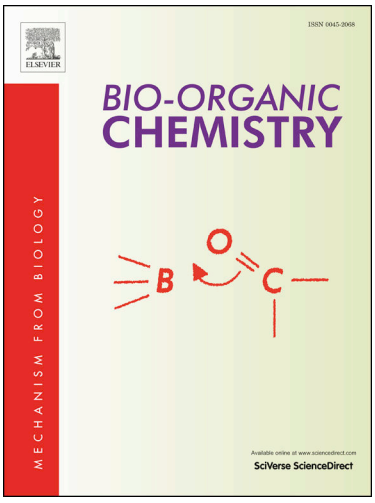
Journal Pre-proofs



Quinoline carboxamide core moiety-based compounds inhibit *P. falcipa-rum*falcipain-2: Design, synthesis and antimalarial efficacy studies

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Quinoline carboxamide core moiety-based compounds inhibit *P. falciparum* falcipain-2: Design, synthesis and antimalarial efficacy studies

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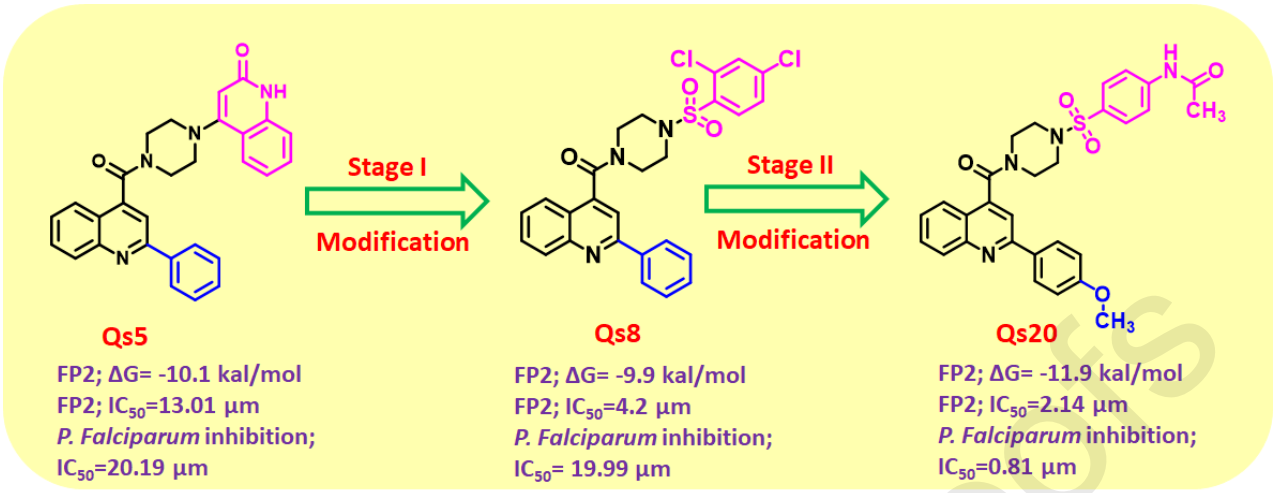


Graphical abstract

Abstract

Targeting Falcipain-2 (FP2) for the development of antimalarials is a promising and established concept in antimalarial drug discovery and development. FP2, a member of papain-family cysteine protease of the malaria parasite *plasmodium falciparum* holds an important role in hemoglobin degradation pathway. A new series of quinoline carboxamide based compounds was designed, synthesized and evaluated for antimalarial activity. We integrated molecular hybridization strategy with *in-silico* drug design to develop FP2 inhibitors. *In- vitro* results of FP2 inhibition by **Qs17, Qs18, Qs20** and **Qs21** were found to be in low micromolar range with IC_{50} 4.78, 7.37, 2.14 and 2.64 µM, respectively. Among the 25 synthesized compounds four compounds showed significant antimalarial activities. These compounds also depicted morphological and food-vacuole abnormalities much better than that of E-64 an established FP2 inhibitor. Overall these aromatic substituted quinoline carboxamides can serve as promising leads for the development of novel antimalarial agents.

Keywords: Quinoline carboxamide; Falcipain-2 (FP2); *P. falciparum*; Malaria; drug development.

1. Introduction

Malaria is one of the most life menacing neglected disease caused by protozoan parasites of genus plasmodium. Approximately two hundred million infections and 438,000 deaths in a year, predominantly children have been reported by WHO due to malaria [1]. Majority of the antimalarial drugs reported till date target the asexual blood-stages of the parasite where it replicates within human erythrocytes [2]. However, liver- and transmission-stage of the parasite do not cause malaria symptoms. Due to the complex life cycle of the parasite and increasing emergence of resistance against conventional antimalarials and artemisinin combination therapy malaria remains a challenging issue for the humanity. The identification of new apt drug targets and the development of effective anti-malarial molecules that target important parasite functions with a potential to act against multi-drug-resistant strains of the parasite is an indispensable need to prevent malaria pathogenesis and its transmission [3].

Proteases are among the attractive drug targets for the development of antimalarial therapy. Falcipain-2 (FP2) and falcipain-3 (FP3) are papain-family cysteine proteases of erythrocytic stages of *P. falciparum* that localize to the food vacuole and readily hydrolyze hemoglobin [4,5]. Disruption of the FP2 gene led to the accumulation of undegraded hemoglobin in trophozoites, confirming a critical role of this enzyme in hemoglobin hydrolysis [6]. Inhibition of FP2 and related proteases led to the inhibition of parasite development [7,8] and cured mice with murine malaria [9,10].

E64 **(Figure 1),** an irreversible inhibitor of cysteine proteases, displayed a significant outcome on growth, adherence and viability of parasite trophozoites [11]. Chiyanzu *et al*. [12] designed a new class of thiosemicarbazones with isatin scaffold as FP2 inhibitors. The most promising FP2 inhibitor of this series **(Figure 1a)** showed an inhibition with IC_{50} 4.4 μM. From such results, chalcones proved to be the robust inhibitors of cysteine proteases. Firstly, Li and co-workers in 1995 started working over the development of antimalarial chalcones as one of the derivatives: 1-(2,5-dichlorophenyl)-3(4-quinolinyl)- 2-propen-1-one **(Figure 1b)** showed an IC_{50} value of 200 nM against both CQ-resistant strain (W2) and CQ-sensitive strain (D6) of *P. falciparum* [13,14]. Several peptide-based antitypanosomal agents such as compound **c (Figure 1c)** have been found to inhibit FP2 of cysteine protease rhodesain and *P. falciparum* of brucei rhodesiense.

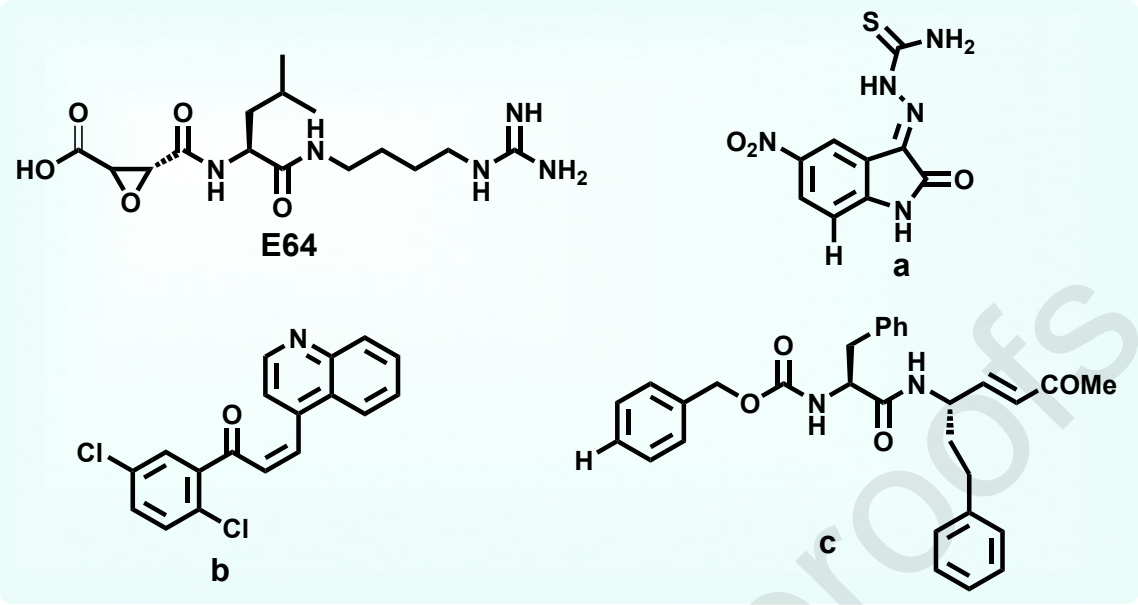


Figure 1. *Structure of some known potential FP2 inhibitors.*

1.1. Design strategy for the new cysteine proteases inhibitors of *P. falciparum*

Recently, (DDD107498), a quinoline-4-carboxamide has been reported with excellent pharmacokinetic and antimalarial properties, including activity against multiple life-cycle stages of the malaria parasite [15]. Compound SC81458 and the clinical development candidate, SC83288 **(Figure 2)**, both containing sulfonamide and piperazine groups, showed noteworthy results to cure a *P. falciparum* infection with tolerable toxicity. Properties like quick parasite killing, good safety margin, a potentially different mode of action and a distinct chemotype support SC83288 for the clinical trials against malaria [16]. The quinoline-4-carboxlic acid and its analogs show diverse range of therapeutic activity such as antimalarial, antifungal and anti-leishmanial effects [17,18].

Dual inhibitors of FP2 and FP3 have been identified through virtual screening of 241000 compounds against homology models of FP2 and FP3 in three consecutive stages of docking [19]. Benzothiazole containing sulfonyl-2-nitrobenzene based compounds, for example compound **1 (Figure 2)** have been found to inhibit FP2 with IC_{50} 11.14 μM. These compounds have been prophesied to bury into the S2 pockets of FP2 and FP3, thereby inhibit both the enzymes significantly [20]. Same way, potential antimalarial agents containing 4-aminoquinoline with natural product isatin scaffold as in compound **2 (Figure 2)** have been discovered with IC_{50} 1.3–0.079 and 2.0–0.050 μM against a chloroquine-sensitive (D10) and two resistants (K1 and W2) strains of *P. falciparum*. Two such isatin based compounds **3** and **4 (Figure 2)** displayed *in-vitro*

activity against K1 and W2 strains with IC_{50} values of 51 and 54 nM, respectively. In addition, these compounds were found to inhibit parasitic cysteine protease FP2 [21]. Keeping these things into consideration, we attempted to incorporate different functional units of reported FP2 inhibitors and different clinical candidate antimalarials in one molecule as represented in **(Figure 2)**. Integration of structure based drug design technique and molecular hybridization was used to incorporate potential scaffolds in the designed molecules.

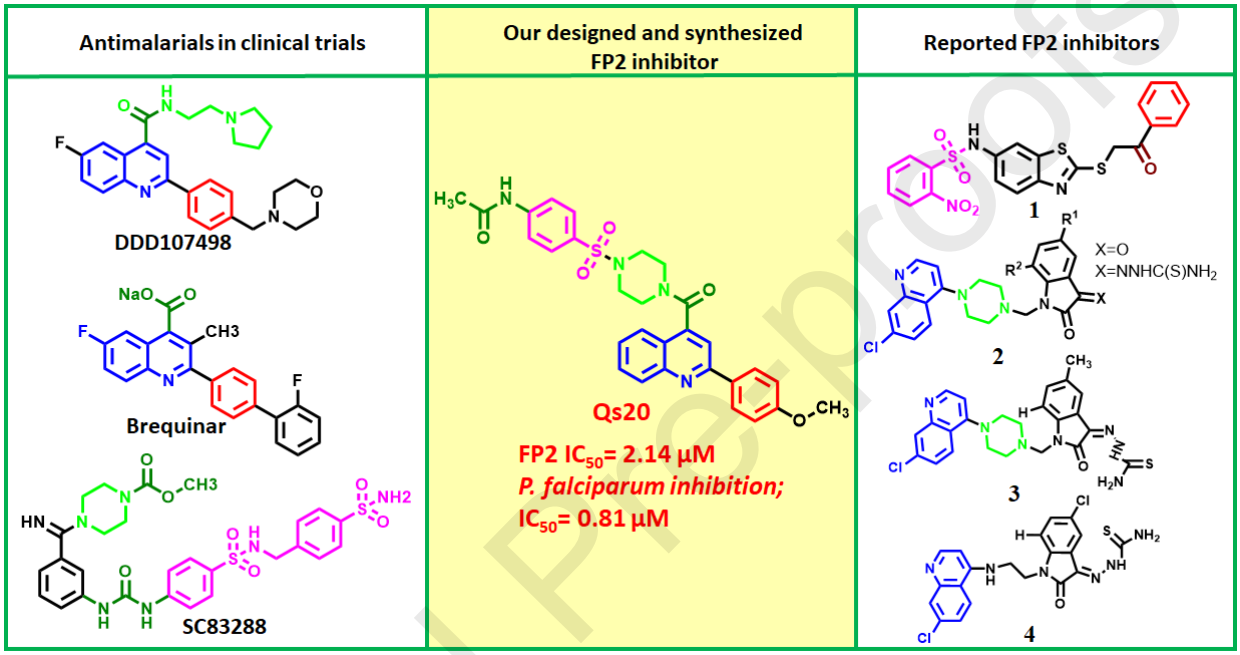


Figure 2. *Rational for the design and synthesis of quinoline-carboxamide as FP2 inhibitors.*

Here, we report the design, synthesis and biological evaluation of quinoline-4-carboxamide based analogues as FP2 inhibitors. The core moiety quinoline was prepared from isatin and acetophenone **(Scheme 1)** using the Pfitzinger reaction. We utilized diversity-oriented route to design and synthesize the target molecules. The 25 synthesized molecules were identified as potential inhibitors of FP2, among which the best compound **QS20** displayed FP2 inhibition with IC_{50} value = 2.14 µM. In addition, the anti-plasmodial activity of these molecules against *P. falciparum* was tested in which **QS20** exhibited better parasite inhibition with IC_{50} = 0.81 µM.

2. Results and discussion

2.1. *In silico* design of potential FP2 inhibitors

In an effort to investigate the plausible modes of action of compounds to act as antimalarials as well as to predict molecular orientation at the active site, the docking simulations

were performed *via* AutoDock Vina program [22]. The docking study was performed on reported crystal structure of FP2 (PDB ID: 3BPF) which was retrieved from RCSB. The computational binding free energy (ΔG) of the designed compounds was calculated and is mentioned in **Table 1**. Docking results suggested that compounds **Qs17, Qs18, Qs20** and **Qs21** could effectively bind to the active site of FP2 **(Figure 3)** with binding energy of **-10.9, -11.3, -11.9** and **-10.9 kcal/mol**, respectively. E64 a well-known FP2 inhibitor was taken as the reference. The binding energy for all these compounds was found to be optimistically favorable than E64 having free energy of complexation -7.9 kcal/mol. As observed from the docking studies, compounds **Qs17** and **Qs18** were found to show π-π interaction with Trp206 and H-bond with Asp170 **(Figure 3a,c)**. In addition, **Qs20** displayed π-π stacking interactions with Trp206, Trp210, Phe156 **(Figure 3e)**. In addition, a strong H-bond was observed between oxygen of p-methoxy benzene of **Qs20** and H of Gln171. Same way, **Qs21** showed π-π stacking interactions with Trp206, Trp210, His174 **(Figure 3g)**. **Qs21** also participated strongly in H-bonding with Asp154 and Gln171. These cumulative effects could be the favorable factor behind high potency of these molecules. Along with that, the docking pose analysis suggested that quinoline-carboxamide scaffold wraps around the key residues of the enzyme and provide perfect snug fit at the active site. In addition to that, geometrical complementary is further strengthened by favorable residue interaction with the molecule. However, the standard FP2 inhibitor, displayed only H-bonds with Trp206, Val152, Gln209 and Asn16, Glu14 Lys37 as shown in **(Figure 3i,j)**. Since E64 lacks an aromatic system and is unable to take part in π-π interactions, may be the reason for its less binding affinity with the enzyme.

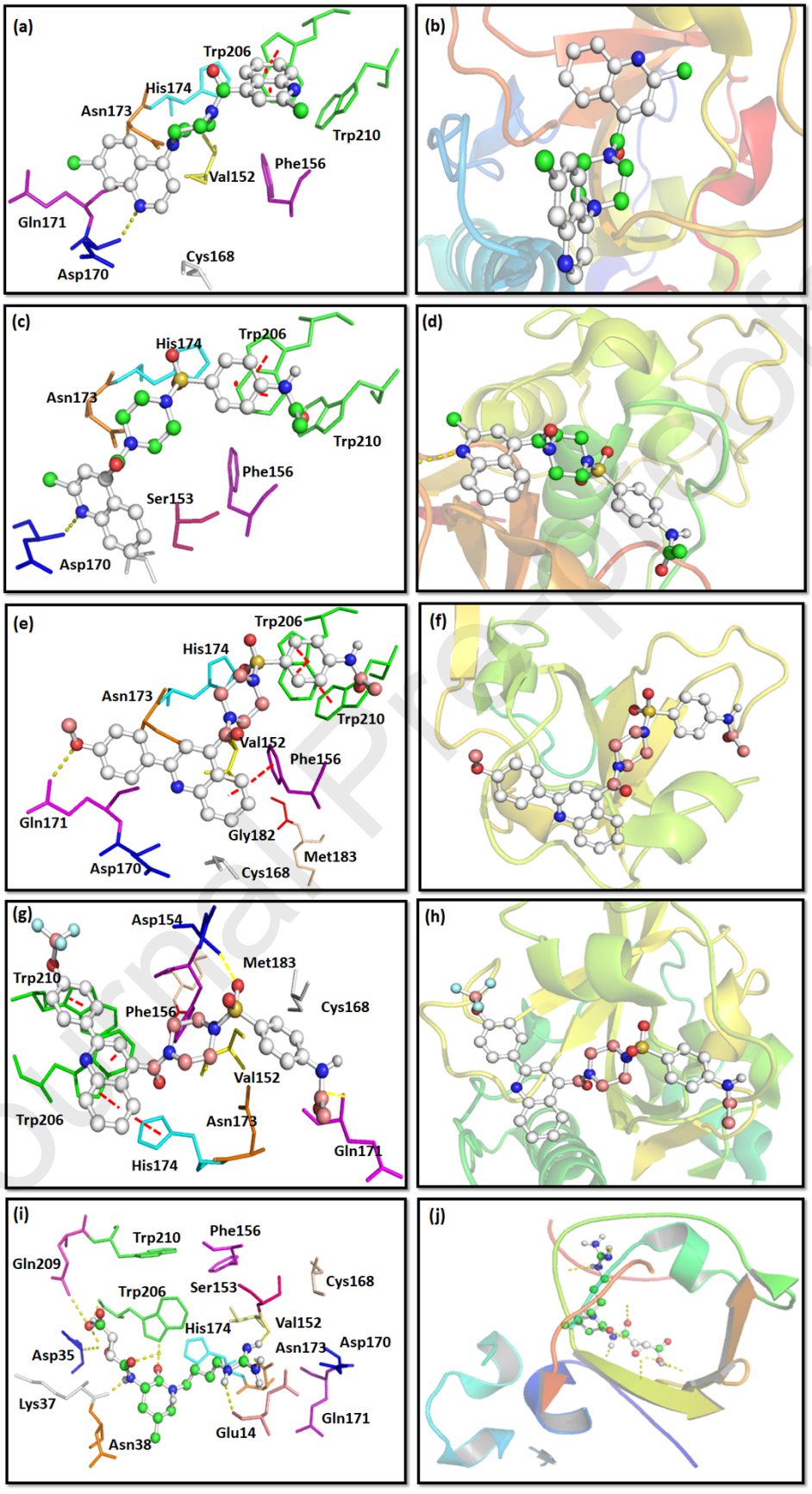

(a)
Trp206
His174
Asn173
Trp210
Phe156
Val152
Gln171
Cys168
Asp170
(b)
(c)
His174
Trp206
Asn173
Trp210
Phe156
Ser153
Asp170
(d)
(e)
Trp206
His174
Asn173
Trp210
Val152
Phe156
Gln171
Gly182
Met183
Asp170
Cys168
(f)
(g)
Asp154
Met183
Trp210
Phe156
Cys168
Val152
Trp206
Asn173
His174
Gln171
(h)
(i)
Trp210
Phe156
Gln209
Cys168
Trp206
Ser153
His174
Val152
Asn173
Asp170
Asp35
Lys37
Glu14
Gln171
Asn38
(j)

Figure 3. *(a) Binding interaction of compound* ***Qs17*** *with active site residues of FP2. (b) One of the docking poses of* ***Qs17*** *with FP2. (c)Binding interaction of compound* ***Qs18*** *with FP2 (d) one the docking poses of* ***Qs18*** *with FP2. (e)(f) Docking interaction of* ***Qs20*** *with FP2 active site. (g)(h) Interaction of* ***Qs21*** *with the active site residues of FP2. (i)(j) Interaction of* ***E64*** *with the active site residues of FP2. Figures were generated with Pymol. H-bonds are indicated with yellow dashed lines and π-π stacking are shown by red dashed lines.*

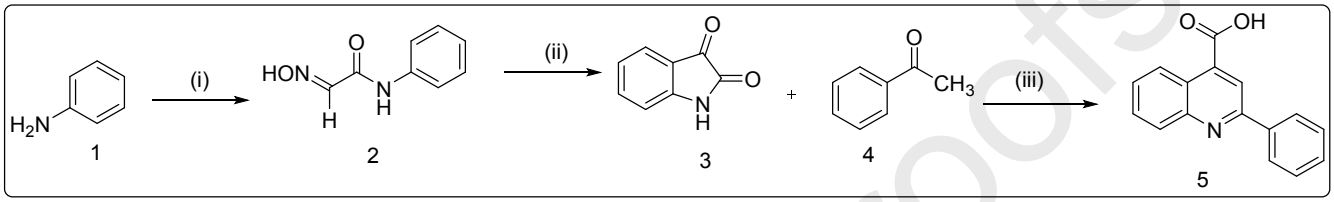
Table 1: *The calculated free energies of selected derivatives in molecular docking using ParDock* [23] *and AutoDock Vina program.* [22].

Compounds	Complexation energy (kcal/mol) of compounds with FP2 using ParDOCK	Complexation energy (kcal/mol) of compounds with FP2 using AutoDock
QS1	-8.81	-9.1
QS2	-10.11	-10.4
QS3	-9.42	-10.1
QS4	-10.07	-10.1
QS5	-10.11	-10.1
QS6	-8.91	-9.8
QS7	-9.01	-8.9
QS8	-8.81	-9.9
QS9	-9.51	-9.3
QS10	-9.98	-10.1
QS11	-8.20	-8.0
QS12	-10.01	-9.0
QS13	-10.89	-11.0
QS14	-7.98	-8.2
QS15	-8.41	-8.6
QS16	-10.01	-9.0
QS17	**-9.98**	**-10.9**
QS18	**-10.45**	**-11.3**
QS19	-9.34	-9.1
QS20	**-10.98**	**-11.9**
QS21	**-10.45**	**-10.9**
QS22	-10.01	-9.2
QS23	-8.01	-8.1
QS24	-7.30	-9.0
QS25	-8.01	-8.1
E64	*-6.14*	-7.9

2.2. Chemistry

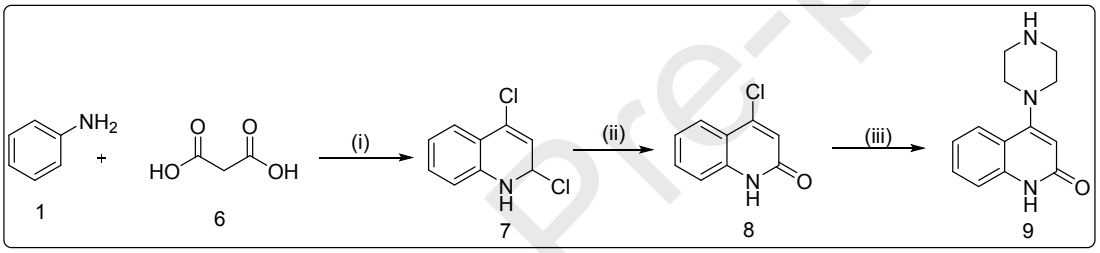
The target compounds were synthesized *via* different organic synthesis routes as depicted in the following reaction schemes. In addition, the synthesis of some substituents is described in the supporting information file.

Scheme 1



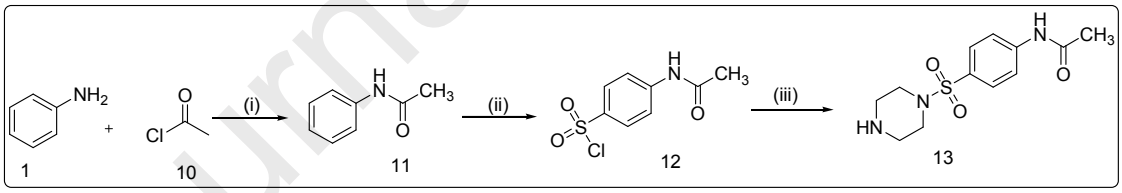
Reagents and conditions: (i) $Cl_3CC(OH)_2$ NH_2OH, HCl, H_2O, Δ (ii) H_2SO_4, 60-80℃(iii) 30% KOH, EtOH/ water, 125℃.

Scheme 2

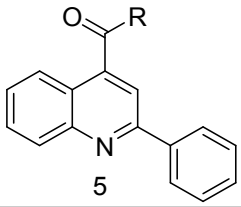


Reagents and conditions: (i) $POCl_3$ (ii) HCl or 1:4 dioxane (iii) piperazine, K_2CO_3, ethylene glycol

Scheme 3



Reagents and conditions: (i) DCM,0℃, 1 h (ii) $ClSO_3H$, 0 C, 1 h (iii) Piperazine, DCM 0℃, 2 h.



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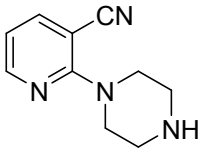
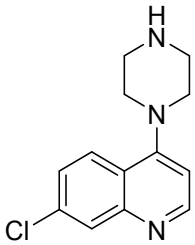
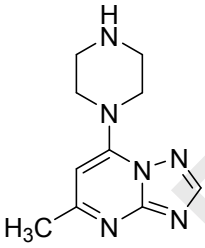
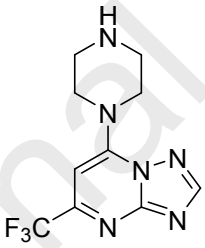
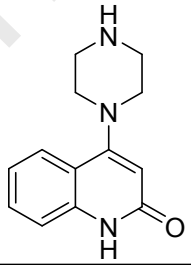
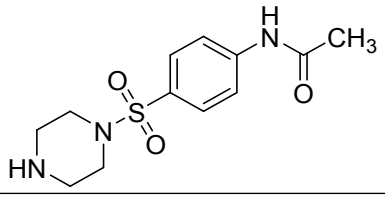
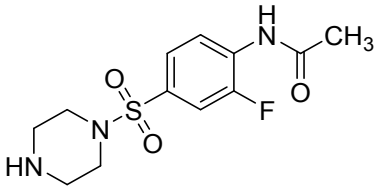
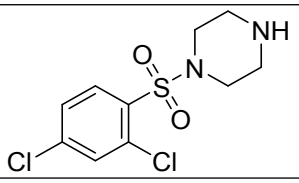
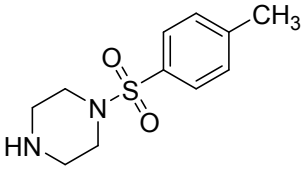
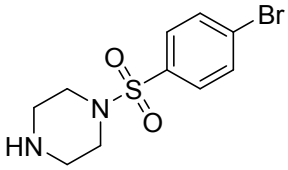
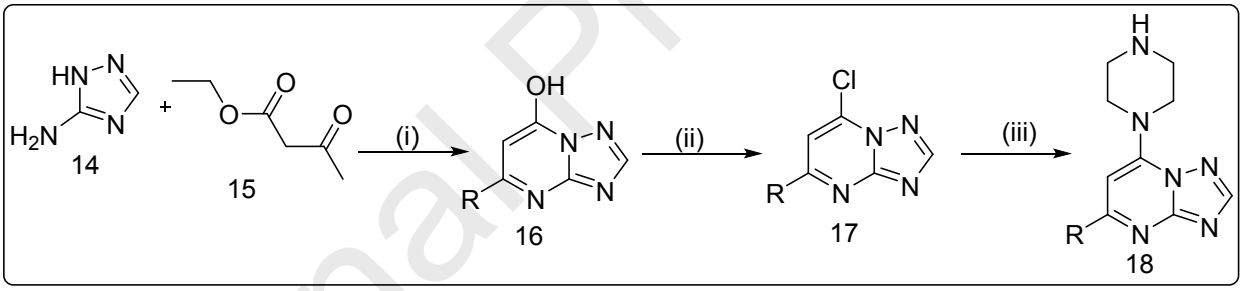
Compounds name	R	Reagents and conditions
Qs1		TEA, HBTU, DMSO, 12 h, rt [24]
Qs2		TEA, HBTU, DMSO, 10 h, rt [25]
Qs3		TEA, HBTU, DMSO, 18 h, rt.
Qs4		TEA, HBTU, DMSO, 18 h, rt.
Qs5		TEA, HBTU, DMSO, 12 h, rt.
Qs6		TEA, HBTU, EDC, DMSO, 7 h, rt.

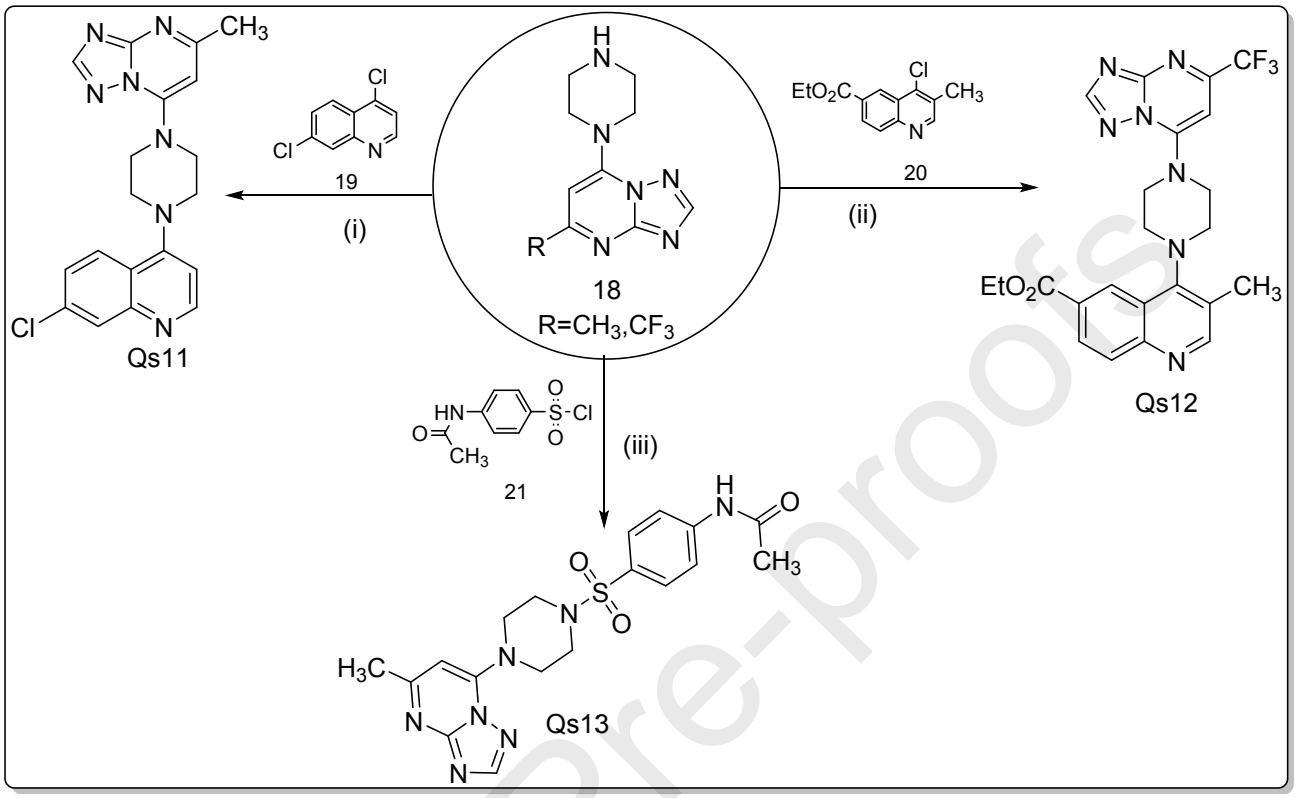
Qs7		TEA, HBTU, EDC, DMSO, 7 h, rt.
Qs8		TEA, HBTU, EDC, DMSO, 6 h, rt.
Qs9		TEA, HBTU, EDC, DMSO, 7 h, rt.
Qs10		TEA, HBTU, EDC, DMSO, 7 h, rt.

Scheme 4



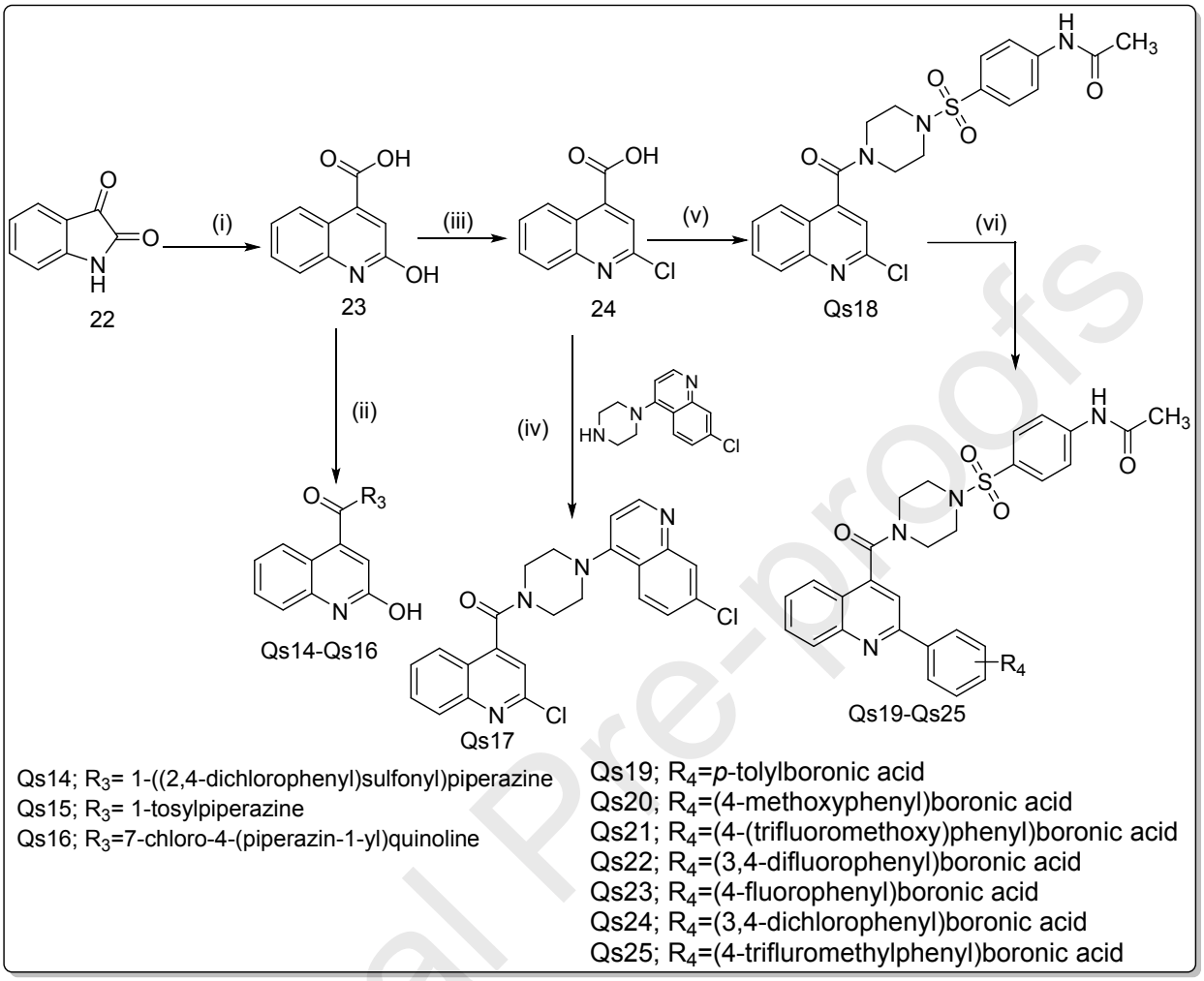
Reagents and conditions: (i) acetic acid, 110℃ (ii) $POCl_3$, 110℃ (iii) 1,4-dioxane, K_2CO_3 piperazine 110℃

Scheme 5



Reagents and conditions: (i)1,4-dioxane, 100℃, K_2CO_3 12h.(ii) 1,4-dioxane, 100℃, K_2CO_3 8h (iii) 1,4-dioxane, 100℃, K_2CO_3 48 h.

Scheme 6



Reagents and conditions: (i) malonic acid, acetic acid, 12 h reflux (ii) R_3, TEA, HBTU, DMSO, 7 h at rt. (iii) $POCl_3$ reflux 2 h (iv) TEA, HBTU, DMSO, 7 h at rt. (v) TEA, HBTU, DMSO, 7 h at rt (vi) Pd $(PPh_3)_4$, K_2CO_3, Aryl boronic acid, Toluene, reflux, 8 h.

2.3. Anti-malarial screening of the synthesized compounds

2.3.1. Inhibition of FP2

The inhibitory activities of the synthesized compounds were analyzed by assessing their ability to block the *in vitro* protease activity of recombinant FP2, as well as their antiplasmodial activities **(Table 2)**. A total of 25 compounds were evaluated in the protease inhibitory assay, and the most potent inhibitors from this enzymatic assay were further evaluated in the parasite cultures as antimalarial agents. In the assays targeting FP2 **(Table 2)**, four compounds, **Qs17**, **Qs18, Qs20** and **Qs21** exhibited micromolar inhibition values ranging from 2.14 to 7.37 μM. Although, all the

compounds inhibited FP2 moderately however few compounds exhibit an average inhibition of FP2.

2.3.2. Effect of Quinoline-4-carboxamide derivatives target compounds on FP2 activity and parasite growth

The inhibition effect of quinoline carboxamide compounds were analyzed on the activity of recombinant FP2 protein as well as on *P. falciparum* growth and development. Recombinant FP2 was produced by following a protocol described by Shenai *et al*.[4] and Kumar *et al* [26] shows the expression, purification and refolding of FP2 protein. The refolded protein was catalytically active as it cleaved the enzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin hydrochloride (ZFR-AMC), a substrate of FP2, in a dose dependent manner. Twenty-five quinoline carboxamide derivatives were evaluated protease assay, as shown in **(Table 2),** all the tested compounds showed the inhibitory activity in micromolar range towards the inhibition of FP2. Compounds **Qs8**, **Qs9**, **Qs10**, **Qs16**, **Qs17**, **Qs18**, **Qs20**, **Qs21** showed the best activity with IC_{50} values less than (10 μM).

Furthermore, the inhibitors were then analyzed for their antimalarial activities in a *P. falciparum* 3D7 culture. Ten compounds (**Qs3**,**Qs4**,**Qs11**,**Qs12**,**Qs16**,**Qs17**,**Qs18**,**Qs20**,**Qs21**, **Qs24**) inhibited *P. falciparum* 3D7 cultures with IC_{50} ranging from 0.81 ± 0.31 μM to 5.47± 0.74 μM. Compound **Qs20** was found to be the most potent parasite growth inhibitor, with an IC_{50} of 0.81 ± 0.31μM, which correlated well with the inhibition of FP2 (IC_{50}= 2.14 ± 0.64 μM). Interestingly the best results for FP2 inhibition was obtained for the two compounds **Qs20** and **Qs21** obtained *via* modification of quinoline carboxamides core moiety containing quinolin-2(1H)-one by the sulfonyl-phenyl-acetamide along with 4-methoxy benzene attached to the quinolin-piperazin-methanone, which specifies the importance of aromatic substitution and sulphonyl actamide incorporation for enhanced anti-malarial activity. The *in-silico* results obtained were in accordance with the *in vitro* studies and indicates the potential of compounds **Qs20** and **Qs21** as effective FP2 inhibitors.

Table 2: *In vitro inhibitory effect of Quinoline-4-carboxamide compounds against recombinant FP2 and P. falciparum 3D7 (IC_{50}) growth in vitro.*

Compounds	Inhibition of FP2 IC_{50} (µM)	Std. error	Inhibition of *P. falciparum* IC_{50} (µM)	Std. error
Qs1	18.20	0.70	19.70	1.05
Qs2	13.79	1.12	NA	NA
Qs3	25.20	1.95	2.82	0.52
Qs4	17.44	0.60	1.47	0.41
Qs5	13.01	1.31	20.19	1.07
Qs6	15.82	1.34	20.45	0.09
Qs7	20.22	1.53	NA	NA
Qs8	4.2	0.57	19.99	1.05
Qs9	3.47	0.50	NA	NA
Qs10	6.14	1.02	23.5	0.24
Qs11	NA	NA	1.87	0.53
Qs12	16.55	1.43	1.07	0.35
Qs13	NA	NA	NA	NA
Qs14	NA	NA	65.53	0.65
Qs15	NA	NA	NA	NA
Qs16	3.19	1.21	5.47	0.74
Qs17	**4.78**	**1.12**	**1.05**	**0.45**
Qs18	**7.37**	**1.13**	**1.95**	**1.95**
Qs19	NA	NA	20.32	0.74
Qs20	**2.14**	**0.64**	**0.81**	**0.31**
Qs21	**2.64**	**0.28**	**1.43**	**0.45**
Qs22	32.72	4.3	25.31	0.90
Qs23	35.3	1.76	NA	NA
Qs24	29.72	2.4	3.82	0.76
Qs25	13.28	2	NA	NA

NA = Not Applicable

2.3.3. Effect of Quinoline-4-carboxamide derivatives on parasite development

Cysteine protease inhibitors are well known to cause morphological and developmental abnormalities, mainly in the food vacuole of the parasites [6]. Based on the overall potency against FP2, *P. falciparum* growth inhibition and *in silico* activity of compounds **Qs17**, **Qs18**, **Qs20** and **Qs21** were selected to study the effect over morphology and development of *P. falciparum* through its asexual stage. Briefly, the ring stage (8-10 h) parasites were treated with these compounds or solvent alone as a control. In control, parasites developed normally from ring to trophozoite stage, and then turned to the schizont stage and merozoites. These merozoites reinvaded fresh RBC to form rings. Cultures treated with a known cysteine protease inhibitor E64 (10 μM) showed severe food vacuole abnormalities at trophozoite stage along with clumps of malarial pigment, indicating abrogation of hemozoin production in these parasites. *P. falciparum* cultures when treated with **Qs17, Qs18, Qs20 and Qs21** morphological and food-vacuole abnormalities were seen followed by developmental arrest at the trophozoite stage. These phenotypes observed were like those depicted in parasites treated with E64. However, the effect for all four compounds was better than the E64 **(Figure 4).**

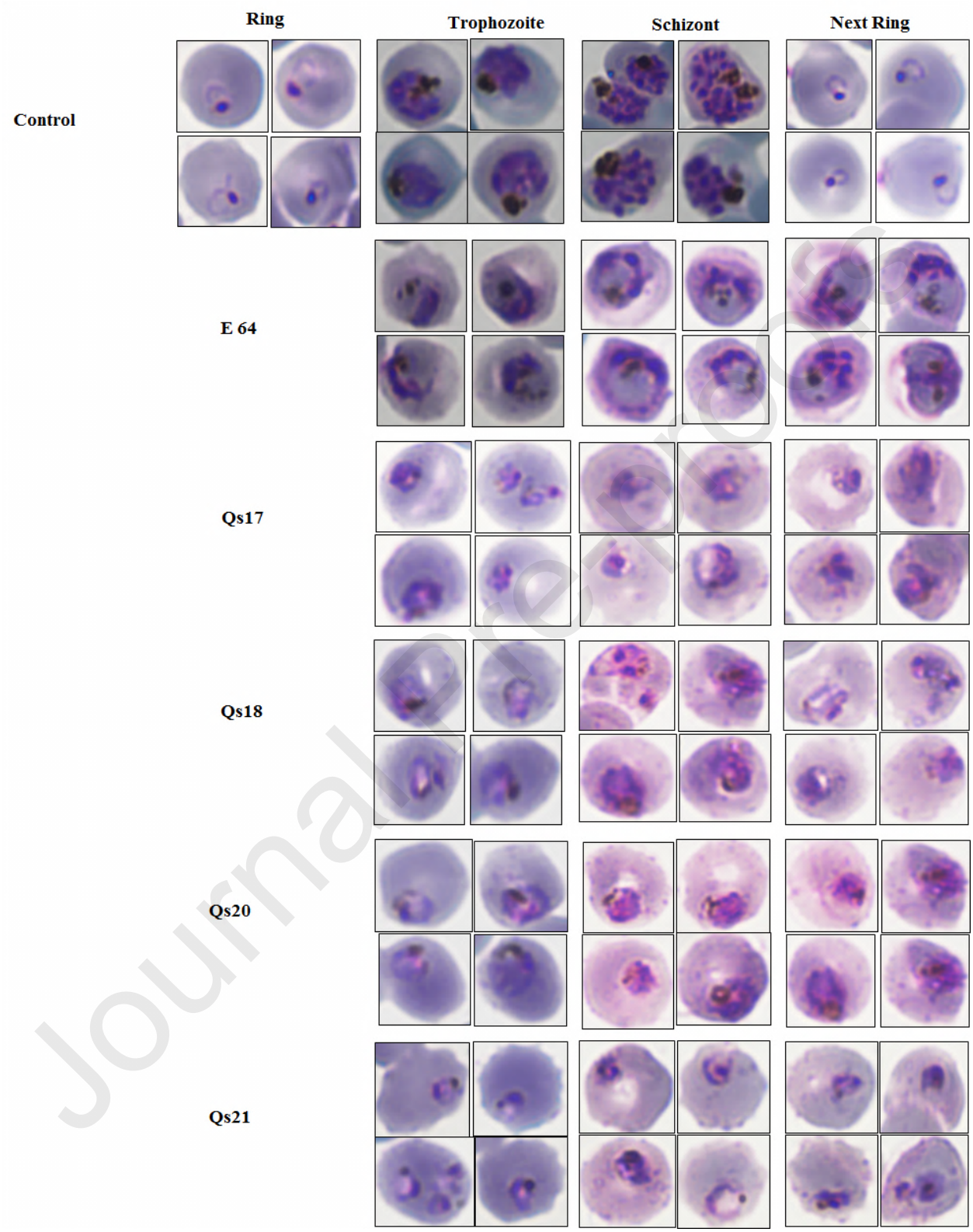


Figure 4. *Effect of* ***E-64****, compounds* ***Qs17, Qs18, Qs20*** *and* ***Qs21*** *on the growth and development of P. falciparum. Light microscopy images of parasitized red blood cells at different time points after the treatment with* ***E64****,* ***Qs17, Qs18, Qs20*** *and* ***Qs21.***

3. Structure activity relationship studies (SAR)

Overall, the effect of various chemical entities attached to the core structure quinolin-piperazin-methanone displayed a considerable role in the FP2 and parasite inhibiting ability of the test compounds (as depicted in **Figure 5**). It was observed that replacement of quinolin-2(1H)-one of **Qs5** with 2,4-dichloro-sulfonylbenzene increased the FP2 inhibiting ability of compound **Qs8** by 3-fold. The FP2 and parasite growth inhibition was found to further improve by the replacement of 2,4-dichloro-sulfonylbenzene of **Qs8** with sulfonyl-phenyl-acetamide in **Qs20**. The parasite growth inhibition by **Qs20** was recorded to be 810 nM. Further the replacement of 4-methoxy benzene of **Qs20** with simple chloro group in **Qs18** resulted in the lowering of FP2 inhibiting capability of the compound by 3.4-fold. The replacement of sulfonyl-phenyl-acetamide of **Qs18** with chloroquinoline in **Qs17** resulted in the enhancement of FP2 inhibition by almost 2-fold, however, the parasite growth inhibiting capacity was found to be unaltered. From the above, we concluded that the presence of both sulfonyl-phenyl-acetamide group and 4-methoxy benzene attached to the quinolin-piperazin-methanone core moiety as in **Qs20** resulted in the most potential antimalarial molecule in the series which can serve as a lead for the further development of novel effective antimalarials.

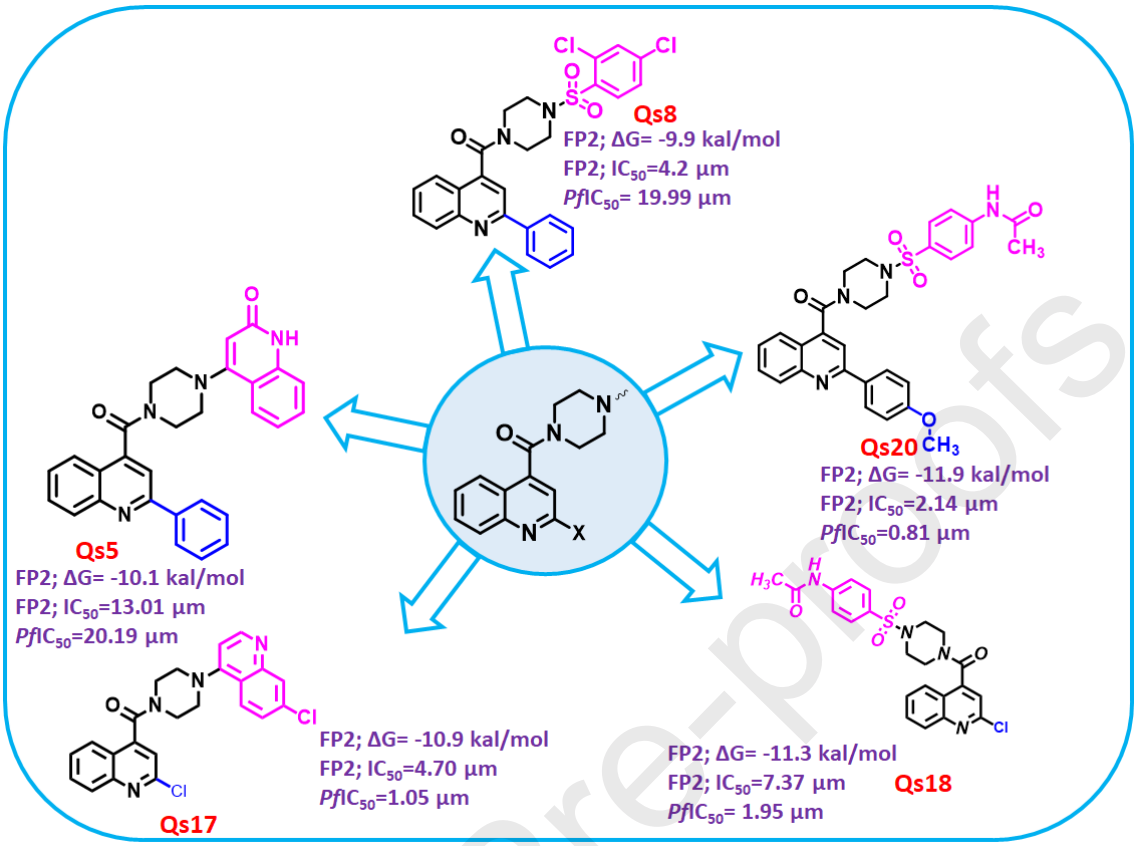


Figure 5. *Diagrammatic illustration of Structure activity relationship studies.*

4. Conclusions

A series of quinoline carboxamide-containing compounds was designed, synthesized and evaluated for its efficacy to inhibit FP2. These molecules arrest the parasite growth at trophozoite stage. Docking simulation and molecular hybridization techniques were used for the design of inhibitors. The designed compounds were synthesized in the wet lab. Computational binding affinity of the compounds was calculated in terms of kcal/mol and it was prophesied that the quinoline carboxamide-based compounds bind to FP2 (PDB ID 3BPF) with high affinity, inhibit its catalytic activity at sub-micromolar concentrations, thereby can arrest the parasite growth. In the series, Compounds **Qs17, Qs18, Qs20** and **Qs21** were predicted to show most effective and favorable interaction with the active site residues of FP2. Later, *in-vitro* results followed almost same trend, as **Qs17, Qs18, Qs20** and **Qs21** inhibited FP2 with IC_{50} values 4.78, 7.37, 2.14 and 2.64 µM, respectively. These compounds also inhibited the growth of *P. falciparum* 3D7 with IC_{50} 1.05, 1.95, 0.81 and 1.43 µM, respectively. From these results, we concluded that compounds containing sulfonyl phenyl acetamide group along with an aromatic substitution to the quinoline

system as in **Qs20** have greater FP2 inhibiting and antimalarial effect than the compounds with mere quinoline structures. Overall, these compounds can be used as promising leads for the development of novel and robust antimalarial agents.

5. Experimental section

5.1. Docking protocol

Crystal structure of FP2 (PDB ID 3BPF) in pdb format was downloaded from Protein Data Bank (www.rcsb.org) [27]. AutoDock Vina version 1.5.6 was used to perform docking with the ligand molecules. PyMOL visualization tool was used to visualize the molecular interactions. Weak non-covalent interactions like π-π stacking interaction and H-bonding were observed between the active site residues of FP2 and the ligand molecules lying within a range of 1.9 Å,2Å and 2.1 Å. In addition, binding free energy was calculated based on which the designed molecules were screened.

5.2. Chemistry

All the chemicals and reagents were purchased from Sigma-Aldrich, Alfa-Aesar, Spectrochem and SD Fine chemicals Pvt. Ltd. India, and used as received. The reactions were monitored and Rf values were determined using analytical thin layer chromatography (TLC) with Merck silica gel 60-120 and F254 pre-coated plates (0.25 mm) thickness. Spot on the TLC plates were visualized using ultraviolet light both at short (254 nm) and long wave (365 nm) UV light. Products were purified by flash chromatography on silica gel (mesh size 200-400). The ^{1}H NMR spectra and ^{13}C NMR were recorded on Bruker 300 spectrometers and 400 spectrometers. Chemical shifts are reported in ppm (TMS,δ 0.00) or with the solvent reference relative to TMS employed as the internal standard ($CDCl_3$,δ 7.26; DMSO-d6 δ 2.54) and multiplicities of NMR signals are designated as s (singlet), d (doublet), dd (double doublet), t (triplet),q (quartet), br (broad coupling), m (multiplet, for unresolved lines). Elemental analyses of the compounds were found to be within ±0.4% of the theoretical values.

5.2.1. General method of preparation of the compound 5

To a solution of chloral hydrate (0.54 mole) in 10 mL water, a solution of sodium sulfate was added (Solution A). On the other hand, to a solution of aniline (0.5 mol) in 5 mL water concentrated hydrochloric acid was added (Solution B). Now the solution B was added to solution B and the reaction mixture could stir at 60–80℃ for half an hour. Formation of isonitrosoacetanilide needle shaped crystals were observed and the reaction was cooled to room temperature. The product

obtained **(2)** was filtered, dried and produced for further reactions without purification [28]. Dry isonitrosoacetanilide (0.46 moles) in a round bottom flask was added with 20 mL concentrated sulfuric acid and the reaction mixture was stirred at 80℃ for half an hour. The mixture was cooled to room temperature and quenched with crushed ice to obtain a yellow colored precipitate. The precipitate was filtered and washed with cold water to remove the excessive sulfuric acid to obtain pure 1H-indole-2,3-dione, **3** (yield 70-75%). To a solution of 1H-indole-2,3-dione (4 mmol) in ethanol (20 mL), acetophenone (5 mmol), water (10 mL), and an aqueous solution of potassium hydroxide (2.80 g, 50 mmol) were added. The reaction mixture was heated to reflux at 80℃ for 5 h. The reaction was monitored by TLC (80 % EtoAc/hexane). After that completion of the reaction, crushed ice was added, and the organic solution was extracted by EtOAc. The combined organic layers were dried over sodium sulphate, filtered and concentrated to give the crude yellow colored product, **(5)** [15], which was purified by column chromatography using 80% EtOAC/Hexane. The yield of the compound was recorded to be 75-80% and LC−MS m/z $(M + H)^+ = 250.2$.

5.2.2. General methods of the preparation of compound 18

A mixture of 3-amino-1,2,4-triazole **(14)** (20 mmol), ethyl acetoacetate **(15)** (20 mmol) and acetic acid (10 mL) was refluxed for 4-5 h. After that, the reaction mixture was cooled to room temperature to obtain a white precipitated which was filtered, washed with acetic acid followed by ethanol, dried under vacuum to obtain the desired product with 65-70% yield. 5- Methyl- [1,2,4] triazolo [1,5-a] Pyrimidin-7-ol **(16)** (10 mmol) was added to 2.75 mL (30 mmol) of phosphorous oxychloride and heated under reflux for 1 h in a round bottom flask. Excessive $POCl_3$ was removed under reduced pressure and the residue was triturated with ice water. The product was extracted from the aqueous mixture with CH_2Cl_2, evaporated, and purified by column chromatographed using 30% MeOH/chloroform. Yield of the product was found to be 55%. A mixture of appropriate compound **17** (10 mmol), piperazine, (10 mmol) and K_2CO_3 (12 mmol) in 1,4-dioxane was refluxed at 100 ℃ for 3 h. The reaction mixture was cooled down to room temperature, filtered and washed with 1,4-dioxane to obtained compound **18** [29][30].

5.2.3. General methods for the preparation of compound 9

6.5 g amine **(1)** and 6.73 g malonic acid **(6)** were dissolved in 39 mL of $POCl_3$ and the reaction mixture was refluxed at 100℃ for 9 h. On the completion of the reaction, the reaction mixture was cooled to room temperature, poured dropwise into ice cold water and then neutralizes by sodium carbonate. White precipitate obtained was filtered, washed with water and dried. The crude product

was purified by column chromatography to obtained pure compound **7**. Then compound **7** was treated with HCl in presence of 1-4 dioxane to obtain 4-chloroquinolin-2(1H)-one **(8)**. A mixture of compound **8** (10 mmol) in ethylene glycol (20 mL) and piperazine (30 mmol) was refluxed for 1.5 h in a round bottom flask. After the completion of the reaction (monitored by TLC) was concentrated under vacuum, washed with water (3 x 20 mL) and the brined organic layer was dried over Na_2SO_4 to obtain 4-(piperazin-1-yl)quinolin-2(1H)-one **(9)** [31].

5.2.4. *Procedure for the preparation of compound 13*

76.0 mmol of aniline **(1)** was dissolved in 120 mL of dichloromethane and cool to 0℃ using an ice bath. Triethylamine (83.6 mmol, 1.10 eq) and the substituted acid chloride **10** (76.0 mmol) were added to the amine solution drop wise for 30 minutes. After the completion of the reaction, the solvents were removed under pressure, cold water was added, and the organic layer was extracted with CH_2Cl_2. The combined organic layers were washed with brine and dried over Na_2SO_4. The resulting off white solid compound **11** was used for the forward reaction step without purification. The yield obtained was 80–85 %. Further, 195 mmol of chloro sulfonic acid was added drop wise to compound **11** (5.0g, 37mmol) in a round bottom flask at 0℃. After the addition of chloro sulfonic acid, the reaction mixture could stir under reflux conditions at 60℃ for 1 h. The reaction mixture was cooled room temperature and poured into crushed ice to obtain white solid precipitate of (4-acetamidobenzene-1-sulfonyl chloride) **12** which was purified by column chromatography [32]. Piperazine (0.01 mmol) was dissolved in 30 mL CH_2Cl_2 and Et_3N (4.4 mL, 0.033 mml) was added to the solution at 0 ℃ for half an hour. 4-acetamidobenzene-1-sulfonyl chloride **12** (2.29 g, 0.01 mmol) was added to the reaction mixture. The reaction was stirred for 2 h, quenched with water and then poured into a separating funnel and extracted with CH_2Cl_2 (3x20 mL) and then the brined organic layer was dried with Na_2SO_4 to obtain N-(4-(piperazin-1-ylsulfonyl) phenyl) acetamide **13** as white colored powder with 60–70% yield.

5.2.5. General procedure for the preparation of compound Qs1-Qs10

To a stirring solution of differently substituted piperazine complexes (20 mmol) in 30 mL DMSO, HBTU (50 mmol), TEA (50 mmol) and EDC were added at room temperature. Then the reaction mixture was added with 2-phenylquinoline-4-carboxylic acid **(5)** (20 mmol). The resulting mixture was stirred at room temperature for 8 h. The reaction monitored *via* TLC. On completion of the reaction, the reaction was quenching with ice and extracted with ethyl acetate (3x50 mL). The combined organic layers were washed with brine solution (50 mL), dried over Na_2SO_4 and

concentrated under vacuum. The crud products **(Qs1–Qs10)** were purified by column chromatography using 70% EtOAc/Hexane.

5.2.5.1. 2-[4-(2-phenylquinoline-4-carbonyl) piperazin-1-yl] pyridine-3-carbonitrile (Qs1)

Light yellow solid, m.p. 180-182 °C, 50% yield obtained after column chromatography. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (dd, *J* = 4.8, 1.8 Hz, 1H), 8.23 (d, *J* = 8.5 Hz, 1H), 8.17 (d, *J* = 6.7 Hz, 2H), 7.86 (m, 4H), 7.54 (m,= 4H), 6.85 (dd, *J* = 7.5, 4.8 Hz, 1H), 4.15 (d, *J* = 26.6 Hz, 2H), 3.88 (s, 2H), 3.55 (d, *J* = 11.5 Hz, 2H), 3.42 (d, *J* = 4.2 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ167.461, 160.875, 157.119, 151.863, 148.498, 143.637, 142.707, 138.919, 130.483, 129.783, 128.966, 127.546, 124.243, 123.072, 117.413, 115.963, 115.401, 96.510, 48.863, 48.407, 46.932, 41.685 HRMS: (ESI, m/z): $[M+H]^+$ calcd for 419.17 found 420.1719. and $[M+Na]^+$: m/z = 442.1703. Anal. Calcd for $C_{26}H_{21}N_5O$ C, 74.44; H, 5.05; N, 16.70; found C, 74.11; H, 5.35; N, 16.40.

5.2.5.2. (4-(7-chloroquinolin-3-yl) piperazin-1-yl) (2-phenylquinolin-4-yl) methanone (Qs2)

Yellow solid, m.p. 200-202 °C, 63 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.89 (s, 1H), 8.74 (d, *J* = 4.5 Hz, 1H), 8.23 (d, *J* = 8.5 Hz, 1H), 8.16 (d, *J* = 8.2 Hz, 1H), 8.09 (s, 1H), 7.87 (t, *J* = 6.7 Hz, 1H), 7.81 – 7.74 (m, 1H), 7.64 – 7.28 (m, 1H), 6.84 (d, *J* = 5.1 Hz, 1H), 3.45 (d, *J* = 33.7 Hz, 1H), 2.80 (s, 1H), 2.08 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.13, 167.32, 156.97, 156.68, 150.96, 148.73, 148.23, 142.38, 138.56, 135.62, 130.45, 130.25, 129.76, 128.85, 127.75, 127.47, 127.40, 126.81, 124.58, 124.03, 122.84, 121.37, 115.91, 109.20, 52.33, 46.93, 41.58, 38.4 HRMS: (ESI, m/z): $[M+H]^+$ calcd found 478.16 found 479.1547 Anal. Calcd for $C_{29}H_{23}ClN_4O$ C, 72.72; H, 4.84; N, 11.70; found C, 72.5.0; H, 4.64; N, 11.60.

5.2.5.3. 4-(4- {5-methyl- [1,2,4] triazolo[1,5-a] pyrimidin-6-yl} piperazine-1-carbonyl)-2phenylquinoline (Qs3)

Dull yellow solid, m.p. 238 -240°C, 53.5 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (s, 1H), 8.22 (s, 1H), 8.17 (d, *J* = 6.9 Hz, 1H), 7.85 (s, 1H), 7.79 (t, *J* =8.7 Hz,1H), 7.59 (t, *J* =7.5 Hz,1H, 1H), 7.53 (d, *J* = 7.5 Hz, 1H), 6.16 (s, 1H), 4.23 (d, *J* = 26.4 Hz, 2H), 3.95 (d, *J* = 5.4 Hz, 2H), 3.70 (d, *J* = 15.9 Hz, 2H), 3.49 (d, *J* = 12.0 Hz, 2H), 2.58 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.43, 165.20, 157.09, 154.44, 149.79, 148.50, 142.21, 138.77, 130.60, 130.56, 129.90, 129.01, 127.60, 127.50, 124.05, 122.97, 115.98, 94.97, 48.30,

47.89, 46.51, 41.13, 25.25 $[M+H]^+$ m/z calcd 449.20 for found 450.1. Anal. Calcd for $C_{26}H_{23}N_7O$ C, 69.47; H, 5.16; N, 21.81; found C, 69.27; H, 5.46; N, 21.41.

5.2.5.4. (2-phenylquinolin-4-yl) (4-(5-(trifluoromethyl)- [1,2,4] triazolo[1,5-a] pyrimidin-6-yl) piperazin-1-yl) methanonein (Qs4)

White solid, m.p. 250-252℃, 55 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.25 (d, *J* = 8.7 Hz, 1H), 8.17 (d, *J* = 7.5 Hz, 1H), 7.83 (*J* = m, 3H), 7.57 (*J* = m, 4H), 7.27 (s, 1H), 6.59 (s, 1H), 4.37 – 3.87 (m, 4H), 3.56 (s, 1H), 2.81 (s, 1H), 2.07 (d, *J* = 13.2 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.53, 157.11, 156.54, 155.58, 150.74, 148.44, 141.96, 138.64, 130.66, 130.57, 129.97, 129.02, 127.72, 127.51, 123.93, 122.88, 116.05, 90.25, 48.61, 48.21, 46.42, 41.15 HRMS: (ESI, m/z): $[M+H]^+$ calcd 503.17 found 504.1682. and $[M+Na]^{+:}$ m/z = 526.1543 Anal. Calcd for $C_{26}H_{20}F_3N_7O$ C, 62.02; H, 4.00; N, 19.47; found C, 61.02; H, 4.51; N, 19.37.

5.2.5.5. 4-(4-(2-phenylquinoline-4-carbonyl) piperazin-1-yl) quinolin-2(1H)-one (Qs5)

Pale yellow solid, m.p. 199-202 ℃, 62 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.29 (s, 1H), 8.24 (d, *J* = 8.4 Hz, 1H), 8.17 (d, *J* = 7.8 Hz, 2H), 7.86 (d, *J* = 5.4 Hz, 2H), 7.82 (t, *J*=7.8Hz, 1H), 7.69 (d, *J* = 8.1 Hz, 1H), 7.64 – 7.46 (m, 5H), 7.37 (d, *J* = 8.1Hz, 1H), 7.22 (dd, *J* = 17.3, 9.7 Hz, 1H), 6.18 (s, 1H), 4.24 (d, *J* = 32.4 Hz, 2H), 3.44 (d, *J* = 36.9 Hz, 4H), 3.07 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.59, 167.49, 165.54, 160.02, 157.15, 148.44, 142.54, 138.80, 134.96, 130.94, 130.57, 130.46, 129.87, 129.02, 127.56, 124.20, 124.08, 123.02, 122.55, 117.29, 116.44, 116.06, 105.97, 52.04, 51.77, 47.03, 41.68. HRMS: (ESI, m/z): $[M+H]^+$ calcd 460.19 for found 461.1902. and $[M+Na]^+$: m/z = 483.1706 Anal. Calcd for $C_{29}H_{24}N_4O_2$ C, 75.63; H, 5.25; N, 12.17; found C, 75.43; H, 5.35; N, 12.25.

5.2.5.6. N-(4-(4-(2-phenylquinoline-4-carbonyl) piperazin-1-ylsulfonyl) phenyl) acetamide (Qs6)

Deep yellow solid, m.p. 200°C, 72 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.50 (s, 1H), 8.21 (d, *J* = 9.00 Hz, 1H), 8.05 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.76 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.72 (s, 1H), 7.70 (s, 1H), 7.61 (m, *J* = 7.7 Hz, 3H), 7.49 (m, *J* = 7.4 Hz, 4H), 7.36 (dd, *J* = 6.0, 3.0 Hz, 1H), 4.00 (m, 2H), 3.30 (t, *J* = 4.9 Hz, 2H), 2.86 (d, *J* = 6.0 Hz, 1H), 2.80 (s, 1H), 2.18 (s, 3H), 2.06 (d, *J* = 6.0 Hz, 1H), 1.26 (t, *J* = 6.00 Hz, 1H). ^{13}C NMR (75 MHz,

$CDCl_3$) δ 169.65, 167.38, 157.21, 148.10, 143.00, 142.16, 138.42, 130.83, 130.12, 129.99, 129.38, 129.00, 128.82, 127.79, 127.58, 126.67, 126.59, 123.93, 122.73, 119.67, 116.13, 46.48, 46.26, 45.80, 41.15, 38.68, 24.48 HRMS: (ESI, m/z): $[M+H]^+$ calcd 514.60 for found 515.1666. and $[M+Na]^+$: m/z = 537.1516 Anal. Calcd for $C_{28}H_{26}N_4O_4S$ C, 65.35; H, 5.09; N, 10.89; found C, 65.55; H, 5.05; N, 10.35.

5.2.5.7. N-(2-fluoro-4-((4-(2-phenylquinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (Qs7)

Crystalline white solid, m.p. 260-262 ℃, 61 % yield obtained after column chromatography**.** ^1H NMR (300 MHz, $CDCl_3$) δ 8.76 (d, *J* = 8.7 Hz, 1H), 8.21 (d, *J* = 8.4 Hz, 1H), 8.13 (d, *J* = 6.6Hz, 1H), 7.77 (d, *J* = 7.7 Hz, 1H), 7.72 (s, 1H), 7.67 (d, *J* = 8.1 Hz, 1H), 7.51 (m = Hz, 5H), 7.26 (t, *J* = 9.4 Hz, 2H), 4.22 (m, 2H), 3.33 (t, *J* = 4.8 Hz, 3H), 3.19 (s, 1H), 2.98 (d, *J* = 26.3 Hz, 2H), 2.24 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.13, 160.87, 158.92, 157.19, 156.16, 154.43, 152.06, 150.19, 135.25, 129.18, 126.73, 124.64, 121.78, 116.77, 111.36, 109.43, 94.94, 51.60, 47.97, 25.32. HRMS: (ESI, m/z): $[M+H]^+$ calcd for found 532.16 found $[M+Na]^+$:m/z = 555.1405 Anal. Calcd for $C_{28}H_{25}FN_4O_4S$ C, 63.15; H, 4.73; N, 10.52; found C, 62.15; H, 4.53; N, 10.72.

5.2.5.8. (4-((2,4-dichlorophenyl) sulfonyl) piperazin-1-yl) (2-phenylquinolin-4-yl) methanone (QS8)

White powder, m.p. 252-254 ℃, 66 % yield obtained after column chromatography**.** ^1H NMR (500 MHz, $CDCl_3$) δ 8.25 (d, *J* = 8.5 Hz, 1H), 8.17 (d, *J* = 7.0 Hz, 2H), 8.07 (d, *J* = 1.0 Hz, 1H), 7.79 (t, *J* = 5.9 Hz, 2H), 7.60 (d, *J* = 7.1 Hz, 1H), 7.56 (s, 1H), 7.54 (d, *J* = 9.0 Hz, 1H), 7.51 (s, 2H), 7.29 (s, 2H), 4.07 (d, *J* = 13.0 Hz, 2H), 3.57 (s, 2H), 3.33 (d, *J* = 2.5 Hz, 2H), 3.26 (d, *J* = 5.5 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.42, 157.08, 138.76, 137.47, 133.99, 133.92, 133.48, 133.35, 131.85, 130.68, 130.58, 130.42, 129.98, 129.90, 129.01, 127.61, 127.52, 124.01, 122.89, 115.93, 47.05, 46.12. HRMS: (ESI, m/z): $[M+H]^+$ calcd 525.07 for found 526.0. and $[M+Na]^+$: m/z = 548.0531 Anal. Calcd for $C_{26}H_{21}Cl_2N_3O_3S$ C, 59.32; H, 4.02; N, 7.98; found C, 58.32; H, 4.46; N, 7.40.

5.2.5.9. (2-phenylquinolin-4-yl) (4-tosylpiperazin-1-yl) methanone (QS9)

Off powder, m.p. 270-272 ℃, 59 % yield obtained after column chromatography**.** ^1H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, *J* = 8.1 Hz, 1H), 8.15 (d, *J* = 7.5 Hz, 2H), 7.79 (t, *J* = 7.6 Hz, 1H), 7.73 (s, 1H), 7.66 (t, *J* = 8.1 Hz, 3H), 7.53-7.30 (m, 4H), 7.38 (d, *J* = 8.0 Hz, 2H), 4.06 (d, *J* = 24.0 Hz, 2H), 3.39 – 3.15 (m, 4H), 2.91 (d, *J* = 32.0 Hz, 2H), 2.50 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$)

δ 167.42, 157.08, 138.76, 137.47, 133.99, 133.92, 133.48, 133.35, 131.85, 130.68, 130.58, 130.42, 129.98, 129.90, 129.01, 127.61, 127.52, 124.01, 122.89, 115.93, 47.05, 46.12. HRMS: (ESI, m/z): $[M+H]^+$ calcd 471.16 for found 472.1624. Anal. Calcd for $C_{27}H_{25}N_3O_3S$ C, 68.77; H, 5.34; N, 8.91; found C, 68.04; H, 5.74; N, 7.91.

5.2.5.10. (4-((4-bromophenyl) sulfonyl) piperazin-1-yl) (2-phenylquinolin-4-yl) methanone (QS10)

Yellowish powder, m.p. 265-267℃, 71 % yield obtained after column chromatography**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, *J* = 8.4 Hz, 1H), 8.15 (d, *J* = 8.0 Hz, 2H), 7.79 (t, *J* = 7.7 Hz, 1H), 7.75 – 7.73 (m, 3H), 7.66 (d, *J* = 8.0 Hz, 1H), 7.62 (d, *J* = 8.5 Hz, 2H), 7.58 – 7.48 (m, 4H), 4.07 (d, *J* = 18.0 Hz, 2H), 3.39 – 3.17 (m, 4H), 2.91 (d, *J* = 17.5 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.31, 157.03, 148.43, 142.03, 138.73, 134.59, 132.71, 130.56, 129.92, 129.17, 129.01, 128.54, 127.55, 127.52, 123.96, 122.84, 115.91, 46.46, 46.31. HRMS: (ESI, m/z): calcd 536.44 found $[M + 2]^+$ = 538.1. Anal. Calcd for $C_{26}H_{22}BrN_3O_3S$ C, 58.21; H, 4.13; N, 7.83; found C, 57.61; H, 4.23; N, 7.09.

5.2.6. General procedure for the preparation of compound Qs11, Qs12, Qs13

1 mmol solution of compound **18** was reacted separately with the compound **19, 20** and **21** to obtain **Qs11,Qs12** and **Qs13**, respectively [33]. Compounds **19, 20** and **21** (1.5 mmol each) were added to compound **18** (1.5 mmol) in presence of K_2CO_3 (3 mmol) and anhydrous 1,4 dioxane (20 mL) and the reaction mixture was refluxed at 100℃ for 12 h. After the completion of the reaction, the mixture was cooled to room temperature. The reaction mixture was concentrated under vacuum and the crude obtained was diluted with chloroform and washed with water (100 mL x 3). The organic layer was separated and dry with Na_2SO_4. The crude mixture was purified by column chromatography using 80% EtOH/ Hexane.

5.2.6.1. 7-chloro-4-(4-(7-methyl- [1,2,4] triazolo[1,5-b] pyridazin-8-yl) piperazin-1-yl) quinoline (Qs11)

Pale yellow , m.p. 200-205℃, 69.2 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.79 (s, 1H), 8.35 (s, 1H), 8.09 (s, 1H), 8.00 (d, *J* = 9.0 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 1H), 6.95 (s, 1H), 6.28 (s, 1H), 4.10 (s, 4H), 3.48 (s, 4H), 2.64 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.13, 157.19, 156.16, 154.43, 152.06, 150.19, 135.25, 132.72, 129.18, 126.73, 124.64, 121.78, 109.43, 94.94, 51.60, 47.97, 25.32. HRMS: (ESI, m/z): $[M+H]^+$ calcd 379.13 for found

380.1368. Anal. Calcd for $C_{19}H_{18}ClN_7$ C, 60.08; H, 4.78; N, 25.81 found C, 60.00; H, 4.45; N, 25.51.

5.2.6.2. Ethyl 7-methyl-4-(4-(5-(trifluoromethyl)- [1,2,4] triazolo[1,5-a] pyrimidin-7-yl) piperazin-1 yl) quinoline-3-carboxylate (Qs12)

Light Yellow solid, m.p. 250-252 ℃, 65 % yield obtained after column chromatography**.** ^{1}HNMR(300 MHz, $CDCl_3$) δ 8.95 (s, 1H), 8.50 (s, 1H), 8.05 (d, *J* = 8.4 Hz, 1H), 7.63 (d, *J* = 8.5 Hz, 1H), 7.27 (s, 1H), 6.70 (s, 1H), 4.47 (q, *J* = 14.2, 7.2 Hz, 2H), 4.33 (s, 2H), 3.65 (t, *J* = 7.3 Hz 4H), 2.61 (s, 3H), 1.44 (t, *J* = 7.2 Hz, 3H), 1.25 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.13, 167.23, 156.97, 156.68, 150.96, 148.73, 148.23, 142.38, 138.56, 135.62, 130.45, 130.25, 129.76, 128.85, 127.75, 127.40, 126.81, 124.58, 124.03, 122.84, 121.37, 115.91, 109.20, 46.93, 41.58, 38.48, 29.55. HRMS: (ESI, m/z): $[M+H]^+$ calcd 485.18 for found 486.0. Anal. Calcd for $C_{23}H_{22}F_3N_7O_2$ C, 56.90; H, 4.57; N, 20.20; found C, 56.40; H, 4.23; N, 20.50.

5.2.6.3. N-(4-((4-(5-methyl- [1,2,4] triazolo[1,5-a] pyrimidin-7-yl) piperazin-1 yl) sulfonyl) phenyl) acetamide (Qs13)

Cotton white, m.p. 270-272 ℃, 74 % yield obtained after column chromatography. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.52 (s, 1H), 8.54 (s, 1H), 8.28 (s, 1H), 7.70 (dd, J = 23.2, 7.6 Hz, 2H), 7.28 (s, 1H), 6.17 (s, 1H), 3.89 (s, 4H), 3.24 (s, 2H), 2.58 (d, J = 1.8 Hz, 2H), 2.21 (s, 3H), 2.10 (s, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ 177.38, 165.65, 153.69, 151.68, 149.64, 142.89, 129.19, 128.94, 119.49, 95.25, 47.30, 45.47, 29.70, 20.91 HRMS: (ESI, m/z): $[M+H]^+$ calcd 415.14 for found 416.1300. Anal. Calcd for $C_{18}H_{21}N_7O_3S$ C, 52.04; H, 5.09; N, 23.60; found C, 51.04; H, 5.49; N, 23.30.

5.2.7. *General procedure for the preparation of compounds Qs14–Qs16*

To a solution of 2-hydroxyquinoline-4-carboxylic acid **23** (2mmol) in (20 mL) DMSO was added with R_3, HBTU (5 mmol), TEA (5mmol) and EDC a round bottom flask, at stirred room temperature overnight. After the completion reaction, the reaction mixture was dissolved in 50 mL ethyl acetate. The organic phase washed with water and dried with Na_2SO_4. The crude product was purified by column chromatography 75 % ethyl acetate/ Hexane to obtain target products Qs14–Qs16.

5.2.7.1. (4-((2,4-dichlorophenyl) sulfonyl) piperazin-1-yl) (2-hydroxyquinolin-4-yl) methanone (QS14)

White, m.p. 240-242℃, 70% yield obtained after column chromatography HRMS: (ESI, m/z): $[M+H]^+$ calcd 466.33 for found $[M+Na]^+$ m/z: 488.0247 Anal. Calcd for $C_{20}H_{17}Cl_2N_3O_4S$ C, 51.51; H, 3.67; N, 9.01; found C, 50.81; H, 3.37; N, 8.81; ^{1}H NMR (500 MHz, DMSO) δ 11.82 (s, 1H), 7.92 (d, *J* = 14.7 Hz, 1H), 7.48 (d, *J* = 14.7 Hz, 2H), 7.41 (d, *J* = 7.6 Hz, 1H), 7.35 – 7.26 (m, 2H), 7.10 (dd, *J* = 14.9, 7.4 Hz, 1H), 6.39 (d, *J* = 16.0 Hz, 1H), 3.84 (d, *J* = 35.0 Hz, 2H), 3.31 (dd, J = 73.9, 40.0 Hz, 4H), 2.51 (d, *J* = 14.0 Hz, 2H).^{13}C NMR (126 MHz, DMSO) δ 165.81, 161.91, 145.65, 139.44, 137.20, 134.15, 133.61, 133.25, 131.57, 131.35, 124.91, 122.70, 119.09, 119.03, 116.54, 116.00, 46.74, 45.99.

5.2.7.2. (2-hydroxyquinolin-4-yl) (4-tosylpiperazin-1-yl) methanone (QS15)

Yellow, m.p. 230-235 ℃, 67 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.75 (s, 1H), 8.23 (d, *J* = 8.5 Hz, 1H), 8.16 (d, *J* = 8.2 Hz, 1H), 8.09 (s, 1H), 7.87 (t, *J* = 6.7 Hz, 1H), 7.81 – 7.70 (m, 1H), 7.61 (d, *J* = 8.1 Hz, 1H), 7.52 (d, *J* = 7.6 Hz, 1H), 7.42 (d, *J* = 9.0 Hz, 1H), 6.84 (d, *J* = 5.1 Hz, 1H), 4.26 (d, *J* = 34.8 Hz, 2H), 3.45 (d, *J* = 33.7 Hz, 2H), 3.09 (s, 2H), 2.80 (s, 2H), 2.08 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 160.87, 157.29, 140.94, 139.53, 134.45, 127.23, 126.63, 125.21, 122.85, 120.14, 118.01, 113.97, 111.81, 111.29, 41.46, 41.04, 36.07 HRMS: (ESI, m/z): $[M+H]^+$ calcd 411.13 for found $[M+Na]^+$ m/z := 434.1065. Anal. Calcd for $C_{21}H_{21}N_3O_4S$ C, 61.30; H, 5.14; N, 10.21; found C, 60.79; H, 5.54; N, 10.51.

5.2.7.3. (4-(7-chloroquinolin-4-yl) piperazin-1-yl) (2-hydroxyquinolin-4-yl) methanone (QS16)

White, m.p. 280-282 ℃, 52 % yield obtained after column chromatography. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.78 (d, *J* = 4.9 Hz, 1H), 8.10 (s, 1H), 7.92 (d, *J* = 9.0 Hz, 1H), 7.59 (d, *J* = 7.7 Hz, 2H), 7.46 (dd, *J* = 10.8, 3.9 Hz, 2H), 7.36 – 7.25 (m, 2H), 6.87 (d, *J* = 5.1 Hz, 1H), 6.72 (s, 1H), 4.19 (d, J = 3.6 Hz, 2H), 3.64 (d, *J* = 10.5 Hz, 2H), 3.37 (s, 1H), 3.14 (d, *J* = 16.8 Hz, 2H).^{13}C NMR (126 MHz, DMSO) δ 161.18, 157.32, 141.12, 134.60, 126.59, 122.25, 120.24, 120.17, 118.00, 114.30, 111.79, 111.37, 104.10, 47.54, 47.23. HRMS: (ESI, m/z): $[M+H]^+$ calcd 418.12 for found 419.1245. Anal. Calcd for $C_{23}H_{19}ClN_4O_2$ C, 65.95; H, 4.57; N, 13.38; found C, 65.45; H, 3.87; N, 13.58.

5.2.8. Procedure for the synthesis of Qs17

To stirred solution of isatin **22** (10.00 g, 61 mmol) in acetic acid (400 mL), malonic acid (18.91 g, 182 mmol) was added and the reaction mixture was refluxed for 16 h. The excessive solvent was removed under vacuum, and the suspended solid was dissolved in water (400 mL), filtered,

and washed with water (300 mL) to obtain brown solid. The brown solid was dissolved in saturated aqueous solution of $NaHCO_3$ (800 mL), and the insoluble material was filtered off. The filtrate was acidified to pH 1−2 with concentrated HCl, the resulting precipitate was filtered, washed with water (300 mL), and dried. The resulting pale-yellow solid **(23)** was used directly for further synthesis without purification. The yield obtained was 60%. Then a solution of 2-hydroxyquinoline-4-carboxylic acid **23** (10.6 g) in $POCl_3$ (70 mL) was refluxed for 2 h. the reaction mixture was cooled to room temperature and the excessive $POCl_3$ was evaporated under reduced pressure. The crude viscous reaction mixture was poured into ice water (400 mL) and stirred for 30 minutes. The resulting precipitate was filtered and washed with water. The solid residue obtained was then stirred in 100 mL of 0.5 M KOH until fully dissolved and pH was adjusted to 2 with HCl (3N). Precipitate obtained was filtered and dried. The desired product **24** was solid and off white in color [15]**.** The product obtained was used for the next step without further purification. **Qs17** was prepared by reacting 2-chloroquinoline-4-carboxylic acid **24** and 7-chloro-4-(piperazin-1-yl) quinoline in presence of DMSO, HBTU and TEA at the room temperature overnight. After the completion of the reaction, the crud product was purified by column chromatography using 65% ethyl acetate/hexane.

5.2.8.1. (2-chloroquinolin-4-yl) (4-(7-chloroquinolin-4-yl) piperazin-1-yl) methanone (QS17)

Yellow, m.p. 220-222 ℃, 58 % yield obtained after column chromatography**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.77 (d, *J* = 5.0 Hz, 1H), 8.17 (dd, *J* = 5.2, 3.1 Hz, 2H), 7.96 (d, *J* = 9.0 Hz, 1H), 7.85 (d, *J* = 10.0Hz, 1H), 7.67 (d, *J* = 5.0 Hz, 1H), 7.53 – 7.49 (m, 2H), 7.35 (s, 1H), 6.93 (d, *J* = 5.0 Hz, 1H), 4.28 (d, *J* = 20.0Hz, 2H), 3.65 – 3.40 (m, 4H), 3.24 (d, *J* = 25.0Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.97, 159.95, 156.91, 147.07, 145.91, 143.58, 131.50, 129.04, 128.68, 127.90, 127.38, 127.17, 124.84, 124.76, 124.41, 120.45, 109.18, 109.02, 106.05, 52.42, 52.15. HRMS: (ESI, m/z): $[M+H]^+$ calcd 436.09 for found 437.0851. Anal. Calcd for $C_{23}H_{18}Cl_2N_4O$ C, 63.17; H, 4.15; N, 12.81; found C, 62.87; H, 4.25; N, 11.85.

5.2.9. Procedure for the synthesis of Qs18

Later, to a solution of 2-chloroquinoline-4-carboxylic acid **24** (10 mmol) in DMSO (10 mL), EDC (528 mg, 2.7 mmol, 2 eq) and HBTU (371 mg, 2.7 mmol, 2 eq) were added. Then, N-(4-(piperazin-1-ylsulfonyl) phenyl) acetamide (10mmol) and TEA (30 mmol) was added to the reaction mixture. The reaction was stirred for 8 h at room temperature. The reacting mixture was

quenched with ice cold water (10 mL) and extracted with ethyl acetate (3x 100 mL). the organic phase was dried over Na_2SO_4 and the solvents were removed under reduced pressure. The product obtained **(Qs18)** was purified by column chromatography using 70% EtOAc/hexane.

5.2.9.1. N-(4-((4-(2-chloroquinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS18)

Crystalline white, m.p. 237-239 ℃, 83 % yield obtained after column chromatography**.** ^{1}H NMR (399 MHz, DMSO) δ 10.46 (s, 1H), 8.19 (d, *J* = 8.4 Hz, 1H), 7.86 (s, 1H), 7.83 (s, 1H), 7.73 (d, *J* = 5.4 Hz, 1H), 7.67 (d, *J* = 8.6 Hz, 1H), 7.64 (d, J = 3.1 Hz, 2H), 7.52 (d, *J* = 4.6 Hz, 1H), 7.44 (d, *J* = 8.3 Hz, 1H), 3.74 (s, 2H), 3.30 (d, *J* = 41.2 Hz, 2H), 2.67 (s, 4H), 2.09 (s, 3H). [M+H] $^+$ m/z calcd 472.10 found [M-H]$^-$ = 471.12 Anal. Calcd for $C_{22}H_{21}ClN_4O_4S$ C, 55.87; H, 4.48; N, 11.85; found C, 54.87; H, 4.68; N, 10.95; ^{13}C NMR (126 MHz, cdcl$_3$) δ 177.66, 167.50, 165.42, 149.00, 140.44, 137.44, 133.78, 130.61, 128.78, 127.21, 126.38, 125.62, 120.04, 118.65, 111.29, 54.06, 48.90, 22.83.

5.2.10. *General Procedure for the synthesis of Qs19–Qs25 involving Suzuki Coupling*

To a solution of N-(4-((4-(2-chloroquinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide **(Qs18)** (1 mmol) in 10 mL toluene, K_2CO_3 (200 mg, 1.5 mmol) was added. Then differently substituted phenyl boronic acids (1.5 mmol) and Pd(pph$_3$)$_4$ were added to the reaction mixture and the mixture was refluxed under argon gas at 100 ℃ for 8 h. After the completion of the reaction, the mixture was cooled to room temperature and the excessive solvent was evaporated in vacuum. The crude products were purified by column chromatography using 70% EtOAc/hexane [34]**.**

5.2.10.1. N-(4-((4-(2-(p-tolyl) quinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS19)

White powder, m.p. 255-257 ℃, 57 % yield obtained after column chromatography**.** ^{1}H NMR (500 MHz, CDCl$_3$) δ 8.21 (s, 1H), 8.15 (d, *J* = 7.5 Hz, 1H), 8.05 (d, *J* = 8.0 Hz, 2H), 7.77 – 7.69 (m, 4H), 7.65 (d, *J* = 8.0 Hz, 1H), 7.54 – 7.47 (m, 2H), 7.36 – 7.32 (m, 2H), 7.29 (s, 2H), 4.06 (d, *J* = 18.5 Hz, 2H), 3.33 (s, 2H), 3.23 (d, *J* = 34.0 Hz, 2H), 2.91 (d, *J* = 18.0 Hz, 2H), 2.46 (s, 3H), 2.27 (s, 3H). ^{13}C NMR (126 MHz, CDCl$_3$) δ 167.39, 157.30, 148.45, 142.95, 142.40, 141.93, 140.09, 136.81, 135.95, 135.71, 135.60, 130.44, 129.76, 129.71, 129.06, 128.78, 128.61, 127.38,

127.33, 123.94, 122.72, 119.50, 115.74, 46.49, 46.32, 24.77, 21.37. $[M+H]^+$ m/z calcd 528.18 for found 528.5006. Anal. Calcd for $C_{29}H_{28}N_4O_4S$ C, 65.89; H, 5.34; N, 10.60; found 65.09; H, 5.54; N, 10.40.

5.2.10.2. N-(4-((4-(2-(4-methoxyphenyl) quinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS20)

Off White solid, m.p. 205℃, 60% yield obtained after column chromatography**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (s, 1H), 8.10 (d, *J* = 8.5 Hz, 1H), 7.76 – 7.69 (m, 4H), 7.68 – 7.58 (m, 4H), 7.50 (d, *J* = 5.5 Hz, 2H), 7.27 (d, *J* = 11.0 Hz, 1H), 7.03 (d, *J* = 8.5 Hz, 1H), 3.88 (s, 3H), 3.12 (d, *J* = 31.0 Hz, 2H), 2.89 (d, *J* = 46.0 Hz, 2H), 2.20 (s, 4H), 2.10 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.55, 169.18, 165.58, 156.43, 150.23, 148.02, 144.29, 142.86, 132.37, 132.05, 131.97, 131.57, 129.28, 128.88, 128.77, 128.67, 128.30, 124.29, 124.00, 122.78, 122.50, 119.61, 119.20, 115.71, 114.48, 55.49, 46.21, 45.77, 24.59. HRMS: (ESI, m/z): $[M+H]^+$ cal:544.18 found $[M+Na]^+$ m/z = 567.1618. Anal. Calcd for $C_{29}H_{28}N_4O_5S$ C, 63.96; H, 5.18; N, 10.29; found C, 63.56; H, 5.58; N, 9.79.

5.2.10.3. N-(4-((4-(2-(4-(trifluoromethoxy) phenyl) quinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS21)

Yellowish white , m.p. 257-259℃, 52 % yield obtained after column chromatography ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.98 (s, 1H), 8.18 (t, *J* = 8.5 Hz, 2H), 7.89 (d, *J* = 7.6 Hz, 1H), 7.75 (d, *J* = 7.5 Hz, 2H), 7.68 – 7.58 (m, 4H), 7.50 (d, *J* = 7.5 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 1H), 7.17 (d, *J* = 7.5 Hz, 1H), 2.83 (s, 8H), 2.17 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.51, 167.22, 155.54, 150.50, 148.31, 143.21, 142.33, 137.22, 135.91, 132.42, 132.03, 131.95, 130.79, 130.47, 129.39, 129.09, 128.82, 128.72, 127.91, 123.96, 122.81, 121.19, 119.67, 46.26, 45.82, 38.67. HRMS: (ESI, m/z): $[M+H]^+$ calcd 598.60 found $[M+Na]^+$ m/z = 621.1320 Anal. Calcd for $C_{29}H_{25}F_3N_4O_5S$ C, 58.19; H, 4.21; N, 9.36; found C, 57.79; H, 4.51; N, 9.14.

5.2.10.4. N-(4-((4-(2-(3,4-difluorophenyl) quinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS22)

Chock White powder, m.p. 275-277℃, 58 % yield obtained after column chromatography**.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 (s, 1H), 7.79 (d, *J* = 8.1 Hz, 1H), 7.72 (dd, *J* = 15.5, 6.5 Hz, 4H), 7.64 (d, *J* = 10.8 Hz, 2H), 7.59 – 7.52 (m, 1H), 7.47 (t, *J* = 6.9 Hz, 1H), 7.25 (d, *J* = 7.8 Hz, 2H), 7.18 (d, *J* = 6.6 Hz, 1H), 4.00 (d, *J* = 39.0 Hz, 2H), 3.28 (d, *J* = 4.2 Hz, 2H), 3.02 (d, *J* = 33.9 Hz,

2H), 2.81 (s, 2H), 2.24 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.65, 167.38, 157.21, 148.10, 143.00, 142.16, 138.42, 130.83, 130.12, 129.99, 129.38, 129.00, 128.82, 127.58, 126.67, 126.59, 123.93, 122.73, 119.67, 116.75, 116.13, 46.26, 45.80, 24.48 HRMS: (ESI, m/z): $[M+H]^+$ calcd 550.15 found $[M+Na]^+$ m/z = 573.1238, $[M+K]^+$ m/z =589.1002 Anal. Calcd for $C_{28}H_{24}F_2N_4O_4S$; C, 61.08; H, 4.39; N, 10.18; found C, 60.98; H, 4.54; N, 10.08.

5.2.10.5. N-(4-((4-(2-(4-fluorophenyl) quinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS23)

White powder, m.p. 288-295℃, 55 % yield obtained after column chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.98 (s, 1H), 8.18 (t, *J* = 8.5 Hz, 2H), 7.89 (d, *J* = 7.5 Hz, 1H), 7.75 (d, *J* = 8.5 Hz, 2H), 7.71 (s, 1H), 7.68 – 7.58 (m, 4H), 7.52 (dd, *J* = 20.5, 7.3 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 1H), 2.83 (s, 8H), 2.17 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.55, 165.58, 150.23, 148.02, 144.29, 142.86, 132.05, 131.97, 131.57, 129.28, 128.88, 128.77, 128.67, 128.30, 124.29, 122.78, 119.61, 119.20, 114.48, 46.21, 45.77, 24.59. $[M+H]^+$ m/z calcd 532.16 for found 532.6 Anal. Calcd for $C_{28}H_{25}FN_4O_4S$ C, 62.15; H, 3.93; N, 10.32; found C, 63.15; H, 4.73; N, 10.52.

5.2.10.6. N-(4-((4-(2-(3,4-dichlorophenyl) quinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS24)

Yellowish powder, m.p. 290-295℃, 60.5 % yield obtained after column chromatography. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 (s, 1H), 7.80 (d, *J* = 7.2 Hz, 1H), 7.72 (dd, *J* = 15.6, 6.3 Hz, 4H), 7.64 (d, *J* = 10.7 Hz, 2H), 7.56 (t, *J* = 7.2 Hz, 1H), 7.48 (t, *J* = 6.9 Hz, 1H), 7.25 (d, *J* = 6.9 Hz, 2H), 7.18 (d, *J* = 6.6 Hz, 1H), 3.99 (d, *J* = 45.9 Hz, 2H), 3.28 (d, *J* = 4.2 Hz, 2H), 3.02 (d, *J* = 33.6 Hz, 2H), 2.81 (s, 2H), 2.24 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.65, 167.38, 157.21, 148.10, 143.00, 142.16, 138.42, 130.83, 130.12, 129.99, 129.38, 129.00, 128.82, 127.58, 126.67, 126.59, 123.93, 122.73, 119.67, 116.75, 116.13, 46.26, 45.80, 24.48. $[M-H]^-$ m/z calcd 582.09 for found 581.10 Anal. Calcd for $C_{28}H_{24}Cl_2N_4O_4S$ C, 57.64; H, 4.15; N, 9.60; found C, 56.64; H, 4.55; N, 9.79.

5.2.10.7. N-(4-((4-(2-(4-(trifluoromethyl) phenyl) quinoline-4-carbonyl) piperazin-1-yl) sulfonyl) phenyl) acetamide (QS25)

White, m.p. 270-272℃, 56.5 % yield obtained after column chromatography**.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.98 (s, 1H), 8.18 (t, *J* = 8.5 Hz, 2H), 7.89 (d, *J* = 7.5 Hz, 1H), 7.75 (d, *J* = 8.5 Hz, 2H), 7.71 (s, 1H), 7.63-7.67 (m, 4H), 7.52 (dd, *J* = 20.5, 7.3 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 1H), 2.83 (s, 8H), 2.17 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.51, 167.22, 155.54, 143.21, 142.33,

137.22, 135.91, 132.44, 132.03, 131.95, 130.79, 130.47, 129.39, 129.09, 128.82, 128.72, 127.91, 123.91, 121.19, 119.67, 46.48, 46.26, 38.67. [M+H] $^{+}$m/z calcd 582.15 for found 583.00 Anal. Calcd for $C_{29}H_{25}F_3N_4O_4S$ C, 59.79; H, 4.33; N, 9.62; found C, 58.89; H, 4.03; N, 9.52.

5.3. Biological assays

5.3.1. Recombinant FP2 protein preparation

Slight modification in the methods reported by Shenai *et al*. [4] and Kumar *et al*. [26] were adopted to prepare recombinant FP2. Bacteria containing the PRSET-A FP2 plasmid at their mid-log phase of growth were treated with isopropyl-1-thio-b-D-galactopyranoside (IPTG, 1 mM) for 5 h at 37 ℃ followed by suspension in ice-cold buffer (50 mM NaH_2P0_4 and 100 mM NaCl). Lysozyme (10ul/1 mL; 1 mg/mL (FC); stock 100 mg/mL) was added and kept in ice for 30 minutes followed by sonication and centrifuge at 15,000 rpm for 45 min at 4 ℃. The pellet was washed twice with native buffer. Lastly, the pellet was solubilized in UB [8M Urea, 20mM Tris, 250mM NaCl, pH 8.0]; (5 mL/g of inclusion body pellet) at room temperature for 60 min with gentle stirring. The mixture was produced for centrifuge at 15,000 rpm for 60 min at 4 ℃ to separate the insoluble material. Imidazole (10 mM) and 1% TritonX-100 were added to the solution. The supernatant was incubated overnight at 4 ℃ with a nickel–nitrilotriacetic acid (Ni–NTA) resin to obtain the purified recombinant protein. The resin was loaded on a column and washed with 10 bed volumes of UB. The bound protein was eluted with 250 mM imidazole in UB and the bicinchoninic acid assay was used for its quantification. For refolding, the fractions containing FP2 protein were pooled in ice-cold refolding buffer and diluted 100-fold using Tris–HCl (100 mM), 1 mM EDTA, 20% glycerol, 250 mM L-arginine, 1 mM GSH, 1 mM GSSG, pH 8.0. The mixture was incubated with moderate stirring at 4 ℃ for 24 h, and concentrated to 25 mL using a stirred cell with a 10 kDa cut-off membrane (Pellicon XL device, Millipore) at 4 ℃. The sample was then filtered using a 0.22-mm syringe filter. Again, bicinchoninic acid assay was used for quantification of the purified and concentrated protein.

5.3.2. Enzyme Assay and Kinetic Analysis

The procedure adopted by Kumar *et al*. was applied to confirm FP2 activity [26]. Briefly, in 96 well plate, 200 µL of buffer (100 mM sodium acetate pH 5.5, 10 mM DTT) containing 15 µM FP2 enzyme, 10 µM fluorogenic substrate benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin hydrochloride (ZFR-AMC) was added and the release of 7-amino-4-methyl coumarin (AMC) was monitored (excitation 355 nm; emission 460 nm) over 30 min at RT using

a LS50B Perkin-Elmer fluorimeter. FP2 was preincubated with different concentration of each inhibitor for 10 minutes at room temperature to determine the effect of the inhibitors (test compounds) on the enzyme activity. The fluorogenic substrate was utilized to confirm the remaining activity of the inhibitors. PRISM software (Graph Pad, San Diego) was used to determine the kinetic constants *k*m, Vmax, and IC_{50} values.

5.3.3. P. falciparum culture and inhibition assay

P. falciparum strain 3D7 treated with human erythrocytes (4% hematocrit) in RPMI media (invitrogen) was incubated and added with 0.5% albumax and 4% hematocrit as per the reported protocol [35]. Cultures were synchronized by repeated sorbitol treatment following Lambros and Vanderberg [36]. The assays performed were done in triplicates and each experiment was repeated twice. Each well holding 0.2 mL of media (RPMI (Invitrogen) with 0.5% albumax), 2% hematocrit, and the parasitemia adjusted to ≤1% was synchronized followed by the addition of the desired final concentration (0–100 μM) of the test compounds, and same amount of solvent (DMSO) as the control. After this, the cultures were allowed to grow for 52 h. SYBR green (DNA fluorescent dye-binding) assay was performed to confirm the parasite growth in presence and absence of the test compounds [37].

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Highlights

- A set of 25 quinoline carboxamide core moiety-based compounds was designed and synthesized to inhibit *P. falciparum* FP2.
- Integration of molecular hybridization strategy with *in silico* drug design was adopted for the development of these FP2 inhibitors.
- Compounds **Qs17**, **Qs18**, **Qs20** and **Qs21** displayed best results in docking and i*n vitro* FP2 inhibition studies.
- These compounds inhibited *P. falciparum* growth with IC_{50} values: 1.05, 1.95, 1.43 and 0.81 μM, respectively.
- Morphological and food-vacuole abnormalities much better than the FP2 inhibitor, E-64 were reported by these test compounds.