Identification of Amino Acid Tethered Triazoles as Potential Antifungal Leads: Appraisal of their Mode of Action

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Abstract

A new set of compounds containing indole nucleus appended with carefully selected amino acids via a triazole linker are identified. These compounds showed substantial antifungal activities against the BWP17 strain of candida albicans. The preliminary screening of the compounds was done by docking in the active site of lanosterol 14-α demethylase enzyme (pdb ID - 3JUS) with ArgusLab.exe software. Subsequently the screened compounds were subjected to in-vivo antifungal assays. The probable mechanism of action of the test compounds was studied by GC analysis of the membrane metabolites. The compounds with tryptophan and histidine substitution were found to be most effective with MIC80 of 50.2 μg/ml and 78.81 μg/ml respectively. In the fungal cells treated with compound 3b the lanosterol levels were found to increase indicating that lanosterol 14-α demethylase enzyme could be the probable target. Additionally, the R6G influx/efflux assay provided the evidence for the fungicidal properties of the test compounds.

Keywords: CA BWP 17- Candida albicans BWP 17; R6G-Rhodamine 6G; Gas chromatography; MIC80-minimum inhibitory concentration for 80% inhibition

Introduction

Diseases caused by fungus are one of the major global challenges today. Starting from local infections to systemic mycosis the fungi are causing more deaths compared to malaria and tuberculosis. Yeasts, primarily the Candida albicans species are the most noxious mediators of invasive mycoses predominantly among the immuno compromised patients [1,2]. The pathogenicity and infectiousness of the fungal diseases therefore needs to be checked and regulated.

Various classes of antifungal drugs have been developed till date to counter the menace created by fungal diseases. The polyene antifungals like amphotericin-B, natamycin primarily target ergosterol [3] which helps in maintaining the fungal membrane integrity. These amphiphilic polynes on interaction with the fungal membrane make it leaky by creating the pores which eventually leads to the membrane disruption and fungal death. The azole antifungal drugs on the other hand target the D-glucan synthase enzyme in fungi by noncompetitive inhibition of antifungals, Echinocandins [6] impair the functioning of beta-1,3-glucan synthase enzyme in the fungal membrane thereby limiting its growth. The newest class of antifungals, allylamines [5] target the enzyme squalene epoxidase which mediates the conversion of squalene to squalene-2,3-epoxide. The allylamines like terbinafine leads to the buildup of squalene causing a pH imbalance in the fungal membrane thereby limiting its growth. The newest class of antifungals, Echinocandins [6] impair the functioning of beta-1,3-D-glucan synthase enzyme in fungi by noncompetitive inhibition eventually leading to the cell death. Despite all the efforts the antifungal therapy is still far off the target because of the efflux pumps operating in the fungal membranes [7]. The present work focuses on the design of the compounds chiefly to target the lanosterol 14-α demethylase enzyme present in the fungal membrane. The biological importance of these compounds is already been checked for their anti-inflammatory property by targeting the enzymes 5-LOX and COX-1/2 enzymes [8]. Interestingly, besides targeting the desired enzyme the compounds were also found to have membrane lysing properties.

The validation of indole moiety andazole nucleus in the antifungal therapeutics for targeting the fungal membrane has been widely acclaimed. The indole nuclei substituted with the carefully selected amino acids via azole linkage has been tested for its antifungal efficacy against the BWP17 strain of candida albicans. The screening of the appropriate amino acids was done by docking the test compounds in the lanosterol 14-α demethylase enzyme (pdb ID – 3JUS) with the help of ArgusLab.exe software. Apart from that, the Lipinski parameters were also calculated for the test compounds theoretically by molinspiration software. From the preliminary investigations, it was observed that the compounds having glycine, tryptophan, histidine, glutamic acid and tyrosine substitution displayed a better docking characteristics in the active site of the enzyme. The hydrophilicity index (ClogP value) for these compounds was also evaluated experimentally by determining the octanol/water partitioning coefficient by the shake flask method and the concentration of analytes was found by quantitative HPLC method. The hydrophilicity index thus obtained was found to be quite similar to that required by the lipinski parameters.

Experimental Section

Synthesis

The L-amino acids (1 mmol) were dissolved in 20 ml of DCM in a round bottom flask kept on an ice bath. The mixture was stirred for a few minutes till the solution becomes clear. Activated K2CO3 (1.2 equivalent, activated by storing at 100°C overnight) was then added to this solution. Afterwards trifluoromethanesulphonic anhydride (0.1 mmol) was very carefully added, dropwise to the reaction mixture. On addition, the colour of the reaction mixture immediately changes from colourless to light green. After stirring the contents of reaction mixture

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for 2-3 minutes was added CuSO₄·5H₂O (2.0 mmol) followed by the addition of sodium azide (1.0 mmol). The progress of the reaction was monitored by thin layer chromatography. The reaction completed in about 6-8 hours. After the reaction completion, the reaction mixture was washed with 15 ml of distilled water and the product was extracted by chloroform using a separator funnel. The final product was azide adduct of the amino acid (Compounds i, ii, iii, iv and v; Scheme 1).

3-methylindole (1) was dissolved in 5 ml DMSO and allowed to stir for a few seconds till a clear solution is obtained. To this solution was added sodium hydride (1.2 equivalent, washed with dry hexane to remove the paraffin coating). The reaction mixture was now stirred for 3-4 minutes till a visible change in the colour of the reaction mixture is observed. The change in colour corresponds to the generation of anion on the indole nucleus. Further, propargyl bromide (1.2 equivalent) was added carefully, dropwise to the reaction mixture. The completion of the reaction was monitored with the help of thin layer chromatography. Reaction completes in 25 minutes to give the product (2). The product (2) was dissolved in 9:1 solution of EtOH: H₂O to get a clear solution. To this solution was immediately added 0.02 mmol CuSO₄·5H₂O. After waiting for 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes.

Scheme 1
Finally, the azidoamino acid obtained in Scheme 1 was added to the reaction mixture. The monitoring of the reaction was done by thin layer chromatography. The reaction generally completes in 10-12 hours to get the products 3a-3e (Scheme 2) in very good yield. The further refining of the products was done by column chromatography using ethylacetate: hexane as eluent. All the reactions were performed in vacum fume hood by using carefully cleaned oven-dried lobachemie glassware. The stirring of the reaction mixtures was done on REMI made magnetic stirrers using teflon coated magnetic beads. Drying of diethyl ether was done over activated anhydrous calcium chloride followed by distillation over anhydrous K₂CO₃. The dried acetonitrile was finally stored over the activated molecular sieves of size 4 Å to prevent the infiltration of moisture. Reactions were monitored by TLC using silica gel GF254.

The chromatograms thus developed were viewed in ultraviolet light and staining was done with iodine. Column chromatography was done to purify the compounds. Silica gel of 100-200 mesh size was used using hexane and ethyl acetate as preferred eluents.

**1H and 13C NMR spectra and DEPT-135**

The spectra were recorded at a frequency 500 MHz and 125 MHz for 1H and 13C spectra respectively. CDCl₃ and DMSO were used as preferred solvents with TMS as an internal standard. The 1H NMR spectral data were reported in form of chemical shifts (δ ppm), multiplicity (s=singlet, br s=broad singlet, d=doublet, dd=double doublet, t=triplet, m=multiplet) and the coupling constant (expressed as J in Hertz). The 13C and DEPT-135 NMR spectral data were represented in terms of chemical shift with +ve signals corresponding to CH₃ and CH carbons and the -ve signals corresponding to CH₂.
carbons while an absent signal points towards a quaternary carbon. SP 300 PYE UNI CAM Infrared Spectrophotometer was used to record the Infrared spectra with anhydrous KBr pellets.

Mass spectra and LCMS procedure

Buker MicroTOF QII mass spectrometer (Buker Daltonik, Bremen, Germany) machine calibrated with sodium formate (internal calibrant) was used to record the mass spectra. KDScientific automated pump with flow rate 180 µL/h was used to inject the sample to electrospray ionization source at a concentration 50 µM in acetonitrile-water-formic acid (7:2:90:1). D2N2 gas heated at 180°C was used for the desolvation of the injected solution. Optimisation of the various parameters of the mass spectrometer was done for obtaining a maximum ion abundance. Specifically, the capillary voltage was adjusted at 4500 V and the vacuum was sustained at 3 x 10^-10 mbar. For LC-MS, Dionex Ultimate 3000 system was coupled to the mass spectrometer. Chirobiotic T 10 µm chiral HPLC column (25 cm x 4.6 mm) was used for the analysis. A solution of Acetonitrile in water (1:1) was used as eluent. 2 µL of sample (injection volume) was loaded to the column. The flow rate was maintained 0.2 ml and the absorbance was set at 200, 220 and 254 nanometers. Sodium formate was used as internal calibrant.

1H/13C/DEPT-135 NMR data of the test compounds

[4-(3-Methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-acetic acid (3a): The compound (2) was dissolved in 9:1 solution of EtOH:H2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of CuSO4.5H2O. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the azidoacetic acid (i) synthesized in Scheme 1, was added to the reaction mixture and the contents were allowed to stir for another 10 minutes. Lastly, the 2-Azido-3-(1H-indol-3-yl)-[1,2,3]triazol-4-yl)- propionic acid (3c): The compound (2) was dissolved in 9:1 solution of EtOH:H2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of CuSO4.5H2O. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-3-(1H-imidazol-4-yl)-propionic acid (ii) obtained in Scheme 2, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to get a yellow coloured solid, mp 121-126°C, 65% yield, [α]D25 = +124 (c 0.01, MeOH), IR (KBr) vmax 3299 (NH), 1600 (C=O) cm-1; 1H NMR (CDCl3, 500 MHz): δ = 8.91 (s, 1H, COOH), 7.45-7.89 (m, 2H, ArH), 6.83-7.39 (m, 5H, ArH), 6.82 (s, 1H, ArH), 4.41 (t, J = 15 Hz, 1H, CH), 2.27 (s, 3H, CH3); ESI-MS (HRMS) calculated for C17H17N7O2 352.1524 Found m/z 352.1523 [M+H]+.

3-(1H-Indol-3-yl)-2-[4-(3-methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-3-(4-Hydroxy-phenyl)-2-[4-(3-methyl-indol-1-ylmethyl)-[1,2,3]triazol-4-yl]-pentanedioic acid (3d): The compound (2) was dissolved in 9:1 solution of EtOH:H2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of CuSO4.5H2O. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-4-carboxy-heptanedioic acid (iv) obtained in Scheme 2, was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to give a dark brown solid, mp 111-116°C, 85% yield, [α]D25 = +23 (c 0.01, MeOH); IR (KBr) vmax 3412 (NH), 1574 (C=O) cm-1; 1H NMR (DMSO d6, 500 MHz): δ = 10.41 (s, 1H, COOH), 9.81 (s, 1H, COOH), 7.91 (s, 1H, ArH), 7.53-7.82 (m, 2H, ArH), 7.22-7.39 (m, 3H, ArH), 4.39 (t, J = 5 Hz, 1H, CH), 4.20 (s, 2H, CH2), 3.63-3.72 (m, 2H x CH2), 2.57 (3H, 3H, CH3); ESI-MS (HRMS) calculated for C17H18N4O4 342.1408 Found m/z 342.1410 [M+H]+.

3-(4-Hydroxyphenyl)-2-[4-(3-methyl-indol-1-ylmethyl)-[1,2,3]triazol-1-yl]-propionic acid (3e): The compound (2) was dissolved in 9:1 solution of EtOH:H2O and allowed to stir till a clear solution is obtained. To this clear solution was immediately added 0.02 mmol of CuSO4.5H2O. After waiting for another 5 minutes, 0.05 mmol sodium ascorbate was added to the reaction mixture and the contents were allowed to stir for 10 minutes. Finally, the 2-Azido-4-(4-hydroxy-benzyl)-pentanedioic acid (v) was added to the reaction mixture. The reaction was monitored by thin layer chromatography technique. The reaction generally completes in 8 hours to give a brown solid, mp 145-153°C, 78% yield, [α]D25 = +35 (c 0.01, MeOH); IR (KBr) vmax 3315 (NH), 1701 (C=O) cm-1; 1H NMR (CDCl3, 500 MHz): δ = 9.15 (s, 1H, COOH), 9.08 (s, 1H, ArH), 7.73-7.94 (m, 2H, ArH), 7.53-7.63 (m, 1H, ArH), 6.87-7.52 (m, 3H, ArH), 4.42 (t, J = 15 Hz, 1H, CH), 4.35 (s, 2H, CH2), 3.33 (d, J = 1H).
3 Hz, 2H, CH2, CH3), 2.20 (s, 3H, CH3); (13C NMR normal/DEPT - 135) (125 MHz, CDCl3): (C) = 173.53 (COOH), 145.30 (C), 144.82 (C), 135.27 (ArCH), 134.94 (ArCH), 130.75 (C), 129.87 (ArCH), 126.81 (ArCH), 126.33 (ArCH), 124.55 (ArCH), 123.30 (ArCH), 121.36 (ArCH), 113.30 (ArCH), 109.04 (C), 107.24 (C), 58.75 (CH), 29.29 (CH2-CH3), 25.19 (CH3), 21.53 (CH3); ESI-MS (HRMS) calculated for C21H20N4O3 377.1615 Found m/z 377.1615 [M+H]+.

Results and Discussion

MIC80

The antifungal activity of the test compounds 3(a-e) was evaluated by performing the MIC80 assay. Ten successive dilutions of the test compounds were prepared along with the solvent control with yeast extract peptone dextrose (YE PD) medium in a 96-well plate. The overnight grown cells of the BWP17 strain of the fungi were resuspended in a 0.9% saline solution to give a resultant optical density of 0.1 at a wavelength of 570 nm (OD570). These cells were further diluted 100 times in SDM medium. 100 μl of these diluted cells were added to each of the wells in the 96 well plate except the column 11 which was taken as media control to examine any kind of contamination while performing the assay. Column 12 which was taken as control positive contained the fungal cells only in the growth medium. The assay was performed in duplicates. After preparing the plate it was incubated at a temperature of 30°C for a duration of 48 hours. Afterwards, the monitoring of OD570 of the cells was done using a micro plate reader. The percent inhibition of the fungal growth in the presence of test compounds 3 (a-e) was determined with respect to the positive control by deducting the percent inhibition due to the solvent control. The MIC80 values were thus calculated by plotting a graph between percent inhibition versus concentration of the test compound using the Graphpad Prism software. The percent inhibition of the fungi was determined at eight different concentrations of the test compounds 3 (a-e). The Compounds 3b, 3c, and 3d containing tryptophan, histidine and glutamic acid substituents appended to the indole through a triazole linker were found to be most effective against CA BWP17 in the presence of 100 µg/ml of compound 3c at concentrations 100 µg/ml and 50 µg/ml. Whereas, the zone of inhibition was much narrower in case of the compounds 3a, 3d and 3e.

Spot assay

The results of MIC80 evaluation and the disc diffusion assay led us to select the compounds 3b and 3c for performing the spot assay. A known concentration of the compounds 3b and 3c was added to the medium containing YEPD and agar. Solvent control plates were also prepared. The plates with naked YEPD-agar medium served as positive controls. The fungal cells growing overnight on the YEPD medium at a temperature of 30°C served as primary culture medium. The secondary culture was prepared from a 4% inoculum of the primary culture medium. From the secondary culture were prepared five serial dilutions of cell suspension with 0.9% saline solution to give a resultant OD of 0.2, 0.04, 0.008, 0.00016 and 0.00032 at 570 nm. A 5 µl volume from each cell suspension was added to all the plates. After incubating the plates at 30°C their images were recorded after 24 hours and 36 hours respectively. The spot assay indicates an impaired fungal growth in the presence of the compounds 3b and 3c. The results were quite comparable with the commercially used drug ketoconazole which showed a similar growth inhibition pattern after 24 hours and 36 hours of treatment (Figure 1).

SEM analysis

The morphological changes occurring in the fungal cells in the presence of test compounds was monitored through the Scanning Electron Microscopy. The CA BWP17 cells were grown in the presence of the most active compound 3b. The cells were pelleted, washed and then fixed into 2.5% glutaraldehyde and 2% Paraformaldehyde in 0.1M phosphate buffer at 4°C. In order to get a superior staining of the fungal cells the assay plates were prepared and 107 cells were spread on it. This plate was then dried overnight at 30°C. A 90 mm diameter YEPD-agar plate was then prepared and 107 cells were spread on it. This plate was then dried for around 10-15 minutes. Small filter paper discs (5 mm diameter) were prepared, sterilized and placed on this plate with the help of clean forceps. A known concentration of each test compound dissolved in an appropriate solvent was spotted on the paper disc. Similar procedure was repeated with the solvent controls. After incubating the assay plates for 48 hours at 30°C the growth of the fungal cells at the periphery of the disc was checked. The diameter of the circle around the disc with no fungal growth corresponds to inhibition zone. In order to maintain an effective concentration of the test compounds in the culture medium the disc diffusion assay was performed at a slightly superior concentration than the MIC80 values. The images of inhibition zone (mm) of fungal culture in the presence of test compounds compared with the corresponding solvent are shown in Tables 1 and 2. The zone of inhibition was highest for the Compound 3b followed by the compound 3c.

Table 1: MIC80 values and experimentally determined hydrophilicity index of compounds 3a-e for CA BWP17 strain.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC80 (µg/ml)</th>
<th>ClogP (Experimental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>617.9</td>
<td>3.45</td>
</tr>
<tr>
<td>3b</td>
<td>50.2</td>
<td>1.89</td>
</tr>
<tr>
<td>3c</td>
<td>78.81</td>
<td>1.78</td>
</tr>
<tr>
<td>3d</td>
<td>188.05</td>
<td>-1.01</td>
</tr>
<tr>
<td>3e</td>
<td>752.78</td>
<td>1.99</td>
</tr>
</tbody>
</table>

Table 2: Diameter (in mm) of the zone of inhibition in the disc diffusion assay of highly effective compounds against CA BWP17.
Osmium tetroxide (OsO₄). The dehydration of the sample was done by sequentially treating the cells with increased concentrations of acetone from 50% to 95%. After drying the sample with critical point drier it was mounted on the stub SCM. Spatter coater was used for the gold coating from 50% to 95%. After drying the sample with critical point drier it was sequentially treating the cells with increased concentrations of acetone 30 KV. The morphological changes were noticeable on the fungal membrane in presence of compound 3b which got augmented with passage of time. The SEM investigations clearly indicate that the membrane integrity leading to the creation of a free passage for the entry and exit of metabolites across the fungal membrane. Overall, highly efficient antifungal leads with membrane bonding interactions were observed for the compounds 3b and 3c which interact with the active site residues principally through N atom of the triazole ring. The ‘R’ stereoisomer of compound 3b interacts with the residues Y576, Y590 and F597 via COOH and N atom of the nitrogen heterocycle by hydrogen bonding interactions of length 1.23 Å, 1.22 Å, 1.77 Å and 1.69 Å respectively. Similarly, the ‘R’ stereoisomer of the compound 3c interacts with the residues Y576, F597, G752 and A756 via N atom of the triazole ring and COOH functional group with hydrogen bonding interactions of length 2.22 Å, 1.88 Å, 1.72 Å and 1.61 Å respectively.

**Study of influx - efflux of R6G in CA BWP17 in presence of compounds**

Several of the commercially available antifungal drugs are subjected to the cellular efflux pumps. To check the vulnerability of the test compounds 3b and 3c towards the fungal efflux pumps, the R6G efflux-influx assay was performed. R6G is a substrate of fungal efflux pumps. It was envisioned that if there is a competition between test compounds and R6G to interact with efflux pump, the transportation of R6G out of the cell will be debarred. Conversely, if there is influx of R6G in presence of compounds it would indicate that there is no interaction between compound and efflux pump protein. Influx-efflux of R6G in fungal cells was monitored by recording the absorbance and fluorescence of extracellular fluid. CA BWP17 primary/secondary culture was grown as described earlier. After centrifugation the cells were taken in PBS solution or non-growing medium. The PBS solution was now divided into three portions. One part was taken positive control, second part was considered as solvent control and the third part was treated with the test compounds. R6G was added to all the three portions. The assay was performed in triplicates. An increase in R6G influx was observed in positive control and solvent control experiments while in presence of compound 3b, most of R6G entered the cell immediately and no change in R6G influx was observed with time. Similarly, the initial efflux of R6G in the presence of compound 3b was found to be much faster compared to positive control and solvent control. Both influx as well as efflux assays were pointing towards rupture of the fungal membrane in presence of compound 3b making free passage for influx and efflux of R6G (Figures 6-8). Similar trends were observed during the efflux of R6G in presence of compound 3c (Figures 9-29).

**Conclusions**

As conclusion, the amino acid tethered triazoles were identified with momentous antifungal activities. The minimum inhibitory concentration assay revealed that the compounds 3b and 3c with tryptophan and histidine substituents were most active inhibitors of the BWP 17 strain of fungi candida albicans. The observation was further confirmed by performing the spot assay and disc diffusion assays. The GC analysis of the metabolites of the fungal membrane revealed an elevated concentration of the ergosterol which led us to conclude that the test compounds actively target the enzyme lanosterol 14-a demethylase. The docking of the test compounds in the active site of the enzyme further strengthened this observation. The study of the R6G efflux/influx pumps in the fungal membrane revealed that the test compounds adversely influence the membrane integrity leading to the creation of a free passage for the entry and exit of metabolites across the membrane. Overall, highly efficient antifungal leads with membrane lysing properties were recognized.

**Acknowledgements**

PP thanks the University of Petroleum and Energy Study for providing the requisite resources for the synthesis of compounds. The state of the art instrumental
Figure 2: SEM images of CA BWP17 culture grown in the presence and absence of compound 3b at different time intervals. D1, D2, D3 and D4 indicate the growth of cells under the optimum conditions in the absence of the compound after 2 h, 6 h, 10 h and 16 h respectively. E1, E2, E3 and E4 indicate the growth of the cells in the presence of compound 3b after 2 h, 6 h, 10 h and 16 h respectively.

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<th>Compd.</th>
<th>Docking Score (Kcal/mol)</th>
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<tr>
<td>3a</td>
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<td>Y576 (1.23 Å), COOH</td>
</tr>
<tr>
<td>3b (S)</td>
<td>-12.34</td>
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<tr>
<td>3c (R)</td>
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<td>Y576 (2.22 Å), N (azole)</td>
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<td>Ketoconazole</td>
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Table 3: The docking characteristics of the different stereoisoemers of the compounds in the active site of the enzyme lanosterol 14-α demethylase.

Figure 3: The figure indicates a depleted levels of ergosterol in the cells treated with compound 3b.

Figure 4: The crystal coordinates of the enzyme lanosterol 14-α demethylase (pdb ID 3jus) in association with the compound 3b. The white dotted lines represent the H-bonding interactions between compound and the amino acid residues in the active site of the enzyme. The interaction are principally through the triazole nucleus and COOH functional group of the compound.
Figure 5: CPK model for showing the placement of the compound 3b in the active site of the enzyme lanosterol 14-α demethylase in the presence of the natural inhibitor (shown in yellow and blue wireframe).

<table>
<thead>
<tr>
<th>Compound</th>
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<th>ClogP</th>
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Figure 6: Lipinski Parameters for the compounds. ClogP - hydrophilicity index, Nviolations - number of violations from Lipinski parameters.

Figure 7: R6G influx in the presence of compound 3b.
Figure 8: (a) R6G efflux in the presence of compound 3b. (b) Differentiation between active and passive efflux.

Figure 9: Influx of R6G in the presence of compound 3c.

Figure 10: (a) R6G efflux in the presence of compound 3c. (b) Differentiation between active and passive efflux.
Figure 11: $^1$H NMR spectra of compound 3a.

Figure 12: $^{13}$C/DEPT-135 of compound 3a.
Figure 13: $^1$H NMR spectra of compound 3b.

Figure 14: $^{13}$C/ DEPT-135 of 3b.
Figure 15: $^1$H NMR of compound 3c.

Figure 16: $^{13}$C/ DEPT-135 of compound 3c.
Figure 17: \(^1\)H NMR spectra of compound 3d.

Figure 18: \(^{13}\)C/ DEPT-135 of compound 3d.
Figure 19: $^1$H NMR spectra of compound 3e.

Figure 20: $^{13}$C/ DEPT-135 of compound 3e.
Figure 21: Compound 3a.

Figure 22: Compound 3b.

Figure 23: Compound 3c.
Figure 24: Compound 3d.

Figure 25: Compound 3e.

Figure 26: Compound 3b (50% ACN:H₂O).
facility at Guru Nanak Dev University is highly acknowledged for characterization of the compounds. Special thanks to the faculty of biotechnology and pharmacology, Lovely Professional University for providing the fungal cultures and designing the in vivo experimental protocols and providing the requisite instrumentation for biological investigation of the compounds.

References


