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Title: A chemical conjugate between HER2-targeting antibody fragment and *Pseudomonas* exotoxin A fragment demonstrates cytotoxic effects on HER2-expressing breast cancer cells

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Running Title: Chemical crosslinking of anti-HER2 scFv and PE24

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ABSTRACT

Conventionally, immunotoxins have been produced as a single polypeptide from fused genes of an antibody fragment and a toxin. In this study, we adopted a unique approach of chemical conjugation of a toxin protein and an antibody fragment. The two genes were separately expressed in *Escherichia coli* and purified to high levels of purity. The two purified proteins were conjugated using a chemical linker. The advantage of this approach is its ability to overcome the problem of low recombinant immunotoxin production observed in some immunotoxins. Another advantage is that various combinations of immunotoxins can be prepared with fewer efforts, because the chemical conjugation of components is relatively simpler than the processes involved in cloning, expression, and purification of multiple immunotoxins. As a proof of concept, the scFv of trastuzumab and the PE24 fragment of *Pseudomonas* exotoxin A were separately produced using *E. coli* and then chemically crosslinked. The new immunotoxin was tested on four breast cancer cell lines variably expressing HER2. The chemically crosslinked immunotoxin exhibited cytotoxicity in proportion to the expression level of HER2. In conclusion, the present study revealed an alternative method of generating an immunotoxin that could effectively reduce the viability of HER2-expressing breast cancer cells. These results suggest the effectiveness of this method of immunotoxin crosslinking as a suitable alternative for producing immunotoxins.

Keywords: scFv, HER2, exotoxin, Pseudomonas, breast cancer

INTRODUCTION

Most anticancer monoclonal antibodies exhibit weak antibody-dependent cytotoxic activity. A recombinant immunotoxin is a genetically engineered antibody fragment conjoined to a protein toxin that reduces the tumor tissues (1). The antibody region of these molecules specifically targets tumor cell surface receptors and then internalizes toward the endocytic compartment. Toxin molecules delivered to the cytosol of the target tumor cells destroy the target cells effectively (2, 3).

Human breast cancers are classified into subtypes depending on their gene expression patterns (4). The overexpression of human epidermal growth factor receptor 2 (HER2), also called HER2/neu, ERBB2, or CD340, has been observed in 20–30% of all breast tumors (5). HER2, a 185-kDa transmembrane tyrosine kinase receptor, belongs to the epidermal growth receptor (EGFR) family 2. The phosphorylation of HER2 dimers results in the activation of various downstream processes, such as cell proliferation, survival, differentiation, angiogenesis, invasion, and metastasis (6). Patients with HER2 overexpression show a significantly poor prognosis and overexpression of HER2 in breast tissues stimulates malignant phenotypic transformation. In addition, HER2-overexpressing tumors are more resistant to general chemotherapy treatment (7).

Trastuzumab, an anti-HER2 antibody, has been approved by the FDA for the treatment of HER2-positive early-stage breast cancer and metastatic breast cancer (8). Trastuzumab blocks HER2 signaling by binding to its extracellular domain and attracts immune cells to tumor sites, resulting in the inhibition of tumor growth (9). Because trastuzumab itself showed weak antibody-dependent cell cytotoxicity, it has been conjugated with various chemical drugs to enhance cytotoxicity for active targeting against HER2-positive breast cancer cells (10).

Pseudomonas exotoxin A (PE) is a bacterial exotoxin from *Pseudomonas aeruginosa* that is expressed as a protein with 613 amino acids (a.a.), and comprises three functional domains (11). The receptor-binding domain Ia (1–252 a.a.) is followed by the translocation domain II (253–364 a.a.). The last four residues (400–404 a.a.) of domain Ib (365–404 a.a.) with domain III (405–613 a.a) is a catalytic subunit of the toxin (12). The catalytic enzyme activity of domain Ib and domain III ADP-ribosylates the elongation factor of the host ribosome, causing apoptotic cell death (13). The 40-, 38-, or 24-kDa portions of the PE without the cell binding domain, designated as PE40, PE38, and PE24, respectively, was fused to the antibody fragment that targets the cancer cell (14).

In this study, we adopted a unique approach of chemical conjugation between an antibody fragment and a toxin instead of the traditional immunotoxins that are recombinant fusion proteins of the two proteins. An advantage of this approach is that it can overcome the problem of low recombinant immunotoxin production that is observed in some immunotoxins. As a proof of concept, the scFv of trastuzumab and the PE24 protein were produced separately using *E. coli* and then chemically crosslinked. The new immunotoxin was tested on the breast cancer cell lines that express HER2.

RESULTS

Cloning the constructs

To fuse three PCR products (i.e., V_H, V_L, and donor vector [pDONR207]) and create pENTR-HER2(scFv), an overlap cloning method was used. The primers were designed for PCR products to have a homologous sequence at both the ends. After overlap cloning, the TEV cleavage site was added at the N-terminal of HER2(scFv), and cysteine residue was added at the C-terminal for crosslinking reaction. A linker was inserted between V_H and V_L.

The attL1 or attL2 site was added at each terminal for the next cloning step, and the expression vector for MBP–HER2(scFv) was obtained using the LR reaction of the gateway cloning method with pENTR–HER2(scFv) and pDEST–HMGWA with MBP tag (Figures 1A, C). For making the PE24 expression vector, a multisite gateway cloning method was used. PE24-encoding gene was amplified by PCR. The attB1 and TEVrs sequence at the N-terminal and attB5 at the C-terminal of PE24 were added. attB site-flanked PE24 was inserted to the donor vector (pDONR221) by BP reaction and pENTR–PE24 was formed. The expression vector for His8–PE24 was created by LR reaction with His8 tag containing pDEST–His8 and pENTR–PE24 (Figure 1B, D).

Expression and solubility analysis of HER2(scFv) and PE24

The expression vector for MBP–HER2(scFv) or His8–PE24 was transformed to *E. coli* BL21. The protein expression and solubility level were determined at different induction temperatures of 37°C or 18°C.

E. coli was grown at 37°C until $O.D_{600}$ = 0.6–0.7. When the O.D value reached the optical value, 0.5 mM IPTG was added and the protein expression was induced at 37°C for 3 h or 18°C for overnight. Then, the cells were sonicated. The total cell fraction, pellet, and soluble fraction were analyzed using SDS-PAGE (Supplementary Figure 1). MBP–HER2(scFv) and His8–PE24 fusion proteins were expressed at both the temperatures. However, when the proteins were induced at 18°C, protein solubility was increased as compared with that at 37°C (Supplementary Table 1).

Purification of HER2(scFv) and PE24

The *E. coli* cells expressing MBP–HER2(scFv) were sonicated, and the soluble fraction of

the cell lysate was applied to the HiTrap FF immobilized metal affinity chromatography (IMAC) column. The MBP–HER2(scFv) fusion protein was eluted at 100 mM imidazole, and TEV protease was added to the elution containing MBP–HER2(scFv) at a ratio of 5:1 (fusion:TEV). After the MBP tag cleavage, HER2(scFv) was purified by the 2nd HiTrap FF IMAC column. The tag-free HER2(scFv) was collected from the flow through (FT) fraction, and the purified HER2(scFv) was dialyzed against phosphate-buffered saline (PBS) (Supplementary Figure 2B). The yield of the final product was 37% (Supplementary Table 2). PE24 also was purified by IMAC chromatography, however, the His8–PE24 fusion protein was eluted at 500 mM imidazole. TEV protease was treated to the eluted His8–PE24 at a ratio of 20:1 with 1 mM DTT addition. After the TEV protease digestion, PE24 was purified from the FT fraction of the 2nd IMAC column in the same manner as HER2(scFv) (Supplementary Figure 2C). After dialysis against PBS, the final yield was 31% (Supplementary Table 3).

Chemical conjugation of HER2(scFv) and PE24

To generate an anti-HER2 immunoconjugate, anti-HER2(scFv) and PE24 were chemically conjugated via N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), a disulfide bond-containing linker. The amino groups of PE24 were modified with SPDP (Figure 2B, lane 1), and HER2(scFv)-Cys was reduced by TECP to make the sulfhydryl group available for conjugation (Figure 2B, lane 2). After a reaction at a ratio of 5:1 (PE24:HER2(scFv)), the HER2(scFv)–PE24 conjugate was formed (Figure 2B, lane 3). Then, this conjugate was purified by size exclusion chromatography using the Hiload 16/600 Superdex 75 pg (Figure 2B, lane 4). The purity of the purified HER2(scFv)–PE24 conjugate was verified by SDS-PAGE under non-reducing condition (10% Tricine gel). The highest yield and purity of the

HER2(scFv)–PE24 conjugate was 58% and 93%, respectively (Supplementary Table 4).

HER2 expression of breast cancer cells

To evaluate the binding capacity of the HER2(scFv)–PE24 conjugate, the HER2(scFv)-GFP conjugate was prepared and flow cytometry analysis was performed. After incubation with HER2(scFv)–GFP, a shift of the fluorescence histogram to the right was observed in the HER2-overexpressing cell lines, SKBR3 and BT-474 (Figure 3A, B). On the contrary, the fluorescence histogram shifted slightly in HER2 low-expressing cells (MDA-MB-231, MCF 7) as compared with that in HER2-overexpressing cells (Figure 3C, D). From the fluorescence-activated cell sorting (FACS) analysis data, we determined that the HER2(scFv)–PE24 conjugate strongly binds to HER2-expressing cells.

Cytotoxicity of HER2(scFv)–PE24 conjugate *in vitro*

To determine the cytotoxicity of the HER2(scFv)–PE24 conjugate, HER2-overexpressing and low-expressing cells were treated with HER2(scFv)–PE24, HER2(scFv), and PE24 at various concentrations. At 72 h of treatment, an MTT assay was performed to measure the cell viability. The HER2(scFv)–PE24 conjugate revealed high toxicity in HER2-overexpressing cell lines (SKBR-3 and BT-474) at the picomolar level. The IC_{50} value of SKBR-3 and BT-474 is 43 pM ± 8 (n = 9) and 6.7 pM ± 3 pM (n = 9), respectively (Figure 4A, B). In contrast, the HER2 low-expressing cell lines (MDA-MB-231 and MCF-7) were 20- to 1.4 × 10^3-fold less affected by the HER2(scFv)–PE24 conjugate. The obtained IC_{50} values of MDA-MB-231 and MCF-7 were 9.44 nM ± 3 nM (n = 9) and 1.01 nM ± 0.38 nM (n = 9), respectively (Figure 4C, D). These data indicate that the cytotoxicity in cells is correlated with the HER2 expression on the cell surface. Treatment with HER2(scFv) alone did not inhibit the

proliferation. Meanwhile, PE24 alone reduced cell viability at high concentration, except for the SKBR-3 cell line.

DISCUSSION

In this study, an antibody fragment and a toxin were produced separately from *E. coli,* and the two proteins were chemically conjugated using a chemical linker. An advantage of this approach is that it could overcome the low recombinant immunotoxin production problem observed in some immunotoxins. Another advantage is that various combinations of immunotoxins can be made with fewer efforts, because the chemical conjugation of the two components is simple. This was attempted previously for anti-CTLA-4 scFv and saporin (15), but has not been explored further.

Inside the cancer cell, the traditional recombinant immunotoxin is digested by the intracellular protease, furin, giving rise to dissociated antibody fragment and the toxin that interferes with intracellular function (16). Since our immunotoxin is in a chemically conjugated form, furin cannot be applied. Instead, the chemical crosslinker in our experiment creates a disulfide-containing linkage between the HER2(scFv) and PE24 such that the link would be cleaved inside the cell because of the reducing intracellular environment.

Four types of breast cancer cell lines were used to test the cytotoxicity of the chemically conjugated HER2(scFv)–PE24. SKBR-3 and BT-474 expressed HER2 higher than MDA-MB231 and MCF-7 did, as per the FACS analysis using HER2(scFv)–GFP (Figure 3). As expected, the cytotoxicity of the chemically conjugated immunotoxin was higher in SKBR-3 and BT-474 with IC_{50} of the picomolar range, whereas the other two cell lines showed IC_{50} of the nanomolar range (Figure 4). This cytotoxicity of the chemically conjugated HER2(scFv)–PE24 on the four breast cancer cells was comparable to that of the conventional

HER2(scFv)–PE24 (unpublished result), demonstrating the feasibility of the chemically conjugated immunotoxin. The efficacy of immunotoxins can be augmented by a hundred- or thousand-fold, but more than a million fold by endosomal escape enhancers in exceptional cases, such as lysosomotropic amines, carboxylic ionophores, calcium channel antagonists, various cell-penetrating peptides, other organic molecules, and light-induced techniques (17). Our immunotoxin already demonstrated IC_{50} of picomolar range, and it remains to be decided how much these endosomal escape enhancers could increase the efficacy.

In patients with solid tumors that have normal immune systems, PE is highly immunogenic because it is a bacterial protein. Anti-drug antibodies (ADA) were detected in all mesothelioma patients who were treated with recombinant immunotoxins (RIT) that contained PE (18). The ADA neutralized the RIT and prevented further treatment. Because immunocompromised hematological patients do not show a strong ADA response, a combination therapy of a RIT with an aggressive immunosuppression regimen was evaluated in patients with advanced chemo-resistant mesothelioma. ADA formation was delayed so that more cycles could be provided, and 40% of the patients showed dramatic tumor responses that substantially increased survival (19). This result demonstrated that RITs could induce major regressions in mesothelioma once immunogenicity was resolved and highlights the need to control immunogenicity to make therapy more effective.

PE38 was at the common fragment form of PE for recombinant immunotoxin (20). However, the nonspecific toxicity and strong immunogenicity of PE38 were the limitations of PE-based immunotoxins (21). For the prevention of immunogenicity, most of the domain II (253–364 a.a.) as B-cell and T-cell epitopes, except for the furin cleavage site, was removed. This fragment, PE24, significantly decreased immunogenicity and the nonspecific toxicity of PE (22). In addition, six amino acids of PE were identified as the B-cell epitope (23, 24). Six

other amino acids of PE were mutated to decrease the immunogenicity of T-cell response (22, 25). Two amino acid positions were overlapped so that 10 amino acids were mutated for dual B- and T-cell de-immunization (26). Our PE24 was derived from 8 amino acid mutations to remove the B-cell epitope (27). Obviously, our PE toxin requires improvements in de-immunization.

Despite immunotoxins showing encouraging effects in clinical or preclinical animal trials, its short *in vivo* half-life hampers its therapeutic efficacy (28). There are several strategies to improve the pharmacokinetic properties of protein-based therapeutics, such as chemical modification with polyethylene glycol (PEGylation) or fatty acid, recombinant fusion with human serum albumin, albumin binding domain, or the Fc domain of the immunoglobulin (29). In a different approach for sustained release, protein or peptide drugs have been encaptured in the poly lactic-co-glycolic acid (PLGA) microsphere, phospholipid gel, or PEG gel (30). These methods may be applied to immunotoxins as well, in order to increase its *in vivo* half-life.

In conclusion, the present study represents another method of generating an immunotoxin. HER2(scFv) and PE24 were produced separately with high purities from *E. coli*. Then, the two proteins were chemically crosslinked. This immunotoxin effectively reduced the viability of HER2-expressing breast cancer cells. Our results suggest that this method of immunotoxin crosslinking is a good alternative to produce immunotoxins.

MATERIALS AND METHODS

Construction of expression vector

To create the expression vector for MBP–HER2(scFv), overlap cloning and multisite gateway cloning were performed. For the expression vector of His8–PE24, multisite gateway cloning

method was used. More detail about this construct is shown in the supplementary method.

Expression and solubility analysis of recombinant fusion protein in *E. coli*, BL21

Expression plasmids were transformed into *E. coli* BL21 strain. *E. coli* was cultured and induced as mentioned in Supplementary Method 2. The fusion protein expression and solubility was analyzed with SDS-PAGE and assessed by an ImageJ image analyzer (http://imagej.nih.gov.ij)

Purification of HER2(scFv)-Cys and PE24

HER2(scFv) and PE24 proteins were expressed in *E. coli* BL21 and purified by IMAC chromatography, as described in the Supplementary Method section.

Crosslinking and purification of HER2(scFv)–PE24 conjugate

Crosslinking between HER2(scFv) and PE24 was performed using sulfo-LC-SPDP, as described in the Supplementary Method. The HER2(scFv)–PE24 conjugate was purified by gel filtration chromatography using the Hiload 16/600 Superdex 75 in PBS at pH 7.4 with 5 mM EDTA.

Purification of GFP and HER2(scFv)–GFP conjugate

HER2(scFv)–GFP was produced, as described in the Supplementary Method section.

Flow Cytometric Analysis

The cells were trypsinized and 2×10^6 cells were centrifuged and resuspended in 1 mL PBS. Then, 5 μg of HER2(scFv)–GFP conjugate was added to each tube and incubated at 4°C for

25 min. The cells were washed with PBS three times, and 1 μg DAPI was added to each tube and incubated at 4°C for 10 min. After washing three times, the cells were analyzed by FACS Canto II flow cytometer (BD Biosciences, San Diego, CA). FlowJo_V10 (FlowJo LLC, Ashland, OR) was used to analyze FACS data.

In vitro cytotoxicity assay

The SKBR-3, BT-474, MDA-MB231, and MCF-7 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were seeded into 24-well plate at a density of 0.5–1 × 10^5 cells per well. After culturing for 24 h, HER2(scFv)–PE24 conjugate was treated to seeded cell at different concentrations (0.002, 0.02, 0.2, 2, 20, and 200 nM). At 72 h of incubation with the conjugate, MTT assay was performed as described in the Supplementary Method.

Statistics

Data are presented as mean ± standard error of n $\geq$ 3 samples. The experiments were performed independently in triplicate.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

FIGURE LEGENDS

Figure 1. Construct design and gateway cloning strategy of the expression vector.

Designed constructs of (A) MBP–anti-HER2(scFv) and (B) His8–PE24. Cysteine residue was added at the C-terminal of anti-HER2(scFv) for crosslinking reaction. The TEV protease cleavage site was included at the N-terminal of both fusion proteins for tag removal. (C) MBP–HER2(scFv) expression vector was created by overlap cloning and gateway cloning methods. (D) The His8–PE24 expression vector was created by the gateway cloning method.

Figure 2. Chemical conjugation with SPDP linker.

(A) A schematic overview and (B) SDS-PAGE analysis of the crosslinking process and purification of the conjugated HER2(scFv)–PE24. Lane 1, SPDP-modified PE24; Lane 2, TECP-treated HER2(scFv); Lane 3, reaction mixture after incubation at 4°C for overnight; Lane 4, purified HER2(scFv)–PE24 conjugate after gel filtration column.

Figure 3. Binding capacity of the HER2(scFv)–GFP conjugate.

Flow cytometry analysis of HER2-overexpressing cell and HER2 low-expressing cell after incubation with DAPI (red) or DAPI and HER2(scFv)–GFP conjugate (blue). The fluorescence histogram indicates that HER2(scFv)–GFP strongly binds to HER2 receptors on (A) SKBR-3 and (B) BT-474 unlike HER2 low-expressing cell lines, (C) MDA-MB-231 and (D) MCF-7.

Figure 4. Cell cytotoxicity of the HER2(scFv)–PE24 conjugate, HER2(scFv) and PE24.

The cytotoxicity of the HER2(scFv)–PE24 conjugate was evaluated on HER2-overexpressing cell lines, SKBR-3 (A) and BT-474 (B), and HER2 low-expressing cell lines, MDA-MB-231

(C) and MCF-7 (D). The cell viability was measured by MTT assay, and the IC_{50} values of HER2(scFv)–PE24 conjugate were as follows: SKBR-3 (43 pM ± 8 pM), BT-474 (6.7 pM ± 3 pM), MDA-MB-231 (9.44 nM ± 3 nM), and MCF-7 (1.01 nM ± 0.38 nM).

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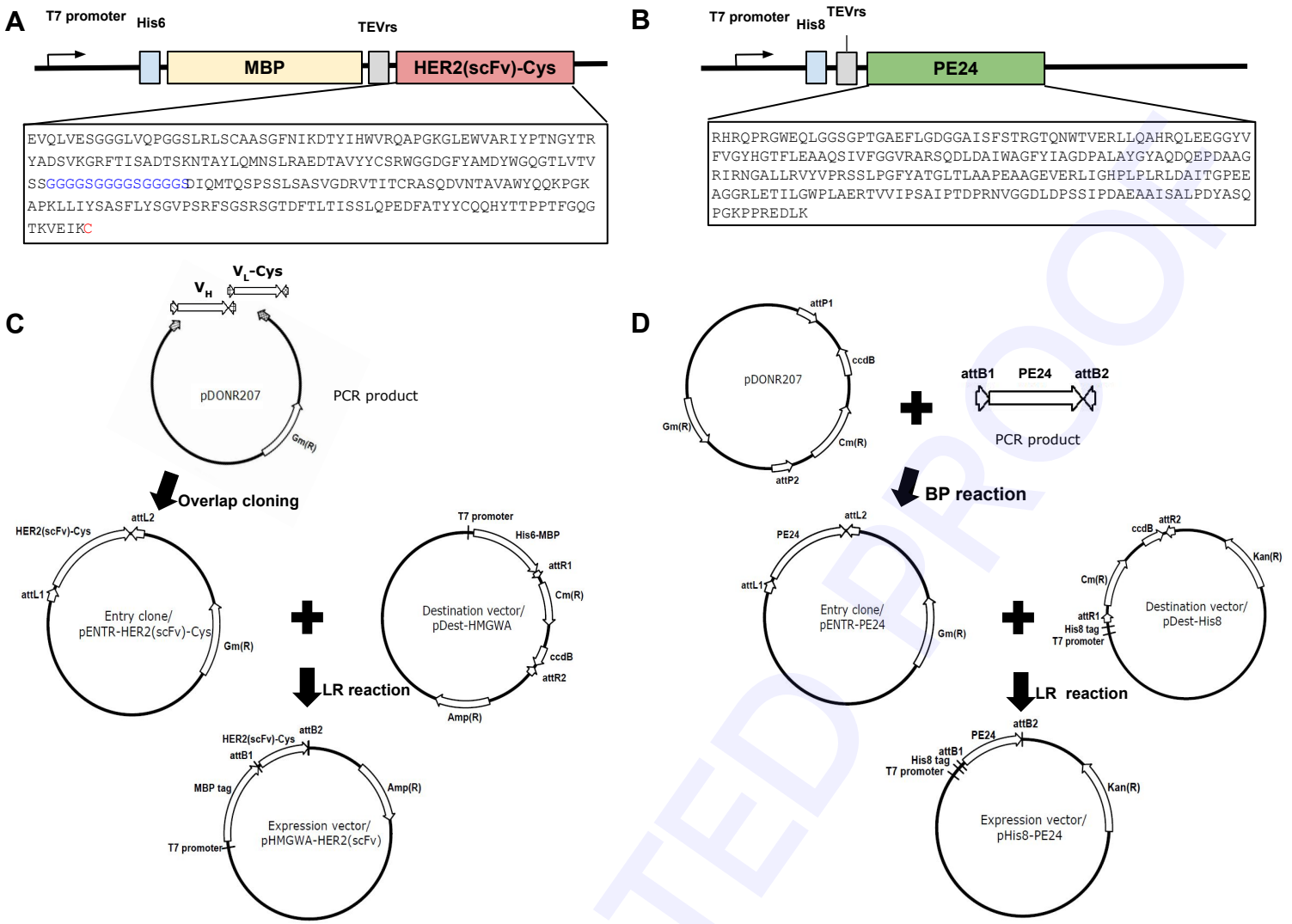


Figure 1. Construct design and gateway cloning strategy of the expression vector.

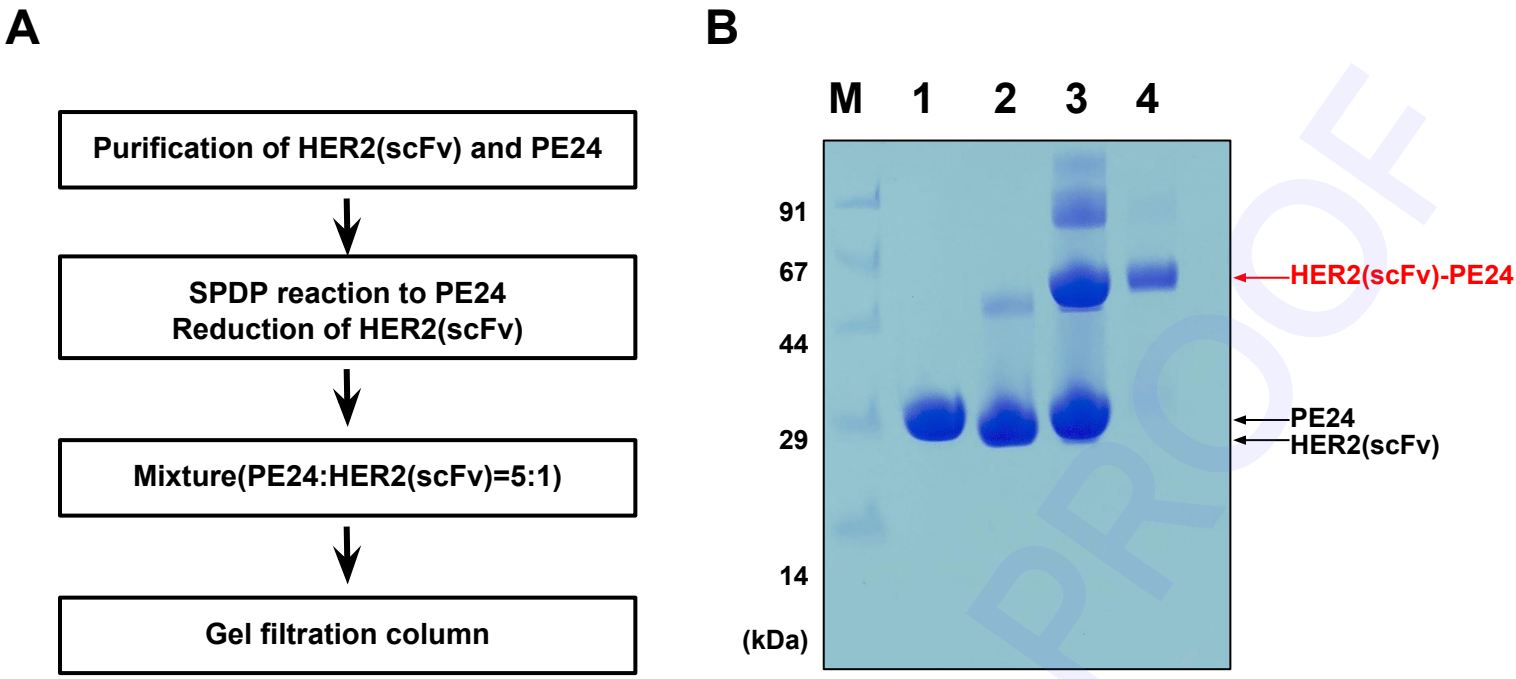


Figure 2. Chemical conjugation with SPDP linker.

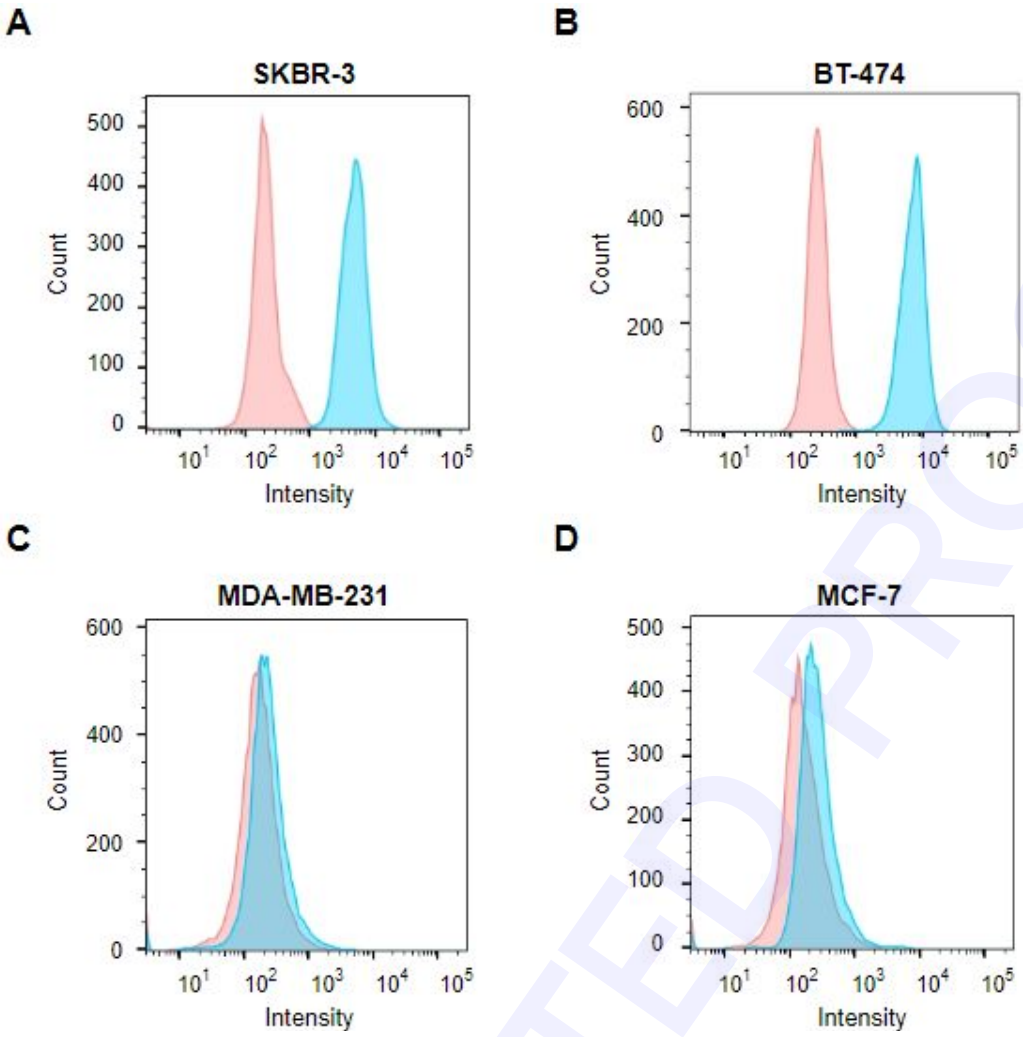


Figure 3. Binding capacity of the HER2(scFv)–GFP conjugate.

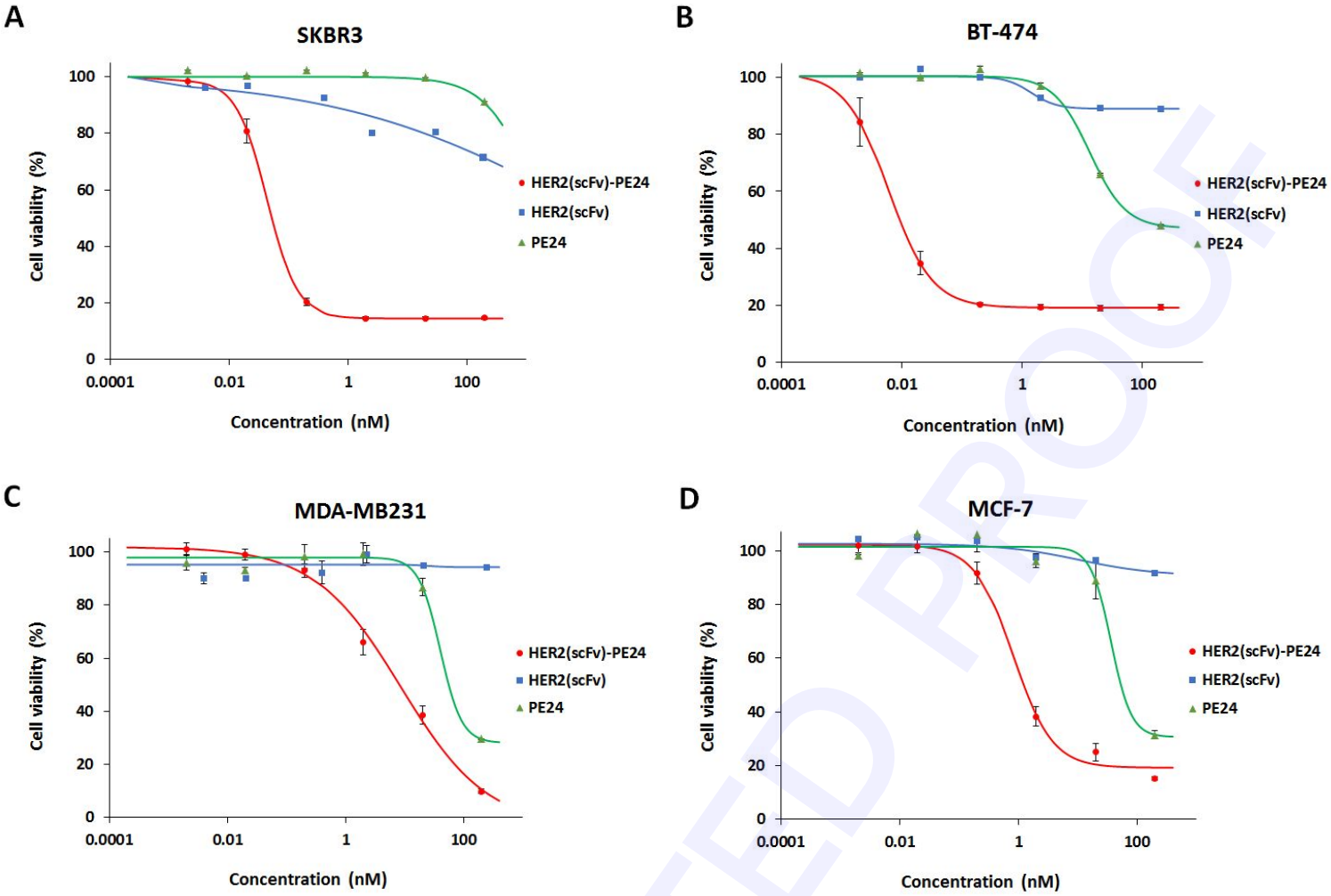


Figure 4. Cell cytotoxicity of the HER2(scFv)–PE24 conjugate, HER2(scFv) and PE24.

Supplementary Information

Materials and Methods

Materials

Sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate (sulfo-LC-SPDP) was acquired from CovaChem (Loves Park, IL, USA) and tris(2-carboxyethyl)phosphine (TCEP) was obtained from Thermo Fisher Scientific Korea (Seoul, Korea). Ampicillin was purchased from Duchefa Biochemie (Haarlem, Netherlands), kanamycin sulfate was obtained from Biosesang (Seungnam, Korea), and NaCl and glycerol were from Samchun Chemical (Pyongtaek, Korea). Overlap cloner DNA cloning kit, lambda integrase and excisionase were acquired from Elpis Biotech (Daejeon, Korea). Dithiothreitol (DTT) and 1-thio-β-d-galactopyranoside (IPTG) were acquired from Anaspec (Fremont, CA, USA). The 0.45 μm pore size filter was purchased from Hyundai Micron (Seoul, Korea). All columns for purification were purchased from GE Healthcare Korea (Seoul, Korea). Waters 600 Controller, Waters 486 Tunable Absorbance Detector, Waters 717 Plus Autosampler and Protein-Pak 300SW SEC 7.5 × 30 mm column were purchased from Waters Korea (Seoul, Korea). Coomassie brilliant blue R-250, and Tris-HCl were from Amresco (Solon, OH, USA). Imidazole was obtained from Daejung Chemicals (Siheung, Korea). Dialysis membranes were purchased from Viskase (Darien, IL, USA), and Amicon Ultra was purchased from Merck Millipore (Billerica, MA, USA). Acrodisc syringe filters were acquired from Pall Korea (Seoul, Korea). *E. coli* BL21(DE3) cell was acquired from Novagen (Madison, WI, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was obtained from Sigma-Aldrich Korea (Yongin, Korea). SKBR3, BT474, MDA-MB-231 and MCF-7 cell lines were acquired from Korea Cell Line Bank (Seoul, Korea). RPMI-1640 Medium, 0.25% trypsin-EDTA, fetal bovine serum (FBS), and penicillin–streptomycin were acquired from Thermo Fisher Scientific Korea (Seoul, Korea). All chemicals were analytical grade.

Construction of expression vector

An overlap cloning was performed to make the entry clone for anti-HER2(scFv). Genes encoding V_H and V_L of trastuzumab were synthesized by Bioneer (Daejeon, Korea). The V_H and V_L regions of trastuzumab were amplified using primers: 5'-GGCTTCGAAAACCTGTATTTTCAGGGCGAAGTACAATTGGTTGAAAGCGGGGGTGG and 5'-CCCGAGCCACCGCCACCTGAGCCACCGCCACCGGAGCTTACAGTTACCAGA GTACCCTGG for V_H, 5'-GCTCAGGTGGCGGTGGCTCGGGTGGCGGTGGCTCAGATATTCAAATGACACAAAGCCCCTCTAG and 5'-GCCTTTGTACAAGAAAGCTGGGTTTAGCATTTAATTTCAACCTTCGTTCCCTGACCAAATG for V_L. Primers were designed for PCR products to have homologous sequence at both ends. attL1 and attL2 sites at each end of V_H and V_L were added, and scFv was formed via the linker $(G_4S)_4$ between V_H and V_L. In additional, the tobacco etch virus protease recognition site (TEVrs) at the N-terminus of HER2(scFv) and Cys residue at the C-terminus were inserted for tag removal and conjugation reaction, respectively. After overlap cloning with amplified donor vector (pDONR207), entry clone was formed. MBP–HER2(scFv) expression vector was created by multisite gateway cloning (LR reaction) with the destination vector (pDEST-HMGWA) and entry clone. In case of PE24, the PE24 sequence [27] was codon-optimized for *E. coli* expression and synthesized. The gene was amplified by PCR with the primers designed to include attB1 and attB2 sites at both ends of PE24: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGAATCTGTATTTCCAAGG and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTTCAGA TCCTCACG. Then, the PCR product was inserted to the vector containing attP1 and attP2 sites by multisite gateway cloning (BP reaction). With the newly formed entry clone (pENTR-PE24) and destination vector (pDest-His8), multi-LR reaction was performed. As a result, the expression vector for His8–PE24 was created. Correct constructs were confirmed by sequencing analysis (Macrogen, Daejeon, Korea)

Expression and solubility analysis of recombinant fusion protein in *E .coli,* BL21

For the transformation of expression plasmid into *E. coli*, BL21 strain was created by the heat shock method. Single colony was inoculated in the Luria–Bertani (LB) medium containing 50 µg/mL overnight at 37°C. The cultured cells were diluted into 4 mL of fresh LB medium at a ratio of 1:50, and the cells were grown at 37°C. When the value of OD_{600} reached 0.6–0.7, 0.5 mM IPTG was added and cell was inducted at 37°C for 3 h or 18°C for overnight. Cells were harvested and resuspended with sonication buffer containing 20 mM Tris, pH 8.0, 1 mM EDTA, and 20% glycerol (v/v). Resuspended cells were sonicated by ultrasonic cell disruptor. To obtain soluble fraction and pellet fraction, sonicated cell was centrifuge at 23,000 g for 10 min.

Purification of HER2(scFv)-Cys and PE24

For 500 mL of culture, one colony was inoculated in 5 mL of LB with 50 µg/mL of ampicillin and grown at 37°C overnight. The cultured cell was diluted with 500 mL of LB containing ampicillin at a 1:100 ratio and grown at 37°C until OD_{600} = 0.6. At this stage, 0.5 mM IPTG was added to the culture and incubated at 18°C overnight to induce protein expression. Cultured and induced cells were harvested by centrifugation at 3,800 g for 20 min at 4°C. After centrifugation, some medium was removed and pellets were resuspended with the remaining medium. Resuspended pellets were collected to 50 mL falcon tube and centrifuged at 3,800 g for 30 min at 4°C. Cell pellets were stored at –20°C until used. For the purification of fusion protein, MBP–HER2(scFv) or His8–PE24, cell pellets were resuspended with 100 mL of buffer A containing 20 mM Tris, pH 8.0, 500 mM NaCl and 5% glycerol(v/v). Suspended pellets were sonicated and homogenized by ultrasonic cell disruptor JY99-IIDN. The cell lysate was centrifuged at 23,000 g for 20 min at 4°C. After centrifugation, the supernatant was filtered with 0.45 µm filter and was applied to 10 mL HiTrap Ni HP that was already equilibrated with buffer A. After applying the cell lysate, 30 mM imidazole was allowed to flow in the column for 5 CV to wash off unbounded proteins. When 100 mM imidazole had flowed in the column for 3–4 CV, MBP–HER2 fusion protein was eluted and collected. In case of his8–PE24, unbounded proteins were washed by 50 mM imidazole and fusion protein was eluted at 500 mM imidazole. TEV protease was treated to remove the MBP tag of MBP–HER2(scFv) (MBP fusion

protein:TEV = 5:1, w/w) or His8 tag of His–PE24 (fusion protein:TEV = 20:1, w/w). Tag cleavage by TEV protease was performed at 18°C overnight. The TEV-treated mixture was dialyzed against buffer A and applied to 10 mL HiTrap Ni HP that was equilibrated with the same buffer. The tag-free HER2(scFv) or PE24 did not bind to the column and was eluted from flow through (FT). The His-MBP tag or His8 tag and His7-TEV protease were eluted at 500 mM imidazole. The purified HER2(scFv) or PE24 was concentrated by Amicon Ultra from Merck Millipore by centrifuging at 3,800 g and dialyzed against PBS at pH 7.4.

Crosslinking of HER2(scFv) and PE24

In this process, 12.5 µL of 20 mM sulfo-LC-SPDP was added to 1 mg of PE24 in PBS (pH 7.4) and 1 mM EDTA. The reaction mixture was incubated at room temperature for 40 min and dialyzed against PBS (pH 7.4) and 5 mM EDTA to remove excess unreacted SPDP reagent. Then, 1 mg/mL of HER2(scFv)-Cys was reduced by 10 mM TCEP to form monomer and was incubated at room temperature for 40 min. Unreacted TCEP was removed by dialysis against PBS (pH 7.4) and 5 mM EDTA. SPDP-modified PE24 was added to reduce HER2(scFv) at a 5:1 molar ratio (PE24:HER2(scFv)) and incubated at room temperature for 30 min or at 4°C overnight.

Purification of GFP and HER2(scFv)–GFP conjugate.

pET28a-sfGFP plasmid was purchased from Addgene (Massachusetts, USA). At the C-terminal of Superfolder GFP (sfGFP), His6-tag was included. sfGFP was purified with IMAC chromatography as mentioned in the purification of MBP–HER2(scFv) fusion protein (Supplementary Method 3). HER2(scFv)and GFP were conjugated and purified as described in crosslinking of HER2(scFv)–PE24 (Supplementary Method).

***In vitro* cytotoxicity assay**

SKBR-3, BT-474, MDA-MB-231, and MCF-7 cells were grown in RPMI 1640 medium supplemented with 10% FBS. Cells were seeded into 24-well plate at a density of 0.5-1 × 10^5 cells per

well. After culture for 24 h, the HER2(scFv)–PE24 conjugate was treated to seeded cell at various concentration (0.002, 0.02, 0.2, 2, 20, 200 nM) in triplicate. At 72 h after incubation with the conjugate, the media was removed, and cells were washed with PBS. Then, 0.5 mL of the MTT solution (0.04 mg/mL in serum-free medium) was added into the wells, and the plate was incubated for 2 hr. Then, the MTT solution was removed, and 0.5 mL of DMSO was added. The plate was incubated for 1 h and absorbance at 595 nm was measured. Cell viability was calculated using the following equation and Microsoft Excel :

$$V = top - (top - bottom)/(1 + (IC_{50} / conc.)^{HC})$$

Where V means cell viability, top is the highest value of cell viability, bottom is the lowest value of cell viability, conc. is the treated conjugate concentration and HC is the Hill coefficient of inhibition.

Tables

Supplementary Table 1. Expression and solubility level of HER2(scFv) and PE24

Protein	Expression level (%)		Solubility (%)	
	37°C	18°C	37°C	18°C
HER2(scFv)	38.1	34.5	64.3	70.1
PE24	53.9	33.3	57.4	91.2

Supplementary Table 2. Production yields of HER2(scFv)

Purification step	Total protein (mg)	Purity (%)	HER2(scFv) (mg)	Yield (%)
Bacterial culture (500 mL)	1400			
Supernatant	107.6	40	15.89	100
First IMAC	21.15	79	6.2	39.02
Second IMAC	4.99	98.01	4.89	30.77

IMAC, immobilized metal affinity chromatography

Supplementary Table 3. Production yields of PE24

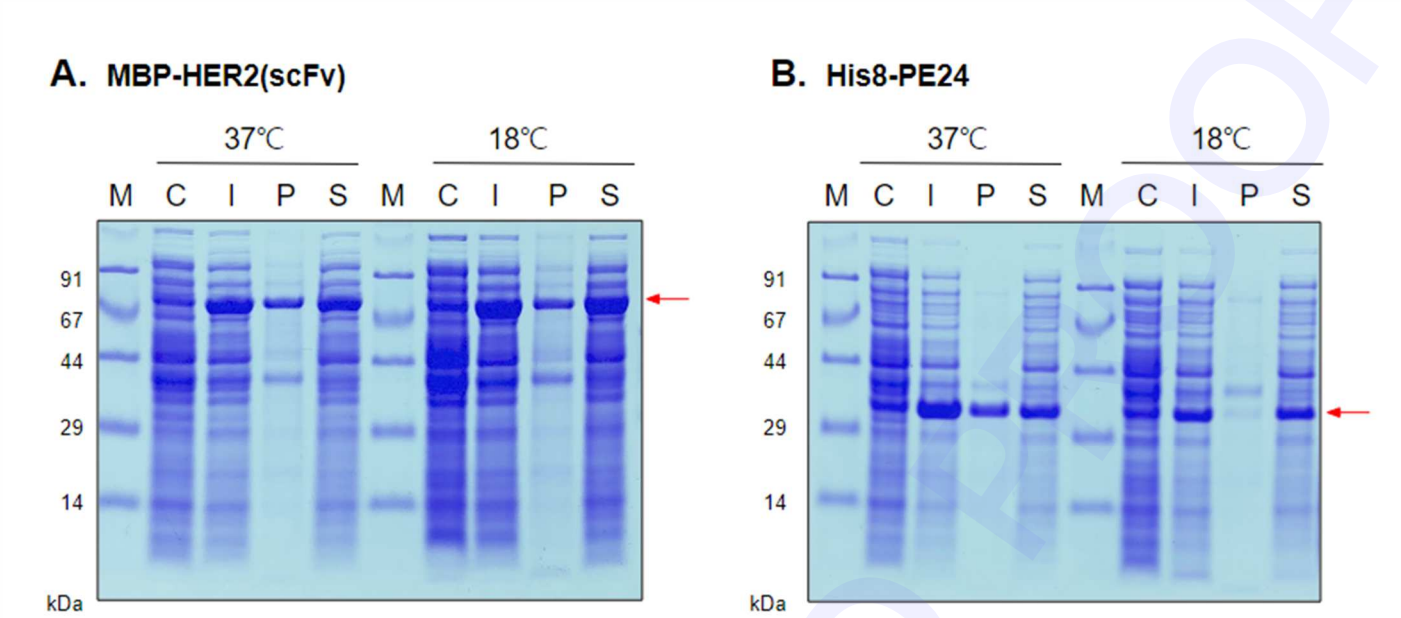
Purification step	Total protein (mg)	Purity (%)	PE24 (mg)	Yield (%)
Bacterial culture (500 mL)	1100			
Supernatant	91.4	31.46	24.78	100
1st IMAC	23.76	97.06	19.88	80.23
2nd IMAC	9.8	99.8	9.78	39.46

Supplementary Table 4. Production yields of the HER2(scFv)–PE24 conjugate.

SPDP-PE24 (mg)	TCEP-treated HER2(scFv) (mg)	HER2(scFv)-PE24 conjugate (mg)	Yield* (%)
1.2	0.24	0.12	25
2.5	0.45	0.34	37
2.84	0.56	0.65	58

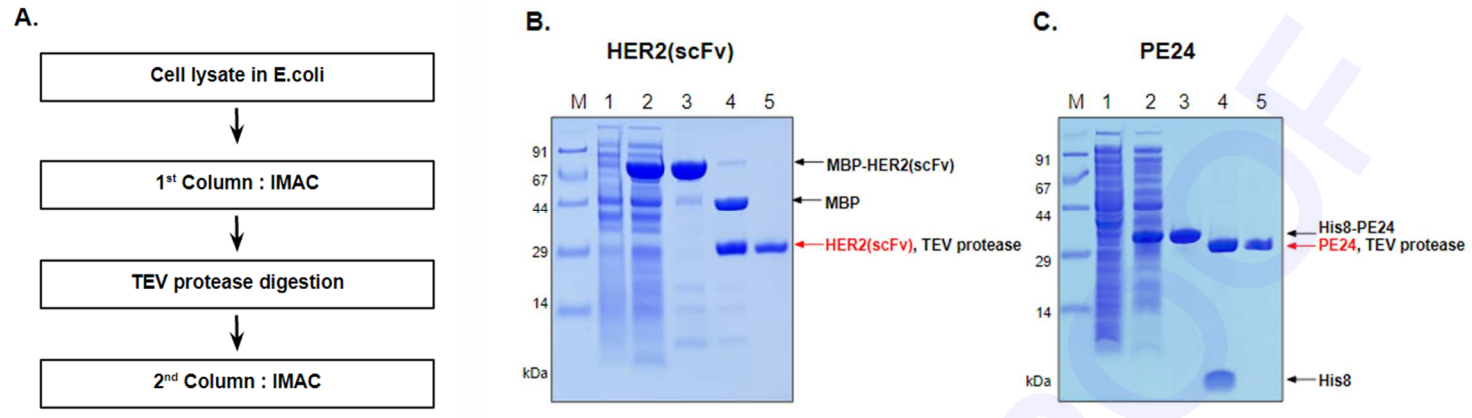
*Yield(%) = mole of conjugate / mole of HER2(scFv)×100 (%)

Supplementary Figures



Supplementary Figure 1. Expression and solubility analysis of MBP–HER2(scFv) and His8–PE24 in *E. coli* BL21 strain.

Expressions of (A) MBP–HER2(scFv) and (B) His8–PE24 were induced at different induction temperatures of 18°C or 37°C. The arrows indicate MBP–HER2(scFv) (70 kDa) and His8–PE24 (29 kDa). M, molecular weight marker; C, IPTG not added-total protein as negative control; I, total cell fraction after induction; P, Pellet fraction after induction; S, soluble supernatant after induction



Supplementary Figure 2. Purification of HER2(scFv) and PE24.

(A) Flowchart of the purification. (B) SDS-PAGE analysis of HER2(scFv) purification. M, molecular weight size marker; lane 1, total cell proteins before induction; lane 2, soluble fraction after induction; lane 3, MBP–HER2(scFv) purified by the first IMAC; lane 4: MBP tag cleavage by TEV protease; lane 5, HER2(scFv) purified by the second IMAC. (C) SDS-PAGE analysis of PE24 purification. lane 1, total cell proteins before induction; lane 2, soluble fraction after induction; lane 3, His8–PE24 purified by the first IMAC; lane 4: His8 tag cleavage by TEV protease; lane 5, PE24 purified by the second IMAC.