The potent Anti-malarial activity of Acriflavine in

\textit{vitro} and \textit{in vivo}

Srikanta Dana,\textsuperscript{1,2,§} Dhaneswar Prusty,\textsuperscript{1, §} Devender Dhayal,\textsuperscript{3} Mohit Kumar Gupta,\textsuperscript{1} Ashraf Dar,\textsuperscript{1} Sobhan Sen,\textsuperscript{4} Pritam Mukhopadhyay,\textsuperscript{2} Tridibesh Adak,\textsuperscript{2} and Suman Kumar Dhar\textsuperscript{1,*}

\textsuperscript{1}Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi 110067, India
\textsuperscript{2}Supramolecular and Material Chemistry Lab, School of Physical Sciences, Jawaharlal Nehru University, New Delhi 110 067, India
\textsuperscript{3}National Institute of Malaria Research, New Delhi 110077, India
\textsuperscript{4}Spectroscopy Laboratory, School of Physical Sciences, Jawaharlal Nehru University, New Delhi 110067, India
\textsuperscript{§}Srikanta Dana and Dhaneswar Prusty contributed equally to this work
\textsuperscript{*}Corresponding author

E-mail: skdhar2002@yahoo.co.in, skdhar@mail.jnu.ac.in

CONTENTS:

1. Supplementary Figures .............................................. 2-5
2. Supplementary Tables .................................................. 6-7
3. Supplementary Materials and Methods ............................. 8-10
1. Supplementary Figures:

**Fig. S1.** The effect of transport inhibitor dantrolene on ACF uptake. Synchronized trophozoite stage parasites were incubated in the absence or presence of dantrolene (25 micromolar) for ten minutes and four hours respectively. Subsequently, the parasites were incubated in the presence of ACF for five minutes and processed for fluorescence microscopy. Mean fluorescence intensity from ten images each from dantrolene untreated and dantrolene treated (ten minutes and four hours respectively) were calculated and plotted graphically. Dantrolene affected the uptake of ACF compared to the untreated parasites although the duration of dantrolene treatment had no visible effect on ACF uptake.
Fig. S2. Synchronized ring stage parasites (12-14 hr) were incubated in drug free media or media supplemented with ACF (100nM) or hydroxy urea (HU) (70µg/ml). Parasite growth was evaluated after 16 hr and 32 hr post drug treatment by giemsa stained thin blood smear and compared with untreated culture. Untreated parasites grew normally whereas both ACF treated and HU treated parasites showed growth arrest in the trophozoite stage.

Fig. S3. The effect of ACF on gyrase mediated DNA cleavage assay. pBR322DNA was incubated with EcGyrA in combination with PfGyrB (A) or EcGyrB (B) in the presence 3µg/ml ciprofloxacin. Different concentrations of ACF were added in the cleavage reaction as indicated on the top. The left most lane does not contain any protein where as the right most lane has only linear pBR322 digested with EcoR1. “OC”, “L” and “S” indicate open circular, linear and supercoiled DNAs respectively. The results indicated that EcGyrA+PfGyrB mediated and
Ciprofloxacin induced DNA cleavage was inhibited in the presence of ACF (A). No DNA cleavage was observed in case of EcGyrA+EcGyrB under the same experimental conditions (B).

**Fig. S4.** Electrophoretic mobility shift assay (EMSA) was performed using radiolabeled double stranded DNA probe and PfGyrB protein in the absence or presence of different concentrations of ACF as indicated on the top as per the protocol described in the materials and methods. The left most lane does not contain any protein. The positions of the free probe and DNA-protein complex are indicated. ACF inhibited the DNA binding activity of PfGyrB in a concentration dependent manner.
Fig.  S5. The uptake of ACF by the parasitized red blood cells in the *Plasmodium berghei* infected mouse. ACF (5mg/kg body weight, (resuspended in PBS) was injected intraperitoneally in the *P. berghei* infected mouse. Blood was collected from the tail vein after 4 hours and subsequently processed for fluorescence microscopy as described earlier. Blood was also collected and processed similarly from control (only PBS injected) mouse. Fluorescence signals of ACF were obtained from parasitized RBC isolated from *P. berghei* infected mouse in all the three stages (ring, trophozoite and schizont) of the parasite (right panel). Fluorescence intensity increased significantly in the schizont stage parasites. No such signal was obtained from parasitized RBC isolated from only PBS injected mouse (Cont) (left panel). The first column from each panel (I) shows the fluorescence signal, the second column (II) shows the DIC images of the parasites while the last column shows the merged images (I+II).
2. Supplementary Tables:

**Table S1.** Peter’s test on Swiss albino mice using *P. berghei* parasites as described in the materials and methods. Effects of drugs on body weight of mouse during the experiment period.

| Doses                                      | Mouse no | Body weight at day 0 (before drug administration) | Body weight at day 4 (after drug administration) | Body weight at day 13 (after drug administration) |
|--------------------------------------------|----------|---------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Control (No drug) + PBS                    | M1       | 25 gm                                             | 25 gm                                           | 22.2 gm                                         |
|                                            | M2       | 30 gm                                             | 30 gm                                           | dead                                            |
|                                            | M3       | 30 gm                                             | 30 gm                                           | 21.3 gm                                         |
|                                            | M4       | 25 gm                                             | 25 gm                                           | 18.0 gm                                         |
| Chloroquine (CQ) 5mg/Kg body weight        | M5       | 30 gm                                             | 30 gm                                           | 28.7 gm                                         |
|                                            | M6       | 30 gm                                             | 30 gm                                           | 28.5 gm                                         |
|                                            | M7       | 25 gm                                             | 25 gm                                           | 26.3 gm                                         |
|                                            | M8       | 20 gm                                             | 20 gm                                           | dead                                            |
| Acriflavine (ACF) 5mg/Kg body weight       | M9       | 35 gm                                             | 35 gm                                           | 33.1 gm                                         |
|                                            | M10      | 30 gm                                             | 30 gm                                           | 29.0 gm                                         |
|                                            | M11      | 35 gm                                             | 35 gm                                           | 32.4 gm                                         |
|                                            | M12      | 30 gm                                             | 30 gm                                           | 33.6 gm                                         |
Table S2. Fluorescence lifetimes ($\tau_i$), amplitude ($a_i$) and average lifetime ($\langle \tau \rangle$) for ACF under different conditions as described below following TCSPC studies. Emission wavelength = 525 nm

| Sample                                  | $a_1$ (ns) | $a_2$ (ns) | $\langle \tau \rangle$ (ns) |
|-----------------------------------------|------------|------------|-----------------------------|
| ACF in Buffer                           | 0.04       | 0.96       | 4.7                         | 4.55                       |
| ACF in presence of PfGyrB               | 0.06       | 0.94       | 4.7                         | 4.47                       |
| ACF in presence of DNA                  | 0.49       | 0.51       | 5.2                         | 2.95                       |
| ACF in presence of DNA and              | 0.49       | 0.51       | 5.2                         | 3.00                       |

*** Fluorescence decay was fitted with function: $F(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$. 

---

7
3. Supplementary Materials and Methods:

**Immunofluorescence assay (IFA) for DNA replication foci studies**

For IFA using the antibodies against PfPCNA, ACF (100 nM) or hydroxyurea (70 µg/ml) treated or untreated trophozoite stage parasites were smeared and fixed with chilled methanol. Samples were permeabilized in 0.005% saponin and blocked in 3% BSA for one hour at room temperature. Fixed samples were incubated overnight with primary antibody (1:3000) at 4°C. After thorough washing with PBS, the cells were incubated with 1:1000 anti-mouse Alexa 594 (Invitrogen) and DAPI (1µg/ml) for 1 hour. After three more washes with PBS, cells were treated with anti-fade and sealed under a coverslip for observation as described above for live fluorescence microscopy.

**Electrophoretic mobility shift assay (EMSA)**

A PCR amplified 240-bp DNA fragment containing strong gyrase site (SGS) from plasmid pBR322 as described previously was radiolabeled using γ-32PATP with the help of polynucleotide kinase enzyme. The purified labeled oligos were incubated with or without PfGyrB in the absence or presence of different concentrations of ACF in binding buffer [10mM Tris-Cl (pH 7.5), 100mM KCl, 5mM MgCl₂, 10 mM DTT, 50µg/ml BSA and 6% glycerol]. Samples were then resolved in a 6% native poly acrylamide gel (6% acryl-amide-bisacrylamide, 1X TGE buffer and 5% glycerol) using 1X TGE buffer (25mM Tris base, 190mM glycine, 1mM EDTA) supplemented with 10mM MgCl₂ for 10 hour at 150V (4°C). The gels were further dried and autographed.
ATPase assay

The ATPase assay of PfGyrB alone or with DNA in the presence or absence of drugs was measured by coupled pyruvate kinase/lactate dehydrogenase (PK/LDH) assay system as per protocol described elsewhere.\textsuperscript{1} The hydrolysis of ATP by PfGyrB was linked to the oxidation of NADH and the reactions were monitored spectrophotometrically at 340nm (Beckman Coulter DU). 70µl reactions were carried out at 22\textdegree C in a reaction buffer containing 35mM Tris-Cl (pH 7.5), 70mM KCl, 5mM MgCl\textsubscript{2}, 250µM NADH, 1.07 mM Phoshoenol pyruvate, 0.56 units of Pyruvate kinase and 0.728 units of lactate dehydrogenase and various concentrations of ATP (ATP-Mg\textsuperscript{2+}). The reaction mixture was incubated at room temperature for 5 minutes before the addition of protein. The rate of ATP hydrolysis was calculated from initial 5 minutes of continuous data collected from decrease in absorbance at 340nm by using the equation:

\[
\text{Rate of ATP hydrolysis} = \frac{\Delta [ADP]}{\Delta \text{time}} = \frac{-\Delta \text{abs} @340\text{nm}/\Delta \text{time}}{(\text{cuvette path length in cm}) \times (6.22 \text{ mM}^{-1}\text{Cm}^{-1})}
\]

Where 6.22 mM\textsuperscript{-1}Cm\textsuperscript{-1} is the molar extinction co-efficient of NADH at 340 nm. The ATPase data was fitted by using non linear regression analysis in GraphPad Prism 5 software.

DNA cleavage and supercoiling assay

DNA cleavage assay was carried out as per the protocol described elsewhere.\textsuperscript{1} In brief, the reaction was carried out in DNA cleavage buffer [35 mM Tris-HCl (pH 7.5), 24 mMKCl, 4 mM
MgCl$_2$, 5 mM DTT, 50 µg/ml BSA and 6.5% glycerol] using plasmid pBR322 DNA as a substrate in the presence of 3µg/ml ciprofloxacin. The samples were incubated for 1 hour for the *E. coli* GyrAB combination and for 2 hour for the EcGyrA and PfGyrB combinations at 25° C in the absence or presence of different concentrations of ACF. The reaction was terminated by the addition of 3 µl of 2% sodium dodecyl sulfate (SDS) and 1 µl of 5 mg/ml proteinase K (Sigma). Samples were further incubated at 37°C for 45 minutes and electrophoresed in a 1% agarose gel. The gel was finally stained with ethidium bromide. Supercoiling reaction was performed following protocol described elsewhere$^1$ in cleavage buffer supplemented with 2mM of spermidine and 2mM of ATP.

**Time resolve fluorescence spectroscopy (TRFS)**

Fluorescence lifetime of ACF was measured in a time correlated single photon counting (TCSPC) setup (Edinburgh instrument, UK). All samples were prepared in DNA binding buffer [35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl$_2$, 5 mM DTT, and 5% glycerol]. Purified pUC18 plasmid and recombinant PfGyrB were used. Each sample was incubated at RT for 30 minutes before collecting the fluorescence lifetime data. The samples were excited with a picosecond diode laser (Edinburgh inst., UK) of wavelength 470 nm, and the emission was collected at 525 nm with magic-angle polarization.

**Reference**

1. Dar, A., Prusty, D., Mondal, N., and Dhar, S. K. (2009) A unique 45-amino-acid region in the toprim domain of Plasmodium falciparum gyrase B is essential for its activity. *Eukaryotic Cell* 8, 1759-1769.