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Improving the capacity of vaccine-induced antibodies to arrest the growth of *Plasmodium falciparum*

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Abstract

Plasmodium falciparum remains a major global health threat, and the efficacy of erythrocyte-stage vaccine candidates may be limited by the rapidity of merozoite invasion, which restricts antibody access to their targets. We investigated whether invasion-inhibitory compounds could enhance the activity of neutralising anti-PfRH5 monoclonal antibodies by potentially prolonging PfRH5 accessibility during invasion. Compounds acting at different stages of egress and invasion were tested in combination with anti-PfRH5 monoclonal antibodies and vaccine-induced polyclonal anti-PfRH5 IgG using Bliss additivity analyses and fixed-ratio isobologram assays. Late-acting invasion inhibitors and egress inhibitors showed additive effects with PfRH5 monoclonal antibodies, suggesting that compounds acting downstream of, or too far upstream from, the PfRH5-basigin interaction do not have enhanced potency when combined with PfRH5 monoclonal antibodies. In contrast, neutralising anti-PfRH5 monoclonal antibodies synergised with early-acting invasion inhibitors, particularly the heparinoids heparin and sevuparin, substantially increasing the heparinoids' potency. Sevuparin also synergised with vaccine-induced polyclonal PfRH5 IgG. Together, these findings demonstrate that vaccine-induced anti-PfRH5 antibodies and compounds that act immediately before the PfRH5-basigin interaction can synergistically inhibit parasite growth. This proof-of-concept study provides new insights to inform vaccine design and demonstrates the potential of chemico-vaccine strategies to improve the efficacy of erythrocytic-stage malaria vaccines.

Introduction

Plasmodium falciparum, the deadliest of the malaria-causing parasites, remains a major global health threat with nearly half of the world's population at risk of infection ¹. In 2024, there were an estimated 282 million cases, resulting in approximately 610,000 deaths from malaria globally ². While vaccines do exist for malaria prevention, they are not highly efficacious ^{3,4}. Work is ongoing to improve the efficacy of malaria vaccines; however, progress is limited by the complexity of the parasite's lifecycle and our incomplete understanding of the host immune response ⁵. While malaria disease is associated with erythrocytic infection, pre-erythrocytic stage vaccines (PEVs) that target the parasite prior to and/or in the liver before clinical symptoms arise have had the most clinical success. In 2021, the World Health Organization (WHO) recommended the widespread use of the first approved malaria vaccine, RTS,S/AS01, for children in areas with moderate to high transmission of *P. falciparum* ⁶. A second and very similar PEV, R21/Matrix-M, was approved by the WHO in 2023. Both vaccines target the highly abundant circumsporozoite protein found on the surface of *P. falciparum* sporozoites, which are injected into the host following the bite of an infected *Anopheles* mosquito ⁷. These vaccines are designed to prevent sporozoites from establishing an infection in the liver, thereby preventing disease. However, due to the suboptimal efficacy of these vaccines, breakthrough infections do occur: sporozoites can evade the immune response, multiply in hepatocytes as liver-stage schizonts, and ultimately release invasive merozoites into the bloodstream, causing clinical malaria symptoms. To prevent breakthrough infections, novel malaria vaccines targeting the erythrocytic stage are needed, and some candidates are now being tested in combination with existing PEVs to potentially enhance protection (ClinicalTrials.gov identifiers: NCT06958198 (2025-04-23), NCT07183371 (2025-07-02) and NCT05357560 (2022-04-13)).

The current leading candidate for an erythrocytic stage malaria vaccine is the reticulocyte-binding protein homolog 5 (PfPRH5) which is an essential protein released onto the surface of the invasive erythrocytic-stage merozoite and is required for binding of the erythrocyte receptor basigin (BSG) ⁸.

PfRH5 forms a pentameric complex, the PCRCR complex, with four other *P. falciparum* proteins: *Plasmodium* thrombospondin-related apical merozoite protein (PfPTRAMP), cysteine-rich small secreted protein (PfCSS), PfRH5-interacting protein (PfRIPR) and cysteine-rich protective antigen (PfCyRPA)⁹. This complex is bound to the merozoite surface via PfPTRAMP's transmembrane domain¹⁰. PfRH5, and by extension the PCRCR complex, is essential for invasion, mediating an irreversible interaction between the merozoite and the erythrocyte that drives the progression of parasite invasion^{8,9}.

Erythrocytic-stage merozoite egress and invasion are highly organised and involve many sequential host-parasite interactions. Activation of parasite protein kinase G (PKG) initiates a proteolytic cascade that triggers the release of subtilisin-like protease 1 (SUB1) from exonemes into the parasitophorous vacuole (PV)¹¹. Within the exonemes, SUB1 is processed by the aspartic protease plasmepsin X (PMX), which is essential for its maturation¹². In the PV, SUB1 cleaves and activates multiple substrates, leading to the rupture of the PV membrane (PVM)¹³ and activation of serine repeat antigen 6 (SERA6). SERA6 subsequently mediates erythrocyte membrane rupture¹⁴, enabling merozoite egress and subsequent invasion of new erythrocytes¹⁵.

Following egress, the initial contact of the erythrocyte by the extracellular merozoite is facilitated by merozoite surface proteins (MSPs)¹⁶. MSP1 is the most abundant protein on the parasite's surface and mediates invasion through low-affinity interactions with host erythrocyte membrane receptors, such as glycophorin A (GPA) and band 3^{17, 18, 19}. Early during invasion, malaria parasites secrete specialised apical organelles termed micronemes and rhoptries^{20, 21}. Before penetrating the erythrocyte, the parasite must reorient itself so that its apical end is in contact with the erythrocyte^{22, 23, 24}. This reorientation is mediated by higher-affinity interactions with erythrocyte receptors, primarily involving the parasite's RHs and erythrocyte-binding antigens (EBAs)²⁵. Following reorientation and PfRH5-BSG binding, the RON complex is secreted from the neck of the rhoptries into

the erythrocyte membrane and cytoskeleton ²⁶. Here, the RON complex acts as the receptor for apical membrane antigen 1 (AMA1), which is already on the surface of the merozoite following egress ²⁷. The binding of AMA1 to RON2 is irreversible and defines the formation of the tight junction between the parasite and erythrocyte and initiates the internalisation of the parasite ²⁸.

Through this tight junction, the remaining rhoptry bulb contents and micronemes are released ²⁷. Using the parasite's actomyosin motor complex, the merozoite actively pushes itself into the erythrocyte ^{29, 30}. Once inside the erythrocyte, the parasite resides in the PV ³¹. The expansion of the PVM as the newly invaded merozoite converts into an amoeboid ring is thought to be facilitated by steroidogenic acute regulatory protein-related lipid transfer protein 1 (START1 or PV6) ^{32, 33, 34}.

Given the parasite's reliance on PfRH5 for establishment of an erythrocytic infection, it has been developed as a novel vaccine antigen in the RH5.1/Matrix-M vaccine candidate, which is currently in Phase 2b trials ^{35, 36, 37}. Interim results indicate that three doses of RH5.1/Matrix-M provide approximately 55% protective efficacy against clinical malaria in children aged 5–17 months over 6 months of follow-up ³⁵. While this is a promising start, for widespread use in areas with ongoing transmission, the WHO recommends a malaria vaccine efficacy of at least 75% against clinical malaria, and ideally with longevity of at least two years before booster doses are required ^{38, 39}. Therefore, the efficacy of RH5.1/Matrix-M needs to be substantially improved before the vaccine can be deployed.

The invasion of erythrocytes by merozoites is a process that occurs within minutes, and PfRH5 binding to BSG occurs later in the invasion process ⁴⁰; therefore, the RH5.1/Matrix-M vaccine-induced antibodies have only a limited window to bind their targets before the parasites become intracellular and essentially inaccessible to the antibodies. Slowing the progression of erythrocyte invasion could provide vaccine-induced antibodies with additional time to bind their target, thereby preventing invasion and improving vaccine efficacy. Previous studies found that interactions between anti-PfRH5

monoclonal antibodies (mAbs) can result in synergistic invasion-inhibitory activity, including combinations involving non-neutralising anti-PfPRH5 mAbs with each other and with neutralising mAbs^{41, 42}. Specifically, anti-PfPRH5 mAbs generated in a Phase 1a clinical trial using a PfPRH5-based vaccine were isolated from individual plasmablasts from vaccinated volunteers⁴³, resulting in 17 distinct anti-PfPRH5 mAbs with well-defined invasion-blocking capacity. Antibodies that bind near the BSG-binding site at the tip of PfPRH5, such as R5.004, R5.016 and R5.008, are the most potent inhibitors of parasite growth and are termed neutralising anti-PfPRH5 mAbs^{42, 44, 45}. Antibodies that bind to other regions of PfPRH5 and poorly inhibit growth are termed non-neutralising. The potentiating anti-PfPRH5 mAbs, such as R5.011, are a subset of non-neutralising mAbs that increase the potency of neutralising mAbs. These mAbs have been reported to prolong the pre-invasion phase, defined as the period from first merozoite contact with the erythrocyte until the start of penetration. In these studies, the pre-invasion phase was approximately three times longer than in controls⁴⁶. The mechanism underlying this delay remains unresolved, but it may increase the opportunity for neutralising mAbs to bind PfPRH5 before invasion proceeds⁴². While this is a promising finding, a challenge for vaccine development is how to induce the most efficacious combination of neutralising and potentiating antibodies to maximise invasion-blocking efficacy. This highlights the need for alternative strategies to achieve a similar effect. Numerous drug-like compounds are known to inhibit or delay merozoite invasion; accordingly, rather than attempting to design a vaccine that elicits specific combinations of neutralising and potentiating mAbs, we propose leveraging invasion-inhibitory compounds to functionally mimic the effects of potentiating mAbs. Slowing or delaying invasion with an inhibitory compound could, in principle, provide neutralising mAbs with additional opportunities to bind their targets, thereby enhancing overall vaccine efficacy. This strategy may also have the potential to inform the design of next-generation multivalent malaria vaccines. By identifying the steps in invasion that can be targeted simultaneously to generate synergistic inhibition, our novel strategy could help to better define which parasite antigens can be combined to optimise vaccine efficacy.

Here, we have paired invasion-inhibitory compounds with anti-PfRH5 neutralising mAbs to investigate whether targeting specific steps in merozoite invasion could synergise with anti-PfRH5 mAbs, providing knowledge to inform future vaccine design. We have demonstrated that there is synergy between specific early acting invasion-inhibitory compounds and anti-PfRH5 mAbs, opening the possibility of using long-acting, invasion-blocking compounds to augment erythrocyte-stage vaccines.

Materials and Methods

***Plasmodium falciparum* culture**

P. falciparum transgenic 3D7_Hyp1-Nanoluciferase (Hyp1-Nluc) parasites ⁴⁷ were cultured at 4% haematocrit (HCT) in human erythrocytes provided by the Australian Red Cross Lifeblood, under low oxygen conditions (1% O₂, 5% CO₂, 94% N₂) at 37°C following established procedures ⁴⁸. Parasites were cultured in complete RPMI medium (cRPMI): RPMI-1640 (Sigma), 0.37 mM hypoxanthine (Sigma), 25 mM HEPES (GIBCO), 31.25 µg/mL gentamicin (GIBCO), 0.5% (w/v) AlbuMAX II (GIBCO) and 0.2% (w/v) NaHCO₃ (Thermo Scientific) and were maintained on 2.5 nM WR99210 (Jacobus) to maintain expression of Hyp1-Nluc. Cultures predominantly composed of ring-stage parasites were regularly synchronised with 5% D-sorbitol (10x RBC pellet volume) (Sigma Aldrich) following standard procedures ⁴⁹. The use of donated human blood to culture *Plasmodium falciparum* was approved by Alfred Hospital Ethics Committee Project 166/24.

Antibody expression and purification

Antibodies were transiently expressed in Expi293F HEK cells (Thermo Fisher Scientific). Cognate heavy and light chain-coding AbVec expression plasmids (a gift from Patrick C. Wilson, University of Chicago, USA) were co-transfected at a 1:1 ratio using Expi293 transfection reagent according to the manufacturer's instructions. Supernatants were harvested via centrifugation. All mAbs were purified using a 5 mL Protein A HP column (Cytiva) on an ÄKTA Pure FPLC system (Cytiva). Equilibration and wash steps were performed with PBS supplemented with 500 mM NaCl, mAbs were eluted in 0.1 M glycine pH 2.7, 100 mM NaCl. The eluates were pH equilibrated to 7.4 using 1.0 M Tris HCl pH 9.0 and

buffer exchanged into Dulbecco's PBS (DPBS) for transport and long-term storage. An alternate purification method used Protein A Sepharose 4B resin (Invitrogen) in gravity flow columns (BIO-RAD). The wash step was performed with PBS, mAbs were eluted with 100 mM citric acid, and pH was equilibrated to 7.4 with 1.0 M Trizma base. Prior to assays, mAbs were buffer exchanged into incomplete RPMI medium (without AlbuMAX II and NaHCO₃) and concentrated using an Amicon ultra centrifugal concentrator (Millipore) with a molecular weight cut-off of 30 kDa.

Clinical trial samples

All human serum samples were from the clinical trial NCT02927145 which has been reported in full elsewhere⁵⁰. In brief, malaria-naïve healthy UK adult participants received three intramuscular doses of the RH5.1 antigen³⁶ formulated in 0.5 mL AS01B adjuvant (GSK) in various dosing regimens. The trial received ethical approval from the UK NHS Research Ethics Service (Oxfordshire Research Ethics Committee A, ref. 16/SC/0345) and was approved by the UK Medicines and Healthcare products Regulatory Agency (ref. 21584/0362/001–0011). Participants signed written consent forms and consent was verified before each vaccination. The trial was registered on ClinicalTrials.gov (NCT02927145) and was conducted according to the principles of the current revision of the Declaration of Helsinki 2008 and in full conformity with the ICH guidelines for Good Clinical Practice (GCP).

Human polyclonal IgG purification

Serum samples from group 4 participants, day 140, vaccinated with three doses of 50 µg RH5.1/ AS01B (ClinicalTrials.gov NCT02927145) were pooled and purified using a 5 mL Protein A HP column (Cytiva) on an ÄKTA Pure FPLC system (Cytiva). Equilibration and wash steps were performed with PBS, mAbs were eluted in 0.1 M glycine pH 2.7. The eluates were pH equilibrated to 7.4 using 1.0 M Tris HCl pH

9.0 and immediately buffer exchanged into Dulbecco's PBS (DPBS) and concentrated using an Amicon ultra centrifugal concentrator (Millipore) with a molecular weight cut-off of 30 kDa.

Growth inhibition assay

Hyp1-Nluc parasites were synchronised to ring stage by sorbitol treatment and growth assays were conducted the following day using trophozoite-stage parasites aged approximately 20-28 hours post-invasion, with a starting parasitaemia of 0.3% and a 2% HCT. Parasites were treated with a 2-fold dilution series of compounds across 10 points in a 96-well round-bottom plate (50 μ L/well final volume) in technical duplicate. Control parasites were treated with the vehicle control at the same maximum concentration as the compound of interest, in technical triplicate. Triplicate wells containing 2% HCT uninfected erythrocytes were included as a background control. Plates were incubated at 37°C for 44 hours and frozen at -70°C. Parasite growth was subsequently determined through measurement of nanoluciferase (Nluc) expression.

Nanoluciferase bioluminescence detection

A Nluc assay was used to measure parasite growth by detecting Nluc production from transgenic Hyp1-Nluc parasites, which export a bioluminescent Nluc reporter enzyme into the erythrocyte compartment⁴⁷. To measure parasite growth using Nluc expression as a proxy, growth inhibition assays were thawed at room temperature to lyse parasite and erythrocyte membranes and release intracellular Nluc. Lysed cells were resuspended and 5 μ L lysate was added to 45 μ L NanoGlo buffer (1:1000 dilution of NanoGlo (Promega) in iRPMI) in a white 96-well luminometer plate. After 3 minutes of incubation, bioluminescence was measured in relative light units (RLU) using the CLARIOstar luminometer (BMG Labtech) at 3600 gain for 1 second/well following standard protocols⁵¹. After subtracting the background bioluminescence of lysed uninfected erythrocytes, values were normalised to the vehicle control (specified in figure legends).

Percentage growth inhibition was calculated as follows:

Formula 1:

$$\left(1 - \frac{RLU_{compound\ treated} - Mean\ RLU_{uRBC}}{Mean\ RLU_{vehicle\ control} - Mean\ RLU_{uRBC}}\right) \times 100$$

Percentage growth inhibition data were plotted in GraphPad Prism (Version 10.2.3 (347)), and non-linear regression curves were fitted using the log(inhibitor) vs. response – variable slope (four parameters) model.

Bliss additivity assay

Parasites were sorbitol synchronised to ring stage and assays were conducted the following day using trophozoite-stage parasites aged approximately 20-28 hours post-invasion, with a starting parasitaemia of 0.3% and 2% HCT. In 96-well round-bottomed plates (75 µL/well final volume), parasites were treated with either: a) a two-fold, nine-point dilution series of the compound of interest; b) a two-fold, nine-point dilution series of the compound of interest in combination with an anti-PfRH5 mAb at a fixed concentration that resulted in 30% inhibition of growth, as previously determined by 44-hour growth assays; or c) the fixed concentration of anti-PfRH5 mAb resulting in approximately 30% growth inhibition, alone. Assays were performed in technical duplicate. As a control, parasites were treated with the vehicle control at the same maximum concentration as the compound of interest, in technical triplicate. Triplicate wells containing 2% HCT uninfected erythrocytes were included as a background control. Plates were incubated at 37°C for 44 hours and frozen at -70°C. Bioluminescence was used as a proxy for growth and analysed as outlined above (Formula 1).

Predicted Bliss additivity calculation

Formula 2:

$$\text{Predicted Bliss additivity} = \left(1 - \left(1 - \left(\frac{x}{100} \right) \right) \times \left(1 - \left(\frac{y}{100} \right) \right) \right) \times 100$$

For a fixed concentration of mAb, the Bliss formula (Formula 2) estimates the percentage inhibition that would be observed at each concentration of the titrated compound, if the two compounds had an additive inhibitory effect (Bliss 1939). In the Bliss equation, x represents the percentage inhibition caused by constant mAb alone, and y represents the percentage inhibition by a given concentration of the drug. The value of y changes at each dilution point, and therefore, the predicted Bliss additivity is calculated for every drug concentration. If the observed inhibition exceeds the predicted additive effect, this indicates synergy. Conversely, if the observed inhibition is lower than the predicted additive effect, this indicates antagonism. The relative half-maximal inhibitory concentration (IC_{50}) for each fitted concentration–response curve was calculated as the concentration producing an inhibitory effect halfway between the fitted upper and lower plateaus. These relative IC_{50} values were then used to compare the predicted additive effect with the measured combined effect.

Fixed-ratio isobologram assay

An alternate method to assess synergy is through performing fixed-ratio isobologram analyses as described in ⁵². Parasites were treated with six ratios of drug/antibody as described in Table 1, where solutions 1 and 6 contained either the drug of interest (at maximum concentration $8 \times IC_{50}$) or the mAb of interest (at maximum concentration 0.5 mg/mL) alone, while solutions 2-4 contained the drug and mAb in fixed-ratio combinations (Table 1).

Parasites were sorbitol synchronised to ring stage, and assays were conducted the following day using trophozoite-stage parasites aged approximately 20-28 hours post-invasion, with a starting

parasitaemia of 0.3%. Solutions 1-6 were prepared at twice the concentration specified in Table 1, and each solution was added to duplicate wells of 96-well U-bottom plates, then serially diluted across the plate (2-fold, 9-point dilution series; 50 μ L per well). 50 μ L 4% HCT trophozoite culture was added to each well, resulting in a halving of the drug and mAb concentrations to a final 1x concentration and bringing the HCT to 2%. Triplicate control wells containing uninfected erythrocytes at 2% HCT were also included. Plates were incubated at 37°C for 44 hours and frozen at -70°C before measuring parasite growth by measuring bioluminescence as previously described.

To calculate the growth inhibition from each fixed ratio of mAb:drug (solutions 2-5), two separate non-linear regression curves were plotted in GraphPad Prism: percentage growth inhibition against $\log_{10}$ [drug] and percentage growth inhibition against $\log_{10}$ [mAb]. A single curve was plotted for each of the solutions 1 and 6. An IC_{50} value was then determined for each curve by drawing a horizontal line at $y=50\%$ growth inhibition and identifying the concentration (*i.e.*, the x coordinate) at which the curve intersects the line. For each fixed ratio, the fractional inhibitory concentration of the mAb

Combination solution number	Concentration of drug	Concentration of mAb
1	$1 \times (8 \times IC_{50})$	$0 \times 0.5 \text{ mg/mL}$
2	$0.8 \times (8 \times IC_{50})$	$0.2 \times 0.5 \text{ mg/mL}$
3	$0.6 \times (8 \times IC_{50})$	$0.4 \times 0.5 \text{ mg/mL}$
4	$0.4 \times (8 \times IC_{50})$	$0.6 \times 0.5 \text{ mg/mL}$
5	$0.2 \times (8 \times IC_{50})$	$0.8 \times 0.5 \text{ mg/mL}$
6	$0 \times (8 \times IC_{50})$	$1 \times 0.5 \text{ mg/mL}$

(FIC_{mAb}) (Formula 3), the FIC of the drug (FIC_{drug}) (Formula 4) and the sum FIC (sFIC) (Formula 5) were

calculated.

Table 1: Solution preparation for fixed-ratio isobologram

Formula 3:

$$FIC_{mAb} = \frac{IC_{50} \text{ of } mAb \text{ in combination solution}}{IC_{50} \text{ of } mAb \text{ alone}}$$

Formula 4:

$$FIC_{drug} = \frac{IC_{50} \text{ of drug in combination solution}}{IC_{50} \text{ of drug alone}}$$

Formula 5:

$$sFIC = FIC_{mAb} + FIC_{drug}$$

For each fixed mAb:drug ratio, the fractional inhibitory concentration of the mAb (FIC_{mAb}) and drug (FIC_{drug}) were summed to generate a sum fractional inhibitory concentration (sFIC). Isobologram curves were generated by plotting FIC_{drug} against FIC_{mAb} , with the dashed line connecting the axial points at 1 representing the expected additive interaction. Interactions were interpreted using both the position of the plotted points relative to this additive line and the sFIC value. Synergy was defined as a curve plotted below the additive line, with sFIC values below 0.75. Additive interactions were indicated by points lying close to the additive line and sFIC values near 1. In contrast, antagonism was indicated by a curve plotted above the additive line and sFIC values greater than 1.25⁵³.

Results

Selection of invasion-inhibitory compounds that target specific invasion-associated processes.

To study if inhibitory compounds combined with anti-PfRH5 mAbs act synergistically to prevent the proliferation of asexual *P. falciparum* parasites, a series of reagents were selected that block downstream (late-acting invasion inhibitors) and upstream (egress inhibitors and early-acting invasion inhibitors) of the PfRH5-BSG binding step (Figure 1). Synergy refers to the phenomenon where the

combined effect of compounds exceeds the sum of their individual effects. In the context of invasion-inhibitory compound interactions, synergy amplifies the overall inhibition to exceed that which would be expected if the compounds' effects were additive.

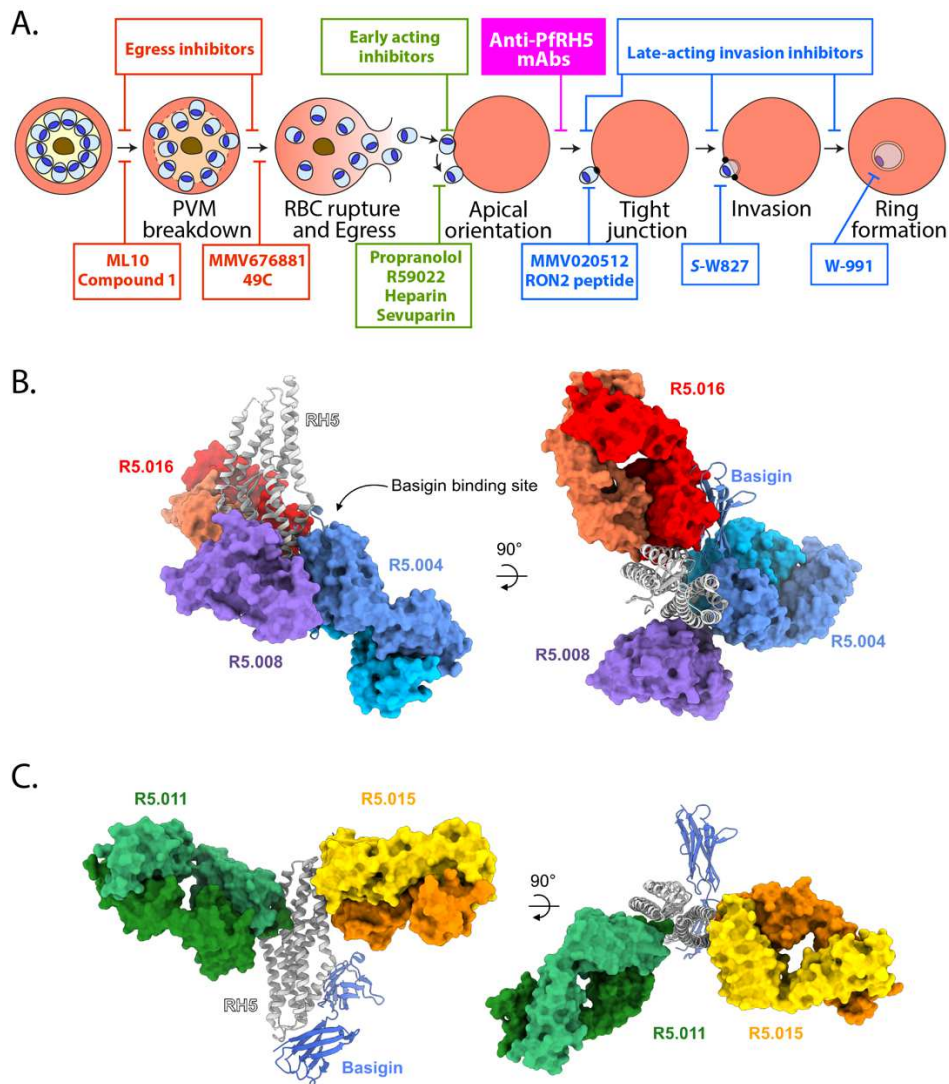


Figure 1. Schematic of the invasion pathway with invasion-inhibitory compounds and their targets, and binding locations of anti-PfRH5 mAbs used in this study. **A.** Invasion of erythrocytes by *P. falciparum* merozoites is a highly sequential process involving multiple parasite-host interactions, beginning with egress from the infected erythrocyte and culminating in ring stage development. Many experimental compounds and antibodies target distinct stages throughout this pathway. The interaction between PfRH5 and BSG occurs midway during invasion and can be inhibited by anti-PfRH5 mAbs (pink box). Herein, we examine both up- and downstream-acting invasion-inhibitory compounds in combination with the anti-PfRH5 mAbs to discover combinations that synergistically inhibit parasite growth. **B.** Structure of neutralising antibodies R5.004 (blue), R5.008 (purple), and R5.016 (red) bound

to PfRH5 (grey). Basigin is shown as a blue cartoon with the binding site indicated by an arrow. Side and top views shown. Composite image of PDB IDs: 4U0Q ⁴⁴, 6RCV ⁴², and 8PWX ⁵⁴. **C.** Structures of non-neutralising antibodies R5.011 (green) and R5.015 (orange) bound to PfRH5 (grey). Basigin is shown as a blue cartoon, with side and top views shown. Composite image of PDB IDs: 4U0Q ⁴⁴, 6RCV ⁴² and 7PHU ⁵⁵.

The antibodies used in this study have previously been shown to affect merozoite invasion by targeting the PCRCR complex ⁴². Specifically, herein we used the PfRH5-specific human neutralising mAbs R5.004, R5.016 and R5.008 and non-neutralising mAbs R5.011 and R5.015 (Figure 1B, C) ⁴², polyclonal human anti-PfRH5 IgG from a Phase 1a clinical trial of the RH5.1 vaccine ⁵⁰ and the PfCyRPA-specific mAb Cy.009 ^{55, 56}. Egress and invasion inhibitory compounds were selected based on their known targets and were grouped as either egress inhibitors (ML10, compound 1, MMV676881, 49C) ^{57, 58, 59, 60}, early-acting invasion inhibitors (propranolol, R59022, heparin and sevuparin) ^{61, 62, 63} or late-acting invasion inhibitors (MMV020512, S-W827, W-991, RON2 peptide) ^{34, 64, 65, 66} in relation to anti-PfRH5 mAb activity. We sought to determine whether synergy with anti-PfRH5 mAbs could be elicited by inhibiting any stage of egress or invasion, or whether inhibition must occur at a defined point relative to PfRH5 exposure to generate synergy. Before use in synergy assays, standard 44-hour dose-response Nluc growth assays were performed, and compound IC₅₀s and mAb IC₃₀s were calculated (Table 2, 3 and Supplementary Figure 1).

Invasion-targeting compounds that work downstream of PfRH5 have additive effects but do not synergise with neutralising anti-PfRH5 monoclonal antibodies.

Synergy was examined by utilising the Bliss predicted additivity model, which predicts the growth inhibition curve that would be generated if a constant concentration of mAb is co-administered with serially diluted compound, provided mAb and compound have purely additive effects. Under additive conditions, the measured combined-treatment curve overlaps with the predicted Bliss additivity curve, and the IC₅₀ of the titrated compound is unchanged by the addition of the fixed mAb. In contrast, synergistic interactions are indicated by an increase in compound potency in the presence of the mAb,

resulting in a leftward shift of the combined-treatment curve and a reduction in the compound IC_{50} . Antagonism between the compound and mAb is indicated by a rightward shift of the combined-treatment curve and an increased IC_{50} , reflecting reduced compound potency in the presence of the mAb. For Bliss analysis, IC_{50} is defined as the concentration of compound corresponding to a response halfway between the fitted upper and lower plateaus of the concentration–response curve. Across all Figures within this paper, the single treatment curve is shown in black, the predicted Bliss additivity curve in teal and the combined treatment curve in pink.

Late-acting invasion inhibitors MMV020512, S-W827 and W-991 which target ROM8/CSC1^{64, 65, 66} (Figure 2A), actin polymerisation⁶⁷ (Figure 2B) and lipid transfer protein PfSTART1^{33, 34} (Figure 2C), respectively, were utilised for Bliss additivity assays with neutralising anti-PfRH5 mAbs R5.016 or R5.004. We sought to determine whether these late-acting invasion inhibitors, which hinder the completion of invasion, could allow the anti-PfRH5 mAbs more time to act, thus acting synergistically when used in combination.

To determine the potency of growth-inhibitory compounds, bioluminescent parasites expressing a nanoluciferase marker were used^{47, 65, 68}. The compounds were serially diluted while the mAbs were held at a concentration previously determined to generate approximately 30% growth inhibition when normalised to the vehicle control (Table 3 and Supplementary Figure 3). In this assay, we used R5.016, which blocks the BSG binding site on the PfRH5 protein, and was shown to synergise with R5.011 in previous anti-PfRH5 mAb studies⁴². R5.016 was held at 0.01 mg/mL, resulting in average growth inhibition of 34.4% (Table 3). Following completion of Bliss additivity assays, we consistently found that each of the late-acting compounds MMV020512, S-W827 and W-991 act additively with R5.016, *i.e.*, the predicted and measured curves overlap, and no significant difference was observed between the IC_{50} of the titrated compound in the measured combined effect compared to its IC_{50} in the

predicted additive effect (Figure 2). We note that slight antagonism was observed between S-W827 and R5.016, but it was not significant (Figure 2B).

The compounds also produced similar effects when combined with the R5.004 mAb (held at 0.02 mg/mL, resulting in average growth inhibition of 37.3% alone) (Supplementary Figure 4A-C). Late-acting invasion inhibitor RON2 peptide, which inhibits the binding of AMA1 to RON2^{69, 70, 71} (Supplementary Figure 4D), was titrated with another neutralising anti-PfRH5 mAb, R5.008, which was held at 0.03 mg/mL, resulting in average growth inhibition of 36.2% alone. This combination similarly revealed an additive effect (Supplementary Figure 4D). Collectively, these results indicate that PfRH5 neutralising mAbs do not enhance the potency of late-acting invasion inhibitors, suggesting that these combinations do not act synergistically and instead act additively.

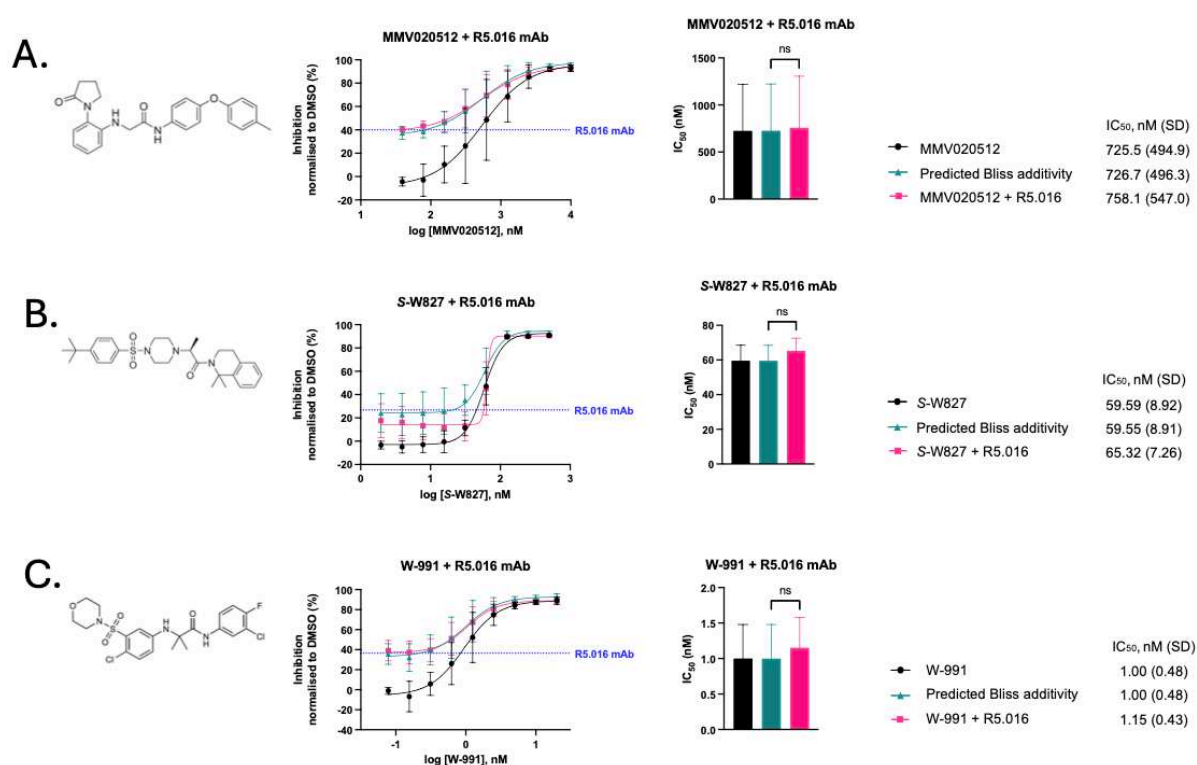


Figure 2. Late-acting invasion inhibitors show an additive invasion-inhibitory effect when combined with anti-PfRH5 mAb R5.016. The structures of late-acting inhibitors **A.** MMV020512, **B.** S-W827 and **C.** W-991 are shown on the left. Growth inhibition was quantified after 44 hours of treatment by measuring Nluc and normalising to growth in the presence of the DMSO vehicle control. Growth curves were generated (middle panels): the compound alone (black), the predicted additive effect (teal), and the measured combined effect of the titrated compound with a fixed concentration of mAb

R5.016 held at 0.01 mg/mL (pink). The bar graphs display the mean IC_{50} of 3 biological replicates of the titrated compound within each condition. The IC_{50} for each fitted concentration–response curve was calculated as the concentration producing an inhibitory effect halfway between the fitted upper and lower plateaus. The observed combined effect was compared to the predicted additive effect using an unpaired t-test and a significance level of 0.05 (GraphPad Prism 10). Data points and error bars represent the mean and SD of three biological replicates, each consisting of two technical replicates.

Egress inhibitors have additive effects but do not synergise with neutralising anti-PfRH5 monoclonal antibody R5.008.

As the observed effect between late-acting invasion-inhibitory compounds and neutralising anti-PfRH5 mAbs was additive, we next applied the Bliss additivity method to detect synergy between known egress inhibitors, which act upstream of PfRH5, and the anti-PfRH5 mAbs. Neutralising anti-PfRH5 mAb R5.008 binds PfRH5 in a similar position to R5.016 (Figure 1B), and as we had insufficient R5.016 to perform additional assays, we used R5.008 to investigate Bliss additivity with egress inhibitors. We speculated that at sub-lethal concentrations, egress inhibitors could partially permeabilise the erythrocyte membrane, allowing anti-PfRH5 mAbs access to merozoites before egress is complete. The egress inhibitors investigated with neutralising anti-PfRH5 mAb R5.008 were ML10 (Figure 3A) and Compound 1 (Figure 3B), which target parasite PKG, preventing the rupture of the PVM^{57, 58}. Plasmepsin IX and X (PMIX/PMX) inhibitor 49C (Figure 3C) and SERA6 inhibitor MMV676881 (Figure 3D), which block rupture of the erythrocyte during egress^{59, 60}, were also examined.

R5.008 was held at a constant concentration of 0.03 mg/mL, which caused an average growth inhibition of 23.9%, while each egress inhibitor was used in a titration. Resultant data revealed that there was no significant difference between the predicted IC_{50} values and the measured IC_{50} values of the titrated compounds in combination with R5.008, demonstrating that these combinations do not act synergistically and instead also have an additive effect (Figure 3).

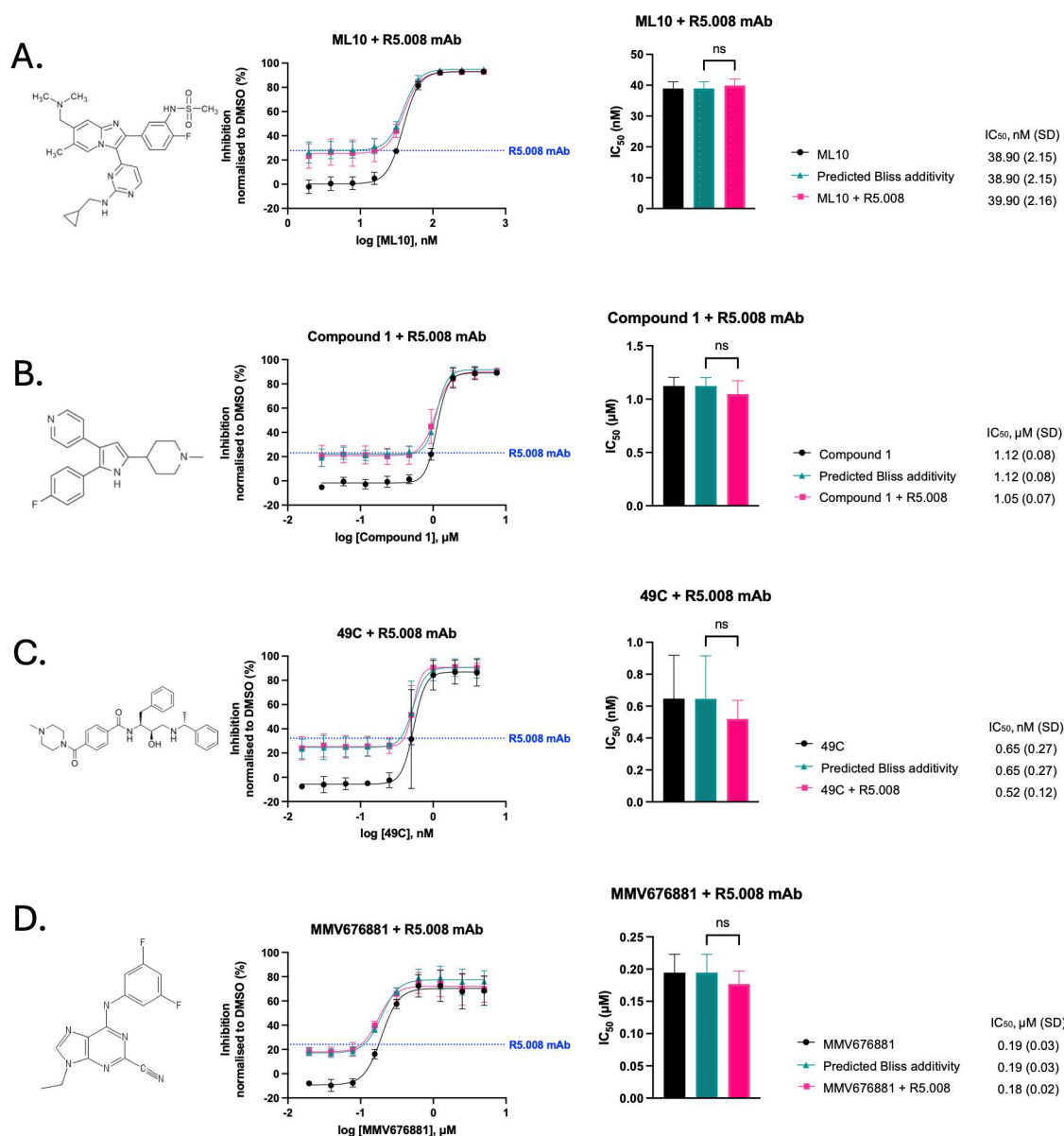


Figure 3. Egress inhibitors and anti-PfPRH5 mAb R5.008 have additive inhibitory effects. The structures of **A.** ML10, **B.** Compound 1, **C.** 49C and **D.** MMV676881 are shown on the left. In the middle is a 2-fold dilution series of the compound alone (black), the predicted additive effect calculated from the Bliss model (teal), and the measured combined effect of the titrated compound with a fixed concentration of mAb R5.008 held at 0.03 mg/mL (pink). The bar graphs display the mean IC₅₀ of 3 biological replicates of the titrated compound within each condition. The IC₅₀ for each fitted concentration–response curve was calculated as the concentration producing an inhibitory effect halfway between the fitted upper and lower plateaus. Growth inhibition was quantified after 44 hours of treatment by measuring Nluc and normalising to growth in the presence of the DMSO vehicle control. The observed combined effect was compared to the predicted additive effect using an unpaired t-test and a significance level of 0.05 (GraphPad Prism 10). Data points and error bars represent the mean and SD of three biological replicates, each consisting of two technical replicates.

Microneme secretion inhibitor (R59022) and stimulator (propranolol) have additive effects but do not synergise with neutralising anti-PfRH5 monoclonal antibody R5.008.

Microneme secretion is an essential step in Apicomplexan parasite invasion, and in the model Apicomplexan parasite *Toxoplasma gondii*, it is known that modulation of phosphatidic acid (PA) levels at the parasite plasma membrane helps to regulate microneme secretion⁶¹. PA levels are modulated by diacylglycerol kinases and phosphatidic acid phosphatases, which can be targeted by R59022 (Figure 4A) and propranolol (Figure 4B), respectively. These inhibitors have previously been shown to either block (R59022) or promote (propranolol) microneme secretion in *T. gondii*⁶¹. Given microneme secretion precedes rhoptry release, we investigated whether either R59022 or propranolol act synergistically with neutralising anti-PfRH5 mAb R5.008 to prevent parasite growth.

We again utilised the Bliss additivity model for these studies and held R5.008 at 0.03 mg/mL, which caused an average growth inhibition of 28.4%, while R59022 and propranolol were titrated into the assay (Figure 4). Following completion of Bliss additivity assays, the measured IC₅₀ values of R59022 and propranolol were higher in the presence of neutralising anti-PfRH5 mAb R5.008 than would be predicted for an additive effect in the Bliss additivity model (Figure 4). This difference, however, was not statistically significant, suggesting that these combinations have an additive interaction under these conditions.

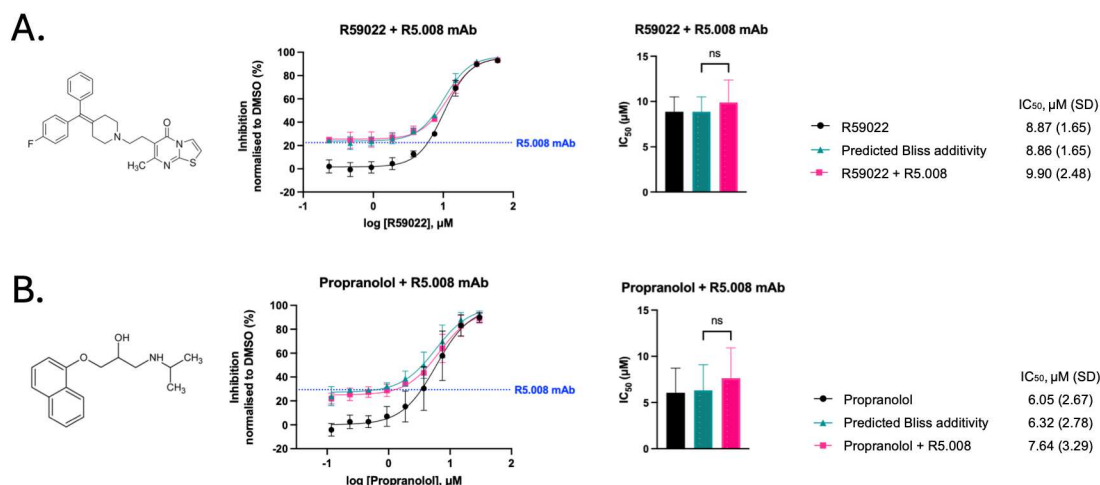


Figure 4. Microneme secretion inhibition (R59022) or stimulation (propranolol) do not show a synergistic effect with neutralising anti-PfRH5 mAb R5.008 when using a Bliss additivity model.

The structures of **A.** R59022 and **B.** propranolol are shown on the left. In the middle is a 2-fold dilution series of the compound alone (black), the predicted additive effect calculated from the Bliss model (teal), and the measured combined effect of the titrated compound with a fixed concentration of R5.008 held at 0.03 mg/mL (pink). The bar graphs display the mean IC₅₀ of 3 biological replicates of the titrated compound within each condition. The IC₅₀ for each fitted concentration–response curve was calculated as the concentration producing an inhibitory effect halfway between the fitted upper and lower plateaus. Growth inhibition was quantified after 44 hours of treatment by measuring Nluc and normalising to growth in the presence of the DMSO vehicle control. The observed combined effect was compared to the predicted additive effect using an unpaired t-test and a significance level of 0.05 (GraphPad Prism 10). Data points and error bars represent the mean and SD of three biological replicates, each consisting of two technical replicates.

Invasion inhibitory compound heparin and neutralising anti-PfRH5 monoclonal antibodies R5.004, R5.016 and R5.008 synergistically inhibit parasite growth.

Previous assays had investigated potential synergistic effects between neutralising anti-PfRH5 mAbs and small molecule inhibitors acting during egress or late stages of invasion. Next, we investigated whether a known early-acting invasion-inhibitory glycosaminoglycan, heparin, could synergise with these mAbs (Figure 5A). Heparin is a polysaccharide of sulphated sugars, previously found to inhibit *Plasmodium* early in the invasion process⁶², likely by disrupting the interaction of MSP1 with the erythrocyte²⁶. As for previous compounds, Bliss additivity assays were performed. Within these assays, R5.004 was held at 0.02 mg/mL, resulting in average growth inhibition of 35.4% (Figure 5B),

R5.016 was held at 0.01 mg/mL, resulting in average growth inhibition of 38.5% (Figure 5C), and R5.008 was held at 0.03 mg/mL, resulting in average growth inhibition of 44.0% (Figure 5D). Following titration of heparin into the assay, the inclusion of a fixed concentration of R5.004 resulted in a 4.7-fold reduction in the IC_{50} of heparin compared to its predicted IC_{50} by Bliss (Figure 5B); however, the reduction in IC_{50} did not quite reach statistical significance (p -value=0.08, n =3). Heparin and R5.016 mAb were found to act synergistically; there was a 5.9-fold reduction in IC_{50} when heparin and R5.016 were used in combination, compared to what was expected for the predicted additive effect (p -value=0.023, n =3) (Figure 5C). Furthermore, a similar effect was seen with heparin and R5.008 (p -value=0.005, n =3) (Figure 5D), where there was a 2.4-fold reduction in heparin's IC_{50} when used in combination with R5.008. Collectively, these results suggest that heparinoid compounds that inhibit early invasion steps could be promising combination partners to synergise with vaccine-induced anti-PfRH5 antibodies.

To examine whether this synergistic effect could be generated by neutralising antibodies raised against other members of the PCRCR complex, we titrated heparin against a constant 0.03 mg/mL of Cy.009 mAb, which alone exhibits on average 34.5% growth inhibitory activity. This antibody recognises the PfCyRPA protein, which binds PfRH5 in the PCRCR complex^{9, 72}. Unlike the neutralising anti-PfRH5 mAbs, heparin and Cy.009 mAb acted additively within the Bliss model (Figure 5E), as the observed reduction in IC_{50} was modest and not significant (p -value=0.47, n =3).

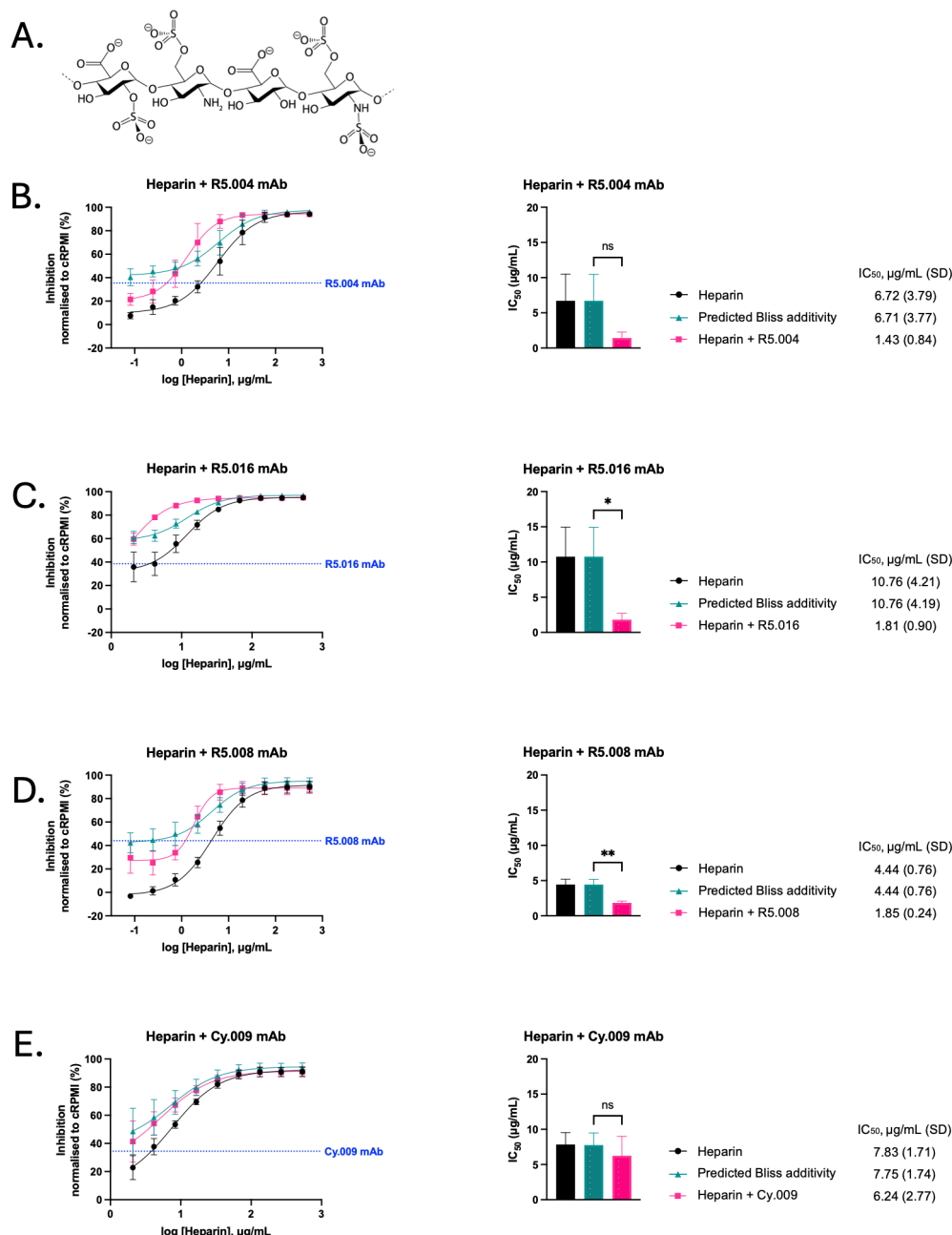


Figure 5. Heparin and neutralising anti-PfRH5 monoclonal antibodies synergistically inhibit parasite growth.

A. The chemical structure of heparin. **B-D.** Line graphs show growth inhibition curves of heparin and either **B.** R5.004 held at 0.02mg/mL, **C.** R5.016 held at 0.01mg/mL, **D.** R5.008 held at 0.03 mg/mL or **E.** Cy.009 held at 0.03 mg/mL. All graphs show heparin alone (black), the predicted additive effect calculated from the Bliss model (teal), and the measured combined effect of the titrated compound with a fixed concentration of anti-PfRH5 mAb (pink). The bar graphs display the mean IC₅₀ of 3 biological replicates of the titrated heparin within each condition. The IC₅₀ for each fitted concentration–response curve was calculated as the concentration producing an inhibitory effect halfway between the fitted upper and lower plateaus. Growth inhibition was quantified after 44 hours of treatment by measuring Nluc and normalising to growth in the presence of the iRPMI vehicle control. The observed combined effect was compared to the predicted additive effect using an

unpaired t-test and a significance level of 0.05 (GraphPad Prism 10). Data points and error bars represent the mean and SD of three biological replicates, each consisting of two technical replicates.

Heparinoid sevuparin acts synergistically with neutralising anti-PfRH5 monoclonal antibodies and polyclonal PfRH5 IgG.

Heparin is an anticoagulant and therefore not suitable as a clinical antimalarial; it is, however, part of a larger family of heparinoid compounds that may share similar anti-malarial invasion-inhibitory effects. One such member of this family, sevuparin, is a chemically modified derivative of heparin that has been shown to block invasion but exhibits minimal anticoagulant activity^{63, 73}. Herein, we sought to determine if sevuparin also synergises with anti-PfRH5 mAbs by performing Bliss additivity assays. Assays were set up as described for heparin with R5.004 held at 0.02 mg/mL, resulting in average growth inhibition of 35.3% (Figure 6A), R5.016 held at 0.01 mg/mL, resulting in average growth inhibition of 37.8% (Figure 6B), and R5.008 held at 0.03 mg/mL, resulting in average growth inhibition of 54.6% (Figure 6C). Sevuparin was titrated against each mAb, and synergy was detected with all mAbs (Figure 6A-C). Specifically, a 7.3-fold reduction in sevuparin IC₅₀ was calculated when used in the presence of R5.004 (p-value=0.018, n=3) (Figure 6A), a 4.9-fold reduction in sevuparin IC₅₀ was calculated in the presence of R5.016 (p-value=0.0006, n=3) (Figure 6B), and a 5.8-fold reduction in sevuparin IC₅₀ was calculated in the presence of R5.008 (p-value=0.0013, n=3) (Figure 6C).

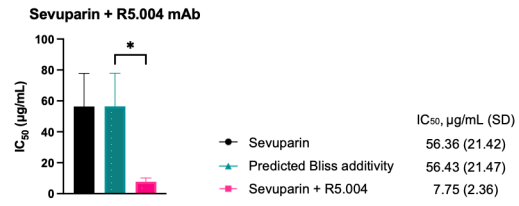
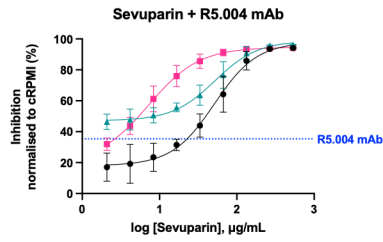
To further explore the relationship between sevuparin and vaccine-induced anti-PfRH5 mAbs, we investigated the effect of non-neutralising anti-PfRH5 mAbs, including the subclass of potentiating anti-PfRH5 mAbs, in a Bliss additivity model with our most PfRH5-synergistic compound, sevuparin. Non-neutralising antibodies would be expected to coexist with neutralising antibodies whether naturally induced by infection or following PfRH5 vaccination. The non-neutralising anti-PfRH5 mAbs tested were R5.011 and R5.015⁴². As non-neutralising anti-PfRH5 mAbs do not directly inhibit parasite growth, we performed Bliss additivity assays with the mAb concentration previously used for

neutralising anti-PfRH5 mAb R5.008 (0.03 mg/mL), that caused approximately 30% growth inhibition (Supplementary Figure 3D). Potentiating anti-PfRH5 mAb R5.011 demonstrated minimal parasite neutralising activity at this concentration, eliciting an average growth inhibition of 9.3% (Figure 6D). Upon completion of Bliss additivity assays, we found that R5.011 and sevuparin act antagonistically, with the parasites growing better in the presence of R5.011 and sevuparin than the predicted additive effect or sevuparin on its own (Figure 6D). This is demonstrated by a 2.1-fold increase in sevuparin IC_{50} when used in combination with R5.011 (p-value=0.0045, n=3) (Figure 6D). We also examined the effect of parasite treatment with the non-neutralising anti-PfRH5 mAb R5.015 at 0.03 mg/mL; alone, this concentration of mAb caused minimal inhibition (3.5%) (Figure 6E, Supplementary Figure 3F), and when combined with sevuparin, no interaction was observed (Figure 6E).

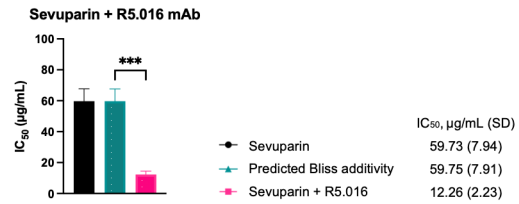
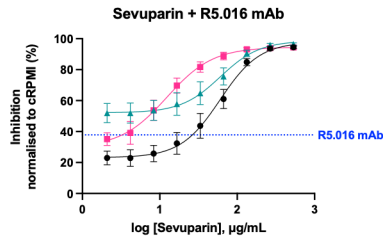
All previous assays within this study have used purified vaccine-induced mAbs with clearly defined PfRH5 binding sites⁴². To determine if the synergistic effect of sevuparin would extend to polyclonal serum containing both neutralising and non-neutralising anti-PfRH5 IgGs, we tested purified total IgG derived from sera of participants vaccinated with the RH5.1 vaccine candidate⁵⁰. Specifically, total polyclonal anti-PfRH5 IgG (pAbs) was purified from volunteer-derived serum⁵⁰ and used in Bliss additivity assays. Within these assays, we titrated sevuparin as we had done previously and held anti-PfRH5 pAbs at a constant concentration of 3 mg/mL, resulting in average growth inhibition of 26.1% (Figure 6F). This resulted in a statistically significant synergistic inhibitory interaction, wherein the IC_{50} of sevuparin was reduced 4.24-fold in the presence of anti-PfRH5 pAbs, compared to the IC_{50} of sevuparin within the predicted additive effect (p-value=0.034) (Figure 6F). This is an important finding because it demonstrates that vaccine-induced anti-PfRH5 pAbs, which comprise a mixture of neutralising and non-neutralising mAbs, can still act synergistically with sevuparin and that the synergistic effects of the neutralising mAbs outweigh any antagonistic effects the potentiating mAbs have.

As sevuparin is not orally bioavailable and has a short half-life, it would not be appropriate for co-administration with PfRH5 vaccines to elicit compound/antibody synergy in human populations. We therefore evaluated the effects of another heparinoid, fondaparinux and orally bioavailable negatively charged sugar polymers, chondroitin sulphate A and hyaluronic acid. Unfortunately, synthetic heparinoid fondaparinux^{74, 75} was a very weak inhibitor of parasite growth (IC₅₀ of approximately 80 μ M) and would therefore be unsuitable for clinical use (Supplementary Figure 2D). Similarly, orally bioavailable chondroitin sulphate A and hyaluronic acid did not inhibit parasite growth (Supplementary Figure 2E and F) and were therefore not pursued further.

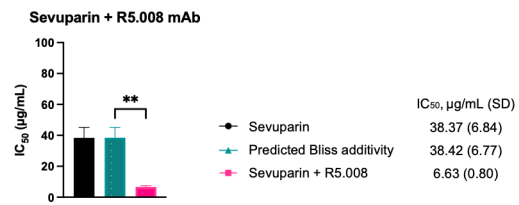
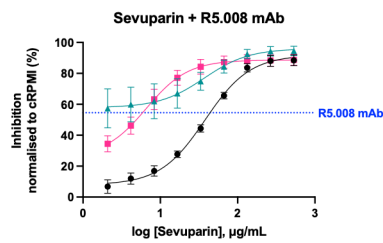
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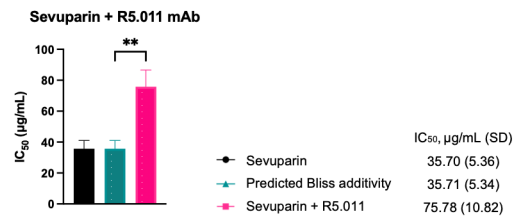
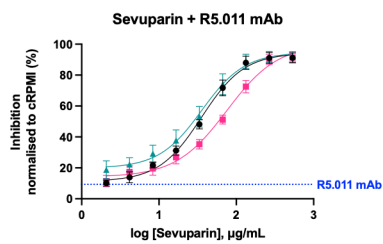
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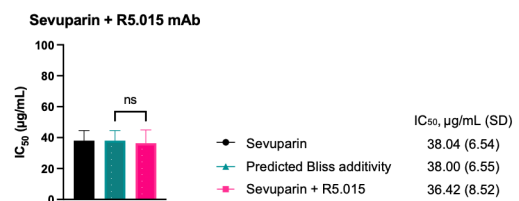
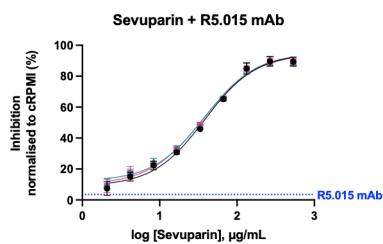
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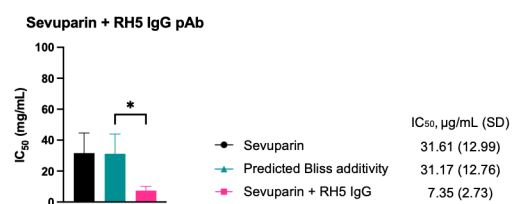
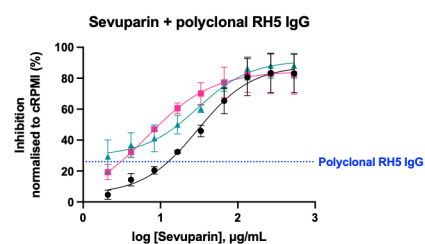


Figure 6. Sevuparin acts synergistically with neutralising anti-PfRH5 monoclonal antibodies and polyclonal PfRH5 IgG.

Line graphs show a 2-fold dilution series of sevuparin alone (black), the predicted additive effect calculated from the Bliss model (teal), and the measured combined effect of titrated sevuparin with a fixed concentration of either **A.** 0.01 mg/mL of R5.004 mAb, **B.** 0.02 mg/mL of R5.016 mAb, **C.** 0.03 mg/mL of R5.008 mAb, **D.** 0.03 mg/mL of R5.011 mAb, **E.** 0.03 mg/mL of R5.015 mAb or **F.** 3 mg/mL of anti-PfRH5 pAb (pink). The bar graphs display the mean IC_{50} of 3 biological replicates of titrated sevuparin within each condition. The IC_{50} for each fitted concentration–response curve was calculated as the concentration producing an inhibitory effect halfway between the fitted upper and lower plateaus. Growth inhibition was quantified after 44 hours of treatment by measuring Nluc and normalising to growth in the presence of the iRPMI vehicle control. The observed combined effect was compared to the predicted additive effect using an unpaired t-test and a significance level of 0.05 (GraphPad Prism 10). Data points and error bars represent the mean and SD of three biological replicates (A-D, F) or two biological replicates (E), each consisting of two technical replicates (E).

Fixed-ratio isobologram analyses demonstrate synergy between early-acting invasion inhibitors and neutralising anti-PfRH5 mAb R5.008.

While Bliss additivity assays efficiently screen compound–antibody interactions at a single antibody concentration, they do not capture full dose–response relationships for both agents. Fixed-ratio isobologram analysis is the gold-standard method for assessing synergy, as it enables full dose–response evaluation across multiple drug:mAb ratios⁵². However, this approach is resource-intensive and impractical for high-throughput synergy screening with limited mAb availability. Therefore, having identified synergistic interactions between early-acting invasion inhibitors and anti-PfRH5 mAbs using Bliss additivity, we applied fixed-ratio isobologram analyses to define full dose–response relationships for selected compounds and anti-PfRH5 neutralising mAb R5.008.

Here, we performed fixed ratio isobologram analyses with R5.008 and one representative inhibitor acting at each stage of the egress/invasion pathway (Figure 1). Compounds were selected for full analysis based on their performance in Bliss additivity assays: those that were synergistic (sevuparin) or showed a slight shift in IC_{50} in the presence of anti-PfRH5 neutralising mAbs (R59022, 49C and S-W827). To perform fixed-ratio isobologram assays, six fixed concentration ratios of compound and

mAb were serially diluted (Table 1)⁵² and growth inhibition assays were performed in the presence of each combination. Growth IC₅₀s for the drug and the antibody within each drug:antibody ratio were then calculated and used to generate the fractional inhibitory concentration (FIC) for the mAb and the compound for each ratio tested. The isobologram curve is constructed by plotting FIC_{drug} vs FIC_{mAb} (n=3) with a dashed line connecting the axial points at 1 (Figure 7). A sum FIC value (sFIC = FIC_{drug} + FIC_{mAb}) below 0.75 indicates a synergistic interaction, around 1 an additive effect and above 1.25 an antagonistic interaction⁵³. Therefore, these data can reveal if a single ratio of drug:antibody is synergistic.

Fixed ratio isobolograms were performed for S-W827, 49C, R59022 and sevuparin as representative compounds, in combination with neutralising anti-PfRH5 mAb R5.008. From these analyses, the sFIC values for S-W827 and R5.008 demonstrated a slightly antagonistic effect, while the sFIC values for both 49C and R59022 combined with R5.008 predominantly demonstrated an additive effect, as anticipated from our Bliss additivity assay data (Figure 4). However, interestingly, we found that the R59022 and R5.008 mAb combination does act synergistically when low levels of R59022 are paired with higher concentrations of R5.008, *i.e.*, combination 5 had an sFIC value of 0.75 (Figure 7C, Supplementary Table 2). Importantly, the synergistic effect of sevuparin and R5.008 detected in Bliss additivity assays was recapitulated in these studies, with synergy detected for every sevuparin:R5.008 mAb combination tested, with sFIC values of 0.41 (combination 2), 0.35 (combination 3), 0.36 (combination 4) and 0.39 (combination 5) (Figure 7D, Supplementary Table 2). These data indicate that the potency of both sevuparin and the anti-PfRH5 neutralising mAb can be improved by co-treatment, supporting our concept that invasion-inhibitory compounds can improve the potency of anti-PfRH5 vaccine-induced antibodies.

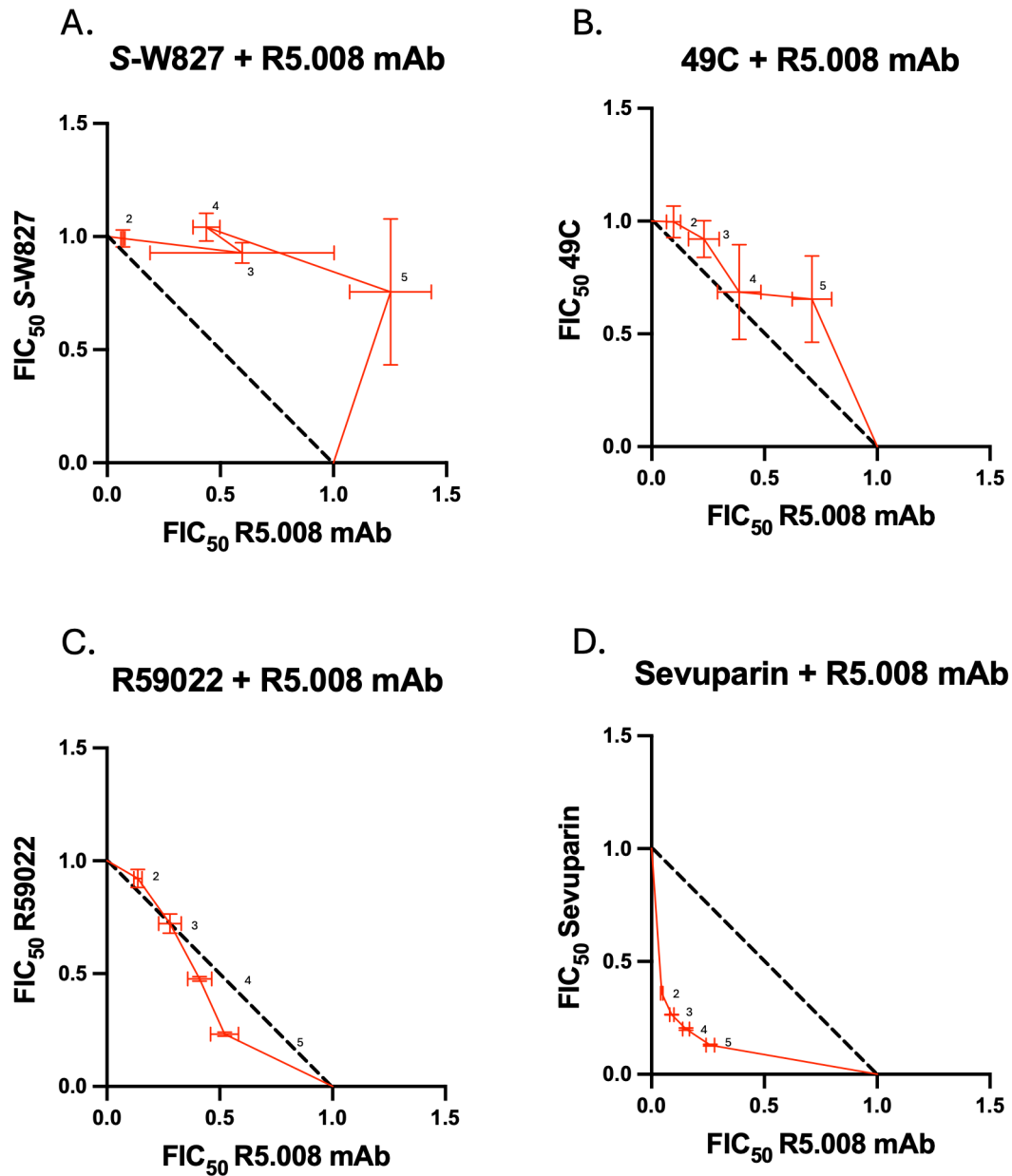


Figure 7. Fixed-ratio isobologram analyses demonstrate synergy between early-acting invasion inhibitors and neutralising anti-PfRH5 mAb R5.008. Isobologram curve showing the interaction between R5.008 and either **A. S-W827**, **B. 49C**, **C. R59022** or **D. sevuparin**. The dashed line connecting the axial points at 1 represents an additive interaction. The interaction is considered synergistic if points fall below the line. Nluc was measured as a proxy for growth inhibition and quantified after 44 hours. Combination solutions were prepared such that the maximum concentration of the DMSO vehicle was less than 0.2%. Data points and error bars represent the mean and SD of three biological replicates, each consisting of two technical replicates.

Discussion

Although substantial progress has been made towards developing erythrocyte-stage malaria vaccines, the current lead candidate, RH5.1/Matrix-M, alone does not provide sufficient protection for widespread use³⁵. Improving the activity of vaccine-induced anti-PfRH5 antibodies, or combining PfRH5 with additional merozoite antigens, is therefore a key goal. Previous research revealed that neutralising anti-PfRH5 mAbs can synergise with potentiating mAbs to enhance the inhibition of merozoite invasion⁴². However, a challenge for vaccine development is how to design a vaccine that induces both strong neutralising antibodies and the right balance of potentiating antibodies. A possible solution for boosting protection against malaria is to combine the RH5.1/Matrix-M vaccine with invasion-blocking compounds or other merozoite antigens to generate synergistic inhibitory activities, as this could enhance protection without requiring redesign of the vaccine itself. Previous work has investigated this concept by combining anti-PfRH5 mAbs with mAbs to other invasion antigens^{76, 77, 78, 79, 80, 81} and has determined that targeting discrete stages of the invasion pathway was the most effective way to uncover synergistic combinations⁷⁹. In this proof-of-principle study, we set out to examine whether invasion-inhibitory compounds could fulfil a similar synergistic role to antibodies, targeting other invasion-associated proteins.

Through plate-based assays, we revealed that invasion-inhibitory compounds can synergise with vaccine-induced anti-PfRH5 mAbs to enhance protection against the erythrocytic stage of malaria, but that this effect depends on the stage of invasion targeted by the compound. Merozoites rapidly invade erythrocytes; therefore, antibody-mediated inhibition of PfRH5 is constrained to a brief and tightly regulated period^{40, 44, 82, 83, 84}. Compounds acting during egress (before PfRH5 is exposed) or late in invasion (after PfRH5–BSG engagement) had an additive effect with anti-PfRH5 mAbs, suggesting that these compounds and antibodies act independently in a stepwise manner. In contrast, compounds acting immediately before PfRH5–BSG engagement synergised with anti-PfRH5 mAbs. This suggests that these inhibitors could extend the period during which PfRH5 is accessible to neutralising anti-

PfRH5 mAbs. These findings demonstrate that synergy with anti-PfRH5 mAbs is determined by the timing of compound action relative to PfRH5 exposure.

We had hypothesised that partial inhibition of egress by sub-lethal concentrations of egress inhibitors may result in partial permeabilisation of the erythrocyte and PVM^{85, 86}, allowing the anti-PfRH5 mAbs to access their protein target before merozoite release, and resulting in compound-mAb synergy. However, we determined that all the anti-PfRH5 mAb/egress inhibitory compound combinations tested had additive effects. This suggests that the erythrocyte and PVM may not have been permeabilised sufficiently to enable the anti-PfRH5 antibodies access to the internal merozoites. Similarly, when the invasion cascade is blocked after the PfRH5-BSG interaction has occurred, no compound/mAb synergy was detected. This is as expected given that the PfRH5-BSG interaction is believed to occur rapidly following PfRH5 release, and slowing the invasion process after this has already occurred will not impact the ability of the anti-PfRH5 mAbs to prevent this interaction from occurring. Despite early and late-acting compounds not producing synergistic effects, their additive effects with anti-PfRH5 neutralising mAbs could still be useful to enhance the efficacy of a PfRH5-based erythrocytic-stage vaccine; however, this effect would be less than what could be achieved with a synergistic interaction. Therefore, the discovery of synergistic compound/mAb combinations is of greater priority.

Within this study, we found that heparinoids act synergistically with anti-PfRH5 mAbs to prevent parasite invasion. Heparinoids are predicted to inhibit invasion by acting upstream of PfRH5²⁸, and while heparin's anticoagulant activity makes it an inappropriate compound for clinical antimalarial treatment, the analogous compound sevuparin is a safer and well-tolerated alternative. Sevuparin is not an anticoagulant and is a promising adjunctive therapy for severe malaria due to its ability to disrupt the sequestration of infected erythrocytes associated with cerebral malaria^{63, 87}. Within our Bliss additivity assays, we found that when these heparinoids are used in combination with

neutralising anti-PfRH5 mAbs, their potency increased 2.4-7.3-fold. Heparin has been shown to act by blocking parasite entry into erythrocytes⁸⁸, through binding invasion ligands such as MSP1, RH1, RH2, RH4, EBA-140, EBA-175 and EBA-181⁸⁹. These ligands are involved in early adhesion to the erythrocyte (MSP1) as well as host-parasite receptor-mediated engagement (EBAs and RHs) in the steps immediately preceding PfRH5-BSG binding. Furthermore, heparin has been shown to bind PfRH5⁹⁰, and this interaction could also increase the time taken for PfRH5 to engage BSG, or it could prevent the formation of the PCRCR complex, which is required for successful invasion. Together, these observations suggest that heparinoids synergise with neutralising anti-PfRH5 mAbs because they act during the narrow invasion window in which PfRH5 is exposed but has not yet completed engagement with BSG.

Following vaccination, both neutralising and non-neutralising antibodies are generated. Therefore, to better model how a chemico-vaccine approach could work *in vivo*, we investigated whether non-neutralising mAbs (including potentiating mAbs) could synergise with sevuparin. As anticipated, given that the non-neutralising mAbs have negligible effect on parasite growth, they did not synergise with sevuparin. Interestingly, however, potentiating mAb R5.011 reduced the potency of sevuparin, indicating antagonism. Given that heparin has previously been shown to bind PfRH5⁹⁰, sevuparin and R5.011 may compete for the same binding site on PfRH5, thereby attenuating each other's inhibitory activity. Indeed, previous research has demonstrated that antibodies targeting the same epitope regions of PfRH5 can exhibit antagonistic effects⁴². Despite this, when combined with polyclonal anti-PfRH5 IgG from previously vaccinated individuals (presumably containing neutralising, non-neutralising and potentiating antibodies), sevuparin was 4.2-fold more potent than when used alone. Therefore, the potentially antagonistic effects of specific anti-PfRH5 potentiating mAbs can be overcome in polyclonal total IgG, suggesting that invasion-inhibitory compounds could potentially increase the potency of vaccine-induced antibodies.

While we have demonstrated that sevuparin works synergistically with neutralising anti-PfRH5 mAbs to prevent parasite invasion, in its current form, it is not an ideal compound for widespread use. This is primarily due to its short half-life (approximately an hour) and its route of administration (either intravenous or subcutaneous) ⁶³. An ideal candidate for this compound-assisted chemico-vaccine approach would have a long half-life and would be administered orally to reduce costs and improve accessibility. Importantly, within this study, we did identify a small molecule inhibitor of invasion (R59022) that was weakly synergistic with anti-PfRH5 mAbs when used at low compound concentrations, suggesting that compounds other than heparinoids could indeed be discovered and adapted for use as vaccine adjuncts. R59022 has previously been shown to inhibit microneme secretion and motility in the model Apicomplexan parasite *Toxoplasma gondii*, and the murine malaria model *Plasmodium berghei* through inhibiting microneme secretion ^{61, 91}. It is possible that at low concentrations, R59022 slows the release of the micronemes without completely blocking it, giving the mAbs more time to bind to PfRH5. While this compound is insufficiently potent in its current form, it does suggest that small-molecule inhibitors of microneme secretion could synergise with vaccine-induced anti-PfRH5 antibodies. This, together with heparinoids also targeting micronemal proteins, justifies further efforts to discover compounds that inhibit microneme secretion and that may synergise with vaccine-induced anti-PfRH5 mAbs. This proof-of-principle study provides the justification for future work towards developing long-acting antimalarials that could be paired with erythrocytic-stage vaccinations to enhance protection against *P. falciparum*.

The chemico-vaccine strategy could be implemented in several ways across both preventive and therapeutic contexts. The compound, when administered at high concentrations, could directly block invasion and greatly reduce parasitemia. As the compound's concentration declines, vaccine-induced antibodies could further extend the compound's effectiveness. At the same time, the compound and the vaccine-induced antibodies could synergistically act to provide greater overall protection against malaria.

Another outcome of this work is that by assessing interactions between anti-PfRH5 mAbs and inhibitors acting at distinct stages of merozoite egress and invasion, we have identified invasion processes that are more amenable to synergistic targeting. Our findings indicate that synergy is not achieved simply by targeting multiple steps within the invasion pathway but is highly dependent on the timing of inhibition relative to PfRH5 exposure. This framework could guide the design of multivalent erythrocytic-stage vaccines by identifying antigen combinations that elicit synergistic antibody responses.

In conclusion, these data demonstrate that invasion-inhibitory compounds can act synergistically with PfRH5 vaccine-induced antibodies. In a clinical setting, this chemico-vaccine synergy could increase the protection that vaccines provide from malaria, thus contributing to more effective malaria prevention strategies and supporting global efforts to reduce malaria-related disease and death. Furthermore, our findings provide new insights into how distinct stages of merozoite invasion can be targeted in combination to maximise inhibitory activity, informing the development of next-generation multivalent malaria vaccines.

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Data Availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Author contribution

A.H.L. – Designed and conducted experiments and analysed data. Wrote the original draft, reviewed and edited the manuscript.

C.B.G.B. – Contributed to supervision and experimental design. Reviewed and edited the manuscript.

M.P.S. – Contributed to supervision and experimental design.

A.T. – Generated reagents and reviewed the manuscript.

B.G.W. – Generated reagents and reviewed the manuscript.

S.E.S. – Generated reagents.

B.C. – Provided funding.

S.J.D. – Generated reagents and reviewed the manuscript.

J.B. – Contributed to supervision. Reviewed the manuscript.

H.E.B. – Provided supervision and experimental design. Reviewed the manuscript and provided funding.

P.R.G. – Provided supervision and experimental design. Reviewed the manuscript and provided funding.

Declaration of competing interests

BGW and SJD are inventors on patent applications relating to PfRH5 and/or RCR-complex malaria vaccines and/or antibodies, and inventors on intellectual property licensed by Oxford University Innovation to Serum Institute of India Pvt Ltd.

Tables

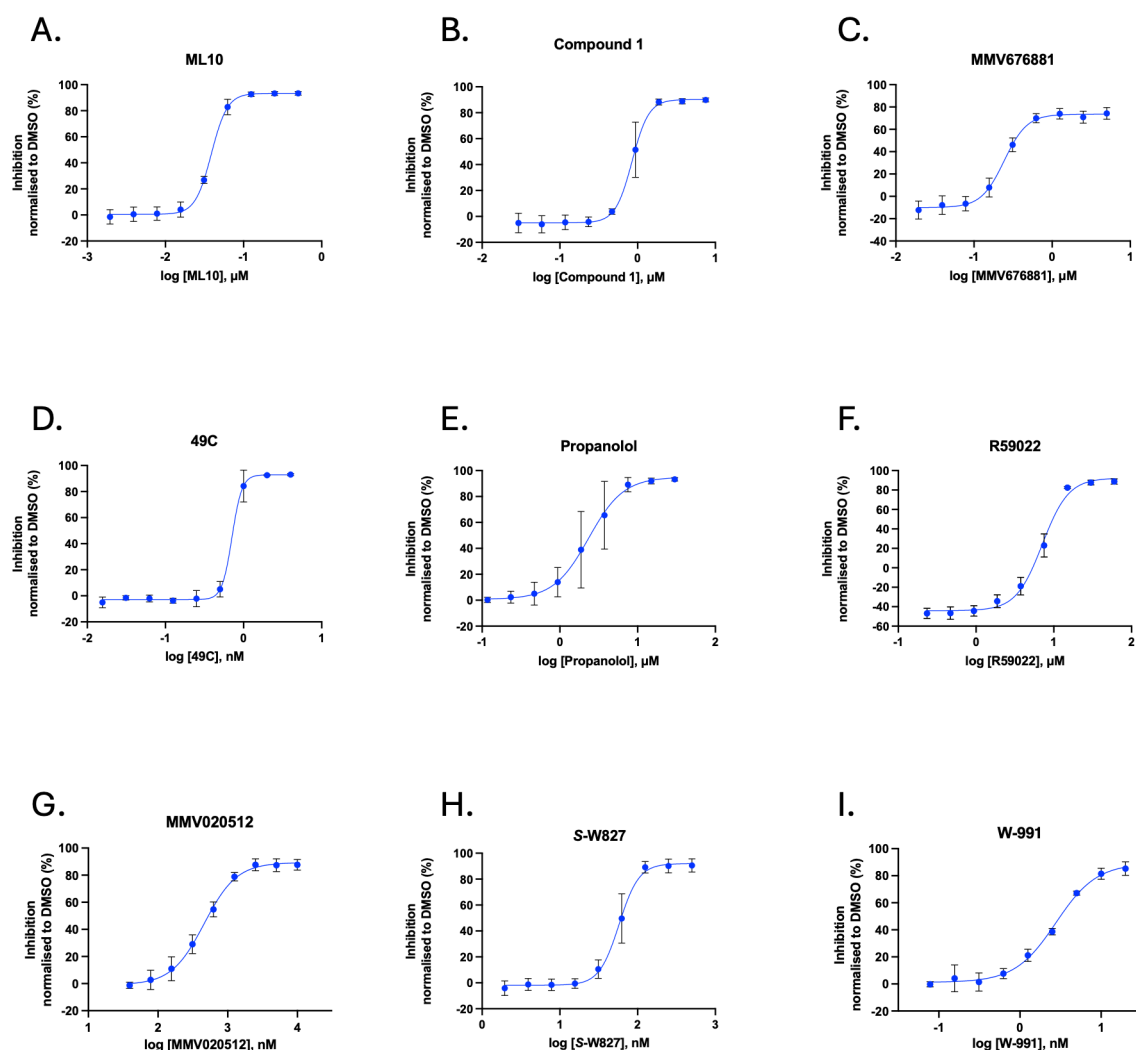
Table 2. Experimental invasion-inhibitory compounds investigated for synergy.

Inhibitor	IC ₅₀	Target	Function
ML10	0.04 µM	PKG	Blocks PVM rupture ⁵⁷
Compound 1	0.85 µM	PKG	Blocks PVM rupture ⁵⁸
MMV676881	0.24 µM	SERA6	Blocks erythrocyte rupture ⁶⁰
49C	0.0008 µM	PMIX/PMX	Blocks erythrocyte rupture ⁵⁹
Propranolol	2.34 µM	PAP	Promotes microneme release ⁹²
R59022	7.12 µM	DGK	Blocks microneme release ⁹²
Heparin	7.7 µg/mL	MSPs/RHs/EBAs	Blocks strong erythrocyte binding ²⁶
Sevuparin	39.54 µg/mL	MSPs/RHs/EBAs	Blocks strong erythrocyte binding ⁶³
RON2 peptide	0.0012 mg/mL	AMA-1	Competes for interaction with AMA1 ⁷⁰
MMV020512	0.46 µM	ROM-8 and CSC1	Inhibits parasite growth ⁶⁴
S-W827	0.05 µM	F-actin	Blocks actin polymerisation ⁶⁷
W-991	0.003 µM	START1	Blocks PVM expansion ⁹³

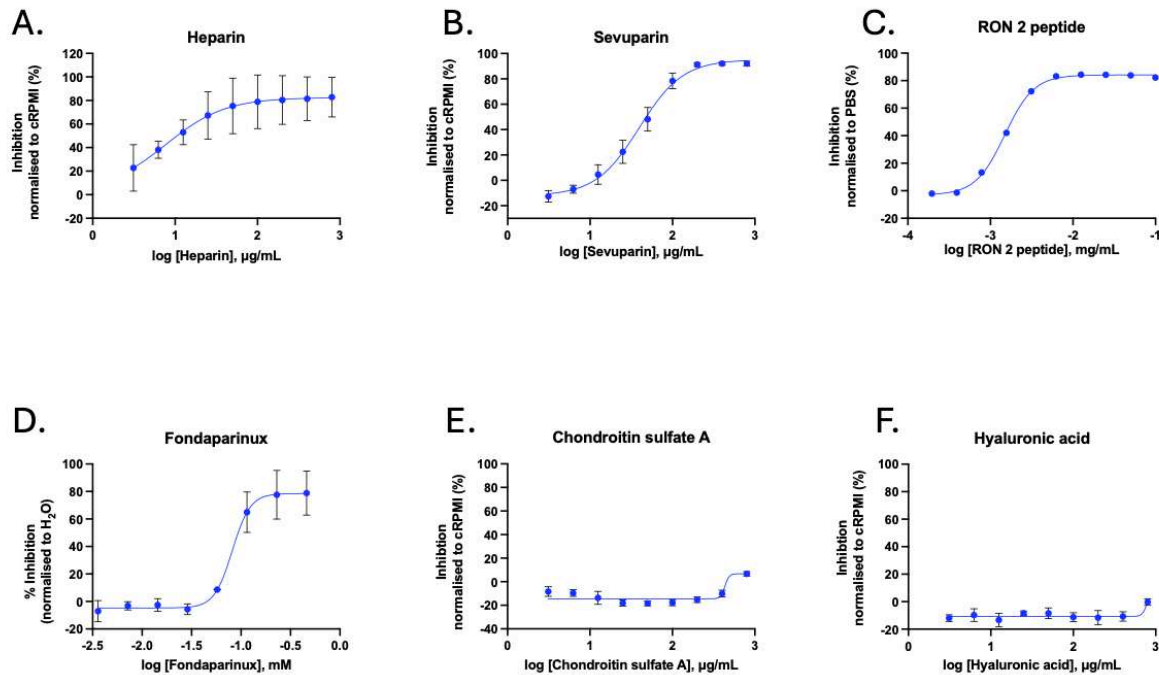
Table 3. Experimental invasion-inhibitory monoclonal antibodies investigated for synergy.

Antibody	IC₃₀	Target	Action
R5.004	0.02 mg/mL	PfRH5	Neutralising mAb ⁴²
R5.016	0.01 mg/mL	PfRH5	Neutralising mAb ⁴²
R5.008	0.03 mg/mL	PfRH5	Neutralising mAb ⁴²
Cy.009	0.13 mg/mL	PfCyRPA	Neutralising mAb ⁸²
R5.011	0.5 mg/mL	PfRH5	Potentiating mAb ⁴²
R5.015	N/A	PfRH5	Non-neutralising mAb ⁴²
Polyclonal PfRH5 IgG	3 mg/mL	PfRH5	50

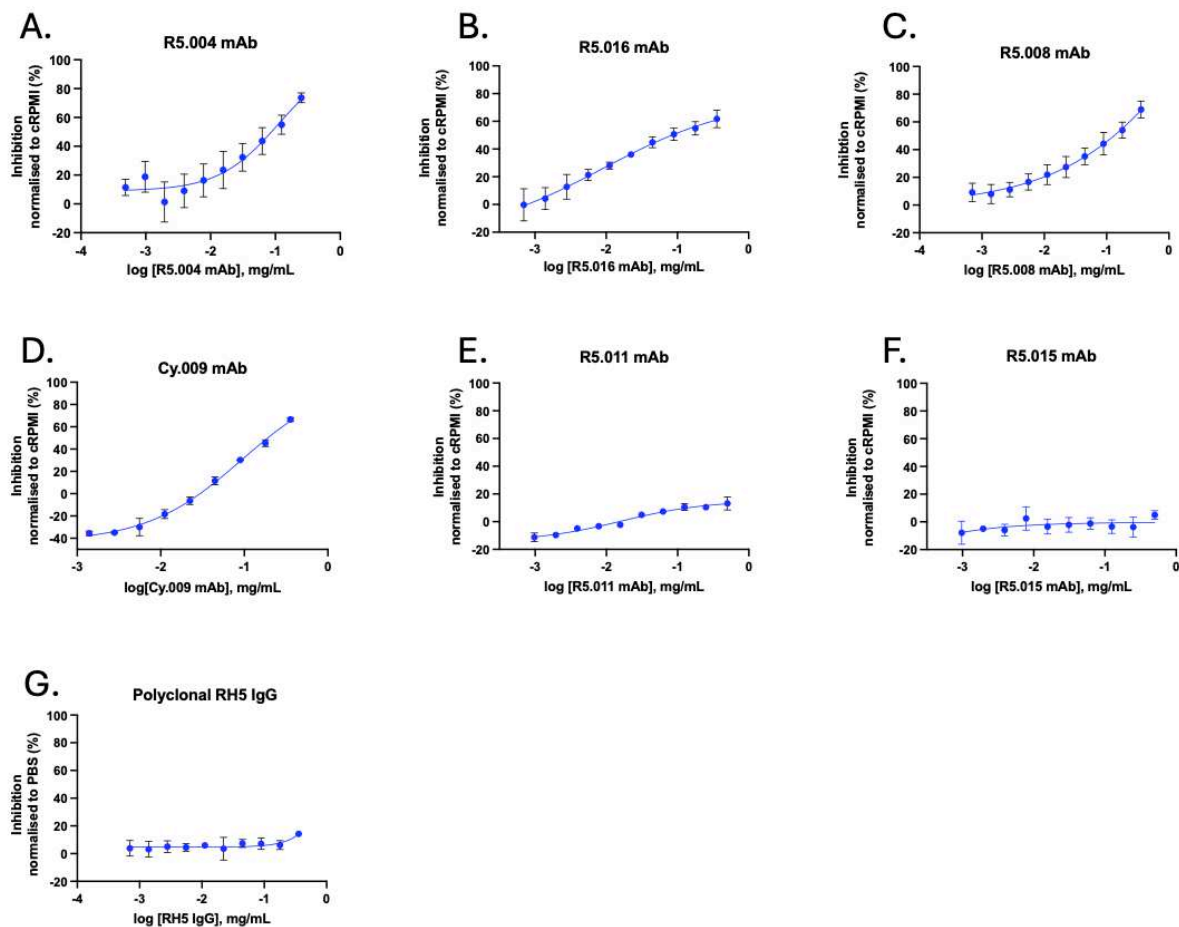
Supplementary figures



Supplementary Figure 1. Dose-response curves for invasion-targeting compounds. Parasites were treated for 44 hours with a 9-point, 2-fold serial dilution of **A.** ML10, starting maximum concentration of 0.5 μ M. **B.** Compound 1, starting maximum concentration of 7.5 μ M. **C.** MMV676881, starting maximum concentration of 5 μ M. **D.** 49C, starting maximum concentration of 0.004 μ M. **E.** Propranolol, starting maximum concentration of 30 μ M. **F.** R59022, starting maximum concentration of 60 μ M. **G.** MMV020512, starting maximum concentration of 10 μ M. **H.** S-W827, starting maximum concentration of 0.5 μ M. **I.** W-991, starting maximum concentration of 0.02 μ M. Data was normalised to DMSO vehicle control. GIA graph lines were fitted using the "log(inhibitor) vs. response – Variable slope (four parameters)" model in GraphPad Prism 10. Data points and error bars represent the mean and SD of three biological replicates, each consisting of three technical replicates.

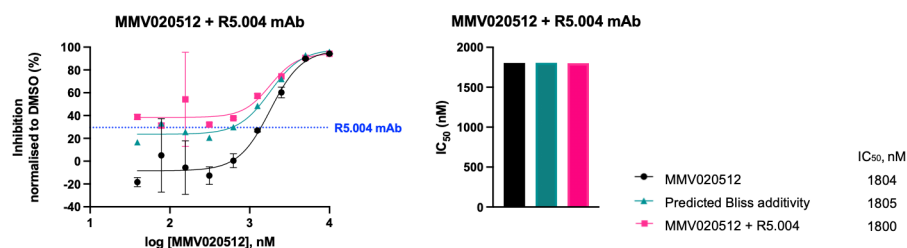


Supplementary Figure 2. Dose-response curves for inhibitory polysaccharides and peptides. Parasites were treated for 44 hours with a 9-point, 2-fold serial dilution of **A.** Heparin, starting maximum concentration of 800 $\mu\text{g/mL}$. **B.** Sevuparin, starting maximum concentration of 800 $\mu\text{g/mL}$. **C.** RON 2 peptide, starting maximum concentration of 0.1 mg/mL. Data normalised to PBS. **D.** Fondaparinux, starting maximum concentration of 0.92 mM. Data normalised to H_2O . **E.** Chondroitin sulfate A, starting maximum concentration of 800 $\mu\text{g/mL}$. **F.** Hyaluronic acid, starting maximum concentration of 800 $\mu\text{g/mL}$. Unless stated data was normalised to cRPMI vehicle control. GIA graph lines were fitted using the "log(inhibitor) vs. response – Variable slope (four parameters)" model in GraphPad Prism 10. Data points and error bars represent the mean and SD of (A,B): three biological replicates, each consisting of three technical replicates. (C): two biological replicates, each consisting of three technical replicates. (D,E,F): two technical replicates.

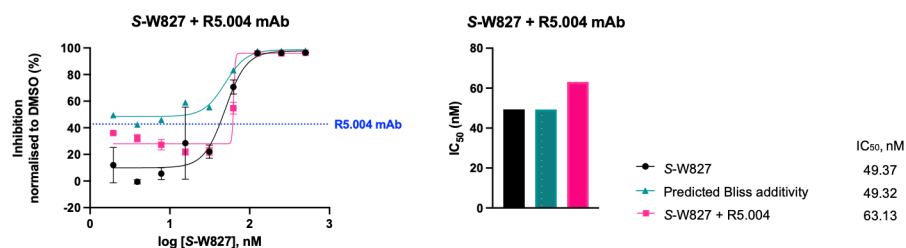


Supplementary Figure 3. Dose-response curves for monoclonal antibodies. Parasites were treated for 44 hours with a 9-point, 2-fold serial dilution of **A.** R5.004 mAb, starting maximum concentration of 0.35 mg/mL. **B.** R5.016 mAb, starting maximum concentration of 0.35 mg/mL. **C.** R5.008 mAb, starting maximum concentration of 0.35 mg/mL. **D.** Cy.009 mAb, starting maximum concentration of 0.5 mg/mL. **E.** R5.011 mAb, starting maximum concentration of 0.5 mg/mL. **F.** R5.015 mAb, starting maximum concentration of 0.5 mg/mL. **G.** Polyclonal anti-PfRH5 total IgG, starting maximum concentration of 0.5 mg/mL. GIA graph lines were fitted using the "log(inhibitor) vs. response – Variable slope (four parameters)" model in GraphPad Prism 10. In panels A, E-G data points and error bars represent the mean and SD of two technical replicates. In panels B-D data points and error bars represent the mean and SD of three biological replicates, each consisting of two technical replicates.

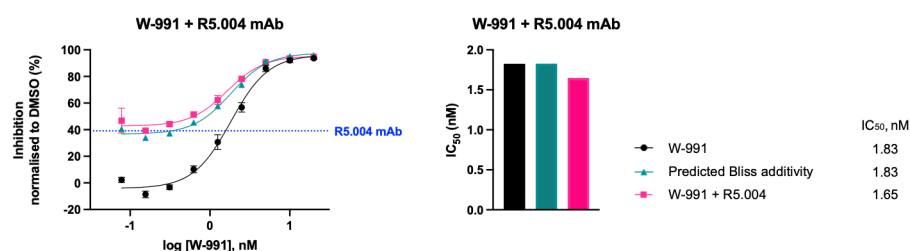
A.



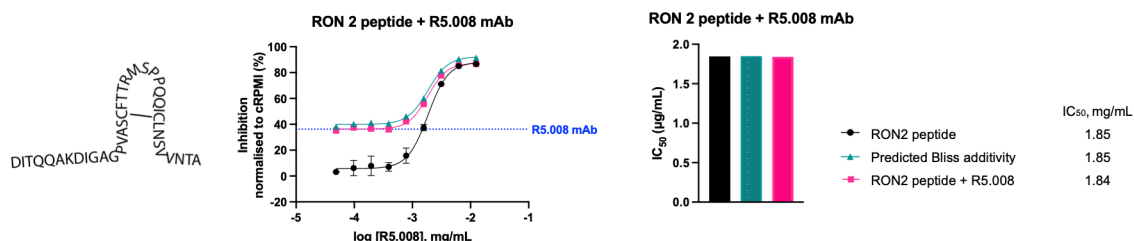
B.



C.



D.



Supplementary Figure 4. Late-acting invasion inhibitors show an additive invasion-inhibitory effect when combined with R5.004 or R5.008 mAbs. Growth inhibition was quantified after 44 hours of treatment by measuring Nluc normalised to growth in the presence of DMSO or PBS vehicle control. Growth curves were generated (middle panels) and analysed by the Bliss additivity model to generate IC₅₀s for the compound alone (black) **A.** MMV020512, **B.** S-W827, **C.** W-991 **D.** RON2 peptide, the structure of which is shown on the left. The predicted additive effect (teal), and the measured combined effect of the titrated compound with a fixed concentration of mAb. R5.004 held at 0.02 mg/mL or R5.008 held at 0.03 mg/mL (pink). The right graphs display IC₅₀ of the titrated compound (calculated from the middle panel) within each condition, represented as a bar graph. The IC₅₀ for each fitted concentration–response curve was calculated as the concentration producing an inhibitory effect halfway between the fitted upper and lower plateaus. Data points and error bars represent the mean and SD of two technical replicates.

Supplementary Table 1. N/A, only one biological replicate done, so p values not available. * p <0.05.

** p <0.01, *** p<0.001

	IC ₅₀ Compound only (SD)	IC ₅₀ Predicted Bliss Additivity (SD)	IC ₅₀ Measured Compound + mAb (SD)	IC ₅₀ fold change (IC ₅₀ predicted/IC ₅₀ measured)	p-value	p-value summary
MMV020512 + IC ₃₀ R5.016 mAb	725.5 (494.9)	726.7 (496.3)	758.1 (547.0)	0.96-fold	0.95	ns
MMV020512 + IC ₃₀ R5.004 mAb	1804	1805	1800	1.0-fold	N/A	N/A
S-W827 + IC ₃₀ R5.016 mAb	59.59 (8.92)	59.55 (8.91)	65.32 (7.26)	0.91-fold	0.43	ns
S-W827 + IC ₃₀ R5.004 mAb	49.37	49.32	63.13	0.78-fold	N/A	N/A
W-991 + IC ₃₀ R5.016 mAb	1.00 (0.48)	1.00 (0.48)	1.15 (0.43)	0.87-fold	0.70	ns
W-991 + IC ₃₀ R5.004 mAb	1.83	1.83	1.65	1.11-fold	N/A	N/A
RON2 peptide + IC ₃₀ R5.008 mAb	1.85	1.85	1.84	1.01-fold	N/A	N/A
ML10 + IC ₃₀ R5.008 mAb	38.90 (2.15)	38.90 (2.15)	39.90 (2.16)	0.97-fold	0.60	ns
Compound 1 + IC ₃₀ R5.008 mAb	1.12 (0.08)	1.12 (0.08)	1.05 (0.07)	1.0-fold	0.43	ns
49C + IC ₃₀ R5.008 mAb	0.65 (0.27)	0.65 (0.27)	0.52 (0.12)	1.0-fold	0.50	ns
MMV676881 + IC ₃₀ R5.008 mAb	0.19 (0.03)	0.19 (0.03)	0.18 (0.02)	1.0-fold	0.43	ns
R59022 + IC ₃₀ R5.008 mAb	8.87 (1.65)	8.86 (1.65)	9.90 (2.48)	0.89-fold	0.58	ns
Propranolol + IC ₃₀ R5.008 mAb	6.05(2.67)	6.32 (2.78)	7.64 (3.29)	0.83-fold	0.62	ns
Heparin + IC ₃₀ R5.004 mAb	6.72 (3.79)	6.71 (3.77)	1.43 (0.84)	4.7-fold	0.077	ns
Heparin + IC ₃₀ R5.016 mAb	10.76 (4.21)	10.76 (4.19)	1.81 (0.90)	5.9-fold	0.023	*
Heparin + IC ₃₀ R5.008 mAb	4.44 (0.76)	4.44 (0.76)	1.85 (0.24)	2.4-fold	0.005	**
Heparin + IC ₃₀ Cy.009 mAb	7.83 (1.71)	7.75 (1.74)	6.24 (2.77)	1.24-fold	0.4690	ns
Sevuparin + IC ₃₀ R5.004 mAb	56.36 (21.42)	56.43 (21.47)	7.75 (2.36)	7.3-fold	0.018	*
Sevuparin + IC ₃₀ R5.016 mAb	59.73 (7.94)	59.75 (7.91)	12.26 (2.23)	4.9-fold	0.0006	***
Sevuparin + IC ₃₀ R5.008 mAb	38.37 (6.84)	38.42 (6.77)	6.627 (0.80)	5.8-fold	0.0013	**
Sevuparin + IC ₃₀ R5.011 mAb	35.70 (5.36)	35.71 (5.34)	75.78 (10.82)	0.47-fold	0.0045	**
Sevuparin + IC ₃₀ R5.015 mAb	38.04 (6.54)	38.00 (6.55)	36.42 (8.52)	1.00-fold	0.85	ns
Sevuparin + IC ₃₀ RH5 IgG pAb	27.12 (6.4)	27.44 (7.2)	5.27 (1.8)	5.2-fold	0.0065	**

Supplementary Table 2. Isobologram analysis of inhibitory compounds with R5.008 mAb. SE, standard error; FIC, fractional inhibitory concentration.

Combination	FIC Compound (SE)	FIC R5.008 (SE)	Sum FIC (SE)	Interaction
1 (100% S-W827)	1 (0)	0 (0)	1 (0)	Additive
2 (80% S-W827 + 20% R5.008)	0.99 (0.04)	0.07 (0.009)	1.06 (0.05)	Additive
3 (60% S-W827 + 40% R5.008)	0.92 (0.04)	0.60 (0.41)	1.52 (0.37)	Antagonistic
4 (40% S-W827 + 60% R5.008)	1.04 (0.06)	0.44 (0.06)	1.48 (0.12)	Antagonistic
5 (20% S-W827 + 80% R5.008)	0.76 (0.32)	1.25 (0.18)	2.01 (0.18)	Antagonistic
6 (100% R5.008)	0	1 (0)	1 (0)	Additive
1 (100% 49C)	1 (0)	0 (0)	1 (0)	Additive
2 (80% 49C + 20% R5.008)	1.00 (0.07)	0.10 (0.03)	1.09 (0.07)	Additive
3 (60% 49C + 40% R5.008)	0.92 (0.08)	0.23 (0.07)	1.15 (0.07)	Additive
4 (40% 49C + 60% R5.008)	0.69 (0.21)	0.39 (0.10)	1.07 (0.21)	Additive
5 (20% 49C + 80% R5.008)	0.65 (0.19)	0.71 (0.09)	1.36 (0.17)	Antagonistic
6 (100% R5.008)	0 (0)	1 (0)	1 (0)	Additive
1 (100% R59022)	1 (0)	0 (0)	1 (0)	Additive
2 (80% R59022 + 20% R5.008)	0.92 (0.04)	0.14 (0.02)	1.06 (0.06)	Additive
3 (60% R59022 + 40% R5.008)	0.72 (0.04)	0.28 (0.05)	1.00 (0.09)	Additive
4 (40% R59022 + 60% R5.008)	0.48 (0.01)	0.41 (0.05)	0.89 (0.06)	Additive
5 (20% R59022 + 80% R5.008)	0.23 (0.008)	0.52 (0.06)	0.75 (0.06)	Synergy
6 (100% R5.008)	0 (0)	1 (0)	1 (0)	Additive
1 (100% Sevuparin)	1 (0)	0 (0)	1 (0)	Additive
2 (80% Sevuparin + 20% R5.008)	0.36 (0.0005)	0.05 (0.005)	0.41 (0.004)	Synergy
3 (60% Sevuparin + 40% R5.008)	0.26 (0.001)	0.09 (0.009)	0.35 (0.008)	Synergy
4 (40% Sevuparin + 60% R5.008)	0.20 (0.005)	0.15 (0.02)	0.36 (0.01)	Synergy
5 (20% Sevuparin + 80% R5.008)	0.13 (0.005)	0.26 (0.02)	0.39 (0.01)	Synergy
6 (100% R5.008)	0 (0)	1 (0)	1 (0)	Additive

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