Virus Research 260 (2019) 114–122

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Evaluation of imidazole and its derivative against Newcastle disease virus infection in chicken: A drug repurposing approach

Moushumee Das, Sangeeta Baro, Sachin Kumar*

Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, 781039, Assam, India

ARTICLE INFO

Keywords:
Repurposing
Newcastle disease virus
Imidazole
Pathogenicity
Replication

ABSTRACT

Despite recent progress in vaccination options, Newcastle disease virus (NDV) stands a severe global epidemic and economic burden. It is difficult to control NDV with vaccination alone due to its evolving genetic variability; therefore, an effective therapeutic must target to resist its replication and further evolution. Drug repurposing is a practical and economical method to develop therapeutics against pathogenic organisms. Applying the knowledge of the broadly used antimicrobial activity of imidazole and its derivatives, we performed repurposing-based design of therapeutics to induce protection against NDV. We checked the ability of the compound at sub-lethal doses to reduce NDV replication *in vitro*, *in ovo* and *in vivo*. Chickens treated with the repurposed drug produced antiviral type I interferon and showed no shedding of the virus. Successful designing of novel NDV drug, in this study empirically demonstrates the principle that repurposing can be used for developing antiviral therapeutics.

1. Introduction

Newcastle disease virus (NDV) is a widely present avian virus. The virus infects a wide range of birds (around 300 different species), both domestic and wild population (Kaleta and Baldauf, 1988). NDV has three pathotypes: lentogenic, mesogenic and velogenic. Virulent strains of NDV causes Newcastle disease (ND). There are no antiviral or therapeutics approved for treating NDV infection in birds. Since its first outbreak in 1926, at least four defined NDV pandemics were reported throughout the continents (Miller et al., 2014). Vaccination and biosecurity measures stay the only available preventive choices (Ganar et al., 2014). The greatest impact of ND is due to the high mortality rates in poultry followed by dwindling egg and meat production. With significant structural improvements in modern intensive production methods, genetic enhancements, improved preventive disease control, biosecurity measures and increasing urbanization, the poultry industry has made major contributions to the livestock production industry worldwide (Steinfeld et al., 2006). Big or small poultry production is a primary source of food and income in many developing countries, and outbreaks of NDV contribute to food insecurity. Significant trade restrictions and increase in the cost of production due to quarantines in outbreak areas drastically affect the economy of those countries.

NDV is single-stranded, nonsegmented, negative-sense RNA virus encoding for six structural proteins. It belongs to the family *Paramyxovirus,* genus *Avulavirus* and species *Avian avulavirus* 1 (Amarasinghe et al., 2007). NDV affects the neurological, gastrointestinal and respiratory systems of birds (Alexander, 2000). Unvaccinated or poorly vaccinated birds often display clinical signs. The severity of NDV varies depending on the species of bird, the strain of the virus and the immunity of the host (Lamb and Kolakofsky, 1996). Constantly evolving NDV strains are genetically divided into two classes, with one genotype of class I and 18 genotypes of class II (Diel et al., 2012; Snoeck et al., 2013). Employment of vaccination, rapid diagnostic assay and culling of the infected flock are the active management used against NDV outbreaks in developed countries.

It is obligatory to report to the OIE in case of a virulent or mesogenic NDV outbreak, resulting in restrictions and trade embargoes being placed on poultry or poultry products from that country. According to the OIE, ND is regarded as the fourth most devastating disease in poultry after highly pathogenic avian influenza, avian infectious bronchitis and low pathogenic influenza (OIE, 2012). ND was ranked 2nd after rabies, among the reported disease outbreaks from 2006 to 2009. Furthermore, several countries reported the NDV outbreaks in domestic poultry during the years 2008–2014 (Das and Kumar, 2017; Dimitrov et al., 2017; Miller et al., 2015). Such widespread distribution and recurrent outbreaks suggest that vaccination and bio-safety measures alone cannot effectively control the disease. In such paradigm, development of an anti-NDV drug could help animal scientist and field

* Corresponding author.
E-mail address: sachinku@iitg.ac.in (S. Kumar).

https://doi.org/10.1016/j.virusres.2018.11.013
Received 26 September 2018; Received in revised form 27 November 2018; Accepted 27 November 2018
Available online 30 November 2018

workers to control the situation.

In the modern age, with the ever-increasing database about the molecular basis of disease, several opportunities lie to translate research findings into new medicines. However, barriers to the therapeutic development processes produce holds and obstacles in the adaptation of a potential molecule into approved drugs. It is important to reduce this time frame, reduce costs and enhance success rates. In such scenario, drug repurposing, use of existing therapeutic to treat a new disease, bears the promise (Nosengo, 2016). Repurposing is a tempting and pragmatic procedure, given pre-approved knowledge regarding the toxicity, pharmacology and formulation. Although the development of a potential drug requires much cost and is tedious, fewer than 15% of these compounds receive approval, despite the majority of them being deemed safe. Since re-purposing embellish upon established research and development efforts, it could speed review by the Food and Drug Administration and, if approved, thereby integrating new targets into healthcare (Corsello et al., 2017). Drug repurposing screens have evolved as an alternative approach to speed up drug development (Pham et al., 2016). Following recent repurposing screen, potential drug candidate for Ebola virus (Kouznetsova et al., 2011), Giardiasis (Chen et al., 2011), amoebiasis (Debnath et al., 2012), hepatitis C virus (HCV) infection (Sun et al., 2013) and, very recently, ZIKV infection (Barrows et al., 2016) have been discovered.

Imidazole is the nitrogen-containing heterocyclic ring that occupies unique importance in biology and pharmacology. Derivatives of imidazole and their versatile properties have attracted considerable interests for more than a century in chemistry and pharmacology. This heterocyclic aromatic compound is polar and ionizable, serving the pharmacokinetic characteristics of lead molecules (Rastogi and Sharma, 1983). Imidazole derivatives offer enormous scope in the field of medicinal chemistry due to their available methods of synthesis and various structures (Boiani and Gonzalez, 2005). To increase egg production and control diseases, poultry producers extensively use imidazole derivatives in feed additives and drugs (Balloun et al., 1969; Mottier et al., 2006). These compounds have large antimicrobial spectra effective against most pathogens of veterinary interest such as *Candida* spp, *Paracoccidioides brasiliensis*, *Aspergillus* and *Penicillium* spp etc (Sheehan et al., 1999). Imidazoles drugs are known to have respectful pharmacokinetic features and are well distributed throughout the body on administration (Hennessy et al., 1983). Among the widely used imidazole drugs used for poultry include Levamisole, Clotrimoxazole, Fluconazole, Tetramisole, Febendazole *etc.*

In our present study, we have evaluated the effect of imidazole on NDV replication. Imidazole could successfully reduce NDV replication in chicken fibroblasts cells when treated with sublethal doses. The finding was further validated by *in-ovo* and chicken experiments wherein an imidazole derivative was shown to reduce NDV infection. Thus, our results suggest repurposing an anti-helminthic drug into potential antivirals in chickens. Besides, the importance of this study could be extended to analyze repurposing of imidazole against other infectious paramyxoviruses to achieve cost-effective and efficient treatment.

2. Materials and methods

2.1. Viruses, cells and compound

We obtained a mesogenic NDV (*m*NDV) strain R2B from the veterinary clinic, College of veterinary sciences, Guwahati, Assam. Recombinant NDV expressing GFP was used to analyze the fluorescent-based study. The recombinant virus was made using reverse genetics approach (available in the lab). The nine-day-old specific pathogen free (SPF) embryonated chicken eggs and the primary chicken embryo fibroblast (CEF) cells were used to propagate NDV. Hemagglutination (HA) titer of NDV stocks was determined using 1% chicken RBC at room temperature. Tissue culture infective dose ($TCID_{50}$) and plaque assay were performed using established protocols (Alexander et al., 1998).

CEF cells were prepared using nine-day-old chicken embryo under aseptic conditions following the standard protocol (Cunningham. Charles H, 1973). The chicken embryo fibroblast (DF1) and baby hamster kidney (BHK21) cells were obtained from ATCC, Manassas, USA. The CEF, DF1, and BHK21 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Invitrogen, USA) with antibiotics and antimycotics (antibiotics/antimycotics; 60 µg of penicillin, 10^5 µg of streptomycin, and 0.25 µg of amphotericin B per ml) (Sigma, USA). The cells were maintained in a 37 °C incubator with 5% CO_2.

A molecular grade imidazole stock solution of 1 M was prepared and filtered for further use (SRL, India). The CEF cells were treated with a serial two-fold dilution of imidazole (starting from 400 mM) and the toxicity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay, 48 h (h) post-treatment. The MTT assay is a colourimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes in live cells are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple colour (Freimoser et al., 1999). Formazan is soluble in DMSO and can be quantified by taking absorbance at 490 nm. Furthermore, a molecular grade albendazole (Sigma, USA) stock solution of 100 mM was prepared and filtered for further use. MTT assay of albendazole was done in DF1 cells. DF1 cells were treated with a sub-lethal dose of imidazole and its effect was observed under the inverted microscope. The albendazole (benzyl derivative of the imidazole) was used for chicken experimentation as per the recommended dose of 5 mg/ kg body weight.

2.2. Infectivity assays and virus titration

CEF cells were infected with *m*NDV and its recombinant expressing GFP. The *m*NDV infection was done in three sets of CEF cells: first treated with a sublethal concentration of imidazole before *m*NDV adsorption, second cells treated during adsorption of the virus, and third treated after *m*NDV adsorption. In all the sets, CEF monolayer was infected with 5×10^5 PFU of *m*NDV for 1 h of adsorption and then washed with PBS thrice to remove the residual virus. The monolayer was then overlaid with 2% DMEM containing 10% fresh sterile allantoic fluid. Treatment of CEF was done with 10 mM, 20 mM, 30 mM and 40 mM of imidazole in all the sets.

To check the effects of imidazole on NDV replication, the infected cells were observed daily for the cytopathic effects (CPE), GFP fluorescence and HA titer was recorded every 24 h until the fifth day post-infection. The *m*NDV titer was quantified by plaque assay in BHK21 cells as described previously (Krishnamurthy et al., 2000). The cells were overlaid with DMEM containing 2% FCS and 0.8% methylcellulose (Sigma, USA). The plaques were visualized by staining with 1% crystal violet fourth day post-infection. Besides, the $TCID_{50}$ titer was calculated using Reed and Muench algorithm (Hierholzer and RA, 1996). The titer of recombinant NDV expressing GFP was monitored under the inverted fluorescent microscope in infected CEF cells. Furthermore, the GFP expression in the different treatment groups was measured by flow cytometry (FACS Calibur BD Biosciences USA).

Total RNA from *m*NDV -infected CEF cells was isolated using TRIZOL reagent, following the manufacturer's instructions (Invitrogen, USA), for its quantification at the transcriptome level. The cDNA was prepared using random hexamers and reverse transcription-PCR (RT-PCR) amplification was performed using NDV gene-specific primers (Kumar and Kumar, 2015). GAPDH was amplified for each of the experimental samples as an internal control.

Western blot experiment was performed for the expression analysis of the viral protein in the different experimental sets (Nayak et al., 2010). Briefly, the infected cells were harvested by trypsinization and lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Invitrogen, USA). Antibodies used were anti–NDV polyclonal (1:5000;

hyperimmune sera raised in chickens) or anti-beta actin (Life Technologies, USA). HRP conjugated secondary antibody was used for all the preparations and signals were detected using ECL chemiluminescence (Novex, USA).

Similar time of addition experiments was conducted to check the effect of sub-lethal dose of albendazole on replication of NDV in DF1 cells.

2.3. In ovo study

Two different experiments with imidazole and albendazole were conducted in embryonated chicken eggs to check antiviral effect. Nine-day-old embryonated chicken eggs were grouped into six groups: uninfected control, drug control, *m*NDV control, treated with drug 12 h before *m*NDV infection, co-treated with drug and infected with *m*NDV, and treated with drug 12 h post-infection. All the sets were infected with a 10^{-5} dilution of 2^9 HA units (~ to 3×10^5 PFU/ml) of egg-grown *m*NDV and 30mM of imidazole and albendazole. The eggs were incubated at 37 °C incubators with regular shaking for 36 h. The growth of *m*NDV in all the sets was determined using HA titer and the presence of pathological lesions in the embryos. The viral titer was also determined using plaque assay in BHK21 cells as described earlier.

2.4. Chicken experiment

The treatment and infectivity studies were further performed in bird model. The SPF birds were housed in isolators in the animal facility at the Khanapara veterinary college, Guwahati, Assam. All the animal experiments were performed following proper protocol of animal ethics, four groups (n = 12 per group) of two week SPF chicks were taken. Albendazole (5 mg/kg body weight) was administered orally 12 h pre-infection, 12 h post-infection and during infection of *m*NDV in three representative groups. The fourth group was taken as *m*NDV infection control. In addition, ten birds were kept as uninfected and untreated control and another eight birds as albendazole control. The first three groups and the infection control group were infected with 10^3 $TCID_{50}$ titer/ml of *m*NDV. Oral and cloacal swabs were obtained on day third and fourth post-infection to measure the *m*NDV shedding. The presence of *m*NDV in the swab samples were determined by its inoculation in embryonated chicken eggs and performing HA assay. From each group, three birds were sacrificed on day 1, 2, 3 and 4 post-infection to quantify the *m*NDV replication. In all the collection a 12 h difference was maintained as per the treatment schedule. The trachea, lungs and spleen in the post-mortem birds were checked for *m*NDV specific pathology. The tissue was fixed in formalin and stained using haematoxylin and eosin following the standard protocol (Culling, 1974a, b). The *m*NDV replications in these tissues were further quantified using qPCR against NDV genes. Total RNA from the tissue samples were isolated and cDNA prepared using random hexamers (as described below).

2.5. Analysis of innate immune response to imidazole treatment

We quantified immune modulator activity of imidazole and its benzyl derivative, albendazole in primary CEF cells and two-week-old chickens, respectively. In the first experiment, the expression of type I interferon (IFN) was checked on *m*NDV infected CEF cells treated with 30 mM imidazole and compared against mock-infected and virus-infected cells. CEF monolayer was overlaid with DMEM containing 30 mM of imidazole and incubated at 37 °C CO_2 incubators for 48 h post-infection with *m*NDV (as mentioned earlier). For chicken experiments, blood samples were collected from the brachial veins from all the groups. PBMCs were isolated from the collected blood samples using histopaque 1077 (Sigma, USA) (Kaiser et al., 2006).

Total RNA from PBMCs was isolated using TRIZOL, following the manufacturer's instructions (Invitrogen, USA). The cDNA was prepared using random hexamers and quantification of type I IFN gene expression was done using real-time PCR. cDNA was prepared using Mu-MLV Reverse Transcriptase (Applied Biosystem, USA). Gene-specific primers were designed against IFN alpha (IFNα), IFN beta (IFNβ) genes to quantify its expression (Applied Biosystems). All real-time PCR reactions were performed using the SYBR Green PCR master mix (Applied Biosystems, USA) in ABI 7500 Fast Real-Time PCR (Applied Biosystems, USA). The thermal cycling conditions were 50 °C for 2 min followed by an initial denaturation step at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. For each data point, experiments were carried out in triplicate, and the relative gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Moreover, the fold change in the gene expression was normalized with an internal control gene (GAPDH). An independent sample *t*-test was done to statistically signify the differences in fold change in IFN gene expression in the different experimental groups. The experimental data were analysed using Microsoft Excel, p-value less than 0.05 was considered significant.

3. Results

3.1. Cytotoxicity assay of imidazole and albendazole

Imidazole, a potent pharmacokinetic compound, was found to have 50% survival concentration values of 50 mM in CEF cells (Fig. 1a). No toxicity was observed in CEF cells at concentrations below 1 mM. The compound showed increasing cytotoxicity in CEF, in the form of vacuolation when treated in a dose-dependent and temporal manner (Fig. 1b). The cell toxicity was linearly related to drug concentration when treated from 10 mM to 40 mM concentration. Furthermore, 50% survival concentration value for albendazole was found to be 40 μM in DF1 cells (Fig. 1c) and its toxicity was temporal and dose-dependent (5 μM to 9 μM) (Fig. 1d).

3.2. Antiviral activity of imidazole and albendazole

Imidazole treatment along with *m*NDV infection was done in CEF monolayer as represented schematically (Fig. 2a). Imidazole treatment at a post-entry step reduced replication of *m*NDV in CEF monolayer by two-fold. The reduction in virus replication was evaluated by plaque assay and GFP expression which showed a reduction of two- and five-fold titers, respectively (Fig. 2b). While in the *m*NDV control group the viral plaques were quantified to be 23×10^5 PFU, in the imidazole treated groups it was approximately 12×10^5 PFU. We observed a dose-dependent reduction in NDV titre in imidazole treated groups using GFP marker as a read-out. A gradual two-fold decrease in virus titer was observed (Fig. 2c). The graphical representation of the reduction in viral titres in the plaque assay and $TCID_{50}$ experiments are shown in Fig. 2d. We conducted experiments to check dose-dependent antiviral activity of imidazole, using independent approaches at transcriptional and translational levels. The expression of intracellular *m*NDV genome showed the reduction in the presence of imidazole. The presence of imidazole suppressed production of infectious *m*NDV by two-fold (Fig. 2e). Western blot analysis showed a two-fold reduction in *m*NDV protein as compared to beta-actin (Fig. 2f). In both cases, CEF cells were treated with an increasing concentration of imidazole from 10 to 40 mM. Besides this, FACS analysis for imidazole treated cells showed almost complete fold reduction in NDV-GFP infected cells, when compared to virus control (Fig. 2g). Moreover, in DF1 cells reduction in GFP expressing NDV infection was clearly visible on treatment with 9 μM albendazole (Fig. 2h). This result was further supported in the plaque assay and $TCID_{50}$ assay (Fig. 2i). About two fold reduction was seen in DF1 cells treated with albendazole post *m*NDV infection. This finding was further supported by western blot (Fig. 2j).

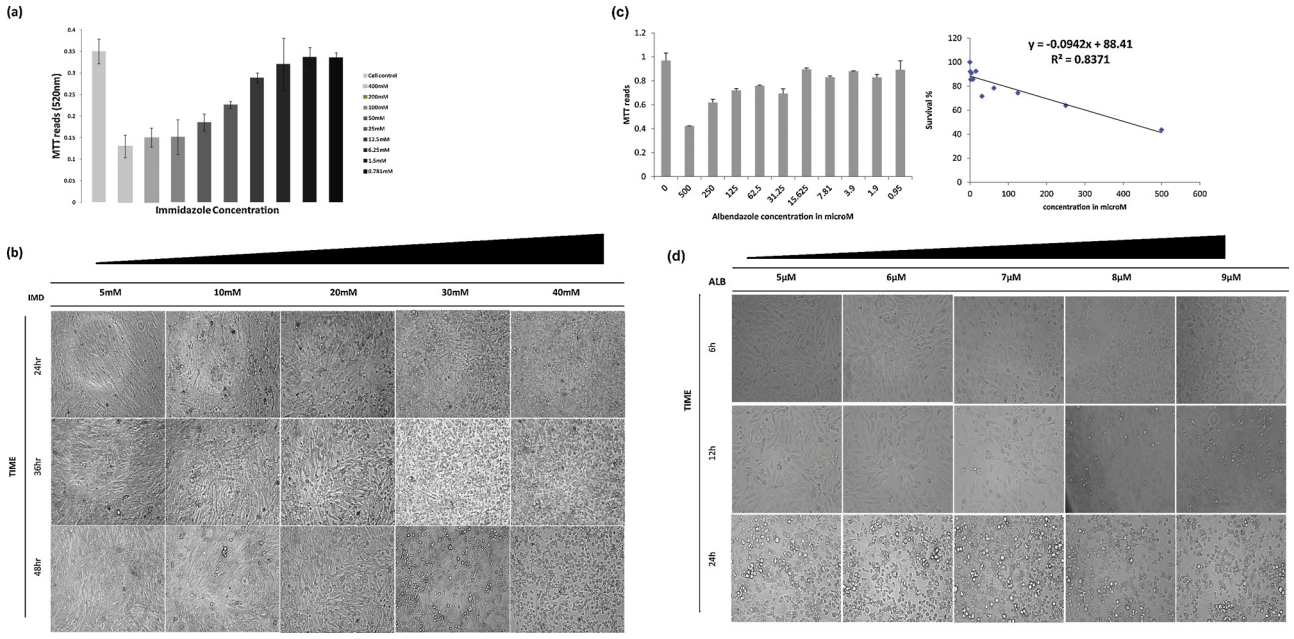

Fig. 1. Toxicity assay for imidazole in CEF cells. Graph representing the determination of 50% survivality using MTT assay (a). The CEF cell monolayer treated in ascending order of sub-lethal concentrations of imidazole (5, 10, 20, 30 and 40 mM) (b). Toxicity assay of albendazole in DF1 cells. Graph representing the determination of 50% survival using MTT assay (c). The DF1 cell monolayer treated in ascending order of concentrations of albendazole (5, 6, 7, 8 and 9 μM) (d).

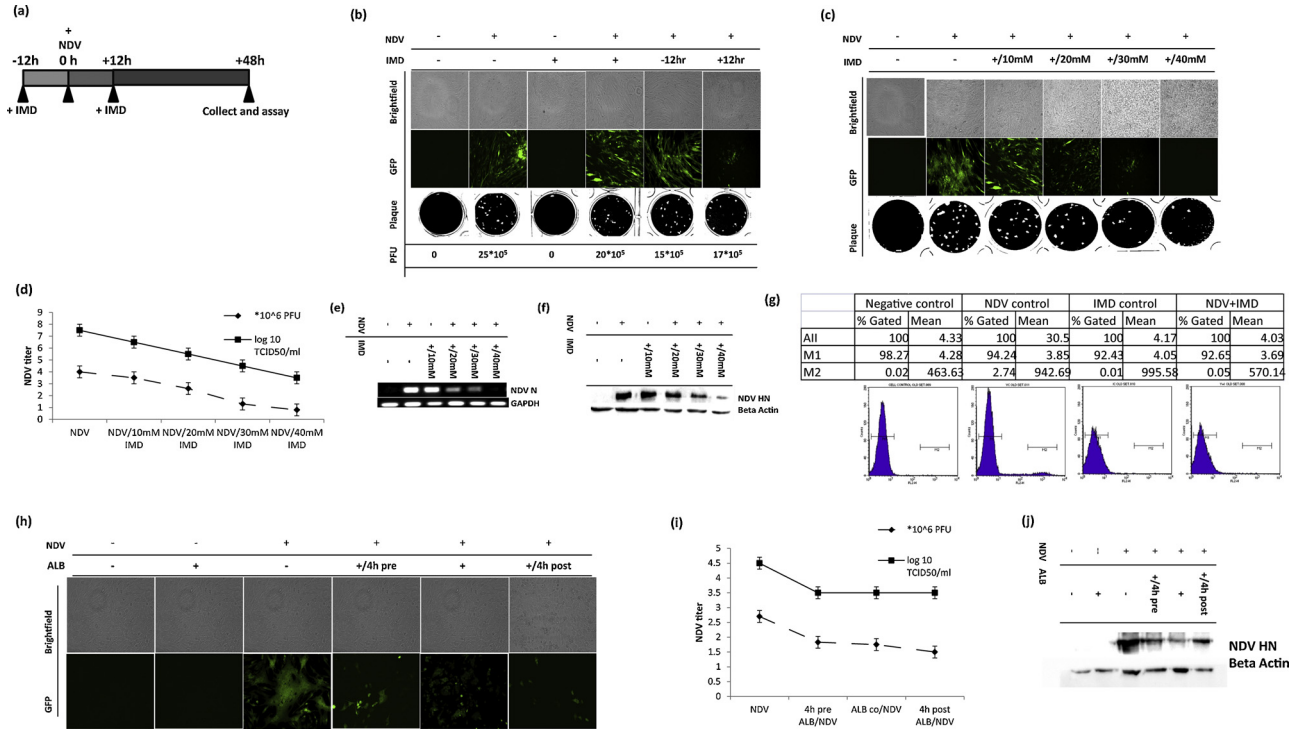

Fig. 2. Identification of imidazole and albendazole as anti-viral agent against NDV *in vitro*. Schematic representation of the time of addition experiment *in vitro* (a). Inhibition of NDV infection by imidazole (30 mM) at pre-entry, co-entry and post-entry step. Assessment was done by GFP measurement under inverted microscope and plaque assay (b). Dose-dependent inhibition of *m*NDV by imidazole treatment at a post-entry step. The assessment of reduction in *m*NDV replication was done using GFP fluorescence under an inverted microscope and plaque assay (c). Reduction in *m*NDV titration in fibroblast cells on imidazole treatment as calculated using plaque and $TCID_{50}$ titer (d). RT-PCR of *m*NDV infected CEF cells treated with increasing concentration of imidazole at post-entry step (e). Western blot of *m*NDV infected CEF cells treated with increasing concentration of imidazole at a post-entry step (f). The assessment of inhibition of recombinant NDV replication was done by GFP measurement using flow cytometer. M1 and M2 represent the percentage of total and fluorescent cells in the gated population in the four representative groups (g). Inhibition of recombinant NDV infection by albendazole (9 μM) at pre-entry, co-entry and post-entry step (h). Reduction in *m*NDV titration in fibroblast cells on albendazole treatment as calculated using plaque and $TCID_{50}$ titer (i). Western blot of *m*NDV infected DF1 cells treated with 9 μM albendazole (*j*).

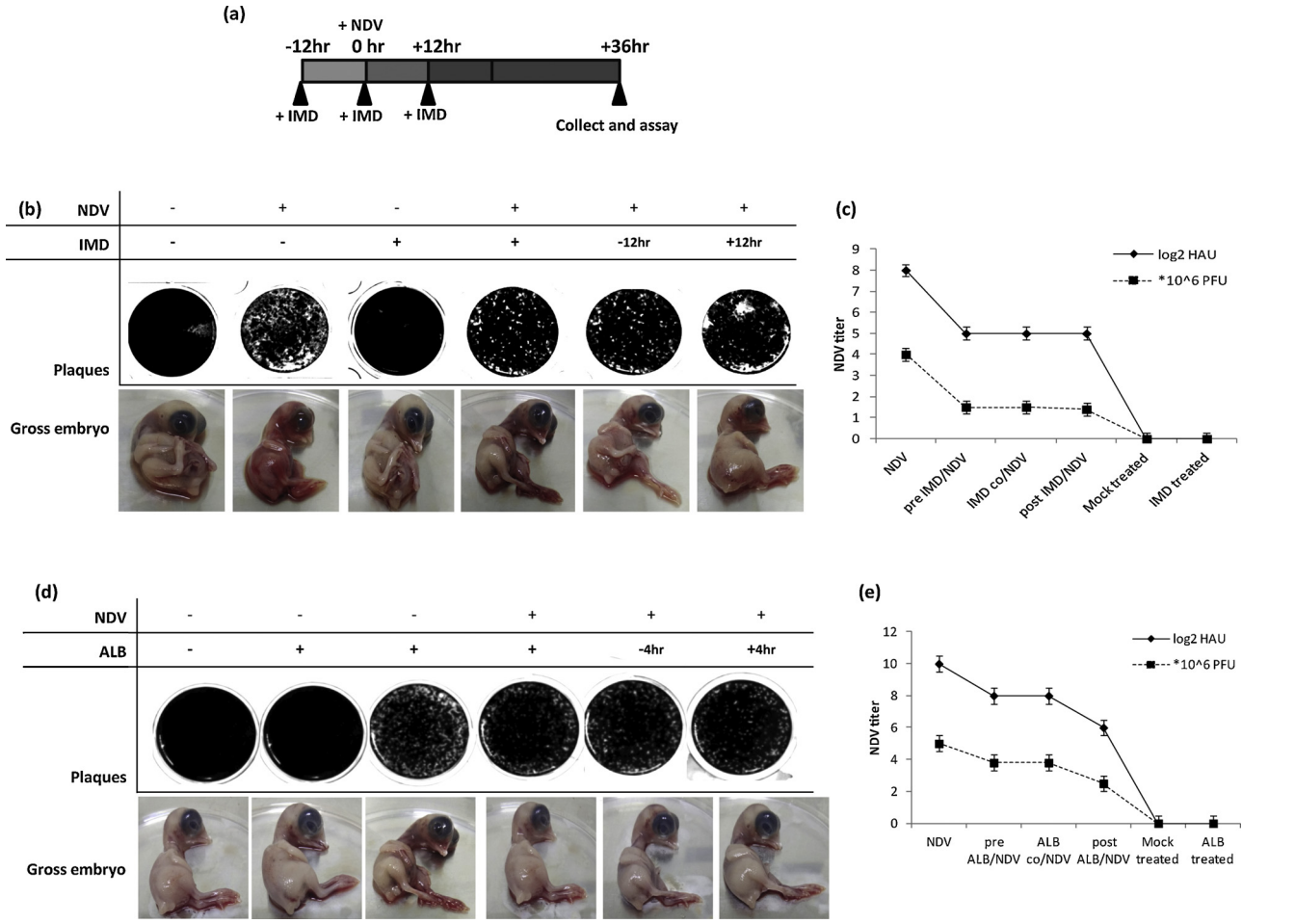

Fig. 3. Identification of imidazole and albendazole as anti-viral agent against *m*NDV *in ovo.* Schematic representation of the time of addition experiment *in ovo* (a). Gross lesions and virus titration in nine-day-old chicken eggs, treated with imidazole and infected with *m*NDV (b). Graph representing HA titer and plaque forming units per ml of *m*NDV in different embryo groups treated and infected with imidazole and *m*NDV 36 h post-infection (c). Gross lesions and virus titration in nine-day-old chicken eggs, treated with albendazole and infected with *m*NDV (d). Graph representing HA titer and plaque forming units per ml of *m*NDV in different embryo groups treated and infected with albendazole and *m*NDV 36 h post-infection (e).

3.3. *In ovo* antiviral activity

The antiviral efficacy of imidazole and albendazole was evaluated in nine-day-old embryonated chicken eggs (Fig. 3a). Allantoic fluid was collected 36 h post-infection from different groups. A two-fold reduction in *m*NDV titer was observed in the egg treated at the post-entry, co-entry and pre-entry steps as compared to the *m*NDV infected control. Clinical lesions observed in the embryo were suggestive of reduced virus replication in the imidazole treated eggs as compared to *m*NDV infected control eggs (Fig. 3b). The fold reduction in the *m*NDV particles was supported with HA titre and plaque titre using infected allantoic fluid (Fig. 3c). The albendazole treated embryos also showed similar pattern of reduction in *m*NDV replication. Reduced plaque of the harvested allantoic fluid and less pathogenicity in the embryos were suggestive of the antiviral effect of albendazole on *m*NDV (Fig. 3d). This was further supported by HA titre and PFU showing about 2 fold reduction (Fig. 3e).

3.4. *Antiviral activity of imidazole derivative in chickens*

To further validate antiviral potency of imidazole compounds we performed experiments with two-week old chicks. Seronegative birds were inoculated by the oculonasal route with *m*NDV or were left uninfected as a control group. All the birds treated with albendazole showed reduced *m*NDV specific pathological symptoms and gross lesions as compared to untreated birds at fourth-day post-infection (Fig. 4a). Internal organs such as trachea, lung and spleen in the infected control group showed distinct inflammation as compared to the treated groups. Tissues such as lungs, trachea and spleen collected from the treated-infected group showed reduced hemorrhages and infiltration of neutrophils as compared to the untreated-infected group (Fig. 4b). A better resolution picture of the same is given as Supplementary figure S1. Moreover, qPCR against *m*NDV specific gene from total RNA from tracheal, lung and spleen tissues collected on the fourth-day post-infection showed a reduction in the expression of viral RNA in the albendazole treated group (Fig. 4c). Furthermore, there was no cloacal or oral shedding observed for any of the treated-infected group till fourth day post-infection (Fig. 4d). All the *m*NDV infected birds showed both oral and cloacal shedding.

3.5. *Upregulation of innate immune response on imidazole treatment*

IFN production is the most prominent host innate immune response during infection with viral infections (Samuel, 2001). In the present study, we analyzed the induction of type I IFN (IFNα and IFNβ) responses in CEF and two-week-old chickens at the early stages of *m*NDV infection. TypeI IFN showed upregulation in CEF monolayer throughout the experimental duration with its peak at 48 h post-infection. The IFNα and IFNβ expression on imidazole treatment showed 413-fold and 29.65 fold increase respectively as compared to mock-infection CEF

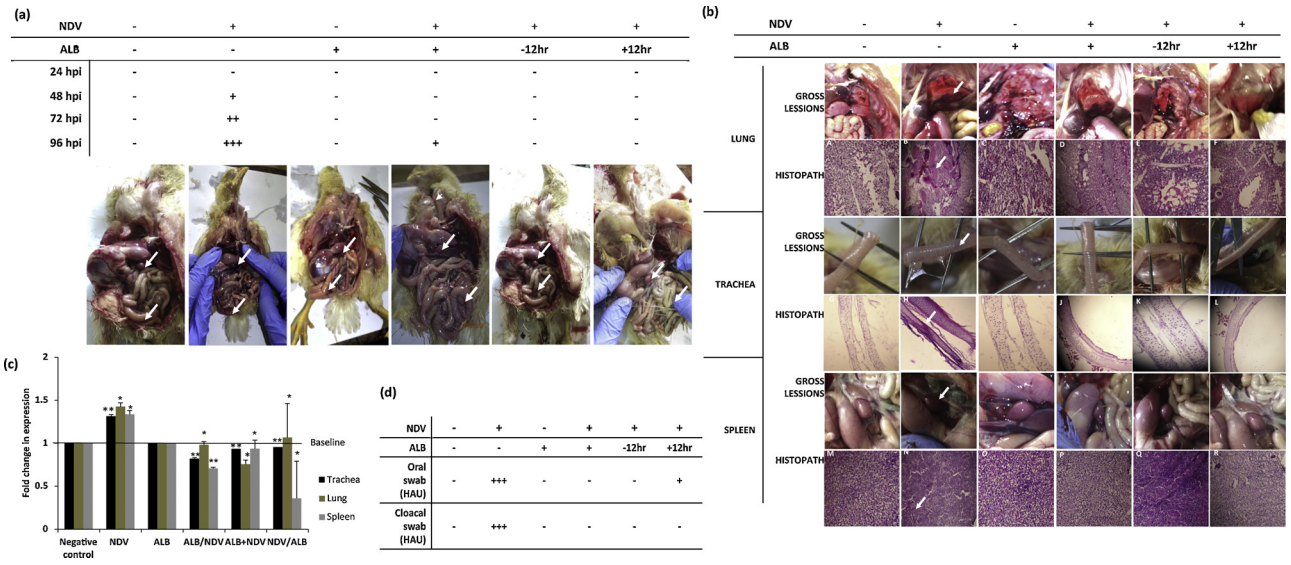

Fig. 4. Identification of albendazole as anti-viral agent against *m*NDV *in vivo*. Reduction in the replication of *m*NDV in presence of albendazole in 2 week old chickens. Gross lesions observed in birds' 96 h post-infection in different groups treated with albendazole. Presence of pathological lesions like inflammation of spleen and lung, haemmorhagic ridges in trachea were represented as "+" and their absence as "-" (a). Gross lesions and histopathology slides on tissues of two-week-old chickens infected with *m*NDV and treated with albendazole at different conditions. *m*NDV specific pathologic signs were seen; B: congestion and hemorrhage in the lung (white arrow); H: dropout and necrosis of mucous epithelial cells in the trachea (white arrow); N: amalgamation of collapsed cell and inflammatory exudates created the homogeneous and pink-staining appearance in the white pulps of spleens (white arrow; A, C, G, I, M and O: Corresponding control tissues. D, J and P: treated with albendazole during NDV infection; E, K and Q: treated with albendazole 12 h pre-NDV infection; F, L and R: treated with albendazole 12 h post-infection. Scale bar = 100 µm (b). Graphical representation of qPCR of *m*NDV gene in trachea, lung and spleen samples collected from the different experimental groups 96 h post experiment (NDV: only *m*NDV infected, ALB: only Albendazole treated, ALB/NDV: pre treated and infected, ALB + NDV: co treated and infected, NDV/ALB: post treated and infected). Values represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.05$ represented as *, $p < 0.005$ represented as **) (c). Haemagglutination assay of virus shed in the oral and cloacal droplets of two-week-old chickens 96 h post-infection in the different experimental groups, amplified in 9 day old embryonated chicken eggs. The "+" and "-" sign represents presence and absence of virus shed post inoculation of swabs in chicken eggs (d).

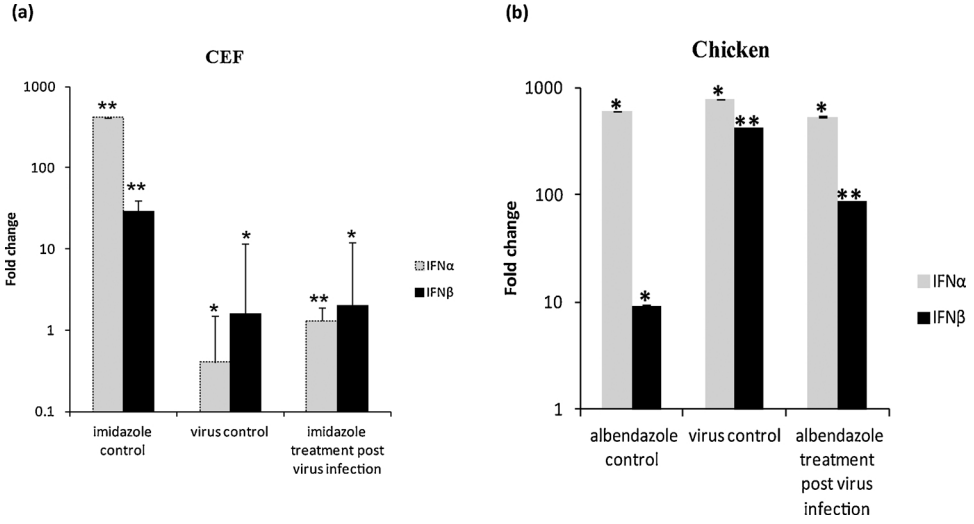

Fig. 5. Expression of innate immune response on imidazole and its derivatives. Up regulation of type I interferon genes on imidazole treatment (30 mM) post-infection in CEF cells was assessed using real time PCR. The graph shows relative fold increase in CEF treated with 30 mM imidazole, plain *m*NDV infected CEF and CEF treated with 30 mM imidazole 12 h post-infection. Increase in expression was compared with negative control CEF (a). Up regulation of type I interferon genes on albendazole treatment (5 mg/kg body weight) post-infection in two-week-old chickens was assessed using real time PCR. The graph shows relative fold increase in birds treated with albendazole, plain *m*NDV infected birds and birds inoculated with albendazole 12 h post-infection. Increase in expression was compared with mock control birds (b). Values represent the mean of three independent experiments ± S.D with standard error bar representation ($p < 0.05$ represented as *, $p < 0.005$ represented as **).

cells. On the contrary, IFNα and IFNβ expression in CEF cells infected with plain *m*NDV showed an increase of 0.4 fold and 1.62 fold respectively. Furthermore, in group infected with *m*NDV and post-treated with imidazole increase of 1.3 fold and 2.05 fold was observed for IFNα and IFNβ gene expression (Fig. 5a). In case of two-week-old chickens, upregulation of type I IFN genes was observed 96 h post-experiment. In the event of chickens treated with plain albendazole, significant IFNα expression of 609 fold was found, whereas IFNβ gene showed a 9 fold increase. In chickens infected with *m*NDV increase of 767 fold and 417 fold was observed in IFNα and IFNβ expression respectively. Furthermore, in birds infected with *m*NDV and post-treated with albendazole IFNα and IFNβ expression showed a significant increase of 535 fold and 87 fold respectively (Fig. 5b).

4. Discussion

The poultry industry is the second largest livestock industries after pork. Chicken meat represents approximately 88% of global poultry meat output. The major impediment to the growth of poultry industries arises from infectious diseases. Among the primary infectious agents

affecting poultry industry, NDV stands to be a major one. Live attenuated NDV vaccines adopted for over centuries do not absolutely block virulent NDV infection or shedding (Read et al., 2015). Besides, there are continuous records of NDV outbreaks in vaccinated flocks being reported from different countries (Khorajiya et al., 2015; Kumar and Koul, 2016; Putri et al., 2017). In our present study, we identified the potential of a commonly used anti-helminthic drug in poultry, for its application as an active antiviral against NDV infection in chicken. The *m*NDV strain R2B used in our study cause a mild respiratory symptom in birds. The *m*NDV shows distinct cytopathic effects and is well characterised in cell culture as compared to lentogenic stains. In addition, *m*NDV mimics the disease condition in young and used as a vaccine in older birds.

In our study, 50 mM concentration of imidazole was found to be the 50% survival dose in primary chicken fibroblast cells. However, the lethal dose of a compound depends on its level of purity (Parasuraman, 2011). We found imidazole specific vacuolation in CEF cells from concentration 10 mM and it increased linearly to 40 mM. In our study, the therapeutic window of imidazole was found to be around 30 mM however, its dose varies in different host cells (Haegler et al., 2017; Raab, 1980).

Our efforts so far have led to the identification of imidazole and its derivatives that suppress *m*NDV replication *in vitro*, *in ovo* and *in vivo*. Imidazole is a potent activator of cellular immune response (Wysocka et al., 2007). Our present work manifests the antiviral property of imidazole and its derivatives in chickens. The drugs when applied in sub-lethal dosages showed a reduction in active virus progenies. Our result suggests the efficacy of the compound as anti-NDV, which is exhibited in a dose-dependent manner. These results implied the inhibitory effect of imidazole and albendazole on *m*NDV replication. Substantial reduction in viral mRNA production and protein synthesis was visible on drug treatment as compared to virus infected cells. Reduced tissue tropism of the virus in the drug treated birds confirmed the efficacy of the drug as an antiviral compound. Previous reports showed the inhibitory effect of drugs on virus transcription and translation (van de Wakker et al., 2017). Our findings suggest the upregulation of IFN genes both *in vitro* and *in vivo* upon imidazole treatment. Our result corroborates with the earlier report where imidazole compounds are shown to induce IFN genes (Sachan et al., 2015). The expression level of IFN is critical in protecting the host against virus infection (Baum and Garcia-Sastre, 2011; Liniger et al., 2012; Pei et al., 2001). In our study, upregulation of IFN and its downstream pathways could be the reason behind the antiviral action. Our results of *in ovo* experiments suggested the ability of compounds to reduce *m*NDV infection in embryonic stages of development. The absence of clinicopathological signs in two-week-old birds supported the effectiveness of oral dose of the compound to reduce *m*NDV pathogenesis. The lack of sterile immunity to block virulent NDV infections and consequent virus shedding is the major hurdle in the development of its vaccine. The amount of *m*NDV shed in the environment by vaccinated birds is considered as an important parameter for vaccine efficacy (Dortmans et al., 2012). Such shedding is considered as a potential reason for evolution and transmission of virulent NDV strains (Kapczynski and King, 2005; Miller et al., 2009). Our result suggests the use of imidazole and its derivative albendazole could diminish replication and thus the transmission of NDV by reducing its shedding. Furthermore, our results showed the reduction of *m*NDV replication in various visceral organs at the transcriptome level. Such inhibition could be attributed to the induction of an antiviral state in the birds on imidazole treatment. Thus, our finding that repurposing of imidazole as a drug reduces NDV replication offers an attractive anti-NDV approach. However, use of these drugs must be regulated so that it could be used in conjunction with conventional NDV vaccination. It will be interesting to see if immunization and imidazole treatment together could improve the efficacy of current vaccines. Such approach might be a real improvement to NDV vaccination strategy.

Imidazole derivatives have broadened the scope in remedying various dispositions in clinical medicines due to their extensive spectrum of biological activities. Such unique properties include anti-cancer (Franchetti et al., 2001), β-lactamase inhibitors (Venkatesan et al., 2008), 20-HETE synthase inhibitors (T. Nakamura et al., 2004), carboxypeptidase inhibitors (Han and Kim, 2001), heme oxygenase inhibitors (Roman et al., 2007), anti-ageing agents (Babizhayev and Yegorov, 2015), anticoagulants (Nantermet et al., 2004), anti-inflammatory (Adams et al., 2001), antibacterial (*Bacillus cereus*) (Varshney et al., 2010), antifungal (Emami et al., 2008), antiviral (Hepatitis C virus) (Ujjinamatada et al., 2007), antitubercular (D. Zampieri et al., 2008) and antidiabetic (Crane et al., 2006). It is an FDA-licensed drug component that is well accepted for use in animals and humans to deal with worm infections for 50 years (Boiani and Gonzalez, 2005; Campbell, 1990). It is recognized to suppress several viruses in culture processes, including the West Nile virus and hepatitis virus (Ujjinamatada et al., 2007). Its broad antiviral action is connected to its desirable electron-rich characteristic, advantageous for imidazole derivatives to promptly bind with a family of enzymes and receptors in biological systems through several weak interactions, thereby presenting broad bioactivities (Zhang et al., 2014). Our time-course studies, showed that inhibition by imidazole takes place at a post-entry step, *in vitro*. On removal of imidazole the antiviral effect was reversed in pre and co infection studies. However, due to limitation in experimental procedures it was impossible to remove it in *in ovo* and *in vivo* studies. The difference in the antiviral effect in cellular and live models can also be attributed to variation of viral kinetics in those systems (Jogler et al., 2006). However, such reversal was not seen in case of imidazole derivative albendazole. Modification in the chemical structure of lead drug molecules has been known to improve their chemical and biological properties (Boiani and Gonzalez, 2005). Thus, subsequent molecular investigations of its molecular mechanism will give us insight into the more active drug development. Imidazole belongs to the category B drug, which means no risk in animal applications. It has little toxicity in avian with an oral median lethal dose (LD_{50}) value of 5 mg/kg body weight (Campbell, 1990). In the present study, the efficacy of imidazole on the impedance of NDV replication was shown to be dose- dependent. Further research on imidazole and its derivatives as anti-NDV therapeutics could help lessen the viral load in infected poultry, thereby reducing transmission during outbreaks.

Despite rapid progress to develop anti-NDV vaccines and presence of established vaccines since time, cases of vaccine failure cannot be overlooked in field conditions (Miller et al., 2013). Therefore, alternative treatments against NDV, comprising small-molecule therapeutics, are also required. The results and conclusions presented in our study should improve current NDV research and have a prompt implement on the development of an anti-NDV drug. Our findings could have implications for fighting epidemics by other paramyxoviruses, such as Nipah virus, Measles virus *etc.* many of which causes ravaging outbreaks worldwide.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We are thankful to all lab members for their constant support and help. We are thankful to Dr K Sharma, Department of microbiology at the College of veterinary sciences, Guwahati, Assam for his assistance in conducting animal experiments. The present work is supported by IIT Guwahati internal support fund and the department of science and technology (IFA-12-LSBM-34).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:https://doi.org/10.1016/j.virusres.2018.11.013.

References

Adams, J.L., Bocham, J.C., Gallagher, T.F., Kassis, S., Webb, E.F., Hall, R., Sorenson, M., Garigipati, R., Grisvold, D.E., Lee, J.C., 2001. Pyrimidinylimidazole inhibitors of p38: cyclic N-1 imidazole substituents enhance p38 kinase inhibition and oral activity. Bioorg. Med. Chem. Lett. 11, 2867–2870.

Alexander, D.J., 1998. Alexander DJ: Newcastle disease and other avian paramyxoviruses. In: Swayne, D.E., G.J, Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. American Association of Avian Pathologists, Philadelphia.

Alexander, D.J., 2000. Newcastle disease and other avian paramyxoviruses. Rev. Sci. Technol. 19 (2), 443–462.

Amarasinghe, G.K., Bao, Y., Basler, C.F., Bavari, S., Beer, M., Bejerman, N., Blasdell, K.R., Bochnowski, A., Briese, T., Bukreyev, A., Calisher, C.H., Chandran, K., Collins, P.L., Dietzgen, R.G., Dolnik, O., Durrwald, R., Dye, J.M., Easton, A.J., Ebihara, H., Fang, Q., Formenty, P., Fouchier, R.A.M., Ghedin, E., Harding, R.M., Hewson, R., Higgins, C.M., Hong, J., Horie, M., James, A.P., Jiang, D., Kobinger, G.P., Kondo, H., Kurath, G., Lamb, R.A., Lee, B., Leroy, E.M., Li, M., Maisner, A., Muhlberger, E., Netesov, S.V., Nowotny, N., Patterson, J.L., Payne, S.L., Paweska, J.T., Pearson, M.N., Randall, R.E., Revill, P.A., Rima, B.K., Rota, P., Rubbenstroth, D., Schwemmle, M., Smither, S.J., Song, Q., Stone, D.M., Takada, A., Terregino, C., Tesh, R.B., Tomonaga, K., Tordo, N., Towner, J.S., Vasilakis, N., Volchkov, V.E., Wahl-Jensen, V., Walker, P.J., Wang, B., Wang, D., Wang, F., Wang, L.F., Werren, J.H., Whitfield, A.E., Yan, Z., Ye, G., Kuhn, J.H., 2007. Taxonomy of the order Mononegavirales: update 2017. Arch. Virol. 162 (8), 2493–2504.

Babizhayev, M.A., Yegorov, Y.E., 2015. An "enigmatic" L-carnosine (beta-alanyl-L-histidine)? Cell proliferative activity as a fundamental property of a natural dipeptide inherent to traditional antioxidant, anti-aging biological activities: balancing and a hormonally correct agent, novel patented oral therapy dosage formulation for mobility, skeletal muscle power and functional performance, hypothalamic-pituitary-brain relationship in health, aging and stress studies. Recent Pat. Drug Deliv. Formul. 9 (1), 1–64.

Balloun, S.L., Miller, D.L., Arends, L.G., Speers, G.M., 1969. Effects of dimetridazole and antibiotics on growth and reproduction in Turkeys. Poult. Sci. 48 (1), 171–176.

Barrows, N.J., Campos, R.K., Powell, S.T., Prasanth, K.R., Schott-Lerner, G., Soto-Acosta, R., Galarza-Munoz, G., McGrath, E.L., Urrabaz-Garza, R., Gao, J., Wu, P., Menon, R., Saade, G., Fernandez-Salas, I., Rossi, S.L., Vasilakis, N., Routh, A., Bradrick, S.S., Garcia-Blanco, M.A., 2016. A screen of FDA-approved drugs for inhibitors of Zika virus infection. Cell Host Microbe 20 (2), 259–270.

Baum, A., Garcia-Sastre, A., 2011. Induction of type I interferon by RNA viruses: cellular receptors and their substrates. Amino Acids 38 (5), 1283–1299.

Boiani, M., Gonzalez, M., 2005. Imidazole and benzimidazole derivatives as chemotherapeutic agents. Mini Rev. Med. Chem. 5 (4), 409–424.

Campbell, W.C., 1990. Benzimidazoles: veterinary uses. Parasitol. Today 4 (6), 130–133.

Chen, C.Z., Kulakova, L., Southall, N., Marugan, J.J., Galkin, A., Austin, C.P., Herzberg, O., Zheng, W., 2011. High-throughput Giardia lamblia viability assay using bioluminescent ATP content measurements. Antimicrob. Agents Chemother. 55 (2), 667–675.

Corsello, S.M., Bittker, J.A., Liu, Z., Gould, J., McCarren, P., Hirschman, J.E., Johnston, S.E., Vrcic, A., Wong, B., Khan, M., Asiedu, J., Narayan, R., Mader, C.C., Subramanian, A., Golub, T.R., 2017. The Drug Repurposing Hub: a next-generation drug library and information resource. Nat. Med. 23 (4), 405–408.

Crane, L., Anastassiadour, M., Hage, S.E., Stigliani, J.L., Mouysset, G.B., Payard, M., Leger, J.M., Bizot-Espiard, J., Ktorza, A., Caignard, D., Renard, P., 2006. Design and synthesis of novel imidazoline derivatives with potent antihyperglycemic activity in a rat model of type 2 diabetes. Bioorg. Med. Chem. 14, 7419–7433.

Culling, C.F.A., 1974a. Chapter 19 - tissues requiring special treatment or techniques. Handbook of Histopathological and Histochemical Techniques, third edition. Butterworth-Heinemann, pp. 405–466.

Culling, C.F.A., 1974b. Chapter 35 - the interference microscope. Handbook of Histopathological and Histochemical Techniques, third edition. Butterworth-Heinemann, pp. 653–656.

Cunningham. Charles H, 1973. A Laboratory Guide in Virology, 7 ed. Burgess Publishing Co, Minneapolis, Minnesota.

Zampieri, D., Mamolo, M.G., Laurini, E., Scialino, G., Banfati, E., Vio, L., 2008. Antifungal and antimycobacterial activity of 1-(3,5-diaryl-4,5-dihydro-1H-pyrazol-4-yl)-1H-imidazole derivatives. Bioorg. Med. Chem. 16, 4516–4522.

Das, M., Kumar, S., 2017. Evidence of independent evolution of genotype XIII Newcastle disease viruses in India. Arch. Virol. 162 (4), 997–1007.

Debnath, A., Parsonage, D., Andrade, R.M., He, C., Cobo, E.R., Hirata, K., Chen, S., Garcia-Rivera, G., Orozco, E., Martinez, M.B., Gunatilleke, S.S., Barrios, A.M., Arkin, M.R., Poole, L.B., McKerrow, J.H., Reed, S.L., 2012. A high-throughput drug screen for Entamoeba histolytica identifies a new lead and target. Nat. Med. 18 (6), 956–960.

Diel, D.G., da Silva, L.H., Liu, H., Wang, Z., Miller, P.J., Afonso, C.L., 2012. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect. Genet. Evol. 12 (8), 1770–1779.

Dimitrov, K.M., Afonso, C.L., Yu, Q., Miller, P.J., 2017. Newcastle disease vaccines-A solved problem or a continuous challenge? Vet. Microbiol. 206, 126–136.

Dortmans, J.C., Peeters, B.P., Koch, G., 2012. Newcastle disease virus outbreaks: vaccine mismatch or inadequate application? Vet. Microbiol. 160 (1–2), 17–22.

Emami, S., Foroumadi, A., Falahati, M., Lotfali, E., Rajabalian, S., Ebrahimi, S., Farahyar, S., Shafiee, A., 2008. 2-Hydroxyphenacyl azoles and related azolium derivatives as antifungal agents. Bioorg. Med. Chem. Lett. 18, 141–146.

Franchetti, P., Marchetti, S., Cappellacci, L., Yalowitz, J.A., Jayaram, H.N., Goldstein, B.M., Grifantini, M., 2001. A new C-nucleoside analogue of tiazofurin: synthesis and biological evaluation of 2-beta-D-ribofuranosylimidazole-4-carboxamide (imidazofurin). Bioorg. Med. Chem. Lett. 11 (1), 67–69.

Freimoser, F.M., Jakob, C.A., Aebi, M., Tuor, U., 1999. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities. Appl. Environ. Microbiol. 65 (8), 3727–3729.

Ganar, K., Das, M., Sinha, S., Kumar, S., 2014. Newcastle disease virus: current status and our understanding. Virus Res. 184, 71–81.

Haegler, P., Joerin, L., KrÃ¤henbÃ¼hl, S., Bouitbir, J., 2017. Hepatocellular toxicity of imidazole and triazole antimycotic agents. Toxicol. Sci. 157 (1), 183–195.

Han, M.S., Kim, D.H., 2001. Effect of zinc ion on the inhibition of carboxypeptidase A by imidazole-bearing substrate analogues. Bioorg. Med. Chem. Lett. 11, 1425–1427.

Hennessy, D.R., Lacey, E., Prichard, R.K., 1983. Pharmacokinetic behaviour and anthelmintic efficacy of 1-n-butyl carbamoyl oxfendazole given by intramuscular injection. Vet. Res. Commun. 6 (1), 177–187.

Hierholzer, J.C., RA, K., 1996. Virus isolation and quantitation. In: Mahy, B.W., HI, K. (Eds.), Virology Methods Manual Academic Press, pp. 25–46 London, United Kingdom.

Jogler, C., Hoffmann, D., Theegarten, D., Grunwald, T., Ãœberla, K., Wildner, O., 2006. Replication properties of human adenovirus in vivo and in cultures of primary cells from different animal species. J. Virol. 80 (7), 3549–3558.

Kaiser, M.G., Cheeseman, J.H., Kaiser, P., Lamont, S.J., 2006. Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to Salmonella enterica serovar Enteritidis. Poult. Sci. 85 (11), 1907–1911.

Kaleta, E., Baldauf, C., 1988. Newcastle disease in free-living and pet birds. In: Alexander, D.J. (Ed.), Newcastle Disease. Kluwer Acedemic Press, Boston, MA.

Kapczynski, D.R., King, D.J., 2005. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. Vaccine 23 (26), 3424–3433.

Khorajiya, J.H., Pandey, S., Ghodasara, P.D., Joshi, B.P., Prajapati, K.S., Ghodasara, D.J., Mathakiya, R.A., 2015. Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. Vet. World 8 (3), 372–381.

Kouznetsova, J., Sun, W., Martinez-Romero, C., Tawa, G., Shinn, P., Chen, C.Z., Schimmer, A., Sanderson, P., McKew, J.C., Zheng, W., Garcia-Sastre, A., 2011. Identification of 53 compounds that block Ebola virus-like particle entry via a repurposing screen of approved drugs. Emerg. Microbes Infect. 3 (12), e84.

Krishnamurthy, S., Huang, Z., Samal, S.K., 2000. Recovery of a virulent strain of newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. Virology 278 (1), 168–182.

Kumar, S., Koul, M., 2016. Newcastle disease virus: a constant threat to the poultry industry in India. Vaccine 34 (5), 597–598.

Kumar, U., Kumar, S., 2015. Molecular characterization of an apoptotic strain of Newcastle disease virus isolated from an outbreak in India. Cancer Gene Ther. 22 (8), 402–409.

Lamb, R.A., Kolakofsky, D., 1996. *Paramyxoviridae*: the viruses and their replication. In: 3 ed. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 1. 2 vols. Lippincott Williams & Wilkins, Philadelphia, pp. 1177–1204.

Liniger, M., Moulin, H.R., Sakoda, Y., Ruggli, N., Summerfield, A., 2012. Highly pathogenic avian influenza virus H5N1 controls type I IFN induction in chicken macrophage HD-11 cells: a polygenic trait that involves NS1 and the polymerase complex. Virol. J. 9.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25 (4), 402–408.

Miller, P.J., Afonso, C.L., El Attrache, J., Dorsey, K.M., Courtney, S.C., Guo, Z., Kapczynski, D.R., 2013. Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. Dev. Comp. Immunol. 41 (4), 505–513.

Miller, P.J., Dimitrov, K.M., Williams-Coplin, D., Peterson, M.P., Pantin-Jackwood, M.J., Swayne, D.E., Suarez, D.L., Afonso, C.L., 2015. International biological engagement programs facilitate Newcastle disease epidemiological studies. Front. Public Health 3, 235.

Miller, P.J., Estevez, C., Yu, Q., Suarez, D.L., King, D.J., 2009. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis. 53 (1), 39–49.

Miller, P.J., Haddas, R., Simanov, L., Lublin, A., Rehmani, S.F., Wajid, A., Bibi, T., Khan, T.A., Yaqub, T., Setiyaningsih, S., Afonso, C.L., 2014. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infect. Genet. Evol. 29, 216–229.

Mottier, P., Huré, I., Gremaud, E., Guy, P.A., 2006. Analysis of four 5-nitroimidazoles and their corresponding hydroxylated metabolites in egg, processed egg, and chicken meat by isotope dilution liquid chromatography tandem mass spectrometry. J. Agric. Food Chem. 54 (6), 2018–2026.

Nantermet, P.G., Barrow, J.C., Lindsley, S.R., Young, M., Mao, S., Carroll, S., Bailey, C., Bosserman, M., Colussi, D., McMasters, D.R., Vacca, J.P., Selnick, H.G., 2004. Imidazole acetic acid TAFIa inhibitors: SAR studies centered around the basic P′1 group. Bioorg. Med. Chem. Lett. 14, 2141–2145.

Nayak, B., Kumar, S., DiNapoli, J.M., Paldurai, A., Perez, D.R., Collins, P.L., Samal, S.K., 2010. Contributions of the avian influenza virus HA, NA, and M2 surface proteins to the induction of neutralizing antibodies and protective immunity. J. Virol. 84 (5), 2408–2420.

Nosengo, N., 2016. Can you teach old drugs new tricks? Nature 534 (7607), 314–316.
OIE, 2012. 7th ed. Newcastle Disease. World Organization for Animal Health, Paris, France.
Parasuraman, S., 2011. Toxicological screening. J. Pharmacol. Pharmacother. 2 (2), 74–79.
Pei, J., Sekellick, M.J., Marcus, P.I., Choi, I.S., Collisson, E.W., 2001. Chicken interferon type I inhibits infectious bronchitis virus replication and associated respiratory illness. J. Interferon Cytokine Res. 21 (12), 1071–1077.
Pham, T.T., Meng, S., Sun, Y., Lv, W., Bahl, J., 2016. Inference of Japanese encephalitis virus ecological and evolutionary dynamics from passive and active virus surveillance. Virus Evol. 2 (1), vew009.
Putri, D.D., Handharyani, E., Soejoedono, R.D., Setiyono, A., Mayasari, N., Poetri, O.N., 2017. Pathotypic characterization of Newcastle disease virus isolated from vaccinated chicken in West Java, Indonesia. Vet. World 10 (4), 438–444.
Raab, W.P.E., 1980. Toxicology of the imidazole derivatives. The Treatment of Mycosis with Imidazole Derivatives. Springer, Berlin, Heidelberg.
Rastogi, R., Sharma, S., 1983. 2-aminobenzimidazoles in organic syntheses. Synthesis 11, 861–882.
Read, A.F., Baigent, S.J., Powers, C., Kgosana, L.B., Blackwell, L., Smith, L.P., Kennedy, D.A., Walkden-Brown, S.W., Nair, V.K., 2015. Imperfect vaccination can enhance the transmission of highly virulent pathogens. PLoS Biol. 13 (7) e1002198.
Roman, G., Riley, J.G., Vlahakis, J.Z., Kinobe, R.T., Brien, J.F., Nakatsu, K., Szarek, W.A., 2007. Heme oxygenase inhibition by 2-oxy-substituted 1-(1H-imidazol-1-yl)-4-phenylbutanes: effect of halogen substitution in the phenyl ring. Bioorg. Med. Chem. 15, 3225–3234.
Sachan, S., Ramakrishnan, S., Annamalai, A., Sharma, B.K., Malik, H., Saravanan, B.C., Jain, L., Saxena, M., Kumar, A., Krishnaswamy, N., 2015. Adjuvant potential of resiquimod with inactivated Newcastle disease vaccine and its mechanism of action in chicken. Vaccine 33 (36), 4526–4532.
Samuel, C.E., 2001. Antiviral actions of interferons. Clin. Microbiol. Rev. 14 (4), 778–809.
Sheehan, D.J., Hitchcock, C.A., Sibley, C.M., 1999. Current and emerging azole antifungal agents. Clin. Microbiol. Rev. 12 (1), 40–79.
Snoeck, C.J., Owoade, A.A., Couacy-Hymann, E., Alkali, B.R., Okwen, M.P., Adeyanju, A.T., Komoyo, G.F., Nakoune, E., Le Faou, A., Muller, C.P., 2013. High genetic diversity of Newcastle disease virus in poultry in West and Central Africa: cocirculation of genotype XIV and newly defined genotypes XVII and XVIII. J. Clin. Microbiol. 51 (7), 2250–2260.
Steinfeld, H., Wassenaar, T., Jutzi, S., 2006. Livestock production systems in developing countries: status, drivers, trends. Rev. Sci. Tech. 25 (2), 505–516.
Sun, W., Park, Y.D., Sugui, J.A., Fothergill, A., Southall, N., Shinn, P., McKew, J.C., Kwon-Chung, K.J., Zheng, W., Williamson, P.R., 2013. Rapid identification of antifungal compounds against Exserohilum rostratum using high throughput drug repurposing screens. PLoS One 8 (8), e70506.
Nakamura, T., Kakinuma, H., Umemiya, H., Amada, H., Miyata, N., Taniguchi, K., Bando, K., M., S, 2004. Imidazole derivatives as new potent and selective 20-HETE synthase inhibitors. Bioorg. Med. Chem. Lett. 14, 333–336.
Ujjinamatada, R.K., Baier, A., Borowski, P., Hosman, R.S., 2007. An analogue of AICAR with dual inhibitory activity against WNV and HCV NTPase/helicase: Synthesis and in vitro screening of 4-carbamoyl-5-(4,6-diamino-2,5-dihydro-1,3,5-triazin-2-yl)imidazole-1- β-d-ribofuranoside. Bioorg. Med. Chem. Lett. 17, 2285–2288.
van de Wakker, S.I., Fischer, M.J.E., Oosting, R.S., 2017. New drug-strategies to tackle viral-host interactions for the treatment of influenza virus infections. Eur. J. Pharmacol. 809, 178–190.
Varshney, V., Mishra, N.N., Shukla, P.K., Sahu, D.P., 2010. Synthesis of nitroimidazole derived oxazolidinones as antibacterial agents. Eur. J. Med. Chem. 45 (2), 661–666.
Venkatesan, A.M., Agarwal, A., Abe, T., Ushirogochi, H.O., Santos, D., Li, Z., Francisco, G., Lin, Y.I., Peterson, P.J., Yang, Y., Weiss, W.J., Shales, D.M., Mansour, T.S., 2008. 5,5,6-Fused tricycles bearing imidazole and pyrazole 6-methylidene penems as broad-spectrum inhibitors of β-lactamases. Bioorg. Med. Chem. 16, 1890–1902.
Wysocka, M., Newton, S., Benoit, B.M., Introcaso, C., Hancock, A.S., Chehimi, J., Richardson, S.K., Gelfand, J.M., Montaner, L.J., Rook, A.H., 2007. Synthetic imidazoquinolines potently and broadly activate the cellular immune response of patients with cutaneous T-cell lymphoma: synergy with interferon-gamma enhances production of interleukin-12. Clin. Lymphoma Myeloma 7 (8), 524–534.
Zhang, L., Peng, X.-M., Damu, G.L.V., Geng, R.-X., Zhou, C.-H., 2014. Comprehensive review in current developments of imidazole-based medicinal chemistry. Med. Res. Rev. 34 (2), 340–437.