New Approach for High-Throughput Screening of Drug Activity on *Plasmodium* Liver Stages

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***Plasmodium* liver stages represent potential targets for antimalarial prophylactic drugs. Nevertheless, there is a lack of molecules active on these stages. We have now developed a new approach for the high-throughput screening of drug activity on *Plasmodium* liver stages in vitro, based on an infrared fluorescence scanning system. This method allowed us to count automatically and rapidly *Plasmodium*-infected hepatocytes, using different hepatic cells and different *Plasmodium* species, including *Plasmodium falciparum*. This new technique is well adapted for high-throughput drug screening and should facilitate the identification of new antimalarial compounds active on *Plasmodium* liver stages.**

Malaria remains one of the major human infectious diseases and is responsible for millions of deaths each year. As resistance to available drugs increases, there is an urgent need to identify new molecules. Targeting *Plasmodium* liver stage development is a valuable strategy for preventing malaria. Indeed, liver stages precede the appearance of blood stages, which are responsible for clinical symptoms and complications. The development of pharmaceutical products inhibiting the growth of *Plasmodium* hepatic forms is relevant for two main reasons: first, such compounds could be used as causal prophylactic agents by people exposed for a limited duration in an area where malaria is endemic (e.g., refugees and travelers) and, second, the emergence of drug-resistant strains is theoretically limited during the liver phase because of the lower parasitic load compared to the blood phase. Although hepatic stages provide attractive targets for antimalarial chemotherapy, the list of effective and widely applied drugs is still limited. The only currently available prophylactic drugs are atovaquone and two related drugs, primaquine and tafenoquine. Atovaquone has been demonstrated to be efficient on the first step of the parasite development (2); however, its use is limited by its unaffordable cost. Because of its hematological toxicity, the use of primaquine is restricted, particularly in Africa because of the frequency of G6PD deficiency (12). The identification of new drugs is slowed down by the lack of a reliable and sensitive method allowing a high-throughput screening. In vitro drug sensitivity assays are largely based on evaluating the number of liver schizonts in sporozoite-infected cultures (7, 10, 15). Even if alternative methods have been proposed (6), the number of

infected cells is usually determined by microscopy analysis. Microscope-based quantification of infected cells is a very time-consuming method. Therefore, we have developed a new approach based on infrared fluorescence detection to automatically and rapidly quantify *Plasmodium* liver schizonts in vitro.

Parasites and cells. *Plasmodium berghei* (ANKA strain), *P. yoelii* (265BY strain), and *P. falciparum* (NF54 strain) sporozoites were obtained from dissection of infected *Anopheles stephensi* mosquito salivary glands. Human hepatocarcinoma HepG2-A16 cells were cultured in Dulbecco modified Eagle medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Biowest, Nuaille, France) and 2 mM glutamine, 50 µg of penicillin/ml, 50 µg of streptomycin/ml, and 100 µg of neomycin/ml (Invitrogen). HepG2 stably expressing CD81 (HepG2/CD81) (13a) were cultured in rat tail collagen I (Becton Dickinson, Le Pont de Claix, France) coated dishes in DMEM supplemented as described above. Primary mouse hepatocytes were isolated as described previously (11) and cultured in William's E medium (Invitrogen) supplemented as described above. Primary human hepatocytes were isolated and cultured as previously described (13).

***Plasmodium* liver stage cultures.** HepG2 cells or primary hepatocytes were plated in eight-chamber plastic Lab-Tek slides (Nalge Nunc International, Cergy Pontoise, France) for 24 h (except for human hepatocytes, 3 to 7 days) prior to inoculation with variable numbers (as indicated) of *P. berghei*, *P. yoelii*, or *P. falciparum* sporozoites. Sporozoite-inoculated culture plates were centrifuged for 5 min at 1,500 rpm at 4°C in order to enhance the infection rate as described by others (4). After 3 h at 37°C, the cultures were washed and further incubated in fresh medium for 48 h (*P. berghei* and *P. yoelii*) or 5 days (*P. falciparum*) before quantification of infected cells in triplicate wells.

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FIG. 1. HepG2-A16 cells (25×10^3) plated in a 96-well microplate were infected with *P. berghei* sporozoites (10^4) and cultured for 48 h before immunolabeling with anti-HSP70 antibodies, followed by the addition of a secondary antibody coupled with Alexa Fluor 680. The microplate was then scanned with the Odyssey infrared imaging system. Typical resulting images are shown for a noninfected well (left) and for a *P. berghei*-infected well (right). Each spot detected by the infrared scanner corresponds to one schizont.

Quantification of infected cells. After fixation of the cultures with cold methanol, intracellular parasites were stained with a mouse polyclonal serum raised against *Plasmodium* heat shock protein 70 (HSP70) (11), followed by goat anti-mouse fluorescein isothiocyanate (FITC) conjugate (Sigma) and goat anti-mouse Alexa Fluor 680 conjugate (Invitrogen, Molecular Probes). The number of parasites was then determined by fluorescence microscopy with 488-nm excitation or by using the Odyssey Infrared Imaging System (LI-COR Biosciences) with a 680-nm wavelength of excitation, a 700-nm wavelength of detection, and a 21- μ m resolution. Infrared fluorescence images generated by the scanner were then analyzed by using a colony counter software (Microtec Niton) to automatically determine the number of fluorescent spots, each spot corresponding to one parasite.

Drug assays. Cultures of HepG2/CD81 cells (25 000 cells/well) infected by *P. yoelii* (20 000 sporozoites/well) were treated with atovaquone (from 0.25 to 64 nM) or chloroquine (from 0.5 nM to 5 μ M). Stock solutions of drugs were prepared at 5 mM (chloroquine in ethanol and atovaquone in 0.1 M NaOH) and diluted in Dulbecco modified Eagle medium supplemented as described above. Diluted drugs were dispensed into triplicate wells in 96-well microplates. Cultures were then analyzed with the odyssey scanner as described above at 48 h postinfection, and the dose-response curves of the two drugs were determined compared to control preparation wells.

Results. The Odyssey Infrared Imaging System is a new scanning system that allows infrared fluorescence quantification (1). This system is particularly well adapted to in-cell quantification because of the absence of cellular autofluorescence background in infrared (1). In order to test whether this system is suitable for detecting *Plasmodium* liver schizonts, we first performed experiments with *P. berghei*-infected HepG2-A16 cells. Two days after infection, cultures were fixed, and the parasites were labeled with anti-HSP70 serum followed by secondary Alexa Fluor 680 antibodies. Infrared fluorescence was then detected by the Odyssey scanner using the maximal resolution (21 μ m), which is consistent with the size of *Plasmodium* liver schizonts obtained in vitro (3, 7). In the images generated by the scanner, schizonts appear as spots that are absent from noninfected wells (Fig. 1). From these images the number of spots can be easily determined automatically by using colony counter software.

FIG. 2. Host cells were infected with sporozoites and cultured for 48 h (A to C) or 5 days (D) before immunolabeling with anti-HSP70 antibodies, followed by secondary antibodies coupled with FITC and Alexa Fluor 680. The cultures were then examined both by fluorescence microscopy and by using the Odyssey scanner to determine the number of schizonts in triplicate wells. The results are expressed as means $\pm$ the standard deviations and were statistically analyzed for correlation. (A) HepG2-A16 (15×10^4) plated in eight-chamber Lab-Tek wells and infected with *P. berghei* sporozoites (correlation coefficient $R^2 = 0.975$; $P < 0.001$). (B) HepG2/CD81 (15×10^4) plated in a 96-well microplate and infected with *P. yoelii* sporozoites ($R^2 = 0.869$; $P < 0.001$). (C) Primary mouse hepatocytes (9×10^4) plated in eight-chamber Lab-Teks and infected with *P. yoelii* sporozoites ($R^2 = 0.921$; $P < 0.001$). (D) Primary human hepatocytes (18×10^4) plated in a eight-chamber Lab-Teks and infected with *P. falciparum* sporozoites ($R^2 = 0.890$; $P < 0.001$).

We then evaluated the concordance of this new quantitative method with the standard fluorescence microscopy method. HepG2-A16 cells plated in eight-chamber Lab-Teks were infected with increasing numbers of *P. berghei* sporozoites and incubated at 37°C for 48 h before fixation. Schizonts were then labeled with anti-HSP70 antibodies (11), followed by two secondary antibodies coupled with FITC (for microscopy quantification) or Alexa Fluor 680 (for infrared detection). The same slides were read by microscopy and using the Odyssey system. With both methods, the number of liver schizonts was correlated with the number of sporozoites inoculated. The number of parasites detected by using the Odyssey scanner closely matched the numbers determined by conventional fluorescence microscopy (Fig. 2A). We also performed experiments with another rodent parasite, *P. yoelii*, using HepG2/CD81 cells as target host cells and 96-well microplates instead of eight-chamber Lab-Teks. Hepatocyte CD81 is required for *P. yoelii* sporozoite infection (14), and expression of CD81 in HepG2 cells was found to be sufficient to confer susceptibility to *P. yoelii* sporozoites (13a). As shown in Fig. 2B, the infrared

FIG. 3. Activity of atovaquone (A) and chloroquine (B) on *P. yoelii* infection in HepG2/CD81 cells. HepG2/CD81 cells (25×10^3) infected by *P. yoelii* (20×10^3 sporozoites) were treated for 48 h before infrared analysis by the Odyssey scanner in triplicate wells. Atovaquone inhibits infection, with an IC_{50} of 3.95 nM, whereas chloroquine is inactive at concentrations up to 5 μ M.

approach allowed the accurate determination of the number of *P. yoelii* schizonts compared to the standard microscope method.

Because metabolic activities may differ in hepatoma cell lines compared to primary hepatocytes (9), we investigated whether our new approach could be used to detect *Plasmodium* liver schizonts in primary hepatocyte cultures, which probably constitute a more suitable experimental system with regard to drug metabolism. Primary hepatocytes were isolated from a mouse liver and infected with increasing numbers of *P. yoelii* sporozoites before analysis by microscopy and infrared detection. No significant autofluorescence background was observed in primary hepatocytes in infrared wavelengths, whereas hepatocytes have a higher autofluorescence background in the visible spectrum compared to cell lines (data not shown). As shown in Fig. 2C, the numbers of *P. yoelii*-infected hepatocytes determined by using the infrared detection system closely matched those obtained by counting the parasites under a fluorescence microscope. This result demonstrates that primary hepatocytes can be used for screening with the infrared detection system.

Finally, since rodent malaria parasites may differ from human parasites in terms of drug sensitivity, we evaluated whether the infrared detection system could be used with the human malaria parasite *P. falciparum*. Unlike rodent parasites, *P. falciparum* sporozoites only infect primary human (or non-human primate) hepatocytes but not hepatoma cell lines (5, 8). Primary human hepatocytes were infected with variable numbers of sporozoites and cultured for 5 days before we quantified the schizonts by both microscopy and the Odyssey system. As shown in Fig. 2D, the numbers of *P. falciparum*-infected cells obtained were similar with both methods.

The results show the ability of the odyssey method to detect and quantify accurately *Plasmodium* liver schizonts in vitro. We then investigated the ability of the new approach to evaluate drug activity. Cultures of HepG2/CD81 cells in 96-well microplates were infected with *P. yoelii* sporozoites and treated with atovaquone or chloroquine. Atovaquone, but not chloroquine, is active on pre-erythrocytic parasites (2, 9). Infection was then monitored by using the Odyssey method. The dose-response curve of atovaquone with a 50% inhibitory concentration (IC_{50}) of 3.95 nM (Fig. 3A) was consistent with previous results obtained by Davies et al. for *P. berghei* in HepG2 cells (2). As expected, chloroquine was inactive on *P. yoelii*

development even at high concentrations up to 5 μ M (Fig. 3B). These results illustrate the ability of the Odyssey method to evaluate drug activity on *Plasmodium* liver stages.

Conclusion. Using the Odyssey infrared imaging system combined with a colony counter, we could automatically and accurately quantify in vitro *Plasmodium* liver infection. This new method is suitable for multiple experimental models, using diverse host target cell types and *Plasmodium* species, including *P. falciparum*. We believe that this method could also be applied to other types of screening, such as high-throughput RNA interference approaches for host-parasite interactions studies and to other pathogens when individual infected cells need to be counted. Moreover, the method is suitable to any culture format that can be read by the scanner (Lab-Teks; 6-, 24-, or 96-well plates). Quantification of a 96-well microplate using the Odyssey system takes about 90 min (including 1 h of automatic scanner detection), whereas fluorescence microscopy analysis would take at least 6 to 8 h. Finally, both methods are nonexclusive since, as shown here, parasites can be immunolabeled with two different secondary antibodies, enabling analysis by both infrared detection and conventional fluorescence microscopy. In conclusion, this rapid, simple and sensitive approach is perfectly adapted for high-throughput drug screening and should greatly facilitate the identification of new antimalarial compounds active on *Plasmodium* liver stages.

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REFERENCES

1. Chen, H., J. Kovar, S. Sissons, K. Cox, W. Matter, F. Chadwell, P. Luan, C. J. Vlahos, A. Schutz-Geschwender, and D. M. Olive. 2005. A cell-based immunocytochemical assay for monitoring kinase signaling pathways and drug efficacy. *Anal. Biochem.* **338**:136–142.
2. Davies, C. S., M. Pudney, J. C. Nicholas, and R. E. Sinden. 1993. The novel hydroxynaphthoquinone 566C80 inhibits the development of liver stages of *Plasmodium berghei* cultured in vitro. *Parasitology* **106**(Pt. 1):1–6.
3. Davies, C. S., A. S. Suhrbier, L. A. Winger, and R. E. Sinden. 1989. Improved techniques for the culture of the liver stages of *Plasmodium berghei* and their relevance to the study of causal prophylactic drugs. *Acta Leiden* **58**:97–113.
4. Gruner, A. C., S. Hez-Deroubaix, G. Snounou, N. Hall, C. Bouchier, F. Letourneur, I. Landau, and P. Druilhe. 2005. Insights into the *P. y. yoelii* hepatic stage transcriptome reveal complex transcriptional patterns. *Mol. Biochem. Parasitol.* **142**:184–192.

5. **Hollingdale, M. R., E. H. Nardin, S. Tharavanij, A. L. Schwartz, and R. S. Nussenzweig.** 1984. Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells: an in vitro assay of protective antibodies. *J. Immunol.* **132**:909–913.
6. **Li, J., J. D. Zhu, A. Appiah, T. F. McCutchan, G. W. Long, W. K. Milhous, and M. R. Hollingdale.** 1991. *Plasmodium berghei*: quantitation of in vitro effects of antimalarial drugs on exoerythrocytic development by a rRNA probe. *Exp. Parasitol.* **72**:450–458.
7. **Mahmoudi, N., L. Ciceron, J. F. Franetich, K. Farhati, O. Silvie, W. Eling, R. Sauerwein, M. Danis, D. Mazier, and F. Derouin.** 2003. In vitro activities of 25 quinolones and fluoroquinolones against liver and blood stage *Plasmodium* spp. *Antimicrob. Agents Chemother.* **47**:2636–2639.
8. **Mazier, D., R. L. Beaudoin, S. Mellouk, P. Druilhe, B. Texier, J. Trosper, F. Miltgen, I. Landau, C. Paul, O. Brandicourt, et al.** 1985. Complete development of hepatic stages of *Plasmodium falciparum* in vitro. *Science* **227**:440–442.
9. **Millet, I. L., and Wallace Peters.** 1986. In vitro testing of antimalarial exoerythrocytic schizonticides in primary cultures of hepatocytes. *Mem. Inst. Oswaldo Cruz* **81**:135–141.
10. **Millet, P., I. Landau, D. Baccam, F. Miltgen, and W. Peters.** 1985. Cultivation of exo-erythrocytic schizonts of rodent *Plasmodium* in hepatocytes: a new experimental model for chemotherapy of malaria. *C. R. Acad. Sci. III* **301**:403–406.
11. **Renia, L., D. Mattei, J. Goma, S. Pied, P. Dubois, F. Miltgen, A. Nussler, H. Matile, F. Menegaux, M. Gentilini, and D. Mazier.** 1990. A malaria heat-shock-like determinant expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by non-parenchymal liver cells. *Eur. J. Immunol.* **20**:1445–1449.
12. **Shanks, G. D., and M. D. Edstein.** 2005. Modern malaria chemoprophylaxis. *Drugs* **65**:2091–2110.
13. **Silvie, O., J. F. Franetich, S. Charrin, M. S. Mueller, A. Siau, M. Bodescot, E. Rubinstein, L. Hannoun, Y. Charoenvit, C. H. Kocken, A. W. Thomas, G. J. Van Gemert, R. W. Sauerwein, M. J. Blackman, R. F. Anders, G. Pluschke, and D. Mazier.** 2004. A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. *J. Biol. Chem.* **279**:9490–9496.
- 13a. **Silvie, O., C. Greco, J. F. Franetich, A. Dubart-Kupperschmitt, L. Hannoun, G. J. van Gemert, R. W. Sauerwein, S. Levy, C. Boucheix, E. Rubinstein, and D. Mazier.** Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the *Plasmodium* species. *Cell. Microbiol.*, in press.
14. **Silvie, O., E. Rubinstein, J. F. Franetich, M. Prenant, E. Belnoue, L. Renia, L. Hannoun, W. Eling, S. Levy, C. Boucheix, and D. Mazier.** 2003. Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. *Nat. Med.* **9**:93–96.
15. **Sinden, R. E., A. Suhrbier, C. S. Davies, S. L. Fleck, K. Hodivala, and J. C. Nicholas.** 1990. The development and routine application of high-density exoerythrocytic-stage cultures of *Plasmodium berghei*. *Bull. W. H. O.* **68**(Suppl.): 115–125.