Persistence and activation of malaria hypnozoites in long-term primary hepatocyte cultures

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Malaria relapses, resulting from the activation of quiescent hepatic hypnozoites of *Plasmodium vivax* and *Plasmodium ovale*, hinder global efforts to control and eliminate malaria. As primaquine, the only drug capable of eliminating hypnozoites, is unsuitable for mass administration, an alternative drug is needed urgently. Currently, analyses of hypnozoites, including screening of compounds that would eliminate them, can only be made using common macaque models, principally *Macaca rhesus* and *Macaca fascicularis,* experimentally infected with the relapsing *Plasmodium cynomolgi*. Here, we present a protocol for long-term *in vitro* cultivation of *P. cynomolgi*–infected *M. fascicularis* primary hepatocytes during which hypnozoites persist and activate to resume normal development. In a proof-of-concept experiment, we obtained evidence that exposure to an inhibitor of histone modification enzymes implicated in epigenetic control of gene expression induces an accelerated rate of hypnozoite activation. The protocol presented may further enable investigations of hypnozoite biology and the search for compounds that kill hypnozoites or disrupt their quiescence.

True malaria relapses are the reappearance of parasites in the blood weeks to years after complete clearance of blood-stage parasites in individuals who had no further infectious mosquito bites. Relapses occur with only a few of the *Plasmodium* species that infect primates, namely *P. vivax*, *P. ovale*, *P. cynomolgi*, *P. fieldi* and *P. simiovale*, and possibly with other *P. vivax*–like species (*P. simium*, *P. schwetzi* and *P. silvaticum*)[1,2]. The long-held notion that relapses originate by parthenogenesis from persisting gametocytes was abandoned after the discovery in 1948 of the hepatic phase of the *Plasmodium* life cycle, first demonstrated with *P. cynomolgi* infections in rhesus monkeys[3]. However, the first detection of uninucleate hepatic forms, termed hypnozoites, which persist for months or more in a state of arrested development (also described as quiescence or dormancy) before resuming their development into mature hepatic schizonts, was not reported until the early 1980s (refs. 4–6). It is thought that hypnozoites are derived from a subpopulation of the sporozoites inoculated by the mosquito[7].

Hypnozoite carriage, which at present cannot be screened for, poses a particular challenge to malaria control and eradication. Relapses not only increase morbidity but also substantially extend the effective duration of the infection and thereby the potential to transmit the infection. The only currently available drug that can eliminate hypnozoites is primaquine, an 8-aminoquinoline introduced in the early 1950s. However, primaquine cannot be used widely because it can lead to potentially life-threatening acute intravascular hemolysis in individuals with severe glucose-6-phosphate deficiency. This undesirable side effect is shared with other 8-aminoquinolines, such as tafenoquine.

The search for compounds with hypnozoitocidal activity suitable for mass administration has become a priority in the current drive to eliminate malaria[8,9]. However, this search is severely limited because, at present, assessing the efficacy of hypnozoite elimination can only be achieved using macaques, generally rhesus, infected with *P. cynomolgi* sporozoites, the acknowledged experimental model for *P. vivax* since the 1950s (refs. 10,11). Moreover, given the practical and ethical limitations inherent to experimental *in vivo* infections, it has not been possible to conduct any cellular, biochemical or molecular investigations that could help elucidate the biology of the hypnozoite.

We have recently described persistent nondividing uninucleate hepatic forms of *P. cynomolgi* in *in vitro*–cultured primary hepatocytes from the natural host *M. fascicularis*[12]. We putatively assumed these forms to be hypnozoites because they displayed a susceptibility to drugs similar to that observed *in vivo* for *P. cynomolgi* and *P. vivax* hypnozoites[12]. In particular, we proposed that the resistance of

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the uninucleate forms to atovaquone could be exploited to obtain cultures enriched for these forms, thereby providing a starting point for further investigations or for screening assays. Nonetheless, it remains important to seek further evidence that the uninucleate forms do indeed represent hypnozoites. The two defining biological features of hypnozoites are their persistence over periods that extend beyond the initial 8–15 d (ref. 13) of normal maturation of *P. cynomolgi*–infected hepatocytes and their subsequent activation to resume development into mature hepatic schizonts. Neither feature could be assessed in our original *in vitro* cultures, as they were maintained for only approximately 10 d (ref. 12).

There are two obstacles to maintaining cultures of primary hepatocytes (simian or human) beyond 1–2 weeks. The first is inherent to the isolated primary hepatocyte. In order to survive, these cells (which do not proliferate *in vitro*) must attach to a substratum where they can aggregate as polarized cells and form bile canaliculus–like structures on their apical side. Close contact between the hepatocytes is crucial if they are to survive and thrive. Under conventional culture conditions, these cells eventually dedifferentiate and lose some of the normal features and functions of primary hepatocytes by approximately 1 week of culture. The second is inherent to the infection by the parasite. *Plasmodium* sporozoites traverse a number of hepatocytes before settling in one where they will develop to form schizonts[14]. The traversed hepatocytes are wounded and, *in vitro*, often die or detach, leaving gaps in the cellular mat. The primary hepatocyte cultures are thereby adversely affected, especially because high numbers of sporozoites are usually inoculated to initiate *in vitro* infections.

Here, we present modified conditions that allow maintenance of *in vitro*–cultured *P. cynomolgi*–infected primary *M. fascicularis* hepatocytes for up to 40 d. Using these long-term cultures, we demonstrate the persistence of the uninucleate hepatic forms for more than 1 month and show that they activate to resume normal development beyond 3 weeks after infection. These observations indicate that the *P. cynomolgi* uninucleate hepatic forms observed *in vitro* are functionally indistinguishable from hypnozoites. Thus, *P. cynomolgi*–infected long-term cultures may be used to screen for compounds with activity against *P. cynomolgi* hypnozoites. In this manner, we assessed a small molecule that inhibits histone methyltransferases and observed that it promotes reversal of hypnozoite dormancy.

RESULTS

Long-term *P. cynomolgi* hepatic cultures

In our experience (J.-F.F. & D.M., unpublished data), many of the modifications in culture conditions that improve the vitality and functionality of cultured primary hepatocytes[15] are incompatible with or do not improve the normal development of hepatic parasites. On the other hand, cocultivation of the primary hepatocytes with another cell type[16], which allows maintaining and extending hepatocyte integrity and vitality, was beneficial to the cultivation of the *P. vivax* hepatic parasites[17]. Here, we opted for cocultivation with a recent human hepatoma cell line, HepaRG. HepaRG is a bipotent progenitor cell line that proliferates *in vitro* at moderate rates, ceasing to do so once confluence is reached, and differentiates into biliary-like or hepatocyte-like cells[18]. The rationale was that the HepaRG cells would not only improve cultured hepatocyte longevity but also plug any gaps consequent to hepatocyte traversal by sporozoites. We employed GFP-expressing HepaRG cells in order to discriminate between them and the primary hepatocytes. We established that neither the *P. cynomolgi*

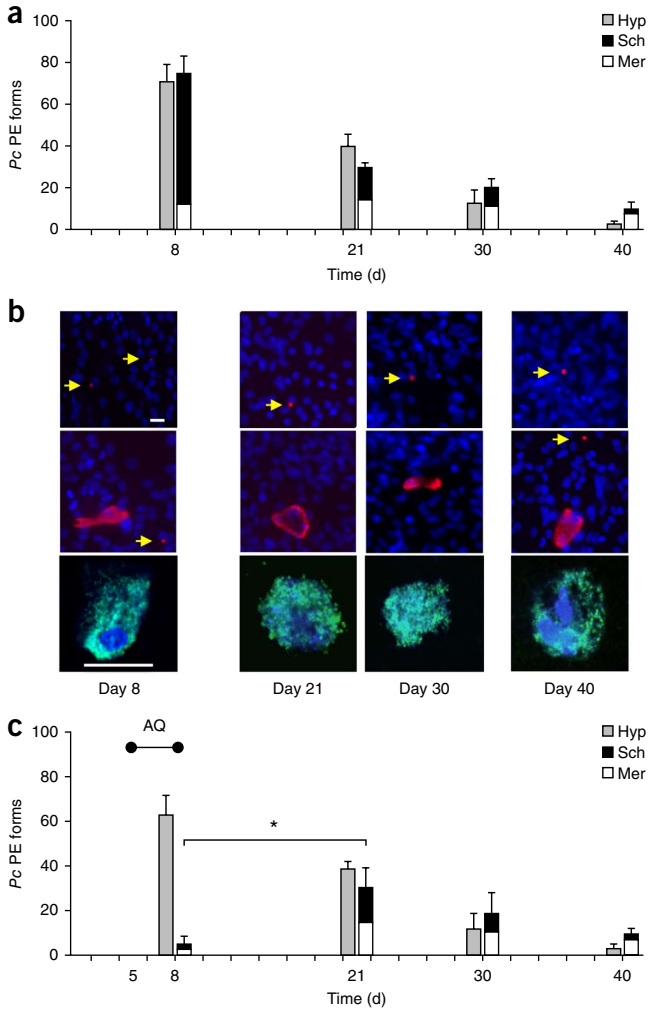

Figure 1 Persistence of *P. cynomolgi* hypnozoites. (**a**) Quantification of the *P. cynomolgi* hepatic forms cultured in *M. fascicularis* primary hepatocytes and HepaRG cells in wells fixed at different times. Gray bars represent uninucleate nondividing hypnozoites (Hyp). Normally dividing preerythrocytic forms (*Pc* PE forms, here and in all figures) are the sum of the schizont forms (Sch, black bars) and the detached infected hepatocytes with fully mature parasites containing merozoites found in culture supernatant (the merosomes) collected at the same time points (Mer, white bars). (**b**) Immunofluorescence of the various hepatic forms observed in the cultures over time, showing typical fields with hypnozoite forms (top), maturing hepatic schizonts (middle) and merosomes recovered from the medium (bottom) from cultures sampled at defined cultivation days (corresponding to those in **a**). Parasites were visualized using antibodies raised against *P. falciparum* HSP70 that cross-react with the orthologs in other *Plasmodium* species (appearing in red in the infected cultures and in green for the merosomes). Nuclei were stained with DAPI (blue). Arrows point to the hypnozoite forms. The scale bar on the top left represents 20 µm and is applicable to all photomicrographs of infected hepatocytes. The scale bar on the merosome picture (bottom left) represents 20 µm and is applicable to all merosomes pictures. (**c**) Quantification of the cultured *P. cynomolgi* hepatic forms subsequent to the treatment with atovaquone (AQ) from day 5 to day 8 after sporozoite inoculation. Data presented in **a** and **c** were derived from two experiments using two independent *P. cynomolgi* sporozoite batches. In each experiment, the total number of parasite forms present at each time point was obtained from duplicate wells. Data are expressed as mean ± s.d. from the two independent experiments. $^*P = 0.0143$, one-tailed Mann-Whitney *U*-test for schizont numbers on days 8 and 21.

nor the *P. falciparum* sporozoites infect the GFP-HepaRG cells (data not shown). We then determined that a ratio of 1 HepaRG cell to 30 primary hepatocytes was optimal for obtaining long-term cultures.

We infected primary *M. fascicularis* hepatocytes cultured with HepaRG cells with *P. cynomolgi* sporozoites and monitored the fate of the preerythrocytic (PE) hepatic parasites over time. We observed equal numbers of multinucleate schizonts and uninucleate hypnozoite-like forms in the wells sampled on days 8, 21 and 30, although their numbers waned steadily such that by day 40, the few parasite forms observed were predominantly schizonts (**Fig. 1a**,**b**). Notably, we observed detached, fully mature schizonts and merosomes, i.e., packets of merozoites released from the fully mature hepatic schizont[19], in culture supernatant collected on days 21, 30 and 40 (**Fig. 1a**,**b**). In simian hosts, maturation of the normal hepatic forms peaks about 9 d after sporozoite inoculation, and very few mature forms have been previously observed from day 15 to day 17 (refs. 1,2,13). Thus, we considered the possibility that the mature forms and merosomes we observed *in vitro* during the latter period of cultivation originated from persisting uninucleate hepatic forms that had activated to resume hepatic development (**Fig. 1a**). Indeed, in our cultures, we noted that the decrease in the number of uninucleate forms observed between two given time points broadly corresponded to the number of multinucleate forms enumerated at the second of these time points (**Fig. 1a**,**c**).

P. cynomolgi hypnozoite persistence and activation *in vitro*

In order to support this interpretation, we exploited the previously established insusceptibility of the uninucleate forms to atovaquone treatment[8,12]. Exposure of the infected cultures to atovaquone between days 5 and 8 led to the death and elimination of the developing schizonts, leaving mostly the hypnozoite-like forms in the cultures (**Fig. 1c**). After removal of the drug, we again observed maturing *P. cynomolgi* hepatic parasites (including schizonts and merosomes) in the cultures sampled from day 21 onwards (**Fig. 1c**). This demonstrates that these forms must have been derived after activation of the uninucleate hypnozoite-like forms that remained on day 8 following atovaquone treatment.

We were concerned that the primary hepatocytes cultured beyond 2 weeks might have gradually lost their ability to sustain parasite infection and development. We sought to determine whether the parasite forms observed beyond 14 d of culture were developing normally or whether their maturation was constrained by the changes that occur in cultured hepatocytes with time. We adopted a strategy that helps maintain primary hepatocyte function: a sandwich configuration, in which the plated cells are sandwiched between two extracellular matrices, collagen[20,21] and Matrigel, a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells[22]. In the current experiments, we were already using collagen as an attachment substrate; thus, we obtained the sandwich configuration by covering the cultures with Matrigel once confluence was reached and after sporozoite invasion.

In our experience (J.-F.F. and D.M., unpublished data), human primary hepatocytes lose their susceptibility to *P. falciparum* sporozoites when they are maintained beyond 1–2 weeks. We tested whether this loss still occurred in the presence of a Matrigel cover and in the presence or absence of HepaRG cells. We maintained the cultures for 15 d before inoculating with *P. falciparum* sporozoites, and we enumerated the infected cells 4 d later. In the absence of the overlaying Matrigel layer, infectivity levels were low, which was in sharp contrast to the high numbers of *P. falciparum* hepatic schizonts that we observed in the Matrigel-covered cultures (**Fig. 2a**). Thus, the use of Matrigel helps maintain the primary hepatocytes' susceptibility to *Plasmodium* parasites beyond 2 weeks. Indeed, the high levels of infectivity observed (**Fig. 2b** and data not shown) were similar to those generally obtained under optimal conditions with fresh human primary hepatocytes and were further enhanced by cultivation with HepaRG cells (**Fig. 2a**).

Given that atovaquone treatment is crucial for the demonstration of hypnozoite persistence and activation, we also confirmed that its differential activity against developing hepatic parasites and the hypnozoite-like forms was not affected by the Matrigel layer (**Fig. 2b**). We could then attempt to establish whether activation of the uninucleate quiescent forms to developing trophozoites was confined to the first days of the infection. We subjected Matrigel-overlaid cultures infected with *P. cynomolgi* sporozoites to two consecutive atovaquone treatments, one from day 5 to day 8 and another from day 15 to day 18. On both occasions, exposure to the drug nearly fully eliminated the hepatic schizonts, leaving only the hypnozoite-like forms. Examination of the cultures 7 d after atovaquone removal (day 15 and day 25) revealed the presence of maturing hepatic schizonts in appreciable numbers (**Fig. 2c**). In each case, the decrease in the number of uninucleate forms was broadly inversely correlated with the increase in the number of schizonts detected 7 d after removal of atovaquone (**Fig. 2c**).

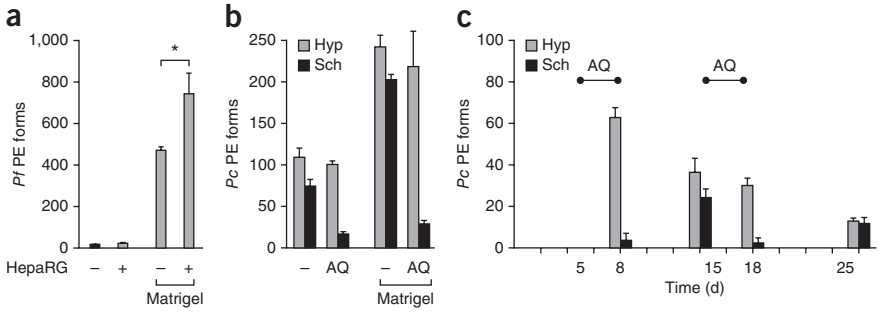

Figure 2 Activation of hypnozoites over time. (**a**) Evaluation of human primary hepatocytes' ability to sustain infection by and development of *P. falciparum* following cultivation for 15 d with (+) or without (–) HepaRG cells, either with or without a Matrigel overlay. The cultures were fixed 4 d after sporozoite addition and the preerythrocytic forms (*Pf* PE forms) enumerated. **P* = 0.05 by one-tailed Mann-Whitney *U*-test for the number of hepatic forms observed under Matrigel in the presence or absence of HepaRG. Data are derived from a single experiment in which each point was derived from triplicate wells. (**b**) Assessment of the influence of Matrigel overlay on the ability of *P. cynomolgi* to infect simian primary hepatocytes cultured with HepaRG when overlaid or not with Matrigel and of the hepatic parasites' susceptibility to atovaquone treatment from day 5 to day 8; control cultures were not treated (–). (**c**) Quantification of the *P. cynomolgi* hepatic forms in Matrigel-overlaid cultures subsequent to treatment with atovaquone on two consecutive occasions, from day 5 to day 8 and then from day 15 to day 18. The gray bars represent the hypnozoites and the black bars represent the schizonts enumerated (mean ± s.d. from duplicate wells) and fixed 7 d later. Data presented in **b** and **c** were derived from duplicate wells of one experiment representative of two experiments initiated with independent *P. cynomolgi* sporozoite batches (data for day 25 were not available for the repeat experiment because of culture contamination).

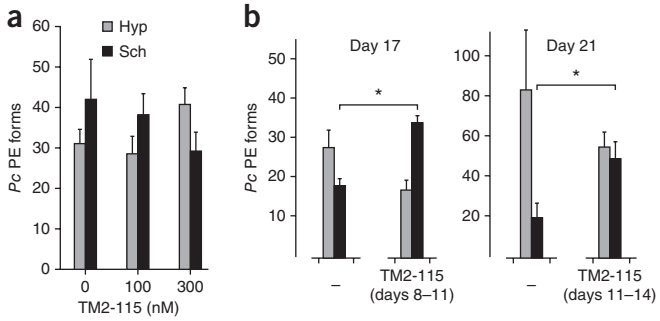

Figure 3 Induction of hypnozoite activation by a histone methyltransferase inhibitor. (**a**) *P. cynomolgi*–infected cultures (under Matrigel) treated from day 5 to day 8 after sporozoite inoculation with 100 nM or 300 nM TM2-115. Data are expressed as mean ± s.d. from triplicate wells of one experiment representative of two experiments initiated with independent *P. cynomolgi* sporozoite batches. (**b**) Effect of treatment with TM2-115 on the fate of cultured *P. cynomolgi* hepatic stages under Matrigel. Two cultures initiated with independent batches of *P. cynomolgi* sporozoites treated with 10 nM TM2-115 from day 8 to day 11 and fixed on day 17 (left) or from day 11 to day 14 and fixed on day 21 (right); control cultures were maintained in normal medium (–). In both **a** and **b**, gray bars represent the hypnozoites and black bars represent the schizonts enumerated (mean ± s.d. from triplicate wells). $^*P = 0.05$ by one-tailed Mann-Whitney *U*-test for the number of schizont forms observed on day 17 or on day 21 after treatment or not with TM2-115.

Taken together, these data demonstrate that the observed uninucleate *P. cynomolgi* hepatic forms are quiescent and nondividing and can persist for more than 30 d, during which time they activate gradually to yield normally developing forms that fully mature to release merozoites. These phenomena, persistence and activation, are the two salient biological characteristics of hypnozoites.

Screening for epigenetic control in hypnozoites

It was recently speculated that epigenetic control, namely repressive histone methylation marks akin to those that silence the *var* gene family in *P. falciparum*[23], might be implicated in the quiescence of hypnozoites[24]. This suggestion was made following the discovery of compounds (BIX-01294 and its derivative TM2-115) that irreversibly arrested the *in vitro* growth of erythrocytic parasites, most probably by inhibiting one or several of the parasite's histone lysine methyltransferases[24]. The availability of *in vitro* cultures in which hypnozoites persist and activate afforded us the opportunity to conduct a preliminary assessment of the effect of such compounds on hypnozoites. The half-maximal inhibitory concentrations (IC_{50}) of BIX-01294 and TM2-115 for the growth of *P. falciparum* blood-stage forms in continuous blood cultures are similar (IC_{50}, around 100 nM)[24]; however, when tested *in vivo*, TM2-115 was nine times more active than BIX-01294 at reducing parasitemia in a *P. berghei* malaria mouse model[24]. Thus, we opted to assess the activity of TM2-115 on the *P. cynomolgi* hepatic cultures.

In a first series of experiments, we added TM2-115 to the cultures from day 5 to day 8 after sporozoite addition, but there was no significant effect on the number and frequency of developing schizonts and quiescent hypnozoites (**Fig. 3a**). The TM2-115 concentrations used were equivalent to or exceeded the IC_{50} values measured for the *P. falciparum* blood-stage forms. Thus, we could not discount the possibility that the treatment with TM2-115 might have led to an arrest of the development of the hepatic forms, which might then have remained detectable at the time of fixation (**Fig. 3a**). We therefore conducted a second series of experiments in which we used a lower concentration of TM2-115 and where we specifically tested for an effect on the activation of the quiescent forms. We treated two *P. cynomolgi* hepatic cultures with atovaquone (from day 5 to day 8) to obtain hypnozoites, which we then subjected to 10 nM TM2-115 for a period of 6–7 d (day 8 to day 11 for one of the cultures and day 11 to day 14 for the other). For both cultures, we observed a significantly higher proportion of maturing hepatic forms in the wells treated with 10 nM TM2-115 as compared to the proportion observed in the nontreated control wells (**Fig. 3b**). Thus, it seems that exposure to sublethal concentrations of TM2-115 accelerated the rate of hypnozoite activation. These observations suggest that histone methylation–dependent changes in transcription may be involved in the control of hypnozoite quiescence.

DISCUSSION

We present a protocol for the long-term *in vitro* cultivation of *P. cynomolgi* hypnozoites in their natural host cell type, *M. fascicularis* primary hepatocytes (**Fig. 4**). Our conclusion that the nondividing PE forms observed in these cultures are indeed hypnozoites is based on the fact that they reproduce the characteristic features displayed by *Plasmodium* hypnozoites *in vivo*. We have previously shown that primaquine is active against the nondividing forms, before and after enrichment by atovaquone treatment[12]. The differential susceptibility of the normal hepatic stages and the quiescent hypnozoites to atovaquone that we previously noted in short-term cultures[12] also occured in the long-term cultures used here. We exploited this differential susceptibility to demonstrate that hypnozoites persisted *in vitro* beyond the initial 8–15 d necessary for the normal hepatic forms to mature fully and that they subsequently activated over the next 2–3 weeks to develop into mature hepatic schizonts containing merozoites. Hypnozoite activation occurred throughout the cultivation period, apparently at a constant rate. However, it is not clear that this is a true reflection of the situation *in vivo*, where factors absent under *in vitro* conditions (for example, immune status, cytokine balance, etc.) might influence hypnozoite activation. Further comparative investigations using sporozoites from strains that differ in their relapse patterns, such as the tropical and temperate *P. vivax* strains[1,2,25], will be needed to address this.

There are very few direct observations of hypnozoites. Morphological descriptions of hypnozoites have been previously confined to a limited number of micrographs from stained sections of livers infected with *P. cynomolgi* and *P. vivax* or electron micrographs of 5-d-old *P. cynomolgi* uninucleate forms presumed to be hypnozoites[6,26]. This is not surprising, given that in a host experimentally massively infected by a few million sporozoites, infected hepatocytes would constitute only an infinitesimal fraction of the hepatocytes (there is an average of 100 million hepatocytes per gram of human liver[27], an organ of about 1.5 kg in adults). A few nondividing forms have been previously observed in human hepatoma-derived HepG2-A16 cells between 6 and 12 d after inoculation with *P. vivax* sporozoites, but no further evidence could be provided that they were hypnozoites[28–32]. To date, the effect of drugs on hypnozoites has only been deduced by monitoring the emergence of blood-stage parasites in experimentally infected humans or monkeys. Interpretation of such data has often been confounded by the administration of blood schizontocides and by acquired immunity. The availability of *in vitro*–cultured hypnozoites may allow researchers to address directly the fundamental biological nature of this hitherto barely accessible parasite stage. Moreover,

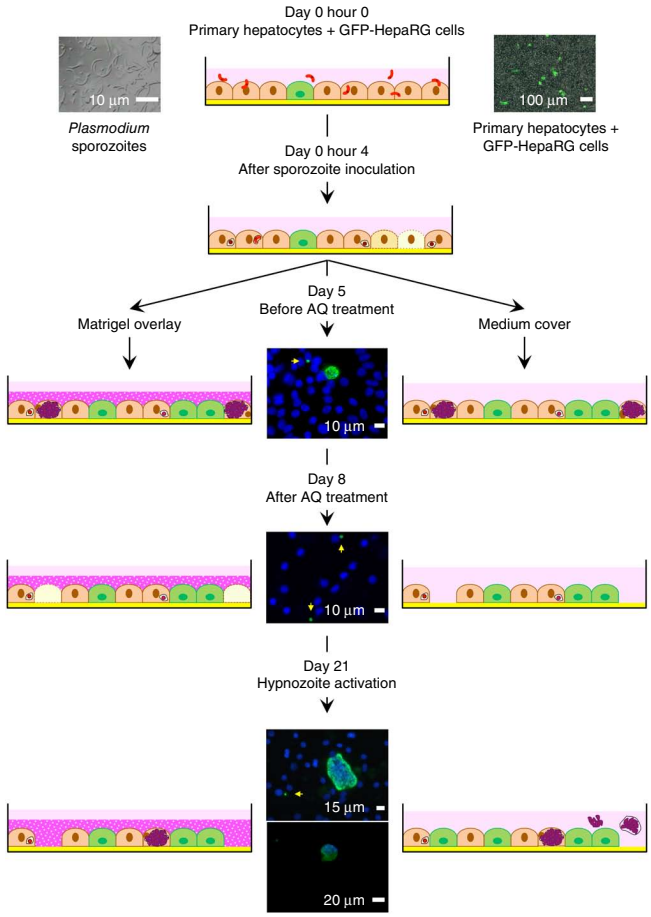

Figure 4 Schematic diagram of the long-term primary hepatocyte cultures. Sporozoites (red) from dissected infected *A. stephensi* salivary glands are added to primary hepatocytes that have been cultured with GFP-HepaRG cells (green) the previous day over a collagen layer (yellow). Four hours later, the cultures are washed free of sporozoites and overlaid with Matrigel (dark pink) or not if merosomes released from hepatic schizonts are to be collected. The sporozoites that invade hepatocyte productively form a parasitophorous vacuole. Over the next 2–4 d, some of the PE parasites initiate their maturation to form multinucleate schizonts (magenta), whereas others remain uninucleate (red) and do not develop further. The proliferating HepaRG cells generally replace hepatocytes wounded by sporozoite traversal or that die off with time, thereby maintaining contact between the cultured cells. On days 5 to 8, the cultures are subjected to atovaquone, a drug that differentially kills maturing PE parasites but not hypnozoites, resulting in cultures with a predominance of uninucleate hypnozoites. The cultures are then maintained for durations that can extend up to 1 month, during which time it is possible to observe and measure the appearance of maturing and fully mature schizonts and of merosomes in the supernatant (the parasites in the four central micrographs are stained green, with the hypnozoites indicated by arrows, and the hepatocyte and the HepaRG cell nuclei are stained blue). These cultures can be subjected to defined compounds or treatments whose activity and influence on the activation, maintenance or survival of the hypnozoites can be measured over the days or weeks that follow.

in vitro cultivation offers distinct advantages over *in vivo* observation, as it allows measuring various parameters such as batch-to-batch variations in sporozoite infectivity, the initial proportion of hypnozoites and, most crucially, the rate of hypnozoite activation. Nonetheless, the use of *in vitro* cultivation for high-throughput screening and other downstream investigations is mitigated by an unpredictable rate of infection inherent to batch-to-batch variations in sporozoite infectivity and in primary hepatocyte susceptibility to infection. It is expected that such variability would be reduced by the use of cryopreserved sporozoites[33] in conjunction with cryopreserved human or simian hepatocytes.

From a practical point of view, access to *in vitro*–cultured hypnozoites will be key to accelerate programs aimed at selecting compounds active against hypnozoites for further development as a radical cure for malaria infection. The cost of assaying selected compounds for activity against hypnozoites *in vitro* is several orders of magnitude lower than that of studies requiring lengthy follow-up of experimentally infected monkeys. Moreover, the readout from the *in vitro* tests provides a robust quantitative estimate of a compound's activity directly on the hypnozoite, an important parameter to guide compound development, that could only be indirectly inferred from blood parasitemia in monkey trials.

From a scientific point of view, *in vitro*–cultured hypnozoites make it possible to investigate the fundamental nature of their quiescence and to identify factors that govern their activation to resume normal hepatic development. A relatively homogeneous population of intact hypnozoites obtained through culturing of infected hepatocytes with atovaquone, or by FACS of hepatocytes infected with transgenic fluorescent *P. cynomolgi*[34], could be subjected to transcriptome analysis to establish whether they are distinguished by a particular expression profile. Admittedly, at present, the number of hypnozoites obtained *in vitro* is rather low. Nonetheless, current yields would be sufficient to probe for molecular processes that govern quiescence and the signals that are speculated to trigger hypnozoite activation[25]. Finally, the application of specific inhibitors of proteins or pathways known to regulate cellular division and differentiation could directly lead to defining the molecular signature of hypnozoite latency.

There is growing recognition that epigenetic control via histone lysine methylation, demonstrated for the variegated expression pattern of the *P. falciparum var* multigene family[23], extends to other processes in the intraerythrocytic cycle, as has been shown for a member of the putative transcription regulator apicomplexan Ap2 family[35]. It is likely that histone modification–mediated epigenetic control also has a role throughout the parasite life cycle, in particular when parasites transform from one morphologically and biologically distinct form to another. The data we present for an inhibitor (TM2-115) of histone methyltransferases, which contribute to the shaping of chromatin domain organization and regulate gene expression, illustrate the potential of *in vitro*–cultured hypnozoites as a system in which both fundamental and practical advances can be achieved. Although preliminary, our results suggest a distinct regulatory mechanism for the maturation of erythrocytic and hepatic parasites, as the former but not the latter were irreversibly arrested by exposure TM2-115. Whether the effect of the compound is directly mediated through a modification of the host or the parasite histone methylation status remains to be investigated. The accelerated rate of hypnozoite activation induced by exposure to low concentrations of TM2-115 was unexpected and suggests that screening strategies should aim at identifying not only compounds that eliminate hypnozoites but also those that induce them to resume their maturation. Indeed, a drug that precipitates hypnozoite activation could be a new treatment strategy: awakened hypnozoites would then become susceptible to drugs active against developing liver forms (for example, atovaquone and proguanil), and any blood-stage parasites that might emerge from surviving hepatic parasites could be

eliminated with drugs active against the erythrocytic stages. Such a 'wake-and-kill' combination therapy could then achieve radical cure.

Long-term *in vitro* cultivation of malarial hypnozoites is a major step forward in the quest to understand the biology of quiescence in *Plasmodium*, though this strategy yields only relatively low amounts of material for molecular and cellular investigations. On the other hand, the cultures do provide a needed tool to screen for and validate new and safe hypnozoitocidal drugs able to effect radical cure, a much-needed addition to the armamentarium essential to achieving sustainable control and elimination of one of humankind's oldest scourges.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.M., G.S. and A.S. designed the study. L.D., J.-F.F., A.L. and A.G. designed and performed the experiments. A.-M.Z., C.H.M.K., R.L.G., N.D.-B., G.-J.v.G., R.S., J.-C.V., L.H., M.J.F., T.T.D. and N.A.M. contributed essential materials. G.S. and D.M. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

1. Garnham, P.C.C. *Malaria Parasites and Other Haemosporidia* (Blackwell Scientific Publications, Oxford, 1966).
2. Coatney, G.R., Collins, W.E., Warren, M. & Contacos, P.G. *The Primate Malarias* (US Government Printing Office, Washington, DC, 1971).
3. Shortt, H.E. & Garnham, P.C.C. Demonstration of a persisting exo-erythrocytic cycle in *P. cynomolgi* and its bearing on the production of relapses. *BMJ* **1**, 1225–1228 (1948).
4. Krotoski, W.A. *et al.* Relapses in primate malaria: discovery of two populations of exoerythrocytic stages. Preliminary note. *BMJ* **280**, 153–154 (1980).
5. Krotoski, W.A. *et al.* Observations on early and late post-sporozoite tissue stages in primate malaria. I. Discovery of a new latent form of *Plasmodium cynomolgi* (the hypnozoite), and failure to detect hepatic forms within the first 24 hours after infection. *Am. J. Trop. Med. Hyg.* **31**, 24–35 (1982).
6. Cogswell, F.B. The hypnozoite and relapse in primate malaria. *Clin. Microbiol. Rev.* **5**, 26–35 (1992).
7. Shute, P.G. *et al.* A strain of *Plasmodium vivax* characterized by prolonged incubation: the effect of numbers of sporozoites on the length of the prepatent period. *Trans. R. Soc. Trop. Med. Hyg.* **70**, 474–481 (1976).
8. Mazier, D., Rénia, L. & Snounou, G. A pre-emptive strike against malaria's stealthy hepatic forms. *Nat. Rev. Drug Discov.* **8**, 854–864 (2009).
9. Wells, T.N.C., Burrows, J.N. & Baird, J.K. Targeting the hypnozoite reservoir of *Plasmodium vivax*: the hidden obstacle to malaria elimination. *Trends Parasitol.* **26**, 145–151 (2010).
10. Schmidt, L.H. *et al. Plasmodium cynomolgi* infections in the rhesus monkey. *Am. J. Trop. Med. Hyg.* **31**, 609–703 (1982).
11. Kocken, C.H.M. *et al.* Statistical model to evaluate *in vivo* activities of antimalarial drugs in a *Plasmodium cynomolgi*-macaque model for *Plasmodium vivax* malaria. *Antimicrob. Agents Chemother.* **53**, 421–427 (2009).
12. Dembélé, L. *et al.* Towards an *in vitro* model of *Plasmodium* hypnozoites suitable for drug discovery. *PLoS ONE* **6**, e18162 (2011).
13. Bray, R.S. *Studies on the Exo-erythrocytic Cycle in the Genus Plasmodium* (Lewis & Co. Ltd, London, 1957).
14. Prudêncio, M., Rodriguez, A. & Mota, M.M. The silent path to thousands of merozoites: the *Plasmodium* liver stage. *Nat. Rev. Microbiol.* **4**, 849–856 (2006).
15. Guguen-Guillouzo, C. & Guillouzo, A. General review on *in vitro* hepatocyte models and their applications. *Methods Mol. Biol.* **640**, 1–40 (2010).
16. Clement, B. *et al.* Long-term co-cultures of adult human hepatocytes with rat liver epithelial cells: modulation of albumin secretion and accumulation of extracellular material. *Hepatology* **4**, 373–380 (1984).
17. Mazier, D. *et al.* Cultivation of the liver forms of *Plasmodium vivax* in human hepatocytes. *Nature* **307**, 367–369 (1984).
18. Gripon, P. *et al.* Infection of a human hepatoma cell line by hepatitis B virus. *Proc. Natl. Acad. Sci. USA* **99**, 15655–15660 (2002).
19. Sturm, A. *et al.* Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science* **313**, 1287–1290 (2006).
20. Dunn, J.C., Yarmush, M.L., Koebe, H.G. & Tompkins, R.G. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J.* **3**, 174–177 (1989).
21. Kono, Y., Yang, S. & Roberts, E.A. Extended primary culture of human hepatocytes in a collagen gel sandwich system. *In Vitro Cell. Dev. Biol. Anim.* **33**, 467–472 (1997).
22. Bissell, D.M., Arenson, D.M., Maher, J.J. & Roll, F.J. Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. *J. Clin. Invest.* **79**, 801–812 (1987).
23. Lopez-Rubio, J.J., Mancio-Silva, L. & Scherf, A. Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. *Cell Host Microbe* **5**, 179–190 (2009).
24. Malmquist, N.A., Moss, T.A., Mecheri, S., Scherf, A. & Fuchter, M.J. Small-molecule histone methyltransferase inhibitors display rapid antimalarial activity against all blood stage forms in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA* **109**, 16708–16713 (2012).
25. White, N.J. Determinants of relapse periodicity in *Plasmodium vivax* malaria. *Malar. J.* **10**, 297 (2011).
26. Atkinson, C.T., Millet, P.G., Collins, W.E. & Aikawa, M. Localization of circumsporozoite antigen in exoerythrocytic schizonts of *Plasmodium cynomolgi*. *Am. J. Trop. Med. Hyg.* **40**, 131–140 (1989).
27. Barter, Z.E. *et al.* Scaling factors for the extrapolation of *in vivo* metabolic drug clearance from *in vitro* data: reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. *Curr. Drug Metab.* **8**, 33–45 (2007).
28. Hollingdale, M.R., Collins, W.E., Campbell, C.C. & Schwartz, A.L. *In vitro* culture of two populations (dividing and nondividing) of exoerythrocytic parasites of *Plasmodium vivax*. *Am. J. Trop. Med. Hyg.* **34**, 216–222 (1985).
29. Hollingdale, M.R., Collins, W.E. & Campbell, C.C. *In vitro* culture of exoerythrocytic parasites of the North Korean strain of *Plasmodium vivax* in hepatoma cells. *Am. J. Trop. Med. Hyg.* **35**, 275–276 (1986).
30. Liu, D., Luo, S., Ye, B., Shu, H. & Fu, R. Demonstration of *in vitro* cultured exoerythrocytic schizonts and hypnozoites of *Plasmodium vivax* (southern China isolate) by an immunoperoxidase antibody technique. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **13**, 86–88 (1995).
31. Shu, H., Lou, S., Liu, D. & Fu, R. [Observation on hypnozoite of different isolates of *Plasmodium vivax* in cultured materials]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **13**, 185–188 (1995).
32. Chattopadhyay, R. *et al.* Establishment of an *in vitro* assay for assessing the effects of drugs on the liver stages of *Plasmodium vivax* malaria. *PLoS ONE* **5**, e14275 (2010).
33. Hoffman, S.L. *et al.* Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria. *Hum. Vaccin.* **6**, 97–106 (2010).
34. Voorberg-van der Wel, A. *et al.* Transgenic fluorescent *Plasmodium cynomolgi* liver stages enable live imaging and purification of malaria hypnozoite-forms. *PLoS ONE* **8**, e54888 (2013).
35. Painter, H.J., Campbell, T.L. & Llinás, M. The Apicomplexan AP2 family: integral factors regulating *Plasmodium* development. *Mol. Biochem. Parasitol.* **176**, 1–7 (2011).

ONLINE METHODS

Ethics. Human hepatocytes were isolated from liver segments taken from adult patients (after oral informed consent was obtained) undergoing partial hepatectomy as part of their medical treatment (Service de Chirurgie Digestive, Hépato-Bilio-Pancréatique et Transplantation Hépatique, Hôpital Pitié-Salpêtrière, Paris, France). The collection and use of this material for the purposes of the study presented here were undertaken in accordance with French national ethical guidelines under article L. 1121-1 of the code de la santé publique. Given that the tissue samples are classed as surgical waste, that they were used anonymously (the patient's identity is inaccessible to the researchers) and that they were not in any way genetically manipulated, article L. 1211-2 stipulates that their use for research purposes is allowed provided that the patient does not express any opposition to this to the surgeon before surgery after being informed of the nature of the research in which they might be potentially involved. Within this framework, the collection and use of this material was furthermore approved by the Institutional Review Board (Comité de Protection des Personnes) of the Centre Hospitalo-Universitaire Pitié-Salpêtrière, Assistance Publique–Hôpitaux de Paris, France.

All the experimental procedures needed to obtain sporozoites from *P. cynomolgi*–infected *Macaca mulatta* that were performed at the Biomedical Primate Research Centre (BPRC) were carried out under protocols approved by the Animal Ethics Committee (DEC) according to Dutch laws. A male adult cynomolgus macaque >6 years old of 9 kg in weight (*M. fascicularis*) was imported from Mauritius and housed in the facilities of the Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA) (Fontenay-aux-Roses, France). Experimental procedures were conducted in strict accordance with the recommendations of the European guidelines for the care and use of laboratory animals (European directive 86/609/EEC, OJ, L358, December 18, 1986). In addition, the CEA and BPRC facilities were accredited (assurance identification numbers A5826-01 and A5539-01) by the US National Institutes of Health (USA) Office of Laboratory Animal Welfare. Furthermore, the Council of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) has awarded BPRC full accreditation. Livers were obtained at necropsy from animals involved in unrelated studies that required euthanasia. The protocols and the use of hepatocytes for the purpose of the work described herein were approved by the Ethical Animal Committee of the CEA (permit number A 92-032-02).

Drugs. Atovaquone: 2-[*trans*-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthalenedione (Laboratoire GlaxoSmithKline, France). TM2-115 was synthesized according to well-established procedures[24,36].

Parasites. *P. falciparum* (strain NF54) sporozoites were obtained from infected salivary glands of *Anopheles stephensi* 14–21 d after an infective blood meal on a membrane-based feeder system (Department of Medical Microbiology, University Medical Centre St. Radboud, Nijmegen, The Netherlands). *P. cynomolgi* (M strain) sporozoites were obtained from infected *A. stephensi* salivary glands collected 14–35 d after a blood meal on the blood of an infected *M. mulatta*, using membrane-based glass feeders (Biomedical Primate Research Centre, Rijswijk, The Netherlands).

Infected salivary glands were removed by hand dissection and crushed in a potter allowing sporozoite extraction. The parasites were recovered after filtration through a 40-µm filter (Cell Strainer, Becton Dickinson) and a 2-min centrifugation at 15,000*g*.

Primary hepatocytes. Human hepatocytes were isolated from liver segments obtained from adult patients undergoing partial hepatectomy. Simian hepatocytes were isolated from liver segments collected from healthy *M. fascicularis*. All hepatocytes were isolated using collagenase perfusion as previously described[37]. Briefly, the hepatic segments were successively perfused at a constant flow rate of around 1 ml per gram of tissue per min, accordingly to the size of the hepatic fragment, with 500 ml of 50 mM HEPES and 0.5 mM EGTA in HBSS buffer (Life Technologies SAS, Saint-Aubain, France), then with 250 ml HBSS-HEPES and finally with 200 ml 0.02% collagenase type IV and 0.05% $CaCl_2$ (Sigma-Aldrich, Saint-Quentin Fallavier, France) in HBSS-HEPES buffer, with recirculation, until appearance of marbling and softening of the tissue, indicating that dissociation is proceeding efficiently. The hepatic fragments were then dissociated in a vessel and viable cells recovered after filtration through a 40-µm mesh Cell Strainer (BD Biosciences) and a centrifugation over a 36% Percoll cushion. Simian hepatocytes were immediately cryopreserved with a Nicool-FREEZAL (Air Liquide Santé, Marnes La Vallée, France) controlled-rate freezer at a rate of −2 °C per min until −4 °C, then at −1 °C per min until −40 °C with an automatic controlled seeding point at −4 °C and finally at −10 °C per min until −140 °C. Once at −140 °C, the cryovials were transferred for storage in liquid nitrogen. They were then used when needed after fast thawing at 37 °C. Human primary hepatocytes were used fresh in the cultures.

HepaRG cell lines. HepaRG cells, derived from a female patient with hepatocarcinoma, are capable of differentiating into biliary epithelial cells and hepatocytes[18]. This cell line was obtained from C. Guguen-Guillouzo at Biopredic International, Rennes, France. The GFP-expressing HepaRG line was obtained by transduction of the HepaRG cell line with the GFP lentiviral vector, pTRIP-CMV-GFP ΔU3 vector[38] (provided by P. Ravassard, le Centre de Recherche de l'Institut du Cerveau et de la Moelle Epinière, UMRS 975, Paris, France). HepaRG cells were transduced by adding 1 µl of concentrated lentiviral preparation per ml of culture. After a 3-h incubation period, cells were washed and incubated at 37 °C and 5% CO_2. After 2 weeks of expansion, the GFP-expressing cells were selected as a nonclonal population by cell sorting based on GFP expression on a BD FACSAria IIu using a 488-nm laser (Flow Cytometry Core CyPS, Université Pierre et Marie Curie, Pitié-Salpêtrière Hospital, Paris, France). The GFP expression allowed easy and fast discrimination of this cell line in the cultures from nonfluorescent primary hepatocytes using fluorescence microscopy.

Medium. William's Medium E (Life Technologies) was supplemented with 10% FCS (Perbio), 5×10^{-5} M hydrocortisone hemisuccinate (Upjohn Laboratories SERB, France), 5 µg per ml insulin (Sigma), 2 mM L-glutamine and 200 U per ml penicillin, 200 µg per ml streptomycin (Life Technologies).

***In vitro* cultures.** Hepatocytes, alone or in culture with HepaRG cells, were seeded at a final cell density of 250,000 cells per cm^2 in 48-well plates coated with collagen I (BD BioSciences). For the cultures, both cell types were seeded together at the ratio of 1 HepaRG cell for 30 primary hepatocytes. The cultures were incubated at 37 °C with 5% CO_2 until infection with the sporozoites.

Infection and drug assays. 1×10^5 *P. falciparum* or *P. cynomolgi* sporozoites resuspended in complete medium were added to their respective host cell cultures (human or simian hepatocytes alone or cultured with HepaRG). The infected culture plates were centrifuged 10 min at 900*g*, allowing fast parasite sedimentation, and then incubated at 37 °C with 5% CO_2. At 3 h after infection, the medium was removed, and Matrigel (BD Biosciences) was added as per the manufacturer's recommendations. After 30 min at 37 °C, medium was added above the polymerized Matrigel layer. The medium was thereafter changed every 48 to 78 h. At defined time points, the cultures were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in 1× PBS for 10 min.

For drug assays, the cultures were treated on the days indicated by adding the drug to the medium (for example, every day from day 5 to day 8 after infection with 551 nM of atovaquone). At the end of all treatment periods, the cultures were maintained in drug-free medium.

Merosome isolation. Culture supernatant was collected at every change of medium and centrifuged 5 min at 2,000*g* at room temperature. The medium was then removed and the pellet potentially containing merosomes was resuspended in 3–5 µl PBS and deposited on IFI slides. Once dry, the slides were fixed for 10 min with cold methanol before proceeding to parasite immunolabeling.

Parasite quantification. Preerythrocytic (PE) parasites were detected by immunofluorescence. Following fixation, the cultures plates and the merosome slides were specifically stained with an anti–*P. falciparum* HSP70

polyclonal serum (raised in our laboratory in BALB/c mice immunized with recombinant *P. falciparum* heat shock protein 70 (HSP70) fused to GST; the serum cross-reacts with the *P. cynomolgi* HSP70), by incubation for 1 hour at room temperature with the serum diluted 1:2,000 in PBS. Binding was revealed with (red) Alexa 594– or (green) Alexa 488–conjugated goat anti-mouse immunoglobulin (A11029, or A11032, respectively, Invitrogen) diluted 1:500. Parasite and cell nuclei were stained with 1 µg per ml of DAPI (Sigma). Parasites were enumerated under a fluorescence microscope with a 200× magnification (Leica DMI3000 B). Images were obtained from a Leica DMI4000 B microscope with a 400× magnification using a Zeiss Axiocam Mrc5 camera and Axiovision software.

Statistical analyses. The numbers of PE parasites observed in different groups were compared using the nonparametric Mann-Whitney *U*-test (GraphPad Prism software). The populations compared were derived from the same initial culture but were considered unrelated because the measurements were conducted on distinct sets of individual wells that were either treated or untreated. A *P* value of 0.05 or less was considered to be statistically significant. No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

36. Liu, F. *et al.* Protein lysine methyltransferase G9a inhibitors: design, synthesis, and structure activity relationships of 2,4-diamino-7-aminoalkoxy-quinazolines. *J. Med. Chem.* **53**, 5844–5857 (2010).
37. Silvie, O. *et al.* Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. *Nat. Med.* **9**, 93–96 (2003).
38. Castaing, M. *et al.* Efficient restricted gene expression in beta cells by lentivirus-mediated gene transfer into pancreatic stem/progenitor cells. *Diabetologia* **48**, 709–719 (2005).

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