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Mini-review

New paradigm of an old target: An update on structural biology and current progress in drug design towards plasmepsin II

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ABSTRACT

Malaria is one of the major parasitic disease whose rapid spreading and mortality rate affects all parts of the world especially several parts of Asia as well as Africa. The emergence of multi-drug resistant strains hamper the progress of current antimalarial therapy and displayed an urgent need for new antimalarials by targeting novel drug targets. Until now, several promising targets were explored in order to develop a promising Achilles hill to counter malaria. Plasmepsin, an aspartic protease, which is involved in the hemoglobin breakdown into smaller peptides emerged as a crucial target to develop new chemical entities to counter malaria. Due to early crystallographic evidence, plasmepsin II (Plm II) emerged as well explored target to develop novel antimalarials as well as a starting point to develop inhibitors targeting some other subtypes of plasmepsins i.e. Plm I, II, IV and V. With the advancements in drug discovery, several computational and synthetic approaches were employed in order to develop novel inhibitors targeting Plm II. Strategies such as fragment based drug design, molecular dynamics simulation, *double drug* approach etc. were employed in order to develop new chemical entities targeting Plm II. But majority of Plm II inhibitors suffered from poor selectivity over cathepsin D as well as other subtypes of plasmepsins. This review highlights an updated account of drug discovery efforts targeting plasmepsin II from a medicinal chemistry perspective.

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1. Introduction

Malaria is one of the oldest parasitic disease, which is still going strong and emerged as a major health risk in several parts of World including South America, Africa, tropical regions of Asia as well documented outbreaks in North America and several regions of Europe. At an average, each year half a billion cases of malaria were documented with a majority of people belong from tropical areas of Asia and several parts of Africa [1,2]. This mosquito borne diseases caused by different species of *Plasmodium* mainly *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium*

ovale. Among all these species of *P. falciparum* is the most common causative agent although *P. vivax* also causes life-threatening symptoms and one of the most prevalent species of Latin America. *P. falciparum* invades erythrocytes and consumes host's hemoglobin as a source of nutrition [3–5]. Till date, several natural products and small molecules inhibitors were identified as a drug candidate which showed significant activity as promising antimalarial candidate [6,7]. Despite the success of artemisinin based antimalarial therapy as well as current FDA approved antimalarial regime (Fig. 1), emergence of drug resistant parasites emerged as a new challenge in developing novel drug candidates [8]. Besides this emergence of different multi-drug resistant strains especially artemisinin resistant strains possessed a great challenge in front of medicinal chemists to develop broad spectrum clinical candidate targeting resistant strains [9,10]. Despite its huge global risk, the neglected status of malaria led to decreased focus from pharmaceutical industry because of its dominancy in third world countries

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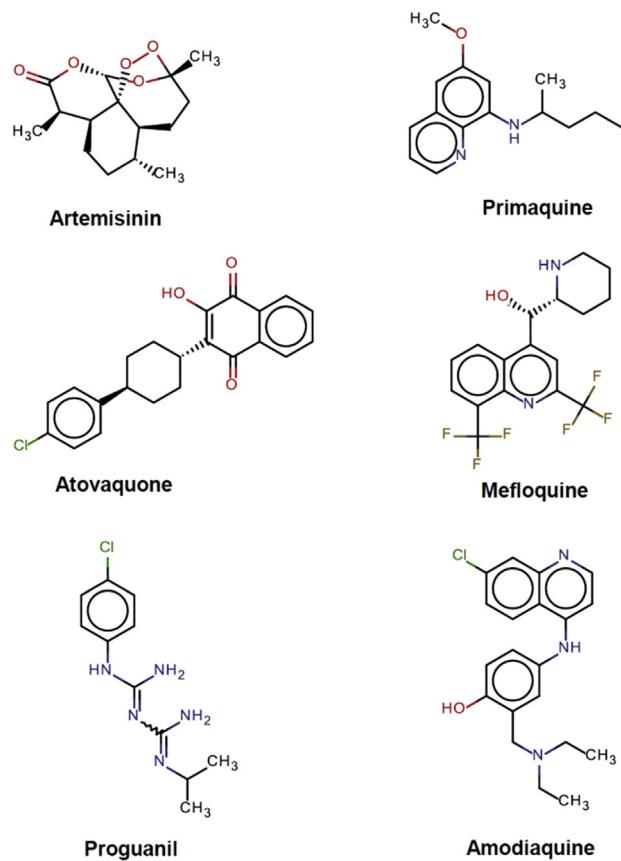


Fig. 1. Chemical representation of currently approved drug candidates to treat malaria.

of Asia and Africa. In order to foster innovation in antimalarial drug discovery, academic research as well as collaborative drug discovery approaches gained momentum in recent years with the discovery of several novel scaffolds as promising antimalarial agents

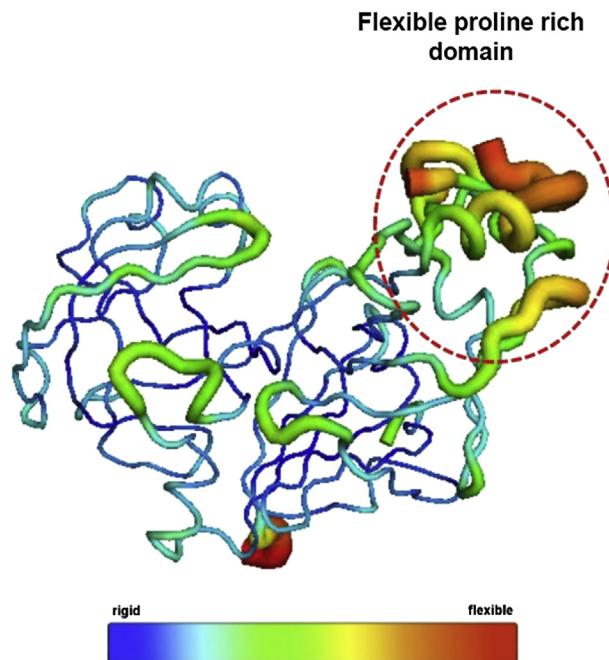


Fig. 3. B-factor representation of apo conformation of Plm II. The highly flexible proline rich domain is highlighted in red (PDB: 1LF4) [24]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[11,12].

Identifying novel drug targets also emerged as an important strategy to develop potent antimalarials with a unique mode of action [8,13]. The role of *P. falciparum* in the process of hemoglobin degradation involves several crucial enzymes and majority of them emerged as a potential drug target because of their crucial role in proteolysis of hemoglobin (Fig. 2). These enzymes include plasmepsins, the aspartic proteases from *P. falciparum*; falcipains (cysteine proteases), Dipeptidyl aminopeptidase 1 (DPAP1),

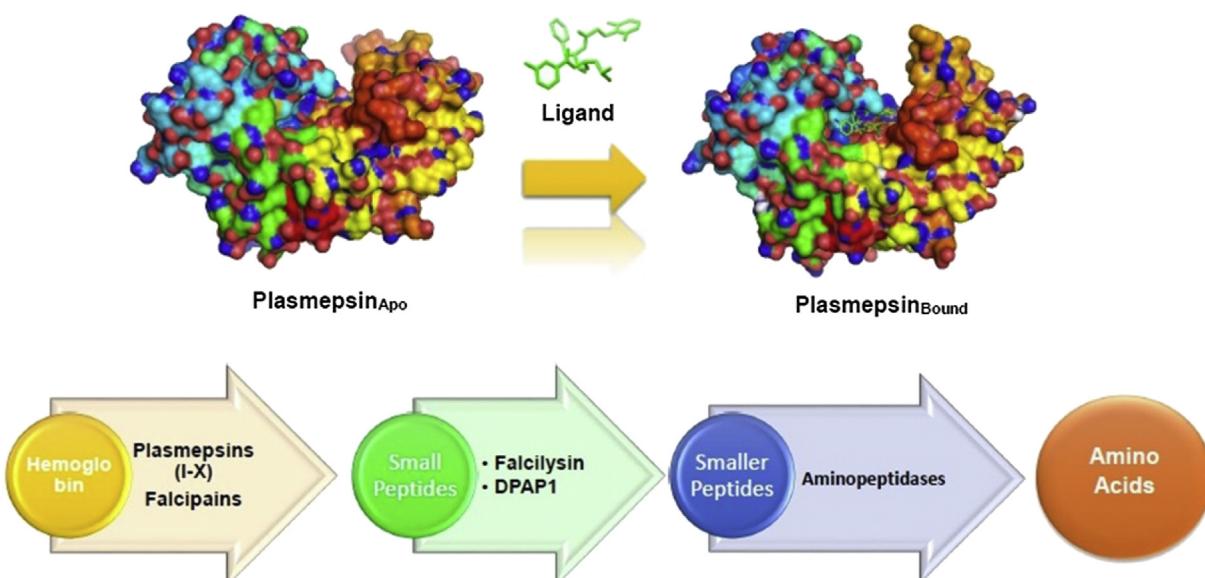


Fig. 2. The process of hemoglobin metabolism in *P. falciparum* and the structure of apo and bound conformations of plasmepsin which plays a key role in hemoglobin metabolism.

falcilysin (metalloprotease), proplasmeprin convertase (which activates proplasmeprin to plasmeprin) [14–16]. With the increasing crystallographic evidences the current effort of rational drug discovery shifted towards targeting these crucial enzymes using novel small molecule/peptide inhibitors.

Plasmeprins, the aspartic proteases of *P. falciparum* are one of the oldest target which still hold significant interest among drug discoverers to develop novel antimalarials. Plasmeprins are classified into 10 subtypes (Plm I, II, IV, V, VI, VII, VIII, IX, X and HAP) based on encoding of 10 different genes with a high inter-genomic similarity [15,16]. Till date, the exact role of several plasmeprins in hemoglobin proteolysis is not well understood. Studies suggest expression of Plm I, II, IV, V, IX, X and HAP occurs in the erythrocytic stage whereas Plm VI, VII and VIII are expressed in exo-erythrocytic stages. To date, Plm I, II, IV and HAP were extensively studied not only because of its prominent role in hemoglobin metabolism but also due to availability of crystal structures in order to develop new chemical entities from a rational viewpoint [17]. Plasmeprin II (Plm II) is one such aspartic protease which gained most attention because early identification of Plm II crystal structure as a starting point to develop novel protease inhibitors as promising antimalarial agents [18]. The sequence of Plm II also emerged as a template to develop *in-silico* models of Plm I, IV and HAP in early years as the binding site regions of Plm I, IV and HAP show 84%, 68% and 39% identity, respectively [16,19]. Similar to other aspartic proteases, the

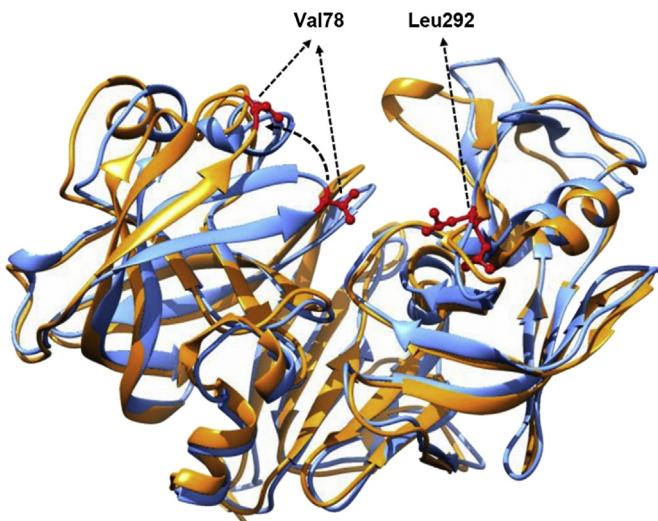


Fig. 5. The open (orange) and closed (cornflower blue) conformations of Plm II. The position of Val78 and Leu292 (red ball and stick representation) were helped in measuring the extent of flap opening and closing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

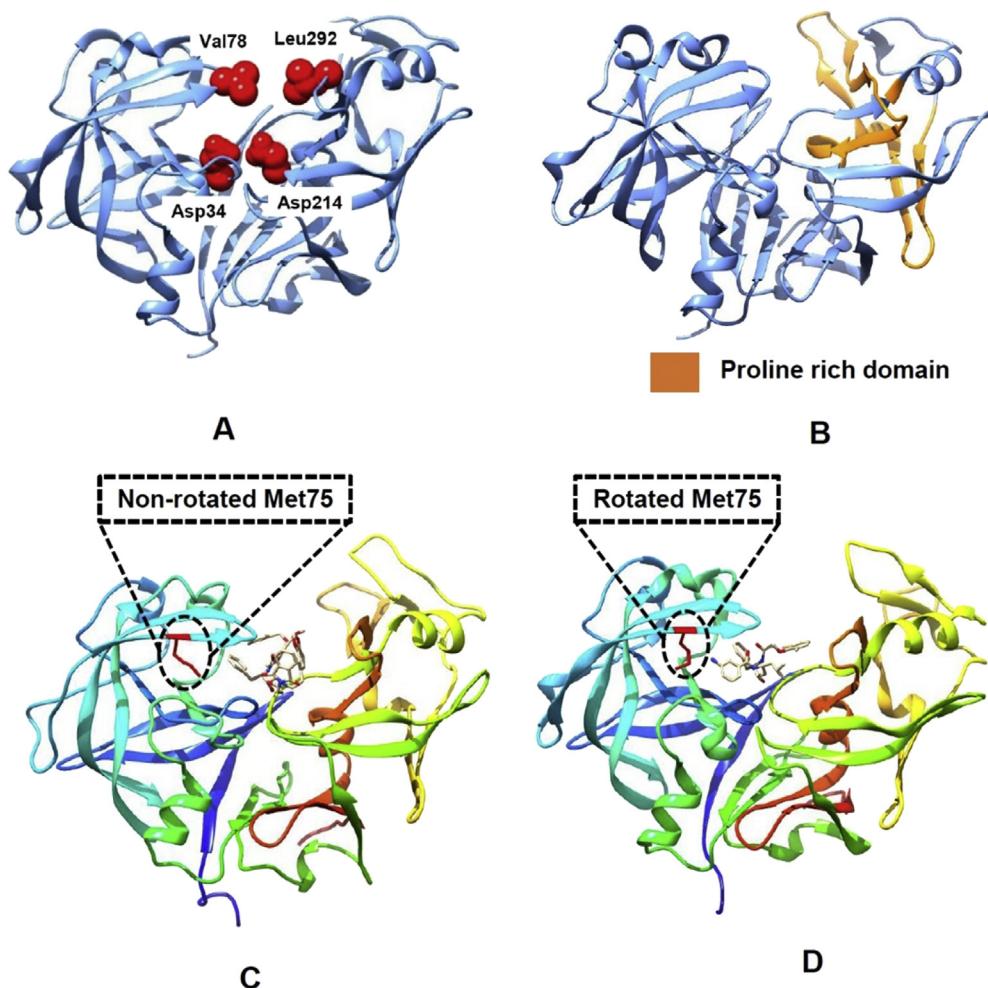


Fig. 4. Pictorial representation of Val78, Leu292, Asp34 and Asp214 (highlighted in red) in Plm II (A). The presence of proline rich domain (B), non-rotated and rotated conformations of Met75 (C) in Plm II. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

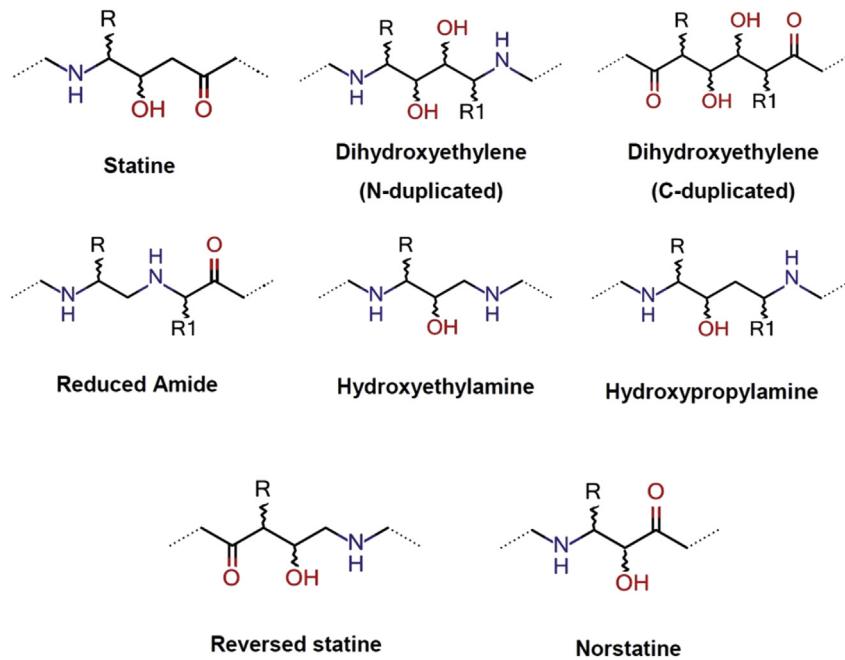
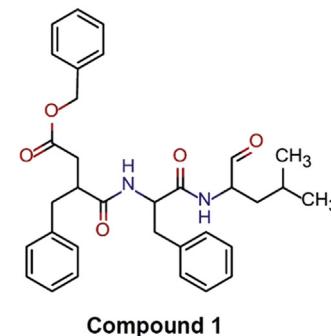


Fig. 6. The peptidomimetics and scaffolds explored in order to identify potent inhibitors targeting Plm II.

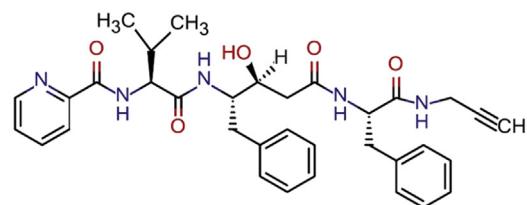
mechanism of Plm II also involves the presence of catalytic water molecule which formed two hydrogen bond interaction between the two catalytic aspartates. The water molecule activated by the aspartic acids thus makes a nucleophilic attack on the amide carbonyl substrate and generated a tetrahedral intermediate which, upon protonation of the nitrogen, collapses into the products [16].

Peptidomimetics and peptides emerged as an obvious choice of inhibitor targeting Plm II [14,16]. Because of this reason cathepsin D (Cat D), renin and HIV-1 protease inhibitors were repositioned in search to find potential lead targeting Plm II. Recent years, repositioning FDA approved HIV-1 protease inhibitors demonstrated activity against Plm II which gives a proof of concept to design peptidomimetic inhibitors targeting Plm II [20–22].

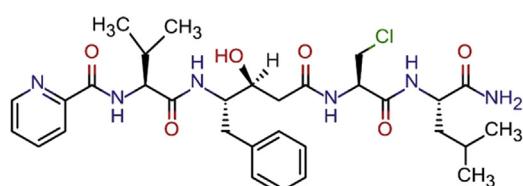
The first wave of plasmeprin inhibitors started in early twentieth century mainly focussing on development of peptidomimetics and small molecule inhibitors [16]. With advancement in synthetic medicinal chemistry as well as computer aided drug design, several peptidomimetics and small molecule inhibitors were emerged as a potential candidate to develop first line aspartic protease inhibitors as antimalarial agent. This review will not only focus on an updated account of efforts directed towards development of novel Plm II



Compound 1



Compound 2



Compound 3

Table 1

Categorisation of different inhibitors described in this review based on class of common scaffolds.

Category of inhibitors	Compound codes
Peptidomimetics	Compound 1
Reduced amides	Compound 4–5
Statines	Compound 2,3,6–19,93–96
Hydroxyethylamines	Compound 20–31,73,74
Hydroxypropylamines	Compound 32–33
Norstatines	Compound 34–36,63–64,70,75,77–78,79,81–82
1,2 dihydroxyethylenes	Compound 37–40,42–44,45,76,90
Non-peptides	Compound 46–48,49–54,58–60,65–69,71–72,80,83–87,89
Bi-functional	Compound 55–57,61–62
Methylenamino	Compound 88

Fig. 7. Structural representation of three potent peptidomimetic inhibitors targeting Plm II, Ro 40-4388 (**compound 1**), **compound 2** and **compound 3**. **Compound 1, 2** and **3** displayed a K_i of 700 nM, 0.02 μ M and 0.025 μ M respectively.

inhibitors but also a detailed report on structural features of Plm II in the context of antimarial drug discovery.

2. Structural features of Plm II

Plm II showed typical characteristics of an aspartic protease similar to that of Cat D, renin, HIV protease etc [23,24]. The mature Plm II consists of 329 amino acid residues and a single chain folded into two topologically similar C and N terminal domains. Similar to other aspartic proteases the apo conformations of Plm II contains catalytic water molecule in the active site which initiates bond cleavage by attacking the carbonyl carbon atom of catalytic aspartic dyads. The catalytic water molecule is located at 2.84 Å and 2.75 Å from the oxygen atoms of the carboxylic acids of the catalytic dyads [24].

The N-terminal domain of Plm II consists of a distinctive single β-hairpin structure, known as flap which played an important role in inhibitor uptake and inhibitor binding just like HIV protease. The opening of flap during inhibitor uptake and later forming hydrogen bond interactions with inhibitor or rigidification in order to keep the inhibitor inside the active site is the main structural property of Plm II which drives inhibitor design [23,24]. Amino acid residue, Leu292 located at the flexible proline rich loop (Ile290-Pro297) (Figs. 3, 4B) in combination with N-terminal flap adapts an open conformation in order to swallow the incoming inhibitor and subsequently leads to a closed conformation in order to hold the inhibitor in its active site [16,23,24].

The extent of flap opening is often measured in terms of measuring the distance of C-α residues of Val78 and Leu292 (Fig. 4A) [24]. To accommodate a larger incoming inhibitor the proline rich loop (Fig. 4B) in combination with flap area displaced from its original position and again adapt a closed conformation in order to keep the inhibitor in the active site. Interestingly Met75, a

residue located at the N-terminal flap region rotated in order to increase the surface area of binding site in order to fit inhibitors with bulky substitution [25]. This hypothesis was further confirmed by molecular dynamics study as well as identification of Met75 in a rotated conformation (Fig. 4D) in case inhibitor bound Plm II X-ray structures 1LEE and 1LF2 [25].

As previously mentioned the relative opening of flap and flexible loop is measured by calculating distance of C-α atoms of Val78 and Leu292 (Figs. 4A, 5). In case of apo conformation of Plm II this distance was found to be 12.6 Å [24] where depending on inhibitor this distance is varied from 9.9 Å (in complexed with pepstatin A) [26] to 12.0 Å (in complexed with small molecule inhibitor EH58) [24]. This varying distance in case of bound conformation suggests that the active site of Plm II can be flexible in order to accommodate inhibitors of different size.

The extent of flap opening, closing and semi-open configurations were monitored and widely studied in HIV-protease using different parameters (distance, dihedral angle, curling) in order to parameterize the important dynamical events during flap dynamics [27,28]. Because of the neglected status of malaria and lack of attention from developed countries these type of studies to generate parameters in order to understand the extent of flap opening and understanding flap motion is yet to be made (Fig. 5). Recently we communicated first such effort which will help assign certain parameters to best describe events such as flap opening as well as overall flap dynamics [29].

3. Chemotherapeutics against Plm II inhibitors: A medicinal chemistry perspective

Similar to other chemotherapeutic agents targeting different protease macromolecules as potential inhibitor/drug candidate, the chemotherapeutics targeting Plm II can be further divided into

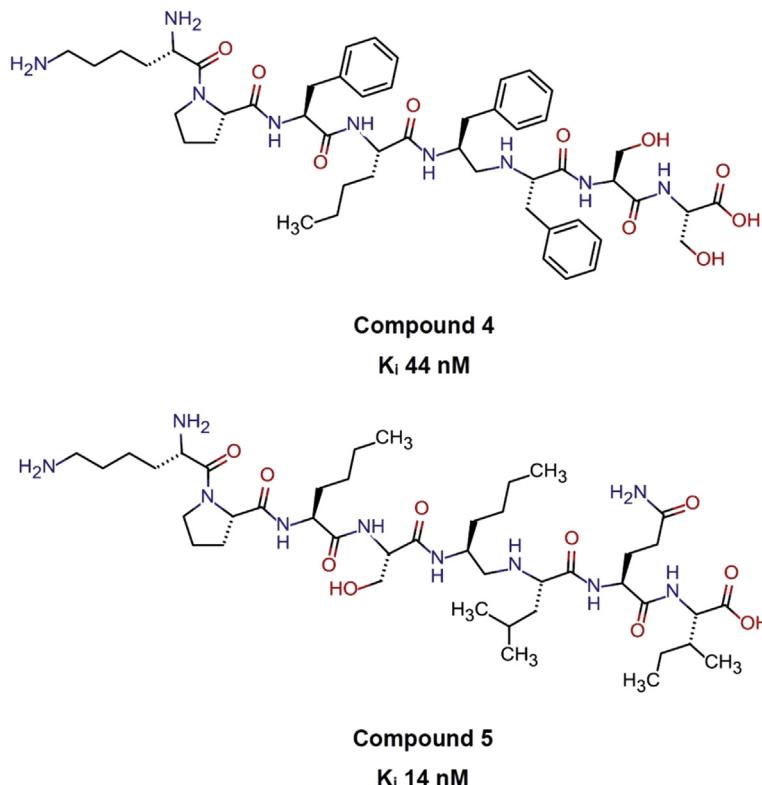
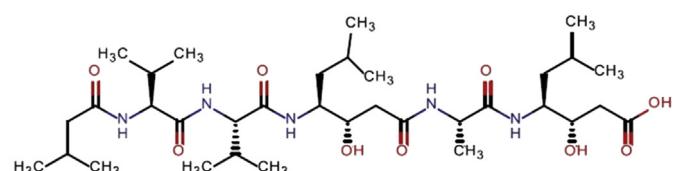


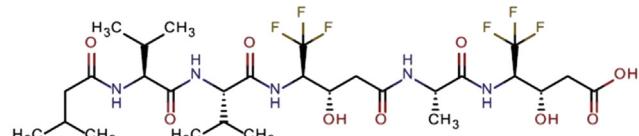
Fig. 8. Structural representation of reduced amide based octapeptide inhibitors (**compound 4, 5**) with their activity profile against Plm II.

peptidomimetics as well as small molecule inhibitors. Peptidomimetics emerged as first choice in order to develop inhibitors targeting proteases because of its isosteric nature with non-cleavable transition-state complex. Till date, several peptidomimetics with different scaffolds e.g. reduced amides, statins, hydroxyethylamines, hydroxypropylamines, norstatines, dihydroxyethylenes, phosphinates etc. (Fig. 6, Table 1) were pursued in order to identify potent inhibitors targeting Plm II [15,30,31]. Additionally several small molecule inhibitors and inhibitors containing a diverse set of scaffolds were identified using a combination of synthetic medicinal chemistry as well as computer aided drug design approach. One major problem encountered in the designing of inhibitors targeting Plm II is lack of selectivity towards a particular class of plasmepsin [16].

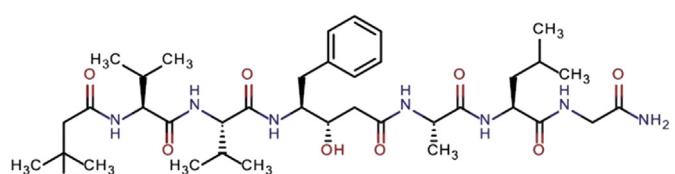
The success of peptidomimetics as HIV protease inhibitors highlighted the potency of pursuing peptidomimetics as potential



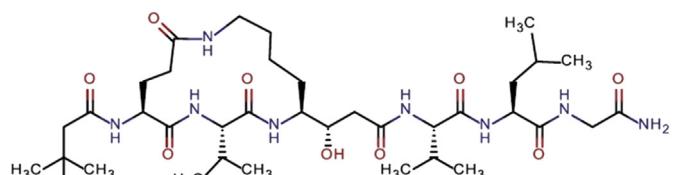
Compound 6
 $K_i = 0.025 \text{ nM}$



Compound 7
 $K_i = 1.3 \text{ nM}$

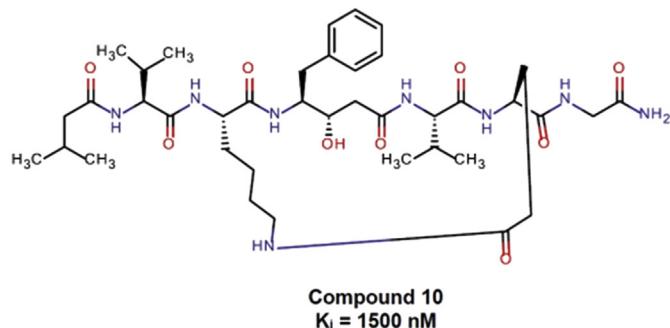


Compound 8
 $K_i = 0.04 \text{ nM}$

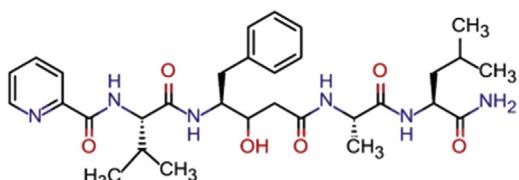


Compound 9
 $K_i = 0.2 \text{ nM}$

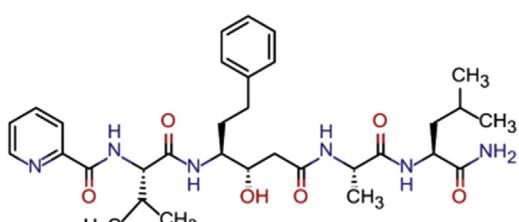
Fig. 9. Structural representation of statine based inhibitors **compound 6–9** targeting Plm II.



Compound 10
 $K_i = 1500 \text{ nM}$

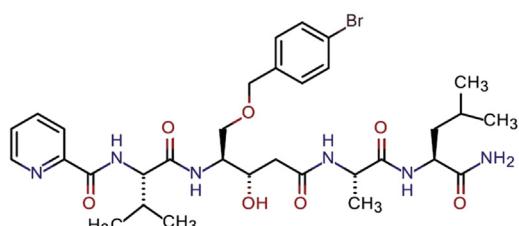


Compound 11
 $K_i = 0.56 \text{ nM}$

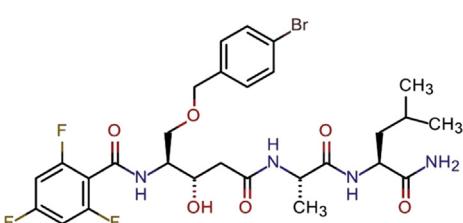


Compound 12
 $K_i = 46 \text{ nM}$

Fig. 10. 2D representation of potent Plm II inhibitors (**compound 10, 11 and 12**) with their activity profile.



Compound 13
 $K_i = 2.2 \text{ nM}$



Compound 14
 $K_i = 10 \text{ nM}$

Fig. 11. Structural representation *p*-bromobenzyl substituted Plm II inhibitors, **compound 13 and 14**.

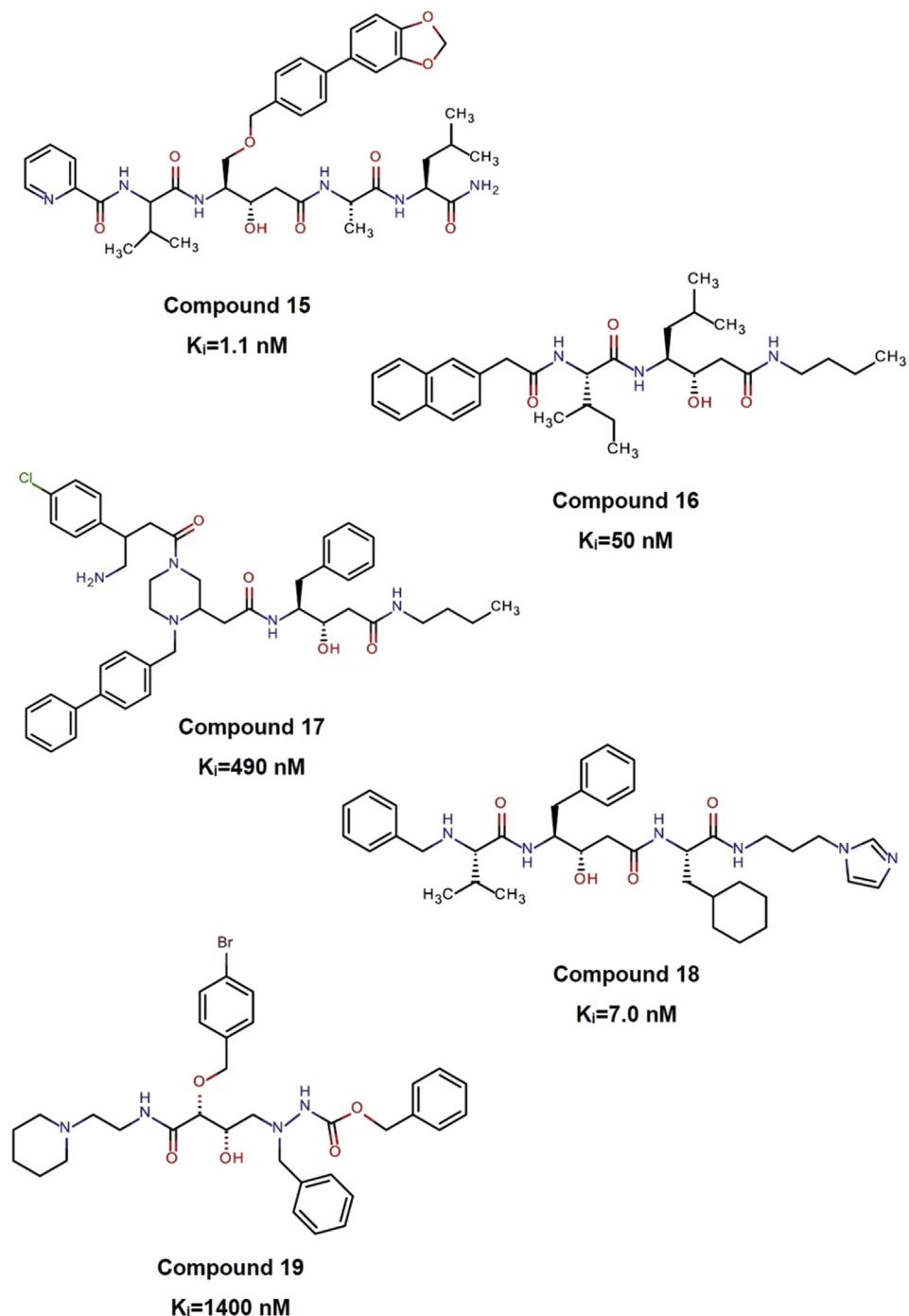


Fig. 12. 2D representation of compound 15–19 and their enzymatic activity (K_i) against Plm II.

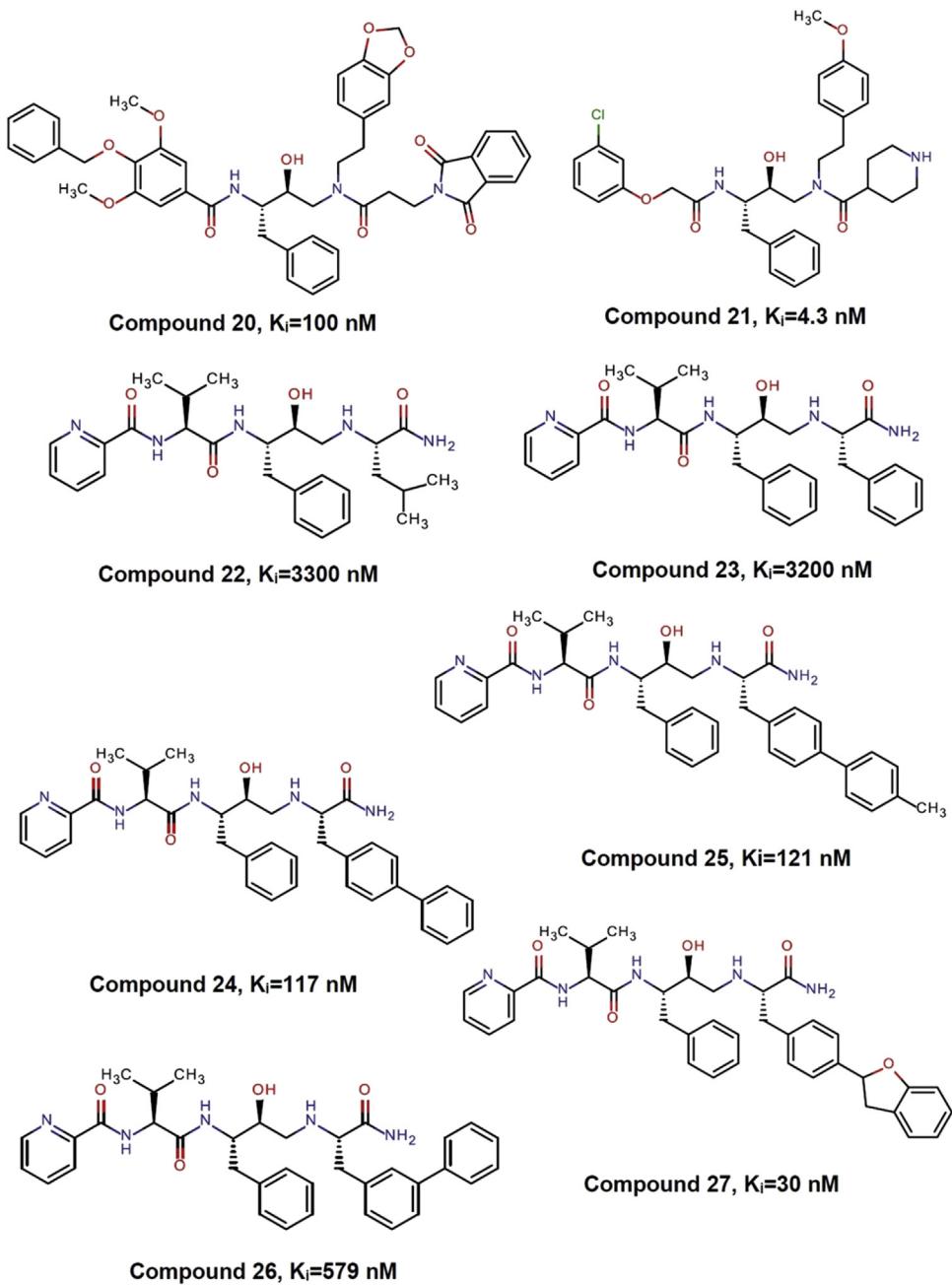


Fig. 13. Structural representation of Plm II inhibitors **compound 20–27** and their enzymatic activity.

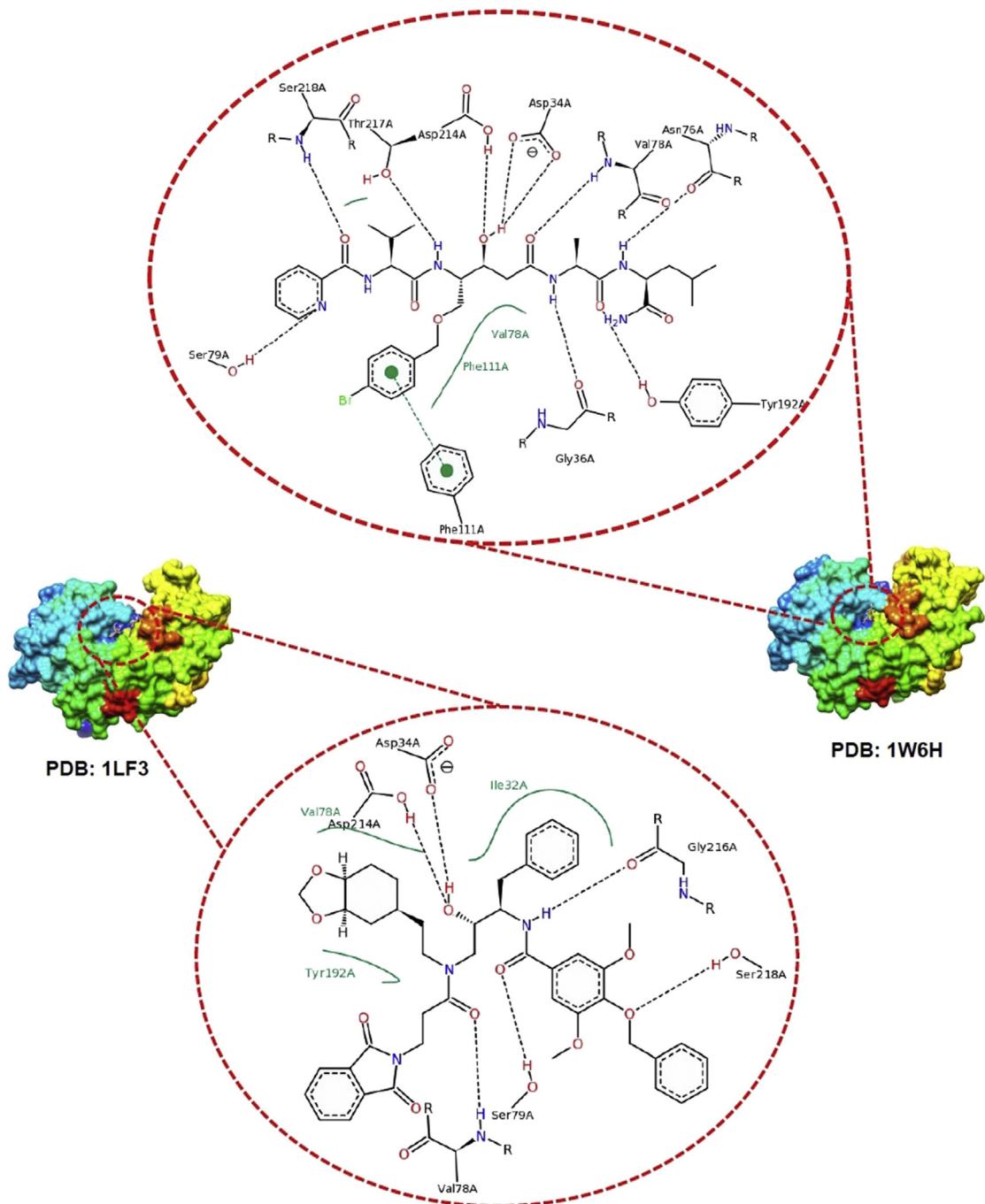


Fig. 14. Protein-ligand interaction plot highlighting interaction of **compound 20** (PDB: 1LF3) and **compound 13** (PDB: 1W6H) with the active site residues of plasmeprin II.

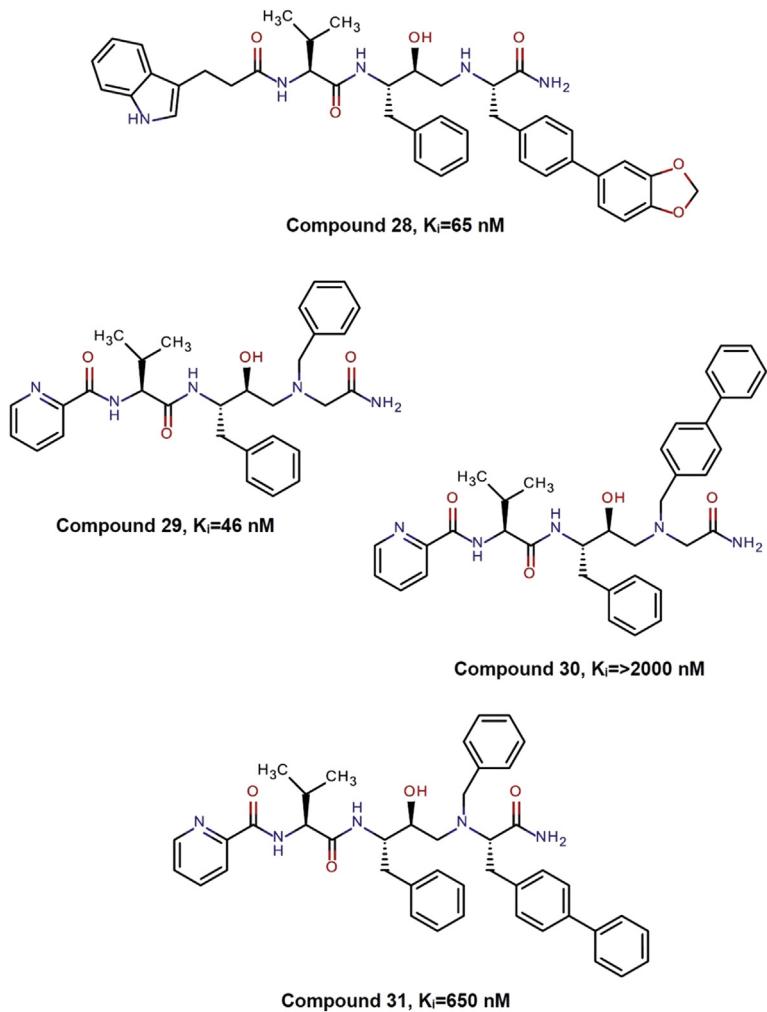


Fig. 15. Structural representation of compound 28–31 with the enzymatic activity targeting Plm II.

inhibitors towards several other therapeutically important proteases. Early years observed a significant number of efforts directed towards discovery peptidomimetics as potential antimalarial agents targeting Plm II. Researchers at Roche discovered one of the potent peptidomimetics, Ro 40-4388 (**compound 1**, Fig. 7) which displayed significant anti Pm-II activity with a K_i at lower micromolar range ($K_i = 700$ nM). The potency and structural features of Ro 40-4388, led to development of several other peptidomimetic inhibitors with an improved activity [32–34]. Gupta et al. identified two potent mechanism based reversible inhibitors (**compound 2**, 3; Fig. 7) of Plm II with an improved enzyme kinetic and inhibitory profile [35]. However the selectivity of these inhibitors towards Plm II needs further optimization in order to develop enzyme specific potent inhibitors of Plm II [35].

Not only peptidomimetics, several groups actively pursued selective octapeptide inhibitors with reduced amide bond isostere. This exploration led to identification of two reduced amides, **compound 4** [36] and **compound 5** [37] (Fig. 8) which displayed enzymatic potency against Plm II at lower nanomolar scale. Structure activity relationship (SAR) highlighted that the occurrence of reduced amide group found to be preferred in the S2 and S3 subsites whereas Nle (norleucine) was found to be suitable for the P1 subsite of Plm II. SAR study also highlighted the branches residues

at P1' position were preferred in case of Plm II.

Statines often considered as general inhibitor of aspartic protease which emerged as a scaffold of choice against a diverse range of therapeutic targets such as plasmeprins, HIV protease, Cat D etc. Pepstatin A [23,38,39] (**compound 6**, Fig. 9) one of inhibitor containing statine scaffold displayed subnanomolar affinity against Plm II and thus emerged as a starting point to develop a variety of statine derivatives, peptidomimetics and peptides targeting Plm II. This exploration led to development of trifluoro containing pepstatin analogue, **compound 7** [40] (Fig. 9) which displayed comparatively less affinity than pepstatin A though but remains highly selective towards Plm II and other plasmeprins. The potency of pepstatin A led to development of another potent statine inhibitor, **compound 8** [26] (Fig. 9) which displayed lower nanomolar affinity ($K_i = 0.04$ nM) against Plm II. Further cyclization between P1 and P3 led to development of novel cyclic statine analogues, **compound 9** [26,41] (Fig. 9) which though displayed decrease in affinity ($K_i = 0.2$ nM) towards Plm II but well tolerated which led to proof of concept in order to develop cyclic or macrocyclic inhibitors targeting Plm II.

It is interesting to observe that cyclization between P2–P3' resulted in identification of a novel statine based analogue (**compound 10**, Fig. 10) with a significant decrease in affinity

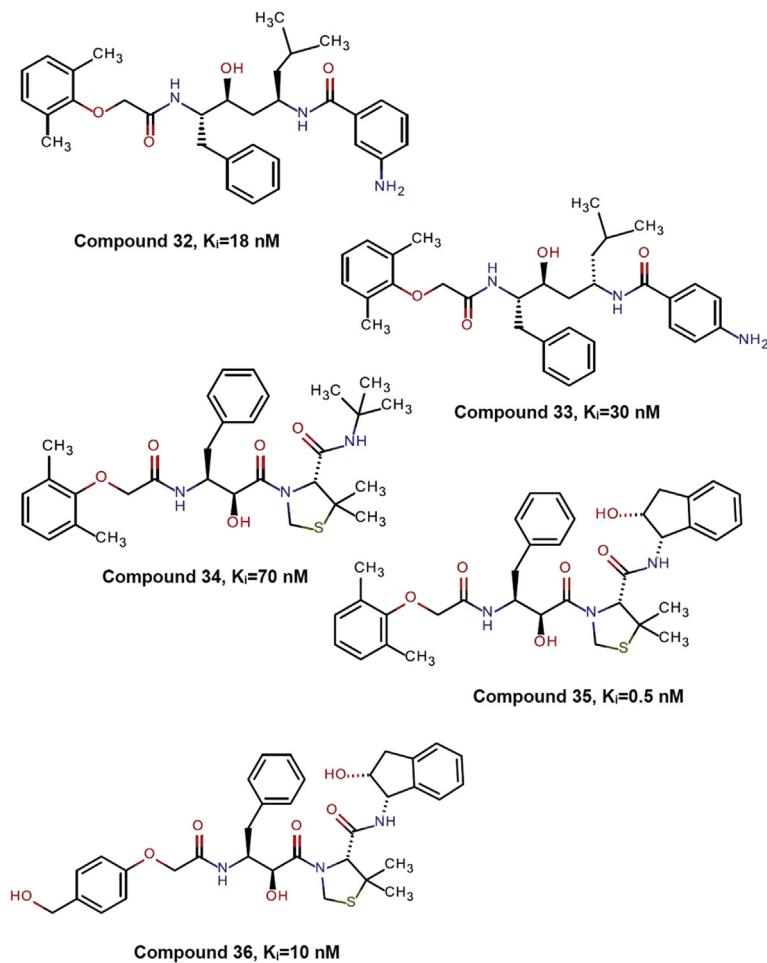


Fig. 16. 2D representation of Plm II inhibitors **compound 32–36** with their enzymatic activity.

($K_i = 1500\text{ nM}$) towards Plm II [23,36,42]. This loss potency might be due to the interference of flap tip residue Val78 with the cyclic portion of **compound 10**. Further a small size of ligand (**compound 11**, Fig. 10) led to an increase in affinity ($K_i = 0.56\text{ nM}$) and selectivity towards Plm II [26,41]. Further substitution at P1 position with bulkier phenethyl group (**compound 12**, Fig. 10) led to significant decrease in affinity ($K_i = 46\text{ nM}$) [43] and thus recognised as sterically unfavourable substitution in the process of structure optimization. Though elongated *p*-bromobenzoyloxy substitution (**compound 13**, Fig. 11) led to an increase in affinity due to perfect fitting in the S1 and S3 subsites. The crystal structure of Plm II complexed with **compound 13** [43] (PDB: 1W6H, Fig. 14) supports the presumption that elongated *p*-bromobenzoyloxy substitution led to a proper fitting and thus an increased affinity. Molecular modelling study further highlighted the importance of flexibility in case of $-\text{CH}_2-\text{O}-\text{CH}_2-$ linkage which played a major role in proper fitting of elongated side chain. Though a shorter substitution at P1 is necessary for high selectivity. In an effort to reduce the peptide character the statine scaffold was modified by a trifluoro substitution (**compound 14**, Fig. 11) which led a decrease in affinity ($K_i = 10\text{ nM}$) despite its selectivity towards plasmepsins [43].

Further improvement in Plm II inhibition of *p*-aryl- substituted analogues was achieved through accessing the hydrophilic cleft between the flap and the S3 α -helix (**compound 15**, Fig. 12) [44]. Carroll et al. and Dolle et al. developed combinatorial statine-based

libraries based on what had been published in the literature at that time for identifying more potent Plm II inhibitors having good selectivity. In this library the most potent inhibitor (**compound 16**, Fig. 12) showed Plm II selectivity six times more than that for Cat D and it's stereogenic centre showed preference towards (S)-configuration like hydroxyl configuration in pepstatin A [45]. Further, molecular docking based identification studies led to development of a cyclic diamino acid containing statine-based combinatorial library containing 18,900 inhibitors. Plm II selectivity was increased by incorporating cyclic diamino acids (**compound 17**, Fig. 12) but inhibitory potency was decreased with a K_i value of 490 nM. P1 side chain of the **compound 17** greatly favoured by the benzyl group suggesting other parts of the inhibitor regulates the overall affinity [46]. Variations on the P2' and P3' side influences the Plasmepsin selectivity. P2' position favoured hydrophobic groups than hydrophilic groups against Plm II (**compound 18**, Fig. 12) [47]. Potency of reversed statine class of inhibitors were remarkably enhanced by incorporation of P1'- substituted hydrazine (**compound 19**, Fig. 12) [48].

Studies highlighted that the central core of the **compound 19** with found to be that with the (R)-configuration of the hydroxyl group and (S)-configuration of the P1 substituent. X-ray crystallographic structure of **compound 20** [49] (Fig. 13) with Plm II (PDB Code: 1LF3, Fig. 14) discloses notable flexibility of the S1' substitute. To improve Plasmepsin selectivity basic piperidine side chains were

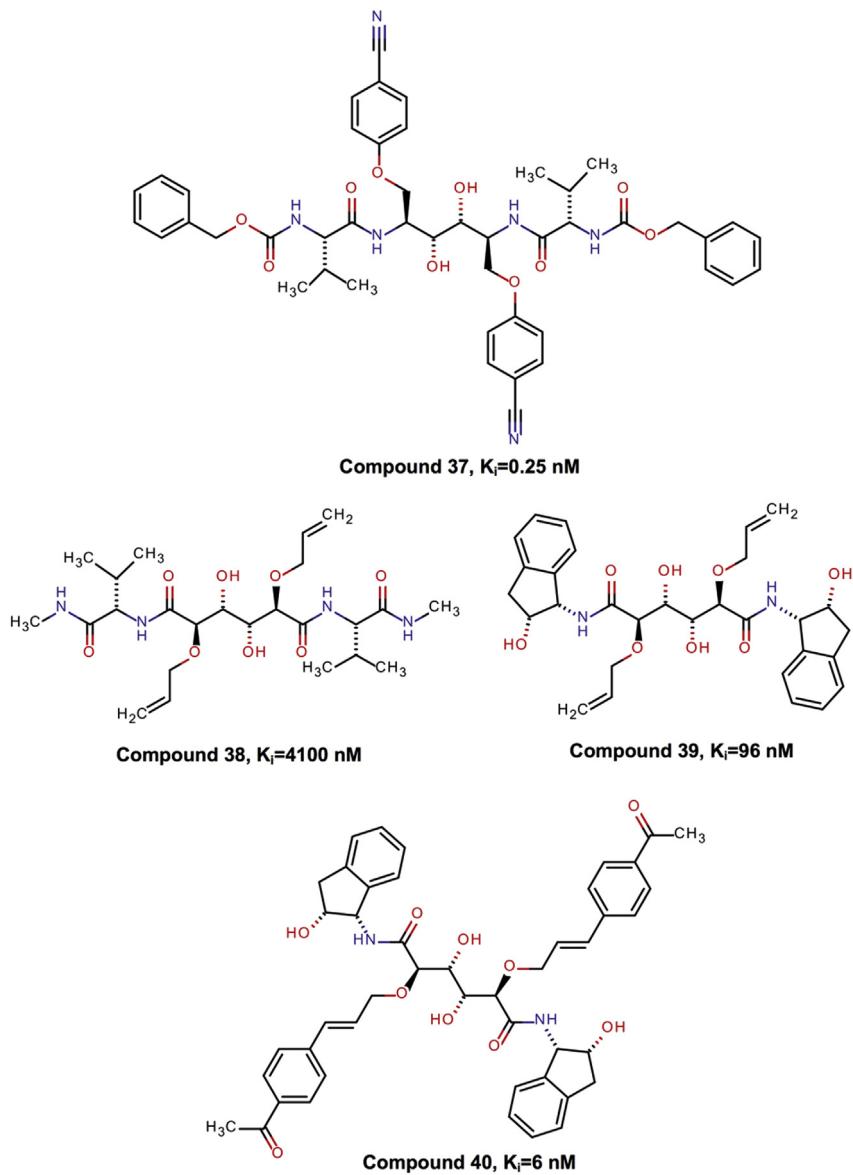


Fig. 17. Chemical representation of potent plasmeplase II inhibitors, **compound 37–40** and their enzymatic activity.

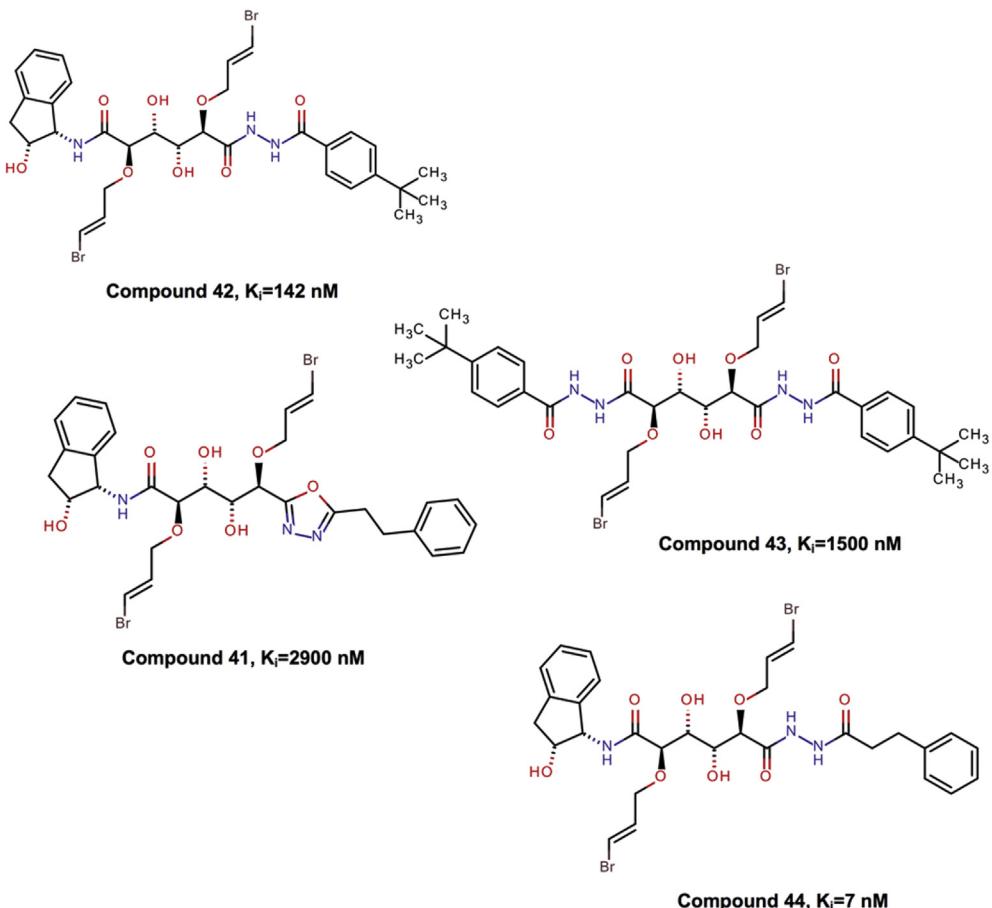


Fig. 18. 2D chemical representation of plasmepsin II inhibitors, **compound 41–44** with their activity profile.

incorporated to improve its selectivity over Cat D. *In vivo* human serum albumin assay of a selected potent inhibitor (**compound 21** [49], Fig. 13) showed no apparent decrease in the inhibitory action and were also in accordance with Lipinski's "Rule of Five". Furthermore structural analysis led to development of potent Plm II inhibitors by introducing secondary basic amine into hydroxyethylamine isostere with divergent P1' side chains to act with the acidic catalytic aspartate residues, Asp34 and Asp214 (**compound 22–26** [50], Fig. 13). Generally in P1' position large meta or para-substituted benzenes were resulted into increase activity and selectivity towards Plm II (**compound 24** and **26**) [50].

Different substituents in the P3 and P1' positions led development of potent Plm II inhibitors (**compound 27** [51], Fig. 13 and **compound 28** [51], Fig. 15) having K_i values at lower nanomolar range with high selectivity over human Cat D. A group of *N*-substituted Plm II inhibitors with diversely sized P1' substitutions were produced by shifting the side chain from C- α to the basic nitrogen of the hydroxyethyl amine core (**compound 29** and **30**, Fig. 15) [52]. In case of **compound 29**, high plasmepsin selectivity achieved by altering the benzyl P1' side chain to isobutyl P1' side chain. Merging the two P1' groups, both on the C- α and on the basic nitrogen led to decreased activity (**compound 31**, $K_i = 650 \text{ nM}$, Fig. 15), showing that the two side chains bind to different subsites in the enzyme [52].

Further research by Asojo and co-workers resulted in development of HIV-1 protease inhibitors with a hydroxypropylamine core, which displayed inhibitory activity against Plm II (**compound 32**

and **33**, Fig. 16) [25]. These inhibitors were developed on the basis of the native cleavage site, Phe33-Leu34, by introducing a benzyl and isobutyl group in the P1' and P1 positions which resulted in low-nanomolar potency ($K_i = 18 \text{ nM}$, $K_i = 30 \text{ nM}$ for **compound 32** and **33** respectively) [25]. A new category of Plm II inhibitors containing the allophenylnorstatine-dimethylthioproline scaffold displayed low toxicity and great bioavailability (**compound 34** and **35**) [53]. **Compound 34** with a t-butyl in the P2' position showed a greater Plm II selectivity over Cat D. Further molecular docking studies of **compound 34** (Fig. 16) displayed a similar kind of binding mode that of pepstatin A [53,54]. Whereas, **compound 35** (Fig. 16) displayed a K_i value of 0.5 nM with indanol hydroxyl group of compound **35** contributed highly towards binding enthalpy [53,55]. Substitution of 1, 3 xylene with 4-hydroxymethyl phenyl (**compound 36**, Fig. 16) led to significant decrease in inhibitory activity though the actual reason for this decrease is not well understood [16].

More research involved remodelling of C_2 -symmetric led to development of 1, 2-dihydroxyethylene transition-state isostere initially developed as HIV-1 protease inhibitors which displayed significant Plm II affinity (**compound 37**, Fig. 17) [56]. **Compound 37** displayed 100-fold plasmepsin selectivity over Cat D and electron withdrawing p-cyano phenoxy P1/P1' substituents appeared to be driving force behind the Plm II affinity [56]. The (*SRRRRS*) isomers (**compound 38**, Fig. 17) emerged from L-mannitol and L-valine were found active in the Plm II assay [57]. MD stimulations of 1, 2-dihydroxyethylene based inhibitors revealed that binding

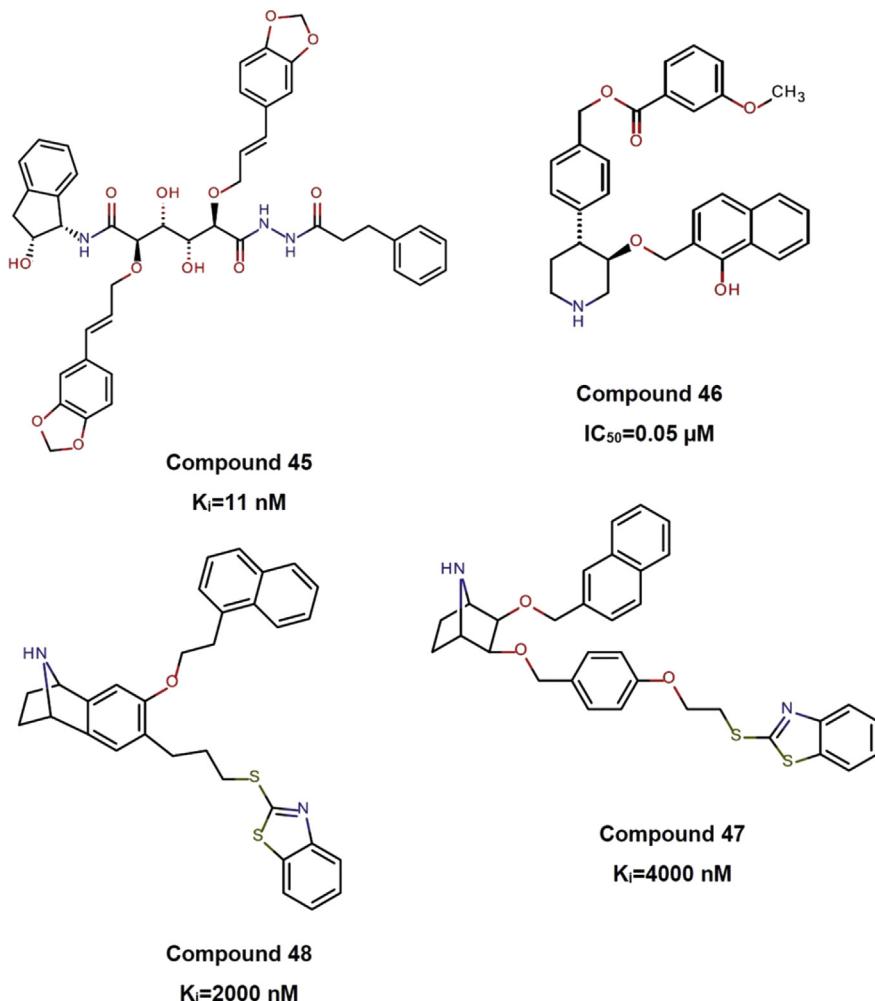


Fig. 19. Chemical representation of novel inhibitors of plasmeprin II, **compound 45–48** and their activity profile.

mode of these inhibitors is quite similar to pepstatin A. Substitution of the P2/P2' valines by (1S, 2R)-1-amino-2-indanol led to development of a Plm II inhibitor (**compound 39**, $K_i = 96\text{ nM}$, Fig. 17) which displayed a 40-fold enhanced Plm II affinity over Cat D [57,58]. Binding mode analysis of **compound 39** predicted intermolecular hydrogen bond interaction between hydroxyls of the 2-indanols with the active site residues of Plm II. A modified *p*-acetylphenyl attached at P1 and P1' side chains led to development of a compound 39 derivative, **compound 40** (Fig. 17) ($K_i = 6\text{ nM}$), which displayed 10-fold greater Plm II affinity than **compound 39** [58].

The C₂ asymmetrical variant of Plm II inhibitor, **compound 42** (Fig. 18) displayed superior potency than C₂ symmetrical variant (**compound 43**, Fig. 18) whereas diacylhydrazine (**compound 41**, Fig. 18) found to be more favourable amide bond substitute than the oxadiazole (**compound 44**, Fig. 18) [59].

Stretching of P1 and P1' side chains of led to development of one of the potent symmetrical diacylhydrazine, **compound 45** (Fig. 19) which led to rotation of Met75 in order to accommodate bulky group of **compound 45** at S2' subsite [59]. A modified 3, 4-disubstituted piperidine led to development of a potent non-peptide inhibitor (**compound 46**, Fig. 19) which displayed higher potency against *P. falciparum* in cell assays [15]. Using the remodeled Plm II structure (PDB Code: 1SME) three main binding sites

were identified: a large hydrophobic S1–S3 pocket (Phe111, Phe120, Met15), a more hydrophilic S2' pocket (Leu131, Tyr192) and a new large lipophilic pocket. Molecular modelling led to recognition of 7-azabicyclo [2.2.1] heptane as the right central scaffold **compound (±) 47** (Fig. 19) [60]. Further modification of 11-azatricyclo [6.2.1.0] undecatriene scaffold led to development of second generation non-peptide Plm II inhibitor, **Compound (±) 48** (Fig. 19) which displayed a good inhibitory activity but a low plasmeprin selectivity over Cat D [61].

Further, high throughput screening led to identification of novel nonpeptide plasmeprin II inhibitors with a 4-aminopiperidine (**compound 49** and **50** [15], Fig. 20) and an ethylene diamine core unit (**compound 51** [62], Fig. 20). **Compound 49** [15] was found highly potent when ring nitrogen was substituted with a lipophilic isobutyl group. Crystal structure of **compound 50** with Plm II (PDB: 2BJU [63]) displayed a water molecule linking a hydrogen bond from the catalytic aspartate to ring nitrogen. Inhibitors with 4-aminopiperidine core unit (**compound 49** and **50**) expressed quite similar SAR for Plm II to that for inhibitors with ethylenediamine core units (**compound 51**). Further, screening of the Walter Reed chemical database led to identification of a small nonpeptide type of inhibitor with a diphenylurea scaffold, **Compound 52** (Fig. 20) which displayed significant potency against Plm

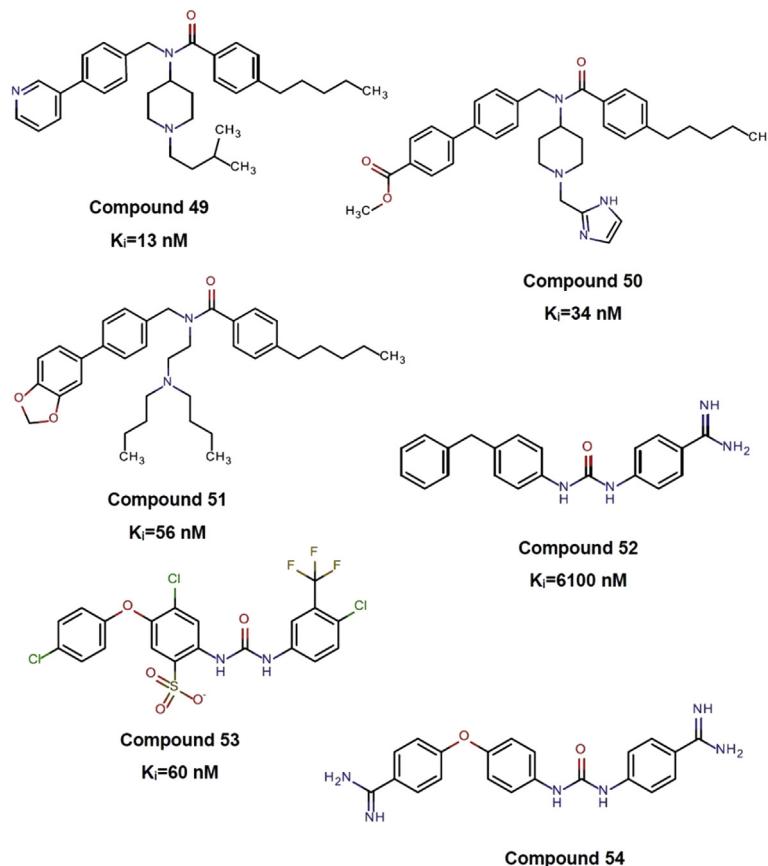


Fig. 20. Chemical representation of novel Plm II inhibitors, **compound 49–54**.

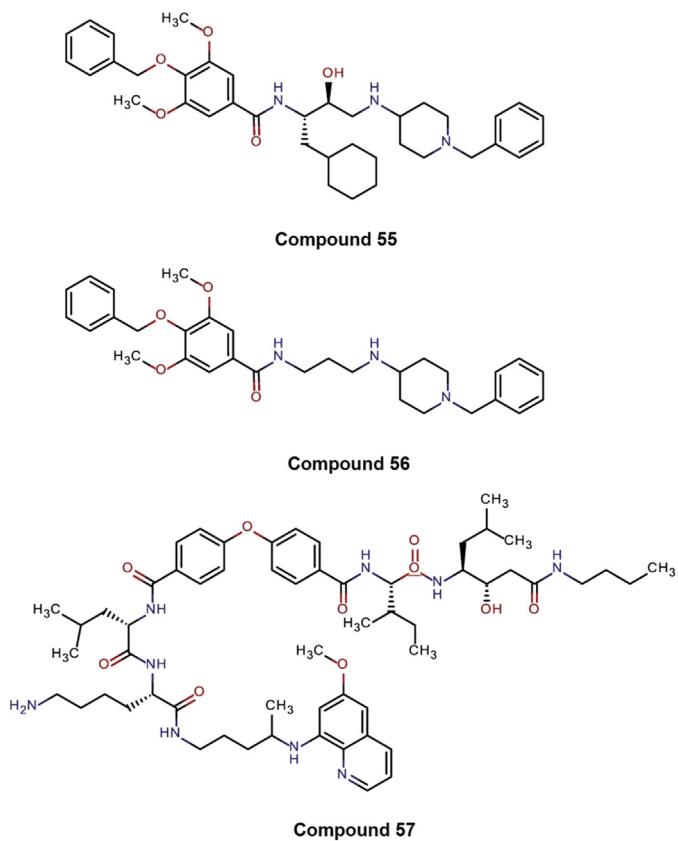


Fig. 21. 2D structural representation of some bifunctional Plm II inhibitors **compound 55, 56** and **57** with K_i values $20\text{ }\mu\text{M}$, $\geq 250\text{ }\mu\text{M}$ and 0.59 nM .

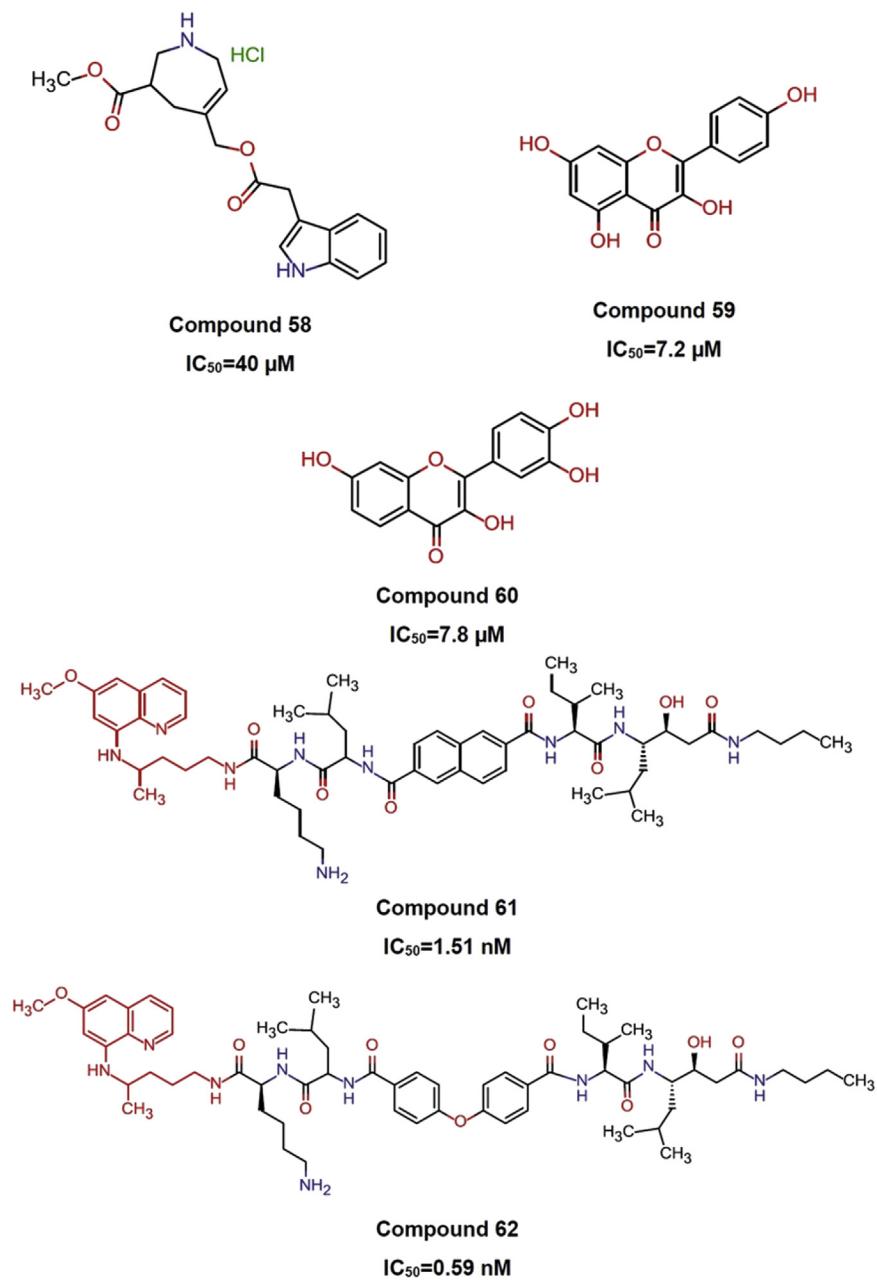


Fig. 22. Structural representation of some new chemical entities (**compound 58–62**) and their Plm II activity.

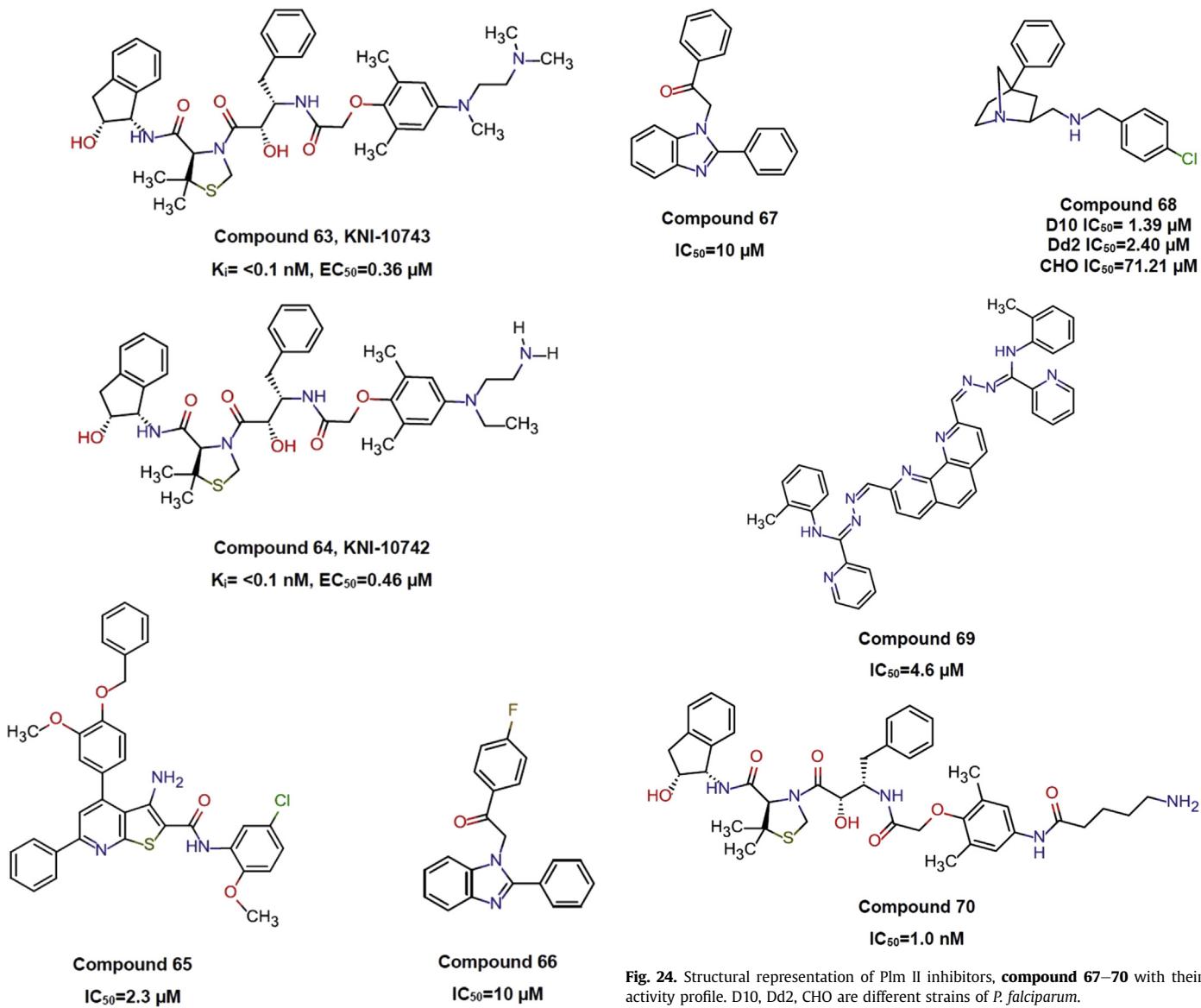


Fig. 23. Chemical representation of novel Plm II inhibitors, **compound 63–66** with their activity profile.

II in screening assays [64].

Subsequent screening of 346 compounds led to identification of a novel inhibitor (**compound 53**, Fig. 20) of diphenylurea category which displayed a high Plm II potency mainly due to the presence of phenoxy substituent and as well as presence of acidic sulphonate group [64]. Further docking analysis of diphenylurea unit containing compounds highlighted that one of the urea nitrogen's linked formed a hydrogen bond interaction with the aspartic acids in the active site in both Plm II (PDB: 1SME) leaving space for the catalytic water molecule. All diphenylurea urea containing Plm II inhibitors displayed high plasmepsin selectivity over Cat D. Bhattacharya et al. identified a nonpeptide amidine-containing inhibitor (**Compound 54**, $IC_{50} = 0.054 \mu\text{M}$, Fig. 20) which displayed significant potency against *P. falciparum* in parasite-infected erythrocytes but its actual mechanism is still unknown [64]. Further concept of double-drug/polypharmacology led to development of some potent bifunctional inhibitors (**compound 55, 56** [65] and **57** [66],

Fig. 21) which displayed potent plasmepsin activity.

Recently, ring-closing metathesis approach led to discovery of a 2, 3, 4, 7-tetrahydro-1H-azepine scaffold containing non-peptidic inhibitor, **compound 58** (Fig. 22) which exhibited excellent activity against Plm II ($IC_{50} = 40 \mu\text{M}$) as well as selectivity [67]. Further, biological assay led to identification of bifunctional inhibitors, **compound 59** (myricetin, Fig. 22) and **compound 60** (Fisetin, Fig. 22) which displayed IC_{50} average inhibitory activity against Plm II [68]. Recently scientists led to development of potent statine based Plm II inhibitors by applying 'double-drug' method. Redesigned statine-based Plm II inhibitors coupled with primaquine (**compound 61** and **62**, Fig. 22) showed great improvement in Plm II inhibition which can be applied in future to develop broad spectrum antimalarial agent [69].

Attachment of 2-aminoethylaminio groups to allophenylnorstatine led to development of two novel inhibitors, KNI-10743 (**compound 63**, Fig. 23) and KNI-10742 (**compound 64**, Fig. 23) which exhibited potent Plm II inhibitory activities ($K_i < 0.1 \text{ nM}$) [70]. Further application of ligand based virtual screening led to

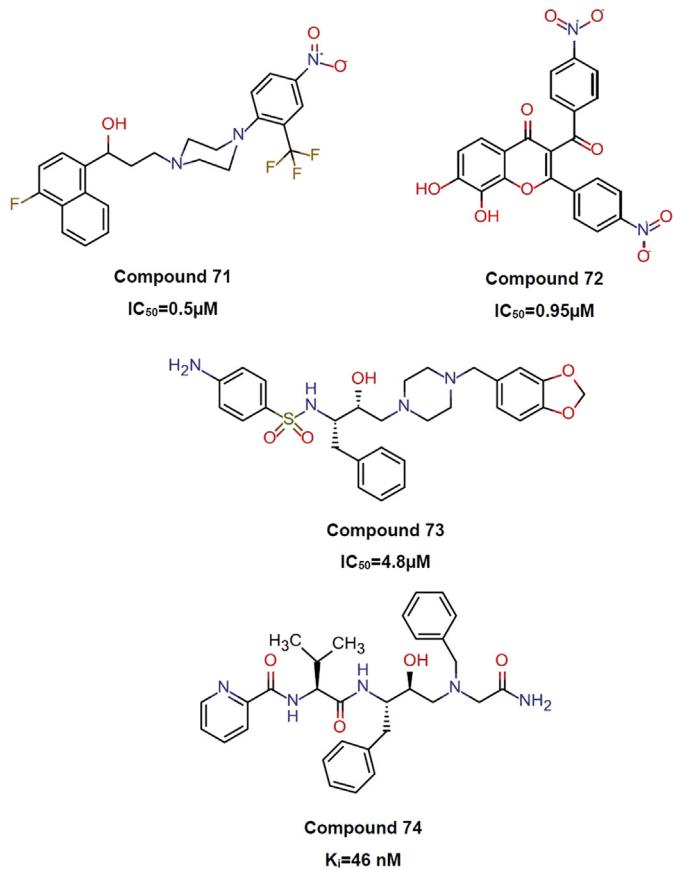


Fig. 25. 2D chemical representation of some novel Plm II inhibitors, **compound 71–74**.

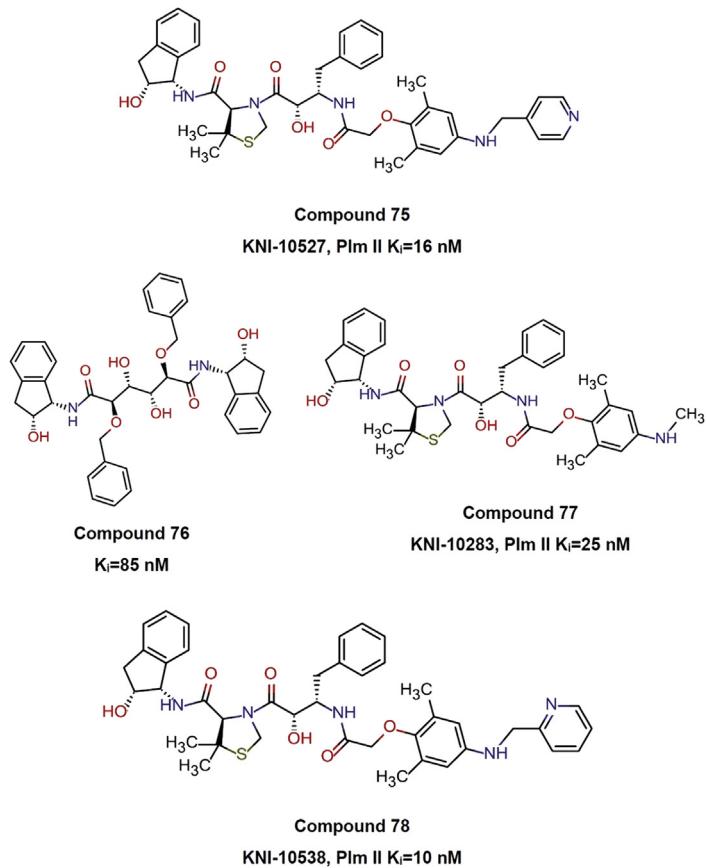


Fig. 26. Inhibitory activity of some potent Plm II inhibitors, **compound 75–78** and their structural representation.

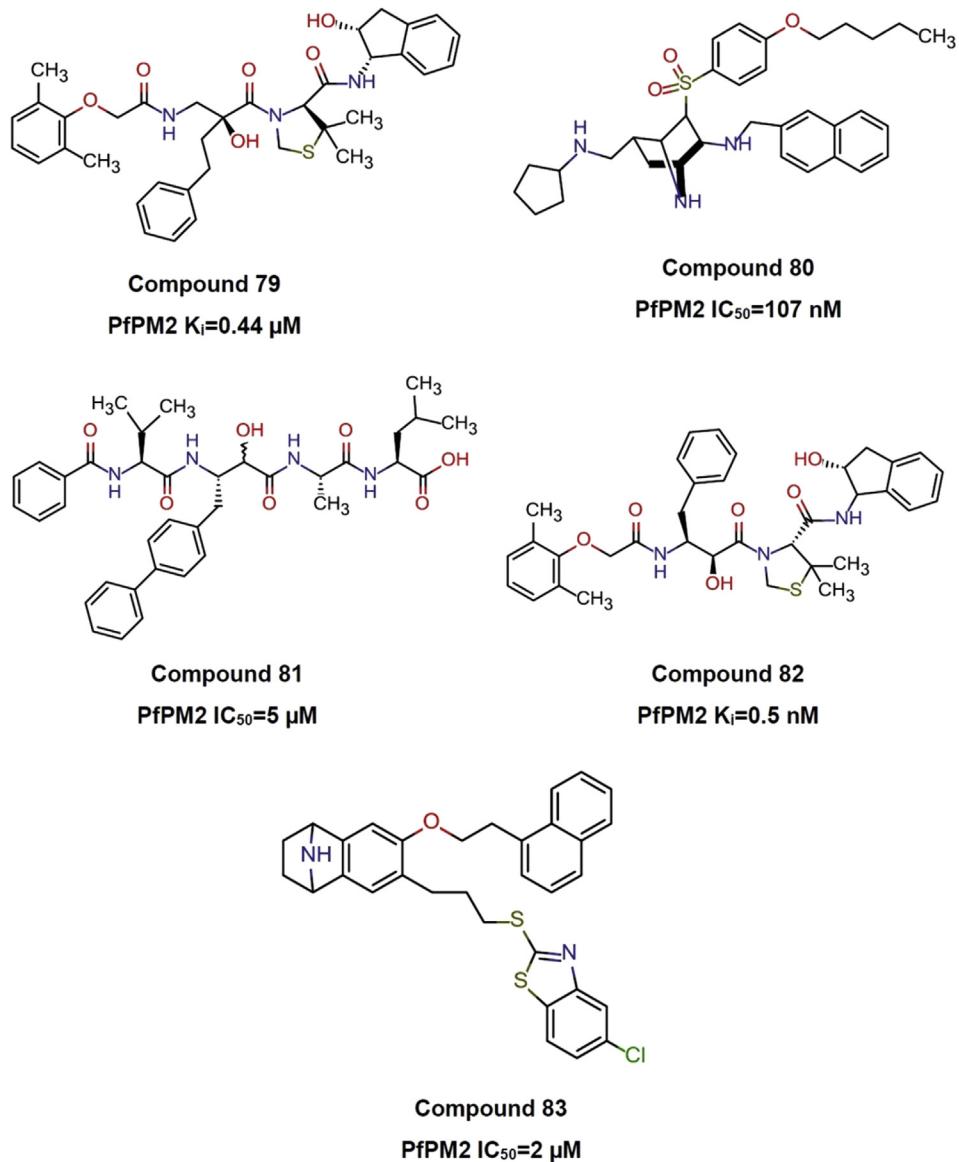


Fig. 27. Inhibitory activity and chemical representation of **compound 79–83** and their Plm II inhibitory activity.

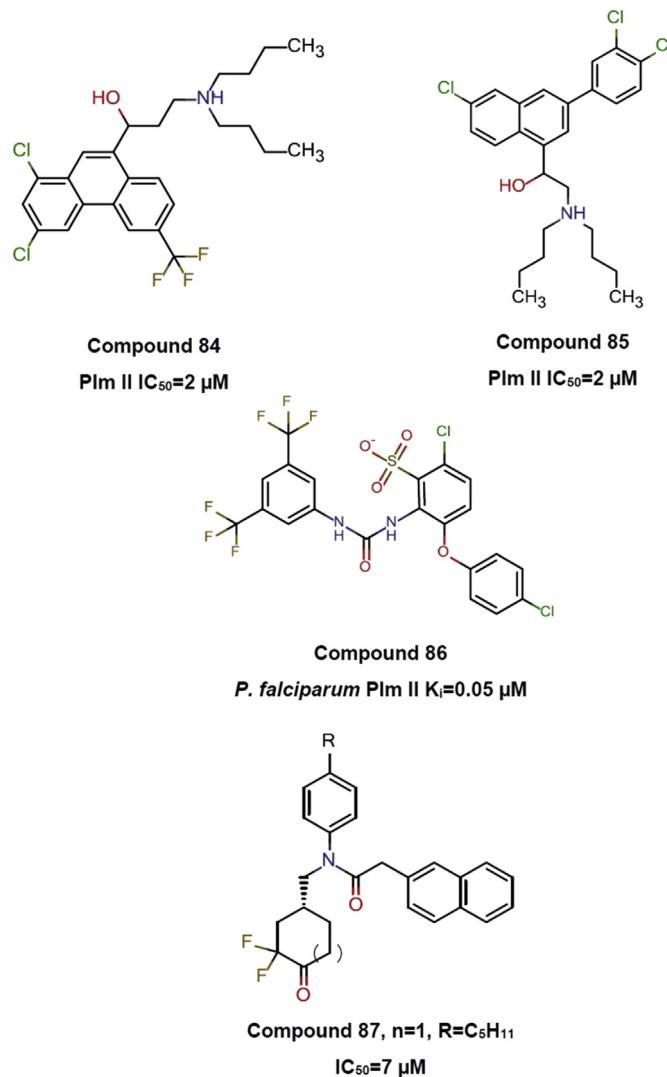


Fig. 28. Structural representation of **compound 84–87** and their Plm II inhibitory activity.

identification of a potent small molecule inhibitor (**compound 65**, Fig. 23) containing thieno [2, 3-b] pyridine scaffold which displayed an IC_{50} of $2.3 \pm 0.6 \mu M$ towards Plm II [71]. Recently synthesized benzimidazole derivatives (**compound 66**, Fig. 23 and **compound 67**, Fig. 24) were found highly active against Plm II exhibiting IC_{50} value at mid micromolar range in enzyme assay [72].

Docking study highlighted the plausible binding mode of these inhibitors where pyridine ring of benzimidazole derivatives was in contact with S10 subsite and the acetophenone moiety was interacted with S1–S3subsite residue. Stereo selective LiAlH₄-induced reductive cyclization of 2-(4-Chloro-2-cyano-2-phenylbutyl)aziridines were employed for development of both endo- and exo-2-aminomethyl-4-phenyl-1-azabicyclo[2.2.1]heptanes (**compound 68**, Fig. 24) as new azaheterobicyclic scaffolds which displayed strong binding interaction with Plm II in *in-silico* studies [73]. Structure based virtual screening led to discovery of a novel non-peptide Plm II inhibitor (**compound 69**, Fig. 24) with moderate potency ($IC_{50} = 4.6 \mu M$) [74]. Thorough binding mode analysis showed that these inhibitors formed numerous hydrogen bonds with catalytic residues (Asp 34 and Asp 214) and also with the other

important residues that led to a stabilized structure. Optimization of plasmeprin inhibitor by focussing on similar structural feature with chloroquine to avoid drug-resistant mechanism of *P. falciparum* led to development of KNI-10823 (**compound 70**, Fig. 24) which showed a K_i at low nanomolar range [75]. Hypothesis suggested that structural similarity between 2-aminoethylamino substituent of KNI-10740 and chloroquine could avoid drug-resistant mechanism of TM91C235 strain.

Inhibitors with piperazine and pyrrolidine moiety were synthesised and tested against the *P. falciparum* chloroquine-resistant (FCR-3) strain in culture. The most potent compound (**compound 71**, Fig. 25) 1-(4-fluoronaphthyl)-3-[4-(4-nitro-2-trifluoromethylphenyl) piperazin-1-yl] propan-1-ol showed high inhibitory activity on *P. falciparum* ($IC_{50} = 0.5 \mu M$) antimarial activity was due to combined existence of a hydroxyl group, a propane chain and a fluorine [76]. Molecular docking studies and electrostatic calculation displayed that this inhibitor could bind to the Plm II active site. In a continuation to find new plasmeprin inhibitor a chromone based ligand (**compound 72**, Fig. 25) which previously showed anti HIV-1 protease activity found to be potent antimarial compound with $IC_{50} = 0.95 \mu M$ [77]. Molecular docking study of chromone based ligands showed to inhibit the activity of Plm II. **Compound 72** displayed a higher binding affinity than the known Plm II inhibitors. Thus chromone series can be further pursued to unveil a new set of ligands targeting plasmeprin II [77]. Recently synthesized hydroxyethylamine derivatives (**compound 73**, Fig. 25) from the intermediated hydroxyethylpiperazines with benzenesulfonyl chlorides or benzoyl chlorides were tested *in-vitro* against a W2 *P. falciparum* clone [52]. Some of the tested derivatives exhibited a remarkable activity *in vivo* and found to be a plausible target on Plm II based on *in-silico* studies. Malaria parasites (*P. falciparum*) digests the host cell hemoglobin with the help of two aspartic proteases Plm I and Plm II (haemeglobinase). In order to combat the hemoglobin degradation a group of inhibitors were designed incorporating a hydroxyethylamine transition state as isostere as central moiety. One of the potent inhibitor (**compound 74**, Fig. 25) of this series displayed moderate potency against Plm II with a K_i of 46 nM [52].

Macrocyclic inhibitors of the Plm II generally designed with a 13-membered or a 16-membered macrocycles and a 1, 2-dihydroxyethylene as transition state mimicking unit. Cyclization was achieved through ring closing metathesis with the second generation Grubbs's catalyst. Macrocyclic inhibitors were found to be moderately selective towards Plm II over human Cat D. One of the potent compound (**compound 76**, Fig. 26) of this series displayed a K_i of 85 nM against Plm II which can be used as a template to design new generation Plm II inhibitors with a better potency and selectivity [78]. Molecular dynamics stimulation with docking studies proposed the binding mode of this newly explored compound with an estimation of binding affinities by the linear interaction energy (LIE) method. Dipeptide type HIV protease inhibitors with an allophenylnorstatine-dimethylthioproline scaffold (**compound 75**, KNI-10527, Fig. 26) exhibited significantly active against PlmII than the most reported Plm II inhibitors [79]. Alkylamino compounds like **compound 77** (KNI- 10283, Fig. 26) and **compound 78** (KNI-10538, Fig. 26) were identified with more than 15-fold improved inhibitory activity with sub micromolar K_i value and low cytotoxicity [79].

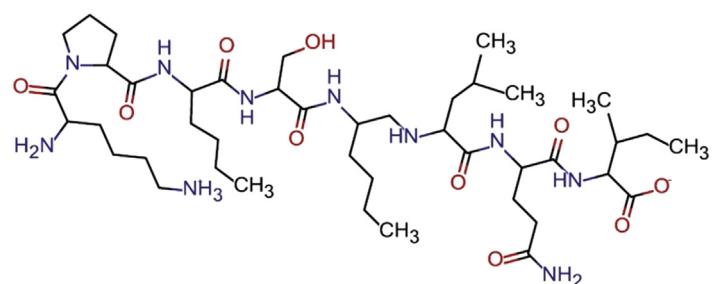
Recently a new class of a-substituted norstatine (**compound 79**, Fig. 27) was developed which displayed Plm II activity at lower micromolar range and can be an effective template to design new class of norstatines based plasmeprin inhibitors [80]. To encounter multidrug-resistant strains of *P. falciparum* a new generation plasmeprin inhibitor (**compound 80**, Fig. 27) was designed with a 7-azabicyclo [2.2.1] heptanes scaffold [81]. **Compound 80** displayed

an IC_{50} of 107 nM against Plm II and can be pursued further to develop more potent and selective Plm II inhibitors [81]. The potency of norstatine inhibitors found to be dependent strongly on the P1 substituent. It was observed that occurrence of large, hydrophobic residues such as 4-bromophenyl, biphenyl, 4-nitrophenyl led to an enhanced inhibitory activity (~70 fold) against Plm II. **Compound 81** (Fig. 27) with biphenyl substitution at P1 position displayed an IC_{50} of 5 μ M which displayed an alternative mode of binding in molecular dynamics simulation [82]. Recently, substrate-based dipeptide type Plm II inhibitors were redeveloped by introducing hydroxymethylcarbonyl isostere as a transition-state mimic. The general redevelopment principle was incorporation of a conformationally restrained hydroxyl group and a bulky unit to fit the S2 pocket. This effort led to identification of a novel Plm II inhibitor, **compound 82** (Fig. 27) which displayed a K_i of 0.5 nM [55]. The general design strategy of this inhibitor can be further explored using computer aided drug design approach in order to develop more potent inhibitors with an activity profile at sub nanomolar range. Structure based de novo design led to development of a potent Plm II inhibitor (**compound 83**, Fig. 27) that showed significant inhibitory activities in micromolar range [83].

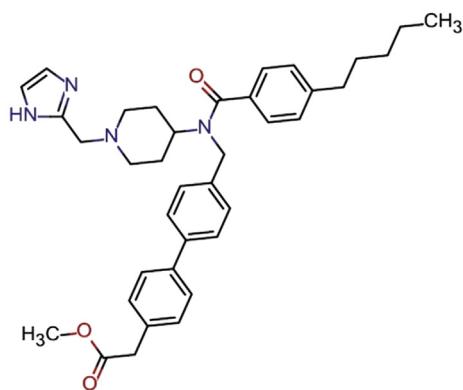
Six potent inhibitors of Plm II were identified with the help of high throughput fragment based docking of ~40,000 molecules using consensus scoring with force field energy functions. This computer aided screening led to discovery of two potent Plm II inhibitors (**compound 84** and **85**, Fig. 28) with moderate activity [84]. Compounds available in Walter Reed chemical database were assessed in an automated assay of *P. falciparum* Plm II. Results showed a diphenyl urea derivative of low molecular mass (**compound 86**, Fig. 28) was found to be active and possessed good selectivity for Plasmepsin II over human Cat D [64]. Recently,

synthesis of hydrated alicyclic α , α -difluoro-cyclopentanones and cyclohexanones, decorated with appropriate substituents led to development of a series of inhibitors targeting Plm II. The biological activity of these molecules were tested against plasmeprin II, revealing an IC_{50} value of 7 μ M for the best molecule (**compound 87**, Fig. 28) [85]. Five methyleneamino-reduced peptide inhibitors were designed from the results of combinatorial approach one of which (**compound 88**, Fig. 29) displayed an IC_{50} of 13.9 nM and can be used as a starting conformation to develop novel Plm II inhibitors [37]. Various X-ray structures of Plm II inhibitors with statine moiety or derivatives were reported. Recent drug discovery research to discover easily synthesizable Plm II inhibitors revealed a highly resolved (1.6 Å) X-ray structure of Plm II with a potent achiral inhibitor (**compound 89**, Fig. 29) in an unprecedented manner, interacting with the catalytic aspartates through the catalytic water indirectly [63].

Recently, a series of novel *C*2-symmetric compounds encompassing the 1, 2-dihydroxyethylene scaffold and a variety of elongated P1/P1c side chains were synthesized via microwave-assisted palladium catalysed coupling reactions which resulted in discovery of a novel Plm II inhibitor (**compound 90**, Fig. 30) which displayed an K_i of 1.2 nM [58]. Recently, novel statine-like derivatives were synthesised using solid-phase synthesis which displayed anti-Plm II activity in nanomolar scale. Two prominent inhibitors of this series (**compound 91** and **92**, Fig. 30) showed a K_i of 2.2 nM and 4.3 nM respectively [43]. This strategy of developing novel statine-like templates with modified amino and carboxy terminus can be further pursued to develop novel set of plasmeprin inhibitors. New potent inhibitors of aspartic proteases Plm II were developed (**compound 93** and **94**, Fig. 30) by employing Suzuki coupling reactions on previously synthesized bromobenzoyloxy-substituted statine based inhibitors [44]. Further studies increased inhibitory



Compound 88

 $IC_{50}=13.9$ nM

Compound 89

Fig. 29. 2D chemical representation of some potent Plm II inhibitors, **compound 88–89**.

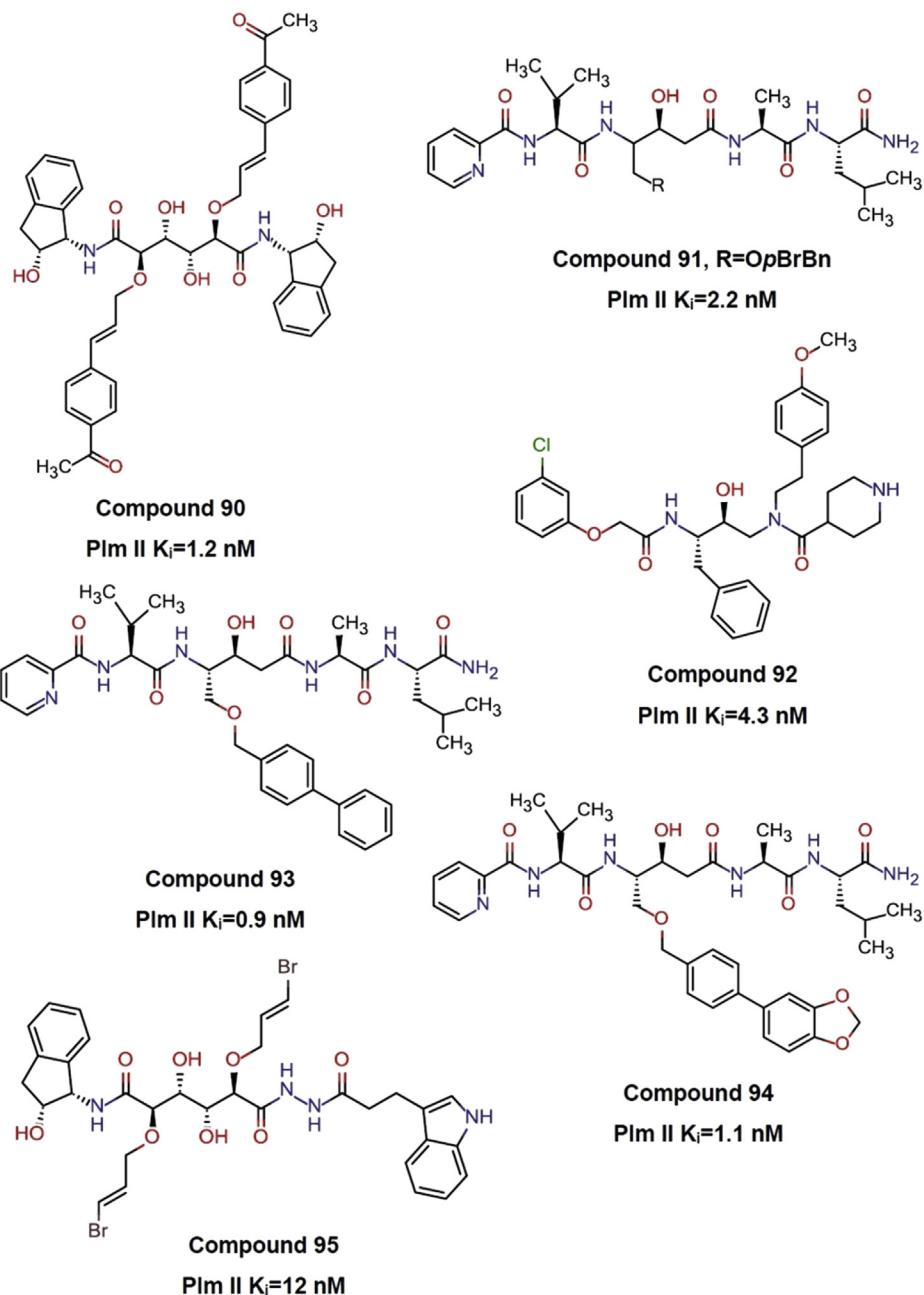


Fig. 30. Chemical representation and enzymatic activity of Plm II inhibitors, **compound 90–95**.

activity eight times by identifying that P1 substituents effectively bind to the S1–S3 cleft of Plm I and II. Various *p*-bromobenzoyloxy-substituted inhibitors of Plm I and II showed low nanomolar K_i values. Using Molecular dynamics stimulations a new series of inhibitors (**compound 95**, $K_i = 12$ nM, Fig. 30) of Plm II were designed with L-mannitol as precursor [59]. These inhibitors were distinguished by either a diacylhydrazine or a five-membered oxadiazole ring replacing backbone amide functions and also possessed good K_i values for Plm II that was in accordance with computationally predicted values. In order to develop mechanism based inhibitors of plasmepsin, peptidomimetic analogues were utilized. One of the analogue (**compound 96**, Fig. 31) was found to be irreversible inactivators of the enzyme with activity at low nanomolar range ($K_i = 35.7$ nM) [35].

From the long history of drug discovery targeting Plm II it can be

understood that throughput the years efforts has been applied in order to improve the selectivity and potency of plasmepsin inhibitors. Also efforts has been directed to apply double drug strategy by coupling the scaffold of primaquine with plasmepsin

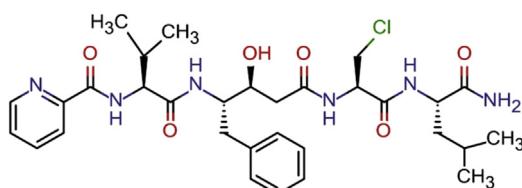


Fig. 31. Structural representation of one of the irreversible inactivators (**compound 96**) of Plm II.

inhibitors which showed admirable antimalarial activity. Despite that, a highly potent small molecule scaffold with tolerable ADME profile is still a long way to go.

4. Conclusion and future prospective

The current regimen of antimalarial therapy gets a major setback with the emergence of multi-drug resistant strains of *P. falciparum* and several other *Plasmodium* species. The ideal antimalarial therapy for future will combine efficacy against multi-drug resistant strains, preferable single dose therapy, and high oral bio-availability as well as less side effects. Till date, no plasmepsin inhibitors were emerged as a drug candidate to combat multi-drug resistant strains as well as an alternative approach of current artemisinin based treatment. The development of plasmepsin inhibitors mainly suffers from poor selectivity over Cat D, insufficient activity in parasite-infected erythrocytes, lack of tested activity on multi-drug resistant strains as well as lack of attention from pharmaceutical majors due to the neglected status of malaria. Furthermore, discovery of a subtype specific plasmepsin inhibitor will be a huge challenge which is not properly addressed till now. But development of a broad spectrum plasmepsin inhibitor or combination of plasmepsin + falcipains inhibitors or plasmepsin inhibitors in combination with other antimalarials e.g. artemisinin, primaquine etc. might be the best strategy to counter the widespread nature of malaria and emergence of drug resistant strains. Development of primaquine coupled plasmepsin inhibitor by the means of double drug approach is one of the starting leap in this regard but the size of inhibitors need to be controlled in order to develop high bioavailable drug-like candidate. Recent identification of plasmepsin V (Plm V) as one of the key drug target might see the future progress in drug discovery but due to unavailability of Plm V crystal structure rational drug design towards Plm V doesn't get much boost from the scientific community. We believe future approaches will take into account the biochemical and biophysical perspectives of different categories of plasmepsins to design broad spectrum small molecule plasmepsin inhibitors with a better ADME profile. In conclusion it can be said that looking at the current scenario the discovery of a drug candidate targeting plasmepsins is a long road to be travelled but future efforts in crystallographic, academic drug discovery, open source approach could help in this regard to travel the road faster.

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