Minor Research Project

Synthesis, in vitro biological activity & molecular docking studies of azine derivatives as potential antifungal agents

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INTRODUCTION

The answers to many problems in medical science have been provided by nature. Most of the earliest therapeutic compounds were isolated from natural sources and administered to patients to treat them for various physiological conditions or against infectious microorganisms. The journey of antifungal agents was no different and began in 1950's with the discovery of polyene antifungals. One of the most celebrated antifungal agents of the mid-20th century was *Amphotericin B.* So much so that it was the sole drug available to control serious fungal infections for as many as 30 years, despite its serious nephrotoxicity.[1] Synthetic chemists started designing other polyene antifungals using *amphotericin B* as the lead. However any reduction in the hydrophobic chain led to loss in selectivity and an increased toxicity. Filipin is an example of a potentially toxic polyene antifungal due to its good affinity for the host cholesterol.

Many reasons contributed to the need for synthetic efforts to combat systemic fungal infections. Widespread use of therapies that depress immune system; indiscriminate use of broad-spectrum antibacterials and chronic immunocompressive infections like AIDS & development of drug resistance are among a few which highlighted the need for discovery of new antifungal agents with novel mechanism of action.[1]

There was a remarkable shift from polyene to azole antifungals in the last quarter of the century as evidenced by the fact that 18 out of 23 antifungal drugs approved between 1980 and 2002 were synthetic of which 83% belonged to the azole class. Fluconazole has been used to treat in excess of 16 million patients, including over 300,000 AIDS patients in the U.S. alone since the launch of this drug.[2]

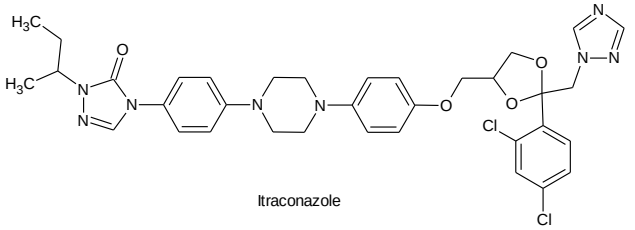
The extensive use of azole antifungals have resulted in development of resistance in fungal strains. The biochemical basis of resistance in fungal strains can be summarised as follows:

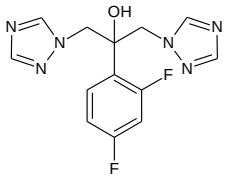
Table 1: Biochemical basis of azole resistance

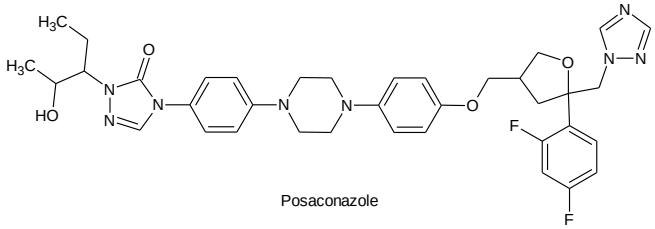
Mechanism	Caused by	Comments
Alteration in drug target (14α-demethylase)	Mutations which alter drug binding but not binding of the endogenous substrate	Target is active (i.e., can catalyse demethylation) but has reduced affinity towards

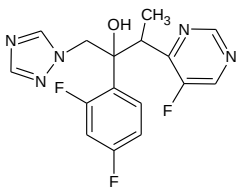
		azoles
Alteration in sterol biosynthesis	Lesions in the $\Delta^{5(6)}$-desaturase	Results in accumulation of 14α-methyl fecosterol instead of ergosterol
Reduction in the intercellular concentration of target enzyme	Change in membrane lipid and sterols; overexpression of specific drug efflux pumps (CDR1, PDR5 &BEN^r)	Poor penetration across the fungal membrane; active drug efflux
Overexpression of antifungal drug target	Increased copy number of the target enzyme	Results in increased ergosterol synthesis; contributes to cross-resistance between fluconazole and itraconazole
*Ghannoum& Rice; Vol.12, **1999,** Clin. Micro. Rev.*		

Apart from resistance, many reports on toxicity of azole antifungals were published in the early 2000's. Schlatter*et al* in 2003 reported that azole fungicides affect mammalian steroidogenesis by inhibiting sterol-14α-demethylase and aromatase enzymes.[3] In the same year, Zhu *et al* demonstrated a potent binding of azole moiety to heme. The group synthesised non-azole based lead molecules using a pioneering de novo design towards novel antifungals. They attributed the affinity of these lead molecules to their non-bonding interaction with the apo protein. This study presented an opportunity to develop novel antifungal agents that specifically interact with the residues in the active site and avoid the serious toxicity arising from co-ordination with the heme of mammalian P450's.[4] The studies by McNicholas *et al* in 2004 on binding of voriconazole, fluconazole, itraconazole (ITZ) and posaconazole (POS) reinstated the importance of hydrophobic interaction in the binding affinities of these molecules to the target. It was shown that POS and ITZ occupy a specific channel within CYP51 leading to non-bonding interaction. These interactions stabilize the binding of the azoles to CYP51 proteins.[5]

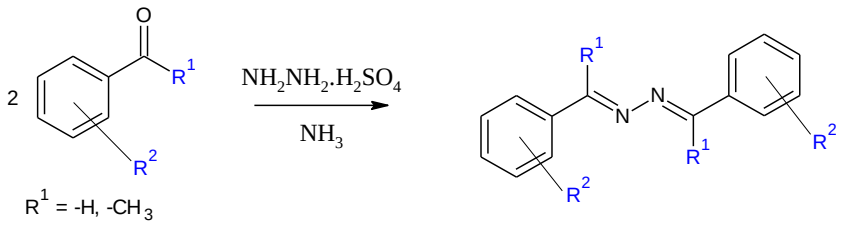








Azines are a class of compounds which have the framework [>C=N-N=C<] in them. In alicyclic chemistry, azines are compounds resulting from the reaction of two molecules of identical carbonyl compounds (symmetrical azines) or from the reaction of two different carbonyl compounds (unsymmetrical azines) with hydrazine. The compounds are called aldazines or ketazines depending on whether the carbonyl compound is an aldehyde or a ketone, respectively. They constitute an important class of stereochemically significant nitrogen donor ligands in organometallic complexes with pharmacological and biological acitivites. Their specific role as binding molecules or modulators of biological receptors makes them suitable candidates for drug development.[6]A few azine derivatives have been shown to possess excellent activities against both bacterial and fungal strains. The vital role of azine functional group in demonstration of antimicrobial property has been established through structure activity relationship.[7]



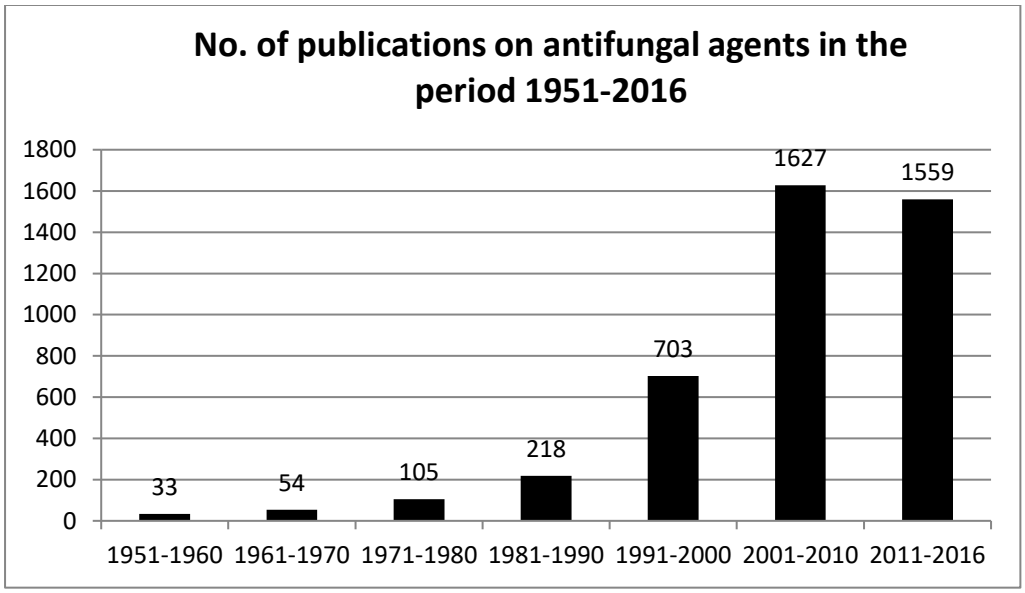
Hence, we propose that substituted azines with groups capable of forming strong non-bonding interactions with the CYP51 protein are likely to exhibit superior antifungal activities.

Advances in computational chemistry has given a new dimension to ration, non-random drug designing. In-silico sreening of molecules that offer good binding affinities to targets can be achieved through molecular docking software. Synthesis of a select few of the screened compounds and determination of biological activities helps in validating the model. This approach has been exploited extensively to design a number of biologically active compounds with a rationalized approach.

International Status and Significance of Study:

There are many research groups around the world who are working in the field of medicinal chemistry & a fair number of them are involved in developing better lead compounds as antifungal agents. Many are working on developing more potent frameworks for existing targets. In 2015, the research group of Maurizio Del Poeta developed a new class of antifungals based on a new molecular target of the microorganism. The research group identified the need for research

in this field owing to the mortality rate of invasive fungal infections being over 50%. Another important contributing factor is the prevalence of fungal infections among individuals with medical conditions that compromise the immune system, such as AIDS, or individuals who are being treated with immunosuppressives, such as those battling cancer.[8] An indication of interest in the field of antifungals among researchers is the number of publications. The number of published work in this field has gone up dramatically in the last three decades and is still on the rise. Hence, a study of this kind is relevant and any contribution to the existing scientific literature crucial in battling these infectious microorganisms.



OBJECTIVES

Primary objectives:

1. In-silico screening of aromatic aldazines and ketazines against the molecular target lanosterol-14α-demethylase (CYP51 protein).
2. Synthesis, purification and characterisation of representative azines.
3. Study of in-vitro biological activity of the representative azines and validate the model established by docking studies.
4. To synthesise a novel antifungal agent with a proposed binding affinity according to the validated docking model.

MATERIALS & METHODS

I. Molecular Docking Studies:

Symmetrical aromatic aldazines have been tested against a wide variety of microorganisms. However, targeted drug design would require identification of a molecular target for systemic infection & model compounds which interact with these targets. There are five major classes of systemic antifungal compounds which are currently in clinical use, viz. the polyene antibiotic, the azole derivatives, the allylamines, thiocarbamates and the fluoropyrimidines. Azole derivatives discovered in the late 1960s, are totally synthetic and are the mot rapidly expanding group of antifungal compounds. They act primarily on ergosterol biosynthesis at the C-14 demethylation stage, a three step oxidative reaction catalyzed by the cytochrome P-450 enzyme, 14-alpha-sterol demethylase ($P450_{DM}$). Azoles disrupt the structre of the plasma membrane, making it more vulnerable to further damage, and alter the activity of several membrane bound enzymes, such as those associated with nutrient transport and chitin synthesis. Most rational drug design efforts have focused on fungal sterols since they are structurally distinct from their mammalian counterpart and their biosynthesis has been studied extensively.[10] Hence the ligands were docked on Lanosterol-14-α-demethylase (CYP51) as the molecular target against pathogenic strains of *Candida tropicalis, vaginalis, albicans* and *gabralta*. Docking studies were carried out on the known crystal structure for *Candida* albicans (5TZ1) available on RCSB site (https://www.rcsb.org/) as a pdb file.

The ligand structures were drawn on chemsketch and saved as mol files. These mol files were imported onto the PyRx software platform which is based on AutoDock vina as the docking program. The imported ligands were converted into PDBQT files and energy minimized before docking onto the molecular target. The ligands were then screened in-silico based on their binding pose values which is a function of the affinity of ligand to the macromolecule and hence reflects on its activity against the target. The proposed docking model was then validated by synthesis of ligands & experimental determination of in-vitro biological activity.

II. Synthesis, purification and characterisation:

Synthesis of substituted aromatic aldazines and ketazines was carried out by one of the following literature protocols:[11] (a) Azine- In a round-bottomed flask, is placed 2.40 g. (18.5 mmol) of powdered hydrazine sulfate, 20 mL of water, and 2.2 cc. (2.06 g., 34 mmol) of 28 per cent aqueous ammonia (sp. gr. 0.90). The mixture is stirred, and, when the hydrazine sulfate has dissolved, 4.4 cc. (4.6 g., 43.5 mmol) of benzaldehyde is added from a separatory funnel during the course of half an hour. After the mixture has been stirred for a further two hours, the precipitated benzalazine is filtered with suction, washed with water, and pressed thoroughly on a Büchner funnel. The product is dissolved in boiling ethyl alcohol, and, on cooling, the azine separates in yellow needles. The azine is freed of ethyl alcohol by drying in a vacuum desiccator over calcium chloride. (b) In a modification to the above, hydrazine hydrate (40% aqueous solution) is used in place of hydrazine sulphate in alcohol medium and refluxed till complete conversion on TLC. On cooling, yellow crystals of azine separate out which is filtered and dried.

III. In-vitro biological activity:

Biological activity of the synthesized compounds were checked against stable virulent ATCC microbial cultures. This was done by determination of Minimum Inhibitory Concentration (MIC) using micobroth dilution method. The determined MIC's were correlated with the docking studies to establish the docking model.

RESULTS & DISCUSSION

I. Molecular Docking Studies:

The docking studies were performed on PyRx platform based on AutoDock vina scoring functions for substituted aldazines and ketazines. The binding pose values from the docking studies are as follows:

Compound	Binding pose	M.W
Benzalazine	-7.2	208
p-hydroxybenzalazine	-7.2	240
Salicylaldazine	-7.2	240
p-N,N-dimethylaminobenzalazine	-7.4	294
Vanillinazine	-7.7	300
p-anisaldazine	-7.4	268
p-tolualdazine	-8.1	236
p-chlorobenzalazine	-7.7	277
1-naphthaldazine	-10.2	308
2-naphthaldazine	-9.8	308
2-tolualdazine	-8.2	236
3-tolualdazine	-7.8	236
1-acetylnaphthaleneazine	-11	336
2-acetylnaphthaleneazine	-10.5	336
3-methylacetophenoneazine	-8.7	264
acetophenoneazine	-8.2	236

The results from docking studies indicate the important role of hydrophobic interactions in binding of the ligand to the molecular target. Ligands with non-polar functional groups around the moiety showed larger binding pose values. The above in-silico observation was validated by in-vitro biological studies.

II. Synthesis, purification and characterisation:

The azines were synthesized according to the literature protocols mentioned above. The following azines were synthesized and the experimental observations of M.P & IR spectrum recorded to validate the formation of the desired product. The IR spectrum showed no peaks corresponding to C=O stretch of the aldehyde/ketone but showed a peak corresponding to C=N stretch of the azine. IR spectra of molecules is attached.

Compound	Molecular Formula	M.W	M.P.
Benzalazine	$C_{14}H_{12}N_2$	208	92 °C
p-hydroxybenzalazine	$C_{14}H_{12}N_2O_2$	240	258 °C
Salicylaldazine	$C_{14}H_{12}N_2O_2$	240	210 °C
p-N,N-dimethylaminobenzalazine	$C_{18}H_{24}N_4$	294	253 °C
Vanillinazine	$C_{16}H_{16}N_2O_4$	300	179 °C
p-anisaldazine	$C_{16}H_{16}N_2O_2$	268	167 °C
p-tolualdazine	$C_{16}H_{18}N_2$	236	155 °C
p-chlorobenzalazine	$C_{14}H_{10}N_2Cl_2$	277	152°C
1-naphthaldazine	$C_{22}H_{16}N_2$	308	156 °C
2-naphthaldazine	$C_{22}H_{16}N_2$	308	233 °C
2-tolualdazine	$C_{16}H_{16}N_2$	236	101 °C
3-tolualdazine	$C_{16}H_{18}N_2$	236	72 °C
1-acetylnaphthaleneazine	$C_{24}H_{26}N_2$	336	117 °C
2-acetylnaphthaleneazine	$C_{24}H_{26}N_2$	336	210 °C
3-methylacetophenoneazine	$C_{18}H_{24}N_2$	264	88 °C
acetophenoneazine	$C_{16}H_{16}N_2$	236	121 °C

III. In-vitro biological activity:

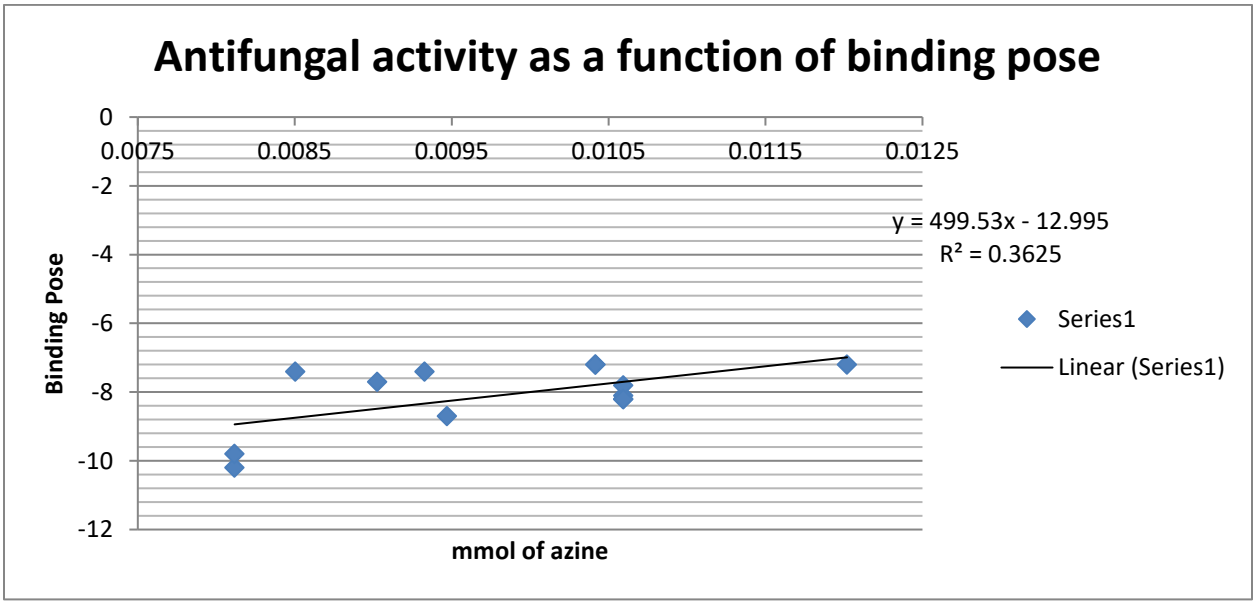
The results from in-vitro antifungal activities are as follows:

Compound		***Candida albicans***		***Candida tropicalis***	
		Range: 2.0 to 10mg/mL	**Range: 0.5 to 4.0mg/mL**	**Range: 2.0 to 10mg/mL**	**Range: 0.5 to 4.0mg/mL**
1A	Benzalazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-7.2	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
2	p-hydroxybenzalazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-7.2	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
3	Salicylaldazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = +	0.5 mg/mL = +
	-7.2	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = +
			2.0 mg/mL = +		2.0 mg/mL = +
			2.5 mg/mL = –		2.5 mg/mL = –
4	p-N,N-dimethylaminobenzalazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-7.4	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
5	Vanillinazine	2.0 mg/mL = –	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-7.7	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = –		1.5 mg/mL = –
			2.0 mg/mL = –		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –

Compound		***Candida albicans***		***Candida tropicalis***	
		Range: 2.0 to 10mg/mL	**Range: 0.5 to 4.0mg/mL**	**Range: 2.0 to 10mg/mL**	**Range: 0.5 to 4.0mg/mL**
7	p-Anisaldazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-7.4	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
8	p-Tolualdazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-8.1	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
9	p-Chlorobenzalazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-7.7	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
14	1-Naphthaldazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-10.2	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
15	2-Naphthaldazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-9.8	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –

Compound		*Candida albicans*		*Candida tropicalis*	
		Range: 2.0 to 10mg/mL	**Range: 0.5 to 4.0mg/mL**	**Range: 2.0 to 10mg/mL**	**Range: 0.5 to 4.0mg/mL**
16	2-Tolualdazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-8.2	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
17	3-Tolualdazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = +	0.5 mg/mL = +
	-7.8	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = +
			2.0 mg/mL = +		2.0 mg/mL = +
			2.5 mg/mL = –		2.5 mg/mL = –
18	1-Acetylnaphthaleneazine	All +	All +	All +	All +
	-11.0				
19	2-Acetylnaphthaleneazine	All +	All +	All +	All +
	-10.5				
20	3-Methylacetophenoneazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = +
	-8.7	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = +
			1.5 mg/mL = +		1.5 mg/mL = –
			2.0 mg/mL = +		2.0 mg/mL = –
			2.5 mg/mL = –		2.5 mg/mL = –
21	Acetophenoneazine	2.0 mg/mL = +	0.5 mg/mL = +	2.0 mg/mL = –	0.5 mg/mL = -
	-8.2	3.0 mg/mL = –	1.0 mg/mL = +	3.0 mg/mL = –	1.0 mg/mL = -
			1.5 mg/mL = +		1.5 mg/mL = -
			2.0 mg/mL = +		2.0 mg/mL = -
			2.5 mg/mL = –		2.5 mg/mL = -

Compound	Binding pose	MIC mg/mL	M.W	MIC (mmol/mL)	LogP[12]	Av. LogP
Benzalazine	-7.2	2.5	208	0.01202	3.24-5.1	4.17
p-hydroxybenzalazine	-7.2	2.5	240	0.01042	3.38-3.85	3.62
Salicylaldazine	-7.2	2.5	240	0.01042	3.37-3.77	3.57
p-N,N-dimethylaminobenzalazine	-7.4	2.5	294	0.00850	3.9-4.11	4.00
Vanillinazine	-7.7	1.5	300		3.53-4.04	3.78
p-anisaldazine	-7.4	2.5	268	0.00933	3.27-5.28	4.28
p-tolualdazine	-8.1	2.5	236	0.01059	3.74-5.67	4.70
p-chlorobenzalazine	-7.7	2.5	277	0.00902	4.23-5.92	5.08
1-naphthaldazine	-10.2	2.5	308	0.00812	4.89-7.2	6.04
2-naphthaldazine	-9.8	2.5	308	0.00812	4.86-7.23	6.04
2-tolualdazine	-8.2	2.5	236	0.01059	3.72-5.65	4.68
3-tolualdazine	-7.8	2.5	236	0.01059	3.73-5.66	4.70
1-acetylnaphthaleneazine	-11	10	336		5.73-6.64	6.18
2-acetylnaphthaleneazine	-10.5	10	336		5.75-6.74	6.24
3-methylacetophenoneazine	-8.7	2.5	264	0.00947	4.37-5.45	4.91
acetophenoneazine	-8.2	2.5	236	0.01059	3.83-4.98	4.40



The roughly linear plot of binding pose values [excluding the biological activity of vanillin azine, acetylnaphthaleneazines] from docking studies with the MIC reinstates the importance of hydrophobic interactions in determining biological activity of the ligands. This validates our docking model.

CONCLUSION & INFERENCES

The proposed docking model was validated within the limitations of a biological system. Hydrophobic interactions are important in binding with the molecular target. This can help in non-random screening of azine ligands for synthesized more potent antifungal agents.

The deviation from the trend was observed for vanillinazine and for acetylnaphthaldazines. This can be attributed to their logP values. Higher the value of logP, lesser will be the diffusion into biological systems. The logP value for more potent analogues according to docking studies are greater than 5 which is beyond the limit according to the Lipinski's rule of 5. Efforts are underway to synthesize salts of these azines by exploiting the basic nitrogen atoms of the azine moiety.

A rational approach can hence be adopted for drug development to combat fungal infections caused by Candida genus for topical application.

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