Construction and Expression of Specificity-Improved Single-Chain Variable Fragments against the Bioactive Naphthoquinone, Plumbagin

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We constructed a single-chain variable fragment (scFv) antibody against plumbagin (PL) with improved specific binding to PL. Variable heavy- and light-chain genes were cloned directly from the cDNA of hybridoma cell line 3A3 and assembled using the splice-overlap extension polymerase chain reaction (SOE-PCR) with specific primers including flexible peptide (Gly₄Ser)₃ linker primers. The constructed scFv gene was ligated into the pET28a expression vector and transformed into *Escherichia coli* BL21 (DE3). The denatured protein expressed as inclusion bodies in *E. coli* was solubilized, purified, and refolded by a stepwise dialysis. Intriguingly, the refolded scFv against PL displayed higher PL-binding specificity than that of its parental monoclonal antibody, MAb 3A3, which suggests the possibility of improving the function by constructing the scFv antibody. These notable properties of the recombinant antibody against PL made it possible to develop an enzyme-linked immunosorbent assay (ELISA) for reliable determination of PL.

Key words single-chain variable fragment; plumbagin; cross-reactivity; enzyme-linked immunosorbent assay; *Plumbago zeylanica*

The 1,4-naphthoquinone derivatives including vitamin K, plumbagin (PL), juglone, and shikonin are well known to possess various pharmacologic properties. Owing to their wide ranging activities such as anti-tumor,¹⁾ radical-scavenging,²⁾ enzyme-inhibitory,³⁾ immunoenhancement,⁴⁾ platelet activation-inhibition,⁵⁾ and RNase H activity inhibition associated with human immunodeficiency virus-1 reverse transcriptase⁶⁾ activity, 1,4-naphthoquinones have attracted much attention, and numerous 1,4-naphthoquinone derivatives have been found in nature or synthesized artificially. Some of them are currently used as medicines or chemical reagents. Among them, PL (5-hydroxy-2-methyl-1,4-naphthoquinone) (Fig. 1) is a naturally occurring yellow pigment mainly isolated from the genus *Plumbago* (Plumbaginaceae), which have especially been attracted much attention as a drug candidate. It has been shown to have various pharmacologic activities against cancer,⁷⁾ fungi,⁸⁾ malaria,⁹⁾ insects,¹⁰⁾ and *Helicobacter pylori*.¹¹⁾ In addition, PL is capable of inhibiting cell proliferation by inducing autophagic cell death.¹²⁾ Furthermore, Sandur *et al.* reported that PL also down-regulates

the expression of nuclear factor (NF)- κ B-regulated anti-apoptotic, proliferative, and angiogenic gene products, committing cells to apoptosis.¹³⁾

In our previous study, we developed an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody against PL (MAb 3A3) and reported the specific reactivity of the assay against PL and menadione.¹⁴⁾ The assay shows potential as a screening method in the breeding of *Plumbago zeylanica* to yield high amounts of PL and the precise monitoring of PL in body fluids. In general, the usefulness of MABs is evaluated based on their uniformity and specific binding to an antigen, which facilitates accurate immunoassays and antibody preparation. However, it is labor intensive and time consuming to obtain MABs prepared from hybridoma cell cultures. Further, hybridoma cells can be contaminated with microorganisms. On the other hand, recent advances in recombinant DNA technology have enabled the production of single-chain variable (scFv) antibodies, which have variable regions of heavy (VH) and light (VL) chains with the short, flexible peptide (Gly₄Ser)₃ in bacteria. Once the gene for construction of recombinant antibodies is established, it can be kept as gene and used to construct various kinds of recombinant antibodies. Thus the scFv strategy has become one of the most common methods in antibody engineering and has been exploited in research, therapeutic, and diagnostic applications including biosensors for the detection of target molecules,¹⁵⁾ cytokines for immunotherapy,¹⁶⁾ and radioisotopes for cancer imaging.¹⁷⁾

In this study, we constructed a recombinant scFv antibody against PL (PL-scFv) derived from a monoclonal antibody against PL (MAb 3A3) for determination of PL in plant. Functional characterization of PL-scFv suggests that it has been improved to have high specific binding to PL, although the character of the scFv antibody is, in general, identical or

Fig. 1. Structure of Plumbagin and Related Compounds

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very similar to that of the parental antibody. Subsequently, a quantitative ELISA using PL-scFv was developed and evaluated as a quality control method to monitor PL content in plants.

MATERIALS AND METHODS

Chemicals and Immunochemicals PL was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Ovalbumin was purchased from Sigma-Aldrich (Steinheim, Germany). T7-Tag horseradish peroxidase (HRP) conjugate was obtained from Novagen (San Diego, CA, U.S.A.). DNA polymerase and DNA restriction enzyme were purchased from Takara (Kyoto, Japan). All other chemicals were standard commercial products of analytical reagent grade.

Preparation of PL-Ovalbumin Conjugates The synthesis of PL-ovalbumin (PL-Ova) conjugates was performed as described in our previous study¹⁴⁾ with some modifications. 1-Ethyl-3-(3'-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; 6 mg, 0.031 mM) and 3'-(5-hydroxy-2-methyl-1,4-naphthoquinone-3-yl) propanoic acid (3 mg, 0.012 mM) were added to a mixture of 35% (w/w) pyridine solution (0.3 ml) and MES buffer consisting of 0.1 M 2-(N-morpholino) ethanesulfonic acid and 0.9% (w/v) sodium chloride (0.3 ml). The reaction mixture was added dropwise to MES buffer 0.3 ml containing ovalbumin 3 mg and then stirred at room temperature for 13 h. Subsequently, the mixture was dialyzed against five changes of H₂O for 2 d at 4 °C and lyophilized to yield 3.2 mg of PL-Ova conjugate, which was used as a coating antigen in ELISA.

Plant Materials Different parts (roots, stems, and leaves) of *P. zeylanica* and parts (stems and flowers) of *Nepenthes* sp. were collected from the herbal garden of Khon Kaen University, Thailand, in 2007 and identified by Dr. Waraporn Putalun, Khon Kaen University. The hairy root of *P. zeylanica* was induced by transformation with the Ri plasmid of wild type *Agrobacterium rhizogenes* ATCC 15834, and cultured in half-strength Murashige and Skoog medium. The plant material was cut into small pieces and dried in a hot-air oven at 50 °C for 2 d and then ground to powder consistency.

These samples are registered as PZ 01-04 and NS 01-04 in the herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University.

Sample Preparation The ground powder (50 mg) was extracted with ethyl acetate 0.5 ml using an ultrasonic bath operated for 15 min. The extract was then centrifuged at 2500 rpm for 1 min and the supernatant was transferred to a microtube. This extraction step was repeated three times. After the final step, the extracted solution was evaporated in a water bath at 60 °C until dryness. The residue was dissolved in methanol 1 ml and diluted appropriately for ELISA.

Construction of PL-scFv Gene DNA and RNA manipulations were performed using standard methods.¹⁸⁾ Total RNA (5 µg) was extracted from 1 × 10⁶ hybridoma cells (3A3) using the Sepasol RNA I super reagent (Nakalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized using random hexamer primers (Amersham Biosciences, Buckinghamshire, U.K.). The VH gene was amplified in the polymerase chain reaction (PCR) using established antibody-specific primers,¹⁹⁾

and the PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.), whereas the VL gene was amplified using PCR with Light Chain Mix Primer (Amersham Biosciences) and cloned into the pMD 20-T vector (Takara, Tokyo, Japan). The cloned VH and VL genes were transformed into *E. coli* JM109 cells. Colony PCR was used to screen positive colonies, and plasmids of positive colonies were purified and sequenced.

The VH and VL domains were assembled and linked together in the splice-overlap extension-PCR (SOE-PCR) to yield the full-length scFv gene. The scFv gene was constructed in a VH-linker-VL format with the standard 15-amino acid linker (Gly₄Ser)₃ and then was subcloned downstream of the His6-tag and T7-tag of pET 28a expression vector (Novagen) to generate a pET28a/scFv plasmid. The pET28a/scFv plasmid was transformed into *E. coli* BL21 (DE3) cells.

The VH- and VL-specific primers for the construction of the scFv gene were:

VH for-*Bam* HI,
5'-CGCGGATCCCAGGTTACTCTAAAAGAATCTGGC-3';
VH rev-linker,
5'-GGAGCCGCCGCCGAGAACACCACCACCGAGGAGACGGTGACTGA-3';
VL for-linker,
5'-GGCGGCGGCGGCTCCGGTGGTGGTTCAGACATTGTGCTGACCCAG-3';
and
VL rev-*Sal*I,
5'-AGCTTTGTCGACCTACCGTTTTATTTCCAGCTT-3'.

Primers VH for-*Bam*HI, VH rev-linker, VL for-linker, and VL rev-*Sal*I represent the forward primer for the VH gene with a *Bam*HI site, reverse primer for the VH gene with the linker sequence, forward primer for the VL gene with the linker sequence, and reverse primer for the VL gene with a *Sal*I site, respectively. In the above sequences, the restriction sites are underlined, and the italicized letters indicate linker coding sequences.

Time-Course Expression of PL-scFv Protein The time course was investigated to optimize the time for expression of the PL-scFv protein. *E. coli* cells were incubated in 100 ml of Luria-Bertani (LB) broth containing 25 µg/ml kanamycin and cultured at 37 °C. The expression of the PL-scFv protein was induced when the optical density at 660 nm reached 0.6 after the addition of 0.1 mM isopropyl-thio-β-D-galactopyranoside (IPTG), and the cells were harvested (15, 30, 45, 60 min and 2, 3, 4.5, 6, 9, 24 h after IPTG induction) by centrifugation, and the pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0). Cells were ultrasonically lysed and centrifuged at 14000 rpm for 5 min at 4 °C. Subsequently, the pellet was resuspended in protein loading buffer [30 mM Tris-HCl, pH 6.8, 0.01% (w/v) bromophenol blue, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 100 mM dithiothreitol], heated at 96 °C for 5 min, and analyzed using 12.5% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE).²⁰⁾

Purification of PL-scFv Protein The PL-scFv protein was purified using immobilized metal ion affinity chromatography (IMAC) which was precharged Ni²⁺ as an affinity

resin. Recombinant PL-scFv antibody was solubilized in washing buffer consisting of 50 mM Tris-HCl (pH 8.0) containing 6 M guanidine HCl (GuHCl), 500 mM NaCl, and 20 mM imidazole. After passing the sample through the column, the column was washed with a 10-fold volume of washing buffer, and eluted with elution buffer consisting of 50 mM Tris-HCl (pH 8.0) containing 6 M GuHCl, 500 mM NaCl, and 500 mM imidazole.

Refolding of PL-scFv Protein The recombinant PL-scFv antibody was solubilized in 50 mM Tris-HCl (pH 8.0) containing 6 M GuHCl, 200 mM NaCl, and 1 mM EDTA and refolded following the method of Umetsu *et al.* with slight modification.²¹⁾ Briefly, the solubilized scFv protein was reduced by the addition of β -mercaptoethanol (β -ME) and β -ME was then removed by dialysis against the same buffer lacking β -ME. Then, the scFv was refolded by gradual removal of GuHCl using stepwise dialysis against Tris buffer containing GuHCl (3, 2, 1, 0.5, 0.1, 0 M). At the 1 M and 0.5 M GuHCl stages, 400 mM L-arginine and of 600 μ M oxidized glutathione (GSSG) were added to facilitate the formation of disulfide bonds. After stepwise dialysis, the refolded scFv solution was centrifuged at 14000 rpm for 20 min at 4°C to remove aggregated protein and used for ELISA as a PL-scFv antibody.

Indirect Competitive ELISA Using Refolded PL-scFv The reactivity of the refolded PL-scFv antibody was determined in the indirect competitive ELISA. A 96-well immunoplate (Nunc, Roskilde, Denmark) was coated with PL-Ova (1 μ g/ml) conjugates in carbonate buffer 100 μ l (pH 9) and incubated at 37°C for 1 h. The plate was washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween 20 (TPBS) and then treated with phosphate-buffered saline 300 μ l containing 5% (w/v) skimmed milk for 1 h to reduce non specific adsorption. PL and plant sample extracts were dissolved with methanol and diluted with H₂O to give 20% (v/v) methanol solutions. Various concentrations of PL or samples (50 μ l) in 20% (v/v) methanol were incubated with 50 μ l of refolded PL-scFv solution (1.4 μ g/ml) for 1 h to observe competition between PL and PL-scFv antibody. The plate was washed three times with TPBS, and then incubated with 100 μ l of a 5000-fold diluted solution of HRP-conjugated anti-T7-Tag antibody (Novagen) for 1 h. After washing the plate three times with TPBS, 100 μ l of substrate solution [0.3 mg of ABTS in 100 mM citrate buffer containing 0.003% (v/v) H₂O₂] was added to each well and incubated for 15 min. Absorbance at 405 nm was measured with a microplate reader (Immuno Mini NJ-2300, Nalge Nunc International).

Cross-reactivities refolded PL-scFv antibody against various compounds were calculated using the method of Weiler and Zenk.²²⁾

RESULTS AND DISCUSSION

Construction and Expression of PL-scFv Protein The VH and VL fragment genes were amplified by PCR using cDNA from hybridoma cell line 3A3. After cloning the amplified genes into a plasmid, clones encoding VH and VL genes were selected according to sequence alignment of the complementary-determining region (CDR) using the Kabat and Chothia numbering scheme (<http://www.bioinf.org.uk/abs>). Cloned VH and VL genes were assembled together to

construct a gene encoding a full-length recombinant antibody with a flexible (Gly₄Ser)₃ linker and restriction enzyme sites at both ends (*Bam*HI and *Sal*I) using SOE-PCR. The assembled scFv antibody gene was digested, cloned into the pET28a vector, and expressed in *E. coli* BL21 (DE3). Analysis of the nucleic acid sequence of the constructed scFv gene on the plasmid vector revealed that it had 717-bp nucleotides encoding 239 amino acids including the (Gly₄Ser)₃ linker as predicted (Fig. 2). This nucleotide sequences were assigned in DDBJ as accession number AB470492.

Subsequently, time-course expression was studied to optimize the time for expression of the PL-scFv protein. It was found that the expression level reached the maximum 3 h after IPTG induction (Fig. 3).

SDS-PAGE analysis of cellular protein extracts from *E. coli* demonstrated that scFv monomer was expressed as an insoluble protein with a molecular mass of 29 kDa, which agreed with the theoretical value for scFv (29539 Da) (Fig. 4).

Purification and Refolding of PL-scFv Protein Expressed PL-scFv present as inclusion bodies in the insoluble fraction was purified using IMAC and then refolded with a dialysis method.²¹⁾ The total weight of purified protein from the inclusion bodies and refolded protein after purification was 5.6 mg and 2.1 mg per 100 ml of cell culture, respectively.

Method Validation Recombinant PL-scFv was characterized in the indirect competitive ELISA using PL-Ova as a solid-phase antigen. After competition, free PL-scFv (1.4 μ g/ml) was bound to a polystyrene micro-immunoplate pre-coated with PL-Ova (1 μ g/ml). After washing the plate, the amount of PL-scFv antibodies bound to the PL-Ova conjugate was measured using HRP-labeled secondary antibody against T7-tag, and a substrate was added to develop color. The range of PL concentration detectable in this assay was 0.2–25 μ g/ml (Fig. 5). It became evident from this experiment that the indirect competitive ELISA using PL-scFv displayed the same sensitivity as that using MAb 3A3.

To evaluate the specificity of PL-scFv, the CRs of PL-scFv antibody with other compounds were determined using the developed ELISA and the calculation described by Weiler and Zenk. Table 1 shows that the CRs of PL-scFv against menadion (2-methyl-1,4-naphthoquinone; vitamin K₃) and juglone (5-hydroxy-1,4-naphthoquinone) were 7.7% and 7.4 %, respectively, whereas other tested compounds didn't exhibit any cross-react with it (<0.005%). It was tantalizing that these data showed that the characteristics of the scFv antibody against naphthoquinones were improved, although it is generally accepted that the specificity of the scFv antibody is identical (or very similar) to that of the parental antibody, especially against low molecular-weight compounds. Since the antigen in this study was hapten, MAb 3A3 recognized menadion with nearly the same specificity as PL (Table 1). However, CR of MAb 3A3 against menadion was lacked in the case of PL-scFv. It is supposed that the conformational difference between the parental antibody (MAb 3A3) and scFv (PL-scFv) had occurred because the conformation of scFv, which does not have constant regions, is altered and different from that of the parental antibody. As a result of the lack of CR against menadion, which was appeared to be 80.5 % in MAb 3A3, the specificity-improved scFv had been constructed. In addition, the low molecular weight (MW 188) of

1	CAG	GTT	ACT	CTA	AAA	GAA	TCT	GGC	CCT	GGG	ATA	TTG	AAG	CCC	TCA	45
1	Q	V	T	L	K	E	S	G	P	G	I	L	K	P	S	15
46	CAG	ACC	CTC	AGT	CTG	ACT	TGT	TCT	TTC	TCT	<u>GGG</u>	<u>TTT</u>	<u>TCA</u>	<u>CTG</u>	<u>AGC</u>	90
16	Q	T	L	S	L	T	C	S	F	S	G	F	S	L	S	30
	<i>HCDR1</i>															
91	<u>ACT</u>	<u>TCT</u>	<u>GGT</u>	<u>GTG</u>	<u>GGT</u>	<u>GTA</u>	<u>GGC</u>	TGG	ATT	CGT	CAG	CCT	TCA	GGG	AAG	135
31	T	S	G	V	G	V	G	W	I	R	Q	P	S	G	K	45
	<i>HCDR2</i>															
136	GGT	CTG	GAG	TGG	CTG	GCA	<u>CAC</u>	<u>ATT</u>	<u>TGG</u>	<u>TGG</u>	<u>AAT</u>	<u>GAT</u>	<u>AAT</u>	<u>AAG</u>	<u>TAC</u>	180
46	G	L	E	W	L	A	H	I	W	W	N	D	N	K	Y	60
181	<u>TAT</u>	<u>AAC</u>	<u>CCA</u>	<u>TCC</u>	<u>CTG</u>	<u>AAG</u>	<u>AGC</u>	CAG	CTC	ACA	ATC	TCC	AAG	GAT	ACC	225
61	Y	N	P	S	L	K	S	Q	L	T	I	S	K	D	T	75
226	TCC	AGA	AAC	GTA	TTC	CTC	CAG	ATC	ACC	AGT	GTG	GAC	ACT	GCA		270
76	S	R	N	Q	V	F	L	Q	I	T	S	V	D	T	A	90
	<i>HCDR3</i>															
271	GAT	ACT	GCC	ACT	TAC	TAC	TGT	GCC	GCT	<u>TTC</u>	<u>TCC</u>	<u>TGG</u>	<u>GCT</u>	<u>ATG</u>	<u>GAC</u>	315
91	D	T	A	T	Y	Y	C	A	A	F	S	W	A	M	D	105
316	<u>TAC</u>	TGG	GGT	CAG	GGA	ACC	TCA	GTC	ACC	GTC	TCC	TCA	GGT	GGT	GGT	360
106	Y	W	G	Q	G	T	S	V	T	V	S	S	G	G	G	120
361	GGT	TCT	GGC	GGC	GGC	GGC	TCC	GGT	GGT	GGT	GGT	TCA	GAC	ATT	GTG	405
121	G	S	G	G	G	G	S	G	G	G	G	S	D	I	V	135
406	GGT	TCT	GGC	GGC	GGC	GGC	TCC	GGT	GGT	GGT	GGT	TCA	GAC	ATT	GTG	450
136	L	T	Q	S	P	T	I	M	S	A	S	P	G	E	K	150
	<i>LCDR1</i>															
451	GTC	ACC	ATG	ACC	TGC	<u>AGT</u>	<u>GCC</u>	<u>AGC</u>	<u>TCA</u>	<u>AGT</u>	<u>GTA</u>	<u>AGT</u>	<u>TAC</u>	<u>ATG</u>	<u>TAC</u>	495
151	V	T	M	T	C	S	A	S	S	S	V	S	Y	M	Y	165
496	TGG	TAC	CAG	CAG	AAG	TCA	GGC	ACC	TCC	CCC	AAA	AGA	TGG	ATT	TAT	540
166	W	Y	Q	K	S	G	T	S	P	K	R	W	I	Y		180
	<i>LCDR2</i>															
541	<u>GAC</u>	<u>ACA</u>	<u>TCC</u>	<u>AAA</u>	<u>CTG</u>	<u>GCT</u>	<u>TCT</u>	GGC	GTC	CCT	GCT	CGC	TTC	AGT	GGC	585
181	D	T	S	K	L	A	S	G	V	P	A	R	F	S	G	195
586	AGT	GGG	TCT	GGG	ACC	TCT	TAC	TCT	CTC	ACA	ATC	AGC	AGC	ATG	GAG	630
196	S	G	S	G	T	S	Y	S	L	T	I	S	S	M	E	210
	<i>LCDR3</i>															
631	GCT	GAA	GAT	GCT	GCC	ACT	TAT	TAC	TGC	<u>CAG</u>	<u>CAG</u>	<u>TGG</u>	<u>CGT</u>	<u>AGT</u>	<u>TAC</u>	675
211	A	E	D	A	A	T	Y	Y	C	Q	Q	W	R	S	Y	225
676	<u>CCA</u>	<u>TTC</u>	<u>ACG</u>	TTC	GGC	TCG	GGG	ACC	AAG	CTG	GAA	ATA	AAA	CGG		717
226	P	F	T	F	G	S	G	T	K	L	E	I	K	R		239

Fig. 2. Nucleotide and Deduced Amino Acid Sequences of PL-scFv

The linker fragment is shown in italics. The nucleotide sequences encoding the complementary-determining regions (CDRs) are underlined. HCDRs, CDRs in heavy chain; LCDRs, CDRs in light chain.

Fig. 3. Time-Course Expression of the PL-scFv Protein Induced by IPTG

Lane 1, marker proteins; lane 2, total protein before IPTG induction; lanes 3–12, total protein 15, 30, 45, and 60 min and 2, 3, 4.5, 6, 9, and 24 h after IPTG induction, respectively.

Fig. 4. SDS-PAGE Analysis of PL-scFv Monomer from *E. coli* BL21 (DE3) Harboring the Construct pET28a (+)

Lane 1, marker proteins; lane 2, total protein before IPTG induction; lane 3, total protein after IPTG induction; lane 4, soluble fraction after induction; lane 5, insoluble fraction after IPTG induction; lane 6, purified and refolded scFv (0.21 μ g).

Fig. 5. Standard ELISA Curve for the Determination of Plumbagin Using Recombinant PL-scFv Protein and Its Parental Monoclonal Antibody MAb 3A3

Red triangles and red curve show the standard curve using PL-scFv. Blue squares and blue curve show the case when MAb 3A3 was used. Concentrations of PL-scFv and PL-Ova coating conjugate were 1.4 μ g/ml and 1 μ g/ml, respectively. The insert shows the linearized calibration curve using the same experimental data.

Table 1. Cross-Reactivities (CRs) of PL-svFv and MAb 3A3 against Various Compounds

Compound	CRs (%)	
	PL-scFv	MAb 3A3
Plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone)	100	100
Menadion (2-methyl-1,4-naphthoquinone)	7.7	80.5
Juglone (5-hydroxy-1,4-naphthoquinone)	7.4	<0.005
Lawson (2-hydroxy-1,4-naphthoquinone)	<0.005	<0.005
1,4-Naphthoquinone	<0.005	<0.005
Sennoside A	<0.005	<0.005
Sennoside B	<0.005	<0.005
1-Naphthol	<0.005	<0.005
2-Naphthol	<0.005	<0.005
Ginsenoside Re	<0.005	<0.005
Ginsenoside Rb1	<0.005	<0.005
Glycyrrhizin	<0.005	<0.005
Solamargine	<0.005	<0.005
Naringin	<0.005	<0.005
Hesperidin	<0.005	<0.005
Rutin	<0.005	<0.005
Colchicine	<0.005	<0.005
Artemisine	<0.005	<0.005
Berberine	<0.005	<0.005
Ephedrine	<0.005	<0.005
Atropine	<0.005	<0.005
Cinnamic acid	<0.005	<0.005
Catechol	<0.005	<0.005

CRs were calculated using the method of Weiler and Zenk²²⁾ as follows:

$$\text{CR (\%)} = \frac{\text{IC}_{50} \text{ for PL}}{\text{IC}_{50} \text{ for compound under investigation}} \times 100$$

PL contributes to the specificity of PL-scFv compared with other previously constructed scFvs. The scFvs against bisphenol A, solamargine, and paeoniflorin display almost identical CRs to low molecular-weight compounds compared with each parental MAb.^{23–25)} It is assumed that the paratope of these scFvs may interact with antigens through multiple moieties in a manner similar to that of each parental antibody, even though their conformation is not completely the same as that of each parental antibody. Meanwhile, CR data of MAb 3A3 against compounds structurally related to PL indicate that its paratope interacts with the methyl group of PL and menadion, but also with other moieties, mainly because of its small size and planar structure. A slight change in the conformation of PL-scFv may evoke a multipoint interaction between PL and PL-scFv at the methyl and hydroxyl groups, improving the specificity for PL.

To validate the indirect competitive ELISA using PL-scFv, intra- and inter assay precision was evaluated by testing nine different PL concentration samples in five assays performed together on the same day and on three consecutive days, respectively. Intra- and inter-assay coefficients of variation (CV) for precision were determined based on the ratios of standard deviations (SD) and means from five assays. From the results shown in Table 2, the maximum intra assay CV was 6.4%, while the maximum inter assay CV was 5.9%. All CV values were less than 10%, indicating that the assay may be used for sample analysis.

Correlation of PL Contents in Samples Determined by ELISA in PL-scFv or MAb 3A3 Table 3 shows the results of quantitative ELISA analysis of PL contents in the root,

Table 2. Intra- and Inter-assay Coefficients of Variation (CV) for Precision of Plumbagin Analysis in ELISA

Plumbagin ($\mu\text{g/ml}$)	CV (%)	
	Intra-assay ($n=5$)	Inter-assay ($n=3$)
0.20	1.11	0.44
0.39	2.23	2.43
0.78	3.03	0.85
1.56	2.75	3.31
3.13	4.17	2.59
6.25	3.98	5.90
12.50	6.42	3.94
25.00	2.14	5.56
50.00	1.22	5.20

All values represent mean $\pm$ standard deviations (S.D.) for three plates and five replicate wells for each concentration within one plate for 3 consecutive days.

Table 3. Determination of PL in *Plumbago zeylanica* and *Nepenthes* sp. Using PL-scFv and MAb 3A3

Sample	Concentration (% w/w, dry wt.)	
	PL-scFv	MAb 3A3
<i>(P. zeylanica)</i>		
Root	0.046 $\pm$ 0.004	0.043 $\pm$ 0.005
Stem	0.028 $\pm$ 0.002	0.027 $\pm$ 0.004
Leaf	0.025 $\pm$ 0.009	0.023 $\pm$ 0.010
Hairy root	0.018 $\pm$ 0.009	0.014 $\pm$ 0.006
<i>(Nepenthes sp.)</i>		
Flower male	0.129 $\pm$ 0.004	0.120 $\pm$ 0.010
Flower female	0.094 $\pm$ 0.003	0.094 $\pm$ 0.007
Stem male	0.047 $\pm$ 0.003	0.043 $\pm$ 0.005
Stem female	0.049 $\pm$ 0.005	0.047 $\pm$ 0.002

leaf, stem, and hairy root of *P. zeylanica*, and the flowers and stems of *Nepenthes* sp., which were reported to contain PL.²⁶⁾ These quantitative analyses of PL using PL-scFv were compared with those obtained in ELISA using MAb 3A3. The results showed that the flower part of *Nepenthes* sp. contained the highest amount of PL among all samples tested. In addition, the PL concentration analyzed using the two methods showed a good correlation, with a coefficient of determination (r^2) of 0.996. These data indicate that PL-scFv could be used as an alternative analysis of PL in plant samples.

In conclusion, successful construction, expression, purification, and refolding of a functional scFv antibody against PL were achieved. To the best of our knowledge, this was the first achievement regarding scFv against bioactive 1,4-naphthoquinone derivatives and it is an extremely rare case that the specificity of scFv was improved compared with that of parental antibody. Indirect competitive ELISA for the determination of PL using specificity-improved PL-scFv affirmed its reliability and utility in surveying low concentrations of PL in a detailed evaluation. Due to the potent pharmacologic activity of PL, it is necessary to monitor its concentration in body fluids precisely from the viewpoint of optimum usage and avoidance of side effects. Since the developed ELISA has sufficient sensitivity and accuracy to detect small amounts of PL in such samples, it may find clinical applications. In addition, our experimental data imply that the specific binding of a recombinant antibody can be improved using a facile procedure.

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REFERENCES

- Kim S. H., Kang I. C., Yoon T. J., Park Y. M., Kang K. S., Song G. Y., Ahn B. Z., *Cancer Lett.*, **172**, 171—175 (2001).
- Sekine T., Masymizu T., Maitani T., Nagai Y., *Int. J. Pharmacol.*, **174**, 133—139 (1998).
- Plyta Z. F., Li T., Papageorgiou V. P., Mellidis A. S., Assimopoulou A. N., Pitsinos E. N., Couladouros E. A., *Bioorg. Med. Chem. Lett.*, **8**, 3385—3390 (1998).
- Abdul K. M., Ramchender R. P., *Immunopharmacology*, **30**, 231—236 (1995).
- Ko F. N., Lee Y. S., Kuo S. C., Chang Y. S., Teng C. M., *Biochem. Biophys. Acta*, **1268**, 329—334 (1995).
- Min B. S., Miyashiro H., Hattori M., *Phytother. Res.*, **16**, S57—S62 (2002).
- Lin L. C., Yang L. L., Chou C. J., *Phytochemistry*, **62**, 619—622 (2003).
- Curreli N., Sollai F., Massa L., Comandini O., Rufo A., Sanjust E., Rinaldi A., Rinaldi A. C., *J. Basic Microb.*, **41**, 253—259 (2001).
- Likhitwitayawuid K., Kaewamatawong R., Ruangrunsi N., Krungkrai J., *Planta Med.*, **64**, 237—241 (1998).
- Medentsev A. G., Akimenko V. K., *Phytochemistry*, **47**, 935—959 (1998).
- Wang Y. C., Huang T. L., *J. Chromatogr. A*, **1094**, 99—104 (2005).
- Hsu Y. L., Cho C. Y., Kuo P. L., Huang Y. T., Lin C. C., *J. Pharmacol. Exp. Ther.*, **318**, 484—494 (2006).
- Sandur S. K., Ichikawa H., Sethi G., Ahn K. S., Aggarwal B. B., *J. Biol. Chem.*, **281**, 17023—17033 (2006).
- Sakamoto S., Putalun W., Tsuchihashi R., Morimoto S., Kinjo J., Tanaka H., *Anal. Chim. Acta*, **607**, 100—105 (2008).
- Kortt A. A., Dolezal Q., Power B. E., Hudson P. J., *Biomol. Eng.*, **18**, 95—108 (2001).
- Penichet M. L., Morrison S. L., *J. Immunol. Methods*, **248**, 91—101 (2001).
- Adams G. P., Shaller C. C., Chappell L. L., Wu C., Horak E. M., Simmons H. H., Litwin S., Marks J. D., Weiner L. M., Brechbiel M. W., *Nucl. Med. Biol.*, **27**, 339—346 (2000).
- Sambrook J., Russell D. W., "Molecular Cloning," 3rd ed., Cold Spring Harbor, New York, 2001.
- Krebber A., Bornhauser S., Burmester J., Honegger A., Bosshard H. R., Pluckthun A., *J. Immunol. Methods*, **201**, 35—55 (1997).
- Laemmli U. K., *Nature (London)*, **227**, 680—685 (1970).
- Umetsu M., Tsumoto K., Hara M., Ashish K., Goda S., Adschiri T., Kumagai I., *J. Biol. Chem.*, **278**, 8979—8987 (2003).
- Weiler E., Zenk M. H., *Phytochemistry*, **15**, 1537—1545 (1976).
- Nishi K., Takai M., Morimune K., Ohkawa H., *Biosci. Biotechnol. Biochem.*, **67**, 1358—1367 (2003).
- Lu Z., Masaki T., Shoyama Y., Tanaka H., *Planta Med.*, **72**, 151—155 (2006).
- Putalun W., Taura F., Qing W., Matsushita H., Tanaka H., Shoyama Y., *Plant Cell Rep.*, **22**, 344—349 (2003).
- Schlauer J., Nerz J., Rischer H., *Acta Bot. Gallica*, **152**, 187—195 (2005).