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Characterization of a single-chain antibody fragment (scFv) specific for *Plasmodium vivax* Duffy binding protein

Running title: HUMAN ANTI-DBP scFvs

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ABSTRACT

Phage display of single-chain variable fragment (scFv) antibodies is a powerful tool for selecting important, useful, specific human antibodies. We constructed a library from three patients infected with *Plasmodium vivax*. Panning on recombinant PvRII enriched a population of scFvs that recognized region II of the *P. vivax* Duffy binding protein (DBP). Three clones of scFvs that reacted with PvRII were selected and their biological functions were analyzed. These scFvs inhibited erythrocyte binding to DBP. Clone SFDBII92 had the greatest affinity ($K_D = 3.62 \times 10^{-8}$ M) and the greatest inhibition activity ($IC_{50} \approx 2.9$ µg/mL) to DBP. Thus, we demonstrated that human neutralizing antibody could be made from malaria patients using phage display and that these neutralizing scFvs should prove valuable for developing both passive and active immunization strategies based on DBP.

INTRODUCTION

Malaria caused by *Plasmodium vivax* is responsible for substantial morbidity in Asia and Central and South America (19). Merozoites of *Plasmodium* must attach to and invade RBC to begin asexual reproduction of the parasite, making this brief event a critical phase in the parasite life cycle. Invasion occurs quickly through a complex, multistep process that follows a distinct sequence of events involving numerous molecules expressed on the surface of the merozoite and in the apical organelles (1, 4, 6). This cascade of events represents potential targets for reducing or eliminating the blood stages of malarial parasites (21, 25, 31).

The Duffy binding protein (DBP) of *P. vivax* interacts with Duffy antigen receptor for chemokines (DARC) on the RBC during junction formation between the merozoite and RBC (1, 2, 16, 34). The *P. vivax* DBP (PvDBP) is a 140-kDa protein that belongs to a family of erythrocyte-binding proteins characterized by a functionally conserved cysteine-rich region (1, 6, 12). This cysteine-rich region is in DBP region II (DBPII), which contains the binding motifs necessary for adhering to DARC on the erythrocyte surface (9, 10, 29). The critical binding motif has been mapped to a 170-amino-acid segment between cysteines 4 and 8 in the cysteines-rich region (26, 28, 29). Studies have shown that although the

cysteine residues are conserved, other regions of DBPII are highly polymorphic (3, 32, 36). However, the hypervariable region of DBP II is located on the sites remote from the DARC-binding site and does not alter the capacity of the protein to bind DARC-positive erythrocytes (28, 33).

Phage display antibodies offer a way to produce high-affinity single-chain variable fragment (scFv) derivatives of human antibodies of "natural host" origin (8). Our goal was to produce human monoclonal antibodies (mAbs) against the DARC-binding region of DBP II of *P. vivax* (PvRII). To do so, we constructed a combinatorial phage display library using peripheral blood mononuclear cells from three patients infected naturally with *P. vivax*. Subsequently, anti-PvRII human scFvs that had neutralizing activity against DBP binding to erythrocytes were identified. These neutralizing scFvs should prove valuable for developing both passive and active immunization strategies based on DBP.

MATERIALS AND METHODS

Constructing the human scFv phage display library

Peripheral blood mononuclear cells were collected from three patients infected by Korean strain of *P. vivax* using Ficoll-Paque. Total RNA was extracted from each sample using TRIzol (Gibco-BRL/Life Technologies, Gaithersburg, MD). First-strand cDNA was generated using Superscript II reverse transcriptase (RT; Invitrogen, Carlsbad, CA). Light- and heavy-chain genes were cloned using polymerase chain reaction (PCR) with the primers described by Barbas *et al*. (5). The variable regions of the light (V_L)- and heavy (V_H)-chain genes were amplified separately from each cDNA and recombined in a second round of PCR. A pool of gene fusions that encoded scFvs of the V_L–spacer–V_H sequence was assembled. Following overlap PCR and gel purification, the amplified products were cloned into the phagemid vector. The ligation mixtures were electroporated into *Escherichia coli* ER2537 cells using Gene Pulser II (Bio-Rad Laboratories, Munich, Germany). Library phages were harvested from the culture supernatant of recombinant *E. coli* and precipitated with 20% PEG/2.5 M NaCl, as described previously (24). The phage pellet was reconstituted in 2 mL of 1% (w/v) bovine serum albumin in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH7.5) before being filtered through a 0.45-μm

filter.

Recombinant PvRII molecules

Recombinant PvRII (C4-C7 cystein rich region of PvDBP II) protein of SK-1 strain isolated in South Korea was produced using a reported method (17). Briefly, the PvRII gene was amplified by PCR with C4-7-F (5'-CGA AGA TAT GAA TTC TGT ATG AAG GAA CTT-3') and C4-7-R (5'-ATT GAT TTC TCG AGC ACA TTT TTC TTT CAG-3') and cloned in the *E. coli* expression vector pET28a+. The expression constructs were transformed in *E. coli* BL21(DE3). The expression of recombinant PvRII was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) in shake flask cultures and purified by metal affinity chromatography using Ni-NTA matrix (Qiagen) from inclusion bodies that had been solubilized with 8 M urea. The recombinant proteins purified under denaturing conditions were refolded by rapid dilution and purified to homogeneity using ion-exchange chromatography with Toyopearl-SP (Sigma) and gel filtration chromatography with Superdex-75 (Amersham-Pharmacia).

Panning the scFv library to select PvRII binder

The phage library was panned for binders using 96-well enzyme-linked immunosorbent assay (ELISA) plates (Costar) coated with PvRII (1 µg/100 µL) at 4°C overnight. Blocking and negative-selection well coating were performed with 10% fetal bovine serum (FBS) in TBS. Antigen-binding clones were eluted by enzyme digestion with 10 mg/mL trypsin in TBS at room temperature for 30 min. The eluted phages were then allowed to infect *E. coli* ER2537 host cells to amplify the selected phages binding to PvRII. After amplification, phages were selected for three additional rounds using the same protocol. An aliquot of each of the polyclonal phages obtained after each round of selection was stored at 4°C until required. After four rounds of selection, 96 single clones were screened for binding to PvRII using ELISA.

Enzyme-linked immunosorbent assay

Microtiter wells were coated with PvRII, as described previously, and blocked with 10% FBS in TBS for 1 h at 37°C. Phages were incubated in the wells for 1 h at 37°C and then washed four times with TBS, 0.05% Tween 20. Bound phages were detected with a 1:3000 dilution of horseradish peroxidase-conjugated mouse anti-M13 antibody (Amersham-

Pharmacia) for 30 min at 37°C and detected by adding ABST substrate (Kirkegaard and Perry).

Production of soluble scFv antibody fragments

We induced scFv expression as described previously (22). Briefly, the *E. coli* Top10F' was infected with selected phages before the cells were grown to the logarithmic phase and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside overnight to produce soluble scFvs. The expressed scFvs were purified using immobilized metal affinity chromatography with a Ni^{2+}-charged HP chelating column, following the manufacturer's protocol (Amersham-Pharmacia).

DNA Sequencing

The DNA that encoded the scFv was sequenced using a BigDye Terminator Ready Reaction Kit (Applied Biosystem) and the products were analyzed on an ABI 3130xl (Applied Biosystem). The sequences of the V_H and V_L genes were compared with the sequences present in the V base Sequence Directory to determine the closest germ line counterpart.

Surface plasmon resonance

A BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden) was used to analyze the kinetic properties of the scFvs. PvRII was immobilized on a 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride/N-hydroxysuccinimide-activated CM5 sensor chip by injecting 10 μg/mL PvRII in 10 mM sodium acetate (pH 4.0) to obtain 1000 resonance units (RU). The scFvs were diluted with running buffer, HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20; Biacore AB), and were injected at 30 μL/min. The rate constants of association (k_{on}) and dissociation (k_{off}) were obtained at five different scFv concentrations (range: 1–100 μg/mL). All assays were repeated at least three times. The dissociation constant (K_D) was calculated from the ratio of the rate constants of association and disassociation (k_{on}/k_{off}). The sensorgrams were analyzed using BIAevaluation 3.2 software. The residual scFv was removed with 50 mM NaOH after each measurement.

Surface expression of PvDBPII in COS-7 cells

The surface expression of region II of PvDBP was carried out as reported previously, with some modifications (14). Briefly, PvDBPII was amplified by PCR using the primers

PvDBPF (5′-ACAATTTGGTAATGTTAGAT-3′) and PvDBPR (5′-TGATTTCCATTTTGACCATC-3′) and cloned into the pDE vector using the *Bgl*II and *Sac*II sites (pDE-PvDBPII). The plasmid DNA used for transfection was purified using an endotoxin-free plasmid MidiPrep kit (Qiagen); 1 µg of DNA was used to transfect COS-7 cells in six-well plates (approximately 5×10^4 cells/well) using FuGene6 (Roche). After 2 days, the transfected COS-7 cells were used for fluorescence and erythrocyte binding-inhibition assays (EBIA). Fluorescence was detected using confocal microscopy (Carl-Zeiss 510Meta, Germany).

Erythrocyte binding-inhibition assay

The EBIA was performed as described previously (14). After 48 h of transfection, COS-7 cells were incubated with various concentrations of purified scFvs for 2 h at 37°C. After washing with PBS, a 10% human erythrocyte suspension of type "O" blood was added to each well, incubated for an additional 2 h, and washed three times with PBS. Binding was quantified by counting the rosettes observed over 20 fields of view at ×100 magnification. Each experiment was performed in triplicate and the data shown are from at least two separate experiments.

RESULTS

A human scFv antibody library was constructed using peripheral blood mononuclear cells from *P. vivax*-infected patients admitted to Busan Paik Hospital in August 2001. The library contained 7.85×10^8 independent clones. The investigation of full-length inserts from 40 clones using PCR and *Bst*NI digestion showed that 70% had inserts and there were no identical digestion patterns in the inserted clones (data not shown).

In *E. coli* BL21(DE3), the recombinant PvRII accumulated mostly in inclusion bodies (Fig. 1). Purified recombinant PvRII showed a single 20.4 kDa band (Fig. 1C). The recombinant PvRII reacted with the pooled sera of the six patients with vivax malaria, but didn't react with an irrelevant antibody, anti-HBV preS1 scFv (Fig. 2) (22).

After the fourth round of selection, 96 clones were analyzed for PvRII binding using ELISA (Fig. 2). Clones which show the higher OD than anti-HBV pre-S1 scFv at least three times. Twelve clones were selected as positive and these were categorized into three groups according to absorbance: low, middle, and high. *E. coli* Top10F' (amber nonsuppressant) was infected with three clones (one from each group: SFDBII12, SFDBII58, and SFDBII92), and these were expressed as soluble scFv forms by IPTG induction for affinity measurements and EBIA. The soluble scFvs were purified using the

IMAC system and were about 34 kDa, as expected (Fig. 3A). In addition, the three clones were sequenced (Table 1). A comparison with the sequences of the germ line V_H genes shows that the clones use a V_H III family-derived germ line segment. Alignment with the V_L germ line sequences showed that these clones use V_K I, V_K III, and V_λ I family segments. The kinetic parameters of these clones were measured using BIAcore (Fig. 3B). SFDBII92 had the greatest affinity ($K_D = 3.62 \times 10^{-8}$ M vs. $K_D = 1.54 \times 10^{-7}$ M for SFDBII12 and $K_D = 1.26 \times 10^{-7}$ M for SFDBII58).

To evaluate the biological effects of anti-PvRII scFvs, region II of PvDBP was expressed on the surface of COS-7 cells in the form of an EGFP fusion protein. Following transfection, we confirmed the cell surface localization of green fluorescence protein and rosette formation under a confocal microscope (Fig. 4). The erythrocyte-binding activity to the transfected cells was measured by counting the number of rosettes. The rosette counts of transfected cells were 53.6 $\pm$ 3.5. Next, to determine whether the anti-PvRII scFvs blocked the adhesion of the PvDBP molecules to human erythrocytes, transfected COS-7 cells were treated with anti-PvRII scFvs and reacted with erythrocytes to form rosettes. All anti-PvRII scFvs showed inhibitory activity, and the number of rosettes decreased as the amount of scFv was increased (Fig. 5). For a negative control, anti-pre-S1 human scFv (22)

was used and had no inhibitory activity. At 90 μg/mL of scFvs, SFDBII92 showed the highest inhibition (91.6% for SFDBII92, 88.4% for SFDBII12, and 80.9% for SFDBII58). The 50% inhibition concentrations (IC_{50}) were 2.9 μg/mL for SFDBII12, 4.0 μg/mL for SFDBII58, and 4.5 μg/mL for SFDBII92.

DISCUSSION

The invasion of erythrocytes by *P. vivax* merozoites is thought to be as complex a process as the invasion of erythrocytes by other *Plasmodium* species, such as *P. falciparum*. Erythrocyte-binding capability of several proteins have examined, including merozoite surface protein 1, reticulocyte binding protein, and DBP, which have high binding affinity to erythrocytes (9, 10, 14, 26). For *vivax* malaria, DBP is considered an important vaccine candidate antigen dues to its high binding affinity and its strong antigenicity in humans (20, 36). The cysteine-rich region of DBP II, especially in the region between C4 to C8, has the binding motifs necessary for adherence to DARC on the erythrocytes; this region of the *dbp* gene is much more variable than other regions (32). Using site-directed mutation analysis, the DARC-binding site of DBP II consists of conserved amino acids (33) and is located on the surfaces distant from the hypervariable region (28). Therefore, we decided to make neutralizing antibody fragments against PvRII from a scFv library of naturally infected malaria patients.

Some human recombinant antibodies against *Plasmodium* have been reported using the MSP protein of *P. falciparum* or AMA protein of *P. chabaudi* as targets (13, 18, 27, 30). We constructed a phage display library from the peripheral blood of human patients, as

reported previously. Roeffen *et al*. (27) showed their biological activity and invasion-inhibition using competitive ELISA with neutralizing antibodies. Lundquist *et al*. (18) showed the neutralizing activity with an antibody-dependent cellular assay. Here, we demonstrated the neutralizing activities of recombinant antibody fragments using EBIA.

Two genotypes of *p. vivax* (SK-1 and SK-2) coexist in the endemic area of the south Korea. Comparing Sal-1, Belem, PNG (Papua New Guinea), COLV and COLT (Colombia) isolates, DBP variations of the SK-1 strain are considered not to affect the binding affinity of PvDBP to RBCs (17).

Using conventional panning, the O/I ratio increased continuously with each panning. After the fourth panning, 96 clones were tested with phage ELISA to determine whether they recognized the PvRII protein. Twelve positive clones showed OD values between 0.2 and 0.8. We grouped these positive clones into three groups, selected three clones (SFDBII12, SFDBII58, and SFDBII92), and analyzed their antibody and functional characteristics.

All the heavy-chain genes of the scFvs were derived from the V_H III germ line gene, whereas the light chains were derived from the kappa I, III, and lambda I germ line genes (Table 1). Surface plasmon response measurements showed that the three antibodies had

affinities (K_D) in the nanomolar range. Previous work on an invasion-inhibiting anti-circumsporozoite antibody ($K_D \approx 300$ nM) (35), HBV neutralizing antibodies ($K_D \approx 160$ nM) (22), and HIV neutralizing antibodies ($K_D \approx 4.6$ nM) (11) showed that the SFDBII antibody series described here indeed has affinities within a biologically relevant range. Hans *et al*. (15) estimated that the binding constant of the PvRII-DARC is 8.7 nM. The affinity of the SFDBII antibodies is lower than that of the PvRII-DARC interaction. If an SFDBII antibody recognizes the binding site of DARC, the affinity can be increased using phage display techniques.

The EBIA results showed that the erythrocyte-binding inhibition activities of the anti-DBP II scFvs were correlated with their affinities. SFDBII92 had the greatest inhibition activity ($IC_{50} \approx 2.9$ μg/mL vs. 4.0 μg/mL for SFDBII12 and 4.5 μg/mL for SFDBII58). The neutralizing activities of SFDBIIs are correlated with ELISA signal and affinity. These suggest that PvRII, C4-C7 region of DBP II is a binding motif as previously reported.

Our anti-DBP antibodies can be candidates for passive immunization to prevent or treat *P. vivax* infection. Unfortunately, we cannot estimate the amount of scFv showing a neutralizing effect *in vivo*. For therapeutic purposes, SFDBII92 should have greater affinity

producing an effective neutralizing activity against vivax malaria, which can be facilitated by using error-prone PCR, chain shuffling, or complementarity determining region (CDR) mutagenesis of the variable regions of SFDBII92 (23, 24).

We successfully made human antibody fragments that neutralized DBP binding to the erythrocyte surface and demonstrated the neutralizing activity of these antibodies *in vitro* using an erythrocyte-binding inhibition assay.

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1 **FIGURE LEGENDS AND TABLES**

2 Figure 1. Expression and purification of recombinant PvRII protein. (A) Recombinant
3 PvRII was expressed as a insoluble protein in *E. coli* BL21(DE3) and electrophoresed using
4 12% SDS-PAGE (1, Bl21[DE3] with pet28a; 2, Bio-Rad low-range protein marker; 3,
5 whole lysates; 4, soluble fraction; 5, insoluble fraction of pet28a- PvRII). (B) The
6 expression of PvRII was confirmed by Western blotting (15% SDS-PAGE) with anti-his
7 monoclonal antibody and horseradish peroxidase-conjugated anti-mouse IgG (1, Bio-Rad
8 low-range protein marker; 2, BL21[DE3]; 3, BL21[DE3] with pet28a; 4, pet28a-PvRII; 5,
9 the soluble fraction of pet28a-PvRII; 6, the insoluble fraction of pet28a-PvRII). (C)
10 Expressed PvRII was purified from the insoluble fraction with a Ni-NTA column under 8 M
11 urea denaturing conditions (1, Bio-Rad low-range protein marker; 2, purified PvRII; 3,
12 insoluble fraction of PvRII; 4, flow-through). Arrows at panel B and C indicate the PvRII,
13 20.4 kDa.

14

15 Figure 2. Selection of binders to PvRII. Each well of the ELISA plate was pre-coated with
16 recombinant PvRII (1 μg/well) and then antibodies from each phage (10^{10} cfu/well) were
17 added to each well. Bound phages were detected with horseradish peroxidase-conjugated

anti-M13 mouse antibody. Anti-HBV pre-S1 scFv (22) was used as an irrelevant antibody, and pooled sera of the six patients with vivax malaria were used for the reactivity of the purified recombinant PvRII. The results are the average of triplicate assays.

Figure 3. Purification and analysis of the binding kinetics of soluble scFvs. (A) ScFvs were expressed in the form of soluble proteins without a p3 phage protein fused to the C-terminal of scFv. Monomeric scFvs were purified with an Ni-NTA affinity column using fast protein liquid chromatography and subjected to 12% SDS-PAGE. (B) The binding kinetics of the scFvs were measured using surface plasmon resonance on a BIAcore biosensor instrument. The graph shows an overlay plot of the sensorgrams obtained for SFDBII92 at six different concentrations (0.5–1 μM) against immobilized PvRII.

Figure 4. Erythrocyte-binding assay. PvDBPII was expressed on the surface of COS-7 cells using the pDE-GFP surface display system. Transfected COS-7 cells were observed under confocal microscopy and reacted with a 10% erythrocyte suspension for rosette formation (A and C, pDE; B and D, pDE-PvDBPII).
Microscope magnification is ×200.

Interactions with receptor of erythrocytes and PvDBPII were observed as rosettes (arrows).

Figure 5. Erythrocyte-binding inhibition assay. Transfected COS-7 cells with pDE-PvDBPII were incubated with various concentrations of soluble scFvs (2-100 μg/mL) for 2 h at 37°C. After washing, a 10% erythrocyte suspension was added for rosette formation. The binding inhibition activity was scored after counting the rosettes in 20 fields at a magnification of ×100. An irrelevant scFv was used as a negative control. The error bars correspond to ± standard error.

Table. 1. Analysis of the complementarity determining region (CDR) and human subgroup assignment for the three anti-PvRII scFvs.

	Clone	CDR1	CDR2	CDR3	Family
V_L	SFDBII12	GFTFGDYAMETH	GISWNSGSIG	GMETAANYYYYYGMETDV	VκIII
	SFDBII58	GFTFGDYGMETH	GISWNSGSIG	DIYSSSWYAAFDI	VκI
	SFDBII92	GFTFSSYWMETS	NIKQDGSEKY	DCSSTSCYGYYYGMETDV	VλI
V_H	SFDBII12	RASQSVSSSYLA	GASSRAT	QQYGSSRLT	V_HIII
	SFDBII58	RASQSISYHLN	AASTLQS	QKYNSAPLT	V_HIII
	SFDBII92	SGSSSNIGSNTVN	SNNQRPS	AAWDDSLNGWV	V_HIII

The V_H and V_L were searched against the Kabat database using the BLAST algorithm and

1 the scFvs groups were analyzed via the World Wide Web

2 (http://acrmwww.biochem.ucl.ac.uk/abs/chothia.html).

3

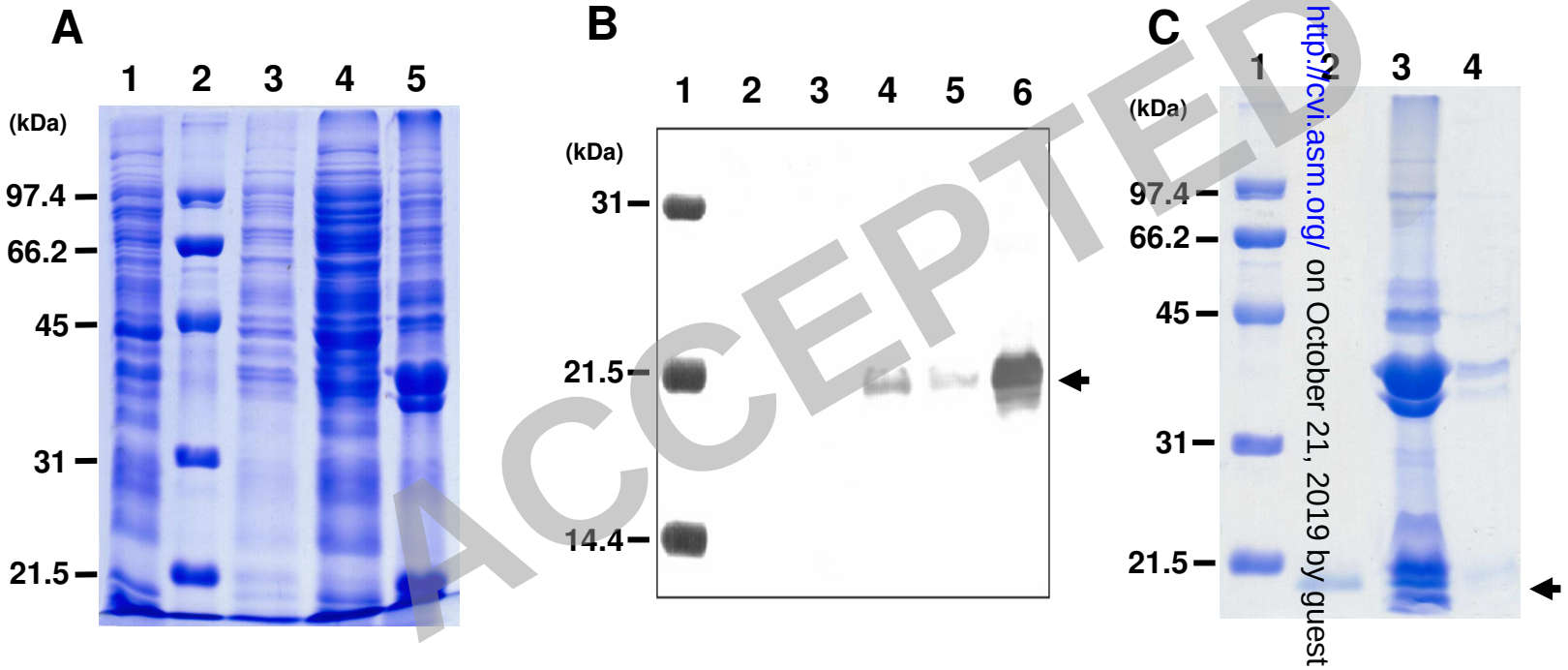

Fig 1. SH Kim et al

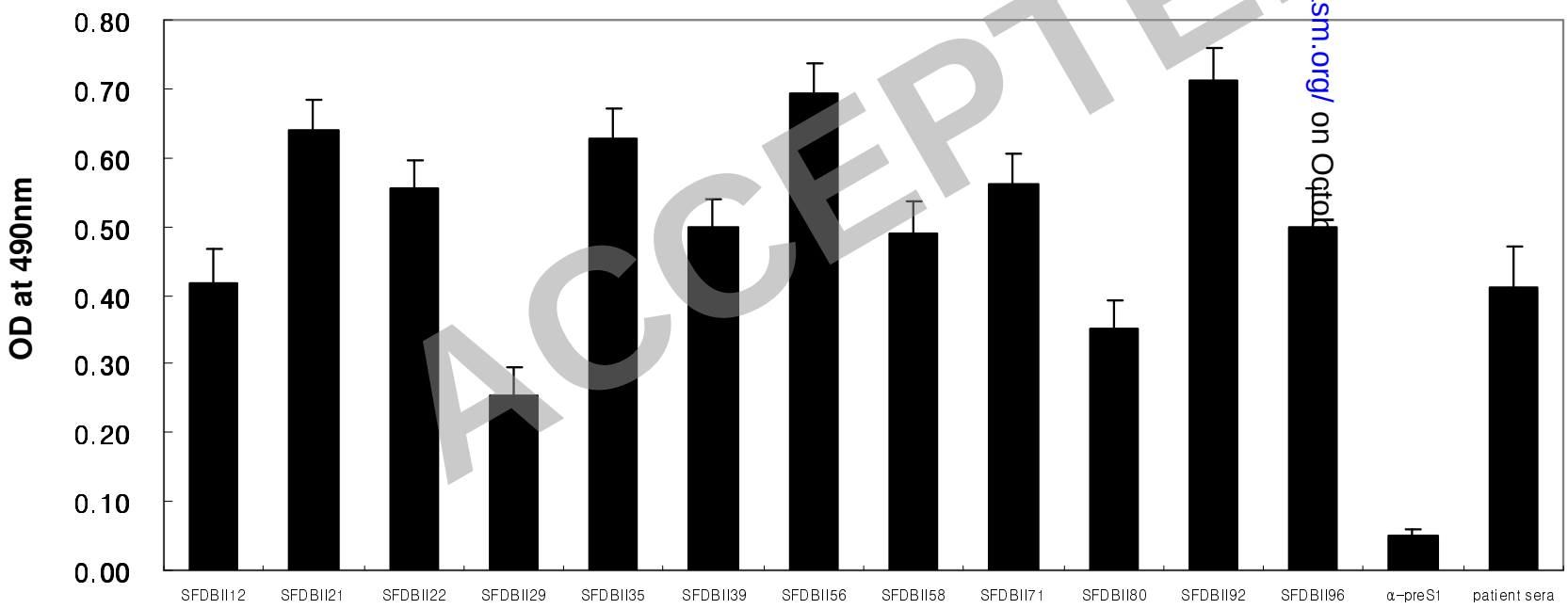

Fig 2. SH Kim et al

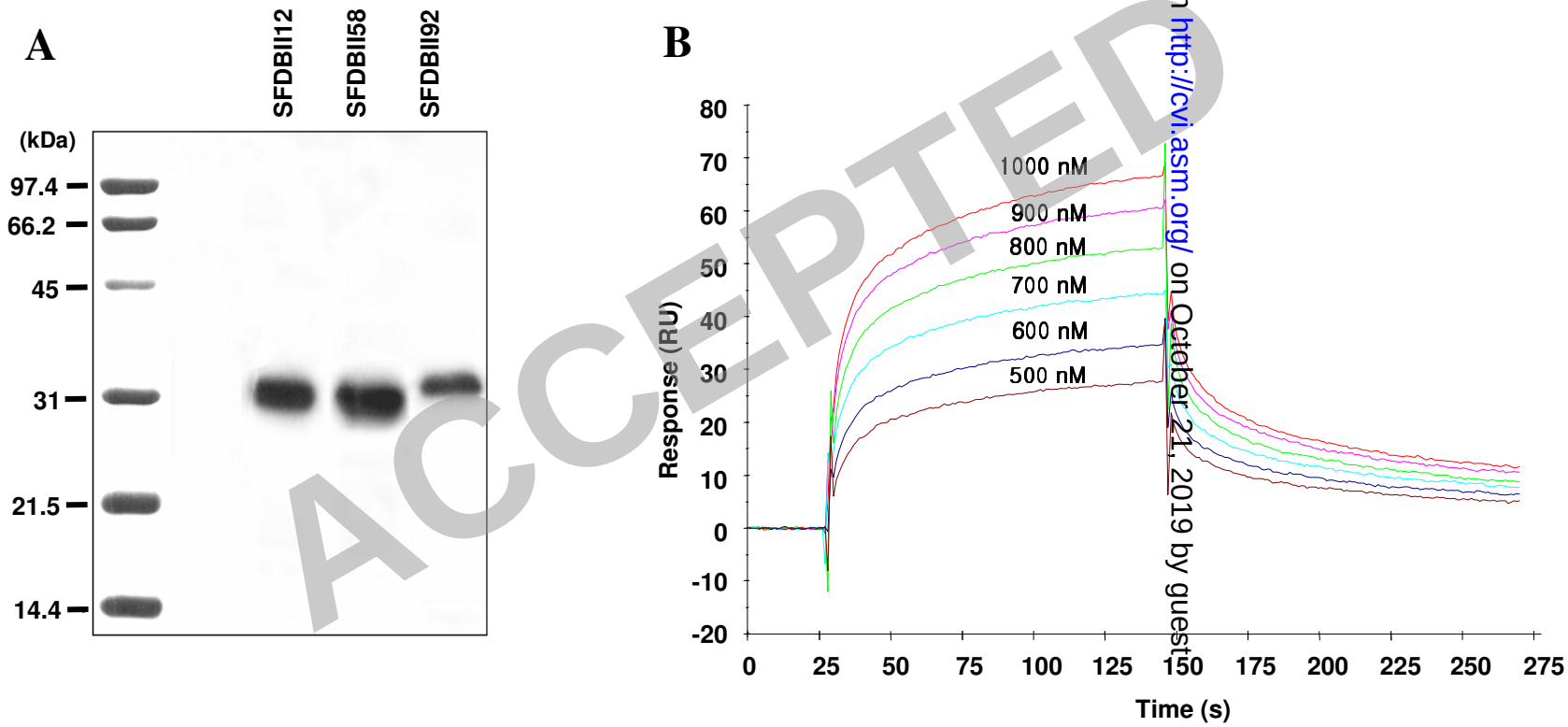

Fig 3. SH Kim et al

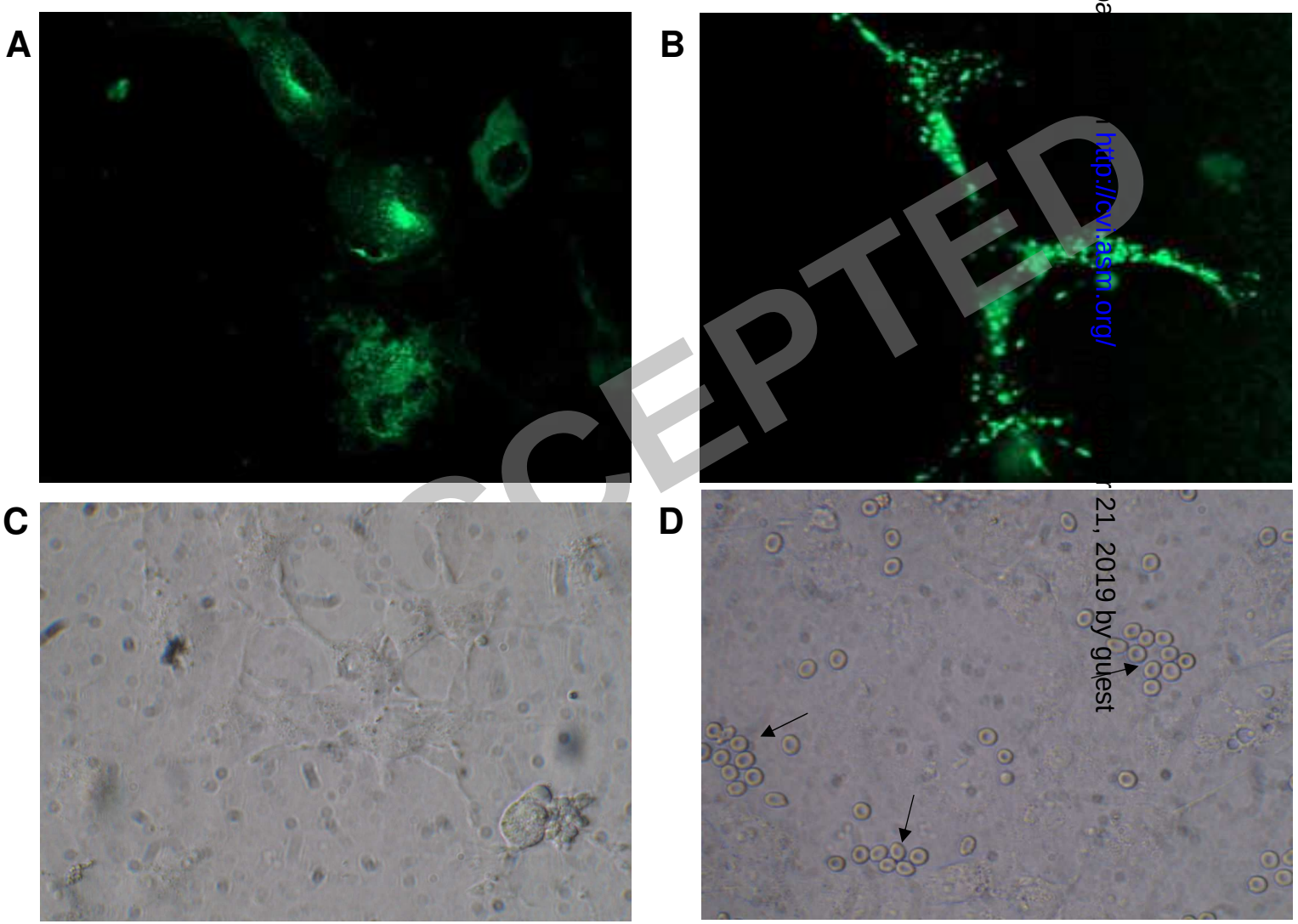

Fig 4. SH Kim et al

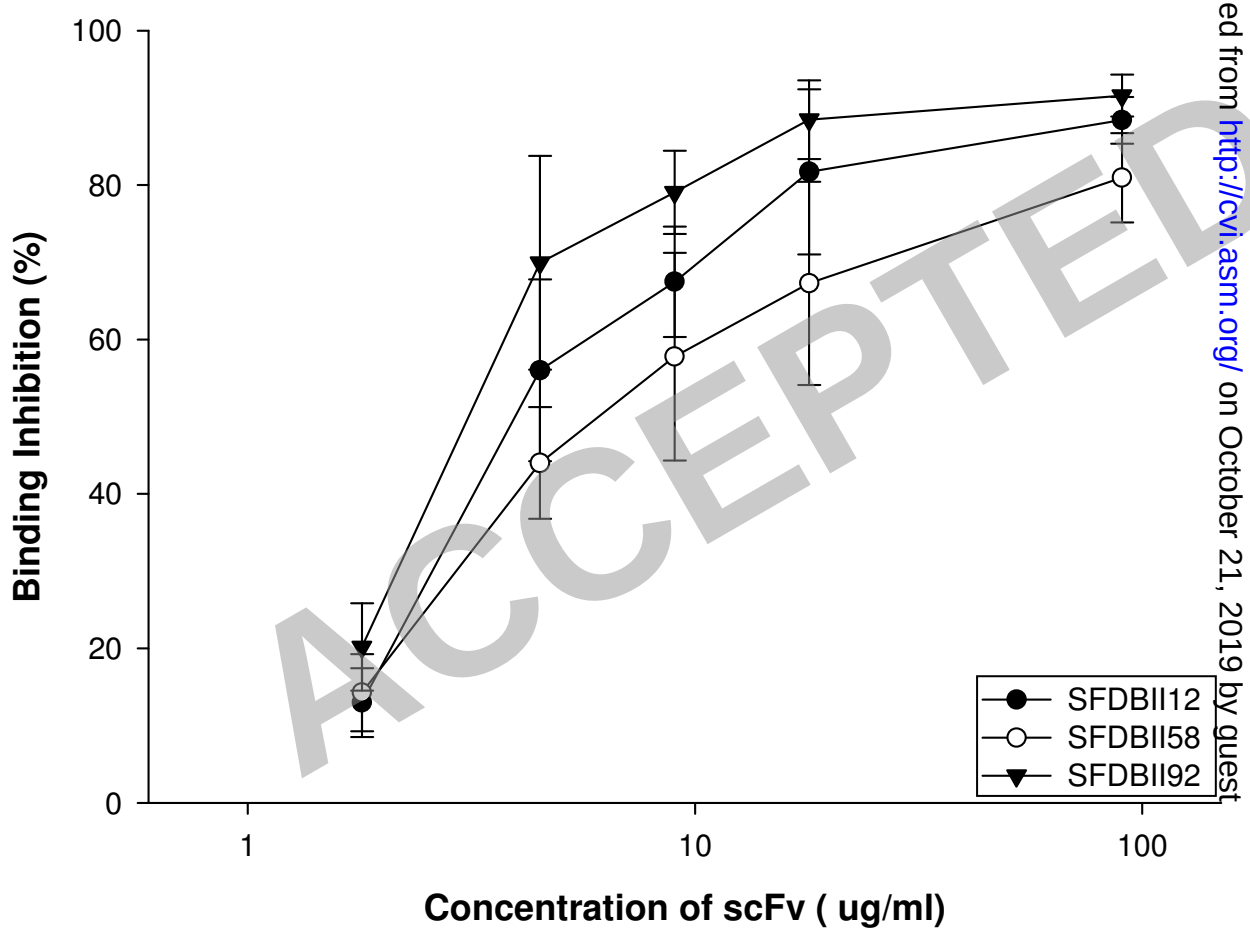

Fig 5. SH Kim et al