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Poly-basic peptides and polymers as new drug candidates against *Plasmodium falciparum*

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Abstract

Background *Plasmodium falciparum*, the malaria-causing parasite, is a leading cause of infection-induced deaths worldwide. The preferred treatment approach is artemisinin-based combination therapy, which couples fast-acting artemisinin derivatives with longer-acting drugs, such as lumefantrine, mefloquine, and amodiaquine. However, the urgency for new treatments has risen due to the parasite's growing resistance to existing therapies. In this study, a common characteristic of the *P. falciparum* proteome—stretches of poly-lysine residues, such as those found in proteins related to adhesion and pathogenicity—is investigated for its potential to treat infected erythrocytes.

Methods This study utilizes in vitro culturing of intra-erythrocytic *P. falciparum* to assess the ability of poly-lysine peptides to inhibit the parasite's growth, measured via flow cytometry of acridine orange-stained infected erythrocytes. The inhibitory effect of many poly-lysine lengths and modifications were tested this way. Affinity pull-downs and mass spectrometry were performed to identify the proteins interacting with these poly-lysines.

Results A single dose of these poly-basic peptides can successfully diminish parasitemia in human erythrocytes in vitro with minimal toxicity. The effectiveness of the treatment correlates with the length of the poly-lysine peptide, with 30 lysine peptides supporting the eradication of erythrocytic parasites within 72 h. PEG-ylation of the poly-lysine peptides or utilizing poly-lysine dendrimers and polymers retains or increases parasite clearance efficiency and bolsters the stability of these potential new therapeutics. Lastly, affinity pull-downs and mass-spectrometry identify *P. falciparum's* outer membrane proteins as likely targets for polybasic peptide medications.

Conclusion Since poly-lysine dendrimers are already FDA-approved for drug delivery and this study displays their potency against intraerythrocytic *P. falciparum*, their adaptation as anti-malarial drugs presents a promising new therapeutic strategy for malaria.

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Background

In 2022, malaria affected an estimated 249 million people around the globe [1]. This figure represents an increase from the 245 million cases reported in 2020 due to the COVID-19 pandemic [2]. Despite a reduction in malaria case incidence (cases per 1000 population at risk) from 82 in 2000 to 57 in 2019, the incidence stayed the same or slightly increased in the following years, with 59 cases per 1000 in 2020 and 2021 [2]. Human malaria, caused by *Plasmodium falciparum,* is responsible for over 600,000 deaths annually, mainly affecting children under five years of age. The *P. falciparum* life cycle includes two different hosts, humans and mosquitoes. By implementing successful control measures, such as the utilization of effective drugs for human treatment and efficient vector control strategies that target mosquitoes responsible for transmitting malaria, it is possible to achieve efficient control over infections and the spread of the disease. While malaria is less prevalent today than two decades ago, unfortunately, current drugs, including artemisinin and combination partners, are confronted with parasitic drug resistance [3, 4]. As such, there is a need for new antimalarials that exploit novel mechanisms.

The current drug treatment paradigm for infected patients is artemisinin-based combination therapy (ACT) [5]. ACT is based on artemisinin derivatives, aggressive short-acting drugs, in combination with multiple long-acting drugs. Artemisinin gets activated by the cleavage of its endoperoxide, which then alkylates hundreds of proteins, potentially causing "proteinopathy", eventually resulting in parasite death [6, 7]. Different strategies were examined in the creation and further advancements of ACT or novel medications targeting malaria parasites. [4, 8]. Several approaches were explored, including using combinations of existing drugs that may not be effective. There was also a focus on modifying existing drugs, with notable examples being artefenomel and ferroquine. Additionally, new potent compounds like KAF156 were discovered [9]. However, it has also been shown that a range of promising anti-malarial peptides, natural and synthetic, exhibit cytotoxic or inhibitory activity on malaria parasites. However, their clinical application has so far been limited [10]. The most attractive feature of peptide-based therapeutics, generally termed antimicrobial peptides (AMPs) [10], is their unique ability to affect multiple targets, thereby reducing the potential for resistance development by the targeted organism [10, 11]. Finally, peptide-based drugs provide a superior platform for generating diverse medications by leveraging various biological and synthetic amino-acid analogs and modifications by non-amino-acid moieties [10, 11]. The simple design, strong therapeutic outcomes, and their intrinsic malleability make anti-microbial peptides a promising avenue for exploratory drug discovery and development.

Polycationic peptides are a member of the AMP family, lethal against many bacteria, fungi, and viruses [12, 13]. The positive charge on the peptides allows them to interact with the negatively charged microbial cell membrane, leading to membrane disruption and cell death [14]. This relatively non-specific mechanism of action can reduce the risk of resistance development compared to traditional antibiotics [15]. Poly-L-lysine (PLL), a highly charged polycationic peptide, has been studied for several applications, including its antimicrobial potential [16].

Plasmodium falciparum has a highly AT-rich genome (81%) with coding sequences close to 75% AT-richness [17]. Previous work has indicated that *Plasmodium* species are among the few organisms that can translate long runs of poly-adenosine in mRNA transcripts to poly-L-lysine peptides with high accuracy [18]. Almost 50% of the *P. falciparum* genome codes for proteins with poly-L-lysine stretches, with some proteins containing 40 lysine residues in a row [18–20]. In most organisms, poly-L-lysine, and more generally poly-basic residue-rich genes, are typically associated with RNA biogenesis, DNA repair, and chromosome segregation [21]. However, the poly-L-lysine motif-containing proteins of *P. falciparum* and other *Plasmodium* species are also affiliated with adhesion and among the secretion repertoire of proteins involved in malaria pathogenesis and invasion [22]. The hypothesis for this enrichment of positively charged amino acid patches in *Plasmodium* adhesion and pathogenesis is that parasites utilize polybasic loops in secreted and membrane-associated proteins to attach and invade host erythrocytes [22–24]. This hypothesis is partially supported by previous studies in which negatively charged heparin or glycosaminoglycan blocked *P. falciparum* growth and invasion [23, 24]. However, charge distribution on *P. falciparum* and the erythrocyte membrane makes a compelling model where peptides of either charge, negative or positive, may interfere with parasitic invasion and infectivity [25].

This study has demonstrated that the parasite's strength in making long poly-lysine peptides can be turned into their weakness. Under the assumption that poly-basic residues are involved in host cell attachment and invasion, the aim was to utilize poly-lysine peptides for clearing erythrocytic *Plasmodium* with the rationale that treating infected cells with poly-basic peptides may block invasion. As such, exogenously added poly-cationic peptides were used to illustrate a specific growth inhibitory effect against *P. falciparum* in human erythrocytes in vitro. The results show that the effects of poly-lysine peptides depend on the length of the peptide, its

stereochemistry, and the link between lysine residues in homo-polymers. The results indicate potent and similar lethal effects of 25–30 residue long α-poly-L and poly-D-lysine peptides on *P. falciparum* with no effect of ε-poly-lysine peptides. Coupling of poly-lysine with non-peptide moieties, as well as the use of poly-basic branching dendrimers, show an increase in the effectivity of poly-lysine peptide-based therapies against *P. falciparum* in vitro with no apparent cytotoxic effects towards human tissue cultures. Using fluorescently labelled and biotinylated poly-lysine probes for live-cell imaging, this study demonstrates that the effects of poly-lysine peptides are associated with specific interactions with the membranes of infected erythrocytes or late-stage erythrocytic parasites. Finally, the pull-down assays from infected erythrocytes on a poly-lysine matrix coupled with mass-spectrometry-based proteomic analyses indicate a set of *P. falciparum* merozoite and erythrocyte membrane and adhesion proteins as the main targets of poly-basic peptides. The results demonstrate a novel route forward for the potential development of new and powerful peptide-based therapeutics against malaria parasites.

Methods

Parasite culture

Plasmodium falciparum strains (Dd2, Nf54, MRA-1238, MRA-1239) were cultured in de-identified human erythrocytes, maintaining ~ 2% parasitaemia and 5% haematocrit. The culture medium consisted of RPMI 1640, enhanced with 5 g/L of Albumax II (Thermo Fisher Scientific, 11,021,037), 0.12 mM hypoxanthine (prepared by adding 1.2 ml of 0.1 M hypoxanthine to 1 M NaOH), and 10 µg/mL of gentamicin [26], Artesunate (Acros) was used to treat *P. falciparum* cells with artemisinin. All parasite cultures were maintained statically, with atmospheric conditions provided by a candle jar.

Parasite sorbitol synchronization

When synchronization was needed, 5% sorbitol (S6021) (by weight/volume) was employed to get the resulting *P. falciparum* culture (primarily ring stage, 5% haematocrit, > 3% parasitaemia). The resulting parasite culture had to be at least 60% synchronized. If synchronization was below 60%, it was repeated in 6 h and 48 h to achieve optimal synchronization [27].

Drug assay

A stock solution for each drug was prepared by diluting 1 mL of a 10 mg/mL drug solution in sterile water, followed by a 10-min sonication. For this study:

Alamanda Polymers Products used:
PLL-20 (000-DKB030-103PLKB20)
PLL-30 (000-KB030-101)
PDL-30 (000-KB050-106PDKB30)
PLL-50 (000-KB050-106)
10-PEG-10 (000-KB020-102PLKC10-b-PEG5K-b-PLKC10).
50-PEG-50 (050-2KC010-101PLKC50-b-PEG1K-b-PLKC50).
PEG-50 (mPEG1K-b-PLKC50).
Sigma products:
POLY-L-LYSINE (4000–15000): P-6516
POLY-D-LYSINE (1000–5000): P-0296
POLY-L-ARGININE: P-4663
POLY-D-LYSINE (4000–15000): P-6403
Poly-L-Ornithine hydrobromide: P-3538–50 MG.
Poly-L-Arginine hydrochloride (10 mg): P4663-10MG.
Poly-D-glutamic acid sodium salt (1 g): P4033.
Artesunate (artemisinin) from Acros.
Heparin from Creative PEGWorks.
Genscript Products:
Rhodamine-AAAYPYDVPDYAAAK$_{25}$: 9.2 mg, costume synthesis LOT: U527JGJ060-3/PE6021.
FiTC-AAAYPYDVPDYAAAK$_{25}$: 9 MG, costume synthesis LOT: U527JGJ060-1/PE6019.

A diluted solution was obtained by combining 392 µL of the designated medium with 8 µL of this stock, which was then chilled on ice. Parasite culture, set at a total volume of 10 mL, was maintained at 4% haematocrit and 1% parasitaemia, which was achieved by adjusting a 400 µL sample of 1% parasitemic blood with the necessary medium. For plating, each well of the plate received 100 µL of DSM1 medium using a multichannel pipet. The first row was treated with 100 µL of the diluted drug solution, with distinct drugs in separate columns, and some columns were kept drug-free for control. Serial dilutions were created by transferring 100 µL from one row to the next and discarding 100 µL from the final row to maintain a consistent volume. Each well was then seeded with 100 µL of erythrocytes and incubated at 37 °C. Additional erythrocytes were used for smear preparation to examine parasitemia initially and post-incubation if required. Control measures included determining parasitaemia at the start (0-h mark) and in the drug-free wells after 72 h. Parasitaemia analysis after the 72-h incubation was conducted using flow cytometry.

Drug and toxicity assay

The protocol for the NYU core drug and toxicity assays can be found at: https://med.nyu.edu/research/scientific-cores-shared-resources/sites/default/files/p-falciparum-in-vitro.pdf [28]. Shortly to describe the procedure: The in vitro growth inhibition assay for *P. falciparum* was conducted using 96-well plates, testing each compound in duplicate and controls in triplicate. *Plasmodium falciparum* 3D7 cultures at 5% haematocrit were maintained

under 1% oxygen, 5% carbon dioxide, and 94% nitrogen. Cultures were synchronized and maintained below 6% parasitaemia. For drug treatment, 100 µL of complete media with the test compound was added to each well, followed by 100 µL of the parasite culture at 0.25% ring-stage parasitaemia and 10% haematocrit. Plates were incubated for 96 h, frozen at − 80 °C overnight, then thawed at 37 °C for 4 h. Cultures were transferred to black 96-well plates, stained with SYBR Green I in lysis buffer, and incubated for 1 h at room temperature, protected from light. Fluorescence was measured at excitation/emission wavelengths of 485/530 nm to assess parasite growth. Statistical analyses were performed to evaluate the significance of the results.

The cytotoxicity assay for high-throughput screening in 96-well plates utilizes Alamar Blue to assess cell viability. Each well contains a final volume of 100 µL of cells and compound, with compounds tested in duplicate. After adding Alamar Blue, the final volume is 110 µL. The assay runs for 4 days in DMEM without phenol red, supplemented with 2% FBS and 1% PSG to prevent interference with the 590 nm absorbance reading. Controls include cells alone, dead cells (treated with 100 µM Ionomycin or another agent), and medium alone. HepG2 cells are trypsinized, harvested, and resuspended in DMEM without phenol red, followed by centrifugation and resuspension. Cells are counted, diluted to 5×10^5 cells/mL, and plated at 100 µL per well. After a 3-h incubation for cell attachment, compounds are thawed, vortexed, and serially diluted across the plate. The assay incubates for 4 days before adding 10 µL of Alamar Blue to each well, and cell viability is measured by absorbance at 590 nm.

Magnet isolation of late-stage schizonts and invasion of pretreated erythrocytes

To isolate late-stage cultures, first create a thin smear to confirm the culture is primarily in the late stage. Prep the column by washing them with 2 mL of Malaria Complete Medium (MCM). Suspend the culture in 7 mL of MCM and allow it to run through the column completely, ensuring no blood remains above the matrix. Wash the column with 5 mL of MCM until the flowthrough is clear. In a new 15 mL tube, place the magnet off the rack and add 1 mL of MCM, letting it run through. Add another 1 mL and gently use a plunger to force it through slowly to avoid rupturing cells. Transfer this elution with blood and MCM to a new flask for late stage isolation, and resuspend the flowthrough in its original flask (QuadroMACS™ Separator (130–091-051), and LS Columns (130–042-401).

Further pretreated erythrocytes with 20 µg/ml PLL-30 and non-treated were used as controls and set up invasion with 0.5% parasitaemia and 2% haematocrit.

Hemolysis assay

The previously described protocol was used to carry out the hemolysis assay [29]. The supernatant was transferred from each well to transparent, flat-bottomed 96-well plates in the hemolysis quantification protocol. Triplicate wells, using 4% haematocrit RBCs in water, ensured maximal haemolysis, with MCM serving as the blank control. Absorbance at 574 nm was recorded using the specified instrument (VICTOR Nivo Multimode Microplate Reader). Data was normalized by subtracting mean blank absorbance and further adjusted against the average of the maximum haemolysis controls to derive relative haemolysis percentages. Mean absorbance values were determined for each experimental set from triplicate measurements.

Cell labelling

Cells with approximately 5% parasitaemia were collected by transferring 100 µL into a 1 mL microcentrifuge tube, followed by centrifugation at 500 *g* for 5 min at room temperature. The pelleted cells were subsequently washed twice with phosphate-free MCM. A staining solution was prepared by diluting 5 µL of Hoechst dye in 10 mL of the same phosphate-free MCM. The cell pellet was then resuspended in 1 mL of this dye solution and incubated in the dark for 5–10 min. After staining, the cells underwent two washes with phosphate-free MCM. For further treatments, a mixture of 10 µL of the selected reagent (FITC/Poly-lysine, short-chain poly-lysine, or glutamate) and 990 µL phosphate-free MCM was added to the cells and incubated for an additional 5–10 min. The treated cells were washed twice with 1 mL MCM and resuspended in a final volume of 100 µL MCM. CellMask orange plasma membrane stain (Invitrogen) was used to stain erythrocyte membranes using a 5 mg/mL concentration as 1000× stock solution. Human tissue cultures were incubated with 10 µL of the selected reagent (FITC/Poly-lysine, short-chain poly-lysine, or glutamate) and 990 µL of opti-MEM for 30 min before imaging.

Imaging and visualization

Imaging and image analysis were performed at the Washington University Center for Cellular Imaging. Cells were visualized on an upright Nikon A1RHD25 confocal platform (Nikon Instruments, Melville, NY) using a CFI SR HP Plan Apochromat Lambda S 100XC silicone immersion objective. Z-series were collected and reconstructed using Imaris 10.0 (Oxford Instruments, Abingdon, UK). Cell count analyses and visualizations were performed using the isosurface generation function in Imaris.

Sporozoites were freshly dissected at day 14 post-infection, as described earlier [30]. Live sporozoites were incubated with 1:500 dilution of 100 mg/ml FITC labeled

Poly-L-Lysine or Rhodamine labeled Poly-L-Lysine for 30 min at room temperature, followed by three washes in PBS. For nuclear staining, 1:1000 dilution of 1 mg/ml DAPI (Roche Diagnostics) was included during Poly-L-Lysine labelling. Sporozoites were then viewed with a Zeiss AxioImager M2 fluorescence microscope equipped with an oil-immersion Zeiss plan Apo 100×/NA 1.4 objective or a Zeiss EC plan Neo 40×/NA 0.75 objective and a Hamamatsu ORCA-R2 camera. Optical z-sections with 0.2 µm spacing were acquired using Volocity software (Quorum Technologies, Puslinch, ON, Canada). For siRNA samples, only a single z-plane was developed. Images were deconvolved with an iterative restoration algorithm using calculated point-spread functions, a confidence limit of 100%, and an iteration limit of 30–35 using Volocity software. Images were cropped and adjusted for brightness and contrast using Volocity software.

Plasmodium falciparum NF54 culture and mosquito infection

Mosquito infection with *P. falciparum* NF54 was performed as previously described [30]. Asexual cultures were maintained in vitro in O^+ erythrocytes at 4% haematocrit in RPMI 1640 (Corning) supplemented with 74 µM hypoxanthine (Sigma), 0.21% (wt/vol) sodium bicarbonate (Sigma), and 10% (vol/vol) heat-inactivated human serum. Cultures were maintained at 37 °C in a candle jar made from glass desiccators. Gametocyte cultures were initiated at 0.5% parasitaemia and 4% haematocrit. The medium was changed daily for up to 15 to 18 days without adding fresh blood to promote gametocytogenesis. Adult *Anopheles stephensi* mosquitoes (3 to 7 days after emergence) were allowed to feed through a glass membrane feeder for up to 30 min on gametocyte cultures at 40% haematocrit containing fresh O^+ human serum and O^+ erythrocytes. Infected mosquitoes were maintained for up to 19 days at 25 °C with 80% humidity and provided a 10% (wt/vol) sucrose solution.

Immuno-precipitation on poly-L-lysine agarose

1 ml parasite pellet was resuspended in 5 mL PBS three times. The pellets were mixed with 2×volume of lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% IGPAL-CA-630, 5% glycerol, a protease inhibitor (PMSF 1 mM), the samples were left on ice for 15 min. Add 100ul of poly-lysine agarose mixture (Sigma P6893-5 ml, resuspended in 400 µl lysis buffer). The mixture was incubated at 4 °C for 2 h on the rocker. After 2 h, the samples were washed with 1 ml wash buffer three times (150 mM NaCl, 50 mM Tris pH 7.5, 5%glycerol). After the washing treatment, the samples were spun down, and the PLL agarose matrix was flash-frozen and sent for mass spec analysis. The negative control was erythrocyte lysate treated the same as infected erythrocytes.

Mass spectrometry and on bead digestion

For the on-bead protein digest, beads were incubated in a buffer containing 2 M urea, 50 mM Tris (pH 7.5), 1 mM DTT, and 5 µg/mL Trypsin for 1 h at 25 °C with shaking. The supernatant was transferred to a new tube, and the beads were washed twice with the buffer, combining the washes with the digest. The eluted proteins were reduced with 4 mM DTT for 30 min, followed by alkylation with 10 mM IAA for 45 min, both at 25 °C with shaking. An overnight trypsin digest was performed at 25 °C. For peptide lysate clean-up, the digest was acidified with 1% formic acid and loaded onto C18-stage tips, prepped with methanol, 80% acetonitrile/0.1% formic acid, and 3% acetonitrile/0.1% formic acid washes. After loading, the stage tips were washed with 3% acetonitrile/0.1% formic acid. Adjustments were made for low protein content, diluting the labelling reagent as necessary.

Statistical analysis

Data analysis and statistics were carried out using GraphPad Prism software (v10). Details of the statistics carried out for each experiment are included in the figure legends.

Results

Polycationic peptides inhibit *Plasmodium falciparum* growth

Recent attention has been drawn to poly-lysine due to its abilities in drug delivery, antimicrobial activity, biocompatibility, anti-cancer activity, and prion propagation prevention [16, 31]. Sulfated polyanions, especially heparin (Fig. 1A), have been shown to inhibit the invasion of erythrocytes by merozoites [23, 32], with an IC50 for heparin noted as 11 µg/ml [23]. To test the potential activity of polycationic peptides against *P. falciparum* parasites, the activity of poly-L-lysine (PLL) and poly-L-ornithine were compared with heparin and poly-L-glutamate in a single dose, 72 h in vitro treatment experiment (Fig. 1B).The poly-amino peptides in mixtures of 20–50 amino acid residues were used,with molecular weights ranging from 3.5 to 6.5 kD. A control set of parasite cultures was incubated without any compound or in the presence of PEG-3550 as an inert and non-charged compound. The activity exhibited from PLLs was comparable to that of heparin—more than 50% inhibition of parasite growth at 12.5 µg/ml for both compounds (Fig. 1B). Poly-ornithine demonstrated similar activity as PLL peptides. At the same time, parasite growth inhibition was not observed in the presence of either poly-L-glutamate or PEG-3550, arguing that the charge and structural

elements of poly-cationic peptides or heparin play an important role in this activity (Fig. 1B).

Following the experiments on parasite growth inhibition of mixed PLL peptides with different lengths (mixture of peptides with 20–50 lysine residues long; molecular weight 3.5–6.5 kDa) (Fig. 1B), the investigation focused on whether the length of poly-L-Lysine peptides plays a role in the efficiency of *P. falciparum* growth inhibition. The PLLs of defined lengths were used (20, 30, and 50 consecutive lysine residues) and followed the survival of the *P. falciparum* Dd2 strain after 72 h incubation with the different compounds (Fig. 1C). The IC50 values for each compound were obtained using acridine orange staining (Fig. 1C). The most potent inhibition was achieved in the presence of PLL-50 and calculated IC50s of 0.355 ± 0.0014 µM (3.550 µg/mL), PLL-30 followed with IC50 of 0.83 ± 0.221 µM (5.229 µg/mL) and PLL-20 IC50 of 2.2 ± 0.155 µM (9.240 µg/mL) (Fig. 1C). The differences in activity indicated that the length of the PLL peptides ostensibly impact *P. falciparum* growth, so future experiments were carried out with PLL-30 and PLL-50.

To determine if the impact of PLLs on parasite growth was due to erythrocyte lysis, a 72-h incubation of both non-infected and infected human erythrocytes with increasing quantities of PLL-30 and PLL-50 was conducted (Fig. 1D, E). Haemoglobin levels released from the lysed erythrocytes were tested at the beginning of the experiment and after 72 h for both infected and non-infected cells. The positive control was sampled with total lysis of erythrocytes, and the negative control was sampled with erythrocytes in the culturing medium without any additional compounds. The effect of PLL-30 on erythrocyte lysis was insignificant, as most erythrocyte cultures exhibited less than 5% lysis, and via unpaired student t-tests, lysis did not differ significantly from non-treated erythrocytes (Fig. 1D). PLL-50 did show significant lysis at the highest concentrations (Fig. 1E). However, at or below $3xIC_{50}$ concentration, lysis was not significant. Moreover, there was no notable difference between infected or non-infected erythrocytes. As such, these results indicate that poly-L-lysine peptides can inhibit the growth of the *P. falciparum* parasites without significantly affecting host cells at relevant concentrations.

Poly-lysine compounds are efficient against artemisinin- and chloroquine-resistant *P. falciparum* strains

As the resistance to artemisinin and ACT is rising in *P. falciparum* isolates worldwide, the effect of poly–lysine peptides on different strains of *P. falciparum*—specifically strains that are resistant to artemisinin was tested. The impact of poly-lysine on the artemisinin-susceptible (MRA-1239) and the resistant (MRA-1238) strains was tested [33]. Peptides with runs of 30 and 50 consecutive lysines (PLL-30 and PLL-50) and the more protease-resistant poly-D-lysine peptide (PDL-30) were utilized. Both PLL-30 and PLL-50, as well as PDL-30 and PDL-50, had similar effects on the growth of *P. falciparum* parasites (Fig. 2). Growth of the MRA-1239 strain was inhibited with an IC50 of approximately 11 ug/ml of poly-lysine peptides (Fig. 2A). In comparison, the artemisinin-resistant strain (MRA-1238) was inhibited at an IC50 of 8 ug/ml (Fig. 2B). The effects of poly-D-Lysine (PDL-30), poly-L-Lysine (PLL-30 and PLL-50), and artemisinin (ART) on the growth of chloroquine-resistant (Dd2) and non-resistant (NF54) strains of *P. falciparum* (Fig. 2C and D) were compared. The IC50s values for the chloroquine-resistant Dd2 strain were lower than those of the chloroquine-sensitive NF54 strain, with average values of 6 µg/ml and 12 µg/ml, respectively. No difference between PLL-30 and PDL-30 peptides, as both were equally effective and active against the chloroquine-resistant (Dd2) and chloroquine-susceptible (NF54) strains. Finally, the toxicity of the PLL-30 peptides was assayed using cultured HepG2 cells (Fig. 2E). PLL-30 peptides did not exhibit any additional toxic effects compared to control conditions (addition of DMSO) within the range of their observed activity against malaria parasites. The experiments indicated that despite the differences in the chirality of the lysine residues, L versus

(See figure on next page.)

Fig. 1 In vitro anti-*Plasmodium* effect of positively charged peptides and their impact on erythrocyte's lysis. **A**. Structure of L-Lysine and D-Lysine and an indication of link in poly-Lysine polymers (n-depicts a different number of residues in homopolymers), L-Ornithine, and heparin. **B** *P. falciparum* Dd2 strain growth assay in the presence of different compounds and amino acid polymers. Data represent endpoint parasitaemia after 72-h treatment with the indicated compound. Results are normalized to non-treated cultures (control) and presented as a percentage of control samples. The average value from three biological replicates is reported with a standard deviation. **C** IC50 values for different poly-L-lysine homo-polymers (PLL). PLL-20, PLL-30, and PLL-50 treat *P. falciparum* Dd2 strain. Results are normalized to non-treated cultures, and the graph presents two biological replicates done in triplicates with a standard deviation. **D**, **E** Effects of PLL-30 and PLL-50, respectively, on lysis of *P. falciparum*-infected (red) and non-infected erythrocytes (black) (technical triplicate with two to three biological replicates, error bars represent SEM). IC_{50} values for *P. falciparum* are indicated on the graph. Via unpaired student t-test, each concentration is compared to its non-treated control of either infected or non-infected RBCs. P-values > 0.05 are not marked; *p-value < 0.05, and **p-value < 0.01

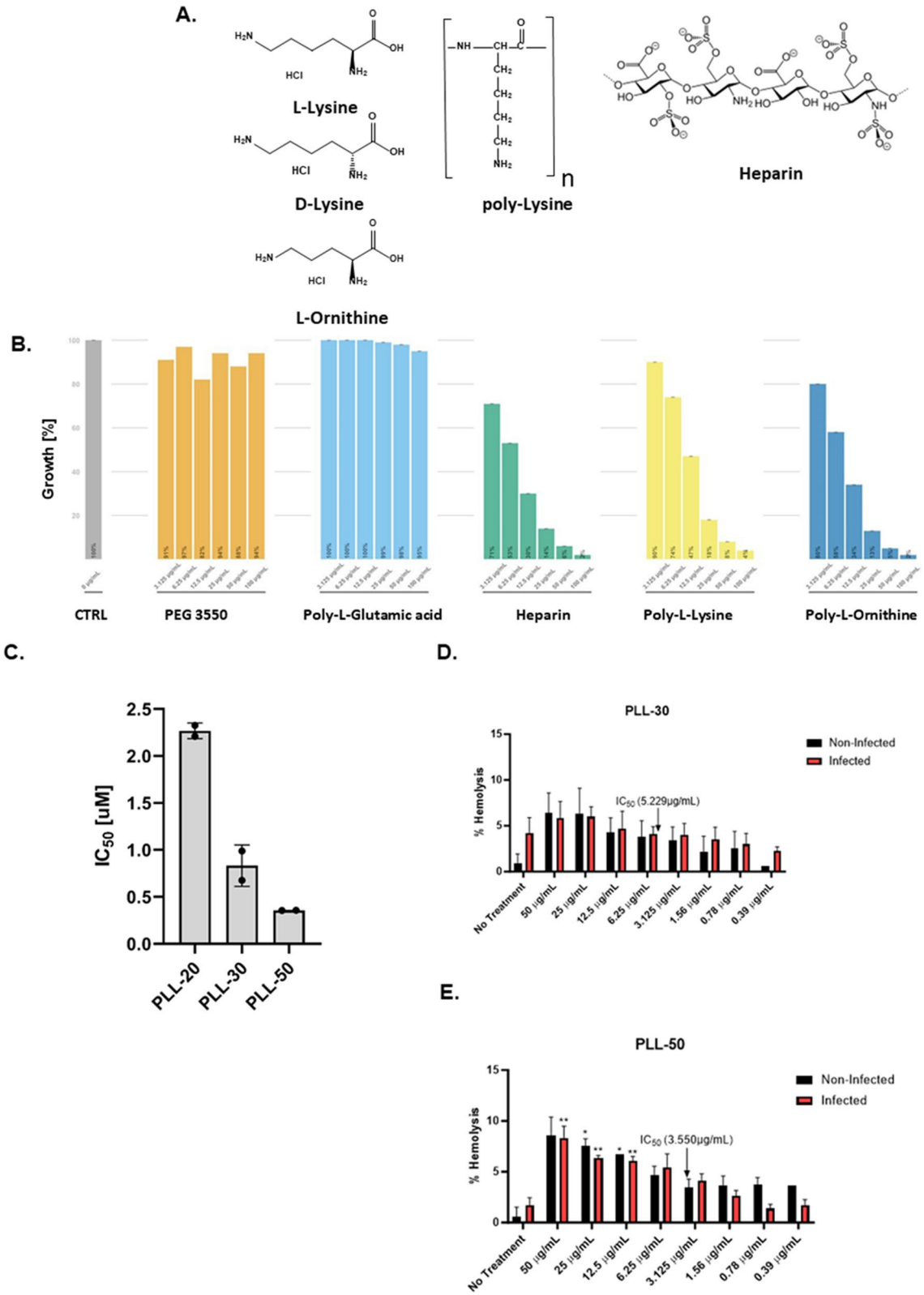


Fig. 1 (See legend on previous page.)

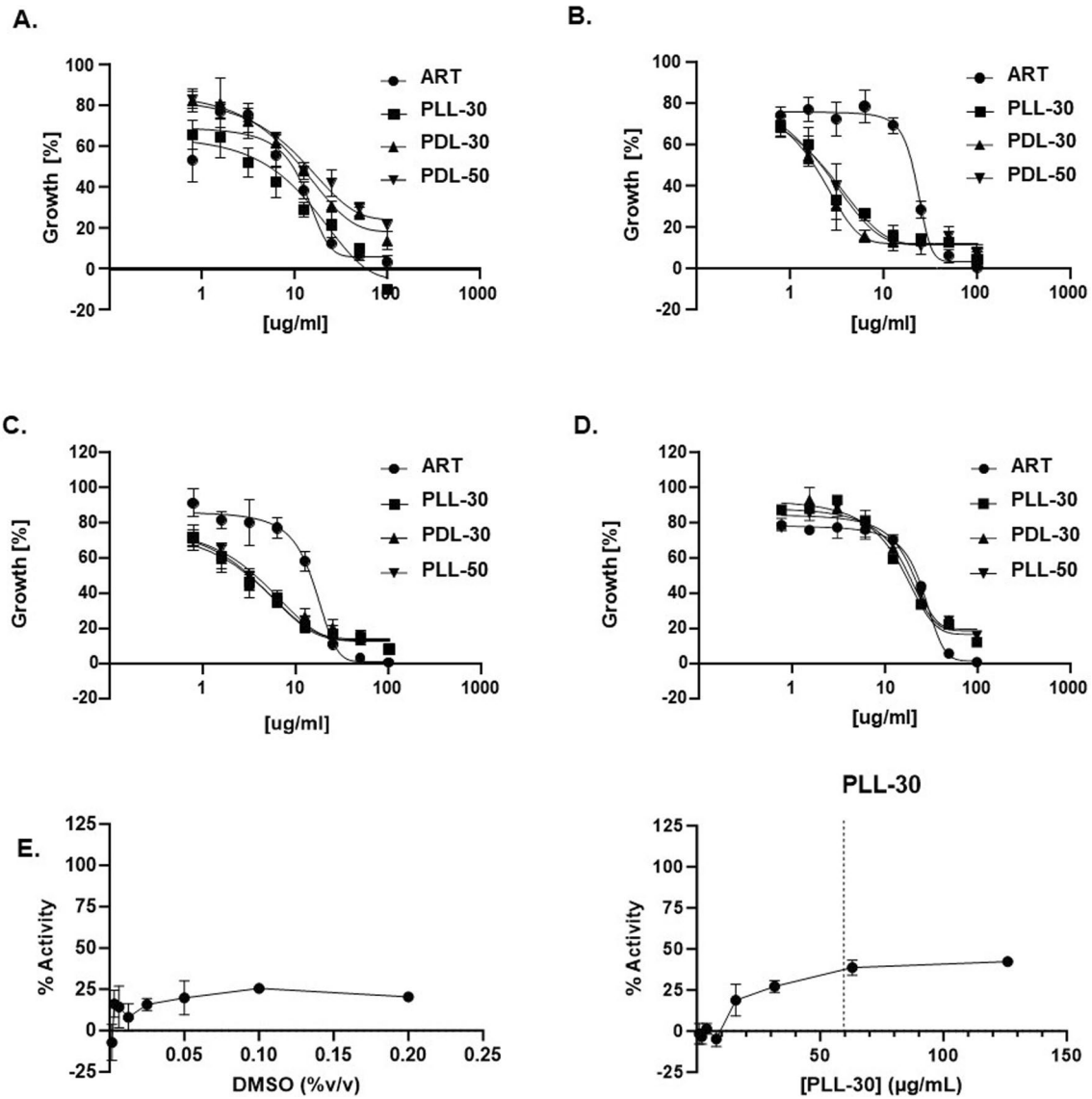


Fig. 2 Different forms of poly-lysine have the same activity on artemisinin or chloroquine-resistant and non-resistant *P. falciparum* strains with low human cell cytotoxicity. **A**, **B** Effects of poly-L-Lysine (PLL-30) and poly-D-Lysine (PDL-30 and PDL-50) compared to (●) artemisinin (ART) on the growth of *P. falciparum* artemisinin-resistant (MRA-1238) and non-resistant strains (MRA-1239), respectively (three biological replicates, with standard deviation). **C**, **D** Effects of poly-L-Lysine (PLL-30) and poly-D-Lysine (PDL-30 and PDL-50) compared to (●) artemisinin (ART) on the growth of *P. falciparum* chloroquine-resistant (Dd2) and non-resistant strains (NF54), respectively (three biological replicates, with standard deviation). **E** Cytotoxicity of PLL-30 within HepG2 cells. Cell viability was presented as a % normalized of untreated control, and dilutions of DMSO were used as a control for dilutions of drugs (three biological repeats). The dashed line indicates IC50 values against *Plasmodium* growth for each tested compound

D, PLL, and PDL peptides had the same growth inhibition effect on different *P. falciparum* strains. Moreover, parasite inhibition was achieved regardless of parasite resistance to artemisinin or chloroquine, arguing for novel parasite targets by poly-lysine peptides.

Modifications of poly-lysine peptides further inhibit the growth of *P. falciparum*

Previous research has indicated that PEG modification of peptides (PEGylation) can enhance the effectiveness of peptide-based therapies (16). PEGylation, the covalent

attachment of PEG molecules to another molecule (often a drug or protein), can improve the solubility, stability, and half-life of the drug or protein [34]. To test the effect of PEG modifications on poly-lysine peptides and their ability to inhibit the growth of *P. falciparum* in erythrocytes, a comparison was first made between PLL-30 peptides PEG-PLL-50 (A–B connection of PEG (B) to peptide (A)) and PLL-50-PEG-PLL-50 (A-B-A connection) copolymers (Fig. 3A). The IC50s for all three compounds were in the sub-micromolar range. The activity of the PEGylated peptides was, on average, 7 to 9 times higher with associated IC50s around 126 nM for PEG-PLL-50 (~2.15 µg/mL) and 94.1 nM for PLL-50-PEG-PLL-50 (1.63 µg/mL) while the IC50 for PLL-30 was at 857 nM (5.5 µg/mL). Previously tested PLL-50, PEG-PLL-50, PLL-50-PEG-PLL-50 and poly-epsilon-lysine (poly-ε-lysine) peptides were utilized. PLL-50, along with longer PEGylated poly-lysine copolymers (PEG-PLL-50 and PLL-50-PEG-PLL-50), all having similar effects on parasite growth inhibition; 50% inhibition at 11 µg/ml (IC50s in the range of 90–1030 nM) (Fig. 3B, Supplementary Fig. S1). A shorter PEGylated poly-lysine copolymer (PLL-10-PEG-PLL-10) was less effective, with 50% growth inhibition at 5.5 µg/ml (IC50 in the range of 1 µM) (Supplementary Fig. 1). Parasite growth inhibition was not observed using the poly-ε-lysine peptides (Supplementary Fig. 1). As in the case of non-modified PLLs, the HepG2 toxicity assays indicated that PEGylated PLL copolymers have relatively low toxicity in the concentration range where they exhibit potent parasite growth inhibition (< 30 µg/mL). However, higher toxicities were associated with high concentrations of PEGylated PLL copolymers (> 80 µg/mL) when incubated for 72 h with HepG2 cells (Fig. 3C).

Finally, given that polyamidoamine (PAMAM) dendrimers, as synthetic mimics of branched poly-lysine peptides, have been investigated for a wide range of biomedical applications due to their low cytotoxicity and potential to be further modified, this study tested their activity against *P. falciparum* parasites [35–38]. Three different PAMAM compounds classified as 2.5-, 4-, and 4.5- generation, based on their distinct architecture, size, shape, and surface functionality (Fig. 3D) were investigated. PAMAM 4.0 generation showed the highest activity in the inhibition of *P. falciparum* growth, with IC50 in the range of 2.5 µg/ml (Fig. 3D). PAMAM of 2.5 and 4.5 generation exhibited lower activity in inhibiting *P. falciparum* growth with IC50s around 13 and 21 µg/ml, respectively. While this observed difference could be due to the distribution of positive and negative charges between PAMAMs of different generations, this study's results with both PEGylated or branched modified poly-lysine peptides and co-polymers strongly implicate the role of positively charged polymers in the effective inhibition of *P. falciparum* parasites growth (image created using BioRender.com).

PLL peptides bind to the membranes of parasites and infected erythrocytes

Previous studies have indicated that *P. falciparum* proteins with poly-lysine repeats are associated with cellular adhesion and are likely to be located at the parasite's surface [22]. To assess the mechanism of action and localization of the poly-cationic peptides and co-polymers against the malaria parasite, a fluorescein isothiocyanate (FITC)-labelled 25 lysines residue-long PLL peptide was designed (Fig. 4A). In addition to the FITC-labelling, HA- and biotin-tagged peptides were engineered. A previously tested PLL-30 peptide by binding one FITC molecule per each 10-lysine residue. The activity of the labeled and modified peptides in inhibiting the *P. falciparum* growth was tested for 72 h. Similar trends in parasitic growth inhibition were observed when FITC-HA-PLL-25-BIO or FITC-labelled PLL-30 peptides were incubated with parasites, albeit without complete 100% inhibition (90% inhibition achieved) at the working concentrations (Fig. 4A). Imaging of the parasite cultures in the presence of the FITC-labeled peptides indicated that the poly-lysine peptides were bound to the membrane of infected erythrocytes or parasites (Fig. 4B and C, Supplementary Fig. 2). Moreover, labeled PLL peptides were bound to specific developmental stages of *P. falciparum* parasites—the merozoite surface (Fig. 4B) as well as the late schizont stage (Fig. 4C). The labelled peptides did not

(See figure on next page.)

Fig. 3 Modifications of poly-Lysine as well as charged polyamidoamine (PAMAM) dendrimers indicate inhibition of *P. falciparum* growth in erythrocytes with low human cell cytotoxicity. **A** IC50 values determined in technical and biological triplicate for non-modified (PLL30) and PEG-ylated forms of PLL (PEG-PLL-50 and PLL-50- PEG-PLL-50) on *P. falciparum* Dd2 strain growth (error bars represent SEM). **B** Relative IC50s of PLL-50, PEG-PLL-50, and PLL-50- PEG-PLL-50 on *P. falciparum* Dd2 parasite growth compared to the non-PEGylated form. Performed in technical triplicate (error bars represent SEM). **C** Cytotoxicity of PLL-50 with PEG modifications on HepG2 cells. Cell viability was presented as a % normalized of an untreated control (three biological repeats are indicated in each graph). The dashed line indicates IC50 values against *Plasmodium* growth for each tested compound. **D** IC50 values for polyamidoamine (PAMAM) dendrimers of different generations (2.5, 4.0, and 4.5 generation) used for the treatment of *P. falciparum Dd2* strain (three biological replicates, with standard deviation). Schematics of the PAMAM molecule of generation 4 with characteristics of the molecule are shown (Biorender)

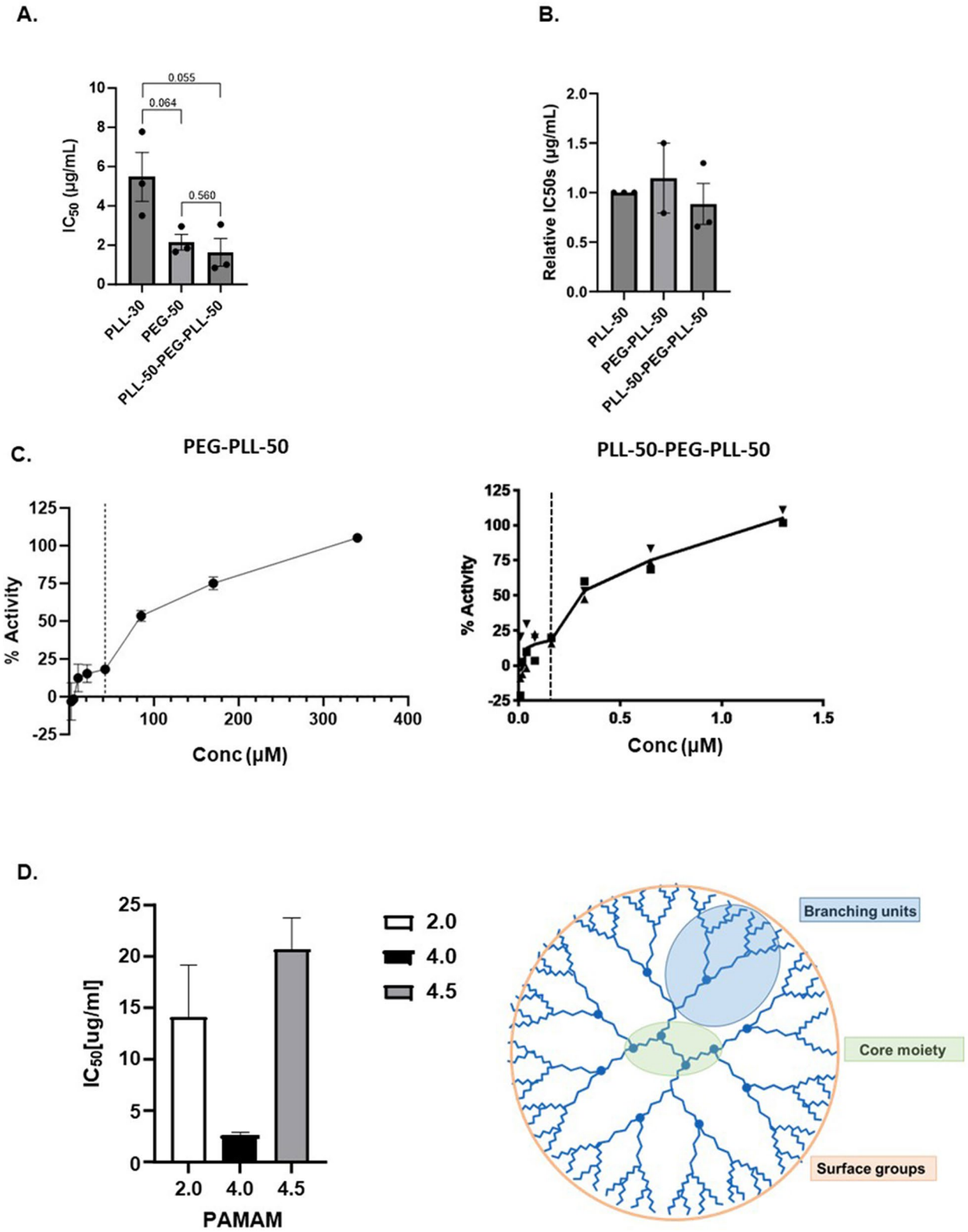


Fig. 3 (See legend on previous page.)

bind to uninfected erythrocytes or human tissue cultures used as controls (Fig. 4B, C, Supplementary Fig. 2) highlighting their specificity towards *P. falciparum* targets or modifications to the human host cell membrane influenced under *P. falciparum* infection.

Since the *Plasmodium* life cycle involves parasite development in mosquitoes, the influence of PLL on parasite growth and development in mosquito guts was tested. No effect of PLL on the oocyst parasite growth stage in mosquito hosts was observed (Fig. 4D). However, imaging of FITC-HA-PLL-25-BIO peptides incubated with *P. falciparum* parasites isolated from mosquito salivary glands indicated that PLL peptides could interact with sporozoite membranes (Supplementary Fig. 3). The binding of FITC-HA-PLL-25-BIO peptide to sporozoites had a similar distribution as previously seen in imaging from parasite merozoites and schizonts. However, some residual PLL peptides could be present on the erythrocyte membranes and could further prevent parasite invasion. To test this possibility, fresh erythrocytes were incubated with 20 µg/ml of PLL-30 for 30 min, followed by washing off the PLL-30 and tested *P. falciparum* invasion by incubation with isolated late-stage schizonts (Supplementary Fig. 3). A decrease in parasite growth by 55% compared to the control was observed, indicating reduced invasion by pre-treatment of erythrocyte membranes with PLL-30 (Supplementary Fig. 3). Results using FITC-labeled PLL peptides indicate that PLL peptides can bind specifically to the membrane of *P. falciparum* parasites or infected cells across multiple parasite developmental stages. Additionally, the pretreatment of erythrocytes with PLL peptides can further prevent infection of new erythrocytes by blocking the invasion of parasites.

Poly-lysine peptides bind to a subset of *P. falciparum* membrane proteins, potentially inhibiting erythrocyte invasion

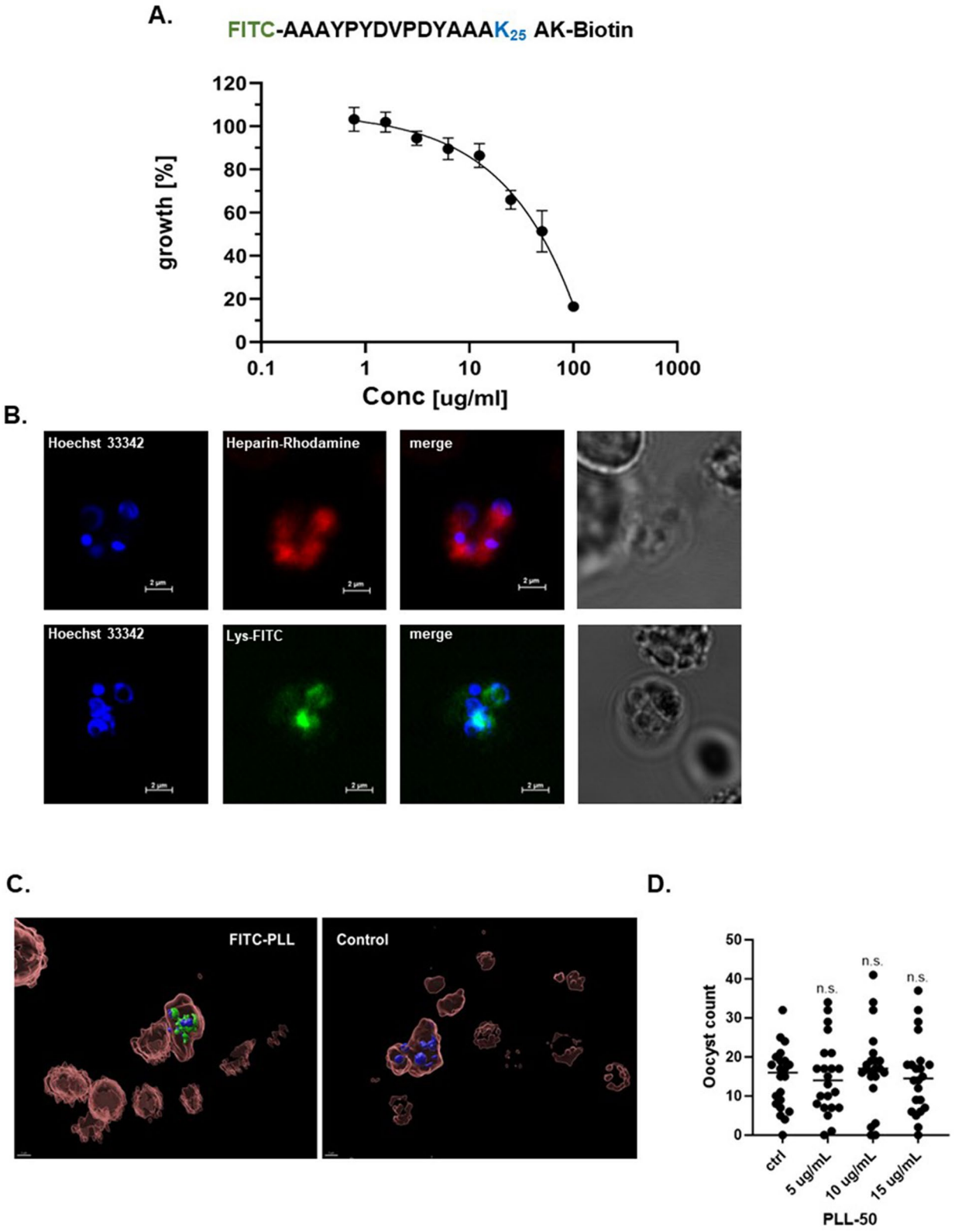
Given that poly-lysine peptides could affect the function of many different proteins, the potential targets of poly-lysine and poly-cationic peptides in *P. falciparum* and parasite-modified human host cells were assesed. The poly-L-lysine agarose, PLL chains with 30–70 residues was incubated with cell lysates from erythrocytes either infected or uninfected with the *P. falciparum* Dd2 strain using non-infected erythrocyte lysate as control (Fig. 4A). Infected erythrocyte cultures were previously developmentally synchronized to increase the number of parasite late schizonts and merozoites from the parasites. Using mass-spectrometry (Fig. 4B), 1295 proteins from either human or parasitic cells were identified (Supplementary Table 1). Of these, 950 proteins were identified with more than two peptides in spectral counts (Fig. 4C, Supplementary Table 2). Six hundred forty-five proteins showed an increase in infected erythrocytes compared to non-infected control (Supplementary Table 2). Most of these proteins were *P. falciparum* proteins associated with various cellular processes, with 423 proteins enriched over 1.5-fold (473 *Plasmodium* proteins, Supplementary Table 2). Since the previous experiments and incubation of the parasite cultures with FITC-labelled PLLs indicated membrane association of the charged peptides, the analyses focused on the *P. falciparum* membrane and surface proteins. Based on gene annotation, 75 *P. falciparum* exported and membrane-associated proteins were identified, from which 57 proteins had significant p-values and enrichment over threefold (Fig. 4C and Supplementary Table 2). These *P. falciparum* proteins were associated with merozoite invasion, host cell surface expression and interaction, and vacuole membranes and extracellular granules. In addition to *Plasmodium* proteins,172 human host proteins were identified, of which 134 were enriched over 1.5-fold in *Plasmodium*-infected cells. Finally, 46 host membrane-associated proteins were identified, of which 25 proteins had significant p-values and enrichment over threefold within host cells (Supplementary Table 2). Considering the data using FITC labelled PLLs and LC–MS experiments on the PLL matrix, multiple surfaces and membrane-associated proteins of *P. falciparum* can be indicated as potential targets of poly-lysine and other poly-cationic polymers.

Invasion of pretreated erythrocytes decreases parasites growth

The FITC labelled poly-lysine showed labelling of parasites without the labelling of erythrocytes. Further incubated the erythrocytes with PLL-30 for 30 min, washed

(See figure on next page.)

Fig. 4 Biotinylated and fluorescence (FITC) labeled poly-lysine peptides indicate the activity of polybasic peptides at membranes of *P. falciparum* in a stage-specific manner. **A** Inhibition of *P. falciparum Dd2* growth using biotinylated and FITC labeled poly-lysine (FITC-HA-PLL-25-BIO (three biological replicates, with standard deviation, are presented in each experiment). **B** Rhodamine-labeled heparin and FITC-PLL binding to *Plasmodium* merozoites. The FITC-PLL-Biotin indicates membrane binding on merozoites. Hoechst 33,342 is used for nuclear staining. The bar indicates 2 um length**. C** Airyscan confocal fluorescence imaging microscopy of FITC-PLL-Biotin treated (FITC-PLL) and non-treated *P. falciparum* Dd2 erythrocytes at late stages of parasite development. Hoechst stain was used to visualize *P. falciparum* nuclei. Bar represents the size of 2 µm. **D** Effect of PLL-50 on oocyst survival. Counts of viable oocysts between control (ctrl) and treated samples (5, 10, and 15 µg per ml of PLL-50) do not show any significant change (n.s. indicates p-value > 0.05 via unpaired student t-test to control)



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off the PLL-30 and did invasion with the late stage schizonts. resulted in a 55% decrease in parasite growth (Supplementary Fig. 4).

Discussion

Anti-microbial peptides (AMPs) have been used as potential therapeutic compounds for multiple single-cell microbes and viruses [12]. AMPs are usually composed of 15–60 amino acids. They can be naturally expressed in multicellular organisms or synthetically made and typically possess a positive charge under physiological pH conditions [39]. As such, the activity of AMPs is usually due to their ability to interact with the negatively charged components of bacterial cell membranes [12, 13, 15]. This interaction can lead to cell membrane disruption, resulting in cell death. The complex structures of AMPs allow them to interact with microbial membranes more efficiently or with higher specificity, and they often contain additional functional elements that can enhance their antimicrobial activity against specific microbes [10, 14, 40]. One such group of naturally- and synthetically-made AMPs represent poly-lysine peptides. Regardless of their link position, α or ε, poly-lysine peptides are potential antimicrobial agents against multiple bacterial species, yeast, or fungi [41, 42]. Interestingly, also *P. falciparum,* and *Plasmodium* spp. Generally, there are numerous poly-L-lysine stretches in proteins involved in the parasites' life cycle functions, mainly focused on cell adhesion and pathogenicity [18]. Previous work exploited heparin, a negatively charged glycosaminoglycan, to interfere with *P. falciparum* invasion [23, 24]. Furthermore, charge distribution and polarization of *P. falciparum* membrane during invasion make a compelling case to test peptides of different charges against parasites [25]. Given that the range of AMPs has shown potential activity against malaria parasites and their mosquito vectors, the possibility of targeting *P. falciparum* parasites with different poly-lysine and poly-cationic polymers was explored.

Previous work from multiple laboratories has focused on inhibiting *P. falciparum* growth or development using naturally occurring or complexed synthetic peptides [11] with a mix of charged and hydrophobic residues. The results indicate a potent anti-malarial activity of compounds based on simple poly-lysine or poly-cationic polymers with additional non-amino acid modifications (Figs. 1–4). These compounds show sub-micromolar potency against *P. falciparum* asexual blood stage and complete inhibition of further growth in a single dose treatment of in vitro parasite cultures in human erythrocytes. The activity of poly-lysine peptides is not dependent on the chirality of the lysine residues (L or D). Still, the activity of poly-lysine peptides is associated with peptide length and residue linkage (Figs. 1 and 2). The results indicate that peptides with a length of 30–50 lysine residues and an α-link between the lysine residues exhibited robust anti-malarial activity. These synthetic poly-lysine peptides, PLLs, and PDLs inhibit the growth of multiple *P. falciparum* strains, including one with known artemisinin resistance (Fig. 2), the compound in the current therapy of choice to treat malaria infection. Importantly, no cytotoxic effects were observed of poly-lysine peptides on cultured HepG2 cells. No significantly increased haemolytic activity of PLLs was observed at concentrations that cause efficient inhibition of parasite growth ($\leq 3xIC_{50}$). Cytotoxicity and hemolytic activity of antimicrobial peptides have previously been described as a reason for the limited use of such compounds in treating various infections and diseases [43, 44].

The results also indicate that the naturally produced antimicrobial peptide and common food preservative poly-ε-lysine did not exhibit any anti-malarial activity (Fig. 3). This is in contrast to studies on Gram-positive and Gram-negative bacteria, where ε-poly-L-lysine show far greater bactericidal activity than α-PLL [45–48]. This discrepancy is likely due to the different structures and distinct modes of action of these poly-lysine polymers, as previously observed in evaluating multiple poly-lysine polymers [49]. Poly-L-lysine's activity might be relatively low compared to other more complex or modified antimicrobial peptides [50–53]. Amphiphilic compounds based on partially PEGylated poly-lysine peptides may offer several advantages, such as reduced cytotoxicity, the induction and stabilization of self-assembled nanoparticles, and extended in vivo circulation time. These nanoparticles demonstrated comprehensive antimicrobial activity against clinically relevant Gram-positive and Gram-negative bacteria while maintaining minimal hemolytic activity [54]. The results using PEGylated poly-lysine co-polymers illustrate the growth inhibition of *P. falciparum* blood stages at lower IC50 concentrations. PEGylated PLL-50 or copolymer of PEG with two PLL-50 peptides showed IC50s 4 to 8 times lower than non-PEGylated shorter PLLs (as μM) and retained inhibition activity comparable to non-PEGylated PLL50 (Figs. 1C, 3A, B).

In addition to PEGylation, further efforts were made to mimic poly-L-lysine polymers or combine them to enhance their antimicrobial activity and stability. These included combinations of charged non-amino acid moieties or the use of branched lysine or non-lysine polymers such as dendrimers [16, 50]. A series of such compounds are already used in multiple biomedical applications, including drug delivery and, more specifically, as vehicles for gene therapy [35, 51, 55, 56]. The experiments tested three generations of poly-amidoamine, or PAMAM, dendrimers with different mixtures of amine and

carboxyl-terminal groups. PAMAMs of 2.5, 4, and 4.5 generations, where a half-generation number indicates that only one type of reaction has been carried out: amination or methylation. The results suggest that PAMAM dendrimers of generation 4, where four complete generation cycles are carried out, resulting in a larger, more complex molecule with more terminal amino groups present on the surface, demonstrate the best growth inhibition against *P. falciparum* parasites (Fig. 3). While PAMAM 2.5 and 4.5 showed reduced activity, likely due to the reduction in the number of charged surface groups, they show the potential for further modification. The variability in modifying these molecules makes them attractive candidates for further investigation as antimalarial drugs.

Previous studies targeting malaria parasites with antimicrobial peptides have indicated activity for some of the tested peptides, mainly in micromolar ranges [11]. However, these studies did not define specific target molecules or the mode of action of tested peptides but rather reported developmental stages influenced by active peptides [11]. Given that most antimicrobial peptides act at the cell wall or the membranes of the targeted microbes, the focus was on finding out how poly-lysine and polycationic peptides target and act on *P. falciparum.* Fluorescently-labelled poly-lysine (FITC-PLLs) as well as fluorescently-labelled and biotinylated HA-tagged PLLs (FITC-HA-PLL-25-BIO) peptides were used to target blood stages of *P. falciparum* (Fig. 4 and Supplementary Fig. 2). Incubation of these labelled and modified PLLs exhibited similar and effective growth inhibition against the tested *P. falciparum* Dd2 strain. Moreover, by imaging parasite cultures in the presence of FITC-labelled PLLs, that the binding of peptides was specific for the parasites in the merozoite and late schizonts stages of the asexual cycle was noted. The FITC-PLLs did not affect the development of the parasites in the mosquito stage as tested oocytes did not show any reduction in number (Fig. 4D). However, it was observed that fluorescently labelled peptides bind to the sporozoite stage parasites isolated from mosquito salivary glands (Supplementary Fig. 3). While FITC-PLLs had a similar pattern of membrane binding, as seen in merozoites, the effect of PLLs on sporozoites and later stages of parasite growth needs to be evaluated by further study.

Previous work using either PLL or PDL with or without FITC labelling indicated that positively charged peptides neutralized the negative charge of the cell surface in different cells [55, 57]. It was noted that PLLs might have a similar effect on *Plasmodium* membranes as FITC-labelled peptides exhibited similar binding patterns to parasite membranes at different stages. Further identification of potential protein targets of PLLs on the surface of infected erythrocytes or the *Plasmodium* membrane was sought. Incubating cell lysates of erythrocytes infected with *P. falciparum* on a poly-L-lysine matrix enabled us to identify a subset of highly enriched parasite proteins (Supplementary Table 2). While both cytoplasmic and membrane proteins were enriched as identified via LC–MS analysis, due to the observed membrane binding of FITC-PLLs, analysis focused on secreted, adherent, and membrane-inserted proteins of *Plasmodium* in the obtained data (Supplementary Table 2). Amongst these proteins, the most significant and potential targets of PLLs were Clag9 (PF3D7_0935800; a merozoite surface protein involved in invasion of human erythrocytes), PHISTb, PHISTc (PF3D7_0401800, PF3D7_0801000 respectively) which are possible vaccine candidates, GARP (PF3D7_0113000; involved in programmed cell death), EMP1-trafficking protein (PTP4; PF3D7_0730900; required in virulence and rigidity), and PIESP2 (PF3D7_0501200) which is considered a possible vaccine candidate [58–62]. Furthermore, additional identification of MDR1 and Kelch13 [63, 64] in LC–MS experiments as potential targets of PLLs indicates further possibility to address the resistance to the ACT, as mutations in these proteins give rise to artemisinin resistance. Finally, some shorter poly-lysine peptides may penetrate the parasite cells and interact with MDR1 and Kelch13 [63, 64], as seen from this study's LC–MS experiments. Studying the interactions and possible implications on molecules like Kelch13 and MDR1 could shed light on strategies to address resistance to ACT, as mutations in these proteins have been recognized as key factors in

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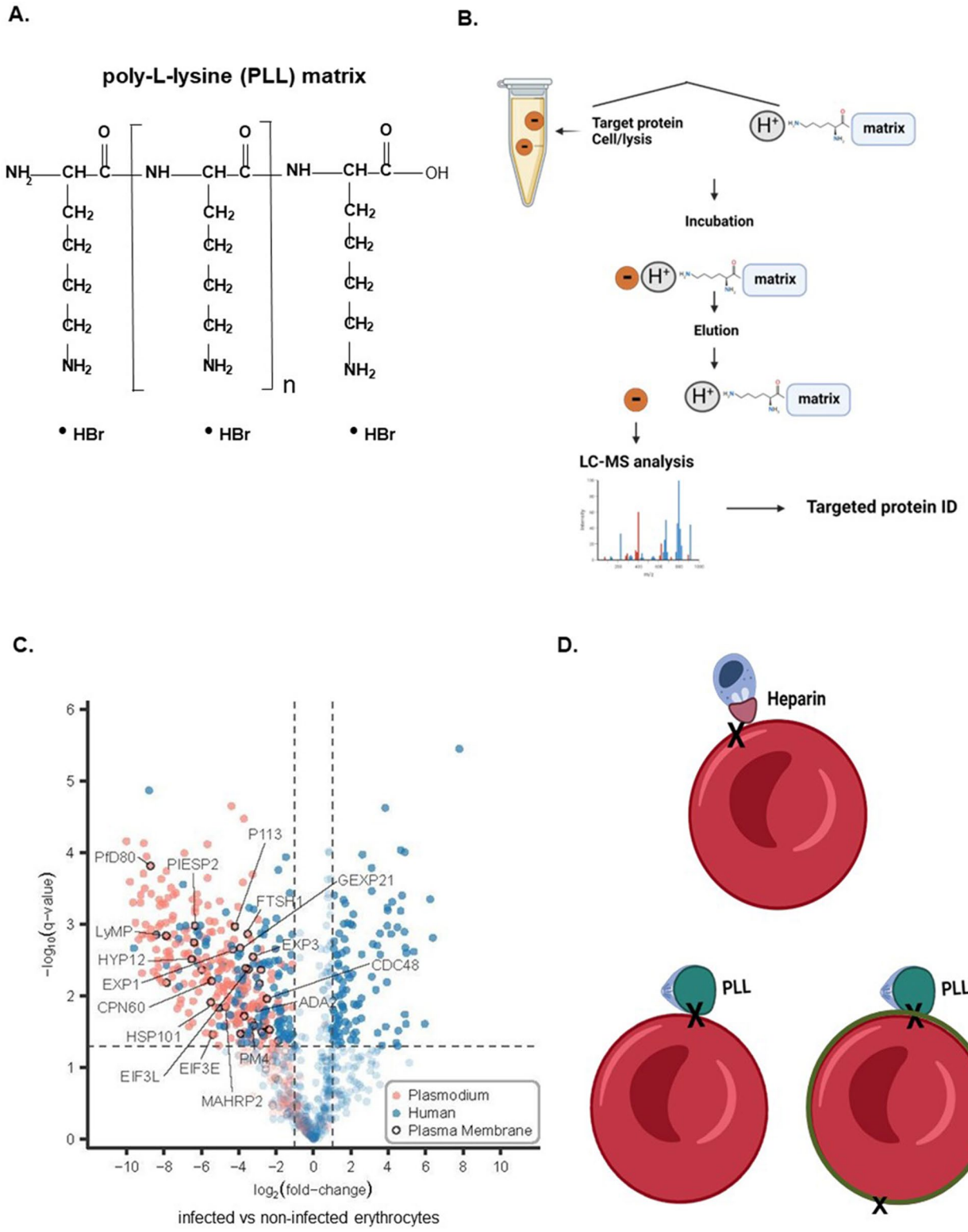
Fig. 5 Identification of poly-L-lysine target(s) and proposed mechanism of *P. falciparum* growth inhibition by polybasic peptides. **A** Poly-L-lysine (PLL) matrix used to identify the target proteins. **B** Erythrocytes infected with P. falciparum were lysed and incubated with a PLL matrix in tri-biological replicates. Non-infected erythrocytes were used as controls. The proteins bound to the matrix were eluted, and LC–MS analysis was performed to indicate potential targets of poly-l-lysine peptides. **C** Volcano plot of PLL matrix enriched *P. falciparum* (red) and erythrocytes (blue) proteins from infected and non-infected human erythrocytes. Some of the potential targets of PLL peptides at *P. falciparum* cells and plasma membrane proteins are indicated (Supplementary Tables 1 and 2). Membrane-associated proteins from *P. falciparum* and human erythrocytes are labeled with closed circles. **D** Proposed mechanism of action for PLL peptides on the invasion of erythrocytes by *P. falciparum*. Positively charged PLL peptides bind to the merozoite body, which is mainly negatively charged compared to the mechanism of heparin binding to the apical part of merozoites. Both compounds act as potential inhibitors of parasite invasion of human erythrocytes (image created using BioRender.com)



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artemisinin resistance. Based on previous studies with heparin [23, 24], this study's PLL pull-down, enrichment of the subset of proteins involved in erythrocyte invasion, those exposed on infected erythrocyte membrane surfaces, as well as late schizont and merozoites stage utilizing fluorescently labelled PLL, a proposed mechanism of action for poly-lysine and poly-cationic peptides against *P. falciparum* is presented (Fig. 5D). The positively-charged peptides appear to bind to the predominantly negatively-charged body of the merozoite, similar to that of the binding of heparin to the apical region of merozoites [23–25]. Such a binding mechanism may inhibit or delay the invasion of human erythrocytes by the parasite, which consequentially leads to parasite death, as is the case with heparin [22, 23]. As such, poly-cationic peptides could impede the progression of the infection and result in the potential clearance of parasites from the host. In addition to these effects of PLLs and poly-cationic peptides on parasites and infected erythrocytes, PLLs may also act on host proteins presented on uninfected cells that may serve as receptors for lysine-rich *Plasmodium* proteins. While imaging with FITC-labelled PLLs did not show specific labelling of uninfected erythrocytes or other tested human cell cultures, pretreatment of erythrocytes by PLL-30 followed by invasion assay with late-stage schizonts resulted in decreased invasion compared to the control (Supplementary Fig. 3). These additional effects on host membranes could also contribute to the proposed mechanism of PLLs targeting the merozoite stage and inhibiting erythrocyte invasion. Moreover, pull-downs on the PLL matrix indicated enrichment of specific human membrane proteins that were previously shown to be invasion receptors for *Plasmodium.* The hypothesis is that the interaction of PLL's with RBC invasion receptors is weak as labelling of uninfected RBCs was not seen via fluorescent microscopy, only via LC–MS. It also needs to be noted that pretreatment of erythrocytes with PLLs reduced the growth of parasites by 55% but at 4 times higher concentration than the observed IC50 for the same compound (Supplementary Fig. 4). Previous studies using PLLs in mice do not report adverse effects of PLLs on haematology during short- or long-term treatment [65]. While further studies are needed, there is no indicaton that simple and pegylated PLLs impact the function of healthy, uninfected RBCs.

Conclusion

In summary, this study characterized the anti-malarial properties of poly-lysine, other poly-cationic peptides, and polymers in this study. A single dose of positively charged peptides was found to inhibit the growth of *P. falciparum* parasites in human erythrocytes in vitro. The poly-cationic peptides and copolymers targeted proteins associated with *P. falciparum* and infected erythrocyte membranes. The hypothesis is that the multivalent interaction of charged peptides and related compounds with multiple membrane proteins of *P. falciparum* leads to a reduced number of newly infected erythrocytes and potential clearance of parasites in one or two cycles of asexual replication by preventing new host cell invasion. This study reveals a possible new direction in creating peptide or peptide mimetic drugs that are potently active against malaria parasite infection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-024-05056-0.

Additional file 1.

Additional file 2.

Additional file 3.

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Author contributions

S.P.D. and S.D. wrote the manuscript R.S., S.P.D, A.J., E.J., D.G., K.F., P.B., A.T. prepared figures J.K. and M.J collected mass spectrometry data all authors reviewed the manuscript R.S. and K.F. share the first authorship.

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Data and materials availability

All data, code, and materials used in the analysis are available to any researcher for reproducing or extending the analysis upon contacting corresponding authors.

Declarations

Competing interests

Authors do not have competing interests. Authors SD, SPD, and JE hold US Provisional Patent Application Serial No. 62/696,868 "Antimalarial Compositions and Methods of Use." Associated with this study.

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