Design, synthesis, antifungal activity, and QM/MM docking study of two azole derivatives with indole ring

Suat SARI 1 *, Didem KART 2

1 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hacettepe University, Sıhhiye 06100 Ankara, Turkey.
2 Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Hacettepe University, Sıhhiye 06100 Ankara, Turkey.
* Corresponding Author. E-mail: suat.sari@hacettepe.edu.tr (S.S.); Tel. +90-312-305 18 72.
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ABSTRACT: Systemic candidiasis is a major health issue for immunocompromised individuals due to the increase in drug-resistance among Candida spp., which are prevalent pathogenic fungi responsible for many types of fungal infections. Azoles are among the most preferred antifungal class for systemic candidiasis with broad antifungal spectrum and systemic availability. In this study, we synthesized and tested antifungal effects of two new indole derivatives to investigate the impact of indole on the activity of azole antifungal compounds and to find potent derivatives against Candida spp. including resistant strains and biofilms. 1-(4-Chlorophenyl)-2-(1H-imidazol-1-yl)ethanol 1H-indole-2-carboxylate (4a) showed excellent antifungal profile with several times more potent activity against the tested species including a fluconazole-resistant C. tropicalis isolate. The minimum inhibitory concentration (MIC) of 4a was 0.03125 µg/ml against C. albicans, which was 0.5 µg/ml for fluconazole. The compound also showed promising biofilm inhibitory effect compared to amphotericin B. The importance of indole was demonstrated through molecular docking studies with the structure of C. albicans CYP51, the established target of azole antifungals, using different protocols. QM/MM docking approach yielded excellent results and accuracy, especially regarding metal interactions. As a result, indole could be a very useful fragment to design new and highly potent antifungal compounds in azole structure.

KEYWORDS: Azole; antifungal; biofilm; Candida albicans; CYP51; indole; induced fit docking; QM/MM docking.

1. INTRODUCTION

Systemic candidiasis is a major health issue for immunocompromised individuals due to increasing drug-resistance among Candida spp., which are prevalent pathogenic fungi responsible for many types of fungal infections. Although C. albicans is the most commonly identified species in candidiasis, the rise of infections caused by non-albicans species with natural and acquired drug resistance is of great concern [1]. Azoles are among the most preferred antifungal class owing to a number of advantages, such as efficacy spectrum and systemic availability, however their common usage comes with the issue of resistance [2]. One of the causes of drug resistance is the ability of pathogens to form biofilms, a form of pathogen colonies found on biotic and non-biotic surfaces featuring structural matrices and extracellular polymers. Most antifungal compounds are effective against biofilms only at higher doses compared to their efficacy against the planktonic forms and there is little known about the antibiofilm properties of azoles [3].

Azoles emerged as a group of antifungal drugs with the introduction of imidazole derivatives such as clotrimazole, miconazole, and oxiconazole. Ketoconazole was the first broad spectrum antifungal with oral availability (Figure 1). Triazole antifungals led to reduced toxicity and side effects with fluconazole, golden standard of azole antifungals, and to improved efficacy with itraconazole. With the second-generation triazole antifungals, such as voriconazole and isavuconazole, and tetrazole antifungals such as VT-1161 (oteseconazole), wider spectrum was obtained including several resistant species [4,5]. Azoles possess three major pharmacophores: an azole ring (imidazole, 1,2,4-triazole, or tetrazole), an aromatic ring (usually halogenated benzene), and a tail group (mainly includes aromatic, heteroaromatic, or heterocyclic moieties) attached to the alkylene linker between the azole and aromatic ring. Azoles inhibit fungal lanosterol 14α-demethylase (CYP51), a cytochrome P450 enzyme that catalyzes biosynthesis of ergosterol, a crucial
component of fungal cell membranes. The azole ring is the pharmacophore responsible for blocking the heme co-factor, which catalyzes the oxidation process, while the aryl and the tail groups occupy the active site and provide a tight binding [6,7]. To date, various crystallographic studies have been reported elucidating CYP51 inhibition by azoles at molecular level [8-11].

![Figure 1. Azole antifungals and the title compounds.](https://doi.org/10.35333/jrp.2020.223)

Previously we reported a number of compounds in azole structure with antifungal effects [12,13], which include an indole derivative showing highly potent antifungal activity with a minimum inhibitory concentration (MIC) of 0.125 μg/ml against C. albicans (Figure 1) [14]. Thus, we designed and synthesized two novel indole derivatives, 1-(4-chlorophenyl)-2-(1H-imidazol-1-yl)ethanol 1H-indole-2-carboxylate (4a) and 2-(1H-imidazol-1-yl)-1-(2-naphthyl)ethanol 1H-indole-2-carboxylate (4b) (Figure 1). The title compounds, which share the typical azole antifungal scaffold, possess imidazole as azole ring, 4-chlorophenyl or 2-naphthyl as aryl group, and indole as tail group. The title compounds were tested for their effects against susceptible and resistant Candida spp., as well as C. albicans biofilms to obtain new potent antifungal molecules. We provided a detailed in silico analysis of C. albicans CYP51 (CAYP51) binding of these compounds using both classical and sophisticated docking approaches such as induced fit docking, which renders limited receptor flexibility to partially represent the dynamic nature of protein-ligand complexes, and quantum mechanics/molecular mechanics (QM/MM) docking, which enables more accurate electrostatic charge assignment in the case of charge polarization, especially for metal interactions, to better model ligand-metalloenzyme complexes.

2. RESULTS and DISCUSSION

2.1. Synthesis of the compounds

Compound 4a and 4b were prepared according to the scheme in Figure 2. Briefly, 1-(4-chlorophenyl)-2-(1H-imidazol-1-yl)ethanone (2a) and 2-(1H-imidazol-1-yl)-1-(2-naphthyl)ethanone (2b) were obtained by N-alkylation of imidazole with 2,4'-dichloroacetophenone (1a) and 2-bromo-1-(2-naphthyl)ethanone (1b) with an excess of imidazole in ice bath. 1a was purchased and 1b was synthesized via bromination of 1-(2-naphthyl)ethanone at the 2nd position in cold acetic acid. By reduction of 2a and 2b with sodium borohydride (NaBH₄), their alcohol derivatives, 1-(4-chlorophenyl)-2-(1H-imidazol-1-yl)ethanol (3a) and 2-(1H-imidazol-1-yl)-1-(2-naphthyl)ethanol (3b), were yielded. These starting materials and their syntheses were previously reported [15-17]. The title compounds were afforded via Steglich esterification of 3a and 3b with indole-2-carboxylic acid in dichloromethane (DCM) in the presence of a coupling agent and a transacylation catalyst, N,N′-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), respectively [18]. 4b was converted to its HCl salt to improve solubility, however 4a was not stable as its salt.
Figure 2. Synthetic scheme for the title compounds. Reagents and conditions: (i) HBrO₃, Br₂, CH₃COOH, 0-5 °C to rt, overnight; (ii) imidazole, DMF, 0-5 °C to rt, overnight; (iii) NaBH₄, CH₃OH, 0-5 °C, 1 h; (iv) indole-2-carboxylic acid, DCC, DMAP, DCM, 0-5 °C to rt, 6 h, aethereal gHCl (for 4b).

The reaction was initiated at 0-5 °C to minimize formation of N,N'-dicyclohexylurea (DCU), an infamous side product of DCC-coupled esterification, which precipitates during the reaction. The reaction starts with cleavage of a hydrogen from the carboxylic acid by DMAP (Figure 3). The resulting carboxylate undergoes a nucleophilic addition to DCC giving the O-acyl intermediate. DMAP makes a nucleophilic attack to the intermediate to trigger an addition-elimination process, which leads to hydrolysis of the O-acyl derivative and formation of the DCU and 1-acyl-DMAP salt. The alcoholic oxygen attacks to the carbonyl carbon of the salt, which leads to an addition-elimination process to finally yield the title esters, DCU, and DMAP unchanged [18].

Figure 3. Esterification mechanism for the title compounds suggested according to Steglich esterification.

The title compounds were afforded as racemates at moderate yields due to DCU removal processes, which was successfully performed according to the spectral data. In the ¹H NMR spectra of the title compounds, the integrations were in accordance with the number of the hydrogen atoms. The chemical shifts and coupling patterns were also confirmative of their structures. The diastereotopic CH₂ protons were observed at ~4.5 and ~5 ppm as doublet and multiplet for 4a and 4b, respectively. The same trend was observed for the chiral CHO hydrogen, which produced a triplet signal for 4a but a multiplet for 4b. This was
probably due to the differing effect of HCl salt on the magnetic environments of the enantiomers of 4b. The deshielding effect of the HCl salt on 4b protons was apparent, too, and this effect was marked the most for the imidazole H2, as expected (see Supporting Information for details). The 1H NMR spectra of the compounds were in accordance with their respective 1H NMR spectrum and their structures. In the LC-MS chromatograms of the compounds, one single and pure signal was observed for each compound, which gave molecular ion peaks in the respective ESI+ spectra (see Supporting Information for details).

### 2.2. Antifungal activity of the compounds

Initially, antifungal activities of the title compounds were evaluated by determining their MIC values against three American Type Culture Collection (ATCC) strains of Candida species (C. albicans, C. krusei, and C. parapsilosis) and one fluconazole-resistant C. tropicalis isolate. The results were impressive, especially regarding 4a, which was found more than 10 times as potent as fluconazole against C. albicans (Table 1). The compound also showed excellent activity against C. krusei and C. parapsilosis, the former being intrinsically resistant to fluconazole. In addition, 4a was highly potent against the C. tropicalis isolate. The MIC values of fluconazole against C. krusei and C. tropicalis was found more than 100-fold higher than those of 4a. Although much less potent than 4a, 4b showed promising antifungal potential with MIC values comparable to those of fluconazole.

| Compound | C. albicans ATCC 90028 (µg/ml) | C. krusei ATCC 6258 (µg/ml) | C. parapsilosis ATCC 90018 (µg/ml) | C. tropicalis (resistant isolate) MBIC (µg/ml) | C. albicans antibiofilm MBEIC (µg/ml) |
|----------|-----------------|-----------------|-----------------|---------------------|-----------------|
| 4a       | 0.03125         | 0.25            | 0.25            | 0.5                 | 8               |
| 4b       | 4               | 64              | 4               | 128                 | 64              |
| Fluconazole | 0.5            | 32              | 0.5             | 64                  | -               |
| Amphotericin B | -               | -               | -               | -                   | 4               |

Pathogenicity of Candida spp. also results from their ability to form biofilms. Factors such as extracellular polymeric substances and dimorphism render biofilms less vulnerable to host immune system and antifungal drugs. Therefore, antifungals show higher MIC values against the biofilms of Candida spp. than their planktonic forms [19]. 4a and 4b were tested for their ability to inhibit and eradicate C. albicans biofilms by determining minimum biofilm inhibitory and eradication concentrations (MBIC and MBEC, respectively). 4a appeared as an effective biofilm inhibitor (MBIC = 8 µg/ml) with a potency close to amphotericin B (Table 1), which is known for its anti-biofilm activity [20]. When the MIC values of the compounds are compared to their MBIC values, the decreased sensitivity of C. albicans biofilms is apparent. Like amphotericin B, however, the tested compounds showed limited biofilm eradication activity, which is also a typical shortcoming ofazole antifungals [21].

These results clearly show that 4-chlorophenyl moiety is much better than 2-naphthyl as the aryl pharmacophore for antifungal activity of imidazole derivatives with an indole tail.

### 2.3. Molecular modelling studies

#### 2.3.1. Molecular descriptor calculations

Poor pharmacokinetics and toxicity cause high attrition rates in late drug discovery stages. Eliminating such compounds in early drug development studies is crucial and in silico methods are widely used for this purpose [22]. A number of descriptors commonly used to identify drug-like chemical space (molecular weight (MW), number of rotatable bonds (RB), hydrogen bond donor and acceptor counts (HD and HA), LogP, and polar surface area (PSA)) [23,24] were calculated for 4a and 4b. These descriptors were found within the values ranges identified by QikProp (2019-4, Schrödinger, LLC, New York, NY, 2019) for drug-like chemical space (Table 2). The reliability of this software for the calculated descriptors was previously established [25]. According to QikProp, the compounds were also free of nonspecific reactive fragments, which are generally checked for identifying pan-assay interference compounds (PAINS). These unwanted fragments are infamous for producing to false positive results [26].

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Table 2. Molecular descriptors\(^a\) calculated for 4a and 4b.

| Compound | MW (130-725 Da) | RB (0-15) | HD (0-6) | HA (2-20) | LogP (-2-6.5) | PSA (7-200 Å\(^2\)) | Reactive fragments |
|----------|-----------------|-----------|----------|-----------|--------------|---------------------|-------------------|
| 4a       | 365.8           | 5         | 1        | 4         | 5.0          | 63.3                | 0                 |
| 4b       | 381.4           | 5         | 1        | 4         | 5.6          | 62.5                | 0                 |

\(^a\) Value range defined for drug-likeness for each descriptor is provided. Number of reactive fragments should be 0 for compounds not to be defined as PAINS.

2.3.2. Molecular docking of the compounds to CACY5P1

The crystal structure of CACY5P1 used in this study (PDB ID: 5TZ1 [8]) includes VT-1161, which represents a typical binding for azole CYP51 inhibitors regarding the orientation of the tetrazole with respect to the heme co-factor, positioning of the 2,4-difluorophenyl group at the bottom of the cavity, and occupation of the active site gorge by its tail (Figure 4A). The key metal coordination between tetrazole N\(^4\) and heme iron, which also coordinates with the nitrogen atoms of protoporphyrin pyroles and one cysteine (Cys470) sidechain S\(^-\), is also apparent in this crystal structure (Figure 4B). The aryl pharmacophore also engages in strong electrostatic interactions with the heme. The tail group interacts with Tyr118 and His377, which are among the conserved residues of eukaryotic CYP51 catalytic sites and known to engage with several other CYP51 inhibitors [8,27]. More importantly, Tyr132 makes a water-mediated H bond with the hydroxyl attached to the ethylene linker of VT-1161, which can be regarded as the fourth pharmacophore since it is conserved among the second-generation triazole and tetrazole antifungals. Actually, H bond interaction with this residue is considered an important molecular determinant for potent inhibition of the enzyme [28,11]. Coordination of azole nitrogen with the heme iron has two key aspects: the distance between the two atoms and the angle between the azole and protoporphyrin planes [29]. The nitrogen-iron distance for coordination is typically lower than 2.5 Å [30]. In the case of 5TZ1, this distance is 2.15 Å (Figure 4C); for other azole-CACY5P1 complexes, it is measured as 2.08 Å (PDB ID: 5FSA [8]) and 2.19 Å (PDB ID: 5V5Z [31]). In order to keep these atoms within coordination distance and provide an effective electrostatic engagement, the azole and protoporphyrin planes should be close to perpendicular orientation [29]. In the case of 5TZ1, the angle between the two rings is 84.0° (Figure 4C); for 5FSA and 5V5Z, it is measured as 79.0° and 79.7°, respectively.

We performed molecular docking studies using four different protocols of Glide (2019-4, Schrödinger, LLC, New York, NY, 2019) [32-34]: standard precision (SP), extra precision (XP), Induced Fit (2019-4, Schrödinger, LLC, New York, NY, 2019) [35], and QM-Polarized Ligand Docking (QPLD) (2019-4, Schrödinger, LLC, New York, NY, 2019) [36], the QM/MM docking protocol of Glide. The first three protocols use conventional MM calculations. SP and XP are typical rigid-receptor docking modes with XP providing high exhaustiveness. Induced fit provides limited receptor flexibility thus better accuracy, since
macromolecule crystal structures usually have energy constrains and ligand-receptor complexes are dynamic hand-glove models rather than rigid key-lock models. Induced fit docking is better at simulating the former at a reasonable computation burden and time cost. In these MM methods, atoms are assigned constant charges, however non-bonded interactions trigger charge polarization. In this respect, QM methods suggest more accurate charge calculations. QPLD offers ab initio method for calculating ligand atom charges polarized by the protein environment. This is especially helpful for metal interactions where charge polarization plays greater role and MM calculations usually fail to model them with high precision [36]. Before running molecular docking simulations of 4a and 4b, we tested the accuracy of the docking protocols used in this study by redocking VT-1161 to the active site of 5TZ1 and calculated the RMSD values of the best pose from each protocol with respect to the co-crystallized binding conformation of VT-1116. The RMSD values were 0.61, 0.64, 0.77, and 0.51 Å for SP, XP, induced fit, and QPLD, respectively, showing good predictive capacity for Glide, especially with QPLD protocol, since lower RMSD means closer alignment of the two conformers (Figure 4D). Docking scores of these poses were -9.4, -6.9, -10.2, and -7.3 kcal/mol, respectively.

In general, molecular docking of 4a and 4b yielded roughly similar docking scores regarding VT-1161 (Table 3). Improvement in the scores of 4a going from SP to QPLD was noteworthy. However, docking scores might be misleading unless associated with fulfilment of key molecular determinants. The binding modes predicted by Glide were in accordance with the general orientation of azoles in the crystallographic studies of CYP51. In the case of 4a, improvement in the binding interactions was parallel to the improvement in the scores (Figure 5A-D). In the SP mode for 4a, Glide predicted a H bond with His377 via indole, with the heme π-cation via 4-chlorophenyl and π-π interactions via imidazole (Figure 5E). In the XP mode, the H bond with His377 disappeared but with Induced Fit, a H bond with the water that mediates with Tyr132 was formed through indole NH, along with a π-π stack with Phe380 through indole (Figure 5F and 5G). However, only with QPLD protocol was Glide able to model the metal coordination with the heme iron (Figure 5H). In the case of 4b, interactions with the key residues such as heme, Tyr132, and His377 were obtained, however no coordination with the iron was predicted by QPLD (See Supporting Information for details). The N3-iron distance and imidazole-protoporphyrin planar angle were especially helpful to better understand the difference between the binding modes of 4a and 4b (Table 4), which probably played a role in the difference of their activity results. Although these two metrics for 4a improved going from SP to QPLD and reached to almost ideal with QPLD (Figure 6A-D), the same was not true for 4b (Figure 6E-H). 4b’s favorable score for the pose predicted by Induced Fit was not reliable when the distance and angle metrics were considered (Figure 6G). These results clearly show that ideal interpretation of docking results is possible when docking scores and binding interactions are considered together and QM/MM approach is more reliable and accurate than MM methods, especially when metal interactions are handled. As for 4a, indole provided a very promising moiety for engaging with the active site gore regarding its H donor NH group, which seems to have replaced the hydroxyl of VT-1161.

Table 3. Docking scores (kcal/mol) of 4a and 4b.

| Compound | SP  | XP  | Induced fit | QPLD |
|----------|-----|-----|-------------|------|
| 4a       | -6.5| -6.7| -7.9        | -8.8 |
| 4b       | -7.3| -4.3| -10         | -6.0 |

Table 4. Imidazole N3-heme iron distance (Å) and imidazole-protoporphyrin angle (°) values for the poses of 4a and 4b predicted by Glide.

| Compound | Metric | SP  | XP  | Induced fit | QPLD |
|----------|--------|-----|-----|-------------|------|
| 4a       | Distance | 3.50| 3.33| 2.56        | 2.45 |
|          | Angle   | 70.6| 68.7| 76.8        | 79.7 |
| 4b       | Distance | 2.97| 3.27| 4.05        | 2.92 |
|          | Angle   | 48.8| 81.3| 63.8        | 89.1 |
4. CONCLUSION

In search of potent antifungal compounds, we synthesized and tested two indole-derivative azole compounds with reference to our previous study showing the potential of indole as a tail substitution. As a result, we obtained 4a, a highly active azole compound against both susceptible and resistant Candida spp., as well as C. albicans biofilms. The compound was several times more potent than the reference drug, fluconazole. Docking studies with CACYP51 structure, the major target for azole antifungals, also confirmed the importance of indole, as well as the importance of QM/MM approach in docking studies. Furthermore, the study demonstrated that 4-chlorophenyl as the aryl pharmacophore was much better than 2-naphthyl. Thus, new indole derivatives will be designed and tested as future studies to make use of this fragment for antifungal efficacy.

Figure 5. Binding modes (A-D) and interactions (E-H) of 4a in STZ1 active site predicted by SP, XP, Induced Fit, and QPLD protocols, respectively.

Figure 6. Coordination distances of imidazole N³ and heme iron and plane angles between imidazole and the protoporphyrin rings for the poses of 4a (A-D) and 4b (E-H) predicted by SP, XP, Induced Fit, and QPLD protocols, respectively.
4. MATERIALS AND METHODS

4.1. Chemistry

All the reagents and chemicals used in this study were purchased from Sigma-Aldrich (USA) and Merck (Germany). For thin layer chromatography (TLC) Merck Kieselgel 60 F254 was used as stationary phase with chloroform-methanol (90:10) solvent system. TLC plates were inspected under 254 nm UV light. Melting points (mp) were determined using a Thomas-Hoover capillary melting point apparatus (USA) and uncorrected. $^1$H NMR (400 MHz) and $^{13}$C NMR (125 MHz) spectra were recorded by a Varian Mercury 400 FT (USA) and Bruker Avance 500 (Germany) NMR spectrometers, respectively. Tetramethylsilane was used as internal reference and the chemical shifts are expressed as δ (ppm) values. The splitting patterns are described as s (singlet), d (doublet), t (triplet), and m (multiplet). LC-MS spectra were obtained using a Micromass ZQ mass spectrometer (USA) connected to Waters Alliance HPLC (USA) with electrospray ionization (ESI+) method and MassLynx 4.1 software.

4.1.1. Synthesis of the compounds

The starting compounds, which were previously reported, were synthesized according to the literature methods. 2,4’-Dichloroacetophenone (1a) was purchased and 2-bromo-1-(2-naphthyl)ethanone (1b) was afforded by bromination of commercially obtained 2-acetonaphthone (1) at the second position, and both of were reduced using NaBH$_4$ to give 1-(4-chlorophenyl)-2-(1H-imidazol-1-yl)ethanol (3a) and 2-(1H-imidazol-1-yl)-1-(2-naphthyl)ethanol (3b), respectively (See Supporting Information for details) [15-17].

The title compounds were synthesized according to Steglich esterification [18]. A mixture of indole-2-carboxylic acid (1 mmol), N,N’-dicyclohexylcarbodiimide (DCC) (1 mmol), and 4-dimethylaminopyridine (DMAP) (0.07 mmol) in dichloromethane (DCM) was added to a mixture of 3a or 3b (1 mmol) in DCM at 0–5 °C dropwise. The mixture was stirred for 20 minutes in ice bath (0–5 °C) then for 6 hours at room temperature. The precipitate was filtered off, the filtrate was kept at ~8 °C overnight, re-filtered, and evaporated under vacuum. The residue was purified via column chromatography (chloroform-methanol 90:10). 4b was converted to its HCl salt form using ethereal solution of gaseous HCl (gHCl). The title compounds were crystallized from methanol. Their structure and purity were validated through $^1$H NMR, $^{13}$C NMR, and LC-MS spectral data.

1-(4-Chlorophenyl)-2-(1H-imidazol-1-yl)ethanol 1H-indole-2-carboxylate (4a): White powder (150.0 mg, 41 % yield); mp: 182-3 °C (methanol); $^1$H NMR (400 MHz, DMSO-d$_6$): δ = 4.51 (d, J = 6.5 Hz, 2H, CH$_2$), 6.22 (t, J = 6.0 Hz, 1H, CHO), 6.84-7.72 (m, 12H, 4-chlorobenzene, indole H$^{2,5}$, imidazole), 11.93 (s, 1H, indole H$^6$); $^{13}$C NMR (125 MHz, DMSO-d$_6$): δ = 51.0 (CH$_2$), 74.5 (CHO), 109.2-138.4 (17C, aromatic), 160.4 (CO); LC-ESI+ m/z: 369 [(M+3)+H]+, 366 (%100) [(M+1)+H]+, 298.

2-(1H-imidazol-1-yl)-1-(2-naphthyl)ethanol 1H-indole-2-carboxylate hydrochloride (4b): White powder (191.9 mg, 46 % yield); mp: 168-70 °C (methanol); $^1$H NMR (400 MHz, DMSO-d$_6$): δ = 4.89-4.99 (m, 2H, CH$_2$), 6.52-6.55 (m, 1H, CHO), 7.10-8.06 (m, 14H, naphthalene, indole H$^{2,5}$, imidazole H$^{3,4,5}$, 9.42 (s, 1H, imidazole H$^7$), 12.18 (s, 1H, indole H$^6$); $^{13}$C NMR (125 MHz, DMSO-d$_6$): δ = 52.9 (CH$_2$), 74.5 (CHO), 109.5-138.2 (21C, aromatic), 160.4 (CO); LC-ESI+ m/z: 383 (%100) [(M+1)+H]+, 314.

4.2. Pharmacology

4.2.1. Microdilution test

The MIC values (µg/ml) of 4a and 4b against the ATCC strains of C. albicans (ATCC 90028), C. krusei (ATCC 6258), and C. parapsilosis (ATCC 90018), and a clinical isolate of fluconazole-resistant C. tropicalis were determined by broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) reference document (M27-A3) [37]. Fluconazole was used as positive control. First, the fungi, which were stored at -80 °C in glycerol, were thawed and subcultured twice onto Sabouraud dextrose agar. RPMI 1640 broth (ICN-Flow, Aurora, OH, USA), with glucose, without bicarbonate and with pH indicator buffered to pH 7.0 with 3-N-morpholinopropanesulfonic acid (Sigma, USA) was used and the inoculum densities were prepared out of 24-hour subcultures. The final test concentration of the fungi was 0.5 to 2.5 × 10$^8$ cfu/ml. Fluconazole was dissolved in sterile deionized distilled water at 64–0.0625 µg/ml. The test compounds were dissolved in dimethyl sulfoxide (Sigma, USA) and diluted using distilled water so that the final twofold concentrations of the compounds prepared in microtiter plate wells were in the range of 1024-
0.25 µg/ml. The plates were incubated at 35 °C for 48 hours and the MIC values were determined as the lowest concentration in which fungal growth was not visible.

4.2.2. Biofilm test

Antibiofilm activities of 4a and 4b were evaluated by determination of their minimum biofilm inhibitory and eradication concentrations (MBIC and MBEC) against C. albicans ATCC MYA-2876 biofilms according to the MBEC™ assay protocol supplied by the manufacturer (Innovotech Inc., Canada) using amphotericin B as positive control. In this standard ASTM method, the biofilms were grown in the Calgary Biofilm Device (Prime and Impact, Prime: 2019-2019). Single molecular determinants of CYP51 inhibit each ligand according to Glide GScore. Each docking pose from all protocols were visually evaluated for the charges of ligands being calculated by Jaguar (2019). Calculation was performed using QSite (2019) files were used directly for the initial docking at XP mode with 20 runs per ligand. In the QPLD protocol, the prepared grid was generated grid files by Glide at SP and XP modes, as well as using Induced Fit and QPLD protocols. At SP and XP modes, docking was performed at 50 runs per ligand, Epik state penalties were added to the scores, and post-docking minimization was enabled. In the Induced Fit docking protocol, each ligand was initially docked to the same coordinates 20 times with ligand and receptor van der Waals radii scaled to 0.5. Then the residues within 5.0 Å to each ligand pose were optimized using Prime and the ligands were redocked to the refined receptor structures within 30 kcal/mol to the best structure and within the best 20 structures at XP mode with 20 runs per ligand. In the QPLD protocol, the prepared grid files were used directly for the initial docking at XP mode with a maximum of 10 poses. Single-point energy calculation was performed using QSite (2019-4, Schrödinger, LLC, New York, NY, 2019) with ab initio charges of ligands being calculated by Jaguar (2019-4, Schrödinger, LLC, New York, NY, 2019) at fast level [42]. Then each pose was redocked at XP mode with the same settings and final 10 poses were selected for each ligand according to Glide GScore. Each docking pose from all protocols were visually evaluated for the molecular determinants of CYP51 inhibition.
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Appendix A. Supplementary Material
Supplementary material related to this article can be accessed at http://doi.org/10.35333/jrp.2020.223.

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