Generating a Bispecific Antibody Drug Conjugate Targeting PRLR and HER2 with Improving the Internalization

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Abstract

Antibody drug conjugate (ADC) therapy has become one of the most promising approaches in cancer immunotherapy. The bispecific targeting could improve the specificity, affinity, and internalization of the ADC molecules. Prolactin preceptor (PRLR) and HER2 have crosstalk signaling in breast cancer, and PRLR undergoes a rapid internalization compared with HER2. To improve the efficacy of HER2 ADCs with enhancing the target specificity and internalization, we constructed a PRLR/HER2-targeting bispecific ADC (BsADC). We evaluated the characterization of PRLR × HER2 BsADC from the affinity and internalization, and further assessed its *in vitro* cytotoxicity in human breast-cancer cell lines (BT474, T47D, and MDA-MB-231) using Cell Count Kit-8 analysis. Our data demonstrated that PRLR × HER2 BsADC kept the affinity to two targeting antigens after conjugating drugs and exhibited higher internalization efficiency in comparison to HER2 ADC. Furthermore, PRLR × HER2 BsADC demonstrated to have superior antitumor activity in human breast cancer *in vitro*. In conclusion, our findings indicate that it is feasible through increasing the internalization of target antibody to enhance the antitumor activity and therapeutic potential that could be further evaluated in *in vivo* animal model.

Keywords
► bispecific ADC
► PRLR
► HER2
► internalization
► antitumor

Introduction

Antibody drug conjugate (ADC), which delivers cytotoxic drugs specifically into targeted cells with antibody, has emerged as an effective cancer therapy.[1,2] The number of ADCs as well as immunotoxins in clinical trials has seen a surge, and they have been approved by the Food and Drug Administration in recent years.[3–5] An ADC comprises a monoclonal antibody conjugated to the cytotoxic payload via a chemical linker.[6] The antibody is the main component of the ADC design. In the development of therapeutic ADCs, strategies for optimizing ADCs about antibody mainly include enhancing specificity, affinity, and internalization.[6] The rapid development of bispecific antibody (BsAb) technology contributes to more choices of antibodies as a partner of ADCs. Conjugating payload into BsAb to improve specificity and internalization, called bispecific ADCs (BsADCs), has been studied in recent years,[7–9] which are mainly being studied at the preclinical and clinical investigations.

Previous reports indicate that prolactin preceptor (PRLR) undergoes rapid internalization as certain class 1 cytokine receptors.[10] PRLRs are expressed slightly elevated in many human breast tumors in comparison to the normal breast tissue. The functional attributes and tumor expression profile of PRLR make it an attractive target in breast cancer.[11] There are already several approaches of immunotherapy using PRLR as a target, including monoclonal antibodies, BsAbs, and ADCs.[11–13] In 2017, Nagini reported that the overexpression of HER2 in human epidermal growth factor receptor (ErbB) family may be the main cause of breast cancer.[14] Moreover, some research studies have confirmed that PRLR and HER2 and their crosstalk signaling have a significant role in breast tumor development, growth, and survival. PRL via PRLR can activate HER2 downstream signaling pathways through JAK2 signal.[15] Andreev and his colleagues had studied that bridging HER2 and PRLR could improve the efficacy of HER2 ADCs.[8] Considering the rapid internalization of PRLR and crosstalk function of PRLR and HER2, we constructed a PRLR × HER2 BsADC to achieve a better therapeutical effect.

"Bispecific Antibody by Protein Trans-splicing (BAPTS)" is a BsAb construction platform in our laboratory, which has been used to generate dozens of BsAbs and applied maturely.[12,16–18] Besides, we have established a number of relevant technology platforms including immunotoxin and bispecific nanobody to facilitate the study on enhancing immunoactivity through bispecific drug conjugates.[19–21] We established a BsADC construction method based on BAPTS, which could conjugate about three payloads into the BsAbs. In this study, we further constructed a PRLR × HER2-targeting BsADC conjugating MMAE as payloads, and characterized its affinity, internalization as well as cytotoxicity *in vitro*. This work will exhibit the considerable value in constructing a BsADC through increasing the receptor internalization to enhance the antitumor activity and therapeutic potential.

Materials and Methods

Cell Lines and Culture Conditions

HEK 293C18 human embryonic kidney cells (HEK 293E), (CRL-10852, ATCC, Manassas, Virginia, United States) were cultured in a growth medium consisting of a 50/50 mix of FreeStyle 293 Expression Medium and SFM4 HEK293 medium (GE Healthcare, Chicago, Illinois, United States), which also contained 100 μg/mL of G418. The BT474 and T47D cells (Chinese Type Culture Collection Cellbank, Shanghai, China) were grown in RPMI-1640 medium and 10% of fetal calf serum was added into the base medium. All cell lines were maintained at 37°C in a 5% (v/v) CO_2 humidified incubator. All cell lines listed were tested negative of mycoplasma. All medium and cell culture reagents were purchased from Invitrogen (Carlsbad, California, United States), except indicated specifically.

BsAb Generated by BAPTS

The BsAb PRLR × HER2 was generated by the BAPTS platform of our laboratory.[16,22] The anti-HER2 antibody sequence came from trastuzumab and the anti-PRLR antibody sequence came from the patent (US2015/0056221 A1). We mutated the amino acid residue valine to cysteine in the position 205 of light chain, for the next conjugation reaction. We set the anti-PRLR antibody as fragment A and the anti-HER2 antibody as fragment B. Transfection into HEK 293E cells was performed according to a published transient transfection procedure.[23] Supernatant was taken for analysis or processing when cell viability dropped to 50% or 1 week posttransfection.[24] The fragment A and fragment B were captured by protein L affinity chromatography (Cytiva, Marlborough, Massachusetts, United States). The PRLR × HER2 was produced through protein trans-splicing of inteins and then purified by a MMC ImpRes Multimodal Chromatography Column (Cytiva).

BsADC Generated by Site-Specific Conjugation

The valine at position 205 of the light chain was mutated to cysteine to achieve site-specific conjugation according to the literature.[25,26] The payload, MC-vc-PAB-MMAE (MMAE, Levena Biopharma, Nanjing, China), was conjugated to BsAb via the maleimidocaproyl linker. The BsAb PRLR × HER2 (1 mg/mL) was added with 1 mmol/L EDTA, and then reduced with TCEP (Sigma-Aldrich, St. Louis, Missouri, United States) at 37°C for 1 hour. The molar concentration of TCEP was 100-fold molar excess of antibody. And then, to reform the interchain disulfide bonds, the reduced antibody was incubated with 30-fold molar excess dhAA (Sigma-Aldrich) for 2 hours at 25°C after ultra-filtration. The conjugating reaction system was PBS/ACN (v/v, 80/20) when MC-vc-PAB-MMAE (20-fold molar excess) conjugated into the antibody with incubating at 37°C for 30 minutes. The excess reagents in the reaction system were purified by G25 desalt column (Cytiva) after conjugating successfully.[27] The coupling efficiency was predicted by the intensities of the Lcs-MMAE bands in SDS-PAGE analysis.

Surface Plasmon Resonance Assay for Affinity Measurement of BsADC

Affinities of the BsAb and BsADC were determined by surface plasmon resonance (SPR; Biacore T200, GE Healthcare). Human HER2-Fc and PRLR-Fc were immobilized to a CM5 chip surface (GE Healthcare) using standard 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide amine coupling protocols. The running flow rate was 10 μL/min. The chip surface was regenerated by 0.1 mol/L glycine, pH 1.5. The binding (K_a) and dissociation (K_d) rate constants were obtained by globally fitting the data to a 1:1 binding model using Biacore T200 Evaluation Software. The equilibrium dissociation constant (K_D) was calculated by dividing the dissociation rate constant (K_d) by the association rate constant (K_a).
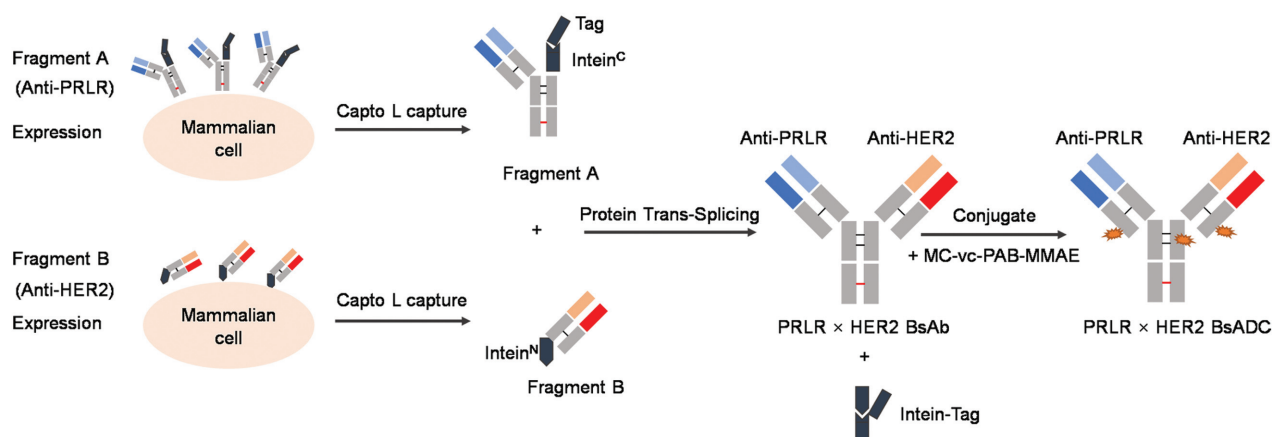
Fig. 1 Schematic representation of fragment expression, the split intein trans-splicing, and drug conjugation process used to generate reactivated BsADC.

To demonstrate simultaneous binding, 100 nmol/L human PRLR-Fc was coupled to CM5 sensor chip as described above. The BsADC was injected for 100 seconds followed by a 100-second injection of HER2-Fc (at a concentration of 50 nmol/L). The PBS was injected as control.

Flow Cytometry-Based Internalization Assay

T47D cells were incubated with BsADC or the control parental ADCs (60 nmol/L each ADC) on ice for 1 hour, and then washed to remove unbound ADCs. An aliquot of cells remained on ice and the rest were incubated at 37°C for different periods of time. Cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature, and then stained with FITC-labeled goat anti-human IgG (H + L) secondary antibody (Invitrogen). The stained cells were tested by CytoFLEX cytometer and analyzed by CytExpert software. Receptor–antibody complex internalization was calculated by the formula[28]:

$$\% \text{ internalization} = (\text{MFI}_{\text{ice}} - \text{MFI}_{37°C})/\text{MFI}_{\text{ice}} \times 100\%$$

Cytotoxicity *In Vitro*

BT474, T47D, and MDA-MB-231 cells were seeded into 96-well plates with 200 µL assay medium and incubated overnight. A gradient dilution concentration of antibodies or ADCs was added into 96-well plates and incubated for 5 days. The PBS was as the control group. The cytotoxicity of antibodies and ADCs was evaluated by measuring cell viability using Cell Count Kit-8 (CCK8) analysis following the manufacturer's instruction (Dojindo, Shanghai, China) and the absorbance was detected using TECAN infinite 200 at a wavelength of 450 nm. Percentage of cell viability was calculated with absorbance as follows:

$$\text{cell viability } (\%) = [(A_{\text{sample group}} - A_{\text{blank group}})/(A_{\text{control group}} - A_{\text{blank group}})] \times 100\%.$$

Data analysis was performed using GraphPad Prism 8.0.2 software (San Diego, California, United States).

Results and Discussion

Molecular Design and Preparation of BsADC

It has been reported that PRLR and HER2 signaling pathways have a crosstalk in breast cancer cells, which are associated with a poor prognosis and therapeutic resistance in breast tumor patients.[15] Furthermore, the PRLRs undergo rapid internalization compared with HER2 antigen.[10] The internalization efficiency is a critical property in overall performance efficiency of an ADC. We hypothesized that a PRLR/HER2-targeting BsADC might improve the efficacy of HER2 ADCs through enhancing the target specificity and internalization. The BAPTS platform, an approach to generate bispecific IgG antibodies with split intein, has been used to generate dozens of BsAbs and applied maturely in our laboratory.[12,18,29] We have designed a procedure to construct BsADC based on the BAPTS, which could conjugate three payloads (data not shown) in one antibody molecule. We employed the BAPTS method to design and construct a BsADC targeting PRLR and HER2 (►**Fig. 1**) and the product was nomenclatural as PRLR × HER2 BsADC.

The PRLR × HER2 BsAb was generated first following the BAPTS procedure. Five vectors were constructed and co-transfected into HEK293E cells for the expression of fragment A (PRLR-3F) and fragment B (HER2–2F). The supernatant of expressing fragments was purified by Capto L affinity chromatography column. From the SDS-PAGE analysis, we found that fragment A and fragment B were expressed and captured by Capto L successfully (►**Fig. 2**). Fragment A (PRLR-3F) was composed of three peptides: PRLR Lc (V205C), PRLR Hc (Knob), and Int^C-Fc (Hole) (►**Fig. 2A**); fragment B (HER2–2F) was composed of two peptides: HER2 V_H-C_{H1}-Int^N and HER2 Lc (V205C) (►**Fig. 2B**). Next, the PRLR × HER2 BsAb was produced by the trans-splicing reaction of PRLR-3F and HER2–2F. The reaction was started with the molar ratio 1:2 (fragment A: fragment B) under reducing conditions of 2 mmol/L DTT. A new band of BsAb was generated in the corresponding molecular weight by nonreduced SDS-PAGE analysis, with the substrates PRLR-3F and HER2–2F decreasing. From the
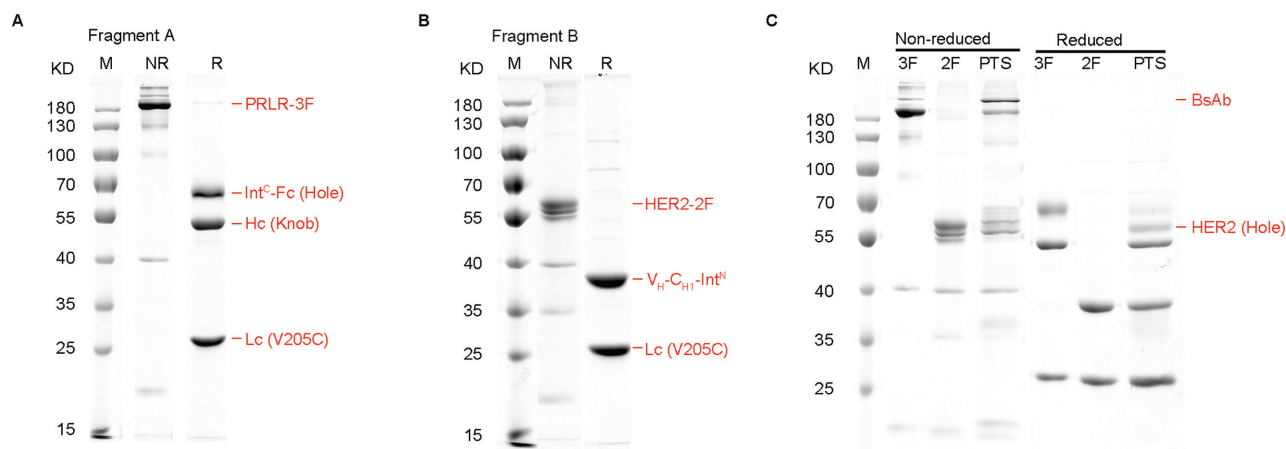
Fig. 2 The construction of PRLR × HER2 BsAb. (**A**) SDS-PAGE analysis of PRLR-3F (fragment A). (**B**) SDS-PAGE analysis of HER2−2F (fragment B). (**C**) SDS-PAGE analysis of PTS reaction. NR, nonreduced; R, reduced; 3F, PRLR-3F; 2F, HER2−2F; PTS, reaction sample.
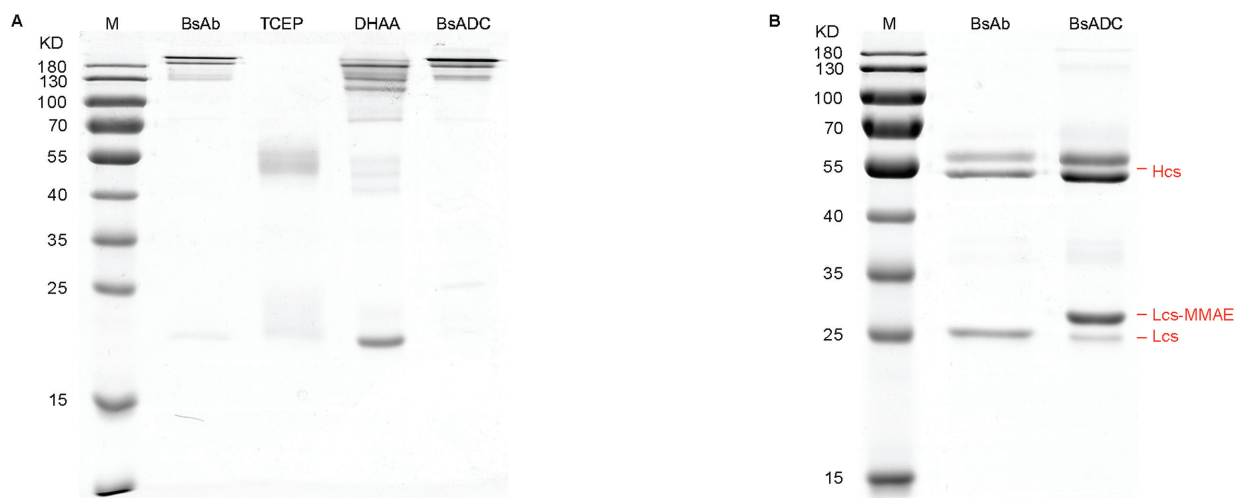

Fig. 3 The construction of PRLR × HER2 BsADC. (**A**) 12% nonreduced SDS-PAGE analysis of conjugating process of PRLR × HER2 BsADC. (**B**) 12% reduced SDS-PAGE analysis of the conjugating efficiency.

reduced SDS-PAGE analysis, the band of HER2 (Hole) was generated with the Int^C-Fc (Hole) peptide disappearing and HER2 V$_H$-C$_{H1}$-Int^N peptide decreasing (►**Fig. 2C**). The kinetics of split intein to generate PRLR × HER2 BsAb under 37°C has been reported in our previous article. The yield could achieve 83% and the rate constant was 0.0644 minutes,[1] which was a relatively high rate for the trans-splicing reaction.[29] The final BsAb product was purified by a MMC ImpRes Multimodal Chromatography Column.

The payload MC-vc-PAB-MMAE was conjugated into BsAb by THIOMAB technology to obtain BsADC.[26] The cysteine and glutathione adducts of BsAb were removed with TCEP (100-fold molar excess of BsAb) followed by diafiltration. The oxidizing agent DhAA was added to reform the reduced samples, and then MC-vc-PAB-MMAE was added to conjugate MMAE on BsAb, as demonstrated by nonreduced SDS-PAGE analysis (►**Fig. 3A**). The molecular weight of the light chain would increase after the MC-vc-PAB-MMAE conjugated into the sites by reduced SDS-PAGE analysis. The conjugation

efficiency of MMAE was ∼85% (►**Fig. 3B**). The PRLR × HER2 BsADC was generated successfully.

Bispecific Binding to HER2 and PRLR Antigens

The affinity of BsADC was tested by SPR to verify whether the BsADC maintained the binding ability after conjugation. For the PRLR antigen, the dissociation constants of BsADC were smaller than BsAb (BsADC 0.54 vs. BsAb 1.48 nmol/L), which indicated that the affinity slightly increased after conjugation. For the HER2 antigen, however, the dissociation constants of BsADC were higher than BsAb (BsADC 2.77 vs. BsAb 0.70 nmol/L) (►**Fig. 4A** and ►**Table 1**). The BsADC could recognize both PRLR and HER2 antigens with excellent affinity. However, the binding kinetics constants of BsADC were little different from BsAb. This may be caused by the conjugation of MMAE.

To determine whether the BsADC kept the capability of binding to HER2 and PRLR antigens simultaneously, we further performed SPR "sandwich" experiments. The BsADC
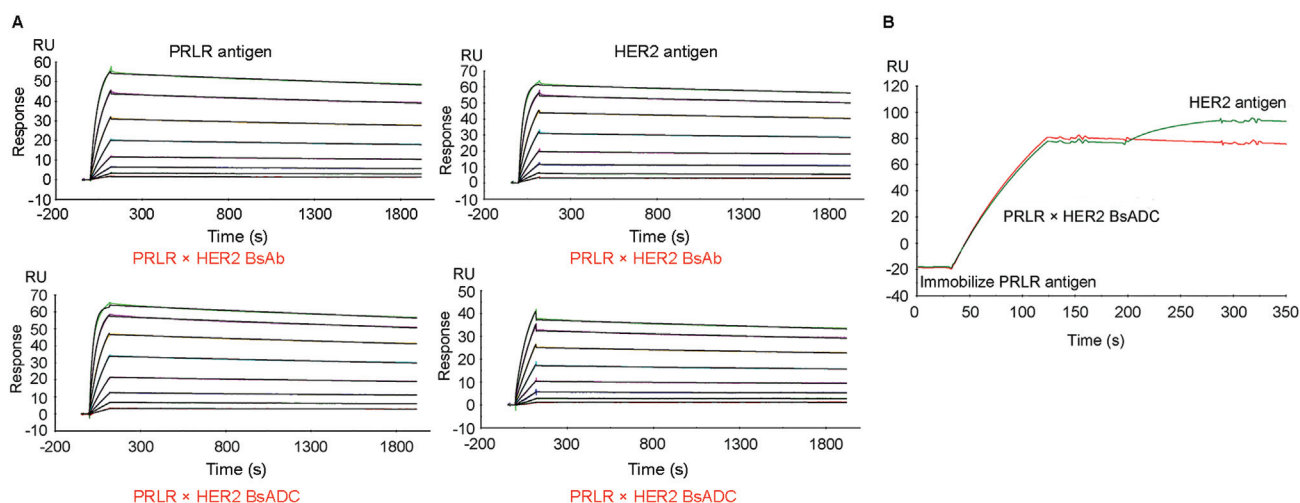
Fig. 4 SPR assay for the affinity measurement of PRLR × HER2 BsADC. (**A**) SPR assay characterizing the binding kinetics of PRLR × HER2 BsAb and BsADC to PRLR antigen and HER2 antigen. (**B**) Bispecific coupling binding to HER2 and PRLR antigens of PRLR × HER2 BsAb. SPR, surface plasmon resonance.

Table 1 Binding rate constants to antigens of BsADC and the control BsAb

Antibody (antigen)	K_a (1/Ms)	K_d (1/s)	K_D (nmol/L)
PRLR × HER2 BsAb (PRLR)	2.15E + 5	3.17E-4	1.48
PRLR × HER2 BsADC (PRLR)	6.45E + 5	3.47E-4	0.54
PRLR × HER2 BsAb (HER2)	1.33E + 5	9.25E-5	0.70
PRLR × HER2 BsADC (HER2)	7.88E + 4	2.18E-4	2.77

showed reactivity to PRLR antigen immobilized on SPR chips and HER2 antigen flowed through chips (►**Fig. 4B**). Thus, BsADC was able to simultaneously bind to PRLR and HER2 antigens.

Internalization

To examine whether the BsADC could induce enhanced internalization, we treated T47D cells with BsADC and its control parental ADCs, and then measured the cell surface level of antigen by flow cytometry. The cell surface level of antigen would decrease when the antibody internalized into cells. The extent of cell surface level reduction of HER2 ADC was far below when compared with those of PRLR ADC and PRLR × HER2 BsADC (►**Fig. 5A**). As shown in ►**Fig. 5B**, at the first hour, the internalization efficiency of PRLR × HER2 BsADC was 42%, that of PRLR ADC was 75%, and that of HER2 ADC was 17%. However, at the fourth hour, PRLR × HER2 BsADC was 86%, PRLR ADC was 89%, and HER2 ADC was 47%. The PRLR × HER2 BsADC had a slower internalization than PRLR ADC; however, they could achieve the same top at the fourth hour. The PRLR × HER2 BsADC elicited a significantly higher level of internalization than HER2 ADC. It is indicated that internalization of HER2 could improve significantly when combined with PRLR to form a BsAb.

In Vitro Cytotoxicity

To evaluate the cytotoxicity of PRLR × HER2 BsADC, human breast cancer cell lines BT474 and T47D were selected as the target cells. First, the cytotoxicity of BsADC and BsAb was detected by the CCK8 kit. ►**Fig. 6(A, B)** shows that PRLR × HER2 BsADC has a stronger killing ability compared with PRLR × HER2 BsAb in BT474 and T47D cells; moreover, a part of cytotoxicity of BsADC is contributed by the conjugated payload, MMAE. The cytotoxicity of BsAb might be caused by inhibition of downstream signaling pathways. Furthermore, the cytotoxicity of PRLR × HER2 BsADC and the ADC controls was detected in T47D cell lines. The PRLR × HER2 BsADC had a better killing activity compared with the parental ADC controls (EC_{50} of PRLR × HER2 BsADC: 0.98 µg/mL vs. EC_{50} of HER2 ADC: 1.66 µg/mL and EC_{50} of PRLR ADC: 8.82 µg/mL) (►**Fig. 6C**). At the same time, we also selected the triple-negative breast cancer cell line MDA-MB-231 as the target cell, which had no PRLR and HER2 expression on the cell surface. The PRLR × HER2 BsADC and HER2 ADC had no killing activity on MDA-MB-231 cells (►**Fig. 6D**). Taken together, these data demonstrate that PRLR × HER2 BsADC has much stronger killing activity than HER2 ADC in HER2-positive breast cancer cells, possibly due to increased internalization of PRLR × HER2 BsAb compared with HER2 antibody.

Andreev and his colleagues had studied that PRLR × HER2 BsADC could improve the internalization of HER2 ADC with conjugating DM1 through an uncleavable linker. In this study, we constructed the PRLR × HER2 BsADC with conjugating MMAE via a cleavable linker through site-specific conjugation in engineered cysteine, which might further improve the therapeutic index. In addition, we constructed the BsADC on the basis of our BAPTS platform, and this method could
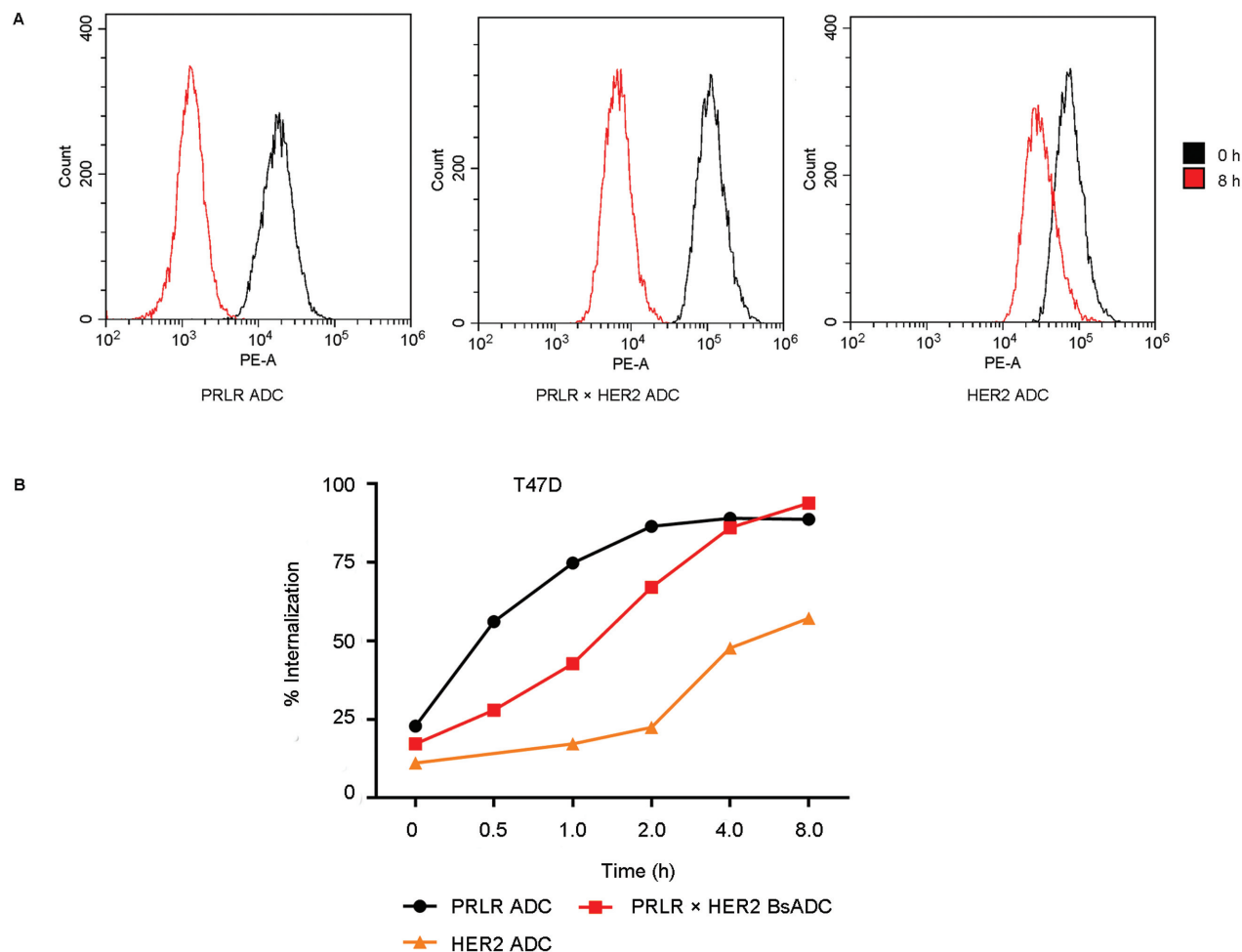
Fig. 5 Internalization of PRLR × HER2 BsADC in comparison with the control parental ADCs in T47D tumor cell lines. (**A**) The cell surface level of antigen detected by flow cytometry. The black line refers to the time at 0 hour; the red line refers to the time at 8 hours. (**B**) Internalization was calculated through the MFI analyzed by CytExpert software.

also be applied to obtain other antibody conjugates. Similar to ADCs, immunotoxins are molecules containing an antibody conjugated with a cytotoxic drug via a linker. The specificity, affinity, and internalization of antibody all need to be considered in the choice of antibodies about immunotoxins and ADCs. Based on the immunotoxin research studies in our laboratory, the PRLR × HER2 BsAb also could apply to the construction of bispecific immunotoxins.

Conclusion

In summary, we constructed a BsADC targeting HER2 and PRLR based on the BAPTS platform. From the SDS-PAGE analysis, the payload MMAE had been conjugated into BsAb. And then, we evaluated the characterization of PRLR × HER2(V205C)-MMAE in terms of the affinity, internalization, and *in vitro* cytotoxicity. The result demonstrated that PRLR × HER2 (V205C)-MMAE retained the affinity to two targeting antigens after conjugating drugs and showed a higher internalization efficiency compared with HER2 (V205C)-MMAE. Furthermore, the BsADC was

demonstrated to have superior antitumor activity *in vitro*. Our findings indicate that it is feasible to enhance the antitumor activity and therapeutic potential through increasing the internalization of the target antibody. Moreover, the result also indicated that BsADC was available in oncotherapy with the construction method based on the BAPTS.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.
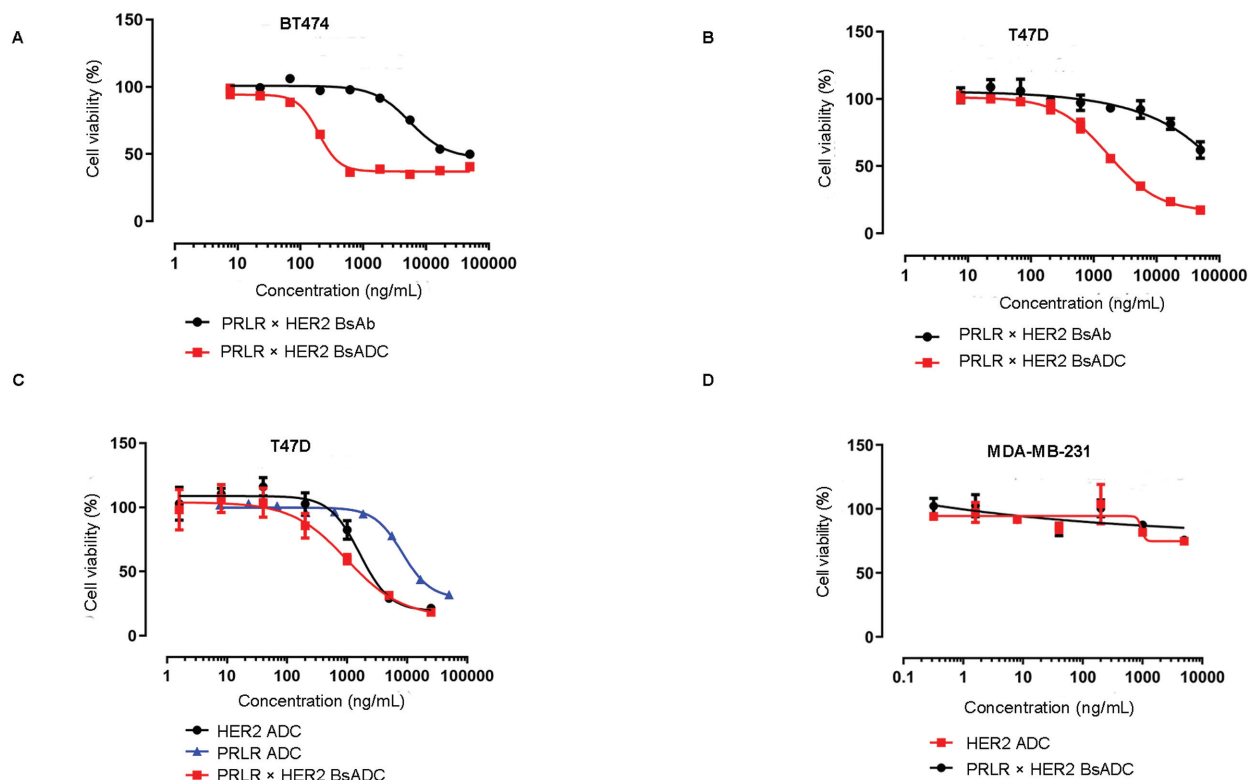
Conflict of Interest
None.

Fig. 6 *In vitro* cytotoxicity of BsADC on tumor cell lines by CCK8 assay. (**A** and **B**) *In vitro* cytotoxicity of BsADC and the control BsAb. (**C**) *In vitro* cytotoxicity of BsADC and the control parental ADCs in T47D cells. (**D**) *In vitro* cytotoxicity of BsADC and the control HER2 ADC in MDA-MB-231 cells. Graphs are representative data derived from different cell lines showing the mean percent growth inhibition $\pm$ SEM ($n = 3$). SEM, standard error of the mean.

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