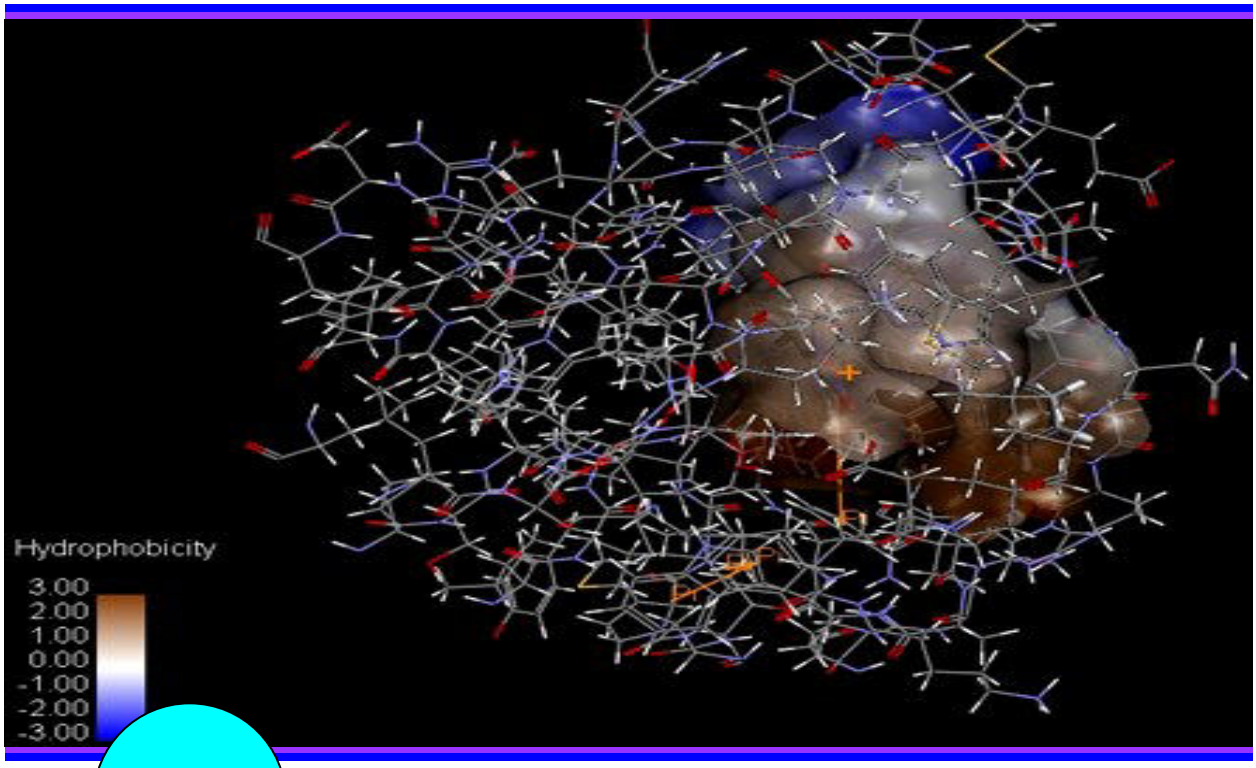

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Pass and Swiss ADME collaborated *in silico* docking approach to the synthesis of certain pyrazoline spacer compounds for dihydrofolate reductase inhibition and antimalarial activity

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Abstract

New series of pyrazoline spacer compounds were prepared by the reaction between benzimidazole chalcones and (2-methyl-5-nitro-imidazole-1-yl)-acetic acid hydrazide by the sensible use of Michael addition. The building blocks used for the synthesis of pyrazoline derivatives were opted by using virtual screening by molinspiration search engine. The hypothetically resulted pyrazoline spacer compounds from this list are checked for their reliability on other *in silico* drug designing online web services like PASS online bioactivity, Swiss ADME predictor. The docking study on final four pyrazoline compounds was carried out using Accelrys Discovery Studio 3.5. These synthesized compounds were, later, characterized with the help of UV, IR, mass and ^{1}H NMR techniques. These compounds were further screened for their *in vitro* antimalarial effect. The PASS, Swiss ADME assisted docking approach and the use of combo heterocyclic ring with pyrazoline scaffold were found to be beneficial to derive and synthesize effective antimalarial agents in the present study.

Introduction

The rule of drug design is more focused into a receptor based design. The structure of the target is usually known to high resolution and the binding site is well defined. Binding of the drug to these hypothetical interaction sites is further influenced by hydrogen bonds, electrostatic and other non-covalent interactions (Wade and Goodford, 1989). The utilization of molecular docking studies made its intervention at this point of drug synthesis. Here comes the scope of utilization of other web based *in silico* screening as a method of the multilevel screening process. The results obtained from Molinspiration and checking for its match on the principles of Lipinski rule are the one of first evolved of this kind (Mabkhot et al., 2014) used as the choice of present study. The resulted compounds were a fusion of two major heterocyclic entities benzimidazole and imidazole connected with a pyrazoline spacer. The nitrogen heterocycles are well-known about their ability to participate in the diverse biological activities (Akhtar et al., 2017). Considerable attention was given here on the synthesizing molecules, which have three nitrogen heterocyclic rings. Chalcones required for synthesis were prepared from Benzimidazole and aromatic aldehydes. The acid hydrazide was prepared from imidazole.

The pictorial representation of this heterocyclic combination, spacer and the way bulky groups can be linked (Figure 1). The bulky group with substitutions is planned on this pyrazoline spacer in an intention of studying

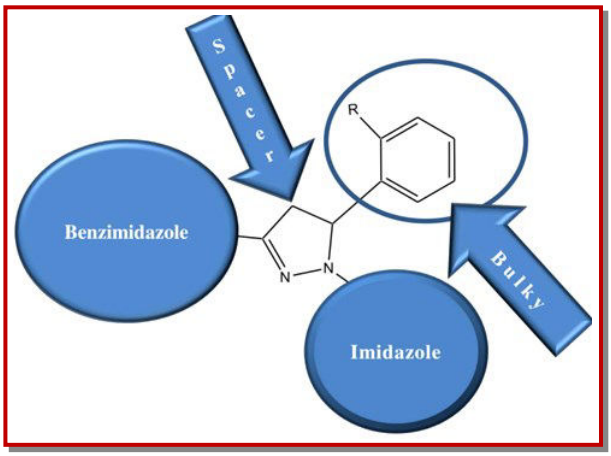


Figure 1: Pictorial representation of molecule with spacer and bulky group

the effect of substitutions in the interaction of this molecule to the binding pockets of the enzyme. The title compounds subjected to PASS online bioactivity prediction (Parasuraman, 2011). The result came as synthesized compounds showed more probabilities to exhibit the antiprotozoal activity.

Our recent study on dihydrofolate reductase enzyme is a good receptor for the lipophilic derivative gave a direction to do a check on these molecules (Krishnakumar et al., 2017). Malarial dihydrofolate reductase (DHFR) is the target of antifolate antimalarial drugs. Inhibition of this enzyme results in disruption of DNA, RNA, and protein synthesis, with consequent cell death. Recent studies on available antimalarials say efficacy of these drugs has decreased because of mutations in the enzyme that have led to drug resistance (Yuthavong et al., 2012). This fostered us to screen the compounds for effective dihydrofolate reductase inhibition.

Further web based *in silico* methods like SWISS ADME calculator, PASS bioactivity tester are implemented to study the efficacy of these compounds in the due course of synthesis. Drug design involves the estimation of absorption, distribution, metabolism, and excretion (ADME) increased in the contemporary discovery process (Daina et al., 2017). In that context, computer models constitute valid alternatives to experiments. The identified molecules are further subjected to Docking study using CDocker program of Accelrys Discovery Studio 3.5 against the dihydrofolate reductase enzyme. Since the *in silico* drug design studies are carried out in an intention to minimize the animal sacrifice and economic burden due to clinical studies, the pharmacological screening in this work performed with non-clinical evaluation of the antimalarial activity.

Materials and Methods

Molinspiration

Various heterocyclic chalcones and acid hydrazide were considered for virtual screening method to check the bioactivity score. When these chalcones were compared, benzimidazole chalcones and imidazole hydrazide were found to have interesting molecular properties, which match with Lipinski prediction. Once a bioactivity model is generated, the actual virtual screening was performed for the predicted pyrazoline molecules.

ADME studies

The ADME study was carried out using SWISS ADME predictor. This is a free web tool to evaluate the pharmacokinetics, drug likeness and medicinal chemistry friendliness small molecules. As mentioned earlier, the attention was given to design the molecules which fit into the rule of drug likeness. The properties like molecular weight less than 500 g/mol, less than 5 numbers of hydrogen bond donors, less than 10 numbers of hydrogen bond acceptors and less than 10 rotatable bonds were chosen as criteria, while the selection of molecules to be synthesized (Doak et al., 2014). The search engine further gave a compiled result on lipophilicity and hydrophilicity of these molecules by integrating results obtained from various Log P and S prediction programs called ILOGP, XLOGP3, WLOGP, ESOL, and SILICOS-IT. Log P, a measure of lipophilicity of the molecule is the logarithm of the ratio of the concentration of drug substance between two solvents in an unionized form. Lipinski rule prescribes an upper limit of 5 for druggable compounds. The lower the log P values the stronger the lipophilicity for the chemical substance. The aqueous solubility of a compound significantly affects its absorption and distribution characteristics. On the other side, low water solubility goes along with a bad absorption, and therefore, the general aim is to avoid poorly soluble compounds. Log S is a unit of expressing solubility in itself, which is the 10-based logarithm of the solubility measured in mol/L. Distribution of Log S in traded drugs reveals a value somewhere between -1 to -4, will be optimum for better absorption and distribution of drugs in the body.

Activity prediction and toxicity

PASS online predicts over 4000 kinds of biological activity, including pharmacological effects been utilized for this study to check the bioactivity score of synthesized compounds as antimalarial drugs. Further preADMET ver. 2.0 toxicity predictor predicts test values for Ames test and rodent carcinogenicity assay from the available database of compounds of similar structure. This web service is used for finding mutagenic and carcinogenicity of synthesized compounds.

Docking studies

Molecular docking study for the inhibition of dihydrofolate reductase was studied. A number of inhibitions made by the different derivatives were studied by evaluating the result obtained after docking the com-

pounds using CDocker program. The scoring functions and hydrogen bonds formed with the surrounding amino acids were used to predict their level of inhibition on binding sites of dihydrofolate reductase receptor. The structure of 1-[3-(1*H*-benzoimidazol-2-yl)- 4, 5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone derivatives were docked using Accelrys Drug Discovery Studio 3.5. The structure of the enzyme dihydrofolate reductase was obtained from Protein Data Bank (PDB code: 7 dfr) and was used for docking. The pose with the most negative CDocker interaction value of the compound was considered to be the most favorable pose having highest inhibition of DHFR.

Synthesis of 1-[3-(1*H*-benzoimidazol-2-yl)-4,5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone derivatives are described here as three schemes.

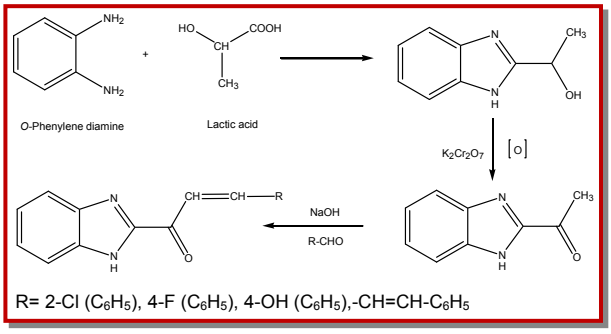
Scheme 1

Step 1: Synthesis of 2-(a-hydroxy ethyl) benzimidazole (Nyati et al., 2006).

A mixture of ortho-phenylenediamine (0.25M) and lactic acid (0.35M) was refluxed for 2.5 hours. The reaction mixture was cooled and made alkaline by gradual addition of 10% NaOH. The residue was collected. The crude product obtained was dissolved in 400 mL of boiling water. To this, 2 g of decolorizing carbon was added and heated for 15 min. The mixture was filtered rapidly at the pump through preheated Buchner funnel. The filtrate was cooled to about 10°C. The product obtained was filtered and washed with 25 mL of cold water and dried at 100°C. The purity of the compound was confirmed by getting a single spot in thin layer chromatography (TLC).

Step 2: Synthesis of 2-acetyl benzimidazole

To the solution of 2-(hydroxy ethyl) benzimidazole (50 mM) in diluted H_2SO_4 (5%, 40 mL), a solution of $K_2Cr_2O_7$ (150 mM) in aqueous H_2SO_4 (25%, 80 mL) was added over a period of 20 min. The mixture was then stirred further for 2 hours. The separated orange solid was washed with water, then suspended in water (50 mL) and treated with aqueous NH_3 (1:1) to a pH 6.0-6.5. The separated solid was washed with water, dried and recrystallized from boiling ethyl acetate. A single spot in the TLC plate confirmed the purity.



Scheme 1: Synthesis of benzimidazole chalcones

Step 3: Synthesis of benzimidazole chalcones

To a solution of step 2 (0.01 M) and aromatic aldehyde (0.01 M) in ethanol (20 mL) was added into a solution of sodium hydroxide (8 mL, 10% solution). The reaction mixture was stirred at room temperature over a period of 24 hours, diluted with water (100 mL) and acidified with concentrated HCl. The product obtained was filtered, washed with water and recrystallized from ethanol (Scheme 1).

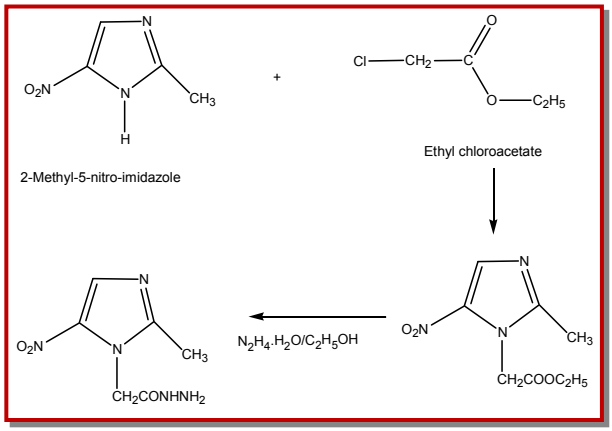
Scheme 2

Step 1: Synthesis of 2-methyl-5-nitro-1-imidazoethyl acetate (Havaldar and Patil, 2008)

A mixture of 2-methyl-5-nitro-imidazole (0.01M), ethyl chloroacetate (12 mL) and potassium carbonate (0.05 mL) in dry acetone were refluxed for 50 hours. The reaction mixture was filtered hot, and the solvent was distilled off from the filtrate. The crude ester thus obtained was purified by recrystallization using methanol.

Step 2: Synthesis of 2-methyl-5-nitro-1-imidazo acethydrazide

A mixture of 2-methyl-5 nitro-1-imidazoethyl acetates obtained from step-1 and hydrazine hydrate (0.1 M) in ethanol (50 mL) was refluxed for 8 hours. The solution on cooling gave the solid mass of hydrazine, which was collected by filtration and recrystallized from the ethanol (Scheme 2).

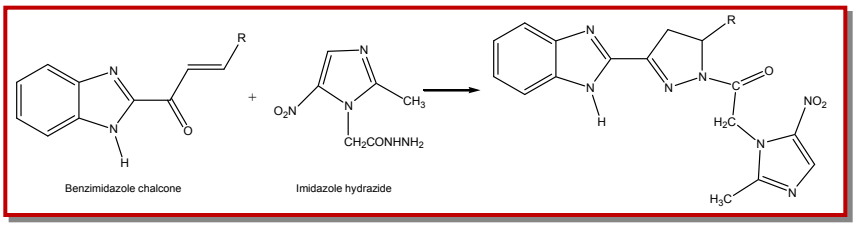


Scheme 2: Synthesis of 2-methyl- 5-nitro-1-imidazo acethydrazide

Scheme 3

Synthesis of 1-[3-(1H-benzoimidazol-2-yl)-4,5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone derivatives (Sharma et al., 2014)

A mixture of various derivatives of synthesized chalcones (0.01 M) and product obtained from the step 2 of Scheme 2 (0.02 M) in 25 mL of ethanol and 0.01 mL of piperidine were added and refluxed for 6 hours. Excess



Scheme 3: Synthesis of 1-[3-(1*H*-Benzoimidazol-2-yl)-4, 5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone

of ethanol was distilled and crystalline solid products obtained were filtered and recrystallized from the ethanol. The purity of the derivatives was confirmed by a single spot on the TLC plate (Scheme 3).

Antimalarial screening

The *in vitro* antimalarial assay was carried out in 96-well microliter plates (Lee et al., 2002). The cultures of *Plasmodium falciparum* RKL-2 strain were maintained in medium RPMI 1640 supplemented with 25 mM HEPES, 1% D-glucose, 0.2% sodium bicarbonate and 10% heat inactivated human serum. 180 mL of 1% D-sorbitol synchronized ring stage parasitemia in 3% hematocrit in a total volume of 200 µL of medium RPMI-1640 was assessed after converting to final dose. A stock solution of 5 mg/mL of each of the test samples was prepared in DMSO, and subsequent dilutions were prepared with culture medium to obtain 5 and 50 mg/mL. The test plates, as well as the positive and negative control (chloroquine and without sample respectively), were incubated at 37°C for 40 hours after treating with 5% CO_2.

Results

Characterization of synthesized compounds

1-{3-(1*H*-benzoimidazole-2-yl)-5-(2-chlorophenyl)-4,5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone (compound **1**): $C_{22}H_{18}ClN_7O_3$, Yellow crystals, M=463.12 g/mol, MP=145°C, IR (KBr, cm^{-1}): 1444.68 (C-C), 1575.84 (N=O), 1631.78 (C=O), 2947.239 (C-H). ^{1}H NMR(500MHz, MeOD, δ/ppm, J/Hz): 2.2 (2H, t, J=5.0, CH_2-Pyrazoline), 2.435 (3H, s, -CH_3), 4.5 (2H, s, -CH_2), 5.0(1H, s, -NH), 7.45-7.68 (9H, m, CH-aromatic). MS (ESI): *m/z* 465.6 $(M + 2H)^+$.

1-[3-(1H-benzoimidazol-2-yl)-5-(4-fluoro-phenyl)-4, 5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone (compound **2**): $C_{22}H_{18}FN_7O_3$, Yellow crystals, M=447.15 g/mol, MP=160°C, IR (KBr, cm^{-1}):1442.20 (C-C), 1580.93(N=O), 1641.02(C=O), 2912.71(C-H). ^{1}H NMR (500MHz, MeOD, δ/ppm, J/Hz):1.7(2H, t, J=7.5, CH_2-Pyrazoline), 2.29(3H, s, -CH_3), 4.5(2H, s, -CH_2), 4.9 (1H, s, -NH), 7.390-8.044(9H, m, CH-aromatic). MS (ESI): *m/z* 448 $(M + H)^+$.

1-[3-(1H-benzoimidazol-2-yl)-5-(4-hydroxy-phenyl)-4,5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone (compound **3**): $C_{22}H_{19}N_7O_4$, White crystals, M=445.15 g/mol, MP=143°C, IR (KBr, cm^{-1}): 1201.65(C-C), 1579.7(N=O), 1647.21(C=O), 3032.1(C-H), 3280.92(O-H). ^{1}H NMR (500MHz, MeOD, δ/ppm, J/Hz): 2.1(2H, t, J=5.0, CH_2-Pyrazoline), 2.48(3H, s, -CH_3), 3.33(1H, s, -OH), 6.3(2H, s, -CH_2), 4.9(1H, s, -NH), 6.652-8.113 (9H, m, CH-aromatic). MS (ESI): *m/z* 447 $(M + 2H)^+$.

1-[3-(1*H*-Benzoimidazol-2-yl)-5-styryl-4,5-dihydro-pyrazol-1-yl]-2-(2-methyl-5-nitro-imidazol-1-yl)-ethanone (compound **4**): $C_{24}H_{21}N_7O_3$, White crystals, M=455.17g/mol, MP=154°C, IR (KBr, cm^{-1}): 1212.04(C-C), 1533.52(N=O), 1605.21(CH=CH-), 1700.77(C=O), 3089.20(C-H). ^{1}H NMR (500MHz, MeOD, δ/ppm, J/Hz):1.4(2H,t,J=11.5, CH_2-Pyrazoline), 2.2(3H, s, -CH_3), 4.59(2H, s, -CH_2), 4.87(1H, s, NH), 6.194-7.955 (9H, m, CH-aromatic). MS (ESI): *m/z* 455 $(M)^+$.

Pass and Swiss ADME

The results obtained from *in silico* studies clearly indicate compound code-3 with hydroxy substitution is the most druggable substance with a minimum violation from any of drug likeness rules discussed above. It was interesting to note that the results from the SWISS ADME predictor values of Log P, molar refractivity, and the total polar surface area in these molecules were in excellent agreement with the most important rules of drug likeness. eg. Lipinski, Ghose, Veber, Egan. Though these compounds were exhibiting good hydrophilic-lipophilic balance and same predicted bioavailability, the hydroxy derivative with high lipophilicity was expecting to show decent GI absorption. This hydroxy derivative with a higher value of probability of antimalarial activity and non-carcinogenic and mutagenic properties were predicted as the lead in the study. The results obtained from the Swiss ADME search engine are listed in Table I. The PASS activity prediction for pyrazoline compounds given a higher value of probability for antiprotozoal action (Figure 2). The results obtained from activity prediction are given in Table I.

Docking

The extent of inhibition of all the synthesized ligands was compared with inhibition produced by standard drug chloroquine. Efforts were made to study and compare the amino acid residues involved in enzyme inhibition. The results obtained were given in Table II.

Table I

Physiochemical properties of synthesized ligands

Physiochemical properties, lipophilicity and water solubility								Antiprotozoal activity prediction	
Compound code	Molecular weight (g/mol)	No. of hydrogen bond donors	No. of hydrogen bond acceptors	No. of rotatable bonds	Total polar surface area (A°)	Log P (iLOGP)	Log S (ESOL)	Pa	Pi
1	463.8	1	6	6	124.9	2.6	-5.1	0.6	0.0
2	447.4	1	7	6	124.9	2.3	-4.6	0.6	0.0
3	445.4	2	7	6	145.2	2.2	-4.3	0.6	0.0
4	455.4	1	6	7	124.9	3.4	-4.9	0.6	0.0

Pa = Probability to active; Pi = Probability to inactive

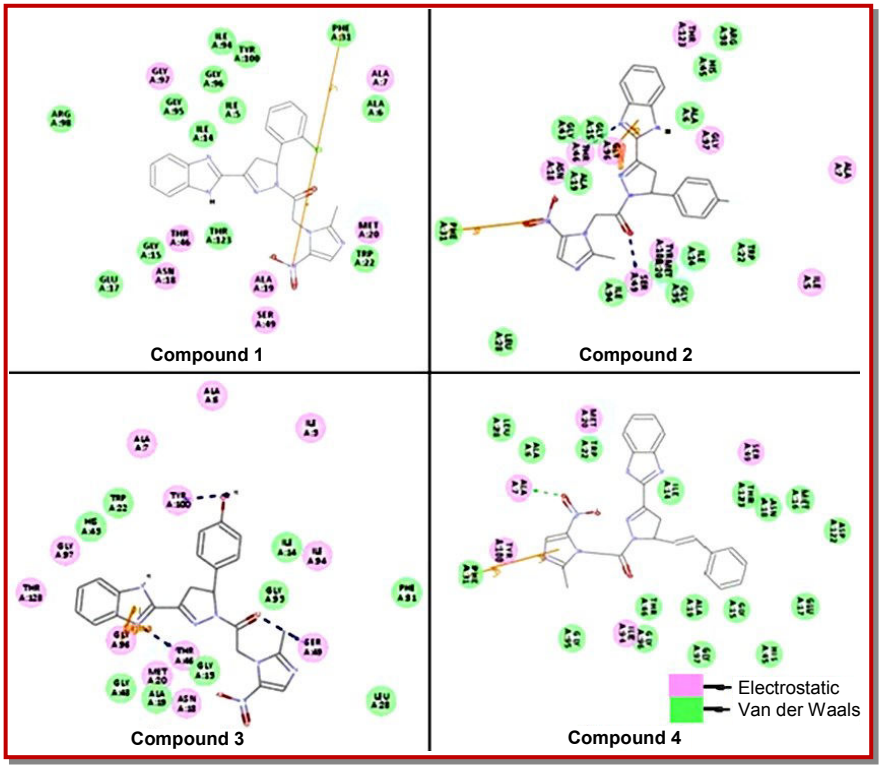


Figure 2: Binding configuration of synthesized derivatives (Discovery Studio 3.5 software)

The four successful synthetic derivatives screened for *in silico* ADME and toxicity studies when subjected to docking studies the most affluent outcome was again found to match with the results obtained from ADME analysis. The compound code-3 exhibits highest –CDocker energy and –CDocker interaction values comparable with standard drug chloroquine. Unlike all other ligands, the hydroxy terminal substitution exhibited a high number of stronger electrostatic interactions. The three hydrogen bond interactions associated with Pi bond between benzimidazole ring and amino acid residue in chain A of dihydrofolate seems to be the reason for the significance of compound-3. The compound code-3 is in good correlation with standard drug chloroquine in binding energy and number of hydrogen bond interaction at the drug receptor. The types and number of interactions between the synthesized ligands and enzyme are shown in Figure 2, and the data are tabulated in Table II. Figure 2 exhibits the prominent lipophilicity of compound 3 by the way it fit into the hydrophobic pocket of the receptor. This clearly represents hydrophobic amino acid terminals is located within that region of receptor and facilitates stronger binding with a lipophilic ligand.

Antimalarial activity

The *in vitro* antimalarial assay results showed that the representative compounds exhibited antimalarial

Table II

Statistical docking results of synthetic ligands (Discovery Studio 3.5 software)

Compound code	CDocker energy	CDocker interaction energy	Nature of hydrogen bond	Distance	Prominent type of interaction
1	18.5	48.3	-	-	Van der Waals
2	6.2	35.9	A:SER49- Mol:O16 A:THR46- Mol:N7	2.3 2.0	Van der Waals
3	23.3	53.4	A:THR46- Mol:N7 A:SER49-Mol:O16 Mol:H51 A-TYR100:OH	2.0 2.4 1.9	Electrostatic
4	10.3	39.4	A:ALA7:HN -Mol:O33	2.3	Van der Waals
Standard	37.0	62.9	Mol:H48 - A:GLU17:O Mol:H48 - A:ASP122:OD Mol:H50 - A:TYR100:OH	2.4 2.4 2.0	Van der Waals

activity at 50 mg/mL (Table III). The smears when accessed for a number of dead cells, as expected the compounds showed good correlation with docking results. The smears were assessed for live and dead parasites. The significance of computer in the prediction of activity can be substantiated by correlating the activity profile with the *in silico* data (Table II). The antimalarial activity is compared with the dock scores (binding energy) of the synthesized compounds using GraphPad Prism 7. The correlation coefficient value "r" 0.9929 found too significant for correlating antimalarial activity and predicted docking score.

Table III

***In vitro* antimalarial assay of synthesized ligands**

Sample code	%Dead parasites (rings + trophozoites + schizont)	
	5 µg/mL	50 µg/mL
1	0	25
2	0	37
3	0	38
4	0	29
Chloroquine	46	

Discussion

The web-based ADME studies, bioactivity prediction, docking and *in vitro* studies are carried out for those molecules, which filtered off from drug rules. The authors are successful in deriving a correlation for synthesized compounds among the bioactive parameters. The correlation coefficient value obtained after comparing the docking score and antimalarial activity clearly reflect a positive equation exist between all tested parameters. It was interesting to note the Swiss ADME prediction and PASS bioactivity prediction results also in coherence with final results. This combination of Pass-Swiss-Docking *in silico* prediction method found to more sensitive and can work with other molecules too.

When it comes to Scaffold chemistry, the docking results are in agreement with the selection of pyrazoline part as a scaffold in the entire molecule. The scaffold is an integral part of the molecule, where a medicinal chemist can try for structural alternation to extract the bioactivity to the molecule in fullest (Mathew et al., 2010). The docking interaction describes none of the cases pyrazoline has much interaction with the amino acid residues in enzyme binding with it. Whereas substitution in pyrazoline with chloro, fluoro, hydroxy and cinnamoyl group found to bring a lot of variations in binding energies as discussed earlier. Assessment of the predictive accuracy of *in silico* prediction tools, alone or in combination was tried by many researchers (Leong et al., 2015). The uniqueness of this work lies with the prediction of a scaffold in a big molecule and its establishment by using binding configuration obtained after docking study. The lead optimization techniques showed that the compound code-3 with hydroxyl substitution showed good drug likeness and bioactivity score in the initial level of *in silico* screening. Further, the same molecule showed good binding to dihydrofolate reductase enzyme with stronger electrostatic interaction. In the later stage of work, this *in silico* results found matching with the results obtained from *in vitro* antimalarial screening.

It was interesting to note that hydroxy derivative showed good binding to dihydrofolate reductase enzyme with prominent electrostatic interactions. Whereas the other molecule, including the standard drug, showed moderate to weak Van der Walls type of interactions. Further, the optimum hydrophilic-lipophilic balance found to exist in molecule contributes to strong receptor binding.

Conclusion

The extended lipophilicity and stronger electrostatic interaction may be a reason for this lead compound to exhibit significant pharmacological action.

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