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## Alkyne modified purines for assessment of activation of *Plasmodium vivax* hypnozoites and growth of pre-erythrocytic and erythrocytic stages in *Plasmodium* spp.

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## ABSTRACT

Malaria is a major global health problem which predominantly afflicts developing countries. Although many antimalarial therapies are currently available, the protozoan parasite causing this disease, *Plasmodium* spp., continues to evade eradication efforts. One biological phenomenon hampering eradication efforts is the parasite's ability to arrest development, transform into a drug-insensitive form, and then resume growth post-therapy. Currently, the mechanisms by which the parasite enters arrested development, or dormancy, and later recrudesces or reactivates to continue development, are unknown and the malaria field lacks techniques to study these elusive mechanisms. Since *Plasmodium* spp. salvage purines for DNA synthesis, we hypothesised that alkyne-containing purine nucleosides could be used to develop a DNA synthesis marker which could be used to investigate mechanisms behind dormancy. Using copper-catalysed click chemistry methods, we observe incorporation of alkyne modified adenosine, inosine, and hypoxanthine in actively replicating asexual blood stages of *Plasmodium falciparum* and incorporation of modified adenosine in actively replicating liver stage schizonts of *Plasmodium vivax*. Notably, these modified purines were not incorporated in dormant liver stage hypnozoites, suggesting this marker could be used as a tool to differentiate replicating and non-replicating liver forms and, more broadly, as a tool for advancing our understanding of *Plasmodium* dormancy mechanisms.

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## 1. Introduction

Malaria is caused by parasites of the genus *Plasmodium* and remains a major global health problem that infects 218 million and kills approximately 409,000 people each year, mostly children under the age of five years. While much effort has been made over the years towards the control and elimination of this disease, recent progress has plateaued (World Health Organization, 2021).

Of the five species affecting humans, *Plasmodium falciparum* and *Plasmodium vivax* account for most cases and pose the greatest threat. Although *P. falciparum* is the deadliest of the human-infecting species, *P. vivax* is more widespread geographically and produces a dormant uninucleate liver stage. Termed hypnozoites, these forms can persist for days, months, and even years, before an unknown mechanism stimulates their reactivation and causes relapsing infections (Krotoski et al., 1982). While it does not produce hypnozoites, *P. falciparum* undergoes a stress-induced growth arrest in the asexual blood stage ring stage when treated with artemisinin (ART) monotherapy (Teuscher et al., 2010; Witkowski et al., 2010; Tucker et al., 2012). This mechanism of induced quies-

Abbreviations: EdA, 7-deaza-7-ethynyl-2'-deoxyadenosine.

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cence is theorised to be a means for drug evasion and subsequent parasite recrudescence once drug pressure is removed. Both species present their own unique obstacles in terms of eradicating malaria, with quiescence being a common thread.

Hypnozoites are insensitive to all currently marketed anti-malarial drugs except the 8-aminoquinolines primaquine and tafenoquine. However, these drugs are contraindicated in patients with a glucose-6-phosphate dehydrogenase (G6PD) deficiency or who are pregnant (Baird, 2012, 2018). This highlights the urgency of discovering novel anti-malarial drugs, the development of which would be aided by a better understanding of the hypnozoite's basic biology. Whilst suitable high-throughput in vitro assays for screening compounds against *P. vivax* liver stage parasites have been recently developed, to date there are no specific markers to exclusively distinguish hypnozoites from liver schizonts (Roth et al., 2018). A recombinant antibody reactive to the parasitophorous vacuole membrane (PVM) resident protein Upregulated in Infectious Sporozoites 4 (*PvUIS4*) has been generated, but it immunofluorescently stains both hypnozoites and liver schizonts (Schafer et al., 2018). Differentiation between the two forms relies on parasite size and specific chemosensitivity, thus much care must be taken to morphologically distinguish hypnozoites from early schizonts (Sylvester et al., 2021). In addition to hypnozoite-specific markers, the identification of markers indicating hypnozoite reactivation would aid in characterising the mechanism of dormancy and resumption of growth. Two such examples are Liver-Specific Protein 2 (LISP2), and acetylated lysine 9 of histone H3 (H3K9Ac) (Mikolajczak et al., 2015; Gupta et al., 2019). LISP2 has been found expressed in early developing liver stage parasites; however, it is limited in that staining is not observed until 3 days post-infection in *P. vivax* and the exact relationship between increased expression of LISP2 and hypnozoite reactivation has not yet been characterised (Voorberg-van der Wel et al., 2020). H3K9Ac has been elegantly used to accurately count individual nuclei in hypnozoites versus liver stage schizonts (Mikolajczak et al., 2015). However, this marker indicates only nuclear division and not necessarily active DNA synthesis, a hallmark of reactivation. As biological processes of reactivation such as DNA synthesis are likely upstream of both LISP2 expression and nuclear division, markers which more closely identify the moment of reactivation are needed.

Acute, uncomplicated *P. falciparum* infections are most often treated with ART combination therapies (ACT) that are active against blood stages (World Health Organization, 2021). ART derivatives are fast acting drugs and are effective in rapidly reducing parasite biomass. While this treatment has been extremely effective in reducing malaria burden, slower parasite clearance times have been reported as resistance to ART treatment begins to rise (Dondorp et al., 2009; Phyo et al., 2012, 2016; Leang et al., 2015). Failure rates of ART monotherapy vary widely, anywhere from 2%-50%, and these have been shown to associate with developmentally arrested ring stages (Teuscher et al., 2010; Codd et al., 2011; Assefa et al., 2021). While in vitro culturing of the *P. falciparum* asexual blood stage and induction of dormancy is possible, elucidating the underlying mechanisms of the induced dormant rings remains technically challenging. These dormant parasites present phenotypically with condensed nuclei and reduced cytoplasm, and thus are difficult to differentiate from dead parasites. Although much work has been done to provide insights into DHA-induced dormancy, the molecular mechanism that allows some parasites to enter this stage and later recrudescence is unknown. Furthermore, these dormant stages recrudescence asynchronously, and we currently lack the tools to differentiate between early versus late activators. The development of a marker that differentiates latent versus active parasites would aid in studying how DHA-induced dormant parasites recover from quiescence.

Modified nucleic acid precursors have been widely used to study the cell cycle. In many organisms, 5-bromo-2'-deoxyuridine (BrdU) and ethynyl-2'-deoxyuridine (EdU), analogs of the nucleoside thymidine have been used to identify proliferating cells versus non-proliferating cells (Gratzner, 1982; Salic and Mitchison, 2008). Previously, attempts were made to adapt these labelling techniques for *Plasmodium*, however they failed due to the parasite's requirement for de novo synthesis of pyrimidines (Janseet al., 1991). Although *P. falciparum* expresses transporters that should allow BrdU to be taken up (Rager et al., 2001; Downie et al., 2008), the parasite lacks a thymidine kinase (TK) and thus cannot convert thymidine from a deoxynucleoside into a deoxynucleotide. Recently, studies showed that transfection with TK can allow BrdU labelling in *P. falciparum*, however parasites became much more sensitive to BrdU toxicity (Merrick, 2015). Furthermore, this technique is not suitable for *Plasmodium* spp. that cannot be easily cultured for transfection, such as *P. vivax*. Alternatively, mammalian cells incorporate alkyne modified purine versions of adenosine, 7-deaza-7-ethynyl-2'-deoxyadenosine (EdA) and guanosine, 7-deaza-2'-deoxyguanosine (EdG), and recent work with a related apicomplexan, *Cryptosporidium parvum*, showed incorporation of EdA (Neef et al., 2012; Pawlowic et al., 2019). *Plasmodium* spp. consist of purine auxotrophs which salvage host cell purines (Cassera et al., 2011), with hypoxanthine being the closest precursor in the parasite's metabolic pathway for all purine nucleotides and deoxynucleotides which are used for nucleic acid synthesis. The sequential steps of *P. falciparum*'s metabolism of adenosine to inosine via an adenosine kinase (AK) followed by the conversion from inosine to hypoxanthine via the purine nucleoside phosphorylase (PNP) suggests parasites could incorporate alkyne labelled inosine and hypoxanthine.

While other biological processes driving hypnozoite reactivation or blood stage recrudescence are likely present before DNA synthesis, synthesis is currently the earliest discernable process specific to replicating forms. To address the need for markers to better differentiate quiescent versus proliferating hypnozoites and dormant versus non-viable blood stage parasites, we hypothesised alkyne modified purines could be synthesised and incorporated into the parasite's nucleus during DNA synthesis and detected using fluorescent chemo-labelling ("click chemistry"). Click chemistry provides the advantage of no animal reactivity, no potential for cross-reactivity, easier production and storage, and increased flexibility in multicolor experiments. In this study, the development and application of clickable nucleoside analogs EdA, 7-deaza-7-ethynyl-2'-deoxyinosine (EdI), 7-deaza-7-ethynylhypoxanthine (EdH), and 8-ethynylhypoxanthine (8eH), collectively termed EdX, as DNA synthesis markers of proliferating parasites, is described. Furthermore, we use EdA staining to help differentiate between dormant and developing liver stage parasites.

## 2. Materials and methods

### 2.1. Chemistry

Given the ability of *P. falciparum* to salvage purines, we hypothesised that alkyne modified derivatives of purine precursors could be developed as tools for the study of DNA synthesis in blood and liver stages of the life cycle. Previous studies with mammalian cells demonstrated that EdA and EdG can be used for cell proliferation studies. For our studies with malaria parasites, we obtained EdA from a commercial source (Carbosynth, United Kingdom), yet similar derivatives of inosine and hypoxanthine required novel synthetic methods (described below). Compound numbers in **bold** refer to chemical structures in Supplementary Fig. S1.

### 2.1.1. Synthesis of EdI (3)

2.1.1.1. 7-(2-Deoxy- $\beta$ -D-erythro-pentofuranosyl)-5-iodo-4-methoxy-7H-pyrrolo[2,3-d]pyrimidine (2). To a suspension of 6-chloro-7-deazapurine (1, 5.0 g, 33 mmol) in dichloromethane (280 mL) was added N-iodosuccinimide (8.5 g, 38 mmol) and the resulting mixture was stirred at room temperature for 6 h. The suspension was filtered and concentrated under reduced pressure, and the residue was recrystallised from MeOH to give 6-chloro-7-iodo-7-deazapurine as an off-white solid (5.77 g, 20.7 mmol; 63%). A solution of this product (4.6 g, 16 mmol) was created in acetonitrile (110 mL), and potassium hydroxide (2.6 g, 46 mmol), and tris[2-(2-methoxyethoxy)-ethyl]amine (0.53 mL, 1.6 mmol) were added. After stirring at room temperature for 15 min, Hoffer's chlorosugar (6.8 g, 18 mmol) was added, and stirring was continued for an additional 15 min. All insoluble materials were filtered off, and the filtrate was evaporated and suspended in 0.5 M sodium methoxide in methanol (55 mL). The suspension was stirred overnight, evaporated and the residue purified by flash chromatography (dichloromethane/methanol 9:1) to give 2 as a colourless solid (4.06 g, 10.4 mmol; 63%).

2.1.1.2. 7-Deaza-7-ethynyl-2'-deoxyinosine (3, EdI). A solution of 2 (0.245 g, 0.626 mmol) in 2 M aqueous sodium hydroxide (19 mL) was heated to reflux for 5 h. After cooling to room temperature, the solution was carefully neutralised with dilute acetic acid to pH 7, yielding a solid that was filtered and washed with water. Recrystallisation from acetonitrile gave 7-(2-deoxy- $\beta$ -D-erythro-pentofuranosyl)-3,7-dihydro-5-iodo-4H-pyrrolo[2,3-d]pyrimidin-4-one (0.2 g, 0.5 mmol; 85%) as a colourless solid. A suspension of this material (0.247 g, 0.655 mmol) was created in dry, degassed dimethylformamide (10.4 mL), and copper(I) iodide (0.025 g, 0.131 mmol) palladium tetrakis(triphenylphosphine) (0.076 g, 0.065 mmol), triethylamine (0.192 mL, 1.38 mmol), and trimethylsilylacetylene (0.907 mL, 6.55 mmol) were added. The reaction was purged with argon before stirring at room temperature for 14 h. The solvents and other volatiles were evaporated under reduced pressure, and the residue was suspended in a mixture of methanol (11 mL) and water (11 mL) with sodium carbonate (0.209 g, 1.97 mmol) and stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (dichloromethane/methanol 9:1) to give 3 (0.110 g, 0.400 mmol; 61%) as a tan solid.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  12.10 (br s, 1H), 7.94 (s, 1H), 7.71 (s, 1H), 6.44 (t,  $J$  = 6.88 Hz, 1H), 5.27 (d,  $J$  = 3.95 Hz, 1H), 4.96 (t,  $J$  = 5.32 Hz, 1H), 4.30–4.36 (m, 1H), 3.99 (s, 1H), 3.82 (q,  $J$  = 3.77 Hz, 1H), 3.48–3.60 (m, 2H), 2.41 (ddd,  $J$  = 13.30, 7.75, 5.75 Hz, 1H), 2.20 (ddd,  $J$  = 13.13, 5.93, 2.86 Hz, 1H) ppm.  $^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )  $\delta$  157.8, 147.3, 145.6, 126.4, 108.2, 98.7, 88.1, 83.7, 81.9, 77.7, 71.2, 62.2, 40.6 ppm. HRMS  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_{13}\text{H}_{14}\text{N}_3\text{O}_4$  276.0984; found 276.0976.

### 2.1.2. Synthesis of EdH (6)

2.1.2.1. 7-Iodo-7-deazahypoxanthine (5). To a solution of 7-deazahypoxanthine (4, 1.0 g, 7.4 mmol) in dry dimethylformamide (20 mL) was added bis(trimethylsilyl)acetamide (3.3 g, 16 mmol), and the resulting solution was stirred at 40 °C for 2 h. The reaction was cooled to room temperature and N-iodosuccinimide (2.0 g, 8.9 mmol) was added in one portion. The reaction mixture was protected from light and stirred at room temperature until completion. The mixture was poured into water (50 mL) with stirring. A mild exotherm was observed, followed by precipitation of the crude product. After stirring for 1–2 h, the product was collected by filtration, washed with water, dried, and purified by flash chromatography (dichloromethane/methanol 9:1) to give 5 (0.65 g, 2.5 mmol; 67%) as a colourless solid.

2.1.2.2. 7-Deaza-7-ethynylhypoxanthine (6, EdH). To a suspension of 5 (0.050 g, 0.19 mmol) in dry, degassed dimethylformamide (3 mL) was added copper(I) iodide (70 mg, 0.038 mmol) palladium tetrakis(triphenylphosphine) (0.022 g, 0.019 mmol), triethylamine (0.056 mL, 0.402 mmol), and trimethylsilylacetylene (0.238 mL, 1.92 mmol). The reaction was purged with argon before stirring at 55 °C for 14 h after which thin layer chromatographic analysis (dichloromethane/methanol, 10:1) showed total consumption of starting material. The reaction mixture was diluted with dichloromethane (20 mL) and extracted with water (2  $\times$  15 mL). The organic phase was separated, dried, and concentrated and the residue was suspended in methanol (5 mL) and water (5 mL) with sodium carbonate (0.061 g, 0.571 mmol) and stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (dichloromethane/methanol 19:1) to give 6 (0.017 g, 0.107 mmol; 55.8%) as a colourless solid.  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta$  12.08 (br s, 1H), 7.86 (s, 1H), 7.39 (s, 1H), 3.93 (s, 1H) ppm.  $^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )  $\delta$  158.6, 148.2, 145.1, 126.6, 107.9, 98.2, 81.1, 78.3 ppm. HRMS  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_8\text{H}_6\text{N}_3\text{O}$  160.0511; found 160.0504.

### 2.1.3. Synthesis of 8eH (9)

2.1.3.1. 8-Iodo-2',3',5'-tris-tertbutyldimethylsilylinosine (8). To a clear solution of tert-butyltrimethylsilyl chloride (2.81 g, 18.6 mmol) and imidazole (2.54 g, 37.3 mmol) in dry dimethylformamide (10 mL) was added inosine (7) (1.0 g, 3.7 mmol) in one portion. The reaction mixture was stirred at room temperature overnight, after which thin layer chromatographic analysis (chloroform/ethyl acetate, 1:1) showed total conversion of starting material to a single product. The white slurry was then partitioned into a mixture of water (100 mL) and dichloromethane (100 mL). The organic layer was separated, and the aqueous phase was extracted with dichloromethane (3  $\times$  50 mL). The combined organic extracts were dried over magnesium sulfate, filtered, and evaporated under reduced pressure to afford a white solid, which was purified by flash chromatography (hexanes/ethyl acetate 9:1) to give 2',3',5'-tris-tertbutyldimethylsilylinosine as a white powder (2.1 g, 3.4 mmol; 92%). To a solution of diisopropylamine (2.3 mL, 16 mmol) in dry, degassed tetrahydrofuran (16 mL) at –78 °C was added dropwise n-butyllithium (11 mL, 18 mmol, 1.6 N in hexane). This mixture was stirred for 10 min at –78 °C, then a solution of 2',3',5'-tris-tertbutyldimethylsilylinosine (2.0 g, 3.3 mmol) in tetrahydrofuran (37 mL) was added dropwise at –78 °C, followed by stirring at –78 °C for an additional 2 h. Iodine (3.32 g, 13.1 mmol) in tetrahydrofuran (12 mL) was added dropwise until a deep-yellow colour persisted. It was stirred for an additional 10 min, after which thin layer chromatographic analysis showed completion of the reaction (chloroform/ethyl acetate 1:1). The mixture was then warmed to room temperature and quenched with a pH 4 buffer of 1 N sodium acetate/acetic acid. The aqueous phase was washed with dichloromethane (3  $\times$  30 mL); the combined organic extracts were washed with 0.5 N sodium bicarbonate, dried over magnesium sulfate, filtered, and evaporated under reduced pressure to give a yellow oil, which was purified by flash chromatography (dichloromethane/ethyl acetate, 9:1) to give 8 as a pale-yellow solid (2.04 g, 2.77 mmol; 85%).

2.1.3.2. 8-Ethynylhypoxanthine (9). To a suspension of 8 (0.5 g, 0.7 mmol) in dry, degassed dimethylformamide (11 mL) was added copper(I) iodide (0.0260 g, 0.136 mmol) palladium tetrakis(triphenylphosphine) (0.078 g, 0.068 mmol), triethylamine (0.199 mL, 1.43 mmol), and triisopropylsilylacetylene (1.24 mL, 6.79 mmol). The reaction was purged with argon before stirring at 55 °C for 14 h, after which thin layer chromatographic analysis (dichloromethane/methanol, 10:1) showed total consumption of starting

material. The reaction mixture was diluted with dichloromethane (50 mL) and extracted with water ( $2 \times 25$  mL). The organic phase was separated, dried, and concentrated to give a brown oil which was suspended in MeOH (15 mL) and 1 N aqueous hydrochloric acid (15 mL) before being heated to reflux overnight. The resultant solution was neutralised with saturated sodium bicarbonate and extracted with dichloromethane ( $3 \times 50$  mL). The organic phase was separated, dried, and concentrated and the residue was dissolved in tetrahydrofuran (15 mL) before slowly adding tetrabutylammonium fluoride (0.355 g, 1.36 mmol). The mixture was stirred overnight and was monitored by thin layer chromatography (dichloromethane/methanol, 10:1). Upon completion, calcium carbonate and Dowex 50WX8-100 resin were added and the mixture stirred for 45 min. The solids were filtered over a pad of Celite and the filtrate evaporated to dryness. The residue was purified by flash chromatography (dichloromethane/methanol, 10:1) to give **9** (0.064 g, 0.400 mmol; 59%) as a colourless solid.  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta$  14.00 (br s, 1H), 12.18 (br s, 1H), 7.96 (s, 1H), 4.52 (s, 1H) ppm.  $^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )  $\delta$  155.6, 152.7, 146.3, 132.8, 121.6, 83.8, 75.1 ppm.  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_7\text{H}_5\text{N}_4\text{O}$  161.0463; found 161.0455.

## 2.2. Biology

### 2.2.1. In vitro culture of intraerythrocytic *P. falciparum* parasites

*Plasmodium falciparum* clone W2 (Indochina II) was cultured using standard techniques (Trager and Jensen, 1976). Briefly, parasites were maintained at 37 °C under hypoxic conditions (90%  $\text{N}_2$ , 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ ) at a hematocrit of 2% A+ human red blood cells (RBCs; Interstate Blood Bank, Memphis, TN, USA). Parasites were cultured in complete medium consisting of RPMI1640 supplemented with 25 mM HEPES, 0.24% (w/v) sodium bicarbonate and either (a) 10% heat-inactivated A+ human plasma (Interstate Blood Bank, Memphis, TN) or (b) 1% (w/v) AlbuMAX II (Thermo Fisher) and 320  $\mu\text{M}$  hypoxanthine (Sigma). Parasite development was monitored with light microscopy of Giemsa-stained blood smears.

### 2.2.2. EdX incorporation and Cu-catalysed azide-alkyne staining of active intraerythrocytic parasites

Asynchronous *P. falciparum* was split to 2% parasitemia in a 2% hematocrit and supplemented with 10  $\mu\text{M}$  EdA (Carbosynth, United Kingdom), EdI, EdH, or 8eH, and incubated for 48 h at 37 °C under hypoxic conditions. After incubation, parasites were briefly centrifuged, and the supernatant removed. Infected cells were then fixed in a solution containing 4% paraformaldehyde and 0.05% glutaraldehyde for 15 min at room temperature, adapted from Balu et al. (2010). Following a wash with PBS (pH 7.4), parasites were prepared for the azide-alkyne click reaction by permeabilisation with 0.1% Triton X-100 for 10 min, followed by a 1 h incubation with 3% BSA at room temperature. Cells were then stained with a freshly prepared staining mix containing 2 mM  $\text{CuSO}_4$  (Sigma), 12  $\mu\text{M}$  Alexa Fluor Azide 488 (Thermo Fisher), and 10 mM sodium ascorbate (Sigma) for 1 h in the dark at room temperature. Parasites were then washed once with PBS and co-stained with 10  $\mu\text{g}/\text{mL}$  of Hoechst 33342 (Thermo Fisher) for 15 min followed by three washes with PBS. They were then mounted onto poly-L-lysine coated slides and imaged with a Zeiss Axio Observer.Z1/7. The Fiji plugin, JACoP, was used to generate Mander's coefficients and Pearson's coefficient,  $r$ , for co-localisation of Hoechst 33342 nuclear staining and EdX incorporation.

### 2.2.3. Cytotoxicity assays of EdX on intraerythrocytic parasites

Cytotoxicity of the modified purines was assessed by measuring the increase in parasitemia over time. Briefly, asynchronous *P. falciparum* was split to 0.5% parasitemia in a 2% hematocrit and supplemented with 10  $\mu\text{M}$  EdX. Parasites were incubated at 37 °C

under hypoxic conditions and allowed to grow for 5 days. On day 3 parasites were split 1:5 to avoid parasite death due to overgrowth. Samples were taken daily and fixed with 4% paraformaldehyde and 0.05% glutaraldehyde, and stored at 4 °C until all samples were collected. Parasites were then stained with 10  $\mu\text{M}$  Hoechst 33342 for 15 min followed by three washes with PBS and the percent parasitemia was analysed via flow cytometry using a Beckman Coulter CytoFLEX. Primary gating was performed based on background fluorescence from uninfected red blood cells to obtain parasite-infected RBCs as an indication of parasitemia. Results were then visualised via a GraphPad Prism 9 plot where statistical analysis was also conducted using a two-way ANOVA multiple comparisons test.

### 2.2.4. Human subjects consideration

*Plasmodium vivax* isolates were collected into a heparin tube (BD) via venipuncture from human volunteers following approval by the Cambodian National Ethics Committee for Health Research (113NHECR). Protocols conformed to the Helsinki Declaration on ethical principles for medical research involving human subjects (version 2002) and informed written consent was obtained from all volunteers or legal guardians.

### 2.2.5. In vitro liver stage *P. vivax* incorporation of EdX

Primary human hepatocytes (PHH) were infected with *P. vivax* sporozoites as previously described (Roth et al., 2018). Briefly, *Anopheles dirus* mosquitoes were fed a bloodmeal containing *P. vivax* gametocytes and maintained on a 12:12 light:dark cycle and 10% sucrose in water. Two days prior to infection, PHHs (lot BGW, BioIVT) were seeded into collagen-coated 384-well plates (Greiner Bio-One) at a density of 18,000 cells per well. Mosquito salivary glands were aseptically dissected on days 16–21 post feeding to obtain *P. vivax* sporozoites. PHH seeded plates were then infected with 5000–18,000 sporozoites per well. Infected PHHs were then exposed to EdX at 5, 6, 7, and 8 days p.i. with 2  $\mu\text{M}$  EdX, 10  $\mu\text{M}$  EdX, and DMSO vehicle control. Media were exchanged daily immediately before EdX exposure. At 9 days p.i., cultures were fixed with 4% paraformaldehyde in PBS. Fixed cultures were stained with recombinant mouse anti-PvUIS4 (rPvUIS4) (Schaffer et al., 2018) diluted 25,000-fold in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) overnight at 4 °C in the dark. Following three washes with PBS, cells were then stained with rabbit anti-mouse Alexafluor488 (Abcam) diluted 1:1000 diluted in staining buffer for 1 h at room temperature in the dark. Cultures were then washed three times with PBS followed by staining with a freshly prepared staining mix containing 2 mM  $\text{CuSO}_4$ , 12  $\mu\text{M}$  Alexa Fluor Azide 594 (Thermo Fisher), and 10 mM sodium ascorbate for 1 h in the dark at room temperature. The cells were then washed three times with PBS and counterstained with 10  $\mu\text{g}/\text{mL}$  of Hoechst 33342 for 15 min at 37 °C. Cultures were washed once with PBS and stored in PBS prior to automated high content imaging with a 20 $\times$  objective on an ImageXpress confocal microscope (Molecular Devices, San Jose, CA, USA). Liver stage parasites and host cell hepatocytes were quantified using MetaXpress software version 6.6.1.42 for ImageXpress. Individual images were also obtained with a 100 $\times$  objective on a DeltaVision II deconvolution microscope (Applied Precision Inc., Currently Leica Microsystems, Buffalo Grove, IL, USA). Analysis was conducted using GraphPad Prism 9 with an ordinary one-way ANOVA multiple comparisons.

### 2.2.6. Human hepatocarcinoma cell line (HepG2) EdX staining

Human hepatocarcinoma cell line (HepG2) cells were cultured in collagen-coated T75 flasks in minimum essential medium eagle with Earle's BSS (MEM Eagle EBSS) from Lonza (Walkersville, MD, USA) supplemented with 10% FBS and 4.4 mM sodium pyruvate at 37 °C with 5%  $\text{CO}_2$ . Cells were seeded at 5000 cells per well in

a collagen coated 384 well plate (Greiner Bio-One). EdX (2  $\mu$ M or 10  $\mu$ M) and 0.1% DMSO vehicle control were added 24 h post seed and allowed to incorporate for 48 h at 37 °C and 5% CO<sub>2</sub>. Cells were then fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were stained with freshly prepared staining mix containing 2 mM CuSO<sub>4</sub>, 12  $\mu$ M Alexa Fluor Azide 488, and 10 mM sodium ascorbate in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) overnight at 4 °C in the dark. Following three washes with PBS, cells were counterstained with 10  $\mu$ g/mL of Hoechst 33342 for 15 min at 37 °C. Cultures were washed once with PBS and stored in PBS prior to automated high content imaging with a 4x objective on a Lionheart imaging system (Biotek). Viability was measured by counting cell nuclei using Gen5 software (Biotek) and statistical analysis was conducted using GraphPad Prism 9 with an ordinary one-way ANOVA multiple comparisons test and unpaired t-test.

### 2.2.7. In vitro liver stage *Plasmodium berghei* incorporation of EdA

*Anopheles stephensi* mosquitos were fed a *Plasmodium berghei* gametocyte-infected bloodmeal and maintained on a 12:12 light:dark cycle and 10% sucrose in water. One day prior to infection with sporozoites, HepG2 cells were seeded at 17,500 cells per well in a collagen-coated 384 well plate (Greiner Bio-One). Mosquito salivary glands were aseptically dissected on day 20–22 post feeding to obtain *P. berghei* sporozoites. HepG2 seeded plates were then infected with 6000 sporozoites per well. Infected and uninfected cultures were then treated 24 h p.i. with 2  $\mu$ M EdA, 10  $\mu$ M EdA, and 0.1% DMSO vehicle control. Media was exchanged before EdA treatment. At 48 h p.i., cultures were fixed with 4% paraformaldehyde in PBS. Fixed cultures were stained with mouse monoclonal antibody 13.3 anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) diluted 10,000-fold in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) overnight at 4 °C in the dark (Roth et al., 2018). Anti-GAPDH was obtained from The European Malaria Reagent Repository. Following three washes with PBS, cells were then stained with rabbit anti-mouse Alexafluor488 diluted 1:1000 diluted in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) for 1 h at room temperature in the dark. Cells were then washed three times with PBS followed by staining with a freshly prepared staining mix containing 2 mM CuSO<sub>4</sub>, 12  $\mu$ M Alexa Fluor Azide 594, and 10 mM sodium ascorbate for 1 h in the dark at room temperature. Cells were then washed three times with PBS and counterstained with 10  $\mu$ g/mL of Hoechst 33342 for 15 min at 37 °C. Cells were washed once with PBS and stored in PBS prior to imaging at 40 $\times$  on a Zeiss Axio Observer.Z1/7 (Pleasanton, CA, USA).

### 2.2.8. In vitro *P. falciparum* EdA, <sup>3</sup>H-hypoxanthine, and <sup>3</sup>H-adenosine incorporation in dihydroartemisinin (DHA)-induced dormant asexual blood stage parasites

*Plasmodium falciparum* (W2) asexual blood stage parasites were synchronised to ring stages using 5% D-sorbitol and were then split to 2% parasitemia in a 2% hematocrit. Dormancy was then induced 48 h post-synchronisation with 700 nM DHA for 6 h while parasites were incubated at 37 °C under hypoxic conditions. Following three washes with RPMI1640, parasites were re-suspended and cultured in complete media containing 10% heat-inactivated A+ human plasma. Parasite recrudescence and development were monitored daily with light microscopy of Giemsa-stained blood smears and media were changed every 48 h. Aliquots of 100  $\mu$ L were transferred daily (up to 10 days post dormancy induction) to a 96 well plate where 5  $\mu$ Ci <sup>3</sup>H-hypoxanthine (Perkin Elmer), 5  $\mu$ Ci <sup>3</sup>H-adenosine (Perkin Elmer), or 10  $\mu$ M EdA was supplemented. These aliquots were incubated for 24 h at 37 °C under hypoxic conditions, after which they were either frozen at –80 °C (for tritiated samples) or fixed with 4% paraformaldehyde

and 0.05% glutaraldehyde, and stored in 4 °C until all samples were collected (for EdA-labelled samples). Uninfected RBCs (uRBCs) and active asynchronous parasites at a starting 2% parasitemia were used as controls. Once all samples were collected, click chemistry was performed on EdA-labelled samples as described above and incorporation was measured via flow cytometry on a Beckman Coulter CytoFLEX (Indianapolis, IN, USA). Tritiated samples were harvested and counted on a Perkin Elmer Microbeta<sup>2</sup> scintillation counter (Waltham, MA, USA). Data was graphed and analysed using GraphPad Prism 9.

## 3. Results

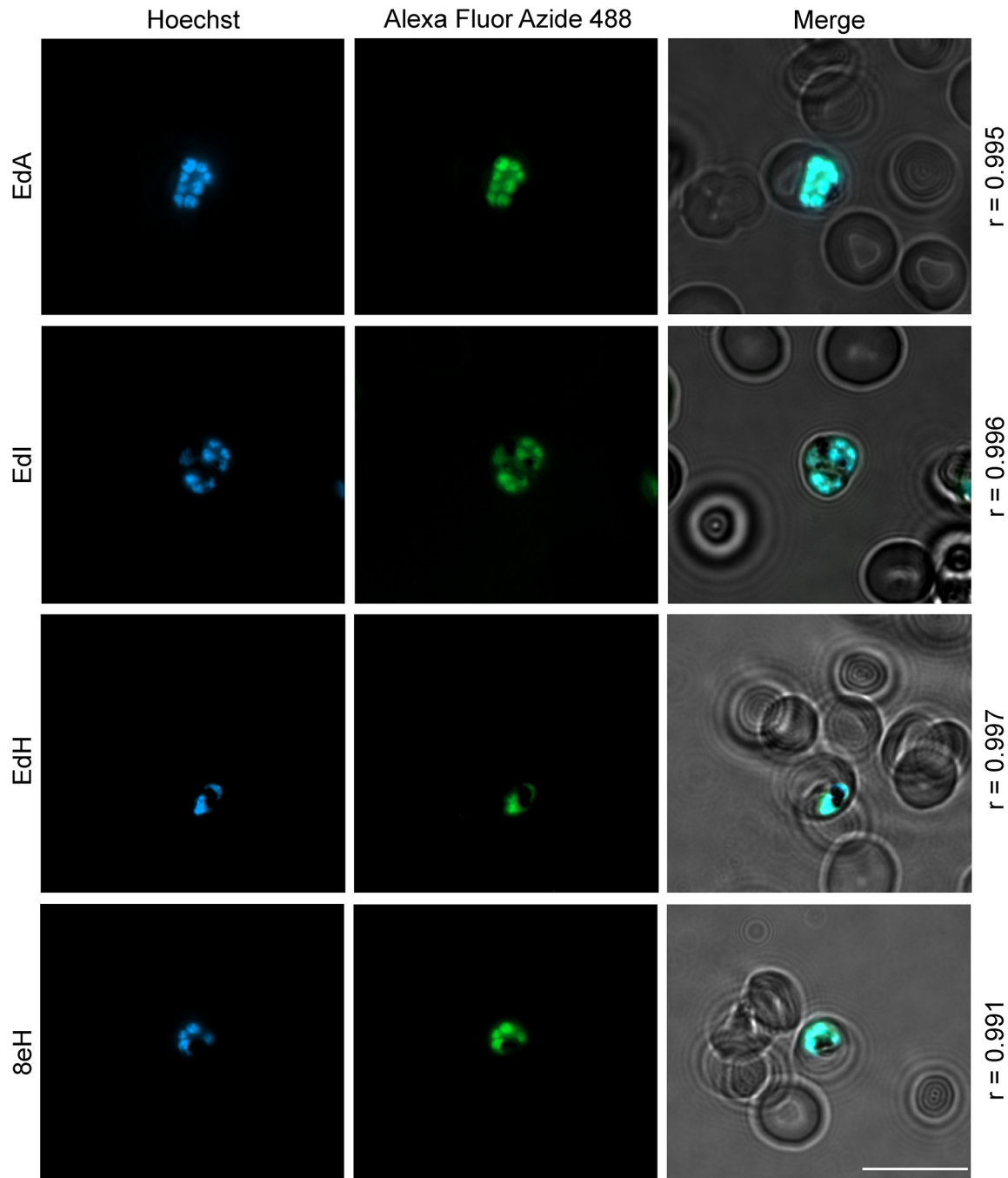
### 3.1. Synthesis and characterisation of EdI, EdH, and 8eH

The synthesis of EdI was initiated from commercially available 6-chloro-7-deazapurine (**1**, Supplementary Fig. S1). Selective iodination at the 7-position followed by addition of Hoffer's chlorosugar and concomitant deprotection and S<sub>N</sub>Ar displacement with sodium methoxide afforded **2**. Following displacement of the methoxy group, Sonogashira installation of the clickable handle with TMS-acetylene and liberation of the terminal acetylene yielded EdI (**3**). In a similar manner, 7-deazahypoxanthine (**4**) was iodinated and affixed with the alkyne to give EdH (**6**). From a cost-effective perspective, it was most pragmatic to prepare 8eH (**9**) from inosine (**7**). Silyl protection of the carbohydrate alcohols allowed smooth iodination at the 8-position to give **8**, which could then be converted to **9** via a modified Sonogashira protocol followed by global deprotection.

### 3.2. Incorporation of EdX analogues into asexual blood stages of *P. falciparum*

To evaluate if EdA, EdI, EdH, and 8eH were incorporated into newly synthesised DNA of *Plasmodium*, we conducted labelling studies using a copper-catalysed azide-alkyne cycloaddition chemical reaction with a fluorescent azide probe. Using an in vitro asynchronous asexual blood stage culture of *P. falciparum*, we observed that the addition of 10  $\mu$ M of each of the alkyne modified purines resulted in nuclear staining after a 48 h incubation period, equivalent to a full asexual blood stage life cycle (Fig. 1). The observed staining co-localised with nuclear Hoechst 33342 staining, which preferentially binds to the A-T regions of DNA, and a Fiji JACoP Pearson's coefficient for EdA, EdI, EdH, 8eH was calculated to range from 0.991 to 0.997, thus indicating that the modified purines were incorporated into parasite DNA. Manders' coefficients for the four images shown in Fig. 1 were also calculated and ranged from 0.925 to 0.999.

Considering the potential cytotoxicity of the modified purines, we assessed parasite growth in the presence of these compounds to quantify their effects on parasite proliferation. Furthermore, because serum supplements contain different levels of purines, we evaluated the cytotoxic effects of EdA, EdI, EdH, and 8eH over a 5 day incubation period in complete media containing albumax II alone (1% w/v), complete media containing albumax II and supplemented with hypoxanthine (10  $\mu$ M), and complete media containing 5% A+ human plasma. The media containing albumax II alone lacks any purines for the parasites to salvage whereas media supplemented with hypoxanthine or human plasma contains purines, although at different levels. In media without hypoxanthine supplementation, all modified purine compounds showed a deleterious effect ( $P < 0.0001$ ) on parasite proliferation compared with the unmodified hypoxanthine media control (Fig. 2A). Cultures with media containing either supplemented hypoxanthine or human plasma were unaffected by the addition of alkyne modified



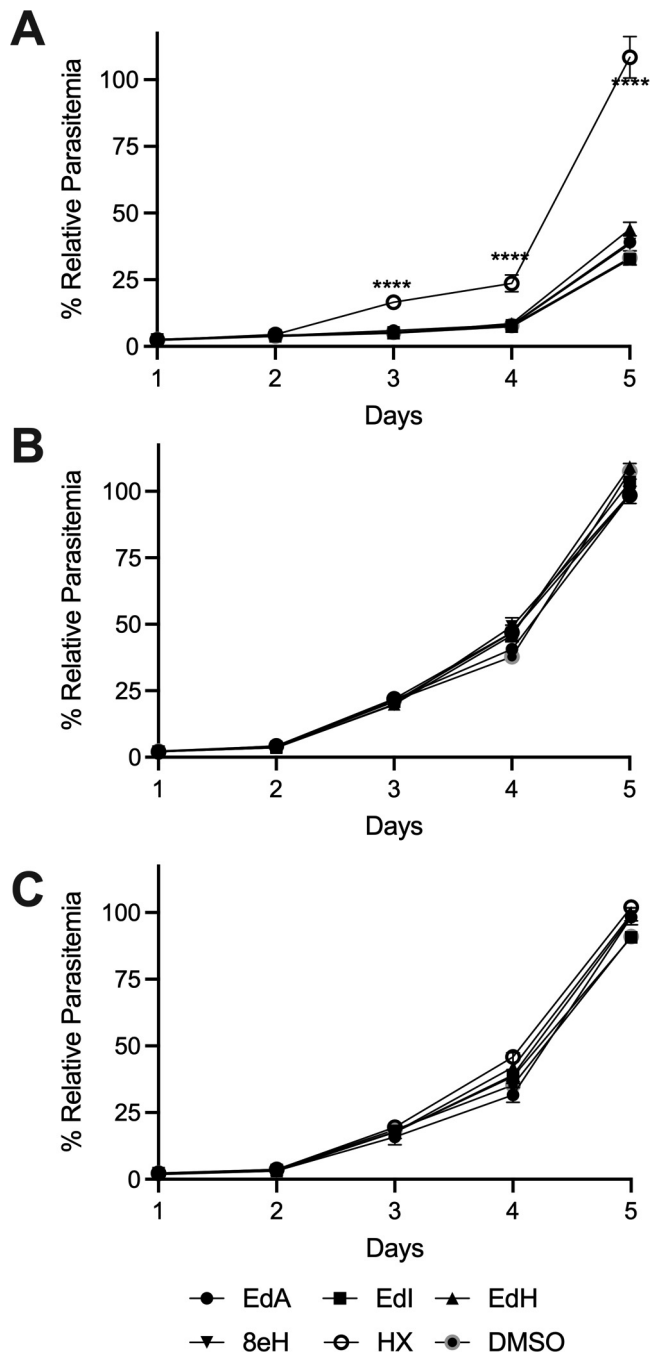
**Fig. 1.** Alkyne modified purines incorporate into the replicating asexual blood stage *Plasmodium falciparum*. Detection of modified purines in *P. falciparum* trophozoites after a 48 h incubation with 10  $\mu$ M of EdA, EdI, EdH, and 8eH (green). Parasites were co-stained with 10  $\mu$ g/mL of Hoechst 33342 (blue). Images were obtained on a Zeiss Axio Observer.Z1/7 microscope with a 100 $\times$  objective. Colocalisation was assessed and Pearson's coefficient ( $r$ ) was calculated using the Fiji JACoP plugin. Scalebar = 10  $\mu$ m.

purines (Fig. 2B-C). These results indicate that while the EdX analogues incorporated into asexual blood stage *P. falciparum* parasite DNA, unmodified purines are necessary for continued parasite growth over time.

### 3.3. *Plasmodium vivax* actively replicating liver stage schizonts incorporate EdA and can be differentiated from dormant hypnozoites via high-throughput content imaging

After invading a hepatocyte, a *P. vivax* sporozoite develops into either an actively replicating liver schizont or a dormant hypnozoite with a single nucleus (Mueller et al., 2009). Over the first

3–5 days of liver stage culture, hypnozoites and schizonts are of similar sizes and are indistinguishable (Sylvestre et al., 2021). Therefore, to verify that EdX analogs incorporate into actively replicating parasite DNA in the liver stage, cultures were supplemented with 2  $\mu$ M or 10  $\mu$ M of EdA, EdI, EdH, and 8eH at 5, 6, 7, and 8 days p.i. and then cultures were fixed at 9 days p.i. By using fluorescent microscopy, we observed that the addition of EdA resulted in nuclear staining of only actively replicating liver stage schizonts, whereas dormant hypnozoites did not incorporate EdA. The EdA fluorescence co-localised with nuclear Hoechst 33342 staining, similar to our *P. falciparum* asexual blood stage staining (Fig. 3). However, unlike in *P. falciparum* asexual blood stage para-



**Fig. 2.** *Plasmodium falciparum* asexual blood stage growth depends on unmodified purines in culture media. *Plasmodium falciparum* asexual blood stages were grown in media supplemented with 10 μM EdA, EdI, EdH, 8eH, hypoxanthine (HX), or 0.1% DMSO vehicle control in (A) complete media supplemented with Albumax II, (B) complete media supplemented with Albumax II and hypoxanthine, or (C) complete media supplemented with A + human plasma. Growth was assessed by flow cytometry using a Beckman Coulter CytoFLEX. Data shown are one representative experiment of three independent experiments. Errors (S.D.) were omitted when smaller than the marker. Significance assessed by a two-way ANOVA and Dunnett's multiple comparisons test, \*\*\*\* $P < 0.0001$ .

sites, *P. vivax* liver stage parasites did not incorporate EdI, EdH, or 8eH (data not shown).

We next evaluated if the EdA staining in *P. vivax* liver stage schizonts could be identified in a high-throughput manner using an automated high content imaging system. We showed that EdA incorporation can be identified and imaged at 20x with a high con-

tent imaging system (Fig. 4A). We tested two concentrations of EdA and observed that separation of liver stage schizonts and dormant hypnozoites can be accomplished using 2 or 10 μM EdA (Fig. 4B-C). Incorporation of EdA was quantified using maximum fluorescence intensity (of EdA) for each parasite in culture compared with parasite net growth area (PvUIS4). Typically, 150 μm<sup>2</sup> is used to demarcate hypnozoites versus liver schizonts based on parasite size alone (Roth et al., 2018). Separate DMSO-treated cultures were used as a negative control for click labelling. This analysis revealed that, while most hypnozoite forms were negative for EdA, some forms of similar size and morphology were positive for EdA and were therefore synthesising DNA at some point between 5–8 days p.i. and these forms were likely reactivating. We noted three of 233 liver forms (1.3%) of a net area less than 150 μm<sup>2</sup> were EdA-positive and when labelled with 10 μM EdA, and four of 197 liver forms (2.0%) of a net area less than 150 μm<sup>2</sup> were EdA-positive when labelled with 2 μM EdA. We also assessed the effect of EdA and its cytotoxicity on PHH, which are non-replicative, and were not found to incorporate EdA. However, we noticed a slight cytotoxic effect of EdA on PHH count compared with the DMSO vehicle control. Cultures treated with 2 μM and 10 μM for three consecutive days had a slight statistically significant decrease in hepatocyte nuclei count, yet the toxic effect did not appear to hinder parasite growth (Fig. 4D-E).

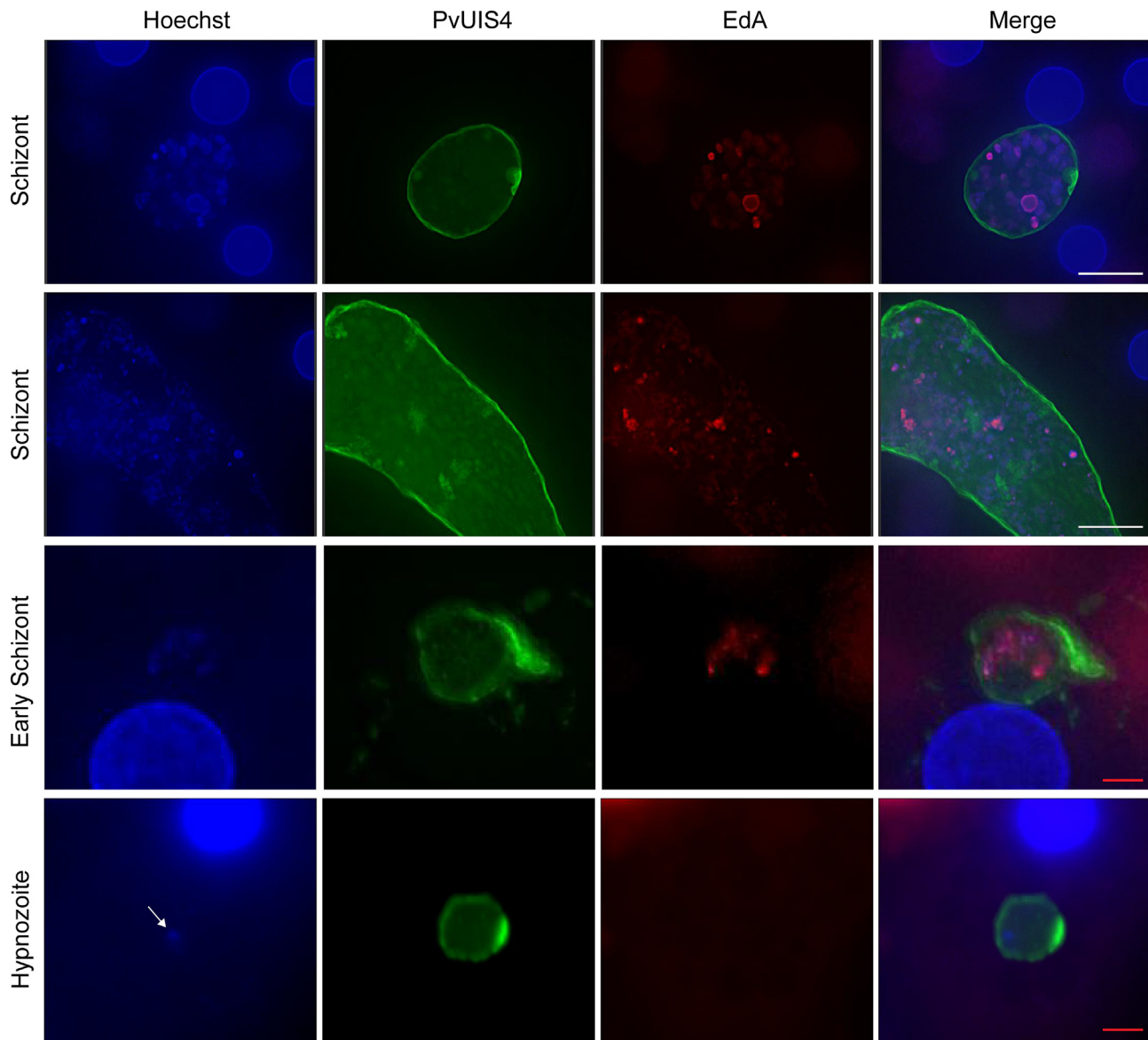
### 3.4. *Plasmodium berghei* liver stage schizonts incorporate EdA

*Plasmodium berghei* does not relapse in vivo and produces only liver stage schizonts. To confirm that all *P. berghei* liver stage parasites are actively replicating, and also confirm the incorporation of EdX in liver stages, we added EdA to *P. berghei*-infected hepatocyte cultures for 24 h prior to fixation. As shown in Fig. 5, all parasites were found to be positive for EdA staining. *Plasmodium berghei* liver stage assays are routinely performed by using HepG2 as host cells (Dorjsuren et al., 2021). Since HepG2 cells are actively replicating, we assessed HepG2 incorporation of EdA at 2 μM and 10 μM alone in the absence of infection for up to 72 h (Supplementary Fig. S2). We noted that while HepG2 cells successfully incorporate EdA, this modified purine is noticeably cytotoxic to the hepatocytes. While 60% of HepG2 cells incorporate EdA at the lower concentration of 2 μM, only 20% of HepG2 cells incorporate EdA at 10 μM (Supplementary Fig. S3). HepG2 nuclei counts were significantly reduced at both concentrations compared with the DMSO control. Altogether, our results suggest that EdA can be used for shorter times at lower concentrations with HepG2 cells and can be used for longer durations with PHH.

### 3.5. *Plasmodium falciparum* asexual blood stage parasites recrudescing from dihydroartemisinin (DHA)-induced dormancy do not incorporate EdA, but do incorporate [<sup>3</sup>H] hypoxanthine and [<sup>3</sup>H] adenosine

Previous studies confirmed that exposure of ring stages to DHA induces a dormant phenotype that is both time of exposure and DHA concentration dependent (Teuscher et al., 2012; Tucker et al., 2012). Due to the successful incorporation of EdA in *P. vivax* liver stage schizonts, we assessed if EdA incorporation could be used to differentiate DHA-induced dormant *P. falciparum* rings from recrudescing parasites. Following exposure to 700 nM DHA, parasites were sampled daily and pulsed with 10 μM EdA for 24 h. We observed that as parasites recrudesced and the number of infected red blood cells (parasitemia) increased, incorporation of EdA did not increase concomitantly (Fig. 6A). In similar experiments, the other purine analogs also were not incorporated in recrudescing parasites. Thus, we assessed if this lack of correlation was due to potential purine storage in the parasite as a response to



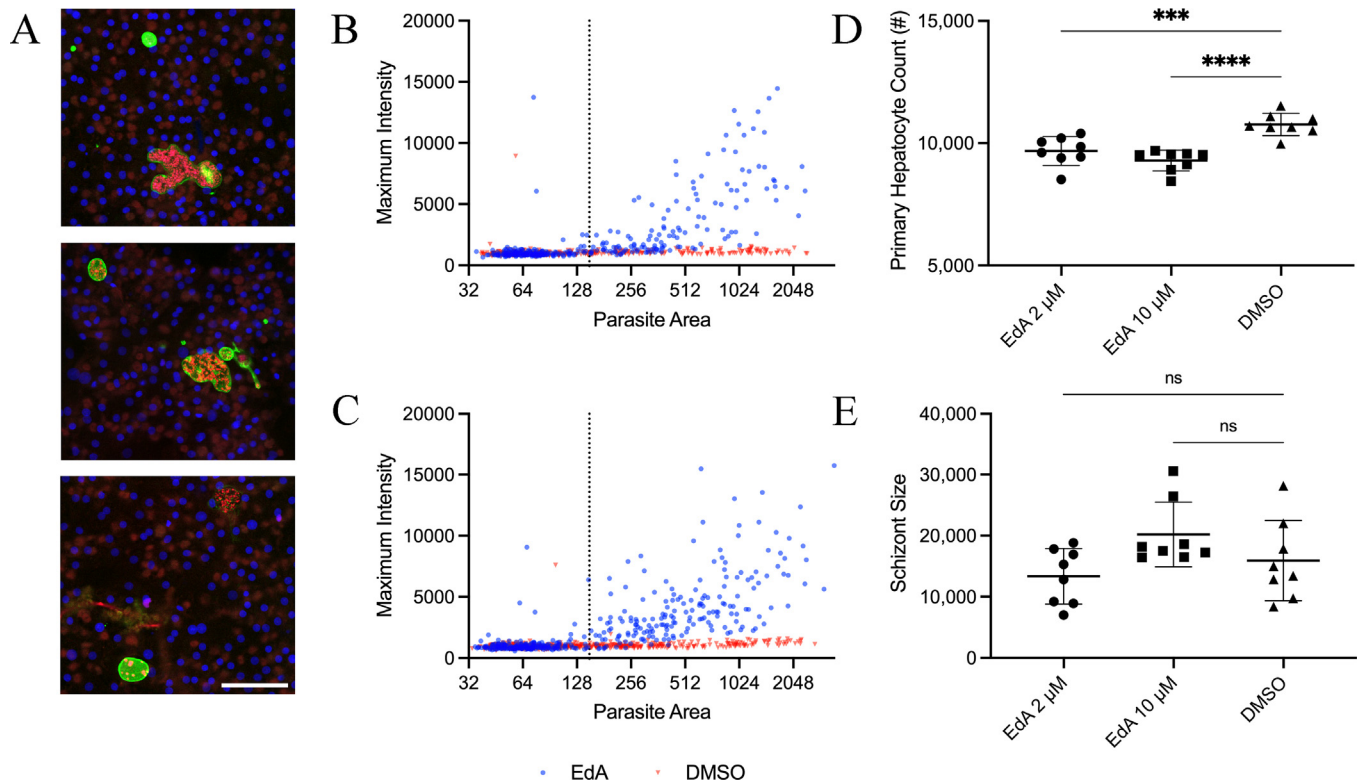


**Fig. 3.** Alkyne modified adenosine (EdA) incorporates in replicating *Plasmodium vivax* liver stage parasites, but not in hypnozoites. Primary human hepatocytes were infected with *P. vivax* sporozoites and incubated with 10  $\mu$ M EdA on days 5–8 p.i. Detection of EdA was assessed via a copper-catalysed click reaction (red). Parasites were co-stained with 1:25,000 PvUIS4, a parasitophorous vacuole membrane stain (green), and 10  $\mu$ g/mL of Hoechst 33342 (blue). The arrow points to a single nucleus in *P. vivax* liver stage hypnozoite. Images were obtained on a DeltaVision II deconvolution microscope at 100x objective. White scalebar = 15  $\mu$ m. Red scalebar = 5  $\mu$ m.

DHA treatment, or if DHA-treated parasites became more sensitive to the alkyne modification to adenosine and thus were unable to take up and incorporate EdA. Parasites were treated with DHA and then sampled daily and pulsed with either  $^3$ H-hypoxanthine or  $^3$ H-adenosine for 24 h. Daily Giemsa smears were also collected to assess morphology of recrudescing parasites. To compare the incorporation of radiolabeled purines in recrudescing parasites, a control sample of parasites that did not undergo DHA treatment was included. The control sample of parasites received 24 h pulses of radiolabeled hypoxanthine or adenosine. Control samples replicated and reached high parasitemia by day 2 and sampling was stopped. We observed that as parasites recrudesced and parasitemia increased, incorporation of radiolabeled purines occurred at the same rate as parasitemia measured by microscopic analysis of Giemsa-stained blood smears (Fig. 6B). Altogether, our data suggest that either DHA-exposed parasites do not store purines or that the alkyne modification affects incorporation into DNA of DHA treated parasites.

#### 4. Discussion

The ability of *Plasmodium* to convert into a dormant phenotype and later reactivate causing recrudescing or relapse infections remains a serious barrier towards malaria eradication. Reactivation occurs both naturally in the liver stages and following a drug-induced growth arrest during the intraerythrocytic life cycle (Barrett et al., 2019). The mechanisms by which the parasite enters dormancy and later recrudesces or reactivates to continue development is not fully understood and we lack tools to study these mechanisms. For *P. vivax* liver stages, a H3K9Ac marker for nuclear division and a LISP2 marker have been developed and associated with reactivation (Mikolajczak et al., 2015; Gupta et al., 2019). However, these markers are downstream of DNA synthesis and replication, which are specific to activated hypnozoites. Since purine and pyrimidine nucleotides are the building blocks of nucleic acids, biosynthesis or incorporation of these building blocks marks initiation of DNA synthesis (Hyde, 2007). Since *Plasmodium* sal-



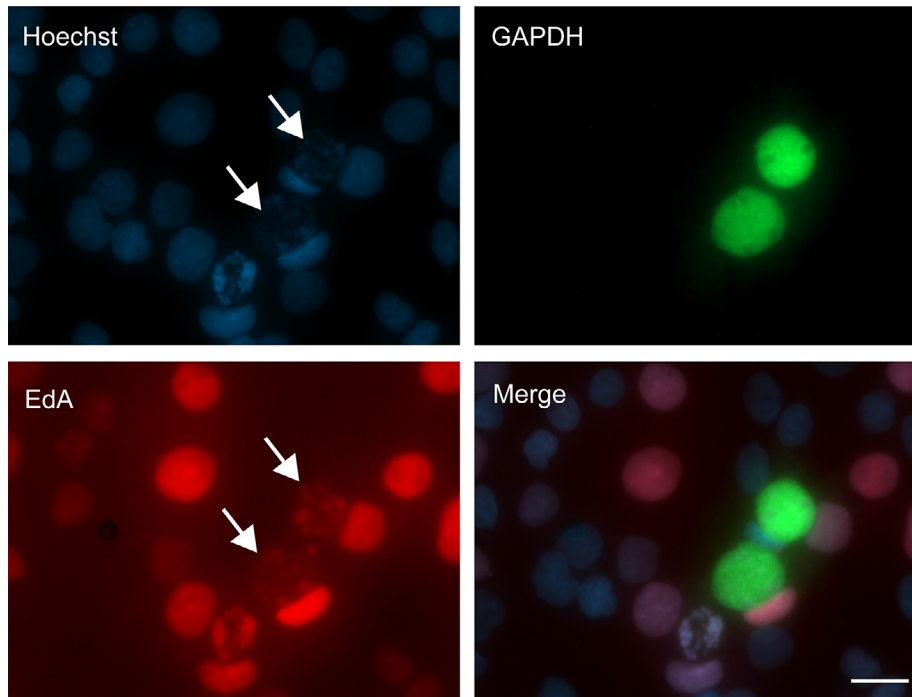
**Fig. 4.** Alkyne modified adenosine (EdA) incorporated into *Plasmodium vivax* hypnozoites and liver stage schizonts can be identified and quantified via high content imaging. *Plasmodium vivax*-infected primary human hepatocytes (PHH) were cultured in media supplemented with EdA and fixed at day 9 p.i. prior to immunofluorescent staining and click labelling. (A) Images acquired of *P. vivax* liver stage parasites (UIS4, green) with incorporated EdA (red) imaged at 20x with an ImageXpress confocal high content imaging system. Bar represents 100  $\mu\text{m}$ . Incorporation of either (B) 2  $\mu\text{M}$  or (C) 10  $\mu\text{M}$  EdA was quantified using maximum fluorescence intensity for each parasite in culture with a net growth area shown on the X-axis. Dotted lines represent the typical cut-off of 150  $\mu\text{m}^2$  used to demarcate hypnozoites versus liver schizonts based on parasite size alone. Separate DMSO-treated cultures represent a negative control for click labelling. Notably, EdA at both concentrations is incorporated in several hypnozoites, indicating DNA synthesis is occurring and these forms are likely reactivating. (D) EdA cytotoxicity was assessed on PHH using the nuclei count as an indicator of toxicity. (E) Liver stage schizont size was assessed for EdA- versus DMSO-treated parasites. The figure is representative of one independent replicate out of two ( $n = 2$ ). An ordinary one-way ANOVA Dunnett's multiple comparisons test was used for D and E. \*\*\* $P = 0.0005$ , \*\*\*\* $P < 0.000$ ; ns, not significant.

vages purines from the host (Downie et al., 2008), we hypothesised that alkyne-containing purine nucleosides could be developed as makers for DNA synthesis markers to differentiate between active and dormant parasites. Our study provides the first known report of a clickable DNA synthesis marker for *Plasmodium* which can easily be integrated into a staining panel design due to its flexibility. We show that EdA, EdI, 8eH, and EdH are incorporated into DNA in *P. falciparum* asexual blood stages, and that EdA is incorporated into actively replicating liver stage schizonts in *P. vivax* and *P. berghei*, but it does not incorporate into dormant *P. vivax* liver stage hypnozoites.

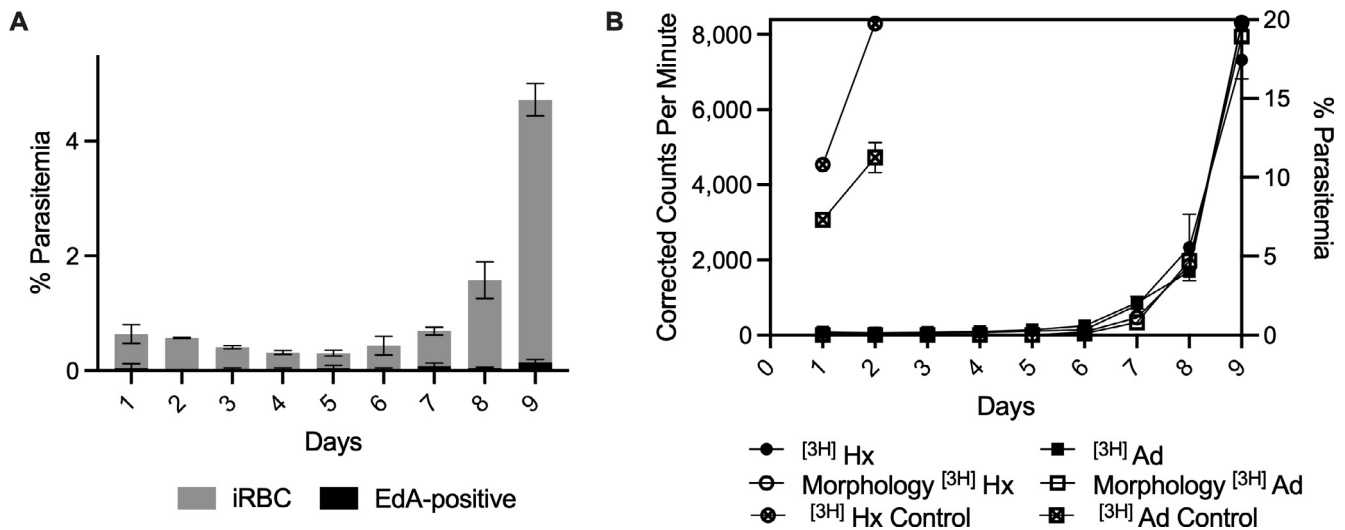
The methodology described in this study represents a valuable tool for *P. vivax* liver stage studies as it is the first to describe a DNA synthesis marker which can be used to distinguish actively replicating and dormant liver stages of the parasite. While the exact criteria defining a hypnozoite in vitro and in vivo is still being resolved, it is generally understood that fully mature hypnozoites survive treatment with phosphatidylinositol 4-kinase inhibitors (PI4Ki) in vivo, allowing for selection of hypnozoites in vitro (Roth et al., 2018). Interestingly, we noted that some (1.3–2.0%) liver forms of similar size and morphology to that of PI4Ki-insensitive hypnozoites (that is, having a growth area less than 150  $\mu\text{m}^2$ ) do incorporate EdA, indicating that these forms could be newly reactivating parasites (Fig. 4). Alternatively, recent reports describe how liver stage parasites must constantly buffer themselves against host cell lysosomes (Niklaus et al., 2019). Thus, it is also possible that these small, EdA-positive liver stage forms are schizonts which failed to develop due to factors such as the

host response to infection. Our data suggest EdA could be used in future studies to identify and characterise newly reactivating parasites and other host-parasite interactions, and to study the reactivation rate at additional timepoints p.i. Interestingly, only EdA was found to incorporate in *P. vivax* and *P. berghei* liver stage parasites. Previous work has shown rapid metabolism of inosine and hypoxanthine into allantoin by rat hepatocytes, which could explain why parasite incorporation of EdI, 8eH, or EdH was not achieved in the liver stage (Vincent et al., 1984).

On the other hand, we observed that active blood stage *P. falciparum* parasites incorporated all the alkyne-modified purines (Fig. 1). This creates a novel opportunity to study DNA synthesis pathways and cell cycling. Unexpectedly, *P. falciparum* recrudescing blood stage parasites coming out of DHA-induced dormancy did not incorporate any of the modified purines (Fig. 6). Moreover, when comparing incorporation of radiolabeled hypoxanthine and adenosine, with EdA incorporation in recrudescing parasites, we found that EdA was not incorporated while the radiolabeled purines were metabolised and incorporated into DNA. Previous work has shown that artemisinin-resistant *P. falciparum* has altered metabolic programming (Mok et al., 2021). Blood stage parasites showed decreased levels of adenosine deaminase and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) in Kelch13 (K13) mutant parasites, suggesting a prior pooling of purines in response to ART drug exposure (Mok et al., 2021). While the work reported here was conducted utilising a K13-wild type *P. falciparum* strain sensitive to ART, it is possible that in response to DHA exposure, even K13 wild type parasites may accumulate



**Fig. 5.** Alkyne modified adenosine (EdA) incorporates in replicating *Plasmodium berghei* liver stage parasites. HepG2 cells were infected with *P. berghei* sporozoites and 10  $\mu$ M EdA were supplemented at 24 h p.i. Cells were then fixed 48 h p.i. Detection of EdA was assessed via a copper-catalysed click reaction (red). Parasites were co-stained with 1:10,000 GAPDH (green) and 10  $\mu$ g/mL of Hoechst 33342 (blue). White arrows point to *P. berghei* liver stage schizont nuclei with incorporated EdA. Images were obtained on a Zeiss Axio Observer.Z1/7 microscope at 40x objective. Scalebar = 20  $\mu$ m.



**Fig. 6.** Asexual blood stage *Plasmodium falciparum* parasites recrudescing from DHA-induced dormancy incorporate [ $^3$ H]-hypoxanthine and/or [ $^3$ H]-adenosine but do not incorporate EdA. (A) DHA dormancy was induced in *P. falciparum* asexual blood stage parasites and daily samples were acquired and pulsed with 10  $\mu$ M EdA for 24 h. Pulsed samples were analysed via flow cytometry on a Beckman Coulter CytoFLEX. (B) DHA dormancy was induced in *P. falciparum* asexual blood stage parasites. Daily samples were acquired and pulsed with [ $^3$ H]-hypoxanthine (Hx) or [ $^3$ H]-adenosine (Ad) for 24 h. Daily Giemsa-stained smears were performed to assess recrudescence (% parasitemia). Control parasites were not treated with DHA. Radiolabeled samples were analysed by a Perkin Elmer Microbeta<sup>2</sup> scintillation counter. Data shown are one representative experiment of two independent experiments, (average  $\pm$  S.D.).

internal stores of purines after exposure to DHA. Alternatively, structural modifications to purine precursors may cause steric hindrance of the purine salvage enzymes (e.g., purine nucleoside phosphorylase). Although the EdX may not be useful for DHA-induced dormancy studies in *P. falciparum*, they remain valuable as an alternative to radiolabeled purines, as these alkyne-modified purines can potentially be used as an inexpensive tool for investigation of the parasite's scavenging mechanisms.

In *P. falciparum* blood stage in vitro cultures, we observed that in the presence of hypoxanthine, the modified purines were not cytotoxic. However, parasites lacking supplementation of hypoxanthine for purine salvage were not able to sustain growth when supplemented with the alkyne modified purines alone. Nevertheless, incorporation of EdA, EdI, 8eH, and EdH was observed in media supplemented with hypoxanthine. These data suggest that there is an upper limit on the proportion of modified purines in

newly synthesised DNA before the proportion is toxic, while a lower proportion allows for sustained replication and parasite growth as well as visualisation of clicked purines via fluorescence microscopy. While testing the cytotoxicity and fluorescent signal of other proportions of modified purine to hypoxanthine may result in a more optimised mixture, our studies were performed with 10  $\mu$ M modified purines as this concentration was used for labelling *C. parvum* (Pawlowic et al., 2019) and preliminary studies with lower concentrations resulted in inconsistent signal (data not shown). In our *P. vivax* and *P. berghei* liver stage experiments, it is important to note that a slight hepatocyte toxicity from EdA was observed which could confound our studies. Nevertheless, parasite growth remained unaffected (Fig. 4). In the present study, long incubation times such as 48–72 h were used. However, to assess reactivation from dormancy, shorter incubation times could be used in future studies to obtain narrower time windows of reactivation and to limit negative impacts on the assay due to hepatocyte toxicity.

This methodology provides many opportunities for the study of malaria parasite biology. While most preliminary in vitro drug screening for liver stage activity is conducted using *P. berghei*, medium throughput platforms using high-content imaging of *P. vivax* liver stages are now being used to confirm and optimise anti-hypnozoite hits (Roth et al., 2018). By incorporating EdA into the high-content pipeline, we can further characterise both the parasite forms quantified during analysis, as well as gain a better understanding of the effect of agonists or antagonists of hypnozoite reactivation. Ideally, future experimentation will involve a *P. vivax* time course with combination of LISP2, EdA, and H3K9Ac staining to better define the timing of DNA replication, nuclear division, and membrane synthesis in reactivating parasites. Similarly, as new techniques such as single-cell RNA sequencing are being used to characterise hypnozoites and their dormancy, we anticipate our markers could be used to better understand the sequencing of DNA synthesis versus other, yet to be discovered processes. Until that time, our EdA staining approach could aid in elucidating the cause of hypnozoite reactivation, which has been hypothesised to include fever, hemolysis, malaria reinfection, and chemical reactivation (Shanks and White, 2013; Adekunle et al., 2015). Recently we reported the recruitment of host aquaporin 3 to the parasite PVM, as well as the formation of a tubulovesicular network around *P. vivax* liver forms; these mechanisms have been hypothesised to be part of the parasite's nutrient-scavenging pathways (Posfai et al., 2020; Sylvester et al., 2021). EdA staining could be used as a bait to further elucidate pathways responsible for purine scavenging. Additionally, we can postulate that EdA would incorporate into *Plasmodium cynomolgi* liver stages and could therefore be useful for *P. cynomolgi* drug discovery platforms. *Plasmodium cynomolgi* produces hypnozoites, although *P. cynomolgi* hypnozoites and liver stage schizonts have been reported to be much smaller than their *P. vivax* counterparts (Dembélé et al., 2011, 2014; Voorberg-van der Wel et al., 2013). Yet, size alone is often used as the defining attribute of hypnozoites versus schizonts during high-content analysis. Given our finding that some hypnozoite-like forms are synthesising DNA, and *P. cynomolgi* liver forms are both relatively smaller than those of *P. vivax*, misclassification of hypnozoites and schizonts is very possible in this model as well, but it could be better characterised using a marker such as EdA.

Herein, we detail the synthesis of new alkyne-modified purines, including inosine (EdI) and hypoxanthine (EdH and 8eH) derivatives, and the first known reported use of alkyne modified purines to study the biology of *Plasmodium*. The use of alkyne-modified purines enables Chemo-labelling (“Click chemistry”), providing advantages over traditional antibody staining in that researchers do not have to consider animal/species cross-reactivity. Further-

more, it offers increased flexibility since the alkyne modified purines can be “clicked” to any azide-linked molecule of choice and can also be leveraged in a high-throughput manner using high-content imaging systems. In addition, the novel purine analogues reported here may have potential uses in other organisms that have yet to be tested and validated. This new tool is inexpensive, easy to incorporate into current workflows, and provides flexibility, making it an ideal tool as a DNA synthesis marker.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpara.2022.03.003>.

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