

## TO DESIGN STRUCTURALLY NOVEL COMPOUNDS TARGETING FALCIPAIN AS ANTI MALARIA DRUG DELIVERY SYSTEM

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### ABSTRACT

The current research work investigates the design, synthesis and biological evaluation of *Plasmodium falciparum* proteases. This is achieved by examining the effect of inhibitor design, especially warheads, on percentage inhibition (IC<sub>50</sub> values) and generation of structure activity relationships (SAR) between cysteine protease falcipain-2 (Series I to IV), and serine protease ClpP (caseinolytic proteases) inhibitors (Series V and VI). Falcipain-2 protease has been a subject of intense research over the past two decades. Inhibition of *Plasmodium falcipain-2* proteases is a strategy to develop novel drugs against malaria. As per the pharmacophoric requirements of falcipain-2

inhibitors, three different non-peptidic small molecule series namely, 2-(4-(substituted benzoyl)-1,4-diazepan-1-yl)-N-phenylacetamide derivatives (Series I), 1-(4-(substituted)piperazin-1-yl)-2-(phenylamino) ethanone derivatives (Series II), and 2-(4-(substituted benzoyl)piperazin-1-yl)-N-phenylacetamide derivatives (Series III), were designed using ligand-based approach. The 'Lipinski's Rule of Five' was adopted while designing the molecules to attain better pharmacokinetic profile. These carboxamides were synthesized from the starting material, aniline (Series I and III), or piperazine (Series II), in a sequence of reactions, respectively.

**KEYWORDS:** *Plasmodium falciparum* proteases, Malaria, Drug Design, Carboxamides, Piperazine.

### INTRODUCTION

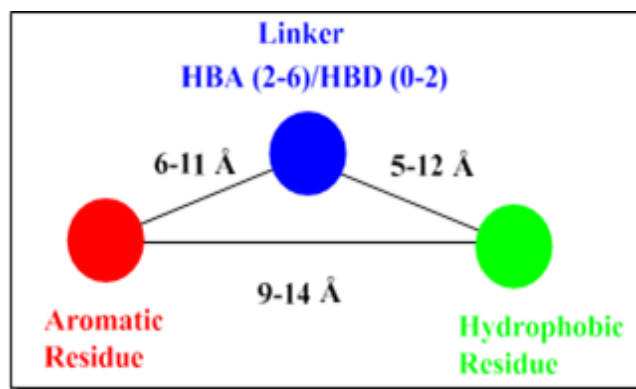
The existing armamentarium of anti-malarial drugs is not sufficient to combat malaria, primarily because of resistance developed by the parasite. The problem got further

compounded due to non-enrichment of anti-malarial drug inventory. Unfortunately, an effective anti-malarial vaccine also could not be developed due to fast and constant mutations in the parasite genome. All these factors led to an urgent need for new research avenues to develop novel and more potent anti-malarial drugs. The current research work investigates the design, synthesis and biological evaluation of *Plasmodium falciparum* proteases. This is achieved by examining the effect of inhibitor design, especially warheads, on percentage inhibition (IC<sub>50</sub> values) and generation of structure activity relationships (SAR) between cysteine protease falcipain-2 (**Series I to IV**), and serine protease ClpP (caseinolytic proteases) inhibitors (**Series V and VI**). Falcipain-2 protease has been a subject of intense research over the past two decades. Inhibition of *Plasmodium falcipain-2* proteases is a strategy to develop novel drugs against malaria. As per the pharmacophoric requirements of falcipain-2 inhibitors, three different non-peptidic small molecule series namely, 2-(4-(substituted benzoyl)-1,4-diazepan-1-yl)-*N*-phenylacetamide derivatives (**Series I**), 1-(4-(substituted)piperazin-1-yl)-2-(phenylamino)ethanone derivatives (**Series II**), and 2-(4-(substituted benzoyl)piperazin-1-yl)-*N*-phenylacetamide derivatives (**Series III**), were designed using ligand-based approach. The 'Lipinski's Rule of Five' was adopted while designing the molecules to attain better pharmacokinetic profile. These carboxamides were synthesized from the starting material, aniline (**Series I and III**), or piperazine (**Series II**), in a sequence of reactions, respectively.

This study is basically focused on To design structurally novel compounds targeting falcipain-2 (FP-2) and casienolytic proteases (ClpP) of Plasmodium falciparum.

## MATERIAL AND METHODS

Several compounds related to FP-2 inhibitors structure, as discussed in the literature review (Section 2), were initially studied to determine the essential pharmacophoric features. Based on the core features, a new pharmacophore model was built (Figure 15). Using this pharmacophore model, novel FP-2 inhibitors (Series I-III) were designed, which may be potent and plausibly with minimum side-effects. In order to attain the better pharmacokinetic profile, molecules were designed according to the 'Lipinski's Rule of Five (1997).



**Figure 1: Proposed pharmacophore model (Green Color: Represents hydrophobic residue; an aromatic group, Red Color: Represents an aromatic residue (monocyclic/bicyclic), Blue Color: Represents the numbers of hydrogen bond donor and acceptor atoms).**

The novel pharmacophore consists of key elements:

- An aromatic residue (monocyclic/bicyclic), which is attached to the hydrophobic moiety; an aromatic residue through a hydrogen bond donor and acceptor atom(s) as linker
- The hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) atom(s) are present as either in heterocyclic/alicyclic, or open chain form
- The distance between the aromatic residue and hydrophobic group was ranged from 9 to 14 Å
- The distances between the centroid of the aromatic residue to linker (HBA/HBD) and centroid of hydrophobic group to linker (HBA/HBD) were ranged from 6 to 11 Å and 5 to 12 Å, respectively

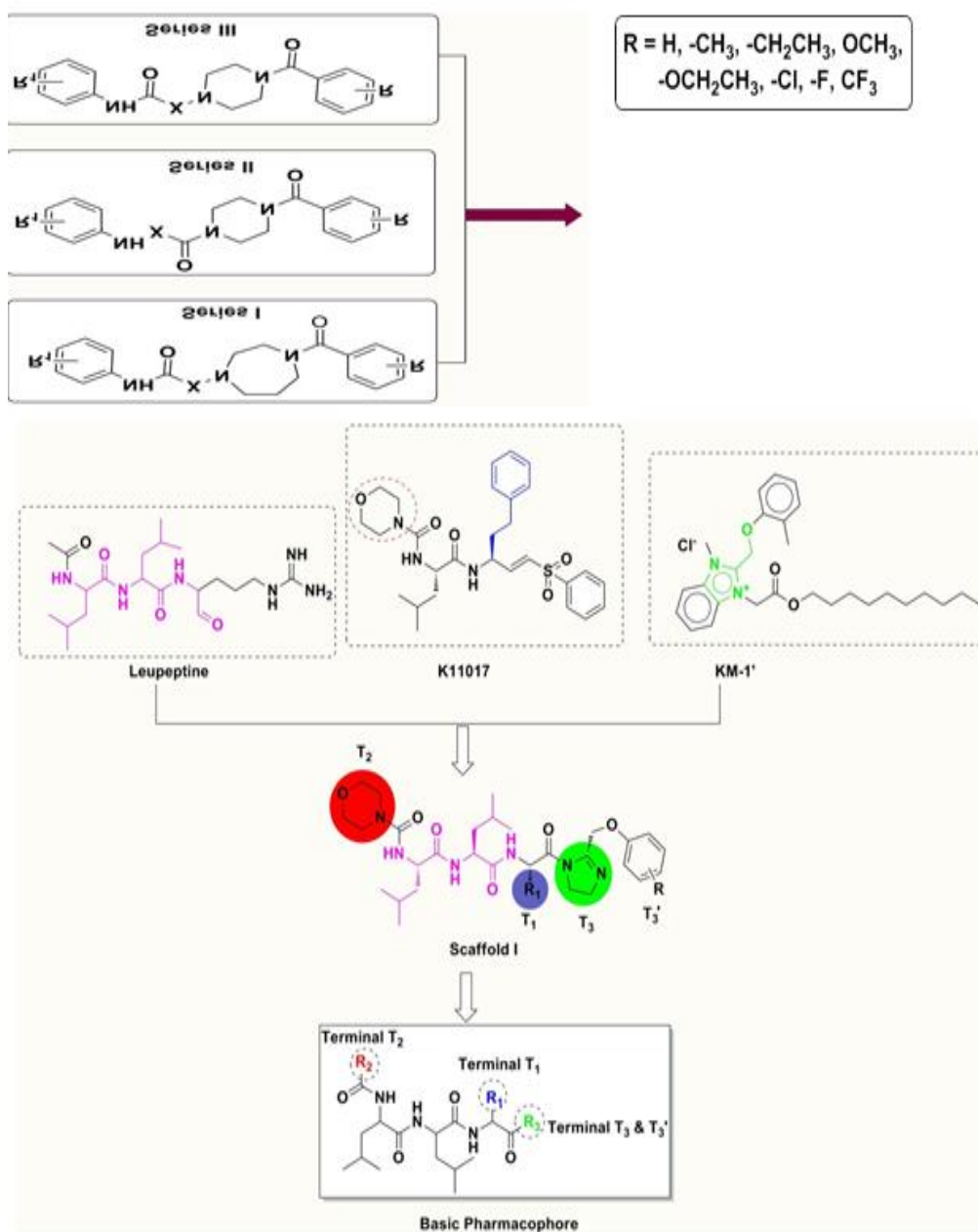
The numbers of hydrogen bond donor and acceptor atoms range from 0-2 and 2-6, respectively. Based on this pharmacophore model, New Chemical Entities (NCE's) were designed for their falcipain-2 inhibitory activity.

A two-step process was used to screen *in silico* small molecule libraries, against the active site of FP-2 employing UNITY suite of software running on maestro version 9.4 installed in a machine on intel xenon W 3565 processor and analogues and structured with the help of chemsketch

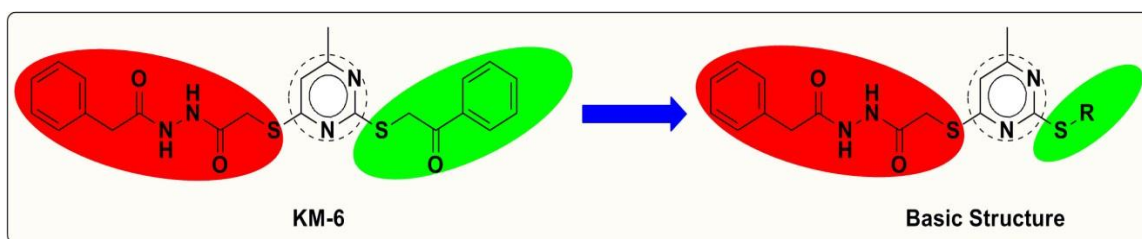
- Hits were selected containing complimentary features to the enzyme active site.
- The hits were docked into the binding pocket as discussed in Section 5

**Series I to III:** Non-peptidic small molecule inhibitors for falcipain-2 enzyme: **Series I:** 2-(4-(Substituted benzoyl)-1,4-diazepan-1-yl)-*N*-phenylacetamide derivatives **Series II:** 1-(4-(Substituted)piperazin-1-yl)-2-(phenylamino)ethanone derivatives **Series III:** 2-(4-(Substituted benzoyl)piperazin-1-yl)-*N*-phenylacetamide derivatives

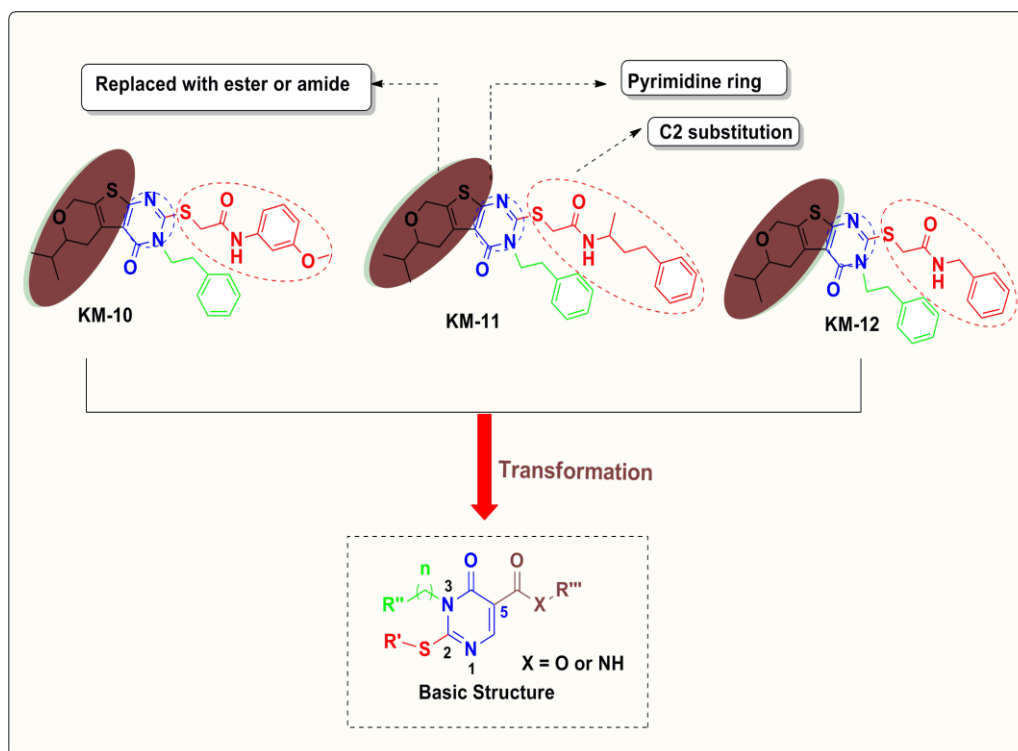
Series	X	R <sub>1</sub>	Compounds
I	CH <sub>2</sub>	H	RM-1 to 20
II	CH <sub>2</sub>	H	RMS-1 to 20
III	CH <sub>2</sub>	H	RMT-1 to 20



**Figure 2:** Representative basic structure of designed compound (Scaffold I) with structure variations (T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>3'</sub>).



**Figure 3: Basic structure of designed compound.**



**Figure 4: Rational design for synthesis of Series VI compounds.**

### Docking studies

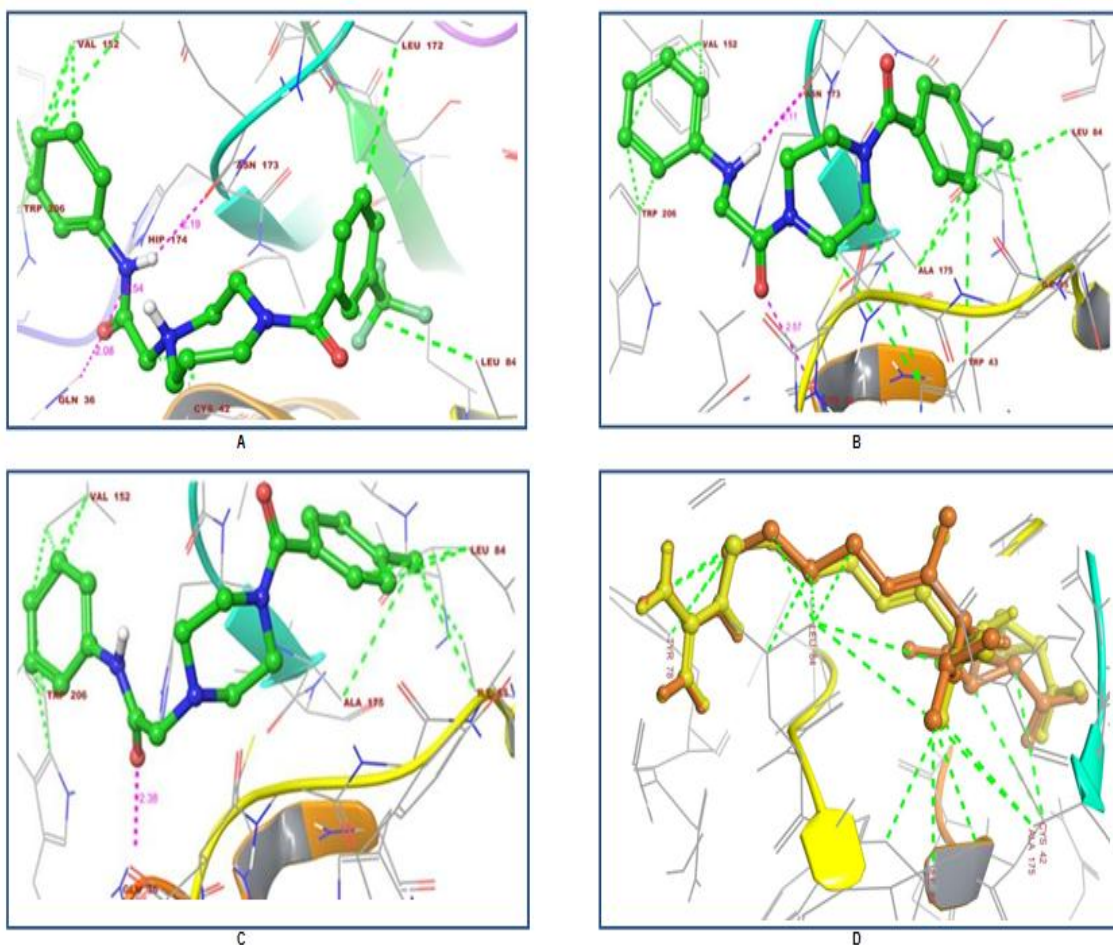
To understand the structural basis for the activities of the inhibitors and to support the *in vitro* activity results, the binding models of the top active analogs as shown by **Figure 5 (A-C)**, was studied with falcipain-2 enzyme using Glide 5.9 (Schrodinger, LLC, New York, NY, 2013) running on maestro version 9.4 installed in a machine on Intel Xenon W 3565 processor and Cent OS Linux Enterprise version 6.3 as the operating system (Friesner et al., 2004; Halgren et al., 2004).

The crystal structure of falcipain-2 (PDB entry 3BPF) from *P. falciparum* was retrieved from the Protein Database Bank with a resolution of 2.9 Å. The downloaded FP-2 protein carries four chains named A, B, C, and D; complexed with epoxysuccinate E64 (Wang et al., 2000). The catalytic triad of Cys 42, Asn 173 and Hip 174 is located in the cleft between the two

structurally distinct domains (Wang et al., 2014). Protein preparation module of Schrodinger suite was used for protein preparation. Proteins were pre-processed separately by deleting the substrate co-factor as well as the observed water molecules were removed from the coordinate set, followed by optimization of hydrogen bonds. Charge and protonation state was assigned and energy was minimized with Root Mean Square Deviation (RMSD) value of 0.3 Å using Optimized Potentials for Liquid Simulations-2005 (OPLS-2005) force field (Jorgensen et al., 1996). Potential of non-polar parts of receptors was softened by scaling van der Waals radii of ligand atoms by 1.00 Å to generate the grid. Analogs and standard drug E-64 structures were drawn using ChemSketch and converted to 3D structure with the help of 3D optimization tool. LigPrep module was used to optimize the geometry of the drawn ligands. The prepared ligands were docked with proteins using extra precision mode (XP). The best docked pose obtained from Glide was analyzed.

Validation of docking protocol was done to govern the reproducibility and reliability of the docking parameters used for the study. In the present study, validation of the docking was done by removing co-crystallized ligand E-64 from the active site and subjecting it again to dock into the binding pocket in the conformation found in the crystal structure (**Figure 5 D**). Furthermore, a set of studies were performed to compute the RMSD value between the pose of co-crystallized inhibitor present in the enzyme and its best ranked pose, in the protein. As a result, the RMSD value calculated for co-crystallized ligand, E-64 was 1.68 Å against the target enzyme. The best scoring pose of the reference drug inside the FP-2 showed that interactions, are in resemblance with the reported X-ray pose interactions. These results suggested that, our docking procedure and software protocol could be relied on to predict the experimental binding mode of the designed analogs.





**Figure 5 (A-C):** Docking pose of compounds RM-7 (*N*-Phenyl-2-(4-(3-(trifluoromethyl) benzoyl)-1,4-diazepan-1-yl) acetamide), RMS-8 (1-(4-(4-Methylbenzoyl)piperazin-1-yl)-2-(phenylamino)ethanone), and RMT-2 (2-(4-(4-methylbenzoyl)piperazin-1-yl)-*N*-phenylacetamide), respectively docked to chain A of falcipain-2 protein (3BPF.pdb). The pink dashed line represents the possible hydrogen bond and green dashed line represents the possible hydrophobic interaction, (D): Redocked mode of E-64 (yellow) superimposed with the co-crystallized ligand (magenta).

## 2. RESULTS AND DISCUSSION

### Pharmacophore and chemistry

#### Series I-III

Existing falcipain-2 inhibitors (Desai et al., 2004; Li et al., 2009; Desai et al., 2006), which hold moderate to potent activities (A-H) as represented in **Figure 21**, were used as a template for designing a pharmacophore model for falcipain-2 inhibitors.

The most common features present in the aforementioned falcipain-2 inhibitors are, an

aromatic residue (monocyclic/bicyclic), which is attached to the hydrophobic moiety; an aromatic residue through a hydrogen bond donor and acceptor atom(s) as linker.

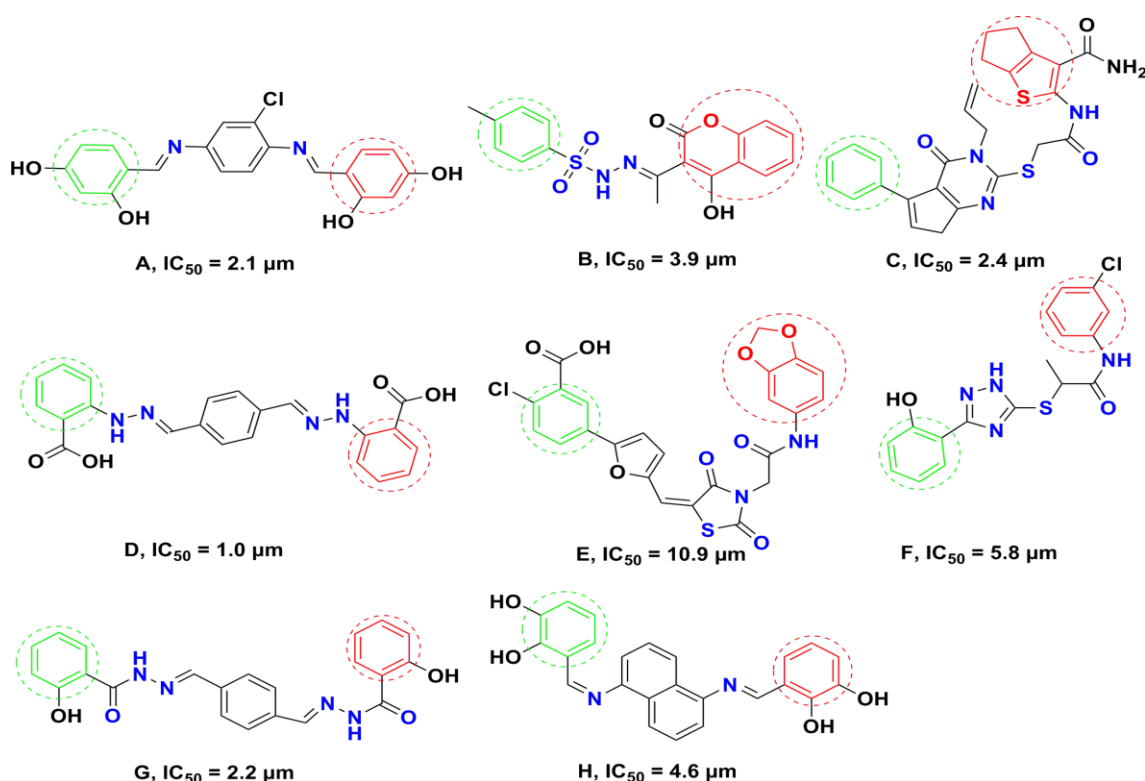
The distance between the aromatic residue to a hydrophobic group, centroid of the aromatic residue to linker (HBA/HBD) and centroid of hydrophobic group to linker (HBA/HBD) were ranged from 9 to 14 Å, 6 to 11 Å and 5 to 12 Å, respectively. The hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) atom(s) are present as either in heterocyclic/alicyclic, or open chain form. The numbers of hydrogen bond donor and acceptor atoms range from 0-2 and 2-6, respectively. The reported molecules are basic in nature due to 2° or 3° amino moiety.

By considering these characteristic features as pharmacophore for falcipain-2 inhibitors, a novel pharmacophore model was built (**Figure 15**). Based on this model, three series (**RM-1 to 20**, **RMS-1 to 20**, **RMT-1 to 20**) of compounds were designed and the corresponding basic structure for each series is represented in synthetic **schemes 1 to 3**, respectively (Experimental Section 4).

The least energy conformation (three minimum energy conformations for each compound) for each designed compound was generated using ACDLABS-12.0 product version 12.01/3D viewer (CHARMM parameterizations), and the pharmacophoric distances were measured from the centroid of an aromatic residue to a hydrophobic residue. The observed distances between the pharmacophoric elements of all the designed compounds are in agreement with our proposed pharmacophore model, and results are summarized in **Tables 7-9**.

To achieve better pharmacokinetic profile, the Lipinski's Rule of Five (Lipinski et al., 1997) i.e., hydrogen bond donor atoms not more than 5, hydrogen bond acceptor atoms not more than 10, molecular weight less than 500, and log P value less than 5, was adopted for the designed molecules. Lipophilicity is an important parameter to be considered while designing ligand to manifest drug-like behavior.





**Figure 6:** Chemical structures of some existing falcipain-2 Inhibitors. (Green Color: represents hydrophobic moiety; an aromatic group, Red Color: represents an aromatic residue (monocyclic/bicyclic), Blue Color: represents the numbers of hydrogen bond donor and acceptor atoms).

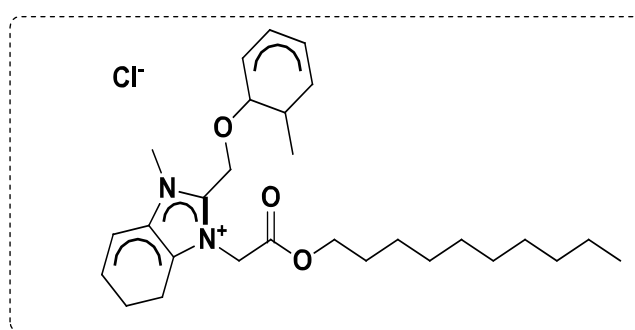
#### Series IV

Structure based drug design is a powerful tool for identification of chemically-diverse set of compounds as novel lead molecules in drug discovery programme. Over the years, many research groups have investigated falcipain-2 inhibitors using *in silico* screening of chemical libraries. Recently, vinyl esters were designed using molecular modeling and their binding to falcipain-2 was evaluated. These inhibitors exhibited antiplasmodial activities (IC<sub>50</sub>) in the low micromolar range, carry a vinyl ester core capable of trapping the active site “Cys” through a covalent bond and function as a Michael acceptor. The poor selectivity for parasitic cysteine proteases over the human cysteine proteases remains a noteworthy concern. Rizzi et. al., 2011, designed peptidomimetics, based on the interactions of cystatin (family of cysteine protease inhibitors) with the active site of falcipain-2. These compounds showed specific inhibition of falcipain-2 activity as well as inhibition of parasite growth at low micromolar range with reduced toxicity and higher selectivity. Structure based drug design for series IV was carried out in collaboration with Dr. Lakshmi Kotra (University Health Network, Toronto-Canada).

### Identification of initial hit

Three-dimensional structures of falcipain-2 (RCSB codes: 1YVB, 3BPF) were investigated to understand the similarities and differences between these three proteases. The catalytic site of this class of cysteine proteases is composed of hydrophobic pockets in close proximity to the catalytic residues (Cys42 and Hip174). Then, using the three-dimensional structure of falcipain-2 from *P. falciparum* (RCSB code: 1YVB) as a template, pharmacophore features for *in silico* screening of commercial chemical compounds libraries were selected. A library of more than 250,000 commercially available compounds, were screened against the active site of falcipain-2 employing UNITY module. A set of 2084 initial hits were obtained following from this screening, which were then subjected to Surflex-Dock™ based docking in the active site of falcipain-2 to generate docked poses. These poses were scored using the empirical scoring function in Surflex-Dock, and the top 200 hit molecules with the corresponding pose based on the best-fit score were selected. The molecules were then inspected individually within the binding pocket of falcipain-2 for their chemical nature, reactive structural elements, and synthetic feasibility.

Overall, one compound (**KM-1'**) was identified and acquired from commercial vendors. This compound was evaluated for their efficacy to inhibit *in vitro* activity of falcipain-2. **KM-1'** (**Figure 7**) exhibited moderate inhibition of falcipain-2 with a  $IC_{50}$  value of  $36.06 \pm 0.61 \mu M$ . Furthermore, this compound inhibited *P. falciparum* growth *in vitro* with an  $IC_{50}$  of  $2.5 \pm 0.2 \mu M$ .



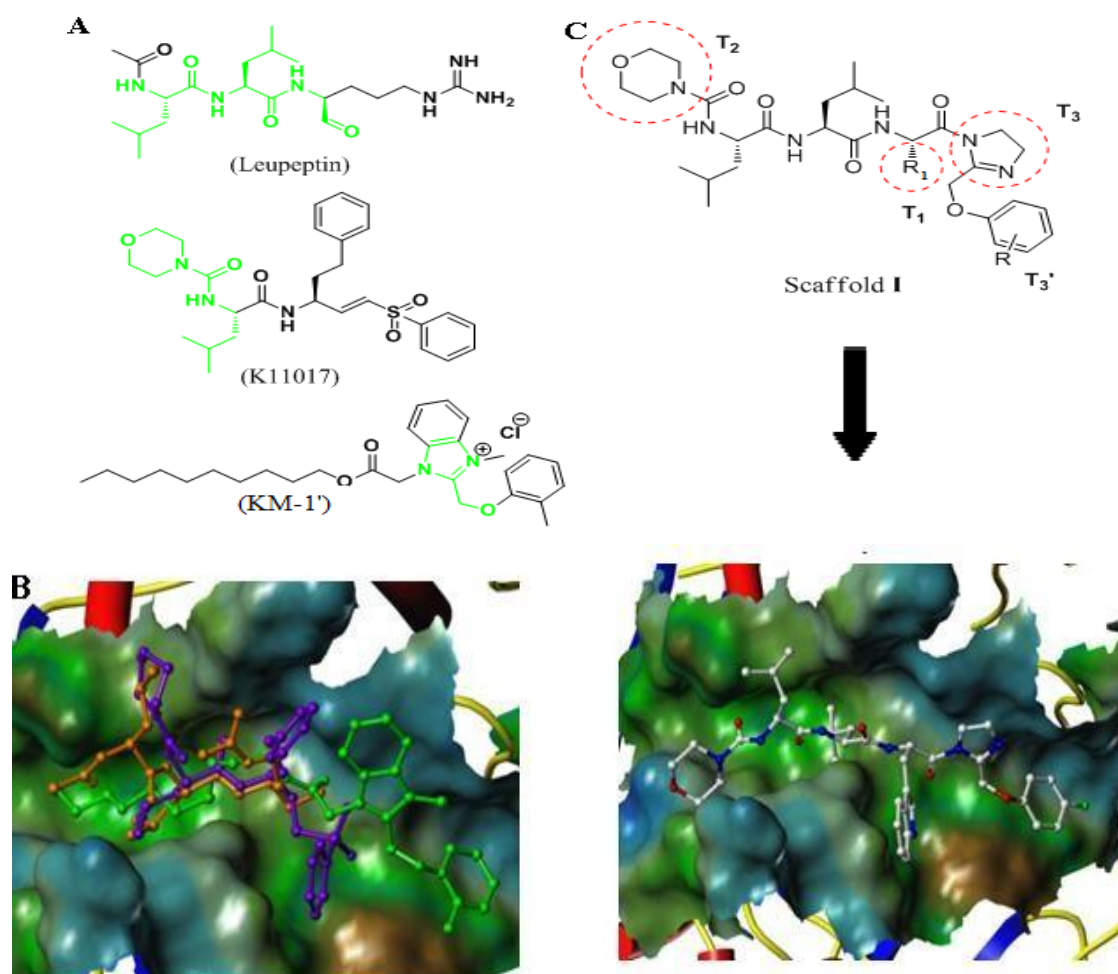
**Figure 7:** Structure of hit compound (**KM-1'**).

Although **KM-1'** exhibited reasonable inhibition of falcipain-2 activity, overall chemical structure required additional modifications. Thus, this hit molecule (**KM-1'**), along with two known falcipain-2 inhibitors, leupeptin and **K11017** (compound **5**; discussed in literature review Section 2) were docked into the binding pocket of falcipain-2 (**Figure 23B**). Then

complementary groups from the three molecules (green portions in **Figure 23A**), and their corresponding interactions with the binding site were carefully considered to finally derive the library of molecules represented in **Figure 23C**, with a new structural element, pharmacophore/scaffold.

This scaffold has four different variable groups  $T_1$ ,  $T_2$ ,  $T_3$  and  $T_3'$  correspond to  $R_1$ ,  $R_2$  and  $R_3$  ( $T_3$  and  $T_3'$ ), substituents as mentioned in the basic structure of **Series IV (Section 3)**. Leu-Leu moiety was retained from the leupeptin structure, as this group provided appropriate binding interactions. One of these Leu moieties is also a substructure in **K11017**.

Other portions of the scaffold included the imidazolyl moiety from **KM-1'**, and various heterocycles at  $T_2$  position, essentially occupying S3 pocket of the target. Benzyl substitutions on the imidazole are designed to occupy S1' subsite (hydrophobic in nature) and  $T_1$  position, occupying S2 pocket of the target. Based on these substitution patterns, synthetic strategies were designed, and the target compounds are featuring scaffold I, were synthesized.



**Figure 8 (A):** Structures of the compounds, leupeptin, K11017 and the active hit (KM-

1') from *in silico* screening, (B): Binding site of falcipain-2 with docked molecules Leupeptin (orange), K11017 (purple) and KM-1' (green), (C): Representative structure of designed compound (scaffold I) with structure variations (T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>3</sub>').

### Identification of initial hits

The two compounds **KM-6** and **KM-11** with IC<sub>50</sub> values  $26.0 \pm 1.6 \mu\text{M}$  and  $23.0 \pm 1.8 \mu\text{M}$ , respectively were identified as moderate inhibitors that served as a solid starting point for the future drug discovery programme. Hence, initially various derivatives (**RMP 1 to 15**) of potential hit **KM-6**, were synthesized to generate an excellent SAR. However, screening results of these compounds were almost similar to the hit compound. Hence, chemical structure required additional modifications to improve the inhibition value.

Compound **KM-11**, which was another hit compound, possessed  $\sim 23 \mu\text{M}$  *in vitro* inhibition value, it was therefore planned to synthesize **KM-11**, and its derivatives, having substitution at C2 position of the pyrimidine ring. However, complex synthetic route for the synthesis of **KM-11**, prompted us to modify our strategy from straight forward synthesis of hit compound towards designing of a new pharmacophore model.

Interestingly, **KM-10** and **KM-12**, were least potent ( $> 100 \mu\text{M}$ ) against *PfClpP* protease which differed from **KM-11** with respect to one pharmacophoric feature, i.e., chain length of substituents present at C2 position (**Figure 9**). Prompted from this fact, these compounds were selected to develop a scaffold that allows for expedient chemical manipulation and subsequent, detailed, SAR studies. Through these modifications, a hybrid pharmacophore was generated (**Figure 9A**). The novel pharmacophore consists of key features:

a) aromatic ring (pyrimidine nucleus), b) substitution at N<sup>3</sup> position with variation in chain length c) variation in substituent's present at C2 position and d) fused ring (**5-isopropyl-5,7-dihydro-4H-thieno[2,3-c]pyran**) which was replaced deliberately with an ester/amide linkage at 5<sup>th</sup> or 6<sup>th</sup> position of the pyrimidine ring, to afford synthetic feasibility and to modulate polarity and bioavailability (Meanwell, 2011).

Based on this pharmacophore, a series of compounds (**RMH 1 to 14**) were designed and synthesized. **Figure 9B** represents the formation of basic compound **RMH-1** from the designed pharmacophore.

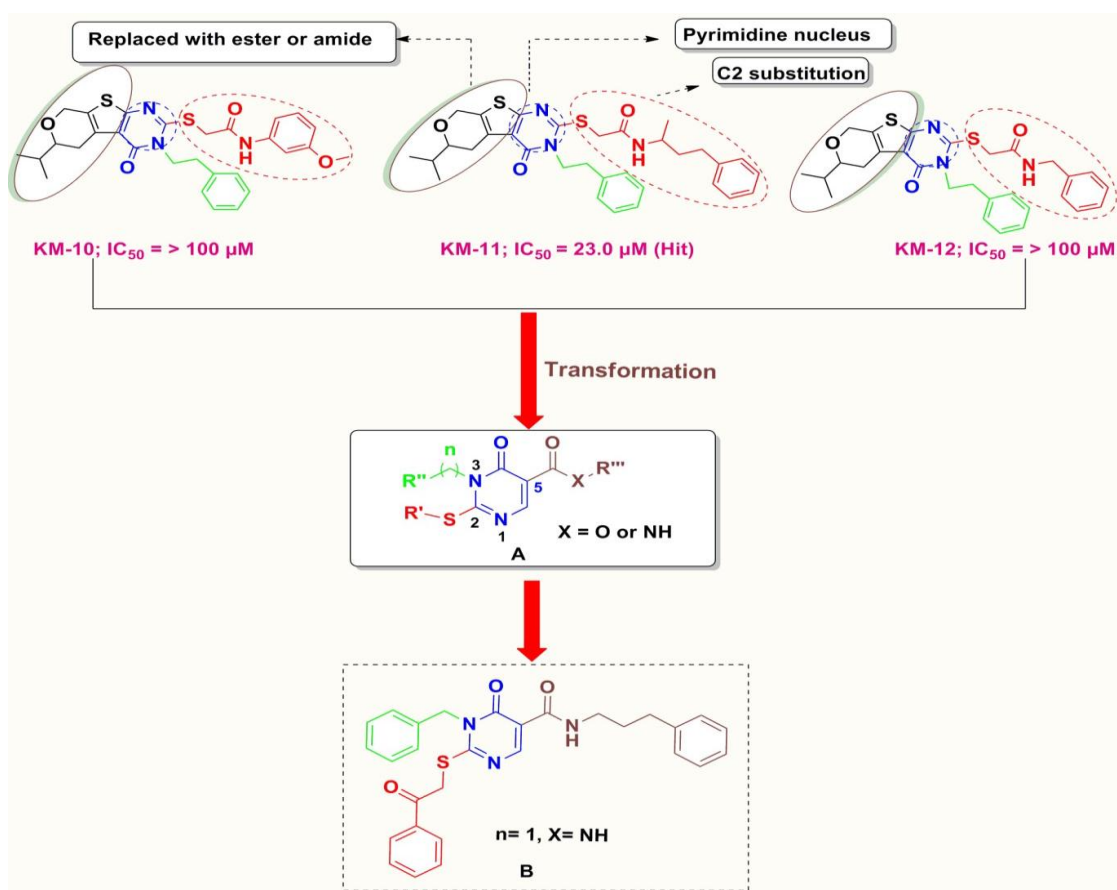


Figure 9 (A): Optimized pharmacophoric model (B): Structure of basic compound.

## 6. SUMMARY AND CONCLUSIONS

In summation, the following conclusions were drawn:

- ❖ Among the three series (**I**, **II** & **III**) of derivatives, homo-piperazine core nucleus with electron withdrawing substituents present on the phenyl ring (**Series I**, **Basic Structure I**), showed potent inhibition indicating that linker played a major role in the designed pharmacophore for the difference in the activity.
- ❖ Molecules of series **IV**, derived from the novel pharmacophore, displayed potent inhibition of FP-2 protease. The substitution at three different positions ( $R_1$ ,  $R_2$  &  $R_3$ ) played a key role for difference in the activity among the series of compounds.
- ❖ Among the peptidomimetics, (**Series IV**), were the most potent compounds from the series against FP-2 enzyme. These compounds could be considered as leads, and an appropriate modification may possibly enhance inhibitory activity.
- ❖ Compound may become the most effective inhibitor of parasite growth, which correlated well with the inhibition of falcipain-2.
- ❖ Overall, among the synthesized moieties against falcipain-2 enzyme (**Series I-IV**), peptidomimetics (**Series IV**) showed better affinity and potent inhibitory activity as

compared to non-peptidic molecules **Series I-III**.

- ❖ In **Series V**, compounds were almost equipotent to the hit compound (**KM-6**) for ClpP inhibition.
- ❖ Molecules of **Series VI**, generated from the novel pharmacophore, displayed prominent inhibition of ClpP protease as compared to the derivatives generated from hit compound **KM-6**. The substitution at N<sup>3</sup> position played a major role for the difference in the activity among the **Series V** and **VI**.
- ❖ Among the **Series VI** was the most potent compound, it can also be utilized as a potential lead compound in the designing of new candidates to optimize the inhibitory potencies of this class of compounds, with potent anti-malarial activity.

Among all series of compounds that were designed (6), the following lead were found to be most promising which could be used for further investigations.

Sl No	Code (Series)	Significant FP-2 inhibition	Significant ClpP inhibition	Significant antiplasmodial activities
1	(Series I)	√		--
2	(Series IV)	√		√
9	(Series VI)		√	√

-- Indicates no significant antiplasmodial activity

√- Indicates significant enzymes (FP-2 or ClpP) and antiplasmodial activity

Future scope

- Based on the affirmative results of the work, it would be fruitful to investigate the effect of selected falcipain-2 and ClpP inhibitors on the morphology and development of *P. falciparum* parasite at asexual stage.
- Drug combination studies using lead ClpP and falcipain-2 inhibitors, along with known anti-malarials could be carried out to develop a path for single-dose combination therapy for malaria.

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