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Original article

Effect of Inducers, Incubation Time and Heme Concentration on IC_{50} Value Variation in Anti-heme Crystallization Assay

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Abstract: Heme detoxification through crystallization into hemozoin has been suggested as a good target for the development of screening assays for new antimalarials. However, comparisons among the data obtained from different experiments are difficult, and the IC_{50} values (the concentrations of drug that are required to inhibit 50% of hemozoin formation) for the same drug vary widely. We studied the effects of changes in heme concentration (precursor of β-hematin), incubation time and three inducers (SDS, Tween 20 and linoleic acid) on the IC_{50} of some antimalarials (chloroquine, quinine, amodiaquine, and clotrimazole). The results showed that increasing both inducer concentration and incubation time raised the IC_{50} of selected antimalarials. Any change in those factors caused the IC_{50} value to vary. Standardization of assay conditions is, therefore, necessary to increase reproducibility and reduce discrepancies in assay performance. Considering all of the variables, the best choice of inducers is in the order of SDS > Tween 20 > linoleic acid.
Key words: antimalarial, β-hematin, heme crystallization, hemozoin, inducer, IC_{50}

INTRODUCTION

Malaria, which is caused by *Plasmodium* parasites, is one of the most common parasitic diseases in tropical countries. Fast-spreading resistance to current antimalarial drugs and the absence of a commercialized vaccine make malaria a global public health priority [1]. The development of a screening method is thus important in the search for new drugs against malaria.

During its intraerythrocytic cycle, the malaria parasite degrades hemoglobin in erythrocytes in order to feed and releases free heme which is very toxic to both the host cells and the malarial parasite [2–4]. In the absence of heme oxygenase, *Plasmodium* cannot cleave heme into an open-chain tetrapyrrole, which is required for cellular excretion [5]. Thus, to protect itself, *Plasmodium* detoxifies free heme following three pathways: neutralization with histidine-rich protein 2 [6, 7], degradation with reduced glutathione [8–10], or crystallization into hemozoin (HZ)—a water-insoluble malarial heme crystal produced in the food vacuole [7, 11]. The last of the three is widely accepted as the main pathway of heme detoxification in the parasite [12, 13]. A new protein that is extremely potent in converting heme into HZ was also recently identified [14].

Several antimalarial drugs have been reported to inhibit HZ formation. Quinoline, such as chloroquine, amodiaquine, quinine, and its derivatives act by decreasing the rate of HZ formation rather than by blocking its formation [15]. Some antifungals (ketoconazole and miconazole) have also been shown to inhibit heme crystallization or to neu-

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Abbreviations: AQ, amodiaquine; BH, β-hematin; cmc, critical micelle concentration; CQ, chloroquine; CLT, clotrimazole; DMSO, dimethyl sulfoxide; HZ, hemozoin; IC_{50}, concentration inhibiting 50% of heme crystallization; LA, linoleic acid; PQ, primaquine; Q, quinine sulfate; SDS, sodium dodecyl sulfate

tralize heme by reducing glutathione and histidine-rich protein 2 [9, 10]. HZ is structurally and chemically identical to β-hematin (BH), an *in vitro* synthesized heme crystal [16–18], and it has been suggested that blocking of BH formation is an ideal target for antimalarial screening [13, 19–22]. Certain factors, such as temperature [23], histidine-rich protein [6, 7], lipids [24, 25], preformed BH [13], alcohols [26], and sodium dodecyl sulfate (SDS) [27] have been deemed to be responsible for promoting BH formation. The surfactant Tween 20 has also been used as an inducer for BH formation assays *in vitro* and for antimalarial candidate screening [28]. Linoleic acid, a commercially available fatty acid, has also been implicated in the initiation of BH formation *in vitro* [29, 30].

Several methods using different catalytic factors have been proposed for the measurement of BH crystallization in the screening of antimalarials [25, 28, 31–33]. However, comparisons among the data obtained from different experiments are difficult, because the methods for performing these assays are not standardized. The IC_{50} values (concentrations required to inhibit 50% of BH formation) of the same drug varied widely, probably due to the use of different catalysts or incubation times [15]. Here, the authors systematically evaluate the effects of substrate heme concentration, incubation time, and the concentration of different inducers on variation in the IC_{50} values of several antimalarials in the BH formation inhibition assay.

MATERIALS AND METHODS

Materials

Hemin chloride (heme), chloroquine (diphosphate salt, CQ), quinine sulfate (Q), primaquine (PQ) and clotrimazole (CLT) were obtained from Sigma (Japan). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemicals (Osaka, Japan), amodiaquine dihydrochloride [21] from MP Biomedical Inc (France), and linoleic acid (LA) from Sigma. All chemicals were of the highest commercially available grade.

Preparation of heme stock solution

A stock solution of heme was prepared by dissolving hemin chloride (16.3 mg) in 1 ml of DMSO and then removing the insoluble heme by centrifugation at 7,000 × *g* for 10 min. Heme concentration was estimated from the absorbance at 400 nm after dilution with 100 mM NaOH-2.5% SDS solution and calculated with a molar extinction coefficient of 10^5 at 400 nm as described previously [34]. The stock reagent was stored in the dark at 4°C until use.

Preparation of antimalarial drugs and inducers

CQ, AQ and PQ, CLT, and Q were dissolved in distilled water, DMSO, and 20 mM H_2SO_4. The concentration of all drug stock solutions was 20 mM. Drugs were diluted twofold in series with distilled water to various concentrations from 0 to 1 mM. SDS and Tween 20 were dissolved in distilled water. LA was dissolved in chloroform, sprayed and dried on the wall of a microtube with nitrogen gas to make a thin layer, then suspended in 100 mM acetate buffer pH 5.2.

Assay of BH formation initiated by Tween 20, SDS and LA

The synthesis of BH was performed as previously described [33]. Various concentrations diluted in serial 1:2 in distilled water with Tween 20, SDS or LA were used. The inducer (Tween 20, SDS or LA) was incubated with 100 μM of heme in 1 ml of 100 mM acetate buffer pH 5.2 at 37°C for 5 and 12 h. The pH of the buffer used in the assay was similar to that of the food vacuole of the malarial parasite where hemozoin formation takes place [35].

After incubation, the sample was centrifuged at 7,000 × *g* for 10 min. The pellet was then suspended in 1 ml of 2.5% SDS buffered with 0.1 M sodium bicarbonate (pH 9.1) and briefly sonicated. After shaking at room temperature for 1 h to dissolve unreacted heme, the remaining insoluble material was recovered by centrifugation. This step was repeated one more time without sonication, thus producing purified BH. In order to determine the amount of heme crystallized into BH, the purified BH was decrystallized into monomer heme and completely dissolved in 0.1 M NaOH-2.5% SDS for 10 min at room temperature. The heme concentration in the solution was then calculated as described above [34].

Inhibition assay of BH formation by antimalarial drugs

Tween 20, SDS or LA was incubated with heme (100 μM) in 1 ml of 100 mM acetate buffer at pH 5.2 in the presence of various drug concentrations of CQ, AQ, Q, PQ or CLT. After incubating at 37°C, BH was purified and its concentration was calculated as described above. Values obtained from triplicate assays were plotted and IC_{50} values were determined by calculating graphically.

Assay of BH formation with various concentration of substrate heme

Heme ranging from 6.25 to 200 μM was incubated at 37°C for 5 h with 0.0025% Tween 20, 1% SDS or 0.25 mg/ml LA. The resulting BH was then purified and its concentration was determined as described above.

Statistical analysis

Data analysis was performed using SPSS 16.0 software. Data are expressed as the mean with standard error of mean (SEM) unless otherwise stated. IC_{50} values obtained from three independent experiments in triplicate of each assay were used in the statistical analysis. Data were logarithm transformed to ensure normal distribution before analysis. A student's t-test for independent samples was performed and values were considered significant when $p < 0.05$. Significant differences in IC_{50} values were analyzed when the inducers' concentration, incubation time or substrate concentration varied.

RESULTS

Promotion of BH formation by different inducers (Figure 1)

The three inducers used in the present study (Tween 20, SDS, and LA) are commercially available and easy to prepare in order to promote BH formation. After 5 or 12 h of incubation, the yield of BH formation was maximal with 0.0025% of Tween 20 (Figure 1A), 2% of SDS (Figure 1B) and 0.25 mg/ml of LA (Figure 1C). The crystal growth curves showed a sigmoidal pattern similar to that of a previous report [28]. A further increase in LA concentration lowered BH formation, probably because hemin tends to accumulate at the surface of the assay mixture [29]. SDS converted heme into BH more efficiently (maximal BH fraction around 50%, Figure 1B) than Tween 20 or LA (40% and 30%, Figure 1A and C respectively) as previously described [28–30].

Effect of inducer concentration on IC_{50} values of antimalarials

To examine the effect of inducer concentration on IC_{50}, the inhibitory effects of antimalarial drugs (CQ, AQ, Q, PQ) and one antifungal (CLT) were studied using two different concentrations of each inducer. Those concentrations were chosen from the result obtained in Figure 1, when BH formation reached around 50% or maximal yield. Tween 20 was used at 0.025% and 0.00125%, SDS at 1% and 0.5%, and LA at 0.25 mg/ml and 1 mg/ml.

Various concentrations of drugs were incubated with 100 μM heme for 5 and 12 h. Three independent experiments in triplicate showed that the antimalarials (CQ, AQ, Q) and CLT were able to inhibit BH formation whatever the inducer used (Table 1, 2 and 3), while PQ had no inhibiting effect on BH formation (IC_{50} value > 2000 μM in any of the assays, data not shown). It has been reported that PQ has no potency to inhibit heme crystallization and no antiplasmodium activity toward intraerythrocytic parasites

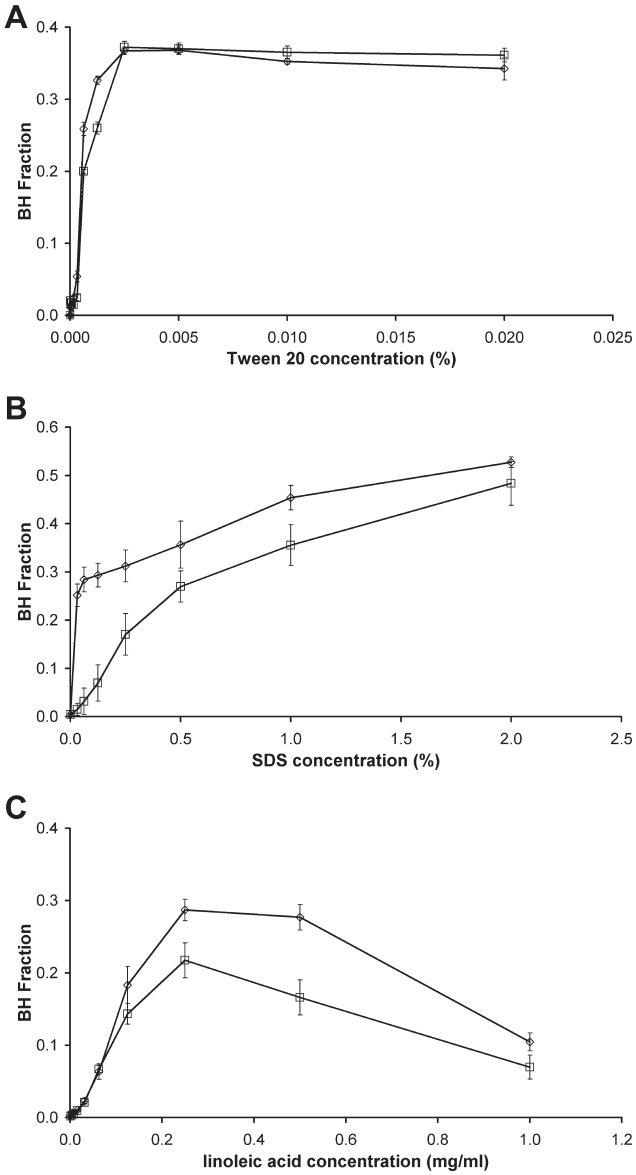

Fig. 1. Follow-up of BH formation with increased concentrations of Tween 20 (A), SDS (B) and linoleic acid (C) after 12 h (open diamond) and 5 h (open square) of incubation

[36]. IC_{50} values were also used to analyze statistical differences in the changes and were considered significant when $p < 0.05$. Increasing the concentration of inducer led to increased IC_{50} values. Increasing the concentration of Tween 20 significantly increased IC_{50} values for all tested drugs, from 1.8 to 2.7 times after 5 h ($p < 0.001$) and 1.2 to 3.6 times ($p < 0.01$) after 12 h of incubation, even though heme concentration was constant (Table 1). With LA, the IC_{50} also increased from 1.4 to 2.3 times after 5 h ($p < 0.05$) and 1.9 to 2.8 times ($p < 0.001$) after 12 h of incubation (Table 3). These findings indicate that the interaction of

Table 1. IC_{50}s (μM) of drugs on BH formation induced by Tween 20.

Drugs	Tween 20 concentration						Incubation time
	0.0025%			0.00125%			
	IC_{50}	SD	CQ index	IC_{50}	SD	CQ index	
CQ	59.0	6.8	1.0	48.5	2.4	1.0	12 h
AQ	45.3	1.2	0.8	25.0	1.7	0.5	
Q	170.0	12.3	2.9	46.8	3.5	1.0	
CLT	105.0	11.6	1.8	75.7	6.1	1.6	
CQ	44.5	5.7	1.0	25.0	2.2	1.0	5 h
AQ	25.3	2.1	0.6	12.8	0.5	0.5	
Q	90.0	9.1	2.0	33.0	1.2	1.3	
CLT	82.3	17.0	1.9	30.3	2.5	1.2	

SD, standard deviation; CQ index, IC_{50} of drug/ IC_{50} of CQ
Mean values from three experiments in triplicate

Table 2. IC_{50}s (μM) of drugs on BH formation induced by SDS.

Drugs	SDS concentration						Incubation time
	1%			0.5%			
	IC_{50}	SD	CQ index	IC_{50}	SD	CQ index	
CQ	376.0	25.2	1.0	245.0	12.3	1.0	12 h
AQ	412.0	17.7	1.1	380.0	17.3	1.6	
Q	201.0	11.1	0.5	180.0	13.2	0.7	
CLT	215.3	10.1	0.6	195.7	7.1	0.8	
CQ	196.7	20.6	1.0	185.3	18.0	1.0	5 h
AQ	223.3	15.8	1.1	146.7	23.1	0.8	
Q	151.7	9.2	0.7	102.7	9.5	0.6	
CLT	192.5	13.5	1.0	187.5	10.6	1.0	

SD, standard deviation; CQ index: IC_{50} of drug/ IC_{50} of CQ
Mean values from three experiments in triplicate

heme and/or drug and inducers (Tween and LA) affected the anti-heme crystallization activities of the drugs. In contrast, the IC_{50} values of the drugs increased only slightly with SDS, i.e. 1.03 to 1.5 times after both 5 ($p = 0.055$) and 12 h of incubation ($p = 0.11$) which was not significant (Table 2). This statistical analysis suggested that SDS is a better inducer, with less variation in IC_{50} values, in inhibiting BH formation assays compared with other tested inducers.

The CQ index value, which is the ratio between the IC_{50} value of a tested drug and the IC_{50} value of CQ under the same reaction conditions [37], was also calculated. This index showed the activity of the tested drug compared with CQ. CQ index values were affected by different inducers used in the assay, a finding that was closely consistent with another study [32]. This might indicate that different inducers interact with heme and/or the drug in different modes.

Table 3. IC_{50}s (μM) of drugs on BH formation induced by linoleic acid.

Drugs	Linoleic acid concentration						Incubation time
	1mg/ml			0.25mg/ml			
	IC_{50}	SD	CQ index	IC_{50}	SD	CQ index	
CQ	90.9	8.2	1.0	32.5	6.4	1.0	12 h
AQ	103.3	12.3	1.1	41.0	7.2	1.3	
Q	62.5	2.9	0.7	27.5	4.0	0.9	
CLT	203.3	13.5	2.2	108.3	13.5	3.3	
CQ	51.0	8.5	1.0	28.7	2.9	1.0	5 h
AQ	46.7	1.2	0.9	27.5	4.1	1.0	
Q	33.3	5.8	0.7	23.0	2.0	0.8	
CLT	185.0	17.1	3.6	80.5	5.3	2.8	

SD, standard deviation; CQ index: IC_{50} of drug/IC_{50} of CQ
Mean values from three experiments in triplicate

CQ index values of Q and CLT in almost all the assays were higher than 1 showing that Q and CLT have a weaker binding coefficient to heme than CQ. In contrast, the CQ index of AQ was lower than 1, suggesting stronger binding, which was consistent with previous reports [32, 37].

Effect of incubation time on IC_{50} value

It has been reported that incubation duration also affects the IC_{50} value [15]. The effects of two different incubation times, 5 h and 12 h, on the IC_{50} values of four antimalarial drugs, CQ, AQ, Q, and CLT, were examined further. These two incubation times were selected because the yield of BH reached the maximum after 12 h incubation, while at 5 h, the BH fraction reached more than half of the maximum with each inducer. A direct correlation was also found between IC_{50} values and incubation times (Tables 1–3). These values of all the tested drugs increased when incubation time was prolonged in all assays with different inducers, a finding consistent with a previous report [15]. When the incubation duration was lengthened from 5 h to 12 h, IC_{50} values were increased in all tested drugs, between 1.4 and 2.5 times ($p < 0.001$) and 1.3 and 1.9 times ($p < 0.05$) when using Tween 20 as an inducer at 0.00125% or 0.0025% of concentration (Table 1); 1.1 and 2.6 times ($p < 0.01$) and 1.1 and 1.9 ($p < 0.01$) with SDS as an inducer at 0.5% or 1% of concentration (Table 2); and 1.2 and 1.5 times ($p = 0.11$) and 1.1 and 2.2 times ($p < 0.05$) with LA as an inducer at 0.25 mg/ml or 1 mg/ml concentration (Table 3). In addition, the statistical analysis showed significant differences between IC_{50}s when incubation time varied ($p < 0.05$), except when using 0.25 mg/ml LA as an inducer. These results also suggested that antimalarial drugs do not completely block BH formation but simply slow the rate [15].

Effect of heme concentration on IC_{50} value

To examine the effect of heme concentration on IC_{50} values, preliminary experiments were performed to explore the yield of BH using various concentrations of substrate heme. BH formation was induced with 0.0025% of Tween 20, 1% of SDS, or 0.25 mg/ml of LA. It was found that with concentrations of heme ranging from 50 μM up to 200 μM, the BH formation increased, whatever the inducer used (Figure 2). However, the yield of BH formation differed from one inducer to another, with SDS and Tween 20 being more efficient than LA.

When the heme concentration increased the IC_{50} values increased (Table 4). With SDS as an inducer, the IC_{50} values of the drugs increased from 3 to 6.5 times ($p < 0.001$) when heme concentration increased from 50 to 100 μM and about 6 to 9 times ($p < 0.01$) when heme concentration increased to 200 μM. It is interesting to note that when heme concentration varied from 50 to 100 μM or 100 to 200 μM, the variation in IC_{50} values was less significant with Tween 20 and LA ($p = 0.18$; 0.001 and 0.94; 0.16, respectively) than with SDS, even though more drugs were needed to bind heme substrate or active sites of crystallization. A clear positive correlation between IC_{50} and heme concentration was observed when using SDS as inducer. Moreover, a lower heme concentration used in the assay reduced the variation in IC_{50} values.

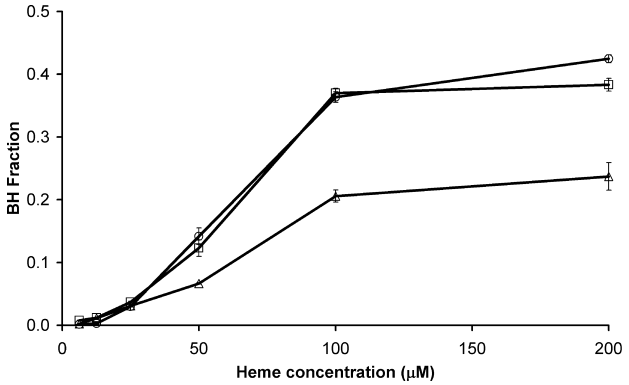

Fig. 2. Follow-up of BH formation with increasing concentrations of heme promoted with 0.0025% Tween 20 (open square), 1% SDS (open circle), and 0.25 mg/ml linoleic acid (open triangle) after 5h of incubation

DISCUSSION

Heme detoxification through crystallization into HZ has been suggested as an ideal target for the development of antimalarial screening methods [13, 19–22]. Recently, new high throughput screening assays have been developed [28, 38]. However, comparisons among the data obtained from different experiments are difficult, and the IC_{50} values for the same drug varied widely, probably due to the use of different catalysts or incubation times [15]. The present study evaluated the effects of heme concentration, incubation time, and different concentration of inducers on variations in the IC_{50} value of selected antimalarials to show the importance of standardization of screening methods.

In another study, parasite extracts were used to promote BH formation [32]. This required tedious preparations and parasite culture. In contrast, we used three inducers

Table 4. IC_{50}s (μM) of drugs on BH formation induced by Tween 20, SDS or linoleic acid after 5 h of incubation at increasing concentrations of heme

Drugs	Heme concentration									Inducer
	50 μM			100 μM			200 μM			
	IC_{50}	SD	CQ index	IC_{50}	SD	CQ index	IC_{50}	SD	CQ index	
CQ	40.0	8.7	1.0	44.5	1.7	1.0	95.0	8.4	1.0	Tween 20 0.0025%
AQ	24.5	2.7	0.6	25.3	2.1	0.6	55.0	2.1	0.6	
Q	80.5	1.6	2.0	90.0	9.1	2.0	198.5	12.7	2.1	
CLT	41.3	3.5	1.0	82.3	17.0	1.9	240.3	19.1	2.5	
CQ	48.0	7.1	1.0	196.7	20.6	1.0	361.7	22.6	1.0	SDS 1%
AQ	34.5	12.1	0.7	223.3	5.8	1.1	290.0	78.1	0.8	
Q	32.5	10.6	0.7	151.7	9.2	0.7	193.3	24.7	0.5	
CLT	60.2	5.0	1.3	192.5	3.5	1.0	353.3	30.6	1.0	
CQ	26.0	2.7	1.0	28.7	2.9	1.0	31.0	6.7	1.0	Linoleic acid 0.25 mg/ml
AQ	13.5	0.6	0.5	14.3	2.1	0.5	23.0	1.8	0.7	
Q	24.3	2.6	0.9	23.0	2.0	0.8	48.4	1.3	1.6	
CLT	81.3	1.4	3.1	80.5	3.5	2.8	116.3	1.0	3.8	

SD, standard deviation; CQ index: IC_{50} of a drugs/IC_{50} of CQ
Mean values from three experiments in triplicate

(Tween 20, SDS, and LA) that are commercially available and easy to prepare. All have been shown to promote BH formation (Figure 1), which is consistent with previous reports [27–30]. IC_{50} values of all drugs in the inhibition assay were calculated and the variation was measured when incubation time, inducer or heme concentration changed. The results showed a direct correlation between IC_{50} values, concentration of inducers and incubation duration (Table 1–3) with any of the inducers used. IC_{50} values of all drugs in the assays using SDS were much higher than those obtained from the assays using other inducers (Table 1–4). This may be due to the ability of SDS to help dissolve monomeric heme in an acid medium [27].

Among the inducers studied, SDS showed the highest impact on IC_{50} variation, while less significant increases were observed with Tween 20 and LA when heme concentration of the assay changed (Table 4). These discrepancies might be due to the different states of inducers. The concentrations of Tween 20 used in this study were slightly lower than its critical micelle concentration (cmc), so it was in monomer or small aggregated form. LA at pH 5.2 may also disperse in monomer or small aggregated form. Therefore, the interaction of heme and/or the drugs with these small forms of inducers might affect the inhibition of heme crystallization by antimalarials. In particular, the interaction of LA and drugs might disturb the heme crystallization by blocking the interaction of heme and LA, as indicated by the less significant increase in IC_{50} regardless of the increase in heme concentration. In contrast, SDS concentration was higher than its cmc, and thus a dynamic equilibrium existed between micelle and monomer. Therefore, heme and drug might be intercalated to the hydrophobic binding site of the SDS micelle, and might bind to inhibit heme crystallization. Further studies are required to elucidate the mechanism of this discrepancy.

Increasing the IC_{50} values is likely to lead to false negativity of tested drugs and could necessitate more testing. Thus, the results suggest that a lower concentration of heme is better for assay performance with low variation in IC_{50} values. In addition, a short incubation time promises to save time and also lower the IC_{50} variation.

Our results showed that the ability of the inducers to achieve the narrowest variation in IC_{50} value was in the order of SDS > LA > Tween 20, when the concentration of inducers and the incubation time increased. Positive correlations between heme concentration and IC_{50} were clearly seen with SDS as an inducer. In contrast, a larger variation in IC_{50} value was seen with SDS than with LA or Tween 20, indicating that more of the drug is required when SDS is used for screening of antimalarial compounds. The ability of inducers to achieve the narrowest variation in IC_{50} values was on the order of LA > Tween 20 > SDS when concentration of heme changed. Considering the stability and easy preparation of SDS and Tween 20 stock solution compared to LA, as well as the advantages of high throughput assay for antimalarial screening, the choice of inducers should be on the order of SDS > Tween 20 > LA.

CONCLUSION

Any change in incubation time, inducer or heme concentration affected the variation in IC_{50} values. Increases in both the inducer concentration and incubation time raised the IC_{50} of some antimalarials used in this study. Therefore, standardization of assay conditions is necessary to increase reproducibility and avoid false negativity in the performance of assays for antimalarial screening based on the inhibition of heme crystallization.

COMPETING INTEREST

There is no competing interest for any of the authors of the manuscript due to either commercial or other affiliations.

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