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Exploring the Utility of Cell-Penetrating Peptides as Vehicles for the Delivery of Distinct Antimalarial Drug Cargoes

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Abstract: The devastating impact of malaria includes significant mortality and illness worldwide. Increasing resistance of the causative parasite, *Plasmodium*, to existing antimalarial drugs underscores a need for additional compounds with distinct modes of action in the therapeutic development pipeline. Here we showcase peptide-drug conjugates (PDCs) as an attractive compound class, in which therapeutic or lead antimalarials are chemically conjugated to cell-penetrating peptides. This approach aims to enhance selective uptake into *Plasmodium*-infected red blood cells and impart additional cytotoxic actions on the intraerythrocytic parasite, thereby enabling targeted drug delivery and dual modes of action. We describe the development of PDCs featuring four compounds with antimalarial activity – primaquine, artesunate, tafenoquine and methotrexate – conjugated to three cell-penetrating peptide scaffolds with varied antiplasmodial activity, including active and inactive analogues of

platelet factor 4 derived internalization peptide (PDIP), and a cyclic polyarginine peptide. Development of this diverse set of PDCs featured distinct and adaptable conjugation strategies, to produce conjugates with *in vitro* antiplasmodial activities ranging from low nanomolar to low micromolar potencies according to the drug cargo and bioactivity of the partner peptide. Overall, this study establishes a strategic and methodological framework for the further development of dual mode of action peptide-drug antimalarial therapeutics.

Introduction

Malaria is a significant global health challenge, particularly within endemic regions in Africa and South-east Asia.^[1] *Plasmodium*, the causative protozoan parasite, was responsible for an

estimated 249 million cases of malaria and 608 000 deaths in 2022, predominantly in African nations.^[2] While the incidence and mortality associated with malaria has significantly declined over the last two decades,^[2] continued progress has been hampered in recent years by the repeated emergence of drug resistant *Plasmodium* strains.^[1, 3] In particular, *Plasmodium falciparum*, the species responsible for the majority of mortality, has evolved partial or complete resistance to nearly all antimalarial therapeutics.^[3-4] The independent development of resistance to artemisinin, a key component of artemisinin-based combination therapies (ACTs), in both Southeast Asia and Africa^[4-5] also highlights the urgent need for new treatment options.

Peptide-drug conjugates (PDCs) have emerged as an effective approach for developing novel therapeutics within the cancer field^[6] but have been underexplored for the treatment of malaria^[7] and other infectious diseases.^[8] In general, drug conjugates have garnered attention as a means of achieving high target-selectivity and reduced off-target effects.^[6c, 9] Such conjugates are comprised of three components; a drug payload that is responsible for therapeutic activity, a carrier molecule for selective delivery of the drug, and a linker to tether the two components, which can be designed to allow programmed release of the drug at a desired location^[6a, 6c] (overview in Figure 1A). Although antibodies have commonly been used as the

targeting moiety,^[6b] interest in PDCs is growing, with two approved PDCs currently on market.^[6e, 10] While the large size of antibodies makes them synthetically costly modalities and primarily limited to the targeting of cell-surface receptors, peptides are much smaller and depending on their properties, can either deliver drug cargo to cell surfaces or cross cell membranes for delivery to intracellular targets.^[6a, 6c]

Peptides capable of crossing cell membranes are called cell-penetrating peptides (CPPs). They utilize a variety of mechanisms to access the intracellular environment, including endocytosis and direct translocation, and can facilitate intracellular delivery of large cargoes including proteins.^[11] Many CPPs are rich in cationic amino acid residues and/or have an amphipathic nature that contributes to their cell-penetrating activity.^[11] We previously developed a novel CPP derived from the active domain of human platelet factor 4 (PF4), a protein with innate antiplasmodial activity that is released by activated platelets in response to *Plasmodium* infection in the bloodstream.^[12] This peptide, here known as PF4-derived internalization peptide (PDIP, formerly cPF4PD^[12]), selectively enters *Plasmodium*-infected red blood cells (RBCs) as opposed to uninfected RBCs, and retains the antiplasmodial activity of PF4 via lysing the *Plasmodium* digestive vacuole.^[12] PDIP has two α -helical regions with amphipathic character and a high proportion

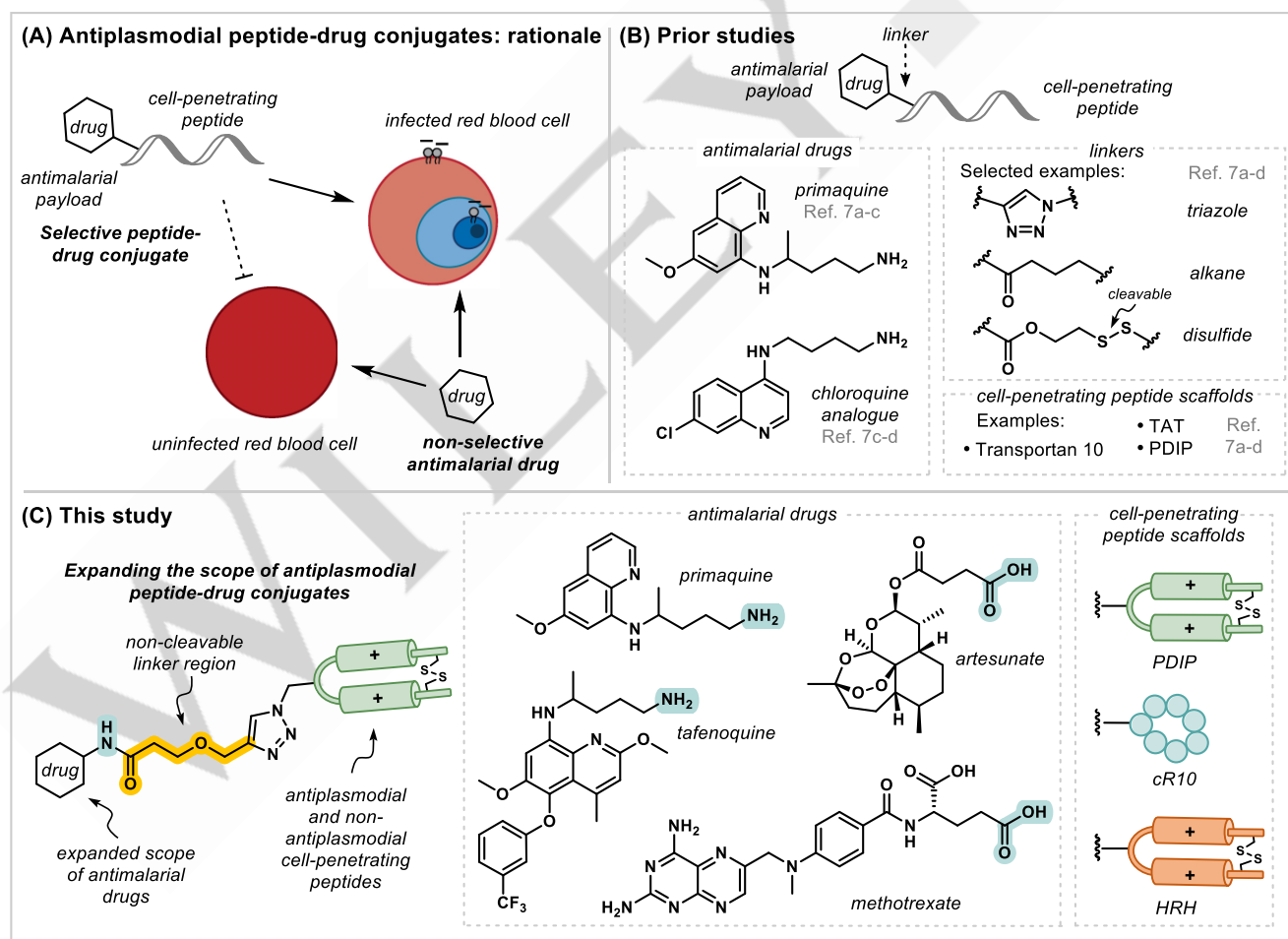


Figure 1. Components for designing and constructing antiplasmodial peptide-drug conjugates (PDCs). (A) Proposed targeted delivery of non-selective small-molecule antimalarial payloads into infected RBCs by cell-penetrating peptides. (B) Previous efforts towards the development of antiplasmodial PDCs.^[7a-d] (C) Antiplasmodial PDCs described in this work contain an expanded scope of antimalarial drugs and cell-penetrating peptide scaffolds.

of cationic residues that facilitate its cell-penetrating ability.^[12] The cationic nature of PDIP also forms the basis of its selectivity for *Plasmodium*-infected RBCs and the parasite digestive vacuole within, as both contain a higher proportion of negatively charged phospholipids within their membranes relative to uninfected RBCs.^[13] For these reasons, the cell-penetrating activity of PDIP has been used in the design of anticancer peptide conjugates,^[14] and we have recently published our preliminary work on antiplasmodial PDIP-based PDCs incorporating the small molecule antimalarial drug primaquine (PQ) and a selection of cleavable and non-cleavable linkers (Figure 1B).^[7a] Other studies have explored the utility of select CPPs for the development of antiplasmodial PDCs incorporating PQ and an analogue of the structurally similar aminoquinoline derivative, chloroquine (Figure 1B).^[7a-d, 15] While these studies highlight PDCs as a promising approach for the design of new therapeutics,^[7a-d] the limited structural diversity of incorporated drug cargoes and in some cases, high hemolytic activity of existing conjugates, warrants further investigation to improve the efficacy and safety profile of PDC candidates.

To address this need and to better understand the contributions of both peptide and drug cargo to overall antiplasmodial activity, we designed and synthesized a library of PDCs incorporating varied cell-penetrating peptide scaffolds and small-molecule antimalarial drug cargoes (Figure 1C). Late-stage conjugation chemistry allowed the construction of the PDC library via a modular approach with variations in peptide, linker, and drug cargo. PDIP and an inactive analogue (PDIP-HRH),^[16] were included as peptide scaffolds, together with cR10, a polyarginine CPP known to facilitate cytosolic delivery of cargo molecules.^[14, 17] Four antimalarial drugs, PQ and tafenoquine (TQ, 8-aminoquinoline derivatives), artesunate (AS, artemisinin derivative) and methotrexate (MTX, antimetabolite), with varying modes of action, efficacy windows, and chemical handles suitable for conjugation, were included as therapeutic cargo with an aim to improve antiplasmodial activity and nonhemolytic properties of PDCs. Examination of antiplasmodial activity using two *in vitro* assay technologies consistently revealed nanomolar to low micromolar activity as dictated by the potency of the cargo drug. Whilst activity of the conjugates did not exceed activity of the parent drug, the most potent PDCs contained peptides that were both cell-penetrating and possessed inherent antiplasmodial activity. This work demonstrates the potential for developing dual action antiplasmodial PDCs, a favorable feature in the design of therapeutics with reduced propensity for acquired drug resistance.

Results and Discussion

Design and Synthesis of Modified Small Molecule Antimalarial Drugs for Conjugation

To investigate the capability of the CPPs to deliver multiple distinct drug cargoes, we chose four representative antimalarial drugs that have either safety or resistance concerns, where incorporation into a PDC for selective delivery to infected cells may prove beneficial. The 8-aminoquinoline derivatives, PQ and TQ, were both chosen for incorporation into PDCs due to their poor safety profile in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency—a common genetic disorder in malaria endemic regions. However, they are still clinically

significant drugs as they are effective treatments against hypnozoites (the dormant stage of certain *Plasmodium* species), preventing relapse of infection.^[18] Additionally, the inclusion of PQ allowed for comparison to our prior studies examining the role of the linker region in the PDIP-PQ molecule.^[7a] We also explored conjugation to AS, a current mainstay antimalarial drug that is at risk of losing effectiveness due to acquired drug resistance,^[5b, 5c] and as such may benefit from incorporation into a PDC, where the combined activity of the peptide and drug is reminiscent of ACTs. Finally, we investigated incorporation of MTX, a dihydrofolate reductase (DHFR) inhibitor frequently used as an anticancer agent. MTX is reported to have antiplasmodial activity, but with limited information about its safety and efficacy as an antimalarial therapeutic, it has yet to be clinically implemented in this context.^[19]

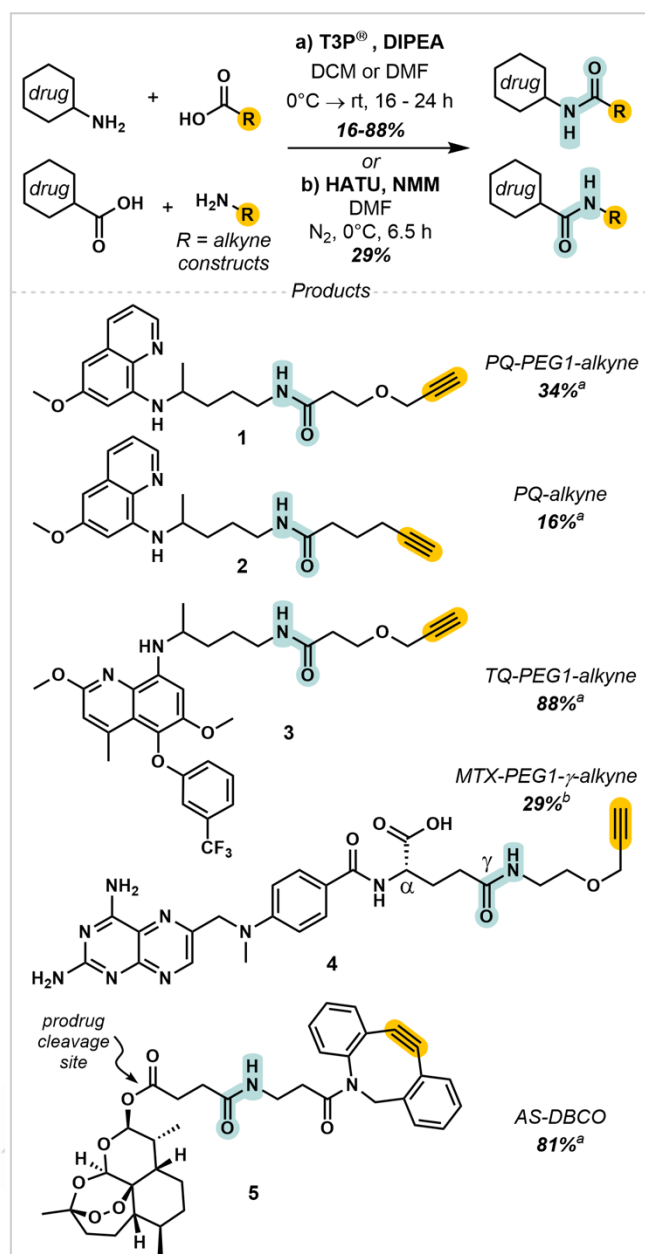
Azide-alkyne cycloaddition click chemistry^[20] was employed to produce the library of PDCs as this method is orthogonal to the disulfide bonds in the PDIP scaffolds and allows late-stage conjugation of peptide and functionalized drug. Both copper-catalyzed azide-alkyne cycloaddition (CuAAC)^[20a, 20b] and strain-promoted azide-alkyne cycloaddition (SPAAC)^[20c] emerged as viable methods to facilitate PDC assembly. However, in the first instance, CuAAC was chosen as the preferred approach given its widely accessible and inexpensive precursors, as well as the smaller chemical footprint associated with the simple triazole linker formed in the final conjugate. PDCs were designed such that the azide handle was incorporated into the peptide, making use of commercially available unnatural amino acids, whilst the drugs were modified by adding a linker with a terminal alkyne handle.

The primary amine of both PQ and TQ serves as an accessible site for introducing the linker-alkyne functionality via amide bond formation. To this end, propargyl-PEG1-acid was coupled onto the primary amine of both PQ and TQ with the aid of the coupling reagent T3P[®] to produce **1** and **3**, in 34% and 88% yield, respectively (Scheme 1). We also synthesized an alkyne bearing analogue of PQ through coupling to 5-hexynoic acid (**2**, Scheme 1), to pair with PEG-azide bearing peptides (see below). Similarly, α - and γ -carboxylic acids within the glutamate moiety of MTX presented convenient sites for amide bond formation to introduce the linker-alkyne functionality. We were particularly interested in incorporation of the alkyne handle onto the γ -carboxylic acid as previous studies have demonstrated greater potency for γ -substituted MTX analogues than α -substituted analogues, owing to the importance of the α -carboxylic acid for the binding of MTX to its target enzyme, DHFR.^[21] Non-selective coupling of propargyl-PEG1-amine to the α - and γ -carboxylic acid of MTX was attained using HATU (Scheme 1), producing a near 1:1 regioisomeric mixture of α -alkyne (33%, see SI) and γ -alkyne (**4**, 29%) functionalized MTX, separable by chromatography.

These modifications to PQ, TQ and MTX thus presented handles for CuAAC conjugation. In contrast, initial investigations identified the poor suitability of alkyne modified AS for CuAAC, which we postulate may be attributable to the instability of the AS ester, cyclic acetal or peroxide functionalities in the presence of CuAAC reagents, including the copper catalyst, which has been previously observed^[22] (see SI Table S2 for further details). Instead, we devised an alternative strategy involving functionalization with a handle suitable for SPAAC conjugation.^[20c] This strategy involved coupling of amine-functionalized dibenzocyclooctyne (DBCO), a highly strained

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alkyne capable of undergoing SPAAC reactions in the absence of copper, onto the carboxylic acid functionality of AS to yield **5** (81%) using T3P® (Scheme 1). Notably, AS is a prodrug form of dihydroartemisinin (DHA), which is released *in vivo* following cleavage of the ester linkage by endogenous esterases (Scheme 1).^[23] Accordingly, this drug-linker moiety was also expected to liberate DHA. Cleavage of the endoperoxide bridge of artemisinin derivatives, predominantly in the presence of heme, further contributes to the activation of this drug.^[24]



Scheme 1. Functionalization of antimalarial drugs with non-cleavable alkyne handles *via* amide bond formation.

Design and Synthesis of CPPs to Act as Delivery Tools

To investigate the role of the peptide, we focused our efforts on the synthesis of three different cell-penetrating scaffolds for incorporation into PDCs. PDIP was utilized as a prototypical delivery candidate given its high selectivity for infected RBCs,

inherent antiplasmodial activity, and minimal hemolytic activity.^[12, 16] Two alternative CPP scaffolds, PDIP-HRH and cR10, that have reduced antiplasmodial activity, were used to understand the relative importance of peptide activity to the overall activity of the PDCs (Scheme 2A).

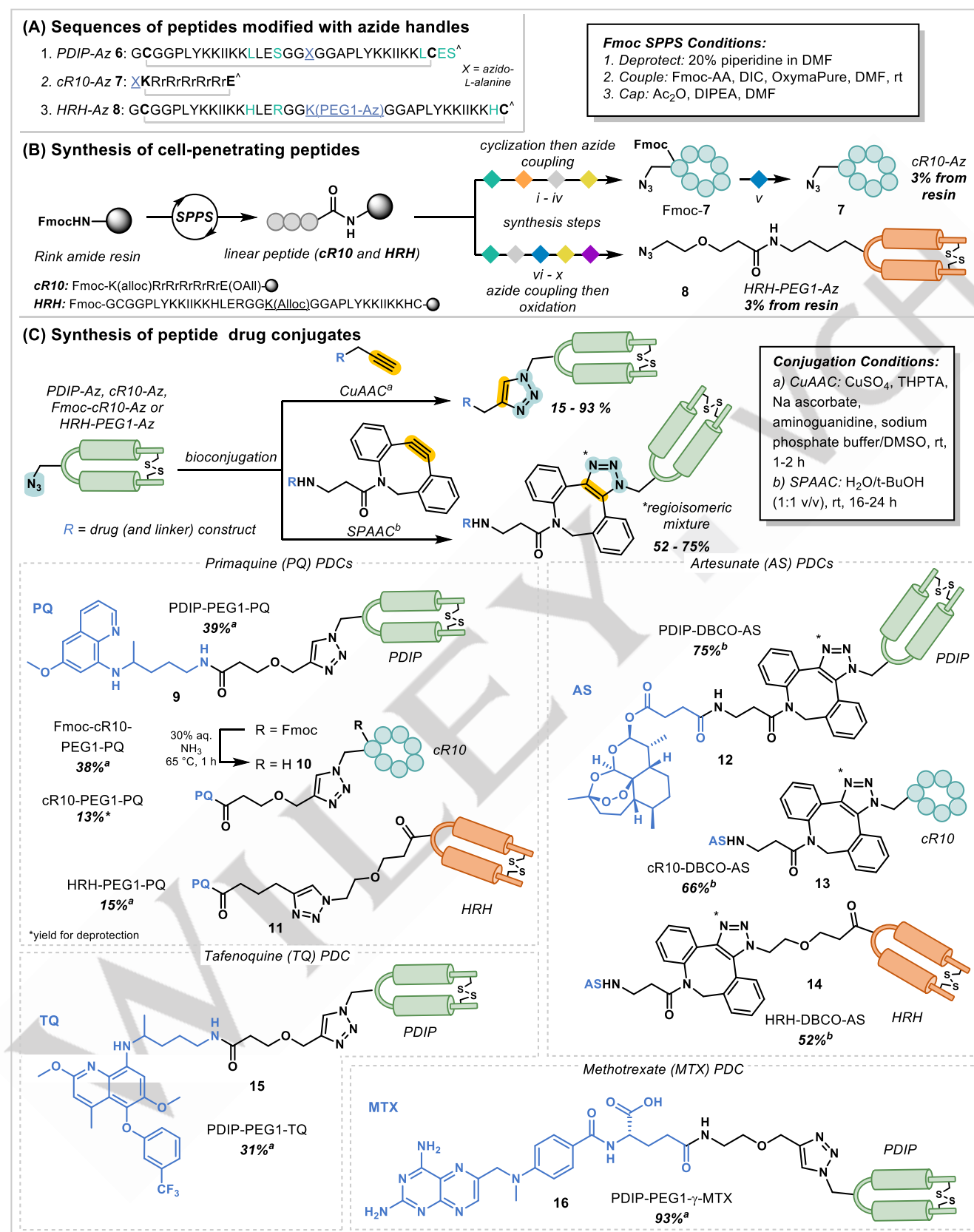
PDIP-HRH, herein referred to as HRH, was identified from activity screening of a library of rationally designed PDIP analogues.^[16] Compared to the PDIP primary sequence, HRH has three amino acid substitutions (L14H, S17R, L33H) and two deletions (E35, S36). These alterations disrupt the structural stability (i.e. helicity) of the peptide and diminish its antiplasmodial activity, although HRH retains cell-penetrating abilities with selectivity for infected RBCs (Supplementary Figure S1). Given these properties and its structural similarity to PDIP, HRH is an ideal CPP scaffold to probe the role of the peptide within PDCs without directly contributing to the antiplasmodial activity.

cR10 is structurally distinct from PDIP and HRH, comprising a sequence of 10 arginine residues (alternating *L*- and *D*-amino acids) that has a +10 charge at physiological pH (Scheme 2A). Polyarginine peptides such as cR10 are highly capable of delivering large cargoes, including proteins and peptides, into cells through direct translocation and without disruption of the plasma membrane of involved cells.^[14, 17] Inclusion of this peptide in our study allowed exploration of PDCs with a structurally diverse peptide scaffold, to further understand the role of the peptide versus drug in the PDCs.

Azide handles were incorporated into PDIP, HRH and cR10 to facilitate the assembly of the PDCs. The sequences of these peptides, with the site of azide handle incorporation highlighted in blue, are presented in Scheme 2A. Derivatives of peptide scaffolds without the azide handle (native PDIP, cR10-Ala) were also synthesized as peptide controls (see SI). As the synthesis of PDIP-Az (**6**) has been previously published,^[7a, 25] the preparation of azide-modified HRH and cR10 (Scheme 2B) are described here.

Solid phase peptide synthesis (SPPS), which involves iterative cycles of deprotection, amino acid coupling and capping of truncated sequences, was utilized to synthesize the resin-bound linear peptide precursors of both cR10 and HRH (Scheme 2B). Consistent with previous syntheses of cR10,^[14, 17] lysine and glutamic acid residues bearing Alloc and allyl side chain protecting groups were incorporated into the cR10 linear precursor. These protecting groups were subsequently removed by selective palladium catalyzed deprotection to then facilitate on-resin cyclization between these positions, accomplished using HATU. Next, Fmoc-azido-*L*-alanine (represented as X in Scheme 2) was coupled onto the *N*-terminal amine to introduce the azide handle for conjugation. To simplify the HPLC purification and analysis given the highly polar and hydrophilic nature of the sequence, the peptide was first cleaved from resin (Fmoc-**7**) and then a solution-phase Fmoc deprotection was performed to yield cR10-Az (**7**) in 3% overall yield.

Initial synthetic approaches to produce an azide-bearing HRH focused on the incorporation of Fmoc-azido-*L*-alanine into the linker region between the two amphipathic helical domains, similar to the strategy previously employed for PDIP-Az^[7a, 25] (Scheme 2B). However, poor SPPS yields were observed for this analogue, presumably owing to instability of the azido group to peptide elongation employing iterative SPPS conditions. To improve the synthetic yield of an azide-bearing HRH, the strategy was redesigned to incorporate an orthogonally protected



Scheme 2. Production of peptide-drug conjugates. (A) Sequence of peptides with azide handles (colored blue) showing cyclization between bolded residues with an amide or disulfide linkage. ^A denotes amidated C-terminus, and differences in PDIP-Az and HRH-PEG1-Az sequences are colored in teal. (B) Synthesis of cR10-Az and HRH-PEG1-Az peptides. cR10-Az synthesis from linear precursor: step i) Alloc deprotection; ii) side-chain cyclization; iii) SPPS to couple Fmoc-azido-alanine; iv) resin cleavage; v) Fmoc deprotection. HRH-PEG1-Az synthesis from linear precursor: vi) Alloc deprotection; vii) Azido-PEG1-acid coupling; viii) Fmoc deprotection; ix) cleavage; x) oxidation. Further details are provided in the Supporting Information. (C) Synthesis of PDCs via CuAAC or SPAAC reactions. Yields are reported with superscripts a or b indicating the conjugation conditions employed; * denotes the position of regioisomers in SPAAC reactions.

Fmoc-*L*-Lys(Alloc) in place of Fmoc-azido-*L*-alanine. On-resin deprotection of this Alloc group allowed for late-stage coupling of a PEG1-azide moiety using standard peptide coupling conditions. Following coupling of the azido-PEG1-acid, subsequent Fmoc deprotection, acidic cleavage from the resin and oxidation to form the macrocyclic disulfide bond afforded HRH-PEG1-Az (**8**) in 3% overall yield from the resin loading.

Conjugation to Produce PDC Library

Eight PDCs were successfully synthesized from the above drug-linker-alkyne and peptide-azide molecules, using copper-catalyzed click chemistry conditions^[26] for PQ, TQ and MTX conjugates, or copper-free, strain-promoted click chemistry conditions^[27] for AS conjugates (see Scheme 2C and SI sections 5-6 for details). The isolated yields ranged from 15-93%. PQ and AS conjugates (**9-11** and **12-14**, respectively) were synthesized with each of the three peptide-azide molecules, whilst TQ (**15**) and MTX (**16**) conjugates were only synthesized with PDIP, given its established antiplasmodial activity and advantageous safety window.^[12, 16] PQ, TQ and MTX-containing conjugates were designed with non-cleavable PEG1 linkers, while the AS conjugates contained a DBCO-amine linker which generated the prodrug AS-DBCO moiety (**5**). Notably, linkers in the HRH conjugates **11** and **14** were slightly longer than their respective PDIP and cR10 analogues, as the peptide-azide construct was synthesized using an extended PEG1 moiety (HRH-PEG1-Az, **8**). However, as we have previously shown that PDCs with linker lengths differing by one PEG unit display similar antiplasmodial activity,^[7a] we predicted this modification would not affect activity. For the synthesis of cR10-PEG1-PQ (**10**), the CuAAC reaction was first performed on Fmoc-cR10-Az (Fmoc-**7**), affording Fmoc-cR10-PEG1-PQ (Fmoc-**10**). Fmoc-deprotection was then carried out with aqueous ammonia, following installation of the drug cargo, to yield **10**. PDIP-PEG1-PQ (**9**), which was also synthesized as part of our original study,^[7a] allowed comparability to prior results.

Temperature and pH Stability of PDCs

Given the observed instability of AS under CuAAC conditions and the prodrug nature of AS, we were particularly interested in examining the stability of AS-conjugates prior to biological evaluation. Selected PDIP-based PDCs were explored as representative examples of PDC stability in aqueous, buffered conditions in comparison to the unconjugated parent drugs and PDIP-Az scaffold. All compounds were incubated at 37 °C in phosphate buffered saline (PBS, pH 7.4) and their stability over a 24-hour period was monitored by LC-MS. Conjugates containing non-cleavable PEG1 linkers (**9** and **15**) were sufficiently stable over the 24-hour period (Table 1). Comparatively, PDIP-DBCO-AS (**12**) was less stable than AS (Table 1). Previous studies have identified numerous decomposition products of AS from thermal and aqueous degradation.^[28] Given these observations, AS-conjugates were prepared immediately prior to compound addition in subsequent biological assays to minimize any pre-assay degradation.

Peptide and Drug Components Influence the Antiplasmodial Activity of PDCs

The synthesized PDCs and their respective parent peptides and drugs (with and without functionalization) were next evaluated

Table 1. Half-lives of PDIP-based PDCs relative to their component peptide and drug in PBS (pH 7.4) at 37 °C.

Compound	t _{1/2} (h)
PBS (pH 7.4), 37 °C, 20 μM	
PDIP-Az 6	>24
PQ	>24
PDIP-PEG1-PQ 9	>24
AS	16.8
PDIP-DBCO-AS 12	1.2, 1.6 ^[a]
TQ	>24
PDIP-PEG1-TQ 15	23.2

[a] Obtained with PBS buffer containing greater concentration of phosphate salts for higher buffer capacity (see SI for details). Standard PBS: 120 mM NaCl, 4 mM Na₂HPO₄, 16 mM Na₂HPO₄, 10× PBS: 120 mM NaCl, 40 mM Na₂HPO₄, 160 mM Na₂HPO₄.

using two different *in vitro* assays, imaging and radiometric analysis, to probe their antiplasmodial activity against asexual blood stage *P. falciparum* strain 3D7. Whilst PDIP is known to act rapidly to disrupt *P. falciparum* digestive vacuole membranes,^[12] a 72 h treatment exposure time was utilized for both growth inhibition assay formats to ensure that effects against all asexual lifecycle stages could be identified. This would ensure that sustained and/or slower-acting parasite killing mechanisms of the PDC were also captured. Initial investigations determined parasite growth inhibition utilizing a well-established high-throughput imaging assay that uses a nuclear stain (DAPI) to detect parasites (Table 2).^[29] Serially-diluted compounds were added to synchronized ring-stage parasites (culture: 2% parasitemia, 0.3% hematocrit) and parasite numbers measured after 72 h.^[29] The high throughput format of this assay allowed direct comparison of peptides, drugs, and PDCs simultaneously. An expanded set of controls were included in this assay, with a focus on examining whether modifications to the drugs (alkyne handles) or peptides (azide handles) produced active compounds and to enable direct comparison of PDCs with their synthetic precursors. Unless otherwise stated we used this data set to compare relative antiplasmodial activities of compounds within the complete set. Results were not obtained for TQ as a free drug or PDC using this method due to the intrinsic fluorescence of TQ,^[30] which interferes with the assay readout at the high concentrations tested. Therefore, activities of TQ drug and PDC, together with select lead compounds, were further validated using a non-fluorescent [³H]hypoxanthine incorporation assay after 72 h treatment of synchronized ring-stage cultures (0.5% parasitemia, 2% hematocrit) with serially diluted compounds (Table 3). Inclusion of lead PQ and AS-based PDCs in the [³H]hypoxanthine incorporation assay additionally allowed identification of any differences between the two assays, with free drug also included to facilitate benchmarking against the clinically used compounds. IC₅₀ values, determined from dose-response curves, represent the compound concentration required to inhibit 50% of *P. falciparum* growth compared to untreated controls, and are shown in Tables 2 and 3 (see Figure 2 for representative dose-response curves). Notably, hemolysis was not observed for compounds in the concentration ranges tested (PQ and TQ conjugates up to 64 μM, AS and MTX conjugates up to 0.4 μM),

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Table 2. Antiplasmodial activity of PDCs and their precursors observed in a high-throughput imaging assay.

Imaging Assay (72 h) ^[a]	
Compound ^[b]	IC ₅₀ (SEM) (μM)
<i>Peptide precursors</i>	
PDIP-Az 6	9.8 (0.3)
cR10-Ala	23.7 (1.1)
cR10-Az 7	~ 47 ^[c]
HRH-PEG1-Az 8	>>64
<i>Primaquine (PQ): drug precursors and PDCs</i>	
PQ-PEG1-alkyne 1	16.7 (0.5)
PQ-alkyne 2	14.0 (0.7)
PDIP-PEG1-PQ 9	9.3 (0.4)
cR10-PEG1-PQ 10	21.3 (0.9)
HRH-PEG1-PQ 11	~ 48 ^[c]
Compound ^[b]	IC ₅₀ (SEM) (nM)
<i>Artesunate (AS): drug precursors and PDCs</i>	
AS	2.8 (0.2)
AS-DBCO 5	3.5 (0.1)
PDIP-DBCO-AS* 12	7.0 (0.2)
Regioisomer 1:	4.1 (0.2)
Regioisomer 2:	<1.6
cR10-DBCO-AS* 13	17.4 (0.5)
HRH-PEG1-DBCO-AS* 14	21.2 (0.6)
<i>Methotrexate (MTX): drug precursors and PDCs</i>	
MTX	73.9 (2.1)
MTX-γ-PEG1-alkyne 4	170 (3.5)
PDIP-PEG1-γ-MTX 16	>400

[a] Half maximal inhibitory concentration (IC₅₀) against *P. falciparum* 3D7 growth determined using imaging assay.^[29] Synchronized parasite cultures were treated with compounds in serum-free growth media (RPMI1640 culture media supplemented with 5 mg/mL Albumax II) for 72 h at 37 °C, 5% CO₂, 5% O₂. IC₅₀ is reported as mean (SEM) of three independent biological replicates. [b] Abbreviations: HRH = PDIP-HRH, Az = azide, DBCO = dibenzocyclooctyne. [c] ~ symbol is used to denote where the dose-response curves did not approach complete growth inhibition, and the value reported is an estimate based off model of best fit. *IC₅₀ values for AS-PDCs describe a 1:1 mixture of regioisomers unless otherwise specified.

with the exception of PDIP-Az (**6**) and PDIP-PEG1-PQ (**9**), for which lysis of RBCs was observed at the highest tested concentrations. However, <10% lysis was detected at the active concentrations for these compounds (see SI Figure S3). Such observations highlight the need to carefully monitor hemolytic activity during PDC development.^[7a, 7c, 7d]

Azide-modified peptides

Comparison of the three azide-modified peptide precursors in the imaging assay (Table 2, Figure 2A) showed that PDIP-Az (**6**) had the greatest antiplasmodial activity (IC₅₀ ~ 10 μM), similar to

Table 3. Antiplasmodial activity of PDCs and their precursors observed in a [³H]hypoxanthine uptake assay.

[³ H]hypoxanthine uptake (72 h) ^[a]	
Compound ^[b]	IC ₅₀ (SEM) (μM)
<i>Peptide precursors</i>	
PDIP	17.7 (0.9)
<i>Primaquine (PQ): drug precursors and PDCs</i>	
PQ	2.7 (0.3)
PDIP-PEG1-PQ 9	5.7 (0.4)
<i>Tafenoquine (TQ): drug precursor and PDC</i>	
TQ	2.33 (0.25)
PDIP-PEG1-TQ 15	4.60 (0.18)
Compound ^[b]	IC ₅₀ (SEM) (nM)
<i>Artesunate (AS): drug precursor and PDC</i>	
AS	6.2 (0.1)
PDIP-DBCO-AS* 12	8.5 (0.3)

[a] Half maximal inhibitory concentration (IC₅₀) against *P. falciparum* 3D7 growth determined from [³H]hypoxanthine incorporation assays.^[31] Synchronized parasite cultures were treated with compounds in serum-free growth media (RPMI1640 culture media supplemented with 5 mg/mL Albumax II) for 72 h at 37 °C, 5% CO₂, 5% O₂. IC₅₀ is reported as mean (SEM) of two independent replicates. [b] Abbreviations: DBCO = dibenzocyclooctyne. *IC₅₀ value for PDIP-DBCO-AS **12** describes a 1:1 mixture of regioisomers.

previously reported values for the unmodified parent peptide in both a 48 h assay with full erythrocytic life cycle coverage,^[7a] and a near-identical 72 h assay.^[16] Corroborating our previous observations of a lack of activity for HRH (near-identical 72 h assay),^[16] HRH-PEG1-Az (**8**) was inactive up to the maximal tested concentration (64 μM). However, cR10-Ala had modest antiplasmodial activity (IC₅₀ ~ 24 μM) which was partially maintained in azide-modified cR10-Az (**7**, IC₅₀ ~ 47 μM).

PQ-based PDCs

Differences in antiplasmodial activity observed for the azide-modified peptides were generally reflected in the PQ-containing series of PDCs (Table 2, Figure 2B). PDIP-PEG1-PQ (**9**) was the most active PDC (IC₅₀ ~9 μM), whilst the cR10-PEG1-PQ PDC (**10**) was 2-fold less active, and HRH-PEG1-PQ (**11**) showed approximately 4-fold reduced activity. Noting that all the conjugates contained a similar, non-cleavable PQ-linker construct (see above synthesis section), these results indicate the importance of the peptide to overall PDC activity.

In most cases, conjugation of PQ to the peptide scaffold led to an increase in activity relative to the parent peptide or peptide-azide, but reduced activity of the drug itself. In the [³H]hypoxanthine uptake assays, PDIP-PEG1-PQ (**9**) was approximately 3-fold more active than the parent PDIP (Table 3). However, similar values were obtained for azide functionalized peptide (PDIP-Az **6**) and PDIP-PEG1-PQ **9** in the DAPI-stained nuclei assay (Table 2). The cR10-PEG1-PQ (**10**) conjugate had 2-fold greater activity than the corresponding cR10 peptide-azide (**7**) and comparable activity to the unmodified cR10 (cR10-Ala) (Table 2). Crucially, HRH-PEG1-PQ (**11**) had measurable

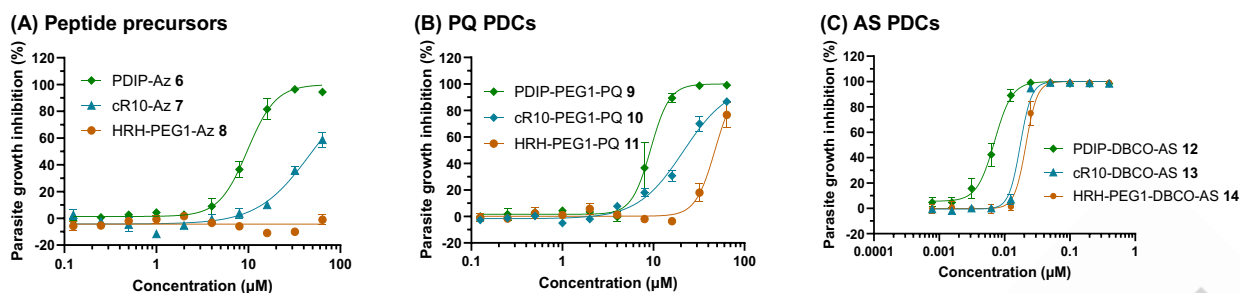


Figure 2. Representative dose-response curves of *P. falciparum* 3D7 *in vitro* growth inhibition by (A) azide-modified peptide precursors, (B) PQ PDCs and (C) AS PDCs, as quantified by imaging assays (SI Protocol A). Data points indicate the mean \pm SD of three independent biological replicates.

antiplasmodial activity despite HRH and HRH-azide (**8**) being inactive across the tested concentrations. That is, PDIP-conjugates were designed to have the greatest intrinsic peptide-derived activity while HRH-conjugates were designed to benefit only from the cell-penetrating ability of the peptide. Thus, the improved activity of PQ-derived PDCs relative to the parent peptides, even if modest in some cases, confirms that the non-cleavable PQ cargo contributes to the overall antiparasmodial activity of the PDCs. This observation builds on our previous studies (48 h assay with full life cycle coverage), which showed that PQ conjugation could recapitulate activity of an N-terminally modified, drug-free PDIP-azide peptide analogue with poor activity.^[7a] The broader suite of peptides examined here confirms that both peptide and drug play a role in the efficacy of the PQ conjugates.

Nevertheless, the antiparasmodial activity of the most potent PQ conjugate, PDIP-PEG1-PQ (**9**), was still less than that of PQ (free drug) in the [³H] hypoxanthine uptake assay (Table 3), and in the DAPI-based assay, where the alkyne-modified PQ compounds **1** and **2** were less potent than the PDIP-PEG1-PQ conjugate (Table 2). These results also corroborate our prior work, which suggested that the permanent modification of the amine in PQ is detrimental to the activity of the drug and that cleavable linkers are a beneficial design element to overcome this limitation.^[7a] We can therefore conclude that in the context of PQ conjugates, peptide, drug and linker region are all important contributors to PDC efficacy. Each component must be carefully optimized when developing the next generation of PDCs, to deliver potency improvements that lead to administering lower therapeutic doses of PQ and reduced side effects.

AS-based PDCs

In contrast to PQ-based PDCs, AS-based PDCs were designed to be inherently cleavable given the prodrug nature of AS resulting from the labile ester functionality,^[23] a structural element which is not altered with our conjugation strategy. We therefore hypothesized that introduction of an alkyne onto the succinate moiety of AS would not interfere with its antimalarial activity, given the succinate moiety acts to improve drug-like properties and would be hydrolyzed by esterases to release active DHA.^[23b] AS-based PDCs were observed to have low nanomolar activity, suggesting at least partial conversion and subsequent release of active DHA had occurred (Tables 2-3). In particular, PDIP-DBCO-AS (**12**) and the alkyne construct AS-DBCO (**5**) had activity that

closely matched AS, suggesting that the modification to the succinate moiety was not detrimental to the innate antiparasmodial mechanism of AS. To determine whether the regioisomers of PDIP-DBCO-AS (**12**) arising from the SPAAC conjugation reaction imparted different activity, both regioisomers were purified separately and screened, revealing very small nanomolar differences (IC_{50} 1-4 nM). Therefore, AS-based PDCs were subsequently tested as mixtures of regioisomers to simplify the synthetic workflow.

Antiparasmodial activity was in the low nanomolar range for all AS-based PDCs (cf. IC_{50} ~ 3 nM for unmodified AS). Since the peptides alone had activity in the micromolar range, this indicates that the activity of the AS-based PDCs is driven by the AS drug cargo (Table 2). Interestingly, the cR10 (**13**) and HRH (**14**) conjugates were 2 to 3-fold less active than the PDIP conjugate (**12**) (Table 2, Figure 2C). However, it should be noted that the linker region in the HRH conjugate (**14**) contained an additional PEG1 moiety compared to those in the PDIP (**12**) and cR10 (**13**) conjugates, and as such, we cannot definitively rule out the possibility that the PEG-containing linker contributed to the observed difference in activity. Nonetheless, both the cR10 and HRH conjugates also exhibited a notable reduction in activity relative to AS itself, suggesting that conjugation to the peptide may have resulted in partial cleavage of the conjugate or otherwise interfered with AS activity.^[28] Therefore as with the PQ conjugates, PDIP emerged as the most effective peptide partner, of those screened in this study, for AS. Further investigation into the mechanism underlying the difference in peptide suitability is required. In particular, it would be of interest to understand whether this difference may be attributed to the selectivity of unmodified PDIP for infected RBCs and/or a result of its inherent antiparasmodial activity.^[12] Ultimately, future work to assess whether incorporation into PDCs is beneficial in the context of overcoming drug resistance, and to further optimize the design of PDCs with greater potency than AS, is required.

TQ and MTX-based PDCs

To further explore alternative drug scaffolds in PDCs designed to have dual bioactivity derived from both peptide and drug, we evaluated the antiparasmodial activity of PDIP-PEG1-TQ (**15**) and PDIP-PEG1- γ -MTX (**16**). Using the [³H]hypoxanthine incorporation assay, we observed that PDIP-PEG1-TQ (**15**) had an IC_{50} value of 4.60 ± 0.18 μ M, which was approximately 2-fold less active than TQ (free drug), yet nearly 4-fold more active than

PDIP ($IC_{50} \sim 18 \mu\text{M}$ in this assay) (Table 3). Thus, analogous to the previous two drug cargoes, the TQ payload contributes to antiparasmodial activity of the PDC despite attachment to the peptide *via* a permanent, non-cleavable modification of its primary amine. Nonetheless, like PQ, TQ-based PDCs ideally need to demonstrate improved potency over the drug alone to lower the necessary therapeutic dose and reduce side effects. Since PDIP-PEG1-TQ is less potent than TQ (Table 3) further PDC derivatives, including those with incorporation of a cleavable linker region, will be required to improve the antimalarial potency.

MTX free drug exhibited nanomolar activity ($IC_{50} \sim 74 \text{ nM}$, Table 2), however, introduction of a PEG1-alkyne handle onto the γ -carboxylic acid of the glutamate moiety to produce MTX- γ -PEG1-alkyne (**4**) appeared to reduce bioactivity. This effect was amplified upon conjugation to PDIP as no inhibition of parasite growth was observed below 400 nM for PDIP-PEG1- γ -MTX (**16**). We hypothesize that the increased steric bulk provided by conjugation of MTX to the peptide carrier might interfere with the ability of the drug to bind to DHFR as a competitive inhibitor. Previous literature has suggested that smaller γ -carboxylic acid alterations are tolerated, whereas larger alterations can impair activity against cancer cell lines.^[21] As such, we are exploring the compatibility of MTX with cleavable linkers to produce antiparasmodial PDCs that allow MTX to benefit from PDIP-targeted delivery into infected RBCs without detrimental effects to its bioactivity.

Uptake of PDIP-DBCO-AS

Given that PDIP-DBCO-AS (**12**) was the most potent PDC candidate, we were interested in evaluating its rate of internalization into parasites, particularly considering its prodrug nature and observed chemical instability (see Table 1, $t_{1/2} = 1.2 \text{ h}$ in PBS pH 7.4, 37 °C). We have previously shown that PDIP exhibits rapid uptake and fast-acting antiparasmodial activity, occurring within 60 minutes following exposure.^[12] We therefore used a "washout" assay to investigate the effect of limited exposure to the PDC on antiparasmodial activity. Parasite cultures (0.5% parasitemia, 2% hematocrit) were treated with 100 nM compounds and the drug-rich media was removed after 15-60 min treatment. Parasitized red blood cells (trophozoites) were washed and replenished with drug-free culture media prior to 48 h growth (37 °C 1% O_2 , 5% CO_2). Therefore, any parasite growth inhibition observed at the end of this time was a reflection of the amount of drug internalized^[32] within the initial treatment period. Comparing AS versus PDIP-DBCO-AS (**12**) treatments, exposures of 15 and 30 min affected parasite growth inhibition to a similar extent, however, an increase in growth inhibition was observed in the cultures treated with the PDC for 60 min (Figure 3). As expected, parasites treated with either equimolar drug-free peptide (PDIP-Az, **6**, 100 nM) or a DMSO control and subjected to the same washing protocol grew similarly to untreated (no drug) and unwashed cells.

Additional studies are required to understand the PDC mechanism that results in increased growth inhibition of the PDIP-DBCO-AS (**12**) compared to AS after 60 min, but we speculate that this observation might correspond to greater uptake of the PDC into infected RBCs either because of an improved rate of uptake or improved selectivity for infected cells. Further investigations are also needed to definitively determine the active intracellular species responsible for this difference in activity at the 60 min treatment time. Activity might be attributed to parasite

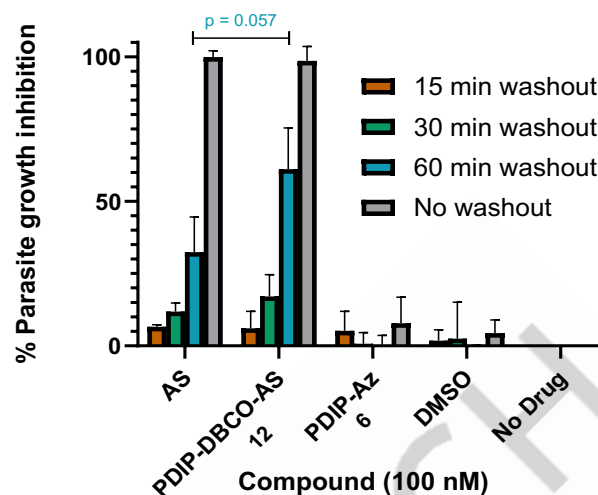


Figure 3. *P. falciparum* 3D7 *in vitro* growth inhibition by PDIP-DBCO-AS (**12**) and AS following washout of compounds from assay media. All compounds were tested at 100 nM concentration, with DMSO (0.4% v/v in media) and assay media (no drug) included as controls. Data points indicate the mean \pm SD of three independent replicates, each composed of two technical replicates. Two-tailed unpaired T-test were conducted to determine statistical significance.

uptake of intact PDC, as mediated by the peptide, or if considerable drug-release from the peptide has already occurred, to an alternative effect. Notably, a previous dual-action artesunate-benzenesulfonamide hybrid compound was designed to act either as an intact species or as mutual prodrugs,^[33] in a similar concept to the widely used artemisinin-based combination therapies. In the present study, analogous conjoint administration of a bioactive peptide and AS may still be beneficial, particularly in the context of circumventing the emergence of drug resistance.^[34]

Conclusion

In this study, we produced a library of eight antiparasmodial PDCs spanning three CPP scaffolds and four antimalarial drug cargoes, the majority of which had not previously been explored in this context. Antiparasmodial activity of the PDCs was cargo-driven and ranged from low nanomolar to low micromolar potency, highlighting the compatibility of this approach for generating future antimalarial therapeutics with clinically relevant activity. Importantly, the low IC_{50} values may also help overcome challenges associated with the safety window of PDCs, including the hemolysis observed in earlier studies employing high concentrations of CPP-derived antiparasmodial PDCs.^[7a, 7c, 7d] Across all peptide scaffolds we determined that the antimalarial drug cargo contributes to the activity of the conjugate, and that maintaining its bioactivity is important to ensure efficacious PDCs, in line with earlier work.^[7a-c] We also demonstrated that CPPs with intrinsic antiparasmodial activity, such as PDIP, can improve the potency of PDCs, ostensibly providing opportunities for the development of conjugates with a dual mechanism of bioactivity and conferring benefits analogous to the co-administration of artemisinin-based combination therapies.

Consistent with our earlier findings,^[7a] we highlighted the importance of linker design in PDCs, with all conjugates containing non-cleavable linkers having reduced activity relative to their parent drug, and only the prodrug cleavable nature of AS allowing AS-containing PDCs to match the activity of the parent drug. However, further investigation is required to determine whether AS-based PDCs remain intact throughout their bioactive lifetime, and therefore, benefit significantly from incorporation into PDCs. Nonetheless, the inherent simplicity of non-cleavable linkers reduces the synthetic cost of PDC manufacture, an outstanding challenge in the field, but an important consideration for the development of cost-effective antimalarial therapeutics that can be widely administered.^[35]

Overall, we identified PDIP as the most efficacious peptide for delivering drug cargoes, although the antiplasmodial potency of the PDCs was not improved compared to the parent drugs. The study revealed limitations in the peptides and/or linker regions investigated that may account for the lack of improved potency. Improvements in the activity of the PDC relative to the parent drug are required for developing new molecules that selectively deliver drug cargoes at reduced therapeutic doses and minimize off-target adverse effects. Further work is therefore required to develop dual-acting molecules that can selectively target infected cells. In addition, future evaluation of lead PDCs against drug-resistant *Plasmodium* strains, and additional developmental stages not examined in the present study, would provide useful insights into the broader applicability of these PDCs as antimalarial drugs. This novel approach therefore suggests a potential pathway for repurposing drug scaffolds, where either safety concerns or resistance development threaten their efficacy or widespread application. Endowed with a greater understanding of how PDC design influences bioactivity, we look towards further exploring their selectivity and safety.

Supporting Information

Supplementary figures and schemes, experimental procedures and characterization data are included for compounds **1** to **16** and **S1** to **S5**. The authors have cited additional references within the Supporting Information.^[7a, 12, 16-17, 21c, 25-27, 29-31, 36]

Author Contributions

C.L.G., N.L., I.R.P. and A.M.W. carried out the synthetic experimental work in this manuscript. C.L.G., N.L., A.L. and M.C. performed the biological assays that used blood and serum. N.L., B.J.M., A.M.W. and L.R.M. conceived of the study, and D.J.C., L.R.M., B.J.M., V.M.A. and M.D.E provided laboratory resources and infrastructure to support peptide synthesis, synthetic chemistry, and parasite growth assays respectively. C.L.G. and L.R.M. wrote the manuscript, which was further edited and reviewed by all authors.

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Ethics Statement

Experimental procedures involving human blood were approved by the Australian National University Human Research Ethics Committee, approval number 2018/398; Griffith University Human Research Ethics Exemption Approval 03/08/11019 (*P. falciparum* culture and activity assays – Imaging); and Australian Departments of Defence and Veterans' Affairs Human Research Ethics Committee (LREP15-014).

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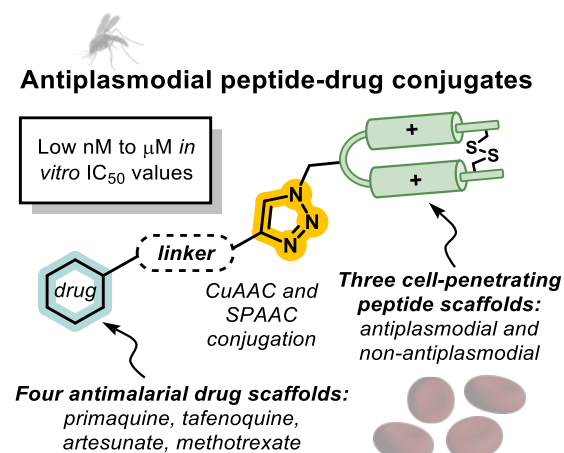
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Keywords: malaria • peptide-drug conjugate • cell-penetrating peptide • bioconjugation • click chemistry

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A library of antiplasmodial peptide-drug conjugates was synthesized, pairing distinct antimalarial drug cargoes with promising cell-penetrating peptide scaffolds. *In vitro* assays revealed low nanomolar to micromolar potency dependent on both drug and peptide composition, providing key insights into the development of peptide-drug conjugates as an alternative class of antimalarial therapeutics.

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