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Short communication

Assessment of the antiviral properties of recombinant surfactant protein D against influenza B virus *in vitro*

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ABSTRACT

The armamentarium of antiviral drugs against influenza viruses is limited. Furthermore, influenza viruses emerge that are resistant to existing antiviral drugs like the M2 and NA inhibitors. Therefore, there is an urgent need for the development of novel classes of antiviral drugs. Here we investigated the antiviral properties of recombinant porcine surfactant protein D (RpSP-D), an innate defense molecule with lectin properties, against influenza B viruses. We have previously shown that porcine SP-D has more potent neutralizing activity against influenza A viruses than human SP-D. Here we show that RpSP-D neutralizes influenza B viruses efficiently and inhibited the binding of these viruses to epithelial cells of the human trachea.

Influenza viruses are an important cause of respiratory tract infections and are responsible for annual epidemics, which constitute a substantial burden on public health (WHO, 2014a). Although influenza A viruses of the H3N2 and H1N1 subtypes are responsible for the majority of infections, influenza B viruses contribute to seasonal influenza outbreaks considerably and predominate one out of every three influenza seasons, causing significant morbidity and mortality every year (Lin et al., 2004). Furthermore, influenza B viruses can cause severe disease although mortality rates of influenza B virus infections are lower than those caused by influenza A (H3N2) viruses (Thompson et al., 2003).

There are two antigenically and genetically distinct lineages of influenza B viruses, represented by the prototypic strains B/Yamagata/16/88 and B/Victoria/2/87. Influenza B viruses of the B/Victoria lineage were the predominant B strains circulating worldwide in the 1980s while viruses of the B/Yamagata/16/88 lineage became the dominant B strain in the early 1990s (McCullers et al., 2004; Rota et al., 1992). However, in most recent influenza seasons, both lineages of influenza B virus co-circulated (WHO, 2013; WHO, 2014b), which complicates selecting influenza B vaccine strains. Between 2001 and 2011, in five out of 10 influenza seasons the lineage of the vaccine strain did not match that of the circulating influenza B virus strains in the USA (Ambrose and Levin, 2012). Since antibodies against viruses of one influenza B lineage display little cross-reactivity with viruses of the other lineage (Belshe, 2010; Camilloni et al., 2009; Shaw et al., 2002), the vaccine afforded suboptimal protection in these seasons. Therefore, quadrivalent vaccines have been developed recently that not only contain components of two influenza A viruses (H1N1 and H3N2), but also components of influenza B viruses of both lineages (FDA, 2012; Traynor, 2012).

Antiviral drugs such as neuraminidase inhibitors (oseltamivir and zanamivir) can be used to treat patients infected with influenza B viruses. Although the majority of influenza B viruses are sensitive to these antiviral drugs, resistant influenza B viruses emerge occasionally (Hatakeyama et al., 2007; Stephenson et al., 2009).

Therefore, the development of novel classes of antiviral drugs that are effective against influenza B viruses of both lineages is desirable. In the present study we investigated the antiviral activity of recombinant porcine surfactant protein D (RpSP-D) against influenza B viruses. SP-D is a member of the collectin family and acts as a component of the innate immune system to protect the respiratory tract against invading pathogens. RpSP-D is of special interest since it displayed stronger antiviral activity against influenza A viruses than human SP-D and other collectins (Hillaire et al., 2013, 2011; van Eijk et al., 2003). Influenza A viruses of various subtypes including A/H1N1, A/H3N2 and A/H5N1 were inhibited by RpSP-D

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Table 1
Influenza B viruses used in the present study.

Lineage	Strain
B/Yamagata/16/88	B/Yamagata/16/88
	B/Jiangsu/10/03
	B/Netherlands/080/02
	B/Netherlands/001/07
	B/Panama/45/90
B/Victoria/2/87	B/Victoria/2/87
	B/Netherlands/138/08
	B/Netherlands/076/06
	B/Malaysia/2506/04
	B/Netherlands/151/01
	B/Hongkong/493/01

although differences in sensitivity were observed. This differential sensitivity of influenza A viruses to RpSP-D was to a large extent dependent on the number and position of glycosylation sites on the hemagglutinin (HA) of the respective viruses (Hillaire et al., 2012). These glycans were recognized by the carbohydrate recognition domain of SP-D.

To date, it remains largely unknown if RpSP-D also displays antiviral activity against influenza B viruses, similar to what is found for influenza A viruses. Therefore we assessed the antiviral potential of RpSP-D against 11 influenza B viruses belonging to the B/Yamagata/16/88 and B/Victoria/2/87 lineage which are listed in Table 1. These viruses were propagated in Madin-Darby Canine Kidney (MDCK) cells as described previously (Rimmelzwaan et al., 1998). RpSP-D was expressed, purified and characterized as described (van Eijk et al., 2004) and RhSP-D was produced based upon the full-length hSP-D clone provided by Dr. E.C. Crouch (Washington University, St. Louis, USA) (Hartshorn et al., 1996).

The minimal concentration of RpSP-D that still prevented hemagglutination by each of the viruses tested was determined as previously described (Hillaire et al., 2011). We only used highly oligomerized forms of RpSP-D as we previously reported that they neutralize influenza A viruses more efficiently than trimers (Hillaire et al., 2011). Each assay was performed in duplicate, repeated three times and the average minimal inhibitory concentration of RpSP-D was calculated for each virus and is displayed in Fig. 1.

Eleven viruses were tested and were all inhibited by RpSP-D and RhSP-D. For each virus tested, less RpSP-D was required than RhSP-D to inhibit the HA activity ($p < 0.05$, Mann–Whitney). On average, a concentration of 6.1 ng/100 μl and 204.4 ng/100 μl was required to prevent hemagglutination by influenza B viruses of RpSP-D and RhSP-D respectively (Fig. 1). RpSP-D prevented hemagglutination by influenza B viruses from B/Yamagata/16/88 and B/Victoria/2/87 lineages to similar extents (5.9 ng/100 μl and 6.3 ng/100 μl respectively). However, RhSP-D tends to inhibit viruses from the B/Yamagata lineage more efficiently than those of the B/Victoria lineage (159.1 ng/100 μl and 242.1 ng/100 μl, respectively) (Fig. 1).

Since RpSP-D inhibited hemagglutination of influenza B viruses more efficiently than RhSP-D, we investigated the inhibitory capacity of RpSP-D in more detail. Using the infection reduction assay, the capacity of RpSP-D to prevent infection of MDCK cells with influenza B viruses was assessed as previously described (Hillaire et al., 2011). Each assay was performed in triplicate, repeated two times and the average minimal inhibitory concentration of RpSP-D was calculated for each virus and is displayed in Fig. 2.

RpSP-D inhibited infection of MDCK cells with all viruses tested in a dose dependent fashion to a certain extent. Viruses from the B/Victoria lineage were neutralized efficiently by RpSP-D as shown in Fig. 2. Almost 90% reduction of infection was observed for all viruses at the highest concentration of RpSP-D tested. Viruses from

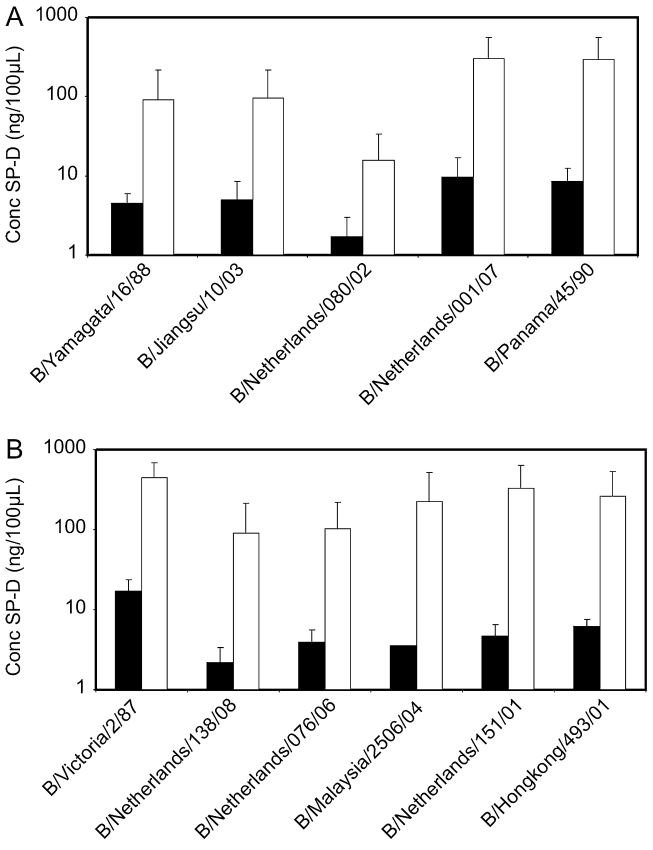

Fig. 1. RpSP-D inhibits influenza B viruses better than hSP-D. The minimal inhibitory concentrations of RpSP-D (black bars) and RhSP-D (white bars) were determined with the HI assay for viruses of the B/Yamagata (A) and B/Victoria lineage (B). The data represent the average of three independent experiments.

the B/Yamagata/16/88 lineage were also inhibited in a range of 50% to 80% at the highest concentration of RpSP-D tested.

It was demonstrated previously that porcine SP-D is more potent in neutralizing influenza A viruses than human SP-D (Hillaire et al., 2011). Also against influenza B viruses RpSP-D displayed stronger neutralizing activity than RhSP-D, indicating that it has the potential of a universal antiviral drug against influenza. The superior antiviral activity of porcine SP-D can be attributed to distinct structural features such as an extra loop in its carbohydrate recognition domain that can facilitate high affinity interactions with distal portions of branched mannoside residues present on the influenza virus HA (van Eijk et al., 2012). Influenza B viruses seemed more sensitive to human SP-D than influenza A viruses, since less RhSP-D was required to achieve full inhibition of HA activity of influenza B viruses than that of influenza A viruses (Hillaire et al., 2011). This may be the result of differences in the number and/or position of putative N-linked glycosylation sites between influenza A and B viruses. Indeed influenza B viruses have more putative N-linked glycosylation sites in the head of HA than influenza A viruses as was demonstrated with prediction algorithms (data not shown, using the online software NetNGlyc).

To be effective as an antiviral drug, RpSP-D must be able to interfere with binding of virus to cells of the respiratory tract. Therefore, we tested the capacity of RpSP-D to inhibit attachment of the two prototype viruses B/Yamagata/16/88 and B/Victoria/2/87 and two seasonal strains, B/Netherlands/080/02 and B/Netherlands/076/06, to human tracheal and ferret lung epithelial cells using virushistochemistry. These viruses were propagated in MDCK cells and purified by sucrose gradient density centrifugation. They were incubated in formalin (10%) for one week and then labeled with

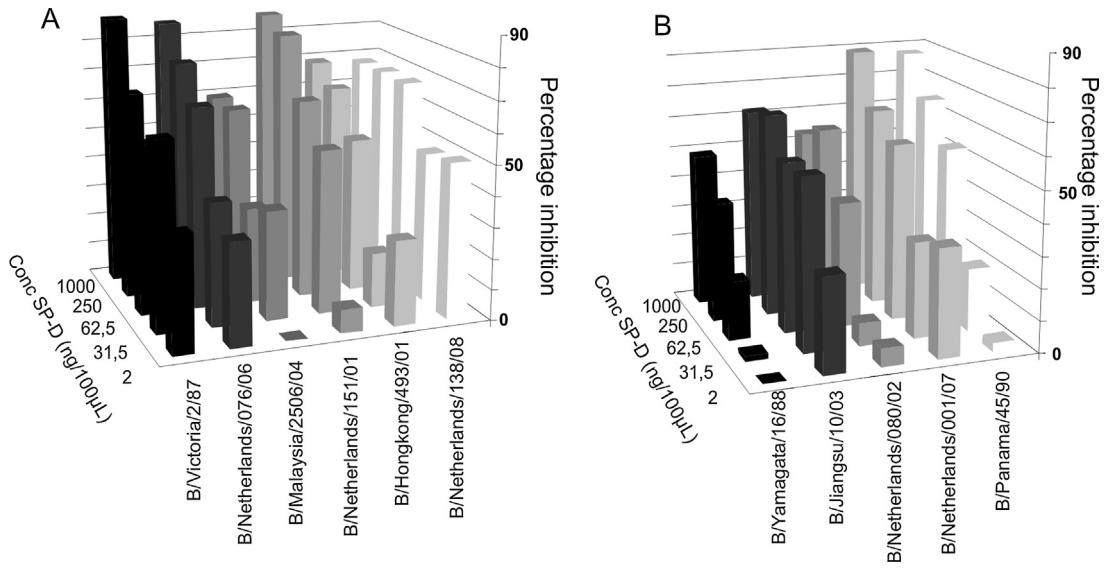

Fig. 2. RpSP-D reduces infection of MDCK cells by influenza B viruses. Using the infection reduction assay, the neutralization of influenza B viruses by various doses of RpSP-D was assessed. The reduction of infectivity was expressed as the relative number of cells that became infected according to the formula: % reduction = 1−(% infected cells in presence of RpSP-D/% infected cells without RpSP-D)×100%. The viruses were grouped by lineage, B/Victoria (A) and B/Yamagata (B). The average of triplicate wells is shown.

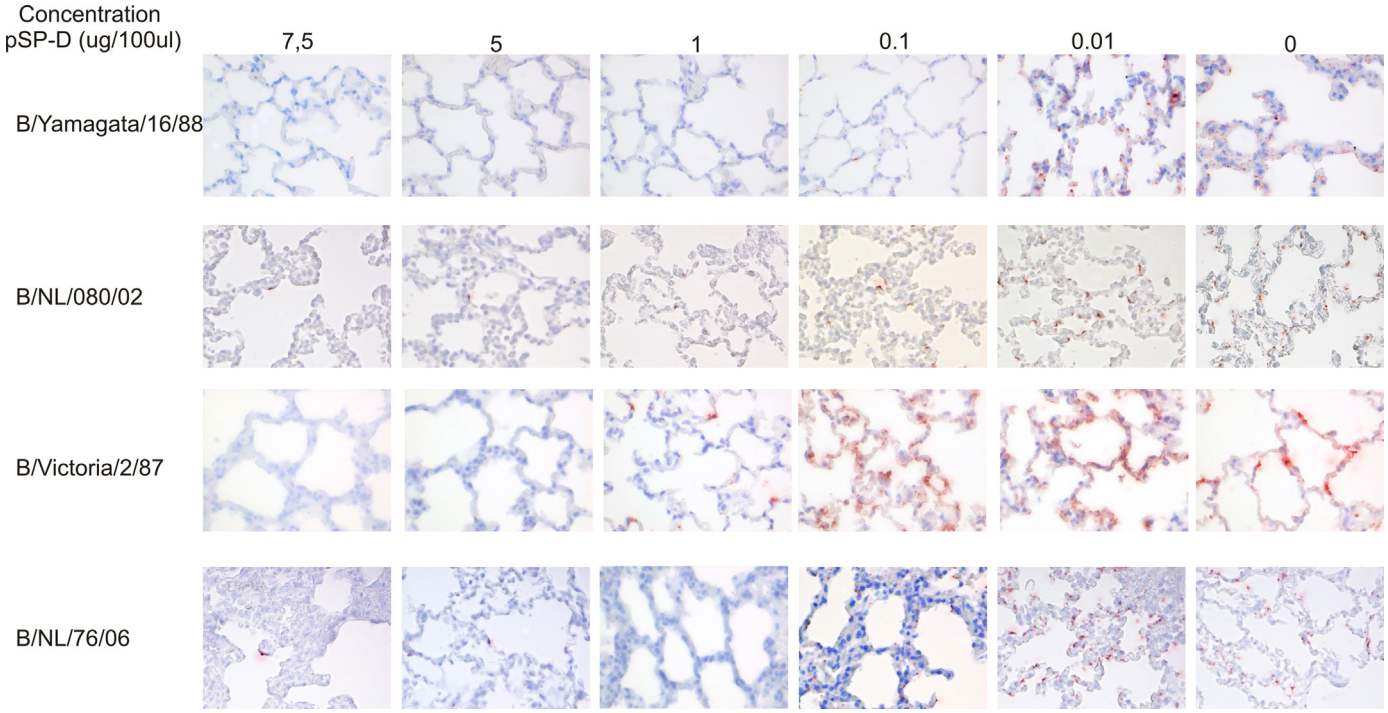

Fig. 3. RpSP-D inhibits binding of viruses of the B/Yamagata/16/88 and B/Victoria/2/87-lineage to ferret type I pneumocytes. Attachment of virus in the absence or presence of RpSP-D was studied by virushistochemistry. Sections of ferret lungs were incubated with FITC-labeled influenza B viruses of the B/Yamagata/16/88 and B/Victoria/2/87 lineage in the absence or presence of various doses of RpSP-D as indicated. Binding of virus is visible as dark-red staining. The tissues were counterstained with hematoxylin (magnification ×100).

FITC (Sigma-Aldrich, Saint Louis, MO) as described previously (van Riel et al., 2006). The assessment of virus binding to the trachea or lungs tissues was performed as described previously (Hillaire et al., 2011).

First we determined the binding pattern of influenza viruses B/Netherlands/080/02 and B/Netherlands/076/06, B/Victoria/2/87 and B/Yamagata/16/88 to ferret trachea and lungs. These four viruses did not bind strongly to ferret trachea (data not shown) but attached to type I pneumocytes of ferret lungs (Fig. 3). As shown in Fig. 3, preincubation of FITC-labeled viruses B/Yamagata/16/88, B/Netherlands/080/02 and B/Netherlands/076/06 with RpSP-D prevented binding to ferret type I pneumocytes at a minimal dose of 1 μg/100 μl, whereas for B/Victoria/2/87 a dose of 5 μg/100 μl was required. Influenza B viruses B/Yamagata/16/88 and B/Victoria/2/87 bound to ciliated cells of the human trachea, which was inhibited completely after preincubation of these viruses with ≥0.1 μg of RpSP-D (Fig. 4). These observations underscored that RpSP-D has potential as antiviral drug against influenza as it can block binding of both influenza A (Hillaire et al., 2011) and B virus (present study) to human

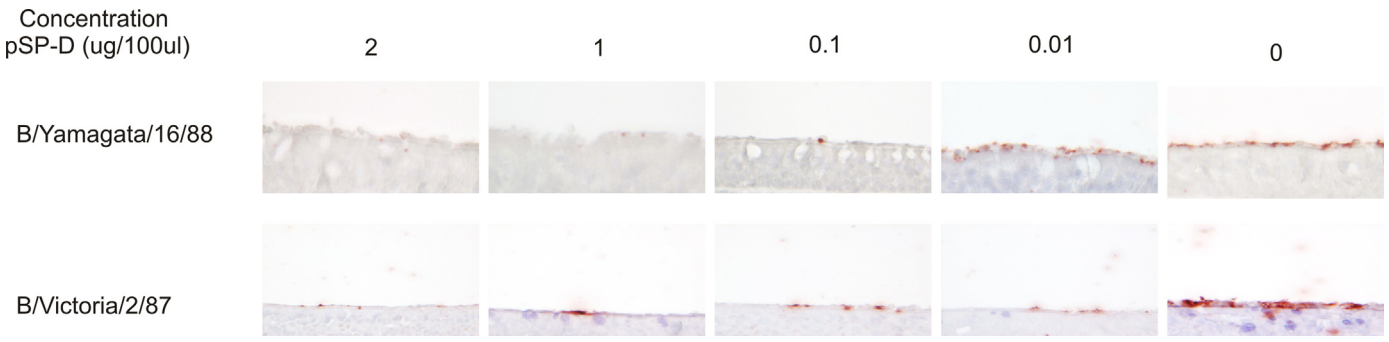

Fig. 4. RpSP-D inhibits binding of influenza viruses B/Yamagata/16/88 and B/Victoria/2/87 to human trachea ciliated cells. Attachment of virus in absence or presence of RpSP-D was studied by virushistochemistry. Sections of human trachea were incubated with FITC-labeled influenza B viruses B/Yamagata/16/88 and B/Victoria/2/87 in the absence or presence of various doses of RpSP-D as indicated. Binding of virus is visible as dark-red staining. The tissues were counterstained with hematoxylin (magnification ×100).

respiratory epithelial cells. Of note, the binding pattern of influenza viruses to respiratory epithelial cells by virushistochemistry corresponds to transmission and pathogenesis of these viruses (Chutinimitkul et al., 2010; van Riel et al., 2006, 2007). The potent antiviral activity of porcine SP-D suggests that this innate immunologic defense molecule has impact on the pathogenicity and incidence of influenza B virus infections in pigs.

In conclusion, RpSP-D displays neutralizing activity against a wide range of influenza A and B viruses. For the development of a SP-D based antiviral drug it may be of interest to investigate properties of pSP-D in more detail. A mutant human SP-D less likely would evoke an immune response. The use of this type of antiviral molecules may be less prone to drive the emergence of resistant virus strains and may increase our armamentarium to combat influenza.

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