

# SC75741 antagonizes vesicular stomatitis virus, duck Tembusu virus, and duck plague virus infection in duck cells through promoting innate immune responses

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**ABSTRACT** Duck Tembusu virus (DTMUV) and duck plague virus (DPV) are typical DNA and RNA viruses of waterfowl, causing drastic economic losses to the duck farm industry in terms of high mortality and decreased egg production. These 2 viruses reappear from time to time because the available vaccines fail to provide complete immunity and no clinical antiviral drugs are available for them. In the present study, we evaluated the antiviral activity of SC75741 for DTMUV, DPV, and the model virus, vesicular stomatitis virus infection in duck cells. SC75741, a nuclear factor-kappa B (NF- $\kappa$ B)-specific inhibitor in mammal cells, revealed the highest antiviral activity among the inhibitors specific to c-Jun NH<sub>2</sub>-terminal kinase, extracellular signal-regulated kinase, p38 mitogen-activated protein

kinase (p38), and NF- $\kappa$ B signaling. The antiviral activity of SC75741 was dose-dependent and showed effects in different duck cell types. Time-addition and duration assay demonstrated that SC75741 inhibited virus infection in the middle of and after virus infection at least for 72 h in duck embryo fibroblast cells. The DPV viral adsorption and genomic copy number were reduced, indicating that SC75741 blocks the phase of the virus life cycle at viral entry and genomic replication. In addition, SC75741 enhanced the expression of interferon only when stimulator of interferon genes (STING) was overexpressed or pre-activated by the virus infection, suggesting that SC75741 acts as a STING agonist. In conclusion, SC75741 is a candidate antiviral agent for DTMUV and DPV.

**Key words:** antiviral agent, SC75741, duck plague virus, duck Tembusu virus, STING agonist

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## INTRODUCTION

Duck Tembusu virus (DTMUV), first identified in China in 2010, mainly causes ovarian hemorrhage and a subsequent substantial decrease in egg production together with a sudden decline in feed uptake and neurological symptom (Chen et al., 2014). DTMUV is a single-stranded positive-sense RNA arbovirus belonging to the

*Flavivirus* genus, *Flaviviridae* family, and shares many conserved motifs with other flaviviruses (Cao et al., 2011). The mosquito Tembusu virus isolate is grouped with duck serum Tembusu virus isolate from China (Yan et al., 2011). Duck plague virus (DPV, also termed as duck enteritis virus), belonging to the Alphaherpesvirinae subfamily of the Herpesviridae, is an acute and highly contagious viral infection causing duck plague that recurrently occurs in ducks, geese, and swans (Converse and Kidd, 2001). Although mandatory inoculation of vaccines can control this disease and reduce the morbidity and mortality of infected ducks, it cannot provide complete immunity against duck plague (Wang and Osterrieder, 2011). DTMUV and DPV have caused

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prevalent epidemics and continue to cause extreme economic losses in the waterfowl industry with high mortality and decreased egg production (Pearson and Cassidy, 1997). No therapeutic option is currently available to treat the infection of DTMUV or DPV. Therefore, the development of antiviral drugs is urgently needed.

The massive release of ILs, tumor necrosis factor alpha, and other circulating mediators of inflammation during critical illness causes a “cytokine storm.” Cytokine storm plays a direct role in the morbidity and mortality rate of viral diseases. It has been proposed that downregulating the inflammatory responses may improve the outcome with or without the combination of antiviral agents (D’Elia et al., 2013). SC75741, a screened broad nuclear factor-kappa B (NF- $\kappa$ B) signaling inhibitor (Leban et al., 2007), blocks the influenza viruses infection and protects mice against highly pathogenic avian influenza A virus by interfering with the expression of cytokines, chemokines, and proapoptotic factors, and inhibiting the caspase-dependent nuclear export of viral ribonucleoproteins (Ehrhardt et al., 2013; Haasbach et al., 2013). Whether SC75741 possesses a promising antiviral potency for viruses, which are highly prevalent in waterfowl, is not clear, but we aimed to investigate this in duck cells.

In the current study, we evaluated the antiviral activity of SC75741 for vesicular stomatitis virus (VSV), DPV, and DTMUV infection in vitro. SC75741 revealed strong antiviral potency in a dose-dependent manner in different duck cell types by inhibiting viral entry into the cell and genomic replication in the cells. Moreover, for the first time, we proposed that SC75741 may potentiate the agonist activity of stimulator of interferon genes (STING) in duck embryo fibroblast (DEF) cells, aiming to develop an agent that could be useful to control the waterfowl viruses causing various diseases.

## MATERIALS AND METHODS

### Ethics Statement

All animal experiments were conducted in accordance with approved guidelines. One-month-old Peking ducklings were purchased from a DTMUV and DPV-free farm, where vaccination against DTMUV and DPV was not implemented. All the ducks were housed in the animal facility at Sichuan Agricultural University, China. The study was approved by the Committee of Experiment Operational Guidelines and Animal Welfare of Sichuan Agricultural University (approved permit number SYXK 2019-187).

### Cells, Virus Strains, and Reagents

DEFs were prepared as previously explained (Zhao et al., 2008; Xin et al., 2009); DEF and baby hamster kidney (BHK21) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA). DEFs were

cultured to 100% confluence for 24 h and then passaged and subcultured for use in the subsequent assays. Duck primary neurons, astrocytes, and monocytes or macrophages were isolated, cultured, and identified as explained earlier (Tian et al., 2019). All cells were cultured in an incubator at 37°C, 5% CO<sub>2</sub>.

The virulent strain of DPV, CHv strain, was isolated and characterized in our laboratory. The recombinant virulent strain of DPV expressing enhanced green fluorescent protein, BAC-CHv-enhanced green fluorescent protein (DPV-GFP), was constructed by our research center basing on the genome of CHv strain (Wu et al., 2017; Zhang et al., 2017; Ma et al., 2018; You et al., 2018). The recombinant VSV strain harboring GFP (VSV-GFP) and DTMUV was stored at our research center (Zhou et al., 2016). Each DPV and DTMUV strain was propagated and titrated on DEFs. VSV-GFP was propagated and titrated on BHK21. SP600125 (c-Jun NH<sub>2</sub>-terminal kinase [JNK] inhibitor, purity 98.82%), LY3214996 (extracellular signal-regulated kinase [ERK] inhibitor, purity 99.87%), TAK-715 (p38 inhibitor, purity 99.93%), SC75741 (NF- $\kappa$ B inhibitor, purity 99.51%), and ruxolitinib (interferon receptor inhibitor) were purchased from MedChemExpress (Monmouth Junction, NJ). All inhibitors were diluted in dimethyl sulfoxide (DMSO).

### Virus Infection and Determination of Fluorescence Formation Units (FFU) and Tissue Culture Infectious Dose 50 (TCID<sub>50</sub>)

For virus infection, the required dose of the virus was diluted in the medium used to culture the various types of cells and incubated with the cells at 37°C for 1 h. The cells were then washed twice with PBS and maintained in the corresponding medium supplemented with or without 2% FBS and 1% penicillin-streptomycin. The cell culture supernatants were collected and titrated to determine the TCID<sub>50</sub> on DEFs for DTMUV, DPV, and DPV-GFP, according to our previously described standardized methods (He et al., 2018; Chen et al., 2019; Li et al., 2019), or FFU on BHK21 for VSV-GFP. All viruses were handled under biosafety level 2 laboratory conditions. For GFP expression observation and FFU determination, a fluorescence microscope was used (excitation wavelength was 460–550 nm, IX71, Olympus Corporation, Tokyo, Japan).

### Cytotoxicity Assay

DEF cells were treated with different concentrations of SC75741, which was a screened NF- $\kappa$ B inhibitor (Leban et al., 2007), and demonstrated inhibition to influenza virus in vitro at 5  $\mu$ mol/mL (Ehrhardt et al., 2013), or the solvent and incubated for 24 h. Afterward, the cell viability of DEF cells was determined using a CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT, Promega Corp., Madison, WI), according to the manufacturer’s instructions.

## Antiviral Activity Assay

Cells were incubated with VSV-GFP, DTMUV, DPV-GFP, or DPV (CHv strain) at 37°C for 1 h; then, the cells were washed twice with PBS and fresh medium was maintained with different concentrations of the inhibitors, SP600125 (10  $\mu\text{mol}/\text{mL}$ ), LY3214996 (5  $\mu\text{mol}/\text{mL}$ ), TAK-715 (5  $\mu\text{mol}/\text{mL}$ ), and SC75741 (0.2–50  $\mu\text{mol}/\text{mL}$ , according to the corresponding assay), or DMSO for 24, 48, or 72 h. Afterward, the cell culture supernatants were collected for TCID<sub>50</sub> or FFU determination. The GFP expression was determined using fluorescence microscopy to observe the viral plaque formation of VSV-GFP and DPV-GFP.

## Time-Addition and Therapeutics Assay

For the time-addition assay, cells were inoculated with 1 multiplicity of infection (MOI) of VSV-GFP, DTMUV, or DPV for 1 h. SC75741 (5  $\mu\text{mol}/\text{mL}$ ) was supplemented before (2 h before viral inoculation), during (in the middle of viral inoculation), and after viral inoculation. At 24 h postinfection (hpi), the cell culture supernatants were collected for TCID<sub>50</sub> or FFU determination.

For the therapeutic assay of SC75741, DEF cells were inoculated with 1 MOI of VSV-GFP, DTMUV, or DPV for 1 h; then SC75741 (5  $\mu\text{mol}/\text{mL}$ ) was added at 0, 2, 4, 8, 12, and 24 hpi. At 48 hpi, the cell culture supernatants were collected for TCID<sub>50</sub> or FFU determination. In order to avoid the leftover of SC75741 in the viral titers assay, the collected cell culture supernatants were incubated with the DEF cells for 2 h, and PBS was used to wash the cells and fresh medium was added.

## Viral Adsorption, Endocytosis, Replication, and Release Assay

In order to investigate the antiviral effects of SC75741 on the viral life cycle, the dissected phase of virus infection was analyzed according to previous reports (Yuan et al., 2019). For the viral adsorption assay, DEF cells were pre-chilled at 4°C for 1 h, following which the cells were incubated with 0.001 MOI of DPV-GFP containing SC75741 (5  $\mu\text{mol}/\text{mL}$ ) or DMSO at 4°C for an additional 2 h. After that, the cells were washed 3 times with ice-cold PBS and blocked with low melting point agar mixed with DMEM containing 2% FBS and cultured at 37°C for 72 h. Then the amount of viral plaque was calculated directly in the well using the mean values from the repeated wells by determining the GFP expressed from the DPV-GFP.

For the viral endocytosis assay, DEF cells were pre-chilled at 4°C for 1 h, and incubated with 0.001 MOI of DPV-GFP at 4°C for 2 h and washed with PBS. Thereafter, fresh DMEM containing SC75741 (5  $\mu\text{mol}/\text{mL}$ ) or DMSO was added and maintained at 37°C for 3 h. After carefully washing 3 times with PBS, the cells were blocked with low melting point agar mixed with DMEM containing 2% FBS and cultured at 37°C for

72 h. Then the amount of viral plaque was calculated directly in the well using the mean values from repeated wells by capturing the GFP expressed from the DPV-GFP.

For the viral genomic replication assay, DEF cells were incubated with 0.1 MOI of DPV for 6 h and washed with PBS, fresh DMEM containing SC75741 (5  $\mu\text{mol}/\text{mL}$ ), or DMSO was added. At 7, 8, 9, and 10 hpi, the cells were collected for genomic DNA extract. The genomic copy numbers of DPV were quantified using an absolute quantitative PCR (Q-PCR) method as described previously (Guo et al., 2009) with primers specific to the sequence of the DPV *UL30* gene (primers are listed in Table 1).

For the virion release assay, DEF cells were infected with 1 MOI of DPV for 18 h. Then DEF cells were washed with PBS and SC75741 (5  $\mu\text{mol}/\text{mL}$ ) or DMSO was added into the culture medium. The cell culture supernatants were harvested at 15, 30, 45, and 60 min after the addition of SC75741 for TCID<sub>50</sub> determination as described above.

## Dual-Luciferase Reporter Gene Assay

Various reporter plasmids, pGL-interferon (IFN)- $\beta$ -luc, pGL-NF- $\kappa$ B-luc, and pRL-TK, were co-transfected into DEF cells with or without plasmids expressing duck STING, retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), mitochondrial antiviral signaling protein (MAVS), TRAF family member associated NFKB activator binding kinase 1, IFN regulatory factor 1, or IRF7 (500 ng/well), which were constructed previously (Chen et al., 2017; Chen et al., 2018; Chen et al., 2019), using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Shanghai, China) according to the instructions provided by the manufacturer. Different doses of SC75741 were added at 12 h post transfection; DMSO was used as a negative control. At 36 h post transfection, cells were harvested and luciferase activity was measured. The luciferase activities were determined with a Dual-Glo Luciferase Assay System (Promega Corp.) and normalized based on the Renilla luciferase activity.

## RNA Isolation and Q-PCR

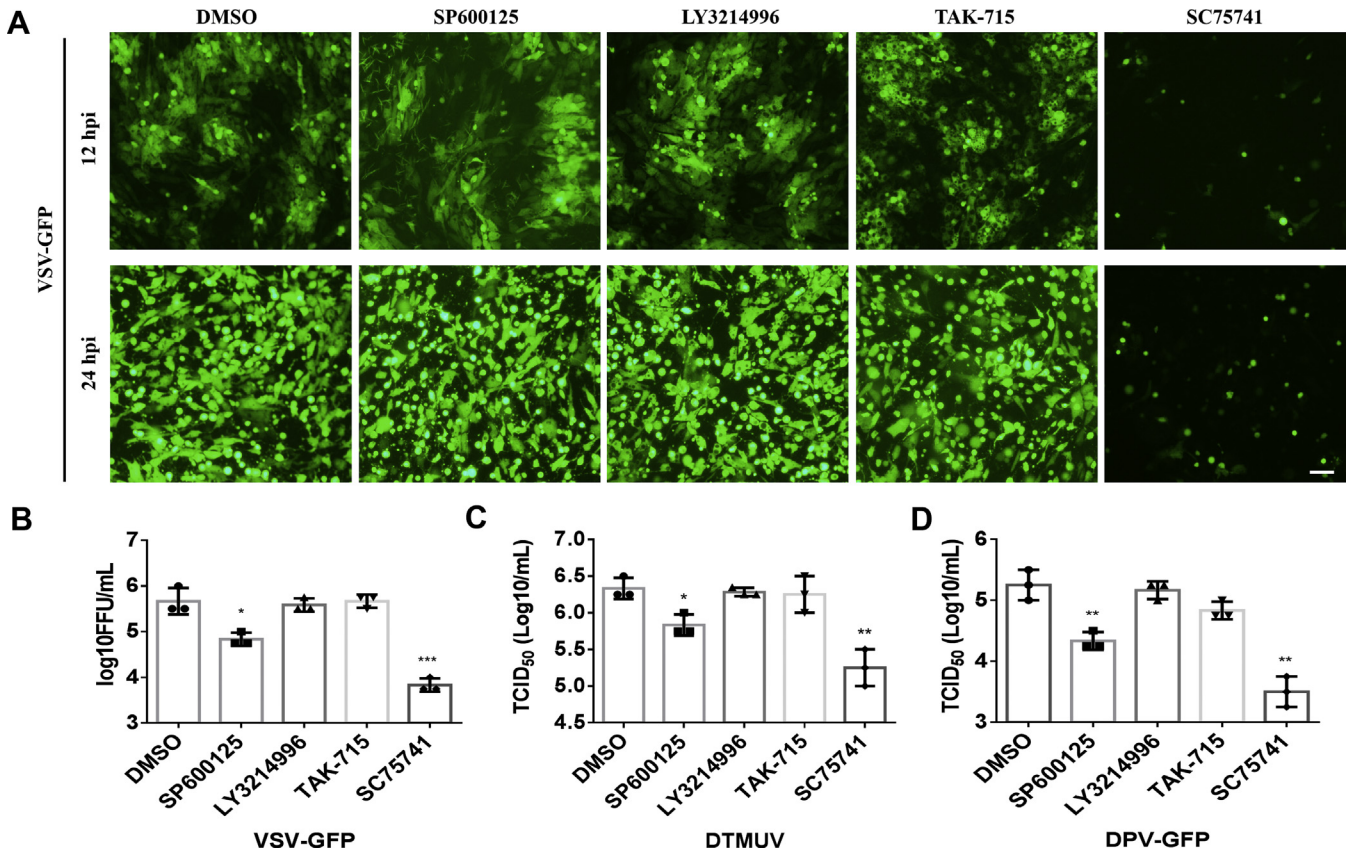
RNA was extracted from the cells using TRIzol reagent (Takara, Kyoto, Japan), according to the manufacturer's instructions; the genomic DNA was digested and excluded with DNase I; total RNAs were reverse transcribed into cDNA with the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan); Q-PCR procedure was performed as described previously (Tian et al., 2019). Expression levels of IL-1 $\beta$ , IL-6, C-C motif chemokine ligand 21 (CCL21), interferon-beta (IFN- $\beta$ ), 2'-5'-oligoadenylate synthetase-like protein (OASL), and myxovirus resistance (MX) mRNAs was detected and presented as relative fold-change according to the 2<sup>- $\Delta\Delta$ CT</sup> method. All primer sequences are listed in Table 1.



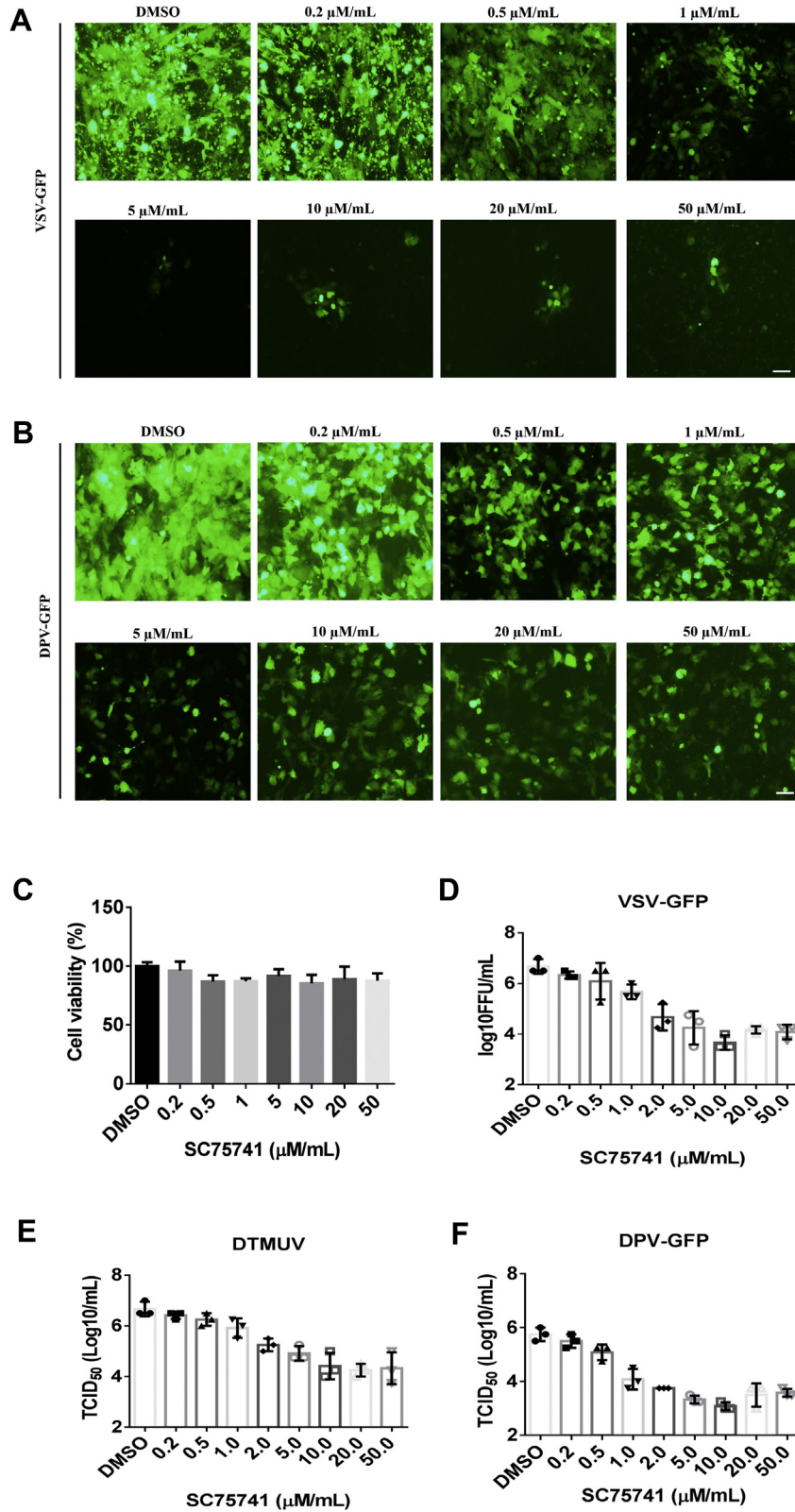
**Table 1.** Q-PCR primers used for quantification of mRNAs and viral genomic DNA.

Primer name	Accession no.	Nucleotide sequence (5'-3')	Use
<i>IFN-β</i> F	KM035791.2	TCTACAGAGCCTTGCCTGCAT	qRT-PCR
<i>IFN-β</i> R		TGTCGGTGTCCAAAAGGATGT	qRT-PCR
<i>MX</i> F	NM_001310409.1	TGCTGTCCTTCATGACTTCG	qRT-PCR
<i>MX</i> R		GCTTTGCTGAGCCGATTAAC	qRT-PCR
<i>IL-6</i> F	XM_013100522.2	TTCGACGAGGAGAAATGCTT	qRT-PCR
<i>IL-6</i> R		CCTTATCGTCGTTGCCAGAT	qRT-PCR
<i>IL-1β</i> F	DQ393268	AAAACGCTCTTCGTGCTGTC	qRT-PCR
<i>IL-1β</i> R		CTCCTGCTGCTCTTCCTCAC	qRT-PCR
<i>OASL</i> F	KY775584	AGTTTGACATTGCCAGTCC	qRT-PCR
<i>OASL</i> R		TCCTCCTCGTGATTCCATTT	qRT-PCR
<i>18S</i> F	AF173614.1	TGTGCCGCTAGAGGTGAAATT	qRT-PCR
<i>18S</i> R		TGGCAAATGCTTTCGCTTT	qRT-PCR
<i>β-actin</i> F	EF667345.1	GCCCTCTTCAGCCATCTTT	qRT-PCR
<i>β-actin</i> R		CTTCTGCATCCTGTCAGCGA	qRT-PCR
DPV <i>UL30</i> F	JQ647509.1	TTTCCTCCTCCTCGCTGAGTG	Absolute RT-PCR
DPV <i>UL30</i> R		CCAGAAACATACTGTGAGAGT	Absolute RT-PCR
Taqman probe to DPV <i>UL30</i>		CGCTTGTACCCAGGG	Absolute RT-PCR

Abbreviations: DEF, duck embryo fibroblast; DPV, duck plague virus; IFN, interferon; MX, myxovirus resistance; OASL, 2'-5'-oligoadenylate synthetase-like protein; Q-PCR, quantitative PCR; qRT, quantitative real time; RT, reverse transcription.



**Figure 1.** SC75741 potentially antagonizes virus infection among the inflammatory pathways. (A) The DEF cells were infected with 1 MOI of VSV-GFP for 1 h; then, the JNK inhibitor (SP600125, 10  $\mu$ mol/mL), ERK inhibitor (LY3214996, 5  $\mu$ mol/mL), p38 inhibitor (TAK-715, 5  $\mu$ mol/mL), and NF- $\kappa$ B inhibitor (SC75741, 5  $\mu$ mol/mL) was added; DMSO was used as control group. At 12 and 24 hpi, the GFP expression was captured by using a fluorescence microscope. The scale bar is 100 nm. (B–D) The DEF cells were infected with 1 MOI of VSV-GFP, DTMUV, and DPV-GFP for 1 h. Then, the JNK inhibitor (SP600125, 10  $\mu$ mol/mL), ERK inhibitor (LY3214996, 5  $\mu$ mol/mL), p38 inhibitor (TAK-715, 5  $\mu$ mol/mL), and NF- $\kappa$ B inhibitor (SC75741, 5  $\mu$ mol/mL) were added; DMSO was used as control group. At 24 hpi, the cell culture supernatants were collected, and the FFU of VSV-GFP, and the TCID<sub>50</sub> of DTMUV or DPV were determined on BHK21 or DEF cells. Abbreviations: BHK21, baby hamster kidney cells; DEF, duck embryo fibroblast; DMSO, dimethyl sulfoxide; DPV, duck plague virus; DTMUV, duck Tembusu virus; ERK, extracellular signal-regulated kinase; FFU, fluorescence formation units; hpi, hours postinfection; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MOI, multiplicity of infection; NF- $\kappa$ B, nuclear factor-kappa B; TCID<sub>50</sub>, tissue culture infectious dose 50; VSV, vesicular stomatitis virus.



**Figure 2.** SC75741 dose dependently inhibits virus growth. (A, B, D–F) The DEF cells were infected with 1 MOI of VSV-GFP, DTMUV, and DPV-GFP for 1 h. Then, different doses of SC75741 were added; DMSO was used as control group. At 24 hpi, the viral plaque of (A) VSV-GFP and (B) DPV-GFP was captured by using a fluorescence microscope. The scale bar is 100 nm. At 24 hpi, the cell culture supernatants were collected, and (D) FFU of VSV-GFP, and (E, F) the TCID<sub>50</sub> of DTMUV or DPV was determined on DEF cells. (C) The uninfected DEF cells were treated with different doses of SC75741 for 24 h; the cell viability in each group was determined by MTT method. Abbreviations: DEF, duck embryo fibroblast; DMSO, dimethyl sulfoxide; DPV, duck plague virus; DTMUV, duck Tembusu virus; FFU, fluorescence formation units; hpi, hours postinfection; MOI, multiplicity of infection; TCID<sub>50</sub>, tissue culture infectious dose 50; VSV, vesicular stomatitis virus.

## Statistical Analysis

Data are presented as SD or SEM, and Student's *t* test, one-way analysis of variance, or Tukey's post hoc test was applied to evaluate significant differences. At least 3 repeats of each group were set in the treatment group and each sample was separately detected. All experiments were repeated 3 times individually. Graphs were plotted and analyzed using GraphPad Prism software, version 6.0 (GraphPad Software, La Jolla, CA). The level of statistical significance was marked with asterisks (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001).

## RESULTS

### SC75741 Potentially Antagonizes RNA and DNA Virus Infection in DEF Cells

In order to testify the impact of mitogen-activated protein kinase and NF- $\kappa$ B pathways on the virus infection in duck cells, we compared the viral plaque formation and titer after blockage of JNK, ERK, p38, and NF- $\kappa$ B pathway with the specific inhibitor. Firstly, the DEF cells were infected with 1 MOI of the model virus, VSV-GFP, for 1 h; after that, SP600125 (JNK inhibitor, 10  $\mu$ mol/mL), LY3214996 (ERK inhibitor, 5  $\mu$ mol/mL), TAK-715 (p38 inhibitor, 5  $\mu$ mol/mL), SC75741 (NF- $\kappa$ B inhibitor, 5  $\mu$ mol/mL), or solvent DMSO was added. The GFP expression from VSV-GFP was captured at 12 and 24 hpi; we observed that the GFP expression was reduced by the treatment of SC75741, at both time points (Figure 1A). The virus titer of VSV-GFP was detected in the cell culture supernatant at 24 hpi; we observed that the FFU of VSV-GFP decreased about one log with the treatment of SP600125, and reduced around 100 times in SC75741-treated cell culture supernatant compared to the DMSO group (Figure 1B). In the same manner, we examined these inhibitors in the infections of a duck RNA virus (Figure 1C), DTMUV, and a duck DNA virus (Figure 1D), DPV. Similar results were observed: the viral titers of DTMUV and DPV were significantly decreased by SP600125 and SC75741, in which SC75741 demonstrated better inhibition among these 4 inhibitors. Altogether, these data demonstrated that the JNK and NF- $\kappa$ B pathway played a key role in VSV, DTMUV, and DPV infection in duck cells, and SC75741 showed antiviral potency for duck virus infection; hence, we focused on the antiviral mechanism of SC75741 in duck cells.

### SC75741 Dose-Dependent Antiviral Infection in DEF Cells

In order to study the inhibitory ability of SC75741 in greater detail, a dose-dependent antiviral activity was examined. The DEF cells were infected with 1 MOI of VSV-GFP (Figure 2A) or DPV-GFP (Figure 2B) for 1 h, and then 0.2, 0.5, 1, 5, 10, 20, 50  $\mu$ mol/mL of SC75741 was added into the cell culture medium and

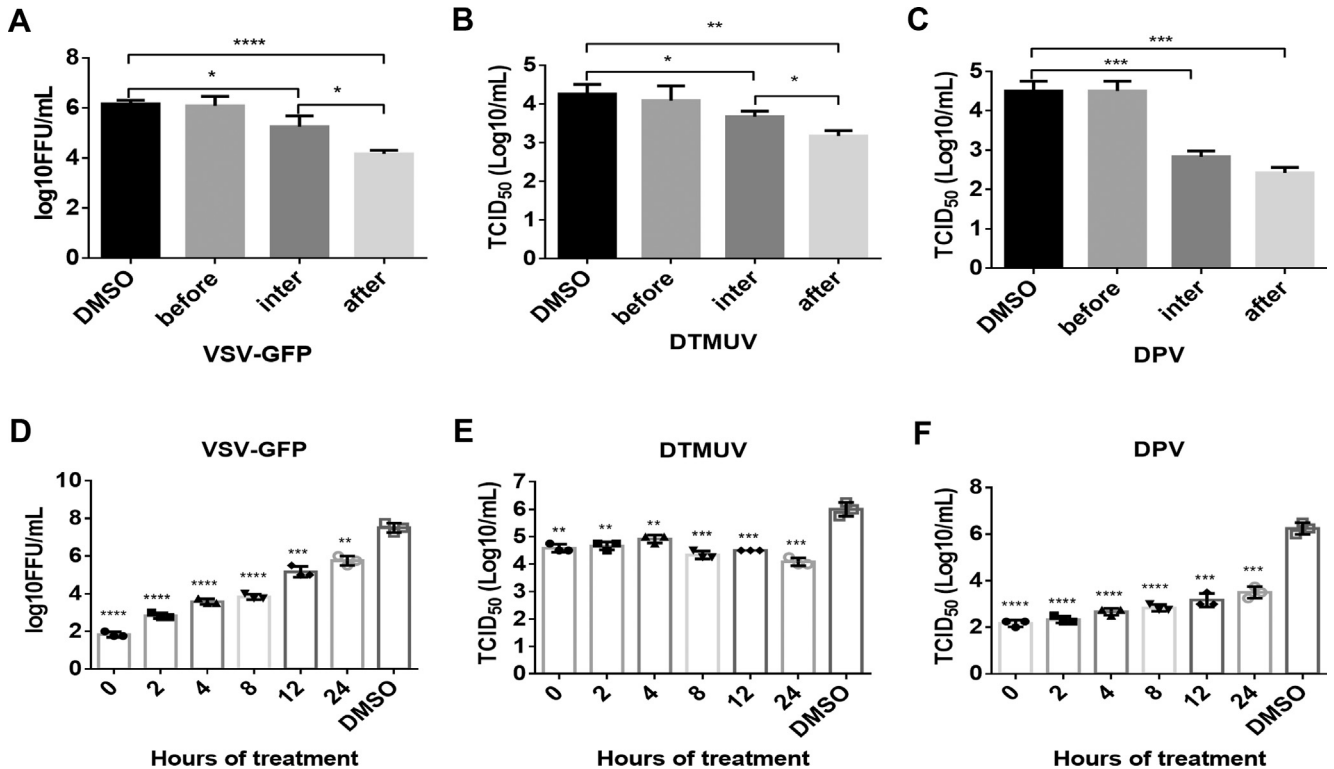
the GFP expression was recorded at 24 hpi. The GFP expressions from both viruses were reduced gradually with increasing concentration of SC75741; above 50% of viral plaque formation was repressed at 0.5  $\mu$ mol/mL of SC75741 (Figures 2A and 2B). Cell viability was observed after the uninfected DEF cells were treated with different doses of SC75741, and the MTT data indicated that the cell viability of SC75741-treated DEF cells was comparable to the mock group (Figure 2C); hence, the antiviral ability in DEF cells did not result from cell death caused by SC75741. The viral titers of VSV-GFP, DTMUV, and DPV-GFP were determined and we observed that the viral titers gradually decreased with increasing doses of SC75741. The viral titers of the RNA viruses, VSV-GFP (Figure 2D) and DTMUV (Figure 2E), decreased at 0.5  $\mu$ mol/mL of SC75741 and significantly reduced at 2 to 50  $\mu$ mol/mL of SC75741. The viral titers of the DNA virus, DPV-GFP, decreased at 0.5  $\mu$ mol/mL of SC75741 and significantly reduced at 1 to 50  $\mu$ mol/mL of SC75741 (Figure 2F). These data demonstrated that SC75741 potentially antagonized a broad spectrum of virus strains in duck cells and the antiviral activity was dose-dependent.

### SC75741 Therapeutically Inhibits Virus Infection

As SC75741 inhibited virus infection in a dose-dependent manner, 5  $\mu$ mol/mL of SC75741 was able to significantly inhibit virus infection without causing cell death. Thus, 5  $\mu$ mol/mL of SC75741 was screened and chosen based on its antiviral activity in murine cells similar to a previous study (Ehrhardt et al., 2013). This dose was chosen as the primary concentration for further assays. We examined whether SC75741 could inhibit virus growth once the infection is established. Firstly, we conducted the time-addition assay of SC75741, before, during, and after the virus infection on DEF cells (Figures 3A, 3C and 3E). No obvious reduction of viral titer of VSV-GFP, DTMUV, and DPV was observed at 24 hpi, when SC75741 was added before the virus infection. The viral titers were reduced when SC75741 was added during and after the VSV-GFP, DTMUV, and DPV infection (Figures 3A, 3C and 3E), and we observed that the viral titers of DPV were most significantly reduced when SC75741 was added during and after virus infection (Figure 3E).

Furthermore, we examined the therapeutic antiviral activity of SC75741; the DEF cells were infected with 1 MOI of VSV-GFP, DTMUV, or DPV for 1 h, SC75741 (5  $\mu$ mol/mL) was added at 0, 2, 4, 8, 12, 24 hpi, and the viral titer in the cell culture supernatant was determined at 48 hpi. We observed that the viral titer of VSV-GFP (Figure 3B), DTMUV (Figure 3D), and DPV (Figure 3F) was significantly reduced when SC75741 was added at different time points, including 24 hpi. With the delay of SC75741 addition, the antiviral effect gradually weakened for VSV and DPV





**Figure 3.** SC75741 therapeutically blocks the growth of viruses on DEF cells. (A, C, E) The DEF cells were infected with 1 MOI of VSV-GFP, DTMUV, and DPV for 1 h. SC75741 (5  $\mu\text{mol}/\text{mL}$ ) was added before, in the middle of (during), or after VSV-GFP, DTMUV, and DPV infection; DMSO was added after infection as control. At 24 hpi, the TCID<sub>50</sub> of DPV in the cell culture supernatants was determined. (B, D, F) The DEF cells were infected with 1 MOI of VSV-GFP, DTMUV, and DPV for 1 h; after infection, SC75741 (5  $\mu\text{mol}/\text{mL}$ ) was added at different time points. At 48 hpi, the cell culture supernatants were collected, and (B) the FFU of VSV-GFP, and the TCID<sub>50</sub> of (C) DTMUV or (D) DPV were determined. Abbreviations: DEF, duck embryo fibroblast; DMSO, dimethyl sulfoxide; DPV, duck plague virus; DTMUV, duck Tembusu virus; FFU, fluorescence formation units; hpi, hours postinfection; MOI, multiplicity of infection; TCID<sub>50</sub>, tissue culture infectious dose 50; VSV, vesicular stomatitis virus.

(Figures 3B and 3F). However, distinct dynamics for DTMUV was observed when the viral titer was reduced more than the former after the addition of SC75741 at 24 hpi (Figure 3D). These data demonstrated that SC75741 can block virus infection when added in the middle of virus infection and function better when it sustained in the culture medium after virus infection, and SC75741 can inhibit virus growth after infection is established for 24 h, indicating the therapeutic antiviral activity of SC75741 in duck cells.

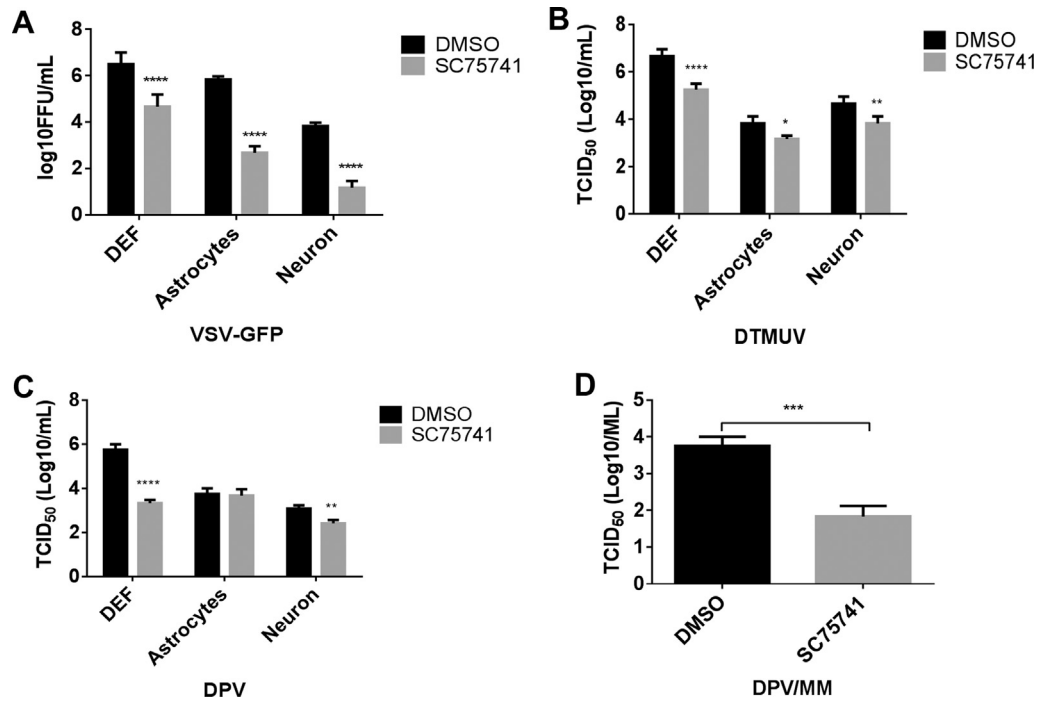
### SC75741 Inhibits Virus Infection in Different Duck Cell Types

We further determined whether SC75741 could inhibit virus infection in different duck cell types. For this reason, we isolated duck primary neurons, astrocytes, and monocytes or macrophages as previously described (Tian et al., 2019). We observed that the viral titers of VSV-GFP (Figure 4A) and DTMUV (Figure 4B) were reduced in duck neurons and astrocytes under the addition of SC75741 as in the DEF cells. The viral titers of DPV were reduced in duck neurons and DEF cells, but not in astrocytes (Figure 4C). As we previously demonstrated that DPV can infect monocytes or macrophages, we examined the antiviral activity of SC75741 in duck monocytes or macrophages. We

observed that the viral titers of DPV in the cell culture supernatant were significantly reduced after the addition of SC75741 (Figure 4D). These data indicated that SC75741 demonstrates broad-spectrum antiviral activity to inhibit virus infection in different duck cell types.

### SC75741 Inhibits the Viral Entry and Viral Genomic Replication of DPV

As we have demonstrated earlier that DPV was inhibited profoundly by SC75741, we further focused on the inhibitory mechanism of SC75741 for DPV. Firstly, we testified the antiviral duration of SC75741 in DPV infection. The DEF cells were infected with 0.01 or 1 MOI of DPV for 1 h, and then SC75741 (5  $\mu\text{mol}/\text{mL}$ ) was added. The viral titers in the cell culture supernatant were detected at 24, 48, and 72 hpi, and we observed that the titers of DPV were significantly reduced by SC75741 at both the multistep infection (0.01 MOI) (Figure 5A) and one-step-infection (1 MOI) (Figure 5B) growth curve. Then the inhibition of SC75741 on the virus life cycle was examined; we observed that SC75741 blocked the cell adsorption (Figure 5C) and genome replication (Figure 5E) stage of the DPV-GFP life cycle, but not at the stage of cell endocytosis (Figure 5D) and progeny virus release



**Figure 4.** SC75741 antagonizes virus infection in different cell types of duck. (A–C) Primary DEF, neurons, and astrocytes cells of duck were infected with 1 MOI of VSV-GFP, DTMUV, and DPV for 1 h; then, SC75741 (5  $\mu\text{mol/mL}$ ) was added at different time points. At 24 hpi, the cell culture supernatants were collected, and (A) the FFU of VSV-GFP, and the TCID<sub>50</sub> of (B) DTMUV or (C) DPV were determined. (D) Duck primary monocytes or macrophages cells were infected with 1 MOI of DPV for 1 h; then, SC75741 (1  $\mu\text{mol/mL}$ ) was added at different time points. At 24 hpi, the cell culture supernatants were collected, and the TCID<sub>50</sub> of DPV was determined. Abbreviations: DEF, duck embryo fibroblast; DPV, duck plague virus; DTMUV, duck Tembusu virus; FFU, fluorescence formation units; hpi, hours postinfection; MOI, multiplicity of infection; TCID<sub>50</sub>, tissue culture infectious dose 50; VSV, vesicular stomatitis virus.

(Figure 5F). These data demonstrated that SC75741 has a lasting inhibitory duration in DPV infection, and it can block viral entry and restrict genome replication of the DPV life cycle.

### SC75741 Enhances IFN and IFN-Stimulated Gene (ISG) Expression, but Inhibits Inflammatory Responses

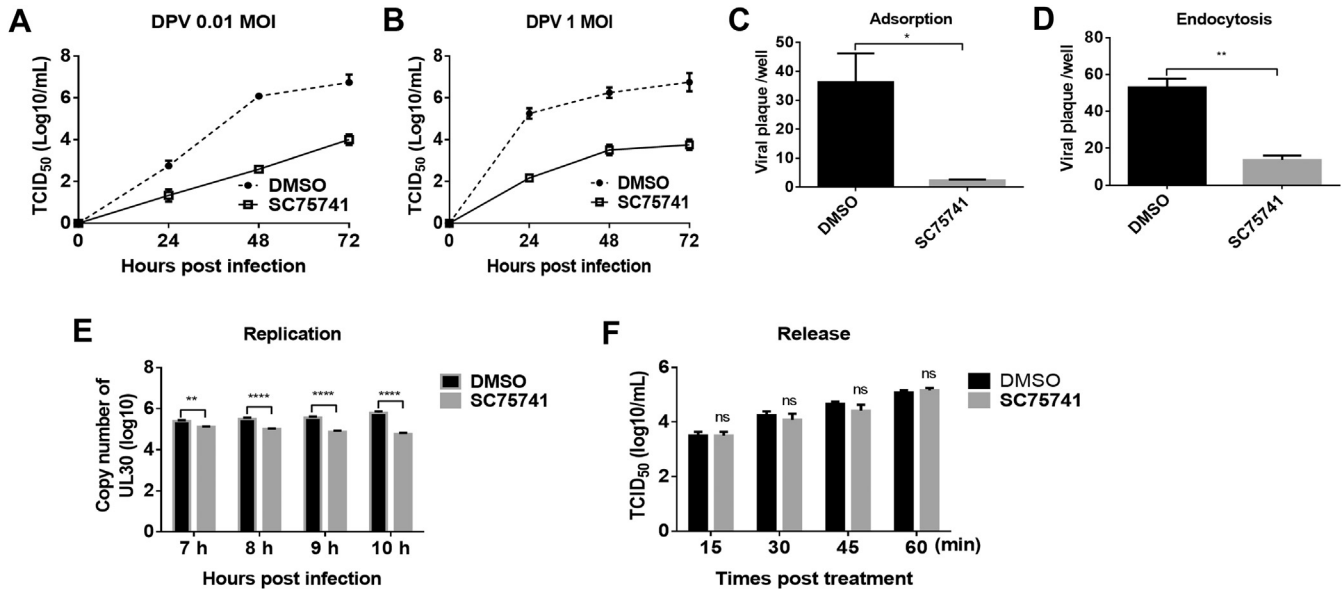
We further examined the impact of SC75741, an NF- $\kappa\text{B}$  inhibitor demonstrated in murine cells (Ehrhardt et al., 2013; Haasbach et al., 2013), on expressions of the inflammatory cytokines, IFN, and ISGs. We observed that SC75741 (1  $\mu\text{mol/mL}$ ) reduced the basal expression level of IL-6 and CCL21 (Figure 6A), but not IL-1 $\beta$ , in duck monocytes or macrophages without virus infection or stimulators. Besides, we found that the basal expression level of IFN- $\beta$ , OASL, and MX was promoted by SC75741 in duck monocytes or macrophages (Figure 6A). Thus, we proposed that SC75741 may promote IFNR signaling in duck cells, and we tested this hypothesis in the DEF cells. We directly added SC75741 at different doses from 0.5 to 20  $\mu\text{mol/mL}$  into the culture medium of DEF cells and collected the cell lysates at 6, 12, 24 h posttreatment; the basal expression level of IL-6, CCL21, IL-1 $\beta$ , IFN- $\beta$ , OASL, and MX was detected by Q-PCR. However, no obvious changes in these genes were detected (data not shown). Our previous study has shown that the basal expression level of STING, RIG-I, MDA5, MAVS, IRF7, IFN- $\beta$ , and MX in

monocytes or macrophages was higher than that in DEF cells, neurons, and astrocytes (Tian et al., 2019). Hence, we hypothesized that SC75741 may promote innate immunity responses relying on the basal level of these genes. We co-transfected the DEF cells with various expressing plasmids, with IFN- $\beta$  luciferase plasmid, to overexpress duck STING, RIG-I, MDA5, MAVS, TANK binding kinase 1, IFN regulatory factor 1, or IRF7 protein, and SC75741 was added at 12 h post transfection. The luciferase value was detected at 36 h post transfection. We observed that SC75741 significantly enhanced the expression of IFN- $\beta$  preactivated by these genes, especially for MAVS, RIG-I, and STING (Figure 6B). As STING is the co-adaptor of cyclic GMP-AMP synthase and RIG-I-like receptors (RLRs) pathway, we overexpressed STING in DEF cells and added SC75741 as a stimulator. We observed that SC75741 promoted the expression of IL-1 $\beta$ , IFN- $\beta$ , OASL, and MX in DEF cells with the STING overexpression (Figure 6C), but not the level of IL-6 and CCL21 that was inhibited by SC75741 in duck monocytes or macrophages (Figure 6A). These data indicate that SC75741 enhances IFN and ISG expression but inhibits inflammatory responses.

### SC75741 Promotes IFN- $\beta$ Expression as a STING Agonist in DPV-Infected DEF Cells

We further examined the expression of STING and IFN- $\beta$  under DPV infection and co-treated with





**Figure 5.** SC75741 inhibits DPV infection by interfering with viral adsorption and replication. (A, B) DEF cells were infected with 0.01 or 1 MOI of DPV. At the indicated time points after infection, culture medium was collected and titrated with a TCID<sub>50</sub> assay. Results were representative of 3 independent experiments. (C–F) SC75741 was added at different time points in the viral life phase, and (C) viral entry, (D) endocytosis, (E) genomic replication, and (F) virion release were analyzed as described in the Materials and Methods section. Abbreviations: DEF, duck embryo fibroblast; DMSO, dimethyl sulfoxide; DPV, duck plague virus; MOI, multiplicity of infection; TCID<sub>50</sub>, tissue culture infectious dose 50.

SC75741. Since SC75741 is extremely good at inhibiting DPV infection, we infected the DEF cells with 5 MOI of DPV for 12 h to establish infection and stimulate the expression of the innate immune gene beforehand, and then SC75741 was added to treat the cell for 6 or 12 h. The expressions of STING and IFN- $\beta$  were determined at 18 and 24 hpi. We observed that the level of STING (Figure 7A) and IFN- $\beta$  (Figure 7B) was enhanced by SC75741, even though the viral genomic copy number and the expression of RIG-I, MAVS, OASL, and MX was significantly lower in SC75741-treated cells (data not shown). Then, we applied the IFNR specific inhibitor, ruxolitinib, to block the IFN signaling in DEF cells. Under such conditions, the viral titer of DPV was recovered in SC75741 and ruxolitinib-treated cells (Figure 7C). The viral titer of DTMUV and VSV inhibited by SC75741 was also restored with ruxolitinib (data not shown). These results demonstrated that SC75741 inhibits virus infection by promoting STING-mediated IFN signaling.

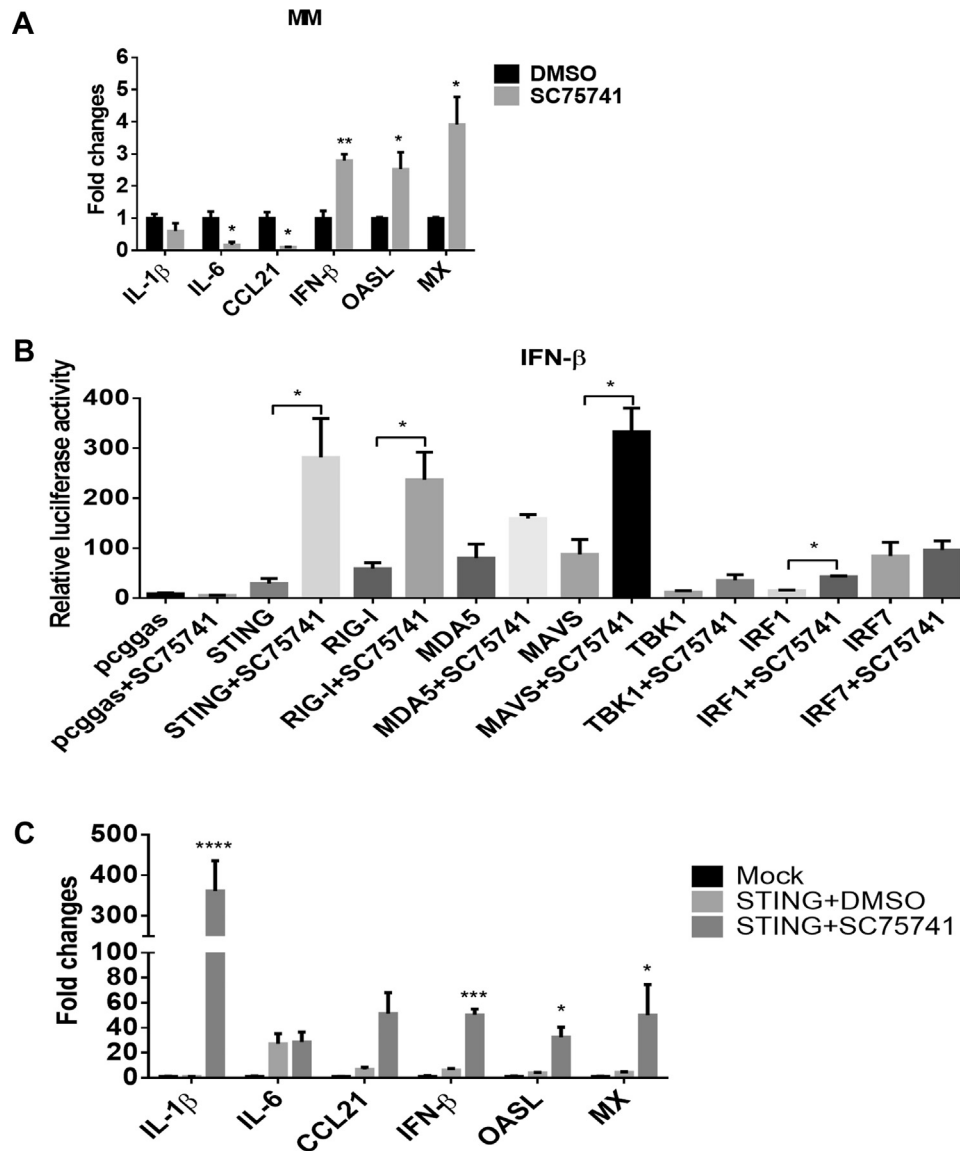
## DISCUSSION

In our current study, we compared the antiviral activity of inhibitors targeting JNK, ERK, p38, and NF- $\kappa$ B signaling, and found that the JNK pathway inhibitor, SP600125, and NF- $\kappa$ B pathway inhibitor, SC75741, restrained the VSV, DTMUV, and DPV infection in DEF cells. Further analysis of the antiviral ability demonstrated that SC75741 reduced VSV, DTMUV, and DPV infection in DEF cells in a dose-dependent manner, and revealed promising therapeutic effects for the established virus infection. SC75741 demonstrates broad-spectrum antiviral activity to inhibit virus infection in different duck cell types. A study demonstrated

that SC75741 blocked cell entry and reduced the genomic replication stage of the DPV life cycle, and inhibited the expression of IL-6 and CCL21 in duck monocytes or macrophages, but promoted the expression of IL-1 $\beta$ , IFN, and ISG as a STING agonist in DEF cells. These results demonstrated that SC75741 may play a pivotal role as a STING agonist to promote the activation of IFNR signaling in a viral infection-dependent manner in DEF cells, where STING is preactivated by virus infection.

SC75741 has been screened as a broad inhibitor of NF- $\kappa$ B signaling in murine cells (Leban et al., 2007). SC75741 not only interferes with the NF- $\kappa$ B subunit (p65) binds to DNA promoter to reduce the expression of cytokines, chemokines, and proapoptotic factors, but also inhibits the caspase activation and the caspase-dependent nuclear export of viral ribonucleoproteins. (Ehrhardt et al., 2013; Haasbach et al., 2013). In our present study, we found that DPV was more profoundly inhibited by SC75741, which is likely because of the genomic replication of DPV occurring in the nucleus that relies on the caspase-dependent nuclear export of viral ribonucleoproteins for viral DNA or protein synthesis, whereas VSV and DTMUV replicate in the cytoplasm. We also observed that SC75741 has the ability to inhibit VSV, DTMUV, and DPV when SC75741 was added in the middle of the virus infection. We speculate that SC75741 may block the cell receptors for these viruses or reduce the cell membrane movement and the endocytosis for viruses entering the cells.

We examined the antiviral activity of SC75741 in duck cells including DEF, neurons, astrocytes, and monocytes or macrophages. We found that SC75741 inhibited VSV and DTMUV infection in DEF, neurons, and astrocytes of duck cells. DPV was inhibited by

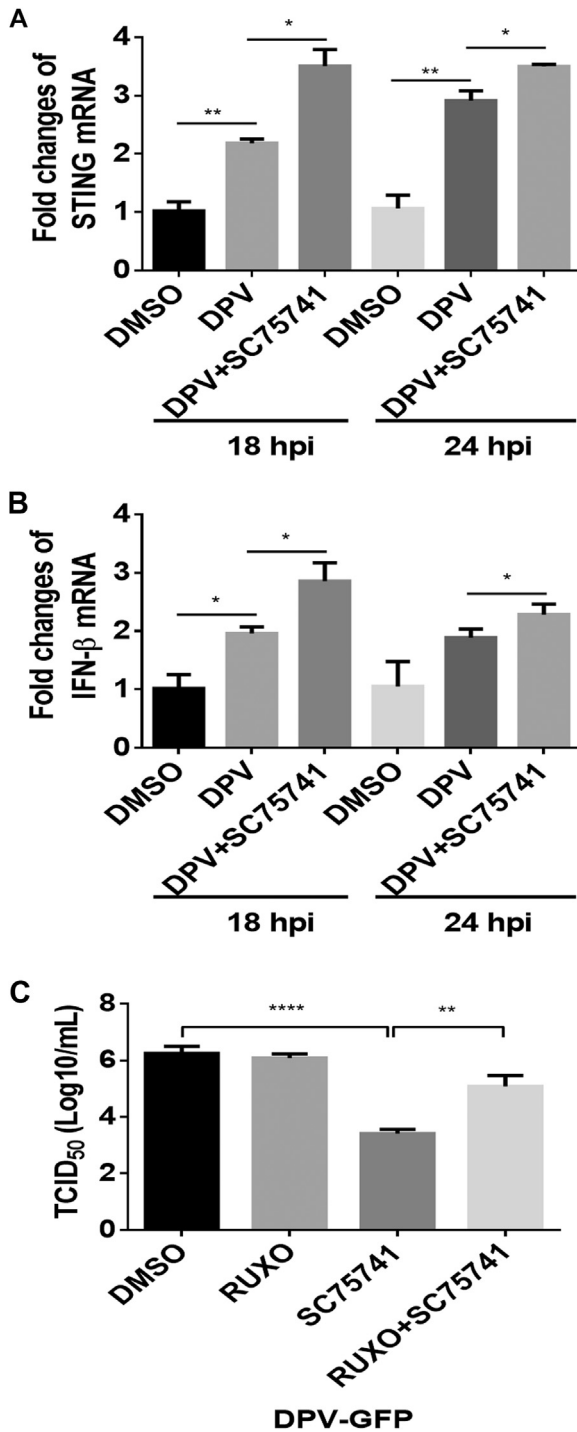


**Figure 6.** SC75741 acts as a STING agonist to promote IFN expression. (A) The duck monocytes-macrophages were treated with SC75741 at a dose of 1  $\mu$ mol/mL; DMSO was used as control group. At 24 h posttreatment, the cells were collected and RNA purified for Q-PCR assays. (B) Various expressing plasmids (500 ng/well) were co-transfected with 100 ng/well reporter plasmids, pGL-IFN- $\beta$  with 50 ng/well pRL-TK plasmid. SC75741 (5  $\mu$ mol/mL) was added at 12 h post transfection, while DMSO was used as negative control. At 36 h post transfection, cells were harvested and luciferase activity was measured. (C) The expressing plasmid pcggas-STING (500 ng/well) was transfected into DEF cells; SC75741 (5  $\mu$ mol/mL) or DMSO was added at 12 h post transfection. At 36 h post transfection, the cells were collected for Q-PCR assays. Abbreviations: DEF, duck embryo fibroblast; DMSO, dimethyl sulfoxide; IFN, interferon; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene 5; MX, myxovirus resistance; OASL, 2'-5'-oligoadenylate synthetase-like protein; Q-PCR, quantitative PCR; RIG-I, retinoic acid inducible gene I; STING, stimulator of interferon genes; TBK1, TANK binding kinase 1.

SC75741 in DEF, neurons, and monocytes or macrophages, but not in astrocytes, suggesting that SC75741 could inhibit RNA and DNA virus infection in different types of duck cells, and could be used to counterwork the infection of DPV and DTMUV due to its tropism for duck cells. For DPV, SC7541 did not show inhibitory activity in duck primary astrocytes, which might be because the receptors for viral entry in astrocytes are different from DEF, neurons, and monocytes or macrophages, or the NF- $\kappa$ B signaling is dispensable for DPV infection in astrocytes.

The cyclic GMP-AMP synthase or STING pathway is the principal intracellular sensor that activates the IFN expression responses and mediates host defense against

various DNA and RNA virus infections (Ma and Damania, 2016). STING-dependent IFN signaling is also activated through RLR and MAVS under DNA or RNA virus infection (Maringer and Fernandez-Sesma, 2014; Liu et al., 2016; Wu et al., 2019). VSV infection is related with the RLR pathway (Crill et al., 2015) and cytokines expression may be mediated by STING. Furthermore, the duck STING gene was previously reported to repress DPV infection through the IFN-dependent pathway (Chen et al., 2018). It is noteworthy that the development of antiviral agents as STING agonists against virus infection attracted attention worldwide (Guo et al., 2015; Sali et al., 2015). In the current study, SC75741 was observed to enhance the expression



**Figure 7.** SC75741 promotes IFN signaling depending on DPV infection. (A, B) DEF cells were infected with 5 MOI of DPV; SC75741 (5  $\mu\text{mol}/\text{mL}$ ) was added at 12 hpi; DMSO was used as negative control. At 18 and 24 hpi, the cells were collected for Q-PCR assays. The relative mRNA level of STING and IFN- $\beta$  were detected with specific primers. Relative expression is presented as fold-change compared to mock treatment. (C) The DEF cells were infected with 1 MOI of DPV-GFP for 1 h; after infection, a different dose of ruxolitinib, SC75741, or both were added; DMSO was used as the control group. At 24 hpi, the cell culture supernatants were collected, and the TCID<sub>50</sub> of DPV-GFP was determined on DEF. Data were means from 3 independent experiments and each experiment was analyzed in triplicate. Student's *t* test was performed to evaluate the differences. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . Abbreviations: DEF, duck embryo fibroblast; DMSO, dimethyl sulfoxide; DPV, duck plague virus; hpi, hours postinfection; hpi, hours postinfection; IFN, interferon; MOI, multiplicity of infection; Q-PCR, quantitative PCR; RUXO, ruxolitinib; STING, stimulator of interferon genes; TCID<sub>50</sub>, tissue culture infectious dose 50.

of IL-1 $\beta$ , IFN, and ISG in DEF only when STING was overexpressed or induced by DPV infection (Figures 6 and 7); hence, we propose that this compound might act as a STING agonist in duck cells, and this may be the reason why genomic replication was inhibited by SC75741 (Figure 5E); however, the molecular mechanism will be further investigated in the future. DTMUV was demonstrated to cleave STING through NS2B protease to impair the IFN expression (Wu et al., 2019). This may be the reason for the different inhibitory outcome among VSV, DTMUV, and DPV, when SC75741 was added at different time points after the virus infection was established.

In conclusion, we illustrated that SC75741 potentiated the antiviral activity for VSV, DTMUV, and DPV infection in a dose-dependent manner, and in different cell types of duck. It has antiviral potential to inhibit virus infection by blocking virus entry and interfering with viral genome replication, even though the virus infection is established. This compound may act as a STING agonist to upregulate the IFN expression in a viral infection-dependent manner. We concluded that SC75741 might be a very promising lead compound and further in vivo studies are warranted to develop antiviral drugs to control the epidemic outbreak of DTMUV and DPV.

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## DISCLOSURES

The authors have no financial conflicts of interest.

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