View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Radboud Repository

Radboud Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/52151

Please be advised that this information was generated on 2017-12-06 and may be subject to change.

Antimicrobial Agents and Chemotherapy

Inhibition of *Plasmodium falciparum* Oocyst Production by Membrane-Permeant Cysteine Protease Inhibitor E64d

S. Eksi, B. Czesny, G.-J. van Gemert, R. W. Sauerwein, W. Eling and K. C. Williamson
***Antimicrob. Agents Chemother.* 2007, 51(3):1064. DOI: 10.1128/AAC.01012-06.**
Published Ahead of Print 18 December 2006.

Updated information and services can be found at:
http://aac.asm.org/content/51/3/1064

These include:

REFERENCES

This article cites 39 articles, 13 of which can be accessed free at: http://aac.asm.org/content/51/3/1064#ref-list-1

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://journals.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2007, p. 1064–1070 Vol. 51, No. 3
0066-4804/07/$08.00+0 doi:10.1128/AAC.01012-06
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Inhibition of *Plasmodium falciparum* Oocyst Production by Membrane-Permeant Cysteine Protease Inhibitor E64d[▽]

S. Eksi,[1] B. Czesny,[1] G.-J. van Gemert,[2] R. W. Sauerwein,[2] W. Eling,[2] and K. C. Williamson[1]*

Department of Biology, Loyola University Chicago, Chicago, Illinois,[1] and Department of Medical Microbiology, University of Nijmegen, Nijmegen, The Netherlands[2]

Received 13 August 2006/Returned for modification 24 September 2006/Accepted 6 December 2006

During asexual intraerythrocytic growth, *Plasmodium falciparum* utilizes hemoglobin obtained from the host red blood cell (RBC) as a nutrient source. Papain-like cysteine proteases, falcipains 2 and 3, have been reported to be involved in hemoglobin digestion and are targets of current antimalarial drug development efforts. However, their expression during gametocytogenesis, which is required for malaria parasite transmission, has not been studied. Many of the available antimalarials do not inhibit development of sexual stage parasites, and therefore, the persistence of gametocytes after drug treatment allows continued transmission of the disease. In the work reported here, incubation of stage V gametocytes with membrane-permeant cysteine protease inhibitor E64d significantly inhibited oocyst production (80 to 100%). The same conditions inhibited processing of gametocyte-surface antigen Pfs230 during gametogenesis but did not alter the morphology of the food vacuole in gametocytes, inhibit emergence, or block male exflagellation. E64d reduced the level of oocyst production more effectively than that reported previously for falcipain 1-knockout parasites, suggesting that falcipains 2 and 3 may also be involved in malaria parasite transmission. However, in this study only falcipain 3 and not falcipain 2 was found to be expressed in stage V gametocytes. Interestingly, during gametocytogenesis falcipain 3 was transported into the red blood cell and by stage V was localized in vesicles along the RBC surface, consistent with a role during gamete emergence. The ability of a membrane-permeant cysteine protease inhibitor to significantly reduce malaria parasite transmission suggests that future drug design should include evaluation of gametogenesis and sporogonic development.

The clinical symptoms of malaria are caused by asexually replicating parasites, but malaria parasite transmission requires that a subpopulation of parasites undergo sexual differentiation. In the species of parasite responsible for the most virulent form of malaria, *Plasmodium falciparum*, complete maturation into mature stage V gametocytes takes 10 to 12 days in the human host. Once stage V gametocytes are taken up by a mosquito, gametogenesis is stimulated and, after fertilization, the zygote differentiates into an ookinete. The ookinete then migrates to the basal surface of the midgut and forms an oocyst, where tens of thousands of infectious sporozoites are produced.

Both asexual and sexual stage parasites are present concurrently in an infected individual and therefore would both be exposed to antimalarial drugs. However, gametocytes have been found to be resistant to many of the commonly used antimalarials, such as the 4-aminoquinolines; and sulfadoxine-pyrimethamine has been reported to increase the production of gametocytes, which could enhance transmission (2, 12, 21, 36). Consequently, even after successful treatment for clinical symptoms, an individual can still transmit the malaria parasite for at least a week. Therefore, it is important to develop therapeutic agents that effectively inhibit both asexual and sexual stage parasites.

Cysteine proteases are currently being developed as drugs that target asexual parasites (27). Treatment of trophozoites with specific cysteine protease inhibitors, such as E64, peptidyl fluoromethyl ketones (FMKs), and peptidyl vinyl sulfones (VSs), block hemoglobin digestion, causing distension of the food vacuole and inhibition of schizont production (25, 30, 34). Cysteine protease inhibitors have also been shown to interfere with, but not completely block, merozoite release and to inhibit *P. berghei* and *P. falciparum* sporozoite invasion (4, 28, 37). Membrane-permeant E64d has also been shown to inhibit the processing of the 360-kDa *P. falciparum* gametocyte surface antigen Pfs230 to 35-kDa and 300-kDa fragments during gametogenesis, while the production of the 47-kDa and the 307-kDa fragments is not affected. When the parasite emerges from the red blood cell (RBC) as a gamete in the mosquito midgut, 35-kDa and 47-kDa sections of the first 555 amino acids (aa) of Pfs230, which includes the immunodominant glutamate-rich repeat domains, are released as soluble fragments (3, 24). In contrast, the 300-kDa and 307-kDa fragments remain associated with the gamete surface and are the targets of malaria parasite transmission-blocking monoclonal antibodies (3, 38).

Four papain-like cysteine proteases have been identified in the *P. falciparum* genome and have been named falcipains 1, 2A, 2B, and 3 (18, 27). The gene for falcipain 1 was cloned first, and although it is expressed in asexual parasites, mRNA levels have been found to increase during sexual differentiation (8, 26). Indeed, targeted gene disruption does not affect asexual growth but significantly decreases oocyst production (8). Falcipains 2A and 2B share 97% amino acid identity and therefore are difficult to differentiate with immunological reagents (18). By using specific oligonucleotide probes, falcipain 2B

* Corresponding author. Mailing address: Department of Biology, Quinlan Life Sciences Building, Rm 317, 6525 N. Sheridan Rd., Loyola University Chicago, Chicago, IL 60626. Phone: (773) 508-3631. Fax: (773) 508-3646. E-mail: kwilli4@luc.edu.

▽ Published ahead of print on 18 December 2006.

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek

mRNA was found to be expressed at a lower level than falcipain 2A. However, falcipains 2A and 2B have similar time courses of expression and their expression peaks at the late trophozoite stage, which suggests that the two falcipain 2 genes may have similar roles (35). Falcipains 2A and 3 have been proposed to be involved in hemoglobin digestion, since they have been located in the food vacuole and the corresponding recombinant proteins have acidic pH maxima and can degrade denatured hemoglobin (31, 34). Additionally, targeted disruption of the falcipain 2A gene was recently reported to cause distention of the food vacuole in trophozoites (33). This phenotype is similar to that induced by treatment with cysteine protease inhibitor E64. The disruption of falcipain 2B had no detectable effect on the food vacuole or asexual growth (32). In contrast, the disruption of falcipain 3 has not been reported, although it has been attempted by our group and other investigators (32). This could indicate that falcipain 3 is essential for asexual growth, as would be expected for a gene that is required for the digestion of hemoglobin, which is a major source of nutrition for asexual parasites and very early gametocytes.

Although the protein expression patterns of falcipain 2A, 2B, and 3 have been well studied in asexual parasites, they have not been characterized in gametocytes. It has been reported that the rate of hemoglobin digestion decreases after day 4 of gametocytogenesis, which corresponds to early stage II, and that hemoglobin digestion may not be necessary for further sporogonic development (14). The work reported here found that falcipain 3 and not falcipain 2A or 2B was expressed throughout gametocytogenesis and was exported into the RBC during gametocyte maturation. Protease inhibitors were then used to evaluate the role of cysteine proteases in malaria parasite transmission and their potential as drug targets.

MATERIALS AND METHODS

***P. falciparum* parasites.** *P. falciparum* strain 3D7 parasites were maintained in culture, and gametocytogenesis was induced as described by Ifediba and Vanderberg (13). Cultures containing mature stage V gametocytes were incubated at 37°C in complete RPMI 1640 (Invitrogen, Carlsbad, CA) for the indicated time with or without protease inhibitors, 100 μM E64d (Sigma, St Louis, MO), 10 μM YA29 (generously provided by M. Bogyo and D. Greenbaum) (10), and 10 μM morpholine urea-leucine-homophenylalanine-phenolvinylsulfone (Mu-Leu-hPhe-VSPh) and 10 μM morpholine urea-leucine-homophenylalanine-fluoromethylketone (Mu-Leu-hPhe-FMK) (generously provided by P. Rosenthal) (29). Gamete/zygote production and exflagellation were induced by resuspending the mature stage V gametocytes in an equal volume of emergence medium (10 μM xanthurenic acid, 1.67 mg ml^{-1} glucose, 8 mg ml^{-1} NaCl, 8 mM Tris-Cl [pH 8.2]) at room temperature (RT). After 1 h the medium and cell pellet were harvested separately by centrifugation at 2,000 × *g* for 3 min. The medium was further clarified by centrifugation at 14,000 × *g* for 10 min. The samples were stored frozen until they were assayed for Pfs230 processing by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (3).

Mosquito feeding assay. Cultures of *P. falciparum* parasites comprising isolate NF54 (Table 1) or; strain 3D7 (Table 1 [see also Fig. 4A]) and containing mature stage V gametocytes incubated with or without protease inhibitors (0.3 ml) were pelleted onto 150 μl packed erythrocytes (19). The supernatant was replaced with 120 μl of normal human serum containing active complement, mixed, and introduced into a water-jacketed membrane feeder maintained at 39°C (20). *Anopheles stephensi* (strain SxK Nij) mosquitoes were allowed to gorge for 10 min and were then grown for 7 more days at 26 ± 1°C and >80% ± 10% humidity. The midguts were then dissected and stained in 1.0% mercurochrome to visualize the *P. falciparum* oocysts (magnification, ×100 to ×200). The 3D7 feedings were performed at Loyola University Chicago, and the NF54 feedings were performed at the University of Nijmegen.

Antibodies. Falcipain 2A/B-specific antibodies were generously provided by P. Rosenthal (34), and glycophorin A-specific antibodies were obtained from Sigma. Pfs230 region A (aa 304 to 378)-specific antibodies were generated as described by Williamson et al. (38). Falcipain 3-specific antibodies against a recombinant maltose-binding protein–falcipain 3 (aa 249 to 502) fusion protein were generated in mice. The coding sequence of falcipain 3 (aa 249 to 502) was amplified by PCR with primer Fal 3.s (5′-TAAT CCC GGG GGT CCA TTC

TABLE 1. E64d-mediated inhibition of oocyst production

Isolate or strain, expt no., and treatment	GM no. of oocysts	Oocyst range	No. of mosquitoes infected/no. dissected	% Mosquitoes infected	% Oocyst reduction
Isolate NF54[a]					
Expt 1					
1 h					
DMSO	94	23–176	20/20	100	
E64d	0.01	0–1	1/20	5	100*
E64d	0.0	0	0/20	0	100*
30 min					
DMSO	111	34–254	20/20	100	
E64d	1.8	0–12	13/20	65	98*
E64d	1.7	0–11	16/19	84	98*
15 min					
DMSO	134	39–367	20/20	100	
E64d	28	3–95	20/20	100	79*
E64d	17.1	0–79	17/20	85	87*
Expt 2					
1 h					
DMSO	11	0–81	16/20	80	
E64d	0	0–0	0	0	100*
E64d	0	0–0	0	0	100*
30 min					
DMSO	21	4–74	20/20	100	
E64d	0.9	0–3	13/20	65	96*
E64d	0.3	0–1	6/16	37.5	99*
15 min					
DMSO	17	0–54	18/20	90	
E64d	5.1	0–21	15/20	75	70*
E64d	1.9	0–11	15/20	75	89*
Strain 3D7[b]					
DMSO	8.74	0–40	15/18	83	
E64d, 10 μM	14.94	0–31	11/12	92	
E64d, 100 μM	1.69	0–4	6/14	43	81*

[a] Mature stage V gametocytes of isolate NF54 were treated for the indicated times with E64d (100 μM) or DMSO alone before being fed to *Anopheles stephensi* mosquitoes. Seven days later the midguts were dissected and analyzed for *P. falciparum* oocysts. For each independent feeding (16 to 20 mosquitoes/feeding), the geometric mean (GM) number of oocysts per midgut was calculated and compared with the geometric mean of the number of oocysts per midgut of mosquitoes fed the control DMSO-treated gametocytes (percent oocyst reduction). For each treatment the range of the numbers of oocysts per mosquito and the number and percentage of the dissected mosquitoes that contained one or more oocysts are listed. *, significant difference ($P < 0.00$) by Kruskal-Wallis analysis.

[b] Mature stage V gametocytes of strain 3D7 were treated for 1 h with DMSO or E64d at the indicated concentrations before being fed to *Anopheles stephensi* mosquitoes. Seven days later the midguts were dissected and analyzed for *P. falciparum* oocysts. The geometric mean of the number of oocysts per midgut was calculated for each treatment and compared with the geometric mean of the number of oocysts per midgut of mosquitoes fed the control DMSO-treated gametocytes (percent oocyst reduction). For each treatment the range of the number of oocysts per mosquito and the number and percentage of the dissected mosquitoes that contained one or more oocysts are listed. *, significant difference ($P < 0.00$) by Kruskal-Wallis analysis.

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek

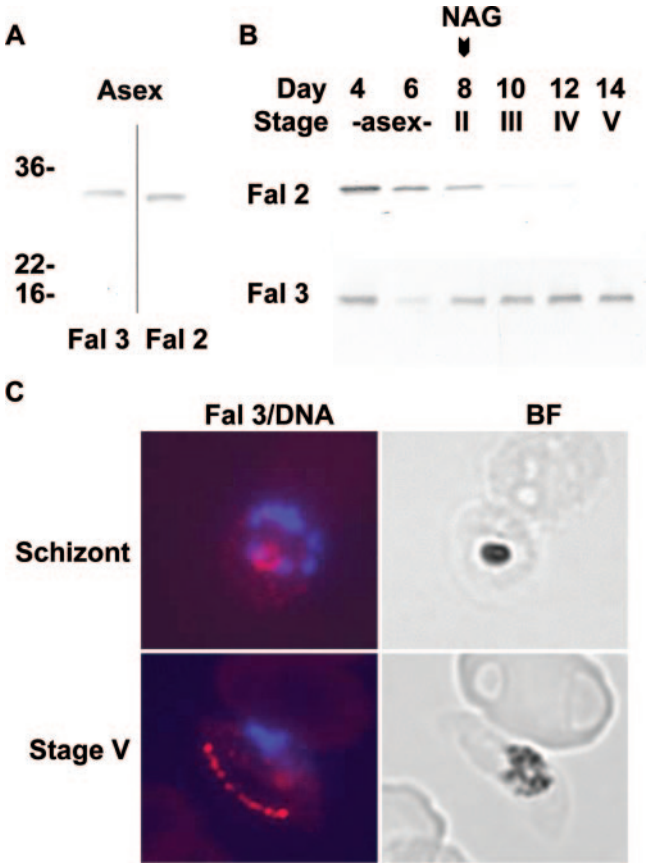

FIG. 1. Falcipain 2A/B and 3 expression. (A and B) Immunoblots of NETT extracts of parasites isolated from cultures containing (A) asexual parasites (Asex) or (B) parasites isolated on the indicated day were probed with anti-falcipain 2A/B (Fal 2)- or falcipain 3 (Fal 3)-specific antibodies, as described in Materials and Methods. *n*-Acetylglucosamine was added on day 8 to block further asexual growth, and the most abundant gametocyte stage (stages II to V) at each time point is indicated. (C) Immunofluorescence assay of a methanol-fixed schizont and stage V gametocyte probed with anti-falcipain 3 antibodies and DNA staining with DAPI. The merged anti-falcipain 3 and DAPI-stained DNA images and the corresponding bright-field (BF) image are shown.

AAA ACA TTA TCA) and primer Fal 3.a (5′ TAAT GTC GAC TTC AAG TAA TGG TAC ATA TGC) and then digested with SmaI and SalI and was then inserted into StuI- and SalI-digested PIH902 (a generous gift of Paul Riggs, New England Biolabs, Beverly, MA). The plasmids were used to transform *Escherichia coli* strain DH10B, and once the sequence was confirmed by automated sequencing (University of Chicago, Chicago, IL), recombinant protein production was induced with isopropyl β-D-thiogalactopyranoside (0.1 mM). The recombinant protein was purified on amylose resin (New England Biolabs) emulsified in monophosphoryl lipid A plus trehalose dicorynomycolate (Sigma) and was used to vaccinate mice (38).

Immunoassays. (i) Immunoblotting. The protein expression patterns of falcipain 2A/B and 3 were tested with asexual *P. falciparum* parasites and throughout the induction and maturation of gametocytes (Fig. 1). Parasites from asexual stock cultures or parasites obtained at the indicated times (days 4 to 14) after the gametocyte cultures were set up were isolated by centrifugation (at 2,000 × *g* for 5 min) after RBC lysis with 0.015% saponin. The cell pellets were washed in phosphate-buffered saline (PBS) and then extracted in NETT (150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 50 mM Tris-Cl [pH 6.8]). SDS sample buffer (final concentrations, 1% SDS, 10% glycerol, and 125 mM Tris-Cl [pH 6.8]) was added to the NETT parasite extract or directly to emergence medium which was isolated by centrifugation (at 2,000 × *g* for 5 min) 1 h after the induction of gamete/zygote formation. The sample was size fractionated on a 12% polyacrylamide gel (Invitrogen) and then transferred to nitrocellulose. The nitrocellulose blot was incubated with the indicated primary antibodies (1:1,000) and visualized with alkaline phosphatase-conjugated secondary antibodies (1:2,000; Sigma), 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, and nitroblue tetrazolium chloride (Promega, Madison, WI).

(ii) Indirect immunofluorescence assay. Monolayers of parasites were air dried and then fixed in dry ice-chilled methanol, rinsed with PBS, blocked in 5% nonfat dry milk–PBS, and incubated for 1 h at RT with the indicated serum or monoclonal antibody. Following two rinses with PBS, the monolayer was incubated for 30 min at RT with a 1:200 dilution of secondary antibody labeled with tetramethylrhodamine isothiocyanate or fluorescein isothiocyanate and then rinsed, mounted in Vectashield with or without 4′,6′-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA), and examined by fluorescence microscopy.

RESULTS

Expression of falcipains 2A, 2B, and 3 during gametocytogenesis. Falcipain 1, 2A/B, and 3 mRNA levels have previously been determined by Northern and microarray analyses to have distinct patterns through the life cycle (8, 39). However, as recently demonstrated by Mair et al. (15), the production of mRNA and protein can be differentially regulated in *Plasmodium*. Therefore, we directly evaluated protein expression at 2-day intervals through gametocytogenesis by immunoblotting using antibodies against falcipain 2A/B (generously provided by P. Rosenthal) and falcipain 3 (produced as described in Materials and Methods) (Fig. 1). Since falcipains 2A and 3 are 68% similar at the amino acid level, samples from an asexual parasite culture were run on the same polyacrylamide gel, and the resulting immunoblot was probed separately with antibodies against falcipain 2B/A or falcipain 3 to establish specificity. Under reducing conditions, distinct bands corresponding to 33 kDa with anti-falcipain 3 antibodies and 32 kDa with anti-falcipain 2A/B antibodies were observed (Fig. 1A). Following the induction of gametocytogenesis and the inhibition of further asexual growth by the addition of *n*-acetylglucosamine to the cultures, the expression of falcipain 2A/B decreased (Fig. 1B). In contrast, falcipain 3 expression remained constant following the induction of gametocytogenesis. The slight decrease in falcipain 3 expression on day 6 corresponds to a marked decrease in schizonts and an increase in ring-stage parasites, which are known to express less falcipain 3. Indirect immunofluorescence assay with the same antibodies indicated that during gametocytogenesis falcipain 3 is transported out of the gametocyte into the RBC (Fig. 1C and 2). In mature stage V gametocytes, falcipain 3 was found to accumulate in vesicles along the RBC membrane.

Effect of cysteine protease inhibitor E64d on gametogenesis and oocyst production. To evaluate the role of falcipain 3 in gametogenesis, we attempted disruption by targeted integration but were unsuccessful. Similar attempts by other groups (32) have also been unsuccessful. It is possible that falcipain 3, unlike falcipain 1, 2A, or 2B, is essential for asexual growth; and thus, knockout parasites cannot be recovered. As an alternative, we tested the effect of the cysteine protease inhibitor E64d on gametogenesis and oocyst production, since it had previously been shown to inhibit *P. falciparum* surface antigen Pfs230 processing during gametogenesis (3). The addition of E64d to mature gametocyte cultures 1 h before the parasites were harvested by centrifugation, mixed with an equal volume

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek

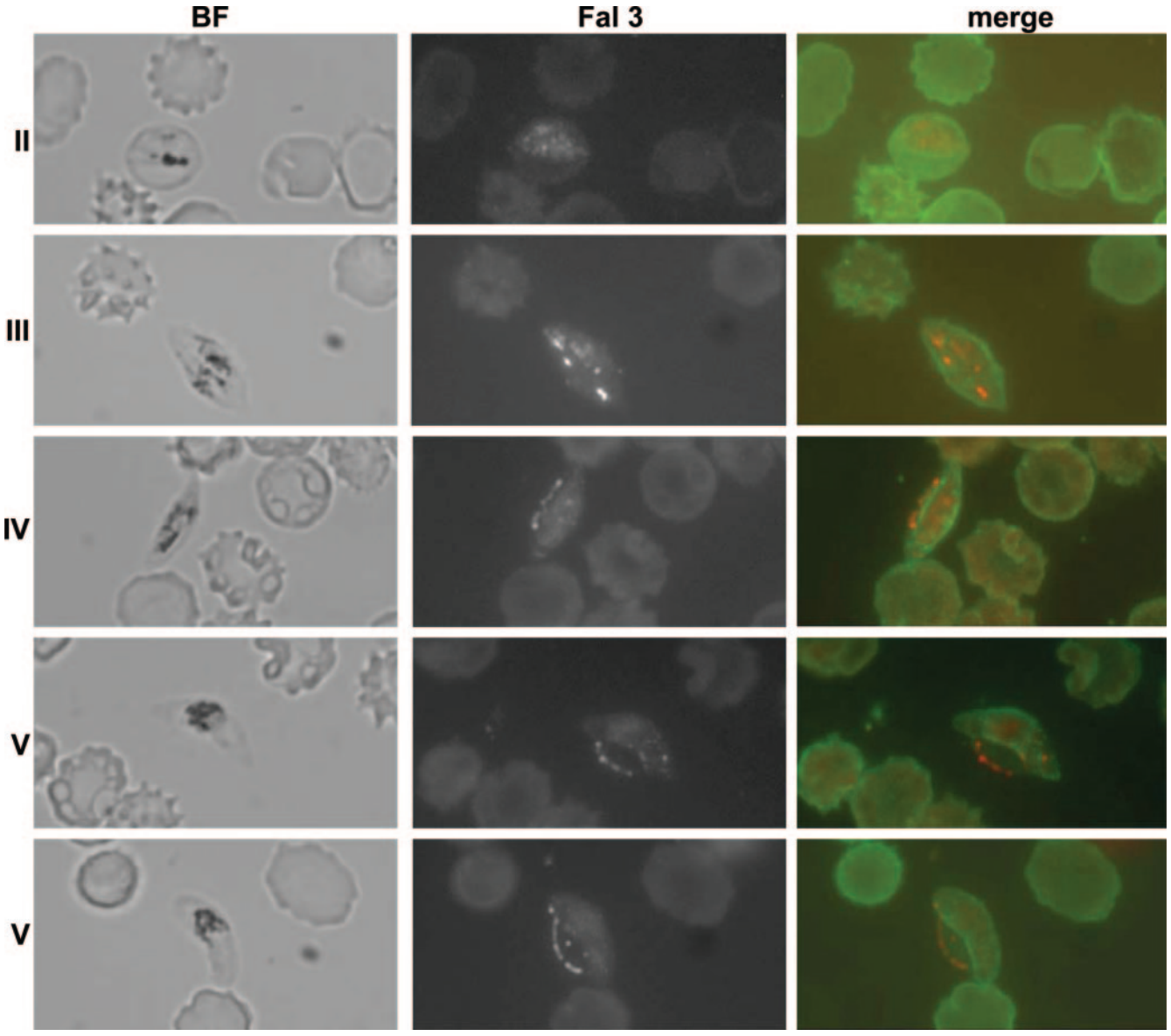

FIG. 2. Localization of falcipain 3 during gametocytogenesis by immunofluorescence assay of stage II, III, IV, and V *P. falciparum* gametocytes. The methanol-fixed gametocytes were probed with anti-falcipain 3 and anti-glycophorin A antibodies and tetramethylrhodamine isothiocyanate- or fluorescein isothiocyanate-labeled secondary antibodies. The bright-field (BF), anti-falcipain 3 (Fal 3), and merged anti-falcipain 3 and anti-glycophorin A (merge) images are shown.

of human serum, and then fed to mosquitoes significantly reduced oocyst production by 80 to 100% (Table 1 [see also Fig. 4A]). A similar 1-h incubation with the carrier dimethyl sulfoxide (DMSO) had no significant effect on mosquito infectivity. The effectiveness of E64d decreased with shorter incubation times, but even a 15-min treatment inhibited oocyst production by 79 to 83% (Table 1). In contrast, there was no inhibition of oocyst production when the concentration of E64d was reduced to 10 μM (Table 1).

Importantly, treatment with 100 μM E64d for an hour did not affect the morphology of the gametocytes or their ability to respond to conditions that simulate the mosquito midgut (<30°C, pH 8.3, 100 μM xanthurenic acid) (Fig. 3). Both DMSO- and E64d-treated gametocytes rounded up and emerged from RBCs, and within 10 min, male gametocytes had completed three rounds of DNA replication and had begun to exflagellate. During exflagellation one male produced up to eight motile flagella. In addition, uninfected RBCs formed clusters around the main body of the exflagellating male, generating exflagellation centers (Fig. 3). The number of exflagellation centers per ×40 field ranged from 0 to 3 for both the DMSO- and E64d-treated gametocytes. However, as shown previously (3), E64d treatment effectively inhibited the production of the 300-kDa and 35-kDa forms of gametocyte surface protein Pfs230 during RBC emergence (Fig. 4B). Consistent with the findings presented in earlier reports (3), there was no change in the production or release of the 47-kDa form of Pfs230 following E64d treatment. These data suggest that the

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek

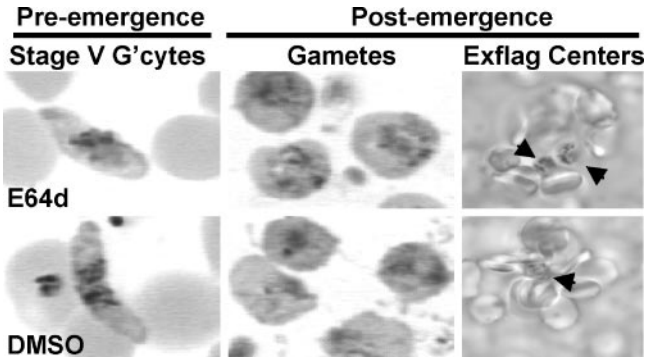

FIG. 3. Gametogenesis following E64d treatment. Mature gametocytes (G'cytes) of strain 3D7 were incubated for 1 h with E64d or an equivalent volume of DMSO. The gametocytes were then pelleted and gametogenesis was induced by adding an equal volume of emergence medium. Giemsa-stained thin smears of samples obtained before the stimulus (preemergence) and 15 min after the stimulus (postemergence) are shown, as are bright-field images of the exflagellation centers that were observed 15 to 30 min after the stimulus. Arrows indicate the exflagellating male parasite(s) in the exflagellation (Exflag) center.

effects of E64d treatment are due primarily to cysteine protease inhibition and not to generalized toxicity.

Effects of additional falcipain inhibitors on gametogenesis and oocyst production. E64d is a broad-spectrum cysteine protease inhibitor, and while it is useful for determination of the functional role of cysteine proteases, it is not specific enough to be used therapeutically. To identify a more specific *P. falciparum* inhibitor, a number of other compounds are being screened for their abilities to block asexual growth and/or falcipain activity (5, 16, 22). Several compounds that effectively inhibit recombinant falcipains 2A, 2B, and 3 have been developed and have been tested for their effects on asexual growth, but they have never been screened against sexual stage parasites (10, 17, 29). Two compounds, Mu-Leu-hPhe-VSPh and Mu-Leu-hPhe-FMK (generously provided by P. Rosenthal), that effectively inhibit falcipain 2B and 3, as well as YA29, which was reported to preferentially inhibit falcipain 1, were used to treat mature gametocyte cultures (10, 17, 29). However, none of these significantly inhibited oocyst production following a 1- to 2-h incubation (Fig. 4A). Under the conditions used for feeding, none of these compounds blocked gametogenesis, exflagellation, or the production of the 35-kDa Pfs230 fragment (Fig. 4B). In some preparations an additional fragment, which is ~2 kDa larger than the 47-kDa form, was present in the same samples that contained the 35-kDa fragment and was absent from the samples that did not contain the 35-kDa fragment. This pattern could suggest that the 35-kDa form is a fragment of the 49-kDa form and that the extent of further processing of the 49-kDa form to the 35-kDa form varies between preparations of supernatants from emerged parasites.

To evaluate whether accessibility to the target protease limited the effectiveness of the drugs, 0.001% saponin was added to permeabilize, but not solubilize, the RBC membrane. In the presence of 0.001% saponin, Mu-Leu-hPhe-FMK, but not the vinyl phenol sulfone derivative, Mu-Leu-hPhe-VSPh, inhibited Pfs230 processing (Fig. 4B). Pretreatment with 0.001% sapo-

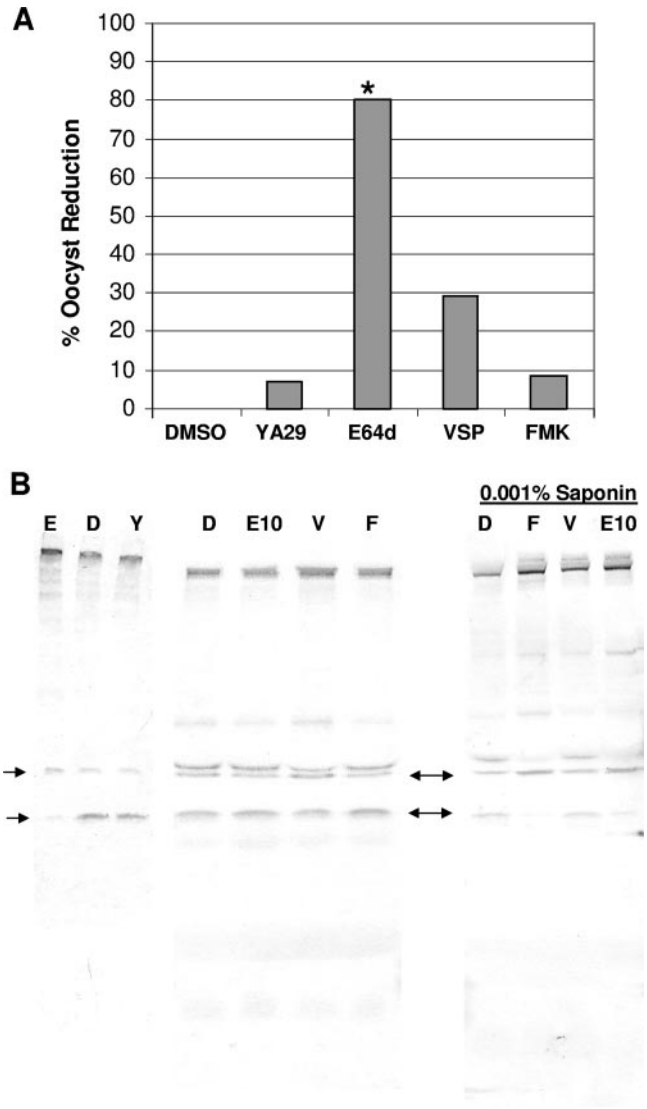

FIG. 4. Effects of cysteine protease inhibitors on oocyst production and Pfs230 processing. (A) Feeding protease inhibitor-treated gametocytes to mosquitoes. Mature gametocytes of strain 3D7 were treated for 1 h with the indicated protease inhibitor, YA29, E64d, Mu-Leu-hPhe-VSPh (VSP), or Mu-Leu-hPhe-FMK (FMK), or DMSO alone before they were fed to *Anopheles stephensi* mosquitoes. Seven days later the midguts were dissected and analyzed for *P. falciparum* oocysts. For each independent feeding (11 to 22 mosquitoes per feeding), the average number of oocysts per midgut was calculated for each treatment and compared with the average number of oocysts per midgut of mosquitoes fed the control DMSO-treated gametocytes (percent oocyst reduction). *, significant difference ($P \leq 0.007$) from the results for the DMSO-treated control by Kruskal-Wallis analysis. (B) Pfs230 processing during emergence following protease inhibitor treatment in the presence and absence of 0.001% saponin. Following a 1-h incubation in the presence or absence of 0.001% saponin with DMSO alone (D) or the indicated protease inhibitor, YA29 (Y), Mu-Leu-hPhe-VSPh (V), Mu-Leu-hPhe-FMK (F), 100 μM E64d (E), or 10 μM E64d (E10), mature gametocytes were pelleted and stimulated to emerge as described in Materials and Methods. The emergence medium was harvested, size fractionated, immunoblotted, and probed with Pfs230 region A (aa 304 to 378)-specific antibodies. The arrows indicate the 47-kDa and 35-kDa fragments.

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek

nin also decreased the concentration of E64d required to inhibit Pfs230 processing to 10 μM but had no effect on gametogenesis or exflagellation. These findings demonstrate that both the inhibitor specificity and the accessibility of the protease(s) involved in Pfs230 processing and oocyst production are distinct from those observed for inhibition of hemoglobin digestion during asexual growth.

DISCUSSION

Pretreatment of stage V gametocytes with the membrane-permeant cysteine protease inhibitor E64d for 1 h was found to significantly inhibit oocyst production and, as reported previously (3), the production of the 300-kDa and 35-kDa forms of gametocyte surface protein Pfs230. Treatment does not affect the morphology of the gametocytes or their ability to round up and emerge from the RBC minutes after being stimulated. Additionally, E64d-treated male parasites were still able to exflagellate within 10 to 15 min of application of the emergence stimulus and to form exflagellation centers. Since exflagellation is one of the most dynamic processes of the parasite life cycle (for a video, see reference 9), maintenance of this function following E64d treatment suggests that the gametocytes remained viable. Therefore, the subsequent reduction in oocyst production is most likely due to the inhibition of cysteine proteases and not to general toxicity.

None of the other more specific inhibitors that have been found to inhibit one or more of the *P. falciparum* falcipains, Mu-Leu-hPhe-FMK, Mu-Leu-hPhe-VSPh, or YA29, significantly reduced oocyst production or Pfs230 processing (Fig. 4). Lower concentrations of E64d also did not effectively inhibit oocyst production or the production of the 35-kDa form of Pfs230 in the absence of saponin (Fig. 1B and 4B). However, an increase in the permeability of the RBC without RBC disruption by addition of 0.001% saponin enhanced the abilities of both Mu-Leu-hPhe-FMK and E64d to inhibit Pfs230 processing. This suggests that there is limited accessibility to the target protease in intact gametocytes, which may contribute to the need for such high external drug concentrations. Accessibility could be a major difference between the targeting of proteases in the food vacuole, which is actively taking up material from the RBC, and the targeting of proteases located in other compartments in the parasite. Target protease access and specificity may be important factors to be considered in future drug optimization studies. As demonstrated by these findings, inhibition of the cysteine proteases involved in hemoglobin digestion has requirements different from those for the inhibition of the cysteine proteases involved in oocyst production.

The inability of Mu-Leu-hPhe-VSPh to inhibit Pfs230 processing even in the presence of saponin may also suggest that the target protease is less sensitive to Mu-Leu-hPhe-VSPh than Mu-Leu-hPhe-FMK or that an active form of Mu-Leu-hPhe-VSPh may not have access to the *P. falciparum* gametocyte compartment where processing takes place. The cysteine proteases involved in oocyst production and/or Pfs230 processing have not yet been identified. Falcipain 2A/B is an unlikely candidate, since the data presented here demonstrate that mature gametocytes lack falcipain 2A/B protein expression. Falcipain 1 may be involved, as its targeted disruption significantly reduces oocyst production; but E64d-induced inhibition is much more effective, suggesting that additional cysteine proteases are involved (8). Falcipain 3 is a possible candidate, as it is expressed at the appropriate time and location and its ability to cleave a fluorogenic substrate is 2×10^2 times more sensitive to inhibition by Mu-Leu-hPhe-FMK (5,850,000 ± 15,800 M^{-1} s^{-1}) than by Mu-Leu-hPhe-VSPh (22,000 ± 1,420 M^{-1} s^{-1}) (17). This differential sensitivity is consistent with the lack of an effect of Mu-Leu-hPhe-VSPh on Pfs230 processing even in the presence of 0.001% saponin. However, the involvement of other E64d-sensitive *P. falciparum* cysteine proteases remains a possibility.

The findings reported here, coupled with the findings of previous work demonstrating an important role of cysteine proteases in trophozoite growth, merozoite release, oocyst rupture, and sporozoite invasiveness, demonstrate the therapeutic potential of cysteine protease inhibitors to significantly reduce both the morbidity from malaria and the transmission of the malaria parasite (1, 4, 10, 23, 25, 28, 30, 34, 37). Identifying the proteases expressed in both the asexual and the sexual stages and their in vivo accessibilities to inhibitors is an important component of drug development. Previous work has implicated falcipains 2A/B and 3 in asexual hemoglobin digestion and, possibly, RBC emergence, and recombinant falcipains 2A and 3 have been used in a number of drug screening assays (6, 7, 11, 29, 31, 33, 34). The data from this study demonstrating that gametocytes express falcipain 3 and not falcipain 2A/B, coupled with the possibility that falcipain 3 could be essential for asexual growth, suggest that falcipain 3 is a prime drug target. Falcipain 1 has also been shown to be expressed by gametocytes and to play a role in oocyst production and therefore should also be included in drug development efforts (8). The differential effectiveness of the three additional falcipain inhibitors compounds tested here, Mu-Leu-hPhe-FMK, Mu-Leu-hPhe-VSPh, and YA29, on asexual growth and oocyst production highlights the importance of including the sexual stages in the evaluation of new therapeutic candidates.

In summary, this work indicates that cysteine proteases are important for sexual stage parasite function. The development of inhibitors that effectively target the asexual and the sexual stages has the potential to effectively block the morbidity caused by the malaria parasite as well as transmission of the malaria parasite.

ACKNOWLEDGMENTS

This investigation received financial support from Public Health Service grants AI40592 and AI48826 from the National Institute of Allergy and Infectious Diseases.

We thank T. Zaya and A. Suri, Loyola University Chicago, and Marga van de Vegte-Bolmer, University of Nijmegen, for technical support; J. Heller, Loyola University Chicago, for critical reading of the manuscript; and M. Bogyo, D. Greenbaum, and P. Rosenthal, University California at San Francisco, for generously providing reagents.

REFERENCES

1. **Aly, A. S., and K. Matuschewski.** 2005. A malarial cysteine protease is necessary for *Plasmodium* sporozoite egress from oocysts. J. Exp. Med. **202:**225–230.
2. **Barnes, K. I., and N. J. White.** 2005. Population biology and antimalarial resistance: the transmission of antimalarial drug resistance in *Plasmodium falciparum*. Acta Trop. **94:**230–240.
3. **Brooks, S., and K. C. Williamson.** 2000. Proteolysis of *Plasmodium falcipa-*

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek

num surface antigen, Pfs230, during gametogenesis. Mol. Biochem. Parasitol. **106:**77–82.
4. **Coppi, A., C. Pinzon-Ortiz, C. Hutter, and P. Sinnis.** 2005. The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion. J. Exp. Med. **201:**27–33.
5. **Desai, P. V., A. Patny, J. Gut, P. J. Rosenthal, B. Tekwani, A. Srivastava, and M. Avery.** 2006. Identification of novel parasitic cysteine protease inhibitors by use of virtual screening. 2. The available chemical directory. J. Med. Chem. **49:**1576–1584.
6. **Dhawan, S., M. Dua, A. H. Chishti, and M. Hanspal.** 2003. Ankyrin peptide blocks falcipain-2-mediated malaria parasite release from red blood cells. J. Biol. Chem. **278:**30180–30186.
7. **Dua, M., P. Raphael, P. S. Sijwali, P. J. Rosenthal, and M. Hanspal.** 2001. Recombinant falcipain-2 cleaves erythrocyte membrane ankyrin and protein 4.1. Mol. Biochem. Parasitol. **116:**95–99.
8. **Eksi, S., B. Czesny, D. C. Greenbaum, M. Bogyo, and K. C. Williamson.** 2004. Targeted gene disruption of *Plasmodium falciparum* cysteine protease, falcipain 1, reduces oocyst production, not erythrocytic stage growth. Mol. Microbiol. **53:**243–250.
9. **Eksi, S., B. Czesny, G. J. van Gemert, R. W. Sauerwein, W. Eling, and K. C. Williamson.** 2006. Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. Mol. Microbiol. **61:**991–998.
10. **Greenbaum, D. C., A. Baruch, M. Grainger, Z. Bozdech, K. F. Medzihradszky, J. Engel, J. DeRisi, A. A. Holder, and M. Bogyo.** 2002. A role for the protease falcipain 1 in host cell invasion by the human malaria parasite. Science **298:** 2002–2006.
11. **Hanspal, M., M. Dua, Y. Takakuwa, A. H. Chishti, and A. Mizuno.** 2002. *Plasmodium falciparum* cysteine protease falcipain-2 cleaves erythrocyte membrane skeletal proteins at late stages of parasite development. Blood **100:**1048–1054.
12. **Hogh, B., A. Gamage-Mendis, G. A. Butcher, R. Thompson, K. Begtrup, C. Mendis, S. M. Enosse, M. Dgedge, J. Barreto, W. Eling, and R. E. Sinden.** 1998. The differing impact of chloroquine and pyrimethamine/sulfadoxine upon the infectivity of malaria species to the mosquito vector. Am. J. Trop. Med. Hyg. **58:**176–182.
13. **Ifediba, T., and J. P. Vanderberg.** 1981. Complete in vitro maturation of *Plasmodium falciparum* gametocytes. Nature **294:**364–366.
14. **Lang-Unnasch, N., and A. D. Murphy.** 1998. Metabolic changes of the malaria parasite during the transition from the human to the mosquito host. Annu. Rev. Microbiol. **52:**561–590.
15. **Mair, G. R., J. A. Braks, L. S. Garver, J. C. Wiegant, N. Hall, R. W. Dirks, S. M. Khan, G. Dimopoulos, C. J. Janse, and A. P. Waters.** 2006. Regulation of sexual development of *Plasmodium* by translational repression. Science **313:**667–669.
16. **Micale, N., A. P. Kozikowski, R. Ettari, S. Grasso, M. Zappala, J. J. Jeong, A. Kumar, M. Hanspal, and A. H. Chishti.** 2006. Novel peptidomimetic cysteine protease inhibitors as potential antimalarial agents. J. Med. Chem. **49:**3064–3067.
17. **Na, B. K., B. R. Shenai, P. S. Sijwali, Y. Choe, K. C. Pandey, A. Singh, C. S. Craik, and P. J. Rosenthal.** 2004. Identification and biochemical characterization of vivapains, cysteine proteases of the malaria parasite *Plasmodium vivax*. Biochem. J. **378:**529–538.
18. **Nielsen, K. M., J. Kasper, M. Choi, T. Bedford, K. Kristiansen, D. F. Wirth, S. K. Volkman, E. R. Lozovsky, and D. L. Hartl.** 2003. Gene conversion as a source of nucleotide diversity in *Plasmodium falciparum*. Mol. Biol. Evol. **20:**726–734.
19. **Ponnudurai, T., A. H. Lensen, A. D. Leeuwenberg, and J. H. Meuwissen.** 1982. Cultivation of fertile *Plasmodium falciparum* gametocytes in semi-automated systems. 1. Static cultures. Trans. R. Soc. Trop. Med. Hyg. **76:** 812–818.
20. **Ponnudurai, T., A. H. Lensen, G. J. Van Gemert, M. P. Bensink, M. Bolmer, and J. H. Meuwissen.** 1989. Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes. Parasitology **98**(Pt 2)**:**165–173.
21. **Puta, C., and C. Manyando.** 1997. Enhanced gametocyte production in Fansidar-treated *Plasmodium falciparum* malaria patients: implications for malaria transmission control programmes. Trop. Med. Int. Health **2:**227–229.
22. **Ramjee, M. K., N. S. Flinn, T. P. Pemberton, M. Quibell, Y. Wang, and J. P. Watts.** 2006. Substrate mapping and inhibitor profiling of falcipain-2, falcipain-3 and berghepain-2: implications for peptidase anti-malarial drug discovery. Biochem. J. **399:**47–57.
23. **Raphael, P., Y. Takakuwa, S. Manno, S. C. Liu, A. H. Chishti, and M. Hanspal.** 2000. A cysteine protease activity from *Plasmodium falciparum* cleaves human erythrocyte ankyrin. Mol. Biochem. Parasitol. **110:**259–272.
24. **Riley, E. M., K. C. Williamson, B. M. Greenwood, and D. C. Kaslow.** 1995. Human immune recognition of recombinant proteins representing discrete domains of the *Plasmodium falciparum* gamete surface protein, Pfs230. Parasite Immunol. **17:**11–19.
25. **Rosenthal, P. J., J. H. McKerrow, M. Aikawa, H. Nagasawa, and J. H. Leech.** 1988. A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium falciparum*. J. Clin. Investig. **82:**1560–1566.
26. **Rosenthal, P. J., and R. G. Nelson.** 1992. Isolation and characterization of a cysteine proteinase gene of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **51:**143–152.
27. **Rosenthal, P. J., P. S. Sijwali, A. Singh, and B. R. Shenai.** 2002. Cysteine proteases of malaria parasites: targets for chemotherapy. Curr. Pharm. Design **8:**1659–1672.
28. **Salmon, B. L., A. Oksman, and D. E. Goldberg.** 2001. Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. Proc. Natl. Acad. Sci. USA **98:**271–276.
29. **Shenai, B. R., B. J. Lee, A. Alvarez-Hernandez, P. Y. Chong, C. D. Emal, R. J. Neitz, W. R. Roush, and P. J. Rosenthal.** 2003. Structure-activity relationships for inhibition of cysteine protease activity and development of *Plasmodium falciparum* by peptidyl vinyl sulfones. Antimicrob. Agents Chemother. **47:**154–160.
30. **Shenai, B. R., A. V. Semenov, and P. J. Rosenthal.** 2002. Stage-specific antimalarial activity of cysteine protease inhibitors. Biol. Chem. **383:**843–847.
31. **Shenai, B. R., P. S. Sijwali, A. Singh, and P. J. Rosenthal.** 2000. Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. J. Biol. Chem. **275:**29000–29010.
32. **Sijwali, P. S., J. Koo, N. Singh, and P. Rosenthal.** 2006. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **150:**96–106.
33. **Sijwali, P. S., and P. J. Rosenthal.** 2004. Gene disruption confirms a critical role for the cysteine protease falcipain-2 in hemoglobin hydrolysis by *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **101:**4384–4389.
34. **Sijwali, P. S., B. R. Shenai, J. Gut, A. Singh, and P. J. Rosenthal.** 2001. Expression and characterization of the *Plasmodium falciparum* haemoglobinase falcipain-3. Biochem. J. **360:**481–489.
35. **Singh, N., P. S. Sijwali, K. C. Pandey, and P. J. Rosenthal.** 2006. *Plasmodium falciparum*: biochemical characterization of the cysteine protease falcipain-2′. Exp. Parasitol. **112:**187–192.
36. **von Seidlein, L., M. Jawara, R. Coleman, T. Doherty, G. Walraven, and G. Targett.** 2001. Parasitaemia and gametocytaemia after treatment with chloroquine, pyrimethamine/sulfadoxine, and pyrimethamine/sulfadoxine combined with artesunate in young Gambians with uncomplicated malaria. Trop. Med. Int. Health **6:**92–98.
37. **Wickham, M. E., J. G. Culvenor, and A. F. Cowman.** 2003. Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte. J. Biol. Chem. **278:**37658–37663.
38. **Williamson, K. C., D. B. Keister, O. Muratova, and D. C. Kaslow.** 1995. Recombinant Pfs230, a *Plasmodium falciparum* gametocyte protein, induces antisera that reduce the infectivity of *Plasmodium falciparum* to mosquitoes. Mol. Biochem. Parasitol. **75:**33–42.
39. **Young, J. A., Q. L. Fivelman, P. L. Blair, P. de la Vega, K. G. Le Roch, Y. Zhou, D. J. Carucci, D. A. Baker, and E. A. Winzeler.** 2005. The *Plasmodium falciparum* sexual development transcriptome: a microarray analysis using ontology-based pattern identification. Mol. Biochem. Parasitol. **143:**67–79.

Downloaded from http://aac.asm.org/ on July 12, 2012 by Universiteitsbibliotheek