Title: Broad spectrum antimalarial activity of peptido sulfonyl fluorides, a new class of proteasome inhibitors

Running title: Antimalarial activity of peptido sulfonyl fluorides

Serena Tschan1, Arwin J. Brouwer2, Paul R. Werkhoven2, Anika M. Jonker2, Lena Wagner3, Sarah Knittel3, Makoah Nigel Aminake4,5, Gabriele Pradel4,5, Fanny Joanny1,3, Rob M. Liskamp2,6, and Benjamin Mordmüller1,3,#

1) University of Tübingen, Institute of Tropical Medicine, 72074 Tübingen, Germany and German Centre for Infection Research
2) Utrecht University, Medicinal Chemistry & Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, NL-3508 TB Utrecht, The Netherlands
3) Centre de Recherches Médicales de Lambaréné, BP 118, Lambaréné, Gabon
4) University of Würzburg, Research Center for Infectious Diseases, 97080 Würzburg, Germany
5) RWTH Aachen University, Institute for Molecular Biotechnology, 52074 Aachen, Germany
6) School of Chemistry, Chemical Biology & Medicinal Chemistry, University of Glasgow, Joseph Black Building, Glasgow G12 8QQ, UK
# Corresponding author:

Benjamin Mordmüller, email: benjamin.mordmueller@uni-tuebingen.de, Tel: +49 7071 2982187, Fax: +49 7071 295189
Abstract

Despite declining numbers of cases and deaths, malaria remains a major public health problem in many parts of the world. Today, case-management relies heavily on a single class of antimalarial compounds: artemisinins. Hence, development of resistance against artemisinins may destroy current malaria control strategies. Beyond malaria control are elimination and eradication programs that will require drugs with good activity against acute infection, but also preventive and transmission blocking properties. Consequently, new antimalarials are needed not only to ensure malaria control but also for elimination and eradication efforts.

In this study we introduce peptido sulfonyl fluorides (PSF) as a new class of compounds with antiplasmodial activity. We show that PSF target the plasmodial proteasome and act on all asexual stages of the intraerythrocytic cycle and on gametocytes. PSF showed activities as low as 20 nM on multi drug resistant and chloroquine sensitive *Plasmodium falciparum* laboratory strains and clinical isolates from Gabon. Structural requirements for activity were identified and cytotoxicity in human HeLa or HEK293 cells was low. The lead PSF PW28 suppressed growth of *P. berghei in vivo*, but showed signs of toxicity in mice. Considering their modular structure and broad spectrum of activity against different stages of the plasmodial life cycle, proteasome inhibitors based on PSF have a great potential for further development as pre-clinical candidate compounds with improved species-specific activity and less toxicity.
Introduction

Malaria is the most important parasitic disease causing an estimated 216 million cases and 655,000 deaths in 2010. Despite many efforts, the development of a malaria vaccine has proven to be difficult and has not led to a registered candidate so far (1). As a consequence, malaria control strongly relies on chemotherapy. In the past, *Plasmodium falciparum*, the parasite causing the most severe form of malaria, has developed resistance against almost all widely used antimalarial drugs, thereby rendering them ineffective (2). Currently, almost all first-line treatments are based on artemisinins due to the lack of other widely efficacious drugs (3). To reduce the risk of resistance against artemisinins, their use is only recommended in combination with other antimalarials (ACT, artemisinin-based combination therapy). However, reduced efficacy, suggesting the rise of resistance against artemisinins, was reported recently (4–6).

Nevertheless, malaria eradication has reappeared on international health agendas (7–10) and expert committees have identified missing knowledge and tools to achieve this goal (7). Discovery of new and improved antimalarial drugs play a key role in sustained malaria control and will be a central part of any future elimination or eradication campaigns. Ideally, new drugs should not only be tools for treatment of acute infection, but rather provide additional benefits, e.g. transmission blocking activities or very short treatment regimens (e.g. single-dose cure).

In light of these demands the search for new antimalarial drug candidates, preferably acting by mechanisms distinct from those of known antimalarials and targeting several stages of the life cycle, is of high relevance.

The 26S proteasome, a 2.5 MDa multimeric enzyme complex is responsible for most of the cellular non-lysosomal, controlled protein degradation (11, 12). It consists of a 20S core...
particle and two 19S regulatory caps. The regulatory caps are responsible for substrate
recognition, unfolding, and translocation into the 20S proteolytic core. The 20S proteasome
is a cylindrical assembly of 28 individual proteins arranged as 4 stacked rings each comprising
7 subunits. The two inner rings consists of seven different β subunits each, three of which are
catalytically active (β1, β2 and β5). Rings of seven different α subunits on each side flank the
two β subunit rings to form a barrel-like structure. According to their proteolytic mechanism,
proteasomes are classified as N-terminal nucleophilic hydrolases (Ntn-hydrolases) or
threonine-peptidases (13, 14). Many known proteasome inhibitors act by covalent
modification of the hydroxyl group of the N-terminal threonine residue, thereby abolishing
catalytic activity (15, 16). On the basis of their reactive head group they are classified as vinyl
sulfones, vinyl ketones, epoxyketones, β-lactones (all resulting in irreversible inhibition),
aldehydes, and boronic acids (reversible inhibition). Reactive headgroups are often combined
with peptide-based backbones, which can confer specificity for the proteasome and its
different catalytic subunits.

Proteasome inhibitors are an emerging class of molecules currently explored for their
potential to modify the human proteasome in various diseases including cancer (17, 18). The
plasmodial proteasome is not an extensively studied protein complex, despite its likely
important function. However, by analogy it can be assumed that it plays important roles in
regulation of cell cycle progression and probably also in other regulatory processes. Such
involvement in critical housekeeping functions would be associated with a broad spectrum of
activity of proteasome inhibitors against different stages of the plasmodial life cycle (19–21).
Indeed, proteasome inhibitors demonstrate activity against all blood stages, including rings
(22–25) and gametocytes (22, 26) as well as hepatic stages (23, 27) in different studies.
Activity against early and late blood stages is advantageous because it offers rapid parasite
clearance during infection, which is especially important in severe malaria. Gametocytocidal activity is particularly important when elimination of malaria is the goal. Most registered drugs and drug candidates have low or no activity against gametocytes. Here, we introduce the recently described peptido sulfonyl fluorides (PSF) (28–30) as a new class of proteasome inhibitors, and report on their antimalarial activity, cytotoxicity, structure–activity relationships, and selectivity.
Materials and Methods

Reagents

MG132, epoxomicin and AdaK[Bio]Ahx₃L₃VS were purchased from Calbiochem (EMD Chemicals Inc., Darmstadt, Germany). Stock solutions of all investigated compounds were prepared in DMSO (10 mM) and further diluted in complete culture medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 0.5% w/v Albumax and 50 µg/ml gentamicin) to obtain appropriate test concentrations. Anti-ubiquitin (P4D1) and anti-proteasome (MCP231) antibodies as well as horseradish peroxidase-linked streptavidin were purchased from Cell Signaling Technology, Calbiochem and Dianova (Hamburg, Germany), respectively and used according to the manufacturers’ instructions.

Parasites and drug sensitivity testing

P. falciparum strains D10, 3D7 and Dd2 were obtained from MR4 (ATCC, VA, USA) and cultured as previously described (31). Clinical P. falciparum isolates were collected at the Centre de Recherches Médicales de Lambaréné, Gabon. Inclusion criteria were uncomplicated malaria due to P. falciparum monoinfection, parasitemia between 1,000 and 200,000 parasites per microliter and no antimalarial drug intake during the preceding two weeks. Informed consent was obtained from all patients or their parents. The study received approval by the regional ethics committee (Comité d’Ethique Regional Indépendant de Lambaréné) and followed the principles of the Declaration of Helsinki (5th revision).

Ninety-six well plates were pre-dosed with drugs in three-fold serial dilutions and stored at -20 °C for no longer than 2 weeks. Venous blood was collected in lithium-heparin tubes (Sarstedt, Germany) immediately before antimalarial treatment was initiated and processed within four hours. Whole blood was centrifuged, plasma and buffy coat were removed and erythrocytes were washed once in complete culture medium.
For both, laboratory strains and clinical isolates, parasitemia and hematocrit were adjusted to 0.05 and 1.5%, respectively with non-infected O+ erythrocytes and complete culture medium. Subsequently, 200 µl of the parasite suspension were added to each well of pre-dosed 96 well plates and incubated for 72 hours in a candle jar at 37 °C. After incubation, plates were freeze-thawed twice and analyzed by histidine rich protein II (hrpII) enzyme-linked immunosorbent assay (ELISA) as described previously (32).

Cytotoxicity

HeLa and HEK 293 T cells (DSMZ, Germany) were cultured in DMEM containing 2mM L-glutamine, 10% fetal calf serum (FCS), 50 units/ml penicillin and 50 µg/ml streptomycin (5% CO₂, 37 °C). Cytotoxicity was determined using the Cytotoxicity Detection Kit Plus (LDH) (Roche, Switzerland). Cells were incubated in culture medium containing 1% FCS and MG132, epoxomicin, PSF or DMSO in 3-fold serial dilutions. After 24 hours, cytotoxicity was assessed according to the manufacturer’s instructions.

Proteasome inhibition assay

The experiment was performed as previously described (33) with minor modifications. Briefly, 3D7 parasites were synchronized by sorbitol treatment (5% w/v for 10 min at room temperature) and schizont-stage cultures were incubated with 0.5 µM epoxomicin, PW28, or equivalent amounts of DMSO for 4 h under standard culture conditions. Erythrocytes were lysed with 0.075% saponin for 5 min at room temperature and parasites were washed with ice-cold PBS until the supernatant was colorless. Parasites were lysed with buffer P (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM MgCl₂, 2 mM ATP) supplemented with 1% NP-40. Lysates were centrifuged for 10 min at 4 °C and 13,000 rpm and the supernatant (30 µg of total protein as determined by Bradford Assay) was incubated with 3 µg of biotinylated vinyl sulfone AdaK[Bio]Ahx₃L₃VS for 2 h at 37 °C. The reaction was stopped by addition of 4x SDS-sample.
loading buffer and heating to 95 °C. Samples were separated by 12% SDS-PAGE and analyzed by streptavidin Western blot.

**Enrichment of ubiquitinated proteins in PSF-treated parasites**

Synchronized, schizont-stage 3D7 parasites were incubated with 0.5 µM epoxomicin, PW28 or equivalent amounts of DMSO for 4 h under standard culture conditions. Erythrocyte lysis was done as described above except for addition of 30 mM N-ethyl-maleimide (NEM) to block de-ubiquitination. Parasites were washed with ice-cold, NEM-containing PBS (20 mM) and were lysed with high salt lysis buffer (350mM NaCl, 1mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP40, 20 mM HEPES, 20% glycerol, pH 7.9, 1 mM DTT, complete protease inhibitor cocktail (Roche)) supplemented with 30 mM NEM. Lysates were centrifuged (13,000 rpm, 4 °C, 10 min) and 20 µg of the soluble protein fraction was separated by 10% SDS-PAGE and analyzed by anti-ubiquitin Western blot. Blots were stripped with 62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM β-mercaptoethanol for 30 min at 56 °C and reprobed with anti proteasome antibody as loading control.

**Effect of PSF on different stages of the *P. falciparum* asexual cycle**

Highly synchronous 3D7 parasites were treated with 0.5 µM PW28 or an equivalent amount of DMSO. After 12 (rings), 6 (trophozoites) or 4 (schizonts) hours the drug was removed by washing and culture was continued without drugs. Viability of parasites was examined by light microscopy of Giemsa-stained thin smears every five to six hours.

**Gametocyte toxicity test**

*P. falciparum* NF54 parasites were cultured at high parasitemia to induce gametocyte formation. Upon appearance of stage II gametocytes, 1 ml of culture was aliquoted in triplicate in a 24-well plate in the presence of compounds at the respective IC₅₀ concentrations (determined in asexual drug sensitivity testing). Gametocytes were cultured...
for 7 days and the medium was replaced daily. For the first 48 h of culture, the gametocytes were treated with compounds; subsequently the medium was compound-free. After 7 days, Giemsa-stained blood smears were prepared and the gametocytemia was evaluated by counting the numbers of gametocyte stages IV and V in a total number of at least 1000 erythrocytes.

**PSF activity on different gametocyte stages**

Samples were taken from the cultures used for the gametocyte toxicity test at day 1, 3, 5, and 7, air-dried on slides and fixed for 10 min in -80 °C methanol. Subsequently, they were incubated in blocking buffer (1% neutral goat serum, 0.01% saponin and 0.5% BSA in PBS) for 30 min at room temperature. After blocking, slides were incubated for 2 h at 37 °C with mouse anti-alpha tubulin antibodies (Sigma-Aldrich, MO, USA) diluted in blocking buffer in order to stain the gametocytes. Samples were washed three times with 0.01% saponin in PBS and incubated with Alexa Fluor 488-coupled goat anti-mouse antibodies (Molecular Probes, OR, USA). Counterstaining of erythrocytes was performed using 0.05% Evans Blue in PBS for 1 min (Sigma-Aldrich). The slides were rinsed twice with PBS and finally stained with Hoechst 33342 nuclear stain (Invitrogen, CA, USA) according to the manufacturer’s protocol. After mounting a cover slip with anti-fade mounting medium (Bio-Rad, CA, USA) slides were examined under an Olympus BX41 fluorescence microscope in combination with a Jenoptik ProgRes Speed XTS camera. The presence of gametocyte stages I to V in drug-treated and DMSO-treated cultures was investigated by counting the numbers of different stages in a total number of 100 gametocytes per setting in duplicate. Digital images were processed using Adobe Photoshop CS software.

**PSF in vivo activity against P. berghei**
Clearance for animal experiments was obtained from the Regierungspräsidium Tübingen.

Female CD1 Swiss mice, 6-8 weeks old, were purchased from Charles River (Köln, Germany) and maintained in the laboratory for one week before starting the experiment. For an initial toxicity assessment three mice received a single dose of 10mg/kg of PW28 diluted in injection solution (7% Tween 80, 3% Ethanol) by intraperitoneal injection (i.p.). Mice were monitored 30 minutes and 2h post-injection and then once a day for four days for signs of toxicity. Subsequently, and after an eventual dose modification, efficacy was assessed using a 4 days suppressive test as previously described (34). Briefly, nine mice were inoculated intravenously (i.v.) with 2x10^7 P. berghei ANKA cl15cy1 (MR4, ATCC) parasitized erythrocytes obtained from a donor mouse. Two hours, 24h, 48h and 72h after inoculation, mice were injected i.p. with PW28 dilution. A control group of nine mice received an equivalent volume of the injection solution. From Day 1 to Day 4 after inoculation, daily blood smears were prepared and stained in 5% Giemsa solution after fixation in methanol. Parasitemia was determined by counting the number of asexual parasites in 1000 erythrocytes. Mice were weighted and observed for symptoms until they were sacrificed. To calculate treatment efficacy, the area under the curve (AUC) until Day 4 was calculated using the trapezoidal rule. The ratio of sums of AUCs was used to calculate antiparasitic efficacy of PW28 in percent.
Results

_In vitro_ activity of PSF against _P. falciparum_ laboratory strains and clinical isolates

A set of 47 different PSF (Figures 1 and S1) was initially tested against _P. falciparum_ 3D7 to examine antiparasitic activity. For those compounds exhibiting IC<sub>50</sub> values below 40 µM, testing was repeated and expanded to the strains D10 and Dd2 (multi drug resistant) (Table 1). Active compounds were stable over the study period except for PW39, which gradually lost activity with each freeze-thaw cycle. The three most active compounds were PW40, PW46, and PW28 with IC<sub>50</sub> values as low as 16 nM for PW28 against D10 parasites. Activity was similar against drug resistant and sensitive strains.

Five of the most active PSF (AJ32, AJ34, AJ38, AJ49, PW28) were selected for testing against _P. falciparum_ clinical isolates from Lambaréné, Gabon to assess the scatter of activities in a set of freshly isolated parasite samples (Figure 2, Table 2). Unexpectedly, we identified 13 parasite isolates with high chloroquine-sensitivity among the 44 samples. PSF were equally active against clinical isolates with heterogeneous chloroquine sensitivities and showed similar activities in clinical isolates compared to laboratory strains except for AJ34, which was more active in clinical isolates. Scatter of IC<sub>50</sub> values was comparable to that of proteasome inhibitors MG132 or epoxomicin and lower than that of antimalarial drugs chloroquine and artesunate.

Structure-activity-relationship

Three different core peptides were chosen for investigation of antimalarial activity of PSF. PheLeu, as in bortezomib, (Leu)<sub>3</sub> as in MG132 and (Ile)<sub>2</sub>ThrLeu as in epoxomicin (Figure 1). The sulfonyl fluoride moiety represents the C-terminal end of the peptide and modifications were introduced towards the N-terminal end. In case of (Ile)<sub>2</sub>ThrLeu the Thr-hydroxyl group was additionally used for variation (Figure 1, Table 1).
PSF based on peptides smaller than three amino acids exhibited no measurable in vitro antimalarial activity (data not shown, structures are given Figure S1). Elongation of peptide backbones from three to four residues led to increased activity regardless of the peptide sequence (AJ38 vs. PW28, AJ41 vs. PW38, AJ30 vs. AJ32, AJ45 vs. AJ47) (Figure 1, Table 1). Introduction of N-terminal groups enhanced antimalarial activity strongly (AJ47 vs. AJ32 and AJ34, AJ48 vs. AJ49 and PW46, PW38 vs. PW39 and PW28, AJ41 vs. AJ38 and PW25). Benzyl-protection of the Thr hydroxyl group reduced IC50 values (AJ48 vs. AJ47, AJ49 vs. AJ34) approximately 5-fold. Interestingly, introduction of an azido-group had an activity-enhancing property as well (e.g. AJ49 vs. PW46). It was also observed that in case of (Ile)2 ThrLeu an acetyl group was superior to the Boc protecting group (AJ34 vs. AJ32) while Cbz- and Boc protecting groups led to equal activities in (Leu)3 sulfonyl fluorides AJ38 and PW25. Comparison of (Leu)4 sulfonyl fluorides (PW39 vs. PW28) revealed a slightly higher activity for the Cbz group as compared to an acetyl group.

The Boc-protected tripeptide (Leu)3 (PW25) was more than 200-fold more active than the Boc- and benzyl-protected IleThrLeu sulfonyl fluoride (AJ30).

**Effect of PSF PW28 on different asexual stages**

We investigated the morphological effect of the most active PSF PW28 on different stages of the asexual cycle by exposing synchronous 3D7 parasites for a short period of time (4-12 h) to the drug. Parasites were subsequently washed to remove the drug and culture was continued. Figure 3 shows Giemsa-stained thin smears of treated parasites compared to DMSO-treated controls. PW28-treated parasites of all stages failed to develop further through the cycle and showed signs of condensation indicating that PW28 treatment results in parasite death even after short exposure.

**Activity against gametocytes**
To assess the activity of PSF against sexual erythrocytic stages, young gametocytes (stages I and II) were exposed to IC50 concentrations (asexual stages) of different PSF, primaquine (positive control), or DMSO (negative control) for two days and subsequently cultured for another five days without drug pressure (22). Out of the tested compounds, all with the exception of PW40 significantly reduced the number of mature gametocytes (p<0.05). The strongest activity was observed for AJ34 and AJ38, which were highly gametocytocidal as no or few mature gametocytes were observed (p<0.01, Figure 4). Noteworthy, the gametocytocidal activities of these two compounds were significantly higher than that of primaquine.

The numbers of gametocyte stages I-V in drug-treated samples were counted by immunofluorescence assays in order to assess which stages are susceptible. The percentages of gametocyte stages that are present in selected drug-treated cultures were determined and compared to those present in DMSO control cultures. The evaluation revealed that the potent proteasome inhibitor AJ34 acted immediately on gametocytes, which were not able to develop further (Figure 5 and S2). Noteworthy, stage I gametocytes were continuously present in the AJ34-treated cultures, indicating that the proteasome inhibitor does not interfere with gametocyte commitment. Primaquine, on the other hand, showed a slightly delayed effect and killed the gametocytes only after two days of drug pressure. The toxic effect of primaquine was best observed in stage III gametocytes, which exhibited a stress-induced spindle-like structure (Figure S2). Stage I gametocytes that were observed several days after the release of drug pressure represent a new generation of gametocytes formed as a response to drug stress. The moderate proteasome inhibitor PW46 was not able to inhibit all gametocytes at IC50 concentrations, and these cells developed into mature gametocytes within the time period of 7 days, though with a delayed maturation time (Figure 5 and S2).
Cytotoxicity against HeLa and HEK 293 T cells

Cytotoxicity of PSF AJ30, AJ32, AJ34, AJ38, AJ47, PW25, and PW28 was assessed in two human cell lines: the cervix carcinoma cell line HeLa and the non-carcinoma human embryonal kidney cell line HEK 293. None of the PSF compounds led to increased lactate dehydrogenase (LDH) release, a sign of cell damage, in neither HeLa nor HEK 293 cells in concentrations up to 500 µM (Figure 6) after 24 h of incubation. In contrast, MG132 was cytotoxic for HEK 293 cells in concentrations higher than 20 µM.

Target identification of PSF

The peptide backbones of the PSF in this study were similar to those of proteasome inhibitors like epoxomicin or MG132. Thus, to test whether the plasmodial proteasome is the target of PSF, 3D7 parasites were treated with epoxomicin, PW28, or DMSO as a control and accumulation of ubiquitinated proteins was examined by anti-ubiquitin Western blot. Figure 7 shows accumulation of ubiquitinated proteins in epoxomicin- and PW28-treated parasites as compared to the DMSO-treated control, indicating that PSF inhibit proteasomal activity.

To investigate which of the three catalytically active subunits are affected by PSF, protein extracts of parasites pre-treated with epoxomicin, PW28 or DMSO were allowed to react with the biotin-labeled vinyl sulfone AdaK[Bio]Ahx₃L₃VS. This proteasome inhibitor reacts with all three β-subunits (33, 35) and can thus be used to label subunits that have not reacted with PSF. Samples were analyzed by streptavidin Western blot shown in Figure 8. For DMSO-treated control parasites three distinct biotin-labeled bands appear on the blot and sizes correspond well to the processed β1 (29.1 kDa), β2 (25.1 kDa) and β5 (23.6 kDa) subunits. In epoxomicin- and PW28-treated parasites biotinylation of subunit β2 and β5 is inhibited, thereby demonstrating that β2 and β5 but not β1 are targeted by PW28 and epoxomicin.

Toxicity of PW28 in vivo
Since PW28 was highly active in vitro its toxicity and activity was tested in vivo in a pilot experiment. Three mice received a single i.p. injection of 10 mg/kg PW28. All mice were healthy until Day 3. On Day 4, one mouse was weak and had a rough hair coat. PW28 treatment had no effect on weight until Day 4.

**In vivo activity of PW28**

The group treated with PW28 had a reduced parasitemia compared with the control group and notably on Day 3 the mean parasitemia was 1.9% and 9.6% in the PW28 group and the control group, respectively (Table 3). The AUC was calculated until Day 3 and the antiparasitic efficacy over time of PW28 was 72%. PW28 treatment reduced activity of mice and 4 days after parasite inoculation five out of the nine mice died, most probably due to PW28-mediated toxicity.
Here we present PSF, a new class of antiplasmodial compounds. Our biochemical studies strongly suggest that the plasmodial proteasome is the target of PSF. We have demonstrated their ability to block development of gametocytes (Figures 4 and 5) and all stages of the asexual cycle of *P. falciparum* (Figure 3) even after short exposure and subsequent removal of the drug. The pattern of antiplasmodial is consistent with the fact that the proteasome is present throughout the whole asexual cycle (33) and correlates with the function of the proteasome which is a key regulator in housekeeping functions like cell cycle progression in most eukaryotes. This is an advantage over most established antimalarial drugs, which are active against distinct stages of intraerythrocytic development, only. Gametocytocidal activity is also rarely observed among established drugs and is increasingly considered important, especially for malaria elimination efforts.

Antiparasitic activity of PSF did not differ among multi drug-resistant and -sensitive laboratory strains (Table 1) as well as clinical isolates (Table 2) and IC50 values as low as 20 nM (Table 1) were observed. Toxicity against HeLa and HEK 293 cells did not increase with increasing antimalarial activity (Table 1, Figure 6). The main structural characteristic associated with activity was a minimum of three amino acids as peptide backbone, while further elongation of the peptide backbone and presence of N-terminal protecting groups led to increasing activity. Interestingly, the exact same peptide backbone present in MG132 or Z-L3VS, which is associated with equally high activity in these two compounds (around 20 nM, (36)), is 10-fold less active when combined with a sulfonyl fluoride headgroup (AJ38, ca 200 nM) instead of aldehyde or vinyl sulfone head groups. This points to a lower reactivity of sulfonyl fluorides when compared to other reactive head groups. However, while backbone elongation in vinyl sulfones seemed to lead to a decrease in activity (36), for sulfonyl...
fluorides backbone elongation led to an increasing activity. Thus, it appears that although vinyl sulfones, epoxyketones and sulfonyl fluorides all act on the same target, different compound properties are required to optimize antiplasmodial activity. The compound with the best in vitro activity, PW28, was tested in vivo against P. berghei ANKA in Swiss CD1 mice. We observed a significant reduction in parasitemia and a 72.1% antiparasitic efficacy. However, 10 mg/kg PW28 was not well tolerated in mice and further dosing schemes, animal studies and structural modifications need to be done before PSF may enter a clinical development path. As a first step, we currently analyze PSF specificity in different tissues with azido-PSFs added to cells and subsequent detection of the molecular complex between target and PSF by click chemistry, an approach that shows great potential as a research tool beyond the identification of species-specific proteasome inhibitors. With this proof-of-principle study we are adding a new class of compounds to the arsenal of antiplasmodial lead structures. Due to the modular design of PSF, large libraries might be generated easily and screened in medium- or high-throughput approaches for antiplasmodial activity and selectivity towards the plasmodial proteasome. In addition, activity against hepatic stages should be assessed in future studies.
Acknowledgments

We thank all study participants and their families. Anne-Marie Nkoma helped with in vitro drug sensitivity testing in laboratory strains and clinical isolates and the European and Developing Countries Clinical Trials Partnership supported the study on clinical isolates (project JP 2008 10800 004). MNA received a fellowship from the International Research Training Group 1522 of the Deutsche Forschungsgemeinschaft. Clemens Unger helped with the in vivo suppressive test.
Literature


9. Kappe SHI, Vaughan AM, Boddey JA, Cowman AF. 2010. That was then but this is now: malaria research in the time of an eradication agenda. Science 328:862–866.


### Table 1: IC₅₀ values of PSF against multi drug resistant and sensitive *P. falciparum* laboratory strains.

<table>
<thead>
<tr>
<th>Peptide backbone</th>
<th>Substituents</th>
<th><em>P. falciparum</em> strain</th>
<th>Compound</th>
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<tr>
<td></td>
<td></td>
<td>3D7</td>
<td>D10</td>
</tr>
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<td>(Ile)₂ThrLeu</td>
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<td>19.1</td>
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Mean IC$_{50}$s are given in nM and are calculated from at least 2 different experiments.

* Compound PW39 lost its activity, therefore values of individual experiments are given.
Table 2: Median IC\textsubscript{50} values of PSF against \textit{P. falciparum} clinical isolates from Lambaréné, Gabon.

<table>
<thead>
<tr>
<th>Compound</th>
<th>AJ32 (25 values)</th>
<th>AJ34 (33 values)</th>
<th>AJ38 (35 values)</th>
<th>AJ49 (24 values)</th>
<th>PW28 (24 values)</th>
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<tbody>
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<td>IC\textsubscript{50} [nM] Median (Range)</td>
<td>429 (98.8-1760)</td>
<td>48.6 (20.2-275)</td>
<td>163 (45.9-360)</td>
<td>908 (207-2410)</td>
<td>27.7 (8.64-81.5)</td>
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<td>Compound</td>
<td>MG132 (38 values)</td>
<td>Epoxomicin (39 values)</td>
<td>Chloroquine (38 values)</td>
<td>Artesunate (29 values)</td>
<td></td>
</tr>
<tr>
<td>IC\textsubscript{50} [nM] Median (Range)</td>
<td>50.9 (9.07-175)</td>
<td>7.72 (2.1-29.6)</td>
<td>160 (2.14-1000)</td>
<td>0.14 (0.03-1.38)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Mean parasitemia and mean weight of *P. berghei* ANKA infected mice treated with either PW28 or the injection solution.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Parameter</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PW28</td>
<td>10mg/kg in 0.3mL</td>
<td>Mean parasitemia [%] (SEM)</td>
<td>NA</td>
<td>0.6 (0.2)</td>
<td>3 (0.5)</td>
<td>9.6 (2.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean weight [g] (SEM)</td>
<td>30.9 (1.1)</td>
<td>30.1 (1.1)</td>
<td>29 (1.1)</td>
<td>28.6 (1.7)</td>
</tr>
<tr>
<td>Control</td>
<td>0.3mL</td>
<td>Mean parasitemia [%] (SEM)</td>
<td>NA</td>
<td>0.4 (0.2)</td>
<td>1 (0.4)</td>
<td>1.9 (1.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean weight [g] (SEM)</td>
<td>30.4 (1.9)</td>
<td>29.7 (1.9)</td>
<td>28.2 (1.7)</td>
<td>28.6 (1.7)</td>
</tr>
</tbody>
</table>
Legends to Figures

Figure 1: Chemical structures of peptido sulfonyl fluorides with high antiplasmodial activity. Details on synthesis and a further chemical characterization were published elsewhere (30).

Figure 2: Distribution of IC<sub>50</sub> values of selected peptido sulfonyl fluorides in clinical isolates from Lambaréné, Gabon in comparison to antimalarial drugs and proteasome inhibitors. (AQ: amodiaquine, AS: artesunate, CQ: chloroquine, DHA: dihydroartemisinin, QN: quinine, Epo: epoxomicin)

Figure 3: Giemsa stained thin smears of synchronous *P. falciparum* 3D7 either treated with DMSO (control) or PW28. Starting from a highly synchronous culture, drugs were added to ring stage parasites (4 hours post invasion), young and mature trophozoites (28h and 36h post invasion) and mature schizonts (44h post invasion). Rings were incubated for 12h, trophozoites for 6 hours and schizonts for 4h with the drugs, which were then removed by washing. Exposure times for trophozoites and schizonts were shorter than for ring stages to avoid transition into the next stage under drug pressure. Indicated time points reflect time after addition of drug. Development was monitored until control parasites had undergone reinvasion and developed into trophozoites.

Figure 4: Inhibition of gametocytes maturation. Compounds at IC<sub>50</sub> concentrations or 0.5 vol% of DMSO were added to stage II gametocyte cultures for 2 days. The numbers of stage IV and V gametocytes in a total number of 1000 erythrocytes were counted after 7 days and compared to the gametocyte numbers in the DMSO control (normalized to 100%). The graph represents results of two independent experiments carried out in triplicate (mean ± SEM). Statistical analysis was performed using one way ANOVA followed by a Tukey test (GraphPad prism 5). Asterisks represent a significant difference between tested compounds and DMSO control, where *** correspond to P < 0.001; ** correspond to 0.001 < P < 0.01; * correspond to 0.01 < P < 0.05 and for p > 0.05 the difference in not considered significant and there is no asterisk. PQ.
Figure 5: Clearance of gametocyte stages following drug treatment. Compounds at IC_{50} concentrations or 0.5 vol% of DMSO were added to stage II gametocyte cultures for 2 days. The cultures were cultivated for another 5 days after release of drug pressure. Samples were taken at day 1, 3, 5, and 7 of the assay. The numbers of gametocytes of stages I to V were counted in a total number of 100 gametocytes in the drug-treated cultures and compared to the DMSO control. Primaquine (PQ) -treated cultures were used as positive control.

Figure 6: Cytotoxicity of selected peptido sulfanyl fluorides compared to MG132, epoxomicin and DMSO against HeLa and HEK 293 cells as assessed by LDH release after 24 h of incubation. Every value represents the mean of two individual tests, which were each performed in duplicate.

Figure 7: Protein extracts of *P. falciparum* 3D7 parasites treated with DMSO, epoxomicin (Epo) or PW28 separated on a 10% SDS gel. A) anti-ubiquitin Western blot. B) anti proteasome Western blot after stripping.

Figure 8: Protein extracts of *P. falciparum* 3D7 parasites treated with DMSO, epoxomicin (Epo) or PW28 were subjected to biotin-labeling of with AdaK[Bio]Ahx3L3VS, separated on a 12% SDS gel, and analyzed by streptavidin Western blot. β1, β2, and β5 refer to the respective catalytically active proteasome subunit.