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# Comparison of rabies virus protection by single chain and leucine zipper Fv fragments cocktail derived from a monoclonal antibody cocktail

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ABSTRACT

Monoclonal antibodies (MAbs) are a unique and attractive class of biologics and are potential substitutes for post-exposure rabies prophylaxis. The safety, tolerance, and broad neutralization efficiency of a MAb cocktail called CL184, composed of the antibodies CR4098 and CR57, was confirmed in a phase I clinical trial. We have prepared a series of single-chain Fv fragments (scFvs) and leucine zipper Fv fragments (zipFvs) from CR57 and CR4098. In this study, we selected and formed scFv and zipFv cocktails and compared their protective effects against the rabies virus. Mice and hamster challenge models demonstrated the improved protection of the zipFv cocktail compared with scFv cocktail, because of its stronger affinity. The results indicate that zipFv production is a promising novel method for the genetic engineering of antibody fragments and improving affinity through systematic screening may be important when designing small molecule antibodies against RV.

## 1. Introduction

Rabies is an infectious disease with high mortality. It is caused by the rabies virus (RV), and has been cause for concern worldwide for human health for more than 4,000 years (Behera et al., 2011). At present, there are no effective therapies against rabies. Approximately 55,000 people die of rabies worldwide each year, most after being scratched or bitten by dogs (Talbi et al., 2011). More than 95% of rabies deaths occur in developing countries, particularly China and India, which lack animal vaccination programs (Knobel et al., 2005). Once humans and animals are exposed to a potential rabies infection, the World Health Organization (WHO) recommends the use of the rabies vaccine and plasma-derived rabies immunoglobulin (RIG). Purified equine rabies immunoglobulin (ERIG) and human rabies immunoglobulin (HRIG) are the only two products approved by the U.S. Food and Drug Administration (FDA) (Kostense et al., 2012). Both have side effects, such as potential disease transmission, adverse serum allergic reactions, high cost, and limited availability (Wang et al., 2012).

Several studies have demonstrated that antibody cocktails containing several monoclonal antibodies might be potential candidates for rabies treatment (Nelson, 2010). Phage display technologies for the in vitro discovery and selection of antibodies are less time-consuming and highly suitable. To date, many antibody candidates have been evaluated for rabies preventative effects (Benedictis et al., 2016; Both et al., 2013; Prosniak et al., 2003). Among these, the most impressive has been a monoclonal antibody cocktail called CL184, composed of the antibodies CR4098 (human IgG1 kappa) and CR57 (human IgG1 lambda) (Bakker et al., 2008). The cocktail displayed the same protection as approved HRIG in a post-exposure prophylaxis challenge study in hamsters (Goudsmit et al., 2006). Phase I experimental results have confirmed its safety, tolerance, and neutralization efficiency. In addition to the full-length IgG-type antibody, many other small molecule antibody fragments have been successfully developed, such as antigen-binding fragments (Fabs) and single chain variable fragments (scFvs) (Sveshnikov et al., 2010; Zhang et al., 2001; Zhao et al., 2009, 2004).

In recent years, a series of small molecule antibody fragments have been successfully expressed in *Escherichia coli*-based gene expression systems, which are quite amenable to gene manipulation and the expression of small non-glycosylated recombinant antibody fragments, including anti-RV scFvs (Weisser and Hall, 2009). In our previous studies, we developed a series of scFvs derived from CR57 and CR4098 (Duan et al., 2014, 2012; Xi et al., 2016; Yuan et al., 2014). Reforming the structure of scFvs, new leucine zipper Fv (zipFv) antibodies were prepared using leucine zippers derived from the transcription factors FOS and JUN (Li et al., 2015; Xi et al., 2017). Structurally, zipFvs strongly resemble IgG, with free heavy chain variable region (VH) and light chain variable region (VL) N-terminal ends.

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In this study, we prepared scFv and zipFv cocktails based on the CL184 cocktail and compared their RV-protective effects in both mice and hamsters. We also investigated the possibility of replacing complete IgG antibodies with scFv or zipFv cocktails.

## 2. Materials and methods

### 2.1. Preparation of scFvs and zipFvs

Four small molecular antibodies (scFv57RN, scFv98H, zipFv57, and zipFv98) were evaluated in this study. The scFv cocktail was composed of scFv57RN (in VL-linker-VH orientation) and scFv98H (in VH-linker-VL orientation) (Xi et al., 2016; Yuan et al., 2014). The zipFv cocktail was composed of zipFv57 and zipFv98, which were described in our previous studies (Li et al., 2015; Xi et al., 2017). Two heterodimers forming "zipper" peptides, FOS/JUN, were respectively linked to their VH/VL C-terminal ends. VH/VL fragments containing FOS/JUN leucine zippers were assembled in vitro to form zipFvs. Antibody expression and preparation methods have been previously described (Li et al., 2015; Xi et al., 2017).

### 2.2. Relative affinity assay

The four small molecular antibodies were added to RV-coated plates (Beijing Midwest Group Co. Ltd) at 0.5 μM. After incubation and washing in phosphate-buffered saline (PBS), the plates were incubated with 200 μL $NH_4SCN$ (0–6 M) and shaken at 500 rpm for 1 h. After five PBS washes, plate-bound proteins were incubated with rabbit polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies. After washing, tetramethylbenzidine was added. After a 20 min reaction, absorbance was measured at 450 nm, and the value of 0 M $NH_4SCN$ was set as 100%.

Dissociation constants (Kd) were measured by non-competitive enzyme-linked immunosorbent assay (ELISA) (Beatty et al., 1987). In brief, serial two-fold dilutions of antibodies were added to ELISA plates coated with inactivated RV at either 0.5 or 1 μg/well. After incubation for 1 h and five PBS washes, plate-bound proteins were incubated with rabbit polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies as described above. The Kd was calculated based on the formula $K = (n - 1)/2(nAb' - Ab)$, where Ab and Ab' corresponded to the antibody concentrations that produced half the absorbance value when the antigen concentrations were Ag and Ag', where $n = Ag/Ag'$.

### 2.3. In vitro neutralization potency test

The rapid fluorescent focus inhibition test (RFFIT) was performed to determine the antibody neutralizing potency according to a previous research (Yu et al., 2013). Serial three-fold dilutions of the four small molecular antibodies, commercial HRIG, and standard serum were added to 96-well microplates. The diluted samples were mixed with 50 μL of the RV strain CVS-11, which caused 80% infection in BSR cells, and incubated at 37 °C for 1 h. Then, $4 \times 10^4$ BSR cells were added to each well and incubated for 24 h. Finally, the plate was fixed with 80% acetone to stain the virus and visualized using a fluorescein (FITC)-conjugated anti-nucleoprotein (NP) monoclonal antibody on a fluorescence microscope. The neutralization potency of the test antibodies was calculated according to the Reed and Muench method (Reed and Muench, 1938).

### 2.4. Antibody clearance in vivo

For in vivo scFv and zipFv clearance tests, each Kunming mouse was injected with 0.3 mg antibody in the quadriceps muscles and bled at different time points ranging from 30 min to 5 h. Serum samples were then added to RV-coated ELISA plates, and processed as described above. In another clearance test, mice were injected with either 200 IU/kg HRIG or scFv cocktail based on their weight. Sera samples were taken at different times over 12 d, and a final blood test was performed by RRFIT.

### 2.5. Neutralization test against RV in a mice model

Kunming mice (10 animals/group) were injected with a mixture of antibody and 2500 LD50 of the CVS-24 strain of RV in the left quadriceps muscles. HRIG served as the positive control, and the negative control was RV alone. The four small molecular antibodies and HRIG were tested at 5, 10, and 20 IU/kg. The mice were evaluated for up to 28 days. Once clinical signs were observed, mice were isolated and monitored daily for death. The in vivo neutralizing potencies of the scFv and zipFv cocktails were also tested. The doses of neutralizing antibody cocktail were equivalent to those of HRIG at 10, 20, and 40 IU/kg.

### 2.6. RV protection in a hamster post-exposure prophylaxis challenge model

The scFv and zipFv cocktails were tested at 80 and 200 IU/kg, using 20 IU/kg HRIG as a positive control. The negative control was RV alone. First, hamsters (6 animals/group) were injected with 100 LD50 CVS-24 in the left quadriceps muscles. After 2 h, the hamsters were injected with HRIG or small molecular antibody cocktail at the same location. At the same time, all hamsters were also vaccinated in the right quadriceps muscles with 100 μL rabies vaccine (0.1 IU). On days 3, 7, 14, and 28, all living hamsters were revaccinated with the same dose of rabies vaccine.

### 2.7. Statistical analysis

Data were obtained from at least three independent experiments and statistical significance ($p < 0.05$) was analyzed using GraphPad Prism version 5.0 in this section.

## 3. Results

### 3.1. Preparation of scFv57RN/98H and zipFv57/98

The scFv57 and scFv98H antibodies were expressed in *E. coli* (BL21) in inclusion bodies. After purification and refolding, sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that both were approximately 25 kDa, consistent with their predicted sizes (Xi et al., 2016; Yuan et al., 2014). VH/VL fusions with zipFv FOS/JUN peptides were also expressed in inclusion bodies. Inclusion bodies containing the two peptides were mixed in 8 M urea at equivalent molar quantities. Assembly and renaturation were performed by dialysis. After renaturation, all antibodies were further purified by gel filtration chromatography. The identification of zipFv57 and zipFv98 was performed as described in our previous reports (Li et al., 2015; Xi et al., 2017).

### 3.2. Relative affinity assay

Usually, the affinities of small molecular antibodies are much lower than their corresponding IgG. However, antigen affinity is a key factor for therapeutic antibodies, so we measured the relative affinities of the four small molecular antibodies. As shown in Fig. 1, the absorbances decreased with increased $NH_4SCN$ concentration. The $NH_4SCN$ concentrations when the absorbance at 450 nm was reduced by 50% were 2.38 and 2.05 M for scFv57RN and scFv98H, respectively (Fig. 1a–b). The Kd values were $(5.1 \pm 0.32) \times 10^6$ L/M for scFv57RN and $(7.70 \pm 0.22) \times 10^6$ L/M for scFv98H. The $NH_4SCN$ concentrations when the absorbance at 450 nm was reduced by 50% were 5.28 M for zipFv57 and 3.33 M for zipFv98. The Kd values of zipFv57 and zipFv98 were $(46.53 \pm 3.91) \times 10^6$ L/M and $(39.31 \pm 2.75) \times 10^6$ L/M,