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Emetine inhibits replication of RNA and DNA viruses without generating drug-resistant virus variants

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ABSTRACT

At a noncytotoxic concentration, emetine was found to inhibit replication of DNA viruses [buffalopoxvirus (BPXV) and bovine herpesvirus 1 (BHV-1)] as well as RNA viruses [peste des petits ruminants virus (PPRV) and Newcastle disease virus (NDV)]. Using the time-of-addition and virus step-specific assays, we showed that emetine treatment resulted in reduced synthesis of viral RNA (PPRV and NDV) and DNA (BPXV and BHV-1) as well as inhibiting viral entry (NDV and BHV-1). In addition, emetine treatment also resulted in decreased synthesis of viral proteins. In a cell free endogenous viral polymerase assay, emetine was found to significantly inhibit replication of NDV, but not BPXV genome, suggesting that besides directly inhibiting specific viral polymerases, emetine may also target other factors essentially required for efficient replication of the viral genome. Moreover, emetine was found to significantly inhibit BPXV-induced pock lesions on chorioallantoic membrane (CAM) along with associated mortality of embryonated chicken eggs. At a lethal dose 50 (LD_{50}) of 126.49 ng/egg and at an effective concentration 50 (EC_{50}) of 3.03 ng/egg, the therapeutic index of the emetine against BPXV was determined to be 41.74. Emetine was also found to significantly delay NDV-induced mortality in chicken embryos associated with reduced viral titers. Further, emetine-resistant mutants were not observed upon long-term (P = 25) sequential passage of BPXV and NDV in cell culture. Collectively, we have extended the effective antiviral activity of emetine against diverse groups of DNA and RNA viruses and propose that emetine could provide significant therapeutic value against some of these viruses without inducing an antiviral drug-resistant phenotype.

1. Introduction

Despite the availability of suitable vaccines, infectious viral diseases with epidemic potential, such as peste des petits ruminants (PPR), foot-and-mouth disease (FMD), sheeppox and goatpox, are leading causes of livestock morbidity and mortality worldwide, thereby threatening food security and the livelihood of marginal farmers and are major hurdles for the animal production industry to achieve optimal production (Jones et al., 2016). The epidemiology of these viruses very much overlaps and mixed infections are quite common (Kumar et al., 2016), leading to an increase in the severity of the disease (Malik et al., 2011). Unfortunately, antiviral drugs against these devastating diseases are lacking. The antiviral therapeutics assume enormous importance in providing instantaneous protection and reduction of the risk of virus transmission from infected animals to the susceptible population during epidemics (Charleston et al., 2011), besides acting as a necessary bridge during the period between animal vaccination and acquiring full immunity (immunity-gap) (Cheng et al., 2008).

Emetine dihydrochloride hydrate (emetine), IUPAC name-(2S,3R,11bS)-2-{[(1R)-6,7-Dimetoxy-1,2,3,4-tetrahydroisoquinolin-

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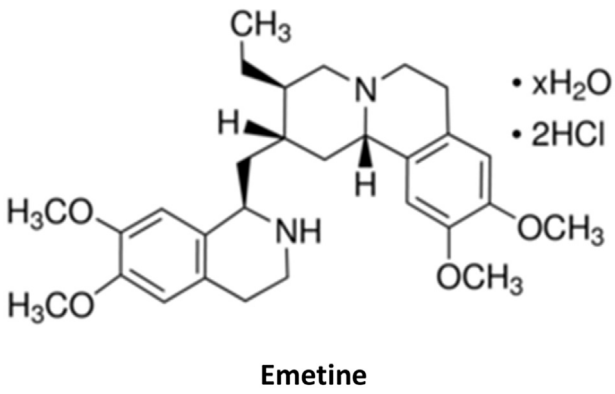


Fig. 1. Structure of emetine.

1-yl]metyl}-3-etyl-9,10-dimetoxy-2,3,4,6,7,11b-hexahydro-1H-pyrido[2,1-a]isoquinoline (Fig. 1), a natural alkaloid as well as its analogs exist in three plant families: *Alangiaceae, Icacinaceae, and Rubiaceae*. Molecular modeling studies of emetine and its analogs have helped to understand the structural basis for their biological activities. The earliest known use of emetine in the traditional medicine was as an emetic and expectorant (Grollman, 1966). Emetine has been shown to inhibit protein synthesis in mammalian, yeast and plant cells but not in bacterial cells (Grollman, 1966, 1968; Han et al., 2014; Smirnova et al., 2003). The mechanism of action of emetine is based on the inhibition of the aminoacyl-sRNA transfer reaction at the 40S ribosomal subunit (Gupta and Siminovitch, 1976; Jimenez et al., 1977). Emetine has also been shown to inhibit DNA synthesis in mammalian cells (Schweighoffer et al., 1991).

Compared to high rate of mutations in the viral genome, the genetic variability of the host is quite low, which makes host targeting agents attractive as they would impose a higher genetic barrier to the generation of resistant viruses (Kumar et al., 2011b). In this regard, various host's kinases have been targeted towards the development of new antiviral therapeutics (De Clercq, 2010), though such studies are still in preclinical stages. Since emetine is believed to affect host cell functions, we anticipated that it should not have a tendency to generate drug-resistant virus variants. Recent studies have shown the antiviral efficacy of emetine against Dengue virus, human immunodeficiency virus type 1 (HIV-1) and cytomegalovirus (Chaves Valadao et al., 2015; Deng et al., 2007; Mukhopadhyay et al., 2016; Ramabhadran and Thach, 1980). In the current study, we have extended the antiviral efficacy and mechanistic insights of emetine against other DNA and RNA viruses and studied the potential development of drug-resistant virus variants on long-term passage of DNA (BPXV) and RNA (NDV) viruses in the presence of emetine.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney (Vero) and Madin-Darby bovine kidney (MDBK) cells available at National Center for Veterinary Type Cultures (NCVTC), Hisar were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, USA) and antibiotics (Penicillin and Streptomycin). Vero cell-adapted Newcastle disease virus (NDV), Accession (Number, VTCC-AVA155), egg-adapted NDV Accession (Number, VTCC-AVA179), buffalo poxvirus (BPXV), Accession (Number, VTCC-AVA90), peste des petits ruminant virus (PPRV) Accession (Number, VTCC-AVA154), and bovine herpesvirus 1 (BHV-1) Accession (Number, VTCC-AVA14) available at NCVTC, Hisar were used in this study. Specific pathogen free (SPF) embryonated chicken eggs were procured from Indovax Pvt. Ltd., Hisar, India. All the viruses listed above were grown in Vero cells, except for BHV-1 which was grown in MDBK cells. The viruses listed above were titrated by plaque assay (Kumar et al., 2016), except that the egg-adapted NDV was titrated by hemagglutination assay (HA) (Kumar et al., 2016).

2.2. Determination of the cytotoxicity (MTT assay) and virucidal activity

The cytotoxicity (Kumar et al., 2008) and virucidal activity (Kumar et al., 2011b) of emetine were determined using previously described assays.

2.3. Egg lethal dose 50 (LD_{50})

LD_{50} of emetine was determined in SPF embryonated chicken eggs of 10th day by inoculating them with five-fold serial dilutions of the virus (concentration ranging from 5000 to 1.6 ng/egg) or PBS (vehicle-control), in triplicates, in a total of 100 μl volumes via allantoic route. Eggs were visualized for the viability of the embryos up to five days post-inoculation to determine the LD_{50} by the Reed-Muench method.

2.4. Egg infective dose 50 (EID_{50})

EID_{50} of BPXV was determined by inoculating 100 μl of serial 10-fold dilution of the virus [stock of 10^7 plaque forming unit (pfu)/ml], in triplicates, in embryonated SPF chicken eggs of 9–10th day via chorioallantoic membrane (CAM). At 5 days post-infection (dpi), eggs were visualized for pock lesions on its CAM. Development of pock lesions on CAM and/or death of the embryo were considered as mortality to determine EID_{50} by the Reed-Muench method.

For determination of EID_{50} for NDV, the serial 10-fold dilutions of the virus were inoculated, in triplicates, in SPF embryonated eggs of 10th day via allantoic route and the eggs were observed daily for the viability of the embryo for a period of 5–7 days. The EID_{50} was determined by the Reed-Muench method. The protective efficacy (duration of survival) of emetine against NDV infection was analyzed by Log-rank (Mantel-Cox) test using GraphPad Prism 7.02 (GraphPad Software, http://en.freedownloadmanager.org/Windows-PC/GraphPad-Prism.html).

2.5. In ovo antiviral efficacy of emetine against BPXV and NDV

SPF embryonated chicken eggs, in triplicates, were inoculated with 5-fold serial dilutions of emetine (50–0.4 ng/egg in 100 μl PBS) via CAM route, followed by infection with BPXV/NDV at 100 EID_{50}. On 5–7 dpi, eggs were visualized for the mortality of the embryos (NDV) or development of pock lesions and/or death of the embryos (BPXV). Effective concentration 50 (EC_{50}) was determined by the Reed-Muench method.

2.6. Time-of-addition assay

Emetine pre-treated (for 0.5 h) or untreated Vero/MDBK cells were infected with respective virus(es) at multiplicity of infection (MOI) of 5 for 1 h followed by washing with PBS and addition of fresh medium. Emetine (200 nM) was periodically applied across the life cycle of the virus. The infectious BPXV, BHV-1, PPRV and NDV, released in the cell culture supernatant respectively at 36 hpi, 12 hpi, 48 hpi, and 10 hpi, were quantified by plaque assay.

2.7. Entry assay

The effect of emetine on viral entry was assessed using a previously described assay (Khandelwal et al., 2014). Briefly, Vero or MDBK cell monolayers were pre-chilled to 4 °C and infected with the respective viruses at MOI of 5 in the emetine-free medium for 1 h at 4 °C to permit attachment, followed by washing and addition of fresh medium containing emetine or vehicle-control. Entry was allowed to proceed at 37 °C for 1 h after which the cells were washed again with PBS to remove any extracellular viruses and incubated with cell culture medium without any inhibitor. The progeny virus particles released in the cell culture supernatants in the treated and untreated cells were titrated by plaque assay.

2.8. Endogenous viral polymerase assay

To demonstrate the direct inhibitory effect of emetine on virion functions, we performed a cell free virion polymerase assay described elsewhere (Aguiar et al., 2007). Briefly, virus particles were purified by ultracentrifugation and resuspended in serum and antibiotic free MEM. Aliquots of purified virus particles were incubated with emetine or vehicle control for 30 min. Viral polymerase was activated by adding various concentrations of dNTPs and 10 mM of $MgCl_2$ in a final volume of 50 µl for 2 h at 37^{O}C. The enzymatic activity was terminated by addition of 22 µl of stop solution (10 mM Tris-HCl, pH7.4, 10 mM EDTA, 50 mg/ml proteinase K). Levels of viral RNA/DNA were measured by qRT-PCR described elsewhere in the manuscript.

2.9. Quantitative real-time RT-PCR

The levels of viral RNA/DNA in the virus-infected cells were measured by quantitative real-time PCR (qRT-PCR). Protocol and sequences of the primers used for amplification of specific viral genes are provided in the supplementary data (Supplementary file 1).

2.10. Western blot analysis

Confluent monolayers of Vero cells in 30 cm cell culture dishes were infected with NDV or BPXV for 2 h, followed by washing with PBS and addition of fresh medium. Cell lysates were prepared in RIPA buffer (Kumar et al., 2011a) and subjected to Western blot analysis to probe the viral proteins. β-actin protein was used as housekeeping control. Anti-NDV and anti-BPXV sera were available in our laboratory. Mouse anti-β-actin primary antibody, Goat Anti-Rabbit IgG HRP and Anti-Mouse IgG (whole molecule)-Alkaline Phosphatase conjugates were procured from Sigma (St. Louis, MO, USA).

3. Results

3.1. In vitro antiviral efficacy of emetine

Emetine at concentrations of ≤400 nM had minimal to no effects on the viability of both Vero (Fig. 2a) and MDBK cells (Fig. 2b), though at higher concentrations (≥2000 nM), it was found to be quite toxic to the cells (Fig. 2a and b). A non-cytotoxic concentration of 200 nM was used for various assays described throughout the

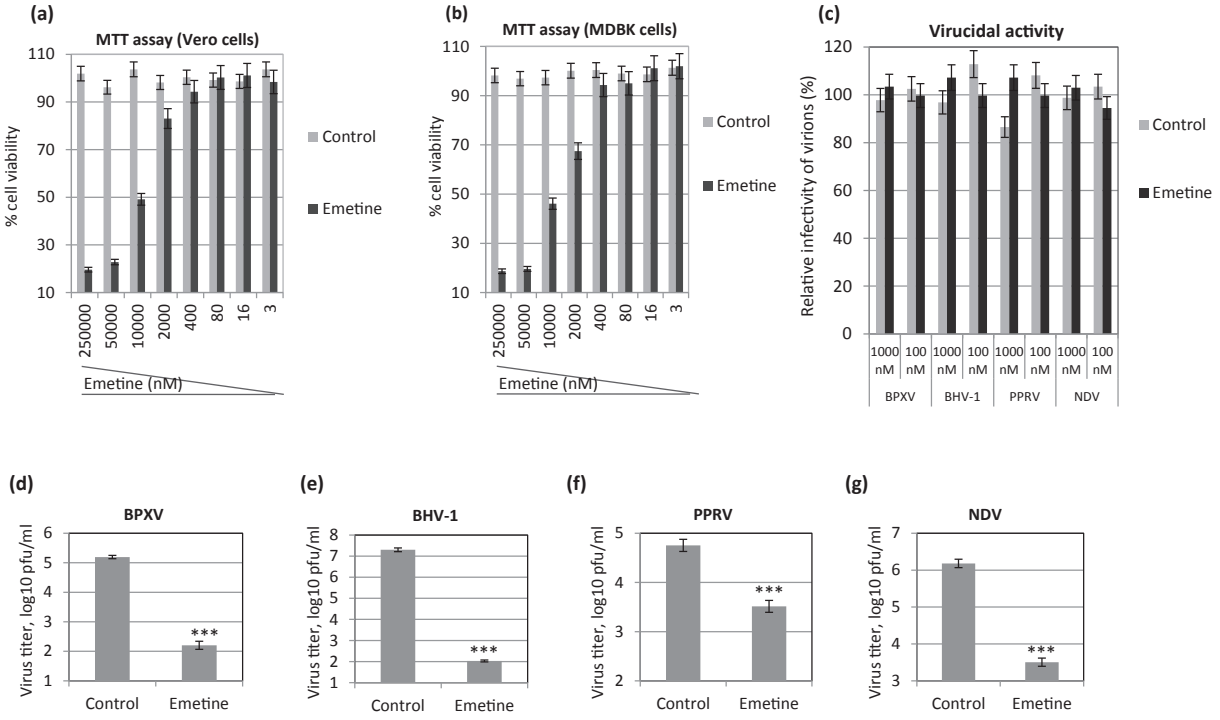


Fig. 2. ***In vitro*** **antiviral efficacy of emetine.** ***Determination of the cytotoxicity (MTT assay):*** Indicated concentrations of emetine or equivalent volumes of vehicle control (PBS) were incubated with cultured Vero **(a)** or MDBK **(b)** cells for 96 h and percentage of cell viability was measured by MTT assay. ***Virucidal activity:*** Indicated concentrations of the emetine or equivalent volumes of vehicle control were mixed with the respective virus and incubated for 90 min at 37 °C after which virus was diluted (1/000) and relative infectivity was determined by plaque assay **(c).** ***In vitro antiviral efficacy of emetine:*** Vero cells were infected with respective virus at MOI of 0.1 in the presence of 200 nM emetine or vehicle-control. The virus particles viz; BPXV **(d)**, BHV-1 **(e)**, PPRV **(f)**, BHV-1 **(g)** released in the cell culture supernatants were quantified by plaque assay. Error bars indicate SD. Pair-wise statistical comparisons were performed using Student's *t*-test (*** = P < 0.001).

current study. To analyze the effect of emetine on cell free virions, infectious virions were incubated with indicated concentrations of emetine followed by determination of the residual infectivity by plaque assay. As shown in Fig. 2c, viral titers were comparable in emetine and vehicle control-treated groups suggesting that emetine has no virucidal effect on the cell-free virions at these concentrations. To determine the *in vitro* antiviral efficacy of emetine, the yields of infectious virions were measured in the presence of emetine or vehicle-control. At a noncytotoxic concentration (200 nM), emetine was found to significantly inhibit replication of BPXV (Fig. 2d), BHV-1 (Fig. 2e), PPRV (Fig. 2f) and NDV (Fig. 2g), suggesting its broad-spectrum antiviral efficacy against these viruses.

3.2. Effect of emetine on specific steps of the viral life cycle

Before evaluating the effect of emetine on viral life cycle, we first analyzed one-step growth curve of the individual virus, where virus released from the infected cells to the culture supernatant at various times post-infection was quantified. In agreement with previous observations (Kumar et al., 2016), a sharp rise in BPXV, BHV-1, PPRV and NDV titers were observed at ~24 hpi (Fig. 3a), ~9 hpi (Fig. 3b), ~24 hpi (Fig. 3c) and ~8 hpi (Fig. 3d), respectively, suggesting the formation of progeny virus particles at these time points and hence the completion of one full virus life cycle. To evaluate the effect of emetine on specific step(s) of the viral life cycle, a time-of-addition assay was performed, where emetine was added at different times post-infection and the resultant virus particles released into the supernatant were quantified by plaque assaying. For BPXV, a similar level of inhibition in viral titers was observed when emetine was applied either at 0.5 h prior to infection or at 3 hpi suggesting that inhibitory effects are unlikely to occur at very early steps of virus replication (Fig. 3e). However, when applied at later time points (i.e., at 6 hpi, 12 hpi, 18 hpi and 30 hpi), similar levels of inhibition were not achieved, suggesting that later stages of virus replication may be affected. Similar findings were recorded with respect to BHV-1 (Fig. 3f), PPRV (Fig. 3g) and NDV (Fig. 3h). Taken together, we conclude that emetine may not

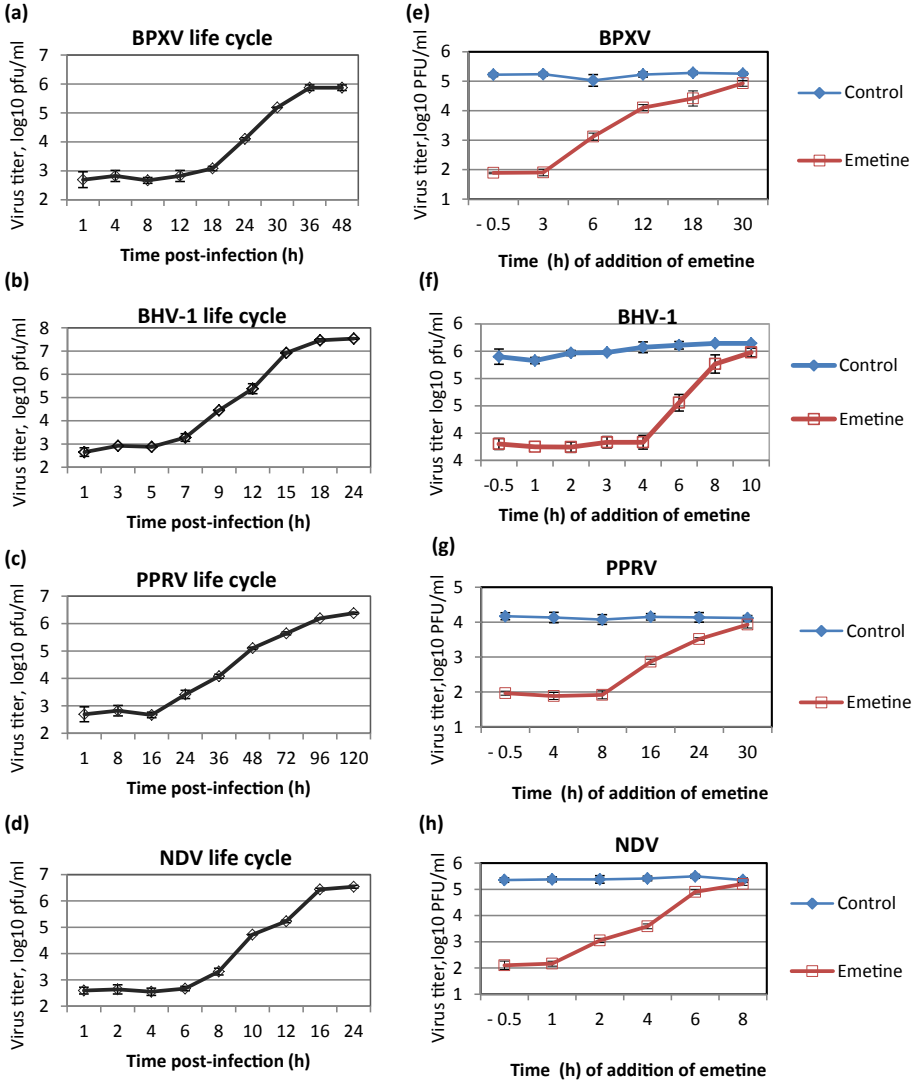


Fig. 3. Effect of emetine on specific steps of viral life cycle. ***One-step growth curve***: Confluent monolayers of Vero/MDBK cells were infected with indicated virus, in triplicates, at MOI of 5, washed with PBS and fresh media was added. Infectious progeny virus particles released in the cell culture supernatant at indicated time points were quantified by plaque assay. Growth curves of BPXV **(a),** BHV-1 **(b),** PPRV **(c)** and NDV **(d)** are shown. ***Time-of-addition assay***: Vero/MDBK cells were infected, in triplicates with indicated virus at MOI of 5, washed with PBS followed by addition of fresh medium containing either 200 nM of emetine or vehicle-control at indicated time points. The infectious BPXV (e), BHV-1 (f), PPRV (g) and NDV (h) released in the cell culture supernatant, respectively at 36 hpi, 12 hpi, 48 hpi, and 10 hpi, were quantified by plaque assay.

inhibit very early steps of the viral life cycle but that the middle-to-late stages of viral cycle may be affected. In attachment-specific assay, we did not observe any difference in the viral titers between emetine and vehicle-control-treated cells in any of the prototype virus tested (Supplementary data, Fig. S1), which further confirmed that emetine did not inhibit virus attachment to the host cells.

To determine whether the pre-attached virus was able to enter the cells in the presence of emetine, a standard entry assay was performed. As compared to the vehicle control-treated cells, no significant reduction in the virus yield was observed in the emetine-treated cells with respect to BPXV (Fig. 4a) and PPRV (Fig. 4b), though a significant inhibition was observed on the entry of BHV-1 (Fig. 4c) and NDV (Fig. 4d).

In order to determine the effect of emetine on the synthesis of viral genome, emetine was applied to the virus-infected cells when early steps of the virus life cycle (attachment/entry) were expected to occur (>2 h). As shown in Fig. 5, a highly significant ($P < 0.001$) reduction in relative viral RNA/DNA copy number (emetine-treated versus vehicle-control) was observed in cells infected with BPXV (Fig. 5a), BHV-1 (Fig. 5b), PPRV (Fig. 5c) and NDV (Fig. 5d), suggesting that emetine strongly inhibits synthesis of viral genome in infected cells.

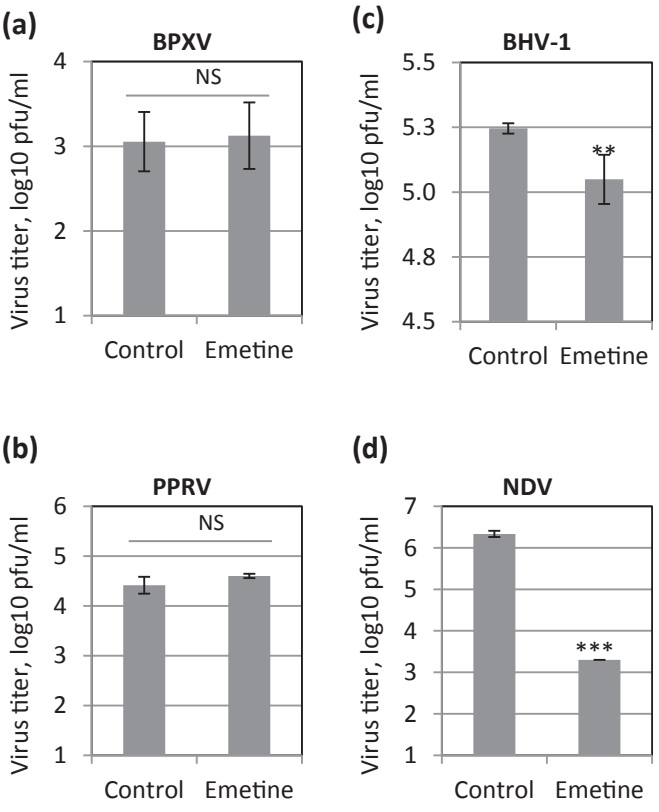


Fig. 4. Effect of emetine on virus entry. The confluent monolayers of Vero/MDBK cells were infected with indicated virus at MOI of 5 in emetine-free medium for 1 h at 4 °C to permit attachment. Thereafter, the cells were washed with ice-cold PBS to remove unattached virus and the fresh medium containing either 200 nM emetine or vehicle control were added. The entry was allowed to proceed at 37 °C for 1 h after which cells were washed again with PBS to remove any extracellular virus and incubated with the cell culture medium without emetine. The cell culture supernatants were titrated for the presence of infections virus particles in treated and untreated cells at 36 hpi, 48 hpi, 10 hpi and 12 hpi respectively for BPXV (**a**), PPRV (**b**), BHV-1 (**c**) and NDV-1 (**d**). Error bars indicate SD. Pair-wise statistical comparisons were performed using Student's *t*-test (** = $P < 0.01$,*** = $P < 0.001$). NS represents no statistical significance.

Emetine-induced decrease in synthesis of viral genome could be due to inhibition in viral protein synthesis. Nevertheless, Western blot analysis of cells infected with NDV (Fig. 5e, **upper panel**) and BPXV (Fig. 5f, **upper panel**) showed decreased levels of viral proteins in presence of emetine. However, there were similar levels of cellular housekeeping protein β-actin in emetine-treated and untreated cells (Fig. 5e and f, **lower panels**) suggesting that emetine does not lead to a general shut off/inhibition of cellular protein synthesis (at least at the concentration we employed).

Using a previously described endogenous polymerase assay, we further evaluated whether emetine can directly act to inhibit virion polymerase functions. In the cell free virion assay, dNTPs were found to induce viral RNA (NDV) synthesis in a dose dependent manner (Fig. 5g) which could be inhibited by emetine (Fig. 5h) at 10 μM but not at 0.2 μM (emetine at 0.2 μM severely blocked virus replication/nucleic acid synthesis in infected cells). In cell free virion polymerase assay with BPXV, dNTPs similarly induced viral DNA synthesis in a dose dependent manner (Fig. 5i), however no significant inhibitory effect of emetine was observed on synthesis of viral DNA (Fig. 5j). Though at a lower level (Ct values in the range of ~30, compared to virion in the range of ~20), we were able to amplify cellular β-actin gene from purified virus particles (data not shown) suggesting, in agreement with others (Shaw et al., 2008; Zhang et al., 2010), that cellular genes may be incorporated in virions, thereby allowing us to use β-actin (housekeeping gene) to normalize the amount of viral RNA/DNA in cell free virion polymerase assay. Moreover, no significant effect of dNTPs was observed in the levels of cellular β-actin gene (data not shown) suggesting that the dNTPs treatment specifically induced viral RNA/DNA synthesis. We also determined the virucidal effect (IC_{50}) of emetine at higher concentration (10 μM) where we observed minimal (~1.5–2 fold reduction respectively in BHV-1 and NDV) to no significant effect (PPRV and BPXV) on virion infectivity (data not shown) suggesting less efficient retention of emetine in the cell free virions; emetine probably lost while measuring residual infectivity of the virions by plaque assay. Taken together, it was concluded that in the virus infected cells, emetine treatment results in a highly significant decrease in synthesis of viral genome. Besides directly inhibiting specific viral polymerases, emetine may have some other targets that regulate replication of the viral genome.

We also analyzed the potential effect of emetine on the release (budding) of progeny virus particles from infected cells. However, no significant effects were observed on any of the viruses tested (Supplementary data, Fig. S2).

3.3. *In ovo* antiviral efficacy of emetine against BPXV and NDV

Members of the family *Poxviridae* can infect chicken embryo and cause distinct visible lesions (pocks) on CAM of embryonated chicken eggs (Gilhare et al., 2015). We successfully exploited this model to evaluate the efficacy of emetine against BPXV. Inoculation of BPXV produced button shaped, yellowish-white and slightly raised necrosed areas of 2–5 mm in diameter (pock lesion), which could be easily counted. These small pock lesions later coalesced to form enlarged pock lesions of 0.5–2.0 cm in diameter. Embryos which exhibited clear pock lesions on its CAM were considered as infected. Among various concentrations (Fig. 6a) tested, BPXV with a titer of <10 pfu/ml did not produce any pock lesions on the CAM, though higher concentrations (>10 pfu/ml) produced typical pock lesions. Using the Reed-Muench method, the egg infective dose 50 (EID_{50}) was calculated as $10^{3.99}$ pfu/ml (Fig. 6a).

Next, we determined the lethal dose of emetine by inoculating it in SPF embryonated chicken eggs. As shown in Fig. 6b, mortality of the embryo was observed at emetine concentration ≥200 ng/egg but not at 40 ng/egg. The LD_{50} was determined to be 126.49 ng/egg.

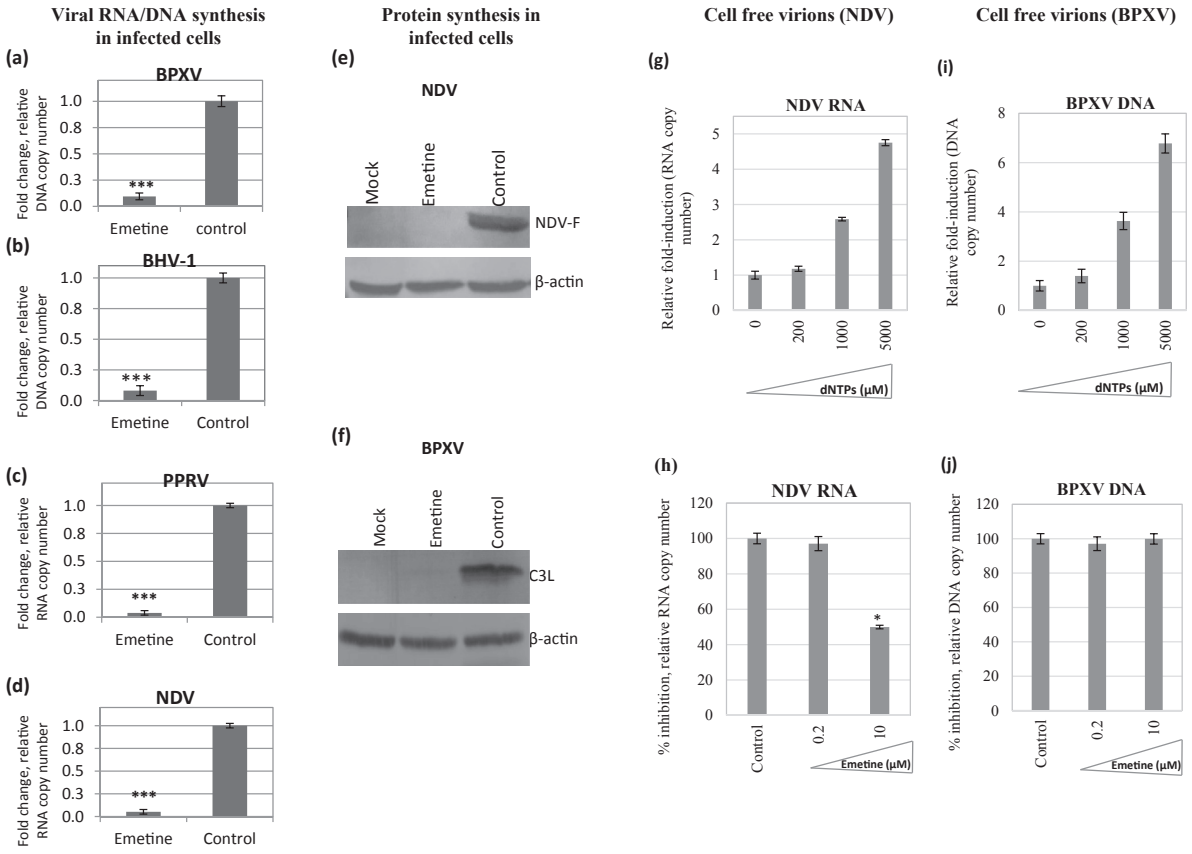


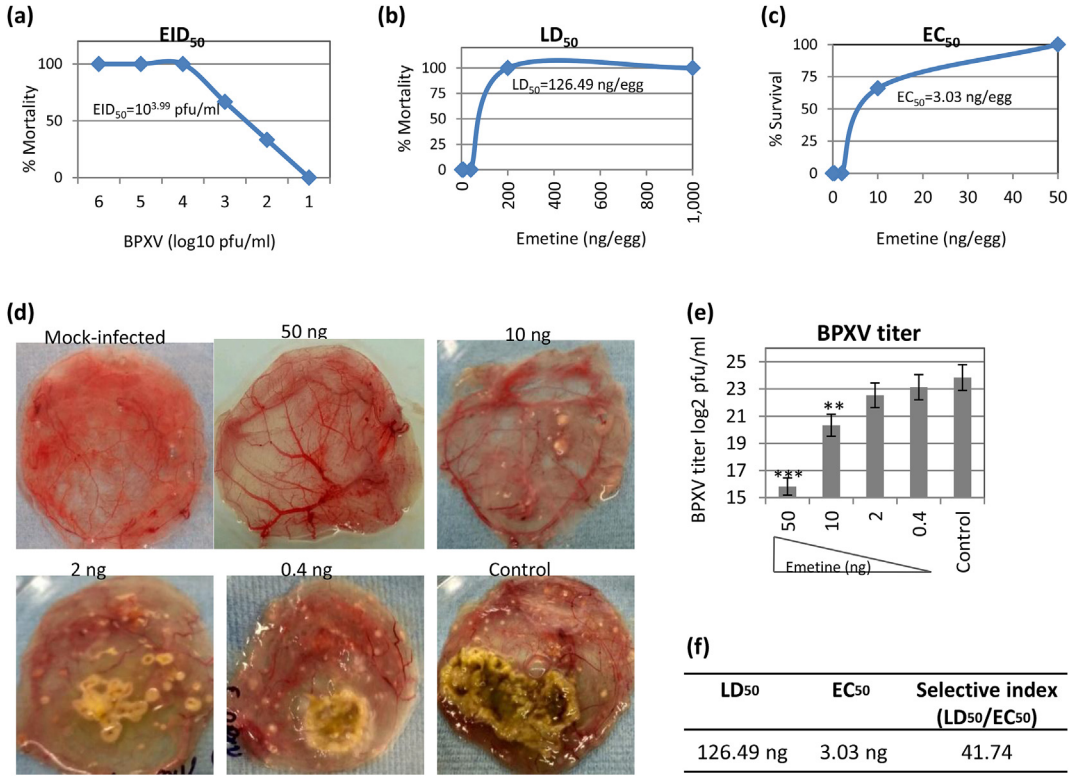
Fig. 5. Effect of emetine on synthesis of viral genome and proteins. ***Viral RNA/DNA synthesis in infected cells:*** Confluent monolayers of Vero/MDBK cells were infected with indicated virus, in triplicates, for 2 h at MOI of 5. Emetine was added at 2 hpi, 2 hpi, 6 hpi and 2 hpi respectively for BPXV, BHV-1, PPRV and NDV. Cells were scrapped for isolation of total RNA/DNA at 30 hpi, 8 hpi, 36 hpi and 8 hpi, respectively for BPXV, BHV-1, PPRV and NDV. Cell lysates were tested for virus-specific genomes by qRT-PCR. The viral RNA/DNA levels, expressed as threshold cycle (CT) values, were analyzed to determine relative fold-change (emetine-treated versus untreated cells) in copy number of BPXV **(a),** BHV-1 **(b),** PPRV **(c)** and NDV **(d)** genome. ***Protein synthesis:*** Vero cells were infected with NDV/BPXV at MOI of 1 followed by washing with PBS and addition of fresh media. Cell lysates were prepared at 24 hpi to detect the viral proteins by Western blot analysis. Representative pictures of three independent experiments for NDV F **(e, upper panel)**, BPXV C3L **(f, upper panel)** along with housekeeping β-actin protein (**e and f, lower panels**) are shown. ***Endogenous viral polymerase assay (cell free virions)***: Purified virion particles, in triplicates, were incubated with indicated concentrations of dNTPs to induce the viral polymerase and resulting replication of viral genome was measured by qRT-PCR. Relative NDV RNA synthesis (over 0 μM dNTPs) at various doses of dNTPs (**g**) and effect of emetine on NDV RNA synthesis (**h**) is shown. Similarly, for BPXV, relative viral DNA synthesis at various doses of dNTPs (**i**) and effect of emetine on viral DNA synthesis (**j**) is also shown. All the values were normalized with β-actin housekeeping control gene. Error bars indicate SD. Pair-wise statistical comparisons were performed using Student's *t*-test (* = $P < 0.05$,*** = $P < 0.001$).

For evaluation of anti-BPXV efficacy of emetine, eggs were infected with BPXV at 100 EID_{50} along with the indicated concentrations of emetine (Fig. 6c and d). As shown in Fig. 6c and d, no obvious pock lesions/embryonic mortalities were observed at emetine concentration of ≥10 ng. The lower concentration of emetine (<10 ng) could not prevent the development of pock lesions on CAM (Fig. 6d). Likewise, at a concentration of ≥10 ng, emetine was also found to significantly inhibit BPXV titer in the CAM in a dose dependent manner (Fig. 6e). At EC_{50} of 3.03 ng/egg, the therapeutic index (LD_{50}/EC_{50}) was determined to be 41.74 (Fig. 6f). Taken together, we conclude that emetine prevents the development of BPXV-induced pock lesions on CAM as well as the associated mortality.

For determination of EID_{50} of NDV, 10-fold serial dilutions of NDV (from a HA unit of 2048–0.002), in triplicates, were inoculated in the SPF eggs via allantoic route and observed daily for death of the embryos. The NDV was found to cause mortality usually within 72 hpi, the EID_{50} was determined to be 0.053 HA unit (Fig. 7a). The inoculation of emetine significantly ($P < 0.001$) delayed the NDV-induced mortality in chicken embryos (Fig. 7b) along with reduced viral titers in the allantoic fluid (Fig. 7c), though it could not provide complete protection against NDV challenge (Fig. 7b).

3.4. Selection of potential emetine-resistant viral mutants

In order to analyze the potential development of emetine-resistant virus mutants, BPXV and NDV were sequentially passaged *in vitro* in a culture medium containing emetine (100 nM) or vehicle-control. Viral supernatants were harvested at 72–120 h (depending on development of >50% cytopathic effect CPE) following each passage and were then used to re-infect a fresh culture of Vero cells, and the viral titer in each successive supernatant was quantified. Throughout the long-term passage, vehicle control-treated cells consistently supported high levels of BPXV (Fig. 8a) and NDV (Fig. 8b) production (~10^6 pfu/ml). The presence of emetine significantly reduced virus production by 1–2 log in BPXV (Fig. 8a) and 2–3 log in NDV infected cells (Fig. 8b) during successive passage from P0 to P25. Further, when we compared the original (P0), P25-emetine and P25-control viral stocks for their sensitivity to emetine inhibition, all showed similar levels of inhibition in viral titers (Fig. 8c, d), suggesting that the P25-emetine stock likely did not contain significant quantities of emetine-resistant virus mutants.



(f)

LD50	EC50	Selective index (LD50/EC50)
126.49 ng	3.03 ng	41.74

Fig. 6. Antiviral efficacy of emetine against BPXV *in ovo*. *EID*$_{50}$: Embryonated SPF chicken eggs, in triplicates were infected via CAM route at indicated concentration of BPXV. Eggs were visualized for development of pock lesions on CAM as well as associated mortality. EID$_{50}$ was determined by the Reed-Muench method **(a)**. ***LD*$_{50}$**: Embryonated SPF chicken eggs, in triplicates were inoculated with indicated concentration of emetine via CAM route. At 5 days post-emetine inoculation, eggs were visualized for viability of the embryo. LD$_{50}$ was determined by the Reed-Muench method **(b)**. ***EC*$_{50}$**: Embryonated SPF chicken eggs, in triplicate were inoculated with indicated concentration of emetine along with infection of BPXV at 100 EID$_{50}$ via CAM route. At 5 days post-emetine inoculation, eggs were visualized for viability of the embryo and/or development of pock lesions on CAM. EC$_{50}$ was determined by the Reed-Muench method **(c).** Typical pock lesions following BPXV infection and its resolution by emetine (**d**), BPXV titers in CAM at various drug-regimes (**e**) as well as selective index (**f**) are also shown. Error bars indicate SD. Pair-wise statistical comparisons were performed using Student's *t*-test (** = P < 0.01,*** = P < 0.001).

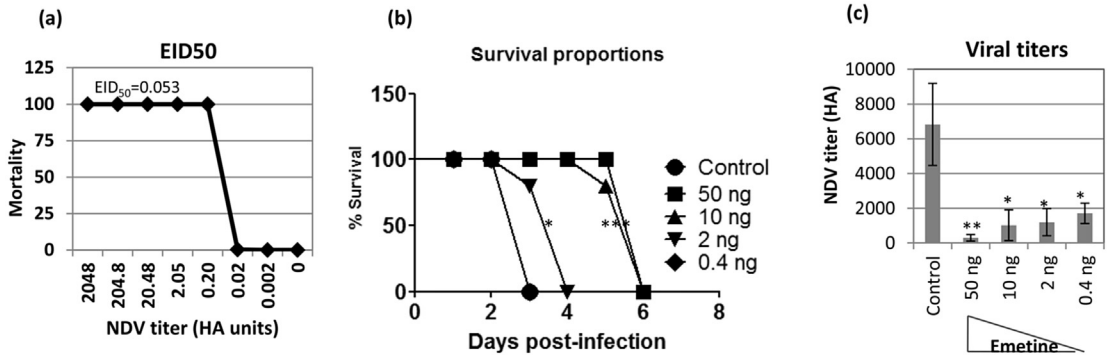


Fig. 7. Antiviral efficacy of emetine against NDV *in ovo*. Embryonated SPF chicken eggs, in triplicates, were infected with serial 10-fold dilutions [from hemagglutinin (HA) unit of 2048–0.002] of NDV and observed daily for death of the embryo followed by determination of EID$_{50}$ by the Reed-Muench method **(a)**. Duration of survival of chicken embryos following NDV challenge as determined by Kaplan-Meier (survival) curve is shown (**b**). Statistical comparisons in survival curve were made using Log-rank (Mantel-Cox) Test using GraphPad Prism 7.02. Viral titers in allantoic fluid at different drug regimes following NDV challenge are also shown **(c).** Pair-wise statistical comparisons of viral titers (emetine-treated and untreated eggs) were performed using Student's *t*-test. * = P < 0.05, * = P < 0.01, *** = P < 0.001.

4. Discussion

Over the years, a range of antiviral agents that directly interfere with viral protein functions were developed (Chacko and Gaglio, 2015; De Clercq, 2010) for human use, however, currently there is no antiviral agent approved to treat viral infections in the livestock sector. It is in this context that we screened for potential antiviral activity of a diverse library of kinase and phosphatase inhibitors for the development of antiviral therapeutics. One such compound-emetine - was found to inhibit replication of poxviruses (BPXV, sheeppox and goatpox viruses), BHV-1, PPRV and NDV suggesting that it has the potential to be developed as a broad-spectrum antiviral drug to treat viral infections in livestock.

A possible inhibitory mechanism of emetine on viral genome replication could be due to direct inhibition of viral protein synthesis. In Western blot analysis we observed that emetine blocked the synthesis of the viral proteins in infected cells. Since virus replication requires the presence of the viral structural and non-

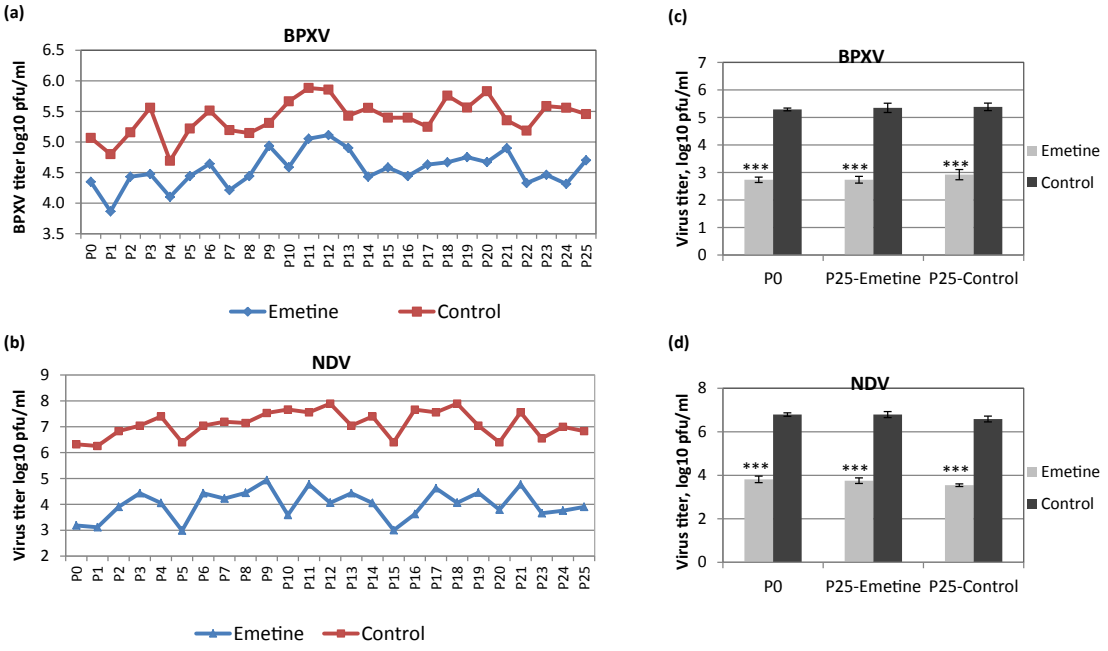


Fig. 8. Selection of potential emetine-resistant virus mutants on long-term *in vitro* culture. BPXV and NDV were passaged in medium containing vehicle-control or 100 nM emetine. Each passage was carried out in fresh cells and with fresh aliquot of emetine but with virus from preceding passage. At each passage, BPXV **(a)** and NDV **(b)** were quantified by plaque assay. The viruses (NDV/BPXV) collected from the original stock (P0), P25-Emetine or P25-Control passages were used to infect Vero cells at an MOI of 0.1 with and without 200 nM emetine. The virus released in the supernatant at 48 hpi was quantified by plaque assay. Emetine-induced reduction in BPXV **(c)** and NDV **(d)** titers (passaged viruses) are shown. Error bars indicate SD. Pair-wise statistical comparisons were performed using Student's *t*-test (*** = $P < 0.001$).

structural proteins, their inhibition may eventually block viral genome replication (Aguiar et al., 2007; Chaves Valadao et al., 2015). We further evaluated the activity of viral polymerase in cell free virions assay where emetine was found to significantly inhibit replication of NDV (Fig. 5h) but not BPXV genome (Fig. 5j) suggesting that besides directly acting on certain viral polymerase, emetine may disrupt some other viral/cellular targets required for efficient replication of the viral genome.

Alternatively, emetine-induced inhibition in viral genome replication can also be explained by the fact that emetine is well known to inhibit biosynthesis of cellular proteins (Leatherman and Middlebrook, 1993; Lietman, 1971), some of these host proteins may be essential for the regulation as well as synthesis of the viral genome in the infected cells. As compared to the virus infected cells, in the cell free virion polymerase assay, we observed inhibitory effect of emetine at modest level (about two-fold instead of >20 fold in virus infected cell) even that at 50-fold higher concentration of emetine. Moreover, emetine did not directly affect BPXV polymerase. In addition, emetine was also found to inhibit at least NDV and BHV-1 entry. These observations tempted us to speculate that besides direct effect on specific viral polymerases, some host factors critically required for virus replication may also be targeted by emetine, a phenomena similar to an another broad-spectrum antiviral compound-Arbidol that has both direct (viral targets) and indirect (host targets) antiviral effects (Wisskirchen et al., 2014).

In agreement with our observations, in a previous study on HIV-1, the IC_{50} of emetine was also found to be 10–100 fold higher for cell free virions than cells, probably due to less efficient retention of emetine in the cell free virions (Aguiar et al., 2007). Additional studies that involve effect of emetine on viral polymerase activity in infected cells within a non-cytotoxic range, global transcriptome/proteome/phospho-proteome/lipidome analysis of emetine-treated and -untreated cells and molecular docking studies (emetine) are required to decipher the detail mechanism of the inhibitory role of emetine on virus replication.

Antiviral drug resistance is a matter of great clinical importance. The inherent ability of the viruses to rapidly develop drug-resistant virus mutants is a major challenge in developing antiviral drugs. Whereas other direct antivirus-acting agents are known to induce a completely resistant phenotype within 5–6 passages (Kumar et al., 2011b), no emetine-resistant NDV and BPXV mutant viruses were observed during long-term ($P = 25$) *in vitro* culture in the medium containing emetine, suggesting that resistance to emetine is unlikely to occur. Since host-targeting antiviral agents show very low tendency in generating drug-resistant virus variants (Kumar et al., 2011b), the reduced tendency of emetine in generating drug-resistant virus mutants might possibly be due to the indirect effects on virus replication (disrupting host factors that supports virus replications). Emetine might possibly be useful as a salvage therapy in clinical settings where the virus has developed resistance to other available drugs.

However, previous reports have shown that emetine can inhibit host's protein synthesis, which raises a potential difficulty of its use due to its potential cytotoxicity *in vivo*. Indeed, we observed that emetine did not induce any mortality at 100 ng/egg, although along with virus infection, a similar concentration leads to shorter survival than was seen in a PBS-treated control group, indicating it's *in vivo* toxic effects at higher dosage (data not shown). Due to the high selectivity index ($SI = 41.74$) of emetine in anti-BPXV activity, we expect that a useful range of concentrations can indeed be identified, though the optimal therapeutic strategies may vary for different viruses and in different clinical settings.

Based on the data of this study, we speculate that emetine might prove to be especially useful for treating acute pathogenic virus infections, such as those caused by BPXV, PPRV, NDV as well as other viruses of zoonotic/biodefense interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2017.06.006.

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