A robust in vitro liver cell model for long-term study of Plasmodium vivax hypnozoites

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Research

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Abstract

Background Plasmodium vivax is the most prevalent species of human malaria parasites, affecting nearly half of the world's population. Eradication of malaria is difficult because of the ability of hypnozoite, the dormant liver-stage form of Plasmodium vivax to tolerate most antimalarials, and later to cause relapse in patients. Research efforts to better understand the biology of P. vivax hypnozoite and design relapse prevention strategies have been hampered by the lack of a robust and reliable model for in vitro culture of liver-stage parasites, including hypnozoites. Experimentally, this form has previously been studied in the primary hepatocyte cell model, but this has some limitations due to its high cost and failures when used for long-term biological studies and high throughput drug screening.

Methods Here, an immortalized human hepatocyte-like cell line (imHC) was infected with P. vivax sporozoites. The infected cultures were maintained for up to 28 days to obtain small liver-stage forms. A novel system to quickly enrich pure small liver-stage forms in culture was also developed. The susceptibility of the enriched small liver-stage forms, presumably hypnozoites, to known antimalarial drugs, atovaquone, primaquine, and tafenoquine, was examined.

Results Small liver-stage forms of P. vivax could persist in long-term imHCs culture. These small forms had a single nucleus and could be enriched by treatment with DSM265, a compound active against growing parasites but not hypnozoites. Resistance to inhibition by atovaquone was consistent with the interpretation that the enriched small parasites represent hypnozoites. Primaquine and tafenoquine displayed poor activity at clearing these putative hypnozoites in vitro.

Conclusions A robust cell-based model, with well-defined dormant liver-stage parasites in long-term stable human liver cells, allows us to follow hypnozoite formation and eventual reactivation to dividing parasites. This model is also well-suited to test radical cure efficacy of compounds against P. vivax hypnozoites. Thus, it will be of value for understanding hypnozoite biology and for drug discovery to eliminate dormant malaria.

Keywords Malaria, Plasmodium vivax, Sporozoite, Liver stage, Hypnozoite, imHCs

Background

Plasmodium vivax is the most widespread species of human malaria, affecting half of the global population outside Africa. *P. vivax* parasites are a major obstacle to malaria eradication because they can remain dormant as hypnozoites in the liver and are responsible for malaria relapses in patients [1]. Parasites in relapse cases not only increase morbidity in a patient but also serve as a potential reservoir for disease transmission in the community [2]. Hypnozoites are not killed by available antimalarial drugs that target blood-stage parasites. The only commercially available effective drug against hypnozoites is primaquine (PQ), and even this is less effective due to increasing PQ resistance [3-5]. Furthermore, PQ cannot be used widely because it can lead to acute hemolytic anemia in individuals with severe glucose-6-phosphate dehydrogenase (G6PD) deficiency [6]. This toxic side effect may be shared with other 8-

aminoquinolines, including the new FDA approved tafenoquine (TQ) [7]. Thus, safe compounds with hypnozoitocidal activity suitable for large-scale administration are urgently needed [1]. Discovery of new drugs effective against hypnozoites is complicated because, at present, very little is known about biological features of the hypnozoites and there are no long-term experimental models to assess potential radical cure efficacy of test inhibitors in vitro.

Development of a reliable long-term human liver-stage model remains limited due to technical challenges. To obtain experimental liver-stage parasites, a researcher has to have access to suitable infected mosquito vectors, which require much specialized efforts and conditions. For *P. vivax*, no robust in vitro culture system exists, thereby hampering the ability to obtain sufficient amounts of mosquito-transmissible stage, gametocytes, to feed female *Anopheles* mosquitoes. Therefore, *P. vivax* gametocytes are generally obtained in limited quantities from *P. vivax* infected-patients [8-11], or from experimentally infected monkeys [12]. Thus, generating large amounts of *P. vivax* sporozoites to support large chemical library screens in liver-stage remains challenging. Also, to date, there is no robust in vitro system to obtain a long-lived *P. vivax* hypnozoites, although a recent attempt has been reported in a phylogenetic related simian malaria parasite *P. cynomolgi* [13]. Currently, humanized liver mouse model of *P. vivax* appears to be the most powerful way to obtain liver stage parasites, including hypnozoites [14]. However, this mouse model is expensive to use as a routine screening platform in a drug discovery pipeline.

The choice of appropriate human liver cells may influence the value of a liver stage assay. In vitro liver stage models to date use both hepatoma cell lines [12, 15-17] as well as primary human hepatocytes [18-23]. Although, hepatoma cell lines (e.g. HC-04) have been widely used to culture *P. vivax* liver-stage parasites, they have their own challenges: Proliferation is not inhibited by cell-cell contact and cells can detach after a long period of culture. This limits its applications in long-term drug assays. Primary human hepatocytes (PHH) are a natural host but, under conventional culture conditions, they gradually lose their hepatic functions within a week [24]. Attempts to prolong functional features of PHH in culture include co-culturing PHH with HepaRG cells [13], organizing PHH among supporting fibroblasts in a micropatterned co-culture (MPCC) platform [21, 22, 25] and, more recently, maintaining PHH in a high throughput microphysiological environment [23]. Nevertheless, donor-to-donor variability [21, 23] remains a major challenge in employing PHH for in vitro assay. Thus, experimentally, both PHH and hepatoma cell lines are limited for long-term cultivation to assess hypnozoite biology and for developing high-throughput screens for anti-relapse compounds.

Recently, a novel ‘immortalized’ hepatocyte-like cell line (imHC) derived from human bone marrow mesenchymal stem cells (hMSCs) [26] has been shown to support the development of *P. vivax* liver-stage parasites [27] and hepatitis B virus infection [28]. Moreover, imHCs retain the cytochrome P-450

functions responsible for drug metabolism, possibly providing a more physiologically relevant model for drug testing [26, 27]. imHCs can also be maintained in culture for months without overgrowth and detachment, thereby offering a suitable model for cultivation of non-dividing *P. vivax* hypnozoites for long periods [27, 29].

Here, persistence of *P. vivax* hypnozoites is demonstrated in long-term cultures of imHCs. Pure hypnozoites in culture were obtained within 7 days by using DSM265 to kill growing liver-stage parasites. Enriched in vitro hypnozoites were characterized using well-defined anti-liver stage compounds. Reactivation from dormancy of the in vitro hypnozoites was also examined.

Materials And Methods

Ethical approval Human P. vivax-infected blood samples were collected in strict accordance with the human use protocol TMEC 11-033, approved by the Institute Ethical Review Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Written approval consents were obtained from donors prior to blood collection. Drugs and compounds Atovaquone (ATQ) and PQ were purchased from Sigma-Aldrich St Louis, MO, USA. PQ as well as TQ were also provided by the WorldWide Antimalarial Resistance Network (WWARN) Reference Standards Programme. DSM265 was synthesized at the University of Washington in Seattle, WA, USA as previously described [30]. P. vivax sporozoites Laboratory-reared female Anopheles dirus were membrane-fed with P. vivax patients' blood samples [8]. Mosquito infection was monitored for midgut oocysts and salivary gland sporozoites on day 7 and day 14 post-feeding, respectively. Salivary glands were dissected from 50 anesthetized infected mosquitoes, placed in 50 µl of ice-cold RPMI 1640 medium (Gibco, Grand Island, NY, USA), pH 8.2, supplemented with 200 U/ml penicillin (Invitrogen, Carlsbad, CA, USA), 200 µg/ml streptomycin (Invitrogen), and 0.25 µg/ml amphotericin B (Invitrogen), and ground with a sterile pestle. The released sporozoites were counted in a hemocytometer and kept on ice for further use. A total of five P. vivax isolates collected from Tak and Yala provinces of Thailand were used in the present study. imHC line imHCs were developed as previously described [31]. Cells were thawed and cultured following our previous published protocol [27]. Briefly, imHCs were cultured in 1:1 DMEM:Ham's F12 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin and were maintained at 37°C in a humidified atmosphere containing 5% CO2. Cells were subcultured, after detachment with 0.125% trypsin-EDTA, every 2-4 days or once they reach approximately 80% confluence. imHCs infection imHCs were seeded at a density of 3 x 105 cells per well in a Matrigel (Corning Corp, Corning, NY, USA)-coated Millicell EZ SLIDE 8-well glass slide (Millipore, Billerica, MA, USA) and maintained at 37oC for 18-20 h prior to sporozoite infection. Culture medium was removed by aspiration, and 2-2.5 x 105 sporozoites in 200 µl of infection medium (1:1 DMEM:Ham F12 media supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) were added to each well of imHC culture. After a 4-h incubation at 37°C, uninvaded sporozoites were removed and then 400-µl aliquot of fresh infection medium was added. The infected imHC culture was maintained at 37°C with daily medium changes prior

to visualization of the liver-stage (LS) parasites on days 7, 14, 21 and 28 by indirect immunofluorescence assay (IFA). Indirect immunofluorescence assay The infected imHCs were washed with PBS buffer, incubated with 4% paraformaldehyde for 20 min, and permeabilized by exposure to 0.1% Triton X-100. Cells then were treated sequentially with 3% BSA (in PBS) solution, followed by mouse anti-PbHsp70 antibodies (clone 4C9, kindly provided by Dr. Fidel P Zavala [32]), then goat secondary IgG Alexa Fluor® 488-conjugated anti-mouse antibodies (1:500 dilution, Invitrogen), followed by Alexa Fluor 568 phalloidin (1:1,000 dilution, Invitrogen), and finally 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). In some experiments, rabbit anti-acetylated histone H3K9 antibodies (1:200 dilution, Millipore) and goat secondary IgG Alexa Fluor® 568-conjugated anti-rabbit antibodies (1:500 dilution, Invitrogen) were used for detecting nuclear histones of the parasites. Samples were covered with ProLong Gold Antifade reagent (Invitrogen), sealed under cover slip, and viewed under a fluorescent microscope (400x magnification; AXIO Observer.A1 equipped with AxioVision Rel 4.8 Software; Carl Zeiss AG, Oberkochen, Germany). DSM265 treatment DSM265, a selective inhibitor of Plasmodium dihydroorotate dehydrogenase, was added to infected imHCs at concentrations ranging from 0.2 to 5 □M 1-day post sporozoite infection. The cultures were maintained up to 6 days with daily changes of medium (including drug supplementation) and harvested on day 7 to determine sporozoite infectivity by immunofluorescence assay. Numbers and sizes of the liver-stage parasites in each well were manually quantified using a fluorescent microscope (ZEISS AXIO Observer.A1). The proportions of small forms (diameter ≤ 10 μm) and growing parasites (diameter > 10 μm) in DSM265-treated cells were determined relative to the 0.1% dimethyl sulfoxide-treated controls. Treatments were conducted over three independent experiments using three batches of sporozoites generated from different P. vivax isolates. The cytotoxicity of DSM265 in a 6-days exposure on imHCs was evaluated using the colorimetric MTT test [33]. Briefly, imHCs were seeded at density of 1 x 104 cells per well in a 96-well plate (Thermo Scientific™, Nunc™; Waltham, MA, USA) and maintained as described above for one day prior to drug treatment. Then, cultures were treated with various concentrations of DSM265 for 6 days, with daily changes of medium (including drug supplementation). After treatment, 100 μl of 0.5% MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphyneltetrazolium bromide) reagent (Sigma-Aldrich) was added in each well. Reaction plates were incubated for 4 h at 37oC and read at 570 nm in EnVision Multi-Mode microplate reader (PerkinElmer, Waltham, MA, USA). The results were expressed as a percentage of cellular viability compared to untreated controls. P. vivax hypnozoite enrichment and drug assays Sporozoite-infected imHC cultures were treated with 5 □M DSM265 from day 1 to day 6 post-infection (pi). The medium supplemented with the compound was renewed daily during the treatment. Some cultures were harvested on day 7 to determine the frequency of hypnozoite production from each isolate. Subsequently the cultures were either left untreated or treated for a further 7 days with either 10 nM ATQ, 5 μM PQ, or 5 μM TQ. The cultures were fixed on day 14 pi. Statistical analysis At least three independent experiments were conducted in triplicate and results of the parasite size are expressed as median and 5th to 95th percentile range. Number and the percent P. vivax liver-stage were shown with mean □ standard deviations. Each individual infection experiment was performed using a different parasite isolate. One-way ANOVA and post-hoc Dunnett multiple comparison test were performed using GraphPad Prism software.

Results

Small *P. vivax* liver stages persist in imHCs

Here, the feasibility of imHCs to support *P. vivax* hypnozoites was examined. imHCs were infected with *P. vivax* sporozoites and the development of the liver-stage parasites was monitored over time. Every week some wells of the cultures were harvested and the numbers and sizes of the liver-stage forms were quantified. The parasites were visualized using antibody raised against *P. berghei* heat shock protein 70 (HSP70) [32], which cross-reacts with *P. vivax* HSP70. As shown in Fig. 1, *P. vivax* liver-stages in imHCs displayed asynchronous features: They ranged in size from ≤ 10 mm in diameter to 45 mm, reflecting mix of developmental parasite stages during the first week of culture in liver cells. Larger liver-stage schizonts were observed on day 14 and, as time progressed, they fully matured and eventually burst leaving very few infected cells in culture. After 28 days of culture, imHCs were only infected with a subpopulation of *P. vivax* parasites that were ≤ 10 mm in diameter (Fig 1). At least 21 days were required to obtain these persistent small forms, which were present at 20.6% ± 3.9% (range, 14 to 27%) frequency compared to all liver-stage parasites formed on day 7 pi. In one 21-day culture of the long-term infection experiments, a few large mature schizonts were also observed (Fig. 1, *P. vivax*#1). It was possible that the late schizonts in the 21-day culture were activated parasites that generated merozoites and resulted in the first relapse episode of this in vitro infection.

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A quick approach to enrich pure non-dividing *P. vivax* liver-stage parasites

DSM265 inhibits the mitochondrial pyrimidine biosynthesis enzyme, dihydroorotate dehydrogenase (DHODH). This compound is active against growing liver-stage parasites, but not hypnozoites. This conclusion is based on an in vitro assay using *P. cynomolgi* liver-stage model (a monkey malaria parasite that also produces hypnozoites) [34]. To assess the effect of DSM265 on *P. vivax* growing forms in imHCs, liver-stage development was monitored in the presence of the compounds in three-point 5-fold dilutions (0.2, 1, and 5 mM final concentrations). The treatment was carried out prophylactically from day 1 to day 6 pi and the cultures were harvested on day 7 pi. MTT assays indicated no toxicity of DSM265 to the imHCs at any concentrations tested in this 6-day treatment (Fig. 2A). Three independent experiments, from three different *P. vivax* isolates, were conducted (Fig. 2C). Experiments using *P. vivax*#1 had to be terminated earlier on day 4 due to bacterial contamination. DSM265 could kill *P. vivax* liver-stages in a dose-dependent manner and only 12.9% ± 5.5% of liver-stage parasites survived the treatment at 5 mM (Fig. 2B). DSM265 effectively eliminated developing forms with sizes > 5 mm and > 10 mm in diameter on day 4 and day 7 at 1 mM, respectively (Fig. 2C). All of the last survivors were small forms with ≤ 10 mm in diameter (Fig. 2C).

To reproducibly obtain pure small forms, 5 mM DSM265 was added to the cultures for 6 days starting from day 1 to day 6 pi to kill growing liver-stage parasites. Cultures were examined at day 7 pi. After DSM265 treatment, nuclear staining using antibody against acetylated forms of histone H3K9 revealed only small forms with a single nucleus (with ≤ 10 mm diameter) in the culture (Fig. 3A). The enriched small forms with a median diameter of 3.74 mm (5th to 95th percentile; 2.8 and 5.0) were at 15.2% ± 4.2% (range, 10 to 24%) relative to the numbers of total liver-stage parasites on day 7 (Fig. 3B-D). Notably, the enriched small forms remained constant in numbers but were clearly growing in size overtime (Fig. 3B-D). The parasites were bigger at week 3 after DSM265 removal with a median size of 7.53 mm (5th to 95th percentile; 4.7 and 10.7) in diameter. Importantly, some large (> 10 mm) multi-nuclei parasites appeared within 2 weeks of culture after DSM265 withdrawal (Fig. 3B-D, red dots and Fig. 4). The formation of growing parasites after small forms enrichment was observed in all *P. vivax* isolates tested.

Small non-dividing liver-stage parasites are resistant to atovaquone

To determine whether the remaining non-dividing forms in cultures were hypnozoites, established differential susceptibility of liver-stage parasites to select antimalarial drugs was exploited [35]. ATQ, an inhibitor of the mitochondrial cytochrome c, does not prevent relapses. Therefore, exposure of the infected cultures to ATQ leads to the death of only developing liver-stage forms but not hypnozoites [35]. Resistance of the non-dividing small forms to ATQ in long-term cultures (Fig. 5A) and in enriched cultures (Fig. 5B) indicated that the persistent liver-stage forms in imHCs were hypnozoites. The results of a single experiment from one *P. vivax* isolate are shown in Fig. 5.

Evaluation of in vitro hypnozoites for anti-relapse assay

To verify the usefulness of the long-term hypnozoite model for anti-relapse assay, susceptibility of the in vitro hypnozoites to known anti-relapse drugs, PQ and TQ, was tested. After DSM265 treatment to remove growing liver-stage forms, PQ was added into the culture for an additional 7 days (Fig. 5B). Surprisingly, PQ at 5 mM was unable to eliminate the remaining small forms in DSM265-treated cultures (Fig 5B). Similar effect was also found in TQ treatment. Similarly, poor activity of PQ and TQ was observed to eliminate small forms in the 28-day long-term cultures (Fig 5A). Furthermore, subsequent work using a different lot or a different source of PQ, or a higher concentration of PQ (10 mM), again showed similar limited effect on the small forms (data not shown).

Discussion

Efforts to eradicate *Plasmodium vivax* are especially challenging due to the dormant form of infection, called hypnozoites, in the liver. To date, biological features of the hypnozoites are still largely unknown. This is mainly due to the lack of a reliable *P. vivax* liver-stage model for biology studies and drug assays. To eliminate global *P. vivax* malaria, it is necessary to establish a robust *P. vivax* hypnozoite model. This should facilitate the identification and developing of a safe, highly effective anti-relapse drug for mass administration without undesirable side effects.

Recently, the immortalized human hepatocyte-like cell line (imHC) has been established to support the development *P. vivax* liver-stage parasites in vitro [27]. imHCs can be maintained in long-term culture, thereby facilitating the development of an in vitro hypnozoites system. In this study, the feasibility of imHCs to support *P. vivax* hypnozoites in culture was studied and the effect of anti-relapse drugs on these parasite forms was investigated.

To assess the feasibility of imHCs to support in vitro hypnozoites, *P. vivax* infected cultures were maintained for 28 days and a population of small forms was identified and examined. The percent sporozoite infection rates in this study ranged from 0.01 to 0.04% (n = 5), which were lower than that observed (~0.1%) in our previous report [27] but it was comparable to the rates (~0.04%) obtained in HC-04 cells [17]. The difference in rate of infectivity between our imHC infections could be due to the inherent capacity of infection by different *P. vivax* isolates. Cultured *P. vivax* liver-stages in imHCs were asynchronous with large variations in size, which is a normal characteristic of liver-stage parasites in cultures [15, 17]. Large schizonts (approximately 20-45 μm) were formed as early as day 7. Mature schizonts, even larger in size, were observed as cultivation progressed. The sizes of these mature schizonts were similar to those reported in PHH cells [21], although they were smaller than those observed in humanized mice [14]. These results indicate that *P. vivax* sporozoite infection in imHCs is reproducible, confirming its potential use as a robust/reliable model for *P. vivax* liver-stage assays. Long-term cultures over 28 days yielded only small parasites, at ~20% frequency. Here, pure small forms were evident and could be enriched after 21 days. This result showed for the first time that small liver-stage forms can form and persist in imHCs.

To obtain small forms even quicker, DSM265 was used to remove all active forms of any size in early stage 7-day culture. The small forms could be completely enriched in 7 days, 2 weeks faster than that used in the standard long-term protocol, and allowed a 3-week period in which to monitor hypnozoites. DSM265 had potent activity against growing forms and yielded only small, single nucleus forms at approximately 15% frequency, similar to that obtained from the long-term culture. Small form frequencies obtained in this study are consistent with yield observed from *P. vivax*-infected cultured primary hepatocytes [22] and in humanized mouse model [14]. Greater proportion of small forms seen in

untreated culture on day 7 (~68% of LS forms), relative to those in DSM265-treated cultures (~15% of LS forms), reflected the presence of slow-growing small parasites (~53% of LS forms). It is difficult to distinguish small, slow–glowing liver forms from hypnozoites based on their microscopic appearance.

A more recent study has identified Liver-Specific Protein 2 (LISP2) as an early molecular marker of liver stage development [36]. Thus, anti-LISP2 antibodies in immunofluorescence analysis offered a potential alternative way to identify slow-growing forms and to differentiate them from true hypnozoites in culture. Similar attempts to enrich in vitro hypnozoites using ATQ [13, 35] and phosphotidylinosiol-4-OH kinase (PI4K) inhibitor-KDU691 [22] have been reported. In our initial experiments, ATQ was used to eliminate the growing parasites in parallel with DSM265. Interestingly**,** approximately 40% of small forms survived the highest dose of ATQ (10 nM) used, whereas only 15% of small forms were observed with 5 mM **DSM265,** indicating that hypnozoites could be quickly and efficiently enriched by DSM265 treatment. Limited efficacy of ATQ to clear slow growing forms in this study may be because of inadequate amounts of the drug used: More than 50 times higher drug concentration was used in *P. cynomolgi* model for the same purpose [13].

To validate that the small forms obtained in imHCs are hypnozoites, it was necessary to demonstrate their reactivation from dormancy. Our initial study in the long-term 28 days culture suggests that hypnozoite activation occurred, as a few schizonts were detected on day 21, similar to what observed in vivo [14]. In imHC hosts, the maturation of *P. vivax* liver-stages peaks 10 days pi and very few mature schizonts are observed beyond day 15 of culture [27]. Thus, it is possible that the mature forms observed later in culture were from activation of the persisted forms. Maintenance of DSM265-enriched small forms in culture medium for a few weeks could also capture the presence of multi-nucleate forms. Some of these growing parasites were > 10 mm in diameter. These schizonts were not fully mature and were similar in size to growing schizonts observed during 7-day culture. These multi-nuclei parasites may represent the initial stage of reactivation.

The present observations are consistent with the earliest relapse observed in other reports. The first relapse of *P. vivax* tropical strain usually occurs within 30 days after the primary infection [37]. In simian malaria model, the earliest relapse observed in vivo with *P. cynomolgi* strain occurred 19-21 days pi [38]. Assuming that reactivated hypnozoites need ~7 additional days of development before merozoites are released from the liver, reactivation of hypnozoites in vivo could start around day 14 pi. This starting period of hypnozoite reactivation is similar to what is observed in the present study. In addition, the persistent small forms were clearly growing in size overtime, which was similar to that observed in *P. vivax* in vivo model [14]. This result may capture a period of hypnozoite development that is required before reactivation is established. Together, persistence of single nucleus small forms and the ability of

these cells to reactivate in culture add confidence in the capacity of imHCs to establish *P. vivax* hypnozoites.

While encouraging, the appearance and morphological observations alone may not be sufficient to prove that the persisted and enriched small forms in imHCs are hypnozoites. Additional pharmacology patterns may be helpful. In some models, differential susceptibility of growing liver-stage forms and of hypnozoites to antimalarial drugs can indicate the presence of hypnozoites in infected hepatocytes cultures [12, 13, 22, 23, 35, 38]. Therefore, the sensitivity of enriched small forms in imHCs to selected antimalarial drugs was evaluated. ATQ inhibits growing liver-stage parasites, but not hypnozoites. As expected, ATQ was unable to eliminate the enriched small forms in both long-term and DSM265-treated cultures, confirming that these persistent forms in imHCs are hypnozoites. PQ and TQ, approved antimalarial drugs that effectively kill hypnozoites in vivo, were then tested as positive controls. Surprisingly, PQ was unable to clear all remaining small forms in DSM265-treated cultures. A similar limited effect was also observed with TQ. PQ and TQ were also unable to clear persistent small forms in the 28-day long-term cultures. Although a range of responses to PQ can be detected in hepatocyte hosts with different CYP450 levels [23, 39], the poor effect of PQ and TQ on these in vitro hypnozoites was unexpected because imHCs have high levels of CYP450 activity that would be sufficient to produce active PQ metabolites [27]. This was confirmed by its potent prophylactic activity against *P. vivax* developing forms (Additional file 1: Fig. S1). Interestingly, poor activity of PQ to clear hypnozoites was also found in the most recent reports of cultured hypnozoites, high-throughput PHH cell-based assay [23] and MPCC model [22]. There is evidence that PQ-treated parasites clear slowly from such cultures [22, 23]. Moreover, PQ treatment can lead to mitochondrial dysfunction but not necessarily clearance of the parasites [40]. Therefore, the remaining parasites after PQ treatment in our system may be a population of residual, dead PQ-treated forms that could be difficult to distinguish from potentially viable hypnozoites.

The ability to culture imHCs over long periods and diverse *P. vivax* strains opens up new avenues for future research. imHCs are susceptible to infection by two variants of Thai *P. vivax* sporozoites, CSP-VK210 and CSP-VK247 [27]. CSP-VK247 sporozoites seem to produce larger numbers of small forms than those of CSP-VK210 genotype [14, 27]. Since one of our primary long-term goals is to develop a robust drug-screening model, obtaining higher numbers of hypnozoites per well is important. Unfortunately, only CSP-VK210 sporozoites were available during the present study. In future, it will be interesting to study hypnozoites generated from CSP-VK247 sporozoites and to examine whether CSP-VK247 sporozoites yield higher proportion of hypnozoites. Furthermore, it is known that *P. vivax* isolates from tropical regions relapse with short time intervals when compared to temperate strains, which show longer relapse frequencies [37]. Given these data, it might be possible to employ our model to further analyze hypnozoite frequencies, relapse patterns, and responses to drugs in *P. vivax* strains from different geographical origins.

Conclusions

To date, there is no robust in vitro laboratory model to study *P. vivax* hypnozoites for long-term biology and drug susceptibility. Our imHC model offers an alternative platform to culture *P. vivax* liver-stage parasites, including hypnozoites. This study demonstrates that *P. vivax* hypnozoites can persist in long-term imHC culture. **The in vitro hypnozoites** can be enriched by DSM265 treatment. Resistance to ATQ strongly suggests that the in vitro hypnozoites are authentic. The imHC model not only supported hypnozoites formation but also eventual reactivation. **A robust long-term *P. vivax* hypnozoite model described in this study is a significant step forward to** enable investigations of the biology of the hypnozoites. This includes questions related to the nature and mechanism of latency, and investigations on compounds that effectively kill dormant parasites, particularly in high-throughput screening formats.

Abbreviations

ATQ, atovaquone; BSA, bovine serum albumin; CYP450s, cytochrome P450 isotypes; DAPI, 4',6-diamidino-2-phenylindole; DHODH, dihydroorotate dehydrogenase; FBS, fetal bovine serum; G6PD, glucose-6-phosphate dehydrogenase; LS, liver-stage; MEM, minimal essential medium; MSCs, mesenchymal stem cells; PBS, phosphate-buffered saline; PHH, primary human hepatocytes; pi, post infection; PQ, primaquine; TQ, tafenoquine

Declarations

Ethics approval and consent to participate

Human *P. vivax*-infected blood samples were collected in strict accordance with the human use protocol TMEC 11-033, approved by the Institute Ethical Review Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Written approval consents were obtained from donors prior to blood collection.

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. The datasets are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RP, OK, and PKR conceived and designed the study. SH provided imHCs. AJ, YP, and PL maintained imHC cultures. JS and WR provided *P. vivax*-infected mosquitoes and sporozoites. AJ, YP, and PL prepared *P. vivax* sporozoites. AJ, YP, PL, and RP conducted sporozoite infection experiments and anti-liver stage assays. AJ, PL, and RP performed IFA and identified liver-stage parasites. YP performed MTT assay. PK, AJ, and RP participated in fluorescence imaging and analysis. SK synthesized DSM265. OK, PKR, RP, SB, and SH contributed reagents/materials/analysis tools. RP, AJ, YP, PK, PKR, and OK wrote and prepared the manuscript. All authors read and approved the manuscript.

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Figures

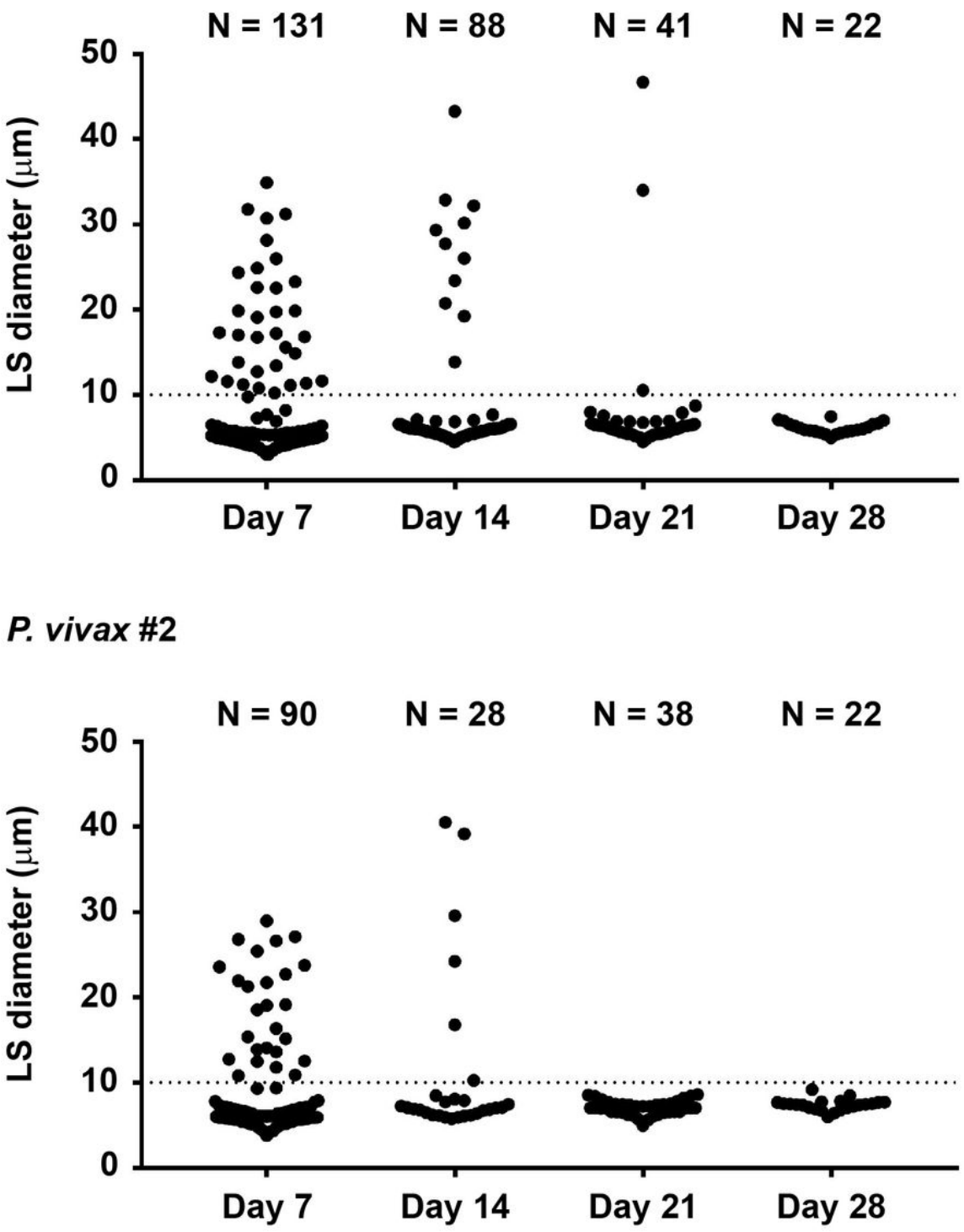

Figure 1

Maturation of P. vivax liver-stage (LS) parasites in imHC culture. P. vivax sporozoites were added to imHCs that had been cultured on Matrigel-coated plates. After four hours of incubation, the cultures were washed to remove un-invaded sporozoites and then maintained for 28 days. Numbers and sizes of the liver stage parasites were quantified every 7 days of development by IFA. Small forms at ≤ 10 □m in

diameter could clearly be distinguished in the cultures after day 21 post-infection. Representative P. vivax infections are shown. Data represent liver-stage forms obtained from triplicate culture wells.

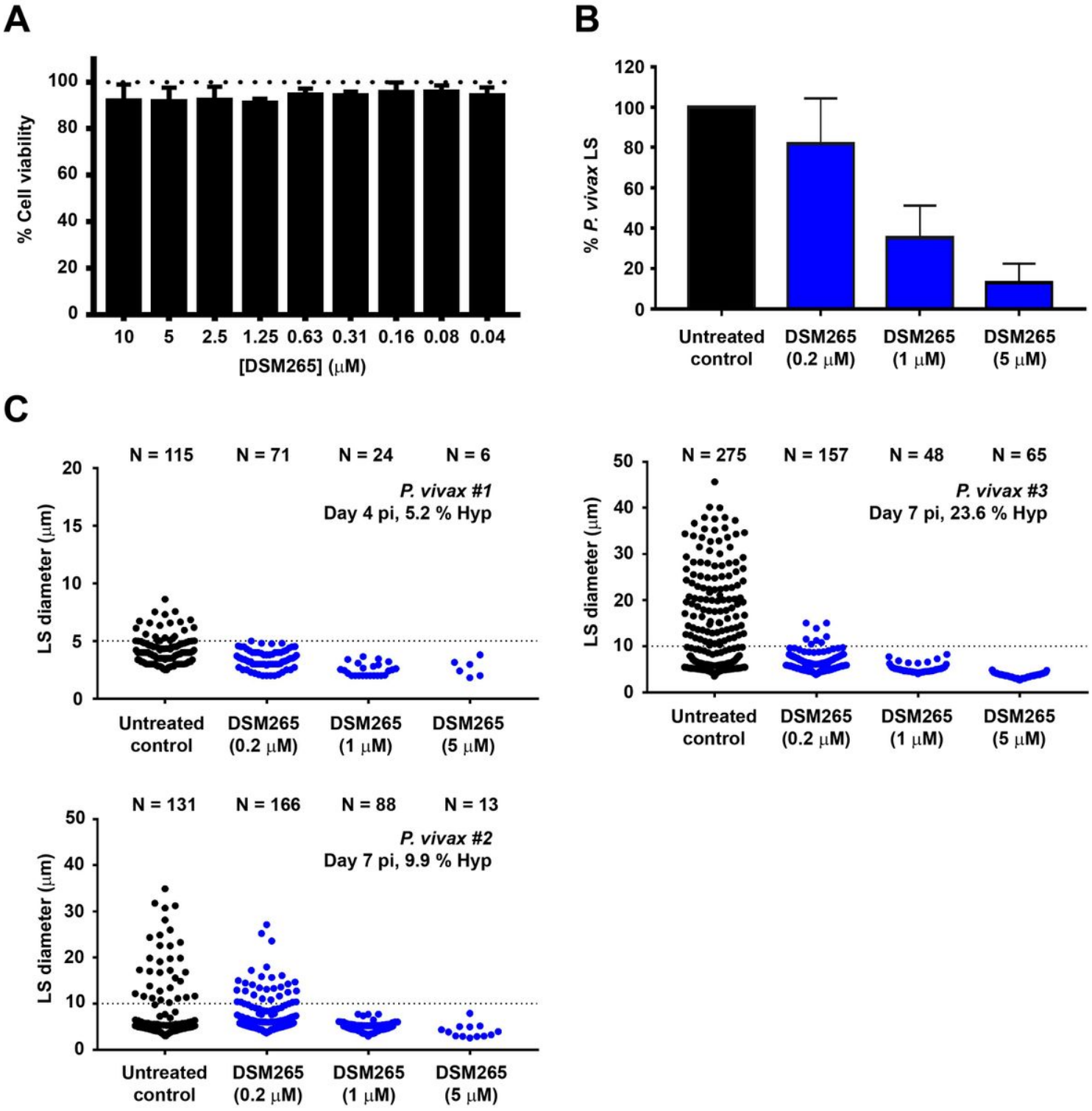

Figure 2

Selection of non-dividing P. vivax small liver-stage parasites with DSM265. (A) DSM265 was not toxic to imHCs as assessed by MTT assays. P. vivax-infected imHCs were exposed to the varying concentrations of DSM265 from day 1 to day 6 post-infection in order to kill the growing liver-stage parasites. The cultures were fixed on day 7 and the remained liver-stage forms were counted. Parasite sizes were

measured. (B) Bars represent treated liver-stage parasites enumerated comparing to untreated control (mean ± SEM from three independent experiments of different P. vivax isolates). (C) Dose dependent effect of DSM265 on P. vivax liver-stage populations, growing and small non-dividing forms (data at day 7 pi for P. vivax isolate#1 were not available due to culture contamination at day 4). Data in each condition represent liver-stage forms obtained from triplicate wells.

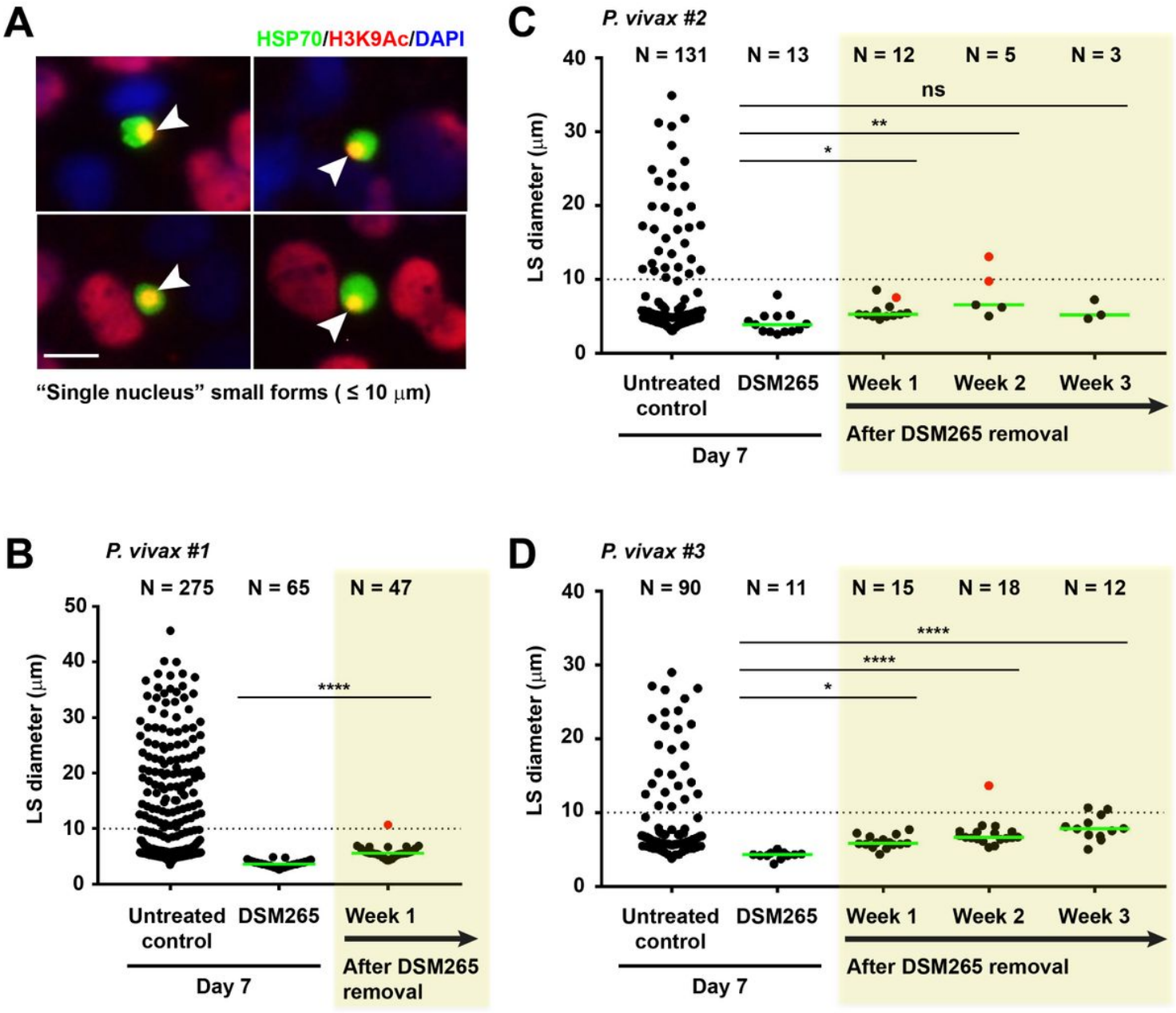

Figure 3

Enrichment of P. vivax hypnozoites with DSM265. (A) The representative IFA images of remained single nucleus liver-stage parasites after DSM265 treatment. The parasite and host nuclei were stained with DAPI, while the parasites were recognized with anti-HSP70. Antibody against H3K9Ac was also used to visualize nuclei. Arrows indicate uni-nucleate feature of the parasites. Scale bar = 10 μm. Cultures were treated with 5 μM DSM265 for 6 days from day 1 to day 6 pi and fixed on day 7. In order to examine activation of enriched hypnozoites over time, subsequently the cultures were left untreated for further 1

week (B) or 3 weeks (C, D). Data represent liver-stage forms obtained from triplicate wells. Green bars on dot plots indicate median values. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test to DSM265 enrichment at Day 7 where values are represented by $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.0001$ (****), and no significance (ns). Multi-nucleate developing parasites are presented in red dots.

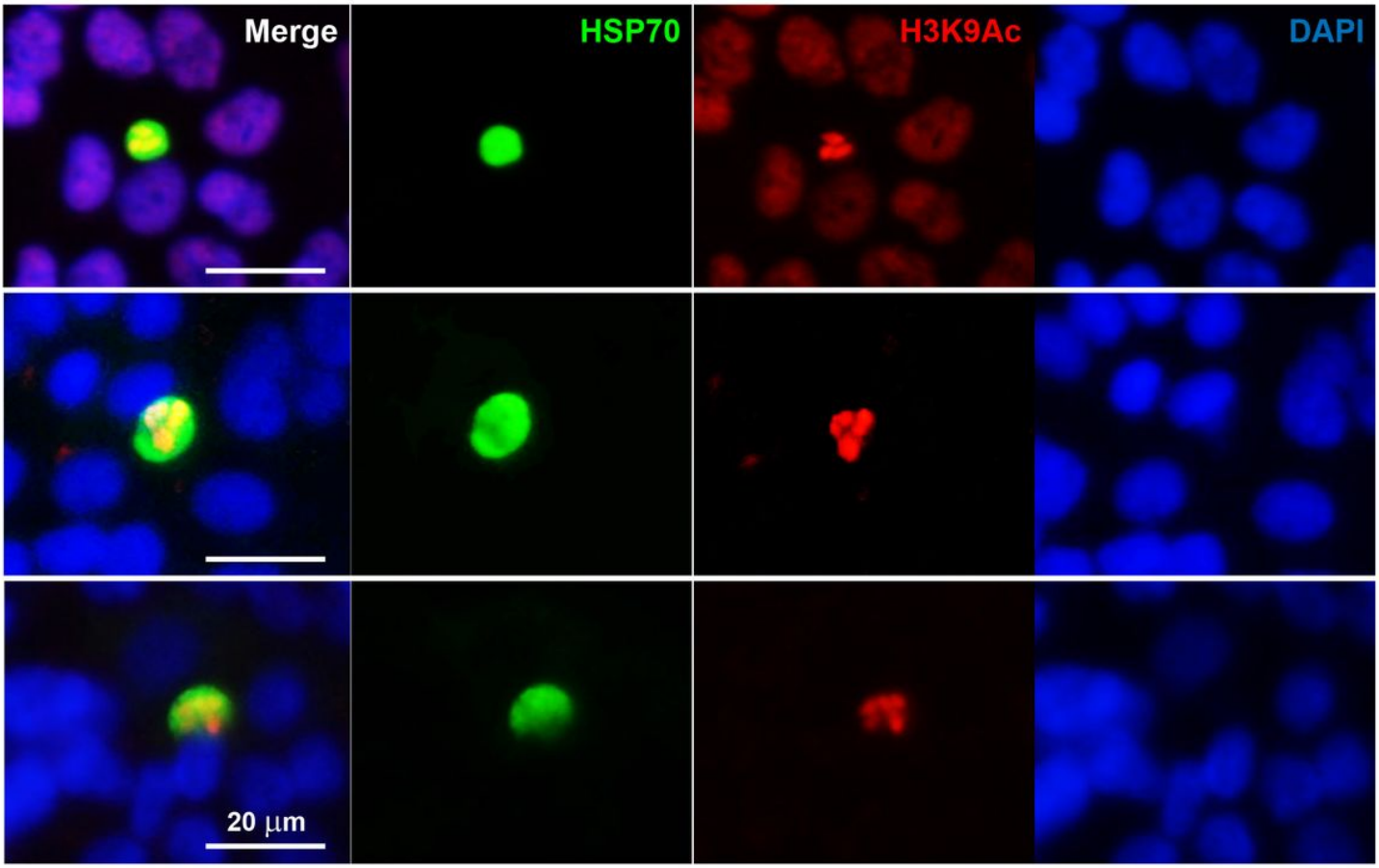

Figure 4

Representative images of multi-nucleate P. vivax liver stage parasites obtained after a week of culture in standard medium without DSM265. The parasite and host nuclei were stained with DAPI, while the parasites were visualized by IFA using anti-HSP70 antibody. Antibody against H3K9Ac was also used to visualize nuclei. Scale bar = 20 m.

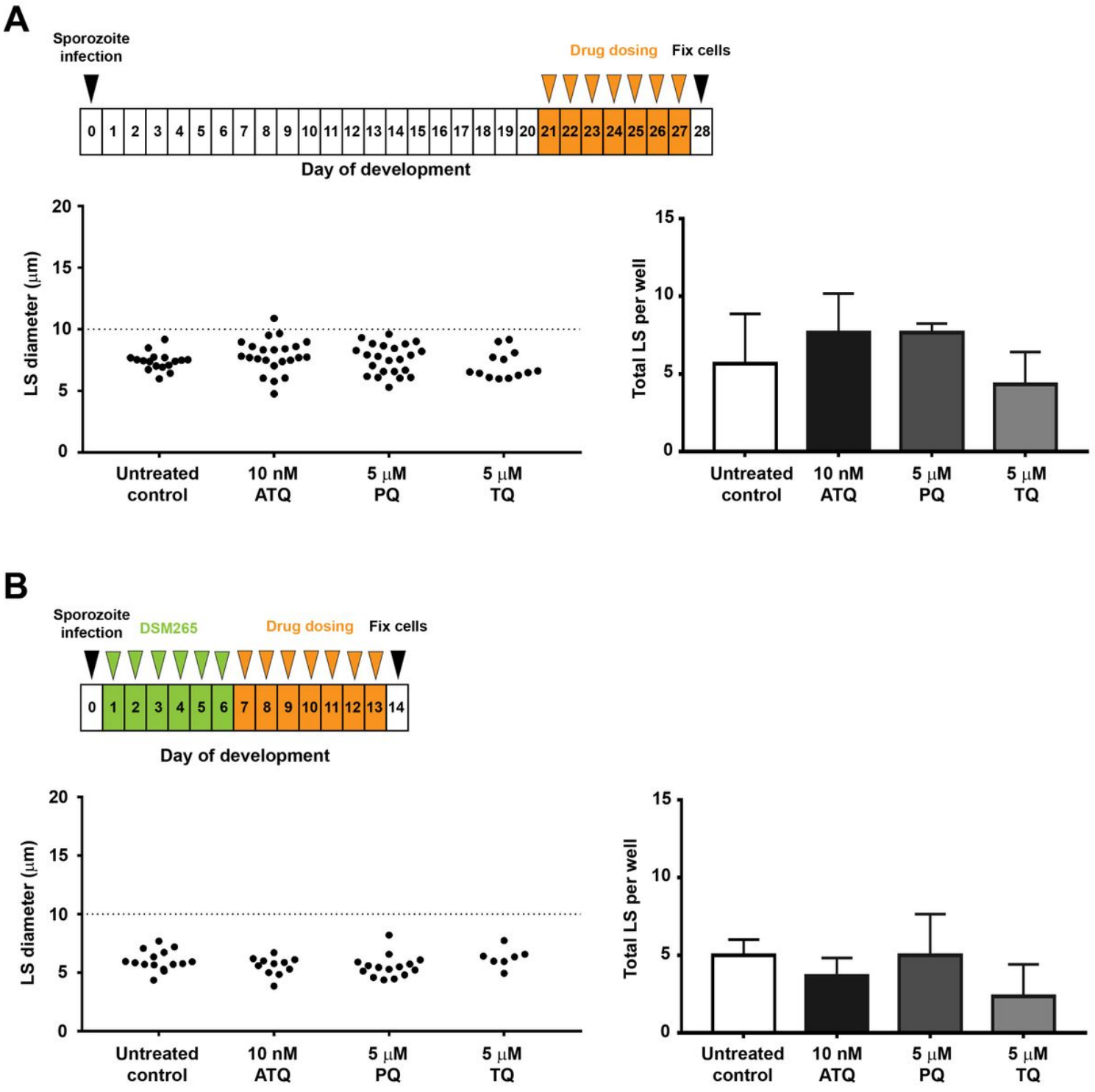

Figure 5

The inhibitory effect of antimalarial drugs on P. vivax hypnozoites in imHCs. (A) P. vivax-infected imHCs were maintained for 20 days prior to one-week treatment of antimalarial drugs. The cells were fixed on day 28. (B) imHCs were infected with P. vivax sporozoites one day prior to 6-days DSM265 treatment. Subsequently the cultures were left untreated or treated with drugs for additional one week and harvested on day 14. Hypnozoites were visualized by immunofluorescence staining. Numbers and sizes of the parasites were measured. Representative data from one P. vivax isolate are presented. Sizes in diameter

of individual LS from triplicate wells are shown on the dot plots. Bar graphs represent mean ± standard deviations for total LS per well from one experiment conducted in triplicate.

Supplementary Files

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