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

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Running Title: SG-based fluorescence assay for cationic peptides and intercalating agents

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Abstract

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

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* further makes the situation more critical and alarming (1, 2). Chemoresistance, drug monotherapies, easy availability of substandard drugs, genetic polymorphism, *etc*. 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, [^{3}H]hypoxanthine incorporation assay has been a gold standard (5), while parasite lactate dehydrogenase (pLDH) (6) and histidine rich protein II (7) based assays are other widely used methods. However, these assays are expensive, involve multistep procedures and thus difficult to utilize for high-throughput screening (8, 9). SG-based fluorescence assay is a recently developed high-throughput screening method which has been reported to be as sensitive as the [^{3}H]hypoxanthine incorporation assay (8, 10). It has been extensively validated and compared with other known methods and is commonly being 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, CPPs, like Tat, Penetratin and TP10, have been demonstrated as efficient agents for delivery of bioactive molecules across the plasma membrane barrier (19). CPPs have also been shown to traffic to the nucleus and bind to the target DNA, forming peptide-DNA complexes (20, 21) . Since SG, CPPs and intercalating agents bind to DNA, the aim of the present study was to evaluate the suitability of a SG-based fluorescence assay for determining antiplasmodial activity of DNA binding agents. CPPs, Tat, Penetratin, TP10, P3, P8 (19, 22) and control peptides (Table 1) were synthesized from USV peptides, Mumbai (India) with more than 95 percent purity. TP10 is a

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broad spectrum antiparasitic CPP (18), while Tat has been shown to be non-toxic (at 50 µM) to *Plasmodium* parasite (23). Synchronized *P. falciparum* 3D7 cultures at 2 percent parasitemia and 2 percent hematocrit were lysed (10), treated with varying concentrations (6.25 µM to 100 µM) of Tat, Penetratin, TP10 and control peptides (CP1 and CP2), followed by incubation with SG dye. Binding of these cationic CPPs with parasite DNA and their effects on SG dye binding was analysed. A dose dependent decrease in SG fluorescence was observed in case of Tat, Penetratin and TP10 (Fig. 1A). In [^{3}H]hypoxanthine incorporation assay, like control peptides, both 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, SG-based fluorescence assay, performed under similar conditions, showed antiplasmodial activity (reflected by SG-DNA binding inhibition) for all peptides, except for control peptide (Fig. 1C). Similar results were also observed with other CPPs, P3 and P8 (Supplementary Fig. S1). As CPP mediated-inhibition is observed at higher concentrations, we analysed whether these anomalous growth inhibition results, obtained for cationic CPPs in SG based fluorescence assay, can be corrected by removal of excess peptide from the wells before addition of the SG dye. After 48 h of treatment with CPPs, supernatant containing excess peptide was removed and cells were washed once with malaria complete medium (24) before adding SG dye. As observed in Fig. 1D, significant correction in false readout could be observed where the results were now comparable to that of [^{3}H]hypoxanthine incorporation assay (Fig 1B). One of the merits of the SG dye-based assay, originally emphasized (25) and subsequently followed in a number of studies (10, 11) is the rapidness of the assay by using a single reagent and avoiding multiple washing steps. Unlike these reports, in the case of cationic CPPs, we noticed that removal of supernatant (containing excess peptide) before addition of lysis buffer containing SG dye significantly reduced the false

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84 readout of antiplasmodial activity of cationic CPP. In one report, *in vitro* antiplasmodial activity
85 of cationic undecapeptides was tested using SG-based fluorescence assay, where before adding
86 lysis buffer containing the SG dye supernatant was first removed after treatment for checking
87 peptide caused hemolysis of the treated cells (26). Although, in that report the antimalarial
88 activity of test peptide was not validated by other methods, it is possible that in line with our
89 observation the results were not affected due to removal of excess peptide in solution. Since the
90 washed wells displayed the results comparable to [^{3}H]hypoxanthine incorporation assay (Fig.
91 1B), it indicates that instead of intracellular peptide, excess peptide in the supernatant interacts
92 with parasite DNA upon lysis and is responsible for false positive readouts by these peptides.

93 Next we wanted to check whether small molecule DNA intercalators, e.g. doxorubicin and
94 actinomycin D, also interfere in SG binding to parasite DNA in solution and *in vivo*. Fig 2A
95 shows that, like CPPs, these intercalators also exhibit a dose dependent inhibition in SG binding
96 to DNA in solution. Both of these intercalators are potent antimalarials (16, 17) and doxorubicin
97 has been shown to exhibit low SG binding signal (due to less DNA) in standard 48 h growth
98 inhibition assay (27). Accordingly, for *in vivo* quantitation of SG dye binding to DNA in
99 presence of these intercalators, we chose an early period of parasite growth where parasitemia is
100 unchanged (18). Synchronized trophozoite stage cells at 1 percent parasitemia and 2 percent
101 hematocrit were cultured for 4 h with varying concentrations (0.1 µM to 100 µM) of
102 intercalators. SG-based fluorescence was measured (Fig. 2B) while simultaneously checking the
103 pLDH activity (Fig. 2C) and parasitemia by Giemsa staining. Doxorubicin and actinomycin D
104 treatment for 4 h, did not affect the parasitemia, reflected by pLDH activity (Fig. 2C) and
105 Giemsa staining (data not shown) but inhibited SG-DNA binding, which occurred in a dose
106 dependent manner with more pronounced effect observed upon doxorubicin treatment. Also,

prior removal of excess drug by washing did not affect significantly this inhibition (Fig 2B). Thus, it can be inferred that; i) both CPPs and intercalators inhibit SG-DNA binding in solution and, ii) *in vivo*, unlike CPPs, intercalators can also exert their inhibitory effect by intercalation with intracellular DNA.

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 the false positive readouts of antiplasmodial activity in an SG dye based growth inhibition assay. This necessitates the validation of the antiplasmodial activity 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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Figure Legends

Fig. 1. Validation of antiplasmodial activity of cationic CPPs assessed by SG-based fluorescence assay. (A) Sorbitol synchronized *P. falciparum* trophozoite stage cells were lysed, treated with varying 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-D)** Synchronized cells at trophozoite stage were cultured in presence of varying 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 [^{3}H]hypoxanthine incorporation assay **(B)** and SG-based fluorescence assay **(C & D)**. For SG dye binding, cells were lysed without washing **(C)** or with washing **(D).** Data represent two independent experiments performed in triplicate with error bars representing standard errors of means**.**

Fig. 2. Effect of small molecule DNA intercalators on SG-based analysis of *P. falciparum* DNA. (A) Sorbitol synchronized trophozoite stage cells were lysed, treated with varying 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 varying 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 activity corresponding to respective bars in panel B. For panels A and B, statistical significance was determined by using unpaired students t-test (two tailed) taking chloroquine as a control. Data represent two independent experiments performed in triplicate with error bars representing standard errors of means**.**

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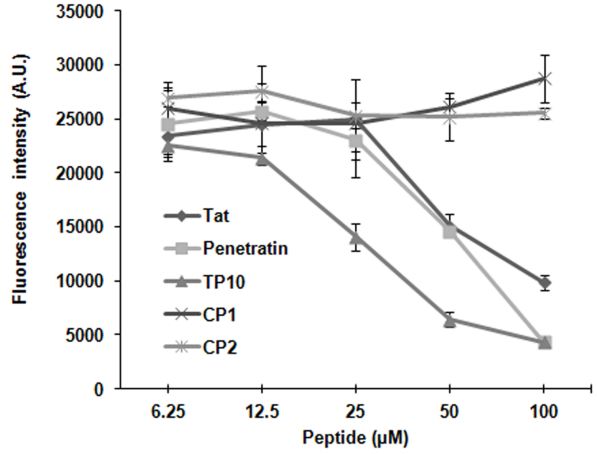
(A)



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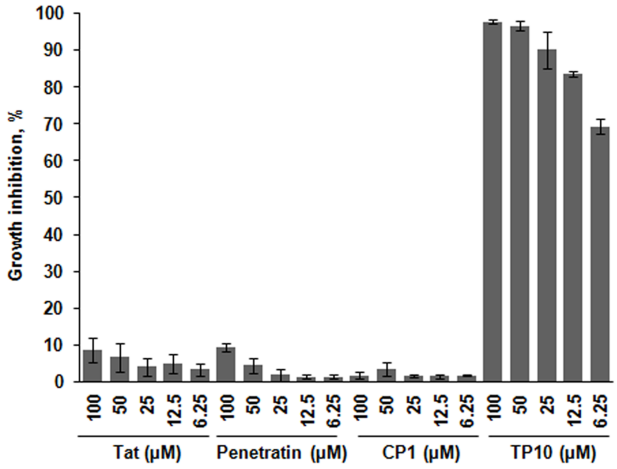

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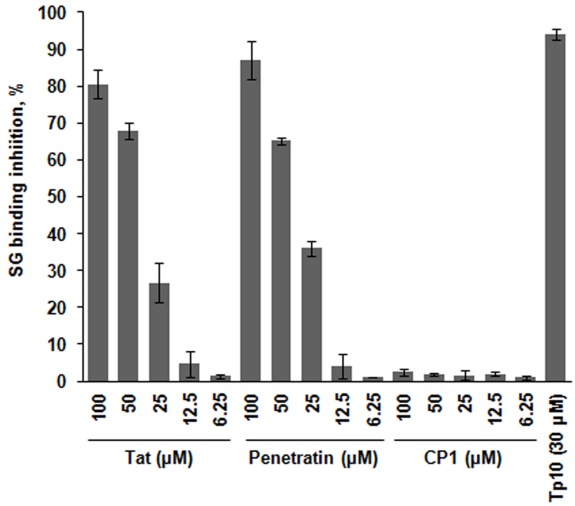

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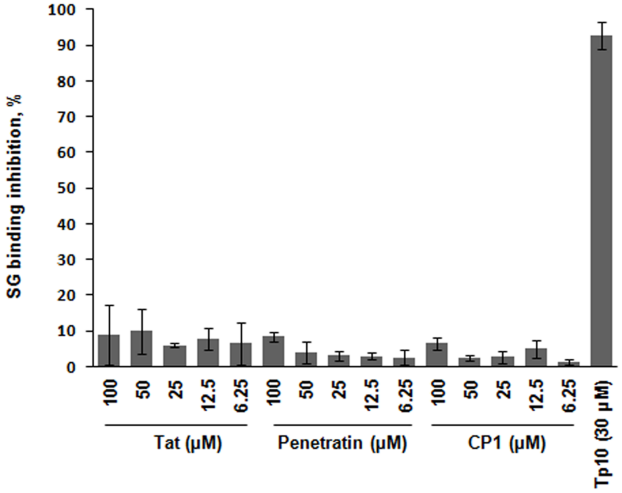

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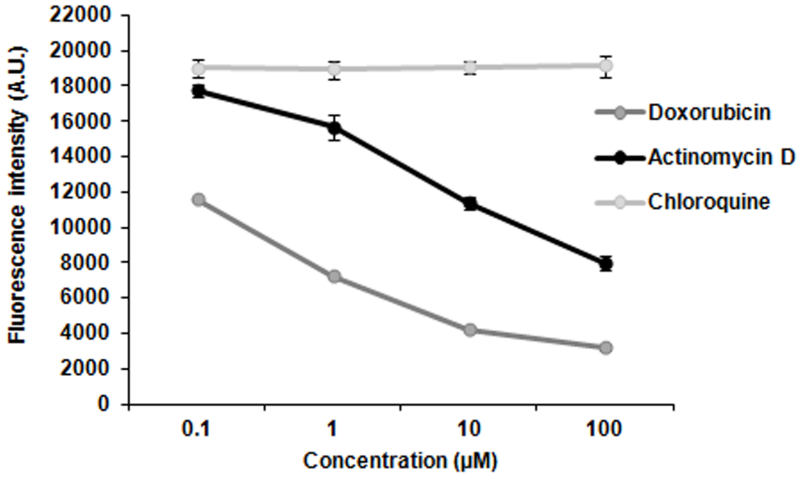

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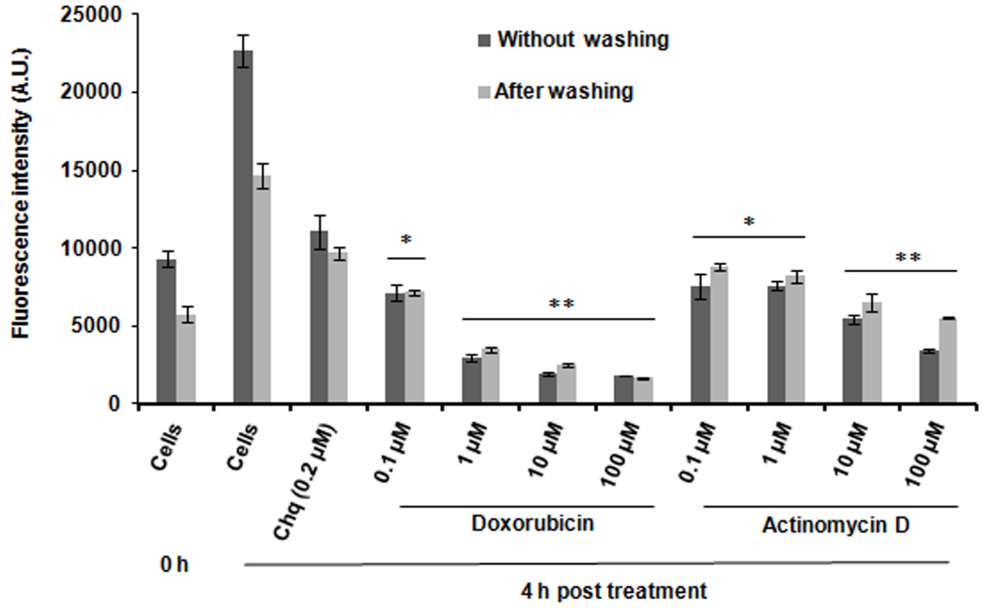

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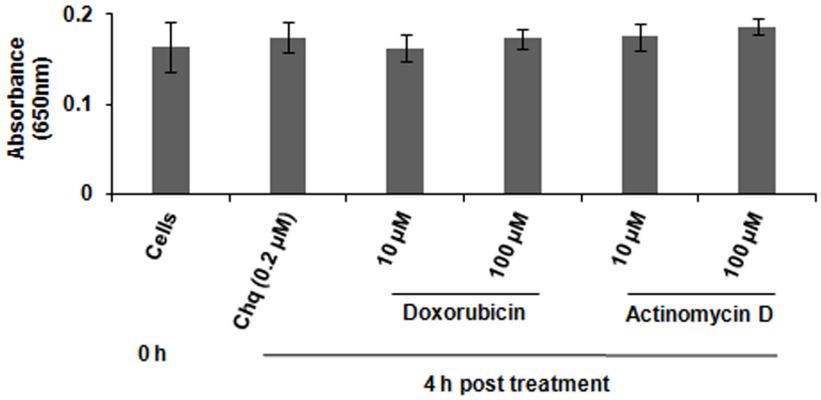

Table 1: List of peptides used in the study.

Name of Peptide	Sequence	[a]pI	Length
Tat	GRKKRRQRRRPPQ	12.70	13
Penetratin	RQIKIWFQNRRMKWKK	12.31	16
P3	RRRQKRIVVRRRLIR	12.90	15
P8	RRWRRWNRFNRRRCR	12.54	15
TP10	AGYLLGKINLKALAALAKKIL	10.18	21
Control cyclic peptide (CP1)	CTHPATSWC	6.72	9
Control linear peptide (CP2)	ITWNEKKSHHLY	8.51	12

[a] pI values were calculated using ProtParam tool (http://web.expasy.org/protparam/).