# Generation and Characterization of a Novel Small Biologic Alternative to PCSK9 Antibodies, DS-9001a, Albumin Binding Domain-Fused Anticalin Protein

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# Supplementary Method

## Phagemid selection of optimized Anticalin proteins against PCSK9

For the selection of optimized PCSK9-specific Anticalin proteins, $2 \times 10^{12}$ phagemids from the biased maturated libraries were used. Phagemids were dissolved in PBS supplemented with 0.1% Tween-20 (v/v) (PBS-0.1%T) containing 50 mM benzamidine and 1% (w/v) casein. To select Anticalin proteins with increased affinity, phagemids were incubated with reduced concentrations of biotinylated PCSK9 proteins that ranged from 0.01 to 10 nM. In several instances, phagemids were incubated at 65° C for 10 min to select for muteins with increased heat tolerance. Dissolved phagemids were incubated for 40 min with biotinylated PCSK9 proteins before 0.3 mM desthiobiotin was added to the solution to saturate free streptavidin binding sites and incubation was continued for 20 min. Subsequently, blocked [1% (w/v) casein in PBS-0.1%T] and drained paramagnetic beads that were coated with either streptavidin or neutravidin were added for 20 min to capture PCSK9-phagemid complexes. Uncomplexed phagemids were removed by washing the beads eight times with 1 mL of PBS-0.1%T by thorough resuspension followed by the collection of beads with a magnet. Bound phagemids were first eluted with 300 µL of 70 mM triethylamine for 10 min, followed by immediate neutralization of the supernatant with 100 µL of 1 M Tris-HCl pH 6.0. After one intermediate wash cycle, remaining phagemids were eluted with 100 mM glycine pH 2.2 for 10 min, followed by immediate neutralization with 50 µL of 0.5 M Tris-base. Both elution fractions were pooled and used to infect 4 mL of log-phase *E. coli* culture ($OD_{550}$ 0.45–0.6) for reamplification. After incubation for 30 min under agitation, bacteria were collected by centrifugation at 5000 × g for 2 min, resuspended in 1 mL of 2xYT medium, and plated on three large LB/Amp agar plates (10 g/L bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5, 15 g/L agar, 100 µg/mL ampicillin). Plates were incubated overnight at 32° C. Infected cells were scraped from the agar plates using 50 mL of 2xYT medium supplemented with 100 µg/mL ampicillin (2xYT/Amp). A total of 50 mL of 2xYT/Amp medium was inoculated with the appropriate volume of bacterial suspension to reach an $OD_{550}$ of 0.08. The culture was incubated at 37° C on a shaker (160 rpm) until an $OD_{550}$ of 0.5 was reached and then infected with helper phages ($1.5 \times 10^{11}$ pfu) by incubation for 15 min with gentle agitation and for 45 min on a shaker at 37° C. Subsequently, kanamycin was added to a final concentration of 70 µg/mL to select for bacteria that had been infected by the helper phages. Finally, expression of the pIII-Anticalin proteins was induced by the addition of 25 ng/mL anhydrotetracycline. PCSK9-specific Anticalin proteins were selected by repeating the above cycle four times. For the specific selection of Anticalin proteins with reduced $k_{off}$ rates, either a more stringent wash protocol was applied by performing 5 additional wash steps after round 1, 10 after round 2, 15 after round 3, and 20 after round 4 or Anticalin protein-PCSK9 complexes were incubated with different amounts (10 nM–5 µM) of purified parental Anticalin protein to allow competition in PCSK9 binding between optimized and parental Anticalin proteins. Additionally, combinations of both methods were applied.

## Evaluation of DS-9001a pharmacokinetic profile in rat

DS-9001a or DS-9001a without ABD was intravenously injected into male SD rats (9 weeks old) at 10 or 7.6 mg/kg, respectively, which are equivalent to 0.45 μmol/kg (n=3). Administration was conducted at 2.0 mL/kg. Blood was collected from the tail vein at 0.5, 1, 2, 4, 6, 8, 10, 24, 48, and 72 h after injection. Plasma was obtained by centrifugation (12,000 rpm, 5 min, 4° C).

Ninety-six-well plates were coated with 25 μL/well of anti-protein 4 IgG, which binds to the Anticalin protein portion of DS-9001a (generated at Immuno-Biological Laboratories Co., Ltd., Fujioka, Japan), diluted to 2 μg/mL with PBS and incubated at 4° C for overnight. The plates were washed three times with 0.05% (v/v) Tween 20 in PBS (PBS-0.05%T) and blocked with 150 μL/well of PBS containing 3% (w/v) BSA for 1–2 h at room temperature. Standards were prepared using PBS-0.05%T containing 20% (v/v) rat plasma and 1% (w/v) BSA (20% rat plasma). Plasma samples were diluted fivefold with PBS-0.05%T containing 1% (w/v) BSA and then further diluted with 20% rat plasma, if necessary. After washing in the same manner as described above, 25 μL/well of the standard and diluted sample were added to the plates and incubated for 1–2 h at room temperature. Detection reagent was prepared by mixing the biotinylated anti-protein 4 IgG and Streptavidin Sulfo-Tag (Meso Scale Diagnostics, LLC, Rockville, MD) to make a concentration of 1 μg/mL each in PBS-0.05%T containing 1% (w/v) BSA; then, the mixture was left on ice for 1 h. After washing, 25 μL/well of the detection reagent was added to the plates and incubated for 1–2 h at room temperature. After the final washing step, 150 μL/well of 2x Read Buffer prepared by dilution of 4x MSD Read Buffer T with surfactant (Meso Scale Diagnostics, LLC) was added to the plates. Luminescence intensity was measured using SECTOR Imager (SI2400; Meso Scale Diagnostics, LLC). The standard curve ranges of DS-9001a and DS-9001a without ABD were 0.685–500 and 0.685–167 ng/mL, respectively. Standard regression was established using a four-parameter logistic curve fit with 1/y2 weighting in the Discovery Workbench 3.0 (Meso Scale Diagnostics, LLC). The measured concentration of DS-9001a or DS-9001a without ABD in each sample was automatically calculated by the software using the calibration curve and then converted to the plasma concentration by multiplying by the dilution factor using Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA).

Pharmacokinetic parameters of DS-9001a and DS-9001a without ABD after administration to rats were calculated using Phoenix WinNonlin (version 6.3; Certara L.P., Princeton, NJ) based on a non-compartmental method. Calculation of the elimination rate constant for DS-9001a was automatically processed by the software. The elimination rate constant for DS-9001a without ABD was calculated using the slope of the regression line of 0.5 to 6 h after dosing.

## Measurement of binding affinity of DS-9001a and DS-9001a without ABD to human PCSK9 and human serum albumin (HSA)

An HBS-EP+ buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.05% surfactant P20) was used as running buffer. A Biotin CAPture kit (GE Healthcare) was used to immobilize the biotinylated PCSK9 ligand to sensor chips. For capture experiments, streptavidin-DNA conjugates were injected to two flowcells for 20 s, and the biotinylated PCSK9 samples were diluted to 1 ng/μL in the running buffer and then injected to one flowcell for 1 min at 10 μL/min, whereas another flowcell was left without captured samples to provide a reference surface. The capture protocol was designed to yield capture levels of ligand samples that resulted in $R_{max}$ values no greater than 20 RU.

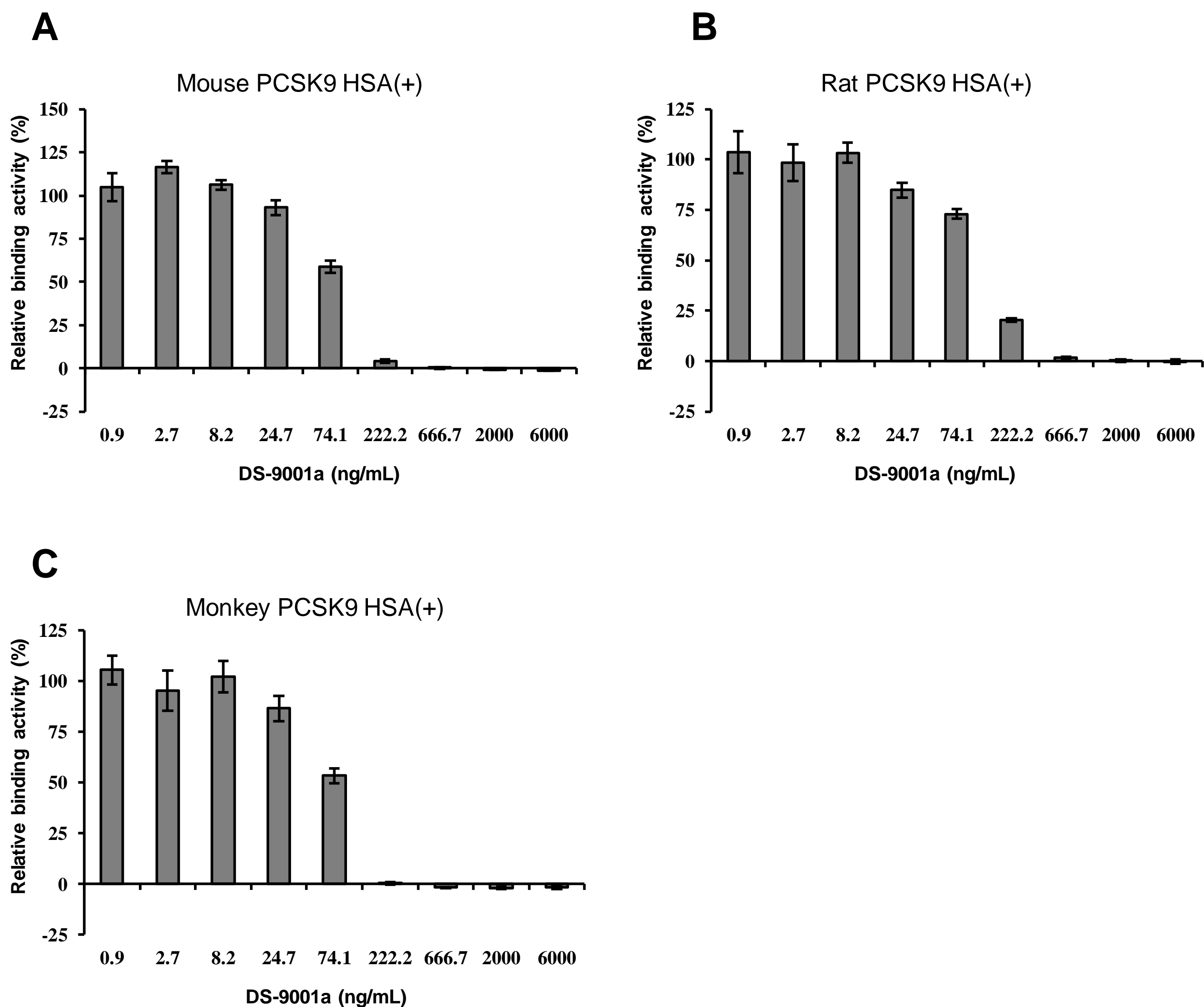
For each kinetic experiment, varying concentrations of purified DS-9001a and DS-9001a without ABD ranging from 0.03 to 100 nM were prepared as the analytes, and injected for 300 s at 30 μL/min followed by 30 min of dissociation. Captured and reference surfaces were regenerated with a 2-min pulse of 6 M guanidine hydrochloride in 0.25 M sodium hydroxide. The binding affinity of DS-9001a to HSA was also measured in the same assay.

The association rate constants ($k_a$), dissociation rate constants ($k_d$), and the resulting dissociation constants ($K_D$) were calculated using a 1:1 Langmuir binding model. The raw data sets were analyzed using Biacore T200 Evaluation Software (version 1.0; GE Healthcare), and the sensorgrams of the reference flowcells were subtracted from the sensorgrams of the sample-captured flowcells.

## Plasma DS-9001a concentrations in cynomolgus monkeys

Plasma DS-9001a concentrations were determined by sandwich ELISA, using anti-DS-9001a antibody (Daiichi Sankyo Co., Ltd.) and biotinylated anti-DS-9001a antibody (Shin Nippon Biomedical Laboratories, Ltd.).

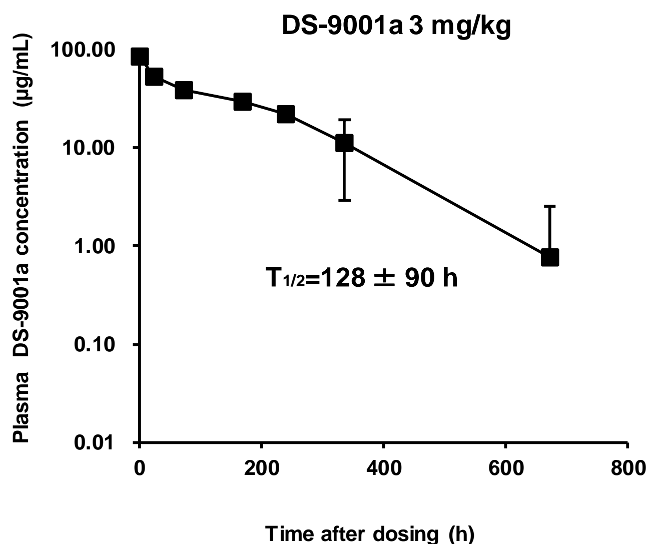
# Supplementary Figures




## Supplementary Figure 1

**DS-9001a inhibits degradation of LDL-R induced by mouse, rat, and monkey PCSK9**

(A–C) Biotin-labeled mouse, rat, and monkey PCSK9 and DS-9001a were added to an anti-LDL-R antibody-coated plate in the presence of human serum albumin (HSA). Then, human LDL-R was added to the plate and the plate was incubated for 2 h. The level of PCSK9 captured by LDL-R was detected with streptavidin-HRP. As positive and negative controls, DS-9001a-deficient and LDL-R-deficient wells were prepared, respectively. The relative binding activity (%) was calculated using the following equation: Relative binding activity = [(luminescent signal in each well) – (luminescent signal in negative control wells)] / [(luminescent signal in positive control wells) − (luminescent signal in negative control wells)] × 100. The results are presented as mean ± SE (n=3).




## Supplementary Figure 2

**Plasma DS-9001a concentrations after single DS-9001a injection into cynomolgus monkeys**

DS-9001a at 3 mg/kg was intravenously administered to cynomolgus monkeys and the blood was collected at specific time points. Plasma total DS-9001a level was measured. The data are presented as mean ± SD (n=6).