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Synthesis, Antifungal Evaluation and Molecular Docking Studies of Novel Pyrazoline Carboxamide Linked 1,2,4-Triazole Hybrid Derivatives as Potential CYP51 Inhibitors

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Abstract: Fungal infections are increasingly causing significant problems for public health around the world, especially due to the rise of resistant strains to current antifungal therapeutics. To identify structurally novel antifungals, we designed, synthesized and characterized a new class of 10 pyrazoline carboxamides with a 1,2,4-triazole as the hybrid scaffold via a convergent, metal-free synthetic process. The hybrid scaffold was created by covalently combining the CYP51 inhibitor triazole pharmacophore with the antifungal-similar pyrazoline carboxamide components into one unified molecular structure – yielding the target compounds at 67-82% collective yields. All of the compounds were evaluated for their in vitro antifungal activity against Aspergillus niger, Candida albicans, Candida glabrata, and Candida tropicalis using the agar disk-diffused method with Amphotericin B serving as the reference standard. Of the 10 compounds, 12e (2,4-dichlorophenyl) showed the most potency, inhibiting growth of A. niger 14.9 mm, C. albicans 21.1 mm, C. glabrata 19.3 mm and C. tropicalis 17.6 mm; therefore, showed greater activity than fluconazole shown against 3 of the 4 organisms, while compounds 12f (4-fluorophenyl) and 12b (4-bromophenyl) also showed exceptional activity against C. albicans or A. niger compared to amphotericin B. In silico predictions using docking techniques for the synthesized compounds against the CYP51 lanosterol 14α-demethylase crystal structure showed statistically significant differences in binding energy that were more favourable to these compounds than to the known antifungal fluconazole (binding energy -7.3 kcal/mol). The most favourable binding affinity was found for compound 12i (4-nitrophenyl; -12.1 kcal/mol) when compared to fluconazole. Furthermore, structure-activity relationship studies indicate that substituents possessing electron-withdrawing properties (halogens) are important structural determinants of the antifungal properties of the synthesized compounds. Vertically positioned halogen substituents at para or in a 2,4-dichlorophenyl configuration relative to the C-5 aryl group of the pyrazoline ring appear to play a major role in the antifungal activity of all synthesized compounds. Predicted docking values were highly correlated with experimental antifungal activity values, thus proving that CYP51 is the primary target for the synthesized compounds and supporting the rationale for our design approach to synthesize these hybridized compounds. Compounds 12e, 12f and 12b are all potential candidates for continuing the preclinical development of drug candidates.

INTRODUCTION

The rising number of fungal infections and resulting invasive infections illustrates the global health crisis represented by fungi worldwide. The CDC estimates that fungus causes over 6.55 million invasive infections yearly and approximately 2.55 million deaths due to those infections annually. Invasive aspergillosis refers to more than 2.1 million cases of infection each year, and the average mortality rate of invasive aspergillosis infection is close to 85% [1]. These numbers show the significance of fungi as a global health problem; consequently, in October 2022, the WHO developed the first fungal priority pathogens list (FPPL). The FPPL includes 19 separate species of fungus that are of critical, high and medium public health interest. Four species on the FPPL are identified as critical priority pathogens: Aspergillus fumigatus, Candida albicans, Candida auris and Cryptococcus neoformans[2]. Candida albicans is the most common opportunistic pathogen; it causes infections that vary in severity from superficial mucosal candidiasis to severe, life-threatening invasive infections in the bloodstream[3]. The emergence of Candida auris has compounded the challenge of preventing healthcare-associated infections by providing a multidrug-resistant species (multidrug resistant) since it was first reported in 2009, by increasing the risk of healthcare-associated transmission, persisting on environmental surfaces in the healthcare environment and having cross-resistance to multiple classes of antifungal medications, with fluconazole, 87-100% resistant across geographic clades[4,5]. Aspergillus fumigatus presents a significant clinical challenge in immunocompromised patients and is intrinsically resistant to fluconazole and has progressively developed resistance to voriconazole[6]. Cryptococcus neoformans is a leading cause of cryptococcal meningitis and is estimated to be responsible for 1 in 5 deaths globally from AIDS-related causes[7]. Currently, there are four classes of antifungal medication available for use, and very few new antifungal medications are being developed; therefore, it is of utmost importance to identify new antifungal compounds that are different in structure to those available today. This is needed in order to combat the escalating problem of resistance to antifungal medications [8].

Azoles are the most common class of antifungal medication used to treat both systemic and superficial infections caused by fungi. The most common azole antifungals, which contain a triazole scaffold, primarily function by selectively inhibiting the enzyme lanosterol 14α-demethylase or CYP51. CYP51 is responsible for catalysing one of the key steps in the biosynthesis of ergosterol (a component of the cell membrane of fungi) and, therefore, when inhibited, will affect the integrity of the fungal cell membrane and stop the growth of the fungus [9,10]. Clinical studies have confirmed the effectiveness of fluconazole, voriconazole, itraconazole, posaconazole, and isavuconazole. However, the extended use of azole antifungals has led to the development of resistance through multiple mechanisms—including point mutations in ERG11 and overexpression of efflux transporters and the production of biofilms that are resistant to antifungal agents [11,12]. Therefore, there is an urgent need for discovery of novel antifungal agents that demonstrate activity against CYP51 (henceforth CYP51) that contain new or unique chemistries in relation to the current class of anti-CYP51 agents in order to evade current mechanisms of resistance.

One of the most frequently utilised pharmacological classes within the field of medicinal chemistry is that of the 1,2,4-triazole ring system, which consists of a chemically unique five-membered aromatic heterocyclic compound (tricyclic) and possesses multiple biological and/or pharmacological activities, including antifungal, antibacterial, antiviral, and anticancer activity [13,14]. The highly privileged characteristic of the pyrazole ring includes its role in the creation of the class of bioactive compounds known as pyrazole carboxamides, which have been demonstrated to be an effective source of antifungal compounds related to interference with the ergosterol biological pathway through inhibition of the succinate dehydrogenase enzymatic activity, as exhibited by commercially available fungicides such as boscalid, fluxapyroxad, and penthiopyrad [15, 16].

The molecular hybridisation strategy, which is the covalent fusion of two or more pharmacophores into one molecule, is widely considered a successful pharmacological design tactic resulting in the modification of market-ready drugs that demonstrate added target interaction affinity, two or more modes of action, decreased susceptibility to drug resistance, and superior pharmacokinetic properties [17]. The synthesis of hybrids containing moieties representative of both the 1,2,4-triazole and pyrazole carboxamide pharmacophores is compelling based upon the fact that both pharmacophores provide well-documented interactions with the enzymes that produce the fungal resistance observed through use of currently available fungicides [18]. Additionally, the carboxamide link between the two pharmacophores increases the affinity of binding with cytochrome P450 enzymes (CYP51) within the substrate channel of the enzyme, and facilitates favourable hydrogen-bonding between the enzyme and the active substrate residue [19].

Furthering the ongoing development of novel

antifungals, this study presents the preparation and characterisation of a library of unique pyrazole carboxamide-linked 1,2,4-triazole hybrid derivatives. All structures have been confirmed via IR, ^{1}H NMR, and mass spectral analyses. Antifungal activity was evaluated in vitro versus pathogenic fungi, and molecular docking studies against fungal CYP51 were performed to determine the binding modes of the most active compounds and to provide a rational mechanistic basis for the observed bioactive properties.

Experimental

Melting points were determined in open capillaries. ^{1}H NMR spectra were recorded at 500 MHz (Bruker Avance) Cryo-magnet Spectrometer in $CDCl_3$ or DMSO Solvent using TMS as an internal standard. IR spectra were recorded on an FT Infra-Red Spectrophotometer Model RZX Perkin Elmer. The products were confirmed by the comparison of their Mass Spectra, IR, and ^{1}H NMR.TLC was carried out on Silica gel G (Merk) plates.

Synthesis

Scheme 1:

Step 1: Preparation of Thiocarbohydrazide

To a vigorously stirred of 25 g 100% hydrazine hydrate (0.5 mole) in 50 ml of water, 7.6 g of carbon disulphide (0.1mole) was added dropwise. The temperature of the solution was raised to 60 ^{0}C. The reaction mixture was then refluxed for 30 minutes, cooled in an ice bath for 30 min, and the precipitated thiocarbohydrazide was filtered off, washed with ethanol and air dried. Compound was recrystallized from a minimum amount of water acidified with a few drops of concentrated hydrochloric acid. The physical data is recorded and correlated with references (Yield-89%, M.P. 171 ^{0}C).[20].

Step 2: Formation of 4-amino-5-(chloromethyl)-4H-1,2,4-triazole-3-thiol

A mixture of 0.1 mole thiocarbohydrazide (10.6 gm) and0.1mole monochloroacetic acid (9.4 gm) in 50 ml water was heated under reflux for 4 hours. Within an hour of refluxing, a solid started separating from the clear solution. After completion of the reaction, the mixture was cooled in an ice bath for 30 minutes. The precipitate was filtered off, washed with diluted base and recrystallized. (Yield-81%, M.P. 205 ^{0}C).

Step 3: Synthesis of 5-(chloromethyl)-4-((2,4-difluorobenzylidene)amino)-4H-1,2,4-triazole-3-thiol

The 0.04 mole of 4-amino-5-(chloromethyl)-4H-1,2,4-triazole-3-thiol (6.58gm) and 0.04 mole of 2,4-difluorobenzaldehyde (4.4 ml) in 30ml DMF containing 3-4 drops of glacial acetic acid was refluxed for 3-4 hours. After that reaction mixture was poured into ice crushed. The product obtained was separated and recrystallzed. A faint yellow coloured powder was obtained (Yield-79%,M.P. 235 ^{0}C).

Scheme 2:

Step 1: Synthesis of(E)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one(10a-j)

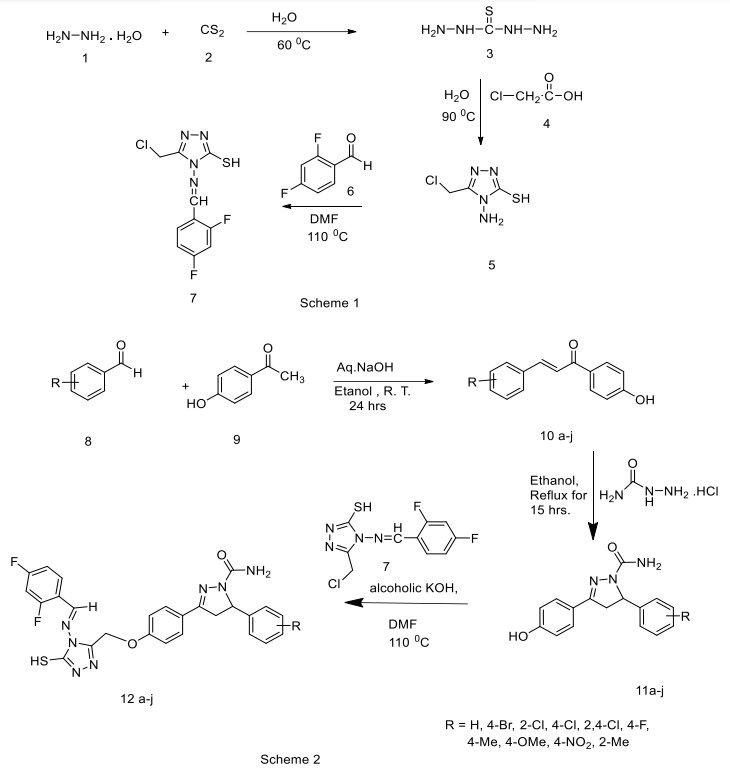
Equimolar quantity of 0.01 mole of 4-hydroxy acetophenone and respective aryl aldehyde were mix and dissolved in required amount of ethanol and add aqueous sodium hydroxide (NaOH 10%) solution. Then reaction mixture was continuously stirred for 24 hours at room temperature and check reaction on TLC (n-hexane: Ethyl Acetate, 8:2). After completion of reaction, the reaction mixture was poured into crushed ice and neutralized with dil. HCl and product was filtered off. The obtained product was recrystallized from ethanol. The physical data is recorded and correlated with references.[21]

Step 2: Synthesis of 3-(4-hydroxyphenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carboxamide (11a-j)

A Mixture of Chalcones (0.01mol) and Semicarbazide hydrochloride (0.01mol) was taken in Ethanol (30ml) and reflex the reaction mixture for 15 hrs. the reactions progress was monitored on thin-layer chromatography (n-Hexane/Ethyl Acetate, 8:2). After completion of the reaction, it was poured on crushed ice, filtered, dried and recrystallized from ethanol. The physical data is recorded and correlated with reference.[22]

Step 3: Synthesis of (E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3yl)methoxy)phenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carboxamide(12a-j)

An equimolar quantity of 0.001 mole of 3-(4-hydroxyphenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carboxamide and 5-(chloromethyl)-4-((2,4-difluorobenzylidene)amino)-4H-1,2,4-triazole-3-thiol were mix and dissolved in required amount of DMF (20ml) as a solvent and added alcoholic potassium hydroxide (KOH 10%) solution. Then reaction mixture was continuously stirred at 110^{0}C for 4 hours whileTLC monitoredthe reactionusing a developing systemof 30% CH_2Cl_2 in ether. After completion of reaction, the reaction mixture was poured into crushed ice and neutralized with dil. HCl and the product was filtered off. The obtained product was recrystallized from ethanol.



Scheme: (E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carboxamide (12a-j)

Spectral analysis and physical data of synthesized compounds 12a-j:

(E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-5-phenyl-4,5-dihydro-1H-pyrazole-1-carboxamide (12a):

Yield 75% , M.P. 164^{0}C, IR (KBr, *V*max ,cm^{-1}): 1595.13(HC=N stretch), 1710 (C=O stretch), 2931.80 (-C-H-stretch in Ar-H), 3329.14(N-H stretch), 1230.94 (-C-O-C- stretch), 1276.88 (N-N stretch).^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm): 12.87 (s,1H, SH), 9.01 (s,1H, HC=N), 7.81(d, 1H), 7.72 (d,2H),7.78(d,1H), 7.41- 7.25 (m, 3H),7.36(dd,2H), 7.29(d,1H), 6.89 (d,2H), 6.87(d,1H), 6.31(s,2H), 5.21(s,2H), 4.05(dd,1H), 3.29(dd,1Ha), 2.89(dd,1Hb). Mass (m/z): 534.15[m+1]. Anal. Cal. for $C_{26}H_{21}F_2N_7O_2S$: C, 58.53; H, 3.97; F, 7.12; N, 18.38; O, 6.00; S, 6.01. Found C:58.16, H:3.67, F:7.04,N:18.14, O: 6.0, S:6.0.

(E)-5-(4-bromophenyl)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide (12b):

Yield 71% ,M.P. 161^{0}C, IR (KBr, *V*max ,cm^{-1}): 1605.04(HC=N stretch), 1700 (C=O stretch), 2935.47 (-C-H-stretch in Ar-H), 3319.01(N-H stretch), 1234.94 (-C-O-C- stretch), 1276.88 (N-N stretch).^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm): 12.86 (s,1H, SH), 9.07 (s,1H, HC=N), 7.89(d, 2H), 7.81 (d,1H),7.79(d,2H),7.29 (d, 2H),7.25(d,1H), 6.89 (d,2H), 6.82(d,1H), 6.34(s,2H), 5.29(s,2H), 4.07(dd,1H), 3.27(dd,1Ha), 2.96(dd,1Hb). Mass (m/z): 612.06[m+1]. Anal. Cal. for $C_{26}H_{20}BrF_2N_7O_2S$: C, 50.99; H, 3.29; Br, 13.05; F, 6.20; N, 16.01; O, 5.22; S, 5.24. Found C:54.0, H:3.01, Br:14.11, F:6.16, N:10.01, O: 5.24, S:5.33,

(E)-5-(2-chlorophenyl)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide(12c):

Yield 78%, M.P. 149^{0}C, IR (KBr, *V*max ,cm^{-1}): 1607.21(HC=N stretch), 1693 (C=O stretch), 2939.13 (-C-H-stretch in Ar-H), 3313.08(N-H stretch), 1239.58 (-C-O-C- stretch), 1276.88 (N-N stretch),744.38 (C-Cl stretch).^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm): 12.91 (s,1H, SH), 9.07 (s,1H, HC=N), 7.86(d, 1H), 7.79 (d,2H),7.70(dd,1H),7.32 (m, 1H),7.28(dd,1H), 7.25(m,1H), 7.23(d,1H), 6.82 (d,2H), 6.79(d,1H), 6.30(s,2H), 5.21(s,2H), 4.33(dd,1H), 3.36(dd,1Ha), 2.99(dd,1Hb). Mass (m/z): 568.11[m+1]. Anal. Cal. for $C_{26}H_{20}ClF_2N_7O_2S$: C, 54.98; H, 3.55; Cl, 6.24; F, 6.69; N, 17.26; O, 5.63; S, 5.65. Found C: 54.47, H: 3.27, Cl: 6.18, F: 6.51, N: 17.02, O: 5.27, S: 5.33.

(E)-5-(4-chlorophenyl)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide(12d):
Yield 74%, M.P. 159^{0}C, IR (KBr,*V*max,cm^{-1}): 1618.13(HC=N stretch),1692.85(C=O stretch), 2945.75(-C-H stretch in Ar-H), 3310.15(N-H stretch), 1514.14(-HC=CH stretch), 1228.18 (-C-O-C- stretch), 1285.17 (N-N stretch), 754 (C-Cl stretch). ^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm): 12.80 (s,1H, SH), 9.03 (s,1H, HC=N), 7.99(d, 1H), 7.89 (d,2H),7.49(d,2H),7.36 (d, 2H),7.26(d,1H), 6.94 (d,1H), 6.72(d,2H), 6.39(s,2H), 5.19(s,2H), 4.23(dd,1H), 3.37(dd,1Ha), 2.99(dd,1Hb). Mass (m/z): 568.11[m+1]. Anal. Cal. for $C_{26}H_{20}ClF_2N_7O_2S$:C, 54.98; H, 3.55; Cl, 6.24; F, 6.69; N, 17.26; O, 5.63; S, 5.65. Found C:58.56, H:3.23, Cl:6.12, F:6.37, N:17.12, O: 5.49, S:5.23.

(E)-5-(2,4-dichlorophenyl)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide(12e) :
Yield 80%, M.P. 159^{0}C, IR(KBr,*V*max ,cm^{-1}):1604.07(HC=N stretch),1692.85(C=O stretch), 2954.75(-C-H stretch in Ar-H), 3319.58(N-H stretch), 1509.18(-HC=CH stretch), 1231.45 (-C-O-C- stretch), 1271.09 (N-N stretch), 748.38 (C-Cl stretch).^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm): 12.97 (s,1H, SH), 9.09 (s,1H, HC=N), 7.99(d, 1H), 7.73 (d,2H),7.71(d,1H),7.32 (dd, 1H),7.23(d,1H), 7.13(d,1H), 6.91(d,1H), 6.85 (d,2H), 6.26(s,2H), 5.28(s,2H), 4.23(dd,1H), 3.23(dd,1Ha), 2.95(dd,1Hb). Mass (m/z): 602.03[m+1]. Anal. Cal. for $C_{26}H_{19}Cl_2F_2N_7O_2S$: C, 51.84; H, 3.18; Cl, 11.77; F, 6.31; N, 16.27; O, 5.31; S, 5.32 Found C:51.58, H:3.08, Cl:11.53, F:6.15, N:16.21, O:5.13, S:5.20.

(E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-5-(4-fluorophenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide(12f):
Yield 77%, M.P. 147^{0}C, IR(KBr,*V*max,cm^{-1}):1607.14(C=N stretch), 1701.94(C=O stretch), 29211.14(-C-H stretch in Ar-H), 1519.39(-HC=CH stretch), 1241.45 (-C-O-C- stretch), 1264.47 (N-N stretch), 897.42 (C-F stretch). ^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm):12.90 (s,1H, SH), 9.01(s,1H, HC=N), 7.82(d, 1H), 7.78 (d,2H),7.33(d,1H),7.29 (d, 2H),7.24(d,2H), 6.95 (d,1H), 6.91(d,2H), 6.25(s,2H),5.23(s,2H), 4.19(dd,1H), 3.24(dd,1Ha), 2.99(dd,1Hb).Mass (m/z): 552.14.11[m+1]. Anal. Cal. for $C_{26}H_{20}F_3N_7O_2S$: C, 56.62; H, 3.65; F, 10.33; N, 17.78; O, 5.80; S, 5.81. Found C:56.48, H:3.31, F:10.19, N:17.46, O:5.52, S:5.69.

(E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-5-(p-tolyl)-4,5-dihydro-1H-pyrazole-1-carboxamide(12g):
Yield 69%, M.P. 139^{0}C, IR(KBr,*V*max, cm^{-1}): 1609.59(HC=N stretch), 1697.35(C=O stretch), 2939.40(-C-H stretch in Ar-H), 3318.23(N-H stretch), 1515.13(-HC=CH stretch), 1215.07 (-C-O-C- stretch), 1247.76 (N-N stretch). ^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm): 12.81 (s,1H, SH), 9.09(s,1H, HC=N), 7.82(d, 1H), 7.74 (d,2H),7.28(d,2H),7.18 (d, 2H),7.11(d,1H), 6.98 (d,2H), 6.93(d,1H), 6.31(s,2H),5.25(s,2H), 4.29(dd,1H), 3.20(dd,1Ha), 2.91(dd,1Hb), 2.78(s,3H).Mass (m/z): 548.16[m+1]. Anal. Cal. for $C_{27}H_{23}F_2N_7O_2S$: C, 59.22; H, 4.23; F, 6.94; N, 17.91; O, 5.84; S, 5.86. Found C:59.08, H:4.07, F:6.78, N:17.67, O: 5.66, S:5.42.

(E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide(12h):
Yield 77%, M.P. 161^{0}C, IR(KBr,*V*max, cm^{-1}):1598.99(HC=N stretch),1711.45(C=O stretch), 2925.79(-C-H stretch in Ar-H), 3321.28(N-H stretch), 1508.33(-HC=CH stretch), 1229.15 (-C-O-C- stretch), 1255.66 (N-N stretch), 1028.06 (Ar-OMe stretch). ^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm) 12.86 (s,1H, SH), 9.04(s,1H, HC=N), 7.89(d, 1H), 7.85 (d,2H),7.28(d,2H),7.19 (d, 2H), 6.99(d,2H), 6.93 (d,1H), 6.89(d,2H), 6.33(s,2H),5.29(s,2H), 4.25(dd,1H), 3.79(s,3H), 3.26(dd,1Ha), 2.99(dd,1Hb). Mass (m/z): 564.16[m+1]. Anal. Cal. for $C_{27}H_{23}F_2N_7O_3S$: C, 57.54; H, 4.11; F, 6.74; N, 17.40; O, 8.52; S, 5.69. Found C:61.49, H:3.68, F:70.28, N:11.00, O: 9.34, S:6.19.

(E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-5-(4-nitrophenyl)-4,5-dihydro-1H-pyrazole-1-carboxamide (12i):
Yield 73%, M.P. 143^{0}C, IR(KBr, *V*max,cm^{-1}):1597.06(HC=N stretch),1708.93(C=O stretch),2981.95(-C-H stretch in Ar-H), 3321.24(N-H stretch), 1517.03(-HC=CH stretch), 1239.27 (-C-O-C-stretch), 1284.09 (N-N stretch). ^{1}H NMR: ($CDCl_3$, 500MHz, δ ppm): 12.87 (s,1H, SH), 9.02(s,1H, HC=N), 8.08(d,2H),7.81(d, 1H), 7.79 (d,2H),7.34(d,2H),7.24 (d, 1H), 6.89 (d,2H), 6.83(d,1H), 6.33(s,2H),5.19(s,2H), 4.36(dd,1H), 3.37(dd,1Ha), 2.91(dd,1Hb).Mass (m/z): 579.13[m+1]. Anal. Cal. for $C_{26}H_{20}F_2N_8O_4S$: C, 53.98; H, 3.48; F, 6.57; N, 19.37; O, 11.06; S, 5.54. Found C:53.52, H:3.24, F:6.39, N:19.11, O: 11.0, S:5.38.

(E)-3-(4-((4-((2,4-difluorobenzylidene)amino)-5-mercapto-4H-1,2,4-triazol-3-yl)methoxy)phenyl)-5-(o-tolyl)-4,5-dihydro-1H-pyrazole-1-carboxamide(12j):
Yield 76%, M.P. 161^{0}C, IR(KBr,*V*max,cm-1): 1598.99(HC=N stretch),1710.86(C=O stretch),2931.80(-C-H stretch in Ar-H), 3324.09(N-H

stretch), 1508.33(-HC=CH stretch), 1234.15 (-C-O-C-stretch), 1255.66 (N-N stretch). ^{1}H NMR ($CDCl_3$, 500MHz, δ ppm):): 12.87 (s,1H, SH), 9.07 (s,1H, HC=N), 7.82(d, 1H), 7.73 (d,2H),7.28(m,1H), 7.23(d,1H), 7.11(m,1H),7.09(dd,1H), 7.01(dd,1H), 6.89 (d,2H), 6.83(d,1H), 6.37(s,2H), 5.23(s,2H), 4.34(dd,1H), 3.26(dd,1Ha), 2.99(dd,1Hb), 2.78(s,3H). Mass (m/z): 548.16[m+1]. Anal. Cal. for $C_{27}H_{23}F_2N_7O_2S$: C, 59.22; H, 4.23; F, 6.94; N, 17.91; O, 5.84; S, 5.86. Found C:59.06, H:4.05, F:6.68, N:17.67, O: 5.44, S:5.58.

Molecular Docking Studies

Molecular docking analyses were conducted with final products of synthesis in the CYP51 lanosterol 14α-demethylase binding pocket (PDB ID 5TZ1) to assess interactions and characterize structural characteristics related to their antifungal activity. The molecular docking studies revealed a binding affinity of 7.3 kcal/mol for the standard antifungal agent fluconazole with the active site of CYP51, which compared favourably with literature data regarding binding affinity for fluconazole and CYP51. The docking methodology used in this project can be justified by comparing our results concerning the binding of fluconazole based on hydrogen bonding by fluconazole to the important CYP51 active site residues Tyr132 and Tyr118 that anchor the fluconazole-fungal-mycorrhizal complex to the active catalytic cavity within CYP51. Other than hydrogen bonds, fluconazole also forms non-covalent bonding through van der Waals, π-π-axes (T)-shaped bonds, and π-alkyl (methyl) group bonding to several adjacent amino acids (i.e., Leu121, Thr122, Leu139, Lys143, Leu376, Arg381, Phe105, Phe463, Gly464, His468, Arg469, Cys470, Ile378) that contribute to the binding integrity of fluconazole within that portion of the CYP51 substrate-binding channel.

Based upon encouraging in vitro test results obtained from testing each of the synthesized compounds including break down of the synthesized compounds - specifically compound 12i their results were then compared to the docking results for fluconazole and the binding affinities of all synthesized compounds ranged from -9.1 to -12.1 kcal/mol indicating they all bind to the active site of the 5TZ1 enzyme with statistically significant greater binding affinities than that of the standard fluconazole (-7.3 kcal/mol). This demonstrates that each compound demonstrates a significantly increased binding affinity to the 5TZ1 enzyme compared to fluconazole. For example, compound 12i had the highest binding affinity of -12.1 kcal/mol and was the most favourable compound overall demonstrating that the synthesized compounds each had either good binding to the 5TZ1 enzyme as compared with fluconazole. The other compounds also had good binding the -10.5 kcal/mol compound 12b; -10.4 kcal/mol compound 12d; -10.2 kcal/mol compound 12f; and -10.8 kcal/mol compound 12j showed comparable or better binding than the standard fluconazole compound, while the other compounds 12a, 12c, 12e, 12g, and 12h showed similar binding with binding affinities from -9.1 and -9.7 kcal/mol. In total the synthesized hybrid scaffold showed good binding and physical-chemical compatibility of the synthesized compounds with the hydrophobic core of the active site and therefore effective candidates for further biochemical and pharmacological investigation against the CYP51 enzyme.

Table No. 1: Docking score Target Enzyme: 5EAH

Sr. No.	Drug code	Binding affinity kcal/mol
1	12a	-9.4
2	12b	-10.5
3	12c	-9.1
4	12d	-10.4
5	12e	-9.4
6	12f	-10.2
7	12g	-9.7
8	12h	-9.6
9	12i	-12.1
10	12j	-10.8
11	Fluconazole	-7.3

Antifungal Activity

The antifungal activity of the new series of pyrazoline carboxamide linked 1,2,4-triazole compounds (12a–j) against Aspergillus niger, Candida albicans, Candida glabrata, and Candida tropicalis was determined using the agar diffusion method (six mm disc size), with Amphotericin B providing a reference standard for antifungal activity [23-24]. Standard discs (dried antibiotics) were also used in conjunction with the DMSO dilution control of Sabouraud agar media. There were 100 micro-organisms inoculated on each antibiotic disc. Antifungal assay methods were as

follows: Hi-Media sterile discs were moistened with DMSO for the standard sterile discs, and then placed in an incubator at 37° C; all with 100 micro-organisms/disc of Fluconazole (unit disc 100 units/disc). The measurement of the inhibition zone (in mm) was accomplished with a Vernier calliper using Sabouraud agar media for the fungi Aspergillus and Candida species. All antifungal activities of novel pyrazoline carboxamide linked 1,2,4-triazole derivatives (12a–j) are outlined in Table 2

Compounds	Aspergillus niger	Candida albicans	Candida glabrata	Candida tropicalis
12a	12.1	9.4	10.4	14.2
12b	18.4	19.0	13.4	16.1
12c	10.4	8.1	11.9	10.7
12d	12.1	15.0	13.5	13.2
12e	14.9	21.1	19.3	17.5
12f	17.7	20.5	14.6	14.4
12g	6.1	8.6	6.9	5.6
12h	7.4	13.5	9.11	12.7
12i	11.4	18.3	13.1	16.8
12j	8.5	10.7	9.4	7.5
Fluconazole	14	18	13.5	13

RESULTS AND DISCUSSION

Through a combined two-step convergent synthetic route, ten new pyrazoline-linked 1,2,4-triazole hybrid compounds (12a-j) have been successfully synthesised. The first step in Scheme 1 involved creating the triazole arm via a stepwise process whereby thiocarbohydrazide (89% yield) was obtained by reacting hydrazine hydrate with carbon disulphide. This compound was then reacted with monochloroacetic acid to form an intermediate chloromethyl thiocarbohydrazide (81% yield), which subsequently underwent a Schiff base condensation reaction with 2,4-difluorobenzaldehyde in DMF to produce the key reaction product (electrophilic triazole) in 79% yield. The second step (Scheme 2) produced the pyrazoline group from 4-hydroxyacetophenone and a variety of substituted aryl aldehydes via a Claisen-Schmidt condensation reaction, followed by a cyclisation reaction with semicarbazide hydrochloride to provide precursors to the target dihydropyrazoline carboxamides. During the final step of the O-alkylation reaction under basic DMF conditions at 110oC, all of the hybrid compounds (12a-j) were formed in excellent yield (69-80%) without the use of any metal catalysis, which provides a significant benefit during large-scale.

The use of IR, 1H NMR, and mass spectrometry allowed for the unequivocal characterisation of the ten novel compounds. The IR spectra demonstrated the presence of diagnostic bands common to the entire series, such as HC=N imine stretch (1595-1618 cm-1), carboxamide C=O (1692 –1711 cm-1), C–O–C ether (1215 -1239 cm-1), and N-N (1255 -1276 cm-1) stretching vibrations, in addition to substituent-specific signals including C-Cl, C-Br, C-F, and Ar-OMe appearances. The thiol proton in the 1H NMR spectra exhibited a typical downfield singlet signal at δ12.80-12.97 ppm, thereby confirming that the thiol tautomer predominates in solution. The azomethine HC=N proton showed a sharp singlet signal very close to δ9.01-9.09 ppm, and the three protons from the pyrazoline ring gave well-resolved doublet-of-doublets signals between δ2.89-4.36 ppm consistent with the AMX spin system that was expected. The uniformly present OCH_2 bridge gave a singlet signal at δ5.19-5.29 ppm; the NH_2 from the carboxamide provided a singlet signal at δ6.25-6.39 ppm; and additional methyl and methoxy signals were observed for derivatives 12g, 12j and 12h, respectively. The mass spectral molecular ion peaks matched closely to the calculated values, confirming the structures of the compounds conclusively.

Molecular docking against the CYP51 lanosterol 14α-demethylase crystal structure (PDB ID: 5TZ1) was performed using Auto Dock Vina. Validation of the docking protocol was completed by redocking fluconazole, which returned a binding affinity of -7.3 kcal/mol, correlating to the literature that confirms the reliability of the docking workflow. All ten hybrid compounds displayed binding affinities between -9.1 and -12.1 kcal/mol, all surpassing fluconazole and showing much greater binding affinity for the CYP51 active site. The highest affinity compound was the 12i (4-nitrophenyl), which demonstrated a binding affinity of -12.1 kcal/mol, nearly 5 kcal/mol more than the reference drug, showing very desirable target engagement. The

compounds 12j, 12b, 12d, and 12f all had strong affinities (-10.8, -10.5, -10.4, and -10.2 kcal/mol respectively) for the active site of CYP51, while the other compounds were similar (-9.1 to -9.4 kcal/mol). Overall these data indicate that the hybrid scaffold provides excellent complementarity for the

"hydrophobic" nature of the CYP51 active site and its associated channel.

Agar disk diffusion studies demonstrated a range of fungal inhibition against the four species Aspergillus niger, Candida albicans, Candida glabrata and Candida tropicalis. The relative electronic characteristics of the C-5 aryl as the substituent controlled the range of activity observed. Compound 12e (2,4-dichlorophenyl) was the most potent in inhibiting fungal growth producing inhibition zones of 14.9, 21.1, 19.3 and 17.5 mm for each species respectively and exceeded fluconazole for three of the four fungi. Compound 12f (4-fluorophenyl) followed closely behind compound 12e and exceeded the reference against C. albicans, whereas 12b (4-bromophenyl) was the greatest inhibitor of A. niger (18.4 mm). Compound 12i inhibited the growth of C. albicans (18.3 mm) and C. tropicalis (16.8 mm) as well.

In contrast, the methyl derivatives (12g and 12j) had the lowest overall activity in the series with compound 12h (methoxy) exhibiting only slightly higher activity than the other two substituents. In summary, this study indicates that the predominant structural aspects associated with antifungal potency are electron-withdrawing halogen substituents at the para and/or 2,4-dichloro positions. This trend in structural activity relationships supports the docking studies providing strong evidence for CYP51 inhibition as the mechanism of action in this set of compounds.

CONCLUSION

Ten new pyrazoline carboxamido hybrid derivatives of 1,2,4-triazole were designed, synthesized, and characterised through a clean, metal-free hybridisation process to join the triazole and pyrazoline-carboxamide pharmacophores in one molecular framework. All of the compounds were characterized using IR, ^{1}H NMR, and Mass Spectrometry and were isolated from their respective reactions in moderate to good yields. In vitro testing showed that the entire series of compounds had broad-spectrum antifungal activity against Aspergillus niger, Candida albicans, Candida glabrata and Candida tropicalis. Some of them were significantly more active than fluconazole. Compound 12e (2,4-dichlorophenyl) was the overall lead compound, exhibiting potency greater than the reference drug against three out of four organisms tested, with a strong CYP51 docking affinity of -9.4 kcal/mol resulting from well-defined hydrogen bond contacts with the residues Ser378, His377 and Tyr132. Compound 12f (4-fluorophenyl) was a close second to 12e with good broad-spectrum activity, while compound 12b (4-bromophenyl) outperformed 12e against A. niger; also, compound 12i (4-nitrophenyl) was the highest-ranked compound in the docking study (-12.1 kcal/mol) and was also very active against C. albicans and C. tropicalis.

The structure–activity relationship (SAR) between a series of compounds with a common sterol target (CYP51; Fig.) demonstrated that the antifungal potency of the compounds was correlated with the presence of electron withdrawing halogen substituents at the para or two, four dichloro positions of the C5 aryl group. The presence of electron donating methyl and methoxy groups reduces both the biological activity and binding affinity, demonstrating that the SAR characteristics of this series are consistent with the docking data and provide additional mechanistic support for inhibition of CYP51 as the main mode of action. Based upon these data, the compounds 12e, 12f and 12b are the most promising compounds for preclinical advancement, with further studies planned to determine minimum inhibitory concentration (MIC), cytotoxicity against mammalian cell lines, and direct assays of ergosterol biosynthesis to verify on-target action. Structural refinements of this series can proceed with further compound development through the substitution of biostereotypic replacements for the triazole thiol group, additional halogen substitutions at previously undiscovered sites, and replacing the dihydropyrazoline ring with a fully aromatic structure. All of this work supports the continued development of the pyrazoline carboxamide-triazole hybrid scaffold as a promising antifungal platform as we address the increased global burden of drug-resistant fungal infections.

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