

Assessment of SYBR Green I Dye-Based Fluorescence Assay for Screening Antimalarial Activity of Cationic Peptides and DNA Intercalating Agents

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The SYBR green I (SG) dye-based fluorescence assay for screening antimalarial compounds is based on direct quantitation of parasite DNA. We show that DNA-interacting cationic cell-penetrating peptides (CPPs) and intercalating agents compete with SG dye to bind to DNA. Therefore, readouts of this assay, unlike those of the [³H]hypoxanthine incorporation assay, for the anti-malarial activity of the above DNA binding agents may be erroneous. In the case of CPPs, false readouts can be improved by the removal of excess peptides.

Malaria continues to be a major public health problem in the 21st century. More than 200 million cases of malaria were reported in 2012, and the emergence of new drug-resistant strains of *Plasmodium* makes the situation more critical and alarming (1, 2). Chemoresistance, drug monotherapies, the easy availability of standard drugs, and genetic polymorphism are some of the major causes of parasite drug resistance (3, 4). Thus, it is imperative to identify new antimalarial agents. For antimalarial drug screening, the [³H]hypoxanthine incorporation assay has been a gold standard (5), while parasite lactate dehydrogenase (pLDH)- (6) and histidine-rich protein II-based (7) assays are other widely used methods. However, these assays are expensive and involve multistep procedures and thus are difficult to utilize for high-throughput screening (8, 9). The SYBR green I (SG) dye-based fluorescence assay is a recently developed high-throughput screening method which has been reported to be as sensitive as the [³H]hypoxanthine incorporation assay (8, 10). It has been extensively validated and compared with other known methods and is commonly used for high-throughput antimalarial drug screening (9, 11–13).

Peptides and DNA intercalating agents have been widely tested for their antimalarial activity (14–17). Some cell-penetrating peptides (CPPs), e.g., TP10 (18), and a number of DNA intercalators have been shown to have antimalarial activity (16, 17). Interestingly, it has been demonstrated that CPPs, like Tat, penetratin, and TP10, are efficient agents for the delivery of bioactive molecules across the plasma membrane barrier (19). It has also been shown that CPPs move to the nucleus and bind to the target DNA, forming peptide-DNA complexes (20, 21). Since SG dye, CPPs, and intercalating agents bind to DNA, the aim of the present study was to evaluate the suitability of an SG dye-based fluorescence assay for determining the antiplasmodial activity of DNA binding agents. CPPs, Tat, penetratin, TP10, P3, P8 (19, 22), and control peptides (Table 1) were chemically synthesized (USV, Mumbai, India) with more than 95% purity. TP10 is a broad-spectrum antiparasitic CPP (18), while Tat has been shown to be nontoxic (at 50 μM) to *Plasmodium* parasites (23). Synchronized *Plasmodium falciparum* 3D7 cultures at 2% parasitemia and 2% hematocrit were lysed (10) and treated with various concentrations (6.25 μM to 100 μM) of Tat, penetratin, TP10, and control peptides

(control cyclic peptide [CP1] and control linear peptide [CP2]) followed by incubation with SG dye. The binding of these cationic CPPs with parasite DNA and their effects on SG dye binding were analyzed. A dose-dependent decrease in SG dye fluorescence was observed in the case of Tat, penetratin, and TP10 (Fig. 1A). In the [³H]hypoxanthine incorporation assay, like control peptides, Tat and penetratin did not show any antiplasmodial activity up to 100 μM, while TP10 exhibited dose-dependent antiplasmodial activity (Fig. 1B). On the other hand, the SG-based fluorescence assay, performed under similar conditions, showed antiplasmodial activity (reflected by SG-DNA binding inhibition) for all peptides, except for the control peptide (Fig. 1C). Similar results were also observed with other CPPs, P3, and P8 (see the supplemental material available at <http://crdd.osdd.net/raghava/figs1.pdf>). As CPP-mediated inhibition was observed at higher concentrations, we analyzed whether these anomalous growth inhibition results, obtained for cationic CPPs in the SG-based fluorescence assay, may be corrected by the removal of excess peptide from the wells before addition of the SG dye. After 48 h of treatment with CPPs, the supernatant-containing excess peptides were removed, and cells were washed once with malaria complete medium (24) before adding SG dye. As shown in Fig. 1D, significant corrections in false readouts were observed, so the results were comparable to those of the [³H]hypoxanthine incorporation assay (Fig. 1B). One of the merits of the SG-based assay, originally emphasized (25) and subsequently followed in a number of studies (10, 11), is the

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TABLE 1 Peptides used in this study

Peptide	Sequence	pI ^a	Length ^b
Tat	GRKKRRQRRRPPQ	12.70	13
Penetratin	RQIKIWFQNRRMKWKK	12.31	16
P3	RRRQKRIVRRRLIR	12.90	15
P8	RRWRRWNRFNRRRCR	12.54	15
TP10	AGYLLGKINLKALAAALAKKIL	10.18	21
CP1	CTHPATSWC	6.72	9
CP2	ITWNEKSKSHLY	8.51	12

^a Isoelectric points (pI) were calculated using the ProtParam tool (<http://web.expasy.org/protparam/>).

^b Number of amino acids.

rapidness of the assay when using a single reagent and avoiding multiple washing steps. Unlike these reports, in the case of cationic CPPs, we noticed that the removal of the supernatant (containing excess peptides) before the addition of a lysis buffer containing SG dye significantly reduced the number of false readouts of antiplasmodial activity of cationic CPP. In one report, the *in vitro* antiplasmodial activities of cationic undecapeptides were

tested using the SG-based fluorescence assay; before adding the lysis buffer containing the SG dye, the supernatant was first removed after treatment to check for peptide-induced hemolysis of the treated cells (26). Although the antimalarial activity of the test peptide was not validated by other methods in that report, it is possible that, in line with our observation, the results were not affected due to the removal of excess peptides in solution. Since the washed wells displayed results comparable to those of the [³H]hypoxanthine incorporation assay (Fig. 1B), this indicates that instead of an intracellular peptide, excess peptide in the supernatant interacts with parasite DNA upon lysis and is responsible for false-positive readouts by these peptides.

Next, we wanted to check whether small-molecule DNA intercalators, e.g., doxorubicin and actinomycin D, also interfere with SG binding to parasite DNA in solution and *in vivo*. Figure 2A shows that, like CPPs, these intercalators also exhibit a dose-dependent inhibition in SG binding to DNA in solution. Each of these intercalators is a potent antimalarial (16, 17), and doxorubicin exhibited a low SG binding signal (due to less DNA) in a standard 48-h growth inhibition assay (27). Accordingly, for *in*

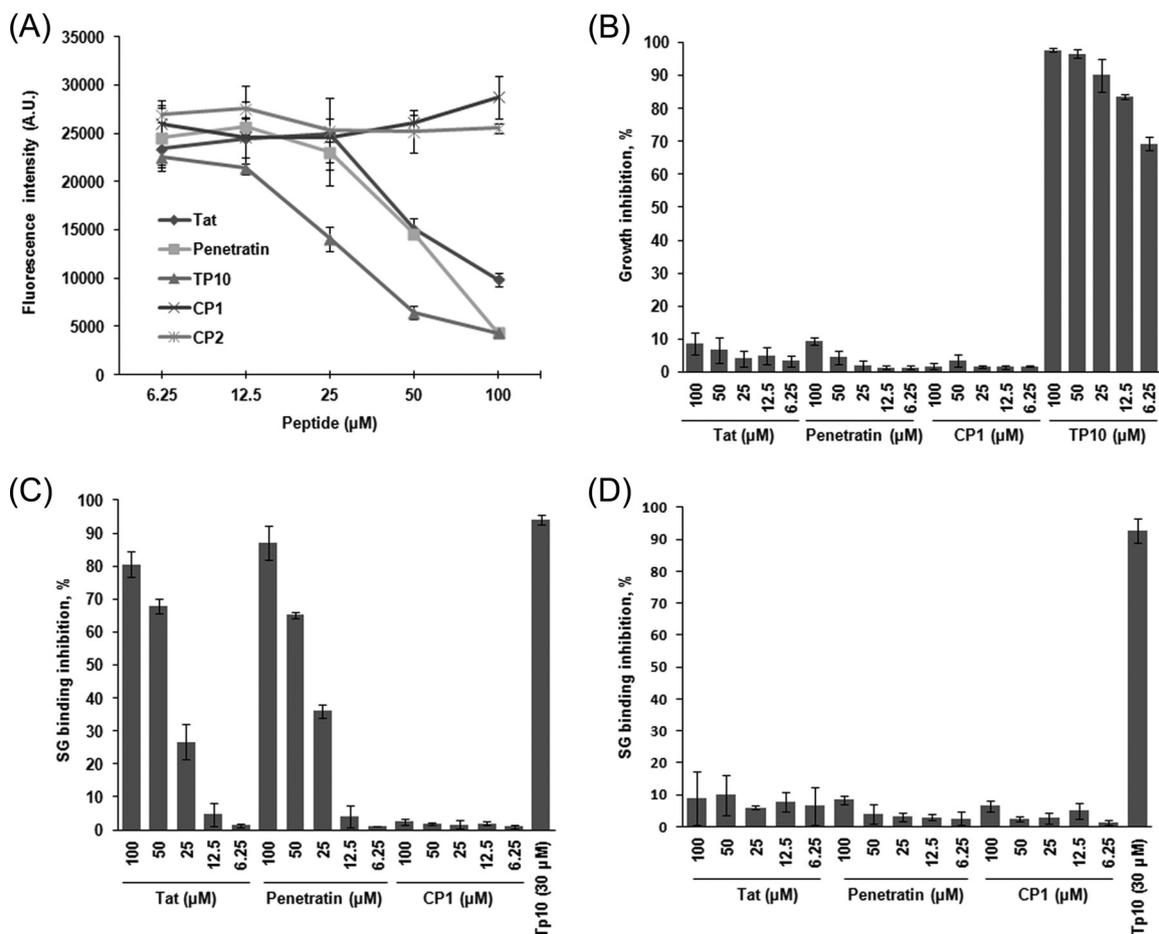


FIG 1 Validation of antiplasmodial activities of cationic CPPs assessed by SG-based fluorescence assay. (A) Sorbitol-synchronized *P. falciparum* trophozoite-stage cells were lysed and treated with various concentrations (6.25 µM to 100 µM) of Tat, penetratin, TP10, and control peptides (CP1 and CP2) for 2 h at 37°C, followed by incubation with SG dye and analysis of fluorescence intensity. (B to D) Synchronized cells at trophozoite stage were cultured in the presence of various concentrations (6.25 µM to 100 µM) of Tat, penetratin, TP10, and control peptide (CP1) for 48 h, and parasite growth inhibition was measured by the [³H]hypoxanthine incorporation assay (B) and the SG-based fluorescence assay (C and D). For SG dye binding, cells were lysed without washing (C) or with washing (D). Data are representative of two independent experiments performed in triplicate; error bars, standard errors of the mean; A.U., arbitrary units.

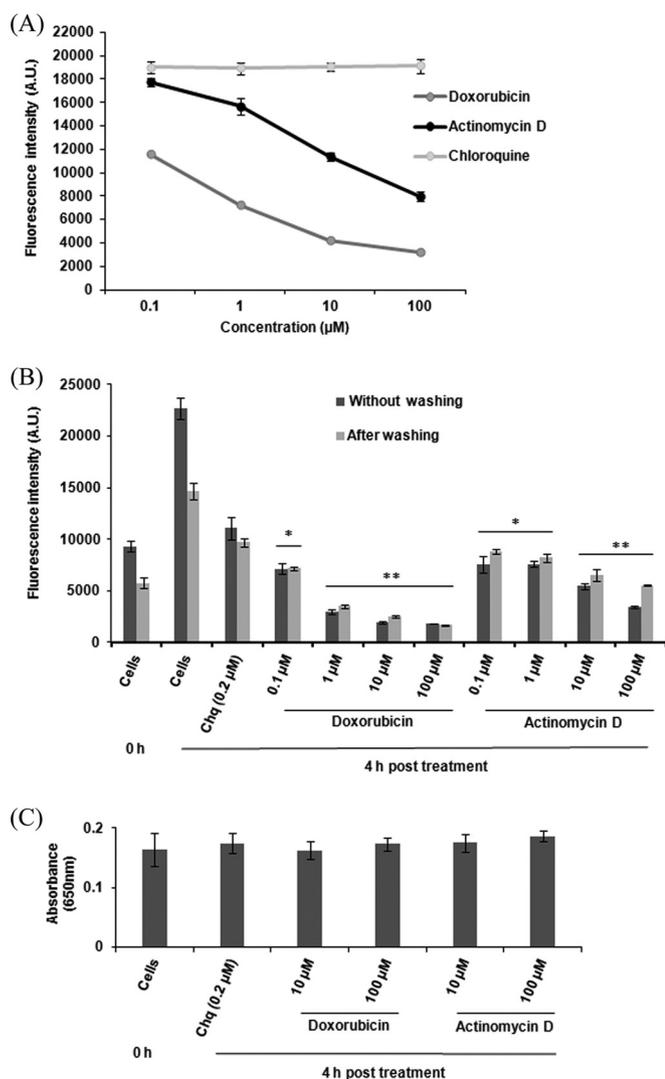


FIG 2 Effect of small-molecule DNA intercalators on SG-based analysis of *P. falciparum* DNA. (A) Sorbitol synchronized trophozoite-stage cells were lysed and treated with various concentrations (0.1 µM to 100 µM) of doxorubicin, actinomycin D, and chloroquine (Chq) for 2 h at 37°C, followed by incubation with SG dye and analysis of fluorescence intensity ($P < 0.0001$ for all concentrations). (B) Sorbitol synchronized trophozoite-stage cells, at 1% parasitemia and 2% hematocrit (0 h), were cultured with various concentrations (0.1 µM to 100 µM) of doxorubicin, actinomycin D, or 0.2 µM chloroquine. After 4 h, cells were lysed without washing or after washing followed by addition of SG dye and analysis for fluorescence intensity (*, $P < 0.05$, and **, $P < 0.005$). (C) pLDH activities corresponding to respective bars in panel B. For panels A and B, statistical significance was determined by using unpaired Student's *t* test (two-tailed) using chloroquine as a control. Data are representative of two independent experiments performed in triplicate; error bars, standard errors of the mean.

in vivo quantitation of SG dye binding to DNA in the presence of these intercalators, we chose an early period of parasite growth where parasitemia was unchanged (18). Synchronized trophozoite-stage cells at 1% parasitemia and 2% hematocrit were cultured for 4 h with various concentrations (0.1 µM to 100 µM) of intercalators. SG-based fluorescence was measured (Fig. 2B) while simultaneously checking the pLDH activity (Fig. 2C) and parasitemia by Giemsa staining. Doxorubicin and actinomycin D treatment for 4 h did not affect the parasitemia, reflected by pLDH

activity (Fig. 2C) and Giemsa staining (data not shown), but inhibited SG-DNA binding, which occurred in a dose-dependent manner with a more pronounced effect observed upon doxorubicin treatment. Also, prior removal of excess drug by washing did not significantly affect this inhibition (Fig. 2B). Thus, it can be inferred that (i) CPPs and intercalators inhibit SG-DNA binding in solution, and (ii) intercalators, unlike CPPs, can also exert their inhibitory effects by intercalation with intracellular DNA *in vivo*.

In conclusion, the present study provides evidence that cationic CPPs and DNA intercalating agents inhibit the binding of SG dye to parasite DNA and, thus, may result in false-positive readouts of antiplasmodial activity in an SG dye-based growth inhibition assay. This necessitates validation of the antiplasmodial activities of these agents with other gold standard assays. Alternatively, as demonstrated in this study for CPPs, the existing SG-based fluorescence assay can be modified by removing the excess cationic peptides prior to the addition of SG dye.

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