A

Dissertation Report on

Novel Framework for Predicting Multitarget Antifungal Drugs against Pathogenic Fungi

Submitted

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by

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THESIS CERTIFICATE

This is to certify that the thesis titled **Novel Framework for Predicting Multitarget Antifungal Drugs against Pathogenic Fungi CLASS FOR DISSERTATIONS SUBMITTED TO IIIT-DELHI**, submitted by **KARTHIKA S**, to the Indraprastha Institute of Information Technology, Delhi, for the award of the degree of **Master of Technology**, is a bonafide record of the research work done by her under our supervision. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

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ABSTRACT

Pathogenic fungal diseases have become a global threat to human health in immunocompromised individuals. With increasing mortality rates due to systemic infections and limited availability of antifungal classes,it is crucial to accelerate antifungal drug discovery by focusing on novel targets. In our research, we aim to target Rho proteins and develop a deep-learning framework for antifungal drug prediction.

Stable structures of plasma membrane conserved Rho proteins of different pathogenic species were generated using ESMFold (Evolutionary scale modeling). Our investigation focused on establishing cavity-specific in silico ligand synthesis with a further emphasis on exploring orthologous cavity sites across pathogenic species. To assess the compounds generated via LigBuilder we applied rigorous statistical methods to segregate them into high and low-affinity binders. Subsequently, we employed graph neural networks for the evaluation.

In addition to targeting novel pathway, we explored drug repurposing as an avenue for therapeutic alternatives against infections. Leveraging the Drug Repurposing Hub for prediction, we incorporated repurposed drugs into our research and conducted docking studies to validate their potential for experimental studies. This approach adds a valuable dimension to our investigation, aligning with the growing interest in repurposing drugs for infections.

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Keywords: *ESMFold, in silico ligand synthesis, Graph neural networks*

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ABBREVIATIONS

ESMFold	Evolutionary Scale Modeling
RMSD	Root Mean Square Deviation
PDB	Protein Data Bank
ECDF	Empirical Cumulative Distribution Function
GROMACS	GROningen Machine for Chemical Simulations
GPCRs	G-Protein coupled Receptors
EF2	Elongation Factor 2
GEF	Guanine nucleotide exchange factor
NCBI	National Center for Biotechnology Information

Chapter 1

Introduction

Fungal diseases threaten human health and, with few antifungal options currently available, cause serious illness and death.There is also the difficulty of identifying new drug targets because of the resemblance between fungal and human cells. (Gintjee et al., 2020). *Candida, Cryptococcus, and Kojima* are the main species of fungal infection, as well as many resistant strains such as *Candida auris*.

The less availability of antifungals and their limited safety margin profiles have led to an increase in complications and deaths due to fungal infections. Antifungal agents in current use can be categorized based on their mechanism of action, encompassing polyenes, flucytosines, azoles, and echinocandins. However, only some of these, such as polyenes, flucytosines, azoles and echinocandins, are clinically applicable to be used in treatment of life-threatening fungal infections(Vanreppelen et al., 2023).

Although effective, treatments using polyenes such as amphotericin B face problems due to high nephrotoxicity, making them second choice. Attempts to reduce nephrotoxicity with lipid-containing formulations are limited due to high cost. Other antifungals, such as flucytosine, are hampered by rapid development and severe hepatic and hematological toxicity.

Additionally, increased resistance in *Kojima fumigatus* has created difficulties in the treatment of aspergillosis, and the mortality rate has reached alarming levels. Early detection is crucial for saving lives worldwide in the case of fungal meningitis. Similarly, prompt identification and intervention are essential for improving outcomes and reducing mortality in chronic pulmonary aspergillosis(Nicola et al., 2019).

The introduction of azoles (including drugs such as clotrimazole and fluconazole) has provided fungistatic type activity with general safety. Echinocandins are the newest

members of the classical antifungal class and are associated with low resistance, although recently there has been difficulty in developing resistance in some fungi, particularly non-albicans Candida species.

In our study, we highlight the importance of introducing new targets in fungal cell wall biology which is necessary for accelerating antifungal drug discovery. In this section, we discuss the outline of how the fungus is dangerous and what are the various modes of action of the existing antifungals and the importance of using rho proteins as a target. Moreover, we delve into the significance of drug repurposing within the realm of drug development.

1.1 Routes of transmission of fungal pathgens

Basidiomycota and Ascomycota are phyla within the sub-kingdom Dikarya, both of which encompass fungal pathogens with the ability to infect humans. Ascomycota is associated with a diverse range of diseases affecting various organs, including oropharyngeal, dermatological, ophthalmic, neuronal, genitourinary, heart, lung, and others. On the other hand, Basidiomycota is known to cause meningitis and dermatological problems, primarily attributed to the genera *Malassezia* and *Cryptococcus*, respectively. Fungal infections

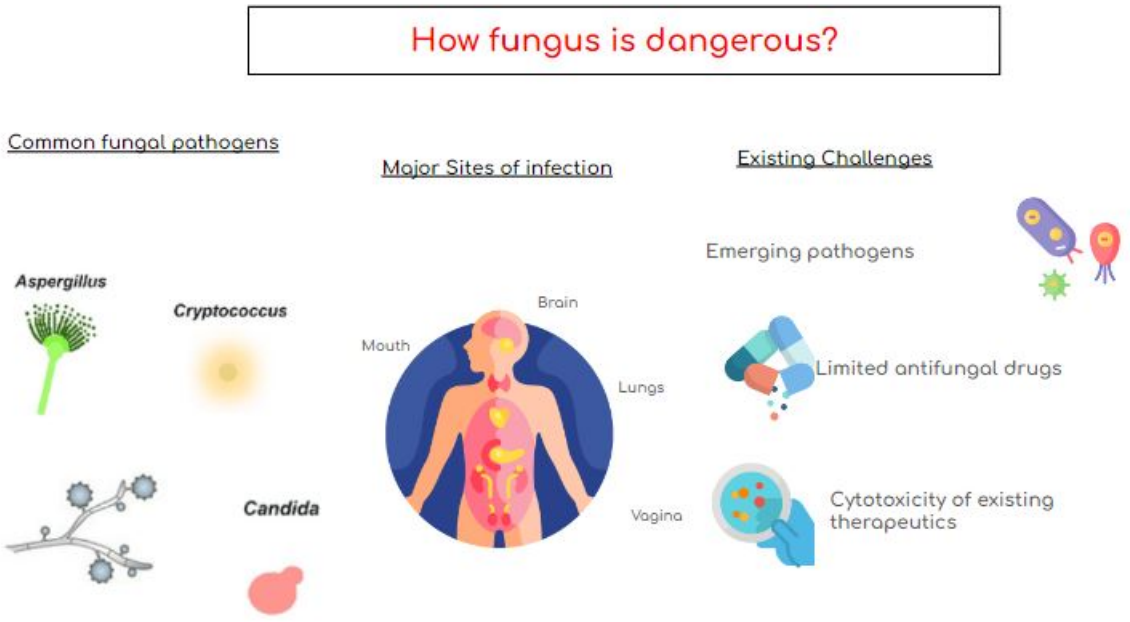


Figure 1.1: How fungus is Dangerous

are usually spread through direct contact or through inhalation. Dermatophytes, including Microsporum, Epidermophyton, Trichophyton, infect the skin through direct contact and produce various proteolytic enzymes to activate keratin tissues to cause mycoses. Another popular method of transmission involves inhaling pneumonia-causing spores.

Blastomyces dermatitidis (causing blastomycosis), Histoplasma capsulatum (causes histoplasmosis), Pneumocystis jiroveci (causes Pneumocystis pneumonia), Aspergillus fumigatus and Cryptococcus neoformans gattii (which causes cryptococcosis) are usually spread through breathing. Talaromyces marneffei (which causes thalomycosis) is transmitted by direct contact and inhalation (Reddy et al., 2022)

1.2 Antifungal Mechanism of Action

Till date, commercially available antifungal agents primarily target components associated with the plasma membrane and the cell wall of fungi. These targets are mainly for the viability and integrity of fungal cells.

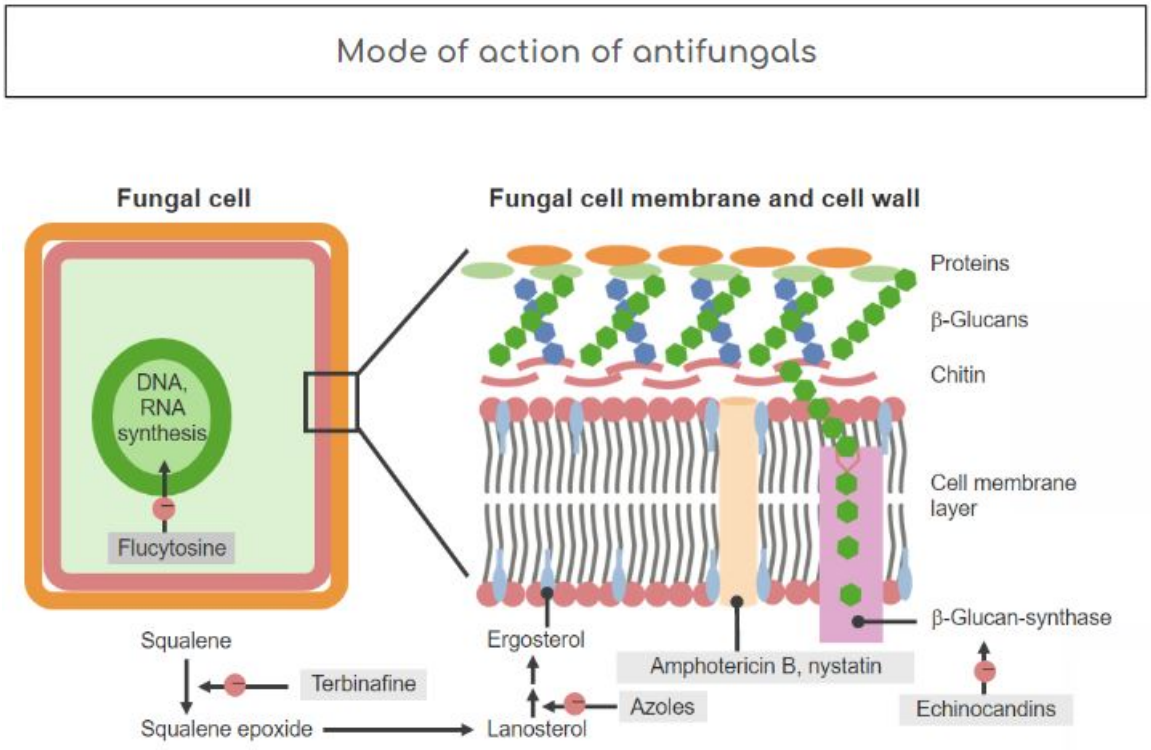


Figure 1.2: Mechanism of action of antifungal agents

1.2.1 Ergosterol

Fungal cell membranes are composed of ergosterol,essential for maintaining the permeability, fluidity, and appropriate functioning of integral membrane proteins. Because of its importance, ergosterol is a popular target for antifungal medications. Most antifungal drugs work on ergosterol by binding to it directly or blocking its synthesis. These mechanisms ultimately lead to the disruption of ergosterol-dependent processes, resulting in the formation of pores in the fungal membrane(Roemer and Krysan, 2014).

Fluconazole, which comes under the class Azoles ,act by inhibiting ergosterol biosynthesis by the 14-α demethylase.It is responsible for the conversion of lanosterol to ergosterol, which destroys the fungal cell wall, and changes the morphology of the fungus and inhibiting its growth.

Allylamines, such as by terbinafine, act as antifungal agents by inhibiting specific early stages of ergosterol biosynthesis. These compounds target squalene epoxidase,the inhibition of squalene epoxidase disrupts the conversion of squalene into lanosterol, a crucial step in the ergosterol biosynthetic pathway.Allylamines are particularly effective against dermatophytes.Dermatophytes are fungi that commonly cause skin, hair, and nail infections.

Polyenes like amphotericin B show anti fungal properties by binding to ergosterol and thus prevent fungal infections. This interaction results in the leakage of monovalent ions and other cytoplasmic components. Additionally, polyenes can interfere with membrane permeability and release free radicals through oxidative reactions and interactions with lipoproteins.

1.2.2 Cell wall

Indeed, the inhibition of chitin and beta-glucan synthesis is a key mechanism of action for many antifungal agents. Chitin and beta-glucan are essential components of the fungal cell wall, providing structural integrity and protection.Antifungals that target these processes disrupt cell wall formation, leading to weakened fungal cell walls and increased susceptibility to environmental stress. Echinocandins, including caspofungin and anidulafungin, inhibit the synthesis of 1,3-β-glucan, eventually disrupting fungal cell wall formation. This targeted approach makes echinocandins effective against certain types of fungal infections. (Prasad et al., 2016)

Chitin, a crucial component of the fungal cell wall, becomes the focus of antifungal

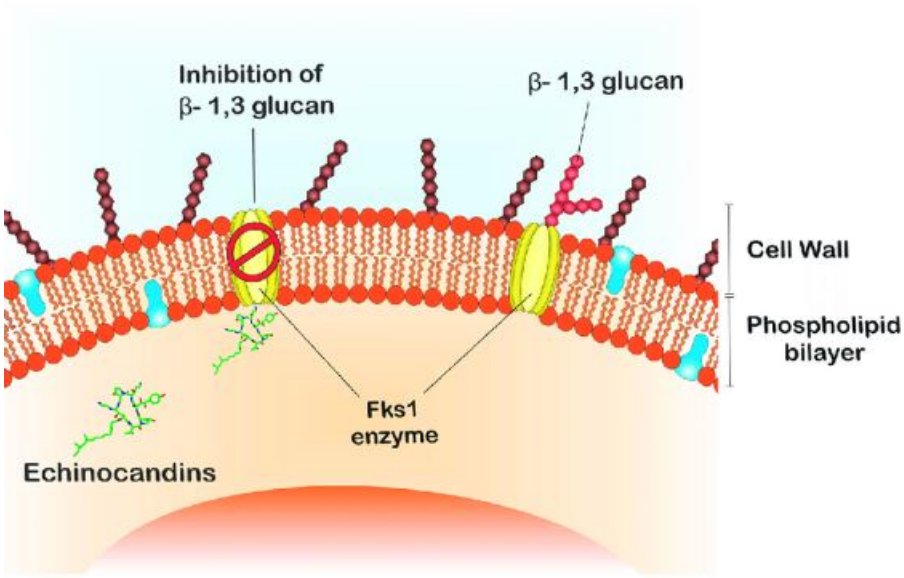


Figure 1.3: Echinocandins targeting the FK506 sensitivity subunit (FKS1 subunit)

agents such as nikkomycin and polyoxins. These agents inhibit the chitin synthase responsible for the elongation of chitin chains.This makes chitin synthase an attractive target for antifungal drug discovery.

1.2.3 Heat Shock Protein 90 Inhibition (Hsp90)

One possible avenue of investigation would be to block Hsp90, a molecular chaperone linked to resistance to antifungal drugs, fungal pathogenicity and phase transition in dimorphic fungi. Various stress conditions have led to the observation of Hsp90's involvement in pathogenesis, susceptibility to dermatophytosis, and regulation of other Hsp in Trichophyton rubrum(Brown et al., 2010).

1.2.4 Nucleic acid synthesis inhibition

The primary factor influencing the inhibition of nucleic acid synthesis is 5-flucytosine, which cytosine deaminase converts to 5-fluorouracil. This is then converted by UMP pyrophosphorylase to 5-fluorouridylic acid. even though 5-flucytosine was synthesized in 1957, its properties were no longer found until 1964.

Sordarins hinder protein synthesis, slowing down cell growth. These compounds target two proteins: translation elongation factor 2 (eEF2) and the large ribosomal subunit protein rpP0.To assess the fungal specificity of sordarins and validate their target, genetic

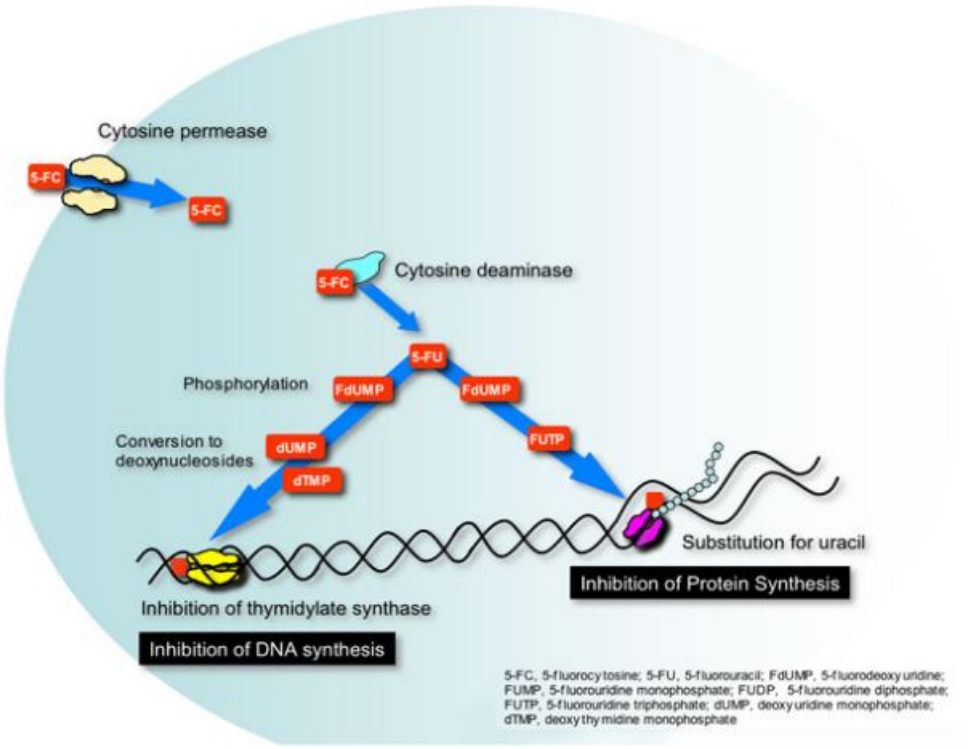


Figure 1.4: Nucleic acid inhibition (5-flucytosine (5FC))

assays were conducted using mutants of Saccharomyces cerevisiae. These assays provided confirmation that the target of sordarins is (eEF2).
Sordarins, although now recently developed for targteing fungal pathogens, deserve attention because of their precise mechanisms of action, that is screening for inhibitors of C. albicans protein synthesis diagnosed sordarins, shedding light on their antifungal effects(Scorzoni et al., 2017).

Further investigations showed EF2 as the potential target of sordarins,the reason that, EF2 in *C. albicans* showed sequence identification with its human counterpart.
Exceptional derivatives within the sordarin show off distinct spectra of susceptibility amongst species, the motives for which aren't completely understood. This variability may be attributed to challenges in the penetration of those agents into target fungi. Despite this, sordarins have a high specificity for the fungal target and the relative ease of synthesizing novel sordarin variations provide promising possibilities for future traits inside this class.

1.2.5 Role of Reactive oxygen species

Certain antifungals, amphotericin B (AmB), can treat fungal infections in a variety of ways. The mitochondria, which naturally generate free radicals, is one important aspect. In unfavorable circumstances, an excess of these radicals is produced, which damages cellular constituents like DNA ,lipids and proteins and ultimately results in cell death. The nitrosative burst is an integral part of the multifaceted antifungal mechanism employed by AmB against the fungal pathogens, ultimately activating fungicidal response(Gonzalez-Jimenez et al., 2023).

1.2.6 Calcineurin signaling

Calcineurin signaling is inhibited against fungal pathogens. Calcineurin, a Ca2+-calmodulin (CaM)-activated protein phosphatase 2B, regulates crucial cellular activity in pathogenic fungi like aspergillus. It performs a vital function in preserving the structural integrity of the cell wall of fungus by the biosynthesis of β-glucans,ergosterol and chitin. (Juvvadi et al., 2017)

1.3 Role of Rho GTPases in the fungal CWI pathway

The signaling pathways modulated by the small GTPases, belonging to Rho family have gained increasing attention due to their involvement in a broad spectrum of diseases, encompassing cardiovascular, various cancers, neurological, and pulmonary conditions. Belonging to the proteins of Ras superfamily, Rho GTPases are pivotal players in various biological processes. They specifically contribute to regulating cellular activities such as cellular migration, cell adhesion, and cytokinesis, underscoring their significance in diverse physiological and pathological contexts.

Functioning as crucial molecular switches, Rho GTPases undergo a cycle of transition as a GTP-bound state when active and a GDP-bound state when inactive. The activation of downstream effector proteins is initiated when Rho GTPases are bound to GTP. This activation process involves guanine nucleotide exchange factors (GEFs), which facilitate GDP release and promote GTP binding. To return Rho GTPases to the GDP-bound state, guanosine triphosphatase activating proteins (GAPs) play a vital role by enhancing intrinsic GTPase activity. Additionally, the guanine dissociation inhibitor (GDI) retains Rho as inactive GDP-bound form within the cytoplasm(Smithers and Overduin, 2016).

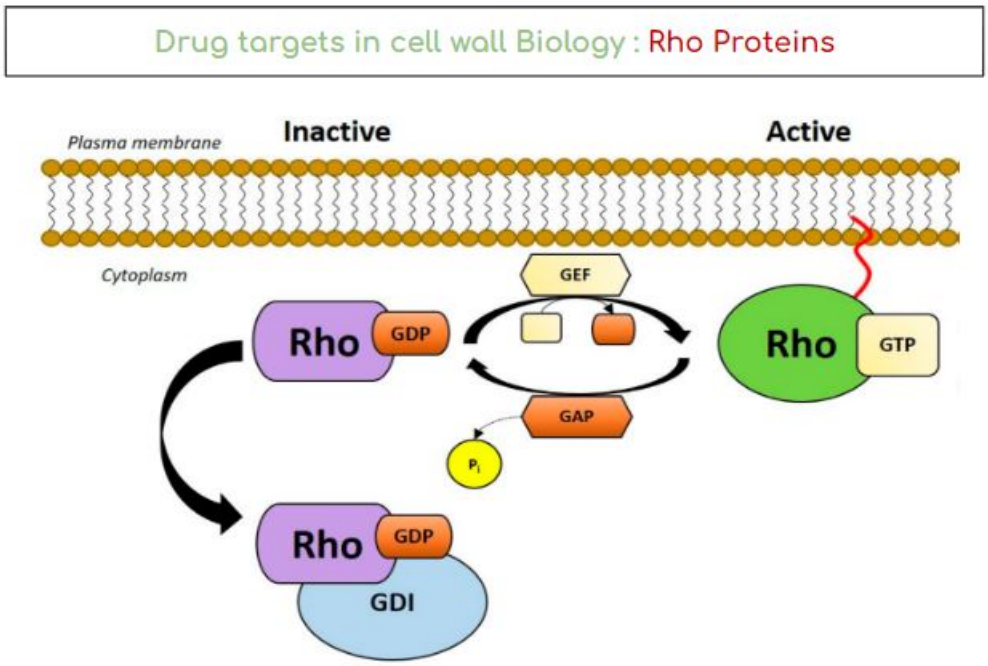


Figure 1.5: Rho Signalling Pathway

During growth and morphogenesis, fungal cells adopt the cell wall integrity pathway (CWI) to respond to external stimuli such temperature fluctuations, osmotic shocks and pH shifts. The cell wall integrity pathway, which was identified and studied in Saccharomyces cerevisiae. Mitogen-activated protein also known as MAP kinase module, transmembrane sensor proteins also known as ScWsc1-3, Rho1 GTPase, guanine nucleotide exchange factors and protein kinase C are organized in this CWI pathway (Dichtl et al., 2016).

Extracellular stressors detected by transmembrane sensors such as Wsc1, Mid2, and Mlt1 initiate the CWI pathway. The Rho1 GTPase is activated by these sensors' stimulation of downstream GEF proteins. Glucan synthase can be directly activated by activated Rho1 GTPase, which can also further activate downstream MAP kinases and protein kinase C (PKC). In the end, this cascade controls cell wall biogenesis by causing transcription of genes via transcription factors like Rlm1.

Studies on the CWI pathway not only highlight how important Rho1 GTPase is for controlling the synthesis of fungal cell walls, but they also point to potential upstream Rho1 regulators. This complicated signaling pathway highlights the complex process of maintaining fungal cell wall integrity by showcasing the dynamic interplay of molecular

components in orchestrating adaptive responses to external environmental stresses.

(Lin and Zheng, 2015)In a recent research the authors meticulously explored recent advancements in the targeted modulation of signaling pathways within 3 pivotal Rho Proteins: Rac1 Cdc42 and RhoA . They targeted both upstream and downstream components of Rho GTPases, along with an insightful exploration of post translational modifications at the molecular level.The paper delved into their crucial regulators in the context of cancer.

A pioneering study was done on elucidating the intricate orchestration of synaptic spine morphology under the dynamic influence of Rac1, Cdc42, and RhoA activities , concluding that a pivotal role of pharmacological modulation of RhoA/ROCK as a promising avenue in drug discovery within the neuro degenerative disease domain. The study offered a comprehensive insight into the function of compounds targeting the pathway i.e RhoA/ROCK, shedding light on their potential in mitigating synaptic dysfunction—a crucial aspect in the context of CNS diseases(Martin-Camara et al., 2021) .

1.3.1 GEFs' role in activating Rho GTPases

Rho GTPases are molecular switches that exist in two different conformational states: the GDP-bound inactive state and the GTP-bound active state. When these GTPases are in their GTP-bound active form, they attach themselves to the plasma membrane, activating downstream effectors and regulating a range of biological functions, such as the control of gene expression and the modification of the actin cytoskeleton.

GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) are two important groups of molecules that delicately govern the dynamic transition of Rho GTPases between different states. As positive regulators that encourage the activation of Rho GTPases, GEFs serve a critical role in promoting the exchange of GDP for GTP. On the other hand, GAPs activate Rho proteins' intrinsic GTPase activity, which causes Rho GTPases to change from an active GTP-bound conformation to an inactive GDP-bound conformation.

In addition to GEFs and GAPs, a third group of regulatory proteins known as guanine nucleotide dissociation inhibitors (GDIs) contributes to the nuanced regulation of Rho GTPases. GDIs exert their influence by inhibiting the activation of Rho GTPases, effectively keeping them in the cytoplasm in their inactive GDP-bound form. This intricate

regulatory network involving GEFs, GAPs, and GDIs highlights the control mechanisms governing the activity of Rho GTPases, essential for their role in diverse cellular functions.

1.4 Drug Repurposing

Drug repurposing entails discovering new applications for clinically approved drugs that might be already recognized. It is a powerful and faster alternative to the conventional drug development method.Aspirin being a classic example for drug repurposing which when launched in 1899 as analgesic and later repurposed in the Nineteen Eighties as anti platelet drug (Stylianou et al., 2014).

Inside the context of infectious fungi, drug repositioning has arised as a promising method to fight various fungal species, inclusive of C. neoformans, Candida, Aspergillus, and dimorphic fungi including Sporothrix. Several research have pinpointed compounds with promising potential for therapeutic application in treating diseases caused by these fungi (Kim et al., 2022) . As an example, sertraline, an antidepressant, exhibited an-

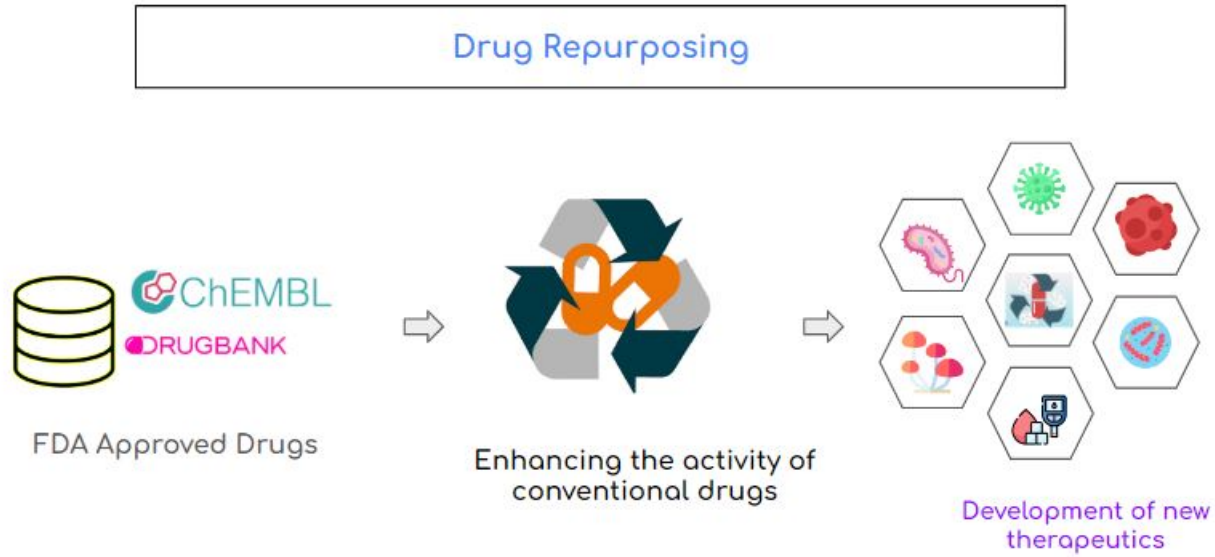


Figure 1.6: Drug Repurposing

ticryptococcal activity in both in vivo and in vitro research. although the exact antifungal mechanism of action remains unknown, sertraline has been advised to result in cell death through disrupting phospholipid membranes, inhibit ergosterol synthesis via binding with the help of an enzyme lanosterol 14-demethylase. (Zhang et al., 2021)clinical testing of sertraline as adjunctive antifungal treatment in HIV patients having cryptococ-

cal meningitis showed improved cerebrospinal fluid clearance but changed into associated with severe unfavorable events.

Despite the challenges faced with sertraline, the improvement of novel derivatives through scaffold hopping validated observed anticryptococcal efficacy in both in vivo and in vitro set up. further, a derivative of haloperidol, when used in combination with fluconazole, precipitated the fungal cell wall resulting in down regulation in specific genes. Ongoing initiatives for drug repurposing towards pathogenic fungal species maintain the promise of providing new therapeutic alternatives within the close to future.(Kim et al., 2020) .This interconnected technique related to drug development and drug repurposing acts as promising strategy involing novel drugs in addressing fungal infections.

In the realm of pioneering screening studies,(JHCCL) known as Johns Hopkins Clinical Compound Library,containing more than thousand five hundred Food and Drug Administration (FDA) and approved foreign drugs was explored Lin group ,targeting the inhibition of A.nidulans growth.This screening helped in identifying the antifungal effect of the polymyxin B, validated by broad spectrum activity.Another noteworthy investigation was looking for drugs in the same library that could inhibit C. albicans species, leading to discovery of 4 compounds without previous recognized antifungal effects , disulfiram, octodrine,fluvastatin and Mycophenolic acid (Wall and Lopez-Ribot, 2020).

Library	**Organism(s)**	**Main "Hits"**	**Comments**
JHCCL	A. nidulans	Polymyxin B, sertraline	Broad spectrum
Prestwick	C. neoformans	Amiodarone	crossing blood-brain barrier
(MMV)Pathogen Box	Candida albicans	MMV688768	distinct activity against biofilm
NIH/NCI Developmental Therapeutics	Multiple species	NSC319726	Synergy with common antifungals
LOPAC	C. neoformans	10058-F4	Screening conducted under nutrient limitation
TargetMol	C. albicans	Robenidine	Action against Aspergillus species and yeasts
McMaster Bioactives	S. cerevisiae	Multiple potentiators	assembling an antifungal combination matrix

Table 1.1: Summary of Screening Studies

Chapter 2

METHODOLOGY

Let us understand the overall workflow in development of antifungal drugs in pathogenic strains.Below is the generalised work flow used in our study.

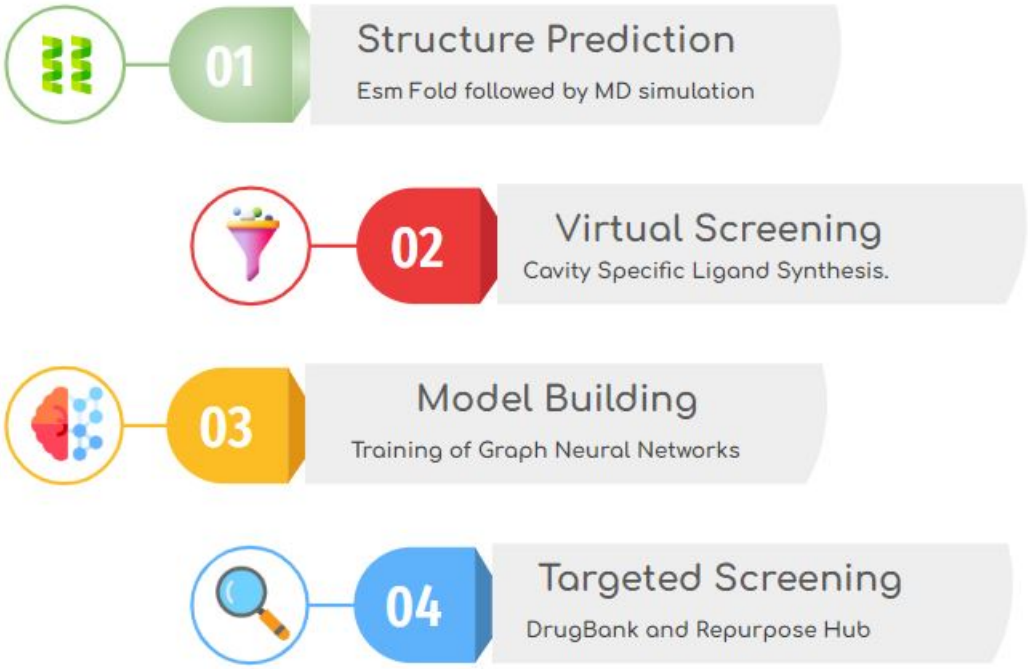


Figure 2.1: Work Flow

2.1 Prediction of Stable structures using ESMFold

2.1.1 ESMFold for Structure Prediction

For our research, we opted to use ESMFold for protein structure prediction due to its distinct advantages over other methods, such as AlphaFold. While AlphaFold employs a

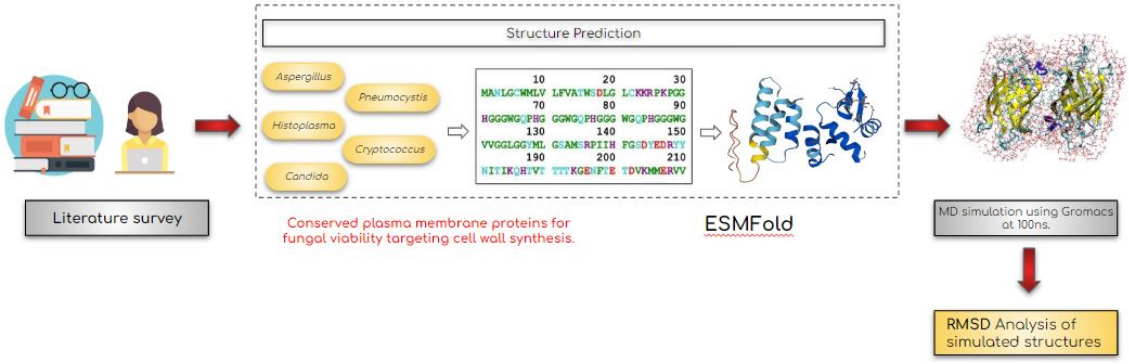


Figure 2.2: Prediction of Stable structures for Rho Proteins

network-based model, ESMFold harnesses the power of a large-scale language model for protein prediction.

ESMFold stands out by generating structure predictions using only a single sequence as input, relying on the internal representations of the language model. This approach contrasts with alternatives like AlphaFold 2, which utilizes multiple sequence alignments (MSAs) and templates of similar proteins. ESMFold's accuracy in atomic-level predictions is particularly noteworthy, surpassing AlphaFold and rivaling RoseTTAFold when provided with full MSAs (Lin et al., 2023).

Another key advantage of ESMFold is its faster prediction speed compared to existing atomic resolution structure predictors. Furthermore, ESMFold offers the opportunity to map structural space in practical timescales. By folding a random sample of one million metagenomic sequences in just a few hours, ESMFold enables the exploration of protein space, particularly in regions distant from existing knowledge.

The adoption of ESMFold in our work is driven by its superior accuracy, efficiency, and capability to address the challenges associated with the ever-expanding protein sequence databases.

Inspired by the article Vaithish Velazhahan et al., Developing novel antifungals: lessons from G protein-coupled receptors Velazhahan et al. (2023). We have chosen Rho proteins identified as conserved plasma membrane proteins crucial for fungal viability, specifically targeting the synthesis of the cell wall.

2.2 Working with GROMACS

Molecular dynamic Simulations facilitates understanding the detailed structure and dynamics at the level of motion of individual atoms. One of the major applications of MD is understanding the intricacies of molecular recognition between proteins and ligands, which facilitates a better understanding of structurally driven drug design. The molecular dynamic simulations of all the proteins has been performed using GROMACS (GROningen Machine for Chemical Simulations). For this simulation, the Lysozyme module was used. Here, we briefly summarize the input files and steps required for a basic MD simulation in GROMACS.

2.2.1 Removal of water molecules:

To remove the crystal water, all the water molecules labeled "HOH" in the PDB file are removed using a basic text editor like vi or emacs on Linux and Mac systems.

2.2.2 Generation of Topology files

In the PDB file, it was essential to check for entries labeled under the comment MISSING, signifying the absence of atoms or entire residues.pdb2gmx may encounter difficulties or fail if the crystal structure contains incomplete internal sequences or amino acid residues with missing atoms. All the structures generated from the ESMFold did not have missing atoms, so pdb2gmx was performed, which is required to generate three files for simulations:

1.Topology for the molecule (.top): Extension is a topology file written in ASCII code. It contains information like the molecule type, atoms, bonds, distance, force-field type,angles, dihedrals, interaction pairs and angle constraints, etc.

2.Position restraint file (.itp): The posre.itp file has valuable informations that is utilized to restrain the positions of heavy atoms.

3.Post-processed structure file(.gro): It serves as a GROMACS-formatted structure file that comprehensively includes all the atoms defined within the force field.(OPLS force field)

2.2.3 Defining the box and solvation of the protein:

The protein was positioned at the center of the simulation box , ensuring a minimum distance of 1.0 nm from the box edge. The box type was specified as cubic. This choice of solute-box distance is crucial due to the implementation of periodic boundary conditions.Once the box was defined, it was filled with solvent (water) using the solvate module. The spc216 model, a generic equilibrated 3-point solvent model, was employed for this process.

2.2.4 Addition of ions

Within GROMACS the tool used for adding ions is genion. This helps in parsing through the topology, where water molecules are replaced with ions. The input for this module originates from running input file with a .tpr extension, generated by the grompp module within gromacs in the pre-processing phase. This .tpr file has parameters for all atoms within the system..

To generate the .tpr using grompp,a molecular dynamics parameter file, is needed.It contains information like integrator-type, time step of integration,total number of steps for simulation run, energy minimization parameters, parameters to set and control temperature and pressure, output control parameters etc.

The integration of parameters specified in the molecular dynamics parameter file file with topology and coordinate information by grompp results in the creation of the .tpr file. The.mdp file serves the purpose of generating an atomic description of the system. It ensures that the addition of only the necessary ions to neutralize the net charge on the given proteins.

2.2.5 Energy Minimization

Energy minimization is the prerequisite step for beginning a dynamics study, whereas equilibration employs the equation of motion for solving a system of atoms. In energy minimization, a force field is used to find the stable point on the potential energy surface using any of the three methods such as steepest descent, conjugate gradient, or Newton-Raphson.

By minimizing the potential energy surface, the system is brought to a stable state, ensuring a starting point for in-depth studies on the motion of individual atoms in molecular dynamics.

So once our electroneutral system was assembled, to begin with dynamics, we ensured that our system had no steric clashes or inappropriate geometry. At the end of the minimization process, three important files are produced:

1.em.log: ASCII-text log file of the EM process
2.em.gro: energy-minimized structure.
3.em.edr: Binary energy file.

2.2.6 Equilibration NVT , NPT

An appropriate initial structure, taking into consideration both geometry and solvent, was ensured through energy minimization (EM). Equilibrating the solvent and ions surrounding the protein is necessary. This is due to the fact that the solvent optimizes independently of the solute. It was crucial to align the protein correctly and bring the solvent to the appropriate simulation temperature. After the system reached the proper temperature, which was established by measuring kinetic energy, pressure was added until the desired density was reached.
Two stages are frequently included in the equilibration process. The first phase, often referred to as "isothermal-isochoric" or "canonical," takes place in an NVT ensemble, meaning that the volume, temperature, and number of particles are all maintained constant. The system's composition determines how long this phase will last, and it is anticipated that the system's temperature will stabilize in the NVT ensemble at the intended value.

The goal of the previous phase, NVT equilibration, was to keep the system's temperature constant. In order to get ready for data collection, the pressure and, by extension, the system's density must be stabilized. Pressure equilibration was done in an NPT ensemble, which maintains consistent temperatures, pressures, and particle counts. This ensemble, which closely mimics experimental circumstances, is referred to as the "isothermal-isobaric" ensemble.

2.2.7 Production

Upon completing the two equilibration phases, the system is now thoroughly equilibrated at the required temperature and pressure. Subsequently, the position restraints were released, marking the commencement of the Molecular Dynamics (MD) production phase. A 100 ns MD simulation was conducted.

2.2.8 RMSD Analysis

Structural stability was assessed by RMSD, which measures the extent to which the simulated structure deviates from the initial reference structure. A low RMSD indicates that the system is relatively stable and has not experienced significant structural changes, whereas high RMSD values may suggest conformational alterations or unfolding.

2.3 Cavity-specific ligand synthesis and virtual screening

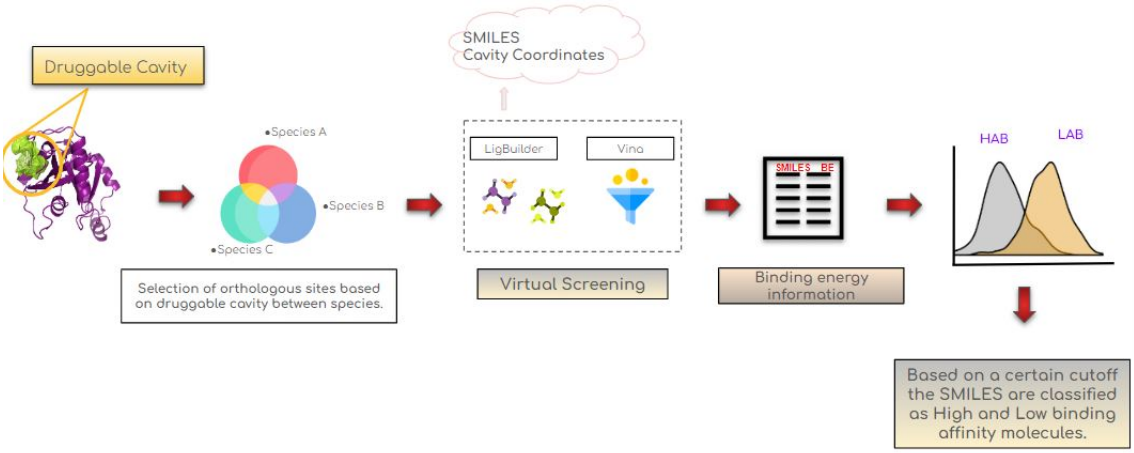


Figure 2.3: Cavity Specific ligand synthesis

2.3.1 Detection of druggable sites using Gcoupler

The Synthesizer Module is designed to identify putative cavities on the protein surface, leveraging input data in the Protein Data Bank (PDB) format. This module requires the protein structure in PDB format, offering flexibility in cavity selection by allowing users to supply critical residues or cavity information.

The Synthesizer Module seamlessly integrates with external tools such as Pocketome, enhancing its functionality and expanding its capabilities. The module produces essential

information, including SMILES data and 3D cavity coordinates. The Ligand Synthesis is done by LigBuilder V3 which uses Genetic Algorithm (GA) for optimal results.

2.3.2 Selection of Orthologous sites

The identification and exploration of orthologous sites, which are characterized by their evolutionary conservation across diverse species, play a pivotal role in various aspects. The preservation of function at these sites, inferred from their conservation, provides insights into the biological processes and functions of genes or proteins.
Additionally, the identification of orthologous sites facilitates the inference of gene or protein function. Moreover, the mapping of druggable residues and their overlap with other species contribute to the recognition of potential drug targets, wherein conserved orthologous sites become crucial step for further investigation and virtual screening.
This approach not only aids in understanding evolutionary relationships but also offers opportunities for cross-species studies, phylogenetic analyses, and the broader implications of genetic variations on functionality.

2.3.3 Virtual Screening

The cavity-specific ligand synthesis was initiated using Ligbuilder, where the synthesis of compounds was done at specific cavities based on their druggability information.
Following this, virtual screening was conducted using AutoDock Vina, employing the Authenticator module provided by Gcoupler. The computation of free binding energies was executed, and compounds were subsequently categorized into high-affinity binders (HABs) and low-affinity binders (LABs). The statistical sub modules employ methods such as Empirical Cumulative Distribution Function (ECDF) and various hypothesis tests, including Kolmogorov-Smirnov, Epps-Singleton, and Anderson-Darling, to enhance the reliability of the classification process.

2.4 Model Building

2.4.1 Building the training data

Once the SMILES were generated, the molecules underwent featurization using MolGraphConvFeaturizer to prepare them for use by models. The data was then divided based on the scaffolds of small molecules using the ScaffoldSplitter. This splitter utilized the data to generate scaffolds through the SMILES information.

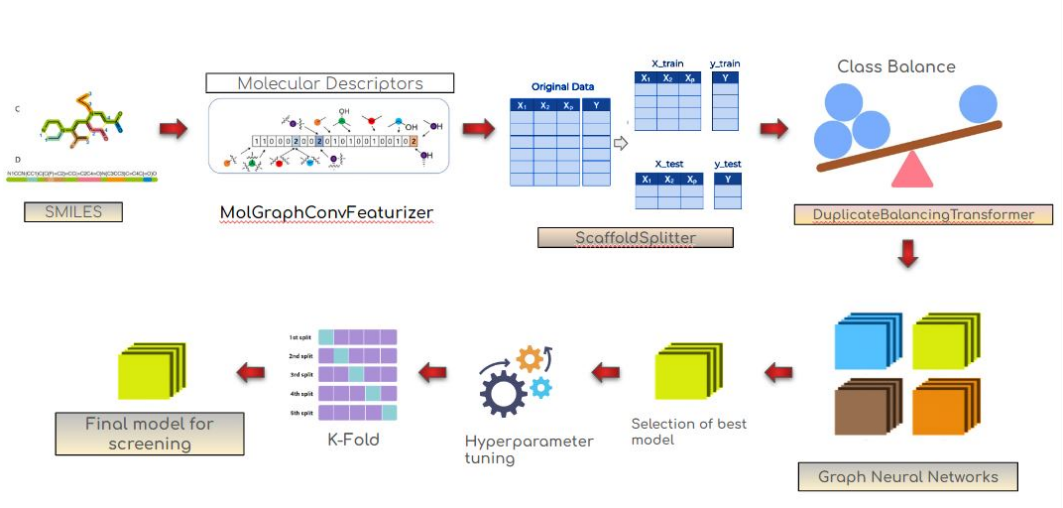


Figure 2.4: Model Building

The molecules were categorized according to BM scaffold (Bemis-Murcko) representation, which identify rings, linkers, frameworks, and atomic properties such as atom type, hybridization, and bond order. Subsequently, splitter organized data into sets based on the identified scaffolds.

2.4.2 Balancing the class balance

The balancing ratio between high and low-affinity molecules is achieved through the utilization of a duplicate balance transformer from Deep Chem. In this process, a dataset is balanced by duplicating samples that belong to the less frequent class. This guarantees an equal sum of example weights across all classes.

2.4.3 Training Graph Neural Networks

The molecules, having undergone training, were utilized to train a Graph Neural Network (GNN), selected specifically for its importance in handling graph data. The performance of GNN was assessed using classification metrics like Accuracy, F1 Score,Matthews Correlation Coefficient,kappa score on both the training and test data.The selection of the best model was based on the performance with the test data, subsequently chosen for further hyper parameter tuning. The final model, refined through this process, was employed for predicting anti-fungal compounds.

2.5 Targeted screening

Targeted screening is conducted using The Drug Repurposing Hub, as described by Beattie et al. (2020) Beattie and Krysan (2020). In an effort to accelerate drug development, the Drug Repurposing Hub, established by researchers from the Broad Institute's Cancer Program, Center for the Development of Therapeutics, and Connectivity Map project,serves as valuable open-access repository. This resource encompasses over 6,000 compounds, including numerous FDA-approved drugs, meticulously curated and verified by researchers over several years. The presented diagram depicts the hierarchy of protein

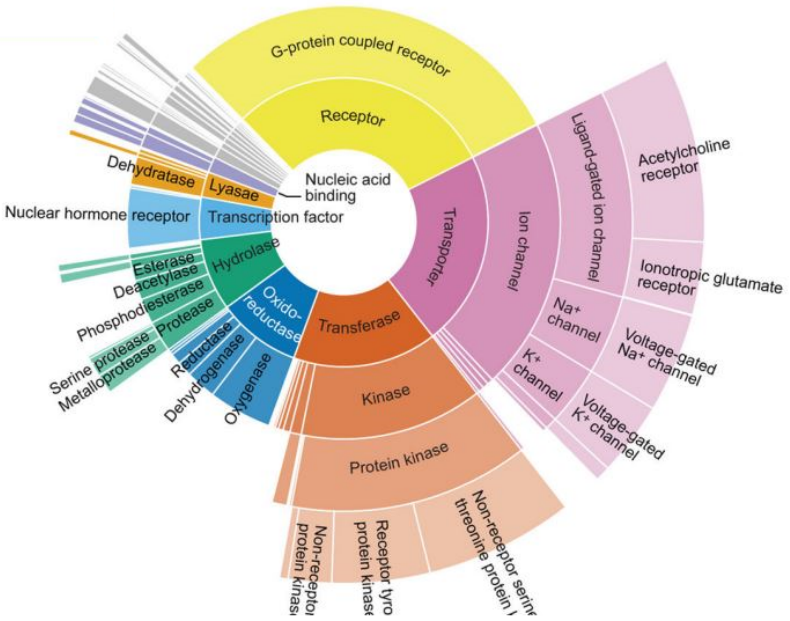


Figure 2.5: Target proteins classified by Repurpose Hub

functions, showcasing a relationship between the degree of specificity in function and the distance from the center of the figure. This visual representation offers an intuitive understanding of how the library is distributed across various protein functional categories, with larger segments indicating a higher emphasis on targeting specific protein functions within the overall repertoire. As anticipated, the analysis reveals an enrichment of drugs within the library that specifically target G protein-coupled receptors,ion channels and kinases. This enrichment underscores the library's focus on these particular protein classes, suggesting a strategic emphasis on modulating signaling pathways, cellular communication, and ion fluxes for potential drug repurposing applications.

The data is utilized, from which the SMILES are converted to open babel canonical ones and employed for predictions. Within the data, numerous known antifungals are also present, allowing for validation of the model's predictive accuracy.

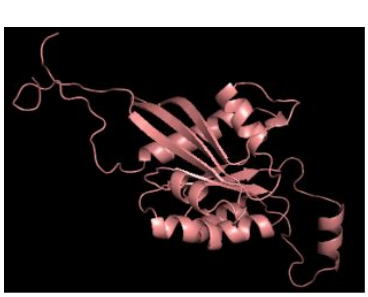
Chapter 3

Results

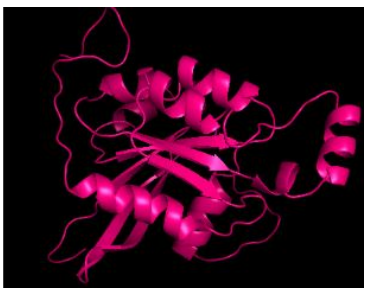
3.1 Prediction of stable structures

3.1.1 Protein structures from ESMFold

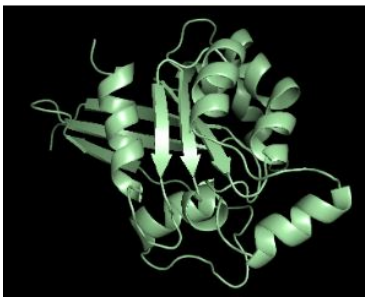
Due to the unavailability of structural information for the corresponding Rho proteins in the majority of pathogenic species, structures were generated using the ESMFold. Initially, structures were generated using AlphaFold, followed by the generation of struc-



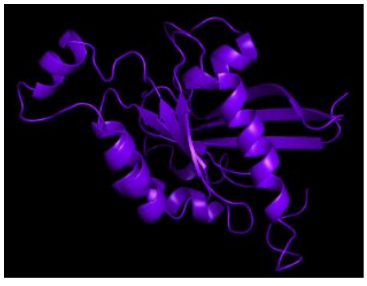
(a) Candida albicans



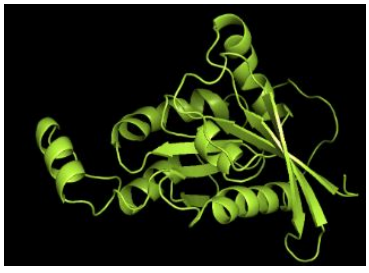
(b) Cryptococcus neoformans



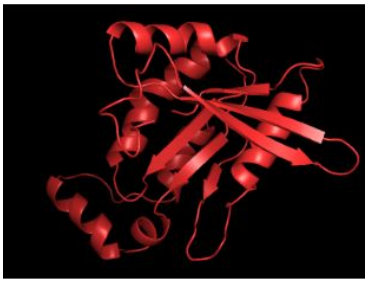
(c) Aspergillus fumigatus



(d) Histoplasma capsulatum



(e) Aspergillus nidulans



(f) Pneumocystis jirovecii

Figure 3.1: Structures from ESMFold of Pathogenic Species

tures using ESM fold. The final structures were aligned, and the RMSD was calculated, revealing a value less than 2 Å, indicating similarity between the two sets of structures. Given that ESM fold, generated using a large language model, found better for shorter

sequences, the decision was made to proceed with ESM fold.

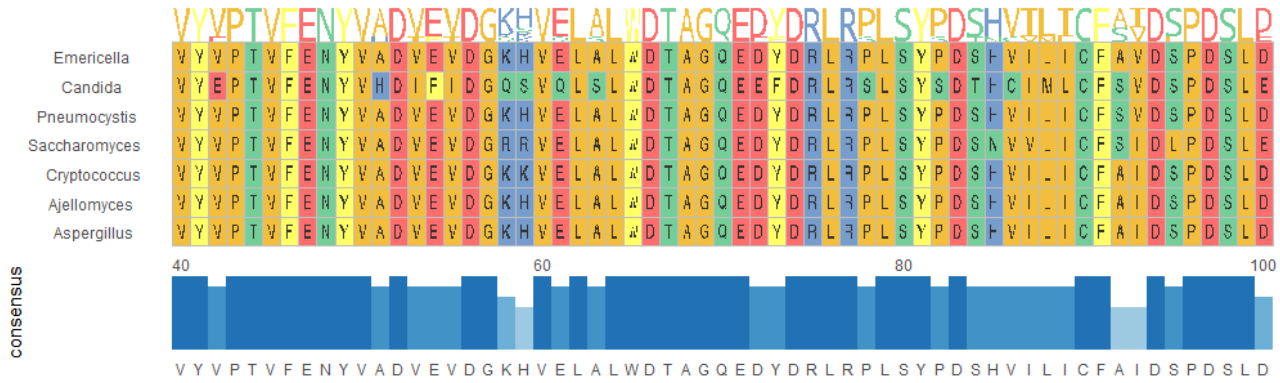


Figure 3.2: MSA from a sequence length of 40 to 100.

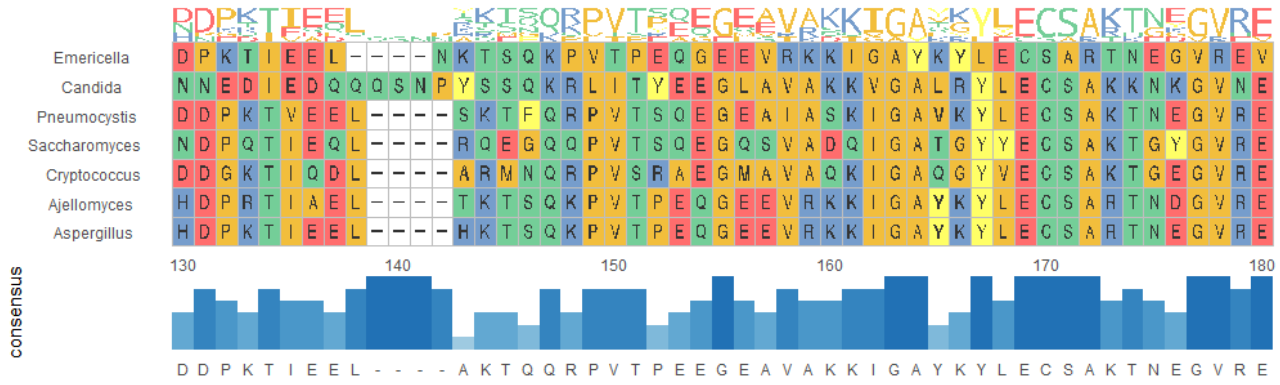


Figure 3.3: MSA from a sequence length of 130 to 180.

All the six sequences of the pathogenic fungi were taken from UniProt and NCBI.The provided Multiple Sequence Alignment (MSA) represents the alignment of Rho protein sequences from different pathogenic fungal species. In the alignment, each row corresponds to a different fungal species, and the sequences are aligned based on their homologous regions.

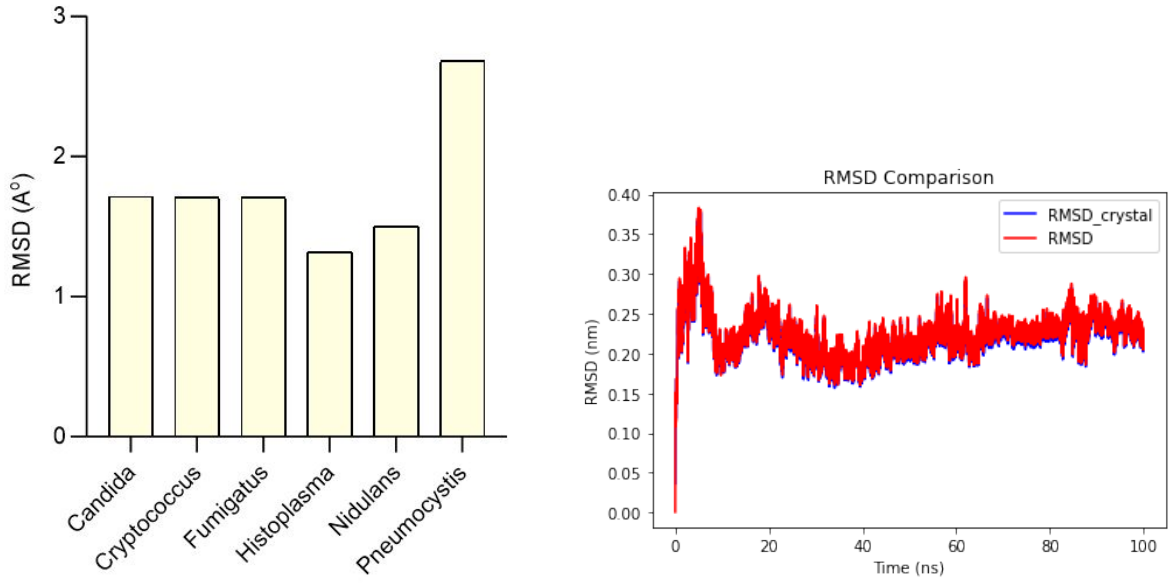
Analysis of MSA was crucial for identifying conserved regions in the Rho proteins among different fungal species, providing insights into potential functional domains and aiding in the interpretation of structural and functional implications in the context of antifungal drug development.

3.1.2 RMSD Analysis

To ensure the structural stability of the generated structures, the final frame post-simulation was extracted. A plot depicting the Root Mean Square Deviation (RMSD) values before and after the simulation is shown in the figure, with a critical consideration that the RMSD values should not exceed 2 angstroms. This threshold is indicative of a desirable level of structural consistency, and deviations beyond this limit may suggest significant structural alterations.

Monitoring the RMSD throughout an MD simulation offered a dynamic perspective on the system's behavior, helping to pinpoint structural changes and ensuring that the system has achieved equilibrium before proceeding to production runs.It helped in Identification of Structural Changes where the RMSD vs. time plot aided in identifying crucial points in the simulation where significant structural alterations occurred. Large conformational changes in a protein throughout the simulation were evident as spikes in the RMSD plot, providing valuable insights into dynamic behavior.

Secondly,Equilibration Convergence;The RMSD plot served as an indicator of the convergence of the structure towards an equilibrium state. A flattening of the RMSD curve signified that the protein has equilibrated, suggesting that the system has reached a stable state. This served as a crucial checkpoint before transitioning to the production phase of the simulation.



(a) RMSD difference in pathogenic species

(b) RMSD vs Time in Histoplasma

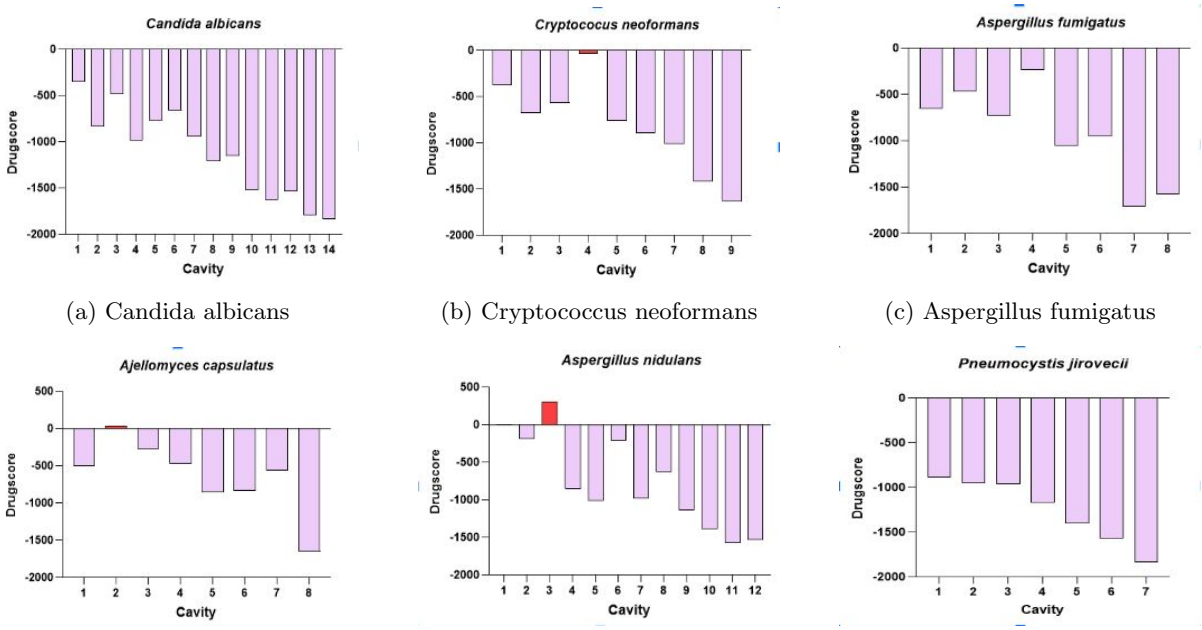
Figure 3.4: RMSD Plots

3.2 Prediction of Cavity in Pathogenic Strains

3.2.1 Detection of Druggable sites

The PDB structure was subjected to Gcoupler to identify druggable sites, and the resulting information about druggability was determined. Notably, **Cavity 3** in *Aspergillus nidulans* exhibited an amphibious nature with a drug score of **302**. The drug score of computed based on the abundance of hydrophobic amino acids and pharmacophore properties within a specific cavity.(Mohanty et al., 2023) The below bar graph includes comprehensive data regarding the druggability in pathogenic strains.

In the analysis of other fungal species, multiple species exhibited amphibious characteristics; however, the corresponding drug scores were comparatively lower. Despite the presence of amphibious behavior, the decision to proceed with *Aspergillus nidulans* was due to its distinctive advantage of yielding a higher drug score.



(a) Candida albicans (b) Cryptococcus neoformans (c) Aspergillus fumigatus

(d) Histoplasma capsulatum (e) Aspergillus nidulans (f) Pneumocystis jirovecii

Figure 3.5: Druggability in Pathogenic strains

3.2.2 Druggable Site Conservation and Ligand Synthesis

Given that Cavity 3 in *Aspergillus nidulans* exhibits amphibious activity, the residues within the cavity have been identified: **[5 ARG, 6 ARG, 7 LYS, 8 LEU, 9 VAL, 39 PHE, 40 GLU, 41 ASN, 42 TYR, 43 VAL, 56 ALA, 57 LEU, 58 TRP, 59 ASP, 60 THR, 61 ALA, 66 TYR, 67 ASP, 68 ARG, 69 LEU, 70 ARG, 71 PRO, 72**

LEU, 73 SER, 74 TYR, 75 PRO, 76 ASP, 77 SER, 78 HIS, 79 VAL, 185 LYS, 186 SER, 187 LYS, 188 LYS, 189 LYS, 190 CYS].

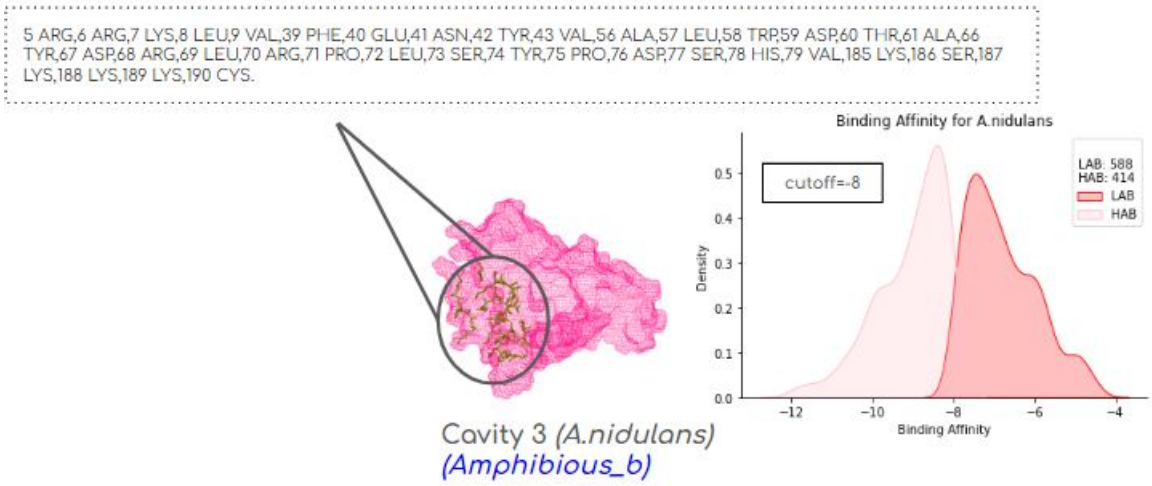


Figure 3.6: Amphibious site in nidulans

To explore the conservation of this site, mapping was performed across other species. Notably, **Cavity 2 in *Histoplasma*** showed 14 overlapping residues, while **Cavity 6 in *Fumigatus*** exhibited 20 overlaps and was selected for ligand synthesis.

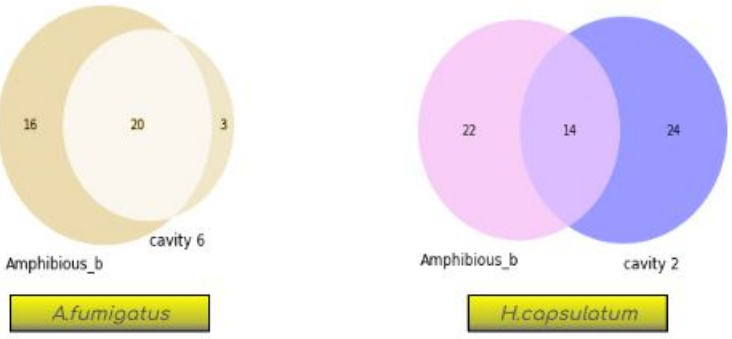


Figure 3.7: Orthologus sites

The ligand synthesis process involved using Lig Builder, and virtual screening was conducted, yielding binding energies for 1000 synthesized SMILES in the specified cavity. Based on a predefined cutoff, these molecules were classified into high and low-affinity binding molecules. This classification serves as a valuable foundation for subsequent analyses and explorations of potential drug candidates.

The distribution provided in the Figure 3.6, outlines the High Affinity Binding (HAB) and Low Affinity Binding (LAB) status corresponding to the generated SMILES. In the

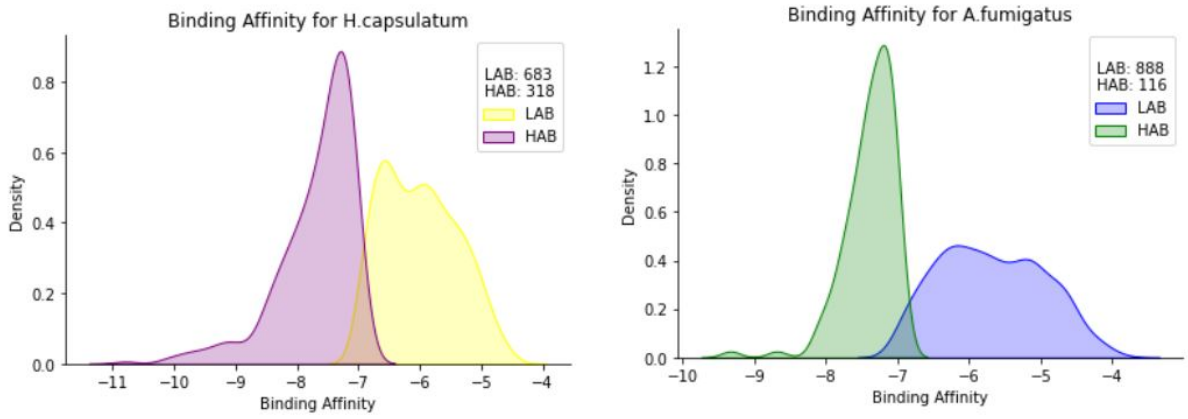


Figure 3.8: Binding Affinity of Histoplasma and Fumigatus

analysis of the remaining species having orthologus sites with specific to nidulans, a cutoff of -7 was implemented. Subsequent attempts with a cutoff of -6 resulted in a significant number being classified as 1 during the screening process. To address this issue, the cutoff was modified to ensure the model is trained with a greater representation of zeros. This adjustment aims to optimize the model's capacity to discern patterns related to both high and low-affinity binding energies in the data during the screening process.

3.3 Performance and Validation of Graph Neural Networks

Four graph-based models GCM (Graph Convolution Model), AFP (Attentive FP), GCN (Graph Convolution Network), and GAT (Graph Attention Network) were trained and tested on binary data labeled as HAB (High Affinity) and LAB (Low Affinity). After pre processing the binary data and conducting a train-test split, we evaluated the models on both training and testing datasets.

The evaluation metrics revealed a high level of performance on the training data for the GCN Network model, with an accuracy of 99.52%, a Matthews Correlation Coefficient (MCC) of 99.05%, and a precision and recall both exceeding 99%.However in the test data the accuracy dropped to 84.58%, and the MCC decreases to 54.29%.The Area Under the Curve (AUC) value, representing the model's ability to distinguish between positive and negative examples, was exceptional in the training set at 99.99% ,while the the Kappa score, which measures agreement between the model and actual values, showed a decrease from 99.04% in training to 86.75% in testing.

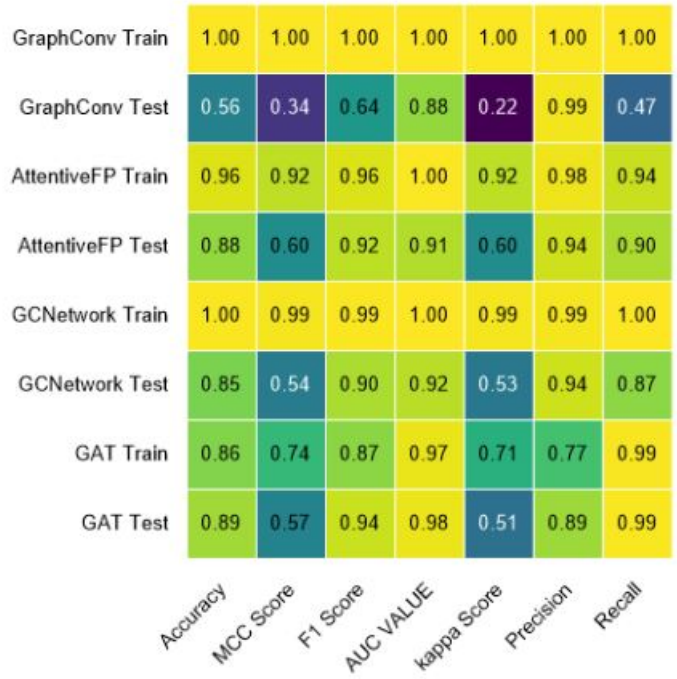


Figure 3.9: Model performance on Cavity 3 (nidulans)

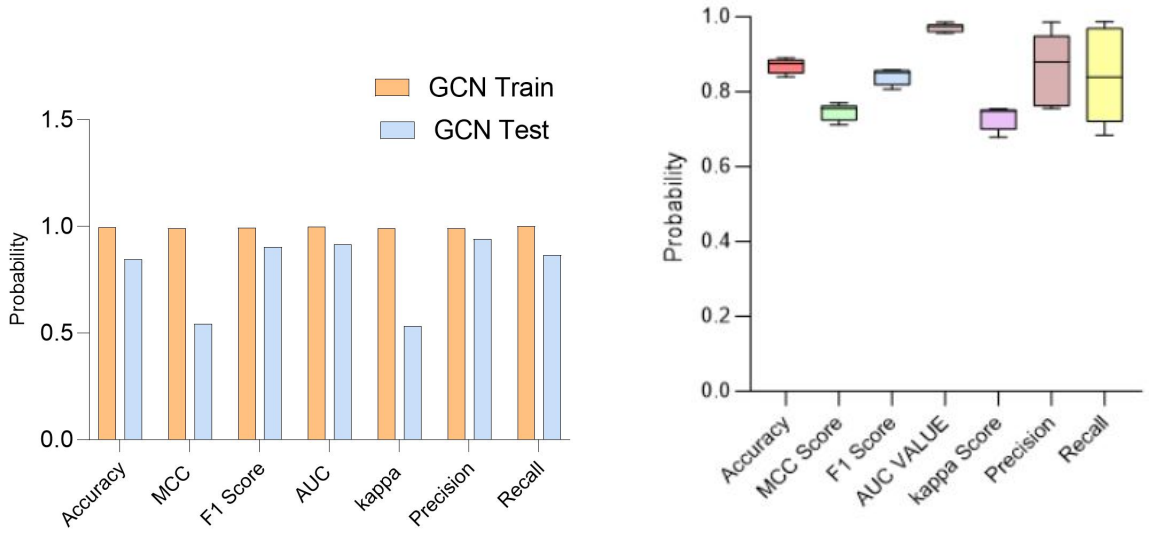
GCN outperformed the other models in terms of predictive performance on both the training and testing sets. Given this superior performance, GCN was selected as the model for further investigation. Subsequently, hyperparameter tuning was employed to optimize the model's parameters and fine-tune its performance.This step aimed to maximize the predictive accuracy of the GCN model.

A batch size of 16 was selected, indicating that weight updates are made more frequently during training. This allowed for faster convergence but also resulted in increased computational overhead in certain cases.

The model architecture comprised two graph convolutional layers with 64 units each. This reflected a moderate level of model complexity, enabling hierarchical feature extraction from the graph structure.

The hidden layer of the predictor network was configured with 128 units, which was preferred for a relatively high-dimensional feature space in the final prediction. This choice enhanced the model's ability to learn detailed patterns and representations from graph-structured data.

The learning rate ranges from 0 to 1.0 smaller learning rate results in smaller changes made to weights each update and requires more training epocs. A learning rate of 0.01



(a) GCN performance on Train and Test data (nidulans)

(b) GCN 5 Fold CV

Figure 3.10: GCN performance on nidulans

was employed. Our chosen hyper parameters indicated a well-considered balance between model complexity, regularization, and computational efficiency.

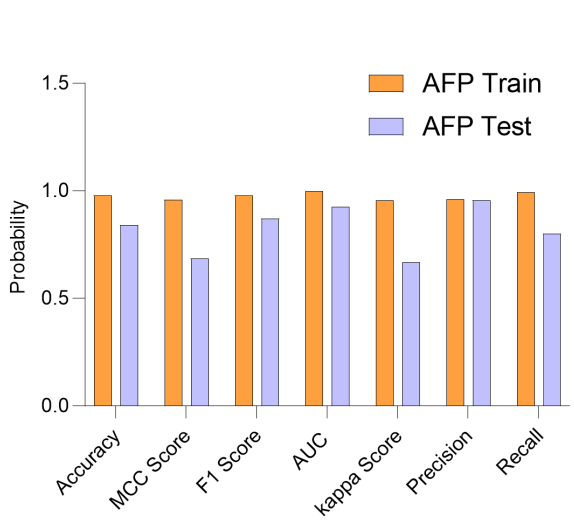
The box plot shows the 5 Fold Cross validation on test data of GCN which outperforms the other models.

Even for the overlapping cavities detected,models were trained.

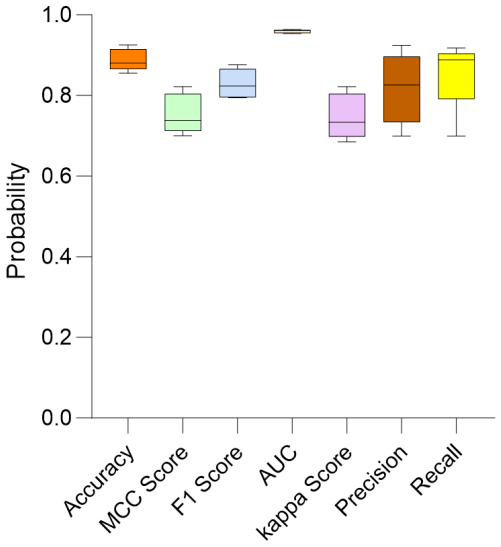
Table 3.1: Performance Metrics of Histoplasma

	Accuracy	**MCC Score**	**F1 Score**	**AUC Value**	**Kappa Score**	**Precision**	**Recall**
GCM Train	1	1	1	1	1	1	1
GCM Test	0.5274	0.2679	0.4920	0.7524	0.1891	0.8846	0.3407
AFP Train	0.9786	0.9576	0.9776	0.9976	0.9571	0.9613	0.9945
AFP Test	0.8408	0.6856	0.8710	0.9241	0.6674	0.9558	0.8000
GCN Train	0.9631	0.9288	0.9623	0.9999	0.9263	0.9274	1.0000
GCN Test	0.6169	0.3580	0.6316	0.7750	0.2974	0.8919	0.4889
GAT Train	0.8816	0.7662	0.8796	0.9574	0.7636	0.8442	0.9180
GAT Test	0.8408	0.6340	0.8832	0.9132	0.6334	0.8705	0.8963

The performance metrics of histoplasma and fumigatus is shown in Table 3.1 and 3.2. AFP Outperformed the models and was further selected for hyper parameter tuning.The final hyperparameters for the AFP model were determined to be having 2 graph neural network layers, a graph feature size of 200, and a dropout rate of 0.These settings were selected based on the optimization process, where various combinations of hyperparameter values were explored.



(a) AFP performance on Train and Test data Histoplasma

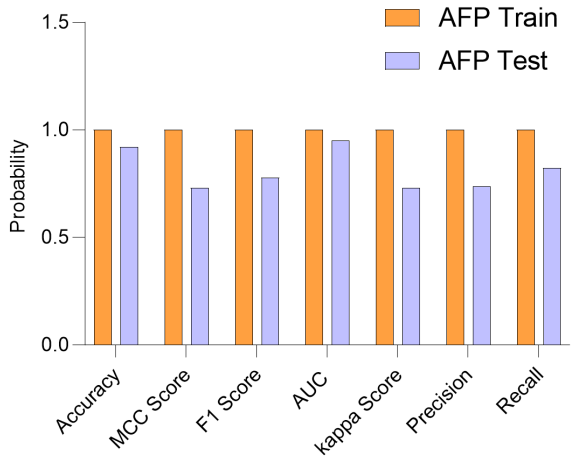


(b) AFP 5 Fold CV

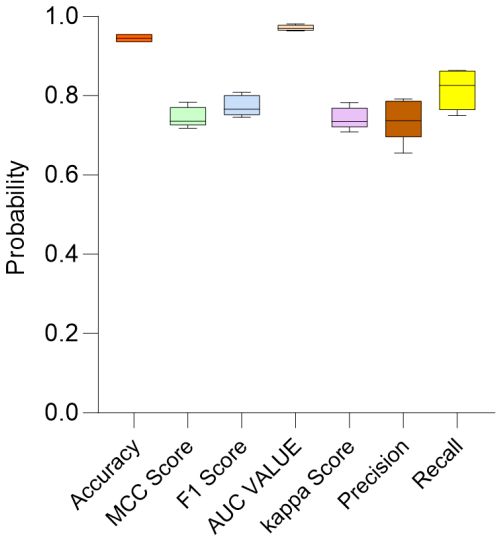
Figure 3.11: AFP performance on histoplasma

Table 3.2: Performance Metrics of fumigatus

	Accuracy	MCC Score	F1 Score	AUC Value	Kappa Score	Precision	Recall
GCM Train	1	1	1	1	1	1	1
GCM Test	0.8955	0.5783	0.5882	0.8815	0.5359	0.8824	0.4412
AFP Train	1	1	1	1	1	1	1
AFP Test	0.9204	0.7311	0.7778	0.9520	0.7295	0.7368	0.8235
GCN Train	1	1	1	1	1	1	1
GCN Test	0.8856	0.5882	0.6567	0.9234	0.5881	0.6667	0.6471
GAT Train	0.9412	0.8847	0.9405	0.9815	0.8825	0.9078	0.9756
GAT Test	0.8408	0.6181	0.6667	0.9613	0.5735	0.5161	0.9412



(a) AFP performance on Train and Test data Fumigatus

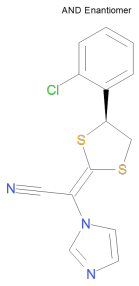


(b) AFP 5 Fold CV

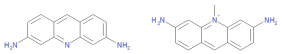
Figure 3.12: AFP performance on Fumigatus

3.4 Screening The Drug Repurposing hub

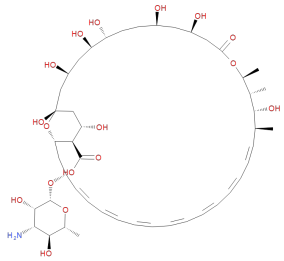
The binding probabilities of query compounds, sourced from the Repurpose Hub comprising 6780 compounds, were predicted using the best model. Among these, 30 compounds are recognized as known antifungals. Additionally, the mean probability of the compounds across all three species was calculated. Using a cutoff of 0.999, 148 compounds were selected for further validation. Subsequently, docking experiments were conducted for these 148 compounds with each of the three target species.



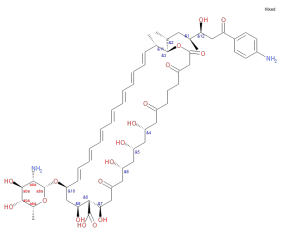
(a) Lanoconazole



(b) Acriflavine



(c) Amphotericin-b



(d) Candicidin

Figure 3.13: Strutural representations of the antifungals

Table 3.3: List of Antifungals in The Drug Repurposing hub

Antifungals	**Mechanism of Action**
Acriflavine	Hypoxia inducible factor inhibitor
Amphotericin-b	Membrane permeability enhancer
Anidulafungin	1,3-β-D-glucan synthase inhibitor
Candicidin	Fungal ergosterol inhibitor
Cerulenin	Fatty acid synthase inhibitor
Chlormidazole	Fungal lanosterol demethylase inhibitor
Butenafine	Fungal squalene epoxidase inhibitor
Lanoconazole	Sterol demethylase inhibitor

In our docking study, the top four hits were identified as MRX-2843, SDZ-NKT-343, NTNCB, and Refametinib. These compounds demonstrated notable Binding energy

(BE) values, with average BE scores of -9.46 ± 1.800333302, -8.7767 ± 0.7695669778 , -8.6333 ± 0.6061627944, and -7.9733 ± 0.9165878754, respectively across species.

To further validate the predictions a comprehensive literature survey was done.As indicated in (Bedoya-Cardona et al., 2023), In the experimental validation phase a series of in vitro assays were carried out to assess the efficacy of reported MEK inhibitors across various fungal species. Docking simulations were performed, specifically targeting proteins F0UAN5 and A0D2XNJ1 from Histoplasma capsulatum and Fusarium oxysporum, respectively. Notably, these proteins were previously identified in complex with MEK1, and the structural information available in the Protein Data Bank informed the docking simulations.The study focused on 25 MEK inhibitors, which were previously characterized in complex with MEK1. These compounds were chosen to explore their potential inhibitory effects on the selected fungal species.

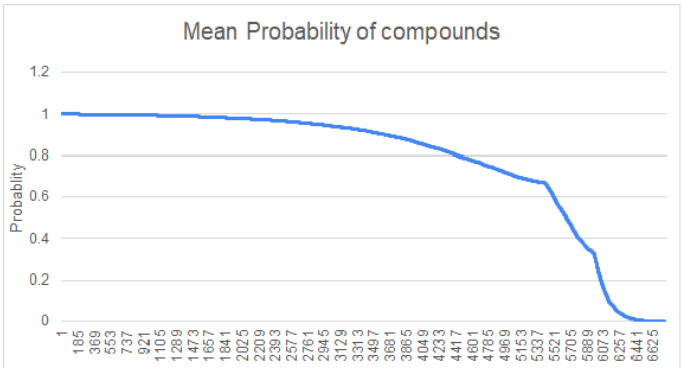
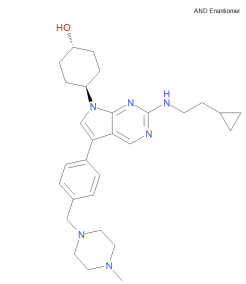


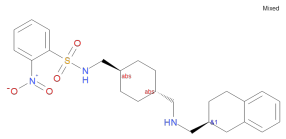
Figure 3.14: Prediction probabilities of compounds from Repurpose hub

Following the docking simulations, growth inhibition experiments were conducted to evaluate the effectiveness of the MEK inhibitors. Histoplasma capsulatum exhibited heightened susceptibility, revealing noteworthy sensitivity to four specific inhibitors: cobimetinib, GDC-0623, myricetin, and refametinib.

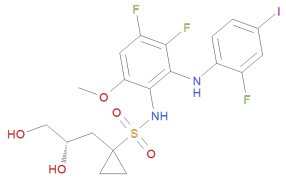
Secondly reported in (Minson et al., 2016),MRX-2843, as type 1 tyrosine kinase inhibitor, represents a groundbreaking approach in acute myeloid leukemia (AML).Its dual inhibition of MERTK and FLT3 pathways, along with demonstrated efficacy in inducing apoptosis and inhibiting colony formation, underscores its potential significance.



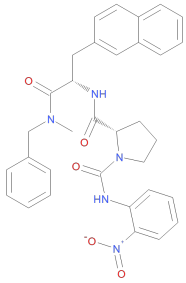
(a) MRX-2843



(b) NTNCB



(c) Refametinib



(d) SDZ-NKT-343

Figure 3.15: Top four hits

NTNCB hydrochloride has shown interactions with neurotransmitter transporters ,GABA receptors and calcium channels.studies have shown SDZ-NKT-343 as novel neurokinin I receptor antagonist. Further it is important to explore and investigate in depth the pathways associated with all these compounds and the selected compounds are required for experimental validation, an essential step to affirm the reliability of the generated results.

Chapter 4

Discussions and Future Perspectives

Opportunistic fungal infections in today's world are observed in immunocompromised patients, causing life-threatening risks. Understanding fungal biology has unveiled numerous potential targets, holding promise for the development of clinically applicable anti fungal agents. In our study, we targeted Rho proteins, which are a vital component of fungal cell wall biology.

Plasma membrane conserved of pathogenic fungal species were utilized to develop stable structures of Rho proteins using ESMFold (Evolutionary scale modeling), which harnesses the power of a large-scale language model for protein prediction and is also considered potential for short sequences. For targeting proteins with larger sequences certain challenges encountered during the validation, have been identified, suggesting areas for potential refinement in future methodologies.

Further, the investigation delved into the effectiveness of cavity-specific ligand synthesis in targeting Rho proteins, within diverse fungal pathogenic species. Gcoupler which was developed to detect druggable sites in a protein structure, was used. Due to its seamlessly designed modules, Gcoupler aided in cavity-specific ligand synthesis and segregating compounds into high and low-affinity binders. At the same time we focused on exploring orthologous cavity sites across the pathogenic species, which constituted a significant aspect of our study.
This methodology helped us understand the importance of evolutionary conservation in identifying potential targets for therapeutic interventions. We developed deep learning algorithms that helped in predicting user-specified compounds for the identification of

novel antifungal drugs.

The exploration of repurposed drugs extends beyond their original therapeutic interventions presenting opportunities for novel acceleration in diverse drug development. In our approach, we performed targeted screening using Repurpose Hub.Out of the initial pool of 6780 compounds predicted using Repurpose Hub, a stringent filtration process with a cutoff of 0.999 yielded 148 compounds. These 148 compounds underwent further evaluation through docking studies using Autodock, focusing on three target species histoplasma, nidulans, and fumigatus.
Notably, the compounds MRX-2843, SDZ-NKT-343, NTNCB, and Refametinib with The average BE scores for these compounds were found to be -9.46 ± 1.800333302, -8.7767 ± 0.7695669778, -8.6333 ± 0.6061627944, and -7.9733 ± 0.9165878754, respectively.

In the future, our goal is to systematically screen extensive databases such as ZINC and DrugBank to enhance the assessment of our prediction accuracy.Moving forward, a crucial aspect involves experimentally confirming the efficacy of the compounds identified, a step to be undertaken following a thorough literature review.

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