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Achiral, Cheap, and Potent Inhibitors of Plasmepsins I, II, and IV**

Christoph Boss, Olivier Corminboeuf, Corinna Grisostomi, Solange Meyer, Andrew F. Jones, Lars Prade, Christoph Binkert, Walter Fischli, Thomas Weller, and Daniel Bur*[a]

Plasmodium species are the causative agents of malaria, infecting 300–660 million people annually and killing 1–2 million.[1] Malaria is undisputedly a disease of poverty and suffers from a lack of business-driven drug research, therefore requiring new initiatives to overcome this absence of market incentives. Low cost-of-goods for antimalarial drugs is a necessity for medical success. Increasing emergence of multiple drug-resistant parasites rapidly decreases the number of effective and cheap antimalarial agents and further aggravates the already devastating social and economic situations in many endemic areas.[2] In the search for new drugable parasite targets, a family of aspartic proteinases (plasmepsins) has been identified[3] in the digesting vacuole of *P. falciparum*.[4] Whereas initial studies have identified only three plasmepsins[5–7] a systematic search in the fully sequenced genome of *P. falciparum*[8] has expanded the family to ten members.[9] Subsequent sequence alignments and knockout studies revealed three aspartic proteinases[10] (PMI, PMII, and PMIV) and the highly related histo-aspartic proteinase HAP[11] (PMIII) located in the food vacuole. These four plasmepsins are mutually redundant[12,13] and engaged in hemoglobin degradation—a process apparently vital for the parasite.

Recently, a new class of achiral, easily synthesizable, and highly potent inhibitors of PMII[14–16] has been identified in our lab and a well resolved X-ray structure of a PMII–inhibitor **1** complex[17] has been determined.

Initial inhibitors were very selective against the human enzymes cathepsin D, E, and renin, however, they were also virtually inactive against the vacuolar PMII paralogs PMI and PMIV. Herein, structure supported design and synthesis of a new class of nonpeptidomimetic compounds is described. Compounds were obtained from a central 4-aminopiperidine with three substituents (A, B, C) (Figure 1 a) yielding cheap, achiral, and highly potent inhibitors of all three vacuolar aspartic proteinases (PMI, II, and IV).

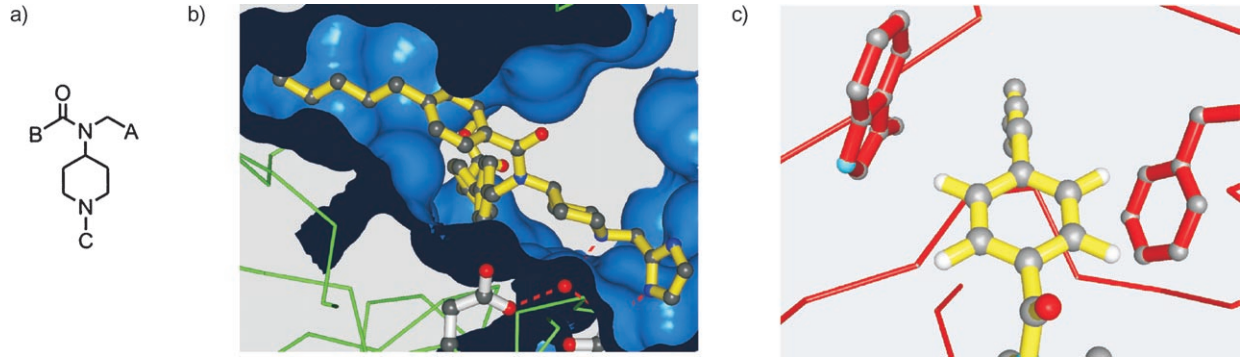

Figure 1. a) Sketch of 4-aminopiperidine template carrying substituents A and B at the exocyclic amino group and substituent C at the ring nitrogen. b) Compound **1** in the active site of PMII. c) Substituent B of **1** in edge-on-face contact with Trp 41 and Phe 111.

Table 1. Inhibition of plasmepsins I, II, and IV and *P. falciparum* (strain K1) growth in red blood cells (RBC).

Compound	IC_{50} [nM]			
	PMI[a]	PMII[a]	PMIV[a]	RBC[b]
1	655	34	10591	3494
2	n.d.[c]	14	330	1415
3	441	11	293	1784
4	n.d.	21580	n.d.	n.d.
13	629	39	1076	1445
14	123	31	253	220
15	1727	146	3038	n.d.
16	537	32	530	2148
17	n.d.	18	635	1817
18	1123	113	19440	126
19	1162	145	3368	45
22	711	54	3214	n.d.
24	1672	236	1447	n.d.
26	113	67	59	847
28	104	47	57	774
30	34	19	7	>500

[a] Values represent the average of 2–3 repetitions of a fluorogenic proteolysis assay; the estimated error for this assay is ±50%. Assay conditions: [enzyme] = 1 nM, [substrate] = 1 μM, acetate buffer (50 mM, pH 5.0), glycerol (12.5%), DMSO (10%), BSA (0.1%). [b] Values were obtained by analysis of the dose-dependent uptake of [^{3}H]hypoxanthine by cultured parasite (strain K1). [c] n.d. = Not determined.

[a] *Dr. C. Boss, Dr. O. Corminboeuf, Dr. C. Grisostomi, Dr. S. Meyer, Dr. A. F. Jones, Dr. L. Prade, Dr. C. Binkert, Dr. W. Fischli, Dr. T. Weller, Dr. D. Bur*
Drug Discovery, Chemistry & Biology,
Actelion Pharmaceuticals Ltd.
Gewerbestrasse 16, 4123 Allschwil (Switzerland)
Fax: (+41) 61-565-6500
E-mail: daniel.bur@actelion.com

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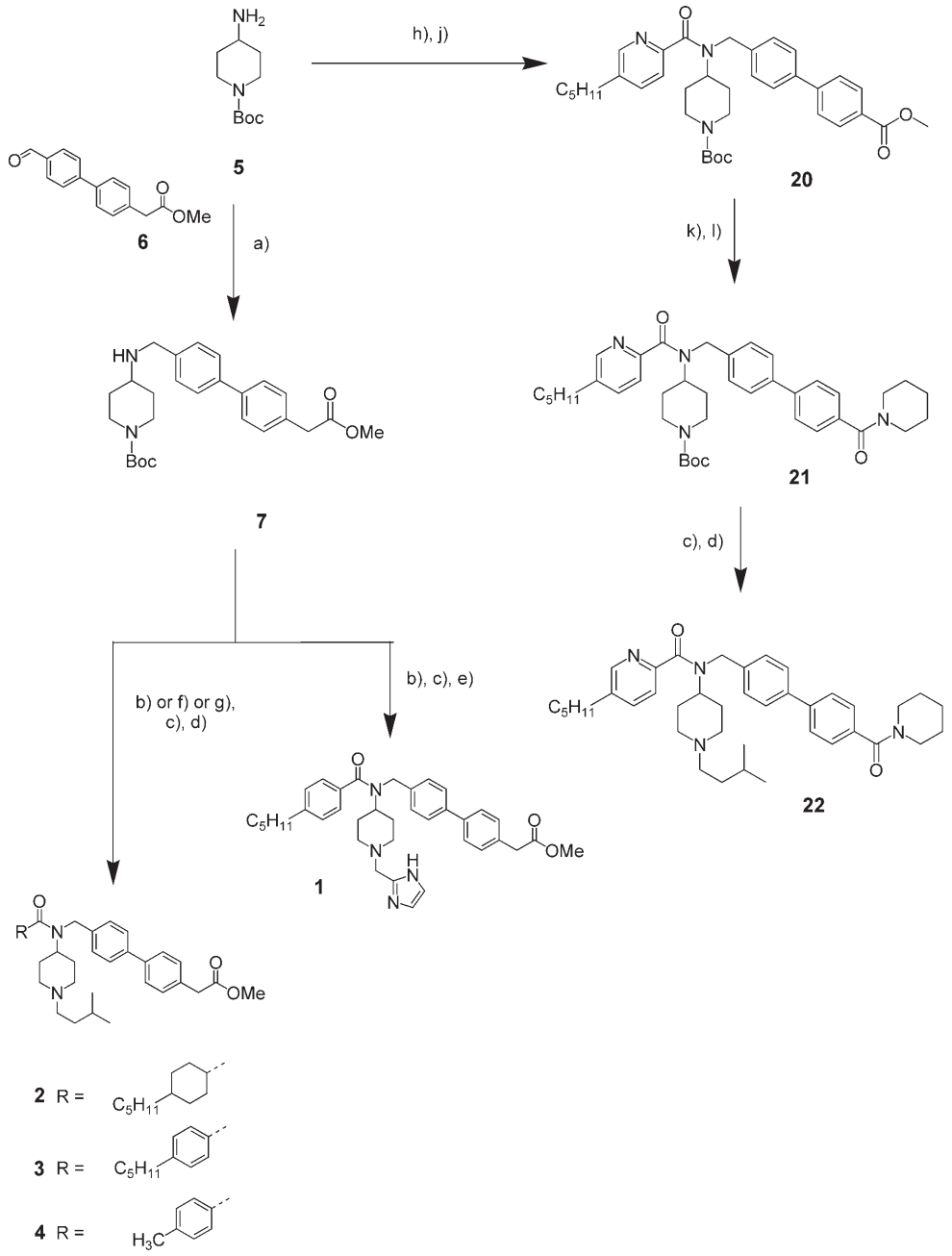
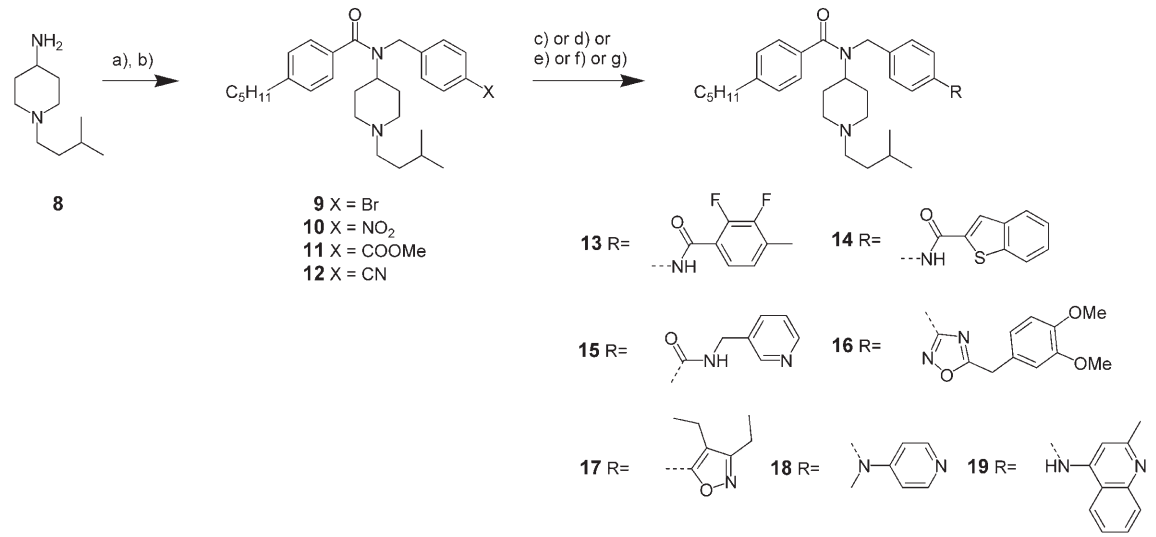


Scheme 1. Synthesis of biphenyl-containing PM inhibitors. a) 1) **6**, MeOH, reflux, 4 h; 2) $NaBH_4$, RT, 15 min. b) 4-Pentylbenzoyl chloride, CH_2Cl_2, DIPEA, RT, 4 h. c) 4 M HCl in dioxane, RT, 1 h. d) Isovaleraldehyde, $Na(OAc)_3BH$, CH_3CN, RT, 8 h. e) 2-Imidazolyl carbaldehyde, $Na(OAc)_3BH$, CH_3CN, RT, 8 h. f) 4-Methylbenzoyl chloride, CH_2Cl_2, DIPEA, RT, 4 h. g) *trans*-4-Pentylcyclohexane carboxylic acid, TBTU, DIPEA, CH_3CN, RT, 8 h. h) 1) 4′-Formylbiphenyl-4-carboxylic acid methylester, MeOH, reflux, 4 h; 2) $NaBH_4$, RT, 15 min. j) 5-Pentylpyridine-2-carboxylic acid, CH_3CN, TBTU, DIPEA, RT, 8 h. k) 2 M LiOH, MeOH/THF, RT, 8 h. l) 1) CH_2Cl_2, oxalyl chloride, DMF, RT, 2 h; 2) CH_3CN, piperidine DIPEA, RT, 8 h. Compounds **1**–**4** and **22** were parts of focused libraries and prepared by means of parallel chemistry. All final compounds were purified by preparative HPLC and analyzed by LC–MS.

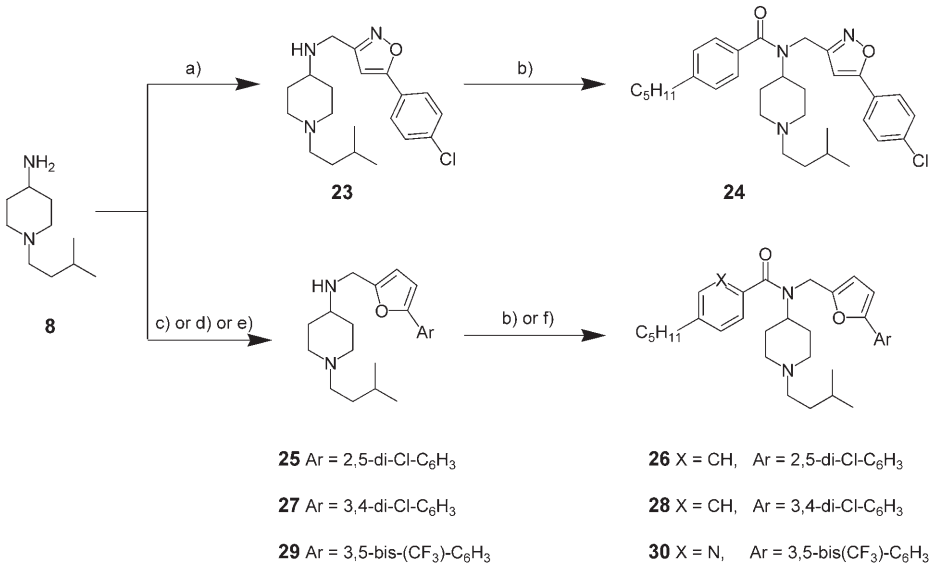
The X-ray structure of the PMII–inhibitor **1** complex revealed an amino acid side chain reorganization in PMII highly reminiscent of a conformational rearrangement previously encountered in renin–inhibitor complexes.[18] The acyl substituent (B) was almost completely buried in a newly formed hydrophobic pocket (Figure 1 b) primarily shaped by aromatic side chains. This part of the inhibitor proved to be crucial for high affinity and the best IC_{50} values are obtained for benzoic acids substituted with an *n*-pentyl chain in *para*-position. Shortening this alkyl chain, introducing unsaturated bonds or heteroatoms led to a significant loss of affinity (data not shown). A diverse set of *para*-substituted phenyl groups was tolerated as A-substituents whereas a 3-methylbutyl residue was superior to benzyl or 2-imidazomethyl groups as piperidine substituent (C).

Supported by the PMII–inhibitor **1** X-ray structure,[17] individual contributions of the central template and its three substituents have been assessed in a qualitative way. The piperidine nitrogen was engaged in a strong H-bond with the catalytic



Scheme 2. Synthesis of PM inhibitors containing biphenyl mimetics. a) 1) OHC-C_6H_4-X, MeOH, reflux, 2 h; 2) $NaBH_4$, RT, 15 min. b) 4-Pentylbenzoyl chloride, CH_2Cl_2, DIPEA, RT, 4–6 h. c) **9**, 4-(Methylamino)pyridine or 4-aminoquinaldine, SK-CC02A*, dioxane, KO*t*Bu, reflux, 8 h; for **18** and **19**. d) 1) **10**, H_2 (1 bar), Pd-C (10%) EtOH, RT, 8 h; 2) 2,3-Difluoro-4-methylbenzoyl chloride or benzo[b]thiophene-2-carbonyl chloride, DIPEA, CH_2Cl_2, RT, 6 h; for **13** and **14**. e) 1) **11**, NaOH 1 M/MeOH (1:1), RT, 4 h; 2) 4-Picolylamine, TBTU, DIPEA, CH_2Cl_2, RT, 6 h; for **15**. f) 1) BuLi, THF, hexane-3-one oxime, −78 °C, 1 h; 2) **11**, RT, 4 h; 3) HCl; for **17**. g) 1) **11**, NaOH 1 M/MeOH (1:1), RT, 4 h; 2) Hydroxylamine·HCl, $NaHCO_3$, EtOH, 90 °C, 2 h; 3) 3,4-Dimethoxy phenylacetic acid, TBTU, DIPEA, DMF, RT, 8 h; 4) 110 °C, 4 h; for **16**. Compounds **13**–**19** were parts of focused libraries and prepared by means of parallel chemistry. All final compounds were purified by preparative HPLC and analyzed by LC–MS. *[SK-CC02 A = 2-(Dimethylamino)-ferrocen-1-yl-palladium(II) chloride dinorbornylphosphine complex; Fluka 44696]

water thereby acting as an anchor and pivot for the inhibitor. The proximal part of the biphenyl moiety in substituent A was involved in hydrophobic interactions whereas the distal part was interacting only weakly with the enzyme. The buried acyl substituent (B) is rigidly linked to the central template and its aromatic ring resides in a perfect position to participate in two favorable edge-on-face contacts with the side chains of Trp41 and Phe111[19] (Figure 1 c). A search in the Cambridge Structural Database[20] (CSD) revealed a preference for a quasiperpendicular orientation of a benzene ring and an attached tertiary amide moiety (data not shown), a conformation also found in the PMII–inhibitor **1** structure ($\theta_{N-C1-C2-C3} = -105°$). Surprisingly, a cyclohexyl ring as found in **2** could equivalently replace the benzene moiety in substituent B without loss of affinity (Table 1). Upon inhibitor binding, the *n*-pentyl chain lost a significant part of its entropy as indicated by very low B values in the X-ray structure.[17] Nevertheless, this *n*-alkyl chain had a strong influence on affinity, reflected by a much stronger protein binding of the 4-pentyl substituted **3** compared to the shorter 4-methyl compound **4** (Table 1). A difference of 112 $Å^2$ in hydrophobic surface between **3** and **4** resulted in a 2000 fold weaker IC_{50} for the smaller compound **4**. The estimated difference in hydrophobic surface free enthalpy was 4.4 kcal mol^{-1} which could be converted to 36.5 cal $mol^{-1} Å^{-2}$ (Lit: 46 cal $mol^{-1} Å^{-2}$ [21]). This value might be smaller because of the



Scheme 3. a) 1) 5-(4-Chlorophenyl)isoxazole-3-carbaldehyde, MeOH, reflux, 4 h; 2) $NaBH_4$, RT, 15 min. b) 4-Pentylbenzoyl chloride, CH_2Cl_2, DIPEA, RT, 4 h. c) 1) 5-(2,5-Dichloro-phenyl)furan-2-carbaldehyde, MeOH, reflux, 4 h; 2) $NaBH_4$, RT, 15 min for **25**. d) 1) 5-(3,4-Dichlorophenyl)furan-2-carbaldehyde, MeOH, reflux, 4 h; 2) $NaBH_4$, RT, 15 min for **26**. e) 1) 5-(3,5-Bis-trifluoromethylphenyl)furan-2-carbaldehyde, MeOH, reflux, 4 h; 2) $NaBH_4$, RT, 15 min for **29**. f) 5-Pentylpyridine-2-carboxylic acid, CH_3CN, TBTU, DIPEA, RT, 8 h for **30**. Compounds **24** to **30** were parts of focused libraries and prepared by means of parallel chemistry. All final compounds were purified by preparative HPLC and analyzed by LC–MS.

neglected loss of entropy of the C_5 chain upon binding to the enzyme. Substituent C was located in the S1′ pocket with a rather wide selection of residues being tolerated.

To optimally guide the synthetic efforts, models of PMI and PMIV were built based on the structure of the PMII–inhibitor **1** complex. Comparison of the X-ray structure with these two models have revealed significant sequence and structural differences in both PMII paralogs near the S1/S3 pockets that are occupied by substituent A. The intention was to improve physicochemical properties (solubility) and to extend inhibitory power of our compounds to all three plasmepsins by 1) keeping the central 4-aminopiperidine moiety and substituent B and 2) by modifying substituents A and C to strengthen protein–inhibitor interactions.

Target compounds were prepared as outlined in Scheme 1–3. Condensing template **5** with aldehyde **6** in a reductive amination yields **7** that was acylated with four different acids (Scheme 1). Deprotection of the piperidine nitrogen and subsequent reductive amination with respective aldehydes led to **1**, **2**, **3**, and **4**.

Reductive amination of **8** with four different *para*-substituted benzaldehydes followed by acylation of the exocyclic amine with 4-pentyl-benzoyl chloride led to **9**, **10**, **11**, and **12** respectively (Scheme 2). Subsequent functional group manipulation yielded compounds **13**–**19** that differ only in the distal part of substituent A.

Precursor **20** was obtained by reductive amination of **5** followed by acylation of the secondary amine. The ester function in **20** was then converted to amide **21** that was subsequently deprotected and alkylated at the piperidine nitrogen to yield **22** (Scheme 1). Template **8** was condensed with substituted aromatic or heteroaromatic aldehydes to give **23**, **25**, **27**, and **29**, respectively, that yielded **24**, **26**, and **28** after acylation with 4-pentyl-benzoyl chloride or **30** after acylation with 5-pentyl-pyridine-2-carboxylic acid (Scheme 3).

Amides **13** and **14** and the inverse amide **15** introduced in substituent A were potent inhibitors of PMII but lack high affinity to PMI and PMIV with **14** being an exception to the rule (Table 1). Introduction of either a monosubstituted oxadiazole **16** or a disubstituted isoxazole **17** led to compounds potently inhibiting PMII and, for the first time, both paralogs in submicromolar concentrations. The two aryl-amines **18** and **19** with potentially basic functionalities for the A substituents display significantly weaker IC_{50} values.

A new X-ray structure of a PMII–**22** complex revealed a strong H-bond between the 2-pyridyl nitrogen and a water molecule (Figure 2a). Furthermore, **22** triggered a side chain rearrangement of Met15 and Tyr17 affecting size and shape of the S3 binding pocket (Figure 2b). The side chain of Met15 rotated similar to earlier findings[22–24] around χ_1 and χ_2 into a subpocket below S3 and allowed side chain rotation of Tyr17 and formation of a H-bond with **22**.

Modeling studies suggested replacement of the proximal benzyl moiety of substituent A with a CH_2-5-ring-heteroaryl unit to obtain slightly different exit vectors for the distal part of the A substituents. Initial attempts with substituted isoxazole units **24** were unsuccessful. However, switching to furan

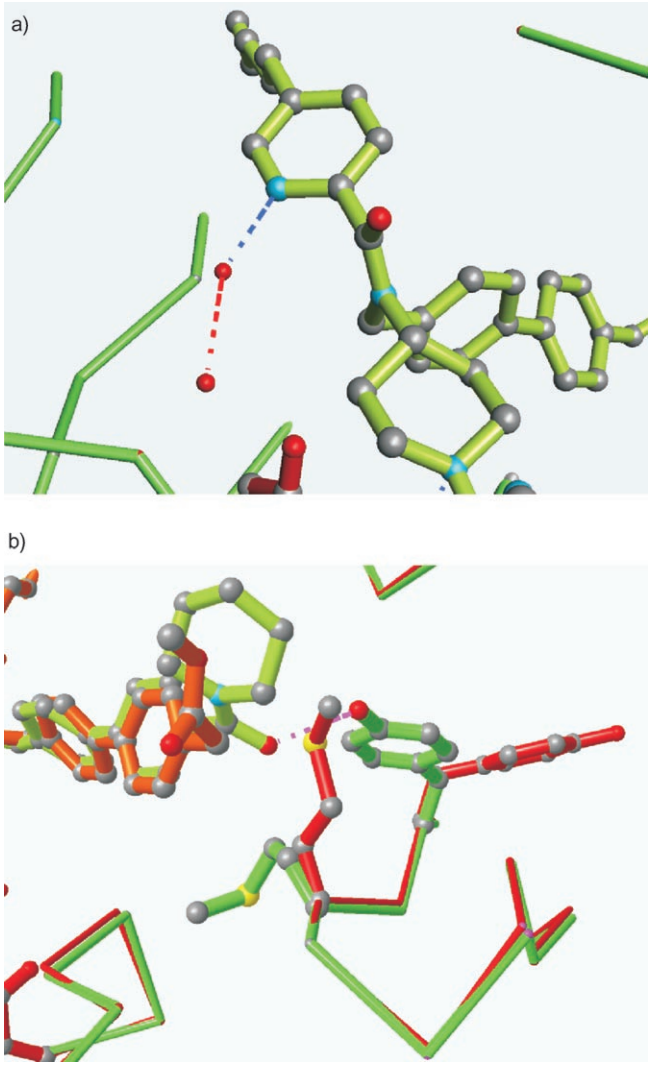


Figure 2. a) X-ray structure (1.7 Å) of PMII–compound **22** (light green, PDB: 2IGX). The pyridine nitrogen atom (cyan) is involved in a H-bond (dotted line) with a water molecule. b) Compound **22** (light green) induces a side chain rearrangement of Met15 and Tyr17 in the green PMII structure compared with the red PMII–inhibitor **1** (orange) complex.

with different ring polarity yielded potent inhibitors with IC_{50} values below 120 nM against all three plasmepsins (**26** and **28**). Even better affinities were obtained by exchanging the benzene ring in substituent B with a 2-pyridyl moiety (in **30**). Shifting the pyridine nitrogen to position 3 had a devastating effect (data not shown) due to replacement of an attractive edge-on-face contact with a repulsive electrostatic interaction (Figure 1c). None of the above described compounds showed significant activity against human enzymes renin, cathepsin D, and E with IC_{50} values >10000 nM, >1500 nM, and >3500 nM respectively.

A structurally diverse selection of compounds was tested for inhibition of parasite growth in red blood cells (RBC) infected with *P. falciparum* (strain K1, chloroquine, and pyrimethamine resistant). Good IC_{50} values were determined for compounds comprising an amine (**18**, 126 nM; **19**, 45 nM) or amide function (**14**, 220 nM) in substituent A (Table 1), however, the most

potent plasmepsin inhibitor **30** performs poorly in this assay. Compounds **18** and **19** displayed no cytotoxic effects up to concentrations of 3000 nM. An X-ray structure of a PMII–**18** complex confirms the expected binding mode of **18** (Figure 3).

In summary, the initial compound class has been modified to produce new achiral molecules potently inhibiting all three vacuolar aspartic proteinases. Structural guidance helped to identify inhibitor parts crucial for binding whereas models of PMI and PMIV supported the design of modified compounds. A new X-ray structure of a PMII–inhibitor **22** complex revealed

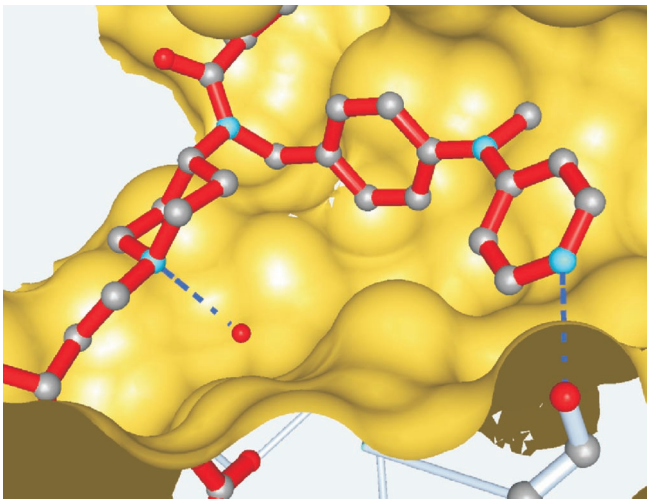


Figure 3. Compound **18** (PDB: 2IGY) located in the active site of PMII as determined by X-ray structure analysis. H-bonds are shown in dotted lines.

a second side chain rearrangement in PMII affecting size and shape of the S3 pocket. Molecules **18** and **19** were identified with up to 1000-fold more potency than pepstatin-A[25] and 100-fold more potent than de novo designed compounds[26] in a RBC assay, efficiently preventing the growth of *P. falciparum*. However, as neither of these two compounds is very potent against all three vacuolar plasmepsins the RBC potency is likely due to significant compound enrichment in the food vacuole or a considerable broad-spectrum activity against other plasmepsins (PMV, IX, and X). Current optimization of these inhibitors focuses on the improvement of in vitro and in vivo activities.

Keywords: aspartic proteases · drug design · inhibitors · malaria · medicinal chemistry

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