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In silico identification of novel inhibitors against *Plasmodium falciparum* dihydroorate dehydrogenase

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ABSTRACT

Plasmodium falciparum causes the most fatal form of malaria and accounts for over 1 million deaths annually, yet currently used drug therapies are compromised by resistance. The malaria parasite cannot salvage pyrimidines and relies on *de novo* biosynthesis for survival. The enzyme dihydrooratate dehydrogenase (DHODH), a mitochondrial flavoenzyme, catalyzes the rate-limiting step of this pathway and is therefore an attractive anti-malarial chemotherapeutic target. In an effort to design new and potential anti-malarials, structure-based pharmacophore mapping, molecular docking, binding energy calculations and binding affinity predictions were employed in a virtual screening strategy to design new and potent *P. falciparum* dihydrooratate dehydrogenase (*PfDHODH*) inhibitors. A structure-based pharmacophore model was generated which consist of important interactions as observed in co-crystal of *PfDHODH* enzyme. The developed model was used to retrieve molecules from ChemBridge database, a freely available commercial database. A total of 87 molecules mapped on the modeled pharmacophore from the database. The retrieved hits were further screened by docking simulation, binding energy calculations and biding affinity predictions using genetic optimization for ligand docking (GOLD) and MOE. Based on these results, finally 26 chemo-types molecules were predicted as new, potential and structurally diverse *PfDHODH* inhibitors.

1. Background

Malaria remains one of the prevalent infectious diseases worldwide. Malaria infects about 900 million people and causes deaths of as many as 2.7 million per year [1,2]. Although, some progress has been made in vaccine developing, only partial immunity is provided by one of the best candidate RTSS [3]. Thus for the treatment and prevention anti-malarial control programs focus on chemotherapy. In recent decades prevalent drug resistance has been encountered for chloroquine, as well as for nearly every other available anti-malarial drug that strictly limited the effectiveness of many of these drugs [4]. Moreover, it has been shown that drug resistance may be emerging at faster rates in some regions of the world [5]. Chemotherapy of multi-drug combinations offer temporary relief [6,7] but given present trends, it is clear that the disease will continue to have an intolerable impact on global health unless novel and potent drugs are developed. So, there is an urgent need for novel and potent anti-malarial drugs.

Pyrimidines are essential components, required for biosynthesis of DNA and RNA as well as for the biosynthesis of phospholipids and glycoproteins. Pyrimidine metabolism has proven to be a vulnerable element in the malarial parasite's biology and is the target of a large number of the clinically effective therapies, together with pyrimethamine and other dihydrofolate reductase inhibitors, and atovaquone, an inhibitor of the bc1 complex [8,9].

The malaria parasite relies entirely on *de novo* pyrimidine biosynthesis to obtain pyrimidine nucleosides, and unlike the mammalian host, it is unable to salvage preformed pyrimidine bases or nucleosides [10] to supply precursors for the biosynthesis of DNA and RNA [11,12]. On the contrary, the human host cells have the enzymatic machinery for both *de novo* pyrimidine biosynthesis and for salvage of preformed pyrimidine bases and nucleosides. The lack of a redundant machinery to obtain pyrimidines in malaria has raised interest in this pathway as a potential source for new therapeutic targets. Dihydroorotate dehydrogenase (*DHODH*) is a flavin mononucleotide (FMN)-dependent present in mitochondria that catalyzes the oxidation of dihydroorotate (DHO) to produce orotate, the fourth step in the *de novo* biosynthesis of pyrimidine [13,14]. Coenzyme Q (CoQ) is the essential component to catalyze the reoxidation of the flavin cofactor, and recent genetic studies suggest that the main function of mitochondrial electron transport in the parasite is to provide CoQ for this reaction [15]. These studies present genetic evidence that *PfDHODH* is an important enzyme to the malaria parasite. These and other studies have proved *PfDHODH*

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as the best validated and new target for the development of novel anti-malarial agents [16–18].

From different species the enzyme *DHODH* can be divided into two classes, those that are localized in cytoplasm and required fumerate or nicotinamide adenine dinucleotide (NADH) for the oxidation of FMN cofactor (class 1) or those that are mitochondrial utilize ubiquinone (coenzyme Q; CoQ) as the final oxidant (class 2). Both human and malarial *DHODHs* belong to class 2 mitochondrial enzymes. Several different series of chemical that are reported as potent inhibitors of *PfDHODH*, were subsequently shown to have anti-malarial activity both *in vitro* and *in vivo* [19–23].

To aid in the development of lead compounds, several groups have reported the X-ray crystallographic structure of *DHODH* in complex with a number of potent inhibitors [24–27]. With the aim to develop novel and potent *PfDHODH* inhibitors, we employed a structure-based virtual screening approach consisting in the application of different sequential filters based on developed pharmacophoric models, calculations of drug-like property, and molecular docking simulations. A pharmacophoric model was generated via structure-based approach, mapping the interactions between ligands and the target protein. The developed pharmacophore was used as a three-dimensional query to screen a commercially available compounds database to design putative hits. The overall work flow of the study is described in Scheme S1 in the supplementary data.

2. Materials and methods

2.1. Generation of structure-based pharmacophore model

In the present study the *PfDHODH*-inhibitor complexed X-ray crystallographic structure (PDB i.d. code 308A, [28] was used as starting structure for the prediction of structure-based pharmacophore models. LigandScout (LS) [29] was utilized for the automatic construction and visualization of 3D pharmacophores from complex structure of *PfDHODH*. LS algorithm described chemical features include hydrogen bond donors and acceptors as directed vectors and positive and negative ionizable areas as well as lipophilic regions characterized by spheres. Furthermore, for increased selectivity, the generated model includes spatial information regarding regions inaccessible to any putative ligand as a result reflecting possible steric restrictions. Especially, excluded volume spheres placed in regions that are sterically hindered are automatically added to the developed pharmacophore model.

Like our previous study [30], first MOE-compatible 3D pharmacophore model was generated by LS with default parameters and then was exported and converted into an MOE, pharmacophore query for virtual screening (www.chemcomp.com). As feature interpretation slightly differs between the two programs so, it was necessary to make a number of adjustments before screening. Prior to virtual screening, necessary adjustments were made, as feature interpretation slightly differs between the two programs. Those aromatic rings which classified simply as hydrophobic groups by LS were classified as either aromatic or hydrophobic in MOE, employing the PPCH_All scheme (which incorporates directionality of hydrogen bond donors and acceptors, and orientation of aromatic rings). Since in LS pharmacophore the aromatic ring is not directly classified per se (due to lack of detection of *p*–*p* stacking or cation–*p* interactions) but rather as a set of hydrophobic atoms, can be interpreted in MOE in a way which is useful in the prediction of correct compounds in virtual screening.

2.2. Pharmacophore-based virtual screening

The ChemBridge database (www.chembridge.com), which allows the downloading of chemicals structures from a range of vendors as SDF files, was used in this preliminary virtual screen. First the database was washed and the 3D structure of each compound was constructed using the MMFF94x force field using MOE. Then the low energy conformers for each compound were generated using Conformation Import methodology implemented in MOE. After evaluating the pharmacophore query, virtual screening was performed by using MOE against ChemBridge database. As, some changes may occur when the pharmacophore is exported from LS to MOE environment, therefore, the pharmacophore query was validated prior to using for virtual screening. To reduce the data of identified compounds, they were docked into binding pocket of *PfDHODH* and visual inspection, binding energies and binding affinities calculations were carried out by MOE to prioritize the final hits.

3. Molecular docking

Docking simulations were performed using GOLD (genetic optimization for ligand docking) from Cambridge Crystallographic Data Center, UK [31] To investigate the full range conformational flexibility of ligand with partial flexibility of the receptor, GOLD uses genetic algorithm for docking flexible small molecules (ligands) into binding site of macromolecules (target proteins). The ligand binding energy with target protein was predicted via GOLD score implemented in GOLD. The total GOLD score, which is represented as “Fitness”, was calculated from the contribution of hydrogen bonds and van der Waals interactions between target protein and ligand as well as the contribution of intra-molecular hydrogen bonds and intra-molecular strain in the ligand. Receptor coordinates of the crystal structure of *PfDHODH*-inhibitor complex [28] was used to define the binding site for docking simulations. All the solvent molecules were removed from the crystal structure and hydrogen atoms were added to the whole protein. The ligand binding site for docking simulation was defined as a collection of amino acids enclosed within a sphere of 10 Å radius around the coordinates of the ligand, which is the inhibitor molecule present in the binding pocket of *PfDHODH*-inhibitor complex. Top 10 docked conformations were allowed to be saved with the early termination option of quitting the genetic optimization calculation for a ligand if the RMSD between any five conformations of the particular ligand is less than 1.5 Å. All other parameters were remained at their default values. Molecular interactions were observed via Ligplot software implemented in MOE.

3.1. Prediction of generalized Born interaction energies and binding affinity

With the aim to identify the most promising hits, the molecular mechanics generalized Born interaction energies and binding affinities of the hits and *PfDHODH* binding pocket were calculated with generalized Born/volume integral (GB/VI) implicit solvent method [32] implemented in MOE. The molecular mechanics generalized Born interaction energy is the non-bonded interaction energy between the receptor protein and the ligand and comprises van der Waals, Coulomb and generalized Born implicit solvent interaction energies; however, ligand and receptor protein strain energies are not taken into account. Solvent molecules are ignored in the calculation. The estimated binding affinity is that of the London dG scoring function reported in units of pki. During calculation the receptor atoms far from the ligand are held fixed (constrained not to move) while receptor atoms in the vicinity of the ligand

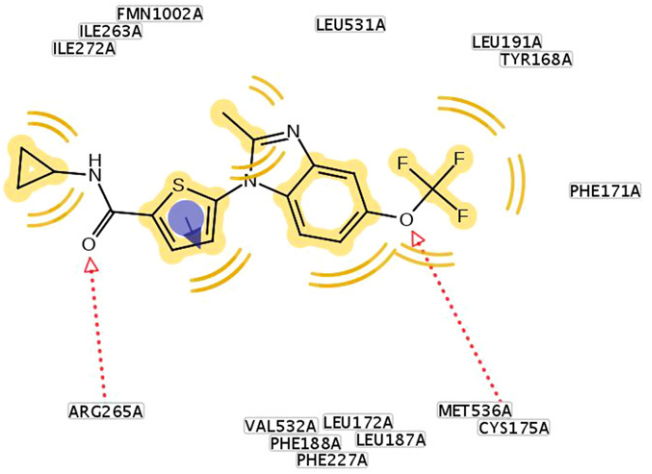


Fig. 1. Two-dimensional pharmacophore model generated by LigandScout from the complex structure of PfDHODH and its ligand. The dotted arrows indicated the hydrogen bond acceptor features, and the yellow sphere represented the hydrophobic feature in the ligand. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(in the binding pocket) are allowed to move but are subject to tether restraints that discourage gross movement. All the calculations were carried out via MOE. The ligand atoms can be configured as either free to be move or subject to similar tethers as the receptor protein. In each case an energy minimization of binding pocket in *PfDHODH*–ligand complex was performed before calculating binding affinity. Ligand and the residues within 10 Å were set to be flexible. After energy minimization, the binding affinity was calculated for each hit and reported in units of pki.

4. Results and discussion

4.1. Structure-based pharmacophore modeling

The pharmacophore model automatically generated by the program LS [30] includes seven features: two hydrogen bond acceptors (HBA) and five hydrophobic groups (Fig. 1). Besides, the program automatically generated several excluded volumes in the pharmacophore model. The HBA features point toward the carbonyl oxygen and ether oxygen of the ligand from the Arg^{265A}, Cys^{175A} and Met^{536A} respectively. The hydrophobic groups are located on the cyclophenyl, thiophene, methyl and trifluoro methoxy moieties of the ligand in the complex structure. The generated pharmacophore model was exported into MOE. Prior to screening, it was necessary to make several adjustments, as feature interpretation varies slightly between the two programs. As in LS pharmacophore model the aromatic ring of the ligand in the complex was not classified as aromatic or hydrophobic features, thus these were interpreted in MOE, by the PPCH_All scheme. Two modifications were made on this model to obtain appropriate model for virtual screening. These modifications were about the phenyl and thiophene rings. It is clear that both are aromatic groups, but the LS could not interpret these rings as aromatic groups automatically. In MOE additional features were developed via MOE pharmacophore query editor. Aromatic features were developed on the phenyl and thiophene rings of the ligand in the complex. The modified pharmacophore model was then validated by screening the test database. In the test database we kept the ligand (reference ligand) present in complex structure. First, the ligand was extracted then hydrogen atoms were added and energy minimized via MOE. The minimized structure of ligand was added to the test database and named as reference compound. After screening, the reference compound was correctly mapped by the modified pharmacophore model (Fig. 2). The result verified the validity of our modified pharmacophore model that can be used for the large databases screening.

4.2. Pharmacophore-based virtual screening

The modified and validated pharmacophore model was then used as *in silico* filter for the screening of ChemBridge database (www.chembridge.com). The ChemBridge database compounds in SDF format were imported into MOE environment where the 3D structure of each compound was modeled using MMFF94x force field. The Conformation Import methodology was applied to

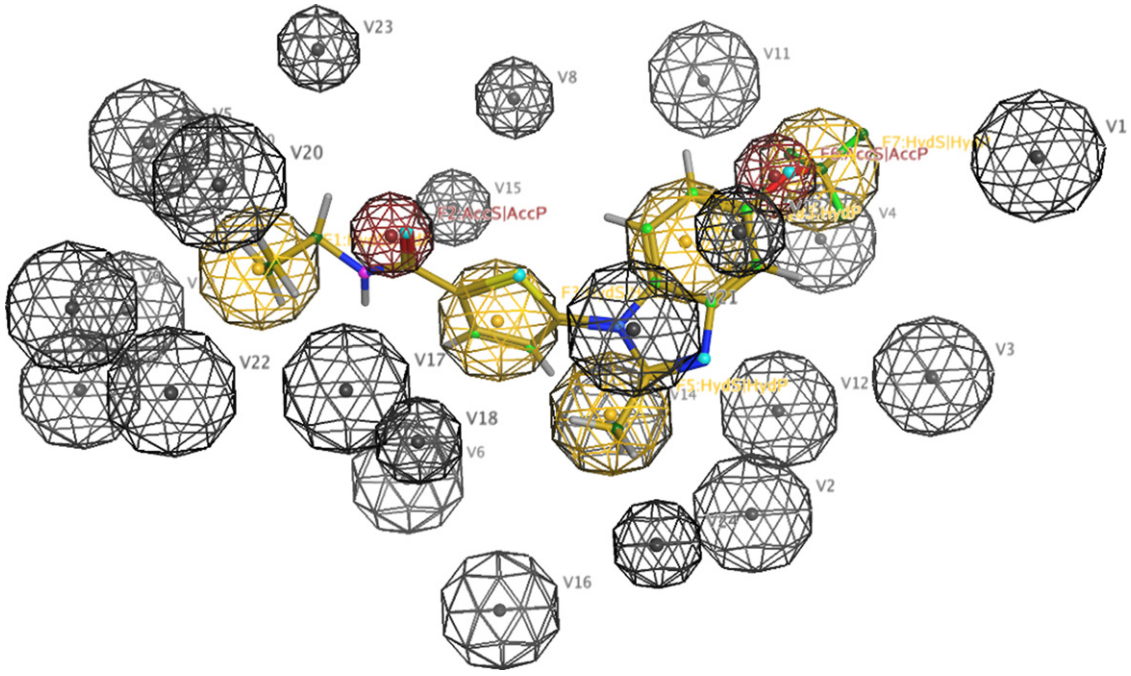


Fig. 2. The fitting of indomethacin on modified three-dimensional pharmacophore model developed by using MOE pharmacophore query editor.

Table 1
GOLD fitness scores, binding energies, and binding affinities of most promising lead compounds. See Section 2 for details.

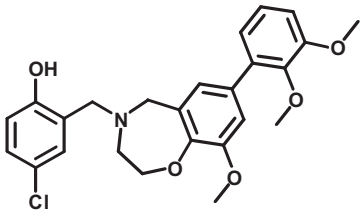
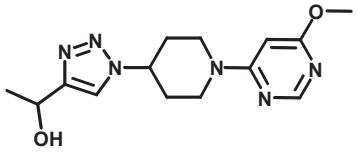
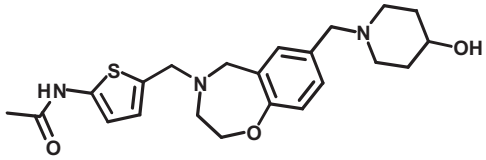
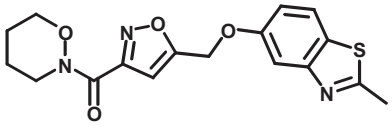
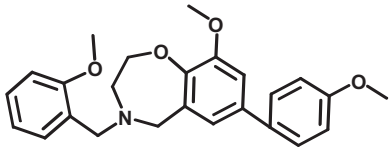
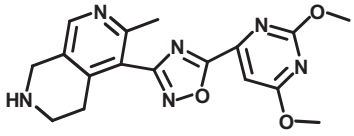
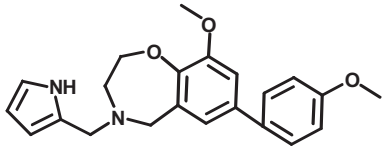
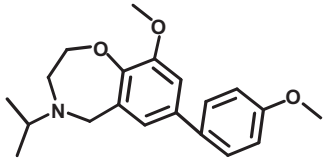
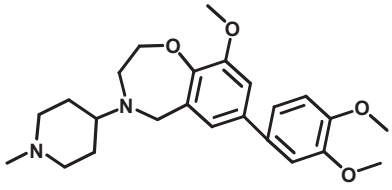
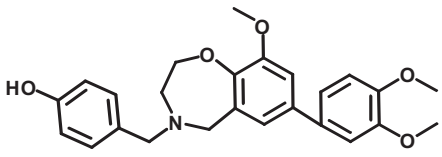
S.no	Compound name	Compound structure	Goldscore	Binding energy (MM/GBV kcal/mol)	Binding affinity (pki)
1	11562591		45.38	−34.54	−9.02
2	12738598		51.19	−25.66	−8.29
3	14132608		50.61	−23.69	−8.74
4	15581393		67.89	−36.41	−10.28
5	18888880		63.30	−23.09	−9.84
6	19339588		55.68	−32.44	−8.69
7	19612301		62.03	−36.44	−11.12
8	19746677		52.21	−24.71	−9.68
9	22630620		42.78	−31.47	−10.58
10	33229335		47.55	−22.10	−8.24

Table 1 (*Continued*)

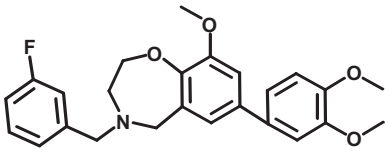
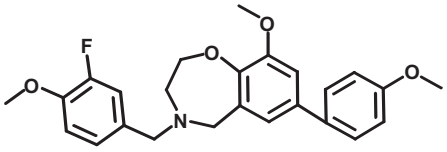
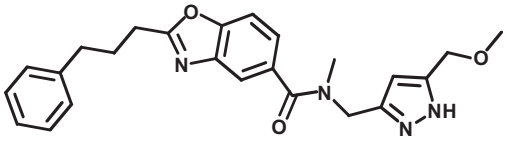
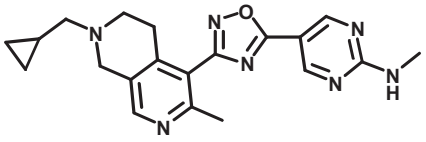
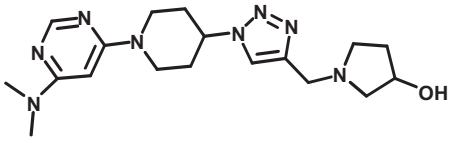
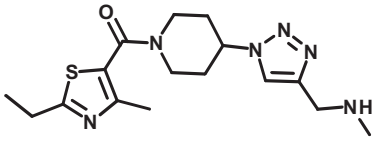
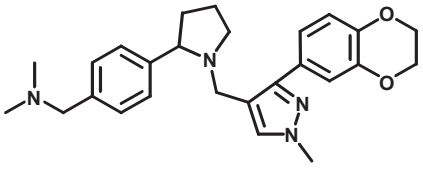
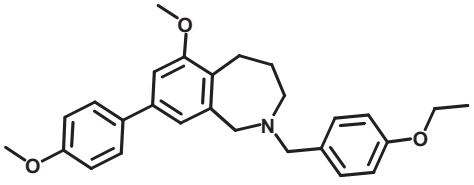
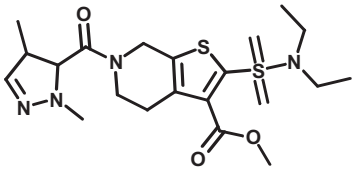
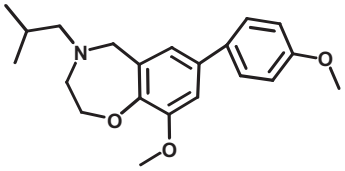
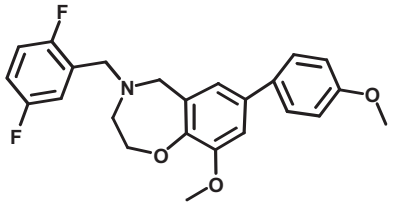
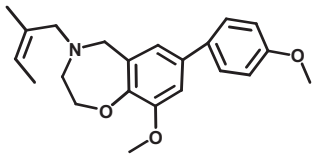
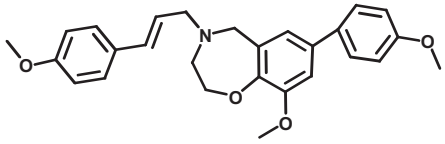
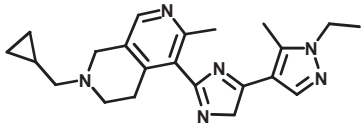
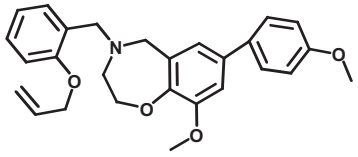
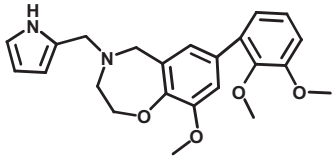
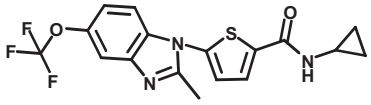
S.no	Compound name	Compound structure	Goldscore	Binding energy (MM/GBV kcal/mol)	Binding affinity (pki)
11	37875399		59.82	−23.72	−8.84
12	48148809		49.08	−37.48	−12.28
13	57267754		69.11	−34.87	−9.95
14	63723187		58.56	−35.40	−13.32
15	66182182		52.19	−37.28	−8.84
16	69738860		54.05	−24.00	−8.30
17	80730650		44.45	−22.58	−10.14
18	81199847		59.16	−33.02	−11.82
19	81412635		62.10	−23.01	−11.73
20	86879915		57.89	−28.27	−9.16

Table 1 (*Continued*)

S.no	Compound name	Compound structure	Goldscore	Binding energy (MM/GBV kcal/mol)	Binding affinity (pki)
21	89927156		69.27	−38.23	−10.71
22	93299057		65.64	−30.29	−8.34
23	94431640		64.50	−33.22	−12.39
24	94775618		61.81	−35.34	−11.13
25	95746526		71.31	−28.73	−9.40
26	99527022		53.00	−23.12	−8.68
	Reference		68.98	−22.73	−8.06

develop low-energy conformations for each compound. All these compounds and their respective conformations were saved in separate MOE database. The conformers of each compound were then screened by the pharmacophore model. To be considered as hit, the compound has to fit all the pharmacophore features. From the pharmacophore-based virtual screening 87 hits were identified that mapped on the developed pharmacophore model (i.e., having the specified requirements). These initially identified hits were selected for further evaluation using molecular docking.

5. Molecular docking

To deduce the promising anti-malarial compounds, all the initial hits were docked into the binding pocket of *PfDHODH* using the automated GOLD docking program. Prior to dock the initial hits, the ligand from the complex structure was extracted and re-docked into the binding pocket to validate the docking protocol. The docked pose corresponding to the high GOLD fitness score was selected as the most promising binding pose. The root mean square deviation (RMSD) between the predicted and experimentally determined conformation was calculated by using SVL script of MOE and found to be equal to 0.55 Å, suggesting that a high docking reliability of GOLD in reproducing the experimentally determined binding mode for *PfDHODH*. The GOLD docking software and the parameters set could be extended to search the *PfDHODH* binding conformations for other compounds accordingly. Using the same docking protocol all the initial hits were docked into the binding pocket of *PfDHODH*. For the top ranked pose of all the compounds, visual inspection was carried out by Ligplot implemented in MOE and those compounds which revealed significant interactions with important residues of binding pocket (Cys^{175A} His^{185A} and Arg^{265A}) of PfDHODH were picked as promising hits. Among the 87 compounds, 56 showed the crucial interaction with the enzyme via visual inspection.

5.1. Computation of binding affinity and expert visual inspection

Finally, binding energy and binding affinity for all the 56 compounds including ligand of the complexed structure were calculated using generalized Born/volume integral (GB/VI) implanted on MOE to prioritize identified promising hits. The criteria for

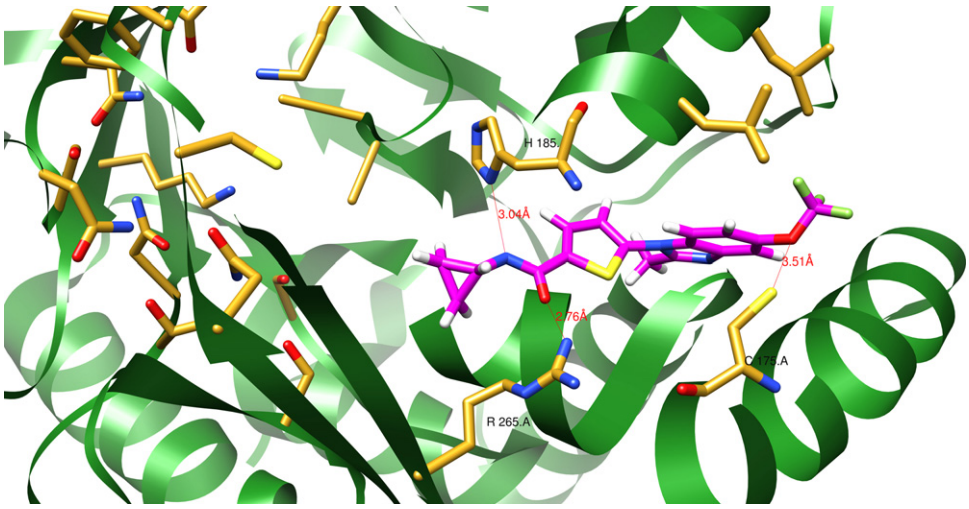


Fig. 3. Binding mode of the reference compound in the active site of *PfDHODH* enzyme.

the selection of the most promising hits were, compounds having binding energy and binding affinity equal to or good as compare to the binding energy and binding affinity calculated for ligand (reference ligand) in the complexed structure, visualization of each compound in the binding pocket and the selection of only those compounds exhibiting interactions with important residues in binding pocket of *PfDHODH*, having hydrophobic moieties to interact with hydrophobic region of the binding pocket and diversity of the compounds. Applying the above mentioned criteria 26 compounds out of 56 fulfill the specific requirements (Table 1). The pharmacophore mapping, visual, binding mode, binding energy and binding affinity prediction showed that these predicted lead hits might be act as potential lead compounds for the development of novel, potent and structurally diverse inhibitors of *PfDHODH* enzyme.

5.2. Binding interactions of finally selected compounds

From the docking studies it was observed that almost all finally selected lead hits showed similar binding mode as that of reference ligand into the binding pocket of *PfDHODH* enzyme. For example, compound **4** for which the strong binding affinity ($-10.28\,pk_i$), lower binding energy (−36.4) and high Gold fitness score (67.89) were observed, showed the binding interaction similar to that of reference ligand (Figs. 3 and 4). From the top-ranked docked conformation, it was also predicted that the nitrogen atom of isoxazole ring and carbonyl oxygen of the compound **4** established interaction with the amino acid residues His^{185A} and Arg^{265A} in a similar manner of reference compound to the same residues. Moreover, compound **4** also established hydrogen bonding to Lys^{229A}. The top-ranked docking pose of compound **2** showed that nitrogen atom of triazole ring, ether and hydroxyl oxygen atoms of the compound formed hydrogen bonds to His^{185A}, Lys^{229A} and Arg^{265A} respectively as shown in supplementary data (Fig. S1). In case of compound **6** the docking results showed (supplementary data Fig. S2) that the nitrogen atom of pyrimidine ring, ether oxygen and nitrogen atom of naphthyridine ring of the compound established hydrogen bonds to His^{185A}, Arg^{265A} and Ser^{447A} respectively. From the docking conformation of compound **24** it was observed that nitrogen atom of oxadiazole, naphthyridine and pyrazole ring form hydrogen bonds to His^{185A}, Arg^{265A} and Cys^{276A} respectively (supplementary data Fig. S3). Beside hydrogen boding these compounds also showed hydrophobic interactions to various residues in the active site of the enzyme. From the docking conformations of the selected compounds, it was observed that there are some specific functional groups that interact to the important residues and fit well in the binding pocket of *PfDHODH* enzyme.

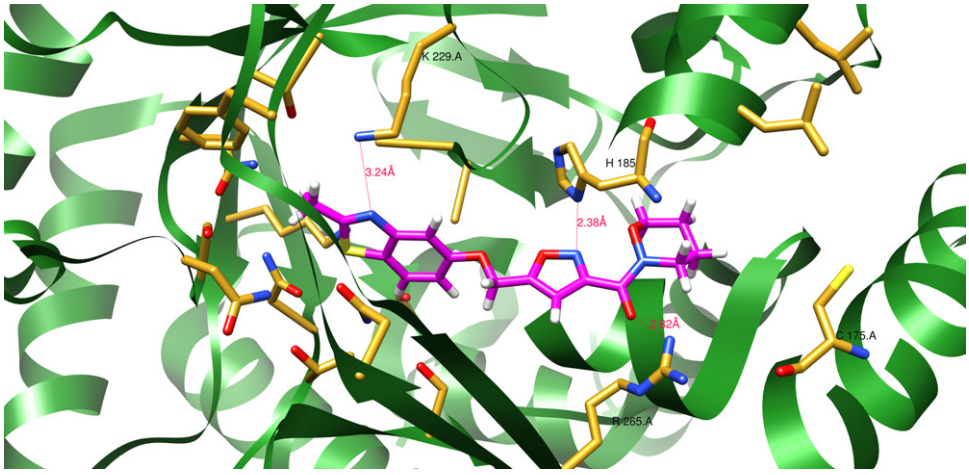


Fig. 4. Binding mode of compound **4** in the active site of *PfDHODH* enzyme.

6. Conclusions

The aim of the present study was to generate a structure-based pharmacophore model to identify structurally diverse lead hits. The identified hits might be used for developing novel and potent inhibitors for *PfDHODH* enzyme. A structure-based pharmacophore model was developed based on the complex structure of *PfDHODH* and ligand present in the complex. The developed pharmacophore model was used for the virtual screening of ChemBridge database. The identified hits were further evaluated by docking simulation, Visual inspection, binding energy calculation and binding affinity prediction. As a result 26 lead hits were reported that fulfilled all the criteria for the design of compounds that might be act as potential leads for development of novel, potent and structurally diverse compounds for inhibition of *PfDHODH* enzyme. From the docking predicted binding mode, it was observed that there are some specific groups that mimics the binding mode of reference ligand and fit well into binding pocket of *PfDHODH* enzyme. Further studies toward the synthesis and structure-activity relationship (SAR) of the above mentioned lead compounds with *PfDHODH* enzyme are in progress and will be reported elsewhere.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmgm.2012.11.010.

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