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Research Letter

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RESEARCH LETTER – Pathogens & Pathogenicity

Antibacterial action of acriflavine hydrochloride for eradication of the gastric pathogen *Helicobacter pylori*

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One sentence summary: Acriflavine hydrochloride as a potent *Helicobacter pylori* therapeutic by itself or as a combination drug against antibiotic-resistant clinical isolates and infections in mice.

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ABSTRACT

Helicobacter pylori, a type 1 carcinogen, accounts for numerous gastric cancer-related deaths worldwide. Repurposing existing drugs or developing new ones for a combinatorial approach against increasing antimicrobial resistance is the need of the hour. This study highlights the efficacy of acriflavine hydrochloride (ACF-HCl) in inhibiting the growth of *H. pylori* reference strain and antibiotic-resistant clinical isolates at low concentrations. ACF-HCl inhibits *H. pylori* growth at MIC value 10 times less than that in *Escherichia coli*, another Gram-negative bacteria. Furthermore, ACF-HCl demonstrates synergistic effect with clarithromycin, a commonly used antibiotic against *H. pylori*. ACF-HCl treatment also eradicates *H. pylori* infection in the mice model efficiently. Our *in vitro* data indicate that bacterial membrane is the prime target. The novel action of ACF-HCl against antibiotic-resistant clinical isolates, synergistic effect with the conventional antibiotic clarithromycin and eradication of *H. pylori* from infected mice highlight the potential of ACF-HCl as a promising therapeutic agent against *H. pylori* by itself as well as for combinatorial therapy.

Keywords: *Helicobacter pylori*; antimicrobial; acriflavine hydrochloride (ACF-HCl); antibiotic resistance; synergistic; mice; infection

INTRODUCTION

Helicobacter pylori, a spiral-shaped Gram-negative microaerophilic pathogen, is known to cause peptic ulcer, gastric mucosa-associated lymphoid tissue lymphoma, gastric adenocarcinoma and chronic gastritis (Farinha and Gascoyne 2005). In 1994 and 2009, *H. pylori* was declared as a carcinogen by International Agency for Cancer Research, WHO. *Helicobacter pylori* infections are widespread infecting ∼50% of world's population and affecting ∼4.4 billion people globally (Savoldi *et al.* 2018). The risk of gastric cancer is associated with *H. pylori* infection and it is the second most leading common cause of cancer-related deaths across the globe (Wroblewski,

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Peek and Wilson 2010). The standard treatment regime for *H. pylori* infections, 'the triple therapy', comprises two antibiotics and a proton pump inhibitor (metronidazole, clarithromycin, levofloxacin or amoxicillin combined with omeprazole or pantoprazole) (Safavi *et al.* 2016). Quadruple therapy, which includes bismuth salts in combination with the existing antibiotics, is an alternative treatment (Gene *et al.* 2003). Emergence of antimicrobial resistance in *H. pylori* against clarithromycin, metronidazole and levofloxacin has led to failure of the treatment therapy worldwide (Wu *et al.* 2000; Jenks and Edwards 2002). Recently, *H. pylori* has been recorded by WHO among 16 antibiotic-resistant bacteria that pose greatest menace to the human health (Dang and Graham 2017).

A recent study has reported ≥15% resistance rates globally toward levofloxacin, metronidazole and clarithromycin (Savoldi *et al.* 2018). In North India, 70.6% drug resistance toward *H. pylori* was reported and the most prevalent among them was metronidazole resistance (Gehlot *et al.* 2016). Emergence of antimicrobial-resistant strains, side effects of the treatment, high costs of therapy and changes in microbiota of the gut have shifted recent research toward developing host immune protection and finding alternative drugs to target the pathogen. While new compounds including curcumin, ellagic acid, fluoroquinolones and arylamino-containing hydroxamic acids are evaluated as potential novel therapeutic candidates, roles of compounds used in other treatment regimens like antimalarial, antitubercular and anticancerous are also examined in eradicating *H. pylori* infections (De *et al.* 2009, 2018; Goswami *et al.* 2012; Kawada *et al.* 2013; Makobongo *et al.* 2013; Abu-Qatouseh *et al.* 2017; Liu *et al.* 2018).

Acridine, its derivatives and flavine-containing compounds offer a wide range of chemical, biological and pharmaceutical properties (Gensicka-Kowalewska, Cholewiński and Dzierzbicka 2017). One of the many acridine analogs is acriflavine (ACF), a mixture of proflavine (3,6-diaminoacridine) and trypflavine (3,6-diamino-10-methylacridinium chloride). During World War I, it was widely used as an antiseptic and to cure sleeping sickness (Browning, Gulbransen and Thornton 1917; Wainwright 2001). It exhibits antimalarial, antibacterial, antiviral, trypanocidal, antitubercular and fungicidal activities and was shown recently to possess anticancer activities as well (Funatsuki *et al.* 1997; Tripathi *et al.* 2006; Kawai and Yamagishi 2009; Lee *et al.* 2009; Manchester *et al.* 2013; Dana *et al.* 2014; Persinoti *et al.* 2014; Pepin *et al.* 2017). In view of the diverse targets and dynamic effects (Funatsuki *et al.* 1997; Lee *et al.* 2009; Cao *et al.* 2016), many acridine derivatives have been synthesized in order to overcome the instability and the toxicity of ACF. There are reports of feeding acriflavine hydrochloride (ACF-HCl), another acridine derivative (structure shown in Fig. 1A), directly to people for treating undulant fever in the 20th century (Thurber 1930). It has been used in clinical trials in combination with other drugs for the treatment of HIV and urinary tract infections (ClinicalTrials.gov; Mathe *et al.* 1998).

Accordingly, this study was conducted to evaluate the antibacterial effect of acriflavine hydrochloride on *H. pylori* strains, including antibiotic-resistant strains under both *in vitro* and *in vivo* infections in mice. The efficacy of this drug as a potential candidate for combination therapy was investigated as the alarmingly increasing rates of resistance toward the existing treatment regime have amounted to the global menace of this deadly pathogen. Our results suggest a potential role of ACF-HCl in anti-*H. pylori* therapeutics.

MATERIALS AND METHODS

In vitro ACF-HCl susceptibility testing

Helicobacter pylori strains used in this study were revived from stock cultures and ACF-HCl (Sigma-Aldrich, St. Louis, Missouri, United States) susceptibility testing was performed using the liquid macro-dilution technique for *H. pylori* reference strain 26695 and Gram-negative *Escherichia coli* K-12 strain W3110 taken as a control. . *Helicobacter pylori* clinical strains used in this study were isolated from biopsy specimens of patients with gastrointestinal disease and were provided by Dr. Asish Mukhopadhyay (NICED, Kolkata, India). For *H. pylori* clinical isolates, the agar dilution technique was followed to determine the minimum inhibitory concentration (MIC) value of ACF-HCl (De *et al.* 2018).

Synergistic effect of ACF-HCl with clarithromycin

The combined effect of ACF-HCl and clarithromycin on *H. pylori* growth was studied by the agar dilution method using two clarithromycin-resistant *H. pylori* strains (Koga *et al.* 2002). All plates were incubated under microaerophilic conditions at 37°C for 3 days after which the readings were taken. MIC was defined as the lowest concentration combinations of the compounds at which there was no visible growth.

Mice infection and ACF-HCl treatment

Mice infection studies were conducted according to protocols approved by the Animal Ethics Committee of NICED (National Institute of Cholera and Enteric Diseases; registration number 68/GO/Rebi/S/1999/CPCSEA; permit number NICED/CPCSEA/68/GO/(25/294)/2020-IAEC/AM/1), which mainly focused on maintenance of standard condition for living, minimum animal suffering and minimum number of mice associated with valid statistical evaluation.

Six-week-old, pathogen-free C57BL/6 male mice weighing 20 ± 5 g were divided into three groups, namely control group, infected group and treated group, comprising six mice each. The mice from the infected as well as the treated group were inoculated with 200 μl of a suspension of 10^8 colony-forming unit/ml (CFU/ml) mouse-colonizing strain, SS1, by oral gavage thrice a week at an interval of 1 day while the mice from the control group received only sterile phosphate-buffered saline (PBS) (Kundu *et al.* 2006). Prior to this, they were fasted for 4 h with free access to water. At 2 weeks post-incubation, the treated group was supplemented with ACF-HCl orogastrically, at a dose of 50 mg/kg body weight once daily for 1 week. Simultaneously, the infected group was given a complementary sterile PBS.

Then, 3 weeks post-infection, all mice from each group were sacrificed by cervical dislocation. Mice gastric tissue was isolated and assessed for polymerase chain reaction (PCR) and histological examination as described elsewhere (De *et al.* 2018).

PCR amplification of DNA isolated from *H. pylori*-infected mice stomach

Genomic DNA from mouse gastric tissues was extracted with a DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The presence or absence of specific bacterial gene, *vacA*, and the specificity for the mouse genome

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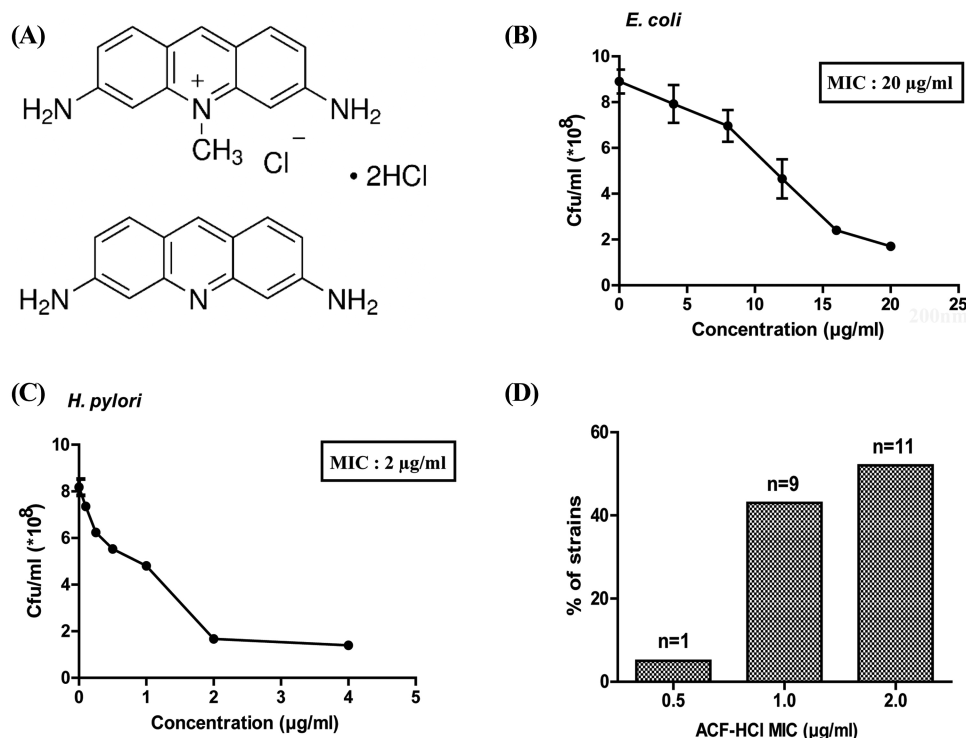

Figure 1. (A) Chemical structure of acriflavine hydrochloride. Dose-dependent killing of laboratory strains of (B) *Escherichia coli* K-12 W3110 strain and (C) reference *H. pylori* strain 26695 by ACF-HCl. The graphs represent mean ± SEM of three independent experiments. (D) Distribution of ACF-HCl MICs among the *H. pylori* clinical isolates from India.

were confirmed by PCR using specific primers (Table S1, Supporting Information) and DNA from the respective tissues (Kundu *et al.* 2011).

Mouse gastric tissue histology

Mouse stomach was taken out aseptically, dissected longitudinally and further into the body and pyloric parts, washed with PBS and finally fixed in 10% formalin buffer solution for histological evaluation. For sectioning, the fixed samples were dehydrated by alcohol and xylene and then embedded in paraffin wax. Five-micrometer sections were cut by a microtome and stained with hematoxylin and eosin (H/E) and observed under a microscope (Kundu *et al.* 2006).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses

Electron microscopy was performed as the described protocol (Kumar *et al.* 2013). Briefly, ACF-HCl treated and untreated *H. pylori* strain 26695 and *E. coli* strain K-12 cells were fixed and suspended in sodium cacodylate buffer. Ciprofloxacin-treated *H. pylori* 26695 strain was taken as control. Further processing of the samples was done in Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (JNU). Finally, ultrathin sections were cut and placed on grids that were examined using JEM-2100F (JEOL) transmission electron microscope.

Membrane permeabilization assay

Membrane permeabilization assay using propidium iodide (PI), a red fluorescent dye that can only traverse cells with loss of membrane integrity, was performed with slight modifications (Tyagi *et al.* 2015; Kwon *et al.* 2019). Briefly, *H. pylori* reference strain 26695 was treated with ACF-HCl at MIC concentration (2 µg/ml) for 2 min, ciprofloxacin (as a control) at MIC concentration and no drug as untreated control. The cells were then harvested and washed by 1× PBS and finally resuspended in 4',6-diamidino-2-phenylindole (DAPI) (1:1000) and propidium iodide (100 ng) in 1× PBS followed by immediate visualization under Zeiss ApoTome Fluorescence Microscope.

Target identification using pull-down and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

To identify the ACF-HCl target(s), pull-down based approach was employed as described elsewhere (Cao *et al.* 2016) with slight modifications. *H. pylori* 26695 strain lysate was prepared in order to extract membrane proteins and allowed to incubate with ACF-HCl bound beads. The proteins bound to beads were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained. The distinct protein band obtained only in the ACF-HCl bound beads lane was excised from the gel and subjected to LC-MS/MS analysis for protein identification.

RESULTS

Acriflavine hydrochloride inhibits the growth of antibiotic-resistant strains of *H. pylori*

In vitro susceptibility of *H. pylori* reference strain 26695 to ACF-HCl was determined by liquid macro-dilution method and compared with Gram-negative bacteria control *E. coli* K-12 W3110 strain. MIC of ACF-HCl for 26695 strain was identified to be 2 µg/ml. Importantly, control *E. coli* strain W3110 exhibited MIC of 20 µg/ml, which is 10 times higher than that

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of *H. pylori* strain 26695 (Fig. 1B and C). Intriguingly, ACF-HCl inhibited the growth of 19 clinically isolated clarithromycin-resistant/sensitive strains (Table 1; Table S2, Supporting Information) and SS1 strain as well with MIC values in the range of 0.5–2 μg/ml. The majority of the strains (95%) showed a MIC of either 1 μg/ml (43%) or 2 μg/ml (52%) (Fig. 1D).

These results clearly confirm that ACF-HCl works as an effective growth inhibitor for Indian *H. pylori* strains irrespective of the disease status. Nevertheless, the roles of polymorphism in target genes and strain-specific differences in the MICs of ACF-HCl require additional studies.

ACF-HCl shows synergism with the conventional *H. pylori* antibiotic clarithromycin

As ACF-HCl showed antimicrobial properties against CLR-resistant *H. pylori* strains, we investigated the potential of ACF-HCl for combinatorial therapy because the combination therapy minimizes risk of antimicrobial resistance and enhances the effects of antibiotics. The combined effect of ACF-HCl and clarithromycin on *H. pylori* CLR-resistant clinical strains growth was determined. In combination, these two compounds caused a drastic reduction in both colony size and colony number compared with that of control plates. Interestingly, at a specific combination of concentration of the two drugs, ∼100 times reduction in growth was observed in comparison with the control bacteria (at a concentration of 0.5A + 0.5C for strain 136 and 0.5A + 1C in case of Bhu8A). The individual MIC values of ACF-HCl and clarithromycin in these strains were greater than the combined concentration. Additionally, the fractional inhibitory concentration index, FICI, indicated that ACF-HCl and clarithromycin together display strong synergistic effect and inhibit bacterial colony formation, and thus bacterial growth (Table 2).

ACF-HCl eliminates *H. pylori* from *H. pylori*-infected mice gastric tissue

The *in vitro* efficacy of ACF-HCl against clinical isolates prompted us to evaluate the effects of ACF-HCl in *in vivo* mice model. The therapeutic effect of ACF-HCl was analyzed by evaluating the presence of *vacA* gene in the gastric tissue samples of all the three groups of mice (uninfected, infected with *H. pylori* strain SS1 and infected-treated with ACF-HCl 50 mg/kg body weight for a week) by PCR amplification using specific primers (Fig. 2A, top panel). Mouse *GAPDH* gene was taken as a control (Fig. 2A, bottom panel). The amplification of *vacA* gene in the SS1-infected mice group specifies the presence of bacteria, whereas no amplification of the *vacA* gene in the uninfected and the SS1 infected-treated with ACF-HCl mice group indicates the absence of *H. pylori* in the respective gastric tissues. Therefore, ACF-HCl treatment completely eradicated *H. pylori* from the gastric tissues of the infected mice group treated with ACF-HCl.

Prior to PCR analysis, stomach tissues from the SS1-infected mice were subjected to rapid urease test (RUT), which showed positive results confirming the colonization of *H. pylori*. Similarly, the effect of ACF-HCl was evaluated by a urease test with the respective mouse gastric tissues derived from the treated mice after infection, which showed negative for RUT (data not shown).

ACF-HCl as anti *H. pylori* agent in mice model: analysis of gastric tissue by histological examination

The 10% formalin-fixed, mice gastric tissue samples underwent microtome sectioning and H/E staining from all the experimental groups as mentioned earlier and were prepared for histological analysis. Microscopic observations also derived the role of ACF-HCl as an anti-*H. pylori* agent following the trends of *in vitro* results. In comparison with the control uninfected mice gastric tissue (Fig. 2B, left panel labeled 1A), the gastric tissue sections from the SS1-infected mice (Fig. 2B, middle panel labeled 1B) showed clear, considerable stripping of the epithelial linings at the surface leading to the formation of deep ulcer. Intriguingly, in infected mice group treated with ACF-HCl (50 mg/kg body weight), we could see healing of the gastric tissue, showing focal restoration and superficial erosions with discontinuous epithelial lining (Fig. 2B, right panel labeled 1C). Moreover, inflammation in the gastric pit cells, as observed in the infected tissues, was reduced in the case of ACF-HCl treated mice. Glandular atrophy was not seen in any of the cases. Similar observations were recorded when the experiment was repeated. Altogether, ACF-HCl has a potential in eliminating *H. pylori* infection.

SEM and TEM analyses demonstrate that ACF-HCl affects the outer membrane of bacteria

Electron microscopy techniques have been employed previously, especially in the case of bacteria, to study the changes induced in the bacterial morphology following drug treatment for elucidating the mechanism of action of the drug. The changes in the ultrastructure of *H. pylori* induced by ACF-HCl treatment were studied by SEM and TEM analyses. Outer morphology of the cells was visualized by SEM analysis. ACF-HCl treated *H. pylori* cells (Fig. 3B) had rough outer surface in contrast to the smooth surface observed for untreated cells (Fig. 3A). Disruption of the uniform smooth outer cell membrane by ACF-HCl treatment suggests that bacterial membrane is targeted by ACF-HCl. In contrast, ACF-HCl treatment of *E. coli* strain, which shows a much higher MIC of ACF-HCl than *H. pylori,* does not show any changes in cell membrane and the outer morphology of the untreated cells (Figure S1A, Supporting Information) looked similar to the treated cells (Figure S1B, Supporting Information). *H. pylori* cells treated with ciprofloxacin were used as a control as it is known that ciprofloxacin targets gyrase and no effect on membrane was observed (data not shown).

To gain insights into the details of alteration of membrane structure, SEM was followed by TEM analysis where we observed similar pattern that the outer membrane of bacterial cells is disrupted following ACF-HCl treatment. The TEM images show detachment of the outer membrane from the cell with some leakage of cytoplasm contents upon ACF-HCl treatment (Fig. 3D) as compared with the intact membrane of the untreated cells (Fig. 3C). Apparently, no changes were observed in the ultrastructure of *E. coli* cells upon ACF-HCl treatment and the untreated cells (Figure S1C, Supporting Information) and treated cells (Figure S1D, Supporting Information) looked similar, thus implying specific inhibition of *H. pylori* cells by ACF-HCl. The disruption of bacterial membrane visible in both SEM and TEM analyses indicated that the underlying mechanism of action of ACF-HCl is by targeting bacterial outer membrane.

To further examine the effect of ACF-HCl treatment on bacterial membrane integrity, we carried out membrane permeabilization assay. As ACF-HCl is an autofluorescent molecule and

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Table 1. Antimicrobial action of ACF-HCl against *H. pylori* antibiotic-resistant clinical isolates.

S. No.	Strain ID	Disease status	Strain status	ACF-HCL MIC (μg/ml)
1	26695	Gastritis	(Reference strain) CLR-sensitive	2
2	D-197	NERD	CLR-resistant	1
3	D-136	NERD	CLR-resistant	2
4	D-148	NERD	CLR-resistant	1

CLR = clarithromycin; NERD = non-erosive reflux disease. Experiments were performed thrice.

Table 2. Synergistic effect of ACF-HCl with clarithromycin.

Strain	Individual MIC (μg/ml)	Effective combination	FIC_A	FIC_C	FICI[a]	Interaction[b]
136	A = 2 C = 4	0.5A + 0.5C	0.25	0.125	0.375	Synergy
Bhu8A	A = 2 C = 4	0.5A + 1C	0.25	0.25	0.5	Synergy

A = ACF-HCl and C = CLR

[a] $FICI = FIC_A + FIC_C$

$FIC_A = \frac{MIC_{A\ \text{in the presence of C}}}{MIC_{A\ alone}}$; $FIC_C = \frac{MIC_{C\ \text{in the presence of A}}}{MIC_{C\ \text{alone}}}$

[b] Interactions = synergy ($FICI \leq 0.5$), additive ($FICI > 0.5$ to ≤ 1), no interaction or neutral ($FICI > 1$ to ≤ 4), and antagonism ($FICI > 4$). Experiments were performed twice.

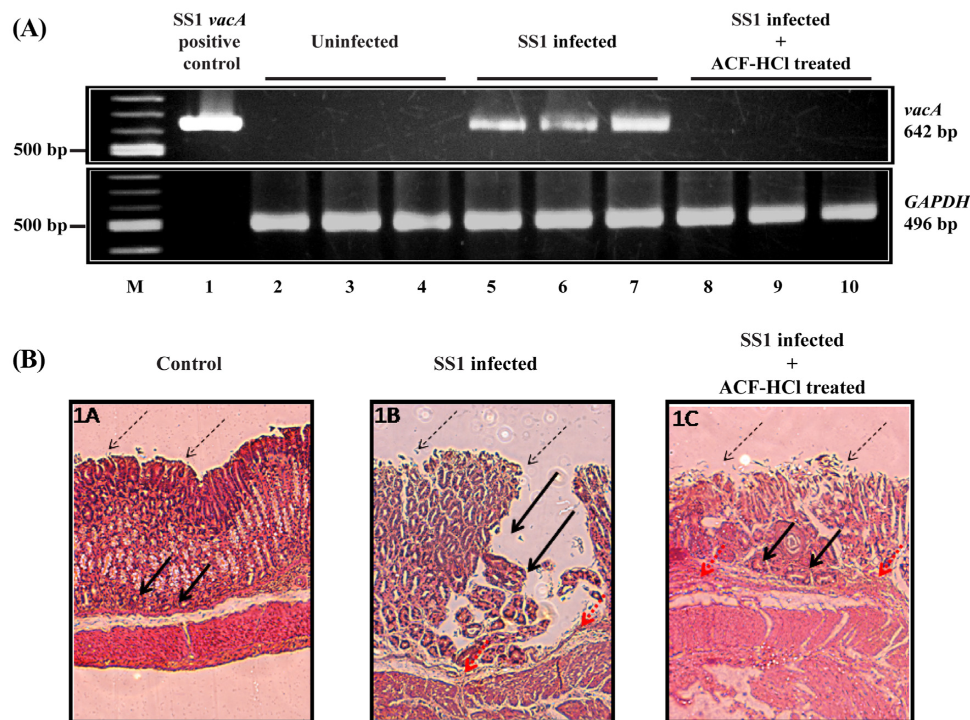

Figure 2. Effect of ACF-HCl on *H. pylori* viability in *H. pylori*-infected C57BL/6 mice. **(A)** Lane 1 illustrates amplification of *vacA* middle region of 642 bp with DNA from the mouse-colonizing strain SS1 as positive control using primers VAG-F and VAG-R. Lanes 2–4 represent amplification of *H. pylori*-specific *vacA* (upper panel) and mouse-specific GAPDH gene (lower panel) using stomach-DNA isolated from uninfected C57BL/C mice. Lanes 5–7 represent amplification of *H. pylori*-specific *vacA* and mouse-specific GAPDH gene using DNA isolated from the gastric tissue of SS1-infected C57BL/C mice 3 weeks post-infection. Lanes 8–10 represent amplification of *H. pylori*-specific *vacA* and mouse-specific GAPDH gene using DNA isolated from the gastric tissue of ACF-HCl treated (50 mg/kg body weight) SS1-infected C57BL/C mice. **(B)** Histology of control, *H. pylori*-infected and ACF-HCl treated C57BL/6 male mice gastric tissues. Histological appearance of gastric tissues from (left panel labeled as 1A) control mice, (middle panel labeled as 1B) mice infected with SS1 for 2 weeks and (right panel labeled as 1C) mice treated with ACF-HCl (50 mg/kg body weight). The gastric mucosal epithelium (dotted black arrows), restoration of epithelial lining over lamina propria (red arrows) gastric glands (black arrows) are shown. The images were taken at 10× magnification. The mice infection studies were performed twice.

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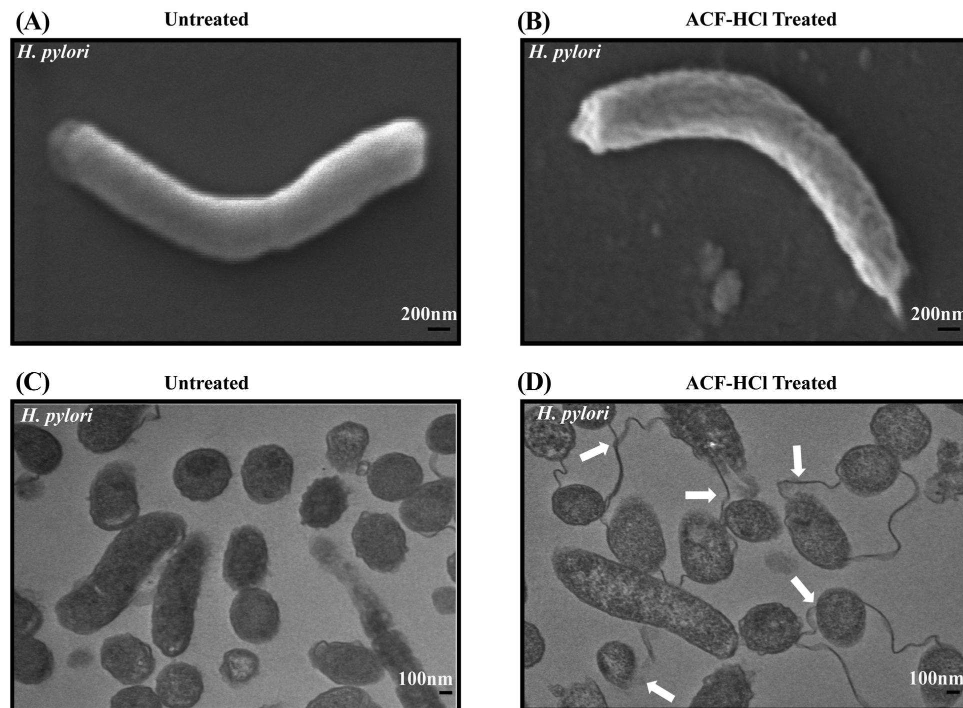

Figure 3. **(A)** Scanning electron micrographs showing morphological changes induced by ACF-HCl treatment in *H. pylori.* The untreated *H. pylori* cells show normal spiral-shaped morphology with smooth shiny outer surface, whereas **(B)** *H. pylori* cells treated with ACF-HCl show rough and distorted outer membrane. Scale bars show 200 nm. **(C)** Visualization at higher magnifications using transmission electron microscopy reveals that ACF-HCl targets outer membrane in *H. pylori*. Untreated *H. pylori* cells had intact membranes as compared with **(D)** ACF-HCl treated *H. pylori* cells that displayed detached outer membranes from the rest of the cell body leading to slight leakage of cytoplasmic contents (shown by white arrows). Scale bars represent 100 nm.

displays green fluorescence when taken up by cells, we used propidium iodide that emits red fluorescence after entering into the cells with compromised membrane integrity.

Helicobacter pylori cells treated with ACF-HCl (Fig. 4A, middle panel) showed a massive increase in red fluorescence as compared with PBS control (Fig. 4A, top panel) or ciprofloxacin control cells (Fig. 4A, bottom panel). The humongous increase in red fluorescence within 2 minutes of ACF-HCl treatment indicated the immediate and efficient permeabilization of bacterial cells by ACF-HCl suggesting that cellular membrane of *H. pylori* is the prime target of ACF-HCl. The brief exposure to ACF-HCl treatment followed by PI staining and immediate visualization might have resulted in the limited PI uptake (red staining) by some cells.

Possible targets of ACF-HCl in *H. pylori*

In order to identify the possible bacterial membrane proteins that are targeted by ACF-HCl, we utilized pull-down based approach with Ni-NTA beads bound ACF-HCl as a bait. Since ACF-HCl is a fluorescent molecule, the beads bound with ACF-HCl displayed green fluorescence in comparison with no detectable fluorescence observed in the control beads under UV illumination (Fig. 4B). Next, *H. pylori* cells were lysed in presence of *n*-dodecyl-β-D-maltoside (DDM) and sarcosine for solubilizing membrane proteins. The bacterial lysate was then allowed to bind overnight to the control and ACF-HCl bound beads. The bound proteins were resolved by SDS-PAGE followed by silver staining (Fig. 4C). A distinct band ~20 kDa molecular mass marker was obtained only in the ACF-HCl beads lane. Subsequently, the region around 20 kDa was excised from both the

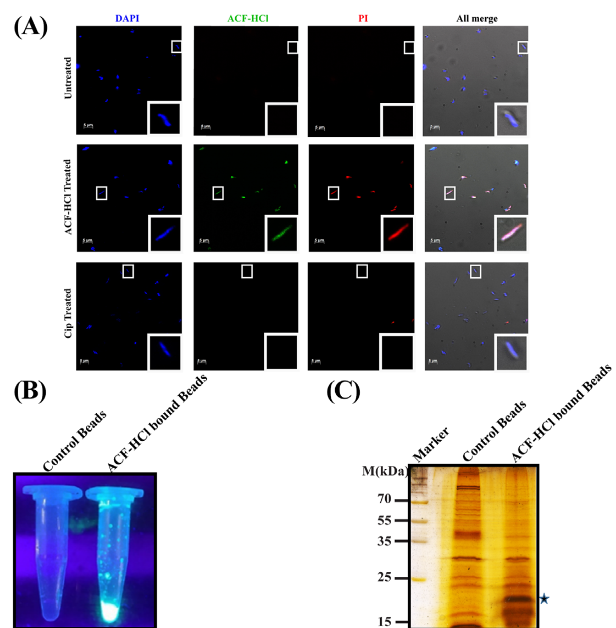

Figure 4. **(A)** ACF-HCl readily permeabilizes the *H. pylori* cell membrane. Membrane permeabilization assay was performed by examining PI uptake after treatment with ACF-HCl (middle panel) or ciprofloxacin, Cip (bottom panel), taken as a control and untreated cells (top panel) using fluorescence microscopy. ACF-HCl treated cells demonstrated more PI uptake as seen by red fluorescence as compared with ciprofloxacin-treated or untreated cells indicating loss of bacterial membrane integrity after ACF-HCl treatment. Scale bars show 5 µm. **(B)** Identification of target(s) of ACF-HCl in *H. pylori*. ACF-HCl bound to Ni-NTA beads under UV illumination exhibits green fluorescence. **(C)** The distinct band obtained only in the ACF-HCl bound beads lane (shown with asterisk) was subjected to LC-MS/MS analysis for the protein(s) identification.

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control and ACF-HCl beads lane and subjected to LC-MS/MS to identify the possible targets.

Remarkably, of the top eight statistically significant hits identified with minimum two peptides of high confidence [1% false discovery rate (FDR)], five were membrane proteins, namely Peptidoglycan-associated lipoprotein Omp18 (Accession number O25750), Maf-like protein (Accession number O25838) and three uncharacterized proteins (Accession numbers: O26089, O25048 and O25995) with transmembrane domains or membrane localization (Table S3, Supporting Information). The outer membrane plays a crucial role in bacteria with regard to cell division, biofilm formation, and in the case of *H. pylori* has been associated with persistent colonization and adhesion of the bacteria to the gut. There are reports of major role of Omp18 in influencing host immune response leading to colonization of bacteria in the gut (Shan *et al.* 2015). Therefore, the specific enrichment of these membrane proteins in the ACF-HCl beads only and not in the control sample suggests that the association of ACF-HCl with these membrane proteins may disrupt the membrane structure leading to bacterial growth inhibition.

DISCUSSION

Infection with *H. pylori* is one of the major causes of the most widespread gastroduodenal diseases among developing countries of the world (Hooi *et al.* 2017). *H. pylori* infection is a potential risk factor for gastric cancer that causes numerous deaths worldwide. This carcinogenic bacterium can survive the harsh acidic conditions of the human gut by virtue of urease gene and other virulence-associated gene clusters. Overuse of antibiotics, mutations arising in the pathogen, changes in the host microbiome and incomplete therapy have led to increased antibiotic resistance. With the emerging antibiotic resistance and failure of the triple therapy regime, there is immediate need of alternative drugs to combat this global deadly disease. Recently, combination drugs are being employed to combat the deadly infections of *H. pylori*. These synergy drugs have two advantages over the already existing therapies. First, individual concentrations of antibiotics in treatment can be lowered, thus minimizing the effects on host microbiome and secondly, the chances of development of antibiotic resistance to the combination are negligible.

In recent years, research has shifted toward evaluating the potential of already known compounds across different microbial species and also to synthesize their analogs. There lies a great potential in flavine and acridine derivatives that were used extensively in the last century. One of the derivatives, acriflavine, was widely used during the World War I as an antiseptic. However, low stability of this drug in the *in vivo* tested systems and high toxicity limits widespread clinical usage of ACF. In our present study, we have used acriflavine hydrochloride orally (50 mg/kg body weight), another acridine derivative that is reported to have a much higher lethal dose 50% (LD50) value as compared with acriflavine. It was used in HIV clinical trial earlier and is recently being used in urinary antiseptic trial (ClinicalTrials.gov Identifier: NCT03379389) and is also proposed as a potential candidate for antitumor clinical trials. It has been reported that the lethal dose 50% (LD50) of ACF-HCl is 1 g/kg body weight of rat given orally G35. The subcutaneous toxicity studies in mice for ACF-HCl demonstrated that the maximum dose for zero mortality is 85 mg/kg (de Bruyn, Robertson and Farr 1950).

It is to be noted that our animal model studies were performed at concentrations (50 mg/kg body weight) given orally (1/20th of LD50 value reported) that are much below the toxicity levels, and no adverse side effects on mice health, physical appearance or mobility were observed throughout the period of study. Therefore, in the context of above published data and our findings, we strongly believe that the dosage reported here for ACF-HCl will have minimal or no toxicity in *in vivo* animal model.

We report that the MIC value of ACF-HCl in *H. pylori* is 10 times less than that of *E. coli*, another Gram-negative bacteria, thus implying that ACF-HCl is highly effective in inhibiting *H. pylori* growth in comparison with *E. coli*. Most intriguing part is the inhibition of growth of antibiotic-resistant clinical isolates of *H. pylori*. Furthermore, ACF-HCl exhibits strong synergistic effect with clarithromycin. The morphological changes induced by ACF-HCl treatment show that the bacterial membrane is primarily targeted by ACF-HCl with slight leakage of cytoplasmic contents. Our TEM images clearly depict the detachment of membrane from the rest of the cell. The integrity of the cellular membrane is compromised by ACF-HCl treatment as measured by increased uptake of propidium iodide, which is otherwise impermeant in the cells with intact membrane integrity. Therefore, we propose that the prime target of ACF-HCl in *H. pylori* is the bacterial membrane. The enrichment of membrane proteins in our LC-MS/MS data further indicated that ACF-HCl associates with the membrane proteins and thus disrupts the membrane structure leading to cell distortion followed by growth inhibition. However, mechanistic details of this process need further studies.

The potential of ACF-HCl as anti-*H. pylori* therapeutic was further established by the findings of the *in vivo* mice infection model. Clear eradication of *H. pylori* from the gastric tissues treated with ACF-HCl was observed following PCR amplification of *vacA* gene. Moreover, in the histological assessment, ACF-HCl was found to be active against *H. pylori* growth at a dose of 50 mg/kg body weight where partial progressive healing of gastric mucosa was found in comparison with the untreated *H. pylori*-infected mouse stomach. Not only ACF-HCl was found to be very much active in the eradication of pathogen from the infected gastric mucosa but it also had a role in initiating restoration of the injured stomach tissue as compared with mice infected with *H. pylori* strain SS1. This information could have a clinical consequence in the development of alternative remedy against *H. pylori*.

CONCLUSION

The present study highlights the potential of ACF-HCl in inhibiting *H. pylori* growth, including antibiotic-resistant clinical isolates *in vitro* at concentrations 10 times less than in another Gram-negative bacteria, *E. coli*. Intriguingly, ACF-HCl demonstrates synergistic effect with the conventional *H. pylori* antibiotic clarithromycin. Additionally, ACF-HCl treatment eradicated *H. pylori* infection in the *in vivo* murine disease model effectively. The *in vitro* experiments indicate that bacterial membrane is the prime target of ACF-HCl. Therefore, ACF-HCl is a potent therapeutic agent against the deadly *H. pylori* infection by itself and for combination therapy as well. The low cost, water solubility and commercial availability are the added advantages. The recent use of ACF-HCl in clinical trials further appends to its potential as a therapeutic drug and it should be employed in *H. pylori* treatment therapy as we have convincingly reported here the inhibitory effect on growth of antibiotic-resistant strains and the effective eradication of the pathogen in the *in vivo* mouse model. The broad-spectrum target across species and the biological and pharmaceutical properties of this wonder drug should

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be exploited further to open a whole new horizon of novel opportunities.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

AUTHORS' CONTRIBUTION

Majority of the experiments (Figs 1, 3 and 4) were performed by AT. BCK, SP and RK performed the MIC determination in antibiotic-resistant strains, drug synergism studies and *in vivo* mice model infection experiments. IK performed LC-MS/MS analysis of ACF-HCl associated proteins. AT, BCK, SP, RK, AKM, IK, AG and SKD analyzed the data and AT, IK, AG, AKM and SKD wrote the manuscript.

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Conflict of Interest. None declared.

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