Assessment of In Vitro Prophylactic and Therapeutic Efficacy of Chloroquine Against Chikungunya Virus in Vero Cells

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The resurgence of Chikungunya virus (CHIKV) in the form of unprecedented and explosive epidemics in India and the Indian Ocean islands after a gap of 32 years is a major public health concern. Currently, there is no specific therapy available to treat CHIKV infection. In the present study, the in vitro prophylactic and therapeutic effects of chloroquine on CHIKV replication in Vero cells were investigated. Inhibitory effects were observed when chloroquine was administered pre-infection, post-infection, and concurrent with infection, suggesting that chloroquine has prophylactic and therapeutic potential. The inhibitory effects were confirmed by performing a plaque reduction neutralization test (PRNT), real-time reverse transcriptase (RT)-PCR analysis of viral RNA levels, and cell viability assays. Chloroquine diminished CHIKV infection in a dose-dependent manner, with an effective concentration range of 5–20 μM. Concurrent addition of drug with virus, or treatment of cells prior to infection drastically reduced virus infectivity and viral genome copy number by ≥99.99%. The maximum inhibitory effect of chloroquine was observed within 1–3 hr post-infection (hpi), and treatment was ineffective once the virus successfully passed through the early stages of infection. The mechanism of inhibition of virus activity by chloroquine involved impaired endosomal-mediated virus entry during early stages of virus replication, most likely through the prevention of endocytosis and/or endosomal acidification, based on a comparative evaluation using ammonium chloride, a known lysosomotropic agent. *J. Med. Virol. 82:817–824, 2010.*
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KEY WORDS: chloroquine; Chikungunya; virus inhibition

INTRODUCTION

The re-emergence of Chikungunya virus (CHIKV) in many parts of the world, with associated severe clinical features, is a significant public health concern. Since 2005, CHIKV infection has assumed epidemic proportions in Asia and sub-Saharan Africa. Several outbreaks of CHIKV fever occurred in 2006, and virus was disseminated among the populations of several islands in the Indian Ocean (the Comoros, Mauritius, Seychelles, Madagascar, La Reunion) prior to outbreaks in India, where an estimated 1.4 million cases have been reported [Charell et al., 2007; Mavalankar et al., 2007; Pialoux et al., 2007]. Recent cases of CHIKV infection in Europe and Italy have occurred as a result of travel to and from infected areas [Rezza et al., 2007]. CHIKV is an arthropod-borne virus of the *Alphavirus* genus of the *Togaviridae* family. It is transmitted primarily to humans by *Aedes aegypti* and *Aedes albopictus* mosquitoes. Like other *Alphaviruses*, the genome of CHIKV consists of a linear, positive-stranded RNA molecule of ~11.8 kb [Jupp and McIntosh, 1998]. CHIKV causes an acute illness characterized by fever, headache, skin rash, vomiting, myalgia, and polyarthralgia [Jupp and McIntosh, 1998].

There is no effective treatment or licensed vaccine available for the clinical management of CHIKV infection. In the absence of an effective vaccine and mosquito control measures, it is necessary to seek effective anti-viral drugs for immediate relief for affected patients and to reduce viremia. The therapeutic application of small interfering RNA (si-RNA) for the inhibition of CHIKV replication has achieved limited success [Dash et al., 2008]. Chloroquine is an effective anti-malarial drug in areas where resistance has not been established. Increasingly, chloroquine is being applied to the clinical management of viral diseases [Savarino et al., 2003, 2006]. Chloroquine as an effective anti-viral therapeutic for the clinical management of viral diseases was first established in the 1990s for

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HIV-1 infection [Savarino, 2005]. Anti-viral effects of chloroquine against SARS-CoV, HIV type 1 and hepatitis B virus have also been reported [Kouroumalis and Koskinas, 1986; Tsai et al., 1990; Vincent et al., 2005], and the use of chloroquine as a therapeutic for HIV-1 infection is currently being evaluated in clinical trials [Savarino et al., 2006]. In light of its availability and cost, and the fact that it is well tolerated, chloroquine offers promise as an anti-viral and immunomodulatory agent for the treatment of emerging viral diseases [Keyaerts et al., 2004]. Increased virulence of CHIKV as a result of evolutionary adaptation during Chikungunya outbreaks has been reported [Schuffenecker et al., 2006; Santhosh et al., 2008]. Thus, it has become increasingly important to develop effective therapeutic approaches for the treatment of CHIKV infection. The goal of the current study was to evaluate the dose- and time-dependent effects of chloroquine on CHIKV replication, and to elucidate the mechanism of viral inhibition in Vero cells.

MATERIALS AND METHODS

Cells, Virus, and Chloroquine Treatment

Vero cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Eagles Minimal Essential Medium (EMEM) supplemented with 1.1 g sodium bicarbonate/l, 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 80 U of gentamycin. The CHIVK isolate DRDE-06, which belongs to the ECS African genotype, was used in the present study [Dash et al., 2007]. Increasing concentrations of chloroquine (5, 10, and 20 μM) were added to cultured Vero cells after determining the maximum non-toxic dose. The cells were treated with chloroquine as follows: for the pre-treatment group, the drug was added to the cells 24 hr prior to infection; for the concurrent treatment group, the drug and CHIKV were administered at the same time; for the post-treatment group, the drug was added at different points from 1–6 hr following infection of cells with CHIKV. In the pre-treatment mode, chloroquine was removed by washing the cells before infection. In the concurrent and post-treatment modes, the drug was maintained in culture until the supernatants were harvested.

Virus Yield Reduction Assay

Cells were seeded in a 25 cm^2 cell culture flask at a density of 1×10^5 cells/ml (1×10^6 cells per flask) and then incubated for 24 hr. Cells were infected with CHIKV at a multiplicity of infection (m.o.i.) of 0.1. Drug was administered to the three different treatment groups (24 hr pre-treatment, concurrent treatment, and post-treatment 1 hpi). Infection was allowed to proceed for 36 hr, at which time cells were scraped and virus was released into the supernatant by freeze thawing the cells three times. Cell pellets were removed

by centrifugation at 1,100g for 10 min. Virus yield was determined by the plaque assay.

Virus Inhibition (Plaque Reduction) Assay

Cells were seeded on 24-well culture plates (Greiner bio-one, Solingen, Germany) at a density of 1×10^5 cells/well and allowed to grow to 95% confluency. The medium was discarded and the cell monolayer was infected with CHIKV (100 pfu/well). Seeded cells were treated with drug 24 hr before infection, concurrent with infection, or 1 hpi. After a period of 1 hr to allow for virus adsorption, cells were overlaid with an overlay medium containing 1.5% methylcellulose, 2% FCS, and the appropriate concentration of drug. After 72 hr, the overlay medium was removed and the infected cell monolayer was fixed in 10% PBS–formaldehyde. Virus plaques that formed on Vero cells were visualized by staining with 1% crystal violet. Percent inhibition was determined relative to untreated control cells.

Cell Viability Assay

Anti-viral activity was assessed by performing cell viability assays on cells that had been infected with CHIKV in the presence of various concentrations of ammonium chloride, ribavirin, or chloroquine. The number of viable cells was quantified 36 hpi by neutral red dye uptake assay [Finter, 1969]. A selectivity index for each test compound for the pre-treatment, concurrent, and post-treatment (1 hpi) groups was determined as the ratio of the concentration of test compound required to reduce cell viability by 50% (CC$_{50}$) to the concentration required to inhibit virus infectivity by 50% as compared to control cells (IC$_{50}$).

Analysis of Genome Copy Number by SYBR Green Real-Time RT-PCR

Vero cell monolayers cultured in 25 cm^2 flasks were infected with CHIKV (m.o.i. of 0.1) to 95% confluence. Increasing concentrations of drug (5, 10, and 20 μM) were added to all treatment groups (pre-treatment, concurrent, and post-treatment 1 hpi). Infection was allowed to proceed for 36 hr, at which time 1 ml of culture supernatant was drawn from each treatment group in triplicate and then pooled. Genomic viral RNA was extracted from 140 μl of pooled supernatant using a QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. The total copy number of CHIKV genomes was analyzed by SYBR green I-based one-step real-time quantitative RT-PCR, as previously described [Santhosh et al., 2007]. A region of the envelope E1 gene was amplified using the following specific primers: 5′-ACGCAATTGAGCGAAG-CAC-3′ (Forward), 5′-CTGAAGACATTGGCCCCAC-3′ (Reverse). Real-time RT-PCR was performed using the MX 3000P quantitative PCR system (Stratagene, La Jolla, CA). Test samples were analyzed following optimization with RNA standards using the Brilliant SYBR Green Single-Step QRT-PCR Master Mix

(Stratagene). After amplification, a melting curve analysis was performed to verify the authenticity of the amplified product according to its specific melting temperature (T_m) using the melting curve analysis software of the Mx3000 system. Analysis of relative cycle threshold (C_t) values was performed and the overall reduction in genome copy number was calculated by plotting C_t versus genome copy number.

Inhibition Kinetics

Subconfluent monolayers of Vero cells in 24-well plates were infected with CHIKV in duplicate, and then treated with chloroquine at a concentration of $20 \,\mu M$ for increasing periods of time post-infection (1 to 6 hpi). Supernatants were collected at each time point and viral load was determined by plaque titration to assess CHIKV growth kinetics.

Effect of Chloroquine on Virus Internalization

The effect of chloroquine on virus internalization was assessed by the immunofluorescence test (IFT). Cultured Vero cells were infected with CHIKV in the presence or absence of drug and infection was allowed to proceed for 14 hr. Cells were washed five times with PBS, and then fixed using chilled methanol. Cells were permeabilized using 0.1% Triton-X100 for the detection of intracellular virus. Fixed cells were incubated with rabbit anti-CHIKV hyperimmune serum (1:2,000 dilution) followed by FITC-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) (1:100). Cells were washed and then observed using a Carl-Zeiss Aximot 2 (Thuringia, Germany) microscope, which was equipped for incident illumination with a narrow band filter combination specific for FITC.

Mechanism of Inhibition of CHIKV Infection by Chloroquine

The mechanism of inhibition of CHIKV activity by chloroquine was assessed by comparing the effects of chloroquine to those of a known lysomotropic agent (ammonium chloride) that interferes with early stages of infection, and a standard anti-viral compound (ribavirin) that inhibits virus replication during late stages of infection. Confluent monolayers of Vero cells were infected at an m.o.i of 0.1 with CHIKV, and then treated with appropriate concentrations of ammonium chloride, ribavirin and chloroquine 24 hr before and 6 hr after CHIKV infection. In the case of pre-treatment, compounds were removed by washing before infection. Cell viability was measured 36 hpi by the neutral red dye uptake assay, as described above.

RESULTS

Prior to screening, we determined the maximum non-toxic dose of chloroquine for Vero cells. A concentration of $20 \,\mu M$ chloroquine was non-toxic to Vero cells. The growth kinetics of CHIKV in Vero cells at different multiplicities of infection was also determined to establish an appropriate time line for harvesting and subsequent analysis of viral activity. The optimum virus yield following infection with a titer of $1 \times 10^8 \,pfu/ml$ was obtained 36 hpi. To determine the anti-CHIKV activity of chloroquine, we analyzed virus yield in Vero cells treated with drug as compared to untreated infected control cells. There was a substantial decrease in viral titer when cells were pre-treated with several different concentrations of chloroquine. Concurrent treatment and post-treatment (1 hpi) with chloroquine also inhibited CHIKV infection at higher concentrations. Viral titer was reduced nearly 99% by $20 \,\mu M$ chloroquine, as indicated by the 2–3 log decrease in virus yield in all treatment groups. These results provided substantial evidence of the anti-CHIKV activity of chloroquine (Fig. 1a).

Anti-CHIKV activity was also evaluated by plaque reduction assay. In the presence of $20 \,\mu M$ chloroquine, plaque formation was inhibited 94%, 70%, and 65% in the pre-treatment, concurrent, and post-treatment (1 hpi) groups, respectively (Fig. 1b). We next evaluated the cell viability of infected Vero cells in the presence of different concentrations of chloroquine by neutral red dye uptake assay. Based on the optical density at 450 nm (OD_{540}) of treated and untreated cells, IC_{50}, IC_{90}, and a selectivity index were calculated (Table I). Pre-treatment with chloroquine was the most effective anti-CHIKV strategy, as indicated by a nearly 2.5-fold higher selectivity index for the pre-treatment group as compared to the post-treatment group.

We analyzed viral genome copy number following infection with CHIKV using real-time RT-PCR. Viral RNA was isolated from the culture supernatants of chloroquine-treated and -untreated cells, and then amplified using E1 gene-specific primers, as described in Materials and Methods Section. The inhibition of CHIKV activity by chloroquine was evaluated by comparing C_t values obtained for each experimental condition, and the specificity of the amplified product was analyzed by T_m curve analysis. As depicted in Figure 2a–d, the amplification curves revealed higher C_t values for the pre-treatment, concurrent, and post-treatment groups at all concentrations of chloroquine as compared to infected cells. These results indicated that chloroquine treatment reduces viral RNA load, thereby inhibiting CHIKV replication. The C_t values for all treatment groups and concentrations of chloroquine are shown in Table II. In addition to relative C_t values, we also determined the absolute values for genome copy number using a standard curve, and observed an overall 2–3 log reduction in viral load in a dose-dependent manner (Fig. 2e).

To determine whether chloroquine inhibited CHIKV internalization, we analyzed the location of intracellular viral antigens by IFT. Infected Vero cells that were treated with chloroquine exhibited lower levels of fluorescence intensity as compared to infected cells and this decrease in fluorescence intensity was dose dependent (Fig. 3). As compared to infected cells, chloroquine pre-
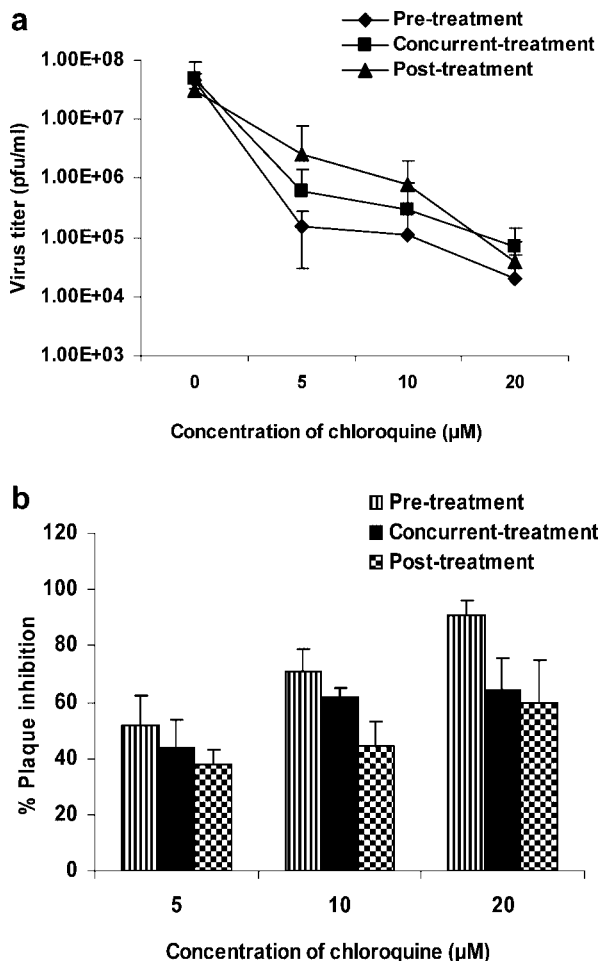
a: Virus yield assay. Evaluation of the anti-CHIKV activity of chloroquine. Vero cells were treated with 5, 10, or 20 μM chloroquine 24 hr before infection with CHIKV, concurrent with infection, and 1 hpi with CHIKV. **b**: Plaque reduction assay. Cells were treated with different concentrations of chloroquine 24 hr before, concurrently and 1 hpi. Plaques were visualized by staining with crystal violet and percent inhibition versus non-treated control cells was calculated. Data represents the means ± standard deviation (SD) of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treated infected cells exhibited lower fluorescence intensity, and in the presence of 20 μM chloroquine, fluorescent cells were undetectable, which indicated a near complete inhibition of virus internalization.

We carried out a time course analysis to determine the kinetics of viral inhibition by chloroquine, and found that the anti-viral effects of chloroquine decreased significantly when the drug was added later than 3 hpi (Fig. 4). The addition of chloroquine during the early stages of viral infection (1–3 hpi) significantly affected viral yield, but at later stages, the drug was ineffective, suggesting that the mechanism of inhibition of CHIKV by chloroquine involves the early stages of virus replication.

To begin to investigate the putative mechanism of action of chloroquine, we compared the effects of chloroquine to those of the anti-viral compounds ribavirin and ammonium chloride. Ammonium chloride was effective against CHIKV only when it was added prior to infection, and did not protect cells when added 6 hpi, based on cell viability (Fig. 5a). In contrast, ribavirin was effective against CHIKV infection only when it was added at the time of infection or after infection, but did not protect cells when it was added prior to infection and then removed by washing (Fig. 5b). Thus, the pattern of protection by chloroquine was similar to that of ammonium chloride, in that pre-treatment of cells inhibited virus replication, but there was no inhibitory effect after 6 hpi. (Fig. 5c).

DISCUSSION

Currently, there is no specific anti-viral treatment for CHIKV infection. We demonstrated that chloroquine is an effective anti-viral agent against CHIKV infection in Vero cells in culture, thus, demonstrating the in vitro prophylactic and therapeutic potential of chloroquine in inhibiting CHIVK infection. Chloroquine treatment significantly reduced virus yield, and reduced plaque forming ability by more than 90% (based on the plaque forming activity of 100 pfu of virus) (Fig. 1b). There was also a significant reduction in viral RNA copy number, based on real-time RT-PCR analysis (Fig. 2), providing strong evidence of the therapeutic potential of chloroquine in inhibiting CHIKV replication. In cell viability assays, chloroquine treatment provided near complete protection of Vero cells against CHIKV infection, which provided further evidence of the anti-viral potential of this drug. Previously, chloroquine was suggested as an effective agent against viral infection [Savarino et al., 2006]. The data obtained from the current study indicate

TABLE I. Effective Concentrations of Chloroquine for Inhibiting the CHIKV Infection in Pre-Treatment, Post-Treatment, and Concurrent Modes

Treatment	IC_{50}[a] (in μM)	IC_{90}[b] (in μM)	SI[c]
Pre-treatment (24 hr)	7.0 ± 1.5	15 ± 1.8	≈ 37.14
Post-treatment (1 hr)	17.2 ± 2.1	30 ± 4.1	≈ 15.29
Concurrent-treatment (0 hr)	10.0 ± 1.2	22 ± 3.8	≈ 26

Values represent the means ± SD of three independent experiments.
[a]Concentration required to inhibit virus infection by 50%.
[b]Concentration required to inhibit virus infection by 90%.
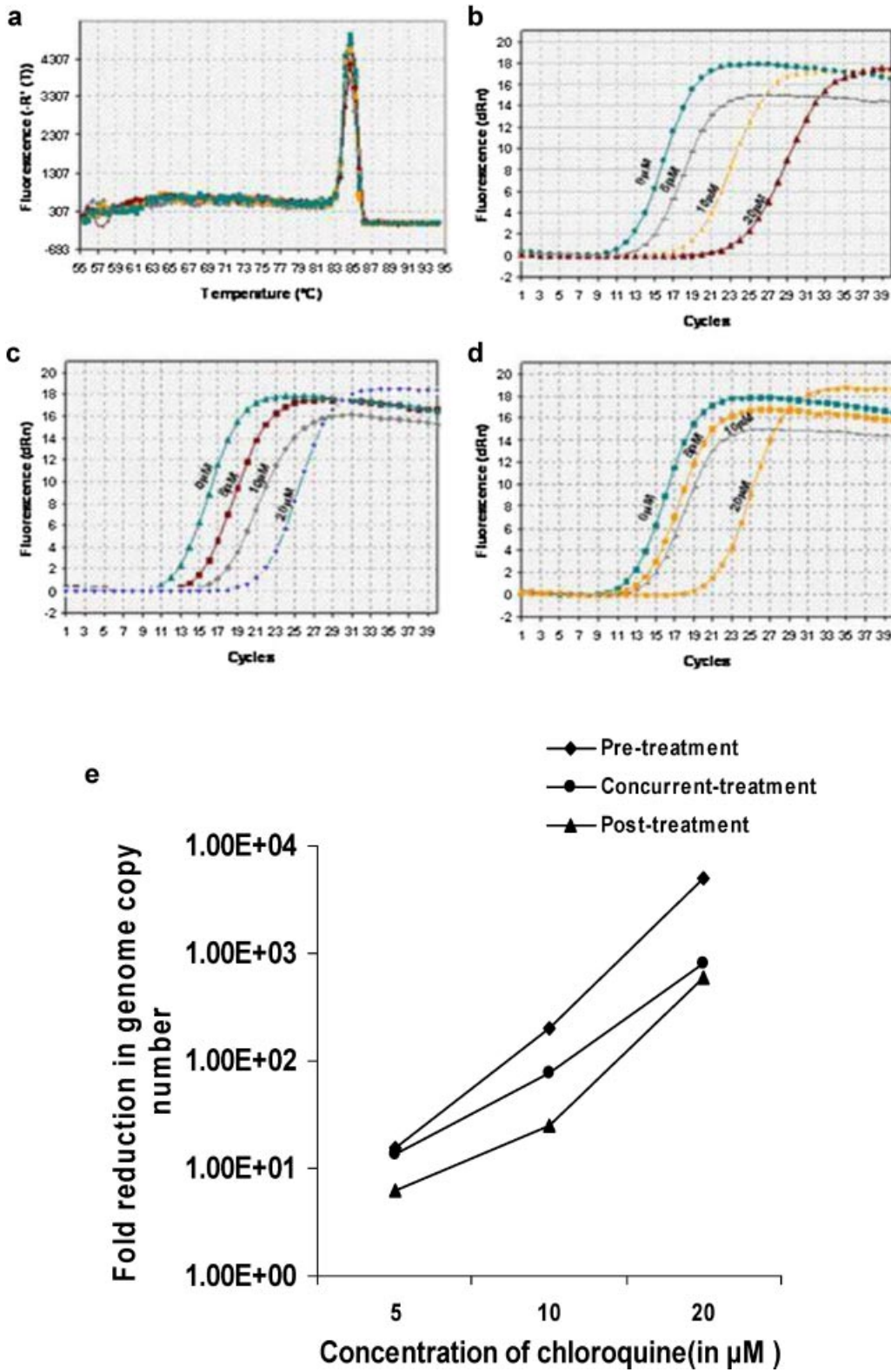[c]Selectivity index is the ratio of CC_{50} to IC_{50}, where CC_{50} is the 50% cytotoxic concentration.

Fig. 2. Real-time RT-PCR analysis of CHIVK genome copy number. Amplification plots (fluorescence vs. Cycle) depicting the relative abundance of CHIKV RNA in the supernatants of infected cells treated with 5, 10, and 20 μM chloroquine. The specificity of the amplified products was analyzed by T_m curve analysis (**a**). Amplification plots for cells treated with chloroquine 24 hr before (**b**), concurrently (**c**), and 1 hpi (**d**). For all treatment groups, the fold-reduction in genome copy number was calculated and plotted against chloroquine concentration (**e**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. Effect of Chloroquine on Viral RNA Copy Number for Pre-Treatment, Post-Treatment, and Concurrent Modes

Treatment	$C_t{}^a$ values (real-time RT-PCR)		
	5 μM	10 μM	20 μM
Pre-treatment (24 hr)	15.8	19.8	24.8
Post-treatment (1 hr)	14.4	15.8	21.5
Concurrent-treatment (0 hr)	15.6	18.2	22.0
Untreated control	12.9	12.6	12.4

$^a C_t$ is defined as the number of cycles required for the fluorescent signal to reach the threshold (i.e., exceed background levels). C_t values are inversely proportional to the amount of target nucleic acid in the sample.

that chloroquine is effective against the novel ECSA genotype of CHIVK that has caused several recent explosive and unprecedented epidemics.

Chloroquine is a weak base that targets acid vesicles, leading to the dysfunction of several proteins. Chloroquine has been shown to inhibit protease activity and affect DNA synthesis [Cassell et al., 1984]. However, our results suggest that the anti-viral activity of chloroquine is not associated with these previously reported activities, since CHIKV infection was unaffected when the drug was added during late stages of viral infection.

Thus, in the case of pre-treatment, the presence of chloroquine might not be essential for viral inhibition, whereas chloroquine is necessary at least up to 1 hpi to significantly inhibit virus yield. The addition of chloroquine at 6 hpi had no effect on viral replication. Our results suggest that chloroquine is effective at early stages of viral infection, and that the effects are dose- and time-dependent.

The mechanism of action of chloroquine appears to depend on the mode of treatment. In pre-treatment mode, cells were rendered refractory to CHIKV infec-
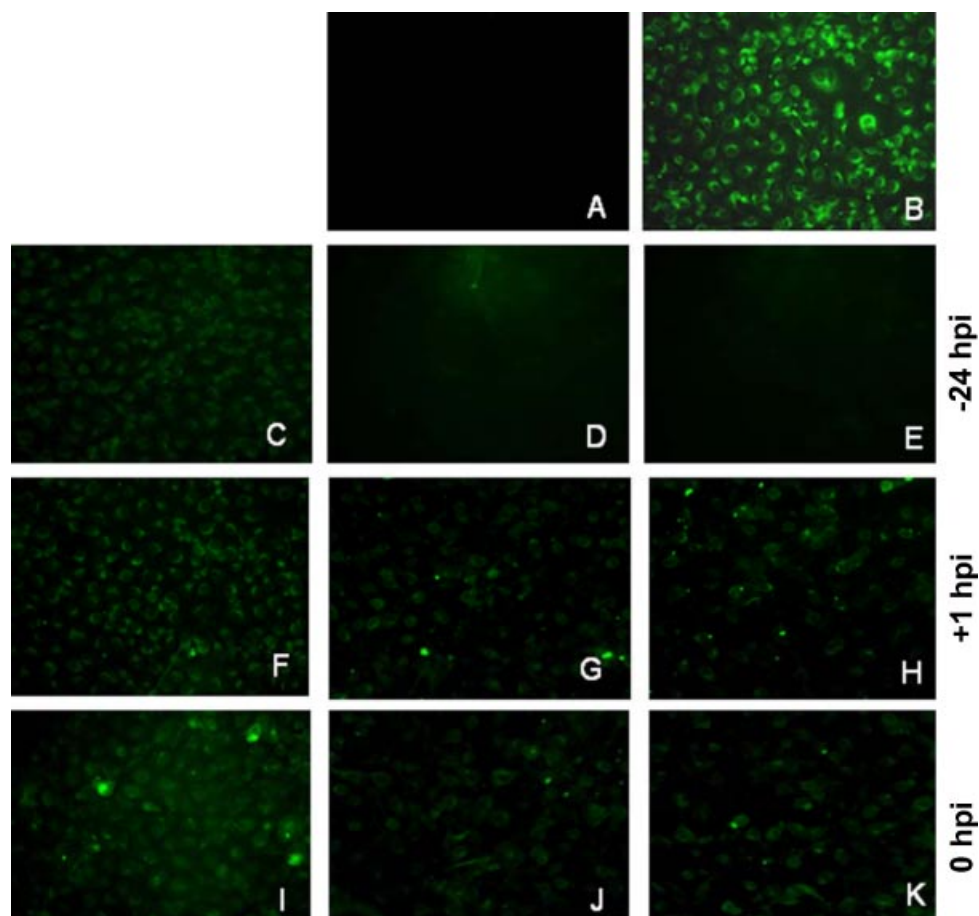

Fig. 3. Immunofluorescence test. Detection of intracellular CHIKV antigen by IFT using anti-CHIKV hyperimmune sera 14 hpi. Note that fluorescence was undetectable in healthy cells (**a**), while CHIKV-infected monolayers exhibited bright fluorescence (**b**). There was a significant decrease in fluorescence intensity upon treatment with 5 μM (**c,f,i**), 10 μM (**d,g,j**), and 20 μM (**e,h,k**) chloroquine. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
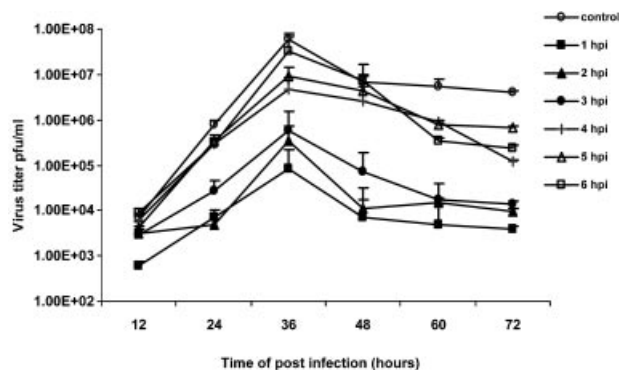
Fig. 4. Inhibition kinetics. Time course analysis of the effect of chloroquine on CHIKV replication. Vero cells were infected with CHIKV and chloroquine ($20\,\mu M$) was administered 1, 2, 3, 4, 5, and 6 hpi. CHIKV replication was analyzed by plaque assay. Data represents the means $\pm$ SD of duplicate assays. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

tion, indicating that chloroquine impairs cell-virus surface interactions. Previously it was shown that pretreatment with chloroquine impairs terminal glycosylation of ACE2, a cell surface receptor for severe acute respiratory syndrome corona virus (SARS-CoV) in Vero cells [Vincent et al., 2005]. A similar mechanism may be responsible for the inhibition of CHIKV infection by chloroquine. In the case of *Alphaviruses* like Sindbis virus (SINV) and Semilink Forest virus (SFV), conformational changes in the viral envelope glycoprotein and subsequent viral fusion are mediated by clathrin-mediated endocytosis by the target cell and the low pH of the endosomal compartment [DeTulleo and Kirchhausen, 1998]. It has been reported that a low endosomal pH is also required for CHIKV entry into cells [Sourisseau et al., 2007]. In the case of concurrent treatment and post-treatment (1 hpi), rapid elevation of endosomal pH and abrogation of virus-endosome fusion might be the primary mechanism by which virus infectivity is inhibited by chloroquine.

The kinetics of inhibition based on a time course analysis clearly imply that the anti-viral effects of chloroquine decline substantially when the drug is added later than 3 hpi (Fig. 4). In the post-treatment group, the addition of chloroquine at an early stage (1–3 hpi) of infection had a marked effect on virus yield, whereas late stage addition (4–6 hpi) was ineffective. The IC_{50} of chloroquine for inhibiting CHIKV in vitro is similar to the plasma concentration of chloroquine reached during the treatment of acute malaria [Charmot and Coulaud, 1990]. Thus, chloroquine might inhibit CHIKV infection and its subsequent dissemination.

The effect of chloroquine on the internalization of CHIKV was investigated by immunofluorescence analysis of intracellular viral antigen. Infected Vero cells treated with chloroquine exhibited markedly lower levels of fluorescence intensity as compared to infected cells, and this effect was dose dependent with complete inhibition at higher concentrations of chloroquine
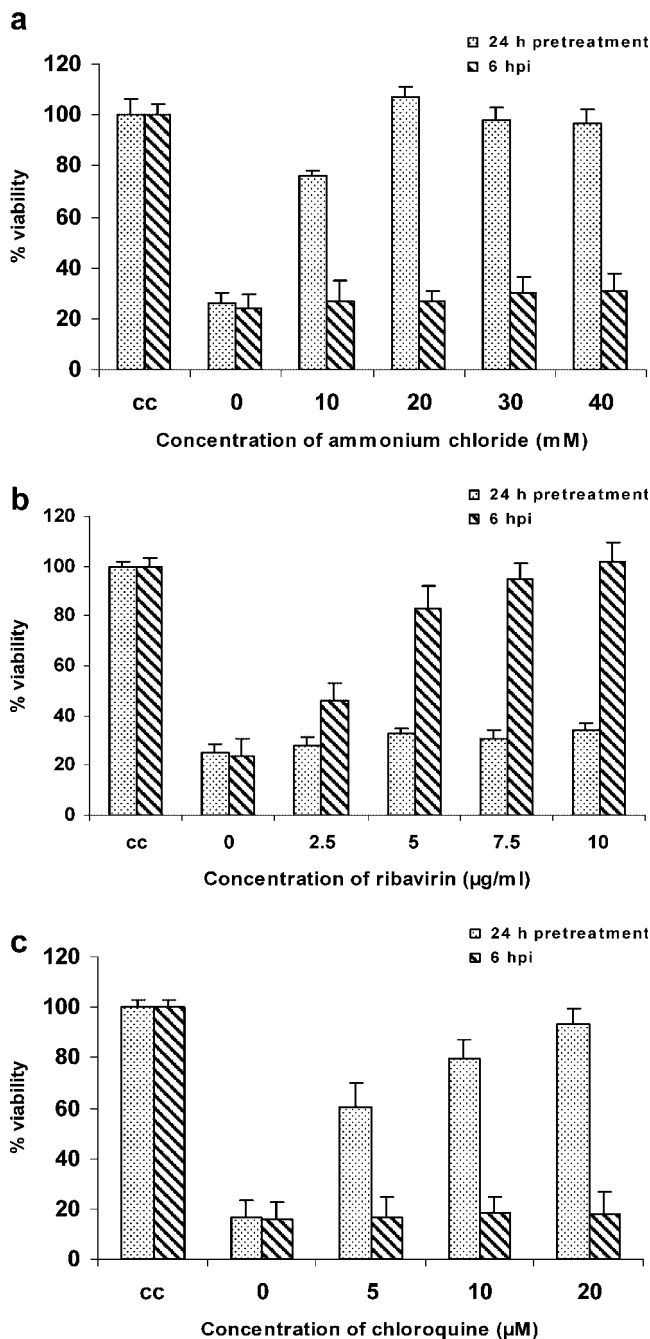

Fig. 5. Vero cells were treated with ammonium chloride (**a**), ribavirin (**b**), and chloroquine (**c**) 24 hr before infection and 6 hpi. Cell viability was analyzed 36 hr after infection, and percent viability was determined relative to healthy control cells (cc). Data represents the means $\pm$ SD of triplicate experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 3). The results of IFT also supported the finding that pre-treatment of cells with 10 or 20 μM chloroquine was more effective than concurrent treatment and post-treatment (1 hpi), which were effective to a lesser extent at higher concentrations of chloroquine. These results suggest that chloroquine treatment prevents or delays virus internalization.

In order to gain an understanding of the mechanism of action of chloroquine, we compared the effects of chloroquine to those of the well-known anti-viral compounds ribavirin and ammonium chloride. Ribavirin is an anti-viral compound that inhibits a number of viruses, including CHIKV, and acts at late stages of viral infection [Gilbert and Knight, 1986]. Ammonium chloride is a lysomotropic agent that blocks early stages of infection, for example, endosome-mediated virus entry, and has no effect during later stages of infection [Cassell et al., 1984]. Ammonium chloride was effective against CHIKV when cells were pre-treated (24 hr before), and retained its anti-viral activity even when it was removed prior to infection. However, administration of ammonium chloride 6 hpi did not protect cells from CHIKV infection (Fig. 5a). In contrast, ribavirin was effective against CHIKV infection only when administered after infection. No inhibitory effect was observed when cells were pre-treated with ribavirin followed by removal of the drug before infection (Fig. 5b). Thus, chloroquine (Fig. 5c) and ammonium chloride exhibited similar patterns of inhibition of CHIKV propagation, suggesting that chloroquine might also target the early stages of CHIKV infection.

In summary, the results of the current study suggest that chloroquine inhibits CHIKV infection in Vero cells though a mechanism that involves the early stages of infection. The fact that chloroquine exerts its anti-viral effects in all the three modes of treatment (pre-treatment, concurrent, and post-treatment) suggests that it has prophylactic and therapeutic potential. Chloroquine blocks the production of proinflammatory cytokines and the proliferation of monocytes, macrophages, and lymphocytes. Thus, it represents a potential drug for the treatment of some of the symptoms of Chikungunya disease. Since immunopathological factors might play an important role in CHIKV infection, it would be relevant to explore the effects of chloroquine on the inflammatory response to CHIKV infection.

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