Antiviral Research 133 (2016) 140–144

Contents lists available at ScienceDirect

Antiviral Research

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

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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ARTICLE INFO

Article history:
Received 7 April 2016
Received in revised form
20 June 2016
Accepted 4 August 2016
Available online 4 August 2016

Keywords:
Newcastle disease virus
Raf kinase inhibitor V
Antivirals
Raf kinase

ABSTRACT

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

Newcastle disease (ND), caused by virulent Newcastle disease virus (NDV), is one of the most economically significant and severe viral diseases affecting commercial and backyard poultry worldwide (Swayne, 2013). Vaccines with live, low-virulence and inactivated oil emulsion viruses are the most accepted prevention and control strategies for combating ND in poultry (Shittu et al., 2016). However, vaccines cannot prevent viral replication and the vaccinated birds still shed virulent NDV at considerable levels (Afonso and Miller, 2013). Furthermore, antigenic differences between vaccines and currently circulating virulent viruses may facilitate the escape and evolution of virulent viruses by allowing higher levels of viral shedding for heterologous virulent strains (Miller et al., 2007). Therefore, only vaccination is not enough to prevent and control ND, and highlighting the urgent need for new and amply available antiviral drugs.

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

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http://dx.doi.org/10.1016/j.antiviral.2016.08.003
0166-3542/

Our previous study demonstrated that targeted overexpression of host RKIP, which subsequently results in decreased Raf/MEK/ERK signaling and IκBa/NF-κB pathway activation, leads to reduced NDV production in cell culture (Yin et al., 2015). This unexpected dependence of NDV replication on the activity of RKIP offers the intriguing possibility to target this signaling pathway for an antiviral intervention. One of the advantages to directly target host intracellular signaling pathways rather than the virus itself would be that the virus cannot easily adapt to the missing cellular function (Dudek et al., 2010; Ehrhardt et al., 2013; Mazur et al., 2007; Scholtissek and Muller, 1991). Moreover, other important prerequisites for such a cell-targeted antiviral drug should be that the compound is safe to use and well tolerated (Collier et al., 2013).

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

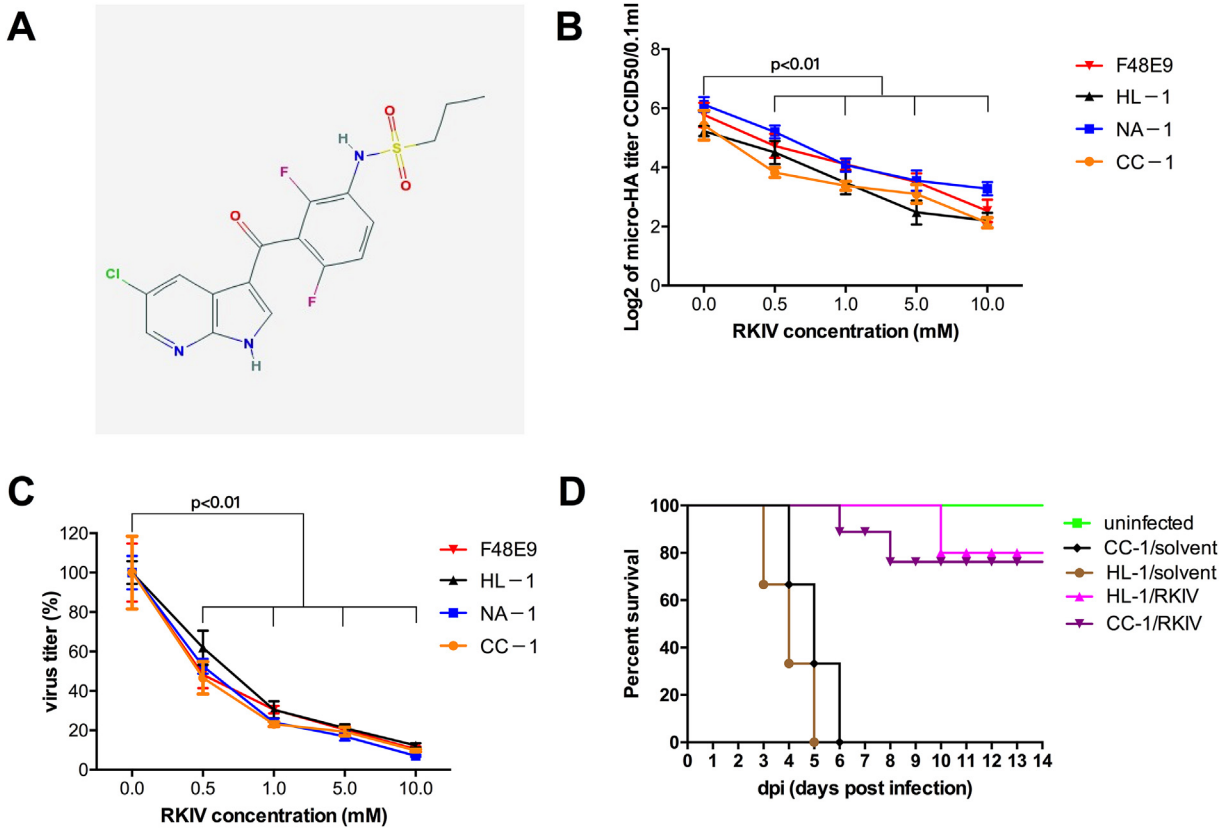

Fig. 1. Structural formulas (A) and anti-NDV activities (B–D) of Raf kinase inhibitor V against virulent NDV infections in 2–3-day old SPF chickens. A: Structural formulas of RKIV; B: *In vivo* dose response of RKIV against four tested NDV. Reduction of virus titers in the lung after treatment with four different RKIV concentrations (0, 0.5, 1, 5, and 10 mM); C: IC50 curve for RKIV against NDV in SPF chickens. D: Survival of NDV-infected chickens (10^6 EID_{50}) after treatment with RKIV compared to NDV-infected chickens treated with *in vivo* solvent; All data are representative of two independent experiments, and the values shown are mean ± SD from triplicate values (Differences were considered significant if $p < 0.05$, $p < 0.01$ as compared to the relative control group, $n = 5–10$).

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 ± 0.86	5.47 ± 0.32
NA-1	goose/China/Jilin/NA-1/1999	VII	3.92 ± 0.41	6.53 ± 0.84
HL-1	chicken/China/Jilin/HL-1/2014	VII	30.19 ± 0.40	52.13 ± 1.21
F48E9	chicken/China/F48E9/1946	IX	2.16 ± 0.64	3.54 ± 0.52