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Antiviral Activity of Emetine Dihydrochloride Against Dengue Virus Infection

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Abstract

Research Article

Dengue fever has become a global concern as dengue infections are prevalent in the tropics and the subtropics, and can lead to a severe life-threatening illness. Currently, there is a widespread interest to search for vaccine or antiviral therapy to combat DENV infection. In this study, we have identified emetine dihydrochloride with potent antiviral activity against DENV infection. Emetine was also shown to inhibit DENV replication consistently in all the dengue serotypes. Experiments were designed to define the stage of viral replication cycle at which emetine blocked DENV infection. Emetine did not target the entry process of DENV into cells as inhibitory effect of emetine on DENV infectivity remained when naked dengue viral RNA were transfected directly into the cells. Thus, we further investigated the inhibitory effect of emetine through timecourse studies and emetine was shown to strongly inhibit DENV infection at early stage of viral replication cycle by either affecting the synthesis viral RNA pathway or viral protein translation pathway. Quantitative RT-PCR assay indicated that emetine strongly reduced the production of positive-strand and negative-strand of DENV RNA. Ultrastructural analysis of emetine-treated cells further revealed that the formations of membranous replication complexes of DENV within cells were aborted in the presence of emetine. Together, these results suggest that emetine can inhibit DENV infection by impeding viral RNA synthesis therefore emetine could be further assessed and developed as a potential antiviral therapeutic agent against **DENV** infection.

Keywords: Viral RNA synthesis; Immunofluorescence assay; Quantitative RT-PCR; Antiviral therapeutics

Introduction

Dengue virus (DENV) is a member of the *Flavivirus* genus of the *Flaviviridae* family of enveloped, positive-strand RNA viruses (Kino et al., 1998). Four distinct serotypes (DENV1-4) of dengue viruses are transmitted to humans through bites of mosquito species, *Aedes aegypti* and *A. albopictus*. DENV causes a spectrum of disease in human, from acute febrile illness dengue fever (DF) to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). DF is a self-limited though debilitating illness characterized by fever, headache, retro-orbital pain, myalgia, arthralgia, and rash. DHF is marked by increased vascular permeability ("plasma leakage"), thrombocytopenia, and hemorrhagic manifestations; DSS occurs when fluid leakage into the interstitial spaces results in shock, which with-

The death rate associated with the more severe form of DHF/ DSS (DHF grades 3 or 4) is approximately 5 %. DENV has gained firm foothold in tropical and subtropical regions worldwide over the past several decades. It has been estimated that about 50-100 million cases of DF, and about 250,000-500,000 cases of DHF occur every year. Furthermore, 2.5 billion of people are at risk for infection in subtropical and tropical regions of the world in the absence of effective intervention (Halstead, 1998; WHO, 2006). Despite the increasing social and economic impact of DENV infection in terms of morbidity and mortality, a prophylactic or therapeutic agent which is effective against dengue virus is currently not available.

out appropriate treatment may lead to death (Halstead, 1989).

In this paper, we report the inhibitory activity of emetine dihydrochloride on DENV infection. Emetine, which belongs to the ipecac alkaloids, has been known to inhibit protein and nucleic acid synthesis (Grollman, 1968; Wink et al., 1998). Emetine has similar protein synthesis inhibitory properties as other alkaloids such as cycloheximide and anisomycin, except that the inhibition effect of emetine is irreversible (Grollman, 1968). Emetine hydrochloride is the hydrochloride of an alkaloid found in the Ipecac root, or prepared by methylation of cephaelin (Lee, 2008). It contains not less than 98 % and not more than 101.5 % of $C_{29}H_{40}N_2O_4$ ·2HCl, calculated on the anhydrous basis (USP Reference Standards). The detailed chemical structure of emetine is shown in Figure 1.

In this study, interesting results were obtained that suggest the potential use of emetine as a potent antiviral against DENV. Emetine treatment of DENV-infected human cell lines substantially reduced viral titer yields as observed from both plaque and immunofluorescence assays. A time-course study also revealed the involvement of emetine inhibition during the early stages of DENV replication. Employing quantitative RT-PCR, emetine treatment was shown to lead to significant reductions in intracellular amounts of negative-strand and positive-strand

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Figure 1: The chemical structure of emetine. Emetine is a complex polycyclic molecule and could be obtained by methylation of cephaelin. The radical CH₃ as circled, is absence in cephaelin (Lee, 2008). The (R)' configuration at C-1' and secondary nitrogen atom at the 2' position are necessary for emetine's activity (Grollman, 1966).

viral RNA in DENV-infected cells. Therefore, our results suggest potent antiviral effects of emetine against DENV and its potential use in the treatment of DENV diseases.

Materials and Methods

Cell culture, virus and antibodies

The cells used in this study were C6/36 cells, Huh-7 human hepatoma cells and BHK21 baby hamster kidney cells. C6/36 is a continuous mosquito cell line isolated from Aedes albopictus embryonic tissue. C6/36 cells were maintained in L-15 medium containing 10 % FCS at 28 °C and were used for the propagation of the four serotypes of dengue strains (DENV1, Singapore isolate S144; DENV2, New Guinea C strain; DENV3, Singapore isolate Eden 130/05; and DENV4, Singapore isolate S8976). Huh-7 hepatoma cells were cultured in DMEM medium with 10 % FCS and BHK21 cells were cultured in RPMI 1640 with 10 % FCS at 37 °C in 5 % CO₂ incubator. For immunofluorescence assay, the monoclonal mouse anti-DENV type 2 virus (US Biological) and the monoclonal mouse anti-DENV virus complex (cross-react with all four serotypes of DENV), clone D3-2H2-9-21 (Chemicon International) were used to detect for DENV infection in cells.

Immunofluorescence Assay

Huh-7 hepatoma cells were seeded at 20,000 cells per well in flat-bottom, 96 well plate (NUNC). The cells were incubated at 37 °C with 5 % CO₂. After 16 h, the cells were infected with DENV at multiplicity of infection (m.o.i.) of 1 for 1 h in the 37 °C, 5 % CO₂ humidifying incubator with gentle rocking every 15 min. The virus suspension was decanted and the wells were washed twice with Phosphate-Buffered Saline (PBS) to remove the remaining unbound virus. The well was replaced with 100 μ l of DMEM with 2 % FCS. After 3 days of infection, the supernatant of each well was harvested for quantitation of infectious virus using plaque assay. The cells were then fixed for 15 min in cold absolute methanol (Sinopharm Chemical). The cells were washed twice and rehydrated with PBS for 30 min. The cells were then incubated with 40 μ l of primary antibody (Mouse

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Anti-Dengue Type 2 Virus, US Biological) diluted 1:1000 for 1 h at 37 °C to detect DENV2 infection in cells. For the detection of DENV1, DENV3 and DENV4 infection, the primary antibody used was the monoclonal mouse anti-dengue virus complex, clone D3-2H2-9-21 (Chemicon International) at the dilution of 1:400. The cells were then washed twice with PBS before addition of 40 μ l of secondary antibody diluted 1:500 for 1 h at 37 °C. The secondary antibody used was goat anti-mouse IgG (H+L) fluorescein conjugated (Chemicon International). Finally, DAPI (Invitrogen) was added for 15 min to stain the cell nuclei.

The images for the immunofluorescence assay were obtained using the Olympus IX81 Motorized Inverted Microscope (Olympus) and the auto-focusing parameters were preset from the Metamorph software. Nuclei of cells counter-stained with DAPI were counted as the total number of cells in the population and the cytoplasm counter-stained with FITC were referred to as the total number of virus-infected cells. Quantitation was performed using CellProfiler software available at www.cellprofiler.org (Carpenter et al., 2006).

Emetine dihydrochloride treatment

The stock concentration of emetine dihydrochloride (BioMol, USA) was prepared at 10 mM in DMSO. In this study, the cells were treated with emetine at the concentration of $10 \,\mu M$ (1:1000 dilution from the stock of 10mM), with a final concentration of 0.1 % DMSO. The cells that were infected with DENV2 and treated with 0.1 % DMSO only in cell culture medium were used as control in this study. For the drug pre-treatment and post-treatment assay, cells were treated with a range of concentration of emetine dihydrochloride at 0.01 µM, 0.1 µM, 0.5 µM, $1 \mu M$, $5 \mu M$ and $10 \mu M$ in 96 well plate before (pre-treatment) or after (post-treatment) DENV2 infection. For the drug pretreatment assay, the Huh7 cells were pre-treated with emetine for 2 h prior to DENV2 infection. The media was then removed and cells were infected with DENV2 at the m.o.i. of 1. The cells were then washed twice before addition of maintenance media. For the post-treatment assay, maintenance media containing appropriate concentrations of emetine was added after the 1 h virus adsorption step.

Time-course studies were also performed at different time points after DENV2 infection to further characterize the antiviral activity of emetine. The time points involved were 2 h p.i., 4 h p.i., 8 h p.i., 12 h p.i., 24 h p.i. and 48 h p.i. . After virus infection at the indicated time point, cells were washed twice with PBS to remove residual virus. Cells were then either treated with DMEM or 0.5 μ M emetine for the respective time point. After 72 h, the cells were stained for immunofluorescence assay.

Plaque assay for infectious virus quantitation

BHK21 hamster kidney cells were seeded in 24-well plates (Greiner Bio-One) and incubated in the 37 °C, 5 % CO₂ humidifying incubator overnight. The cell culture media was first decanted before adding the appropriate dilutions of the virus. Virus samples were diluted in ten-fold serial dilution in DMEM 2 % FCS. 100 µl of the diluted virus was then added to the 24 well plates in triplicate for each dilution. The cells were incubated for 1 h in the 37 °C, 5 % CO₂ incubator with gentle rock-

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ing every 15 min. After 1 h, the cells were washed twice and overlaid with 1 % CMC in RPMI with 2 % FCS. The cells were then fixed and stained with 10 % paraformaldehyde/1 % crystal violet (Sigma-Aldrich) solution after 4 days for plaque visualization. The crystal violet solution was removed after 1 h and the plates were washed and left to dry in the incubator. Virus titer was quantified as plaque forming unit per milliliter (pfu/ml).

Cell viability assay

Cell viability assay was done to assess the cytotoxicity of emetine by incubating the Huh-7 cells with emetine (concentration range of 0.01 μ M to 10 μ M) in DMEM 10 % FCS for 3 days; 0.1 % DMSO was used as a non-treated control. Cell viability was then measured using alamarBlue reagent (Invitrogen). One tenth volume of alamarBlue reagent was added with the cells and was incubated for 2 h at 37 °C. The fluorescence measurements were performed at excitation wavelength of 570 nm and emission wavelength of 585 nm as recommended by the manufacturer. The percentage of cell viability was then determined by comparing with the non-treated 0.1 % DMSO control.

Cell transfection with DENV2 RNA

Viral RNA was first isolated and purified from DENV2 viral supernatants (5 x 10⁸ PFU) using the PureLink Viral RNA extraction kit (Invitrogen) and eluted in RNAse-free water. For the transfection of DENV2 viral RNA into cells, Huh-7 cells were first plated (1x10⁴ cells/well) in a 24-well plate, pre-treated or post-treated for 6 h with 0.5 μ M of emetine at 37 °C upon transfection of the DENV2 RNA. DENV2 RNA was prepared according to the manufacturer's instructions of TransIT RNA transfection kit (Mirus) and transfected into Huh-7 cells. The cells and supernatants were harvested 72 h.p.i. for viral antigen detection by immunofluorescence assay and viral plaque assay.

RT-PCR detection of DENV2 positive and negative-sense RNA

In brief, the positive and negative strand of the DENV2 RNA within the Huh-7 cells was assayed by real-time PCR using the the ABI 7000 Sequence Detection System (Applied Biosystems). The primers and RT-PCR conditions are used in accordance to a previously published protocol by (Wang et al., 2002).

Sample preparation for transmission electron microscopy

The emetine-treated and DENV2-infected cell samples were first washed twice with PBS in the 75 cm² flask. The cell monolayer was then fixed with primary fixative (2.5 % glutaraldehyde, Agar) overnight at 4 °C. Following the primary fixative, the cells were washed and scraped off the cell culture flasks before post-fixed with 1 % osmium tetroxide (Ted Pella) for 2 h. Few grains of potassium ferrocyanide were added to enhance the contrast of the membranous structure within cells. After 2 h, the cells were washed and dehydrated with ethanol in ascending percentages (25 %, 50 %, 75 %, 95 % and 100 %). The dehydration step was enhanced by another two rounds of absolute acetone treatment for 10 min each. The dehydrated cell pellet then underwent a series of infiltration with increasing ratio of araldite 502 (Ted Pella) to acetone at increasing temperature before embedding in fresh araldite for 24 h at 60 °C. Ultrathin section were cut and trimmed with an ultramicrotome (Reichert-Jung) to the size of approximately 50 nm–70 nm. Freshly cut sections were then picked up onto a 200 mesh copper grid before staining with 2 % uranyl acetate and post-fixed with lead citrate. The stained sections were viewed under the transmission electron microscope, Philip EM 208 and captured digitally with a dual view digital camera (Gatan).

(A)



Figure 2(A, B): Effect of emetine on DENV2 inhibition in dose-dependent manner (Pre-treatment). Huh-7 cells were pretreated at different concentrations of emetine for 2 h before DENV2 infection. For the IFA images, cell nuclei were stained with DAPI (blue) and the presence of DENV infection is represented by FITC staining (green). Values shown are means of triplicate experiments with error bars representing standard errors of the means.

Results

Emetine inhibits DENV replication

Little is known about the potential antiviral mechanism of emetine. Hence, in this study, we investigated the possible antiviral effect of emetine on the DENV replication. To determine the antiviral activity of emetine to inhibit DENV, emetine was



Figure 2(C, D): Effect of emetine on DENV2 inhibition in dose-dependent manner (Post-treatment). Huh-7 cells were post-treated with emetine for 3 days upon DENV2 infection with different concentrations of emetine. For the IFA images, cell nuclei were stained with DAPI (blue) and the presence of DENV infection is represented by FITC staining (green). Values shown are means of triplicate experiments with error bars representing standard errors of the means. Generally, emetine shows dose-dependent reduction in DENV2 infectivity for both pre and post-treatment. However, the cells post-treated with emetine display greater inhibition of DENV2 infection compared to the pre-treated cells at different concentrations of emetine.

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initially tested for dose-dependent reduction of DENV2 replication. Huh-7 cells were either pre-treated for 2 h with emetine and subject to DENV2 infection or post-treated for 3 days with different concentration of emetine upon DENV2 infection. The pre-treatment assay with emetine will enable us to interrogate if the early process of DENV replication (virus entry) is affected while the post-treatment assay will indicate if post-entry virus replication mechanism (viral RNA replication, viral protein translation and assembly) is affected.

The emetine-treated and DENV2-infected cells were processed for immunofluorescence assay to stain for the presence of DENV2 envelope protein with specific antibody. Figures 2A and Figure 2C showed the immunofluorescene images of pretreated or post-treated cells at different concentrations of emetine, respectively. The number of DENV-infected cells were counted, expressed as the percentage of viral antigen positive cells and plotted against the different concentrations of emetine treatment as shown in Figure 2B and Figure 2D for both the pre-treatment and post-treatment assays, respectively. Generally, emetine exhibited dose-dependent inhibition of DENV2 infection of Huh-7 cells for both pre and post-treatment. Compared to the 0.1 % DMSO control, cells post-treated with emetine at 0.1 µM shows 33 % virus inhibition. With 0.5 µM to 10 µM of emetine post-treatment, the level of DENV2 infection was significantly inhibited by more than 90 % when compared to the 0.1 % DMSO control (Figure 2D). In contrast, cells pretreated with emetine showed significant inhibition of more than 90 % only at much higher concentrations of 5 μ M and 10 μ M (Figure 2B). Thus, post-treatment with emetine was revealed to be more effective in reducing DENV2 replication than pre-treatment with emetine.

In addition, we have also assessed the effect of emetine on the direct treatment with DENV particle with different concentration of emetine. We have carried out the experiments of direct incubation of 5000 PFU of dengue virus particles with emetine at the concentrations of 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M and



Figure 2(E): Virus titer was quantitated using plaque assay for non-treated (0.1 % DMSO) and emetine treated (0.01 μ M, 0.1 μ M, 0.5 μ M, 1 μ M, 5 μ M, 10 μ M) DENV2-infected Huh-7 cells. DENV2 titer was shown to reduce in dose-dependent manner for cells post-treated with emetine, correlate with the IFA images results. DENV2 infection was reduced by more than 2.5 log units in cells treated with emetine at 0.5 μ M and 1 μ M. At higher concentrations of 5 μ M and 10 μ M respectively, there was complete inhibition of DENV2 production.

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0.1% DMSO for 1 h. The excess emetine was washed off using a spin column with 100,000MW cut-off and the virus was subjected to plaque assays for quantification. The direct treatment of emetine on DENV particles did not seem to affect DENV infection of cells (data not shown). Hence, this experiment further support that emetine inhibition of DENV infection is not affecting the initial interaction of DENV particles with host cells.

The inhibitory effect of emetine on DENV2 replication upon post-treatment was further verified using plaque assay as shown in Figure 2E. Consistent with the results obtained by immunof-luorescence assay, a dosage-dependent reduction of DENV2 titer was also observed for cells treated with emetine at concentrations from 0.01 μ M to 10 μ M. Treatment of cells with emetine at 0.5 μ M and 1 μ M reduced DENV2 titer by more than 2.5 log units when compared to the 0.1 % DMSO control. Interestingly, there was complete inhibition of infectious DENV2 production at high concentrations of emetine treatment at 5 μ M and 10 μ M, respectively.

In addition, cell viability assay using alamarBlue assay was performed to ensure that the DENV2 infectivity reduction was not caused by the cytotoxicity of the different concentrations of emetine used in these experiment. Percentages of cell survival were plotted against the different concentrations of emetine from 0.01 μ M to 10 μ M as shown in Figure 3. Compound is considered toxic from the cell viability assay if the compound showed more than 50% decrease in signal when compared to 0.1% DMSO control, and p < 0.05 as determined by one-tailed student's t test. From the data obtained, emetine did not show any significant cytotoxicity for all the concentrations used in this study. Thus, we could deduce that the dose-dependent reduction of DENV2 infection by emetine was not complicated by cytotoxic effects.

To further determine if the inhibitory effect of emetine is also applicable to other serotypes of DENV, Huh-7 cells were infected with dengue serotype 1, 3 and 4 and subjected to emetine treatment at different concentrations (0.01 μ M to 1 μ M). Dosage-dependent inhibition of all the DENV serotypes were observed (Figure 4). These data therefore suggested that emetine



Figure 3: Cell viability assay. Cell viability assay using alamarBlue to determine compound cytotoxicity for different concentration of emetine from 0.01 μ M to 10 μ M, as well as cells treated with 0.1 % DMSO. The assay results indicate that emetine did not show any significant cytotoxicity for the concentrations used in this study.



Figure 4: Inhibitory effect of emetine on different DENV serotypes. Dosage-dependent inhibition of DENV serotype 1, 3 and 4 was observed when Huh-7 cells were treated with different concentrations of emetine. Thus, antiviral activity of emetine is highly active against all four serotypes of DENV.

has antiviral activity against all serotypes of DENV.

The results from the post-treatment assays may suggest that emetine is not affecting the early steps (attachment, entry, nucleocapsid escape, and uncoating) of the DENV replication cycle. This was further confirmed by utilizing an indirect assay whereby naked dengue viral genomic RNA was transfected into Huh-7 cells that were either pre-treated or post-treated with 0.5 µM and 1 µM of emetine. Pre-treatment of cells with emetine did not inhibit the replication of DENV2 upon transfection of the dengue viral RNA as revealed by the significant levels of viral proteins accumulating within the cells (Figure 5A). In contrast, post-treatment of Huh-7 cells with the different concentrations of emetine upon transfection of the purified dengue viral RNA virtually abolished the replication of DENV2 (Figure 5B). Hence, these data may indicate that the antiviral action of emetine may occur at a step after the release of the viral genome into the cytoplasm for virus replication.

Time-course studies of emetine effect on DENV2 infection

Further experiments were performed to elucidate the exact involvement of emetine within the stages of DENV replication (viral RNA replication, viral protein translation or viral assembly and release). In our first approach, we investigated the effect of emetine added to the cells across different time points after DENV infection of Huh-7 cells. After exposure of DENV2 to Huh-7 cells, cells were treated with 0.5 µM emetine at different time points ranging from 2 h p.i. to 48 h p.i.. From the immunofluorescence images as shown in Figure 6A and Figure 6B, Huh-7 cells treated with emetine at early time points up to 12 h p.i. showed significant inhibition of DENV2 infection. Emetine treatment markedly reduced DENV2 replication by more than 80 % for cells treated within 8 h p.i. and approximately 65 % inhibition for treatment 12 h p.i. As compared to the emetine treatment at earlier time points, the level of DENV2 inhibition was not significant with emetine treatment at the later stages of infection. Emetine added at 24 h and 48 h after DENV2 exposure had minimal inhibition of DENV2 infection (Figure 6C). The data may therefore suggest that emetine does act early on the virus replication process and this may suggest that the

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Figure 5: Transfection of DENV2 viral RNA into Huh-7 cells. Cells were either (A) pre-treated or (B) post-treated with different concentrations of emetine and transfected with equivalent amounts of purified DENV2 RNA. Three days after transfection, the cells were processed for antigen expression by immunoflourescence assay. The data are representative of three independent experiments. Cells pre-treated with emetine did not inhibit the DENV2 infection upon transfection of DENV2 RNA. However, cells post-treated with emetine showed drastic reduction in DENV2 replication. We can hence deduce that inhibitory action of emetine occurs after the release of viral genome into the cytoplasm.

synthesis of dengue viral RNA or viral protein translation could be affected.

Emetine inhibits DENV2 RNA synthesis

In order to assess the effect of emetine on viral RNA synthesis, quantitative RT-PCR was performed on emetine-treated and untreated DENV2-infected Huh-7 cells to compare the amounts of positive-strand and negative-strand DENV2 RNA present with cells as infection progressed. At 24 h p.i., treatment of cells with $0.5 \,\mu$ M of emetine reduced positive-strand viral RNA by 1000-fold and negative-strand viral RNA by 10,000-fold when compared against non-treated, DENV2-infected cells (Figure 7). Similarly, at 48 and 72 h p.i., the level of positive-strand and negative-strand of dengue RNA remained significantly lower in emetine-treated, DENV2-infected cells as compared to the non-treated DENV2-infected cells (Figure 7). As such, these interesting data suggested that emetine could act by impeding the synthesis of dengue viral RNA.

To complement the possibility of emetine in inhibiting DENV replication by affecting the synthesis of viral RNA, transmis-

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sion electron microscopy was performed on DENV2-infected cells treated with 0.5 μ M of emetine or 0.1 % of DMSO as a negative control. By observing the cells at the ultrastructural level, we could determine if specific DENV-induced structures are formed in DENV2-infected cells upon treatment with emetine. The formation of typical membranous structures of DENV replication complexes within cells is essential for the synthesis of viral RNA (Ng and Chu, 2002). As shown in DENV2-infected cells treated with 0.1 % DMSO (Figure 8A and 8B), cells exhibited membranous replication structures (arrows) which are



Figure 6(A, B): Effect of emetine on DENV2 replication for time-course studies. IFA images of (A) DENV2-infected Huh-7 cells and (B) DENV2-infected Huh-7 cells treated with 0.5 μ M emetine. Emetine was added at the stated timepoints after DENV2 infection. Cells treated with emetine show higher infectivity reduction at early time points up to 12 h.p.i.

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Figure 6(C): Bar chart representation of percentage of viral antigen positive cells for DENV2-infected cells and DENV2-infected cells treated with $0.5 \,\mu$ M emetine at 2 h.p.i. to 48 h.p.i.. Values shown are means of triplicate experiments with error bars representing standard errors of the means. Emetine added from 2 h.p.i. to 8 h.p.i show more than 80% reduction in virus activity. In contrast, no drastic reduction was observed for addition of emetine at later time points of 24 h and 48 h.



Figure 7: Quantification of positive and negative strand dengue viral RNA within the cells. Huh-7 cells were infected with DENV2 at m.o.i. of 1 in the presence and absence of emetine. The infected cells were then collected at different time points and subjected to real-time RT-PCR assay for quantification for positive and negative-sense DENV2 RNA species. Amounts of positive and negative-sense RNA per million cells are shown. The data are obtained from duplicate set of experiments. Emetine-treated cells exhibited reduction of positive-strand viral RNA by 1000-fold and negative-strand viral RNA by 10,000-fold when compared against 0.1 % DMSO non-treated, DENV2-infected cells at 24 h.p.i. The level of positive-strand and negative-strand of dengue RNA also remained significantly lower at later time point of 48 h.p.i. and 72 h.p.i..

characteristic of flavivirus infection. In Figure 8B, large numbers of DENV particles (arrowheads) were also observed within the ER lumen and secretory vesicles. In contrast, there were minimal DENV-induced membranous replication structures observed within the infected cells that were treated with emetine (Figure 8C). Furthermore, no virus particles were observed within the emetine-treated cells. The cellular morphology of DENV2-infected cells that were treated with emetine was also observed to be similar to cells that were treated with emetine but not subjected to DENV2 infection (Figure 8D). Together, these results further supported the notion that emetine may act to inhibit DENV at the early stages of viral replication. JAA/Vol.1 Issue.1



Figure 8: Ultrastructural analysis of DENV2-infected and emetine treated Huh-7 cells. (A, B) DENV2-infected Huh-7 cells treated with 0.1 % of DMSO. (A) DENV2 induced membranous structures and replication complexes (arrows) can be observed. DENV2 particles (arrowheads) can also be observed in close association with the replication complexes (arrow). (B) DENV2 particles (arrowheads) are observed to accumulate within the ER lumen in close association with the virus replication complexes (arrows). (C) DENV2-infected Huh-7 cells that were treated with 0.5 μ M of emetine. Distinct membranous structures induced by DENV2 infection are not obvious even though there is extensive proliferation of the ER membranes (arrows). (D) Mock-infected Huh-7 cells that were treated with 0.5 μ M of emetine. These cells exhibited typical ultrastructral morphology of non-infected cells.

Discussion

Currently, there is no available vaccine or antiviral drug for clinical application and treatment of DENV infection despite the increasing risk of DENV infection in tropical and subtropical regions of the world. There is limited number of known compounds that were able to inhibit dengue virus infectivity in vitro. Some of the well known compounds are mycophenolic acid (MPA) and ribavirin (Diamond et al., 2002). Ribavirin acts as antiviral by inhibiting the inosine monophosphate dehydrogenase (IMPDH) that disrupt the biosynthesis process of guanine nucleotide (Leyssen et al., 2001), while mycophenolic acid acts by inhibiting of dengue viral RNA replication (Diamond et al., 2002). In clinical aspect, ribavirin is currently only used for Hepatitis C treatment with side effects of causing anemia on patients (Hong et al., 2002). Other inhibitory compounds that prevent DENV infection are cycloheximide (Shukla and Chaturvedi, 1981), and glucosidase inhibitors such as castanospermine (Whitby et al., 2005) and deoxynojirimycin (Wu et al., 2002). Nevertheless, none of these compounds has been further established as antiviral drugs for treatment in dengue patients. Therefore, there are current demands to identify and evaluate new inhibitors for treatment of DENV infections.

Thus, the aim of the current study is to identify novel compound which could act as inhibitor for DENV infection. We have focused our interest on ipecac alkaloid - emetine dihydrochloride that is extracted from the South American plant ipecac (*Cephaelis ipecacuanha*) and has been used in traditional

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phytomedicine for centuries which was subsequently introduced to Western medicine during the 1600s (Lee, 2008). The main pharmacologically active alkaloids found in the ipecac root are emetine (methylcephaelin) and cephaelin (Möller et al., 2007). One of the primary pharmacological usages of ipecac is in the induction of vomiting (emesis) in patients who ingested poison and is available as a non-prescription drug in many countries (Garrison et al., 2003). However, the clinical benefit of emesis in toxin removal has been found to be highly variable given the type of poisons ingested ("Position paper: Ipecac syrup," 2004). Emetine was also used as an effective antibiotic for amoebiasis caused by *Entamoeba histolytica*, although side effects, such as cardiotoxicity, have led to its replacement in amoebiasis treatment with metronidazol (Knight, 1980).

Despite the replacement of emetine in its traditional medicinal roles with safer and more effective alternatives, the diverse bioactivity of emetine has led to continue research into novel medicinal applications for emetine. Emetine has been shown to inhibit protein synthesis in eukaryotic cells through interactions with ribosomes (Grollman, 1968). It has also been observed to inhibit nucleic acid synthesis, intercalate DNA and target the enzymes DNA polymerase I and reverse transcriptase (Grollman, 1968; Wink et al., 1998). Emetine has been widely reported as a potential chemotherapy agent with strong cytotoxicity demonstrated against several human cancer cell lines, e.g. U937 (histocytic lymphoma), A549-S (lung adenocarcinoma) (Watanabe et al., 2002) and Jurkat T (T cell leukemia) (Moller et al., 2007; Moller et al., 2006). The inhibition of protein synthesis was thought to be the main cause of apoptotic cytotoxicity observed in emetine-treated cells (Wink, 2007).

However, recent studies also point to the induction of apoptosis by emetine through the regulation of pro-apoptotic factors (Shultz and Chalfant, 2007). Emetine was described to regulate the alternative splicing of *Bcl-x* pre-mRNA through a protein phosphatase-1 (PP1) mediated pathway. Emetine-treated cells showed increased levels of the pro-apoptotic variant, *Bcl-xS*, and reduced levels of the anti-apoptotic *Bcl-xL* (Boon-Unge et al., 2007). A DNA assay of emetine-treated Jurkat cells also identified the upregulation of several pro-apoptotic and anti-survival genes, e.g. capsase 8 (*CASP8*), caspase 9 (*CASP9*) and deathassociated protein 6 (*DAXX*) (Moller et al., 2007). Recently, emetine has also been proposed as a synergistic compound for use in drug-induced apoptosis of leukemia cells (Moller et al., 2007).

Although the apoptotic properties of emetine is much desired in the treatment of cancer, it would present a strong case arguing against the potential use of emetine as an antiviral. However, the experience from amoebiasis treatment can provide us with much insight into the management of emetine as a therapeutic agent. In the treatment of amoebiasis, emetine is administered intramuscularly or subcutaneously (deep) daily at 1mg of emetine per kg with a maximal dose of 60mg for a maximum period of 10 days (Knight, 1980). Severe side effects and death occur rarely and were only observed at high doses of emetine, e.g. in a fatal case, a total dose of 13g of emetine was administered in 65mg increments (Knight, 1980). Emetine was also shown to be well tolerated when delivered intravenously at 1.5mg/kg doses twice a week in clinical trials of emetine as an anti-tumour agent (Panettiere and Coltman, 1971). In comparison, our results demonstrated strong inhibition of DENV by emetine at a much lower concentration of 0.5μ M (277ng/ml).

At the cellular level, characterization studies of emetine seem to indicate cell-type specific activity and a differential dosage requirement for apoptosis and protein synthesis inhibition. Emetine (1µg/ml) was observed to result in 98% inhibition of protein synthesis in MCF-7 cells (human breast cancer) without inducing apoptosis (Beidler et al., 1999). In demonstrating emetine's novel function in regulating *Bcl-x* splicing, the authors also noted that at concentrations of up to 10µM, emetine was not able to induce apoptosis in MCF-7, PC3 (prostate cancer), A549 and C33A cells (cervical carcinoma) (Boon-Unge et al., 2007).

The dose-dependent and cell-type specific physiological effects of emetine described above demonstrate that the safe therapeutic use of emetine can be achieved through dosage management and method of drug administration.

Currently, there has been minimal research into the potential use of emetine as an antiviral against the virus members of the Flaviviridae family. In this paper, we are able to demonstrate the ability of emetine to inhibit DENV replication and virus production at pharmacologically relevant concentration with minimal cytotoxicity. Our investigations on the efficacy of emetine treatment to reduce DENV infection was initially tested in human Huh-7 cell lines. From the data obtained, emetine-treated cells show dose-dependent reduction of DENV replication. Posttreatment assay with emetine has successfully demonstrated that emetine can result in almost complete inhibition of dengue virus infectivity at a lower dosage of 0.5 µM when compared to the pre-treatment assay (Figure 2). Emetine thus probably inhibits DENV at the post-entry process of the virus replication cycle. Indirect assay of transfection of naked dengue viral genomic RNA was also performed for both pre-treatment and posttreatment of emetine on the cells. Inhibitory effect of emetine remained even when naked dengue viral RNA was transfected directly into the cells. As such, the inhibitory action of emetine is not affecting the entry pathway of the DENV but occurs most probably after the release of DENV viral genome into the cytoplasm.

To further map out the antiviral mechanism of emetine against DENV, time-course studies with emetine treatment have demonstrated that emetine-treated cells caused drastic reduction in DENV infection for treatment at early stages of virus replication. Addition of emetine at later stages of infection had minimal inhibition of DENV infection in the cells (Figure 6C). From these results, we could deduce that emetine inhibits the DENV infection at the early stages of viral replication life cycle, either affecting the viral RNA synthesis pathway or viral protein translation pathway. Quantitative RT-PCR assay was further used to detect the positive and negative strands of viral RNA within emetine-treated cells for different time points post-infection. Emetine treatment was shown to strongly inhibit the synthesis of positive-strand and negative-strand viral RNA (Figure 7). These data may therefore suggest that emetine may act on the inhibition of viral RNA synthesis. Furthermore, ultrastructural analysis of emetine-treated, DENV-infected Huh-7 cells revealed that the formation of the typical membranous structures of DENV replication complexes which is essential for viral RNA synthesis was also aborted (Figure 8). Collectively, these observations

further support the evidence that emetine acts by obstructing the synthesis of DENV RNA.

A plausible mechanism of emetine in inhibition of DENV RNA synthesis is by direct inhibition of the DENV protein synthesis. Emetine could possibly block the translation of the viral polyprotein precursor which is essential for formation of the structural and non-structural proteins. Hence, this disrupts the DENV replication since viral replication requires the presence of the viral non-structural proteins. Alternatively, the inhibition of DENV RNA synthesis by emetine can also be explained by the fact that emetine may have inhibitory actions on the biosynthesis of cellular proteins that are known to be essential for mediating the synthesis of DENV RNA. A number of cellular proteins such as elongation factor-1A (eEF1A), T-cell intracellular antigen 1 (TIA-1/TIAR) and polypyrimidine tract-binding protein (PTB) have been shown to interact with both the positive and negative strands of flavivirus RNA. The interactions between these host proteins and viral RNA are essential for the regulation as well as the synthesis of the viral RNA within flavivirus-infected cells (Davis et al., 2007; Emara et al., 2008; Anwar et al., 2009). More studies will be needed to decipher the detail mechanism of the inhibitory role of emetine on DENV replication. Besides, we have also extended our findings on the inhibitory effect of emetine on other DENV serotypes (DENV 1, 3 and 4) in dose-dependent manner. The antiviral action of emetine is consistent across the four serotypes of DENV (Figure 4).

Nonetheless, additional studies are now being conducted to determine if the antiviral activity of emetine is effective against other flaviviruses and possibly other RNA viruses. Furthermore, we are now in the process of evaluating the drugs in the murine model to ensure that the compounds will also reduce dengue replication *in vivo* within non-cytotoxicity concentration. In summary, emetine dihydrochloride might be a promising inhibitor for DENV infection.

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