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In SILICO Computer Simulation Risk Assessment of Triazole Fungicides on Human Cytochrome P450 Aromatase Enzyme: *CYP19A1* Inhibition by Triazoles Using Autodock Software

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Abstract: Inhibitory effect of triazole fungicides were evaluated on the human aromatase enzyme and compared with the Letrozole (LTZ), the most potent inhibitor of aromatase, which is used as anti-estrogen for breast cancer treatment. For this study was used software AUTODOCK to calculate inhibition energy (IE) of triazoles on aromatase enzyme CYP19A1. Those compounds with minimal binding energy are safer in terms of toxicity and resistance of other prescription drugs like non-steroid AIs. In our study we found that four triazole fungicides compounds, Triticonazole, Tebuconazole, Metconazole and Fluquinconazole, exhibited minimal inhibition constant (IC).

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Keywords: in silico, risk assessment, triazoles, pesticide, aromatase inhibitors, CYP19 enzyme

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1. Introduction

Biosynthesis of estrogens from androgens is catalyzed by cytochrome P450 aromatase. Aromatase inhibition by the triazole compounds Letrozole (LTZ) and Anastrozole is a prevalent therapy for estrogen-dependent postmenopausal breast cancer.

Azoles are widely used as agricultural fungicides and antimycotic drugs that target 14α-demethylase. Some were previously shown to inhibit aromatase, thereby raising the possibility of endocrine disruptive effects. However, mechanistic analysis of their inhibition has never been undertaken.

We have evaluated the inhibitory effects of 15 common fungicides in human aromatase enzyme in comparison with the Letrozole (LTZ), the most potent inhibitor of aromatase used as anti-estrogen for breast cancer treatment using AUTODOCK software for calculation of inhibition energy on CYP19 aromatase enzyme. [i]

Triazole containing compounds as systemic fungicides are widely used in agriculture due to its high efficiency, broad spectrum, low toxicity and long effectiveness [ii]. Currently 16 triazole fungicides: bitertanol, cyproconazole, difenoconazole, epoxiconazole, fluquinconazole, flusilazole, flutriafol, hexaconazole, metconazole, myclobutanil, penconazole, propiconazole, tebuconazole, triadimefon, triadimenol and triticonazole, are approved by Swiss Federal Office of Public Health (Zürich, Switzerland). Switzerland no longer allows the use of many chemicals that are still sprayed on American fields (Rosensteil, 2015). By 2005 was set the goal to halve the pesticide pollution of water bodies [iii]. Although, by 2014 report was released that in the five rivers in Switzerland's found heavily polluted in spring and summer by a cocktail of different pesticides [iv].

Target enzymes of triazoles in steroidogenesis are the sterol *14-alfa-demethylase* (encoded by the CYP51 gene) and the *aromatase* (encoded by the CYP19 gene).

The human aromatase enzyme is a member of the cytochrome P450 family and is the product of the *CYP19A1* gene, located on chromosome 15 [v,vi]. Aromatase is the only known vertebrate enzyme that can aromatize a six-membered ring; aromatase is, therefore, the sole source of estrogen in the body [vii].

Nevertheless, since aromatase was first characterized, research has been impeded by the lack of its three dimensional structure. In 2009, Ghosh *et al.* successfully solved the crystallized structure of human aromatase enzyme and provides a structural basis for the specificity to androgen [viii, ix].

The catalytic site of aromatase is located at the juncture of the I and F helices, β-sheet 3, and as the B-C loop. Androstenedione binds into the steroid binding pocket such that its β-face orientates towards the heme group of aromatase, placing C19 within 4.0 Å of the Fe atom. This binding site is only possible if the I-helix backbone is moved 3.5 Å, creating a binding pocket that is approximately 400 $Å^3$. This important distortion is created by residue P308, without which N309, steric hindrance would prevent catalytic activity.

This crystal structure of aromatase will not only allow better structure-based drug design than previous models, but it has also allowed a direct analysis of why some currently available aromatase inhibitors function better than others [x].

As triazole moieties are widely used in fungicides, some studies reported that agricultural triazole pesticides are culprits for the development of resistance to other triazole containing drugs [xi] e.g. triazole aromatase inhibitor antiestrogens. Among them are Anastrozole and Letrozole, the third generation nonsteroidal aromatase inhibitors (AIs), which are now used as first-line therapy in the treatment of breast cancer in postmenopausal women (Scheme 2) [xii, xiii, xiv]. In recent years, some triazole residues have been found in agricultural

products, including fruits, wheat, tea leaves and wine and water [xv,xvi,xvii,xviii,xix]. In one study was concluded that many azole compounds developed as inhibitors of fungal sterol 14-alfa-demethylase are inhibitors also of mammalian sterol 14-alfa-demethylase and mammalian aromatase with unknown potencies [xx].

To avoid the risk of possible development of resistance to other triazole drugs and to reduce toxicity of aromatase inhibitors in the treatment of breast cancer, there are used different methods in order to find out new preventive strategies.

Hypothesis of this study is that common agricultural triazole fungicides may express inhibitory effect on human aromatase enzyme Cyp19A1, in this way contributing to drug resistance and increased risk of cumulative toxicity of anti-estrogen medications.

In our study we performed virtual screening of 15 fungicides and AI reference drug Letrozole to measure inhibitory effect on human aromatase. In this way we aim to range which pesticides are most potent inhibitors of Cyp19A1 enzyme to predict and prevent possible summative cumulating effect of fungicide undesirably overlapping with the activity of anticancer drugs.

The publication of a high resolution X-ray structure of human aromatase has opened the way to a greater understanding of the structural basis for estrogen synthesis and substrate/inhibitor recognition [xxi]. Triazole aromatase inhibitors (AIs) bind to the active site of CYP19 by coordinating the heme iron atom of the enzyme through a heterocyclic nitrogen lone pair.

In our docking study we used together the X-ray structure of human cytochrome P450 aromatase Cyp19A1 (PDB code 3S79, resolution 2.75 Å) [xxii] associated with the metabolism of estrogens and carcinogens with breast cancer, with a collection of commercially available compounds, particularly, 15 triazole fungicides and anticancer drug Letrozole as reference standard (Table 1).

Molecular docking is established method for analysis of molecular associations, which is mostly used in the drug discovery field to study the binding of small molecules (ligands) to macromolecules (receptor) [xxiii].

Cytochrome P450 aromatase homology models were published and used to perform docking and molecular dynamics simulations on known AIs [xxiv, xxv]

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. Materials and methods:

The availability of X-ray structure of human aromatase enables us to set up docking protocol by AutoDock software to identify iron - ligand interactions between heme protein and 16 different triazole ligands, as chemical scaffolds able to inhibit aromatase, thus testing interactions within the aromatase binding site.

Computational ligand docking methodology, AutoDock 4.0, based on Lamarckian genetic algorithm [xxvi] was employed for virtual screening of a compound library with 16 entries including reference compound as Letrozole, the 3rd generation aromatase inhibitors for the treatment of breast cancer, with the enzyme Cytochrome P450 aromatase(Cyp19A1), a potential drug target.

Autodock 4.0 uses GA as a global optimizer combined with energy minimization as a local search method [xxvii].

The macromolecule, Cytochrome P450 aromatase or Cyp19A1 (PDB code 3S79, resolution 2.75 Å) was retrieved by using AutoDock 4 (The Scripps Research Institute, Molecular Graphics Laboratory, 10550 North Torrey Pines Road, CA, 92037) running on

operative system Windows 7 (Miscosoft corporation 2007)

PRODRG was used to draw the 2D structures of different ligands. All the structures were written in protein database (PDB) format. Input molecules files for an AutoDock experiments must confirm to the set of atom types supported by it. Therefore, PDBQT format was used to write ligands, recognized by AutoDock.

Torsional degree of freedom (TORSDOF) is used in calculating the change in the free energy caused by the loss of torsional degree of freedom upon binding. In the AutoDock 4.0 force field, the TORSDOF value for a ligand is the total number of rotatable bonds in the ligand.

The 3D crystal structure of Cytochrome P450-aromatase Cyp19A1 (Picture 1) PDB code 3S79, resolution 2.75 Å was downloaded from Brookhaven Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB; http://www.rcsb.org/pdb).

The nonbonded oxygen atoms of waters, present in the crystal structure were removed. After assigning the bond orders, missing hydrogen atoms were added, then the partial atomic charges was calculated using Gasteiger–Marsili method (Gasteiger). United atom charges were assigned, non-polar hydrogens were merged, and rotatable bonds were assigned, considering all the amide bonds as non-rotatable. The receptor file was converted to PDBQT format, which is PDB plus "q" charges and "t" AutoDock type. (To confirm the AutoDock types, polar hydrogens should be present, whereas non-polar hydrogens and lone pair should be merged, each atom should be assigned Gasteiger partial charges). Amino acids which form target pocket or inhibition cite of aromatase

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Molecular Docking Study:

In the present work, we have studied the in silico binding affinities to the active pocket (Pic. 2.) of enzyme 3S79 (Pic. 1.) to the selected 15 triazole fungicides (Scheme 1.) and the standard anti-aromatase drug Letrozole.

Of the three different search algorithms offered by AutoDock 4.0, the Lamarckian Genetic algorithm (LGA) based on the optimization algorithm was used in favor to other two – simulated annealing and genetic algorithm.

For all dockings, 10 independent runs with step sizes of 0.2Å for translations and 5Å for orientations and torsions were used. AutoDock tools along with AutoDock 4.0 and AutoGrid 4.0 was used to generate both grid and docking parameter files (i.e., gpf and.dpf files) respectively.

A grid box size of 42 x 42 x 42 Å points with a grid spacing of 0.375 Å was generated using AutoGrid [xxviii]. The grid was centered at x,y,z coordinates of 85.51, 52.282, 48.114, which was reported as the binding site residues.

For each docking experiment, the lowest energy docked conformation was selected from 10 runs. The successful completion of docking experiment took from 1 to 4 hours, on a 2.0 GHz Intel (R) core 2 duo machine with 3.0 GB of RAM and Windows 7 operating system.

Prior to actual docking run, AutoGrid 4.0 was introduced to precalculate grid maps of interaction energies of various atom types.

The energy of interaction of this single atom with the protein is assigned to the grid point. An affinity grid is calculated for each type of atoms in the substrate, typically carbon, oxygen, nitrogen, and hydrogens as well as grid of electrostatic potential using a point charge of 1 as the probe. Autodock 4.0 uses these interaction maps to generate ensemble of low energy conformations. It uses a scoring function based on AMBER force field, and estimates the free

energy of binding of a ligand to its target. For each ligand atom types, the interaction energy between the ligand atom and the receptor is calculated for the entire binding site which is discretized through a grid. This has the advantage that interaction energies do not have to be calculated at each step of the docking process but only looked up in the respective grid maps.

Since a grid map represents the interaction energy as a function of the coordinates, their visual inspection may reveal the potential unsaturated hydrogen acceptors or donors or unfavorable overlaps between the ligand and the receptor.

Results:

The binding affinity was evaluated by the binding energies, docking energy, inhibition constant, intermolecular energy, and RMSD values. It was demonstrated that the docking protocol could reliably reproduce the interaction of aromatase with its substrate with an RMSD of 0 Å.

The results of LGA docking experiments of the triazoles using AutoDock 4.0 and AutoGrid 4.0 are summarized in Table 1.

Binding energy for reference compound Letrozole (Fig.3) in our docking study is comply with other studies and is in agreement with them [xxix].

Triazole compounds (Fig 1a,b,c) 1, 3, 10 are chosen as possessing aromatase inhibitory potency based on obtained algorithmic parameters docking: highest binding energy, highest inhibition constant, and hydrogen bonds.

Compound 1 (Bitertanol) exhibits RMS equivalent to zero in 7th orientation to heme molecule of protein, and at this position binding energy is - 6,19; IC – 2,9X10-5; and two hydrogen bonds with amino acids of target pocket ASP371 and LEU372.

Compound 3 (Difenoconazole) demonstrated best compliance of inhibitory bound in 7th orientation to RMS=0 posing heme molecule, binding energy -7,36, IC – 4,03X10-6, and one hydrogen bond with target pocket amino acid THR310.

Compound 10 (Penconazole) exhibits its highest binding energy -7.71 at orientation 6th to heme molecule parallel alignment at RMS zero point, IC – 2.22X10-6 and one H-BOND with THR310.

In our study we found that four triazole fungicides compounds 15, 12, 8, 5 exhibited minimal inhibition constant (IC). Those are Triticonazole, Tebuconazole, Metconazole and Fluquinconazole. (Fig. 4 a,b,c,d).

Compound 15 exhibits its binding energy -21.65 at orientation 2nd to heme molecule parallel alignment at RMS zero point, IC – 1.35X10-016 and no H-BOND.

Compound 12 exhibits its binding energy -21.09 at orientation 7th to heme molecule parallel alignment at RMS zero point, IC – 3.5X 10-016 and no H-BOND.

Compound 8 exhibits its binding energy -19.69 at orientation 5th to heme molecule parallel alignment at RMS zero point, IC – 3.68X10-015 and no H-BOND.

Compund 5 exhibits its binding energy -17.25 at orientation 9th to heme molecule parallel alignment at RMS zero point, IC 2.29 X10-013 and no H-BOND.

Conclusions:

Those compounds with minimal binding energy are safer in terms of toxicity and resistance of other prescription drugs like non-steroid AIs. Those with higher binding energies may cause drug resistance or toxicity in cases of simultaneous administration and it should be taken cautiously during treatment with other triazole containing drugs like Letrozole.

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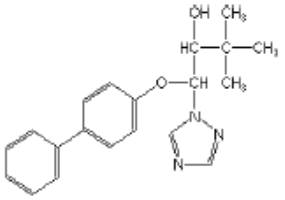
Table 1. Results of docking of 15 fungicides and one non-steroid Aromatase inhibitor (NSAI) Letrosole.

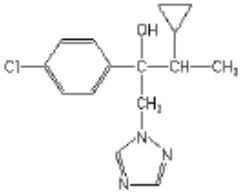
SL. NO.	MOLECULE	ORIENTATION	BINDING ENERGY Kcal/mole	DOCKING ENERGY Kcal/mole	INHIBITATION CONSTANT (Ki) (nM)	INTERMOL ENERGY	TORSIONAL ENERGY	INTERNAL ENERGY	RMSD	HYDROGEN BOND
1.	Bitertanol	7th	-6.19	-5.09	2.9 e-005	-8.06	1.87	2.97	0.0	2 H-BONDS WITH **ASP371, LEU372**
2.	Cyproconazole	10th	-9.25	-8.73	1.66 e-007	-10.81	1.56	2.08	0.0	1 H-BOND WITH **MET374**
3.	Difenoconazole	7th	-7.26	-6.83	4.03 e-006	-8.92	1.56	2.09	0.0	1 H-BOND WITH **THR310**
4.	Epoxiconazole	8th	-10.83	-12.58	1.04 e-008	-12.14	1.25	-0.44	0.0	1 H-BOND WITH **MET374**
5.	**Fluquinconazole**	9th	-17.25	-17.83	**2.29 e-013**	-17.87	0.62	0.04	0.0	
6.	Flutriafol	2nd	-8.7	-7.94	4.18 e-007	-9.95	1.25	2.01	0.0	
7.	Hexaconazole	7th	-10.72	-11.52	1.38 e-008	-12.59	1.87	1.07	0.0	1 H-BOND WITH **MET374**
8.	**Metconazole**	5th	-19.69	-19.92	**3.68 e-015**	-20.95	1.25	1.01	0.0	
9.	Myclobutanil	1st	-8.5	-8.1	5.92 e-007	-10.36	1.87	2.27	0.0	1 H-BOND WITH THR310
10.	Penconazole	6th	-7.71	-9.07	2.22 e-006	-9.27	1.56	0.2	0.0	1 H-BOND WITH THR310
11.	Propiconazole	3rd	-8.64	-10.17	4.64 e-007	-10.2	1.56	0.03	0.0	
12.	**Tebuconazole**	7th	-21.09	-21.91	**3.5 e-016**	-22.95	1.87	1.04	0.0	
13.	Triadimefon	4th	-8.46	-9.97	6.29 e-007	-10.02	1.56	0.04	0.0	
14.	Triadimenol	9th	-10.59	-11.37	1.72 e-008	-12.15	1.56	0.78	0.0	
15.	**Triticonazole**	2nd	-21.65	-21.96	**1.35 e-016**	-22.89	1.25	0.94	0.0	
16	**Letrozole**	7^{th}	-9.54	-8.77	**1.01 e-007**	-10.48	0.93	1.71	0.0	

Scheme 1. Downloaded from free the public domain http://www.alanwood.net/pesticides.

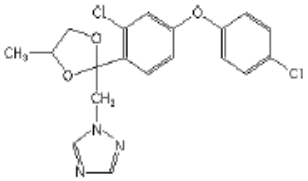
1-Bitertanol



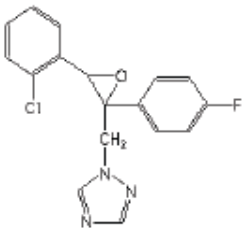
2-Cyproconazole



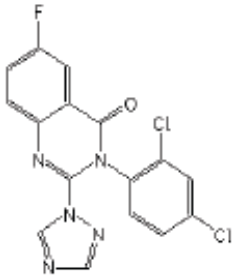
3-Difenoconazole



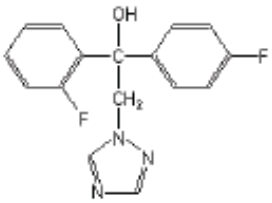
4-Epoxiconazole



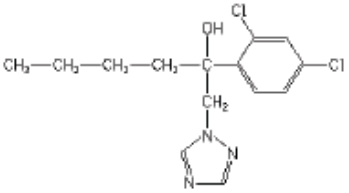
5-Fluquinconazole



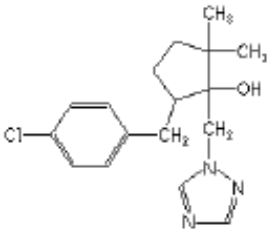
6-Flutriafol



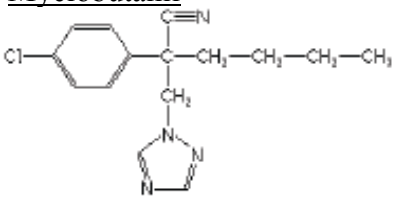
7-Hexaconazole



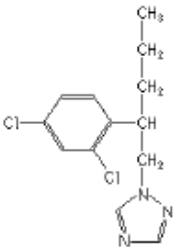
8-Metconazole



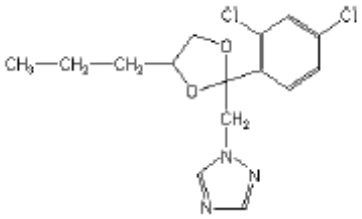
9-Myclobutanil



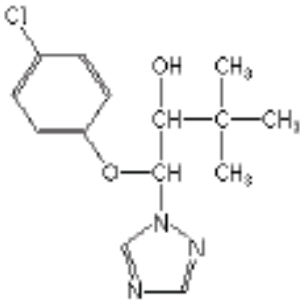
10-Penconazole



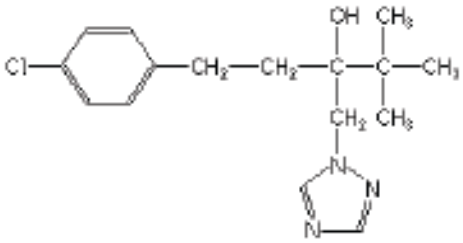
11-Propiconazole



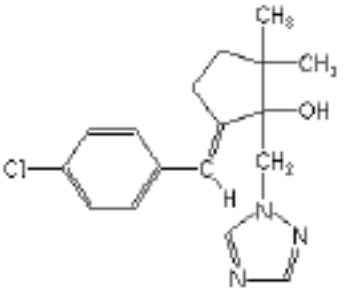
14-Triadimenol



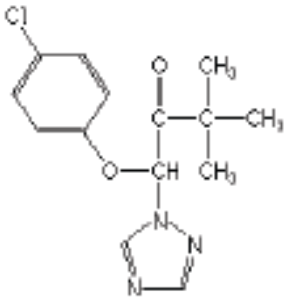
12-Tebuconazole



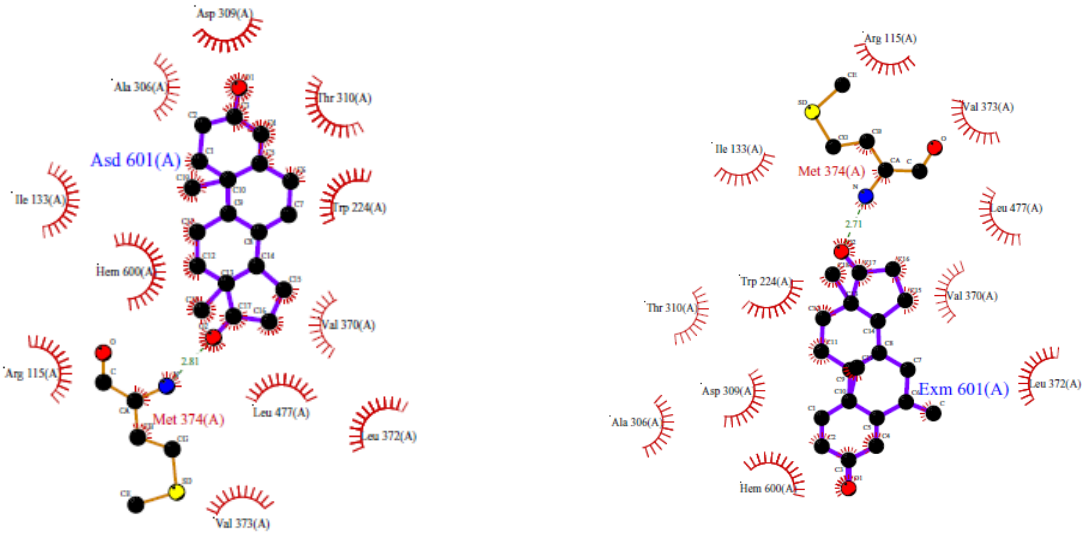
15-Triticonazole



13-Triadimefon



Pic. 1. Human placental aromatase cytochrome P450 aromatase (CYP19A1) refined at 2.75 angstrom 3S79 (ribbon model). [source: http://www.rcsb.org/pdb/explore/jmol.do?structureId=3S79].



Pic 2. Target pocket surrounding Heme-molecule of aromatase *CYP19A1*.

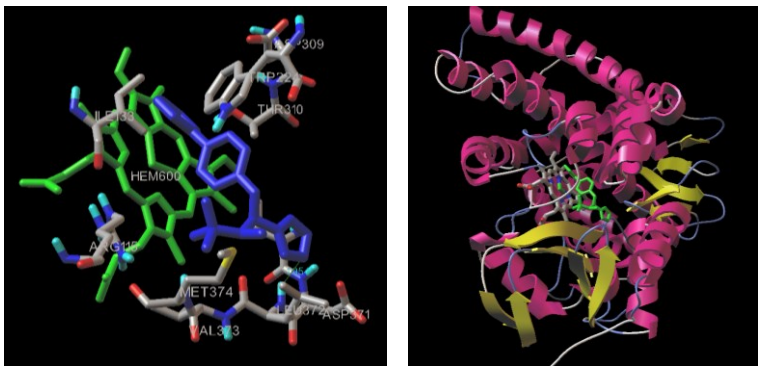


Fig. 2a: Compound 1 docked within the binding pocket of the enzyme 3S79. Predicted binding mode of compound 1 (). On the left, stick and ball model, and on the right, ribbon model of enzyme 3S79 in the binding pocket of which compound 1 is forming hydrogen bond with the amino acids **ASP371** and **LEU372**.

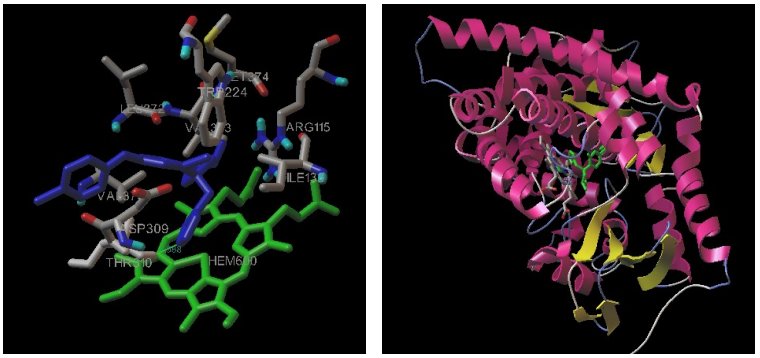


Fig. 2b: Predicted binding mode of compound 3. On the left, stick and ball model, and on the right, ribbon model of enzyme 3S79 in the binding pocket of which compound 3 is forming hydrogen bond with the amino acid **THR310**.

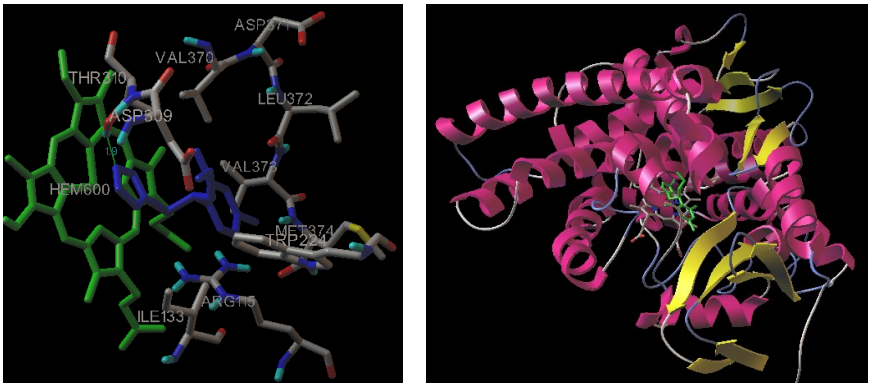


Fig. 2c: Predicted binding mode of compound 10. On the left, stick and ball model, and on the right, ribbon model of enzyme 3S79 in the binding pocket of which compound 3 is forming hydrogen bond with the amino acid **THR310**.

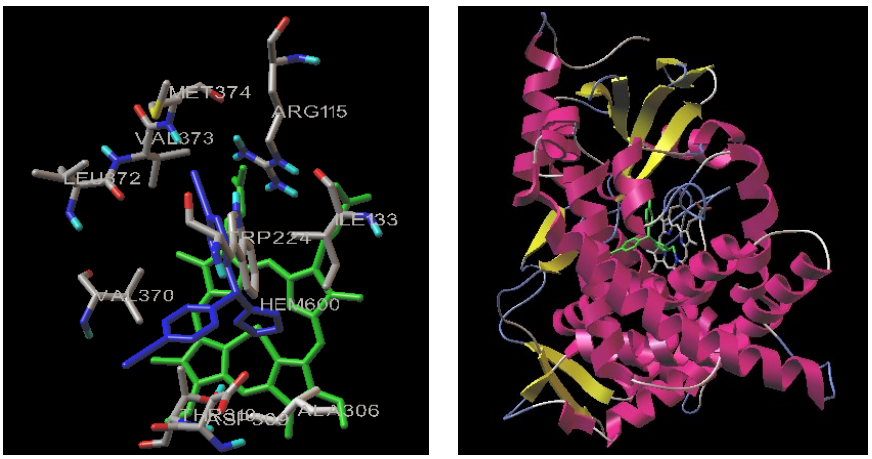
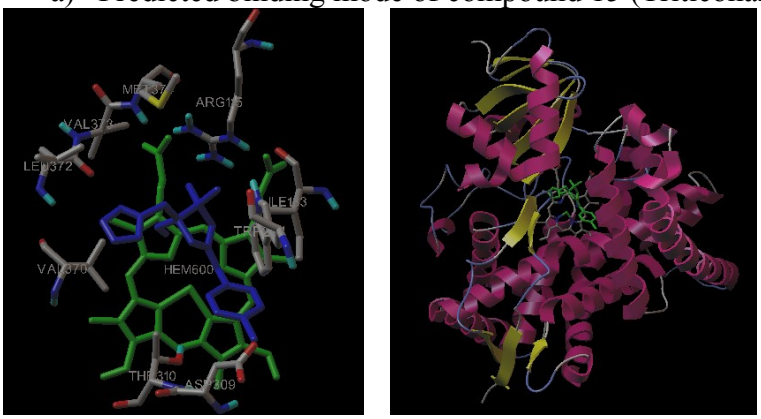
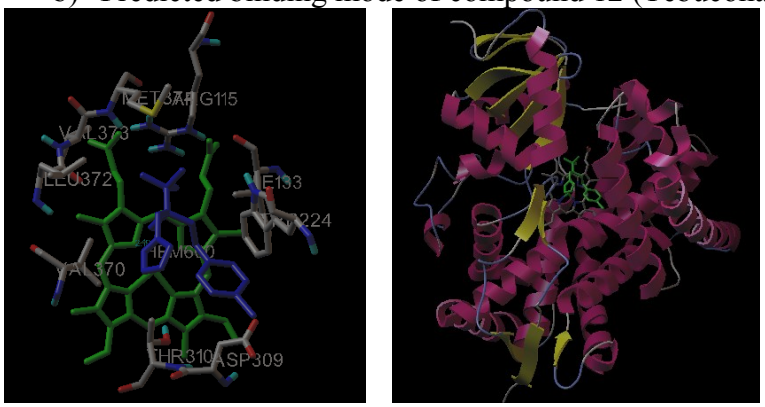


Fig. 3: Reference compound, Letrozole docked in the binding pocket of the enzyme 3S79. (Ball and stick model and ribbon model).

a) Predicted binding mode of compound 15 (Triticonazole).



b) Predicted binding mode of compound 12 (Tebuconazole)



c) Predicted binding mode of compound 8 (Metconazole)

d) Predicted binding mode of compound 5 (Fluquinconazole)

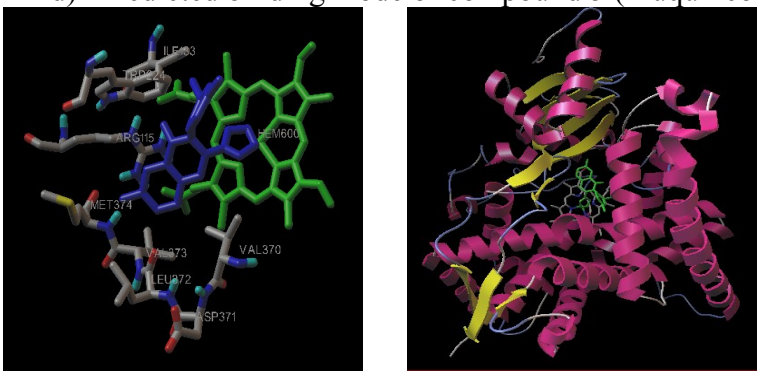


Fig. 4. Molecular surface view of compounds **15, 12, 8, 5** docked within the binding pocket of the enzyme 3S79 without H-bonds.

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