**A Raf kinase inhibitor demonstrates antiviral activities both *in vitro* and *in vivo*
against different genotypes of virulent Newcastle disease virus**

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21

22 **Abstract**

23 Newcastle disease (ND) is still one of the major plagues of birds worldwide. Combat actions are
24 limited to vaccines, highlighting the urgent need for new and amply available antiviral drugs.
25 Previous results have shown that Newcastle disease virus (NDV) downregulates the intracellular Raf
26 kinase inhibitor protein (RKIP) expression for efficient replication, suggesting that this molecular
27 may be a suitable target for antiviral intervention. In the present work, we investigated whether or
28 not the Raf kinase inhibitor V (RKIV), which functions in the same way as RKIP by targeting the
29 intracellular Raf kinase, is able to suppress the propagation of enzootic virulent NDV *in vitro* and *in*
30 *vivo*. *In vitro* antiviral activity of RKIV was assessed by cell-based assay, and *in vivo* activity was
31 determined in the chicken model. Our results clearly showed that RKIV treatment protected the
32 cells from NDV-induced CPE with the effective concentrations on nM level, and inhibited virus
33 replication in the lungs of infected chickens in a dose-dependent manner and protected chickens
34 from the lethal infection by NDV. Thus, we conclude that the Raf kinase inhibitor compound RKIV, by
35 inhibiting the host cellular target Raf kinase, might be very promising as a new class of antivirals
36 against the enzootic virulent NDV infection.

37 **Keywords**

38 Newcastle disease virus; Raf Kinase Inhibitor V; antivirals; Raf Kinase

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42 Newcastle disease (ND), caused by virulent Newcastle disease virus (NDV), is one of the most
43 economically significant and severe viral diseases affecting commercial and backyard poultry
44 worldwide (Swayne, 2013). Vaccines with live, low-virulence and inactivated oil emulsion viruses are
45 the most accepted prevention and control strategies for combating ND in poultry (Shittu et al.,
46 2015). However, vaccines cannot prevent viral replication and the vaccinated birds still shed virulent
47 NDV at considerable levels (Afonso and Miller, 2013). Furthermore, antigenic differences between
48 vaccines and currently circulating virulent viruses may facilitate the escape and evolution of virulent
49 viruses by allowing higher levels of viral shedding for heterologous virulent strains (Miller et al.,
50 2007). Therefore, only vaccination is not enough to prevent and control ND, and highlighting the
51 urgent need for new and amply available antiviral drugs.

52 Infection of the host cell by NDV results in the induction of the interferon (IFN) response and related
53 intracellular signaling pathways that are, in part, suppressed by the virus to ensure efficient
54 replication (Cheng et al., 2014; Krishnamurthy et al., 2006). This dependency also was observed in
55 other viruses, such as the influenza virus (Pinto et al., 2011), human immunodeficiency virus (HIV)
56 (Zhu et al., 2011), and human cytomegalovirus (Mathers et al., 2014). Therefore, the dependency of
57 this virus may be useful for developing novel antiviral strategies.

58 Our previous study demonstrated that targeted overexpression of host RKIP, which subsequently
59 results in decreased Raf/MEK/ERK signaling and I κ Ba/NF- κ B pathway activation, leads to reduced

60 NDV production in cell culture (Yin et al., 2015). This unexpected dependence of NDV replication on
61 the activity of RKIP offers the intriguing possibility to target this signaling pathway for an antiviral
62 intervention. One of the advantages to directly target host intracellular signaling pathways rather
63 than the virus itself would be that the virus cannot easily adapt to the missing cellular function
64 (Dudek et al., 2010; Ehrhardt et al., 2013; Mazur et al., 2007; Scholtissek and Muller, 1991).
65 Moreover, other important prerequisites for such a cell-targeted antiviral drug should be that the
66 compound is safe to use and well tolerated (Collier et al., 2013).

67 Complementing our earlier findings (Yin et al., 2015), we now addressed the question whether or
68 not the Raf kinase inhibitor compound RKIV (Figure 1A), [which functions in the same way as RKIP by](#)
69 [targeting the intracellular Raf kinase \(Tsai et al., 2008\)](#), is also effective against the current enzootic
70 virulent NDV isolates. As representative virulent NDV strains, we used the chicken origin classical
71 genotype IX virulent F48E9 strain (F48E9), two genotype VII virulent strains
72 goose/China/Jilin/NA-1/1999 (NA-1) and chicken/China/Jilin/HL-1/2014 (HL-1), and a genotype VI
73 virulent strain pigeon/China/Jilin/CC-1/2014 (CC-1). The primary chicken embryo fibroblast (CEF) and
74 the immortalized chicken fibroblast cell line DF-1 were grown in Dulbecco's modified Eagle's
75 medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Life Technologies) and maintained
76 with 5% FBS. Specific pathogen-free (SPF) chickens, aged 2-3-days, were purchased from Merial
77 China (Beijing, China). RKIV (Santa Cruz Biotechnology, USA) was dissolved in DMSO at a stock
78 concentration of 100 mM. Cell viability was significantly decreased to 15-20% when DF-1 and CEF
79 cells incubated with an *in vivo* solvent consisting of 10% DMSO, 30% Cremophor EL, and 60% PBS for
80 3 days; on the contrary, no obvious pathological changes and dysfunctions were observed in the

81 eyes and respiratory systems of chickens administered oculonasally with 200 μ l *in vivo* solvent daily
82 for 5 days (data not shown). Therefore, RKIV was dissolved further in either PBS solution for *in vitro*
83 studies or *in vivo* solvent for *in vivo* treatments. All experimental protocols used in this work were
84 reviewed and approved by the Experimental Animal Council of Jilin University, China.

85 The antiviral activity of RKIV against the panel of NDV strains (multiplicity of infection, 0.001) *in vitro*
86 was assessed in a multicycle virus cell-based cytopathic effect (CPE) reduction assay with a
87 colorimetric readout using the

88 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfphenyl)-2H-tetrazolium/phenazin
89 e methosulfate (MTS/PMS) method as described previously (Sun et al., 2015). In brief, for the CPE
90 reduction assay, log₁₀ dilutions of RKIV (1,000–0.1 nM) were combined with 0.001 multiplicity of
91 infection (MOI) of NDV and added into a monolayer of DF-1 or CEF cells in 96-well, white,
92 flat-bottom cell cultural plates. Plates were incubated at 37°C and 5% CO₂ for 3 days, and then cell
93 viability was measured with the MTS/PMS method. The results showed that RKIV treatment
94 efficiently reduced the CPE of the DF-1 and CEF cells infected by the NDV strains. In detail, the EC₅₀
95 values for RKIV against CC-1 and F48E9 were from 1.24 ± 0.86 to 5.47 ± 0.32 nM in both cells (Table
96 1). RKIV also was effective against the both genotype VII NDV viruses, while better effective
97 inhibitory concentration were found against NA-1 (3.92 ± 0.41 nM in DF-1 cells and 6.53 ± 0.84 nM
98 in CEF cells) compared to HL-1 (30.19 ± 0.40 nM in DF-1 cells and 52.13 ± 1.21 nM in CEF cells). As
99 compared to the same genotype VII virulent strain NA-1, this less susceptibility of HL-1 replication
100 for RKIV also was observed in RKIP stable overexpressed (ovRKIP) DF-1 cells (data not shown).

101 Furthermore, RKIV is well tolerated by DF-1 and CEF cells; when concentrations up to 100 μ M were

102 used, no toxic side effects were found (CC50: 10.65 ± 1.29 mM in DF-1 cells and 15.95 ± 3.65 mM in
103 CEF cells). Therefore, our data demonstrate that RKIV protects cells from NDV-induced CPE at
104 effective concentrations in the nM range.

105 Next, we investigated whether or not RKIV would act as an antiviral agent against NDV *in vivo*. Viral
106 titers were determined at 24 hours post infection (hpi) to minimize the influence of the innate or
107 adaptive immune response, since remarkably severe clinical signs and lesions were monitored after
108 24 hpi of virulent NDV strains without RKIV treatment (including conjunctivitis, comb hemorrhages,
109 edema, depression, and neurological signs) (data not shown). To test this, at least five SPF chickens
110 per group administered oculonasally with 200 μ l of four different concentrations of RKIV (0.5, 1, 5,
111 and 10 mM). Sixty minutes after the RKIV treatment, chickens were infected by oculonasal
112 treatment with 10^6 EID₅₀ different NDV strains. For RKIV *in vivo* dose-dependent response
113 determination, chickens were sacrificed 24 hpi, and lungs were shredded with an amount of the
114 10-fold volume PBS. To remove the cell debris, the homogenates were centrifuged for 15 minutes at
115 2,000 rpm, and the supernatant was collected for the virus titration. The virus titer was quantified
116 by the micro-HA method as described previously (Yin et al., 2015). In line with the antiviral effects *in*
117 *vitro*, treatment with a single dose of 10 mM RKIV resulted in a strong suppression for the virus
118 replication in the lungs of the F48E9-, NA-1-, CC-1-, and HL-1-infected chickens at 24 hpi (Figure 1B).
119 Nevertheless, even though 5 and 1 mM RKIV a titer reduction in the lungs of RKIV-treated chickens
120 was found, this reduction was less than 3 log of magnitude. Less than 2 log of magnitude reduction
121 of NDV titer was found when the chickens were treated with 0.5 mM RKIV. This set of data allowed
122 us to draw an IC50 curve and to calculate the IC50 value for RKIV against F48E9, NA-1, CC-1, and

123 HL-1 in chickens (0.52 ± 0.21 , 0.55 ± 0.25 , 0.49 ± 0.13 , and 0.68 ± 0.10 mM, respectively) (Figure 1C).

124 Taken together, these results show that RKIV treatment reduces NDV titers isolated from the lungs
125 of infected chickens in a dose-dependent manner.

126 We next questioned whether or not RKIV treatment would also result in prolonged survival of SPF
127 chickens infected with virulent NDV isolates. Therefore, chickens were treated daily with 200 μ l of
128 10 mM RKIV for 5 consecutive days beginning 1 h prior to infection with 10^6 EID₅₀ genotype VI CC-1
129 or genotype VII HL-1 viruses. While all 10 solvent-treated chickens died between 3 and 6 days post
130 infection (dpi), 7/10 and 8/10 of the RKIV-treated chickens were survived upon CC-1 and HL-1
131 infection, respectively (Figure 1D). To evaluate the toxic side effects of RKIV and *in vivo* solvent in
132 chickens, at least five non-NDV-infected SPF chickens per group were treated with 200 μ l of either
133 10 mM RKIV or *in vivo* solvent daily for 5 days. Results showed that treatment of either 10 mM RKIV
134 or *in vivo* solvent did not result in obvious toxic side effects at 5, 7 and 9 dpt (days post treatment)
135 as compared to untreated chickens (as indicated by measuring the ratios of liver/body weight and
136 spleen/body weight, Figure 2A and 2B). However, the ratios of both liver/body weight and
137 spleen/body weight were significant decreased when chickens treated with either 10mM RKIV or *in*
138 *vivo* solvent daily for more than 5 days as compared to unthreatened chickens (Figure 2C and 2D).
139 Furthermore, obvious toxic side effects were also observed in chickens treated with 15, 20 or 40
140 mM of RKIV daily for 5 days (Figure 2E and 2F). Taken together, the results indicate that RKIV
141 treatment protects chickens from lethal NDV inoculation.

142 This was the first time that this new anti-NDV strategy of targeting intracellular signaling pathways

143 instead of the virus itself was discussed. On the one hand, the great advantage of using inhibitors of
144 host intracellular signaling rather than the virus itself as antivirals is the extremely low tendency of
145 these inhibitors to develop resistance, which has been already proven for several compounds
146 (Debing et al., 2015; Ludwig et al., 2004; Mazur et al., 2007). On the other hand, one has to consider
147 toxic side effects because these compounds interfere with the host cell machinery. Besides strong
148 antiviral activity, RKIV showed surprisingly little toxicity *in vitro* and *in vivo*. Results from K.
149 Droebner indicated that the weight of the spleen of solvent-treated (10% DMSO, 30% Cremophor EL,
150 and 60% PBS) mice was reduced significantly compared to untreated mice (Droebner et al., 2011).
151 However, such observation was only seen when chicken treated with either 10mM RKIV or *in vivo*
152 solvent daily for more than 5 days or overdose of RKIV (such as 15, 20, 40 mM) daily for 5 days
153 (Figure 2C-2F).

154 Although currently we proved the RKIV is a strong anti-NDV compound both *in vitro* and *in vivo*,
155 RKIV is not an inexpensive compound when used with chicken flocks compared to vaccines.
156 Cheaper compound ways of targeting the host intracellular Raf kinase or Raf kinase inhibitor
157 protein should be developed in the further. However, RKIV can be used in rare and valuable birds
158 for combating ND. In summary, our data clearly demonstrate that the Raf kinase inhibitor
159 compound RKIV might be very promising as a new class of antivirals against virulent NDV.

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Transparency declarations

None to declare.

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236

237 **Figure legend**

238 **Figure 1.** Structural formulas (A) and anti-NDV activities (B-D) of Raf kinase inhibitor V against
239 virulent NDV infections in 2–3-day old SPF chickens. A: Structural formulas of RKIV; B: *In vivo* dose
240 response of RKIV against four tested NDV. Reduction of virus titers in the lung after treatment with
241 four different RKIV concentrations (0, 0.5, 1, 5, and 10 mM); C: **IC50** curve for RKIV against NDV in
242 SPF chickens. D: Survival of NDV-infected chickens (10^6 EID₅₀) after treatment with RKIV compared to
243 NDV-infected chickens treated with *in vivo* solvent; All data are representative of two independent
244 experiments, and the values shown are mean $\pm$ SD from triplicate values (Differences were

considered significant if $p < 0.05$, $p < 0.01$ as compared to the relative control group, $n = 5-10$).

Figure 2. Toxic side effects of Raf kinase inhibitor V and *in vivo* solvent in 2–3-day old SPF chickens.

A-B: Non-NDV-infected chickens were treated with 200 μ l of either 10 mM RKIV or *in vivo* solvent daily for 5 days. Then the ratio of liver or spleen to body weight was calculated by liver or spleen weight divided by body weight $\times 100$ at 5, 7 and 9 dpt (days post treatment). C-D: The ratio of liver or spleen to body weight was calculated from non-NDV-infected chickens were treated with 200 μ l of either 10 mM RKIV or *in vivo* solvent daily for 6, 8 and 10 days. E-F: The ratio of liver or spleen to body weight was calculated from non-NDV-infected chickens were treated with 200 μ l of either 15, 20 or 40 mM RKIV daily for 5 days. All data are representative of two independent experiments, and the values shown are mean $\pm$ SD from triplicate values (Differences were considered significant if $p < 0.05$, $p < 0.01$ as compared to the relative control group, $n = 5-10$).

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Table 1. Antiviral activity (EC_{50}) of RKIV against different genotype virulent NDV in virus cell-based assays

Symbol	NDV strains	Genotype	RKIV EC_{50} (nM)	
			DF-1	CEF
CC-1	pigeon/China/Jilin/CC-1/2014	VI	1.24 $\pm$ 0.86	5.47 $\pm$ 0.32
NA-1	goose/China/Jilin/NA-1/1999	VII	3.92 $\pm$ 0.41	6.53 $\pm$ 0.84
HL-1	chicken/China/Jilin/HL-1/2014	VII	30.19 $\pm$ 0.40	52.13 $\pm$ 1.21
F48E9	chicken/China/F48E9/1946	IX	2.16 $\pm$ 0.64	3.54 $\pm$ 0.52

A **B** **C** **D**

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