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Enhancing the antiviral activity of chimeric canine interferon with ricin subunit B by using nanoparticle formulations

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Despite interferon alpha having a broad spectrum of antiviral activity and strong antiproliferative activity, its applications are severely limited due to the intrinsic properties of proteins, such as poor stability and short serum half-life. In our study, canine interferon alpha (*CaIFN α*) gene was fused with the ricin toxin B chain (RTB) to form *rCaIFN α /RTB*, which encodes a 463-amino acid protein containing a 15-amino acid encoded (G₄S)₃ flexible linker. After expression in prokaryote, purification and renaturation, the cytotoxicity and antiviral activity of *rCaIFN α /RTB* were investigated in Madin–Darby canine kidney (MDCK) cells. *rCaIFN α /RTB* exerted a superior anti-vesicular stomatitis virus (VSV) activity on MDCK cells. Furthermore, we have developed a nanoparticle formulation of *rCaIFN α /RTB* by using polyethylenimine (PEI) through electrostatic interaction. *rCaIFN α /RTB@PEI₁₀₀₀₀* is more stable than *rCaIFN α /RTB* at various pH and temperature levels, and it possesses enhanced antiviral activity. Our findings facilitate further research on the role of type I IFN in antiviral defense responses in *Canis lupus familiaris*.

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Introduction

Interferons (IFNs) were first reported as a substance that interferes with vaccinia and influenza virus replication.¹ Soon after confirmation of their broad spectrum of antiviral activity, several studies also demonstrated their strong antiproliferative and immune-modulatory activities. Type I IFNs bind to ubiquitous membrane receptors,² which are formed by a heterodimer of two transmembrane proteins (IFN α R1, IFN α R2), triggering the IFNAR-JAK-STAT signaling cascade, thus inducing the production of a wide array of antiviral proteins, including products of the “classical” IFN-stimulated genes (ISGs).^{3–5}

The production of the first recombinant human (rh) type I IFN proteins in *Escherichia coli* in 1980 raised hope in the therapy of different neoplastic and viral diseases.⁶ Especially in recent years, interferon therapy in viral diseases such as hepatitis B, hepatitis C and the treatment of cancer has achieved good curative effects.^{7,8} However, because of its poor stability and short serum half-life, the usual dose for unmodified IFN α is required to be once daily or three times weekly to achieve its

medical performance, which leads to an increase in the frequency and severity of adverse effects. To improve its pharmacokinetics and decrease the dosing frequency, several formulations have been developed to protract the effect of IFN, including biodegradable microspheres,⁹ multivesicular liposomes,¹⁰ and molecular modification with poly(ethylene glycol) (PEG; PEGylation).¹¹ Several PEGylated rhIFN products are already in the market because of their prolonged half-life.¹² However, the chemical conjugation process of PEGylation is rather complex and involves generating new molecular entities, and their safety evaluation is a long process. It has been revealed that the bioactivity of the protein may be affected to some extent. Hence, some studies presented an alternative strategy to PEGylation. A genetic fusion chimeric protein was expressed, composed of recombinant interferon α and recombinant human serum albumin, and it exhibits similar antiviral and antiproliferative activities and a protracted serum half-life compared with unmodified interferon α .^{13,14}

In this study, we first expressed a chimeric canine interferon alpha fused with ricin toxin B subunit (*rCaIFN α /RTB*) in *E. coli* and tested its biological activity *in vitro*. Ricin toxin B subunit is one subunit of the ricin toxin, which possesses extremely fatal toxicity. RTB is a 34-kD galactose-binding lectin which could bind to cell-surface glycoproteins or glycolipids, mediate cell endocytosis, and deliver the ricin A subunit into cytosol to exert its toxic activity.^{15–17} Studies have shown that RTB is non-toxic and could be a superior drug carrier when fused with other molecules.^{18–22} Furthermore, our present study investigated the immune-modulatory effects of RTB on the murine macrophage

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cell line RAW264.7, finding that RTB could activate macrophages and release downstream cascades of JAK-STAT. Phosphorylation levels were increased at 20 sites in the RTB-treated macrophage cell line,²³ including JAK1/TYK2 and STAT1/2, which are the docking sites and specific adapter protein of the type 1 interferon signaling pathway.

Polyethylenimine (PEI) is a cationic polymer that has been used for many years in typical industrial processes, such as paper production, shampoo production, and water purification.²⁴ Since 1995 when PEI was first introduced as a multipurpose vector for gene delivery, it has been widely utilized as nanocarrier for gene delivery systems, such as RNA delivery^{25–28} and chemotherapy.^{29–32} Our study investigated the potential of PEI as a nanocarrier for rCaIFN α /RTB, and the antiviral activity and physiochemical characteristics of the nanoparticle formulation were evaluated as well.

As shown in Fig. 1, recombinant CaIFN α /RTB chimeric protein was expressed and co-assembled with polymer PEI₁₀₀₀₀. The formed rCaIFN α /RTB@PEI₁₀₀₀₀ nanoparticles disintegrated after entering the inner environment of the organism, and rCaIFN α was slowly released, recognized by the cell surface type I interferon receptor to activate the

downstream IFN-JAK-STAT signaling cascade, which leads to the expression of the interferon-stimulated gene to develop interferon α 's broad-spectrum antiviral effect.

Results and discussion

Prokaryotic expression of rCaIFN α /RTB

To ensure optimal flexibility or separation of the adjacent domains and to promote intermolecular interactions of engineered fusion proteins,³³ glycine-serine (GS) linkers were introduced to express canine interferon fusion protein, which was fused with the ricin subunit B chain. Glycine-serine (GS) linkers have no ordered secondary structure and could be adjusted by the number (n) of G₄S-units. (G₄S) n -linkers are frequently used in recombinant fusion proteins and antibody engineering to generate loops that connect domains and do not interfere with their folding.^{34,35} The gene fragment of interest encoding the rCaIFN α /RTB protein was modified as shown in Fig. 2 and cloned into the pET28a expression vector. Then, the recombinant expression vector was transformed into *E. coli*/BL21(DE3) and induced with 1 mmol L⁻¹ IPTG. After denaturation-purification-renaturation processes, the rCaIFN α /RTB

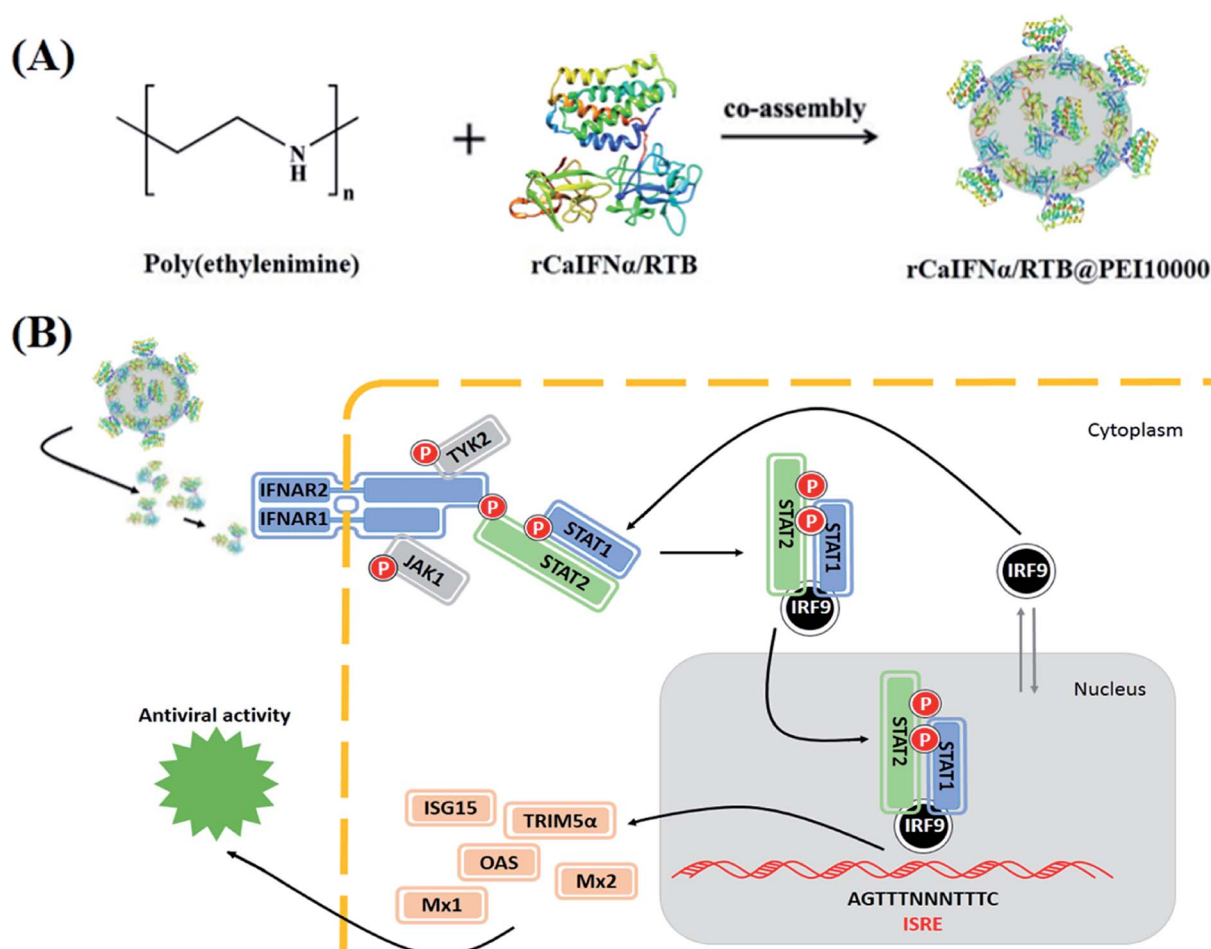


Fig. 1 Schematic illustration of rCaIFN α /RTB@PEI₁₀₀₀₀. (A) Development scheme of rCaIFN α /RTB@PEI₁₀₀₀₀. (B) Antiviral mechanism of rCaIFN α /RTB@PEI₁₀₀₀₀.



Gene Processing

Feature Key	Position	Description	Graphical View	Length
Gene I	1-435	Canine Interferon α		435
Gene II	436-540	(G ₄ S) ₃ Linker		45
Gene III	541-1329	Ricin B chain		789

Fig. 2 Gene processing of recombinant CalFN α /RTB.

protein was folded in the correct structure, which coded for 463 amino acids. SDS-PAGE analysis revealed that the rCaIFN α /RTB was mainly expressed as an inclusion body form, with an apparent molecular weight of 51 kD (Fig. 3A). Western blot analysis of the inclusion bodies incubated with 6-polyhistidine rabbit monoclonal antibody and HRP goat anti-rabbit IgG indicated that the CalFN α /RTB gene was expressed, consistent with the SDS-PAGE results (Fig. 3B).

Synthesis and characterization of rCaIFN α /RTB@PEI₁₀₀₀₀ NPs

Despite the main applications of PEI polymer as a gene vector for medical applications,^{36–40} our study assembled the recombinant protein and PEI to enhance the antiviral activity of rCaIFN α /RTB. The rCaIFN α /RTB@PEI₁₀₀₀₀ NPs were synthesized by mixing rCaIFN α /RTB and PEI₁₀₀₀₀ in a water solution, followed by centrifugation and washing. The recombinant protein was dialyzed in Tris–HCl buffer with pH value 9.5, which is over the theoretical PI of rCaIFN α /RTB, to display negative charge and maximize the electrostatic absorption effect of PEI polymer. rCaIFN α /RTB@PEI₁₀₀₀₀ NPs were further characterized by TEM and zeta potential techniques. The morphology and structure of rCaIFN α /RTB@PEI₁₀₀₀₀ NPs were firstly investigated by TEM. As shown in Fig. 4A, as expected, the rCaIFN α /RTB@PEI₁₀₀₀₀ NPs presented a uniform spherical shape, with the average size of 332.4 nm, as shown in Fig. 4D.

Zeta potential of rCaIFN α /RTB@PEI₁₀₀₀₀ NPs was also monitored to estimate whether the rCaIFN α /RTB was co-assembled with PEI. Our results showed that compared with -16.7 mV of rCaIFN α /RTB, $+30.4$ mV zeta potential was observed for the rCaIFN α /RTB@PEI₁₀₀₀₀ (Fig. 4C). Then, the successful co-assembly of rCaIFN α /RTB with PEI polymer was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Free rCaIFN α /RTB without PEI was used as parallel control. Fig. 4B shows the two proteins displaying the main band with same molecular weight at 51 kD. All of these results mentioned above indicate that the rCaIFN α /RTB was successfully assembled with the PEI polymer.

The stability of rCaIFN α /RTB@PEI₁₀₀₀₀ NPs

The stability of rCaIFN α /RTB@PEI₁₀₀₀₀ NPs was tested by monitoring their size and size distribution in aqueous solution at 4°C for 6 days. As shown in Fig. 4E, rCaIFN α /RTB@PEI₁₀₀₀₀ NPs remained stable and monodispersed for up to 6 days in aqueous solution at 4°C , guaranteeing the feasibility of long-term storage.

Cellular uptake of rCaIFN α /RTB@PEI₁₀₀₀₀ NPs

To better observe the cellular uptake process of NPs, FITC was selected as a fluorescence probe to label rCaIFN α /RTB, and FITC-rCaIFN α /RTB@PEI₁₀₀₀₀ was prepared. The green fluorescence emitted under excitation was used to better observe the uptake of

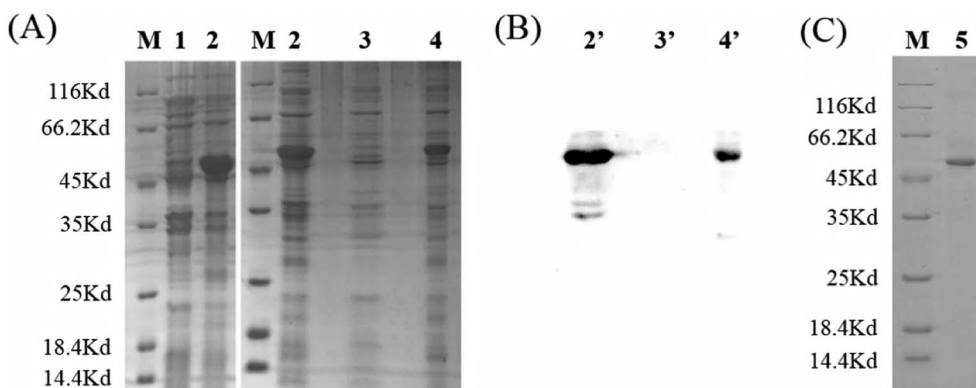


Fig. 3 (A) Protein expression and verification of rCaIFN α RTB. M: unstained protein marker; lane 1: empty vector pET28a; 2: rCaIFN α RTB with 1 mM IPTG induction; 3: supernatants of rCaIFN α RTB; 4: precipitates of rCaIFN α RTB. (B) Western blot analysis of rCaIFN α RTB by anti-6 \times His Tag monoclonal antibodies. 2': rCaIFN α RTB with 1 mmol L⁻¹ IPTG induction; 3': supernatants of rCaIFN α RTB; 4': precipitates of rCaIFN α RTB. (C) rCaIFN α RTB after purification and renaturation.



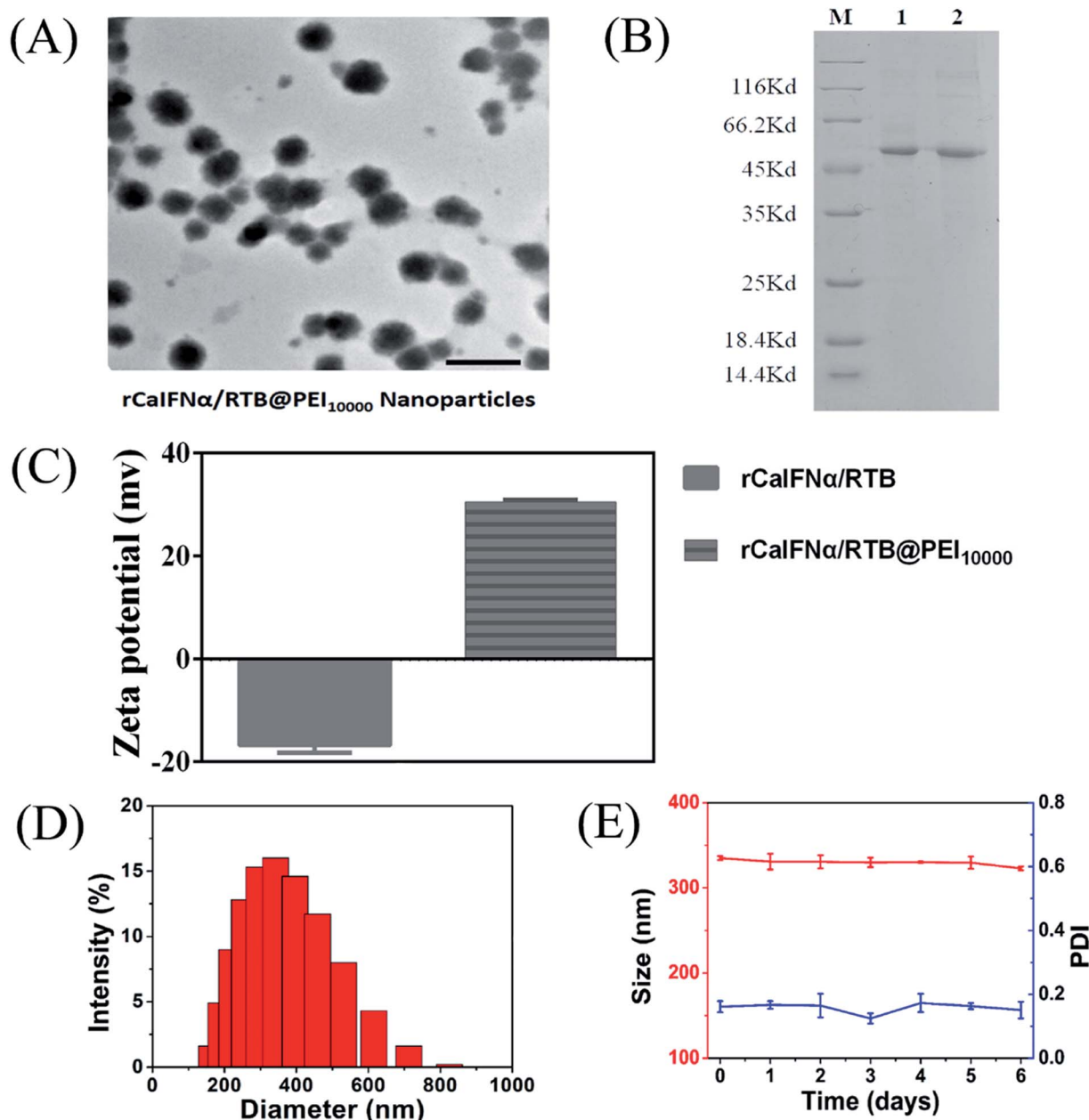


Fig. 4 (A) TEM image of rCaIFN α /RTB@PEI₁₀₀₀₀; the scale bar indicates 500 nm. (B) SDS-PAGE analysis of rCaIFN α /RTB@PEI₁₀₀₀₀: lane 1, free rCaIFN α /RTB as a control; lane 2, rCaIFN α /RTB@PEI₁₀₀₀₀. (C) Zeta potential of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀. (D) Size distribution of rCaIFN α /RTB@PEI₁₀₀₀₀. (E) Size change of rCaIFN α /RTB@PEI₁₀₀₀₀ in aqueous solution at 4 °C for 6 days.

NPs by cells, and Hoechst 33258 was used as the nuclear dye (Fig. 5). First, we diluted FITC-rCaIFN α /RTB@PEI₁₀₀₀₀ NPs (rCaIFN α /RTB: 50 μ g mL⁻¹). After incubation with MDCK cells for 2 h, we found that NPs could barely enter the cell membrane; the rCaIFN α /RTB@PEI₁₀₀₀₀ nanoparticles disintegrated, and rCaIFN α was slowly released. The uptake behavior showed a time-dependent manner. To determine whether the released rCaIFN α /RTB could be combined with cell membrane surface receptor IFNARs and activate the downstream cascade, the cell membrane dye Dil was used to locate the cell membrane. The results showed that after 2 hours of incubation, the uptake of rCaIFN α /RTB occurred. The green fluorescence signal of FITC-

rCaIFN α /RTB was overlapped by the red fluorescence signal of the cell membrane around the blue nucleus, indicating that the rCaIFN α /RTB released from NPs combined with the cell surface receptors, and the NPs were able to develop their antiviral effect through the IFNAR-JAK-STAT signaling cascade. The above results illustrate that rCaIFN α /RTB@PEI₁₀₀₀₀ NPs have a sustained-release effect, which can prolong the half-life of the drug and better enable its biological role.

Antiviral activity of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀

To analyze the antiviral activity of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀, cytopathic inhibition assay was operated with



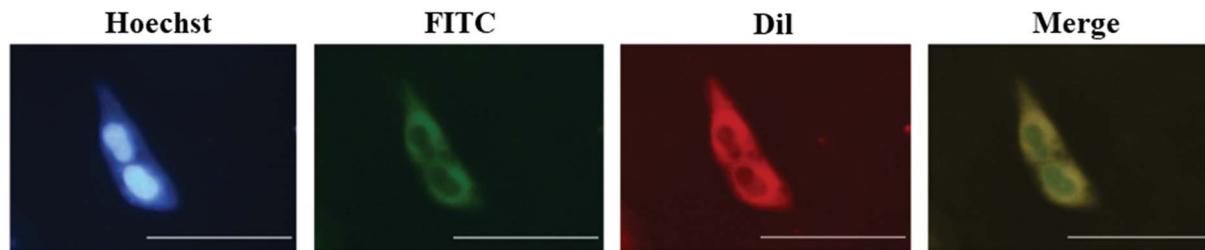


Fig. 5 Fluorescence imaging of MDCK cells incubated with FITC-rCaIFN α /RTB@PEI₁₀₀₀₀ NPs for 2 hours; the scale bar indicates 100 μ m.

MDCKs, and the antiviral activity of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ was analysed and compared with commercial CaIFN α (Fig. 6). rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ were serially diluted and pre-incubated with MDCKs for 24 h, then $100\times$ TCID₅₀ VSV was challenged for another 24 h. Results show that while the viral control displayed complete cytopathicity, the rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ protein exerted a superior antiviral activity compared to commercial CaIFN α . The recombinant fusion protein and its NPs were able to develop protective effects with the MDCK cell line from the $100\times$ TCID₅₀ VSV challenges. The antiviral activity against VSV reached 1.2×10^9 IU mL⁻¹ with the recombinant protein concentration at 1.4 mg mL⁻¹. We also conducted a cytopathic inhibition assay with WISH cell lines, but results show that the recombinant fusion protein could not develop its antiviral activity in WISH cell lines, mainly because the antiviral ability of rCaIFN α /RTB was species-specific.

Primary physicochemical characteristics of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀

We tested the stability of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ at pH 2, 4, 6, 7, 9, and 11, as well as at 42 °C, 56 °C, and 65 °C, respectively. Therefore, rCaIFN α /RTB was treated at different conditions, then antiviral assay was conducted in the MDCK/VSV system in parallel for rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀. Results showed that rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ were stable at changing pH conditions, but when incubated in 65 °C for 4 h, the rCaIFN α /RTB antiviral activity was lost, but that of rCaIFN α /RTB@PEI₁₀₀₀₀

was not (Fig. 7A and B). In addition, physicochemical characteristics of rCaIFN α /RTB were analyzed using the MDCK/VSV system, including pH and temperature sensitivity. rCaIFN α /RTB was verified to be sensitive to temperature and pH variation, but after rCaIFN α /RTB was assembled with the poly-ethylenimine polymer, the rCaIFN α /RTB@PEI₁₀₀₀₀ was remarkably stable at various temperature and pH levels. Given the substantial antiviral activity and low cytotoxicity of rCaIFN α /RTB@PEI₁₀₀₀₀, it is a potential candidate for a novel, effective therapeutic agent.

Conclusion

In conclusion, a novel fusion protein rCaIFN α /RTB was expressed, and its biological activity was estimated. rCaIFN α /RTB presented moderate biological activity and low cytotoxicity. This research provides a new idea and developed direction for the biological modification of canine interferon α , and it demonstrates a new proof that biotoxin subunits can be used as an excellent carrier protein. Furthermore, rCaIFN α /RTB@PEI₁₀₀₀₀ NPs were synthesized, and their biological activities were estimated as well. The NPs enhance the stability of rCaIFN α /RTB and enable greater tolerance to pH and temperature, while maintaining significant antiviral activity in extremely acidic conditions. This work provides a new idea for the study of new dosage forms of canine interferon α and shows the great potential of PEI₁₀₀₀₀ as a simple and effective nano-drug carrier in the study of drug stability. All of these findings lay a foundation for future research into rCaIFN α /RTB and

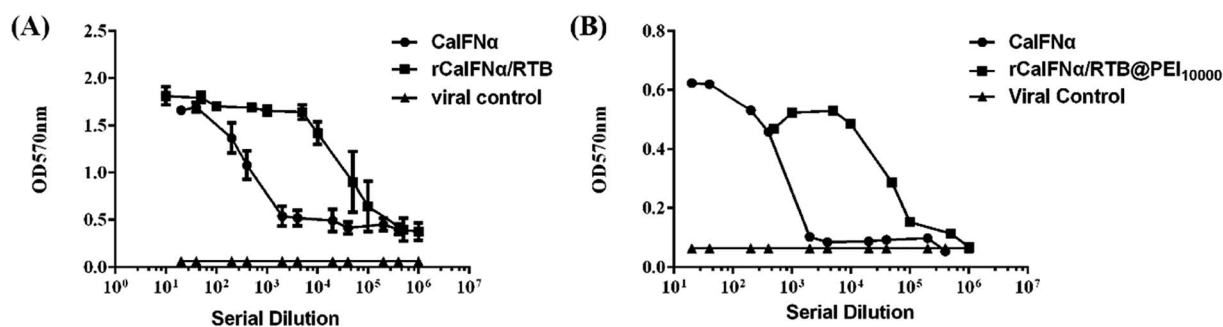


Fig. 6 Antiviral activity of (A) rCaIFN α /RTB and (B) rCaIFN α /RTB@PEI₁₀₀₀₀. The protective effects of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ were evaluated in antiviral assays compared with commercial CaIFN α using MDCK cell lines challenged with $100\times$ TCID₅₀ VSV, as described under Materials and methods.

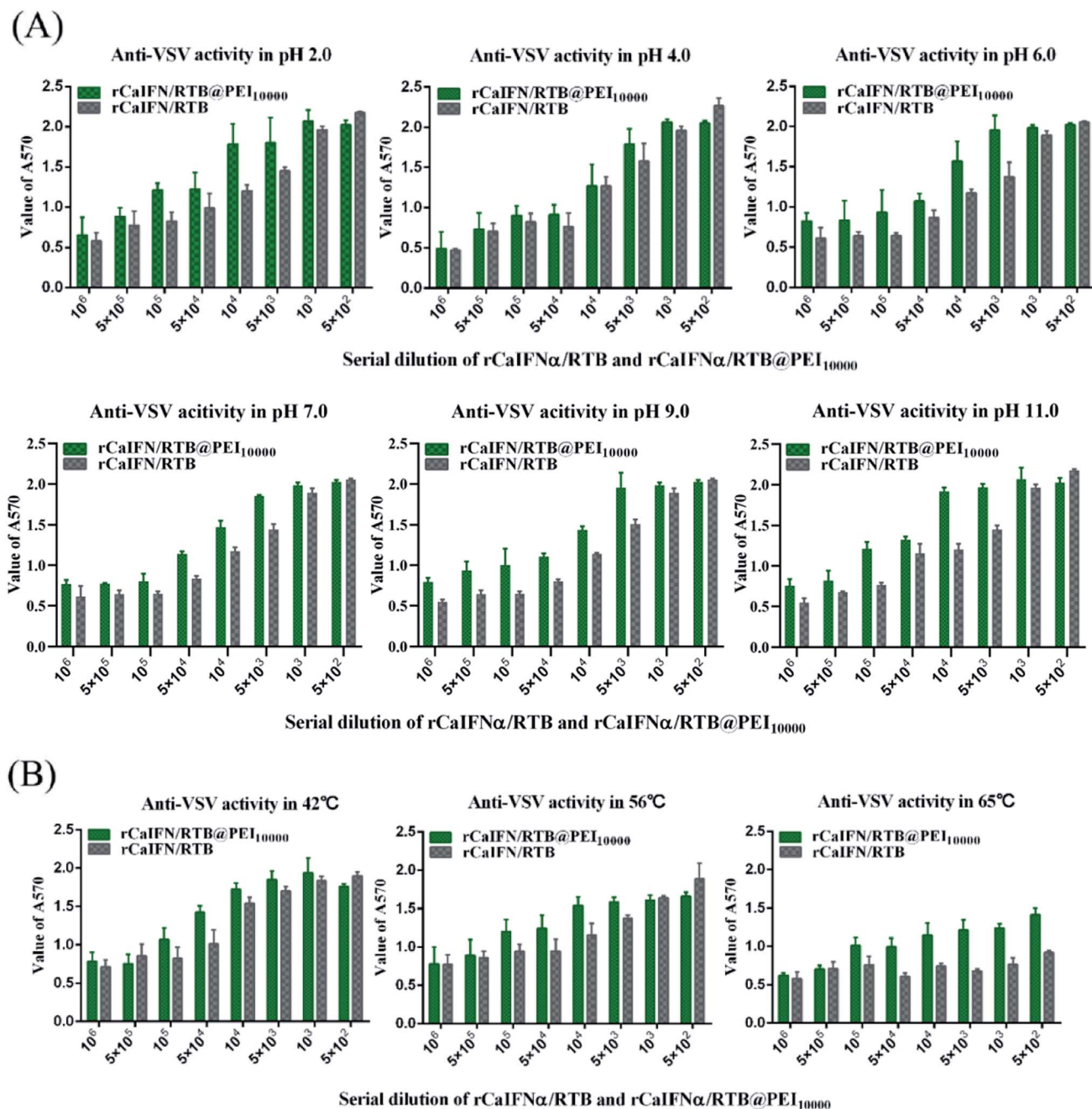


Fig. 7 Comparison of antiviral activity of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ on MDCK cell lines challenged with 100 \times TCID₅₀ VSV after treatment at various pH conditions and temperatures.

might also provide insights on the use of rCaIFN α /RTB as an effective antiviral agent against viral dog infections and diseases.

Materials and methods

Virus and vector

Vesicular stomatitis virus (VSV) was kindly provided by Dr Zhang Guo Li from Changchun Military Veterinary Research Institute. The recombinant *E. coli* cloning vector for canine IFN α and RTB was constructed and preserved in our laboratory.

Overlap PCR method was used to link the 3' end of the canine IFN α gene and 5' end of the RTB gene with linker sequence, which is N-GGGGSGGGGSGGGG-C ((G₄S)₃ linker).

Prokaryotic expression of CaIFN α /RTB and renaturation process

The recombinant cloning vector *pMD19T-CaIFN α /RTB* was digested with restriction enzymes Nde I and Xho I. The gene fragment of interest was inserted into the expression vector pET28a (Novagen, Darmstadt, Germany), which included an N-



terminal histidine hexamer tag (6×His tag). The recombinant expression vector was constructed and transformed into *E. coli*/BL21(DE3). The recombinant protein rCaIFN α /RTB was expressed mainly in the form of inclusion bodies and then resolved in denaturation buffer with a high concentration of urea. The denatured rCaIFN α /RTB was purified with chelating sepharose, which was charged with nickel and renatured in urea gradient dialysis. The final concentration of rCaIFN α /RTB was determined with the BCA protein assay and then analyzed with SDS-PAGE.

Western blot analysis of rCaIFN α /RTB

After SDS-PAGE analysis, proteins were transferred to PVDF membrane without staining. Then, the membrane was blocked with PBS containing 5% BSA. Rabbit monoclonal antibody against 6×His tag (1 : 3000 diluted) was used as the primary antibody, and HRP-conjugated goat anti-rabbit IgG (1 : 40 000 diluted) was used to detect the immune complex.

Analysis of antiviral activity *in vitro*

Antiviral activity was assayed by the ability of rCaIFN α /RTB to inhibit the cytopathic effects (CPE) of vesicular stomatitis virus (VSV) on canine kidney cells (MDCK) according to the protocols below. MDCK was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 8% fetal bovine serum. Cells were digested, then seeded onto 96-well plates to a concentration of 5×10^4 cell per mL for the antiviral activity assay. Cells were treated with commercial canine IFN α and rCaIFN α /RTB, then filter-sterilized and serially diluted with DMEM supplemented with 2% FBS. The plates were incubated at 37 °C with 5% CO₂ for 24 h. Then, the supernatant was discarded, and cells were washed with DMEM without FBS three times and challenged with VSV (100× TCID₅₀). When the CPE reached 100% in the viral control, whole cells were stained with crystal violet for 30 min, then washed with ddH₂O three times. The crystal violet was eluted with 100 μ L elution buffer (50% ethanol, 0.1% acetic acid), and absorbance was measured at 595 nm on a microplate reader (Tecan infinite F500). The antiviral activity of rCaIFN α /RTB was also estimated in the human amniotic cell line (WISH), cultured in DMEM supplemented with 8% FBS. All antiviral assays were repeated three times.

Detection of primary physicochemical characteristics of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀

The physicochemical characteristics of rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ were analyzed using the MDCK/VSV system, including pH and temperature sensitivity. In detail, the rCaIFN α /RTB and rCaIFN α /RTB samples were analyzed as follows: (1) combined with hydrogen chloride or sodium hydroxide to adjust pH levels of 2.0, 4.0, 6.0, 7.0, 9.0, 11.0 or 4 h at 4 °C, after which they were adjusted back to the original pH 7.0; (2) placed in a 42 °C, 53 °C, and 65 °C water bath for 4 h, then rapidly placed in an icebox for cooling, after which the antiviral activity was determined by the MDCK/VSV system. The

antiviral activities of the rCaIFN α /RTB and rCaIFN α /RTB@PEI₁₀₀₀₀ samples were compared.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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