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Cellular IMPDH enzyme activity is a potential target for the inhibition of Chikungunya virus replication and virus induced apoptosis in cultured mammalian cells

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ABSTRACT

Inosine monophosphate dehydrogenase (IMPDH) catalyzes an essential step in the de novo biosynthesis of guanine nucleotide, namely, the conversion of IMP to XMP. The depletion of the intracellular GTP and dGTP pools is the major event occurring in the cells exposed to the inhibitors such as mycophenolic acid. The present study was undertaken with an objective to assess the antiviral potential of mycophenolic acid (MPA) against Chikungunya virus via inhibition of IMPDH enzyme in Vero cells. The inhibitory potential of MPA on CHIKV replication was assessed by virus inhibition assay (cytopathic effect, immunofluorescence), virus yield reduction assay and cell viability assay. Inhibition of virus induced apoptosis was analyzed by Hoechst staining, DNA fragmentation, immunoblotting of Caspase-3, PARP and Bcl-2. Percentage apoptotic cell population was determined by flow cytometry. Total genome infectivity was determined by analyzing the ratio of total infectious viral particles to the genome copy number. Non-toxic concentration of MPA (10 μM) reduced ≥99.9% CHIKV titre in Vero cells. MPA via depletion of substrate for polymerase (GTP), inhibited CHIKV induced apoptosis. By limiting the rate of de novo synthesis of guanosine nucleotide, MPA could apparently block the formation of the CHIKV progeny. The antiviral activity of MPA against Chikungunya virus is mediated through depletion of GTP pool via inhibition of IMPDH as demonstrated by Immunoblotting and different microscopic analysis.

1. Introduction

Chikungunya is one of the most important re-emerging human arboviral infections of global significance (Weaver and Barrett, 2004). In 2005–2007 outbreaks, it emerged in many parts of Indian Ocean islands and India affecting more than a million persons (Dash et al., 2007; Schuffenecker et al., 2006). The first European outbreak of Chikungunya was recorded in Italy in 2007, causing huge public health concern in most parts of Europe (Rezza et al., 2007). A large number of imported cases of Chikungunya were reported in many countries among travelers returning from epidemic areas (Lanciotti et al., 2007). First described in Newalla province of Tanzania, outbreaks occurred in most of the parts of Africa (Briolant et al., 2004). The sheer magnitude of the outbreaks with rapid adaptation to an unusual vector *Aedes albopictus* renewed the interest of the scientific community in *Chikungunya virus*.

Chikungunya illness is caused by *Chikungunya virus* (CHIKV), belonging to genus *Alphavirus* of the family *Togaviridae*. CHIKV is an enveloped virus containing a single-stranded positive-sense RNA of approximately 11.8 kb, which is capped at 5′ end and polyadenylated at the 3′ end. Its genome has two open reading frames coding for four non-structural proteins (nsP1–4), three structural proteins (capsid, E1, and E2) and two small cleavage products i.e. E3 and 6K (Strauss and Strauss, 1994). Phylogenetically CHIKV isolates are classified into three genotypes, viz., Asian, East Central South (ECS) African, and West African (Powers et al., 2000). A novel clade of ECS African genotype was implicated in the recent Chikungunya epidemic around the world (Dash et al., 2007).

Chikungunya illness in humans is often characterized by abrupt onset of fever, headache, fatigue, nausea, vomiting, rash, myalgia, and severe arthralgia. The painful polyarthralgia is the typical symptom and may persist in ∼10% of cases for several months causing serious economic and social impact on both the individual and the affected communities in some instances (Jupp and McIntosh, 1988). However, there is no vaccine against CHIKV infection licensed for human use and therapy for CHIKV infection is still limited to supportive care as no effective antiviral agents are developed yet. Different clinically approved antiviral agents including alpha interferon and chloroquine showed some degree of inhibition for CHIKV in vitro however, none of them proved to be

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effective in vivo (Khan et al., 2010; De Lamballerie et al., 2008). Interestingly, Alphaviruses are known to induce apoptosis in various cells in vitro and in vivo, suggesting that apoptosis inhibition could be a possible novel therapeutic strategy to restrict CHIKV induced tissue injury (Li and Victor, 2004). Additionally, virus induced inflammatory response and arthritis like characteristics are associated with CHIKV infection therefore immunosuppressive and anti-inflammatory agents can be investigated further (Jaffar-Bandjee et al., 2009).

Mycophenolic acid (MPA) is a weak organic acid and well-known immunosuppressive agent. It has been investigated as a effective agent against growth and multiplication of several microbial pathogens including viruses (Kitchin et al., 1997). MPA inhibits the replication of several viruses in vitro to varying degrees (Gong et al., 1999). MPA is a non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH) thereby blocking the synthesis of xanthine monophosphate and subsequently depleting intracellular guanosine pool. MPA is used clinically in the prevention of rejection of transplanted organs (Lipsky, 1996). Various studies have reported that MPA blocked the cytopathic effect and replications of several viruses (Lanford et al., 2001).

In the present study, inhibitory potential of MPA on CHIKV replication through inhibition of IMPDH enzyme was analyzed. Initial evaluation of anti-CHIKV activity was based on virological methods like virus yield reduction assay, cell viability assay, nuclear morphology and fragmentation assay. Inhibitory effect was found to be reversible when exogenous GMP was supplemented to the medium. This suggested that the anti-CHIKV activity of MPA was associated with the depletion of intracellular guanosine pool. Apart from the inhibition of CHIKV replication, MPA also inhibited virus induced apoptosis as revealed through absence of caspase-3 and PARP cleavage.

2. Materials and methods

2.1. Cells and virus propagation

Vero cells were obtained from National Center for Cell Science (NCCS), Pune, India and were maintained by regular sub-culturing. The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma, St. Louis, USA) and 2 mM L-glutamine. Cells were incubated at 37 °C with 5% CO_2 for normal growth. The Chikungunya virus isolate DRDE-06 (EF210157), belonging to ECS African genotype, isolated from the 2006 Indian epidemic was used in the present study (Dash et al., 2007). The complete genome sequence of this isolate was recently reported (Santhosh et al., 2008). The propagation of the isolate (DRDE-06) was carried out in Vero cells following the standard virus adsorption technique (Gould & Clegg, 1991).

2.2. Drug, reagents and antibodies

Mycophenolic acid and guanosine monophosphate from Sigma, St. Louis, USA were used at non-toxic concentrations. Quantitative analysis of apoptosis was done by propidium iodide (Invitrogen, Carlsbad, USA) staining followed by flow cytometric analysis. Nuclear fragmentation was visualized by Hoechst stain (Invitrogen, Carlsbad, USA). Anti-Bcl-2, Anti-PARP, Anti-Caspase-3 and Anti-actin antibodies were used from Calbiochem, Darmstadt, Germany.

2.3. Measurement of apoptosis

2.3.1. DNA fragmentation assay

Qualitative measurement of apoptosis was done by analysis of fragmentation of cellular DNA by visualizing oligonucleosomal-sized DNA fragments (DNA laddering) and quantitatively by FACS analysis. DNA fragmentation study was performed by extracting intracellular DNA as described earlier (Gong et al., 1994). Cells were grown in 25 cm^2 culture flasks and infected with or without treatment of MPA. Cells were pelleted and washed with PBS by centrifugation at 800 × *g* for 10 min. Cells were resuspended in HBSS (Hanks' Balanced Salt Solution), diluted with 5 ml of ice cold 70% ethanol and stored at −20 °C for a period of minimum 7 days. The cells were then pelleted by centrifugation at 800 × *g* for 10 min and air dried to remove ethanol completely. The pellet was resuspended and lysed in 50–100 μl of phosphate citrate buffer (192 parts of 200 mM Na_2HPO_4 and 8 parts of 100 mM citric acid pH 7.8). After incubation for 2 h at 37 °C, the cell lysate was centrifuged at 3000 rpm for 10 min and supernatant was concentrated using Speed-Vac concentrator. Concentrate was resuspended in 10 μl of Tris-EDTA buffer, 3 μl of 0.25% NP-40 and 5 μl of RNase A (1 mg/ml) and suspension was incubated at 37 °C for 90 min. To this 5 μl of proteinase K (1 mg/ml) was added and incubated overnight at 37 °C. To the above suspension, 20–30 μl of Tris EDTA buffer was added; the samples were vortexed and incubated at 65 °C for 90 min to dissolve DNA. DNA isolated from control and infected cells was electrophoresed on 1.8% agarose gel with 1 kb DNA ladder along side as standard.

2.3.2. Flow cytomery

Flow cytometric analysis of apoptosis was performed at 36 and 48 hpi according to previous report (Li et al., 2004). Briefly, adherent and detached cells were collected by centrifugation at 250 × g, washed in PBS and the cell pellet was lysed in sodium citrate pH 6 ± 0.5, 1% Triton X-100 and 25 μg/ml propidium iodide (PI). Nuclei were analyzed after 30 min incubation at 4 °C in the dark with a flow cytometer (Partec, Munster, Germany) and minimum of 2×10^4 nuclei were analyzed.

2.4. Analysis of cytopathic effect (CPE), nuclear morphology and nuclear fragmentation

Confluent Vero cell monolayers grown in 24-well plates (Greiner bio-one, Germany) were infected with CHIKV at an MOI of 0.1. Following 1 h virus incubation period, the medium was removed and infected cells were incubated in medium containing different concentrations of drugs. The virus-induced cytopathic effect (CPE) was recorded at 48 hpi by analyzing cell and nuclear morphology by inverted light & phase contrast microscope.

DNA condensation or presence of apoptotic cells was assessed by using intercalating agent bis-benzimide (Hoechst). Cells were grown at a density of 10^4 cells/ml and infected with CHIKV. At 48 h of post infection (hpi), cells were washed with PBS, fixed with ice cold methanol for 10 min at −20 °C and then washed with PBS. Hoechst 33342 was added in the ratio 1:10 of culture medium and incubated for 15 min at 37 °C in dark. After incubation cells were gently washed with PBS and mounted on microscopic slides. Fluorescence was visualized under fluorescence microscope equipped with UV compatible filters.

2.5. Immunofluorescence assay

The effect of MPA on accumulation of intracellular 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 till 48 hpi. Cells were washed five times with PBS, and then fixed using chilled methanol. Cells were permeabilized using 0.1% TritonX-100 for the detection of intracellular virus. Fixed cells were incubated with rabbit anti-CHIKV hyper-immune serum (1:2000 dilution) followed by FITC-conjugated anti-rabbit IgG (Sigma, St. Louis, USA) (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.

2.6. Cell viability assay

Non-toxic concentration of MPA was determined by analyzing the cell viability of uninfected cells treated with various concentration of MPA. The concentration required to reduce 50% cell viability (CC_{50}) was determined.

Additionally, the antiviral activity was assessed by performing cell viability assays on cells that had been infected with CHIKV in the presence of various non-toxic concentrations of MPA. The number of viable cells was quantified at 48 hpi by neutral red dye uptake assay (Finter, 1969). Selectivity index for MPA 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 infected cells (IC_{50}).

2.7. Virus yield reduction assay

Cells were seeded in a 25 cm^2 cell culture flask (Greiner bio-one, Solingen, Germany) at a density of 1×10^5 cells/ml and then incubated for 24 h. Cells were infected with CHIKV at a multiplicity of infection of 0.1. Drug was added at different concentrations. Infection was allowed to proceed for 48 h, cells were scraped at different hours of post infection and virus was released into the supernatant by freeze thawing the cells three times. Cell pellets were removed by centrifugation at $1100 \times g$ for 10 min. Virus yield was determined by the plaque assay. Briefly, serial 10-fold dilutions of the harvested supernatant from each experimental group were prepared. From each dilution 0.1 ml supernatant was used to infect the vero cell monolayer. Following 1 h of virus adsorption, cells were overlaid with an overlay medium containing 1X EMEM, 1.5% methylcellulose, 2% FCS. After 72 h, the overlay medium was removed and the infected cell monolayer was fixed in cold methanol. Virus plaques that formed on Vero cells were visualized by staining with 0.1% crystal violet.

2.8. Analysis of genome copy number by qRT-PCR

Vero cell monolayers cultured in 25 cm^2 flasks were infected with CHIKV (MOI 0.1) at 95% confluency. Increasing concentrations of drug were added to all treatment groups after infection. Infection was allowed to proceed for 48 h; thereafter 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 RNAmini kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. The total copy number of CHIKV genome was analyzed by SYBR green I-based one-step real-time quantitative RT-PCR (Santhosh et al., 2007). A region of the envelope E1 gene was amplified using the following specific primers: 5′-ACGCAATTGAGCGAAGCAC-3′ (Forward), 5′-CTGAAGACATTGGCCCCAC-3′ (Reverse). Real-time RT-PCR was performed using the Mx 3000P quantitative PCR system (Stratagene, La Jolla, USA). Test samples were analyzed following optimization with RNA standards using the Brilliant SYBR Green Single-Step qRT-PCR Master Mix (Stratagene, La Jolla, USA). After amplification, a melting curve analysis was performed to verify the authenticity of the amplified product according to its specific melting temperature (Tm) using the melting curve analysis software of the Mx3000 system. Analysis of relative cycle threshold (C_t) values was performed and the genome copy number was calculated by using standard graph plotted against C_t vs. genome copy number.

For the preparation of standard graph, an amplicon (of same primer set) was cloned in pGEMT-Easy vector (Promega, USA) and a serial 100 fold dilutions (ranging from 10 log 10–2 log 10 copies) were prepared. After amplification, C_t values for all the dilutions were analyzed and standard graph was plotted against the C_t values and copy numbers. Total viral genome copy number was calculated by using straight line equation of the standard graph.

2.9. Caspase activation and Bcl-2 expression

For studies on caspase and Bcl-2, mock and CHIKV infected cells were lysed in lysis buffer (10 mM HEPES pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM EDTA, 5 mM DTT, 2 mM PMSF, containing 0.5% CHAPS). Cellular debris was spun down at $2000 \times g$ for 5 min, and supernatant was used as whole cell protein extract. Protein concentration of cell extracts was determined by BCA kit (Sigma, St. Louise, MO, USA).

2.10. PARP cleavage

For PARP cleavage cells were washed in PBS and lysed in PARP sample buffer (62 mM Tris HCl pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol with bromophenol blue). To effectively dissociate PARP–DNA interactions, samples were sonicated on ice (6 cycles for 5 s) and heated for 20 min at 65 °C. Separation of the proteins by SDS-PAGE, blotting and blocking of the membranes was performed. The blot was incubated overnight with the anti-PARP antibody (dilution 1:2000) and stained with HRP-conjugated anti-mouse antibodies (dilution 1:25000) and ECL detection was done according to the manufacturer's protocol (Life technologies, Carlsbad, CA, USA). The quantity of the loaded protein was normalized by uniform expression of ß-actin.

2.11. Statistical analysis

Results are expressed as mean values ± SD of at least three experiments. Comparisons between two groups were performed using Student's *t*-test; *p*-values less than 0.05 were considered to be significant.

3. Results

3.1. MPA inhibits CHIKV replication and CHIKV induced cell death

To evaluate the effect of MPA on CHIKV replication, a virus yield reduction assay was performed to compare the infectious virus titer of infected culture soup treated with various concentration of MPA. It was demonstrated that MPA inhibited the CHIKV replication and virus induced cell death in Vero cells (Fig. 1a and b). Additionally the inhibition kinetics of MPA for CHIKV was found to be dose dependent and MOI dependent (Table 1). The virus yield was decreased by the factor of $4\log_{10}$ (≥99.99%) at the non-toxic concentration of

Table 1
Inhibitory activity of MPA on CHIKV in Vero cells infected at different MOI ($p < 0.05$).

MOI	CC_{50}[a] (μM)	IC_{50}[b] (μM)	SI[c]
0.01	30 ± 3.1	0.10 ± 0.02	~300
0.1	30 ± 3.1	0.21 ± 0.06	~150
1.0	30 ± 3.1	1.80 ± 0.52	~16.6
10	30 ± 3.1	3.21 ± 1.10	~9.34
100	30 ± 3.1	7.81 ± 1.82	~3.84

[a] Determined as a concentration that reduced the viability of 50% control uninfected cells.
[b] Determined as a concentration required to reduced virus induced CPE to 50% of untreated infected cells.
[c] CC_{50}:IC_{50}.

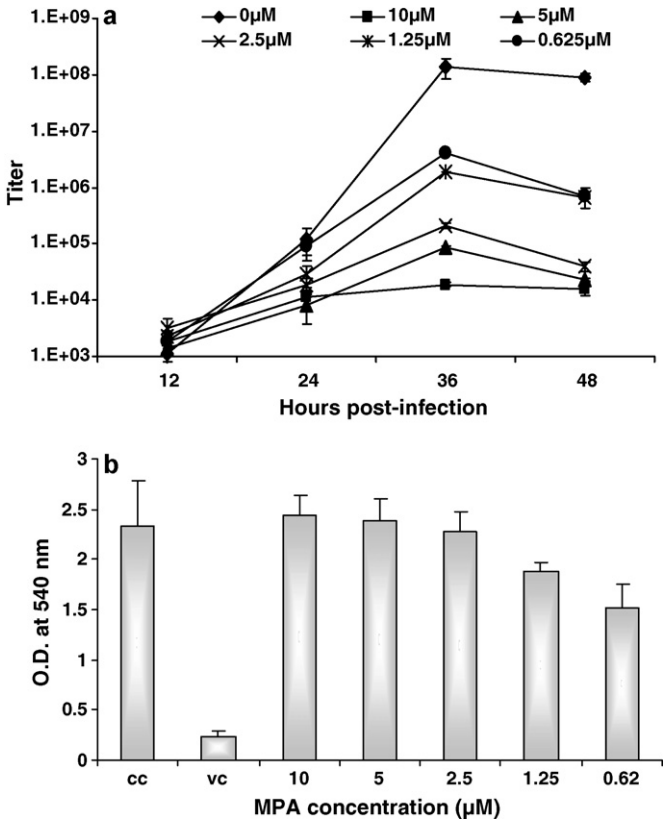

Fig. 1. Evaluation of protective efficacy of MPA against CHIKV multiplication as shown by virus yield reduction assay (a), and viability assays (b). Both the results showed a dose dependent protective response against CHIKV. Uninfected cell control and untreated virus control are represented by CC and VC, respectively.

10 μM MPA. IC_{50}, CC_{50} and selectivity index was calculated based on cell viability assay. A regression curve (Dose vs. Viability) was analyzed and IC_{50}, CC_{50} and selectivity index for MPA was found to be 0.2 μM, 30 μM and 150, respectively, at MOI of 0.1(Table 1).

In some of the previous studies of Alphaviruses, pathogenesis was found to be mediated through apoptosis and cell death (Levine et al., 1993; Lewis et al., 1996). Therefore, the study was planned to evaluate the anti-CHIKV activity of MPA at the level of morphological changes associated with apoptosis and cell death. The inhibition of CHIKV induced apoptosis and cell death by MPA was assessed through inhibition of CPE, nuclear blebbing, nuclear fragmentation and localization of intracellular viral antigen. All the four parameters were analyzed for the MPA treated and untreated infected cells at 48 hpi (since the maximum destruction was observed at this point). As depicted in Fig. 2c, no CPE was observed when cells were treated with 10 μM of MPA. MPA mediated inhibition of CHIKV replication was further correlated with the absence of viral antigen inside the cells when analyzed by immunofluorescence (Fig. 2g). When treated and untreated cells were analyzed by Hoechst staining at 48 hpi, no nuclear fragmentation was observed in MPA treated cells (Fig. 2k) compared to infected control (Fig. 2j). Similarly nuclear morphology was also found to be protected in CHIKV infected cells by MPA (Fig. 2o). As compared to infected cells, MPA treated cells showed almost similar nuclear morphology as mock infected. In order to evaluate the mechanism of anti-CHIKV activity of MPA, exogenous guanosine (GMP) was added. When CHIKV infected cells treated with MPA were supplemented with exogenous guanosine, the cells could no more prevent the CHIKV induced cell death as depicted in Fig. 2(d, h, l, and p) and the morphological characteristics were similar to infected cells, Fig. 2(b, f, j, and n).

Table 2
Relative infectivity of CHIKV RNA detected by qRT-PCR in Vero cell's supernatant at 48 hpi with or without MPA treatment.

MPA(in μM)	PFU[a]	Genome copy[a]	% Genome infectivity[b]
00	1.21×10^8	3.9×10^9	3.33
1.25	3.1×10^6	1.5×10^8	2.00
2.5	8.1×10^5	1.26×10^8	0.64
5.0	3.5×10^5	1.20×10^8	0.29
10	4.6×10^4	3.1×10^7	0.14
15	1.2×10^4	9.1×10^6	0.131

[a] Copy number or plaque forming unit were shown in per ml volume.
[b] Percent genome equivalents calculated that were infectious.

3.2. MPA rescued the cell population from CHIKV induced apoptosis

In order to further define the inhibitory effect of MPA on CHIKV induced apoptosis, total infected cell population was analyzed by DNA fragmentation and nuclei staining by propidium iodide for qualitative and quantitative analysis of apoptosis respectively. As depicted in Fig. 3, cells were protected from CHIKV induced DNA fragmentation when treated with MPA. For quantitative analysis of apoptosis, treated and untreated cell populations were harvested at 36 and 48 hpi. The total apoptotic population (in form of sub-G1 phase) at 36 and 48 hpi was found 1.25% and 1.57%, respectively when treated with MPA. This obtained proportion was significantly lower than the one obtained in untreated control i.e. 16% and 43% (Fig. 4).

In addition, the cleavage of the nuclear 116 kDa PARP protein to a smaller 85 kDa fragment is regarded as a hallmark of caspase mediated apoptosis. Therefore CHIKV induced cleavage of PARP was analyzed in order to demonstrate the effect of MPA on caspase mediated apoptosis (Fig. 5a and b). It was found that caspase and PARP cleavage were inhibited by MPA treatment and exogenous addition of GMP could reverse its inhibitory effect. Also in the presence of MPA, CHIKV-induced down regulation of Bcl-2 (anti-apoptotic protein) was suppressed (Fig. 5c). Collectively the above analysis of apoptotic biomarkers in CHIKV infected cells suggested the potential inhibition of CHIKV replication by MPA.

3.3. MPA mediated inhibition of genome infectivity

Infectious virus present in cell supernatant of 48 hpi was titred by plaque assay. In parallel, genome copy number of viral RNA was also determined by extrapolating the sample C_t value on the standard graph (Fig. 6a). For this, viral RNA from same sample was extracted and quantified by SYBR-Green qRT-PCR (Fig. 6b). The infectious viruses and the viral genome copy number decreased in parallel with increasing dose of MPA but reduction in infectious viral progeny was greater than the reduction in genome copy number (Table 2). MPA at a concentration of 10 μM, reduced infectious virus by 4 log 10 compared to untreated control while it decreased the genome copy number by only 2 log 10. Thus, the total genome infectivity (percent of infectious genome copies) was 24 fold lower (3.33%/0.148%) in the presence of 10 μM MPA compared to untreated control.

4. Discussion

Viruses have evolved as remarkably successful intracellular pathogens. Indeed, most of the viruses employ so much of their host cell machinery that it has historically proven difficult to separate virus' function from cellular functions. Few decades ago only five drugs were licensed for the treatment of viral infection. Presently, because of better understanding and elucidation of virus specific events for antiviral agents, there are more than 30 drugs for the

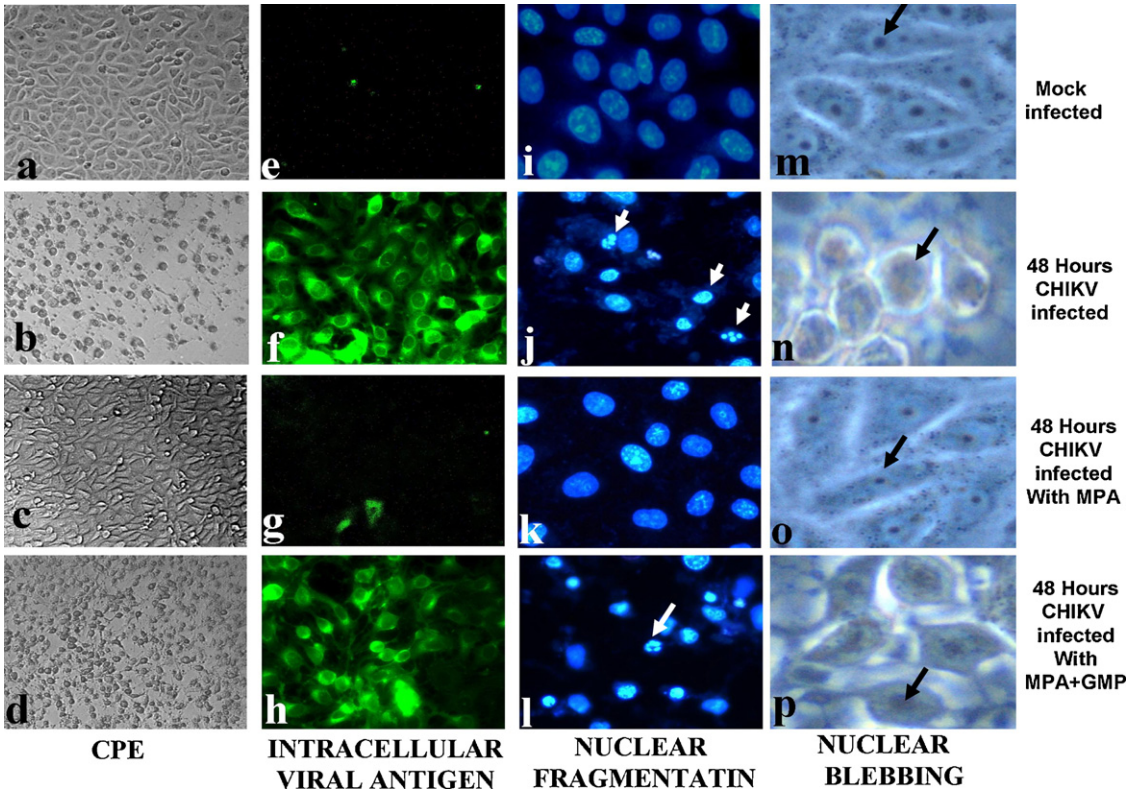

Fig. 2. Microscopic examination of CHIKV induced CPE (a–d) at 10×, accumulation of intracellular CHIKV antigen (e–h) at 20×, nuclear fragmentation by Hoechst stain (i–l) at 20× and analysis of nuclear morphology by phase contrast microscope (m–p) at 40×. In every examination, the protective efficacy of MPA (10 μM) could be revealed. Reversal of MPA mediated inhibition of CHIKV replication by exogenous addition of 0.5 mM GMP (d, h, l, and p) was also examined to prove that MPA had no effect on CHIKV replication other than inhibition of GMP synthesis via IMPDH.

treatment of viral infection. However, the effective therapies for several important viral infections are lacking and the current treatments are not always well tolerable (De Clercq et al., 2001).

In last few years, repeated outbreaks of Chikungunya were reported in Indian Ocean and other tropical regions of the world (Dash et al., 2007; Schuffenecker et al., 2006). Although the morbidity rate was very much lower than that of other alphaviruses but the continuous evolution of CHIKV genome harboring this pathogen as a successful invader highlight the need for further refinement of antiviral drug design and development against CHIKV (Santhosh et al., 2008; Schuffenecker et al., 2006). On the way of viral therapeutics, there is always a problem of limited selectivity and broadening the selectivity will often be a challenge for researchers. Selection of an important biochemical step that can be targeted in order to have higher selectivity index is one of the potential way to inhibit viral infection. Depletion of substrates required for the continuous multiplication of the virus can be an effective strategy and has been proved effective in earlier studies (Ichimura and Levy, 1995).

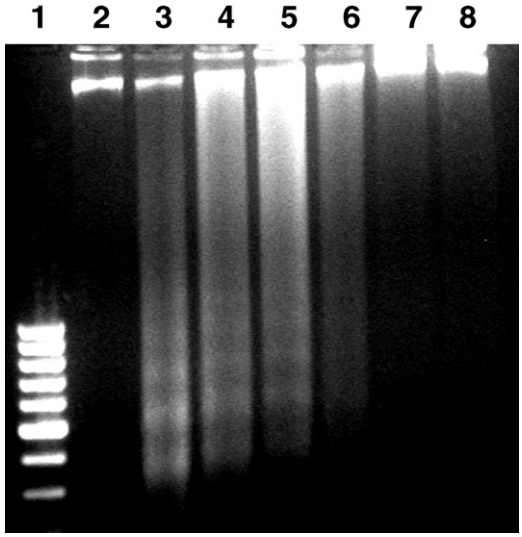

Fig. 3. Qualitative measurements of CHIKV induced apoptosis by DNA fragmentation assay .Cells were totally protected from DNA damage on MPA treatment (10 μM) when analyzed by DNA ladder assay. Molecular weight marker 1, mock infected 2, untreated CHIKV infected panel 3 (10 MOI), 4 (1 MOI) and 5 (0.1 MOI). MPA (10 μM) treated panel 6 (10 MOI), 7 (1 MOI) and 8 (0.1 MOI) at same time points.

By giving particular attention to Chikungunya virus, it was hypothesized that depletion of the substrate for the polymerase enzyme can affect the viral propagation. The enzyme IMPDH was selected as a target in order to prevent the viral multiplication and thereby cell damage. This enzyme catalyzes the conversion of IMP to XMP leading eventually to produce GMP/GTP that is an essential substrate for RNA/DNA polymerase. According to the given hypothesis there are two possible outcomes that will lead to prevention of CHIKV infection in in vitro model. (1) Depletion of the substrate for polymerase enzyme i.e. GTP, can prevent viral genome multiplication that is an essential process for the production of new viral progeny. (2) RNA viruses exist as quasispecies which are a mixture of related genome that share a consensus sequence and a broad mutant spectrum (Ruiz-Jarabo et al., 2000). Therefore, RNA viruses function on the edge of mutation crisis and an increase in average error frequency can result in loss of genetic information and decreased viability; this is termed “error catastrophe” (Crotty et al., 2001). So depletion of the substrate for polymerase may probably lead to production of mutant progeny that are lacking in

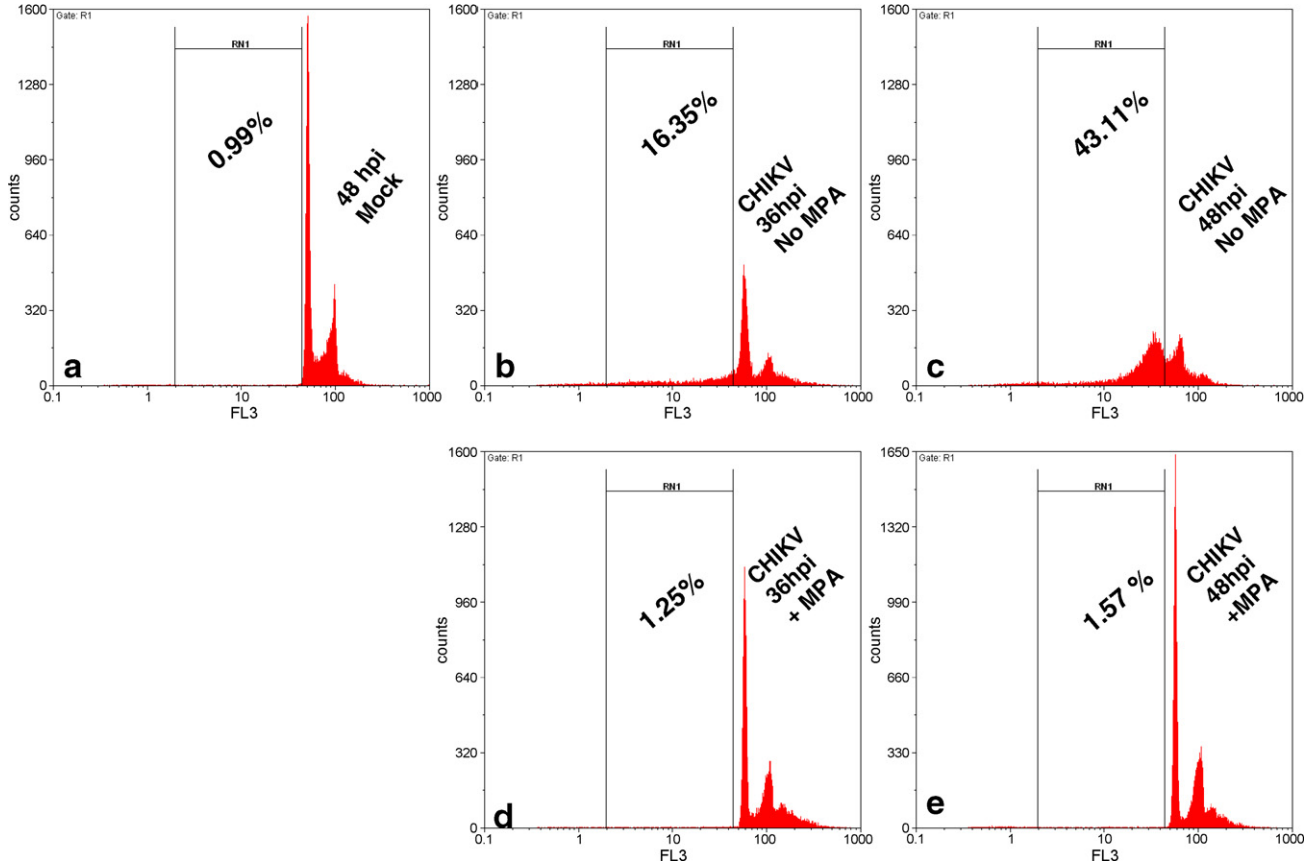

Fig. 4. Quantitative measurements of CHIKV induced apoptosis by propidium iodide (pi) staining followed by flow cytometry. Extent of protection was determined by infecting the cells at MOI of 0.1 in presence of 10 μM MPA and analyzed for presence of sub-G1 population with pi staining.

genetic information and viability. Precisely, in GTP depleted condition either the polymerase will stall at particular site (due to lack of availability of required nucleotide) leading to incomplete replication or polymerase can lead to incorporation of any irrelevant nucleotide at the site. In both the cases there are enough possibilities of reduced viral multiplication and infectivity.

In the present study, the effect of the inhibition of cellular IMPDH enzyme to deplete cellular GTP pool that is an essential substrate for viral replication was analyzed and MPA was chosen as a suitable candidate to target this activity. This study demonstrated that inhibition of cellular IMPDH activity is capable of inhibiting the CHIKV infection in vitro. Inhibition of CHIKV replication by MPA was virtually observed to be complete, as seen by lack of detectable rise in viral titer in the presence of MPA during a 48 hpi incubation (Fig. 1b) and as seen by inability to detect intracellular viral antigen in immunofluorescence assay (Fig. 2g). Molecular analysis of infected cell population also revealed high protective efficacy of MPA against CHIKV.

Assessment of CHIKV induced cellular damage was also taken as one of the parameter for the evaluation of virus propagation. Since Bcl-2 is critical for the commitment of cells to undergo apoptosis in viral infection, the expression level of this molecule in MPA treated and untreated infected cells was checked. Additionally, Bcl-2 down regulation has been reported in case of many alphavirus infection (Grandgirard et al., 1998). Although similar findings in case of CHIKV infection are still lacking but the direct involvement of caspases in alphavirus induced apoptosis has been shown in many reports and this plays a central role in apoptotic cell death. Therefore, it was decided to assess CHIKV induced caspase-mediated apoptosis which could be a direct reflection of viral growth and multiplication. Inhibition of IMPDH activity could prevent the CHIKV induced apoptosis which seems to be mediated via inhibition of virus multiplication as the exogenous addition of GMP could revert the MPA provided cell protection (Fig. 2d, h, l, and p).

Recent studies showed that MPA reduces intracellular GTP/GMP level in Vero cells which can be correlated with its anti-CHIKV activity in this study (Kitchin et al., 1997; Gong et al., 1999). Extent

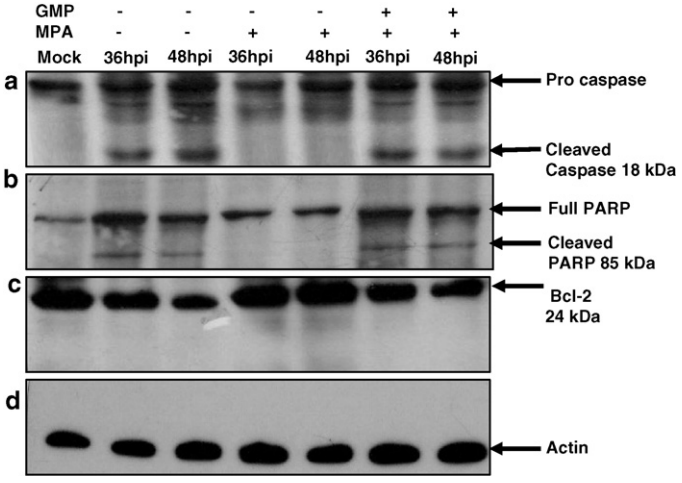

Fig. 5. Influence of MPA on the activation/expression of key molecules of apoptosis. Representative blots showing the effect of MPA on level of caspase-3 activation (a), PARP cleavage (b) and bcl-2 down regulation(c). Total cell lysate containing 50 μg protein per sample was loaded per lane and β-actin was used as an internal quantity marker (d). MPA and GMP were used at concentration of 10 μM and 0.5 mM, respectively.

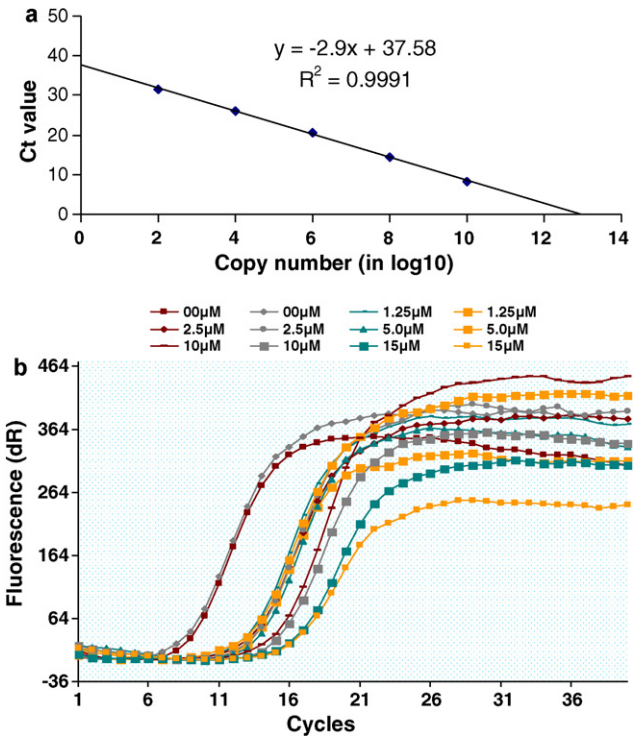

Fig. 6. Analysis of viral genome copy number by qRT-PCR. Serial 100-fold dilutions were prepared and C_t values for known copy number were obtained. A standard graph was plotted for copy number (ranging from 10^{10} to 10^2) vs. C_t values obtained (a). Amplification curves were then obtained for MPA treated and untreated samples (b). Genome copy number was analyzed with standard plot.

of GTP/GMP depletion by MPA can vary from cell to cell. Similar results were obtained when the study was performed in HeLa cells (Data not shown). This inhibitory activity was reversed by addition of exogenous GMP (0.5 mM). Moreover, present study showed that infectivity of the virus released into the medium was sensitive to the MPA concentration. The data indicated that without MPA treatment the total genome infectivity was found 3.33% whereas at higher concentration (10 μM) it was reduced drastically and found to be 0.148% (Table 2). The results showed that MPA at higher concentration affected the total genome infectivity of virion released into the medium significantly and it was concluded that apart from the prevention of virus replication, mutation can also be a reason for reduced infectivity. Results of the above experiment provide substantial evidences pertaining to the probable outcome of polymerase substrate depletion. Since it is difficult to characterize the MPA induced mutations (transitions or transversions) in natural infective viral population due to the presence of a complex mixture of mutated genome population (Jarabo et al., 2000), similar study will be of interest if infectious clone can be used as described earlier (Day et al., 2005).

In conclusion, it was demonstrated that CHIKV utilizes IMPDH activity for its growth and multiplication which is a potential and effective target to prevent its infection. Inhibition of IMPDH can be accomplished by using any potent IMPDH inhibitor like MPA at well tolerated dose. It would be useful to explore similar findings by targeting IMPDH in case of other alphaviruses which are more lethal than Chikungunya like Sindbis virus, Semliki forest virus, etc.

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Conflict of interest

Authors do not have any conflict of interest.

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