An Accessible 3D HepG2/C3A Liver Spheroid Model Supporting the Complete Intrahepatocytic Lifecycle of *Plasmodium falciparum.*

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Abstract

Current liver-stage *Plasmodium falciparum* models are complex, expensive, and largely inaccessible, hindering research progress. Here, we show that a 3D liver spheroid model grown from immortalized HepG2/C3A cells supports the complete intrahepatocytic lifecycle of *P. falciparum*. Our results demonstrate sporozoite infection, development of exoerythrocytic forms, and breakthrough infection into erythrocytes. The 3D-grown spheroid hepatocytes are structurally and functionally polarised, displaying enhanced albumin and urea production and increased expression of key metabolic enzymes, mimicking *in vivo* conditions – relative to 2D cultures. This accessible, reproducible model lowers barriers to malaria research, promoting advancements in fundamental biology and translational research.

Background

Malaria remains a global health emergency. Despite intensive efforts, the WHO's 2023 World malaria report estimated that the number of global malaria cases in 2022 remains stubbornly high at 249 million, exceeding pre-COVID-19 pandemic levels in 2019 [1].

Chemotherapy remains a cornerstone to the multifaceted approach for malaria control and elimination and whilst the past two decades has delivered a robust pipeline of promising candidate drugs and several new treatments that act on the asexual blood stages (categorised as target candidate profile 1 [TCP-1]), there has been little progress in the development of effective therapies active against liver-stage hypnozoites (*Plasmodium vivax* and *P. ovale*) or hepatic *P. falciparum* schizonts (TCP-3 and TCP-4, respectively [2]).

The establishment of a stable *in vitro* culture system for the asexual blood stages of *P. falciparum* [3] has been vital in unlocking our understanding of the intraerythrocytic stages of malaria infection and in advancing antimalarial drug discovery efforts for malaria treatment (TCP-1). Conversely, the relative paucity in our understanding of the biology of the hepatic stage of development and limited TCP3/4 pipeline has, in part, been attributed to the difficulty of establishing in vitro human liver stage malaria models, with current methods deemed expensive, laborious and impractical [2].

Initial studies attempting to establish stable in vitro models of malaria life stages using immortalized hepatoma cell lines, such as HepG2, HSS-102, and HC-04, achieved limited success and were unable to demonstrate breakthrough infection into erythrocytes. While these human hepatoma cell lines, allowed partial development of Plasmodium parasites, significant limitations persisted. For instance, HepG2-A16 cells could support sporozoite invasion but failed to sustain full parasite development. Similarly, HC-04 cells, though capable of supporting the liver stages of *P. falciparum* and *P. vivax*, exhibited reduced parasite viability and did not facilitate merozoite egress into erythrocytes due to their limited culture lifespan [4-6].

More sophisticated approaches have achieved greater success in developing *in vitro* models for malaria research. Techniques such as using induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (iHCLs), immortalised hepatocyte-like cell (imHC) lines, co-culture systems, 2D primary human hepatocyte (PHH) monolayer systems, and 3D cell cultures have shown considerable promise [6]. These advanced models have allowed large-scale drug screening campaigns [7, 8], and have improved our understanding of *Plasmodium* liver stage biology. However, challenges remain, including limited expression of drug metabolism enzymes, difficulty in maintaining cultures long-term, and low sporozoite infection rates. Additionally, these advanced technologies are not widely accessible, particularly to laboratories in malaria-endemic low- and middle-income countries (LMICs) that often face resource and capability constraints. This disparity highlights the need for more accessible and robust *in vitro* models to support global malaria research efforts.

Multiple studies have shown that immortalised hepatocarcinoma cell lines cultured in 3D have superior liver-specific functionality when compared to monolayer cultures [9]. A simple, high-throughput method of generating 3D cultures is through the creation of 'spheroids'. Unlike more complicated 3D cell culture techniques, spheroids are simple to generate, in-expensive to culture, reproducible and may be analysed using a variety of methods [9].

Here, we investigated whether a previously reported method of generating 3D liver spheroid from the immortalised C3A cell line, a derivative of HepG2 cells, displaying *in vivo*-like morphology and phenotype [10], could be used to support the complete intrahepatocytic lifecycle of Plasmodium. Data is presented that demonstrate the C3A liver spheroid model supports initial *P. falciparum* sporozoite infection, the development of exoerythrocytic forms (EEFs), and the subsequent egress of merozoites and breakthrough infection of erythrocytes.

These data are discussed in the context of a method that is reproducible, inexpensive and accessible.

Methods

HepG2/C3A cell culture

HepG2/C3A cells were maintained in EMEM (LGC standards, Middlesex, UK), supplemented with 10% FBS (Sigma, UK) and 1% penicillin/streptomycin (Fisher Scientific, UK) at 37°C with 5% CO_2. Cell culture medium was replaced every 3 to 4 days and cells were not allowed to exceed 80% confluency.

HepG2/C3A spheroid formation and growth

HepG2/C3A spheroids were grown using the liquid overlay technique, described elsewhere [10, 11]. Spheroid growth was monitored using a phase-contrast microscope (ECLIPSE TS100/100-F, Nikon) at 10×.

Quantification of protein, albumin, and urea production

Cell lysates were obtained by disrupting 2D monolayer cultures and HepG2/C3A spheroid cultures using lysis buffer (250mM sucrose, 50mM Tris-HCl pH7.4, 5mM $MgCl_2$, 1mM β-mercaptoethanol and 1% Triton X-100) and stored at -80°C. Protein concentrations from spheroid and monolayer cell lysates were quantified using Bio-Rad Protein Assay (Bio-Rad Laboratories), according to the manufacturer’s instructions.

Albumin and urea were quantified using the Albumin Human ELISA Kit (Abcam) and the Urea Assay Kit (Abcam), respectively, according to manufacturer's instructions. Supernatants for spheroid cultures were collected 4 days after media change and then every 4 days over 28 days. 2D monolayer supernatants were collected 4 days after media change only.

Measurement of CYP3A4 and CYP2D6 activity

CYP3A4 and CYP2D6 activity was measured using P450-GLO assay (Promega), according to manufacturer's instructions. Data was normalised based on the viabile cell mass to account for any differences between cultures, which was measured using CellTitre-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer’s instructions. Luminescence was measured using POLARstar OMEGA plate reader (BMG Labtech).

Immunofluorescence analysis of spheroids

Spheroids were fixed with 4% paraformaldehyde for 1h at 4°C, washed three times with PBS and permeabilised with 0.5% Triton X-100 in Tris-Buffered Saline with 0.05% Tween20 (TBST) overnight at 4°C. Spheroids were then blocked with 0.1% Triton X-100/5% BSA in TBST for 2h at room temperature, anti-*P. falciparum* GAPDH (1:100) and anti-*P. falciparum* HSP70 (1:200) was then added, diluted in 0.1% Triton X-100/1% BSA in TBST and incubated overnight at 4°C. Spheroids were washed three times with 1% Triton X-100 in TBST incubated with goat anti-rabbit-alexa fluor (AF)564 (HSP70) and goat anti-mouse- AF488 (GAPDH) (1:1000) or AF-488 (Pg-P) (1:1000) conjugated antibody in in 0.1% Triton X-100/5% BSA in TBST and incubated overnight at 4°C. DAPI (1:5000) and/or phalloidin (1:250) in 0.1% Triton X-100/5% BSA in TBST was added to the spheroids and incubated for 1h at room temperature in the absence of light. Finally, spheroids were mounted with Prolong Gold (Life Technologies) onto a glass microscope slide. Images were acquired at 40× using a Zeiss LSM880 confocal microscope (Zeiss, Germany).

Statistical analyses

Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, USA).

Infection of HepG2/C3A spheroids with *P.falciparum* NF54

P.falciparum NF54 parasites were maintained in continuous culture using established method [3]. Sexual stages of the parasites were cultured at 4% haematocrit in complete medium (RPMI 1640 with L-glutamine, 25mM HEPES pH7.4 (Gibco), 2g/L sodium bicarbonate, 50mg/ml hypoxanthine (Sigma) and 10% human heat-inactivated AB+ serum) from 0.5% mixed stage asexual cultures. Media was changed daily for 16-17 days and appearance of gametocyte stages was monitored every 48-72 h by microscopic analysis of thin blood smears [11].

Female *Anopheles coluzzii* mosquitoes aged between 5-7 days were deprived of sugar for 18-20h prior to infection. The standard membrane feeding assay was used to provide the mosquitoes with *P.falciparum* NF54 cultures containing stage V gametocytes for 30min [12]. Afterwards, engorged mosquitoes were incubated in insectary conditions and offered *ad libitum* 10% sucrose solution. On day 16 post-infection with *P. falciparum* NF54, mosquitoes were collected and washed with 70% v/v ethanol and dissected to obtain salivary glands. Salivary glands were crushed in PBS and sporozoites stored in liquid nitrogen until required.

Each HepG2/C3A spheroid, grown as described above, were infected with *P. falciparum* NF54 $25x10^3$ sporozoites and incubated at 37°C with 5% CO_2 for 6 days. Human O+ red blood cells were then introduced to infected HepG2/C3A cultures at a final haematocrit of 0.1% in complete EMEM and incubated at 37°C with 5% CO_2. 50 µL samples were taken from each well daily for 5 days and gassed with 3% O_2, 4% CO_2 and 93% N_2 and incubated at 37°C. To monitor asexual stage development, thin blood smears were prepared from the blood sample plate and stained with 10% Giemsa. Slides were examined under oil immersion at 100× using a Zeiss LSM880 confocal microscope (Zeiss, Germany).

Results

HepG2/C3A spheroid formation and spheroid structure

HepG2/C3A spheroids were grown and their structure and function assessed ahead of use in the *Plasmodium* infection model. Spheroids aggregated into a single structure between day 3 and day 5 and their diameter increased between day 5 and day 25 (Supplementary Figure 1). Spheroid polarisation and localisation of hepatocyte transporters was assessed by phalloidin staining of F-actin filaments and immunofluorescent imaging of Pg-P. At 4 days, F-actin filaments started to form between cells within the spheroid and at day 15 (Figure 1A). At 4 and 15 days secondary Pg-P structures were visible alongside F-actin filaments (Figure 1A).

Confirmation of liver-like function in spheroids

Spheroid albumin and urea production was quantified over 28 days and compared with 2D monolayer albumin and urea production at day 4 (Figure 1 B and C). Albumin production from the spheroids steadily increased over time from 1.56 ± 0.49 ng/mg at day 4 to 11.7 ± 1.03 ng/mg at day 28. Spheroid albumin production at day 24 was significantly higher than 2D monolayer albumin production at day 4 (Figure 1B). Similarly, urea levels also steadily increased from 0.7 ± 0.29 nmol/mg cell protein at day 4 to 1.7 ± 0.30 nmol/mg at day 28, with Similarly, spheroid albumin production from day 16 onwards was significantly higher than 2D monolayer albumin production at day 4 (Figure 1C). CYP3A4 activity was 7-fold higher and CYP2D6 10-fold greater in 3D spheroids (Figure 1D; 2878 ± 2.6 RUL/10^6 cells, 1315 ± 2.0 RUL/10^6 cells, respectively) compared to 2D culture (Figure 1D; 405 ± 0.7 RUL/10^6 cells, 122 ± 1.03 RUL/10^6 cell respectively).

HepG2/C3A spheroid infection with *P. falciparum* NF54 sporozoites and *Plasmodium* asexual blood-stage breakthrough from HepG2/C3A spheroids to human erythrocytes

Spheroids were infected with *P. falciparum* NF54 at an MOI of 4 and incubated for 4 days. Spheroids were then fixed and stained with antibodies to the sporozoite liver stage proteins GAPDH and HSP70. The presence of *P. falciparum* infection during the exoerythrocytic

(hepatic) stages was confirmed by antibody immunofluorescence staining of GAPDH (Figure 2A and 2B) and infection burden of liver spheroids quantified as a percentage of infected hepatocytes relative to uninfected hepatocytes (Supplementary Figure.2).

Following successful infection of HepG2/C3A spheroids by *P. falciparum* sporozoites, human erythrocytes were introduced to n=5 infected spheroids 6 days post-infection and incubated for 8 days. A thin blood film was then prepared, and erythrocytes were observed to contain ring staged parasites (Figure 2C). After 12 days, another thin blood film was then prepared – this blood film contained a greater proportion of mature gametocytes and schizonts (Figure 2D). Blood stage breakthrough was observed from all five infected spheroids.

Discussion

The HepG2/C3A liver spheroid model developed here represents a low-cost, accessible means to explore the complete intrahepatocytic lifecycle of *P. falciparum*, addressing significant limitations faced by many laboratories in adopting advanced *in vitro* models for malaria research.

3D cell culture technologies have increasingly been recognised for their ability to more accurately model *in vivo* cellular *behaviour in* vitro relative to 2D cultures. The hepatocytes within the 3D-grown HepG2/C3A spheroid were shown to be structurally and functionally polarised, recapitulating the *in vivo* condition [13]. Secondary structures composed of actin were observed forming enhanced networks throughout the spheroids, that were confirmed as Pg-P, transporters localised to the canalicular membrane of hepatocytes, as described elsewhere [14]. Further, HepG2/C3A spheroids were shown to exhibit enhanced CYP metabolism, as exemplified by raised CYP 3A4 and CYP 2D6 expression, as well as enhanced synthesis and secretion of albumin and urea. These data support previous observations [14] and confirm that cells cultured in 3D have superior functionality to those cultured in 2D [9, 15].

Previous attempts to create stable *in vitro* models of malaria life stages using immortalised hepatoma cell lines, such as HepG2, have had limited success and failed to demonstrate breakthrough infection into erythrocytes [4-6]. However, the structural and functional attributes of 3D-grown HepG2/C3A cells appear to offer the necessary physiological conditions for *P. falciparum* intrahepatocytic development. Our findings suggest these conditions are essential for completing the parasite's lifecycle within hepatic cells.

The current study used a high MOI of 4 to obtain spheroid liver stage infection, but future studies are required to optimise the precise methodology and establish limits of detection and minimum MOI required. Further investigation is also needed to identify the specific conditions that make 3D-grown HepG2/C3A cells permissive to *P. falciparum* development. However, one recent global gene expression study of human malaria parasite liver stages revealed significantly increased expression of genes in central metabolic pathways and redox homeostasis [16]. It is noteworthy that previous studies have shown that HepG2/C3A 3D spheroids replicate the liver's spatiotemporal oxygen and nutrient zonation [9, 14]. It will be important to determine whether the critical contributing factor to the permissiveness of the 3D spheroid model to intrahepatocytic parasite development is that the specific nutrient and redox needs of the parasite can be met by the unique spatiotemporal microenvironment of the spheroid.

In summary, the HepG2/C3A liver spheroid model lowers barriers to malaria research by offering a simpler and more accessible method to study the hepatic stages of *P. falciparum* infection. This model addresses a critical gap in malaria research tools and can significantly enhance our ability to study fundamental aspects of Plasmodium biology and develop effective liver-stage interventions. This approach not only broadens the scope of research possibilities but also provides a platform for high-throughput screening of antimalarial drugs and vaccines, promoting advancements in both fundamental biology and translational research.

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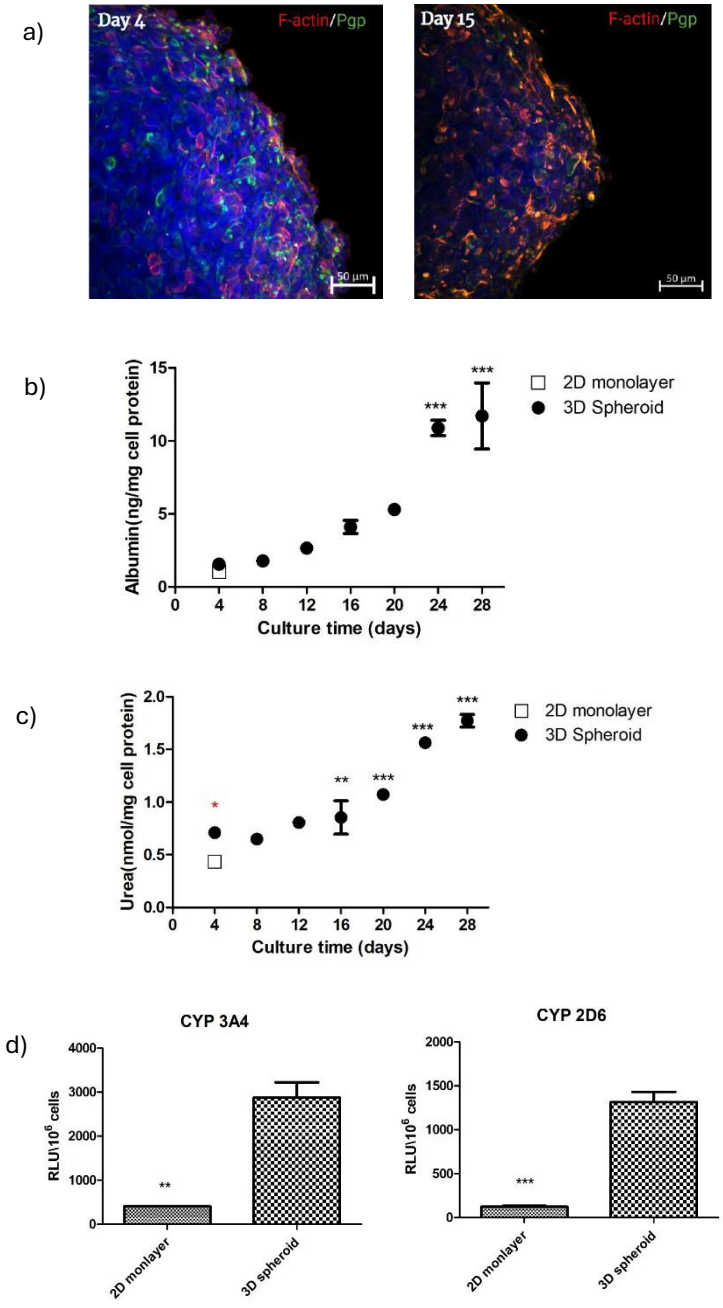

Figure 1. Spheroid formation and characterisation. a) Secondary structure formation. Phalloidin (red) to stain F-actin and immunofluorescent staining was performed for the canalicular transporter Pg-P (green) with Hoechst (blue) to stain the nuclei on Day 4 and 15. Quantification of b) Albumin and c) Urea from HepG2/C3A grown in 2D and as 3D spheroids supernatants, normalised by mg cell protein. Data analysed by One-way ANOVA, with Dunnett's multiple comparison d) Comparison of CYP 3A4 and CYP 2D6 expression from HepG2/C3A grown in 2D and as 3D spheroids, normalised by cell number, data analysed by unpaired t-test. All Data represents the mean ± SEM of three independent experiments, performed with technical replicates. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

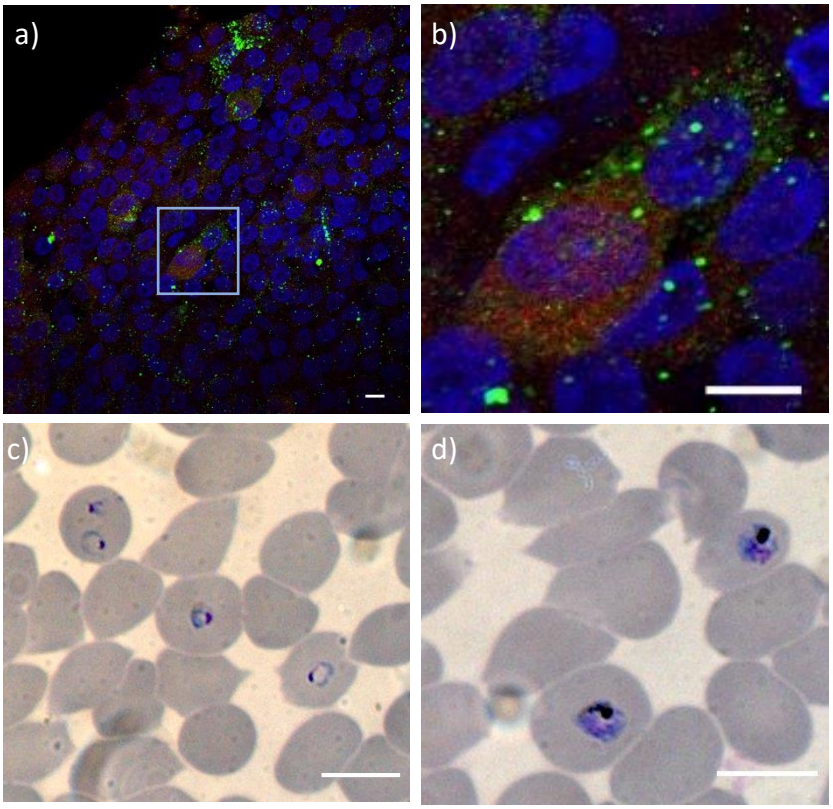

Figure 2. Assessment of HepG2/C3A 3D spheroid culture infection by *P.falciparum* NF54 sporozoites and asexual blood stage breakthrough from infected HepG2/C3A 3D spheroids. a) Liver stage parasites were detected by immunofluorescence on day 4 post-infection and stained with antibodies towards GAPDH (green) and HSP70 (red), cell nuclei are visualised with DAPI stain (blue). Region of interest (ROI) is shown in blue box. b) Close-up of ROI showing staining of GAPDH (green) and HSP70 (red) surrounding cell nuclei (blue).c) Giemsa-stained thin blood films were performed on day 8 and d) day 12 post-infection with *P.falciparum* NF54 n=5 HepG2/C3A spheroids. On day 8 ring stage parasites were observed (c) and by day 12 trophozoite/early schizont parasites could also be detected (d). Liver stage images are representative of n = 2 biological replicates conducted in duplicate. White scale bar represents 10 µm for all images.

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