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Development of selective Inhibitors of crucial drug target Phosphoethanolamine methyltransferase of *Plasmodium falciparum* based on Chemo informatics and *in vitro* experiments

Jagbir Singh, Rani Mansuri, Pravin Kumar Atul, Mahesh Kumar and Arun Sharma

Abstract
The chemo informatics and simulation approaches are highly preferred to develop drug candidate, applied in the study to develop a combinatorial library to meet, the need of new potent antimalarial. Combinatorial library was developed based on information of competitive inhibitors of *Plasmodium falciparum* phosphoethanolamine methyltransferase essential for membrane development for rapid multiplication of parasite. Combinatorial library was developed using LeadGrow (vLifeMDS4.6 software) by replacing the substitution sites (R1 and R2) with different aromatic and hetero-aromatic rings, and R_3 with Oxygen and Sulfur. Library was screened based on druglike properties, ADMET using Discovery Studio 3.2 and also binding affinity from Schrodinger v9.6. The best scored analogs were got synthesized for *in vitro* testing. The thermodynamic stability was studied through dynamic simulation using Desmond. Among the druglike analogs aromatic (benzoic acid) ring at R_1interacted through one hydrogen bond acceptor and donor. At position R_2 hetero aromatic rings (Pyrimidine, Pyrazine and Imidazole) containing two hetero atoms could score better, actively participated in non-covalent interactions within the binding site. All the synthesized three analogs showed Schizonticidal and gametocidal activity but Comp.1 could show better IC_{50} 3.4 µM and LD_{50} 192.91µM with ten folds higher (56.73) selectivity index. Comp.1 inhibited protein with EC_{50} 2.1 µM was also found thermodynamically stable till 30ns.The Comp.1 has better affinity and drug-score as well; it may have the possibilities of potent antimalarial. *In-vitro* observations and the thermodynamic stability of Comp.1inhibitor also established the target (*Pf*PMT) specificity. Hence; theComp.1 may further be optimized and validated for being potent antimalarial candidate. Identified Comp.1 and other two analogs may also further be served as lead for further rational drug designing for more potent antimalarial.

Keywords: Chemoinformatics, combinatorial library, *Pf*PMT, Molecular dynamics, ADMET

1. Introduction
Mosquito borne disease malaria in humans is caused by unicellular microorganism called parasitic *Plasmodium protozoa*. According to the WHO fact sheet millions of malaria cases found and thousands of people are killed by malaria globally [1]. The increase in antimalarial drug resistance produces the thrust of new potent antimalarial to come over the resistance problem where computational drug designing approaches (Structure and Ligand based drug designing) are being applied to identify potent drug target or potent druggable compounds, which may be optimized through designing combinatorial library to meet appropriate druglike properties [2]. In this paper, a combinatorial library was developed and analyzed against an excellent malaria drug target *Plasmodium falciparum* phosphoethanolamine methyltransferase (*Pf*PMT) protein for the biosynthesis of phosphocholine an important component for rapid membrane synthesis [3, 4] through the ADMET, binding affinity and *in vitro* validations.
The combinatorial library based *Pf*PMT competitive inhibitors NSC 158011 (N-naphthalen-1-yl-2-phenylsulfanylethanethioamid) [5], identified Zinc [6] and Asinex inhibitors [7] was developed using LeadGrow module of vLifeMDS software [8] and shortlisted based on the Lipinski rule of five, ADMET and docking score. The compound analogs interacting with crucial amino acids of catalytic dyad and tyrosine residues important for triple methylation were selected for binding energy calculation. Compounds which were found to have more stable energy than the docking control inhibitor were analyzed through the dynamics simulation for 30 ns.
There finally, the analog Comp.1 containing Pyrimidine found thermodynamic stable out of Imidazole and Pyrazine rings within the active site throughout the 30ns of simulation was synthesized.

In vitro experiments proved it nontoxic even at 192.91µMand showed better schizontcicidal IC50 (3.4µM) and protein EC50 (2.1µM) tested. Hence, the Comp.1 analog have great probability of being inhibitors of *Pf*PMT and may prevent growth of *Plasmodium falciparum* to overcome the problem of drug resistance. Studied compounds may also serve as the lead molecule for the rational drug designing and further modifications may be achieved for more potency.

2. Materials and method

2.1 Combinatorial library development and ADMET analysis

The combinatorial library designing is technique is very important tool for large library enumeration in a single run. The huge number of novel diverse compounds may easily be developed. The experimentally proved competitive inhibitors of *Pf*PMT with better affinity for *Pf*PMT were used as lead for combinatorial library development. The compound modification was carried out using Lead Grow of module MDS4.6vLifesciences which enables the diverse number of substitutions and also facilitates the physicochemical properties calculation and Lipinski screen of designed library [9]**.** ADME and toxicity of designed combinatorial compound library was predicted from discovery Studio3.5 (DS3.5) [10] uses quantitative structural relationship and similarity searching of the model's database to identify the physiochemical properties of compounds as a query compound.

For a drug to be absorbed and systemic bioavailable for its action there should be optimum balance between the solubility and liphophilicity of a drug. So, the computational ADME and toxicity analysis (Lipinski' rule of five, carcinogenicity, mutagenicity, Cyp2d6 prediction) of combinatorial library was done using discovery Studio 3.5 (DS3.5) [6]. The compounds with unfavorable ADMET and toxic properties were removed prior to the docking [11, 12]**.**

2.2 Docking analysis

The combinatorial library was screened virtually using Virtual screening module of Schrodinger *v9.6* through three successive docking stages based on precision and accuracy because as the precision level increases the dataset size decreases to smaller with more accuracy. The phosphocholine binding site of *Pf*PMT was used as active site for docking. The phosphocholine binding site (Grid) was used for docking of *Pf*PMT inhibitors was again used for docking of combinatorial library. Compounds better XP score were scored for their binding energy using PrimeMM-GBSA (Schrodinger *v9.6)* under OPLS_2005 force field including ligand strain energies and analyzed [13]**.** The competitive inhibitor NSC 158011 was used as docking control**.** The best three analogs got custom synthesized from GCC Biotech (www.gccbiotech.net).

2.3 Compound synthesis

Retro-synthesis designed

The retro synthesis approach [14] was used to synthesize three analogs were broken into two synthons which were then converted to retrons heterocyclic substituted propionic acid [3-(pyrimidin-4-yl)propanoic acid, 3-(pyrazin-2-yl)propanoic acid and 3-(1*H*-imidazol-4-yl)propanoic acid] and 4-amino benzoic acid as per their synthesis feasibility and availability of the retrons. In the reaction capital A shown in blue circle denotes the heterocyclic rings (Pyrimidine, Pyrazine and Imidazole rings). Reaction was broken into four steps shown in Fig.1.

Step.1: Oxidation

The A-CH_3(4-methylpyrimidine [I], 2-methylpyrazine [II] and 4-methyl-1*H*-imidazole [III]) were oxidized to carboxylic acid in presence of strong and best oxidizing KMnO4 which keeps the capability of converting methyl to carboxylic group (Strassner and Houk 2000) and reaction was conformed through TLC.

Step.2: Carbon incorporation (Carbon chain elongation)

At step second, the reduction of formed carboxylic acid was carried out in presence of LiAlH4 to methanol because LiAlH4 generally does not affect the carbon-carbon double and triple bonds. To incorporate first carbon methanol was treated with HI and KCN followed by hydrolysis into substituted acetic acid. One more carbon was incorporated too by treating substituted acetic acid again with LiAlH4 into substitute ethanol followed by HI and KCN again provided the substituted propanoic acid, the required retron [15]. The reactions were confirmed through TLC.

Step.3: Amide bond formation

At this step compounds substituted propanoic acid was mixed with 4-amino benzoic acid in presence of DIC (N,N'-diisopropylcarbodiimide) which is a good dehydrating agent and helps in amide bond formation. DIC is soluble in organic solvents and easily be removed by extraction [16]. The target compounds were prepared and crystallized**.**

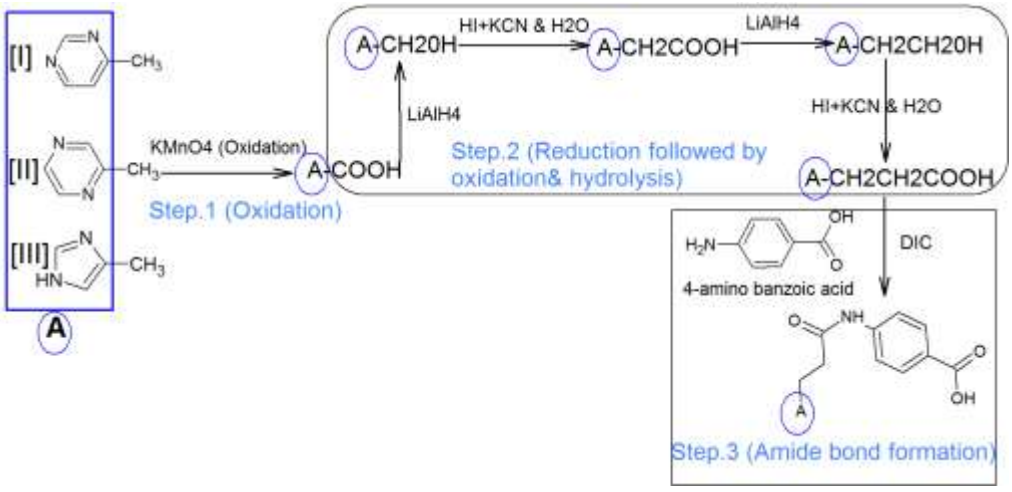


Fig 1: The schematic diagram of steps employed for synthesis of selected analogs

Spectral analysis

Comp. 1: Mp: 189-191; Rf value: 0.66; Yield:68%, Ft-IR(KBr^{-1})

C-H str. (3062), C=Cstr. (1595), N=CHstr.(1682), C-Nstr. (1280), C=Ostr. (1755,1672), N-H (3322)

^{1}H NMR (δ DMSO):2.74 - 3.08 (s, 4H, C-H), 6.2 - 8.5(m, 3H, Pyrimidine), 7.0 - 7.1 (2H, Ar), 7.8 - 8.1 (2H, Ar).^{13}C NMR (δ DMSO):Aromatic nucleus (110.3, 109.6, 121.5, 128.8, 129.3, 134.5), Pyrimidine nucleus (104.3, 156.3, 157.8, 160.3), C=O (171.2), CH_2-CH_2 (31.9, 33.6)

Comp. 2: Mp: 184-188; Rf value: 0.62; Yield:65%, Ft-IR(KBr^{-1})

C-H str. (3060), C=Cstr. (1592), N=CHstr.(1684), C-Nstr. (1282), C=Ostr. (1752,1673), N-H (3324)

^{1}H NMR (δ DMSO):2.72 - 3.06 (s, 4H, C-H), 6.5 - 8.2(m, 3H, Pyrazine), 7.1 - 7.3 (2H, Ar), 7.7 - 8.3 (2H, Ar).

^{13}C NMR (δ DMSO):Aromatic nucleus (110.6, 108.8, 121.1, 127.6, 130.1, 135.1), Pyrazine nucleus (105.1, 157.6, 155.9, 159.6), C=O (169.8), CH_2-CH_2 (33.3, 35.4)

Comp. 3: Mp: 174-178; Rf value: 0.68; Yield:63%, Ft-IR(KBr^{-1})

C-H str. (3065), C=Cstr. (1590), N=CHstr.(1679), C-Nstr. (1278), C=Ostr. (1750,1669), N-H (3319)

^{1}H NMR (δ DMSO): 2.70 - 3.04 (s, 4H, C-H), 6.1 - 8.3(m, 2H, Imidazole), 6.9 - 7.1 (2H, Ar), 7.8 - 8.1 (2H, Ar).^{13}C NMR (δ DMSO):Aromatic nucleus (110.4, 109.3, 120.4, 128.1, 128.8, 134.8), Imidazole nucleus (103.8, 155.4, 158.7), C=O (168.8), CH_2-CH_2 (30.9, 32.8)

2.4 Schizonticidal and Gametocidal activity

The parasite culture of *P. falciparum* was carried out for *in vitro* schizonticidal analysis of synthesized compound in RPMI1640 and culture was synchronized and 1% ring stage was used for test**.**To obtain only ring stage parasite, culture was synchronised using 5% sorbitol. For antimalarial activity assay each compound was dissolved in 1% DMSO to prepare stock solution. Synchronized parasite without any test compound used as control and synchronized parasites incubated with test compound considered as Test wells. 100 µl of test compound was added in 1 well which was made to ten serial dilutions. After incubation period of 24 hours, numbers of schizonts were counted in control. When 10% or more (i.e. 10 schizonts per 100 asexual parasites) mature schizonts were observed in the control, thick and thin blood smear of each well of the test plate (control & test wells) were made. The gametocidal activity was done on gametes of RKL-9 strain of *P. falciparum* which developed from 1% synchronized rings in the RPMI 1640 media supplemented with hypoxanthine needed for gametes development and their maturation. The gametes were microscopically examined for their development on 5th or 6th day of incubation. 1st stage of gametes was incubated with the test analogs in serial dilution. The gametocidal activity was performed according the Singh J. *et al* 2019. The primaquine was used as reference drug. Thin smears were made and microscopy was done for next stages development and inhibition was calculated in terms of % inhibition [17, 7]**.** Schizonticidal and gametocidal IC_{50} of test compound was generated using Microsoft excel.

2.5 Cell Cytotoxicity

The culture of Human embryonic kidney cells 293 (HEK293) was carried out in DMEM high with glucose penicillin and fetal bovine serum as additives and supplements. The colorimetric based MTT assay (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was performed for cyto-toxicity. The trypsin washed 98% viable five thousand cells were incubated with the test compound in different concentrations (10µM, 50µM, 100µM, 150µM, 200µM, 250µM) for 24 hours. 5 mg/ml MTT added to the test plate and the supernatant was replaced with DMSO and recorded at 580nm using Synergy/HTX MultiScan reader (BioTek). The selectivity index of the test compound was calculated based on the lethal dose LD_{50}antimalarial IC_{50} [8, 7]**.**

2.6 Protein Inhibition and Molecular dynamics simulation

The *Pf*PMT protein native form was confirmed through SAM-dependent methyltransferase assay Kit prior performing the inhibition assay. In order to investigate the inhibitory effect of the test compound the 1% DMSO solution was used for dissolving the test compound. The test compound was assayed with the native *Pf*PMT protein assay to calculate the inhibition of protein quantified at 510 nm. The native protein functional assay absorbance at 510 nm was used as *Pf*PMT 100 % functional activity and the absorbance with the test compound at different concentrations was also observed at 510 nm which were converted into % protein activity inhibition. The graph was plotted for the % *Pf*PMT inhibition against the concentration of test compound to establish EC_{50} [7].

Dynamics simulation of the best scored compound (Comp.1) was done and analyzed the intrinsic stability of the compound within the binding site using the Desmond *v 4.2* using the OPLS-AA/ 2005 force field. The protein-ligand complex was prepared prior the simulation. The prepared protein was solvated in TIP3P water model within the orthorhombic box and simulation system was neutralized by adding Na+ and Cl- ions [6]**.** Finally, simulation was run for 30ns using the NPT ensemble where the temperature 300K and pressure of 1ATM were kept constant. The trajectory was saved at every 30ps time interval. The trajectory was visualized in Maestro [19, 20]**.**

3. Results and discussion

3.1 Combinatorial library development and ADMET analysis

Docking score and binding energy of known inhibitors confirmed the docking affinity of these towards *Pf*PMT and showed the inhibitors 39225, 109268, 310551 and 641296) could not score well towards the target protein *Pf*PMT. The inhibitors 125034 and 158011 scored highest dock score -8.7 and -8.4 respectively, showed good docking affinity of both for the protein. The inhibitor NSC 158011 interacted amino acids crucial, where the thioacetamide scaffold also participated in Vander Waals for catalytic dyad formation Tyr19 and His132 through the Vander Waals interaction. There both the benzene and bicyclic aromatic ring naphthalene provided π-alkyl interaction which may be responsible for the better dockscore than other inhibitors (Fig.2). The inhibitor NSC 158011 has also been experimentally proved as competitive inhibitor hence was used as lead for modification and optimization through combinatorial library design for the better binding affinity, ADMET and inhibition.

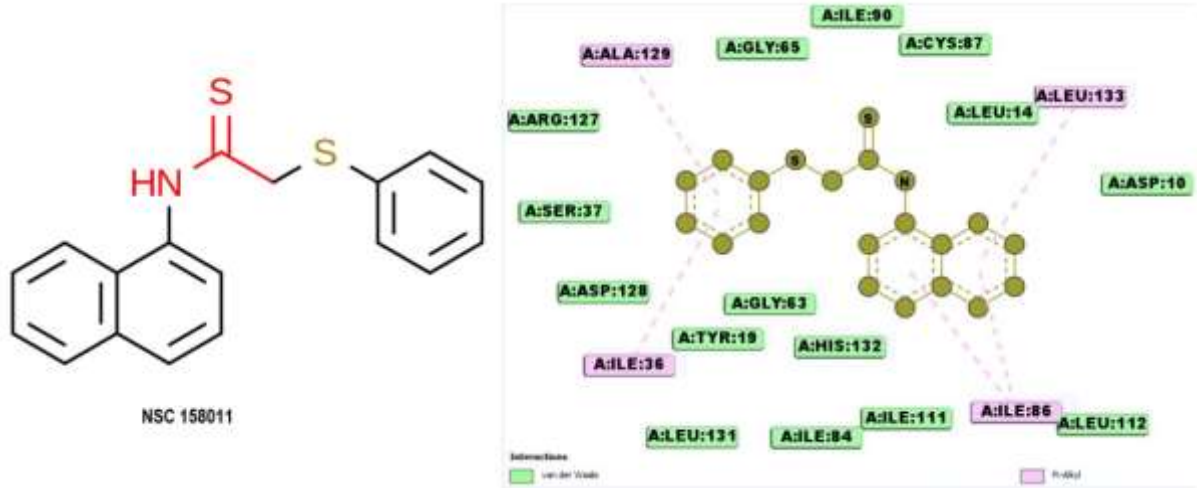


Fig 2: The interaction of NSC 158011 with *Pf*PMT with catalytic amino acids and other important amino acids

The previous study of Zinc compounds provided the ZINC12882412 (IC_{50} 2.1µM) and ZINC02103914 (IC_{50} 3.0µM) with better Schizonticidal activity. Both the hits were found to have one common scaffold acetamide which interacted with crucial amino acids through non-covalent interactions [6]. The *Pf*PMT competitive inhibitors ASN.1 and ASN.3 with the better Schizonticidal were also found to have common one scaffold acetamide like zinc hits. The acetamide interacted through the either noncovalent bonding [7] (Fig.3). Thus, the *Pf*PMT focused inhibitors given below; all four have acetamide alike thioacetamide of competitive inhibitor NSC 158011. It showed that if the druglike compound has acetamide/thioacetamide may have little better probability of being focused and competitive inhibitor of *Pf*PMT.

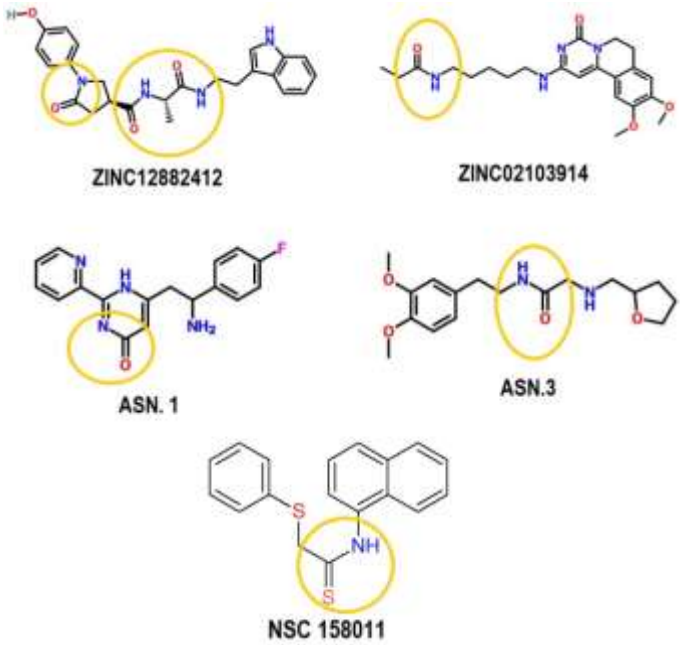


Fig 3: Drug likeness and toxicity analysis of lead compound for optimization. The scheme for modification was shown with modification sites

Hence, the combinatorial library was built replacing both the aromatic rings Naphthal-1-yl and phenyl of thioacetamide scaffold of NSC 158011 (N-naphthalen-1-yl-2-phenylsulfanylethanethioamid) were replaced with different five, six member heterocyclic rings. Large number of FDA approved drugs has heterocyclic rings pyrimidine [21, 22, 23], pyrazine [24, 25] and imidazole [26, 27, 28] scaffold because of their medicinal importance as antiparasitic, antibacterial, antiviral and anticancer etc. as well as their metabolic resistant behavior makes these scaffolds medicinally effective. The modification template substructure thioacetamide was kept constant in the modification from Lead Grow (MDS v Life sciences) and optimized for drug likeness and ADMET. The combinatorial library of 1800 analogs was developed using substitutes in Table 1.

Table 1: Substituent used for modification in combinatorial library design

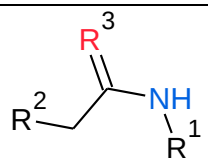
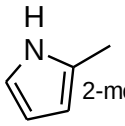
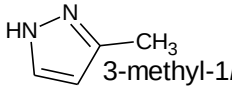
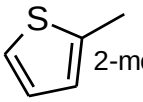
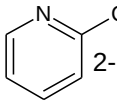
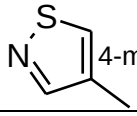
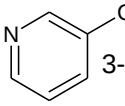
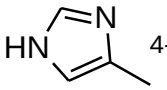
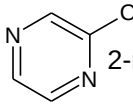
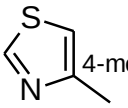
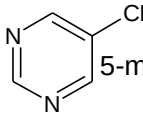
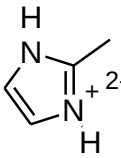
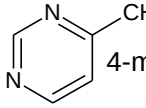
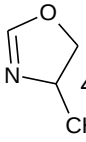
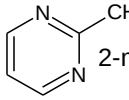
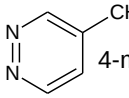
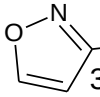
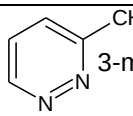


Table of Substituent for both substitution **(R_1& R_2)** sites			
1.	Benzene	16.	para-Benzaminde
2.	para-Banzoic acid	17.	para-Phenylamine
3.	ortho-Banzoic acid	18.	para-Flourobenzene
4.	para-Aceto-phenone	19.	ortho-Flourobenzene
5.	para-Ethyl phenyl ketone	20.	para-Chlorobenzene
6.	furan	21.	ortho-Chlorobenzene
7.	 2-methyl-1*H*-pyrrole	22.	 3-methyl-1*H*-pyrazole
8.	 2-methylthiophene	23.	 2-methylpyridine
9.	 4-methyl-1,2-thiazole	24.	 3-methylpyridine
10.	 4-methyl-1*H*-imidazole	25.	 2-methylpyrazine
11.	 4-methyl-1,3-thiazole	26.	 5-methylpyrimidine
12.	 2-methyl-1*H*-imidazol-3-ium	27.	 4-methylpyrimidine
13.	 4-methyl-4,5-dihydro-1,3-oxazole	28.	 2-methylpyrimidine
14.	 5-methyl-1,2-thiazole	29.	 4-methylpyridazine
15.	 3-methyl-1,2-oxazole	30.	 3-methylpyridazine
R_3 : O and S			

The library of screened through lipinski rule where the six physicochemical filters (Molecular weight, H bond acceptor, H bond donor, x LogP, rotatable bonds, and polar surface area) were analyzed. All analogues of combinatorial passed the Lipinski rule with zero violation. The toxicity screening of library revealed that the analogues containing sulfur at R_3 were found to have thioacetamide substructure as toxic and were eliminated from further analysis. The oxygen containing at R_3 could pass the toxic level and none of these found showing carcinogenic as well as mutagenic toxic properties. Since, the dissolution of an oral drug before action depends upon its solubility and absorption of a drug depend on lipophilicity (Alogp98) as well as polar surface area (PSA) for good intestinal absorption and to be systemic bioavailable to reach to the site of action. So their solubility and lipophilicity both should be within the range of physiochemical parameters. So, all the compounds obeyed Lipinski rule of five was further analyzed for computational ADMET and druglikeness. According the DS3.5 ADME prediction solubility levels 4 and absorption level 0 suggested the optimal solubility and good absorption level respectively**.** Selected compounds did not show affinity towards cytochrome enzyme (Cyp2d6), implied there is no probability of drug drug interaction within the body. Compounds did not

show Plasma proteins affinity signified the possibility of compounds to be free of retention of compounds within the body.

Therefore, the selected three analogs found to have solubility and absorption within the range and may be absorbed in the intestine and permeate the cells to reach the site of action without any drug interaction. To the TopKat module for toxicity found the compound analogs free of mutagenic and carcinogenic properties hence, selected compounds found non carcinogen, non-mutagen, and nontoxic were also found to have good druglikeness (Table 2).

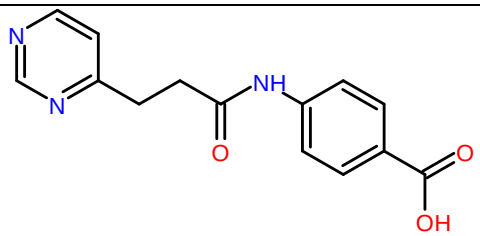
Table 2: Shown ADME, toxic physicochemical parameters analysis and Schizonticidal activity of selected top dock scoring analog compound of combinatorial compound library

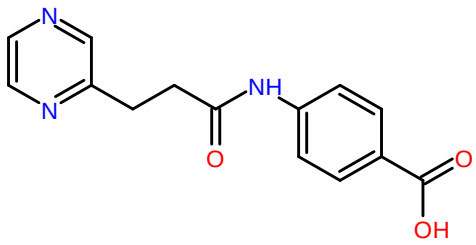
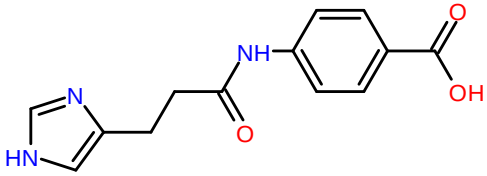
	Comp.1	**Comp.2**	**Comp.3**
IUPAC Name	4-[3-(pyrimidin-yl) propanamido] benzoic acid	4-[3-(pyrazin-2-yl) propanamido] benzoic acid	4-[3-(1*H*-imidazol-4-yl) propanamido] benzoic acid
Lipinski rule			
H-bond acceptor	4	4	4
H-bond donor	2	2	3
Rotatable bonds	7	7	7
XlogP	0.706	-0.005	0.731
Molecular weight	271.275	271.275	259.264
Polar surface area	92.18	92.18	95.08
Lipinski rule violation	0	0	0
Toxicity analysis			
Carcinogenicity(Mouse/Rat Male /Female NTP_Prediction	False	False	False
Mutagenicity(AmesPrediction	False	False	False
Toxicity	False	False	False
ADME properties			
Solubility	4	4	4
Absorption	0	0	0
Cyp2D6 Inhibition	False	False	False
Plasma Protein binding	False	False	False

3.2 Docking analysis

The known competitive inhibitor of *Pf*PMT NSC158011 was used as docking control with dock score = -8.4 and binding energy = -42.62, which was kept cutoff value for screening of combinatorial library. The analogs from ADMET filtered library which scored better docking score (>-10.0) than control were counted as good analogs with better docking score for *Pf*PMT. The binding energy of these analogs was subjected where the analogues with better binding energy (>-50.0) than the control showed better binding affinity. The compound analogs which failed to show the docking and binding energy better than the docking control were eliminated at this stage. Finally, three analogs which could show the better binding energy which also formed hydrogen bonds with either crucial tyrosine residues or catalytic dyad (Tyr19 and His132) residues of *Pf*PMT were selected. Among the analogs Comp.1 showed little more docking score -13.9 but the binding energy -78.63is quite higher than the control which showed its better affinity within the pocket But rest two analogs Comp.2 and Comp.3 showed docking score (-10.2 and -10.9) and their binding energy (-52.82 and -55.68) very close to the cut off value. The scoring implied that these may be the focused inhibitors of *Pf*PMT and may give better anti-Schizonticidal effects with good probability of being antimalarial candidate. The lower stable binding energy indicated that all these analogs are thermodynamically stable within the binding pocket (Table 3). The acetamide core, mainly C=O group of each compound analog actively participated in non-covalent interactions and formed H bonds (Fig.4) implied the reliability of designed compounds for being better antimalarial. Hence, these analogs may restrict the formation of pCholine from pEth through blocking the formation of catalytic dyad and binding of pEth (phosphoethanolamine), analogs may interestingly, give better activity.

Table 3: Substituent groups at substitution points R_1 and R_2 using vLifescinces MDS software. Docking and binding energy of the compound analogs

S. No	**Structures**
Comp.1	 XP Glide Score: **-13.9**Binding Energy: **-78.63**

Comp.2	 XP Glide Score: **-10.2**Binding Energy: **-52.82**
Comp.3	 XP Glide Score: **-10.9**Binding Energy**: -55.68**

Among the substituent, at R_1, *p*-benzoic acid could only give the acceptable XP Dock score and showed the better non-covalent interaction with important tyrosine amino acids which may be enhancing the interaction affinity towards the *Pf*PMT protein. The C=O and OH of *p*-benzoic acid provided H-bond donor as well as acceptor to the crucial residues of *Pf*PMT important for binding of pEth signified that presence of strong electro withdrawing/donating group or strong organic acid at R_1 position may be increasing the affinity (XP glide score) of analogs towards the *Pf*PMT. At the substitution position R_2, no aromatic and heterocyclic ring containing single hetero atom could show better dockscore but interestingly, heterocyclic ring with 2 N (Pyrimidine, Pyrazine and Imidazole) only illustrated the better binding affinity towards the *Pf*PMT through the vanderwaal and other non-covalent interactions with crucial residues (Fig.4). Hence, these selected three compound analogs found to have packed complementarily within the active pocket with better protein affinity as well as better druglikeness. These three analogs were got synthesized from GCC biotech (https://gccbiotech.net/) for their in vitro testing and validation.

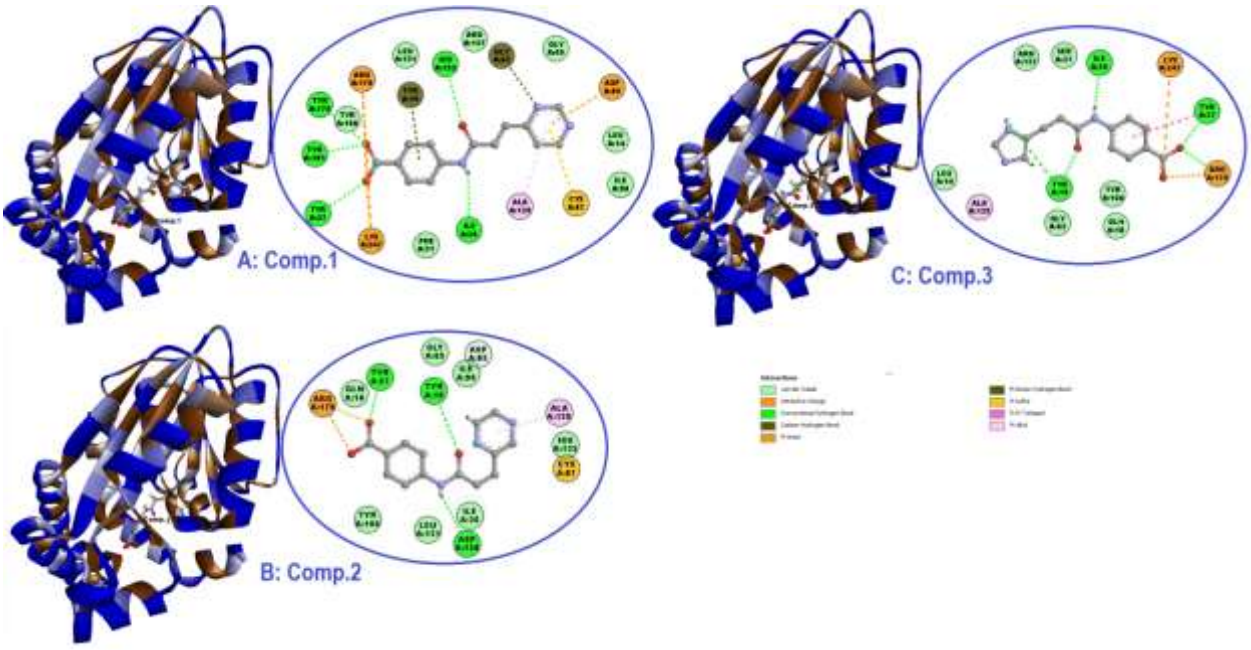


Fig 4: 2D interaction of selected drug like compounds with *Pf*PMT, showing conserved residues participating in interaction

3.3 Schizonticidal and gametocidal activity

The synthesized analogs were tested for its Schizonticidal activity against the *Plasmodium falciparum3D7* culture. On evaluating the antimalarial activity, at serial dilution revealed the schizont inhibitory effect test analogs on the growth of *Plasmodium falciparum* in blood stage. The analog Comp.1 inhibited the schizont at 3.4 µM concentration. The Comp.2 and Comp.3 inhibited the schizont at concentration 9. 65 µM and 7.18 µM respectively more than five µM of concentration nearly about 10µM IC_{50} which is not acceptable according to WHO guidelines for allowed chemical compounds concentration in blood. For being safer the analogs which showed IC_{50}Schizonticidal and gametocidal activity at concentration below 5.0µM were selected as better active hits. Fortunately, the Comp.1 showed good Schizonticidal activity against the *Pf*PMT 3D7 atIC_{50} 3.4 µM. The inhibition against the *P. falciparum* culture for all the tested analog was calculated in MS-Excel (Table 4& Fig.5).This also led us to the knowledge that the test Comp.1 may be a potent hit for further testing and inhibitor of *Plasmodium falciparum*.

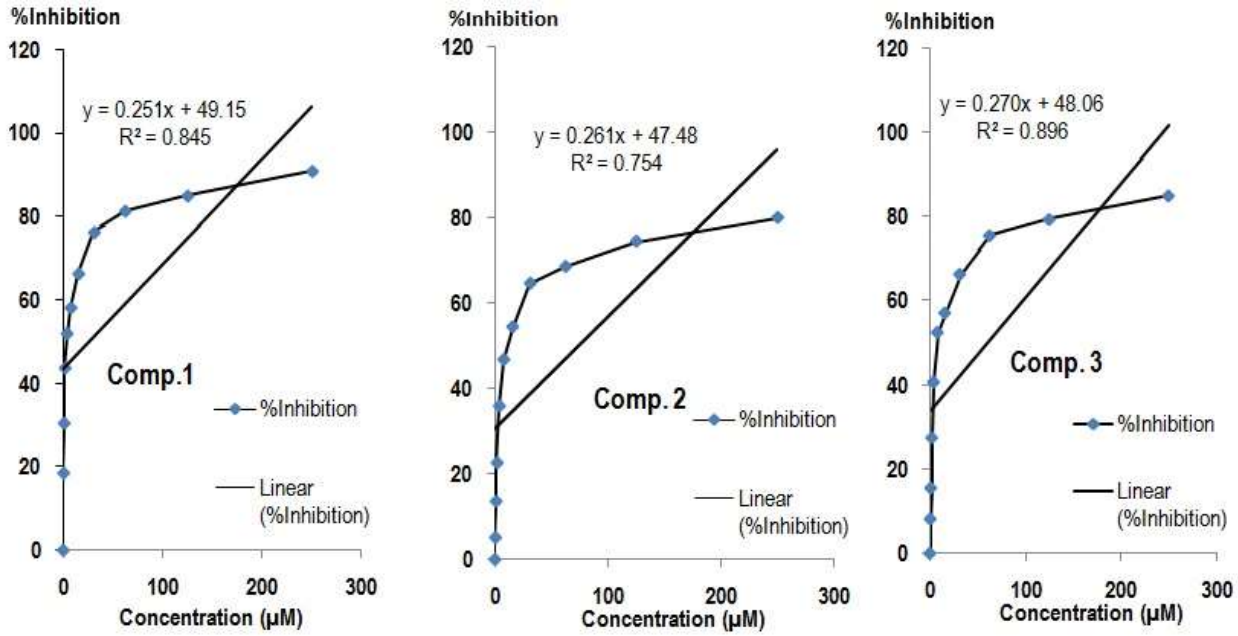
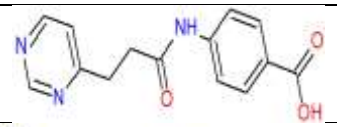
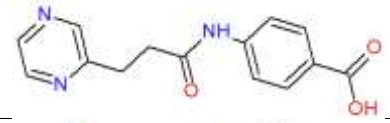
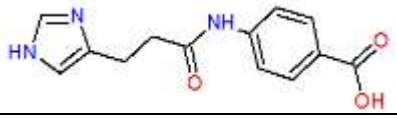


Fig 5: Schizonticidal IC_{50} graphs of the analogs against schizont *P. falciparum*3D7

These three analogs were also tested for their gametocidal activity for establishing the dual activity profile (schizonticidal and gametocidal as well) of these tested compounds. The *RKL-9* rings were cultured for gametocytes development. When the 1st stage of gametocytes is formed the three analogs were added to each well in serial dilution and incubated till the further stages developed in the control. The % inhibition of next stages of gametocytes, used to calculate the gametocidal IC_{50}. The three Analogs (Comp.1, 2 and 3) inhibited the gametocytes 4.2 µM, 12.37 µM and 9.17 µM respectively. Comp.1 was found capable of inhibiting the gametocyte at IC_{50} 4.2 µM. Analog which showed gametocytes inhibition below 5 µM were counted as better hits. These analogs showed gametocidal activity but the only Comp.1 showed the significant inhibition of 2nd and 3rd stages of gametes at 4.2 µM concentration <5 µM **(Table 4).**

Table 4: *In vitro* data and safety index of hits

	Structure	Schizonticidal IC_{50}	Gametocidal IC_{50}	LD_{50}	Selectivity index
Comp.1		3.4 µM	4.2 µM	192.91 µM	56.73
Comp.2		9. 65 µM	12.37 µM	85.74 µM	8.88
Comp.3		7.18 µM	9.17 µM	164.97 µM	22.97

Hence, Schizonticidal and gametocidal analysis implied analog Comp.1 may have the dual effects which may inhibit growth (asexual stage) and transmission (gametocyte) of the *Plasmodium falciparum* and found potent hit for consideration as antimalarial candidate. However, the analogs Comp.2 and 3 have higher gametocidal and Schizonticidal IC_{50} but these led to the information about potential of its substituted heterocyclic rings and still these three may also be further modified for activity enhancement.

3.4 Cytotoxicity testing

The three analogs found nontoxic and free of mutagenic, carcinogenic, tumorgenic and reactive functional groups in *insilico* toxicity analysis and these also possess the better ADME properties for being orally absorbed**.** The *in-vitro* toxicity and selectivity analysis of the three analogs was calculated through incubated with the 5000 HEK-293 viable cells at different dilutions as shown in the figure and % non-viability was calculated for LD_{50}192.91µM, 85.74µM and 164.97µM for Comp.1, Comp.2 and Comp.3 respectively. It was observed that the Comp.1 approximately 91 percent of the HEK-293 cells was viable at the 50 µM and even 80 % of cells were found viable at 100 µM with LD_{50} of the 192.91µM. The Comp.2 found lethal for the approx. 50% and 60% of the cells at 50 µM and 100 µM respectively and even approximately 100% cells were found nonviable at highest test concentration 250 µM. The Comp.3 was also found lethal for approximately 15% and 28% cells at 50 and 100 micromolar respectively. Test analogs were selected safer if, 50% cells were viable at selectivity index at least ten folds of their IC_{50}.

Hence, the cyto-toxicity implied that the Comp.2 and Comp.3 may be lethal at lower concentration and found toxic with selectivity index 8.88 and 22.97 respectively which found very less than the ten folds of Schizonticidal IC_{50}. But the analog Comp.1 was safer among these as 50% cell were

found viable at ten folds of IC_{50} Schizonticidal (3.4µM) activity. So the selectivity index (LD_{50}/IC_{50}) was found 89.7 µM (Fig.6, Table 4), confirmed the therapeutic safety of the analog. Thus the analog Comp.1 may have the elevated probability of being a safer antimalarial and selected for *Pf*PMT inhibition assay.

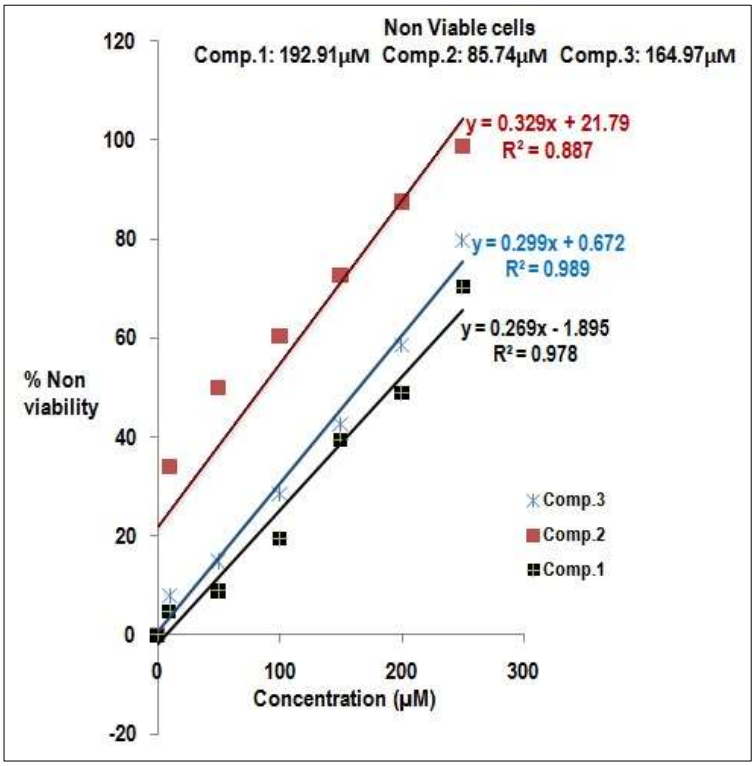


Fig 6: The*in-vitro* toxicity against HEK-293.Green dots define the no toxicity of the compounds (Toxicity Risks)

3.5 Protein Inhibition and molecular dynamics simulation

The activity of protein *Pf*PMT was assayed and increase in absorbance at 510 nm for H_2O_2 production was recorded. *Pf*PMT was also assayed in presence of different concentration of test hit Comp.1 (10µM, 8 µM, 6 µM, 4 µM, 2 µM, 1.0 µM, 0.5 µM, 0.1 µM). The 50% of protein function was inhibited at the EC_{50} calculated as 2.1 µM (Fig.7) with p value < 0.05 implied potential probability for *Pf*PMT specificity. Even the hit was capable of inhibiting the protein function at 0.1 µM concentration. The better inhibition of the *Pf*PMT may be due to the good drug-score, solubility and lipophilic profile of the Comp.1. The better antischizont activity of Comp.1 may be because, its six membered heterocyclic ring Pyrimidine (R_2) buried within the hydrophobic core of binding pocket, enhancing the hydrophobic profile of the analog and the protein affinity through the π-π shaped, π alkylation and π-donor bonding. The benzoic acid at N- terminal of the analog interacted through the hydrogen bonds with crucial tyrosine residues Tyr27, Tyr175, Tyr181 and the Tyr160 formed vander waals interaction, these residues are crucial for the binding of substrate. It also formed H-bonding with Tyr19 through the carbon hydrogen bond, where the oxo group of acetamide formed H-bond with a crucial residue His132 for formation of catalytic dyad for the triple methylation. Unfortunately, there analogs Comp.2 and Comp.3 inhibited the schizont at IC_{50} 9. 65 µM and 7.18 µM respectively**,** higher than 5.0 were not counted as active hits but these two may also be further modified to enhance their activity.

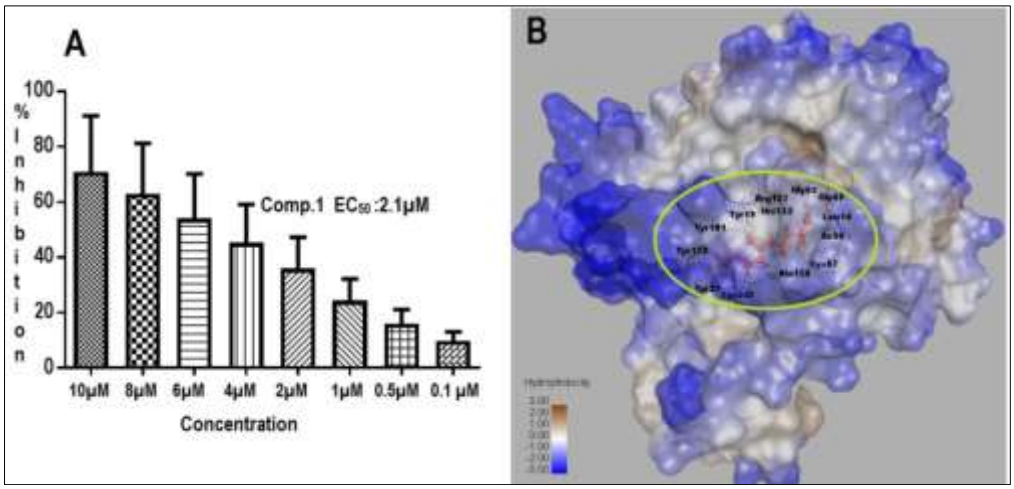


Fig 7: A Inhibition of *Pf*PMT at varying concentration of test compound Comp.1, **B** The crucial tyrosine and other interacting amino acids are shown

The molecular dynamics of protein-inhibitor complex was run for 30ns study the behavior of the complex stability of complex and their interactions during the simulation time. The protein-inhibitor complex counted total atoms 28195 were TIP3P solvated using 7970 of water. Salt (Na-29 & Cl-22) was added to the system for neutralization of the system for simulation. The distant electrostatic interactions (cutoff of 9.0 Å) were analyzed through particle-mesh Ewald method. A distance was used for short-range electrostatics and Lennard-Jones interactions. The trajectories were visualized in Desmond Maestro.

The protein ligand graph RMSD showed that the protein ligand complex got stabilized till reaching the end of simulation. The backbone (alpha helix and beta sheets) of the protein did not show much fluctuation and got stable till the simulation end. The sidechain showed little more RMSD fluctuation than backbone which also got stable till the end of simulation at 28Å RMSD (Fig.8). The ligand (Comp.1) showed no fluctuations till 10ns but after that ligand showed sudden fluctuation in RMSD from 1.5Å to 2.5Å which got stable at 2.1Å till the end of simulation. As per the ligand fit on protein and ligand fit ligand graph, the Comp.1 found to go under the conformational changes within the binding pocket but till the simulation end the Comp.1 was found to attain stability or stable conformation within the pocket. There the RMSF graph also showed the fluctuations of amino acids throughout the simulation between the range of RMSD (0.4Å-2.7Å). The sidechains and the heavy atoms underwent more fluctuations then the backbone of residues (Fig.8).The protein and ligand showed thermodynamic stability individually and in complexed state as well till the end of simulation for 30ns.

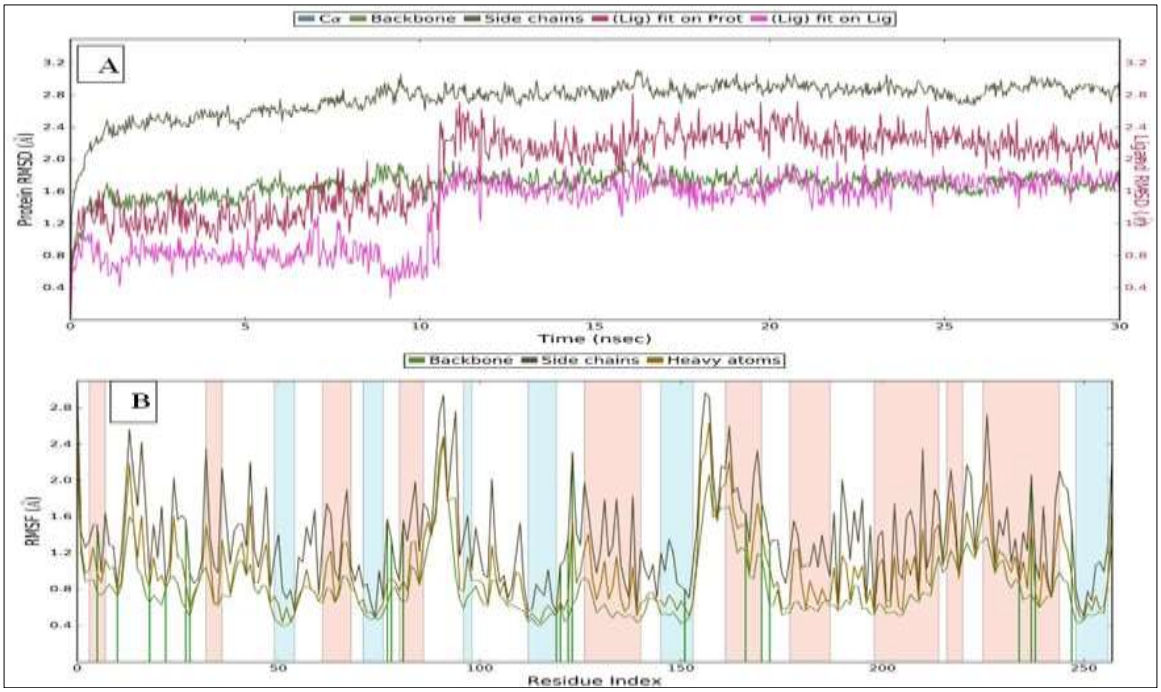


Fig 8: A The RMSD of protein and ligands with respect to the time of simulation, **B** The RMSF (root mean square fluctuation) of backbone, sidechain and heavy atoms throughout the simulation

The Comp.1 had six rotatable bonds shown in different colors. All the bonds were found to undergo conformational adjustment. But the two rotatable bonds, one in bonds connecting benzene and carboxylic acid, another is bond connecting benzene and nitrogen of amide substituent showed more conformational changes of the torsion throughout the course of the simulation implied that the benzoic acid underwent more conformational adjustments to achieve lowest possible energy for stable interaction within the pocket. The contacts of Comp.1 with crucial residues Tyr19, Tyr160, Tyr181 and Lys247 were remained intact throughout the simulation. The His132 showed interaction with the Comp.1 till the 10ns. Hence the good EC_{50} of the protein at 2.1µM and the thermodynamic stability of Comp.1 within the binding pocket implied that the analog may be having the protein specificity and may be utilized further as lead molecule for rational drug designing (Fig.9).

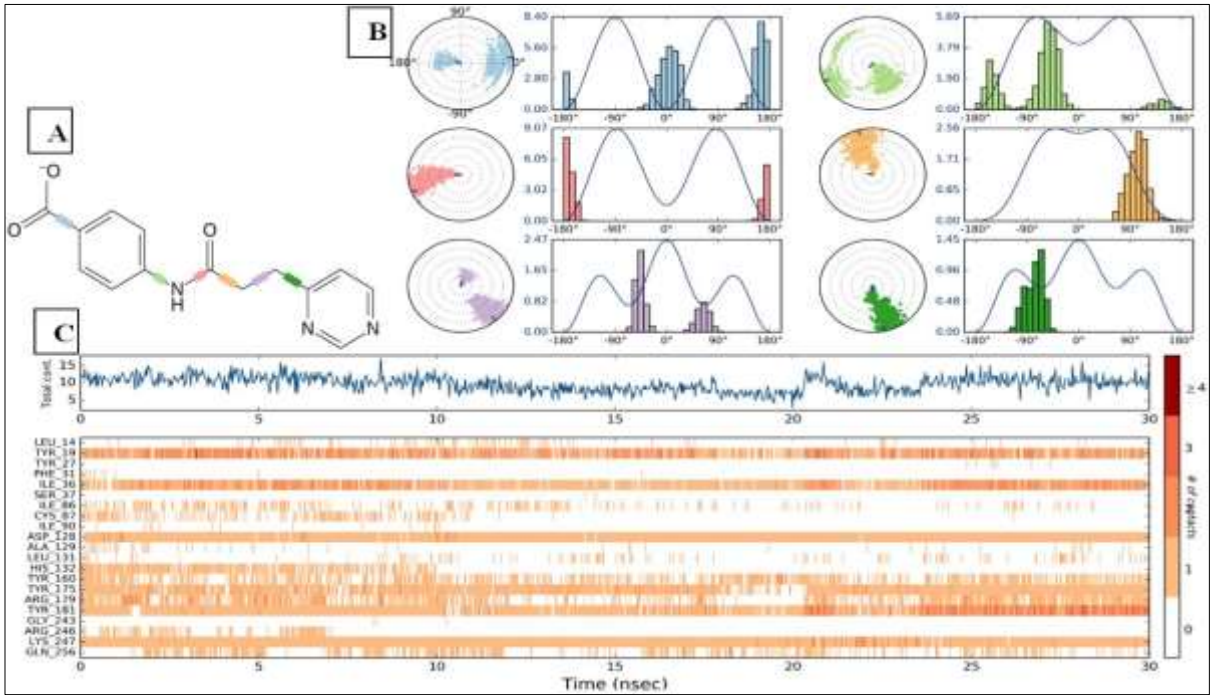


Fig 9: A The rotatable bond of structure of Comp.1 in different colors, **B** Describe the conformational of the torsion throughout the course of the simulation, **C** A timeline representation of the interactions and contacts during the dynamic simulation

4. Conclusion

The phosphatidyl choline is a phospholipid essentially required for the rapid membrane development for growth, development and rapid multiplication as well. Moreover, the absence of this protein in human host makes it an excellent drug target enzyme for rational development of new antimalarial. Hence, the *Pf*PMT and its known inhibitor were used for chemo informatics based identification of inhibitors of the potential target. The combinatorial library developed of based on the information of competitive inhibitors of *Pf*PMT screened through Lipinski rule, ADMET physicochemical properties and binding affinity. Approach successfully led to identify of druglike compound analogs which occupied the crucial catalytic dyad (Tyr19 & His132) and also interacted with other important tyrosine residues (Tyr160, Tyr175 & Tyr181).

According to the computational analysis the benzoic acid at R_1 could participate in better dockscore and binding energy. At R_2 Pyrimidine, Pyrazine and Imidazole (containing two hetero atoms) heterocyclic rings could show better complementary profile helped to attain the lowest energy conformation into the binding pocket for better packing of the analogs. All the synthesized three analogs showed Schizonticidal and gametocidal activity but Comp.1 could show better druglikeness profile with Schizonticidal IC_{50} 3.4µM and Gametocidal IC_{50} 4.2 µM (<5 µM) and LD_{50}192.91µM which implied the better safety index of 56.37 µM, ten folds higher than even the gametocidal IC_{50}. The protein inhibition (EC_{50})at 2.1µM lower concentration than its IC_{50} revealed its better protein specificity where MD simulation also added the confirmation that the Comp.1 ligand formed thermodynamically stable interact with protein into binding site and interaction with crucial residues were also sustained through the whole simulation. As per the medicinal compounds information the Pyrimidine and substituted benzene rings is the part of many of FDA approved drugs. Hence, the analog Comp.1has druglike properties may prevent the biological function of *Pf*PMT which may be validated as antimalarial candidate. The studied analogs may also further be led to the rational drug discovery for a more potent antimalarial which may act as potent inhibitors of *Pf*PMT and orthologues globally to overcome the problem of multi drug resistance.

5. Declarations

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Ethical approval: The approval from Human Ethical Committee of ICMR-National Institute of Malaria Research, New Delhi was received(ECR/NIMR/EC/2015/206).

Consent to participate: Not applicable

Competing interests: We have no conflicts of interest to declare.

Availability of data and materials: Not applicable

Code availability: Not applicable

Authors' contributions: JS, AS and MK- Computational and *in vitro* studies, RM- analysis of computational results, SV, PKA - help out in *in-vitro* result analysis. All the authors of the Manuscript approved the submitted manuscript.

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