A SYNOPSIS REPORT ON

"Design, Synthesis and Biological Evaluation of Some Novel Antimalarial Agents"

For submission of Ph.D. Thesis

By

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FOPH/26

Research Guide

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1. INTRODUCTION

The World Health Organization (WHO) in its World malaria Report-2021 from 84 malaria endemic countries on the prevalence, advances, treatment and diagnosis of Malaria[1]has presented a report indicating 247 million malaria cases and 619000 deaths globally, it shows an increase in overall malaria cases from 245 million in 2020 to 247 million in 2022.But the situation is still frightening with rise in drug resistant cases in the recent years. Children's below age 5 and pregnant women are the most affected ones. In 2021 12 million women were found to be exposed to malaria during pregnancy which resulted into low birth weight children's.

Out of 84 countries, twenty nine countries are accounted for malaria cases for 95% burden. East African countries are accounted for about 51% of all cases globally. The WHO African region accounted for the 94% malaria cases in 2022. The south-Asia WHO region is accounted for 3% of malaria cases burden globally. The main causative agent of malaria is *Plasmodium, a genus* belonging to the phylum protozoa. *Plasmodium* has four main species *i.e. Plasmodium falciparum*, *P. vivax*, *P. malarie* and *P. ovale* which have the potential of inducing malaria disease in humans. Among these species, *P. falciparum* contributed for 90% of malaria cases worldwide and was responsible for the highest number of malaria deaths annually[2]. The fifth species of *Plasmodiumi.e.P. Knowlesi*is responsible for malarial infection in both humans as well as monkeys in certain areas of Southeast Asia[3]. The transmission of malaria occurs mainly through the bites of infected female anopheles mosquitoes and depends on a number of climatic factors (rain fall patterns, temperature and humidity), and tropical regions are more favorable than temperate ones for its spread.

2. LIFE CYCLE OF MALARIA[4]

The life cycle of *Plasmodium* parasite is complex one and in order to eradicate the disease, every stage should be considered for the treatment of malaria. The life cycle of *Plasmodium* parasite mainly divided into four stages *i.e.* liver stage, blood stage, transmission stage and mosquito stage (**Fig**. **1**).

The human malaria parasite has complex life cycle and it differs from species to species as shown in **Figure 1.** It is a multistage life cycle that involves two living beings, half in mosquito vector and other half in human host. It starts when the plasmodium infected female Anopheles mosquito hunting for the blood meal bites the skin, probes for a blood vessel to suck the blood and spills saliva inside the blood stream which prevents blood clotting. The motile infectious form of Plasmodium in its developing stage is already present in salivary glands and thus enters the blood stream along with saliva. The parasite passes through several stages of development starting from sporozoites to merozoites, trophozoites and gamatocytes.

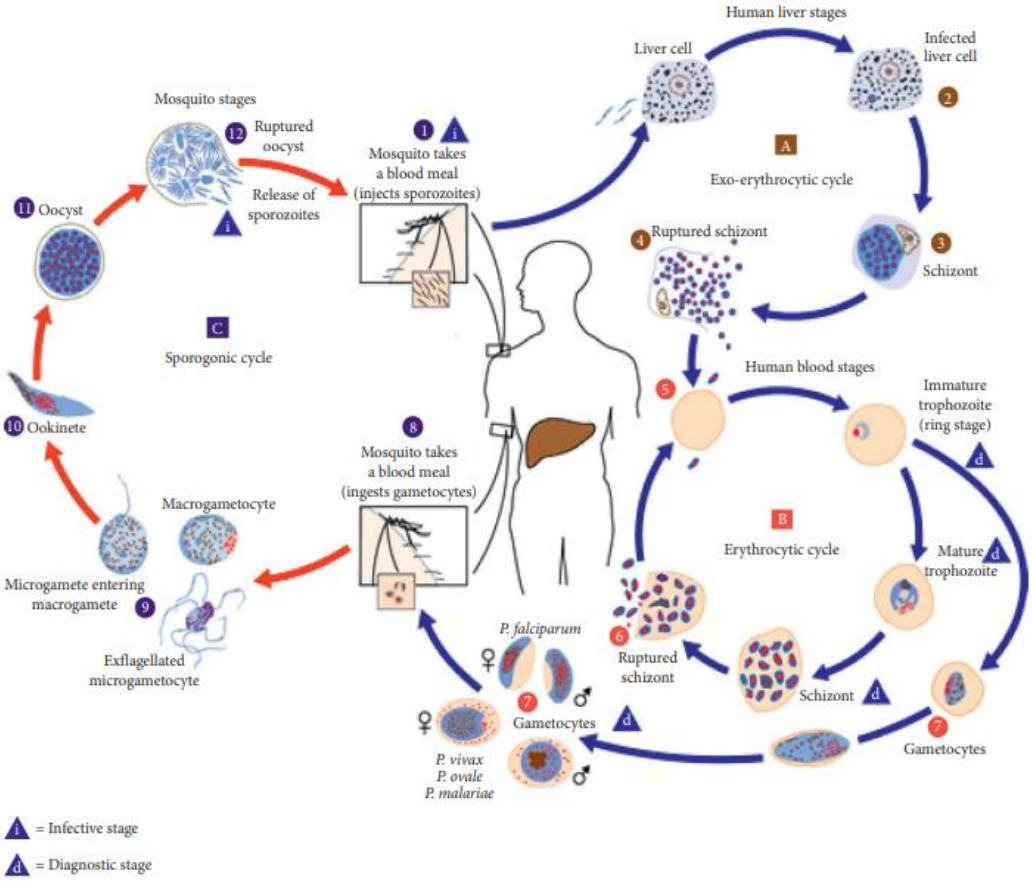


Figure 1: Life cycle of Malaria Parasite[4]

Within 30–60 min of inoculation, the thread-like sporozoites are carried to the liver by the circulatory system. Over a period of 7–12 days, the sporozoites grow into schizonts and can develop up to 30,000 merozoites. The circumsporozoite protein creates and maintains a favourable environment for the growth and development of sporozoite within the parasitophorous vacuoles, where it asexually forms merozoites. The pre-erythrocytic stage remains as a silent phase. All the merozoites, collectively known as schizonts ruptures the hepatocyte and enters back into blood stream to find new target called RBC. A single infected hepatocyte can form 1000 of new parasites which infects the RBCs inside the blood which is termed as centre stage for asexual development of malarial parasite. On the other hand, some vivax and ovalesporozoites turn into hypnozoites, a form that can remain latent in the liver for months or years and cause relapses in infected individual.

After invading into RBCs by multiple receptor–ligand interactions, the merozoites undergoes development from the early ring stage to late trophozoite and then after mitotic divisions to the schizont stage by consuming haemoglobin. As the erythrocyticschizont ruptures, it again releases the merozoites that continues the life cycle by invading into other RBCs. At the time of RBC lysis, the cyclical fever occurs as schizonts rupture to release new infectious merozoites. (It occurs every 48 hr in tertian malaria and every 72 hr in quartan malaria infection). During this repeated cycle, several merozoites get developed into two sexual forms i.e., male, and female gametocytes known as erythrocytic gametes with single nucleus and waits for the arrival of blood seeking female anopheles.

The gametocytes, male (microgametocyte) and the female (macrogametocytes), are ingested by an anopheles mosquito during a blood meal in order to become sporozoites. Sporozoites multiply in mosquito and this phase of cycle is known as sporogonic cycle, which are later stored in salivary glands of female *Anopheles* mosquito that again bite another individual and life cycle continues.

3. Therapeutic target sites for malaria[5]

Among the various approaches for the treatment of malaria, following are the most promising:

- Food vacuole
- Apicoplast

- Mitochondria
- Cytosolic
- Plasma membrane
- Transporter
- **Food vacuole as a drug target:**

When the malaria parasite develops inside human erythrocytes, digestive vesicles fuse to form a sizable digestive vacuole or food vacuole. This cellular component oversees the breakdown of approximately 60–80 percent of the host's red blood cell haemoglobin offers a source of amino acids necessary for parasite formation and proliferation. A group of proteases that break down haemoglobin into tiny peptides starts this route. Heme is produced from haemoglobin during proteolysis as a hazardous by-product that is detoxified by being changed into hemozoin. According to some research, hemozoin includes up to 95% of the free iron formed during the breakdown of haemoglobin. Two other mechanisms for haemoglobin degradation are glutathione-dependent degradation in the cytoplasm and hydrogen peroxide-mediated degradation of haemoglobin within the food vacuole.

- **Inhibiting hemozoin crystals formation**

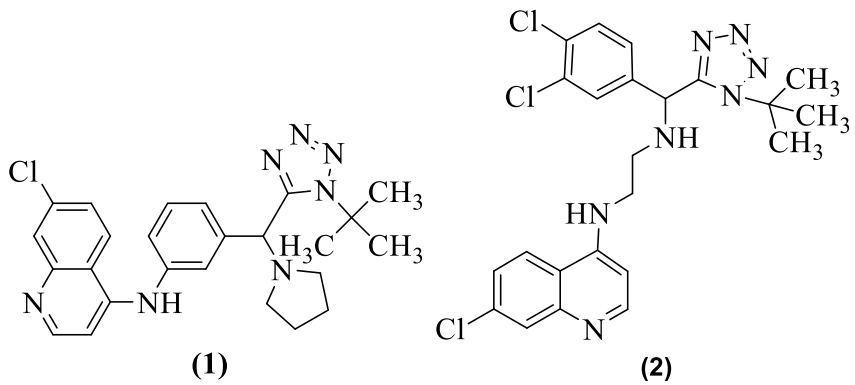
Heme (Fe (II) PPIX), a by-product of haemoglobin degradation, and is poisonous to the parasite and is produced in huge quantities. This heme'speroxidative characteristic causes membrane damage. The most crucial step in P. falciparum's detoxication of the heme group is the formation of the hemozoin pigment, which is produced by the oxidation of heme to hematin (Fe(III)PPIX) and subsequent conversion of hematin to hemozoin by histidine-rich protein (HRP-2). Haemozoin is a crystalline polymer that is identical to haematin and has co-ordinately bound heme units together by the propionate carboxyl groups on one unit and the ferric ion on the other one. The different cyclic dimers are connected by the hydrogen bond between the remaining carboxylate groups. Hematin is known to be the target of chloroquine and other blood schizonticidals like chloroquine, halofantrine, mefloquine, quinidine, and 8-aminoquiniolines analogues like tafequine as well as bisquinoline analogues.

- **Target heme detoxification**

The toxic heme must be eliminated in order for *P. falciparum* to live. Hazardous heme is both converted into and broken down by hydrogen peroxide, which is produced during the transition from the oxyhemoglobin (Fe II) to methemoglobin (Fe III) stage. Reactive oxygen species such superoxide anion, H2O2, and hydroxyl radicals cause oxidative stress. Some of the heme is degraded by reduced glutathione, which seems to diffuse into the parasite's cytoplasm.

4. Literature Survey

Gao*et.al.,* found that a class of medications containing a quinoline and tetrazole hybrid moiety were more effective against plasmodial parasites. The hybrid (1) in the disclosed series shown its potential to be a strong inhibitor of CQ-sensitive (3D7) and resistant (K1 and W2) strains of *P. falciparum* with matching IC50 values of 0.010, 0.0096, and 0.077 M.The hybrid (1) had a high metabolic clearance rate, and the study also concluded that tertiary amine nitrogen substituents are susceptible to metabolism. Additionally, compound (1) showed significant CYP450 inhibitionenzyme, but it had the disadvantages of poor pharmacokinetics and limited bioavailability.

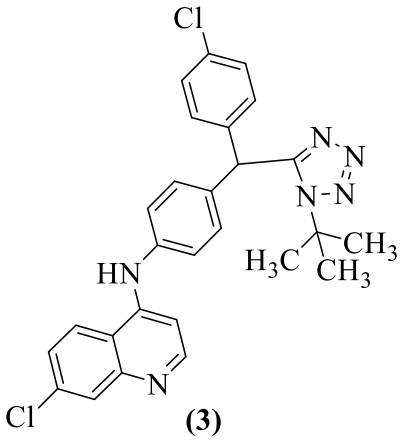


(1) **(2)**

In a different study, Tulukula*et al*. unveiled a string of aminoquinoline-clubbed tetrazole hybrids that were effective *P. falciparum* strain inhibitors. In that work, the authors have substituted an alkyl bond for the previously reported aryl linkage found in (1).The hybrid (2) had IC50 values against the 3D7, K1, and W2 strains of the malaria parasite of 0.0004, 0.008, and 0.030 M, respectively, and it also displayed a resistance index value of 20. The cytotoxicity of compound (2) was under control, and it strongly inhibited the CYP3A4 enzyme isoforms. *P.*

berghei ANKA mice model hybrid (2) showed 47% inhibition of parasitaemia load on day 7 of in vivo research.[7]

Pandey *et al*., reported on a novel series of aminoquinoline-tetrazole hybrids that were tested for possible antimalarial activity. The hybrids (3 and 4) from the described series were shown to be powerful inhibitors of CQ-sensitive (3D7) and resistant (K1) parasite strains that cause malaria, with IC50 values of 10.6, 142.9, and 11.78, 233.7 nM, respectively. When given intraperitoneally or orally, both the drugs (3 and 4) in in vivo trials shown a 99.99% decrease in parasitaemia load on day 4.[8]



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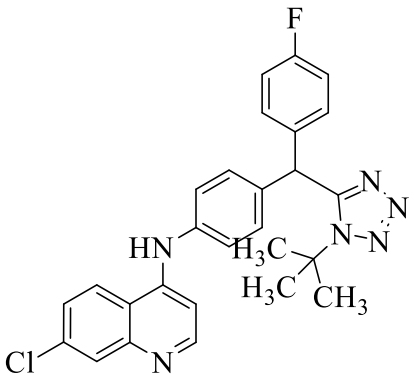
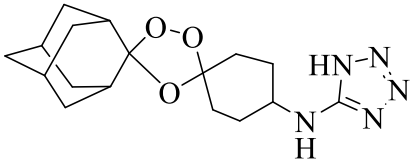


(4)

2.1.2 Tetrazole- Peroxide hybrids

According to Gao *et al.*, series of tetrazole- peroxide hybrids showed potential antiplasmodial activity. It was noted that the antiplasmodial activity of tetrazole-peroxide hybrids against multidrug-resistant Dd2 *P. falciparum* was similar to that against chloroquine-susceptible 3D7, indicating their have the potential to treat multidrug-resistant malaria.Two hybrids **5** (IC50: 2.5 and 2.8 nM) and **6** (IC50: 2.1 and 2.9 nM) were comparableor better than the references artemisinin (IC50: 2.5 and 4.0 nM), artesunate (IC50: 4.6 and 5.1 nM) and dihydroartemisinin (IC50: 4.2 and 4.7 nM) against multidrug-resistant Dd2 and chloroquine-susceptible 3D7 *P. falciparum* strains.[9]



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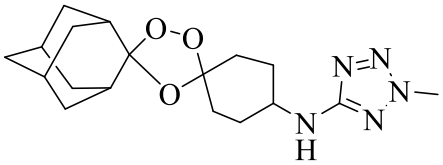
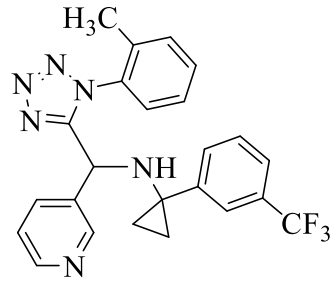


(6)

2.1.3 Tetrazole - pyridyl agents

Lawong *et al.,* gave the series of compounds showing good antimalarial activity against *P. falciparum* strains *Pf 3D7* and *Pf Dd2.* the best compound 7 was found having IC_{50} of 0.23μM[10].

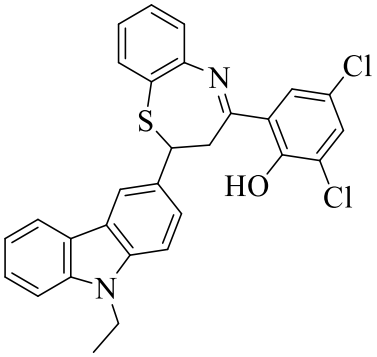


(7)

2.2 Carbazole containing antimalarials

2.2.1 Carbazole-thiazepine derivatives[11]

A variety of 1,4-benzothiazepine and pyrazoline ring containing carbazole-based compounds have been synthesised by Kandor V.A*., et al*(2019).Derivatives weresynthesized, and FT-IR, 1HNMR, 13NMR, and Mass spectral analyses were used to confirm the structures of the newly synthesized compounds. All fresh derivatives underwent in vitro testing for antimalarial activity. Three different compounds (8, 9 and 10) showed positive antimalarial activity.



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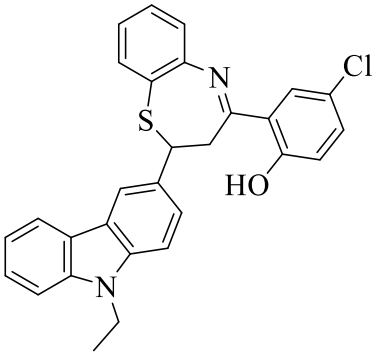

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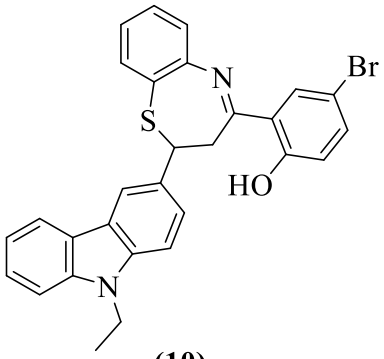
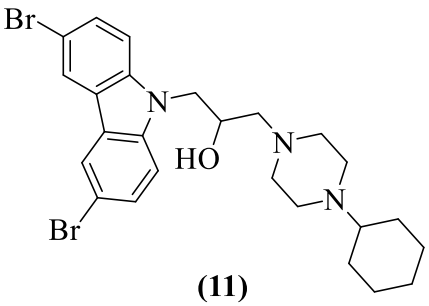


(10)

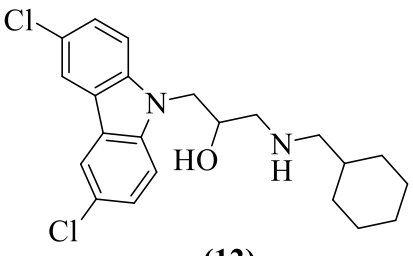
2.2.2 Carbazole- piperazine[12,13]

Agata *et al.,* synthesised several compounds that contains carbazole ring and are showing potential antimalarial activity having IC_{50} value of 9 nM against *Pf*-K1.



(11)

Another compounds showing good antimalarial activity against *P. falciparum* strains *Pf*-K1,the best compound **12** were found having IC_{50} of 16nM respectively.



(12)

5. AIMS AND OBJECTIVES

- Degradation of host red cell hemoglobin within the food vacuole of parasite is the most essential step for the formation of amino acids necessary for the protein synthesis. Degradation of hemoglobin leads to the formation of heam as toxic byproduct and parasite tends to detoxify this heam by converting it into the haemozoin. Hence, the blockade of heam to being converted into the haemozoin is the potential target for the development of antimalarial agents.
- Another crucial target for antimalarial agents is inhibition of hemozoin formation which played a key step as it involves biomineralization of ferriprotoporphryin IX within the acidic digestive food vacuole as a way to combat the toxicity of this hemoglobin degradation byproduct and its inhibition leads to increase in heam concentration in the parasite therefore it could be regarded as another important target for the synthesis of novel antimalarial lead molecules.
- Keeping in mind, the importance of these two biological targets, we thought logically synthesizing the novel lead molecules containing the two drugs active against both targets i.e. heam in the food vacuole and β-hematin. As per the literature, carbazole-containing compounds have been discovered to be responsible for the inhibition of β-hematin formation. So we planned to use two individual active pharmacophores of two marketed drugs (having heme as a target) i.e. TDR30137 (I) Hit of carbazole and choloroquine-tetrazole scaffold (II) for the preparation of novel antimalarial agents. Both of these pharmacophores were separately integrated with the help of two or three carbon/piperazinyl linker to offer final hybrid compounds (III; Fig.2)

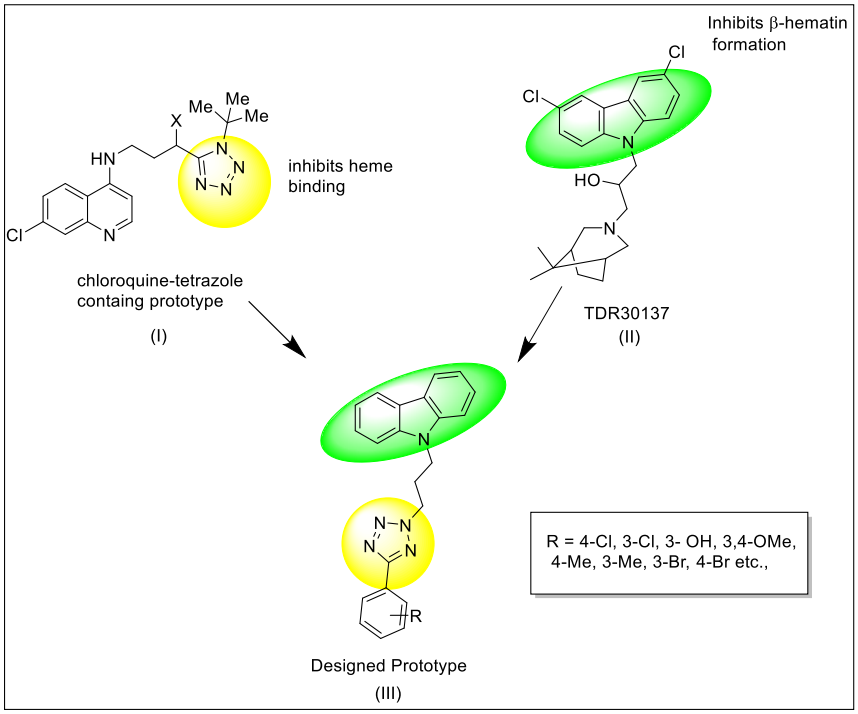


Figure 2: Designed Prototype for the synthesis of compound 16

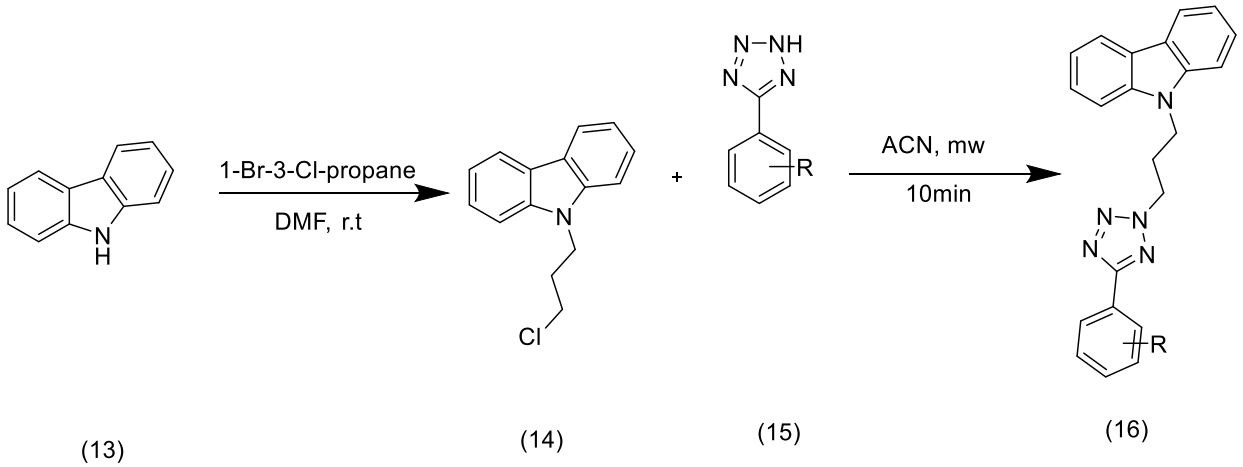
So, the broader objectives of the current research work is

- To design novel agents active against both heam and β-hematin in food vacuole
- To establish the mechanism of action for inhibitory activity of new molecules through molecular docking, molecular dynamics etc.
- Synthesis of designed prototypes having potential activity against the malarial strains.
- *In vitro* biological evaluation of synthesized compounds

6. EXPERIMENTAL WORK DONE

Designed prototype was synthesized using following procedure as depicted in synthetic scheme 1.

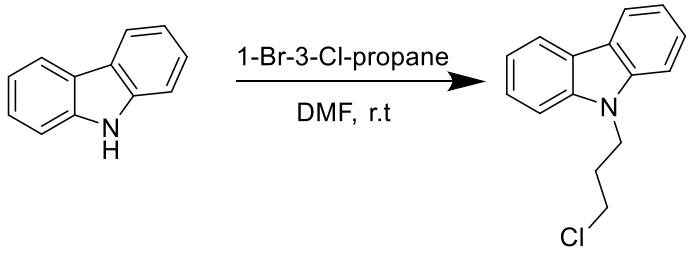
Synthesis of 9-(3-(5-phenyl)-1*H*-tetrazol-1-yl)propyl)-9*H*-carbazole has been carried out by using scheme 1



(13) (14) (15) (16)

Scheme 1:- Synthesis of 9-(3-(5-phenyl)-1*H*-tetrazol-1-yl)propyl)-9*H*-carbazole (16)

Step:1 Synthesis of 9-(3-chloropropyl)-9*H*-carbazole (14)

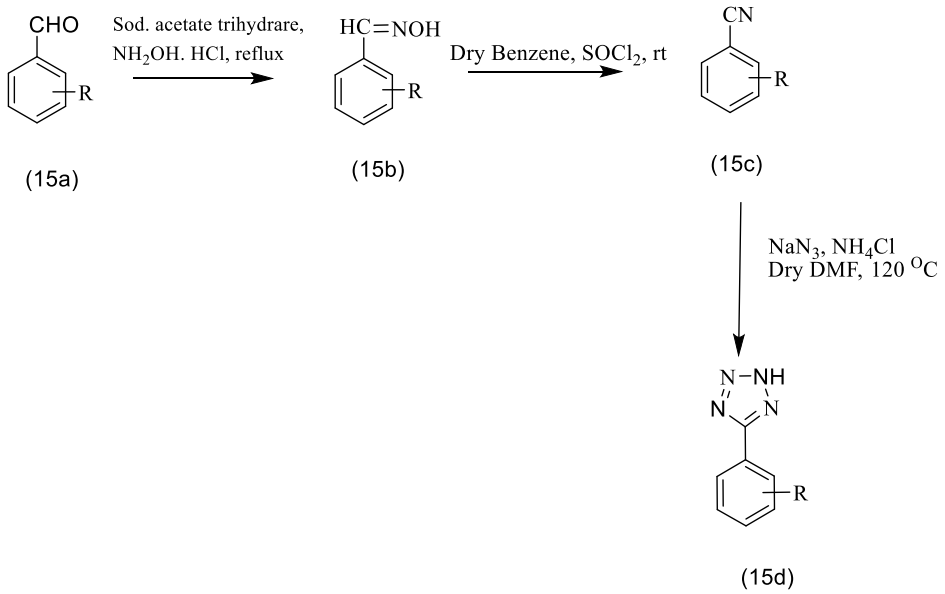


(13) (14)

In a doubled necked RBF, n-hexane was taken and sodium hydride (1.07gm, 0.044M) was added into it. RBF was kept aside for 5 minutes and then excess of n-hexane was removed in methanol. The RBF was then dried and Carbazole (49, 5gm, 0.0299M) and DMF were added with constant stirring condition for 2 hrs. After 2 hrs, 1-Bromo-3-chloro-propane was added drop wise into the flask under dry condition. Nitrogen gas was continuously passed into the flask with the help to balloon and septum. The reaction was monitored with the help of TLC. After completion of the reaction, crushed ice was added. The product was extracted using ethyl acetate and dried over

rotavapour. The IR spectra of compound shows NH starching at 3419 and CN stretching at 1450cm^{-1}.

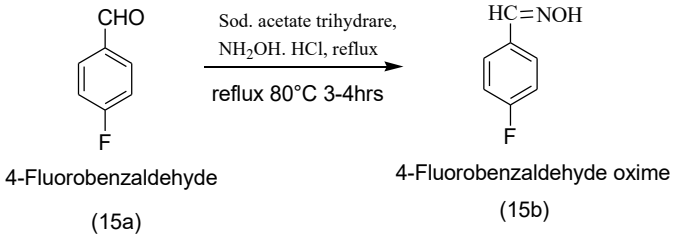
Synthesis of tetrazole derivatives has been carried out by using scheme 2



R= 4-Cl, 4-F, 2-NO_2, 4-Br, 3,4-OMe, 3-Cl, 3-OH, 4-OMe, Phenyl, 4-Me, 3-Me, 3-NO_2 etc.

Scheme 2:- Synthesis of Tetrazole (15d)

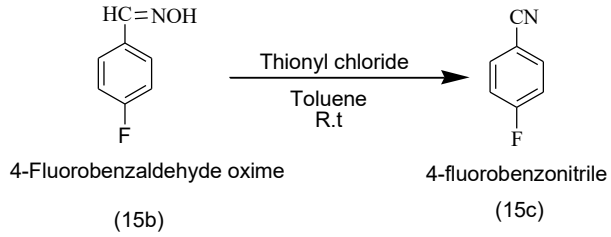
Synthesis of benzaldooxime(15b)



In 25 ml RBF, 4-fluorobenzaldehyde was dissolved in methanol. Sodium acetate trihydrate and hydroxylamine hydrochloride were added to the reaction mixture and refluxed at 90 ^{o}C for 3-4 hrs. After completion of reaction, reaction mixture was poured into the cold water. The precipitated solid was then filtered, dried to obtain desired product.In the IR spectrum of

compound, the -OH stretching was observed at 3191 cm^{-1} and C=N peak was observed at 1604cm^{-1}.

Synthesis of benzonitrile (15c)

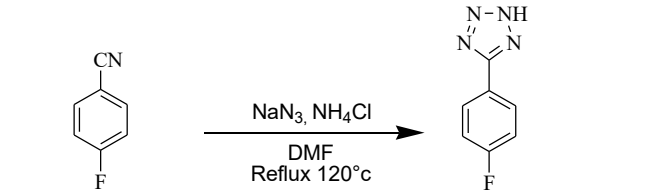


4-Fluorobenzaldehyde oxime (15b)

4-fluorobenzonitrile (15c)

4-Fluorobenzaldoxime was dissolved in dry benzene in a 10 ml RBF and to this solution thionyl chloride was added drop wise at room temp with stirring. Completion of the reaction was monitored by TLC and after completion of the reaction; excess of thionyl chloride was removed under vacuum. Cold water was added to the remaining reaction mass and washed with $NaHCO_3$ solution (5 %), extracted with chloroform and dried over Na_2SO_4. Chloroform was evaporated with the aid of rotary evaporator offering product as brown liquid.In the IR spectrum of compound, the C=N stretching was observed at 2232cm^{-1}.

Synthesis of 5-(4-fluorophenyl)-2H-tetrazole (15d)



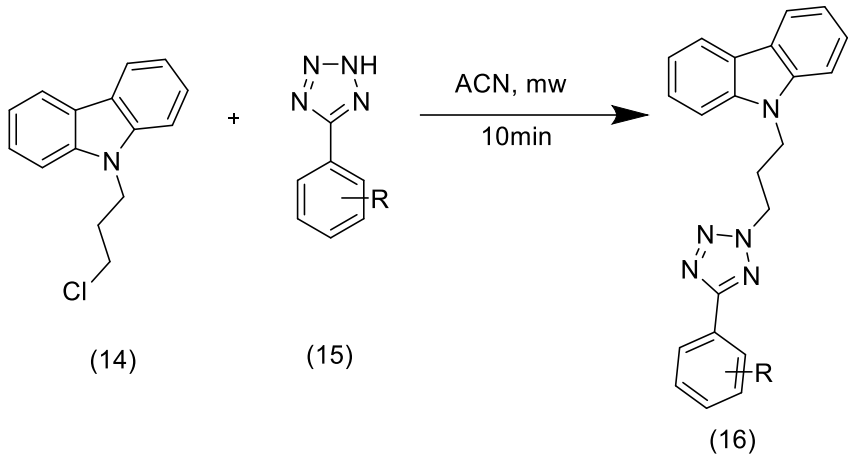
4-Fluorobenzonitrile (15c)

5-(4-Fluorophenyl)-2*H*-tetrazole (15d)

In a 50 ml RBF, benzonitrile (140, 0.5 ml, 0.0048M), sodium azide (0.93 gm, 0.014 M) and ammonium chloride (0.77 gm, 0.014 M) were taken in dry DMF (3 ml). The reaction mixture was heated on oil bath at 120 °C for 6-8 hrs. Completion of the reaction was monitored by TLC. After completion of the reaction, crushed ice was added to the reaction mixture and acidified (pH: 2) with dilute HCl. The obtained precipitate was filtered under vacuum and dried offering

compound (141) as white crystals. In the IR spectrum of compound, the-NH streching was observed at 3420cm^{-1} and tetrazole ring peaks were observed at 1248, 1164, 1052cm^{-1}. Its mass spectrum showed molecular peak at 165(M)$^{+}$.

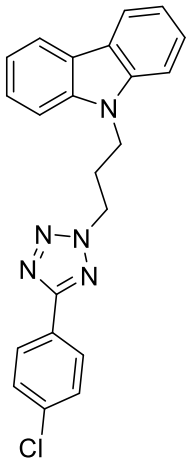
Synthesis of 9-(3-(5-phenyl)-1*H*-tetrazol-1-yl)propyl)-9*H*-carbazole (16)



(14) (15) (16)

R = 4-Cl, 3-Cl, 3- OH, 3,4-OMe, 4-Me, 3-Me, 3-Br, 4-Br etc.,

In 25ml of single-neck RBF, 9-(3-chloropropyl)-9*H*-carbazole,5-phenyl-1*H*-tetrazole, TEA were added in ACN and kept in microwave with the Power 8 for 10 mins. Reaction mixture was monitored by TLC. After completion of reaction, crushed ice was added. The mixture was then extracted with chloroform. Further purification was done by column chromatography using 20% pet ether : ethyl acetate that offered a colorless compound.



9-(3-(5-(4-chlorophenyl)-2*H*-tetrazol-2-yl)propyl)-9*H*-carbazole

(16)

The IR spectrum of compound shows NH stretching at 3419 cm^{-1}, C-N stretching at 1450 cm^{-1}. Its PMR spectra shows 8.15 (d, J = 2.0 Hz, 1H), 8.11 (d, J = 7.7 Hz, 2H), 8.05 (dt, J = 6.4, 1.9 Hz, 1H), 7.52 – 7.40 (m, 4H), 7.40 (d, J = 8.2 Hz, 2H), 7.26 (dd, J = 14.8, 1.0 Hz, 2H), 4.68 (t, J = 6.7 Hz, 2H), 4.51 (t, J = 6.7 Hz, 2H), 2.70 (p, J = 6.7 Hz, 2H). The mass spectrum of compound shows molecular ion peak at 389 $(M)^{+2}$.

DESIGNING OF COMPOUND 22

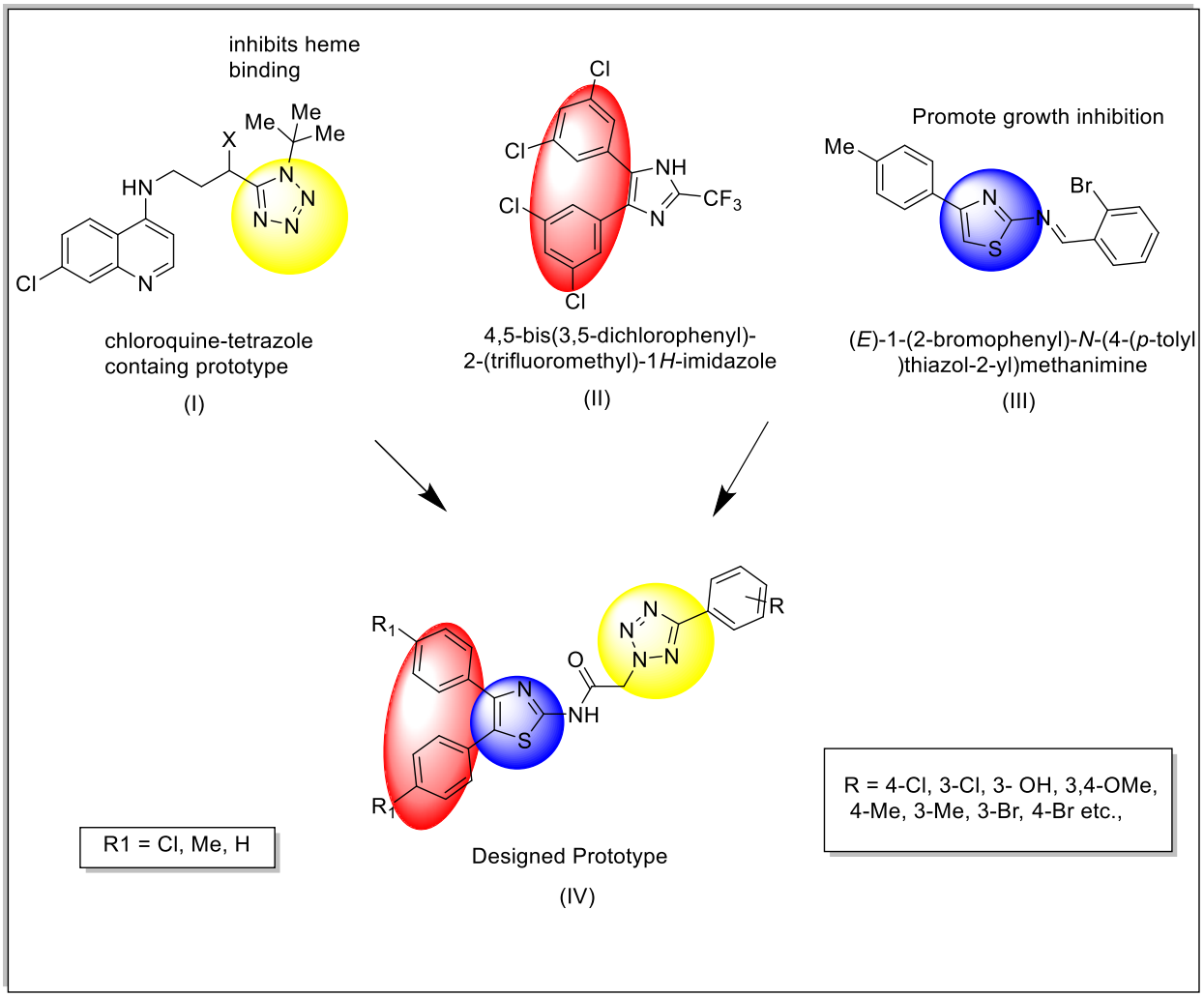
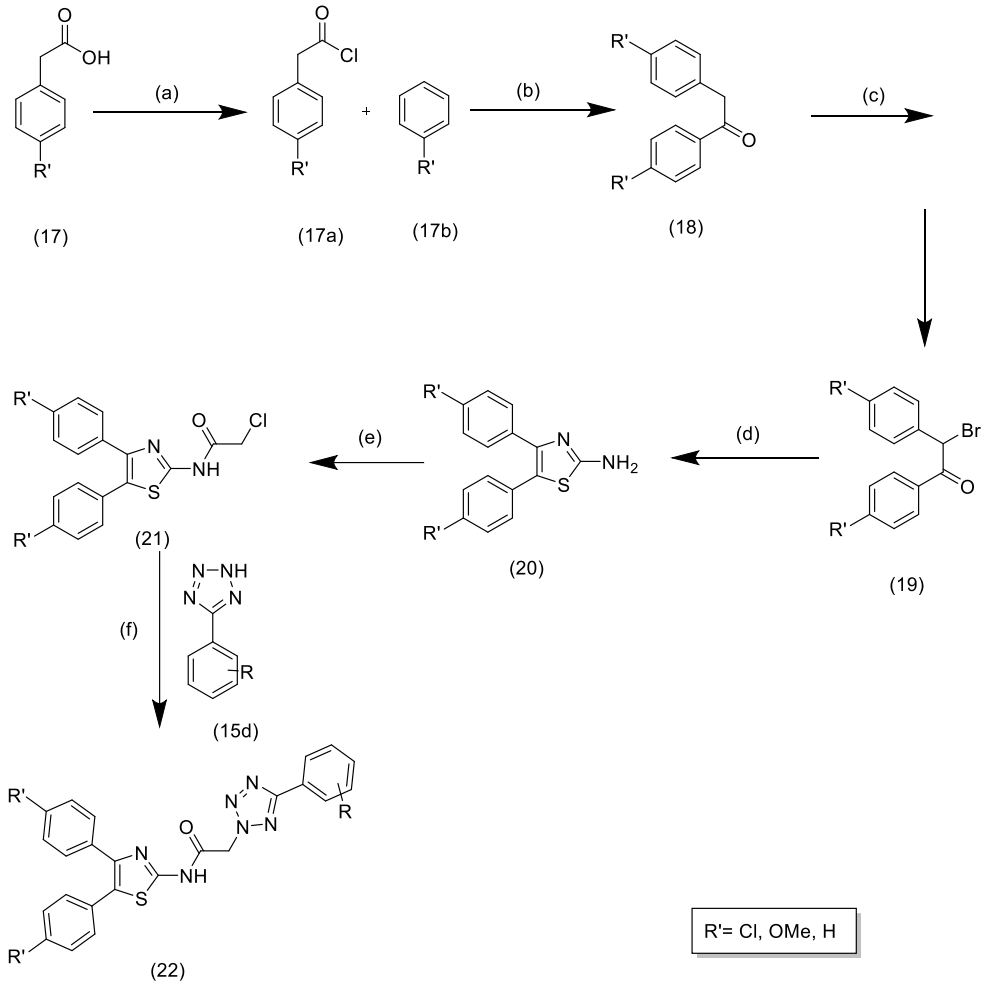


Figure 3: Designed Prototype for the synthesis of compound 22

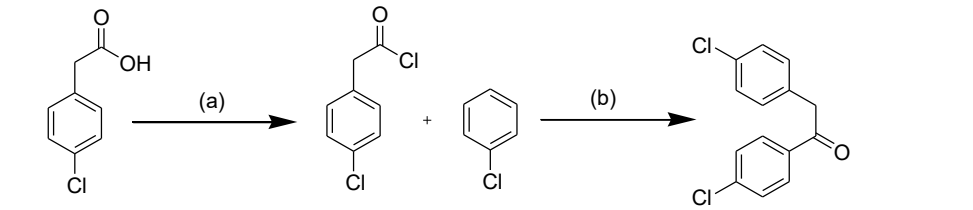
Synthesis of Designed prototype (22) has been carried out by using scheme 3



Reagents used: (a) thionyl chloride, (b) aluminium chloride, DCM, (c) bromine, acetic acid, (d) thiourea, methanol, (e) chloroacetyl chloride, DCM, (f) ACN, potassium carbonate

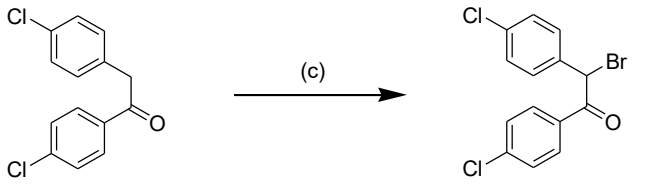
Scheme 3:- Synthesis of N-(4,5-bis(4-chlorophenyl)thiazol-2-yl)-2-(5-(4-(R)phenyl)-2*H*-tetrazol-2-yl)acetamide (22)

Synthesis of 1,2-bis(4-chlorophenyl)ethan-1-one



4-Chlorophenylacetic acid (17) (2 g, 13.33 mM) was converted to acid chloride using thionyl chloride (3 ml) under refluxing for 3 hrs under anhydrous conditions. Excess of thionyl chloride was removed by vacuum. In another RBF, dry DCM (10 ml) and anhydrous $AlCl_3$ (1.74 g) were taken. The contents were stirred under anhydrous conditions. To this solution (1.26 ml) of chlorobenzene was added drop wise. The acid chloride was added to the contents of the RBF and stirring was continued for 2 hrs maintaining the temperature between -5 to -10 °C. The contents were finally poured over crushed ice containing conc. HCl and then extracted with successive quantities of chloroform. The organic extract was washed with sodium bicarbonate solution (5%) and water. It was then dried over anhydrous sodium sulfate, filtered and subjected to solvent recovery. The crude product so obtained was recrystallized from methanol to afford 1,2-bis(4-chlorophenyl)ethan-1-one (18) as white crystals.In the IR spectrum of compound the C=O stretching of carbonyl was seen 1689 cm^{-1}. Its mass spectrum showed molecular ion peak at 265.14 $(M)^+$.

Synthesis of 2-bromo-1,2-bis(4-chlorophenyl)ethan-1-one

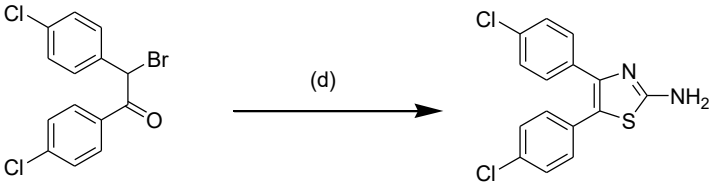


1,2-Bis(4-chlorophenyl)ethan-1-one (18)

2-Bromo-1,2-bis(4-chlorophenyl)ethan-1-one (19)

1,2-Bis(4-chlorophenyl)ethan-1-one (18) (2.0 g, 8.92 mM) was taken in a 100 ml RBF and dissolved in sufficient quantity of glacial acetic acid (10 ml) by warming. Bromine (0.5 ml) was added drop-wise into the stirred solution and the reaction was monitored by TLC until completion. The reaction mixture was poured into the ice cold water (200 ml) containing sodium metabisulphite to neutralize the excess bromine. The white precipitate so obtained was extracted with chloroform (3 x 20 ml) and the separated chloroform layer was dried, the solvent distilled off and the resulting residue was crystallised in methanol to yield pure pale yellow crystals of compound.In the IR spectrum of compound, the C=O stretching of carbonyl was seen at 1680 cm^{-1} . Its mass spectrum showed molecular ion peak at 344.77 $(M)^{+}$.

Synthesis of 4,5-bis(4-chorophenyl)thiazol-2-amine



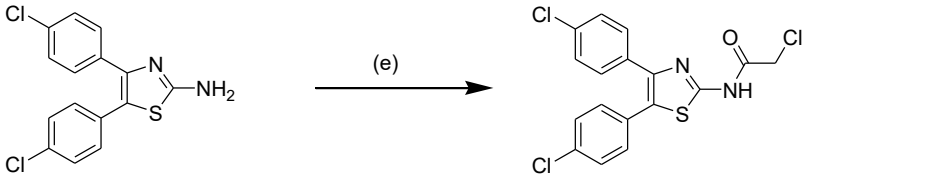
2-Bromo-1,2-bis(4-chlorophenyl)ethan-1-one (19)

4,5-Bis(4-chlorophenyl)thiazol-2-amine (20)

2-Bromo-1,2-bis(4-chlorophenyl)ethan-1-one (1.0 g, 3.29 mM) was dissolved in sufficient quantity of methanol in a 100 ml round bottom flask. Thiourea (0.35 g, 4.59 mM) and 3-4 drops of water were added into the reaction mixture and refluxed for 4-6 hrs. The reaction was monitored by TLC. After completion of the reaction, it was poured onto ice-cold water and the

resulting solution was basified with ammonia. The product so precipitated was filtered, dried and crystallized in methanol to obtain the desired compound as a white solid.In the IR spectrum, compound showed N-H stretching vibrations as two strong peaks at 3444 and 3274 cm-1 and N-H deformation as moderate peak at 1630 cm^{-1}. Its mass spectrum showed molecular peak at 321.32 $(M)^{+}$.

Synthesis of N-(4,5-bis(4-chlorophenyl)ethan-1-one

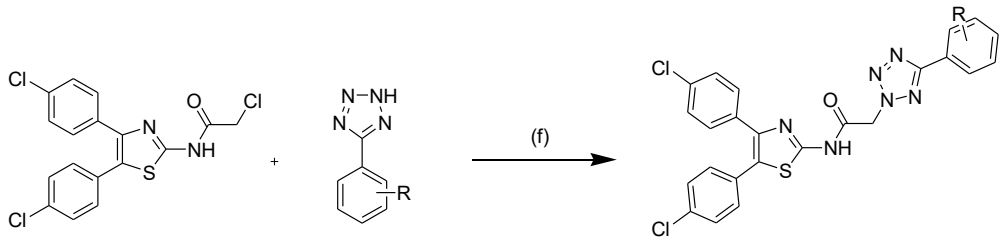


4,5-Bis(4-chlorophenyl)thiazol-2-amine (20)

N-(4,5-Bis(4-chlorophenyl)thiazol-2-yl)-2-chloroacetamide (21)

4,5-Bis(4-chorophenyl)thiazol-2-amine was dissolved in sufficient quantity of DCM in a 100 ml round bottom flask. Triethylamine was added to the reaction mixture and stirred for 10-15min in ice bath. After 15min chloroacetyl chloride was added drop wise to the reaction mixture and stirred for 2-4 hrs. The reaction was monitored by TLC. After completion of the reaction, it was poured onto ice-cold water. The product so precipitated was filtered, dried and crystallized in methanol to obtain the desired compound.In the IR spectrum of compound, the C=O stretching of the amide was observed at 1677 cm^{-1}. Its mass spectrum showed molecular ion peak at 398.70 $(M)^{+}$.

Synthesis of final compound (22-66)

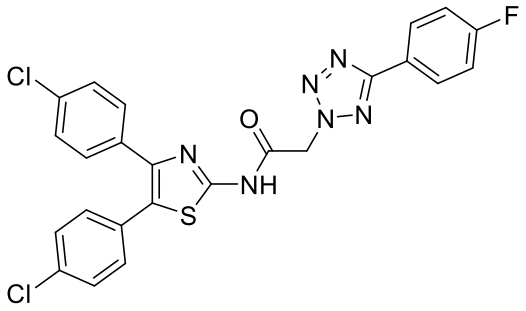


N-(4,5-Bis(4-chlorophenyl)thiazol-2-yl)-2-chloroacetamide (21)

(15d)

(22-66)

4-(2*H*-tetrazol-5-yl)benzene-1,2-diol was taken in a 50 ml RBF and dissolved in sufficient quantity of Acetonitrile. Potassium carbonate was added to the reaction mixture and stirred for 10mins. After stirring N-(4,5-bis(4-chlorophenyl)thiazol-2-yl)-2-chloroacetamide was added to the reaction mixture and the reaction mixture was refluxed for 8-10hrs. The reaction was monitored by TLC until completion. On completion the reaction mixture was poured into the ice cold water (200 ml). The product so precipitated was filtered, dried and purified using pet ether, ethyl acetate (16:4) to obtain the desired compound as a white solid.



N-(4,5-bis(4-chlorophenyl)thiazol-2-yl)-2-(5-(4-fluorophenyl)-2*H*-tetrazol-2-yl)acetamide

(22)

IR spectrum of compound for the C=O stretching of the amide was observed at 1677 cm^{-1},while signals for the tetrazole ring were present at 1245, 1144 and 1040 cm-1 . Its PMR spectrum offered signals for aromatic protons at δ 8.11 (m, 1H), 7.51-7.53 (m, 2H),7.35-7.37 (m, 2H), 7.31 (d, 4H), 7.26 (s, 2H), 7.13-7.18(t, 2H). Other signals for aliphatic protons were observed at δ 4.57 (s, 2H). . Its mass spectrum showed molecular peak at 526$(M)^{+2}$.

7. Computational studies

Docking studies were performed for the synthesized compounds. Autodock of MGL tools 1.5.6 were used for protein preparation, ligand preparation and grid generation and autodock4 software for docking. Docking pair of protein and ligand generated from autodock vina was saved in pdbqt format and were visualized in Discovery studio. In Discovery studio the molecular interactions in the form of hydrogen bonds between protein and ligands were characterized. The PDB ID used for the docking studies is 2GHU. Ligand-enzyme interaction for compound 16 is shown in Figure 4. Docking studies of compound 16 have shown a number of pi-pi T shaped

interactions with docking score -7.9. The phenyl ring present in the compound was interacting with various amino acid residues via pi-pi stacking and pi-alkyl interaction.

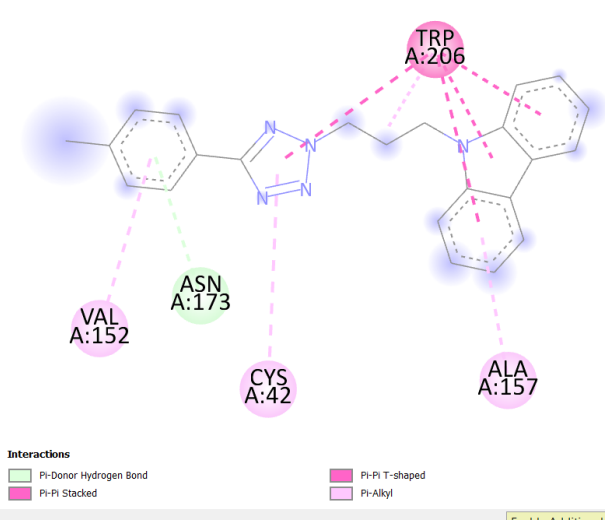


Figure 4: Protein Ligand interactions of compound 16

Docking studies of compound 22 have shown two strong hydrogen bonding interactions with docking score -8.8. The H-bond can be seen between nitrogen of compound 22 and amino acid residue Cys 42. Such hydrogen bonding interactions serves as 'anchor' guiding the 3D orientation of the ligand in the active site. The phenyl ring present in the compound was interacting with various amino acid residues via pi-pi stacking, pi-alkyl and amide-pi interaction. The protein ligand interactions of compound 22 is shown in figure 5.

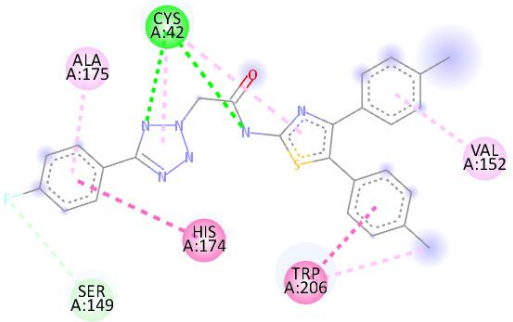


Figure 5: Protein-Ligand interactions of compound 22

WORK TO BE CARRIED OUT

8. Biological evaluation

These following evaluations will be done to check the potency of synthesized compounds against malaria

- **Kinetic affinity determination of ligands with GAP50 using surface plasmon resonance (SPR)**

  Surface plasmon resonance (SPR) is a technique that can be utilized to study diverse types of biophysical interactions in order to determine both steady-state and kinetic binding affinities between relevant binding partners.
- **Measurement of Oxidant level using CM-H2DCFDA dye**

  CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA, useful as an indicator for reactive oxygen species (ROS) in cells. This indicator exhibits much better retention in live cells than H_2DCFDA. CM-H_2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus facilitating long-term studies.
- **Antiplasmodial activity and cytotoxicity determination**

  Screening of piperazinylquinoline-aryltetrazoles against chloroquine resistant *Pf*INDO and chloroquine sensitive *Pf*3D7 using SYBR green I lysis method. SYBR Green I is a cyanine dye routinely used in the biological screening as a nucleic acid stain for the staining of double stranded DNA (most sensitive) and RNA. This dye is mostly used for the in-vitro antimalarial screening of novel heterocyclic compounds which are supposed be act as antimalarial agents. The precedence of SYBR Green I dye is that host cells (i.e. human erythrocytes) are devoid of DNA and parasites infected RBC's are clearly differentiated when observed under fluorescent plate reader. When SYBR Green I dye intercalates with the DNA of parasite cells it forms DNA-dye-complex which absorbs blue light at λmax of 497 nm and emits green light at λmax of 520 nm.
- **Ring-stage survival of ART-resistant strain in presence of compounds**

In order to elucidate the activity of the proposed compounds against ART-resistant strain (*Pf*Cam3.1^{R539T}), ring-stage survival assay will be performed using chloroquine-resistant strain (*Pf*INDO) as control.

- **Evaluation of ROS production in presence of compounds**

  Increase in ROS levels inside the cell is detrimental to the cellular organelles and inhibit the essential enzymes for maintaining various cellular process non-specifically.

- **In Vitro Stage-Specific Inhibition Assay**

  To determine the potential of the test compound on different stages of life cycle of malaria parasite i.e. ring (R), trophozoite (T) and schizont (S) stages, it was treated with sorbitol synchronized P. falciparum (INDO) cultures of individual stages i.e. R, T and S. Giemsa stain and SYBR Green I assay were used for the determination stage specificity potential of the proposed compounds.

- **Cytotoxicity Assay**

  Cytotoxicity will be assessed using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) assay.

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