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

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The zoonotic malaria parasite *Plasmodium knowlesi* has recently been established in continuous *in vitro* culture. Here, the *Plasmodium falciparum* [^{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_{50}s) 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 ~438,000 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 [^{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 [^{3}H]hypoxanthine results in the incorporation of this radiolabeled 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 [^{3}H]hypoxanthine incorporation assay remains a gold standard approach and is used as a reference for other approaches. In this study, the [^{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 [^{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 [^{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 −20°C. [^{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 [^{3}H]hypoxanthine incorporation. Z-factors were calculated to assess assay quality (15).

No significant difference in [^{3}H]hypoxanthine incorporation was observed for cultures seeded at 1% (Fig. 1A) versus 2%

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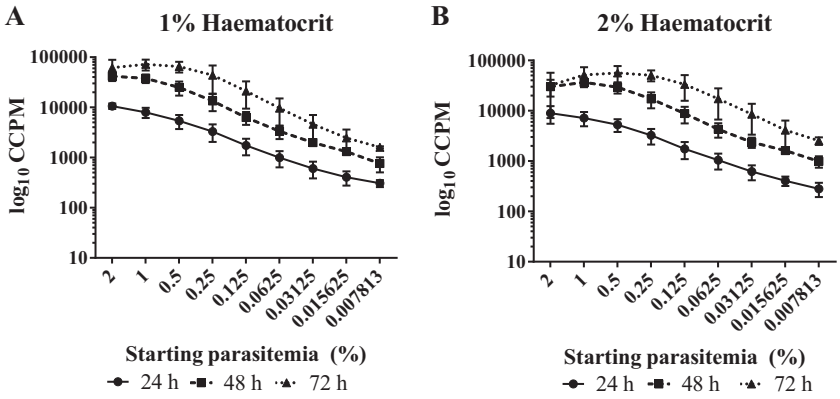

FIG 1 Comparison of the effects of starting parasitemia, hematocrit, and assay duration on *in vitro* [^{3}H]hypoxanthine incorporation by *P. knowlesi* A1H.1. [^{3}H]hypoxanthine incorporation (Log_{10}-corrected counts per min) was determined for asynchronous *P. knowlesi* A1H.1-infected erythrocytes 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 ± standard deviations for three independent experiments, each carried out in triplicate wells. The proportions of rings, trophozoites, and schizonts (as percentages of total parasitemia) in starting cultures are shown in Fig. S1 in the supplemental material.

(Fig. 1B) hematocrit over 24 h ($P > 0.45$), 48 h ($P > 0.06$), or 72 h ($P > 0.09$). As would be expected, the 24-h assays yielded comparatively low levels of [^{3}H]hypoxanthine incorporation compared to the levels obtained in the 48-h and 72-h assays (Fig. 1A and B), with a starting parasitemia of below 0.0625% resulting in Z-factors of <0.5 (marginal assay conditions). For the 48-h and 72-h assays, Z-factors of 0.5 to 1.0 were obtained for all starting parasitemias, indicating excellent assays (15). However, when assessed for 48 h or 72 h, the levels of [^{3}H]hypoxanthine incorporation of cultures with a starting parasitemia of ≥0.5% were seen to plateau and decline in some instances, suggesting overgrowth and/or parasite death. For this reason, assays to assess drug activity were performed using a 0.25% starting parasitemia and 2% hematocrit (Z-factor of 0.87 ± 0.07 [mean ± standard deviation]).

The [^{3}H]hypoxanthine incorporation assay described above was used to assess the activities of chloroquine, cycloguanil, and clindamycin against *P. knowlesi* A1H.1. Chloroquine and cycloguanil are fast-acting drugs which act in the first *P. falciparum* asexual intraerythrocytic developmental cycle (16, 17), while clindamycin is a slow-acting drug with a "delayed-death" phenotype that is not observable until after two asexual intraerythrocytic developmental cycles (16). The activity of all three drugs was assessed against *P. knowlesi* A1H.1 using asynchronous parasites (0.25% parasitemia and 2% hematocrit) over 24 h, 48 h, or 72 h. Three independent assays (in triplicate wells) were carried out, and the resulting 50% inhibitory concentrations (IC_{50}s) determined by log-linear interpolation (18) (Table 1). When comparing the effects of drugs over time (24 h versus 48 h, 24 h versus 72 h, and 48 h versus 72 h), there was no significant difference in IC_{50}s for chloroquine ($P > 0.05$) (Table 1), consistent with this compound exerting its activity in the first asexual intraerythrocytic developmental cycle, as previously reported for *P. falciparum* (48-h IC_{50} of 0.006 ± 0.002 μM and 96-h IC_{50} of 0.008 μM ± 0.003 μM for *P. falciparum* strain 3D7) (16). The *P. knowlesi* A1H.1 chloroquine data are also consistent with previously reported data for *P. knowlesi* strain H (19) (from which *P. knowlesi* A1H.1 is derived [11]) grown in rhesus monkey erythrocytes (IC_{50} of 3.2 nM [95% confidence interval, 2.2 to 4.7] in parasite lactate dehydrogenase 22-h maturation assay) (20). While there were significant differences in the IC_{50}s for cycloguanil at 24 h versus 48 h and 72 h (Table 1) ($P < 0.05$), there was no significant difference between the 48-h and 72-h IC_{50}s (Table 1) ($P = 0.540$). In contrast to the *P. knowlesi* A1H.1 chloroquine and cycloguanil data, clindamycin had 24-h and 48-h IC_{50}s of >10 μM, compared to an IC_{50} of 0.0030 ± 0.001 μM in the 72-h assay, consistent with the delayed-death phenotype reported for *P. falciparum* with this drug (21, 22). This translates to a >670-fold-lower IC_{50} at 72 h than at 24 h or 48 h, more than 2 orders of magnitude higher than the difference at 72 h versus 24 h or 48 h for the fast-action compounds chloroquine and cycloguanil.

To our knowledge, this is the first study to use [^{3}H] hypoxanthine uptake as a method to assess drug activity against *in vitro* culture-adapted *P. knowlesi* grown in human erythrocytes and the first to report on the *in vitro* sensitivity of clinically used antimalarial drugs against this culture-adapted *Plasmodium* species. This work paves the way for additional studies with culture-adapted *P. knowlesi* as an *in vitro* model for drug discovery, until now limited to the use of *P. falciparum* due to the inability to easily and routinely culture sufficient quantities of other *Plasmodium* species *in vitro*. Such studies include those designed to better understand

TABLE 1 *In vitro* IC_{50}s of antimalarial drugs against *P. knowlesi* A1H.1

Compound	Mean IC_{50} (±SD) (μM) at indicated time point (h)			*P* value for time point (h) comparison		
	24	48	72	24 vs 48	24 vs 72	48 vs 72
Chloroquine	0.0109 (±0.0031)	0.0069 (±0.0022)	0.0065 (±0.0030)	0.147	0.159	0.888
Cycloguanil	0.0039 (±0.0007)	0.0015 (±0.0005)	0.0012 (±0.0005)	0.004	0.002	0.540
Clindamycin	>10	>10	0.0149 (±0.005)	ND[a]	ND	ND

[a] ND, not determined.

growth dynamics and drug action, including *in vitro* drug combination studies (isobolograms) and adaptation of assays such as the *P. falciparum* parasite reduction ratio (PRR) assay (23) to assess the speed of drug action on the parasite.

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