Adaptation of the [\textsuperscript{3}H]Hypoxanthine Uptake Assay for In Vitro-Cultured Plasmodium knowlesi Malaria Parasites


The zoonotic malaria parasite Plasmodium knowlesi has recently been established in continuous in vitro culture. Here, the Plasmodium falciparum [\textsuperscript{3}H]hypoxanthine uptake assay was adapted for P. knowlesi and used to determine the sensitivity of this parasite to chloroquine, cycloguanil, and clindamycin. The data demonstrate that P. knowlesi is sensitive to all drugs, with 50% inhibitory concentrations (IC\text/sub{50}) consistent with those obtained with P. falciparum. This assay provides a platform to use P. knowlesi in vitro for drug discovery.

In 2015, there were an estimated 214 million clinical cases of malaria which resulted in \textasciitilde438,000 deaths (1). Substantial funds have been invested in producing a malaria vaccine; however, the efficacy of experimental vaccines has been poor (2, 3), and as a result, vector control and drugs remain the mainstays for the prevention and treatment of malaria. While there has been a significant reduction in malaria-associated mortality and morbidity in recent years (1), there is concern that lack of sustained funding, together with insecticide and antimalarial drug resistance, will affect this progress (4). To prevent backward momentum in disease control and push toward the endgame strategy of malaria elimination, new chemotherapeutics with novel modes of action and activity against multiple species and life cycle stages are needed (5).

The ability to easily and rapidly assess the activity of new lead compounds against multiple Plasmodium species has been limited to date, as only Plasmodium falciparum has been amenable to routine, long-term, continuous in vitro culture (6). While there have been some recent improvements to in vitro culture techniques for Plasmodium vivax, the culture of this parasite is still limited by the requirement of reticulocytes, and the parasite density over long-term culture is low (7). However, the recent adaptation of the zoonotic malaria species Plasmodium knowlesi (8) to continuous in vitro culture in human erythrocytes (9–11) has changed this position. Although a routine drug sensitivity assay for P. knowlesi has not yet been established, the ability to culture this parasite species in vitro provides researchers with an unprecedented opportunity to rapidly test new drug leads against two human-infecting Plasmodium species.

In vitro assays for assessing malaria parasite growth inhibition are indispensable tools for the screening and evaluation of potential new drug leads and, also, for the surveillance of parasite drug resistance. A “gold standard” approach for assessing P. falciparum growth inhibition is the incorporation of [\textsuperscript{3}H]hypoxanthine into parasite nucleic acids (12). As Plasmodium parasites are unable to synthesize purines de novo, they must scavenge these metabolic precursors for growth. Thus, supplementation of parasite cultures with [\textsuperscript{3}H]hypoxanthine results in the incorporation of this radio-labeled purine into nucleic acids, permitting growth to be quantitated using a scintillation counter. While there are a number of other methods available to assess in vitro proliferation and growth inhibition of Plasmodium parasites (e.g., enzymatic assays, such as the parasite lactate dehydrogenase assay (13), and dye-based fluorescence assays, such as those that use SYBR green I or 4’,6-diamidino-2-phenylindole [DAPI] (14)), the [\textsuperscript{3}H]hypoxanthine incorporation assay remains a gold standard approach and is used as a reference for other approaches. In this study, the [\textsuperscript{3}H]hypoxanthine incorporation assay was assessed for use with P. knowlesi strain A1H.1 in vitro–adapted parasites (11).

The effect of starting parasitemia at two different hematocrits (1% versus 2%) was assessed by seeding P. knowlesi A1H.1 parasites into 96-well tissue culture plates, followed by the addition of [\textsuperscript{3}H]hypoxanthine (0.5 μCi/well). Cultures were then maintained under standard culture conditions (11) for 24 h. The length of the assay was also assessed by preparing additional plates labeled with [\textsuperscript{3}H]hypoxanthine at 24 h or 48 h and incubating these plates under standard culture conditions for a further 24 h. These assay durations correspond to approximately one (24 h), two (48 h), and three (72 h) asexual intraerythrocytic developmental cycles. The assays were stopped by freezing the assay plates at \textasciitilde20°C. [\textsuperscript{3}H]Hypoxanthine incorporation was then assessed by thawing and harvesting the well contents onto 1450 MicroBeta filter mats (Wallac, USA). Once air dried, the mats were analyzed using a Trilux MicroBeta liquid scintillation counter (PerkinElmer, USA). Each assay condition was assessed by performing three independent experiments in triplicate, and each assay plate included uninfected erythrocyte control wells (1% and 2% hematocrit) to account for background [\textsuperscript{3}H]hypoxanthine incorporation. Z-factors were calculated to assess assay quality (15).

No significant difference in [\textsuperscript{3}H]hypoxanthine incorporation was observed for cultures seeded at 1% (Fig. 1A) versus 2%
parasitemias, indicating excellent assays (15). However, when as-
72-h assays, Z-factors of 0.5 to 1.0 were obtained for all starting
matocrit (Z-factor of 0.87 were performed using a 0.25% starting parasitemia and 2% he-
and/or parasite death. For this reason, assays to assess drug activity
plateau and decline in some instances, suggesting overgrowth
in starting cultures are shown in Fig. S1 in the supplemental material.

FIG 1 Comparison of the effects of starting parasitemia, hematocrit, and assay duration on in vitro [3H]hypoxanthine incorporation by P. knowlesi A1H.1.
[3H]hypoxanthine incorporation (Log10-corrected counts per min) was determined for asynchronous P. knowlesi A1H.1-infected erythrocytes over a range of starting parasitemias at 1% (A) versus 2% (B) hematocrit. The assay durations were 24 h, 48 h, and 72 h. Data are presented as mean results
for 48 h or 72 h, the levels of [3H]hypoxanthine incorpora-

 Starting parasitemia (%) A 1% Haematocrit B 2% Haematocrit 24 h 48 h 72 h 24 h 48 h 72 h

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IC50 (±SD) (µM) at indicated time point (h)</th>
<th>P value for time point (h) comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.0109 (±0.0031)</td>
<td>0.0069 (±0.0022)</td>
</tr>
<tr>
<td>Cycloguanil</td>
<td>0.0039 (±0.0007)</td>
<td>0.0015 (±0.0005)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

a ND, not determined.
growth dynamics and drug action, including in vitro drug combination studies (isobolograms) and adaptation of assays such as the *Plasmodium falciparum* parasite reduction ratio (PRR) assay \(23\) to assess the speed of drug action on the parasite.

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**REFERENCES**