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Design and Synthesis of new fluconazole analogues

Vandana S. Pore, Sandip G. Agalave, Pratiksha Singh, Praveen K. Shukla, Vikash Kumar, Mohammad I. Siddiqi

Abstract: We have synthesized new fluconazole analogues containing two different 1,2,3-triazole units in the side chain. Synthesis of new amide analogues by using a variety of acids is also described. All the compounds showed very good antifungal activity. Hemolysis study of the most active compounds 6e and 13j showed that both compounds did not cause any hemolysis at the dilutions tested. These compounds did not exhibit any toxicity to L929 cells at MIC and lower concentrations. In docking study, overall binding mode of 6e and 13j appeared reasonable and provided a good insight into the structural basis of inhibition of Candida albicans Cyp51 by these compounds.

Introduction

Invasive fungal disease continues to be a problem associated with significant morbidity and high mortality in immune compromised and, to a lesser extent, immune competent individuals. In many cases, it is not the AIDS or cancer itself but the mycoses that are lethal to these patients. Serious fungal infections are caused mostly by Candida albicans, Cryptococcus neoformans and Aspergillus fumigates. The common antifungal agents currently used in clinic are polyenes, allylamines, azoles and echinocandins. The current antifungal drugs like amphotericin B are highly toxic. Some drugs such as fluconosine and azoles are becoming ineffective due to appearance of resistant strains.

Triaizole antifungals have emerged as front-line drugs for the treatment and prophylaxis of many systemic mycoses because of their broad antifungal spectrum, high potency and low toxicity against most yeasts and fungi. A large number of triazole compounds as clinical drugs or candidates have been frequently employed for the treatment of various types of diseases, which have shown their large development value and wide potential as medicinal agents. There are several reports on current developments ofazole-based antifungal drugs. Unfortunately, the excessive use of azoles has led to development of severe resistance, which significantly reduced the efficacy of them. Several new triazoles such as voriconazole, posaconazole, ravuconazole, albacazole etc. containing 1,2,4-triazole and difluorobenzene moieties are marketed or in the late stages of clinical trials (Figure 1).

Fluconazole, α-(2,4-difluorophenyl)-α-(1H-1,2,4 triazol-1-yl-methyl)-1H-1,2,4 triazol-1-ethanol (Figure 1) plays an excellent role in prophylaxis, empirical therapy, and the treatment of both superficial and invasive yeast fungal infections. It is a potent inhibitor of the cytochrome P450 (CYP)-mediated metabolism of the antiepileptic agent phenytoin, a well-known human and animal tetratogen. Fluconazole is well absorbed and exhibit high oral bioavailability. It is an antifungal agent of choice for the treatment of infections by Candida albicans and Cryptococcus neoformans due to its potent activity, excellent safety profile, and favourable pharmacokinetic characteristics. However fluconazole is not fungicidal and is ineffective against invasive aspergillosis. Extensive use of fluconazole has increased the number of fluconazole-resistant C. albicans isolates. Therefore, toxicity concerns, limited
antifungal spectrum, development of resistant strains and observed nephrotoxicity to fluconazole have created a need to modify its activity. Several reports on the synthesis and biological activity of structurally modified new analogues of fluconazole are available in the literature. The cytochrome P450 (CYP) enzymes play an essential role in the metabolism of xenobiotics by catalyzing the monooxygenation of a broad diversity of substrates. Some nitrogen-containing heteroaromatic xenobiotics such as pyridine, imidazole, and triazole derivatives are known to inhibit CYP enzymes by direct coordination with the heme iron (type II ligands). Based on the structure of the active site of CYP51 and the extensive investigation of the structure activity relationships of azole antifungals, we designed and synthesized a novel series of 1-(1H-1,2,4-triazol-1-yl)-2-(2,4-difluorophenyl)-3-(substituted or unsubstituted 1,2,3-triazol-1-yl)-propan-2-ols containing a 1,2,4-triazole ring, a difluorobenzene group and a 1,2,3-triazole ring having long side chain or bile acid. Some of these molecules showed much better activity than fluconazol and amphotericin B and were found to be less toxic at lower dose (0.001mg/mL) to human cancer cells Hep3B and A431.

Results and Discussion

Though the 1,2,4-triazole ring, the difluorophenyl group, and the hydroxyl group are the pharmacophores of antifungal agents, the side chain also plays an important role and the optimization of the side chain attached to the pharmacophore remains attractive to the current research. Application of the isosterism concept for the development of new compounds with therapeutic potential leads to significant advances in molecular diversity and allows covering chemical space in the important areas of medicinal chemistry. It is reported that triazoles and amide group are isosteres. It is found that the antifungal activity of fluconazole can be enhanced by replacing one of the 1,2,4-triazole ring of fluconazole by isosteres 1,2,3-triazole, thiocarbamate, 1,2,4-triazolone and by incorporating amide group or 1,2,3-triazole in the side chain.

Chemistry:

In continuation of our work on fluconazole analogues we designed and synthesized new fluconazole analogues featuring complex side chain having one 1,4-disubstituted 1,2,3-triazole ring and the one 2,4-disubstituted 1,2,3-triazole ring as shown in Schemes 1 and 2.

Synthesis of fluconazole analogues in which 2, 4-disubstituted 1,2,3-triazole ring is in side chain component:

Ketone was reacted with propargyl bromide in the presence of zinc to get acetylenic compound as per our earlier reported procedure (Scheme 1). One pot reaction of alkynes with formaldehyde solution and sodium azide in glacial AcOH/1,4-dioxane afforded alcohols. Mesylation of alcohols followed by reaction with sodium azide gave azides. Click reaction of azido compounds with alkyn 2 under microwave irradiation afforded 1,4 and 2,4-disubstituted 1,2,3-triazole containing compounds 6. All the compounds in Scheme 1 were fully characterized by spectroscopic method and tested for antifungal activity. The results of biological activity of these compounds are summarized in Table 1.

Synthesis of fluconazole analogues in which 2, 4-disubstituted 1,2,3-triazole ring is in fluconazole component:

By using alkyn we synthesized azido compound using similar reaction protocol and click reaction of compound with different alkynes under microwave conditions afforded compounds which are new analogues of fluconazole containing 1,4 and 2,4-disubstituted-1,2,3-triazole units in the side chain (Scheme 2).
**Table 1. In Vitro antifungal activity of test compounds 4-9 and standard antifungal agents**

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Ca = Candida albicans; Cn = Cryptococcus neoformans; Ss = Sporothrix schenckii; Tm = Trichophyton mentagrophytes; Af = Aspergillus fumigatus; Cp = Candida parapsilosis (ATCC-22019)

Scheme 3: Synthesis of compounds 13 (a-j)

Racemic intermediate azide 11 was synthesized from ketone 1 by our earlier reported procedure through epoxide 10 as shown in Schemes 3. Reduction of Azide 11 by using hydrogen on Pd/C to amine 12 and its coupling with different types of acids (Schemes 3) afforded a variety of amides 13. These amides were fully characterized by spectroscopic analysis and were tested for their antifungal activity. The results of biological activity are summarized in Table 2.

**Biology:**

In Vitro antifungal activity of compounds 4-9 and compound 13:

All the newly synthesized compounds were tested for their antifungal activity. The results of biological activity are summarized in Tables 1 and 2.

The biological activity results in Table 1 show that intermediate compounds 4 and 5 were inactive against all the tested fungal strains.

Most of the other compounds 6 to 9 were active against C. albicans, C. neoformans and C. parapsilosis (ATCC-22019) than...
flucanazole. Some compounds were found to be more active than amphotericin B also. Specifically compound 6b (MIC 0.00038 µg/mL for C. neoformans) and 6e (MIC 0.00015 µg/mL for C. neoformans) showed very good antifungal activity and compound 6e was found to show highest activity against Cryptococcus neoformans (MIC 0.00015 µg/mL) and Candida parapsilosis (ATCC-22019) (similar morphology was observed for compounds 6b, 6c, 6e and 13j at their MIC).

The cytotoxicity study of lead compounds 6a, 6b, 6c, 6e and 13j was performed against mammalian fibroblast cell line (L929) by MTT assay. Figure 2A-Control showing normal morphology of L929 cells at resting stage, Figure 2B- Morphology of L929 cells treated at MIC of compound 6a (similar morphology was observed for compounds 6b, 6c, 6e and 13j) at their MIC, Figure 2C-Morphology at higher concentration (50 and 25 µg/ml) of compound 6a which was similar to compounds 6b, 6c, 6e and 13j.

The biological activity results in Table 2 show that all the amides 13a-13j are active against C. albicans and C. neoformans. Compounds 13c and 13d showed good activity against all the strains except A. fumigatus. Some compounds showed better activity than standard drugs flucanazole and amphotericin B. Compound 13j showed highest activity against all the strains, particularly against C. albicans and C. neoformans (70 fold more active than flucanazole and 17 fold more active than amphotericin B against C. albicans while highly active than flucanazole and amphotericin B against C. neoformans) and hence hemolysis and docking study was carried out on this compound.

Among all the compounds tested for the antifungal activity, compounds 6a, 6b, 6c, 6e and 13j were found to exhibit the best activity particularly against yeast like fungi. These compounds were tested for their toxicity using mouse fibroblast cell line L929. Morphological anomalies in L929 cells exposed to compounds 6a, 6b, 6c, 6e and 13j (25 µg/ml) were evident under phase contrast microscope. L929 cells in control were fairly transparent and attached to the surface of the wells of tissue culture plate (Figure 2A). The compounds did not exhibit any toxicity to L929 cells at MIC and lower concentrations, as was evident from their normal morphology (Figure 2B) however when exposed to higher concentrations (50 and 25 µg/ml), the L929 cells lost their normal morphology (Figure 2C) whereas the MTT assay revealed >90% viability of L929 cells even at higher concentrations.

Among all the compounds 6e and 13j were found to be most active and may be treated as lead molecules and hence hemolysis and docking study of these molecules was carried out.

Hemolysis Study:

Hemolysis assay results were evaluated by observing the effect of test compounds on mammalian RBCs (Figure 3). Hemolysis assay of compounds 6e and 13j was determined at 560 nm by using SOFTmax Pro 4.3 Software (Molecular Devices, Sunnyvale, USA) and both compounds did not cause any hemolysis at the dilutions tested.
Ile131, Phe228 and Leu300. Overall binding mode of Cyp51. This stabilization was due to the residues Phe126, placed near His310 and Val509. However, in case of structural basis of inhibition of was used.25 These compounds. For image generation UCSF Chimera 1.7 conformation of coordination bond with Fe of heme group. In docked the molecules azole ring is optimally oriented to make binding site of mostly stabilized by hydrophobic interactions contributed by hydrophobic channel of Cyp51. We observed that binding is analagous. Proposed binding mode of with the reported binding mode of flucanazole and its analogues. Analyses of 50 possible binding poses of 6e and 13j each in the binding site of Candida albicans Cyp51 revealed that many of the conformations of the bound ligand are in good agreement with the reported binding mode of flucanazole and its analogues. Proposed binding mode of 6e and 13j in the active site of Candida albicans Cyp51 (Figure 4) showed that in both the molecules azole ring is optimally oriented to make coordination bond with Fe of heme group. In docked conformation of 6e (Figure 4A), difluorophenyl group was placed near His310 and Val509. However, in case of 13j (Figure 4B), difluorophenyl group was found to orient towards Tyr118 and Tyr132. Long hydrophobic tails of 6e and 13j occupied the hydrophobic channel of Cyp51. We observed that binding is mostly stabilized by hydrophobic interactions contributed by Cyp51. This stabilization was due to the residues Phe126, Ile131, Phe228 and Leu300. Overall binding mode of 6e and 13j appeared reasonable and provided a good insight into the structural basis of inhibition of Candida albicans Cyp51 by these compounds. For image generation UCSF Chimera 1.7 was used.25

**Docking Study**

Analyses of 50 possible binding poses of 6e and 13j each in the binding site of Candida albicans Cyp51 revealed that many of the conformations of the bound ligand are in good agreement with the reported binding mode of flucanazole and its analogues. Proposed binding mode of 6e and 13j in the active site of Candida albicans Cyp51 (Figure 4) showed that in both the molecules azole ring is optimally oriented to make coordination bond with Fe of heme group. In docked conformation of 6e (Figure 4A), difluorophenyl group was placed near His310 and Val509. However, in case of 13j (Figure 4B), difluorophenyl group was found to orient towards Tyr118 and Tyr132. Long hydrophobic tails of 6e and 13j occupied the hydrophobic channel of Cyp51. We observed that binding is mostly stabilized by hydrophobic interactions contributed by Cyp51. This stabilization was due to the residues Phe126, Ile131, Phe228 and Leu300. Overall binding mode of 6e and 13j appeared reasonable and provided a good insight into the structural basis of inhibition of Candida albicans Cyp51 by these compounds. For image generation UCSF Chimera 1.7 was used.25

**Figure 3:** Hemolytic assay of compounds 6e and 13j

**Experimental Protocols:**

**Chemistry**

All chemicals were obtained from commercial sources and used as received without further purification. Solvents were dried according to literature procedures. All the reactions were carried out under nitrogen atmosphere. Column chromatography was carried out by using silica gel (60-120 mm, Merck). All reactions were monitored by TLC with silica gel coated plates; spots were visualized by UV light and/or with dipping in a phosphomolybdic acid solution or anisaldehyde solution and charring on a hot plate. 1H, 13C NMR were recorded in CDCl3 using TMS as internal standard on AC 200 MHz or AV-400 MHz Bruker NMR spectrometers. Chemical shifts are reported in ppm. Microwave reactions were carried in CEM Discover, Model number: 908010. FTIR spectra were recorded on Shimadzu FTIR-8400 spectrophotometer on KBr plate using CHCl3 or Nujol. Only diagnostic bands are reported on cm⁻¹ scale. The ESI ion trap mass spectra were measured by a Finnigan MAT LCQ mass spectrometer. The HRMS spectra were acquired on thermoscientific Q-Exactive spectrometer.

**Conclusion:**

In conclusion we have synthesized a series of new fluconazole analogues having complex side chain with two 1,2,3-triazole rings. In addition we have also synthesized amide analogues of fluconazole. All the newly synthesized molecules were found to show good antifungal activity. Compound 6e with two 1,2,3-triazole rings with cyclohexymethyl group in the side chain and amide analogue of decanoic acid 13j having long chain were found to show highest antifungal activity. These compounds did not exhibit any toxicity to L929 cells at MIC and lower concentrations, however when exposed to higher concentrations (50 and 25 μg/mL), the L929 cells lost their normal morphology. The MTT assay revealed >90% viability of L929 cells even at higher concentrations. Among all the compounds 6e and 13j were found to be the most active and may be treated as lead molecules and hence hemolysis and docking study of these molecules was carried out. Both compounds 6e and 13j did not cause any hemolysis at the dilutions tested. In docking study, overall binding mode of 6e and 13j appeared reasonable and provide a good insight into the structural basis of inhibition of Candida albicans Cyp51 by these compounds. This is first report of fluconazole analogues containing 2,4-disubstituted-1,2,3-triazole unit (Compounds 6 and 13).

**General procedure for the Synthesis of 2-Substituted-1,2,3-triazoles**

CAUTION: Any experiments which may result in the formation of hydrazoic acid should be performed in a well ventilated fume hood and behind a blast shield. Sodium azide should not be mixed with strong acids.

The mixture of 37% HCHO aq. (10 equiv), glacial AcOH (1.5 equiv), and 1, 4-dioxane (5 mL) was stirred for 15 min. To this mixture was added NaN3 (1.5 equiv), followed by alkyne (1.5 equiv). At this point pH of the reaction mixture was 6.5. After additional 10 min of stirring, sodium ascorbate (20 mol %) was added, followed by...
CuSO₄ solution (5 mol %) in 4 mL of H₂O. The mixture was stirred for 18 h at 25 °C, diluted with H₂O (30 mL) and it was extracted using DCM (3 x 50 mL). Combined organic layer was filtered through celite, dried over Na₂SO₄, and concentrated on a rotary evaporator to give crude product. The crude product was sufficiently pure to be used for the next step without further purification.

**(4-Phenyl-1,2,3-triazol-1-yl)methanol (4a)**

Yellowish solid (92%), IR – 3276, 1640, 1456, 1296, 1073 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.46 (s, 1H), 7.75 (m, 1H), 7.71 (m, 1H), 7.49 (m, 1H), 7.45 (m, 1H), 5.85 (d, 1H), 4.92 (t, 1H), 1.36 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 150.0, 133.7, 131.4, 129.0, 128.9, 126.1, 124.4, 122.7, 111.5, 103.9, 75.2, 64.8, 56.8, 33.9; HRMS calcd for C₁₉H₂₀N₄O₂ [M+H⁺]: 342.1579, found: 342.1575.

**White solid (83%) IR – 3112, 2927, 1618, 1426, 1242 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.93 (s, 1H), 7.77 (m, 1H), 7.73 (m, 1H), 7.51 (m, 1H), 7.46 (m, 1H), 5.65 (2H), 1.36 (3H); ¹³C NMR (100 MHz, CDCl₃) δ 152.2, 149.4, 132.8, 126.7, 125.8, 67.3, 34.7, 31.2.

**2-(azidomethyl)-4-(4-fluorophenyl)-2H-1,2,3-triazole (5c)**

White solid (8%). IR – 3129, 2929, 2110, 1673, 1486, 1235, 978 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.93 (s, 1H), 7.82-7.78 (m, 2H), 7.70-7.17 (m, 2H), 7.20 (s, 1H), 5.65 (2H), 1.36 (3H); ¹³C NMR (100 MHz, CDCl₃) δ 132.6, 127.9, 115.0, 67.4.

**General method for the synthesis of the compound 6 (a-e).**

2-(2,4-difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)pent-4-yn-2-ol (6a)

Yellowish solid (88%). IR – 2929, 2108, 1519, 1242 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.97 (s, 1H), 7.85-7.80 (m, 2H), 7.48-7.38 (m, 3H), 5.65 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 149.4, 132.8, 129.5, 128.9, 126.0, 67.3

**2-(azidomethyl)-4-(4-fluorophenyl)-2H-1,2,3-triazole (5b)**

Yellowish liquid (88%). IR – 2929, 2108, 1519, 1242 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.94 (s, 1H), 7.56 (s, 2H), 2.70 (t, 2H), 1.75-1.68 (m, 2H), 1.43-1.26 (m, 6H), 0.89 (t, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 51.0, 134.6, 66.8, 31.3, 28.8, 28.7, 25.3, 22.4, 13.9.

**2-(azidomethyl)-4-(4-fluorophenyl)-2H-1,2,3-triazole (5c)**

Yellowish solid (87%). IR – 3129, 2929, 2110, 1673, 1486, 1235, 978 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.93 (s, 1H), 7.82-7.78 (m, 2H), 7.70-7.17 (m, 2H), 7.20 (s, 1H), 5.65 (2H), 1.36 (3H); ¹³C NMR (100 MHz, CDCl₃) δ 132.6, 127.9, 115.0, 67.4.

**2-(azidomethyl)-4-(4-fluorophenyl)-2H-1,2,3-triazole (5d)**

White solid (91%). IR – 2964, 2107, 1618, 1486, 1242 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.94 (s, 1H), 7.77 (m, 1H), 7.73 (m, 1H), 7.51 (m, 1H), 7.46 (m, 1H), 5.65 (2H), 1.36 (3H); ¹³C NMR (100 MHz, CDCl₃) δ 152.2, 149.4, 132.8, 126.7, 125.8, 67.3, 34.7, 31.2.

**2-(azidomethyl)-4-(cyclohexylmethyl)-2H-1,2,3-triazole (5e)**

Yellowish solid (58 %), IR – 3421, 1617, 1500, 1426, 1242 cm⁻¹, ¹H NMR (400 MHz, CDCl₃) δ 7.97 (s, 1H), 7.85-7.80 (m, 2H), 7.48-7.38 (m, 3H), 5.65 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 149.4, 135.4, 66.9, 38.0, 33.1, 33.0, 26.3, 26.1.

**General procedure for the Synthesis of 2-azidomethyl-1,2,3-triazoles**

The solution of 2-substituted-1,2,3-triazoles obtained in first step in dry DCM (5 mL) was cooled to 0 °C. Triethyl amine (1.5 equiv) was added and the reaction mixture was stirred for 5 min at 0 °C. This mixture methane sulfonyl chloride (3 equiv) was added and the reaction mixture was stirred at 0 °C for 2 h. It was then diluted with cold H₂O and extracted with DCM. Organic layer was washed with dilute NaHCO₃, brine and dried over Sodium sulfate. It was then filtered and concentrated under vacuum to give crude product which was used for next step without further purification.

Compound obtained in the above step was dissolve in dry DMF. Sodium azide (3 equiv) was added and the reaction mixture was stirred at 65 °C for 6 h. After cooling to room temperature, the reaction mixture was diluted with cold water and was extracted with DCM (3X50 mL). Combined organic layer was washed with water and brine, dried over sodium sulfate and concentrated on rotary evaporator. The crude mixture obtained was purified by column chromatography on silica gel using EtOAc: Hexane (3:7) to give pure desired products.
Glassy solid (55%); IR - 3418, 1617, 1502, 1145, 965 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.02 (s, 1H), 7.73 (s, 1H), 7.40-7.35 (m, 2H), 7.24-7.29 (m, 2H), 6.66-6.54 (m, 2H), 5.18 (s, 1H), 4.64 (d, \(J = 14.34\) Hz, 1H), 4.45 (d, \(J = 14.34\) Hz, 1H), 3.37 (d, \(J = 13.84\) Hz, 1H), 3.05 (d, \(J = 13.84\) Hz, 1H), 2.56 (t, 2H), 1.55 (m, 2H), 1.25-1.19 (m, 6H), 0.81 (t, 3H)); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) C 164.7, 160.8, 159.7, 155.7, 150.9, 150.3, 142.9, 134.8, 129.4, 123.9, 122.8, 111.0, 103.6, 74.3, 64.05, 56.9, 33.8, 31.0, 29.3, 28.4, 24.9, 22.1, 13.5; HRMS calcld for \(\text{C}_{22}\text{H}_{28}\text{N}_{9}\text{O}_{2}\) [M + H]\(^+\): 472.2379, found: 472.2377.

Glassy solid (62%); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.09 (s, 1H), 7.86 (s, 1H), 7.79-7.70 (m, 3H), 7.52 (s, 1H), 7.36-7.28 (m, 1H), 7.19-7.10 (m, 2H), 6.72 (d, 2H), 6.68-6.55 (m, 2H), 5.23 (s, 3H), 4.73 (d, \(J = 14.15\) Hz, 1H), 4.54 (d, \(J = 14.15\) Hz, 1H), 3.46 (d, \(J = 15.79\) Hz, 1H), 3.15 (d, \(J = 15.79\) Hz, 1H)); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) C 164.3, 163.5, 162.3, 161.6, 19.4, 157.4, 151.6, 149.1, 143.7, 133.1, 129.1, 128.0, 125.4, 124.4, 122.7, 116.1, 111.4, 104.0, 75.2, 64.8, 56.8, 33.9; HRMS calcld for \(\text{C}_{22}\text{H}_{19}\text{N}_{9}\text{O}_{2}\) [M + H]\(^+\): 482.1659, found: 482.1655.

Yellowish solid (96%); IR - 3132, 3016, 2112, 1618, 1501, 1216 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.02 (s, 1H), 7.85 (s, 1H), 7.83 (s, 1H), 7.40 (s, 1H), 7.37-7.32 (m, 1H), 7.80-7.70 (m, 2H), 5.49 (s, 1H), 4.94 (bs, 1H), 4.80 (d, \(J = 14.20\) Hz, 1H), 4.53 (d, \(J = 14.20\) Hz, 1H), 3.46 (d, \(J = 15.75\) Hz, 1H), 3.19 (d, \(J = 14.20\) Hz, 1H)); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) C 151.5, 144.8, 144.2, 136.3, 129.7, 124.1, 111.5, 104.0, 75.0, 68.8, 56.6, 34.3; HRMS calcld for \(\text{C}_{22}\text{H}_{19}\text{N}_{9}\text{O}_{2}\) [M + H]\(^+\): 362.1284, found: 362.1280.

General method for the synthesis of the compound 9 (a-e):

By using alkyne 8 (1 mmol) and azide 3 (a-e) (1.2 mmol) and following procedure for click reaction as for compounds 6, compound 9 (a-e) were obtained.

Glassy solid (50%); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.01 (s, 1H), 7.85 (s, 1H), 7.83 (s, 1H), 7.79 (d, \(J = 7.27\) Hz, 2H), 7.44-7.41 (m, 3H), 7.37-7.33 (m, 1H), 7.25-7.20 (m, 1H), 6.76-6.69 (m, 3H), 6.61-5.76 (m, 1H), 4.99 (bs, 1H), 4.77 (d, \(J = 14.03\) Hz, 1H), 4.52 (d, \(J = 14.03\) Hz, 1H), 3.43 (d, \(J = 14.03\) Hz, 1H), 3.17 (d, \(J = 14.03\) Hz, 1H)); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) C 163.9, 161.4, 159.5, 157.0, 152.0, 148.8, 145.5, 137.0, 129.8, 128.9, 128.6, 128.4, 124.0, 119.2, 111.5, 104.1, 75.0, 64.2, 56.6, 34.4; HRMS calcld for \(\text{C}_{22}\text{H}_{19}\text{N}_{9}\text{O}_{2}\) [M + H]\(^+\): 464.1753, found: 464.1749.

Yellowish solid (75%); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.01 (s, 1H), 7.83 (s, 1H), 7.37 (d, \(J = 9.96\) Hz, 2H), 6.80-6.63 (m, 4H), 4.94 (s, 1H), 4.77 (d, \(J = 14.15\) Hz, 1H), 4.49 (d, \(J = 14.15\) Hz, 1H), 3.42 (d, \(J = 14.78\) Hz, 1H), 3.15 (d, \(J = 14.78\) Hz, 1H), 2.67 (t, 2H), 1.62-1.58 (m, 2H), 1.29-1.25 (m, 2H), 0.87 (t, 3H)); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) C 163.8, 161.3, 159.5, 157.1, 149.4, 145.3, 136.7, 132.8, 124.1, 111.5, 104.0,77.3, 74.9, 64.4, 60.4, 56.6, 34.3, 31.4, 29.1, 28.8, 25.4, 22.3, 14.0; HRMS calcld for \(\text{C}_{22}\text{H}_{19}\text{N}_{9}\text{O}_{2}\) [M + H]\(^+\): 472.2377.

Sticky solid (71%); IR - 3419, 2925, 1616, 1499, 1231, 966 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.01 (s, 1H), 7.84-7.70 (m, 2H), 6.80-5.54 (m, 4H), 4.96 (bs, 1H), 4.78 (d, \(J = 14.02\) Hz, 1H), 4.51 (d, \(J = 14.02\) Hz, 1H), 3.44 (d, \(J = 14.78\) Hz, 1H), 3.16 (d, \(J = 14.78\) Hz, 1H); HRMS calcld for \(\text{C}_{22}\text{H}_{19}\text{N}_{9}\text{O}_{2}\) [M + H]\(^+\): 482.1659, found: 464.1655.
Yellowish solid (69 %), IR – 3130, 2963, 1667, 1617, 1500, 1272, 1138 cm\(^{-1}\), \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.09 (s, 1H), 7.87 (s, 1H), 7.78 (s, 1H), 7.68 (d, \(J = 8.24\) Hz, 2H), 7.50 (s, 1H), 7.47 (d, \(J = 8.24\) Hz, 2H), 7.32-7.28 (m, 1H), 6.61 (dd, \(J = 13.73, 20.45\) Hz, 2H), 6.68-6.57 (m, 2H), 5.26 (s, 1H), 4.72 (d, \(J = 14.04\) Hz, 1H), 4.55 (d, \(J = 14.04\) Hz, 1H), 3.47 (d, \(J = 14.91\) Hz, 1H), 3.15 (d, \(J = 14.95\) Hz, 1H), 1.35 (s, 9H); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) C\(_{163.6}\), 161.5, 151.5, 159.3, 157.4, 152.5, 149.9, 143.6, 133.2, 129.9, 126.2, 125.9, 124.4, 122.6, 111.4, 103.9, 75.1, 64.8, 56.8, 34.7, 33.9; HRMS calcd for C\(_{11}H_{13}N_4OF_2\) [M + H]+: 287.4.

General procedure for the synthesis fluconazole containing amide:

To a solution of 1-azido-2-(2,4-difluorophenyl)propan-2-ol (3.5 g, 12 mmol) in 40 mL of ethanol was added 10% active charcoal-supported palladium (350 mg). The solution was stirred overnight at room temperature under a hydrogen atmosphere (60 psi) and filtered through celite. It was then concentrated and the residue was washed with ethyl acetate-pet ether (50:50) and oven dried to get the desired product 12.


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**In Vitro antifungal activity**

All the newly synthesized compounds were tested for their *in vitro* antifungal activity against different fungal strains such as *Candida albicans*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Candida parapsilosis* (ATCC-20219) using fluconazole and amphotericin B as standard drugs. Minimum inhibitory concentration (MIC) values were determined using standard broth microdilution technique as per NCCLS guidelines. The results of biological activity are summarized in Tables 1 and 2.

**Materials and Methods**: MIC determination: Minimum inhibitory concentration (MIC) of compounds was tested according to standard microbroth dilution technique as per NCCLS guidelines. Briefly, testing was performed in flat bottom 96 well tissue culture plates (CELLSTAR_ Greiner bio-one GmbH, Germany) in RPMI 1640 medium buffered with MOPS (3-[N- morpholino]propanesulfonic acid) (Sigma Chem. Co., MO, USA) for fungal strains. The concentration range of test compounds was 50–0.36 μg/mL and for standard compounds 32–0.0018 μg/mL. The plates were incubated in a moist chamber at 35 °C and observed absorbance were after 24 h for *C. albicans*, 48h for *C. parapsilosis* and *Cryptococcus neoformans*, 72 h for *Aspergillus fumigatus*, *S. schenckii*, and *Trichophyton mentagrophytes*. MIC was determined as 80% inhibition of growth with respect to the growth control.

**Toxicity Study:**

Compounds 6a, 6b, 6c, 6e and 13j were found to exhibit the best in vitro activity against all the fungi. These compounds were tested for their toxicity using mouse fibroblast cell line L929.

**Material and Method**

To test the toxicity of lead compounds 6a, 6b, 6c, 6e and 13j against mammalian cells, mouse fibroblast cell line L929 was used. Stock solutions (1mg/mL) of the test compounds were prepared in DMSO. The cell line L929 was grown in RPMI 1640 medium supplemented with 10% FBS and 1 X antimiticotic solution (sigma, USA) at 37 °C in humidified atmosphere having 5% CO₂. One hundred μL (1x10⁶ cells μL in RPMI) of the confluent fibroblast stock suspension (1x10⁶ cells/mL) was dispensed in 96 well tissue culture plates. The original medium from the wells was replaced with 100 μL serum free RPMI when the cells reached 80% confluence after incubation in a CO₂ incubator at 37 °C. Various concentrations of the test compounds (25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09 μg/mL) were added to the growing cells along with control (with no compounds) and incubated for 24 hours. 200 μL of MTT solution (0.5 mg of MTT in RPMI 1640 medium) was added to each well after removing the media completely and incubated for 4 hours at 37 °C to allow MTT metabolism. An aliquot of 100 μL of DMSO solvent was added to each well and the plate was incubated for 30 minutes at room temperature. Response of L929 cells to the test compounds was determined spectrophotometrically at 570 and 630 nm. The morphology of the cells was observed using Giemsa stain under Phase contrast microscope. After fixation of the cells in the wells of 96 well tissue culture plates, Giemsa stain was added to each well and incubated for 30 min at 37 °C. The excess stain was removed by thorough washing with phosphate buffer saline
and the culture plates were air dried and observed under a phase contrast microscope.

**Hemolytic Study:**
Hemolytic assay was done by observing the effect of test compounds on mammalian RBCs.

**Materials and methods:**
Hemolytic activity was evaluated by observing the effect of test compounds on mammalian RBCs. Briefly, fresh blood of rabbit was collected in heparinized tubes, and red blood cells (RBCs) were collected by centrifugation at 1000g for 5 min. Supernatant was discarded and sedimented RBCs were washed three times in normal saline (0.9%) by centrifugation. Cell pellet was resuspended as 5% V/V in normal saline supplemented with 10% fetal bovine serum. For each compound, experiment was performed in duplicate with positive as well as negative controls. The test compounds were dissolved in 10% Dimethylsulfoxide (in 0.9% saline) and diluted two fold in saline. Equal volume of diluted compounds and RBCs suspension were mixed for test solution while 0.9% saline (0.9%) by centrifugation. Cell pellet was centrifuged at 1000 g for 5 min, triton X with RBC as positive control, and incubated at 37 ºC for 30 min. After incubation mixture was centrifuged at 1000g for 5 min, supernatant was collected and diluted with normal saline in 1:1 ratio. Absorbance was taken at 560 nm by spectrophotometrically and result was calculated by

\[
\text{% Hemolysis} = \frac{(\text{Absorbance of sample}) - (\text{Absorbance of blank})}{\text{Highest absorbance of positive control}} \times 100
\]

**Docking Study**
Putative analysis of 50 possible binding modes of the most active molecules 6e and 13j in the binding site of *Candida albicans* Cyp51 active site was carried out.

**Materials and methods:**
Previously developed homology model of *Candida albicans* Cyp51 was used to dock 6e and 13j. Optimized 3D coordinates of 6e and 13j were generated with the help of Marvin sketch (http://www.chemaxon.com). Before flexible docking, input files for Autodock 4.2 were generated using MGLtools1.5.4 (http://mgltools.scripps.edu). Docking grid was set to 75, 75 and 70 points in x, y and z directions respectively, enclosing the heme group. Lamarckian genetic algorithm was used to generate 50 conformations each of 6e and 13j.

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**Notes and references**


