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# Kinase inhibitor roscovitine as a PB2 cap-binding inhibitor against influenza A virus replication

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## ARTICLE INFO

### Article history:

Received 15 March 2020

Accepted 9 April 2020

Available online 13 April 2020

### Keywords:

Influenza A virus

Roscovitine

Kinases inhibitor

Viral polymerase

PB2cap-binding inhibitor

## ABSTRACT

In this study, we examined the impact of roscovitine, a cyclin-dependent kinase inhibitor (CDKI) that has entered phase I and II clinical trials, on influenza A viruses (IAVs) and its antiviral mechanism. The results illustrated that roscovitine inhibited multiple subtypes of influenza strains dose-dependently, including A/WSN/1933(H1N1), A/Aichi/2/68 (H3N2) and A/FM1/47 (H1N1) with IC<sub>50</sub> value of 3.35 ± 0.39, 7.01 ± 1.84 and 5.99 ± 1.89 μM, respectively. Moreover, roscovitine suppressed the gene transcription and genome replication steps in the viral life cycle. Further mechanistic studies indicated that roscovitine reduced viral polymerase activity and bound specifically to the viral PB2cap protein by fluorescence polarization assay (FP) and surface plasmon resonance (SPR). Therefore, we believed roscovitine, as a PB2cap inhibitor, was a prospective antiviral agent to be developed as therapeutic treatment against influenza A virus infection.

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## 1. Introduction

Human influenza is one of the most common infectious diseases affects billions of people around the globe annually. It tends to come during the winter season and often times swing across countries over the course of months as an epidemic, causing significant morbidity and mortality as the patients may develop severe respiratory infections, pneumonia, meningitis, etc. In addition to a direct impact of the virus itself, influenza also may bring complications such as bacterial super-infections and cardiovascular

events that cause numerous infected individuals death [1]. New strains of the influenza virus can be transmitted from animals to human, and the virus often evolved rapidly over time, which makes vaccine development more difficult in general. On the other hand, because of continuous antigenic drift and antigenic shifts in the influenza viral surface glycoproteins, a vaccine formulated for one subtype of influenza maybe ineffective in the following year due to a new epidemic strain. Hence, human influenza viruses continue to pose important threats to human health in the modern society. Various prevention and treatment methods of influenza have been widely studied around the world.

Currently, the use of antiviral drugs is regarded as the first line to defense the influenza epidemic and pandemic. M2-ion channel inhibitor amantadine, the neuraminidase (NA) inhibitors zanamivir and oseltamivir are the FDA-approved antiviral drugs. However, both classes of drugs face the increased drug resistance challenge. Thus, despite the utility of these antiviral drugs, novel antivirals are in demand due to the emergence of adverse effects and the increased resistance of new influenza strains. For example, the US FDA approved Xofluza (baloxavir marboxil), an influenza virus polymerase inhibitor, for the treatment of influenza with symptoms of less than 48 h in people at the age of 12 or over.

Exploring the antiviral activity of existing drugs with known

**Abbreviations:** CC<sub>50</sub>, 50% cytotoxic concentration; CDKI, Cyclin-dependent kinases inhibitor; CPE, Cells cytopathic effect; CTD, Carboxy-terminal domain; DMEM, Dulbecco's modified Eagle's medium; FP, Fluorescence polarization assay; IAV, Influenza A virus; IC<sub>50</sub>, 50% inhibitory concentration; MDCK, Madin-Darby canine kidney; NP, Nucleoprotein; PA, Polymerase acidic protein; PB1, Polymerase basic protein 1; PB2, Polymerase basic protein 2; RdRp, RNA-dependent RNA polymerase; RNAP-II, RNA polymerase II; SPR, Surface plasmon resonance; vRNPs, Viral ribonucleoproteins.

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safety profiles is feasible and cost-saving, such example including cyclin-dependent kinases inhibitor (CDKI) [2]. For instance, FIT-039 (CDK9 inhibitor) suppresses the replication of several viruses such as human papillomavirus and hepatitis B virus [3]. Flavopiridol and dinaciclib, both have the abilities against the replication of influenza virus by inhibiting the host's RNA polymerase II (RNAP-II) activity, which results in viral mRNA transcription decreased [4].

Roscovitin is a purine derivative CDKI that inhibits CDK1, CDK2, CDK5, CDK7 and CDK9. It has been reported that roscovitin blocked the replication of a variety of viruses *in vitro*, such as herpes simplex virus (HSV) and human immunodeficiency virus (HIV) [5], while proving high safety in human clinical trials in the research of its effects against cancer.

In this study, we first identified the inhibitory activity of roscovitin against influenza A virus (IAV) replication, of which had not yet been reported in the past. Further investigation demonstrated that roscovitin specifically bound to highly conserved PB2cap region while inhibiting viral gene transcription and genome replication. These findings suggest that roscovitin is a promising PB2cap inhibitor for the therapeutic treatment of influenza A virus infection.

## 2. Materials and methods

### 2.1. Chemicals, cells, viruses and plasmids

Roscovitin was purchased from Bidepharm (Shanghai, China) with a purity of 99.35%. Zanamivir and peramivir were purchased from Sigma-Aldrich (St. Louis, MO, USA). D715-2441 was synthesized with a purity of more than 98% in our laboratory.

Madin Darby canine kidney (MDCK), human embryonic kidney (293T) cells and human lung bronchial epithelial (Beas-2B) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin. IAV subtypes including A/WSN/1933 (H1N1), A/FM-1/1/47 (H1N1), and A/Aichi/2/68 (H3N2) were stored at  $-80^{\circ}\text{C}$ .

Plasmids pHW2K-NP, pHW2K-PA, pHW2K-PB1, pHW2K-PB2 and pPolI-Fluc (firefly luciferase reporter plasmid) were generously supplied by Professor Bojian Zheng (University of Hong Kong, Hong Kong, China). The hRluc-TK (Renilla luciferase plasmid) was purchased from Promega (Madison, WI, USA).

### 2.2. Antiviral assay and microscopy

MDCK cells were infected with viruses ( $100\text{ TCID}_{50}$ ) in 96-well plates at  $37^{\circ}\text{C}$  for 1 h. Then the supernatant was replaced by roscovitin at 2-fold gradient dilutions containing  $1\ \mu\text{g}/\text{mL}$  TPCK (Sigma-Aldrich, MO, USA). At 48 h pi, cytopathic effects (CPE) in virus-infected cells were observed through microscopy and the antiviral level was detected by MTT-based assay.

### 2.3. Plaque assay

MDCK cells were treated with A/WSN/1933 (H1N1) (MOI = 0.01) for 1 h, followed by the addition of  $2\times\text{DMEM}$  (Sigma-Aldrich, MO, USA) mixed medium containing  $1\ \mu\text{g}/\text{mL}$  TPCK-trypsin (Sigma-Aldrich, MO, USA), 1% penicillin/streptomycin liquid, 2% microcrystalline cellulose and roscovitin at serial concentrations each well, then continued to culture for 72 h in a condition of 5%  $\text{CO}_2$ ,  $37^{\circ}\text{C}$ .

### 2.4. Immunofluorescence microscopy

MDCK cells in 48-well plate were infected with viruses (MOI = 0.01) as described above. After 24 h incubation, cells were

fixed by 4% paraformaldehyde for 20 min and blocked by 3% BSA for 1 h. After incubation with nucleoprotein (NP) antibody at  $4^{\circ}\text{C}$  overnight, cells were incubated with FITC-labelled secondary antibody for 1 h (Santa Cruz, CA, USA). DAPI nuclear dye was added and stained for 5 min. The fluorescence was observed at fluorescence microscope (Nikon, Japan).

### 2.5. Western blotting and quantitative real-time PCR

MDCK or Beas-2B cells were infected with viruses (MOI = 0.01) for 1 h, followed by the addition of roscovitin at different concentrations. After incubation, the cells were lysed by RIPA or RNA isolation kit. The antibodies were shown in Table S1 and the qRT-PCR primer sequences were shown in Table S2.

### 2.6. Time-of-addition assay

As previous reported [6], Beas-2B cells were infected with viruses (MOI = 0.01) for 1 h, then treated with roscovitin at the concentration of  $10\ \mu\text{M}$  in different time intervals, turn for 0-2 h, 2-5 h, 5-8 h, 8-10 h, and 0-10 h. Western blotting and quantitative real-time PCR were used to analyze the result.

### 2.7. Mini-replicon assay

The mini-replicon system was used to detect the cellular RNA-dependent RNA polymerase activity of influenza virus. 50 ng of PB1, PB2, PA and NP plasmids and 10 ng of pPolI-Fluc (a firefly luciferase reporter plasmid) together with 10 ng of hRluc-TK (Renilla luciferase plasmid) were transfected into 293T cells by Lipofectamine 3000. After 10 h transfection, the supernatant was replaced with fresh medium containing roscovitin. After culturing for 24 h, cells were lysed for 20 min, and luciferase activity was measured by luciferase reporter gene detection kit provided by Promega (Madison, WI, USA).

### 2.8. Fluorescence polarization assay

$50\ \mu\text{L}$  of the PB2cap was serially diluted with reaction buffer ( $100\ \text{mM}$  KCl,  $1\ \text{mM}$  DTT,  $0.5\ \text{mM}$  EDTA,  $50\ \text{mM}$  HEPES and 1% DMSO, pH = 7.2) and added into 96-well black plate. The FITC-labelled m7GTP (EDA-m7GTP-ATTO 488; Jena Bioscience, Germany) as a cap analog detection probe,  $20\ \text{nM}$  of probe was incubated with serial-diluted PB2cap at room temperature for 30 min. Then, roscovitin at 2-fold gradient dilutions were added into the plate for 30 min. The plate was measured by Infinite M1000 Pro (Tecan, USA).

### 2.9. Molecular docking assay

PB2cap protein structure was derived from the RCSB protein data bank (PDB code; 4CB4) [7]. The binding domain and ligand was assigned AMBER ff14SB force field and AM1-BCC charges, respectively. Docking was performed with UCSF Dock 6.7. The electrostatic interactions and van der Waals forces between roscovitin and the binding sites of PB2cap were acquired and constituted the Grid scores. Then the optimal model was obtained by cluster analysis (RMSD threshold of  $2\ \text{\AA}$ ).

### 2.10. Surface plasmon resonance (SPR) analysis

SPR data for roscovitin binding with the PB2cap protein was analyzed by PlexArray HT A100 system. Roscovitin was fixed on a sensor chip external by photo-cross-linking. Then PB2cap protein was injected by  $250\ \text{nM}$ ,  $500\ \text{nM}$  and  $1000\ \text{nM}$  at a flow of  $2\ \mu\text{L}/\text{s}$ .

The contact time and dissociation time were both 300 s. The running buffer was PBS and phosphate buffer was glycine-HCl (pH 2.0). The data was export by Plexera DE software and BIA evolution software was used to adjust the data and calculate the  $K_D$  value.

### 2.11. Statistical analysis

All statistical analyses of the data were carried out using GraphPad 5.0 Prism software. All experiments were performed three times and the results were expressed as the mean  $\pm$  standard deviation (SD). Statistical significance between two groups was analyzed by *t*-test. *P*-values < 0.05 were regarded as statistically significant.

## 3. Results

### 3.1. Roscovitine interferes with influenza replication

Before studying the antiviral activity of roscovitine, we first examined the effect of roscovitine on cell viability. Highest concentration evaluated for cytotoxicity assays was 200  $\mu$ M, and roscovitine reduced the cell viability by ~30% in MDCK or Beas-2B cells at this concentration (Fig. S1). Besides, roscovitine showed broad-spectrum inhibition against different subtypes of virus strains, including A/FM-1/1/47 (H1N1), A/WSN/1933(H1N1) and A/Aichi/2/68 (H3N2) (Table 1). The  $IC_{50}$  was approximately 3–10  $\mu$ M in different virus strains, implying the future derivatives of roscovitine would improve the antiviral properties while maintaining the lower cytotoxic effects. We conducted the further experiments with A/WSN/1933(H1N1) virus strain. Compared with viral infection group, the cytopathic effect (CPE) was decreased significantly (Fig. 1b). Besides, the number of plaques, the expression of viral mRNA and fluorescent spots showed the analogous downward trend in a dose-dependent manner (Fig. 1c–e). Furthermore, at 12 h and 24 h post-infection, the expression of viral NP and PB2 protein were reduced obviously (Fig. 1f). Simultaneously, an obvious reduction in the progeny viral particles of the roscovitine-treated supernatant was observed by plaque assay (Fig. 1g). All the results above demonstrated roscovitine could effectively inhibit influenza virus replication.

### 3.2. Roscovitine is unable to inhibit neither viral entry nor release

Hemagglutinin (HA) and NA are both the viral surface glycoproteins and relate to virus entry and release stages, respectively. Therefore, we exerted three experiments, hemagglutinin inhibition assay, H5N1 pseudovirus neutralization assay and NA inhibition assay, to confirm whether roscovitine has effect on viral entry and release. The result demonstrated roscovitine could not prevent red blood cells from hemagglutination (Fig. S2a) nor H5N1 pseudovirus infection, indicated it had no effect on virus entry (Fig. S2b). Meanwhile, no significant inhibition effect was observed on enzyme activity of NA, implying that the compound hardly affected

virus release (Fig. S2c). Therefore, we estimated that roscovitine might suppress influenza virus through other mechanisms.

### 3.3. Roscovitine blocks viral genome transcription and replication stage

First of all, a time-of-addition assay was performed to detect the antiviral stage of roscovitine. With the treatment of post-infection and entire-infection, the inhibition effect of roscovitine was improved obviously, especially post-infection mode, while had inconspicuous effect on the pre-treatment or pre-infection modes (Fig. S3), suggesting that the compound exerted inhibition effect after the virus entered target cells. As reported previously [8], one life cycle of the influenza virus was around 8–10 h, and was divided into three main steps: virus entry (0–2 h), viral gene transcription and genome replication (2–8 h) and progeny viral release (8–10 h). To explore the period of viral life cycle disturbed by roscovitine, Beas-2B cells were treated with roscovitine at different time intervals during one viral life cycle (Fig. 2a). As shown in Fig. 2b and c, the compound could greatly lessen the expression level both viral protein and mRNA at 2–5 h or 5–8 h time interval, indicating it blocked viral gene transcription and genome replication stage. Because of viral RNA-dependent RNA polymerase (RdRp) playing an important role in viral genome transcription and replication stage, we conjectured the antiviral ability was relevant to interfere with viral RdRp activity.

### 3.4. Roscovitine affects influenza viral polymerase activity

As reported previously [9], drugs that inhibit host's RNAP-II and CDK9 show anti-influenza activity. Under the treatment of above host factors inhibitors, the process of influenza virus snatching 5'-cap structures from host's pre-mRNAs synthesized by RNAP-II will be affected. Therefore, we supposed roscovitine may exert antiviral effect by inhibiting RNAP-II activity. To confirm this, we explored the phosphorylation level of carboxy-terminal domain (CTD) to estimate the effect on RNAP-II. As shown in Fig. 3a, roscovitine played weakly inhibition ability of the CTD-ser2 domain up to 20  $\mu$ M, whereas the viral M2 protein expression markedly reduced at 2.5  $\mu$ M. Therefore, we considered its major antiviral effect might target to virus itself, not inhibiting the activity of RNAP-II.

Next, we conducted the mini-replicon system, a cellular level of viral RdRp activity assay. Polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA) and NP plasmids were transfected into 293T cells, acting as viral RdRp and NP complexes. The reporter plasmids would transcribe into corresponding viral mRNA mediating luciferase expression. As showed in Fig. 3b, the activity of reporter gene decreased in a dose-dependent manner, revealing that roscovitine inhibited polymerase activity. The viral RdRp is one part of viral ribonucleoproteins (vRNPs). Once translocated in the nucleus, the primary roles of vRNPs are to transcribe viral mRNAs for the production of viral proteins and to replicate full-length complementary genomic RNA (cRNA) for amplification of vRNA and generation of progeny vRNPs [10]. Therefore, the synthesis of vRNA and cRNA will be affected when the activity of viral RdRp is cut down. As depicted in Fig. 3c–f, a significant inhibitory effect on the synthesis of cRNA and vRNA was observed. Taken together, these results revealed roscovitine blocked influenza viral RdRp activity.

### 3.5. Roscovitine shows a specific interaction with the viral PB2cap protein

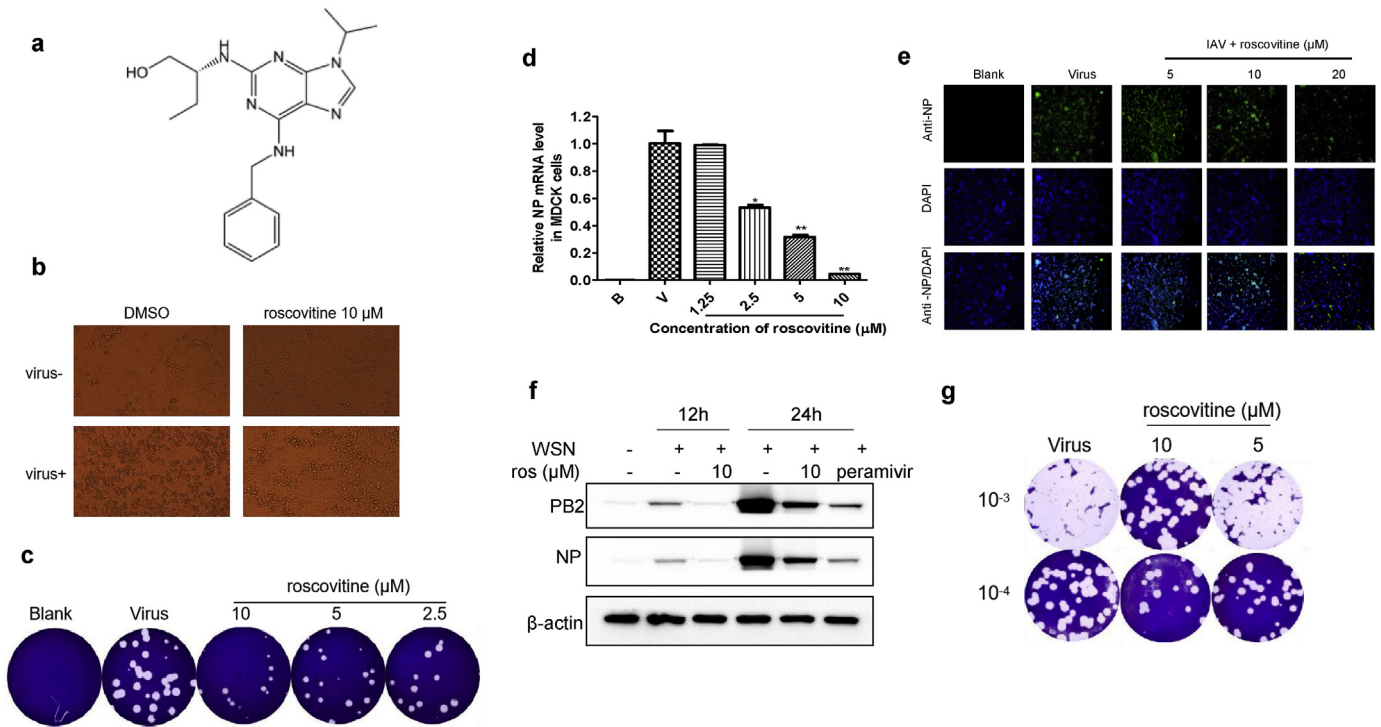
To further investigate the antiviral mechanism, we conducted a series experiments associated with cap-snatching process of RdRp,

**Table 1**  
Inhibitory activities of roscovitine against different subtypes influenza virus infection.

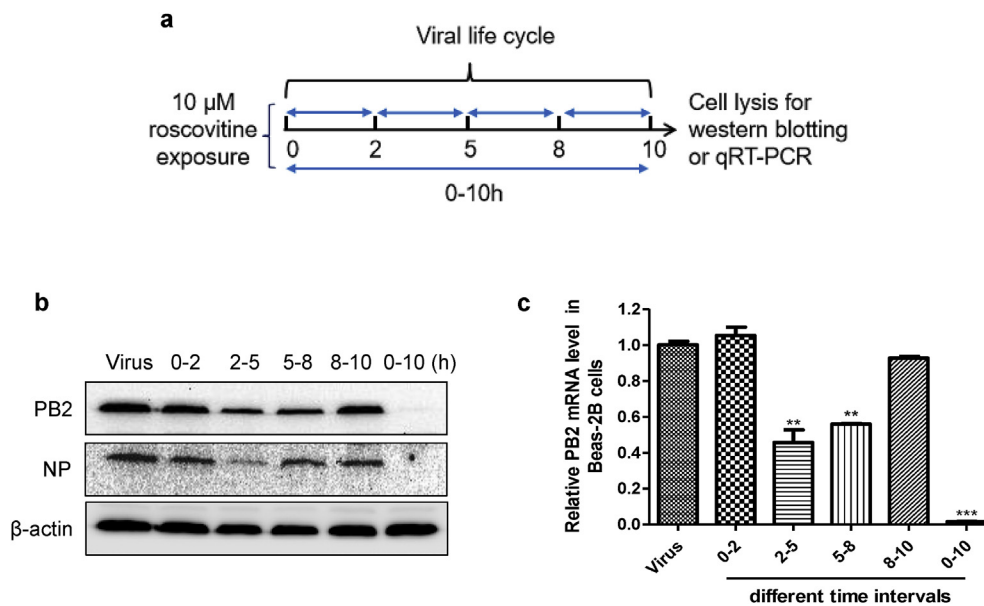
IAV strains	Inhibition activity of Roscovitine <sup>a</sup>	
	$IC_{50}$ ( $\mu$ M)	$CC_{50}$ ( $\mu$ M)
A/WSN/1933(H1N1)	3.35 $\pm$ 0.39	>200
A/FM1/47 (H1N1)	5.99 $\pm$ 1.89	
A/Aichi/2/68 (H3N2)	7.01 $\pm$ 1.84	

<sup>a</sup> Tested in triplicate. The data was depicted as mean  $\pm$  S.D.





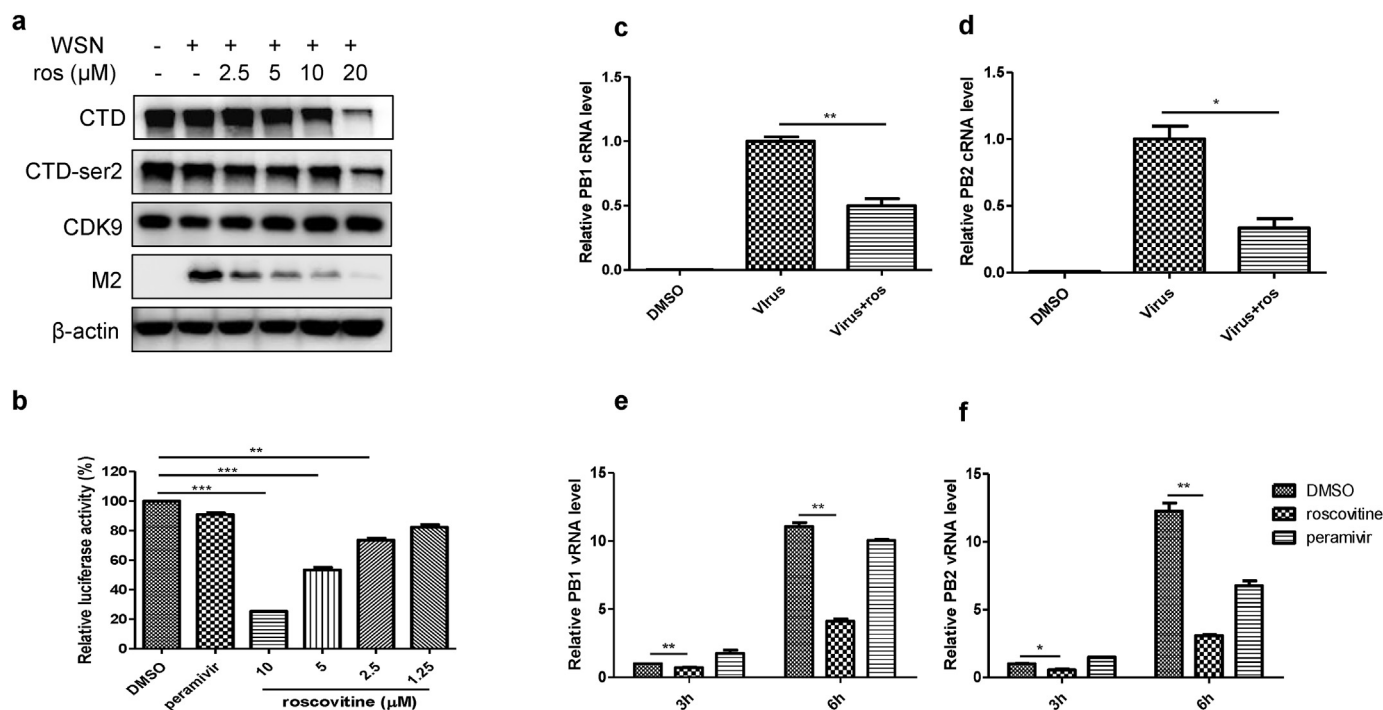
**Fig. 1.** Roscovitine interfered with influenza replication. (a) Chemical structure of roscovitine. (b) Roscovitine reduced virus-induced CPE in MDCK cells (100TCID<sub>50</sub>). (c) Roscovitine reduced the formation of viral plaques (MOI = 0.01). (d) Viral NP mRNA expression was detected at 24 h pi. (e) The expression of viral NP protein (green foci) was detected by immunofluorescence microscopy. (f) The expression of viral NP and PB2 protein, peramivir as a positive control. (g) The production load of progeny virus in the supernatant. The supernatant was diluted 1000 or 10,000 times. Data were shown as mean ± SD (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ ).



**Fig. 2.** Roscovitine interfered with viral gene transcription and genome replication. (a) Exposure times of roscovitine during a single cycle of influenza virus replication. (b) Roscovitine was added to Beas-2B cells at different indicated time intervals and the expression of the viral NP and PB2 protein was detected at 10 h pi. (c) The viral mRNA expression in Beas-2B cells was detected by qRT-PCR at different exposure time intervals after virus infection. Data were shown as mean ± SD (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ).

involving with PB2cap domain. We expressed and purified PB2cap-binding domain (Fig. S4a and S4b). FP assay was used to detect the interaction between roscovitine and PB2cap protein. As depicted in Fig. S4c and S4d, the binding ability and specificity were verified firstly with unlabeled m7GTP as a positive control. Using FP assay, we found that roscovitine suppressed the interaction between

FITC-m7GTP with PB2cap in a dose-dependent manner (Fig. 4a). Next, we used SPR assay which had high sensitivity to confirm the result above, and the PB2cap binding inhibitor D715-2441 was used as a positive control [11]. According to Plexera DE analyzed, the  $K_D$  value between roscovitine and PB2cap was 1.18  $\mu\text{M}$ , while D715-2441 was 60.9  $\mu\text{M}$  (Fig. 4b and Fig. S5). Overall, these data



**Fig. 3.** Roscovitine inhibited influenza viral polymerase activity. (a) The expression level of the CTD domain phosphorylation of RNAP-II in Beas-2B cells by Western blotting at 24 h pi. (b) Roscovitine inhibited viral polymerase activity. Peramivir was used as a negative control. (c–d) Inhibition effect of roscovitine on intracellular cRNA by qRT-PCR in MDCK cells after 6 h pi. (e–f) Inhibition effect of roscovitine on intracellular vRNA by qRT-PCR in MDCK cells at 3 and 6 h pi. Data were shown as mean  $\pm$  SD (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ).

demonstrated that roscovitine could strongly interact with PB2cap.

### 3.6. Molecular docking of roscovitine with PB2cap protein

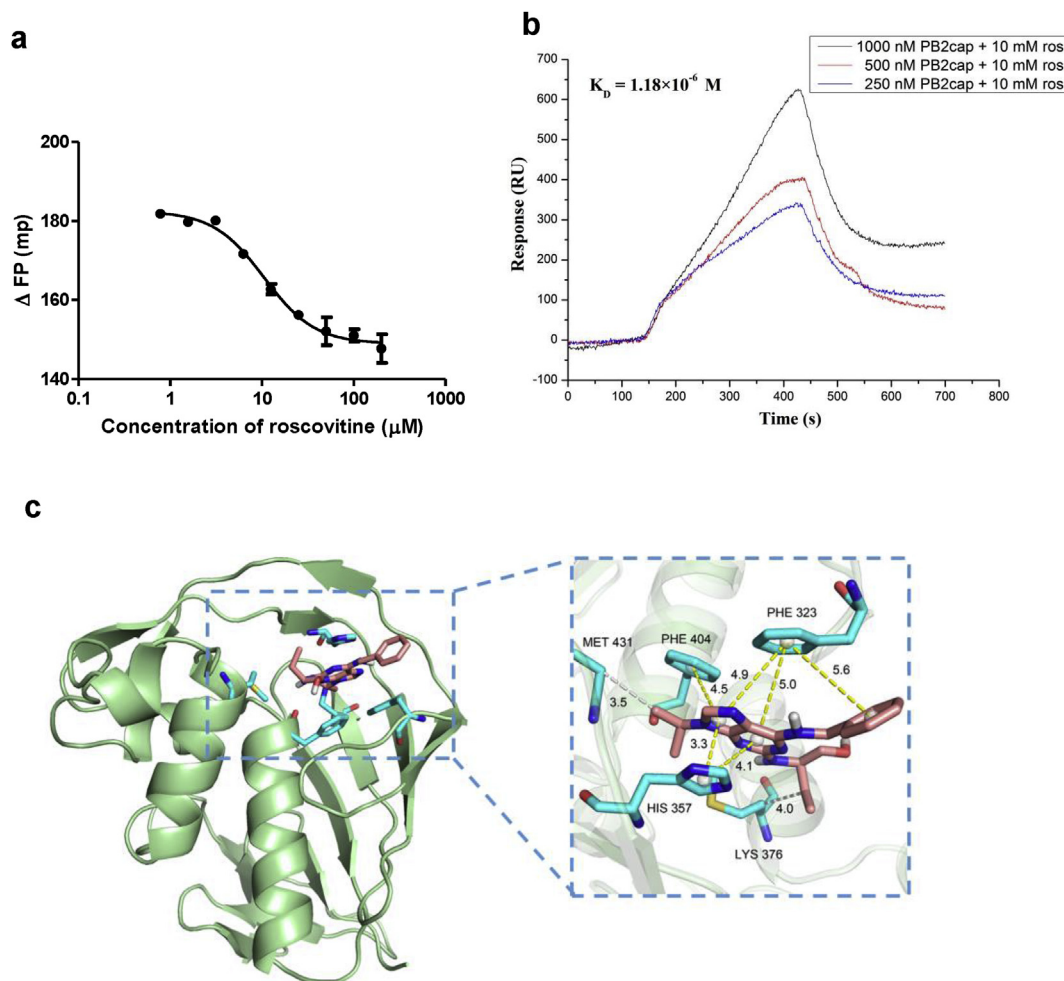
We next investigated the amino acid binding sites on the PB2cap protein via molecular docking. The structure of the PB2cap was consistent with that previously described for complexes with m7GTP [12]. We found that roscovitine could bind to PB2cap domain through imidazole ring of His-357, the benzene rings of Phe-404 and Phe-323 by  $\pi$ - $\pi$  interaction, also through Met-431 and Lys-376 by hydrophobic interactions (Fig. 4c). In summary, the present results indicated that roscovitine, which had a strong interaction with PB2cap, was a possible drug candidate targeting to PB2cap.

## 4. Discussion

With the rapid evolving and stealthy spreading mechanism of different subtypes of influenza, it is essential to explore possible antiviral chemicals against the viruses. Meanwhile, novel antiviral drugs development is an important complementary tool in addition to vaccines, aiming to improve therapeutic efficiency and reduce the drug resistance. However, drug development is an expensive, risky, labor-intensive and time-consuming process. It usually takes 10–15 years on average and costs a billion dollars or more to complete a development process [13]. Therefore, it is indispensable to exploit alternative use of existing drugs that have been on the market and approved for indications on other diseases, as well as drug candidates under clinical trials. Roscovitine has been reported to act on a wide variety of cancer cell lines and induce apoptosis. Moreover, it is currently considered as a potential drug to treat neurodegenerative and retinal degeneration diseases, lung inflammation and kidney diseases. Besides, the compound was also

shown to inhibit HSV and HIV infection [14]. Thus, it is regarded as an efficient potential drug candidate. In this paper, we focused on the antiviral effect of roscovitine on IAVs and demonstrated its antiviral activity as a promising PB2cap inhibitor.

Generally, IAVs possess a single-stranded, eight-segmented RNA genome of negative polarity (vRNA) and accomplish one life cycle including viral attachment, entry, gene transcription and genome replication, packaging of progeny viruses and release period [15]. After virus entry in the host cell, vRNP complexes are imported in the nucleus, where transcription and replication take place. The vRNP complexes constitute the core of the virus and consist of RdRp, NP and vRNA segments, representing the minimal transcriptional and replicative machinery of influenza [10,16]. RdRp of IAVs is highly conserved among all strains and subtypes during evolution. As a new drug class for antiviral therapy in patients with influenza, the RdRp inhibitors are characterized by continuous effective activity and great patient compliance [17]. For instance, Xofluza inhibits viral replication by targeting the endonuclease function encoded by the PA subunit of the viral RdRp and was approved by the FDA in October 2018, which represents a novel mechanism of action being studied and approved in almost two decades [18]. In this article, we found roscovitine exerted the antiviral effect by targeting to virus itself, and as a competitive inhibitor, it could diminish the combination of FITC-m7GTP with PB2cap (Fig. 4a). PB2 subunit is one part of RdRP and plays a critical role in the initiation of transcription by binding to 5'-cap domain of host pre-mRNAs, called cap-snatching process. The PB2 subunit cap-binding domain (PB2cap, residues 318–483) is responsible for the binding ability and considered a valid target for development of new anti-influenza compounds [19]. VX-787 presents broad-spectrum anti-influenza activity by disrupting PB2 cap-binding activity [20]. Furthermore, the PB2cap antivirals may considerably minimized the emergence of escape mutants, which are urgently



**Fig. 4.** Roscovitine showed a specific interaction with the viral PB2cap protein. (a) The specific binding between roscovitine and PB2cap protein was detected using the FP assay. (b) Affinity of roscovitine for PB2cap analyzed by SPR assay. (c) Molecular docking model of roscovitine with PB2cap domain.

indispensable on the edge of a new devastating pandemic. According to this study, roscovitine might influence PB2cap function to exert antiviral activity by FP assay. Next, SPR assay was used to further confirm the result. And the binding ability of roscovitine was approximately 60-fold stronger with PB2cap than that of positive compound D715-2441, a PB2cap inhibitor we reported previously (Fig. 4b and Fig. S5). Molecular docking in this study demonstrated that it bound to the cap-binding pocket of PB2 via the residues Phe-323, His-357, Lys-376, Phe-404, Met-431 (Fig. 4c), consistent with a part of m7GTP-binding residues [19].

In summary, this study has showed strong evidence of potent anti-influenza activity of roscovitine *in vitro*. What's more, roscovitine disturbs viral replication by targeting viral conserved sites of PB2cap. Notably, as a drug candidate, roscovitine has gone through several phase I and II clinical trials with good safety profiles for cancer therapy, and there are plenty of antiviral activity studies conducted on roscovitine as well. Thus, exploring the therapeutic effect of roscovitine in the treatment of influenza is feasible and promising. Furthermore, re-investigating the existing kinase inhibitors as potential antivirals and finding its potential mechanism may provide a wide application prospect against viral infection disease, such as influenza A virus infection, as well as the coming pandemic COVID-19 disease, which caused by another RNA virus, the SARS-CoV-2.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This study was financially supported by grants from the National Natural Science Foundation of China (81728007), Major scientific and technological projects of Guangdong Province (2019B020202002), Chinese Academy of Traditional Chinese Medicine (ZZ13-035-02) and Evidence based capacity building project of Traditional Chinese Medicine (2019XZZX-LG04) to S. L.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbrc.2020.04.034>.

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