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In silico and *in vitro* screening to identify structurally diverse non-azole CYP51 inhibitors as potent antifungal agent

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ABSTRACT

The problem of resistance to azole class of antifungals is a serious cause of concern to the medical fraternity and thus there is an urgent need to identify non-azole scaffolds with high affinity for lanosterol 14α-demethylase (CYP51). In view of this we have attempted to identify novel non-azole CYP51 inhibitors through the application of pharmacophore based virtual screening and *in vitro* evaluation. A rigorously validated pharmacophore model comprising of 2 hydrogen bond acceptor and 2 hydrophobic features has been developed and used to mine NCI database. Out of 265 retrieved hits, NSC 1215 and 1520 have been chosen on the basis of Lipinski's rule of five, fit and estimated values. Both the hits were docked into the active site of CYP51. In view of high fit value and CDocker score, NSC 1215 and 1520 have been subjected to *in vitro* microbiological assay. The result reveals that NSC 1215 and 1520 are active against *Candida albicans, Candida parapsilosis, Candida tropicalis,* and *Aspergillus niger.* In addition to this the absorption characteristics of both the hits have also been determined using the rat sac technique and permeation in order of NSC 1520 > NSC 1215 has been observed.

1. Introduction

In the past two decades, the mortality and morbidity rate has dramatically increased in patients suffering with fungal infections like *Candidosis*, *Aspergillosis* and *Cryptococcosis* [1,2]. Several antifungal agents have been developed and clinically used but still cure rate is low probably due to high resistance to current antifungals.

The role of lanosterol 14α-demethylase (CYP51) in fungal growth makes it a relevant target for the development of drugs active against different fungal strains. CYP51 catalyses the oxidative removal of 14α-methyl group of lanosterol to give desaturated intermediates required in ergosterol biosynthesis and this step is considered crucial for fungal growth. [3]

The administration of azole class of CYP51 inhibitors leads to accumulation of lanosterol and other 14-methyl sterols along with the depletion of ergosterol [4]. Mechanistically, the nitrogen atom at 3rd position of azole ring binds the ferric atom in heme prosthetic group, which prevents the binding of lanosterol [5,6]. The activity scale of azoles depends upon their binding capability to heme iron as well as the affinity of the N-1 substituent for the protein part of the cytochrome [7,8]. Though azoles have played a very important role in controlling fungal infection, but the problem of resistance and toxicity have restricted their clinical use. Undoubtedly the situation is alarming and there is an imperative need for non-azole CYP51 inhibitors [9].

Diverse *in silico* techniques such as pharmacophore modeling, virtual screening and molecular docking has played an important role in drug discovery [10] and it is a well known fact that many new drugs have their origin from Computer Aided Drug Designing (CADD) [11–13].

Prompted by the role of pharmacophore based virtual screening in lead identification [14–16] we have employed a sequential work flow comprising of varied *in-silico* tools and wet lab experimentation to identify novel antifungal agents.

2. Materials and methods

2.1. Data set preparation

A structurally diverse data set of 28 triazole derivatives [17] with inhibitory activity against CYP51 was selected from the literature. Out of 28 triazole derivatives, 20 compounds were selected in training set taking utmost care to ensure scaffold diversity and wide range of activity values (highly active, active, moderately

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active, and inactive). Structures of all the compounds were sketched and energy was minimized to the closest local minima. Conformational analysis was carried out using the BEST conformation module which considers the spatial arrangement of chemical features rather than simply the arrangement of the atoms. A maximum of 255 conformers were generated for each molecule within an energy threshold of 20.0 kcal/mol above the global energy minimum using CHARMM force-field parameters [18]. Instead of using lowest-energy conformer of each compound, all conformers were used for pharmacophore model generation.

Prior to quantitative pharmacophore model development, common-feature pharmacophore study was carried out to elucidate the important features which led to the identification of two hydrogen bond acceptor (HBA) and two hydrophobic (HY) features. All the training set compounds and their conformers along with CYP51 inhibitory activity were used to generate quantitative pharmacophore models employing the features identified from the common feature pharmacophore analysis. The minimum number for each feature was kept '0′ and maximum to '5′, allowing the algorithm to generate an equitable model. Out of various generated pharmacophore models, best model was selected on the basis of correlation coefficient, root-mean-square deviation (RMSD) and cost difference [19]. The chosen pharmacophore model was rigorously validated using Fischer's randomization test, rm^2 matrices test, internal and external test set prediction.

2.2. Rm^2 metrics test

In order to understand the proximity in predicted and observed response data, the rm^2 metrics (average rm^2 and delta rm^2) developed by ROY et al. was calculated and analyzed. The recommended values of 'Average rm^2, and 'Delta rm^2, are >0.5 and <0.2 [20,21].

2.3. Fischer's randomization test

The objective of this test is to check whether there is a strong correlation between chemical structures and biological activity. This test focuses on randomly assigning activity values to the molecules in the training set. If these activity values generate pharmacophores with analogous or better cost values and correlations than the original model is considered to be generated by chance. Nineteen different HypoGen runs were applied at 95% confidence level using the same features and parameters as used during original pharmacophore model generation [22]. All the pharmacophore hypothesis generated during Fischer's randomization test were evaluated for their statistical significance.

2.4. Güner–Henry scoring method

The goodness of the pharmacophore model depends on its ability to differentiate between active and inactive compounds, thus, Güner–Henry (GH) scoring method has been employed to accurately assess the virtual screening capability of the generated pharmacophore model [23–25]. A database of 312 structurally diverse known CYP51 inhibitors from ten publications was constructed and used for GH scoring [26–35]. This method is considered as an appropriate metric, since it includes the calculation of the percent yield of actives in a database (%Y, recall), the percent ratio of actives in the hit list (% A, precision), the enrichment factor (E) and GH score. These parameters are computed using Eqs. (1–4).

$$\%A = \frac{H_a}{A} \times 100 \quad \ldots..(1)$$

$$\%Y = \frac{H_a}{H_t} \times 100 \quad \ldots..(2)$$

$$E = \frac{H_a/H_t}{A/D} \quad \ldots..(3)$$

$$GH = \frac{H_a\,(3A + H_t)}{4H_tH_a} \times \left(\frac{1 - H_t - H_a}{D - A}\right) \quad \ldots..(4)$$

%A is the percentage of known active compounds retrieved from the database (precision); H_a, the number of actives in the hit list (true positives); *A*, the number of active compounds in the database; *%Y*, the percentage of known actives in the hit list (recall); H_t, the number of hits retrieved; *D*, the number of compounds in the database; *E*, the enrichment of active compounds in the virtual screening hit list in comparison to the non-filtered database and GH is the Güner–Henry score.

2.5. Internal and external test set prediction

The utility of any pharmacophore model lies in its ability to correctly predict the activity of compounds outside the training set. In light of this the chosen model was used to predict the activity of structurally diverse 8 internal and 12 external tests set compounds. The correlation coefficient value for each set was observed to ascertain the validity of model [36–38].

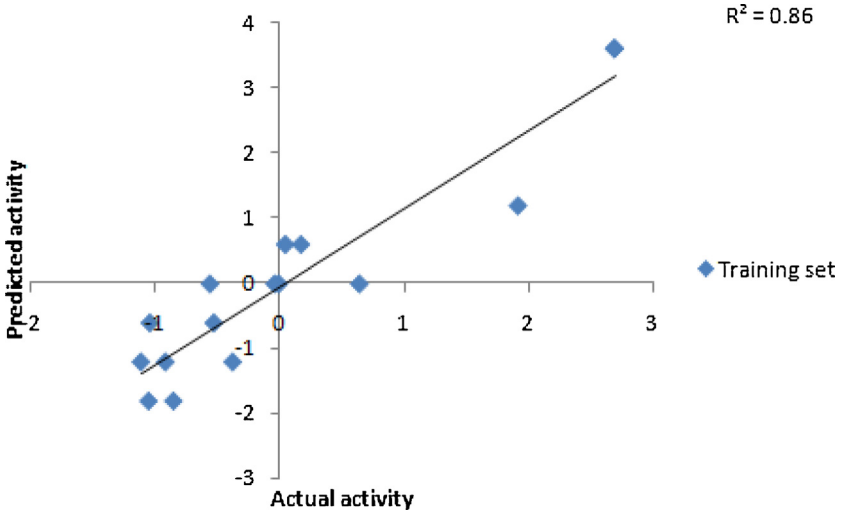

Fig. 1. Plot of predicted versus the corresponding actual activity (IC_{50}) for training compounds.