs. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs. non-treated controls.

C. albicans [13]; therefore, abolishment of wrinkles on colonies by aripiprazole could be considered to mirror its ability to prevent biofilms. Also, aripiprazole inhibited yeast-to-hyphal transitions in liquid RPMI under aerobic conditions at concentrations as low as 5 or 10 μg/mL (Fig. S4). Under anaerobic conditions, it is difficult to limit *C. albicans* hyphae, and several antifungal azoles or polyenes at their active concentrations have failed to annihilate filamentations [14]. In our study, aripiprazole effectively inhibited the hyphae under anaerobic conditions of two azole-resistant strains at its biofilm inhibitory concentration (BIC; 25 μg/mL) (Fig. 1g). Similarly, ketoconazole also suppressed filamentations at 50 μg/mL (Fig. 1g).

3.4. Aripiprazole and ketoconazole suppressed yeast flocs

Flocs, or aggregates, are cohesive yeast clusters that enable cells to evade stress and protect cells from harsh environments [15]. Flocculation has been shown to enable long-term survival and facilitate mating. In the presence of 3% human serum, strong dense flocculation was observed in non-treated controls but not in aripiprazole- or ketoconazole-treated groups (Fig. 1h). Aripiprazole was significantly better than ketoconazole at preventing floc formation at similar dosages.

3.5. Aripiprazole and ketoconazole did not suppress the cAMP pathway or activated ROS

Biofilm formation by *C. albicans* usually depends on its achieving a hyphal form, and Ras/cAMP/PKA signalling pathway in *C. albicans* is important in this [16]. Supplementation of db-cAMP (5 mm) did not recover biofilm or hyphae production in aripiprazole- or ketoconazole-treated *C. albicans* ATCC 10231 (Fig. 2a i and ii). In addition, ROS-induced cell killing was not achieved by aripiprazole or ketoconazole (300 μg/mL). Fluorescence was only observed in cells treated with 50 μg/mL hydrogen peroxide (positive control) following staining with Carboxy-H2DCFDA (Fig. 2b). These findings indicate that neither aripiprazole nor ketoconazole triggered antioxidant response in yeasts cells.

3.6. Aripiprazole averted lipid raft polarization of hyphal tips at lower doses and damaged cell membranes at higher doses

Filipin is a quantitative biological marker of ergosterol in fungi [17] and staining with this marker was used to observe sterol distributions in *C. albicans* [18]. Filipin staining of non-treated *C. albicans* ATCC 10231 revealed pseudohyphal elements with fluorescent hyphal tips (Fig. 2c). In treated groups, pseudohyphal formation

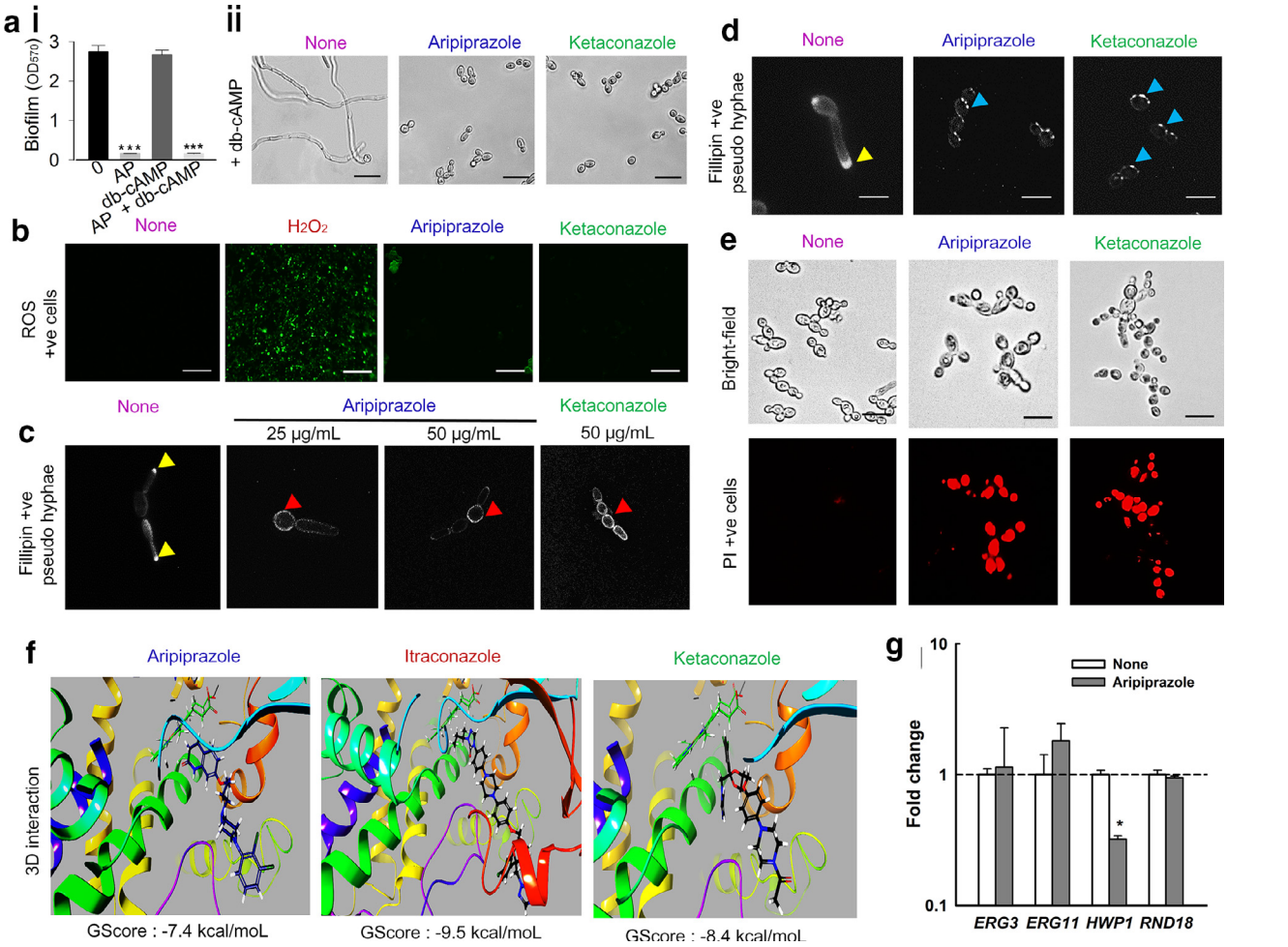

Fig. 2. Mechanistic study of the modes of action of aripiprazole and ketoconazole. (a) Effects of exogenous db-cAMP (5 mM) on *C. albicans* biofilms (i) and hyphal morphogenesis (ii), scale bar=20 µm, (b) H2DCFDA staining was used to estimate numbers of reactive oxygen species (ROS)-positive cells in aripiprazole- or ketoconazole-treated cultures, scale bar=100 µm, (c) filipin staining of pseudohyphal elements after incubation for 90 min with aripiprazole or ketoconazole (yellow arrowhead denotes concentrated filipin staining on emerging hyphal tips; red arrowhead indicates uniform filipin staining across yeast cell membranes), scale bar=20 µm, (d) filipin staining of pseudohyphal elements after incubation for 90 min with aripiprazole (250 µg/mL) or ketoconazole (250 µg/mL) (yellow arrowhead denotes concentrated filipin staining on emerging hyphal tips; blue arrowheads indicate filipin spots on cell membranes), scale bar=20 µm, (e) microscopic visualization of yeast cells stained with PI, scale bar=20 µm, (f) 3D interactions of aripiprazole, itraconazole, or ketoconazole with lanosterol 14-alpha-demethylase (CYP51), 3D interaction diagram shows the heme^{601} prosthetic group in green, aripiprazole in blue, and itraconazole and ketoconazole in black, and (g) expression profiles of *ERG3*, *ERG11* and *HWP1* genes in the presence and absence of aripiprazole (50 µg/mL). *RND18* is a housekeeping gene.

was completely blocked, and sterol polarisation to hyphal tips was not observed in 80% of cells (Fig. 2c). Similar findings were also reported for thiazolidinedione and succinamide derivatives, which are known to function by inhibiting sterol distribution [19]. At high dose (250 µg/mL), sterols concentrated at one spot (Fig. 2d) rather than being distributed throughout the cell membrane, indicating aripiprazole disrupted sterol distribution at high dose. PI staining of *C. albicans* ATCC10231 treated with sub-MIC concentrations (250 µg/mL) of aripiprazole or ketoconazole confirmed membrane damage in 70% of cells (Fig. 2e). Therefore, aripiprazole irreversibly stalled sterol synthesis and damaged cell membrane.

3.7. Molecular docking confirmed binding between aripiprazole and lanosterol 14-alpha-demethylase

Azole drugs are classified as lucid inhibitors of CYP51 [2]. Aripiprazole interacted efficiently with the heme group (Heme^{601}), and exhibited π–π face-to-edge interactions with Tyr^{118}, a feature shared with the other azoles tested (Table S2, Fig. 2f, and Fig. S5). The GScore and binding energy scores of aripiprazole were -7.4 and -73.48 kcal/moL, respectively; the scores of aripiprazole were similar to those of itraconazole and ketoconazole and far better than those of the other azoles tested (Fig. 2f and Table S2). As expected, all azoles interacted well with Heme^{601} and similarly exhibited Pi-Pi stacking with Tyr^{118} in the catalytic site of CYP51 (Table S2, Figs. S6 and S7). These data corroborated the binding modes depicted by Haitao et al., 2000 [20]. Gene expression analysis revealed that the mRNA levels of *ERG11* gene that encodes CYP51, as well as *ERG3*, were not altered by aripiprazole, which indicates aripiprazole is an enzyme inhibitor that binds heme prosthetic group, eventually blocking the catalytic cycle. However, the expression of *HWP1* (hyphal development gene) was significantly (3-fold) reduced, indicating its hyphal inhibitory activity (Fig. 2g). The interaction of a few aripiprazole derivatives with CYP51 was also tested (Table S2). An aripiprazole dimer bound significantly better than itraconazole (GScore: -9.5 kcal/moL, ΔG: -110.66 kcal/moL) with a GScore and binding energy of -10.95 and -131.77 kcal/moL, respectively (Table S3). The ring structure of aripiprazole contains di-chlorine atom like itraconazole and ketoconazole (Fig. S6 and Fig. 3) and is thought to be crucial for positive binding interactions. Further in-depth study of the

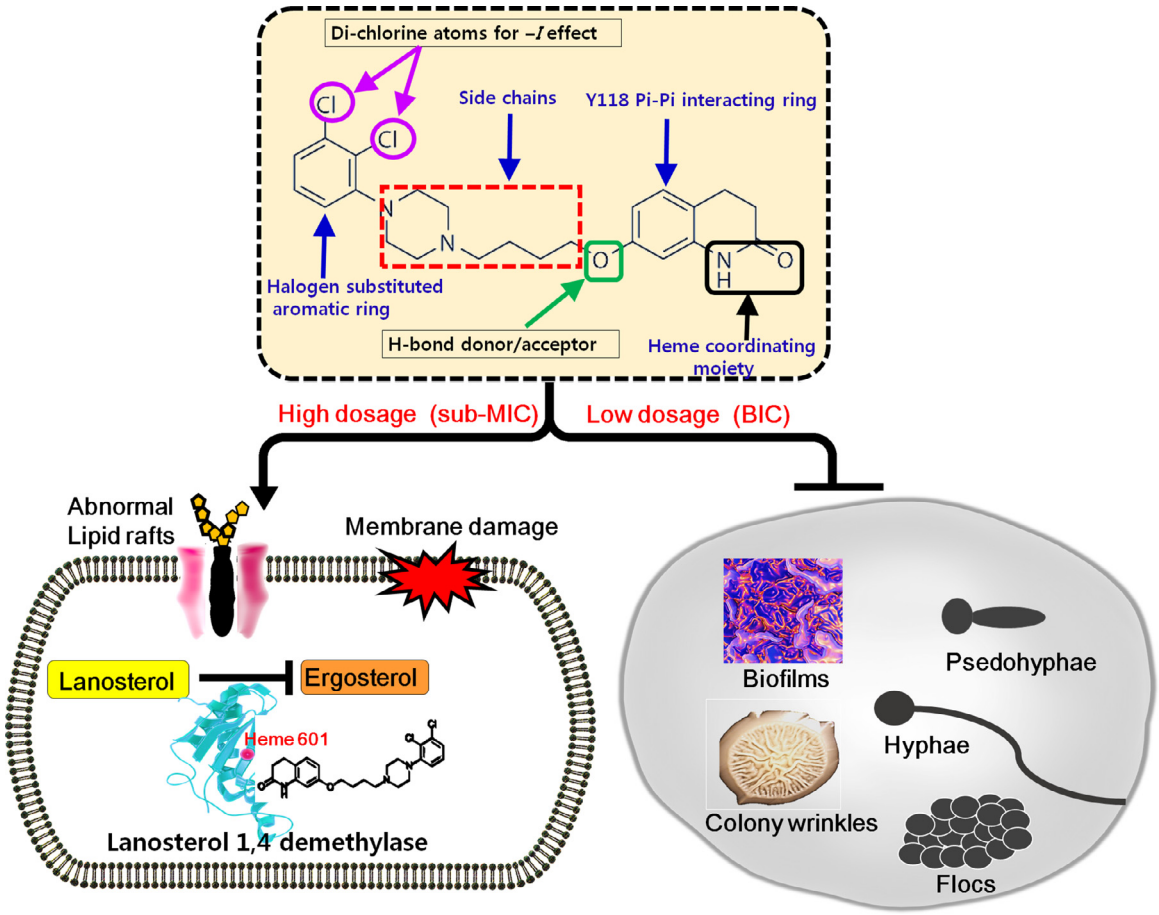

Fig. 3. Pharmacophore model and proposed mode of action of aripiprazole. Pharmacophore structure of aripiprazole showing moieties that mimic those of standard azoles. Pink arrows indicate the two chlorine atoms (pink circles) on the aromatic ring, the green arrow shows the H-bonding (donor/acceptor) oxygen (green box), and the black arrows indicate the heme-containing moiety (black box), side chains (red dotted box), and the Tyr^{118} π–π face-to-edge interacting ring, and the diagram depicts the differential mode of action of aripiprazole at low and high dosage. At low dosage (at BIC; 25 or 50 μg/mL), aripiprazole effectively suppressed biofilm-associated virulence factors whereas at high dosage (<sub-MIC; 250 μg/mL) aripiprazole mimicked the action mechanism of antifungal azoles by inactivating CYP51, thus disrupting lipid rafts and causing membrane damage.

aripiprazole derivatives/CYP51 interaction is warranted to discover other inhibitors of biofilm formation and sterol biosynthesis.

4. Conclusion

The results of this study indicate aripiprazole is a potent inhibitor of *C. albicans* biofilm formation and warrants point-of-care testing. At BIC, aripiprazole did not affect the planktonic cells of *C. albicans*, thus ruling out the chance of inducing selection pressure and plausible development of drug resistance. Molecular docking and in vitro assays provided an explanation of the mode of action of aripiprazole (Fig. 3). The costs of novel drug development are enormous and the times required are staggering. The repurposing of existing drugs offers possibilities of inexpensive, readily available solutions. It is hoped this study on the repurposing of aripiprazole might be viewed as an example and used to screen other clinically approved drugs for antifungal and antibiofilm activity.

Declarations

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Competing Interests

None

Ethical Approval

Not required.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.05.016.

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