

Phenotypic and transcriptomic effects of the antimalarial compound GBPS340 on Plasmodium falciparum intraerythrocytic development.

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Abstract

Background. The global spread of multidrug-resistant *Plasmodium falciparum* has become a major obstacle to malaria control, threatening progress toward elimination. Innovative therapeutic strategies are therefore urgently needed. We developed a new 6-arylpyridine series, from which GBPS340 emerged as a promising candidate based on its *in vitro* activity against multidrug-resistant *Plasmodium* strains, its *in vivo* efficacy comparable to that of mefloquine in a mouse model of *Plasmodium berghei* ANKA infection, and its favourable safety profile.

Methods. We investigated the phenotypic and transcriptomic effects of GBPS340 on *P. falciparum* 3D7 parasites. Ring- and trophozoite-stage parasites were treated independently and monitored for 48 h, alongside DMSO-treated controls, in three independent biological replicates. Parasite morphology, parasitemia, percentage of pyknotic forms, and intraerythrocytic stage distribution were characterized by microscopy, and transcriptomic responses were analysed using RNA sequencing.

Results. GBPS340 markedly impaired parasite development, with effects depending on the developmental stage at treatment initiation. Following ring-stage exposure, parasitemia was significantly reduced at both 24 and 48 h, with delayed progression from rings to trophozoites and impaired schizogony, resulting in failure to establish a new ring-stage population. Pronounced morphological alterations and an apparent absence of hemozoin in the mature stages were also observed. Trophozoite-stage exposure induced a weaker and delayed phenotypic response.

RNA sequencing revealed significant transcriptional changes following ring-stage exposure, with more than 600 genes differentially expressed at both 24 and 48 h, predominantly downregulated. Gene Ontology analysis highlighted alterations in pathways associated with the inner membrane complex, parasite motility, and erythrocyte invasion. The transcriptional response following trophozoite-stage exposure was considerably weaker.

Conclusions. Overall, these findings indicate that GBPS340 disrupts the intraerythrocytic developmental cycle of *P. falciparum* in a stage- and time-dependent manner, with ring-stage parasites showing the greatest susceptibility. The observed transcriptional modifications highlight candidate pathways that warrant further investigation to elucidate the molecular targets and mechanism of action of GBPS340.

Background

Malaria remains the leading parasitic disease affecting humans worldwide. In 2024, approximately 282 million new malaria cases and more than 610,000 malaria-related deaths were reported across 80 countries [1]. Among the five *Plasmodium* species affecting humans, *P. falciparum* remains the most prevalent and deadliest worldwide [1]. The World Health Organization recommends a three-day course of artemisinin-based combination therapy as the first-line treatment for uncomplicated *P. falciparum* malaria [2]. However, the emergence of resistance to artemisinin and partner drugs, along with the limited efficacy of currently available vaccines, may compromise global malaria control efforts. This situation highlights

the urgent need to preserve existing antimalarial drugs while identifying new compounds with novel mechanisms of action to address this growing threat.

For several years, our team has been developing new arylaminoalcohol derivatives as alternative therapeutic agents. We previously reported the optimized asymmetric synthesis of these compounds, incorporating a variety of aryl cores, including quinoline, pyrrolo[1,2-a]quinoxaline, fluorene, 3-arylpyridine, and 6-arylpyridine [3–13]. Among more than 200 synthesized molecules, 50 exhibited IC_{50} values below 50.0 nM against both *P. falciparum* (Pf) 3D7 and PfW2 strains, with high selectivity indices (> 100). The 6-arylpyridine derivatives are particularly promising, showing consistent *in vitro* activity against multidrug-resistant *Plasmodium* strains (HB3, BRE1, and Dd2), *in vivo* efficacy comparable to that of mefloquine in a mouse model of *P. berghei* ANKA infection at a dose of 5 mg/kg, and a favourable safety profile [14]. Interestingly, their sustained activity against mefloquine-resistant strains suggests a mechanism of action distinct from that of mefloquine, which still remains to be elucidated. Among these series, GBPS340 has emerged as a particularly promising candidate (Fig. 1).

However, the biological response of *P. falciparum* to this compound remains poorly characterized. In this study, we investigated the stage- and time-dependent phenotypic and transcriptional responses of *P. falciparum* to GBPS340 exposure. By combining morphological and transcriptomic analyses of ring- and trophozoite-stage parasites, we aimed to characterize the effects of GBPS340 on parasite development and identify the cellular processes potentially affected by the compound.

Methods

Parasite cultures

Plasmodium falciparum 3D7 parasites, a cloned line derived from the NF54 isolate [15], were used in this study. They were kindly provided by the Muséum national d'Histoire naturelle, Paris, France. The parasites were maintained *in vitro* in human erythrocytes at 3.3% hematocrit in RPMI 1640 (Sigma–Aldrich) supplemented with 25 mM HEPES, 25 mM $NaHCO_3$, 0.5% AlbuMAX I lipid-rich bovine serum albumin (Life Technologies), 0.5 mM hypoxanthine (Sigma–Aldrich), and 50 μ g/mL gentamicin (Sigma–Aldrich). Cultures were incubated at 37°C under a controlled atmosphere containing 5% CO_2 , following the method originally described by Trager and Jensen [16]. Parasite cultures were tightly synchronized at the ring stage by two successive treatments with 5% (w/v) D-sorbitol applied 4 h apart, according to the method originally described by Lambros and Vanderberg [17].

Preparation of GBPS340 solutions

On the basis of a 50% inhibitory concentration (IC_{50}) of 8.7 ± 4.31 nM against *P. falciparum* 3D7 cultures [14], GBPS340 was used at a working concentration of 18 nM, corresponding to approximately twice the IC_{50} . Stock solutions of GBPS340 were prepared in DMSO at a concentration of 2 mM and stored at -20°C, and working solutions were obtained by dilution in RPMI 1640 medium.

GBPS340 treatment in ring-stage cultures

Following double sorbitol synchronization, the first part of the parasite culture was allowed to complete one intraerythrocytic replication cycle (Fig. 2). At the subsequent ring stage, GBPS340 exposure was initiated, and this time point was defined as H0. Immediately before exposure, a 5-mL aliquot of culture was collected for thin blood smear preparation and preservation in TRIzol™ reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The remaining culture was divided into two conditions: GBPS340 treatment and DMSO supplementation as a negative control. Samples from treated and untreated control cultures were collected after 24 h (H24) and 48 h (H48) of exposure, processed for thin blood smear preparation, and preserved in TRIzol™ reagent at -80°C until transcriptomic analysis.

The protocol was performed in triplicate. Squares indicate 5-mL aliquot collection for thin blood smears and TRIzol™ preservation. IDC, intraerythrocytic developmental cycle. h, hours.

GBPS340 treatment in trophozoite-stage cultures

A second part of sorbitol-synchronized parasites was maintained under standard incubation conditions until maturation to the trophozoite stage. GBPS340 treatment was then initiated when most parasites displayed trophozoite morphology, and the same experimental protocol as that used for the ring-stage cultures was applied at H0, H24, and H48.

For both parasite stages, experiments were performed in triplicate.

Phenotypic characterization of the effect of GBPS340

Phenotypic characteristics, including parasite morphology, parasitemia, percentage of pyknotic forms, and intraerythrocytic stage distribution, were assessed via microscopic examination (×1,000 magnification) of Giemsa-stained thin blood smears. Each smear was independently examined by four readers, and the mean value of the four readings was used for analysis. To account for the differences in initial parasitemia (H0) among biological triplicates, parasitemia at H24 and H48 was normalized to the corresponding initial value and expressed as the ratio of parasitemia at H24 or H48 to that at H0.

A one-sided paired Student's *t* test was used to test the a priori hypothesis that GBPS340 reduces normalized parasitaemia ratios. Differences in the proportion of rings between GBPS340-treated and untreated ring-stage cultures were assessed at H24 and H48 using two-sided paired Student's *t* tests. The same test was used to assess differences in the proportion of trophozoites between treated and untreated trophozoite-stage cultures at both time points. A significance threshold of $\alpha = 0.05$ was applied throughout.

Transcriptional analysis of the effect of GBPS340

RNA extraction, library construction, and sequencing.

RNA was extracted from erythrocyte pellets preserved in TRIzol™ reagent using the RNeasy Mini Kit on the QIACube (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA concentration and quality were assessed using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared using the Illumina Stranded mRNA Prep Ligation Kit according to the manufacturer's protocol. The cDNA library concentration was assessed using a Qubit 4 fluorometer (Thermo Fisher Scientific), and transcriptome profiling was performed using high-throughput RNA sequencing on a NextSeq 550 platform (Illumina, San Diego, CA, USA) in paired-end mode (2×75 bp reads).

Bioinformatic analysis of RNA-sequencing data.

The raw sequencing data were analysed using the open-access Galaxy Europe platform [18]. Adapter sequences and low-quality reads were removed using Cutadapt [19], and read quality was assessed using FastQC [20] based on Phred quality scores. Reads with a Q30 score or higher, corresponding to a 99.9% probability of accurate base calling [21], were retained for downstream analyses. High-quality reads were aligned to the *P. falciparum* 3D7 reference genome available in PlasmoDB [22, 23] using RNA STAR [24], and gene-level read counts were generated using FeatureCounts [25]. Differential gene expression analysis was performed using DESeq2 [26] with a multifactorial design incorporating the developmental stage of the parasite, time points and GBPS340 treatment. The read counts were modelled using a negative binomial distribution, and statistical significance was assessed using the Wald test. Genes with an adjusted p value < 0.05 and an absolute log2-fold change > 1 ($|\log_2FC| > 1$) were considered differentially expressed. Heatmaps and principal component analysis (PCA) plots were generated using Galaxy Europe and R software (version 4.4.3; R Core Team, 2025), respectively. Finally, differentially expressed genes (DEGs) were functionally classified according to Gene Ontology (GO) terms using the GOseq package [27] to identify enriched biological processes across experimental conditions.

Results

Aliquots collected at different time points (H0, H24, and H48) and under the different experimental conditions previously described (ring- or trophozoite-stage cultures, untreated controls or GBPS340-treated cultures) were used to prepare 30 thin blood smears and 30 samples preserved in TRIzol™ reagent.

Effect of GBPS340 on parasitemia and intraerythrocytic stage distribution in ring-stage cultures

At H0, the mean parasitemia was 5.08%, with 88.16% of parasites at the ring stage (Table 1, Fig. 3). In the untreated control cultures, the mean parasitemia was 4.86% at H24 and 4.52% at H48. The parasites progressed normally through the intraerythrocytic cycle, with trophozoites representing the predominant stage at H24 (57.55%). At H48, following schizont rupture and erythrocyte reinvasion, a new generation of ring-stage parasites predominated (58.28%), while schizonts accounted for 21.27% of the parasite population.

Table 1

Assessment of parasitemia, pyknotic forms, and parasite stages in ring-stage cultures at different time points.

Ring-stage cultures	Replicate	Parasitemia ^a (%)	Ratio ^b (%)	Pyknotic forms ^a (%)	Ring stage ^c (%)	Trophozoite stage ^c (%)	Schizont stage ^c (%)
H0	R ₁	6.01	100	0.00	82.70	7.88	9.40
	R ₂	2.18	100	0.00	92.60	2.95	4.40
	R ₃	7.06	100	0.00	89.18	5.25	5.55
	Mean ± SD	5.08 ± 2.57	100 ± 0	0.00 ± 0	88.16 ± 5.03	5.36 ± 2.47	6.45 ± 2.62
H24, untreated cultures	R ₁	5.23	87.02	0.00t	40.38	58.73	0.90
	R ₂	1.85	84.86	0.00	28.78	64.28	6.95
	R ₃	7.50	106.23	0.00	50.18	49.65	0.18
	Mean ± SD	4.86 ± 2.84	92.71 ± 11.76	0.00 ± 0	39.78 ± 10.71	57.55 ± 7.39	2.68 ± 3.72
H24, GBPS340-treated cultures ^d	R ₁	4.00	66.56	1.32	67.65	30.80	1.55
	R ₂	1.25	57.34	0.30	78.98	21.03	0.00
	R ₃	5.70	80.74	0.37	87.55	10.93	1.50
	Mean ± SD	3.65 ± 2.25	68.21 ± 11.79	0.66 ± 0.57	78.06 ± 9.98	20.92 ± 9.94	1.02 ± 0.88
H48, untreated cultures	R ₁	6.29	104.66	0.00	69.63	18.15	12.23
	R ₂	0.79	36.24	0.00	59.05	13.73	27.20
	R ₃	6.48	91.78	0.00	46.15	29.48	24.38
	Mean ± SD	4.52 ± 3.23	77.56 ±	0.00 ± 0	58.28 ±	20.45 ± 8.12	21.27 ± 7.95

H0 = time of GBPS340 exposure; H24 and H48 = 24 and 48 h after treatment initiation; biological triplicates are indicated by R₁, R₂, and R₃. ^aPercentage of red blood cells infected with viable parasites or pyknotic forms; ^bParasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%); ^cPercentage of parasites at a given developmental stage ^dGBPS340 was used at a concentration of 18 nM.

The percentages shown for each replicate (R₁, R₂, and R₃) represent the mean values obtained from four independent readers. The bold percentages represent the means of three replicates.

Ring-stage cultures	Replicate	Parasitemia ^a (%)	Ratio ^b (%)	Pyknotic forms ^a (%)	Ring stage ^c (%)	Trophozoite stage ^c (%)	Schizont stage ^c (%)
			36.36		11.76		
H48, GBPS340-treated cultures ^d	R ₁	2.52	41.93	2.44	30.50	66.38	3.10
	R ₂	0.38	17.43	0.40	29.90	70.10	0.00
	R ₃	3.10	43.91	2.18	62.70	36.55	0.73
	Mean ± SD	2.00 ± 1.43	34.42 ± 14.75	1.67 ± 1.11	41.03 ± 18.77	57.68 ± 18.39	1.28 ± 1.62
H0 = time of GBPS340 exposure; H24 and H48 = 24 and 48 h after treatment initiation; biological triplicates are indicated by R ₁ , R ₂ , and R ₃ . ^a Percentage of red blood cells infected with viable parasites or pyknotic forms; ^b Parasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%); ^c Percentage of parasites at a given developmental stage ^d GBPS340 was used at a concentration of 18 nM.							
The percentages shown for each replicate (R ₁ , R ₂ , and R ₃) represent the mean values obtained from four independent readers. The bold percentages represent the means of three replicates.							

The data represent the mean percentages from three biological replicates (see Table 1).

In contrast, GBPS340 treatment markedly impaired parasite development. At H24, parasitemia was 3.65%, with parasites remaining predominantly at the ring stage (78.06%), whereas only 20.92% had progressed to the trophozoite stage. At H48, parasitemia was 2%, with trophozoites representing the predominant developmental stage (57.68%), whereas schizonts accounted for only 1.28% of the parasite population. GBPS340 significantly reduced the normalized parasitaemia ratios at both H24 ($t(2) = -11.67$, $p = 0.004$) and H48 ($t(2) = -3.34$, $p = 0.034$; one-sided paired Student's t test) (Fig. 4, Additional file 1). Furthermore, at H24, GBPS340 exposure was associated with a significantly greater proportion of ring-stage parasites compared with matched untreated controls (two-sided paired t test, $t(2) = 5.77$, $p = 0.029$). Taken together, these results suggest delayed intraerythrocytic parasite maturation following GBPS340 treatment. In parallel, the proportion of pyknotic forms increased over time, reaching 0.66% at H24 and 1.67% at H48.

Parasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%). The bar length represents the normalized parasitaemia ratio, whereas the segments represent the relative contribution of each parasite stage. The values represent the means of four independent readings per smear from three biological replicates.

The normalized parasitaemia ratios differed significantly between GBPS340-treated and untreated cultures at H24 and H48 (one-sided paired Student's t test; $p = 0.004^{**}$ and $p = 0.034^*$, respectively).

The proportions of ring-stage parasites differed significantly between GBPS340-treated and untreated control cultures at H24 (two-sided paired Student's t test, $p = 0.029$ $\dagger$).

Effect of GBPS340 on parasitemia and intraerythrocytic stage distribution in trophozoite-stage cultures

At H0, the mean parasitemia was 4.58%, with trophozoites accounting for 71.66% of the parasite population (Table 2, Fig. 5). In the untreated control cultures, the mean parasitemia was 5.28% at H24 and 2.85% at H48. Parasites progressed normally through the intraerythrocytic cycle, with ring-stage parasites predominating at both H24 (74.69%) and H48 (65.19%) following schizont rupture and erythrocyte reinvasion.

Table 2

Assessment of parasitemia, pyknotic forms, and parasite stages in trophozoite-stage cultures at different time points.

Trophozoite-stage cultures	Replicate	Parasitemia ^a (%)	Ratio ^b (%)	Pyknotic forms ^a (%)	Ring stage ^c (%)	Trophozoite stage ^c (%)	Schizont stage ^c (%)
H0	R ₁	5.17	100	0.00	26.72	72.95	0.33
	R ₂	2.11	100	0.00	27.5	71.52	0.98
	R ₃	6.46	100	0.00	29	70.52	0.48
	Mean ± SD	4.58 ± 2.23	100 ± 0	0.00 ± 0	27.74 ± 1.16	71.66 ± 1.22	0.60 ± 0.34
H24, untreated cultures	R ₁	6.70	129.59	0.00	75.45	11.08	13.45
	R ₂	1.52	72.04	0.00	76.80	12.68	10.53
	R ₃	7.63	118.11	0.00	71.83	15.75	12.43
	Mean ± SD	5.28 ± 3.29	106.58 ± 30.46	0.00 ± 0	74.69 ± 2.57	13.17 ± 2.37	12.14 ± 1.48
H24, GBPS340-treated cultures ^d	R ₁	2.98	57.64	2.24	30.25	50.63	19.13
	R ₂	0.82	38.86	0.18	24.38	60.20	15.40
	R ₃	6.26	96.90	1.30	51.43	40.38	8.20
	Mean ± SD	3.35 ± 2.74	64.47 ± 29.62	1.24 ± 1.03	35.35 ± 14.23	50.40 ± 9.91	14.24 ± 5.56
H48, untreated cultures	R ₁	4.09	79.11	0.00	51.70	47.35	1.18
	R ₂	1.02	48.34	0.00	52.90	45.18	1.93
	R ₃	3.43	53.10	0.00	90.97	6.83	2.20
	Mean ± SD	2.85 ± 1.62	60.18 ± 16.56	0.00 ± 0	65.19 ± 22.33	33.12 ± 22.79	1.77 ± 0.53

H0 = time of GBPS340 exposure; H24 and H48 = 24 and 48 h after treatment initiation; biological triplicates are indicated by R₁, R₂, and R₃. ^aPercentage of red blood cells infected with viable parasites or pyknotic forms; ^bParasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%); ^cPercentage of parasites at a given developmental stage ^dGBPS340 was used at a concentration of 18 nM.

The percentages shown for each replicate (R₁, R₂, and R₃) represent the mean values obtained from four independent readers. The bold percentages represent the means of three replicates.

Trophozoite-stage cultures	Replicate	Parasitemia ^a (%)	Ratio ^b (%)	Pyknotic forms ^a (%)	Ring stage ^c (%)	Trophozoite stage ^c (%)	Schizont stage ^c (%)
H48, GBPS340-treated cultures ^d	R ₁	0.85	16.44	2.61	29.38	54.00	16.55
	R ₂	0.35	16.59	0.21	48.51	39.99	11.50
	R ₃	2.85	44.12	2.36	74.60	22.15	3.25
	Mean ± SD	1.35 ± 1.32	25.72 ± 15.94	1.73 ± 1.32	50.83 ± 22.70	38.71 ± 15.96	10.43 ± 6.71
H0 = time of GBPS340 exposure; H24 and H48 = 24 and 48 h after treatment initiation; biological triplicates are indicated by R₁, R₂, and R₃. ^aPercentage of red blood cells infected with viable parasites or pyknotic forms; ^bParasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%); ^cPercentage of parasites at a given developmental stage ^dGBPS340 was used at a concentration of 18 nM. The percentages shown for each replicate (R₁, R₂, and R₃) represent the mean values obtained from four independent readers. The bold percentages represent the means of three replicates.							

The data represent the mean percentages from three biological replicates (see Table 2).

In contrast, GBPS340 treatment impaired parasite development. At H24, the mean parasitemia was 3.35%, with trophozoites remaining the predominant stage (50.4%), whereas only 35.35% of parasites had progressed to the ring stage. At H48, parasitemia was 1.35%, with ring-stage parasites accounting for 50.83% of the parasite population, while trophozoites and schizonts still represented 38.71% and 10.43% of parasites, respectively. GBPS340 was associated with a lower normalized parasitaemia ratio compared to matched untreated controls at both H24 (one-sided paired Student's *t* test, *t*(2) = - 2.75, *p* = 0.055) and H48 (*t*(2) = - 2.22, *p* = 0.078), although neither difference reached statistical significance (Fig. 6, Additional file 1). GBPS340 exposure was associated with a significantly higher proportion of trophozoite-stage parasites at H24 (two-sided paired *t* test, *t*(2) = 5.55, *p* = 0.031). The proportion of pyknotic forms increased over time, reaching 1.24% at H24 and 1.73% at H48.

Parasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%). The bar length represents the normalized parasitaemia ratio, whereas the segments represent the relative contribution of each parasite stage. The values represent the means of four independent readings per smear from three biological replicates.

*The proportions of trophozoite-stage parasites differed significantly between GBPS340-treated and untreated control cultures at H24 (two-sided paired Student's *t* test, *p* = 0.031†).*

GBPS340 effect on parasite morphology

Microscopic examination revealed early-onset abnormalities in GBPS340-treated parasites, which were detectable as early as H24 and persisted at H48 (Fig. 7). Pronounced cytoplasmic vacuolization was the predominant feature of ring-stage parasites and was also observed, to a lesser extent, in trophozoites. As trophozoites mature, cytoplasmic fragmentation becomes the predominant alteration, indicative of progressive parasite degeneration. In schizonts, GBPS340 treatment induced marked disorganization, with merozoites scattered throughout the cytoplasm. Notably, mature parasites exposed to GBPS340 consistently lacked hemozoin.

Morphological alterations in parasites cultured in the presence of GBPS340 (bottom panel) compared with untreated parasites (top panel). A, cytoplasmic vacuolization; B, cytoplasmic fragmentation; C, scattering of merozoites throughout the cytoplasm; D, hemozoin. Note the absence of hemozoin in GBPS340-treated parasites.

Transcriptional analysis of the effect of GBPS340

To characterize the transcriptional response to GBPS340, RNA sequencing was performed on all culture conditions and replicates, followed by a quality-control assessment of sequencing depth. Two samples with fewer than 1 million reads were excluded from downstream analyses because of insufficient sequencing depth (the H0 sample from the first replicate of the trophozoite-stage culture and the H48 sample from the third replicate of the untreated trophozoite-stage culture). Among the remaining samples ($n = 28$), the number of raw sequencing reads per sample ranged from 1.4 to 20.4 million (mean $\pm$ SD: 12.6 ± 3.4 million), and uniquely mapped reads accounted for 58–91% of the total reads (Additional file 2).

Principal component analysis.

Following quality control and normalization of read counts using DESeq2, PCA revealed that biological replicates clustered together according to parasite developmental stage and experimental conditions, supporting the reproducibility of the RNA-seq dataset (Fig. 8).

Ring- and trophozoite-stage cultures clearly presented distinct transcriptional profiles at H0, highlighting the parasite developmental stage as a major source of transcriptomic variation.

In the absence of GBPS340, transcriptomic profiles also changed markedly over time in cultures initiated at each developmental stage, which is consistent with progression through the intraerythrocytic developmental cycle. More specifically, transcriptomes from ring-stage cultures at H24 clustered closely with those from trophozoite-stage cultures at H0. Transcriptomes from trophozoite-stage cultures at H24 clustered closely with those from ring-stage cultures at H0. At H48, transcriptomes from cultures initiated at a given developmental stage tended to cluster with H0 transcriptomes from the same stage. These observations are consistent with the expected transcriptional dynamics over approximately one intraerythrocytic developmental cycle.

GBPS340 exposure altered this transcriptional trajectory. Within each developmental stage, GBPS340-treated and untreated control cultures formed distinct clusters, suggesting treatment-induced transcriptional changes. At H48, the transcriptomes from GBPS340-treated cultures tended to cluster more

closely with those of untreated cultures at H24 within the same developmental stage and consequently with H0 transcriptomes corresponding to the alternate developmental stage. This pattern is consistent with a delay in the normal transcriptional progression induced by GBPS340.

As early as H24, the divergence between GBPS340-treated and untreated ring-stage cultures was apparent, becoming particularly pronounced at H48. In contrast, for trophozoite-stage cultures, GBPS340 exposure altered the global transcriptomic profiles at H24 and H48, with separation dynamics that differed across time points. Overall, these findings indicate that the transcriptional response to GBPS340 depends on both the exposure duration and developmental stage, with a more pronounced divergence in ring-stage cultures at H48.

PCA plot based on DESeq2-normalized gene expression profiles across ring-stage and trophozoite-stage parasites at baseline (H0), 24 hours (H24), and 48 hours (H48) post-exposure. Each point represents an individual biological replicate. The first two principal components (PC1 and PC2) are shown. The dashed lines are shown for visual guidance.

GBPS340-treated ring-stage parasites showed marked separation from untreated controls at H24 (orange open circles), whereas at H48 (green open circles), their transcriptional profiles shifted toward those of untreated H24 controls (orange filled circles) and H0 trophozoites (blue squares), which is consistent with delayed transcriptional progression.

Differential gene expression induced by GBPS340.

As a first step, a comparative analysis of ring- and trophozoite-stage cultures at H0 identified 2,452 genes that were significantly differentially expressed between the two developmental stages, reflecting the parasite's developmental transcriptional program (Additional file 3). Because these stage-dependent transcriptional differences could confound the identification of treatment-induced changes, these genes were excluded from subsequent analyses to focus specifically on the transcriptional response to GBPS340.

In ring-stage cultures, GBPS340 exposure resulted in the identification of 622 differentially expressed genes (DEGs) at H24, 253 of which lacked functional annotation (Additional file 4). At H48, 600 DEGs were identified, including 241 unannotated genes. At both time points, the majority of DEGs were downregulated upon GBPS340 exposure (Fig. 9A). The hierarchical clustering dendrograms show consistent clustering of biological triplicates (#1, #2, and #3) by treatment condition (control vs. GBPS340) and time point (H0, H24, and H48) (Fig. 9B). This finding supports the reproducibility of the transcriptomic profiles, which is consistent with the PCA results. Furthermore, hierarchical clustering of transcriptomic profiles revealed that H24 cultures treated with GBPS340 clustered more closely with their untreated counterparts than with H0 cultures. In contrast, at H48, untreated cultures clustered more closely with H0 cultures than with their GBPS340-treated counterparts.

In trophozoite-stage cultures, GBPS340 exposure was associated with 86 DEGs at H24, including 37 genes with no currently annotated function. At H48, the transcriptional response was markedly reduced, with only three DEGs identified (Additional file 4).

Gene Ontology enrichment analysis.

GO enrichment analysis of the GBPS340-treated ring-stage transcriptome revealed enrichment of invasion-related categories among the downregulated transcripts (Additional file 5, Fig. 9C). At H24, genes annotated as belonging to the inner membrane pellicle complex (GO:0070258) were significantly enriched among the downregulated transcripts (adj. $P = 4.21 \times 10^{-5}$). The core components of the glideosome, including gliding-associated protein (GAP)40, GAP50, and the glideosome-associated protein with multiple membrane spans (GAPM)1–3 family, were among the most strongly downregulated genes ($\log_2FC$ ranging from -2.46 to -3.46). At H48, the inner membrane pellicle complex remained the most enriched category (adj. $P = 3.95 \times 10^{-5}$), encompassing genes encoding photosensitized INA-labelled protein 1 (PhIL1)-interacting proteins (PIP)2 and PIP3, while two additional invasion-related terms were also significantly enriched: cell surface (GO:0009986) (adj. $P = 3.95 \times 10^{-5}$) and entry into host (GO:0044409) (adj. $P = 2.00 \times 10^{-3}$).

The genes assigned to these categories included rhoptry proteins, such as high-molecular-weight rhoptry proteins 2/3 and rhoptry neck protein 3; the apical asparagine-rich protein AARP; and the motor-associated proteins actin I and profilin, which were markedly downregulated ($\log_2FC$ ranging from -2.90 to -4.30). Several 6-cysteine family members (P36 and P41) and regulatory proteins involved in motility, including calmodulin, calcineurin subunit B, and protein phosphatase PP1, were also downregulated. Conversely, a small number of surface antigens, including surface-associated interspersed protein (SURFIN) family members and reticulocyte-binding protein 2 homologue b, were upregulated at H48 within the cell surface category, whereas *Plasmodium* Helical Interspersed Subtelomeric (PHIST) family exported proteins were upregulated at H24 within the host cell category. No GO terms were significantly enriched in GBPS340-treated trophozoite-stage cultures at either H24 or H48.

Furthermore, as microscopic examination revealed an apparent absence of hemozoin in GBPS340-exposed parasites, GO terms related to hemoglobin catabolism (GO:0042540) and heme detoxification (GO:0042167) were also examined. Among the seven genes assigned to the GO term GO:0042540, only PF3D7_1407900, encoding plasmepsin I, and PF3D7_1458000, encoding cysteine proteinase falcipain 1, were significantly differentially expressed in the presence of GBPS340. Similarly, among the two genes assigned to the GO term GO:0042167, only PF3D7_0831800, encoding histidine-rich protein II, was significantly downregulated following GBPS340 exposure. (Additional file 6).

Discussion

The work presented herein is part of an ongoing research program aimed at elucidating the mechanism of action of GBPS340 using complementary phenotypic and transcriptomic approaches. Microscopic analysis was used to characterize alterations in parasite development and stage distribution following GBPS340 exposure, whereas transcriptomic profiling was performed to identify the molecular pathways potentially underlying these phenotypic effects. Ring- and trophozoite-stage parasites were investigated separately to determine whether the response to GBPS340 depended on the developmental stage at the time of drug

exposure. This experimental design therefore enabled us to distinguish stage-dependent effects from more general responses to drug exposure.

Although GBPS340 is a structural analogue of mefloquine, this study aimed primarily to characterize its stage- and time-dependent phenotypic and transcriptional effects rather than to assess its similarity to mefloquine or establish a shared mechanism of action. Direct comparative studies are needed to determine whether the structural similarities between the two compounds are reflected in their transcriptional responses and molecular mechanisms. Importantly, GBPS340 retains activity against mefloquine-resistant parasites, including the Dd2 strain, with an IC_{50} of 19.9 ± 9.4 nM [14]. Although these findings do not establish distinct mechanisms of action, they suggest that the two compounds may affect parasite development through different molecular pathways. This observation is consistent with the limited cross-resistance reported between the two compounds and further supports the need for direct comparative studies.

The experimental design also incorporated a full intraerythrocytic developmental cycle between sorbitol synchronization and the initiation of GBPS340 exposure. This approach minimized potential confounding effects of the synchronization procedure and reduced the likelihood that the phenotypic or transcriptional changes observed were attributable to residual effects of sorbitol treatment rather than to GBPS340 exposure.

GBPS340 markedly impaired intraerythrocytic parasite maturation, with the magnitude and timing of the effects depending on the developmental stage at which treatment was initiated. In ring-stage cultures, GBPS340 significantly reduced normalized parasitemia at both H24 and H48 and caused a pronounced delay in progression from rings to trophozoites at H24. This was followed by impaired progression through schizogony and failure to establish a new ring-stage population at H48. A comparable delay in parasite maturation has been reported following mefloquine exposure, particularly when treatment is initiated at the ring stage, supporting the hypothesis that antimalarial activity may involve disruption of developmental progression rather than immediate parasite death or complete developmental arrest [28]. More broadly, stage-dependent differences in antimalarial activity have been reported for several compounds, emphasizing that parasite susceptibility can vary substantially throughout the intraerythrocytic cycle [29].

The developmental delay observed following GBPS340 exposure was accompanied by a progressive accumulation of pyknotic forms, suggesting that impaired maturation was followed by a gradual loss of parasite viability rather than immediate and complete developmental arrest. Morphological abnormalities, including cytoplasmic vacuolization and fragmentation, schizont disorganization, and the apparent absence of hemozoin, further support the profound disruption of parasite development and viability. However, such morphological features are not specific to GBPS340 and have been described following exposure to other antimalarial compounds [30, 31].

In trophozoite-stage cultures, GBPS340 also reduced normalized parasitemia, although this effect was less pronounced and/or delayed than that observed following ring-stage exposure. At H24, treatment resulted in an altered stage distribution characterized by an accumulation of trophozoites, whereas this difference was no longer apparent at H48. Thus, GBPS340 was active when exposure was initiated at either stage, but

the magnitude and temporal profile of the response differed according to the developmental stage at drug exposure.

Overall, these findings argue against strict stage specificity while indicating marked stage-dependent susceptibility to GBPS340, with early intraerythrocytic stages appearing particularly sensitive. Taken together, the phenotypic data support a model in which GBPS340 disrupts parasite maturation early in the intraerythrocytic cycle, with subsequent consequences for schizogony, parasite viability, and establishment of the next ring-stage population.

This stage-dependent phenotypic response was also reflected at the transcriptomic level. PCA highlighted the parasite developmental stage as a major source of transcriptional variation, which is consistent with the tightly regulated temporal cascade of gene expression throughout the intraerythrocytic developmental cycle of *P. falciparum* [32–35]. The transcriptomic profiles also varied according to sampling time, reflecting the extensive transcriptional changes associated with parasite maturation. The temporal progression observed in untreated cultures was consistent with the expected dynamics of the intraerythrocytic cycle and therefore validated the experimental design.

Against this developmental background, GBPS340 altered the normal transcriptional trajectory of the parasite. Following ring-stage exposure, a marked divergence was observed as early as H24 between treated and untreated cultures. These findings suggest that the compound triggered rapid and profound transcriptional perturbation in ring-stage parasites within 24 h of exposure. The treated cultures continued to diverge from their untreated counterparts, with the most pronounced differences observed at H48. Rather than following the expected transcriptional trajectory through the trophozoite and schizont stages and subsequent erythrocyte reinvasion, GBPS340-treated parasites retained transcriptional features characteristic of earlier developmental stages. This pattern is consistent with the phenotypic evidence of delayed maturation and suggests that prolonged GBPS340 exposure progressively disrupts the transcriptional progression associated with the intraerythrocytic cycle. By contrast, trophozoite-stage exposure resulted in a substantially weaker transcriptional response, which is consistent with the more limited phenotypic effect observed in these cultures. Thus, the transcriptional response to GBPS340 appears to depend not only on exposure duration but also on the developmental stage at which treatment is initiated.

This interpretation is further supported by previous studies showing that drug-induced transcriptional responses in *P. falciparum* can be strongly influenced by parasite developmental stage. In particular, mefloquine exposure has been shown to delay parasite maturation and consequently alter the expression of stage-regulated genes [28]. More generally, comparative transcriptomic analyses have established that PCA can be used to identify similarities and differences between the responses induced by antimalarial compounds and to generate hypotheses regarding shared cellular effects [36].

Because parasite developmental stage is a major determinant of transcriptional variation [32–35], genes showing significant differential expression between ring- and trophozoite-stage cultures at H0 were excluded from subsequent analyses. This strategy was intended to reduce the confounding effect of stage-dependent transcription and to facilitate the identification of transcriptional changes associated with

GBPS340 exposure. However, this filtering step may also have removed genes whose expression was affected indirectly by GBPS340 through altered parasite progression. The resulting analysis should therefore be interpreted as a conservative approach to identifying drug-associated transcriptional changes rather than as a complete representation of the transcriptional consequences of treatment. Despite this limitation, GBPS340 induced extensive transcriptional alterations in ring-stage parasites, with more than 600 genes differentially expressed at both H24 and H48, indicating that its effect was sustained rather than restricted to an early transient response.

The progressive transcriptional divergence observed following ring-stage exposure was consistent with the phenotypic evidence of impaired maturation, reduced parasitemia, and accumulation of pyknotic forms. These findings suggest that GBPS340 induces a progressive disruption of the intraerythrocytic developmental cycle rather than an immediate, generalized transcriptional response to drug exposure. Conversely, the comparatively limited transcriptional response following trophozoite-stage exposure reinforces the stage-dependent nature of the response to GBPS340. Taken together, the PCA, clustering, and differential expression analyses support a dynamic effect of GBPS340 on parasite gene expression that varies with both the duration of exposure and the developmental stage at which treatment is initiated.

The enrichment pattern revealed a coherent disruption of parasite motility and invasion-related functions. In particular, the coordinated downregulation of genes encoding components of the inner membrane complex, such as GAP40, GAP50, and GAPM proteins, as well as components of the glideosome—a multiprotein complex located between the parasite plasma membrane and the inner membrane complex that is essential for parasite motility and erythrocyte invasion [37, 38]—suggests a broad alteration of the molecular machinery underlying parasite motility and host–cell invasion following GBPS340 exposure. Previous studies have highlighted the potential of this pathway as a promising target for antimalarial drug development. Moussaoui et al. demonstrated that their synthetic antimalarial compound KNX-002 inhibits the ATPase activity of myosin A, thereby disrupting glideosome function [39]. Moreover, Dans et al. identified sulfonylpiperazine compounds, including MMV020291, that prevent *P. falciparum* erythrocyte invasion by interfering with actin-1 and profilin dynamics, thereby disrupting the polymerization processes required for parasite motility and invasion [40]. The potential impairment of the molecular machinery underlying parasite motility and erythrocyte invasion is further supported by the altered expression of PhIL1-interacting proteins, including PIP2 and PIP3. The PhIL1-associated complex is localized to the inner membrane complex and contributes to merozoite reorientation and invasion of host erythrocytes [41]. The marked repression of PIP2 and PIP3 following GBPS340 exposure is therefore consistent with potential disruption of the processes required for efficient merozoite motility and erythrocyte invasion. Interestingly, PIP1 was significantly upregulated, in contrast to PIP2 and PIP3. Although the biological significance of this differential regulation remains unclear, it may reflect partial remodelling or compensatory regulation of the PhIL1-associated complex. Similarly, the concomitant downregulation of rhoptry and actin-associated proteins is consistent with a potential disruption of the molecular processes underlying merozoite motility and host-cell invasion, in which rhoptry secretion and actin–myosin-dependent motility play critical roles. [38, 42]. Several 6-cysteine proteins, including P36 and P41, were also markedly downregulated following GBPS340 exposure, whereas P230p was upregulated, indicating a heterogeneous response within this protein family. Given the strong stage-dependent expression of 6-Cys proteins and their roles in parasite

development, host–cell interactions, and transmission [43], their differential expression may primarily reflect the developmental perturbation induced by GBPS340 rather than a direct effect on the 6-Cys family. Nevertheless, their altered expression is consistent with the broader disruption of parasite surface-associated functions observed following treatment. The altered expression of a limited number of surface antigens and PHIST family proteins may similarly reflect changes in parasite–host interactions or compensatory responses to cellular stress rather than direct effects on the invasion machinery. PHIST proteins constitute a large family of exported *P. falciparum* proteins involved in remodelling infected erythrocytes and interacting with host-cell cytoskeletal components and parasite virulence-associated proteins [44, 45]. The differential expression of these genes following GBPS340 exposure may therefore reflect broader alterations in host-cell remodelling associated with parasite developmental disruption. Taken together, the GO enrichment results revealed a coherent pattern of transcriptional alterations affecting parasite motility and invasion-related functions. These changes could contribute to impaired parasite egress and/or invasion and may therefore be related to the reduced parasitemia observed following GBPS340 exposure. However, because the transcriptomic analysis was performed on cultures in which parasite development was already perturbed, these changes should be interpreted as molecular correlates of the GBPS340-induced phenotype rather than as evidence that the invasion machinery constitutes a direct molecular target of the compound. Functional studies will be needed to determine whether these transcriptional alterations translate into measurable defects in merozoite motility, egress, or erythrocyte invasion.

GBPS340 is a mefloquine-derived compound belonging to the arylaminoalcohol family. Although the primary molecular targets proposed for these antimalarials are predominantly cytosolic, interference with hemozoin formation has also been reported [46]. It was therefore relevant to investigate whether GBPS340 might affect this process, particularly in view of the microscopic observations suggesting an absence of hemozoin following treatment. However, no significant enrichment was detected for GO terms associated with hemoglobin catabolism or heme detoxification. Nevertheless, three individual genes involved in these processes, encoding plasmepsin I, falcipain 1 and histidine-rich protein II, were differentially expressed following GBPS340 exposure. These isolated transcriptional changes do not support major transcriptional alterations in pathways associated with heme metabolism, although the posttranscriptional or metabolic effects of GBPS340 on hemozoin formation cannot be excluded. Whether the observed absence of hemozoin reflects a direct effect on heme detoxification or is secondary to the broader disruption of parasite development remains to be determined. A further limitation of the present study is that GBPS340 exposure was initiated at the ring or trophozoite stage, whereas schizont-stage parasites were not directly investigated. Thus, although the present results indicate a marked disruption of parasite maturation and progression through schizogony, they do not establish whether schizonts display a distinct susceptibility to GBPS340 or whether the observed effects on late-stage development are secondary to the earlier perturbation of the intraerythrocytic cycle. Direct exposure of synchronized schizonts would therefore be necessary to define the stage-specific activity of GBPS340 more comprehensively.

Conclusions

Overall, the phenotypic and transcriptomic data converge on a model in which GBPS340 disrupts the normal progression of the *P. falciparum* intraerythrocytic developmental cycle. The magnitude and kinetics of this response depend on the developmental stage at which drug exposure begins, with ring-stage parasites showing the most pronounced phenotypic and transcriptional alterations. Prolonged exposure is associated with progressive developmental delay, impaired progression through schizogony, reduced parasite viability, and failure to establish the next ring-stage population. At the transcriptional level, GBPS340 induces broad changes in gene expression, with GO enrichment indicating altered parasite motility and invasion-related functions. These findings provide a basis for further investigation of the biological processes underlying the antimalarial activity of GBPS340. Complementary functional, proteomic, biochemical, and genetic approaches, including studies across additional parasite stages, will be needed to define the molecular targets, mechanism of action, and stage-specific activity profile of this compound. Together, these complementary approaches help refine our understanding of the GBPS340 mechanism of action and support its further development as a candidate antimalarial compound.

Abbreviations

DEGs	Differentially expressed genes
IC	Inhibitory concentration
GAP	Gliding-Associated Protein
GAPM	Glideosome-associated protein with multiple membrane spans
GO	Gene Ontology
PCA	Principal component analysis
PhiL1	Photosensitized INA-labelled protein 1
PIP	PhiL1-interacting protein
PHIST	<i>Plasmodium</i> Helical Interspersed Subtelomeric
SURFIN	Surface-associated interspersed protein

Declarations

Ethical approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare no competing interests.

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Author contributions

IBG curated and analysed the data, conducted the investigation, provided resources, validated the results, visualized the data, and wrote the original draft of the manuscript. YPL conducted the investigation, curated the data, provided resources, and validated the results. AA curated and formally analysed the data, contributed to the methodology, and provided resources. PA conducted the investigation and provided resources. RM provided resources. ADK, PS, YLG, CD and AT contributed to the conceptualization of the study. CD and AT contributed to the methodology and project administration. PS and AT supervised the work. AT obtained funding. ADK, PS, PA, AA, YLG, CD and AT reviewed and edited the manuscript. All the authors read and approved the final manuscript.

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Figures

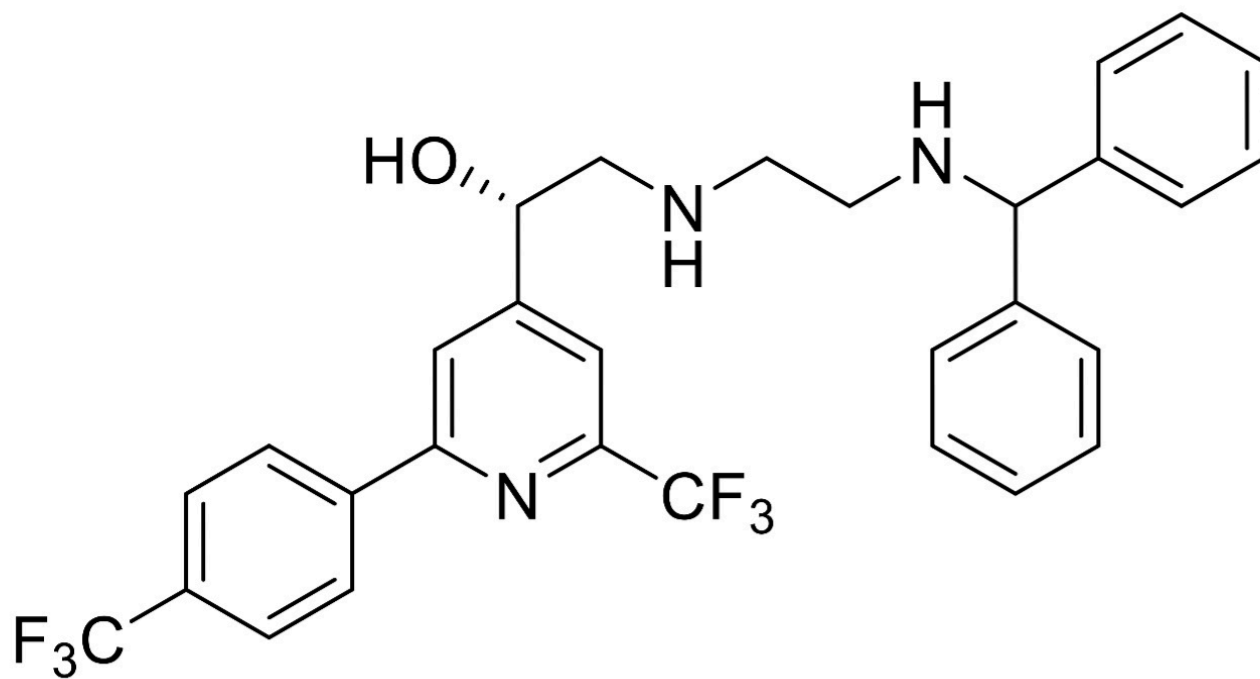


Figure 1

Chemical structure of GBPS340.

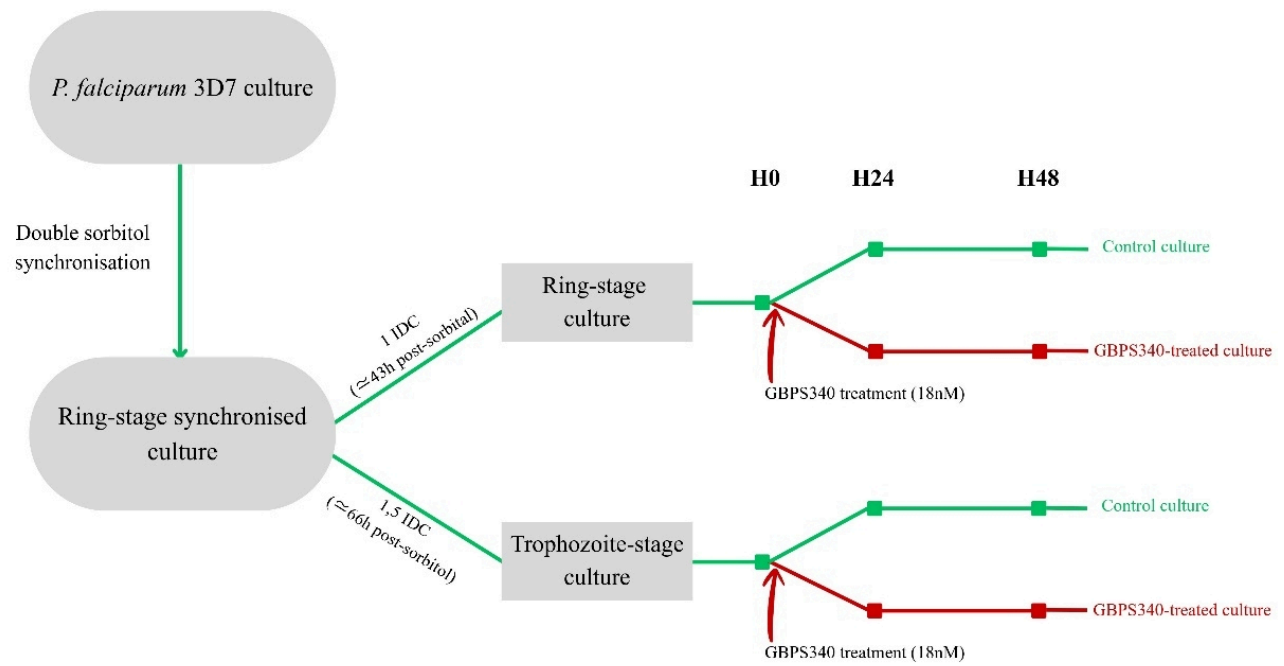


Figure 2

Overview of the experimental design

The protocol was performed in triplicate. Squares indicate 5-mL aliquot collection for thin blood smears and TRIzol™ preservation. IDC, intraerythrocytic developmental cycle. h, hours.

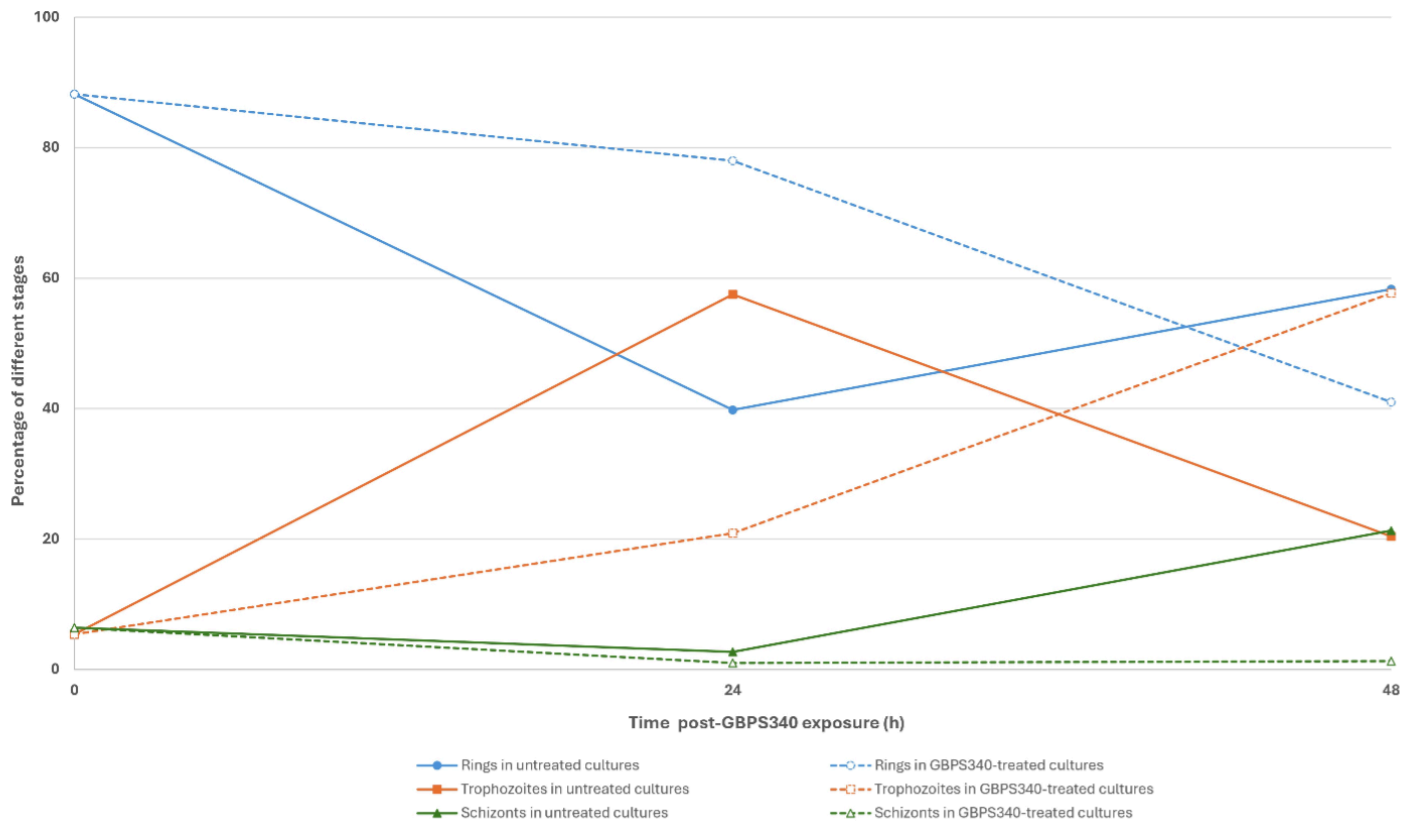


Figure 3

Dynamics of parasite stage distribution over time in GBPS340-treated and untreated ring-stage cultures.

The data represent the mean percentages from three biological replicates (see Table 1).

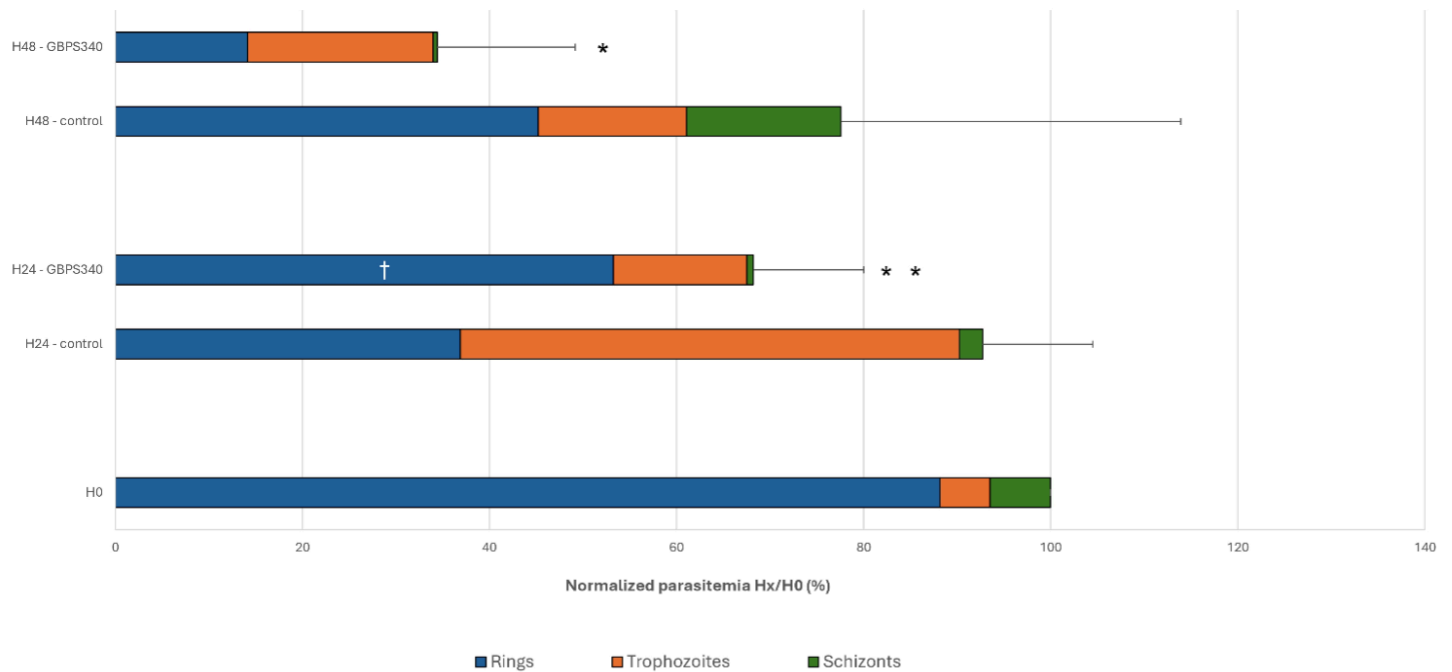


Figure 4

Normalized parasitaemiaratios and parasite stage distributions in GBPS340-treated and untreated ring-stage cultures.

Parasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%). The barlength represents the normalized parasitaemia ratio, whereas thesegments represent the relative contribution of each parasite stage. The valuesrepresent the means of four independent readings per smear from three biological replicates.

The normalized parasitaemiaratios differed significantly between GBPS340-treated and untreated cultures at H24 and H48 (one-sided paired Student’s t test; $p = 0.004^{**}$ and $p = 0.034^{*}$, respectively).

The proportions of ring-stage parasites differed significantly between GBPS340-treated and untreated control cultures at H24 (two-sided paired Student’s t test, $p = 0.029 \uparrow$).

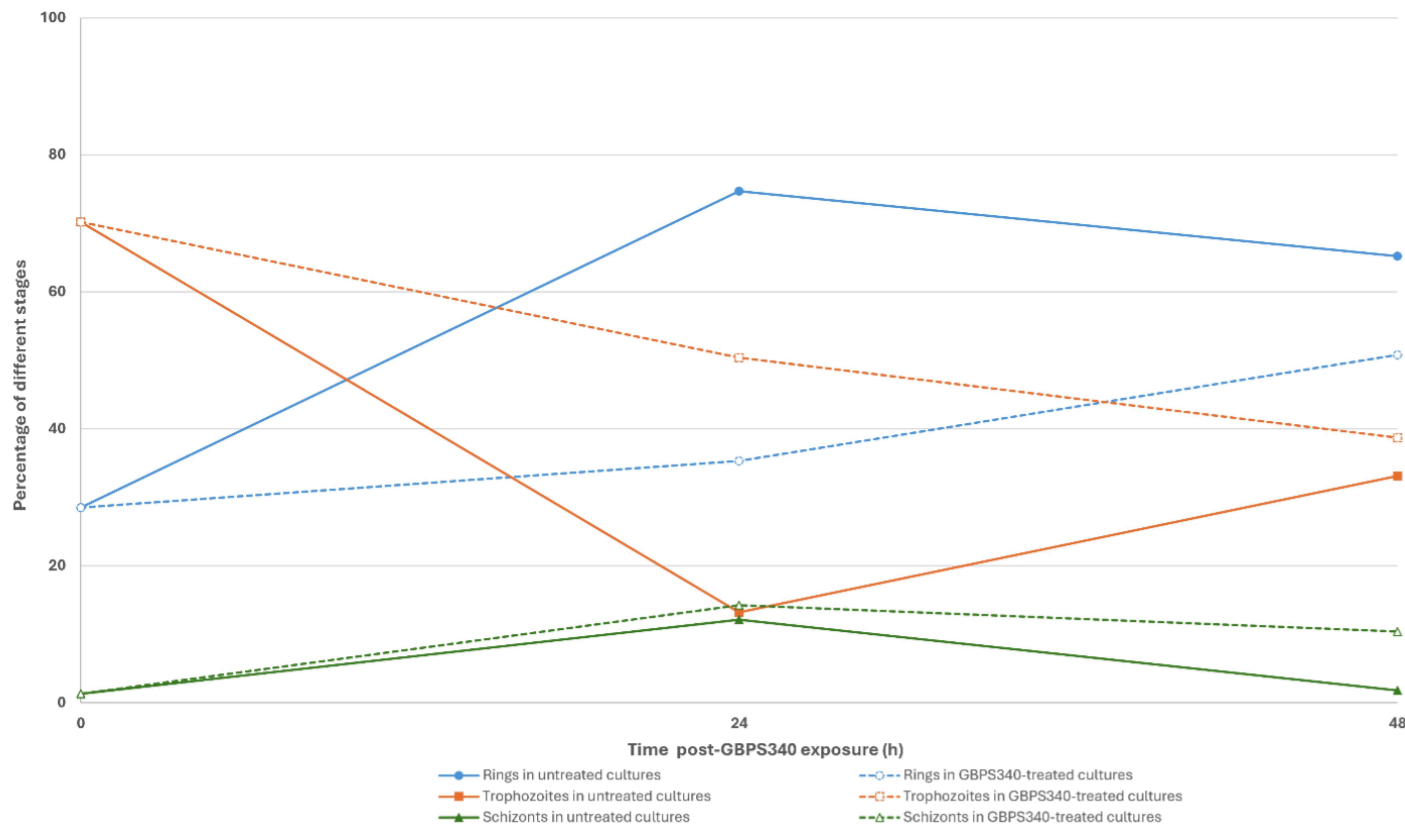


Figure 5

Dynamics of parasite stage distribution over time in GBPS340-treated and untreated trophozoite-stage cultures.

The data represent the mean percentages from three biological replicates (see Table 2).

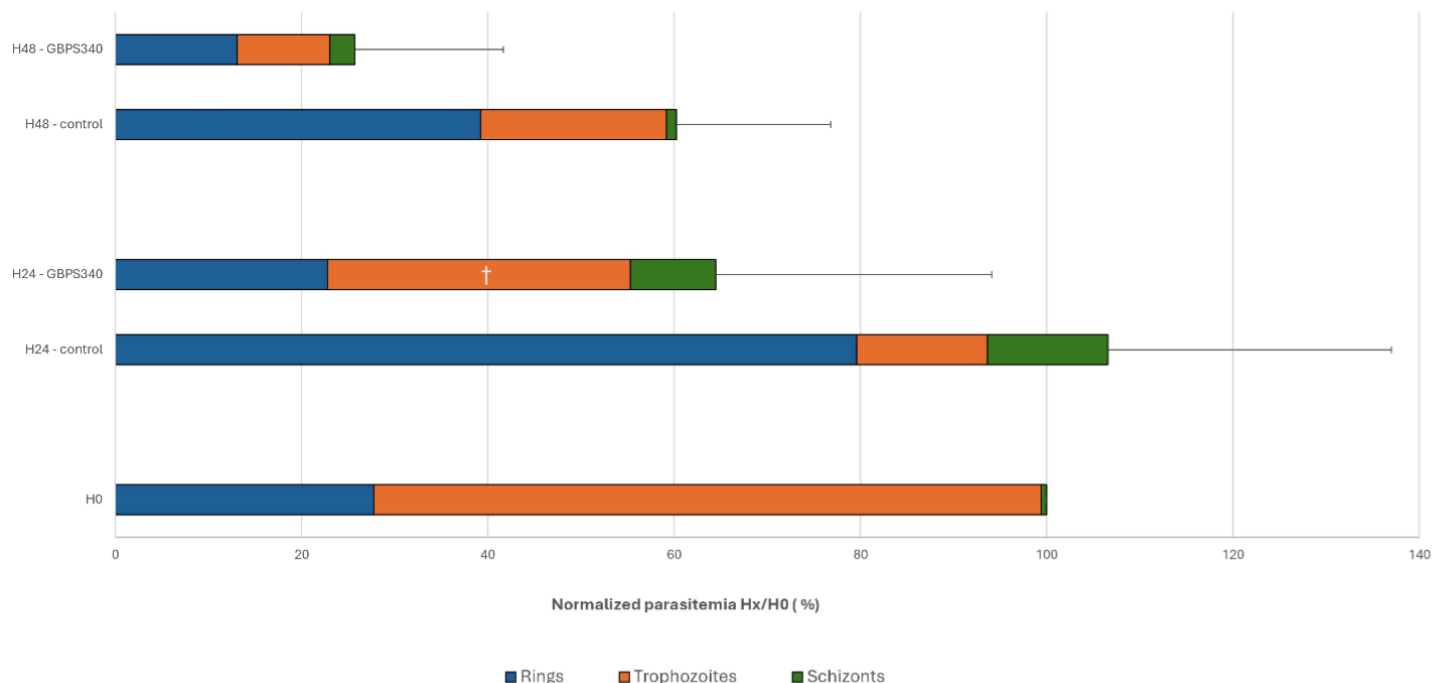


Figure 6

Normalized parasitaemia ratios and parasite stage distributions in GBPS340-treated and untreated trophozoite-stage cultures.

Parasitemia at H24 and H48 was normalized to the corresponding H0 value and expressed as a ratio (%). The barlength represents the normalized parasitaemia ratio, whereas thesegments represent the relative contribution of each parasite stage. The valuesrepresent the means of four independent readings per smear from three biological replicates.

The proportions of trophozoite-stage parasites differed significantly between GBPS340-treated and untreated control cultures at H24 (two-sided paired Student's t test, $p = 0.031†$).

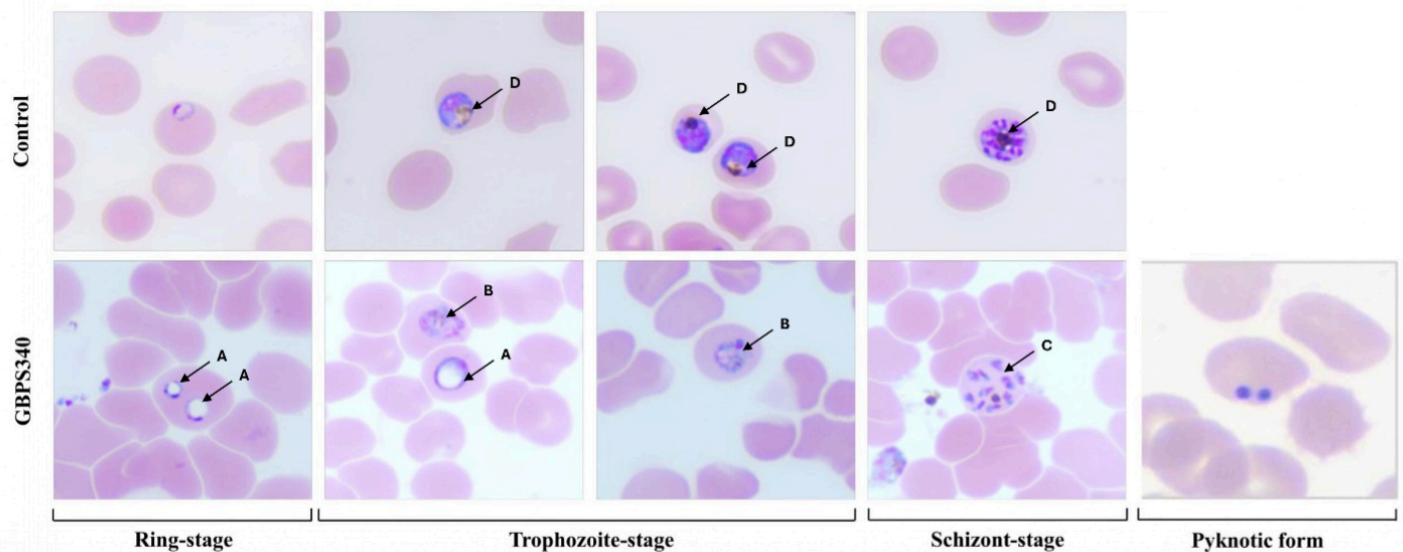


Figure 7

Effect of GBPS340 on parasite morphology.

Morphological alterations in parasites cultured in the presence of GBPS340 (bottom panel) compared with untreated parasites (top panel). A, cytoplasmic vacuolization; B, cytoplasmic fragmentation; C, scattering of merozoites throughout the cytoplasm; D, hemozoin. Note the absence of hemozoin in GBPS340-treated parasites.

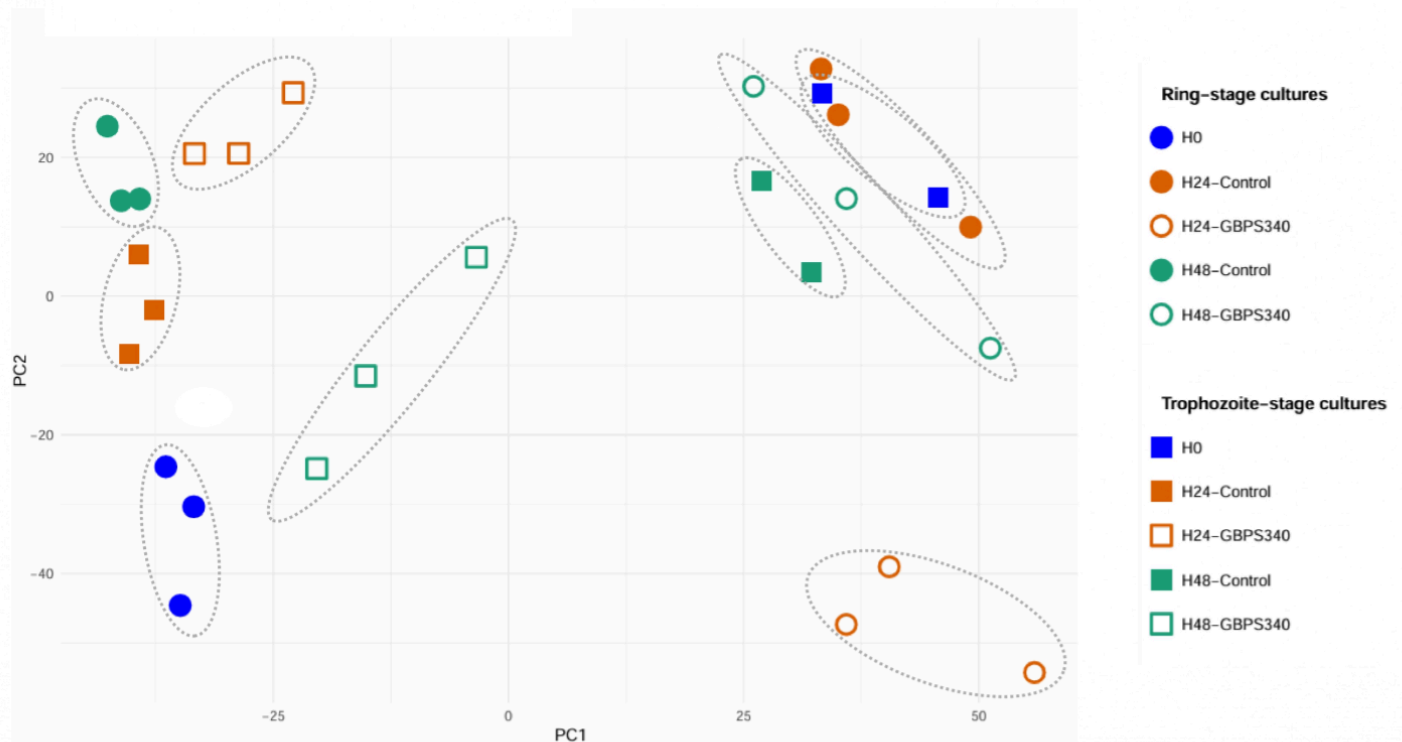


Figure 8

Principal component analysis of *Plasmodium falciparum* transcriptomes following GBPS340 exposure in ring- and trophozoite-stage cultures.

PCA plot based on DESeq2-normalized gene expression profiles across ring-stage and trophozoite-stage parasites at baseline (H0), 24 hours (H24), and 48 hours (H48) post-exposure. Each point represents an individual biological replicate. The first two principal components (PC1 and PC2) are shown. The dashed lines are shown for visual guidance.

GBPS340-treated ring-stage parasites showed marked separation from untreated controls at H24 (orange open circles), whereas at H48 (green open circles), their transcriptional profiles shifted toward those of untreated H24 controls (orange filled circles) and H0 trophozoites (blue squares), which is consistent with delayed transcriptional progression.

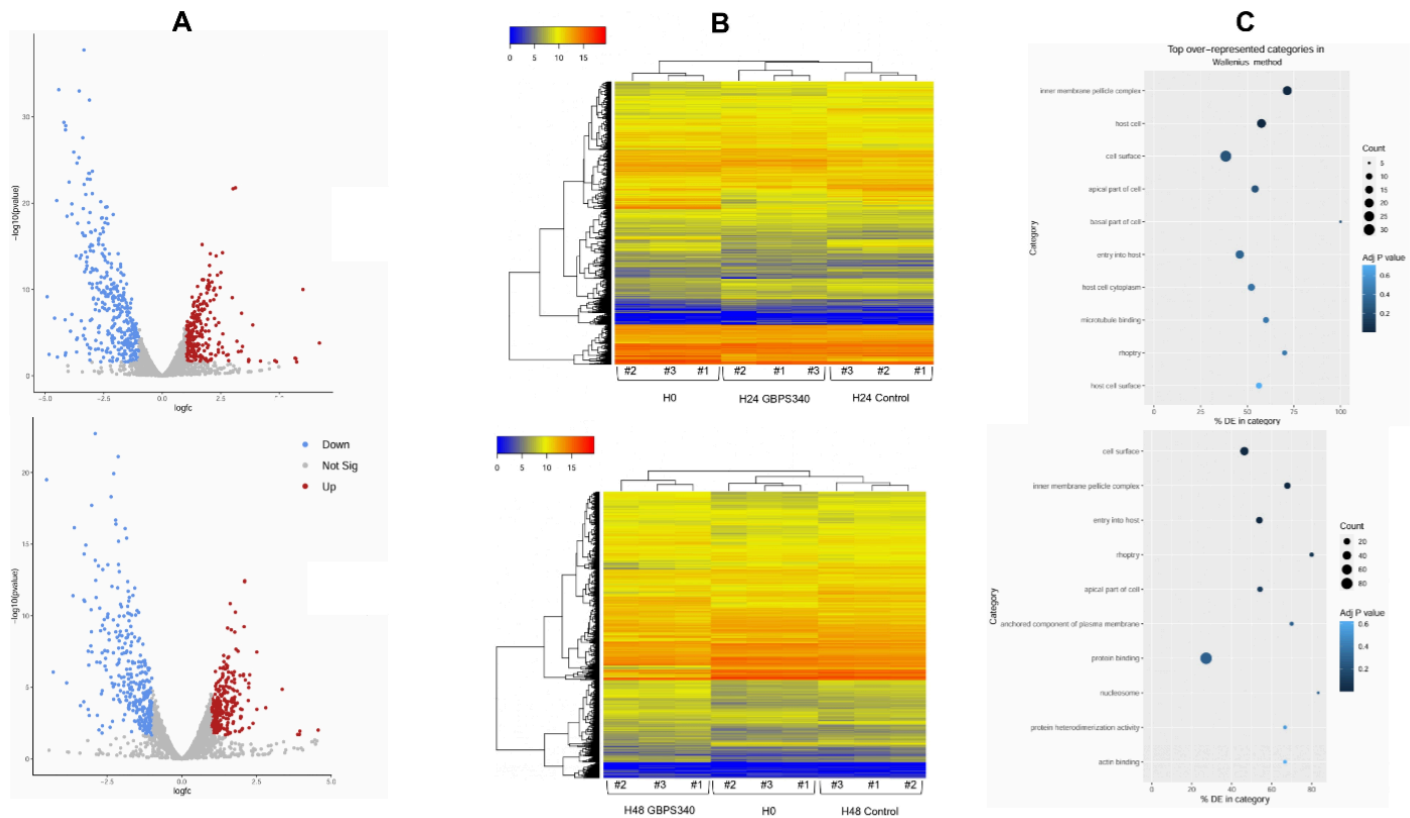


Figure 9

Transcriptomic profiling and Gene Ontology enrichment analysis of *Plasmodium falciparum* ring-stage parasites treated with GBPS340.

(A) Volcano plots showing differentially expressed genes between GBPS340-treated and control cultures at H24 (upper panel) and H48 (lower panel). Downregulated genes are shown in blue, upregulated genes in red, and genes with non-significant differential expression in gray.

(B) Hierarchical clustering heatmaps showing the expression profiles of differentially expressed genes across

biological replicates at H24 (upper panel) and H48 (lower panel). The H0 samples are shown as a reference for the initial ring-stage transcriptome. The biological replicates are numbered #1, #2, and #3. The colorscales represent the relative gene expression levels (blue to orange/red) and statistical significance ($-\log_{10}P$ -value or adjusted P -value) versus the fold change ($\log_2FC$).

(C) Gene Ontology enrichment analysis of differentially expressed genes using Wallenius' non-central hypergeometric test at H24 (upper panel) and H48 (lower panel). The dot size represents the number of genes associated with each GO category, and the dot position indicates the percentage of differentially expressed genes within each category. The color indicates the adjusted p value.

Supplementary Files

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