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ORIGINAL RESEARCH PAPER

# Retrocyclin 2: a new therapy against avian influenza H5N1 virus in vivo and vitro

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**Abstract** We tested the ability of retrocyclin 2, a type of $\theta$ defensin, to protect cells and chicken embryos from infection by H5N1 highly pathogenic avian influenza virus. A gene fragment of retrocyclin 2 was designed based on the protein sequence of retrocyclin 2 and cloned into the eukaryotic expression vector pcDNA4.01 (HismaxA), named pcDNA4-RC2. The expression vector pcDNA4-RC2 protected MDCK cells and chicken embryos from infection by the H5N1 virus through inhibition of virus replication and viral mRNA transcription. Retrocyclin 2 is therefore effective in preventing H5N1 virus infection *in vivo* and *in vitro* and could be considered as a new therapy for H5N1 influenza and other diseases.

**Keywords** Avian influenza virus · Chicken embryos · H5N1 virus · Influenza virus · MDCK cells · Retrocyclin 2

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## Introduction

The highly pathogenic H5N1 virus, which was first isolated from a farmed goose in Guangdong Province, China, in 1996, has become one of the most devastating bird and human's catastrophes in history. In 1997, human infections with avian influenza H5N1 were first reported in Hong Kong. Altogether, 433 cases (262 fatal) are reported in the first known instance of human infection with this virus (WHO 2009).

Existing vaccines and therapy for H5N1 infection have only a limited value which could make the threat of a new H5N1 pandemic enduring, despite intensive efforts to control it. Current vaccines, consisting of either killed virus or recombinant surface glycoproteins, induce only a weak IgG response. In addition, the existing vaccines have to be reformulated almost every year because the viral antigens that elicit protective antibodies usually undergo changes, rendering the previous year's vaccine ineffective against any new virus subtype. The use of drugs for avian influenza is limited because of severe side effects and the possible emergence of resistant viruses (Ge et al. 2003).

Defensin peptides belonging to three subfamilies, designated $\alpha$, $\beta$, and $\theta$ defensins, have been identified in leukocytes and other cells of humans or nonhuman primates. $\theta$ defensins are circular octadecapeptides with two antiparallel $\beta$-sheets that are bridged by a tri-disulfide ladder and connected by two $\alpha$-turns

(Trabi et al. 2001). They are only expressed in nonhuman primates. In vivo, their formation entails ligation of two nona-peptides, each derived from the *C*-terminal domain of a pre-pro-peptide (Tang et al. 1999) that is encoded by a $\theta$-defensin gene. Certain $\theta$ defensins, including retrocyclin 2, can protect cells from infection by HIV-1 in vitro (Cole et al. 2002) and that they are miniature lectins (Wang et al. 2003) that bind gp120 and block the entry of HIV-1. They have been also shown to block steps of herpes virus infection, neutralize anthrax toxin and inhibit low pathogenic influenza virus (Yasin et al. 2004; Kim et al. 2005; Doss et al. 2009).

The present experiments examined the ability of recombinant eukaryotic expression plasmid, pcDNA4-RC2, to protect cells and chicken embryos from infection by H5N1. In this study, we construct a eukaryotic expression plasmid pcDNA4-RC2 to evaluate its antivirus effects in vitro and in vivo. Our data indicate that recombinant RC2, expressed intracellularly by the pcDNA4-RC2, exhibits antivirus activities.

## Materials and methods

### Viruses and assays

Avian influenza viruses (A/environment/Qinghai/1/2008(H5N1)), subtypes H5N1, were stored in National Research Center for Wildlife Born Diseases, Institute of Zoology. The viruses were grown in the allantoic cavity of 10-day-old embryonated chicken eggs (Chinese Academy of Agriculture, Beijing, China) at 37°C. Allantoic fluid was harvested 48 h after inoculation and stored at −80°C. Virus titer was measured by hemagglutination (HA) assays. The HA assay was carried out in V-bottom 96-well plates. Serial 2-fold dilutions of virus samples were mixed with an equal volume of a 0.5% suspension (v/v) of chicken erythrocytes (Chinese Academy of Agriculture, Beijing, China) and incubated on ice for 1 h. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive.

### Construction of eukaryotic expression vector pcDNA-RC2

The nucleotide sequence encoding the mature region of retrocyclin-2 was amplified by PCR using the following primers: (sense: 5′-CGGGATCCATGGGTATTTGTAGATGTATTTGTGGTAGAAGAATTTGT-3′; antisense: 5′-CCGCTCGAGTCATCTACCACAAATACATCTACAAATTCTTCTACCACA-3′). The PCR amplification began with denaturation at 94°C for 5 min and then performed at 94°C for 50 s and 55°C for 50 s for 30 cycles. The amplified mature retrocyclin-2 fragment was cloned into eukaryotic expression vector pcDNA4.01HismaxA (Invitrogen). The recombinant plasmid was named pcDNA4-RC2 and verified by DNA sequencing. The empty vector pcDNA4.01 (HismaxA) was used as a control and named pcDNA4.

### Cell culture and virus infection

MDCK cells were grown in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco), at 37°C under 5% (v/v) $CO_2$. pcDNA4-RC2 was transfected into MDCK cells using SuperFect transfection reagent (Qiagen) according to the manufacturer's instructions. MDCK cells transfected with pcDNA4 were used as a control. Transformed cells were cultured in six-well plates in DMEM for 24 h. The culture medium was then removed and 100 µl H5N1 virus [5,000 pfu/ml] in infection medium, consisting of DMEM, 2% (v/v) heat-inactivated fetal bovine serum, was added to each well. After incubation for 1 h at room temperature, 2 ml infection medium containing 4 µg trypsin/ml was added to each well and the cells were cultured at 37°C under 5% $CO_2$. Supernatants were harvested from infected cultures after 48 h and assayed for virus titers and real-time RT-PCR.

### Chicken embryos inoculation and virus infection

For each inoculation, 100 µg pcDNA4-RC2 or pcDNA4 was mixed with 30 µl SuperFect transfection reagent and the mixture was incubated at room temperature for 30 min. The mixture was then combined with 100 µl of H5N1 virus [5,000 pfu/ml] and immediately injected into the allantoic cavity of 10-day-old chicken eggs. Chicken embryos transfected with pcDNA4 were used as control. The embryos were incubated at 37°C for 24 h and allantoic fluid was harvested to measure virus titers

and mRNA relative expression with real-time RT-PCR.

### Hemagglutination assay

The HA assay was carried out in a conventional manner. Influenza solution from MDCK and chicken embryo allantoic fluid (50 μl) were mixed in serial two-fold dilutions in PBS. Subsequently, 50 μl 1% (v/v) chicken red blood cells were added to the above mixture and further incubated at room temperature for 30 min.

### RNA extraction and real-time RT-PCR

DNase-treated total RNA was isolated by using RNApure total RNA extraction kit according to the protocol of the manufacturer (Bioteke, China). Reverse transcription (RT) was carried out by using an Omniscript reverse transcriptase kit (Qiagen) in a 20 μl reaction mixture, containing 200 ng total RNA and primers of oligo(dT)15 and Uni12(5′-AGC AAAAGCAGG-3′), at 42°C for 1 h. One μl of RT reaction mixture was then used for quantitative real-time PCR by using gene-specific primers, SYBR green PCR Master Mix (Takara, JP). Before the PCR, the mixture was incubated at 50°C for 2 min and 95°C for 10 min. The reaction was then performed at 95°C for 15 s and 55°C for 1 min for 40 cycles. All reactions were done in triplicate. The levels of PCR products were monitored with an ABI PRISM 7000 sequence detection system and analyzed with ABI PRISM 7000SDS software (Applied Biosystems). Cycle times were analyzed at a reading of 0.2 fluorescence unit. Cycle times that varied by >1 unit between duplicates were discarded. The duplicate cycle times were averaged and normalized to the cycle time of $\beta$-actin. Sense and antisense primers for the $\beta$-actin gene were 5′-GCACGGCATCGTCA CCAACT-3′ and 5′-CATCTTCTCGCGGTGGCC T-3′, respectively.

### Statistical analysis

SPSS 11.5 was used for statistical analysis. The statistical significance of results in all of the experiments was determined by Student's t-test and ANOVA. The findings were regarded as significant if $P < 0.05$.

## Results

### Design of pcDNA4-RC2 expression plasmid against avian influenza H5N1 virus

The gene fragment of retrocyclin 2 was designed based on retrocyclin 2 protein sequence (GICRC ICGRRICRCICGR) (Yasin et al. 2004). Retrocyclin 2 gene fragment,flanked by *Bam*HI and *Xho*I sites (5′-ATGGGTATTTGTAGATGTATTTGTGGTAG AAGAATTTGTAGATGTATTTGTGGTAGATGA-3′) was successfully obtained by overlap-extension PCR. These oligonucleotides were annealed and ligated to the *Bam*HI and *Xho*I sites of pcDNA4 to get plasmid pcDNA4-RC2 and the construct was confirmed by DNA sequencing (Invitrogen) (Fig. 1).

### Inhibition of influenza virus production in MDCK cell line

To test whether the RC2 from plasmids inhibited H5N1 virus production, we examined their effects in MDCK cells. Two results show that RC2 can potently inhibit highly pathogenic H5N1 virus production in MDCK cells. Firstly, as shown in Fig. 2a, in mock transfection (pcDNA4), virus titers in the infected cultures were about 60 times than those transfected with pcDNA4-RC2, which indicated that RC2 were effective in interfering with H5N1 virus production in MDCK cells. Secondly, RC2 from pcDNA4-RC2 significantly inhibited about 80% of virus production in the real-time RT-PCR, and this result showed that

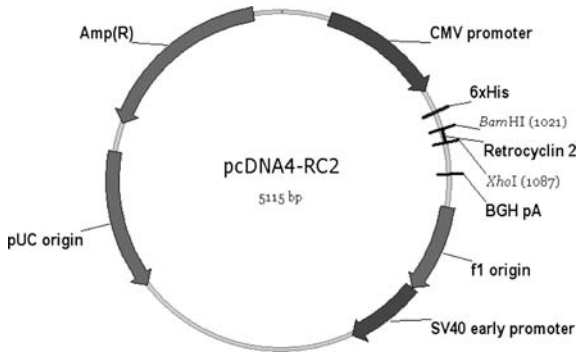


**Fig. 1** A pcDNA4.01 (HismaxA) vector was used for construction of retrocyclin 2 expression vector. Expression of the gene of interest was under the control of the CMV promoter for a regulated recombinant protein expression. An ampicillin resistance gene was used for selection

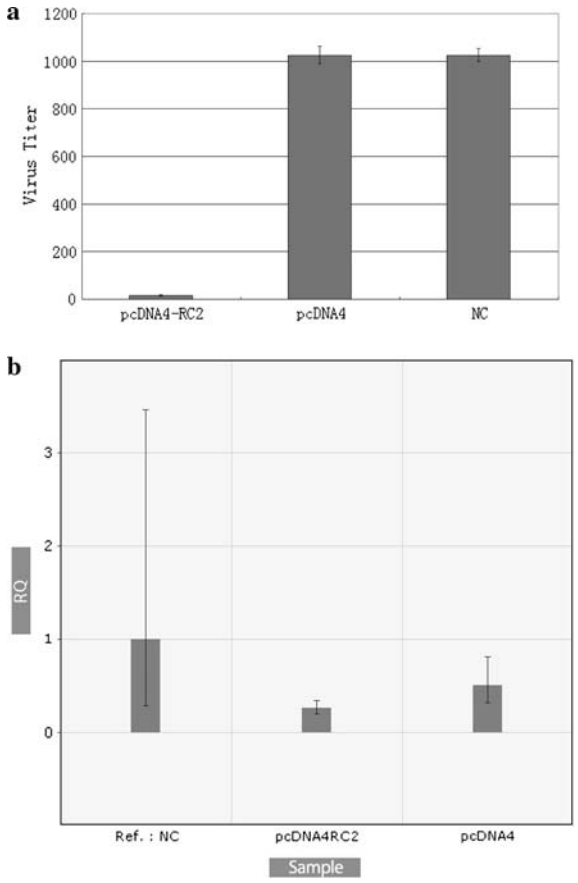


**Fig. 2** Inhibition of H5N1 virus production in MDCK cells. MDCK cells were first transformed with pcDNA4 or pcDNA4-RC2 expression plasmids and then infected with H5N1 virus [5,000 pfu/ml]. **a** Viral titers in the culture supernatants were measured by HA assay. HA units are arithmetic means based on titer endpoints of arithmetic dilutions. Virus titers (HA units) from three groups (Negative control, pcDNA4 and pcDNA4-RC2) of cell cultures are shown. Data represent the means ± S.D. ($n = 3$). **b** pcDNA4-RC2 interferes with H5N1 virus production in MDCK cells with statistical diagram of Real-time PCR. The mRNA relative expression levels from three groups (Negative control, pcDNA4 and pcDNA4-RC2) of cell cultures are shown. Data represent the means ± S.D. ($n = 3$)

mRNA level of H5N1 virus was significantly reduced by pcDNA-RC2 (Fig. 2b). Together, these results show that RC2 could potently inhibit H5N1 virus production in MDCK cells.

Inhibition of virus production in chicken embryos

To extend the results in MDCK cells, we tested the ability of RC2 to inhibit H5N1 virus production in developing chicken embryos, a widely used in vivo model of influenza virus infection. SuperFect transfection reagent was introduced for pcDNA4-RC2 transfection in the embryos. H5N1 virus alone and virus plus pcDNA4 or virus plus pcDNA4-RC2 were injected with SuperFect into the allantoic cavity of 10-day-old chicken embryos. Allantoic fluids were collected 24 h later for measurement of virus titers and were assayed by real-time RT-PCR. When virus was injected alone or plus pcDNA4, higher virus titers were detected. Co-injection of pcDNA4-RC2 expression plasmids and H5N1 virus, however, reduced virus titers significantly (Fig. 3a). The real-time RT-PCR result showed that RC2 from pcDNA4-RC2 significantly inhibited over 90% of virus production (Fig. 3b). These results were concordant with those in MDCK cells. Thus, RC2 also interfered with H5N1 virus production in chicken embryos.

## Discussion

In this study we developed a simple RC2 delivery strategy by combination of conventional pcDNA4-RC2 eukaryotic expression system. Our results demonstrate RC2 expression plasmids can potently inhibit H5N1 highly pathogenic avian influenza virus production in MDCK cells and chicken embryos.

H5N1 virus infection is considered as a dangerous disease of birds and humans, due to its easy transmission, frequent antigenic shift and drift of the virus, and the limited efficacy of current vaccines and therapy. It was confirmed that RC2 expression plasmids potently inhibited H5N1 virus production in cell lines and chicken embryos in present experiments. These results provide a basis for further development of RC2 expression plasmids for prophylaxis and therapy of H5N1 virus infection in birds and humans. H5N1 virus naturally infects epithelial cells in the upper respiratory tract and lungs in birds, and RC2 expression plasmids could be conveniently administrated via intranasal or pulmonary routes. Considering that the number of virions is probably small at the beginning of a natural infection, sufficient amounts of RC2 may be taken up by epithelial cells in the upper airways and lungs to inhibit virus replication, thus, potentially achieving preventive or therapeutic effects.

Although phylogenetic analysis suggests that $\theta$-defensins have been around for over 30 million years

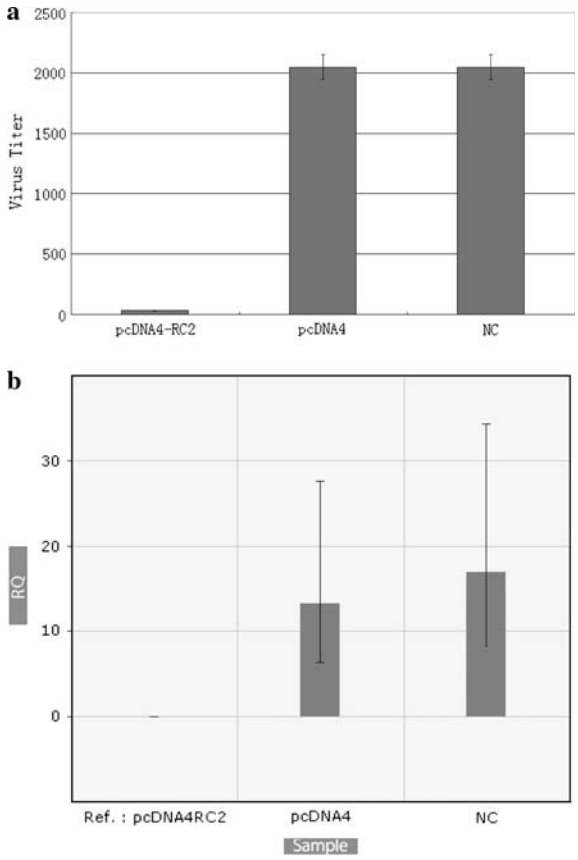


**Fig. 3** Inhibition of virus production in embryonated chicken eggs. A mixture of pcDNA4-RC2 or pcDNA4 (100 μg), Superfect (30 μl), and 100 μl H5N1 virus [5,000 pfu/ml] was injected into the allantoic cavity of 10-day-old chicken embryos. Allantoic fluid was collected 24 h later and assayed for virus titers and real-time RT-PCR. **a** Viral titers in the allantoic fluid were measured by HA assay. HA units are arithmetic means based on titer endpoints of arithmetic dilutions. Virus titers (HA units) from three groups (Negative control, pcDNA4 and pcDNA4-RC2) of allantoic fluid are shown. Data represent the means ± S.D. ($n = 3$). **b** pcDNA4-RC2 interferes with H5N1 virus production in chicken embryos with statistical diagram of real-time PCR. The mRNA relative expression levels from three groups (Negative control, pcDNA4 and pcDNA4-RC2) of allantoic fluid are shown. Data represent the means ± S.D. ($n = 3$)

(Nguyen et al. 2003), they are "novel" molecules for many reasons. Whereas circular peptides have been identified in various plants (Barry et al. 2003; Rosengren et al. 2003) and bacteria (Craik et al. 2003), θ-defensins are the only known circular peptides of animal origin. They were first described in 1999 (Tang et al. 1999), but as yet only a few publications (Trabi et al. 2001; Wang et al. 2003; Doss et al. 2009) describe their provenance and properties. There is a remarkable parallelism between the effects of retrocyclins on HIV-1 and those on HSV. In both cases, the peptide binds with very high affinity to a viral surface glycoprotein involved in viral entry, either gp120 (56) or gB2 (Yasin et al. 2004), and it is able to prevent cellular entry of HIV-1 (Munk et al. 2003) and HSV-2 (Yasin et al. 2004).

Besides HIV-1 and HSV-2, whether θ-defensins will be effective against other virus remains to be verified. Their inactivity against lactobacilli and their negligible cytotoxicity are desirable properties for a potential topical microbicide. While additional questions remain to be addressed, retrocyclins and other θ defensins are interesting molecules whose chemical and biological properties deserve increased attention.

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