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Article

Assessment of Biological Role and Insight into Druggability of the *Plasmodium falciparum* Protease Plasmepsin V

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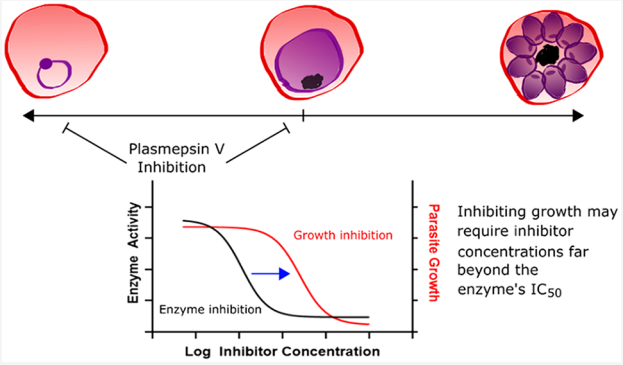
ABSTRACT: Upon infecting a red blood cell (RBC), the malaria parasite *Plasmodium falciparum* drastically remodels its host by exporting hundreds of proteins into the RBC cytosol. This protein export program is essential for parasite survival. Hence export-related proteins could be potential drug targets. One essential enzyme in this pathway is plasmepsin V (PMV), an aspartic protease that processes export-destined proteins in the parasite endoplasmic reticulum (ER) at the *Plasmodium* export element (PEXEL) motif. Despite long-standing interest in this enzyme, functional studies have been hindered by the inability of previous technologies to produce a regulatable lethal depletion of PMV. To overcome this technical barrier, we designed a system for stringent post-transcriptional regulation allowing a tightly controlled, tunable knockdown of PMV. Using this system, we found that PMV must be dramatically depleted to affect parasite growth, suggesting the parasite maintains this enzyme in substantial excess. Surprisingly, depletion of PMV arrested parasite growth immediately after RBC invasion, significantly before the death from exported protein deficit that has previously been described. The data suggest that PMV inhibitors can halt parasite growth at two distinct points in the parasite life cycle. However, overcoming the functional excess of PMV in the parasite may require inhibitor concentrations far beyond the enzyme's IC_{50}.



KEYWORDS: *aspartic protease, malaria, CRISPR/Cas9, knockdown, protein export, dense granule*

Malaria remains a scourge of the developing world, causing nearly 500 000 deaths per year, with the overwhelming majority due to *Plasmodium falciparum* infection.[1] While the life cycle of *P. falciparum* includes replication in both the liver and blood, symptomatic human disease is caused by infection of red blood cells (RBCs).[2] Upon infection of a host RBC, the parasite executes a dramatic program of protein export, sending hundreds of proteins through the secretory system, across the surrounding vacuole (parasitophorous vacuole, PV) through a parasite-encoded translocation complex, and into the host cytosol.[3,4] These exported effectors drastically remodel the host cell, setting up new solute permeability pathways, modifying the RBC shape and rigidity, and reconstituting trafficking machinery in the RBC cytosol to send parasite-encoded adhesins to the RBC surface.[3,4] These adhesins mediate binding of infected RBCs to vascular endothelia allowing parasites to avoid splenic clearance. Adherent parasites in the brain can cause vascular leakage leading to death in severe cases.[2]

Due to the central role of protein export in the survival and virulence of *P. falciparum*, there has been interest in this pathway as a source of potential drug targets.[5] One essential enzyme in the pathway is the parasite aspartic protease plasmepsin V (PMV).[6,8,9] PMV processes exported proteins in the parasite ER[13] by cleaving them cotranslationally in a variant signal recognition particle complex.[7] Cleavage occurs at the conserved amino acid motif RxLxE/Q/D, termed the *Plasmodium* export element (PEXEL).[8−12] PMV is highly specific for RxL in the PEXEL and cleaves after the leucine.[10,13,14] PEXEL processing is a critical step in protein export, as mutations in PEXEL that block PMV processing also block protein export.[11,12] Furthermore, processing of PEXEL proteins is likely an essential function in the parasite, as PMV is essential for survival in both *P. falciparum* and the related rodent parasite *P. berghei*, and treatment with a PEXEL peptidomimetic is lethal to intraerythrocytic parasites.[6,15−17]

A number of tools have been used to study PMV, including peptidomimetic inhibitors that block its function *in vitro* and are lethal to parasites in culture, a DiCre-mediated inducible excision of the gene, and crystallographic studies of *P. vivax* PMV.[6,16−18] However, study of PMV function has been hindered by an inability of previous depletions to yield a

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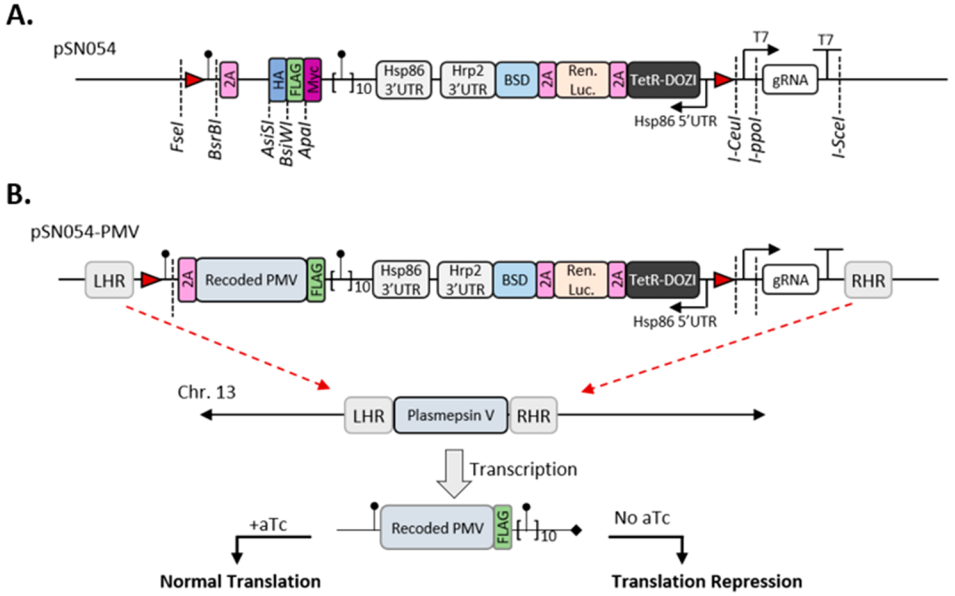

Figure 1. Architecture of pSN054 and application to editing PMV. (A) Schematic of pSN054 showing restriction sites (dashed lines), loxP (red triangles), aptamers (black lollipops), and 2A skip peptide (pink rectangle). Restriction sites allow the choice of 3x-HA (blue), FLAG (green), or Myc (purple). An expression cassette drives production of the Tet repressor-DOZI helicase fusion (TetR-DOZI, black), *Renilla* luciferase (Ren. Luc.), and blasticidin-S deaminase selectable marker (BSD). The T7 expression cassette drives transcription of CRISPR guide RNAs (gRNA). (B) Cloning strategy for editing of the PMV locus. Left and right homologous regions (LHR and RHR) were inserted at FseI and I-SceI respectively, while the recoded gene sequence was inserted into plasmid cut with AsiSI and BsiWI. The endogenous PMV sequence was disrupted by CRISPR/Cas9 gene editing. When transcribed, aptamers are bound by TetR-DOZI in the absence of aTc, repressing translation. In the presence of aTc, TetR-DOZI does not bind the aptamers and translation occurs as normal.

phenotype in RBC culture. The most robust knockdown described used the *glmS* ribozyme system, reducing PMV levels 10-fold with no measurable effect on parasite growth or PEXEL processing.[7,16] Here, we sought to apply the recently described TetR-DOZI aptamer system for stringent and tunable regulation of PMV.[19] This system can deplete a reporter gene 45 to 70-fold when aptamers are installed in both the 5′ and 3′ untranslated regions (UTRs) of the target gene.[19] However, cloning such a construct in traditional plasmid systems requires the assembly and maintenance of large circular plasmids that are prone to deletions and vector rearrangements during propagation in *E. coli*.[20] To overcome this technical challenge, we assembled a number of tools for genetic manipulation onto a previously described linear vector. Using this new vector system, we achieved substantially greater depletion of PMV than had been reported. By tuning the degree of knockdown, we confirmed that PMV is maintained in substantial excess during RBC infection and must be suppressed to nearly undetectable levels to affect parasite growth. Finally, we found that PMV-depleted parasites die immediately after invasion in a manner distinct from that of disruptions of other protein export machinery, suggesting PMV may have additional roles beyond those previously described.

RESULTS

Construction of a Linear Vector for Aptamer Knockdowns. To overcome the challenges associated with maintaining *P. falciparum* genomic material in circular plasmids, we utilized the pJAZZ linear vector system[20] as a chassis for DNA assembly. This system has previously been used to manipulate large [A+T]-rich genomic fragments, including those derived from the rodent malaria parasite, *P. berghei*.[20,21] We constructed a plasmid ("pSN054") to allow facile cloning, endogenous tagging, robust regulation of expression and inducible knockout of *P. falciparum* genes (Figure 1A, Figure S1). pSN054 has the following features: a single 5′ aptamer, 10× array of 3′ aptamers, regulatory protein (TetR-DOZI complex),[19] parasitemia-tracking component (*Renilla* luciferase), drug selection marker (*Blasticidin S deaminase*),[22] cassette for generation of sgRNA for CRISPR/Cas9 genome editing, cloning sites for inserting homologous sequences for genome repair, modularized affinity tags for tagging genes of interest at the N- or C-terminus, and loxP sites for gene excision (Figure 1A).

To utilize pSN054 for genome editing, a gene of interest must be recodonized to replace the current gene, preventing aberrant homologous repair from truncating the construct. To facilitate repair, 400−600 bp of homologous sequence corresponding to the 5′ and 3′ UTR of a gene, are cloned into the FseI and I-SceI restriction sites respectively (Figure S1). This intermediate vector can be used to create gene knockouts since it does not possess the coding sequence of the gene of interest. A subsequent Gibson assembly inserts the coding sequence of the gene. pSN054 contains the 2A skip peptide[23] such that cloning into the AsiSI site produces a protein with no tag on the N-terminus. If an N- or C-terminal tag is desired, the relevant restriction site is used for gene insertion (Figure S1). This donor plasmid can be adapted for use with the T7-RNAP CRISPR/Cas9 system[24] by cloning an sgRNA into the I-ppoI site. Alternatively, a U6 promoter-containing gRNA plasmid[25] can be used and cotransfected with a finished pSN054 donor plasmid.

TetR-DOZI Aptamer System for Tagging and Regulation of PMV. To apply pSN054 to PMV, we cloned pieces of the 5′ UTR and 3′ UTR into FseI and I-SceI, respectively, as well as a recodonized PMV coding sequence into a plasmid cut

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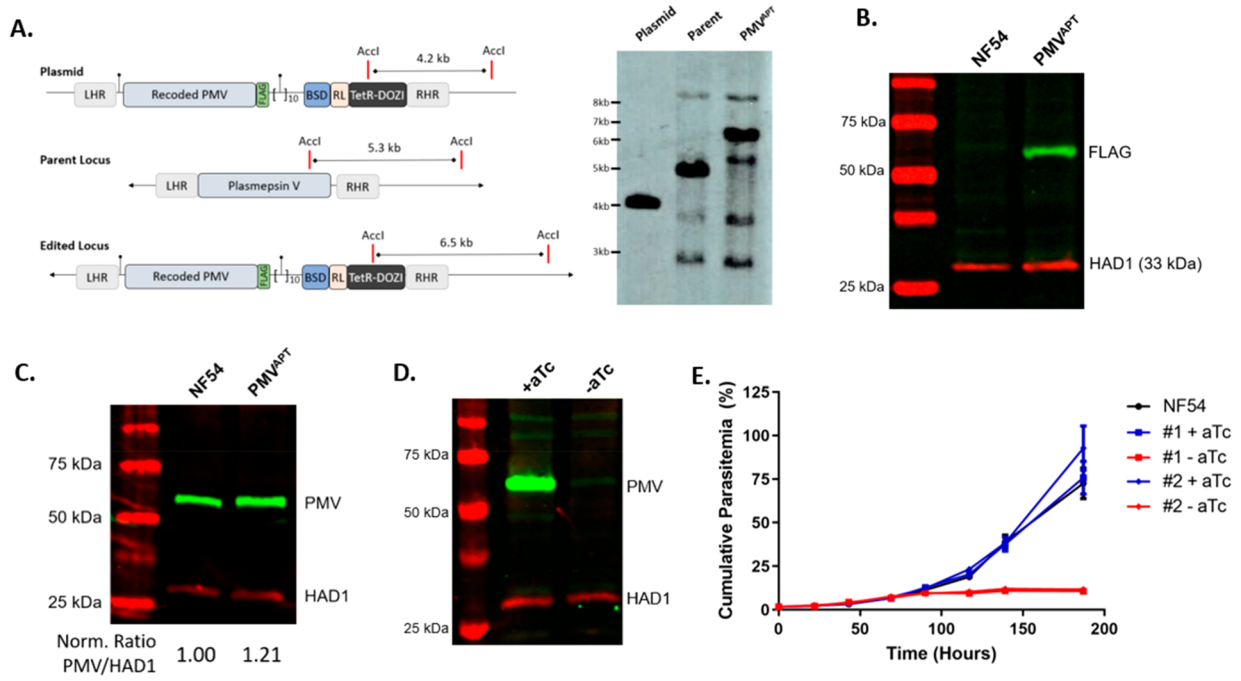

Figure 2. Depletion of PMV demonstrates its essentiality in parasite culture. (A) Tagging was verified by Southern blot with right homologous region (RHR) used as probe. Digest schematic shows expected size of bands. The topmost and bottom three bands in the "PMV^{APT}" lane are detected in the parent as well and are likely nonspecific. (B) Protein tagging was verified by Western blot using an anti-FLAG antibody as well as an antibody to a cytosolic sugar phosphatase HAD1 as loading control. (C) Expression levels were compared to the parent strain by Western blot using anti-PMV and anti-HAD1 antibodies. An average ratio of PMV to HAD1 signal from two experiments is shown. (D) Knockdown of PMV was initiated by washing aTc from ring-stage cultures and adding either 500 nM aTc (+aTc) or an equal volume of DMSO (−aTc). After 72 h, knockdown was assessed by Western blot, probing with anti-PMV and anti-HAD1. Uncut gels used to generate panels (C) and (D) are shown in Figure S2. (E) Two separate transfected PMV-regulatable lines ("#1" and "#2") were split +/− aTc as above and growth monitored daily by flow cytometry. The experiment was done twice in technical triplicate. A representative experiment is shown, with data points representing mean and error bars standard deviation.

with AsiSI and BsiWI (Figure 1B). This plasmid enabled us to replace the endogenous PMV gene with a FLAG-tagged recodonized PMV flanked by aptamers. The construct was cotransfected into the *P. falciparum* strain NF54^{attB}[26] (referred to as "NF54" throughout) along with a separate gRNA-containing plasmid pAIO,[25] and parasites selected and cloned. Incorporation of the construct into the genome was verified by Southern blot (Figure 2A). Expression of a FLAG-tagged protein of the expected size was verified by Western blot (Figure 2B). Similarly, a Western blot with anti-PMV antibody verified that modification of this locus did not substantially change PMV expression levels of the edited line relative to the parent (Figure 2C).

PMV Is Maintained in Substantial Excess during Infection. We then utilized the TetR-DOZI aptamer system to post-transcriptionally deplete PMV. Knockdown was initiated by washing out anhydrotetracycline (aTc) from young ring-stage parasites in RBC culture. In the absence of aTc, PMV levels were depleted about 50-fold (Figure 2D) and parasite growth was arrested after 96 h (Figure 2E). This confirms previous reports that PMV is essential for intra-erythrocytic growth[6,16,27] and showcases the ability of the TetR-DOZI aptamer system to drive more substantial depletion of proteins than previously possible.

We next utilized the tunability of the TetR system to determine the amount of PMV required for parasite survival. To this end, we titrated aTc levels and followed parasite growth by flow cytometry. Parasites maintained in 3 nM aTc or above grew normally. Parasites maintained in DMSO or 1 nM aTc were arrested by 96 h, while parasites maintained in 2 nM aTc survived an additional cycle before arresting at 120 h (Figure 3A). Given this, we sought to quantify the PMV depletion necessary to affect parasite growth. We synchronized parasites and washed out aTc from cultures containing predominantly young ring-stage parasites, then harvested samples for Western blot at 72 h following washout to compare the PMV levels in parasites that would go on to survive another cycle (2 nM aTc) to those that would arrest in the next 24 h (1 nM aTc). Over three independent experiments, we estimate that approximately 8% of wildtype PMV (2 nM aTc) supports another cycle of growth, while 3% (1 nM aTc) was insufficient and led to death upon reinvasion (Figure 3B,C). Since these parasites presumably divide their PMV, diluting it among their daughter cells before the next cycle begins, our data suggest that PMV is maintained in enormous functional excess in parasite culture, with perhaps less than 1% of wildtype PMV likely sufficient to support growth in parasite culture.

PMV-Depleted Parasites Arrest Early in their Life Cycle. Given the canonical role of PMV in protein export, we expected PMV depletion to phenocopy disruption of other critical export machinery such as components of the *Plasmodium* translocon for exported proteins (PTEX) which mediates translocation of effectors across the PV membrane.[3,28,29] PTEX components Hsp101, PTEX150, and EXP2 are all essential in parasite culture, and depletion of any of these caused parasite arrest during the early trophozoite stage.[30−32] Therefore, we were surprised to find that PMV

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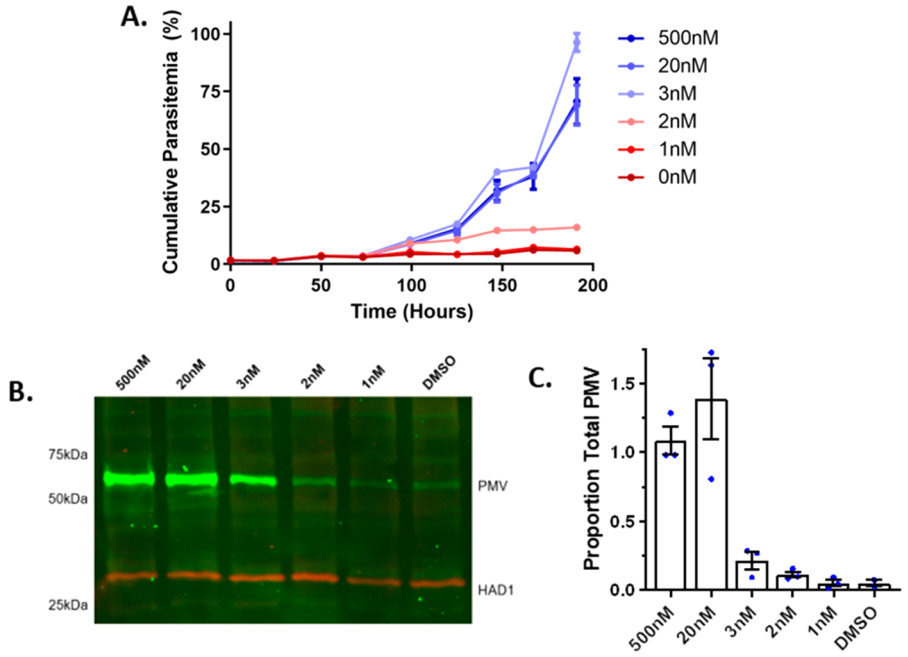

Figure 3. PMV is maintained in substantial excess. (A) Knockdown of PMV was initiated by washing aTc from ring-stage cultures, and parasites maintained in 500, 20, 3, 2, 1, or 0 nM (DMSO) aTc. Growth was monitored daily by flow cytometry. Three experiments were performed with each sample done in technical triplicate. A representative experiment is shown with points representing mean and error bars the standard deviation. (B) Samples were prepared as in (A) but harvested at 72 h for Western blot. Three experiments were performed. A representative blot is shown. Uncut gels are shown in Figure S3. (C) Quantification of the three Western blots. Blue dots represent mean PMV signal relative to the 500 nM signal, as quantified on a diluted standard curve for each experiment (see Materials and Methods); bar height represents the mean of the three experiments; error bars represent the standard error of the mean.

depletion caused growth arrest very early in the intraerythrocytic development cycle, arresting as "dots" shortly after invasion (Figure 4A,B). Arrested parasites were further investigated by transmission electron microscopy and were found to have gross structural abnormalities (Figure 4C). PMV-depleted parasites generally showed more electron density throughout the parasite cytosol, failure to expand much beyond the size of merozoites, and large unidentified vacuolar structures within the parasite. In contrast, when parasites were fixed as schizonts from the preceding cycle, there was no obvious morphological defect (Figure 4D). This suggests that PMV plays some critical role(s) in successfully initiating the intraerythrocytic development cycle, distinct from its canonical protein export role.

To assess whether PEXEL processing was defective at the time of parasite death, we purified PMV-depleted schizonts and allowed them to invade fresh RBCs for 4 h, then probed the status of the early exported protein RESA by Western blot and immunofluorescence. PMV depletion led to an accumulation of unprocessed RESA as well as a corresponding defect in RESA export to the surface of the infected RBC (Figure S4).

PMV Inhibitors Kill Parasites at Multiple Points in the Intraerythrocytic Development Cycle. Previous work indicated that PMV inhibitors were lethal at the ring-trophozoite transition, consistent with depletion of PTEX components.[17] Since this differs from our finding that PMV depletion arrests parasite growth early after invasion, we sought to recapitulate the previously described early trophozoite death as well as our early postinvasion death with the peptidomimetic PMV inhibitor WEHI-842.[17,18] We treated synchronized ring-stage parasites or schizonts with 5 μM WEHI-842 for an 8-h window, then monitored parasites by thin smear. We found that ring-stage parasites treated with WEHI-842 arrested as early trophozoites as previously described (Figure 5A). However, parasites treated with WEHI-842 beginning in schizogony arrested immediately after invasion, as was seen in our genetic PMV depletion line (Figure 5B). Taken together, our data suggest that parasites are sensitive to PMV inhibition at two points during asexual growth in RBCs. The first is immediately after invasion. The second is in early trophozoites and is phenocopied by PTEX disruptions.

DISCUSSION

We report the first regulatable knockdown that lowers PMV levels enough to reveal a lethal phenotype. This work overcomes the technical limitations of past knockdown systems by utilizing the TetR system with aptamers on both the 5′ and 3′ end of the gene of interest. This manipulation was facilitated by the plasmid described here, pSN054, which enables a suite of previously described genetic tools to be utilized with relative ease for gene editing to achieve protein tagging, regulation of gene expression, parasite growth monitoring and inducible knockout as required.

An unexpected result is that PMV depletion does not phenocopy the disruption of PTEX components.[30−32] The fact that the early death phenotype described here is not seen in disruption of other export machinery suggests that PMV has a role independent of protein export that is essential for parasite survival in RBCs. A reasonable hypothesis is that this role could be the cleavage of a critical substrate early in the life cycle to allow it to perform an essential activity. In this case, the substrate would likely be acting within the parasite or parasitophorous vacuole, since disruption of Hsp101 function with a destabilization domain blocked nearly all exported

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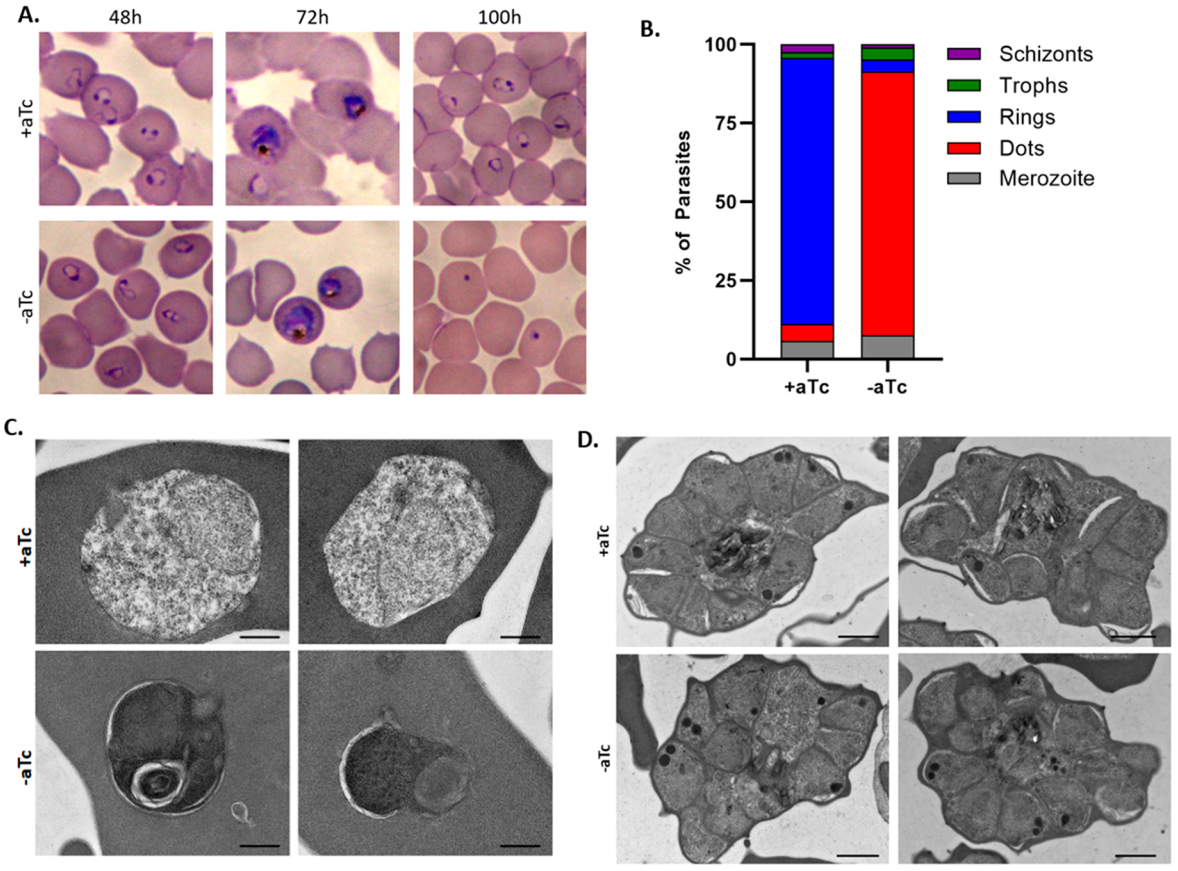

Figure 4. PMV-depleted parasites arrest early in their life cycle. (A) aTc was washed from ring-stage parasites, which were then maintained in 500 nM aTc (+aTc) or an equal volume of DMSO (−aTc). Parasite growth was monitored by Hemacolor-stained thin smear. Experiment was performed three times (B) Phenotype quantification for 0- to 4-h rings (N = 208 parasites +aTc; 105 parasites −aTc). (C) Parasites maintained as above. At 96 h, parasites were fixed, and early ring-stage parasites visualized by transmission electron microscopy. (D) Parasites were maintained as in (C) except schizonts were harvested at 90 h following aTc washout. Scale bars represent 1 μm.

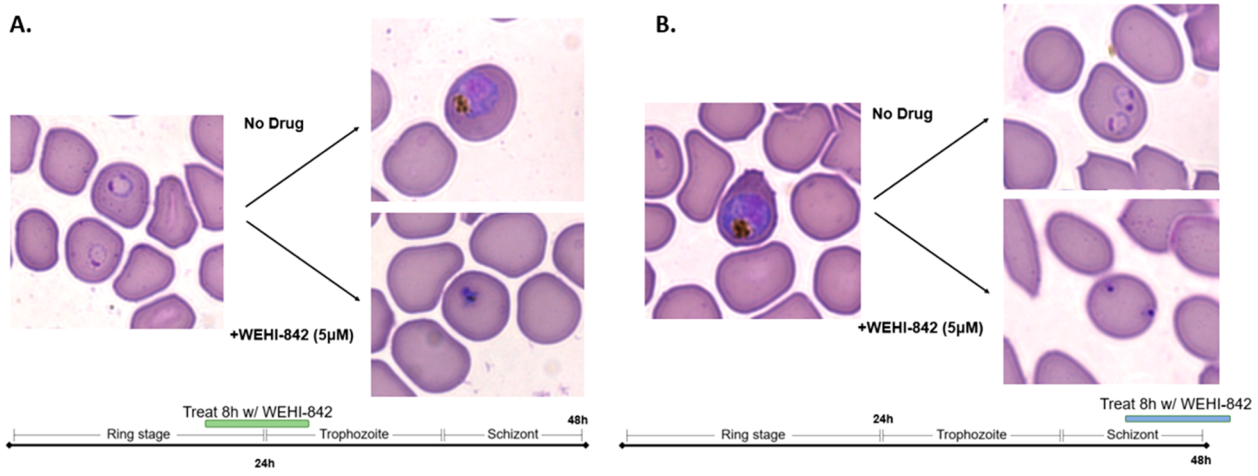

Figure 5. PMV inhibition arrests parasite growth at two distinct points in the life cycle. Parasites were synchronized to within 3 h, then treated with 5 μM WEHI-842 for an 8-h window beginning either in (A) ring-stage (12 to 15 h after invasion) or (B) early schizont (41 to 44 h after invasion). At the beginning and end of each window, parasites were monitored by Hemacolor-stained thin smear. The experiment was performed twice; representative images from one experiment are shown.

effectors within the vacuole but only arrested growth in early trophozoites.[30] One possible source of essential PMV substrates is parasite secretory organelles called dense granules that are involved in establishing the PV.[33] In the Apicomplexan parasite *Toxoplasma gondii*, the PMV ortholog Asp5 primarily cleaves dense granule effectors at a PEXEL-like sequence near their N-termini.[34,35] Similarly, in *Plasmodium*, the dense granule protein RESA is cleaved at a "relaxed" PEXEL sequence of RxLxxE[10] by PMV (Figure S4). It is then rapidly secreted into the PV during parasite invasion.[10] While RESA is dispensable for intraerythrocytic growth,[36] other PMV substrates may follow a similar trafficking route and may be

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ACS Infect. Dis. 2020, 6, 738−746

required early in the intraerythrocytic development cycle. Alternatively, early death could be a nonspecific result of PMV deficiency, such as a buildup of uncleaved PEXEL proteins in the ER. Consistent with this, treatment with the canonical ER-stress inducer DTT arrested growth in *P. falciparum* with similar morphology by Giemsa stain to that caused by PMV depletion described here.[37]

One encouraging note for the development of PMV inhibitors as antimalarials is our finding that PMV inhibition can lead to parasite death at two distinct points within intraerythrocytic development. Knockdown of PTEX components seems to cause growth arrest only at the early trophozoite stage in blood-stage parasites.[30−32] Due to this, drugs inhibiting the function of PTEX components may take up to a full intraerythrocytic cycle (48 h) to reach the point in the cycle where growth arrest occurs. In contrast, PMV inhibitors may arrest growth more quickly by acting upon intraerythrocytic parasites at more than one point in the life cycle. Recent work has also shown that PMV inhibitors can block development of early stage gametocytes, and have a transmission-blocking effect.[38] Together, these findings bolster the case that PMV inhibitors can have properties in line with the target antimalarial profiles put forward by the Medicines for Malaria Venture.[39] However, these beneficial characteristics are counterbalanced by our finding that PMV must be suppressed to barely detectable levels to affect parasite growth. Peptidomimetic inhibitors of PMV that have been developed are generally greater than 100-fold less potent in culture than on isolated enzyme.[16,17,40] It has been presumed that potency against parasites is limited by cellular permeability. Our functional genetics data would suggest that an additional, and possibly major, component of the potency drop-off is the need to inhibit nearly all the cellular enzyme to kill parasites. It is possible that a potent compound with good biochemical and pharmacokinetic properties could overcome this barrier to sufficiently inhibit PMV during human infection to achieve high antimalarial efficacy.

Taken together, our study provides further data on the proposed antimalarial drug target plasmepsin V. Future work is needed to determine if PMV is maintained at excessive levels *in vivo* as it is *in vitro*, and to elucidate the cause of growth arrest after invasion in PMV-depleted parasites.

MATERIALS AND METHODS

Parasite Lines and Culture. *P. falciparum* strain NF54^{attB} (referred to as NF54 throughout) was used as a parent strain for transfections.[26] Asexual parasites were cultured in RPMI 1640 (Gibco) supplemented with 0.25% (w/v) Albumax, 15 mg/L hypoxanthine, 110 mg/L sodium pyruvate, 1.19 g/L HEPES, 2.52g/L sodium bicarbonate, 2 g/L glucose, and 10 mg/L gentamycin. Deidentified RBCs were obtained from the Barnes-Jewish Hospital blood bank (St. Louis, MO), St. Louis Children's Hospital blood bank (St. Louis, MO), and from American Red Cross Blood Services (St. Louis, MO).

Generation of Knockdown Line. The construct for aptamer regulation of PMV was constructed using pSN054, described above. The right homologous region (3′ UTR) was amplified from NF54 genomic DNA using primers AGTGGTGTACGGTACAAACCCGGAATTCGAGCTCGGGGAATCAACATAGAAACGTTAAAG and GATTGGGTATTAGACCTAGGGATAACAGGGTAATGTACTAGGTCATTTTCTTTATTTTAC, and cloned into the I-SceI site using Gibson Assembly (NEB). The left homologous region (5′ UTR) was amplified from NF54 genomic DNA using primers TTGGTTTTCAAACTTCATTGACTGTGCCGACATTAATTTGTGTAACATATAAATATGTAG and AAGTTATGAGCTCCGGCAAATGACAAGGGCCGGCCCTTTCCTTAAAAAATAATTATTGAT, and cloned into the FseI site. PMV was codon-optimized for expression in *Saccharomyces cerevisiae* and synthesized as gene blocks by Integrated DNA Technologies (Coralville, IA) then cloned into the vector at the AsiSI and BsiWI sites. The plasmid was grown in BigEasy Electrocompetent Cells (Lucigen) with 12.5 μg/mL chloramphenicol and 0.01% (w/v) arabinose.

CRISPR/Cas9 editing was performed as previously described.[25] Guide RNA sequences were inserted into the pAIO vector by annealing oligonucleotides of the sequences ATTAAGTATATAATATT**TGTAATGGTTGTAAAGATTG**GTTTTAGAGCTAGA and TCTAGCTCTAAAAC**CAATCTTTACAACCATTACA**AATATTATATACTTAAT and inserting them into BtgZI-cut pAIO by In-Fusion HD Cloning (Clontech). pAIO was maintained in XL10 Gold cells (Agilent Technologies). Bold sequences represent the gRNA site.

For each transfection, 100 μg of donor vector and 50 μg of pAIO were transfected into early ring-stage parasites in 2 mm gap electroporation cuvettes (Fisher) using a BioRad Gene Pulser II. Transfectants were maintained in 0.5 μM anhydrotetracycline (aTc; Cayman Chemical) and were selected beginning 24 h post-transfection with Blasticidin S (2.5 μg/mL; Fisher). Parasites were obtained from several independent transfections and clones obtained by limiting dilution.

Validation of PMV^{APT} Line. Proper integration of our construct was verified by Southern Blot as in ref 24. For a probe, the right homologous region was amplified from NF54 genomic DNA using primers described above.

To verify tagging of protein, schizonts of NF54 and PMV^{APT} were first synchronized by purifying on magnetic columns (Miltenyi Biotech) then allowed to invade fresh uninfected RBCs for 3 h before remaining schizonts were cleared with 5% sorbitol. Parasites were then allowed to progress for 40 h, then RBCs lysed with cold PBS + 0.035% saponin. Samples were centrifuged to pellet parasites and remove excess hemoglobin, then parasites lysed in RIPA (50 mM Tris, pH 7.4; 150 mM NaCl; 0.1% SDS; 1% Triton X-100; 0.5% DOC) plus HALT-Protease Inhibitor Cocktail, EDTA-free (Thermo Fisher). Lysates were centrifuged at high speed to pellet and remove hemozoin. Cleared lysates were then diluted in SDS sample buffer (10% SDS, 0.5 M DTT, 2.5 mg/mL bromophenol blue, 30% 1 M Tris pH 6.8, 50% glycerol) and boiled for 5 min. Lysates were separated by SDS-PAGE, then transferred to 0.45 μm nitrocellulose membrane (BioRad). Membranes were blocked in PBS + 3% bovine serum albumin, then probed with primary antibodies mouse anti-PMV 1:25[41] or anti-FLAG 1:500 (M2, Sigma), and rabbit anti-HAD1 1:1000.[42] Membranes were washed in PBS + 0.1% Tween 20, then incubated with secondary antibodies goat antimouse IRDye 800CW 1:10,000 (Licor) and donkey antirabbit IRDye 680RD 1:10 000 (Licor). Membranes were then washed in PBS + 0.1% Tween 20 and imaged on a Licor Odyssey platform.

Assessment of Knockdown. To assess the effect of PMV knockdown on parasite growth, aTc was removed from cultures by washing 3 times for 5 min each in media without aTc, then either 500 nM aTc ("+aTc") or DMSO ("−aTc") was added and parasites split into 1 mL triplicate cultures for

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ACS Infect. Dis. 2020, 6, 738−746

each condition. Parasite growth was monitored daily by flow cytometry (BD FACSCanto) using acridine orange (1.5 μg/mL in PBS). Parasites were subcultured every 48 h to prevent overgrowth. "Cumulative parasitemia" was back-calculated based on the subculture schedule. Flow cytometry data is plotted with each point representing the mean of three technical replicates with error bars showing the standard deviation. Experiments were done three times unless otherwise noted.

For titrations of aTc, parasite cultures were washed as above to remove aTc, then cultures were maintained in media containing the shown concentrations of aTc or DMSO (aTc stocks were diluted to ensure each culture received equal volumes of the solvent DMSO in order to attain the desired final aTc concentration). To determine the effect of aTc concentration on PMV levels, cultures were prepared as above, and samples taken for Western blot 72 h after aTc was removed. Sample preparation and Western blotting was done as above (see the Validation of PMV^{APT} Line section). To quantitate PMV titration blots, the 500 nM sample was diluted out by factors of two to draw a standard curve correlating a PMV signal to a relative amount of the 500 nM sample (see Figure S3). Blots were quantitated using Licor Image Studio. The experiment was performed three times, and the mean for those three experiments is plotted with error bars representing the standard error of the mean.

WEHI-842 Treatment. $NF54^{attB}$ parasites were synchronized to within 3 h as above (see the Validation of PMV^{APT} Line section above, paragraph 2). Then either 5 μM WEHI-842, or an equal volume of DMSO was added to late rings (15 h after invasion initiated; parasites 12−15 h old), or late trophozoites (44 h after invasion initiated; parasites 41−44 h old). Parasites were incubated with drug or DMSO for 8 h, then assessed by thin smear. The experiment was performed twice, with similar results in each. Representative images from one experiment are shown.

Microscopy. Parasites monitored by thin smear were dyed using Harleco Hemacolor stains (MilliporeSigma). Images were taken using a Zeiss Axio Observer.D1 at the Washington University Molecular Microbiology Imaging Facility. For transmission electron microscopy, infected RBCs were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM sodium cacodylate buffer, pH 7.2 for 1 h at room temperature. Samples were washed in sodium cacodylate buffer at room temperature and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h. Samples were rinsed extensively in deionized water prior to *en bloc* staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h. Following several rinses in deionized water, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) equipped with an AMT 8-megapixel digital camera and AMT Image Capture Engine V602 software (Advanced Microscopy Techniques, Woburn, MA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.9b00460.

Figure S1: Cloning schematic for using pSN054; Figure S2: Uncut gels used to generate Figure 2; Figure S3: Uncut gels used to generate Figure 3B,C; Figure S4: PMV is required for processing and export of RESA (PDF)

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Author Contributions

§A.J.P. and A.S.N. contributed equally to this work. A.S.N. and J.C.N. conceived the plasmid platform. A.S.N. designed and built pSN054 and the pSN054 construct for PMV with supervision from J.C.N. A.J.P. designed and performed PMV experiments under the supervision of D.E.G.

Notes

The authors declare no competing financial interest.

Sequences for genes used in this study were obtained from PlasmoDB (Release 38) using the following gene IDs: plasmepsin V (PF3D7_1323500), HAD1 (PF3D7_1033400) and IspD (Pf3D7_0106900). Also discussed were Hsp101 (PF3D7_1116800), Exp2 (PF3D7_1471100), PTEX150 (PF3D7_1436300), plasmepsin IX (Pf3D7_1430200), and plasmepsin X (Pf3D7_0808200).

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