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Original Article

Viability of *in-silico* analysis of CYP51s in Aspergillus species for identifying novel azole resistance mechanisms

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ABSTRACT

From the perspectives of evolutionary history and drug resistance, the classification and characterization of cytochrome P450 monooxygynases in Aspergillus species have shown numerous structure-function correlates of the enzyme and its variations. Aspergillus is one of the major pathogens of the respiratory tract that deteriorate the prognosis of various pulmonary diseases. The prognosis of the target diseases is further impacted due to the emergence of azole resistance. This article explores the attributes of azole resistance of Aspergillus strains with regard to point mutations and polymorphisms in *CYP51* and CYP51 from an *in-silico* perspective. Studies reveal that Aspergillus requires CYP51A and CYP51B for full functionality. The CYP51 in Aspergillus is chemically 14-alpha demethylase which converts lanosterol to ergosterol. Ergosterol helps in the membrane fluidity and virulence of the fungal isolate. The enzyme is also the target of azoles, especially the active heme molecule within the active site. The current study shows that deletion mutations in CYP51 could lead to over expression of 14-alpha demethylase which could be considered a novel mechanism for azole resistance. Moreover, the deletion mutations could also potentiate the over activity of the ABC transporter proteins (efflux pump) because the alignment of the amino acid residues in mutated CYP51 nearly matched that of ABC transporter proteins in the same fungal isolate. It was further contended that deletion mutations in *CYP51* promoter could prevent the inhibitory effects of transcription factors on CYP51 expression which could be considered as another method for azole resistance.

KEYWORDS: CYP51, Aspergillus, *in-silico* azole, resistance.

1. INTRODUCTION

Efforts are now being made to decipher the molecular basis of complex diseases using the high-throughput strategies that are available for identifying the dynamics of the bio-molecules that drive such diseases [1]. In this regard, *in-silico* methods have generated wide interest among microbiologists, molecular biologists, and pharmacologists, in identifying novel drug targets through computational analysis [2]. Different species of fungi are widely recognized for their pathogenic profile and economic importance. Moreover, such pathogens are continuously evolving new strategies for adapting to diverse ecological niches with negative selection pressures as well as in hostile host environments. One such adaptation in Aspergillus species is the development of azole resistance. Azole resistance is mediated by various mechanisms among which point mutations in the target enzyme cytochrome p450 monooxygenase of the variant CYP51 is most common [3]. The

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mutations in the cytochrome p450 enzyme are attributed to the selection pressure caused by the widespread use of triazole fungicides in agriculture as well as in health care settings. The mutations similar to that observed in clinical isolates of the Aspergillus species during the 1990s in *Candida albicans* and *Aspergillus fumigatus* were also isolated in the field of agriculture [4]. However, the number and the location of the point mutations have significantly increased and changed significantly over the last decade. Interestingly, the current trend indicates that triazole antifungal mutations emerge from the plant agriculture and manifest as pathogenic and resistant fungi in clinical infections [5]. It is speculated that the prevalence of azole resistance fungi and diseases will significantly increase over the next two decades. This is because there would be a significant increase in the use of triazole antibiotics both in agriculture as well as in clinical settings. Azoles are antifungal compounds that target the CYP51 variant of cytochrome P450 monooxygenases across a wide range of fungal species. CYP51 in Aspergillus is chemically 14-alpha demethylase that is required for converting lanosterol to ergosterol [6]. Ergosterol is responsible for the fluidity of the fungal plasma membrane and virulence of the fungal strain. Aspergillus is a life-threatening pulmonary pathogen, which causes bronchopulmonary Aspergillosis. More than 10 million individuals are at the risk of developing invasive aspergillosis as a result of the increased use of corticosteroid anticancer therapies. Studies suggest that almost 200,000 individuals across the globe develop invasive aspergillosis with the mortality rate of 50% even with triazole therapy [3]. On the other hand, more than 4 million asthmatics across the globe develops bronchopulmonary aspergillosis, while another 3 to 13 million of the target population suffer from severe asthma due to fungal sensitization [7]. The prognosis of Aspergillosis deteriorates due to an increase in the prevalence of azole resistance.

Although CYP51 is widely distributed across the fungal kingdom, with high conservation from species to species, differences still exist in types and subtypes in terms of phylogeny. Aspergillus contains two CYP51 proteins, A and B [8]. Certain species of Aspergillus could also contain a third variant of the CYP51 protein which is paralogous (extra copy) to either protein A or B. Studies on *Aspergillus fumigatus* suggest that CYP51B protein remains constitutively expressed while the A variant has to be induced for its functional activity [9]. Resistance to azoles might be depicted by amplification of CYP51 genes within the fungus, or other strategies [10]. It could be possible that deletion mutations (that were observed in this study) in CYP51 could result in overproduction of ERG-11.

These findings suggest that it is necessary to identify novel mechanisms of azole resistance in pathogenic Aspergillus. Such initiatives would help to develop novel target molecules that could mitigate the risk of azole resistance. Interestingly, studies suggest that pathogenic Aspergillus resistant to one group of azoles could be sensitive to another group of azoles, while it could also be inferred that mutation in one of the amino acid residues in the CYP51 variants could cause mutations in other amino acid residues in the same variant of CYP51. Therefore, understanding the probable mutational hotspots of CYP51 could help to design novel azoles that would mitigate the challenge of azole resistance in pathogenic fungi. The present study explores the research question “Does *in-silico* analysis of CYP450s in Aspergillus species promise identification of putative targets that have turned resistant to azole group of drugs?”.

2. METHODS

2.1. Study design and sampling

The present study was carried out using secondary data and a quantitative approach. The study was based on various *in-silico* methods that ranged from basic local alignment search tool (BLAST) to modeling of the protein (CYP51) of interest.

2.2. Procedure

2.2.1. BLAST

BLAST analysis was initially carried out using CYP51 from Aspergillus species. The reason for carrying out the BLAST analysis was to identify the nucleic acid sequence and amino acid sequence of the CYP51 from different strains of Aspergillus. The Aspergillus strains were then explored based on their historical data on azole resistance which was obtained from evidence-based literature and recent publications. Another objective of the BLAST

analysis was to find proteins that were most similar to CYP51 with the highest identity. However, the sequence identity was chosen with a cut off of at least 98 percent. The structural configuration of the CYP51 genome was studied in terms of chain characteristics, sequence pattern and tandem repeats, and the chemical properties of the peptide chain such as hydrophobicity. The peptide chain CYP51 was also studied from the perspective of evidence-based literature that explained azole resistance in Aspergillus species. The azole-resistant strains along with the homologous proteins were next subjected to fast and structured task analysis (FASTA).

2.2.2. FASTA

The FASTA was carried out for multiple sequence analysis (MSA) of the homologous species of CYP51. The BLAST sequences were selected for the FASTA analysis. The MSA was done by comparing the first twenty species (with 98% to 100% fit-identity) with the subsequent nineteen species in decreasing order of identity that was obtained from the Uniprot database. The ALIGN process was applied to the twenty species, which resulted in the FASTA format. The FASTA analysis was run on the twenty species to provide information on the similarity and identity of various parts of the genome. The FASTA alignment was further subjected to phylogenetic analysis that revealed the evolutionary trend and cladistics from where the species diverged over a period. The FASTA analysis was done using appropriate template selection of the ABC transporter protein because it is one of the major proteins that are implicated in drug resistance of various microbes to antimicrobials including the azole group. The FASTA sequences were compared on the basis of chain lengths, DNA binding ability, and transmembrane helices orientation.

The amino acid properties of the respective chains were also compared to investigate homology, as well as the hydrophobic properties, and the orientation of the negative and positive charge residues, the assembly of the aliphatic and aromatic groups along with polar residues and serine –threonine assembly. The similar positions were noted to study the conservativeness of the sequences based on consensus sequences and conserved sequences. The similar sequences would be searched for single nucleotide polymorphisms to extrapolate azole sensitiveness and azole resistance of the species of Aspergillus and the allied FASTA proteins.

2.2.3. Phylogenetic analysis

Phylogenetic analysis was carried out next, to evaluate the evolutionary divergence of the respective proteins based on their structure function correlates. The structure-function correlates were evaluated from evidence-based literature and was compared with the phylogenetic traits related to the parsimony principle. The cladistic analysis along with the divergence pattern helped to identify whether a specific protein of the CYP51 (and the organism that housed it) was becoming sensitive or resistant to the azole group of antibiotics. The resistance pattern was also correlated with the mutational hotspots in the similar sequences. Individual nucleotide and protein FASTA similarities and identities were examined. The nucleotide FASTA helped to identify the single nucleotide polymorphisms along with the mis-sense or non-sense mutations that might have translated into the azole resistance. The phylogenetic tree was also used to forecast evolutionary changes in azole resistance and other antifungal resistances.

2.2.4. Protein modelling

Protein modelling was carried out using the PyMol software to understand the structure function correlates of the mutated versions of the CYp51 protein and non-mutated versions of the CYP51 protein to understand the putative targets for azoles and other anti-fungals. The protein modelling was also undertaken to evaluate the possible structure and class of antifungal that would be effective in mitigating antimicrobial resistance in Aspergillus species.

2.2.5. *In-silico* and statistical software

The *in-silico* software that was used for this study to conduct the BLAST, FASTA, and phylogenetic analysis was the UniProt and CLUSTAL-W software while the SWISS-MODEL software was used for modelling the proteins of interest. The correlation analysis was conducted using different variables such as the length of the similar sequences, the mutational hotspots, and the mis-sense mutations in the conserved and consensus sequences. The parsimony attributes and cladogram

distances are an estimate of the predictable mutations or sensitivity of the CYP51 protein to azole and other antifungals.

3. RESULTS

3.1. FASTA format strategy

TR|Q9P8R0|Q9P8R0_ASPFM
MVPMLWLTAYMAVAVLTAILLNVVYQLF
FRLWNRTEPPMVFHWVPFLGSTISYGIDP
YKF 60

TR|A0A0H4U5P5|A0A0H4U5P5_ASPFL -
MSWPRIGAYALLAFVAIMALNVTYQFLFRM
LNKTRPPLVFHWIPFIGSTIHYGTDPYGF 59

TR|G4WW88|G4WW88_ASPTU
MAYLAVAGVYAFAALLVAIVLNVARQLLV
R--NEKEPPVVFHWIPFLGSTISYGMDPYAF
58

TR|G4WWA2|G4WWA2_ASPAW
MALLAVAGVYAFAALLVAIVLNVTRQLLFR
--NEKEPPVVFHWIPFLGSTISYGMDPYAF 58

TR|G4WWA3|G4WWA3_ASPNG
MALLAVAGVYAFAALLVAIVLNVTRQLLFR
--NEKEPPVVFHWIPFLGSTISYGMDPYTF 58

TR|G4WW93|G4WW93_9EURO
MAYLAVAGAYAFAALLVAIVLNVARQLLV
R--NEKEPPVVFHWIPFLGSTISYGMDPYAF
58

TR|G4WW85|G4WW85_ASPNG
MALLAVAGVYAFAALLVAIVLNVTRQLLFR
--NEKEPPVVFHWIPFLGSTISYGMDPYTF 58

TR|G4WW98|G4WW98_9EURO
MAYLAVAGAYAFAALLVAIVLNVARQLLV
R--NEKEPPVVFHWIPFLGSTISYGMDPYAF
58

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3.2. Phylogenetic analysis

The phylogenetic analysis (Figure 1a) reflected that the out-group was ASPFL, while the Aspergillus strain (ASPFM) evolved from the common ancestral origin of ASPFL. The phylogenetic analysis further revealed that the Aspergillus strains ASPNG, ASPAW, and Eurotiomycetes in general might have common ancestral origin to ASPFM (which is the most common Aspergillus strain) but they formed distinct in-groups over the evolutionary period. As ASPNG and EURO had common ancestral origins, these strains may be the mutated versions of ASPFM and could house the genes for azole resistance.

The FASTA analysis with CYP450s revealed that the Aspergillus strains as well as those of the Eurotiomycetes family shared a common ancestor (Figure 1b), with the eurotiomycete strains being the out-group. The number of identical positions in CYP450 was 331 while that for CYP51 variant was 338. These findings suggest that the CYP51 had more conserved sequences than CYP450, over the course of evolution. Such findings are not surprising because CYP51 in Aspergillus or other fungal strains is associated with specific functions such as membrane fluidity and virulence while CYP450 accomplishes a plethora of functions from metabolism to drug transport. In contrast, the number of similar positions in CYP51 was 90 compared to that of CYP450 which was 112. These findings suggested that CYP51 might have conserved sequences but they are more vulnerable to point mutations and single nucleotide substitutions. Therefore, it could be contended that azole resistance could stem from point mutations in CYP51 because it is the enzyme (14-alpha demethylase) that causes membrane fluidity and virulence of the fungal strain. Likewise, the cytochrome p450 enzyme (especially the active heme) is the major target of azoles.

3.3. Protein modelling

The two most similar members of azole-resistant fungi CP51A_ASPFU and ATRR_ASPFU were compared. The former is the azole-resistant fungi and the latter is the ABC drug transporter protein obtained from Candida. The most significant deletion mutations were found in the amino acids between 200 and 240 nucleotide bases, and are also found in the folded chains of CYP51.

SP|Q4WNT5|CP51A_ASPFU ----------

SP|Q4WI89|ATRR_ASPFU
QQNSSSHYSTPRLESQSSPRTAATSPESQKES
ETEVEGLSDMMCSLVTNNCGETRYIGSS 240

The protein modelling (Figure 2) revealed that the mutational hotspots are present both within the folded chains that are mainly conserved regions (red)

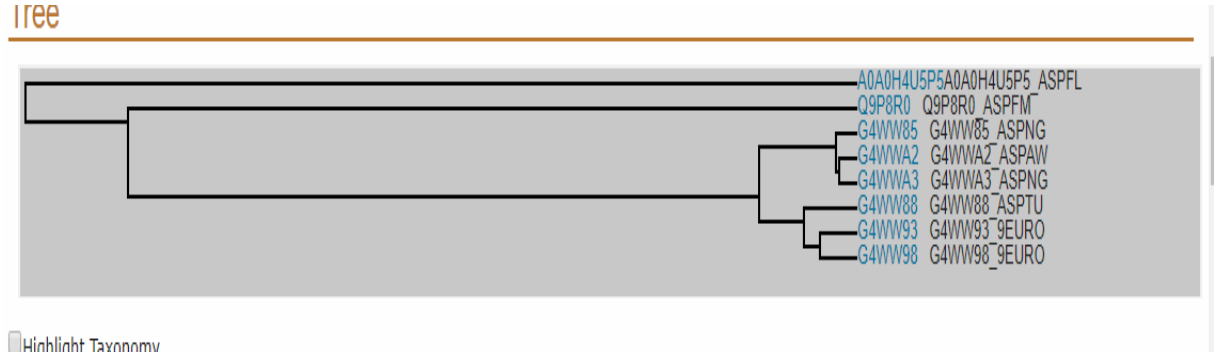

Figure 1a. Phylogenetic tree.

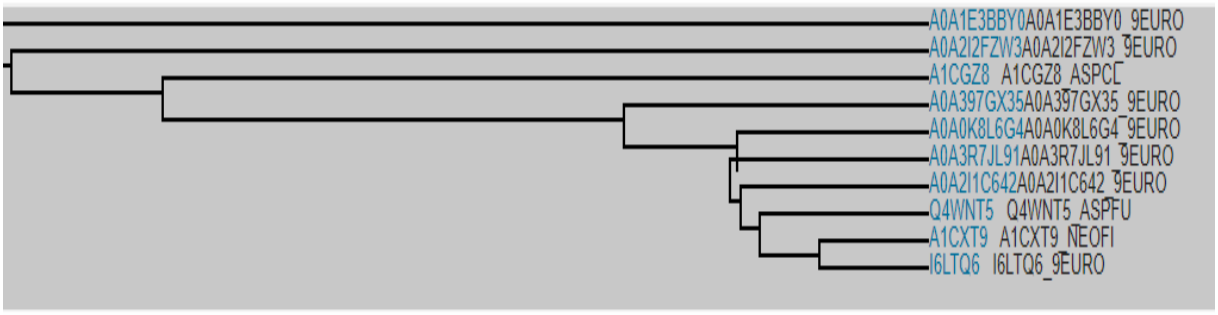

Figure 1b. Phylogenic analysis with Eurotiomycetes CYP51.

Figure 2. Protein model.

as well as in the N-terminal portions. Interestingly, this is the first study that showed that mutations in specific hotspots increase the likelihood of mutations in other hotspots for the same gene and protein. These assumptions were substantiated by local quality estimation which showed that predicted local similarity fluctuates in a rhythmic manner.

It could be possible that mutations (fluctuations) in one residue or a set of residues could cause mutations (fluctuations) in other residues within

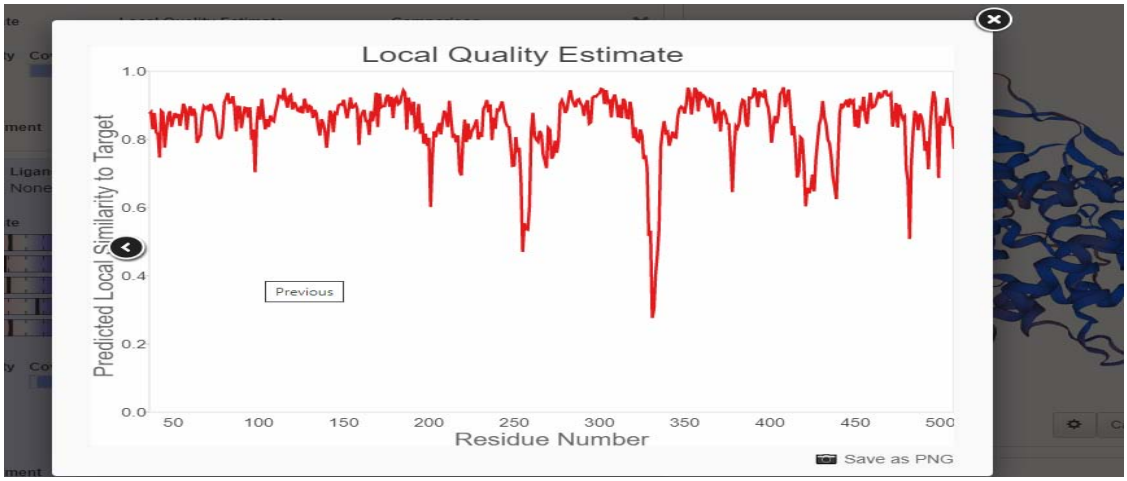

Figure 3. Local quality estimation.

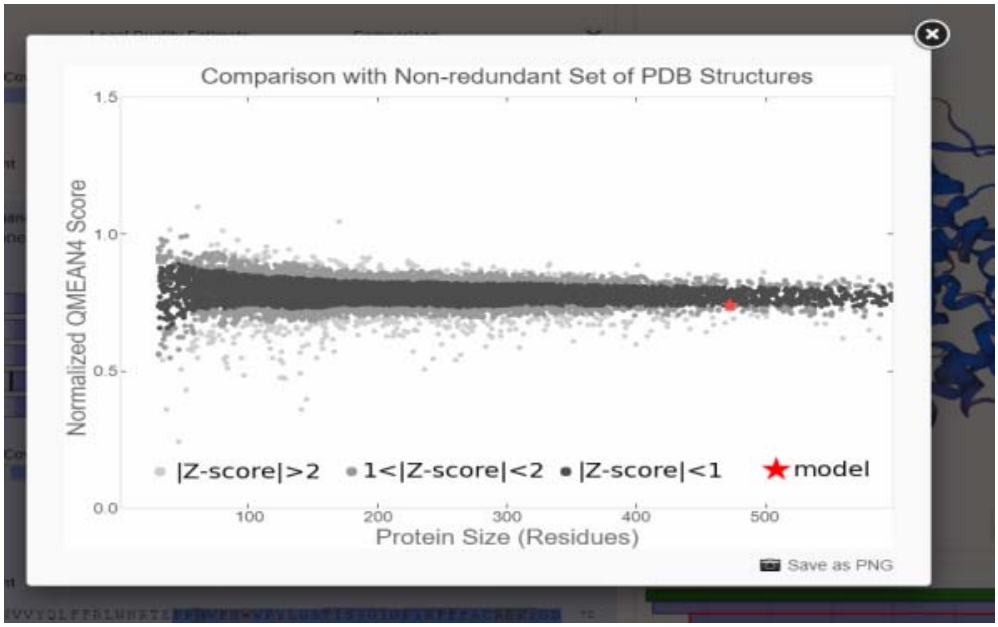

Figure 4. PDB estimation.

the same molecule (Figure 3). These assumptions are substantiated by previous studies which had endorsed that the CYP51 is subjected to specific mutations and some of them are dependent on each other. However, there was no evidence till date that CYP51 of Eurotiomycetes also exhibit the same characteristics, especially in *Aspergillus fumigatus*. The conserved nature of CYP51 was further substantiated by the non-redundant Protein Data Bank (PDB) structures.

The non-redundant PDB structure (Figure 4) showed that the 14-alpha demethylase (azole resistance) (model) marginally differed (yet significantly), in terms of QMEAN4 score, from the normalized QMEAN4 score. This finding suggested that even minor fluctuations in PDB structure of CYP51 could attribute to azole resistance.

Crystal structure of sterol 14-alpha demethylase (CYP51B) from a pathogenic filamentous fungus Aspergillus fumigatus (Figure 5) in complex with

VNI showed that it was different from the azole-resistant variant. The detailed structural correlates of azole resistance CYP51 in *Aspergillus fumigatus* is provided in the following Ramachandran plot:

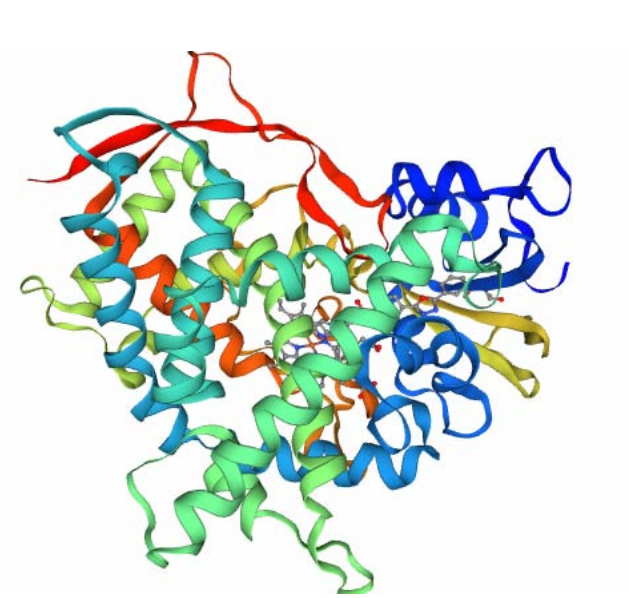

Figure 5. Crystal structure of the model protein.

The Ramachandran plot in Figure 6 endorsed that most of the CYP51 molecules are B-pleated sheets and as a result there is a chance of finding folded chains and conserved regions. However, it could be possible that mutations in the molecule could change its conformation from Beta-pleated sheets to right-handed and left-handed alpha helix. In the case of azole-sensitive species, the change in conformation could disrupt the orientation of the active site, whereas in the case of azole-resistant isolates, mutations could enter a more beta-pleated sheet conformation to ensure that the active site is capable of converting lanosterol into ergosterol.

4. DISCUSSION

The present study showed that azole resistance in CYP51A and CYP51B of Aspergillus species occur at the gene and protein level. The gene changes include single nucleotide polymorphisms and deletions while point substitutions are observed in amino acid sequences in the CYP51. The changes in the three-dimensional conformation of the active site of the 14-alpha demethylase (CYP51A) protein impair the formation of ergosterol from lanosterol.

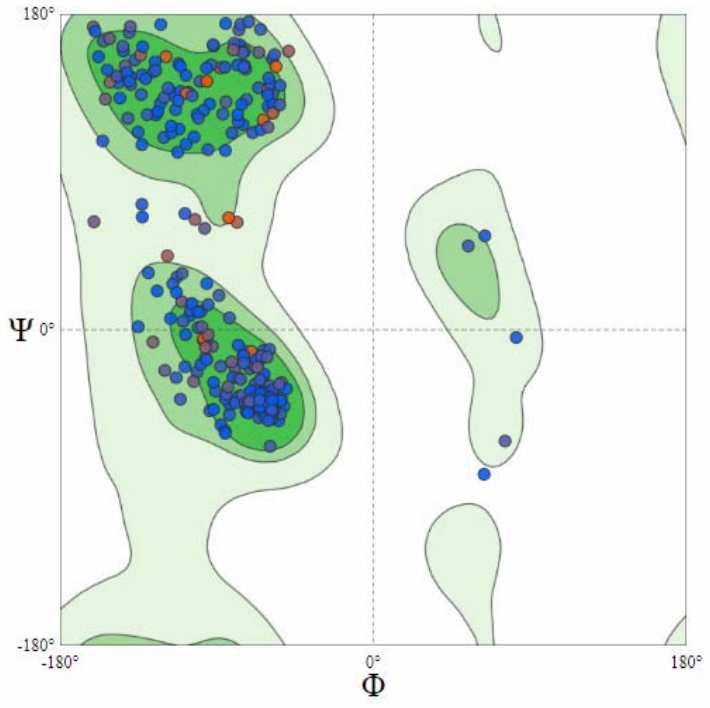

Figure 6. Ramachandran plot.

As a result, there is a marked decrease in membrane fluidity and mycelial growth that is essential for virulence of the fungal isolates. This study also showed that deletions in the tandem repeats of the promoter of CYP51 could have resulted into reduced expression of the14-alpha demethylase. Another possibility of azole resistance is that single nucleotide polymorphisms in the tandem repeats of the promoter might have increased its sensitivity to the transcription factors that causes down regulation of expression of CYP51A which again results in reduced titer of 14-alpha demethylase.

The single nucleotide polymorphisms in the CYP51 could have resulted in the folding of the heme active sites, which is necessary for binding with the ligands (azole). Finally, this is the first study that showed that mutations in CYP51 and azole resistance could be an alteration in the function of 14-alpha demethylase as an overactive ABC transporter. The sequence identity of the CYP51 protein and the ABC transporter coupled with previous evidence that azole resistance could occur through non-CYP51A mechanisms substantiated such assumptions.

The present FASTA analysis reflected that there were deletions in the CYP51 in comparison to the ABC drug transporter protein. Since ABC drug transporter is an important mediator of efflux pump for antimicrobials that enter the microbes, deletion mutations might convert CYP51 to overactive ABC drug transporters that efflux the azole out of the fungal cell. Such assumptions need to be substantiated experimentally by assaying the amount of azoles that came out of the fungal cell. The CYP51 is located in the inner face of the endoplasmic reticulum as a membrane mono-spanning enzyme, while its N-terminus is an amphipathic helix that links the catalytic unit of 14-alpha demythylase with the lipid bilayer.

The multiple sequence alignment of CYP51 proteins indicates that they exhibit an identity between 36.5% and 93.9% across people and fungal isolates. The evidence suggests that the amino acid residues that are present within the folding of chains play a crucial role in enzymatic function. Therefore, it could be contended that changes in the amino acid residues within the folded chains might contribute to azole sensitivity. In addition, further folding of the protein chains that contain the conserved amino acid sequences might contribute to azole resistance. On the other hand, the residues that formed the surface of the CYP51 protein active site remained highly conserved, especially the Y118, F126, G127, V130, and T311 in the beta helix, beta helical turn and helix-1 signature regions.

Amino acid sequences that are conserved were classified into six substrate recognition sites and three motifs. Amongst them, the most conserved motif is a heme- binding domain that contains the heme axial Cys ligand and the E-R-R triad imposed by the EXXR and PER motifs. These motifs contributed to the locking of the heme pocket, which ensured stabilization of the core structure. On the other hand, the six putative targets that contain the substrate recognition site serve as landmarks for fungal CYP51s. Previous studies indicate that the CYP51B complex containing the tetrazole-based inhibitor VT-1598 has an optimized hydrogen bond between the phenoxymethyl oxygen of the inhibitor and imidazole ring nitrogen of HIs374 of CYP51. Studies further suggest that binding of azoles with CYP51 truncated its structure which led to the inhibition of fluidity of the cell membrane of the fungal isolate as well as its virulence [5].

The widespread use of CYP51-targeting molecules has resulted in the production of drug-resistant isolates. It is suggested that most of the resistance mechanisms associated with azoles are related to structural and genomic changes in CYP51. On the other hand, the binding of transcription factors such as Pdr1 and Stb5, as well as insertion elements such as Aft1 into the promoter region of CYP51 could reduce its sensitivity to the azoles [11]. It could be possible that deletion mutations in CYP51 could promote the binding of the transcription factors to the CYP51 promoter which causes reduced expression of CYP51 or changes the functional conformation of the ERG-11 [12]. Therefore, changes in ERG-11 interaction with azoles could occur at the genomic level and at the protein level. Aspergillus has a unique ability to thrive inside mammalian hosts as well as in the external environment, which is critical for azole treatment failure and the emergence of azole-resistant species. Previous studies suggest that non-synonymous substitution of amino acids;

transcription factors, tandem repeats, and Dap proteins are the major causes of azole resistance in Aspergillus. The substitutions in the amino acids due to single nucleotide polymorphisms or deletion mutations might result in truncated proteins which are unable to convert lanosterol into ergosterol. It might also be possible that the truncated proteins could behave as ABC transporter proteins that gained efflux function after such deletion mutations or substitutions. Indeed, point mutations have been observed in lanosterol 14-alpha demethylase (CYP51) in G54, L98, G138, M220, and G448 [13]. These amino acid positions are considered as mutational hotspots. Interestingly, most of these amino acids are present in the folded chains of the protein which suggests that mutations could have led to changes in the three-dimensional structure of the active site which is necessary for enzymatic conversion of lanosterol to ergosterol (Figure 6).

Mutations in G54R/E/V and 138S led to cross-resistance with itraconazole and posaconazole. These findings further support that there is a conserved position within the enzyme for binding of ligands (azoles) which mediated their antifungal action. This is because mutations in the G448S were associated with voriconazole tolerance. When the substitution at G138s was reversed, the fungal isolate was susceptible to both itraconazole and posaconazole. The evidence further suggests that a single mutation in the CYP51 could increase the risk of multiple mutations and different azoles for the same fungal isolate. For example, heterologous expression of G54W significantly reduced susceptibility of Aspergillus isolates to itraconazole and posaconazole but had no effect on voriconazole. These findings suggest that the group of azoles should be changed if the fungal isolates are refractory to another group of azoles instead of considering treatment failure with azoles. In another study, the authors showed that mutations in the tandem repeat of the CYP51 promoter and enzyme leads to broad spectrum azole resistance [14]. Hence, the broad-spectrum azole resistance is a function of point mutations in the CYP51 gene while narrow-spectrum azole resistance could be attributed to the CYP51 protein. The point mutations (deletions) in the tandem repeats of the promoters could have reduced the transcription efficiency of the CYP51, which reduces the titer of the functional enzyme. Studies have depicted that L98H could cause flexible changes in the BC loop and IH loop of *A. fumigatus* CYP51. As a result, the position of Tyr 107 and 121 side chains also changes. Such changes modify the ligand-biding (azole binding) channels in CYP51A of Aspergillus which prevents the binding of azole to the active heme. The lack of binding of azole to the active heme ensures constitutive action of the CYP51 enzyme.

The mutations are carried over in subsequent generations because they do not confer survival disadvantage to *Aspergillus.* In contrast, Y121F substitution could disrupt the hydrogen bonding between tyrosine and heme center of CYP51A of *Aspergillus fumigatus*, which results in the instability of the active center of the enzyme. The instability of the active center results in poor functionality of the CYP51A enzyme and reduces the conversion of lanosterol into ergosterol. Although most of the mechanisms associated with azole resistance in Aspergillus species are related to CYP51, other resistance mechanisms than CYP51 confer azole resistance to *Aspergillus*. For example, mutations in the efflux pump (ABC transporter protein) could also lead to azole resistance. These findings are aligned with the findings of the present study because it also showed that the CYP51 azole-resistant variant has remarkably similar identity with the ABC transporter of the fungal isolates in *Aspergillus*. Such findings suggest that point mutations in the ABC transporter have increased its effect, and it is also possible that point mutations or deletions in the 14-alpha demethylase have functionally converted it into an efflux pump capable of ejecting azoles from the fungal cell membrane.

5. CONCLUSION

The major strength of this study was that it implemented all the possible *in-silico* methods to elucidate the structure-function correlates of azole resistance in CYP51 of Eurotiomycetes**.** The *in-silico* analysis was not only limited to the polymorphisms in the CYP51 gene but was extrapolated to the structural anomalies in the CYP51 protein. However, the major limitation of this study was that it did not evaluate the *in-silico* analysis in terms of protein expression or protein structure.

Future studies should explore altered mRNA expression through quantitative reverse transcription (RT-qPCR) to evaluate the point mutations in CYP51. The future studies should also assay the amount of ergosterol produced by azole-resistant and azole-sensitive Aspergillus. Such analysis would help to identify whether azole resistance primarily is a function of single nucleotide polymorphisms or point mutations in the amino acid sequence of CYP51. To substantiate the findings further, X-ray crystallographic structures of azole-resistance and azole-sensitive CYP51 of Aspergillus should be studied.

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CONFLICT OF INTEREST STATEMENT

The authors have no financial conflicts of interest related to this work.

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