Third Meeting Proposal

Discovery of Novel Fungal CYP51 Inhibitors as Anti-Fungal Drugs

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Submitted to

Advancement to Candidacy Committee

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Abstract

A new era of infectious disease has emerged where previously effective medicines are losing their efficacy due to microbial resistance. This presents a danger in both developed and developing countries where pathogens which previously were routinely treated can become persistent and deadly infections. One of the deadliest types of infections are due to fungi, with especially high mortality in immunocompromised individuals. Thus, a demand for novel drugs which can overcome the resistance of pathogens to existing medications is great.

Sterol 14∝-demethylases (CYP51s) are key enzymes located in the endoplasmic reticulum which remove a methyl group from cyclized sterol precursors. CYP51s are highly conserved across phyla, belonging to the cytochrome P450 superfamily. CYP51s lead to the formation of cholesterol in animals and ergosterol in fungi and protozoa. CYP51 is a well validated anti-fungal drug target due to its inhibition preventing fungal synthesis of vital sterols which comprise the fungal cell membrane. The biological role of sterols is to provide moisture control and rigidity in the plasma membrane. Without sterols, fungi cannot grow effectively and frequently die as a result of administering CYP51 inhibitors.

Many current anti-fungal drugs are inhibitors of CYP51s but suffer from several weaknesses. In certain species of fungi, such as *Cryptococcus neoformans*, CYP51 inhibitors such as Fluconazole are only fungistatic. In the case of broad spectrum azoles such as Itraconazole, Voriconazole, and Posaconazole, poor absorption and penetration of the central nervous system greatly limit clinical efficacy. In the current proposed research, a structure based approach to discovery of novel CYP51 inhibitors was performed. A virtual screen using the recently solved structures of *Aspergillus fumigatus* CYP51 with the potent, broad-spectrum anti-fungal drug VT-1598 was performed. The ligands prioritized from the ZINC database were molecules with heteroaromatic rings capable of coordinating to a key heme group in the CYP51 binding site. 5 promising compounds with predicted interaction energies greater than VT-1598 were discovered and visually verified to coordinate to the heme group in the CYP51 binding site. A plan for the spectrophotometric and MIC study of these compounds was thus planned for Hit A, whose facile synthesis will enable screening of analogs towards the key anti-fungal drug CYP51.

Background:

Fungi:

Fungi are eukaryotic organisms that form the bedrock of nutrient cycling on Earth[1]. As such, they are ubiquitous organisms whose kingdom contains over 100,000 species. Fungi have been naturally selected to extract nutrients from their environment via hyphae, branching filaments that can penetrate solid substances. These hyphae enable efficient digestion of complex plant matter including lignin and cellulose. Fungi refresh soil by digesting old plant material and enriching the soil with their metabolic byproducts. Fungi can reproduce sexually or asexually depending on environmental conditions, and fungal spores are pervasive in most ecosystems. This fungal presence means many lost profits to the agricultural industry, where fungi spoil many fruits and vegetables prior to reaching the consumer. It is estimated that around 20 % of pre-harvest agriculture losses can be traced back to fungi, which equals billions of dollars in lost productivity[2].

Although many fungi attack plants, only about 0.2% of species are human pathogens[3]. Despite this small fraction of fungal pathogens, natural selection and evolutionary common heritage with humans make fungi pathogens that can be difficult to treat. Fungal infection of humans typically occurs following inhalation of spores or exposure to compromised skin. Other fungi, such as *Candida* species, are commensal to humans and thus their infections reflect uncontrolled growth of an otherwise innocuous organism. The types of fungal infections can be categorized by their localization; cutaneous infections occur on the skin whereas subcutaneous infections occur below the skin due to exposed wounds. Lastly, systemic infections occur when fungi enter the body, often through the lungs, and begin colonizing the host. In healthy humans, the fungi trigger a powerful immune response via polymorphonuclear leucocytes, macrophages, and natural killer cells, however the specific immune response varies by fungus. Depending on whether an immunocompromised individual lacks T-cells or neutrophils, certain species of fungi will be more problematic than others. The mortality of fungal infections is undoubtedly correlated with the prevalence of fungal infections in individuals who already have compromised immune systems. This population includes organ transplant recipients, acquired immunodeficiency syndrome (AIDS) sufferers, and those undergoing immunosuppressive therapies in general. The medical complications that occur with fungal infection include internal bleeding via vessel erosion and organ failure. These complications are typically the ultimate causes of death.

Fungal infections of humans have become a significantly more pressing public health issue in the last 20 years. With advanced medical procedures becoming more common, more individuals are subject to states of immunocompromization[4]. This increase in the number of immunocompromised persons and the high mortality rate of invasive fungal infections renders the lifesaving technologies developed in the last 20 years null and void. Some estimates place the number of individuals affected by fungal diseases at around 1.2 billion individuals, with around 1.6 million deaths a year. To further compound the issue, many fungal species have become resistant to existing anti-fungal drugs, which already suffered from limited efficacy due to poor pharmacokinetic properties. It is therefore highly advantageous to mankind’s wellbeing to create

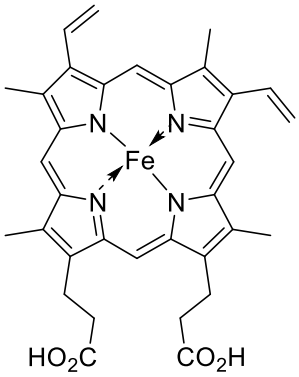
new anti-fungal drugs, specifically with improved drug-like properties. In this proposal, the inhibition of a specific fungal metabolic enzyme will be the basis of an antifungal drug.

Cytochrome P450 Oxidases

CYP51 is a classical target in antifungal drug design. CYP51 is part of the larger family of cytochrome P450s. These enzymes are named after the wavelength (450 nm) of spectrophotometric absorption maxima under reducing conditions[5]. Cytochrome P450s carry a heme group that enables mono-oxygenation reactions. These reactions serve various roles *in vivo*, including xenobiotic metabolism, steroid oxidation, and fatty acid oxidation. Cytochrome P450s require some type of reduction upon reacting with their substrate, which often means a companion protein delivers electrons to reduce iron at the end of the cycle. A generic version of the reaction can be summarized as below.

$$RH + O_2 + \text{Reducing species} + H^+ \rightarrow ROH + NADP^+ + H_2O$$

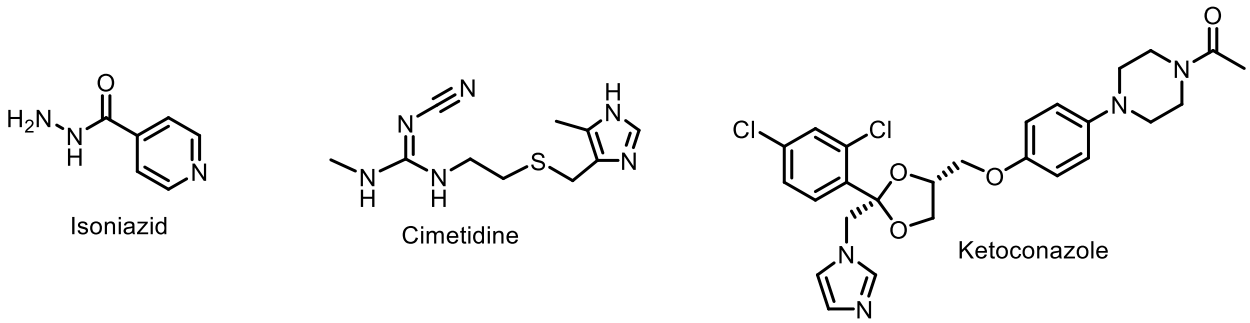
Where R is the organic substrate. Molecular oxygen from the atmosphere serves as the source of oxygen in the reaction. Because practically all kingdoms of life contain CYP450, there are many variations to equation 1, however the concept remains the same. The most common reducing species are nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH)



Heme-Fe II Complex

Iron complexed with heme plays a key role in stabilizing intermediates in CYP450 mediated oxidation, transitioning to a high oxidation state to aid reduction by an organic substrate. Cysteine plays the other key role via interaction with the iron moiety. Many inhibitors of human CYP450 have been elucidated, some of which result in life-threatening drug-drug interactions. Some are shown below.

Figure 1. Assortment of CYP450 family inhibitors, from right to left (ketoconazole CYP2A6, Cimetidine (broad spectrum CYP450 inhibitor), and Isoniazid (CYP2C9))



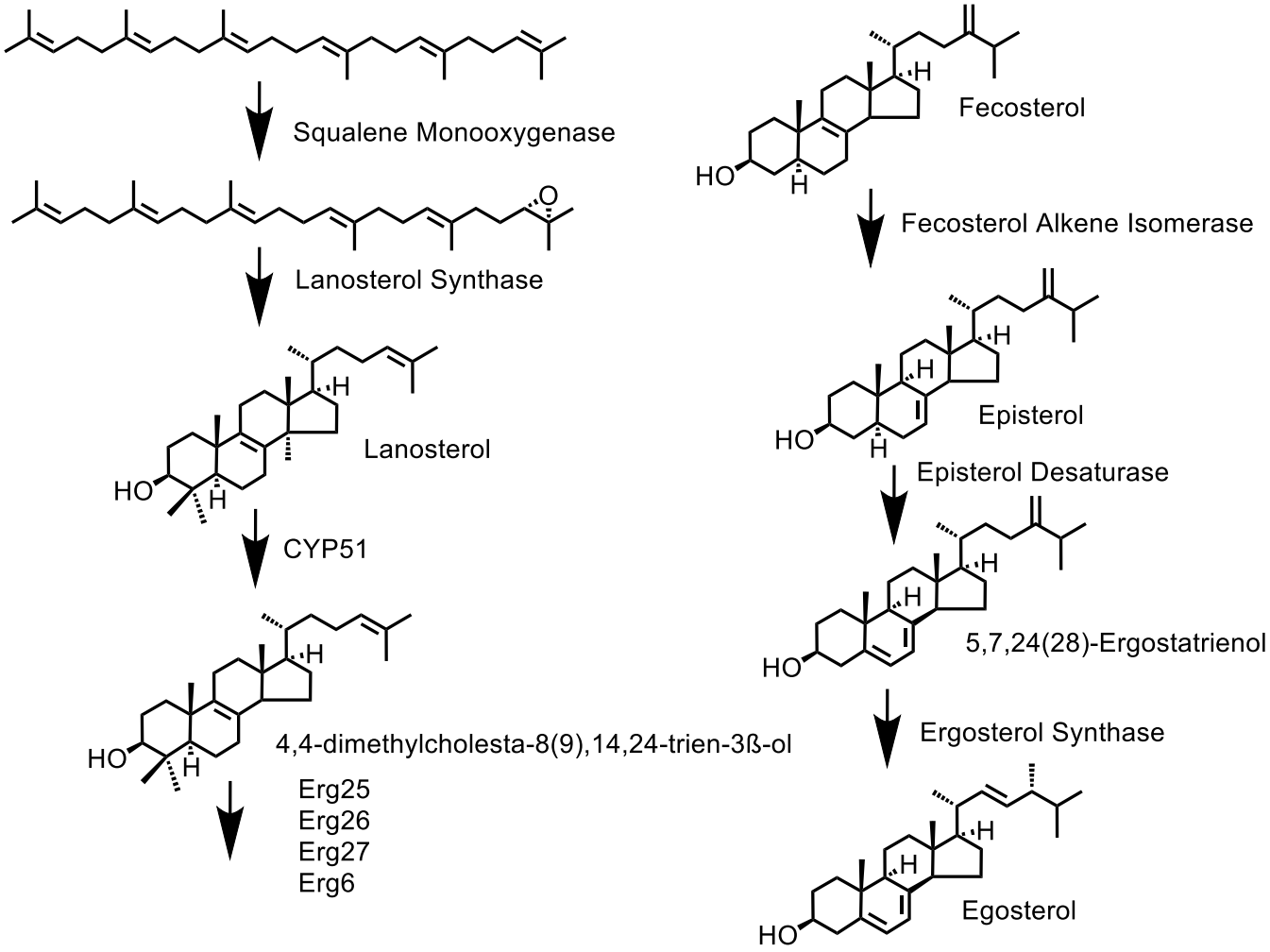
Because of the broad range of substrates that CYP450 metabolize, there are dramatic differences in the binding sites of each respective protein, thereby enabling the design of molecules which preferentially inhibit one subtype of oxidase enzyme[6-9]

Fungal Metabolism

When controlling microbial infections, conventional medicinal chemistry targets a metabolic pathway or biochemical process that is present in the microbe but not the host. In the case of fungal pathogens, they contain greater genetic similarity to humans meaning there are fewer fundamental differences in cellular arrangement and biochemical processes to target. However, many enzymes of fungi which may demonstrate sequence similarity to those of humans may have drastically different structural features, especially within the binding site. These types of proteins have been successfully targeted in antifungal drug design, particularly the cytochrome P450 superfamily of enzymes[10].

Fungal CYP51, a type of CYP 450 enzyme, is a classical drug target in antifungal drug design due to its vital role in cell wall synthesis. CYP51, which is also referred to as sterol 14∝-demethylase, processes the ergosterol precursor lanosterol in a demethylation reaction. The overall steroid pathway for ergosterol starting from squalene is shown below

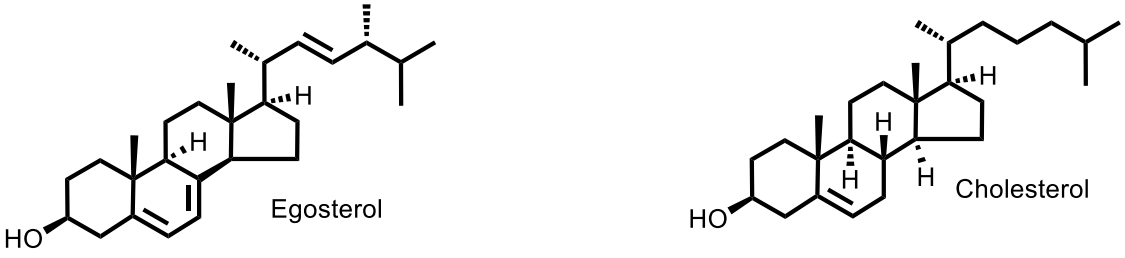
Figure 2. Fungal Biosynthesis towards ergosterol



Ergosterol, like other steroids, is necessary to maintain the fluidity of cellular membranes. Therefore, depletion of ergosterol can have fungistatic or fungicidal effects. Additionally, accumulation of some of the precursors in ergosterol biosynthesis can actually disrupt the fungal cell membrane. CYP51 is not the only step in the biosynthesis that can be inhibited for fungicidal effects. Several clinical medicines target other enzymes in the same pathway.

The main structural difference between cholesterol and ergosterol is that ergosterol bears two more alkene groups, one in the sterol core and one on the aliphatic chain. The structures are shown below.

Figure 3. Ergosterol structure compared with cholesterol



The result is a steroid which more effectively preserves the cellular membrane of fungi under diverse climatic conditions. Without ergosterol, fungi cannot grow effectively, and in many species the result of inhibiting ergosterol biosynthesis is in fact fungicidal. Additionally, drugs which directly interact with membrane ergosterol such as Amphotericin B also have fungistatic or fungicidal properties. Inhibitors of ergosterol biosynthesis have been created for different steps of the biosynthesis. Examples include the squalene epoxidase inhibitor terbinafine (Trade name Lamisil) and the inhibitor of sterol C7-C8 isomerase and sterol reductase amorolfine. It should be noted not all antifungal drugs interfere with ergosterol biosynthesis. A table is shown below detailing the antifungal drugs deemed most important by the World Health Organization alongside their mechanisms of action.

Table 1. Medically relevant anti-fungal drugs.

Compound/Drug (Year of Discovery/Legal Approval)	Mechanism	Indications	Limitations
Terbinafine (1991)	Squalene Epoxide inhibitor	Jock Itch, Athlete's foot, Fungal infections of nails	Diverse side effects, particularly with oral treatment
Flucytosine (1957)	Protein Synthesis Inhibitor, DNA synthesis inhibitor	Systemic Fungal infections	Rapid resistance development, side effects including leukopenia and hepatotoxicity
Griseofulvin (1939)	Cell division inhibitor	Fungal infections of nails and scalp	Patients with liver issues
Tavaborole (2014)	Protein Synthesis Inhibitor	Fungal infections of nail	Application site irritation
Micafungin (2005)	Beta 1,3-glucan synthesis inhibitor	Candidemia, esophageal candidiasis	Intravenous administration
Nystatin (1950)	Cell Wall Destabilizer (Ionophore)	Vaginal yeast infection, esophageal candidiasis	Diarrhea, Skin burning (topical), abdominal pain
Natamycin (1995)	Cell Wall Destabilizer (Ergosterol)	Fungal infections adjacent to eye	Allergic reactions, very poor absorption
Amphotericin B (1958)	Cell Wall Destabilizer (Ergosterol)	Systemic fungal infections	Multiple organ toxicity, particularly in the kidney
Clotrimazole (1969)	CYP51 Inhibitor	Topical fungal infections	Narrow spectrum of efficacy
Miconazole (1971)	CYP51 Inhibitor	Topical fungal infections	Topical use only
Butoconazole (1974)	CYP51 Inhibitor	Vaginal *Candida* infections	Topical use only

Ketoconazole (1977)	CYP51 Inhibitor	Topical fungal infections	Short half-life, long term side effects, drug-drug interactions
Itraconazole (1984)	CYP51 Inhibitor	Systemic fungal infections	Multiple drug-drug interactions, high lipophilicity
Fluconazole (1988)	CYP51 Inhibitor	Systemic fungal infections	Development of resistance
Voriconazole (2002)	CYP51 Inhibitor	Invasive fungal infections	Visual problems, nausea, vomiting, abdominal pain,
Posaconazole (2005)	CYP51 Inhibitor	Invasive fungal infections	Nausea, Vomiting, Diarrhea, headache, insomnia, fever, shaking
Efinaconazole (2014)	CYP51 Inhibitor	Nail infection	Topical use only
Isavuconazonole (2015)	CYP51 Inhibitor	Invasive Aspergillus and Mucormycosis infections	Allergic reactions, drug-drug interactions, short QT syndrome

The limited diversity of mechanisms of anti-fungal drugs can be rationalized by the genetic and biochemical similarity between humans and fungi. Both organisms are eukaryotic and therefore arrange their cellular compartments very similarly, in addition to more similar structures of enzymes and proteins. The result is fewer unique mechanisms of action for drugs to capitalize on relative to anti-bacterial drugs. In any case, the most clinically important class of antifungal drugs target the CYP51 protein. This prevents the demethylation of lanosterol in fungi and causes fungistatic or fungicidal conditions within the fungi. It appears much of the efficacy of CYP51 drugs is due to a combination of a lack of cellular ergosterol as well as accumulation of ergosterol precursors which have disrupting effects on fungal membranes. The affinity constants for several CYP51 inhibitors is shown below[8].

Table 2. Affinities of Clinical drugs towards *Candida albicans* CYP51 and their selectivity over the human analog.

CYP51 Inhibitor	K_d (*Candida albicans*)(nM)	K_d (*H. sapiens*) (nM)
Clotrimazole	26	55
Fluconazole	56	30,400
Itraconazole	19	92
Ketoconazole	12	42
Voriconazole	10	2,290

Additionally, the opportunistic fungal pathogen *A. Fumigatus* CYP51 was expressed and the affinity of several antifungal drugs was measured.

Table 3. Affinities of experimental and FDA approved drugs towards CYP51 of *Aspergillus fumigatus*[6, 11]

CYP51 Inhibitor	K_d (*Aspergillus fumigatus*) (nM)
VNI	203
Posaconazole	131
VT 1598	13
Voriconazole	56

The refinement of techniques for characterizing small molecule affinity for CYP51 has enabled high throughput screening (HTS) techniques to identify new small molecule inhibitors of CYP51. These techniques, pioneered by *Lepesheva et al* and *Konkle et al*, take advantage of the spectrophotometric properties correlated with ligand binding in the CYP51 binding site. The interaction between heme iron and water in the CYP51 binding has a characteristic absorption maximum at 417 nm. This ligand free form of the enzyme bears iron in its low spin, hexa-coordinated state. As a strong coordinating substrate enters, water is expelled and the absorption maximum shifts depending on the ligand strength. Although this method is far from sufficient in determining fungal response to the drug, it provides a clear method for testing structure based drug design techniques based on new X-ray structures of CYP51 determined for pathogenic fungi.

Structure-Based Drug Design of Fungal CYP51 inhibitors

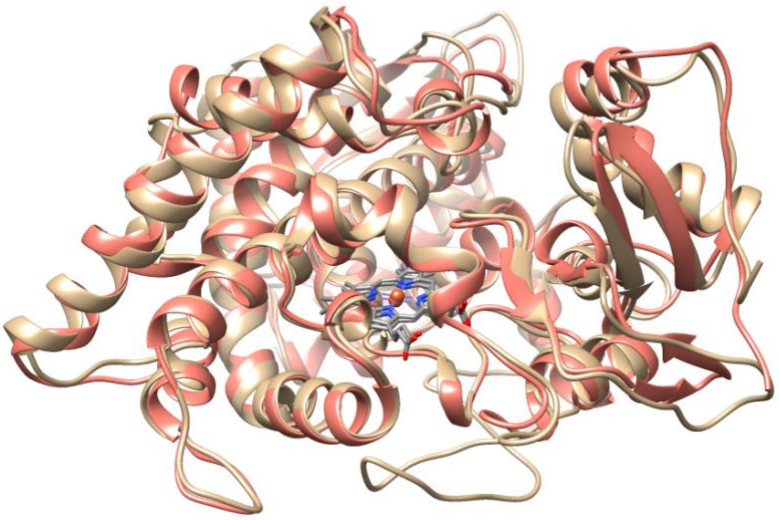
X-ray structures of fungal CYP51 from a variety of species have recently been solved, enabling the use of docking algorithms to find new inhibitors. A table of structures and their descriptions is shown below

Table 4. CYP51 X-ray structures of fungal species/humans as well as their resolution

Species (PDB Code)	Ligand	Resolution (Angstroms)	Year of Publication
H. Sapiens (3JUV)	Ligand-free	3.12	2010
H. Sapiens (3LD6)	Ketoconazole	2.80	2010
Saccharomyces cerevisae (4WMZ)	Fluconazole	2.05	2015
Saccharomyces cerevisae (6E8Q)	Posaconazole		2019
Candida glabrata (5JLC)	Itraconazole	2.40	2016
Candida albicans (5TZ1)	VT-1161	2.00	2017
Candida albicans (5V5Z)	Itraconazole	2.90	2017
Candida albicans (5FSA)	Posaconazole	2.86	2017
Aspergillus fumigatus (5FRB)	VT-1598	2.99	2017

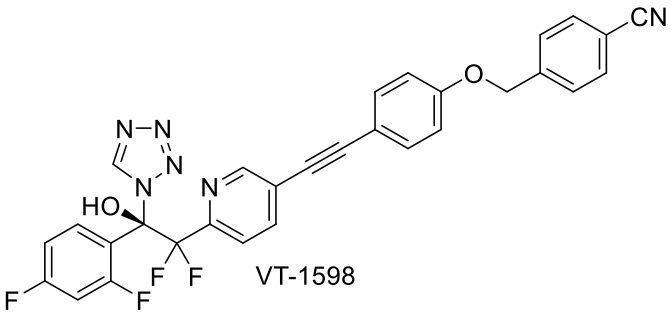
Several relevant crystal structures have been deposited in the protein data bank in the past 5 years. Indeed, the table above is by no means exhaustive. Across all models, the heme position and secondary structure is almost entirely retained. A comparison of human and *aspergillus* CYP51 is shown below.

Figure 4. *A. fumigatus* (5FRB) CYP51 (tan) versus human (3JUV) CYP51 (red) is shown below.



However, a vital distinction is observed in structural properties between human and fungal varieties. Fungal varieties of CYP51 bear far less flexibility in their substrate entry portals relative to the human version. The rigidity in the fungal enzyme partially explains why certain drugs, such as Posaconazole, function as irreversible inhibitors of fungal CYP51. In molecular dynamics simulations, human CYP51 demonstrates far more flexibility, thus explaining the intrinsically lower affinity of human CYP51 to various small molecule inhibitors. This does not mean an antifungal drug targeting CYP51 will not have affinity to other human enzymes within the CYP450 family. However, the X-ray structures of various human CYP450 enzymes have been determined and enable a method to use structural biology to guide selectivity of hit compounds during drug development.

In terms of virtual screening, a highly appealing structure is that of *A. fumigatus* CYP51 (PDB ID 5FRB)[12-15]. The experimental drug in which it was complexed, VT-1598, bears potent, broad spectrum antifungal activity, even within azole resistant fungal strains[14-16].

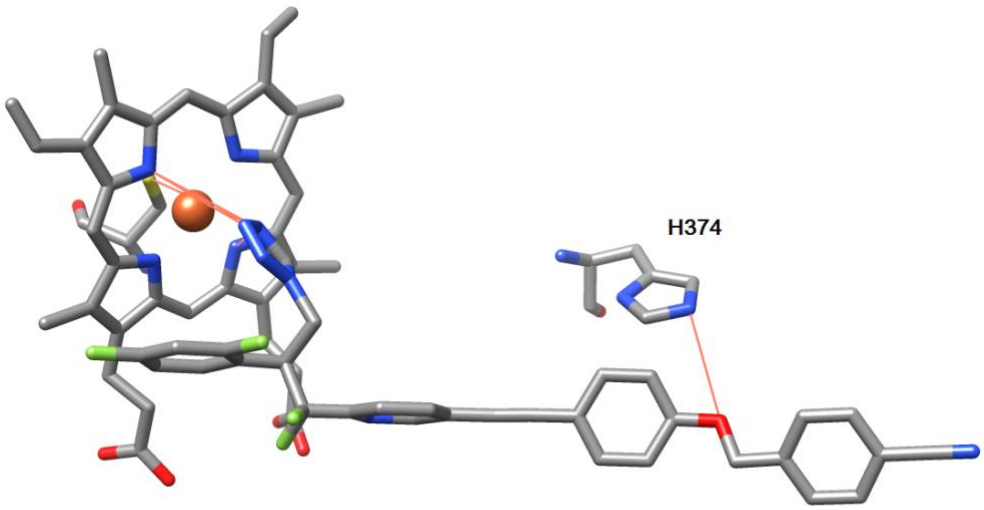


VT-1598

These properties suggest that the binding site model of CYP51 complexed with VT1598 are representative of several fungi and may yield new molecules with similar potency. Specifically, a hydrogen bond between a conserved Histidine residue in fungal CYP51 and the

ether moiety of VT1598 is proposed to enhance interactions in the binding site across many fungal species.

Figure 5. Co-crystal structure interaction between CYP51 and VT1598 providing broad-spectrum anti-fungal properties of VT1598 (Visualized in *A. fumigatus* CYP51)



A docked molecule which makes similar contacts in the binding site, especially the hydrogen bond with the conserved histidine of fungal CYP51, should have broad spectrum activity as well. Therefore, the choice of lead compounds will be predicated on both strong chelation to the heme moiety of CYP51 as well as the potential for hydrogen bonding with the conserved histidine.

Specific Aims

Aim 1: Identify a novel broad-spectrum inhibitor of pathogenic fungal CYP51 through docking

Historically, ergosterol biosynthesis inhibitors have provided the greatest value in controlling fungal growth agriculturally and medically. Within this vital fungal pathway, CYP51 has emerged as the most druggable target, yielding drugs with long term safety due to structural differences in human and fungal CYP51. The issues that remain with existing drugs are their ability to function in multiple fungal species and their drug-like properties. The solution of the *Aspergillus fumigatus* CYP51 X-ray structure complexed with a broad spectrum antifungal drug allows the testing of an interesting hypothesis; if virtual screening is done on a receptor-ligand complex reflecting a broad spectrum drug, then the hits may also bear broad spectrum activity, especially if key interactions are shared between the cognate ligand and virtual screen hit.

Aim 2: Chemical synthesis of congeneric series of compounds analogous to hit compounds for CYP51 affinity assay

Compounds which emerge from the virtual screen will be tested *in vitro* for their ability to bind to *A. fumigatus* CYP51. The assay, which is based on spectrophotometric shifts in optical absorbance of CYP51-ligand complexes, is well characterized. Once compounds are confirmed as binding to CYP51, they will serve as starting points in chemical space for identifying even more potent CYP51 inhibitors. Since drug-like properties are of essential importance, the compounds prioritized for synthesis and biological assays will be those calculated to have low affinity to efflux pumps to ensure minimal resistance liability via medicinal chemistry algorithm. Another layer of prioritization will be based on docking score to wild type and mutant models of 5FRB. Several clinical isolates of azole resistant *A. fumigatus* demonstrate key mutations in the binding site including L98H, Y121F, and T289A. The effect of these mutations on ligand binding can be computationally screened through docking prior to biological evaluation. Since these mutations are presumed to arise from environmental and agricultural use of azole drugs, it is reasonable to assume patients may bear these resistance mutations even without CYP51 inhibitor administration.

Aim 3. Minimum Inhibitor Concentration (MIC) assay of hit compounds and their analogs

Upon verifying that compounds predicted to bind to *A. fumigatus* CYP51 have high affinity for the enzyme, an assay assessing the hit compound's fungistatic or fungicidal properties will be performed. The fungicidality of a compound can be assessed by standardized methods outlined by the Clinical and Laboratory Standards Institute (CLSI). Once fungicidality of hit compounds or their analogs is verified for *A. fumigatus*, other species of fungi will be tested and the efficacy spectrum of hit compounds will be assessed.

Research Plan

Aim 1. Identify a novel broad-spectrum inhibitor of pathogenic fungal CYP51 through docking

Prior to the 21st century, lead compounds for medicinal chemistry generally came from natural products or high throughput screening (HTS). Developments in computational chemistry within the last 30 years have yielded a new method for lead discovery referred to as virtual screening, where molecular docking programs rapidly evaluate compounds in a binding site model created by X-ray crystallography, NMR, or cryo-EM methods. DOCK is a program developed since the 1980s and now includes many different methods for evaluating a small molecule in a binding site[17-18]. For the current work, classical library screening will be performed. Library screening is the *in silico* alternative to *in vitro* HTS for discovery of new hit compounds. The appeal of *in silico* methods is multifold over HTS. HTS requires advanced chemical facilities to automate assays and large inventories of drugs. Furthermore, HTS does not guarantee that 'hits' discovered are not pan assay interference compounds (PAINS). Docking of compounds from databases enables their prioritization for *in vitro* or *in vivo* assays based on the complementarity of a small molecule to a respective binding site. As simplified as the docking assessment may be, it is more rational and less expensive than HTS.

For sampling a binding site, DOCK applies an anchor and grow algorithm. As molecules are "grown" in the binding site, an empirically based force field evaluates the favorability of the hypothetical interactions. Briefly, the force field that will be used can be summarized as follows.

$$DOCK\ score = \sum_{i=1}^{ligand} \sum_{j=1}^{receptor} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} + 332 \frac{q_i q_j}{D r_{ij}} \right)$$

The force field calculation is ultimately a summation of interactions between every atom of a ligand and every atom of a receptor. Of course, as the distance between each atom pair increases, their interaction energy becomes negligible. The first two terms in the summation represent the Van der Waal interaction where A and B represent constants related to atomic radii. The Lennard-Jones potential shown is the 6-12, which has a "sharper" repulsion curve than the 6-9 potential. The third term is the electrostatic interaction between an atom pair. Molecules are not treated quantum mechanically but rather have charge distributions assigned based on quantum mechanics via programs such as Antechamber.

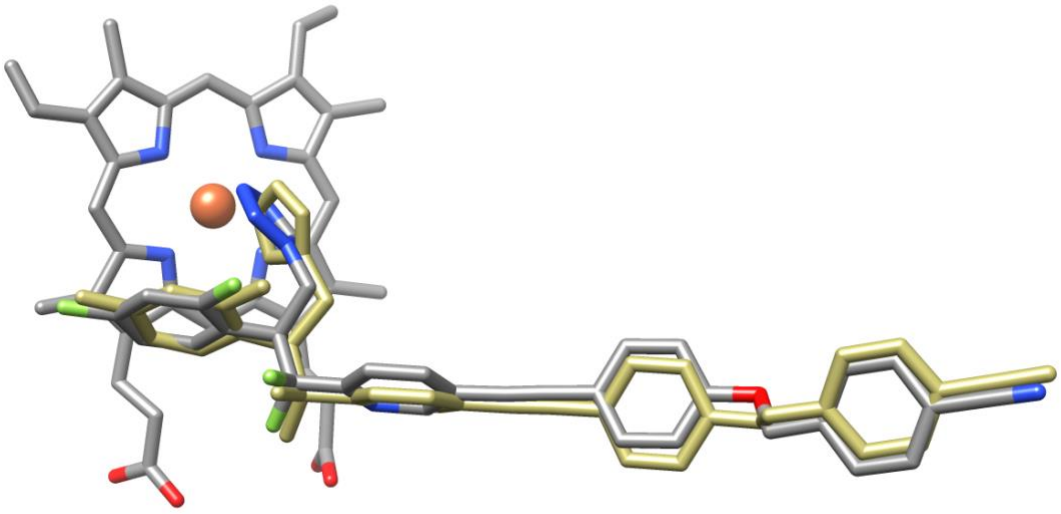
The ZINC database provides millions of compounds in a format that can immediately be evaluated by DOCK[19]. This database will serve as a key resource in the first section of the computational work. Once high scoring compounds are found based on the grid score, the next step is to rescore compounds based on molecular fingerprint overlap. From this recalculation it should become clear whether the compounds scoring well are actually making similar contacts as the cognate ligand. It is predicted that mimicking these contacts may improve the chance that the drug will have broad spectrum fungicidal activity.

The control docking experiment proceeded as expected, with the cognate ligand achieving its best DOCK score in a conformation less than 2 $Å^2$ off of the X-ray conformation. The information is summarized below.

Table 5. Control experiments prior to virtual screening for the 5FRB ligand-receptor complex

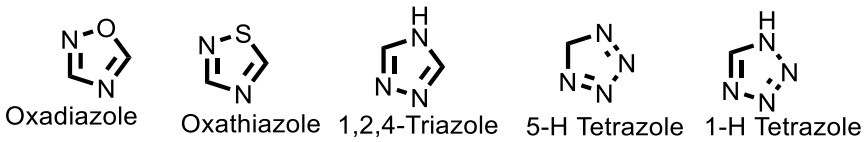
Method	DOCK score	RMSD (Hungarian) ($Å^2$)
Minimization	-48.858	1.34
Rigid	-61.253	1.77
Flexible	-64.617	1.60

Figure 6. Comparison of the flexible docking result (Brown) with the X-ray conformation of VT-1598 (Gray) in the CYP51 binding site (Visualized by UCSF Chimera)



The poorer score of the minimization method may be due to the less lenient 6-12 Lennard-Jones potential detecting a modest steric 'clash' which the crystallographic modelers did not consider significant. When docking the model through the anchor and grow algorithm, the score improves while retaining an RMSD below 2 $Å^2$, implying the system is reliable for finding new ligands of this key protein. It should be noted that the predominant contribution of docking score is from van der Waal interactions (-3.644 electrostatic grid score).

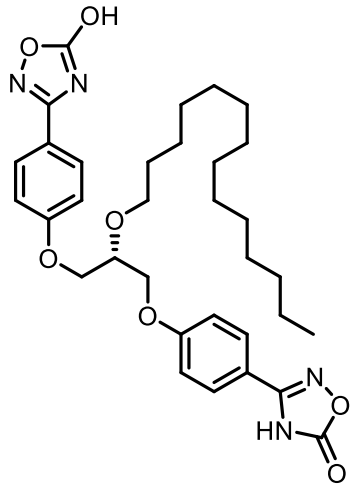
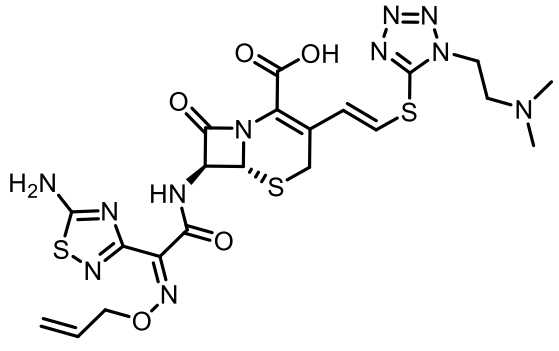
Amongst the compounds that underwent scoring, the library was heavily enriched with heteroaromatic rings that could presumably coordinate to the heme group. This includes compounds in the ZINC database that have oxathiazole, oxadiazoles, or tetrazole moeities.



Empirically, it is clear that compounds with nitrogen that can coordinate to the CYP51 iron have therapeutic effects in the clinic. Indeed, the entire class of these compounds are referred to as the azoles. In total, 91,698 compounds from this subset have been scored so far. Alongside these compounds, another molecular library referred to as “biogenic” was added from the ZINC database to the docking experiment, adding another 18,309 compounds and bringing the total scored so far to 109,917. The compounds in the biogenic subset exhibit a Tanimoto similarity score of 0.8 or greater to known metabolites or natural products. These compounds will serve as an interesting starting point for the virtual screen but will be expanded upon in future work. Due to the biochemical competition between various organisms and fungi, it is reasonable to assume many natural products and their analogs may double as antifungal agents.

Once docking was completed, the top performing compounds were rescored via Cartesian minimization and rank ordered. The top compounds were then visually inspected for heteroaromatic coordination to the heme group and presence of a hydrogen bond acceptor at the key His365 position. Compounds bearing these two features were tabulated.

Table 6. Top compounds from heteroaromatic ZINC database subset

Compound (ZINC ID)	DOCK Score (Cartesian minimization)	C Log P
 44018411	-98.686	9.88
 59211274	-87.266	-1.09

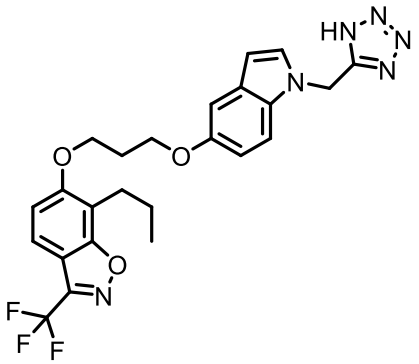
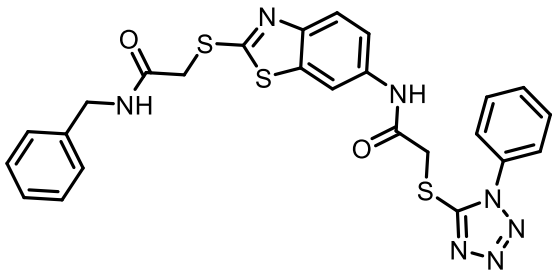
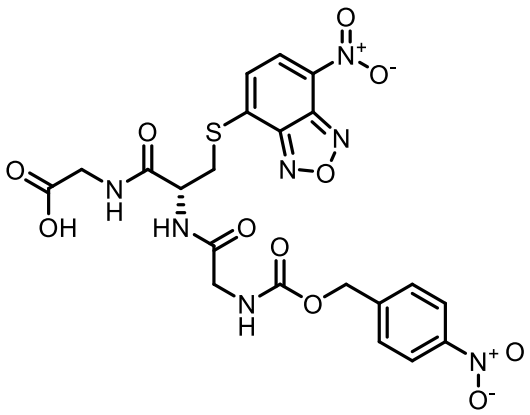
 13677996	-77.062	6.12
 02842243	-76.240	4.54

Table 7. Top compound from biogenic ZINC database subset docking

Compound (ZINC ID)	DOCK Score (Cartesian minimization)	C Log P
 71788496	-85.873	2.57

A notable observation from initial virtual screening is the highly favorable scores of very hydrophobic compounds. This is logical since CYP51's natural substrate is lanosterol, a very hydrophobic compound. More generally, the CYP450 family of proteins generally metabolize very hydrophobic compounds into more polar, oxidized molecules. Thus, the binding sites of CYP51 are dominated by hydrophobic residues. Upon visual inspection, the excessively hydrophobic or non-coordinating compounds were ignored from curation into the hit compound table. Indeed, only one compound from the biogenic subset bore a coordination interaction with heme, strongly discouraging further attempts to virtual screen using this subset of the ZINC database. The predicted binding modes of tabulated compounds versus the co-crystal of VT1598 is shown below.

Figure 7. ZINC ID 44018411 (gray) predicted binding mode in *A. fumigatus* CYP51 relative to the co-crystal of VT1598 (red)

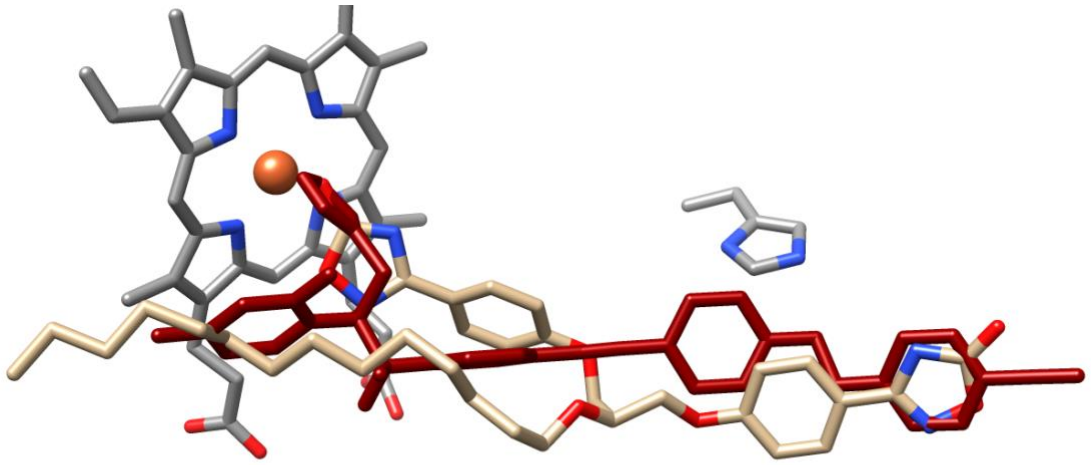


Figure 8. ZINC ID 59211274 (gray) predicted binding mode in *A. fumigatus* CYP51 relative to the co-crystal of VT1598 (red)

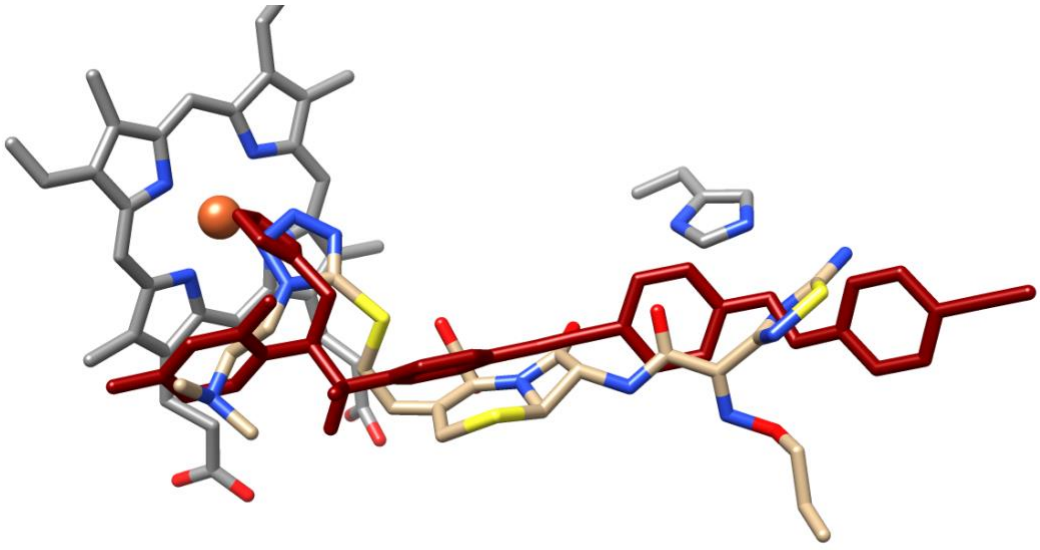


Figure 9. ZINC ID 13677996 (gray) predicted binding mode in *A. fumigatus* CYP51 relative to the co-crystal of VT1598 (red)

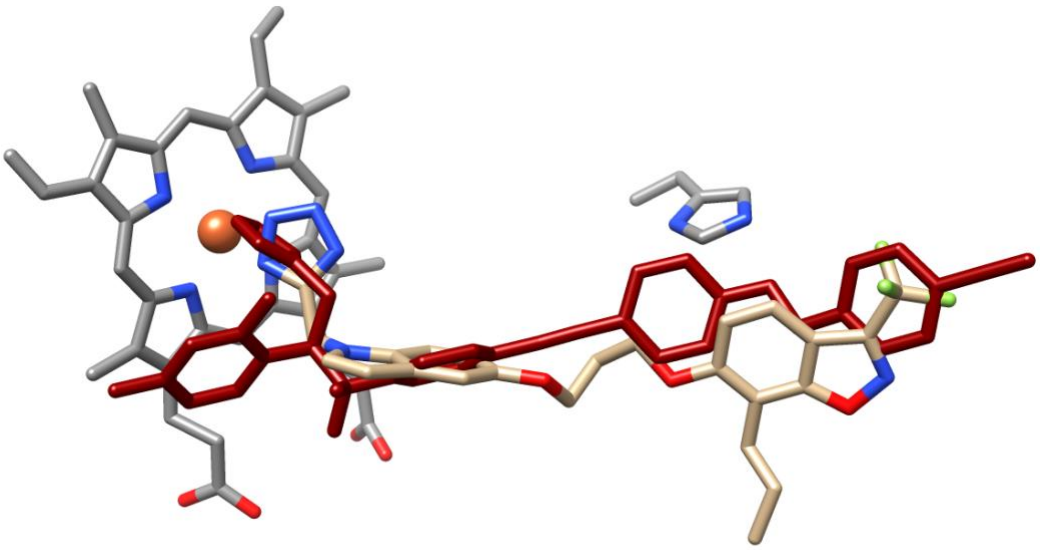


Figure 10. ZINC ID 02842243 (gray) predicted binding mode in *A. fumigatus* CYP51 relative to the co-crystal of VT1598 (red)

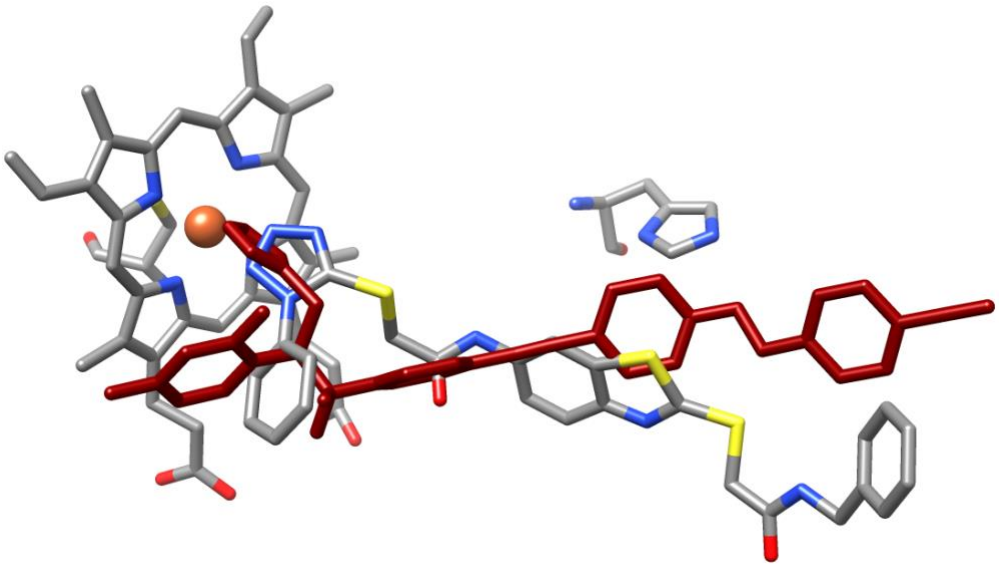
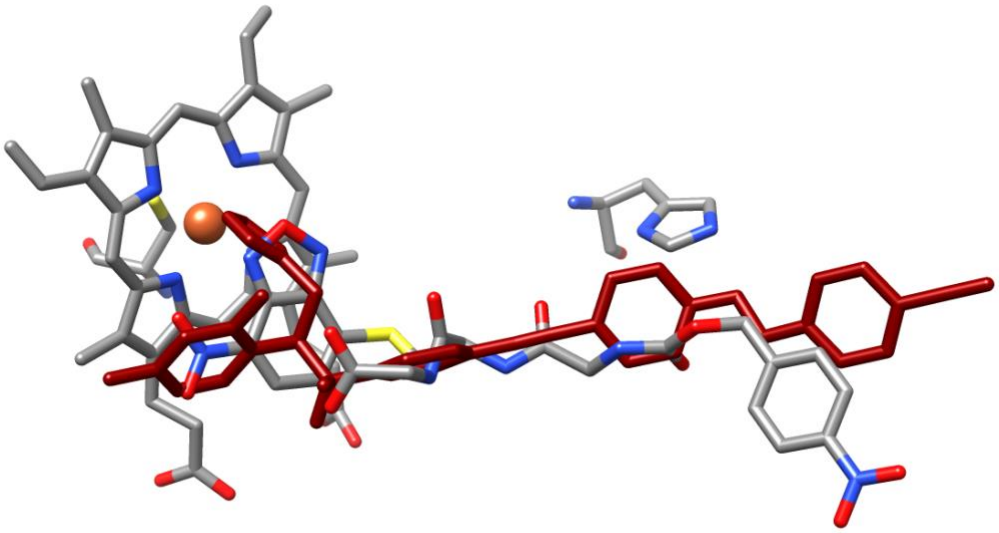


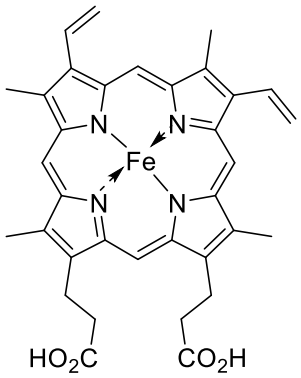
Figure 11. ZINC ID 71788496 (gray) predicted binding mode in *A. fumigatus* CYP51 relative to the co-crystal of VT1598 (red)



Virtual screening thus far has found interesting scaffolds that may yield anti-fungal drugs. Clearly, the molecular profile of compounds docked is very important to finding realistic lead compounds. This is because the binding site is very hydrophobic and will bias results towards very heavy, non-polar, non-drug like molecules as was observed with the biogenic subset of the ZINC database. Future virtual screening will continue to focus on groups that can coordinate effectively to heme. Additionally, incorporation of fingerprint rescoring will increase likelihood of finding hits that can hydrogen bond with H374.

Aim 2: Chemical synthesis of congeneric series of compounds analogous to hit compounds for CYP51 affinity assay

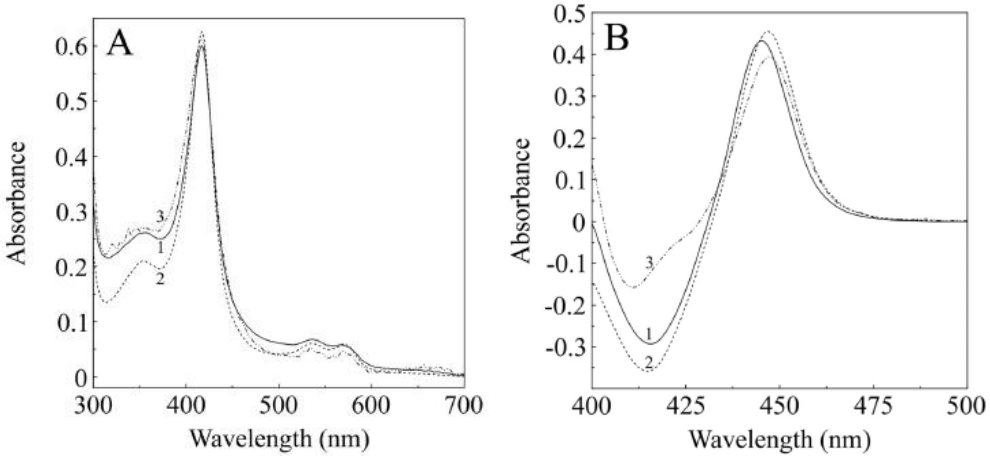
A rapid validation of docking results can be performed via expression, purification, and spectral assay of fungal CYP51 with hit compounds. The Cytochrome P450 superfamily has been well studied in terms of spectral properties enabling a relatively straightforward assessment of a molecule's interaction within the binding site.



Heme-Fe II Complex

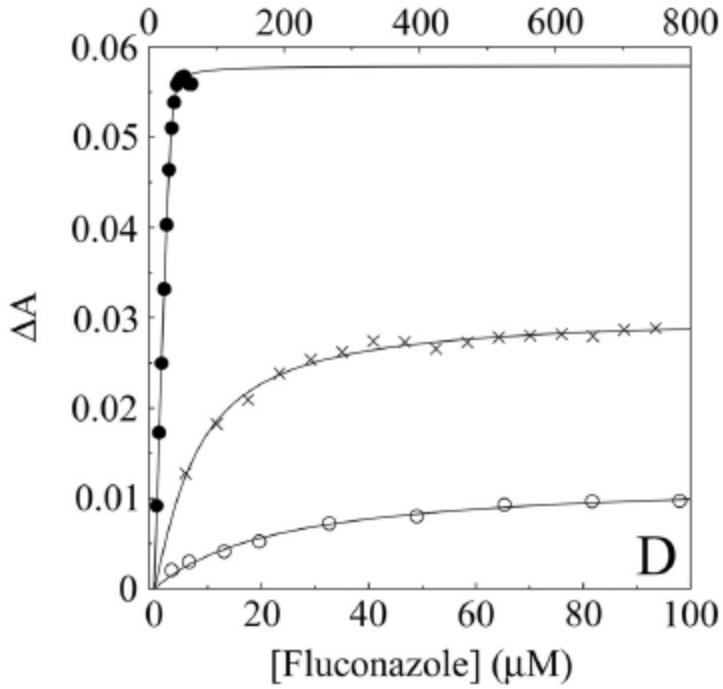
The key physical phenomenon is the change in the heme electronic properties as ligands enter or leave the binding site. These phenomena can be classified into type I and type II. The medically relevant azoles and carbon monoxide are type II ligands since they strongly coordinate to the heme and serve as π electron acceptors. The result is a "red-shift" in the maxima of the absorbance spectra. Carbon monoxide's spectral shift is shown below.

Figure 12. Adapted from [8]. Change in absorbance maxima between CYP51 in its oxidized form (left) versus coordinated carbon monoxide (right). The maxima transitions from approximately 420 nm to 450 nm.



With CYP51 in hand alongside hit compounds, the affinity constants can be determined for various compounds. The change in spectrophotometric absorbance with titration of molecule will serve as a measure of affinity. For example, a high affinity, highly selective fungal CYP51 inhibitor such as fluconazole will shift absorbance with very small quantities while not shifting human CYP51 absorbance under the same conditions.

Figure 13. Adapted from [8]. Azole saturation of 5 μM CYP51 of human (O) and fungal (●) with fluconazole for affinity calculation.



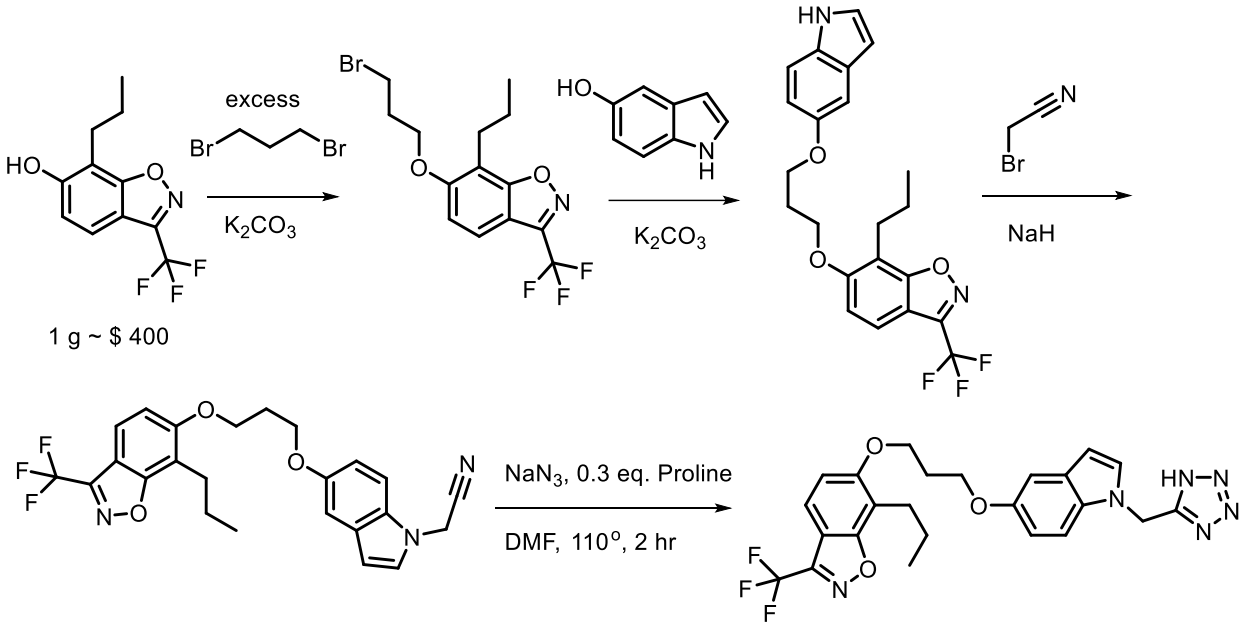
The equation that will be applied for determination of affinity is as follows.

$$\Delta A = \left(\frac{\Delta A_{max}}{2E}\right)\{(L + E + K_d) - \sqrt{[(L + E + K_d)^2 - 4LE]}$$

Where A represents absorbance, A_{max} represents maximum absorbance, L represents ligand concentration, and E represents enzyme concentration. Affinity can be measured for many species of fungal CYP51 as well as human cytochrome P450 enzymes.

Amongst the top scoring compounds, several bear extremely complicated structures limiting the ability to synthesize them. Unfortunately, this includes the beta-lactam ZINC ID 59211274 which has a very low c Log P value suggesting good medicinal properties in addition to hydrogen bonding ability with H374. However, the molecule may be chemically simplified and reassessed computationally prior to beginning a synthesis. A simpler hit compound, ZINC ID 13677996 (Hit A), was chosen as the first candidate for biological evaluation. The synthesis of the compound can be completed as follows.

Figure 14. Synthetic route of Hit A and its potential analogs



There are several clear potential modifications that may be explored with Hit A. In order to improve hydrogen bonding with H374, the linker aliphatic chain may need to be extended or shortened. Docking can be used to prioritize which modification to try first. Additionally, the trifluoromethyl group may be removed to reduce the price of the starting material if it is not key to binding. The tetrazole group can also be N-methylated or acylated at the end of the synthesis to change coordination properties towards the iron of the heme group. These modifications can be synthetically accessed easily based on the above synthetic route.

Aim 3. Fungal growth assay of hit compounds and their analogs

The minimum inhibitory concentration (MIC) of a drug towards a pathogen is a good reflection of how effective a small molecule will eliminate a pathogen *in vivo*. Much data has been gathered on *A. fumigatus* MIC values, especially clinical isolates reflecting resistance to certain azoles. The University of Texas Health Center at San Antonio has compiled many experimental results on the emerging resistance of *A. fumigatus* via changes in MIC value from clinical isolates. Some of these values are shown below.

Table 8. MIC_{100} values from the University of Texas Health Center at San Antonio for *A. fumigatus*.

Isolate ID	Source (Year)	Mutations	Itraconazole	Posaconazole	Voriconazole	Isavuconazole
DI15-110	Connecticut (2013)	None	>16	2	8	4
DI15-114	California (2014)	G54R	>16	>16	4	4
DI15-104	Ohio (2011)	G448S	4	1	16	8
DI15-111	Reference Lab (2013)	G54W	>16	>16	0.25	0.125

DI15-98	Reference Lab (2008)	None	16	2	16	16

Interesting observations arise from this data. First, newer azoles are still not robust to resistance mechanisms of fungi, as evidenced by the elevated MIC for Voriconazole and Isavuconazole for fungi isolated from patients. Even without mutations in the CYP51 gene, fungi can gain resistance to azoles via other mechanisms, presumably xenobiotic efflux pumps and overexpression of CYP51 itself. As expected, older azoles have even less ability to hinder fungal growth, however certain mutations make fungi more vulnerable to older azoles such as the 2011 *Aspergillus* sample from Ohio with the glycine to serine mutation at position 448.

One procedure for testing anti-fungal properties of a molecule *in vivo* are cellular growth inhibition assays. The method outlined in involves the growing of standard *A. fumigatus* in potato-dextrose broth at 27^{o} for 2 days, followed by dilution of fungi. The fungi will then be diluted by a factor of 5 in a buffered solution of 0.05 % Tween 20 and phosphate buffered solution (PBS), placed onto another potato-dextrose agar plate and incubated at 37^{o} for 2 days. With the fungi growing, the conidia will be separated and diluted into 0.05 % Tween/PBS buffered solution. At this point the solution should be standardized to 1,000 spores and can be added to a 96 well plate for addition of the respective molecule being tested. Each molecule must be tested at multiple concentrations and in triplicate to ensure reproducibility. Upon incubation with the chemicals for a day at 37^{o}, 10 % alamarBlue will be added for cell viability measurement. Six hours after addition of alamarBlue, fluorescence will be measured on a fluorescence microplate reader for cell survival. The excitation and emission wavelengths will be 570 and 600 nm respectively. From this experiment, the effect of drug on fungal survival will be assessed. This experiment provides robust preclinical evidence that a molecule has antifungal properties.

Another method of assessing *in vivo* inhibition of fungal growth is the Etest®. The method has demonstrated consistency with the Clinical Laboratory Standards Institute (CLSI) in determination of MIC values of known drugs. The principle is based on growing the fungi on an agar plate with a concentration gradient of antifungal agent being tested. The method's minor issues are determination of endpoint, which may be difficult if a trailing effect is seen for the fungal growth. In such cases, a higher interpretation of the MIC may result. However, as long as the experiment is done with controls such an issue should not be significant.

Expected outcome and potential impact

It is reasonable to assume that a virtual screen of over a million compounds will yield at least one molecular scaffold with the key physicochemical properties needed to be a potent, broad-spectrum CYP51 inhibitor with selectivity to fungal CYP51 and minimal liability to resistance via efflux pumps and CYP51 binding site mutations. From here, pharmacokinetic properties can be optimized through medicinal chemistry for a truly revolutionary anti-fungal drug.

Timeline

The project outlined in this proposal requires multiple specializations to complete properly. Therefore, the project will take at least a year and possibly more depending on the competency of the individuals in their respective field. The *in silico* aspects are not excessively complicated and therefore a graduate student with dual specialization in computational methods and chemical synthesis and another with dual specialization in biological assays and chemical synthesis should be sufficient for completing the project in a year.

Aim 1:

Depending on supercomputer allocations, the docking process time frame can vary dramatically. With decent allocations, virtual screening and analysis can be done in less than two months for a million compounds. As promising compounds emerge, chemical synthesis or biological assays can begin without full completion of the library scoring.

Aim 2:

Chemical synthesis or purchase of hit compounds and their *in vitro* assessment via fungal CYP51 spectrophotometric assay will be a more time consuming process, especially if a complicated scaffold emerges as the most promising. However, once the expression and purification of CYP51 in bacteria is optimized, the process should be routine. Thus, approximately 6-8 months will be needed for this aim to be completed

Aim 3:

The key hypothesis of this work is based on the fact that broad-spectrum antifungal drugs can be derived from one crystallographic model from one fungal species, *A. fumigatus*. Therefore, multiple strains of pathogenic fungi must be grown to assess how a small molecule can affect the fungi. In order to acquire and perform the proper *in vivo* assays, at least 4 months will be needed for a competent biologist.

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