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Potent Antimalarial Activity of Acriflavine *In Vitro* and *In Vivo*

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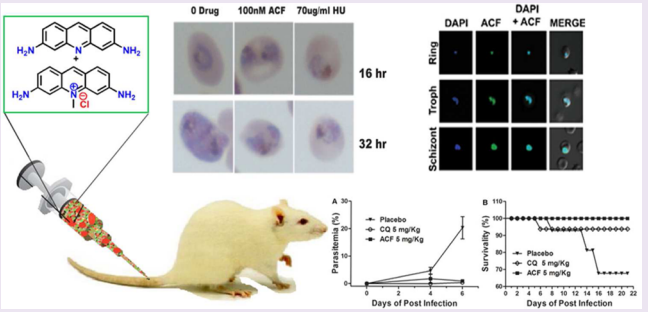
Supporting Information

ABSTRACT: Malaria continues to be a major health problem globally. There is an urgent need to find new antimalarials. Acriflavine (ACF) is known as an antibacterial agent and more recently as an anticancer agent. Here, we report that ACF inhibits the growth of asexual stages of both chloroquine (CQ) sensitive and resistant strains of human malarial parasite, *Plasmodium falciparum in vitro* at nanomolar concentration. ACF clears the malaria infection *in vivo* from the bloodstreams of mice infected with *Plasmodium berghei*. Interestingly, ACF is accumulated only in the parasitized red blood cells (RBCs) and parasite specific transporters may have role in this specific drug accumulation. We further show that ACF impairs DNA replication foci formation in the parasites and affects the enzymatic activities of apicoplast specific Gyrase protein. We thus establish ACF as a potential antimalarial amidst the widespread incidences of drug resistant *Plasmodium* strains.



Each year, malaria kills 1 to 2 million human beings.[1] The primary chemotherapeutic drugs, such as chloroquine (CQ) and pyrimethamine, are of little or no use because the malarial parasite developed resistance against them.[2] Recent reports of resistance to artemisinin, the only effective antimalarial drug at present, have become the cause of concern.[3,4] Therefore, it is important to search for new antimalarial drugs and also to test the efficacies of some of the old drugs whose antimalarial potential has not been verified in depth.

Acriflavine (ACF), a mixture of 3,6-diamino-10-methylacridinum chloride (trypaflavine) and 3,6-diaminoacridine (proflavine), is an old drug that was previously used as a trypanocidal agent during World War II.[5] However, due to the preferential use of CQ for the treatment of malaria, the antimalarial activity of ACF was not investigated further.

ACF has been recently shown to have potential anticancer activity in mice[6] and has been approved by FDA for clinical trials. Beside anticancer action, ACF is an antibacterial acridine used in topical antiseptics.[7]

Gyrase is a type II topoisomerase present in bacteria with two subunits (A and B). Two naturally occurring point mutations in gyrase B (*acrB*) enzyme (S759R; R760C) result in loss of gyrase binding to DNA and make *E. coli* susceptible to ACF.[8] The apicoplast of malarial parasite, *P. falciparum* is an essential organelle housing both the subunits of bacterial type gyrase enzyme. The functional complementation of *P. falciparum* GyrB (PfGyrB) with *E. coli* GyrB (EcGyrB),[9] the conservation of one of the *acrB* residues in the PfGyrB (R965),[10] and the previous use of different acridine derivatives as antimalarials as well as antiprotozoal agents[11] prompted us to investigate the potency of ACF as an antimalarial agent and elucidate its mechanism of action.

We find that ACF not only kills CQ sensitive and resistant malarial parasites *in vitro* in nanomolar range, it also suppresses parasite growth significantly *in vivo* in the mouse model system. Interestingly, we find that ACF is accumulated preferentially in the parasitized RBC and not in uninfected RBC possibly through its uptake via parasite specific transporters. ACF impairs the nuclear metabolic pathways, such as DNA replication. We further used *P. falciparum* gyrase as a reference DNA metabolic enzyme to show that ACF inhibits the topological activity of this enzyme, possibly by interacting with the substrate DNA. These findings establish ACF as a potent therapeutic molecule against malaria, an infection often associated with resurgence of drug resistant parasitic strains.

RESULTS

ACF Inhibits *Plasmodium* Growth *In Vitro*. ACF is a mixture of trypaflavine and proflavine with a ratio of 2:1 (Figure 1A). The presence of proflavine stabilizes the mixture. To determine the antimalarial activity of ACF *in vitro*, CQ sensitive

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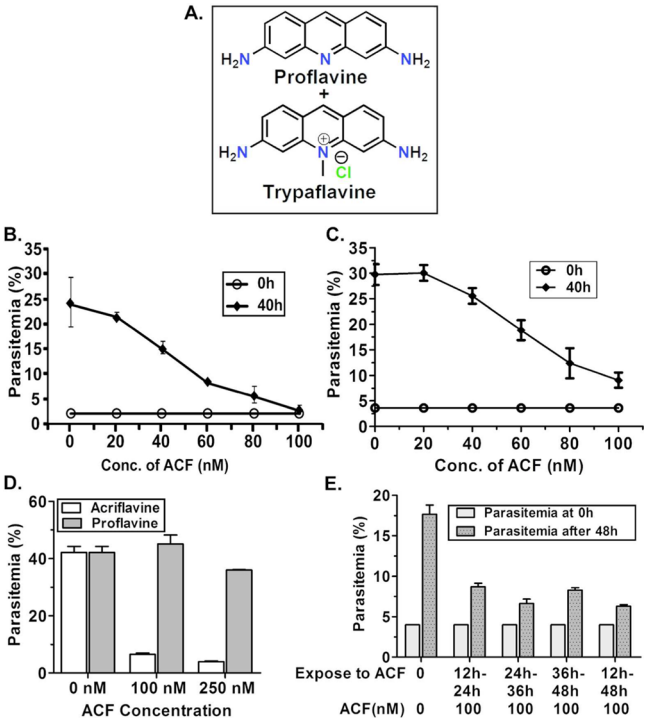

Figure 1. Effect of ACF on 3D7 and W2 strains of *P. falciparum* grown *in vitro*. (A) Structure of ACF: It is a mixture of 3,6-diamino-10-methylacridinum chloride (Trypflavine) and 3,6-diaminoacridine (Proflavine). Synchronized chloroquine (CQ) susceptible 3D7 (B) and chloroquine (CQ) resistant W2 (C) parasites at ring stage were treated with different concentrations of ACF as indicated. Calculation of percent parasitemia (mean of triplicate experiments) showed that ACF inhibited the growth of both the parasites efficiently. (D) 3D7 parasites were incubated in the absence or presence of different concentrations of ACF or proflavine as indicated. (E) Effect of ACF on different stages of 3D7 parasites was established by treating the parasites for 12 hours with 100 nM ACF at three different stages as ring (~12 h), trophozoite (~24 h), and schizont (~36 h) of first life cycle. Ring stage parasites were also incubated continuously for ~36 h. In each case, parasitemia (mean of triplicate experiments) was determined at the end of the first life cycle and plotted against different concentrations of ACF/Proflavine. Error bars shown in parts B–E correspond to standard error of mean (SEM).

(3D7) and CQ resistant (W2) *P. falciparum* parasites were synchronized in ring stages followed by treatment with a range of ACF concentrations (0–100 nM). After 40 h of the ACF treatment, the parasitemia was calculated in each case. There was a drastic decrease in parasitemia with >90% inhibition of 3D7 parasites at 100 nM ACF concentration (Figure 1B). Similarly, the growth of CQ resistant parasites (W2) was reduced considerably (>70%) at 100 nM ACF (Figure 1C). The IC_{50} value of ACF lies in between 40 and 60 nM for the CQ sensitive 3D7 strain whereas 60–80 nM for CQ resistant W2 parasites (Figure 1B and C). Further, we evaluated the antimalarial property of ACF vs proflavine at 100 nM and 250 nM drug concentrations (Figure 1D). The results indicate that proflavine is ineffective against malaria parasite *in vitro* suggesting that trypaflavine is the active antimalarial component in ACF.

The intraerythrocytic asexual life cycle of *P. falciparum* consists of three developmental stages including ring, trophozoite, and schizont. To determine which of these developmental stages is specifically targeted by ACF, different stages of synchronized parasites such as ring (~12 h post invasion), trophozoite (~24 h post invasion), and schizont (~36 h post invasion) were treated with 100 nM ACF for 12 h. Parasites were washed and resuspended in fresh complete RPMI media after 12 h treatment and grown to complete the first life cycle. Interestingly, the growth of parasites was inhibited at all the three stages of parasite development when treated with ACF. However, the impact was more prominent at the trophozoite stage compared to the other stages. Interestingly, the effect of ACF in trophozoite stage parasites (~12 h treatment) was similar to continuous treatment of ACF for ~36 h, beginning at the ring stage (Figure 1E). Altogether, these data indicate that ACF is effective at all the stages of asexual life cycle, and suggest that it targets either a conserved function or multiple functions throughout asexual life cycle.

ACF Inhibits the *Plasmodium* Growth *In Vivo*. The antiparasitic activity of ACF in the *in vitro* culture prompted us to examine the antimalarial activity of ACF in mouse model infected with *Plasmodium berghei*. The mice were divided into three groups (each group had 4 mice). For four consecutive days, group I was intraperitoneally injected with 5 mg ACF/kg body weight; group II received 5 mg CQ/kg body weight and the group III received placebo (PBS) and no drug. Every alternate day the parasitemia was calculated from blood samples

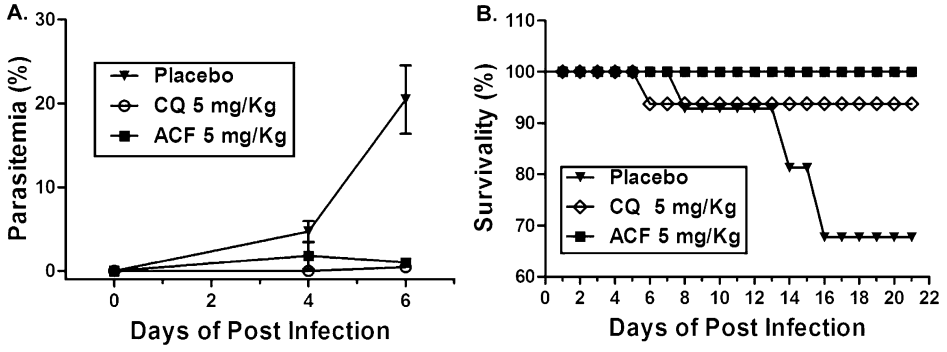

Figure 2. Effect of ACF in mouse model of *P. berghei* infection. (A) Graph shows average parasitemia at different days of post infection for placebo (PBS), chloroquine (CQ), and ACF treated *P. berghei* infected mice as mentioned in the materials and methods. Inset shows the details of the drugs used placebo (PBS only), 5 mg/kg body weight ACF, and 5 mg/kg body weight chloroquine (CQ) (both drugs were resuspended in PBS), respectively. (B) It demonstrates rate of surviability of chloroquine (CQ) and ACF treated mice in comparison with placebo (PBS) treated mice plotted against days postinfection.

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collected from the tail of placebo (control) and drug treated mice. The placebo (control) mice developed 22.33% parasitemia at the end of day 6, where as parasitemia in ACF and CQ treated mice was calculated as 1.04% and 0.35%, respectively, as indicated in Figure 2A. The mouse groups were kept under observation for 3 weeks after the drug treatment. The survivability rate of the ACF treated mice group was 100% and that of CQ treated mice was 75%, whereas only 25% survivability rate was observed in untreated control mice under the same experimental conditions (Figure 2B). No drug-related effects on body weight or general condition of animals were noticed during the dosing and recovery periods (Supporting Information Table S1). We also used up to 10 mg ACF/kg body weight, which also showed potent antimalarial activity *in vivo* with no apparent effect on the body weight and physiological conditions of the animals during the course of the experiment (data not shown). These results confirm the potent *in vivo* antimalarial activity of ACF.

Localization of ACF in Live Malaria Parasites. The antimalarial activity of ACF both *in vitro* and *in vivo* encouraged us to study its uptake and localization into the parasite. Generally, ACF is known to have preference for interaction with the regions of DNA which are rich in AT base pairs.[12] Since *Plasmodium* DNA is ~80% AT rich,[13] we examined the localization of ACF within the parasite by fluorescence microscopy. For this purpose, mixed stage parasites were treated with 100 nM ACF for 5 min and subsequently observed under fluorescence microscope. The nuclei were stained with DAPI in order to determine the localization and accumulation of ACF with respect to nuclear signal. Merge panels of ACF and DAPI indicate that ACF is specifically accumulated in the parasitized RBC and not in the uninfected RBC. Within the infected RBC, ACF signal was often merged with DAPI stained nuclei (Figure 3A). These results show that ACF is specifically accumulated in the infected parasites.

Uptake of ACF in the Presence of Different Transport Inhibitors. The specific accumulation of ACF in the infected RBC only raises the possibility that the parasite specific transporters may play a role for the accumulation of ACF in the parasites. To examine this possibility, we used different NPP (New permeation pathway) and PSAC (Plasmodial surface anion channel) inhibitors such as furosemide, TP-52, and dantrolene.[14−17] NPP stands for any pore or channel-like transport mechanism that changes the permeability of the host plasma membrane with the maturation of the parasite inside the host.[18,19] Multiple distinct transport mechanisms may be induced by the parasites. However, more specific nomenclature for individual ion channels has been proposed in some cases (PSAC).[20,21] The parasites in culture were incubated in the absence and presence of different transport inhibitors for 10 min followed by ACF treatment for 5 min. The accumulation of the drug into the parasite nuclei was analyzed by fluorescence microscopy and the fluorescence intensity of the accumulated ACF was measured by densitometry scanning. We find that both furosemide (NPP inhibitor) and TP-52 inhibit the uptake of ACF significantly as compared to the inhibitor free ACF treated control parasites (Figure 3B). Dantrolene (PSAC inhibitor) showed moderate effect on the uptake of ACF under the similar experimental conditions (Figure 3B). In order to investigate whether the moderate effect of dantrolene on ACF uptake was due to the time of incubation in the presence of dantrolene, we incubated the parasite for 10 min and 4 h with dantrolene (25 μM) respectively before ACF uptake studies. We found that the effect of dantrolene was not dependent on the duration of the incubation time (Supporting Information Figure S1). The fold decrease in ACF uptake was similar in both the cases compared to the untreated parasites. The effect of dantrolene was discussed previously where 10 μM of dantrolene was enough to inhibit increased permeability of some solutes (anions, sugars, amino acids, and bulky organic cations) within an hour of treatment by specific inhibition of the plasmodial surface anion channel.[22] These results suggest that some parasite specific transporters are possibly involved in the uptake of ACF into the parasite.

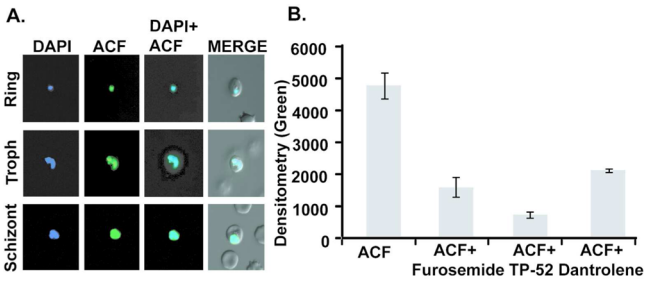

Figure 3. Localization of ACF in the parasites. (A) The localization of ACF in the live parasites was tracked using fluorescence microscopy at the excitation spectra of 488A^0. DAPI shows the nuclei and merge panels include bright field images. ACF is accumulated only in the infected red blood cells nuclei. (B) Effect of different parasite transporter inhibitors on the accumulation of ACF in the parasites. The figure shows comparison of ACF accumulation in the parasites in the absence and presence of different inhibitors as indicated below following densitometry analysis of fluorescence microscopy images. In each case, the average (mean) intensity was calculated from ten different images. Error bars represent SEM.

Effect of ACF on Parasite DNA Replication. Since ACF is accumulated in the parasite nuclei, we hypothesized that ACF may affect the global DNA metabolic processes such as DNA replication in the nucleus of the parasites. To investigate the effect of ACF on the parasite DNA replication, we used hydroxyurea (HU),[23] a known inhibitor of parasite DNA replication in parallel with ACF. We found that both ACF (100 nM) and HU (70 μg/mL) arrested the parasite growth at the early trophozoite stage that corresponds with the time of initiation of DNA synthesis (Supporting Information Figure S2). Further, we investigated the pattern of active replication foci formation in the presence of HU and ACF following immunofluorescence assay using antibodies against *P. falciparum* PCNA. PCNA has previously been shown to form distinct DNA replication foci in the parasites during replicating trophozoite stage.[24] Immunofluorescence results indicate the presence of diffused signals of PCNA in ACF as well as HU treated parasites compared to the distinct nuclear foci found in untreated parasites (Figure 4). These results indicate that the uptake of ACF in the parasite nucleus disrupts the replication foci formation and thus may abrogate the process of DNA replication.

Effect of ACF on Activity of DNA Metabolic Enzymes. ACF has been reported to inhibit the activity of the DNA topoisomerases.[25] We previously characterized the apicoplast targeted bacterial type DNA topoisomerase enzyme gyrase from *Plasmodium falciparum*.[9] Moreover, PfGyrase contains one of the *acrB* mutations that make EcGyrB sensitive to ACF. Gyrase is a two subunit (A and B) enzyme, where the A subunit (GyrA) is responsible for DNA cleavage and religation reaction

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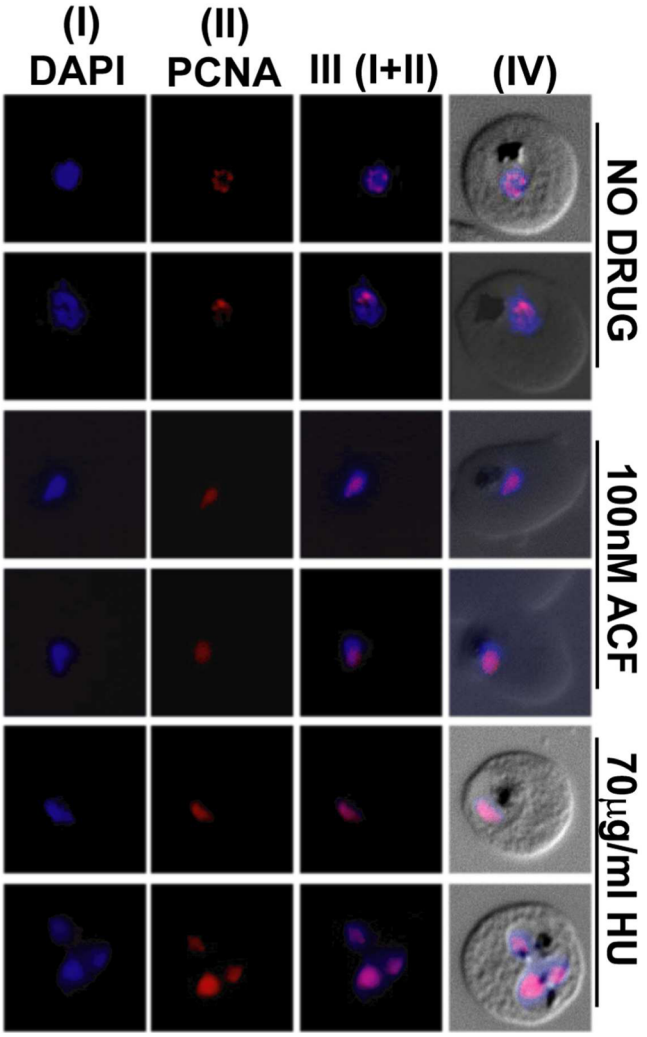

Figure 4. Effect of ACF on replication foci formation. Immunofluorescence assay to show pattern of replication foci (PCNA foci) formation during parasite developmental stages in untreated, ACF (100 nM) and HU (70 μg/mL) treated parasites. DAPI was used for nuclear staining. Panel III shows the merged images of DAPI (I) and PCNA (II) signals whereas panel IV represents the merged images of PCNA signal, DAPI and DIC images.

and is targeted by quinolones class of drugs such as ciprofloxacin.[26] The B subunit (GyrB) is an ATPase and is targeted by coumarins (Coumeramycin A1, novobiocin).[27,28] Since ACF is known to interact with DNA, we examined whether ACF would affect the DNA dependent ATPase activity of PfGyrB. PfGyrB showed basal ATPase activity, which was reduced in the presence of coumermycin but not in the presence of ACF (Figure 5A). The presence of AT rich DNA stimulated the PfGyrB ATPase activity significantly. However, the presence of ACF reduced the stimulation of ATPase activity drastically even at 5 μM ACF concentration. At ~80 μM ACF concentration, DNA stimulated ATPase activity was reduced to the basal level observed in the absence of DNA (Figure 5A). These results show that ACF inhibits the DNA stimulated ATPase activity of PfGyrB. PfGyrB complements the EcGyrB function.[9] PfGyrB in association with *E. coli* GyrA (EcGyrA) shows ciprofloxacin mediated DNA cleavage activity as well as DNA supercoiling activity.[29] We tested the effect of ACF on ciprofloxacin mediated DNA cleavage of pBR322 substrate DNA by PfGyrB−EcGyrA complex and EcGyrB−EcGyrA complex. We found that the presence of ACF inhibited the DNA cleavage activity of PfGyrB−EcGyrA complex in a concentration dependent manner while the DNA cleavage activity of EcGyrB−EcGyrA complex was not affected at all under our experimental conditions (Supporting Information Figure S3).

Next, we examined the effect of ACF on the supercoiling activity of EcGyrB−EcGyrA complex and PfGyrB-EcGyrA complex, respectively. We found that ACF inhibited the supercoiling activity PfGyrB−EcGyrA complex very efficiently whereas the same activity of EcGyrB-EcGyrA complex was not affected at all under our experimental conditions (Figure 5B). These results clearly indicate that ACF specifically inhibits PfGyrB specific activity.

The inhibition of DNA dependent ATPase activity and supercoiling activity of gyrase raises the issue whether ACF inhibits the gyrase enzyme activities by binding to PfGyrB or the inhibition is mediated through the interaction of ACF with DNA. For this purpose, we studied the fluorescence lifetime decays (Figure 5C) of ACF using time-correlated single photon counting (TCSPC) setup in the absence and presence of DNA and PfGyrB protein. We found that nanosecond lifetime decay of ACF alone markedly differed in the presence of DNA, whereas the presence of PfGyrase B did not change it at all (Supporting Information Table. S2). These results strongly suggest that the inhibition of gyrase activity by ACF is mediated through interaction of the drug with the DNA substrate. This interaction may lead to conformation change in DNA unsuitable for binding of PfGyrase with DNA. Indeed, we found that ACF inhibited the DNA binding activity of PfGyrB in Electrophoretic Mobility Shift Assay (EMSA) as shown in Figure S4 (Supporting Information). These results confirm that parasite gyrase may be one of the targets of ACF as shown above by inhibition of DNA dependent activities of gyrase in the presence of ACF.

DISCUSSION

The drug resistance is a major impediment in the eradication of malaria infection. In the late 1950s, *Plasmodium* became resistant to CQ and by early 1970s the drug was completely replaced by a combination of sulphadoxine and pyrimethamine (SP) for malaria therapy. Because of rapid resistance by the *Plasmodium* parasite, SP had to be replaced with mefloquine and later in 1990s mefloquine resistance gave way to introduction of artimisinin therapy.[30] Now artimisinin, considered as most effective antimalarial drug, is becoming ineffective against malaria in South East Asia.[31]

The reports of artmisinin tolerance by *Plasmodium falciparum* prompted us to revisit ACF, an old and neglected drug, for antimalarial therapy. Any new or old therapeutic antimalarial drug should have the property of killing wild type and drug resistant parasite strains and should be effective against the blood stages of the parasite that are mainly responsible for the malaria symptoms in infected patients. Here, we show that ACF kills both CQ sensitive and resistant forms *P. falciparum in vitro*.

ACF remarkably cleared the malaria infection from the blood circulation of the mouse models infected with rodent specific *P. berghaie* (Figure 2A). No major side effects on the physiology of the animals were observed (Supporting Information Table S1). The ACF treated mice were as healthy as their control untreated counterparts. This emphasizes the potential of ACF as an antimalarial drug. It is intriguing that under the same

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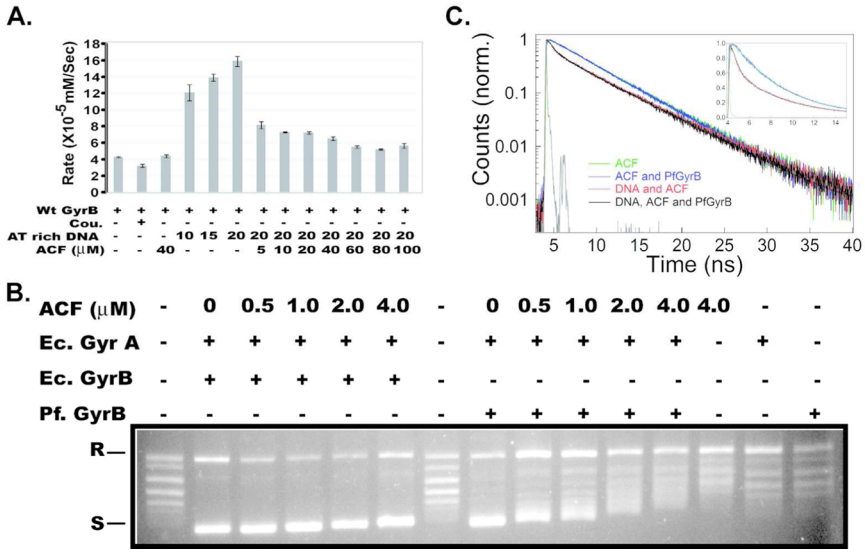

Figure 5. Effect of ACF on *Plasmodium* gyrase activity. (A) Analysis of DNA dependent ATPase activity of PfGyrB with different concentrations of ACF. ATPase assays were carried out by NADH-coupled enzymatic assay (as discussed earlier in ref 8). The reaction rates of PfGyrB with or without DNA were plotted against coumermycinA1 (PfGyrB inhibitor) or different concentrations of ACF. The experiments were performed in triplicate and the error bars represent SEM. (B) Effect of ACF on supercoiling activity of *E. coli* gyrase (A+B) (Lane 1−6) or EcGyrA-PfGyrB (Lane 7−15) using relaxed pUC18 DNA as substrate. Lane 1 and 7, relaxed pUC18 DNA; Lane 2, pUC18 DNA+EcGyrAB; Lane 3−6, ACF 0.5−4 μM, respectively; Lane 8, pUC18DNA+EcGyrA-PfGyrB; Lane 9−12, ACF 0.5−4 μM, respectively; Lane 13, pUC18DNA+ACF; Lane 13−14, EcGyrA, PfGyrB respectively. ACF inhibited EcGyrA+PfGyrB mediated supercoiling activity but no effect on EcGyr mediated activity was found under the same experimental conditions. (C) Interaction of ACF with DNA. Fluorescence transients of ACF (green), ACF+DNA (red), ACF+PfGyrB (blue), ACF +DNA+PfGyrB (black) measured with TCSPC technique as described in the materials and methods (Supporting Information) with an excitation at 470 nm. Inset shows decays in initial time-range.

experimental conditions and drug concentration (5 mg/kg body weight) ACF shows better survivability (100%) than CQ treated parasite infected mice (75%) (Figure 2B). The animal experiment results are really promising although further experiments will be required to ascertain whether ACF indeed has better potential over CQ as antimalarial therapy.

ACF is accumulated in the parasite nuclei but not in the uninfected RBC or within the intact region of RBC in the infected RBC (Figure 3A). Previously, it has been shown that ACF preferentially interacts with the AT regions of the minor grooves of DNA double helix.[12] The high AT richness (>80%) of the *Plasmodium* genome[13] may explain the specific accumulation of the drug into the parasite nuclei. We also show that *Plasmodium* specific membrane transporters have a role in the influx and accumulation of drug in the parasite nuclei (Figure 3B). It will be interesting to find out what factors determine these parasite membrane transporters to import ACF specifically into the parasite from the host erythrocytes.

The interaction of ACF with the parasite DNA is likely to interrupt the DNA metabolic process such as DNA replication. The ACF treatment disrupts the punctuate PCNA foci formation, which normally represent the DNA replication factories (Figure 4). How does ACF prevent the formation of replication foci remains to be elucidated further. It is possible that the interaction of ACF with DNA may alter the structure of the DNA affecting the DNA binding activity of various proteins. It may explain why the growth of parasites is inhibited at all the three stages of parasite development when treated with ACF (Figure 1E). ACF may modulate the function of the DNA binding proteins throughout different stages of the parasite life cycle. While during trophozoite stage it may affect the parasite DNA replication by modulating the DNA binding activity of replication factors, parasites from other stages (ring/schizont) may be affected by the inhibition of transcription, recombination, and many other DNA mediated processes that require proper DNA−protein interaction.

Interestingly, ACF could inhibit the DNA stimulated ATPase activity of PfGyrB and DNA supercoiling activity of PfGyrB in complex with EcGyrA whereas it failed to inhibit the supercoiling activity of EcGyrB in complex with EcGyrA. ACF did not interact with PfGyrB directly whereas it interacted with DNA. We have shown earlier that, unlike EcGyrB, PfGyrB interacts with DNA directly.[29] Therefore, ACF inhibits the PfGyrB activity by altering the conformation of DNA conducive for PfGyrB binding. Although we have taken PfGyrB as a model for the proof of mechanism of action of ACF, it can be extended to other DNA binding proteins.

There is some evidence related to the DNA interacting property of Acridine ring containing compounds that limit its widespread use.[32] UV exposure of *E. coli* cells in the presence of micromolar level of Acriflavine (1 μg/mL = 3.8 μM) results in higher rate of cell death, mutation frequency, and blockage of DNA, RNA, and protein synthesis.[33] No measurable incidence of mutation in non-UV exposed ACF treated cells was found in the same study.[33] The increased lethality and mutation rate in the presence of ACF may occur due to the interaction of ACF with UV damage site (thymine dimer, which is otherwise repairable). Therefore, ACF may not be mutagenic by itself. The concerns over the DNA intercalating and DNA damaging activity of ACF may be overspeculative considering the IC_{50} value of ACF for effective killing of the parasites lying within nanomolar range. The efficient uptake and retention of ACF by the parasites may also add to the potent antimalarial effect of ACF.

The *in vivo* antimalarial activity of ACF is intriguing. However, as per a previous report,[34] ACF showed short

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pharmacokinetic half-life, since the concentration in the blood of iv-administered ACF (200 mg) was found to be decreased by 90% over 5 min and undetectable after 30 min.[5] The above finding raises the issue regarding the stability and *in vivo* antimalarial activity of ACF. The *in vivo* antimalarial activity of ACF can be explained as follows. First, 5 mg/kg body weight of ACF is equivalent to ∼320 μM (considering the approximate blood present in mouse is 60 mL/kg body weight and the molecular weight of ACF is ∼260). The effective concentration of ACF will be 32 μM after 5 min, which is ∼1000 times more than the IC_{50} value of ACF (∼30 nM) obtained from *in vitro* culture. We believe that rapid efficient uptake of ACF (Figure 3AB) through parasite specific transporter and the presence of 1000 times more ACF than IC_{50} value are the key determinants of ACF action *in vivo* to clear the parasites. Second, we have shown that ACF interacts with DNA efficiently (Figure 5C; Supporting Information Figure S4) and it is accumulated in the nucleus very rapidly (Figure 3A). Therefore, once ACF is accumulated in the nucleus, the effective concentration of ACF outside the cell may not compromise its activity. To substantiate our claim, we have injected ACF in the parasite infected mice and followed up the uptake and retention of ACF in the parasitized red blood cells 4 h following injection. We find efficient uptake and retention of ACF by the parasites in the infected red blood cell compared to the untreated parasites (Supporting Information Figure S5). Finally, a previous study[6] has shown that 2 mg/kg body weight of ACF is sufficient to prevent tumor growth and tumor vascularization in mouse model. If a similar dose of ACF is effective against a tumor, which is solid mass of tissue that may restrict the entry of ACF at the core, ACF may clear parasites from the red blood cells that are in circulation.

Taken together, it is demonstrated convincingly that ACF shows potent antimalarial activity both *in vitro* and *in vivo* working in the nanomolar range. Moreover, ACF is accumulated specifically in the infected RBC containing parasites and not in the uninfected RBC. Further, it is shown that gyrase is a potential target of Acriflavine *in vitro*. As per our knowledge, *in vitro* and *in vivo* antiplasmodial activity of ACF has not been reported so far. In this report, we convincingly demonstrated that ACF, which is in clinical use as antibacterial and antifungal drug and recently promoted for anticancer therapeutic trails, could prove to be a potential antimalarial drug. Additionally, ACF is commercially available, has a very low cost, and is water-soluble, thus meeting the criteria required for an antimalarial drug.

METHODS

***P. falciparum* Culture.** *P. falciparum* strains, 3D7 (CQ sensitive) and W2 (CQ resistant) were cultured in human erythrocyte in RPMI 1640 medium with 0.5% (w/v) Albumax (Invitrogen-Gibco) in 90% N_2, 5% CO_2 and 5% O_2. W2 strain was kindly provided by Dr. Pawan Malhotra (ICGEB, New Delhi). Synchrony was maintained by sorbitol treatment at early ring stage in each life cycle. Parasite pellets were obtained by 0.05% saponin treatment and stored at −80 °C until use.

***In Vitro* Antimalarial Activity of ACF.** The antimalarial activity of different compounds was evaluated by using classical Giemsa staining followed by microscopic method with CQ sensitive and resistant strains. Synchronized ring-stage parasite culture was incubated with different compounds (ACF and proflavine) either for the entire duration of the first life cycle or for12 h duration as mentioned in the legend of Figure 1. Growth inhibition activity of the compounds was determined by plotting the drug concentration versus the average percentages of parasitemia of the triplicate culture with standard deviation after one complete life cycle. For morphological analysis of antimalarial action, samples were taken out from treated and untreated culture at different time intervals and Giemsa stained parasite pictures were captured using Nikon light-microscope.

***In Vivo* Antimalarial Efficacies Studies Using Mouse Model.** *In vivo* antimalarial activity of acriflavine was determined against rodent strain *P. berghei* according to Peter's 4-day suppressive test.[35] Swiss albino mice (four mice in each group) were inoculated with parasitized red blood cells. Thereafter, acriflavine/CQ was injected intraperitoneally with a fixed dose (5 mg/kg body weight) daily for four consecutive days beginning on the day of infection. The control group of mice was injected with phosphate buffer saline (PBS) as the drug was resuspended in PBS. Parasitemia was monitored by Giemsa stained thin blood smear. Mean values and standard deviations of parasitemia for each group were calculated on fourthand sixth days after inoculation. Survivability of animals along with intermittent assessment of body weight was followed up to 21 days. Survival curves were drawn in GraphPad prism 5 software using the method of Kalpan and Meier survival analysis.

Live Fluorescence Microscopy to Study Uptake of ACF in the Absence and Presence of Transport Inhibitors. Mixed stage parasites at high parasitemia were prepared for live fluorescence microscopy by incubating with 100 nM ACF for 5 min at 37 °C. After washing with RPMI media, nuclei were stained by 1 μg/mL of DAPI (Sigma) treatment. Distribution of ACF in live parasites was monitored using fluorescence microscopy. To determine the effect of transport inhibitors on ACF uptake, trophozoite stage parasites were incubated in the absence and presence of different transport inhibitors (furosemide, TP-52, and dantrolene, respectively)[14−17,22] for 10 min followed by 100 nM ACF treatment for 5 min as above. Subsequently fluorescent signals contributed by ACF were captured under similar exposure conditions as above. ACF uptake was also monitored after 4 h treatment with dantrolene. Average fluorescent intensity with standard deviation of ten different parasites was analyzed against different transport inhibitors. Images from the fluorescent microscope were collected in Axiovision and prepared in Adobe Photoshop. All steps are carried out at room temperature except for the incubation of drugs at 37 °C.

Additional experimental details of immunofluorescence assay (IFA) for DNA replication foci studies, electrophoretic mobility shift assay (EMSA), ATPase assay, DNA cleavage and supercoiling assay, and time resolved fluorescence spectroscopy (TRFS) are available in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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