Broad-Spectrum Antimalarial Activity of Peptido Sulfonyl Fluorides, a New Class of Proteasome Inhibitors

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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 with 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 antiparasitodal 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 at concentrations as low as 20 nM against multidrug-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 HEK 293 cells was low. The lead PSF PW28 suppressed growth of *Plasmodium 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 preclinical candidate compounds with improved species-specific activity and less toxicity.

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 relies strongly 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 recommended only 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). The discovery of new and improved antimalarial drugs plays 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 also 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 nonlysosomal, 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 consist of seven different β -subunits each, three of which are catalytically active (β 1, β 2, and β 5). 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 groups, they are classified as vinyl sulfones, vinyl ketones, epoxyketones, β -lactones (all resulting in irreversible inhibition), aldehydes, and boronic acids (reversible inhibition). Reactive head groups are often combined with peptide-based backbones, which can confer specificity for the proteasome and its different catalytic subunits.

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FIG 1 Chemical structures of peptido sulfonyl fluorides with high antiplasmodial activity. Details on synthesis and further chemical characterization were reported previously (30).

Proteasome inhibitors are an emerging class of molecules currently being 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 an 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, 38) as a new class of proteasome inhibitors (30) 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 dimethyl sulfoxide (DMSO) (10 mM) and further diluted in complete culture medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 0.5% [wt/vol] Albumax, and 50 µg/ml gentamicin) to obtain appropriate test concentrations. Antiubiquitin (P4D1) and antiproteasome (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 the Malaria Research and Reference Reagent Resource Center (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* mono-infection, parasitemia levels of between 1,000 and 200,000 parasites per µl, and no antimalarial drug intake during the preceding 2 weeks. Informed consent was obtained from all patients or their parents. The study received approval by the regional ethics committee (Comité d'Ethique Régional Indépendant de Lambaréné) and followed the principles of the Declaration of Helsinki (fifth revision).

Ninety-six-well plates were predosed with drugs in 3-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 4 h. 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 levels were adjusted to 0.05 and 1.5%, respectively, with noninfected O⁺ erythrocytes and complete culture medium. Subsequently, 200 µl of the parasite suspension was added to each well of predosed 96-well plates and incubated for 72 h in a candle jar at 37°C. After incubation, plates were freeze-thawed twice and analyzed by a 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 Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 10% fetal calf serum (FCS), 50 units/ml penicillin, and 50 µg/ml streptomycin (in 5% CO₂ at 37°C). Cytotoxicity was determined by using the Cytotoxicity Detection Kit Plus (lactate dehydrogenase [LDH]) (Roche, Switzerland). Cells were incubated in culture medium

TABLE 1 IC₅₀s of PSF against multidrug-resistant and sensitive *P. falciparum* laboratory strains^a

Peptide backbone	Substituent(s)	IC ₅₀ (nM) against <i>P. falciparum</i>			Compound
		3D7	D10	Dd2	
(Ile) ₂ ThrLeu	R ¹ =Boc, R ² =Bn	712	378	408	AJ32
	R ¹ =Ac, R ² =H	966	605	668	AJ49
	R ¹ =H, R ² =Bn	3,687	4,150		AJ47
	R ¹ =H, R ² =H	>40,000			AJ48
	R ¹ =Ac, R ² =Bn	355	247	228	AJ34
	R ¹ =N ₃ Ac, R ² =Ac	63.5	37.8	109	PW40
	R ¹ =N ₃ Ac, R ² =H	75.0	22.9	85.1	PW46
(Leu) ₃	R ¹ =Cbz	308	200	167	AJ38
	R ¹ =Boc	284	208	215	PW25
	R ¹ =H	>40,000			AJ41
	R ¹ =PheN ₃	337	101	362	PW35
(Leu) ₄	R ¹ =H	9,600	11,870	11,800	PW38
	R ¹ =Ac	25	88.7	198	PW39 ^b
		176	1,670	1,900	
		483	>5,500	2,600	
	R ¹ =Cbz	19.1	16.2	20.2	PW28

^a Mean IC₅₀s were calculated from at least 2 different experiments.

^b Compound PW39 lost its activity; therefore, values from individual experiments are given.

FIG 2 Distribution of IC_{50} s of selected peptido sulfonil 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.

containing 1% FCS and MG132, epoxomicin, PSF, or DMSO in 3-fold serial dilutions. After 24 h, cytotoxicity was assessed according to the manufacturer's instructions.

Proteasome inhibition assay. The proteasome inhibition assay was performed as previously described (33), with minor modifications. Briefly, 3D7 parasites were synchronized by sorbitol treatment (5% [wt/vol] for 10 min at room temperature), and schizont-stage cultures were incubated with 0.5 μ M 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 phosphate-buffered saline (PBS) until the supernatant was colorless. Parasites were lysed with buffer P (50 mM Tris [pH 7.4], 1 mM dithiothreitol [DTT], 5 mM $MgCl_2$, 2 mM ATP) supplemented with 1% NP-40. Lysates were centrifuged for 10 min at 4°C at 13,000 rpm, and the supernatant (30 μ g of total protein, as determined by a Bradford assay [34]) was incubated with 3 μ g of biotinylated vinyl sulfone AdaK[Bio]Ahx₃L₃VS for 2 h at 37°C. The reaction was stopped by addition of 4× SDS sample loading buffer to the mixture and heating to 95°C. Samples were separated by 12% SDS-PAGE and analyzed by streptavidin Western blotting.

Enrichment of ubiquitinated proteins in PSF-treated parasites. Synchronized, schizont-stage 3D7 parasites were incubated with 0.5 μ M epoxomicin, PW28, or equivalent amounts of DMSO for 4 h under standard culture conditions. Erythrocyte lysis was done as described above, except for the addition of 30 mM *N*-ethyl-maleimide (NEM) to block deubiquitination. Parasites were washed with ice-cold, NEM-containing PBS (20 mM) and were lysed with high-salt lysis buffer (350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 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 at 4°C for 10 min), and 20 μ g of the soluble protein fraction was separated by 10% SDS-PAGE and analyzed by antiubiquitin Western blotting. Blots were stripped with a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β -mercaptoethanol for 30 min at 56°C and reprobed with antiproteasome antibody as a loading control.

Effects of PSF on different stages of the *P. falciparum* asexual cycle. Highly synchronous 3D7 parasites were treated with 0.5 μ M PW28 or an equivalent amount of DMSO. After 12 h (rings), 6 h (trophozoites), or 4 h (schizonts), 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 5 to 6 h.

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 into a 24-well plate in the presence of compounds at the respective 50% inhibitory concentrations (IC_{50} s) (determined by 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 gametocytemia was evaluated by counting the numbers of stage IV and V gametocytes in a total number of at least 1,000 erythrocytes.

PSF activity in different gametocyte stages. Samples were taken from the cultures used for the gametocyte toxicity test at days 1, 3, 5, and 7; air dried on slides; and fixed for 10 min in methanol at −80°C. Subsequently, the samples were incubated in blocking buffer (1% neutral goat serum, 0.01% saponin, and 0.5% bovine serum albumin [BSA] in PBS) for 30 min at room temperature. After blocking, slides were incubated for 2 h at 37°C with mouse anti- α -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,

TABLE 2 Median IC_{50} s of PSF against *P. falciparum* clinical isolates from Lambaréné, Gabon

Compound	Median IC_{50} (nM) (range)
AJ32 (25 values)	429 (98.8–1,760)
AJ34 (33 values)	48.6 (20.2–275)
AJ38 (35 values)	163 (45.9–360)
AJ49 (24 values)	908 (207–2,410)
PW28 (24 values)	27.7 (8.64–81.5)
MG132 (38 values)	50.9 (9.07–175)
Epoxomicin (39 values)	7.72 (2.1–29.6)
Chloroquine (38 values)	160 (2.14–1,000)
Artesunate (29 values)	0.14 (0.03–1.38)

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

OR, USA). Counterstaining of erythrocytes was performed by 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 coverslip with antifade mounting medium (Bio-Rad, CA, USA), slides were examined under an Olympus BX41 fluorescence microscope in combination with a Jenoptik ProgRes Speed XT5 camera. The presence of stage I to V gametocytes in drug-treated and DMSO-treated cultures was investigated by counting the numbers of gametocytes at different stages in a total number of 100 gametocytes per setting, in duplicate. Digital images were processed by 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 to 8 weeks old, were purchased from Charles River (Köln, Germany) and maintained in the laboratory for 1 week before the start of the experiment. For an initial toxicity assessment, three mice received a single dose of 10 mg/kg of body weight of PW28 diluted in injection solution (7% Tween 80, 3% ethanol) by intraperitoneal (i.p.) injection. Mice were monitored 30 min and 2 h postinjection and then once a day for 4 days for signs of toxicity. Subsequently, and after an eventual dose modification, efficacy was assessed by using a 4-day suppressive test, as previously described (35). Briefly, nine mice were inoculated intravenously (i.v.) with 2×10^7 *Plasmodium berghei* ANKA cl15cy1 (MR4,

ATCC) parasitized erythrocytes obtained from a donor mouse. At 2, 24, 48, and 72 h after inoculation, mice were injected i.p. with the 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 1,000 erythrocytes. Mice were weighed and observed for symptoms until they were sacrificed. To calculate treatment efficacy, the area under the curve (AUC) until day 4 was calculated by using the trapezoidal rule. The ratio of sums of AUCs was used to calculate the percent antiparasitic efficacy of PW28.

RESULTS

***In vitro* activity of PSF against *P. falciparum* laboratory strains and clinical isolates.** A set of 47 different PSF (Fig. 1; see also Fig. S1 in the supplemental material) was initially tested against *P. falciparum* 3D7 to examine antiparasitic activity. For those compounds exhibiting IC_{50} s below 40 μ M, testing was repeated and expanded to strains D10 and Dd2 (multidrug 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,

FIG 4 Inhibition of gametocyte maturation. Compounds at IC_{50} s or 0.5% (vol/vol) of DMSO was added to stage II gametocyte cultures for 2 days. The numbers of stage IV and V gametocytes in a total number of 1,000 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 $\pm$ standard error of the mean). Statistical analysis was performed by using one-way analysis of variance followed by a Tukey test (GraphPad Prism 5). Asterisks represent a significant difference between tested compounds and the DMSO control, where *** corresponds to a P value of <0.001 , ** corresponds to $0.001 < P < 0.01$, and * corresponds to $0.01 < P < 0.05$, and for a P value of >0.05 , the difference is not considered significant, and there is no asterisk. PQ, primaquine.

with IC_{50} s as low as 16 nM for PW28 against D10 parasites. Activities were similar against drug-resistant and -sensitive strains.

Five of the most active PSF (AJ32, AJ34, AJ38, AJ49, and 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 (Fig. 2 and Table 2). Unexpectedly, we identified 13 chloroquine-sensitive parasite isolates among the 44 samples. PSF were equally active against clinical isolates with heterogeneous chloroquine sensitivities and showed activities against clinical isolates similar to those against laboratory strains, except for AJ34, which was more active against clinical isolates. The scatter of IC_{50} s was comparable to that of the

proteasome inhibitors MG132 and epoxomicin and lower than that of the 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)₃, as in MG132; and (Ile)₂ThrLeu, as in epoxomicin (Fig. 1). The sulfonyl fluoride moiety represents the C-terminal end of the peptide, and modifications were introduced toward the N-terminal end. In the case of (Ile)₂ThrLeu, the Thr-hydroxyl group was additionally used for variation (Fig. 1 and Table 1).

PSF based on peptides smaller than 3 amino acids exhibited no measurable *in vitro* antimalarial activity (data not shown; structures are given in Fig. S1 in the supplemental material). Elongation of peptide backbones from 3 to 4 residues led to increased activity regardless of the peptide sequence (AJ38 versus PW28, AJ41 versus PW38, AJ30 versus AJ32, and AJ45 versus AJ47) (Fig. 1 and Table 1). Introduction of N-terminal groups enhanced antimalarial activity strongly (AJ47 versus AJ32 and AJ34, AJ48 versus AJ49 and PW46, PW38 versus PW39 and PW28, and AJ41 versus AJ38 and PW25). Benzyl protection of the Thr-hydroxyl group reduced the IC_{50} s (AJ48 versus AJ47 and AJ49 versus AJ34) approximately 5-fold. Interestingly, introduction of an azido group had an activity-enhancing property as well (e.g., AJ49 versus PW46). It was also observed that in the case of (Ile)₂ThrLeu, an acetyl group was superior to the *t*-butoxycarbonyl (BOC)-protecting group (AJ34 versus AJ32), while benzyloxycarbonyl (Cbz)- and Boc-protecting groups led to equal activities in (Leu)₃ sulfonyl fluorides AJ38 and PW25. Comparison of (Leu)₄ sulfonyl fluorides (PW39 versus PW28) revealed a slightly higher activity for the Cbz group than for 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).

Effects of PSF PW28 on different asexual stages. We investigated the morphological effects of the most active PSF, PW28, on different stages of the asexual cycle by exposing synchronous 3D7 parasites to the drug for a short period of time (4 to 12 h). Parasites

FIG 5 Clearance of gametocyte stages following drug treatment. Compounds at IC_{50} s or 0.5% (vol/vol) of DMSO was 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 days 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 numbers of gametocytes in the DMSO control. Primaquine (PQ)-treated cultures were used as positive controls.

FIG 6 Cytotoxicity of selected peptido sulfonyl 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.

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 activities of PSF against sexual erythrocytic stages, young gametocytes (stages I and II) were exposed to IC_{50} s (asexual stages) of different PSF, primaquine (positive control), or DMSO (negative control) for 2 days and subsequently cultured for another 5 days without drug pressure (22). Of the tested compounds, all with the exception of PW40 significantly reduced the number of mature gametocytes ($P < 0.05$). The strongest activities were observed for AJ34 and AJ38, which were highly gametocytocidal, as no or few mature gametocytes were observed ($P < 0.01$) (Fig. 4). Of note, the gametocytocidal activities of these two compounds were significantly higher than that of primaquine.

The numbers of stage I to V gametocytes 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 (Fig. 5; see also Fig. S2 in the supplemental material). Of note, 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 2 days of drug pressure. The toxic effect of primaquine was best observed in stage III gametocytes, which exhibited a stress-induced spindle-like structure (see Fig. S2 in the supplemental material). The 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 IC_{50} s, and these cells developed into mature gametocytes within a time period of 7 days but with a delayed maturation time (Fig. 5; see also Fig. S2 in the supplemental material).

Cytotoxicity against HeLa and HEK 293 T cells. Cytotoxicities of the PSF AJ30, AJ32, AJ34, AJ38, AJ47, PW25, and PW28 were assessed in two human cell lines: the cervix carcinoma cell line HeLa and the noncarcinoma 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 either HeLa or HEK 293 cells at concentrations of up to 500 μ M (Fig. 6) after 24 h of incubation. In contrast, MG132 was cytotoxic for HEK 293 cells at concentrations higher than 20 μ M.

Target identification of PSF. The peptide backbones of the PSF in this study were similar to those of proteasome inhibitors such as epoxomicin or MG132. Thus, to test whether the plasmodial proteasome is the target of PSF, 3D7 parasites were treated

FIG 7 Protein extracts of *P. falciparum* 3D7 parasites treated with DMSO, epoxomicin (Epo), or PW28 separated on a 10% SDS gel. (A) Antiubiquitin Western blot; (B) antiproteasome Western blot after stripping.

with epoxomicin, PW28, or DMSO as a control, and accumulation of ubiquitinated proteins was examined by antiubiquitin Western blotting. Figure 7 shows accumulation of ubiquitinated proteins in epoxomicin- and PW28-treated parasites 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 pretreated 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, 36) and can thus be used to label subunits that have not reacted with PSF. Samples were analyzed by streptavidin Western blotting, as shown in Fig. 8. For DMSO-treated control parasites, three distinct biotin-labeled

FIG 8 Protein extracts of *P. falciparum* 3D7 parasites treated with DMSO, epoxomicin (Epo), or PW28 were subjected to biotin labeling with AdaK[Bio]Ahx₃L₃VS, separated on a 12% SDS gel, and analyzed by streptavidin Western blotting. β 1, β 2, and β 5 refer to the respective catalytically active proteasome subunits.

TABLE 3 Mean parasitemias and mean weights of *P. berghei* ANKA-infected mice treated with either PW28 or the injection solution^a

Day	Control treatment at a dose of 10 mg/kg in 0.3 ml		PW28 treatment at a dose of 0.3 ml	
	Mean parasitemia (%) (SEM)	Mean wt (g) (SEM)	Mean parasitemia (%) (SEM)	Mean wt (g) (SEM)
0	NA	30.9 (1.1)	NA	30.4 (1.9)
1	0.6 (0.2)	30.1 (1.1)	0.4 (0.2)	29.7 (1.9)
2	3 (0.5)	29 (1.1)	1 (0.4)	28.2 (1.7)
3	9.6 (2.5)	28.6 (1.7)	1.9 (1.4)	28.6 (1.7)

^a NA, not applicable.

bands appeared on the blot, and the sizes correspond well to the processed β 1 (29.1-kDa), β 2 (25.1-kDa), and β 5 (23.6-kDa) subunits. In epoxomicin- and PW28-treated parasites, biotinylation of subunits β 2 and β 5 is inhibited, thereby demonstrating that β 2 and β 5 but not β 1 are targeted by PW28 and epoxomicin.

Toxicity of PW28 *in vivo*. Since PW28 was highly active *in vitro*, its toxicity and activity were 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 reduced parasitemia compared with that of the control group, and notably, on day 3, the mean parasitemias were 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 of PW28 over time was 72%. PW28 treatment reduced the activity of mice, and 4 days after parasite inoculation, five out of the nine mice died, most probably due to PW28-mediated toxicity.

DISCUSSION

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 (Fig. 4 and 5) and all stages of the asexual cycle of *P. falciparum* (Fig. 3), even after short exposure and subsequent removal of the drug. The pattern of the antiplasmodial compounds 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, such as 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 multidrug-resistant and -sensitive laboratory strains (Table 1) as well as clinical isolates (Table 2), and IC₅₀s 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 and Fig. 6). The main structural characteristic associated with activity was a minimum of 3 amino acids as the peptide backbone, while further elongation of the peptide backbone and the 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 [37]), is 10-fold less active when combined with a sulfonyl fluoride head group (AJ38, ca. 200 nM) instead of aldehyde or vinyl sulfone head groups. This points to a lower reactivity of sulfonyl fluorides than of other reactive head groups. However, while backbone elongation in vinyl sulfones seemed to lead to a decrease in activity (37), for sulfonyl fluorides, backbone elongation led to 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 antiparasitodal 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% 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 are currently analyzing PSF specificity in different tissues with azido-PSF 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 antiparasitodal lead structures. Due to the modular design of PSF, large libraries might be generated easily and screened by medium- or high-throughput approaches for antiparasitodal activity and selectivity toward the plasmodial proteasome. In addition, activity against hepatic stages should be assessed in future studies.

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